AN ABSTRACT OF THE THESIS OF Deborah A. Grosenbaugh for the Master of Science Degree in Biology presented May 2, 1979.

Title: Role of the blue-green alga Nostoc muscorum as a possible nitrate source to the groundwaters of Guam.

Approved: Roy T. Tsuda
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An investigation of the nitrogen-fixing blue-green alga Nostoc muscorum Ag. as a possible contributor of nitrate to the groundwater of Guam was carried out from October 1977 to January 1979. Total potential nitrate contribution was determined by combining data obtained for ammonia excretion (a maximum of 1.39 μg/NH_4/g Nostoc/hr), percent nitrogen (3.38%) and growth rates (40% increase/wk) with algal biomass estimates for Guam (2.6×10^6 kg) to obtain a value of 1.5 μg NO_3-N/mℓ rainwater. Soil percolate studies were similarly extrapolated to produce a corresponding value of 6.5×10^{-4} μg NO_3-N/mℓ rainwater. Both values are well below those obtained from the groundwater, i.e., ca. 2 μg NO_3-N/mℓ. It is concluded that Nostoc muscorum does not contribute significantly to the high nitrate content of Guam's groundwater.

ROLE OF THE BLUE-GREEN ALGA *NOSTOC MUSCORUM* AS A POSSIBLE NITRATE SOURCE TO THE GROUNDWATERS OF GUAM

by

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INTRODUCTION

Ever since Mink (1976) noted that the nitrate (NO_3) concentration of the groundwater on Guam often exceeds 9 mg/ℓ NO_3 (2 µg/mℓ NO_3-N), there has been a concern over the high nitrate content of Guam's groundwater, its major source of potable water. Recent analyses from the Guam Environmental Protection Agency (GEPA) have shown that this value often exceeds 5 mg/ℓ NO_3-N. The United States Environmental Protection Agency (EPA) has set 10 mg/ℓ NO_3-N as the maximum acceptable standard for potable water.

Nitrate itself is not toxic, but nitrate is readily converted to nitrite (NO_2) in the human body. Upon conversion to nitrite, the molecule is then instrumental in the conversion of hemoglobin to methemoglobin. Methemoglobin is rendered incapable of carrying oxygen to the tissues, the resulting condition being referred to as methemoglobinanemia (Phillips and Todd 1978).

Serious and occasionally fatal poisonings in infants have been reported following the ingestion of well waters containing nitrates. The 1958 International Standards for Drinking Water (World Health Organization) states that ingestion of nitrates in excess of 50-100 ppm (11.3-22.6 µg/mℓ NO_3-N) by infants less than one year old may give rise to methemoglobinemia (Camp and Meserve 1974).

Nitrite has also been implicated as a possible cancer-causing agent. Nitrite, in the body, combines with other organic compounds to form nitrosoamines, some of which are potent carcinogens (Phillips and Todd 1978).

The source of groundwater nitrate has been attributed to biological surface phenomena (Mink 1976). The groundwater originates as rainwater, which percolates through surface soils to a Ghyben-Herzberg lens. Mink (1976) states that if the groundwaters were in equilibrium with the natural growth and decay cycle of biological matter, then one would expect a maximum of 1.0 mg/ℓ NO_3 (0.23 μg/mℓ NO_3-N) to be present in the water. Since this value is often exceeded, we must seek alternative sources for the cause of the high levels. One possible contributor is the heterocystous blue-green alga *Nostoc muscorum* Ag. The legume *Leucaena leucocephala* (Lam.) DeWit (Chamorro name: tangentangen), with its nitrogen-fixing root nodules, has also been suggested as contributing fixed nitrogen to the groundwater (Mink 1976).

The purpose of this study is to determine what possible effect, if any, *Nostoc muscorum* has on the nitrate content of Guam's groundwater. If the total estimated biomass of the alga is sufficient to account for the intrusion of large amounts of combined nitrogen into the lens systems, then it might be possible to control nitrate concentrations by keeping the biomass of *N. muscorum* to a minimum. On the other hand, if it is shown that *N. muscorum* plays no more than a minor role in nitrate contribution, alternative sources must be sought.

Nostoc muscorum is a heterocystous blue-green alga which is abundant on the limestone soils above Guam's northern aquifer. *Nostoc muscorum* has been shown capable of reducing atmospheric dinitrogen to ammonia. It is among the most common of the nitrogen-fixing algae and is most abundant in tropical soil (Stewart 1973). Stewart et al. (1967), using the acetylene reduction technique, found that a species of Nostoc was capable of converting acetylene to ethylene at the rate of 1.62 Mμ moles C_2H_2/mg protein/min. If the conversion ratio (3.2:1) for C_2H_2 reduced:N_2 fixed of Stewart et al. (1968) is used, a value of 0.51 Mμ moles N_2 fixed/mg protein/min is obtained.

Nitrogenase, the enzyme responsible for the reduction of dinitrogen to ammonia, is common to all nitrogen-fixing organisms, differing only in minor chemical and physical characteristics (Stewart 1973). Nitrogenase consists of two metalloproteins that are inactive separately (Fogg 1974). Together they are about 275,000 MW and possess two molybdenum atoms at the active site. The presence of combined nitrogen inhibits the synthesis of nitrogenase, and various other compounds such as H_2, N_2O, NO and CO act as competitive inhibitors.

Nitrogenase requires ATP in order to reduce nitrogen. This requirement may be rate-limiting. The ATP requirement may also account for light dependency in nitrogen fixation. Other factors that have been shown to limit nitrogen fixation by nitrogenase are insufficient molybdenum, lack of phosphorus and low pH (Fogg 1974). Nitrogen fixation is also prevented by desiccation.

The overall equation for nitrogen fixation by nitrogenase is as follows:

$$3N_2 + 3H_2 + 12ATP \rightarrow 2NH_3 + 12ADP + 12P_i$$

Ammonia is thus the first stable product of nitrogen fixation. It is incorporated into the cellular metabolism as glutamine (Stewart 1973). Release of fixed nitrogen into the environment is accomplished primarily through decay of the plant tissue. Watanabe and Kiyohara (1960) reported that Bacillus subtilis Cohn was responsible for the release of 40 percent of the cell nitrogen as ammonia in ten days at 30°C in N. muscorum. It has also been shown that blue-green algae liberate combined nitrogen during the course of healthy growth (Fogg 1974).

Once the atmospheric nitrogen has been fixed in the form of ammonia, it then undergoes the process of nitrification to NO_2 and NO_3 by indigenous bacteria in the soil. Two different populations of soil bacteria are responsible for the different steps involved in this conversion (Alexander 1961). First, the Nitrosomonas population oxidizes ammonia to nitrite. This nitrite is then further oxidized to nitrate by the Nitrobacter population. The nitrate ions then percolate down through the permeable limestone into the fresh-water lens where they can be detected in the drinking water pumped up from wells.

MATERIALS AND METHODS

Both field and laboratory studies were conducted to derive information on the contribution of *Nostoc muscorum* to the nitrate content of Guam's groundwater. The field study consisted of harvesting the blue-green alga in selected areas above Guam's northern aquifer in order to derive values on biomass. Laboratory studies were designed to estimate the potential total nitrogen contributed by a given amount of the alga under partially controlled conditions and to estimate the total nitrogen contributed by a *Nostoc*-bacteria soil system under simulated in-situ conditions.

Estimates of Biomass

Estimates of the biomass of *Nostoc muscorum* on Guam were made on the basis of transects run in a selected area (Harmon) over the groundwater lens and on observations made in four ponding basins (Dededo, Barrigada, Latte Estates and Marianas Terrace) that discharge surface-storm runoff into the lens system.

A 1.0-km^2 area (Fig. 1) in Harmon was selected where *N. muscorum* was found in abundance and conditions for growth were favorable. Biomass studies made in this area would presumably give an upper limit estimate. The number of kilometers of roadway in the study area was determined from an aerial photograph obtained from the Guam Bureau of Planning. On the basis of these observations, the roads were divided into three types: type 1, well traveled, little to no *N. muscorum*; type 2, used only by local traffic, moderate

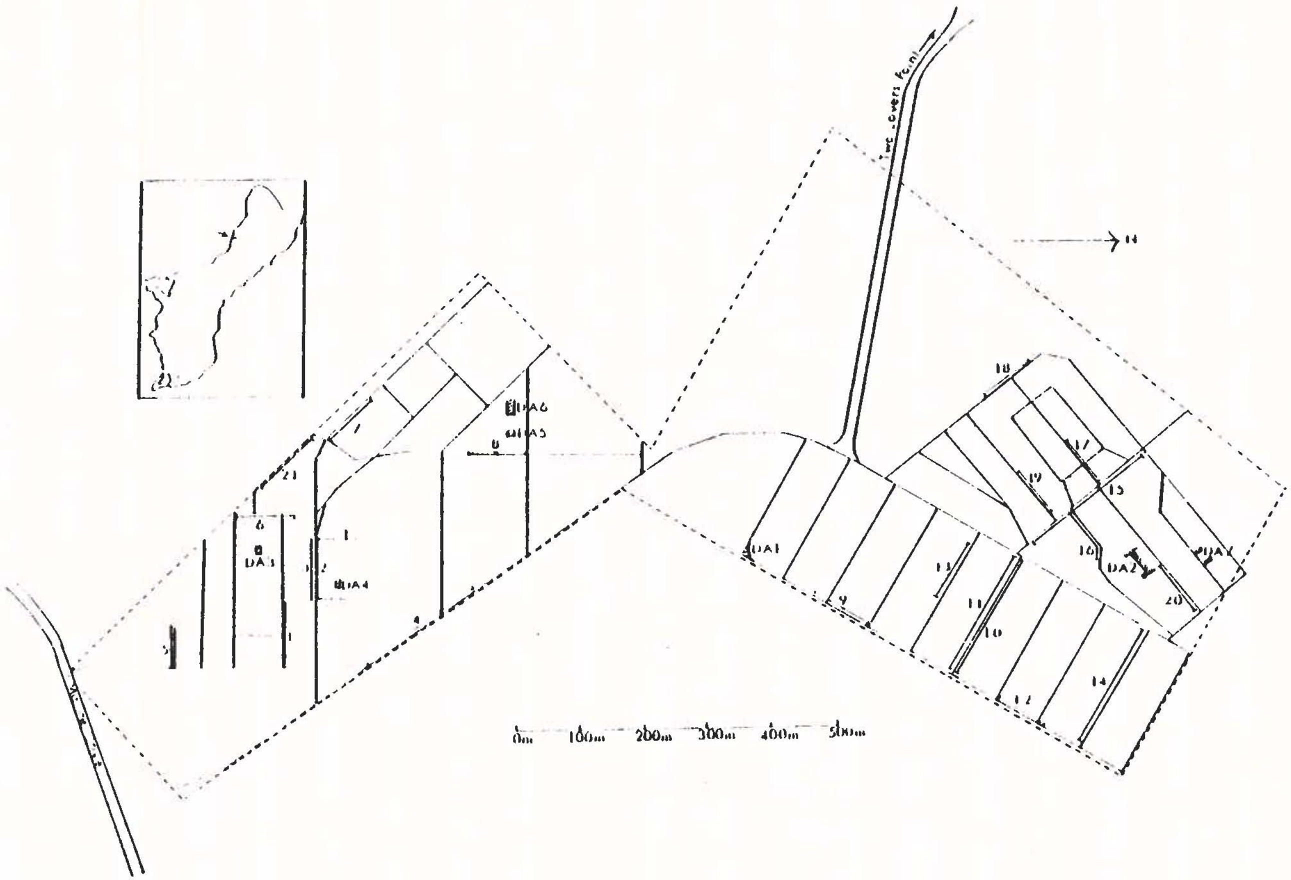

Figure 1. Harmon study area showing the location of the transects and "disturbed areas" from which biomass estimates were obtained. Dashed lines indicate the boundary of the study area and the hatched areas (designated as DA) show the location of the "disturbed areas".

amounts of *N. muscorum* present along the sides; type 3, little-used, overgrown, *N. muscorum* abundant over the entire road surface.

