BENTHIC NITRIFICATION IN MANGROVE ECOSYSTEMS

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

In

MARINE SCIENCES

to the

GOA UNIVERSITY

By

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December 2009

Statement

As required under the University Ordinance 0.19.8 (vi), I state that the present thesis entitle "**BENTHIC NITRIFICATION IN MANGROVE ECOSYSTEMS**" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities and suggestions have been availed of.

K.P. Krishnan

Certificate

This is to certify that the thesis entitled "**BENTHIC NITRIFICATION IN MANGROVE ECOSYSTEMS**" submitted by **K.P.Krishnan** for the award of the degree of Doctor of Philosophy in Department of Marine Sciences is based on his original studies carried out by him under my supervision. The thesis or any part thereof has not been previously submitted for any degree or diploma in any University or Institution.

Place: Dona Paula

Date: 9/7/10

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Certified that all corrections suggested by examiners have been incorporated.

Srivaja

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Acknowledgements

With heart-felt gratitude, I sincerely thank my mentor Dr. P.A.Loka Bharathi for her valuable guidance, patience and encouragement. I express my sincere thanks to Drs. E. Desa, former Director, NIO, S. R. Shetye, Director, NIO and Rasik Ravindra, Director, NCAOR for their support, facilitation and continuous encouragement. My sincere thanks to Dr. Shanta Achuthankutty for her support and encouragement. I am thankful to Prof. G.N.Nayak, Goa University for his kind consideration to be the co-guide and member of the faculty research committee. I sincerely thank Drs. M.V.M.Wafar, Kamesh Raju, V.K. Banakar, B.N.Nagendernath, B.Ramalingeshwara Rao for giving me an opportunity to work with researchers across various disciplines. I acknowledge the support rendered by Prof. H.B.Menon, Head, Marine Sciences, Goa University for all his timely actions. I gratefully acknowledge the help rendered by Drs. Krishnakumari, Mohandas, Savita and Judith. I am highly obliged to Dr.C.T.Achuthankutty for going through the drafts of this thesis. I express my sincere thanks to Dr M. R. Tapaswi, Librarian, for his efficient support in procuring the best literature and journals. The financial support from UGC, Government of India is greatly appreciated for providing me the opportunity to work at NIO. I thank Mr.T.K.Ramankutty and family who were always there for any help especially during my initial days at NIO. My life at NIO wouldn't be have been complete without my friends who were there at any moment of time. I thank Ramesh, Nuncio, Sreekumar, Sudheesh, Rajani, Reny, Roxy, Yatheesh, Thomas, Anil, Kuldeep, Niaz and other friends whose names I have not

mentioned here for want of space. My colleagues at NIO microbiology lab and here at NCAOR were there always for any help. Sheryl, Christabelle, Santosh, Chandan, Yeshwant, Daphne and Neil have been very supportive. I thank Mr.Poi for keeping a track on the tide and planning the monthly field work at Divar and Tuvem.

I owe to my wife Biji for everything she has shared with me in this journey. I am indebted to my beloved grandmas, parents and sister for everything they have done to help me reach here. Jill and Prince left me midway to join the 'Holy Spirit'. Lenin, Stalin and Monu keeps me happy and makes this life worth living.

Without 'His' blessings nothing would have been possible!

Thank you all forever!!!

K.P.Krishnan

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Chapter 1. Introduction

General introduction

The tidal forests of coastal wetlands, existing in the intertidal zones of sheltered shores, estuaries, tidal creeks, backwaters, lagoons, marshes and mud-flats of the tropical and sub-tropical regions are commonly referred to as '*Mangroves*'. Though, mangroves generally prefer shallow sheltered intertidal swampy regions, they are capable of establishing and growing in the shallow sheltered sandy and rocky coasts. They form an important ecological asset and economic resource of the coastal environment. The mangroves are the most productive and ecologically sensitive ecosystems, which can efficiently fertilize the sea and potentially protect the coastal zone. Mangrove regions being rich in detritus (organic matter), serve as a natural nursery and feeding grounds for a variety of fishes and shellfishes, and hence are also used for aquaculture practices [McIntosh, 1982; Achuthankutty and Nair, 1983]. The mangroves exist under very hostile and inhospitable conditions like higher salinity, tidal extremes, wind velocity, high temperature and muddy anaerobic soil. The plants have peculiar adaptations such as support roots, viviparous germination, salt-excreting leaves, breathing roots, knee roots, *etc.*, by which the plants are well-adapted to water-logged, anaerobic saline soils of coastal environment. The mangrove flora can also adapt to climatic changes (precipitation and temperature), sea level rises and to the incidence of solar ultraviolet–B radiation [Rahaman, 1990; Swaminathan, 1991; Moorthy, 1995; Moorthy and Kathiresan, 1996]. They play a significant role in sedimentation, helping in land building process, and also

protect the same by reducing erosion with the help of their specialized root network.

The mangrove area in Asia equals more than 5.8 million hectares and accounts for some 38 percent of global mangrove area, representing the highest percentage of mangroves worldwide. Indonesia is the country with the largest extent of mangroves in the region (and in the world), accounting for about half the regional extent of mangrove area. Other Asian countries with a significant extent of mangroves are (in order of mangrove area) Malaysia, Myanmar, Bangladesh and India, which, together with Indonesia, account for more than 80 percent of total Asian mangrove area. Asia has the largest mangrove area of any region, and the mangroves are exceptional for their high biodiversity (especially in South and Southeast Asia). The edaphic and coastal features of South and Southeast Asian countries, together with the high rainfall and significant riverine inputs, are particularly favorable to the development of well-structured mangrove forests. Some of the largest mangrove forests in the world are found in Asia, the best known being the Sundarbans, a transboundary forest covering approximately 1 million hectares in Bangladesh and India.

Distribution and ecology of mangroves in Goa (south-west coast of India):

Mangroves are restricted to the lower latitude 32ºS-38ºN in the tropical regions of which the maximum diversity and area cover lies between 25ºS-25ºN. Indian mangroves are distributed in about 6,740 km^2 [Krishnamurthy *et al*, 1975] which constitute 7% of the total Indian coastline [Untawale, 1987]. Along the

central west coast, approximately 21,000 hectares of mangrove area have been estimated, while along Goa it was estimated to be ~ 2,000 hectares [Jagtap 1985; Jagtap *et al*, 1993,1994]. The Goa coastline is approximately 110 km long and within the latitude 15^0 00'N - 15^0 52'N and longitude 73^0 30'E - 74^0 44'E. The inter-tidal zones of two major (Mandovi and Zuari) and seven minor estuaries in Goa are mostly flanked on both sides by rocky cliffs formed with silty-sand and silty-clay along with copious amounts of organic matter. Mandovi and Zuari are the two major estuaries that flow over an area of 2500 km^2 that is about 68% of the total geographical area and are important for the economy of the territory. They flow through the mining areas and are heavily used for transporting ferromanganese ores to the Marmugao harbor (Goa). About two-third of the total ferromanganese ores of Goa come from the mines located in the basins and watersheds of these two estuaries. In fact, 90% of ferromanganese ores are transported through these estuaries in barges [Nair *et al*, 2003].

The mangroves grow luxuriantly in alluvial soil substrate, which are fine textured, loose mud or silt, rich in humus and sulphides [Rao, 1987]. Their distribution is limited by temperature [Duke, 1992] and they prefer moist atmosphere and freshwater inflow, which brings in abundant nutrients and silt from terrestrial sources. Repeatedly flooded but well-drained soils support good growth of mangroves, but impeded drainage is detrimental [Gopal and Krishnamurthy, 1993]. The Indian mangrove flora is comprised of more than 60 species belonging to 41 genera and 29 different families and of these; about 50% are reported from the west coast [Deshmukh, 1991]. About 25 species reported

from east coast are not found along the west coast. Similarly, about 8 species that characterize the west coast are absent on the east coast. *Rhizophora , Sonneratia, Avicennia, Excoecaria.* etc are some of the dominant mangrove genus found along the Mandovi and Zuari estuaries, while *Bruguiera, Acanthus, Derris, Clerodendrum* etc are less abundant.

Nitrogen cycling in mangrove ecosystem:

An overall perspective of the nitrogen cycle is summarized and illustrated in Figure 1. In mangrove ecosystems, the nitrogen flux is dynamic and partitioned between terrestrial, aquatic and benthic compartments. Studies on the seasonal variation in nitrogen fluxes in mangrove sediments and waters along the west coast of India have been done by Dham (2000) and Heredia (2000) respectively. Though mangroves are considered to be productive coastal marine ecosystems [Qasim and Wafar, 1990], nutrient measurements, especially that of nitrogen, an important factor sustaining this production has been sparse Dham *et al* [2002]. Additionally, the concepts of new and regenerated production [Dugdale and Goering, 1967], has triggered an entire gamut of elemental flux studies, principally that deals with the key element, nitrogen. Separate estimates for new and total production are crucial for quantifying carbon and nitrogen fluxes into the sea [Platt *et al*, 1991]. Most of the nutrient flux studies in mangroves have been confined to a limited period of the annual cycle [Boto and Wellington, 1988; Trott and Alongi, 1999; Harrison *et al*, 1983; Rivera-Monroy *et al*, 1995; Krishnamurthy *et al*, 1975]. Recently, Dham *et al* [2002] have reported the seasonal changes in

uptake of nitrogenous nutrients and regeneration in the plankton fraction of a mangrove ecosystem on the west coast of India.

Benthic nitrification: Process and Controls

Nitrification occupies a central position within the global nitrogen cycle. It is a microbial process by which ammonium is sequentially oxidized to nitrite and nitrate. It is an important process in the nitrogen cycle, particularly because it links nitrogen mineralization to potential nitrogen loss from the benthic system through denitrification [Seitzinger, 1990; Sloth *et al*, 1992]. It is the dominant process converting reduced inorganic nitrogen to its oxidized form and mitigating ammonium levels from being toxic [Hall, 1986; Sloth *et al.*, 1992], thus maintaining homeostasis. The oxidation of ammonium is a two-step process catalyzed by ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). AMO catalyzes the oxidation of ammonium to hydroxylamine and HAO catalyzes the oxidation of hydroxylamine to nitrite. HAO is located in the periplasm and is a homotrimer with each subunit containing eight C-type hemes [Daniel *et al*, 2002].

Despite the potential importance of nitrification, only a few studies have explored the factors regulating this process in mangroves [eg. Dham, 2000 and Heredia, 2000], and no single set of factors has emerged consistently as the regulator of nitrification rates.

Diversity and abundance of nitrifiers:

Nitrifying bacteria are the only organisms which are capable of converting the most reduced form of nitrogen (ammonium), to the most oxidised form (nitrate) and also can carry out a range of other processes within the nitrogen cycle. Though nitrification is an autotrophic process, heterotrophic nitrification is also reported to occur in various groups of bacteria and fungi, though at a slower rate than that found among autotrophic organisms [Verstraete and Alexander 1973; Watson *et al*, 1981]. *Nitrosomonas, Nitrosococcus, Nitrosospira* etc. are the most frequently observed genus associated with the process of ammonium oxidation and *Nitrobacter, Nitrospina*, *Nitrococcus*, *Nitrospira* etc. are involved in nitrite oxidation [Watson *et al,* 1981]. In the recent times, several studies have reported the use of molecular probes by Fluorescence *In Situ* Hybridization (FISH) for detection and enumeration of the nitrifying community. 16S rRNA probes for FISH have been successfully used for identification and quantification of nitrifier populations in nitrifying fluidized bed reactors [Wagner *et al*, 1998] and autotrophic nitrifying biofilms [Kindaichi *et al*, 2004]. Most Probable Number (MPN) method is also employed [Whitby *et al*, 2001] to quantify nitrifiers in fresh water lake sediments.

Impact of abiotic parameters on nitrification:

Nitrification was traditionally considered to be restricted to aerobic environments [e.g. Froelich *et al,* 1979], but recent studies [Mortimer *et al*, 2004] have shown that nitrification does happen in anoxic environments at the expense

of elements like manganese and/or iron. In general, benthic nitrification rate is regulated by the availability of dissolved oxygen [Caffrey *et al,* 2003] and ammonium [Henriksen and Kemp, 1988]. It also depends on ammonium regeneration rates, which in turn is positively influenced by temperature [Nixon, 1981]. Ammonium oxidation is also controlled by light intensity; light stimulates ammonium assimilation while it inhibits oxidation [Ward *et al*, 1984]. Caffrey *et al,* [2003] have also shown that nitrification rates are negatively influenced by hypersaline conditions. *In vitro* studies have shown that several compounds like valine, hydroxyproline, threonine, thiourea, thiosinamine, *dl*-Methionine, chloromycetin, nitrourea and nitromethane [Quastel and Scholefield, 1949] are inhibitory in nature at various concentrations, while a number of organic amendments, including yeast extract, vitamin free casamino acids, acetic acid and some amino acids can stimulate growth and nitrification rates. On the other hand the presence of glucose or glycerol, does not enhance the rate of nitrification, and may diminish the rate and the yield of nitrate formed, by diverting nitrogen from the nitrifiers to the heterotrophs proliferating at the expense of easily assimilable carbon [Delwiche and Finstein, 1965].

From the previous sections it is obvious that nitrification, though a key reaction in the environment is influenced by an array of factors. Some have positive influence while some are negative. Hence, the health and sustainability of the 'Mangrove-Buffer Zones', and there by coastal environments needs to be studied from the perspective of nitrogen cycling and nitrification in particular. To understand this aspect the following aim and objectives were set forth.

Aims and Objectives of the present study:

The aim of the present study is to understand the principle factors influencing nitrification rates in mangrove ecosystems and to delineate the taxonomy and nutritional status of the nitrifier community therein. This study has been conducted with the following objectives:

- to quantify the abundance of nitrifying populations
- to identify the nitrifiers at cellular and molecular level
- to delineate their trophic status
- to quantify nitrification rates and understand the influence of environmental parameters.

Significance:

Mangrove forests that once covered more than 200,000 km^2 of sheltered tropical and subtropical coastlines is disappearing worldwide at a rate of 1 to 2% per year. This is very much comparable or greater than the loss in adjacent coral reefs or tropical rainforests. Most of this happens in the developing countries where >90% of the world's mangroves are located. As mangrove areas are becoming smaller or fragmented, their long-term survival is at great risk, and essential ecosystem services may be lost. Therefore, any further decline in mangrove area is likely to be followed by accelerated functional losses. Mangroves act as a CO_2 sink as well as an essential source of oceanic carbon. The decline also affect mangrove-dependent fauna, as well as physical benefits like the buffering of seagrass beds and coral reefs against the impacts of river-

borne siltation, protection of coastal communities from sea-level rise, storm surges, and tsunamis.

This study is probably the first of its kind addressing the nitrification issue in mangroves in conjunction with the bacterial flora mediating this process. Very little research has been carried out on benthic nitrification in the marine environment, especially from the mangroves. This study on down core variability in nitrifiers and nitrification rates at a monthly resolution is probably the first of its kind in any mangrove ecosystem. Also, a systematic account on the occurrence and significance of heterotrophic bacteria in nitrification is recorded in a comprehensive way. Though some reports are available on the impact of certain abiotic factors on nitrification rates, this study addresses the influences of key anthropogenic inputs like liquid hydrocarbons, fertilizers and pesticides besides the other well known factors. This work adds a new dimension to ecological management in coastal zones by demonstrating the elements functioning and governing nitrification in these environments. More over, the nitrifiers isolated from these environments could be used for bioremediation processes in an efficient and environmentally safe way.

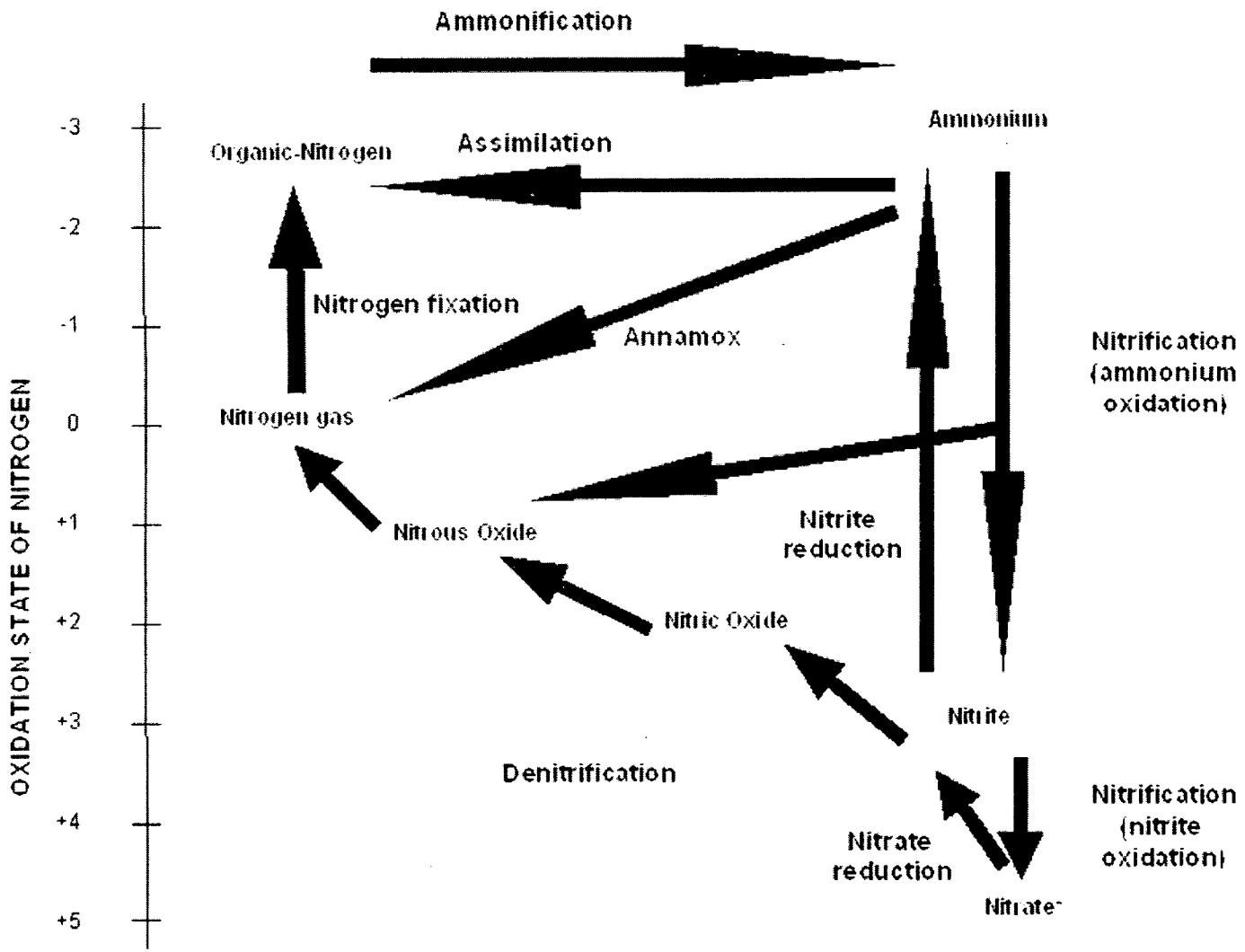


Figure 1.Key redox transformations in the nitrogen cycle

Chapter 2.
Review of Literature

2.1. Definition and distribution of mangroves

Mangroves are the tidal forests of coastal wetlands, existing in the intertidal zones of sheltered shores, estuaries, tidal creeks, backwaters, lagoons, marshes and mud-flats of the tropical and sub-tropical regions of the world. They form an important ecological asset and economic resource of the coastal environment. They are the most productive ecosystems, which fertilize the sea, and also protect the coastal zone. The word "Mangroves" refer to the plants and also the forest community. According to Macnae [1968] "Mangal" refers to the habitat or the forest community and "Mangroves" to the plant species. Duke [1992] has also reported the use of "Mangrove" as an adjective for "mangrove tree" or "mangrove fauna". The origin of the word "mangrove" could be traced to the Portuguese word 'mangue' (= a type of trees) and the English word 'groves' (= a group of trees). In French, the word 'manglier' is similar to 'mangue'. Probably all these words originated from the Malay word, 'Manggi-manggi' [Macnae, 1968]. The mangrove forests are also referred to as "tidal forests", "oceanic rain forests" and "coastal woodlands". Changing wind velocity and patterns, tides, fluctuating temperature and salinity have resulted in the evolution of varied adaptive strategies in the natural mangrove flora and fauna. So far there are no reports on any terrestrial plant that can survive these adverse conditions [Kathiresan, 1991; Kathiresan and Bingham, 2001]. The 'Mangal' are constantly exposed to water-logged and anaerobic saline soils. Occurrence of Support roots, viviparous germination, salt-excreting leaves, breathing roots, knee roots, *etc* makes them well-adapted to coastal environment. Other aspects

like change in climate (precipitation and temperature), sea levels rise and incidence of solar ultraviolet–B radiation also pose ecological challenges to the costal flora and fauna [Rahaman, 1990; Swaminathan, 1991; Moorthy, 1995; Moorthy and Kathiresan, 1996].

Alluvial soil is an excellent substrate facilitating successful growth of mangroves. These substrates are fine textured, loose mud or silt, rich in humus and sulphides [Rao, 1987]. They develop in low lying and broad coastal plains where the topographic gradients are very small and the tidal amplitude is large. Their distribution is governed by temperature [Duke, 1992] and they prefer moist atmosphere and freshwater inflow, which brings in abundant nutrients and silt from terrestrial sources. Unsheltered shores pose a potential threat to mangrove seedlings from waves and currents. Periodically flooded but well-drained soils support good growth of mangroves. Improper drainage is detrimental for mangrove vegetation [Gopal and Krishnamurthy, 1993]. Indian mangroves are distributed in about 6,740 sq.km [Krishnamurthy *et al*, 1987] which constitute 7% of the total Indian coastline [Untawale, 1987]. In general, there are three different types of mangroves in India viz., deltaic, backwater-estuarine and insular. The backwater-estuarine type is characterized by funnel-shaped estuaries of major rivers (Indus, Narmada and Tapti) or backwaters, creeks, and neritic inlets. The insular mangroves can be found in Andaman and Nicobar islands where many tidal estuaries, small rivers, neritic islets, and lagoons support rich mangrove flora [Gopal and Krishnamurthy, 1993]. Majority (70%) of the mangrove vegetation is encountered in the east coast while the west coast accounts for

only 12%. The rest is found along the bay islands of Andaman and Nicobar [Krishnamurthy *et al*, 1987; Kathiresan, 1995].

Natural regeneration of mangroves in Goa have been studied [Kumar, 2000] and various methods of regeneration of mangroves were described [Kumar, 1999]. In Maharashtra and Goa, mangroves exist mainly as large patches along the Mandovi estuary, the Vasishta estuary, the Savithri estuary, the Kundalika estuary, the Dharamtar creek, the Panvel creek, the Vasai creek, the Thane creek and the Vaitarana creek [RSAM, 1992]. The mangroves occur over an area of 5 sq.km in Goa. Mangroves in the Mandovi estuary of Goa spread to an area of 2,000 ha with distinct zones, which differ in environment, species composition and growth [FSI, 1997]. Mascarenhas and Chauhan [1998] have reported that Goa once had a luxuriant mangrove swamp of around 20 km inland from the open sea coast during the recent geological past, when the sea level was 1 to 3 m lower than present.

2.2. Ecology of mangroves

2.2.1. Microbial aspects in Indian mangroves

Mangroves have been ecologically well-studied [Gopal and Krishnamurthy, 1993] along the Sundarbans [Naskar and Guha Bakshi, 1989], the Andaman-Nicobar Islands [Singh *et al,* 1986, 1987; Ellis, 1987; Dagar, 1987; Rao and Chakrabarti, 1987], the Mahanadi delta [Banerjee and Choudhury, 1987], the Krishna estuary [Prasad, 1992], the Cauvery delta [Kathiresan, 2000] and the Mumbai coasts [Ghosh *et a.,*1994]. Mangroves provide a unique

ecological niche to a variety of microorganisms [Agate, 1991] and about 125 species of microorganisms (bacteria, fungi, algae) have been identified [Kathirvel, 1996]. The photosynthetic microorganisms behave like heterotrophs in the mangrove environment. The cyanobacteria and photosynthetic bacteria survive in low light or partially dark conditions by utilizing the suspended organic matter, which are available abundantly in the mangrove waters [Rao and Krishnamurthy, 1994]. This unique heterotrophic adaptation of photoautotrophs, is a mechanism of survival in hostile coastal anaerobic and anoxic conditions of mangrove habitat [Rao and Krishnamurthy, 1994]. Hydrocarbonoclastic bacterial isolates have been reported from mangals of Andaman [Shome *et al*, 1996]. The sulphate reducing bacteria have been isolated from the mangrove swamps of Goa [Saxena *et al,* 1988; Lokabharathi *et al,* 1991]. Purple photosynthetic bacteria are reportedly isolated from Pichavaram mangrove sediments: two major groups viz., purple sulphur bacteria (family- Chromatiaceae, strains belonging to *Chromatium* sp.) and purple non-sulphur bacteria (family- Rhodospirillaceae, strains belonging to *Rhodopseudomonas* spp.) [Vethanayagam, 1991]. Besides sulphur bacteria, the iron oxidizing and iron reducing bacteria do exist in mangrove habitat. This type of bacteria is higher in mining areas of Goa than in non-mining mangroves areas of Konkan [Panchanadikar, 1993]. The methanogenic bacteria have been studied for the first time for their distribution and ecology in mangrove sediments of Pichavaram [Ramamurthy *et al,* 1990]. In general, the bacterial counts are maximum during

the post-monsoon months and the counts of fungi and actinomycetes are maximum during the monsoon months [Mini Raman and Chandrika, 1993].

2.2.2. Value of mangroves

The value of mangrove is a measure of its importance to society. Value of mangroves can be considered of three hierarchical levels: population, ecosystem and global. At the population level, mangrove-dependent fish, shellfish, animals and timber provide important and valuable harvests and recreational fishing and hunting. At the level of the whole ecosystem, mangroves have value to the public for flood mitigation, storm abatement, aquifer recharge, water quality improvement, aesthetic and general subsistence. At the regional and global level, mangroves contribute to the stability of available nitrogen, atmospheric sulfur, carbon dioxide and methane [Mitsch and Gosselink, 2000]

Mangroves may be important in returning the 'excess nitrogen' to the atmosphere through denitrification. Denitrification requires the proximity of an aerobic and a reducing environment as well as a source of organic carbon, something abundant in most mangroves. As most tropical mangroves are the receivers of fertilizer- enriched agricultural runoff and are an ideal environment for denitrification, they are likely to be important to the world's available nitrogen balance. Also, ammonia for fertilizer production is manufactured from nitrogen gas at more than double the rate of all natural fixation. Wetlands in general have been recommended as a key ecosystem in providing a solution to this eutrophication [Mitsch and Gosselink, 2000]

2.3. Dynamics of nitrogen

Nitrogen cycle is of great concern because, together with carbon, hydrogen and oxygen, it is intimately associated with reactions carried out by living organisms. The cycling of other essential nutrients, especially phosphorous and sulphur is closely linked with biochemical nitrogen transformations. Nitrogen is considered as one of the major limiting factors in coastal waters, making the nitrogen dynamics in mangroves particularly significant. Nutrient flux measurements in mangroves have been widely reported [Boto and Wellington, 1988; Trott and Alongi, 1999; Harrison *et al*, 1983; Rivera-Monroy *et al*, 1995; Krishnamurthy *et al*, 1975]. Since most of the studies have been restricted to certain periods of the year, there exists a dearth in understanding these processes on an intra-seasonal/annual scale. New and regenerated production in ecological systems trigger and gamut of elemental flux studies, principally that deals with the key element, nitrogen [Dugdale and Goering, 1967]. The major nitrogen pools in mangroves are total sediment nitrogen (mostly organic nitrogen), total plant nitrogen, and available inorganic nitrogen in sediments. Organic nitrogen consists of compounds from amino acids, amines, proteins and humic compounds with low nitrogen content. Inorganic nitrogen consists of ammonium nitrogen, nitrate and nitrite nitrogen. In sediments, nitrites occur in trace quantities; whereas ammonium and nitrate nitrogen is the predominant form of inorganic nitrogen and is mainly derived through mineralization of organic nitrogen and further oxidation. The gaseous form of nitrogen includes ammonia, dinitrogen and nitrous oxide [Vymazal,1995]. The total sediment pool is the

largest, ranging from 100 to 1000g N/m^2. The total plant nitrogen pool is less than total sediment nitrogen while inorganic sediment nitrogen is the lowest [Faulkner and Richardson, 1989].

The original source of soil nitrogen is atmospheric nitrogen which occupies as much as 79% of the air composition and is believed to have originated from fundamental rocks of the earth's crust and mantle [Miller, 1999]. Gains in mangrove nitrogen occur by fixation of N_2 into organic nitrogen and by addition of ammonia, nitrate and nitrite in rainwater. Losses occur through plant removal, leaching and volatilization in terms of organic nitrogen, nitrate and ammonia, respectively. Organic nitrogen is converted to ammonium and nitrate ions by mineralization and to ammonia gas by ammonification. Ammonium ions are oxidized to nitrate ions by nitrification while nitrate ions are reduced to nitrogen by denitrification, thus completing the cycle.

2.3.1. States of nitrogen

Nitrogen appears in both oxidized and reduced states. A single nitrogen atom can serve as a terminal electron acceptor for eight electrons, from N (+5) of nitrate ions to N (-3) of ammonium ions. In most compounds nitrogen is either bonded to carbon and hydrogen, where the oxidation state of the nitrogen is negative (such as amines, amides, proteins and urea), or bonded to oxygen (such as nitrate, nitrite and nitrous oxide), where the oxidation state is positive.

The mechanisms involved in nitrogen cycling in mangroves include N_2 fixation, ammonia volatilization, ammonification, nitrification, denitrification and nitrous oxide production [Wen et al, 1997].

2.3.2. Nitrogen transformations

Nitrogen fixation is a process where atmospheric nitrogen is converted into organic nitrogen, either chemically by lightning or biologically by microorganisms. The covalent triple bond of the N_2 molecules is highly stable and can only be broken artificially at elevated temperature and pressure. Ammonia volatilization is a process where ammonium- nitrogen is in equilibrium between gaseous and hydroxyl form. Ammonia loss to the atmosphere is related to both pH and ammonium ion concentration. About 0.036, 0.36, 3.6, and 36% of the total reduced nitrogen in the soil solution is present as ammonia at pH values of 6, 7, 8 and 9 respectively [Stevenson and Cole, 1999]. Reddy and Patrick [1984] pointed out that losses of ammonia through volatilization from flooded soils and sediments are insignificant when the pH value is above 9.3. Losses also increase when the temperature and wind speed over the soil surface increase. Ammonification is the biological transformation of organic nitrogen to ammonium or ammonium ions. The majority of the reduced nitrogen produced in this way stay within the sediment despite a small portion being volatilized. The optimal pH range for ammonification process is between 6.5 and 8.5 [Patrick and Wyatt, 1964]. The large fraction of the organic nitrogen in many wastewaters is readily converted to ammonia [Kadlec and Knight, 1996]. The rate of aerobic ammonification doubles with a temperature increase of $10^{O}C$ [Reddy *et al,* 1979]. Nitrification is the biological oxidation of ammonium to nitrate with nitrite as an intermediate in the reaction. Firstly, ammonium is oxidized to nitrite. This step is executed by strictly aerobic bacteria which are entirely dependent on the

oxidation of ammonia for the generation of energy for growth. The second step is the oxidation of nitrite to nitrate that is performed by facultative chemolithotrophic bacteria which at the same time can utilize organic compounds for energy generation. Optimal temperature range for nitrification in soils is from 30 to 40^0 C whereas the optimal pH value is from 7.5 to 8.6. Denitrification is the reduction of nitrate to molecular nitrogen under anoxic conditions, where nitrogen is used as an electron acceptor. The end product of denitrification is N_2 but nitrogen oxides can be produced if electron donors are insufficient. Presence of suitable electron donors, such as organic carbon compounds, reduced sulphur compounds and molecular hydrogen, are major limits for denitrification. Denitrification is also sensitive to pH because denitrifying enzyme reductase breaks down at low pH. The optimal pH range for denitrification lies between 7 and 8. Denitrification increases at temperatures of 25°C and above, proceeding at a progressively slower rate at lower temperatures, and finally ceases at 2°C.

Almost all process in the conventional nitrogen cycle can occur in close proximity, either spatially or temporally, in the ecosystem structure of mangroves. Seasonal drying and wetting cycles allow efficient ammonification and nitrification [McLachlan, 1970]. At the same time, ideal conditions for denitrification are provided by the slow oxygen diffusion rates in hydric soils combined with an oxygen demand generated from the high primary production in mangroves. Not all transformations of soil nitrogen are mediated by microorganisms but some are chemical in nature. Ammonia can be fixed by the soil organic fraction which is not readily available to plants or microorganisms. Nitrite can react with organic

constituents, including humic and fulvic acids, with part of them being converted to organic forms and part of them being lost as gases. Ammonium ions can be fixed on interlamellar surfaces of clay minerals and the hydrated micas. The magnitude of these non-biological processes varies from one soil to another and influences the fate of inorganic forms of nitrogen in soils [Stevenson and Cole, 1999].

2.4. Factors governing nitrification rates

The benthic compartment together with the overlying pelagic system forms an important avenue for a plethora of reactions in the nitrogen cycle. It has been recognized that nitrogen plays an important role in fertility of the benthic system. A major fraction of the nitrogen in sediment is bound to organic matter and very little mineral nitrogen is present at any given time [Chu *et al,*1998]. Inputs of nitrogen to terrestrial and aquatic ecosystems have increased several-fold over the last one hundred and fifty years, with a very large increase during the last forty years [Holland *et al,*1999]. Higher magnitude of fertilizer production and its indiscriminate use together with increased fossil fuel combustion and widespread cultivation of nitrogen fixing crops have contributed to the striking increase in nitrogen inputs [Smil, 1990; Galloway *et al,*1995 and Vitousek *et al,* 1997].

Triska *et al* [1990] and Jones *et al* [1995] have reported that in streams, nitrification rates were primarily governed by the supply of ammonium and oxygen. In sediments these processes occur close to the sediment-water interface and in the oxidized lining of animal burrows [Boto, 1982] and is mainly

controlled by the availability of ammonium, dissolved oxygen as well as the population dynamics of nitrifying bacteria [Hansen *et al*, 1981]. It has been reported that bioturbation enhances the nitrifying activity down the sediment [Aller, 1988] while benthic micro algae can become strict competitors with nitrifiers for the nitrogen source [Nielsen *et al*, 1990]. Trace metals like iron has a well-established role in the process of nitrification both in the aerobic and anaerobic regions. While laboratory studies have shown that anoxic nitrification to be thermodynamically possible [Anschutz *et al*, 2000; Hulth *et al*, 1999; Luther *et al*, 1997], Mortimer *et al* [2002] found significant evidence for such a reaction during high-resolution analysis of sediments. Apart from its role in respiration, iron also serves as the integral part of the enzymatic system involved in nitrification. Studies suggest that iron is capable of forming a catalytic component of ammonium monoxygenase (associated with the cell membrane) of *Nitrosomonas europaea* and possibly a part of the oxygen-activating center [Zahn *et al*,1996]. Nitrification was traditionally considered to be restricted to aerobic environments [e.g. Froelich *et al*, 1979], but recent studies [Mortimer *et al*, 2004] have shown that nitrification does happen in anoxic environments at the expense of elements like manganese and/or iron. In general, benthic nitrification rate is regulated by the availability of dissolved oxygen [Caffrey *et al*, 2003] and ammonium [Henriksen and Kemp, 1988]. It also depends on ammonium regeneration rates, which in turn is positively influenced by temperature [Nixon, 1981].

2.5. Benthic nitrogen cycling in mangroves – National and International scenario

The Indian mangrove ecosystems like its other tropical counterparts form an integral part of the costal buffer zone. In spite of its ecological significance, due to several logistic constraints, studies, especially that on nutrient chemistry and associated biological processes have been confined to a few months in an annual cycle and very few studies have traced key nitrogen conversions with respect to the various phases of the monsoon cycle. Dham (2000), Heredia (2000) and Dham *et al* [2002] had studied the seasonal variation in nitrogen fluxes in mangrove sediments and waters along the west coast of India. But these studies have been limited to the involvement of various fractions of algae on several aspects of the nitrogen cycle. The nitrogen fixation by microorganisms has been investigated in mangroves. Nitrogen-fixing bacteria, *Azotobacter* species have been isolated from sediments of Pichavaram mangroves and their counts were more in the mangrove habitat than in marine backwaters and estuarine systems [Lakshmanaperumalsamy, 1987]. Nitrogen fixing bacteria in the rhizosphere of mangrove plant community have been quantified in the Ganges river estuary and the bacterial counts were reported to be high in inundated swamps and low in occasionally inundated ridges and degraded areas of mangroves [Sengupta and Chaudhuri, 1990].

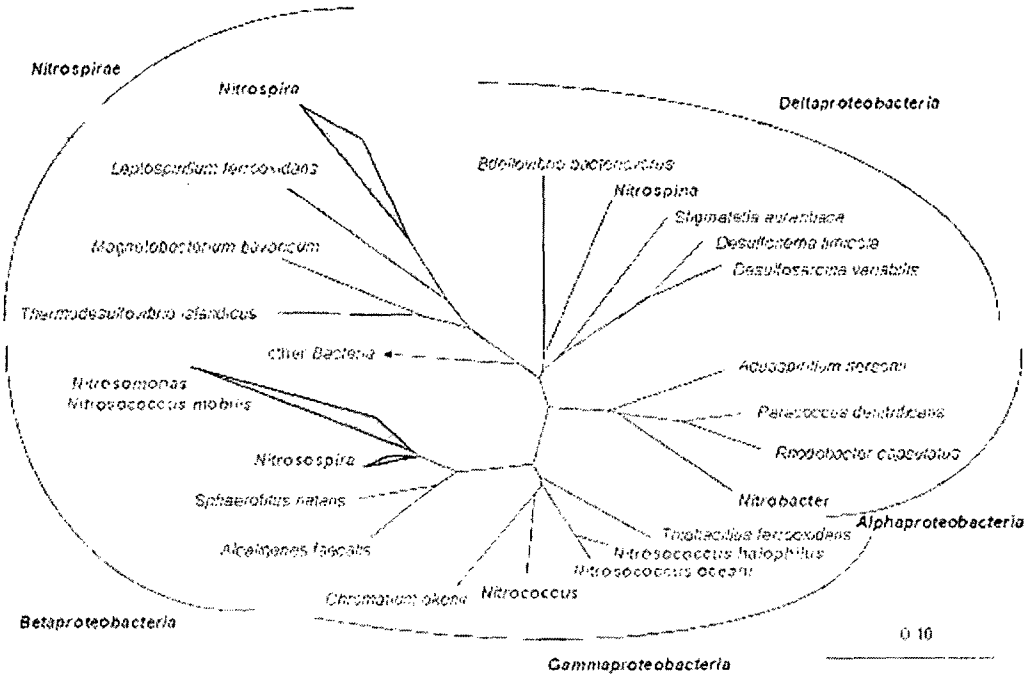
The international scenario is not much different from the national contributions. In fact most of the aspects we know in general come from work published on Indian mangroves. In other tropical mangroves, most of the nutrient

flux studies have been confined to a limited period of the annual cycle [Boto and Wellington, 1988; Trott and Alongi, 1999; Harrison *et al*, 1983; Rivera-Monroy *et al*, 1995].

2.6. Biodiversity of nitrifiers

Based on comparative 16S rRNA gene (rDNA) sequence analysis, cultured ammonia-oxidizing bacteria comprise two monophyletic groups within the Proteobacteria. *Nitrosococcus oceanus* and *N. halophilus* belong to the gamma subclass of the class Proteobacteria [Woese *et al,* 1985], while the members of the genera *Nitrosomonas* and *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus* (the latter three being closely related to each other [Head *et al,* 1993]), as well as *Nitrosococcus mobilis* (actually a member of the genus *Nitrosomonas*) constitute a closely related assemblage within the beta subclass of Proteobacteria [Head *et al,* 1993; Pommerening-Röser *et al*, 1996; Stehr *et al*, 1995; Teske *et al*, 1994; Utåker *et al,* 1995; Woese *et al*, 1984] Based on ultra structural properties, cultivable nitrite-oxidizing bacteria have been assigned to the four recognized genera, viz *Nitrobacter*, *Nitrospina*, *Nitrococcus*, and *Nitrospira*. Comparative 16S rRNA sequence analyses revealed that one of these genera, *Nitrobacter* [Winogradsky, 1892], with its four species namely *N. vulgaris, N. alkalicus, N. hamburgensis* and *N. winogradskyi* [Bock *et al*, 1983 and 1990], is a member of the alpha subclass of Proteobacteria [Orso *et al,* 1994; Teske *et al*, 1994]. The genera *Nitrospina* (*N.gracilis*) and *Nitrococcus* (*N.mobilis*) [Watson and Waterbury, 1971], with one species each, belong to the delta and gamma subclass of Proteobacteria, respectively [Teske *et al*, 1994].

The remaining genus, *Nitrospira* [Watson *et al*, 1981],encompassing the species *Nitrospira moscoviensis* [Ehrlich *et al*, 1995] and *N. marina* [Watson *et al*, 1986], is a member of the *Nitrospira* phylum of the domain *Bacteria* Ehrlich *et al*, 1995].



16S rRNA based tree showing the phylogenetic affiliation of ammonia and nitrite oxidizing bacteria. The scale bar indicates 0.1 estimated change per nucleotide. (Image Courtesy: http://www.microbial-ecology.net/nitrifiers.asp)

2.7. ^{15}N isotope as a tracer for nitrogen distribution

2.7.1. Natural abundance of ^{15}N and its uses

There are six known isotopes of N, but only ^{14}N and ^{15}N are stable. Most of the Earth's N occurs as the stable isotope ^{14}N (99.634% of atmospheric N) whereas the natural abundance of ^{15}N at atmosphere is only 0.366%. The advantage of using ^{15}N as a tracer is that it is non radioactive. Therefore

experiments can be carried out over long period of time. Unlike those radioactive isotopes, ^{15}N has no health hazards and so permission is not necessary for experiments carried out in fields and research labs.

^{15}N is being used in the studies related to1) nitrogen balance in sediments and waters, 2) stabilization of N through immobilization, 3) uptake of soil and fertilizer N by plants and fate of residual fertilizer N in soil, 4) losses of soil and fertilizer N through leaching and denitrification, 5) biological N_2 fixation, 6) fixation of ammonium ions by clay and ammonia by organic matter and the availability of the fixed N to plants and microorganisms, and 7) relative use of ammonium and nitrate ions by microorganisms and higher plants.

2.7.2. Assumptions and possible inaccuracies

There are some key assumptions for the usage of ^{15}N in soil N studies. First, the isotope composition of N in the natural soil is expected to remain constant over time. Second, living organisms are believed to use ^{14}N and ^{15}N isotopes in a indiscriminate manner. Third, the chemical reactivity and response to physical factors of the two isotopes are assumed as identical and remain constant over time.

However, slight variations occur in the N isotope composition of soil. In general, ^{15}N values increase with depth in soil profiles while tree tissues and fresh litter are slightly depleted in ^{15}N relative to soils. ^{15}N values also increase with soil age, organic matter age and extent of decomposition. The divergence is mainly due to small difference in mass of ^{14}N and ^{15}N isotopes. For instance, the oxygen-nitrogen bond of ^{14}N-NO_3 is weaker than that of ^{15}N-NO_3, so

denitrification of $^{14}N\text{-}NO_3$ occurs more readily than of $^{15}N\text{-}NO_3$. Preferential sorption of $^{15}NH_4^+$ on clays and other cation-exchange surfaces than $^{14}NH_4^+$ leads to comparatively high ^{15}N depletions in soil solution. Consequently, less ^{15}N is available for plant uptake and in turns, results in ^{15}N depletion in plants than in soil. ^{15}N content of mineralized N derived from humus is not constant. Feigin *et al* [1974] showed that the ^{15}N content of soil-derived nitrate increased with incubation time. Nitrogen inputs that are depleted in ^{15}N is possible for the crucial cause of lower ^{15}N values in vegetation and litter at the soil surface, while the discrimination against ^{15}N during mineralization and the relative isolation of soil N from atmospheric input may result in higher ^{15}N values at deeper soils [Lajtha and Michener, 1994]. Consistent differences in ^{15}N between plant species are also demonstrated, through the mechanism behind is not yet known [Robinson, 2001].

2.7.3. Nitrogen balance studies with ^{15}N isotope

As N cycle is a very complex system, it is very different to prepare reliable balance sheets where all gains and losses are accounted for. It is complicated to estimate whether the losses are through leaching, incomplete nitrification, complete or incomplete denitrification or ammonia volatilization. Also, it is intricate to determine whether the N increment in soil is owing to N_2 fixation, N deposition or addition of N fertilizer. The ^{15}N-enrichment approach takes an advantage over these problems as it has the ability to quantitatively trace a given N input through the various pools.

Natural abundance studies of ^{15}N can provide information on overall patterns of N cycling, sources of N inputs and even perturbations in nutrient cycles from decades or centuries. A novel and rather new approach is to use ^{15}N labels in large scales. The use of ^{15}N tracers in an adequate scale is to cancel out small but detectable shifts in ^{15}N natural abundances in nitrogen pools. In other words, it can counteract that previously mentioned inaccuracies when quantifying the ^{15}N balance in ecosystems.

Several conditions must be met to obtain trustable tracing sensitivity in N addition balance work. First, reliable estimates should be obtained for the size of the various N pools. Second, representative samples have to be collected for analysis. Third, the experiment must be performed with an adequate replication and proper local controls.

Chapter 3.
Materials and Methods

3.1. Description of the study area

The Mandovi and the Chapora are two tropical estuaries lying in close geographic proximity on the west coast of India (Figure 2). The hydrological characteristics of these two estuarine systems are governed by the monsoon regime. The physical characteristics of the Mandovi and the Chapora estuaries have been described by Varma and Rao, 1975; Varma and Cherian, 1975; Murthy *et al*, 1976. Based on the environmental characteristics the Mandovi estuarine system is classified as a tide dominated coastal plain estuary and geo-morphologically identified as drowned river valley estuaries [Murty *et al*, 1976]. The estuarine channel of the Mandovi is used to transport large quantities of ferromanganese ores from mines located upstream to the Murmagao harbor (Arabian Sea), while the Chapora is free from the movement of ferromanganese ore bearing barges. Lush mangrove vegetation fringes both the estuarine systems.

To study the influence of metal contamination on nitrification process, two sites were selected (Figure 2). The control site which is relatively free and less affected from metal pollution is located at Tuvem in the Chapora estuary at 15° 38.28' N and 73° 47.71' E. The experimental site which is exposed to enrichment of metal ores is located at Diwar in the Mandovi estuary at 15° 30.42' N and 73° 52.28' E. The Mandovi estuary is longer and the estuarine system is complex. The river has its origin from the Parwa Ghat of the Karnataka part of Sahyadri hills and joins the Arabian Sea through Aguada Bay, after traversing a stretch of about 70 km. In both the estuaries the pre- and post-monsoon flow is regulated

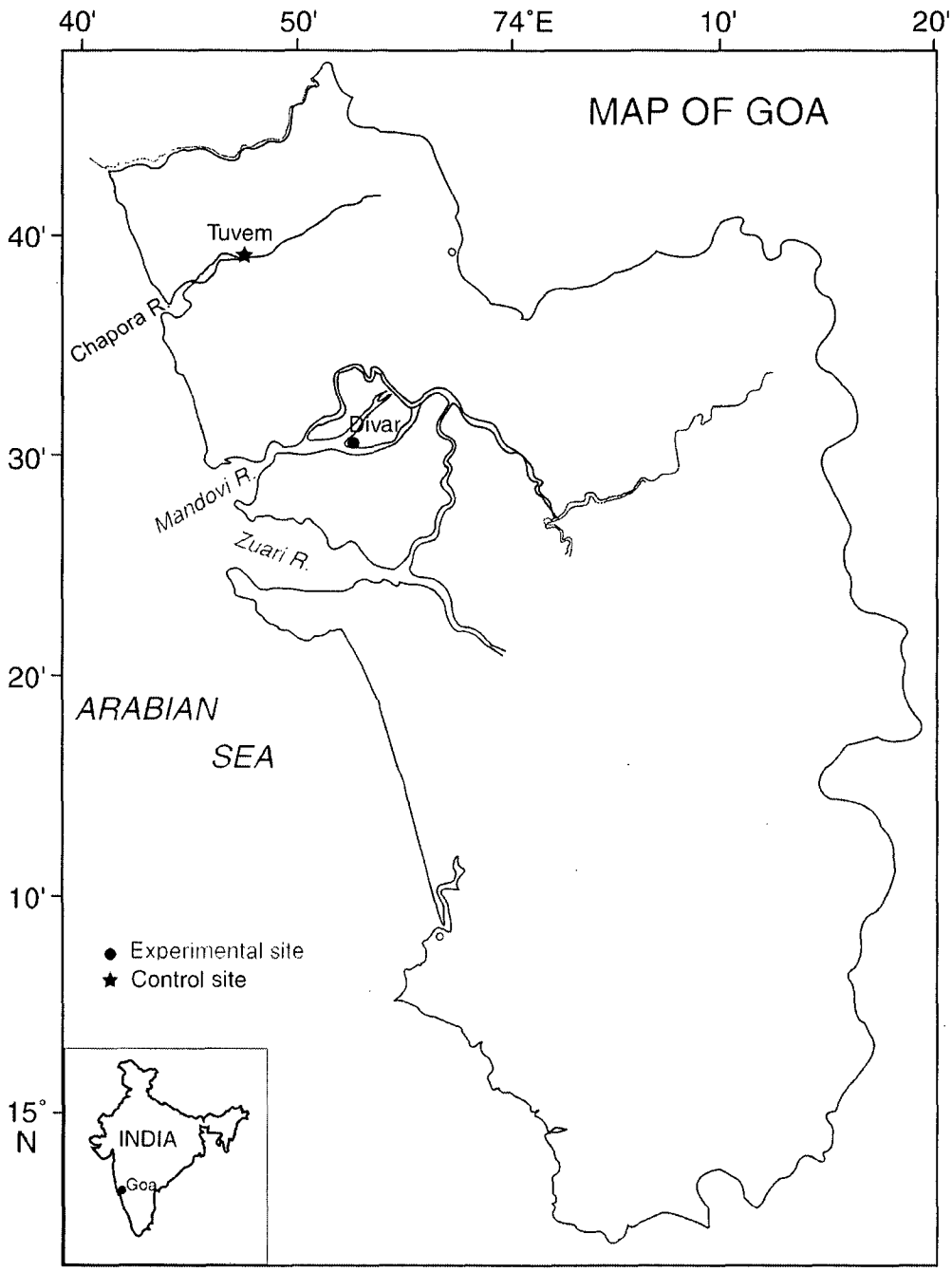


Figure 2: Location of sampling sites in the Chapora and the Mandovi estuary.

by the semi-diurnal tides. The position of the stations was fixed using the determined Global Positioning System (GPS) (Magellan GPS NAV 5000^{TM}, USA).

3.2. Sampling programme and sample processing

Field observations were carried out for a period of one year at both the control and experimental sites from April 2005 to March 2006. Sampling was carried out on monthly basis during the low tide period. The study period was grouped according to three distinct seasons based on the south west monsoon, namely Pre-monsoon (February to May), Monsoon (June to September) and Post-monsoon (October to January).

Sediment cores were collected from fringing mangrove areas along the Chapora (control site) and the Mandovi (experimental site) estuaries (Figure 2). A PVC hand-held sediment corer was used to retrieve sediment cores of 12-15 cm in length and 8 cm diameter. The cores were transported to the laboratory in cold condition for analyzing physico-chemical and microbiological parameters, taking necessary precautionary measures. In the laboratory, sub samples were taken at 2 cm intervals from surface to 10 cm, by carefully sectioning the core with a sterile blade in a laminar flow.

Water samples from the adjoining streams were collected for measuring salinity, temperature and dissolved oxygen. Water samples were also collected in clean carboys for preparing bacteriological media.

3.3. Analytical techniques

3.3.1. Hydrological parameters

3.3.1.1. Temperature and Salinity

Water temperature was measured by dipping the stainless steel temperature probe of a hand held traceable mini digital thermometer with a precision of 0.1 ^{o}C into ambient water. Salinity was likewise measured using a hand refractometer (ATAGO 2442-W01) calibrated to zero with distilled water.

3.3.1.2. Dissolved oxygen

The dissolved oxygen (DO) concentration in the water samples was estimated using Winkler's titrimetric method [Carpenter, 1965]. Water samples were collected in 125 ml acid washed (10% HCl) glass-stoppered bottles and fixed immediately with 1 ml of manganous chloride (3M) and 1 ml of alkaline-iodide (8M-4M) solution (Winkler's reagents). Samples were mixed well and the precipitate was allowed to settle. In the laboratory, 1 ml of sulphuric acid (10N) was added to dissolve the precipitate and the samples were titrated with 0.01N sodium thiosulphate using starch as indicator. The procedure was standardised by using potassium iodate. Results are expressed as ml l^{-1}.

3.3.2. Ambient nitrogen concentrations

3.3.2.1. Pore water extraction

Extraction of interstitial waters is usually done with pressure–operated squeezes or centrifugation. In the first method, the sediment core is crushed mechanically under high pressure to expel the water [Manheim, 1966; Kriukov and Manheim, 1982; Zimmermann *et al*, 1978; Bender *et al*, 1987; Nath *et al*,

1988]. Alternatively the pressure is generated by passing an inert gas through the core that displaces the pore water [Reeburg, 1967]. In the centrifugation method, the sediment core is placed in a tube and centrifuged at high speed to expel the pore water, which then is siphoned off. High vacuum suction has also been used to recover pore waters [Manheim, 1966]. An advantage of these methods is that since the sections of the core are well defined, it would be possible to obtain profiles of distribution of elements.

Pore water in this study was collected by centrifugation method. After sectioning the cores at 2 cm interval, each fraction was made into slurry with a known volume of saline and then loaded separately into centrifuge tubes. The tubes were spun at low R.P.M (5000) at 4 ^{o}C for 10 minutes (REMI Cooling Centrifuge). The water was then carefully siphoned out into a pre-cleaned 100 ml polyethylene bottle and allowed to stand for 15 minutes in cold conditions in order to sediment out the coarse particles. Further, the diluted pore water was filtered on GF/F and then subsequently filtered on 0.22 μ membrane filter. The filtrate was stored in cold (~4^{o}C) until further analysis.

By spinning at low temperature and RPM it was made sure that minimal disturbance was caused to the benthic organisms, which on lysis could change the pore water chemistry. The advantage of using this technique was that it enhanced the possibility of profiling without compromising much on changes arising during the handling. Thus the measurements of nitrogen concentrations in interstitial waters or the rates of nitrogen transformation were least affected by external factors associated with handling.

3.3.2.2. Ammonium

In the method employed here [Koroleff, 1969] dissolved ammonia reacts with hypochlorite at basic pH to form a monochloramine and in the presence of phenol, forms indophenol blue colour. The reaction is catalysed by sodium nitroprusside and requires six hours for colour development at room temperature. Freshly prepared distilled water was taken as blank. Samples were analyzed every time with freshly prepared reagents. Ammonium chloride was used a standard. The optical density was measured at 630 nm (precision:±0.05 μM N-$NH_4^+ l^{-1}$).

3.3.2.3. Nitrite

Nitrite was measured by the method described by Bendschneider and Robinson [1952]. In this method, nitrite reacts with sulphanilamide in an acid solution (pH <2) and the resulting diazo-compound reacts with N- (1-naphthyl)-1-ethylenediamine to form a highly coloured azo-dye. The optical density was measured at 543 nm (precision: ±0.01 μM N-$NO_2^- l^{-1}$).

3.3.2.4. Nitrate

The method of Wood *et al* [1967] was employed for measuring nitrate. The nitrate in the sample was reduced almost quantitatively to nitrite in a cadmium-copper column and the nitrite was measured by the method described earlier for nitrite (precision: ±0.1 μM N-$NO_3^- l^{-1}$).

An earlier method for nitrate measurement was that described by Morris and Riley [1963], where copper was used as the cathode instead of mercury. More recently, Jones [1984] has described an alternative method for nitrate

reduction, in that reduction of nitrate is achieved by shaking of samples with spongy cadmium.

3.3.3. Bulk sediment parameters

3.3.3.1. Sediment temperature, pH and Eh

As soon as the sediment cores were brought to the laboratory the sediment temperature was measured at an interval of 2 cm by inserting a stainless steel temperature probe of a hand held traceable mini digital thermometer with a precision of 0.1 °C. At 2 cm interval pH and Eh were also measured using a digital pH/Eh meter (Thermo-Orion)) after calibrating it with the standard buffers of pH 4, 7 and 9.2. The calibration standard used for Eh was equimolar (M/300) solutions of potassium ferricyanide and potassium ferrocyanide in 0.1 M potassium chloride. The system has an Eh of 0.430 mV at 25°C [Zobell, 1946].

3.3.3.2. Total Organic Carbon

Total organic carbon (TOC) in the 2 cm sub-sample of core was measured by wet oxidation with chromic acid followed by titration with ammonium ferrous sulfate [El Wakeel and Riley, 1957] and expressed as percentage. This method has a precision of 0.01%.

3.3.3.3. Iron and manganese

Sediment cores sectioned at 2 cm intervals were prepared for metal analysis according to Balaram *et al* [1995]. Metal concentrations (Fe and Mn) were measured using a flame atomic absorption spectrophotometer (AAS, PerkineElmer Model 5000). The accuracy of the analytical procedures was

assessed using the certified reference material MAG-1 (USGS) that yielded results within the reference value range [Flanagan, 1967, 1976]. MAG-1 is a fine grained gray-brown clayey mud with low carbonate content, from the Wilkinson Basin of the Gulf of Maine. The collection site was approximately 125 km east of Boston, Massachusetts. The age of the sediment is Holocene, but probably includes reworked Pleistocene sediment from surrounding areas. Element concentrations were determined by cooperating laboratories using a variety of analytical methods. Certificate values are based primarily on international data compilations [Abbey, 1983; Gladney and Roelandts, 1987; Govindaraju, 1994]. USGS reports [Flanagan, 1967, 1976] provide background information on this material.

3.3.4. Tracer technique

Research on nitrogen dynamics in sediments using tracer techniques has been rather scanty till recently owing to the difficulties of handling of samples and analytical techniques. A majority of the ^{15}N tracer studies conducted on sediments have only focused on ammonium regeneration rather than on the concurrent assimilation of nitrogen [Blackburn *et al*, 1988]. ^{15}N isotopes dilution techniques employed for these studies were also extended to measurements of nitrification rates in marine sediments by [Koike and Hattori ,1978]. Several works on nitrification have since been carried out using these techniques [e.g. Henriksen *et al*, 1981]

Research on nitrogen cycling in mangrove sediments is rare, even using simple techniques such as colorimetry. In a study carried out in a Southeast

Asian mangrove forest, Kristensen *et al* [1988] examined the transformation and transport of inorganic nitrogen in the sediments. Nitrification was measured in aerobic sediment slurries as the accumulation of NO_2^- after addition of chlorate. Chlorate inhibits NO_2^- oxidation and results in its accumulation which can then be measured [Belser and Mays, 1980]. After the incubation period, the increase in NO_2 was measured spectrophotometrically [Strickland and Parsons, 1972].

3.3.4.1. Measurement principle for nitrification rates

One of the first direct measurements of nitrification rates in marine sediments employed ^{15}N isotope dilution technique with sediment slurries [Koike and Hattori, 1978]. This approach involved addition of $^{15}N\text{-}NO_3^-$ to a mixture of sea water and sediment. This was incubated for 48 hours open to atmosphere but without shaking. The observed changes in concentration and atom% enrichment of NO_3^- over time were then used to calculate nitrification rate. Other reports of nitrification in sediments using ^{15}N include those of Chaterpaul *et al* [1980]; Jenkins and Kemp [1984] and Nishio *et al* [1983]. In the present study, nitrification rates were measured by the method of Schell [1978]. In this method, the nitrite in the sample is extracted as a dye (1-benzene-azo-2-napthol) by using an organic solvent like CCl_4.

3.3.4.2. Experimental protocol of nitrite extraction

Each 2 cm intact section of the sediment core was transferred to a beaker (500 ml capacity) and 250 ml of filtered estuarine water was added. Samples were then incubated with $^{15}N\text{-}NH_4^+Cl^-$ in the dark for 24 hours. At the end of the incubation period, samples were gently mixed and pre-screened through a 200

μm mesh net. Samples were then filtered onto Whatman GF/F filter pads (pre-ignited at 400^0C for 4 hours) and 200 ml of the filtrate was recovered for the extraction of nitrite.

A part of the filtrate recovered (200 ml) was transferred to a separating funnel of 250 ml capacity and unlabeled ^{14}N-$NaNO_2^-$ (1cc =1.25 N-$Na^+NO_2^-$ and 1cc =12 N-$Na^+NO_2^-$) were added to duplicate samples. Two concentrations of the vector were added to the filtrate as it was difficult to predict the nitrite concentration in the sample after incubation. This ensured that there was sufficient N for detection by emission spectrometry. The initial step involves the formation of a diazonium compound with 3 ml of aniline sulfate solution (5 ml/l of aniline sulfate in 1N HCl). After 5 minutes, 3 ml of β-napthol (5 g/l of β-napthol in 3N NaOH) was added to the separating funnel and the contents were well mixed. This resulted in the formation of a complex coloured compound - azo dye: 1-benzene-azo-2-napthol. The dye was then acidified with 1 ml of concentrated HCl to protonate the dye and allowing its efficient extraction. It was further extracted thrice using the organic solvent carbon tetrachloride (CCl_4).

In the first extraction, 5 ml of CCl_4 was added and the separating funnel was shaken vigorously for about 10 seconds. The phases were allowed to separate and the organic phase was drained into a clean, dry separating funnel taking care so as to avoid the passage of the organic film or to retain any traces of dye. The organic film present between the two phases is a potential source of organic nitrogen contamination and therefore was avoided. The subsequent extractions were carried out in the same way using 3 ml of CCl_4 each time. The

organic phases recovered at the end of each extraction were carefully pooled into a 10 ml beaker.

3.3.4.2.1. Isotopic analysis by emission spectrometry

There are two techniques for isotopic analyses of samples involving nitrogen tracers viz. emission spectrometry and mass spectrometry. Emission spectrometry was preferred since it is less complicated and requires no high vacuum for sample preparation; it requires nitrogen gas in the order of 0.2-10 µg in comparison to 30 µg – 3 mg for mass spectrometry and is less expensive. The precision of emission spectrometry is in the order of 0.01 atom % ^{15}N compared to that of mass spectrometry, which is 0.001 atom% ^{15}N.

3.3.4.2.2. Principle of detection

The ^{14}N and ^{15}N atoms in nitrogen gas are paired to form the nitrogen molecules $^{14}N^{14}N$ (^{28}N), $^{14}N^{15}N$ (^{29}N), $^{15}N^{15}N$ (^{30}N). When an external energy source is supplied, the nitrogen molecules in the tube containing the sample get excited and on returning to the ground state, emit electromagnetic radiations of specific energy. These radiations are emitted in the ultra-violet region at different wavelengths (297.7 nm, 298.3 nm and 298.9 nm for ^{28}N, ^{29}N and ^{30}N respectively). When the emitted light is resolved by a monochromator, the light intensities corresponding to the three wavelengths are detected by a photomultiplier-amplifier system and recorded as peaks. The measurement of the peak heights allows the ^{15}N% abundance in the sample to be calculated as

$\%^{15}N$ abundance = 100/2R+1

Where, R = peak height of ^{28}N / peak height of ^{29}N

3.3.4.2.3. Preparation of samples for emission spectrometry

Samples (particulate matter retained on the filters) were first processed by Kjeldahl digestion. The Kjeldahl method encompasses three steps: digestion, distillation, and titration. Digestion is accomplished by boiling the sample in concentrated sulfuric acid. The end result is an ammonium sulfate solution. This is further distilled by adding excess base (eg. sodium hydroxide) to the digestion product to convert NH_4^+ to NH_3. Further, NH_3 is recovered by distilling the reaction product. Back titration with sulfuric acid quantifies the amount of ammonia in the receiving solution. The amount of nitrogen in the sample is then calculated from the quantified amount of ammonia ion in the receiving solution. In the present study, at the end of the titration step, a few drops of 0.01N standard HCl solution were added to the flask containing the distillate to make it acidic. The distillate was then evaporated to dryness by placing the flask at low temperature in the oven. The residue in the flask was re-dissolved in 10 ml deionized water and transferred to a stoppered tube (5 ml capacity). A second washing was necessary to remove all the traces of the residue. The tubes were dried at low temperature until the contents reached dryness, after which the PON was recovered in a known quantity of deionized water in such a way that 1 µl contained 1µg PON. Finally, the sample in each tube was withdrawn by capillary action into capillary tubes (5 cm in length) and these were placed on a rack and dried in the oven at low temperature.

3.3.4.2.4. Conversion to nitrogen gas

The determination of $^{14}N/^{15}N$ isotope ratios is carried on nitrogen gas generated from a sample. Hence for the analyses, it is necessary to convert the nitrogen atoms in the sample to molecular nitrogen gas. There are three methods by which this conversion can be achieved: a) Kjeldahl-Rittenberg method; b) modified Dumas method or c) Kjeldahl-Dumas method.

