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CITY UNIVERSITY OF NEW YORK, PH.D., 1979

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NITROGEN FIXATION ASSOCIATED WITH THE MACROALGA *ENTEROMORPHA INTESTINALIS* LINK AT TOWD POINT, A LONG ISLAND SALT MARSH

by

PATRICIA SULLIVAN SCHNEIDER

A dissertation submitted to the
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1979

Patricia Sullivan Schneider

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

NITROGEN FIXATION ASSOCIATED WITH THE MACROALGA *ENTEROMORPHA INTESTINALIS* LINK AT TOWD POINT, A LONG ISLAND SALT MARSH

by

Patricia Sullivan Schneider

Adviser: Professor John J. Lee

Nitrogen fixation associated with *Enteromorpha intestinalis* Link ranged from 0.15 to 14.4 μg N/g dwt/hr. Ten heterotrophic N_2 fixing bacterial epiphytes, identified as members of the genera: *Azotobacter*, *Azomonas*, *Klebsiella* and *Enterobacter* have been isolated from the *Enteromorpha* community. Axenic culture experiments with N_2 fixing isolates and multiple regression analysis of field data demonstrated the sensitivity of nitrogen fixation to temperature, salinity, pH and combined inorganic nitrogen. The study indicates that epiphytic bacterial nitrogen fixation is important for the development of the *Enteromorpha* community and suggests a possible symbiosis.

Nitrogen fixation, predominantly photosynthetic, was associated with decaying *Spartina* in experimental microcosms. This nitrogen input is probably important to the salt marsh detrital food web.

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I. Introduction

Approximately 73.8% of the United States commercial landing consists of estuarine dependent species (McHugh, 1976). This dependence of coastal fisheries upon estuaries has focused attention on estuarine food webs (Odum, 1971). There are two basic types of estuarine food webs: the detritus food web based upon vascular plant tissue and the grazing food web based upon phytoplankton (Odum and Heald, 1975). Both types are always present but there is a tendency for one to dominate.

The detritus food web is important wherever there are extensive wetlands. The salt marshes along the eastern coast of the United States are usually characterized by luxuriant growth of *Spartina* spp. The marsh grass is very productive but there is little direct grazing. Instead, the major energy flow between the first and second trophic level is by way of the detritus food chain. In the northern marshes frost usually kills the grass at the end of October, but it remains standing till the spring thaw when it is sheared off by tidal action (Schultz and Quinn, 1973). Bacteria, yeasts and fungi attack the *Spartina*, and as decomposition proceeds, cellulose and other complex structural compounds are dissimilated into simpler molecules. The animal food value is increased as the marsh grass with a C:N ratio of ~15:1 is converted to high-protein bacterial protoplasm with a C:N ratio of 5.7:1 (Teal, 1962; Burkholder,

1957; Harrison and Mann, 1975). As the microbes mineralize the organic carbon in the detritus, they must also assimilate nitrogen (Gosselink and Kirby, 1974). In the case of decomposing red mangrove leaves, a significant portion of the required nitrogen is supplied by nitrogen fixation (Gotto and Taylor, 1976). But at present, no such evidence exists for *Spartina* mineralization.

The *Spartina* detritus becomes available to the other trophic levels either by direct consumption or indirectly by consumption of detrital feeders. The tides remove approximately 45% of this detrital material before the marsh consumers can utilize it and transport it to the estuary (Teal, 1962). This influx of vascular plant detritus enhances the secondary production of the estuary.

Nitrogen is needed for primary productivity and is considered the principal limiting element in both the open ocean and coastal marine waters (Thomas, 1966; Ryther and Dunstan, 1971). Most marine plants satisfy this requirement by absorbing dissolved, combined nitrogen (nitrite, nitrate, ammonia and organic nitrogen) from the water column. The supply of available nitrogen, therefore, can have a tremendous effect on the coastal ecosystem. The factors influencing the supply of available nitrogen merit careful study.

Combined nitrogen enters marine waters through rainfall, land runoff, transport from adjacent water masses and *in situ* nitrogen fixation (Stewart, 1966). These processes replace nitrogen lost through denitrification and export. The relative importance of each phase of the nitrogen cycle

differs from one community to another and seasonally within the same community. The value of the contribution made by marine nitrogen fixing organisms is largely uncertain. But, Hardy and Holstem (1972) estimate that 20% of global nitrogen fixation or 10 - 36 metric tons per year occurs in the sea.

The organisms involved in denitrification are strict anaerobes. While no denitrification occurs if the oxygen level exceeds 0.2 mg O_2/l, localized anoxic zones of active denitrification may exist if the oxygen level is only a few tenths ml/l (Goering, 1968). The sediments may contain extensive anaerobic zones, and while sulfate reduction seems to be the dominant process, denitrification also occurs (Fenchel and Reidly, 1970).

Salt marsh sediments have characteristics which favor denitrification : low oxygen tension and oxidizable organic matter. Oxygen is consumed as the marsh grasses decompose and the sediment becomes anaerobic below the surface. Anaerobic decomposition proceedes at a much slower rate and organic matter accumulates in the sediment. Payne (1973) estimated that 2/3 of the nitrate flowing over rich organic sediments is lost by denitrification. Denitrification has been shown to be an important component of the nitrogen cycle in Cape Cod salt marshes where denitrification rates of 0.3 - 1.5 µg N_2/g sediment/hr were recorded (Kaplan _et al_., 1977).

The salt marsh loses nitrogen through denitrification

and export. Even if efficient recycling of combined nitrogen took place within the marsh, a shortage of nitrogen would occur unless the supply of combined nitrogen was replenished. Unless combined nitrogen import occurs, nitrogen fixation by members of the salt marsh community may be necessary to counteract nitrogen loss.

Most published studies of nitrogen fixation in the coastal marine environment have concentrated on sand dune slacks, rocky littoral shores (Stewart, 1967), sea grass epiphytes (Goering and Parker, 1972; McRoy *et al*., 1973; Capone and Taylor, 1977) and estuarine waters and sediment (Brooks *et al*., 1971); Sugahara *et al*., 1974; Werner *et al*., 1974; Herbet, 1975; Lakshmanaperumalsmay *et al*., 1975; Marsho *et al*., 1975).

Nitrogen fixation has also been studied in mangrove communities (Zuberer and Silver, 1978) and in coral reefs (Hanson and Gundersen, 1976). Until recently, little data have been available on N_2 fixation in the salt marsh. Salt marsh sediments and blue-green algae have been shown to fix significant amounts of nitrogen (Jones, 1974; Whitney *et al*., 1975; Hanson, 1977a; Carpenter *et al*., 1978) and nitrogen fixation was found associated with *Codium fragile* along the New England coast (Head and Carpenter, 1975).

Many of these marine coastal studies are primarily field measurements. Field data are needed to document the extent of nitrogen fixation in natural systems like the salt marsh. But, before the full ecological significance of marine nitrogen fixation can be evaluated, a fuller understanding of the

fixation process must be obtained. Physiological laboratory studies can provide insights that might not be obvious in natural multivariant systems.

A preliminary survey detected nitrogen fixation associated with *Enteromorpha intestinalis* Link, a macroalga, in a Towd Point, Long Island salt marsh. Since the ability to fix nitrogen is limited to procaryotes, the *Enteromorpha* cannot be responsible for the observed fixation. Are the agents of fixation bacteria, blue-green algae or a combination of both? What factors influence the rate of nitrogen fixation? How significant is this fixed nitrogen? The present investigation was designed to answer these questions.

An additional objective was to determine if a significant portion of the nitrogen needed for the increase in nitrogen during the decay of *Spartina* could be provided by *in situ* nitrogen fixation.

II. Materials and Methods

Study Area

Samples were collected and field observations were made at Towd Point, North Sea Harbor, Southampton, Long Island, New York, U.S.A. (N 40°55' W 72°25'; Lee *et al.*, 1966, 1970; Fig. 1). The area is adjacent to a wildlife sanctuary and as yet, not greatly disturbed by pollution.

Measurement of Nitrogen Fixation

Nitrogen fixation was measured by the acetylene reduction technique (Schollhorn and Burris, 1967; Stewart *et al.*, 1967; Hardy *et al.*, 1968; Bunt *et al.*, 1970; Waugman, 1971; Blandreau and Dommergues, 1973; Carpenter, 1973). The nitrogenase enzyme has rather low substrate specificity and one of the analogues for N_2 is acetylene. The acetylene reduction technique is sensitive, rapid and economical. Its only drawback seems to be the indirect method of measurement (Hardy, 1968).

For *in situ* measurements, clumps of *Enteromorpha* (1 g dwt) were collected with sterile forceps and immediately placed in 25 ml serum bottles containing 10 ml sterile sea water. The bottles were sealed with rubber serum caps and their edges coated with silicone sealant to prevent gas leakage. One bottle of sterile sea water served as a control. For laboratory experiments, axenic organisms or experimental mixtures were inoculated into serum bottles or serum tubes containing sterile media. Controls were uninoculated media. Acetylene, 0.2 atm, was then introduced into each vessel with a gas tight syringe. Knowles *et al.* (1973) demonstrated that nitrogen fixation is

markedly inhibited by even low levels of acetylene and at 0.2 atm of acetylene, nitrogenase will be saturated by acetylene. In the field the bottles were incubated in the marsh water for one hour; half of the samples in the light and half in the dark (bottles were wrapped in aluminum foil). In the lab vessels were incubated at 25°C in an illuminated (18 hr light/ 6 hr dark) Sherer environmental chamber (model Cel 4-4) for one week. Vessels were agitated before and after inoculation to equilibrate the vapor and aqueous phases (Flett et al., 1976).

In the field the equilibrated gas phase was immediately removed after incubation. The field procedures developed by Waugman (1971) were followed. At the end of the incubation period the neddle of a 10 ml syringe with the plunger fully in was pushed through the rubber cap into the incubation atmosphere. Then 10 ml of water was injected into the vessel from another syringe, so raising the internal pressure that the gas was displaced into the first syringe. The syringe containing the gas sample was then removed and the gas injected upwards into an inverted 10 ml serum bottle filled with water. The displaced water was vented through another syringe. The serum bottle was finally covered with silicone sealant to minimize leakage and the gas sample returned to the lab for analysis by gas chromatography. In laboratory experiments, the gas samples were analyzed immediately after incubation.

A Hewlett Packard gas chromatograph (model 5750B) was used with a Supelco Porapak T 50/80 mesh column and hydrogen flame ionization detector. Instrument operating conditions were as follows: injection port temperature of 260^{0}C,

column oven temperature of 60^oC, flame detector temperature of 120^oC, N_2 carrier gas flow rate of 25 cc/min, H_2 flow rate of 40 cc/min and air flow rate of 300 cc/min. Volume of sample injected (0.5 to 3 cc) and signal attenuation setting were adjusted to obtain reasonable peaks. Chromatograms were characterized by two ideal Gaussian shaped peaks with good separation (Fig. 2). Sample components were identified by comparison of their retention times with the retention times of ethylene (T_r=20 sec) and acetylene (T_r=42 sec) standards injected under identical conditions. A third, unidentified peak (T_r=10 sec) was observed on the chromatograms but this peak was small and well separated from the ethylene (Fig. 2). Strict qualitative analysis would require trapping of the individual peaks for spectroscopic analysis.

Ethylene standards were prepared by serial dilution in sealed experimental vessels containing sterile media and a gas phase at 1.2 atm. Because of this procedure, it was unnecessary to adjust experimental calculations for ethylene solubility and the pressure of the gas phase inside the experimental vessels. These diluted ethylene standards were injected into the gas chromatograph under standard conditions to product a reference curve. A compact linear curve was obtained by plotting log of ethylene concentration vs log of peak area (Fig. 3). Reasonable quantitation can be achieved by estimating peak area by counting squares on the graph paper (Burris, 1974). Strict quantitation would require integration of the area under the curve. The concentration of ethylene in experimental samples was ascertained by reference to the standard

curve. Standards were run routinely each time the chromatograph was operated to check retention time and the standard curve.

Since 22.4 l of any gas at STP ($0^{o}C$ or $273^{o}K$, 760 mm) constitutes 1 mole of that gas, ethylene volume was converted to moles of ethylene using the appropriate correction factors. Ethylene production was then converted to the equivalent nitrogen fixation using the theoretical ratio of 3 moles to 1 mole for *in vitro* systems of acetylene and nitrogen respectively (Schollhorn and Burris, 1967). Table 1 presents a sample step by step conversion of ethylene production to N_2 fixed.

Blue-Green Algal Studies

Blue-green algal members of the *Enteromorpha* community were isolated using the enrichment-culture technique of Van Baalen (1961) who succeeded in isolating a number of blue-greens from coastal marine environments, many from the Long Island Area. A specimen of *Enteromorpha* (5 g dry weight) was transferred into a 500 ml flask of sterile enrichment medium (Van Baalen's modification of Provasoli's (1957) ASP-2 medium, silicate omitted, nitrate and phosphate enriched, pH increased to 8.2) and incubated at room temperature ($25^{o}C$ maintained by air conditioning) in a light bank (16hr light/8 hr dark) with aeration. Three of these flasks were set up. Periodically solidified agar (2%) plates (Van Baalen's medium modified by addition of actidione, cyclohexamide, which inhibits eucaryotic growth; Table 2) were inoculated with 0.1 ml of the enrichment culture, sealed in Fisher Whirl-Pack bags (#18125B) and incubated in the light bank for up to three months (McCurdy

and Hodgson, 1973). After growth, algal colonies were picked and transferred. Unialgal cultures were easily obtained.

Plates of Van Baalen's medium (no actidione) were also inoculated in similar manner with 0.1 ml of the enrichment culture, sealed and incubated in the light at 35°C. Incubation at 35°C has been reported to suppress eucaryotic algal development, thus facilitating the isolation of blue-greens (Allen and Stainer, 1968).

Axenic cultures of the blue-greens were difficult to obtain because bacteria live in the gelatinous sheaths. Techniques for isolation tried included: 1) ultraviolet irradiation (Gerloff *et al*., 1950); 2) sonication (McCurdy and Hodgson, 1974); 3) antibiotics (penicillin and dihydrostreptomycin; (Pinter and Provasoli, 1958); 4) repeated subculturing on nitrogen free media (Table 2); and 5) differential growth and migration on solidified media (Allen and Stainer, 1968; Stainer *et al*., 1971). The latter technique proved to be most effective. Purity was judged by phase microscopy and by plating cultures on solidified basal medium (Lee *et al*., 1975) enriched with acidicase (1 g/liter) and yeast extract (1 g/l) and incubated in the dark.

Bourrelly's (1970) wet mount procedures for optical microscopy were used to identify the blue-green algae.

The population of blue-greens in the field was examined weekly. A small sample of *Enteromorpha* and its epiphytes (0.2 g dry weight) was aseptically inoculated into a test tube of 20 ml of sterile sea water and vigorously agitated to

dislodge the epiphytes (Lee _et al._, 1975). Appropriate volumes (10 to 100 ml) of diluted or undiluted epiphyte suspension were filtered through sterile Millipore HA filters (0.45 um pore size). Filters were then placed on plates of Van Baalen's Media with actidione(Table 2). McCurdy and Hodgson (1973) demonstrated that the membrane filter technique is superior to surface plating for blue-green enumeration because it permits larger sample size and restricts colony spreading. The plates were incubated in a light bank at room temperature (25°C) for up to three months. Blue-green colony counts were made using a dissecting microscope. The viable counts obtained are estimates of the relative numbers of blue-green population. But, this technique does not produce total or absolute counts, such data requires direct microscopic counts. The membrane filter technique produces only relative numbers because in order for a cell to be counted it must grow on the selected medium and because of the filamentous structure of the blue-green each colony does not represent growth from a single cell. Within this restriction the technique is useful in ecosystem studies and the method also permits isolation of blue-green cultures (McCurdy and Hodgson, 1973).

Blue-green algal vegetative growth in nutritional culture experiments was determined by direct cell counts according to the technique developed by Burnham _et al._ (1973) for counting filamentous algae and bacteria. The procedure is independent of filament length or individual cell size. Filaments were dispersed by sterile glass beads (1.0-1.5mm), then counts were made of cells (from any filament or part of

a filament) using a hemocytometer.

In anaerobic blue-green experiments oxygen-free conditions were established by the addition of sodium thioglycolate, a nontoxic reducing agent (0.5 g/l), to the culture medium and by replacing air in the culture vessels with 99.96% Ar/0.04% CO_2 (Brock and Brock, 1973). Tubes were sealed with gas-tight rubber stoppers.

Bacterial Studies

A modified version of Sugahara's (1974) nitrogen free marine bacterial medium was used for the enumeration and isolation of the bacterial nitrogen fixers. The original medium utilizes sucrose (0.058 M/l) as its sole carbohydrate source. The medium was modified by reduction of the amount of sucrose (0.01 M/l) and inclusion of four additional carbohydrates (0.01 M/l), fructose, glucose, lactose and mannitol fixing bacteria capable of utilizing sucrose, the modified medium promoted growth of nitrogen fixing bacteria capable of utilizing a variety of carbohydrates.

In order to obtain reasonable estimates of nitrogen fixing bacterial population, serial dilutions were used for inoculating plates with the epiphyte suspension. The plates were sealed and incubated in a light bank at room temperature. After growth, replica plating (Lederberg, 1952) was done to eliminate bacteria which grew by utilizing stored nitrogen and were incapable of nitrogen fixation. Cultures obtained by this procedure were tested for nitrogen fixation by the acetylene reduction technique. In addition, each of the 128 bacterial cultures

isolated by E. Kennedy (unpublished) using a variety of natural and artificial sea water media (Lee *et al*., 1975) were tested for ability to fix nitrogen.

The nitrogen fixing bacteria were classified on the basis of morphological and biochemical tests according to the scheme of Bergey's Manual of Determinative Bacteriology (1974). Biochemical tests were conducted as described by Skerman (1969). Carbohydrate reactions and protein reactions were tested by E. Kennedy, City College of New York.

In laboratory experiments with the nitrogen fixing isolated, media was adjusted to pH 8.0 at a salinity of $20^{o}/oo$ (optimum culture conditions for many marine microorganisms: (Provasoli *et al*., 1957) and close to conditions at Towd Point) when pH or salinity were not variables. For salinity experiments, the medium was slowly evaporated to $80^{o}/oo$ to prevent the formation of precipitates. The salinity of the evaporated media was measured by a Beckman induction salinometer (model RS-7B). Media was then diluted to lower salinities with distilled water. Nitrogen fixation was studied in media with a range of $5^{o}/00$ to $80^{o}/oo$. The pH tolerance was studied at 0.5 intervals from pH 4.0 to 9.5 and was measured on a Corning research pH meter (model 12). An illuminated aluminum gradient block similar to that developed by Thomas *et al*. (1963) was used in temperature studies. Test temperatures ranged from 10-40°C at 2°C intervals.

Scanning Electron Microscopic Studies

Samples of *Enteromorpha* were fixed (60 min) for electron

microscopical study in 2.5% glutaraldehyde diluted in sea water. The samples were then washed in three changes of sea water and dehydrated in a gradual series of alcohol baths. Isoamyl acetate was added as an indicator of completion for critical point drying. A Denton vacuum DCPI critical point dryer was used. Specimens were loaded into the drying chamber which was then flushed with liquid CO_2 at 900 psi. The CO_2 was bled off until isoamyl acetate odor (banana oil) was undetectable (5-10 min). The chamber was then full of liquid CO_2 which had displaced isoamyl acetate from the specimens. The chamber was immersed in 50°C water and as the chamber warmed, the pressure rose to 1650 psi; during the rise it passed through the critical point ($\sim$1060 psi). After drying the samples were attached to stubs using conducting silver paint (Ladd #6300), coated with platinum-carbon overlay (double coating) and viewed with a Kent Cambridge Sterioscan S4 at magnifications of 1400X and 3500X.

Nutrient Analysis

Once a week during the summer, samples of marsh water were collected in a clean beaker. Water for dissolved nutrient (nitrite, nitrate, ammonia, phosphate, silicate and organic nitrogen) analysis was filtered through Millipore (HA) filters. Samples were frozen and stored in carefully washed polyethylene bottles until analyses were performed. Analyses were done on a Technicon Auto Analyzer as described by Strickland and Parsons (1968). The instrument determines

nutrients by modifications of the sensitive spectrophotometric methods used in conventional sea water analysis, except that both sample and reagents flow continuously through the system. Nitrite was allowed to react with sulphanilamide in an acid solution. The resulting diazo compound reacted with N-(1-naphthyl)-ethylenediamine and formed a highly colored azo dye. Nitrate was reduced to nitrite on a column of copperized cadmium. Ammonia was reacted with hypochlorite to form chloramine, which, in turn, condensed with phenol to give an indophenol blue complex. Orthophosphate and silicate were converted to phosphomolybdic acid and silicomolybdic acid, respectively, by reaction with ammonium molybdate and acetic acid. Organic nitrogen was converted into ammonium sulfate by sulfuric acid; the solution was then made alkaline and ammonia liberated (Kjeldahl method). The ammonia was then analyzed as described above. When colored solutions were obtained by the chemical reactions just described, they were pumped through colorimeters. Transmission of a solution was measured by a pen recorder from which the concentration of a nutrient was evaluated after suitable standardization.

Water for particulate carbon and particulate nitrogen analysis was filtered through pre-combusted (2 hr at 200°C) Gelman glass fiber filters (#61631). The filters were then dried, weighed and analyzed on a Hewlett-Packard C-N-H analyzer (model 185) following the procedures detailed by Sharp (1974). The elemental analyzer combines a micro-Dumas controlled pyrolysis step with gas measurement on a thermal conductivity gas chromatograph. Flow rate of helium, the

carrier gas, was 110-120 ml/min and the combustion cycle was 20 sec. For organic C-N analysis the oxidation furnace temperature was set at 850°C and for total C-N analysis the oxidation furnace temperature was set at 1025°C. The analyzer was standardized with acetanilide (10.36% N, 71.09% C, 6.71% H). Weighed samples of acetanilide were analyzed under standard conditions to product reference curves. Linear curves were obtained by plotting mg C vs peak height and mg N vs peak height. The concentration of carbon and nitrogen in the experimental samples was ascertained by reference to the standard curves. Standards were run routinely each time the instrument was operated to check the standard curves.

