NITROGEN-FIXING AEROBIC BACTERIA AND THEIR NITROGENASE ACTIVITY IN NORTH MUMBAI COASTAL WATERS

P. K. Pandey and N. S. Sreelu
Central Institute of Fisheries Education, Versova, Mumbai – 400 061, India

ABSTRACT

The effect of the physicochemical parameters of water and soil on the distribution of nitrogen-fixing bacteria and their nitrogen-fixing capacity was studied. Four species of nitrogen-fixing bacteria, *e. g., Azotobacter chroococcum, A. vinelandii, A. beijerinckii* and *A. armeniacus* were recorded from water and soil samples of Mumbai coast. A higher number of bacterial populations were observed in sediment than in water samples. A positive correlation was observed between the dissolved organic matter and nitrogen-fixing bacterial populations of water as well as between available phosphorus and the nitrogen-fixing bacteria of sediment. The nitrogen-fixing capacity of *A. chroococcum* was found to be 1.076 nmol C_2H_4/l/d and that of *A. vinelandii* was 0.965 nmol C_2H_4/l/d. Station 1 showed higher level of nitrogenase activity in comparison to other four stations.

Keywords: Nitrogen-fixing bacteria, *Azotobacter*, nitrogenase activity

INTRODUCTION

Nitrogen availability is a key factor regulating primary productivity in pelagic, coastal, marine and estuarine waters. The quantitative role of nitrogen fixation in the ocean's nutrient budget is of considerable scientific interest (Karl *et al.*, 1997). Chronic nitrogen deficiencies in such systems would appear to be of selective advantage to nitrogen-fixing microorganisms to convert nitrogen to ammonia, a biologically utilizable form of nitrogen. The mechanism of nitrogen fixation has been studied in several free-living aerobic and anaerobic bacteria (Quispel, 1974), in the root nodules of leguminous plants (Bergersen, 1971, 1977; Mortenson, 1978), in *Azolla* containing nitrogen-fixing blue-green algal endophyte (Peters and Calvert, 1982), in free-living photoautotrophic blue-green algae (Venketaraman, 1993) and nitrogen-fixing phototrophic bacteria (Sprent and Sprent, 1990). In recent years, research on nitrogen fixation in marine as well as freshwater habitats has expanded due to its recognition as an important process from a budgetary point of view.

The present investigation was undertaken to enumerate, isolate and

characterize nitrogen-fixing bacteria in coastal waters and soils, and to study the influence of different physicochemical parameters on their nitrogen-fixing capacity.

MATERIAL AND METHODS

Five stations (S1 to S5) were selected in and around the creek discharging drainage water near Juhu beach along the coast of Mumbai for a period of three and a half months (105 days) from 01 October 2002 to 15 January 2003. S1 was a creek connected to sea in which all the drainage water from the nearby locality was drained and got mixed with seawater. The other stations were selected on either side of the creek at a distance of 1 km from each other. S2 was at Seven Bungalows coast and S3 was at Versova coast; these two stations were located on the right-hand side of the creek. S4 was at Chaupatty and S5 at Juhu on the left-hand side of the creek. Water and soil samples were collected at fortnightly intervals during the study period. For microbial studies, water and sediment samples were collected aseptically using sterile test tubes and Petri plates. The heterotrophic and nitrogen-fixing bacterial populations were enumerated using standard pour plate method. For the total aerobic heterotrophic bacterial count, nutrient agar in seawater was used as the medium. For the total nitrogen-fixing bacterial count, Jensen's medium in seawater was used. Fifty bacterial isolates were taken on to agar slants from Jensen's medium and maintained at room temperature. Based on the different morphological observations and biochemical tests such as Gram's staining, starch hydrolysis, hydrogen sulphide production, peroxidase reaction, nitrate reduction and antibiotic sensitivity, the bacterial isolates were identified following Krieg (1984).

Water quality parameters such as temperature, salinity, dissolved oxygen, total alkalinity, dissolved organic matter, ammonia-nitrogen, nitrite-nitrogen, nitrate-nitrogen, phosphate-phosphorus, etc. were estimated following APHA (1998). Moisture content, pH and organic carbon of sediment were analysed following Walkley and Black (1934). Available nitrogen (Jhingran *et al.*, 1969) and phosphorus (Olsen *et al.*, 1954) in sediment were also estimated.