Twenty-one 100-m transects were run along the various road types during the wet months of October through December, 1978. *Nostoc muscorum* cover was noted at 0.5 m intervals, referring to it as sparse (clumps at widely spaced intervals), medium (even, but not total cover), or heavy (even, total cover). Samples from various transects were obtained to determine an average dry weight for sparse, medium and heavy coverage. Average biomass for the three road types was then determined and extrapolated to the entire area, taking into consideration large abandoned foundations (referred to as disturbed areas, DA in Fig. 1) where algal growth was particularly abundant. The biomass value for the study area was then expanded to include the entire surface area over Guam's northern aquifer, estimated at 2.6×10^2 km^2 (ca. 100 square miles).

Since the ponding basin system plays a significant role in recharging the lens system, four ponding basins (Fig. 2) were investigated to determine if the *N. muscorum* inhabiting the basins was abundant enough to contribute a significant amount of fixed nitrogen. Since each ponding basin varies in configuration, drainage and vegetation, different transect methods were employed for each of three of the ponding basins, Marianas Terrace having no apparent *N. muscorum* biomass (Fig. 3). The ponding basins under study were selected on the basis of their location over the lens, their soil type and accessibility.

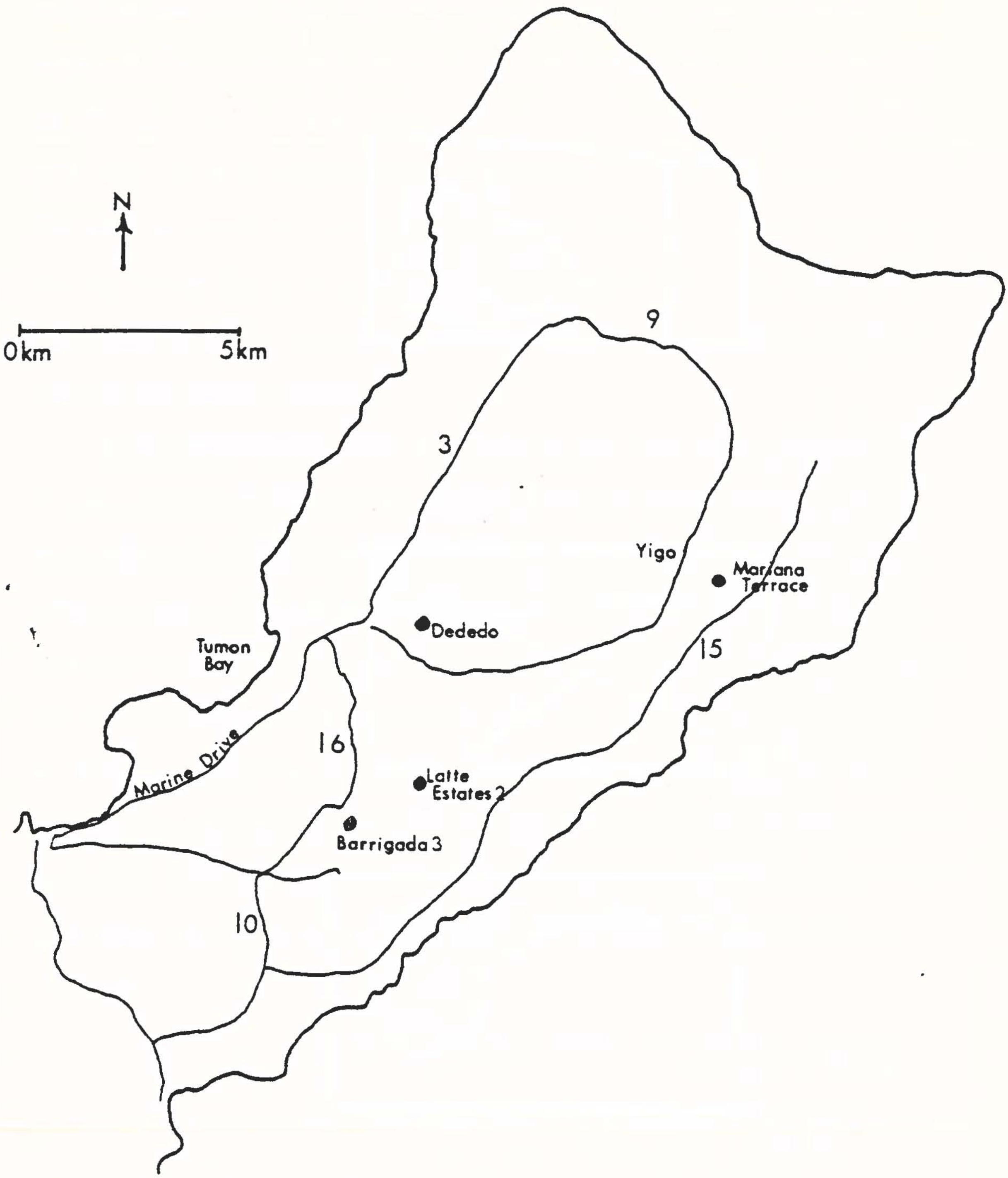

Figure 2. Location of ponding basins analyzed for Nostoc muscorum cover. Map from Zolan et al. (1978).

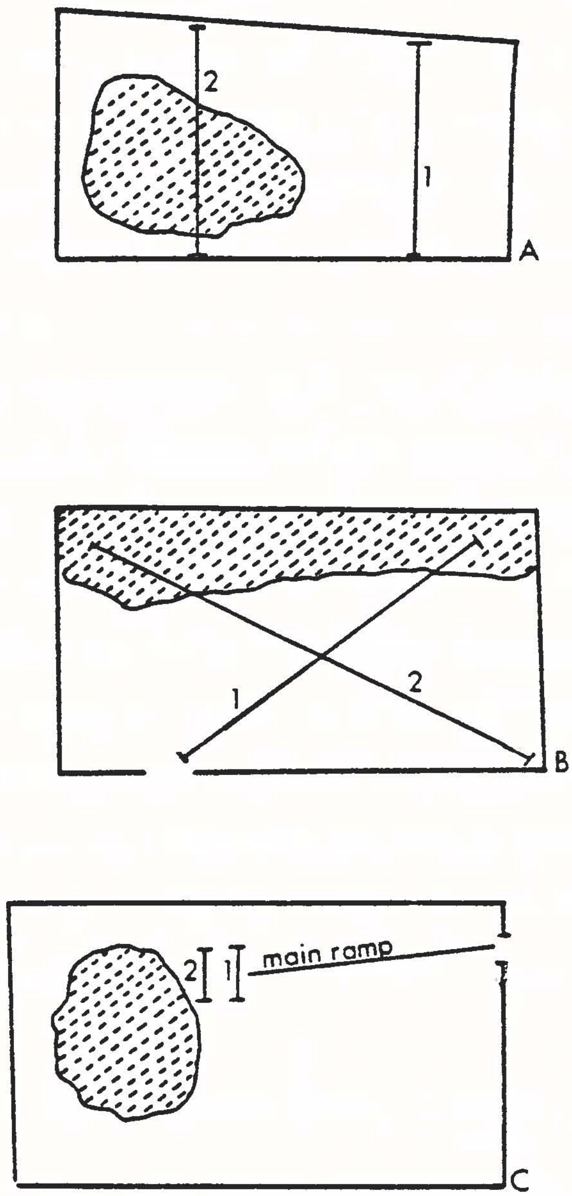

Figure 3. Location of transects at the three ponding basins. The ponding basins illustrated are A. Dededo, B. Barrigada No. 3, and C. Latte Estates No. 2. Hatched areas denote location of standing water.

Biomass estimates were derived primarily by running transects as shown in Fig. 3 and collecting all of the algae in a 0.25-m^2 quadrat on either side of the transect tape every 5m. The dry weight of the alga was then extrapolated to include a 0.5 m wide strip along the entire length of the transect. From this value algal biomass/m^2 was estimated for the entire 0.5 m wide strip and multiplied by the appropriate factor to account for the bottom surface area of the respective ponding basin.

A different method was employed in the case of Latte Estates No. 2. The occurrence of _N. muscorum_ was restricted to an area approximately 100 m wide surrounding the main ponding area. Algal cover in the ramp area was determined by running two 10-m transects and calculating biomass as stated above. _Nostoc muscorum_ surrounding the standing water was associated with low grass and thus difficult to sample. The area involved was estimated and the mean value for sparse coverage along the road transects was used to derive a biomass estimate. The summation of the estimates from the two areas provided a biomass value for the ponding basin. Transect lengths were as follows:

A. Dededo Ponding Basin - Transect 1 (70 m)
- Transect 2 (110 m)

B. Barrigada No. 3 - Transect 1 (80 m)
- Transect 2 (80 m)

C. Latte Estates No. 3 - Transect 1 (10 m)
- Transect 2 (10 m)

D. Marianas Terrace - No _Nostoc muscorum_

Potential Total Nitrogen Contributed by *Nostoc muscorum*

To obtain an estimate of total nitrogen that could potentially be converted to nitrate by nitrifying bacteria, three parameters were examined: (1) excretion of ammonia, the product of nitrogen fixation, directly into the surrounding medium by living tissue, (2) percent nitrogen present in the organism that would be released into the soil upon degradation, (3) growth rates of *Nostoc muscorum* to determine maximal rates at which degradation could occur.

Ammonia excretion. Excretion of ammonia was examined by using an ammonia ion electrode (Orion, Model 95-10) connected to a Beckman Expandomatic pH meter (Model SS-2). In all cases replicate samples of four were obtained.

Algal samples were washed thoroughly with deionized water to remove excess combined nitrogen. The samples were blotted dry and placed in covered Pyrex containers (no. 3250, approximately 350-mℓ capacity) so as to cover the bottom completely. Double-distilled, ammonia-free water (40 mℓ), containing 1 mℓ of a trace element mixture (2.86 g H_3BO_3, 1.80 g $MnSO_4 \cdot H_2O$, 0.222 g $ZnSO_4 \cdot 7H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, 0.018 g MoO_3 in 1 liter of H_2O adjusted to pH 6.7) was added so that the algae was partially immersed. In additional experiments, distilled water containing known amounts of ammonia was used to determine the effects of initial ammonia concentration on ammonia excretion. The preparations were then allowed to incubate for a 24-hr period under ambient light and temperature conditions or, in some cases, in the dark entirely. The water was then poured off and analyzed for ammonia concentration with the ammonia ion electrode. Controls were obtained by incubating the algal preparation for 10 min in order to determine initial contribution of ammonia by the algae. The change in ammonia concentration due to washing was monitored in separate experiments where it was found that the initial ammonia concentration decreased by an average of only 0.02 μg/mℓ as a result of washing. Ammonia excretion is expressed as μg NH_4/g <u>Nostoc</u>/hr, obtained by the following equation:

$$\frac{(\mu g/m\ell\ NH_4\ \text{sample}) - (\mu g/m\ell\ NH_4\ \text{control}) /\ (\text{g alga, dry wt})}{24\ \text{hrs incubation time}} \times 40\ m\ell$$

<u>Percent nitrogen</u>. Percent nitrogen content of the alga (dry wt) was determined by digesting about 0.1 g of algal material over heat with 3 mℓ of conc. H_2SO_4 and a 1 cm length of copper wire (no. 12) until the preparation turned white (about 5 hrs). The products were then quantitatively transferred to 100-mℓ volumetric flasks and diluted to that volume. Aliquots of 5 mℓ were basified with 1 mℓ of 10 N NaOH and subjected to microkjeldahl steam distillation (Bremner 1965) to determine TKN (total Kjeldahl nitrogen). Percent nitrogen was determined by the following equation:

$$\%\ N = \frac{(m\ell\ \text{titrated} - \text{blank})\ \ (.014)\ \ (N\ H_2SO_4)}{\text{weight of sample}}$$

<u>Algal growth</u>. Small pieces (.13 - 1.65 g wet wt) of freshly gathered <u>Nostoc muscorum</u> were placed in 50-mℓ beakers partially covered with Parafilm (American Can Co.) to allow gas exchange without excessive evaporation of the media. Initially, 2 mℓ of distilled water containing 1 mℓ/ℓ of the trace element mixture was introduced

to the bottom of the beaker. The beakers were then placed in a Biotronette Mark III environmental chamber (Lab-Line Instruments, Inc.) and incubated at 32°C and 350 ft-c for a 12-hr day. The samples were weighed weekly on an analytical balance. Media was added every few days to replace that which was lost due to evaporation.