Measurement of Field Environmental Conditions

Environmental conditions in the marsh were monitored weekly throughout the summer. Dissolved oxygen was determined by the Winkler method (Strickland and Parsons, 1968). Salinity was measured with a Beckman induction salinometer (model RS-7B). pH was measured in the field using a pH test kit (Carolina Biological Supply Co., #65-2925). Illumination was monitored with a Gowsen Luna-Pro light meter.

Enteromorpha Biomass in the Study Area

The biomass of *Enteromorpha* in the study area was estimated by quadrat sampling along transects. A wooden frame with an area of 1 m^2 was moved along the transects to facilitate sampling. The capture and release method was used in order to disturb the *Enteromorpha* population as little as possible.

The mean mass of *Enteromorpha* in the quadrat samples was then converted into a value (g wet wt/m^2) that estimated the amount present in the study area.

Multiple Regression Analysis

Stepwise multiple regression technique was used to analyze the data collected on the fourteen field trips during the summer. This analysis was carried out to find the relationship between nitrogen fixation (dependent variable) and all the other variables (ecological parameters) using the maximum degrees of freedom for each analysis (Horne and Goldman, 1972). The regression was performed in step-wise fashion and the independent variables entered into the regression in order of their importance from most to least important. The results of the analysis show which of the independent variables (temperature, salinity, pH, O_2, light, inorganic nitrogen, PO_4, *Enteromorpha* primary production, isolate B3711 (bacterial nitrogen fixer) population, isolate C11X (bacterial nitrogen fixer) population) best describes nitrogen fixation in the *Enteromorpha* community during the study period. Calculations were done by CUNY's IBM 360/50 using an available Statpack program, Step-wise Regression.

Experimental Microcosms

Six Laboratory model ecosystems were set up using BioFlo chemostats (New Brunswick Scientific Co., model C30). 450 g of glass beads (0.45-0.5mm) were placed on the bottom of each flow-through reaction vessel with 100 ml of sea water and

sterilized. Then 100 mg of radioactive detritus ^{14}C (Batch #4, specific activity: 34.72 μ Ci/mg) was mixed with 10 g (approximate dry wt) of unlabeled aged (76 days) detritus and placed on top of the glass beads.

Unlabled aged detritus was prepared in the following manner: 96 g of ground (#40 mesh) non-radioactive detritus was added to 1 liter of Millipore HA filtered sterile sea water (8.0 μm) allowed only fungal, bacterial and blue-green propagules to remain in the filtrate. The mixture was placed in a light bank at room temperature with aeration for 76 days.

Sterile sea water was pumped from the medium reservoirs to the reaction vessels (described above) into the product reservoir at a rate of 200 ml/day. Air bubbled through the reaction vessel into the product reservoir to a beaker containing phenethylamine to capture radioactive CO_2. The temperature was 24°-$25^{o}C$. Illumination was 12 hr light/12 hr dark cycle.

Reaction vessels 1 and 2 were set up exactly as described. Reaction vessels 3 and 4 differed only in that they were kept in the dark. Reaction vessels 5 and 6 were illuminated and were inoculated with 3 ml of ten stock algal cultures (Table 4).

After 3 months, samples of detritus were taken from each vessel for carbon-nitrogen analysis. All exit and entry tubes were sealed and 0.2 atm of acetylene was injected into each reaction vessel. After 24 hr, samples were taken of the atmosphere in each vessel and analyzed for ethylene.

III. Results

N_2 Fixation at Towd Point

In the Towd Point study area (354.88 m^2; Fig. 4) the macrophytic standing crop is seasonal. From late May to early August *Enteromorpha intestinalis* is the dominant macrophyte (Fig. 5). Nitrogen fixation was measured weekly in the *Enteromorpha* community throughout the summer of 1974. Values ranged from 0.15 to 14.40 µg N/g dry weight *Enteromorpha*/hr with a mean of 7.53 µg N/g/hr. Maximum fixation was observed during July (Fig. 6). The rate of nitrogen fixation associated with *Enteromorpha* incubated in the dark was not significantly different ($P<0.25$) from the rate of light incubated samples (Fig. 6).

Environmental Conditions at Towd Point

The amount of water exchanged between high and low tides ($\sim 3.14 \times 10^5$ l) was about 97.3% of the high tide volume. This tidal flux directly affected temperature and salinity in the marsh. During high tide, the water temperature of the study area was generally homogeneous and ranged from 24°C to 29°C during the summer (Table 4). However, at slack low tide thermal gradients of 5°C were often observed (Matera, 1972). Similarly, salinity was homogeneous at high tide ranging from 25.0°/oo to 31.1°/oo but gradients of 4°/oo could be observed at slack low tide (Table 5).

The marsh water was supersaturated with oxygen. The

capacity for water to hold oxygen depends upon salinity and temperature. In all cases the field value (6.4 - 16.5 ml O_2/l) exceeded the saturation value for the corresponding temperature and salinity (Fig. 7).

pH was mildly alkaline ranging from 7.8 to 8.4. Illumination varied from a low of 2000 lux to a high of 9800 lux (Table 5).

Chemical analysis of the inorganic nutrients within the marsh indicated that levels were high (Table 6) and thus do not seem growth limiting to the *Enteromorpha* community (Lee *et al*., 1975). Nitrate, nitrite, ammonia, phosphate and silicate were measured for three summers during ebb high tide. Mean values obtained were: NO_3^- 4.51 µg at N/l; NH_4^+ 4.66 µg at N/l; NO_2^- 0.49 µg at N/l; $PO_4^{\equiv}$ 1.93 µg at P/l; SiO_2 12.55 µg at Si/l. Dissolved organic nitrogen had a mean of 32.2 µg at N/l. The mean value for particulate organic nitrogen was 17.04 µg at N/l and for particulate organic carbon 192.55 µg at C/l.

Nutrient levels fluctuated from year to year and throughout the summer. In general, 1974 levels of inorganic nitrogen and phosphate were low and silicate was high in comparison to 1970 and 1973 levels (Table 5). Fig. 5 shows the weekly mean levels of NO_2^-, NO_3^-, NH_4^+ and $PO_4^{\equiv}$ for the summer of 1974. Ammonia was the dominant form of inorganic nitrogen and exhibited the greatest fluctuation in concentration. Ammonia levels were lowest (~1.7 µg at N/l) from late June through mid July.

Highest levels of ammonia (3-3.5 µg at N/l) were recorded during the second half of the summer. Nitrate (mean: 0.36 µg at N/l) and nitrite (mean: 0.26 µg at N/l) were present in low concentrations and only slight variations in concentration were observed during the summer. Phosphate levels (0.25 - 3.02 µg at P/l) fluctuated sharply during the course of the summer. A broad peak in phosphate level was observed during midsummer.

Other Sources of Combined Nitrogen

In order to estimate the amount of nitrogen entering the marsh from runoff (fresh water drainage) and transport from the inlet channel, nutrient levels were measured at five stations in the study area and two (6 and 7) in the channel which flows past the study area. Stations 1 and 2 are at the mouths of two small rivulets and were considered possible sites of runoff. Analysis of variance showed no significant difference ($P=0.25$) between the nitrite, nitrate and ammonia levels at the seven stations (Table 7). Therefore, there is no evidence of nitrogen influx from either land runoff or transport from the adjacent channel.

Analysis of rainfall collected in the study area gave the following mean values: NO_3^- 3.35 ug at N/l; NO_2^- 0.15 ug at N/l; NH_4^+ 3.1 ug at N/l. The amount of combined nitrogen entering the marsh through precipitation was calculated using these average values and the monthly levels of rainfall (Table 8).

Nitrogen Uptake

Carbon-nitrogen analysis revealed that the natural mature *Enteromorpha* community had a mean C:N ratio of 8:1 (n=10; range=5.3:1 to 9.6:1). Uptake of combined nitrogen was calculated from community (*Enteromorpha* + epiphytes) ^{14}C uptake data (Lee *et al.*, 1973; Fig. 9), assuming a C:N ratio of 8:1. Community nitrogen uptake increased gradually during May and reached a peak in July (2.4×10^2 mg N/g) before declining in August (Fig. 10; Table 8).

Blue-Green Algal Studies

The blue-green algal population declined rapidly from May to the beginning of August and then increased through September (Fig. 11). The enrichment culture technique combined with the use of cyclohexamide proved to be an effective method to isolate blue-green algae. On the other hand, incubation at 35°C failed to suppress eucaryotic algal development due to the ability of many of these organisms to tolerate high temperatures (Saks and Lee, 1972).

Identification of the blue-green cultures was difficult because the taxonomic literature emphasizes the gross morphological characteristics observed in natural samples (Stainer *et al.*, 1971; McCurdy and Hodgson, 1973). Accordingly the isolates were assigned to genera but could not be assigned to particular species as they are presently described. Ten non-heterocystous, non-branching filamentous blue-green algal isolates were obtained from the *Enteromorpha*

community. None of the isolates formed akinetes and all of the isolates reproduced by short filaments called hormogonia. Thus all of the isolates belong to the Family Oscillatoriaceae. They were classified as follows:

Oscillatoria - thin sheaths, both trichome and hormogonia motile, terminal vacuoles, non-granular cytoplasm
isolates: y,17,2

Lyngbya - firm sheaths, only hormogonia motile, with or without terminal vacuole, homogeneous or granular cytoplasm but not granular at cross walls
isolates: v,z,20,w,37^{h}

Pseudabanea - thin sheaths, both trichome and hormogonia motile, with or without terminal vacuole, non-granular cytoplasm, constricted at cross walls, cells not adjacent to each other
isolates: 37^{g},37^{d}

None of the isolates grew on nitrogen free media. Soon after transfer to nitrogen free media, the isolates exhibited nitrogen chlorosis (yellowing as the nitrogen containing pigments are utilized) and eventually died. The acetylene reduction technique failed to demonstrate nitrogen fixation by the blue-greens under aerobic conditions and this is consistant with other reports for these genera (Fogg *et al.*, 1973). The isolates were also tested under anaerobic conditions, since certain species of *Lyngbya* and *Oscillatoria* have been reported to fix nitrogen anaerobically (Kenyon *et al.*, 1972). However, the results were negative.

The nutritional requirements of the blue-green algae were also examined. 0.2 mM (0.2 mg at N/1) was the minimum level for growth on nitrate as the sole source of nitrogen (Fig. 12). Vegetative growth increased rapidly above the minimum level. Since nitrate assimilation was accompanied by an increase in pH (pH values of 9 were observed), growth decreased at high levels of nitrate. Isolates v, z, 37^h, 37^d and 37^8 grew poorly above 20 mM (20 mg at N/1) $NaNO_3$ but the remaining blue-greens (y, 17, w, 20, 2) grew well at ten times the concentration or 0.2 M (0.2 g at N/1) $NaNO_3$.

Nitrite also supported blue-green growth (1 X 10^3 to 1 X 10^4 cells/ml) when the concentration of $NaNO_2$ was between 2 μM (2 μg at N/1) and 20 mM (20 mg at N/1) (Fig. 13). The maximum growth (0.2 mM (0.2 mg at N/1) to 2 mM (2 mg at N/1) $NaNO_2$) was less than that observed when $NaNO_3$ was the sole nitrogen source.

Ammonia was a good nitrogen source over a wide range of concentrations (0.2 μM (0.4 μg at N/1) to 20 mM (40 mg at N/1) $(NH_4)_2SO_4$). Optimum growth levels (0.2 to 2 mM $(NH_4)_2SO_4$) were comparable to those supported by $NaNO_3$. As could be expected in a buffered media, ammonia assimilation was accompanied by a corresponding decrease in pH (to pH 5 after 1 month incubation). This restricted growth at high levels of ammonia (Fig. 14).

Bacterial Studies

No correlation was found between the number of bacteria isolated on nitrogen-free media and nitrogen fixation in

the Enteromorpha community. The nitrogen free media produced high colony counts ($\sim 1X10^5$ colonies/g dry wt Enteromorpha) but when the colonies were isolated none were able to fix nitrogen. This technique was employed for two summers without success. Carry over and high internal nitrogen pools enabled some isolates to survive five replica platings on nitrogen free media even though they were unable to fix nitrogen when tested with the acetylene reduction technique.

Only ten of the 128 bacterial clone cultures isolated from the Enteromorpha community fixed nitrogen: B16, B3P5, B3711, C11X, C13, C18, D21, D41 and D58 (the letter and first digit refer to the isolation medium (Lee et al., 1975)). Isolate B3711 fixed nitrogen at the fastest rate (8 g at N/g dry wt bact./wk). Under similar conditions, C11X fixed nitrogen approximately half as fast (4 g at N/g/wk). The other eight isolates fixed nitrogen at comparatively low rates (Table 9), often requiring long periods of incubation before ethylene production was detected by gas chromatography. The two cultures with the highest activity were chosen for physiological testing.

Only five of the ten isolates grew in deep agar butts indicating that five were aerobes and five were facultative anaerobes (Table 9). Since B16, C13, C18, D41 and D58 are facultative anaerobes which fix nitrogen only under low P_{O_2} or anaerobic conditions (Mulder, 1975), long incubation was probably necessary to reduce O_2 supply to a level that permitted fixation.

Table 10 lists the characteristics of the five aerobic nitrogen fixing isolates. B3711, C11X and B22 were identified as species of *Azotobacter*. B3P5 and D21 were identified as species of *Azomonas*.

Table 11 lists the characteristics of the five facultative anaerobic nitrogen fixing isolates. B16, C13 and C18 were identified as species of *Enterobacter* and D41 and D58 as species of *Klebsiella*.

Specific classification of the isolates would require further physiological and biochemical testing as well as the use of more specialized techniques such as serology, bacteriophage typing and genetic analysis, including measurement of G-C and DNA-DNA homology analysis (Buchanan and Gibbons, 1974).

Both B3711 and C11X were mesophiles having an optimum temperature range of 20-25°C for nitrogen fixation (Fig. 15). C11X had a temperature range of 10°-35°C, while B3711 had a slightly broader range of 10°-40°C. At 15°C which is close to the lower thermal limit of both bacteria, the two fixation rates were identical (1 g at N/g/wk). At 20°C, however, B3711 fixed 8 g at N/g/wk and C11X fixed only 3.4 g at N/g/wk. Near the upper thermal limit the rates converge again but B3711 has a slightly greater thermal tolerance. At 30°C B3711 fixed $\sim$0.75 g at N/g/wk more than C11X.

Both B3711 and C11X were alkalophiles with ph optimum of 8 for nitrogen fixation (Fig. 16). The two bacteria had identical ranges of pH tolerance (6.5 to 9.5). Throughout

the pH range, B3711 fixed more nitrogen than C11X but rates were quite close near the upper and lower limits of tolerance. At pH 8 (average pH in the natural community was 8.1), C11X fixed at approximately half the rate of B3711 (8 g at N/g/wk), while at pH 7.5, B3711 fixed 1.25 g at N/g/wk more than C11X and at pH 9 only 0.25 g at N/g/wk more. At neutral pH (7), the difference is only 0.125 g at N/g/wk.

The niches of the two bacteria overlapped in temperature and pH tolerance but they diverged in regard to salinity. B3711 was better adapted to fresh water; nitrogen fixing capacity decreased rapidly as salinity increased (Fig. 17). At 5°/oo fixation was 13.75 g at N/g/wk but at 45°/oo, which is near its upper saline limit, fixation was only 0.25 g at N/g/wk. C11X was a brackish water organism with a narrower range of salinity tolerance (5°/oo to 35°/oo). At 15°/oo, the optimum salinity for C11X, the two bacteria had almost identical fixation rates (8.5 g at N/g/wk). Above its salinity optimum, C11X was more sensitive to increased salinity than B3711. At 25°/oo, B3711 (5.7 g at N/g/wk) fixed almost six times as much as C11X (1 g at N/g/wk) and at 30°/oo, B3711 fixed approximately ten times as much.

The presence of combined inorganic nitrogen suppressed the fixation rates of both species (Fig. 18 & 19). Fixation by both bacteria was completely inhibited by 2 mM (2 mg at N/l) of nitrate, 20 µM (20 µg at N/l) of nitrite or 2 mM (4 mg at N/l) of ammonia. Below these levels, fixation was related to nutrient concentration. $NaNO_2$ was the least inhibiting form of combined nitrogen. At 0.02 µM (0.02 µg at

N/l) $NaNO_2$, fixation was unaffected, but when the concentration was increased to 0.2 µM (0.2 µg at N/l) $NaNO_2$, fixation fell to less than half. At 2 µM (2 µg at N/l) $NaNO_2$, the two organisms fixed at approximately the same slow rate (1 g at N/g/wk). Nitrogen fixation by both bacteria was more easily repressed or inhibited by NO_3^- or NH_4^+ than NO_2^-. Fixation by B3711 was more sensitive to nitrate than ammonia, while C11X was identically sensitive to both nutrients.

The population structure of the nitrogen fixing bacteria in the _Enteromorpha_ community was quite complex (Fig. 20). Changes in the species composition of the community was reflected in the numbers and types of nitrogen fixing bacteria growing on the isolation media. The number of nitrogen fixing bacteria growing on the isolation media fluctuated from 3.24 X 10^2/g dry wt _Enteromorpha_ in early summer to a peak of 5.01 X 10^5/g in early August. Isolates C13, C18 and D58 did not appear until late June. The other seven species were consistently isolated throughout the summer.

B16 (isolated on basal medium enriched with phosphate and amino acids) had a sigmoid growth curve; it rapidly increased in numbers from 20/g in mid May to a peak of 1 X 10^5/g in early August. This peak was maintained through early September at which time it became the most abundant species. The numbers of B22 isolated on basal medium enriched with phosphate and peptone steadily increased throughout the summer from a low of 4/g to a peak of 4 X 10^2/g. B3711 and B3P5 were both isolated on a phosphate and urea enriched medium but exhibited vastly

different growth trends. B3711 was the dominant bacteria through most of the summer with a peak of 3 X 10^5/g in early August after which it gradually declined. B3P5, however, maintained a fairly constant population level of 10/g throughout the sampling period.

Medium C1 (basal medium enriched with phosphate, nitrate and vitamin B_{12}) was the most successful medium for isolating nitrogen fixing bacteria (3 species). Isolates C13 and C18 did not appear in the population until late June. At the peak of their abundance, they were still rare organisms (10/g). C18 was present through September but C13 disappeared in mid August. C11X was unique in regard to seasonal trends. It was the most abundant bacterium at the beginning of the season (1.5 X 10^2/g). In early July it dropped to 40/g and remained constant throughout the rest of the summer.

Peak growth (9.5 X 10^2/g) of D22 (basal medium enriched with phosphate, nitrate, B_{12}, biotin, thiamine and glutamate) occurred in August and September. D41 (basal medium enriched with phosphate, nitrate, B_{12}, biotin, thiamine and glycerol) maintained a population level of 10/g all summer. D58 was isolated on basal medium enriched with phosphate, nitrate, B_{12}, biotin, thiamine and mannitol. D58 first appeared in late June and increased steadily until it peaked at 20/g in early August. It maintained that level through September.

Scanning Electron Microscopic Studies

Scanning electron microscopy (Fig. 21) revealed the presence of numerous rod-shaped bacteria, some of which might have been nitrogen fixing, attached to the surfaces of the *Enteromorpha*.

Multiple Regression Analysis

The relationship between N_2 fixation (g at N/g dry wt *Enteromorpha*) and specific environmental conditions was analyzed by a multiple regression technique which considers the influence of the independent variables (environmental conditions) on a single dependent variable (nitrogen fixation). Ten environmental factors were considered, seven of which were physical parameters (temperature, salinity, pH, light, oxygen, total inorganic nitrogen and phosphate) and three of which were biological parameters (*Enteromorpha* primary production, B3711 population and C11X population). The environmental variables were entered into the regression in order of their importance from most to least important. Results of the regression are shown in Tables 12 and 13.

The analysis showed that N_2 fixation was most significantly correlated (R^2=0.817) to the primary production (μMC/mg/hr) of the *Enteromorpha* community (*Enteromorpha* + epiphytes). The primary productivity data used in the regression was the ^{14}C uptake data from the summer of 1971. Lee *et al*. (1973) found that carbon fixation by *Enteromorpha* and its epiphytes gradually increased during

May and reached a peak (130 µMC/mg/hr) in early July before declining in August (Fig. 9). It was assumed that the seasonal pattern of primary productivity was the same in 1974.

The second most important environmental parameter was water temperature (°C). There was a significant positive correlation between nitrogen fixation and temperature. The third variable most closely related to N_2 fixation was oxygen (ml/l). This variable was also significantly positively correlated with nitrogen fixation. Light (lux) and salinity (°/oo) were significantly and negatively correlated with fixation. PH had a significant positive correlation with fixation. Phosphate (µg at P/l) was the seventh variable in terms of importance and was positively correlated with fixation but the correlation was of low significance (P=0.15). The eight and ninth variables, C11X population (colonies/g dwt Enteromorpha) and inorganic nitrogen concentration (µg at N/l), were negatively correlated with fixation but significance was low (P=0.15; P=0.20). The least important variable, B3711 population (colonies/g dwt Enteromorpha), was positively correlated but of low significance (P=0.15). The final multiple regression equation (Table 12) indicates a strong relationship between the environmental parameters tested and nitrogen fixation since these parameters explain 98.4% (R^2=0.984) of the variation in fixation.