Nitrogenase activity was determined following the acetylene reduction assay (ARA) in a gas chromatograph (Shimadzu Class-GC10) using Porapak-N column and flame ionization detector (FID). The temperature regime was: injection temperature - 100°C, column temperature - 80°C and FID temperature 150°C. The carrier gas (nitrogen) flow rate of 40 ml/min was maintained. A standard chromatogram was obtained by injecting 1 µl of standard ethylene. Further, 1 µl of gaseous phase in the headspace from different samples was injected, and the ARA value was calculated and expressed as nmol C_2H_4/l/d.

RESULTS AND DISCUSSION

Physicochemical Parameters of Water

The water temperature at the study sites remained below 30°C. Since all known heterotrophic nitrogen-fixing bacteria belong to the mesophilic group (Jensen, 1981), the temperature observed was ideal for the growth and natural occurrence of the nitrogen-fixing bacteria.

Salinity values during the study period were more or less $35^0/_{00}$. During the first half of November, the salinity values of S1, S2 and S3 stations were low due to mixing of seawater with land drainage. Kawai and Sugahara (1971a) reported nitrogen-fixing bacteria at salinity of $34^0/_{00}$; however, the nitrogen-fixing bacterial count was independent of the salinity value. The present study also showed the same result as the nitrogen-fixing bacterial count was independent of salinity. During the present study, the value of dissolved oxygen ranged from nil to 6.4 mg/l. However, the nitrogen-fixing bacterial count showed no correlation with the dissolved oxygen content in the present study as reported by Kawai *et al.* (1971). The total alkalinity values recorded during the course of study were in the range of 85-142 mg $CaCO_3$/l. A significant difference in the levels of total alkalinity was recorded both between different stations ($F_{4, 24}$=2.9; p<0.05) as well as between periods ($F_{6,24}$=14.39; p<0.05).

According to Paerl *et al.* (1987), deficiencies in organic matter may play key roles in limiting and regulating marine nitrogen fixation. The dissolved organic matter values recorded during the course of study were in the ranges of 3.6-24.8, 0.4-13.6, 2.0-16.0, 2.8-18.0 and 1.6-14.8 mg/l at S1, S2, S3, S4 and S5, respectively (Fig. 1). A significant

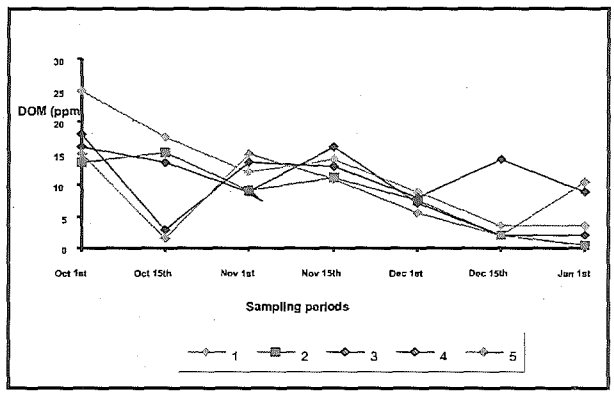

Fig. 1: Variations in dissolved organic matter (ppm) of water

difference in the levels of dissolved organic matter was observed between periods ($F_{6, 24}$=5.44; p<0.05). A positive correlation was observed between the dissolved organic matter and the nitrogen-fixing bacteria of water (r=0.45; p<0.05). The dissolved organic matter contents in different waters indicated the levels of degradation of organic matter taking place.

Ammonia-nitrogen levels recorded during the study were in the range of 0.03-3.72 mg/l. A significant difference in the levels of ammonia-nitrogen was observed between different stations ($F_{4, 24}$=5.07; p<0.05). According to Dash (1993), the ammonia-nitrogen values obtained in freshwater ponds were in the range of 0.16-14.82 g-at N/l. But in the present

study, high values of ammonia-nitrogen (0.03-3.72 mg/l) were obtained. This may be due to the domestic drainage coming to the coastal waters at the site of study. The nitrite-nitrogen values recorded during the study period were in the ranges of 0.04-0.10, 0.03-0.17, 0.05-0.50, 0.036-0.18 and 0.02-0.17 g-at N/l at stations S1, S2, S3, S4 and S5, respectively. The nitrate-nitrogen of water was noted in the ranges of 1.69-20.70, 3.50-21.39, 4.50-20.35, 12.87-26.46 and 14.81-26.36 g-at N/l at stations S1, S2, S3, S4 and S5, respectively. A significant difference in the levels of nitrate-nitrogen was recorded both between different stations ($F_{4, 24}$=3.14; p<0.05) as well as between periods ($F_{6, 24}$=4.19; p<0.05). Nitrite-nitrogen level was comparatively less than the value obtained by Kawai and Sugahara (1971a) at Kumihama Bay. The nitrate-nitrogen value was very high compared to the value obtained by Kawai and Sugahara (1971a). This indicates that active oxidation of ammonia is taking place converting it to nitrate which is less toxic and more useful as a nutrient to the aquatic ecosystem.

