STUDIES ON CERTAIN NITROGEN CYCLE BACTERIA IN THE PRAWN CULTURE FIELDS OF KERALA

THESIS SUBMITTED
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
OF THE
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

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JULY 1988

DECLARATION

I hereby declare that this thesis entitled **"STUDIES ON CERTAIN NITROGEN CYCLE BACTERIA IN THE PRAWN CULTURE FIELDS OF KERALA"** has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Cochin-31,
July, 1988

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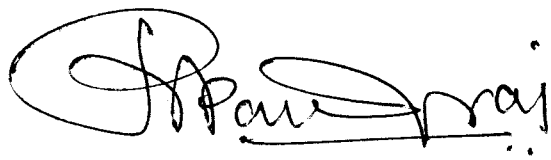
CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON CERTAIN NITROGEN CYCLE BACTERIA IN THE PRAWN CULTURE FIELDS OF KERALA"** is the bonafide record of the work carried out by Shri. ARUN SHIVNATH NINAWE under my guidance and supervision and that no part thereof has been presented for any other Degree.



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PREFACE

In the South-west coast of India, certain paddy fields found in the vicinity of Cochin backwaters, traversed by tides, are used for a traditional extensive type of prawn culture operation. There are two types of prawn culture fields: the perennial fields, where prawns alone are cultured throughout the year; and the seasonal fields, known as "Pokkali fields", where prawns and paddy are cultivated on a rotation basis depending upon the prevailing ecological conditions. These fields have a total brackishwater area of about 26,000 hectares; of this only about 5,100 hectares are currently utilized for growing paddy during the South-west monsoon season and prawns during the rest of the year. The individual fields have an area, generally, ranging from 0.5 hectare to 10 hectares.

In the Pokkali fields, paddy is cultivated during the monsoon season from June to September-October. As soon as the South-west monsoon becomes weak the salinity of the Cochin backwater increases due to influx of sea water and this coincides with the ingress of large number of prawn post-larvae into the backwater, which are being trapped into ponds and grown for three to four months before hearvesting (Muthu, 1978). Usually, prawn culture is done from October to April-May period.

The productivity of these brackishwater ponds depends a great extent on the physico-chemical properties of the water and sediments particularly the availability of the nutrients such as nitrogen and phosphorus and the production of prawn food organisms. The salinity of the water in these brackishwater ponds does not remain constant throughout the year but fluctuates widely.

during the different seasons of the year, attaining maximum values during the summer and minimum during the monsoon. Such changes in the water salinity during the different seasons are likely to bring about profound influence on the bacteria associated with the biogeochemical transformation of the nutrient elements. A series of chemical and biochemical reactions continuously takes place within the bottom soil resulting in the release of nutrient elements to the overlying water which are mediated by specific groups of microorganisms including bacteria. The dynamics of these processes influence greatly the growth and population of microorganisms in these ponds.

Despite the extensive studies on the Cochin backwaters detailed investigations on the microbial ecology of these prawn culture ponds, are lacking. Recognising the importance of nitrogen in the productivity of aquatic ecosystems and the involvement of various groups of microorganisms in the turnover of nitrogenous matter, the present study was carried out on the nitrogen cycle bacteria by selecting two perennial and two seasonal ponds.

Enumeration of total heterophic, proteolytic, ammonifying, nitrifying, denitrifying and nitrogen fixing bacterial populations was carried out for a period of two years; enumeration of Azotobacter was done for one year. In order to understand the factors which contribute to the variations in the bacterial biomass, 13 environmental parameters were regularly monitored. Thirty Azotobacter strains isolated from the prawn culture fields were identified. Detailed studies were made on the nitrogen fixing capacity, and the influence of parameters such as salinity, pH, certain trace elements and vitamins on the growth or nitrogen fixation on selected strains. Data were properly analysed using appropriate statistical methods and valid conclusions have been drawn.

The thesis has been organised into two parts; Part I dealing with distribution and ecology of nitrogen cycle bacteria and Part II with studies on isolated Azotobacter strains. Each of the parts has an introduction, material and methods, results and discussion sections relevant to each Part. A summary follows the discussion of the Part II. All the references are pooled and presented after the summary.

The study carried out herein, I hope precedes many more detailed investigations on this prawn culture system.

ACKNOWLEDGEMENTS

With deep sense of gratitude I wish to express my sincere thanks to Dr. R. Paul Raj, Senior Scientist and my Supervising Teacher for his unstinted guidance, sustained interest, constructive criticism and affectionate treatment during the course of this investigation, but for which this piece of work would not have materialized. Dr. E. G. Silas, former Director, C.M.F.R.I. and Sub-Project Co-ordinator, C.A.S. in Mariculture, took a keen interest in my work programme and gave valuable advice, help and suggestions throughout my research work. I express my sincere gratitude to him for providing excellent facilities for the laboratory studies and field work. I also thank Dr. P.S.B.R. James, present Director for providing facilities to complete my thesis.

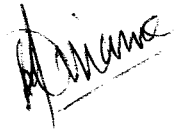
I wish to recall with gratitude all the help and encouragement given by Dr. A. Noble, Dr. P.V. Ramchandran Nair, and Dr. P.V. Rao, Senior Scientists, C.M.F.R.I., Cochin during the course of the investigation.

Mr. M. Srinath, Senior Scientist (Statistics) spared his valuable time and advice in the statistical analysis of the data, using the Hewlett Packard Computer on board 'FORV' "Sagar Sampada". I sincerely appreciate this gesture and express my gratitude to him.

It is my previlege to thank Dr. C.T. Pillay, Dr. V. Chandrika, Dr. A.D. Diwan for their immense help while doing my research work. My thanks are also due to the staff of C.M.F.R.I. and staff and scholars at C.A.S. in Mariculture at Cochin, for their timely help.

I express my gratitude to the Indian Council of Agricultural Research, New Delhi for offering me a Senior Research Fellowship to carry out this work at the ICAR/UNDP/FAO "Project Centre of Advanced Studies in Mariculture", Central Marine Fisheries Research Institute, Cochin.

Finally, I owe much more than I can express to my parents, Smt. Subhadra and Shri. Shivnath Ninawe, whose charisma and blessings enabled me to bring out this piece of work.



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PART I

DISTRIBUTION AND ECOLOGY OF NITROGEN CYCLE BACTERIA

INTRODUCTION

Nitrogen is an important constituent of all living matter, and consequently, its available form and concentration have frequently been implicated as factors limiting primary productivity in aquatic environments (Thomas, 1970; Skelef et al., 1971). In the aquatic environments, nitrogen is found primarily in five oxidation states: NH_4, N_2, N_20, NO_2 and NO_3. By far the most abundant species of nitrogen is N_2, but it is essentially unreactive. The next most abundant species and a biologically active one is the nitrate ion (NO_3^-). The other bioactive inorganic ions, nitrite (NO_2^-) and ammonium (NH_4^+), are less abundant overall, but are of local significance.

The concentration of various natural nitrogen species in the aquatic environment is primarily the result of biological fixation of atmospheric nitrogen and recycling of nitrogenous compounds by microorganisms. Thus, nitrogen cycle is the most complex of the nutrient cycles because of the many forms in which nitrogen is present and because many of the steps in the cycle are mediated by specific groups of microorganisms.

The atmosphere is the main exchange pool of nitrogen, but it is unavailable in gaseous form to majority of organisms. Nitrogen principally enters the biosphere through activities of nitrogen fixing bacteria and blue-green algae. Nitrogen fixers convert gaseous nitrogen to proteins, and the nitrogen becomes available to higher plants after the prokaryotes decompose. Nitrogen in plant proteins is utilised by the herbivore sub-system or passes directly to the decomposer sub-system together with animal bodies and excretory products.

The process of decomposition of proteins by microbes play an important role in the mineralisation of organic material. Many bacteria convert protein compounds to end products such as ammonia, hydrogen sulphide and mercaptans (Rodina, 1972). Thus the biodegradation of proteins is principally effected by protein mineralising bacteria. Protein mineralisation occurs in both the water mass and the sediments under aerobic as well as anaerobic conditions. Ammonification results in the formation of ammonia from amino acids. The dissolved ammonia may be utilized by plants from the soil in solution or undergo nitrification, so that the nitrate formed is absorbed.

The reverse process of nitrification, denitrification is carried out by bacteria, which, under anaerobic conditions use nitrogen oxides as terminal electron acceptors instead of oxygen. The activity of denitrifying bacteria which reduce nitrate, NO_3^- to N_2, N_20 and NH_3, is of considerable ecological significance, since this represents potential loss of nitrogen from the system. Nitrogen, however, shows no evidence of continuous accumulation and immobilization under natural conditions; so denitrification rates are assumed to balance fixation rates.

A number of researchers, in the recent past, have presented models for regeneration of nitrogen in the aquatic environments. A review of models for investigating the behaviour of nitrogen in soil was given by Frissel and Van Veen (1982). Models that have been made to obtain a better understanding of the nitrogen cycle are usually based on a mechanistic description of processes such as leaching, volatilization of ammonia, mineralisation, immobilization, nitrification, denitrification and uptake by plants. Sharp et al. (1982) evolved a model for nutrient regeneration in the oceans with an aquarium system.

Experimental measurement of nitrogen mineralization in coastal waters was carried out by Harrison (1978). Budgets for nitrogen recycling have been

presented for North Sea sediments by Billen (1982). The distribution and biological transformation of nitrogen in the Baltic sea have been investigated by Gundersen (1981). Stewart et al. (1982), studied the nitrogen cycling in eutrophic freshwaters. Studies on nitrogen mineralization have been made in the sediments of a freshwater tidal marsh (Aziz and Nedwell, 1977) and in a eutrophicated mangrove estuary (Nedwell, 1975).

Smith et al. (1981) studied the rates of ammonification and nitrogen fixation associated with North Carolina Sea-grasses and concluded that bacterial nitrogen cycling is an important process during both the active and the reduced periods of growth. Wetzel et al. (1981) studied the interaction of sediment and water column processes on estuarine nutrient cycles. Particular attention in most of the above studies is paid to key microbiological processes involved in nitrogen cycling and the environmental factors that affect them.

Greenland (1977) explained the contribution of microbes to the nitrogen status of tropical soils. Araragai et al. (1979) surveyed the microflora related to the nitrogen cycle in tropical upland farm soils. Savant et al. (1982) studied nitrogen transformations in wetland rice soils. Sethunathan et al. (1983) reviewed the information relating to microflora, several microbial transformations and the factors influencing these transformations in a rice soil under submerged and upland conditions.

Thus, most of the studies on nitrogen mineralization brought to light the involvement of a number of groups of bacteria, and this resulted in systematically investigating the distribution and abundance of specific groups of bacteria associated with the nitrogen cycle, as reviewed in the following pages.

Total Heterotrophs:

A great deal of information exists on the involvement of heterotrophic bacteria in the decomposition of organic matter and in the regeneration of nitrogen in the aquatic ecosystems, just as in the terrestrial ecosystem. In the marine environment also the importance of heterotrophic bacteria as decomposers of particulate organic matter has been well established (Newell, 1965; Harrison and Mann, 1975). Marine heterotrophic bacteria, however, have extremely diverse biochemical activities and potential growth rates on various organic substrates (Sieburth, 1971), and so it is suspected that during the decomposition of organic matter in seawater there are fluctuating communities of bacteria.

Extensive studies have been conducted on the distribution of bacteria in the marine environment. The presence of bacteria in the deep ocean and differences in the bacterial density in relation to depth have been reported by Zobell (1968) and in the seawater samples from Bengal Bay and the South-China Sea by Simidu et al. (1982).

In the Mediterranean basin Lebedeva et al. (1963) found that the estuarine areas of different rivers were characterized by richer microflora than the high seas. Denis et al. (1972) studied the microbiological composition in a few aquatic mediums from the Atlantic Ocean and the Mediterranean Sea.

Zhukova and Fedosov (1963) studied the significance of microbes in the upper sediment layer of a shallow water-basin in the transformation of organic matter. Anthony (1963) examined the regional variation of bacteria in North-Atlantic sediments. According to Bianchi (1971) in the Eastern Mediterranean deep-sea sediments microbial population is only important in the superficial

sediment layer. Novitsky and Kepkay (1981) explained the pattern of microbial heterotrophy through changing environments in the marine sediments of Halifax Harbour.

The distribution and temporal fluctuations in the density of bacteria in the water, covering a high salinity marsh and large creeks near the mouth of the marsh system were investigated by Wilson and Stevenson (1980).

Microbiological study of a hypersaline lake in French Somaliland indicated that 90% of the isolated strains were euryhaline and 10% were anaerobes (Brisou et al., 1974). The distribution of heterotrophic bacterial populations in hypersaline environments in the South-eastern Spain was dependent on the salt concentrations (Rodriguez-Valera et al., 1981). Rodriguez-Valera et al. (1985) observed little seasonal variation, except for a slight decrease in total number during winter in a hypersaline environment.

Daft and Fallow Field (1978) found seasonal variations in algal and bacterial populations in Scottish freshwater habitats. Yoshikura et al. (1980) studied the distribution and seasonal fluctuations of heterotrophic bacteria in the rivers and estuaries in Japan. Decomposition, population density and standing crop of bacteria and fungi in a freshwater swamp were studied by Tezuka et al. (1982). They found seasonal fluctuations in bacterial density, with an abundance of bacteria immediately after the monsoonal rains, especially the North-East monsoon from the freshwater swampy region. Franklin et al. (1978) reported the seasonal and diurnal population shifts in the aerobic heterotrophic bacteria of the Meduxnekeag River. Bacterial distribution, biomass and heterotrophic activity in a wood stream of the Lneneburger Heide was reported by Meyer (1982). Emiliani (1984) surveyed the oligotrophic bacteria, their seasonal fluctuations and

correlations with environmental variables from the middle Panama River, Argentina.

Walker et al. (1975) made a survey of Rhode River estuary of the Chesapeake Bay and reported the heterotrophic bacterial density in water and sediment. Palumbo and Ferguson (1979) surveyed the distribution of suspended bacteria in the Newport River Estuary, North Carolina and found that bacterial distribution was influenced by salinity with an abundant population in the low saline areas in the upper estuary, when compared to the high saline lower estuarine region. Hakim et al. (1981) surveyed the bacterial population found in the intertidal sediments of Karnafuli estuary in the Bay of Bengal.

Seasonal changes in some physiological groups in microorganisms and their relationship have been studied from waters of Ilawa lakes by Niewolak and Zmyslowsky (1971). Fjerdingstad and Berg (1973) studied some characteristic features of the bacterial decomposition in sediment from lakes and ponds in South-west Greenland (The Narssaq area) for estimating the decomposition of Cellulose and the oxidation of sulfur. Maksimova et al. (1974) surveyed the heterotrophic microbes in lake Baikal water and found that the heterotrophic bacterial number was significantly high in regions with high content of easily oxidizable organic matter, and found much variation in the number of heterotrophic bacteria at the different sampling sites of the lake. Survey of aerobic heterotrophic bacteria from water, mud and macrophytes in lake Grasmere, New Zealand was carried out by Ramsay (1976). Koleshko et al. (1982) studied the number and species composition of heterotrophic bacteria in lake Noroch, one of the largest lakes in Belorussia during 1979-80.

Vaatanen (1980) studied the effect of environmental factors on microbial populations in brackishwaters of the South Coast of Finland. In his observation organic carbon, water temperature, chrolophyll 'a' and salinity have shown maximum contribution, but rainfall and winds also explained part of the variation during most part of the year. In winter the variation, was largely governed by parameters such as humic acid, salinity, water temperature and rainfall. Austin (1983) observed seasonal variation in the bacterial population from the coastal marine fish rearing units, with relatively low numbers in winter compared to counts in summer. Taber and Neihof (1984), surveyed the distribution of total counts in 11 sampling sites from the Chesapeake Bay and found a seasonal pattern in distribution with significant dependence on in situ water temperatures.

Ezura et al.(1974) surveyed the microbial counts and heterotrophic bacterial flora to determine the relationship between bacterial populations and environmental factors in Akeshi Bay. The horizontal and vertical distribution of heterotrophic bacteria in relation to temperature, pH, dissolved oxygen, salinity and available nutrients has been investigated in Tolo Harbour, Hongkong, during summer months by Chan and Kuch (1976). Bell et al. (1980) reported the seasonal variation in the predominant bacterial populations including bacteria in two Canadian rivers. Correlations between predominant heterotrophic bacteria and physico-chemical water quality parameters in two Canadian rivers were also revealed by Bell et al. (1982).

The influence of pH on the growth and reproduction of heterotrophic bacteria was reported by Thiamann (1964). Baker and Innis (1982) observed that low pH markedly reduce heterotrophic microbial activity in sediment lake water system.

Velankar, (1955) observed that there was not much difference in the qualitative and quantitative bacterial population from Mandapam and Madras (Bay of Bengal, East Coast) and Cochin and Malabar coasts (West coast) of India. Studies were conducted by Venkataraman and Sreenivasan (1956) in the marine environments of Tuticorin, in which water samples were analysed for total bacterial counts. Qualitative and quantitative studies of the bacterial population in two stations in the West Coast viz., Karwar and Vengurla, have been made by Gore (1974) for a period of eight months, which revealed that the pattern of distribution to be almost similar to that of the East Coast.

Shanta Nair and Loka Bharati (1980) studied the distribution pattern of halotolerant and limnotolerant heterotrophic bacterial flora of three sandy beaches of the West Coast of India, and found more or less a similar pattern of distribution among the beaches, though seasonal variations in the distribution was observed. Microbiological studies on the sediments of Andaman Sea (Matondkar, 1981) revealed that there was no significant correlation between organic carbon values and heterotrophic activity.

Total viable bacterial counts of sediment and water samples from a mangrove swamp, situated in Zuari estuarine belt were studied by Matondkar et al. (1981). Matondkar (1981) reported that the heterotrophic bacterial flora from mangrove swamps of Goa exhibit certain physiological activities in association with detritus.

Jana et al. (1980) studied the growth characteristics of the heterotrophic bacterial population of water and bottom sediments in tanks under different trophic conditions. Jana and Roy (1983) estimated the microbial populations involved in the nitrogen cycle and their activity in water and sediments of fish farming ponds under mono-and polyculture systems in India

and reported that seasonal changes in the microbial populations involved in the nitrogen cycle were almost the same in all four ponds.

Proteolytic bacteria

The protein degrading ability of heterotrophic bacteria has been well known in the aquatic environments and the proportion of proteolytic bacteria within the marine microflora is probably even greater than that found in inland water and soil (Skerman, 1963; ZoBell, 1964). ZoBell and Upham (1944) found that of the sixty strains of marine bacteria isolated from the coast of South California all could release ammonia from peptone and forty seven could liquefy gelatin; where proteins become available they multiply very rapidly, as the generation time of most putrefying bacteria is relatively short. According to ZoBell(1964a) almost all heterotrophic marine bacteria may release ammonia from peptone, and almost three quarters of them liquefy gelatin.

Kawai and Sugahara (1971) surveyed some standing crops of the nitrogen cycle bacteria in freshwater, inland bays, offshore regions and open-seas of Japan and reported that proteolytic bacteria form a dense bacterial biomass in the freshwater environments, and the bacterial population decrease gradually from coastal to offshore regions.

Bianchi (1971) reported that proteolytic bacteria are widely distributed among the microbial population of marine sediments in Eastern Mediterranean deep-sea sediments. Miyoshi et al.(1982) studied the proteolytic activity of marine bacteria from Uranouchi Bay, with special reference to the decomposition of dead organisms and contributed some information on bacterial decomposition of particulate organic matter in the marine environment.

The distribution, enumeration, isolation and activity of proteolytic bacteria in the North Inlet estuarine environment were given by Sizemore et al. (1973). It was experimentally proved that 44 to 62% of the bacteria isolated from the North Inlet estuary were capable of extracellular protein degradation. Approximately the same number of organisms was found in various localities within the estuary and during different seasons; however, differences in the vertical stratification within the upper 15 cm of sediment was observed (Sizemore et al., 1973). Walker et al. (1975) reported high counts of heterotrophic bacteria in the water and sediments of Chesapeake Bay, of which a significant portion was proteolytic bacteria.

Sugahara and Hayashi (1974) reported the population density of gelatin liquefiers in the water and filtersands of the ayu-fish breeding ponds. Sugahara et al. (1974) reported the proteolytic bacterial density in the water and sediments of experimental aquaria. Protein mineralizing bacterial population in water and sediments of the monoculture pond was reported to be about three times as large as in the polyculture and traditionally managed fish ponds of USSR (Antipchuk and Jana, 1978). Jana and Roy (1983) studied the protein mineralizing bacteria from water and sediments of four fish ponds being used for traditional, mono and polyculture systems of fish farming and they compared the population sizes of the bacterial flora in the polyculture and monoculture ponds. Seasonally, bacterial numbers were generally higher in the winter in both the water and the sediments, and lower in the summer.

Ammonifying bacteria

Ammonification is of particular importance for aquatic life as a means to generate ammonia through the conversion of organic nitrogen to the more mobile inorganic state. Ammonification in soil and water is brought about mostly by spore-

forming bacteria, actinomycetes and fungi. These can degrade proteinaceous matter with concomitant production of ammonia. Bacteria and Fungi break protein molecules rapidly, while actinomycetes do it slowly (Waksman, 1922).

The process of ammonification has been the object of numerous investigations in the lake regions (Trifonova, 1961; Niewolak, 1965) rivers (Rheinheimer, 1959; Braune and Uhlemann, 1968) and in other inland water bodies (Fischer, 1960; Ritter et al., 1971; Daubner and Ritter 1973).

In studies of Zurich Lake, Minder (1920) has observed a relation between the numbers of ammonifying bacteria and the amount of NH_4-N accumulated in water. He also found that organic matter content of bottom sediments of the lake significantly influenced the density of ammonifying bacteria. In the freshwater soft sediments of Neusiedlersee, Austria the population of ammonifiers represented 15 to 40% of the total number of bacteria (Muhlhauser, 1980). Stewart et al. (1977) reported highers number of ammonifying bacteria in the water columns of three Scottish freshwarer habitats than the sediment-water interface and in the sediments. Niewolak et al. (1978) studied the fluctuations in the numbers of ammonifying bacteria and the ammonification rates in water and bottom sediments of a few lakes in Poland during 1971-1974 and observed that more of the populations occurred in near-bottom water than that of the surface waters. Kawai and Sugahara (1972) found that the population of ammonifying bacteria decrease gradually from coastal to off-shore regions, with relatively higher numbers in the sediments of Japan Bays.

Seasonal changes in population sizes of ammonifying bacteria in the water and sediments of four fish ponds being used for traditional, mono and poly-culture systems of fish farming were studied (Jana and Roy, 1983) in India. Jana and Roy (1980), have demonstrated the growth of Indian Major carps grown

in these ponds as direct functions of the population size of ammonifying bacteria occurring in water.

In the Elbe River, Rheinheimer (1959) has observed changes in the ammonification rate according to the site of sampling and the season. The greater number of ammonifiers present during winter in the river water is known to offset to a large degree the lower ammonification rates, so that also during the coldest time of the year ammonification in the river is quite considerable (Rheinheimer, 1965a). Ammonification rate is more intense in the summer season, when the numbers of ammonifying bacteria in water and bottom sediments in the four lakes of the Masurian lake land attained maximum temperature, but at lower temperature it does not inhibit their biochemical activity (Rheinheimer, 1959; Neiwolak, 1965; Braune and Uhlemann 1968; Ritter et al., 1971; Daubner and Ritter 1973).

Nitrifying bacteria

Nitrification is defined as the process of reducing nitrogen compounds to an oxidized state. In the past, the presence of high concentrations of nitrite in seawater was assumed to be the result of nitrifying bacteria (Brandhorst, 1959). Early experiments with cultures of marine nitrifying bacteria, however, resulted in very low rates of nitrite production which could not be ecologically significant (Carlucci and Strickland, 1968). Carlucci et al. (1970) concluded that nitrification might be significant in certain microenvironments with high ammonia concentrations; but they also showed that nitrite could be released by phytoplankton under certain conditions.

Nitrifying bacteria are chemolithotrophs and can obtain all the energy necessary for growth and carbon assimilation from the aerobic oxidation of ammonium to nitrite or nitrite to nitrate. In the marine environment, two genera of bacteria, Nitrosomonas and Nitrosococcus, mediate the first

step in nitrification NH_4^+ to NO_2^-); while in the second step, Nitrobacter, Nitrospira and Nitrosococcus groups oxidize NO_2^- to NO_3^- (Schmidt, 1978).

Pioneering work by Waksman and co-workers (Waksman, 1933; Carey and Waksman, 1934) indicated that marine nitrifying activity was primarily confined to the sediments rather than the water column. Carey (1937) found that raw seawater apparently could not produce "nitrite or nitrate" during 52 days of incubation. Laboratory experiments (Carlucci and Strickland, 1968; Yoshida, 1967) have shown that marine nitrifying bacteria grow and oxidize their substrates very slowly, while pure cultures of marine nitrifiers show a fairly low affinity for their substrates, which are often environmentally unrealistic. Thus, the ecological importance of heterotrophic nitrification has been difficult to assess because of the low level of products. Under axenic conditions, the rate of heterotrophic NO_2^- + NO_3^- formation is 10^3-10^4 times slower than in autotrophs (Focht and Verstraete, 1977).

Some decades ago, nitrifying organisms were isolated from the Baltic and the North Sea sediments (Brandt, 1902). Later, nitrification was also demonstrated in various coastal waters (Vargues and Brisou 1963; Rheinheimer, 1963). Watson (1965) observed that in the shallow areas of the sea, nitrification occurred both in water and sediments; but in the open ocean it occurred only in the body of water. Carlucci and Strickland (1968) isolated several nitrifiers from the northern Pacific ocean. Castellviy and O'Shanahan (1977) isolated a heterotrophic bacteria from the Peruvian upwelling system, capable of growing on sodium citrate and ammonium sulfate, which liberated nitrate and nitrite. Carlucci et al. (1970) were unable to demonstrate nitrification in seven isolates of open-ocean heterotrophic bacteria.

Recent estimates of direct counts of nitrifiers (living or dead) are on the order of only 10^4 litre^{-1} (Ward et al., 1982). Estimates of MPN abundance (Watson, 1965; Ezura et al., 1974) would suggest that nitrifying bacteria in the water column of oceans and bays are low, on the order of $1\text{-}10^3$ litre^{-1}. Standing crop of nitrifying bacteria in water and bottom sediments of open seas and the bays of Japan ranged from 10^0 to 10^3 as ammonia-oxidizers and nitrite-oxidizers (Kawai and Sugahara, 1972). Yoshida and Kimata (1967) reported that marine nitrifying bacteria always occur in the sea water but the bacterial counts are very low, in the offshore regions. Kawai and Sugahara (1972) demonstrated that the number of nitrifying bacteria in the water is of the order from 10^2 to 10^4 cells/l in freshwaters. The number of nitrifying bacteria in the bottom sediments ranged from 1 to 10^3 cells/10 g in offshore regions and from 10 to 10^4 cells per 10 g in fresh waters and inland bays.

Researches on the nitrifying bacteria in Ocean depths on the coast of Algeria indicated active nitrification in waters less than 100 m depth, which gets reduced with the increase in depth(Vargues and Brisou, 1963).

In the beach sands at Machiya-ura, Japan the upper layer of sand had more nitrifying bacteria than those of the lower layer (Tanaka et al., 1979). Monib et al. (1980) observed ammonia oxidation and nitrite accumulation were affected by the initial densities of nitrifying bacteria in soils and that nitrification in sandy-loam was more rapid than in other soils. The importance of sediment derived ammonium for nitrifying bacteria and the fate of nitrate produced by nitrification were discussed by Christofi et al. (1981) in a Lund enclosure in Bletham Tarn, English Lake District.

In the lower Elbe, near Bleckede (Km 500), the number (MPN) of nitrifying bacteria was mostly higher than that of the nitrate bacteria and the

difference was greatest (by a factor of 10, atleast) during the warm season (Rheinheimer, 1965). Henriksen et al. (1981) studied the distribution of nitrifying bacteria in different types of sediments from Danish waters (Henriksen et al., 1981). In the bottom deposits of the Ilawa lakes seasonal fluctuations in the development of nitrifiers was noticed with maximum numbers in the vegetative period and minimum number in winter (Niewolak, 1970).

Schultz (1981) studied the distribution of nitrifying bacteria in the tidal Potamac River and reported decrease in Nitrosomonas densities but increase in Nitrobacter densities with time. Ardakani et al (1974) showed that the numbers of ammonium oxidizing bacteria (about 10^4/g) were highest in the surface 0-2.5 cm, while nitrite oxidizing bacteria were 50 times higher.

Enrichment, enumeration and characterization of nitrate reducing bacteria present in sediments of the River Tay estuary and the physiology of nitrate assimilatory bacteria from the Tay estuary were studied by Herbert et al (1980). Jones and Hood (1980.) isolated Nitrosomonas sp. from a freshwater marsh and estuarine bay, but, their estuarine isolates lost all activity at 0 ppt Na^+, with a requirement of 7.5-10 ppt Na^+ for optimal activity.

Nitrifying capacity in nitrification of both sediments and water in relation to ammonia-oxidizing bacteria and nitrite oxidizing bacteria were estimated in four zones of Vellar estuary, covering marine and limnetic environments, on the South-east coast of India by Rajendran and Venugopalan (1977). They found that nitrification process was more in sediments than water column and the distribution of marine and limnetic forms of nitrifying bacteria was chiefly controlled by salinity. Surveys made on the quantitative distribution of nitrifying bacteria in the inshore environment at Karwar and Vengurla showed that the numerical abundance of nitrifying bacteria ranged between 10 to 15 per ml in surface

and bottom seawaters at Karwar, while that at Vengurla ranged from 5 to 7 per ml (Gore, 1974). In the mud samples they occurred in the range 50 to 100 per gram both at Karwar and Vengurla. The nitrate reducers varied between 100 to 300 per ml. of seawater and 1000 to over a lakh per gram of mud at both the stations (Gore, 1974).

Kawai et al. (1964) reported the numbers of nitrifying bacteria in an aquarium with a circulating system, in breeding waters and in filter-sands. Sugahara and Hayashi (1974) studied the distribution of ammonia-oxidizing and nitrite-oxidizing bacteria which are responsible for nitrification in the filter-sands and in breeding pond water from February to March 1971 at Hashima and Mino Auy Fish hatcheries. Studies on the nitrifying bacteria in water and sediments from four traditional fish farming ponds (Jana and Roy, 1983) indicated the natural and potential capacity to generate both nitrite and nitrate in these water bodies. Jana et al. (1982) studied the relationship between nitrification and denitrification rates and fish growth in fish ponds under polyculture system.

Surveys were made on the nitrifying bacterial populations of flooded rice soils in the different regions of the world. Conversion of ammonia to nitrate at the oxidized soil layer and reduction of nitrate to nitrogen in the reduced layer, resulted in loss of nitrogen (Shioiri, 1941). Number of ammonium-oxidizers in the surface soil layer of 0 to 0.5 cm and in the deeper layers were reported by Suzuki (1967).

The density of nitrifiers occurring in the sediments of fish culture ponds were negatively correlated with NO_3-N, but positively with pH and dissolved oxygen of the bottom water (Kawai et al., 1971; Kawai and Sugiyama, 1977).

Nitrification occurs even in submerged rice soils used for growing rice, however, because oxygen diffusing from the atmosphere through the water phase keeps the upper few millimeters of soil oxygenated below the oxygen containing zone, no nitrate is synthesized (Yoshida and Padre, 1974). Sugahara et al. (1974) showed that nitrifying activity decreases under low oxygen tension, however, nitrification still occurs to a fair degree even under extremely low oxygen tension.

The effects of the environmental factors on nitrification is reviewed by Painter (1970), Focht and Chang (1975) and, Focht and Verstraete (1977). The pH values are particularly important and the favourable range for both Nitrosomonas sps. and Nitrobacter sps. is pH 7.0 to 9.0 with inhibition below pH 6.0, although slow rates of nitrification have been reported at pH values as low as 4.5 (Anthonison et al., 1976). Aleem and Alexander (1958) reported that high ammonia concentrations inhibits both ammonia and nitrite oxidizers. The optimum temperature for nitrification lies in the range 25° to 35°C, with no significant growth below 5°C or above 40°C and reported maximum growth of Nitrobacter winogradsky at 42°C and same growth upto 49°C was observed in the natural environment (Laudeloud and Van Tichelen, 1960).

The effect of temperature, pH, salinity and inorganic nitrogen on the rate of ammonium oxidation by nitrifiers was studied in wetland environments (Jones and Hood, 1980). Maximum density was observed for the freshwater isolates at 35°C, pH 8.5, salinities of 0.3 to 0.5% Na^+ and K^-, and ammonium concentrations greater than 0.5 g/l. For the estuarine isolates maximum activity was observed at 40°C, pH 8.0, salinities of 0.5 to 1.0% Na^+ and K^-, and 0.2 g/l ammonium.

Denitrifying bacteria:

Denitrification reaction, i.e. the complete reduction of nitrate to a gaseous product (N_2 or N_2O) in the marine environment occurs in two separate steps. As nitrate disappears from anoxic seawater, nitrite accumulates in nearly a 1:1 correspondence until nitrate concentrations are low or exhausted (Georing and Cline,1970). Nitrite is then further reduced to gaseous nitrogen. Dinitrogen appears to be the major form of gaseous nitrogen produced by aquatic denitrifiers (Goering 1968; Koike and Hattori, 1975). N_2O supersaturation has, however, been detected in the water column of the North Atlantic Ocean and Caribbean Sea (Yoshinari, 1976) and it was negatively correlated with oxygen.

Relatively few numbers of bacteria can reduce nitrate to N_2 through a series of consecutive reduction steps, and N_2O is occasionally accumulated. According to ZoBell (1946), about 40% of the marine bacteria he had isolated, could reduce nitrate to nitrite in the presence of sufficient organic matter, but fewer than 5% could reduce nitrate to N_2.

Experimental evidence for nitrate reduction to ammonium in coastal marine sediments has been presented by Koike and Hattori (1978a) and Sorensen (1978a). This process might be mediated by strictly anaerobic bacteria such as Clostridium or facultative anaerobes (Cole,1978) and the portion of the ammonium yielded is further utilized as a nitrogen source for the growth of denitrifiers.

Standing crop of the denitrifying bacteria which are responsible for the nitrogen loss in nitrogen cycle was studied by Kawai and Sugahara (1972) in various water regions of inland bays of Japan and their population ranged from 0 to 10^4 cells/ml in water region, and from 10^2 to 10^6 cells/g in the bottom

sediments. Denitrifiers were recorded in the seawater and mud samples from Karwar and Vengurla by Gore (1974). Numerical abundance varied between 1 and 10 per ml. of seawater at both the places and that of mud was between 100 to 1000 per gm. Denitrifiers were recorded abundantly in the sediments, their population decreasing from some 10^6/g in the surface material to only a few in the sub-sediments of various newly discovered depths in the Red Sea (Heitzer and Ottow, 1976). Koike and Hattori (1978) found that in anaerobic coastal sediments population densities of denitrifying bacteria decreased with the depth of sediments.

Vanderboght and Billen (1975) failed to detect nitrification in reduced estuarine sediments. They also maintained that the rapid decrease of nitrate in the interstitial water could be due to the presence of denitrification. While studying the inorganic nitrogen metabolism in a eutrophicated tropical mangrove estuary, Nedwell (1975) observed the ability of the heterotrophic bacterial population to reduce nitrate. Aziz and Nedwell (1977) studied the microbial nitrogen transformations in the salt-marsh environment and concluded that nitrification is absent from these salt-marsh sediments, probably because of the reduced nature of the environment, while the bacterial populations of the sediments have large capabilities for reduction of nitrate.

In rivers like the Elbe, denitrifiers are usually higher in winter than in the summer months. In the lower Elbe the counts (MPN) of denitrifiers were found to vary between several hundred to several tens of thousands per ml. water (Rheinheimer, 1965).

Thr processes of reduction of nitrates to nitrites (Partial denitrification) and molecular nitrogen (complete denitrification) and their role in the nitrogen balance in lakes have been studied by various authors (Jannasch, 1960,

Niewolak, 1970; Goering and Dugdale, 1966; Chen et al., 1972a; Daubner and Ritter, 1972; Andersen, 1977; Jones, 1979). The bacteria responsible for the reduction of NO_3-N to NO_2-N were found to be more numerous in the near-bottom water and in sediment layers at the depth of 0-5 cm and 6-10 cm, than at the depth of 11-15 cm. While studying the seasonal changes of denitrifying bacteria in the bottom deposits of the Ilawa lakes, Niewolak (1970) found that the bacteria display a relatively high sensitivity to temperature, and that sandy grounds contain much less denitrifying bacteria than the muddy deposits in deeper parts of the Ilawa lake.

In the submerged rice soil, nitrate is quickly denitrified, and almost all of the added nitrate is finally transformed to N_2 gas (Yamane, 1957). Gamble et al.(1977) reported the numbers of denitrifying bacteria in 19 soils, including 4 rice soils. The minimum and the maximum numbers of denitrifiers in the 19 soils were 1.2×10^4 and 7.0×10^6/g soil. Ishizawa and Toyoda (1964) obtained an average of 3×10^5 denitrifying bacteria/g soil from the plot layer of its Japanese paddy soils. Araragi and Tangcham (1975) surveyed 98 Thailand paddy soils and obtained an average of 6×10^5/g; however in the studies of Garcia et al. (1974) , the average was 2×10^3.

In the fish ponds in Japan the number of denitrifying bacteria ranged from 10^3 to 10^4 cells/g in the filter-sands, while from 10^2 to 10^4 cells/ml or less in the waters, and was found to be 0.1-6% of that of heterotrophs (Sugahara and Hayashi, 1974). The number of bacteria capable of reducing nitrate was found to be more than one twentieth of heterotrophic bacteria existing in the waters and the filter-sands of the ayu-fish culturing ponds at Hasima (Sugahara and Hayashi, 1974).

Many of the bacteria that bring about denitrification are sensitive to high hydrogen-ion-concentrations and hence various acid soils contain a sparse denitrifying population. However in some soils denitrification is still rapid at pH values of 4.7 (Ekpete and Cornfield, 1965) so the pH range applicable to nitrogen volatilization from all soils cannot be clearly defined. Georing and Cline (1970) found that the production of nitrite and nitrate in ocean waters coincided with a depletion of oxygen and at low concentrations of nitrogenous oxide, nitrate was reduced faster than nitrite.

Aerobic nitrogen fixing bacteria and nitrogen fixation:

Biological nitrogen fixation transforms molecular nitrogen into ammonia or organic nitrogen and through this process, the atmospheric nitrogen enters the biosphere and gets involved in the nitrogen cycle Recent estimates indicate that approximately 255 x 10^6 metric tons of N_2 is fixed annually. With some contribution of nitrogen fixed from sources such as lightning and combustion, biological nitrogen fixation and industrial ammonia synthesis support the nitrogen needs of the entire biological community (Skinner, 1976). Biological nitrogen fixation accounts for approximately 70% of the total N_2 fixed (Burns and Hardy, 1975).

In the process of fixing atmospheric nitrogen a variety of microorganisms and algae take active role. The ability to fix dinitrogen aerobically seems to be restricted to the Cyanophyceae, Azotobacteriaceae (genera Azotobacter, Azomonas, Azotococcus, Beijerinckia and Derxia) and two other obligate aerobic bacteria Mycobacterium flavum 301 and Pseudomonas methanitrificans (Coty, 1967). While Winogradsky (1893) showed that certain clostridia were capable of fixing nitrogen anaerobically, Beijerinck (1901) observed that the azotobacters

were representative of aerobic microorganisms which were capable of nitrogen fixation. Recent observations, however, indicate that biological nitrogen fixation is not confined to Azotobacter, Clostridium and Rhizobium species. Nitrogen fixation among the non-sporulating sulfate-reducing bacteria earlier observed by Sisler and ZoBell (1951) have been further confirmed by many authors (LeGall et al., 1959; Reider-Henderson and Wilson, 1970; Postgate, 1970b). Biological nitrogen fixation has also been observed in a number of hydrogenase producing bacteria (Rodina, 1956; and Pshenin, 1963).

The distribution and ability to fix molecular nitrogen amongst prokaryotes of particular importance to marine, brackish and freshwater environments has been considered recently by Postgate (1971), Benemann and Valentine (1972) and Stewart (1973). Benecke (1933) reported that Keutner (1903) was the first person to make an extensive search for nitrogen fixing bacteria in the Baltic Sea, the North Sea, and off the African coast. Species of aerobic nitrogen fixers, Azotobacter chroococcum and also the anaerobic Clostridium pasterianum were found (Benecke and Keutner, 1903). Waksman et al. (1933 b) surveyed the abundance of nitrogen fixing Azotobacter and Clostridium species around American Samoa and Tortugas, Florida seas. Survey on the distribution of nitrogen fixing bacteria in sea water at all depths in the Central Pacific Ocean showed that their numbers ranged from 0 to 10^4 per ml of the seawater (Maruyama, 1975; Maruyama et al.,1970). Sugahara et al. (1971) studied some ecological aspects of nitrogen fixing bacteria and nitrogen fixation in water regions of Maizura Bay and Kumihama Bay in situ. The standing crop of nitrogen fixing bacteria was often found to be quite small in surface waters of the Mid-Pacific Ocean and Japan Sea, their number ranged from 200 to 400 cells/l respectively and the nitrogen fixing bacteria occurred more abundantly in bottom sediments than in overlying water (Kawai and Sugahara, 1971b).

Niewolak (1970) observed that the distribution of nitrogen fixing bacteria in Ilawa lakes depends mainly on the bottom types and organic matter contents. Sarlov et al. (1980), observed that the number of Azotobacter ranged from 0×10^3 to 10×10^3 per ml in the surface layer of bottom deposits of Estonian lakes and Rybinsk reservoir. Pshenin (1963) isolated both Azotobacter and Clostridium species from water, sediment and algal thallai in the Black Sea.

Sarlov and Babanazava (1982), studied the characteristics of the microflora and nitrogen fixation in the Takyr soils of the rice fields in the Karaklpak ASSR. It has been established that the nitrogen fixation in the sulfate-carbonate soil is mainly preferred by different nitrogen fixing microbes in addition to Azotobacter chroococcum .

Heterotrophic nitrogen fixation in paddy soils was reviewed by Watanabe (1978), Yamaguchi (1979) and Matsuguchi (1979). Little is known about the survival of aerobic N_2-fixing heterotrophic bacteria in anaerobic soils. As nitrogen fixation is very sensitive to high oxygen tensions, reduced paddy soils appear to be a medium for most anaerobic or microanaerobic N_2-fixers. Magdoff and Bouldin (1970) suggested that Azotobacter develops in the aerobic-anaerobic interface close to the soil surface by utilising products of anaerobic metabolism and oxygen, however, nitrogen fixing clostridia are more abundant than aerobic N_2- fixers. Takeda and Fukusaka (1975a) showed that the dominant clostridia in flooded rice soils are proteolytic and not saccharolytic species such as Clostridium pasteurianum. However, these nitrogen fixing bacteria show higher population densities when an energy source was added to the flooded soil (Rice and Paul, 1972). Rinaudo and Dommergues (1971) presented evidence that the wetland rice root has the ability to fix atmospheric nitrogen owing to bacteria living on its surface or in its tissue.

Sen (1929)suggested that nitrogen fixing heterotrophic bacteria were present at the surfaces of wetland rice-roots and De (1936) attributed the maintenance of nitrogen fertility of the submerged rice soil to the nitrogen fixing activity of photo trophic N_2- fixers in the flooded soils of India. Biological nitrogen fixation by Azospirillum from acid, saline and two unique acid-sulfate-saline soils "Pokkali and Kari" from Kerala has been investigated by Sethunathan et al (1977).

Studies on the nitrogen fixation, the relationship between nitrogen fixation and in situ extractable ammonium concentrations, and the contribution by diazotrophs in a stream side marsh was established as 15 g N m^{-2} $year^{-1}$ (Casselman et al., 1981). Inland marshes exhibited considerably lower N_2-fixation rates equivalent to 4.5 g N $m^{-2}year^{-1}$. Nitrogen fixation by photo-synthetic bacteria and their role in soil fertility has been reported by Kobayashi and Haque(1971) and a significant role of photosynthetic bacteria to soil fertility and improvement of the plant growth condition was observed.

Extensive surveys were made on the rate of nitrogen fixation from North Pacific Ocean (Magne et al., 1974), in open Pacific Ocean (Maruyama, 1975), in Antarctic deep sea and coastal sediments (Hartwing and Stanley, 1978) and in intertidal environments of the Texas Gulf Coast (Gotto et al., 1981). Non biogenic N_2 fixation and some ecological implications on non-biogenically fixed nitrogen in Antarctic surface waters was given by Parker et al. (1978).

Pshenin (1980), studied the processes of heterotrophic and photoauto-trophic nitrogen fixation in near surface waters of the Sevatopol Bay, where heterotrophic nitrogen fixation occurs by means of bacteria and spirochaetes abundant in the near surface water. Ahmad (1981) reported that the rate

of nitrogen fixation fluctuated during the seasonal cycles and also varied with respect to sampling dates, temperature, depth and the transparancy of lake water.

Olah et al. (1983) studied the N_2 fixation in the sediment of a shallow lake basin, a reservoir and a fish pond and observed that the amount of nitrogen fixed in the sediments of the basin was close to the fixation in the water column, while that fixed in the sediments of reservoir was considerably greater. In fish ponds nitrogen fixation was relatively low, when compared with natural lakes. The turnover rate of amonium nitrogen in a Gold fish pond water was 141.2%/h (i.e. 42.5 min), which was extremely fast as compared with other aquatic regions (Kawai and Sugiyama, 1977).

Studies on the nitrogen fixation by nitrogen fixing bacteria from marine, estuarine and mangrove regions at Porto Novo (India) revealed that all the isolates collected from these environments were capable of fixing atmospheric nitrogen both in the liquid media and sediments and the period of incubation had exerted a marked influence on the amount of nitrogen fixed in the liquid (Lakshmana-perumalswamy et al., 1975). Biological nitrogen fixation by free-living bacteria in rice soils has been reported from alluvial, laterite, acid-saline and two unique acid-sulfate-saline soils, "Pokkali and Kari" from Kerala (Sethunathan et al.,1977). Studies on the nitrogen fixing bacteria in fish culture ponds and rates of nitrogen fixation in situ were also carried out from Japan (Sugahara and Kawai, 1971).

Several agroclimatic factors are found to influence the nitrogen fixation ability of bacteria. According to Fedorov (1947), Azotobacter fixed nitrogen within a temperature ranging from 10 to 30°C. He also found that nitrogen

fixation remained almost unchanged within the pH range of 6.24 to 8.34. At more acid or alkaline pH nitrogen fixation markedly decreased and complete inhibition could be detected above 8.75 and below 5.0. One of the factor which limits nitrogen fixation by Azotobacter is oxygen. Meyerhof and Burk (1928) demonstrated the higher rate of nitrogen fixation in culture with a low oxygen content (0.03-0.04 atmos). However at 0.6 atmos. partial pressure of oxygen, nitrogen fixation was inhibited. Wilson and Wilson (1940) and Fedorov (1952) have pointed out that more than 60% oxygen in gaseous mixture competes with nitrogen resulting in inhibition of nitrogen fixation. According to Fedorov (1952); Gaur and Mathur (1966), humus and humic acid substances stimulate the nitrogen fixing activity of Azotobacter.

Despite the great deal of information available on the microbial nitrogen fixation, very few studies have been conducted on the Azotobacter from aquatic environment. In the USSR lakes the number of Azotobacter cells ranged from 0-10 per litre of water (Kuznetsov, 1959). Deufel (1965) found that the Azotobacter counts in lake Constance increased from a few cells to 1,000-3,000 per litre water between 1958 and 1962 and he attributed this variation to the progressive eutrophication resulting to an increase in the concentration of carbon compounds. Distribution of Azotobacter in the bottom sediments of Ilawa lakes depends mainly on the bottom type (Niewolak, 1970).

The occurrence of an abundant population of nitrogen fixing Azotobacter and Clostridium in the sea was reported by Waksman et al. (1933b). Reinke (1903) reported that Azotobacter occurs abundantly on the surface of marine algae, and suggested the symbiosis between Azotobacter and marine algae, in which the latter supply the former with carbohydrates as the source of energy and utilised the nitrogen by the former. Issatchenko (1924) isolated Azotobacter and Clostridium

from cultures inoculated with the algal thalli fucus and suggested that this may be the reason of their occurrence in the ocean. Azotobacter was reported to be associated with volvox (Reinke, 1903), algae and plankton (Keutner, 1905) and seaweeds. Pshenin (1963), who investigated the distribution of nitrogen fixing bacteria in the Black Sea isolated both Azotobacter and Clostridium from water, sediments and algal thallai. In a three year study off Wales, Wynne-Williams and Rhodes (1974) were able to isolate a "few" aerobic or facultative anaerobic N_2- fixing bacteria but could not isolate any species of Azotobacter. The above authors (1974) suggested that Pshenin's work should be reappraised since he isolated the Azotobacter in 50%, rather than full strength seawater. Azotobacter found in the whole water column of the Black Sea is distributed microzonally, being associated with the microzonal distribution of organic matter in the water (Kriss, 1959) and their numbers varied with the seasons of the year (Wynne-Williams and Rhodes, 1974).