Experimental Microcosms

Differences in the appearance of the reaction vessels

of the three sets of microcosms became apparent several days after inoculation. In the vessels with bacteria and blue-green algae incubated in the light, mats of bacteria and blue-greens built up on the sides of the vessels and on the surface of the detritus. Just below the surface of the glass beads, purple sulfur bacteria (possibly *Chromatium*) appeared. A layer of green sulfur bacteria (possibly *Chlorobium*) developed directly below the *Chromatium*. In the middle of the sediment a grey layer (pyrite, FeS_2, production) formed and near the bottom a black layer (monosulfuric iron sulfide production) developed (Jorgensen and Fenchel, 1974). The second set of microcosms (bacteria and blue-greens; dark) was distinguished by a lack of photosynthetic organisms. There was a buildup of bacteria on the walls and sediment surface but no blue-green algae. The photosynthetic purple and green sulfur bacteria were absent from the sediment; only the grey and black layers developed. In the third set of microcosms (bacteria, blue-greens and eucaryotic algae; light) the sediments were identical to those of the first set (procaryotes; light). However, they were easily distinguished by dense diatom growth on the sides of the vessels and on the detritus surface. Bacteria were included in this diatom mat but blue-greens were absent.

Detritus mineralization in the microcosms was measured after three months incubation (Fig. 22). The fresh dead *Spartina* contained 150 mg organic carbon/g dwt. After decomposition, organic carbon in the

detritus of the first set of microcosms (procaryotes; light) decreased 16.66% (125 mg OC/g dwt). The rate of carbon mineralization in the remaining two sets of microcosms was approximately twice as fast. In the second set of microcosms (dark; procaryotes only) there was a 32% decrease in organic carbon (102 mg OC/g dwt). In the microcosms with eucaryotic algae added (set III) there was a 34.66% decrease in organic carbon (99 mg OC/g dwt).

Fig. 23 summarizes the data on nitrogen gain in the *Spartina* detritus during this experiment. The fresh, dead *Spartina* contained 12 mg organic nitrogen/g dwt. There was a 51.6% increase in organic nitrogen (18.2 mg ON/g dwt) in the first set of microcosms (procaryotes; light). There was only a 33.33% increase (16 mg ON/g dwt) in the microcosms incubated in the dark. The greatest increase in organic nitrogen (73.3% or 20.8 mg ON/g dwt) was found in the third set (bacteria, blue-greens and eucaryotic algae; light).

The C:N ratio of incubated detritus decreased from 9.2:1 to 7.46:1 in the microcosms with eucaryotic algae; indicating an increase in potential food value. In the first set of microcosms (procaryotes; light) the C:N ratio remained virtually the same (9.24:1). There was an increase in C:N ratio (11.58:1) in the second set of microcosms (procaryotes; dark).

Fig. 24 shows the rates of nitrogen fixation in the microcosms after three months incubation. Fixation was

highest (70 g N/day/microcosm) in the microcosms where photosynthetic and heterotrophic procaryotes grew (set I). Without photosynthetic organisms (set II), fixation fell to only 3 g N/day/microcosm. The addition of eucaryotic algae (set III) supressed the rate of nitrogen fixation to only 7 g N/day/microcosm (2.3 times the rate of set II).

IV. Discussion

Rate of Nitrogen Fixation

In contrast to Renault *et al.* (1975) who found no nitrogen fixation associated with *Enteromorpha intestinalis* in Morocco, a high rate of fixation was observed in the Towd Point *Enteromorpha* community; higher than rates reported in association with other marine macroalgae. The *Enteromorpha* bacterial epiphytes fixed nitrogen (14.4 µg N/g dwt *Enteromorpha*/hr) at approximately twice the rate reported for *Codium fragile* (7.3 µg N/g dwt/hr (Head and Carpenter, 1975)) and about four times the rate observed for *Microdictyon* sp. (3.8 µg N/g dwt/hr (Capone *et al.*, 1977)).

Significance of Nitrogen Fixation

One way of determining the significance of the fixed nitrogen is to compare it to nitrogen entering the community through other sources, i.e., precipitation, runoff and transport from adjacent water masses.

Precipitation

Junge's (1958) concise survey of the observations of the trace-substance content in rain included a summary of data on ammonia and nitrate content of rainfall at coastal stations around the world. In most nitrate analysis, nitrite was included. The mean ammonia concentration was 8.3 µg at N/1 and the mean nitrate level was 4.7 µg at N/1. At

Towd Point the ammonia level was only 3.1 µg at N/l but this is consistent with other U.S. rainfall analysis. Haines (1975) reported ammonia levels of 0.7, 2.5 and 8.0 µg at N/l in rainfall along the Georgia coast. The Towd Point nitrate + nitrite value of 3.5 µg at N/l agrees well with Junge's mean of 4.7 µg at N/l.

During July the new nitrogen fixed in the marsh *Enteromorpha* community is thirteen times the amount entering through rainfall (Table 8). In September when the *Enteromorpha* population was declining, the situation is reversed; rainfall contributes four times the amount provided by fixation. Taking the summer as a whole, fixation contributed 4.67 times more nitrogen per hour than rainfall.

Freshwater Runoff

Freshwater runoff could not be demonstrated since there was no significant difference between the nutrient levels at stations 1 and 2 and the rest of the marsh water (Table 7). Sites 1 and 2 were not areas of true runoff and actually represented tidal flush. No water flowed into the marsh from these sites at slack tide and at low tide the sediment in these areas was often exposed. The land behind the marsh was relatively level and densely populated with *Spartina*. Nutrient inputs with the exception of phosphorous are not sufficient to meet the production rate of *Spartina* and *S. alterniflora* production is apparently nitrogen limited in the high marsh (Hanson, 1977a). Therefore, any nutrients released by leeching would be rapidly taken up by the *Spartina*. Also, the adjacent land is part of a wild life

preserve which would preclude the use of fertilizers and the dumping of sewage.

Transport From the Channel

Transport of nutrients from the adjacent channel could not be demonstrated, since the nutrient levels at stations 6 and 7 were not significantly different from the water inside the marsh (Table 7). The channel water and the marsh water are well mixed by tidal flush. The entire channel is surrounded by dense marsh which would raise the nutrient level of the channel water. Therefore, it is unrealistic to expect to find different levels of nutrients in the channel and in the marsh and to quantitatively measure the nutrient flow.

Nitrogen Uptake

Another way to gauge the importance of nitrogen fixation, is to compare fixation with nitrogen uptake. Nitrogen uptake by the *Enteromorpha* community as calculated from the C:N ratio (8:1) and ^{14}C uptake data (Lee *et al*., 1973) was very high. Head and Carpenter (1975) estimated *Codium* uptake as 1.04×10^{-1} mg N/g dwt/hr. *Enteromorpha* community nitrogen uptake ranged from 75 to 195 mg N/g dwt/hr (Fig. 6). The maximum rate of nitrogen fixation (1.08×10^{-1} mg N/m^2/hr) would provide only 0.055% of the hourly nitrogen requirement during July. This comparison probably underestimates the importance of nitrogen fixation, according to Head and Carpenter (1975), because nitrogen fixation represents a new source of nitrogen which may be repeatedly recycled by

the processes of excretion, cell lysis and grazing (Jones and Stewart, 1969 ; Mauge and Burris, 1972).

Since this nitrogen uptake was calculated from ^{14}C uptake by the *Enteromorpha* community, it includes nitrogen uptake by the algal epiphytes as well as by the macrophyte. Just how much of this nitrogen is required by the *Enteromorpha* and how much is required by the algal epiphytes is uncertain. Lee *et al*. (1973) found that when the epiphytes were incubated by themselves, they fixed much more carbon than the undisturbed *Enteromorpha* community for most of the summer. *Enteromorpha* by itself fixed slightly more than the mixed community (Lee *et al*., 1973; Fig. 9). The fact that the macrophyte and its algal epiphytes are more productive alone than they are together is probably due to increased surface area, circulation of nutrients and decrease in shading. Since the data indicates that each of these community members fix more carbon alone than they do together, this data cannot be used to compare photosynthetic rates under natural conditions in a mixed community. Therefore, the only nitrogen uptake calculated was that of the mixed community.

Bound Nitrogen

The level of bound nitrogen in the community can also be used to assess the importance of nitrogen fixation. Assuming a 14 hr day and maximum fixation, it would take about a month (33.6 days) to double the nitrogen content of *Enteromorpha* (5.07×10^1 mg N/m^2) in July. This relationship is significant when compared to the actual growth rate; *Enteromorpha* biomass doubles between mid June and mid July

(Fig. 5).

Fate of Fixed Nitrogen; Symbiosis

The fate of the fixed nitrogen is an important consideration when analyzing the importance of the fixation process. *Azotobacter* and presumably all of the other free living bacteria use the main part of fixed nitrogen for cell synthesis (Mulder, 1975). But 7 to 13% of the total amount of nitrogen fixed by *Azotobacter* is excreted by the cells (Brotonegoro 1974). This fixed nitrogen is liberated in the form of ammonia and amides (Newton *et al.*, 1953). Jones (1974) demonstrated that ^{15}N labeled products of bacterial nitrogen fixation were directly utilized by salt marsh plants. This transfer of nitrogen may be via extracellular products but it may be also via grazing and cell lysis. Since nitrogen is the principal limiting nutrient for primary production, any nitrogen provided by fixation could stimulate *Enteromorpha*. In addition, Mishustin and Shilinkova (1971) concluded that for some plants *Azotobacter* provides a stimulant not related to nitrogen fixation and the subsequent release of nitrogenous compounds.

Reinke (1903) suggested that *Azotobacter* may have a symbiotic relationship with its host plant; the algae supply the bacteria with carbohydrates as a source of energy and the algae use nitrogen fixed by the bacteria. Prior to this study, the only known example of such a relationship was with *Codium* and its epiphytes. The addition of glucose stimulates nitrogen fixation by its bacterial epiphytes (Head and Carpenter, 1975).

Enteromorpha and its epiphytic community is very productive; the annual net production is ~0.5 to 1 gC/m^2/yr or ~440 gC/yr in the Towd Point marsh (Lee *et al.*, 1973). *Enteromorpha* in the Black Sea released ~30% of dissolved organic matter/m^2 or ~26% of its annual gross production (Khailov and Burlakov, 1969). In addition, experiments with axenic cultures of prominent algal *Enteromorpha* epiphytes demonstrated excretion of hexoses and pentoses (Saks *et al.*, 1976). The nitrogen fixing isolates are able to utilize a wide range of carbohydrates (Tables 10 and 11) and could assimilate these extracellular carbohydrates. Field studies have demonstrated rapid glucose and lactose uptake by the bacterial epiphytes (Lee *et al.*, 1973). Half of the nitrogen fixing bacteria utilize lactose and all except two (C18 and D58) metabolize glucose (dextrose) (Tables 10 and 11). So the glucose and lactose uptake data could indicate uptake by the nitrogen fixing bacteria. One of the primary requirements for nitrogen fixing bacteria is the presence of adequate amounts of assimilable carbon compounds (Mudler, 1975). The carbon excreted by *Enteromorpha* and its algal epiphytes undoubtedly helps satisfy this requirement. Jones (1974) concluded that the limiting factor for salt marsh bacterial nitrogen fixation was the availability of carbohydrates. The excretion of carbohydrates could explain why nitrogen fixation occurs in the *Enteromorpha* and *Codium* communities but is absent from the marsh water column (Whitney *et al.*, 1975).

There is no direct experimental evidence that the

Enteromorpha community at Towd Point provides the reducing ability for the nitrogen fixing bacterial epiphytes. However, the feasibility of this theory can be tested by comparing rough estimates of Enteromorpha community carbon excretion and nitrogen fixing bacterial carbon requirements. Calculations were based upon the following assumptions: 1) Enteromorpha community primary production = 1.82 X 10^6 µg C/g/hr (Lee et al., 1973); 2) Enteromorpha community carbohydrate excretion = 26% of primary production (Kahailov and Burlakov, 1969); 3) nitrogen fixation = 14.4 µg N_2/g/hr; 4) efficiency of nitrogen fixation = 10 mg N_2/g glucose or 1 µg N_2/40 µg C (Mulder, 1975). Enteromorpha community carbohydrate excretion was estimated at 4.7 X 10^4 µg C/g/hr and the nitrogen fixing bacterial carbohydrate requirement was estimated at 5.76 X 10^2 µg C/g/hr. If these assumptions are valid, the Enteromorpha community excretes about eight times the carbon required for maximum fixation and only a very small percentage (0.03%) of the carbon fixed by the Enteromorpha community is required by the bacterial epiphytes to fix atmospheric nitrogen. Therefore, the Enteromorpha community could feasibly provide the carbon needed for nitrogen fixation, but differences in epiphyte population, physiological condition and environmental conditions make such extrapolations uncertain.

Multiple regression analysis provided additional evidence of a symbiotic relationship between Enteromorpha and its nitrogen fixing bacterial epiphytes. A significant positive correlation was found between primary production and nitrogen fixation in the Enteromorpha community. Primary production

was the most important of the ten environmental parameters tested in multiple regression. The data on primary productivity of *Enteromorpha* by itself and of the epiphytes alone did not enter into the regression analysis. This data cannot be used to estimate relative rates of photosynthesis in the mixed community since the data indicates that each of these two community members alone fix more carbon than they do together.

Marsh Nitrogen Budget

While the results of this study indicate that nitrogen fixation is important for the development of the *Enteromorpha* community, it is difficult to extrapolate the value of fixation associated with *Enteromorpha* to the nitrogen budget of the whole marsh. A salt marsh is a very complex, dynamic ecosystem and construction of a nitrogen budget would require measurement of all major nitrogen pools or standing stocks and estimation of fluxes between these pools or compartments as well as nitrogen fluxes into and out of the marsh. No such values for a salt marsh are yet available and such quantitation is beyond the scope of this study.

However, Fig. 25 presents a partial conceptualization of the nitrogen cycle in the Towd Point marsh. Runoff and transport from the channel were assumed to be insignificant since nutrient analysis failed to demonstrate nitrogen input from these sources. Particulate organic nitrogen (2.16×10^2 mg N/m^2), dissolved organic nitrogen (4.09×10^2 mg N/m^2)

and inorganic nitrogen (1.23×10^2 mg N/m^2) are mean values for 1970, 1973 and 1974. Nitrogen fixation, bound nitrogen and precipitation were calculated using maximum rates and assuming maximum *Enteromorpha* biomass.

This model estimates the quantity and quality of nitrogenous nutrients available to marsh organisms at any given moment. But, the data can be deceptive because it does not reflect the turnover rate of compounds. For example, the model indicates that N_2 fixation associated with *Enteromorpha* could double the inorganic nitrogen pool in one month assuming a 14 hr day, but the rate at which inorganic nitrogen is being produced and utilized in the marsh is unknown. Therefore, the significance of the fixed nitrogen to the inorganic nitrogen pool is unknown.

The contribution of fixation to the marsh budget cannot be assessed without measurement of denitrification. No denitrification occurs in the *Enteromorpha* community because oxygen levels are high. But, denitrification occurs in the marsh sediments and it is an important component of the marsh nitrogen cycle (Kaplan, *et al.*, 1977).

The Towd Point marsh lacks blue-green algal mats and *Codium* sp. is rare. But other potential sources of nitrogen fixation are present: sea grass epiphytes, *Spartina* mud and marsh sediment (Goering and Parker, 1972; McRoy *et al.*, 1973; Hanson, 1977a; Whitney *et al.*, 1975). Fixed nitrogen entering the marsh from these sources must be quantified in order to get a true estimate of the value of fixation associated with *Enteromorpha*.

It is difficult to measure total nitrogen flux in a salt marsh and no such values are yet available (Horne, 1977). However, nitrogen fixation values per unit area of a salt marsh have been estimated. With reservations, it seems fair to assume that for analytical purposes the data from a comparable marsh can be used for general extrapolation. Whitney _et al_. (1975) reported a mean nitrogen fixation rate of 0.1926 mg $N/m^2/hr$ for sediment in Flax Pond, a salt marsh on the north shore of Long Island. These workers compared this fixation value with the total flux of nitrate nitrogen on each tide (1.3 to 5.4 kg) and concluded that sediment nitrogen fixation was an important segment of a large nitrogen budget. During July, nitrogen fixation associated with _Enteromorpha_ at Towd Point (0.108 mg $N/m^2/hr$) was comparable to the sediment fixation rate at Flax Pond (0.1926 mg $N/m^2/hr$). Therefore, at its peak fixation by _Enteromorpha_ epiphytes may be as important as sediment fixation to the marsh nitrogen budget.

Agents of Fixation

Blue-Green Algae

No nitrogen fixing blue-green algae were found in the _Enteromorpha_ epiphytic community. This is similar to that observed with _Codium_ (Head and Carpenter, 1975) but in contrast to the situation in _Microdictyon_ sp. (Capone _et al_., 1977). Perhaps the differences are climatic, _Codium_ and _Enteromorpha_ grow in temperate coastal waters while _Microdictyon_ grows in coral reefs. All three of the genera

(*Oscillatoria*, *Lyngbya* and *Pseudanabaena*) isolated from the *Enteromorpha* community are common to salt marshes (Fogg *et al.*, 1973). None of the isolates were able to fix nitrogen aerobically, an observation consistent with other reports (Fogg *et al.*, 1973). Certain species of *Lyngbya* and *Oscillatoria* have been reported to fix nitrogen anaerobically (Kenyon *et al.*, 1972) but the *Enteromorpha* isolates were unable to do so. The ecological advantage of anaerobic fixation in an environment super-saturated with oxygen is hard to imagine.

Nutritional studies suggest that the blue-green isolates have higher optimum nitrogen requirements than the levels observed in the field. Based on field samples taken in 1970, 1973 and 1974, summer nutrient levels ranged from: 0.15 to 33.10 µg at N/l NO_3^-; 0.15 to 1.90 µg at N/l NO_2^- and 0.15 to 11.10 µg at N/l NH_4^+. Optimum growth of blue-greens in culture occurred at 0.02 to 0.2 g at N/l NO_3^- or 0.2 to 2.0 mg at N/l NO_2^- or 0.4 to 4.0 mg at N/l NH_4^+.

Bacteria

Of the 128 clones of bacteria isolated from the *Enteromorpha*, ten (7.8%) were able to fix nitrogen. In contrast, only 41 types of bacteria were isolated from *Codium*, four of which fixed nitrogen. The difference in numbers may be due to differences in media and technique but it could also be due to actual differences in epiphytic bacterial populations.

The bacterial assemblage of the *Enteromorpha* community is highly complex and contains a variety of genera (*Azotobacter*, *Azomonas*, *Enterobacter* and *Klebsiella*)

capable of fixing nitrogen in marine environments particularly in sediments (Werner *et al*., 1974; Herbert, 1975; Lakshmanaperumalsamy, 1975).

Three of the aerobic N_2 fixing isolates (B22, B3P5, D21) fixed only trace amounts of nitrogen at average field conditions, pH 8, $20^{o}/oo$, $25^{o}C$ (Table 9). Fixation rates were so low that ethylene production was barely detectable. The fixation rates of B3711 and C11X (*Azotobacter*) were much higher. These two bacteria were chosen for physiological testing because they are probably the most important nitrogen fixers in the field.

The data on the physiological requirements of clones B3711 and C11X suggests that abiotic aspects of the niches overlap in regard to temperature, pH, O_2 and combined inorganic nitrogen concentration but diverge sharply in salinity tolerance (Table 14). Conditions in the field are not optimum for fixation by either isolate, but they are within the tolerance range of both species.

Factors Affecting Fixation

Light

There was no significant difference in nitrogen fixation between *Enteromorpha* communities incubated in the light and those incubated in the dark. Since light is required by photosynthetic nitrogen fixers, the lack of difference between light and dark incubation could indicate that fixation is heterotrophic (Stewart, 1965). Heterotrophic fixation, however, may also decrease in the dark if it is

symbiotically linked to photosynthetic organisms (Head and Carpenter, 1975). But, the one hour incubation period was too short to observe this symbiotic effect (Head and Carpenter, 1975).

A significant negative correlation was shown between nitrogen fixation and light, indicating that the nitrogen fixers are not photosynthetic and supporting the conclusion that blue-greens are not the agents of fixation (Tables 12 and 13). Light ranked fourth in terms of importance out of the ten environmental parameters tested. This high correlation was probably due to the interaction between light and other variables such as temperature and salinity.

Oxygen

The *Enteromorpha* community is supersaturated with oxygen (Fig. 7; Table 14). In all probability the isolates belonging to *Klebsiella* and *Enterobacter* do not fix nitrogen in the *Enteromorpha* community during the summer, since nitrogen fixation by organisms of the *Enterobactereaceae* occurs only under anaerobic conditions of at a low P_{0_2} (Mulder, 1975).

Multiple regression analysis showed a significant positive correlation between nitrogen fixation and oxygen concentration, indicating that the agents of fixation are aerobic. Oxygen level ranked third in terms of importance out of the ten environmental variables tested. But, this high correlation was probably linked either directly to the primary production (the most important environmental factor)

or the release of organic metabolites from the primary producers. Being reductive the nitrogen fixing reaction is anaerobic, but oxygen is required by aerobic nitrogen fixing bacteria for ATP formation. These bacteria solve the problem by excluding oxygen from the site of fixation. The *Azotobacter* possess a number of features which enable them to accomplish this: nitrogenase is associated with a membrane; the cytoplasm contains 0_2 resistant particles (confirmational protection); augmented respiration removes excess 0_2; slime and large cell size (small surface area) impedes 0_2 uptake (Dalton and Prostgate, 1969; Brotonegoro, 1974; Mulder, 1975).