Soil Percolation Experiments

A series of experimental soil flats were designed to obtain estimates of the ability of Nostoc muscorum, in association with nitrifying bacteria, to contribute nitrate to the groundwater system. Each fiberglass-coated plywood flat (Fig 4A) measured .65 m x 1.33 m x 10 m and had a small drain situated at one end for collecting the soil percolate. Eighteen flats were used and arranged in the manner as shown in Fig. 4B: two control flats having no soil, and eight each of two soil types, referred to as Barrigada clay and San Agustin clay (Fig. 5). Both soils are classified as Guam clays (Dr. J. L. Demeterio, personal communication). At approximately 1-month intervals, water percolate samples were obtained from the flats and analyzed for total nitrogen by microkjeldahl steam distillation (Bremner 1965).

Soil composition data for the experimental soil types were obtained as follows. Organic content was determined by the Walkley and Black method (Jackson 1958). Soil pH was determined by mixing 10 g of soil with 10 mℓ distilled water and reading the pH of the preparation after a standing period of 15 min. Physical soil composition data was obtained from Ms. Marylou Baccay (personal communication). Percent sand, silt and clay was determined by

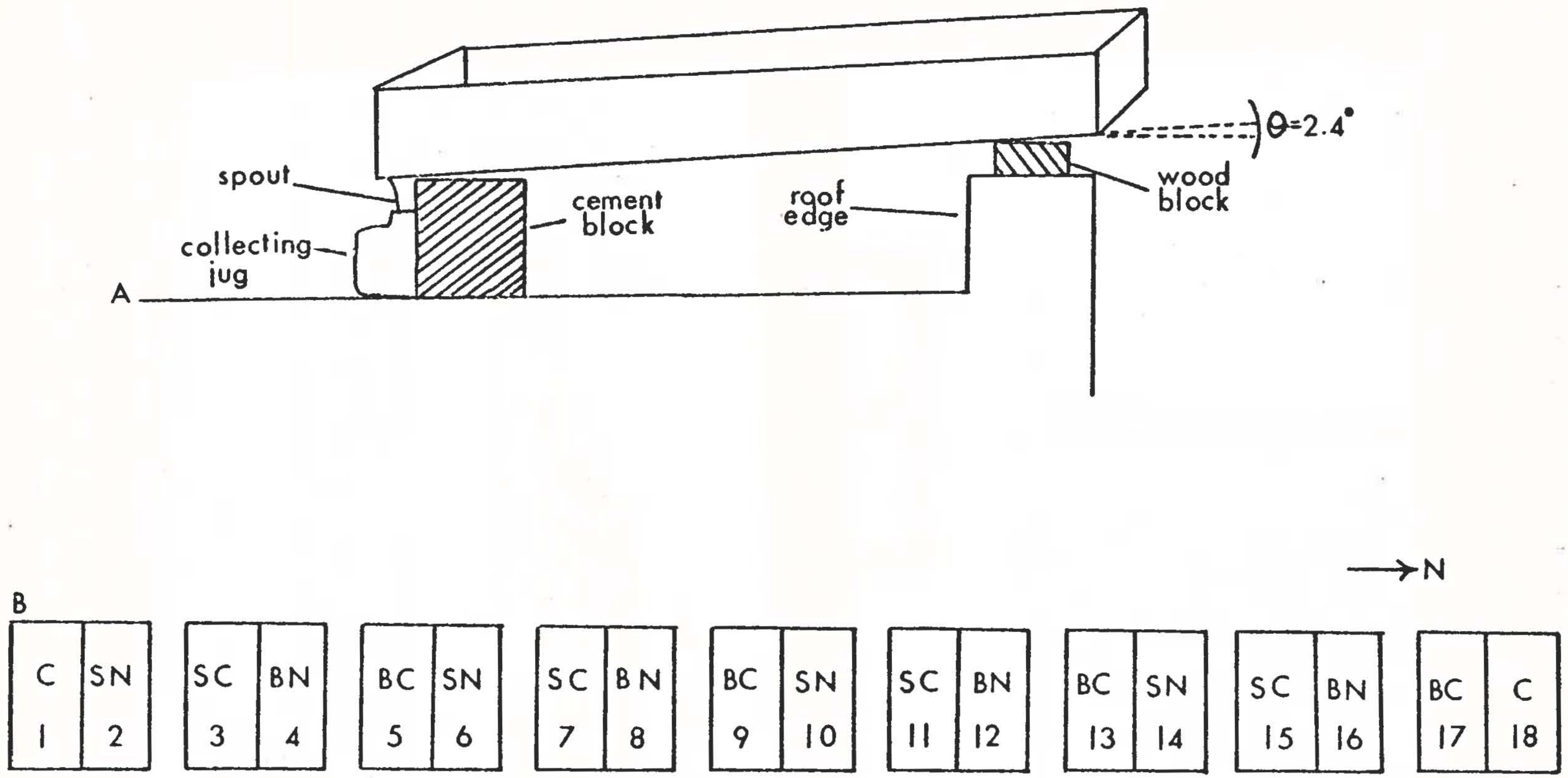

Figure 4. Experimental design for the soil percolate studies. A. Set-up of individual soil flats. B. Arrangement of soil flats. Explanation of symbols: C, control, no soil; SN, San Agustin clay with Nostoc; SC, San Agustin clay, bare soil; BN, Barrigada clay with Nostoc; BC, Barrigada clay, bare soil.

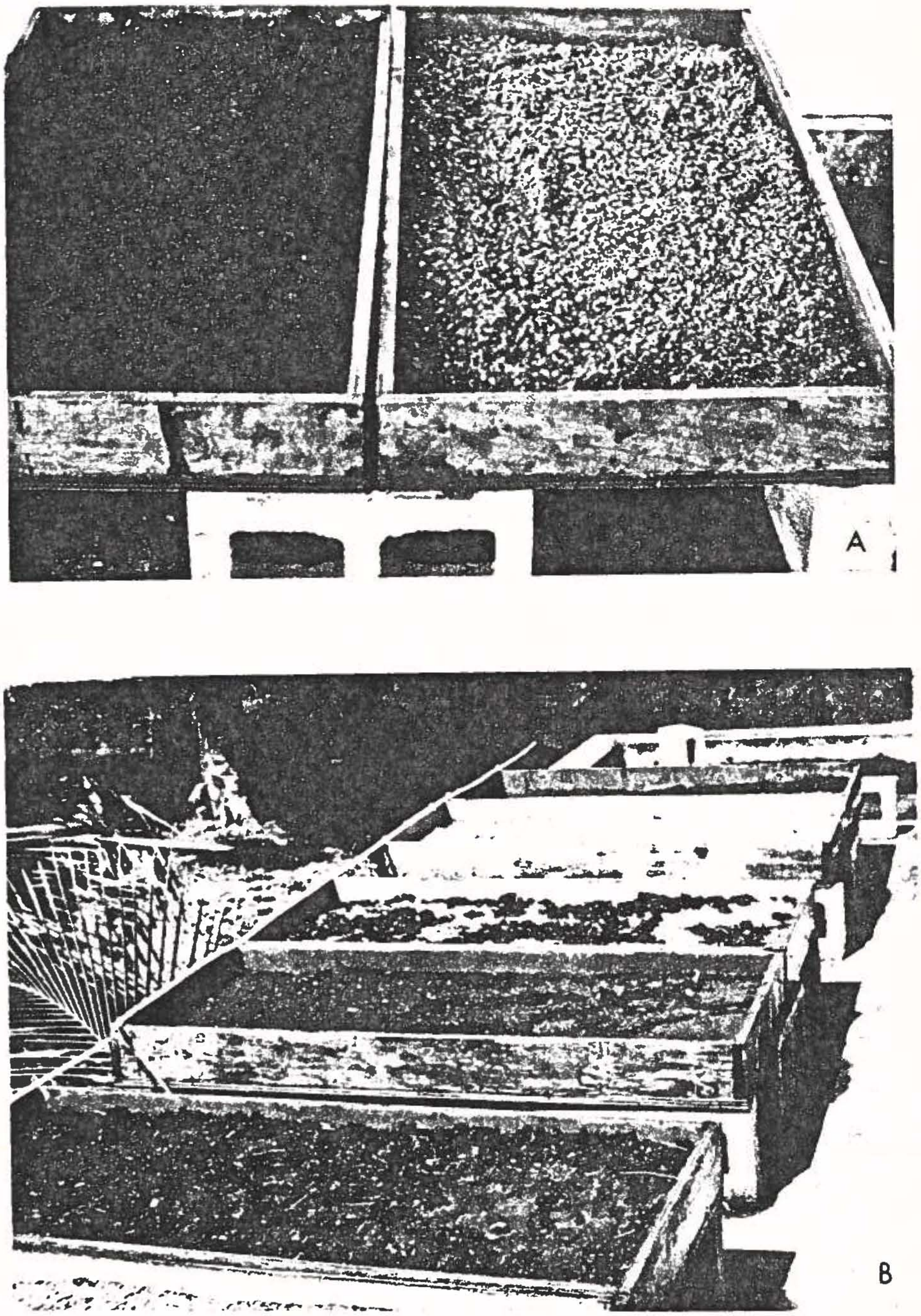

Figure 5. Experimental design for soil percolate studies before (A) and after (B) the addition of Nostoc muscorum.

hydrostatic means, where 50 g of the soil was thoroughly mixed with a known volume of water to which Calgon was added as a dispersing agent. Hydrometer readings were taken at 0 sec, 10 sec and 2 hr to determine percent sand, percent silt and clay and percent clay, respectively.

RESULTS

Biomass Estimates of Nostoc muscorum

Nostoc muscorum, on Guam, is found primarily along lightly traveled roads and in disturbed areas where ground cover is generally sparse, such as abandoned roads (Fig. 6A), old foundations, unused runways and on hard-packed clay surfaces (Fig. 6B) that are exposed to surface run-off.

Table 1 lists the dry weights of Nostoc muscorum for samples typical of sparse, medium and heavy coverage along various transects. Average dry weights per 100 m of roadway obtained using the values from Table 1, are listed in Table 2. The total biomass estimates are obtained by multiplying the average for each road type by 2 (to account for the other side of the road), divided by the transect length 0.1 km, and multiplied by the number of kilometers of each road type as determined from aerial photographs. Table 3 shows these calculations. An estimate of 10^{-2} kg/m^2 is obtained by adding the values obtained from the disturbed areas (see Table 1). If the area over the lens is 2.6×10^2 km^2, then an upper estimate of 2.6×10^6 kg for the biomass of N. muscorum is derived.

Guam's Department of Public Works (1969) requires that ponding basins be in existence where the natural seepage of groundwater is retarded due to development. These ponding basins provide a means for recharging the lens. Therefore, any Nostoc muscorum growing in these basins would directly affect the soil percolate originating as runoff from urban areas. The biomass estimates for these ponding basins are listed in Table 4.