Ishizawa and Toyoda (1964), and Ishizawa et al. (1975) made surveys on the distribution of N_2 - fixing bacteria in Japanese paddy soils, and the numbers of Azotobacter were found to seldom exceed 10^4/g soil. In Egypt and Iran (Mahmoud et al., 1978; Hamdi et al., 1978), the population of Azotobacter was more than 10^5/g and sometimes upto 10^6/g from the flooded-rice soil. The distribution of Azotobacter species in Indian paddy soils was reported by Paul and Sen (1961), Rangaswami and Sadasivan (1964).

The presence of Azotobacter chroococcum in the root system of tropical plants, its nitrogen fixation and ammonia assimilation rates were studied by Purushothaman et al. (1979) and the occurrence of Azotobacter chroococcum in 'Pothos scandens' commonly known as many plants which can grow in plain water was reported by Sharma et al.(1985) and in more or less

aerobic phyllosphere field by Ruinen (1961). De and Bhattacharya (1961) observed that though Azotobacter chroococcum was the most predominating spp. in West Bengal soils, it was absent in about 40% of the soils of the State Addition of organic matter to the soils, while stimulating the growth of other bacteria and fungi, had a depressing effect on the growth of Azotobacter (1961). Rangaswami and Subbaraj a (1962) reported six species of Azotobacter in the rice field soils of Annamalainagar viz. A. chroococcum, A. indica, A. lacticogenus, A. beijerinckii and A. vinelandii. A. indica and A. lacticogenus were reported from North Indian soils also (Starkey and De, 1939; Kauffmann and Toursaint, 1951). At Annamalainagar over 4000 Azotobacter cells were found/gram of soil, when the soil was dry from March to June and the population get reduced to 10 or less/gram of soil, when there was water-logging in July (Rangaswami and Subbaraja, 1962). Above 40% moisture, Azotobacter population increased in the soil, while higher moisture levels had a suppressing effect (De , 1936; De and Bose, 1938; Rangaswami and Subbaraja, 1962). In Japanese rice fields, Azotobacter occurred in some non-volcanic soils but not in volcanic soils, except after water logging (Ishizawa and Toyoda, 1964). The cellular biology, distribution and the agricultural implications of Azotobacter have been exhaustively reviewed by Rubinchek (1963).

MATERIAL AND METHODS

Description of the culture fields selected for the study and sampling sites

The present investigation was carried out at Narakkal (76° 14'E long. 10° 03'N lat.), a fishing hamlet in the Vypeen Island, about 15 Km North-West of Cochin, on the South-West coast of India. Four ponds were selected for regular sampling; of which two were perennial prawn culture systems, located within the

premises of the Prawn Culture Laboratory of the Central Marine Fisheries Research Institute, and the other two were seasonal prawn culture systems, where prawn and paddy are cultivated during the inter-monsoon (October to April-May) and monsoon periods (June-September), respectively.

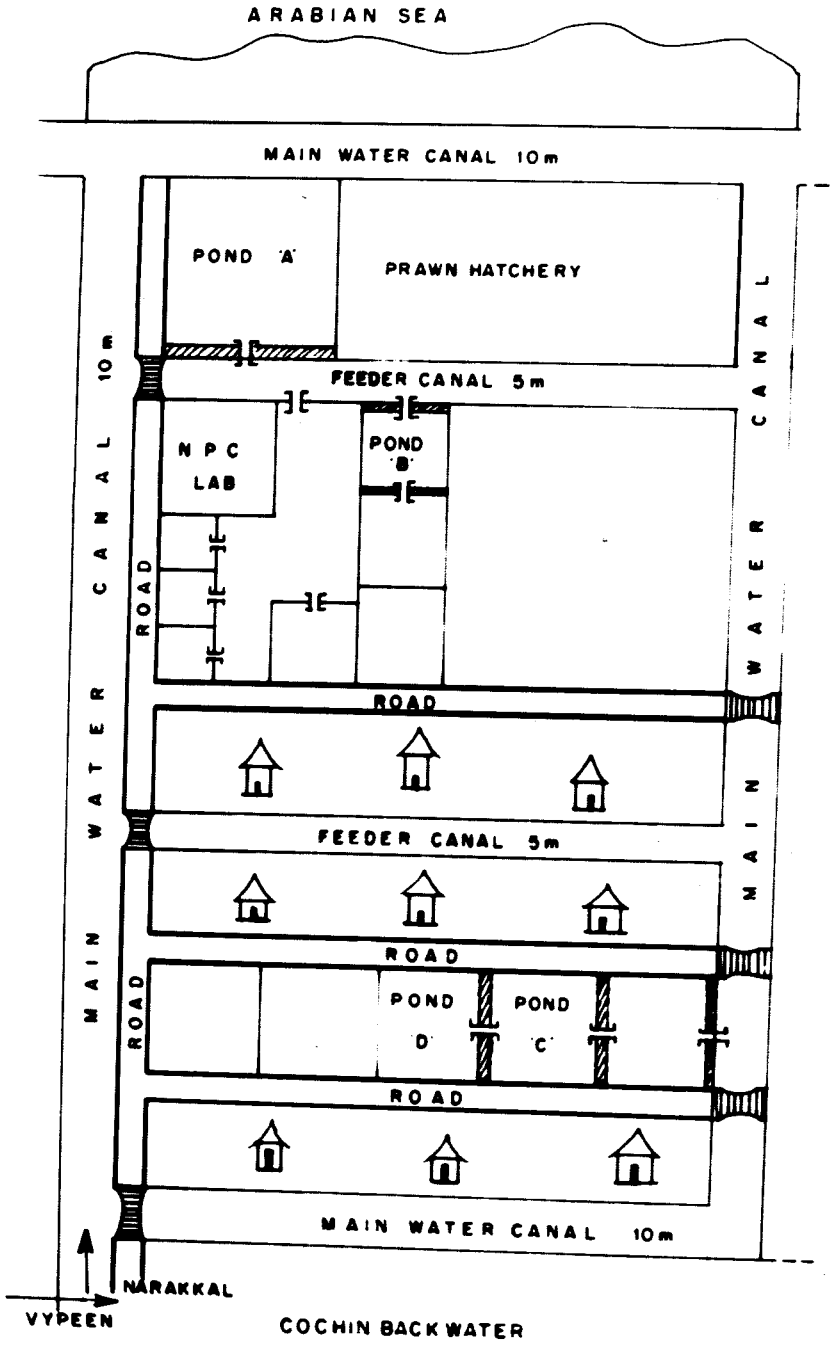
The four culture ponds selected for the present study were designated as pond A, B, C and D (Fig. 1).

Pond A and B: Pond A and B are perennial prawn culture systems. Pond A is situated on the western side of the Prawn Culture Laboratory, having an area of 0.6 hectare, with a muddy bottom and connected to the main feeder canal through a sluice gate. There was exchange of water throughout the period of study, in the pond, influenced by tides and its depth ranged from 0.5 to 1.5 metres, the minimum during neap tides and the maximum during spring tides. The maximum depth was observed in the monsoon season and the minimum in the summer season.

Pond B, with an area of 0.1 ha and muddy bottom, is situated at the northern side of the laboratory. It has a depth of 0.5 to 1.5 metres; and other conditions of pond B are similar to that of Pond A. Pond B was utilized for scientific prawn farming and periodically fertilized with inorganic fertilizer, according to the farm manager.

Pond C and D: Pond C and D are the "Pokkali fields" connected to the main feeder canal through similar types of fields. These seasonal fields are relatively shallow with a clay substratum and during the summer months of April and May the fields become dry, except for a small stretch, where water was present throughout the year. The water level in the fields during paddy cultivation varied from 0 to 0.5 m and during prawn culture period it varied from 0.5 to 1.2 m. The area of each one was 0.5 ha.

FIG. 1



LAYOUT OF THE STUDY AREA AND LOCATION OF PONDS

Study Area	Dimension	Area
A	100 x 60 m	0 6 ha
B	33 x 33 m	0 1 ha
C	100 x 50 m	0 5 ha
D	100 x 50 m	0 5 ha

A & B Perennial ponds

C & D Prawn-cum paddy fields

N P C LAB Narakkal Prawn Culture Laboratory

Bunds

Sluice gate

Houses

Over bridge

Foot path road

N

Sampling procedure

Water and sediment samples were collected, fortnightly, for a period of two years from October 1982 to September 1984, from four fixed sites in each pond for enumeration of the nitrogen cycle bacteria and for monitoring the environmental parameters. Surface water samples were aseptically collected from the four fixed sites of each pond in a sterilized 100 ml. capacity polyethylene container. After thorough mixing, the samples were preserved in 300 ml capacity BOD bottles for bacterial analysis. Sediment samples were collected from the four fixed sites, with an impact corer (100 cm x 4 cm diameter) made of perspex material, from the upper 10 cm layer. About 5 cm of surface sediment layer was removed aseptically from each core sample and thoroughly mixed with a sterile spatula in a sterile bottle, followed by vigorous shaking. Sediment samples from each pond were separately preserved in wide-mouthed sterile glass bottles . After sampling, all the samples were transported to the laboratory in a ice-box.

Water and surface sediment samples for chemical analyses were collected separately in 500 ml plastic containers. Successive collections were made in the fields starting from pond A to pond D between 0800 hours and 1000 hours throughout the two years of sampling.

Environmental parameters

Air and water temperatures, from the sampling sites, were recorded using a 1 to 50°C high precision mercury thermometer prior to the collection of water and sediment samples.

Dissolved oxygen:

Borosilicate glass reagent bottles (125 ml), with BOD stoppers were used for collecting samples for dissolved oxygen estimation. Three, replicates,

surface water samples were taken from each pond and the bottles were stoppered inside the water without allowing any air bubble. Using a graduated pipette, 1 ml of Winkler A and 1 ml of Winkler B reagents were added immediately after removing the bottles from water. The fixed bottles were kept in a covered rack to prevent evaporation and transported to the laboratory. Traditional Winkler method, with azide modification (Ann, 1975), was used to determine the dissolved oxygen content in 100 ml sample by standardizing the sodium thiosulfate solution against potassium dichromate. The dissolved oxygen content was expressed as mg/litre.

Salinity:

Water samples were collected in 500 ml polyethylene narrow mouthed bottles and preserved in ice-box till analysis in the laboratory. Salinity was determined by Mohr's Knudson titration method (Strickland and Parsons, 1968) using 10 ml of water sample, after standardizing the silver nitrate solution using the standard sea water supplied by the Institute of Oceanographic Sciences, Wormley, England (I.A.P.S.O.), every fortnight. Each sample was titrated till concordant values were obtained and the salinity content was expressed as ppt.($\%_o$).

pH and Eh:

pH of the water and sediment samples were determined in a Elico model LI-10T pH meter (Elico Pvt Ltd. Hyderabad, India), after standardizing the equipment using test buffers of pH 4.0 and 9.2 by adjusting the temperature, using a glass electrode along with a reference electrode.

Eh of the water and sediment samples were determined using the same Elico pH meter with a platinum electrode and values were recorded in mV units.

Nutrients:

Nitrite was determined in 50 ml of water sample by the Azo-Dye method (Bendschneider and Robinson, 1952). The determination of nitrite is based on the classical Griess's reaction in which the nitrite ion at pH 1.5 and 2.0 is diazotized with sulfanilamide, resulting in a diazo compound, which in turn is completed with N-(1-naphthyl)-ethylene-diamine to form a highly coloured azo-dye with an absorption maxima at 545 nm that is measured colorimetrically and the nitrite content was expressed as /ug at.NO_2-N/l.

The methodology used was the same for the determination of nitrate after reducing 50 ml of the sample through an amalgamated cadmium reduction column (Ann, 1975). Nitrate is reduced to nitrite almost quantitatively in the amalgamated cadmium column (Solyom and Carlberg, 1975). The nitrite is then determined according to the classical Griess's reaction (Ann, 1975) and the nitrate content was expressed as /ug at.NO_3-N/l.

Ammonia:

Ammonia content was determined in a 50 ml of water sample following the phenol hypochlorite method (Solorazano, 1969) with the successive addition of phenol solution, sodium nitroprusside solution and oxidizing reagents. They were mixed thoroughly after each addition. The colour was allowed to develop at room temperature in dark for one hour, and after the incubation period the absorbance was measured at the wave length of 640 nm with a ECIL spectrophotometer and the ammonia content was expressed as /ug at.NH_3-N/l.

Sediment sampling and analysis

As soon as the surface sediment samples were collected using the corer, they were filled in 200 ml wide-mouthed polyethylene bottles, in triplicate, with

minimum aeration and closed airtight. These bottles were shaken well after closing and the samples were preserved in an ice-box.

Sediment organic carbon:

Sediment samples were dried in an oven at 100°C for 24 hours and organic carbon content was determined in 100 mg of the dried, seived sample after potassium dichromate and sulfuric acid digestion, according to the procedure followed by Holme and McIntyre (1971) and Ann,(1975). In this method hot chromic acid is used to oxidize any organic carbon present, and the excess acid not reduced by organic matter is determined volumetrically with ferrous salt and the organic carbon content was expressed as µg at.C/g.

Sediment total phosphorus:

Sediment samples were dried in an oven at 105°C for 24 hours. Total phosphorus was determined in 0.5 g of the dried sample, after prolonged nitric-perchloric acid hot digestion. This brings all P into solution except that held in resistant silicate lattice structural sites. The outline of the method suggested by Ann,(1975) was followed and the results on the absorbance measured at 882 nm against distilled water spectrophotometrically and the total phosphorus was expressed as µg at.P/g.

Sediment kjeldahl nitrogen:

Sediment dried at 110°C for 24 hours was powdered and 0.1 g sample was processed for determining ammonia according to Ann,(1975). The nitrogen compounds in the mud were converted to ammonium sulfate by sulfuric acid digestion. The ammonia liberated was distilled and determined using indophenol blue method and the absorbance was measured after 45 minutes at 625 nm against distilled water spectrophotometrically and the total nitrogen content was expressed as µg at.N/g.

Bacterial enumeration:

Water and sediment samples were collected at fortnightly intervals, from the four ponds, for a period of two years from October 1982 to September 1984, for the enumeration of total heterotrophic, proteolytic, ammonifying, nitrifying (ammonia-oxidizing), denitrifying and nitrogen fixing bacterial populations. Enumeration of Azotobacter population was done for a period of one year from October 1983 to September 1984. Bacterial enumeration was carried out by employing two methods, viz., pour plate method and most probable number (MPN) method.

The pour plate count technique was followed for the enumeration of total aerobic heterotrophic, proteolytic, nitrogen fixing and Azotobacter bacterial populations. Enumeration of total aerobic heterotrophic bacteria was done using ZoBell's 2216 E Marine agar medium (Supplied by Hindustan Dehydrated Media, Bombay). Frazier gelatin medium (Table 1) was used for the enumeration of proteolytic bacteria (Rodina, 1972). The media were prepared with filtered aged sea water (75% seawater and 25% distilled water bringing out the approximate salinity of 30 ppt) for proteolytic bacterial enumeration. Free-living aerobic nitrogen fixing bacteria were enumerated in nitrogen-free agar medium (Rodina, 1972) and the Azotobacter by using Mannitol Agar medium (Norris and Ribbons, 1972). Media for free-living aerobic nitrogen fixing bacteria and for Azotobacter were prepared using double distilled water with 3% NaCl.

The enumeration of ammonifying, nitrifying (ammonia-oxidizing) and denitrifying bacterial groups were carried out by following the Most Probable Number (MPN) technique (Rodina, 1972). The MPN method is normally used because of the difficulties in culturing the above bacterial groups on solid media and the relative ease with which growth may be assessed in liquid culture. Dilution plate count is rarely used for these groups because of the tedious nature of the

Table 1: Composition of Media

*ZoBell Marine Agar 2216E medium for Total heterotrophic bacteria

Formula	Contentration per l.
Peptone	5.0 g
Yeast extract	1.0 g
Ferric citrate	0.1 g
Sodium chloride	19.45g
Magnesium chloride (dried)	8.8 g
Sodium sulfate	3.24g
Calcium chloride	1.8 g
Potassium chloride	0.55g
Sodium bicarbonate	0.16g
Potassium bromide	60mg
Strontium chloride	34mg
Boric acid	22mg
Sodium silicate	4mg
Sodium fluoride	2.4mg
Ammonium nitrate	1.6mg
Disodium phosphate	8.0mg
Agar	15.0 g

pH 7.6 ± 0.2

* Commercially available media prepared only with doubled distilled water.

Composition of Frazier Gelatin Medium (Rodina, 1972) for Proteolytic bacteria

NaCl	3 g
$K2HPO_4$	1.5 g
KH_2PO_4	0.5 g
Gelatin	4 g
Dextrose	0.05 g
Peptone	0.1 g
Beef extract	5ml
Agar	15 g
*Water	100ml

pH 7.2 ± 0.2

* Having approximately salinity of 30 ppt with the addition of 75% Aged water and 25% doubled distilled water.

Peptone water (Rodina, 1972) for Ammonifying bacteria

K_2HPO_4	1 g
KH_2PO_4	1 g
$MgSO_4.7H_20$	0.5 g
NaCl	30 g
Doubled distilled water	1000 ml

pH 7.2 ± 0.2

Meiklejohn Medium (Rodina, 1972)
for Nitrifying(ammonia-oxidizing bacteria)

$(NH_4)_2SO_4$	0.66 g
NaCl	30 g
KH_2PO_4	0.1 g
$MgSO_4.7H_20$	0.14 g
$FeSO_4.7H_20$	0.03 g
Double distilled water	1000 ml
* Trace elements mixture	1 ml

pH 7.2 ± 0.2

Fedorov Medium (Rodina, 1972)
For Nitrogen fixing bacteria and for nitrogen fixation.

Double distilled water	1000 ml
Mannitol	20 g
K_2HPO_4	0.3 g
$CaHPO_4$	0.2 g
$MgSO_4.7H_2O$	0.3 g
K_2SO_4	0.2 g
NaCl	30.0 g
$FeCl_3.6H_20$	0.1 g
$CaCO_3$	5 g
* Trace elements solution	1 ml
Agar	20 g

pH 7.0 ± 0.2

Giltay Medium (Rodina, 1972)
for Denitrifying bacteria

Two solutions are prepared:

1.	Distilled water	500 ml
	Asparagine	0.4 g
	Glucose	10.0 g
	KHO_3	2.0 g
2.	D.D.W	500 ml
	Sodium citrate	2.5 h
	KH_2PO_4	2.0 g
	$MgSO_4.7H_20$	1.0 g
	$CaCl_2$, $6H_20$	0.2 g
	$FeCl_2.4H_20$	Trace
	NaCl	30.0 g

Mannitol Agar medium (Conn, 1957)
for Azotobacter

Mannitol	20 g
K_2HPO_4	1.0 g
$MgSO_4.7H_20$	02 g
$CaC0_3$	1.0 g
$FeS0_4$	Trace
Na_2Mo0_4	5 g
NaCl	30.0 g
Agar	20.0 g
Double distilled water	1000 ml

pH7.0 ± 0.2

*Trace element solution (Rodina, 1972)

Distilled watrer	1000 ml
H_3BO_3	5 g
$(NH_4)_2$ $Mo0_4$	0.5 g
KI	0.5 g
NaBr	0.5 g
$ZnSO_4.7H_20$	0.2 g
$Al_2(S0_4)_3$ 18 H_20	0.3 g

techniques, the small size of the ammonifiers, nitrifiers and denitrifier colonies and overgrowth by heterotrophs in organic compounds, problems of lengthy incubation, and, media selectivity (Burns and Slates, 1982).

Peptone water medium (Table 1) was used for the enumeration of ammonifying bacterial population and Giltay medium (Table 1) was used for the denitrifying bacterial population; whereas Meiklejohn medium (Table 1) was used for the nitrifying (ammonia-oxidizers) bacterial population (Rodina, 1972). All these media were prepared using double distilled water with 3% sodium chloride as suggested by Lakshmanaperumalsamy et al. (1975) and Maruyama et al. (1970). To each 90 ml of the sterilized dilution blank (prepared with 0.85% NaCI solution), 10 ml of the water sample and ten gram of wet mud sample were inoculated aseptically and serial dilutions were prepared from 10^1 to 10^7 for both water and sediments separately. Since the bacterial numbers were very high in lower dilutions, higher dilutions were preferred for the enumeration of bacterial population after standardizing the methodology. For the enumeration of total heterotrophic and proteolytic bacterial populations in water 10^4 to 10^5 and in sediments 10^5 to 10^6 dilutions were used; whereas for nitrogen fixing and Azotobacter populations in water 10^2 to 10^3 dilution, and in sediments 10^3 to 10^4 dilution were used.

For ammonifying bacterial populations 10^3, 10^4 and 10^5 dilutions in water and 10^4, 10^5 and 10^6 dilutions in sediment were used. Enumeration of nitrifying (ammonia-oxidizers) and denitrifying bacterial populations were made using dilutions of 10^2, 10^3 and 10^4 for water, and 10^3, 10^4 and 10^5 for sediment, after the standardization of the enumeration method. For each dilution three replicates were kept and all the cultures were incubated at room temperature ($28 \pm 2°C$) with controls in triplicate.

ginal water sample or ten gram of the wet sediment was inoculated into each the flasks containing 100 ml nitrogen-free liquid medium and the flasks were ubated at room temperature (28 ± 2°C) for a period of fifteen days in the dark. he amount of nitrogen fixed was estimated with the help of mi crokjeldahl method s described in the methods of aquatic microbiology (Rodina, 1972) and the results ere expressed in mg NH_3-N/10 ml in water and in mg NH_3-N/10 g of the ediment.

Statistical analysis of data:

To study the effect of environmental parameters on bacterial population and itrogen fixation, the following linear multiple regression model was fitted (Snedecor nd Cochran, 1967):

$$Y = a + \sum_{i=1}^{n} bi.xi$$

Y = bacterial population

a = constant

bi = partial regression coefficient of Y on xi

xi = ith explanatory variable

n = No. of explanatory variables.

The data were processed in a Hewlett Packard computer on board the vessel FORV'Sagar Sampada' provided by the Department of Ocean Development, Government of India.

To study the influence of environmental parameters on the distribution of Azotobacter partial regression analysis was carried out (Snedecor and Cochran, 1967).

RESULTS

The period of observation extended from October 1982 to September 1984. In order to present the data, the period from October 1982 to September 1983 has been considered as the first year, and that from October 1983 to September 1984 as the second year. For graphical representation of the data monthly mean values were used. Ponds A and B were designated as the perennial fields and ponds C and D as the seasonal fields.

ENVIRONMENTAL PARAMETERS

Atmospheric temperature

Atmospheric temperature (Table 2) recorded in the study area ranged from 27.75 to 32.0°C during the two years, and not much variation was observed among the four ponds. Atmospheric temperature recorded during the second year was slightly higher than that of first year. Besides, during the first year temperatures were relatively higher in the postmonsoon and premonsoon seasons than that in the monsoon season. However, no definite seasonal trend was observed during the second year, as there was wide fluctuations in the temperature. The highest temperature (32°C) was recorded in the month of November 1982 and lowest (27.75°C) in September 1983 in the first year. In the second year also the highest temperature (32°C) was noticed in November 1983 but the lowest (28.75°C) was in June 1984.

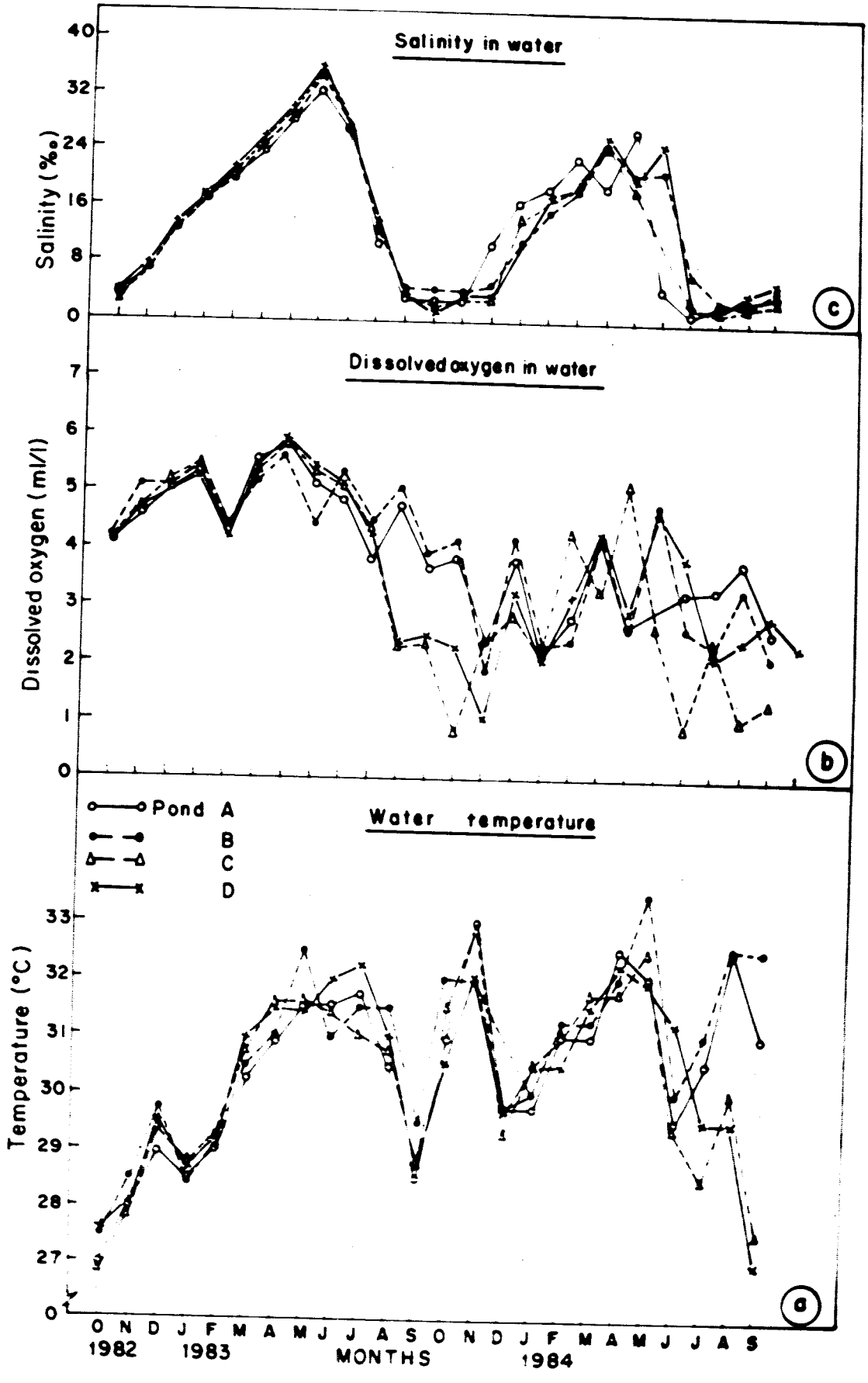
Water temperature

Figure 2a, shows the mean monthly water temperatures recorded during the period of investigation from the four ponds. In the two years of study, the water temperature ranged from 27.0 to 33°C in pond A, 27.5 to 33.75°C

Table 2: Atmospheric temperature data from the study area

1982-1983		1983-1984	
nth	Temperature(in °C)	Month	Temperature(in °C)
ctober	28.0	October	29.25
ovember	28.0	November	32.0
ecember	28.5	December	30.75
nuary	28.0	January	29.75
ebruary	28.0	February	31 0
arch	28.0	March	30.0
pril	29.0	April	31.25
lay	30.0	May	31.25
une	30.5	June	28.75
uly	30.5	July	29.5
ugust	29.75	August	31.5
eptember	27.75	September	30.05

FIG. 2


Salinity in water
Salinity (‰)
40
32
24
16
8
0
c
Dissolved oxygen in water
Dissolved oxygen (ml/l)
7
6
5
4
3
2
1
0
b
Pond A
B
C
D
Water temperature
Temperature (°C)
33
32
31
30
29
28
27
0
a
O N D J F M A M J J A S O N D J F M A M J J A S
1982
1983
MONTHS
1984

in Pond B, 27.0 to 33.0°C in pond C and 27.5 to 33.75°C in pond D. Water temperatures were relatively high during the second year compared to the first year. A similar pattern in the distribution of water temperature was noticed in all the ponds during the post-monsoon and premonsoon seasons of the first year. The water temperature steadily increased from the month of October, showed a slight decline in January and reached the maximum in April in pond C, May in pond B, and July in ponds A and D. In September, a sharp decline was observed in all the ponds. During the late premonsoon and early monsoon seasons, water temperatures in all the ponds were consistently high. Unlike during the first year, when only one temperature maximum peak could be noticed in all the four ponds, during the second year, ponds A and B showed three peaks during October-November, April-May and in August. However in ponds C and D no peak was noticed in August. During the late postmonsoon months (Dec-Feb) temperatures were low. After the second peak noticed during the premonsoon season in ponds C and D a steady decline in temperature was observed, with minor variations. But in ponds A and B temperature showed a decline till June, but again steadily increased.

Dissolved Oxygen

During the first year, a clear trend was observed from October to July in all the four ponds, with minor differences between ponds (Fig. 26). During the postmonsoon months, beginning October, there was a steady increase in the dissolved oxygen (DO) content untill January. This was followed by a decline in DO levels in February. Thereafter, the DO levels increased steadily, and the maximum values were recorded during April. After this premonsoon maximum, a steady decline was noticed in the seasonal ponds (C and D) during the monsoon months. In pond B, the DO showed a decline during May; but again showed an increase during June, followed by a

decline in July. During the late monsoon months of August and September during the first year, and early postmonsoon months (Oct-Nov) of second year the DO levels in the perennial ponds (Ponds A and B) remained at a relatively higher level than that in the seasonal ponds (C and D). From December to September in the second year, there was no consistent trend in the distribution of dissolved oxygen in the various ponds.

Salinity

Data for salinity (Fig. 2c) showed significant differences between the two years. In both the seasonal and perennial ponds, the salinity ranged from 1.67‰ to 34.22‰ during the first year and from 1.87‰ to 28.22‰ during the second year, thus indicating brackish conditions through out the second year in all the ponds. Salinity exhibited a similar trend in all the ponds during the first year. A steady increase was noticed during the post-monsoon season, from October, resulting in a salinity maxima during the late premonsoon month of May in all the ponds. Thereafter, salinity exhibited a steady decline. Among the ponds, pond A alone had relatively lower salinity levels during certain months. In the late monsoon months of the first year and early postmonsoon months of the second year (from August to November) the lowest salinity levels were found in all the ponds. From the late postmonsoon months of November in the second year, salinity showed a steady increase in the ponds with the exception of pond A, and the maximum concentrations were observed in March. However, in pond A salinity showed an increase from October with a slight decline during March and exhibited a peak in April. From May salinity showed a decline resulting in minimum levels during the monsoon season in all the ponds.

pH of the water and sediment

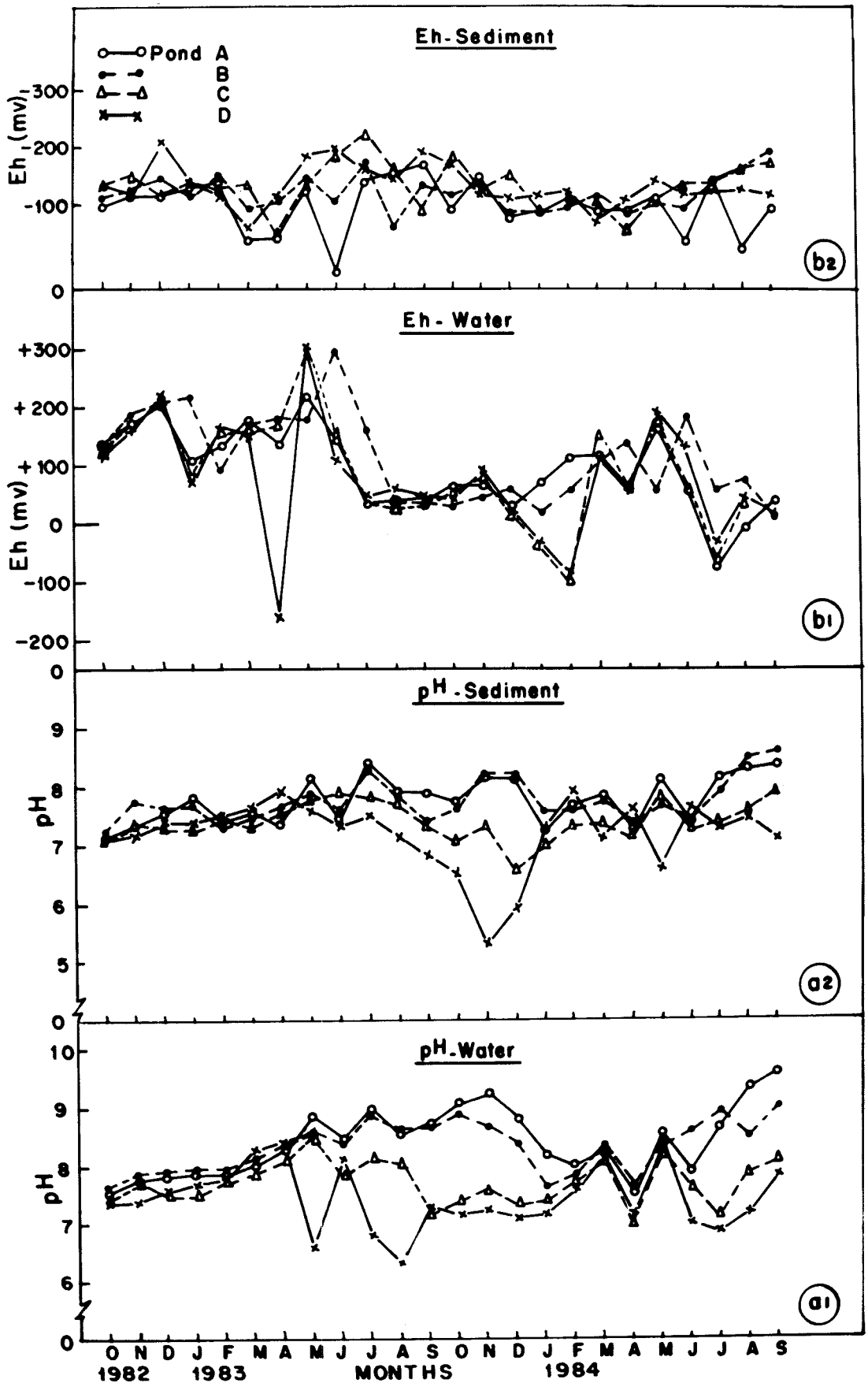
Water pH:

Water pH (Fig. 3a1) significantly differed between the seasonal and the perennial ponds. No striking difference could be observed in the pattern of pH distribution between the two perennial ponds (A and B) during most part of the investigation, though minor variations in the actual pH values were noticed in certain months. The water pH ranged from 7.5 to 9.6 in pond A, 7.6 to 9.5 in pond B, 7.0 to 8.15 in pond C and 6.57 to 8.47 in pond D.

In the first year, during the postmonsoon and early premonsoon seasons, pH was relatively low in all the ponds, though the perennial ponds had slightly higher pH than the seasonal ones. From the late premonsoon months pH showed an increase in ponds A, B and C with a peak value in May. In contrast, in pond D an abrupt decrease in pH was noticed in May. Similarly, while a decline in pH was observed in ponds A, B and C during June, in pond D, there was a steep increase in the pH. This inconsistent trend in pH of the pond D was noticed till the end of the first year (September, 1983). During the monsoon months of July and August, pH in ponds A, B and C exhibited almost a similar pattern, but a inverse trend was observed in pond D.

During the second year, unlike in the first year, pH distribution differed markedly between the seasonal and perennial ponds. During the postmonsoon season pH was significantly lower in the seasonal ponds than the perennial ponds. Throughout this season pH remained almost at a

FIG. 3


Eh-Sediment
Pond A
B
C
D
Eh (mv)
300
200
-100
0
b2
Eh - Water
+300
+200
+100
0
-100
-200
Eh (mv)
b1
pH - Sediment
9
8
7
6
5
pH
a2
pH - Water
10
9
8
7
6
pH
a1
O N D J F M A M J J A S O N D J F M A M J J A S
1982
1983
MONTHS
1984

constant level in ponds C and D, and within the range 7.1 to 7.5. During the same period in ponds A and B, pH was in the range 7.6 to 9.3. Among the two perennial ponds pH was relatively more in pond A. After exhibiting a low level in January, pH showed an increase in all the ponds, a peak was noticed in March, followed by a decline in April and again an increase in May. Thus during the premonsoon season pH showed wide fluctuations.

Sediment pH:

Figure 3a2 indicates the distribution of sediment pH in the four ponds during the period of investigation. The sediment pH ranged from 7.2 to 8.45 in pond A, 7.2 to 8.6 in pond B, 6.52 to 7.95 in pond C and 5.3 to 7.67 in pond D. During most part of the first year, sediment pH, was found within the range 7-8, except for slightly higher sediment pH in July in ponds A and B and slightly lower pH in pond D. But during the second year, wide seasonal fluctuations in the sediment pH were observed, with the pond D showing maximum variation. In pond D, sediment pH showed uncharacteristic low values during the postmonsoon, particularly in November-December months. When compared to the seasonal ponds, the perennial ponds had markedly higher pH during the post-monsoon season. After this period, there was no consistent trend in sediment pH in any of the ponds.

Eh of the water and sediments

Water Eh:

The Eh of water (Fig. 3b1) showed striking difference between the ponds. With the exception of a single instance of negative value (-155 mV) during April, in pond D, water Eh was found to be positive throughout the

first year. But during the second year, negative values were observed in certain months particularly in the seasonal ponds. Water Eh was found to vary from -75 to + 247 mV in pond A, + 15 to + 295 mV in pond B, -95 to + 300 in pond C and - 155 mV to + 300 mV in pond D. During the onset of the postmonsoon season a gradual but steady increase in water Eh was noticed with a peak in December in ponds A, C and D, and in January in pond B. After a decline in January in ponds A, C and D and February in pond B water Eh was found to inconsistently increase in all the ponds, with the notable exception of pond D in which a steep decline in Eh (- 155 mV) occurred in April. In ponds A, C and D high values occurred during the premonsoon month of May and in pond B in June. Thereafter, a steady decline resulted in low values in the monsoon season. Besides, the declining trend continued till the early premonsoon season in ponds C and D giving negative values, as against the gradual increasing trend in the perennial ponds. During the second year too, a premonsoon increase followed by a monsoon decline leading to negative values in most of the ponds was recorded. In general, Eh values observed in the first year were found to be much higher than that in the second year, and the seasonal ponds were in a much reduced state than the perennial ponds during the monsoon season of the second year.

Sediment Eh:

Data depicted in Figure 3b2 indicates that there was much variation in the Eh of the sediment among the ponds. Sediment Eh ranged from -165 to -25 mV in pond A, -180 to -33.5 mV in pond B, -227.5 to -50 mV in pond C, and -290.5 to -60 mV in pond D. Thus, the sediment was in a reduced condition throughout the period of study. The overall picture of sediment

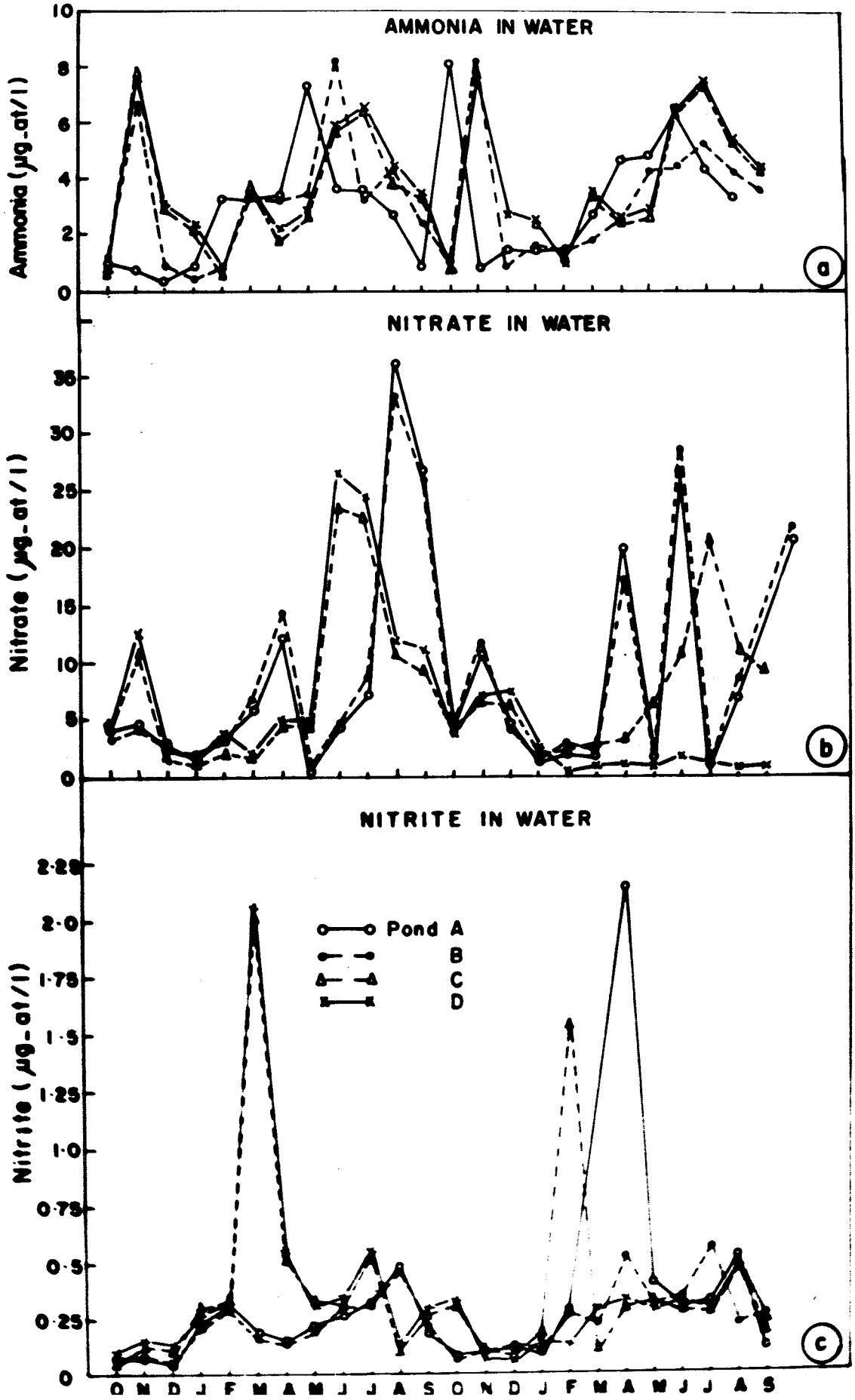
pH indicates that there was no specific seasonal pattern, though the monsoon season provided highly negative values in both the years. Besides, compared to the first year, the variation between ponds was relatively narrow in the second year.

Nitrite-N

Fig. 4c, shows the distribution pattern of nitrite in the sediments during the two years of study. The nitrite content ranged from 0.045 to 2.04 ug at/l in pond A, 0.05 to 2.07 ug at/l in pond B 0.06 to 2.04 ug at/l in pond C and 0.065 to 2.11 ug/at/l in pond D. In both the years the lowest nitrite levels were recorded during the postmonsoon months. A sharp, unusual increase in nitrite level was noticed in the seasonal ponds (C and D) in March 1983, while in the same month nitrite levels were significantly low in the perennial ponds (A and B). Similarly, during the second year high nitrite levels were encountered in pond C in February and in pond A in April.

During the first year there was no striking difference in the nitrite content of the water between the ponds up to the month of February, though the seasonal ponds had relatively high nitrite levels. After this period, in the premonsoon reason, nitrite levels showed a similar trend in both the perennial ponds but with relatively low levels when compared to the seasonal ponds, which had higher nitrite levels, with an uncharacteristic peak in March. The highest nitrite levels in the perennial ponds occurred during the monsoon season. Whereas in the seasonal ponds nitrite levels showed a premonsoon peak in July, but decreased abruptly in August and again showed a minor peak during the late monsoon and early postmonsoon months (Sep-Oct). As in the case of perennial ponds, the seasonal ponds also had the lowest nitrite levels in the remaining post-monsoon months of the second year.

FIG. 4


AMMONIA IN WATER
Ammonia (µg-at/l)
10
8
6
4
2
0
a
NITRATE IN WATER
Nitrate (µg-at/l)
35
30
25
20
15
10
5
0
b
NITRITE IN WATER
Nitrite (µg-at/l)
2·25
2·0
1·75
1·5
1·25
1·0
0·75
0·5
0·25
0
Pond A
B
C
D
c
O N D J F M A M J J A S O N D J F M A M J J A S

Except for the unusual peaks observed in pond C in February and pond A in April, nitrite levels remained low and inconsistent during the premonsoon season in all the ponds when compared to the mosnoon season of the second year. During the latter season, in ponds A, C and D, a similar trend was observed in the nitrite levels with peak values in August. Whereas, in pond B a peak occurred during July, which was followed by an abrupt decline during August.

Nitrate-N

Fig. 4b illustrates the distribution of nitrate in the sediments. Nitrate distribution exhibited almost a similar pattern with higher levels during the monsoon months and relatively low values during late postmonsoon and early premonsoon months in both the years, except in pond D, which showed extreme low values during most part of the second year. The nitrate content ranged from 0.27 to 36.84 µg at/l in pond D, 0.52 to 36.12 µg/at/l in pond B, 1.52 to 26.96 µg at/l in pond C and 0.12 to 27.11 µg at/l in pond D. During the postmonsoon season of first year, in the perennial ponds relatively low levels of nitrate were found when compared to the seasonal ponds. But during the premonsoon season nitrate levels were relatively high in the perennial ponds with a peak in April. Even though monsoon peaks were exhibited by all the ponds, in the seasonal ponds it was observed during the early monsoon season as against the late monsoon period in the perennial ponds. During the second year, there was a minor postmonsoon peak followed by a premonsoon low in all the ponds. The seasonal pond D had extremely low nitrate levels during the monsoon season in contrast to the peak nitrate levels in all other ponds, with the perennial ponds A and B showing greater variations.

Ammonia-N

Figure 4a, illustrates the distribution of ammonia in the sediments of ponds during the investigation period. The ammonia content ranged from 0.33 to 8.03 µg at/l in pond A, 0.43 to 8.12 µg at/l in pond B, 0.66 to 7.86 µg at/l in pond C and 0.72 to 7.75 µg at/l in pond D. There was an identical pattern in the distribution of ammonia in the seasonal ponds (and D), but there were differences between the two perennial ponds (A and B)., The seasonal ponds exhibited four major peaks and two minor peaks in the ammonia levels during the period of investigation. A sharp postmonsoon peak was observed during both the years in the seasonal ponds. Another major peak was evident during the early monsoon season during both the years. The minor peaks were noticed during the early premonsoon season in the seasonal ponds. The lowest ammonia levels were recorded during the months of October and February in both the years.