Temperature

The effects of temperature on nitrogen fixation in marine environments are variable. Thermal optima have been found for nitrogen fixation in the sediment of Tokyo Bay (30°C), Sagami Bay (20°C) and Rhode River estuary (20°C) (Maruyama *et al*., 1974; Marsho *et al*., 1975). In contrast, Whitney *et al*. (1975) found that heterotrophic fixation in blue-green mats at a Long Island salt marsh was insensitive to diurnal temperature changes: night temperatures (17 - 18°C) were 15 - 18°C lower than day temperatures. As might be expected by extrapolation from the majority of reports, *Enteromorpha* isolates B3711 and C11X grew well within the temperature ranges observed in the field, and their highest fixation rates occurred at temperatures found during most of the summer (20° - 25°C) (Tables 5 and 14). Multiple regression analysis of the 1974 field data provided additional confirmation

of the positive relationship between temperature and nitrogen fixation in the *Enteromorpha* community. Water temperature was the second most important of the ten environmental parameters analyzed (Tables 12 and 13).

pH

Those isolates of *Azotobacter* which have been studied, fix nitrogen in neutral or alkaline environments (Mulder, 1975). Maruyama *et al*. (1974) reported that fixation in the sediment was highest at pH 8 but that it occurred over a wide range (pH 5 to pH 10). Isolates B3711 and C11X were also physiologically well adapted to fix nitrogen over a wide pH range (pH 6.5 to pH 9.5). Both fixed maximally at pH 8. The level of pH in the field was very close to optimal with a range of pH 7.8 - 8.4. Optimal pH was observed during five of the fourteen field trips in 1974 (Table 5). Multiple regression analysis showed a significant positive correlation between fixation and pH which was the sixth variable in terms of importance (Tables 12 and 13). The effect of elevated pH may be indirect (Torrey and Lee, 1976). Photosynthesis removes CO_2 from the water, reducing the amount of carbonic acid and H^+ ions and increasing pH.

Salinity

Nitrogen fixing bacteria of both terrestrial and marine origins have been found in the marine environment. Maruyama *et al*. (1974) concluded that fixation in marine sediment was due to marine bacteria since fixation increased with increased salt concentration. Anaerobic fixation was optimal at 20 - $30^{o}/oo$ and aerobic fixation was optimal at 30 - $40^{o}/oo$. The

four *Azotobacter* strains isolated by Lakshmanaperumalsamy *et al*. (1975) from marine sediments were also identified as marine bacteria because they fixed nitrogen optimally at a salinity of $30^{o}/oo$. In contrast, the 30 strains of *Azotobacter* and 9 strains of *Klebsiella* and *Enterobacter* isolated by Hubert (1975) from Kingoodie Bay, Scotland did not fix nitrogen in the presence of salt or sea water. Judging from its salinity tolerance, isolate B3711 was best adapted to fresh water but it was halotolerant since it fixed nitrogen in inverse proportion to salinity up to $50^{o}/oo$. Clone C11X was a brackish water bacterium with an optimum salinity of $15^{o}/oo$. It was more sensitive to salinity changes than B3711 and had a tolerance range of only 5-$35^{o}/oo$. Salinity at the Towd Point marsh was not optimum for either bacterium. But the natural salinity range observed in the field (25-$31^{o}/oo$) was below the upper limit of tolerance of both isolates. Multiple regression analysis showed a significant negative correlation between fixation and salinity which was the fifth variable in terms of importance (Tables 12 and 13). *In situ* nitrogen fixation decreased as salinity increased. Thus, the field data corroborates the salinity sensitivity observed in axenic lab experiments.

Combined Nitrogen

Nitrogen fixation was repressed by the presence of combined nitrogen; this was expected since fixing nitrogen is energetically costly. Repression by combined nitrogen has been demonstrated before in axenic cultures (Brotonegoro, 1974; Wilson *et al*., 1943) and in salt marshes (Van Raalte

et al., 1974; Head and Carpenter, 1975). Isolates B3711 and C11X were almost identical in their physiological responses to combined inorganic nitrogen (Fig. 18 and 19). Nitrogen fixation was completely inhibited by 2 mg at N/1 NO_3^- or 2 X 10^{-2} mg at N/1 NO_2^- or 4 mg at N/1 NH_4^+. These critical levels exceed the natural concentrations observed during the summer of 1974 (1.0 to 13.19 μg at N/1 NO_2^-+NO_3^- +NH_4^+). Analysis of field data often shows an apparent, though not necessarily significant, inverse relationship between inorganic nitrogen concentrations and nitrogen fixation (Horne and Goldman, 1972; Horne and Fogg, 1970). This situation was found for nitrogen fixation in the *Enteromorpha* community. A negative correlation between inorganic nitrogen concentration and nitrogen fixation was shown by multiple regression analysis but it was not significant (Table 12). A similar and significant inverse relationship between nitrogen fixation and combined nitrogen concentrations (NO_2^-+NO_3^-+NH_4^++urea) has been found in the *Codium* community (Head and Carpenter, 1975).

Field experiments conducted by Hanson (1977b) suggested that nitrogen enrichment indirectly stimulated nitrogen fixation associated with *Spartina alterniflora* roots by enhancing *Spartina* production and root exudation. A similar increase in *Enteromorpha* production could counteract the inhibitory effect of combined nitrogen on fixation. This could explain the low significance of the negative correlation between the level of combined nitrogen and nitrogen fixation in the field.

Phosphate

A weak positive correlation (P=0.15) between phosphate concentration and nitrogen fixation was found. No laboratory experiments were carried out to study the effect of phosphate levels on fixation by isolates B3711 and C11X. *Azotobacter* spp have high phosphorous requirements; this is partially due to their high metabolic activity (Mulder and Brotonegoro, 1974). Lees and Postgate (1973) demonstrated that adequate phosphate supply is very important for *Azotobacter* in the presence of high O_2 levels. Thus, the positive relationship between phosphate and N_2 fixation by *Azotobacter* spp associated with *Enteromorpha* corroborates the data of other workers.

Population of Nitrogen Fixing Bacterial Isolates

Nitrogen fixation and the populations of isolates B3711 and C11X were also weakly correlated (P=0.15). While isolate C11X was negatively correlated, a positive correlation between the population density of isolate B3711 and fixation was found. This indicates that isolate B3711 is probably the most important nitrogen fixer in the *Enteromorpha* community. However, the inclusion of this variable in the regression did not increase the percent of explained variation in nitrogen fixation.

N_2 Fixation and *Spartina* Decomposition

Models of ecosystems are of necessity generalizations and simplifications of complex natural systems. These inherent qualities prevent direct extrapolation of results to field conditions. But the microcosm approach may provide valuable

insights not obvious in natural multivariant systems.

The results of the microcosm experiments (Table 15) were in agreement with the data of previous workers who found a build-up of organic nitrogen, microbial protein, associated with the mineralization of carbon (Harrison and Mann, 1975; Gosselink and Kirby, 1974).

This study indicates that nitrogen fixing organisms associated with the decaying Spartina may contribute significantly to the observed increase in nitrogen of the detritus. Similar results were reported for decomposition of Red Mangrove leaves by Gotto and Taylor (1976) who estimated that approximately 2/3 of the nitrogen fixation was photosynthetically driven. In the microcosm experiments with Spartina decomposition, photosynthetic procaryotes, purple sulfur bacteria (Chromateacae); purple non-sulfur bacteria (Rhodospirillaceae); green sulfur bacteria (Chlorobiaceae) and blue-green algae, were responsible for most of the fixation.

The addition of eucaryotic algae had a depressing effect upon nitrogen fixation; possibly by competing with the photosynthetic procaryotes for essential nutrients (low molecular weight substrates, phosphorous, vitamins, metals, etc.) or by efficiently recycling available nitrogen thus decreasing the need for new nitrogen. This latter possibility is suggested by the fact that the reaction vessels with eucaryotes had a greater increase in organic nitrogen than the vessels with photosynthetic and heterotrophic procaryotes alone; even though nitrogen fixation without the

eucaryotic algae was ten times higher. The microcosm with procaryotes and eucaryotes was the only system to have a decrease in C:N ratio or increase in food value, indicating the beneficial effect of the eucaryotic algae. Therefore, while nitrogen fixation probably accounts for some of the observed nitrogen increase in *Spartina* detritus, the rate of nitrogen fixation cannot be used to predict the increase in detrital food value.

The sterile sea water flowing through the experimental microcosms was collected at Towd Point. Nitrogen fixation was detected in all the reaction vessels; this indicates that ambient concentrations of dissolved nitrogen at Towd Point are insufficient for *Spartina* decomposition. Thus, nitrogen fixation is probably of considerable importance to the marsh detrital system.

Study Limitations and Future Goals

The present study has obvious limitations and leaves many questions unanswered. To obtain a valid conversion factor for extrapolating C_2H_2 reduction to actual N_2 fixation, parallel ^{15}N tracer experiments are required. Instead, this study utilized the theoretical ratio 3:1 which is based upon a 6 e^- transfer for conversion of N_2 to $2NH_3$ and a 2 e^- transfer for reduction of C_2H_2 to C_2H_4. An experimental value of 3:1 is seldom found (Burris, 1974). Nevertheless, utilization of the customary 3:1 ratio can provide extremely good estimates (Hanson, 1977b).

More extensive field data is desirable before estimating

the total amount of nitrogen fixed during the summer by the Enteromorpha epiphytes. No attempt was made to assess the effects of the tidal cycle on fixation or to determine if there was any diurnal variation in fixation rates.

Multiple regression analysis does not prove a cause and effect relationship. But the analysis was useful in providing confirmation of physiological laboratory data. One weakness of the analysis was that historical effects could not be considered. There was no way to ascertain how past conditions may have influenced the observed fixation rate. An ideal analysis would allow for a lag period and luxury nutrient consumption (Torrey and Lee, 1976).

The high correlation between the primary production of the Enteromorpha and nitrogen fixation suggests symbiosis. But both variables are influenced by abiotic environmental parameters and this may partially account for the strong correlation between the two. The significance of the analysis is also limited by the fact that the carbon fixation and nitrogen fixation were not measured simultaneously. Conclusive evidence regarding symbiosis could be obtained in tracer experiments with ^{14}C and ^{15}N using axenic Enteromorpha and isolates B3711 and C11X.

The possibility of a specific relationship between Enteromorpha and its nitrogen fixing epiphytes remains to be investigated. Azotobacter paspali, for example, is restricted to a few ecotypes of Paspalum notatum, a South American subtropical grass (Dobereiner et al., 1972). A similar association might exist between Enteromorpha and

its Azotobacter epiphytes, B3711 and C11X.

The nitrogen fixing bacterial isolates should be identified to species. Species identification can be determined on the basis of mathematically analyzed overall similarities in morphological, biochemical and physiological characteristics. The most serious disadvantage inherent in the use of numerical taxonomic analysis is the relatively large number of tests required (Skerman, 1967).

It would be interesting to compare the characteristics of the Enteromorpha - Azotobacter isolates and the Codium - Azotobacter isolates. If the same species are found on both macroalgae, then locally adapted populations, ecotypes, may exist. Such compensation may involve genetic strains or merely physiological acclimation. reciprocal transplantation might be an effective experimental approach.

In this study the population of bacterial nitrogen fixers was estimated by viable counts obtained with the spread plate method. Subsequent work could include direct counts using the immunoflorescence technique (Daley and Hobbie, 1975). The specific population of each nitrogen fixing isolate could be quickly and easily traced spatially and seasonally through the Enteromorpha community. Studies could be undertaken to examine the relationship between the rate of fixation and the population density of the nitrogen fixers.

The efficiency of nitrogen fixation by the Enteromorpha isolates remains to be studied. The efficiency of nitrogen fixation is calculated as the amount of nitrogen

fixed per g of carbon compound consumed. Some values have been calculated by Mulder (1975): *Klebsiella* fix ~5 mg N/g glucose and *Azotobacter* fix ~10 mg N/g glucose. In the case of *Azotobacter* efficiency of nitrogen fixation depends largely on oxygen supply. At high pO_2 a large amount of substrate is respired to exclude O_2 from the nitrogenase system (respiratory protection) and less is available for cell synthesis and nitrogen fixation. *Azotobacter* cultures fixed 46.5 mg N/g glucose when pO_2 was reduced to 0.01 atm (Mulder and Brotonegoro, 1974). Mishustin and Yemtsev (1975) studied *Clostridium* species isolated from a variety of different soil habitats and found a variety of different ecological strains with different efficiencies of nitrogen fixation. The effect of environment upon the efficiency of marine nitrogen fixation is unknown. The relationship between niche and efficiency merits thorough study.

The *Enteromorpha* community consists of clumps of interwoven filaments suspended in the marsh water, isolated from the nutrient rich sediments. Thus, the *Enteromorpha* community is actually a microenvironment within the salt marsh. This approach can be used in studying the individual organisms, community and flow of nutrients. The majority of *Enteromorpha* algal and bacterial epiphytes are in intimate surface contact with each other. Some substrate molecules undoubtedly pass from one organism to another. Under such conditions a major pathway for recycling or regeneration of nitrogen within the *Enteromorpha* community may be direct

cycling from organism to organism. Lee *et al*. (1973) found rapid uptake of ^{14}C labeled urea and amino acids by both the algal and bacterial components of the *Enteromorpha* community. Concentrations of dissolved nitrate, ammonia and organic nitrogen were found to be significantly higher within the *Enteromorpha* community than in the water surrounding it (Lee *et al*., 1973). These are clues to the complexity of nitrogen cycling within the *Enteromorpha* community. More study is needed to explore the subtle relationships between the community members and to determine if direct nitrogen cycling is operationally significant. ^{15}N tracer studies could determine the fate of the fixed nitrogen and quantify direct cycling in the community.

The experimental microcosms show that chemostats are good tools for the study of the relationship between nitrogen fixation and *Spartina* detritus decomposition. If the N_2 was tagged with an ^{15}N tracer, the fate of the fixed nitrogen could be followed and effects of environmental alterations on fixation rates could be evaluated.

V. Concluding Remarks

Many investigators have suggested that marine heterotrophic nitrogen fixation is ecologically trivial. (Stewart, 1969; Brenzonick, 1973; Jones, 1974). But this does not apply to the salt marsh where heterotrophic fixation occurs in a variety of microhabitats (Goering and Parker, 1972; McRoy *et al.*, 1973; Hanson, 1977a; Whitney *et al.*, 1975). While this nitrogen fixation may not be of universal or year round occurrence, it can be locally significant.

This local significance may be directly linked to the marsh tidal cycle. As high tide floods the marsh combined nitrogen is made available to macrophytic plants, algae and bacteria but these nutrients are largely utilized by the time slack low tide occurs (Aurand and Daiber, 1975). In addition, marsh water microbial biomass reaches a maximum near low tide due to resuspension of fine sediments (Erkenbrecher and Stevenson, 1975). These microbes increase the demand for available nutrients at a time when nutrient levels are already low. Under these conditions, the ability to fix nitrogen would be an ecological advantage. Direct nutrient cycling and epiphytic nitrogen fixation could enable the *Enteromorpha* community to maintain high primary productivity at low tide. Investigations into the effect of tidal cycle upon nitrogen fixation and primary productivity are necessary to successfully evaluate this theory.

Detailed studies of the structural-functional relationships

in the *Enteromorpha* community are also needed. Recent success in the development of differential media which led to the isolation of a diversity of algae and bacteria from the *Enteromorpha* community gives promise that realistic assessments of the nitrogen budgets of members of the community can be made. ^{15}N tracer techniques combined with culture studies can determine the fate of the fixed nitrogen and the operational significance of direct nitrogen cycling.

Appendix 1, Tables

Table 1. Step by step conversion of ethylene production (using the gas chromatogram in Fig. 1) to equivalent N_2 fixed

1. C_2H_4 peak indentified by retention time (20 sec)
2. peak area estimated by counting the small squares on the chromatogram paper; peak area = 80 (squares)
3. peak area adjusted by multiplying by attenuation
   peak area = 80 X 1 = 80
4. C_2H_4 peak area converted to ml c_2H_4 using standard curve (Fig. 2) 80 peak area = 0.28 ml C_2H_4
5. calculate ml C_2H_4 in the total sample
   0.28 ml C_2H_4 X (15 ml(total sample vol.)/ 3 ml (injected sample vol.)) = 1.4 ml C_2H_4
6. divide by 22.4 l (2.24 X 10^4ml) to convert ml C_2H_4 to moles of C_2H_4 at STP (^oC, 760 mm)
   1.4 ml C_2H_4 X (M/2.24 X 10^4ml) = 6.3 X 10^{-5}M C_2H_4 = 63 µM C_2H
7. correct for temperature and pressure
   temp = 20^oC = 293^oK ; pressure = 740 mm
   63 µM C_2H_4 X (293^oK / 273^oK) X (760 mm / 740 mm) = 69 µM C_2H_4
8. divide by 3 to convert to moles of nitrogen fixed
   69 µM C_2H_4 X 1/3 = 23 µM N_2 fixed
9. divide by 2 to convert moles of N_2 to g at N
   23 µM N_2 / 2 = 12 µ g at N
10. divide by g dry weight of bacteria in the vessel and by incubation time
    12 µ g at N X (1 / 5 X 10^{-5}g) X 1 / wk = 0.24 g at N/g/wk

Table 2. Medium for the growth of blue-green algae.

COMPONENTS	STRENGTH/LITER
NaCl	18.0 g
$MgSO_4$-$7H_2O$	5.0 g
KCL	.6 g
Ca(as Cl^-)	.1 g
$NaNO_3$*	1.0 g
K_2HPO_4	.05 g
TRIS (THAM)	1.0 g
B_{12}	2 µg
Vitamin mix S3**	10 ml
Na_2EDTA	.03 g
Fe(Cl^-)	.8 mg
Zn(Cl^-)	.15 mg
Mn(Cl^-)	1.2 mg
Co(Cl^-)	.003 mg
Cu(Cl^-)	.024 mg
B(H_3BO_3)	.012 g
Bring up to volume with distilled water	
Actidione (added after autoclaving)	.02 g

* Omitted in Nitrogen free media

** see Provasoli *et al.*, 1957

Table 3. Nitrogen free bacterial medium

COMPONENTS	STRENGTH (GRAMS/LITER)
KH_2PO_4	.2
Na_2MoO_4-$2H_2O$	.01
$FeSO_4$-$7H_2O$	.001
$MnSO_4$-$6H_2O$	.001
Sucrose	3.4
Glucose	1.8
Fructose	1.8
Lactose	3.4
Mannitol	1.8
Artificial sea water*	1 liter

* see Lee *et al.*, 1975

Table 4. Algal cultures added to microcosm 5 and 6 (innoculated with 3 ml. of each culture).

STRAIN	CELLS/ml	ORGANISM
13	4.75×10^6	*Dunaliella salina*
9	3.75×10^6	*Cylindrotheca closterium*
38	1.45×10^6	*Chlorococcum sp*
B1-714	8.25×10^6	*Fragilaria construens*
B1- 45	4.25×10^6	unidentified chrysophyte
B1-717	2.50×10^6	*Fragilaria construens*
W 509	8.80×10^5	*Amphora sp*
41	6.80×10^7	*Nannochloris sp*
W 500	1.90×10^5	*Amphora* (cymbelloides-like)
W 506	2.00×10^5	*Navicula salinarum*

Table 5. Environmental data for field trips during the summer, 1974.

Field Trip #	Date	H_2O Temp. (°C)	pH	Light (lux)	°/oo	Oxygen (ml/l)
1	6/2	24	7.9	4000	25.0	8.0
2	6/9	26	8.2	5000	25.5	7.3
3	6/19	29	7.8	3500	26.0	8.5
4	6/24	24	8.4	3200	27.0	6.4
5	7/3	26	8.4	8800	27.0	16.5
6	7/13	26	7.8	2000	27.0	10.8
7	7/21	24	8.4	4000	31.1	16.1
8	7/28	26	8.2	7500	26.0	10.5
9	8/6	25	8.0	5400	25.4	9.2
10	8/13	27	7.9	4200	25.7	8.7
11	8/20	26	8.3	9800	26.1	10.2
12	8/27	28	7.8	6000	27.2	12.1
13	9/3	27	8.1	8000	25.6	8.5
14	9/13	25	8.2	3600	25.2	10.8

Table 6. Concentration of selected nutrients (μg at/l) in the study area.

Year		NO_3^-	NO_2^-	NH_4^+	SiO_2	$PO_4^{\equiv}$	Diss. O. N	Part. O. N	Part. O. C
1970	min	0.72	0.18	4.30	10.40	2.25	12.95		
	max	3.18	0.50	11.10	18.30	3.01	73.15		
1973	min	0.15	0.15	0.15	2.00	0.36			
	max	33.10	1.90	1.90	8.90	2.30			
1974	min	0.30	0.40	0.30	0.30	0.25		2.50	44.41
	max	0.90	1.19	11.10	11.00	3.02		41.34	385.20
no.	1970	20	20	20	20	20	20		
deter.	1973	72	72	72	72	72			
	1974	70	70	70	70	70		35	35
mean	1970	2.96	0.33	6.35	14.18	2.74	32.2		
	1973	10.22	0.90	4.94	18.73	1.60			
	1974	0.36	0.26	2.69	28.91	1.45		17.04	192.55
Std.	1970	1.09	0.12	2.76	3.11	0.35	24.74		
Dev.	1973	19.50	0.06	2.27	7.5	0.75			
	1974	0.17	0.12	2.87	13.05	0.49		0.17	1.15

Table 7. Concentration of selected dissolved nutrients (μg at N/1) at seven different sites within the study area.

Nutrient	Station						
NO_3^-	1	2	3	4	5	6	7
maximum	.43	.98	.74	.76	.19	.27	.72
minimum	.07	.50	.17	.17	.08	.12	.29
mean	.24	.50	.38	.41	.20	.31	.49
# determinations	70						
Sum of Squares							
between	3.358						
within	22.759						
F ratio	1.549						
p	.25						
Significant	NO						
NO_2^-							
maximum	.28	.46	1.19	.23	.27	.24	.25
minimum	.20	.23	.20	.19	.17	.14	.14
mean	.24	.32	.41	.21	.22	.21	.21
# determinations	70						
Sum of Squares							
between	2.945						
within	20.433						
F ratio	1.513						
p	.25						
Significant	NO						

Table 7. continued.

Nutrient	Station						
NH_4^+	1	2	3	4	5	6	7
maximum	2.97	10.39	3.66	5.57	21.12	2.10	2.87
minimum	.69	.96	.66	1.02	.73	.60	.48
mean	1.68	4.29	2.08	2.22	5.87	1.29	1.45
# determinations	70						
Sum of Squares							
between	56.375						
within	463.861						
F ratio	1.175						
p	.25						
Significant	NO						
$PO_4^{\equiv}$							
maximum	2.20	3.02	2.41	2.55	2.12	1.90	1.81
minimum	0.35	1.05	0.06	0.75	0.93	0.25	0.57
mean	1.84	2.03	1.40	1.44	1.35	1.38	1.43
# determinations	70						
Sum of Squares							
between	4.167						
within	14.794						
F ratio	2.957						
p	.025						
Significant	YES						

Table 7. continued.