Ecologically, phosphorus is often considered as the most critical single element in the maintenance of aquatic productivity (Banerjea, 1967). In the present study, phosphate-phosphorus was found in the range 12.5-93.0 g-at P/l. This is attributed to the domestic sewage supply from the adjacent locality.

Sediment Characteristics

Highly alkaline sediment in the pH range 8.6-9.34 was observed at the sampling stations. Carbon is an important constituent of all organic matter. According to Banerjea (1967), 1.5 to 2.5% organic carbon appears to be optimal for a productive freshwater body. The present study showed a much lower value of organic carbon (0.01 to 0.52%).

Nitrogen is of prime importance for energetic and synthetic processes, and sediments are the main source of nitrogen in an aquatic ecosystem. The present study showed comparatively low content of available nitrogen in sediment, values being in the range of 10.08 to 23.52 mg N/100 g of soil. Available phosphorus was recorded in the ranges of 1.69-2.59, 1.53-2.43, 1.70-2.68, 2.03-2.73 and 1.88-2.75 mg/100 g of sediment at S1, S2, S3, S4

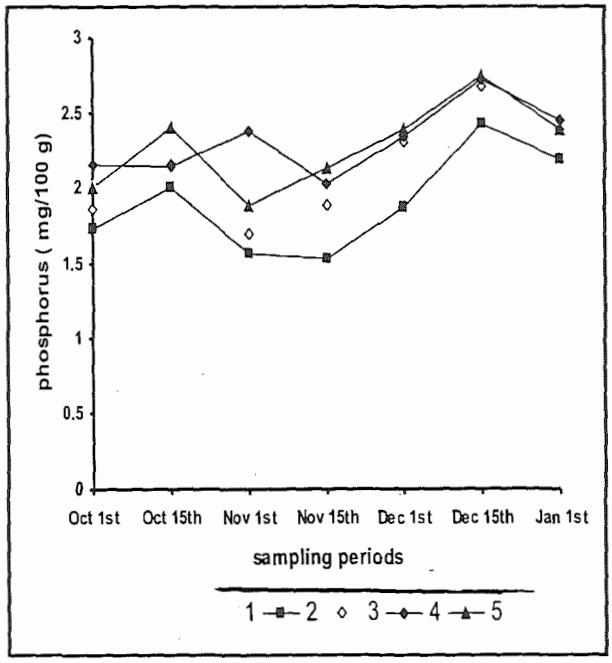

Fig. 2: Variations in available phosphorus (mg/100g) of soil

and S5, respectively (Fig. 2). The present study showed a positive correlation between the available nitrogen content and available phosphorus.

Total Heterotrophic Bacterial Population

The bacterial coenoses serve as a link between the abiotic environment and the biotic communities in an aquatic ecosystem. Kawai and Sugahara (1971a) reported that the number of total heterotrophic bacteria in Maizuru Bay was 10^2 to 10^3 cells/ml, and in Surugama and Sagami Bay, were about 10^3 to 10^5 cells/l. In the present study, the total heterotrophic bacterial count at sampling sites was comparatively higher than that reported by them (0.004-2.34 x 10^6 cfu/ml), which may be attributed to the higher levels of nutrient availability. Kawai and Sugahara (1971b) reported 10^4 to 10^5 cells/g mud in Surugama Bay and Sagami Bay. The present study also showed a comparatively high value of total heterotrophic bacterial count of 0.03 to 32.22 x 10^6 cfu/g dry weight of soil. The observation that bacterial count in sediment is higher than that in water is consistent with this finding.

Aerobic Nitrogen-fixing Bacteria

The heterotrophic free-living aerobic nitrogen-fixing bacteria have been considered to be significantly contributing to the productivity of natural and agricultural systems. Guerinot and Colwell (1985) reported 0.4 to 1.0 x 10^3/l nitrogen-fixing bacteria in the water samples collected off the coast of Puerto Rico and 2.0 to 5.5 x 10^2/l in the Chesapeake Bay, which were quite comparable with the number of nitrogen-fixing bacteria recorded in water, *i.e.,* 0.03 to 4.65 x 10^3 cfu/ml (Fig. 3) and was higher than the value reported for nitrogen-fixing bacteria in

