Development of a microbial process leading to the conversion of ammonia to molecular nitrogen: Characterization and optimization of the process

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DECLARATION

Statement under O. Ph.D.8/ (iii) of The M. S. University of Baroda, Vadodara, India

The work presented in the thesis has been carried out by me under the guidance of Prof. Anjana Desai, Department of Microbiology and Biotechnology Centre, Faculty of Science, The M. S. University of Baroda, Vadodara, Gujarat, India. The data reported herein is original and has been derived from studies undertaken by me.

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Date: — Candidate

This is to certify that the above declaration is true.

Place: Vadodara — Prof. Anjana Desai

Date: — Research Guide

.....Dedicated to my family

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The major problems in the world are the result of the difference between how nature works and the way people think.

- *Gregory Bateson.*

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LIST OF ABBREVIATIONS

*amo*A	-	Ammonia monooxygenase alpha subunit
anammox	-	Anaerobic ammonia oxidation
ANOVA	-	Analysis of variance
AOA	-	Ammonia oxidizing archaea
AOB	-	Ammonia oxidizing bacteria
ARDRA	-	Amplified ribosomal DNA restriction analysis
BABE	-	Bio-Augmentation Batch Enhanced
CANON	-	Complete autotrophic nitrogen removal over nitrite
CFU	-	Colony forming unit
cm	-	Centimeter
C/N	-	Carbon/Nitrogen
COD	-	Chemical oxygen demand
DEMON	-	DEamMONification
DNA	-	Deoxy ribonucleic acid
DO	-	Dissolved oxygen
EDTA	-	Ethylenediamine tetra acetic acid
GC	-	Gas chromatography
HAO	-	Hydroxylamine oxidoreductase
HRT	-	Hydraulic retention time
hao	-	Hydroxylamine oxidoreductase gene
MLSS	-	Mass liquor suspended solids
MLVSS	-	Mass liquor volatile suspended solids
nir	-	Nitrite reductase gene
nos	-	Nitrous oxide reductase gene
OLAND	-	Oxygen limited autotrophic nitrification and denitrification

PBS	-	Phosphate buffered saline
ppm	-	Parts per million
RFLP	-	Restriction fragment length polymorphism
RMSD	-	Root mean square deviation
rRNA	-	Ribosomal RNA
SBR	-	Sequential batch reactor
SEM	-	Scanning electron microscopy
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHARON	-	Single reactor system for high activity ammonia removal over nitrite
SMP	-	Soluble microbial products
SNAD	-	Simultaneous nitrification, anammox and denitrification
SNAP	-	Single stage nitrogen removal using anammox and partial nitrification
SND	-	Simultaneous nitritation denitrification
SSC	-	Single strand conformers
SSCP	-	Single strand conformation polymorphism
SVI	-	Sludge volume index
Ti/Tv	-	Transitions/Transversions
TS	-	Total solids
TDS	-	Total dissolved solids
TSS	-	Total suspended solids

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Chapter 1

Introduction

Chapter 1

Introduction

1.1 Nitrogen cycle

Nitrogen is one of the primary nutrients critical for the survival of all living organisms. It is an important component of many biomolecules, including proteins, DNA and chlorophyll (Bernhard, 2010). Nitrogen (N_2) is the most abundant gas in the earth's atmosphere (constituting 78% of it) and is an element with 9 oxidation state (-3 to +5) (Fig 1.1) (Egli, 2003). Interconversion of these molecules, mediated through different groups of microorganisms under varied set of environmental conditions, forms the nitrogen cycle (Fig 1.1) (Egli, 2003). Nitrogen fixation is the first reaction in the nitrogen cycle in which molecular nitrogen is converted to ammonia by symbiotic and free living nitrogen fixers (Kasap and Chen, 2005; Miller, 2007). Nitrogen in this form becomes available to primary producers, such as plants (Bernhard, 2010).

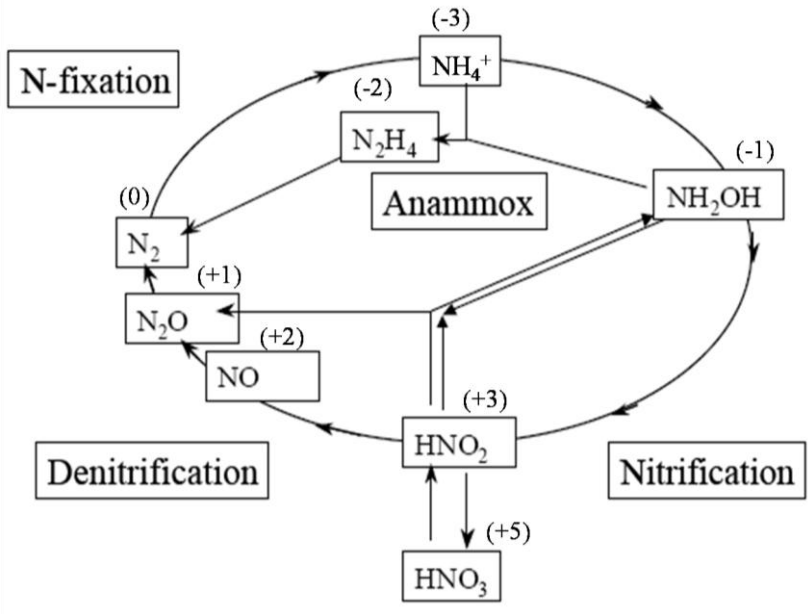

Fig 1.1 The nitrogen cycle (Egli, 2003). Values given in parenthesis are the oxidation state of nitrogen.

Ammonia gets assimilated into cells to form glutamate or glutamine by glutamate dehydrogenase (GDH) or glutamine synthetase (GS) and glutamate synthase (GOGAT) pathway (Meti et al., 2011). Nitrification is the first step in mineralization where NH_3 is microbiologically converted to nitrate. This process is essentially chemoautotrophic and is carried out by two groups of organisms, autotrophic Ammonia Oxidizing Bacteria (AOB) like *Nitrosomonas* spp. oxidizing NH_3 to NO_2^- and autotrophic nitrite oxidizing bacteria (NOB) like *Nitrobacter* spp. oxidizing NO_2^- to NO_3^- (Kowalchuk and Stephen, 2001). Heterotrophic denitrifiers anaerobically reduce nitrate to molecular nitrogen completing the nitrogen cycle (Zumft, 1997). More recently, a new microbial process called the anammox process involving anaerobic ammonia oxidation with nitrite as the electorn acceptor, carried out under strict anaerobic conditions has been added in the nitrogen cycle (van de Graaf et al., 1995; 1996; Strous et al., 1997; Egli et al., 2001). The major nitrogen cycling pathways and relevant genes involved in the different processes are summarized in Table 1.1.

1.2 Ammonia

Ammonia (NH_3) is a gas with a characteristic pungent odor. Ammonia contributes significantly to the nutritional needs of terrestrial organisms by serving as a precursor to foodstuffs and fertilizers. Ammonia, either directly or indirectly, also is a building block for the synthesis of many pharmaceuticals. Although it is widely used, ammonia is both caustic and hazardous. Industrially it is manufactured by different methods (http://www.epa.gov/osweroe1/docs/chem/ammonia.pdf). When dissolved in water it gets ionized to form NH_4^+. The degree to which ammonia forms ammonium ion depends on pH and temperature. Ammonium ion concentration increases 10 fold by increasing pH by one unit and a 2 fold increase in ammonium ion concentration is observed with each 10°C rise in temperature from 0-30°C temperature (Erickson, 1985). It is important to maintain pH and temperature both during ammonia oxidation because undissociated form of ammonia (NH_3) is the substrate for ammonia oxidizers rather than ammonium ion (NH_4^+) (Suzuki et al., 1974).

Table 1.1 Nitrogen cycling pathways and relevant genes (Zehr and Kudela, 2011).

Reaction Name	Chemical Reaction	Genes
Nitrogen fixation	$N_2+8H^++8e^-+16ATP \rightarrow 2NH_3+H_2+16ADP+16Pi$	*nifH, nifD, nifG, nifK*
Ammonium oxidation	$NH_3+O_2+2H^++2e^- \rightarrow NH_2OH+H_2O$ $NH_2OH+H_2O \rightarrow HNO_2+4H^++4e^-$ $0.5O_2+2H^++2e^- \rightarrow H_2O$	*amoC, amoA, amoB, hao*
Nitrite oxidation	$2NO_2^-+H_2O \rightarrow NO_3^- +2H^++2e^-$ $2H^++2e^-+0.5O_2 \rightarrow H_2O$	*norA, norB*
Heterotrophic nitrification	$R\text{-}NH_2 \rightarrow NO_2$ $R\text{-}NH_2 \rightarrow NO_3$	*narH, narF*
Anaerobic ammonia oxidation	$HNO_2+4H^+ \rightarrow NH_2OH+H_2O$ $NH_2OH+NH_3 \rightarrow N_2H_4+H_2O$ $N_2H_4 \rightarrow N_2+4H^+$ $>HNO_2+NH_3 \rightarrow N_2+2H_2O$ $>HNO_2+H_2O+NAD \rightarrow HNO_3+NADH_2$	9 *hao* like genes, hydrazine hydrolase (*hzf*) and hydrazine dehydrogenase
Dissimilatory nitrate reduction and denitrification	$5[CH_2O]+4NO_3^-+4H^+ \rightarrow 5CO_2+2N_2+7H_2O$ $5H_2+2NO_3^-+2H^+ \rightarrow N_2+6H_2O$ $NO_3 \rightarrow NO_2^-$ $NO_2^- \rightarrow NO+N_2O$ $N_2O \rightarrow N_2$	*narD,G,H,I,F; napA,B,D,E; nirB,C,K,U,N,O,S; norB; nosZ*
Assimilatory nitrate and nitrite reduction	$NAD(P)H+H^++NO_3^-+2e^- \rightarrow NO_2^-+NAD(P)^++H_2O$ 6 ferredoxin (red)$+8H^++6e^-+NO_2^- \rightarrow NH_4^++6$ferredoxic (ox)$+2H_2O$	*nasA, nasB, nasC, nasD* (noncyanobacterial bacteria); *narB* (cyanobacteria); *nrtA, nrtB, nrtC, nrtD* (or nap) permeases (cynobacteria)
Dissimilatory nitrate reduction to ammonia	$NO_3^-+2H^++4H_2 \rightarrow NH_4^++3H_2O$	*nir, nar, nap, nrfABCDE*
Ammonification/regeneration/remineralization	$R\text{-}NH_2 \rightarrow NH_4^+$	-
Ammonium assimilation	NH_3+2-oxoglutarate+NADPH+$H^+ \leftrightarrow$ Glutamate+NADP+(glutamate dehydrogenase) NH3+glutamate+ATP $\rightarrow$ glutamine+ADP+Pi glutamine+2-oxoglutarate+NADPH+$H^+ \rightarrow$ 2glutamate+$NADP^+$(glutamine synthetase and NADH-dependent glutamine:2-oxoglutarate amidotransferase	gdhA, gltB

1.2.1 Industrial uses of ammonia (Modak, 2002)

Ammonia is used in several industries including 1) fertilizer industries towards production of ammonium sulfate, ammonium phosphate, urea; 2) chemical industries for the synthesis of nitric acid; in making explosives such as TNT and ammonium nitrate; 3) in nitroglycerine formation which is

used as a vasodilator; 4) in hydrazine formation which is used in rocket propulsion systems; 5) in fibers and plastics industries for the synthesis of Nylon and other polyamides; 6) in refrigeration it is used for making ice, large scale refrigeration plants, air-conditioning units in buildings and plants; 7) in pharmaceuticals it is used in the manufacture of drugs such as sulfonamide; 8) in paper and pulp industries, ammonium hydrogen sulfite enables some hardwoods to be used; 9) in mining and metallurgy industries it is used in nitriding (hardenning) steel and in zinc and nickel extraction and finally 10) ammonia in solution form is also used as a cleaning agent.

1.2.2 Release of ammonia from different sources

Around 131,000 thousand metric tons of ammonia is produced per year world over. With 11,500 thousand metric tons being produced per year, India holds second position in the world after China in ammonia production (data provided is for the year - 2010) (http://www.indexmundi.com/en/commodities/minerals/nitrogen/nitrogen_t12.html) (See appendix 1). Putrefaction of nitrogenous animal and vegetable matter produces ammonia in small quantities which is released in the atmosphere. Ammonia and ammonium salts are found in small quantities in rainwater, whereas ammonium chloride and ammonium sulphate are found in volcanic districts (Shipley, 1919). Ammonium salts are also found distributed in all fertile soil and in seawater.

High concentrations of ammonia observed in water are usually due to effluent discharges from sewage treatment plants or industrial processes, or runoff from fertilized fields or livestock areas. Depending on the contact with residential, industrial, or farming effluents, ammonia concentrations vary widely in aquatic environments. In unimpacted water bodies, ammonia concentrations range from 8.5 to 43 ppb, whereas in impacted water bodies, concentrations as high as 16 ppm have been reported.

1.2.3 Toxicity of ammonia (Pritchard, 2007)

Ammonia is an essential metabolite for DNA, RNA, and protein synthesis and is necessary for maintaining acid-base balance. It is excreted primarily as urea and urinary ammonium compounds through the kidneys. In healthy humans levels of ammonia in blood ranges from 0.7 to 2 mg/L. Most important injurious effects of exposure to excessive amounts of ammonia on human are due to its irritant and corrosive properties. Exposure to ammonia gas causes burning sensation in respiratory tract, skin, and eyes. Ammonia dissolves in the water present in skin, mucous membranes, and eyes and becomes ammonium hydroxide, which is responsible for necrosis of the tissues. Contact with liquid ammonia (not ammonium salts) results in cryogenic injury in addition to the alkali burns. Hepatic and renal effects have also been reported in animals and humans; however, ammonia does not appear to be a primary liver or kidney toxicant.

1.2.4 Effect of ammonia on the environment

Ammonia is released in wastewaters through municipal solid waste landfills (500–3000 mg/L), starch production (800–1100 mg/L), domestic sewage (100 mg/L), swine wastewater (115–175 mg/L), sludge liquor (100–2000 mg/L), yeast effluent (180–450 mg/L), fertilizer manufacture, and agricultural activities (500–1000 mg/L) (Berge et al., 2005; Schmidt et al., 2003). Discharge of ammonia into receiving waters stimulates algal and aquatic plant growth leading to eutrophication of subsurface water. These, in turn, reduce oxygen concentration in the receiving water bodies which adversely affects fish and other aquatic life. Nitrous oxide is emitted during oxidation of ammonia which is toxic to aquatic invertebrate and vertebrate species (Philips et al., 2002). Ammonia increases oxygen demand in receiving waters as oxidation of 1 mg ammonia requires 4.6 mg O_2 resulting in oxygen depletion in the receiving water bodies and adversely affects aquatic life. Concentrations of ammonia exceeding 1 mg/L can cause corrosion of copper pipes (Dean and Lund, 1981). Presence of ammonia also affects chlorination as chlorine combines with ammonium to form

chloramines, which have a lower germicidal effect than free chlorine and thereby reduces the efficacy of water and wastewater disinfection (Bitton, 2005)

1.2.5 National permissible limits for ammonia

The Environmental Protection Agency (EPA) identifies the most serious hazardous waste sites in the nation. These sites are then placed on the National Priorities List (NPL) and are targeted for long-term federal clean-up activities. Ammonia has been found in at least 137 of the 1,647 current or former NPL sites. Accordingly the permissible levels of ammonia to be released into the natural reservoir should not exceed 100 ppm according to Gujarat Pollution Control Board (GPCB) whereas that as per Central Pollution Control Board (CPCB) should not be more than 50 ppm. (Pollution standards: dpcc.delhigovt.nic.in/down/standards.pdf and CPCB, Ministry of Environment & Forests, July 2000).

1.3 Removal of ammonia from wastewater treatment plants

Ammonia from the wastewater treatment plants mainly involve either physiochemical or biological processes depending on the cost effectiveness of the process and concentration of ammonia required to be treated. According to Mulder (2003) the choice of the process depends on three levels of ammonia concentrations. I) Wastewater with concentration of ammonia up to 100 ppm, as present in domestic wastewater, biological activated sludge processes are preferred. II) Wastewater containing ammonia concentration between 100-5000 ppm, biological processes are preferred based on their cost effectiveness. III) Wastewater containing concentrated ammonia 5000 ppm and above, physicochemical methods become economically feasible.

1.3.1 Physicochemical methods

Removal of ammonia by physicochemical methods include ion exchange by zeolite reverse osmosis; MAP process consisting of the precipitation of the ion $MgNH_4PO_4$ (MAP) through the addition of phosphoric acid and magnesium oxide; electrodialysis; air and steam stripping processes

(Siegrist, 1996). The main disadvantages of the physicochemical processes include cost and generation of secondary products like brine which are difficult to dispose off.