Nutrient	Station						
SiO_2	1	2	3	4	5	6	7
maximum	59.49	50.17	59.52	40.71	46.49	51.27	58.59
minimum	3.09	3.80	11.40	5.97	4.51	7.21	4.20
mean	31.87	31.50	36.61	22.83	27.36	25.14	27.08
# determinations	70						
Sum of Squares							
between	1722.43						
within	10331.4						
F ratio	1.751						
p	.25						
Significant	NO						

Table 8. Community bound nitrogen and calculated rates of nitrogen uptake, nitrogen fixation and precipitation for the Towd Point marsh.

Month	*Enteromorpha* community bound N (mg N/m^2)	*Enteromorpha* community uptake (mg $N/m^2/hr$)	*Enteromorpha* community N_2 fixation (mg $N/m^2/hr$)	Precipitation (mg $N/m^2/hr$)
June	31.0	4.95×10^{2}	3.15×10^{-2}	1.46×10^{-2}
July	50.7	1.36×10^{3}	1.08×10^{-1}	8.28×10^{-3}
Aug.	26.5	6.60×10^{2}	3.41×10^{-2}	1.34×10^{-2}
Sept.	6.48	1.13×10^{2}	2.39×10^{-3}	1.14×10^{-2}

Table 9. Comparison of nitrogen fixation by ten isolates from the *Enteromorpha* epiphytic community (25°C; 20°/oo; pH 8.0; aerobic).

Isolate	O_2 Response	Incub. Time	N_2 Fixed (g at N/g bact)
B3711	aerobe	1 wk.	8.0
C11X	aerobe	1 wk.	4.0
B22	aerobe	1 wk.	0.5
B3P5	aerobe	1 wk.	0.4
D21	aerobe	1 wk.	0.25
B16	f. anaerobe	4 wk.	0.25
C13	f. anaerobe	4 wk.	0.25
C18	f. anaerobe	4 wk.	0.25
D41	f. anaerobe	4 wk.	0.25
D58	f. anaerobe	4 wk.	0.25

Table 10. Morphological and biochemical characteristics of five nitrogen fixing aerobic isolates (Azot., _Azotobacter sp._; Azom., _Azomonas_ sp.).
* OW, off white; B, brown; Y, yellow

Characteristic	Isolates				
	B22	B3P5	B3711	C11X	D21
N_2 fixation	+	+	+++	++	+
Gram Stain	−	−	−	−	−
Motility	+	+	+	−	+
Capsule	−	−	−	+	+
Cysts	+	−	+	+	−
PHB globules	−	−	−	−	−
Pigmentation *	OW	B	OW	OW	Y
Fluorescence	−	−	−	−	−
Carbohydrate Reactions					
Lactose	+	−	−	+	+
Dextrose	+	+	+	+	+
Sucrose	+	−	+	+	+
Galactose	+	−	−	−	+
Maltose	+	+	+	−	+
Arabinose	−	−	+	+	+
Sorbitol	+	−	−	+	+
Xylose	+	−	−	+	−
Insitol	−	+	+	+	−
Mannose	+	+	+	−	+
Mannitol	−	−	−	−	+
M.R.	−	−	+	−	+
V.P.	−	−	−	−	−
Protein Reactions					
Indole	−	−	−	−	+
Arginine	+	+	+	−	−
Lysine	+	+	+	−	−
Orinithine	−	+	−	−	+
Identity	Azot. 1	Azom. 1	Azot. 2	Azot. 3	Azom. 2

Table 11. Morphological and biochemical characteristics of five nitrogen fixing facultative anaerobic isolates (K, Klebsiella sp.; E, Enterobacter sp.).
* OW, off white; W, white; P, pink

Characteristic	B16	C13	C18	D41	D58
N_2 fixation	+	+	+	+	+
Gram stain	−	−	−	−	−
Motility	+	+	+	−	−
Capsule	−	−	+	+	+
Pigmentation *	OW	OW	OW	P	W
Carbohydrate Reactions					
Lactose	−	+	+	+	−
Dextrose	+	+	−	+	−
Sucrose	+	+	−	+	−
Galactose	+	+	−	+	+
Maltose	+	+	+	+	+
Arabinose	−	+	+	−	−
Sorbitol	+	+	+	+	+
Xylose	+	+	+	−	+
Insitol	−	−	+	−	−
Mannose	+	+	+	+	+
Mannitol	+	+	+	+	−
M.R.	+	+	−	−	−
V.P.	−	−	−	−	−
Protein Reactions					
Indole	+	−	−	−	+
Arginine	+	+	−	−	−
Lysine	+	−	−	+	+
Orinithine	−	−	−	+	+
Identity	E1	E2	E3	K1	K2

Table 12. Independent variable entered (in order of decreasing importance) in step-wise multiple regression analysis with nitrogen fixation/g dwt *Enteromorpha*/hr as the dependent variable.

Step No.	Variable Entered	Unit	Coefficient of Multilple Determinism R^2
1	*Ent.* Primary Production	μM C/mg/hr	.817
2	Temperature	^{o}C	.856
3.	Oxygen	ml/l	.881
4.	Light	lux	.891
5.	Salinity	$^{o}/oo$	.904
6	pH	pH	.923
7	PO_4	μg at P/l	.974
8	C11X Pop.	cells/g dwt	.980
9	NO_2^-+NO_3^-+NH_4^+	μg at N/l	.984
10	B3711 Pop.	cells/g dwt	.984

Table 13. Multiple regression equation for the final step of the regression shown in Table 12.

N_2 Fixation =	0.025481 Enteromorpha Primary Production	+ 0.92258 Temperature	+ 0.61086 O_2	
t value*	6.152	1.635	2.330	
significant	yes	yes	yes	
	-0.00128 Light	- 1.64242 Salinity	+ 13.88629 pH	+ 1.32679 $PO_4^{=}$
t value*	-4.094	-3.128	3.510	1.395
significant	yes	yes	yes	yes
	-0.02498 C11X Population	- 0.90659 (NO_2^- + NO_3^- + NH_4^+)	+ 0.00006 B3711 Pop.	
t value*	-1.026	-0.836	0.114	
significant	no	no	no	
	-109.45			

*t values for testing significance of partial coefficient.
Not Significant = P for t = 10%

Table 14. Physiological tolerances for nitrogen fixation by two *Azotobacter* isolates and field conditions at Towd Point. (NO_2^-, NO_3^- and NH_4^+ in µg at N/l)

	B3711		C11X		Field
	Optimum	Range	Optimum	Range	Range
Temp.	20°-25°C	10°-40°C	20°-25°C	10°-35°C	24°-29°C
pH	8	6.5-9.5	8	6.5-9.5	7.8-8.4
°/oo	5°/oo	5-50°/00	15°/oo	5-35°/00	25-31°/oo
NO_3^-	0	0-2X10^3	0	0-2X10^3	.15-33.1
NO_2^-	0-.02	0-20	0-.02	0-20	.15-1.9
NH_4^+	o	0-40	0	0-40	.15-11.1
O_2	aerobic		aerobic		6.4-16.5 ml/l

Table 15. Detritus Decomposition in three model microcosms.

	Light Procaryotes	Dark Procaryotes	Light Procaryotes Eucaryotes
% dec. organic carbon	16.66%	32.0%	34.6%
% inc. organic nitrogen	51.6%	33.3%	73.3%
C:N ratio	9.24:1	11.58:1	7.46:1
N_2 fixation ($g\ N_2$/day/chem)	70	3	7

Appendix 2, Illustrations

Figure 1. Location of the study area; Towd Point, North Sea Harbor, Southampton, Long Island, New York, USA (N 40°55', W72°25').

FIG. 1

N
W
E
S

Long Island Sound

Little Peconic Bay

Great Peconic Bay

North Sea Harbor

*study area

0 1 2 3 Miles

Atlantic Ocean

Figure 2. Typical chromatogram of 3 ml of the gas phase of an experimental incubation after N_2ase catalyzed reduction of 0.2 atm C_2H_2.

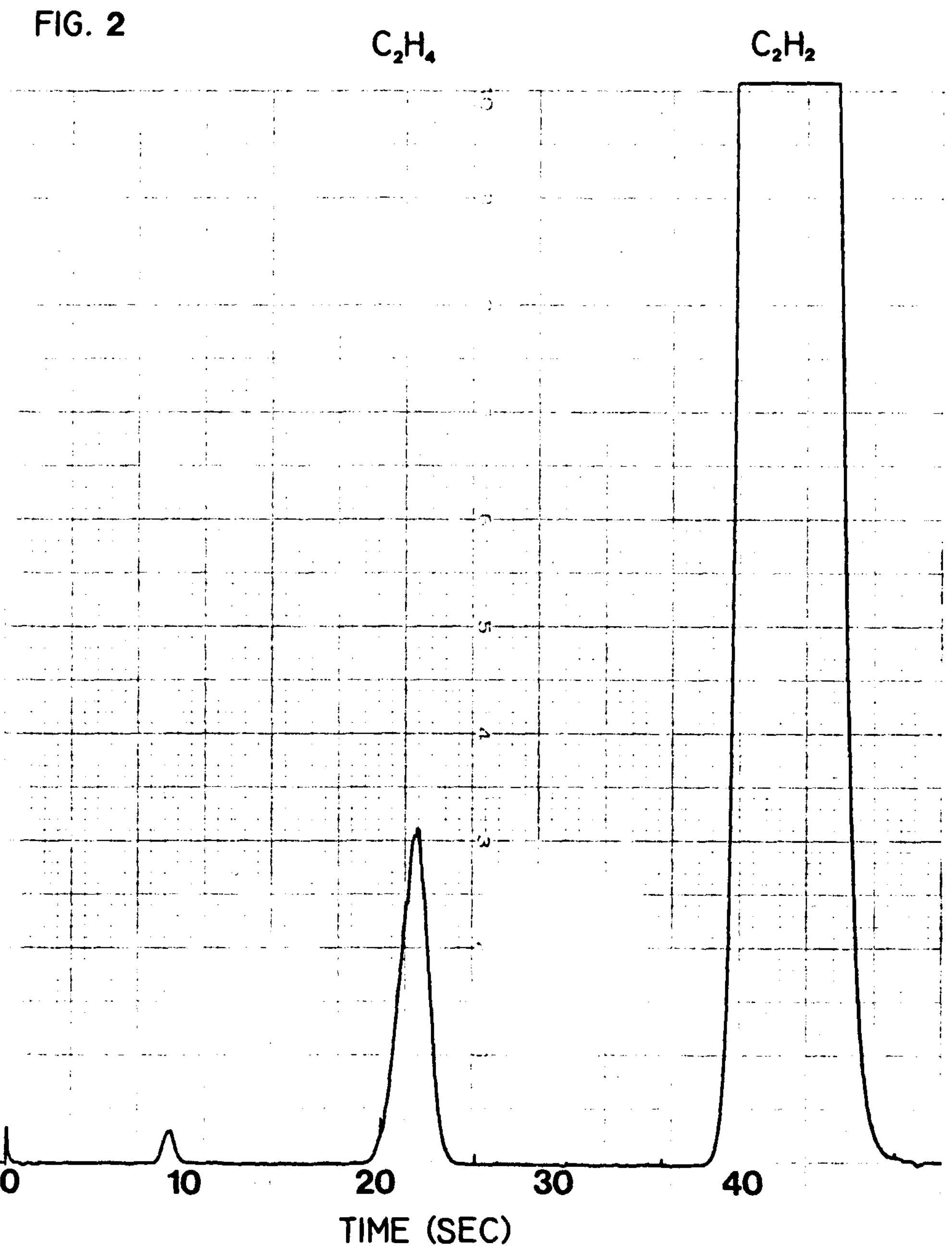


FIG. 2
C_2H_4
C_2H_2
0
10
20
30
40
TIME (SEC)

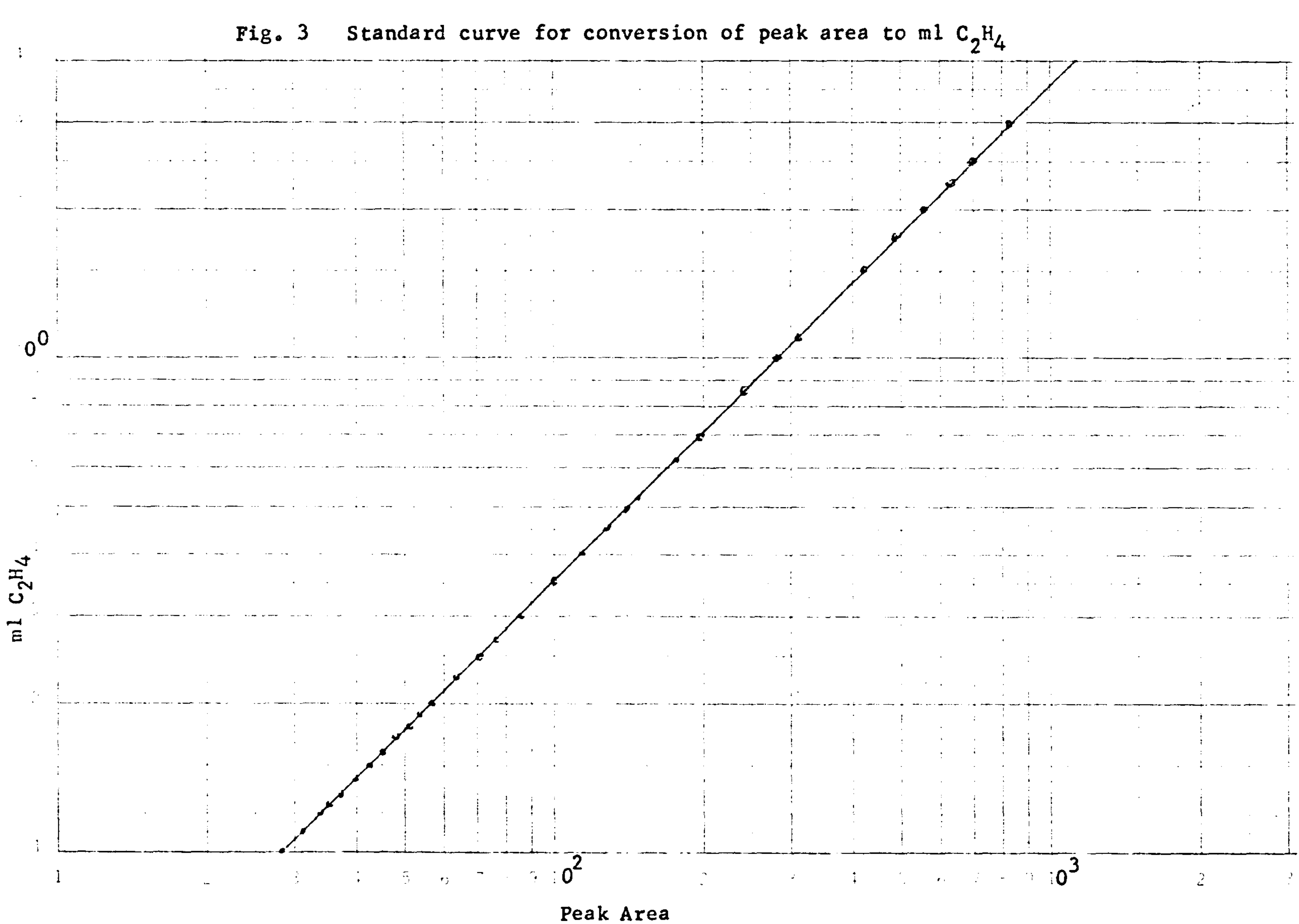


Fig. 3 Standard curve for conversion of peak area to ml C_2H_4

Figure 4. Location of sampling stations in the study area, Towd Point Marsh (354.88 m^2).

FIG. 4

2

1

3

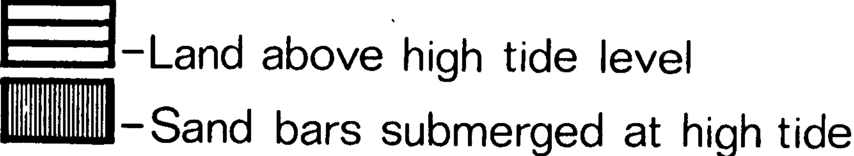
5

4

6

7

0 10 20 30 M



-Land above high tide level

-Sand bars submerged at high tide

1,2,..7–Sampling stations

6,7–Inlet channel

Figure 5. The biomass of *Enteromorpha* in the study area. Biomass (g wet wt/m^2) estimated by quadrat sampling along transects.

FIG.5

g wwt/m^2

g dwt/m^2

Enteromorpha Biomass

300 200 100

9 6 3

JUNE JULY AUG SEPT

Figure 6. Nitrogen fixation in the *Enteromorpha* community at Towd Point - light and dark incubation. Samples (1 g dwt) were incubated 1 hr in marsh water. Ranges are indicated by vertical line through mean datum point (three replicates).

FIG. 6

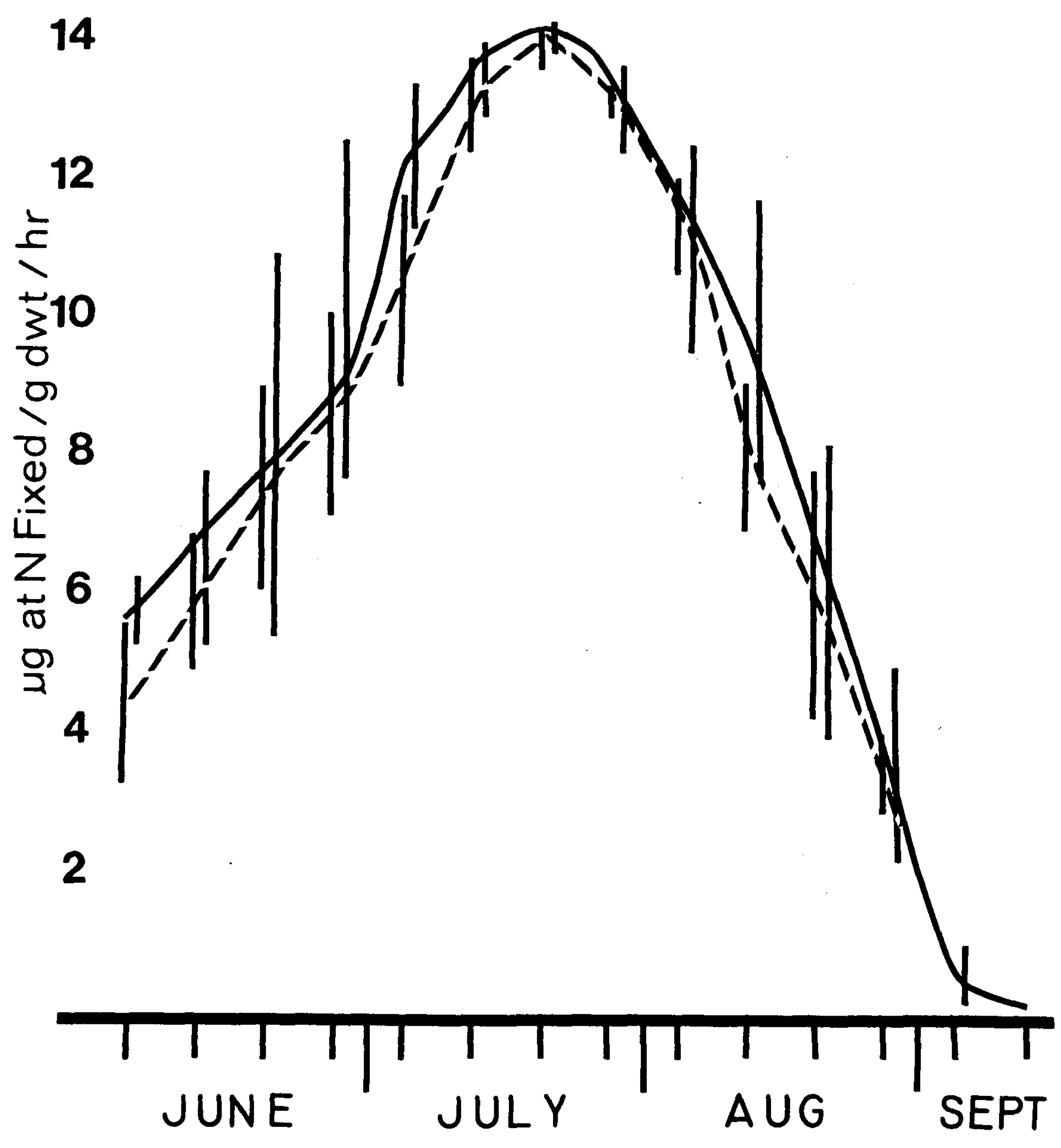


Figure 7. Field O_2 values vs O_2 saturation values. Dissolved oxygen (ml/l) was determined by the Winkler method.