In the present study, the modified Dumas method was used for conversion of bound nitrogen to nitrogen gas. Samples containing the extracted dissolved nitrogen (nitrite from nitrification experiments) were directly processed by the modified Dumas combustion method. This involves the dry combustion of organic and/or inorganic nitrogen with an oxidant, copper oxide resulting in the complete reduction of ammonia to molecular nitrogen. The reaction takes place in vacuum in a sealed tube. The vacuum is necessary so that there is no dilution of the ^{15}N in the sample with atmospheric nitrogen. Vacuum is also necessary for discharge of the tubes (4 torr). The capillaries were transferred to discharge tubes (volume = 4 cc) with an upper constricted end, and approximately 15 mg of copper oxide was added. Since the nitrogen content of the sample in the discharge tube should yield a pressure of around 4 torr, it is necessary to know the approximate nitrogen content of the sample to at least ± 20%. The total N content required depend on the volume of the discharge tube and was estimated to be approximately 26 μg. The discharge tubes were connected to a vacuum line in a vertical position and evacuated until the vacuum exceeds 10^{-3} (atmospheric pressure). At a time, eight tubes could be evacuated on the vacuum system. The

adsorbed gases on the inner walls of the tubes were removed by heating with a hand torch. After degassing the tubes while monitoring with a vacuum gauge, the tubes were carefully sealed at the constricted end with a hand torch. The conversion of sample nitrogen to nitrogen gas was then carried out by combusting the evacuated discharge tubes in the muffle furnace at 500^0C for 6 hours followed by cooling to room temperature. The other gases produced during the combustion i.e. CO_2, H_2O and oxides of nitrogen were frozen out with liquid nitrogen, allowing the nitrogen gas in the tubes to be analyzed for its ^{15}N content by emission spectrometry. This was done in a Jaco NIA-1 N-15 analyzer. Although the light intensity of the discharge is the highest at a pressure of 1.5 torr [Sommer and Kick, 1965], the discharge lasts longer when more nitrogen is present, and a more stable discharge is obtained at 4 torr. Enrichments were determined using a calibration curve (Figure 3) made using standards provided by the manufacturer. A detailed account on analyses of ^{15}N:^{14}N ratios have been documented by Fiedler and Proksch [1975].

The discharge tubes, before being connected to the vacuum line were washed with chromic acid, rinsed well with deionised water and heated in a muffle furnace at 500^0C overnight to remove all traces of nitrogen. These were then wrapped in aluminium foil and stored in a desiccator. Copper oxide was also pre-heated at 500^0C and stored in a desiccator.

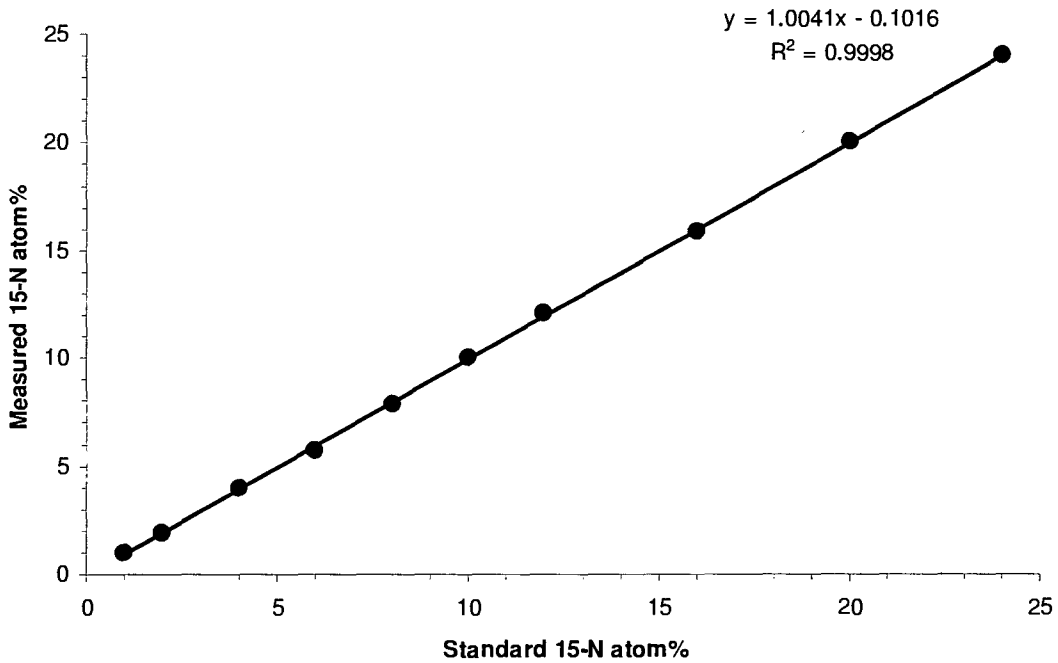


Figure 3: Calibration curve for measurement of isotope ratios

3.3.4.2.5. Calculations

Nitrification rates were calculated using equations developed by Schell [1978].

Initial atom% excess of ^{15}N in NO_2^- in the dissolved fraction,

$$^{15}NDI = \frac{[(0.000365 \times Nc) + (0.95 \times Tc) + (0.00365 \times Ac) \times 100]}{TN} - {}^{15}Na$$

Final atom % excess of ^{15}N in NO_2^- in the dissolved fraction (NDF),

$$^{15}NDF = 2\left[\left({}^{15}Nex \times \frac{(Nc + Vc)}{Nc} - \frac{({}^{15}Na \times Vc)}{Nc}\right) - {}^{15}Na\right]$$

Where,

Nc = Ambient nitrite concentration (μg at N l^{-1})

Vc = Vector concentration (μg at N l^{-1})

Tc = Tracer concentration (μg at N l^{-1})

Ac = Ambient ammonium concentration (μg at N l^{-1})

TN = Total nitrogen concentration (Nc + Tc + Ac)

^{15}Na = Natural abundance of ^{15}N (0.0365%)

^{15}Nex = Atom% excess of ^{15}N in the azo dye fraction

Since it is measuring uptake of $^{15}NH_4^+$ into $^{15}NO_2^-$,

$$V\ (h^{-1}) = \frac{^{15}NDF}{^{15}NDI} \times \frac{1}{T}$$

Where, T = incubation duration (24 hours)
Nitrification rate, R (ηg at N l^{-1} h^{-1}) = (V x Nc) x1000

3.4. Bacteriological techniques

3.4.1. Total and viable bacterial counts

As soon as the sediment cores were brought to the laboratory it was sectioned in sterile condition at 2 cm intervals up to a depth of 10 cm. The sub-samples were serially diluted to 10^{-2} with physiological saline and were fixed immediately with buffered formalin for total bacterial counts. Bacterial abundance was determined by acridine orange direct count method [Hobbie *et al* 1977]. Sediment dilutions (2 ml) preserved with 2% (final concentration) buffered formalin were stained with acridine orange (Hi-Media, Mumbai) (final concentration 0.01% w/v) for five minutes before filtering it through 0.22 µm black Nucleopore track etched membrane filter (Whatman). Samples were enumerated at 1250x magnification in an Olympus (BH) epifluorescence microscope, using a 515 nm barrier filter and at least 10 fields of >30 bacteria field^{-1} were counted. Bacterial abundance was expressed as numbers per g wet weight of the sediment. Direct viable counts were made following the method of Kogure *et al* [1984]. Serially diluted sediment samples were incubated at 28± 2 °C for 6 h with nalidixic acid (0.02% w/v), piromidic acid (0.001% w/v), pipemidic acid (0.01%w/v) (Sigma, USA) and yeast extract (0.01%). Incubation was terminated by the addition of buffered formalin (final concentration 2%). Samples were stained with acridine orange (as mentioned for Total Counts) and observed under epifluorescence microscope. Only enlarged and elongated cells were enumerated as viable bacterial cells and their counts are expressed as numbers per g wet weight of the sediment.

3.4.2. Most Probable Number method

Nitrifiers were also enumerated by the most probable number (MPN) method of Alexander and Clark [1965] on both inorganic and organic media. Inorganic nitrifying media (seawater amended with ammonium chloride of 2 mM final concentration) was distributed in 5 ml quantities in 15 ml screw capped tubes. The organic nitrifying media was prepared by adding 0.01% final concentration of glucose to the inorganic nitrifying media. From each dilution, ranging from 10^{-1} to $10^{-6/7}$, 500 µl was inoculated in triplicate in both the nitrifying media (inorganic and organic) until the inoculation from the highest dilution into the culture tubes yielded negative results. The culture tubes were incubated in the dark for a period about 60 days at 28(±1) °C. After incubation, the tubes were tested for the presence of NO^{-}_{2} and/or NO^{-}_{3}. The combinations of positive and negative tubes were scored and MPN was assessed from McCready's table [Rodina, 1972].

3.4.3. Plate counts

Sub samples of approximately 5 g wet weight sediment from each of 2 cm sediment core were sampled using sterile syringe cores. The sub samples were transferred to 45 ml of full strength sterile seawater (10^{-1} dilution). Tween80 (50 µl) was added and the mixture was sonicated at 40 mHz for 10 seconds. Serial dilutions of the sediment samples were made in autoclaved seawater to yield dilutions from 10^{-1} to $10^{-6\ to\ 7}$. Medium for the isolation of heterotrophic bacteria was prepared using nutrient strength of 25%, which correspond to 25% nutrient broth + 2% agar. A concentration of 100% corresponds to 8 g nutrient broth

(HiMedia Laboratories Pvt. Ltd., Bombay, India) per 1000 ml seawater. Medium for the enumeration and isolation of nitrifying bacteria was prepared with a mineral medium which is essentially a modified Winogradsky medium [Rodina, 1972] with pure agar (Difco) as gelling agent. The medium was substituted with ammonium chloride at 2 mM (final concentration). About 100 µl from 10^{-2} dilution was plated onto each medium. Inocula from positive MPN tubes, representing both the inorganic and organic enrichments from various sections were plated on respective solid medium. Bacterial counts in the form of colony forming units (CFU) formed on the medium were recorded after a 15-day incubation period at 28±1°C. Bacterial colonies were enumerated and is expressed as colony forming units (CFU) per g wet weight of sediment.

3.4.4. Isolation and purification techniques

Representative cultures of nitrifying bacteria representing different colony morphologies on the nitrifying plates were isolated. Each isolate represented a definite fraction of the plate count having similar colony and cell morphology. These were transferred to plates with nitrifying medium and upon growth they were checked for purity on the basis of microscopic examination. The isolates were stored in nitrifying medium slant tubes at 4 °C for taxonomic identification and characterization.

3.4.5. *In vitro* measurement of nitrification activity of the isolates

Nitrifier isolates were checked for their activity (ammonium oxidation and nitrite oxidation) in both inorganic and organic nitrifying media [Section 3.4.2 for media composition]. Aliquots of various media were dispensed in sterile,

disposable microplates. Further a 100 µl of the culture suspension in saline of 0.1 OD_{600} was inoculated into the wells. The cultures were incubated in the dark for a period about 10 days at 28±1 °C. After incubation, the wells were tested colorimetricaly (qualitative) for the fall of NH_4^+ as well as the rise and fall of NO_2^-. Care was taken to have uninoculated controls wherever possible. Further, based on the fall of ammonium as well as the rise/fall of NO^-_2 with respect to the controls, the cultures were grouped into three viz. 1) ammonium oxidizers [AO], 2) nitrite oxidizers [NO] and 3) coupled ammonium-nitrite oxidizers [CAoNo]. Based on visual (colorimetric) observations on the changes in levels of ammonium and nitrite in the microplates with respect to controls, irrespective of whether the activity was noted in inorganic or organic nitrifying media, ten isolates each of AO, NO and CAoNo were selected for detailed kinetic studies.

For preparing the inoculum, the isolates were grown in aged offshore seawater amended with 0.01% glucose for a period of 120 hours. The cells were then spun at 8000 RPM at 4°C and washed with physiological saline and suitable aliquots were used as inocula for subsequent experiments. Growth of all the isolates was monitored over a period of 120 hours at an interval of 24 hours under various experimental conditions. Direct cell counts for bacterial growth measurements were made using a cell counting chamber. Growth of AO. NO, CAoNo cultures were monitored during ammonium oxidation, nitrite oxidation and coupled ammonium-nitrite oxidation, respectively. The media used for each isolate was same one in which it was tested positive. [Section 3.4.2 for media composition].

Kinetics of ammonium, nitrite, and coupled ammonium-nitrite oxidation by batch cultures of various isolates in their respective medium [Section 3.4.2 for media composition] were monitored for a period of 120 hours under 28±1°C and at 1 atm pressure in triplicate. Ammonium concentration at the beginning of AO and CAoNo experiments was ~20 μM NH_4^+-Nl^{-1}. For nitrite oxidation experiments, an initial concentration of ~2 μM NO_2^--Nl^{-1} was maintained. Incubations were done in dark and changes in the level of ammonium, nitrite and nitrate were monitored as previously described (section 3.3.2.2 to 4) along with the increase in cell numbers at an interval of 24 hours for a period of 120 hours.

3.4.6. Phenotypic characterization of isolates in BIOLOG plates

Since, in the present study all the thirty isolates were Gram negative, the Biolog plate used was Biolog GN2 MicroPlate. The Biolog GN2 MicroPlate is designed for identification and characterization of a very wide range of aerobic gram-negative bacteria. Biolog's MicroPlates and databases were first introduced in 1989, employing a novel, patented redox chemistry. This chemistry, based on reduction of tetrazolium, responds to the process of metabolism (i.e. respiration) rather than to metabolic by-products (e.g. acid). Since the GN2 MicroPlate is not dependent upon growth to produce identifications, it provides superior capability for all types of gram negative organisms: fermenters, non-fermenters, and fastidious organisms all are identified in a single panel. The Biolog GN2 MicroPlate performs 95 discrete tests simultaneously and gives a characteristic reaction pattern called a "metabolic fingerprint".

Bacterial cultures (thirty isolates) showing enhanced nitrification activity (Section 3.4.5) were grown on the respective medium of isolation. However, use of Biolog Universal Growth w/5% Sheep Blood agar media enhanced the speed of growth of some strains. Further, the isolates were swabbed from the surface of the agar plate, and suspended in GN/GP Inoculating Fluid (Biolog); 150 µl of bacterial suspension was pipetted into each well of the GN2 MicroPlate. The MicroPlate was then incubated at 30°C for about 48 hours and then read using a micro plate reader (BMG Optima).

3.4.7. Biochemical Characterization

The physiological and biochemical properties were examined according to standard methods of Gerhardt *et al* [1981]. General cell morphology was studied under an Olympus inverted microscope using young cultures of the strains grown on mineral media plates.

3.5. Molecular techniques

3.5.1. Fluorescence *In Situ* Hybridization (FISH)

Determining the structure and dynamics of bacterial communities is a core component of microbial ecology. Fluorescence *In Situ* Hybridization (FISH) with rRNA-targeted nucleic acid probes is a molecular tool for rapid and cultivation independent monitoring of phylogenetically defined bacterial populations in environmental samples. rRNA molecules are ideal target molecules for detection of prokaryotes because they are ubiquitously distributed, contain conserved and

variable sequence regions and are naturally amplified within microbial cells as integral parts of the ribosome.

In the present study, the sediment cores collected during the months of April (Pre-Monsoon), July (Monsoon) and October (Post-Monsoon) were sectioned at 2 cm intervals in sterile conditions to obtain representative samples up to 10 cm depth. Sub samples were re-suspended in phosphate-buffered saline (1x PBS), pH 7.4, consisting of 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter of distilled water. The samples were fixed with 4% paraformaldehyde (in 1x PBS) for one hour at room temperature. , The samples were then washed twice with 1x PBS by centrifuging for 5 minutes at 4°C at 10000 RPM. This was done to remove residual paraformaldehyde. The fixed samples were suspended in a solution of 50% PBS and 50% ethanol and stored at -20°C. Before the commencement of FISH, aliquots of the samples were subjected to ultrasonic cleaning for dispersing cells adhered to sediment particles. For FISH, 10 µl of this sample was applied on a gelatin coated glass slide and dried for 1 h at 46°C, and subsequently dehydrated in solutions of 50%, 80% (vol/vol, in 10 mM Tris-HCl, pH 7.5), and 96% ethanol for 3 minute each. To start hybridization, 10 µl of hybridization buffer (with a composition dependent on the used probe) (Table 1) with 1 µl of fluorescently labeled probe (at a concentration of 50 ng/µl) were applied on the dehydrated sample and when necessary an unlabeled competitor (at 50 ng/µl) was added to the mixture (eg. when probed for *Nitrobacter spp.*). The samples were hybridized at 46°C for 1 h in a humidified chamber. Following hybridization, a stringent washing step was

Probe	Sequence (5'-3')	Flurochrome	Target organisms	Conc*		Tm (°C)	Reference
				Formamide (%)	NaCl (M)		
EUB338	GCTGCCTCCCGTAGGAGT	Alexa fluor 350	Bacteria	0	0.9	55	Amann *et al* [1990]
Nso1225	CGC CAT TGT ATT ACG TGT GA	Fluorescein - isothiocyanate (FITC)	Betaproteobacterial ammonia-oxidizing bacteria	35	0.08	50	Mobarry *et al* [1996]
NIT3	CCT GTG CTC CAT GCT CCG	Fluorescein - isothiocyanate (FITC)	Nitrobacter spp.	40	0.056	55	Wagner *et al* [1996]
CNIT3	CCTGTGCTCCAGGCTCCG		Competitor to NIT3				Wagner *et al* [1996]

*Concentrations presented as percentage of formamide in hybridization buffer or molar concentration of NaCl in wash buffer.

Table 1: Probes used for FISH and the corresponding hybridization and washing conditions

performed for 10 min at 48°C in a buffer with the appropriate NaCl concentration (Table 1). Care was taken to include positive control for all the probes. All oligonucleotide probes were obtained from *Molecular probes* (Invitrogen). Microscopic observations were made with an Olympus BX51 microscope, equipped with mirror units U-MWU2, U-MWB2 and U-MWB2. Digital images were taken with an Olympus DP-70 12.5 million-pixel cooled digital color camera. Details on the flurochrome used for each probe is given in the Table 1. A detailed version of this method was reported by Egli *et al* [2003]. The type cultures, *Escherichia coli* (ATCC:9637), *Nitrosomonas europaea* (ATCC:19718) and *Nitrobacter winogradkyi* (ATCC:25391) were used as positive control for the probes EUB338, Nso1225 and NIT3 respectively.

3.5.2. Bacterial taxonomy – 16SrDNA based identification

Five cultures each showing enhanced ammonium oxidation, nitrite oxidation and coupled ammonium-nitrite oxidation capability were checked for purity and further identified. A brief protocol for the same is given below:

16S rDNA sequencing based bacterial identification can be divided into three phases: i) extraction of bacterial DNA, ii) amplification of 16S rRNA gene by polymerase chain reaction (PCR) and iii) DNA sequencing of the amplified product and phylogenetic analyses of the sequence.

Bacterial DNA was extracted by repeated freezing (-80°C) and heating (at 95°C) of the bacterial cells for 30 min each in Tris buffer at pH 8. The cells were centrifuged at 10,000 x g for 10 min at 4°C to separate the cells. To the

supernatant, few microlitres of proteinase K, DNAase free RNAase and SDS were added and incubated at 37°C for half an hour. Further extraction and precipitation was done following Sambrook *et al* [1989]. An approximately 1500 bp-segment of the 16S rRNA gene was amplified by PCR with a forward primer pA (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer pH' (5'-AAGGAGGTGATCCAGCCGCA-3'). The PCR cycle involved denaturing of the strand at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing (for 60 seconds at 55°C), extension (for 90 seconds at 72°C) and denaturing at 95°C (for 1 min). A final extension for 10 min at 72°C was carried out before the amplified sequences were loaded on 0.8% agarose gel and separated by gel electrophoresis. The gel was then immersed in ethidium bromide for 2 h and the products were detected using a transilluminator. The PCR products were then purified. DNA sequence and phylogenetic analyses were carried out using a DNA sequencing machine (Applied Biosystems). The sequences obtained were aligned and compared with those available in the Genebank of the National Centre for Biotechnology Information (NCBI), USA [Altschul *et al* 1990].

3.6. Experiments – factors influencing nitrification

Sediment cores from both the control and experimental sites were collected during the post-monsoon season (October) as described earlier [Section 3.2]. Each sediment core was transferred to a separate sterile beaker (500 ml capacity) and 250 ml of autoclaved estuarine water was added to form

slurry. For dissolved oxygen amendment studies the beaker capacity was 1000 ml and the volume of autoclaved estuarine water used was 600 ml. In the present study ammonium [section 3.6.1.], nitrite [section 3.6.2.], nitrate [section 3.6.3.], organic loading [section 3.6.4.], dissolved oxygen [section 3.6.5.], liquid hydrocarbon - diesel [section 3.6.6.], pesticide - chloropyrifos [section 3.6.7.] and fertilizer - NPK 80:40:30 [section 3.6.8.] were used as amendments in the slurry described above to study their potential impacts on nitrification rates. All the amendments were made at three final concentrations (levels) as given in sections 3.6.1-3.6.5 with controls (unammended). After amendments adequate care was taken to gently mix the slurry to homogenize the contents.

For all the three levels of amendments and the control, three sets of incubations were carried out viz., - 1) Nitrification was inhibited by adding Allyl thio urea to a final concentration of 86 μM [Ginestet *et al*, 1998], 2) Denitrification was inhibited by adding Chloramphenicol to a final concentration of 300 mg L^{-1} [Brooks *et al*, 1992] and 3) Sub-samples of third set were monitored without adding any inhibitors. All incubations were closed (without exchange of water or other nutrients) and conducted in the dark at 28±2°C. Flow chart for the experimental set up is shown in Figure 4.

The initial concentrations of ammonium, nitrite and nitrate in the pore water were analyzed. Further, all the experimental and control systems, except for dissolved oxygen amendment studies [section 3.6.5] were sampled at 2-day interval for 20 days. For dissolved oxygen amendment studies, samples were collected at 24-hour intervals for 8 days. A poly vinyl chloride (PVC) cylinder (2.5

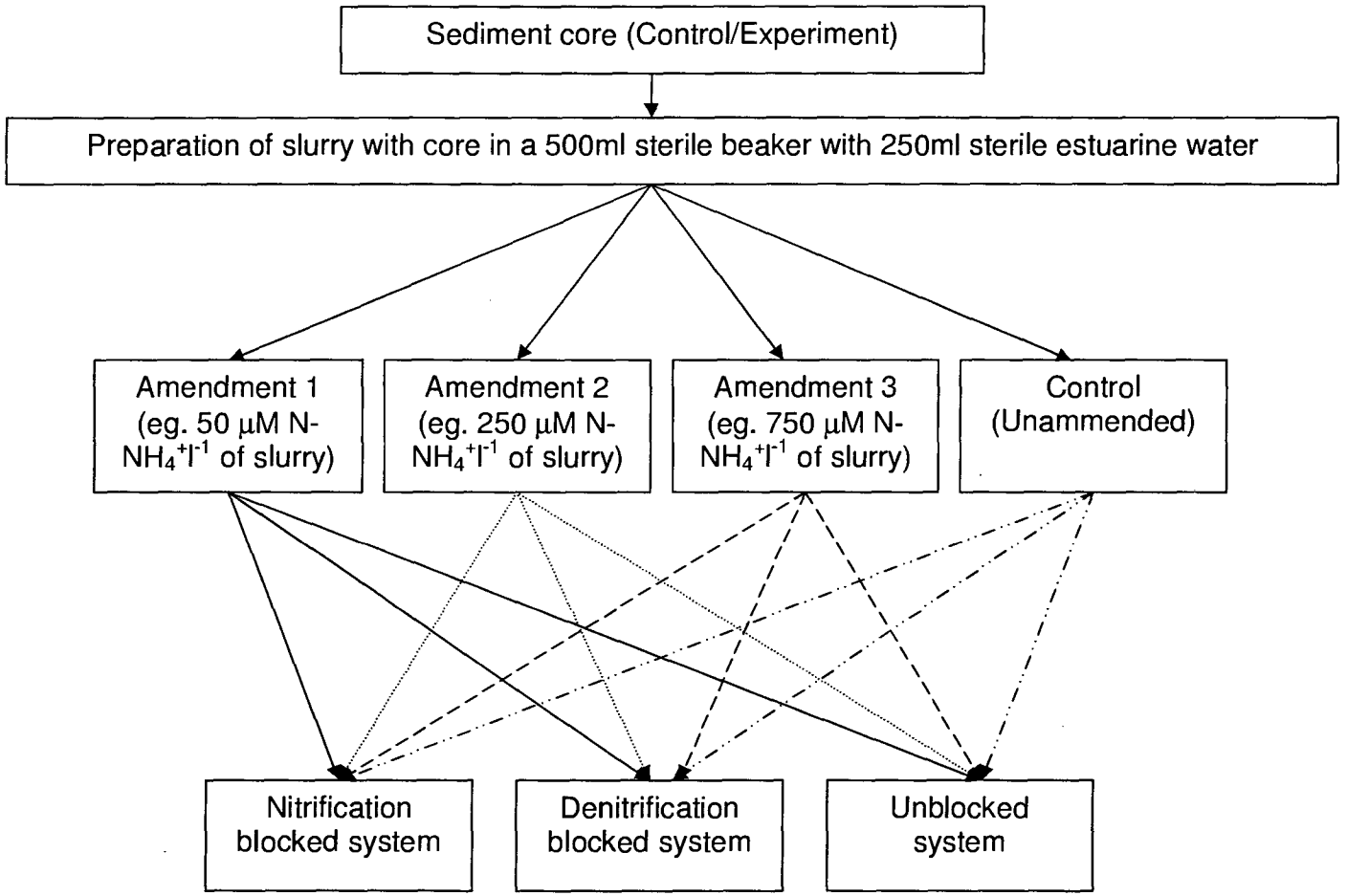


Figure 4: Flowchart of protocol for experiments designed to understand the influence of various abiotic parameters on nitrification rates

cm diameter and 10 cm height) was pushed into the sediment of the microcosms where it remained till the end of the experiment. Inside this PVC cylinder a second cylinder of polymethylmethacrylatye (1 cm diameter and 10 cm height) was pushed and used to take a sediment core. In this way the sediment profile in the system was not disturbed. Pore water was extracted [as mentioned in section 3.3.2.1] and concentrations of ammonium [as mentioned in section 3.3.2.2], nitrite [as mentioned in section 3.3.2.2] and nitrate [as mentioned in section 3.3.2.2] were measured. Variations in the abundance of nitrifiers were monitored in the incubations where denitrification was blocked as previously describe in section 3.4.2. Only the rates are presented in the results section.

3.6.1. Ammonium

In addition to the ammonium present in the pore water at the beginning of incubation, ammonium was added to the system as ammonium chloride solution at three different concentrations viz, ~50, 100 and 150 μM $N\text{-}NH_4^+ l^{-1}$ of slurry.

3.6.2. Nitrite

In addition to the nitrite present in the pore water at the beginning of incubation, nitrite was added to the system as sodium nitrite solution at three different concentrations viz, ~5, 15 and 20 μM $N\text{-}NO_2^- l^{-1}$ of slurry.

3.6.3. Nitrate

In addition to the nitrate present in the pore water at the beginning of incubation, nitrate was added to the system as sodium nitrate solution at three different concentrations viz, ~10, 25 and 50 μM $N\text{-}NO_3^- l^{-1}$ of slurry.

3.6.4. Dissolved organic carbon

Glucose is one of the most assimilable forms of organic carbon having immediate impact on growth and metabolism of microbes in the experiments defined in the previous section [3.6]. Hence glucose was added to the system at three different concentrations viz. 10, 25 and 50 mg glucose-C l^{-1} of the sediment slurry.

3.6.5. Dissolved oxygen

For altering the levels of dissolved oxygen in the overlying water of the sediment slurry, differential aeration process was employed. One set was left as it is without any aeration till the end of the experiment (8 days). The second set was aerated with a one-hour on/off cycle and the third set was aerated continuously till the end of the experiment. Aeration was done using commercially available aerators and care was taken to switch off the aerators approximately one minute prior to sediment sampling.

3.6.6. Liquid hydrocarbon

Commercial diesel (density of 0.820 kg/L at 15°C) was used to study the effects of liquid hydrocarbon on nitrification rates. Diesel was preferred in the study as it is used as fuel in fishing trawlers, barges and other vehicles used for water transport. The different concentrations at which diesel was used were 10, 50 and 100 mg/g wet weight of the sediment.

3.6.7. Pesticide

Chloropyrifos is a toxic crystalline organophosphate insecticide that inhibits acetylcholinesterase and is being used to control insect pests. It is one of the most widely used organophosphate insecticides in India. Chloropyrifos used in the present study has a minimum purity level of 94% and exists as a semi solid mass. The different concentrations at which the pesticide was used were 10, 50 and 100 ppm of the sediment slurry.

3.6.8. Fertilizer

The fertilizer used in the present study is NPK 80:40:30, which contain ~53% nitrogen, 27% phosphates and 20% potassium. The different concentrations at which the fertilizer was used were 1, 5 and 10% w/w the sediment in the slurry.

3.7. Statistical tools

Measures of Central Tendency, Analysis of Variance (ANOVA), and Correlation coefficient were done using the statistical package of Microsoft Office Excel 2003 and Multiple Regression Analysis was done using SPSS for Windows (Release 7.5.1). Guidelines for interpretation of statistical data were obtained from Bailey [2004].

Chapter 4.
Results

4.1. Hydrological parameters and Sediment geochemistry

4.1.1. Hydrological parameters - salinity and dissolved oxygen

Variation of seasonal averages in salinity at both the control and experimental sites were very large. The seasonal variations followed a well defined seasonal cycle with minimum salinity during the monsoon season, gradually increasing during the post-monsoon and attaining the maximum during the pre-monsoon season (Figure 5a). At control site it ranged from 1.2 (SD± 0.9; n=4) during the monsoon season to 23.7 (SD±6.1; n=4) psu during the pre-monsoon season, while at the experimental site it ranged from 3.2 (SD±3.2; n=4) during the monsoon season to 28.7 (SD± 2.2; n=4) during the pre-monsoon season. With the onset of monsoon the salinity decreased sharply at both the sites. In general, the variation in salinity at both the control and experimental sites were quite comparable.

The changes in dissolved oxygen (DO) concentrations during the study period are expressed as seasonal averages of absolute values. The seasonal arithmetic mean of DO level at the control site ranged from 0.8 during the post-monsoon (SD±1.3, n=4) to 3.6 mlL^{-1} (SD±1.2, n=4) during the monsoon season while at the experimental site it ranged from 1.1 (SD±1.1, n=4) to 3.8 mlL^{-1} (SD± 1.3, n=4), respectively for the same seasons (Figure 5b). In general, it was observed that the DO levels at both the control and experimental sites were higher during the monsoon months in contrast to the pre and post-monsoon months. Irrespective of the sites, the lowest dissolved oxygen was observed during the post-monsoon period at the control site (Figure 5b).

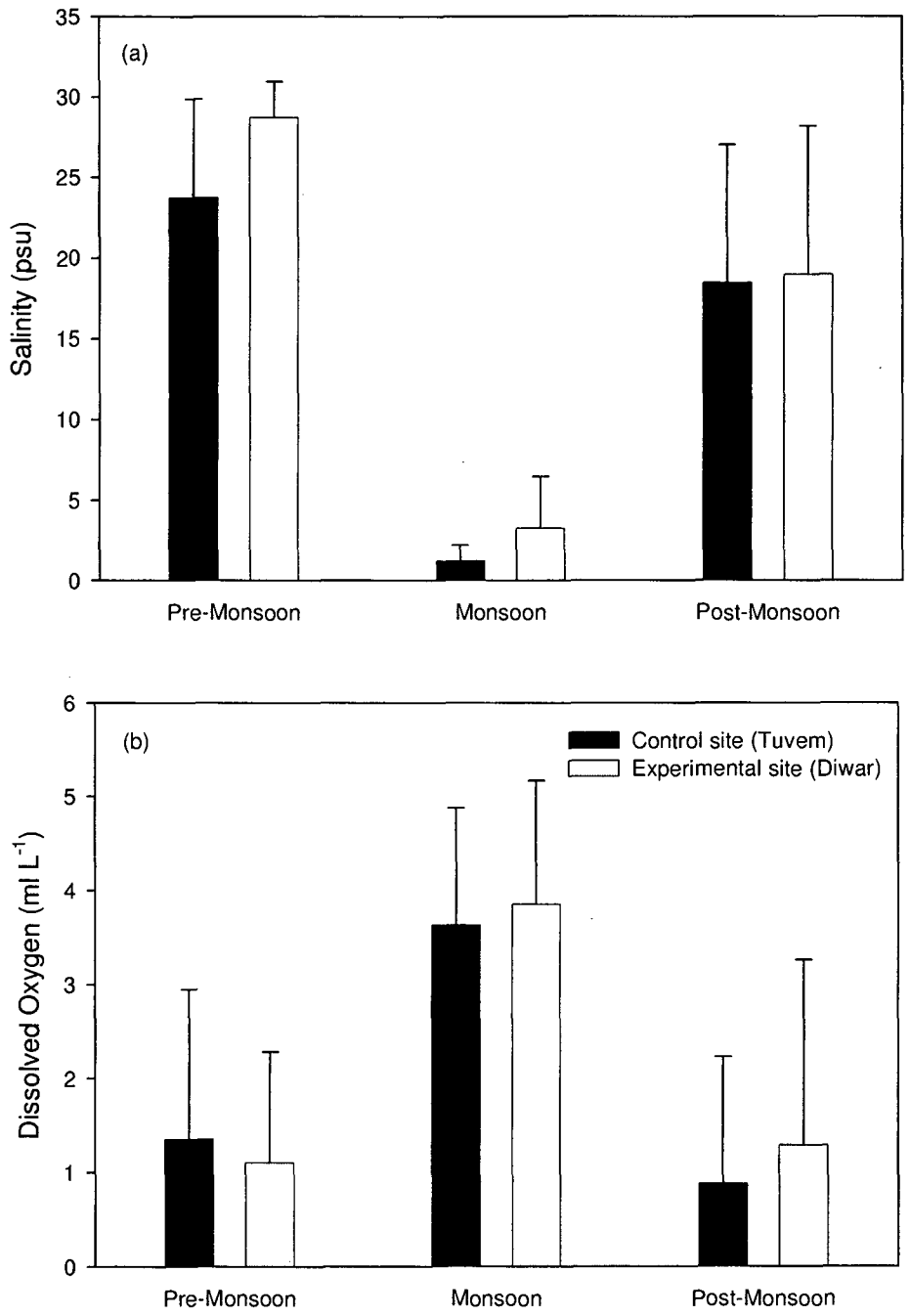


Figure 5: Seasonal variation in salinity (a) and dissolved oxygen (b) of waters overlying the sediment at the control and experimental site. Error bars indicate ± Standard Error.

4.1.2. Sediment geochemistry

4.1.2.1. Temperature, pH and Eh

During the entire period of study, at the control site the down core variation in temperature (Figure 6) ranged from 23.6 to 31.1°C (mean= 27.7 ± 1.8°C; n= 60) while at the experimental site it varied from 25.6 to 31.6 °C (mean= 28±1.7°C; n=60). Analysis of variance (ANOVA) showed that there was a very high significant monthly variation in sediment temperature profiles at both the control ($F = 323.1$, $df = 11$, $p = 4.66 \times 10^{-41}$) and experimental sites ($F = 328.9$, $df = 11$, $p = 3.06 \times 10^{-41}$). However, no statistically significant down core variation (annual) was observed in temperature at both the control and experimental sites. Moreover, throughout the period of sampling there was no significant variation in temperature between the control and experimental sites for any of the measured depth intervals. Seasonal and annual arithmetic mean of temperature for different depth intervals at both the control and experimental site are given in Table 2. In general it could be observed that at both the control and experimental sites, the pre-monsoon months were warmer than the monsoon and post-monsoon months. The post-monsoon months were the coolest.

Monthly, down core variation in pH is illustrated in Figure 7. At the control site the down core variation in pH ranged from 5.57 to 7.32 (mean = 6.52±0.56; n=60), while at the experimental site it varied from 6.08 to 7.49 (mean = 6.77±0.26; n=60). ANOVA showed a highly significant monthly variation in sediment pH profiles at both the control ($F= 70.2$, $df = 11$, $p = 9.84 \times 10^{-26}$) and experimental ($F = 6.2$, $df = 11$, $p = 2.82 \times 10^{-6}$) sites. There was no statistically

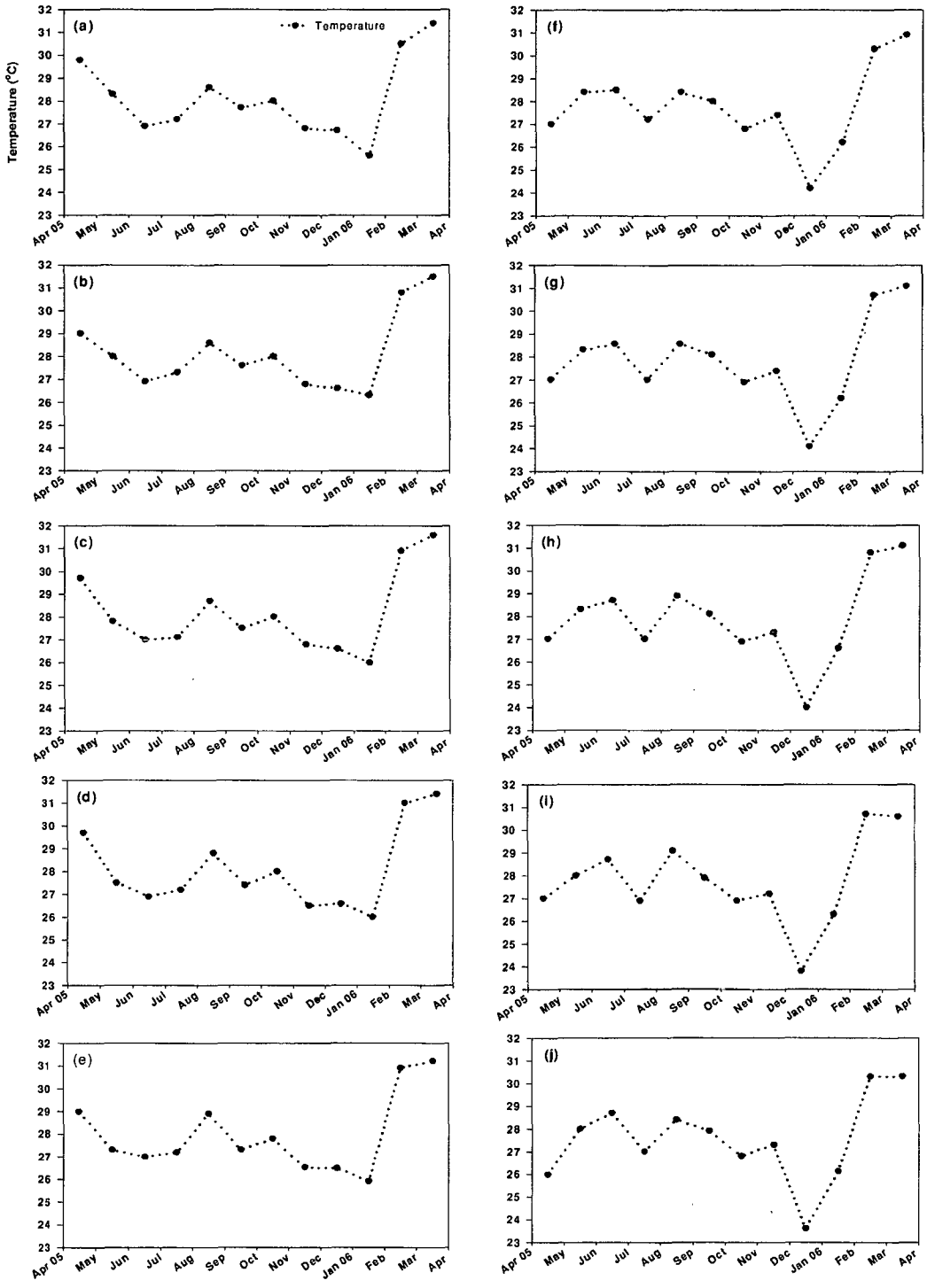


Figure 6: Monthly down core variation of temperature at control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm.

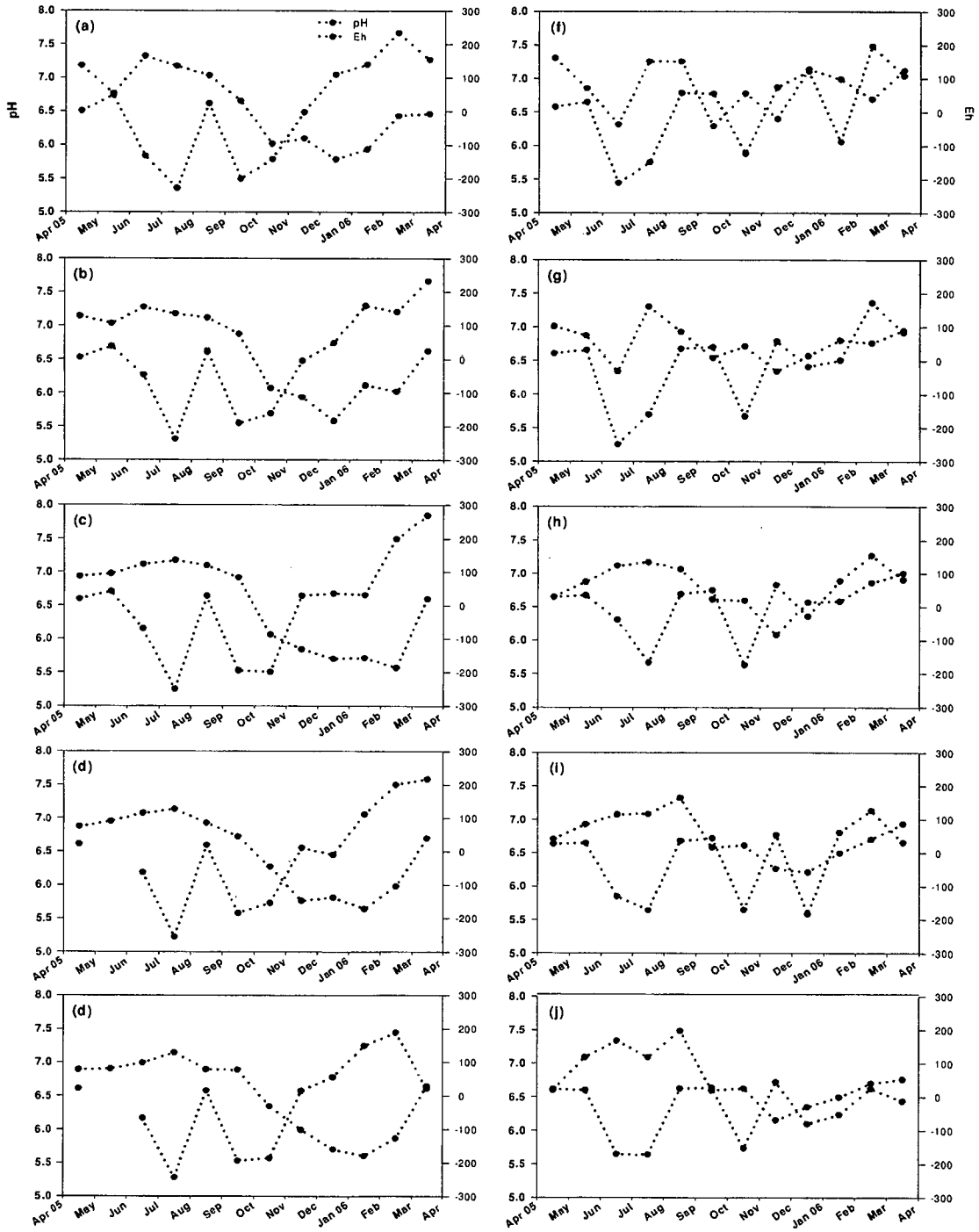


Figure 7: Monthly down core variation of pH and Eh (mV) at control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm. Primary y and secondary y axis in all cases refer to pH and Eh respectively.

Table 2: Seasonal and annual arithmetic mean±standard error of temperature (°C) at different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	30.0 ± 1.3	27.6 ± 0.7	26.7 ± 0.9	28.1 ± .6
2 to 4	29.8 ± 1.6	27.6 ± 0.7	26.9 ± 0.7	28.1 ± 1.5
4 to 6	30 ± 1.6	27.5 ± 0.7	26.8 ± 0.8	28.1 ± 1.6
6 to 8	29.9 ± 1.7	27.5 ± 0.8	26.7 ± 0.8	28.0 ± 1.6
8 to 10	29.6 ± 1.8	27.6 ± 0.8	26.6 ± 0.8	27.9 ± 1.6
EXPERIMENTAL SITE (Diwar)				
0 to 2	29.1 ± 1.7	28.0 ± 0.5	26.1 ± 1.3	27.7 ± 1.7
2 to 4	29.2 ± 1.9	28 ± 0.7	26.1 ± 1.4	27.8 ± 1.8
4 to 6	29.3 ± 1.9	28.1 ± 0.8	26.2 ± 1.4	27.8 ± 1.8
6 to 8	29 ± 1.8	28.1 ± 0.9	26 ± 1.5	27.7 ± 1.8
8 to 10	28.6 ± 2	28 ± 0.7	25.9 ± 1.6	27.5 ± 1.7

Table 3: Seasonal and annual arithmetic mean±standard error of pH at different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	6.7 ± 0.3	7.0 ± 0.2	5.9 ± 0.1	6.5 ± 0.5
2 to 4	6.7 ± 0.5	7.1 ± 0.1	5.9 ± 0.2	6.5 ± 0.5
4 to 6	6.5 ± 0.6	7.0 ± 0.1	5.8 ± 0.1	6.4 ± 0.6
6 to 8	6.6 ± 0.4	6.9 ± 0.1	5.8 ± 0.2	6.4 ± 0.5
8 to 10	6.5 ± 0.4	6.9 ± 0.1	5.9 ± 0.3	6.4 ± 0.5
EXPERIMENTAL SITE (Diwar)				
0 to 2	6.9 ± 0.2	6.7 ± 0.5	6.8 ± 0.3	6.8 ± 0.3
2 to 4	6.8 ± 0.1	6.7 ± 0.4	6.6 ± 0.2	6.7 ± 0.2
4 to 6	6.9 ± 0	6.9 ± 0.2	6.4 ± 0.2	6.7 ± 0.3
6 to 8	6.8 ± 0.1	7.0 ± 0.3	6.3 ± 0.1	6.7 ± 0.3
8 to 10	6.7 ± 0.2	7.1 ± 0.3	6.4 ± 0.2	6.7 ± 0.3

significant down core variation at both the control and experimental sites. No significant variation was also seen between the control and experimental sites for any of the measured depth intervals. Seasonal and annual arithmetic mean of pH for different depth intervals at both the control and experimental site are given in Table 3. At the control site, pH appears to be comparatively on the alkaline side during the monsoon months and acidic during non monsoon months. The post - monsoon season is characterized by a considerable decrease in pH. In contrast, at the experimental site, pH values were slightly on the acidic side and comparable throughout the period of sampling.

Down core variation in Eh (Figure 7) at the control site ranged from -256.5 to 266.5 (mean = -5.64±140.9; n = 58), while at the experimental site it varied from -249.4 to 196.7 (mean = -11.66±98.64; n=60). ANOVA showed that there was a very high significant monthly variation in sediment Eh profiles at both the control ($F= 66.7$, df = 11, $p = 1.72 \times 10^{-24}$) and experimental sites ($F= 18.4$, df = 11, $p = 1.12 \times 10^{-13}$) whereas there was no statistically significant down core variation in Eh at both and between the control and experimental sites. Moreover, throughout the period of sampling there was no significant variation in Eh between the control and experimental sites for any of the measured depth intervals. Seasonal and annual arithmetic mean Eh for different depth intervals at both the control and experimental sites are given in Table 4.

4.1.2.2. Ammonium

Pore water ammonium concentrations (Figure 8) varied widely at both the control (5.5 to 32.6 µg at NH_4^+-Nl^{-1}; mean = 17.9±5.2 µg at NH_4^+-Nl^{-1}; n = 60) and

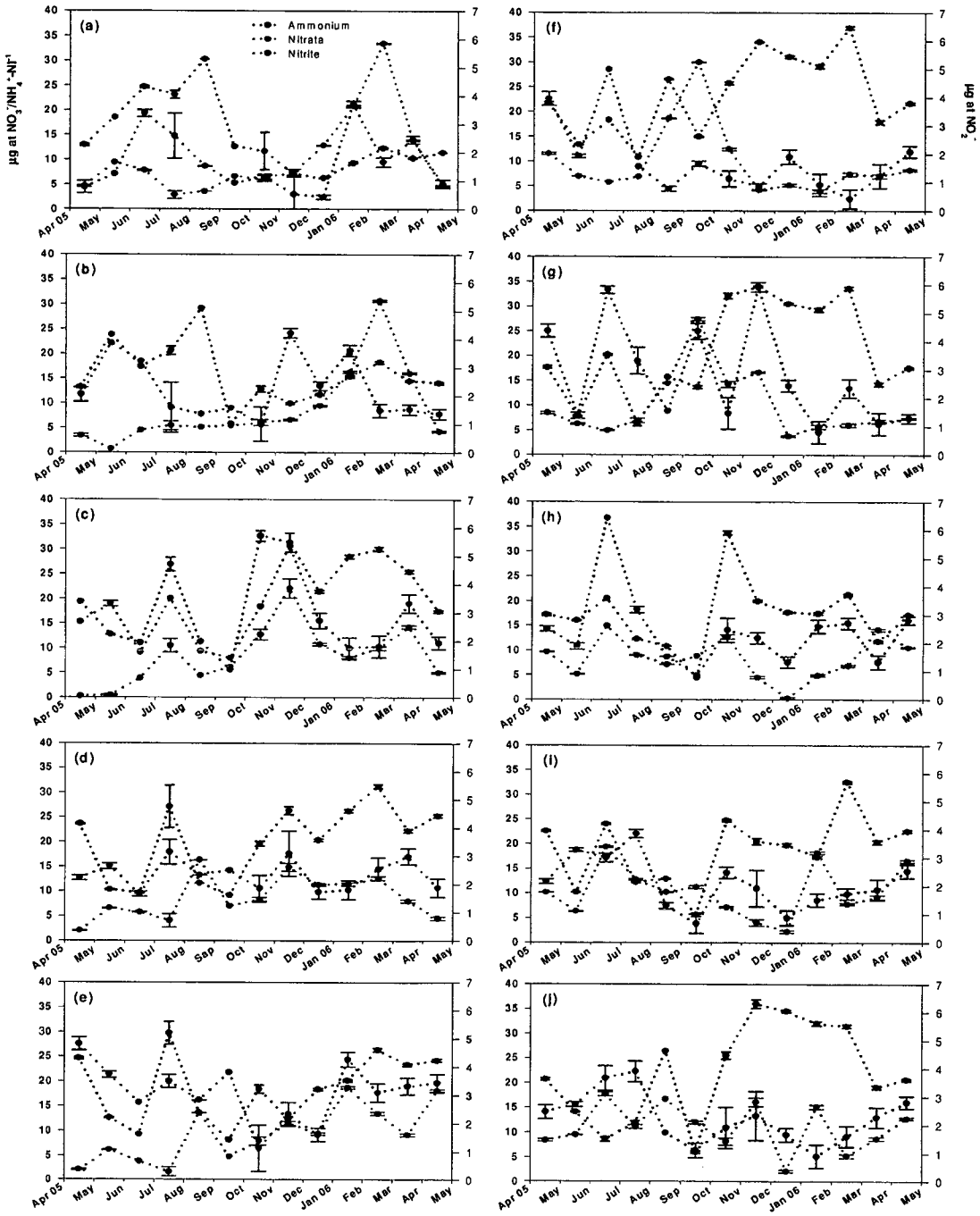


Figure 8: Monthly down core variation of ammonium, nitrite and nitrate at control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm. Error bars ± represent stand error of the mean (n=3). Primary y refers to ammonium and nitrate; and secondary y refers to nitrite.

Table 4: Seasonal and annual arithmetic mean±standard error of Eh (mV) at different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	108.4 ± 105.0	136.4 ± 113.0	24.5 ± 128.2	-1.1 ± 142.8
2 to 4	103.1 ± 102.7	113.8 ± 122.4	9.1 ± 133.6	-0.5 ± 136.9
4 to 6	130.9 ± 120.5	122.9 ± 124.3	-26.8 ± 115.5	-6.3 ± 147.6
6 to 8	145.7 ± 107.5	-122.2 ± 122.4	-11.3 ± 109.5	-8.8 ± 143.7
8 to 10	76.9 ± 96.0	-123.15 ± 118.2	8 ± 141.5	-20.8 ± 132.9
EXPERIMENTAL SITE (Diwar)				
0 to 2	86.8 ± 84	-62.8 ± 138.9	-4.5 ± 120.2	6.4 ± 118.3
2 to 4	76.6 ± 69	-83.5 ± 144.7	-32.1 ± 95.7	-13.0 ± 114.8
4 to 6	74.0 ± 58	-29.9 ± 99.5	-16 ± 115.5	9.3 ± 93.7
6 to 8	52.7 ± 48.7	-56.1 ± 111.6	-60.1 ± 134.8	-21.1 ± 104.8
8 to 10	13.4 ± 17.8	-72.4 ± 114.8	-61.1 ± 81.8	-40.0 ± 80.6

Table 5: Seasonal and annual arithmetic mean±standard error of ammonium concentration (µg at NH_4^+-NL^{-1}) at different intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	13.1 ± 3.1	22.6 ± 7.3	8.5 ± 2.4	14.7 ± 7.1
2 to 4	16.7 ± 4.3	18.2 ± 9.6	12.4 ± 2.2	15.7 ± 3.0
4 to 6	20.9 ± 6.7	13.6 ± 9.1	28.4 ± 5.0	21.0 ± 7.3
6 to 8	22.5 ± 7.6	14.3 ± 8.5	23.1 ± 3.6	19.9 ± 4.9
8 to 10	22.2 ± 5.5	15.1 ± 9.9	17.3 ± 3.4	18.2 ± 3.6
EXPERIMENTAL SITE (Diwar)				
0 to 2	22.2 ± 8.8	17.6 ± 6.6	30.7 ± 4.0	23.5 ± 6.6
2 to 4	18.1 ± 9.5	13.9 ± 5.8	32.3 ± 2.2	21.4 ± 9.6
4 to 6	16.6 ± 3.3	12.0 ± 6.5	22.7 ± 7.4	17.1 ± 5.4
6 to 8	21.6 ± 7.9	13.6 ± 7.6	20.7 ± 3.0	18.6 ± 4.3
8 to 10	21.2 ± 6.3	13.2 ± 9.1	32.4 ± 4.9	22.2 ± 9.6

experimental sites (4.4 to 36.8 µg at NH_4^+-Nl^{-1}; mean = 20.6±7.1 µg at NH_4^+-Nl^{-1}; n=60). ANOVA showed no significant monthly or down core variation at the control site. At the experimental site, although the monthly variation is very significant (F= 8.9, df = 11, p = 5.6 x 10^{-9}), the down core variability was insignificant. Throughout the sampling period there was a significant variation in ammonium concentration between the control and experimental sites only at the depth interval 0-2 cm (F = 7.9, df = 1, p = 0.009). Seasonal and annual arithmetic mean of ammonium concentrations for different depth intervals at both the control and experimental site are given in Table 5. It was observed that at the experiment site higher ammonium levels were encountered during the post-monsoon months compared to monsoon and pre-monsoon months, where as such a clearly defined trend was not seen at the control site.

4.1.2.3. Nitrite

Pore water nitrite concentrations (Figure 8) varied widely at both the control (0.4 to 5.3 µg at NO_2^--Nl^{-1}, mean = 2.3±0.4µg at NO_2^--Nl^{-1}, n=60) and experimental sites (0.4 to 6.4 µg at NO_2^--Nl^{-1}, mean = 2.2±0.5 µg at NO_2^--Nl^{-1}, n = 60). ANOVA did not show any significant monthly variation at the control site, but showed a significant down core variation (F = 3.9, df = 4, p-=0.0065). At the experimental site, on the other hand a significant monthly variation (F = 4.5, df = 11, p = 6.1 x 10^{-5}) was seen but no down core variation was seen. Throughout the period of sampling a significant variation in nitrite concentration between the control and experimental sites was observed at 8-10 cm (F = 5.4, df = 1, p = 0.028) depth interval. Seasonal and annual arithmetic mean of nitrite

concentrations for different depth intervals at both the control and experimental site are given in Table 6.

4.1.2.4. Nitrate

Pore water nitrate concentrations (Figure 8) varied widely at both the control (0.2 to 33.4 μg at NO_3^--Nl^{-1}; mean = 9±3.3 μg at NO_3^--Nl^{-1}; n = 60) and experimental sites (0.2 to 29.8 μg at NO_3^--Nl^{-1}; mean = 10±3.1μg at NO_3^--Nl^{-1}, n=60). ANOVA showed a significant monthly variation (F=4.9, df = 11, p = 2.9 x 10^{-5}) at the control site, with no significant down core variation. Similarly, at the experimental site too there was significant monthly variation (F = 2.84, df = 11, p = 0.004), with very little down core variation. Throughout the period of sampling no significant variation in nitrate concentration was observed between the control and experimental sites for any of the measured depth intervals. Seasonal and annual arithmetic mean of nitrate for different depth intervals at the control and experimental sites are given in Table 7. In general, there was a decrease in nitrate values at the control site during the monsoon months when compared to pre and post-monsoon values.

4.1.2.5. Total Organic Carbon

Total Organic Carbon (TOC) varied widely (Figure 9) at both the control (0.02 to 6.9%; mean = 2.4±1.1%; n = 60) and experimental sites (0.1 to 6.5%; mean = 2.7±0.8; n = 60). ANOVA showed a very significant monthly variation (F = 10.4; df = 11; p = 4.8 x 10^{-10}) at control and experiment sites, but with low down core variation (F = 13.6; df = 11; p = 3.7 x 10^{-12}) Throughout the period of sampling, TOC differed significantly between the control and experimental sites

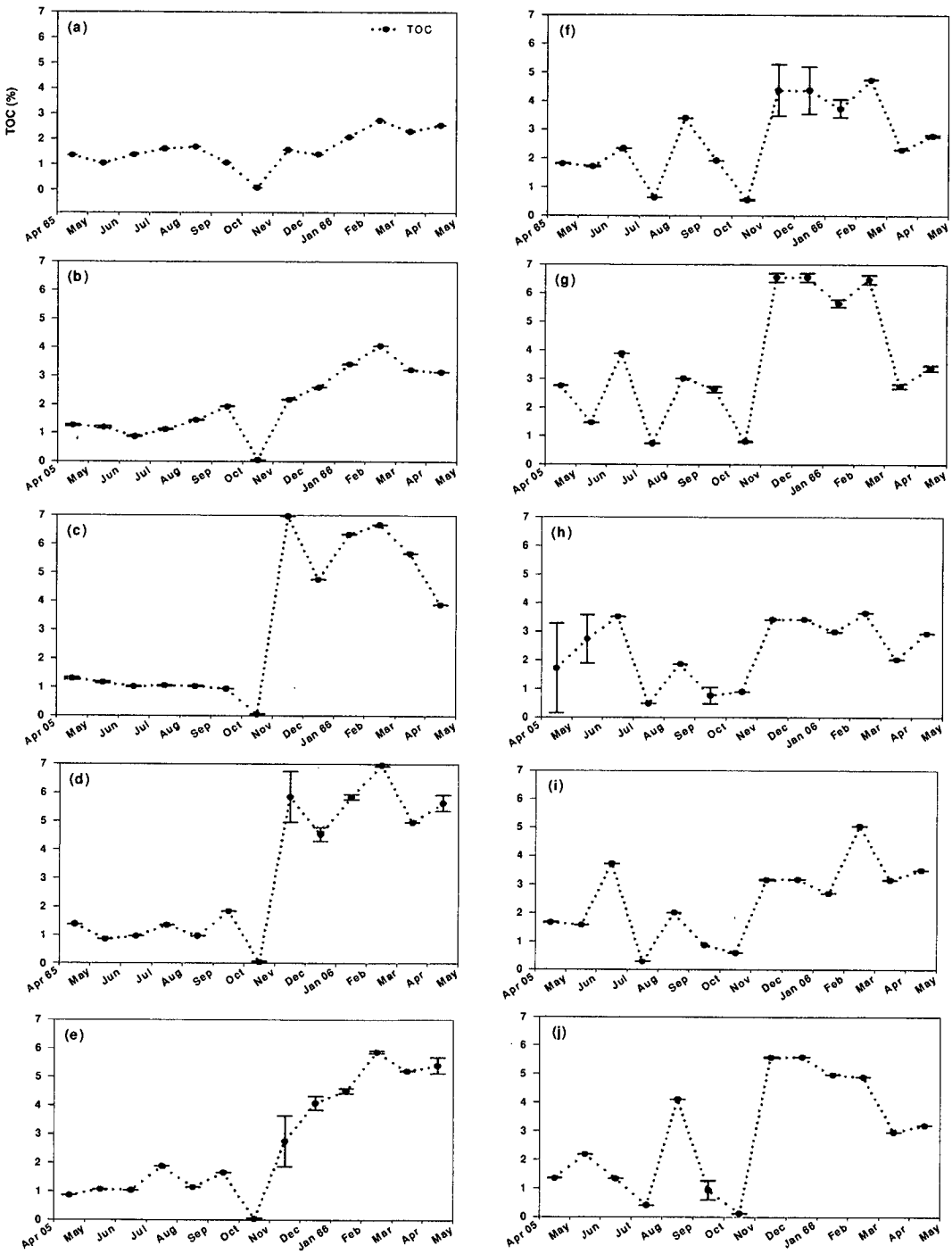


Figure 9: Monthly down core variation of total organic carbon at control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm. Error bars ± represent stand error of the mean (n=3).

Table 6: Seasonal and annual arithmetic mean±standard error of nitrite concentration (µg at NO_2^--NL^{-1}) at different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	1.4 ± 0.6	2.0 ± 1.0	1.4 ± 1.5	1.6 ± 0.3
2 to 4	2.0 ± 1.0	1.9 ± 0.8	2.7 ± 1.4	2.2 ± 0.4
4 to 6	2.6 ± 0.7	2.0 ± 0.9	3.2 ± 1.5	2.6 ± 0.6
6 to 8	2.4 ± 0.4	2.3 ± 0.6	2.1 ± 0.6	2.3 ± 0.1
8 to 10	3.6 ± 0.6	3.2 ± 0.5	2.3 ± 1.3	3.0 ± 0.6
EXPERIMENTAL SITE (Diwar)				
0 to 2	1.9 ± 1.3	2.2 ± 1.8	1.1 ± 0.4	1.7 ± 0.5
2 to 4	2.0 ± 1.3	3.7 ± 1.7	2.6 ± 2.2	2.8 ± 0.8
4 to 6	2.2 ± 0.6	2.9 ± 2.4	2.1 ± 0.5	2.4 ± 0.4
6 to 8	2.3 ± 0.6	2.2 ± 1.4	1.6 ± 0.6	2.0 ± 0.3
8 to 10	2.3 ± 0.4	2.6 ± 1.3	1.7 ± 0.6	2.2 ± 0.4

Table 7: Seasonal and annual arithmetic mean±standard error of nitrate concentration (µg at NO_3^--NL^{-1}) at different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-Monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	13.1 ± 11.9	5.1 ± 2.3	11.7 ± 6.6	10.0 ± 4.2
2 to 4	10.9 ± 12.4	5.0 ± 0.4	9.5 ± 4.6	8.5 ± 3.0
4 to 6	5.9 ± 6.08	6.2 ± 2.9	13.3 ± 6.0	8.5 ± 4.1
6 to 8	6.7 ± 3.9	8.2 ± 5.4	11.4 ± 2.6	8.7 ± 2.4
8 to 10	9.7 ± 6.2	5.9 ± 5.2	11.8 ± 4.8	9.1 ± 3.0
EXPERIMENTAL SITE (Diwar)				
0 to 2	8.1 ± 1.9	15.2 ± 11.3	6.4 ± 4.0	9.9 ± 4.6
2 to 4	6.9 ± 0.9	13.4 ± 10.2	10.0 ± 6.3	10.1 ± 3.2
4 to 6	9.2 ± 3.4	9.9 ± 3.4	5.6 ± 5.2	8.2 ± 2.3
6 to 8	9.9 ± 3.9	13.3 ± 4.0	7.8 ± 7.1	10.4 ± 2.7
8 to 10	8.9 ± 2.7	14.4 ± 3.3	10.4 ± 6.5	11.2 ± 2.8

Table 8: Seasonal and annual arithmetic mean±standard error of Total Organic Carbon content (%) at different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	1.9 ± 0.7	1.4 ± 0.2	1.2 ± 0.8	1.5 ± 0.3
2 to 4	2.5 ± 1.2	1.3 ± 0.4	2.0 ± 1.4	1.9 ± 0.6
4 to 6	3.7 ± 2.4	1.0 ± 0.0	4.5 ± 3.1	3.0 ± 1.8
6 to 8	3.9 ± 2.6	1.2 ± 0.4	4.0 ± 2.7	3.0 ± 1.5
8 to 10	3.6 ± 2.4	1.4 ± 0.3	2.8 ± 2.0	2.6 ± 1.1
EXPERIMENTAL SITE (Diwar)				
0 to 2	2.6 ± 1.2	2.0 ± 1.1	3.2 ± 1.8	2.6 ± 0.5
2 to 4	3.3 ± 1.8	2.5 ± 1.3	4.8 ± 2.7	3.6 ± 1.1
4 to 6	2.6 ± 0.7	1.6 ± 1.3	2.6 ± 1.2	2.3 ± 0.5
6 to 8	2.9 ± 1.4	1.7 ± 1.5	2.4 ± 1.2	2.3 ± 0.6
8 to 10	2.9 ± 1.3	1.6 ± 1.6	4.0 ± 2.6	2.8 ± 1.1

at depth intervals 0-2 ($F = 6.1$; $df = 1$; $p = 0.02$) and 2-4 cm ($F = 15.6$; $df = 1$; $p = 0.02$). Seasonal and annual arithmetic mean of TOC at different depth intervals at both the control and experimental sites are given in Table 8. It could be observed that in general there was a more defined decrease in TOC values at the control site during the monsoon months when compared to pre and post-monsoon values when compared to experimental station.