1.3.2 Biological methods

Biological removal of ammonia is conventionally carried out by the nitrification-dentrification system

1.3.2.1 Nitrification

Nitrification is an important step in the nitrogen cycle in soil and was discovered by the Russian microbiologists, Sergei Nikolaievich Winogradsky. It is the biological oxidation of ammonia to nitrate. Nitrification is carried out in two steps by two different groups of nitrifying bacteria. Oxidation of ammonia to nitrite is usually the rate limiting step of nitrification (Winogradsky, 1891).

Step 1: Conversion of ammonia to nitrite by Ammonia Oxidizing Bacteria (AOB):

i) **Ammonia to hydroxylamine carried out by Ammonia MonoOxygenase (AMO):**

$$NH_3 + O_2 + 2H^+ + 2e^- \xrightarrow{\textbf{AMO}} NH_2OH + H_2O$$

ii) **Hydroxylamine to nitrite carried out by Hydroxylamine Oxydoreductase (HAO):**

$$NH_2OH + H_2O \xrightarrow{\textbf{HAO}} NO_2^- + 4H^+ + 4e^-$$

Overall reaction:

$$NH_3 + O_2 \longrightarrow 2H^+ + NO_2^- + H_2O + 2e^-$$

$\Delta G^o = -275 KJmol^{-1}\ N$

$E^o = 343mV$

Oxidation of ammonia to nitrite is performed by two different groups of organisms, AOB belonging to the family *Nitrobacteraceae* and ammonia oxidizing archaea (AOA) (Kowalchuk and Stephen, 2001; Venter et al., 2004;

Lu et al., 2012). AOB form monophyletic group belonging to β- and γ-Proteobacteria (Head et al., 1993; Purkhold et al., 2000). Most AOA belong to two distinct clusters: CGI.1a (the marine and sediment lineage) and CGI.1b (the soil lineage) in the phylum *Crenarchaeota* (Zenpichler et al., 2008). AOA dominate in acidic soils with pH<5.5 whereas AOB are predominantly found in soil with alkaline or neutral pH (Prosser and Nicol, 2012; Shen et al., 2012). Recent studies have shown that both AOB and AOA resist high concentration of ammonia but AOA are more sensitive to ammonia inhibition than AOB (Prosser and Nicol, 2012).

Nitrification also plays an important role in the removal of nitrogen from wastewater treatment plants. The cost of this process resides mainly on aeration required for the growth of nitrifiers. Recent studies have shown presence of both AOB and AOA in wastewater treatment plants with the help of *amo*A gene (alpha subunit of the enzyme ammonia monooxygenase specific to AOB and AOA respectively) as a marker (Limpiyakorn et al., 2011; Zhang et al., 2011). AOA identified in the activated sludge showed close identity with AOA found in soil (Zhang et al., 2011) except for activated sludge obtained from bioreactors treating saline sewage (Jin et al., 2010). In industrial wastewater treatment plants where concentration of inlet ammonia is high (36.1–422.3 mgN l^{-1}), AOB dominante (their number is four times higher than AOA) whereas in municipal wastewater treatment plants where ammonia concentration is less (5.6–11.0 mgN l^{-1}), AOA dominate in number (Limpiyakorn et al., 2011). Amongst AOB, *Nitrosomonas* spp. and *Nitrosospira* spp. are the predominant population found in wastewater treatment plants (Juretschko et al., 1998; Schramm et al., 1998; Rowan et al., 2003; van der Wielen et al., 2009).

Nitrifying organisms are chemoautotrophs, and use carbon dioxide as their carbon source for growth (see overall equation below).

Overall equation for *Nitrosomonas*:

$$55NH_4^+ + 76O_2 + 109HCO_3^- \longrightarrow C_5H_7O_2N + 54NO_2^- + 5\ 7H_2O + 10\ 4H_2CO_3$$

Ammonia (NH_4^+) combined with oxygen and hydrogen carbonate (HCO_3^-) to produce bacterial cell mass ($C_5H_7O_2N$), nitrite (NO_2^-), water (H_2O) and carbonic acid (H_2CO_3) (Ahn, 2006). Bacterial cell mass and carbonic acid constitute as soluble microbial products (SMP).

Owing to the extremely slow growth of ammonia oxidizers and release of organic matter (SMP) (see equation above) during their growth, heterotrophs tend to build up rapidly along with the autotrophs making their isolation and maintenance as pure culture not just difficult but also time consuming (Schmidt and Belser, 1982; Rittmann et al., 1994; Ohashi et al., 1995). High levels of heterotrophs are reported to coexist with nitrifiers in autotrophic nitrifying biofilms cultured even without an external organic carbon supply (Okabe et al., 1999; 2002). Understanding of the ecophysiological interactions between nitrifiers and heterotrophs is required to reveal factors that control the efficiency and stability of microbial nitrification towards improving the performance of the process (Kindaichi et al., 2004). Sparse information is available about the pathways, a biofilm community uses to maximize utilization of the metabolites produced by nitrifiers and how its utilization prevents buildup of waste materials to a significant level.

AOB have high iron requirement as it is used in the transfer of electrons in several cytochromes and enzymes like hydroxylamine oxidoreductase (Prince and George, 1997). In the natural niche of AOB, availability of iron is less as it is present in the insoluble ferric form leading to iron deficient condition. An efficient iron transport and scavenging system is required in the microorganisms to survive under such condition (Andrews et al. 2003). Genome of *Nitrosomonas* spp. show presence of several genes (9 to 42) that code for siderophore receptor but genes for siderophores production are either incomplete or absent in most AOB except *Nitrosococcus oceani* which has a complete set for siderophore synthesis but studies on the functionality of the genes are not proven (Vajrala, 2008). It is therefore speculated that in nature AOB depend on siderophores produced by common soil microorganisms to suffice their iron requirement (Wei et al., 2006). A combination of microautoradiography (MAR)-fluorescence in situ

hybridization (FISH) approach was developed by Lee et al. (1999) and Ouverney and Fuhrman (1999) that allowed analysis of specific microbial activities and functions in various microbial communities like activated sludge, marine samples, freshwater sediments and sewer biofilms (Nielsen et al., 2000; 2002;2003). A detailed investigation is required to understand such ecophysiological interactions between AOB and heterotrophs found in nature.

Step 2: Conversion of nitrite to nitrate by Nitrite oxidizing bacteria (NOB):

The second step of nitrification (oxidation of nitrite into nitrate) is carried out by NOB by nitrite oxidoreductase.

$$NO_2^- + \tfrac{1}{2}O_2 \xrightarrow{\textbf{NXR}} NO_3^-$$

$\Delta G^o = -74 KJmol^{-1}$ N
$E^o = 434mV$

Overall equation for *Nitrobacter*:

$$400NO_2^- + NH_4^+ + 4H_2CO_3 + 195O_2 \longrightarrow C_5H_7O_2N + 3H_2O + 400NO_3$$

Nitrite (NO_2^-) combines with ammonia (NH_4^+), carbonic acid (H_2CO_3), hydrogen carbonate (HCO_3) and oxygen (O_2) to produce bacterial cell mass ($C_5H_7O_2N$), water (H_2O) and nitrate (NO_3^-).

Unlike AOB, nitrite oxidizers are more scattered phylogenetically. They belong to four genera of which members of the genus *Nitrobacter* belong to *Alphaproteobacteria* (Stackebrandt et al., 1988), members of the genus *Nitrococcus* are affiliated with the *Gammaproteobacteria* (Woese et al., 1985), members of genus *Nitrospina* are affiliated with the *Deltaproteobacteria* (Teske et al., 1994) whereas members of the genus *Nitrospira* represent a new phylum *Nitrospirae,* in the domain Bacteria (Ehrich et al., 1995). Amongst these four genera *Nitrobacter* and *Nitrospira* are capable of growing as heterotrophs and mixotrophs (Aharon, 2006) and are considered most important in nitrite oxidation in wastewater treatment plants (Henze, 1997; Burrell, 1998). Dominance of *Nitrobacter* and *Nitrospira* in wastewater

treatment plants has been revealed by FISH and 16S rRNA targeted probes (Wagner, 1996). According to Schramm (1999) *Nitrobacter* are considered as r-strategist with low affinity towards nitrite and oxygen and therefore survive better under high oxygen and nitrite concentrations whereas *Nitrospira* are considered as k-strategist with low μmax and are well-adapted to low nitrite and oxygen concentrations. Therefore, *Nitrospira* will out-compete *Nitrobacter* in reactors fed with wastewater containing low nitrite concentrations whereas in reactors fed with wastewater containing high nitrite concentrations, for example in nitrifying sequencing batch reactors *Nitrobacter* would dominate and this dominance cannot be reverted by decreasing nitrite concentration (Wagner et al., 2002).

The main factors controlling nitrification in wastewater treatment plants and other environments include

1) A constant source of ammonia is required to be given to the activated sludge for the continuation of nitrification process (Sahrawat, 2008).
2) Elimination or minimization of organic load from the aerated nitrifying reactor. Organic carbon though may not inhibit nitrification process directly, it increases heterotrophic growth that causes oxygen depletion and consequently nitrification gets inhibited (Barnes and Bliss, 1983).
3) pH between 7.5 to 8.5 is reported to be optimum for *Nitrosomonas* and *Nitrobacter* and the process completely stops below pH 6.0 (U.S. EPA, 1975; Painter and Loveless, 1983).
4) Dissolved oxygen is one the most important factor that controls nitrification. To oxidize 1 mg of ammonia, 4.57 mg of O_2 is required (Christensen and Harremoes, 1978). In activated sludge system bulk DO concentration should not be less than 2 ppm (van Haandel and van der Lubbe, 2007).
5) BOD5/TKN ratio should be between 1 and 3 (Metcalf and Eddy, 1991)

6) Larger detention time should be provided. For activated sludge plant: 6-48 hours and for Lagoons: 30 days or longer with Mean Cell Residence Time (MCRT): 4 to 15 days.
7) Wastewaters originating from synthetic chemicals manufacturing industries sometimes contain potential inhibitors like acetonitrile, hence removal of such inhibitor from the effluent is must for the proper functioning of nitrification process (Blum and Speece, 1991).
8) Nitrifiers are very sensitive to near UV, visual and fluorescent light (Wolfe et al., 2001). Ammonia monooxygenase gets inactivated when nitrifiers are exposed to light.
9) The size and type of system and degree of ammonia present will influence the dosage rate. Higher concentrations of ammonia have inhibitory effect on nitrifiers.
10) A sufficient population of nitrifying bacteria must be present for efficient nitrification process to occur. These bacteria generally are found to have attached growth on the sludge floc particles in an activated sludge plant.

1.3.2.2 Denitrification

Denitrification is the microbial reduction of nitrate to molecular nitrogen (N_2) through a series of gaseous nitrogen oxide as intermediates (Hayatsu et al., 2008). This respiratory process reduces oxidized forms of nitrogen through the oxidation of organic matter as electron donor. Thermodynamically favorable nitrogen electron acceptor are nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2), in order of highest to lowest (Fig 1.2).

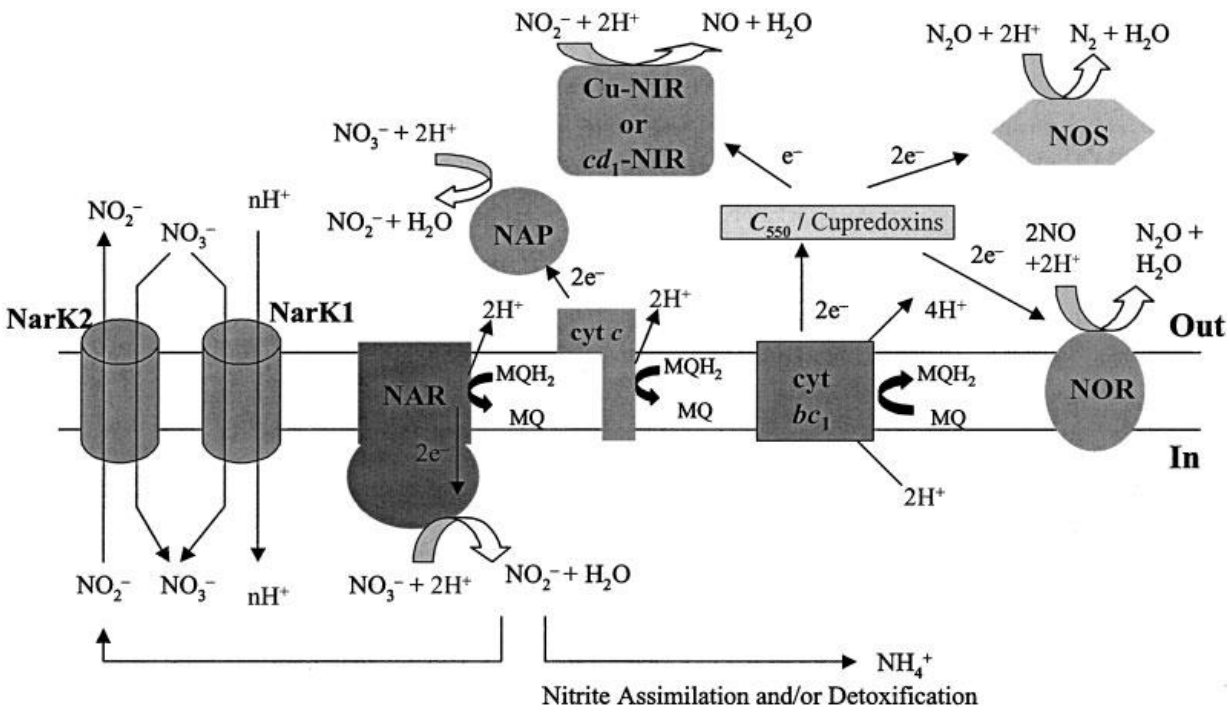

Fig 1.2. Schematic representation of the denitrification pathway in bacteria (Cabello et al., 2004). Periplasmic nitrate reductase (NAP), membrane-bound nitrate reductase (NAR), periplasmic nitrite reductases (Cu-NIR or cd1-NIR), membrane-bound nitric oxide reductase (NOR) and periplasmic nitrous oxide reductase (NOS) are represented without indicating their subunit composition and cofactors. cyt bc1, proton-pumping cytochrome bc1 complex; cyt c, NapC membrane-bound tetrahaem cytochrome c; cyt c550, cytochrome c550; MQH2/MQ, menaquinol/menaquinone pool.

With the development of culture independent molecular techniques in studying the diversity and distribution of denitrifiers, it has become evident that the distribution of denitrifiers does not follow a distinct pattern (Zumft, 1997; Henry et al., 2008; Hayatsu et al., 2008; Enwall and Hallin, 2009). The ability of carrying out denitrification is sporadically distributed amongst taxonomically different groups of archaea, bacteria and fungi (Tiedje, 1988; Zumft, 1997; Hayatsu et al., 2008). Owing to this their identification based on 16S rRNA gene is difficult (Philippot et al., 2007). Therefore, functional genes like nitrite reductase genes (*nir*K and *nir*S) and nitrous oxide reductase genes (*nos*Z) have been used frequently as markers for identifying

and studying the diversity of denitrifiers apart from 16S rRNA gene sequences (Braker et al., 1998; Throbäck et al., 2004; Henry et al., 2008; Ishii et al., 2011). Fungi capable of denitrification include ascomycota such as *Fusarium solani, Fusarium oxysporum, Cylindrocarpon tonkinense* and *Gibberella fujikuroii* and the basidiomycota *Trichosporon cutaneum* (Bollag and Tung, 1972; Shoun et al., 1992; 2006). Archaea capable of denitrification include halophiles like *Haloarcula, Halobacterium*, and *Haloferax* and hyperthermophiles like *Pyrobaculum* (Cabello et al., 2004). These follow the denitrifying pathway similar to bacteria but have been reported to have differences in the arrangement, structure and regulation of the enzymes (Philippot, 2002). The versatile group of bacteria capable carrying out denitrification include organotrophic, phototrophic, lithotrophic, diazotrophic, thermophilic, psychrophilic, halophilic, magnetotactic, pathogenic and others. To name a few, bacteria belonging to the genus: *Pseudomonas, Paracoccus, Rhodopseudomonas, Alcaligenes, Bradyrhizobium, Magnetospirillum, Sphingobacterium, Neisseria* and others are capable of carrying out denitrification (Zumft, 1997; Ishii et al., 2011).

The main factors controlling denitrification in wastewater treatment plants and other environments include (Barnes and Bliss, 1983; Hawkes, 1983).

i) ***Nitrate concentration.*** Since nitrate serves as an electron acceptor for denitrifying bacteria, the growth rate of denitrifiers depends on nitrate concentration.

ii) ***Anoxic conditions.*** Denitrification being a respiratory process where nitrate is used as an ultimate electron acceptor instead of oxygen, the process is well carried out under anoxic conditions. Denitrification may occur inside activated sludge flocs and biofilms despite relatively high levels of oxygen in the bulk liquid. Thus, presence of oxygen in wastewater may not preclude denitrification at the microenvironment level (Christensen and Harremoes, 1978).

iii) ***Presence of organic matter.*** Denitrifying bacteria require organic matter as an electron donor to carry out denitrification process

which include pure compounds e.g., acetic acid, citric acid, methanol, ethanol; raw domestic wastewater; wastes from food industries (brewery wastes, molasses) and bio-solids. The preferred source of electrons for denitrifiers is methanol, which is also used as a carbon source to drive denitrification (Christensen and Harremoes, 1978) as observed through the equation:

$6NO_3+5CH_3OH \rightarrow 3N_2+5CO_2+7H_2O+6(OH)$. Methane present in biogas, (approximately 60%), can also serve as a sole carbon source in denitrification (Werner and Kaiser, 1991).