FIG.7

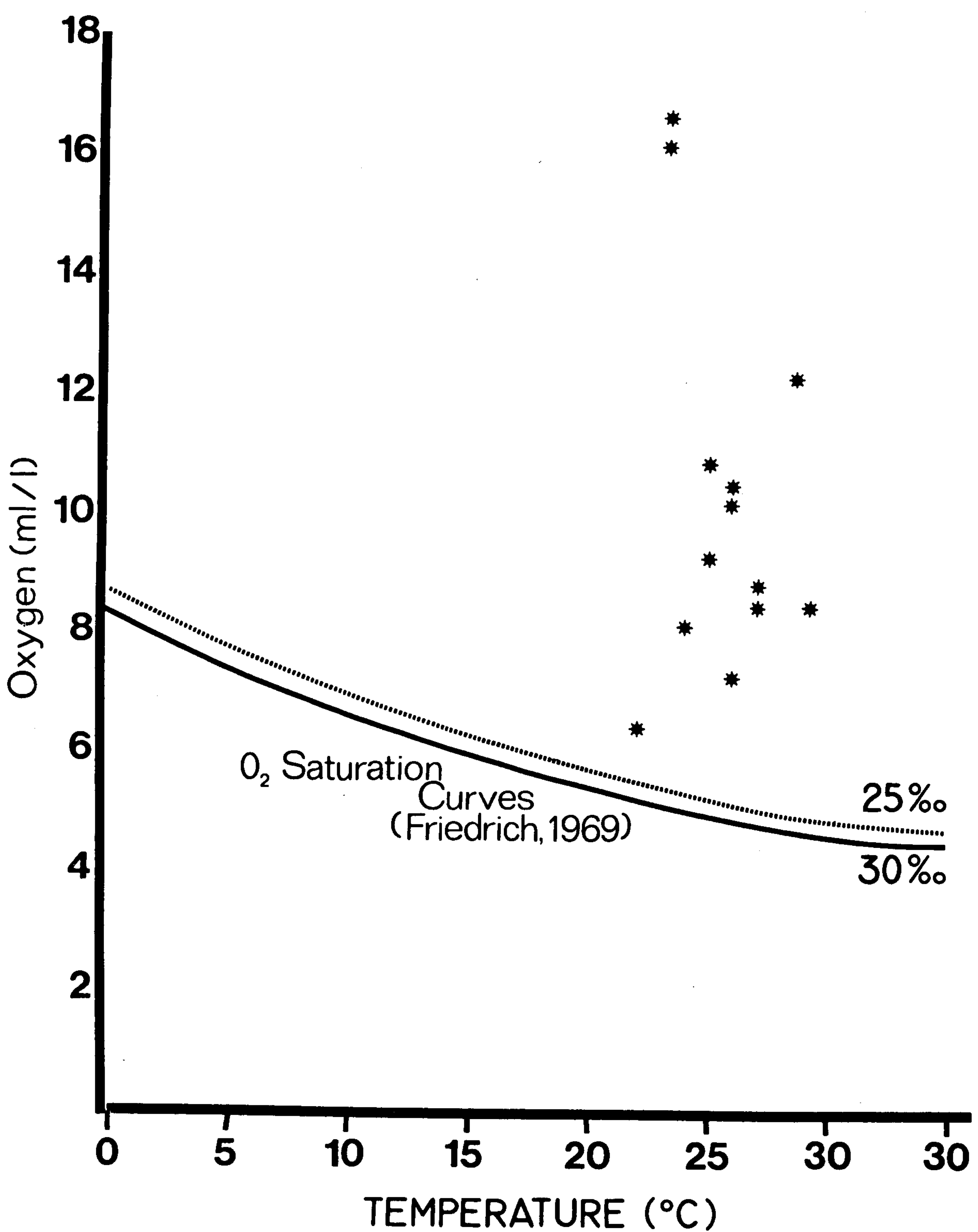


Figure 8. Concentration of phosphate, nitrite, nitrate and ammonia in the study area during the summer.

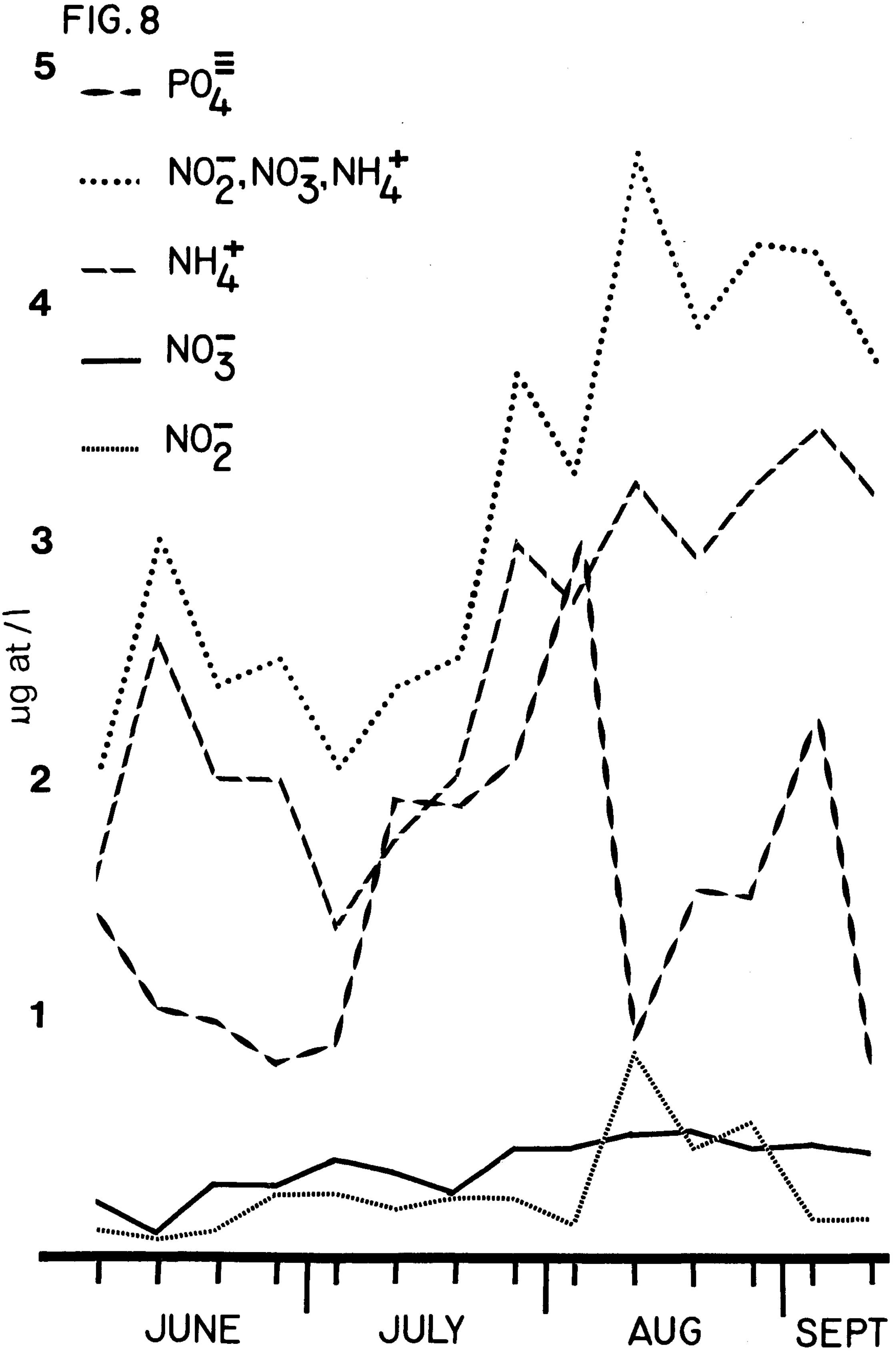
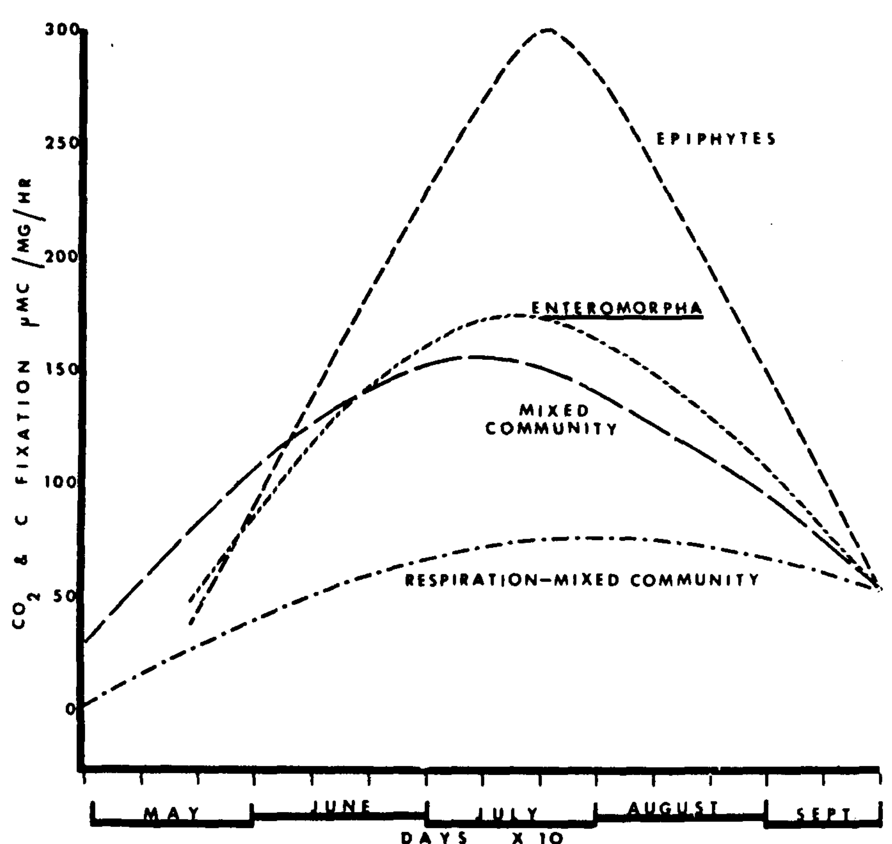


FIG. 8
5
4
3
2
1
$PO_4^{\equiv}$
NO_2^-, NO_3^-, NH_4^+
NH_4^+
NO_3^-
NO_2^-
ug at / l
JUNE
JULY
AUG
SEPT

Fig. 9



Primary production and respiration during the summer by a natural Enteromorpha community, washed Enteromorpha, and its epiphytes. Figure summarizes data from 50 replicate experiments.

(Lee et al., 1973)

Figure 10. Nitrogen uptake by the Enteromorpha community. N_2 uptake was calculated from community (Enteromorpha + epiphytes) ^{14}C uptake data (Fig. 9) assuming a C:N ration of 8:1.

FIG. 10

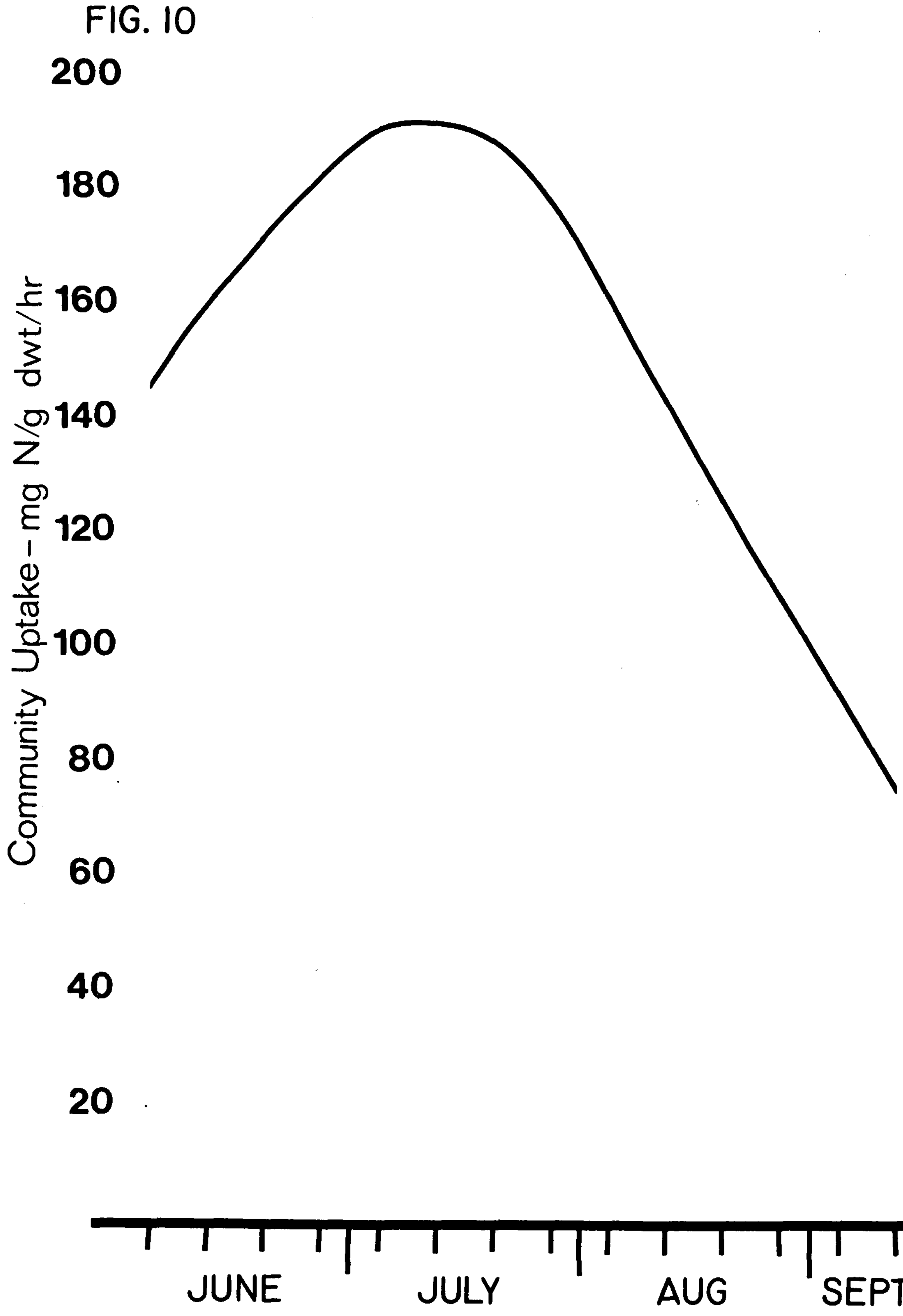


Figure 11. Population of Blue-green algae in the *Enteromorpha* community during the summer. Population was estimated by colony counts using the membrane filter technique. Ranges are indicated by vertical line through mean datum point (three replicates).

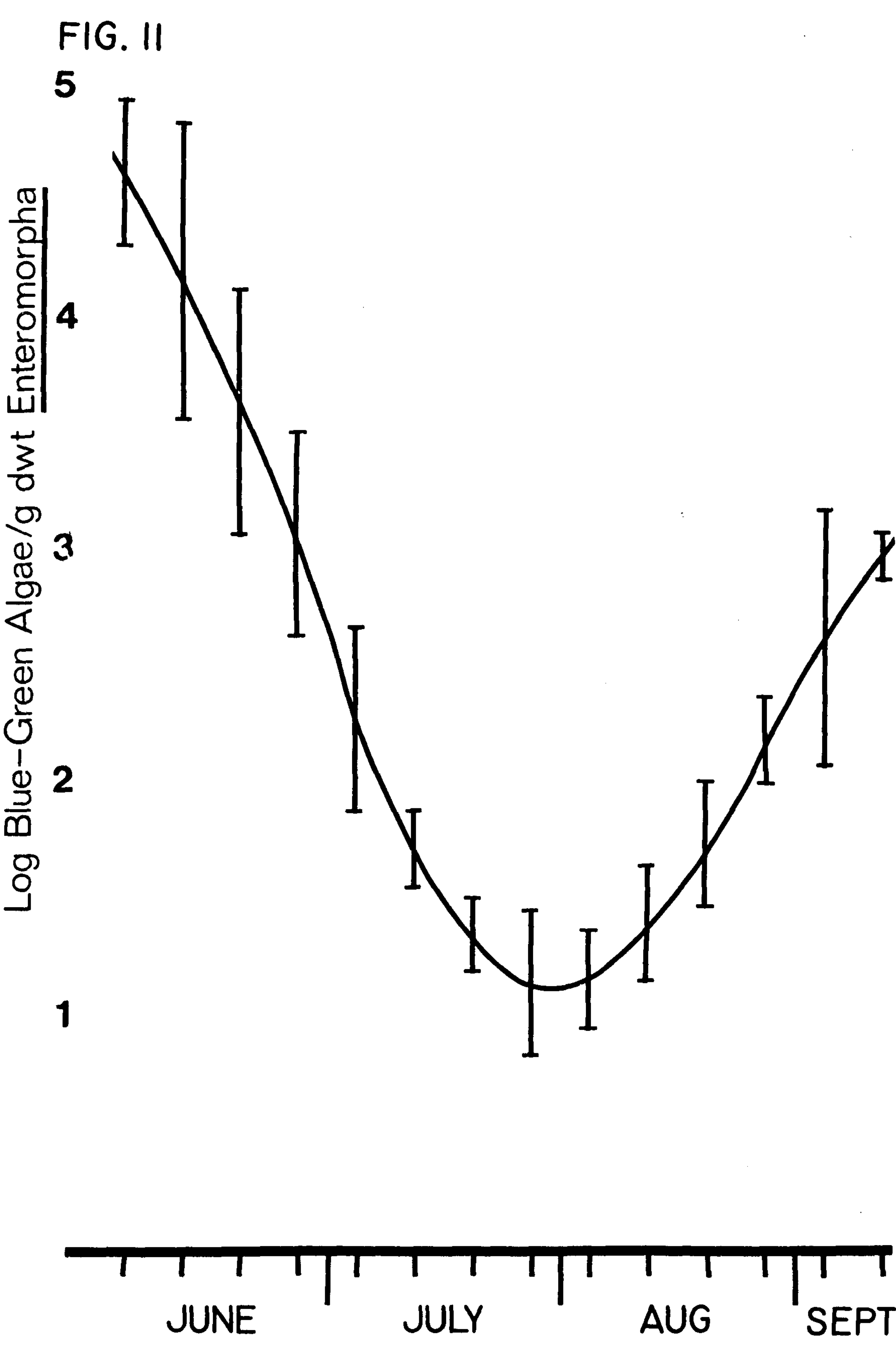


FIG. II
Log Blue-Green Algae/g dwt Enteromorpha
5
4
3
2
1
JUNE
JULY
AUG
SEPT

Figure 12. The effect of nitrate ($NaNO_3$) concentration on the growth of blue-green algal isolates. (pH 8.2, 25°C, 20°/oo, 16L/8D) Growth was determined by direct cell counts. Data points are means of three replicates.

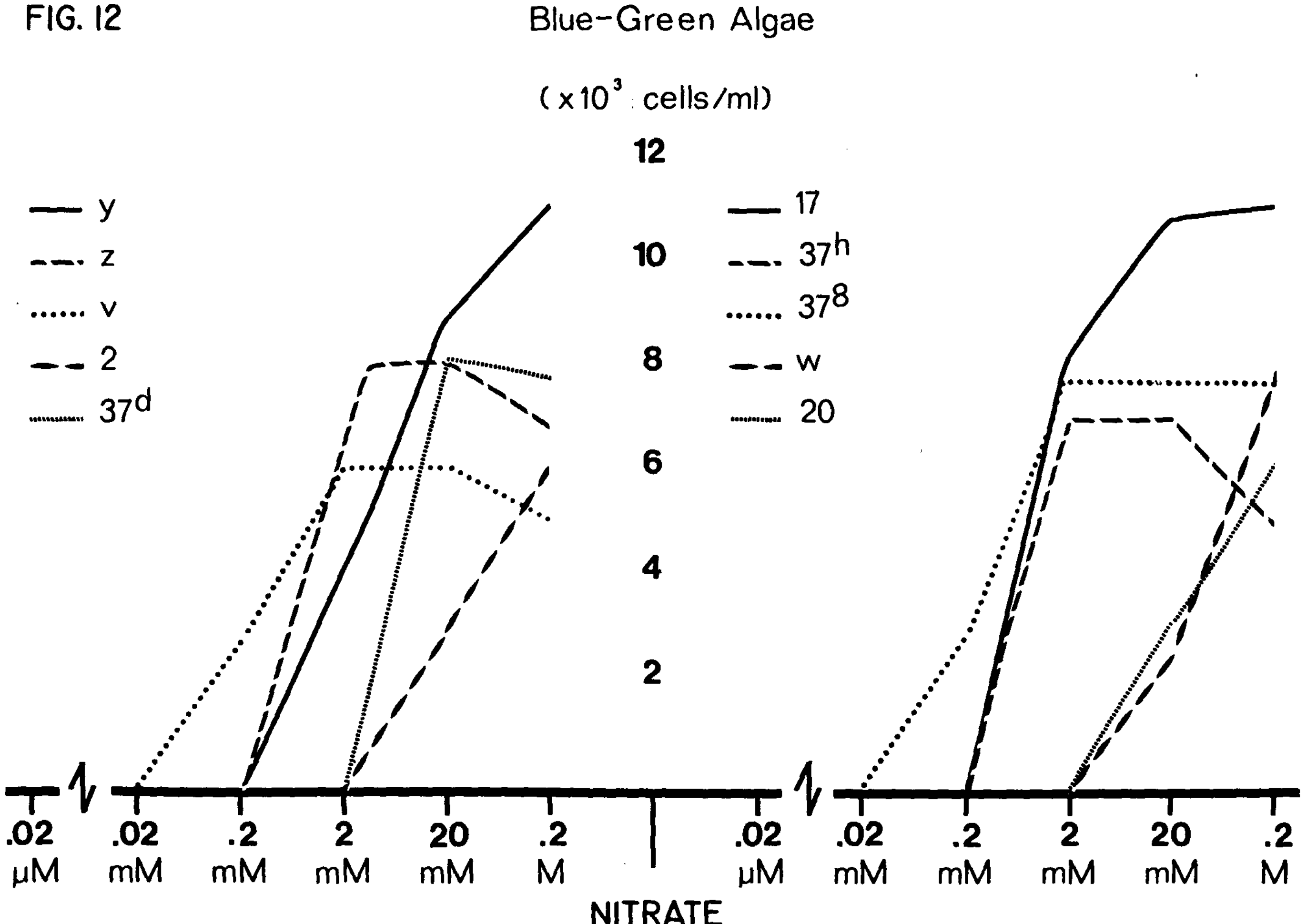


FIG. 12
Blue-Green Algae
(x 10^3 cells/ml)
12
10
8
6
4
2
y
z
v
2
37^d
17
37^h
37^8
w
20
.02 µM
.02 mM
.2 mM
2 mM
20 mM
.2 M
.02 µM
.02 mM
.2 mM
2 mM
20 mM
.2 M
NITRATE

Figure 13. The effect of nitrite ($NaNO_2$) concentration on the growth of blue-green algal isolates. (pH 8.2, 25°C, 20°/oo, 16L/8D) Growth was determined by direct cell counts. Data points are means of three replicates.