4.1.2.6. Iron

Iron varied widely (Figure 10) at both the control (1.1 to 12%; mean = 6.7±1.0%; n = 60) and experimental sites (2.9 to 46%; mean = 17.9±7.6%; n = 60). ANOVA showed no significant monthly variation at the control site, but significant down core variation was seen ($F = 3.2$; $df = 4$; $p=0.017$). At the experimental site, on the other hand there was very high significant monthly variation was seen ($F = 11.6$; $df = 11$; $p = 6.5 \times 10^{-11}$) with no significant down core variation. Throughout the period of sampling, there was very highly significant variation in iron content between the control and experimental sites for the all the depth intervals viz. 0-2 ($F = 26.6$; $df = 1$; $p = 2.7 \times 10^{-5}$), 2-4 ($F = 17.4$; $df = 1$; $p = 3.4 \times 10^{-4}$), 4-6 ($F = 13.1$; $df = 1$; $p = 1.3 \times 10^{-3}$), 6-8 ($F = 20.0$; $df = 1$; $p = 1.5 \times 10^{-4}$) and 8-10 cm ($F = 13.5$; $df = 1$; $p = 1.1 \times 10^{-3}$). Seasonal and annual arithmetic mean of iron content for different depth intervals at both the sites are given in Table 9. It could be observed that in general there is an enrichment of iron (a maximum of >160% during the pre-monsoon season) at the experimental site when compared to the control site. In addition at the experimental site, the

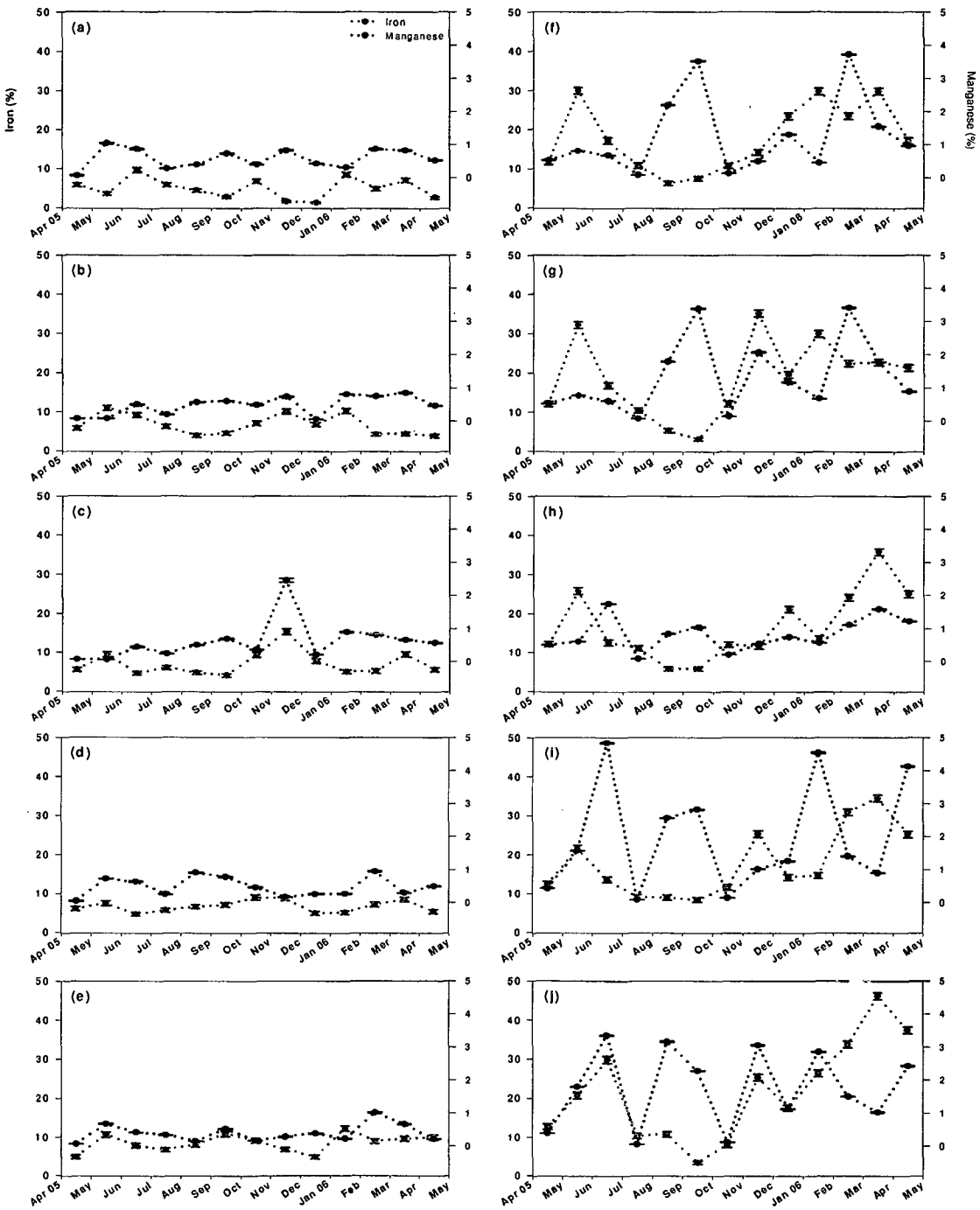


Figure 10: Monthly down core variation of iron and manganese at control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm. Error bars ± represent stand error of the mean (n=3). Primary y and secondary y in all cases refer to iron and manganese respectively.

Table 9: Seasonal and annual arithmetic mean±standard error of iron content (%) at different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	4.6 ± 1.7	5.5 ± 2.9	4.4 ± 3.7	4.8 ± 0.5
2 to 4	5.7 ± 2.9	5.8 ± 2.3	8.4 ± 1.8	6.7 ± 1.5
4 to 6	6.9 ± 2.2	4.7 ± 0.9	9.2 ± 4.3	7.0 ± 2.2
6 to 8	6.8 ± 1.2	5.9 ± 1.0	6.9 ± 2.2	6.5 ± 0.5
8 to 10	8.6 ± 2.2	8.3 ± 1.7	8.0 ± 3.2	8.3 ± 0.2
EXPERIMENTAL SITE (Diwar)				
0 to 2	22.3 ± 7.9	10.3 ± 4.8	19.4 ± 8.6	17.3 ± 6.2
2 to 4	22.0 ± 7.1	8.7 ± 6.1	24.0 ± 10.3	18.3 ± 8.3
4 to 6	24.4 ± 8.4	8.6 ± 3.5	14.4 ± 4.4	15.8 ± 7.9
6 to 8	24.8 ± 8.5	9.9 ± 2.3	16.3 ± 5.9	17.0 ± 7.4
8 to 10	30.0 ± 13.3	13.4 ± 11.3	19.2 ± 8.4	20.9 ± 8.4

Table 10: Seasonal and annual arithmetic mean±standard error of manganese content (%) at different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	0.6 ± 0.3	0.5 ± 0.2	0.4 ± 0.2	0.5 ± 0.0
2 to 4	0.4 ± 0.3	0.4 ± 0.1	0.5 ± 0.3	0.4 ± 0.0
4 to 6	0.4 ± 0.3	0.4 ± 0.1	0.9 ± 1.0	0.6 ± 0.3
6 to 8	0.5 ± 0.3	0.6 ± 0.2	0.2 ± 0.1	0.4 ± 0.1
8 to 10	0.5 ± 0.3	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
EXPERIMENTAL SITE (Diwar)				
0 to 2	1.5 ± 1.2	1.6 ± 1.5	0.5 ± 0.4	1.2 ± 0.5
2 to 4	1.4 ± 1.1	1.4 ± 1.4	1.0 ± 0.8	1.3 ± 0.2
4 to 6	1.0 ± 0.4	0.9 ± 0.6	0.5 ± 0.2	0.8 ± 0.2
6 to 8	1.6 ± 1.4	2.5 ± 1.9	1.7 ± 1.9	1.9 ± 0.4
8 to 10	1.4 ± 0.7	2.2 ± 1.5	1.7 ± 1.4	1.8 ± 0.3

highest enrichment can be seen in the pre-monsoon season, followed by post-monsoon and the monsoon.

4.1.2.7. Manganese

Manganese varied (Figure 10) both at the control (0.04 to 2.4%; mean = 0.5±0.1%; n = 60) and experimental sites (0.06 to 4.8%; mean = 1.4±0.3%; n=60). ANOVA showed that there was a significant monthly variation both at control (F = 2.5; df = 11; p=0.009) and experimental sites (F = 3.2; df = 11; p=0.001) but no significant down core variation at both the sites. Throughout the period of sampling no significant variation was seen between the control and experimental sites for the depth intervals 0-2 and 4-6 cm, but a significant variation existed in other depth intervals, viz. 2-4 (F = 7.4; df =1; p = 1.1 x 10^{-2}), 6-8 (F = 10.2; df =1; p = 3.8 x 10^{-3}) and 8-10 cm (F = 17.3; df =1; p = 3.4 x 10^{-4}). Seasonal and annual arithmetic mean of manganese content for different depth intervals at both control and experimental sites are given in Table 10. It could be observed that in general the enrichment is higher at the experimental site.

4.2. Bacterial abundance and distribution

4.2.1. Total and viable cell counts

Total and viable cell counts are presented in the Figures 11 and 12 respectively. It was observed that both at the control and experimental sites the total cell counts were higher during the monsoon months compared to the non monsoon months and ranged from 10^{9-10} cells g^{-1} wet weight sediment. It was also seen that the abundance, as recorded by total cell counts was the lowest during the post-monsoon season. Seasonal and annual arithmetic mean (± standard error) of total cell counts is presented in Table 11. There was significant monthly variability at both the control ($F=5.7$, $df = 11$, $p = 9.1 \times 10^{-6}$) and experimental sites ($F=3.6$, $df = 11$, $p = 9.3 \times 10^{-4}$) with no down core variation at either of the sites. As was the case with the total cell counts, the abundance of viable fraction (Figure 12) also showed no significant down core variation throughout the period of study. The monthly variability was profound at both the sites with the experimental site showing more variability ($F=8.8$, $df = 11$, $p = 2.8 \times 10^{-8}$) than the control site ($F=4.9$, $df = 11$, $p = 5.1 \times 10^{-5}$). A similar trend was not profound at the control site. However at both the sites, the viable counts were generally higher in the monsoon months and lower during the post-monsoon months as was observed in the case of total counts.

4.2.2. Total heterotrophic bacterial abundance and distribution

Total heterotrophic bacterial counts (Figure 13) varied widely at both the control (non detectable to 5.5×10^{5} CFU g^{-1} wet weight sediment; mean = $0.7 \pm 0.2 \times 10^{5}$ CFU g^{-1} wet weight sediment; $n = 60$) and experimental sites (non

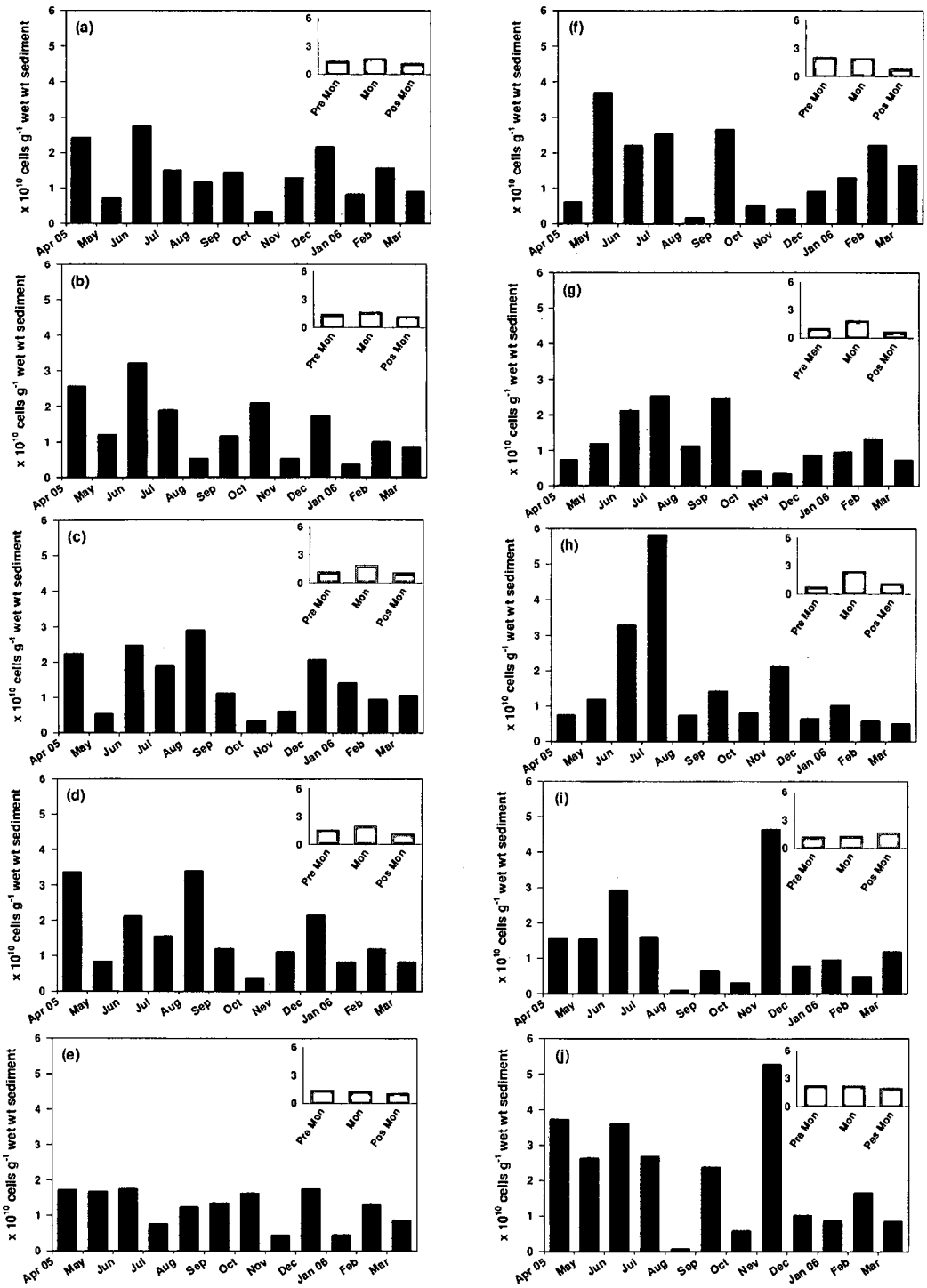


Figure 11: Monthly down core variation of total cell counts at control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm. Seasonal averages are given in the inset.

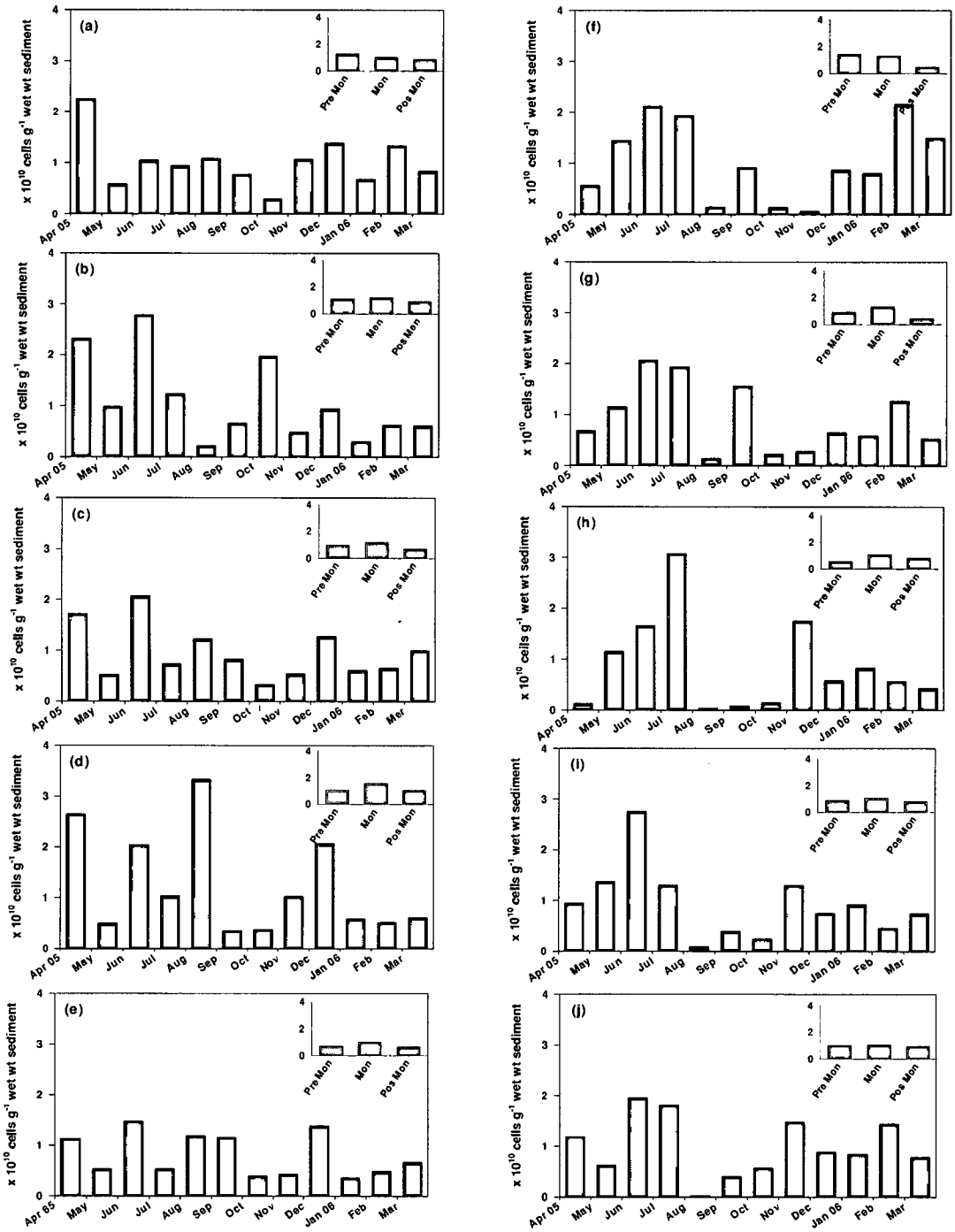


Figure 12: Monthly down core variation of viable cell counts at control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm. Seasonal averages are given in the inset.

Table 11: Seasonal and annual arithmetic mean (± standard error) of Total Bacterial Counts (x 10^{10} cells g^{-1} wet weight sediment) for different depth intervals at the control and experimental site.

CONTROL SITE (Tuvem)				
Depth interval	Pre-monsoon	Monsoon	Post-monsoon	Annual
0 to 2	1.4 ± 0.7	1.7 ± 0.7	1.1 ± 0.7	1.4 ± 0.7
2 to 4	1.4 ± 0.7	1.7 ± 1.1	1.1 ± 0.8	1.4 ± 0.8
4 to 6	1.1 ± 0.7	2.1 ± 0.7	1.1 ± 0.7	1.4 ± 0.8
6 to 8	1.5 ± 1.2	2.0 ± 0.9	1.1 ± 0.7	1.5 ± 0.9
8 to 10	1.3 ± 0.3	1.2 ± 0.4	0.9 ± 0.5	1.2 ± 0.4
EXPERIMENTAL SITE (Diwar)				
0 to 2	2.0 ± 1.2	1.9 ± 1.1	0.7 ± 0.4	1.5 ± 1.1
2 to 4	0.9 ± 0.3	2.0 ± 0.6	0.6 ± 0.3	1.2 ± 0.7
4 to 6	0.7± 0.3	2.8 ± 2.2	1.1 ± 0.6	1.6 ± 1.5
6 to 8	1.2 ± 0.5	1.3 ± 1.2	1.6 ± 2.0	1.4 ± 1.2
8 to 10	2.2 ± 1.2	2.1 ± 1.5	1.9 ± 2.2	2.2 ± 1.5

Table 12: Seasonal and annual arithmetic mean (± standard error) of Total Viable Counts (x 10^{10} cells g^{-1} wet weight sediment) for different depth intervals at the control and experimental site.

CONTROL SITE (Tuvem)				
Depth interval	Pre-monsoon	Monsoon	Post-monsoon	Annual
0 to 2	1.2 ± 0.7	0.9 ± 0.1	0.8 ± 0.4	1.0 ± 0.4
2 to 4	1.1 ± 0.8	1.2 ± 1.1	0.9 ± 0.7	1.1 ± 0.8
4 to 6	0.9 ± 0.5	1.2 ± 0.6	0.6 ± 0.4	0.9 ± 0.5
6 to 8	1.0 ± 1.0	1.6 ± 1.3	1.0 ± 0.7	1.2 ± 1.0
8 to 10	0.7 ± 0.2	1.0 ± 0.3	0.6 ± 0.5	0.8 ± 0.4
EXPERIMENTAL SITE (Diwar)				
0 to 2	1.4 ± 0.6	1.2 ± 0.9	0.4 ± 0.4	1.0 ± 0.7
2 to 4	0.9± 0.3	1.4 ± 0.8	0.4 ± 0.2	0.9 ± 0.6
4 to 6	0.5 ± 0.4	1.2 ± 1.4	0.8 ± 0.6	0.9 ± 0.9
6 to 8	0.8 ± 0.3	1.1 ± 1.2	0.8 ± 0.4	0.9 ± 0.7
8 to 10	1.0± 0.3	1.0 ± 0.9	0.9 ± 0.3	1.0 ± 0.5

detectable to 8.4 x 10^{5} CFU g^{-1} wet weight sediment; mean = 1.3± 0.4 x 10^{5} CFU g^{-1} wet weight sediment; n = 60). ANOVA showed that there was significant monthly variation (F=6.7, df = 12, p = 4.3 x 10^{-7}) at the control site, while there was no significant down core variation. Similarly, at the experimental site also there was significant monthly variation (F = 9.4, df = 12, p = 2.3 x 10^{-9}), while there was no significant down core variation. Throughout the period of sampling no significant variation was noticed in total heterotrophic bacterial counts between the control and experimental sites for any of the measured depth intervals. Seasonal and annual arithmetic mean (± standard error) of total heterotrophic bacterial counts for different depth intervals at both the control and experimental site are given in Table 13. It could be seen that in general there was a decrease in total heterotrophic bacterial counts at the control site during the post-monsoon months as compared to pre-monsoon and monsoon months. Moreover, the total heterotrophic bacterial counts at the experimental site were higher than the control site during all the seasons.

4.2.3. Abundance and distribution of nitrifying bacteria

4.2.3.1. Plate count method

Retrievable nitrifier counts (Figure 14) varied widely at both the control (1.0 to 8.4 x 10^{2} CFU g^{-1} wet weight sediment; mean = 4.3 ± 0.5 x 10^{2} CFU g^{-1} wet weight sediment; n = 65) and experimental sites (1.2 to 7.1 x 10^{5} CFU g^{-1} wet weight sediment; mean = 3.8 ± 0.5 x 10^{2} CFU g^{-1} wet weight sediment; n = 65). ANOVA showed that there was significant monthly variation (F=9.2, df = 12, p = 3.4 x 10^{-9}) at the control site, while there was no significant down core

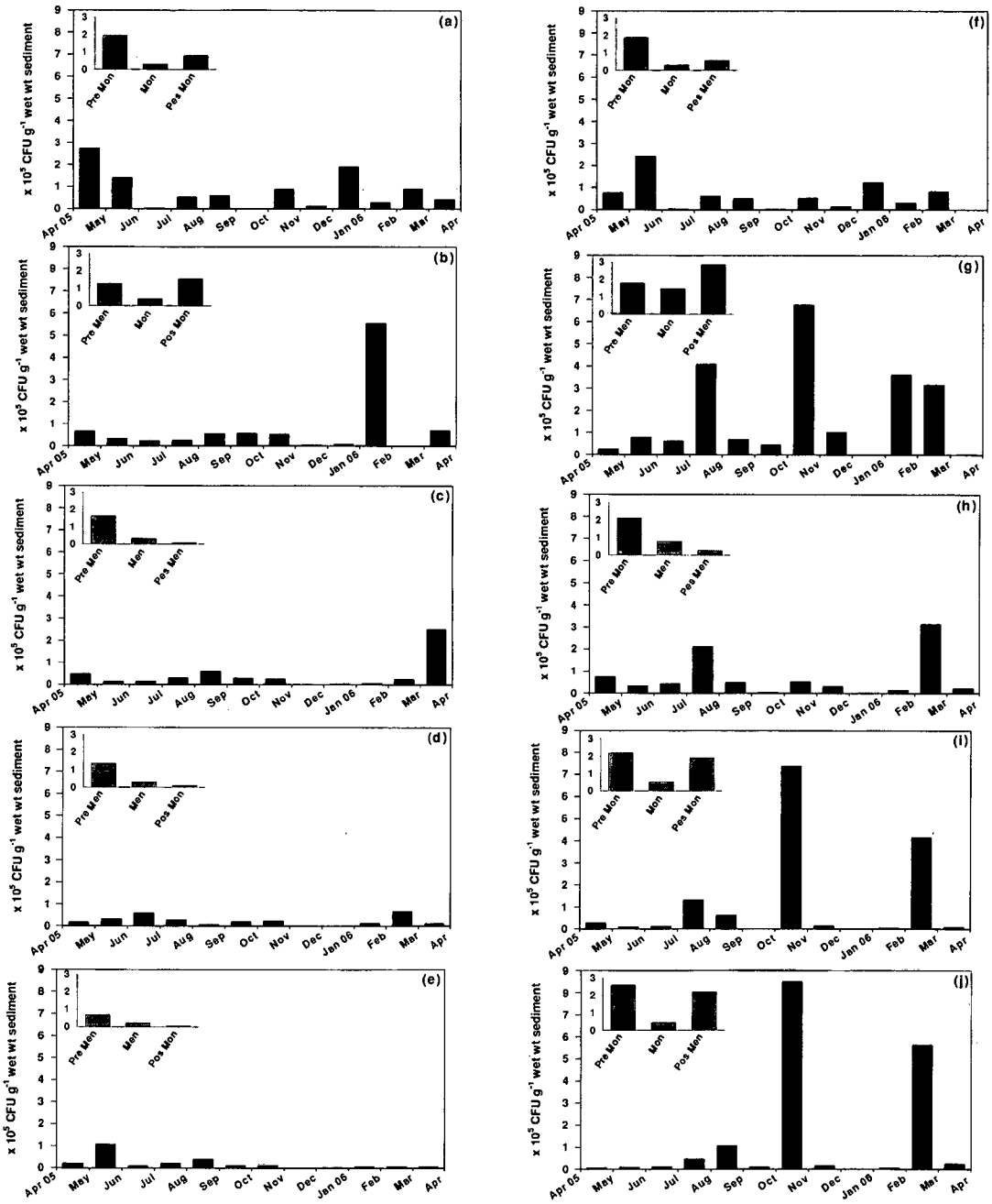


Figure 13: Monthly down core variation in total heterotrophic bacterial counts at control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm. Seasonal averages are given in the inset.

Table 13: Seasonal and annual arithmetic mean (± standard error) of retrievable counts of total heterotrophic bacterial counts (x 10^5 CFU g^{-1} wet weight sediment) for different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	1.9 ± 1.5	0.2 ± 0.3	0.7 ± 0.7	1.0 ± 1.2
2 to 4	1.2 ± 1.9	0.3 ± 0.1	1.5 ± 2.6	1.0 ± 1.8
4 to 6	1.6 ± 1.9	0.3 ± 0.1	0.08 ± 0.1	0.7 ± 1.3
6 to 8	1.3 ± 2.3	0.2 ± 0.2	0.09 ± 0.1	0.6 ± 1.4
8 to 10	0.6 ± 0.8	0.2 ± 0.1	0.05 ± 0.05	0.3 ± 0.5
EXPERIMENTAL SITE (Diwar)				
0 to 2	1.8 ± 2.1	0.2 ± 0.3	0.5 ± 0.4	0.9 ± 1.4
2 to 4	1.7 ± 2.0	1.4 ± 1.7	2.8 ± 3.0	2.0 ± 2.2
4 to 6	2.1 ± 2.5	0.7 ± 0.9	0.2 ± 0.2	1.1 ± 1.7
6 to 8	2.1 ± 2.8	0.5 ± 0.6	1.8 ± 3.6	1.5 ± 2.5
8 to 10	2.5 ± 3.3	0.4 ± 0.4	2.1 ± 4.2	1.7 ± 3.0

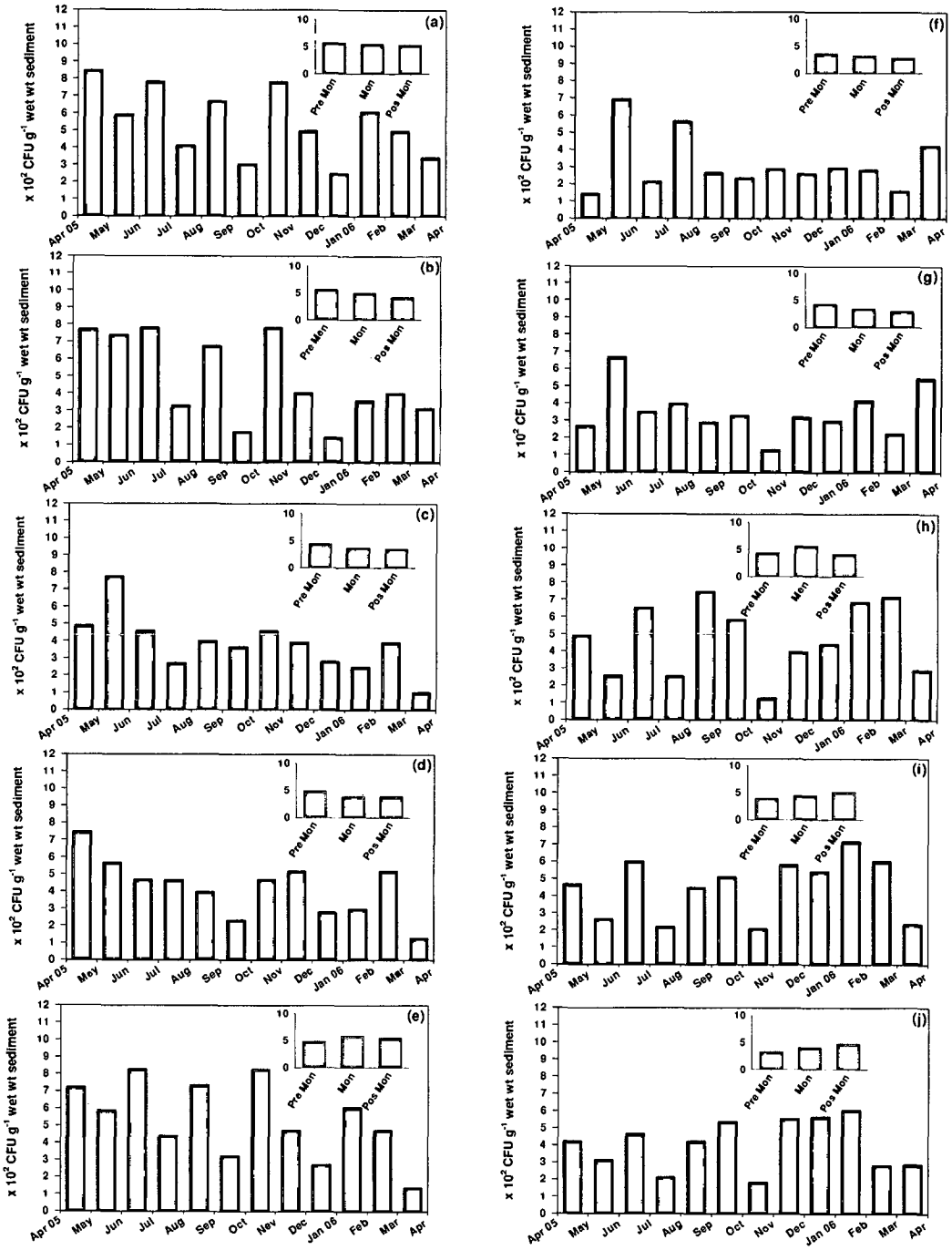


Figure 14: Monthly down core variation of nitrifiers (retrievable plate counts) at the control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm. Seasonal averages are given in the inset.

variation. At the experimental site, there was no significant monthly and down core variations. Throughout the period of sampling there was no significant variation in retrievable nitrifier counts between the control and experimental sites for the measured depth intervals, except for 0-2 cm ($F=8.1$, $df = 1$, $p = 8.8 \times 10^{-3}$). Seasonal and annual arithmetic mean of retrievable nitrifier counts for different depth intervals at both the control and experimental sites are given in Table 14. It could be observed that in general the retrievable nitrifier counts at the control site were higher than the experimental site for all the seasons.

4.2.3.2. Most Probable Number method (MPN)

MPN counts of autotrophic nitrifying bacteria (Figure 15a) varied widely at both the control (0.02 to 3.5×10^{2} cells g^{-1} wet weight sediment; mean = $0.6 \pm 0.2 \times 10^{2}$ cells g^{-1} wet weight sediment; $n = 65$) and experimental sites (0.01 to 10.1×10^{2} cells g^{-1} wet weight sediment; mean = $1.0 \pm 0.3 \times 10^{2}$ cells g^{-1} wet weight sediment; $n = 65$). ANOVA showed no significant monthly and down core variation at the control site, while a significant monthly variation was seen at the experimental site ($F=6.1$, $df = 12$, $p = 1.3 \times 10^{-6}$), but no significant monthly nor down core variation. Throughout the period of sampling there was no significant variation in MPN counts of autotrophic nitrifying bacteria between the control and experimental sites for any of the measured depth intervals. Seasonal and annual arithmetic mean (± standard error) of MPN counts of autotrophic nitrifying bacteria for different depth intervals at both the control and experimental site are given in Table 15a.

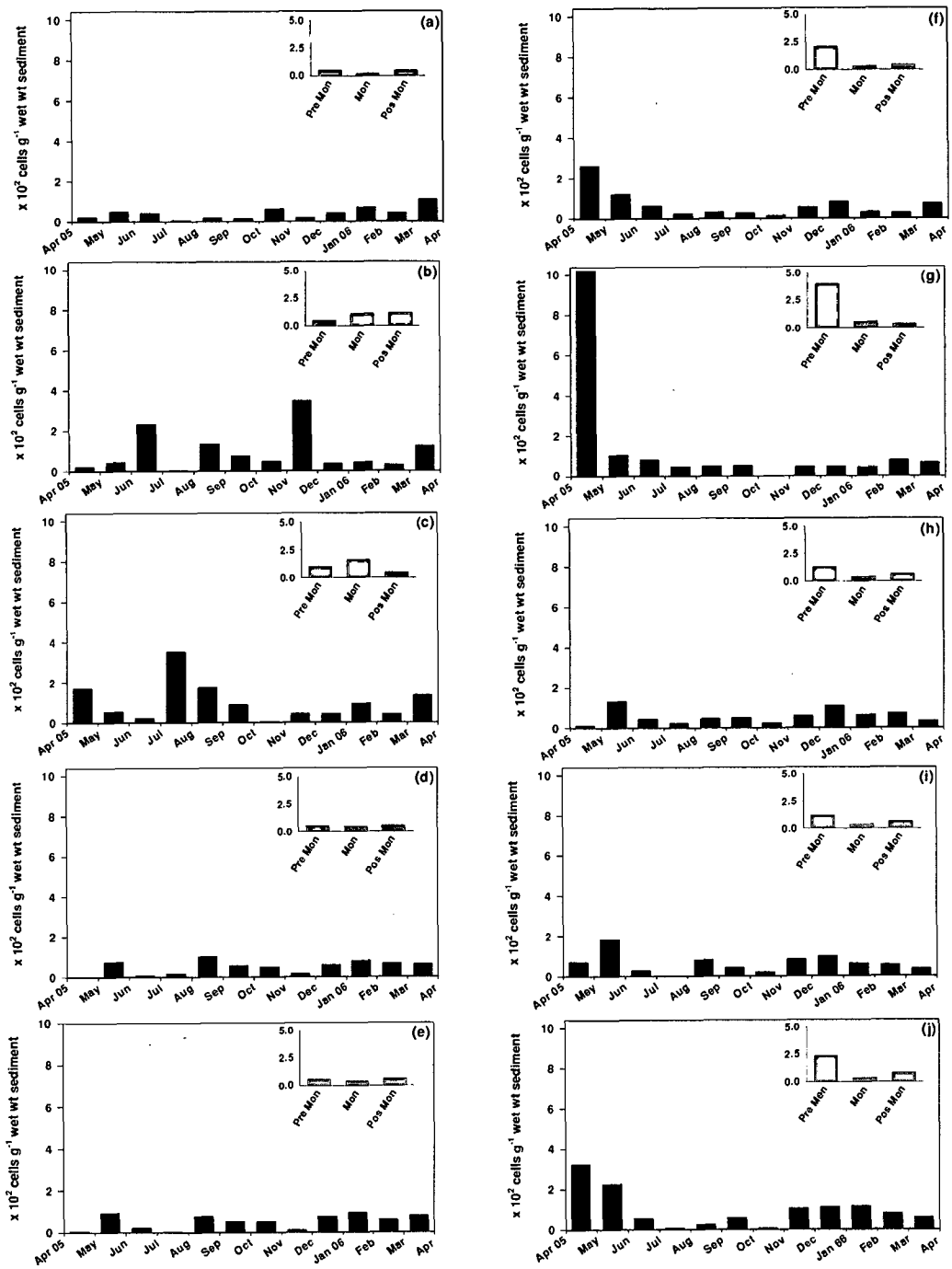


Figure 15a: Monthly down core variation of autotrophic nitrifiers (MPN) at control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm. Seasonal averages are given in the inset.

Table 14: Seasonal and annual arithmetic mean (± standard error) of retrievable counts of nitrifying bacteria (x 10^2 CFU g^{-1} wet weight sediment) for different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	5.4 ± 1.9	5.4 ± 2.2	5.3 ± 2.2	5.3 ± 1.9
2 to 4	5.3 ± 2.0	4.9 ± 2.8	4.2 ± 2.6	4.8 ± 2.3
4 to 6	4.4 ± 2.4	3.7 ± 0.7	3.4 ± 0.9	3.9 ± 1.5
6 to 8	5.0 ± 2.2	3.9 ± 1.1	3.9 ± 1.2	4.3 ± 1.6
8 to 10	4.2 ± 2.5	5.8 ± 2.3	5.4 ± 2.3	5.1 ± 2.3
EXPERIMENTAL SITE (Diwar)				
0 to 2	3.9 ± 2.3	3.2 ± 1.6	2.8 ± 0.1	3.3 ± 1.6
2 to 4	4.3 ± 1.8	3.4 ± 0.4	2.9 ± 1.1	3.6 ± 1.4
4 to 6	4.7 ± 2.0	5.6 ± 2.1	4.1 ± 2.2	4.8 ± 2.0
6 to 8	4.3 ± 1.8	4.4 ± 1.6	5.1 ± 2.1	4.6 ± 1.7
8 to 10	3.9 ± 1.6	4.0 ± 1.4	4.7 ± 1.9	4.2 ± 1.5

Table 15a: Seasonal and annual arithmetic mean (± standard error) of Most Probable Number counts of autotrophic nitrifying bacteria (x 10^2 CFU g^{-1} wet weight sediment) for different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	0.4 ± 0.3	0.1 ± 0.1	0.4 ± 0.2	0.3 ± 0.2
2 to 4	0.4 ± 0.4	1.1 ± 0.9	1.1 ± 1.5	0.8 ± 0.9
4 to 6	0.9 ± 0.5	1.6 ± 1.4	0.4 ± 0.3	1.0 ± 0.9
6 to 8	0.5 ± 0.2	0.4 ± 0.4	0.5 ± 0.2	0.4 ± 0.2
8 to 10	0.5 ± 0.3	0.3 ± 0.3	0.5 ± 0.3	0.4 ± 0.3
EXPERIMENTAL SITE (Diwar)				
0 to 2	2.0 ± 2.1	0.3 ± 0.1	0.4 ± 0.2	1.0 ± 1.5
2 to 4	3.9 ± 4.4	0.5 ± 0.1	0.3 ± 0.2	1.8 ± 3.1
4 to 6	1.2 ± 1.5	0.4 ± 0.1	0.6 ± 0.3	0.8 ± 0.9
6 to 8	1.1 ± 0.9	0.3 ± 0.3	0.6 ± 0.3	0.7 ± 0.6
8 to 10	2.3 ± 1.7	0.3 ± 0.2	0.8 ± 0.5	1.2 ± 1.4

MPN counts of heterotrophic nitrifying bacteria (Figure 15b) showed wide variation at both the control (0.4 to 6.6 x 10^3 cells g^{-1} wet weight sediment; mean = 2.2 ± 0.4 x 10^3 cells g^{-1} wet weight sediment; n = 65) and experimental sites (0.3 to 5.9 x 10^3 cells g^{-1} wet weight sediment; mean = 2.2 ± 0.4 x 10^3 cells g^{-1} wet weight sediment; n = 65). A significant monthly variation was seen at the control site (F=4.0, df = 12, p = 1.3 x 10^{-6}), while no significant down core variation was noticed. Similarly at the experimental site also there was a significant monthly variation (F=3.2, df = 12, p = 1.5 x 10^{-3}), with no down core variation. As in the case of MPN counts of nitrifying bacteria, no significant variation in retrievable nitrifier counts was also seen between the control and experimental sites for any of the measured depth intervals throughout the sampling period. Seasonal and annual averages of retrievable nitrifier counts for different depth intervals at both the control and experimental site are given in Table 15b.

4.2.3.3. FISH based enumeration of eubacteria and nitrifiers

The abundance of eubacteria was highest in April (9.9±2.8x10^9 cells g^{-1} sediment) at the control and experimental site (5.3±4.6x10^9 cells g^{-1} sediment) (Table 16). Down core variation at the control site showed that the general trend during the months of April and July was tending towards lower values down the core while during October there were two maxima encountered at 2-4 and 8-10cm (Figure 16). The pattern was quite different at the experimental site (Figure 17), with the July month recording a profound maximum at 4-6 cm. There was no conspicuous down core variation during October, while the population tends to

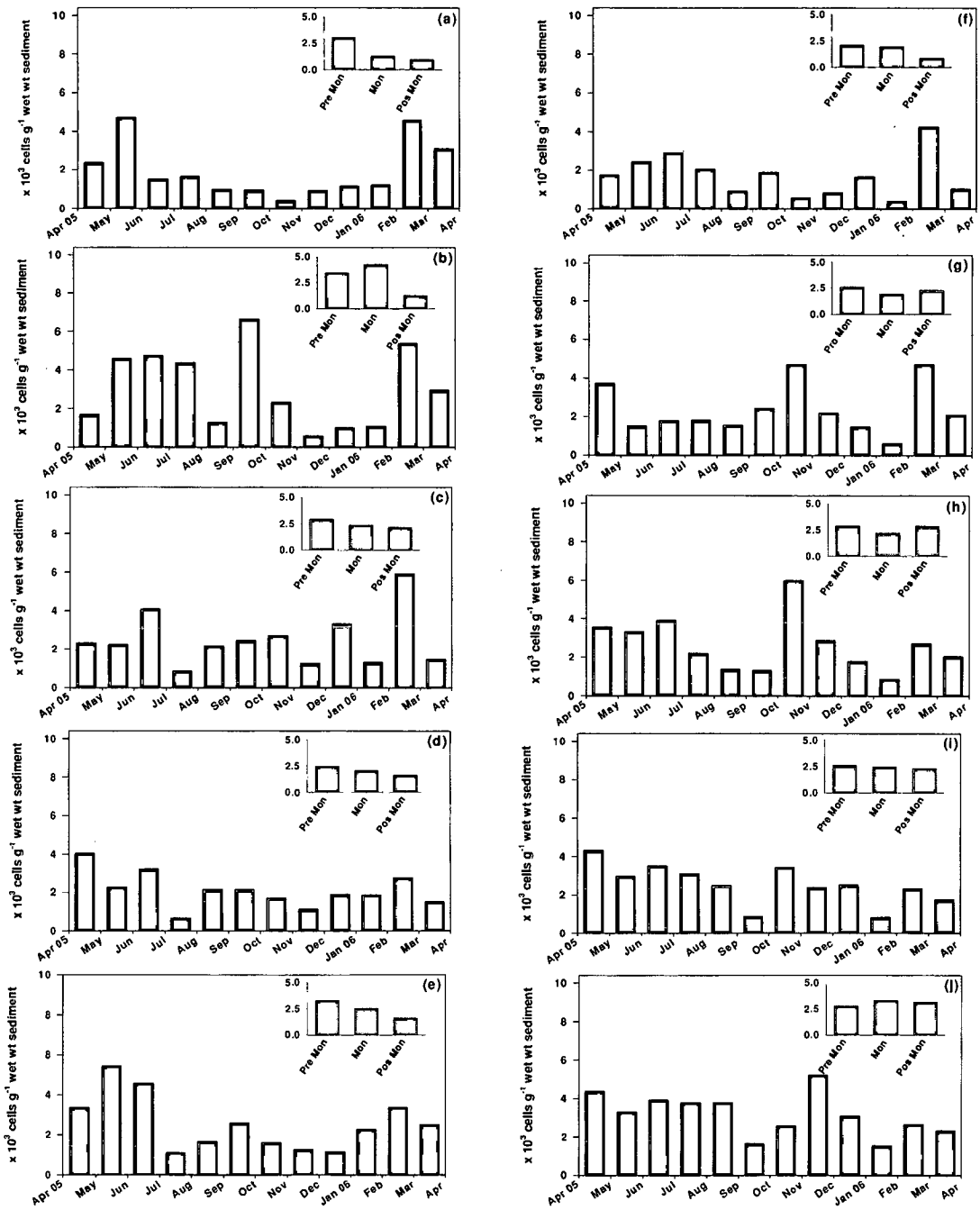


Figure 15b: Monthly down core variation of heterotrophic nitrifiers (MPN) at control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm. Seasonal averages are given in the inset.

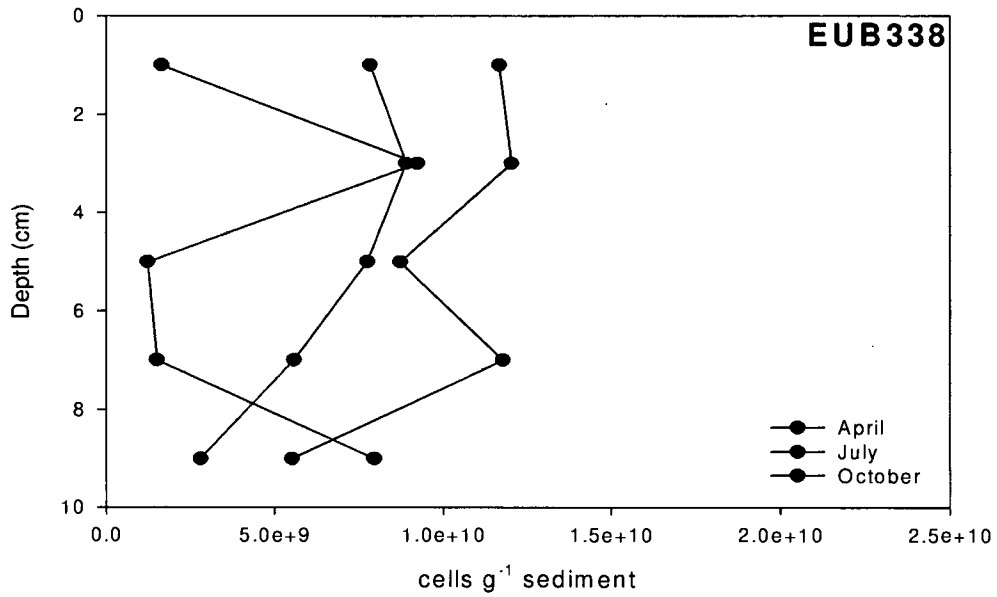


Figure 16: Down core variation of eubacteria at the control site for the three representative months April (Pre-Monsoon), July (Monsoon) and October (Post-Monsoon).

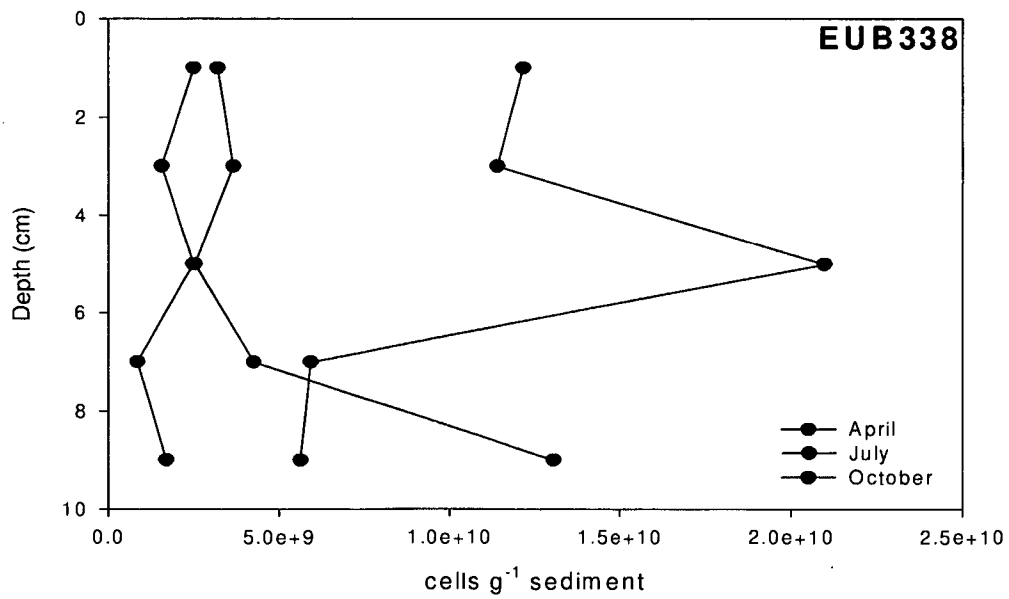


Figure 17: Down core variation of eubacteria at the experimental site for the three representative months April (Pre-Monsoon), July (Monsoon) and October (Post-Monsoon).

Table 15b: Seasonal and annual arithmetic mean (± standard error) of Most Probable Number of heterotrophic nitrifying bacteria (x 10^3 cells g^{-1} wet weight sediment) for different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval (cm)	**Pre-monsoon**	**Monsoon**	**Post-monsoon**	**Annual**
0 to 2	3.0 ± 1.7	1.2 ± 0.3	0.9 ± 0.3	1.8 ± 1.4
2 to 4	3.3 ± 1.5	4.2 ± 2.2	1.2 ± 0.7	3.0 ± 1.9
4 to 6	2.9 ± 1.7	2.3 ± 1.3	2.1 ± 1.0	2.5 ± 1.3
6 to 8	2.4 ± 1.0	2.0 ± 1.0	1.6 ± 0.3	2.0 ± 0.8
8 to 10	3.2 ± 1.4	2.4 ± 1.5	1.5 ± 0.5	2.5 ± 1.3
EXPERIMENTAL SITE (Diwar)				
0 to 2	2.0 ± 1.3	1.9 ± 0.8	0.8 ± 0.5	1.6 ± 1.0
2 to 4	2.5 ± 1.5	1.8 ± 0.3	2.2 ± 1.7	2.2 ± 1.3
4 to 6	2.8 ± 0.5	2.2 ± 1.2	2.8 ± 2.2	2.6 ± 1.3
6 to 8	2.5 ± 1.1	2.4 ± 1.1	2.2 ± 1.0	2.4 ± 1.0
8 to 10	2.7 ± 1.2	3.2 ± 1.0	3.1 ± 1.5	3.0 ± 1.2

Table 16: Abundance of Eubacteria (as probed with EUB338) in the depth interval 0-10 cm at the control and experimental sites. Arithmetic mean (± standard error) values (for 0-10 cm) are given for months representing pre-monsoon (April), monsoon (July) and post monsoon months (October).

	EUB338 (Eubacteria) x 10^9 cells g^{-1} sediment	
	Control	Experiment
April	9.9 ± 2.8	5.3 ± 4.6
July	6.5 ± 2.4	1.2 ± 6.2
October	4.3 ± 3.9	1.8 ± 6.9

generally increase down the core during the month of April (Figure 17). The lowest abundance (Table 16) at the control sites was encountered in October (Post-monsoon) while it was lower during the monsoon and post-monsoon seasons at the experimental site.

The abundance of β proteobacterial ammonia oxidizers is presented in Table 17. The abundance of these oxidizers was higher during April at the control site while it was comparatively very high during October at the experimental site. Both the sites recorded the lowest values during the monsoon months (1.2±1.0 to $2.1\pm0.6\times10^{2}$ cells g^{-1} sediment). Down core profiles for the control site (Figure 18) showed a clear downward trend in the abundance of ammonia oxidizers irrespective of the season. At the experimental site, a profound subsurface maxima (Figure 19) could be observed in the month of October, while both June and July profiles were similar. In general, the β proteobacterial ammonia oxidizers contributed to $\sim10^{-6\ to\text{-}5}$% of the total eubacterial assemblage in mangrove sediments (Table 17). The abundance of *Nitrobacter sp.*, a representative nitrite oxidizer showed an inverse trend in abundance (Table 18) when compared to its ammonia oxidizer counterpart (Table 17). The highest abundance was observed during the monsoon month (July) at both the sites, while the lowest was observed during April and October for Control and Experimental sites respectively. The *Nitrobacter sp.* (nitrite oxidizer) contributed to $\sim10^{-6\ to\text{-}5}$% of the total eubacterial population in mangrove sediments (Table 18). Figures 20 and 21 illustrate the down core variation of *Nitrobacter sp.* at the control and experimental sites respectively. At both the sites the abundance

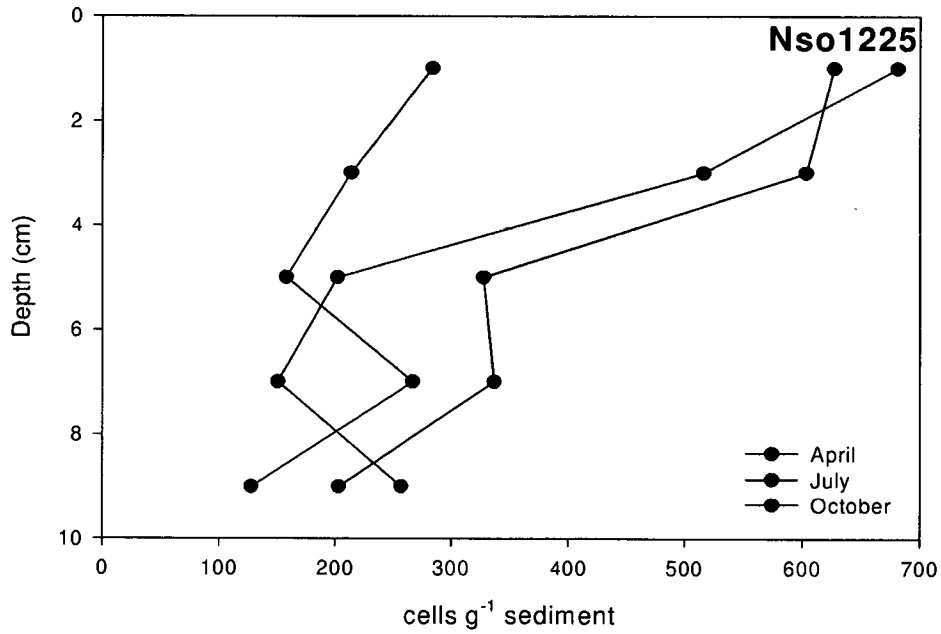


Figure 18: Down core variation of β proteobacterial ammonia oxidizers at the control site for the three representative months April (Pre Monsoon), July (Monsoon) and October (Post-Monsoon).

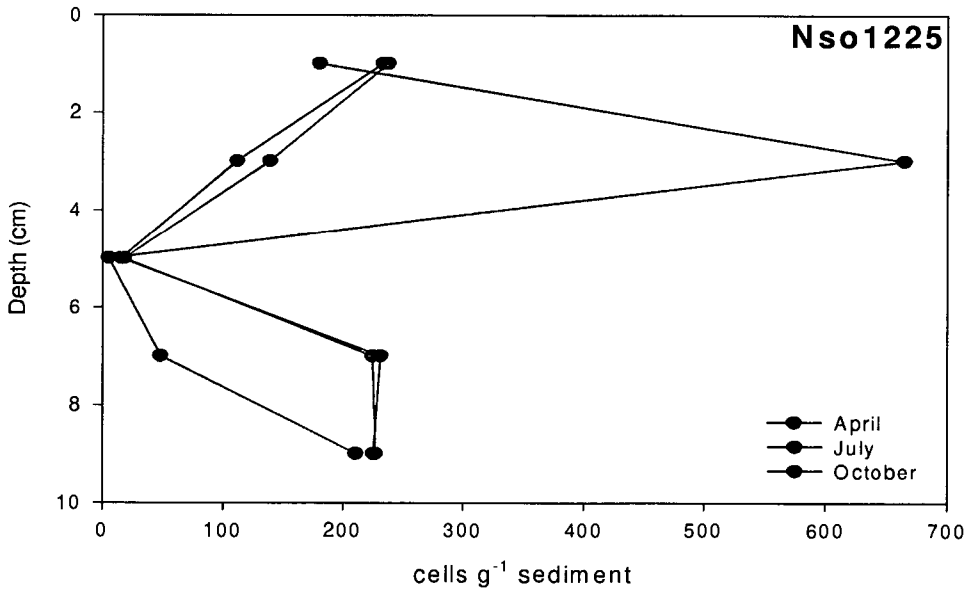


Figure 19: Down core variation of β proteobacterial ammonia oxidizers at the experimental site for the three representative months April (Pre-Monsoon), July (Monsoon) and October (Post-Monsoon).

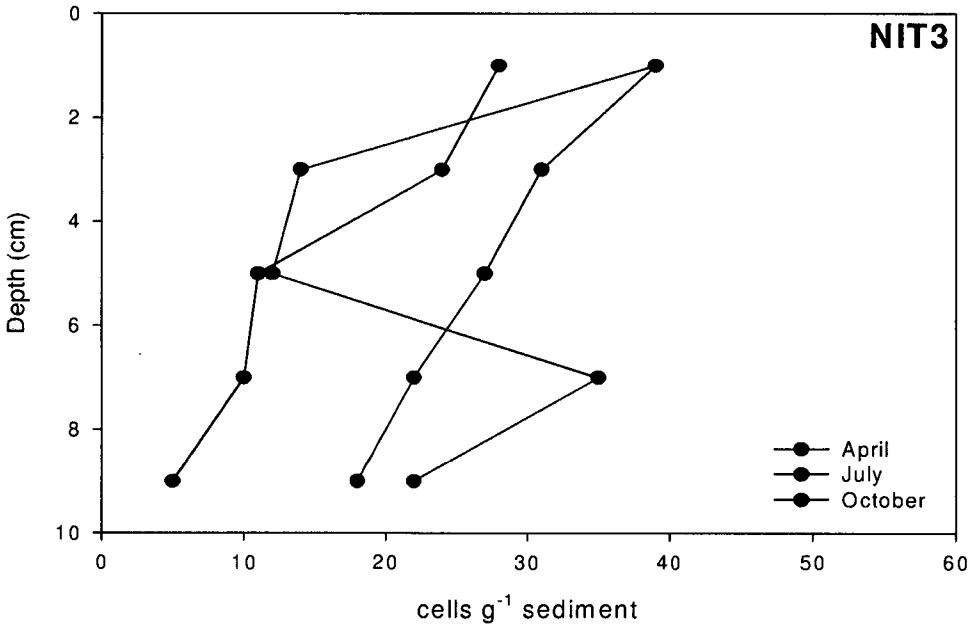


Figure 20: Down core variation of *Nitrobacter sp.* at the control site for the three representative months April (Pre-Monsoon), July (Monsoon) and October (Post-Monsoon).

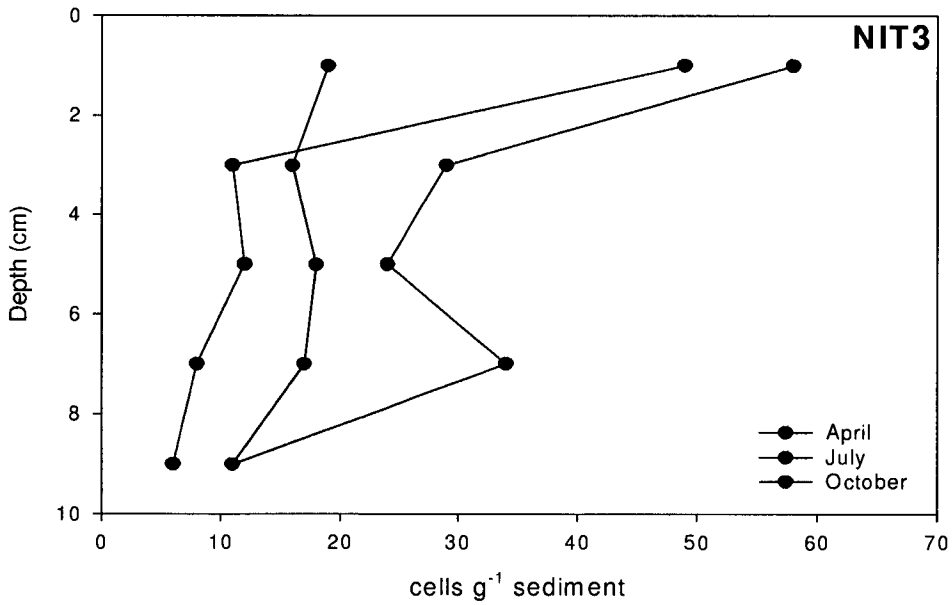


Figure 21: Down core variation of *Nitrobacter sp.* at the experimental site for the three representative months April (Pre-Monsoon), July (Monsoon) and October (Post-Monsoon).

Table 17: Abundance of β proteobacterial ammonium oxidizers (as probed with Nso1225) in the depth interval 0-10 cm at the control and experimental sites. Arithmetic mean (± standard error) values (for 0-10 cm) are given for months representing pre monsoon (April), monsoon (July) and post monsoon months (October).

	Nso1225 (β proteobacterial ammonium oxidizers) 10^2 cells g^{-1} sediment			
	Control	% of total Eubacterial population	Experiment	% of total Eubacterial population
April	4.1 ± 1.8	$4.1x10^{-6}$	2.2 ± 2.6	$4.1x10^{-6}$
July	2.1 ± 0.6	$3.2x10^{-6}$	1.2 ± 1.0	$1.0x10^{-5}$
October	3.6 ± 2.2	$8.3x10^{-6}$	6.4 ± 4.6	$3.5x10^{-5}$

Table 18: Abundance of *Nitrobacter sp.* (as probed with NIT3) in the depth interval 0-10cm at the control and experimental sites. Arithmetic mean (± standard error) values (for 0-10 cm) are given for months representing pre-monsoon (April), monsoon (July) and post-monsoon months (October).

	NIT3 (*Nitrobacter sp.*) 10^2 cell g^{-1} sediment			
	Control	% of total Eubacterial population	Experiment	% of total Eubacterial population
April	1.6 ± 1.1	$1.6x10^{-6}$	2.0 ± 7.0	$3.7x10^{-6}$
July	2.7 ± 0.8	$6.2x10^{-6}$	2.4 ± 8.5	$2.1x10^{-5}$
October	2.3 ± 1.0	$5.3x10^{-6}$	1.7 ± 4.9	$6.0x10^{-6}$

tends to decrease in general with increase in depth. Also, a secondary maximum could be observed at 4-8 cm in some of the cases. However, the variation in abundance differed with season, having the most profound peaks in the October and July for control and experiment, respectively.

4.3. Nitrification rates

4.3.1. ^{15}N based estimations (Tracer technique)

Nitrification rates (Figure 22) varied widely at both the control (0.3 to 22.7 nM g (sediment)$^{-1}$ h^{-1}; arithmetic mean = 9.2± 0.8 nM g (sediment)$^{-1}$ h^{-1}; n = 65) and experimental sites (0.4 to 22.5 nM g (sediment)$^{-1}$ h^{-1}; arithmetic mean = 8.9 ± 0.5 nM g (sediment)$^{-1}$ h^{-1}; n = 65). ANOVA showed a significant monthly variation ($F=35.7$, df = 12, $p = 7.2 \times 10^{-21}$) at the control site, but did not show such a down core variation. Similarly, at the experimental site there was significant monthly variation ($F = 43.9$, df = 12, $p = 6.5 \times 10^{-23}$), and no down core variation. Throughout the period of sampling there was no significant variation was seen in nitrification rates between the control and experimental sites for any of the measured depth intervals. Seasonal and annual averages of nitrification rates for different depth intervals at both the control and experimental site are given in Table 19. It could be seen that in general nitrification rates at the control and the experimental sites were comparatively higher during the pre-monsoon season and the lowest was during the monsoon season.