1.3.2.3 Limitations of the conventional nitrification-denitrification system

The conventional nitrification-denitrification reactions have been known for a long time (Winogradsky, 1890; Beijerinck and Minkman, 1910; Kluyver and Donker, 1926). As nitrification and denitrification are carried out under different conditions and by different group of microorganisms, these processes need to be separated in time or space to function effectively (Khin and Annachhatre, 2004). The main limitations of the conventional system are: i) Nitrification reaction consumes a large amount of oxygen, requiring 4.2 g of oxygen for the nitrification of each gram of ammonia (Gujer and Jenkins, 1974). ii) Denitrification, requires an external organic carbon source. For example, 2.47 g of methanol is required for complete denitrification of one gram nitrate nitrogen (McCarty et al., 1969) which increases the cost of the process. Methane is low cost substitute for methanol, generally readily available in large amounts in wastewater treatment facilities through the anaerobic digestion of sludge (Costa et al., 2000; Lee et al., 2001). iii) The process produces a large amount of sludge.

1.4 Recent advances in the removal of ammonia from industrial effluents

Owing to the limitation of the conventional nitrification-denitrification systems several new processes have been developed for the treatment of

ammonia containing effluent to meet the rising demand of environment protection. These include:

1.4.1 SHARON (single reactor system for high activity ammonia removal over nitrite)

This new process for biological removal of nitrogen is operated without biomass retention in a single aerated reactor at a relatively high temperature (35°C) and pH (above 7). These are simple continuous stirred tank reactor ideally suited for removing nitrogen from waste stream with a high ammonium concentration (>500 ppmN) (Brouwer et al., 1996; Hellinga et al., 1997; 1998; Jetten et al., 1997; van Dongen et al., 2001). The process was first developed at the Technical University Delft, the Netherlands (Hellinga et al., 1998), and its full-scale reactors have also been developed (Mulder et al., 2001; van Kempen et al., 2001). It is the first successful process in which nitrification/denitrification with nitrite as an intermediate was achieved under stable conditions (van Kempen et al., 2001).

1.4.2 ANAMMOX (Anaerobic ammonia oxidation) process

Anaerobic **AMM**onium **OX**idation **(Anammox)** is a globally important microbial process of the nitrogen cycle (Arrigo, 2005). The simultaneous removal of ammonium and nitrite under anoxic conditions was first observed in a pilot scale denitrifying reactor in Delft, The Netherland (Mulder et al., 1995). In this biological process, nitrite and ammonium are converted directly into dinitrogen gas (Fig 1.3).

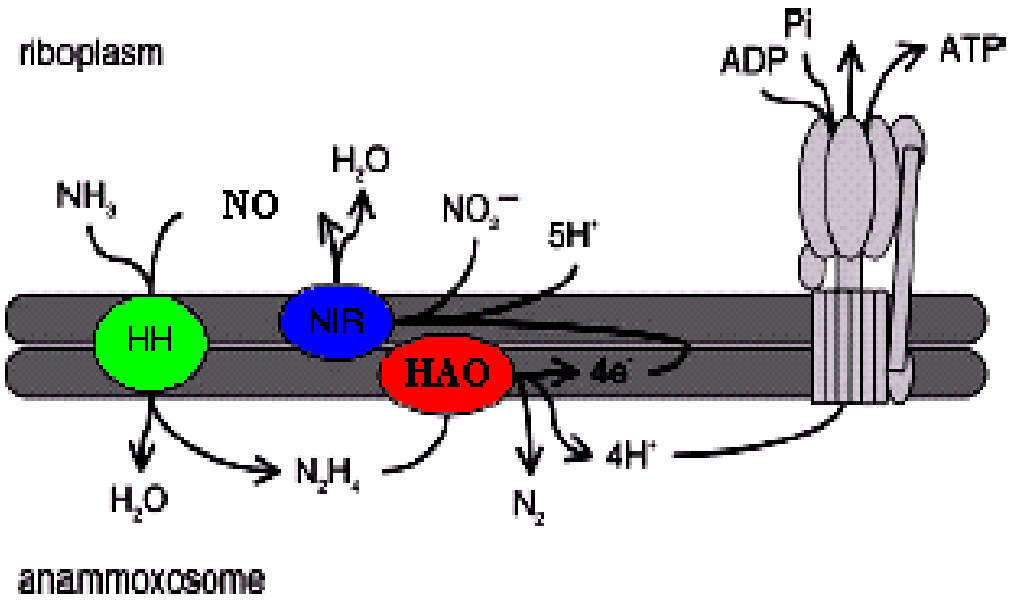

Fig 1.3. Anammox reaction carried out under anoxic conditions by the anammox bacteria (Niftrik et al., 2004)

This process contributes up to 50% of the dinitrogen gas produced in the oceans (Arrigo, 2005). The overall catabolic reaction is:

$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$

Biomass production with CO_2 as the sole carbon source is:

$CO_2 + 2NO_2^- + H_2O \rightarrow CH_2O$ (biomass) $+ 2NO_3^-$

NO_2^- not only acts as an e^- acceptor in anaerobic ammonia oxidation but also as an e^- donor for CO_2 fixation in biomass formation (see reactions above) (Kuenen and Jetten, 2001). Bacteria that perform the anammox process belong to phylum Planctomycetes (Kuenen, 2008).

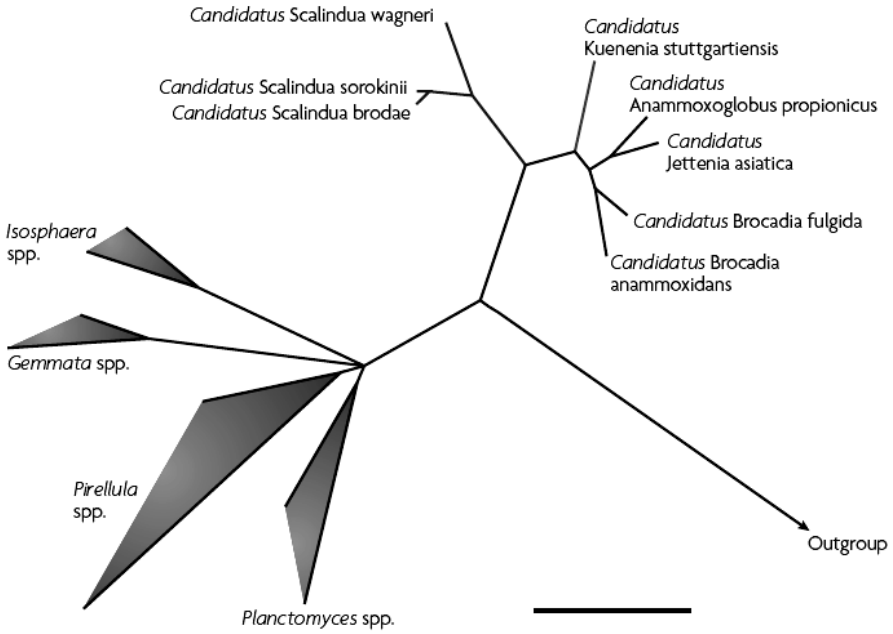

Fig 1.4 Phylogenetic tree of anammox bacteria based on 16S rRNA gene (with bar representing 10% sequence divergence) (Kuenen, 2008)

Currently five genera of anammox bacteria have been (provisionally), defined: *Brocadia*, *Kuenenia*, *Anammoxoglobus*, *Jettenia* (all fresh water species), and *Scalindua* (marine species) (Kuenen, 2008) (Fig 1.4). Anammox are small coccoid cells with a diameter of approximately 800 nm. They possess anammoxosome, a membrane bound compartment inside the cytoplasm which is the catalytic site for the anammox reaction to take place (Fig 1.5) (Jetten et al., 2009). They do not have peptidoglycan in their cell wall, possess crateriform structures on cell surfaces and reproduce by budding (Fig 1.5 C) (Kuenen and Jetten, 2001). Their membranes mainly consist of ladderane lipids unique to these microorganisms (Boumann et al., 2006; Niftrik et al., 2009). Hydrazine (used as high-energy rocket fuel and poisonous to most living organisms) is produced as an intermediate (Kuenen and Jetten, 2001). They have extremely slow growth rate with doubling time of nearly two weeks (Kuenen and Jetten, 2001). Optimum temperature for their growth is 37°C and optimum pH is around 8.0 (Kuenen and Jetten, 2001).

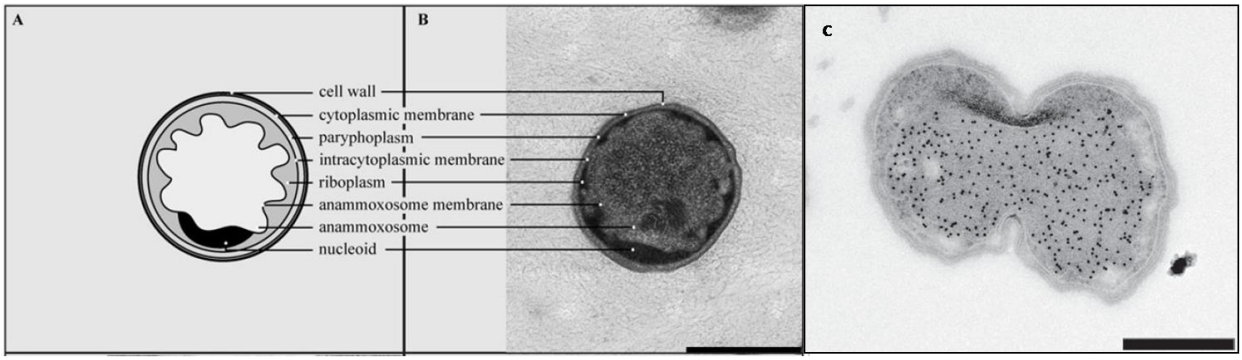

Fig 1.5. A) Schematic diagram of a non-dividing cell showing cell plan to be divided into three cytoplasmic compartments separated by single bilayer membranes (Lindsay et al., 2001; Fuerst, 2005) and B) Transmission electron micrograph showing *Candidatus* Kuenenia stuttgartiensis (Niftrik et al., 2009). Scale bar: 500 nm. C) Budding *Candidatus K. stuttgartiensis* Scale bar: 500nm (Jetten et al., 2009)

1.4.2.1 Enrichment of anammox bacteria (Jetten et al., 2009)

Sequencing batch reactor (SBR) has been shown as a promising tool to obtain stable enrichment of anammox bacteria in lab (Strous et al., 1998). The reactor could be operated stably for >1 year under stable conditions as well as in substrate limiting conditions. Biomass can be efficiently retained and homogeneous distribution of substrates, products and biomass aggregates can be achieved. The reactors are inoculated with a suitable environmental sample (wastewater sludge, river or marine sediment); fed with ammonium, nitrite, bicarbonate and sparged with oxygen-free gas. Nitrite and ammonium inlet concentrations are gradually increased, such that *in situ* reactor levels were maintained in the micromolar range by microbial activity. Stable enrichments are generally obtained after a period of 90–200 days from SBRs during which its content slowly turns red (as haem proteins constitute approximately 20% of the cellular protein mass) and the anammox bacteria increase to at least 70% of the population as biofilm aggregates (Strous et al., 1999; Kartal et al., 2007; 2008; van de Vossenberg et al., 2008). Membrane reactors are recently applied for the successful cultivation of anammox bacteria as single cell suspensions (van der Star et al., 2008).

1.4.2.2 Advantages of the anammox process over the conventional nitrification-denitrification system

The anammox process has several advantages in removing ammonia from wastewater compared to the conventional removal of ammonia as reported by Fux et al., (2003).

1. Reduction in energy and cost as oxygen addition can be reduced by 60%
2. Cost of the process can be further reduced as external supply of organic carbon is not required.
3. Very little amount of sludge is produced during the process.
4. Emission of CO_2 is eliminated in the system as it is utilized as carbon source by the chemolithoautotrophic bacteria

Its comparison with the conventional system is as shown in Table 1.2 as reported in Plaques (2008).

Table 1.2 Comparison of conventional and anammox system

Requirement	Conventional treatment	Anammox process	Units
Power	3-5	1-2	kWh/kgN
Methanol	2.5-3	0	kg/kgN
Sludge production	0.5-1.0	0.1	kgVSS/kgN
CO_2 emission	> 4.7	0.7	kg/kgN
Total Costs[1]	3-5	1-2	€/kgN

[1]Total cost includes both operational and capital costs.

1.4.3 Combined SHARON and ANAMMOX process

Combined SHARON and ANAMMOX processes is based on the principle that wastewater containing ammonium is oxidized to nitrite in the SHARON reactor using only 50% of the influent ammonium (Jetten et al., 1997). The

effluent from SHARON reactor containing a mixture of ammonium and nitrite is used as the influent for the ANAMMOX process where ammonium and nitrite are anaerobically converted to dinitrogen gas and water in the ratio 1:1 (van Dongen et al., 2001). This ratio can be achieved in sludge liquor without any pH control, because the sludge liquor contains bicarbonate as the counter ion for ammonium. When half of the ammonium in the sludge liquor is converted to nitrite, alkalinity of the water gets depleted that leads to drop in pH which prevents further nitrification, thereby maintaining the ratio of ammonia to nitrite as 1 (Jetten et al., 2002). The combined SHARON and ANAMMOX treatment is ideally suitable for concentrated sludge reject waters (sludge liquor) and industrial wastewaters containing high concentration of ammonia and low amount of organic carbon and occurs in either two separate reactors or a single vessel (Dijkman and Strous, 1999). The combined process requires less oxygen (1.9 kgO_2/kgN instead of 4.6 kgO_2/kgN), no carbon source (instead of 2.6 kgBOD/kgN) and has a low sludge production (0.08 instead of approximately 1 kgVSS/kgN) (van Loosdrecht and Jetten, 1998).

1.4.4 SNAD (simultaneous nitrification, anammox and denitrification) process

Anammox based treatment processes are generally applied to ammonia containing wastewater without COD, but most ammonia laden wastewater contain some portion of COD e.g. opto-electronic industrial wastewater containing 100 mgCOD/L and 567 mgNH_4^+-N/L (Daverey et al., 2012). To solve this problem denitrification was added to nitritation – anammox process occurring in a single reactor and the process so developed was called simultaneous partial nitrification, anammox and denitrification (SNAD) process (Chen et al., 2009). It is a one reactor configuration process making use of partial nitrification converting ammonia partially to nitrite, anammox reaction converting ammonia and nitrite produced by AOB to molecular nitrogen and denitrifiers, reducing nitrate produced by anammox bacteria to molecular nitrogen with the help of COD present in the wastewater treatment plant. The process therefore has the advantage of

complete nitrogen removal along with reduction in the levels of chemical oxygen demand (COD). In a full-scale landfill-leachate treatment plant in Taiwan, granules, capable of carrying out the SNAD process, were identified (Wang et al., 2010). Owing to the requirement of longer start-up time and slow growth rates of anammox bacteria (the doubling time was reported to be approximately 11 d), developing a SNAD process in the laboratory becomes highly difficult and in addition, the reactor carrying out anammox process must be efficient in retaining the biomass (Strous et al., 1998).

1.4.5 Simultaneous nitrification and denitrification (SND)

1.4.5.1 SND by autotrophic aerobic ammonia oxidizers

Nitrosomonas europaea and *Nitrosomonas eutropha* can nitrify and denitrify simultaneously when grown under oxygen limitation wherein in addition to oxygen, nitrite acts as an electron acceptor. Simultaneous nitrification and denitrification resulted in significant formation of the gaseous N-compounds like nitrous oxide and dinitrogen which cause significant nitrogen loss. Pure cultures of *N. eutropha* are able to denitrify with molecular hydrogen as electron donor and nitrite as the only electron acceptor in a sulfide-reduced complex medium under anoxic conditions (Schmidt and Bock, 1997). In the process, increase in cell number is directly coupled to nitrite reduction with nitrous oxide and dinitrogen as the only detectable end products (Bock et al., 1995; Schmidt and Bock, 1997). Genes for denitrifying enzymes nitrite reductase (*nir*K) and nitric oxide reductase (*nor*) have been identified in the genome of *Nitrosomonas europaea* ATCC 19718 by Chain et al., (2003). A recent study has shown that nitrifier denitrification is a major source of N_2O produced by *N. europaea* ATCC 19718 (Schmidt et al., 2004).