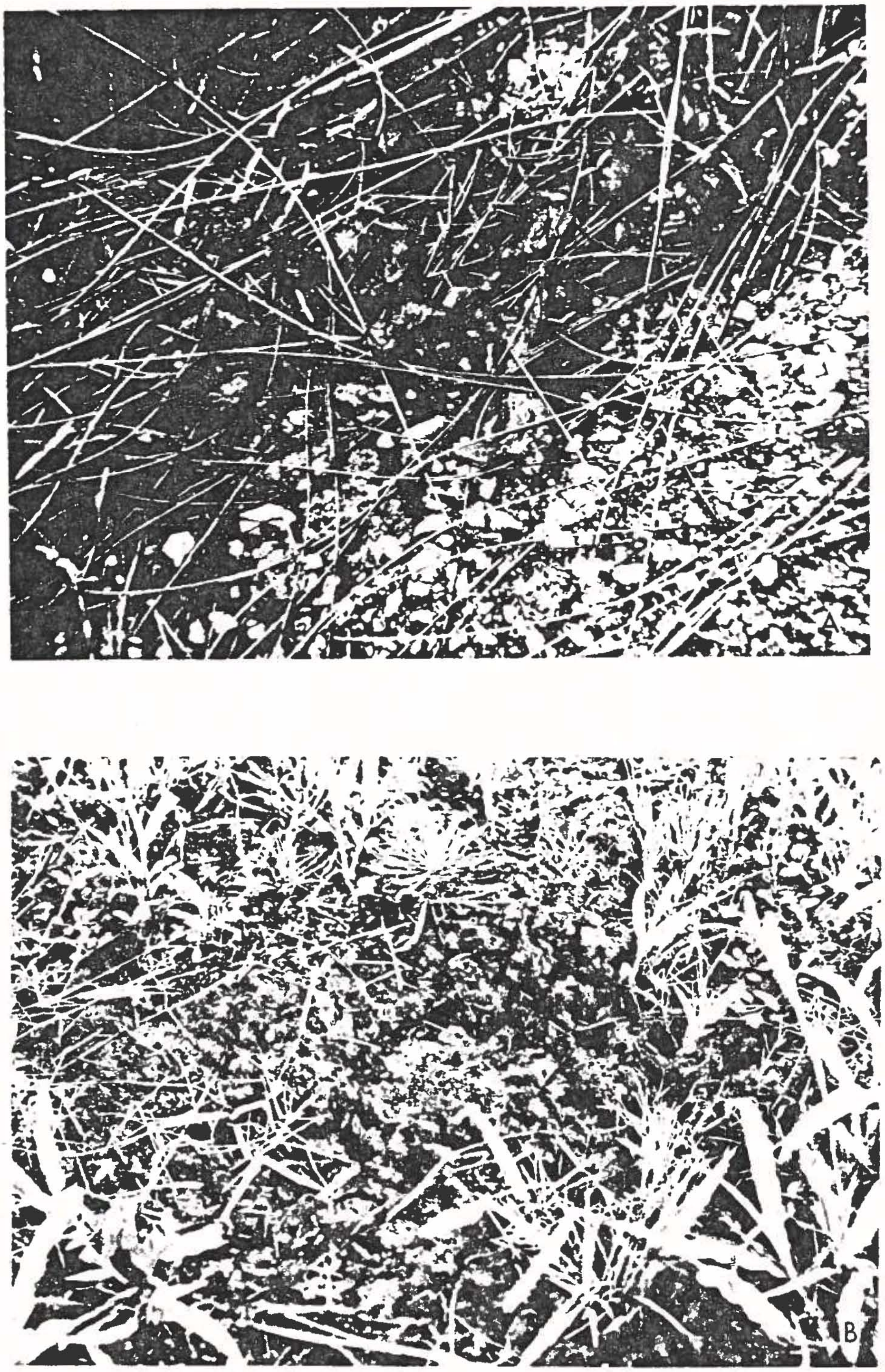

Figure 6. Habitat of Nostoc muscorum. A. abandoned asphalt road, B. hard-packed Guam clay.

Table 1. Dry weights of Nostoc muscorum for samples obtained from transects during October, November, and December 1978. See Fig. 1 for location of transects.

Transect	Date	Dry Weight (g)	Sample Size (m^2)	g/m^2
		SPARSE COVERAGE		
1	10-10	15.1	2.50	6.0
5	10-10	15.2	2.50	6.1
6	10-11	9.0	2.50	3.6
12	10-17	14.9	2.50	6.0
8	11- 8	18.0	2.50	7.2
3	12- 4	16.7	2.50	6.7
7	12- 4	12.6	2.50	5.0
2	12- 4	9.6	2.50	3.8
18	12-18	6.1	2.50	2.4
19	12-18	13.3	2.50	5.3
			$\bar{Y}$ = 5.2 g/m^2	
			s = 1.52	
		MEDIUM COVERAGE		
2	10-10	52.7	2.50	21.1
6	10-11	46.6	2.50	18.6
1	10-10	35.4	2.50	14.2
15	10-17	158	2.50	63.2
17	10-17	182	2.50	72.8
9	10-17	35.4	2.50	14.2
20	10-17	114	2.50	45.6
19	11- 8	148	2.50	59.2
8	11- 8	128	2.50	51.2
7	12- 4	48.8	2.50	19.5
			$\bar{Y}$ = 38.0 g/m^2	
			s = 22.7	
		HEAVY COVERAGE		
2	10-10	226	2.50	90.4
21	11- 8	122	1.25	97.6
8	11- 8	186	1.25	149
2	11- 8	172	1.25	138
20	10-19	188	1.25	150
7	12- 4	150	1.25	120
6	12- 4	126	1.25	101
1	12- 4	215	1.25	172
18	12-18	165	1.25	132
15	12-18	237	1.25	190
			$\bar{Y}$ = 134 g/m^2	
			s = 32.7	
		DISTURBED AREAS		
1	10-19	375	2.50	150
2	12-18	238	1.25	190
3	10-19	636	1.0	636
			$\bar{Y}$ = 325	

Table 2. Dry weight (g) of Nostoc muscorum per 100 m on different types of roads.

Transect No.	Sparse $\bar{Y}$ = (5.2 g/m^2)	Medium $\bar{Y}$ = (38 g/m^2)	Heavy $\bar{Y}$ = (134 g/m^2)	Total
		ROAD TYPE 1		
2	16	342	3216	3.57×10^3
3	130	-	-	1.30×10^2
4	-	-	-	-
				1.23×10^3
		ROAD TYPE 2		
7	109	4522	804	5.44×10^3
9	109	38	-	1.47×10^2
10	-	-	-	-
11	10	-	-	1.00×10^1
12	5	1140	268	1.41×10^3
13	36	1216	-	1.25×10^3
14	-	-	-	-
15	21	304	402	7.27×10^2
16	36	1216	134	1.39×10^3
17	78	1254	268	1.60×10^3
18	88	266	-	3.54×10^2
19	52	570	-	6.22×10^2
21	5	874	4288	5.17×10^3
				1.61×10^3
		ROAD TYPE 3		
1	42	76	8308	8.43×10^3
5	78	38	-	1.16×10^2
6	57	304	6164	6.52×10^3
8	26	3534	5226	8.79×10^3
20	135	228	9648	1.00×10^4
				6.77×10^3

Table 3. Total biomass of Nostoc muscorum for a selected 1 km^2 area.

TOTAL ALONG ROADS

Type 1 Roads -

$$\frac{(1.23\ kg)\ (2)}{(0.1\ km)} \times (34\ km) = 836\ kg$$

Type 2 Roads -

$$\frac{(1.61\ kg)\ (2)}{(0.1\ km)} \times (70\ km) = 2254\ kg$$

Type 3 Roads -

$$\frac{(6.77\ kg)\ (2)}{(0.1\ km)} \times (35\ km) = 4739\ kg$$

total 7829 kg / 10^6 m^2

TOTAL IN DISTURBED AREAS

Total calculated area of disturbed areas: 4709 m^2

Average dry weight for disturbed areas: 0.325 kg/m^2

Total biomass in disturbed areas: 1530 $kg/10^6\ m^2$

TOTAL FOR 1 KM^2 STUDY AREA

Road + Disturbed areas = 9359 $kg/10^6\ m^2$ = $9.36 \times 10^{-3}\ kg/m^2$ = $10^{-2}\ kg/m^2$ = $10^4\ kg/km^2$

Table 4. Dry weight (kg) estimates for Nostoc muscorum in four ponding basins situated over the groundwater lens.

Site	Soil Type	Area	Dry Weights of Algae
Dededo	Barrigada limestone; overlying Guam clay*	31,600 m^2	423 kg
Barrigada No. 3	Mariana limestone; overlying Guam clay	2,500 m^2	22 kg
Latte Estates No. 2	Barrigada limestone; overlying Guam clay	2,000 m^2	7.3 kg
Mariana Terrace	Mariana and Barrigada limestone; overlying Chacha-Saipan and Saipan-Yona-Chacha clay*	25,000 m^2	0.0 kg

*The U.S. Soil Taxonomy method describes Guam clay as belonging to the Inceptisol order, subgroup Lithic Ustropepts; and Chacha clay as belonging to the Inceptisol order, subgroup Oxic Ustropepts (Park 1979).

In most cases, the flora in the ponding basins is of sufficient density to exclude any large amounts of *N. muscorum* growth. One ponding basin (Dededo) did support a biomass of 423 kg of *N. muscorum* presumably attributable to a scarcity of dense plant growth. This particular ponding basin is a possible influx point for NO_3-N into the lens which will be discussed later in the discussion section. Since it has been shown that NO_3-N levels of water entering the ponding basins is low at an average of 0.033 $\mu g/m\ell$ NO_3-N (Zolan et al. 1978) and the biomass of *N. muscorum* in these basins is not generally high, the ponding basins in themselves are probably not a significant source of NO_3 contribution with respect to *N. muscorum*.

Potential Total Nitrogen Contributed by *Nostoc muscorum*

Ammonia excretion. The results of the ammonia excretion experiments are presented in Fig. 7. The change in the ammonia concentration in the media is plotted against initial ammonia concentration. Data points from samples incubated in the dark for the entire 24-hr period were grouped together with those samples exposed to daylight after an analysis of covariance (Sokal and Rohlf 1969) showed them to be on the same regression line.

This lack of light dependency is not in accordance with nitrogen fixation studies (Steward 1973) that indicate a definite positive correlation between light and rates of nitrogen fixation. However, since excretion, rather than fixation, is being measured, the same correlation would not be expected to exist, as excretion rates are more strongly dependent on the ammonia concentration of the surrounding media (Fogg 1971).

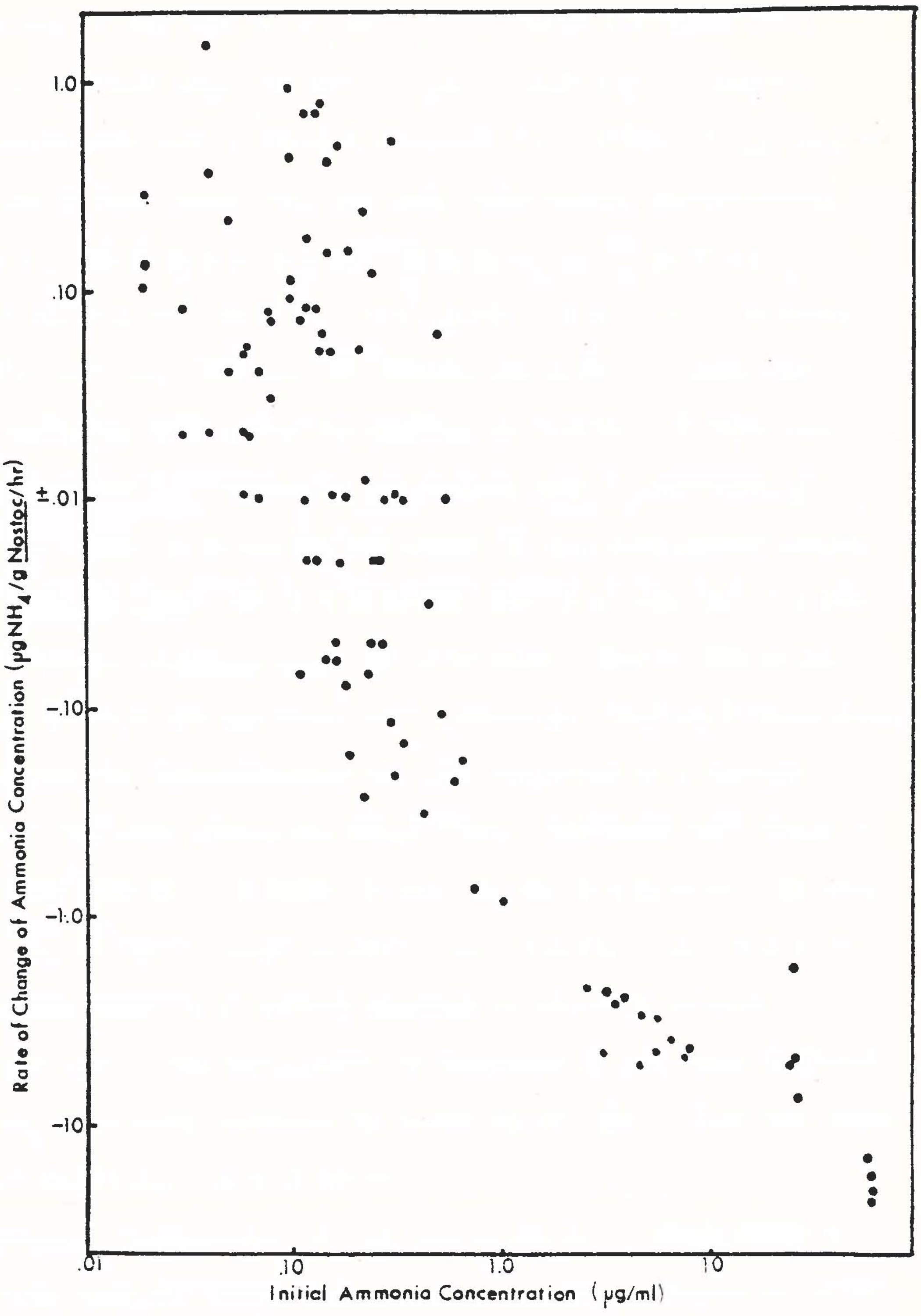

Figure 7. Initial ammonia concentration versus rate of ammonia uptake or excretion by Nostoc muscorum.