4.3.2. Culture based assay (*In vitro*)

One hundred fifty six strains were isolated, representing 13 different colony morphotypes of which one hundred twelve (71%) tested positive for ammonium oxidation (AO), nitrite oxidation (NO) or both (CAoNo) (Figure 23). Sixty two isolates were positive for ammonium oxidation with 28 in inorganic, 6 in organic and the rest testing positive in both types of nitrifying media. Twenty nine isolates were positive for nitrite oxidation of which 16 tested positive in inorganic,

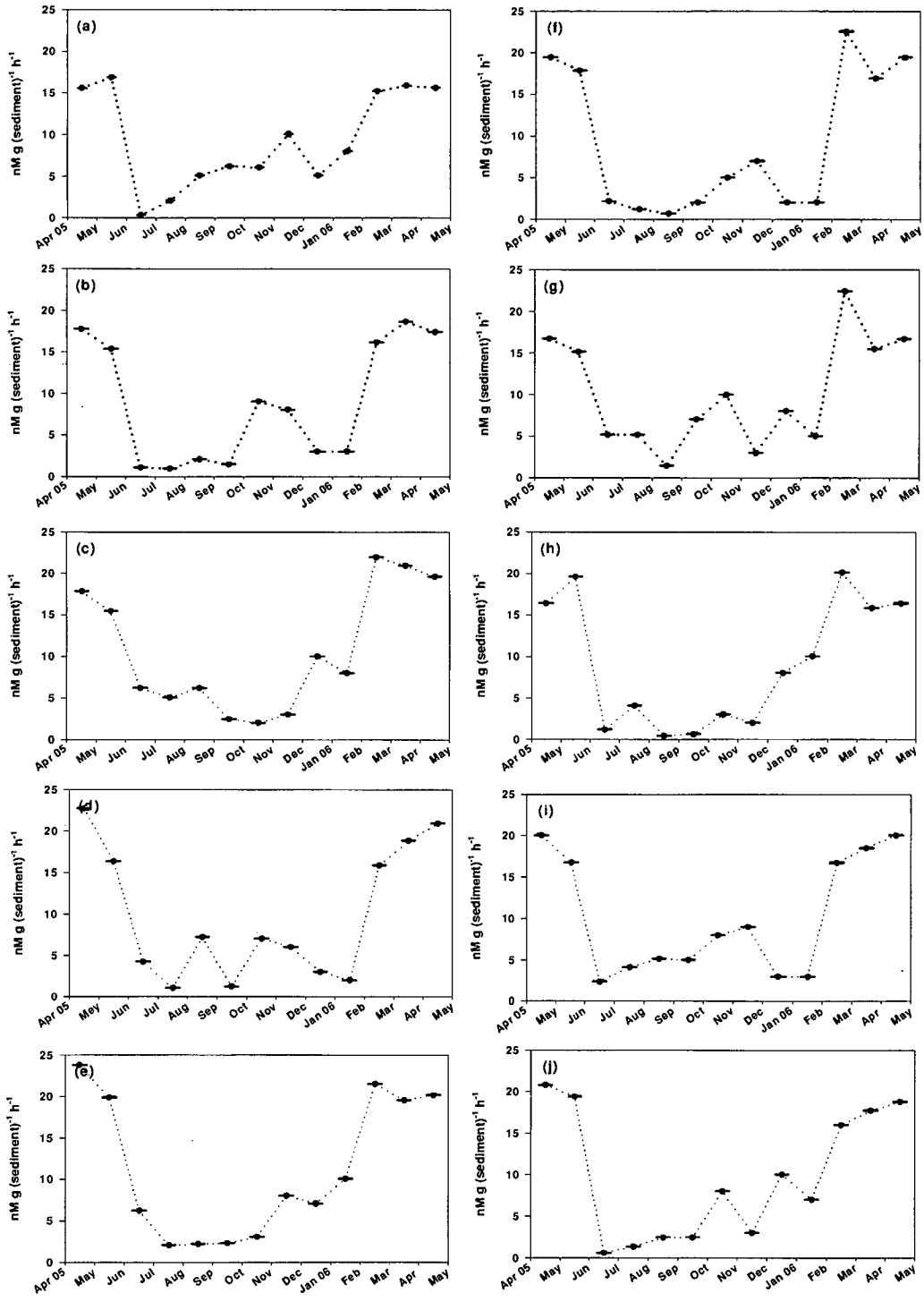


Figure 22: Monthly down core variation in nitrification rates at the control site – (a) 0-2 cm, (b) 2-4 cm, (c) 4-6 cm, (d) 6-8 cm, (e) 8-10 cm and at experimental site - f) 0-2 cm, (g) 2-4 cm, (h) 4-6 cm, (i) 6-8 cm, (j) 8-10 cm.

Table 19: Seasonal and annual arithmetic mean (± standard error) of nitrification rates (ng at-N g (sediment)$^{-1}$ h^{-1}) for different depth intervals at the control and experimental sites.

CONTROL SITE (Tuvem)				
Depth interval	Pre-monsoon	Monsoon	Post-monsoon	Annual
0 to 2	15.8 ± 0.6	3.4 ± 2.6	7.2 ± 2.2	8.8 ± 6.3
2 to 4	17.0 ± 1.3	1.3 ± 0.4	5.7 ± 3.2	8.0 ± 8.0
4 to 6	19.1 ± 2.5	4.9 ± 1.7	5.7 ± 3.8	9.9 ± 7.9
6 to 8	18.9 ± 2.9	3.4 ± 2.8	4.5 ± 2.3	8.9 ± 8.6
8 to 10	20.9 ± 1.7	3.1 ± 2.0	7.0 ± 2.9	10.3 ± 9.3
EXPERIMENTAL SITE (Diwar)				
0 to 2	19.2 ± 2.1	1.5 ± 0.7	4.0 ± 2.4	8.2 ± 9.5
2 to 4	17.2 ± 2.9	4.7 ± 2.3	6.5 ± 3.1	9.5 ± 6.8
4 to 6	17.6 ± 2.0	1.5 ± 1.6	5.7 ± 3.8	8.3 ± 8.3
6 to 8	18.4 ± 1.6	4.1 ± 1.2	5.7 ± 3.2	9.4 ± 7.8
8 to 10	18.5 ± 1.8	1.6 ± 0.9	7.0 ± 2.9	9.0 ± 8.6

1 in organic and the rest in both types of nitrifying media. All (21) the isolates that exhibited both ammonia as well as nitrite oxidation (CAoNo) were in organic nitrifying media (Figure 23).Ten isolates each from these groups (AO, NO and CAoNo) were short listed for detailed study on the kinetics and growth based on the intensity of colorimetric reactions employed to detect the fall in level of ammonium and appearance of nitrite and nitrate. Table 20 gives a comparison of all the isolates in terms of its morphology, abundance, growth and kinetics of AO, NO and nitrate production in the case of CAoNo. Ammonia oxidizers which showed enhanced activity in the primary screening came from both organic and inorganic media in equal proportions, while most of the nitrite oxidizers (90%) were from organic media. There were 7 different colony morphologies with most of them being pinpoint colonies (13/30) which was cream in color having regular edges. The next dominant morphotype was small round colonies with white pigmentation. Specific growth rate for ammonium oxidizers ranged from 0.03 to 0.8 divisions per hour while for the nitrite oxidizers and coupled ammonia-nitrite oxidizers, it ranged from 0.03 to 0.9 and 0.14 to 1.2 divisions per hour, respectively. It could be observed that in general the CAoNo isolates had higher specific growth rates compared to ammonia and nitrite oxidizers and the specific growth rates of the latter two were comparable.

The ammonia oxidizers from inorganic media showed enhanced rates than their heterotrophic counterparts (Table 20). The nitrite oxidizer (NO15), the lone isolate that was from inorganic media topped the rates compared to all the nitrite oxidizing heterotrophs. The ammonia oxidation ranged from 2.1 to 7.4 µg

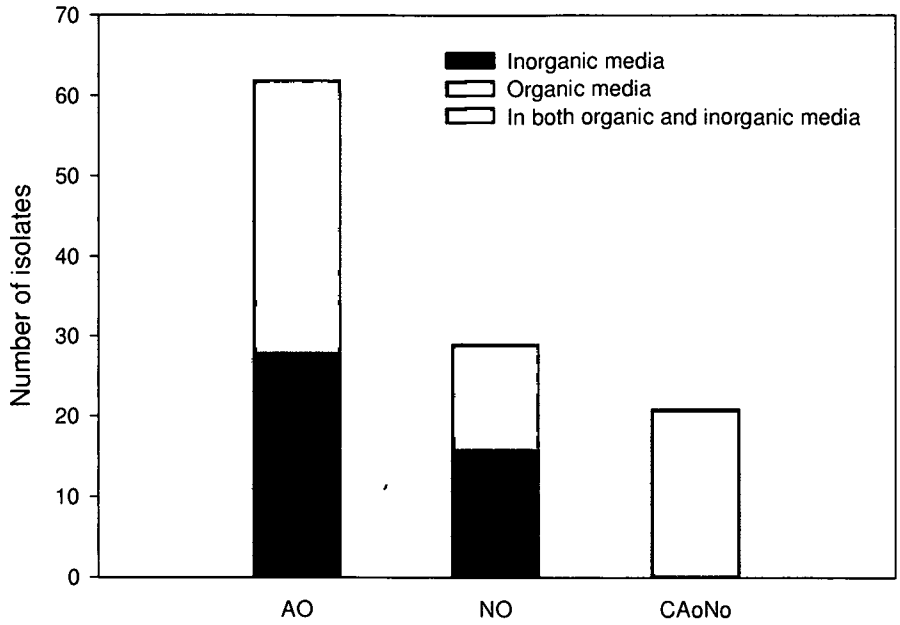


Figure 23: The number of isolates that were exclusively ammonia oxidizer (AO), nitrite oxidizer (NO) and coupled ammonia-nitrite oxidizer (CAoNo). The stacks in each bar illustrate the organic nature of the media in which the isolates showed the activity.

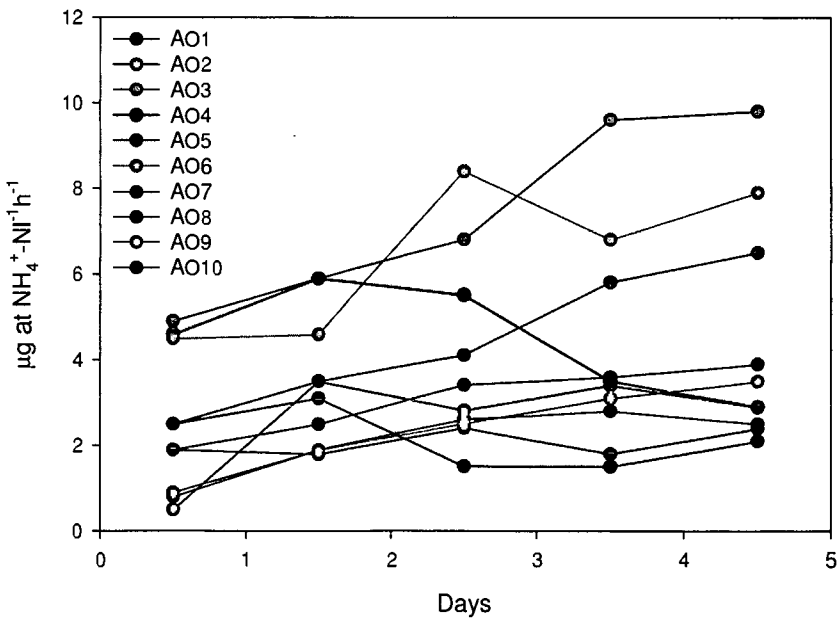


Figure 24: Kinetics of ammonia oxidation among the ten ammonia oxidizing isolates tested in the present study.

Table 20: Characteristics of nitrifiers studied. AO, NO and CAoNo refer to ammonia oxidizer, nitrite oxidizer and coupled ammonia-nitrite oxidizer respectively. Ammonium oxidation rate is presented in μM NH_4^+-$Nl^{-1}h^{-1}$. Nitrite oxidation is presented in μM NO_2^--$Nl^{-1}h^{-1}$. CAoNo is presented as nitrate production (μM NO_3^--$Nl^{-1}h^{-1}$). #Values in parenthesis corresponds to CFU x 10^2 g/wet weight sediment corresponding to 100%. * indicates that test was done on inorganic nitrifying media while ** indicates that test was done on organic nitrifying media.

Isolate	Nature	Rate	Specific Growth rate (Divisions per hour)	Colony morphology	Abundance % of the isolate among the total CFU#
AO1	AO	2.1**	0.2	Small/Cream/Regular	42.8 (8.3)
AO2	AO	2.6**	0.8	Pinpoint/Cream/Regular	34.7 (7.9)
AO3	AO	7.4*	0.04	Small/Cream/Irregular	48.5 (7.7)
AO4	AO	4.5*	0.05	Small/Pale yellow/ Regular	14.2 (5.5)
AO5	AO	2.1**	0.3	Pinpoint/Cream/Regular	33.4 (4.9)
AO6	AO	6.4*	0.05	Small/Pale yellow/ Regular	52.6 (2.7)
AO7	AO	2.1*	0.4	Pinpoint/Cream/Regular	64.8 (2.9)
AO8	AO	3.1**	0.5	Small/Cream/Irregular	28.6 (6.6)
AO9	AO	2.4**	0.4	Small/Cream/Irregular	25.4 (5.4)
AO10	AO	4.5*	0.03	Pinpoint/Cream/Regular	18.6 (4.5)
NO11	NO	0.8**	0.9	Medium/Brown/Regular	43.7 (3.1)
NO12	NO	0.12**	0.4	Pinpoint/Cream/Regular	21.5 (1.6)
NO13	NO	0.3**	1.1	Small/White/Regular	32.5 (7.9)
NO14	NO	0.8**	0.9	Small/White/Regular	35.6 (2.8)
NO15	NO	1.1*	0.03	Pinpoint/Cream/Regular	65.4 (2.6)
NO16	NO	0.5**	0.5	Pinpoint/Cream/Regular	26.7 (2.1)
NO17	NO	0.6**	0.6	Pinpoint/Cream/Regular	47.6 (6.0)
NO18	NO	0.44**	0.5	Small/Cream/Irregular	68.7 (1.4)
NO19	NO	0.7**	0.8	Pinpoint/Cream/Regular	45.9 (7.8)
NO20	NO	0.5**	0.4	Small/White/Regular	82.3 (1.4)
CAoNo21	CAoNo	1.3**	0.6	Pinpoint/Cream/Regular	41.6 (4.2)
CAoNo22	CAoNo	1.4**	0.4	Large/Cream/Irregular	61.4 (3.9)
CAoNo23	CAoNo	0.1**	0.85	Pinpoint/Cream/Regular	37.4 (1.4)
CAoNo24	CAoNo	0.4**	0.6	Medium/Brown/Regular	22.8 (8.3)
CAoNo25	CAoNo	0.5**	0.14	Small/White/Regular	64.6 (2.9)
CAoNo26	CAoNo	1.8**	1.2	Small/White/Regular	64.3 (2.4)
CAoNo27	CAoNo	0.6**	1.1	Pinpoint/Cream/Regular	54.6 (6.8)
CAoNo28	CAoNo	0.5**	0.9	Large/Cream/Irregular	41.6 (5.3)
CAoNo29	CAoNo	1.1**	0.54	Pinpoint/Cream/Regular	39.6 (5.3)
CAoNo30	CAoNo	0.7**	0.5	Large/Cream/Irregular	24.9 (3.2)

at NH_4^+-N l^{-1} h^{-1} while the nitrite oxidation and nitrate production ranged from 0.12 to 1.1 µg at NO_2^--N l^{-1} h^{-1} and 0.1 to 1.8 µg at NO_3^--N l^{-1} h^{-1}, respectively. Figure 24 illustrates the detailed kinetics of ammonium oxidation among the ten isolates for a period of five days. AO3, AO6 and AO10 were conspicuously different from the rest by virtue of their enhanced rates of ammonia oxidation. The other isolates showed more or less similar trend in the kinetics of ammonia oxidation. In the case of nitrite oxidation experiments (Figure 25), isolates NO15 and NO11 showed enhanced nitrite oxidation activity compared to the others. Isolates NO14, NO16, NO19 and NO20 showed similar trend in kinetics exhibited by a gradual and slow increase in oxidation rates. Isolates NO13, NO17 and NO18 were characterized by a significant dip in oxidation rates during the end of the incubation period. The lowest rates were encountered with NO12. The activity of coupled ammonia and nitrite oxidation measured as nitrate production is illustrated in Figure 26. From the end point kinetics the isolates could be grouped into three with the group one showing enhanced activity than group two and group three showing the least activity. The first group encompasses isolates CAoNo22 and CAoNo26 while the second group is formed by CAoNo21, CAoNo29 and CAoNo30. The other CAoNo isolates fall in the third group. The general trend in isolates, except for CAoNo22 and CAoNo23 is that at the end of the incubation the rates tend to dip down. The lowest nitrate production rate was observed for CAoNo23.

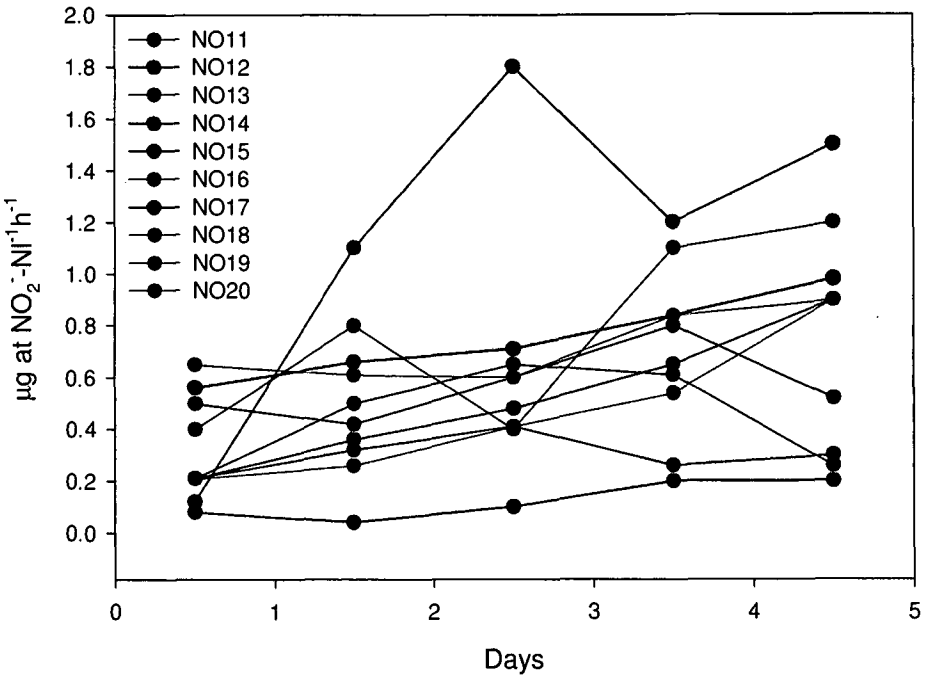


Figure 25: Kinetics of nitrite oxidation among the ten nitrite oxidizing isolates tested in the present study

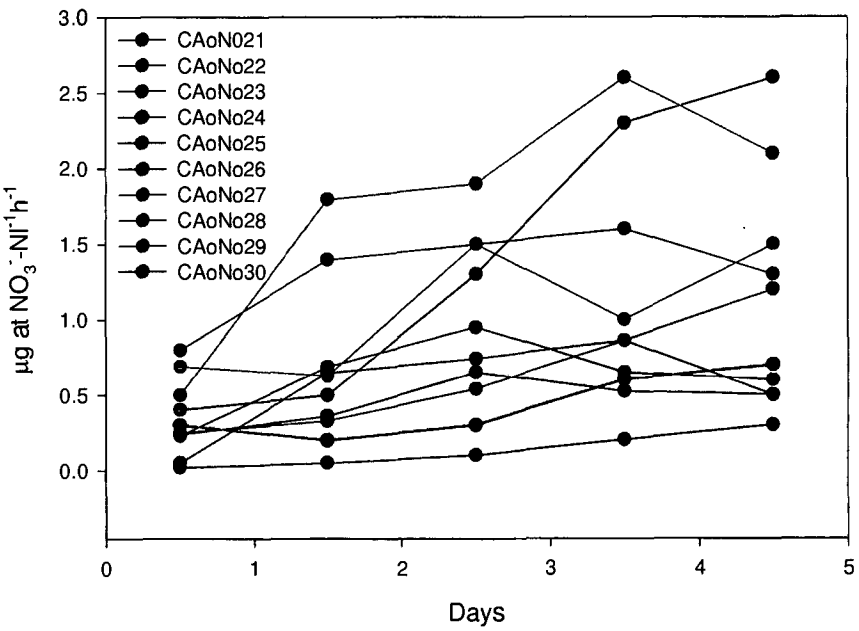


Figure 26: Kinetics of nitrate production among the ten ammonia and nitrite oxidizing isolates tested in the present study

4.4. Interrelationships of bacteria and environmental parameters

Control site: Irrespective of the month and depth, nitrification rates positively correlated with autotrophic nitrifiers ($r = 0.43$, $p<0.001$) and iron ($r = 0.47$, $p<0.001$), while it had a weak negative relation with nitrate concentration ($r=-0.25$, $p<0.05$). The heterotrophic nitrifers increased with increasing depth ($r=0.33$, $p<0.01$) and had a positive correlation with the nitrite concentration ($r=0.31$, $p<0.01$). The total organic carbon in the sediments correlated positively with both manganese ($r=0.36$, $p<0.01$) and iron ($r=0.45$, $p<0.001$). The concentration of pore water nitrate had a significant correlation with the concentration of manganese ($r=0.59$, $p<0.001$) but had a less but negative correlation with iron ($r=-0.22$, $p<0.1$).

During the pre-monsoon season, the nitrification rates increased with depth ($r=0.67$, $p<0.001$) and ammonium ($r=0.57$, $p<0.001$). The nitrate pool was positively regulated) by the manganese concentration ($r=0.58$, $p<0.001$). In the monsoon season, nitrification rates and heterotrophic nitrifiers had a negative relation with organic carbon ($r=-0.45$, $p<0.05$) and ammonium ($r=0.57$, $p<0.01$) respectively. During the post-monsoon season, the nitrification rates do not have any significant correlation with any of the parameters studied. However, the abundance of heterotrophic nitrifiers was positively governed by ammonium ($r=0.45$, $p<0.05$).

Depth wise analysis of biotic and abiotic factors showed that manganese controlled the distribution of heterotrophic nitrifiers at 0-2 cm ($r=0.5$, $p<0.05$) and 8-10 cm ($r=0.4$, $p<0.1$); and that of autotrophic nitrifiers at 6-8 cm ($r=0.55$,

$p<0.05$). The autotrophic nitrifers also had a significant correlation with iron ($r=0.54$, $p<0.05$).

Experimental site: In general, throughout the period of sampling, the nitrification rates were governed by the abundance of iron ($r=0.47$, $p<0.001$) and autotrophic nitrifiers ($r=0.43$, $p<0.001$). Pore water nitrate retarded nitrification rates ($r=-0.25$, $p<0.05$). Heterotrophic nitrifiers were positively influenced by depth ($r=0.33$, $p<0.01$), ammonium ($r=0.23$, $p<0.1$) and nitrite concentration ($r=0.31$, $p<0.1$). The concentration of ammonium was dependent on the concentration of organic carbon ($r=0.67$, $p<0.001$) and iron in these sediments ($r=0.25$, $p<0.05$).

Seasonal analyses showed that during the pre-monsoon season, nitrification rates were primarily governed by availability of ammonium ($r=0.55$, $p<0.001$), heterotrophic nitrifiers ($r=0.46$, $p<0.02$) and manganese ($r=0.4$, $p<0.05$). The autotrophic nitrifiers regulated the concentration of nitrite ($r=0.49$, $p<0.01$). It was observed that nitrification rates were not governed by any of the parameter studied during the monsoon and post- monsoon season. In the monsoon, though the population of heterotrophic nitrifiers were controlled by depth ($r=0.47$, $p<0.02$) and the availability of iron ($r=0.55$, $p<0.05$), the autotrophic nitrifiers appeared to be unaffected by any of the parameters. During the post-monsoon season, both the autotrophic and heterotrophic nitrifiers increased with depth ($r=0.4$, $p<0.05$).

Analysis of various sections of the core showed that at 8-10 cm, nitrification rates had a negative influence on pore water nitrate ($r=-0.54$, $p<0.05$).

The abundance of autotrophic nitrifiers had a positive correlation with nitrification rates at both 6-8 ($r=0.4$, $p<0.1$) and 8-10 cm ($r=0.68$, $p<0.01$). At deeper sections, the nitrate concentration has a positive correlation with manganese content in the sediments ($r=0.7$, $p<0.001$).

4.5. Phenotypic characteristics and molecular Identity of nitrifiers

4.5.1. Metabolic profiles and biochemical characteristics

Metabolic profiles of all the 30 nitrifiers were obtained on BIOLOG plates having 26 sugars, 24 carboxylic acids, 20 amino acids, 8 amines, 5 polymers and 12 miscellaneous compounds. Tables 21, 22 and 23 give a detailed account of the carbon utilization profiles of the ammonium, nitrite and coupled ammonium-nitrite oxidizers, respectively.

In general, it was observed that most of the isolates were able to oxidize 43-70% of the sugars tested. Amino acids and carboxylic acids were used in the range 56-80% and 10-23% respectively. Amides were utilized in the range 23-67%, while polymers and miscellaneous compounds were utilized with an efficiency of 12-45% and 16-34% respectively. It was found that α D-Glucose was one of the most preferred sugars in addition to sugars like by D-Galactose and L-Arabinose. The other preferred sugars were D-Mannose, L-Rhamnose, D-Cellobiose, D-Fructose and D-Pscicose. Similarly, Glycyl-L-Aspartic acid, Glycyl-L-Glutamic acid and L-Serine were the most preferred amino acids. The most preferred carboxylic acid used by all the strains was Propionic acid, while the amide and polymer were Succinamide and Tween 40, respectively.

Specifically, all the ten ammonium oxidizers studied preferentially used α D-Glucose and Lactulose followed by D-Galactose, Adonitol and L Arabinose. The least preferred sugars were Turanose and m-Inositol. Dextrin was the least preferred polymer while the most preferred was Tween 40. L-Asparagine and L-Alanine were the least preferred amino acids while the most preferred were D-

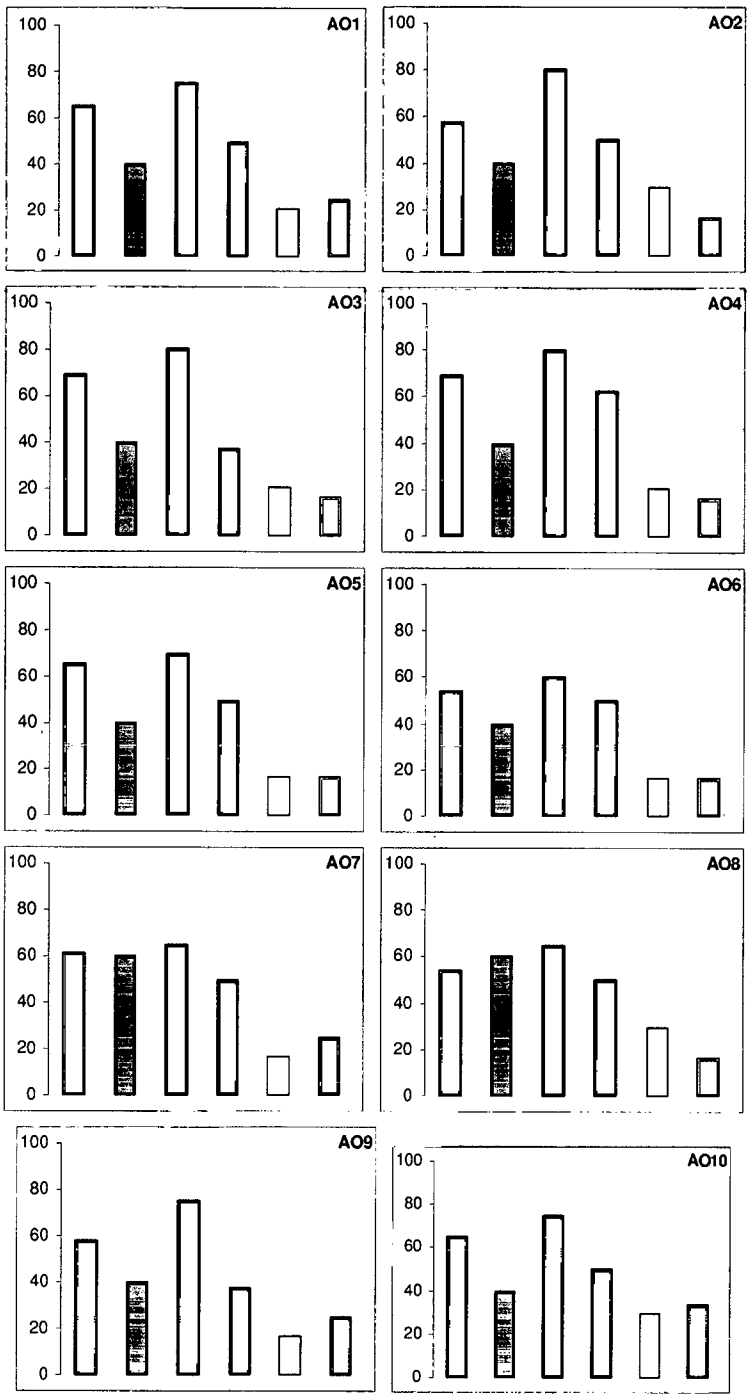


Figure 27: Summary of the percentage utilization of carbon substrates by Ammonium Oxidizers. ◘ Sugars (26), ◘ Polymers (5), ◘ Amino Acids (20), ◘ Amides (8), □ Carboxylic Acids (24) ◘ Miscellaneous (12). Values in brackets indicate the number of substrates tested. % utilization is given on y axis.

Table 21: Difference in carbon source utilization according to Biolog GN2 for the different Ammonium Oxidizer (AO) isolates.

Carbon source	AO1	AO2	AO3	AO4	AO5	AO6	AO7	AO8	AO9	AO10
Sugars										
D-Arabitol	-	+	+	+	+	-	+	-	-	+
Adonitol	+	+	+	+	-	+	+	+	+	+
D-Cellobiose	+	-	+	+	+	+	+	+	-	+
D-Fructose	-	+	+	+	+	-	+	+	+	+
L-Fucose	+	-	+	-	+	-	+	-	+	+
D-Galactose	-	+	+	+	-	-	-	+	+	-
Gentiobiose	-	-	+	+	+	-	-	-	+	+
α-D-Glucose	+	+	+	-	+	-	+	+	+	+
m-Inositol	-	+	-	-	+	-	-	-	-	-
α-D-Lactose	+	+	+	+	-	-	+	+	-	+
Lactulose	+	+	+	+	+	+	+	+	+	+
Maltose	+	-	+	+	-	-	+	+	+	-
L-Arabinose	+	-	+	-	+	-	-	-	+	+
D-Mannitol	-	-	-	-	+	+	+	-	+	-
D- Mannose	-	+	-	+	+	+	-	+	+	-
i-Erythritol	+	+	-	+	-	-	+	-	-	+
L-Rhamnose	+	-	+	+	+	+	-	-	-	-
D-Sorbitol	+	-	+	-	-	+	-	+	+	+
Sucrose	+	-	-	-	+	+	-	-	-	+
D-Trehalose	+	+	+	-	+	-	-	-	-	+

Turanose	-	-	+	-	-	-	-	-	-	-
Xylitol	+	-	+	+	-	+	-	-	+	-
D-Melibiose	+	+	-	+	+	+	-	+	-	-
β-Methyl-D-Glucoside	-	+	-	+	-	-	+	-	+	+
D-Psicose	+	+	-	-	+	+	+	+	-	-
D-Raffinose	-	-	+	+	+	+	+	+	+	+
Polymers										
α-Cyclodextrin	+	+	+	-	-	-	-	+	-	-
Dextrin	-	-	-	+	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	+	-	-	+
Tween 40	+	+	+	+	+	+	+	+	+	+
Tween 80	-	-	-	-	+	+	+	+	+	-
Amino acids										
D-Alanine	+	+	+	+	+	+	+	+	+	+
L-Alanine	+	-	+	+	-	+	+	+	+	+
L-Alanyl-Glycine	+	+	+	-	+	+	-	+	+	-
L-Asparagine	-	-	-	+	-	-	+	-	+	+
L-Glutamic Acid	-	+	+	+	+	+	-	-	-	+
Glycyl-L-Glutamic Acid	+	+	+	+	+	+	+	+	+	+
L-Aspartic Acid	+	+	+	+	-	+	+	+	+	-
L-Orinithine	-	-	-	+	+	-	+	-	+	+
L-Histidine	+	+	+	+	+	+	-	+	+	-
Hydroxy-L-Proline	+	+	+	-	-	-	+	+	+	+
L-Leucine	+	+	-	+	+	+	+	-	-	+
D-Serine	+	+	-	+	+	-	-	+	+	-
L-Threonine	+	+	+	+	+	-	-	+	-	+
L-Phenylalanine	+	-	+	-	-	-	-	-	+	+

Table 21 Continued

D,L-Carnitine	+	+	+	+	+	-	+	-	-	-
Glycyl-L-Aspartic Acid	+	+	+	+	+	+	+	+	+	+
L-Pyroglutamic Acid	-	+	+	-	+	+	-	+	-	+
L-Proline	+	+	+	+	-	+	+	-	+	+
γ-Aminobutyric Acid	+	+	+	+	+	-	+	+	+	+
L-Serine	-	+	+	+	+	+	+	+	+	+
Amides										
L-Alaninamide	+	-	-	-	-	+	-	-	-	+
N-Acetyl-D-Glucosamine	-	+	+	+	-	-	-	+	+	-
N-Acetyl-D-Galactosamine	+	-	-	+	+	+	+	-	+	-
Succinamide	+	+	-	+	+	+	+	+	+	+
Glucoronamide	-	+	+	-	-	+	+	-	-	+
Phenylethylamine	-	+	-	+	+	-	-	-	-	-
2-Aminoethanol	+	-	+	-	+	-	-	+	-	+
Putrescine	-	-	-	+	-	-	+	+	-	-
Carboxylic acids										
D-Gluconic Acid	-	-	-	-	-	-	-	-	-	-
D-Galacturonic acid	-	-	-	-	-	-	-	-	+	-
Acetic Acid	-	-	-	-	-	-	-	-	-	+
α-Hydroxybutyric Acid	-	-	-	-	-	-	-	+	-	-
β-Hydroxybutyric Acid	-	-	-	-	-	-	-	-	-	-
γ-Hydroxybutyric Acid	-	-	-	-	-	-	-	-	+	-
p-Hydroxyphenylacetic Acid	-	-	-	-	-	-	+	-	-	+

Table 21 Continued

α-Ketoglutaric Acid	-	+	-	-	+	-	-	+	-	+
α-Ketovaleric Acid	-	+	-	+	-	-	-	-	-	-
α-Ketobutyric Acid	-	-	-	-	-	-	-	-	-	+
D,L-Lactic Acid	-	-	-	-	-	-	-	-	-	-
D-Glucosaminic acid	-	+	+	-	-	+	-	-	-	-
D-Glucuronic acid	+	-	-	-	-	+	+	+	-	-
Propionic Acid	+	+	+	+	+	+	+	+	+	+
Formic Acid	-	+	+	-	-	-	-	-	-	+
Citric Acid	+	-	+	+	-	-	-	-	-	-
Succinic Acid	-	+	+	-	-	-	+	+	-	+
Cis-Aconitic Acid	+	-	-	-	+	-	-	+	+	-
D-Galactonic Acid Lactone	-	+	-	+	+	-	-	-	-	-
Itaconic Acid	-	-	-	-	-	-	-	-	-	-
D-Saccharic Acid	-	-	-	-	-	-	-	-	-	-
Sebacic Acid	-	-	-	+	-	+	-	+	-	-
Quinic Acid	+	-	-	-	-	-	-	-	-	-
Malonic Acid	-	-	-	-	-	-	-	-	-	-
Miscellaneous										
2,3-Butanediol	-	-	-	-	-	-	-	-	-	+
Glycerol	-	-	-	-	+	-	-	-	-	-
Inosine	-	-	-	-	-	-	+	-	-	-
Thymidine	+	-	-	-	-	-	-	-	-	+
Uridine	-	-	+	-	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	+	-	+	+	-	-	+	+
α-D-Glucose-6-Phosphate	+	+	-	-	-	+	+	+	-	-

Table 21 Continued

Urocanic acid	+	-	-	+	-	-	-	+	-	-
Pyruvic Acid Methyl Ester	-	-	-	-	-	-	-	-	+	-
Succinic Acid Mono-Methyl ester	-	+	-	+	-	-	+	-	+	-
D-L-α-Glycerolphosphate	-	-	-	-	-	-	-	-	-	+
Bromosuccinic Acid	-	-	-	-	-	-	-	-	-	-

Table 21 End

Alanine, Glycyl-L-Aspartic acid and Glycyl-L-Glutamic acid. Though Propionic acid was used by all the isolates, carboxylic acids like D-Gluconic Acid, β-Hydroxybutyric acid, L Lactic acid, Itaconic acid, Saccharic acid and Malonic acid were not used by any of the ammonium oxidizers. Some of the miscellaneous compounds, except for Bromosuccinic acid were used by all the isolates. α D-Glucose-10-Phosphate and α D-Glucose-6-Phosphate were the most preferred among the miscellaneous compounds. Isolate AO3 and AO4 showed the highest metabolic flexibility (69%) in terms of sugar utilization while AO6 and AO8 were able to utilize only 53% of the sugars provided. Isolate AO3 was the only ammonium oxidizer capable of oxidizing Turanose and Uridine. AO2 and AO5 were characterized by their ability to use m-Inositol. The polymer Dextrin was only utilized by the isolate AO4. Quininc acid was preferred only by AO1, while Glycerol, α Hydroxybutyric acid, D-Galacturonic acid, Inosine and Acetic acid were used only by AO5, AO8, AO9, AO7 and AO10, respectively. Figure 27 illustrates the percentage of the different substrates utilized by ammonium oxidizers. Isolates AO7 (60%) and AO8 (60%) could be characterized by their higher capacity to use more number of polymers. Isolates AO8 (29%), AO2 (29%) and AO10 (29%) performed better in terms of the number of carboxylic acids utilized. Most of the isolates studied were able to utilize most of the amino acids tested. The efficiency with which the amines were used by the isolates ranged from 37-62%.

The most preferred sugars by nitrite oxidizers were α D-Glucose and D-Galactose, while the least preferred was D-Melibiose (Table 22). Glycogen was

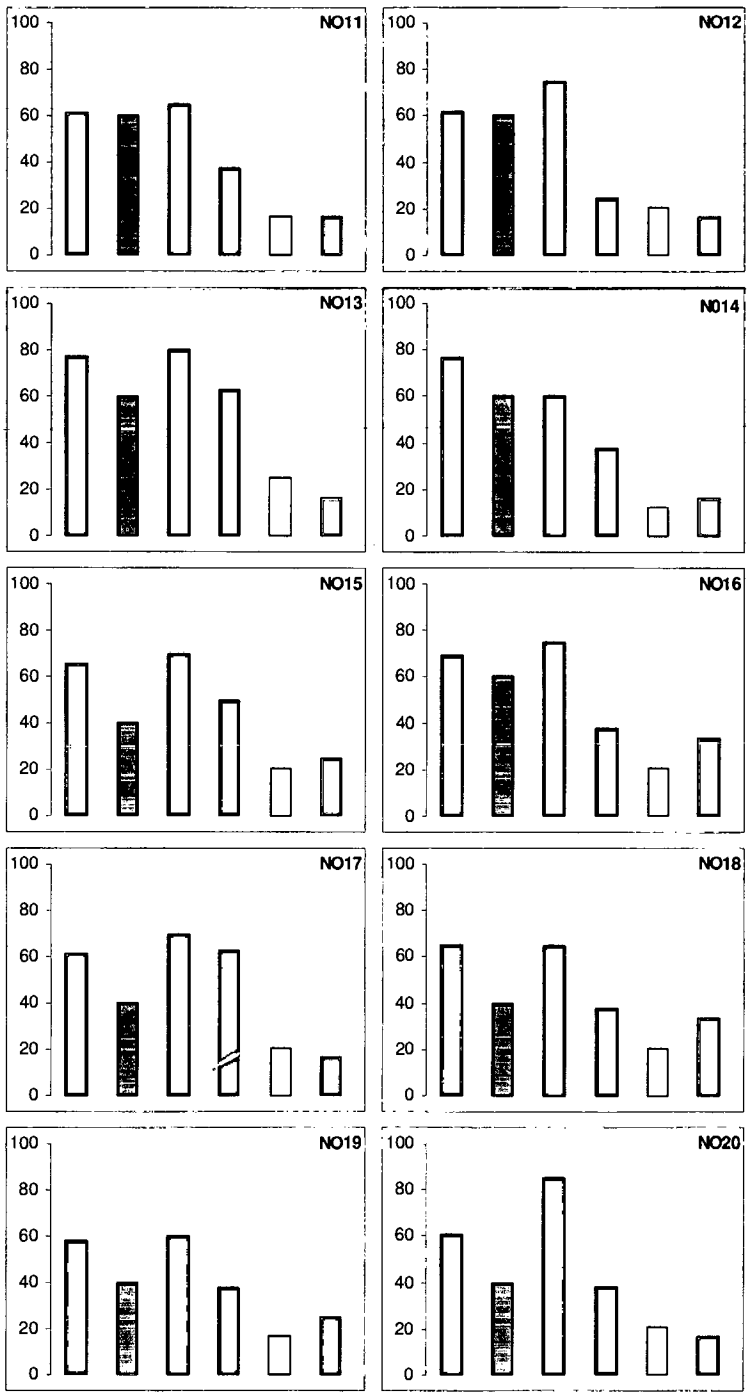


Figure 28: Summary of the percentage utilization of carbon substrates by Nitrite Oxidizers. ◘ Sugars (26), ◘ Polymers (5), ◘ Amino Acids (20), ◘ Amides (8), □ Carboxylic Acids (24) ◘ Miscellaneous (12). Values in brackets indicate the number of substrates tested. % utilization is given on y axis.

Table 22: Difference in carbon source utilization according to Biolog GN2 for the different Nitrite Oxidizer (NO) isolates.

Carbon source	NO11	NO12	NO13	NO14	NO15	NO16	NO17	NO18	NO19	NO20
Sugars										
D-Arabitol	-	+	-	-	+	+	+	+	-	+
Adonitol	-	-	+	+	+	-	-	+	+	-
D-Cellobiose	-	-	+	+	-	+	+	+	-	+
D-Fructose	-	+	+	-	+	+	-	-	+	-
L-Fucose	-	+	+	+	+	-	-	-	-	+
D-Galactose	+	+	-	+	-	-	+	-	-	+
Gentiobiose	-	-	+	+	+	+	+	+	+	-
α-D-Glucose	-	+	+	+	+	+	+	+	+	+
m-Inositol	-	+	+	+	+	-	+	+	-	+
α-D-Lactose	-	-	-	+	+	+	-	+	-	-
Lactulose	+	+	+	+	+	+	+	-	+	-
Maltose	-	+	-	+	-	+	+	+	+	+
L-Arabinose	-	+	+	+	+	+	+	+	-	-
D-Mannitol	-	+	+	+	+	-	-	-	+	+
D- Mannose	+	-	+	+	+	+	-	-	+	+
i-Erythritol	-	+	-	+	+	-	-	+	-	-
L-Rhamnose	-	-	+	+	-	+	+	+	-	+
D-Sorbitol	-	+	-	+	-	+	+	+	+	+
Sucrose	-	+	+	-	-	-	+	+	+	+
D-Trehalose	-	-	+	+	+	+	-	-	+	+
Turanose	-	-	-	+	-	+	+	+	+	-

Xylitol	-	+	+	+	+	-	+	-	+	-
D-Melibiose	-	-	+	-	-	+	-	-	-	+
β-Methyl-D-Glucoside	-	-	+	-	-	-	+	+	+	+
D-Psicose	-	+	+	+	+	+	-	-	+	+
D-Raffinose	-	+	+	-	-	+	+	+	-	-
Polymers										
α-Cyclodextrin	+	+	+	+	-	+	-	+	-	+
Dextrin	-	-	-	-	+	-	+	-	+	-
Glycogen	+		+	-	-	-	-	-	-	-
Tween 40	+	+	+	+	+	+	+	+	+	-
Tween 80	-	+	-	+	-	+	-	-	-	+
Amino acids										
D-Alanine	+	+	+	+	+	+	+	+	+	+
L-Alanine	+	+	+	+	+	-	+	+	+	+
L-Alanyl-Glycine	+	-	+	+	+	+	+	-	-	+
L-Asparagine	+	+	-	+	+	-	+	+	+	+
L-Glutamic Acid	+	+	+	-	-	-	+	+	-	+
Glycyl-L-Glutamic Acid	+	+	+	+	+	+	+	-	+	+
L-Aspartic Acid	+	+	+	+	-	+	-	+	+	+
L-Orinithine	-	+	+	+	+	-	+	-	-	+
L-Histidine	+	+	-	-	-	+	+	-	-	+
Hydroxy-L-Proline	-	-	+	-	+	+	+	+	+	+
L-Leucine	+	+	-	+	+	-	-	-	+	+
D-Serine	+	+	-	-	+	+	+	+	-	+
L-Threonine	-	-	+	-	-	+	-	-	+	-
L-Phenylalanine	-	+	+	-	-	+	+	+	-	+
D,L-Carnitine	+	-	+	-	+	+	-	+	-	-
Glycyl-L-Aspartic Acid	+	+	+	+	+	+	+	+	+	+
L-Pyroglutamic Acid	-	-	+	-	+	+	-	+	+	-

Table 22 Continued

L-Proline	-	+	+	+	+	+	+	-	-	+
γ-Aminobutyric Acid	-	+	+	+	-	+	-	+	+	+
L-Serine	+	+	+	+	+	+	+	+	+	+
Amides										
L-Alaninamide	+	-	-	-	-	-	+	-	-	-
N-Acetyl-D-Glucosamine	-	-	+	-	-	-	-	-	-	-
N-Acetyl-D-Galactosamine	+	-	+	-	+	-	+	-	+	-
Succinamide	+	+	+	+	+	+	+	+	+	+
Glucoronamide	-	+	-	+	-	+	+	+	-	+
Phenylethylamine	-	-	-	-	+	-	-	-	-	+
2-Aminoethanol	-	-	+	+	+	-	-	+	+	-
Putrescine	-	-	+	-	-	+	+	-	-	-
Carboxylic acids										
D-Gluconic Acid	-	-	-	-	-	-	-	-	-	-
D-Galacturonic acid	-	-	-	-	-	-	-	-	-	-
Acetic Acid	-	-	-	-	-	-	-	+	-	-
α-Hydroxybutyric Acid	-	-	-	-	-	-	-	-	-	-
β-Hydroxybutyric Acid	-	-	-	-	-	-	-	-	-	-
γ-Hydroxybutyric Acid	-	-	-	+	-	+	-	-	-	-
p-Hydroxyphenylacetic Acid	-	-	-	-	-	-	-	+	-	-
α-Ketoglutaric Acid	-	-	-	-	-	-	+	+	-	-
α-Ketovaleric Acid	-	-	+	-	+	-	-	-	-	-
α-Ketobutyric Acid	-	-	-	-	-	+	-	-	-	-
D,L-Lactic Acid	-	-	-	+	-	-	-	-	-	-
D-Glucosaminic acid	-	+	+	-	-	-	-	-	+	-
D-Glucuronic acid	+	-	-	+	+	+	-	-	+	+
Propionic Acid	+	+	+	-	+	+	+	+	+	+

Table 22 Continued

Formic Acid	-	-	-	-	+	-	-	-	-	-
Citric Acid	-	+	-	-	-	-	-	-	-	-
Succinic Acid	-	-	+	-	-	-	+	+	-	+
Cis-Aconitic Acid	+	-	-	-	-	-	-	-	-	-
D-Galactonic Acid Lactone	-	+	-	-	-	+	+	-	+	+
Itaconic Acid	-	-	+	-	+	-	-	-	-	-
D-Saccharic Acid	-	-	-	-	-	-	+	-	-	-
Sebacic Acid	-	-	-	-	-	-	-	-	-	-
Quinic Acid	+	-	+	-	-	-	-	-	-	+
Malonic Acid	-	+	-	-	-	-	-	-	-	-
Miscellaneous										
2,3-Butanediol	-	-	-	-	-	-	-	-	-	-
Glycerol	+	-	+	-	-	-	-	+	-	-
Inosine	-	-	-	-	+	+	-	-	-	-
Thymidine	-	+	-	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-	-	+	-
α-D-Glucose-1-Phosphate	+	-	-	-	+	+	-	+	-	+
α-D-Glucose-6-Phosphate	-	+	+	+	-	-	+	+	+	+
Urocanic acid	-	-	-	+	-	-	-	-	+	-
Pyruvic Acid Methyl Ester	-	-	-	-	-	+	-	-	-	-
Succinic Acid Mono-Methyl ester	-	-	-	-	+	-	+	+	-	-
D-L-α-Glycerolphosphate	-	-	-	-	-	-	-	-	-	-
Bromosuccinic Acid	-	-	-	-	-	+	-	-	-	-

Table 22 End

the least preferred polymer but as in the case of ammonium oxidizers Tween 40 was the most preferred one. All the isolates were able use D-Alanine, L-Serine and Glycyl L-Aspartic Acid while the least preferred amino acid was L-Threonine. Among amides, Succinamide was used by all the isolates while N-Acetyl D Glucosamine, was used only by the isolate, NO13. The following list gives the isolates and one of the signature carbon substrates which is used only by the isolate. Isolate NO20 had no carbon source unique to its utilization profile.

NO11: Cis Aconitic acid

NO12: Citric acid and Malonic acid

NO13: N-Acetyl D Glucosamine

NO14: D, L-Lactic acid

NO15: Formic Acid

NO16: α Ketobutyric acid

NO17: D-Saccharic acid

NO18: p-Hydroxyphenylactic acid

NO19:Uridine

Many carboxylic acids like D Gluconic acid, D Galacturonic acid, Sebacic acid, α-Hydroxybutyric acid and β-Hydroxybutyric acid were not used by any of the nitrite oxidizers. Also, nitrite oxidizers in this study do not have the capability to utilize compounds like 2,3-Butanediol and D, L-α-Glycerol phosphate. The percentage utilization of various substrates by nitrite oxidizers is presented in Figure 28. Most of the isolates showed an enhanced efficiency (60-85%) in utilizing many amino acids. NO11, NO12, NO13, NO14 and NO16 (all 60%) were the best among the ten nitrite oxidizers for its ability to use more number of polymers. In terms of amide utilization, NO13 and NO17 (both 62%) showed the highest capability in the number of substrates utilized.

The different substrates tested were utilized by one or the other coupled ammonium-nitrite oxidizers (Table 23). Carboxylic acids such as D Galacturonic acid, Acetic acid,α β and γ Hydroxybutyric acids and α Ketoglutaric acid were not used by any of the isolates. Like the nitrite oxidizers none of them were able to use 2, 3 Butanediol. The most preferred sugars were α D-Glucose and D-Galactose, while the least preferred was L-Fucose as only three of the CAoNo isolates used it. As was the case with ammonium and nitrite oxidizers, the most preferred polymer was Tween 40, while the least preferred was Glycogen. Glutamic acid was used by only three of the ten isolates while Glycyl L-Aspartic acid was used by all the isolates. Succinamide and Propionic acid were the most preferred amide and carboxylic acid, respectively. The following list gives the isolates which has one of the signature carbon substrate which is used only by the isolate.

CAoNo21: D-Gluconic acid

CAoNo24: Thymidine

CAoNo25: p-Hydroxyphenylactic acid and Malonine

CAoNo26: α Ketobutyric acid

CAoNo27: Pyruvic acid Methyl Ester

CAoNo28: D-Glucosaminic acid

Figure 29 illustrates the percentage utilization of carbon substrates by the coupled ammonium- nitrite oxidizers in utilizing the different carbon substrates. Isolates CAoNo22 and CAoNo25 (75%) showed the maximum ability to use more number of amino acids than any other isolate, while for sugars it was

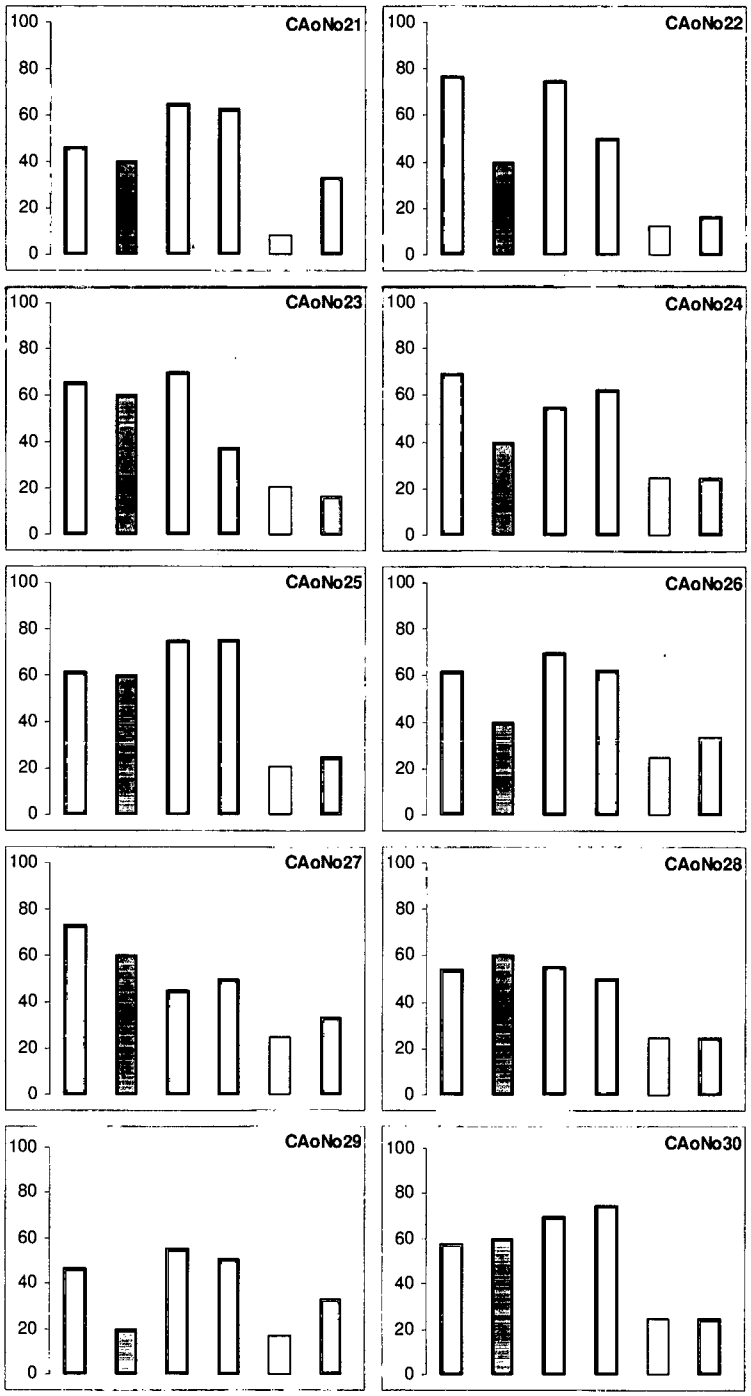


Figure 29: Summary of the percentage utilization of carbon substrates by Coupled Ammonium-Nitrite Oxidizers. ◘ Sugars (26), ◘ Polymers (5), ◘ Amino Acids (20), ◘ Amides (8), □ Carboxylic Acids (24) ◘ Miscellaneous (12). Values in brackets indicate the number of substrates tested. % utilization is given on y axis.

Table 23: Difference in carbon source utilization according to Biolog GN2 for the different Coupled Ammonium-Nitrite Oxidizer (CAoNo) isolates.

Carbon source	CAo No21	CAo No22	CAo No23	CAo No24	CAo No25	CAo No26	CAo No27	CAo No28	CAo No29	CAo No30
Sugars										
D-Arabitol	-	+	+	+	-	-	+	+	-	-
Adonitol	-	+	+	+	+	+	+	-	+	-
D-Cellobiose	-	-	-	+	-	-	+	+	-	-
D-Fructose	-	-	+	-	+	+	-	-	-	-
L-Fucose	-	-	-	+	-	-	-	-	+	-
D-Galactose	-	+	+	+	-	-	+	+	+	-
Gentiobiose	-	+	-	+	+	+	-	-	-	-
α-D-Glucose	-	+	+	+	+	+	+	+	+	-
m-Inositol	-	-	+	-	-	+	+	-	-	-
α-D-Lactose	-	-	-	+	+	-	-	-	-	-
Lactulose	-	+	+	+	-	-	+	+	+	-
Maltose	-	+	+	-	+	+	-	-	+	-
L-Arabinose	-	+	+	+	+	+	+	+	+	-
D-Mannitol	-	-	-	+	-	+	-	+	-	-
D- Mannose	-	+	-	-	+	-	+	-	-	-
i-Erythritol	-	+	+	-	-	-	+	+	-	+
L-Rhamnose	-	+	+	+	+	-	+	+	+	-
D-Sorbitol	-	+	+	-	+	+	-	+	+	-

Sucrose	-	+	-	+	-	-	+	-	-	-
D-Trehalose	-	+	+	+	+	+	+	-	+	-
Turanose	-	+	-	-	+	+	+	+	-	+
Xylitol	+	+	+	-	-	-	+	+	-	-
D-Melibiose	-	+	+	+	+	+	+	+	-	-
β-Methyl-D-Glucoside	+	+	+	+	+	+	+	-	-	-
D-Psicose	-	+	+	+	-	+	+	+	+	-
D-Raffinose	-	+	-	+	+	+	+	-	+	-
Polymers										
α-Cyclodextrin	-	-	+	+	-	-	+	+	-	-
Dextrin	+	+	+	-	-	+	-	-	-	-
Glycogen	-	-	-	-	+	-	-	-	-	+
Tween 40	+	+	+	+	+	+	+	+	+	+
Tween 80	-	-	-	-	+	-	+	+	-	+
Amino acids										
D-Alanine	+	+	+	+	+	-	+	+	+	+
L-Alanine	-	+	-	-	+	+	+	-	+	+
L-Alanyl-Glycine	-	+	-	-	+	+	+	+	-	+
L-Asparagine	+	+	+	-	+	+	-	-	-	-
L-Glutamic Acid	-	-	+	-	+	-	-	-	-	+
Glycyl-L-Glutamic Acid	+	+	+-	+	+	+	+	+	+	+
L-Aspartic Acid	-	+	+	-	+	+	+	-	-	+
L-Orinithine	+	-	+	+	-	+	-	-	+	+
L-Histidine	+	+	+	-	-	-	-	+	-	+
Hydroxy-L-Proline	+	-	-	-	+	-	-	+	+	+
L-Leucine	-	+	+	+	+	+	+	-	-	+

Table 23 Continued

D-Serine	+	+	+	+	-	+	-	-	+	-
L-Threonine	+	+	+	-	+	+	+	+	+	+
L-Phenylalanine	-	-	+	+	-	+	-	-	+	-
D,L-Carnitine	+	+	-	+	+	-	-	+	-	+
Glycyl-L-Aspartic Acid	+	+	+	+	+	+	+	+	+	+
L-Pyroglutamic Acid	+	-	+	+	+	+	-	+	-	-
L-Proline	-	+	-	+	-	+	-	-	+	+
γ-Aminobutyric Acid	+	+	+	-	+	-	+	+	-	-
L-Serine	+	+	+	+	+	+	-	+	+	-
Amides										
L-Alaninamide	+	-	-	+	-	+	-	-	-	-
N-Acetyl-D-Glucosamine	+	+	-	+	+	-	+	-	-	+
N-Acetyl-D-Galactosamine	-	-	+	-	+	+	+	-	+	+
Succinamide	+	+	+	+	+	+	+	+	+	+
Glucoronamide	-	-	-	-	+	-	-	-	+	+
Phenylethylamine	+	+	-	+	-	+	-	+	-	+
2-Aminoethanol	-	+	+	+	+	+	+	+	+	+
Putrescine	+	-	-	-	+	-	-	+	-	-
Carboxylic acids										
D-Gluconic Acid	+	-	-	-	-	-	-	-	-	-
D-Galacturonic acid	-	-	-	-	-	-	-	-	-	-
Acetic Acid	-	-	-	-	-	-	-	-	-	-
α-Hydroxybutyric Acid	-	-	-	-	-	-	-	-	-	-
β-Hydroxybutyric Acid	-	-	-	-	-	-	-	-	-	-

Table 23 Continued

γ-Hydroxybutyric Acid	-	-	-	-	-	-	-	-	-	-
p-Hydroxyphenylacetic Acid	-	-	-	-	+	-	-	-	-	-
α-Ketoglutaric Acid	-	-	-	-	-	-	-	-	-	-
α-Ketovaleric Acid	-	-	+	+	-	-	-	-	-	-
α-Ketobutyric Acid	-	-	-	-	-	+	-	-	-	-
D,L-Lactic Acid	-	-	+	+	-	-	-	-	-	+
D-Glucosaminic acid	-	-	-	-	-	-	-	+	-	-
D-Glucuronic acid	-	-	+	-	-	-	+	+	-	-
Propionic Acid	+	+	+	+	+	+	+	+	+	+
Formic Acid	-	+	-	+	-	+	+	-	-	-
Citric Acid	-	-	-	+	+	-	-	-	+	-
Succinic Acid	-	-	-	-	-	-	-	+	-	+
Cis-Aconitic Acid	-	-	+	-	-	-	+	-	-	-
D-Galactonic Acid Lactone	-	+	-	-	-	+	-	-	+	+
Itaconic Acid	-	-	-	-	-	-	+	+	-	+
D-Saccharic Acid	-	-	-	-	-	+	+	-	-	-
Sebacic Acid	-	-	-	+	+	-	-	+	-	-
Quinic Acid	-	-	-	-	-	+	-	-	+	+
Malonic Acid	-	-	-	-	+	-	-	-	-	-
Miscellaneous										
2,3-Butanediol	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	+	+	-	-	-	-	-	+
Inosine	-	+	-	-	-	+	+	+	+	-
Thymidine	-	-	-	+	-	-	-	-	-	-
Uridine	-	-	-	-	+	+	-	-	+	-

Table 23 Continued

α-D-Glucose-1-Phosphate	+	-	-	+	-	+	-	-	+	+
α-D-Glucose-6-Phosphate	-	-	-	-	+	-	+	-	-	-
Urocanic acid	+	-	+	-	-	-	-	+	-	-
Pyruvic Acid Methyl Ester	-	-	-	-	-	-	+	-	-	-
Succinic Acid Mono-Methyl ester	+	+	-	-	+	-	+	-	-	+
D-L-α-Glycerolphosphate	-	-	-	-	-	+	-	+	-	-
Bromosuccinic Acid	+	-	-	-	-	-	-	-	+	-

Table 23 End

CAoNo22 (76%) and CAoNo27 (73%). CAoNo23, CAoNo25, CAoNo27, CAoNo28 and CAoNo30 had the capacity to use the maximum number (60%) of polymers, as compared to the other isolates. CAoNo25 and CAoNo30 (both 75%) had the best metabolic flexibility in terms of the utilization of amides.

The biochemical characteristics of the isolates are given in Table 24. All the isolates were gram negative and positive for oxidase and catalase tests. Only the isolates AO8, CAoNo21 and CAoNo30 were able to grow with 4%NaCl. Almost 50% of the isolates were able to grow at 37°C. It could be noted that most of the nitrite oxidizers (4/5) were able to grow at 37°C. All except AO6, AO4 and AO8 were able to utilize citrate. The ability to utilize nitrate was more prevalent (4/5) in CAoNo isolates. Many of the isolates used glucose oxidatively while isolate AO8 showed fermentative metabolism. Gelatinase (5/15) and amylase activity (3/15) was not widespread in these isolates. AO3, AO6, CAoNo26 and CAoNo21 were positive for urease production. Production of indole (1/15) and hydrolysis of arginine (2/15) were not common in the isolates studied.

4.5.2. Molecular identity by 16SrDNA

Table 25 gives an account on the identity of some of the nitrifier strains showing enhanced activity in *in vitro* experiments. It was interesting to observe that the nitrifers were distributed in the classes α, β and γ of proteobacteria. Thirteen of the 15 isolates belonged to the β and γ proteobacteria. The α, β and γ proteobacteria were encountered at Divar while at Tuvem it was restricted to the β and γ classes of proteobacteria. Though some of the well known nitrifiers

Table 24: Some of the biochemical characteristics of the isolates. + (positive); - (negative); O (oxidative); F (fermentative)

Culture ID	Grams reaction	Oxidase	Catalase	Urease	Gelatinase	Amylase	Tween 20	Indole production	Arginine hydrolysis	Glucose metabolism	Nitrate reduction	Growth on Citrate	Growth at 37°C	Growth in presence of 4% NaCl
AO3	-	+	+	+	+	-	+	-	-	O	+	+	+	-
AO6	-	+	+	+	-	-	-	-	-	-	-	-	-	-
AO4	-	+	+	-	-	-	-	-	-	-	-	-	-	-
AO10	-	+	+	-	-	-	+	-	-	O	+	+	-	-
AO8	-	+	+	-	+	+	-	+	-	F	+	-	+	+
NO15	-	+	+	-	-	-	-	-	-	O	+	+	+	-
NO11	-	+	+	-	-	-	-	-	-	-	+	+	+	-
NO14	-	+	+	-	-	-	-	-	+	O	-	+	+	-
NO19	-	+	+	-	+	-	+	-	-	O	+	+	-	-
NO17	-	+	+	+	-	-	+	-	-	O	-	+	+	-
CAoNo 26	-	+	+	-	+	-	+	-	-	O	+	+	-	-
CAoNo 22	-	+	+	+	-	+	+	-	+	O	+	+	-	-
CAoNo 21	-	+	+	-	+	+	-	-	-	-	+	+	+	+
CAoNo 29	-	+	+	-	-	-	+	-	-	O	+	+	-	-
CAoNo 30	-	+	+	-	-	-	-	-	-	-	-	+	+	+

like *Nitrosomonas halophila, Nitrosomonas marina* and *Nitrobacter winogradskyi* were encountered among the identified isolates, most of the isolates (40%) belonged to the genus *Pseudomonas.* Other genera represented were *Alcaligenes*, *Janthinobacterium* and *Aeromonas.* It could also be observed that of the six genera encountered, only *Pseudomonas* was found to have the ability to oxidize ammonium, nitrite and both simultaneously. *Alcaligenes* was the other genus which was capable of oxidizing nitrite and ammonium and nitrite in CAoNo incubations. It was noted that the strains capable of oxidizing ammonium and nitrite (CAoNo) simultaneously, were all heterotrophs. However, in ammonium oxidation experiments, both the *Pseudomonas* strains oxidized ammonium in inorganic conditions while *Aeromonas sp* was capable of nitrification in organic media. *Nitrobacter winogradskyi*, a known nitrite oxidizer showed very high activity in inorganic nitrifying media while the rest of the nitrite oxidizers were heterotrophs and oxidized nitrite in organic nitrifying media. The *Nitrosomonas* genus contributed upto ~53% of the nitrifiers while nitrite oxidizers were up to 65% and was represented by *Nitrobacter winogradkyi.* In the present study, under given conditions *Nitrosomonas marina* could be the dominant species compared to its counterpart *N. halophila* involved in the oxidation of ammonium in mangrove sediments. This gives additional insights on the trophic status of nitrifiers, which are originally known to be strict lithotrophs.