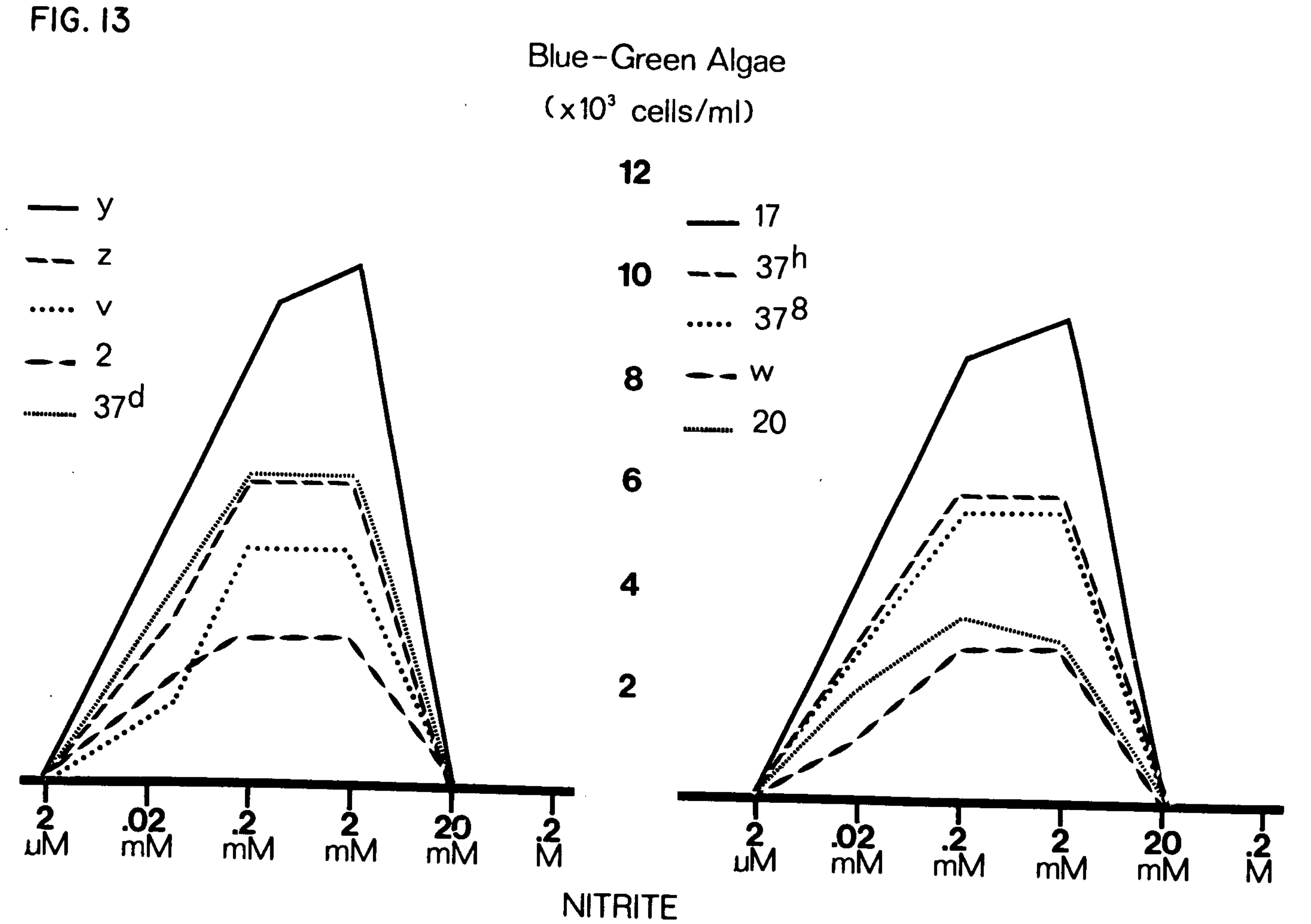


FIG. 13
Blue-Green Algae
(x10³ cells/ml)
12
10
8
6
4
2
y
z
v
2
37d
17
37h
378
w
20
2 uM
.02 mM
.2 mM
2 mM
20 mM
.2 M
NITRITE

Figure 14. The effect of ammonia ($(NH_4)_2SO_4$) concentration on the growth of blue-green algal isolates. (pH 8.2, 25°C, 20°/oo, 16L/8D) Growth was determined by direct cell counts. Data points are means of three replicates.

FIG. 14

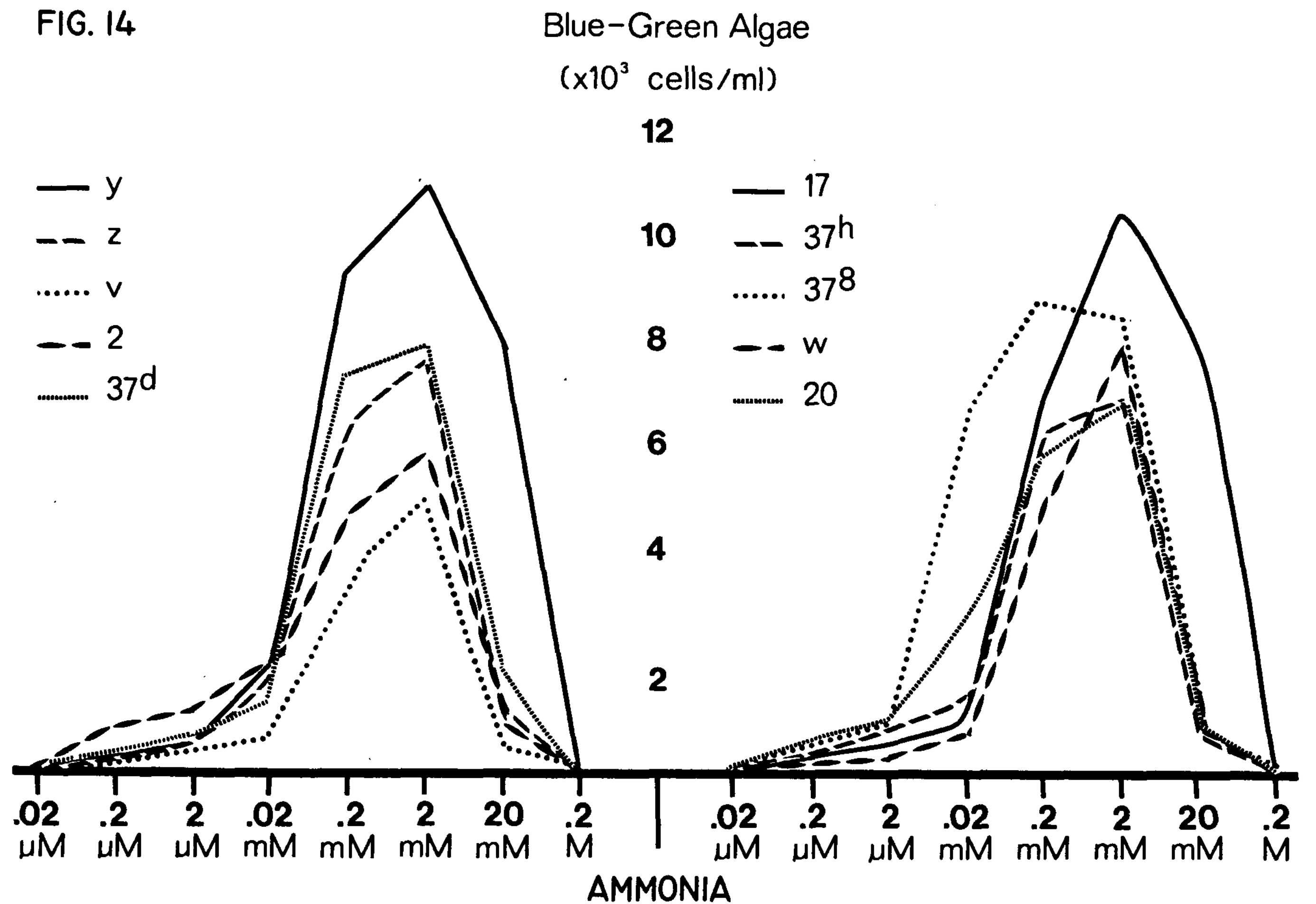


Figure 15. The effect of temperature on nitrogen fixation by isolates B3711 and C11X. (ph 8, $20^{o}/oo$) Ranges are indicated by vertical line through mean datum point (three replicates).

FIG. 15

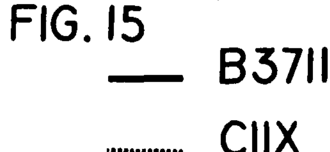


Figure 16. The effect of pH on nitrogen fixation by isolates B3711 and C11X. (25°C, 20°/oo) Ranges are indicated by vertical line through mean datum point (three replicates).

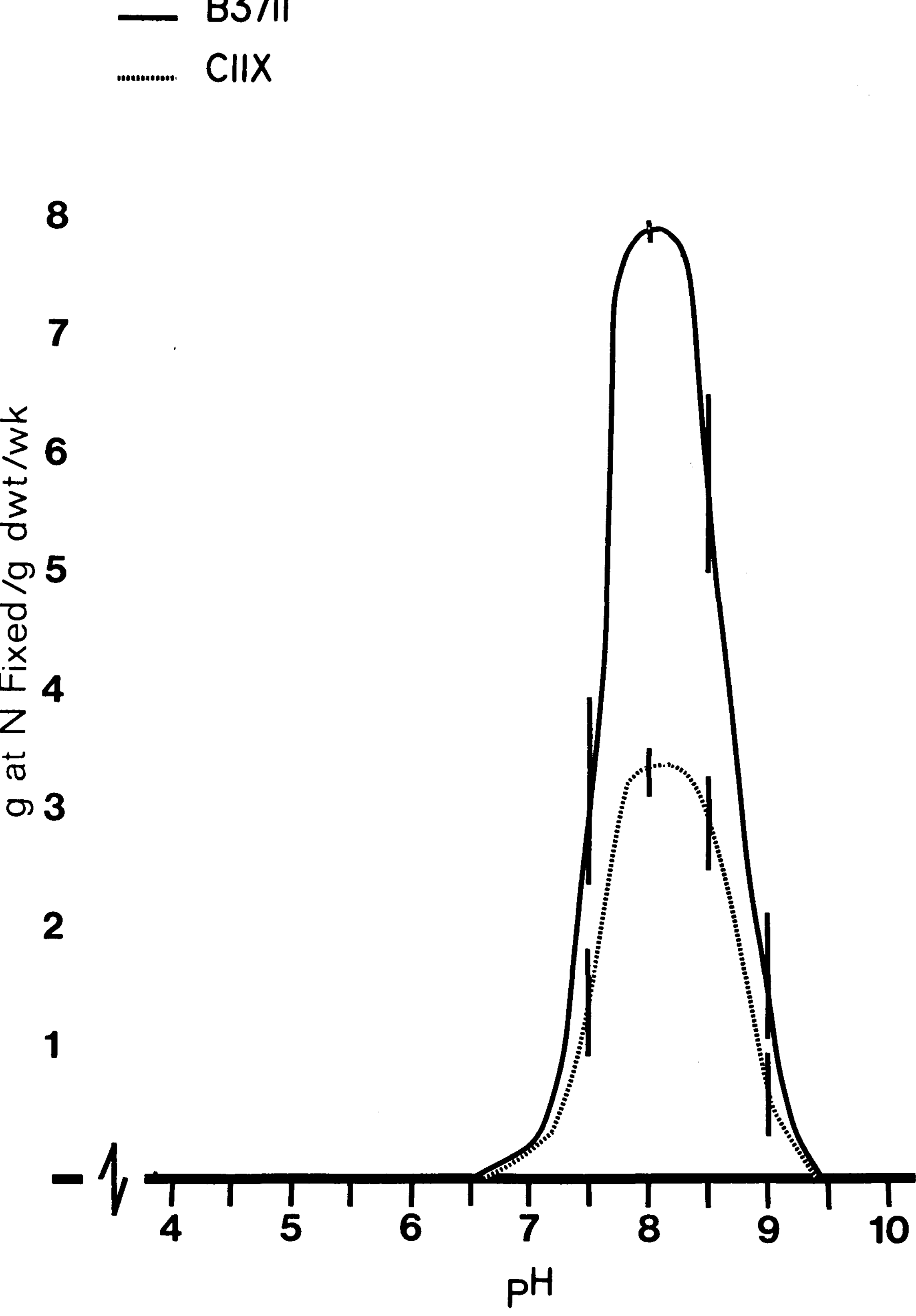


FIG. 16
B37II
CIIX
g at N Fixed /g dwt /wk
8
7
6
5
4
3
2
1
4
5
6
7
8
9
10
pH

Figure 17. The effect of salinity on nitrogen fixation by isolates B3711 and C11X. (ph 8, 25°C) Ranges are indicated by vertical line through mean datum point (three replicates).

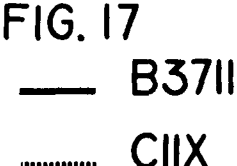


FIG. 17
B3711
C11X
g at N Fixed/g dwt/wk
16
14
12
10
8
6
4
2
5
10
15
20
25
30
35
40
45
50
SALINITY (‰)

Figure 18. The effect of combined inorganic nitrogen on nitrogen fixation by isolate B3711. (25°C, 20°/oo, pH 8) Ranges are indicated by vertical line through mean datum point (three replicates).

FIG. 18

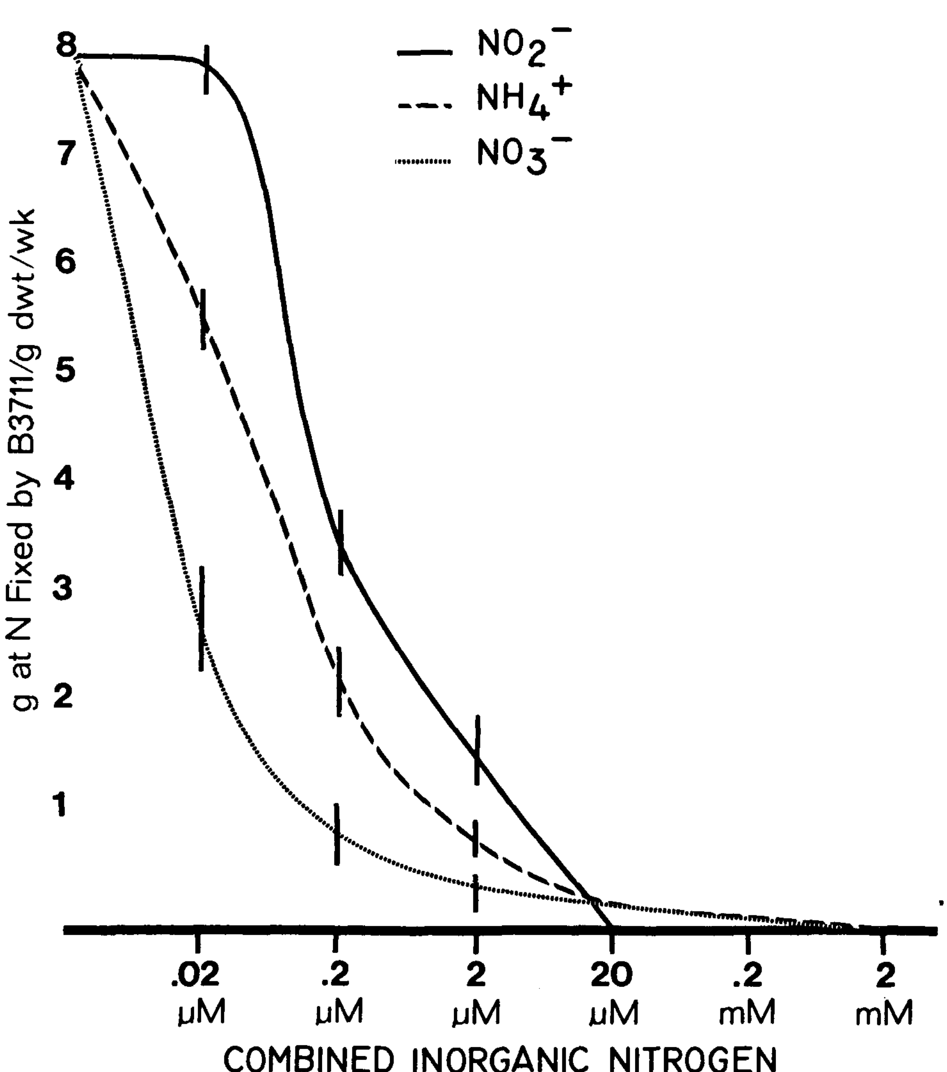


Figure 19. The effect of combined inorganic nitrogen on nitrogen fixation by isolate C11X. (25°C, 20°/oo, pH 8) Ranges are indicated by vertical line through mean datum point (three replicates).

FIG. 19

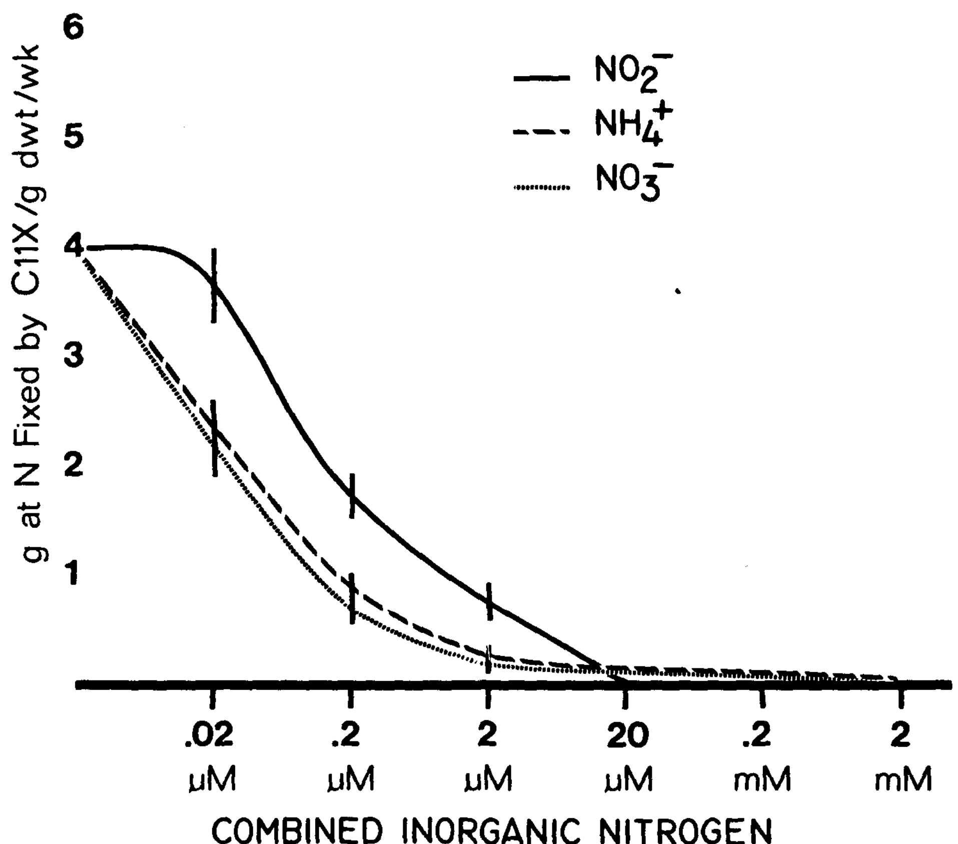


Figure 20. Population structure of nitrogen fixing bacteria in the *Enteromorpha* community during the summer. Populations were estimated by colony counts of plates inoculated with serial dilutions of epiphyte suspension. Values are means of three replicates.

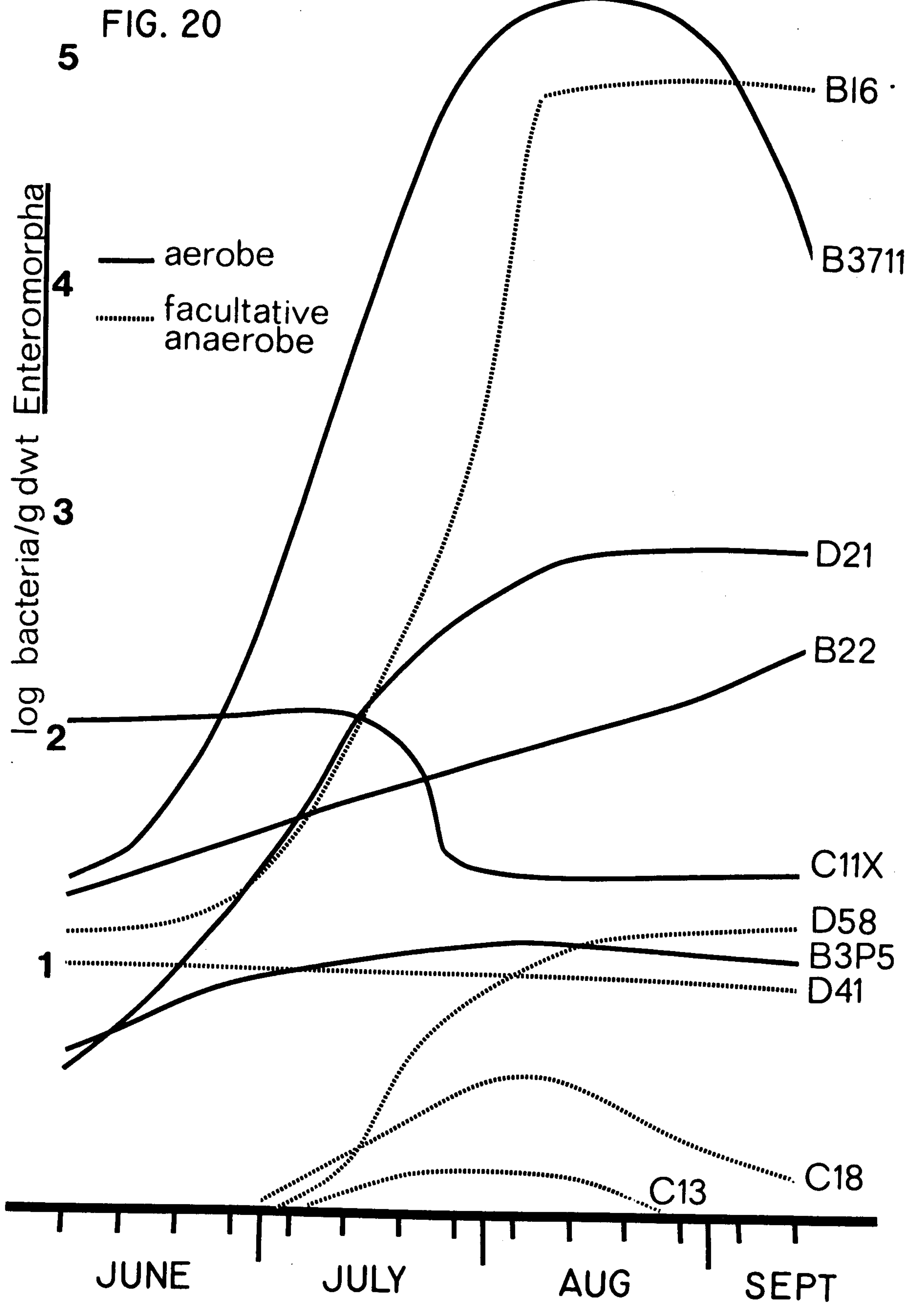


FIG. 20
5
4
3
2
1
log bacteria/g dwt Enteromorpha
aerobe
facultative anaerobe
B16
B3711
D21
B22
C11X
D58
B3P5
D41
C18
C13
JUNE
JULY
AUG
SEPT

Figure 21. Scanning electron micrographs of the surface of *Enteromorpha intestinalis*.