Regression analysis of 96 data points (Sokal and Rohlf 1969) shows a significant negative correlation between initial ammonia concentration and rate of ammonia excretion ($t = -13.21$; $t_{.001}$ $(60) = 3.460$), and further shows that the best relationship approximating the data is logarithmic with the y-intercept at 1.39 μg NH_4/g Nostoc/hr and the x-intercept at 1.05 μg/mℓ. In Fig. 7, it appears that the x-intercept (the initial ammonia concentration where no ammonia excretion exists) may be somewhat to the left of this value, but the y-intercept (the theoretical maximum ammonia excretion at zero initial ammonia concentration) seems to be a good approximation.

Percent nitrogen. Table 5 presents results of analysis for the nitrogen content in Nostoc muscorum in various stages of hydration. Since the raw data are expressed as a percentage, they were transformed using the arcsine transformation and then subjected to a one-way analysis of variance (Sokal and Rohlf 1969). The means were found to differ significantly to a level of $\alpha = 0.001$ which represents the area to the right of the critical value of the F distribution. The data were then subjected to a two-way analysis of variance without replication (Sokal and Rohlf 1969) to determine if the means differed significantly between locations or condition of algae. This was found to be not significant in both cases.

A possible explanation for the random variation among means is that certain samples contain differing amounts of extraneous materials. Although every effort was made to remove the materials, the small size of the sample (0.1 g) would allow any small amount of debris to enter into the calculations.

Table 5. Percent nitrogen in Nostoc muscorum plant tissue from various sites, sampled while in various stages of hydration. The data represented are means of the actual data points, where the number of samples from which they were derived is given in parentheses.

	Dededo Ponding Basin	Harmon Transect 21	Harmon Transect 8	Nimitz Hill
Moist Bloom	3.15%(n=2)	3.05%(n=3)	2.67%(n=3)	4.14%(n=6)
Dry Top Crust	2.74%(n=3)	3.46%(n=3)	3.10%(n=6)	3.40%(n=3)
Completely Desiccated	3.55%(n=3)	3.96%n=3)	3.40%(n=4)	3.34%(n=4)

The means were determined by multiplying each mean by its respective value of n, then dividing the resulting sum by the sum of n to obtain a value of 3.38 percent nitrogen or 33.8 g per kg dry weight of Nostoc muscorum. In terms of potential nitrate contribution, this is equal to 150 g NO_3 per kg N. muscorum if the ratio of the molecular weights of NO_3:N is taken to be 4.43.

Algal growth. Table 6 presents the results of the Nostoc muscorum growth experiments, which are graphed in Fig. 8. It can be seen readily in Fig. 8 that biomass increase per week is a function of initial biomass. The percent increase per week for each of 18 samples is listed in Table 6. The average of the means is 42 percent with a standard error of the mean of 0.306, setting the 95 percent confidence limits between 27 percent and 57 percent for weekly algal biomass increase (Sokal and Rohlf 1969).

Since frequent observations of field areas indicate that the yearly increase in the biomass of N. muscorum is not noticeable, it is assumed that approximately 40 percent of the algal biomass per week undergoes degradation during the periods of algal bloom.

Nitrate-Nitrogen Percolation Through Soil

The characteristics of the two experimental Guam clays used in this phase of the study are presented in Table 7. Table 8 lists the values obtained for NO_3-N in μg/mℓ for those soil flats containing Nostoc muscorum. The mean values for each soil type minus their respective controls are presented in Fig. 9 with the mean daily rainfall for each sampling period (Table 9). As expected, the NO_3-N

Table 6. Weekly wet weights (g) of Nostoc muscorum growth samples and their respective mean percent weekly increase in parenthesis.

	Sample Number																	
Date	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
11/21/78	.164			.201		.286	.266			.250			.233		.222		.289	
11/28/78	.254 (55)			.435 (116)		.451 (58)	.391 (73)			.309 (24)			.335 (44)		.283 (28)		.542 (88)	
12/5/78	.260 (2)			.462 (6)		.537 (19)							.403 (20)				.589 (9)	
12/12/78	.375 (44)					.581 (8)							.483 (20)				.627 (6)	
12/19/78		.237			.340			.574			.367					.520		.389
12/26/78		.429 (81)			–			1.43 (149)			.735 (100)					–		.461 (18)
1/2/79		.561 (31)			.663 (48)			1.78 (24)			.830 (13)					.992 (46)		.925 (101)
1/9/79		.661 (18)			.973 (47)			1.89 (6)			.948 (14)			.115		1.44 (45)		1.02 (10)
1/16/79			1.65		1.09 (10)				1.58			1.12		.134 (16)		1.76 (22)		1.07 (5)
1/23/79			3.27 (98)		1.83 (71)				2.24 (42)			2.70 (141)		.139 (4)		2.36 (34)		
$\bar{Y}$ % Weekly Increase	34	43	98	61	45	28	73	60	42	24	42	140	28	10	28	38	34	34

% Weekly Increase for 18 Samples $= \frac{\bar{Y}_i n_i}{n_i} = 40\%$

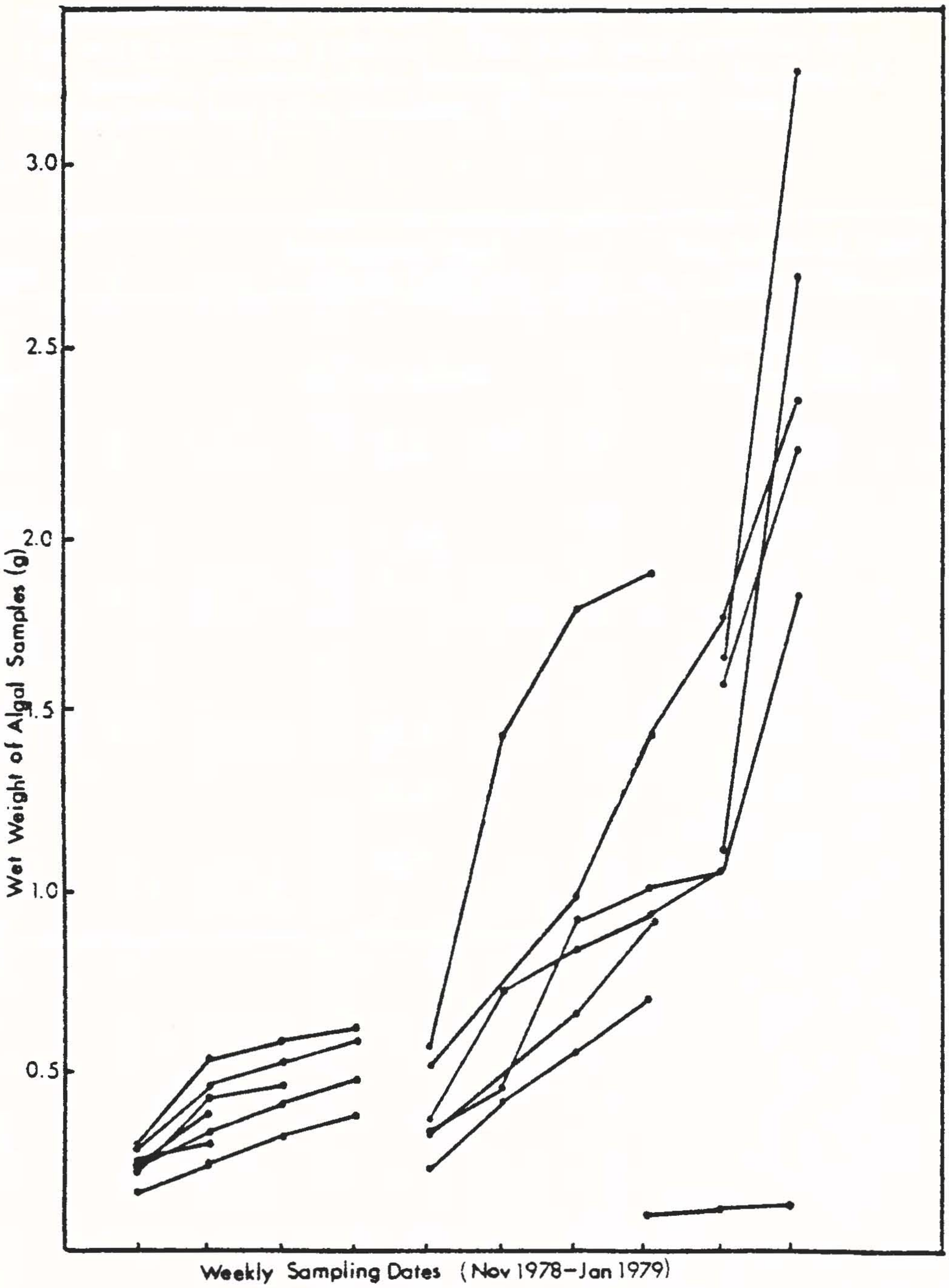

Figure 8. Growth rates for <u>Nostoc</u> <u>muscorum</u>.

Table 7. Characteristics of the two experimental Guam clays used for soil percolate studies. The data were provided by Ms. Baccay, chemist, Soil Testing Laboratory, College of Agriculture and Life Sciences, University of Guam.

Parameter	Barrigada Type Guam Clay	San Agustin Type Guam Clay
Drainage	well-drained	moderately well-drained
pH	6.4	7.3
Organic Matter (% total)	1.39	5.07
Physical Composition (% total)		
Sand	36.7	22.0
Silt	14.6	30.0
Clay	48.7	48.0

Table 8. Nitrate-nitrogen (μg/mℓ) in rainwater percolates from soil flats containing <u>Nostoc</u> <u>muscorum</u>.