Ammonia distribution pattern in the perennial ponds significantly differed from that of seasonal ponds. There was also substantial differences between the two perennial ponds as for as ammonia distribution is concerned. In pond B, as in the case of the seasonal ponds, a peak concentration of ammonia occurred during the post-monsoon season of November in the first year, in contrast to the extremely low levels of ammonia in pond A during the early postmonsoon. After a late postmonsoon decline in the ammonia levels in the perennial ponds, an inconsistent increase was noticed, resulting in peak levels during the late premonsoon month of May in pond A and early monsoon month of June in pond B. This was followed by a steady decrease resulting in low levels in September in pond A and October in pond B. In October of the second year, when lowest ammonia levels occurred in ponds B, C and D in pond A a peak was observed. The other three ponds exhibited ammonia peaks in

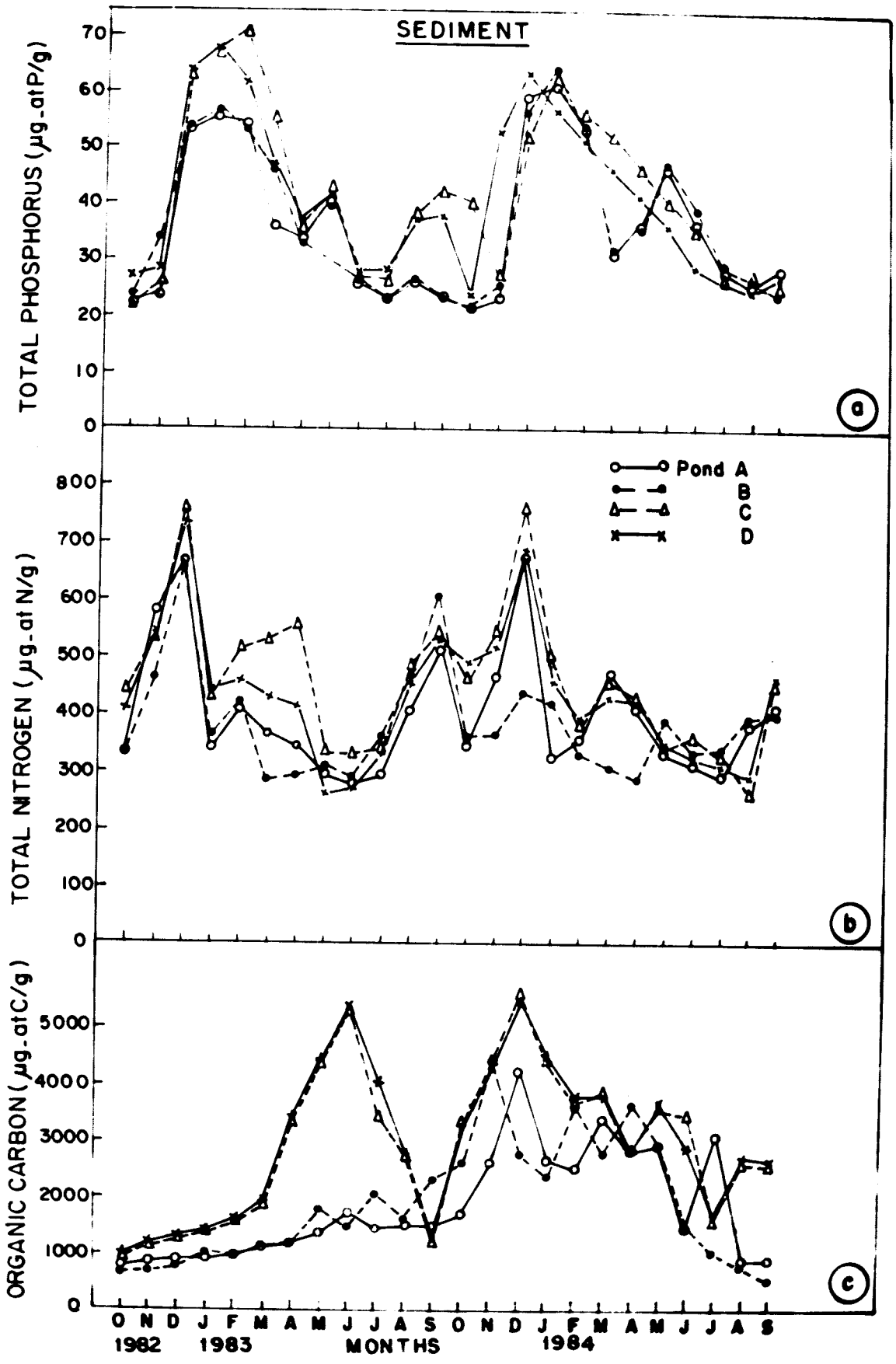
November. From late postmonsoon to early premonsoon ammonia levels were found to be low. During the subsequent period, during the second year, a similar pattern was observed in both the perennial ponds, though pond A had relatively higher levels of ammonia during the premonsoon season.

Organic Carbon

The distribution patterns of organic carbon in the sediments of the seasonal and perennial ponds are shown in Fig. 5c. The organic carbon content ranged from 758.33 to 3474.98 µg at/g in pond A, 774.99 to 4391.64 µg at/g in pond B, 1008.32 to 5691.64 µg at/g in pond C and 1007.37 to 5864.64 µg at/g in pond D. Throughout the period of investigation, organic carbon was found to be considerably higher in the seasonal ponds, than that in the perennial ponds, with minor deviations. In all the ponds, organic carbon levels were consistently low during the postmonsoon and early premonsoon seasons of the first year. While a marked increase was evident in the seasonal ponds resulting in a very prominent premonsoon peak, in the perennial ponds a marginal inconsistent increase was observed, without any prominent peak till the end of monsoon season. After the premonsoon increase in the seasonal ponds, there was a steady decline during the monsoon reaching a low level in September.

Consistently higher carbon levels were evident during the second year than the first year, particularly in the perennial ponds. Besides, unlike during the first year, there was a postmonsoon increase in the carbon content in all the ponds. The seasonal ponds had significantly higher carbon contents than the perennial ones. From the late postmonsoon season a steady but inconsistent decline was observed in the seasonal ponds towards the monsoon season. But the perennial ponds showed greater variation throughout most part of the second year.

FIG. 5


SEDIMENT
TOTAL PHOSPHORUS (µg.atP/g)
70
60
50
40
30
20
10
0
a
Pond A
B
C
D
TOTAL NITROGEN (µg.at N/g)
800
700
600
500
400
300
200
100
0
b
ORGANIC CARBON (µg.atC/g)
5000
4000
3000
2000
1000
0
c
O N D J F M A M J J A S O N D J F M A M J J A S
1982
1983
MONTHS
1984

Total nitrogen

Figure 5b represents the pattern of nitrogen distribution in the sediments of the perennial and seasonal ponds. The total nitrogen content ranged from 278.56 to 676.34 µg/at/g in pond A, 284.34 to 652.42 µg at/g in pond B, 268.47 to 763.27 µg at/g in pond C and 262.98 to 728.11 µg at/g in pond D. A linear increasing trend was observed with the onset of postmonsoon during both the years in all the four ponds till December (except in pond D in the second year), which was followed by a sudden decline in January However, in the premonsoon season considerable quantitative differences were observed between ponds. Among the ponds, the seasonal ones had relatively higher levels of nitrogen in the sediment, the pond C having distinctly higher amounts than pond D. During the late premonsoon month of May and the early monsoon months of June and July nitrogen levels were consistently low in all the ponds. In the subsequent monsoon months, in all the ponds, the nitrogen content showed an increase with a peak in September.

As in the case of first year, a prominent increase in nitrogen, with a peak, was noticed in all the ponds during the postmonsoon season of the second year, though pond B had relatively low nitrogen levels. After a late post-monsoon decline, nitrogen increased steadily during the premonsoon season, except in pond B which showed a steady decline in the same period. In the monsoon season nitrogen was relatively low in all the ponds.

Total phosphorus

The phosphorus content in the sediment (Fig. 5a) showed almost a similar seasonal pattern in all the ponds with minor fluctuations. The seasonal ponds had relatively higher amounts of phosphorus than the perennial ponds throughout the period of investigation except during May-June period in the

second year. The phosphorus levels ranged from 21.17 to 61.63 µg at/g in pond A, 21.28 to 64.17 µg at/g in pond B, 26.31 to 71.44 µg at/g in pond C and 23.41 to 67.27 µg at/g in pond D. In both the years, during the early postmonsoon season, phosphorus level was low in all the ponds, except in pond D, which had relatively high values in November of the second year. In both the years, peak level of phosphorus was observed in the late postmonsoon and early premonsoon period (Dec-Feb). In ponds A and B a steady decline was observed, after this period, reaching the lowest values during the late monsoon months, but for a slight increase in pond A in the month of May. In the seasonal ponds also low levels of phosphorus were recorded during the early monsoon season; but a minor peak was noticed during the late monsoon season.

BACTERIAL ANALYSIS

Bacterial density has been represented in the figures as their log number per ml of water or per gram of sediments using monthly mean values.

Total heterotrophs

Figure 6a and 6b show the monthly mean number of aerobic heterotrophs recorded in the water and sediments from the ponds Relatively more numbers of bacteria occurred in the sediments when compared to water in all the ponds throughout the period of investigation, with the notable exception in pond D in the month of September 1983 in which lesser numbers occurred in the sediment.

During the first year of study almost a similar pattern was observed in the occurrence and distribution of aerobic heterotrophs in the

FIG. 6a

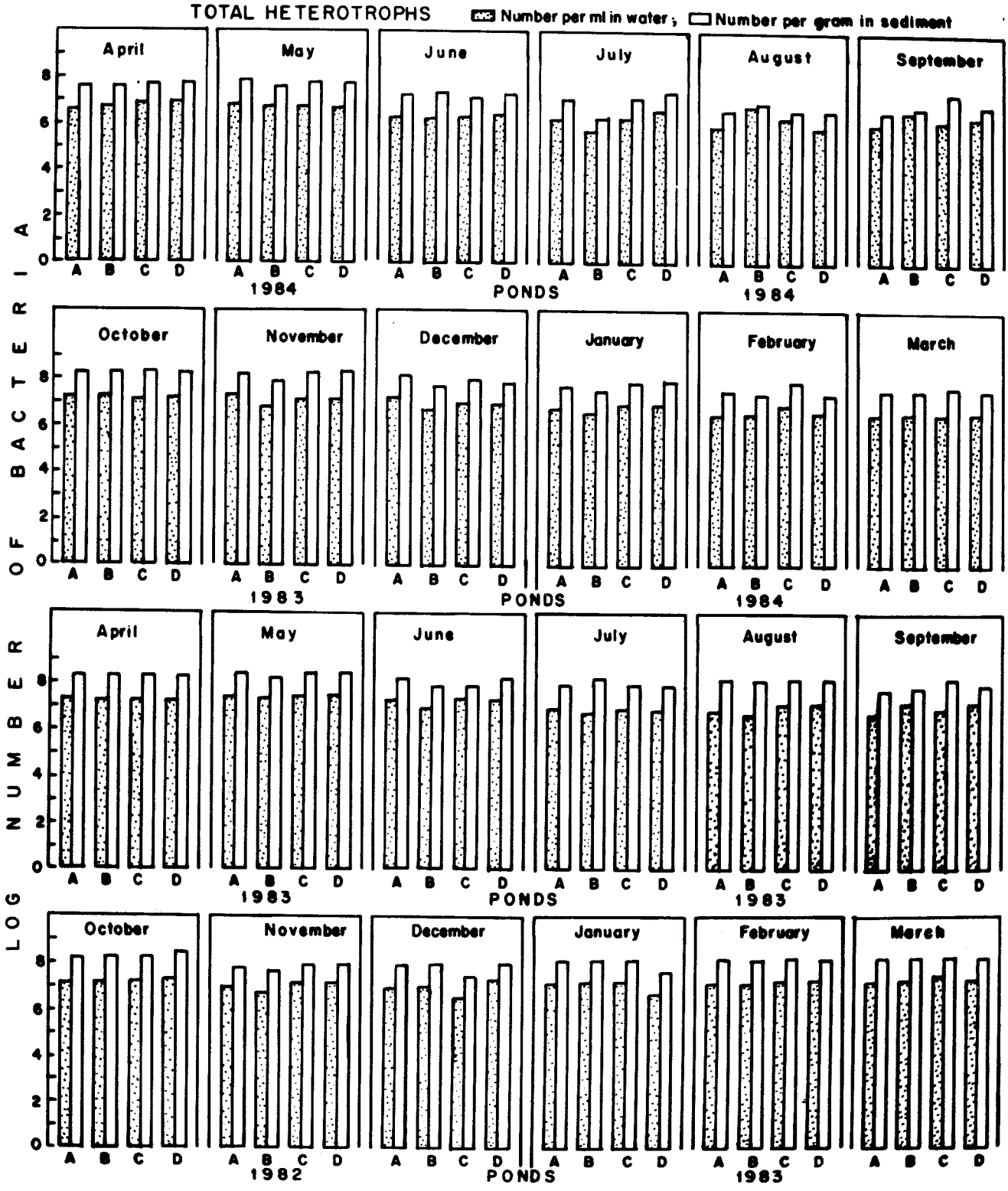
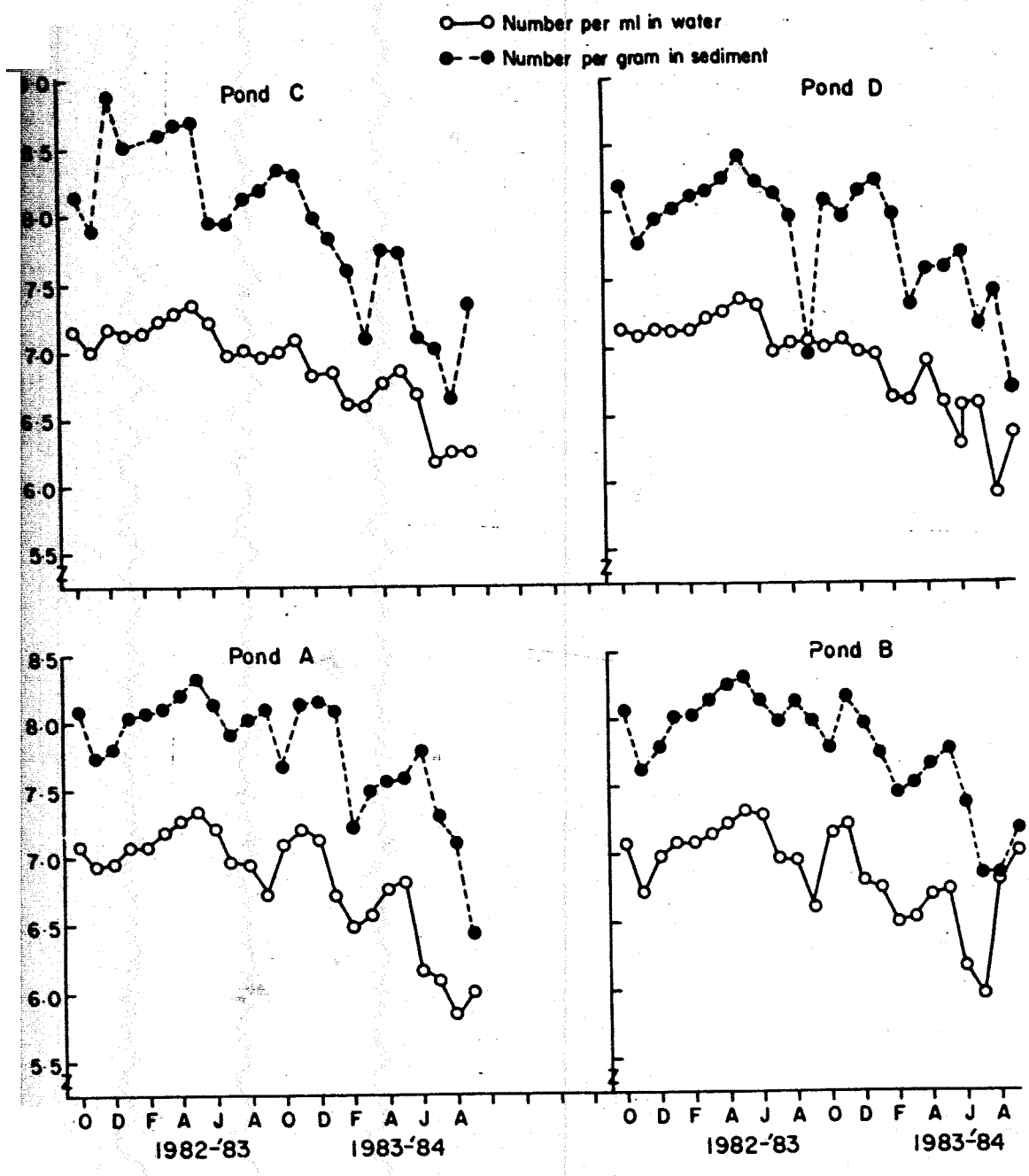

TOTAL HETEROTROPHS
Number per ml in water; Number per gram in sediment
LOG NUMBER OF BACTERIA
April
May
June
July
August
September
October
November
December
January
February
March
A B C D
1984
1983
1982
PONDS

Fig. 6b TOTAL HETEROTROPHS



water of all the ponds, with minor variations. In ponds A, B and C a drop in the bacterial numbers occurred during November which was followed by a consistent increase during the late postmonsoon and premonsoon seasons. In pond D, however, there was no significant variation from October to February, though there was a steady increase in the heterotrophs during the premonsoon months. In all the 4 ponds a peak abundance of heterotrophs was noticed in the month of May with a steady decline during the monsoon months.

During the second year of observation the mean number of heterotrophs recorded from the water were significantly less than that of the first year in all the seasons. Besides, a drop in the population was observed during the late post monsoon and early premonsoon seasons. During the early premonsoon season an increase was observed in all the ponds. As in the case of first year, a peak occurred during May in ponds A, B and C, and April in pond D. During July-August period relatively few numbers were found in the samples. After this period, in pond B and D a minor peak was noticed.

Almost a similar pattern was observed in the bacterial abundance in the sediments as in the case of water during the first year, with the exception of pond D which showed deviation from the trend observed in other ponds. In all the 4 ponds, maximum bacteria occurred during the premonsoon month of May in the first year. Following this period, there was a reduction in the bacterial population during the early monsoon season in ponds A, B and C, and with a prominent decline in pond D in which very low numbers occurred in the month of September.

In contrast to the first year, during the second year a peak was noticed during the postmonsoon months of October-November in pond C,

November-January in pond A, November in Pond D and October-January in pond B. A second peak was noticed during the premonsoon months of April-May in pond C, May in pond B, and in the monsoon months of June in ponds A and D. This was preceded by a drop in bacterial numbers during February in ponds A and B, and March in ponds C and D.

The mean log numbers of aerobic heterotrophs recorded from the water and sediments during the different months are compared in figure 6b. The average log values in water ranged from 5.83 to 7.31 in pond A, 5.84 to 7.32 in pond B, 6.20 to 7.34 in pond C and 5.84 to 7.35 in pond D. Whereas, in the sediments it ranged from 6.60 to 8.31 in pond A, 6.84 to 8.3 in pond B, 6.69 to 8.96 in pond C, and 6.60 to 8.41 in pond D. Pond C had consistently higher number of bacteria, both in water and sediments, than the other ponds.

In order to identify factors which influenced the variation in the bacterial density, both in water and sediments, in the ponds, multiple linear regression analysis was performed on the data on water and sediment characteristics with that of the bacterial number and the results are given in Table 4. Of the 13 environmental parameters considered, water Eh, sed. Eh, Sed. organic carbon and total nitrogen did not have any significant influence on the distribution of heterotrophs in both the water and sediments in any of the ponds.

In pond A, sedi. pH $>$ water temp. $>$ dissolved oxygen content (DO) water pH $>$ nitrite-N $>$ NH_3-N in the water (R^2= 94.438; $P<0.05$) and sedi. pH $>$ water temp. $>$ D.O. $>$ water pH $>$ NH_3-N $>$ NO_2-N in the sediments (R^2=92.84; $P < 0.05$) were found to have significant influence on

Table 3: Monthly rainfall data from October 1982 - September 1984

*1982 - 1983		**1983 - 1984	
Month	Rainfall(in mm)	Month	Rainfall (in mm)
October	89.0	October	183.1
November	23.9	November	102.5
December	9.4	December	85.1
January	5.0	January	156.5
February	Trace	February	170.1
March	Trace	March	59.0
April	0.0	April	113.1
May	109.8	May	136.6
June	25.0	June	785.0
July	273.0	July	601.4
August	646.4	August	243.7
September	605.8	September	131.1

* Total rainfall - 2448.9 mm

** Total rainfall - 3207.0 mm

In (1982-83) the first year

Postmonsoon - 127.3

Premonsoon - 134.8 mm

Monsoon - 2186.8 mm

In (1983-84) the second year

Postmonsoon 525.2 mm

Premonsoon - 479.3 mm

Monsoon - 1761.0 mm

Table 4: Estimates of the parameters in multiple linear-regression analysis for total heterotrophic bacteria

Symbol	ENVIRONMENTAL PARAMETERS	TOTAL HETEROTROPHIC BACTERIA							
		WATER				SEDIMENTS			
		A	B	C	D	A	B	C	D
a		0.9449	-0.8513	0.6134	0.6957	1.6226	-1.2593	1.8846	-2.0144
b1	Water temp.	0.2128	NS	0.1124	0.1493	0.2416	NS	0.2116	0.1903
b2	Water pH	0.2807	0.2120	0.5234	0.1139	-0.3965	-0.1552	0.5474	0.1541
b3	Sedi. pH	-0.5364	0.8221	-0.2673	0.1310	-0.7164	1.2670	-0.6435	0.0818
b4	Water Eh	NS	NS	NS	NS	NS	NS	NS	NS
b5	Sedi. Eh	NS	NS	NS	NS	NS	NS	NS	NS
b6	Diss. O_2	0.2628	NS	0.0902	NS	0.3342	NS	0.0864	NS
b7	Salinity	NS	-0.3981	NS	NS	NS	-0.3674	NS	NS
b8	Niitrite-N	0.3876	NS	0.0379	NS	-0.3135	NS	0.0258	NS
b9	Nitrate-N	NS	-0.1331	NS	0.0312	NS	-0.1459	NS	0.0333
b10	Ammonia-N	-0.0569	NS	-0..0460	-0.0653	-0.0635	NS	-0.0948	-0.0942
b11	Org. carbon	NS	NS	NS	NS	NS	NS	NS	NS
b12	Tot. nitrogen	NS	NS	NS	NS	NS	NS	NS	NS
b13	Tot. phosphorus	NS	0.0377	NS	NS	NS	0.2606	NS	NS
R^2		94.38%	95.53%	97.67%	96.87%	94.84%	95.85%	97.63%	96.45%

NS - not significant A, B, C, D - Ponds

the bacterial numbers. Sedi. pH and NH_3-N in water and sedi. pH, NH_3 -N and NO_2-N in the sediments showed a negative effect on the abundance of heterotrophic bacteria in the respective water and sediment regions.

In pond B, salinity > NO_3-N > total P > sedi. pH > water pH in the water (R^2 = 95.53; $P < 0.05$) and salinity > total P > NO_3-N > sedi. pH > water pH in the sediments (R^2 = 95.85; $P < 0.05$) explained the variation in bacterial density. Salinity and NO_3-N had a negative influence in water, salinity, NO_3-N, water pH showed negative influence on the occurrence of heterotrophs in the sediments.

The factors which significantly influenced the density of heterotrophs in pond C were water temp. > sedi. pH > water pH > NO_2-N > D.O. > NH_3-N in the water (R^2 = 92.67; $P < 0.05$) and sedi. pH > water temp. > NH_3-N > water pH > D.O. > NO_2-N in the sediments (R^2 = 97.63; $P < 0.05$). Sediment pH and NH_3-N showed a negative correlation with total heterotrophs in both the water and the sediments.

In pond D, NO_3-N > water temp. > NH_3-N > water pH > sedi. pH in the water (R^2 = 96.87; $P < 0.05$) and water temp. > NO_3-N > NH_3-N > water pH > sedi. pH in the sediments (R^2 = 96.45; $P < 0.05$) were factors influencing the density of heterotrophs. The number of heterotrophs were negatively correlated with ammonia nitrogen in water and sediments.

Proteolytic bacteria

The distribution pattern and relative abundance of proteolytic bacteria during the different months are shown in figure 7a and b. During the first year of study there were no striking differences in the seasonal distribution pattern of proteolytic bacteria in both the water and sediments

FIG. 7a

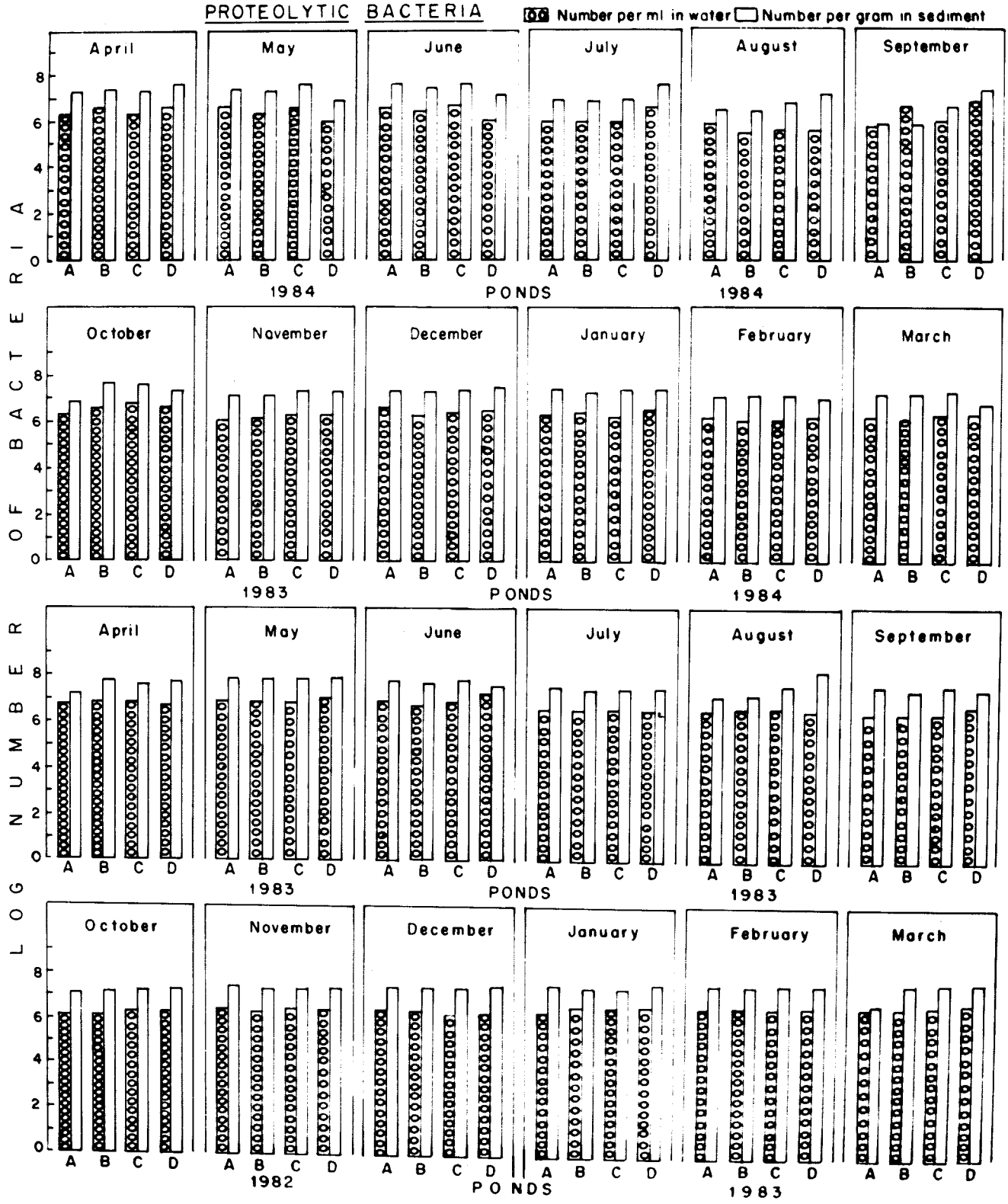
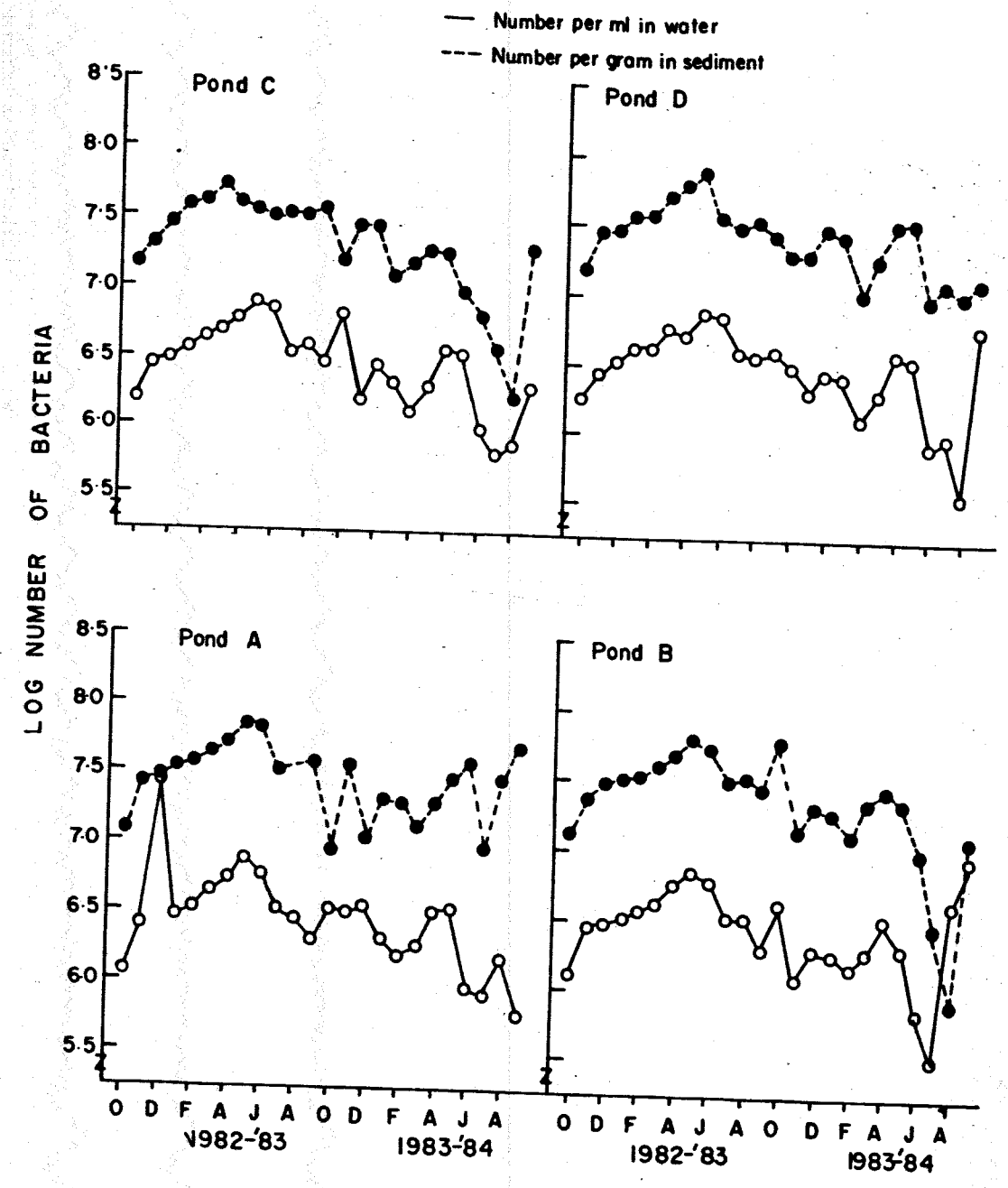

PROTEOLYTIC BACTERIA
Number per ml in water
Number per gram in sediment
LOG NUMBER OF BACTERIA
April
May
June
July
August
September
October
November
December
January
February
March
A B C D
1984
1983
1982
PONDS

Fig. 7b PROTEOLYTIC BACTERIA



in most of the ponds. A steady increase in the bacterial population was observed from the postmonsoon season in all the ponds and peak occurrence of bacteria was noticed during the premonsoon months, particularly in May 1983. In pond D, however, a steep increase in the bacterial numbers was noticed in the water in the postmonsoon month of December 1982. During the monsoon season there was a steady decline in the bacterial population and the lowest numbers were recorded during August-September in most of the ponds. Besides, there was also a close relationship between the seasonal pattern of distribution, in both water and sediments,in the first year in all the ponds.

In the second year, there was no consistent trend in the bacterial population in the ponds. In spite of this, a peak occurred during the premonsoon months, April-May of 1984 in sediments and water in most of the ponds. During the monsoon months of July-September 1984 there were marked differences in the pattern of distribution of bacteria in the ponds. Thus the seasonal influence of bacteria was more apparent during the first year than during the second year.

The average log numbers in the water ranged from 6.17 to 7.46 in pond A, 6.11 to 6.86 in pond B, 6.23 to 6.86 in pond C and 7.2- to 7.87 in pond D. Whereas, in the sedimentsit ranged from 6.66 to 7.85 in pond A, 7.14 to 7.85 in pond B, 7.20 to 7.86 in pond C and 6.25 to 6.87 in pond D. Pond D water and pond C sedimentswere relatively more productive than the sediment and water zones of other ponds (Fig. 7a and b).

Among the parameters which were considered for studying their influence on the abundance of proteolytic bacteria (Table 5) Eh of the sediment, organic carbon and total nitrogen did not have any significant

Table 5: Estimates of the parameters in multiple linear regression analysis for the proteolytic bacteria

Symbol	ENVIRONMENTAL PARAMETERS	PROTEOLYTIC BACTERIA							
		WATER				SEDIMENTS			
		A	B	C	D	A	B	C	D
a		0.9881	0.9050	0.2593	0.7600	1.1799	0.8045	1.1856	0.7804
b1	Water temp.	0.2133	0.0658	0.0580	0.1232	0.2485	0.0245	0.0656	0.1208
b2	Water pH	0.1226	NS	-0.1195	NS	0.2339	0.1839	-0.472	NS
b3	Sedi. pH	-0.3567	0.5345	0.5773	0.2601	-0.5363	0.6116	0.5896	0.4190
b4	Water Eh	NS	NS	NS	NS	NS	NS	NS	NS
b5	Sed. Eh	NS	NS	NS	NS	NS	NS	NS	NS
b6	Diss. O_2	0.2150	NS	0.1665	-0.0461	0.2088	NS	0.1200	NS
b7	Salinity	NS	-0.2857	NS	NS	NS	-0.1331	NS	NS
b8	Nitrite-N	0.0776	NS	0.0780	0.1453	-0.0402	NS	0.0397	0.0965
b9	Nitrate-N	NS	-0.0678	NS	0.0264	NS	-0.0605	NS	NS
b10	Ammonia-N	-0.0449	NS	-0.0537	NS	-0.0460	NS	-0.0500	NS
b11	Org. carbon	NS	NS	NS	NS	NS	NS	NS	NS
b12	Total nitrogen	NS	NS	NS	NS	NS	NS	NS	NS
b13	Total phosphorus	NS	0.0724	NS	NS	NS	0.2925	NS	NS
R^2		98.16%	97.57%	98.43%	96.65%	95.43%	94.96%	98.43%	97.45%

NS - not significant

A, B, C, D - Ponds

influence either in the sediments or in the water in any of the ponds.

The factors which significantly influenced in pond A were sedi. pH water temp. > D.O. > NH_3-N > water pH > NO_2-N in the water (R^2 = 98.16; $P < 0.05$) and sedi. pH > water temp. > D.O. > water pH > NH_3-N > NO_2-N in the sediments (R^2 = 95.43; $P < 0.05$). Sediment pH and NH_3-N in the water and sedi. pH, NH_3-N and NO_2-N in the sediments showed negative influence.

In pond B, salinity > total P > NO_2-N > sedi. pH > water temp. in the water (R^2 = 97.57; $P < 0.05$) and total P > salinity > NO_3-N > sedi. pH > water pH > water temp. in the sediments (R^2 = 94.96; $P < 0.05$) significantly influenced the abundance of proteolytic bacteria. While salinity, total P and nitrite nitrogen had significant negative influence in the water, salinity, nitrate and water temp. had negative influence in the abundance of proteolytic bacteria in the sediments in this pond.

In pond C, sedi. pH > D.O. > NH_3-N > water temperature > water pH > NO_2-N affected the distribution and abundance in the water (R^2 = 96.65; $P < 0.05$) Whereas, sedi. pH > D.O. > NH_3-N > water temp. > water pH > NO_2-N influenced the distribution and abundance of proteolytic bacteria in the sediments (R^2=98.43; $P < 0.05$). Both in water and sediments, NH_3-N and water pH showed negative influence.

In pond D, NO_3-N > water temp. > sedi. pH > D.O. > NO_2-N were the factors which contributed to the variation in the bacterial numbers in the water (R^2 = 95.43; $P < 0.05$). But, in the sediments, water temp. > sedi. pH > NO_2-N (R^2=97.43; $P < 0.05$) significantly influenced the density of proteolytic bacteria. Among the factors D.O. had a negative influence on the distribution of proteolytic bacteria in the water region.

Ammonifying bacteria

Ammonifying bacterial numbers (Fig. 8a and b) were relatively low during postmonsoon months in the water in pond A and pond C and during early postmonsoon in pond B. In contrast, in pond D there was a peak occurrence of ammonifiers in the month of November, though during the months of October and December relatively low numbers occurred. In all the ponds a peak occurred during the early premonsoon season. In pond A and C the peak was noticed in the month of February, whereas in pond B and D it was observed during the months of January and February during the first year. There was a sharp decline in the density of ammonifiers in all the ponds during the late premonsoon season. However, in the early monsoon month of June a secondary peak was evident in all the 4 ponds. Thereafter, a steady decline was recorded in all the ponds, with the lowest numbers occurring during the post-monsoon month of November in pond A, C and D and October in pond B. In December 1983 (Second year) a sharp increase in the population was noticved in ponds A, C and D. However, in pond B the peak occurred in November. In general, pond B was found to show striking difference in the seasonal abundance with that of other ponds. During the second year in the premonsoon season a slight increase was noticed in all the ponds with minor variations during the different months. Following this, during the monsoon season there was significant reduction in most of the ponds, except in pond C.

Almost a similar pattern of distribution occurred in both the water and sediments in pond A throughout the period of investigation. In pond B, minor variations were observed during the first year, but during the second year marked variations were noticed particularly during the postmonsoon season. In fact very low number of ammonifiers was recorded from the sediments in pond B

FIG. 8a

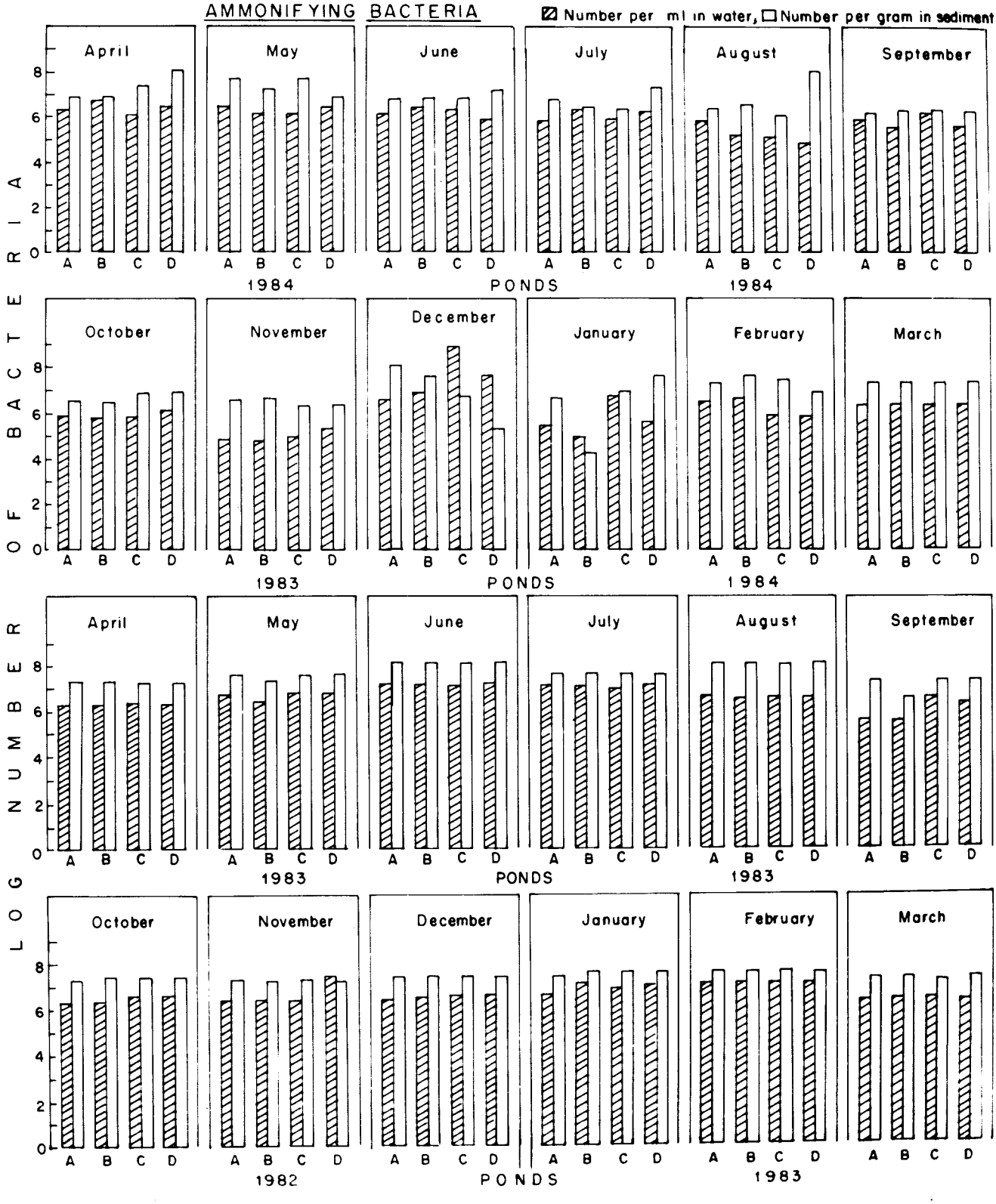
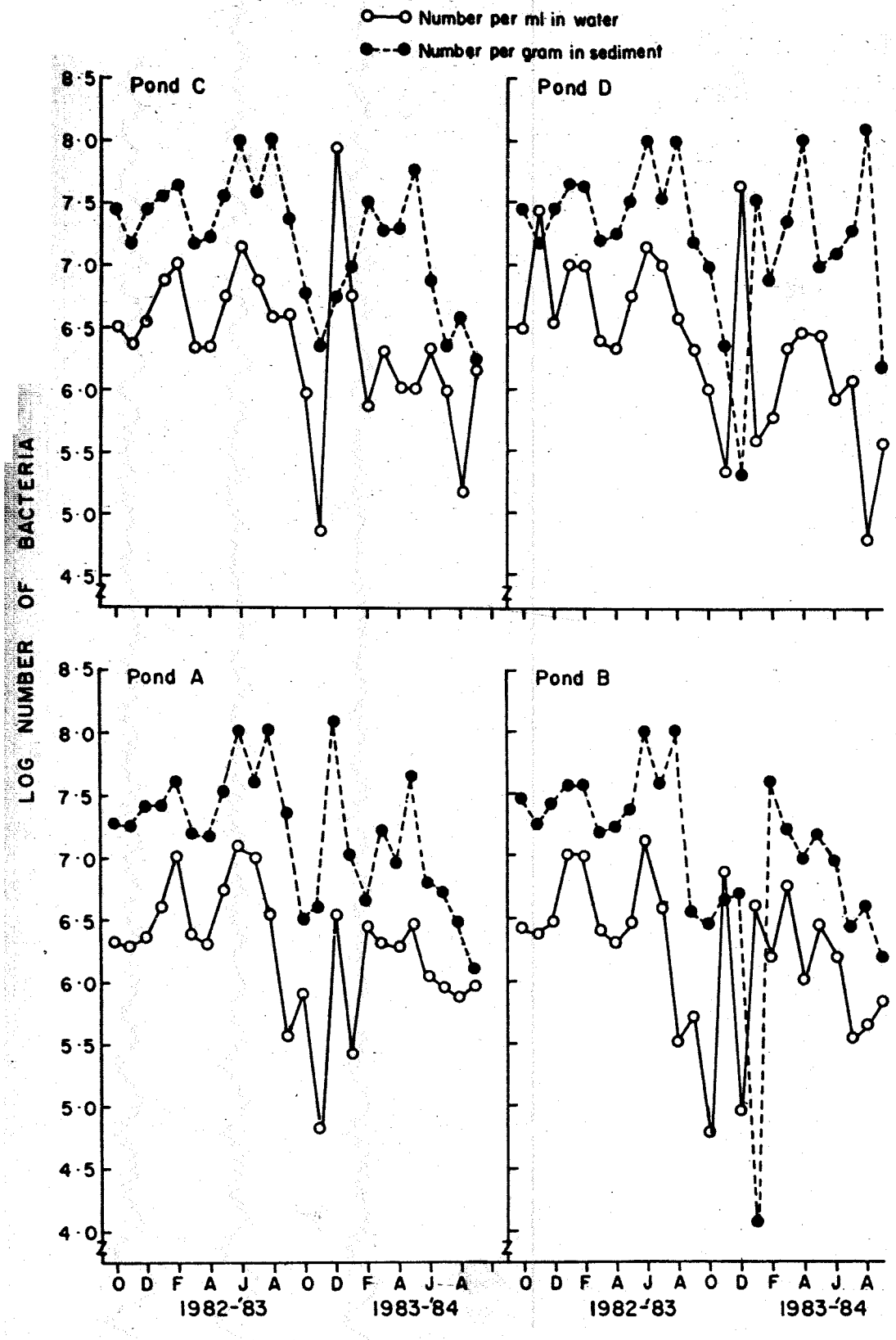

AMMONIFYING BACTERIA
Number per ml in water, Number per gram in sediment
LOG NUMBER OF BACTERIA
April
May
June
July
August
September
October
November
December
January
February
March
A B C D
1984
1983
1982
PONDS

Fig. 8b AMMONIFYING BACTERIA



in the month of January 1983. Immediately after this period a sharp increase was noticed in the month of February. Thereafter, the number of ammonifiers showed a gradual but inconsistent decline during the rest of the premonsoon and monsoon months.

In pond C, during the first year, in the sediments a similar trend was observed in the bacterial abundance as in the case of water. During the second year minor variations were noticed between the water and sediments. While in the water a peak was noticed during December, relatively low numbers occurred in the sediments.

In pond D during most of part of the first year ammonifiers exhibited a similar pattern in their occurrence in the water and sediments, except in November 1982, when a reverse trend in the abundance was noticed. But, during the second year significant differences were noticed between the water and the sediments, with the sediments showing greater fluctuations than the water. Besides, in December 1983 a reverse trend was noticed with the sediments showing a sharp decline in contrast to the peak in the water.

The average log values of ammonifiers in the water ranged (Fig. 8a and b) from 5.57 to 7.14 in pond A, 5.5 to 7.14 in pond B, 6.34 to 7.14 in pond C and 6.34 to 7.42 in pond D. Whereas, in the sediments, it ranged from 7.26 to 8.04 in pond A, 6.54 to 8.04 in pond B, 7.17 to 8.04 in pond C, and 7.22 to 8.04 in pond D. Water region of pond C and D were more productive than pond A and B. However, sediments of pond A, C and D harboured more ammonifiers than pond B.

Among the environmental parameters considered water Eh, sediment Eh, organic carbon and total nitrogen did not have any significant influence on

the distribution and abundance, both in water and sediments, in any of the ponds (Table 6).

In pond A, the pH of the sediment was the most influencing factor both in the water and sediments. The other factors which influenced the distribution of ammonifiers in the order of importance are water pH > D.O. > water temp. > NH_3-N (R^2 = 91.11, $P < 0.05$) in the water and in the sediments D.O. > water pH > water temperature (R^2= 93.50; $P < 0.05$). The abundance of ammonifiers in the water was inversely related to the water pH and NH_3-N; but in the sediments water pH alone had a negative influence.

In pond B, total phosphorus > salinity > nitrate > sediment pH > water pH influenced the bacterial density in the water (R^2 = 87.13; $P < 0.05$); whereas, total phosphorus > salinity > sediment pH > water temp. > water pH affected the distribution in the sediments (R^2 = 87.41; $P < 0.05$). In the water, nitrate and water pH and in the sediments water pH and water temperature showed negative influence on the abundance of ammonifiers.

In pond C, sediment pH > water temp. > D.O. > NH_3-N > water pH (R^2 = 93.20; $P < 0.05$) were the factors which contributed to the variations in bacterial density in the water. Whereas in the sediments, sediment pH > water temp. > D.O. > NH_3-N > water pH > NO_2-N (R^2 = 97.85; $P < 0.05$) explained the variation. Besides, the ammonia concentration had a significant negative influence on the abundance of ammonifiers in water. However, in the sediments, water temp. NH_3-N and water temp. showed negative effect on the ammonifying bacteria.