Table 25: Molecular identity of the three groups of nitrifiers; Ammonium Oxidizer (AO), Nitrite Oxidizer (NO) and Coupled Ammonium-Nitrite Oxidizer (CAoNo). #Values in parenthesis correspond to CFU x 10^2 g/wet weight sediment corresponding to 100%.

Culture ID	16SrDNA Identity	Abundance of the isolate among the total CFU#	Area of isolation	Depth of isolation
AO3	*Pseudomonas sp.*	48.5 (7.7)	Tuvem	0-2 cm
AO6	*Nitrosomonas marina*	52.6 (2.7)	Tuvem	8-10 cm
AO4	*Nitrosomonas halophila*	14.2 (5.5)	Divar	4-6 cm
AO10	*Pseudomonas sp.*	18.6 (4.5)	Tuvem	4-6 cm
AO8	*Aeromonas sp.*	28.6 (6.6)	Divar	2-4 cm
NO15	*Nitrobacter winogradskyi*	65.4 (2.6)	Divar	0-2 cm
NO11	*Alcaligenes sp.*	43.7 (3.1)	Tuvem	2-4 cm
NO14	*Pseudomonas sp.*	35.6 (2.8)	Divar	0-2 cm
NO19	*Janthinobacterium sp.*	45.9 (7.8)	Tuvem	2-4 cm
NO17	*Pseudomonas sp.*	47.6 (6.0)	Tuvem	0-2 cm
CAoNo 26	*Janthinobacterium sp.*	64.3 (2.4)	Tuvem	0-2 cm
CAoNo 22	*Pseudomonas sp.*	61.4 (3.9)	Divar	2-4 cm
CAoNo 21	*Alcaligenes sp.*	41.6 (4.2)	Divar	0-2 cm
CAoNo 29	*Pseudomonas sp.*	39.6 (5.3)	Divar	8-10 cm
CAoNo 30	*Alcaligenes faecalis*	24.9 (3.2)	Divar	8-10 cm

4.6. Experiments – factors influencing nitrification

Laboratory experiments were conducted to assess the influence of environmental parameters like dissolved oxygen, dissolved organic carbon, ammonium, nitrite and nitrate on nitrification rates. The impact of anthropogenic factors like liquid hydrocarbons, pesticides and fertilizers were also studied on this process. In general, dissolved oxygen and ammonium had a stimulatory effect, while nitrite, nitrate and dissolved organic carbon had a retarding effect. Though pesticides, at a lower concentration showed stimulatory effect, liquid hydrocarbons and fertilizers had a profound negative influence at all the concentrations tested.

In unamended and unblocked (UB) control incubations (Figure 30 A), ammonium was produced at a rate of 23.5±6.0 nM NH_4^+ g (sediment)$^{-1}$ h^{-1}. Henceforth the unit nM NH_4^+ g (sediment)$^{-1}$ h^{-1} shall be referred to as nM NH_4^+ h^{-1}. The rates were found to increase up to the 12th day. This suggests that the net ammonium pool is dominated by ammonium production/nitrite reduction. The nitrite production rates in the system varied from 1.6 to 5.6 nM NO_2^- h^{-1} (Figure 30A). The unit nM NO_2^- g (sediment)$^{-1}$ h^{-1} shall be referred to as nM NO_2^- h^{-1}. The nitrate production rates in the system showed a constant decreased with time at a rate of 14.4±3.6 nM NO_3^- g (sediment)$^{-1}$ h^{-1}. From now on the unit nM NO_3^- g (sediment)$^{-1}$ h^{-1} shall be referred to as nM NO_3^- h^{-1}. In nitrification blocked (NB) control systems (Figure 30B), ammonium production/nitrite reduction was lower and occurred in the range 2.5 to 4.8 nM NH_4^+ h^{-1}. The rate of nitrate disappearance was almost uniform thought the period of incubation (13.2±1.1 nM

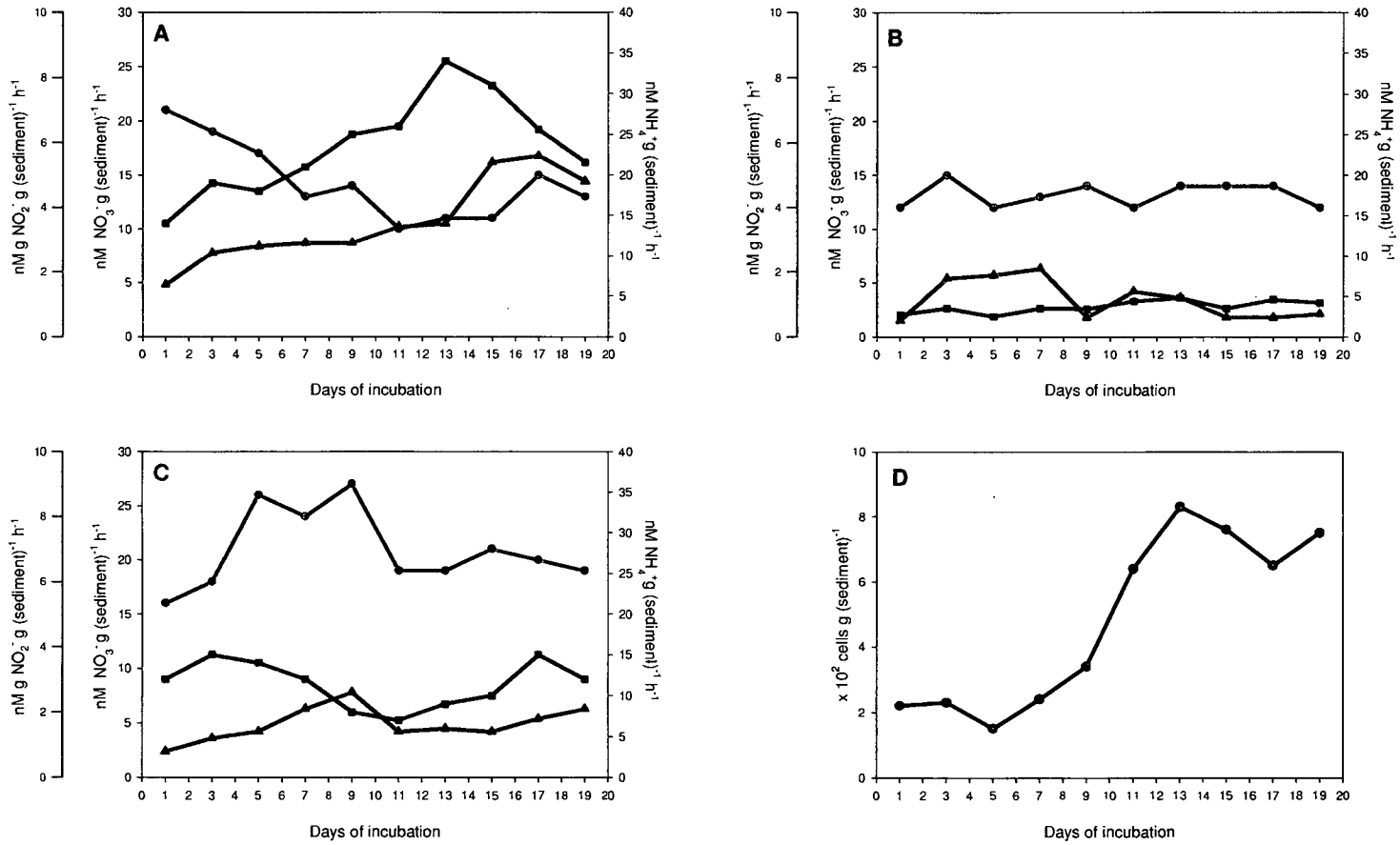


Figure 30: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems in control incubations. Nitrifier counts in denitrification blocked system is given in (D).

NO_3^- h^{-1}). The nitrite production rates in the system varied from 0.5 to 2.1 nM NO_2^- h^{-1} (Figure 30B). In denitrification blocked (DB) control systems (Figure 30C) nitrification occurred at a rate of 21±3.6 nM NO_3^- h^{-1}. The peak rates were encountered between the 5th and 9th day. A dip in nitrate production beyond the 9th day indicates that nitrification rates are substrate dependent. The nitrite production rates in the system varied from 0.8 to 2.6 nM NO_2^- h^{-1} (Figure 30C). The pattern of evolution of the abundance of nitrifier in the DB system almost resembled a sigmoid curve with its peak on the 13th day (Figure 30D).

4.6.1. Dissolved oxygen

Figures 31 A, B and C illustrate the changes in pulse mode incubations in UB, NB and DB systems. In UB system, the ammonium pool is probably controlled by net ammonium production, which occurred at a rate of 64.7±7.2 nM NH_4^+ h^{-1}. There was not much change in the nitrite production rates, which ranged from 1.1-2.9 nM NO_2^- h^{-1}. The nitrate production rates increased up to the 4th day and declined sharply till the 7th day. The nitrate production rate was 27±5.6 nM NO_3^- h^{-1}. In NB system (Figure 31B), the denitrification rates were retarded to a magnitude of 5.6±1.7 nM NO_3^- h^{-1}, while the net change in NO_2^- was 2.3±0.4 nM NO_3^- h^{-1}. The variation in the changes in ammonium pool almost resembled that of the UB system, which is probably controlled by net ammonium production/nitrite reduction. In DB system (Figure 31C) a profound increase in ammonium utilization rates (100.9±10.8 nM NH_4^+ h^{-1}) was observed along with a sharp increase in nitrate production rates (108.5±31.1 nM NO_3^- h^{-1}). This clearly

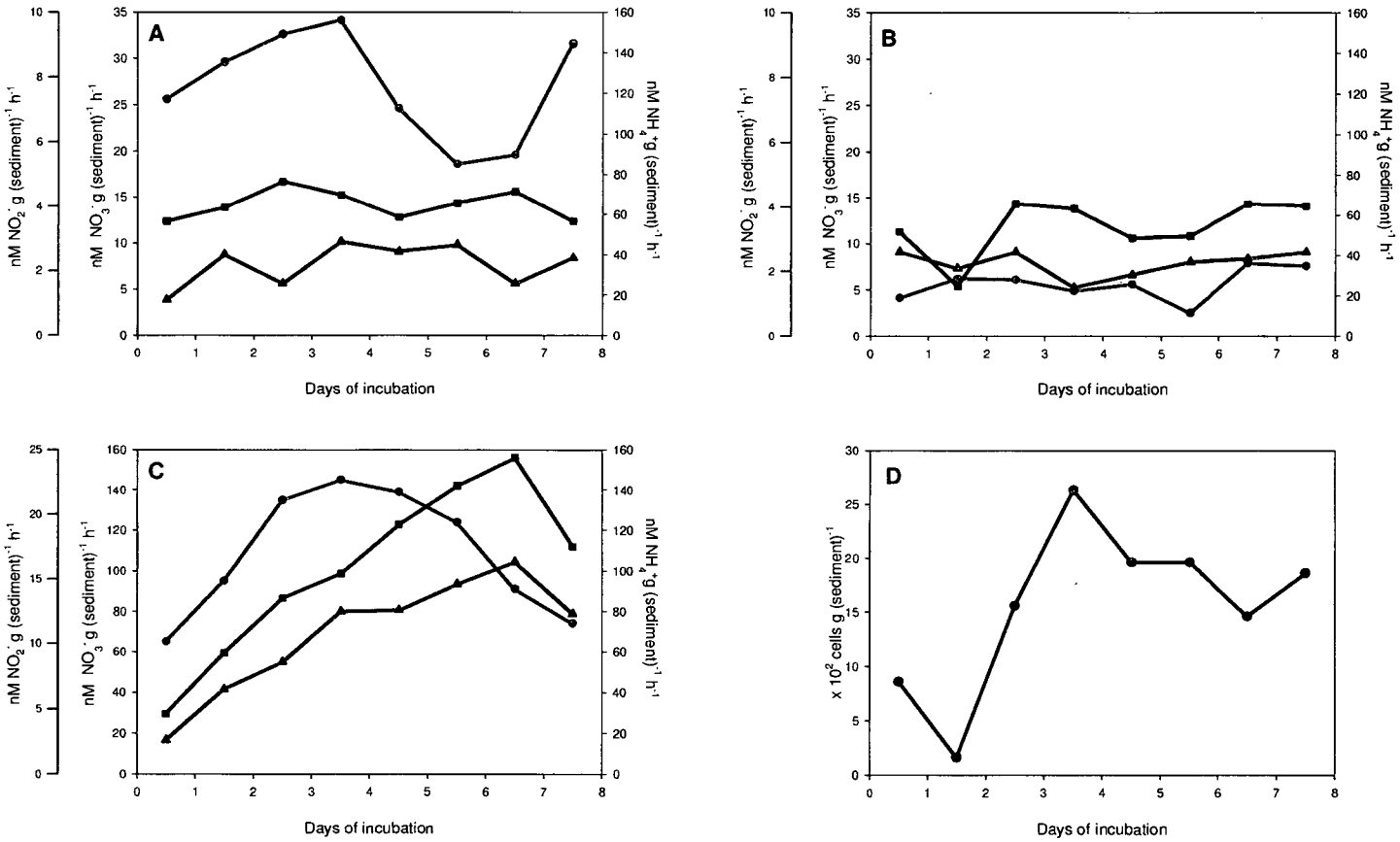


Figure 31: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems in pulse mode incubations. Nitrifier counts in denitrification blocked system is given in (D).

indicates that the pulse mode triggers the production of ammonium at the same time catalyses the rates of nitrification to a large extend. The pattern of net nitrite production (Figure 31C) more or less resembles that of ammonium, while nitrate production pattern was more parabolic when illustrated. The nitrifier counts in DB system (Figure 31D) supports the nitrate production rates with both peaking at the end of 4 days with the nitrifier counts attaining a maximum growth of ~27 x 10^2 cells g (sediment)$^{-1}$.

In continuous mode incubations, the variations in nitrite production rates were higher in the UB system (Figure 32A) than NB (Figure 32B) and DB systems (Figure 32C). In general, it ranged from 1.3 to 6.2 nM NO_2^- h^{-1}. There was not much change in the ammonium high rates in UB and NB system, while the ammonium utilization was profound in DB systems. In NB system, the denitrification rates ranged from 1.6 to 6.4 nM NO_3^- h^{-1}. These rates are considerably lower than the control NB system (Figure 30C), probably due to the negative impact of dissolved oxygen on nitrate reduction. In DB systems the nitrification rate of 94.7±25 nM NO_3^- h^{-1} occurred with concomitant net ammonium utilization, which was 127±34 nM NO_3^- h^{-1}. The nitrification rates increased with time, indicating that ammonium production could be tightly coupled with this process, thereby making the substrate available. The population of nitrifiers in DB system (Figure 32D) was relatively lower than its counter part in pulse mode. The cell counts increased from 6.5 to 14.5 x 10^2 cells g (sediment)$^{-1}$ on the 7th day. In general, continuous aerations appear to retard efficient conversion of ammonium to its oxidized products through nitrification.

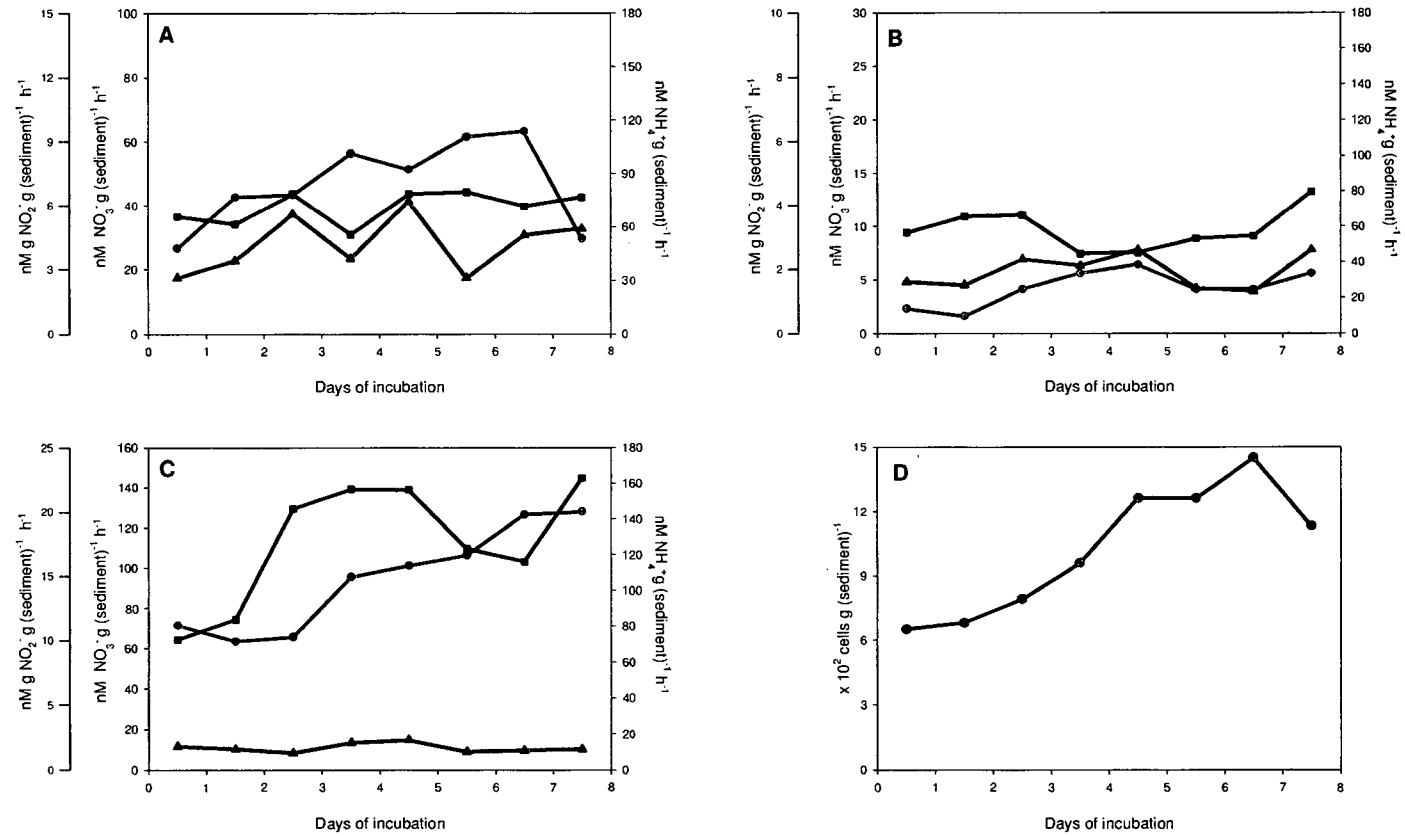


Figure 32: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems in continuous mode incubations. Nitrifier counts in denitrification blocked system is given in (D).

4.6.2. Dissolved Organic Carbon

In UB 10 mg C L^{-1} amendments (Figure 33A), the rate of ammonium production (15.8±3.5 nM NH_4^+ h^{-1}) gradually increased with time, but nitrate production rates retarded over time (16.2±2.1 nM NO_3^- h^{-1}). The nitrite production rate ranged from 0.6 to 6.4 nM NO_2^- h^{-1} and peaked on the 12th day, while for the other days the changes were comparable. In NB (Figure 33B) incubations, the ammonium production rates (8±2 nM NH_4^+ h^{-1}) were comparatively lower and the variation was marginal. The denitrification rates, which ranged from 6.6 to 21.3 nM NO_3^- h^{-1}, were found to increase gradually up to the 10th day, but beyond that it decreased. In DB (Figure 33C) systems, nitrification rates (8.1±1.6 nM NO_3^- h^{-1}) were found to be retarded compared to the NB controls (Figure 30C), though there was no much hourly variation. The nitrate production and ammonium utilization (15±2.4 nM NH_4^+ h^{-1}) patterns were in phase up to the 12th day, beyond which they were almost inverse. The dip in nitrification rates is also reflected in the pattern of nitrifier counts, which showed a very sharp fall of cell counts from 3.5 to 0.8 x 10^2 cells g $(sediment)^{-1}$ within the first 8 days.

In UB 25 mg C L^{-1} amendments (Figure 34A), the net ammonium production rates (16±4.1 nM NH_4^+ h^{-1}) were found to decrease up to the 8th day but beyond that the rates steadily increased until the 14th day. The nitrate production (12.8±5 nM NO_3^- h^{-1}) in the system on the other hand increased, gradually overtime with a marked spike on the 11th day. This spike coincided with the sharp increase in net ammonium and also peak in nitrite production. There were multiple peaks in nitrite production (1.3±1.1 nM NO_2^- h^{-1}) till the end of the

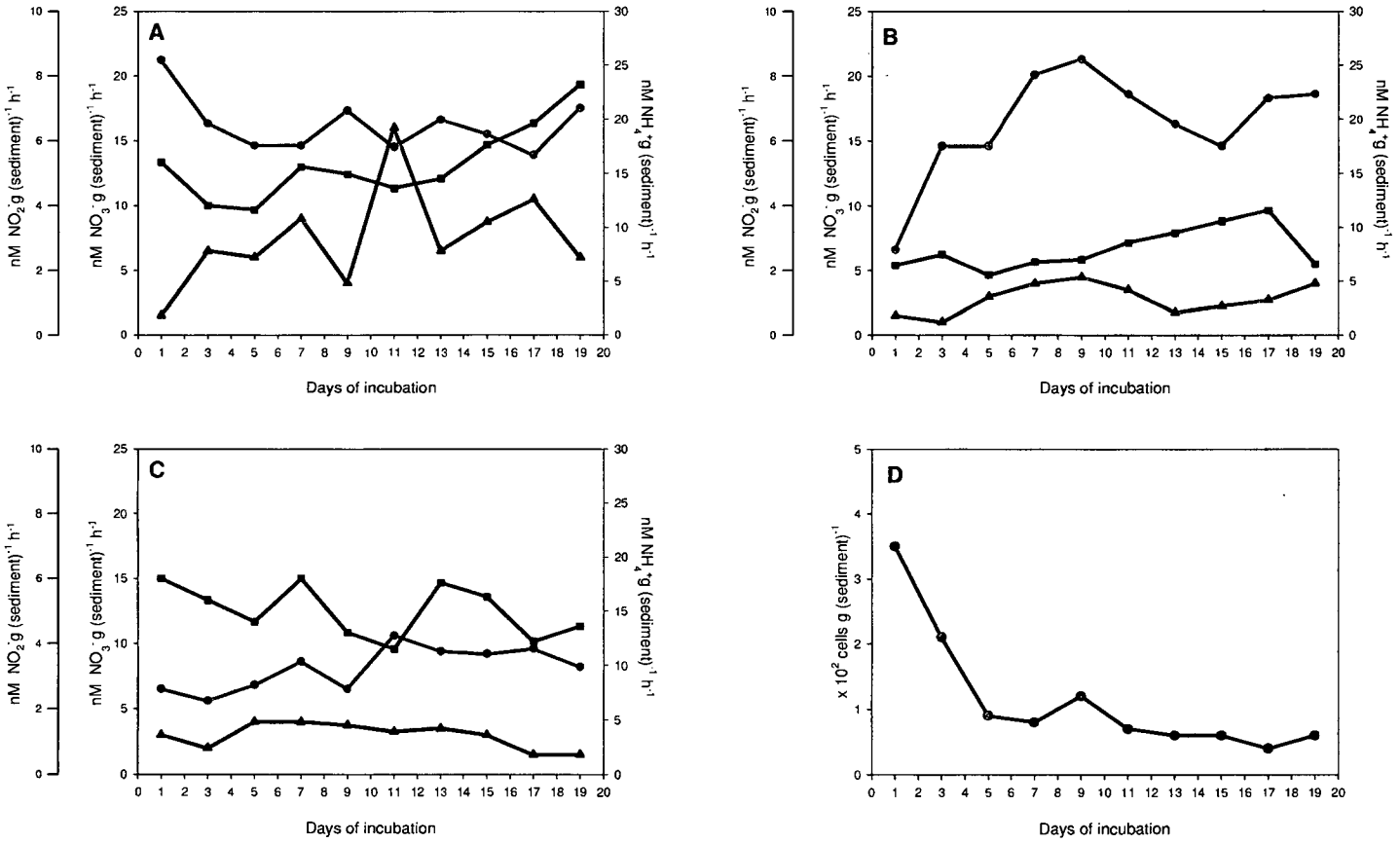


Figure 33: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked systems (C) with 10 mg Glucose-CL^{-1}. Nitrifier counts in denitrification blocked system is given in (D).

period of incubation. In NB (Figure 34B), much variation was not seen in the ammonium (3.6±1.1 nM NH_4^+ h^{-1}) and nitrite (2.2±0.6 nM NO_2^- h^{-1}) production patterns, but nitrate reduction (21.9±6.1 nM NO_3^- h^{-1}) was dominant with the maximum reduction rates observed between 10th and 12th days. The nitrification rates (5.2±1.9 nM NO_3^- h^{-1}) in DB (Figure 34C) were lower than the control DB without much change throughout the period of incubation. Ammonium utilization rates (14.7±1.5 nM NH_4^+ h^{-1}) were higher than the control system. However, since the nitrite production was low, the increase observed in ammonium utilization could be due to stimulated assimilation or inefficient nitrite oxidation. The nitrifier population (Figure 34D) was lower than control and peaked twice (6th and 16th day) during the 20 day long incubation period.

Net ammonium production in 50 mgCL^{-1} amended UB incubations (Figure 35A) was the highest (18.3±4.0 nM NH_4^+ h^{-1}) among all the three dissolved organic carbon amendments. Though initially the rates dipped, it peaked half way through the incubation period. Beyond this point the rates gradually decreased. Much variation was not seen in the nitrate production rates (14.9±2.0 nM NO_3^- h^{-1}), indicating that the processes governing the same are regulated. The nitrite production rates were the highest in (5.5±3.8 nM NO_2^- h^{-1}) in UB systems, peaking on the 8th and 18th day. In NB system (Figure 35B), denitrification rates (28.6±8.5 nM NO_3^- h^{-1}) were found to have a net increase up to the 10th day, and thereafter fell to very low values until the 14th day and then sharply increased. Variation in nitrite (1.1±1.0 nM NO_2^- h^{-1}) and ammonium production (8.2±1.7 nM NH_4^+ h^{-1}) in the system was marginal. In DB system (Figure 35C), the nitrification

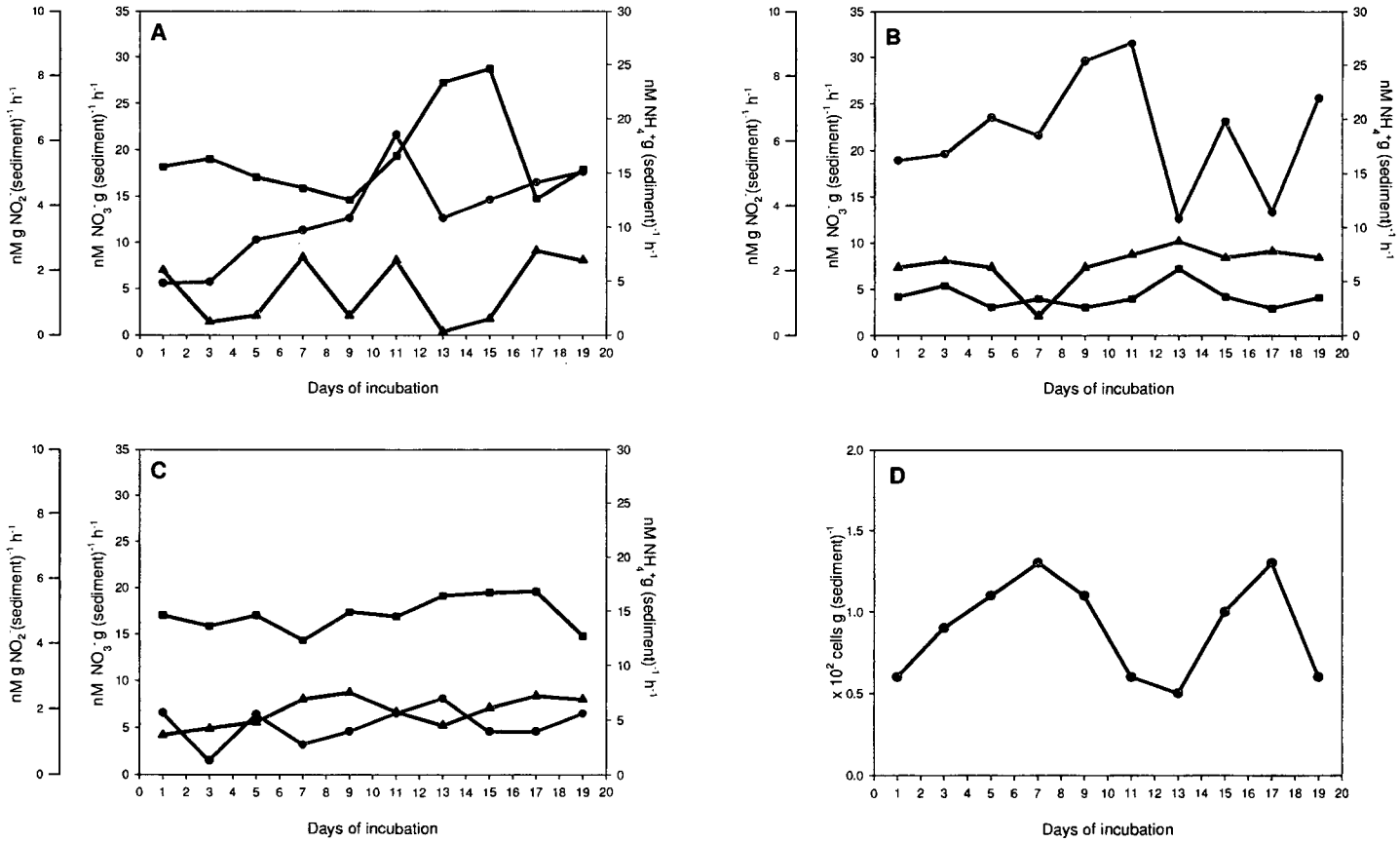


Figure 34: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 25 mg Glucose-CL^{-1}. Nitrifier counts in denitrification blocked system is given in (D).

rates (3.7±0.7 nM NO_3^- h^{-1}) were found to be extremely low and was the lowest among the three carbon amendments. Unlike that of 25 mgC amendment, the ammonium utilization rates (in 50 $mgCL^{-1}$ amendment) were found to be in line with nitrate production rates. The range of nitrifier counts (Figure 35D) was more or less comparable to that of lower amendments but a well defined peak was evident on the 8^{th} day (2.1 x 10^2 cells g (sediment)$^{-1}$.

In brief, the addition of dissolved organic carbon retarded nitrification rates at all concentrations tested.

4.6.3. Ammonium

Figure 36A illustrates various nitrogen conversion rates in the UB system spiked with 50 μM ammonium. The net ammonium assimilation (22.5±9.4 nM NH_4^+ h^{-1}) was found to increase in the second half of the incubation period with two prominent peaks occurring on the 10^{th} and 14^{th}-16^{th} day. The nitrite and nitrate production rates were 2.1±0.6 nM NO_2^- h^{-1} and 20.9±4.8 nM NO_3^- h^{-1}, respectively with both patterns showing with intermittent spikes. The intermittent nitrite spikes were also encountered in NB incubations (Figure 36B). The nitrate reductions (5.9±2.0 nM NO_3^- h^{-1}) as well as ammonium production rates (4.1±1.3 nM NH_4^+ h^{-1}) were comparatively lower and uniform throughout the period of incubation. It appears that addition of ammonium has a negative impact on denitrification rates. In DB systems (Figure 36C), nitrification rates (95.4±36.6 nM NO_3^- h^{-1}) increased over incubation period with concomitant increase in ammonium disappearance (88±45.1 nM NH_4^+ h^{-1}) and nitrite production (9.6±5.2

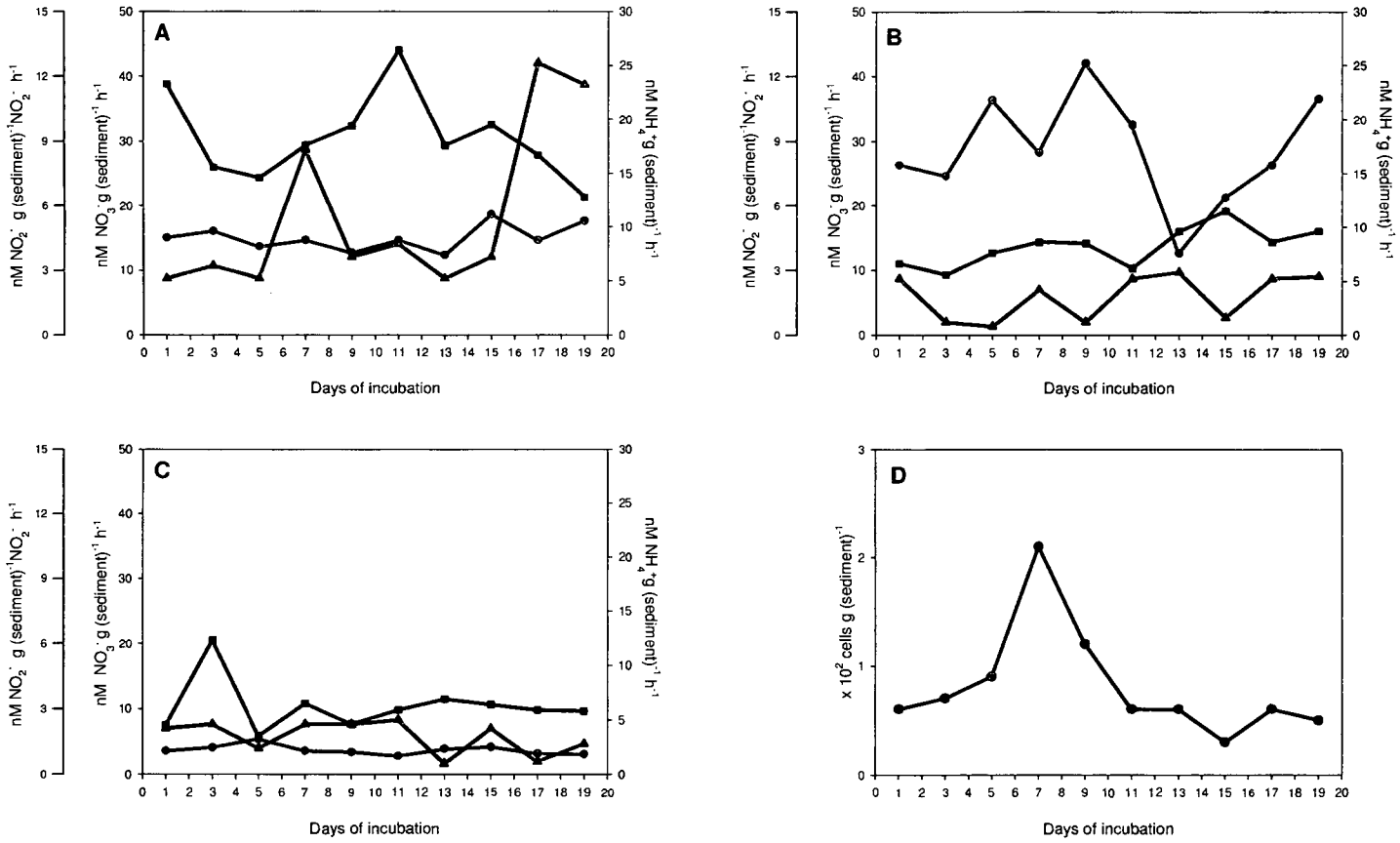


Figure 35: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked systems (C) with 50 mg Glucose-CL^{-1}. Nitrifier counts in denitrification blocked system is given in (D).

nM NO_2^- h^{-1}). Nitrate peaked much earlier (at 10 hr) than ammonium (at 14 hr) and nitrite (at 18hr). The nitrifier counts in DB system (Figure 36D) showed a steady increase over time till. The nitrifier counts at the beginning of the incubation was $3.8x10^2$ cells g $(sediment)^{-1}$ while at the end of the incubation it was $5.8x10^2$ cells g $(sediment)^{-1}$.

100 µM amendment with ammonium in UB system (Figure 37A) triggered the production of nitrate in the system up to 12 days and ranged from 86-186 nM NO_3^- h^{-1}. The production dropped later, but at a very low pace. Net ammonium assimilation (143±16.8 nM NH_4^+ h^{-1}) in the system was comparatively higher indicating that the addition of ammonium has enhanced the utilization of ammonium. It could also be observed in general, that the rates were lower during the second half of the incubation period. The net nitrite production rate (2.0±1.4 nM NO_2^- h^{-1}) was also lower during the second half of the incubation period. Denitrification rates (Figure 37B) in NB systems (20.2±6.2 nM NO_3^- h^{-1}) had a distinct peak on the 10^{th} day and further the rate showed a decreasing trend with time. The net changes in ammonium production (16.6±4.0 nM NH_4^+ h^{-1}) were not profound throughout the period of incubation. Nitrite production (2.4±1.3 nM NO_2^- h^{-1}) attained its maxima, coinciding with the nitrate reduction maxima on the 10^{th} day. In DB system, (Figure 37C), both nitrate production (159.4±21 nM NO_3^- h^{-1}) and ammonium utilization (152.2±24.1 nM NH_4^+ h^{-1}) appear to be stimulated to the maximum throughout the period of incubation. A more or less similar trend with low intensity could also be observed in the nitrite production rates (7.9±2.2

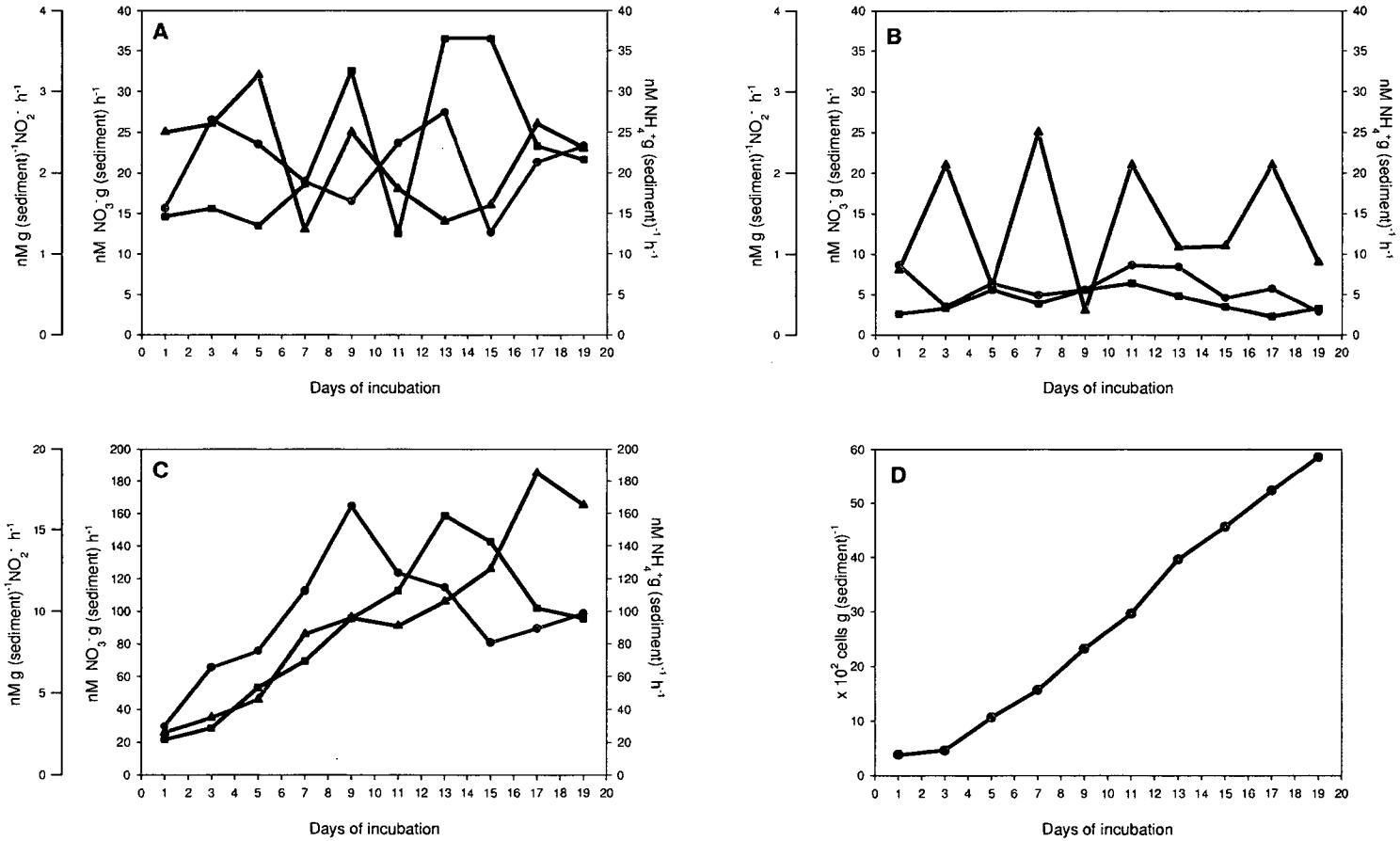


Figure 36: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 50 μM NH_4^+. Nitrifier counts in denitrification blocked system is given in (D).

nM NO_2^- h^{-1}). The elevated number of nitrifiers in the DB incubations (Figure 37D); almost throughout the incubation period fortifies the above observations.

The pattern of rates of net nitrate (163.4±31.6 nM NO_3^- h^{-1}) and nitrite (4.7±2.4 nM NO_2^- h^{-1}) produced in 150 µM ammonium amendments in UB system (Figure 38A) was almost similar. Overall, the rates of production were higher for both the ions. However, approximately after 75% of the incubation period, both rates declined sharply. The ammonium utilization rates were high (142.4±14.6 nM NH_4^+ h^{-1}) throughout the period of incubation with little change. The denitrification rates (24.7±5.8 nM NO_3^- h^{-1}) in the NB system (Figure 38B) showed a gradual increase up to the 14th day, but were erratic thereafter. A peak in the rate of nitrite production (3.2±0.8 nM NO_2^- h^{-1}) was observed on the 6th day while the ammonium production rates (15.3±2.1 nM NH_4^+ h^{-1}) were comparatively low with little variation through the period of incubation. Figure 38C (DB system) shows the enhancement of nitrification rates (142.0±24.5 nM NO_3^- h^{-1}) throughout the experiment but showed a gradual decrease from the 8th day onwards. However, the rates were less compared to 100 µM amendments. Nitrite production rates (4.7±2.9 nM NO_2^- h^{-1}), as in some other cases had multiple peaks with ammonium utilization (157.7±21.1 nM NH_4^+ h^{-1}) being high and almost uniform throughout the period of incubation. The nitrifier counts (Figure 38D) ranged from 12 to 34 x10^2 cells g (sediment)$^{-1}$. The abundance of nitrifiers in the experimental set up showed that after an initial increase, the counts declined until the 10th day and thereafter increased almost until the end of the incubation.

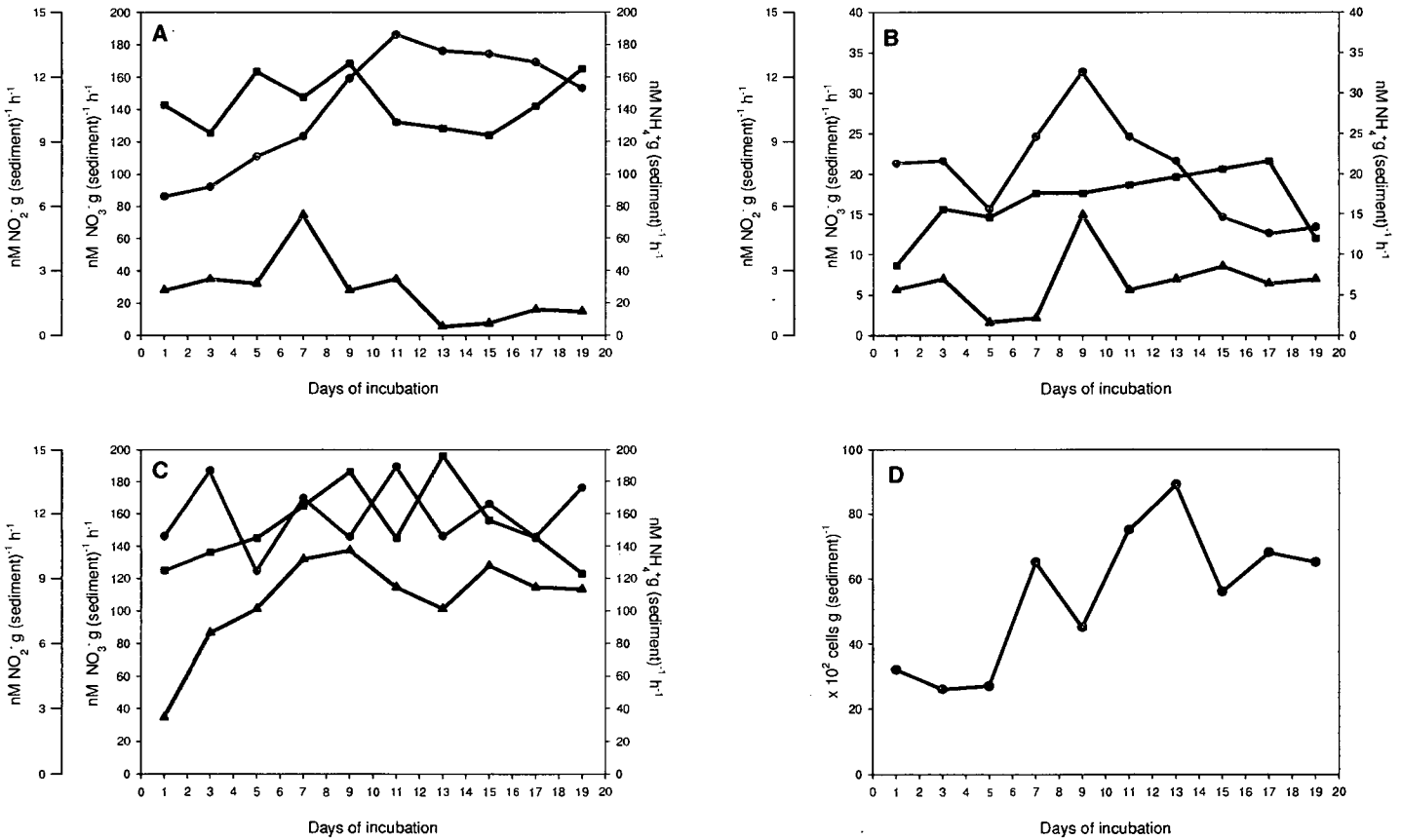


Figure 37: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 100 µM NH_4^+. Nitrifier counts in denitrification blocked system is given in (D).

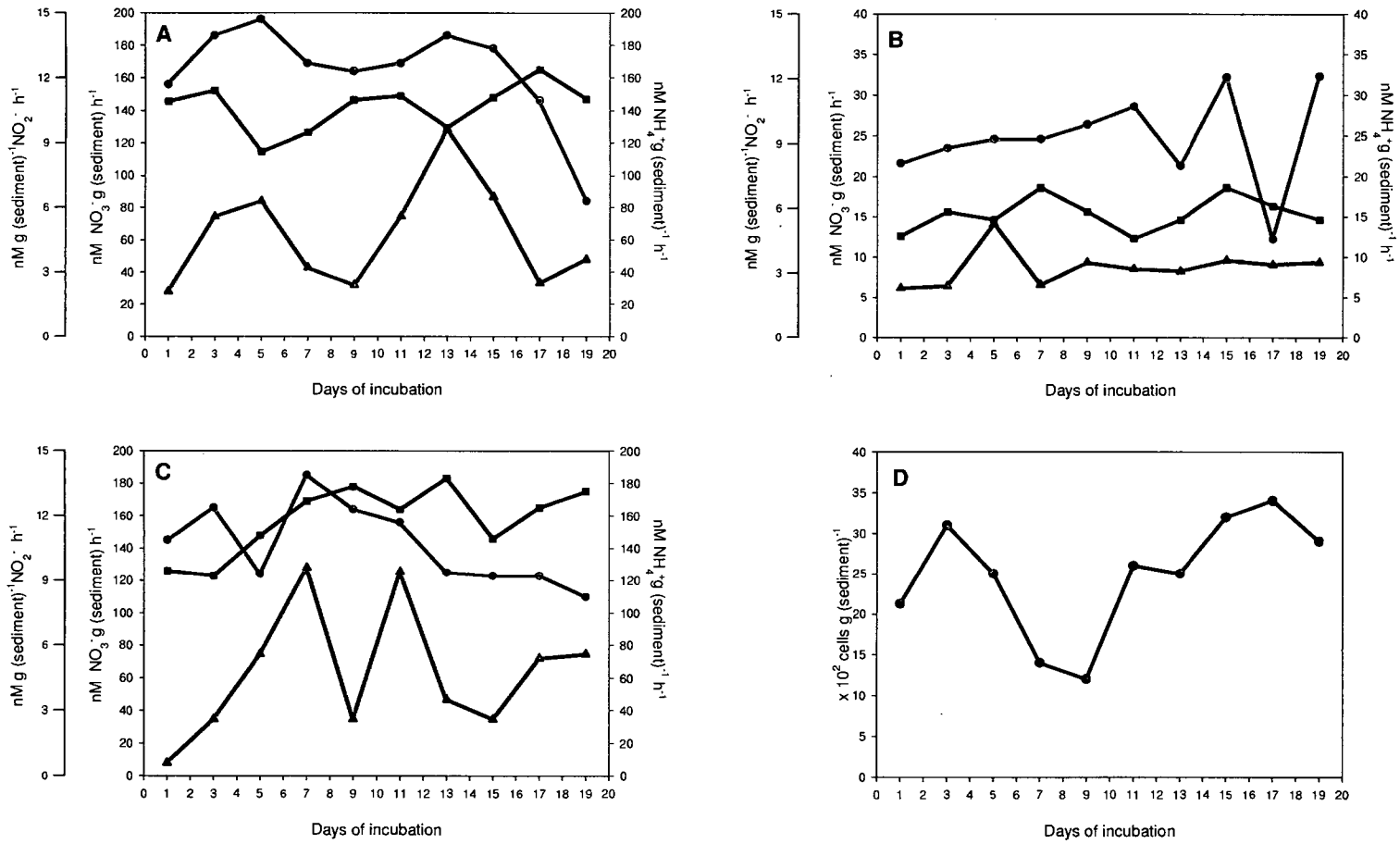


Figure 38: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 150 µM NH_4^+. Nitrifier counts in denitrification blocked system is given in (D).

In general, amendments of ammonium favored nitrification rates. Maximum stimulation in nitrification rates occurred when the sediments were amended with 150μM of ammonium.

4.6.4. Nitrite

In UB 5 μM nitrite enriched system (Figure 39A), the net ammonium production rates were low (1.4±0.9 nM NH_4^+ h^{-1}) and showed very little variation. In general, the nitrite production rates increased with time with the peak production on the 10th day (3.2 nM NO_2^- h^{-1}). The nitrate reduction rates (14.6±4.7 nM NO_3^- h^{-1}) decreased with time almost throughout the period of incubation. Figure 39B shows the change in various nitrogen conversion rates in NB system. The ammonium production rates (11.3±4.2 nM NH_4^+ h^{-1}) after an initial dip, increased up to the 12th day beyond which it decreased sharply and attained 5.1 nM NH_4^+ h^{-1} at the end of the incubation period. The denitrification rates in the system showed a decrease on the 8th and14th day, while for the rest of the days it was comparable (6.2±2.1 nM NO_3^- h^{-1}). The pattern of change in nitrite production and denitrification rates were similar in the system beyond the 10th day, while until the 10th day both processes showed almost opposite trend. In DB system (Figure 39C), there was not much net change in the rate of nitrate produced in the system. The rate of nitrate production ranged from 2.6 to 6.2 nM NO_3^- h^{-1}. Except for a sharp decline in ammonium utilization (11.6±3.2 nM NH_4^+ h^{-1}) on the 6th day, the rate showed a net increase in the system. Nitrite production (1.4±1.0 nM NO_2^- h^{-1}) declined sharply till about 12th day, beyond which the rates were

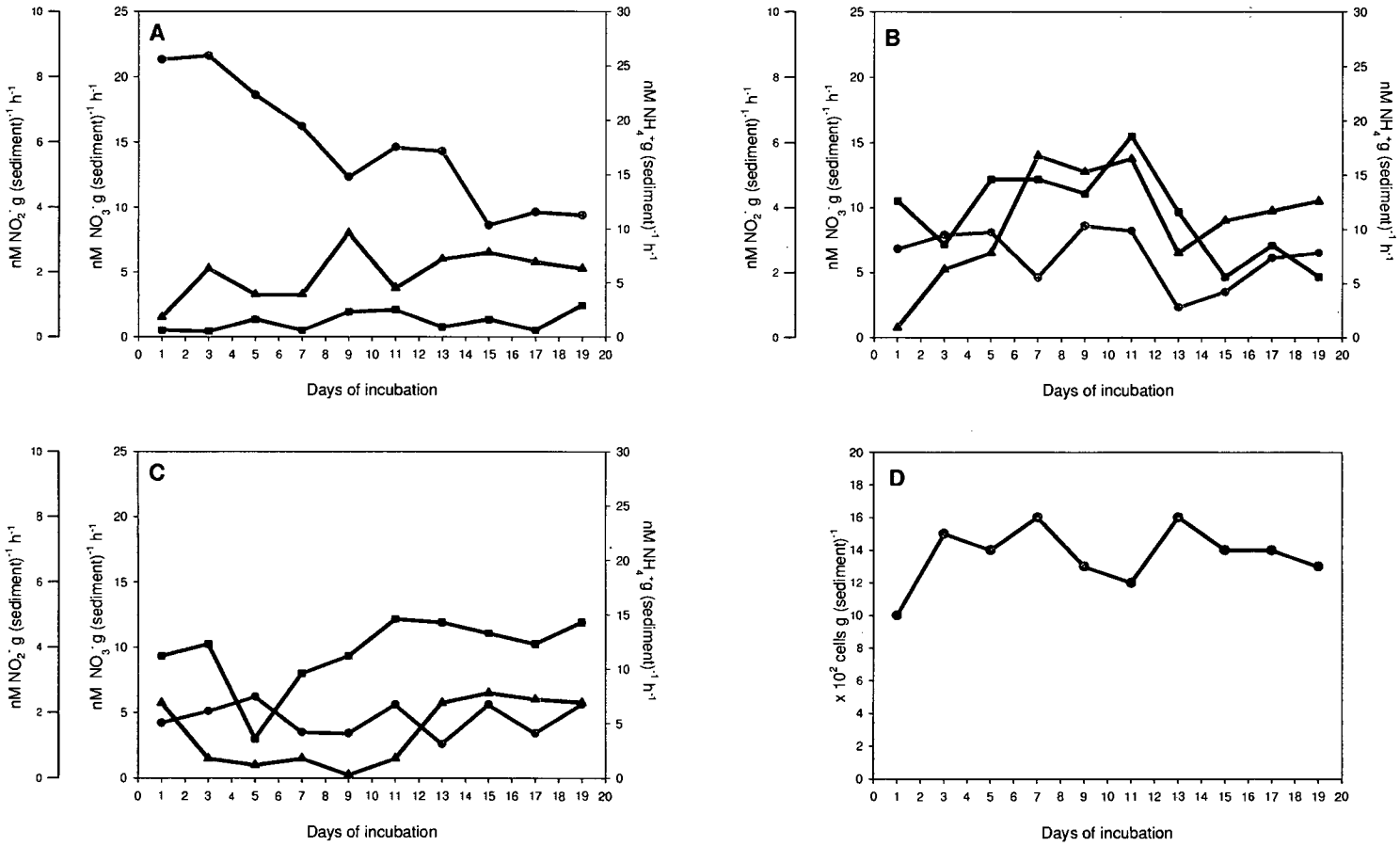


Figure 39: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 5 µM NO_2^-. Nitrifier counts in denitrification blocked system is given in (D).

relatively higher and consistent till the end of the incubation. The nitrifier counts in the DB system showed no definite trend throughout the period of incubation. Maximum stimulation of counts ($16x10^2$ cells g $(sediment)^{-1}$) occurred on the 8^{th} and 14^{th} day.

In 15 µM nitrite enriched system (Figure 40A) without any blocks, the net ammonium production rates (14.9±1.8 nM $NH_4^+ h^{-1}$) did not show much variability in the system. The same was also true for both the nitrite production (0.8±0.5 nM $NO_2^- h^{-1}$) and nitrate reduction rates (13.9±2.8 nM $NO_3^- h^{-1}$). However, the nitrite production and nitrate reduction rates had a prominent peak on the 14^{th} and 12^{th} day, respectively. In NB system (Figure 40B), the net ammonium production rates were comparatively higher than the UB incubations throughout the period of incubation. The ammonium production rates ranged from 21.3 to 25.1 nM $NH_4^+ h^{-1}$. There was a profound increase in denitrification rates, which ranged from 29.3 to 37.3 nM $NO_3^- h^{-1}$. The nitrite production (1.1±0.8 nM $NO_2^- h^{-1}$) in the system did not show any definite pattern, but a couple of peaks were observed on the 10^{th} and 14^{th} day of incubation. In DB system (Figure 40C), the nitrification rates were comparatively lower and ranged from 2.4 to 6.5 nM $NO_3^- h^{-1}$. This is very conspicuous from the fall in the rate of ammonium utilization (11.2±7.1 nM $NH_4^+ h^{-1}$) as well as nitrate production patterns. The rate of change in nitrite (1.06±0.6 nM $NO_2^- h^{-1}$) produced in the system was similar to its DB counterpart in 5 µM enriched system. The evolution of nitrifying population in the system did not show any clear trend and hardly any variation was observed (16.6±2.9 $x10^2$ cells g $(sediment)^{-1}$).

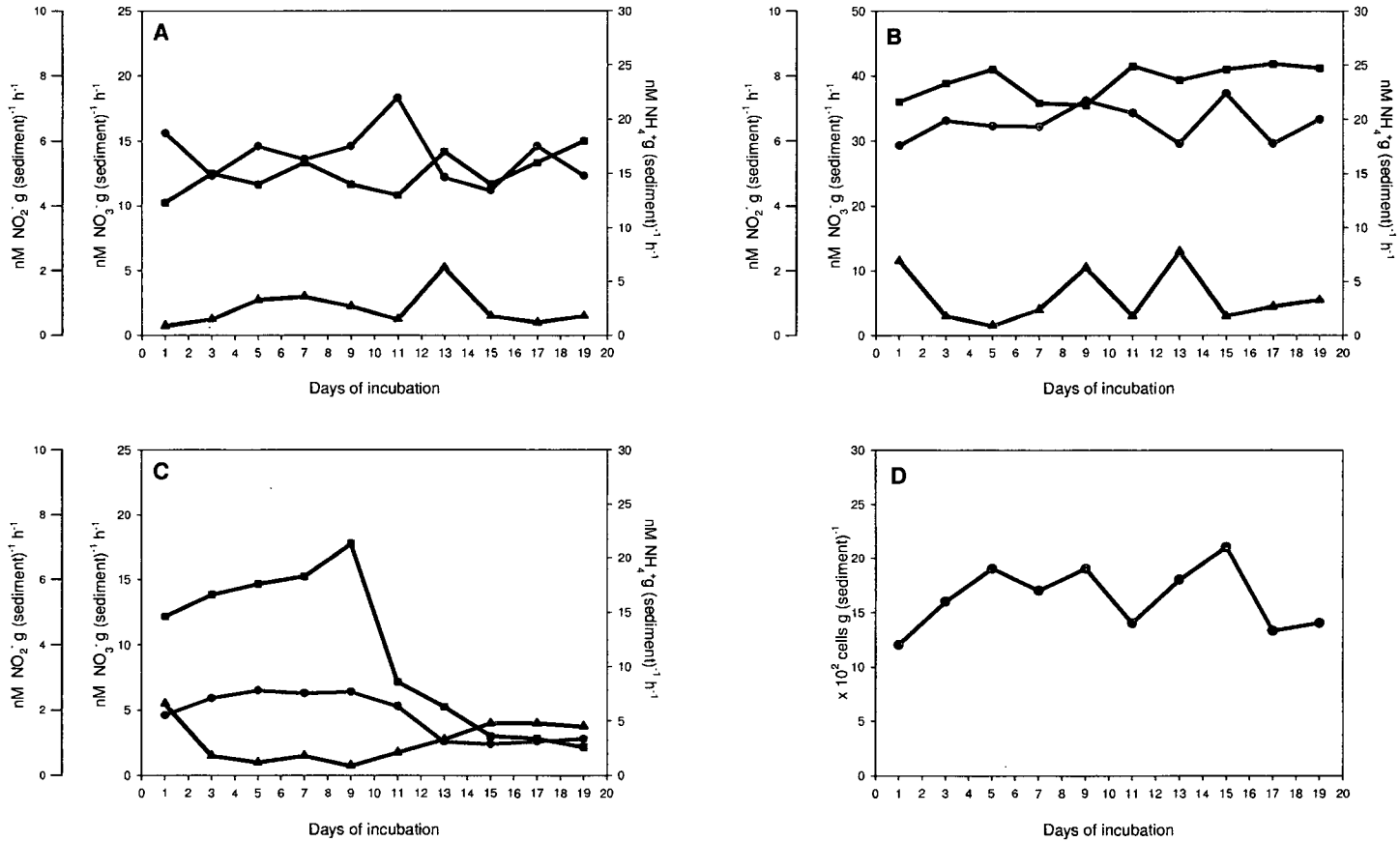


Figure 40: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 15 µM NO_2^-. Nitrifier counts in denitrification blocked system is given in (D).

The Figure 41A illustrates the net change in nitrogen conversion rates in the UB system spiked with 20 μM nitrite. The ammonium production rates (16.7±3.2 nM NH_4^+ h^{-1}) increased in the system until about 12 days of incubation, but it slowed during the second half of the period of incubation. The nitrate reduction (13.9±2.2 nM NO_3^- h^{-1}) did not show any particular trend, but the rates were found to decrease from 6th to the 16th day. The pattern and rate of nitrite produced (1.3±0.6 nM NO_2^- h^{-1}) throughout the period of incubation was very similar to the enrichments of 5 and 15μM. The ammonium production rates (3.2±1.4 nM NH_4^+ h^{-1}) in NB system (Figure 41B) were comparatively much lower than UB system and ranged from 1.3 to 6.5 nM NH_4^+ h^{-1}. The denitrification (14.6±2.4 nM NO_3^- h^{-1}) and nitrite production rates (1.7±0.6 nM NO_2^- h^{-1}) and pattern were more or less similar to the UB incubation. In DB system (Figure41C), the nitrification rates (11.3±4.8 nM NO_3^- h^{-1}) were found to increase with time with a very sharp increase during the end of the incubation period. In general, the net ammonium utilization (13.6±3.0 nM NH_4^+ h^{-1}) rates showed a downward trend. The rates of nitrite oxidation were comparable with other incubations, but the pattern was almost opposite that of the ammonium utilization. Nitrite oxidation ranged from 0.8 to 2.6 nM $NO_2^-h^{-1}$.The addition of 20 μM nitrite, after an initial rise, appears to have adversely affected the nitrifier population, which showed a steady decrease over the period of incubation (Figure 41D). In general the nitrifier counts dropped from 21.6 to 11.5 x10^2 cells g $(sediment)^{-1}$.

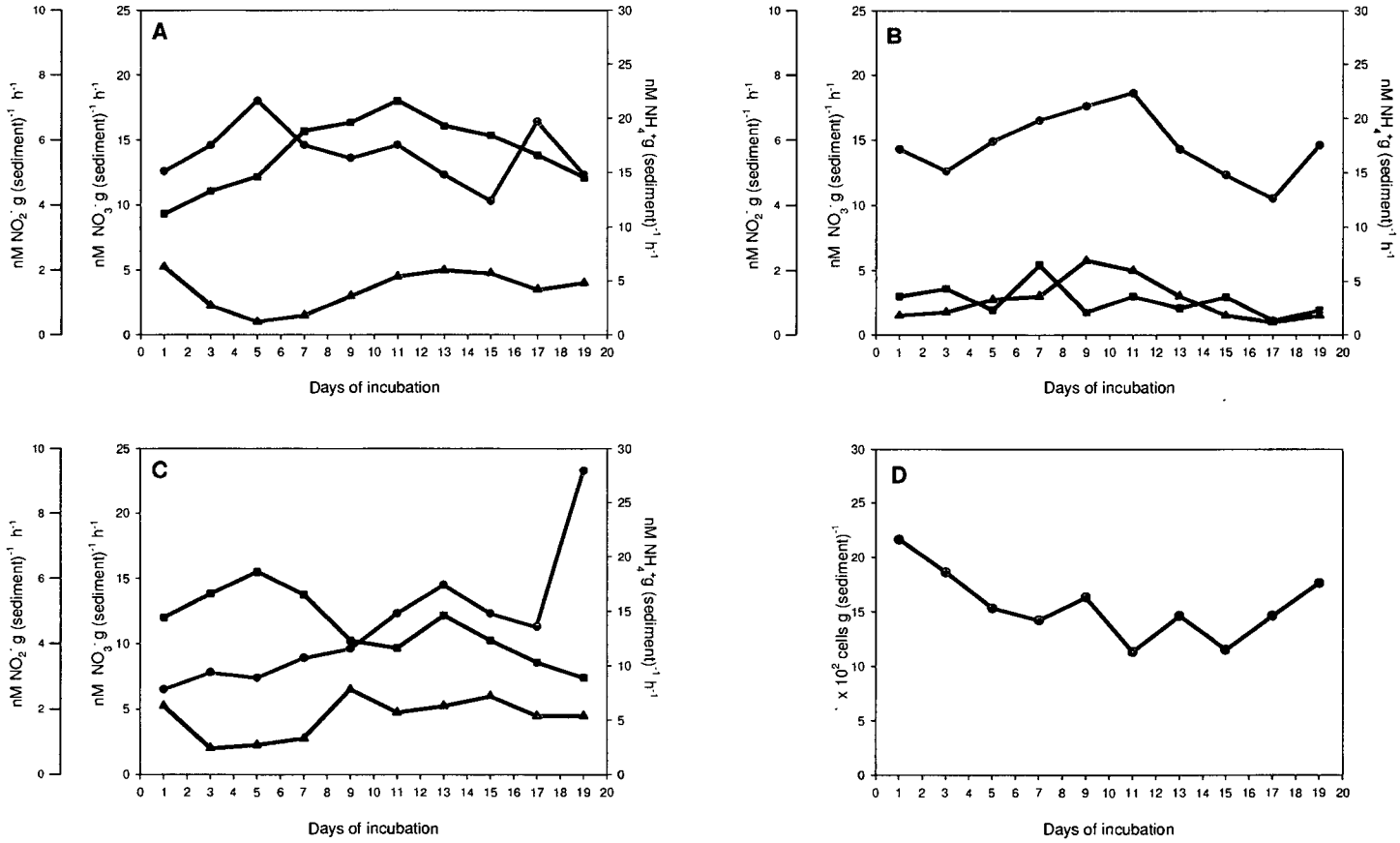


Figure 41: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 20 µM NO_2^-. Nitrifier counts in denitrification blocked system is given in (D).