Sampling Dates		12/29	2/1	3/14	6/30	7/20	9/1	10/6	11/18	12/28
Barrigada Guam Clay	No. 4	5.84	5.61	9.17	8.78	11.38	10.99	15.42	8.92	15.41
	8	11.86	6.51	9.69	1.40	-0.29	-5.73	-0.13	-0.15	1.63
	12	0.19	-1.18	11.38	4.38	4.89	9.31	8.55	6.74	16.42
	16	-7.50	-0.54	0.10	5.93	0.23	1.40	8.16	4.13	10.04
$\bar{Y}$ ($s_{\bar{Y}}$)		10.87(4.12)	7.63(2.01)	12.28(2.54)	9.04(1.54)	8.62(2.71)	17.50(3.86)	13.06(3.18)	16.03(1.58)	19.25(3.39)
$\bar{Y}$ control ($s\bar{Y}$)		8.27(3.32)	5.03(1.11)	4.70(1.93)	3.92(0.97)	4.57(1.06)	13.51(5.27)	5.06(2.40)	10.08(3.34)	8.38(1.93)
$\bar{Y}$ - $\bar{Y}$ control		2.60	2.60	7.58	0.13	4.05	3.99	8.00	5.95	10.87
San Agustin Guam Clay	No. 2	6.31	-51.39	47.06	-0.55	5.67	15.36	7.96	5.15	20.38
	6	-19.34	41.46	44.08	-2.11	5.15	17.69	-1.94	9.65	21.25
	10	94.92	48.13	15.30	-3.02	4.24	-0.46	4.28	2.10	5.88
	14	-6.38	19.91	-2.20	-0.42	9.82	13.15	6.62	11.82	15.16
$\bar{Y}$ ($s_{\bar{Y}}$)		54.76(25.88)	102.98(22.78)	82.19(11.83)	17.70(0.63)	14.29(0.30)	25.51(4.07)	10.05(4.22)	9.97(2.03)	33.00(3.53)
$\bar{Y}$ control ($s_{\bar{Y}}$)		35.88(29.47)	88.45(35.28)	56.13(13.98)	19.22(2.33)	9.24(1.22)	14.07(2.98)	5.83(1.65)	5.15(1.62)	17.33(4.68)
$\bar{Y}$ - $\bar{Y}$ control		18.88	14.53	26.06	-1.52	5.05	11.44	4.22	4.82	15.67

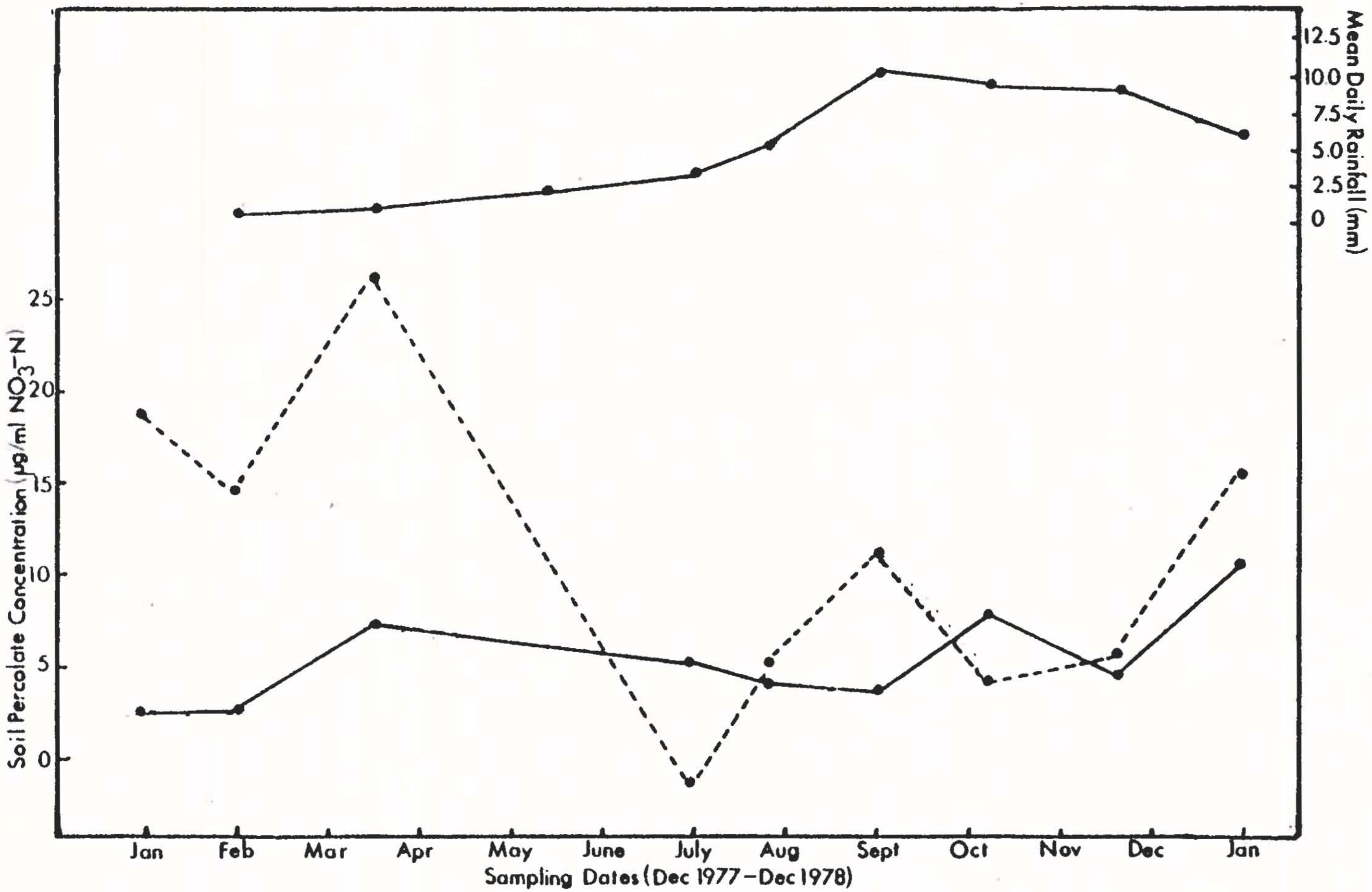

Figure 9. Mean nitrate-nitrogen percolate concentrations in excess of controls for Barrigada (——) and San Agustin (----) Guam clays and the corresponding mean daily rainfall.

Table 9. Compiled rainfall data for each sampling period.*

Dates	Total Rainfall (mm)	Mean Daily Rainfall (mm)	Days With No Rainfall (%)	Days Exceeding 13 mm Rainfall (%)**
12/29/77 2/1/78	37.6	1.02	51	0
2/2/78 3/14/78	54.6	1.27	60	2
3/15/78 6/30/78	378	3.56	34	8
7/1/78 7/20/78	106	5.33	40	10
7/21/78 9/1/78	441	10.4	17	26
9/2/78 10/6/78	323	9.40	6	26
10/7/78 11/18/78	380	9.14	12	26
11/19/78 12/28/78	238	6.10	18	13

*Data from the first three sampling periods were obtained from Fleet Weather Central (NAS rain gauge). Subsequent data were the result of daily monitoring of a rain gauge situated at the study site.

**Amount of rainfall required to saturate flats.

in the percolate from the San Agustin clay was initially higher than for the Barrigada clay because of the relatively high organic content of the San Agustin clay; but these values dropped to levels comparable to the Barrigada clay after an increase in mean daily rainfall, presumably due to leaching of nutrients by excess rainwater.

In Fig. 10, the absolute values for NO_3-N levels in the percolate are obtained, indicative of the high organic content of the San Agustin clay. If mean control values are subtracted, obtaining net NO_3-N production values, then the mean of the two soils becomes 5.50 μg/mℓ NO_3-N and 6.61 μg/mℓ NO_3-N for Barrigada and San Agustin clays, respectively. Any discrepancy in these values is probably due to a greater degradation of <u>Nostoc muscorum</u> on the San Agustin clay discussed later.

During the first six months (three sampling periods of the experiment), little rain fell. Samples were obtained by artificial flooding. Therefore, values from these flats most likely reflect residual NO_3-N in the soils as the alga was almost continuously in a desiccated state (Table 9). Also, it can be noted in Table 8 that the standard error is large for these points indicating that several outside factors were operating. For this reason, the analysis of experimental results will be confined to the months of July through December when sufficient rain fell for the <u>Nostoc muscorum</u> to remain in a hydrated state for a large percentage of the time (Table 9). Also, standard errors drop sharply at the time of this sampling (Table 8).

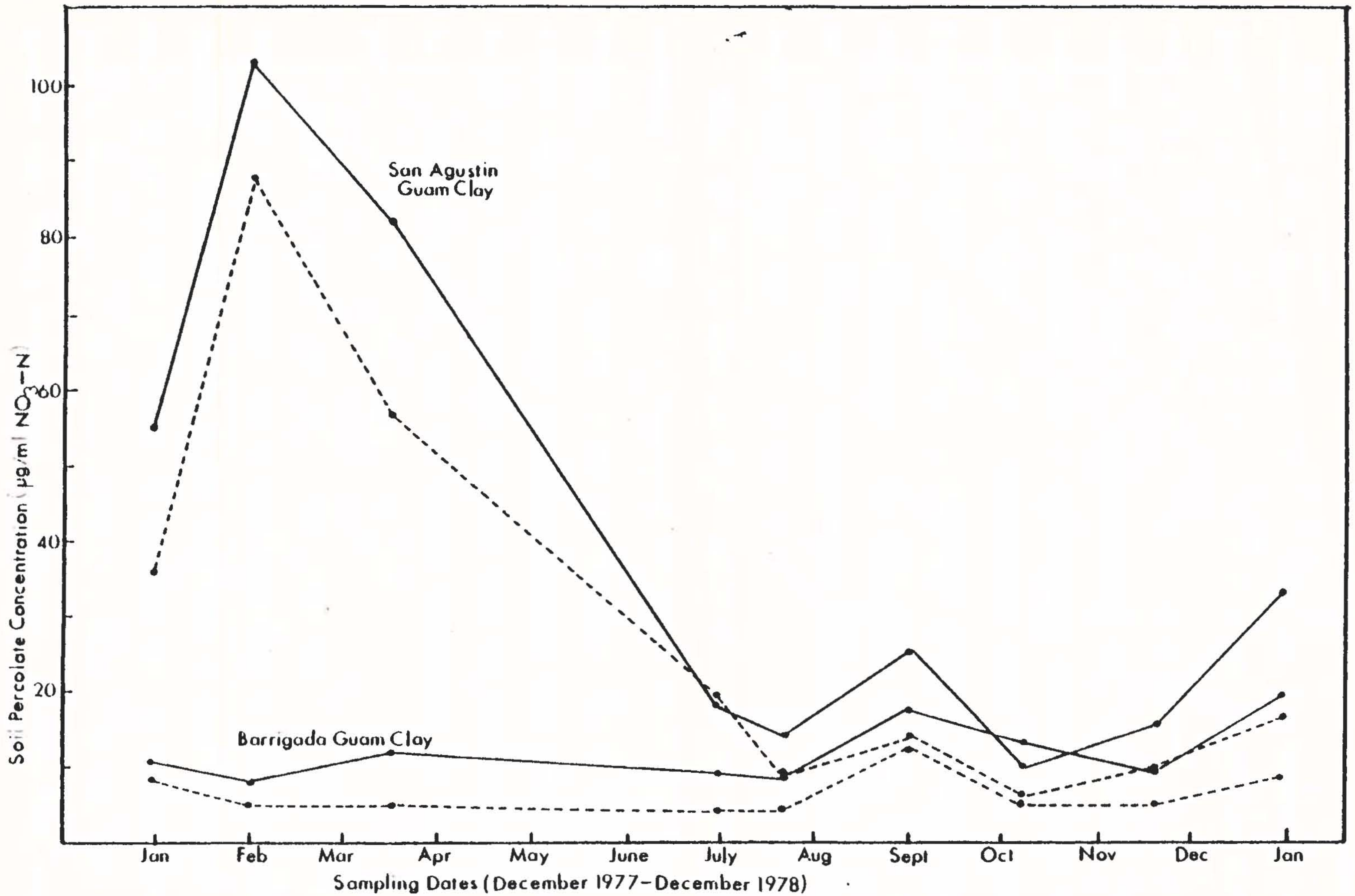

Figure 10. Mean nitrate-nitrogen percolate concentrations (µg/mℓ) for Barrigada and San Agustin Guam clays (——) and their respective controls (---).

It can be seen from Fig. 10, in all cases but one (San Agustin clay in July), that the control flat percolates are lower than the percolates from those flats containing Nostoc; the means of the ratios (Nostoc: control) for July through December are 2.06:1 for San Agustin soil and 2.05:1 for the Barrigada soil. Also evident is the fact that values from the San Agustin clay percolates are more erratic than those from the Barrigada clay. Since Nostoc is not normally seen growing on San Agustin clay it is possible that a stable microbe-Nostoc ecology is prevented, as evidenced by the fact that significant algal decay occurred throughout the experiment on that soil, while the algae on the Barrigada clay appeared healthy throughout the experiment (Fig. 11).

Figs. 12 and 13 show the NO_3-N content of the percolate from the separate flats; Fig. 11 illustrates the condition of the flats on November 18, 1978, toward the end of the experiment. The fact that significant decay occurred on the San Agustin clay and not on the Barrigada clay could account, in part, for the fact that NO_3-N levels in the soil percolates from San Agustin clay flats are generally higher than in their Barrigada counterparts.