In pond D, D.O. > NO_2-N > water temp. > sediment pH > NH_3-N NO_3-N in the water (R^2 = 92.02; $P < 0.05$) and sediment pH > water temp. >

FIG. 9a

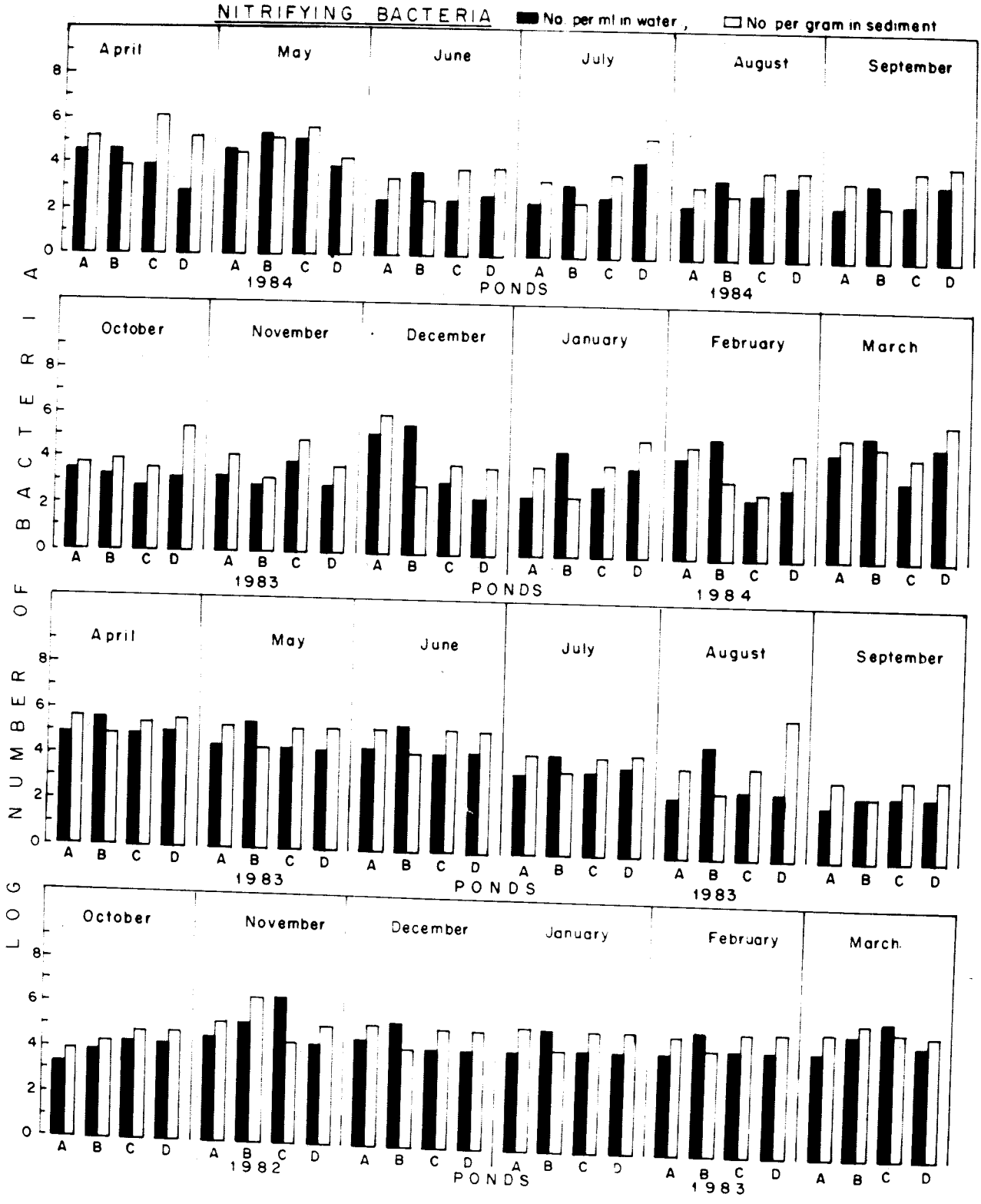
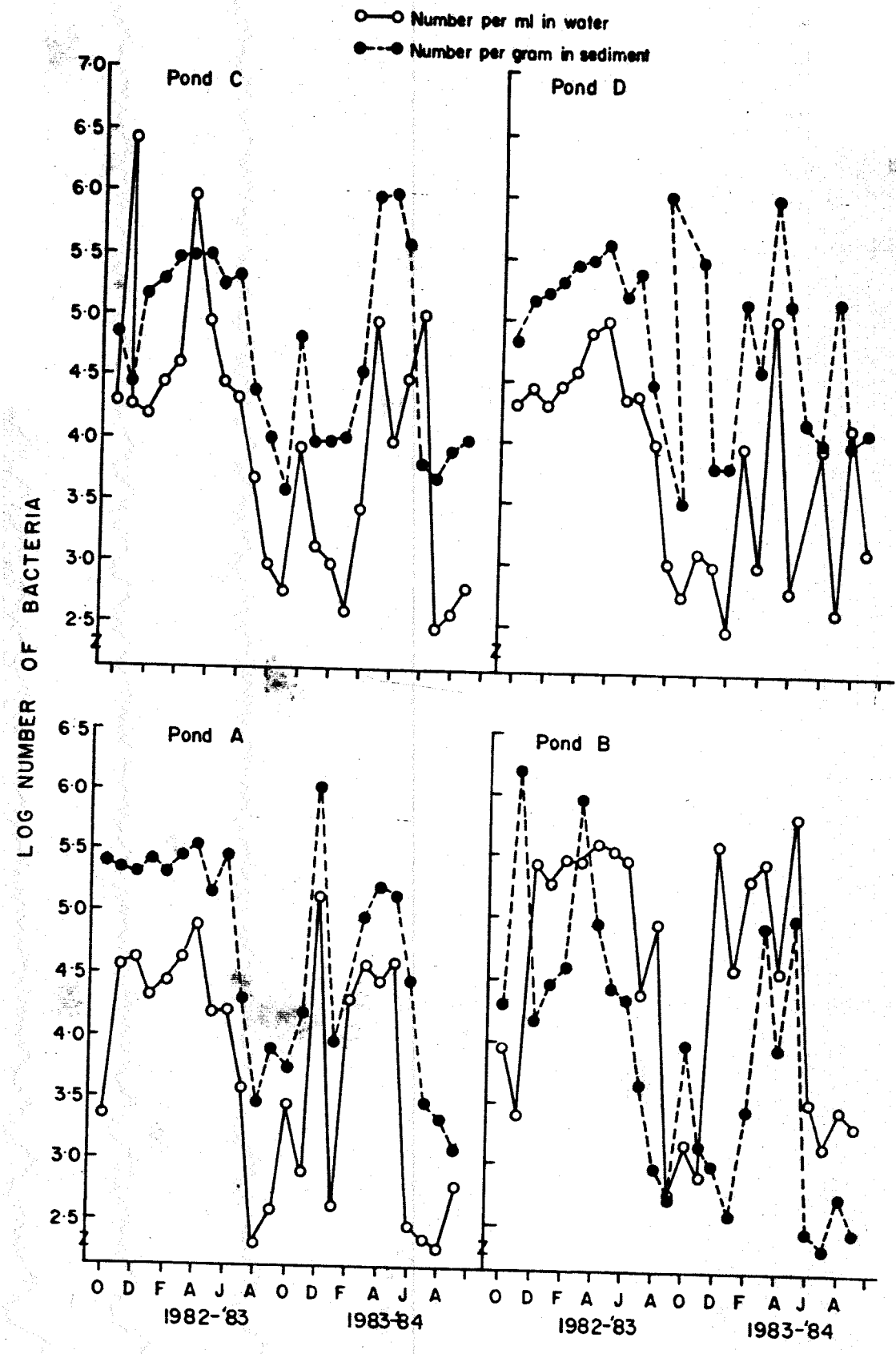

NITRIFYING BACTERIA
No per ml in water,
No per gram in sediment
LOG NUMBER OF BACTERIA
April
May
June
July
August
September
October
November
December
January
February
March
A B C D
1984
1983
1982
PONDS

Fig. 9b NTIRIFYING BACTERIA



this peak a steady decline in the number of ammonia oxidizers occurred during late premonsoon and monsoon seasons. During the second year also, in the water, high numbers of ammonia-oxidizing bacteria were encounterced during the late post-monsoon and premonsoon reasons. However, in the monsoon months ammonia oxidizers were low Whereas, the sediments harboured relatively few numbers during the postmonsoon and monsoon months compared to the premonsoon season.

In pond C and D, ammonia-oxidizers were consistently higher in the sediments than in the water, except for minor variations as recorded in pond C. In pond C, except for a steep increase in November-1982, the bacterial numbers were relatively low during the postmonsoon months. In the sediments however, relatively more numbers occurred during the premonsoon than post-monsoon and monsoon months during the first year. During the second year a minor peak occurred in October 1983 and a major peak during March-April 1984. During most of the postmonsoon, and entire monsoon months low numbers were encountered.

In pond D, a well defined seasonal pattern was noticed during most part of the first year in both the water and the sediments. The number of ammonia-oxidizers steadily increased in the water and the sediments from the postmonsoon months and reached the maximum during the early premonsoon months which was followed, by a steady decline resulting in lowest numbers during the monsoon months of August in the sediments and September in the water. During the second year no trend could be delineated due to tremendous inconsistent variations during the different seasons.

The average log numbers of ammonia-oxidizers in water ranged from 2.28 to 4.85 in pond A, 2.75 to 5.65 in pond B, 2.75 to 6.42 in pond C,

and 2.75 to 5.04 in pond D, and in the sediments from 3.47 to 5.53 in pond A, 2.75 to 6.42 in pond B, 3.58 to 5.5 in pond C and 3.53 to 6.04 in pond D. Pond C water and pond B sediments were found to be more productive than the water and sediments of other ponds (Fig. 9a and b).

Among the 13 environmental parameters, water Eh, sediment Eh, organic carbon and total nitrogen did not show any significant influence on the distribution and abundance of ammonia-oxidizers either in the water or in the sediments in any of the four ponds (Table 7).

The abundance of ammonium oxidizers in the water of pond A was influenced by water pH > sediment pH > D.O. > water temp. > NO_2-N (R^2 = 95.45; $P < 0.05$); whereas in the sediments, sediment pH > water pH > D.O. > water temp. NO_2-N (R^2 = 92.11; $P < 0.05$) were the influencing factors. While water pH, nitrite and ammonia had a negative influence on their abundance in the water, water pH and nitrite showed a significant negative influence in the sediments.

In pond B, salinity > total P > water pH > water temp. > sediment pH (R^2 = 94.12; $P < 0.05$) contributed to the observed variations in the water and total P > salinity > water pH > water temp. > sediment pH (R^2 = 83.94; $P < 0.05$) explained the variation in the sediments. The environmental factors which had a negative influence were water pH and sediment pH in the water, and water pH in the sediments.

In pond C, sediment pH > water temp. > D.O. > water pH > NO_2-N (R^2 = 87.96; $P < 0.05$) significantly influenced the abundance of the bacteria in the water; and in the sediments, sediment pH > water temp. > NH_3-N > D.O. > NO_2-N > water temp. (R^2 = 95.50; $P < 0.05$) had significant effect. The bacterial abundance was inversely related to sediment pH and NO_2-N in the water and sediment pH, NH_3-N, NO_2-N in the sediments.

Table 7: Estimates of the parameters in multiple linear regression analysis for nitrifying (ammonia-oxidizing) bacteria

Symbol	ENVIRONMENTAL PARAMETERS	NITRIFYING BACTERIA							
		WATER				SEDIMENTS			
		A	B	C	D	A	B	C	D
a		1.8351	1.2226	-1.7399	0.1225	2.3662	0.4328	1.1213	0.4701
b1	Water temp.	0.1904	0.1265	0.3545	-0.1835	0.1137	0.0750	0.4494	0.1203
b2	Water pH	-0.9425	-0.8416	0.9669	NS	-0.6895	-0.9060	-0.1544	-0.8755
b3	Sed. pH	0.3713	0.4664	-1.8318	1.1838	0.4910	0.9532	-0.8913	1.1717
b4	Water Eh	NS	NS	NS	NS	NS	NS	NS	NS
b5	Sedi. Eh	NS	NS	NS	NS	NS	NS	NS	NS
b6	Diss. O_2	0.3699	NS	0.3173	0.1703	0.2051	NS	0.1402	NS
b7	Salinity	NS	0.4528	NS	NS	NS	0.1779	NS	NS
b8	Nitrite-N	-0.5500	NS	-0.3321	NS	-0.2299	NS	-0.4926	-0.1994
b9	Nitrate-N	NS	NS	NS	NS	NS	NS	NS	NS
b10	Ammonia-N	-0.0534	NS	NS	NS	NS	NS	-0.1224	-0.1640
b11	Org. carbon	NS	NS	NS	NS	NS	NS	NS	NS
b12	Total nitrogen	NS	NS	NS	NS	NS	NS	NS	NS
b13	Total phosphorus	NS	0.6899	NS	NS	NS	0.5235	NS	NS
R^2		95.45%	94.12%	87.96%	88.22%	92.11%	83.94%	95.50%	84.18%

NS - not significant. A, B, C, D - Ponds

The factors which contributed to the variation in the abundance of the nitrifiers in pond D were sediment pH > water temp. > D.O. in the water (R^2 = 88.22; $P < 0.05$) and water pH > NH_3-N > water temp. > NO_2-N > sedi. pH (R^2 = 84.18; $P < 0.05$) in the sediments. Water temp. in water and water pH, NH_3-N, NO_2-N in the sediments showed negative influence in the distribution of ammonia-oxidizers.

Denitrifiers

In pond A, during both the years peak occurrence of denitrifiers was noticed in the water during the premonsoon, which was very distinct during the month of April 1983 and May 1984 (Fig. 10a and b). The lowest numbers were recorded in the postmonsoon season in both the years. During the first year a clear seasonal trend was observed with a low density during the postmonsoon season, followed by a peak in the premonsoon and a decline in the monsoon seasons. Whereas, in the second year, though there was low numbers during the postmonsoon, in the other two seasons there was an inconsistent trend. The trend in abundance of the denitrifiers in the sediments followed as that of water, except for slight fluctuations.

In pond B, two peaks were noticed in the water and sediments during the first year, a major peak in the premonsoon month of March and a minor peak in the early monsoon month of June. Relatively, low numbers occurred during the postmonsoon season. However, a distinct variation was observed during the late monsoon months, when in the water the density of denitrifiers was markedly lower than that in the sediments. During the second year, in both the water and the sediments relatively low numbers of denitrifiers occurred during the early postmonsoon months. But during the late postmonsoon months there was a peak occurrence of denitrifiers. Following this period, an inconsistent decline was evident during

FIG. 10a

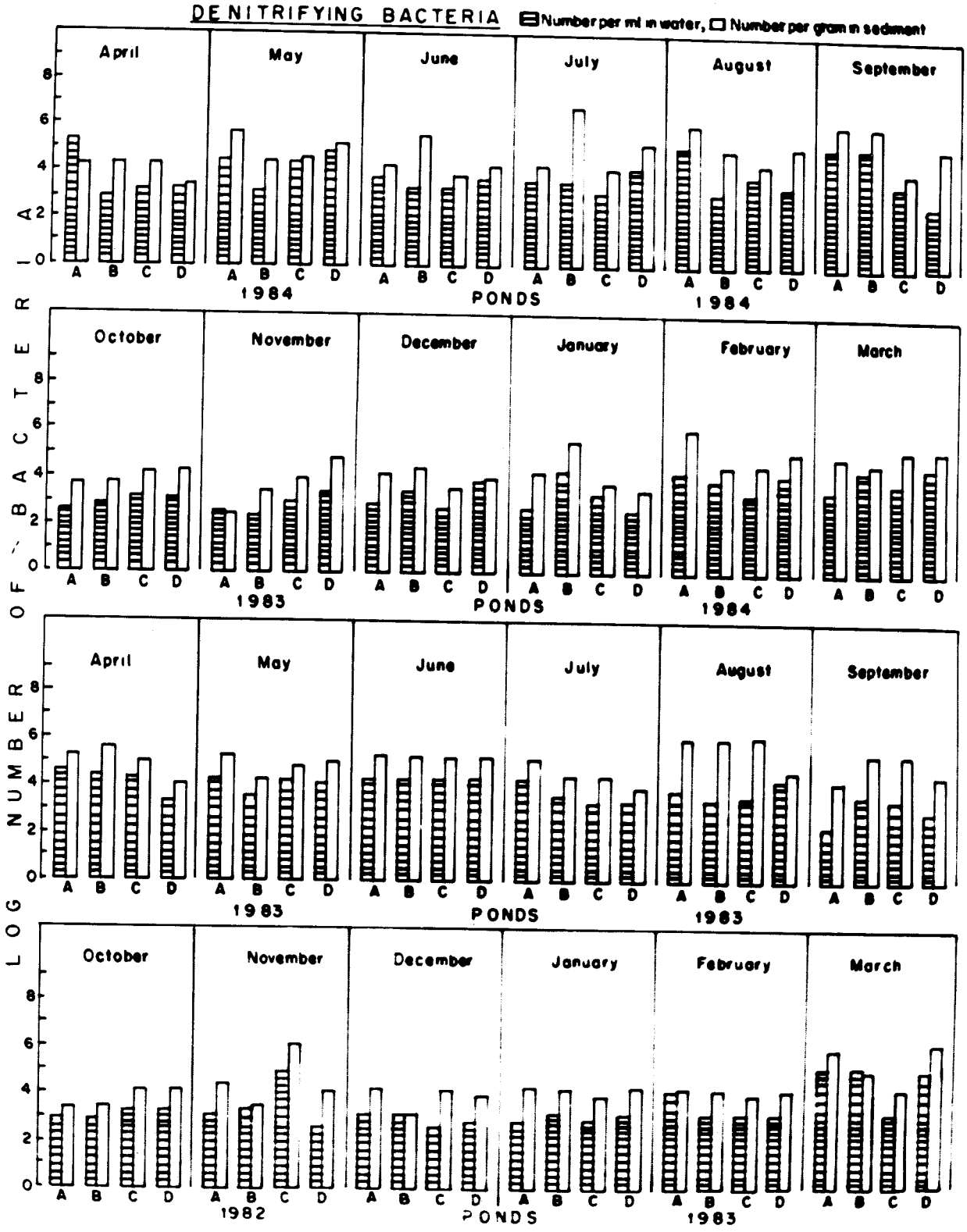
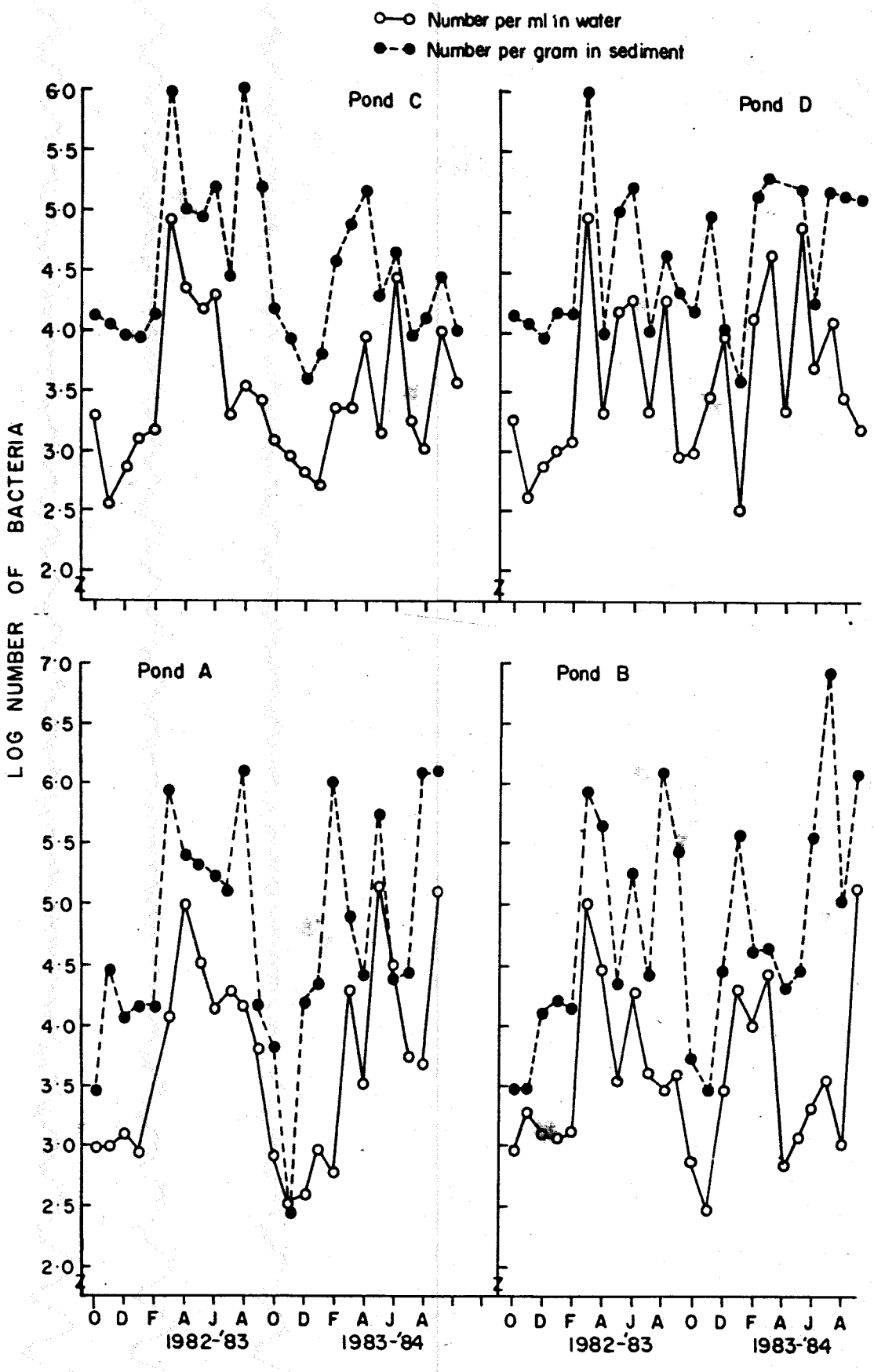

DENITRIFYING BACTERIA
Number per ml in water, Number per gram in sediment
LOG NUMBER OF BACTERIA
April
May
June
July
August
September
October
November
December
January
February
March
A B C D
1984
1983
1982
PONDS

Fig. 10b DENITRIFYING BACTERIA



the premonsoon season. Very high numbers of denitrifiers, occurred in the sediments in July (early monsoon) as against low numbers in the water. In August a sharp decline was observed, which was followed by an increase in September in both the water and the sediments.

In both the water and the sediments of pond C, in both the years, denitrifiers occurred in less numbers during the postmonsoon season than that of the other two seasons. Besides, the distribution pattern was almost similar in both the water and the sediments, though consistently higher numbers were encountered in the sediments. A premonsoon peak was evident in both the water and the sediments in March during the first year and in April during the second year. While a monsoon peak occurred in August in the sediments during the first year, there was a steady but inconsistent decrease in the number of denitrifiers in the water from March till January of the following year.

As observed in the case of other ponds in pond D during the postmonsoon months of the first year relatively low numbers of denitrifiers occurred in both the water and the sediments. Besides, three peaks in their abundance was recorded. The first peak was in the premonsoon month of March; the second and third peaks occurred in the monsoon months of June and August respectively in both the water and the sediments. Thus, during most part of the first year there has been an inconsistent trend in the occurrence of denitrifiers. During the late monsoon month of September during the first year and in the early postmonsoon month of October in the second year denitrifiers were found to be very low. After this period during the second year, there was a wide variation in their occurrence, thereby no clear-cut pattern of distribution could be established especially in the water.

The average log numbers of denitrifiers in the water ranged from 2.28 to 5.04 in pond B, 2.63 to 4.96 in pond C and 2.67 to 4.98 in pond D. In the sediments it ranged from 3.47 to 6.14 in pond A, 3.47 to 6.14 in pond B, 3.94 to 6.04 in pond C and 3.95 to 6.04 in pond D (Fig 10a and b).

Among the environmental parameters water Eh, sediment Eh, organic carbon and total nitrogen in the sediments did not show any significant influence on the abundance of denitrifiers in both the water and the sediments in any of the ponds (Table 8).

In pond A water, sediment pH > water pH > water temp. > NH_3-N NO_2-N (R^2 = 86.23; $P < 0.05$) and in the sediments, sediment pH > salinity > water pH > D.O. > NH_3-N > water temp. > NO_2-N (R^2 = 78.38; $P < 0.05$) had significant influence. The factors which negatively influenced in the water are sediment pH, water pH and nitrite content, and water pH and water temperature in the sediments.

Total P > water pH > water temp. > sediment pH > D.O. were the factors significantly (R^2 = 78.61; $P < 0.05$) influencing the distribution of denitrifiers in the water of pond B; whereas in the sediments salinity > total P > NO_3-N > water pH > water temp. > sedi. pH (R^2 = 84.48; $P < 0.05$) contributed significantly to the variation. While total P and water temp. showed negative influence in the distribution of denitrifiers in the water, total P and sedi. pH showed a negative influence in the sediments.

Sediment pH > water temp. > NO_2-N > water pH were the significantly (R^2 = 95.86; $P < 0.05$) influencing factors in water of pond C; whereas, sedi. pH > water temp. > water pH > NO_2-N > NO_3-N > D.O. were the significantly (R^2 = 92.43; $P < 0.05$) influencing factors in the sediments. Water temp. and

Table 8: Estimates of the parameters in multiple linear regression analysis for denitrifying bacteria

Symbol	ENVIRONMENTAL PARAMETERS	DENITRIFYING BACTERIA							
		WATER				SEDIMENTS			
		A	B	C	D	A	B	C	D
a		-2.0112	-1.1228	-0.3833	-0.4042	-1.8948	0.7483	-0.1073	0.6472
b1	Water temp.	0.2489	-0.0971	-0.2819	NS	-0.0772	0.2278	-0.4241	-0.1465
b2	Water pH	-0.7018	0.6749	-0.7414	-0.4322	-0.867	0.6826	-1.1001	-0.2070
b3	Sed. pH	1.5825	0.1933	2.3912	0.9152	1.5911	-0.8200	3.6488	1.3230
b4	Water Eh	NS	NS	NS	NS	NS	NS	NS	NS
b5	Sed. Eh	NS	NS	NS	NS	NS	NS	NS	NS
b6	Diss. O_2	0.1100	0.0535	NS	0.1334	0.1588	NS	-0.2078	NS
b7	Salinity	NS	0.3088	NS	NS	0.0493	0.4220	NS	NS
b8	Nitrite-N	-0.1935	NS	1.1101	0.5459	0.1867	NS	1.0529	0.9064
b9	Nitrate-N	NS	NS	NS	NS	NS	NS	NS	NS
b10	Ammonia-N	0.05019	NS	NS	NS	0.0635	NS	-0.1462	NS
b11	Organic carbon	NS	NS	NS	NS	NS	NS	NS	NS
b12	Total nitrogen	NS	NS	NS	NS	NS	NS	NS	NS
b13	Total phosphorus	NS	-0.0799	NS	NS	NS	-0 2699	NS	NS
R^2		86.23%	78 61%	95 86%	81 96%	78 38%	84 48%	92.43%	89 47%

NS - not significant A, B, C, D - Ponds

water pH had negative influence on the distribution of denitrifiers.

In pond D, sedi. pH > NO_2-N > water pH > D.O. in the water (R^2 = 81.96; $P < 0.05$) and sedi. pH > NO_2-N > water temp. > water pH in the sediments (R^2 = 89.47; $P < 0.05$) significantly contributed to the variations in abundance. Water pH in the water, and water temp. and water pH in the sediments showed a negative influence on the distribution of denitrifiers in the respective regions.

Nitrogen fixing bacteria

The distribution pattern and relative abundance of nitrogen fixing bacteria in the ponds are shown in Fig. 11 a and b. During the first year there were no striking differences in the seasonal distribution pattern of nitrogen fixers in most of the ponds. In both the water and sediments of all the 4 ponds there was an increase in the nitrogen fixers from the postmonsoon season showing a peak during April in pond A, June in pond B, and in May in the seasonal ponds C and D. Following the peak occurrences their numbers declined in the water region of all the ponds during the monsoon season. However, in the sediment there was no consistency in the declining trend.

In the second year an early premonsoon peak was recorded in ponds A, C and D. Whereas, in pond B there was no distinct peak during the same period, but a prominent peak was observed during the late postmonsoon season. In pond A low numbers were recorded during December. Conversely, in all other ponds, low numbers occurred in November. In ponds C and D a late post-monsoon peak was recorded particularly in the water; the peak in pond D was uncharacteristically high, far exceeding that of the sediments.

FIG. 11a

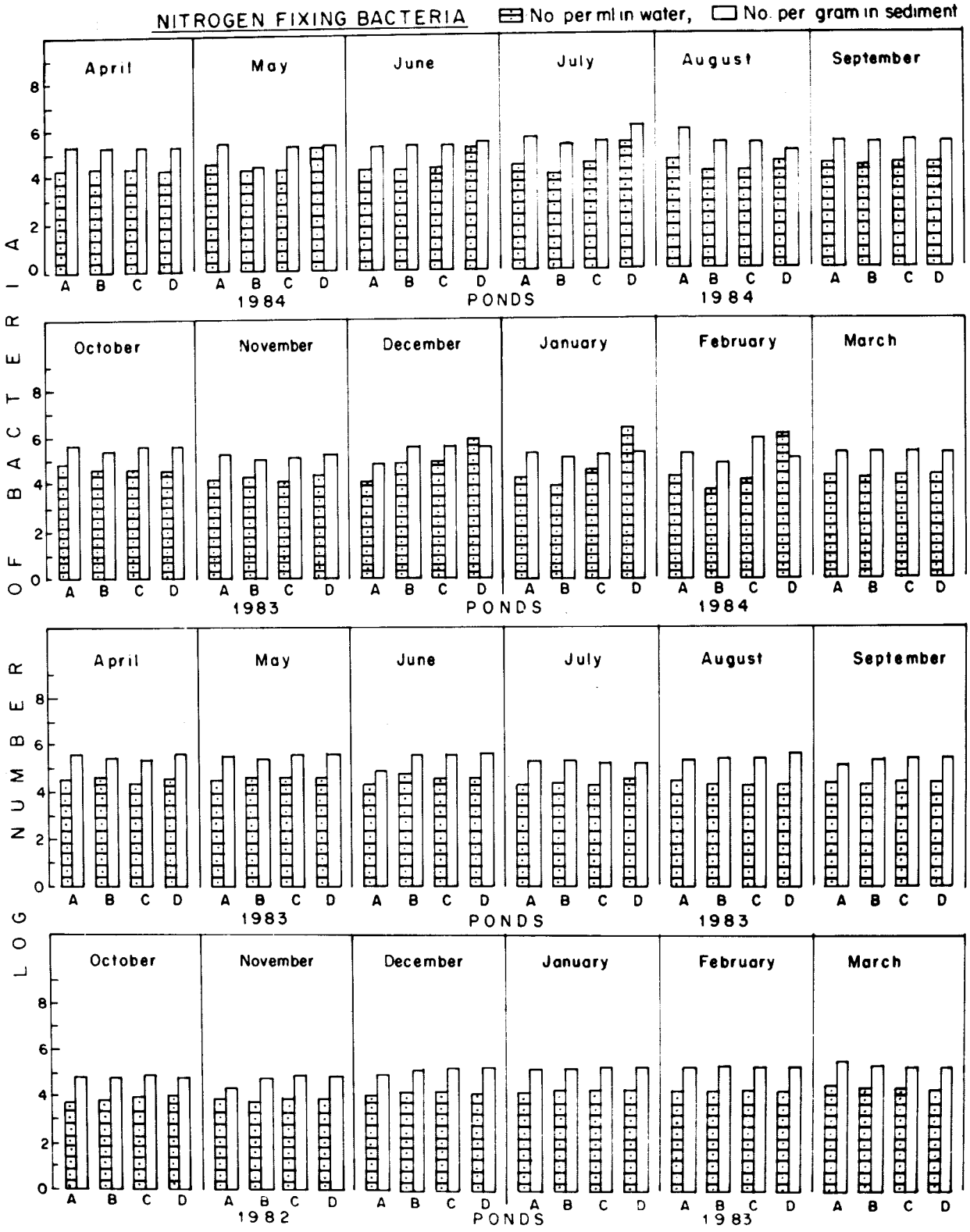
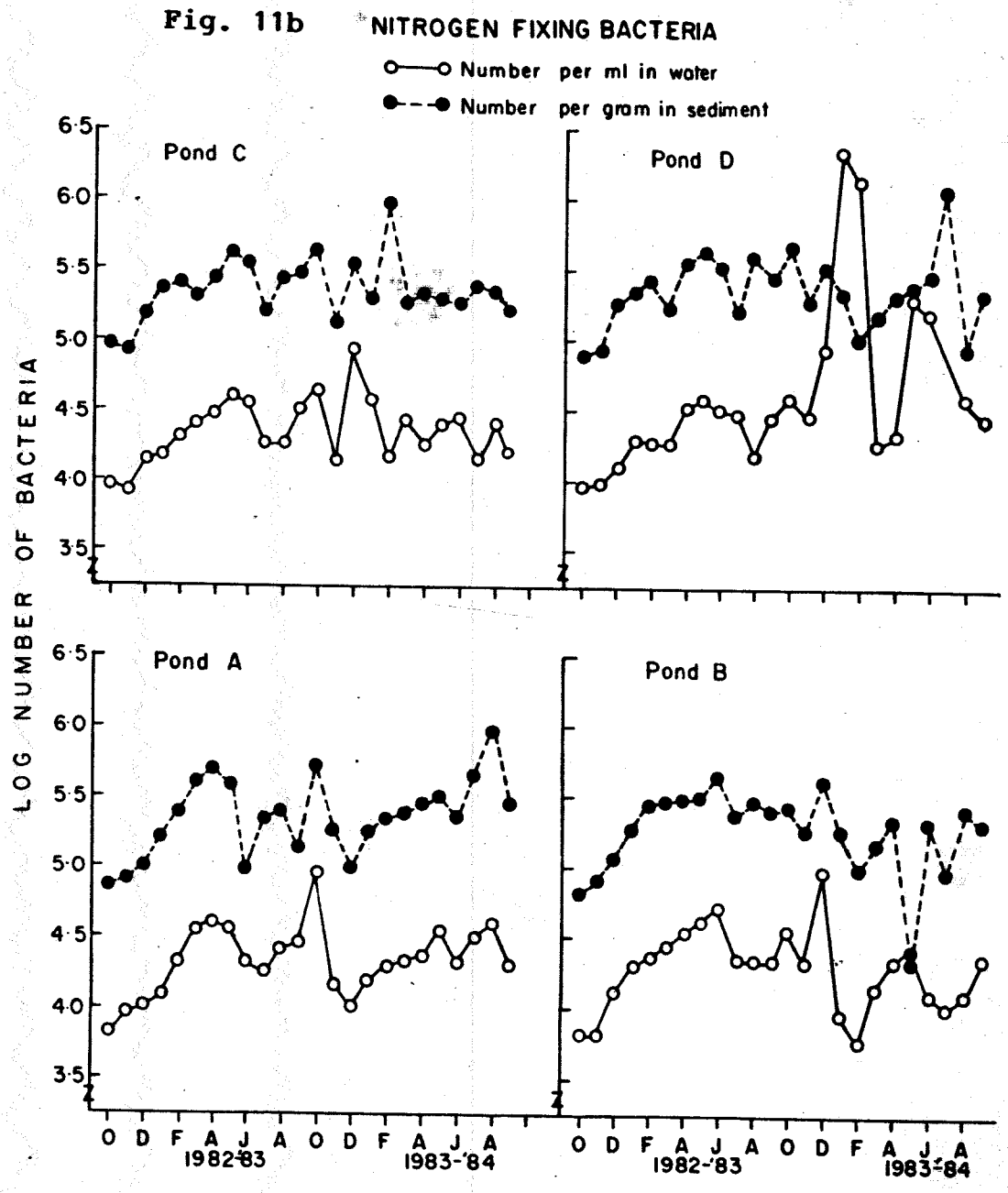

NITROGEN FIXING BACTERIA
No per ml in water,
No. per gram in sediment
LOG NUMBER OF BACTERIA
April
May
June
July
August
September
October
November
December
January
February
March
A B C D
1984
1983
1982
PONDS
0
2
4
6
8

Fig. 11b


NITROGEN FIXING BACTERIA
Number per ml in water
Number per gram in sediment
LOG NUMBER OF BACTERIA
Pond C
Pond D
Pond A
Pond B
6·5
6·0
5·5
5·0
4·5
4·0
3·5
O D F A J A O D F A J A
1982-83
1983-84

The average log numbers of nitrogen fixers in water ranged from 3.69 to 4.67 in pond A, 3.77 to 4.70 in pond B, 3.89 to 4.61 in pond C and 3.95 to 4.58 in pond D. In the sediments it ranged from 4.84 to 5.69 in pond A, 4.77 to 5.69 in pond B, 4.84 to 5.65 in pond C and 4.86 to 5.65 in pond D. (Fig. 11a and b).

Among the 13 environmental parameters (Table 9) water Eh, sediment Eh, organic carbon and total nitrogen did not have any significant influence on the distribution of nitrogen fixers in the ponds.

Of the factors significantly influencing, sedi. pH > water pH > water temp. > D.O. > NH_3-N > NO_2-N (R^2 = 97.60; $P < 0.05$) in the water, and sedi. pH > water temp. > water pH > D.O. > NH_3-N NO_2-N (R^2 = 98.22; $P < 0.05$) in the sediments were important. Sediment pH and ammonia concentration in water showed negative influence on the distribution of nitrogen fixers in both the water and the sediments.

In pond B, salinity > total phosphorus > water pH > sediment pH > (R^2 = 97.04; $P < 0.05$) in the water and total P > salinity > water pH > sedi. pH > water temp. (R^2 = 96.03; $P < 0.05$) in the sediments contributed significantly to the variations. While salinity and sediment pH affected the distribution of nitrogen fixers negatively in the water, total P and salinity in the sediments, showed a negative influence on the sediment nitrogen fixers.

The factors which could explain the variation in the bacterial populations in pond C were sedi. pH > NO_2-N > NH_3-N > D.O. in the water (R^2 = 96.25; $P < 0.05$) and sedi. pH > NH_3-N > NO_2-N > D.O. > water pH in the sediments (R^2 = 98.71; $P < 0.05$). Among these factors, in the water, ammonia and in the sediments ammonia and D.O. showed a negative influence on the distribution of nitrogen fixers.

Table 9: Estimates of the parameters in multiple linear-regression analysis for nitrogen fixing bacteria

Symbol	ENVIRONMENTAL PARAMETERS	NITROGEN FIXING BACTERIA							
		WATER				SEDIMENTS			
		A	B	C	D	A	B	C	D
a		-0.0743	0.1094	0.0048	1.0509	-0.2271	0.1371	0.4816	0.1370
b1	Water temp.	0.1277	NS	NS	NS	0.1640	-0.0761	NS	NS
b2	Water pH	0.4478	0.5513	NS	0.2915	0.2817	0.5753	0.1160	0.1189
b3	Sedi. pH	-0.4837	-0.1058	0.5166	NS	-0.2454	0.3994	0.5673	0.1765
b4	Water Eh	NS	NS	NS	NS	NS	NS	NS	NS
b5	Sedi. Eh	NS	NS	NS	NS	NS	NS	NS	NS
b6	Diss. 0_2	0.1280	NS	0.0166	0.0894	0.1202	NS	-0.0429	0.0320
b7	Salinity	NS	-0.1383	NS	-0.0429	NS	-0.0867	NS	NS
b8	Nitrite-N	0.1023	NS	0.2415	0.0831	0.1239	NS	0.1978	0.0951
b9	Nitrate-N	NS	NS	NS	-0.0319	NS	NS	NS	NS
b10	Ammonia-N	-0.0359	NS	-0.0319	0.1078	-0.0546	NS	-0.0431	0,0195
b11	Organic carbon	NS	NS	NS	NS	NS	NS	NS	NS
b12	Total nitrogen	NS	NS	NS	NS	NS	NS	NS	NS
b13	Total phosphorus	NS	0.0960	NS	0.0359	NS	-0.0913	NS	NS
R^2		97.60%	97.04%	96.25%	90.38%	98.22%	96.03%	98.81%	94.82%

NS - not significant

A, B, C, D - Ponds

The factors influencing the distribution of nitroten fixers in pond D were total P > salinity > NO_2-N > NO_3-N > NH_3-N > water pH in the water (R^2 = 90.38; $P < 0.05$) and sedi. pH > water pH > D.O. > NH_3-N > NO_2-N in the sediments (R^2 = 94.82; $P < 0.05$). Salinity and nitrate content of the water showed a negative influence in the abundance of nitrogen fixers.

Azotobacter

Figure 12a and b illustrates the distribution of Azotobacter during the period of investigation of one year from October 1983 to September 1984. There were much differences between ponds in the seasonal distribution of Azotobacter in both the water and the sediments. Except for pond A in which there was a consistent decrease in the Azotobacter numbers during the postmonsoon months in the water, in all other ponds a postmonsoon peak was found in the water - December in ponds B and C and January in pond D. While a similar trend was observed in the sediment in ponds B and C there was a decline in December in ponds A and D and a sharp increase in January in pond A. In pond A during the premonsoon season there was a inconsistent increase in the bacteria, and there was not much variation during the monsoon months. In ponds B and C, water and sediments also, a gradual increase in the Azotobacter numbers occurred during the premonsoon season with a peak in late premonsoon. But during the early monsoon a sharp decline and during the late monsoon a peak in abundance was also observed. Pond D showed a strikingly different pattern to that of other ponds. Unlike in other ponds, where low numbers were encountered in the sediments during February, in pond D sediments showed a peak occurrence of Azotobacter. However, in the water the distribution of bacteria was almost similar in other ponds during the early premonsoon season. A late postmonsoon decline in the population occurred in both the water and sediments in pond D, which was followed by a sharp increase in population showing a prominent peak during early monsoon months.

FIG. 12a

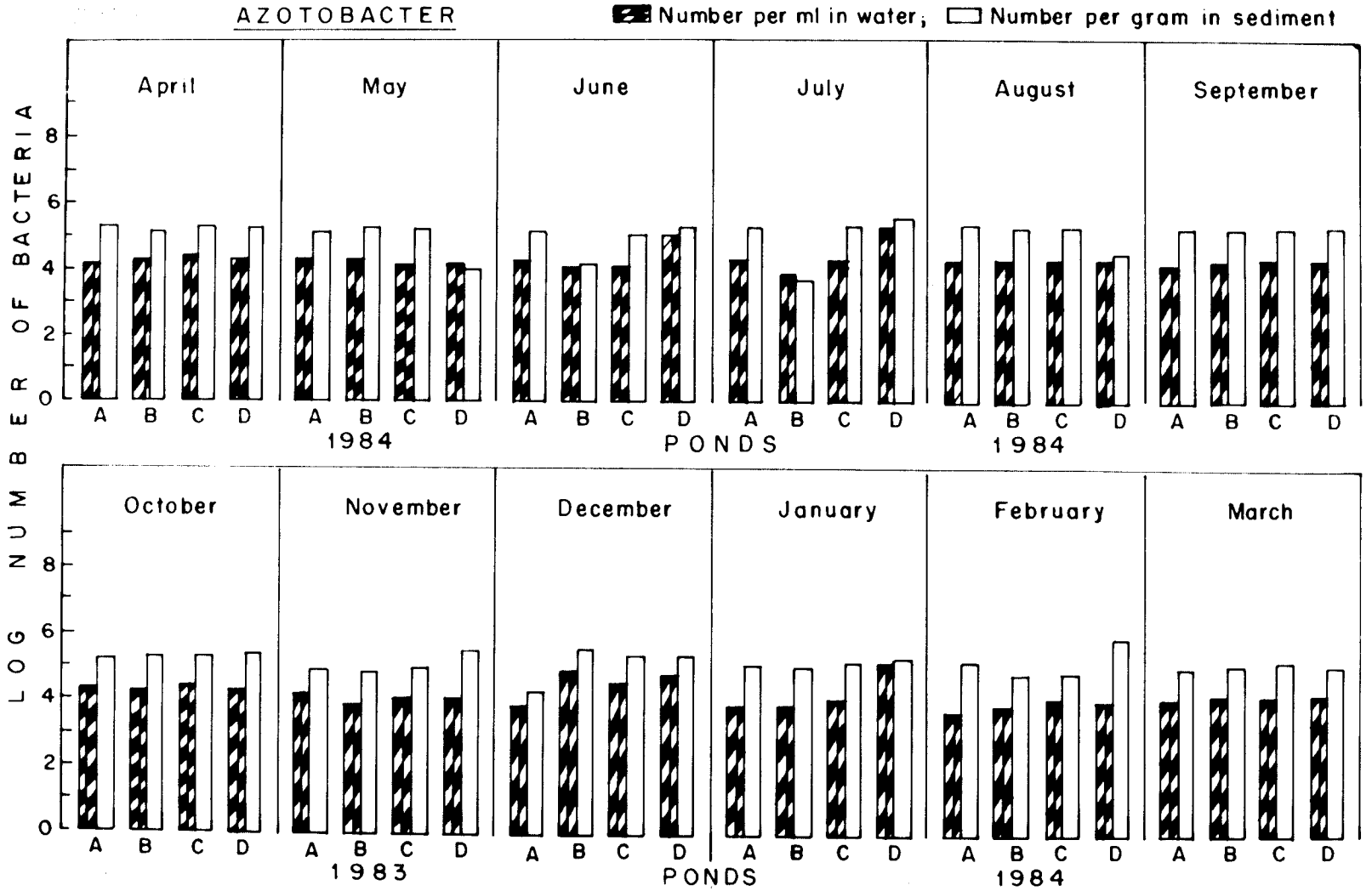
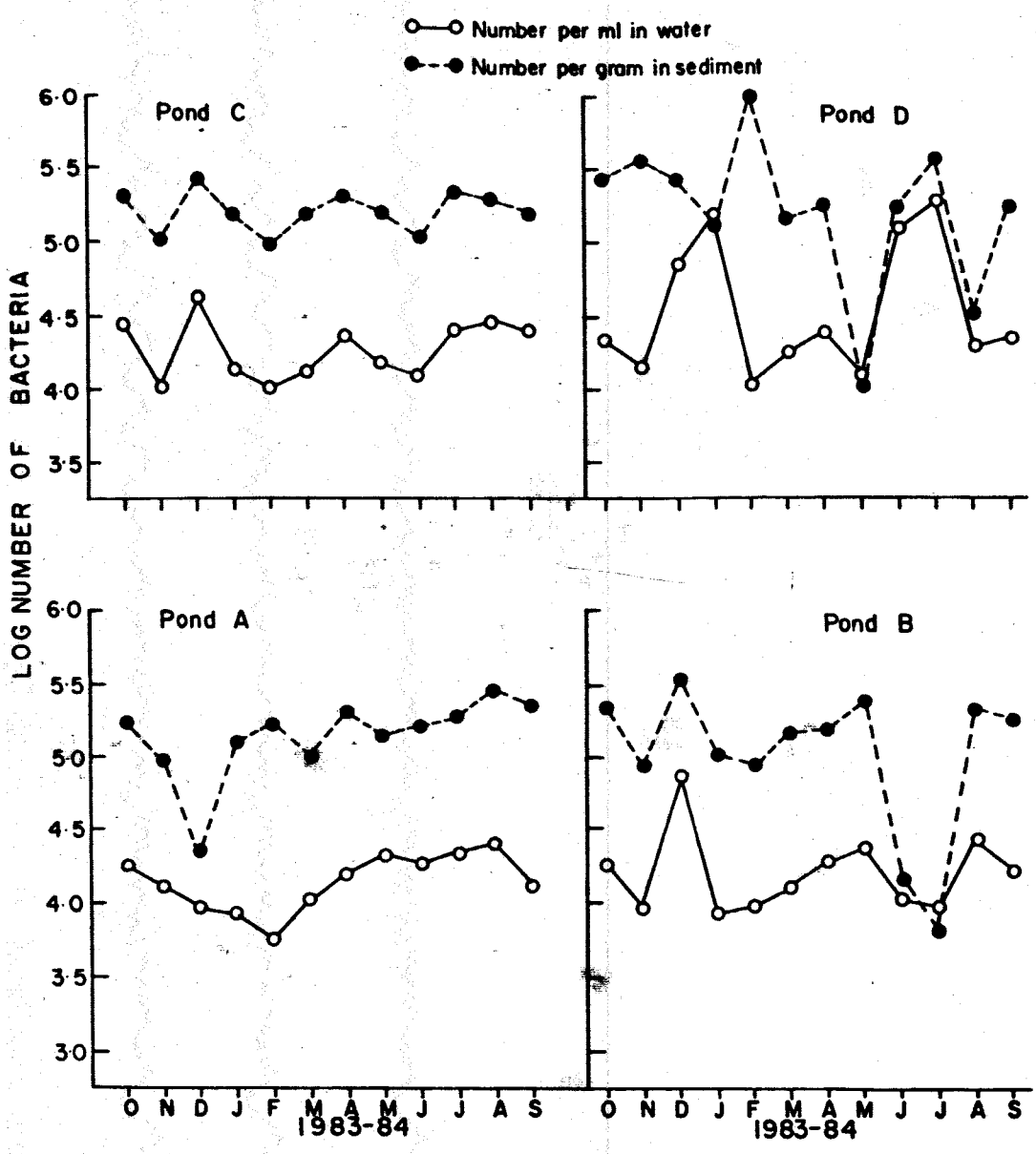

AZOTOBACTER
Number per ml in water; Number per gram in sediment
LOG NUMBER OF BACTERIA
0
2
4
6
8
April
May
June
July
August
September
October
November
December
January
February
March
A B C D
1984
1983
PONDS

Fig. 12b AZOTOBACTER



The average log numbers of Azotobacter in the water ranged from 3.94 to 4.38 in pond A, 3.90 to 4.86 in pond B, 4.04 to 4.63 in pond C, 4.02 to 5.28 in pond D and in the sediments it ranged from 4.37 to 5.47 in pond A, 3.84 to 5.56 in pond B, 4.95 to 5.40 in pond C and 4.06 to 5.95 in pond D. Ponds B and D were relatively more productive than the other two ponds (A and C) (Fig. 12a and b).