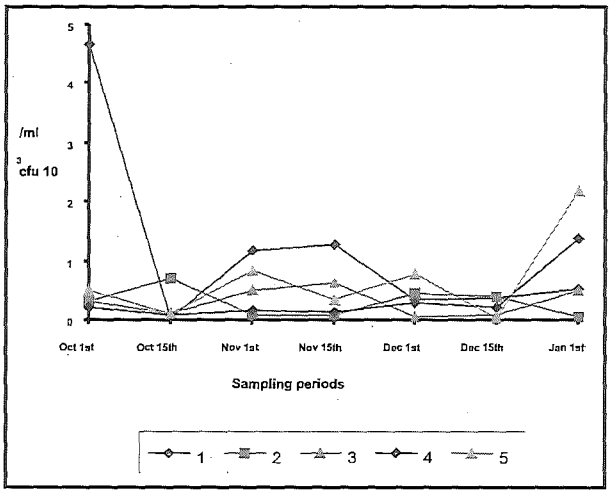

Fig. 3: Variations in total nitrogen-fixing bacteria (cfu10^3/ml) of water

Surugama Bay and Sagami Bay by Kawai and Sugahara (1971b). The present study showed nitrogen-fixing bacterial count in the range of 0.3-12.7 x 10^3 cfu/g dry weight of soil (Fig. 4). Nitrogen-fixing bacterial population

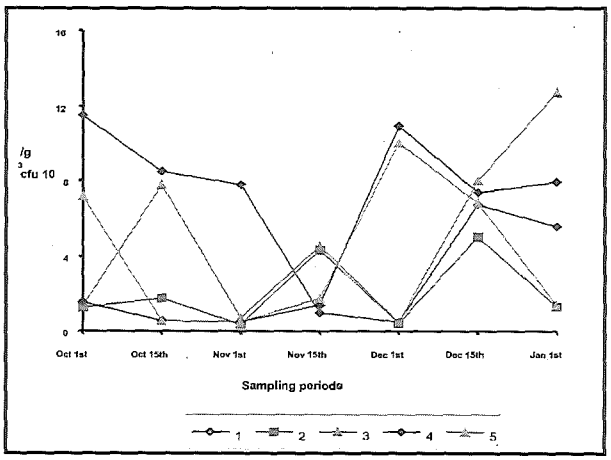

Fig. 4: Variations in total nitrogen-fixing bacteria (cfu10^3/ml) dry weight of soil

was higher in sediments than those in overlying waters as recorded by Jana and Roy (1986).

According to Guerinot and Colwell (1985), significant correlation was not observed between numbers of the nitrogen-fixing bacteria present at any given locality with the physical and chemical parameters. However, a positive correlation was observed between the dissolved organic matter and nitrogen-fixing bacteria of water at the present study sites. Dash (1993) attributed higher aerobic nitrogen-fixing bacterial population in water to higher dissolved organic matter. A positive correlation between the available phosphorus and nitrogen-fixing bacteria in soil was also observed in the present study.

Four nitrogen-fixing bacterial species occurred in the water and soil samples. These were *Azotobacter chroococcum, A. vinelandii, A. beijerinckii* and *A. armeniacus*. Pandey (2002) reported *A. chroococcum, A. vinelandii, A. beijerinckii, A. armeniacus* and *A. nigricans* in freshwater fish ponds. Dash (1993) also reported *A. chroococum, A. vinelandii, A. beijerinckii, A. armeniacus* and *A. nigricans* in freshwater ponds.

Nitrogenase Activity Studies by Acetylene Reduction Assay

ARA value was 1.076 nmol C_2H_4/l/d when each millilitre contained 48 colony-forming units of *A. chroococcum* and 0.965 nmol C_2H_4/l/d for *A. vinelandii* when each millilitre contained 35 colony-forming units. *A. chroococcum* showed higher level of nitrogenase activity than *A. vinelandii*.

S1 (creek) was found to have high number of *A. chroococcum*. The maximum ARA value was observed at S1 followed by S3, S4, S5 and S2. Higher level of nitrogenase activity at S1 is attributed to the higher load of nutrients through domestic sewage, mainly dissolved organic matter and available phosphorus, which showed positive correlation with aerobic heterotrophic nitrogen-fixing bacteria. The influx of nutrient-rich sewage water favours greater number of nitrogen-fixing bacteria enhancing the productivity of the seawater through the higher level of nitrogen.

REFERENCES

APHA, 1998. Standard Methods for the Examination of Water and Waste Water (20th edition). American Public Health Association, Washington, DC.