Since the ammonia excretion data show that, at most, only a small amount of fixed nitrogen can be contributed by metabolizing Nostoc muscorum, the decaying alga on the San Agustin clay would contribute more fixed nitrogen than would the healthy alga on the Barrigada soil. This is supported by the data in Table 10 which show that the San Agustin clay flats, where decay was greatest, produce high concentrations of nitrate in their respective soil percolates.

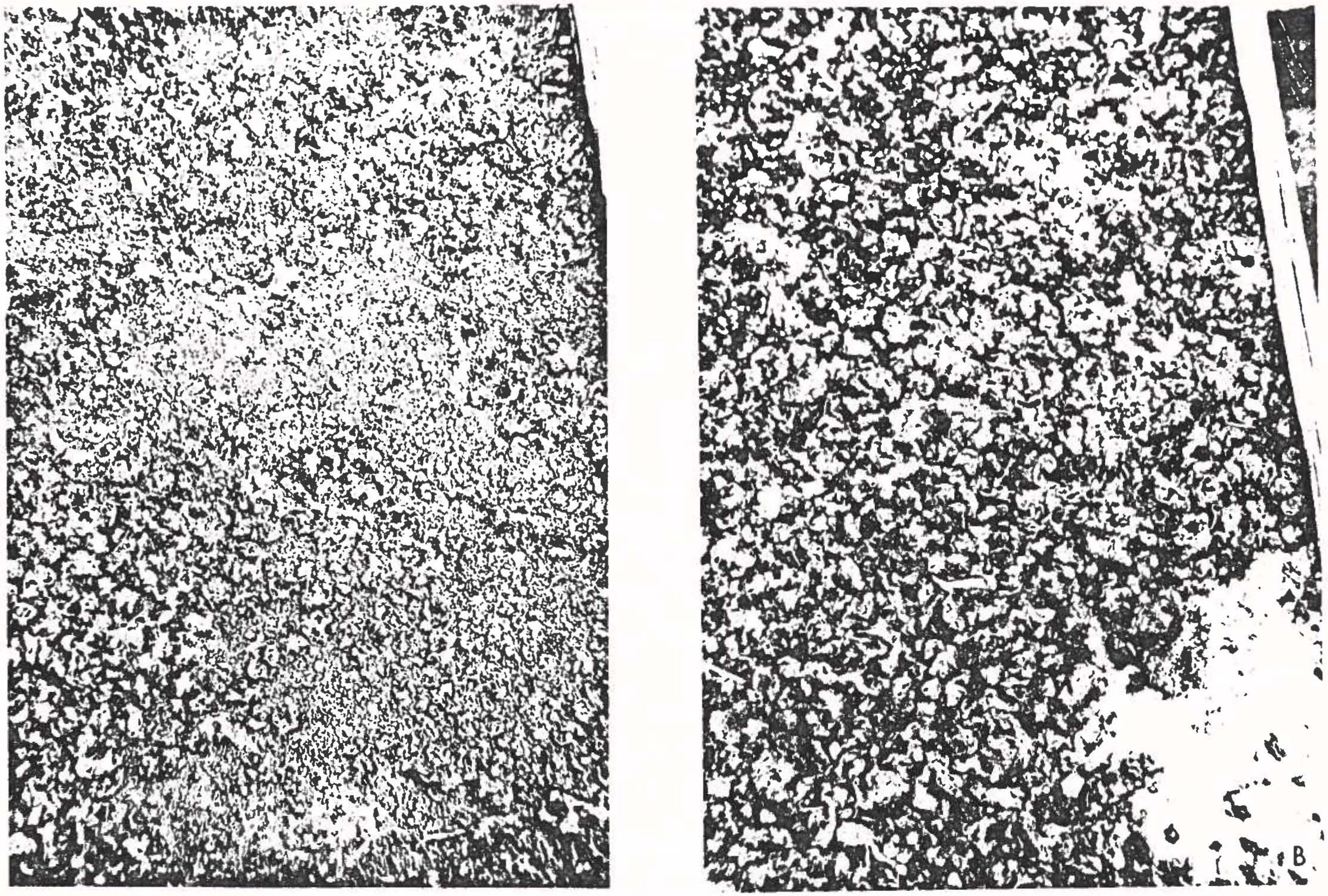

Figure 11. Representative soil flats showing the condition of <u>Nostoc muscorum</u> on San Agustin Guam clay (A) and on Barrigada Guam clay (B).

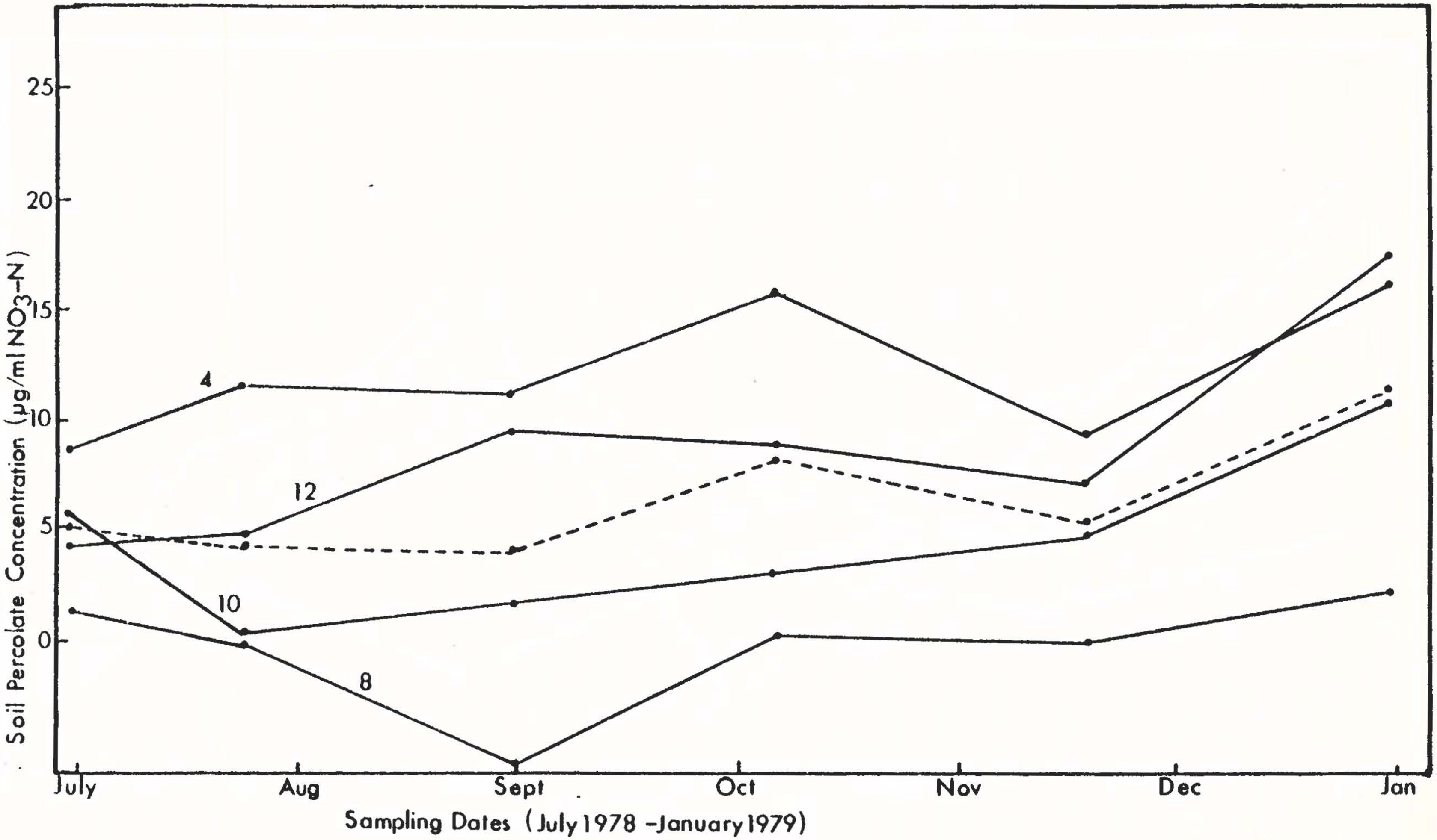

Figure 12. Nitrate-nitrogen percolate concentrations in excess of controls for individual flats containing Barrigada Guam clay. The mean of the four values is indicated by the dashed line.

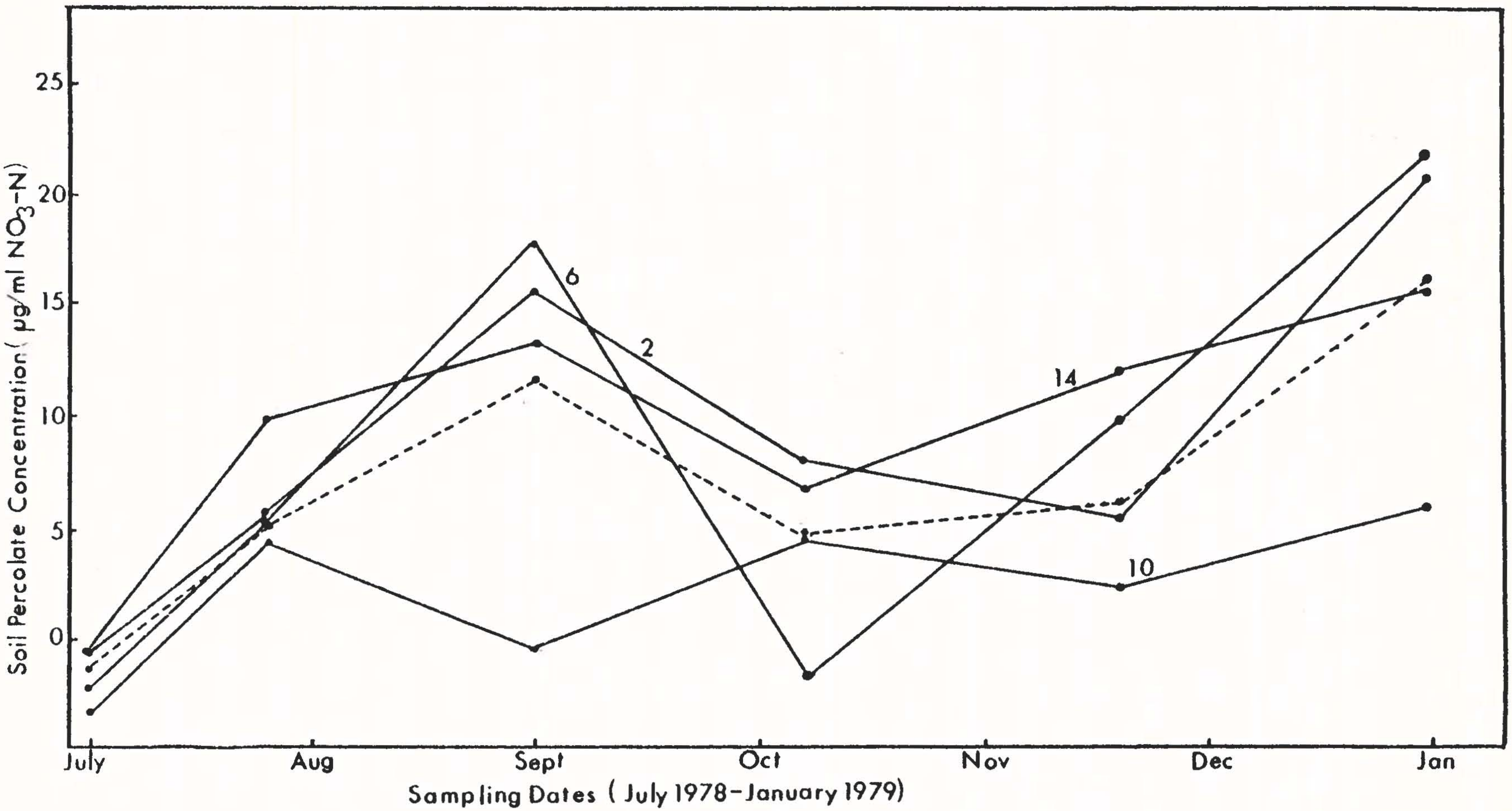

Figure 13. Nitrate-nitrogen percolate concentrations in excess of controls for individual flats containing San Agustin Guam clay. The mean of the four values is indicated by the dashed line.