In order to identify the factors which influenced the distribution and abundance of Azotobacter density in both the water and sediments in various ponds partial correlation coefficient analysis was performed on the data on water and sediment characteristics with that of the Azotobacter numbers. The results are given in Table 11, after finding out the significance level at $P < 0.05$. Of the 13 environmental parameters considered only the sediment phosphorus affected the density of Azotobacter in water; whereas sediment organic carbon, total nitrogen and total phosphorus showed significant influence in the sediments in pond A. In pond B, sediment P and water D.O. showed significant influence on the distribution of Azotobacter in the water, and total nitrogen, organic carbon and total N showed a significant influence on their distribution in the sediments. In pond C, NO_2-N and sediment Eh showed significant influence in the water and NO_2-N was the only factor found significant in the sediment Azotobacter distribution. In pond D, sediment Eh, NO_2-N, water temperature and water Eh were the influencing factors in water and only NO_2-N was found to be the influencing factor in the sediments.

Nitrogen fixing rates:

The mean rate of nitrogen fixation by the nitrogen fixing bacteria during the different months are shown in Figure 13a and b. Bacterial nitrogen

Table 11: Results of the correlation analysis on *Azotobacter* population

a	ENVIRONMENTAL PARAMETERS	WATER				SEDIMENTS			
		A	B	C	D	A	B	C	D
b1	Water temp.	0.3662	0.3728	0.4178	-0.5316*	0.3636	0.3724	-0.3068	-0.3624
b2	Water pH	0.3751	0.3568	-0.3323	-0.3217	-0.0632	0.0648	0.005	0.007
b3	Sedi. pH	0.2836	0.3052	-0.378	-0.416	-0.0428	-0.0369	-0.4041	-0.4219
b4	Water Eh	-0.1709	-0.1817	-0.4869	-0.5162*	-0.0348	-0.0363	-0.1174	-0.1217
b5	Sedi. Eh	0.3295	0.3417	0.786*	0.772*	-0.1065	-0.1273	0.0578	0.0613
b6	Diss. 0_2	0.4296	0.5013*	-0.097	-0.092	-0.1563	-0.1629	0.023	0.024
b7	Salinity	-0.4069	-0.4127	-0.2772	-0.2772	-0.2554	-0.2258	-0.1695	-0.1795
b8	Nitrite-N	-0.0362	-0.0296	0.6672*	0.6423*	-0.0227	-0.0325	0.5628*	0.5627*
b9	Nitrate-N	0.1763	0.1892	0.2853	0.2952	0.2673	0.2768	0.34	0.33
b10	Ammonia-N	0.4598	0.4724	-0.1072	-0.1213	0.2226	0.2429	-0.0253	-0.0251
b11	Organic carbon	-0.3922	-0.3422	0.0184	0.0179	-0.7778*	-0.7624*	-0.0442	-0.044
b12	Total nitrogen	0.3525	-0.3626	0.2216	0.2612	-0.8124*	-0.8236*	-0.3227	-0.3137
b13	Total phosphorus	-0.6764*	-0.6863*	-0.1896	-0.1884	-0.5401*	-0.5608*	-0.1776	-0.2173

* Significant ($P < 0.05$) A, B, C, D - Ponds

FIG. 13a

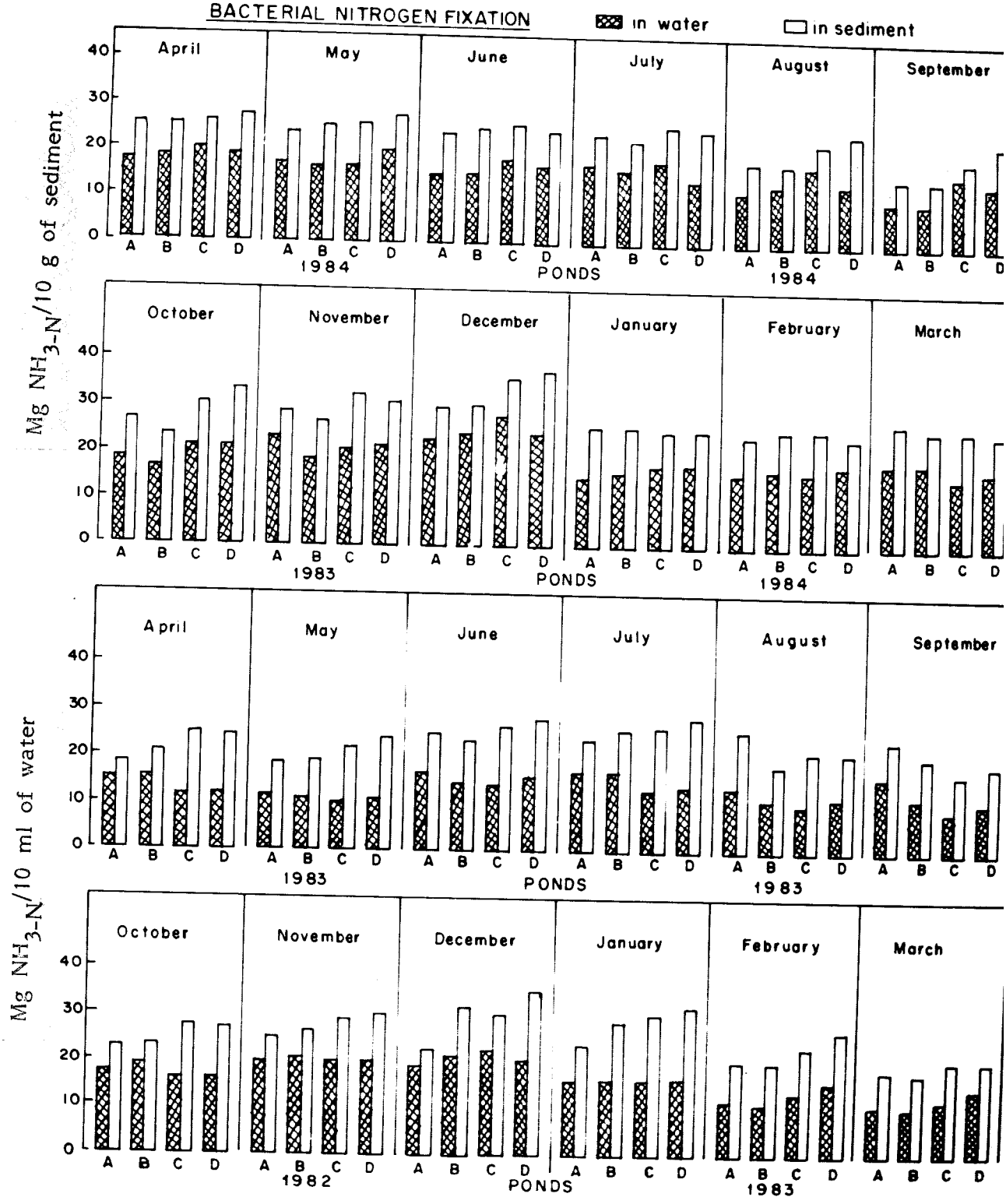
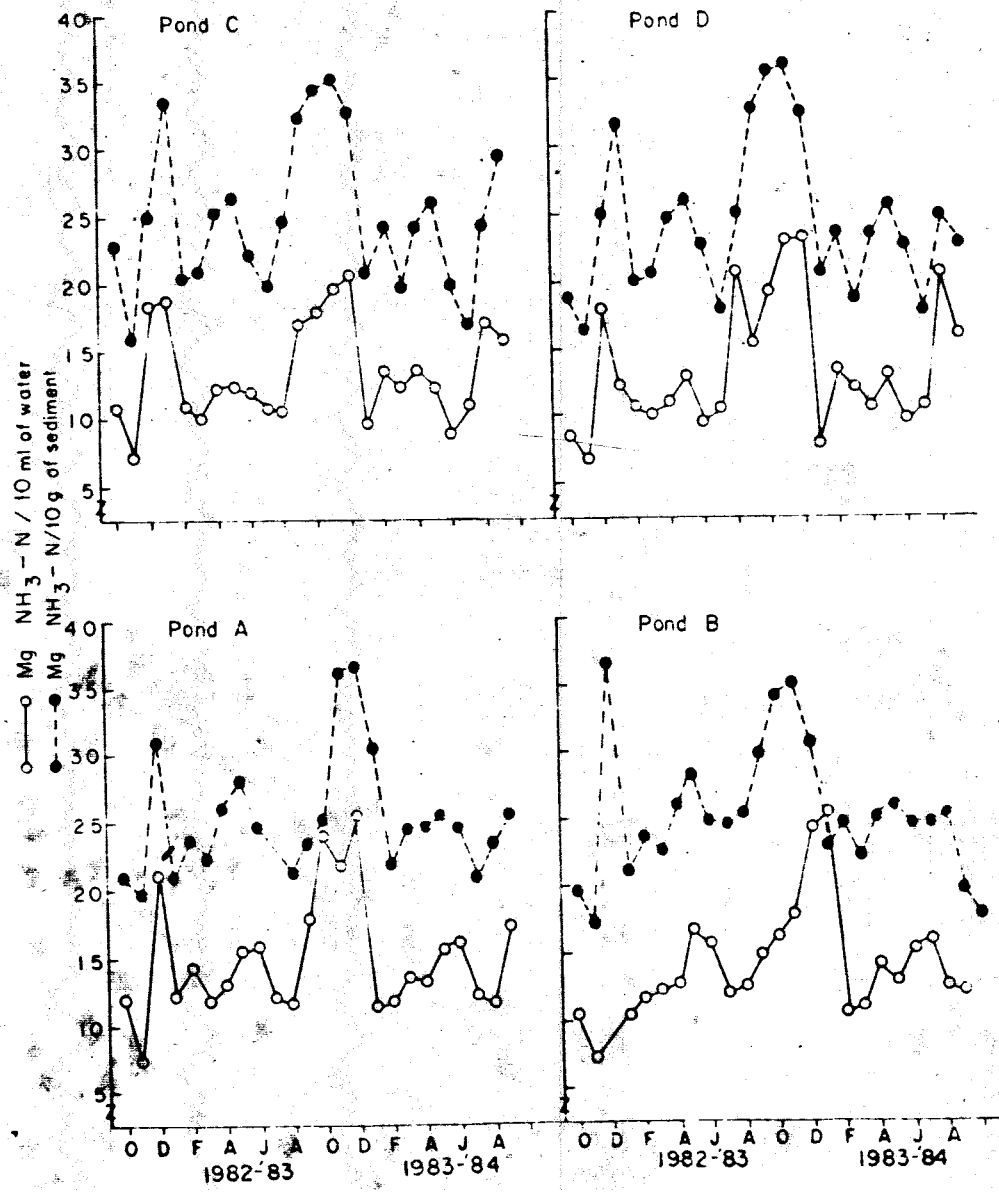

BACTERIAL NITROGEN FIXATION
in water
in sediment
April
May
June
July
August
September
October
November
December
January
February
March
Mg NH3-N/10 g of sediment
Mg NH3-N/10 ml of water
0
10
20
30
40
A B C D
1984
1983
1982
PONDS

FIG.13b. BACTERIAL NITROGEN FIXATION



fixation was consistently higher in the sediments than that in water. The postmonsoon and premonsoon months showed relatively higher nitrogen fixation rates than the monsoon season.

In pond A, except for the month of December, nitrogen fixation was relatively low in the postmonsoon season in both the water and the sediments during the first year, in contrast to the second year, in which the postmonsoon season provided the highest rates of nitrogen fixation.

In pond B a steady increase was noticed from the postmonsoon season in the water showing peak values during late premonsoon season in the first year. In the sediments also a similar trend was noticed, except during December when an uncharacteristic maximum value was recorded. A significant decline in the nitrogen fixation rate occurred during the monsoon season in both the water and the sediments. Consistently high nitrogen fixation was observed during the post-monsoon and premonsoon months in the second year in the water when compared to be first year. But in the sediments, rate of nitrogen fixation was more in the first year than that of the second year.

The trend observed in pond C and D, water and sediments, was similar, except for minor variations. In pond C postmonsoon and monsoon seasons showed higher nitrogen fixation rates than the premonsoon season, in both the years. But in pond D, during the first year, higher nitrogen fixation rates were obtained in the monsoon months followed by the postmonsoon and relatively low rates were recorded during the premonsoon season in both the water and the sediments. Whereas, during the second year, in both the water and sediments postmonsoon season provided greater nitrogen fixation rates followed by the monsoon, and the premonsoon season showed low values.

The mean rates of nitrogen fixation in the water ranged from 7.21 to 21.18 mg in pond A, 7.21 to 19.18 mg in pond B, 9.13 to 21.74 mg in pond C 8.73 to 23.67 mg in pond D and in the sediments it ranged from 19.8 to 30.77 mg in pond A, 17.23 to 37.23 mg in pond B, 18.11 to 36.01 mg in pond C and 19.17 to 36.69 mg in pond D (Fig. 13a and b).

Among environmental parameters considered to determine their effect on nitrogen fixation water Eh and organic carbon did not show any significant influence in both the water and the sediments in any of the four ponds (Table 10).

Of the influencing parameters, in pond A sedi. pH> salinity > water pH > D.O. > NH_3-N > NO_2-N > water temp. (R^2 = 57.82 $P < 0.02$) significantly influenced the nitrogen fixation rates in the water; whereas total P > salinity > sedi. pH > D.O. > NH_3-N > NO_2-N > water pH (R^2 = 60.27; $P < 0.05$) affected the sediment nitrogen fixation rates. The factors which had a negative influence are sedi. pH, salinity, NH_3-N, NH_2-N in water; and salinity, sedi. pH, NH_3-N, NO_2-N in the sediments.

In pond B, NO_2-N > total P > total N_2 > salinity > NO_3-N > water pH > sedi. pH > water temp. in the water (R^2 = 67.42; $P < 0.05$) and total P > totl. N_2 > salinity > NO_3-N > water pH > water temp. > sedi. pH > NO_2-N D.O. in the sediments (R^2 = 67.65, $P < 0.05$) had significant influence on the nitrogen fixation. While nitrite, salinity, sedi. pH, water temp. and D.O. had negative influence in the water, salinity, water temp., sedi. pH, NO_2-N, D.O. regatively affected the sediment nitrogen fixation.

The factors influencing the nitrogen fixation in pond C water were sedi. pH > water pH > NH_3-N > total P > salinity > water temp. > NO_2-N > D.O. (R^2 = 58.01; $P < 0.05$); whereas sedi. pH > sedi. Eh > NH_3-N > NO_3-N >

Table 10: Estimates of the parameters in multiple linear regression analysis for nitrogen fixing rate

Symbol	ENVIRONMENTAL PARAMETERS	NITROGEN FIXATION RATES							
		WATER				SEDIMENTS			
		A	B	C	D	A	B	C	D
a		11.1738	11.0304	13.5160	17.7910	9.8257	14.4850	6.7260	16.0751
b1	Water temp.	0.2193	-1.2363	0.7417	-0.8646	NS	-1.2020	0.2450	NS
b2	Water pH	4.6962	8.5397	8.9024	-0.3436	3.0519	5.8641	10. 5891	NS
b3	Sedi. pH	-5.5525	-6.4697	-13.8498	3.5493	-2.0460	-3.3403	-14.9072	-3.0476
b4	Water Eh	NS	NS	NS	NS	NS	NS	NS	NS
b5	Sedi. Eh	NS	NS	NS	NS	NS	NS	-0.1698	-0.1289
b6	Diss. 0_2	2.1715	-0.1770	0.3467	0.7636	2.7080	-0.1695	1.0177	3.2671
b7	Salinity	-0.3386	-0.5788	0.1861	-0.4825	-0.3890	-2.7767	0.3126	-0.4062
b8	Nitrite-N	-3.8948	-0.2493	12.4155	-5.4752	-0.910	-3.0682	3.3615	–
b9	Nitrate-N	NS	0.5671	NS	-0.1568	NS	0.3280	-0.5500	-0.4520
b10	Ammonia-N	-0.8045	NS	1.1075	-0.1270	-0.9634	NS	2.2204	0.5194
b11	Organic carbon	NS	NS	NS	NS	NS	NS	NS	NS
b12	Total nitrogen	NS	0.2753	NS	NS	NS	0.3508	NS	NS
b13	Total phosphorus	NS	3.1173	0.1308	0.1549	0.1687	4.0974	0.1916	0.2296
R^2		57.82%	67.42%	58 01%	5346%	60.27%	67.65%	55.10%	66.01%

NS - not significance

A, B, C, D - Ponds

water pH > salinity > total P_2 > water temp. > NO_2-N (R^2 = 55.10; $P < 0.05$) had influence in the sediments. Among these sedi. pH and NO_2-N in the water and sedi. pH, NO_3-N, NO_2-N in the sediments showed negative influence on the nitrogen fixation.

In pond D, D.O. > salinity > total P > sedi. pH > water pH > water temp > NO_3-N > NH_3-N in the water (R^2 = 53.46; $P < 0.05$) and sedi. Eh > D.O. > salinity > NO_3-N > total P > sedi. pH in the sediments (R^2 = 66.01; $P < 0.05$) contributed to the variation in the nitrogen fixation rates. While salinity, water pH, water temp., NO_3-N and NH_3-N in water had negative influence, sedi. Eh, salinity, NO_3-N, sedi. pH had negatively influence on the sediments nitrogen fixation.

DISCUSSION

Among the bioelements present in the hydrosphere nitrogen is one of the most important, being a component in all living things (Kuznetsov, 1959). It is by now well established that bacteria are very important as nitrogen fixers and as nitrogen liberators from organic matter. These attributes called for intensive investigations on the bacteria associated with the nitrogen cycle in the terrestrial and the aquatic ecosystems. Within the aquatic ecosystems, a great deal of research on nitrogen cycle bacteria has been carried out on the macrosystems, such as rivers, lakes, reservoirs, estuaries, bays and oceans. The microsystems, such as the fish ponds received relatively little importance, particularly in India, and the present study is on one such unique system, where prawns and paddy are cultivated in the same field on a rotation basis. Besides, a comparison is also made with the adjoining perennial prawn culture fields.

The present investigation clearly reveals the considerable variation in the relative abundance of the selected nitrogen cycle bacterial groups between ponds, between sediments and water, and between the different seasons of the year. A number of environmental variables have significantly contributed to the observed variations indicating that the microbial productivity in these prawn culture systems depends largely upon the physical and chemical conditions of the water and the sediments.

Physico-chemical characteristics of water and sediments:

The perennial and the seasonal prawn culture systems selected for the study are dynamic ones, being continuously subjected to changes in physical and chemical properties of the sediments as well as the overlying water. Marked

fluctuations in the levels and kinds of nutrients, temperature, salinity, dissolved oxygen, pH and Eh have been observed in these systems during the period of study. These changes occur principally, as a result of freshwater intrusions, changes in the tidal flow, evaporation, seasonal and diurnal variations, as well as by the biogeochemical processes within the systems.

In the opresent study, both in the seasonal and perennial prawn culture systems, temperature, salinity and dissolved oxygen content (D.O.) of the water showed a decrease during the monsoon period as a result of fresh water run-off from rivers and local precipitation. Shynamma and Balakrishnan (1973) also observed that dissolved oxygen values remain low during the monsoon period in the Cochin backwaters. The low D.O. contents encountered during the monsoon and postmonsoon in the ponds may be due to increased decomposition of debris at the bottom. The increased content of silt-load in the water and increased respiration by biomass during monsoon and premonsoon can be additional causes for dissolved oxygen depletion (Shynamma and Balakrishnan, 1973). Ramamirtham and Jayaraman (1963) are of the opinion that apart from the influence of monsoon rains and the considerable amount of evaporation during the hot months, the influence of various types of phenomena in the Arabian Sea, such as "upwelling", "sinking", "coastal piling" etc. considerably affects the hydrological conditions in this area. Sankaranarayanan and Qasim (1969) noted that in the Cochin backwaters changes in water temperature, salinity and dissolved oxygen were small during the premonsoon period and large during monsoon and postmonsoon periods. As the culture ponds are located in the vicinity of the Cochin backwaters they are also exposed to the alterations in the physico-chemical conditions in the backwaters to a greater extent.

The role of monsoon in lowering the salinity and temperature in the inshore waters has been reported by Annigeri (1968). Nagarajaiah and Gupta (1983) have also reported that the monsoon and the freshwater discharge are the main factors which regulate different physico-chemical parameters of brackishwater ponds along Nethravathi Estuary, Mangalore. The greater fluctuations in water temperature and dissolved oxygen in the prawn culture ponds may be attributed to the relative shallowness of the ponds and the regular mixing of water due to tidal influence, particularly in the perennial ponds. The relatively low dissolved oxygen values recorded in the seasonal ponds as compared to the perennial ponds during the non-monsoon period can be related to the reduction of increased amounts of dead and decaying matter particularly the paddy stumps.

Easwaraprasad (1982) and Sreenivasan (1982) while investigating the ecology of brackishwater culture ponds near Cochin, have reported the water and the sediments to be more alkaline during the monsoon and postmonsoon periods. In the present case, pH did not behave conservatively and marked variations in both the culture systems in different seasons occurred. The increased hydrogen ion concentration of water and sediments recorded in the seasonal and perennial prawn culture systems during the monsoon and post-monsoon periods may be related to the removal of CO_2 which is assimilated due to increased photosynthetic activity of the systems during that time (Sreenivasan, 1982). Relatively inconsistent pH noticed in the water and the sediments during the monsoon season may be due to increased sedimentation and subsequent reduction of organic debris resulting in relatively more reducing conditions near the bottom. Low pH values as recorded in the present study have also been reported during the monsoon period by Sankaranarayanan and

Qasim (1969) for the inshore area of Cochin and by Radhakrishnan (1978) during the southwest monsoon periodin the inshore waters of Mangalore.

The differences in the seasonal behaviour of pH observed between the two culture systems during monsoon and postmonsoon periods may be due to the prevalence of freshwater conditions in the backwaters and incursion or poorly oxygenated off-shore waters which are bound to affect the perennial ponds as they are connected to the backwater system, unlike the seasonal ones.

The low redox potential (Eh) values of water and sediments recorded in the present investigation during monsoon and in the postmonsoon period, in both the culture systems, are consistent with the occurrence of relatively reduced conditions due to the increased rate of death and decay of organic debris during that period as already discussed. While studying the ecology of prawn-culture brackishwater ponds around Cochin, Paulinose et al. (1981) have also reported seasonal redox potential trends similar to the present findings.

The extent of increase of nitrate and ammonia in the premonsoon was considerable; whereas, nitrite, organic carbon, total nitrogen and total phosphorus showed fluctuation. Nair et al. (1975) described nutrients and primary productivity in the Vembanad Lake and emphasized that the distribution of nutrients is largely dependent on the marine influence and the freshwater discharge.

The fluctuating values of nitrite recorded during the present study seems to be related to its transitional nature in the nitrogen cycle (Rakestraw, 1936). The nitrite values recorded by Sreenivasan (1982), while investigating the ecology of culture ponds in the Vypeen Island were extremely higher

(976 µg/l) than the present observation in the prawn culture ponds, though the seasonal trend was similar in both the cases. The relative shallowness (depth 40 cm) of the ponds studied by Sreenivasan (1982) may be the cause of higher values noted by him.

Sankaranarayanan and Qasim (1969) reported fluctuating but higher values of nitrite during monsoon and postmonsoon period in the inshore area off Cochin. Subrahmanyam (1959) and Annigeri (1968, 1972) also reported values similar to the present findings in the inshore areas of the Arabian Sea.

Frequent oscillation of total phosphorus noted during the present study are indicative of the regeneration of phosphate phosphorus from the bottom sediments (Sankaranarayanan and Qasim, 1969). Increase of total phosphorus noted during the late premonsoon and early postmonsoon may be due to the greater silt load in the Cochin backwaters at that time (Sankaranarayanan and Qasim, 1969). According to Ansari and Rajgopal (1974) large quantity of allochthonous phosphate will find its way into the backwaters and this may be responsible for high values of phosphorus, even in the prawn culture ponds during the monsoon period. Nagarajaiah and Gupta (1983) also recorded a nutrient peak in the brackishwater ponds along Nehravati Estuary, Mangalore, during June which is consistent with the present findings in the prawn culture systems. Ansari and Rajgopal (1974) noted wide variations of mud phosphate with relatively high concentrations during monsoon and low levels in the premonsoon and postmonsoon periods.

In the prawn culture systems low phosphorus was recorded during the monsoon season as compared to the premonsoon and postmonsoon seasons. This may be due to the seasonal changes in the hydrobiological features of the overlying waters and the texture and mineralogical composition of sediments.

Sankaranarayanan and Panampunnayil (1979) also reported low phosphorus values during the monsoon months of June-September followed by an increase in the following months. They attributed the low values during the monsoon months to the leaching of the phosphorus, both the interstitial and adsorbed forms from the mud to the overlying water.

In the prawn culture systems a seasonal distribution pattern was evident for the total nitrogen content of the sediments, with the postmonsoon season exhibiting higher values than the premonsoon and early monsoon period; but in the late monsoon months nitrogen values were high. In contrast to the present observations Sankaranarayanan and Panampunnayil (1979) reported that the seasonal variation of nitrogen does not follow any definite pattern.

More organic carbon was recorded during the postmonsoon months than the premonsoon and monsoon seasons in all the ponds. Seasonal variation in organic carbon may be related to the plankton productivity in the overlying water, the settling of humic material brought in from land and also the oxidation of organic matter by organisms living in the bottom. In the seasonal ponds the decomposition of paddy stumps may be an additional factor. The reduction of organic carbon in the monsoonal months is mainly due to the tidal currents and due to the leaching of organic nutrients from the sediments into the overlying water. A comparison of the organic carbon content of the sediments of the seasonal ponds with that of the perennial ponds clearly indicates the greater fertility of the seasonal ponds. Along the different brackishwater ecosystems adjoining the Cochin backwater, the seasonal fields(pokkali)have the highest fertility status followed by the perennial fields (Eswaraprasad, 1982). In contrast to the present observations, Sankaranarayanan and Panampunnayil (1979) reported increase in organic carbon during southwest monsoon compared to the premonsoon and postmonsoon seasons.

As elucidated by the forgoing discussion, it is clear that when rainfall occurs during monsoon and postmonsoon period, the increased freshwater run-off from the catchment area alters the physicochemical characteristics of the water and the sediments in the present ecosystems. Incursion of offshore waters into the inshore area during premonsoosn period govern the variations of physicochemical parameters during the premonsoon season.

Bacterial distribution and abundance in water and sediments:

Analysis of the bacterial data indicates that throughout most part of the investigation sediments in most of the ponds harbour more number of bacteria than the water. This can be attributed to the gradual deposition of bacteria from the overlying water in the vegetative stage or in the form of spores or by the growth and propagation of the bacteria indigenous to the sediments or finally by the combination of both the factors as suggested by Russell (1967). It is also possible that the specific substrates and nutrients required by the bacteria for their propagation may be available in greater concentrations in the sediments. Besides, particulate substrates necessary for favourable development of large attached populations may tend to settle, and carry large numbers of bacteria into the mud during sedimentation (Renn, 1937). In addition to these, there is the possibility of the transport and deposition of bacterial populations from the adjoining brackishwater feeder canals, particularly in the case of perennial ponds, as these ponds are provided with sluices for proper exchange of water, throughout the year.

Sediments from the upper 5 cm was taken for bacterial enumeration for the present study. In these ponds, where water is quite stagnant, it would be reasonable to expect greater number of microbes at the organic matter rich surface layer of the sediments than in the deeper layers. According to Reuszer 1933) in the topmost layers, at the mud-water interface, the bacterial population

is more dependent upon the degree of decomposition of the organic matter than upon the quantity of organic matter present. However, any movements which cause vertical mixing from one layer to another may induce short term variation in the bacterial content of the surface of the sediments as well as the overlying water. Conversely, in the water column, availability of nutrients may be less than that of sediments, thereby the propagation of bacteria will be affected. Besides, the water column is susceptible for greater fluctuations in the environmental conditions, resulting in poor propagation of the bacteria. Thus, the greater abundance of the bacteria in the sediments could be attributed to all the above mentioned factors.

The important deviations observed in the abundance relate to ammonifiers, nitrifiers and denitrifiers in certain months when the sediments harboured less bacteria than water because of fluctuations in dissolved oxygen content, water temperature, sediment pH and anoxic conditions. Certain anerobic bacteria such as Desulfovibrio sp. in the soils have been known reduce the Eh, which affects the distribution of nitrifiers in the sediments (ZoBell, 1975).

The occurrence of more numbers of nitrifiers in water than the sediments during February-August period in pond B, in both the years, is due to low contents of phosphorus and nitrogen in the sediments, besides the low dissolved oxygen levels in the water .

The occurrence of relatively low numbers of nitrogen fixers in February 1984 in pond B and in September 1984 in pond D sediments is associated with the sharp decline in water temperature and salinity, and wide fluctuations in pH as compared to the other ponds. Stanley and Morita (1968) demonstrated that the maximum growth temperature varies with the salinity. Acidic pH in the soils is known to reduce the carbonate contents inhibiting the growth and

multiplication of nitrogen fixing bacteria. The relation between Ca CO_3 and rate of nitrogen fixation by the nitrogen fixing bacteria has been found by Lakshmanaperumalswamy et al. (1975) who reported that with the reduction in content of Ca CO_3 in the medium the amount of nitrogen fixed by the nitrogen fixers got reduced. So the reduction in the sediment bacterial numbers could be due to this factor.

In the present study, counts of heterotrophs ranged from 4.0×10^5 to 23.1×10^6 in the water and 5×10^6 to 23.1×10^7 in the sediments of perennial ponds; from 10×10^5 to 24×10^6 in the water and 5.0×10^6 to 23×10^7 in the sediments of the seasonal ponds. These are considerably higher than the observations made by Jana and Roy (1983) in freshwater fish culture ponds of Bengal.

Proteolytic numbers ranged from 60×10^4 to 83×10^5 in the water and 50×10^5 to 69×10^6 in the sediments in the seasonal ponds; from 40×10^4 to 89×10^5 in the water and 49×10^5 to 83×10^6 in the sediments of the perennial ponds. In the water and filter-sands of Ayu fish culture ponds of Japan the proteolytic numbers were relatively low, being in the range 3.2×10^4 to 1.6×10^6 cells (Sugahara and Hayashi, 1974). Similarly, proteolytic density was relatively low 8.11×10^5 in the marsh ecosystem located in Chesapeake Bay (Walker et al., 1975). Thus the proteolytic bacterial productivity in the prawn culture systems is greater than many other aquatic systems.

The density of ammonifiers ranged from 0.5×10^5 to 14×10^6 in the water and from 1.5×10^6 to 14×10^7 in the sediments of perennial ponds; and it ranged from 0.6×10^5 to 14.0×10^6 in water and 1.6×10^6 to

11 x 10^7 in the sediments of the seasonal ponds. The seasonal and perennial prawn culture systems are more productive for ammonifiers than that of the fresh water fish culture ponds of Bengal which had a population ranging from 0.2 x 10^6 to 1.9 x 10^6 in the water and the sediments respectively (Jana and Roy, 1983).

Population densities observed in the decreasing order are heterotrophs ammonifiers proteolytic in the ponds during the present investigation. The distribution of heterotrophs are more pronounced in both the years but proteolytic and ammonifying bacteria showed inconsistency in distribution in the postmonsoon months in most of the ponds.

In the present findings the number of nitrifiers (ammonia-oxidizers) ranged from 0.4 x 10^3 to 10 x 10^4 in the water and 0.6 x 10^4 to 11 x 10^5 in the sediments of the perennial ponds. In the seasonal ponds the range was 0.3 x 10^3 to 11.0 x 10^4 in the water and 0.3 x 10^4 to 11.0 x 10^5 in the sediments. Ammonia oxidizers ranged from 10 x 10^3 cells/ml in the water and from 10^3 to 10^5 cells/g in the filter-sands, at the Mashima and Minu Ayu fish Hatchery (Sugahara and Hayashi, 1974). The number of nitrifying bacteria in an aquarium with a circulatory system ranged from 10 x 10^3 cells/g in the filter sands (Kawai et al., 1964). Thus the present observations indicate that the brackishwater prawn culture systems harbour more ammonia-oxidizers probably due to more favourable environmental conditions and substrate availability.

The density of denitrifiers observed in the present study are in the range 0.3 x 10^3 to 1 .0 x 10^4 in the water and 0.3 x 10^4 to 14.0 x 10^5 in the sediments of the perennial ponds; and in the seasonal ponds their numbers

range from 0.3 x 10^3 to 14.0 x 10^4 in the water and 0.5 x 10^4 to 11.0 x 10^5 in the sediments. Seasonal ponds had relatively less denitrifiers in the sediments than the water. Standing crop of denitrifying bacteria which are responsible for the nitrogen loss in the nitrogen cycle in various water regions of inland bays such as Maizura Bay and Kumihama Bay in Japan ranged from 0 to 10^4 cells/ml in the water region and from 10^2 to 10^6 cells/g in the bottom sediments (Kawai and Sugahara, 1972). Gamble et al. (1977) reported the numbers of denitrifying bacteria from 19 soils including 4 paddy soils to range from 1.2 x 10^4 and 7.0 x 10^6/g soil. Ishizawa and Toyoda (1964) obtained an average of 3 x 10^5 denitrifiers per gram soil in 18 Japanese paddy soils. Araragi and Tangachan (1975) obtained an average of 6 x 10^5/g in Thailand paddy soils. However, the studies of Garcia et al. (1974) fave an average of 2 x 10^3. Denitrifiers ranged from 10^3 to 10^4 cells/g of the filter sands and from 10^2 to 10^4 cells/ml or less in the water (Sugahara and Hayashi, 1974).

The density of nitrogen fixers ranged from 40 x 10^2 to 130 x 10^3 in the water and 60 x 10^3 to 63 x 10^4 in the sediments of the perennial ponds; and from 70 x 10^2 to 113 x 10^3 in the water and 50 x 10^3 to 74 x 10^4 in the sediments of the seasonal ponds. In the fish culture ponds of Japan (Kawai et al. 1971) nitrogen fixers occur in less numbers from 1.2 x 10^2 to 3.5 x 10^3 in the water and from 4.8 x 10^2 to 9.2 x 10^4 in the bottom sediments. Maruyama et al. (1970) reported nitrogen fixers from nil to 10^4 per 100 ml of sea water. In general it is observed that the prawn culture ponds are more productive than the other systems.

In the present findings nitrogen fixing Azotobacter ranged from 6 x 10^3 to 12.6 x 10^4 in the water and 4 x 10^4 to 4.9 x 10^5 in the sediments of the perennial ponds; and from 7 x 10^3 to 6.8 x 10^4 in the water and 7 x 10^4 to

4.9×10^5 in the sediments of the seasonal ponds. Niewolak (1970) found Azotobacter in small numbers rarely exceeding 100 cells/g dry weight of sediments. Saslow et al. (1980) observed the Azotobacter to range from 0×10^3 per ml in the bottom sediments of Estonian lakes. Deufel (1965) found that Azotobacter counts in the Lake Constance increased from 1000-3,000 cells per litre. Thus the prawn culture systems are more productive than many of the water and soils compared here.

In general, the perennial pond A is less productive for heterotrophs and proteolytic bacteria than pond B, C and D. Ammonifiers showed greater fluctuations in the perennial ponds (in pond A and B). Pond A is relatively large and is connected to the main feeder brackishwater canal, thereby, exposed to the dynamic changes in the physico-chemical factors in the feeder canal. The fluctuations in salinity, temperature and nutrients composition of the sediments and the overlying water may shift the pH, besides supplementary factors like tide, freshwater run-off, seasonal and even periodic temperature changes might contribute to the fluctuations in bacterial numbers as suggested by Liston (1968). Pond B, on the other hand, is smaller in size and though subjected to similar characteristics, is more productive because of the prawn farming management practices, such as organic and inorganic fertilization and supplementary feeding, thereby more energy sources are available for the multiplication of bacteria. By contrast the high productivity recorded in the seasonal ponds in mainly due to the rich organic matter and nutrients which gets accumulated as a result of decomposing paddy stumps and weeds.

asonal variation in bacterial density:

A well defined monsoon based seasonal shift in the abundance of heterophic and proteolytic bacteria in the ponds was evident from the study. The ve two groups were prodominent during the premonsoon season compared to the nsoon and postmonsoon seasons. Heavy rainfall and freshwater discharge bring

down the salinity during the monsoon, resulting in the death of both the marine as well as estuarine forms which are susceptible for sudden changes in salinity and associated changes in the environmental factors. The effect of rainfall on the bacterial density is obvious from the data obtained for the first year of study when there was a distinct decline in the population during monsoon. Besides, heavy rainfall in the second year led to more reduction in bacterial numbers as compared to the first year even during postmonsoon and premonsoon seasons. The occurrence of relatively greater numbers of heterotrophic and proteolytic groups in the monsoon season during the second year can be attributed to the comparatively low rainfall in the monsoon months of that year. Thus rainfall seems to have a negative influence on the propagation of the heterotrophs and proteolytic bacterial groups.

Tezuka et al. (1981) showed seasonal fluctuations in bacterial density with an abundance of bacteria immediately after the monsoonal rains, especially the North-east monsoon and the consequent freshwater discharge. Mantondkar et al. (1980) reported that due to heavy monsoon rainfall, considerable reduction in salinity and low values of nutrients occur because of continuous wash out and dilution of estuarine water resulting in low counts of bacteria. In contrast, Taylor (1940) has observed a correlation between high bacterial counts and periods of rainfall, and some relations between the total numbers of phytoplankton and the bacterial populations of water samples in a freshwater system.

Taber and Neihof (1984) reported that the seasonal distribution of heterotrophs are significantly dependent on the water temperature. According to Vaatanen (1980) more variation in the heterotrophs found out during winter was governed by humic acid, salinity and water temperature and rainfall. Austin (1983) reported variations in bacterial population from the coastal rearing units of fish ponds with relatively low numbers in winter and high

counts in summer. In the present observations the abundance of total heterotrophs, to some extent, varied in different seasons due to intensity of changes in temperatures, rainfall and local variations in the nutrient contents. The decline in water temperature leads to more reduction of total heterotrophs in monsoon than postmonsoon and the premonsoon seasons.

Fred et al.(1924) recognised the complexity of factors which influenced the seasonal distribution of bacteria. Drainage from land was one factor which operated intermittently at regular intervals. Jana and Roy (1983) reported that bacterial numbers were generally higher in the winter in both the water and sediments and low in the summer in freshwater fish ponds from Bengal. Though the seasonal temperature fluctuations are not marked in the present study area, minor changes in temperatures seem to significantly affect some of the groups.

The greater density of ammonifiers in both the water and the sediments, noticed in the postmonsoon season, may be due to greater accumulation of detritus at the bottom. Detritus provide bacteria not only with a surface for attachment, but also the source of nitrogenous matter (Jones, 1976). The decrease in ammonifiers density during certain months can be attributed to the poor availability of organic matter and accumulation of toxic products of metabolism of other microbes in the bottom (Niewolak et al., 1978) or due to more negative redox potential (Kuznetsov, 1958). Rheinheimer (1959) reported low ammonifiers, in the winter in the river water due to large degree of cold water which affects the ammonification process due to low water temperatures. Niewolak (1965) found that the rate of ammonification is more intense in the summer season when the number of ammonifiers in the water and sediments were maximum. Since the temperature variations in the prawn culture systems during the period of study are not marked, temperature-induced population

fluctuations may not be substantial, but, other factors may contribute substantially to the population fluctuations.

In most of the ponds more nitrifiers (ammonia-oxidizers) occurred in the first year than the second year due to heavier rainfall in the second year (3207.0 mm). Due to the depletion of ammonia in the system low ammonia oxidation takes place resulting in the reduction of nitrifiers. Besides, rainfall affects the contents of nutrients such as NO_3-N, NO_2-N and NH_3-N, and, the observed fluctuations in the nitrifiers density are mainly due to the variations in the concentrations of the nitrogen nutrients (NO_2-N, NH_3-N) water temperature, D.O., and, water and sediment pH.

The decline in nitrifiers in pond A during November 1982 is due to low nitrogen (NH_3, NO_2 and NO_3 nitrogen) nutrients; in March 1983 due to low NO_2, from February-April due to low NH_3, and in June 1984 due to low NH_3, NO_3, sediment Eh and dissolved oxygen. Low nitrogen levels are predominent in certain months perhaps due to the specific nitrogen uptake by phytoplankton. The influence of phytoplankton growth on nitrifiers has been demonstrated by Dugdale (1977) and McCarthy (1981). Chen (1968) and Fouden (1969) proposed that the nitrifying bacteria were affected by the level of such nutrients as nitrogen and phosphate. The density of nitrifiers occurring in the sediments of the fish culture ponds also were negatively influenced by NO_3-N, and positively influenced by pH, and D.O. of the bottom water (Kawai et al., 1971; Kawai and Sugiyama, 1977). Salinity is also the factor which affects nitrifiers due to its fluctuations from the month of March to September 1982-83. During the late monsoon and premonsoon seasons low salinities, low dissolved oxygen content and less oxidation-reduction potential seems to contribute to the decline of nitrifying bacteria. Billen (1978) found that the activities of the nitrifying bacteria were inhibited by low oxygen/Eh conditions.

Denitrifiers occurred in greater numbers in the premonsoon season than the postmonsoon and monsoon season in the sediments during both the years. Denitrifiers occurred in low numbers in November 1983 in the sediments of seasonal ponds (D) due to high dissolved oxygen levels and low water temperature.

The increased activity of nitrifying bacteria results in NO_2-accumulation in the ponds. This coupled with depletion of dissolved oxygen in the water accelarates the processes of denitrification thereby an increase in the denitrifiers number occurs in some seasons Brandhorst 1959). Garcia et al. (1974) concluded that the denitrifying capacity has a significant correlation with the organic carbon content. The occurrence of greater numbers of denitrifiers during the premonsoon in the second year can be attributed to the relatively high organic carbon levels in the sediments.

The occurrence of greater numbers of nitrogen fixers in the premonsoon season than the postmonsoon and monsoon may be due to the presence of more inorganic nitrogenous compounds which have accumulated in the seasonal ponds due to the decomposition of paddy stumps leading to the production of more humus and humic acid. Gaur and Mathur (1966) studied the stimulating effect of humus and humic acid on the nitrogen fixing activity of the Azotobacter. Minerals and organic substances also may be available in greater concentrations to support more numbers of nitrogen fixing bacteria during the premonsoon season than the monsoon and postmonsoon seasons as indicated by Niewolak (1970). Rice and Paul (1972) have suggested that increased nitrogen fixing bacterial density can be seen when the energy source is added to the flooded soil. So the increased availability of the required energy source might have been one of the factors contributing to their increase in the premonsoon season. The occurrence

of less numbers of nitrogen fixing bacteria in some months is due to the freshwater run-off from rivers and local precipitation that brings down the salinity and temperature. It can also be due to the increased consumption of the nitrogen fixers by zooplankton (Rodina, 1958)

The observed seasonal shifts in the population of nitrogen cycle bacteria and the variations in their abundance necessitated identification of parameters which could explain the variations. Multiple linear regression analysis of the data gave substantial indications as to the factors responsible for the variations.

Among the 13 parameters, water temperature, dissolved oxygen, salinity, water pH, sediment pH, nitrite, nitrate, ammonia and sediment phosphorus levels significantly influenced the distribution of most of the groups of bacteria in most of the ponds in both the water and sediments. Organic carbon, total nitrogen and Eh of the water and sediment did not contribute to the variations of any of the bacterial groups either in the water or in the sediments in any of the ponds.

Among the parameters which significantly contributed to the variations, also, there was no consistent trend; for example some of the factors which showed an inverse relationship with certain bacterial groups in one pond, showed a direct relationship in another pond. So the response to the bacteria to a particular factor is dependent to some extent on that pond type probably because of the differences in their prevailing environmental conditions.

A number of earlier workers also reported the contribution of various environmental factors to the distribution of nitrogen cycle bacteria.

The contribution of temperature to bacterial abundance has been shown by Vaatanen (1980) for brackishwater bacteria; Niewolak (1965) for ammonifiers and Jones (1978) for total heterotrophs and proteolytic bacteria groups. All these workers have shown a significant positive correlation with the water temperature. The contribution of dissolved oxygen was demonstrated by Goering (1968) who showed a negative correlation with denitrifiers. Fedorov (1947) showed that oxygen limits the growth and metabolisn of nitrogen cycle bacteria. While Kawai and Sugiyama (1977) and Sugahara et al. (1974) showed a positive correlation with nitrifiers Bell et al . (1981) showed a positive correlation for total heterotrophs.

Palumbo and Ferguson (1979) and Rheinheimer (1968) while studying the relationship between salinity and bacteria showed the preference of low salinity by bacteria for their optimum growth. In the present study also salinity contributed to the variation with some of the groups of nitrogen cycle bacteria showing an inverse relationship; whereas other groups showed a positive correlation.

According to Waring and Bremner (1964) mineralization is significantly influenced by the pH of the environment. In the present study pH was found to have very significant role on the microbial productivity. Authorison et al. (1976) showed that the inhibition of nitrification and growth and distribution of nitrifiers is dependent on pH. Federov (1947) explained that more acidic or alkaline pH of the ecosystem inhibits the growth of nitrogen fixing bacteria. Khullar and Chahal (1975) observed that the increase of soil pH results in low bacterial numbers. Lipman (1922) reports that nitrifiers survive at high pH but survival gets reduced at low pH level. In contrast to this Ekpete and Cornfield (1967) showed that denitrifiers were sensitive to high pH. According to Thiamann

(1964) the growth and reproduction of the heterotrophic bacteria is dep endent upon the pH. Baker and Innis (1982) observed low pH values reduced microbial activity. But Kawai et al. (1971) and Kawai and Sugiyama (1977) reported the density of nitrifiers to be correlated with the pH. Thus low pH seems to be detrimental for the growth of various groups of bacteria. Similarly alkaline pH levels also does not seem to be conducive for the growth and reproduction of the nitrogen cycle bacteria.

According to Aleem and Alexander (1958) high ammonia concentration inhibit the process of nitrification by lowering the number of nitrifiers. But Gameson (1958) and Stratter and McCarthy (1967) reported the introduction of nitrogenous compounds leads to higher degree of nitrification and mineralisation, showing that the nitrogenous compounds augment the process of nitrification and mineralisation. Brandhorst (1958) found that increasing concentration of NO_2-N augment the nitrifiers and the nitrogen fixers. But in the present study as observed by Kawai and Sugiyama (1977) a negative correlation of nitrifiers with NO_2-N was also observed in some of the ponds.

Franklin (1981) reported that NO_3-N was a major physiochemical parameter which affects the distribution of bacteria. In the present study an inverse relationship was mostly observed for NO_3-N with nitrifiers as reported by Kawai et al. (1971) and Kawai and Sugiyama (1977) from the fish culture ponds in Japan and for the nitrogen fixing bacteria by Stratter and McCarthy (1967)in the aquatic ecosystem. However, Brandhorst (1959) has shown that the nitrifiers and the heterotrophic acticity increases with the increase of NO_2-N concentration.

According to Bell et al. (1981) bacterial counts are primarily affected by the level of organic C, N and P, with the reduction in any of these elements is accompanied by a decrease in bacterial counts in both the water and sediments. But in the present study, organic carbon is not found to be a limiting factor for the abundance of nitrogen cycle bacteria; perhaps organic C concentcation in the prawn culture fields may be adequate for the bacterial groups. But as far as phosphorus is concerned, it has shown a definite positive influence only on the bacterial population in pond B.

According to Bell et al (1981) and Brouzes et al. (1971) total heterotrophs and nitrogen fixing bacteria show a significant influence with the amount of organic carbon and N but in the present findings organic C and N did not show any significant influence on the distribution of total hecterotrophs and nitrogen fixing bacteria probably due to their inadequate proportion in relation with phosphorus.

The sediment pH was found to be the most influencing environmental parameter on the distribution and abundance of total hecterotrophs, proteolytic, ammonifying, nitrifying, denitrifying and nitrogen fixing bacteria in both the water and the sediments in most of the ponds with minor variations as given below. Salinity is found to be the most influencing factor on the denitrifiers in the sediment region of pond B. Certain groups in Pond B are mostly influenced by the sediment phorphorus levels. Water temperature was the most influencing parameter on the abundance of proteolytic and heterotrophic groups in pond D; whereas dissolved oxygen was the most influencing factor for ammonifiers and nitrifiers, in pond D.

Partial regression analysis indicates that the sediment P, N and C and sediment and water Eh, dissolved O_2, water temperature and nitrite had a significant influence on the distribution and abundance of Azotobacter in the present ecosystem. Sediment pH did not show any significant influence on the distribution of Azotobacter in contrast to the observations made by Raju et al. (1974). Khullar and Chahal (1975) found out a positive correlation with sediment pH for Azotobacter. Organic carbon significantly influenced the distribution of Azotobacter. In contrast to this De and Bhattacharya (1961) reported depressing effect of organic carbon on the growth of Azotobacter. This was probably ~~be~~ due to the ~~selection of~~ presence higher level of organic carbon ~~in the~~ present in the ecosystem.