Amendments of nitrite had a negative impact on nitrification rates at all the concentrations tested. The impact was higher with an increase in concentration.

4.6.5. Nitrate

Net ammonium production (9.1±4.4 nM NH_4^+ h^{-1}) in 10 µM nitrate enriched UB system (Figure 42A) showed a steady decline from the beginning to the end of incubation. However, nitrate production in the system though did not show any definite trend, had multiple peaks and ranged from 14 to 32 nM NO_3^- h^{-1}. The nitrite production rates ranged from 0.6 to 2.1 nM NO_2^- h^{-1}. Figure 42B illustrates the changes in NB system. In NB system, the denitrification rates showed steady increase overtime (18.3±5.2 nM NO_3^- h^{-1}) with ammonium production rates showing alternate increase and decrease for almost throughout the period of incubation. The nitrite production rates (3.2±0.9 nM NO_2^- h^{-1}) were higher than that in the UB system and had a pattern of rate of change almost opposite to that of ammonium production. The nitrification rates (11.9±4.3 nM NO_3^- h^{-1}) in the DB system (Figure 42C) was marked by large variations in nitrate production rates throughout the period of incubation, while not much variation was seen in ammonium utilization (14.8±1.1 nM NH_4^+ h^{-1}). The rate of nitrite production (6.1±2.3 nM NO_2^- h^{-1}) increased in a very prominent manner till the 12^{th} day and thereafter decreased sharply. The nitrifier counts (Figure 42D) after an initial decrease, increased till the 12^{th} day. The addition of 10 µM nitrate stimulated the nitrifiers to attain the population maximum of 16 $x10^2$ cells g $(sediment)^{-1}$ on the 8^{th} day.

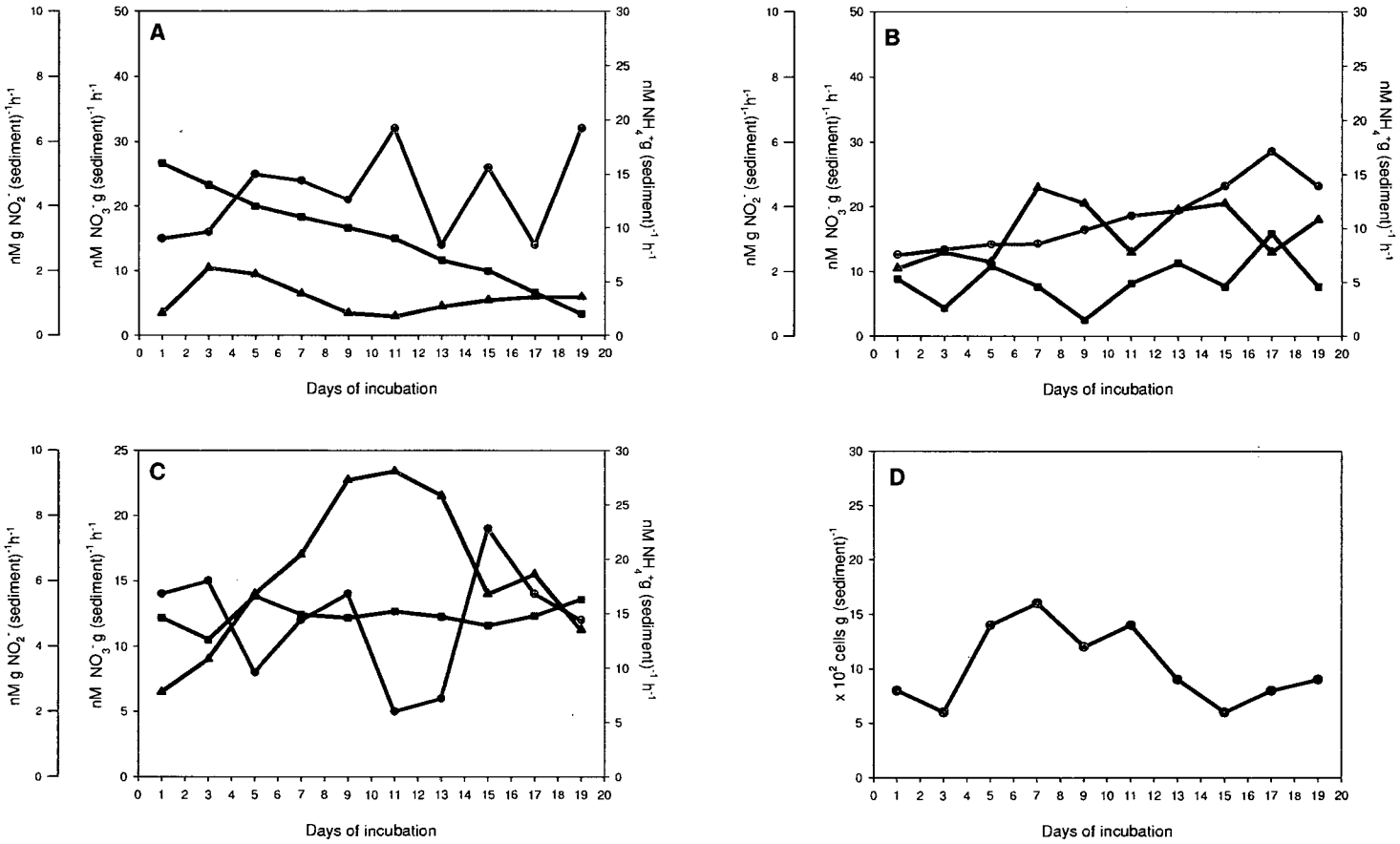


Figure 42: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 10 μM NO_3^-. Nitrifier counts in denitrification blocked system is given in (D).

Figure 43A, B and C illustrate the kinetics of nitrite, nitrate and ammonium in UB, NB and DB systems, respectively with 25 μM nitrate amendment. In UB system, the net ammonium production rates (18.1±4.4 nM NH_4^+ h^{-1}) increased throughout the experiment. The variations in nitrate reduction and nitrite production followed almost the same trend. Production of nitrite and reduction of nitrate ranged from 0.2 to 3.1 nM NO_2^- h^{-1} and 12 to 26 nM NO_3^- h^{-1} respectively. The nitrifier counts in DB system (Figure 43D) showed a decrease over time indicating that probably nitrate excess could have a negative impact on the nitrifying population. The counts were found to decrease from 12 to 4 x 10^2 cells g (sediment)$^{-1}$ by the end of incubation. Even though the counts were decreasing, the nitrification rates (DB system) were found to increase at a very slow pace (18.7±3.9 nM NO_3^- h^{-1}) till about the 12th day, beyond which it decreased. In general there was an overall increase in ammonium utilization (16.9±3.6 nM NH_4^+ h^{-1}) and nitrite production (4.0±2.2 nM NO_2^- h^{-1}) in the system. Two prominent peaks between 6th and10th day and 14th and 16th day characterized nitrite production. Ammonium utilization reached maxima on the 16th day, which almost coincided with the second nitrite peak. In NB system, there was very large variation in denitrification rates, (38.5±12.7 nM NO_3^- h^{-1}) especially in the first half of the incubation period. The ammonium production rates, which ranged from 2.3 to 8.6 nM NH_4^+ h^{-1} were lower in this system compared to UB system. Nitrite production (2.6±1.6 nM NO_2^- h^{-1}) was high with a very prominent peak on the 9th day.

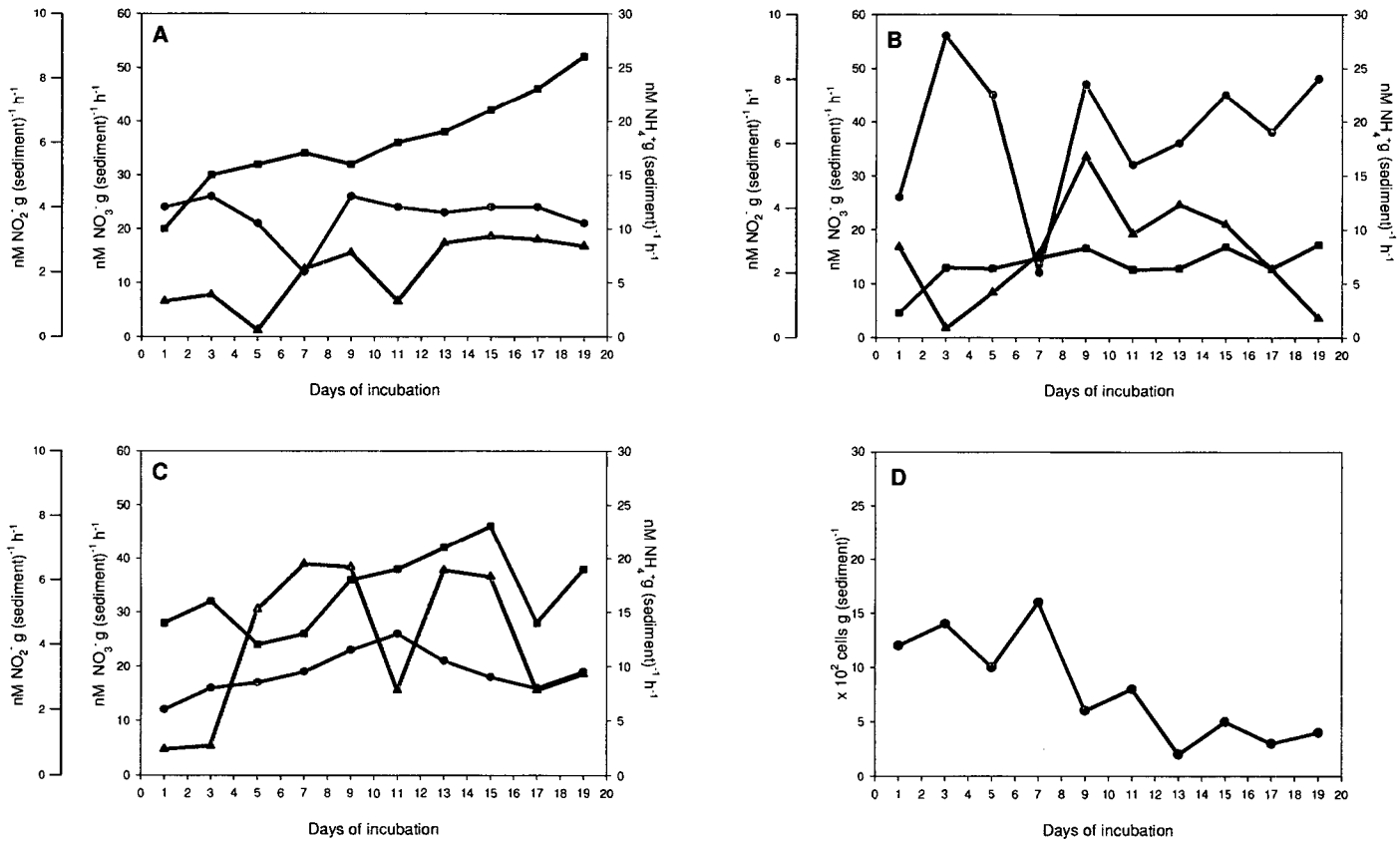


Figure 43: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 25 µM NO_3^-. Nitrifier counts in denitrification blocked system is given in (D).

The net variation in ammonium production was minimal (16.4±2.4 nM $NH_4^+ h^{-1}$) in UB incubation amended with 50 µM nitrate (Figure 44A). The rate of change in nitrate reduction (16.6±4.4 nM $NO_3^- h^{-1}$) seen initially was minimized over time. The change in rate of nitrite production (1.5±0.8 nM $NO_2^- h^{-1}$) was low but showed conspicuous variability with three phases of high and two phases of low production rates. In NB incubations (Figure 44B), the denitrification rates (57.7±17.1 nM $NO_3^- h^{-1}$) were high with very high variability. As was the case with 25 µM enrichment; the variation was comparatively low in the second half of the incubation. Maximum ammonium production (10±3.1 nM $NH_4^+ h^{-1}$) occurred between 8th and 12th day while nitrite production rates were higher from 6th to 14th day. Nitrification rates (Figure 44C) in DB incubation decreased with time (65.6 to 43.7 nM $NO_3^- h^{-1}$). However, ammonium utilization and nitrite reduction increased with time. The abundance of nitrifier population changed little throughout the period of study, except for an increase to $26x10^2$ cells g $(sediment)^{-1}$ on the 10th day.

In general, all the amendments of nitrate impeded nitrification rates. The impact was more intense at higher concentrations.

4.6.6. Liquid hydrocarbons

Figures 45A, B and C illustrate the changes of ammonium, nitrite and nitrate in UB, NB and DB experimental incubations spiked with 10 mg/g liquid hydrocarbon, respectively. The variability in the rate of nitrite production was minimum in the UB incubation (0.4±0.1 nM $NO_2^- h^{-1}$) and the highest was seen in

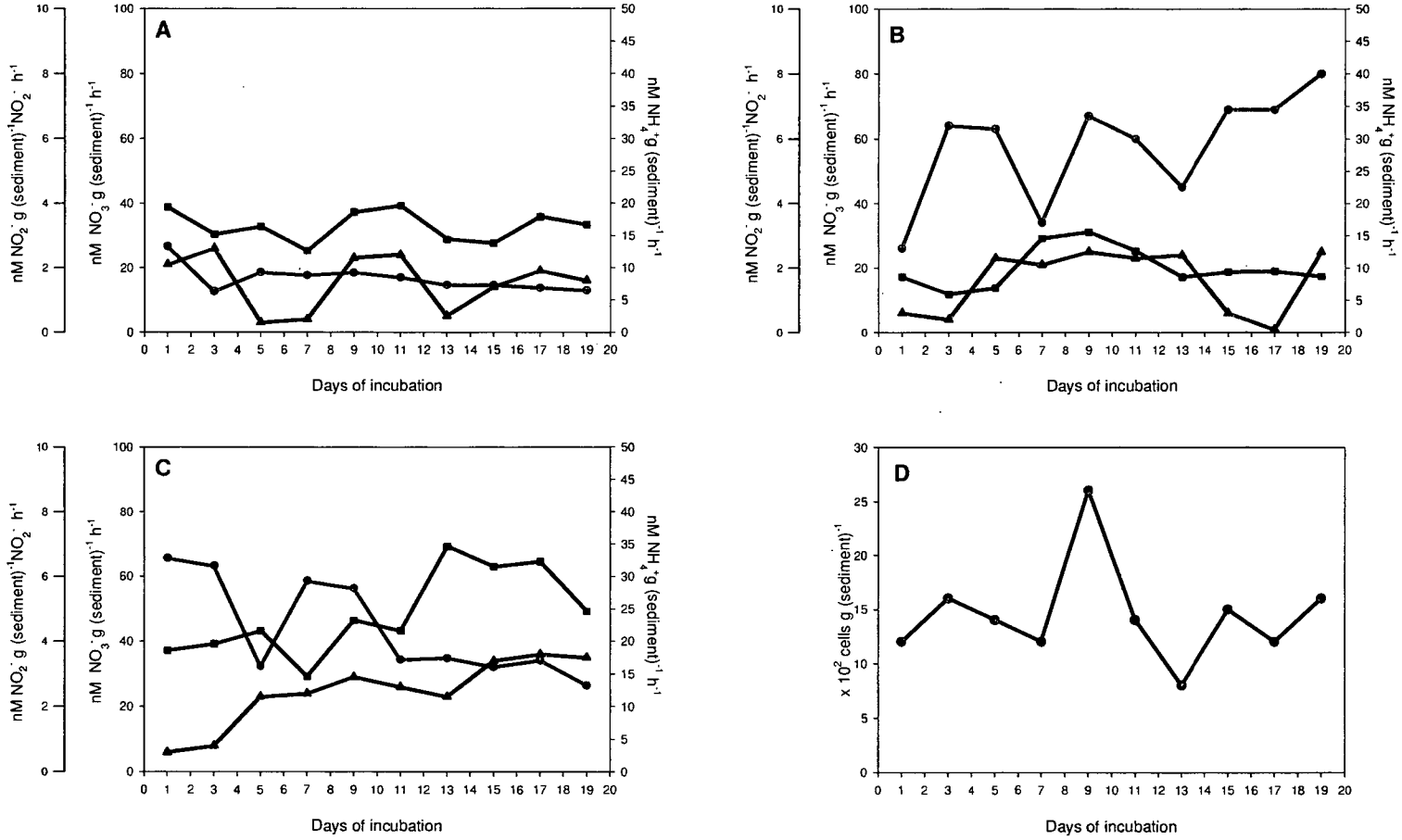


Figure 44: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 50 μM NO_3^-. Nitrifier counts in denitrification blocked system is given in (D).

NB system (1.6±1.1 nM NO_2^- h^{-1}). In the latter system, the nitrite production was characterized by a sharp decrease from 10th to 14th day. The denitrification rates (9.3±2.3nM NO_3^- h^{-1}) was found to increase over time with a prominent decrease coinciding with decrease in nitrite production (Figure 45B). Ammonium production (4±2 nM NH_4^+ h^{-1}) in NB system was found to have intermittent peak rates throughout the incubation period. The effect of net ammonium utilization was higher in the DB system (14±2.4 nM NH_4^+ h^{-1}), governing the ammonium pool when compared to that of ammonium production in NB. However, the pattern was more or less similar to that in the NB system. Nitrate production rates (1.6±0.5nM NO_3^- h^{-1}) were very low as was the case in UB system, indicating that net ammonium assimilation governs the ammonium pool in the presence of liquid hydrocarbons. The nitrite production rates gradually increased in this system. It ranged from 0.2 to 2.3nM NO_2^- h^{-1}. It was interesting to observe that the peaks in nitrifier counts ($0.6x10^2$ cells g (sediment)$^{-1}$) almost matched with the dips in net ammonium utilization. This provides additional proof on the possibility of the role of net ammonium assimilation in the system governing ammonium availability.

There was very little variation in ammonium production (0.5±0.2 nM NH_4^+ h^{-1}) and nitrate production (0.7±0.3nM NO_3^- h^{-1}) in UB incubations spiked with 50 mg/g liquid hydrocarbon (Figure 46A). The nitrite production rates, though low showed significant variability with two prominent dips on the 5th and 11th day. The nitrite production rates ranged from 0.1 to 0.8 nM NO_2^- h^{-1}. Denitrification rates (23.2±5.9nM NO_3^- h^{-1}) in the NB system were found to increase with time (Figure 46B). This is also supported with the prominent increase in nitrite production,

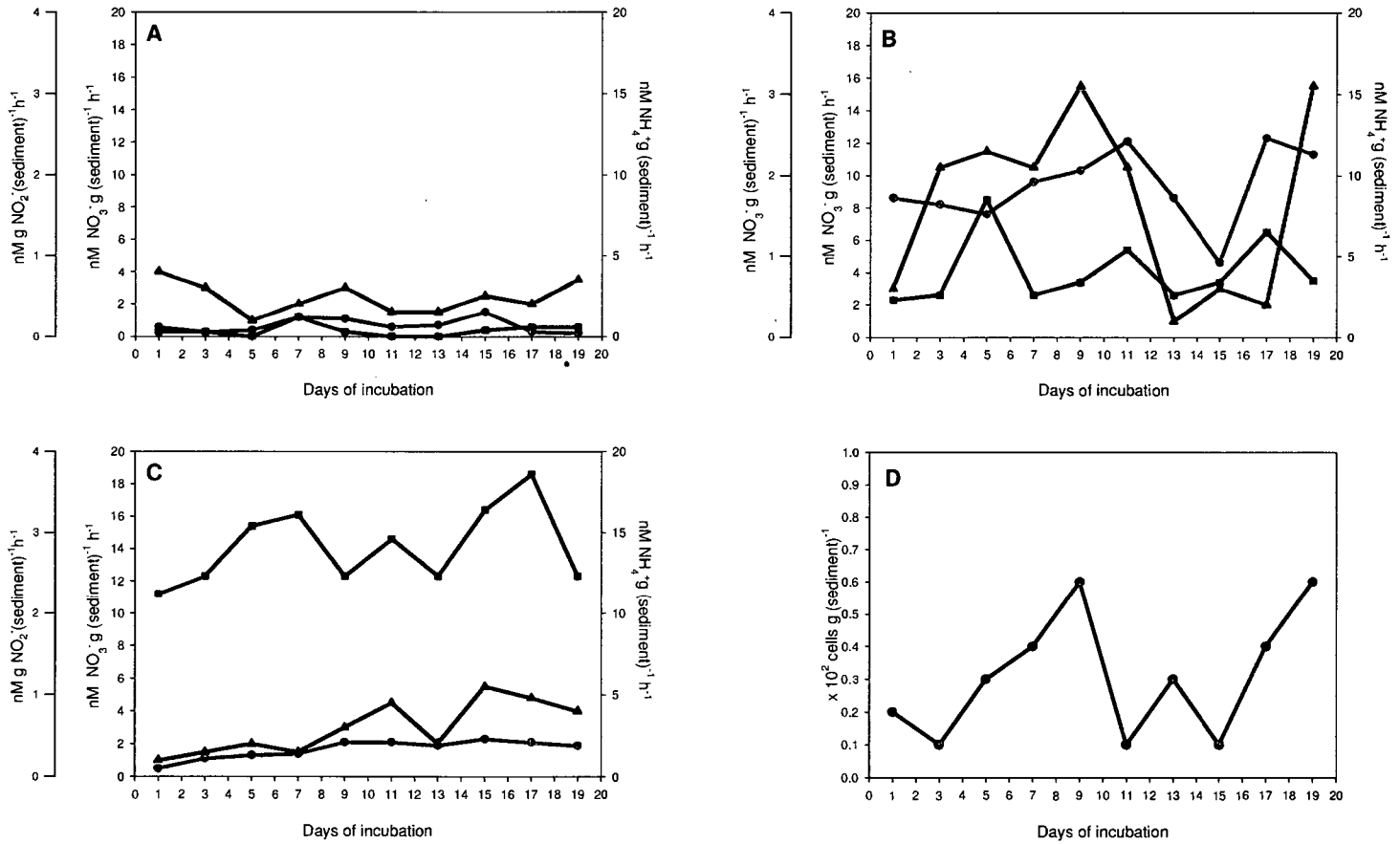


Figure 45: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked systems (C) with 10 mg/g liquid hydrocarbon. Nitrifier counts in denitrification blocked system is given in (D).

especially till the 10th day. Ammonium production was low (1.2±0.3 nM NH_4^+ h^{-1}) and uniform throughout the period of incubation. Net nitrification rates in the DB system (Figure 46C) was low (1.2±0.7nM NO_3^- h^{-1}) as in the case of 10 mg/g liquid hydrocarbon amendments However, ammonium was found to be assimilated in the system almost throughout the period of incubation, indicating the enhanced role of assimilation. The nitrite production rates (0.5±0.5 nM NO_2^- h^{-1}) were high on the 4th day, however subsequently the rates decreased sharply and were found to be almost stable till the end. The nitrifier counts which ranged from levels of non detection to $1.1x10^2$ cells g $(sediment)^{-1}$ except for the two peaks on the 8th and 14th day decreased with time(Figure 46D).

Addition of 100 mg/g liquid hydrocarbon completely blocked nitrification. Nitrifier counts were below the limits of detection throughout the period of incubation. In UB system (Figure 47A), the net change in nitrate production (0.2±0.1 nM NO_3^- h^{-1}) and net ammonium assimilation (0.2±0.1 nM NH_4^+ h^{-1}) was very low and did not vary with time. However, the nitrite production (1.9±1.0 nM NO_2^- h^{-1}) showed extreme variability with multiple peaks throughout the period of incubation. In NB system (Figure 47B), ammonium production (1.8±0.7 nM NH_4^+ h^{-1}) increased with time, while denitrification rates (5.1±2.4 nM NO_3^- h^{-1}) exhibited very high degree of variability. The nitrite production rates ranged from 0.1 to 0.6 nM NO_2^- h^{-1}. In DB system, no net ammonium decrease was detected indicating that the addition of 100 mg/g liquid hydrocarbon has completely blocked the nitrification in this system. This was also reflected in the net production rates of

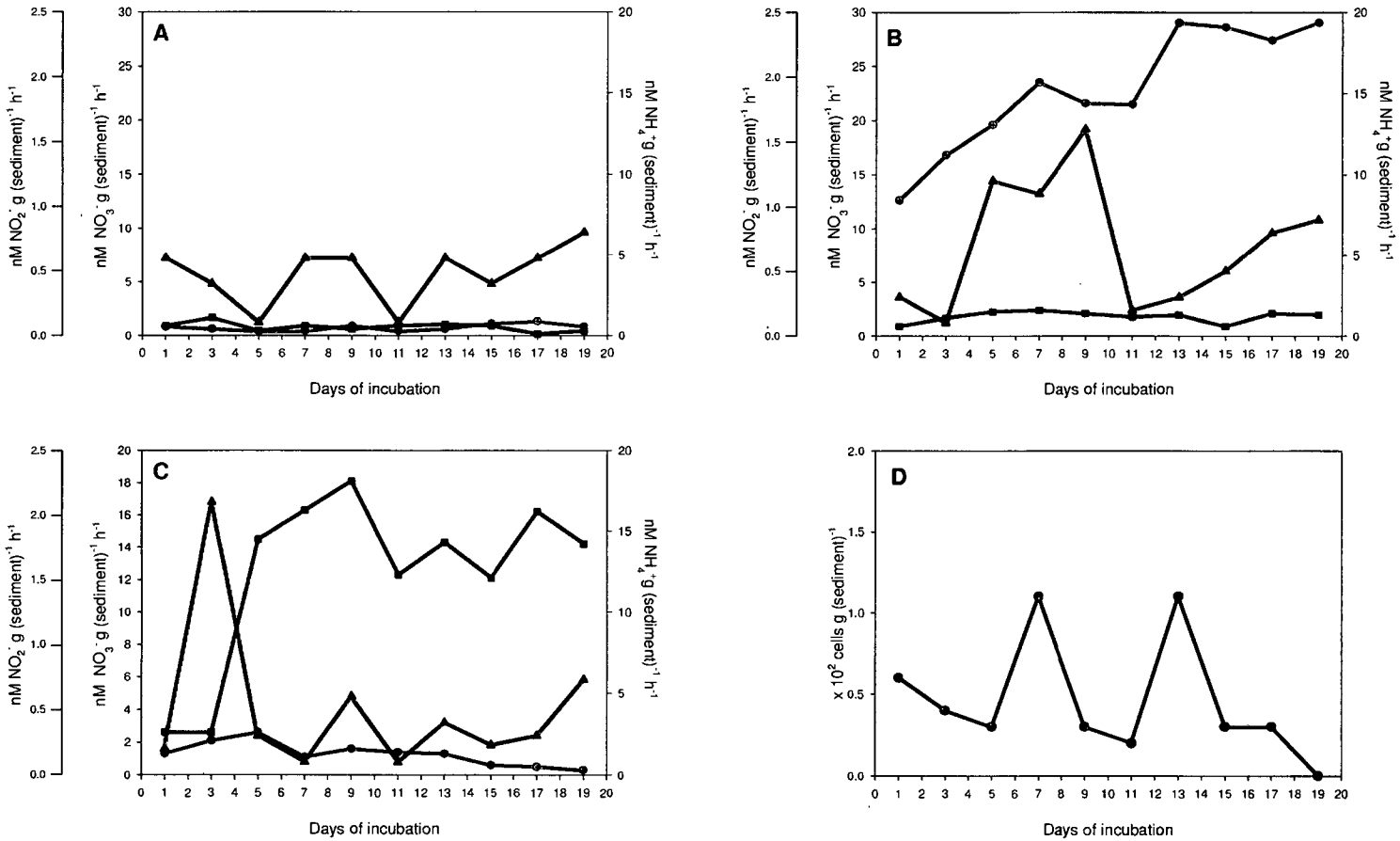


Figure 46: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked systems (C) with 50 mg/g liquid hydrocarbon. Nitrifier counts in denitrification blocked system is given in (D).

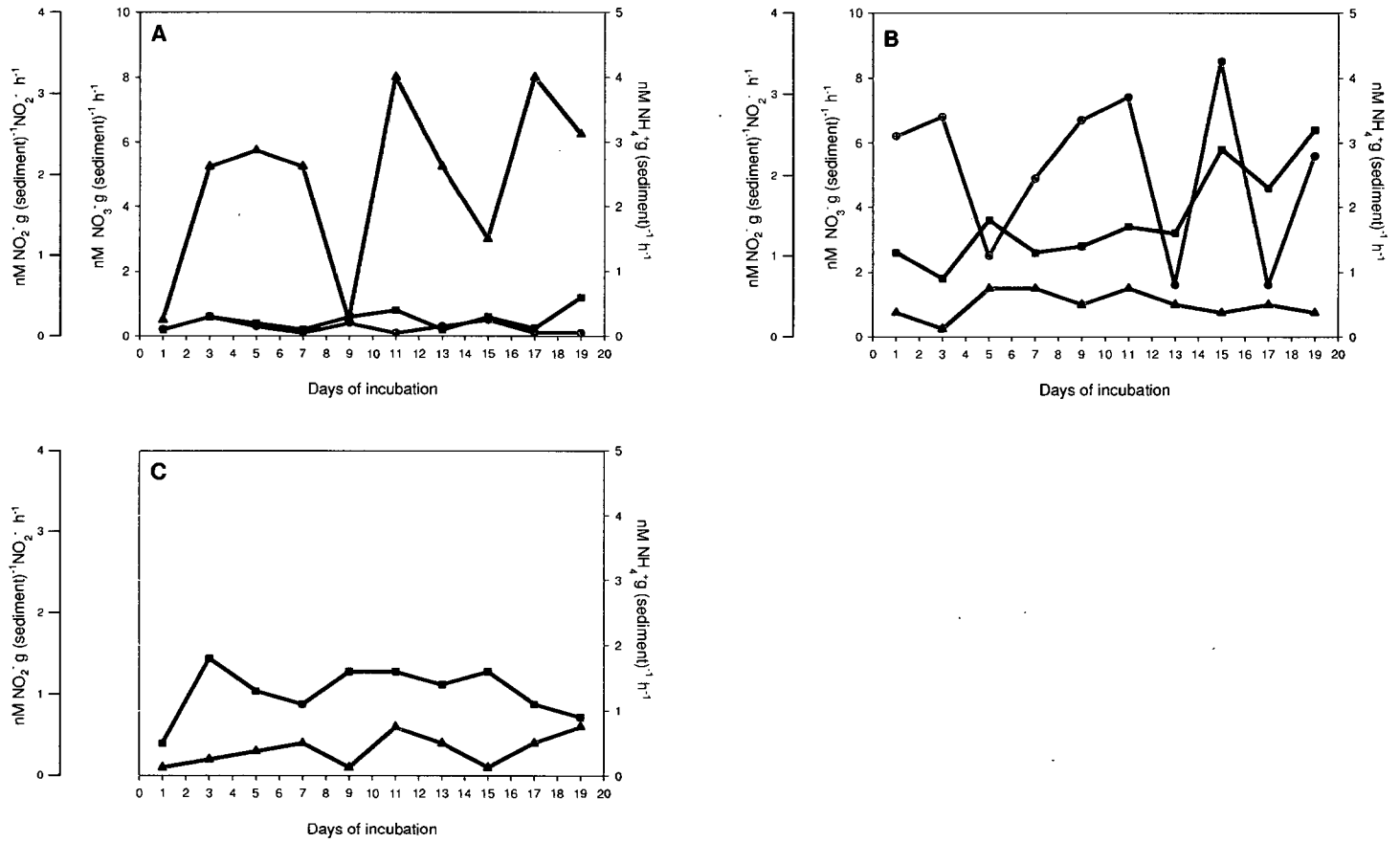


Figure 47: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 100 mg/g liquid hydrocarbon.

nitrite and disappearance rate of ammonium which ranged from 0.1 to 0.6 nM $NO_2^- h^{-1}$ and 0.5 to 1.8 nM $NH_4^+ h^{-1}$, respectively.

Liquid hydrocarbons retarded nitrification rates at 10 and 50mg/g concentrations, while it was totally inhibited at 100mg/g amendment.

4.6.7. Pesticides

The addition of pesticide at 10 ppm in UB (Figure 48A) experimental incubations had minor effect on the nitrate production rates (14.4±1.7 nM $NO_3^- h^{-1}$). The nitrite production rates (1.2±0.5 nM $NO_2^- h^{-1}$), however increased over time while the net ammonium assimilation/oxidation (3.5±1.4 nM $NH_4^+ h^{-1}$) increased until the 12th day and later decreased to very low rates. Denitrification rates (13.8±1.4 nM $NO_3^- h^{-1}$) in NB system were almost similar throughout the incubation period, while nitrite production rates (2.0±1.4 nM $NO_2^- h^{-1}$) increased almost logarithmically (Figure 48B). As in the case with denitrification, the ammonium production rates also did not vary much (2.3±1.0nM $NH_4^+ h^{-1}$) till the end of the incubation. In DB system, nitrification rates (25.4±8.7 nM $NO_3^- h^{-1}$) showed an increase till the 8th day (Figure 48C), but, the rates showed a downward trend and had very high variability with a couple of peaks. The utilization of ammonium (14.7±2.3nM $NH_4^+ h^{-1}$) also followed almost the nitrate production rates trend with an initial increase followed by a dip. The nitrite production (0.7±0.6 nM $NO_2^- h^{-1}$) in the system did not vary much with time, except for a very strong peak on the 8th day. The nitrite production rates ranged from 0.2 to 2.6 nM $NO_2^- h^{-1}$. The enhancement of nitrification rates and its dip

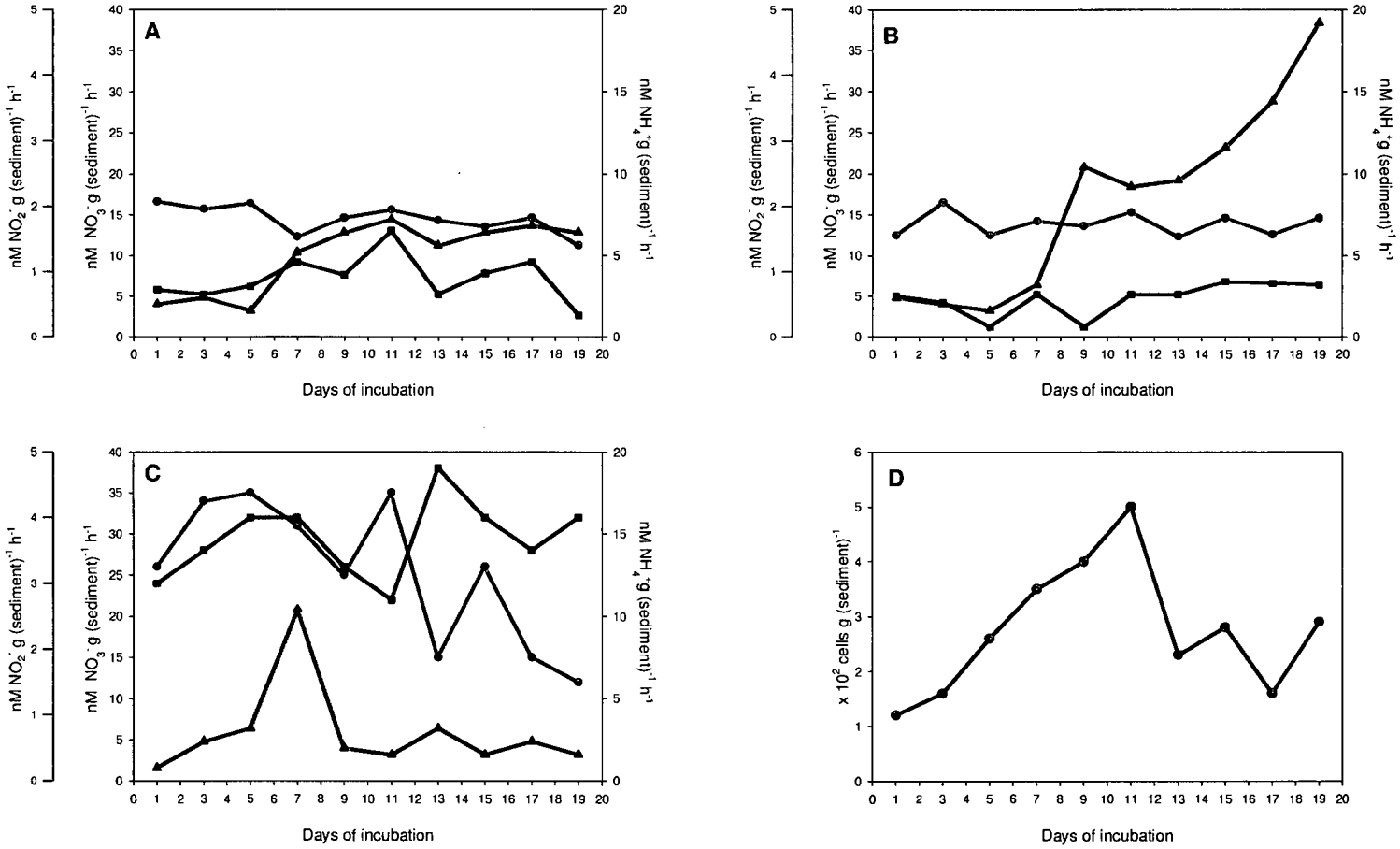


Figure 48: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 10 ppm pesticide. Nitrifier counts in denitrification blocked system is given in (D).

could also be observed in the nitrifier cell counts (Figure 48D) which showed a very significant increase up to the 12th day and a decrease later on. It could be observed that the addition of pesticide at 10 mg/g concentration stimulated the nitrifier cell counts from 1.2 to 5 x10^2 cells g (sediment)$^{-1}$.

Addition of 50 ppm pesticide into the system was found to stimulate nitrite production (1.6±0.7 nM NO_2^- h^{-1}) in UB incubations (Figure 49A). Also, ammonium production (2.9±2.3nM NH_4^+ h^{-1}) and net nitrate production (15.6±2.5 nM NO_3^- h^{-1}) showed marginal increase during the second half of the incubation period. The denitrification rates (Figure 49B) in the NB system ranged from 12.3 to 25.6 nM NO_3^- h^{-1} with a peak on the 12th day. The nitrite production rates were also stimulated up to the 14th day (1.7±0.8 nM NO_2^- h^{-1}), and beyond which it declined to very low levels, and again improved after the 14th day. The ammonium production rates (1.4±0.6nM NH_4^+ h^{-1}) were extremely slow and did not show much variability. Though nitrification rates in DB system though were stimulated (23.8±5.1 nM NO_3^- h^{-1}), it did not exhibit any specific trend (Figure 49C). The ammonium utilization rates were also found to increase after a brief dip but further the rates were lower and comparable with the rates at the beginning of the incubation. The nitrite production rates (1.5±0.7 nM NO_2^- h^{-1}) almost followed the pattern of ammonium utilization rates (24.5±5.8nM NH_4^+ h^{-1}) except that in the beginning where both of them followed the opposite trend. There was not much change in the number of nitrifiers (Figure 49D) in DB incubations. The nitrifiers ranged from 1.4 to 2.6 x10^2 cells g (sediment)$^{-1}$.

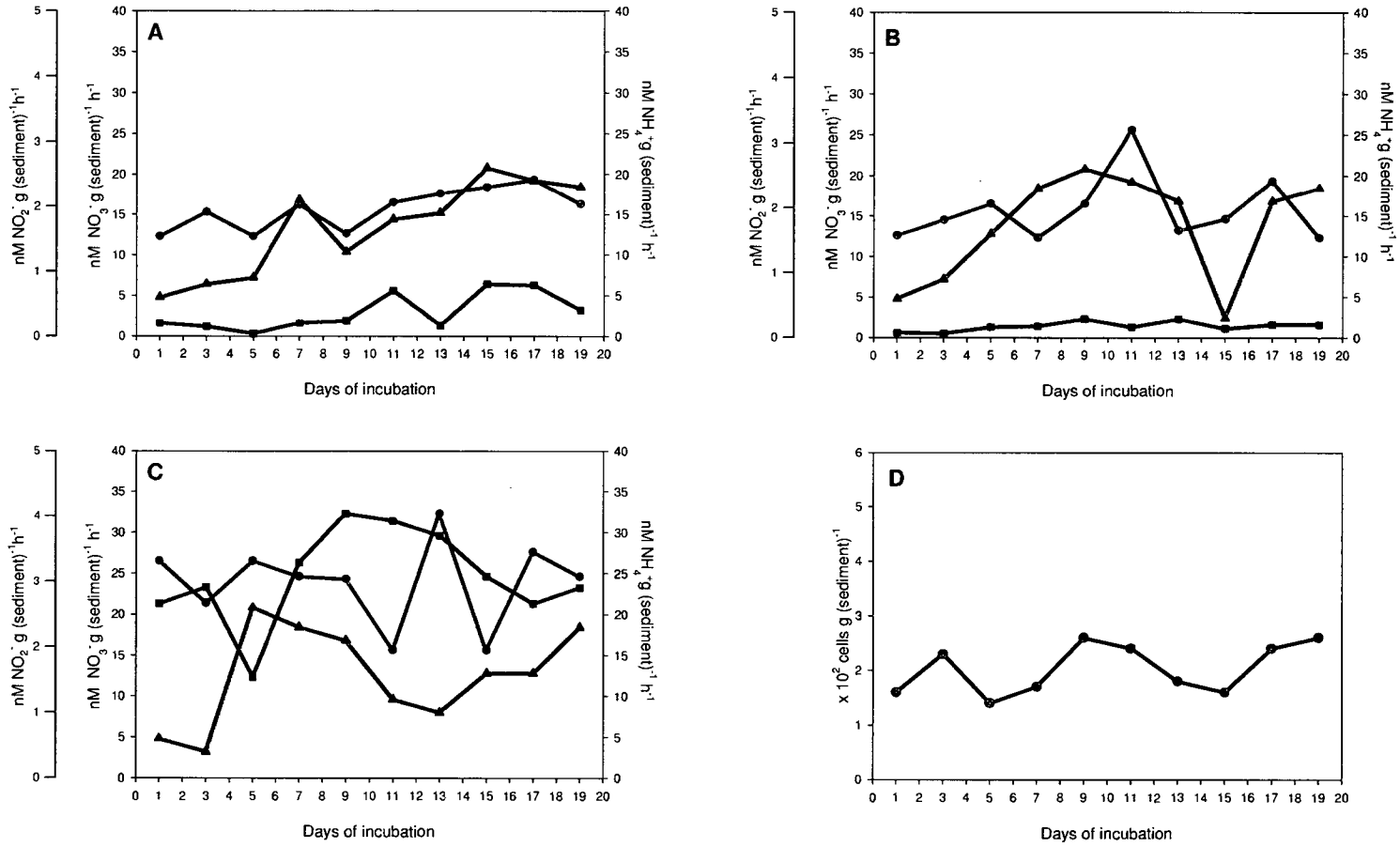


Figure 49: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 50 ppm pesticide. Nitrifier counts in denitrification blocked system is given in (D).

Figures 50A, B and C illustrate the various nitrogen conversion rates in incubations with amendment of 100 ppm pesticide in UB, NB and DB systems, respectively. The nitrate production rates (9.0±5.5 nM $NO_3^- h^{-1}$) in the UB system were almost uniform until the 12th day and increased sharply to 4.9 nM $NO_3^- h^{-1}$ on the 14th day and further, the rates declined. The net ammonium production rates (1.6±1.5nM $NH_4^+ h^{-1}$) were low and showed a slight increase from the 8th to the 12th day. In general, the nitrite production rates (1.8±0.7 nM $NO_2^- h^{-1}$) increased over time with a sharp increase on the 4th day and the level was maintained throughout. The denitrification (8.5±2.1nM $NO_3^- h^{-1}$) and net ammonium production rates (1.2±0.7nM $NH_4^+ h^{-1}$) in the NB system followed almost a uniform trend throughout the incubation period in the NB system. However, the denitrification rates were found to decrease slightly after the 16th day. There was very clear variability observed in nitrite production rates and it ranged from 0.6 to 2.9 nM $NO_2^- h^{-1}$. The nitrification (12.8±2.4nM $NO_3^- h^{-1}$) and net ammonium utilization rates (14.0±1.8nM $NH_4^+ h^{-1}$) in the DB system showed very little variability while nitrite production rates (1.0±0.7 nM $NO_2^- h^{-1}$) were found to decrease with time after the initial rise. Even though, ammonium conversion rates were not reflected, the nitrifiers were found to increase steadily till the 14th day and decreased thereafter (Figure 50D).

Pesticides were found have a stimulatory effect at lower concentrations while at 100ppm, nitrification rates were retarded by almost half the magnitude (10nM $NO_3^- h^{-1}$) when compared to the control DB system.

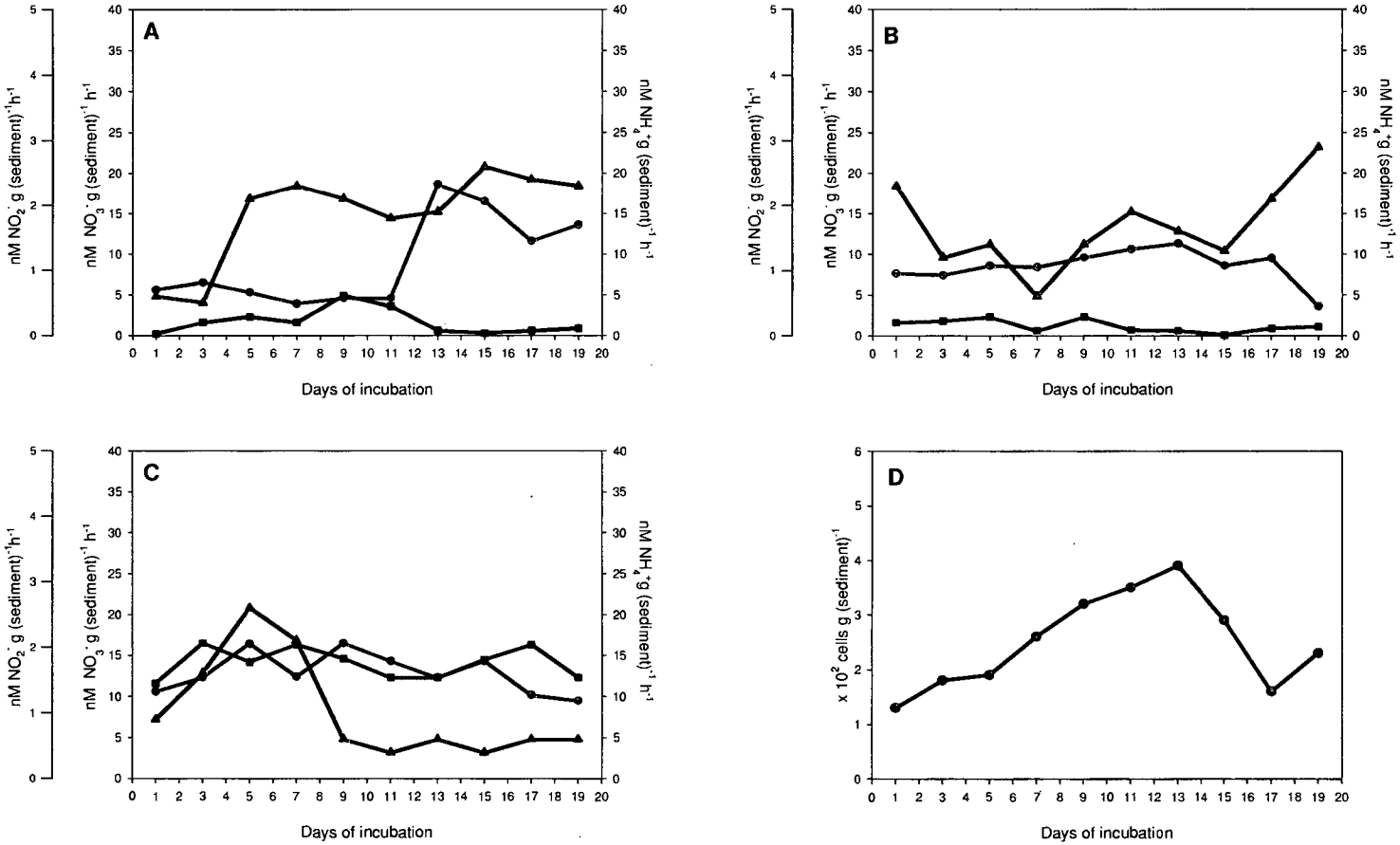


Figure 50: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 100 ppm pesticide. Nitrifier counts in denitrification blocked system is given in (D).

4.6.8. Fertilizers

The addition of fertilizer at a concentration of 1% w/w into the UB incubations resulted in very strong variations in the nitrite (5.1±1.7 nM $NO_2^- h^{-1}$) and nitrate production (48.6±15.6 nM $NO_3^- h^{-1}$) in the system (Figure 51A). Both the profiles were marked by multiple peaks of elevated rates of production. On the contrary, the ammonium production rates (23.5±6.8nM $NH_4^+ h^{-1}$) decreased with time. A similar trend for ammonium production rates was observed in the NB incubations (24.0±12.2nM $NH_4^+ h^{-1}$). However, denitrification (76.1±11.1 nM $NO_3^- h^{-1}$) increased over time (Figure 51B). Nitrite production (3.8±1.1 nM $NO_2^- h^{-1}$) varied considerably in the system in a range of 2.8 to 6.5 nM $NO_2^- h^{-1}$. Nitrification rates in the DB system are illustrated in Figure 51C. Very little variation was encountered in the profile of nitrification rates (20.4±4.3 nM $NO_3^- h^{-1}$) while there was considerable variation on ammonium utilization (21.2±4.5nM $NH_4^+ h^{-1}$). The nitrite production showed little variation except for a strong peak on the 16th day of the incubation (6.5nM $NO_2^- h^{-1}$). The nitrifier counts (Figure 51D) unlike the nitrification rate profiles showed a sharp increase in cell numbers till the 10th day. The cells were stimulated from to 1.1 to 6.4 x10^2 cells g $(sediment)^{-1}$.

Enrichment of the sediment slurry with 5% w/w fertilizer in UB incubations (Figure 52A) resulted in the decrease of net ammonium production rates (41.0±6.9nM $NH_4^+ h^{-1}$) in the system over time. Though the nitrate production rates (52.8±18.9 nM $NO_3^- h^{-1}$) showed an increasing trend, the variability observed was high. The nitrite production rates (7.0±1.1 nM $NO_2^- h^{-1}$) increased with time till it peaked on the 10th day; after that the rates declined. In NB system

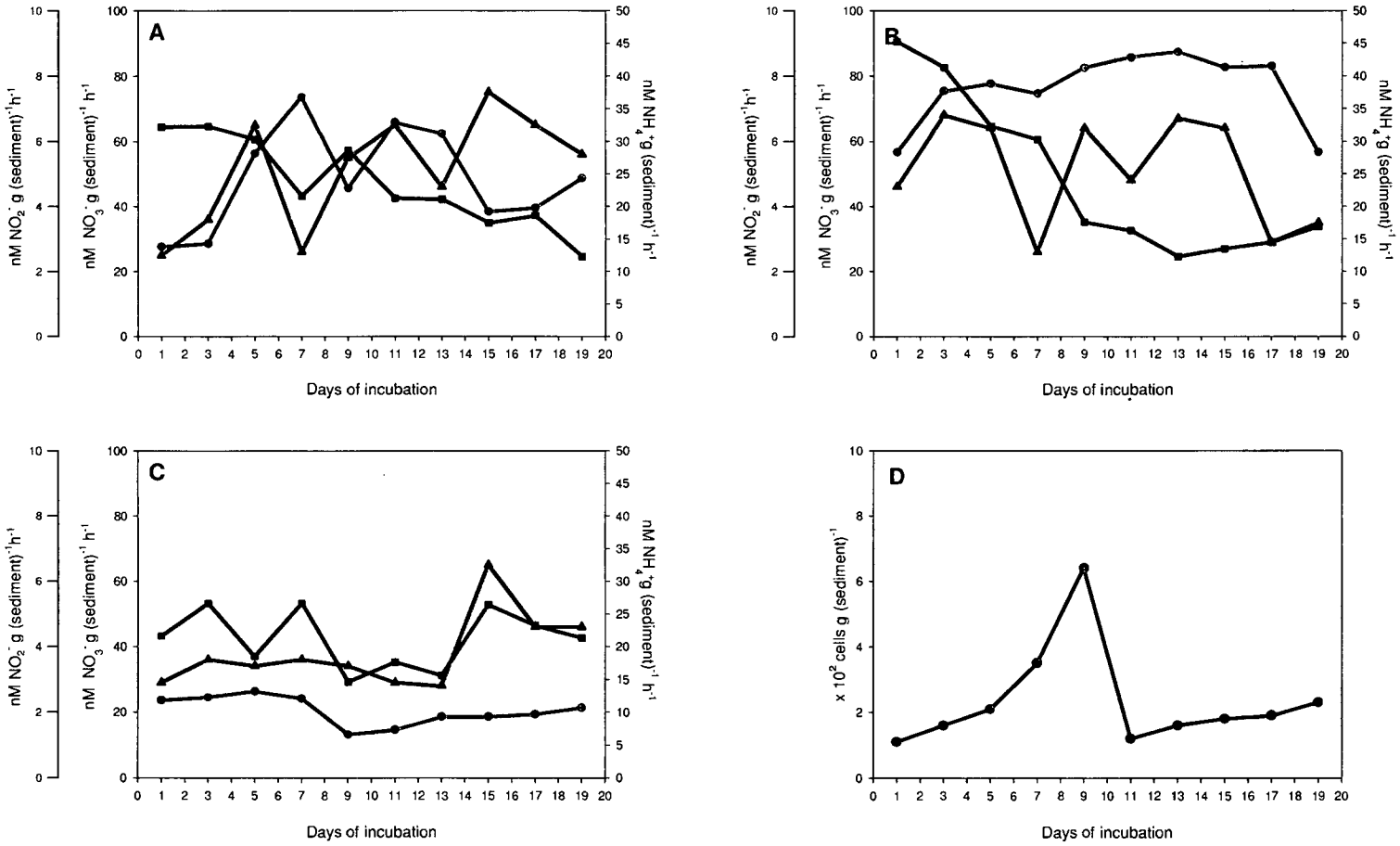


Figure 51: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 1% w/w fertilizer. Nitrifier counts in denitrification blocked system is given in (D).

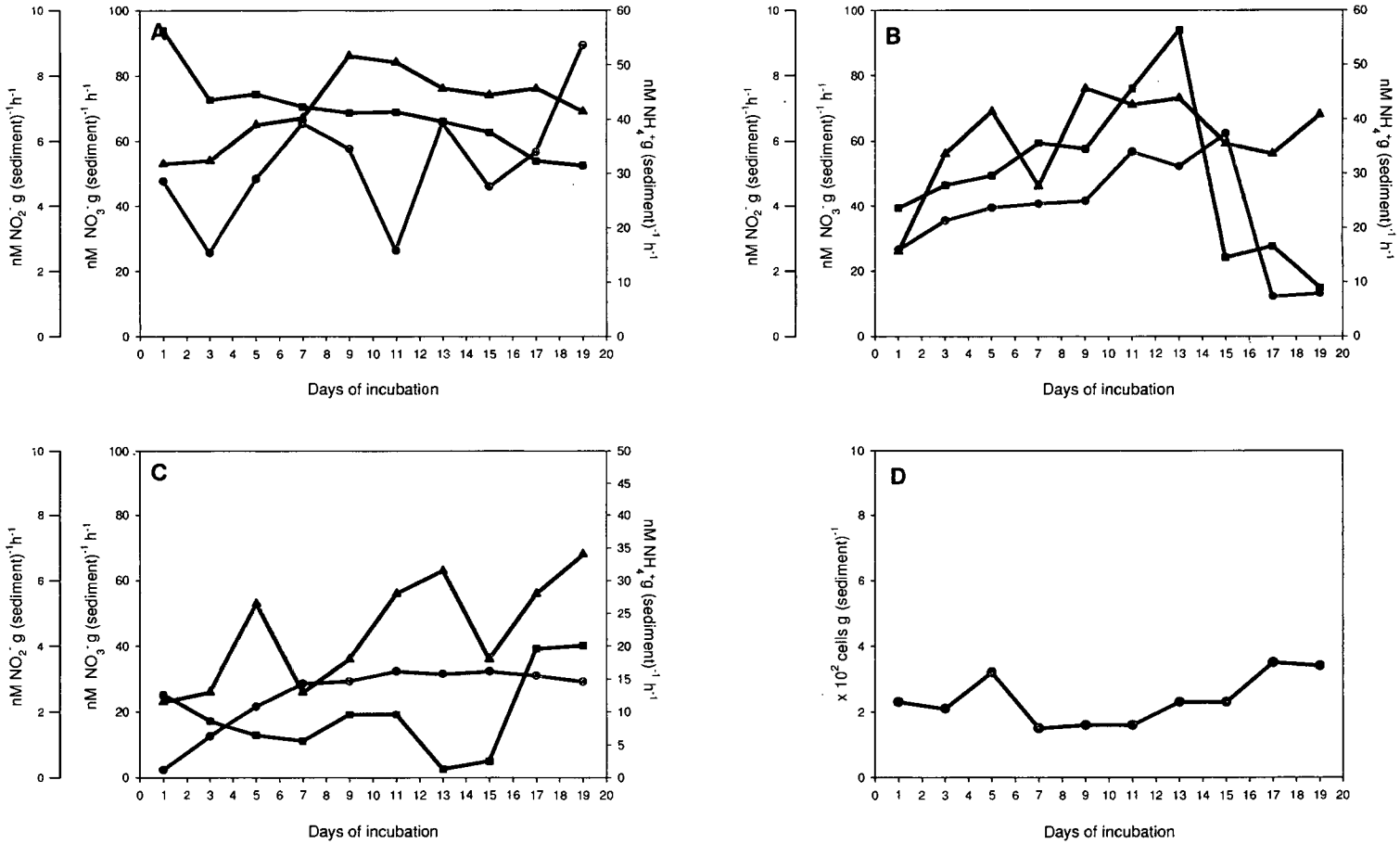


Figure 52: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 5% w/w fertilizer. Nitrifier counts in denitrification blocked system is given in (D).

(Figure 52B) the denitrification rates (37.9±16.9 nM NO_3^- h^{-1}) were found to be stimulated till the 16th day. A similar trend was also observed for the net ammonium production rates (29.3±14.5nM NH_4^+ h^{-1}) with a sharp increase till the 14th day followed by a steep fall. The nitrite production (6.0±1.1 nM NO_2^- h^{-1}) varied strongly throughout the period of incubation. However, in general the nitrite production rates were found to increase with time supporting the hike observed in denitrification rates. The nitrification rates in the DB system (Figure 52C) were found to increase till the 8th day (25.0±10.0 nM NO_3^- h^{-1}) and then maintained a uniform profile throughout the period of incubation. Though nitrite production rates (4.4±1.6 nM NO_2^- h^{-1}) were on a higher side, the variability was also on the higher side. The rate of ammonium utilization (9.6±6.3nM NH_4^+ h^{-1}) decreased with time but showed a prominent increase by the end of the incubation period. There was no visible variation in the nitrifier counts (2.3±0.7x10^2 cells g (sediment)$^{-1}$) throughout the period of incubation (Figure 52D).

In UB system (Figure 53A) with 10% w/w fertilizer, the nitrate production (52±16.4 nM NO_3^- h^{-1}) and ammonium production (49.9±18.2nM NH_4^+ h^{-1}) increased with time and followed a similar trend. There was also no much variation in nitrite production (2.9±1.4 nM NO_2^- h^{-1}), except for a conspicuous peak on the 8th day. There was no specific trend in nitrite production (2.3±0.6nM NO_2^- h^{-1}) in NB incubation (Figure 53B) although denitrification rates increased with time (46.2±14.9 nM NO_3^- h^{-1}). Similarly ammonium production (66.9±17.3nM NH_4^+ h^{-1}) also showed a profound increase with increase in time. Nitrification and

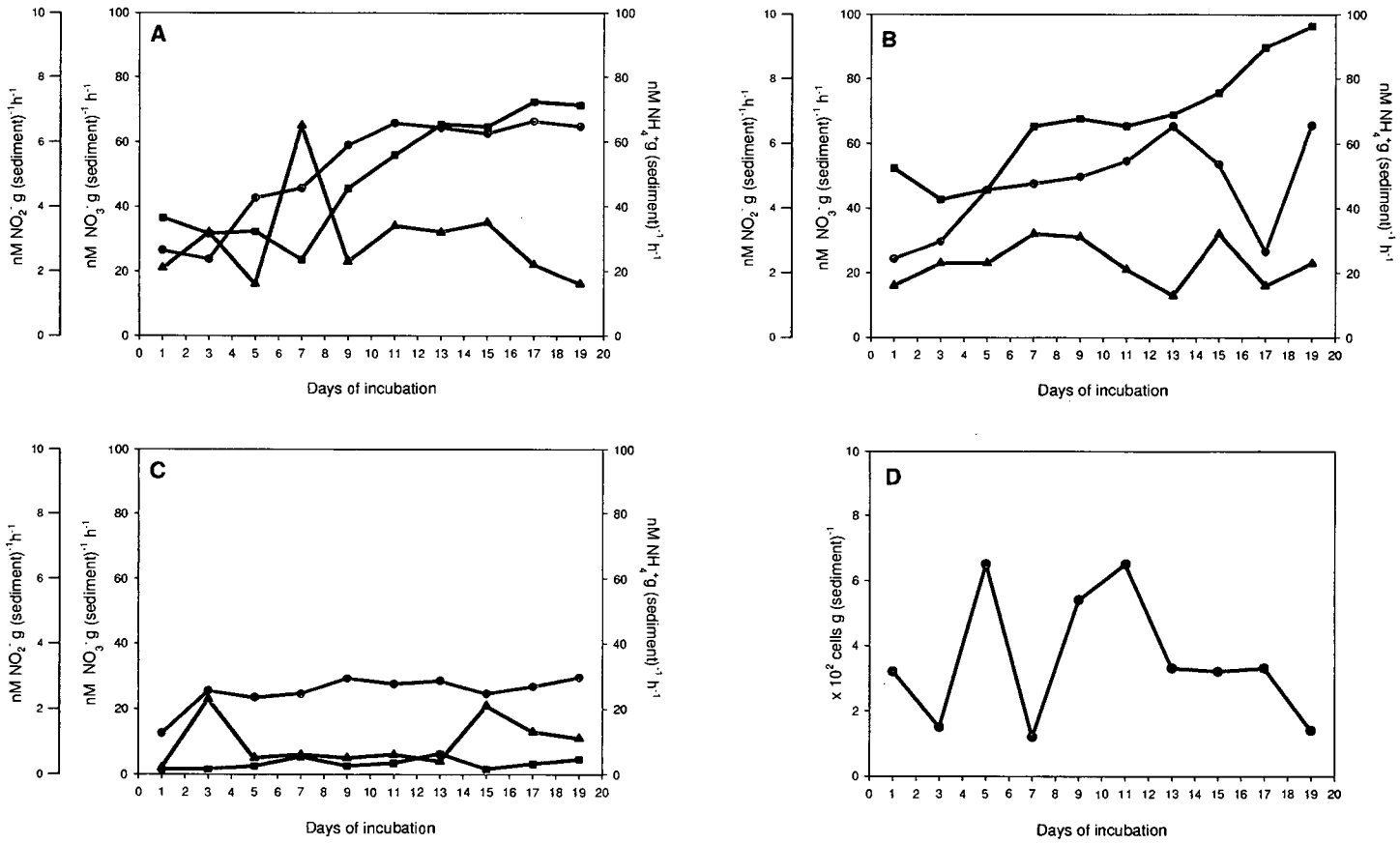


Figure 53: Rate of change of ammonium (-), nitrite (-) and nitrate (-) in unblocked (A), nitrification blocked (B) and denitrification blocked (C) systems with 10% w/w fertilizer. Nitrifier counts in denitrification blocked system is given in (D).

ammonia utilization rates in DB system were uniform throughout the period of incubation with nitrite production (0.9±0.7 nM NO_2^- h^{-1}) varying little with two peaks, one in the beginning and one at end of the incubation. As was in earlier cases, the nitrifying population abundance showed significant variability ($3.5\pm1.9\times10^2$ cells g $(sediment)^{-1}$)), contrary to the uniformity shown in rate of nitrification and ammonia utilization.

On the whole, fertilizers had a very high negative impact on nitrification rates for all the amendments tested. Higher the amount of fertilizer amended in the system, lower was the nitrification rate.

Chapter 5. Discussion

This section begins with a brief introduction on the role of bacteria on the biogeochemical cycling of nitrogen in mangrove environments. Further, the discussions have been done in three sections. The first section (5.1) discusses the ambient nitrogen, organic carbon and metals in the mangrove sediments. The second section (5.2) dwells into the abundance and identity of nitrifiers with specific reference to their trophic status in mangrove sediments. The final section (5.3) discusses both field and laboratory results and aims to understand the factors regulating nitrification in mangrove sediments.

Microbial activity controls the mineralization of organic matter in intertidal soils and sediments thereby influencing pore water nutrient availability and the speciation of redox sensitive ions [Paerl and Pinckney 1996]. Organic matter oxidation is coupled to the reduction of an oxidant by terminal metabolism and produces a variety of products, including nitrogen and nitrous oxide gases, reduced iron and manganese, sulfide and methane which are indicative of denitrification, metal reduction, sulfate reduction and methanogenesis, respectively. This process recycles complex organic matter back to inorganic forms such as bicarbonate, ammonium, and phosphate, which are critical for primary production in oligotrophic mode.