1.4.5.2 SND by the combination of nitrifiers and denitrifiers

Nitrification is combined with aerobic denitrification that occurs in a single reactor under overall identical conditions. This process when operated continuously saves cost as the second anoxic tank is not required and saves organic energy upto 40% (Turk and Mavinic, 1986; Abeling and Seyfried,

1992). This process is especially useful in the treatment of ammonia from low COD:nitrogen ratio containing effluent (Yoo et al., 1999). Aerobic denitrifying bacteria including *Mesorhizobium* sp. and *Burkholderia cepacia*, well adapted to fluctuating oxic–anoxic conditions, have been isolated from diverse natural and managed ecosystems and have been characterized (Patureau, 2000; Matsuzaka et al., 2003; Okada et al., 2005). The SND process is effective in maintaining neutral pH in the reactor even without the addition of external acid or base source as during nitrification alkalinity is reduced whereas it is produced during denitrification (Yoo et al., 1999).

1.4.6 Partial nitritation and anammox in one single reactor

This is a process in which partial nitritation and anammox processes are applied in a single reactor**.** This basic process has several names like **SNAP (Single stage nitrogen removal using anammox and partial nitritation), DEMON (DEamMONification) OLAND (oxygen limited autotrophic nitrification and denitrification).** Under oxygen limited conditions autotrophic aerobic ammonium-oxidizing bacteria oxidize ammonia to nitrite, and anoxic ammonium-oxidizing bacteria or anammox bacteria combine this nitrite with the remaining ammonia to form dinitrogen gas and some nitrate (Kuai and Verstraete, 1998; Pynaert et al., 2003). From an economical point of view, these processes are preferred over conventional nitrification/denitrification for wastewaters with a low biodegradable organic content. The processes save 30-40% of the overall nitrogen treatment costs (Fux et al., 2004), as they have lower requirement of aeration, lesser sludge production, and no requirement of external organic carbon addition.

1.4.7 CANON (complete autotrophic nitrogen removal over nitrite)

The process is highly suited for removal of nitrogen from wastewaters that are highly loaded with ammonium and contain low concentrations of organic carbon (Helmer et al., 1999, 2001; Koch et al., 2000). It consists of a combination of nitritation and anaerobic ammonium oxidation which occur under completely autotrophic conditions and hence the process is called

completely **a**utotrophic **n**itrogen removal **o**ver **n**itrite (or CANON). Like SHARON process, the CANON process also completely eliminates the need for the addition of organic carbon to achieve denitrification (Strous, 2000; Egli et al., 2001; Third et al., 2001). In the first step of the process, ammonium is partly oxidized to nitrite, whereas in the second step the remaining ammonium is oxidized anaerobically with nitrite to form dinitrogen. These reactions can occur in a single reactor under oxygen limited conditions. The two groups of microorganisms interact and perform the two sequential reactions simultaneously and for optimal efficiency, the anaerobic phase must be fed with slightly more nitrite than ammonium (Strous, 2000; Egli et al., 2001). A technical scale reactor with the CANON process is already operating in the Netherlands (van Dongen et al., 2001).

1.4.8 BABE (Bio-Augmentation Batch Enhanced)

This process removes nitrogen from concentrated stream and nitrification capacity of the activated sludge system is increased by seeding it continuously with the nitrifiers produced in the aerator basin. The process was developed and designed based on model simulation and is especially useful in systems with limited tank volume which have restricted nitrification capacity and is applicable even in colder climates (van Haandel and van der Lubbe, 2007).

1.5 Appendix

Table 1.3: Ammonia: Estimated World Production, By Country (http://www.indexmundi.com/en/commodities/minerals/nitrogen/nitrogen_t12.html)

AMMONIA: ESTIMATED WORLD PRODUCTION, BY COUNTRY[1, 2]

(Thousand metric tons, contained nitrogen)

Country	**2006**	**2007**	**2008**	**2009**	**2010**
Afghanistan	20 -	20	20	20	20
Albania	10 -	10	10	10	10
Algeria	470 -	524	500	614	822
Argentina	727 [3]	726	726	570 [r]	600
Australia	1,200	1,200	1,200	1,200	1,200
Austria	400 [r]	400 [r]	400 [r]	300 [r]	350
Bahrain	370 [3]	344 [3]	350	350	350
Bangladesh[4]	1,250	1,300	1,300	1,300	1,300
Belarus	815	830	805 [3]	750	800
Belgium-	850	850	850	850	850
Bosnia and Herzegovina	1	1	1	1	1
Brazil	950	950	950	950	950
Bulgaria-	309	310	310	310	310
Burma	30	30	30	30	30
Canada	4,100	4,498 [3]	4,781 [3]	4,000	4,000
China[3]-	40,660	42,480	41,140	42,290	40,870
Croatia-	319 [3]	320	320	320	300
Cuba	42 [3]	47 [3]	42	27 [r]	27
Czech Republic-	250	225	200	200	200
Denmark	2	2	2	2	2
Egypt	1,800	1,750	1,750	2,000	3,000
Estonia	170	170	170	170	170
Finland	62	62	62	62	60
France	616 [3]	800	800	800	800
Georgia	140	150	150	150	150
Germany[3]	2,718	2,746	2,819	2,363	2,677
Greece-	130	130	130	125	120
Hungary	275	300	300	300	300
India[5]	**10,900**	**11,000**	**11,100**	**11,200**	**11,500**

Indonesia-	4,300	4,400	4,500	4,600	4,800
Iran	1,020	2,000	2,000	2,000	2,500
Iraq	10	10	10	10	550
Italy	480 [3]	460	460	460	460
Japan[3]	1,091	1,114	1,244 [r]	1,021 [r]	1,000
Korea, North	100	100	100	100	100
Korea, Republic of	90	--	--	--	--
Kuwait	495	485	485	470 [r]	480
Libya[3]	518	523	417	488 [r]	580
Lithuania------	453 [3]	936 [3]	950	950	950
Malaysia	950	960	950	950	950
Mexico[3]	487	714 [r]	826 [r]	895 [r]	826
Netherlands	1,800	1,800	1,800	1,800	1,800
New Zealand-	120	125	125	125	125
Norway	350	350	350	350	350
Oman	1,000	1,000	1,000	1,000	1,000
Pakistan-	2,200	2,250	2,300	2,300	2,400
Peru	5	5	5	5	5
Poland[3]	2,007	1,995	1,995	1,697 [r]	1,800
Portugal-	244	244	244	244	244
Qatar	1,500 [r]	1,500 [r]	1,600 [r]	1,700 [r]	1,700
Romania	1,300	1,300	1,300	1,100 [r]	1,100
Russia	10,500 [3]	10,500	10,425 [3]	10,441 [3]	10,400
Saudi Arabia	2,000	2,600	2,600	2,600	2,600
Serbia	80	85	47 [r]	53 [r]	55
Slovakia-	241 [3]	260	260	260	260
South Africa	480 [-]	480	480	430	450
Spain-	400	400	400	400	400
Switzerland--	32	32	32	32	32
Syria	120	120	120	210	260
Taiwan	12 [-]	12	12	12	12
Tajikistan	35	25	25	20	20
Trinidad and Tobago	5,111 [3]	5,129 [3]	5,130	4,946 [r, 3]	5,000
Turkey	92 [3]	-- [3]	50	100	100
Turkmenistan	250	270	270	270	270
Ukraine	4,200	4,200	4,000 [r]	2,500 [r]	3,400
United Arab Emirates	380	380	380	380	400
United Kingdom	750	1,050	1,100	1,100	1,100

United States[3, 6]	8,190	8,540	7,870	7,700	8,290 [p]
Uzbekistan	940	1,000	1,000	1,000	1,000
Venezuela	1,160	1,160	1,160	1,160	1,160
Vietnam	230	300	300	300	300
Zimbabwe	10	35	25	25 [r]	25
--- Total	125,000 [r]	131,000	130,000	127,000 [r]	131,000

[p]Preliminary. [r]Revised. -- Zero.

[1]World totals, U.S. data, and estimated data have been rounded to no more than three significant digits; may not add to totals shown.-

[2]Table includes data available through June 24, 2011.

[3]Reported figure.

[4]May include nitrogen content of urea.

[5]Data are for years beginning April 1 of that stated.

[6]Synthetic anhydrous ammonia; excludes coke oven byproduct ammonia.

1.6 Present investigation

Most of the wastewater treatment plants (WWTPs) world over, make use of conventional nitrification (conversion of ammonia to nitrate) and denitrification processes (conversion of nitrate to nitrogen) for ammonia removal. In terms of process operation, nitrification and denitrification are mutually opposite, the former require autotrophic and aerobic microorganisms while the latter involves heterotrophic and anaerobic microorganisms, making the entire operation environmentally unsustainable and requires high energy and space. The rise in the demands for environmental protection has forced the development of more efficient nitrogen-removing technologies in wastewater treatment such as SHARON, ANAMMOX, SNAD, CANON, OLAND, DEMON. The new technologies developed are mostly based on the anaerobic ammonium oxidation (ANAMMOX) process in combination with partial nitrification. These processes are more efficient than the conventional nitrification-denitrification processes in terms of time, space and cost economics. Anammox process needs a preceding nitritation step carried out by autotrophic ammonia oxidizing bacteria (AOB) that converts half of the wastewater ammonia to nitrite. Proper implementation of anammox process for the removal of ammonia from industrial effluents therefore requires combination of partial nitrification with the anammox process. Since, the industrial effluent being addressed in the present study contained high amount of ammonia and low organic carbon, simultaneous nitrification, anammox and denitrification (SNAD) was chosen for the treatment of the effluent. Of the three groups of organisms involved in ammonia removal using SNAD process, two groups, autotrophic ammonia oxidizers (AOB) and anammox being slow growing require enrichment so as to generate sufficient biomass. Isolation and identification of the organisms from the respective enriched biomass using conventional 16S rRNA gene as a phylogenetic marker and *amo*A and *hao* genes as functional markers was the first step in the investigation.

Physiological properties shown by isolated strains does not often represent their actual activities in in-situ environmental conditions (Kowalchuk and Stephen, 2001) as laboratory conditions do not mimick natural conditions and isolated pure cultures may not represent the dominant population in those environments (Amann et al., 1995). 16S rDNA and *amo*A gene based identification systems have been used to study the diversity and abundance of AOB in natural niche and wastewater treatment plants (Bernhard et al., 2010; Junier et al., 2010). Since 16S rRNA gene does not state about the physiology of the microorganisms and mutation in the gene often lead to detection of phylogenetically close by physiologically unrelated microorganisms and *amo*A gene used is smaller in size than 16S rRNA gene and generally does not give resolution at par with 16S rRNA gene, there is a need for a better molecular marker for studying AOB (Purkhold et al., 2003), *hao* gene being a potential candidate, has been exploited in the present study.

Observations of coexistence of heterotrophs with autotrophic ammonia oxidizers (AOB) are reported in literature. AOB are readily found in aerobic environments rich in ammonia but owing to their extremely slow growth and release of soluble microbial products (SMP) during their growth, heterotrophs tend to build up rapidly along with the autotrophs making their isolation and maintenance as pure culture not just difficult but also time consuming (Schmidt and Belser, 1982; Rittmann et al., 1994; Ohashi et al., 1995). Present investigation also showed persistent coexistence of heterotrophs with AOB. Since detailed investigation on physiological significance of such co-existing microorganisms is lacking in literature, an attempt was made to unravel the mechanism of the coexistence of autotrophs and heterotrophs.

In view of the above, the present investigation was undertaken towards developing a process for anoxic removal of ammonia from the effluent of a fertilizer industry. The study was extended further towards identifying the major group of microorganisms present and studying their diversity and

defining their role in the process of ammonia removal. The major objectives set for the work accordingly are listed as under.

1. Enrichment and isolation of ammonia oxidizing (AOB) consortia from various sludge samples and exploring *hao* as an alternative functional and molecular marker towards studying the diversity of AOB.

2. Identifying physiological significance of heterotrophs co-existing with AOB.

3. Enrichment and identification of anammox bacteria from various sludge samples and developing an effective consortium of AOB and anammox capable of carrying out anoxic removal of ammonia.

4 Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for anoxic removal of ammonia and molecular characterization of the biomass developed.

5. Kinetics of ammonia removal in a 5 L open reactor: An aerobic solution to high strength ammonia containing wastewater of a fertilizer industry.

Chapter 2a

Comparison of polymorphism in ammonia monooxygenase and hydroxylamine oxidoreductase for analyzing Nitrosomonas spp. diversity

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Comparison of polymorphism in ammonia monooxygenase and hydroxylamine oxidoreductase genes for analyzing *Nitrosomonas* spp. diversity

ABSTRACT

Autotrophic Ammonia Oxidizing Bacteria (AOB), key players in the global nitrogen cycle, are confined to two monophyletic lineages belonging to the beta and gamma-subclass of *Proteobacteria*. *Nitrosomonas* genus belongs to beta-subclass of *Proteobacteria* encompassing closely related species. Single Strand Confirmation Polymorphism (SSCP) analysis of ammonia monooxygenase (*amo*A) and hydroxylamine oxidoreductase (*hao*) gene fragments and hydroxylamine oxidoreductase (HAO) enzyme zymogram were used to resolve *Nitrosomonas* spp. These sequencing independent methods were highly sensitive. *Nitrosomonas europaea* ATCC19178 (standard strain) and twelve isolates, obtained by enriching sludge from wastewater treatment plants and paddy field soil, were differentiated into six and four groups by SSCP analyses of *amo*A and *hao* gene fragments respectively, whereas they could be resolved into six distinct groups through HAO enzyme activity staining. SSCP analysis of *amo*A gene fragment, amongst the three, could best resolve the studied AOB, based on richness and evenness in the population (Simpson's Index of Diversity–0.85). However, the ensembled use of these molecular methods (SSCP of *amo*A and *hao* gene fragments) and HAO enzyme zymogram in fingerprinting of AOB provided better resolution and evenness, contributing significantly in AOB diversity studies.

2a.1 Introduction

Autotrophic Ammonia Oxidizing Bacteria (AOB) play an important role in oxidizing ammonia to nitrite aerobically in the global nitrogen cycle (Kowalchuk and Stephen, 2001). This oxidation occurs in two steps: ammonia is first oxidized to hydroxylamine by ammonia monooxygenase that is subsequently oxidized to nitrite by hydroxylamine oxidoreductase enzyme (HAO). Ammonia monooxygenase (*amo*A) gene fragment and 16S rDNA have been widely used to fingerprint AOB group (Nyberg et al., 2006; Bernhard et al., 2010) but very few studies have employed hydroxylamine oxidoreductase gene (*hao*) as a marker to study AOB diversity. The use of *hao* gene as a molecular marker for detecting AOB was first suggested by Shinozaki and Fukui (2002) and recently Schmid et al., (2008), developed primers for the amplification of *hao* gene fragment. Of the two genes *amo*A and *hao* unique to AOB, *hao* is expected to show edge over *amo*A being larger in size (850bp) than *amoA* gene fragment (491bp) and hence may have higher resolution. It was therefore important to compare its efficiency as a molecular marker with *amo*A gene in differentiating AOB.

Single-Strand Conformation Polymorphism (SSCP) is a simple, reliable, nonradioactive technique to identify variability in a gene within and between various groups of organisms (Hayashi, 1991; Bastos et al., 2001). Differences in the nucleotide sequence of the PCR products lead to variation in electrophoretic mobility of single-stranded conformers developed during SSCP analysis. The technique is extremely useful in the study of evolutionary biology and molecular ecology and has been reported not only to match the resolution of sequencing but also follows an order of magnitude more efficient in terms of fastness, labor and resources (Sunnucks et al., 2000). It has been used to differentiate species of a single genus (Delbe`s and Montel, 2005) and can therefore be applied to study the diversity of AOB. The technique being highly sensitive and able to detect even a single base substitution (Hayashai, 1991), has been used, in the present study, as a tool to analyze *hao* and *amo*A gene fragments towards differentiating AOB.

HAO enzyme is a complex hemoprotein of α2 or α3 oligomer containing eight haem units per 63kDa subunit with molecular mass of 125-175kDa for the holoenzyme (Nejidat, 1997). Gene sequence based diversity studies applying molecular tools generally rely on the primers designed to amplify the gene of interest from all the microorganisms under study. Mutations in the gene of interest at the primer binding site would inhibit binding of the primers affecting amplification of the gene in question. Consequently, some microorganisms, in spite of being present, will not to be detected from the system. The use of enzyme zymogram based fingerprinting methods to differentiate microorganisms can overcome this problem as it depends on the enzyme activity (Shukla et al., 2009). This novel approach was therefore used in the present study to differentiate *Nitrosomonas* spp. (members of a single genus) and the results have been compared with the diversity profiles of *amoA* and *hao* genes obtained through SSCP analyses. The concerted use of all these three methods not only enabled better resolution of AOB being studied in the present investigation, but also resolved them with higher evenness.

2a.2 Materials and Methods

2a.2.1 Standard Strain

Nitrosomonas europaea ATCC 19178 was used as the standard strain. It was obtained from Dr. Daniel J. Arp, Daniel J. Arp Laboratory, Department of Botany and Plant Pathology, Oregon State University, Corvallis OR 97331, USA.