Nitrogen fixation in water and sediments:

A distinct seasonal variation was observed in the amount of nitrogen fixed by the aerobic nitrogen fixing bacteria, both in the water and sediments of the prawn culture systems. In all the ponds, the amount of nitrogen fixation was found to be more in the sediments than the water throughout the period of investigation. This may be because of the greater numbers of nitrogen fixing bacteria present in the sediments than in the water. It is also possible that the essential substrates and nutrients required by nitrogen fixers for their propagation and nitrogen fixation may be available in greater concentrations in the sediments. Sugahara et al. (1971) while examining the nitrogen fixation rate in the waters of Suyama fish culture ponds also reported more nitrogen fixation activity in the sediments than in the water.

In the prawn culture systems low nitrogen fixation was recorded during the monsoon season than the premonsoon and the postmonsoon seasons. Rainfall

may affect the propagation of nitrogen fixers and their nitrogen fixation capacity in the monsoon months as a result of changes in hydrological and hydrochemical characteristics of the water and the sediments. In the monsoon months, excretion of zooplankton and phytoplankton may also increase the levels of ammonia and nitrates which along with low organic carbon contents can lower the nitrogen fixing bacterial numbers and their nitrogen fixation capacity. Besides, the consumption of the nitrogen fixing bacteria by zooplankton may considerably reduce the nitrogenase activity thereby the nitrogen fixation (Rodina, 1958). Aziz and Nedwell (1977) while examining the microbial nitrogen transformations in salt marsh ecosystem revealed that low nitrogen fixation occurs during winter, and the rates increase during spring and reach the maximum in summer.

Low nitrogen fixation as observed in the monsoon may also be due to the presence of more inorganic nitrogen levels in both the water and the ~~soils~~ sediments and more ammonia nitrogen which may inhibit the rate of nitrogen fixation in the prawn culture systems. Yoshida et al. (1973) observed nearly complete inhibition of nitrogenase activity with an amendment of inorganic nitrogen in rice soils. Teal et al. (1979) observed that high levels of ammonium-nitrogen resulted from added fertilizers into the marsh soil inhibited bacterial nitrogen fixation. Casselman et al. (1981) reported the adverse effect of relatively high NH_3-N on the nitrogen fixation. The efficiency of nitrogen fixation had also been reported to be drastically affected by the presence of combined nitrogen (Wilson, 1958).

Due to the reduced oxidation-reduction potential and acidic nature of the soil in the premonsoon and postmonsoosn months, nitrogen fixation was

considerable in these seasons than the monsoon, in the prawn culture systems. According to Yoneyamma et al. (1977) nitrogen fixation occurs only in soil with Eh lower than - 0.15 mV, when the flooded soil was mixed with straw; but in the prawn culture systems the sediments always was in a reduced state (-25 mV). This may be due to the increased density of some of the micro-aerophilic bacteria which can tolerate and fix atmospheric notrogen even under reduced dissolved oxygen levels, low oxidation reduction potentials and in a slight acidic nature of the soil. According to Ishizawa et al. (1975) and Watanabe et al (1978) low Eh may allow certain microaerophilic nitrogen fixers in the fields to grow favourably in the summer months. Thus the number of nitrogen fixing bacteria that are counted under aerobic or microaerophilic conditions is sometimes higher than that of Azotobacter or Beijerinckii.

During the premonsoon and postmonsoon months more organic carbon present in the sediment may also be the cause of higher nitrogen fixation. Brouzes et al. (1971) observed that the substantial amount of available carbon compounds can significantly increase the number of nitrogen fixers resulting in more nitrogen fixation,.

Increased rainfall during the postmonsoon months of the second year (525.2 mm) resulted in relatively lower nitrogen fixation in that season than in the postmonsoon season of the first year.

In the prawn culture ponds the rate of nitrogen fixation in the water ranged from 7.21 to 21.18 mg NH_3-N/10 ml in the perennial ponds and from 8.73 to 23.67 mg NH_3-N/10 ml in the seasonal ponds. However, in the sediments it ranged from 17.24 to 37.23 mg NH_3-N/10 g in the perennial ponds and from 18.11 to 36.23 mg NH_3-N/10 g in the seasonal ponds. Thus the sediments of the ponds have higher potential for nitrogen fixation than the water.

Among the parameters considered, water temperature, salinity, dissolved oxygen, water pH, sediment pH, nitrite, ammonia, total nitrogen, total phosphorus and sediment Eh significantly influenced the nitrogen fixation both in the water and the sediments of most of the ponds. In the perennial prawn culture system (A and B) nitrogen fixation had an inverse relationship with dissolved oxygen, sediment pH, NO_3-N, NO_2-N and salinity, both the water and the sediments. In the seasonal ponds (C and D) nitrogen fixation in the water was affected inversely due to NO_3-N, NH_3-N, sediment pH; and in the sediments, nitrogen fixation was affected inversely with the sediment pH, sediment Eh, NO_3-N, and NO_2-N. Sediment pH is found to be the most significant parameter influencing the nitrogen fixation.

In most of the ponds dissolved oxygen showed a direct relationship with nitrogen fixation. However, in pond B in both the water and the sediments an inverse relationship was found with low dissolved oxygen. According to Ashton (1981) oxygen rich water leads to better fixation of atmospheric nitrogen. Wilson and Wilson (1940) and Fedorov (1952) reported that more than 60% oxygen in gaseous mixture competes with nitrogen of the environment which inhibits the nitrogen fixation.

PART II

STUDIES ON ISOLATED AZOTOBACTER STRAINS

INTRODUCTION

Beijerinck (1901) observed that azotobacters are the representatives of aerobic microorganisms capable of fixing nitrogen. The obligatory aerobic Azotobacter species can utilise many organic substances as energy sources. They require approximately 50 g glucose to bind 1 g nitrogen.

Various suggestions have been made for classifying the bacteria in the genus Azotobacter. Krassilnikov (1949) included the genus Azotobacter in the family Bacteriaceae. Some investigators (Jensen, 1954, Norris, 1959) distinguished the Azotobacteriaceae as an independent family which was regarded as the best approach. Johnstone (1975) gave an outline classification of the genus Azotobacter into different species.

The following species of Azotobacter have now been described A. chroococcum, A. beijerinckii, A. vinelandii, A. agilis, A. nigricans, A. woodstowni, A. macrocytogenes, A. galophilus etc. Voets and Debacker (1956) classified A. chroococcum, A. beijerinckii and A. vinelandii into the genus Azotobacter and A. agilis having characteristic round shaped cells, in a separate genus Azomonas. The identification of a particular species of Azotobacter is difficult and this led to the search of criteria for their classification. Anon (1974) has grouped all the species so far recognised based on different morphological and physiological features. However the properties of a particular species may change substantially under the influence of various external factors (Van Schreven, 1966). Morphological and biochemical characteristics of different species of Azotobacter have been studied and well documented in the Berge's Manual of Determinative

bacteriology (Buchanan & Gibbon, 1974).

A wealth of data obtained on studies with Azotobacter showed convincingly that bacterial variability is influenced by external conditions. All the species of Azotobacter including A. chroococcum are gram negative during all stages of their development (Peterson, 1961). Bisset et al (1967) obtained gram positive variants of A. chroococcum by growing it on potato media, but these variants on usual media led to the reappearance of gram negative forms. Bacteria of family Azotobacteriaceae, with the exception of genus Beijerinckii, are large sized organisms. They show their most regular and constant cell size and shape only during their exponential growth phase when supplied with a suitable carbon source and nitrogen source. According to Jensen (1955), during the exponential growth phase A. chroococcum formed short rods with accumulated ends while A. beijerinckii were oval with rounded ends. A. chroococcum were 2.0-2.4 x 3.7-5 μ while A. beijerinckii were 2.8-3.1 x 3.7-6.6 μ, cells of A. paspali were slender having size about 1.2 x 4 - 10 μ. Alternation of cells from the several Azotobacter species may occur upon aging (Jensen, 1955).

Azotobacter chroococcum, A. vinelandii, A. beijerinckii and A. paspali types formed large number of cysts during early stationery phase. Formation of highly irregular cells of doubtful importance for reproduction may occur under certain nutritional conditions, particularly in the chroococcum - beijerinckii - vinelandii group. During 16 to 48 hrs incubation, cells of A. chroococcum underwent changes from short rods to coccal forms (Bachinskaya, 1935). such cells were usually large and swollen and sometimes smaller than normal cells. Such aberrant cell forms, regarded as "pleomorphism" has been carefully studied in case of A. vinelandii by Winogradsky (1952), A. chroococcum by Peterson (1961) and A. paspali by Dobereiner, (1966).

The degree of motility among the different species, and within the species among different strains of Azotobacter was found to vary. Some strains were found to be non-motile (Sen, 1955). The motility was found to depend upon composition of the medium. They were weakly motile in media containing mannitol or sugars (Omelyanskii, 1923). Beijerinck (1901), showed that the majority of A. chroococcum cells possessed one flagellum while Hofer (1944) and Krassilnikov et al. (1952) on the basis of flagella medium studies concluded that Azotobacter was peritrichate.

A. chroococcum was characterised by the formation of an insoluble black-brown pigment (melanin like) which arises on aging of cultures as a result of oxidation of tyrosin by tyrosinase, a copper containing enzyme. Blinkov (1962) reported that Azotobacter obtained from various soils and water in the Soviet Union was represented by two forms: one forming a dark-brown pigment and the other forming colourless colonies under all conditions. However, both the forms were alike, where growth and nitrogen fixation were concerned. Sen (1955) observed that pigment forming strains of Azotobacter fixed more nitrogen than the strains which did not form the pigment. Omelyanskii (1923) showed that only living cells were capable of pigment formation; but Prazmowskii (1913) showed the dead cells of Azotobacter were capable of forming brown pigment.

The carbon requirement of Azotobacter for the growth is supposed to be very high. The carbon source for the organisms can be provided by various organic compounds. The requirement for specific carbon sources in the medium for Azotobacter species was reported by Collins (1963) and Jensen (1961). In Jensen's view a lack of organic substance is the chief factor limiting the spread of Azotobacter in soils with a favourable pH and sufficiently high phosphorus content.

Azotobacter is characteristically able to assimilate molecular nitrogen as ell as bound forms of the element. The amount of nitrogen assimilated depends rimarily on the properties of the particular strain of Azotobacter. The nitrogen ssimilated by Azotobacter is released into the media in the form of protein, amino cids and partly in the form of ammonia (Fedorov, 1952 and Sandrak, 1958). Most ultures assimilate no more than 10 mg of mol. N_2/g of carbon source consumed. n Kluyver and Becking's experiments (1955) individual strains of A. chroococcum ixed as much as 15 mg of nitrogen and in Lopatina's experiment (1949) s much as 30 mg of nitrogen were fixed. High and low fixation had been eported in the case of A. chroococcum by several workers (Zinoveva, 1962; James, 970; Sundara Rao et al., 1959). Commonly obtained nitrogen fixation values for zotobacter in pure cultures were in the range of 10-20 mg N fixed per gram sugar onsumed (Stewart, 1969); however, it was recently shown that in continuous cultures t low 0_2 values, Azotobacter chroococcum fixed 38-43 mg N/g mannitol consumed Dalton and Postgate, 1969). It would thus, appear that efficiencies of N_2- fixation n natural systems may be 2-3 times the values commonly reported on the basis of ure culture studies. Marine nitrogen cycle reviewers concluded vigorously that itrogen fixation by bacteria must be negligible because of energy limitations Vaccaro, 1965), and that it may be of much greater important than has been ealised hitherto (Wood, 1965).

The nitrogen fixing capacity of Azotobacter may vary greatly with onditions of its cultivation and with the composition and acidity of the nutrient nedium (Burk et al., 1934), temperatures (Iswaran and Sen, 1960), aeration Butkevich and Kolesnikova, 1941) ; the presence of bound source of nitrogen Iswaran, 1960); the character of carbon source (Fedorov, 1952), the presence n the medium of trace elements and biologically active substances (Esposito and

Several of the marine isolates could not be "trained" or "adapted" to grow on freshwater media, and freshwater isolates had been previously shown not to require such elevated concentrations of either Na^+ or K^+ (Macleod et al., 1954). Further evidence of the salt requirement of marine bacteria was provided by Markel et al. (1957). The growth of marine bacteria in chemically defined medium has been shown to be due to the ability of seawater to supply the inorganic ions required for growth by the organisms (Macleod and Onofrey, 1956).

Modern halophiles were first described by Baxter and Gibbons (1956). Several food-spoilage organisms have been classed as moderate halophiles, being able to grow in the range of 0.5 to 3.5 M NaCl. This definition also fits marine bacteria, many of which require about 0.5 M (3%) NaCl for growth (Macleod, 1965) and have been found on further examination to withstand, 20, 25 or even 30% NaCl (Forsyth et al., 1971). If the temperature is raised to 25°C or higher, atleast 0.5 M NaCl is needed for growth and NaCl cannot be replaced by KCL or by non-ionic solutes (Novitsky and Kushner, 1975).

Most microorganisms that can grow in high salt concentrations can also grow in much lower ones. This was brought out by a survey of a number of marine bacteria isolated (on agar containing 3% NaCl) off the coast of New Braunswick (Forsyth et al., 1971). Most of these bacteria could grow in NaCl concentrations of 20% and 30%. These studies implies that many or most marine bacteria can grow in much higher salt concentrations than those of the ocean, and such bacteria may be considered moderately halophilic or atleast very salt tolerant microbes.

Pratt (1974) studied the salt requirements for growth and function of marine bacteria. Kakimoto et al. (1974) studied the mineral requirements of marine bacteria by using various media in which the growth of marine isolates was

affected mainly by the inorganic constituents, and concluded that the mineral requirement of marine isolates depends not only upon the individual species, but rather upon the specific mineral requirements of each genus. Pratt and Tedder (1974) studied the variation in salt requirement for the optimum growth of marine bacterial culture and observed that marine halophiles have both a specific requirement for Na^+ and a non-specific requirement for solute which could be satisfied by KCl; growth rate was enhanced by the addition of KCl to sub-optimum concentrations of NaCl but none of the isolates grew without the addition of Na^+.

There are also few studies on the influence of salinity on nitrogen fixing microbes. Beijerinck (1901) was the first to note that Azotobacter was resistant to high salt concentrations. Though Lipman (1912) had shown that NaCl in concentrations higher than 0.5-0.6% was toxic to Azotobacter, Krasnikov (1958) found that Azotobacter from saline soils of Central Asia grew in the presence of 11.6% NaCl. James and Shende (1972) studied the salt tolerance of strains of A. chroococcum from the rhizosphere of agricultural crops and found that at low salt concentration (as NaCl), the optical denisty of the culture can vary, but at high concentration it gradually falls and the rate of decrease in optical density calculated per unit increase in salt concentration in the medium varies from 0.09 to 0.59, whereas the lethal concentration of salt was within the limit 3.24 to 3.9%. Azotobacter isolates from the rhizosphere of barseem were most resistant to NaCl, while the strains isolated from rhizosphere of maize and wheat were most sensitive.

According to Babak(1965) soil salinization upto a certain extent did not inhibit the development of Azotobacter but its number was usually higher in slightly saline than in strongly saline soil. Tenatin (1954), reported that in certain soils Azotobacter was absent due to inadequate water content rather than effect of soil salts.

The effect of salinity on the quantity of nitrogen fixed was studied by Lakshmanaperumalswamy et al. (1975) and it was observed that all the isolates of Azotobacter surveyed from 13 different stations representing marine, estuarine and mangrove region at Porto-Novo preferred a salinity of 30%₀ for their optimum growth and for maximum fixation of nitrogen. Greaves et al. (1942) found that excessive concentrations of salts in the soil rendered Azotobacter inactive; with the removal of salts due to rain or irrigation, Azotobacter started functioning again. Iswaran and Sen (1958) from the agricultural soils of India reported that an increasing concentration of salt had a depressing effect on nitrogen fixation by Azotobacter. Iswaran et al. (1965) found a fall in the nitrogen fixation activities at varying degrees of salt in the four strains of A. chroococcum.

Effect of pH

Roy et al. (1962) were of the opinion that the distribution of Azotobacter in soils mostly depends upon the nature of the soil and climatic conditions. Further it was shown that soil temperature (Taha et al., 1967), organic matter (Markov, 1969) the soil humidity (Emiliani and Priano, 1971), the soil pH and the soil air (Kreshlin and Valauzene, 1973) affected the population of Azotobacter. Raju et al. (1974) showed that the soil pH around 8.5 supported the maximum population of Azotobacter. Khullar and Chahal (1975) found that Azotobacter count was maximum between soil pH 7.8 and 8.8 and that the count decreased with the increase in pH. The efficiency of nitrogen fixation in all the isolates was not affected adversely with the increase of soil pH from 7.0 to 8.7. Very high concentrations of hydrogen-ions (acid) or extremely low concentrations (alkaline) are normally toxic to most organisms. Environments more acid than pH 6.0 are free

of the organism or contain very few Azotobacter cells. Similarly, the bacteria will neither grow nor fix N_2 in culture media having a pH below 6.0 (Jurgensen and Davey, 1968). Sethunathan et al. (1977) experimentally proved that the nitrogen fixing activity of the enrichment cultures obtained from the Indian rice soils has a certain degree of relation with pH of the soil.

Yamagata and Itano (1923), Smalii (1939) and Blinkov (1955) came to the conclusion that the optimum pH for the growth of Azotobacter was near or slightly above neutrality. Fred (1918) reported that, Azotobacter was extremely sensitive to slight changes in pH; the narrow limits for its growth being pH 6.6-8.4 or 8.8. Gainey (1923) reported that the average pH of soils showing no Azotobacter growth was 5.71 and the nitrogen fixed 3.88 mg per culture; while the average pH of these soils showing the growth of Azotobacter was 6.78 and the average amount of nitrogen fixed was 8.1 mg.

Burk et al. (1934) showed that the growth of some A. chroococcum strains was arrested at pH 6.0. According to Peterson (1925) in his experiments A. chroococcum could not grow in a medium with pH 6.0 and below. Caskey (1926) observed weak growth in a medium having ammonium salts at pH 5.8; the strain could grow at pH 5.0 in the presence of nitrate.

Starkey (1940) obtained an acid tolerant Azotobacter actively fixing nitrogen over the pH range of 3.1-8.0. Blinkov (1953) isolated acid-resistant strains from Siberian soil's and assumed that they had originated from ordinary sensitive strains of Azotobacter chroococcum. Rangaswami and Sadasivan (1965) found Azotobacter chroococcum to be predominant in both alkaline and neutral soils.

ffect of trace elements

A wide range of trace elements cycle naturally through the biosphere. ome of these are essential micronutrients at low concentrations but toxic at igher levels, others have no known biological function. Microbes respond to race elements in different ways, depending on the kind of microorganisms and epending on the concentration of trace elements in the environment. All icrobes require certain metals, including Co, Cu, Fe, Mn and Zn, in their utrition (Brock, 1974; Enoch and Lester, 1972). Some also require Mo, V and Ni (Bartha and Ordel 1965; Bertrand, 1974; Bertrand De Wolff, 1973; Ehrlich, 1976; Esposito and Wilson, 1956). All these metals are mainly involved in enzyme function, and they are needed only in very low concentrations in the nutrient medium, usually in the range of micrograms litre^{-1}. Some microorganisms are able to take some of these elements into the cell by active transport (Silver and Kralovic, 1969; Wang and Newton, 1969; Bhattacharyya, 1970; Silver et al., 1970; Eisenstadt et al., 1973; Khovrycher, 1973).

MacLeod and Onofrey (1957), demonstrated that the requirement of marine bacteria for seawater was not just for the sodium ion or a total ionic effect, but that different bacteria had different requirements, which could be either for the chloride ion, sodium or potassium ions (Macleod and Hori, 1960) or sulfate (Macleod and Onofrey, 1956).

Many inorganic nutrients are necessary for the development of the microorganisms but only a select few are specifically implicated in the metabolism of nitrogen, that is they are indispensable for nitrogen linked proliferation. Some are required in lesser amounts for growth. Molybdenum, iron, calcium and cobalt are critical for the fixation of nitrogen reaction. A requirement of

calcium has been demonstrated during nitrogen assimilation by blue-green algae and some species of Azotobacter but the calcium can sometimes be replaced by strontium. Calcium is also required for growth (Evans and Kliewer, 1964). Similarly, organisms making use of nitrogen must have cobalt available for them, although a lesser concentration of this element may be essential for growth on combined nitrogen and the role of cobalt in the nitrogen assimilation process has been established for Azotobacter, Beijerinckia, Clostridium, and several algal genera (Jakobsons et al., 1962). Molybdenum (Mo) was found to be essential for nitrogen fixation (Fedorov, 1952 and Kovalskii et al., 1967) Krzemieniewski and Kovats (1936) showed that the stimulating effect of Mo on the nitrogen fixing activity of Azotobacter cultures was only manifested in the presence of iron. Vanadium had the same effect as Mo but was less effective (Bortels, 1936; Burk and Horner, 1940). The higher nitrogen fixation activity of A. chrococcum was noted at a certain ratio of vanadium to iron (Bortels, 1939). Manganese was also found to stimulate nitrogen fixation (Mirotvorskii and Grigoryan, 1945; Gribanov 1954).

Toxic levels of certain metals have also been revealed by different authors in some natural environment and found the toxicity level of cobalt is 23-26 mg litre^{-1} in Azotobacter (Avakyan, 1967) as CoCl. $6H_2O$ (Den Dooren Dc Jong and Roman, 1971). Iron and zinc toxicity was studied by using $FeCl_3$ and $ZnSO_4.7H_2O$ compound and found the toxic level 10^{-3} M of Fe in Bacterium communis, (Winslow and Hotchkiss (1922), whereas 1 mg litre^{-1} toxicity of zinc in Aspergillus niger (Adiga et al., 1961).

Vitamin requirement

Macleod et al. (1954) found that several marine bacteria required the addition of vitamins to the medium for growth. Requirements for biotin, thiamine and niacin were demonstrated in the case of three organisms. Surface active

agents stimulated the growth of two others. One organism a Flavobacterium required six amino acids, biotin, thiamine, a combination of three nucleosides, and a surface active agent in the medium to provide appreciable growth in the absence of Yeast extract (Macleod et al., 1958). Burkholder (1963), reported on the general growth requirements of 1,748 aerobic heterotrophic bacteria isolated from marine muds and found that biotin and thiamine were the vitamins most frequently required for growth. Cobalamine and nicotinic acid stood next, and pantothenate and riboflavin requirements occurred infrequently.

MATERIAL AND METHODS

Isolation, characterization and identification of Azotobacter:

Isolates from the Azotobacter counting plates were purified by repeated streaking on nitrogen-free agar medium and maintained at room temperature in the same medium throughout the period of investigation. The identification of Azotobacter isolates was based on the Bergey's Manual of Determinative Bacteriology (1974); and the classification was made according to Norris and Chapman (1968). The identity was confirmed by studying the morphological and physiological characteristics of Azotobacter suggested by Gibbs and Shapton (1968).

The various morphological attributes studied were size, shape, colony character, thickness of the capsule, presence of spores, and motility. The size of the organism was measured with the help of an occular micrometer, after susbjecting the smear to Gram staining by using 48 hours old culture following Hucker's modification given by Rodina,(1972). The thickness of capsule, in a four days old culture, was also determined with the help of an occular micrometer, after the

smear was stained by Anthony's method with Tyler's modification for capsule staining (Anthony, 1931). To ascertain the presence of spores, if any, spore staining was done by Bartholomew and Mittwer's "cold" method (1950) in 15 days old culture. The motility was studied by growing the organism on slants for 24 hours, after which a small quantity of sterile water was added to the test tube and allowed to stand for half an hour without shaking the test tube. A drop of the suspension was then taken and tested for motility using the "Hanging drop preparation" and the flagella staining was done by following Leifson's method (Rodina, 1972). Pigment production was noted in all the isolates after growing them on Jensen's nitrogen-free agar medium, in petridishes, for 48 hours or more at 28 ± 2°C.

Biochemical and physiological characteristics:

Utilisation of carbohydrates: Basal medium used to study the comparative utilization of different sources of carbohydrates is given in Table 12. The carbohydrates used were glucose, sucrose, fructose, starch, mannitol, lactose, rahmnose, mannose, maltose, arabinose, xylose and raffinose. Ten ml of basal medium containing 1% sugar was dispensed in each of the test tubes containing an inverted Durham's tube. The tubes were sterilised by steaming them in autoclave for 30 minutes for 3 consecutive days. The tubes were inoculated with the selected Azotobacter isolates and incubated at 28 ± 2°C for 48 hours to 72 hours, and, acid and gas production in each tube if any was noted.

Starch hydrolysis: Nutrient agar medium (Rodina, 1972) containing 0.2% starch powder was prepared, sterilised and poured into sterile petri dishes. The plates were spotted with the cultures and incubated at 28 ± 2°C for 48 hours. After 48 hours of incubation, they were flooded with Gram's iodine solution (Rodina, 1972) and presence or absence of halos around the colonies of organism was noted.

Table 12: Composition of Media

Jensen's nitrogen free medium
(Rodina, 1972)

Sucrose	1.5 g
$MgSO_4.7H_2O$	0.2 g
$(FeSO_4)_3.9H_2O$	0.05 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
NaCl	0.2 g
$Na_2MoO_4.2H_2O$	0.05 g
$CaCl_2.6H_2O$	0.05 g
Agar	20.0 g
*Double Distilled water	1000 ml

pH 7.0

*For nitrogen fixing ability and the effect of salinity double distilled water was replaced by artificial sea water and for other experimental studies it was replaced by 3% Nacl instead of 0.02% NaCl

Basal medium

Peptone	10 g
NaCl	5.0 g
Distilled water	1000 ml
pH	7.0

Nutrient Agar medium

Beef extract	3.0 g
Peptone	5.0 g
Agar	20 g
Distilled water	1000 ml

pH 6.8 6 7.2

Glucose phosphate broth

D. glucose	5.0
K_2HPO_4	5.0
Peptone	5.0
Distilled water	1000 ml

pH 7.5

Simon's Citrate agar medium

NaCl	5.0 g
$MgSO_4.7H_2O$	0.2 g
Ammonium dihydrogen phosphate	1.0 g
K_2HPO_4	1.0 g
Sodium citrate	5.0 g
Agar	2.0 g
Bromothymol blue	0.08 g
Distilled water	1000 ml

pH 6.8 - 7.0

Dubos medium (Rodina, 1972)

$NaNO_3$	0.5 g
K_2HPO_4	1.0 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
$Fe_2(SO_4)3.9\ H_2O$	Trace
Distilled water	1000 ml

pH 7.5

Liquefication of gelatin: Nutrient broth was supplemented with 12% gelatin and sterilised. The medium was poured into sterile petri dishes in aseptic condition. After the medium was solidified cultures were spotted on plates in duplicate. The plates were incubated at 28 ± 2°C for 48 hours and then flooded with 1.5% mercuric chloride with 20% HCl. The presence of clear halos around the colonies due to liquefication was noted.

Utilization of citrate as the sole carbon source: Simon's citrate agar medium (Conn, 1957) was prepared and sterilized. Bacterial inoculum was added aseptically into a sterile medium in a test tube. The inoculum was properly mixed by shaking and poured into sterile petri dishes at 45°C in an aseptic condition. The cultures were plated in duplicate and the plates were incubated at 28 ± 2°C for 48 hours. The utilization of citrate and the growth on citrate-agar medium resulting in the colour change of bromothymol blue into green or bright blue was noticed.

Action on litmus milk: Litmus solution was added to separated milk to get a pale-mauve colour. The milk was distributed in test tubes and steam sterilised for 30 minutes for 3 consequtive days. The tubes were then incubated at 28 ± 2°C. The appearance and change in colour of the milk was periodically examined after 24 hours of inoculation.

Methyl Red and Voges Proskauer (U.P) test: For both the tests glucose phosphate broth (Conn, 1957) was prepared and dispensed (5 ml in each test tube) and was steam sterilised for 30 minutes. The tubes were inoculated and incubated for 72 hours at 28 ± 2°C. For methyl red test 5 drops of the indicator (0.1 g methyl red into 300 ml of ethanol + 200 ml distilled water) were added to each tube. For V.P. test 0.5 ml of 6% α-napthol solution and

0.5 ml of 16% potassium hydroxide were added to each tube containing 2% of the liquid culture. Development of red colour indicated a positive reaction in both the tests.

Production of hydrogen sulfide:

Peptone water (Table 12) containing 0.01% cysteine was dispensed in 5 ml lots in each test tube. Dry filter paper strip saturated with lead acctate solution was put in each test tube. The test tubes were sterilized, inoculated and incubated for 48 hours to 72 hours at 28 ± 2°C. Blackening of lead acetate paper indicated H_2S production.

Reduction of nitrate to nitrite: Basal medium (Table 12) was fortified with 0.2% potassium nitrate. The medium was distributed in tubes containing an inverted Durham's tube. The tubes were sterilised, inoculated and incubated at 28 ± 2°C for 72 hours. The formation of nitrite was detected by the development of red colour by addition of Griess-Ilosvay's reagent (Conn, 1957) to the culture tube.

Production of HN_3 from peptone: Peptone water (Table 12) was prepared and 5 ml lots were distributed in each test tube and sterilised, and when cool inoculated and incubated for 48 hours at 28 ± 2°C. One ml of Nessler's reagent was added to the culture tube when the incubation was over. The development of orange to brown colour indicated the presence of ammonia.

Indole test: Tryptone broth (Table 12) containing 1% tryptone was dispensed in 10 ml lots in each test tube and sterilized. After inoculation, incubated for a period of 48 hours at 28 ± 2°C, and tested by adding reagents (Conn, 1957). The appearance of red colour in 5 minutes was noticed.

Catalase reaction: In this reaction the inoculum was skipped from a petri dish and flooded with one drop of 10% solution of H_2O_2. The evolution of gas bubbles from the colonies was noted.

Cellulose decomposition: Dubos medium (Table 12) was added in each test tube (less than 2 cm) and several narrow strips of filter paper were placed into each of the test tubes. Then sterilised, inoculated and incubated for 8 days at 28 ± 2°C. After incubation the growth of cellulose - degrading bacteria was observed on the filter paper.

Pectinase decomposition: To screen out pectinolytic activity of the bacteria, culture media suggested by (Table 12) Rodina (1972) were used. Two ml of the solution containing pectin was dispensed with a sterile pipette into each tube containing agar. The test tubes were inoculated with the isolates and incubated for 8 days at 28 ± 2°C. The action of the organism on the pectin was detected by breakdown of pectin as shown by liquefication.

Nitrogen fixing ability:

The relative efficiency of thirty isolated strains (Az_1 to Az_{30}) of Azotobacter to fix atmospheric nitrogen was studied by growing them in Jensen's medium of neutral reaction (Rodina, 1972). The medium was prepared with artificial seawater without any nitrogen source. The experiment was conducted in triplicate. The amount of nitrogen fixed in liquid culture was determined after the incubation periods of 15, 30 and 45 days by following the modified kjeldahl micromethod (Rodina, 1972).

Effect of salinity on the growth and nitrogen fixation in Azotobacter strains:

Experimental studies were carried out on the 30 isolated strains (Az_1 to Az_{30}) of Azotobacter to elucidate the effect of salinity on the growth

and nitrogen fixation by growing them in the Jensen's medium (Rodina, 1972). Salinity levels used for the experiment were 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60‰. Artificial seawater for the media was prepared according to standard methods (A.P.H.A., 1971). Artificial seawater was diluted to prepare the desired salinity level from 5‰ to 35‰, and salinity levels above 35‰ were prepared with the help of double strength saline water. The flasks were incubated for a period of 15 days in dark and the growth was measured as absorbance with the help of a colorimeter (Erma Photoelectric colorimeter Model AE-11N, Erma Optical Works, Ltd., Japan) at 420 nm and the amount of fixed nitrogen was estimated by using modified kjeldahl micromethod (Rodina, 1972).

Effect of pH on the growth and nitrogen fixation ability of Azotobacter strains:

Experiments were conducted to study the effect of pH on the growth and nitrogen fixation efficiency of nine (Az_1 to Az_9) selected strains of Azotobacter. The pH levels tested were 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. Strains were grown in 100 ml of Jensen's medium (Rodina, 1972) in sterilized Erlenmeyer flasks. The inoculated flasks were incubated for a period of 15 days in dark and the growth of cells was determined by measuring the absorbance at 420 nm with an Erma colorimeter and the nitrogen fixation was determined by modified kjeldahl micromethod (Rodina, 1972).

Effect of trace elements on the growth of Azotobacter strains:

To study the requirement of trace elements for the growth of Azotobacter strains (Az_1 to Az_9) four trace metals viz. cobalt ($CoCl_2.6H_20$) copper ($CuSO_4.5H_20$), zinc ($ZnSO_4.7H_20$) and iron ($FeCl_3$) were used in the concentration range of 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/l. The experiment

was conducted in triplicate. These concentrations were mixed in the nitrogen free broth of Jensen's medium and one ml of inoculum was added in each culture tube into the sterilized broth. After the incubation period of fifteen days at room temperature (28 ± 2°C), the absorbance was measured at 420 nm with the help of a colorimeter.

Effect of vitamins on the growth of Azotobacter strains:

The effect of vitamins viz., ascorbic acid, biotin, thiamine, and cyanocobalamine were studied on the growth of Azotobacter strains (Az_1 to Az_9) by using increasing concentrations of 20 to 200 µg/l in the case of ascorbic acid, 10 to 100 µg/l in the case of biotin and thiamine, and 1.00 to 10.0 µg/l in cyanocobalamine. The above concentrations were prepared with the help of double distilled water, and mixed in the nitrogen free Jensen's broth. The broth was filled in the culture tubes (10 ml amounts) and sterilized. In each culture tube, one ml of inoculum was added and incubated at room temperature (28 ± 2°C). The experiment was conducted in triplicate. The turbidity was measured with the help of colorimeter at 420 nm, after the incubation period of fifteen days.

To study the response of different levels of pH, salinity, trace elements and vitamins on the growth and nitrogen fixation of Azotobacter cells the following second degree polynomial response function was fitted (Snedecor and Cochran, 1967):

$$Y = a + bx + cx_2$$

Where Y = Growth of the Azotobacter or its nitrogen fixation

x = level of pH, salinity etc.

a, b, c = are variable constants.

The data were processed in a H.C.L. computer at the National Informatics Centre, New Delhi.

RESULTS

Bacterial identification and characterization

All the 30 isolates were collected either from the water or from the sediments of the perennial and seasonal ponds (Table 13). Of the 30 isolates, 13 isolates were identified as A. chroococcum; 9 as A. vinelandii and 8 as A. beijerinckii.

All the 13 isolates of A. chroococcum were gram negative, oval shaped cells occurring individually or in pairs, or sometimes in groups. Size ranged from 2.9 to 5.2 μm in diameter (Plate 1 a,b,c) with peritrichous flagella and were motile. They were of the cyst forming and capsule producing types, and were capable of producing brown and blackish-brown water insoluble pigments. They utilized starch and mannitol as carbon source, but were unable to utilise rhamnose (Table 13).

The isolates of A. vinelandii were also gram negative, elongated oval shaped cells; occurred individually or sometimes in groups (Plate 2a,b,c) their size ranged from 2.8 to 4.5 μm in diameter and were motile with peritrichous flagella. They were cyst forming, produced capsular slime and capable of producing green and greenish-yellow water soluble pigments. As a carbon source, they utilized mannitol and rhamnose, but were unable to utilise starch (Table 13).

All the eight isolates of A. beijerinckii were also gram negative, circular in shape; occurring singly and their size ranged from 5.4 to 7.4 μm in diameter (Plate 3a,b,c) larger than both A. chroococcum and A. vinelandii. They were non-motile; non-flagellated; producing cyst and also had capsular

Table 13: General characteristics of the Azotobacter strains

	A. chroococcum	A. beijerinckii	A. vinelandii
Gram reactions	-	-	-
Shape	Round cells occurring singly, in pairs, in chains and in groups	Circular cells occurring singly	Elongated oval shaped cells, singly and few in groups.
Size (dia /um)	2.9 µm to 5.2 µm	5.4 µm to 7.4 /um	2.8 /um to 4.5 µm
Motility	+	-	+
Flagella	Peritrichous		Peritrichous
Cyst formed	+	+	+
Capsular slime presence	+	+	+
Pigments: Water soluble-fluorescence	None	None	Green
Water insoluble in cells	Black brown	Yellowish	None
Carbohydrates utilisation:			
Starch	+	-	-
Mannitol	+	-	+
Rhamose	-	-	+
Habitat	Soil and water	Soil and water	Soil and water
Isolates designated	Azc1 to Azc13	Azb1 to Azb8	Azv1 to Azv9

+ = Positive; - = Negative

me. They had water insoluble yellowish pigment. They were unable to ilise starch, mannitol and rhamnose as their carbon source (Table 13).

All the 30 Azotobacter isolates (Az_1 to Az_{30}) were catalase positive, dole producing, showed negative reaction to Vogues Proskaur test and methyl d test (except in isolate Azb_2 and Azb_6); citrate utilisers, showing positive action in litmus milk in which the curdling of milk and the colour change as observed; unable to decompose cellulose and pectin (Table 14).

Of the 13 isolates of A. chroococcum (Azc1 to Azc13) only one Azc5 as found to hydrolyse gelatin. All the 9 isolates of A. vinelandii (Azv1 to Azv9) nd 8 isolates of A. beijerinckii (Azb1 to Azb8) were also unable to hydrolyse elatin (Table 14).

While all the 13 isolates of A. chroococcum hydrolysed starch, all the isolates of A. vinelandii did not hydrolyse starch. Among the isolates of A. beijerinckii only Azb2 was able to hydrolyse starch, and all others showed egative response to the test of starch hydrolysis. All the 13 isolates of A. chroococcum and six isolates of A. vinelandii produced H_2S from cysteine; whereas in A. beijerinckii only two isolates Azb4 and Azb6 were unable to produce H_2S; but in the remaining six isolates H_2S production was observed.

In the ammonia production test, four isolates of A. chroococcum viz. Azc3, Azc4, Azc10 and Azc12) have shown negative test; the remaining solates have shown a positive test. Among the 9 isolates of A. vinelandii (except isolate Azv6 and Azv7) seven isolates produced ammonia; but in A. beijerinekii all the 8 isolates produced NH_3 from peptone.

Date	Isolate No	Pond	Habitat	Salinity (‰)	Shape	Length & Breadth (in μm)	Pigment Formation	Hydrolysis of: Gelatin	Hydrolysis of: Starch	Catalase Production	Indole production	H_2S Production	Ammonia production	Curdling	Colour changing	NO_3 reduced to NO_2	MR	VP	Citrate utilization	Cellulase activity	Pectinase activity	Isolates designated as
20.01.83	1	C	M	18.73	Elongated oval	3.4 x 4.5	Greenish yellow	-	-	+	+	-	+	+	+	-	-	-	+	-	-	Azv1*
21.04.83	2	C	M	31.87	Round oval	3.2 x 5.1	Brownish Black	-	+	+	+	+	+	+	+	-	-	-	+	-	-	Azc1*
02.08.82	3	C	W	36.40	Elongated oval	3.2 x 4.2	Greenish	-	-	+	+	+	+	+	+	+	-	-	+	-	-	Azv2*
08.06.83	4	B	W	36.30	Circular	6.4 x 5.7	Yellowish	-	-	+	+	-	+	+	+	-	-	-	+	-	-	Azb1*
15.06.83	5	C	M	18.91	Round oval	3.6 x 4.7	Brownish	-	+	+	+	-	+	+	+	-	-	-	+	-	-	Azc2*
16.07.83	6	D	M	3.90	Round oval	3.4 x 3.9	Brownish	-	+	+	+	-	-	+	+	-	-	-	+	-	-	Azc3*
08.12.83	7	B	M	11,83	Round oval	2.9 x 4.8	Brownish Black	-	+	+	+	-	-	+	+	+	-	-	+	-	-	Azc4
05.02.83	8	C	M	20.13	Round oval	2.9 x 3.4	Brownish	+	+	+	+	+	+	+	+	-	-	-	+	-	-	Azc5
22.02.83	9	A	M	20.23	Elongated oval	2.8 x 3.9	Greenish yellow	-	-	+	+	+	+	+	+	-	-	-	+	-	-	Azv3*
03.03.83	10	D	M	19.40	Round oval	3.9 x 4.7	Brownish	-	+	+	+	+	+	+	+	-	-	-	+	-	-	Azc6
01.04.83	11	B	W	28.19	Circular	6.2 x 7.4	Yellowish	-	-	+	+	-	+	+	+	+	+	-	+	-	-	Azb2*
10.05.83	12	A	M	34.20	Round oval	5.2 x 4.9	Brownish	-	+	+	+	+	+	+	+	-	-	-	+	-	-	Azc7
21.05.83	13	D	W	35.13	Round oval	4.7 x 5.2	Brownish	-	+	+	+	-	+	+	+	-	-	-	+	-	-	Azc8
05.07.83	14	D	M	16.77	Round oval	4.6 x 4.9	Brownish	-	+	+	+	+	+	+	+	+	-	-	+	-	-	Azc9
04.08.83	15	C	M	4.44	Elongated oval	3.7 x 2.9	Greenish yellow	-	-	+	+	-	+	+	+	+	-	-	+	-	-	Azv4
14.08.84	16	D	M	4.41	Round oval	4.2 x 4.9	Brownish black	-	+	+	+	-	-	+	+	-	-	-	+	-	-	Azc10
28.08.83	17	B	W	4.35	Circular	6.9 x 6.4	Yellowish	-	-	+	+	-	+	+	+	-	-	-	+	-	-	Azb3*
22.09.83	18	D	M	1.28	Elongated oval	3.2 x 4.8	Greenish yellow	-	-	+	+	+	+	+	+	-	-	-	+	-	-	Azv5
25.10.83	19	C	W	3.59	Round oval	3.9 x 4.2	Brownish Black	-	+	+	+	+	+	+	+	-	-	-	+	-	-	Azc11
07.12.83	20	B	W	8.45	Circular	5.8 x 6.1	Yellowish	-	-	+	+	+	+	+	+	-	-	-	+	-	-	Azb4
29.12.83	21	A	W	14.39	Circular	6.2 x 7.4	Yellowish	-	-	+	+	-	+	+	+	-	-	-	+	-	-	Azb5
16.01.84	22	B	W	14.88	Round oval	3.9 x 4.3	Greenish	-	-	+	+	+	-	+	+	+	+	-	+	-	-	Azv6
08.02.84	23	B	M	18.95	Round oval	2.9 x 3.8	Greenish	-	-	+	+	-	-	+	+	+	-	-	+	-	-	Azv7
27.04.84	24	A	M	20.76	Round oval	3.7 x 4.2	Brown	-	+	+	+	+	-	+	+	-	-	-	+	-	-	Azc12
15.05.84	25	C	M	33.91	Elongated oval	2.9 x 3.7	Greenish yellow	-	-	+	+	+	+	+	+	-	-	-	+	-	-	Azv8
28.05.84	26	D	M	15.53	Circular	5.4 x 4.9	Yellowish	-	-	+	+	+	+	+	+	-	-	-	+	-	-	Azb6
14.06.84	27	B	M	8.17	Circular	5.4 x 5.7	Yellow	-	-	+	+	-	+	+	+	-	-	-	+	-	-	Azb7
28.06.84	28	B	M	5.31	Elongated oval	4.3 x 3.8	Greenish yellow	-	-	+	+	-	+	+	+	-	-	-	+	-	-	Azv9
14.08.84	29	C	M	3.25	Circle	6.4 x 4.9	Yellow	-	-	+	+	-	+	+	+	-	-	-	+	-	-	Azb8
28.07.84	30	D	M	1.67	Round oval	4.9 x 5.2	Brownish	-	+	+	+	-	+	+	+	-	-	-	+	-	-	Azc13

+ Positive; - negative

* Strains used for experimental studies - pH, trace elements, vitamins

VP - Voges Proskauer test, MR - Methyl red test.

Of the 13 isolates of A. chroococcum, two isolates viz., Azc4 and Azc9 had the ability to reduce nitrate into nitrite and the remaining 11 isolates showed no response to this test. Four isolates of A. vinelandii viz. Azv2, Azv4, Azv6 and Azv7 reduced nitrate to nitrite and the remaining 5 isolates had not shown any response to the test; whereas in A. beijerinckii only one isolate (Azb2) showed a positive response to nitrate reduction.

All the 30 isolates utilised glucose and sucrose as their sole carbon source with acid production (Table 15) but were unable to ferment lactose. Of the 13 isolates of A. chroococcum; 12 isolates fermented fructose, exception being isolate Azc5; whereas, among A. vinelandii isolates Azv7 and Azv8 did not ferment fructose and all other 7 isolates produced acid from fructose. In A. beijerinckii isolates Azb1, Azb3, Azb6 and Azb7 were unable to produce acid from fructose, but in the remaining 4 isolates acid production was observed. All the 13 isolates of A. chroococcum hydrolysed starch but in A. vinelandii (9 isolates) and in A. beijerinckii (8 isolates) starch fermentation was not noticed. Mannitol was fermented by all the isolates of A. chroococcum, 7 isolates of A. vinelandii (except isolate Azv_2 and Azv_8) and 7 isolates of A. beijerinckii (except isolate Azb5).

None of the isolates of A. chroococcum and A. beijerinckii were able to utilise rhamnose, whereas 7 isolates of A. vinelandii were able to utilise rhamnose as their carbon source. Mannose was utilised by 5 isolates of A. chroococcum (viz. Azc1, Azc7, Azc8, Azc9 and Azc10) four isolates (Azv4, Azv5, Azv7 and Azv9) of A. vinelandii and four isolates (Azb5, Azb6, and Azb8) of A. beijerinckii.

	Utilisation of											
Isolate	Glucose	Sucrose	Fructose	Starch	Mannitol	Lactose	Rhamnose	Mannose	Maltose	Arabinose	Xylose	Raffinose
Azv1	+(+)	+(+)	+(+)	-(-)	+(+)	-(-)	+(-)	-(-)	+(-)	-(-)	+(-)	+(-)
Azc1	+(+)	+(+)	+(+)	+(-)	+(+)	-(-)	-(-)	+(-)	-(-)	-(-)	+(-)	+(-)
Azv2	+(+)	+(+)	+(-)	-(-)	+(+)	-(-)	+(-)	-(-)	+(-)	+(-)	-(-)	+(-)
Azb1	+(+)	+(+)	+(-)	-(-)	+(-)	-(-)	-(-)	-(-)	-(-)	+(-)	+(-)	+(-)
Azc2	+(+)	+(+)	+(-)	+(-)	+(-)	-(-)	-(-)	-(-)	-(-)	+(-)	+(-)	+(-)
Azc3	+(+)	+(+)	+(-)	+(-)	+(+)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)
Azc4	+(+)	+(+)	+(-)	+(-)	+(+)	-(-)	-(-)	-(-)	+(-)	-(-)	+(-)	-(-)
Azc5	+(+)	+(+)	-(-)	+(-)	+(+)	-(-)	-(-)	-(-)	+(-)	-(-)	+(-)	-(-)
Azv3	+(+)	+(+)	+(+)	-(-)	+(+)	-(-)	+(+)	-(-)	+(-)	-(-)	-(-)	+(-)
Azc6	+(+)	+(+)	+(-)	+(-)	+(+)	-(-)	-(-)	-(-)	-(-)	-(-)	+(-)	-(-)
Azb2	+(+)	+(+)	+(+)	-(-)	+(-)	-(-)	-(-)	-(-)	-(-)	+(-)	+(-)	+(-)
Azc7	+(+)	+(+)	+(-)	+(-)	+(+)	-(-)	-(-)	+(-)	-(-)	-(-)	-(-)	-(-)
Azc8	+(+)	+(+)	+(-)	+(-)	+(+)	-(-)	-(-)	+(-)	+(-)	+(-)	+(-)	+(-)
Azc9	+(+)	+(+)	+(-)	+(-)	+(+)	-(-)	+(-)	+(-)	+(-)	-(-)	+(-)	+(-)
Azv4	+(+)	+(+)	+(+)	-(-)	+(+)	-(-)	-(-)	+(-)	+(-)	-(-)	+(-)	+(-)
Azc10	+(+)	+(+)	+(-)	+(-)	+(+)	-(-)	-(-)	+(-)	-(-)	-(-)	-(-)	-(-)
Azb3	+(+)	+(+)	-(-)	-(-)	+(-)	-(-)	+(-)	+(-)	+(-)	+(-)	+(-)	+(-)
Azv5	+(+)	+(+)	+(+)	-(-)	+(+)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	+(-)
Azc11	+(+)	+(+)	+(-)	+(-)	+(+)	-(-)	-(-)	-(-)	-(-)	+(-)	+(-)	-(-)
Azb4	+(+)	+(+)	+(+)	-(-)	+(-)	-(-)	-(-)	+(-)	-(-)	-(-)	-(-)	+(-)
Azb5	+(+)	+(+)	+(+)	-(-)	-(-)	-(-)	-(-)	-(-)	+(-)	-(-)	-(-)	-(-)
Azv6	+(+)	+(+)	+(+)	-(-)	+(+)	-(-)	+(-)	+(-)	+(-)	-(-)	-(-)	+(-)
Azv7	+(+)	+(+)	-(-)	-(-)	+(+)	-(-)	+(-)	+(-)	-(-)	+(-)	-(-)	-(-)
Azc12	+(+)	+(+)	+(-)	+(-)	+(+)	-(-)	-(-)	-(-)	-(-)	+(-)	-(-)	+(-)
Azv8	+(+)	+(+)	-(-)	-(-)	-(-)	-(-)	+(-)	-(-)	-(-)	+(-)	-(-)	+(-)
Azb6	+(+)	+(+)	-(-)	-(-)	+(-)	-(-)	-(-)	+(-)	+(-)	-(-)	-(-)	+(-)
Azb7	+(+)	+(+)	-(-)	-(-)	+(-)	-(-)	-(-)	-(-)	-(-)	+(-)	+(-)	+(-)
Azv9	+(+)	+(+)	+(-)	-(-)	+(-)	-(-)	-(-)	+(-)	+(-)	-(-)	+(-)	+(-)
Azb8	+(+)	+(+)	+(+)	-(-)	+(-)	-(-)	-(-)	+(-)	+(-)	-(-)	+(-)	+(-)
Azc13	+(+)	+(+)	+(-)	+(-)	+(+)	-(-)	-(-)	-(-)	-(-)	+(-)	+(-)	+(-)

+ acid formation; - no acid formation; (+) gas formation; (-) no gas formation

Maltose was utilised by four isolates of A. chroococcum (Azc_5, Azc6, Azc9, Azc10) four isolates (Azv4, Azv6, Azv7 and Azv9) of A. vinelandii and only two isolates (Azb6 and Azb8) of A. beijerinckii resulting in acid production. Six isolates of A. chroococcum (Azc2, Azc3, Azc7, Azc9, Azc12 and Azc13), 3 isolates of A. vinelandii(Azv2, Azv5, and Azv8) and three isolates A. beijerinckii (Azb1, Azb4 and Azb7) were able to utilise arabinose with acid production.