Environmental pollution by inorganic nitrogen is the result of disequilibrium between input of fixed nitrogen (biotic and abiotic), usually in the form of ammonia, and its output, usually in the form of dinitrogen, the only form of nitrogen which can be considered as environmentally safe. All other forms of nitrogen, which can accumulate due to the establishment of unbalanced fluxes,

may create problems ranging in their severity from mild nuisances to serious ecological hazards. Only by manipulating and maintaining environmental factors to allow microorganisms to transform fixed nitrogen to dinitrogen gas, can we avoid the consequences of the accumulation of biologically active inorganic nitrogen species. Success depends on an in depth understanding of the processes involved, at all levels, from biochemistry to ecology, as with any other biological activity. Pollution of the environment with nitrogen differs from pollution by other biogenic pollutants such as carbon and phosphorous, because not only can it trigger eutrophication of water bodies or affect soils and atmosphere, but also cause toxicity to various life forms, depending on the organisms exposed and upon environmental factors such as pH and temperature. When compared to other environmentally important biogenic inorganic ions, the major nitrogenous end products of degradation of organic except for dinitrogen are very soluble, and can therefore reach high concentrations. Of the various forms of nitrogen, ammonia and nitrite are the most toxic to aquatic wildlife (fish, crustaceans, mollusks). It is therefore obvious that for all practical reasons nitrogen assimilation and dissimilatory denitrification must be optimally balanced. If not, either excess assimilation will cause accumulation of toxic inorganic nitrogen, or, on the other hand, denitrification might reduce primary productivity, affecting agriculture and the capacity of any ecosystem to sustain life. Main inputs of ammonia and nitrite into the environment are through microbial nitrogen fixation and anthropogenic sources such as wastewater discharge and the widespread

use of industrial fertilizers. Ammonification resulting from the degradation of organic matter could also contribute substantially.

Mangrove soils are usually nutrient deficient [Alongi 1996; Alongi and Sasekumar, 1992; Boto and Wellington, 1984] though rich in organic matter, suggesting highly throughput recycling of the inorganic nutrients [Holguin *et al* 2001]. Nitrogen fixation is an important source of new nitrogen but this process is influenced by spatial and temporal variability [Lee and Joye, 2006]. The high productivity of mangroves is thus sustained by internal nutrient recycling, which could be coupled to organic matter mineralization. Mangrove soils can be both hypersaline and at the same time biochemically reducing [Kathiresan and Bingham, 2001]. Increased rainfall during the wet season can affect pore water salinity, redox potential, and pH and other soil biogeochemical processes [Alongi *et al*, 1999;2004]. Earlier studies of benthic metabolism and nutrient transformations in mangrove soils fringing oceans or rivers have a marked relationship between organic matter availability, elemental cycling and mangrove density [Nedwell *et al*, 1994, Sherman *et al*, 1998]. This study focuses on the nitrification pathway, understanding the factors regulating and modulating this process in the mangrove sediments.

5.1. Ambient nitrogen, organic carbon and metals

Ammonium: The significance of ammonium in the marine environment is recognized from the following facts. These include 1) the most of preferred form of dissolved inorganic nitrogen (DIN) by the phytoplankton [McCarthy and

Goldman, 1979]; Billen, 1984] as well as heterotrophs 2) major form of biogenic input to the ocean [Sharp, 1983] and 3) central component in the regeneration pathway [Boucher *et al*, 1994]. Ammonium is produced in sediments through different processes: (i) decomposition of organic matter by various heterotrophic organisms [Billen, 1978]; Boynton *et al*, 1980], (ii) primary excretory product of microheterotrophs [Blackburn and Henriksen, 1983; Svensson, 1997], (iii) nitrogen fixation by prokaryotic organisms [Zuberer and Silver, 1979; Potts, 1979; Boto and Robertson, 1990], (iv) turnover of urea [Lomstein and Blackburn, 1992] and (v) dissimilatory reduction of nitrate to ammonium [Cole, 1988; Koike and Hattori, 1978; Sorensen, 1978]. Ammonium produced by these processes can be reoxidized, reincorporated into the organisms, adsorbed onto the particles, and diffused along concentration gradients to other regions of the sediment or to the overlying water. However, high concentrations of ammonium could also reprecipitate as a constituent of authigenic minerals [Martens *et al,* 1978]. Almost all the measurements of pore water ammonium in mangrove sediments show that the concentrations are higher than in sediments of other ecosystems [Alongi, 1996]. Generally, mangrove sediments are considered as anaerobic and ammonium is the major form of inorganic nitrogen [Alongi *et al*, 1992; Kristensen *et al,* 1988]. Hulth *et al* [1999] suggested that, due to lack of oxygen, the transformation of ammonium into oxidized or gaseous forms by the activity of microorganisms becomes slower and therefore the ammonium gets accumulated in the sediments. However, studies conducted by Lizumi [1986] on the nutrient pools in mangrove soils showed that ammonium concentrations are low in the

mangrove zone compared with the non-mangrove zone and suggested that this low level of ambient ammonium could be due to uptake of mangrove plants. Boto and Wellington [1984] also reported variations in ammonium concentrations with depth as well as season in mangrove sediments, and opined that the variation in ammonium uptake by mangrove plants could be a major controlling factor. In a Bermuda mangrove system, ammonium concentrations ranged from 31 to 114 µM [Hines and Lyons, 1982]. Carlson *et al* [1983] noted that the ammonium concentrations were reported to range from 5-11 µM in an *Avicennia germinans* dominated sediment. Rosenfeld [1979] reported that pore water ammonium in eutrophicated Florida mangroves ranged from 390 to 760 µM, which was much higher than those recorded from other mangroves areas. In the present study, depth compromised ammonium concentrations in pore water during the pre-monsoon season at the control site was 19.1±4 µg at NH_4^+-NL^{-1}. Similar values were encountered in the pore water at the experimental site with ammonium averaging 19.9 ±2.4 µg at NH_4^+-NL^{-1}. However, the monsoon season was characterized by lower levels of ammonium in the pore water at both the control (16.8 ±3.6 µg at NH_4^+-NL^{-1}) and experimental sites (14.1±2.1 µg at NH_4^+-NL^{-1} pore water). Physical processes also significantly influence the nutrient pool. In a study carried out in the Ems estuary, de Jonge and Colijn [1994] observed that high water movement and wave action resulted in higher efflux of pore water nutrients. Similar conditions were also observed in the present study. This resulted in the loss of nutrients from the sediment during the monsoon season. Also, the Mandovi is characterized by a large seasonal influx of freshwater into

the estuary, with unchanged tidal amplitude over large distances [Unnikrishnan *et al,* 1997]. During the monsoon season the concentrations of ammonium decreased, supporting this observation. This fall could be explained by the advection of fresh water during these months. In this season, due to high speed of freshwater, the erosion of the surface sediments and the efflux of pore water ammonium to the overlying waters led to the fall. In this study, the post-monsoon values at the control site showed an increase in ammonium accumulation (17.9±7.9 µg at NH_4^+-NL^{-1} pore water) as compared to the monsoon, but they were still lower than the pre-monsoon values. Ammonium accumulation at the experimental site was the highest (27.8±5.6 µg at NH_4^+-NL^{-1} pore water) during post-monsoon season as compared to monsoon and pre-monsoon seasons. This probably due to the effect of higher organic load coming into the estuary due to a wider catchments area or due to higher anthropogenic impact in the Mandovi estuary. Most of these values, except for the lower range (monsoon value) fall within the range recorded at Phuket, Thailand [Kristensen *et al,* 1988].

During the non-monsoon seasons, the physical conditions favor physiological and regenerative process of ammonium production in the sediment and, as water movement is low, there is accumulation of ammonium in the sediment. Throughout the sampling period there was a significant variation in ammonium concentration between the control and experimental sites only at the depth interval 0-2 cm ($F = 7.9$, $df = 1$, $p = 0.009$). This could be attributed to sediment composition (percentage of sand, silt and clay) and adsorption, terrigenous input and biological processes. Since it is dominated by clay, the

fluxes could be more restricted to the sediment water interface. Also, Adsorption experiments on mangrove sediments described by Rosenfeld [1979] indicate that ammonium adsorption was low compared with other marine sediments. The low absorption capacity of ammonium by mangrove sediments may be attributed to the low sediment porosity, density and cation exchange capacity [Boto, 1982]. The differences in the particle size and the porosity of the sediment are also important factors which can influence ammonium concentrations significantly [Rocha, 1998]. At the mangrove zones, the sediment is composed mostly of clay and silt. As a result the sediments are compact and less porous and hence the diffusion of ammonium ion from sediments is less. Terrigenous input also plays an important role in influencing ammonium concentrations. In shallow mangrove-lined estuaries, litter forms a significant fraction of particulate organic matter [Wafar *et al*, 1997].

Nitrite: Nitrite is a well studied transient compound in the nitrogen cycle. It is an intermediate product in many nitrogen transformation processes such as: nitrification, denitrification, dissimilatory nitrate reduction and assimilatory nitrate reduction. The first three processes are mediated by bacteria and the fourth one by autotrophic organisms and also a number of aerobic bacteria and fungi [Hattori, 1983]. In assimilatory nitrate reduction nitrite is released when light is not adequate for photosynthesis and when nitrate is abundant. However, the extent of nitrite production by this process is generally not significant enough to affect regenerated production [Bronk *et al*, 1994; Collos *et al*, 1996]. Nitrite produced

during any of these processes would get released into the pore water. The rates at which these processes proceed vary in the sediment, depending on the amount of substrate available and its supply (either by biogenic or external sources). The rate of these processes also depends on the geomorphology as well as the biochemical composition of the sediments. Availability of oxygen in the sediments and the quantity of organic load also influence the nitrite concentrations in the sediment. The benthic microorganisms oxidize organic nitrogen, using at first, the oxygen dissolved in pore water when oxygen is insufficient, nitrite and nitrate are used as electron acceptors, as in the first three process mentioned above [Cartaxana and Lloyd, 1999]. In mangroves the distribution of nitrite has not been studied extensively. In most of the studies, the estimation of nitrite has been made in connection with nitrification or denitrification processes. Morell and Corredor [1993] estimated the pore water nitrite at various depths (1-9cm) in different localities. They reported concentrations ranging from 0.02 to 0.35 μM in the mangrove lagoon. Alongi [1996] compared the nitrite concentrations in different locations (mangroves and mudflats) and found that the concentrations were significantly lower (0.02-0.05μM) in sediments in which mangrove roots were found.

In the present study, the average pre-monsoon NO_2^- concentration at control and experimental sites were 2.4±0.8 and 2.1±0.1 μg at NO_2^--NL^{-1} pore water respectively. During monsoon season, NO_2^- values at control (2.3±0.5 μg at NO_2^--NL^{-1} pore water; n=20) and experimental sites (2.7±0.6 μg at NO_2^--NL^{-1} pore water; n=20) were comparable to their pre-monsoon counterparts. The post-

monsoon nitrite levels at the control site (2.3±0.6 µg at NO_2^--NL^{-1} pore water; n=20) showed a similar trend as that of the other seasons, but experimental site recorded the lowest values (1.8±0.5 µg at NO_2^--NL^{-1} pore water; n=20) during the post-monsoon season. As described in the previous section, the ammonium levels were higher at the experiment site during the post-monsoon season which in conjunction with low nitrite could mean that the conditions prevailing are not very conducive for nitrification.

In addition, the present study, there is no significant correlation between nitrite and nitrate, suggesting that these two species of nitrogen had different controlling mechanisms. Nitrite showed a significant monthly variability at the experimental site ($p < 0.1$, $df = 11$) where as the control site was marked by significant down-core variability ($p < 0.01$, $df = 11$). The monthly variability in pore water nitrite at the experimental site is probably due to the following reasons. The physical process that is fresh water advection and diffusion of nitrite from the overlying waters are the major sources of nitrite and the variations in the supply leads to the monthly changes. The down core variability observed at the control site could be due to the variations in sediment composition and utilization of nitrite by the autotrophic biomass and mangrove vegetation. Maximum down-core variation was observed during the pre-monsoon season ($p < 0.01$, $df = 4$). Even though the inter seasonal variability occurred neither at the experimental nor at the control site, the bottom most layer of the core (8-10cm) showed significant variation in nitrite both at the control and experimental sites. This could be due to the differential impact of denitrification. Denitrification being a

respiratory pathway promotes quick fluxes there by bringing in profound variation in various niches.

This study shows that the supply of oxidized forms of nitrogen is from different sources. Nitrite may also have been produced through denitrification as the mangrove sediment is generally with low oxygen concentrations and this may facilitate the nitrite production [Cartaxana and Lloyd, 1999; Alongi *et al*, 1999]. The utilization of nitrite by the autotrophs is also an important factor that could influence the nitrite pool significantly. Nitrite uptake rates of phytobenthos were reported to be low and insignificant considering the ambient concentrations in mangrove sediments by Dham [2000]. However, Boto and Wellington [1983] observed that mangrove roots can take up nitrite efficiently and may influence the nutrient pool significantly. This phenomenon is further discussed with respect to spatial variation in nitrite concentrations.

Spatial variations in pore water nitrite concentrations could be controlled by factors such as: 1) sediment composition and oxygen levels 2) topography of the area and 3) flux processes or diffusion.

Nitrate: Nitrate is thermodynamically stable and the most oxidized form of inorganic nitrogen. The availability of nitrate has been attributed a special status in marine primary production [Eppley *et al*, 1979]. Its importance lies in its abundance as a species of nitrogen (more than any inorganic dissolved form), next to N_2 and secondly its abundance as a biologically assimilable form in the dissolved nitrogen pool in the seas. Nitrate is also involved in reduction pathways

and serves as the terminal electron acceptor in microbially-mediated processes such as denitrification and dissimilatory nitrate reduction. Nitrate production and its utilization in the benthic component are much less understood. The production of nitrate in the benthic pool could be linked with physical as well as biological processes. Among the physical processes, nitrate could be supplied by external sources mainly through overlying waters *via* percolation [Henriksen *et al*, 1984] and advection of fresh water [Smith *et al*, 1985]. The biological processes involved in nitrate production include bioturbation and other microbial processes [Nixon *et al*, 1976]. The *in situ* production of nitrate in the sediment *via* nitrification [Kristensen *et al,* 1988] is considered as a major biological process that adds nitrate into the dissolved pool. Equally important as the supply and production of nitrate in the sediment is its loss or removal by biological means that determines the net ambient nitrate levels in the dissolved pool [Alongi *et al*, 1999; Cartaxana and Lloyd, 1999]. Nitrate uptake [Boto *et al*, 1985; Alongi, 1996], denitrification [Sorensen, 1978; Cartaxana and Lloyd, 1999] and dissimilatory nitrate reduction [MacFarlane and Herbert, 1982;1984] are the major processes through which nitrate can be lost significantly from the sediments or transformed into another compound. In denitrification and dissimilatory nitrate reduction, nitrate replaces oxygen as the terminal electron acceptor when oxygen diffusion rates in the soil or sediment are insufficient to fulfill the demand for microbial respiration [Jensen *et al*, 1994]. Measurement of nitrate from mangrove sediments by several workers have shown that nitrate concentrations are generally lower than those of ammonium [Boto and

Wellington, 1984; Carlson *et al*, 1984], in comparison with coast, estuarine and sea grass ecosystems.

In this study, the concentration of nitrate at the control site were 9.3±2.9 µg at NO_3^--NL^{-1} pore water during the pre-monsoon season, while, the monsoon values were lower (6.1±1.2 µg at NO_3^--NL^{-1} pore water). In contrast, highest nitrate values at the experimental site (13.2±2.0 µg at NO_3^--NL^{-1} pore water) were recorded during the monsoons. This could be due to enhanced river runoff in monsoon seasons with more nitrates flushed down from the catchment areas. The pre and post-monsoon values were 8.6±1 µg at NO_3^--NL^{-1} pore water and 8±2.1 µg at NO_3^--NL^{-1} pore water respectively. However highest levels were recorded at the control site (11.5±1.3 µg at NO_3^--NL^{-1} pore water; n=20) during the post-monsoon season. A similar range was reported by [Kristensen *et al,* 1988] from the mangrove sediments of Phuket, Thailand.

Nitrate showed significant monthly variability at both the control ($p= 2.9x10^{-5}$, df = 11) and the experimental site ($p= 4.4x10^{-3}$, df = 11). The monthly variability in the present study can be explained in the context of the physical and biological processes. In the monsoon season, the high nitrate concentrations were noticed at the experimental site. This sharp increase in the nitrate concentrations can be mainly attributed to transport with fresh water as a result of heavy river discharge into the estuary caused by southwest monsoon rainfall. Nitrate concentrations in the overlying waters at this time are ten times higher than in the non monsoon months [Heredia, 2000] and this could have influenced

the pore water concentrations. Alongi [1988] made a similar observation during the rainy season in a mangrove forest of Australia.

There was no down core variation neither at the control nor at the experimental site. In spite of having a well packed nature, the sediment pore water dissolved nitrogen variability is less probably indicating that the microbes have a profound role which is similar or even more than physical processes. There exists considerable variability in nitrate at both the control and experimental sites during the monsoon and post-monsoon seasons ($p < 0.1$, $df = 1$). The nitrate concentrations reported in this study is similar to the range reported by [Kristensen *et al,* 1988] from the mangrove sediments of Phuket, Thailand.

In the present study there was no significant correlation between nitrification rates and nitrate concentrations at the control site, while there was a significant negative correlation ($r = -0.25$, $p < 0.05$, $n = 65$) ($p<0.05$ at the experimental site probably indicating a mechanism of feedback inhibition at the experimental site. In the control system the nitrate pool could be either non limited by nitrification or there are alternative local sources for nitrate input. The nitrification rates are higher in the non monsoon seasons at both the sites. It can, therefore, be suggested that in the non-monsoon months, the pore water nitrate concentrations are controlled by microbial activity (nitrification) whereas, in the monsoon season it may have been influenced by overlying waters. The rate at which the nitrate is utilized could also be a factor influencing the monthly variations of nitrate concentrations. Uptake studies revealed that the preference

of the autotrophs to utilize a particular nutrient leads to the variations in the dissolve pool [Stanley *et al*, 1987; Alongi, 1988; 1996; Boto and Wellington, 1988; Revera-Monroy *et al*, 1995]. Boto *et al* [1985] reported significant uptake of nitrate by mangrove roots and suggested that pore water nitrate could be depleted due to high uptake. Gilbert and McCarthy [1984] showed that in shallow ecosystems, variation in the uptake rates and nitrogen demand of the autotrophs are the major causes for the seasonality in nitrate concentrations.

Organic carbon: Mangroves are known to be highly productive ecosystems (global litter fall of 100 Tg C y^{-1} [Jennerjahn and Ittekot, 2002]). Recent estimates show that as much as 11% of the total organic carbon inputs across the coastal zone (i.e. through riverine transport) is of mangrove origin show that the carbon fixed by mangroves is could be significant in the carbon budget of the coastal zone [Jennerjahn and Ittekot, 2002]. It has become clear that a strong interaction exists between the intertidal zone and the adjacent aquatic environment. Both import (of terrestrial material, phytoplankton, sea grasses, etc.) and export (of mangrove-derived organic matter) are expected to have major consequences for the carbon dynamics in both compartments [Hemminga *et al*, 1994; Bouillon *et al*, 2003; Marchan *et al*, 2003]. The imported carbon sources have an important trophic role in sustaining macro-invertebrate communities of the estuarine zones [Bouillon *et al*, 2002]. Mineralization, however, could represent a major fate for exported organic matter [Bouillon *et al*, 2002; Borges *et al*, 2003]. Similarly, estimates of iron available [Alongi, 1988;

Alongi *et al*, 1993; Middelburg *et al*, 1996; Alongi *et al*, 2000] indicate that intense mineralization takes place in intertidal region of mangrove sediments. A continuous range of mangrove ecosystem types exists, from 'retention' systems where sediments are rich in organic carbon which is almost entirely of local origin, to 'flow-through' systems with mineral sediments (i.e. relatively low in organic carbon) where the organic matter can be dominated by imported sources [Bouillon *et al*, 2003].

In this study, organic carbon revealed lowest values of 1.2 (±0.1) and 1.9 (±0.3) %, at the control and experimental sites respectively during the monsoon season. This could again be attributed the higher flow rates and lower residence times associated with the southwest monsoon. Both the sites showed difference in pre and post-monsoon organic carbon accumulation. Highest accumulation at the control site was in the pre-monsoon season (3.1±0.8%) while at the experimental site it was in the post-monsoon season (3.4±1%). This compliments the earlier observation made on the levels of ammonium which was higher at the experimental site during the post-monsoon season. In contrast, intermediate values were observed at the control site during post-monsoon (2.9±1.3%) and experimental site during pre-monsoon (2.9±0.3). Though there was no monthly variability at the control and experimental sites there was considerable inter seasonal variability at both the sites (control, $p<0.01$, $df = 2$; experiment, $p<0.1$, $df = 2$).

Organic carbon related positively to iron ($r = 0.67$, $p < 0.01$) and manganese ($r = 0.92$, $p < 0.001$, $n = 15$) at the experimental site, whereas the

relationships were negative at the control site ($r = -0.72$, $p < 0.001$) for iron and ($r = -0.51$, $p < 0.05$) for manganese. These relationships could imply that at the experimental site there was considerable extraneous input of organic carbon favoring the accumulation of iron and manganese. Wangersky [1986] has reported that coatings of organic matter prevalent in fine grained sediments bind a variety of trace elements.

Iron and Manganese: Depth compromised average values of iron at the control site ranged from 6-7.4% for the entire sampling period. There was no inter-seasonal variability at the control site but there was considerable down-core variation ($p < 0.1$, $df = 4$) irrespective of the month. In contrast, high inter-seasonal variability was observed at the experimental site ($p<0.001$, $df = 2$), with pre-monsoon showing the highest accumulation of Iron (24±3.2%) followed by post-monsoon (18.7±3.6%) and monsoon (10.2±1.9%) seasons respectively. This is because of the fact that the experimental site is under the influence if the movement of iron ore bearing barges which traverse the estuary in large numbers especially in the pre and post-monsoon season. Maximum disparity between the control and experimental sites was observed during the pre-monsoon season ($p<0.001$, $df = 2$) while the lowest was during the monsoon ($p<0.01$, $df = 2$). The post-monsoon values depicted a transitional stage with considerable variability ($p<0.001$, $df = 2$). The values reported in the present study are comparatively higher to those reported by Ray *et al* [2006] from the Godavari estuarine mangrove ecosystem on the eastern coastline of India.

These authors reported that the average sedimentary iron and manganese values were 0.004% and 0.001%, respectively. Studies by Alagarsamy [2006] showed that the concentrations of iron varied from 2.2% to 49.7% on the surface sediments of the Mandovi estuary, while the concentration of manganese ranged below detection limit to 1.61%. Though reports from the Mandovi estuary [Alagarsamy, 2006] showed that metal concentrations were generally low during monsoon, compared to the pre and post-monsoon seasons, at the adjoining mangrove sediments, they were found to vary. In the present study, the highest accumulation of iron was observed during pre-monsoon, while the values in the monsoon and post-monsoon seasons were lower and comparable. The variation of Manganese values was similar to observations made by Alagarsamy [2006] in the surface sediments of the adjoining Mandovi estuary.

Even though there was significant monthly variability in the accumulation of manganese at both the control ($p<0.01$, $df = 12$) and experimental site ($p<0.01$, $df = 12$), there was no significant inter seasonal variability at both the sites. In general the Manganese levels remained ~0.5% at the control site throughout the sampling period, whereas at the experimental site it ranged from 1.1(±0.6)% in the post-monsoon to 1.7(±0.6)% in the monsoon season with intermediate values of 1.4 (±0.2%) in the pre-monsoon season. Notable variation between the control and experimental site was observed in the monsoon season ($p<0.001$, $df = 1$). The degree of similarity appears to increase as it progresses to post-monsoon season ($p<0.01$, $df = 1$) and further decreases in the pre-monsoon season ($p<0.0001$, $df = 1$).

In general, the enrichment of iron increases with depth to reach values >165% at an 8–10 cm interval during pre-monsoon. During monsoon, the general enrichment pattern is reversed with the highest enrichment (85.6%) at 0–2 cm. There was no significant correlation between iron and manganese in the monsoon months, neither at the control nor at the experimental site. However, a direction is suggested in the relationship. It is negative at the experimental site and positive at the control site, perhaps suggesting that under a lower concentration of iron, manganese concentration tends to increase. With a higher concentration of iron, this trend changes, suggesting that the increase of both of the elements do not get coupled after a threshold. These observations are contrasted by very high enrichment (393–773%) of manganese at the experimental site during the pre-monsoon season, especially in the depth range of 4–6 cm. A positive relation between iron and manganese during the non-monsoon months at the control site and the absence of such a relation at the experimental site showed that, though the chemistry of iron and manganese are closely related, they could be differentially preferred by organisms, which in turn is influenced by the prevailing environment.

The geoaccumulation index (I_{geo}) was originally defined by Müller [1979] for metal concentrations in the <2 μ fraction and developed for global standard shale values. The choice of the background value plays an important role in the interpretation of geological data. I_{geo} has been widely utilized as a measure of pollution in freshwater [eg. Müller, 1980; Singh *et al*, 1997; Kralik, 1999] and marine sediments [e.g. Stoffers *et al*, 1986; Bryan and Langston, 1992; Dickinson

et al, 1996]. I_{geo} of iron and manganese in the experimental site with control as reference showed that the sediments in the depth range 0–10 cm fall in the 'uncontaminated to moderately contaminated by iron' category during the pre-monsoon and monsoon season. While, in the post-monsoon season, though the 0–4 cm still remains 'uncontaminated to moderately contaminated by iron', the 4–10 cm layer has recovered from Iron contamination and could be termed as 'Uncontaminated'.

The contamination due to manganese is more acute than iron during the pre-monsoon season. During the pre-monsoon, the 0–8 cm section falls under the 'Moderately to strongly contaminated' category, while the 8–10 cm section falls under the 'Moderately contaminated category'. All the depths fall in the 'Uncontaminated' group during the monsoon and post-monsoon and hence could be assessed as free from the manganese pollution. These observations could again suggest that, though the elements are closely related, the biogeochemical cycling of manganese could be more efficient and rapid when compared to iron in the mangrove sediments. Moreover, the mangrove ecosystems play a buffering role by reducing the enrichment levels of iron and manganese in the sediments. The overall assessment could be that, though the Mandovi estuary is under the influence of ferromanganese ore mining with significant impact on the estuarine sediments, the sediments of the adjoining mangroves are comparatively less contaminated, but when strongly contaminated, could be self-regulatory and recover in the time scale tested.

5.2. Nitrifiers –Insights into their trophic structure, abundance and diversity

Occurrence of heterotrophic nitrification have been widely reported [Verstraete and Alexander, 1972; Castignetti and Holocher, 1984; Barraclough and Puri, 1995; Gupta, 1997; Lu *et al*, 2008; Ahmad *et al*, 2008], though most of them categorize nitrification as an exclusively chemoautotrophic process. Specifically, the potential of heterotrophic nitrification in sediments has been previously reported by Schimel *et al* [1984]. In this study, it was observed that nitrite/nitrate was produced by nitrifiers even when the media was supplemented with 0.01% glucose favoring higher organic carbon: dissolved inorganic nitrogen ratio. Hence, the total dissolved inorganic nitrogen pool could be significantly governed by heterotrophic nitrification. The abundance of heterotrophic nitrifiers increased with depth at the experimental site and was governed by concentration of ammonium in the pore water ($r=0.23$, $p < 0.1$). Since the autotrophic nitrifiers were dominant at the experimental site it might pose as a strict competitor for their heterotrophic counterpart for ammonium. Retrievable nitrifier counts showed more significant monthly variation at the control site than the experimental site. In general, the retrievable nitrifier counts at the control site were higher than the experimental site for all the seasons.

At the control site, in the top 2 cm, nitrification could be primarily governed by the abundance of heterotrophic nitrifiers ($r=0.55$, $p <0.05$), while manganese levels in turn govern ($r =0.5$, $p <0.05$) the abundance of these nitrifiers. Another significant relationship at the control site was observed at 4–6 cm, where both the groups of nitrifiers were found to be mutually exclusive ($r=0.5$, $p <0.05$) with

nitrite having a feedback inhibition on the former ($r=0.48$, $p<0.1$). Iron and manganese governed the abundance of autotrophic nitrifiers in the depth intervals of 6–8 ($r=0.55$, $p< 0.05$) and 8–10 cm ($r = 0.54$, $p <0.05$). The autotrophic nitrifiers governed the production of nitrate at 6–8 cm ($r=0.56$, $p <0.05$). The concentration of manganese had significant impact on the abundance of heterotrophic nitrifiers ($r=0.47$, $p < 0.1$). As was the case at the control site, the abundance of heterotrophic nitrifiers at the experimental site (0–2 cm) was controlled by the levels of manganese in the sediment. The heterotrophic nitrifiers at 4–6 cm were governed by the levels of ammonium ($r=0.79$, $p< 0.001$) which could be linked to nitrification ($r=0.46$, $p < 0.1$).

Lack of seasonal variability in manganese indicate that manganese turnover time could be much less, as it is actively removed both by physical processes and biogeochemical sequestration. The positive correlations observed between the nitrifiers and manganese could be due to the fact that manganese could be actively used as a co-factor for the ammonia monoxygenase enzyme. More importantly, it could also serve as an alternate terminal electron acceptor in anaerobic respiration [Hulth *et al*, 1999]. Absence of a significant relationship between iron with the heterotrophic and autotrophic nitrifiers at the both the control and experimental sites suggested that iron was present in excess and perhaps non-limiting. Moreover, manganese (IV) reduction precedes that of Iron (III) because of reduction energetics of the solid phases [Burdige *et al*, 1992].

The relationship between manganese and general heterotrophs indicate that the latter has a considerable influence in regulating the levels of Manganese.

During post-monsoon, the variation in heterotrophs affected the variation in manganese concentration up to 90% ($r = 0.949$, $p < 0.001$). These relationships demonstrate that the nitrifiers and general heterotrophs could be actively involved in maintaining the level of manganese on par with the reference levels at the control site.

In a seasonal perspective, at the control site the availability of ammonium is the most important factor governing the abundance of autotrophic nitrifiers during all the seasons, except for the post-monsoon season when there is a feedback inhibition by nitrate. At the experimental site, only ammonium and organic carbon were found to regulate the abundance of heterotrophic nitrifiers during the monsoon season. However, iron and depth were the two parameters governing the abundance of autotrophic nitrifiers during pre-monsoon and monsoon seasons.

The above observed function diversity is not well supported by the taxonomic diversity of nitrifers hitherto recorded. After the first reports on successful isolation of chemolithoautotrophic ammonia oxidizers at the end of the 19th century [Frankland *et* al, 1890; Winogradsky, 1890], researchers continued to investigate the diversity of ammonium oxidizers in natural and engineered environments by applying enrichment and isolation techniques. These efforts resulted in the description of sixteen ammonium oxidizing bacterial species [Watson,1965; Jones *et al*, 1988; Koops *et al*, 1976; 1990; 1991]. Furthermore, DNA-DNA hybridization studies provided evidence for the existence of at least fifteen additional species [Koops and Harms, 1985; Koops *et al*, 1991;Stehr *et* al,

1995]. However, low maximum growth rates and growth yields of ammonium oxidizers render cultivation-based analysis of their environmental diversity extremely time-consuming and tedious. Furthermore, all culture techniques are potentially selective and thus bear the risk of incomplete coverage of the actually existing bacterial diversity [Wagner *et al*, 1993; Amann *et al*, 1995; Juretschko *et al*, 1998]. Comparative 16S rRNA sequence analyses of cultured ammonium oxidizing bacteria revealed that members of this physiological group are confined to two monophyletic lineages within the *Proteobacteria*. *Nitrosococcus oceani* [Watson, 1965; Trüper and de Clari,1997] is affiliated with the gamma-subclass of the class *Proteobacteria*, while members of the genera *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio* form a closely related grouping within the beta-subclass of *Proteobacteria* [Woese *et al*, 1984; Head *et al*, 1993; Teske *et* al, 1994; Stehr *et al*, 1995; Utåker *et al*, 1995; Princic *et al*, 1998]. It has been suggested [Head *et al*, 1993] and subsequently questioned [Teske *et* al, 1994] that the latter three genera should be reclassified in the single genus *Nitrosospira*. The availability of 16S rRNA sequences also provided a basis for the development of cultivation-independent methods to investigate the diversity and community composition of these microorganisms in complex environments. PCR-mediated preferential amplification of ammonium oxidizing bacterial 16S rDNA and subsequent cloning and sequencing have been extensively applied to create phylogenetic inventories of various environments [Kowalchuk *et al*, 1997; 1998; 2000; Pomnerening-Röser *et al*, 1996; McCaig *et al*, 1999; Mendum *et al*, 1999; Phillips *et al*, 1999;

Whitby *et al*, 1999; Bano and Hollibaugh, 2000], which led to the recognition of seven 16S rRNA beta-subclass ammonium oxidizing bacterial sequence clusters. Recently, the battery of molecular tools to infer the presence of ammonium oxidizing bacteria in the environment has been supplemented by PCR primers for specific amplification of the ammonia monooxygenase structural gene *amoA* [Holmes *et al*, 1995; Mendum *et al*, 1999; Rotthauwe *et al*, 1997; Sinigalliano *et al*, 1995]. While environmental 16S rDNA and *amoA* libraries significantly extended our knowledge on the natural diversity of ammonium oxidizing bacteria, biases introduced by DNA extraction, PCR amplification, and cloning methods [Reysenbach *et* al, 1992; Farrelly *et al*, 1995; Suzuki and Giovannoni, 1996; Chandler *et al*; 1997; Wintzigerode and Goebel, 1997; Polz and Cavanaugh,1998; Suzuki *et al*, 1998] have impacts on quantitative information on the community composition. In contrast to PCR-based methods, quantitative information on ammonium oxidizing bacterial population structure and dynamics in the environment is obtainable via membrane or *in situ* hybridization techniques in combination with ammonium oxidizing bacteria specific oligonucleotide probes [Wagner *et al*, 1995;1996; Mobarry *et al* 1996; Schramm *et al*, 1996;1998; Juretschko *et al*, 1998; Logemann *et al*, 1998]. The latter approach also allows one to directly relate community structure with the morphology and spatial distribution of the detected organisms.

Enumeration of total eubacterial counts by fluorescence *in situ* hybridization showed that the probe used had detected 34-43% of acridine orange counts. This probably has some implications. One being that the range of

the probe, though considered universal is limited when used on mangrove sediments. The other reason for the underestimate could be due the detrital load in the sediments which could give false signals. The probe Nso1225 is known to cover most of the β proteobacterial ammonium oxidizers, however the detection in the range 52-62% probably signal the existence of novel groups of nitrifiers including the heterotrophs. Incomplete coverage of cultured ammonium oxidizing bacteria in the current 16S rRNA and *amoA* gene data sets hampered the design and evaluation of specific primers. It was also interesting to note that the sequence match for many of the isolates was less than 98% (16SrDNA) probably indicating its genetic novelty. In addition, this study shows that well known heterotrophs like *Pseudomonas, Janthinobacterium, Alcaligenes* etc have potential to convert ammonium to its oxidized forms, a process which was thought to be exclusively due to autotrophic nitrifiers. Heterotrophic nitrification, not coupled to any energy yielding metabolic pathways, has been demonstrated in many organisms, including bacteria, such as *Arthrobacter* sp. [Witzel and Overback 1979], *Alcatigenes faecalis* [Papen *et al*,1989],*Thiosphaera pantotropha* [Robertson and Kuenen 1988], and fungi [Killham, 1986]. A possible mechanism for the accumulation of nitrite by heterotrophic organisms is the co oxidation of ammonia to nitrite, coupled to the xanthine oxidase reaction [Nagano and Fridovich, 1985].The most active heterotrophic nitrifier as yet reported belongs to the genus Alcaligenes isolated from soil [Castignetti and Gunner, 1980; 1981; Castignetti and Hollocher, 1982; Castignetti *et al*, 1983]. It is also capable of the assimilation of nitrate [Castignetti and Gunner, 1980] and

denitrification [Castignetti and Hollocher, 1982]. The ability to carry out both heterotrophic nitrification and denitrification has been established with certainty among bacteria only with the genus Alcaligenes, but this dual ability can be presumed to apply also to a strain of *Pseudomonas aeruginosa* which can oxidize acetaldoxime nitrogen to nitrite [Amarger and Alexander, 1968; Obaton *et al*, 1968]. While Poth and Focht [1985] have stated that *Nitrosomonas* releases nitrous oxide only by denitrifying reaction, Hooper *et al* [1990] also showed that some of the nitrous oxide may be produced through oxidation of ammonium. Also, the data presented by Kim and Craig [1990] suggested that nitrification is the origin of some nitrous oxide in deep ocean water. A possible mechanism of accumulation of nitric and nitrous oxides during nitrification by *Nitrosomonas* is that under certain conditions (e.g. the presence of organic reductants) excess hydroxylamine is released by the cells and reacts chemically with nitrite to form nitric and nitrous oxides. The data presented by Klemendtsson *et al* [1988] and Downs [1988] suggested that nitrifiers play a major role in nitrous oxide production in soils and freshwater lakes, according to Yoshida [1988] the role of nitrifiers in the production of nitrous oxide in the oceans has been overestimated.

The present study gives provides additional proof to this process and thereby strengthens the argument on the existence of heterotrophic nitrification and also the possibility of denitrification to coexist with nitrification in the same organism. The functional diversity of the existing as well as the new genera of nitrifiers could also be very high and this is probably reflected in the metabolic finger prints [details given is section 4.5]. Most of the isolates (22 of the 30

isolates) had signature metabolic profiles which are tuned to a particular carbon substrate. For eg. In the present study, *Nitrobacter winogradskyi* was the only nitrite oxidizer that was capable of using formic acid, while the use of Turanose, Uridine and Acetic acid were restricted to the genus Pseudomonas among the ammonium oxidizers. Incidence of signature substrates was comparatively less among the coupled ammonium-nitrite oxidizers that were identified in this study, however α-Ketobutyric acid and D-Gluconic acid was signature to Janthinobacterium and Alcaligenes respectively.

This work also throws light on the occurrence of wide variety of microbes catalyzing critical reactions which were hitherto considered as a strictly autotrophic process. Also, many isolates generally known to be heterotrophs were able to oxidize ammonium up to its most oxidized product nitrate, a feature that could be subject to further studies in the future. Also, most of the exclusive nitrite oxidizers encountered in this study except for *Nitrobacter winogradskyi* showed higher activity in the presence of organic carbon.

5.3. Regeneration of nitrogen by nitrification: Process and Controls

5.3.1. Inter parameter relationships

Benthic nitrogen regeneration may supply an important, but variable fraction of the nitrogen requirements for plankton production in marine coastal and shelf regions. The amount of nitrogen recycled by the benthos depends on the pelagic-benthic coupling, i.e. the quantity and quality of the organic matter sinking to the sediments, but also on the nitrogen transformations taking place in

the sediments. In the sediments nitrification plays a key role. Its coupling with N-mineralization and denitrification, together with nitrate, determines the concentrations in the overlaying water and the species of inorganic nitrogen effluxing from the sediment and the amount of nitrogen shunted into the denitrification sink. Numerous investigations have described benthic nitrogen exchange in estuarine and coastal sediments [Klump and Martens, 1983; Seitzinger, 1988]. The variability in temporal and seasonal patterns indicates a complex relationship between physical-chemical and biological control factors, but there are some general trends: higher inputs of organic nitrogen to the sediments generally increase both nitrification and denitrification. However, at high organic inputs and/or increasing temperatures, nitrification becomes low or zero due to limited oxygen penetration and sulfide inhibition [Hansen *et al,* 1981; Kemp *et al,* 1982; 1990; Jensen *et al,* 1988; Billen, 1988; Seitzinger, 1988].The increase in nitrification rate at higher ammonium availability is usually small due to increased oxygen uptake by other heterotrophic processes. A higher organic input to the benthos is often associated with increased macrofaunal abundance [Nixon 1981; Kemp *et al,* 1982; Grebmeier *et al,* 1988]. The macrofauna may further contribute to increased rates of nitrification and denitrification and to a stronger coupling between the two processes due to the effect of ventilated macrofaunal burrows [Kristensen *et al,* 1988; Aller, 1988]. Macrofaunal activity also tends to increase the total rate of nitrogen mineralization [Kristensen and Blackburn, 1987; Lomstein *et al,* 1989] and the flux of ammonium from the sediments.

The benthic chamber in mangroves has been least studied in terms of nitrogen cycling. This has hampered our understanding on these productive coastal marine ecosystems which are vulnerable to human impacts. Most of the studies reported from mangrove forests are sporadic measurements, though some studies conducted in Australia [Boto and Wellington, 1988; Trott and Alongi, 1999], Pakistan [Harisson *et al*, 1997], Mexico [Rivera-Monroy *et al*, 1995], and India [Krishnamurthy *et al*, 1975; Dham *et al*, 2002] discuss seasonal cycles. Nitrifying bacteria and nitrification rates in general, may be regulated by many factors including ammonium [Triska *et al*, 1990; Jones *et al*, 1995), pH [Saractchandra, 1978], temperature [Paul and Clark, 1989], oxygen concentration [Stenstromm and Podlska, 1980; Triska *et al*, 1990], competition for ammonium [Verhagene and Laanbroek, 1991], and organic carbon availability [Verhagene and Laanbroek, 1991]. Irrespective of season or depth of sampling, nitrification rates were predominantly governed by the availability of organic carbon ($r=0.32$, $p< 0.01$) at the control site. This is supported by a positive correlation of organic carbon with heterotrophic nitrifiers ($r=0.28$, $p <0.05$). Nitrification rates at the top layer (0–2 cm) of the core from the control site was governed by the abundance of heterotrophic nitrifiers ($r=0.58$, $p < 0.02$); whereas at the bottom (8–10 cm) it appears to be regulated by both the abundance of heterotrophic nitrifiers ($r =0.46$, $p < 0.1$) as well as organic carbon ($r=0.45$, $p< 0.1$). A positive correlation with nitrite ($r= 0.48$, $p < 0.1$) probably indicates that the ammonium oxidation might be of a higher magnitude than nitrite oxidation. In the pre-monsoon season nitrification rates increased with depth ($r=0.67$, $p <$

0.001) and were limited by the availability of ammonium ($r = 0.57$, $p < 0.001$). This favors enhanced nitrification rate [Straus and Lamberti, 2000]. Bulk of the carbon could be labile as the nitrification rate is negatively linked to organic carbon ($r = 0.4$, $p < 0.05$). In addition, there was no single set of factors governing nitrification rate during the monsoon season. However, the recalcitrant fraction of organic carbon is considered to favor enhanced nitrification rates by reducing the utilization pressure on ammonium between heterotrophs and nitrifiers [Straus and Lamberti, 2000]. Even though the organic carbon: dissolved inorganic nitrogen ratio is high there was no relation between organic carbon and nitrification rates for the pre and post-monsoon season indicating that organic carbon is either unlimiting or dominated by the recalcitrant fraction. In general, the system evolved from a 'low organic carbon: dissolved inorganic nitrogen – low nitrification rate' system in the monsoon season to a 'high organic carbon: dissolved inorganic nitrogen – high nitrification rate' system in the pre-monsoon season with the post-monsoon representing a transition period where the organic carbon: dissolved inorganic nitrogen ratio gradually increased. This study indicates that the quality of organic carbon could be a more important proxy to nitrification rate rather than organic carbon: dissolved inorganic nitrogen ratio. Further, it could also be inferred that maximum fraction of recalcitrant carbon during the pre and post-monsoon seasons accumulated at 4–6 cm.

Nitrification rates at experimental site showed a positive relation with iron ($r = 0.47$, $p < 0.001$) and autotrophic nitrifiers ($r = 0.43$, $p < 0.001$) which is indicative of anaerobic autotrophic nitrification. An inverse relation with nitrate (r

=-0.25, p <0.05) signifies feedback inhibition indicating that at the experimental site, nitrification rate at the optimum. Hence it appears that the control site is dominated by heterotrophic nitrification whereas autotrophic nitrification governs the experimental site. In addition, organic carbon (3.4±1%) and ammonium (27.8±5.6 µg at NH_4^+-NL^{-1}) peaked at the experimental site during the post-monsoon season while at the control site it was during the pre-monsoon season (organic carbon 3.1±0.8% and ammonium 27.8±4 µg at NH_4^+-NL^{-1}). However, the pre-monsoon of the experimental site was marked by highest levels of nitrification rates (18.2±0.6 ng at-N g (sediment)$^{-1}$ h^{-1}) indicating a decoupling with organic carbon and ammonium. These parameters remain coupled during the pre-monsoon season at the control site. Ammonium plays ($r= 0.55$, $p< 0.001$) plays a limiting role in the pre-monsoon season even though the organic carbon: dissolved inorganic nitrogen ratio is comparatively higher. Further, the production of nitrite appears to govern the nitrification rates during the monsoon season. The abundance of autotrophic nitrifiers is the major factor governing the nitrification rate for most of the depth intervals sampled. Positive relations with autotrophic nitrifiers at 0–2 cm ($r =0.54$, $p< 0.05$), 2– 4 cm($r=0.47$, $p <0.1$), 6–8 cm ($r=0.49$, $p< 0.1$) and 8–10 cm ($r =0.68$, $p< 0.01$) is in accordance with the earlier observation. In addition, iron appears to be the next critical factor regulating nitrification at the experimental site with maximum coupling observed at 4–6 cm ($r =0.7$, $p < 0.001$) and 6–8 cm ($r =0.6$, $p <0.01$) intervals. The feedback inhibition of nitrate is restricted to the bottom layer of 8–10 cm depth indicating enhanced nitrification potential down the core till this depth.

5.3.2. Experimental studies

The mangrove environments are subject to several influences, the most profound could be those with an anthropogenic origin. The impact on nitrification can be significant even when pollution sources are remote and contact indirect. For instance, although there is very little human activity on the shores of Lake Superior, its levels of nitrates increased 4-fold during the past century, primarily due to loading of nitrogenous compounds from the atmosphere [Bennet, 1986]. When the ecosystem is disturbed certain key reactions like nitrification is disturbed resulting in ecosystem malfunction. When nitrite accumulates, due to incomplete nitrification or denitrification, it reacts readily with chlorine, decreasing effective residual chlorine concentrations. It also interferes with chloramination [Wolfe *et al*, 1988]. Heavy agricultural fertilization frequently results in massive nitrification and leaching of nitrates to ground water, increasing concentrations of nitrates to levels beyond permitted standards [Soares *et al*, 1991].

The experimental studies [details given in section 4.6] reported in the present study have been conducted in dark, as light has a detrimental effect on nitrification. Hooper and Terry [1973, 1974] have shown that visible light (420 nm) inhibits oxidation of ammonia but not of hydroxylamine, at a rate constant proportional to its intensity. Inhibition by light also abolishes the ability of the putative ammonia monooxygenase peptide to bind acetylene [Hyman and Arp, 1992].

Although nitrification is considered to be sensitive to excess ammonia and nitrite, Blouin *et al* [1989] have reported a complete oxidation of very high

concentrations of ammonia nitrogen in farm wastes in 5 days to nitrite, and another 10 days were needed for oxidation of all nitrite to nitrate. In the present study, the addition of ammonium was found to have a positive influence on nitrification rates with maximum stimulation occurring at 100 µM ammonium (159±21 nM NO_3^- h^{-1}). Though denitrification rates retarded in 50µM ammonium amendment, higher amendments stimulated the reduction of nitrate. Unlike that of ammonium, addition of nitrite had a very strong negative impact on nitrification rates. Even additions of lower concentrations brought down the nitrification rates by an order of ten. Higher additions of nitrite had a stimulatory effect on nitrite reduction. The addition of 50µM nitrate triggered the increase of nitrification rates by almost double to reach ~65 nM NO_3^- h^{-1}, however on prolonged incubation the rates came down to ~43 nM NO_3^- h^{-1}. All the nitrate enrichments facilitated the proportional increase of denitrification rates in the system, the increase as compared to the controls were from ~18 to 60 nM NO_3^- h^{-1}. At a concentration of 2-3 mM, ammonia will cause 50-90% inhibition in the rate of oxygen generation by any algal population. Un-ionized ammonia penetrates freely through the cell membrane and, unless kept below ~2 mM, reaches intracellular concentrations which strongly inhibit photosynthesis and oxygenation. This can be interrupted only by enhancing nitrification [Abeliovich, 1983].

The majority of nitrogen fertilizer added in agricultural practice is in the form of ammonia, which is adsorbed by the soil particles and then slowly released. However, in the presence of nitrifiers, ammonium is also rapidly oxidized to nitrate which is then lost either through leaching to groundwater

(polluting aquifers) or to surface waters (polluting lakes and rivers) or it is oxidised to nitrous and nitric oxides. Under anaerobic conditions, if electron donors are available in sufficient concentrations, nitrates may be lost by their reduction to dinitrogen. Therefore, direct measurements of nitrification potential, number of nitrifiers, and actual nitrification rates, in coastal ecosystems are needed for accurate estimates of nitrogen fluxes through these habitats. In the present study, in contrast to expectations, the addition of fertilizers only marginally increased the nitrification rates in the system (~25 nM NO_3^- h^{-1}). Addition of 1% w/w had a profound influence on triggering denitrification rates in the system to reach values close to 80 nM NO_3^- h^{-1}.

The addition of dissolved organic carbon (glucose) had a negative impact on nitrification rates while it supported enhanced denitrification rates. Nitrification rates were found to decrease with increasing dissolved organic carbon to reach very low values of ~3 nM NO_3^- h^{-1} at 50 mgC/l amendment. Probably, the addition of dissolved organic carbon into the system resulted in enhanced heterotrophic activity which resulted in enhanced competition for ammonium between the nitrifiers and other heterotrophs. Similar observations have been made by Strauss and Lamberti [2000] in stream sediments.

Contamination of mangrove soils with liquid hydrocarbons is yet another aspect of anthropogenic activity. Addition of liquid hydrocarbon had tremendous negative impact on nitrification. An addition of 10 and 50 mg/g retarded nitrification rates to as low as ~1 nM NO_3^- h^{-1} while 100mg/g was found to block nitrification completely. The same was also true for denitrification rates but the

extent of inhibition was much lower. Unlike the present report, Drozd [1976] had demonstrated that hydrocarbons are metabolized by a wide range of chemolithotrophic ammonia oxidizers [Hyman and Wood 1983; Jones and Morita 1983; Voysey and Wood 1987], apparently due to the non-specificity of ammonia monoxygenase. Other hydrocarbons were also co-metabolized by ammonia oxidizers. Ethylene is oxidized by *N. europaea* to ethylene oxide, a reaction sensitive to inhibitors of ammonia oxidation. Ethylene oxide is also further metabolized [Hyman and Wood 1984]. Hyman *et al* [1988] also demonstrated that n-alkanes (C~-C8) were oxidized to their respective alcohols, with increasing rates from C1 to C4.Halogenated hydrocarbons were also degraded by ammonia oxidizers Vannelli *et al* [1990].

Much is known about the possible toxicity of pesticides to microorganisms growing in laboratory media and to microbial populations and communities in soils. For example, the effects and fate of propoxur [Kuseske *et al*, 1974; Gupta *et al*, 1975], dichlorvos [Ballington *et al*, 1978], chlorpyrifos [Miles *et al*, 1979] and carbaryl [Rodriguez and Dorough, 1977] in soil have been investigated. Butcher *et al* [1977] suggested that chlorpyrifos enhanced algal blooms in pond water. Studies of the effects of a number of pesticides on nitrification in soil have shown some inhibition at high concentrations (usually greater than 50 ppm) of the test compounds [Kuseske *et al*,1974; Gupta *et al*, 1975]. In the present study, the pesticide chloropyrifos was found to have a marginal stimulatory effect on nitrification in both amendments of 10 and 50ppm. As was the case in earlier reports concentrations above 50ppm retarded nitrification rates to almost 13 nM

NO_3^- h^{-1}. The case was almost different for the denitrification mechanism, addition of chloropyrifos at concentrations of 50 and 100ppm retarded denitrification rates in the system to ~ 12 and 8 nM NO_3^- h^{-1}.

Schoberl and Engel [1964] evaluated the effect of dissolved oxygen concentration by observing dissolved oxygen uptake rates as a function of its concentration. They found that the growth rate for Nitrosomonas was independent of the dissolved oxygen concentration above 1 .0 mg/l, and that for Nitrobacter, growth rate was independent above 2 .0 mg/l. Limiting amounts of dissolved oxygen (concentrations below 2 mg/l) inhibit nitrification and cause nitrite accumulation or nitrous and nitric oxide production [Goreau *et al*, 1980; Painter, 1986]. Ammonia oxidizing bacteria are the key functional group in removing ammonium from wastewaters. Knowledge of the effect of oxygen on nitrification and nitrifying populations has economic importance since aeration of activated sludge is one of the most costly items in the operation of a wastewater treatment plant [Painter, 1986]. In environments with high inputs of ammonium, such as wastewaters, bio-oxidation of this substrate increases the oxygen uptake and lowers the pH. Such modifications of the environment not only affect the production of nitrite and nitrate but can also select a different nitrifying community that is perhaps specialized for these new conditions. Nitrification does occur in extreme environments that pure cultures of nitrifiers cannot tolerate [Bock *et al,* 1986]. The effects of extremely high dissolved oxygen concentration have received less attention than low dissolved oxygen concentrations. Okun [1949] and Haug and McCarty [1972] investigated high dissolved oxygen concentration

and report no adverse effect for dissolved oxygen concentrations of 33 and 60 mg/l respectively. Since there was a lacuna on the understanding of the impact of elevated oxygen levels in the system on nitrification, differential aeration experiments were conducted which yielded results in favor for nitrification. Though both the aeration strategies could lower the ammonium levels by nitrification, the pulse mode incubations were found to have a better effect compared to the continuous mode. This could mean that in continuous mode there could be a possibility of supersaturation of dissolved oxygen which could be limiting nitrification.

Chapter 6.
Summary and Conclusion

The thesis entitled 'Benthic nitrification in mangrove ecosystems' deals with the oxidative pathway, nitrification in mangrove sediments.

The highly productive mangrove environments are an ecological asset and an economic resource. Being ecologically sensitive and any loss of these buffer zones could have severe impacts on several biogeochemical cycles. The biogeochemical cycle of nitrogen being very complex could be one of the most affected.

Nitrification occupies a central position within the global nitrogen cycle. It is the pre dominant process converting reduced inorganic nitrogen to its oxidized form. The conditions in sediment due to its low oxygen content pose a challenge for nitrifiers which are primarily aerobic. Despite the importance of nitrification, only a few studies have explored the factors regulating this process in mangroves and no single set of factors has emerged consistently as the regulator of nitrification rates.

Hence, the aim of the present study is to understand the principle factors influencing nitrification rates in mangrove ecosystems. In order to address this aim, the study had the following objectives.

- to quantify the abundance of nitrifying populations
- to identify the nitrifiers at cellular and molecular level
- to delineate their trophic status
- to quantify nitrification rates and understand the influence of environmental parameters.

To fulfill the above objectives, both field observations and laboratory experiments were carried out. The observations covered a one year period at Divar influenced by ferromanganese ores mining (experimental site) and Tuvem (control site), relatively pristine and free from mining influence. The experimental site at Divar facilitated the study on the effect of metals on benthic nitrification.

Sediment cores were analyzed at 2 cm intervals up to 10 cm. Ambient physico-chemical parameters like salinity, dissolved oxygen, sediment temperature, pH and Eh were measured along with pore water ammonium, nitrite and nitrate. Bulk sediment properties like total organic carbon, iron and manganese were measured using standard techniques. Nitrification rates were measured by ^{15}N stable isotope enrichment technique.

Total and viable microbial counts, general heterotrophs, and nitrifiers were enumerated in all the sections of the core. Representative cultures of nitrifying bacteria were checked for their activity (ammonium oxidation and nitrite oxidation) in both inorganic and organic nitrifying media. Metabolic profiles were obtained for these cultures on BIOLOG plates; growth and ammonium/nitrite oxidation kinetics of some of the potential isolates were recorded for a period up to 120hrs. Isolates showing high activity were identified by both biochemical and molecular techniques.

Experiments were conducted to quantify the effect of various abiotic factors on key nitrogen conversions. Differential aeration, amendments of ammonium, nitrite, nitrate, dissolved organic carbon, liquid hydrocarbons,

fertilizers and pesticides were used to understand environmental and anthropogenic impacts on nitrification and the nitrifier population.

Salient results:

1. Benthic nitrification rates at the control site was 9.2 ± 0.8 nM g sediment^{-1}h^{-1}, while at the experimental site it was 8.9 ± 0.5 nM g sediment^{-1}h^{-1}. Nitrification rates peaked at both the sites during the pre-monsoon season. There was no significant down core variation in nitrification rates at either of the sites.
2. The concentration of iron and the abundance of autotrophic nitrifiers significantly correlated with nitrification rates at the experimental site, while at the control site organic carbon were important. Even though the organic carbon: dissolved inorganic nitrogen ratio is high there was no relation between organic carbon and nitrification rates for the pre and post-monsoon season indicating that organic carbon is either unlimiting or dominated by the recalcitrant fraction. It is therefore inferred that both the quality of organic carbon and quantity of iron govern nitrification rates in these mangrove swamps.
3. The control system evolved from a 'Low Organic Carbon: Dissolved Inorganic Nitrogen – Low nitrification system' in the monsoon season to a 'High Organic Carbon: Dissolved Inorganic Nitrogen – High nitrification system' in the pre-monsoon season with the post-monsoon season representing a transition period where the ratio gradually increased.

4. The total microbial counts in these sediments ranged from 10^{9-10} cells g^{-1} sediment while the viable fraction was 10^{8-9} cells g^{-1} wet sediment. FISH (Flourescence *in situ* Hybridization) analyses for eubacterial abundance at both the sites showed that the population in sediments ranged from 10^{9-10} cells g^{-1} wet sediment. The β proteobacterial ammonia oxidizing and *Nitrobacter* population in the sediments ranged from 10^{2-3} cells g^{-1} wet sediment.
5. At both the sites, the abundance of heterotrophic nitrifiers by MPN was an order more than their autotrophic counterpart which was ~10^2 cells g sediment^{-1}. The abundance of retrievable nitrifers on modified Winogradky's plates at both the sites ranged from 2.3 to 3.3 x 10^2 cells g wet sediment^{-1}.
6. Seventy one percent of the 112 strains isolated were tested positive for nitrification, of which 55% were ammonium oxidizers, 25% were nitrite oxidizers and 20% were able to oxidize both. Fifty eight percent of the isolates were able to nitrify only in presence of organic carbon which encompass all the nitrifiers that were able to oxidize both ammonium and nitrite.
7. Most of the prominent isolates (8/15) were identified by both biochemical and molecular techniques as *Pseudomonas, Aeromonas* and *Alcaligenes*. 16SrRNA sequencing also established the identity of well known nitrifiers like *Nitrosomonas* and *Nitrobacter*. This study shows that heterotrophic nitrification could be more ubiquitous and significant in mangroves.

8. During the pre-monsoon season, the sediments at the experimental site fall under 'moderately to strongly contaminated' category with respect to iron and manganese enrichment. However, the quality of the sediment gets restored during the monsoon and post-monsoon seasons. The nitrifiers apart from regulating ammonium work in conjunction with heterotrophs regulating the availability of dissolved metal ions.
9. Laboratory experiments on natural and anthropogenic factors regulating nitrification rates show that ammonium and dissolved oxygen were the two critical factors whose concentration had a positive impact. Dissolved organic carbon and nitrite had a retarding effect on this process. Surprisingly, pesticide stimulated at lower concentration, while at higher concentrations they were inhibitory. Liquid hydrocarbons and fertilizers were inhibitory to nitrification process at all the concentrations tested.

Thus, heterotrophic nitrification was found to be ubiquitous in the mangrove sediments. Well known heterotrophs like *Pseudomonas*, *Alcaligenes*, *Aeromonas* and *Janthinobacterium* also participate in this process, which was thought to be exclusively governed by autotrophic nitrifiers. Though mangrove systems are carbon rich, it is the degree of lability that negatively affects the nitrification process. At the control site it was the extent of recalcitrance of organic carbon, while at the experimental site it was iron and autotrophic nitrifiers that positively controlled nitrification.

Nitrification modulates ammonium concentrations which otherwise could tend to build-up to toxic levels in these suboxic systems. The process also ensures mobilization of metals like iron and manganese, thus increasing their bioavailability.

The overall assessment is that, though the Mandovi estuary is under the influence of ferromanganese ore mining, the sediments of the adjoining mangroves were comparatively less contaminated. Most importantly, it regulates nutrient flux into coastal waters. This study highlights the trophic nature of nitrifiers prevailing in mangrove sediments thus enabling efficient management of these systems.

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Annexure II

Available online at www.sciencedirect.com
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Marine Pollution Bulletin 54 (2007) 1427–1433
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Bacterial contribution to mitigation of iron and manganese in mangrove sediments

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Abstract

The Mandovi and Chapora are two tropical estuaries lying in close geographic proximity on the west coast of India. Seasonal changes in down core variation of Fe, Mn and Total Organic Carbon (TOC) in the mangrove sediments adjoining these estuaries were studied to assess their influence on some of the representative benthic bacteria belonging to heterotrophic and autotrophic groups. Heterotrophic bacteria (HB) cultured on different nutrient concentrations (0.01%, 0.1% and 25%) together with nitrifiers (NtB; representating autotroph) were chosen to assess the influence of the above-mentioned abiotic parameters on the former. The experimental site located along the Mandovi is under the influence of extensive ferromanganese ore mining, while the control site at Chapora is relatively free from such influences. Geoaccumulation index computed for Mandovi showed that sediments (0–10 cm) were 'uncontaminated to moderately contaminated' by Fe during the pre monsoon and monsoon seasons, while in the post monsoon season the 4–10 cm fraction was almost completely restored from contamination. Similar computations for Mn showed that in pre monsoon, sediments fell in the 'moderately contaminated' and 'moderately to strongly contaminated' categories, while in the monsoon and post monsoon seasons all the sections were 'Uncontaminated'. The difference observed in correlation between Fe and Mn with the various fractions of heterotrophs and nitrifiers indicated that though these two elements shared a similar chemistry in the environment, microbes involved in biogeochemical processes might prefer them differentially. The relationship between TOC and HB enumerated on 0.01% dilute nutrient agar remained at $r = 0.50$, $p < 0.05$ throughout the year. Hence, it could be apparently linked to their preferred concentration of organic carbon requirement. A relationship of $r = 0.61$, $p < 0.01$ between manganese concentration and heterotrophs recovered on different strengths of nutrient agar is suggestive of their response to the metal enrichment. They could thus contribute towards maintaining the level of Mn at par with reference levels at Chapora. A positive correlation between Mn with NtB ($n = 10$, $p < 0.05$, $r = 0.58$) at the experimental site during the non-monsoon months is suggestive of the latter's contribution to regulation of the metal concentration in the sediment probably through anaerobic nitrification at the expense of manganese. The study therefore supports our hypothesis that both autochthonous autotrophs and heterotrophs work in tandem to mitigate concentration of Mn and related metals in mangrove sediments.

Keywords: Iron; Manganese; Mangroves; Heterotrophs; Nitrifiers; Geoaccumulation

1. Introduction

The Mandovi and Chapora are two tropical estuaries lying in close geographic proximity to each other on the west coast of India, but the latter is relatively distanced from pollution. The hydrological characteristics of these two estuarine systems are governed by the monsoon regime. The physical characteristics of the Mandovi and Chapora estuaries have been described earlier (Varma and Rao, 1975; Varma and Cherian, 1975; Murthy et al., 1976). The estuarine channel of the Mandovi is used to transport large quantities of ferromanganese ores from mines located upstream to the Marmagao harbour (Arabian Sea), while the Chapora is free from movement

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0025-326X/$ - see front matter © 2007 Published by Elsevier Ltd.
doi:10.1016/j.marpolbul.2007.05.023

of ferromanganese ore bearing barges. Lush mangrove vegetation fringes both of the estuarine systems. Though the impacts of iron ore processing on the surface sediments of the Mandovi estuary have been documented earlier by Alagarsamy (2006), its influence on the benthic bacterial flora is sparsely understood.

In this study, an attempt has been made to understand the downcore distributory patterns of heterotrophic and autotrophic bacteria together with total Fe and Mn during monsoon and non-monsoon periods. Retrievable heterotrophic bacteria were enumerated on various nutrient strengths to estimate the various fractions requiring different carbon and nitrogen requirements. Nitrifiers were enumerated as a representative autotrophic community as they are one of the most important physiological groups in this ecosystem responsible for converting ammonia to its oxidized forms.

Geoaccumulation index (I_{geo}, Müller, 1979) was used to assess the impact of mining on the mangrove sediments. Although I_{geo} was originally devised for use with global standard shale values as background metal levels, Rubio et al. (2000) have shown the use of regional background values to give more appropriate results. In this study, I_{geo} has been calculated using regional metal concentrations in Chapora mangrove sediments. A comparison between the Mandovi and Chapora mangrove sediments was therefore made to assess the probable influence of mining on bacterial groups in this region. We hypothesize that bacterial populations in the mangrove sediments could play an important role in preventing the concentration of the metal species from accumulating.

2. Materials and methods

2.1. Site description and sampling procedure

Sediment cores were collected from fringing mangrove forests along the Chapora and Mandovi estuaries. The control site in the Chapora estuary was located at Tuvem, whereas the Divar mangrove ecosystem was selected as the experimental site along the Mandovi estuary (Fig. 1). The experimental site is under the influence of ferromanganese ore mining. Samples were collected during the months of April (Pre Monsoon), July (Monsoon) and October (Post Monsoon) of 2005 using a PVC hand-held sediment corer. Sub samples were taken at 2 cm intervals from the surface to 10 cm by carefully sectioning the core in the lab.