2a.2.2 Enrichment of AOB and growth conditions

The inorganic medium used for the enrichment of AOB was prepared as under Solution 1: To 900 ml of DDH_2O the following reagents were added in sequence: 3.3 g $(NH_4)_2SO_4$, 0.41 g KH_2PO_4, 0.75 ml from 1 M $MgSO_4$ stock solution, 0.2 ml from 1 M $CaCl_2$ stock solution, 0.33 ml from 30 mM $FeSO_4$/50 mM EDTA stock solution (pH7.0), 0.02 ml from 50 mM $CuSO_4$ stock solution. Solution 2: To 400 ml DDH_2O, 40.82 g KH_2PO_4 and 3.6 g

NaH_2PO_4 were added and pH was adjusted to 8.1 with 10 N NaOH, and volume was made to 500 ml with DDH_2O. Solution 3: 500 ml 5% (w/v) Na_2CO_3 (anhydrous). Preparation of the standard inorganic medium: All the three solutions were separately autoclaved. 100 ml of media contained 90 ml of solution 1, 10 ml of solution 2 and 0.8 ml of solution 3 (Hyman and Arp, 1992). Soil and sludge samples (5 g) were inoculated in 100 ml of the medium and incubated in dark for 1 month at 30°C. Ammonia oxidizing activity of the samples was calculated by measuring the amount of nitrite produced according to Griess-Romijn (1996). The enriched samples were streaked on Nylon 66 membrane filter (45 mm diameter, 0.2 µm pore size) placed on the same medium containing 1% agar to get isolated colonies. Filters were transferred on fresh plates every third day for six transfers consecutively till visibly large red colored, isolated colonies appeared on the membrane.

2a.2.3 DNA extraction, PCR amplification, cloning, and sequencing

Isolates were identified by 16S rRNA gene amplification, cloning and sequencing. Log phase cultures, with O.D. at 600 nm in the range of 0.120 - 0.134 (50 ml), were centrifuged at 19,200 X g for 15 min and DNA was extracted according to Schmidt et al., (1991). Amplification of 16S rRNA gene was carried out with *Taq* polymerase using universal primer 27F and 1541R. PCR protocol included an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 45 s and extension at 72°C for 1.5 min and a final extension at 72°C for 10 min. *amo*A gene fragment was amplified using modified amoA1F (5'-TGGGGTTTCTACTGGTGGT-3') and reported amoA2R (5'-CCCCTCKGSAAAGCCTTCTTC-3') primers designed by Rotthauwe et al., (1997), targeting 331-349 and 802-822 positions respectively of *Nitrosomonas europaea amo*A gene open reading frame (Rotthauwe et al., 1997). *hao* gene fragment was amplified as described by Schmid et al., (2008) using haoF4 and haoR2 primers (binding positions 451–467 and 1283–1299 respectively relative to *N. europaea hao* gene) and protocol. Primer haoR3 (binding position 1174–1189 relative to *N. europaea hao* gene)

was used as reverse primer to amplify *hao* gene fragment from DN3, which failed to give amplification with haoR2 as the reverse primer (Schmid et al., 2008). Purification of the amplified 16S rRNA gene was carried out using PCR Clean-up gel extraction NucleoSpin Extract II Kit (Macherey – Nagel GmbH and Co.KG, Germany) according to the manufacturer's instructions. These were then cloned in pTZ57R/T vector using INSTA cloning kit (Fermentas). Clones of 16S rRNA gene were differentiated based on amplified ribosomal DNA restriction analysis using *Alu*I restriction enzyme. These clones were grouped based on ARDRA pattern obtained. Representative 16S rRNA gene clones from each group were sequenced commercially by Single Pass Analysis (Bangalore Genei). *amo*A and *hao* genes from all the isolates were similarly cloned sequenced and submitted in NCBI.

2a.2.4 Single Strand Conformation Polymorphism

SSCP of *amo*A and *hao* genes was carried out, to fingerprint the enriched AOB, according to Sambrook and Russel, (2001) with modifications. Purified PCR product (2 μl) was denatured by mixing in 18 μl of a solution containing 98% formamide, 0.01 M ethylenediaminetetraacetic acid (EDTA) and bromophenol blue. The mixture was incubated at 95°C for 10 min and immediately cooled on ice. Single stranded DNA molecules (conformers) were resolved in 15 cm gels with 0.75 mm thickness (DCode Universal Mutation Detection System - Bio-Rad). The mixture was loaded onto 8% nondenaturing gel containing 30% acrylamide-0.8% bisacrylamide and was run in 1X TBE (90 mM Tris-borate at pH 8.3, 4 mM EDTA). The run was carried out at constant 70 V and 25°C temperature. SSCP was carried out for 18 h in case of *amo*A gene and 20 h for *hao* gene. Silver staining technique was used to visualize the bands. Electrophoretic mobility of the single strand conformers was calculated by the AlphaEaseFC version 4.0 software.

2a.2.5 Preparation of cell free extract

Cell free extracts were prepared to get a crude enzyme lysate for HAO enzyme polymorphism study. Cells (100 ml) grown to log phase were harvested by centrifugation (19,200 X g for 10 min), washed once with 10 mM sodium phosphate-buffered saline (PBS) pH 7.4 and resuspended in sterile distilled water. The cell free extract was prepared by passing the cells through French press at 1000 psi followed by centrifugation at 19,200 X g for 10 min at 4°C. Protein concentration was determined as per Bradford's method (Bradford, 1976).

2a.2.6 HAO enzyme activity staining

HAO enzyme activity on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), under non-reducing condition was detected by formazan formation as described by Nejidat et al., (1997). Protein (10 µg) was mixed with loading buffer containing SDS but without beta-mercaptoethanol and loaded onto 7.5% SDS-PAGE gel. The run was carried out for 6 hr at 25°C at 70 V (1 hr after the dye front starts coming out) after which the gel was immersed in to a solution containing 50 mM Tris-HCl, pH 8.0, 0.1 mM phenazine methosulphate, 0.2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide and 5 mM hydroxylamine (prepared fresh and mixed just before immersing the gel). Dark blue to black colored formazan formed over the HAO enzyme activity band just within 15 min of incubation in dark. The protein marker lane was cut and stained separately with coomassie brilliant blue G250 stain. Electrophoretic mobility of HAO enzyme activity staining band was calculated using AlphaEaseFC version 4.0 software. Reaction controls used were i) substrate control without hydroxylamine in the staining solution and ii) system containing 2-mercaptoethanol (reducing agent) for inactivating the enzyme. Cell free extract from *Escherichia coli* DH5α was used as a negative control.

2a.2.7 Statistical analysis

All experiments were carried out in triplicate to check their reproducibility. The electrophoretic mobility of *amo*A and *hao* gene fragment single stranded conformers and HAO enzyme activity stained bands were statistically analyzed using SigmaStat version 3.5 software.

2a.2.8 Calculations for diversity

Simpson's Diversity Index (Simpson, 1949) was used as a measure to study the diversity of AOB as it takes into account both richness (the number of species detected per technique and in combination) and evenness (relative abundance of the different species). Simpson's Index 'D' was initially calculated by:

$$D = [\Sigma n(n-1)]/[N(N-1)]$$

Where n = total number of organisms belonging to a particular species and N = total number of organisms of all the species analyzed.

Simpson's Index of Diversity = 1 – D where D was Simpson's Index.

Evenness in the population was calculated according to Pielou Index according to Pommier et al., (2010).

2a.3 Results and Discussion

2a.3.1 Enrichment and isolation of AOB from various sludge and soil samples

Amongst the 22 industrial sludge and soil samples enriched under aerobic, autotrophic conditions, six samples (DnrA, DnrB, CETP, DN, N and PF) showed significantly higher nitrite production (P value ≤ 0.05) (Table 2a.1). Owing to the slow growth rates of AOB, they were initially enriched for one month in inorganic medium that led to substantial increase in their number.

Table 2a.1. Ammonia oxidizing activity of consortia enriched from different environmental samples

Sr. No.	Samples	Nitrite produced (µg/ml)
1.	D1 (effluent, DNR A reactor, GNFC, Bharuch))	3.6
2.	D2 (effluent, DNR B reactor, GNFC, Bharuch)	0.2
3.	**DnrA (solid sludge, DNRA reactor, GNFC, Bharuch)**	**108.6**
4.	**DnrB (solid sludge, DNRB reactor, GNFC, Bharuch)**	**97.3**
5.	**CETP (CETP collector, GNFC, Bharuch)**	**101.6**
6.	N1 (CEPT, Nandesari)	8.0
7.	N2 (Nandesari)	2.9
8.	**DN (Deepak Nitrite, Nandesari)**	**103.7**
9.	**N (Municipal waste water treatment plant, Nandesari)**	**99.5**
10.	SR9A (SR9A reactor, GNFC)	29.0
11.	Garden soil (Model farm, Vadodara)	0.11
12.	Pulses soil (Model farm, Vadodara)	0.07
13.	Cereal soil (Model Farm, Vadodara)	0.13
14.	Cereal and pulses grown in rotation (Model Farm, Vadodara)	0.12
15.	Aquarium water (Kamati Baug, Vadodara)	2.0

16.	Domestic sludge (Vadodara)	1.0
17.	Aquarium water (Railway station, Vadodara)	3.0
18.	Pond water (Alkapuri, Vadodara)	3.0
19.	**PF (Paddy field, Mandya)**	**103.68**
20.	Alembic sludge (Vadodara)	12.0
21.	Sindhrot river water (Vadodara)	2.0
22.	Gotri Pond water (vadodara)	4.0

Further studies were carried out with the six samples (DnrA, DnrB, CETP, DN, N and PF) that showed higher nitrite formation. Amplification of *amo*A gene (492bp) confirmed the presence of autotrophic Ammonia Oxidizing Bacteria in these six enriched samples (Fig 2a.1).

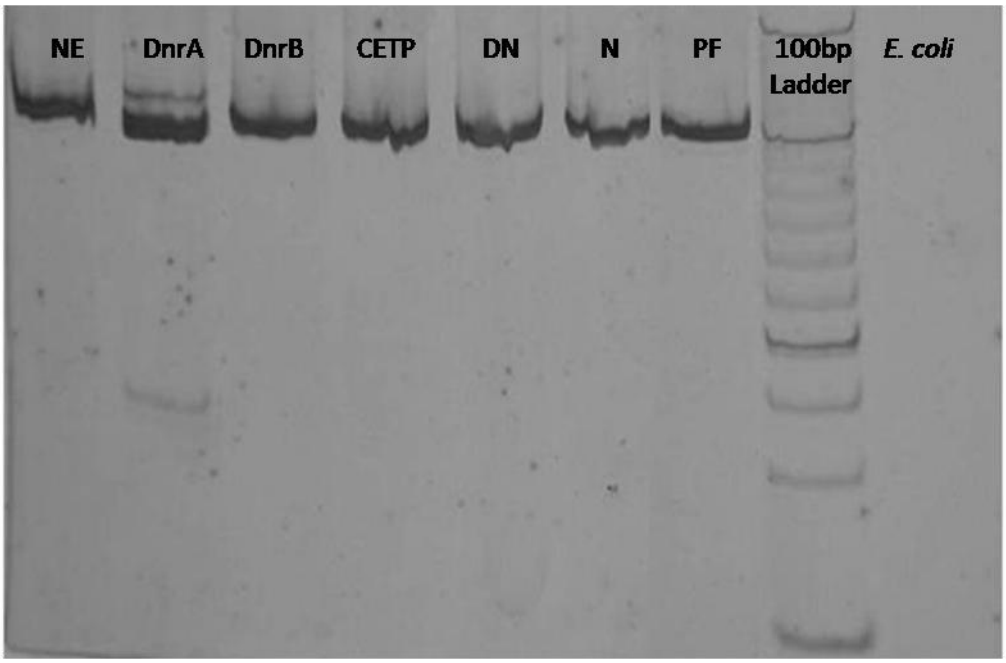


Fig 2a.1. Amplification of *amo*A from the enriched samples and *Nitrosomonas europaea* (NE)

Isolation of AOB from these enriched samples was carried out by spreading the samples on Nylon66 membrane filters kept on inorganic media. Twelve different kinds of isolated colonies (based on colony morphology) appeared within two weeks of incubation [which included six transfers on fresh media plates releasing AOB from nitrite inhibition (Stein and Arp, 1998)] (Fig. 2a.2).

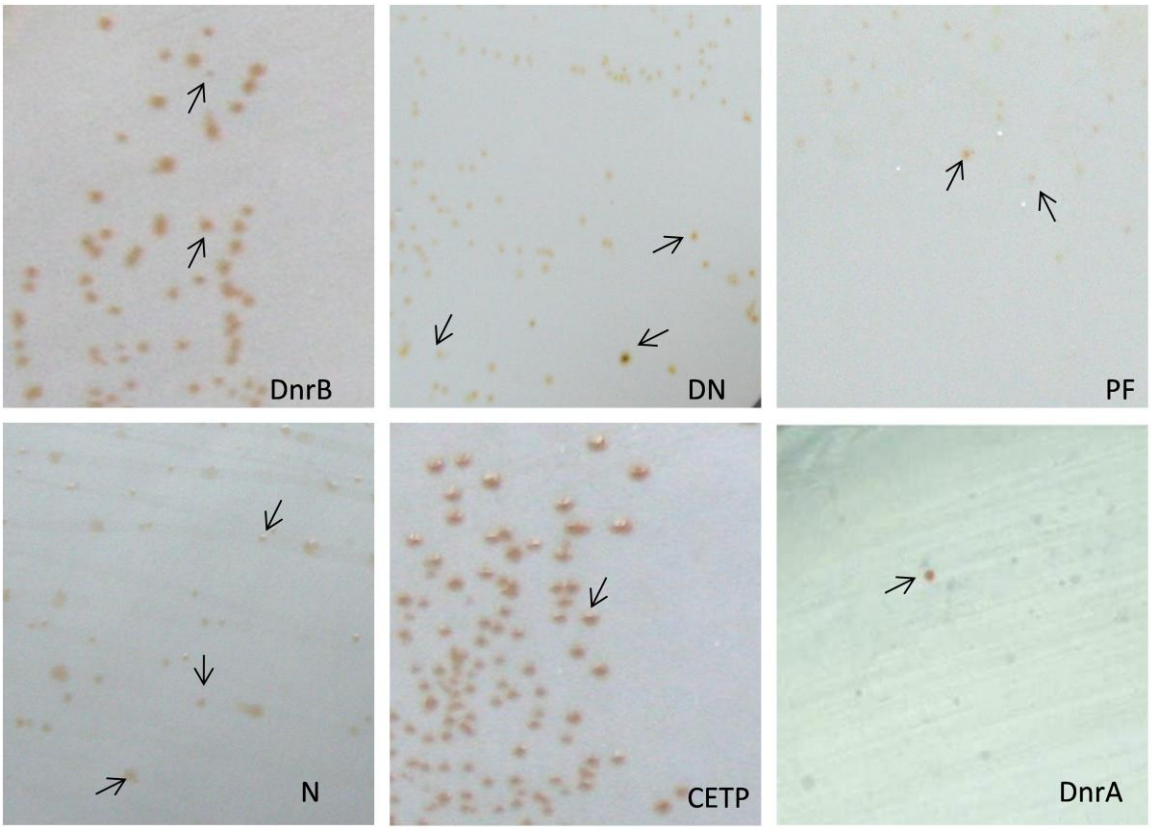


Fig 2a.2. Red colored colonies of AOB obtained on the Nylon 66 membrane filters. The different kinds of colonies are indicated by the arrow mark

Colonies were picked and maintained individually. Purity of these colonies was checked by spreading them on Luria Bertani plates. Heterotrophs were observed along with the autotrophs in most of the isolated colonies. Detailed study regarding the coexistence of these heterotrophs and autotrophs has been discussed in chapter 3.

2a.3.2 Identification of microorganisms present in the red colored AOB colonies

Identification of AOB based on 16S rRNA gene partial sequence revealed presence of *Nitrosomonas* species in 11 of the 12 isolates obtained from the enriched samples (Table 2a.2). N1 showed 87% similarity with *Nitrosospira* sp. NSP12 and hence was phylogenetically very different from the other isolates obtained. Dominance of *Nitrosomonas* spp. amongst AOB has been reported and found to be primarily present in waste water treatment plants and enriched soil and ground water samples (Stephen et al., 1996; van der Wielen et al., 2009). As presence of only one kind of AOB was observed in each colony, hence it can be considered as pure with respect to AOB.

Table 2a.2. Identification of AOB isolated from enriched consortia based on 16S rDNA analysis.