A. 1400X
B. 3500X

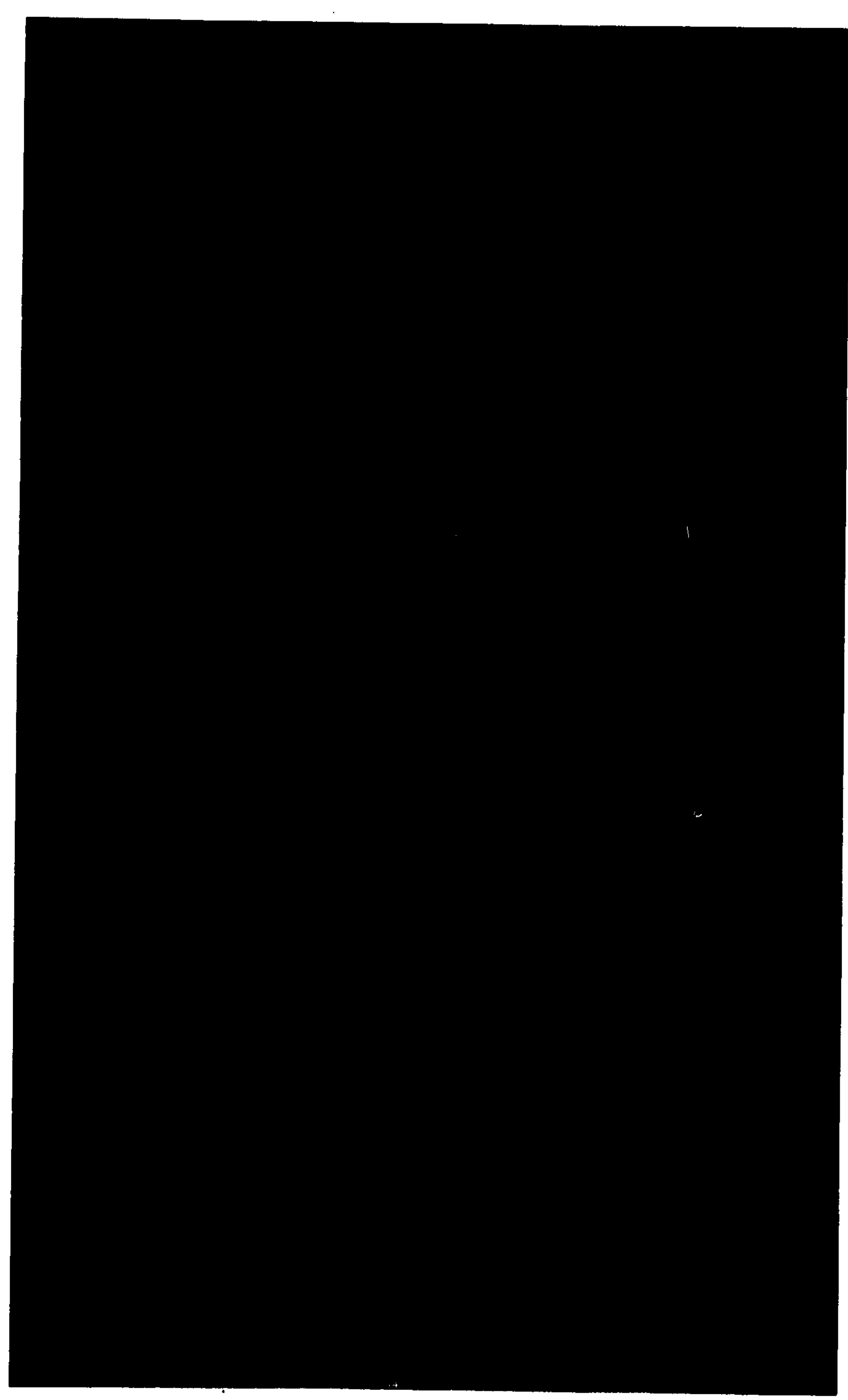


Figure 22. Carbon content of ground *Spartina* detritus before and after 3 months incubation in the experimental microcosms. (25°C, 12L/12D) Sea water flowed through the vessels at 200 ml/day. Values are means of two replicates.

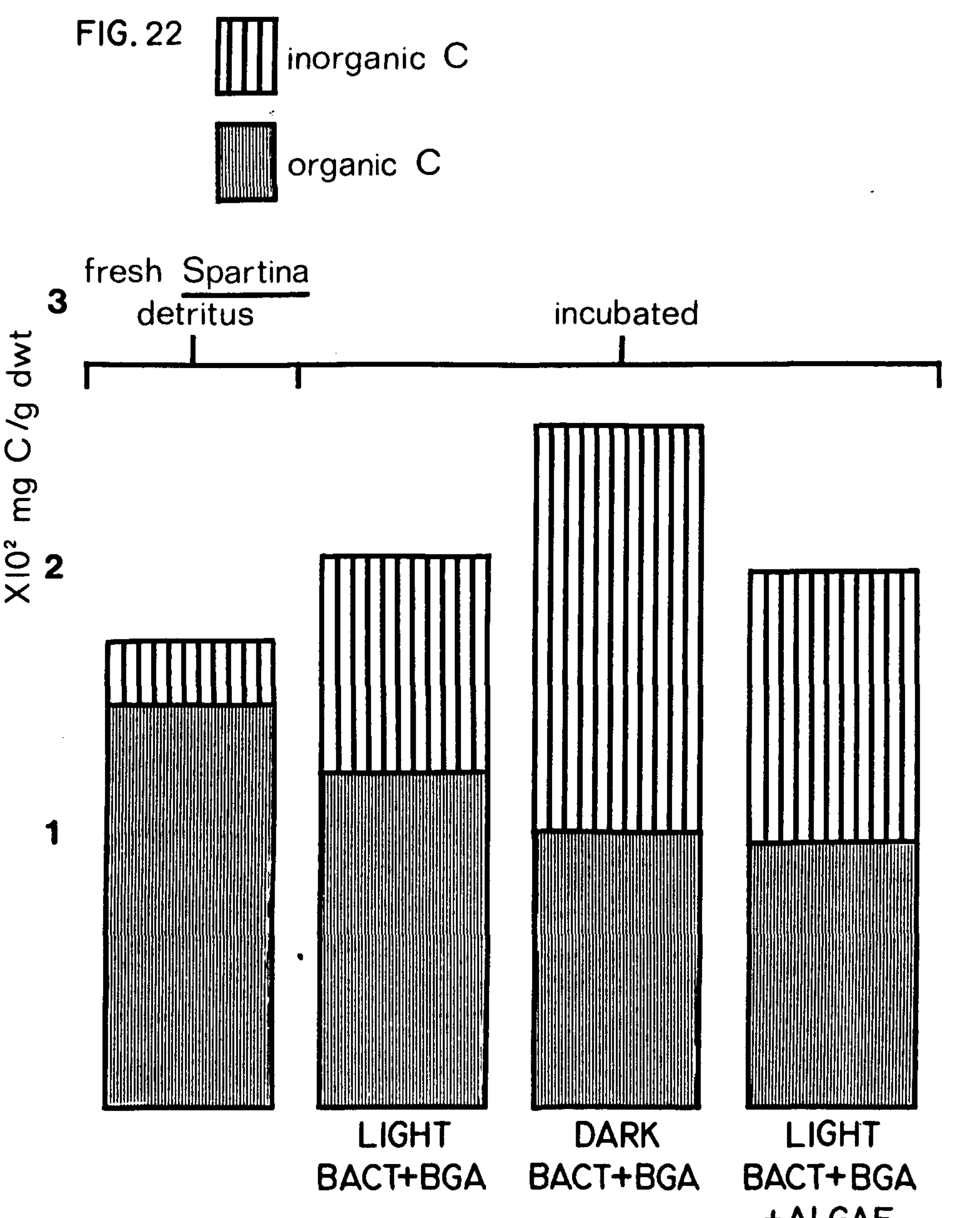


FIG. 22
inorganic C
organic C
fresh Spartina
detritus
incubated
X10² mg C/g dwt
3
2
1
LIGHT
BACT+BGA
DARK
BACT+BGA
LIGHT
BACT+BGA
+ALGAE

Figure 23. Nitrogen content of ground *Spartina* detritus before and after 3 months incubation in the experimental microcosms. (25°C, 12L/12D) Sea water flowed through the vessels at 200 ml/day. Values are means of two replicates.

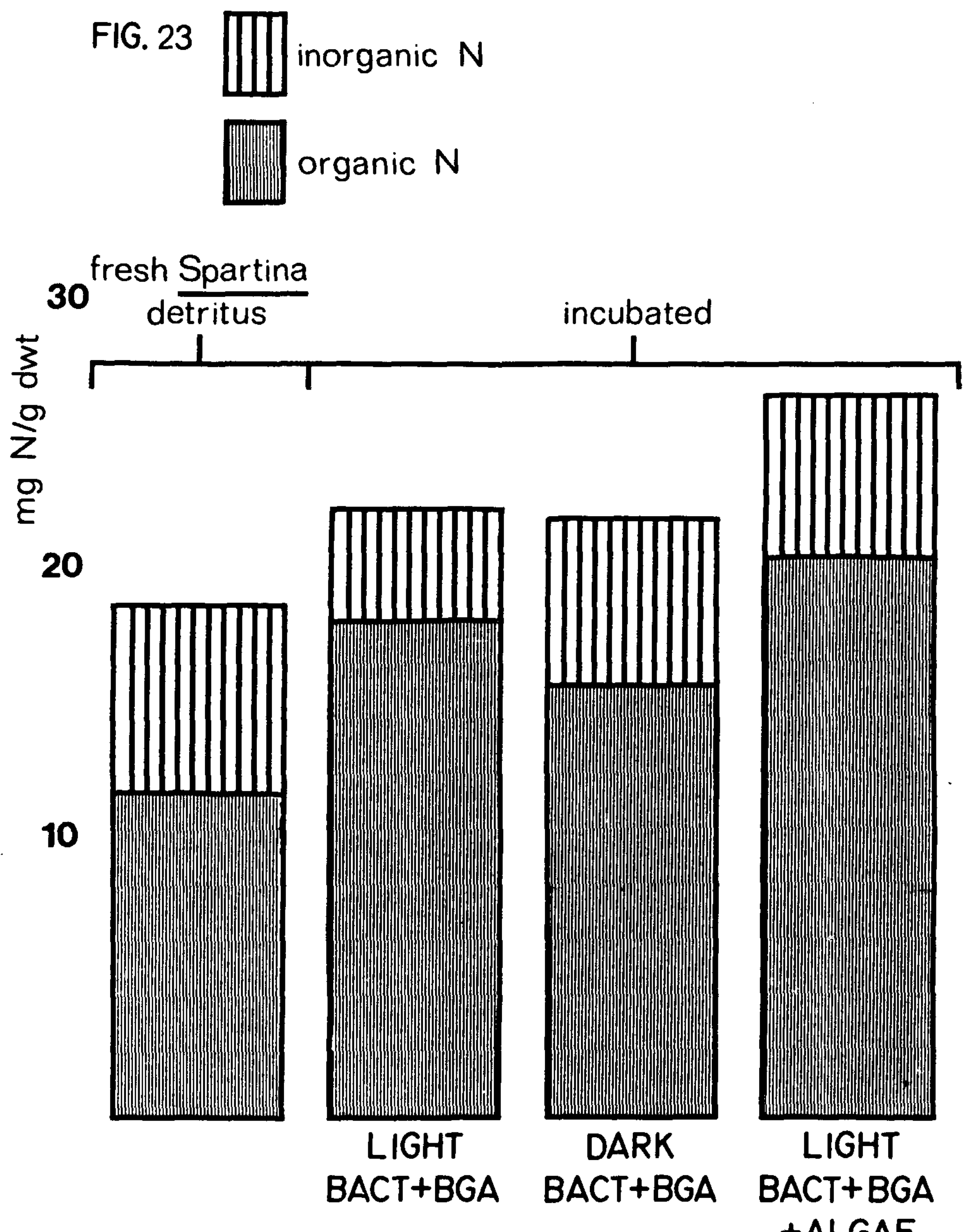


FIG. 23
inorganic N
organic N
fresh Spartina detritus
incubated
mg N/g dwt
30
20
10
LIGHT BACT+BGA
DARK BACT+BGA
LIGHT BACT+BGA +ALGAE

Figure 24. The rate of nitrogen fixation in the experimental microcosms. (25°C, 12 hr L/12 hr D) Sea water flowed through the vessels at 200 ml/day. Ranges are indicated by vertical line through mean datum point (two replicates).

FIG. 24

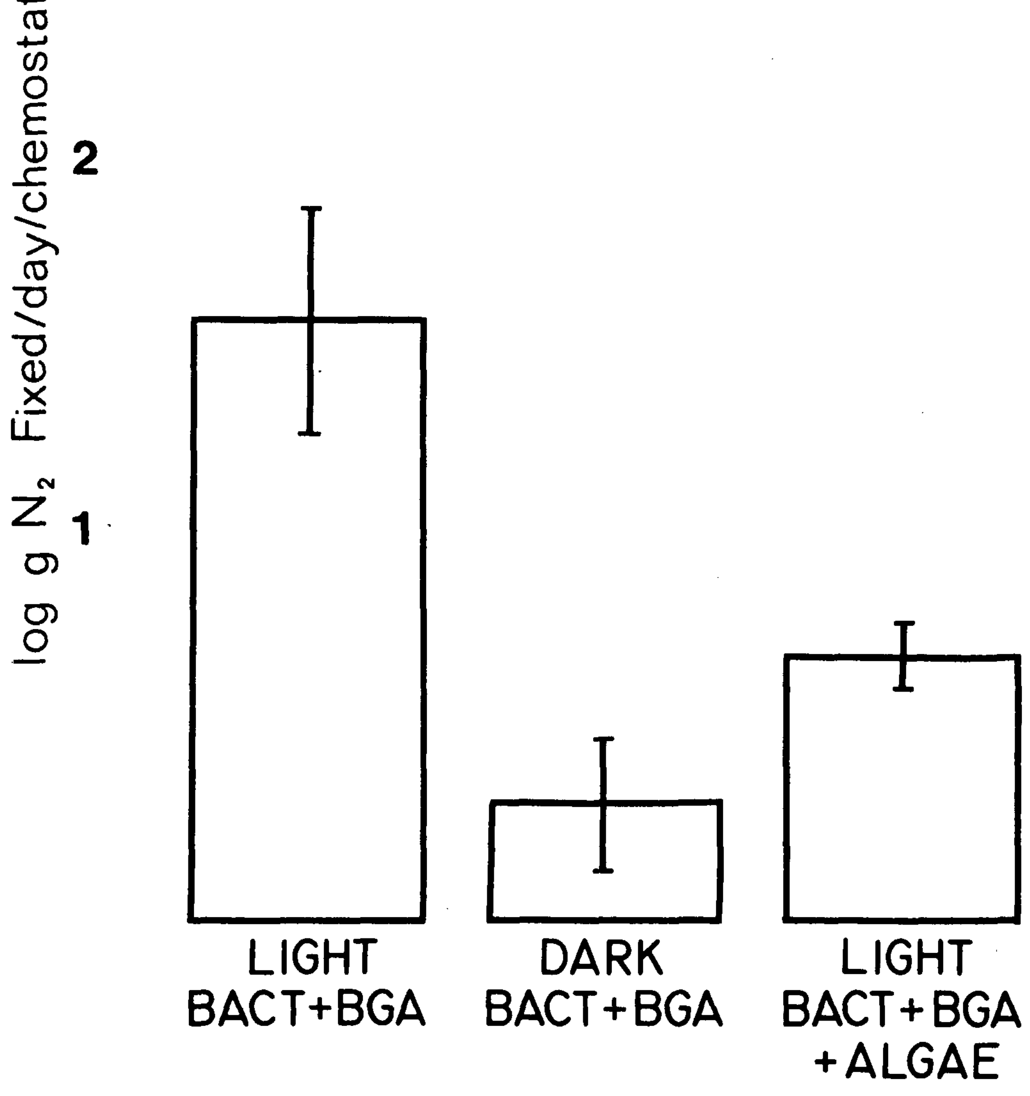
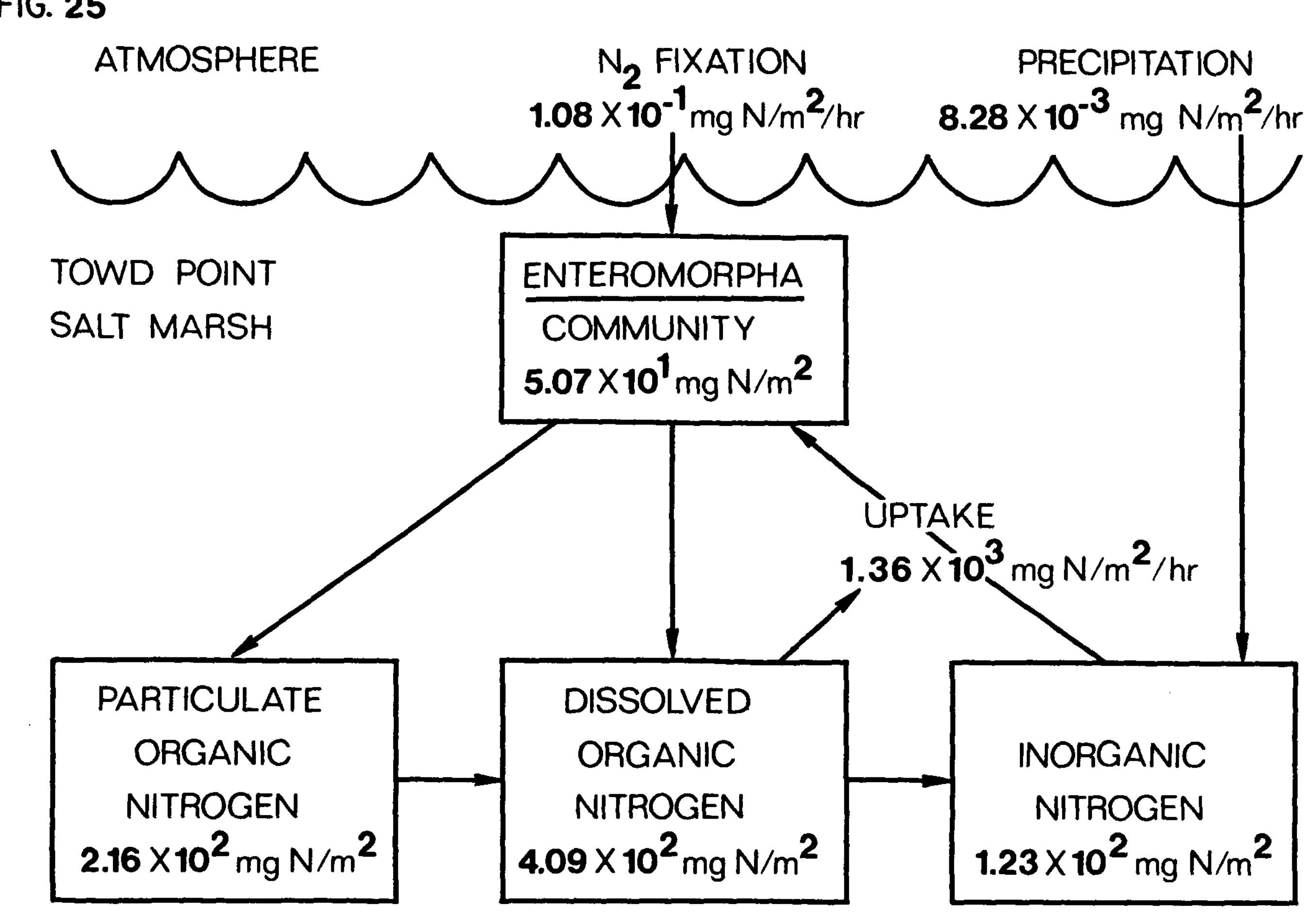


Figure 25. A schematic representation of parts of the nitrogen cycle for the Towd Point salt marsh.

FIG. 25



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