Table 10. Mean drainage, biomass and combined nitrogen data for the individual soil flats.

Barrigada Type Clay	Drainage (ℓ/min)	Final Biomass (g dry wt.)	NO_3-N/Sampling (μg/mℓ)	$\frac{NO_3\text{-}N}{kg}$	$\frac{NO_2\text{-}N}{kg}$	$\frac{NH_4\text{-}N}{kg}$
#4	0.84	308	11.8	38	2.1	0.81
#8	1.2	214	-0.54	-2.0	1.7	0.23
#12	0.096	430	8.4	20	0.32	0.070
#16	0.34	302	5.0	16	1.5	0.30
San Agustin Type Clay						
#2	0.044	270	9.0	33	0.56	0.71
#6	0.18	47	8.3	180	0.94	0.45
#10	0.28	205	2.2	11	2.7	0.66
#14	0.78	80	9.4	120	0	0

Referring to the NO_3-N levels in percolates from San Agustin clay flats (Fig. 13), the trends of the individual flats are erratic in comparison to the NO_3-N concentration profiles on the Barrigada soils (Fig. 12). Again, it is possible that the conditions are not optimal for an algae-Nitrobacter system and that the upward trend at the end of the experiment is due to algal decay.

Since Nostoc muscorum was most often observed growing on either concrete, asphalt or hard packed Guam clay, of the Barrigada type, it is the results of Fig. 12 that are probably most significant as far as nitrate contribution to the groundwater lens is concerned. Three of the four flats had NO_3-N percolate levels higher than the control levels. Flat 8 was consistently below control levels except for an apparent "attempt" at recovery during the last sampling period. Referring to Table 10, this can perhaps be explained by the drainage rate on this particular flat. Post-flooding drainage from this flat was 1.2 ℓ/min, the fastest for any of the flats. Since water was not retained during any length of time, it could be that the $NH_4^+ \rightarrow NO_2 \rightarrow NO_3$ conversion could not take place; or that simply an artifact of sampling was introduced, i.e., that NO_3-N in the soil was immediately washed from the soil and subsequently diluted.

Another factor evident in Table 10 is that besides having the highest drainage rate, flat 8 has the lowest algal biomass for its soil type. An attempt to correlate drainage rate with biomass was undertaken. If both soil types are taken together, there appears to be no correlation (r_{12}=-0.005) where r_{12} is the product-moment correlation coefficient of Sokal and Rohlf (1969). If the two soil types are analyzed separately, the data points fall into definite groups. Nostoc muscorum on the Barrigada clay maintained or increased its biomass in three out of four cases; the correlation between drainage and final dry weight was r_{12}=-0.90. This value for the product-moment correlation coefficient must be viewed with caution, since the small sample size dictates that $\alpha = 0.1$ for the t distribution (Rohlf and Sokal 1969).

Still, it does seem that a correlation between drainage rate and final biomass does exist at least on the Barrigada soil. That is, where drainage is slow resulting in standing water, N. muscorum is increased.

Contribution of Nitrate to the Groundwater by Nostoc muscorum

From the data obtained, two estimates for nitrate contribution by all Nostoc muscorum affecting the lens were made. The first estimate was derived from the potential total nitrogen contribution based on the ammonia excretion data (in µg NH_4/g/yr) which was converted to g NO_3-N/yr assuming total conversion of NH_4-N to NO_3-N and considering the total biomass of Nostoc muscorum to be 2.6×10^6 kg. Nitrogen released by decaying algae was calculated in g NO_3-N/yr considering that 3.38 percent of the algae was nitrogen (again assuming total conversion) and that there was a steady-state growth and decay rate of 40 percent of the biomass per week. These two values were combined and divided by the amount of yearly rainfall (mℓ) calculated from Mink's (1976) mean yearly rainfall for the northern plateau (235 cm) and the area over the lens ($2.6x10^8$ m^2). The final value is expressed as μg/mℓ.

Soil percolate data were handled in the following manner: positive NO_3-N values (flat nos. 4, 12, 16) were expressed in terms of (μg/mℓ)/(kg/m^2) and the mean divided by the total density of Nostoc muscorum over the lens (kg/m^2), obtaining the value in μg/mℓ. Similar manipulations were performed for the Dededo Ponding Basin. The estimated nitrate contribution of Nostoc muscorum to the groundwater is $2.6x10^{-2}$ μg NO_3-N/mℓ by ammonia excretion and 1.5 μg NO_3-N/mℓ through algal decay for a combined estimate of 1.5 μg NO_3-N/mℓ if the parameters of total potential nitrogen are considered. Since the contribution by ammonia excretion is two orders of magnitude lower than the decay value, it can be considered to be negligible.

If the soil percolate data are extrapolated to account for the entire area of northern Guam, a value of $6.5x10^{-4}$ g NO_3-N/mℓ is obtained. The significance of these results and possible reasons for the large discrepancy between the methods is discussed in the next section. The same calculations in relation to the Dededo ponding basin yield 2.1 μg NO_3-N/mℓ and $8.7x10^{-4}$ μg NO_3-N/mℓ for total potential nitrogen and soil percolate derived estimates, respectively.

DISCUSSION

The results indicate that the total estimated Nostoc muscorum biomass over Guam's lens could potentially account for 1.5 μg NO_3-N/mℓ if all algal nitrogen were converted to NO_3 and found its way into the lens. Extrapolating the soil percolate data, a more conservative figure of 6.5×10^{-4} μg NO_3-N/mℓ is derived. These figures are well below the NO_3-N concentrations of groundwater from the various wells compiled by the Guam Environmental Protection Agency (Table 11).

The large discrepancies between potential NO_3 contribution and extrapolated soil percolate data are not surprising. Potential NO_3 contribution estimates should be considerably higher than those extrapolated from percolate data since in the former, uptake of combined nitrogen by soil bacteria and plants is not considered. The potential NO_3 estimate may also be inherently high because the 40 percent weekly algal growth rate is based on the assumption that the alga is hydrated constantly six months of the year.

Even through N. muscorum appears not to be a major contributor of NO_3 to the lens, it could possibly be a factor in some areas where growth is particularly high. The ecology of this particular alga is such that, in general, it is either found in abundance or not at all. Since this study was concerned only with estimates of algae actually affecting the lens, no quantitative ecological studies were attempted; although, in the course of running transects, numerous observations were made.

Table 11. Mean nitrate-nitrogen (NO_3-N) levels (in $\mu g/m\ell$) of the various wells on Guam compiled from Guam Environmental Protection Agency monitoring.

Date	Tumon-Maui Tunnel	Marbo Well (Air Force)	Mangilao	Marbo (Gov-Guam)	Finegayan	Dededo	Agafo Gumas	Yigo
3/14/78						2.32	1.85	2.77
3/15/78				1.59	1.40			
3/16/78			2.64					
3/30/78	2.07	1.95						
4/27/78	2.46	1.96						
6/27/78				1.83	1.56			
9/12/78			3.84					
9/25/78						3.50	2.77	3.83
9/28/78	3.06	3.48						
11/2/78	2.97	3.15						
12/26/78	2.88	2.94				3.33	2.64	
1/25/79	2.91	2.76						

The type of clay surface on which *N. muscorum* is normally found corresponds closely to the Barrigada Guam clay soil type on which the alga appeared healthy and did not undergo the noticeable decay as seen on the San Agustin variety. It is evident that the only differences in soil characteristics are in soil pH, organic content, and physical soil composition. The higher organic content of San Agustin clay can account for the higher moisture retention (Brady 1974) which in conjunction with physical composition would affect drainage rates. While drainage on Barrigada type clay was negatively correlated with algal biomass, no such correlation exists for San Agustin clay. This is probably a result of the fact that more moisture is retained in contact with the alga for extended periods of time, contributing to its decay. Organics in the soil also yield ammonia and nitrate upon decomposition (Brady 1974). This may give plants that require nitrogen a competitive edge over *N. muscorum* that is not present on the organic-deficient Barrigada clay types.

Drainage seems to be a prime ecological factor in relation to the growth of *N. muscorum*. The alga requires frequent drenching to maintain its hydrated state, yet if allowed to stand for several days in puddles, it undergoes rapid decay. This characteristic is especially evident in the ponding basins where no *N. muscorum* is seen in the main ponding area or on the steep sloping sides, but rather in areas that receive run-off which does not accumulate for extended periods of time.

The drainage data from the experimental flats indicate that a negative correlation between drainage and biomass exists where slower draining flats had a higher final algal biomass. In all cases, drainage rates were such that each flat was completely drained within a 24-hr period. This correlation probably breaks down when drainage rates are low enough that significant algal decay occurs.

The fact that N. muscorum is hardly ever seen along well-traveled roads is something of a curiosity. It may be that stress due to the likelihood of automobiles pulling off the roads onto the shoulder is a factor, or possibly, carbon monoxide, a competitive inhibitor of nitrogenase (Fogg 1974), is indirectly retarding growth by preventing nitrogen fixation.

Nostoc muscorum also seems to have trouble competing with other vegetation for space and sunlight. Off roadways and in ponding basins, there is a noticeable decrease in algal density as other types of ground cover become dominant. Basically, N. muscorum is mostly to be found in areas where surface run-off collects but not for more than a couple of days, and where competition from other forms of vegetation is limited. This condition exists in several of the ponding basins, Dededo in particular. If the same operations are performed on the data as for total lens area with respect to algal biomass and surface area of Dededo ponding basin, the estimates show 2.1 μg/mℓ and 8.7×10^{-4} μg/mℓ for potential and extrapolated NO_3-N contribution, respectively. These values are higher than those which take the entire area of northern Guam into account, yet still too low to be considered significant. Still, if N. muscorum were of sufficient density in a localized area, its nitrate contribution might be a significant factor for that particular area.

In any case, contribution of combined nitrogen by Nostoc muscorum appears to be almost entirely through algal decay. The regression equation (see Fig. 7) estimates a maximum possible ammonia excretion rate of 1.4 μg NH_4/g Nostoc/hr if the surrounding media contains no combined nitrogen. Even under such hypothetical conditions, nitrate contribution through ammonia excretion would be two orders of magnitude lower than that which would be contributed through algal decay. Since ammonia release is most likely determined by an equilibrium between the cell and its surrounding media (Fogg 1971), nitrate concentrations resulting from excretion are most likely negligible.

Nostoc muscorum can take up ammonia from the media if provided in adequate concentrations. The findings here support Ohmori and Hattori (1974), who, in studying nitrogen-fixation in Anabaena cylindrica Lemm., found that a concentration of 1×10^{-3}M ammonia (18 μg/mℓ) was sufficient to completely inhibit nitrogenase activity.

Since excretion is inhibited at about 1 μg/mℓ, it may be that N. muscorum nitrogenase is more sensitive to ammonia inhibition than A. cylindrica nitrogenase. More likely, nitrogenase is only partially inhibited by 1 μg/mℓ ammonia in the media since these experiments measure excess ammonia excreted. This partial inhibition is most likely enough to slow down nitrogen fixing activity to where an excess of ammonia is not being produced.

It is interesting that Stewart et al. (1967), using the acetylene reduction technique, found that a species of Nostoc was capable of reducing 0.51 Mμ moles N_2/mg protein min. Converting units, this works out to be $7x10^4$ μg NH_4/g Nostoc/hr. Since the results show that a maximum of 1.4 μg NH_4/g Nostoc/hr is excreted, it can be seen that only a very small fraction of nitrogen that is fixed by Nostoc muscorum is excreted. Thus, the major nitrogen source will not be a direct result of nitrogen excretion, but rather, the degradation of complexes (primarily protein) that contain nitrogen. Even so, algal biomass of N. muscorum is not of sufficient density to contribute a significant amount of fixed nitrogen to the groundwater system.

CONCLUSION

Nostoc muscorum is not a major contributor to the high nitrate content of Guam's groundwater. The alga, however, may contribute small amounts of nitrate in areas where it is particularly abundant.

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