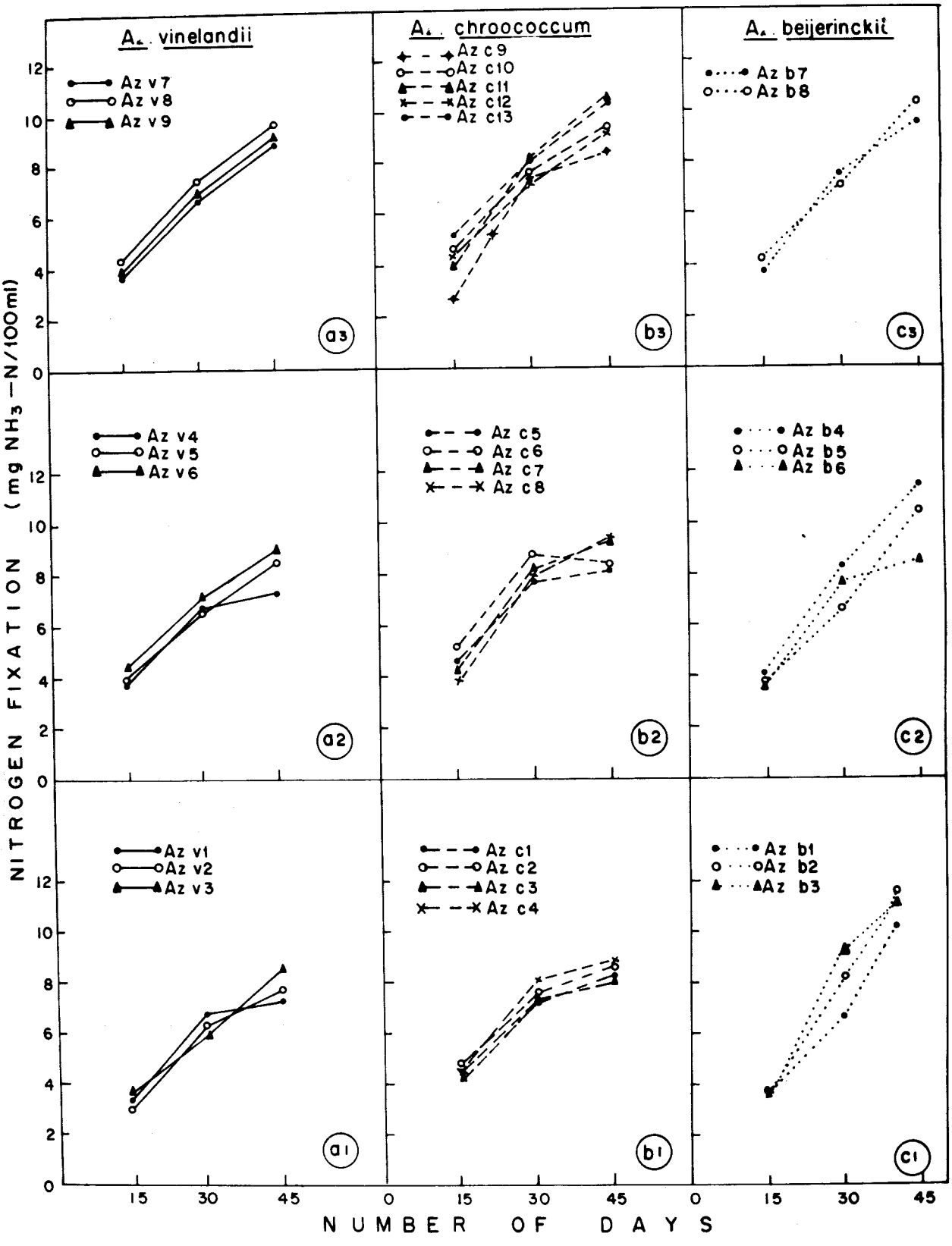
Xylose was utilised by 8 isolates (Azc1, Azc2, Azc3, Azc5, Azc7, Azc9, Azc10, Azc13) of A. chroococcum, 6 isolates of A. vinelandii (Azv1, Azv3, Azv4, Azv5, Azv9) and by 5 isolates of A. beijerinckii (Azb1, Azb2, Azb4, Azb7 and Azb8) Raffinose was utilised by 9 isolates of A. chroococcum (excepting isolates Azc4, Azc5, Azc8 and Azc12) two isolates of Vinelandii (Azv3 and Azv6) and three isolates (Azb2, Azb3 and Azb4) of A. beijerinckii.

Effect of incubation time on nitrogen fixation by Azotobacter:

The nitrogen fixing capacity of all the 30 strains of Azotobacter isolated from the perennial and seasonal prawn culture ponds was studied by culturing them for 15, 30 and 45 days, under the culture room temperature (28 ± 2°C). The results are illustrated in Fig. 14.

All the 30 strains were found to fix nitrogen, when they were cultured in the nitrogen-free medium, as indicated by the increase in the nitrogen content of the medium on the 15th, 30th and 45th day. Among the three species, most isolates of A. beijerinckii fixed relatively more nitrogen at the end of 45 days of incubation. isolates of A. vinelandii were observed to fix relatively less nitrogen, compared to the isolates of A. chroococcum

FIG. 14


A. vinelandii
A. chroococcum
A. beijerinckii
Az v7
Az v8
Az v9
Az c9
Az c10
Az c11
Az c12
Az c13
Az b7
Az b8
Az v4
Az v5
Az v6
Az c5
Az c6
Az c7
Az c8
Az b4
Az b5
Az b6
Az v1
Az v2
Az v3
Az c1
Az c2
Az c3
Az c4
Az b1
Az b2
Az b3
a3
b3
c3
a2
b2
c2
a1
b1
c1
NITROGEN FIXATION (mg NH3—N/100ml)
NUMBER OF DAYS
0
2
4
6
8
10
12
15
30
45

and A. beijerinckii at the end of 45 days. Of the 3 species of Azotobacter, A. beijerinckii was found to be the most efficient in fixing nitrogen, which was followed by A. chroococcum and A. vinelandii (Fig. 14).

All the isolates of A. vinelandii fixed nitrogen in the range of 3.0 to 4.5 mg NH_3-N/100 ml of the medium during the first fifteen days of incubation. The amount of nitrogen fixed ranged from 6.0 to 7.5 mg on the 30th and from 7.4 to 9.6 mg on the 45th day. Among the 9 A. vinelandii strains, there was a decline in the nitrogen fixing activity in Azv1, Azv2 and Azv4 after 30 days of incubation. The remaining 6 strains showed a linear increase in the amount of nitrogen fixed with the incubation time.

Except for one strain of A. chroococcum (Azc9) which fixed only 2.7 mg NH_3-N/100 ml, all the other 12 strains fixed nitrogen ranging from 3.9 to 5.18 mg after 15 days of incubation. However, on the 30th day, the nitrogen fixed ranged from 7.3 to 8.8 mg in all the 13 strains. Thereafter, a linear increase in nitrogen fixation was observed only in three strains of A. chroococcum (Azc11, Azc12, Azc13). In strain Azc6 there was a decline in the amount of nitrogen fixed after 30 days; whereas in all other strains the amount of nitrogen fixed increased on a non-linear proportion.

In all the 8 strains of A. beijerinckii the amounts of nitrogen fixed ranged from 3.5 to 4.1 mg, 3.5 to 8.25 mg and 6.6 to 11.5 mg NH_3-N/100 ml of media after 15, 30 and 45 days of incubation, respectively. Seven of the strains showed a linear increase in nitrogen fixation with time. Only the strain Azb6 showed a linear increase upto 30 days, followed by a non-linear increase in nitrogen fixation in the subsequent period of incubation.

Effect of salinity on the growth and nitrogen fixation

To elucidate the effect of selected salinity levels on the growth and nitrogen fixing capacity of the 30 Azotobacter isolates an experimental study was made for a period of 15 days. The results of the experiment are presented in figs. 15, 16 and 17. Bacterial growth has been represented based on the optical density recorded in a ERMA colorimeter; whereas, the amount of nitrogen fixed by each of the isolate has been denoted as mg NH_3-N/100 ml of the media.

The growth of all the 9 strains of A. vinelandii were observed to be significantly ($P < 0.05$) influenced by the salinity concentration of the medium (Fig. 16). Besides, the strains preferred optimal salinity levels for maximum growth. In most of the strains the maximum growth occurred at the salinity levels between 15 ppt and 35 ppt. In strain Nos. Azv2, Azv6 and Azv9 faster growth rate was observed at a salinity of 20 ppt. In strain Nos. Azv3 and Azv8 maximum growth occurred at 25 ppt. In strain Nos. Azv1, Azv4 and Azv7 maximum growth occurred at the salinity of 30 ppt. However, only in one strain Azv5 maximum growth occurred at 35 ppt. In general salinity levels above 40 ppt and below 10 ppt resulted in reduced growth of most of the A. vinelandii strains. In strain Azv3 there was no significant difference in the growth between the salinity range 25 ppt and 35 ppt. In all the isolates of A. vinelandii there was a steady increase in the growth with the increasing salinity level, showing the maximum at optimal salinity levels, as indicated earlier, and thereafter a steady decline occurred at supraoptimal salinity levels.

The amount of nitrogen fixed by the 9 strains also showed a similar pattern with the maximum at the optimum salinity level, which ranged from 25 ppt to 35 ppt, with the exception of only one strain (Azv1). Nitrogen

FIG. 15

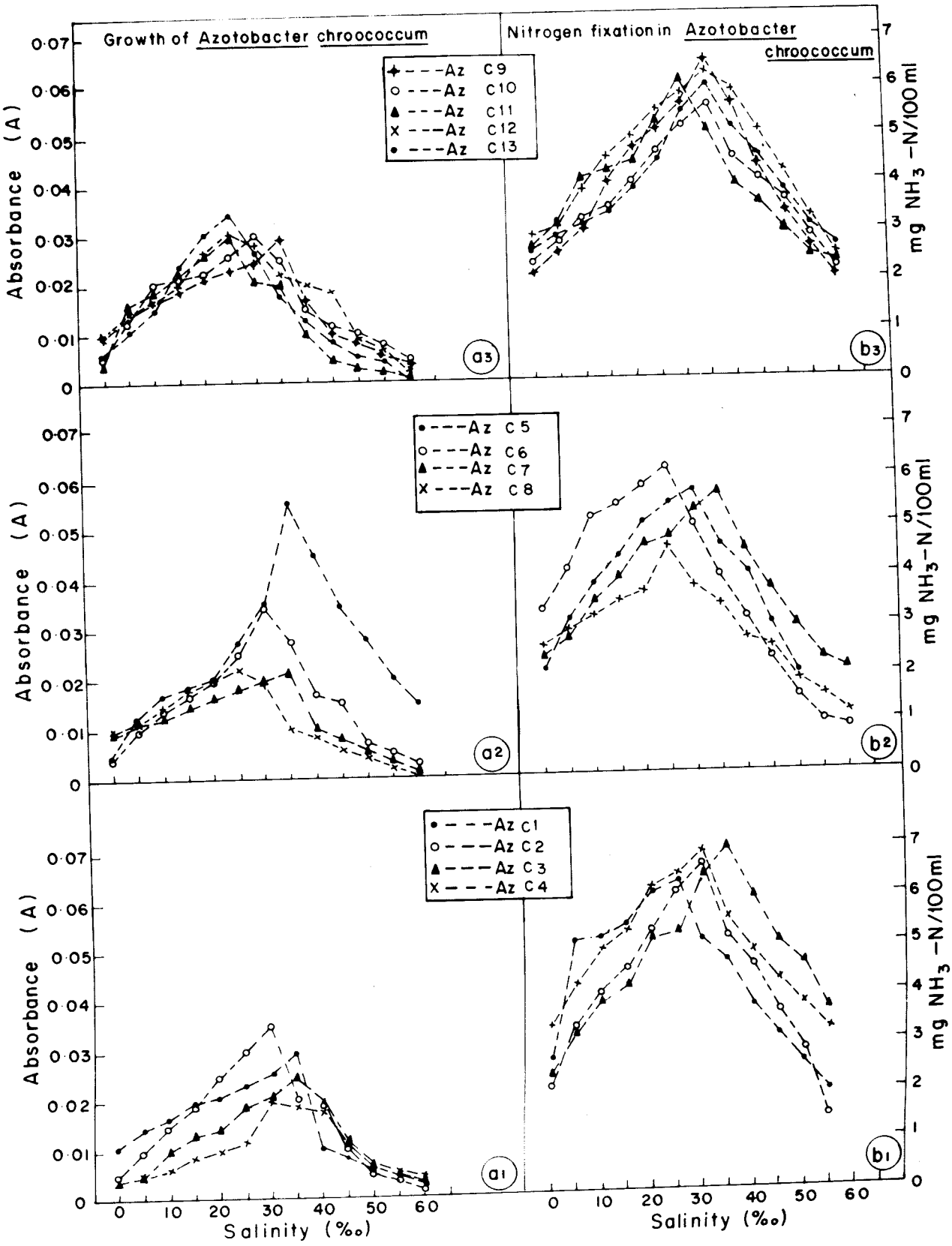

Growth of Azotobacter chroococcum
Nitrogen fixation in Azotobacter chroococcum
Absorbance (A)
mg NH3 -N/100ml
Salinity (‰)
Az C9
Az C10
Az C11
Az C12
Az C13
Az C5
Az C6
Az C7
Az C8
Az C1
Az C2
Az C3
Az C4
a3
b3
a2
b2
a1
b1

FIG. 16

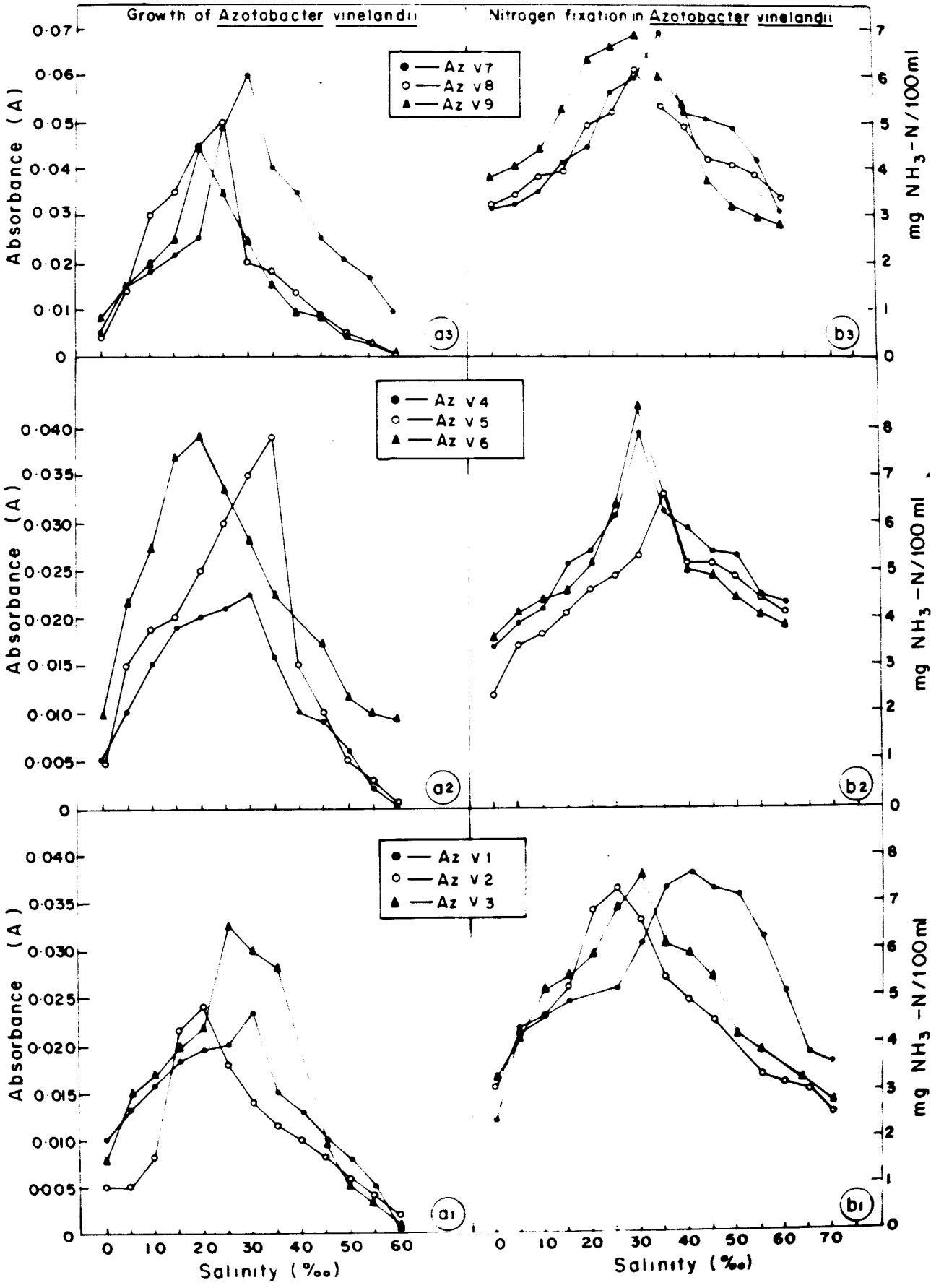
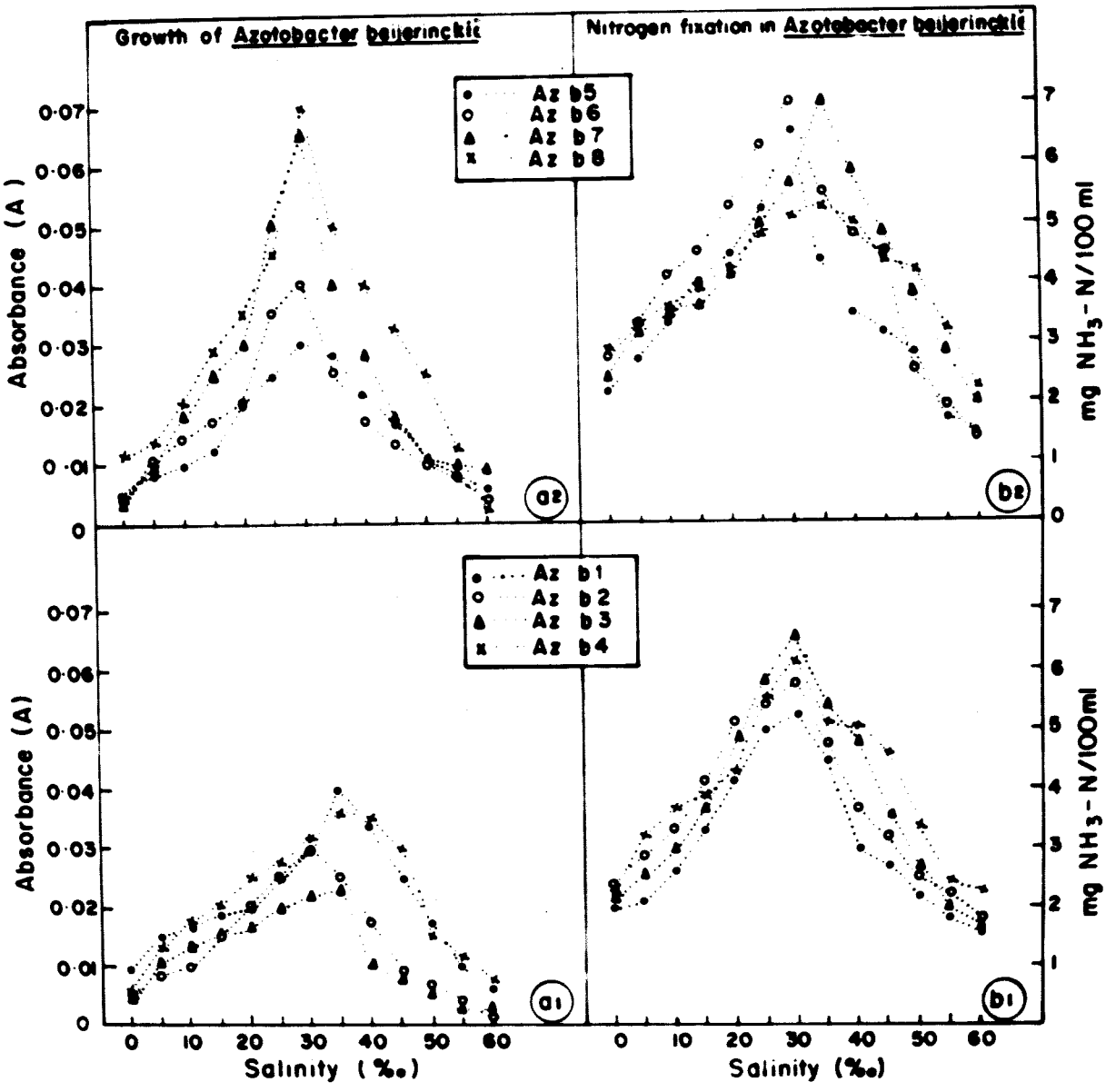

Growth of Azotobacter vinelandii
Nitrogen fixation in Azotobacter vinelandii
Az v7
Az v8
Az v9
Az v4
Az v5
Az v6
Az v1
Az v2
Az v3
Absorbance (A)
mg NH3-N/100 ml
Salinity (‰)
a3
b3
a2
b2
a1
b1

FIG. 17


Growth of Azotobacter beijerinckii
Nitrogen fixation in Azotobacter beijerinckii
Az b5
Az b6
Az b7
Az b8
Az b1
Az b2
Az b3
Az b4
Absorbance (A)
mg NH3-N/100ml
Salinity (‰)
a2
b2
a1
b1

fixation was relatively less in most of the strains in salinities between 15 ppt and 50 ppt, with minor variations. At the optimum salinity range the amount of nitrogen fixed was found to be almost double (two times) than that fixed at salinities below 15 ppt or above 45 ppt in most of the strains.

An interesting observation is that there has been some variation in the salinity level at which the maximum growth, as well as nitrogen fixation occurred in some strains. For instance, in strain Azv1 while the maximum growth occurred at 30 ppt, the maximum nitrogen fixation occurred at 40 ppt; in Azv2 the maximum growth occurred at 20 ppt, but the maximum nitrogen fixation was noticed at 25 ppt; in Azv8 maximum growth occurred at 25 ppt and maximum nitrogen fixation at 30 ppt; in Azv7 maximum growth occurred at 30 ppt and maximum nitrogen fixation at 35 ppt. In some strains maximum growth as well as nitrogen fixation occurred at the same salinity level.

In order to determine the optimal salinity levels for achieving maximum growth and nitrogen fixation, the growth and nitrogen fixation data were processed and the second degree polynomial equation of the form $Y = a + bx + cx^2$ has been fitted and the results are given in Tables 16 and 17. The results indicate that the strains Azc6 and Azc7 require relatively higher salinities of 29.52 and 30.82 ppt, respectively, for their maximum growth In strains Azv1, Azv3 and Azv9 the salinity level required for maximum growth ranged from 23.52 to 24.83 ppt. In the strains Azv5 and Azv8 there were no significant differences in the optimal salinity levels (26.56 and 26.58). However only in strain (Azv2) no significant response was obtained by statistical analysis.

In contrast to the growth of A. vinelandii, the optimum salinity at which the maximum nitrogen fixation occurred differed significantly between the strains and in most of the strains of A. vinelandii there was no direct relationship between

growth and the amount of nitrogen fixed with that of salinity level The salinity level for maximum nitrogen fixation in the increasing order was Azv2 (27.17 ppt), Azv3 (29.28 ppt), Azv8(30.71 ppt), Azv1 (31.18 ppt), Azv7 (31.53 ppt) Azv4 (32.6 ppt), Azv5 (36.17 ppt), Azv6 (39.69 ppt) and Azv9 (40.83 ppt).

In A. chroococcum with the increasing salinity level of the medium all the 13 strains showed a steady increase in growth upto the optimal salinity levels, with slight differences (Fig. 15). In most of the strains the maximum growth occurred within the salinity range of 20 to 35 ppt; the exceptions being strain nos. Azc1, Azc4, Azc5 and Azc9, in which the maximum growth was recorded within the salinity range of 25 to 45 ppt. In general, the salinity levels above 45 ppt and below 5 ppt were found to be non-conducive for the growth of most of the A. chroococcum strains. Strain No Azc3, Azc4 and Azv8 were found to be more tolerant to salinities below 10 ppt; whereas, in strain nos. Azc8, Azc11 and Azc12 growth steadily declined after the maximum at 25 ppt salinity. In contrast strain nos. Azc2, Azc4, Azc6 and Azc12 showed relatively better growth in higher salinity levels, and the differences noticed in the growth among these strains within the salinity range of 30 to 45 ppt were not significant.

The nitrogen fixation by all the 13 strains also showed the maximum values at optimum salinity levels. The optimum observed salinity levels ranged from 20 to 40 ppt. Nitrogen fixation was found to be relatively less, in most of the strains, in salinities less than 10 ppt and salinities above 45 ppt with slight variations. Differences were also noticed in the salinity level at which maximum growth and maximum nitrogen fixation occurred For instance, in strain nos. Azc6 and Azc10 maximum growth was at 30 ppt, but maximum nitrogen fixation was at 25 ppt in Azc6, and at 35 ppt in Azc10. There was no significant difference in the nitrogen fixation between salinities within the range 5 to 15 ppt

in Azc1, 10 to 20 ppt in Azc11, and 20 to 25 ppt in strain nos. Azc1, Azc3 and Azc7.

The optimum salinity level at which the maximum growth occurred in the strains are given in Table 16. The optimum levels ranged from 21.91 to 35.34 ppt in the various strains. Among the strains, Azc5 required relatively high salinity (35.34 ppt) for its maximum growth. The optimum salinity for five of the strains (Azc1, 4, 8, 11 and 12) was within 20-25 ppt. The remaining seven strains required 25-30 ppt.

In contrast to the growth of A. chroococcum strains, the salinity at which the maximum nitrogen fixation occurred differed significantly between strains (Table 17) and it varied from 28.42 to 35.88 ppt. In most of the strains there was no direct relationship between the growth and nitrogen fixation with that of the salinity level.

In A. beijerinckii strains also salinity had significant influence on the growth and nitrogen fixation (Fig. 17). In most of the strains, maximum growth occurred at the salinity level between 25 and 40 ppt. In general, salinity levels above 40 and below 5 ppt were found to inhibit the growth of most of the strains of A. beijerinckii. Unlike in A. chroococcum and A. vinelandii there was not much variation in the salinity level at which the maximum nitrogen fixation took place. In the first six strains of A. beijerinckii (Azb1 to Azb6) the maximum nitrogen fixation was noticed at 30 ppt and in the other two strains (Azb7 and Azb8) the maximum was at 35 ppt though in Azb8 there was no significant difference between 30 and 45 ppt. Nitrogen fixation was relatively less in most of the strains in salinity < 20 ppt was well as > 50 ppt with little variations.

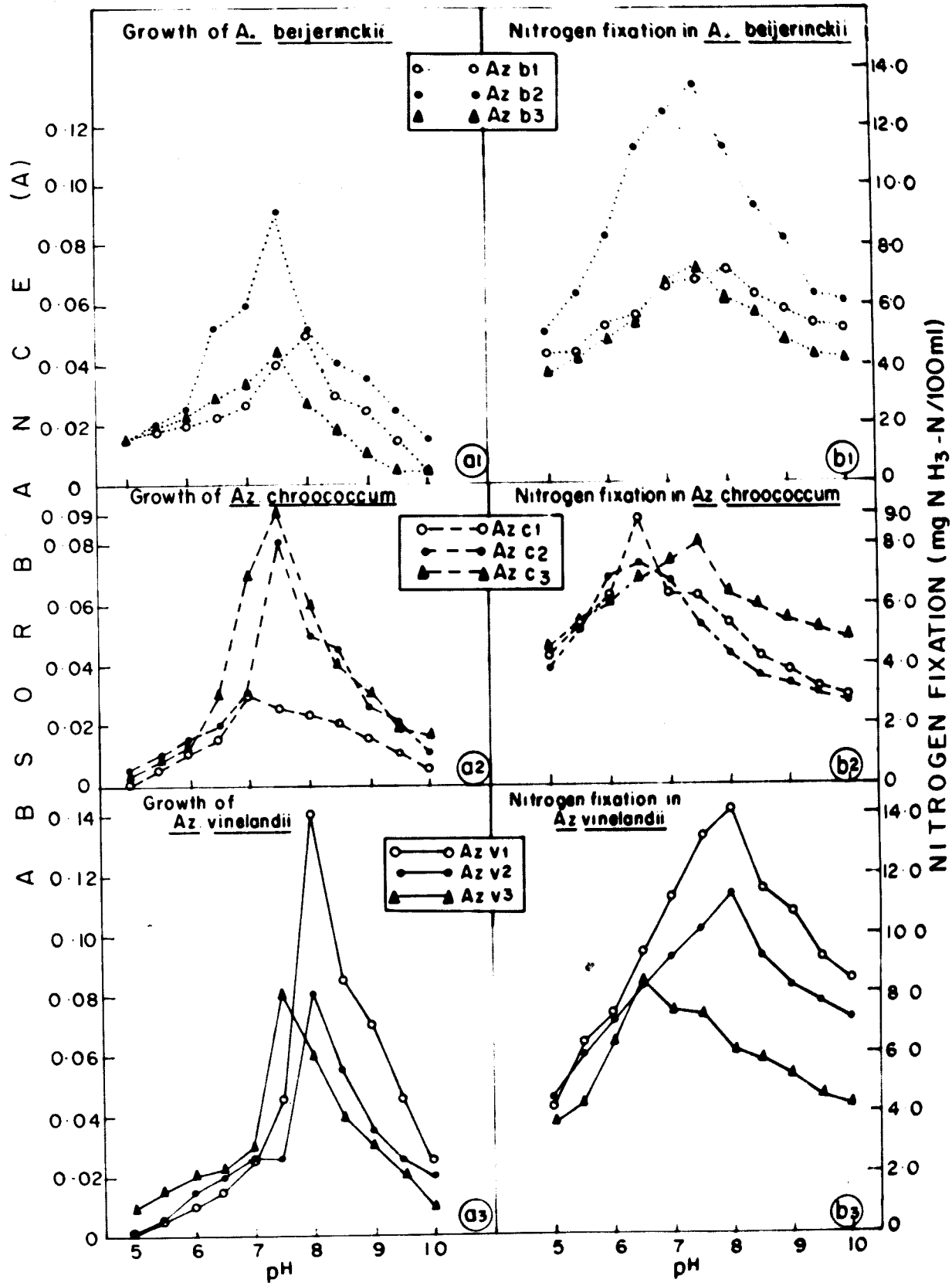
Statistically, determined optimal salinity levels for maximum growth and nitrogen fixation are presented in Table 16 and 17. Strain Nos. Azb5 and Azb7 required relatively higher salinity of 29.51 and 29.96 ppt respectively; strain Nos. Azb6 and Azb8 required more or less same salinity (28.72 in Azb6 and 28.96 ppt in Azb8) for their maximum growth. In strains Azb2, Azb3 and Azb4, the maximum growth was noticed at the optimum salinity of 26.66 ppt, 26.41 ppt and 26.63 ppt respectively. However in Azb1 the maximum growth occurred at the salinity of 24.01 ppt. The optimum salinity for maximum nitrogen fixation ranged from30.15 to 39.78 ppt.

Effect of pH on the growth and nitrogen fixation in Azotobacter

With a view to examine the influence of pH of the medium on the growth and nitrogen fixation by three species of Azotobacter an experiment was conducted, in which the nitrogen-free culture medium was adjusted to various pH levels, and the isolates were inoculated and incubated at culture room temperature (28 ± 2°C) for 15 days, after which the nitrogen content of the broth was estimated. The results are summarized in Fig. 18.

In Azotobacter vinelandii, a gradual increasing trend was observed with the increasing level of pH in strains Azv1 and Azv3 (upto pH7) which was followed by a sharp increase resulting in maximum growth at pH 7.5 in Azv3 and at pH 8.0 in Azv1. However, in isolate Azv2, there was do difference in growth between pH 7 and 7.5, though the maximum growth was noticed at pH 8.0. After the maximum, with further increase in pH a sharp decline was noticed for all the strains. Nitrogen fixation also increased with the increasing level of pH, with the maximum levels in Azv1 and Azv2 at pH 8.0 and in Azv3 at pH 6.5. Thereafter a declining trend was evident.

FIG. 18


Growth of A. beijerinckii
Nitrogen fixation in A. beijerinckii
Az b1
Az b2
Az b3
Growth of Az. chroococcum
Nitrogen fixation in Az chroococcum
Az c1
Az c2
Az c3
Growth of Az. vinelandii
Nitrogen fixation in Az vinelandii
Az v1
Az v2
Az v3
ABSORBANCE (A)
NITROGEN FIXATION (mg N H3-N/100ml)
pH
a1
b1
a2
b2
a3
b3

Tanle 16: Results of the response curve analysis for the data salinity on the growth of Azotobacter strains

Isolates	Isolates designated as	Growth constants			R^2	X max	Y max (expected)
		a	b	c			
1	Azc1	0.1151648350	0.0009028970	-0.000018821	71.1	23.98	0.020
2	Azc2	0.0043956043	0.0015305694	-0.000028311	81.67	27.03	0.036
3	Azc3	0.0011538461	0.0012472527	-0.000021318	82.2	29.25	0.012
4	Azc4	-0.0340879100	0.0025477520	-0.000058121	46.0	21.98	0.014
5	Azc5	0.0006153840	0.0019985014	-0.000028271	60.3	35.34	0.065
6	Azc6	0.0029670329	0.0015676323	-0.0000278121	46.2	28.18	0.079
7	Azc7	0.00641758241	0.0008282616	-0.0000160239	79.8	26.15	0.019
8	Azc8	0.0060549450	0.0008851148	-0.000017902	76.1	24.72	0 017
9	Azc9	0.01013186813	0.0009594405	-0.000018701	83.5	26.65	0.024
10	Azc10	0.0084615384	0.0012141850	-0.000022617	83.9	26.84	0.028
11	Azc11	0.0217472527	0.0025552440	-0.000051708	62.6	24.70	0.068
12	Azc12	0.0135494505	0.0015097900	-0.000031628	62 3	23.86	0.058
13	Azc13	0.0041208791	0.0015404590	-0.00027652	93.46	27.85	0.019
14	Azv1	0.0103076920	0.0007341658	-0.000015404	93.0	23.83	0.019
15	Azv2	0.0183626373	-0.0003965030	0.000003256	33.0	NS	-
16	Azv3	0.0084158200	0.0013147852	-0.00002647	73.6	24.83	0.030
17	Azv4	0.0066813186	0.0009880110	-0.00001958	84.7	25.23	0.028
18	Azv5	0.0055604395	0.0017029970	-0.000032027	77.6	26.58	0.030
19	Azv6	0.0010549450	0.0015871128	-0.000026873	86.7	29.58	0.030
20	Azv7	-0.0069560430	0.0042501490	-0.000068931	59 2	30.82	0 022
21	Azv8	0.0090989010	0.0012810189	-0.000024115	98.8	25.56	0.037
22	Azv9	0.0124835164	0.001204950	-0.000025614	64.3	23.52	0.027
23	Azb1	0.0120219780	0.0010794205	-0.000022477	77.91	24.01	0.028
24	Azb2	0.0025054945	0.0014939060	-0.000028011	99.6	26.66	0.020
25	Azb3	0.0055494500	0.0010049950	-0.000019020	8.15	26.41	0.021
26	Azb4	0.0084285714	0.0013345650	-0.000026033	86.6	26.63	0.032
27	Azb5	0.0024164830	0.0016147852	-0.000027352	98.22	29.51	0.029
28	Azb6	0.0035494500	0.0016128871	-0.00002807	70.59	28.72	0.029
29	Azb7	0.0008351648	0.0026627370	-0.000044635	66.7	29.82	0.025
30	Azb8	0.0012329670	0.0032242300	-0.000055660	78.0	28.96	0.023

salinity on the nitrogen fixation of Azotobacter.

Isolates	Isolates designated as	Growth constants			R^2	X Max	Y Max (expected)
		a	b	c			
1	Azc1	2.976923076	0.27214685	-0.00461398	87.79	29.49	6.41
2	Azc2	1.94626337	0.27325674	-0.003904095	87.29	34.99	7.24
3	Azc3	2.02483516	0.260128871	-0.00394665	91.83	32.95	7.32
4	Azc4	3.18373626	0.22898101	-0.003718081	90.52	30.51	7.43
5	Azc5	2.114725274	0.216346653	-0.003177022	95.3	34.04	7.21
6	Azc6	3.32208791	0.222156843	-0.003811588	87.17	29.14	7.68
7	Azc7	2.69076923	0.1703476523	-0.0026101898	92.17	32.63	6.92
8	Azc8	3.301678351	0.168346653	-0.002961638	86.78	28.42	5.93
9	Azc9	2.7802197802	0.132221778	-0.002009190	76.78	32.9	6.63
10	Azc10	2.52043956	0.25541358	-0.003953046	90.39	32.3	6.23
11	Azc11	3.25637362	0.190219780	-0.003134065	66.9	30.34	6.23
12	Azc12	2.8112087912	0.150252747	-0.00238241	77.7	31.53	6.23
13	Azc13	2.62131868	0.143577422	-0.00205614	91.8	35.88	6.68
14	Azv1	2.37868131	0.31262037	-0.00501198	93.77	31.11	7.62
15	Azv2	3.33021978	0.20053446	-0.003689	81.91	27.17	6.67
16	Azv3	3.244615384	0.227101898	-0.0038775	87.58	29.28	6.21
17	Azv4	3.011318681	0.20342157	-0.00311948	79.49	32.6	5.73
18	Azv5	2.32318681	0.174782217	-0.00241578	87.61	36.17	5.98
19	Azv6	1.927472527	0.242105894	-0.00304975	89.2	39.69	5.28
20	Azv7	2.369560439	0.200859140	-0.003055344	78.5	31.53	6.22
21	Azv8	2.73571428	0.16809990	-0.002754045	72.5	30.51	6.51
22	Azv9	3.181098901	0.21903496	-0.002681718	92.5	40.83	5.29
23	Azb1	2.70241758	0.2718291708	-0.00439660	92.42	30.91	7.12
24	Azb2	2.571318681	0.211655344	-0.00350909	89.79	30.15	7.23
25	Azb3	2.16516483	0.193838161	-0.00285994	70.55	33.88	7.58
26	Azb4	3.011648351	0.255270729	-0.0039920	83.0	30.91	7.75
27	Azb5	0.578021978	0.29886913	-0.004477122	96.2	33.37	7.25
28	Azb6	2.22406593	0.250604395	-0.00314945	88.4	39.78	5.21
29	Azb7	2.06362637	0.20033766	-0.00290269	76.3	34.50	7.37
30	Azb8	2.50087912	0.145263736	-0.002182417	91.8	31.28	8.27

Statistically, (Table 18 and 19) the maximum growth was observed at the optimum pH of 7.82 in Azv1, pH 7.77 in Azv2, and at pH 7.75 in Azv3; and the maximum nitrogen fixation was recorded at the optimum pH 6.81 in Azv1, 6.64 in Azv2 and 7.40 in Azv3.

Among the three strains of A. chroococcum, strain Azc1 showed relatively poor growth at all the tested pH levels. In this strain though maximum growth occurred at pH 7, there was not much variation in growth up to pH 8.5. Conversely, this strain fixed maximum nitrogen at 6.5 pH, which was higher than the maximum recorded in the other two strains. Strains Azc2 and c3 grew to the maximum level in pH 7.5. Interestingly in Azc2 nitrogen fixation was found to be maximum at 6.5 pH. The third strain Azc3 showed maximum nitrogen fixation at 7.5 pH.

Statistical analysis of the data (Table 18 and 19) showed that maximum growth occurs in pH 7.64 in Azc1, pH 8.28 in Azc2 and 7.59 in Azc3, whereas the maximum nitrogen was fixed at pH 7.91 in Azc1, 7.85 in Azc2 and 7.79 in Azc3.

In the case of A . beijerinkii, growth and nitrogen fixation were markedly higher in strain Azb2 than the other two strains, in most of the pH levels tested, with the maximum growth and nitrogen fixation at pH 7.5. Besides, the growth and nitrogen fixation in Azb2 at pH 6.5 exceeded the maximum recorded in the other two strains (Azb1 and Azb3). In the latter strains, both the growth and nitrogen fixation were found to be relatively higher at pH 7.5 (Azb3) and 8(Azb1), than other pH levels.

Statistically determined optimum pH values (Table 18 and 19) for maximum growth were 7.52 for Azb1, 7.10 for Azb2 and 7.67 for Azb3 and for

Table 18: Results of the response curve analysis for the data on the influence of pH on the growth of nine Azotobacter strains

Strains	Growth constants			R^2	X max	Y max (expected)
	a	b	c			
Azc1	-2.789	0.7759	-0.0508	87.18	7.64	0.173
Azc2	-0.289	0.0812	-0.0049	90.0	8.28	0.048
Azc3	-0.214	0.0668	-0.0044	87.03	7.59	0.040
Azv1	-0.1863	0.0547	-0.0035	69.68	7.81	0.028
Azv2	-0.3788	0.0689	-0.0070	64.10	7.77	0.049
Azv3	-0.3665	0.0585	-0.007	65.48	7.75	0.039
Azb1	-0.4099	0.0649	-0.0083	83.48	7.52	0.042
Azb2	-0.1795	0.0597	-0.0042	80.5	7.10	0.039
Azb3	-0.4921	0.1542	-0.0094	65.23	7.67	0.040

Table 19: Results of the response curve analysis for the data on the influence of pH on the nitrogen fixation of nine strains of Azotobacter

Strains	Growth constants			R^2	X max	Y max (expected)
	a	b	c			
Azc1	-47.9421	15.2221	-0.9621	51.3	7.91	12.26
Azc2	-33.4962	11.0505	-0.7030	57.28	7.85	9.93
Azc3	-15.3313	5.6499	-0.3622	68.96	7.79	6.70
Azv1	-14.0910	6.0273	-0.4419	67.23	6.81	6.46
Azv2	- 9.5448	4.6535	-0.3502	61.0	6.64	5.91
Azv3	-23.2428	8.2129	-0.5548	58.97	7.40	7.15
Azb1	-51.4570	16.8568	-1.1249	70.49	7.49	11.69
Azb2	-18.6899	6.6738	-0.4436	79.05	7.52	6.41
Azb3	- 1.2718	2.1544	-0.1511	68.18	7.12	6.40

e maximum nitrogen fixation pH 7.49 in Azb1, pH 7.52 in Azb2 and at
7.12 in Azb3.

ffect of Trace elements on the growth of Azotobacter

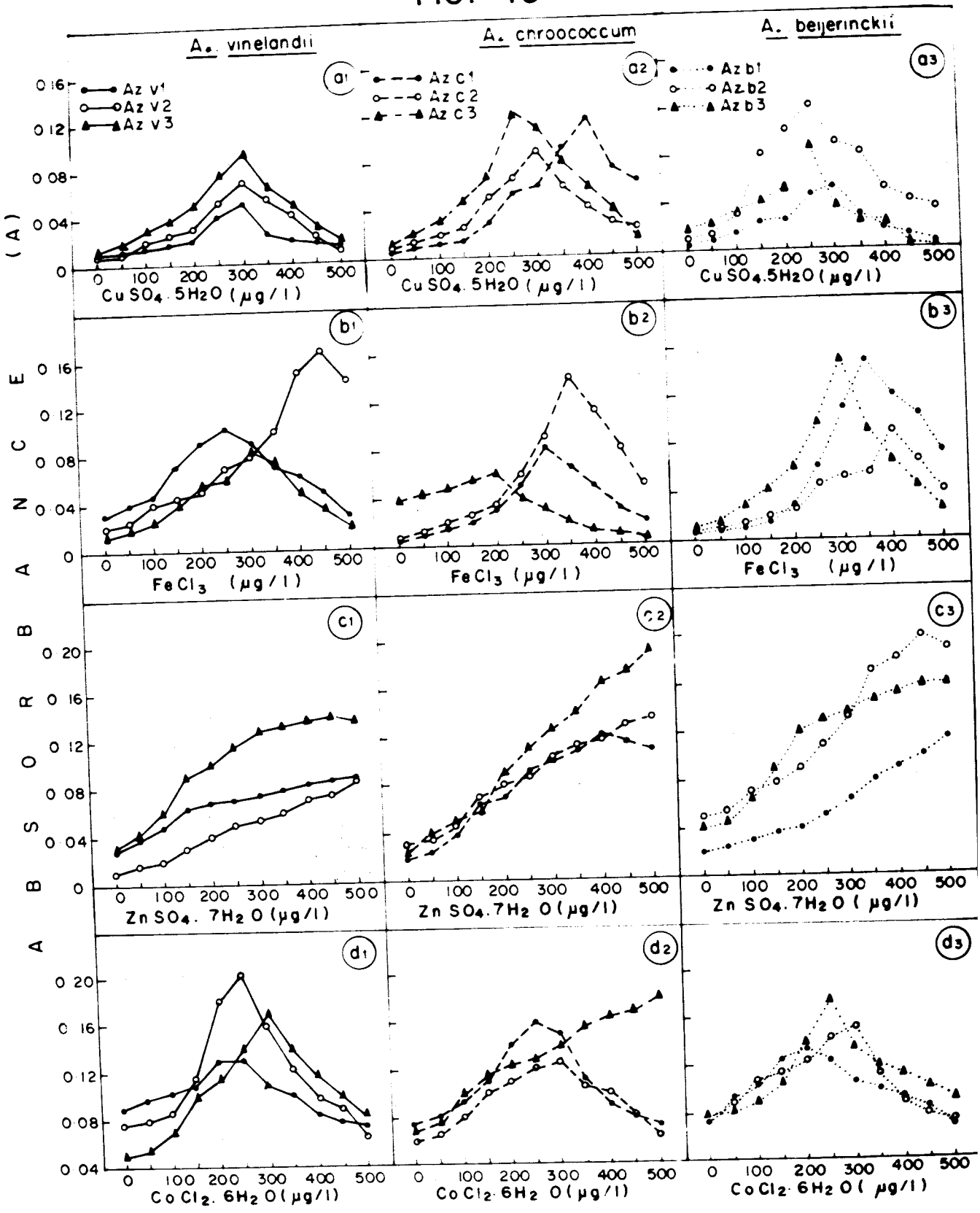
obalt:

Cobalt showed a pronounced effect on the growth of all the Azotobacter rains (Fig.19). Of the nine Azotobacter strains,only one strain of A. chroococcum Azc3) showed almost a linear increase in growth with the increasing concentration cobalt chloride upto the highest test level of 500 µg/l. In all the other eight rains, distinctly, an increasing trend was evident, resulting in maximum growth optimal cobalt chloride levels, followed by a decreasing trend showing lower rowth response at cobalt chloride levels in excess of 350 µg/l. The statistically Table 23) determined optimal level of cobalt chloride promoting maximum growth A. vinelandii strains were 251.65 µg/l for Azv1, 266.63 µg/l for Azv2, 298.88 g/l for Azv3 and for A. chroococcum 219.3 µg/l (Azc1), 265 µg/l (Azc2), and 63.63 µg/l (Azc3). In the case of A. beijerinckii the optimal levels were 241.87 g/l for Azb1 271.4 µg/l in Azb2 and 391.42 µg/l in Azb3.