Sr. No	Isolates used in the study	Origin	Blast result with 16S rDNA (%) identity
1	*Nitrosomonas europaea ATCC*19178 standard strain (NE)	Dr. Daniel Arp, Corvallis.	-
2	DnrA	Solid sludge from (Denitrifying reactor) DnrA reactor, GNFC, Bharuch,	*Nitrosomonas eutropha* C91 (93)
3	DnrB1	Solid sludge from (Denitrfying reactor 2) DnrB reactor,GNFC, Bharuch	*Nitrosomonas sp.* DYS317 (99)
4	DnrB2		*Nitrosomonas sp.* DYS317 (98)
5	CETP	Biofilm from Central effluent treatmentplant, GNFC, Bharuch	*Nitrosomonas sp.* DYS317 (99)
6	DN1	Effluent from Deepak Nitrite, Nandesari	*Nitrosomonas sp.* ENI-11 (99)
7	DN2		*Nitrosomonas sp.* DYS317 (98)
8	DN3		*Nitrosomonas eutropha* C91 (99)
9	N1	Activated sludge from Municipal waste water, Nandesari	*Nitrosospira sp.* NSP12 (87)
10	N2		*Nitrosomonas sp.* ENI-11 (99)
11	N3		*Nitrosomonas sp.* ENI-11 (99)
12	PF1	Surface soil (2cm from the surface) Paddy field, Mandya	*Nitrosomonas sp.* DYS317 (99)
13	PF2		*Nitrosomonas sp.* ENI-11 (99)

2a.3.3 Diversity of AOB using *amo*A and *hao* gene fragments as markers

*amo*A and *hao* genes are exclusively present in AOB and therefore reduce the possibility of detecting non-specific organisms compared to 16S rRNA gene which is a universal phylogenetic marker (Rotthauwe et al., 1997). These genes are therefore considered to be better marker compared to 16S rRNA and hence were used in the present study to differentiate the obtained AOB isolates. *amo*A and *hao* gene fragments were amplified from the isolated AOB population (Fig 2a.3 A and B respectively).

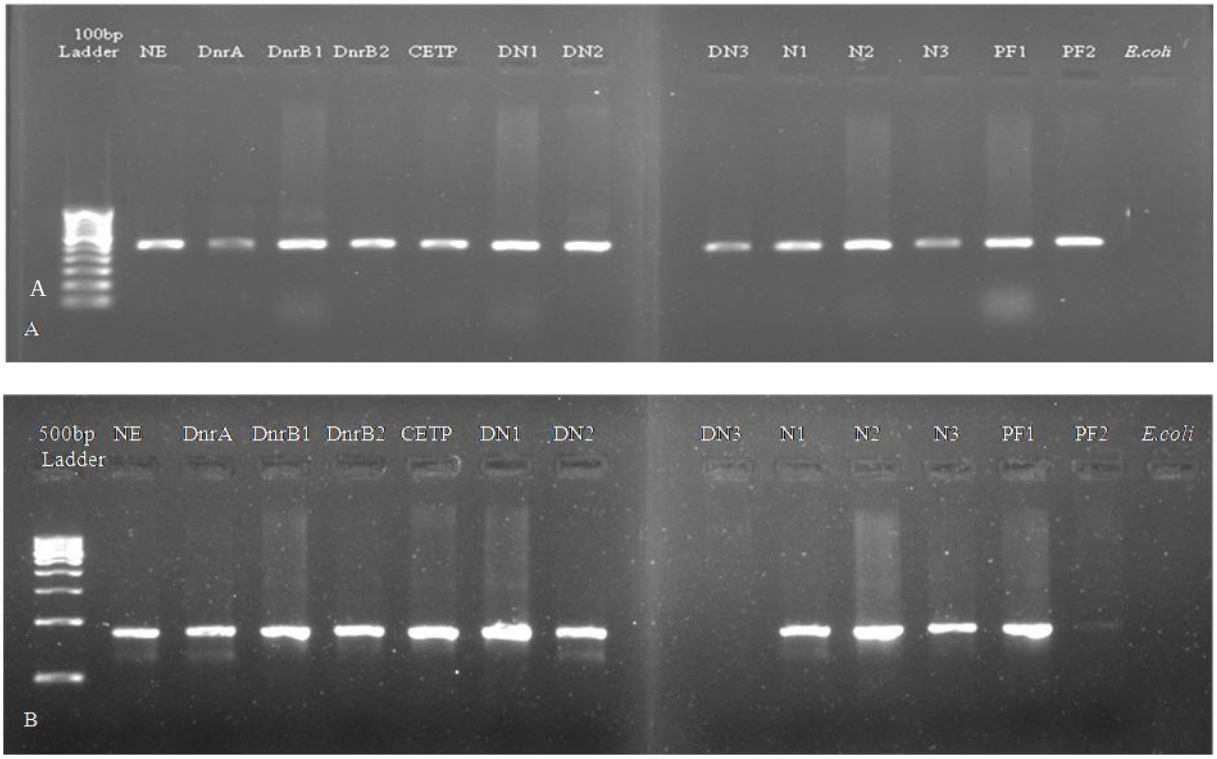


Fig 2a.3 Amplification of A) *amo*A and B) *hao* gene fragments from all isolates. NE- *Nitrosomonas europaea*.

*amo*A gene fragment amplification was observed in DN3 but it failed to show *hao* gene fragment amplification (Fig 2a.3). The amplified *amo*A and *hao* fragments were cloned and differentiated based on RLFP (Fig 2a.4).

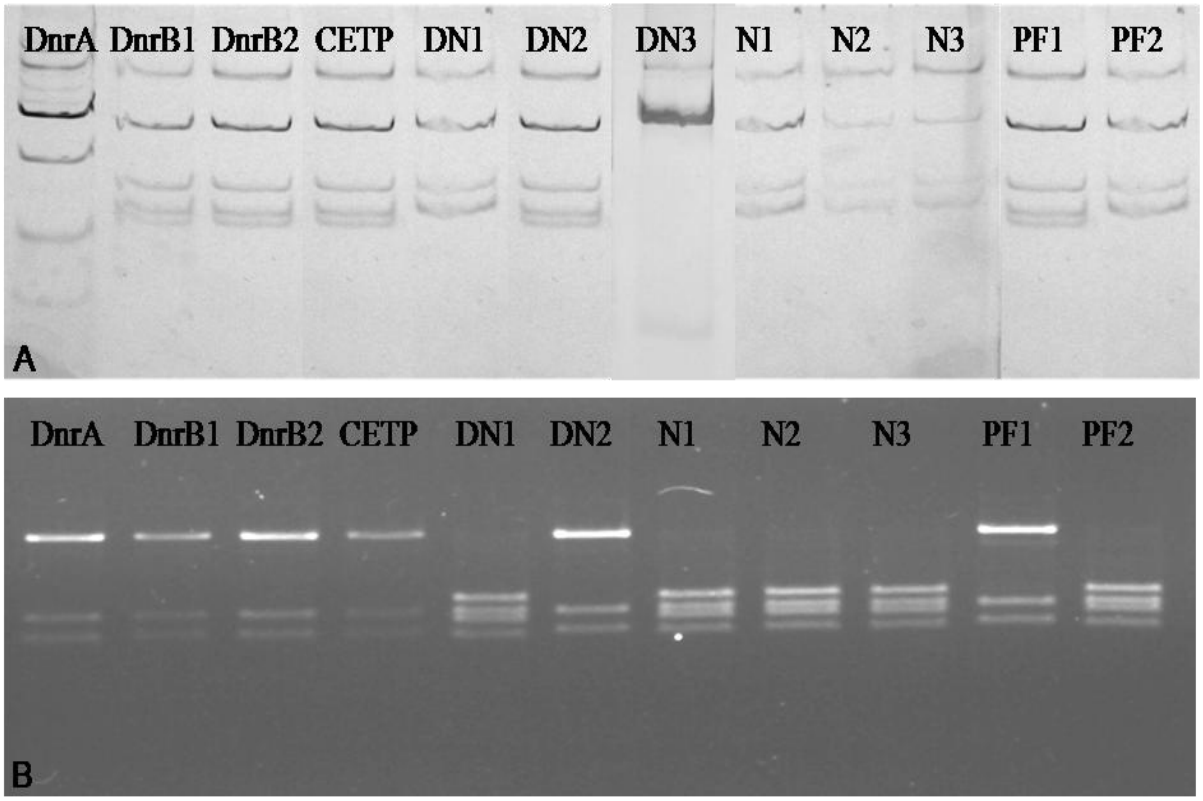


Fig 2a.4 RFLP patterns observed by digesting A) *amo*A gene fragment with *Hha*I *and Hae*III and B) *hao* gene fragment with *Msp*I restriction enzymes.

Four distinct patterns were observed by digesting *amo*A gene fragment with *Hha*I and *Hae*III restriction enzymes. DnrB1, DnrB2, CETP, DN2 and PF2 having (98 to 99% similarity) with *Nitrosomonas* sp DYS317 according to 16S rRNA gene analysis (Table 2a.2), showed similar *amo*A gene RFLP pattern. Similarly, DN1, N2, N3 and PF2 having (99%) similarity with *Nitrosomonas* sp. ENI11 (Table 2a.2) showed similar *amo*A gene RFLP pattern. N1 showing 87% similarity with *Nitrosospira* sp. NSP12, showed *amo*A gene RFLP pattern similar to DN1, N2, N3 and PF2 (Fig 2a.4A). DnrA and DN3 having (93 and 99%) similarity with *Nitrosomonas eutropha* according to 16S rRNA respectively, showed distinct *amo*A gene fragment RFLP patterns (Fig 2a.4B). RFLP of *hao* gene fragment showed only two patterns. DnrA, DnrB1, DnrB2, CETP, DN2, and PF1 grouped in one and DN1, N1, N2, N3 and PF2 formed a second group (Fig 2a.4B). Resolution of studied AOB obtained through *amo*A gene RFLP was similar to 16S rRNA gene fragment, whereas technique with

higher sensitivity is required for the proper resolution of AOB using *hao* gene fragment. SSCP analysis of both the gene fragments was therefore adapted.

Through SSCP analyses, *amo*A and *hao* gene fragments were resolved in to six and four groups respectively (Fig 2a5A and B). Statistical analysis showed significant differences in the SSCP patterns amongst samples with P values of <0.001.

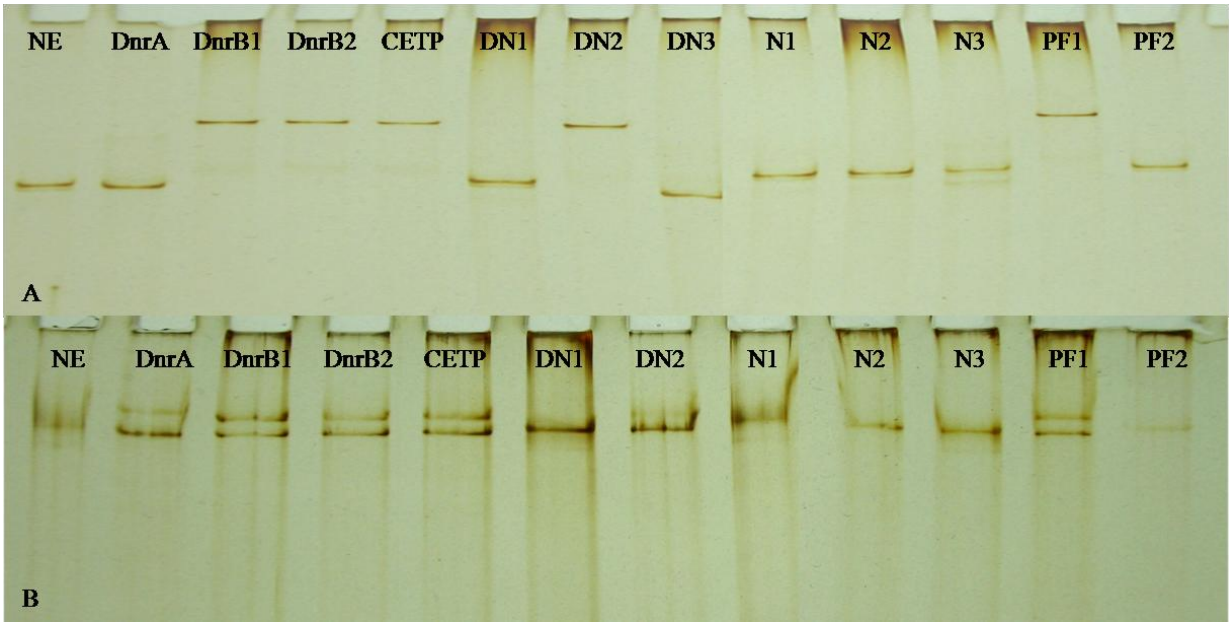


Fig 2a.5 Diversity of *Nitrosomonas* spp. A) *amo*A gene fragment (492bp) SSCP profiles B) *hao* gene fragment (850bp) SSCP profiles

According to the electrophoretic mobility of the single strand conformers of *amo*A gene fragment, *N. europaea*, DnrA and DN1 formed one group; DnrB1, DnrB2, CETP and DN2 formed a second group; and N1, N2 and PF2 formed third group; whereas DN3, N3 and PF1 showed distinct patterns (Fig 2a.5A). SSCP analysis of *hao* gene fragment grouped *N. europaea*, DN1, DN2, N2, N3 and PF1 together; DnrB1, DnrB2, CETP, and PF1 formed another group; and DnrA and N1 showed distinct patterns (Fig 2a.5B). Some nitrosomonads that failed to get resolved through *amo*A gene fragment SSCP, like DnrA from *N. europaea*; DN2 from DnrB1, DnrB2, and CETP; and N1 from N2 and PF2; were easily distinguished through SSCP analysis of *hao* gene fragment (Fig 2a.5A and B). Similarly, PF2 and N3 that could not be resolved through

SSCP analysis of *hao* gene fragment were distinguished through *amo*A gene fragment SSCP (Fig 2a.5A and B). DN3 *hao* gene fragment that could not be amplified using haoF4 and haoR2 primer set was subsequently amplified using reverse primer haoR3. However, owing to the difference in the size of *hao* amplicon obtained from DN3, it could not be included in the *hao* gene SSCP analysis. N1 grouped with N2 and PF2 as per *amo*A gene analysis but according to 16S rRNA gene sequence analysis, N1 showed 87% identity with *Nitrosospira* sp. Nsp 12 whereas N2 and PF2 showed 99% identity to *Nitrosomonas* sp. ENI-11, (Table 2a.2), indicating N1 to be phylogenetically very different from the other member of this group. Similar results were also obtained by Böttcher (1996) and Juretschko et al., (1998) where unexpected high similarity in *amo*A gene observed between different genera resulted from lateral *amo*A gene transfer events between species of ammonia oxidizers of different genera. A similar phenomenon may be occurring with N1, residing in close vicinity with N2 in the same niche. Overall 53.8% resemblance was observed between both the genes in fingerprinting of enriched soil and sludge samples for AOB diversity studies.

2a.3.4 Diversity of AOB observed through HAO enzyme activity staining

HAO enzyme activity staining was used for the first time to study the diversity of the enriched *Nitrosomonas* spp. To begin with, HAO enzyme activity staining technique was validated by using appropriate controls (Fig 2a.6). HAO activity bands were not observed in substrate control (without hydroxylamine) and *E. coli* cell free extract (Fig 2a.6). Loss of *N. europaea* HAO activity was observed upon addition of 2-mercaptoethanol (Fig 2a.6) [being a reducing agent, it cleaved disulfide bonds at the active site of the enzyme (Arcerio and Hooper, 1993)]. These controls confirmed that the activity bands were specific to HAO enzyme and were not mere artifacts.

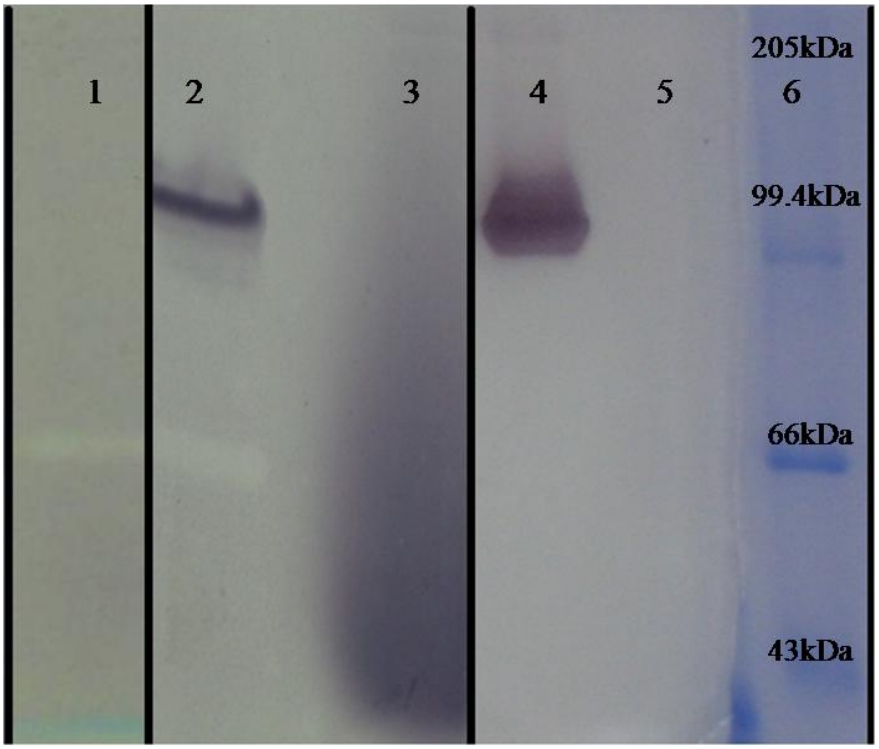


Fig 2a.6 Validation of HAO activity staining using different controls. Lane 1. Substrate control without hydroxylamine. Lane 2 and 4. *N. europaea*, positive control. Lane 3. Loss of activity in the presence of 2-mercaptoethanol. Lane 5. *E. coli* DH5α, negative control and Lane 6 Protein marker stained with coomassie brilliant blue G250.