Banerjea, S. M., 1967. Water quality and soil condition of fish ponds in some states of India in relation to fish production. *Indian J. Fish.*, **14:** 115-141.

Bergersen, F. J., 1971. Biochemistry of symbiotic nitrogen fixation in legumes. *Ann. Rev. Plant physiol.*, **22:** 121-140.

Bergersen, F. J., 1977. Physiological chemistry of dinitrogen fixation by legumes. In: Hardy, R. W. F. and Silver, W. S. (ed.), A Treatise on Dinitrogen Fixation, Section III, Biology. Wiley, New York, pp. 519-555.

Dash, B., 1993. Biological nitrogen fixation in freshwater fish pond ecosystems with reference to hydrobiological conditions. Ph. D. Thesis, The Orissa University of Agriculture and Technology, Bhubaneswar, 325 pp.

Guerinot, M. L. and Colwell, R. A., 1985. Enumeration, isolation and characterization of N_2-fixing bacteria from seawater. *Appl. Environ. Microbiol.*, **50(2):** 350-355.

Jensen, V., 1981. Heterotrophic micro-organisms. *In*: Broughton, W. J. (ed.), Nitrogen Fixation. Vol. 1. Ecology, Clarendon Press, pp. 30-56.

Jhingran, V. G., Natarajan, A. V., Banerjea, S. M. and David, A., 1969. Methodology of Reservoir Fisheries Investigation in India. Bulletin of Central Inland Fisheries Research Institute, Barrackpore.

Karl, D., Letelier, R., Tupas, L., Dore, J., Christian, J. and Hebel, D., 1997. The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. *Nature,* **388:** 533-538.

Kawai, A. and Sugahara, I., 1971a. Microbiological studies on nitrogen fixation in aquatic environments. II. On the distribution of nitrogen fixing bacteria in seawater regions. *Bull. Jap. Soc. Sci. Fish.*, **37:** 747-754.

Kawai, A. and Sugahara, I., 1971b. Microbiological studies on nitrogen fixation in aquatic environments. III. On the nitrogen fixing bacteria in offshore regions. *Bull. Jap. Soc. Sci. Fish.*, **37:** 981-985.

Kawai, A., Sugiyama, M. and Sugahara, I., 1971. Microbiological studies on nitrogen fixation in aquatic environments. IV. On the nitrogen fixing bacteria in culture ponds. *Bull. Jap. Soc. Sci. Fish.*, **37:** 987-991.

Krieg, N. R. (ed.), 1984. Bergey's Manual of Determinative Bacteriology, Vol. I. The Williams and Wilkins Co., Baltimore, 1268 pp.

Mortenson, L. E., 1978. Regulation of nitrogen fixation. *Curr. Topic. Cell. Regul.*, **13:** 179-227.

Olsen, S. R., Cole, C. V., Watanabe, F. S. and Dean, L. A., 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. *Circ. U. S. Dep. Agric., Circ. No.* 939.

Paerl, H. W., Crocker, K. W. and Prufert, L. E., 1987. Limitation of N_2 fixation in coastal marine waters: Relative importance of molybdenum, iron, phosphorus and organic matter availability. *Limnol. Oceanogr.*, **32(3):** 525-536.

Pandey, P. K. 2002. Nitrogenous Biofertilization with *Azolla* in Freshwater Fish Pond Ecosystem. Ph. D. Thesis, Central Institute of Fisheries Education, Mumbai, 274 pp.

Peters, G. A. and Calvert, H. E., 1982. The *Azolla-Anabeana* symbioses. *In:* Rao, N. S. S. (ed.), Advances in Agricultural Microbiology. Oxford and IBH Publishing Company, New Delhi, pp. 191-218.

Quispel, A., 1974. The endophyte of the root nodules in non-leguminous plants. *In:* Quispel, A. (ed.), The Biology of Nitrogen Fixation. North-Holland Publishing Company, Amsterdam, pp. 599-620.

Sprent, J. P. and Sprent, P., 1990. Nitrogen-fixing Organisms, Pure and Applied Aspects. Chapman and Hall, London, 256 pp.

Venketaraman, G. S., 1993. Blue-green algae (cyanobacteria). *In:* Rao, N. S. S., Venketaraman, G. S. and Kannaiyan, S. (ed.) Biological Nitrogen Fixation. Indian Council of Agricultural Research, New Delhi, pp. 43-63.

Walkley, A. J. and Black, I. A., 1934. Estimation of soil organic carbon by the chromic acid titration method. *Soil Sci.*, **37:** 29-38.