Zinc:

Zinc had a significant effect on the growth of all the nine Azotobacter trains (Fig. 19). In most of the strains, within the zinc levels tested (50-500 µg/l) growth inhibition was not observed. The deviations noticed were in Azv3. Azc1 and Azb3, wherein growth inhibition resulted at zinc chloride concentrations exceeding 450 µg/l (Azv3 and Azb3) and 400 µg/l (Azc1) respectively. A gradual, but steady increase was observed in all other strains, exhibiting the highest growth at the maximum zinc chloride in the medium. The estimated optimum levels of zinc chloride providing maximum growth in the various strains are shown in Table 22.

FIG. 19


A. vinelandii
A. chroococcum
A. beijerinckii
a1
a2
a3
b1
b2
b3
c1
c2
c3
d1
d2
d3
Az v1
Az v2
Az v3
Az c1
Az c2
Az c3
Az b1
Az b2
Az b3
ABSORBANCE (A)
CuSO4.5H2O (μg/l)
FeCl3 (μg/l)
ZnSO4.7H2O (μg/l)
CoCl2.6H2O (μg/l)
0 100 200 300 400 500
0 0.04 0.08 0.12 0.16 0.20

The optimal levels inducing maximum growth was at 408·16 µg/l in Azv1, 463.43 µg/l in Azv2 and 269.89 µg/l in Azv3. In A. chroococcum the optimum levels were 459·18 µg/l for Azc1 and 269·68 µg/l for Azc2 In Azc3 a linear increase in growth with the zinc chloride concentration of the medium occurred thereby even the maximum zinc concentration used in the study was insufficient to meet the requirement. The optimum concentrations for A. beijerinkii strains were 132·32 µg/l for Azb1 435.08 µg/l for Azb2 but in Azb3 there was linear increase in growth, with the increase in zinc concentration of the media.

Iron:

The concentration of Iron (50 to 500 µg/l) also significantly influenced (Figure 19) the growth of all the strains and the maximum growth occurred at optimal levels. The concentrations in which maximum growth occurred were 250 µg/l for Azv1, at 300 µg/l in Azv3 and 450 µg/l in Azv2. While iron concentration in A. vinelandii above these levels decreased the growth, those below these resulted in reduced growth. Among the three A. vinelandii strains, Azv2 was found to require markedly higher (450 µg/l) iron concentration in the medium for proper growth. In A. chroococcum one of the strains (Azc3) showed maximum growth at relatively low iron (200 µg/l) concentration in the medium, when compared to other strains, which grew to the maximum at 300 µg/l (Azc1) and 350 µg/l (Azc2) respectively. When iron was excluded from the medium relatively good growth occurred in Azc3. In A. beijerinkii the iron concentration the medium which provided maximum growth was 300 µg/l for Azb3, 350 µg/l for Azb1 and 400 µg/l for Azb2.

Results of the statistical analysis of the data furnishing optimum iron levels in the medium for maximum growth are presented in table 21. The

Table 20: Results of the response curve analysis for the data on the influence of copper on the growth of Azotobacter strains

Strains	growth constants			R^2	X max	Y max (expected)
	a	b	c			
Azc1	0.0028676	0.0002055	0.00000037	87.3	277.70	0.45
Azc2	0.006682	0.00008763	-0.0000000174	85.5	251.81	0.0216
Azc3	-0.0016961	0.00015194	-0.000000217	73.0	350 09	0.036
Azv1	-0.0002053	0.0001460	-0.000000193	86.7	378.26	0.04
Azv2	0.0263975	0.00017263	0.000000339	85.6	NS	-
Azv3	0.00278129	0.00007176	-0.00000011	83.0	326.18	0.018
Azb1	-0.00491911	0.0007358	-0 00000137	80 3	268 90	0.012
Azb2	0.0087593	0 00008266	-0.00000016	89.2	258.31	0.026
Azb3	0 0027736	0.00007992	-0.000000147	78 6	271 83	0.017

NS - not significant

Table 21: Results of the response curve analysis for the data on the influence of Iron on the growth of Azotobacter strains

Strains	Growth constants			R^2	X max	Y max (expected)
	a	b	c			
Azc1	0.020122	0.0005149	-0.00000099	87.3	260.05	0.08
Azc2	0.033970	-0.0001220	0.00000112	91.5	NS	-
Azc3	0.011589	-0.0000423	0.00000051	68.13	NS	-
Azv1	0.001864	0.0000939	-0.00000015	77 5	313.00	0.03
Azv2	0.003145	0.0000766	-0.000000091	95.2	420.87	0.025
Azv3	0.002284	0.0001023	-0.000000130	77,2	393.46	0.023
Azb1	-0 09217	0.00126499	-0.000002124	73 5	297.78	0 08
Azb2	0.006449	-0.00003816	-0.000000488	99.5	NS	-
Azb3	0.094442	-0.0007128	0.00000130	64 90	NS	-

NS - not significant

Table 22: Results of the response curve analysis for the data on the influence of zinc on the growth of Azotobacter strains

Strain	Growth constants			R^2	X max	Y max (expected)
	a	b	c			
Azc1	0 0309681	0 0002239	-0.0000002438	98 1	459 18	0.09
Azc2	0.0065737	0.00015507	0.0000000116	98.,8	NS	-
Azc3	-0.1744746	0.0026585	-0.000004928	99.4	269 68	0.186
Azv1	0.0109584	0 000400	-0 00000049	97 4	408 16	0 174
Azv2	0.0251366	0.0002966	-0 000000320	98 5	463 43	0.098
Azv3	-0.1892298	0.0032981	-0.00000611	96.3	269 89	0.44
Azb1	0.048666	0.000131	0.000000495	99.2	132 32	0.36
Azb2	0.02955122	0.000496	-0.00000057	97.8	435 08	0.09
Azb3	0.026715039	0.0002871	0.000000100	98.8	NS	-

NS - not significant

Table 23: Results of the response curve analysis for the data on the influence of cobalt on the growth of Azotobacter strains

Strains	Growth constants			R^2	X max	Y max (expected)
	a	b	c			
Azc1	0.08879	0.0002851	-0.00000065	81.90	219.30	0.18
Azc2	0.040117	0.0001061	-0.0000020	64 73	265 0	0.18
Azc3	0.006315	0.0012127	-0.0000023	66.85	263.63	0.116
Azv1	0.060439	0.0005939	-0.00000118	75.12	261.65	0.140
Azv2	0.048682	0.0005066	-0.00000095	92.45	266.63	0.148
Azv3	0.028090	0.0007532	-0.00000126	85.55	298.88	0.146
Azb1	0.071744	0.0005031	-0.00000104	78.0	241 87	0.134
Azb2	0.066638	0.0005211	-0.00000096	63.30	271.40	0.141
Azb3	0.0731092	0.00004328	-0.00000055	95.70	391.45	0.165

calculated optimum iron concentrations producing maximum growth were 313.0 µg/l in Azv1, 420.87 µg/l in Azv2 and 293.43 µg/l in Azv3. In A. chroococcum, it was noticed at 260.05 µg/l for Azc1. No significant response on the growth was noticed for Azc2 and Azc3. Whereas in A. beijerinckii the maximum growth was noticed at 297.78 µg/l in Azb1, but in Azb2 and Azb3 there was no significant growth maximum.

Copper:

The influence of copper on the growth of Azotobacter is illustrated in fig. 19. In all the strains, a gradual increase in growth was observed with the increasing level of copper in the medium showing maximum at 300 µg/l in all the three strains of A. vinelandii. The maximum growth in A. chroococcum occurred at 250 µg/l, 300 µg/l and 400 µg/l in Azc3, c2 and c1 respectively In the case of A. beijerinkii two strains (Azb2 and Azb3) showed maximum growth at 250 µg/l, while the third strain showed maximum at 300 µg/l. There was little difference between the growth at 300 and 250 µg/l. Copper concentrations greater than the said levels induced growth reduction in the strains, with only two strains Azc1 and Azb2 showing better tolerance to the higher copper concentrations in the media.

The optimum concentration of copper for maximum growth (Table 20) was at 378.26 µg/l for Azv1 and 326.18 µg/l for Azv2; but no significant response was observed in Azv2. Whereas the optimum concentration producing the maximum growth was at 277.70 µg/l for Azc1; 251.81 µg/l for Azc2 and 350.05 µg/l for Azc3. In A. beijerinckii the optimum copper levels were 268.90 µg/l for Azb1, 258.31 µg/l for Azb2 and 271.83 µg/l for Azb3.

Effect of vitamins on the growth of Azotobacter

Ascorbic acid:

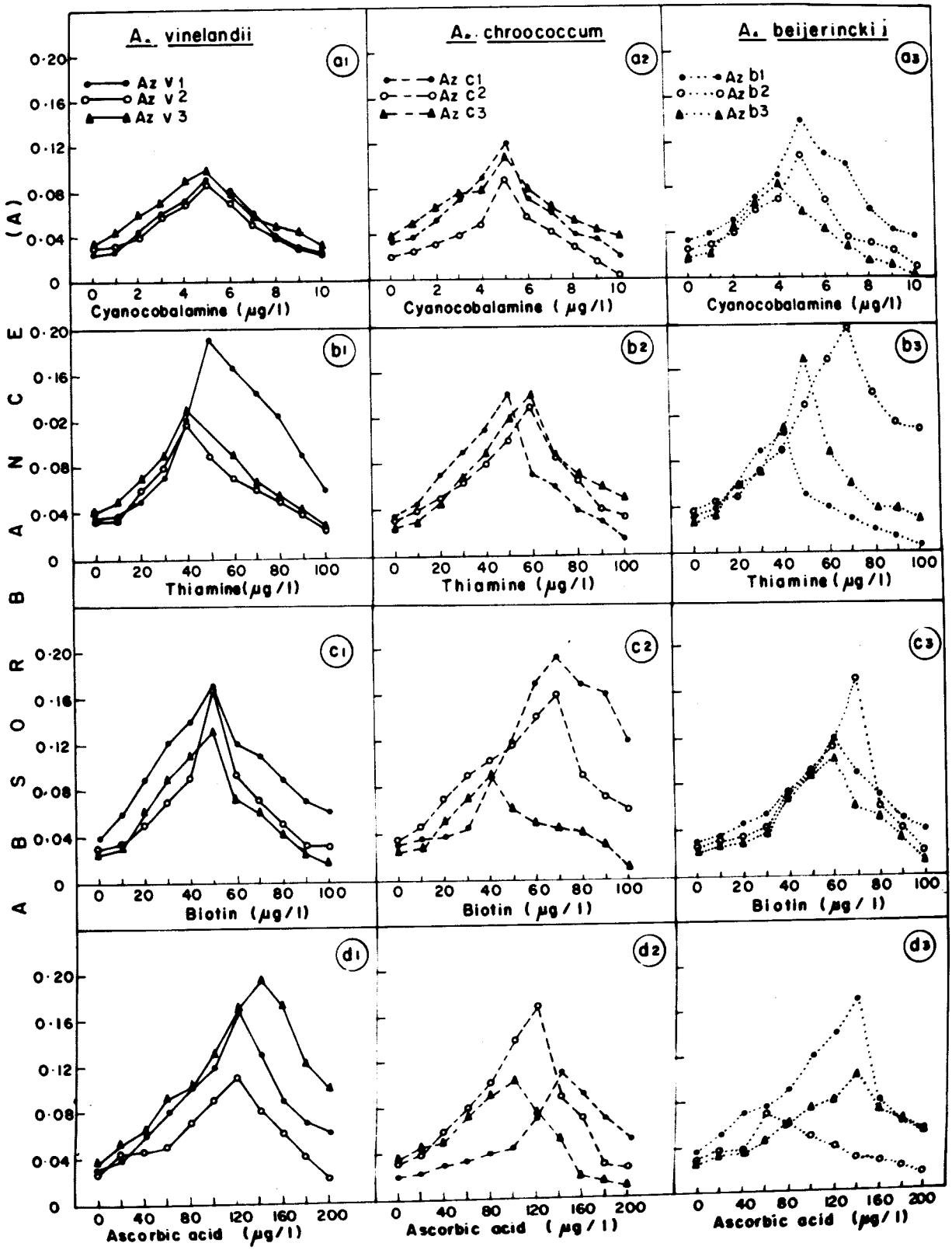
The influence of ascorbic acid concentration in the growth of Azotobacter strains is shown in fig. 20. All the nine strains of Azotobacter were found to require ascorbic acid for their growth; but considerable differences were observed between the strains in their ascorbic acid requirement to induce maximum growth. Strains of A vinelandii required 120-140 µg/l ascorbic acid; strains of A. chroococcum 100-140 µg/l, and in the case of A. beijerinkii two strains required 140 µg/l, whereas the third one (Azb2) required relatively low (60 µg/l) level of ascorbic acid. Ascorbic acid concentrations above the recorded levels resulted in reduced growth.

The calculated optimum concentrations for maximum growth are given in table 27. The optimum level inducing maximum growth was 125.63 µg/l in Azv1, 100.95 µg/l in Azv2 and 172.36 µg/l in Azv3; 110.0 µg/l in Azc1, 102.82 µg/l in Azc2 and 107.95 µg/l in Azc3; whereas, optimum for A.beijerinckii was 78.25 µg/l in Azb1, 130.94 µg/l in Azb2 and 87.69 µg/l in Azb3.

Biotin:

Fig. 20 shows the effect of graded levels of biotin on the growth of Azotobacter strains All the strains required biotin for their growth at adequate levels The biotin levels which gave the highest growth were 50 µg/l for the strains of A. vinelandii, 40 µg/l for Azc3 and 70 µg/l for Azc1 and c2; whereas in A. beijerinckii, two strains showed maximum growth at 60 µg/l (Azb1 and Azb3) but in Azb2 it was noticed at 70 µg/l. Thus, in general, strains of A. vinelandii grew well at relatively lower biotin levels, though strain Azc3 showed the lowest requirement.

FIG. 20


A. vinelandii
A. chroococcum
A. beijerinckii
Az v1
Az v2
Az v3
Az c1
Az c2
Az c3
Az b1
Az b2
Az b3
a1
a2
a3
b1
b2
b3
c1
c2
c3
d1
d2
d3
ABSORBANCE (A)
Cyanocobalamine (μg/l)
Thiamine (μg/l)
Biotin (μg/l)
Ascorbic acid (μg/l)

In table 26 the estimated optimum biotin levels for maximum growth of the strains are given. The optimum levels found were 18.0 µg/l in Azv3 but no significant growth was noticed in Azv1, whereas in A. chroococcum, the maximum growth was observed at 13.14 µg/l, for Azc1; 13.80 µg/l for Azc2; but in Azc3 no significant growth was noticed. In A. beijerinckii, the maximum growth was found to be at 21.91 µg/l in Azb2, 11.42 µg/l in Azb3; but in Azb1 no significant growth occurred

Thiamine:

Thiamin was also found to be essential for all the nine Azotobacter strains (Fig. 20) However, there was considerable differences of thiamine requirement in both the isolates of Azv2 and Azv3 in which the maximum growth was noticed at 40 µg/l but in Azv1 a gradual decline was followed by a sharp increasing trend with a peak at 50 µg/l. In A. chroococcum, a gradual increasing trend was observed in all the 3 isolates with the maximum growth at 50 µg/l for Azc1, and, at 60 µg/l for Azc2 and Azc3; but a sharp decline was noticed with the increasing level of concentration. In A. beijerinckii, the maximum growth was recorded at 40 µg/l in Azb1, 50 µg/l in Azb3 and at 70 µg/l in Azb2 and thereafter a decline ~~trend~~ was noticed in each of the strains

Statistically, the maximum growth was observed (Table 25) in Azv2 at 26.26 µg/l, in Azv2 at 15.49 µg/l and in Azv3 at 17.55 µg/l. In A. vinelandii and in A. chroococcum it was observed at 22.4 µg/l in Azc2, 43.39 µg/l in Azc3, but in Azc1 no significant growth was noticed Whereas in A beijerinckii the maximum growth was noticed at 14.38 µg/l in Azb2 but no significant growth was noticed in Azb1 and Azb3

Table 24: Results of the response curve analysis for the data on the influence of cyanocobalamine on the growth of Azotobacter strains

Strain	Growth constants			R^2	X max	Y max (expected)
	a	b	c			
Azc1	0.015041958	0.02289627	-0.00226689	81.70	5.57	0.08
Azc2	0.021454545	0.0193666	-0.001984848	76.8	4.87	0.07
Azc3	0.0144055944	0.01289627	-0.001357808	77.6	4.74	0.09
Azv1	0.020937062	0.026060139	-0.002713286	72.9	4.80	0.06
Azv2	0.01755944	0.00885734	-0.00108391	72.5	4.08	0.05
Azv3	0.032272727	0.02013636	-0.00204545	79.1	4.92	0.07
Azb1	0.02520979	0.012811655	-0.0015011655	77.8	4.26	0.04
Azb2	0.020034965	0.018922144	-0.00217948	69.9	4.34	0.027
Azb3	0.040055944	0.06211188	-0.00169930	87.43	4.76	0.07

Table 25: Results of the response curve analysis for the data on the influence of thiamine on the growth of Azotobacter strains

Strains	Growth constants			R^2	X max	Y max (expected)
	a	b	c			
Azc1	0.06830371	0.000958739	0.000002944	53.6	NS	-
Azc2	0.08228461	-0.000156096	-0.000003488	75.7	22.50	0.08
Azc3	0.065813318	-0.003391071	-0.000003907	68.2	43.3	0.115
Azv1	0.095265681	-0.000267296	-0.000005088	74.4	26.26	0.09
Azv2	0.0583119849	0.000182252	-0.00000588	71.54	15.49	0.105
Azv3	0.101741591	-0.000163437	-0.000004656	61.7	17.55	0.049
Azb1	-0.032328963	0.0020505701	0.0000181248	82.2	NS	-
Azb2	0.087985619	0.000121260	-0.000004237	65.4	14.38	0.06
Azb3	0.052268861	0.0003480174	0.000000743	77.2	NS	-

NS - not significant

Table 26: Results of the response curve analysis for the data on the influence of biotin on the growth of Azotobacter strains

Strain	Growth constants			R^2	X max	Y max (expected)
	a	b	c			
Azc1	0.101800333	0.000071152	-0.000002569	87.2	13.14	0 09
Azc2	0.075417315	-0 00008457	-0.000003063	63,6	13.80	0 087
Azc3	0.04774878	0.000269617	0.000000583	73.2	NS	-
Azv1	-0.02063936	0.001804847	0.00001460	65.0	NS	-
Azv2	0.08662337	-0.00009201	-0.000003344	84.9	13.75	0.105
Azv3	0.081469460	-0.00014585	-0.000004050	74.5	18.0	0.069
Azb1	0.040493656	0.000372706	0.0000008202	50.7	NS	-
Azb2	0.225404608	-0.000678045	-0.000015468	18.6	21.91	0.089
Azb3	0.043330573	0.0000874655	-0.000003829	67.0	11.42	0.05

NS - not significance

Table 27: Results of the response curve analysis for the data on the influence of ascorbic acid on the growth of Azotobacter strains

Strains	Growth constants			R^2	X max	Y max (expected)
	a	b	c			
Azc1	0.00678321	0.00213601	-0.0000097	98.27	110.0	0.13
Azc2	0.016769230	0.00129769	-0.0000063	75.33	102.82	0.12
Azc3	0.00728321	0.00216783	-0.00001004	61.87	107.95	0.13
Azv1	0.010653846	0.000551538	-0.000002194	69.23	125.63	0.165
Azv2	0.010524475	0.002162062	-0.000010708	73.53	100.95	0.006
Azv3	0.00603496	0.00213944	-0.000006206	70.23	172.36	0.09
Azb1	0.0325734	0.00498310	-0.000003184	75.67	78.25	0.07
Azb2	0.0174755	0.000925512	-0.000003534	72.77	130.94	0.115
Azb3	0.02944056	0.001145617	-0.000006532	76.17	87.69	0.105

Cyanocobalamine:

The influence of graded levels of cyanocobalamine on the growth of Azotobacter strains is illustrated in Fig 20. All the strains required cyanocobalamine in the medium for their normal growth Except for the A. beijerinkii strain Azb3, all other strains showed maximum growth at 5 µg/l In Azb3 maximum growth occurred at 4 µg/l. Above and below these levels growth was relatively poor.

The calculated optimum concentrations of cyanocobalamine for maximum growth (Table 24) were 4.80 µg/l for Azv1 and Azv2; 4.92 µg/l for Azv3; 5.57 µg/l for Azc1; 4.87 µg/l for Azc2 and 4.74 µg/l for Azc3. In A. beijerinckii the maximum growth was noticed at 4.26 µg/l for Azb1,, 4.34 µg/l for Azb2 and 4.76 µg/l for Azb3.

DISCUSSION

Characteristics of the isolated Azotobacter strains:

Morphological, cultural and physiological characteristics studied in respect of 30 isolates revealed that the isolates belong to the following three species: Azotobacter chroococcum, A. vinelandii and A. beijerinckii. Although the strains had shown considerable variability in some of the important physiological attributes, they possessed common morphological and biochemical similarities. All the strains were gram negative confirming the observation of Peterson (1961) who observed that species of Azotobacter including A. chroococcum, A. vinelandii, and A beijerinckii were gram negative during all stages of their development. They were large sized and heavily capsulated.

Different strains had shown marked variation in their size. In shape also these strains varied, being round, oval short rods and elongated ones. According to Jensen (1955), during exponential growth phase A. chroococcum formed short rods with cell dimensions of 2.0 - 2.4 x 3.7 - 5.0. Strain variation in A. chroococcum had been studied in great detail by Zinoveva (1962) and James (1970) who had described different forms of the organisms. Bachinskaya (1935) had reported changes from rod to coccal forms of the cells of the A. chroococcum during 15 to 48 hours of incubation. The degree of motility, among different strains of A. chroococcum examined, was found to vary, although all of them were motile forms. A. vinelandii strains also showed motility, but A. beijerinckii strains were non-motile. Non-motile forms of A. chroococcum were earlier observed by Sen (1955) and Lohnis and Smith (1916).

Observations of pigment formation as recorded in Table 11 indicate that all the 13 strains of A. chroococcum are chromogenic, forming pigment

ranging from brown to brownish black. A. chroococcum is characterized by the formation of an insoluble black-brown pigment (melanin like) which arises on ageing cultures as a result of oxidation of tyrosin by tyrosinase, a copper containing enzyme (Beijerinck, 1901 and Zinoveva, 1962). A. vinelandii forms a water soluble greenish yellow pigment, whereas A. beijerinckii forms yellow colour water insoluble pigment. The existence of non-pigment forming strains was mentioned by Sen (1955) and Blinkov (1962). According to Mulder (1939) ongoing culture did not form the Black-brown pigment but kept their white colour. Prazmowskii (1913) reported that dead cells of Azotobacter were capable of producing brown pigment.

All the 13 strains of A. chroococcum were able to utilize starch and mannitol as their carbon source but were unable to utilize rhamnose. Nine strains of A. vinelandii utilized mannitol and rhamnose but not starch, but all the 8 strains of A. beijerinckii were unable to utilize any of these 3 carbon sources. It must be emphasized that the individual species and even strains of the same species of Azotobacter differed in respect of their source of carbon nutrition as observed by Jensen (1951) and Shethna and Bhat (1962). According to them the strains were able to assimilate lactose, mannitol, benzoic acid and other sugars.

Jensen (1961) while characterizing different strains of Azotobacter came out with an excellent enrichment technique, using rhamnose for isolating A. vinelandii. On the contrary A. beijerinckii and A. chroococcum could not grow in it at all. The results obtained during the present investigation are in agreement with the findings of most of the above workers. All the strains were able to utilize sucrose but not rhamnose, and acid and gas formation was noticed in most of the strains of Azotobacter. In respect of other biochemical properties tested a little variation was observed in the present

investigation while characterising the Azotobacter isolates as indicated in Table 14. All the biochemical tests conducted for the 30 strains confirmed their identity as A. chroococcum, A. vinelandii and A. beijerinckii as per specification given in Bergey's Manual of Determinative Bacteriology (Anon,1974).

Effect of incubation time on nitrogen fixation:

Substantial differences were observed in the efficacy of the thirty Azotobacter strains to fix nitrogen. Among the 3 species, strains of A. beijerinckii were the most efficient with relatively greater nitrogen fixation rates. Most of the A. vinelandii strains were found to fix relatively less nitrogen than the strains of A. chroococcum and A. beijerinckii. This may be due to the inherent variations between the species. It is also probable that the utilization efficiency of the substrates provided may differ between species. Strain differences in nitrogen fixation has also been earlier reported by Batra and Rao (1980) in Azotobacter like isolates from the rhizosphere of grasses growing in sodic soils which fix different amounts of nitrogen with a equal amount of carbon consumed in Jensen's medium.

The period of incubation had a significant influence on the quantity of nitrogen fixed as demonstrated by Lakshmanaperumalsamy et al. (1975). Although no significant variations were evident during the first 15 days of incubation, distinct differences were noticed in the subsequent periods. Comparing the rate of nitrogen fixed at different intervals, strains of A. beijerinckii are found to be the most vigorous strains than A. chroococcum and A. vinelandii. These results indicate that in nature various strains of Azotobacter may fix nitrogen at different levels with reference to time. Certain strains of A. vinelandii showed a decline in nitrogen fixation after

30 days of incubation. It is probable that the experimental conditions provided to them may be inadequate to sustain further growth and nitrogen fixation after 30 days.

Consistent increase in nitrogen fixation was noticed from the incubation period of 15 days to 45 days for the strains of A. chroococcum, except for only one strain (Azc9), in which nitrogen fixation was relatively low after 15 days of incubation. The poor response observed in the latter strain may be due to the rapid utilization of the energy source in the medium, thereby the energy nutrients gets exhausted.

In the case of A. beijerinckii strains, as the period of incubation increased nitrogen fixation activity also showed a linear increase upto the 30th day and a non-linear increase after that period. These results suggest that the nutrients in the media are utilized at a faster rate as the growth seems to be faster during the first 30 days. Due to the reduction in the nutrients level in the medium further incubation leads to relatively less efficiency.

Effect of salinity on growth and nitrogen fixation:

Most of the A. vinelandii strains preferred a salinity range of 15 to 35 ppt for their optimal growth, though they were obtained from ponds with salinities ranging from 1.28 to 36.40 ppt. This indicates that the genera existed in the brackishwater were compared of various strains originated from the estuarine and marine environments and perhaps include halo-tolerant freshwater forms. Rheinheimer (1980) stated that brackishwater carry not only the genera of marine bacteria and salt-tolerant freshwater forms, but also halophilic bacteria whose specific habitat is brackishwater. In all the strains of A. vinelandii a steady increase in growth and nitrogen fixation was noticed

with the increase in salinity showing maximum growth and nitrogen fixation at the optimum salinity levels (23.52 to 30.42 ppt for growth and 27.17 to 40.83 ppt for nitrogen fixation). In certain strains, while the optimum growth occurred at one salinity level; its maximum nitrogen fixation was at another salinity level. These results suggest that growth efficiency and nitrogen fixation efficiency of the Azotobacter need not have a direct relationship and that the strains require specific salinity requirements for growth, which is independent of the need for nitrogen fixation. None of the strains could attain maximum growth at salinities of 5 ppt and 60 ppt. Certain strains isolated from sediments with a salinity from 1.28 to 36.40 ppt were resistant to high salt contents (more than 35 ppt).

In most of the A. chroococcum strains the optimum growth and nitrogen fixation occurred within the salinity range 23.98 to 27.85 ppt; a few of the strains preferred a salinity range 29.49 to 35.88 ppt for their optimum growth and nitrogen fixation. Due to the influence of tides, some marine forms enter the ponds through the Cochin backwaters and adapt into it. On the other hand, James and Shende (1972) reported that in the case of A. chroococcum strains from the rhizosphere of agricultural crops the growth of the Azotobacter culture can vary at low salt concentrations, but at high salt concentrations it gradually falls. The differences observed above are mainly due to the environmental situations from which the Azotobacter strains were collected. In general, the salinity levels above 45 ppt and below 5 ppt does not seem to be conducive for the propagation of A. chroococcum and A. beijerinckii strains.

While salinity levels above 40 ppt seldom occurred in the prawn culture systems from where the bacteria were isolated, salinity levels below 5 ppt. are usually found during the monsoon season. In the prawn culture systems

seawater from the Arabian Sea gradually blends with the freshwater from rivers or rain and become brackish, and such a change in the water quality would be deleterious to the growth and propagation of bacterial forms which prefer narrow salinities. This may partially explain the reduction in the occurrence of bacterial population in the sediments and water in the ponds during the monsoon as discussed in Part I. Morita et al (1973) concluded that marine bacteria are always vulnerable to the salinity and temperature changes that may significantly affect their normal functions. So it is clear that the marine populations of Azotobacter may prefer steady saline conditions and drastic changes may reduce their population. Blinkov (1955) is of the opinion that the presence of the salt alone is not enough for the formation of halophilic forms, and that soil salt composition and strain property are of great importance for the formation of halophilic strains.

Besides, in most of the species, there was a considerable difference in the salinity level at which maximum growth and nitrogen fixation occurred suggesting that aerobic nitrogen fixing bacteria tend to have different fixation potential under varying salinity regimes as discussed by Lakshmanaperumalsamy et al. (1975). Iswaran and Sen (1958) reported that an increasing concentration of salt had a depressing effect on nitrogen fixation by Azotobacter but in the present study depressing effect is exhibited after reaching the level of salinity at which the optimum growth and nitrogen fixation occurred.

The temperature optimal and temperature tolerance of microbes is known to be influenced by salinity (Ritchie, 1959). As the experiments were carried out under identical laboratory conditions and same temperature, the response achieved relates mainly to salinity. Though the strains are isolated from the same brackishwater ecosystems, they preferred different salinities for their optimum growth and nitrogen fixation.

Effect of pH on growth and nitrogen fixation:

In the present study most of the strains of A. vinelandii preferred a pH range 7.75 to 7.82 for normal growth and decline in growth was noticed in all the strains with further increase or decrease of pH. Nitrogen fixation also increased with the increasing level of pH but the optimum nitrogen fixation occurs within the pH range 6.64 to 7.4 for the three strains.

In A. chroococcum relatively poor growth was noticed at all the tested pH levels and the maximum growth occurred between pH 7.59 and 8.28 Conversely, the maximum nitrogen fixation was noticed between the pH levels of 7.79 and 7.91. Thus in most cases the pH levels providing maximum nitrogen fixation differed significantly with that of growth.

In A. beijerinckii the growth and nitrogen fixation were found to be higher compared to A. vinelandii and A. chroococcum. But pH levels below 6.0 was detrimental to the growth as well as nitrogen fixation in A. beijerinckii. Some authors isolated A. beijerinckii in situ conditions with its growth and nitrogen fixation at pH 5.6 (Jensen, 1955; Tchan, 1953a). Koleshko (1961), Boyd and Boyd (1962) established that the acidification of the medium leads to a sharp decline in viability and nitrogen fixation ability. According to them at pH 4.5 the growth of the strains was completely inhibited. However in the present findings growth and nitrogen fixation was completely suppressed at pH below 7.75 in two of the strains and at pH 6.5 in the third strain which is more resistant to the acidic conditions. The optimum pH for these strains was found to lie near or slightly above neutrality as supported by the works of many other authors (Yamagata and Itano, 1923; Samalii, 1939; and Blinkov, 1955).

According to Rubenchik (1963) the optimum pH should be only determined in young cultures, as the initial pH of the medium gets altered during growth;

acid media becomes alkaline and vice versa. Khullar and Chahal (1975) found that Azotobacter was found maximum between the soil pH 7.8 and 8.0 and that the counts decreased with the increase in pH. In their observations sediment pH above 9.0 resulted in a decrease in nitrogen fixation in all the strains of A. chroococcum and A. vinelandii. This reveals a wide variation of capacity to fix atmospheric nitrogen by different isolates of Azotobacter and confirms with the observations recorded by many other workers (Blinkov, 1953, Sulaiman, 1973).

Jurgensen and Davey (1968) are of the opinion that Azotobacter will neither grow nor fix nitrogen in culture media having a pH below 6.0. According to Sethunathan et al. (1977) if pH 6.0 was not an absolute limit for the growth of Azotobacter it was definitely a limit for its nitrogen fixation.

From the present observations and the observations made by the other workers it is clear that the optimum pH for the growth and nitrogen fixation of Azotobacter was near or slightly above neutrality.

Effect of trace elements on growth:

Several earlier workers have reported the essentiality of trace elements such as cobalt, copper, iron, manganese and zinc in the nutrition of bacteria (Enoch and Lester, 1972; Brock, 1974). Bacteria respond to trace elements in different ways, depending upon the kind of bacteria and the concentration of trace elements in the environment. In the present investigation out of the nine strains of Azotobacter only one (Azb3) showed a linear growth with increasing concentration of cobalt upto the highest test level of 500 µg/l, but its statistically calculated optimum level was at 263.63 µg/l. However, in others, distinctly increasing growth was noticed upto the optimum level of

391.42 µg/l and thereafter a decline was observed. These results suggest that cobalt is an essential trace element and required at optimal levels for the optimum growth of Azotobacter strains.

In the case of zinc, growth inhibition was not observed with the concentrations used and the observed response suggested that zinc requirement may be higher than the highest level (500 µg/l) tested in the present investigation. However, the response curve analysis indicates that the optimum growth of Azotobacter strains at the zinc level of 463.43 µg/l. In most of the strains, except in one strain, (Azc3) linear increase in growth with the increasing level of zinc. Though the maximum zinc concentration used in the medium was quite high, the growth and multiplication of the Azotobacter cells were not adversely affected.

Iron also showed a significant influence on the growth of all the strains of Azotobacter. Besides, all the strains were found to show maximum growth at optimum level. While the iron concentrations below 293.43 µg/l are inadequate for the growth of the strains, the one above 420.87 µg/l depressed the multiplication and growth of all the three strains of A. vinelandii. In A. chroococcum optimum growth was recorded at the iron level of 260.05 µg/l in Azc1 and no significant growth response was evident in Azc2 and Azc3 when observed statistically. In A. beijerinckii iron level above 297.78 µg/l seems to have a detrimental effect on the growth.

In all the 9 strains of Azotobacter, increasing level of copper induces growth and reproduction. Some of the strains of Azotobacter had a better level of tolerance to the increasing level of copper in a growing medium; while few of them showed a decline in growth due to its toxic effect.

A. vinelandii are more tolerant than A. chroococcum and A. beijerinckii. The inhibitory level of copper is found to be 378.26 µg/l and the optimum requirement is found to be 250.05 µg/l for normal growth.

Many inorganic nutrients are necessary for the growth and multiplication of the bacteria but only a select few are specifically implicated in the metabolism of nitrogen. Trace elements like zinc are mainly involved in the enzyme action and able to take part in the active transport (Bhattacharya, 1970; Silver et al. 1970; Wang and Newton, 1969). Some are required in lesser amounts for the growth; while some require more concentration of trace elements and some of the trace elements are even replaced by the others depending upon the dynamic changes occurring in the ecosystem and depending upon their availability for the Azotobacter cells to grow in the system concerned (Jakobson et al., 1962). Organisms making use of nitrogen must have cobalt available for them, although a lesser concentration of this element may be essential for growth on combined nitrogen and the role of cobalt in the nitrogen assimilation process has been established for Azotobacter, Beijerinckia, Clostridium and several other genera (Jakobson et al. 1962). Winslow and Hotchkiss (1922) and Avakyan and others (1967) reported that some of the trace elements are essential micronutrients at low concentrations but toxic at higher levels, others have no known biological functions when they studied the toxicity level of cobalt, copper, zinc and iron on different microorganisms.

Effect of water soluble vitamins on growth:

Essentiality of ascorbic acid, biotin, thiamine and cyanocobalamine for normal growth and multiplication by all the nine strains of Azotobactor is confirmed from the present study. However, differences have been noticed between the strains as far as their optimal requirements are concerned, Macleod et al. (1954) found that several marine bacteria required the addition

of vitamins to the medium for sufficient growth and its multiplication. Chalifour and Holder-Franklin (1981) also reported many of the isolates required growth factors; the most common one was cyanocobalamine.

The different strains of Azotobacter required ascorbic acid levels in the range of 78.25 to 172.36 µg/l for their optimum growth. The level of biotin required for the essential cell growth of Azotobacter was found to be 21.90 µg/l and the optimum level ranged from 14.38 to 43.39 µg/l in all the three strains of Azotobacter. The optimum requirement of thiamine for growth of Azotobacter strains were in the range of 14.38 to 43.39 µg/l. Beyond this level there was no significant response for thiamine. Certain microlevel of cyanocobalamine is essential for the growth of Azotobacter strains. They seem to promote growth even after the level of 4.26 µg/l and growth inhibitors beyond 5.57 µg/l in the present study; so is concluded that all the strains require essential level of cyanocobalamine for their normal growth.

Burkholder (1963) reported that biotin and thiamine are the vitamins most frequently required for growth. Cyanocobalamine stands next to the biotin. In the present observations in addition to the above vitamins, vitamin C is shown to promote growth. Requirements of these essential vitamins in the medium for the growth of different strains of Azotobacter may be due to the absence of yeast extract as a source of protein in the culture medium.

SUMMARY

SUMMARY

The present investigation was undertaken during the period 1982-85 to study the ecology of certain groups of bacteria involved in the nitrogen cycle in selected perennial and seasonal prawn culture ponds of Kerala. Four ponds were selected for the study: two of which are perennial prawn culture ponds, located within the premises of the Prawn Culture Laboratory of the Central Marine Fisheries Research Institute at Narakkal and the other two are seasonal prawn culture ponds, where prawn and paddy are cultivated during the inter-monsoon (October-May) and monsoon (June-September) periods, respectively.

Water and sediment samples were collected fortnightly from each of the ponds for enumeration of the nitrogen cycle bacteria and for monitoring the environmental parameters.

Enumeration of bacterial groups involved in the nitrogen cycle, such as, aerobic total heterotrophs, proteolytic, ammonifying, nitrifying (ammonia-oxidizing), denitrifying and nitrogen fixing bacteria, was carried out for two years (October 1982 to September 1984) using standard microbiological methods, with a view to studying the seasonal variations in their distribution and abundance. Enumeration of Azotobacter was done for one year (1983-84). Bacterial enumeration was carried out by employing two methods, viz., the pour plate method and the Most Probable Number (MPN) method. The pour plate count technique was followed for the enumeration of total aerobic heterotrophs, proteolytic, nitrogen fixing and Azotobacter bacterial populations by employing various selective media with a suitable salinity. Ammonifiers, nitrifiers (ammonia-oxidizers) and denitrifiers were enumerated by following the MPN method with various liquid media prepared with a suitable salinity.

Environmental parameters, such as, water temperature, dissolved oxygen, salinity, pH, Eh, nitrate, nitrite, ammonia, organic carbon, total nitrogen and total phosphorus were determined, fortnightly from all the ponds, to study their influence on the distribution and abundance of the selected bacterial groups in the water and the sediments.

Studies were also carried out on the in situ bacterial nitrogen fixation, fortnightly, using microkjeldahl method, and the effect of environmental factors on nitrogen fixation was computed.

Data were analysed to find out if there were any significant differences in the bacterial population and nitrogen fixation rates among the ponds, between the seasons, and also between the water and sediments in each of the ponds.

Linear multiple regression analysis of the data was carried out to determine the influence of environmental factors on the distribution of each of the bacterial groups, as well as on the aerobic bacterial nitrogen fixation rates in the water and the sediments.

Thirty nitrogen fixing Azotobacter strains isolated from the water and sediments were identified based on their morphological, physiological and biochemical characteristics.

Experimental studies were made to elucidate the effect of incubation period and salinity on the growth and nitrogen fixation ability of all the 30 isolated strains of Azotobacter in the laboratory. Experimental studies were also conducted on nine selected strains of Azotobacter (A. chroococcum - 3 strains; A. vinelandii - 3 strains; A. beijerinckii - 3 strains) to evaluate the effect of pH, certain vitamins (cyanocobalamine, biotin, ascorbic acid and thiamine)

and trace elements (cobalt-$CoCl_2$ $6H_20$, copper-$CuSO_4.5H_20$), zinc-$ZnSO_4.7H_20$ and iron-$FeCl_3$) on the growth of the strains. Data obtained from the experiments were statistically analysed, following standard procedures to ascertain if there were any significant influence of the test parameters on the growth of the bacteria. The optimum requirements for salinity, pH, selected vitamins and trace elements for growth of the nine strains have also been found out.

Data on the environmental parameters for the two years (October 1982 to September 1984) showed that the physico-chemical conditions, in general, remain more or less steady in both the perennial and seasonal culture systems during the premonsoon period, but major noticeable variations occur during the monsoon and postmonsoon months as a result of heavy rainfall. The physico-chemical parameters, generally, recover to their premonsoon level by the end of postmonsoon period. Freshwater run-off from catchment areas and local precipitation during monsoon and postmonsoon have enormous dilution effect resulting in decreased salinity of the water.

Water temperature and Eh of both the water and the sediments show a decrease during the monsoon and postmonsoon periods. But the quantum of decrease in temperature is considerably higher in the seasonal ponds. The pH of water and sediments is quite stable in the perennial ponds, with a little decrease during the monsoon period; whereas considerable increase of pH occurs during monsoon and postmonsoon periods in the seasonal prawn culture systems. The Eh in both the systems behaved in a similar fashion throughout the period of study, except for more reduction in oxidation-reduction potential in the seasonal prawn culture fields in the premonsoon season. Depletion of dissolved oxygen content is more pronounced during the premonsoon season in the

seasonal ponds as compared to the perennial ponds. The considerable increase in nutrient levels in water (nitrite, nitrate, ammonia) observed during the monsoon and early premonsoon periods results in increased primary production and consequently the culture systems become more alkaline as evident from high values of pH during the same period.

Erratic fluctuations in nitrite, nitrate and ammonia are recorded. Total phosphorus and organic carbon show increased values during the post-monsoon and in the early monsoon, also in certain months of early pre-monsoon seasons. Nitrogen level is more in the premonsoon and monsoon than in the postmonsoon season. The dissolved oxygen level shows more or less uniform values, but for a slight decrease during monsoon.

During most part of the investigation, in all the ponds, sediments were found to harbour more number of bacteria than the water. This is attributed to various factors, such as, gradual deposition of bacteria from the overlying water; increased propagation of the bacteria indigenous to the sediments; settlement of the particulate substrates during the process of sedimentation.

In general, seasonal ponds are found to be more productive than the perennial ponds due to the death and decomposition of more organic matter and desintegration of paddy-stumps after harvesting the paddy, and also due to the application of organic manure for the paddy crop.

Heterotrophic and proteolytic bacteria are more abundant in the premonsoon season than the monsoon and postmonsoon seasons; but ammonifiers are more in the postmonsoon season than the monsoon and the premonsoon

seasons in both the culture systems. Nitrifiers (ammonia-oxidizers) did not show any consistent trend during the period of investigation. Denitrifiers are abundant in premonsoon than the postmonsoon and monsoon seasons.

In general, total heterotrophs, proteolytic and ammonifiers were more abundant than the nitrifiers, denitrifiers and nitrogen fixers.

The linear multiple regression analysis of the data reveals that water temperature, dissolved oxygen, salinity, nitrate, nitrite, ammonia, water pH, sediment pH and total phosphorus have a significant influence on the distribution of total heterotrophs, proteolytic,. ammonifying, nitrifying, denitrifying and nitrogen fixing bacteria in both the sediments and the overlying water of all the ponds, except for minor changes occurring in some of the groups in certain ponds. Water Eh, sediment Eh, organic carbon and total nitrogen have not shown any significant influence on the different groups of nitrogen cycle bacteria, except for Azotobacter. Sediment and water Eh and organic carbon have significant influence on the distribution of Azotobacter in addition to other parameters.

The abundance of ammonifiers, nitrifiers and denitrifiers is negatively influenced by water and sediment pH and NH_3-N in both the water and the sediments. Water temperature also had a negative influence on the distribution and abundance of denitrifiers in most of the ponds; and ammonifiers in ponds B and C, and only proteolytic in pond B sediments. Ammonia showed an inverse relationship with heterotrophs, proteolytic, ammonifying and nitrogen fixing bacteria only in pond C. Salinity had also a negative influence on the distribution of total heterotrophs, proteolytic and nitrogen fixers only in pond B water and sediments.

Partial regression analysis indicates that the abundance of Azotobacter is mostly influenced by nitrogen, phosphorus and NO_2-N compared to other parameters like dissolved oxygen, water and sediment Eh, and water temperature.

Nitrogen fixation in the ponds is more in the premonsoon season than the postmonsoon and monsoon seasons during the first year; but, in the second year, more nitrogen fixation occurred in the postmonsoon than the premonsoon and monsoon seasons. Besides, nitrogen fixation is found to be more in the seasonal ponds than the perennial ponds; and nitrogen fixation rates during the first year are superior to that of the second year.

Multiple regression analysis of the environmental parameters with that of nitrogen fixation shows that bacterial nitrogen fixation in water is significantly influenced by water temperature, water pH, sediment pH, dissolved oxygen, salinity and NO_2-N, and in the sediments by factors like sediment pH, salinity, nitrate and total phosphorus, in all the ponds.

Morphological, physiological and biochemical characterization of 30 different free-living aerobic nitrogen fixing Azotobacter strains was carried out to identify them upto the species level. Of these: 13 strains belong to A. chroococcum; 9 strains to A. vinelandii and 8 strains to A. beijerinckii.

Experimental studies were carried out on all the 30 strains to assess their nitrogen fixation capacity for a period of 45 days. Relative nitrogen fixation efficiency of Azotobacter strains reveals that good nitrogen fixation occurs even upto the incubation period of 45 days in A. beijerinckii and A. vinelandii; but only in one strain of A. chroococcum (Azc6) a decline is noticed in nitrogen fixation after 30 days of incubation. Nitrogen fixation is found to be highest in two strains belonging to A. beijerinckii which fixed 11.74 and 11.42 mg NH_3-N/100 ml

after the maximum incubation period of 45 days, followed by two strains belonging to A. chroococcum which fixed 10.6 mg NH_3-N/100 ml and 10.70 mg NH_3-N/100 ml of medium.

Salinity showed a significant influence on the growth and nitrogen fixation in all the strains of Azotobacter. Salinity levels above 40 ppt and below 10 ppt in general, results in reduced growth of A. vinelandii and its nitrogen fixation is limited at salinity levels < 15 ppt and > 50 ppt. The estimated optimum salinity levels for the growth range from 23.52 to 30.82 ppt and the nitrogen fixation from 27.17 to 40.83 ppt for the 9 different strains of A. vinelandii.

Salinity levels below 5 ppt and above 50 ppt are found to be non-conducive for the growth of A. chroococcum and its nitrogen fixation is depressed at salinity levels <10 ppt and >45 ppt. The estimated optimum salinity levels for growth range from 23.98 to 27.85 ppt and for nitrogen fixation range from 24.49 to 35.88 ppt for the 13 different strains of A. chroococcum.

For A. beijerinckii salinity levels below 5 ppt and above 40 ppt seems to be detrimental and their nitrogen fixation also is very low at salinity levels <20 ppt and > 50 ppt. The estimated optimum salinity levels for growth range from 24.01 to 29.96 ppt and for nitrogen fixation range from 30.15 to 39.78 ppt for the 8 different strains of A. beijerinckii.

pH showed a significant influence on the growth and nitrogen fixation in all the strains of Azotobacter. The estimated optimum pH levels, for the growth vary from 7.75 to 8.82 and for nitrogen fixation from 6.64 to 6.81 in A. vinelandii strains. In A. chroococcum strains optimum growth is noticed

at pH 7.59 to 8.28 and nitrogen fixation at pH 7.79 to 7.91. However, in A. beijerinckii strains optimum growth is at pH 7.10 to 7.67 and nitrogen fixation at pH 7.12 to 7.52. In general, pH below 6.5 and above 8.5 results in reduced growth and nitrogen fixation of the Azotobacter.

Various trace elements (viz., zinc, iron, cobalt and copper) showed a significant influence on the growth of Azotobacter strains. The estimated optimum levels of various trace elements range from 219.3 to 391.0 µg/l of cobalt, from 132.32 µg/l to 463.43 µg/l of zinc, from 260.05 to 420.87 µg/l of iron, and from 251.81 to 378.26 µg/l of copper for the nine selected strains.

The tested vitamins also showed a significant influence on the growth of Azotobacter strains. The estimated optimum levels of various vitamins for growth range from 78.25 to 172.36 µg/l of ascorbic acid, 11.42 to 21.91 µg/l of biotin, 14.38 to 43.39 µg/l of thiamine and 4.26 to 5.57 µg/l of of cyanocobalamine.

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* Not referred the original publication