Variation in the electrophoretic mobility of the enzyme was observed on the SDS gel (Fig. 2a.7) contradicting to the results of Nejidat et al., (1997) where no difference was observed in HAO of *Nitrosomonas europaea* ATCC 19718, *Nitrosomonas europaea* LMD 86.25, *Nitrosomonas eutropha* N904, *Nitrosomonas* spp. and *Nitrosolobus* spp. The resolution of enzymes in the present study could be attributed to the extended electrophoresis time (1 hr after the removal of the dye front from the gel, at 70V), resulting in significant resolution in the HAO enzyme activity staining bands of *Nitrosomonas* spp. (P values < 0.001).

The zymogram pattern of HAO enzyme (Fig 2a.7) was analyzed and compared with *amoA* and *hao* gene fragment resolution. DN1 that could not be differentiated from *N. europaea* by SSCP analysis of both genes was resolved

through HAO enzyme zymogram (Fig 2a.7). *amo*A gene fragment SSCP showed 61.5% similarity with HAO enzyme zymogram while *hao* gene fragment SSCP showed 46.1% similarity with HAO enzyme zymogram in differentiating AOB. Although *hao* gene from DN3 failed to get amplified using the reported primers (haoF4 and haoR2), its HAO enzyme activity band appeared in the zymogram that was distinctly different from the other HAO enzyme of the same genus (Fig 2a.7). Since the technique is independent of the sequence variations, it could also be used to obtain information for DN3 isolate. The method thus takes into consideration more diverged species of AOB that may not be considered by general PCR based fingerprinting methods, thus providing additional information in the diversity study of AOB, however, the method cannot be applied directly to environmental samples.

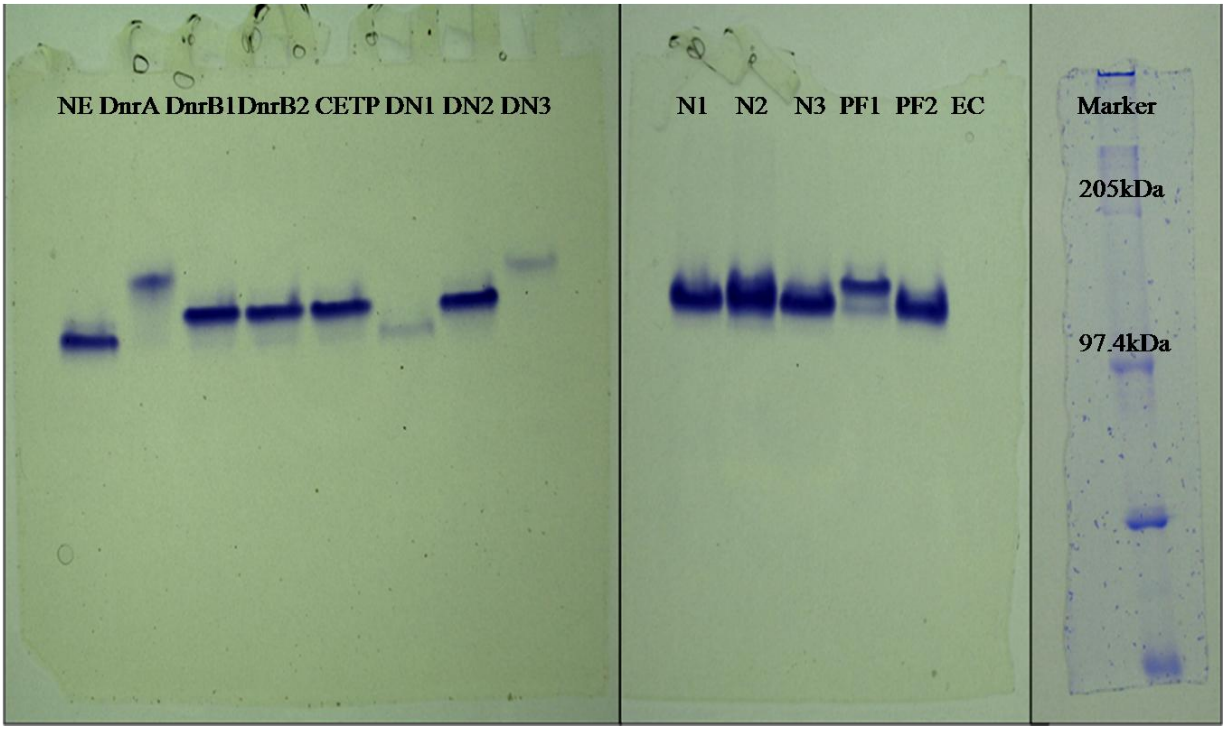


Fig 2a.7 Hydroxylamine oxidoreductase (HAO) zymogram pattern of *Nitrosomonas* spp. (NE-*N. europaea* and EC - *E. coli* DH 5α).

2a.3.5 Statistical analysis of AOB diversity

Simpson's Index of Diversity (Simpson, 1949) calculated for all the three methods was found to be 0.64 for HAO enzyme zymogram, 0.85 for *amo*A gene fragment SSCP and 0.68 for *hao* gene fragment SSCP.

Richness and evenness in the population as depicted by the SSCP analysis of *amo*A and *hao* gene fragments and HAO enzyme zymogram are shown in Fig 2a.8. Higher AOB species richness and evenness was observed in SSCP analysis of *amo*A gene fragment, compared to *hao* gene fragment (Fig 2a.8A and B). Some AOB that were not differentiated through SSCP analysis of *amo*A gene fragment, got resolved through *hao* gene fragment SSCP and vice versa. Therefore, when *amo*A and *hao* gene fragments SSCP patterns were analyzed together, the thirteen AOB got differentiated into eight groups (Simpson's diversity index – 0.9) with not more than 31% population belonging to a single group, thereby indicating higher richness and evenness (Pielou index of evenness 0.93) in the diversity of AOB compared to their individual analysis.

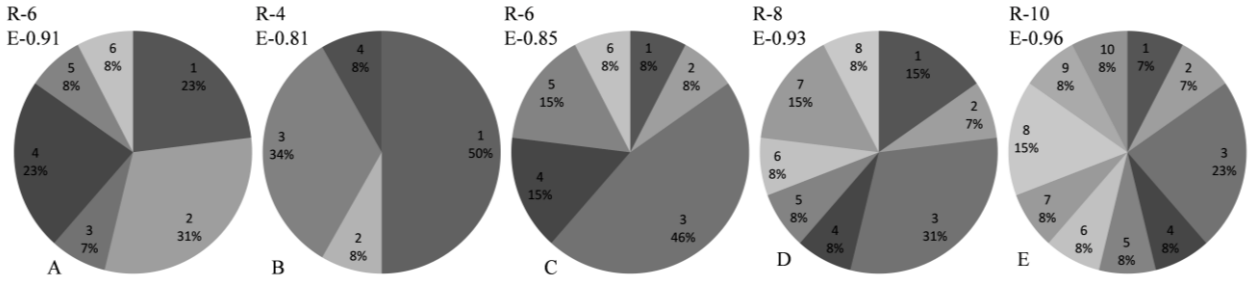


Fig 2a.8 Richness and evenness in the diversity of *Nitrosomonas* spp. A) *amo*A gene fragment SSCP B) *hao* gene fragment SSCP C) HAO activity staining D) concerted analysis of *amo*A and *hao* gene fragment SSCP E) concerted analysis of *amo*A and *hao* gene fragment SSCP together with HAO activity staining zymogram. (R-richness and E-evenness).

Through HAO enzyme activity staining, the studied AOB could be differentiated into six groups (Fig 2a.8C), with one of the groups predominantly representing 46% of the total AOB studied (Fig 2a.8C).

Species richness observed through HAO enzyme activity staining was comparable to *amo*A gene fragment SSCP analysis but evenness in the population was less (Pielou index of evenness 0.85), whereas when HAO enzyme activity staining was compared with *hao* gene fragment SSCP analysis, higher richness and evenness in the diversity of AOB was observed (Fig 2a.8 B and C). *hao* gene fragment under study constitutes just 39.1% of the hydroxylamine oxidoreductase complete coding DNA sequence (CDS) (Sayavedra-Soto et al., 1994; Schmid et al., 2008). Therefore, the diversity of AOB represented here through *hao* gene fragment is just a small fraction of the total diversity that can be represented by hydroxylamine oxidoreductase complete CDS. This justifies the higher diversity observed in the AOB population by HAO enzyme activity staining (involving complete enzyme) than SSCP analysis of *hao* gene fragment. Albeit, SSCP of *hao* gene fragment cannot be ignored, as some AOB that could not be differentiated through *amo*A gene SSCP and HAO enzyme activity staining e.g. N1 could be differentiated from N2 only by *hao* gene fragment SSCP (Fig 2a.5 and 2a.7). Interestingly, the concerted use of these three techniques differentiated the thirteen AOB into ten groups (Simpson's index of diversity – 0.95) (Fig 2a.8E), projecting higher richness and evenness (Pielou index of evenness 0.96) in the studied population.

In conclusion, these methods differentiated *Nitrosomonas* spp. with resolution in the following order: *amo*A gene fragment SSCP > *hao* gene fragment SSCP > HAO enzyme zymogram. Hence, amongst all the techniques used in the present study to resolve AOB based on single genus, *amo*A gene fragment SSCP exhibited highest potential and therefore is a superior functional marker than *hao* gene fragment. Better primers are required to be designed for *hao* gene to be used analogous to *amo*A gene SSCP and HAO enzyme activity staining, so that *hao* can be amplified from all AOB. HAO enzyme zymogram technique being simple to perform can be used as a preliminary method to study diversity. The conserted use of these polyphasic approaches provides a better understanding of their pivotal role in metabolic and functional diversity in the biogeochemical cycle of varied ecosystem.

Chapter 2b

Assessing hao as a molecular and phylogenetic marker in comparison with amoA and 16S rRNA genes for analyzing autotrophic Ammonia Oxidizing Bacteria

Chapter 2b

Assessing *hao* as a molecular and phylogenetic marker in comparison with *amo*A and 16S rRNA genes for analyzing autotrophic Ammonia Oxidizing Bacteria

ABSTRACT

Hydroxylamine oxidoreductase (*hao*) gene fragment was used as a functional and phylogenetic marker to study diversity in 21 autotrophic Ammonia Oxidizing Bacteria (AOB). It was further compared with 16S rRNA gene and ammonia monooxygenase (*amo*A) gene fragment which are conventionally used as phylogenetic and functional markers to study the diversity of AOB. Phylogenetic relationship amongst AOB observed through the Neighbor-Joining method revealed similar tree topologies for 16S rRNA, *amo*A and *hao* gene fragments. Grouping of AOB according to the three genes was statistically analyzed and no significant difference was observed between them. Magnitude of genetic variation in AOB was studied for the first time by comparing 16S rRNA, *amo*A and *hao* genes fragments. Transition bias was observed in the three genes on studying their mutational patterns. Negative association observed between transitions/transversions (Ti/Tv) ratio and sequence distance indicated lower sequence divergence in 16S rRNA gene than *amo*A and *hao* gene fragments. The patterns of synonymous and non-synonymous substitution within AOB through *amo*A and *hao* gene fragments were found to be similar. The non-synonymous mutations did not cause major changes in the structure of the protein. The present study for the first time reports co-evolution of *amo*A and *hao* gene in AOB through simple linear regression (r^2 value 0.9) and Pearson's correlation coefficient (r-0.949, p value <0.001). Similarity in the phylogeny of AOB, based on 16S rRNA, *hao,* and *amo*A gene fragments and its co-evolution with *amo*A gene fragment irrefutably suggests high potential of *hao* gene fragment to be used as a functional and phylogenetic marker to study AOB.

2b.1 Introduction

16S rRNA gene is the most conventionally used phylogenetic marker to study the diversity and phylogeny of autotrophic Ammonia Oxidizing Bacteria (AOB) (Head et al., 1993; McCaig et al., 1994; Purkhold et al., 2000; 2003) but there are few limitations to this, i) it fails to state the physiology of the microorganisms ii) change in specificity caused by mutations in 16S rRNA gene leads to detection of phylogenetically close but physiologically and ecologically unrelated microorganisms and iii) detection of microorganisms present in very low number is difficult. These limitations can be easily overcome by using genes exclusively present and unique to the microorganisms of interest reducing the possibility of detecting non-target organisms (Aakra et al., 2001a and b; Junier et al., 2010).

Oxidation of ammonia to hydroxylamine, the first step of aerobic ammonia oxidation by AOB, is catalyzed by the ammonia monooxygenase (AMO). Its α-subunit (*amo*A) has been used as a fine scale molecular marker in studying the diversity of AOB (Rotthauwe et al., 1997; Kowalchuk et al., 2000; Qui et al., 2010; Wang et al., 2011). Existence of *amo*A ortholog is also documented in autotrophic ammonia oxidizing archaea (AOA) through metagenomic studies (Könneke et al., 2005) and has been used in several studies to check the abundance and diversity of both AOB and AOA (Bernhard et al., 2010; Junier et al., 2010; Jin et al., 2011). The main drawbacks of using *amo*A gene fragment are that it is relatively short and in some studies it has provided lesser resolution than 16S rRNA gene (Purkhold et al., 2003). This emphasizes on the need for an alternate marker (Purkhold et al., 2003).

An octahaem enzyme, hydroxylamine oxidoreductase (HAO), catalyzes the conversion of hydroxylamine to nitrite in AOB (Arp et al., 2002). An ortholog of the enzyme though not present in AOA (Hallam et al., 2006) is reported in anammox bacteria oxidizing hydroxylamine as well as hydrazine (Shimamura et al., 2008). The use of *hao* as a molecular marker for identifying AOB was suggested by Shinozaki and Fukui (2002), and subsequently Schmid et al., (2008) used it to detect AOB from environmental

samples. Nevertheless, its use as a phylogenetic and functional marker in comparison with *amo*A and 16S rRNA genes needs detailed investigation.

Holmes et al., (1995) showed evidences of evolutionary relationship of AMO with particulate methane monooxygenase and later Klotz and Norton (1998) showed that the concerted relationship between these occurred under AT/GC mutational pressure. HAO on the other hand has been reported to have evolved from octahaem cytochrome *c* nitrite reductase (Klotz et al., 2008). Evolutionary relationship between these enzymes (AMO and HAO) involved in the same pathway has not been studied till date. *amo*A gene fragment has been widely used as a marker for studying AOB, if *hao* coevolves with *amo*A than it can be used as a marker in equivalence with *amo*A in studying AOB. This emphasizes the need to study co-evolution of both the genes. The present study therefore was aimed to apply *hao* gene fragment for the phylogenetic studies of AOB. Its efficiency as a phylogenetic marker was compared with *amo*A and 16S rRNA genes based phylogeny. Co-evolution of *amo*A and *hao* genes was studied for the first time to espouse the use of *hao* as a phylogenetic marker. Effect of mutations on the structure of hydroxylamine oxidoreductase enzyme of all reported AOB were also examined.

2b.2 Materials and Methods

2b.2.1 Sequence data

Sequences of 16S rRNA, *amo*A and *hao* genes of 21 AOB (9 AOB strains belonging to β and γ – subclass of proteobacteria and 12 uncultured AOB from this study) were analyzed (Table 2b.1). Sequences for the AOB strains were obtained from GenBank, whereas information from the 12 uncultured AOB were derived by cloning and sequencing the genes in author's lab as mentioned in Chapter 2a. The sequences have been submitted by the authors to GenBank (unpublished data). The size of *hao* genes used ranged from 654bp to 850bp. Two strains (*Nitrosospira* sp. 40KI and *Nitrosospira* sp. III7) were not considered in the study as their *hao* sequences were too short

(243bp). Size of *amo*A gene ranged from 453bp to 883bp whereas the size of 16S rRNA gene ranged from 1207bp to 1529bp. Accession numbers of all the genes are mentioned in the phylogenetic tree constructed to examine the evolutionary relationship of the AOB.

2b.2.2 Mutation and phylogenetic analysis

Multiple sequence alignments were performed using ClustalW. Maximum likelihood estimate of the patterns of nucleotide substitution and the mean diversity of the entire population was calculated independently for the three genes using MEGA 4 software.

Neighbor-Joining trees for 16S rRNA, *amo*A and *hao* genes were generated based on the nucleotide sequence divergence using MEGA 4 software. Phylogenetic trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons (pair wise deletion option).