2.2. Chemical analyses

Organic carbon was determined by the wet oxidation method with a precision of 0.01% (El Wakeel and Riley, 1957). Sub samples for metal analyses were dried at 60(±2) °C for 48 h and disaggregated in an agate mortar before chemical treatment for Fe and Mn analysis. For each sample, a known quantity (~0.2 g) of sediment was digested in a teflon vessel with a solution (10 ml) of concentrated HF (48% GR; Merck), HNO_3 (69% GR; Merck) & $HClO_4$ (35% GR; Merck) in a ratio of 7:3:1. The sediment was then dried on a hot plate in a fume hood chamber.

An aliquot of 5 ml of the above acid mixture was added and dried on the hot plate for 1 h. Further, 2 ml of concentrated HCl (35% GR; Merck) was added, followed by 10 ml of HNO_3 (69% GR; Merck). The residue was warmed and then transferred to a clean, dry standard flask to make a final volume of 100 ml with double distilled water. The detailed procedure of sediment digestion is given in Balaram et al. (1995). Trace metal concentrations (Fe and Mn) were measured using a flame atomic absorption spectrophotometer (AAS, PerkineElmer Model 5000). The accuracy of the analytical procedures was assessed using the certified reference material MAG-1 (USGS) that yielded results within the reference value range (Flanagan, 1967, 1976).

2.3. Microbial analyses

Sediment core was sectioned at 2 cm intervals in sterile conditions to obtain representative samples at 0–2, 2–4, 4–6, 6–8 and 8–10 cm depths. Sub samples of approximately 5 g wet weight sediment were sampled using sterile syringe cores. The sub samples were transferred to 45 ml of full strength sterile seawater (10^{-1} dilution). Tween80 (50 μL) was added and the mixture was sonicated at 40 mHz for 10 s. Serial dilutions of the sediment samples were made in autoclaved seawater to yield dilutions from 10^{-1} to $10^{-6/7}$.

Medium for the isolation of heterotrophic bacteria (HB) was prepared using various nutrient strengths of 0.01, 0.1 and 25%, which correspond to 0.01%, 0.1% and 25% nutrient broth + 2% agar, respectively. A concentration of 100% corresponds to 8 g nutrient broth (HiMedia Laboratories Pvt. Ltd., Bombay, India) per 1000 ml seawater. About 100 μl from 10^{-2} dilution was plated onto the medium. The plates were incubated for 15 days at room temperature. Bacterial counts in the form of colony forming units (CFU) formed on the medium were recorded after a 15-day incubation period at 28(±1) °C. Dry weight of the sediment used for the dilutions was determined by drying the filtered sediment in an oven at 60 °C for 48 h.

Nitrifiers (NtB) were enumerated by the most probable number (MPN) method of Alexander and Clark (1965). Nitrifying media (seawater amended with NH_4Cl of 2 mM final concentration) was distributed in 5 ml quantities in 15 ml screw capped tubes. From each dilution ranging from 10^{-1} to $10^{-6/7}$, 500 μl was inoculated in triplicate in the nitrifying media until the inoculation from the highest dilution into the culture tubes yielded negative results. The culture tubes were incubated in the dark for a period ~60 days at 28(±1) °C. After incubation, the tubes were tested for the presence of NO_2^- and/or NO_3^-. The combinations of positive and negative tubes were scored and MPN was assessed from McCready's table (Rodina, 1972).

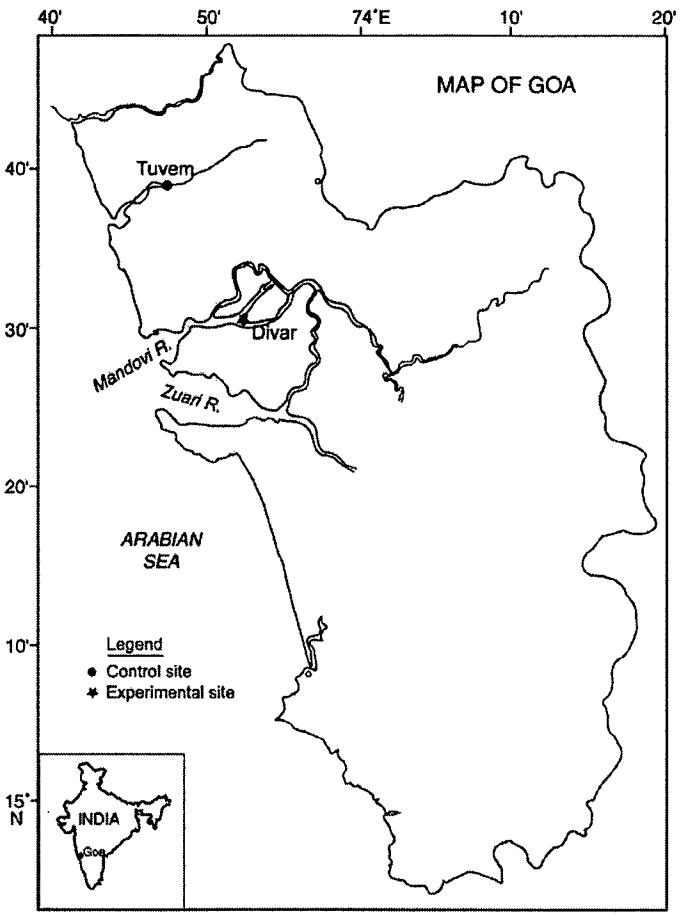

Fig. 1. Location of sampling sites in the Chapora and Mandovi estuary.

Statistical analysis was carried out using Pearson's Correlation Coefficient in order to explore the possible associations existing between different variables. The analysis was done using Microsoft Excel 2000. The bacterial parameters were normalized by log transformation before analyses.

3. Results and discussion

3.1. Down core variation and index of geoaccumulation for iron and manganese

Figs. 2a, b and 3a, b show the down core variation of Fe and Mn during pre monsoon, monsoon and post monsoon seasons. The overall range in sedimentary Fe values ranged between 8% and 12.6% at the experimental site, while it ranged between 4.7% and 9.3% at the control site. The Mn values varied from 0.06% to 0.52% at the experimental site and 0.05–0.48% at the control site. The values reported

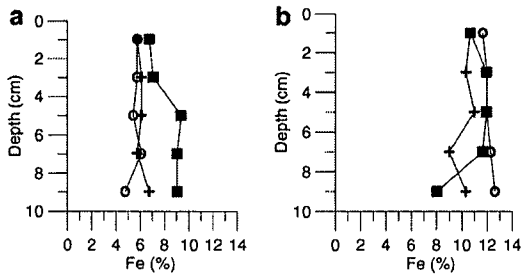

Fig. 2. Down core variation in Fe. (a) refers to the control site while (b) refers to experimental site (○ – Pre Monsoon, + – Monsoon and ■ – Post Monsoon).

in the present study are comparatively higher to those reported by Ray et al. (2006) from the Godavari estuarine mangrove ecosystem on the eastern coastline of India. These authors reported that the average sedimentary Fe

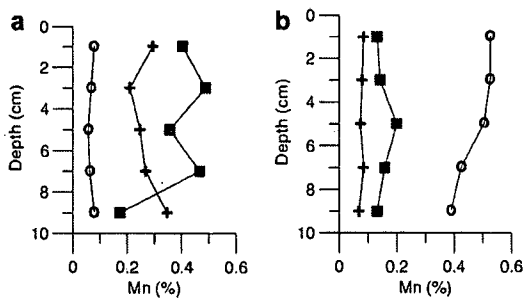

Fig. 3. Down core variation in Mn. (a) refers to the control site while (b) refers to experimental site (O – Pre Monsoon, + – Monsoon and ■ – Post Monsoon).

and Mn values were 0.004% and 0.001%, respectively. Studies by Alagarsamy (2006) showed that the concentrations of Fe varied from 2.2% to 49.7% on the surface sediments of the Mandovi estuary, while the concentration of Mn ranged below detection limit to 1.61%. Though reports from the Mandovi estuary (Alagarsamy, 2006) showed that metal concentrations were generally low during monsoon, compared to the pre and post monsoon seasons, at the adjoining mangrove sediments, they were found to vary. In the present study, the highest accumulation of Fe was observed during pre monsoon, while the values in the monsoon and post monsoon seasons were lower and comparable. The variation of Mn values was similar to observations made by Alagarsamy (2006) in the surface sediments of the adjoining Mandovi estuary.

Irrespective of the depth, the experimental site showed an enrichment of Fe > 100% of the control site during pre monsoon. Percentage of enrichment has been calculated using the formula:

If $a > b$:

Percentage of enrichment $= (a - b)/b * 100$

If $b > a$ under washout conditions:

Percentage of enrichment $= (b - a)/a * 100$

where a is the concentration of Fe/Mn at experimental site and b is the concentration of Fe/Mn at control site.

In general, enrichment increases with depth to reach values >165% at an 8–10 cm interval during pre monsoon. During monsoon, the general enrichment pattern is reversed with the highest enrichment (85.6%) at 0–2 cm. Moreover, observations from statistical analysis indicate that there is no significant correlation between Fe and Mn in the monsoon months, neither at the control nor at the experimental site. However, a direction is suggested in the relationship. It is negative at the experimental site and positive at the control site, perhaps suggesting that under a lower concentration of iron, manganese concentration tends to increase. With a higher concentration of iron up to 12–13%, this trend changes, suggesting that the increase of both of the elements do not get coupled after a threshold.

During post monsoon, it could be observed that, though the trend in down core variation fairly resembled the monsoon season, the magnitude fell sharply to show negative enrichment. It was interesting to note that, although the control site was free from the influence of mining, the experimental site showed negative Mn enrichment in all sections during monsoon and post monsoon. This could be due to enhanced fresh water supply to the Mandovi compared to the Chapora. These observations are contrasted by very high enrichment (393–773%) of Mn at the experimental site during the pre monsoon season, especially in the depth range of 4–6 cm. A positive relation between Fe and Mn during the non-monsoon months ($r = 0.64$, $p < 0.02$, $n = 10$) at the control site and the absence of such a relation at the experimental site showed that, though the chemistry of Fe and Mn are closely related, they could be differentially preferred by organisms, which in turn is influenced by the prevailing environment. Alternatively, the concentration of iron is too high to warrant any bacterial dependance.

The geoaccumulation index (I_{geo}) was originally defined by Müller (1979) for metal concentrations in the <2 μ fraction and developed for global standard shale values, which is expressed as follows:

$$I_{geo} = \log_2(C_n/1.5B_n)$$

where C_n is the measured concentration of metal 'n' in the sediment and B_n is the background value for metal 'n'.

The factor 1.5 is used for the possible variations of the background data due to lithological variations.

The choice of the background value plays an important role in the interpretation of geological data. I_{geo} has been widely utilized as a measure of pollution in freshwater (e.g. Müller, 1980; Singh et al., 1997; Kralik, 1999) and marine sediments (e.g. Stoffers et al., 1986; Bryan and Langston, 1992; Dickinson et al., 1996). Geoaccumulation index (I_{geo}) of Fe and Mn in the experimental site (control site metal values were taken as reference values) was computed based on Müller (1979) for all the depths and seasons. The results are plotted in Fig. 4a and b.

According to the I_{geo} classification (Table 1), it could be inferred that the sediments in the depth range 0–10 cm fall in the 'uncontaminated to moderately contaminated by iron' category during the pre monsoon and monsoon season. While, in the post monsoon season, though the 0–4 cm still remains 'uncontaminated to moderately contaminated by iron', the 4–10 cm layer has recovered from Fe contamination and could be termed as 'Uncontaminated'. The contamination due to Mn is more acute than Fe during the pre monsoon season. During the pre monsoon, the 0–8 cm section falls under the 'Moderately to strongly contaminated' category, while the 8–10 cm section falls under the 'Moderately contaminated category'. All the depths fall in the 'Uncontaminated' group during the monsoon and post monsoon and hence could be assessed as free

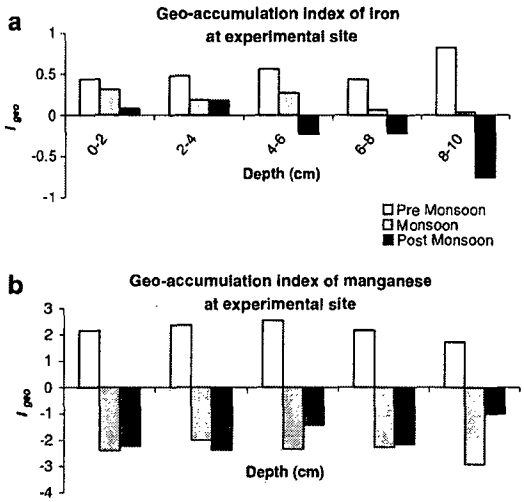

Fig. 4. (a) and (b) I_{geo} values of Fe and Mn, respectively, for the experimental site at Divar. The y-axis scale and legend to (b) is the same as that of (a).

Table 1
Description of sediment quality by geoaccumulation index (Müller, 1979)

I_{geo} Value	I_{geo} Class	Description of sediment quality
>5	6	Extremely contaminated
4–5	5	Strongly to extremely strongly contaminated
3–4	4	Strongly contaminated
2–3	3	Moderately to strongly contaminated
1–2	2	Moderately contaminated
0–1	1	Uncontaminated to moderately contaminated
<0	0	Uncontaminated

from the Mn pollution. These observations could again suggest that, though the elements are closely related, the biogeochemical cycling of Mn could be more efficient and rapid when compared to Fe in the mangrove sediments. Moreover, the mangrove ecosystems play a buffering role by reducing the enrichment levels of Fe and Mn in the sediments. The overall assessment could be that, though the Mandovi estuary is under the influence of ferromanganese ore mining with significant impact on the estuarine sediments, the sediments of the adjoining mangroves are comparatively less contaminated, but when strongly contaminated, could be self-regulatory and recover in the time scale tested.

3.2. Total organic carbon (TOC) and bacterial trophic structure

Down core variability in TOC for the different seasons are illustrated in Fig. 5a and b. TOC varied from 0.02 to 1.8% in the control site, whereas at the experimental site, it varied from 0.12 to 2.74%. Lowest TOC values were recorded during the post monsoon season at the control

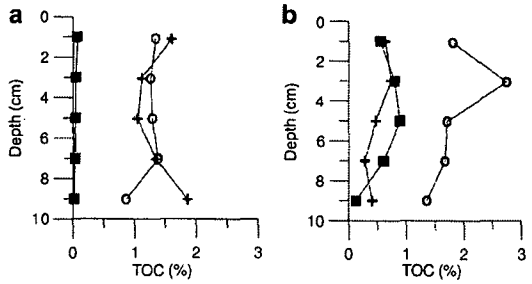

Fig. 5. (a) Control site and (b) Experimental site show the down core variation in Total Organic Carbon (TOC) in sediments. (○ – Pre Monsoon, + – Monsoon and ■ – Post Monsoon).

site and the highest values were recorded during the pre monsoon at the experimental site.

TOC varied with different fractions of retrievable heterotrophic bacteria (Figs. 6 and 7). In the pre monsoon season, TOC and HB retrieved on all three strengths of nutrient agar showed a positive relationship at the control site. However, only on 0.01% HB was the correlation significant at $r = 0.80$, $p < 0.05$. Despite the season, it could be observed that at the control site the abundance of HB retrieved on 0.01% dNA is limited ($r = 0.50$, $p < 0.05$, $n = 5$) by the availability of organic carbon. The extent of limitation due to carbon availability for HB retrieved on 0.01% ($r = 0.89$, $p < 0.01$, $n = 5$) and 0.1% dNA ($r = 0.96$, $p < 0.001$, $n = 5$) is maximum during the monsoon. Though there is a heavy organic flux into the estuary during the monsoon, the higher dependency of HB on TOC could be due to the lower lability of the organic carbon. Reduced residence time during monsoon could be responsible for the delayed degradation of complex organic matter into simpler labile forms. Short residence time could also be due to the large seasonal influx of freshwater into the estuary, with unchanged tidal amplitude over large distances (Unnikrishnan et al., 1997). As in the control site, as well as at the experimental site, TOC showed a positive relation with 0.01% HB ($r = 0.72$, $p < 0.1$) during pre monsoon. There does not exist any significant relationship between the 25 and 0.1% fraction of HB with TOC at the experimental site during the pre monsoon and monsoon seasons.

The scenario in post monsoon is very different from the preceding seasons at both the control and experimental sites. It was observed that the 0.01% dNA fraction of HB had a positive relationship of $r = 0.889$ ($p < 0.02$) and $r = 0.79$ ($p < 0.05$), respectively, with TOC. The other fractions bore an insignificant, negative relationship. However, it is suggestive of enhanced utilization compared to production of organic carbon. Though the estuarine environment is eutrophic with considerable organic loading, a persistent relationship between TOC and the 0.01% dNA fraction of HB could be due to a dominant fraction of heterotrophs, which constantly require an optimal organic carbon concentration of 0.01%. Similar observations have

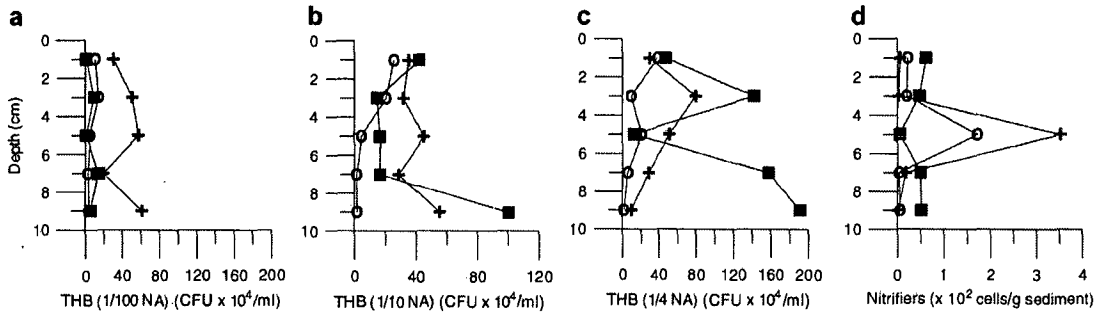

Fig. 6. Control site shows the down core variation of bacterial groups with different nutritional requirements (○ – Pre Monsoon, + – Monsoon and ■ – Post Monsoon).

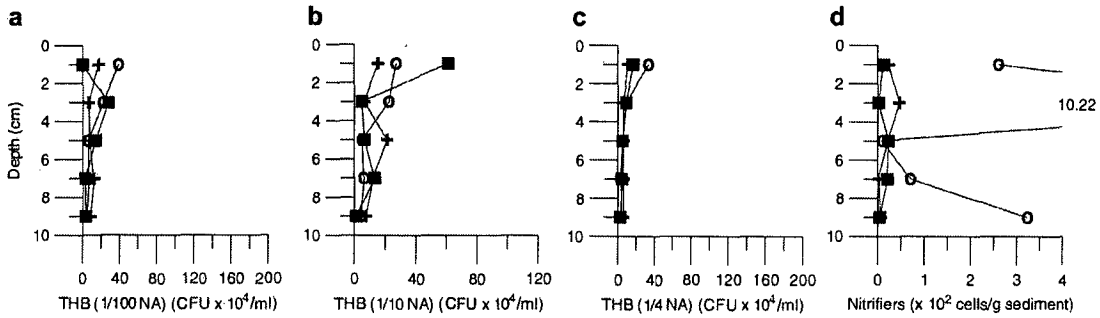

Fig. 7. Experimental site shows the down core variation of bacterial groups with different nutritional requirements (○ – Pre Monsoon, + – Monsoon and ■ – Post Monsoon).

been made earlier by Goltekar et al. (2006) in the Mandovi estuary, which indicate that bacteria from relatively more dynamic estuarine systems seemed less resilient compared to the coastal and offshore populations, as they were best retrieved only on 1% nutrient strength.

3.3. Relation of sediment geochemistry with bacterial trophic status

It was interesting to note that TOC related positively to Fe ($r = 0.67$, $p < 0.01$, $n = 15$) and Mn ($r = 0.92$, $p < 0.001$, $n = 15$) at the experimental site, whereas the relationships were negative at the control site ($r = -0.72$, $p < 0.001$, $n = 15$ for Fe and $r = -0.51$, $p < 0.05$, $n = 15$ for Mn). These relationships could imply that at the experimental site there is considerable extraneous input of TOC favoring the accumulation of Fe and Mn. Wangersky (1986) has reported that coatings of organic matter prevalent in fine-grained sediments bind a variety of trace elements. Enrichment of Mn in the pre monsoon and its subsequent removal in the monsoon indicate that Mn turnover time could be much less, as it is actively removed both by physical processes and biogeochemical sequestration. It could be observed that at the experimental site NtB are higher at the depth ranges of 2–4 cm and 8–10 cm. Irrespective of the season, a positive correlation between Mn and NtB ($r = 0.61$, $p < 0.01$, $n = 15$) at the experimental site suggests that Mn could be used actively as a co-factor for the ammonia monoxygenase enzyme. More importantly, it could also serve as an alternate terminal electron acceptor in anaerobic respiration (Hulth et al., 1999). This relationship disappeared when the Mn levels decreased during the monsoon season and was re-established during the post monsoon season ($r = 0.62$, $p < 0.02$, $n = 10$) when the Mn levels increased. Absence of a significant relationship between Fe with various fractions of heterotrophs and nitrifiers at both the control and experimental sites suggested that Fe is present in excess and perhaps non-limiting. Moreover, Mn(IV) reduction preceeds that of Fe(III) because of reduction energetics of the solid phases (Burdige et al., 1992).

The relationship between Mn and various fractions of HB indicate that the latter has a considerable influence in regulating the levels of Mn. At the experimental site, HB cultured on 0.01% dNA related to Mn during pre monsoon ($r = 0.826$, $p < 0.05$, $n = 5$) and further strengthened during the monsoon ($r = 0.968$, $p < 0.001$, $n = 5$). HB on 0.1% dNA also showed a relationship to Mn concentrations ($r = 0.951$, $p < 0.001$, $n = 5$) during the monsoon. However, during post monsoon, the variation in HB on a higher

strength of 25% nutrient agar affected the variation in Mn concentration up to 90% ($r = 0.949$, $p < 0.001$, $n = 5$). All these observations show that the different fractions of HB could respond in sequence to the Mn enrichment taking place in the pre monsoon season. These relationships demonstrate that HB are actively involved in maintaining the level of Mn on par with reference levels at the Chapora estuary.

A very strong positive correlation between NtB and TOC in the pre monsoon season ($r = 0.987$, $p < 0.001$, $n = 5$) showed that heterotrophic nitrification could be a dominant process controlling the cycling of ammonia in sediments. The potential of heterotrophic nitrification in sediments has been previously reported by Schimel et al. (1984). Furthermore, the absence of a relationship in monsoon and a negative relationship in post monsoon ($r = -0.673$, $p < 0.1$, $n = 5$) could indicate a shift from a relatively heterotrophic to relatively autotrophic mode of nitrification.

4. Conclusion

The present study shows that the heterotrophs respond to the Mn enrichment and could be active in maintaining its level and the other associated metal concentrations like that of Fe on par with the reference site. A positive correlation between Mn with NtB at the experimental site during the non-monsoon months is indicative of the latter's contribution to regulation of the metal concentration, especially of Mn in the sediment, probably through its use as a terminal electron acceptor in respiration at the expense of manganese oxide. The study therefore lends support to our hypothesis that both autochthonous autotrophs and heterotrophs work in tandem to mitigate manganese and perhaps related metals like iron in mangrove swamps. Though the Mandovi estuary is under the influence of ferromanganese ore mining with relatively higher sediment metal concentrations, the adjoining mangroves are comparatively less contaminated due to the self-regulation facilitated by the native bacterial flora.

Acknowledgements

We are thankful to the Dr. S.R. Shetye, Director, NIO, for providing facilities to carry out this work. We also acknowledge the help rendered by Dr. Maheshwari Nair, Mr. Vineesh and Mr. Martin in trace metal analysis. KPK and SOF record their gratefulness to UGC and CSIR (Government of India) for the award of Senior Research Fellowship. This is NIO contribution no. 4246.

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Estuarine, Coastal and Shelf Science 84 (2009) 419–426

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

Journal homepage: www.elsevier.com/locate/ecss

Organic carbon and iron modulate nitrification rates in mangrove swamps of Goa, south west coast of India

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ARTICLE INFO

Article history:
Received 18 February 2009
Accepted 19 July 2009
Available online 23 July 2009

Keywords:
nitrification
nitrifiers
mangroves
organic carbon
manganese
iron

ABSTRACT

Nitrification, fuelled by ammonium is the pivotal oxidative pathway to nitrogen cycling. In spite of its ecological significance, the factors regulating nitrification rates in the benthic realm remain poorly understood. The present study therefore examines some of the factors like ammonium, nitrite, nitrate, organic carbon, iron and manganese on down-core variability in benthic nitrification rates in two different mangrove ecosystems, one under the influence of ferromanganese ore mining (experiment) and the other relatively undisturbed (control). We hypothesize that besides organic carbon, iron could also influence the rate of nitrification. The study also contrasts the distributive pattern of autotrophic and heterotrophic nitrifiers in the two regions. The concentration of iron at the control site ranged from 1.1% to 15.1% while at the experimental site it ranged from 2.9% to 46%. The levels of organic carbon at control and experimental sites ranged from 0.02% to 6.9% and 0.1% to 6.5%, respectively. The nitrification rates at the control and experimental sites are comparable and ranged from 3.2 ± 1.2 to 18.4 ± 1.9 ng at-N g(sediment)$^{-1}$ h^{-1} and 2.7 ± 1.5 to 18.2 ± 0.6 ng at-N g(sediment)$^{-1}$ h^{-1}, respectively. While the abundance of heterotrophic nitrifiers at both the sites ranged from 10^{2-3} cells g^{-1} sediment, the autotrophic nitrifiers at the experimental site was higher by an order at $\sim 10^3$ cells g^{-1} sediment reflecting the relatively higher refractile nature of organic carbon at the experimental site (Straus and Lamberti, 2000). Though organic carbon and nitrification rates are similar in both the sites, the underlying mechanisms governing the processes could be different. Our studies suggest that at the control site, heterotrophic nitrifiers govern nitrification rates ($r = 0.28$, $p < 0.05$, $n = 64$) using organic carbon ($r = 0.32$, $p < 0.01$, $n = 64$). At the experimental site, nitrification was governed more by autotrophic nitrifiers ($r = 0.43$, $p < 0.001$, $n = 64$) at the expense of iron ($r = 0.47$, $p < 0.001$, $n = 64$). Therefore at the experimental site with higher load of iron, autotrophic nitrification could be more important. It is therefore inferred that both the quality of organic carbon and quantity of iron govern nitrification rates in these mangrove swamps.

1. Introduction

Nitrification is a microbial process that converts ammonium (NH_4^+) to nitrate (NO_3^-) via nitrite (NO_2^-) and occupies a central position within the global nitrogen cycle. Hence, the factors regulating this process are vital to eutrophication as well as to health concerns related to enhanced nitrate levels in aquatic ecosystems. Nitrification also serves as a significant link between nitrogen mineralization and potential nitrogen loss from the benthic system through denitrification (Seitzinger, 1990; Sloth et al., 1992). Nitrification was traditionally considered to be restricted to aerobic environments (e.g. Froelich et al., 1979), but studies (Mortimer et al., 2004) have shown that nitrification does happen in anoxic environments at the expense of elements like manganese (Mn) and/or iron (Fe). In a recent study, Krishnan et al. (2007) had suggested the possibility of anaerobic nitrification in mangroves as a mechanism to ward off the excess manganese entering the sediments as a result of pollution. Nitrification also depends on NH_4^+ regeneration rates, which in turn is positively influenced by temperature (Nixon, 1981). The presence of organic carbon (OC) compounds may diminish the rate and the yield of nitrate formed, by diverting nitrogen from the nitrifiers to the heterotrophs proliferating at the expense of easily assimilable carbon (Delwiche and Finstein, 1965). However, this depends mainly on the

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doi:10.1016/j.ecss.2009.07.015

magnitude of C:N ratio and the quality of organic compound (Straus and Lamberti, 2000). Straus and Lamberti (2000) have hypothesized that high C:N and higher levels of labile organic carbon does inhibit nitrification, whereas low C:N and higher levels of refractory organic carbon enhance nitrification rates. Moreover, though mangroves are considered to be productive coastal marine ecosystems (Qasim and Wafar, 1990), nutrient measurements, especially those of nitrogen which is an important factor sustaining this productivity has been sparse (Dham et al., 2002). Hence, in order to understand some of the important factors regulating nitrification rates (N_{rate}) two different mangrove sites with different ecological setup were studied (south-west coast of India, Fig. 1). We hypothesize that besides organic carbon, iron could also influence the rate of nitrification.

2. Materials and methods

2.1. Site description and sampling procedure

Sediment cores were collected from mangrove swamps along the Chapora and Mandovi estuaries. The control site (15° 38.28′ N and 73° 47.71′ E) which is relatively free from metal pollution is located at Tuvem in the Chapora estuary, while the experimental site which is under the influence of ferromanganese ore mining is exposed to enrichment of metal ores of Fe and Mn is located at Diwar (15° 30.42′ N and 73° 52.28′ E) in the Mandovi estuary (Fig. 1). Monthly sampling was carried out at both the control and experimental sites from April 2005 to April 2006 using a PVC hand-held sediment corer. Sediment core was sectioned at 2 cm intervals in sterile conditions to obtain representative samples at 0–2, 2–4, 4–6, 6–8 and 8–10 cm depths.

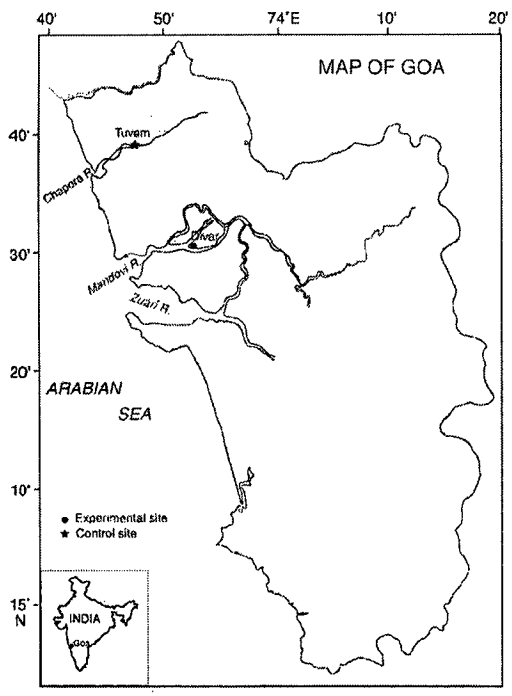

Fig. 1. Location of sampling sites in the Chapora and Mandovi estuary.

2.2. Pore water extraction and chemical analyses

After sectioning the cores, each fraction was made into slurry with a known volume of saline and then loaded separately into centrifuge tubes. The tubes were spun at low RPM (5000) at 4 °C for 10 min. The water was then carefully siphoned out into a pre-cleaned 100 ml polyethylene bottle and allowed to stand for 15 min in cold conditions in order to sediment out the coarse particles. Further, the diluted pore water was filtered on GF/F and then subsequently on 0.22 μ membrane filter. The filtrate was stored in cold for further analysis. By spinning at low temperature and RPM it was ensured that minimal disturbance was caused to the benthic organisms, which on lysis could change the pore water chemistry. NH_4^+, NO_2^- and NO_3^- were measured spectrophotometrically as described by Koroleff (1969), Bendschneider and Robinson (1952) and Wood et al. (1967), respectively. OC was determined by the wet oxidation method with a precision of 0.01% (El Wakeel and Riley, 1957). Sub samples for metal analyses were dried at 60(±2) °C for 48 h and disaggregated in an agate mortar before chemical treatment for Fe and Mn analysis. The detailed procedure of sediment digestion is given in Ref. Balaram et al. (1995). Trace metal concentrations (Fe and Mn) were measured using a flame atomic absorption spectrophotometer (AAS, Perkin Elmer Model 5000). The accuracy of the analytical procedures was assessed using the certified reference material MAG-1 (USGS) that yielded results within the reference value range (Flanagan, 1967, 1976).

2.3. Measurement of nitrification rates

N_{rate} was measured by the method of Schell (1978). Each intact section was transferred to a beaker (500 ml capacity) to which filtered estuarine water was added. Samples were then incubated with ^{15}N-NH_4Cl (10% of the ambient) in the dark for 24 h. At the end of the incubation period, samples were gently mixed and pre-screened through a 200 μm mesh net. Samples were then filtered onto Whatman GF/F filter pads (pre-ignited at 400 °C for 4 h) and 200 ml of the filtrate was recovered for the extraction of nitrite. NO_2^- in the sample was extracted as a dye (1-benzene-azo-2-napthol) by using an organic solvent. ^{15}N:^{14}N isotope ratios of the NO_2^- extracted from the incubation medium were measured by emission spectrometry using a Jasco N-150 Nitrogen analyzer. N_{rate} was calculated according to Lipschultz (1984). All measurements were done in triplicate.

2.4. Microbial analyses

Sub samples of approximately 5 g wet weight sediment were sampled using sterile syringe cores. The sub samples were transferred to 45 ml full strength sterile seawater (10^{-1} dilution). Tween80 (50 μL) was added and the mixture was sonicated at 40 mHz for 10 s. Serial dilutions of the sediment samples were made in autoclaved seawater to yield dilutions from 10^{-1} to $10^{-6/-7}$. Nitrifiers were enumerated by the most probable number (MPN) method of Alexander and Clark (1965) on both inorganic and organic media. Winogradsky's inorganic nitrifying media (seawater amended with NH_4Cl of 2 mM final concentration) was distributed in 5 ml quantities in 15 ml screw capped tubes. The organic nitrifying media was prepared by adding 0.01% final concentration of glucose to Winogradsky's inorganic nitrifying media. From each dilution ranging from 10^{-1} to $10^{-6/-7}$, 500 μl was inoculated in triplicate in the nitrifying media until the inoculation from the highest dilution into the culture tubes yielded negative results. The culture tubes were incubated in the dark for a period ~60 days at 28(±1) °C. After incubation, the tubes were tested for the presence of NO_2^- and/or NO_3^-. The combinations of positive and negative

tubes were scored and MPN was assessed from McCready's table (Rodina, 1972).

Statistical analysis was carried out using Pearson's Correlation Coefficient and Analysis of Variance in order to explore the possible associations and variances existing between/within different variables. The analysis was done using Microsoft Excel 2000. The bacterial parameters were normalized by log transformation before analyses.

3. Results

3.1. Pore water chemistry

Monthly down-core variation in NH_4^+, NO_2^- and NO_3^- at both the control and experimental sites have been illustrated in Fig. 2a, b and c, respectively. Depth compromised pre-monsoon (February–April) average of NH_4^+ at the control site was 19.1 ± 4 μg at NH_4^+-NL^{-1} pore water ($n = 25$). Similar values were encountered at the experimental site with NH_4^+ averaging 19.9 ± 2.4 μg at NH_4^+-NL^{-1} pore water ($n = 25$). However, the monsoon season (June–September) was characterized by lower levels of NH_4^+ at both the control (16.8 ± 3.6 μg at NH_4^+-NL^{-1} pore water; $n = 20$) and experimental sites (14.1 ± 2.1 μg at NH_4^+-NL^{-1} pore water; $n = 20$). Interestingly, the post-monsoon (October–January) values at the control site showed an increase in NH_4^+ accumulation (17.9 ± 7.9 μg at NH_4^+-NL^{-1} pore water; $n = 20$) as compared to the monsoon, but they still remained lesser than the pre-monsoon values. NH_4^+ accumulation at the experimental site was highest (27.8 ± 5.6 μg at NH_4^+-NL^{-1} pore water; $n = 20$) during post-monsoon season as compared to monsoon and pre-monsoon seasons. NH_4^+ showed significant monthly variability at the experimental site ($p < 0.001$, df = 12), while there was no significant variability at the control site. Similar trend was observed inter-seasonally with no variability at the control site but with a significant variation at the experimental site ($p = 1.5 \times 10^{-6}$, df = 2). When compared, the control and experiment sites differed only in the post-monsoon season ($p < 0.001$, df = 1) and this could be attributed to the variability observed at 0–2 cm ($p < 0.001$, df = 1).

Average pre-monsoon NO_2^- concentration at control and experimental sites were 2.4 ± 0.8 and 2.1 ± 0.1 μg at NO_2^--NL^{-1} pore water ($n = 25$), respectively. During monsoon season, NO_2^- values at control (2.3 ± 0.5 μg at NO_2^--NL^{-1} pore water; $n = 20$) and experimental sites (2.7 ± 0.6 μg at NO_2^--NL^{-1} pore water; $n = 20$) were comparable to their pre-monsoon counterparts. The post-monsoon nitrite levels at the control site (2.3 ± 0.6 μg at NO_2^--NL^{-1} pore water; $n = 20$) showed a similar trend as that of the other seasons,

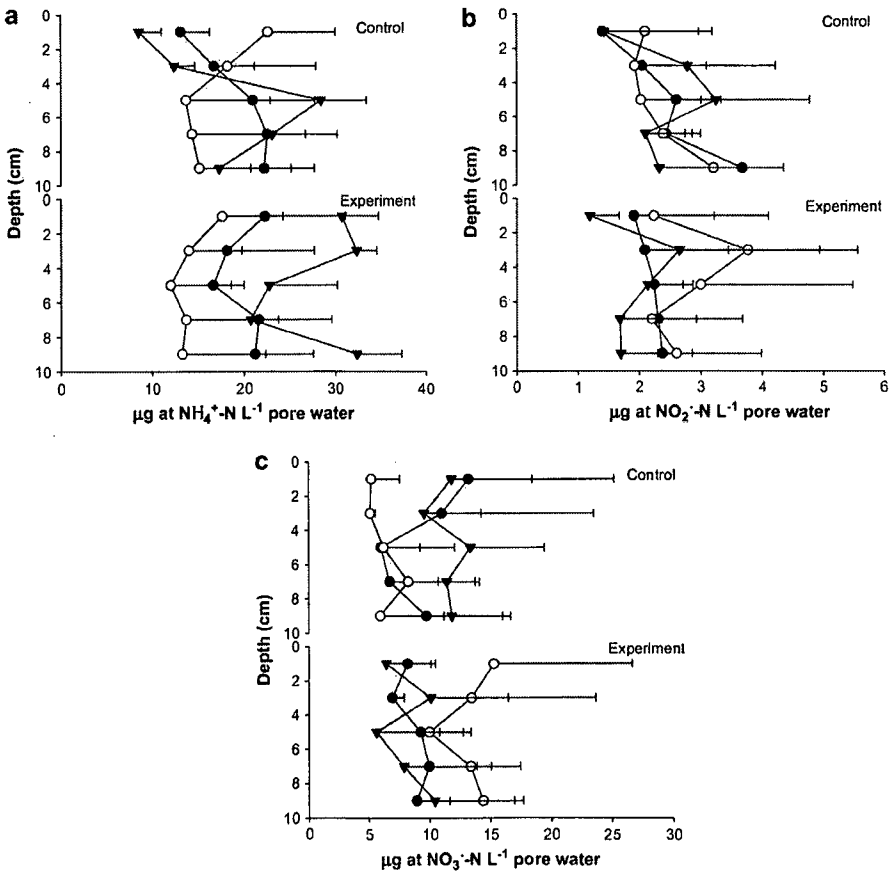

Fig. 2. Seasonal down-core variation in pore water ammonium (a), nitrite (b) and nitrate (c) at both the control and experimental sites for the pre-monsoon (●), monsoon (○) and post-monsoon (▼) seasons. Only positive standard deviation is shown in the figure.

but experimental site recorded the lowest values (1.8 ± 0.5 μg at NO_2^--NL^{-1} pore water; $n = 20$) during the post-monsoon season. NO_2^- showed a significant monthly variability at the experimental site ($p < 0.1$, df = 12) where as the control site was marked by significant down-core variability ($p < 0.01$, df = 12). Maximum down-core variation was observed during the pre-monsoon season ($p < 0.01$, df = 4). Even though the inter-seasonal variability occurred neither at the experimental nor at the control site, the bottom most layer of the core (8–10 cm) showed significant variation in NO_2^- both at the control and experimental sites.

Levels of NO_3^- at the control site were 9.3 ± 2.9 μg at NO_3^--NL^{-1} pore water ($n = 25$) during the pre-monsoon season, while the monsoon values were lower (6.1 ± 1.2 μg at NO_3^--NL^{-1} pore water; $n = 20$). In contrast, highest NO_3^- values at the experimental site (13.2 ± 2.0 μg at NO_3^--NL^{-1} pore water; $n = 20$) were recorded during the monsoons. The pre- and post-monsoon values were 8.6 ± 1 μg at NO_3^--NL^{-1} pore water; $n = 25$ and 8 ± 2.1 μg at NO_3^--NL^{-1} pore water; $n = 20$, respectively. However highest levels were recorded at the control site (11.5 ± 1.3 μg at NO_3^--NL^{-1} pore water; $n = 20$) during the post-monsoon season. NO_3^- showed significant monthly variability at both the control ($p = 2.9 \times 10^{-5}$, df = 12) and the experimental sites ($p = 4.4 \times 10^{-3}$, df = 12). Contrary to the pattern of inter-seasonal stability in NO_2^-, NO_3^- values were significantly variable at both the control ($p < 0.1$, df = 2) and experimental sites ($p < 0.01$, df = 2). There exists considerable variability at both the control and experimental sites during the monsoon and post-monsoon seasons ($p < 0.1$, df = 1).

3.2. Iron, manganese and organic carbon

Monthly down-core variation in Fe, Mn and OC at both the control and experimental sites have been illustrated in Fig. 3a, b and c, respectively. Depth compromised average values of Fe at the control site ranged from 6% to 7.4% for the entire sampling period. There was no inter-seasonal variability at the control site but there was considerable down-core variation ($p < 0.1$, df = 4) irrespective of the month. In contrast, high inter-seasonal variability was observed at the experimental site ($p < 0.001$, df = 2), with pre-monsoon showing the highest accumulation of Fe ($24 \pm 3.2\%$) followed by post-monsoon ($18.7 \pm 3.6\%$) and monsoon ($10.2 \pm 1.9\%$) seasons, respectively. Maximum disparity between the control and experimental sites was observed during the pre-monsoon season ($p < 0.001$, df = 2) while the lowest was during the monsoon ($p < 0.01$, df = 2). The post-monsoon values depicted a transitional stage with considerable variability ($p < 0.001$, df = 2).

Even though there was significant monthly variability in the accumulation of Mn at both the control ($p < 0.01$, df = 12) and experimental sites ($p < 0.01$, df = 12), there was no significant inter-seasonal variability at both the sites. In general the Mn levels remained ~0.5% at the control site throughout the sampling period, whereas at the experimental site it ranged from $1.1(\pm 0.6)\%$ in the post-monsoon to $1.7(\pm 0.6)\%$ in the monsoon season with intermediate values of $1.4(\pm 0.2\%)$ in the pre-monsoon season. Notable difference between the control and experimental sites was observed in the monsoon season ($p < 0.001$, df = 1). The degree of similarity appears to increase as it progresses to post-monsoon season ($p < 0.01 = 3.4 \times 10^{-2}$, df = 1) and further decreases in the pre-monsoon season ($p < 0.0001$, df = 1).

A study of the OC at the control and experimental sites revealed lowest values of $1.2(\pm 0.1)\%$ and $1.9(\pm 0.3)\%$, respectively, during the monsoon season. Both the sites showed differences in pre- and post-monsoon OC accumulation. Highest accumulation at the control site was in the pre-monsoon season ($3.1 \pm 0.8\%$) while at the experimental site it was in the post-monsoon season ($3.4 \pm 1\%$). In contrast, intermediate values were observed at the control site during post-monsoon ($2.9 \pm 1.3\%$) and experimental site during pre-monsoon (2.9 ± 0.3). Though there was no monthly variability at the control and experimental sites there was considerable inter-seasonal variability at both the sites (control, $p < 0.01$, df = 2; experiment, $p < 0.1$, df = 2). The control and experimental sites showed similar values during non-monsoon seasons but different levels during the monsoon ($p < 0.1$, df = 1). This variability could be attributed to that observed in the depth intervals 0–2 cm ($p < 0.1$, df = 1) and 2–4 cm ($p < 0.1$, df = 1).

3.3. Nitrification rates

N_{rate} (Fig. 4a) at the control site ranged from 3.2 ± 1.2 ng at-N g(sediment)$^{-1}$ h^{-1} in the monsoon to 6 ± 1.1 and 18.4 ± 1.9 ng at-N g(sediment)$^{-1}$ h^{-1} in the post- and pre-monsoon, respectively. The trend was similar at the experimental site with pre-monsoon showing the highest rates (18.2 ± 0.6), followed by post-monsoon (5.8 ± 1.1) and monsoon (2.7 ± 1.5) seasons. There was significant monthly and inter-seasonal variation at both the control ($p = 7.2 \times 10^{-21}$, df = 12 and $p = 9.3 \times 10^{-26}$, df = 2) and experimental sites ($p = 6.5 \times 10^{-23}$, df = 12 and $p = 6.3 \times 10^{-28}$, df = 2). There was no down-core variability in N_{rate} for both the control and experimental sites.

3.4. Autotrophic and heterotrophic nitrifiers

The abundance of autotrophic (A_{Ntb}) and heterotrophic nitrifiers (H_{Ntb}) is illustrated in Fig. 4b and c. The A_{Ntb} abundance at control site fot all the seasons were ~10^6 cells g^{-1} dry weight of sediment, where as at the experimental site highest abundance was encountered during the pre-monsoon season [$2.1(\pm 1) \times 10^7$ cells g^{-1} dry weight of sediment] and the lowest abundance was during the monsoon season [$3(\pm 7) \times 10^5$ cells g^{-1} wet weight of sediment]. The post-monsoon recorded a marginal increase [$7(\pm 2) \times 10^5$ cells g^{-1} wet weight of sediment]. The seasonal average of H_{Ntb} showed that they were one to two orders higher than the A_{Ntb}. At the control site, highest abundance of H_{Ntb} was during the pre-monsoon season [$3.0(\pm 1.0) \times 10^8$ cells g^{-1} wet weight of sediment], while the lowest was in the post-monsoon season [$1.5(\pm 0.4) \times 10^8$ cells g^{-1} wet weight of sediment]. The monsoon season recorded intermediate values [$2.4(\pm 2.3) \times 10^8$ cells g^{-1} wet weight of sediment]. The abundance of H_{Ntb} at the experimental site was uniform ($2.2–2.5 \times 10^8$ cells g^{-1} wet weight of the sediment) throughout the monsoon cycle with highest in the pre-monsoon and lowest in the post-monsoon. There was no inter-monthly variation in the abundance of A_{Ntb} at the control site while there was significant variability at the experimental site ($p = 1.31 \times 10^{-6}$, df = 12). In contrast, the H_{Ntb} abundance showed significant monthly variability at both the control ($p = 1.7 \times 10^{-4}$, df = 12) and experimental sites ($p = 1.5 \times 10^{-3}$, df = 12). Neither A_{Ntb} nor H_{Ntb} showed any significant down-core variation at the control and experimental sites. There was considerable similarity between the control and experimental sites for all the depth intervals for both the A_{Ntb} and H_{Ntb}. A comparison of inter-seasonal abundance revealed variability at the experimental site for A_{Ntb} ($p = 4.2 \times 10^{-3}$, df = 2) where as the abundance was uniform at the control site. In contrast, the H_{Ntb} showed significant monthly variability at the control site ($p = 1.4 \times 10^{-3}$, df = 2), while their inter-seasonal abundance at the experimental site was uniform. On comparing the control and experimental sites for the different seasons, the A_{Ntb} varied during both the pre-monsoon ($p = 4.6 \times 10^{-3}$, df = 1) and monsoon ($p = 4.8 \times 10^{-2}$, df = 1) seasons while the post-monsoon season was comparable. Similar comparisons for H_{Ntb} yielded contrasting results. There was variability only in the post-monsoon season ($p = 3.6 \times 10^{-2}$, df = 1)

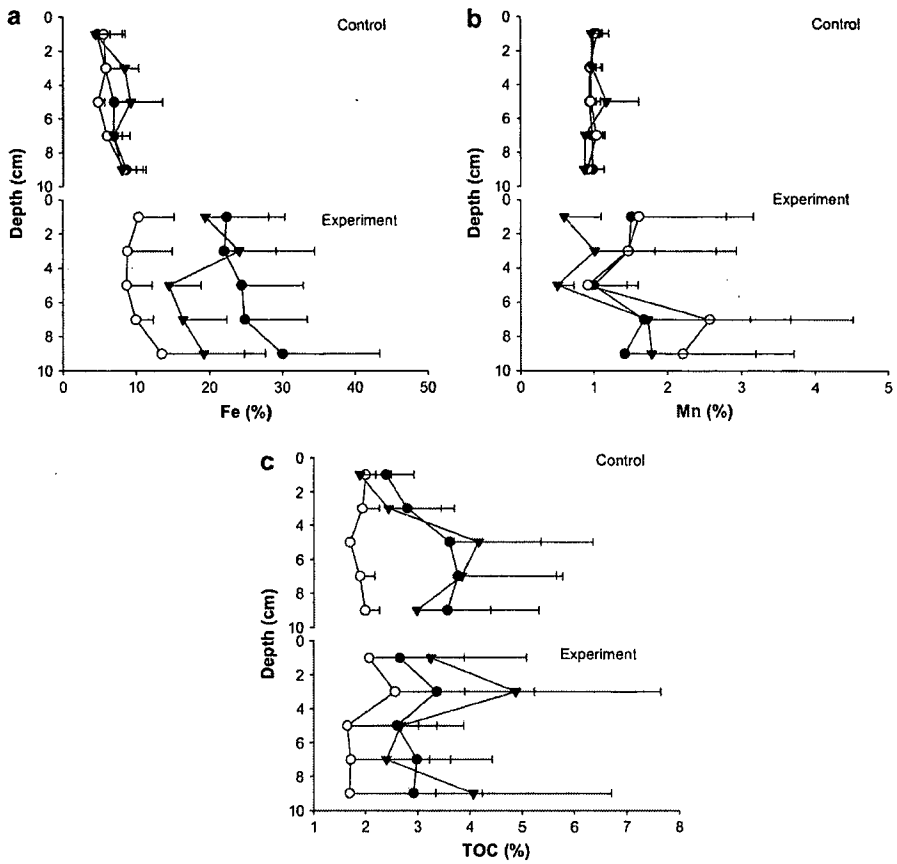

Fig. 3. Seasonal down-core variation in iron (a), manganese (b) and OC (c) at both the control and experimental sites for the pre-monsoon (●); monsoon (○) and post-monsoon (▼) seasons. Only positive standard deviation is shown in the figure.

whereas the abundance was uniform for both the pre-monsoon and monsoon seasons.

4. Discussion

4.1. Nitrification rates: process and controls

Lack of seasonal monitoring especially in the benthic realm in mangroves has hampered our understanding on these productive coastal marine ecosystems which are vulnerable to human impacts. Most of the studies conducted in the mangrove forests report sporadic measurements, though some studies conducted in Australia (Boto and Wellington, 1988; Trott and Alongi, 1999), Pakistan (Harisson et al., 1997), Mexico (Rivera-Monroy et al., 1995), and India (Krishnamurthy et al., 1975; Dham et al., 2002) discuss seasonal cycles. Further, very few studies have attempted to investigate benthic nitrification and factors governing the same. Nitrifying bacteria and N_{rate} in general, may be regulated by many factors including NH_4^+ (Triska et al., 1990; Jones et al., 1995), pH (Saractchandra, 1978), temperature (Paul and Clark, 1989), oxygen concentration (Stenstromm and Podlska, 1980; Triska et al., 1990), competition for NH_4^+ (Verhagene and Laanbroek, 1991), and OC availability (Verhagene and Laanbroek, 1991). In the present study, multiple regression analysis was employed along with simple correlation analysis to elucidate the factors governing N_{rate}. The results of the multiple regression analysis are given in Table 1. In general, N_{rate} was governed by the availability of OC ($r = 0.32$, $p < 0.01$, $n = 64$) at the control site irrespective of season or depth of sampling. This is further supported by a positive correlation of OC with H_{Ntb} ($r = 0.28$, $p < 0.05$, $n = 64$). Depth wise correlation analysis gives additional insights into these relationships. N_{rate} at the top layer (0–2 cm) of the core from the control site was governed by the abundance of H_{Ntb} ($r = 0.58$, $p < 0.02$, $n = 12$); whereas at the bottom of the core (8–10 cm) it appears to be regulated by both the abundance of H_{Ntb} ($r = 0.46$, $p < 0.1$, $n = 12$) as well as the OC ($r = 0.45$, $p < 0.1$, $n = 12$). A positive correlation with NO_2^- ($r = 0.48$, $p < 0.1$, $n = 12$) probably indicates that the NH_4^+ oxidation might be of a higher magnitude than NO_2^- oxidation. In the pre-monsoon season N_{rate} increased depth ($r = 0.67$, $p < 0.001$, $n = 25$) and was limited by the availability of NH_4^+ ($r = 0.57$, $p < 0.001$, $n = 25$). A scatter plot of OC:DIN vs nitrification rate (Fig. 5) represents three well defined clusters. The cluster representing the pre-monsoon is well differentiated from the other two clusters. In the monsoon season, OC to DIN ratio is low (Fig. 5). This favors enhanced N_{rate} (Straus and Lamberti, 2002), N_{rate} is negatively linked to OC ($r = -0.4$, $p < 0.05$, $n = 25$) suggesting that the bulk fraction of the carbon is labile. In

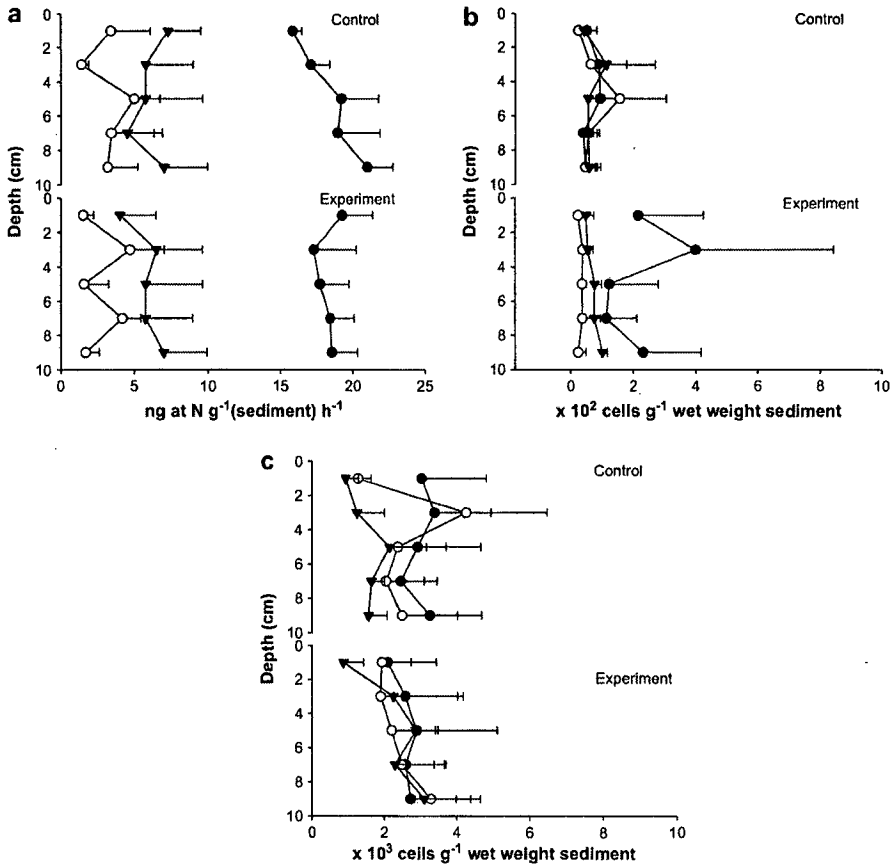

Fig. 4. Seasonal down-core variation in nitrification rates (a), autotrophic nitrifiers (b) and heterotrophic nitrifiers (c) at both the control and experimental sites for the pre-monsoon (●); monsoon (○) and post-monsoon (▼) seasons. Only positive standard deviation is shown in the figure.

addition, there was no single set of factors governing N_{rate} during the monsoon season. However, the recalcitrant fraction of organic carbon is considered to favor enhanced nitrification rates by reducing the utilization pressure on ammonium between heterotrophs and nitrifiers (Straus and Lamberti, 2000). Though the OC:DIN ratio is high there is no relation between OC and N_{rate} for the pre- and post-monsoon season indicating that OC is either unlimiting or dominated by the recalcitrant fraction (Fig. 5). In general the system evolves from a 'Low OC:DIN – Low N_{rate}' system in the monsoon season to a 'High OC:DIN – High N_{rate}' system in the pre-monsoon season with the post-monsoon representing a transition period where the OC:DIN ratio gradually increased. Hence, our study

Table 1
Summary of multiple regression analysis: only the significant factors are listed ($p < 0.05$) in the descending order of their significance; 'r' value is given in brackets.

Season	Factor under consideration	Control (parameters influencing the factor under consideration)	Experiment (parameters influencing the factor under consideration)
Pre-monsoon	Nitrification rate	Depth (0.67)	NH_4^+ (0.55)
	Autotrophic nitrifiers	OC (0.71) > NH_4^+ (0.64) > Mn (0.61)	Fe (0.34)
	Heterotrophic nitrifiers	Nil	Nil
Monsoon	Nitrification rate	Nil	NO_2^- (0.67)
	Autotrophic nitrifiers	NH_4^+ (0.58)	Depth (0.54) > NO_2^- (0.47)
	Heterotrophic nitrifiers	Nil	NH_4^+ (0.71) > OC (0.62)
Post-monsoon	Nitrification rate	NH_4^+ (0.69)	Nil
	Autotrophic nitrifiers	NH_4^+ (0.74)	Nil
	Heterotrophic nitrifiers	NO_3^- (0.50)	Nil
Full monsoon cycle	Nitrification rate	OC (0.76) > H_{mtb} (0.68)	Fe (0.82) > A_{mtb} (0.71)
	Autotrophic nitrifiers	N_{rate} (0.82)	NH_4^+ (0.73) > NO_2^- (0.62) > Depth (0.61) > OC (0.58) > N_{rate} (0.51)
	Heterotrophic nitrifiers	NO_2^- (0.74)	N_{rate} (0.65)

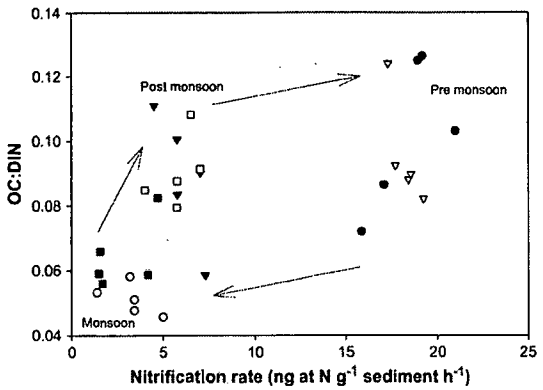

Fig. 5. OC: DIN vs nitrification rate for both the control and experimental sites. Pre-monsoon control (●), monsoon control (○) and post-monsoon control (▼), and pre-monsoon experiment (▽), monsoon experiment (■) and post-monsoon experiment (□).

indicates that the quality of OC could be a more important proxy to N_{rate} rather than OC:DIN ratio. In addition multiple regression analysis (Table 1) shows that NH_4^+ is probably the only ion involved in the regulation of N_{rate}. Further, it could also be deduced that maximum fraction of recalcitrant carbon during the pre- and post-monsoon season is accumulated at 4–6 cm.

N_{rate} at experimental site showed a positive relation with Fe ($r = 0.47$, $p < 0.001$, $n = 64$) and A_{Ntb} ($r = 0.43$, $p < 0.001$, $n = 64$) which is indicative of anaerobic autotrophic nitrification. Further, a negative relation with NO_3^- ($r = 0.25$, $p < 0.05$, $n = 64$) signifies feedback inhibition indicating that at the experimental site, N_{rate} is at the optimum. Hence it appears that the control site is dominated by heterotrophic nitrification whereas autotrophic nitrification governs the experimental site. In addition, OC ($3.4 \pm 1\%$) and NH_4^+(27.8 ± 5.6 μg at NH_4^+-NL^{-1}) peak at the experimental site during the post-monsoon season while at the control site it was during the pre-monsoon season (OC $3.1 \pm 0.8\%$ and NH_4^+ 27.8 ± 4 μg at NH_4^+-NL^{-1}). However, the pre-monsoon of the experimental site was marked by highest levels of nitrification rates (18.2 ± 0.6 ng at-N g(sediment)$^{-1}$ h^{-1}) indicating a decoupling with OC and NH_4^+. These parameters remain coupled during the pre-monsoon season at the control site. Multiple regression analysis (Table 1) shows that NH_4^+ ($r = 0.55$, $p < 0.001$, $n = 25$) plays a limiting role in the pre-monsoon season even though the OC:DIN ratio is comparatively higher (Fig. 5). Further, the production of nitrite appears to govern the nitrification rates during the monsoon season. Depth wise correlation analysis shows that the abundance of A_{Ntb} is the major factor governing the N_{rate} for most of the depth intervals sampled. Positive relations with A_{Ntb} at 0–2 cm ($r = 0.54$, $p < 0.05$, $n = 12$), 2–4 cm ($r = 0.47$, $p < 0.1$, $n = 12$), 6–8 cm ($r = 0.49$, $p < 0.1$, $n = 12$) and 8–10 cm ($r = 0.68$, $p < 0.01$, $n = 12$) is in accordance with the earlier observation. In addition, Fe appears to be the next critical factor regulating N_{rate} at the experimental site with maximum coupling observed at 4–6 cm ($r = 0.7$, $p < 0.001$, $n = 12$) and 6–8 cm ($r = 0.6$, $p < 0.01$, $n = 12$) intervals. The feedback inhibition of NO_3^- is restricted to the bottom layer of 8–10 cm depth indicating enhanced nitrification potential down the core till this depth??

4.2. Factors governing the abundance and distribution of nitrifiers

Even though there are reports on the occurrence of heterotrophic nitrification (Verstraete and Alexander, 1972; Castignetti and Holocher, 1984; Barraclough and Puri, 1995; Gupta, 1997; Lu et al., 2008; Ahmad et al., 2008), most of the studies consider nitrification as a purely chemoautotrophic process. In the present study, we were able to observe the production of NO_2^-/NO_3^- even when the nitrifying media was amended with 0.01% glucose favoring higher ratios of OC:DIN. Hence, heterotrophic nitrification could contribute to the total NO_2^-/NO_3^- pool. Though quantification of heterotrophic nitrification is yet to be done, we tried to explore the factors governing the abundance of H_{Ntb}. The abundance of A_{Ntb} and H_{Ntb} at the control and the former at the experimental site were found to be unlimited by any of the factor measured in this study. However, the H_{Ntb} at the experimental site increased with depth ($r = 0.33$, $p < 0.01$, $n = 64$) and were governed by concentration of NH_4^+ ($r = 0.23$, $p < 0.1$, $n = 64$). Since the A_{Ntb} fraction is dominant at the experimental site it might outcompete the H_{Ntb} for NH_4^+. At the control site in the depth interval 0–2 cm, the NO_3^- production is principally governed by the abundance of H_{Ntb} ($r = 0.55$, $p < 0.05$, $n = 12$), while Mn levels in turn govern ($r = 0.5$, $p < 0.05$, $n = 12$) the abundance of these nitrifiers. Another significant relationship at the control site was observed at 4–6 cm, where H_{Ntb} and A_{Ntb} were found to be mutually exclusive ($r = -0.5$, $p < 0.05$, $n = 12$) with NO_2^- having a feedback inhibition on the former ($r = -0.48$, $p < 0.1$, $n = 12$). The abundance of A_{Ntb} in the depth intervals of 6–8 and 8–10 cm was governed by the levels of Mn ($r = 0.55$, $p < 0.05$, $n = 12$) and Fe ($r = 0.54$, $p < 0.05$, $n = 12$). Moreover the autotrophic nitrifiers govern the production of NO_3^- at 6–8 cm ($r = 0.56$, $p < 0.05$, $n = 12$). Similarly the abundance of H_{Ntb} in the deepest section of the core is governed by the availability of Mn ($r = 0.47$, $p < 0.1$, $n = 12$). Similar to the control site, at the experimental site (0–2 cm) the abundance of H_{Ntb} is governed by the levels of Mn in the sediment. The H_{Ntb} at 4–6 cm were governed by the levels of NH_4^+ ($r = 0.79$, $p < 0.001$, $n = 12$) and they could be actively linked to NO_3^- production ($r = 0.46$, $p < 0.1$, $n = 12$). In a seasonal perspective it was found that at the control site the availability of NH_4^+ is the most important factor governing the abundance of A_{Ntb} during all the seasons, except for the post-monsoon season when there is a feedback inhibition by NO_3^-; the abundance of H_{Ntb} is probably not limited by any factor under consideration in the present study. At the experimental site, only NH_4^+ and OC were found to regulate the abundance of H_{Ntb} during the monsoon season. However, Fe and depth were the two parameters governing the abundance of A_{Ntb} during pre-monsoon and monsoon seasons. Their abundance was not limited by any factor during the post-monsoon season.

5. Conclusion

The role of iron in governing N_{rate} in mangrove sediments has been demonstrated. Our studies suggest that heterotrophic nitrification could be more ubiquitous and significant in mangrove ecosystems. In spite of having similar levels of OC and N_{rate} at both the sites, the underlying mechanisms governing the processes could be different. Higher abundance of H_{Ntb} at both the sites (10^{2-3} cells g^{-1}) and the A_{Ntb} at the experimental site ($\sim 10^3$ cells g^{-1}) reflect the relatively higher refractile nature of organic carbon at the experimental site. We conclude that both the quality of organic carbon and quantity of iron govern nitrification rates in these mangrove swamps.

Acknowledgements

Authors wish to express their gratitude to Dr. Satish R. Shetye, Director, NIO and Dr. Rasik Ravindra, Director, NCAOR for their interest in this work. Our sincere thanks to Prof. McLusky and an anonymous reviewer for critically reviewing the manuscript. KPK records his gratefulness to UGC (Government of India) for the award of Senior Research Fellowship.

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