Codon-based evolutionary diversity for the entire population was calculated for *amoA* and *hao* genes. Total effective and non-effective mutations and rates of synonymous and non-synonymous mutations were calculated using MEGA 4.0 software. Correlation plots based on percentage sequence similarity between species were plotted for 16S rRNA, *amo*A and *hao* where simple linear regression was applied. Pearson's correlation coefficient was determined using SigmaStat version 3.5 for the sequence similarity between the three genes.

2b.2.3 Statistical analyses

Shannon's diversity index (*H'*) (that takes into consideration both richness and abundance) was used to measure the diversity of the studied AOB using 16S rRNA, *amo*A and *hao* genes according to the equation (Shannon, 1948).

$$H' = -\sum_{i=1}^{s} (\mathrm{Pi}) \log (\mathrm{Pi})$$

Where S is the number of AOB groups obtained through the three gene fragments.

Evenness in the population was measured by calculating Shannon's *E*, $E=H'/\ln S$ where *S* represents maximum diversity for a community with S observed species. To find whether the diversity of AOB calculated through Shannon's diversity index using the three genes was statistically different from each other, Shannon's t-test was carried out which included calculating variance of the estimator for the Shannon's index (*VarH')* and degrees of freedom (*df*). All calculations were carried out according to Abundance Curve calculator by Dr. James A Danoff-Burg and X. Chen.

2b.2.4 Structure comparison of hydroxylamine oxidoreductase

Hydroxylamine oxidoreductase protein sequence alignment was carried out in Clone Manager 7 version 7.11. According to the alignment the *hao* sequences were grouped and protein structure of one representative from each group was obtained by homology modeling using SWISS-MODEL workspace, an integrated Web-based modeling expert system (Arnold et al., 2006) and their pdb files were generated. These structures were aligned with hydroxylamine oxidoreductase of *Nitrosomonas europaea* (pdb code 1FGJ) and the 3D structure alignments were analyzed in Accelrys Discovery studio Visualizer v2.0.1.7347 (Accelrys Software Inc.).

2b.3 Results and Discussion

Oxidation of ammonia to nitrite in AOB is a two step process, wherein the first reaction of the oxidation of ammonia to hydroxylamine is catalyzed by ammonia monooxygnase and the second step of the oxidation of hydroxylamine to nitrite is catalyzed by hydroxylamine oxidoreductase (Hooper et al., 1997). Gene coding for the active site of ammonia monoxygenase (*amo*A) has been widely used as a marker for studying the

diversity and phylogenetic analysis of AOB (Rotthauwe et al., 1997), but *hao* has not been widely studied as a functional and phylogenetic marker. After the development of primers for *hao* and its amplification by Schmid et al., (2008), quite a few *hao* sequences have been deposited in the databanks, but their number is relatively less as compared to *amo*A and 16S rRNA gene fragments. With the aim to compare *hao* as marker with *amo*A (a well established functional marker) and 16S rRNA gene (a conventional phylogenetic marker) the study was initiated by obtaining *hao* sequences from GenBank of those AOB whose *amo*A and 16S rRNA gene sequence data were also available (Table 2b.1).

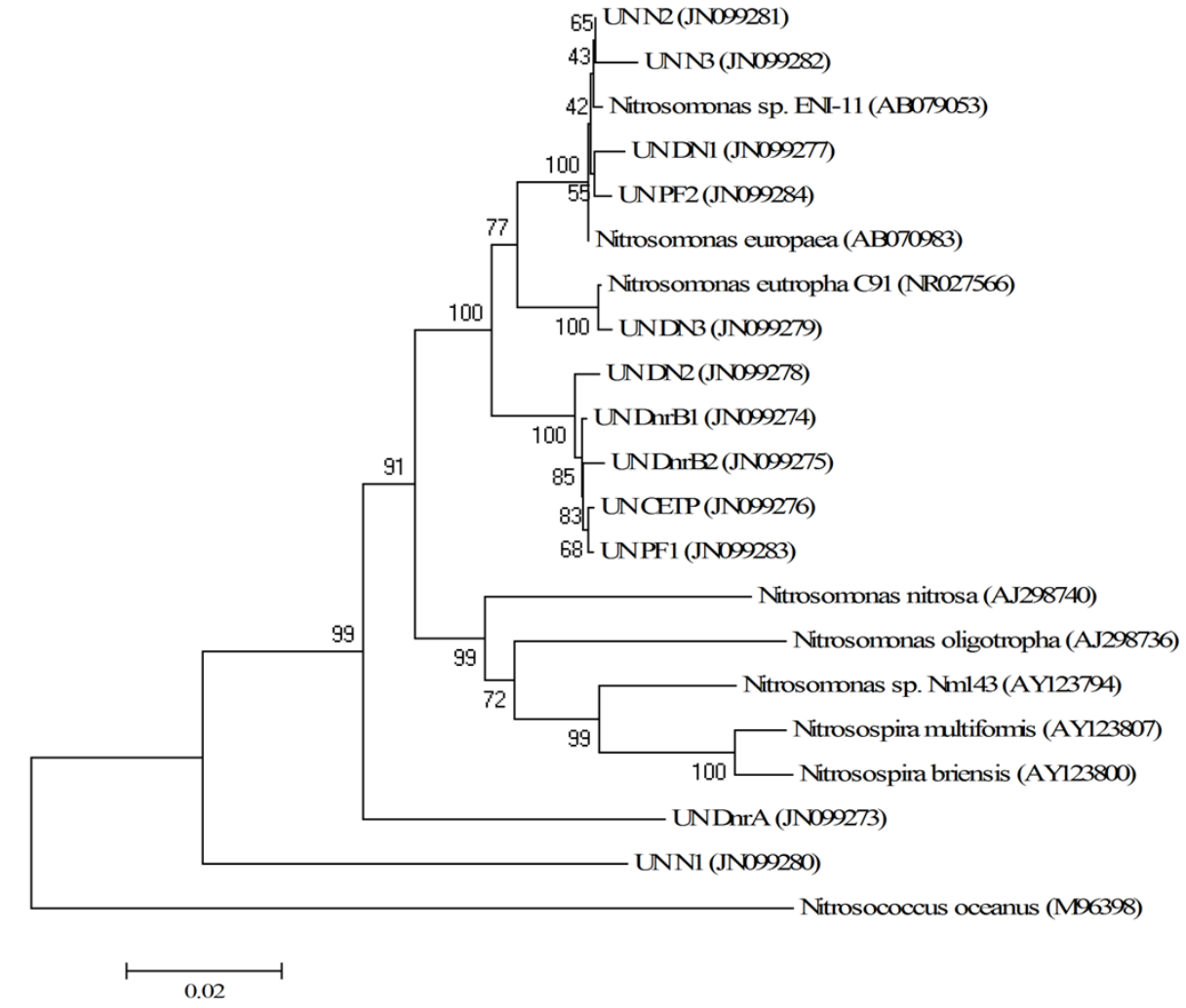
Table 2b.1 Sources of 16S rRNA, *amo*A and *hao* genes analyzed in this study.

	16S rRNA		*amo*A		*hao*	
	Accession No.	Reference	Accession No.	Reference	Accession No.	Reference
Nitrosomonas sp. ENI 11	AB079053	Hirota et al. 2000	AB079054	Hirota et al. 2000	AB030387	Hirota et al. 2000
Nitrosomonas europaea	AB070983	Shinozaki and Fukui 2002	JN099309	Keluskar and Desai *	U04053	Sayavedra- Soto et al. 1994
Nitrosomonas nitrosa	AJ298740	Aakra et al. 2001	AF272404	Purkhold et al. 2000	FM163624	Schmid et al. 2008
Nitrosomonas oligotropha	AJ298736	Aakta et al. 2001	AF272422	Purkhold et al. 2000	FM163625	Schmid et al. 2008
Nitrosomonas sp. Nm143	AY123794	Purkhold et al. 2003	AY123816	Purkhold et al. 2003	FM163622	Schmid et al. 2008
Nitrosomonas eutropha C91	NR027566	Stein et al. 2007	U51630	Norton et al. 2002	CP000450	Stein et al. 2007
Nitrosococcus oceanus	M96398	Head et al. 1993	U96611	Norton et al. 2002	AY858555	Bergmann et al. 2005
Nitrosospira multiformis	AY123807	Purkhold et al. 2003	DQ228454	Ida et al. unpublished	AB070980	Shinozaki Fukui 2002
Nitrosospira briensis	AY123800	Purkhold et al. 2003	U76553	Norton et al. 2002	FM163621	Schmid et al. 2008
Uncultured Bacterium (UN) DnrA (This study)	JN099273	Keluskar and Desai*	JN099285	Keluskar and Desai *	JN099297	Keluskar and Desai *
UN DnrB1 -do-	JN099274	Keluskar and Desai*	JN099286	Keluskar and Desai *	JN099298	Keluskar and Desai *
UN DnrB2 -do-	JN099275	Keluskar and Desai *	JN099287	Keluskar and Desai *	JN099299	Keluskar and Desai *
UN CETP -do-	JN099276	Keluskar and Desai *	JN099288	Keluskar and Desai *	JN099300	Keluskar and Desai *
UN DN1 -do-	JN099277	Keluskar and Desai *	JN099289	Keluskar and Desai *	JN099301	Keluskar and Desai *
UN DN2 -do-	JN099278	Keluskar and Desai *	JN099290	Keluskar and Desai *	JN099302	Keluskar and Desai *
UN DN3 -do-	JN099279	Keluskar and Desai *	JN099291	Keluskar and Desai *	JN099303	Keluskar and Desai *
UN N1 -do-	JN099280	Keluskar and Desai *	JN099292	Keluskar and Desai *	JN099304	Keluskar and Desai *
UN N2 -do-	JN099281	Keluskar and Desai *	JN099293	Keluskar and Desai *	JN099305	Keluskar and Desai *
UN N3 -do-	JN099282	Keluskar and Desai *	JN099294	Keluskar and Desai *	JN099306	Keluskar and Desai *
UN PF1 -do-	JN099283	Keluskar and Desai *	JN099295	Keluskar and Desai *	JN099307	Keluskar and Desai *
UN PF2 -do-	JN099284	Keluskar and Desai *	JN099296	Keluskar and Desai *	JN099308	Keluskar and Desai *

*Indicates unpublished work of the authors

2b.3.1 Phylogenetic analysis of AOB with respect to 16S rRNA, *amo*A and *hao* genes

Detailed phylogeny of AOB was determined by sequence analyses of 16S rRNA, *amo*A and *hao* gene fragments. Trees constructed using Neighbor-Joining method were found to be similar for all the three genes with 100 bootstrap replicates, clustering *Nitrosomonas* and *Nitrosospira* lineages (betaproteobacterial AOB) together separated from *Nitrosococcus* lineage (gammaproteobacterial AOB) (Fig 2b.1). Of the AOB examined in the present study, *Nitrosomonas* sp. Nm143, *Nitrosospira multiformis* and *Nitrosospira briensis* always grouped together separated from the *Nitrosomonas* lineage in all the genes (Fig 2b.1). *Nitrosomonas oligotropha* showed higher similarity with *Nitrosospira* lineage in *amo*A and 16S rRNA gene phylogenetic trees and separated from the *Nitrosomonas* lineage whereas in *hao* phylogenetic tree, it was included in the *Nitrosomonas* lineage. *Nitrosomonas nitrosa* too was not included in the *Nitrosomonas* lineage in the 16S rRNA phylogeny whereas according to *amo*A and *hao* phylogeny it was included in the *Nitrosomonas* lineage (Fig 2b.1).


UN N2 (JN099281)
UN N3 (JN099282)
Nitrosomonas sp. ENI-11 (AB079053)
UN DN1 (JN099277)
UN PF2 (JN099284)
Nitrosomonas europaea (AB070983)
Nitrosomonas eutropha C91 (NR027566)
UN DN3 (JN099279)
UN DN2 (JN099278)
UN DnrB1 (JN099274)
UN DnrB2 (JN099275)
UN CETP (JN099276)
UN PF1 (JN099283)
Nitrosomonas nitrosa (AJ298740)
Nitrosomonas oligotropha (AJ298736)
Nitrosomonas sp. Nm143 (AY123794)
Nitrosospira multiformis (AY123807)
Nitrosospira briensis (AY123800)
UN DnrA (JN099273)
UN N1 (JN099280)
Nitrosococcus oceanus (M96398)
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100
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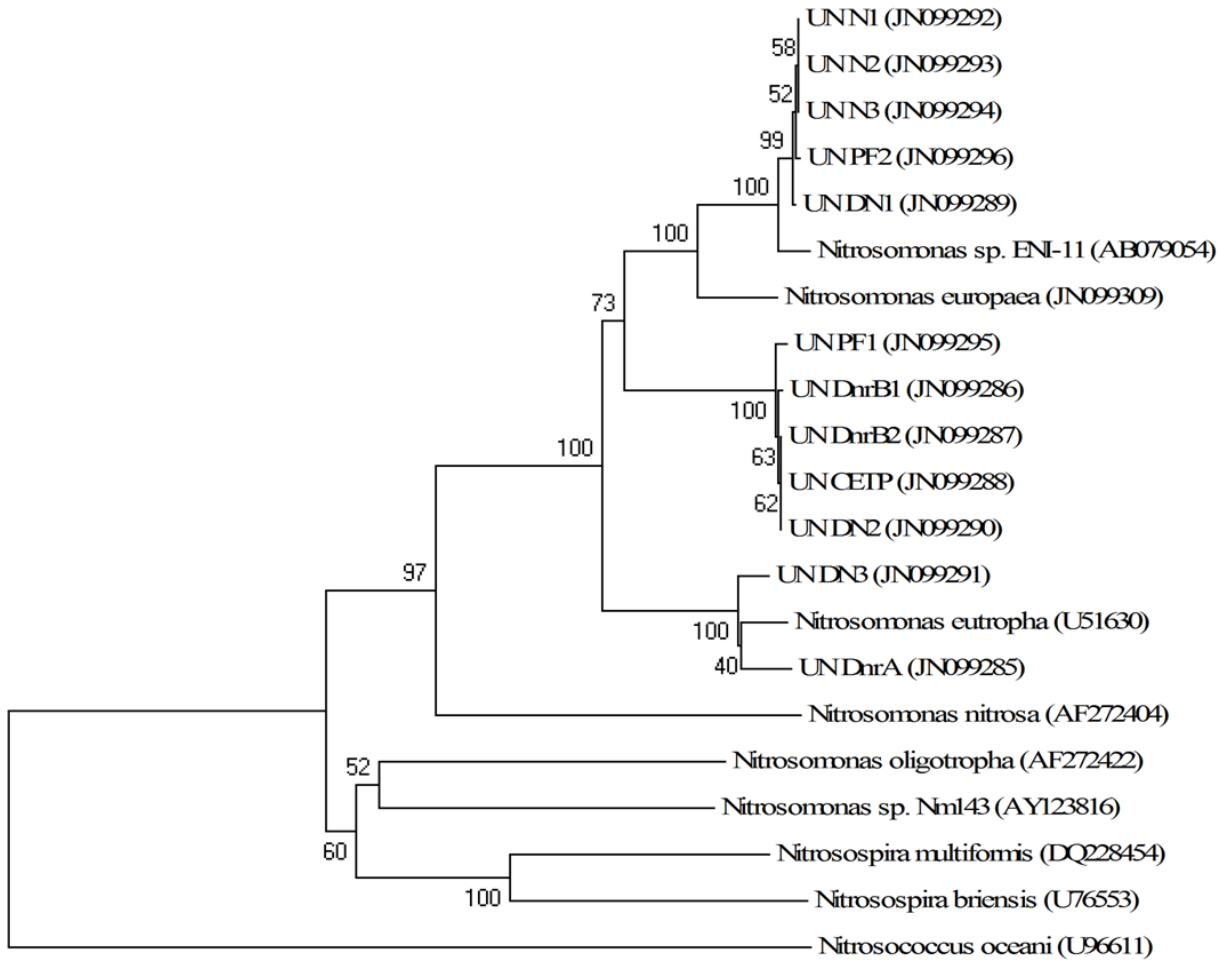
83
68
99
99
72
99
100
0.02

A


UN N1 (JN099292)
UN N2 (JN099293)
UN N3 (JN099294)
UN PF2 (JN099296)
UN DN1 (JN099289)
Nitrosomonas sp. ENI-11 (AB079054)
Nitrosomonas europaea (JN099309)
UN PF1 (JN099295)
UN DnrB1 (JN099286)
UN DnrB2 (JN099287)
UN CETP (JN099288)
UN DN2 (JN099290)
UN DN3 (JN099291)
Nitrosomonas eutropha (U51630)
UN DnrA (JN099285)
Nitrosomonas nitrosa (AF272404)
Nitrosomonas oligotropha (AF272422)
Nitrosomonas sp. Nm143 (AY123816)
Nitrosospira multiformis (DQ228454)
Nitrosospira briensis (U76553)
Nitrosococcus oceani (U96611)
58
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B

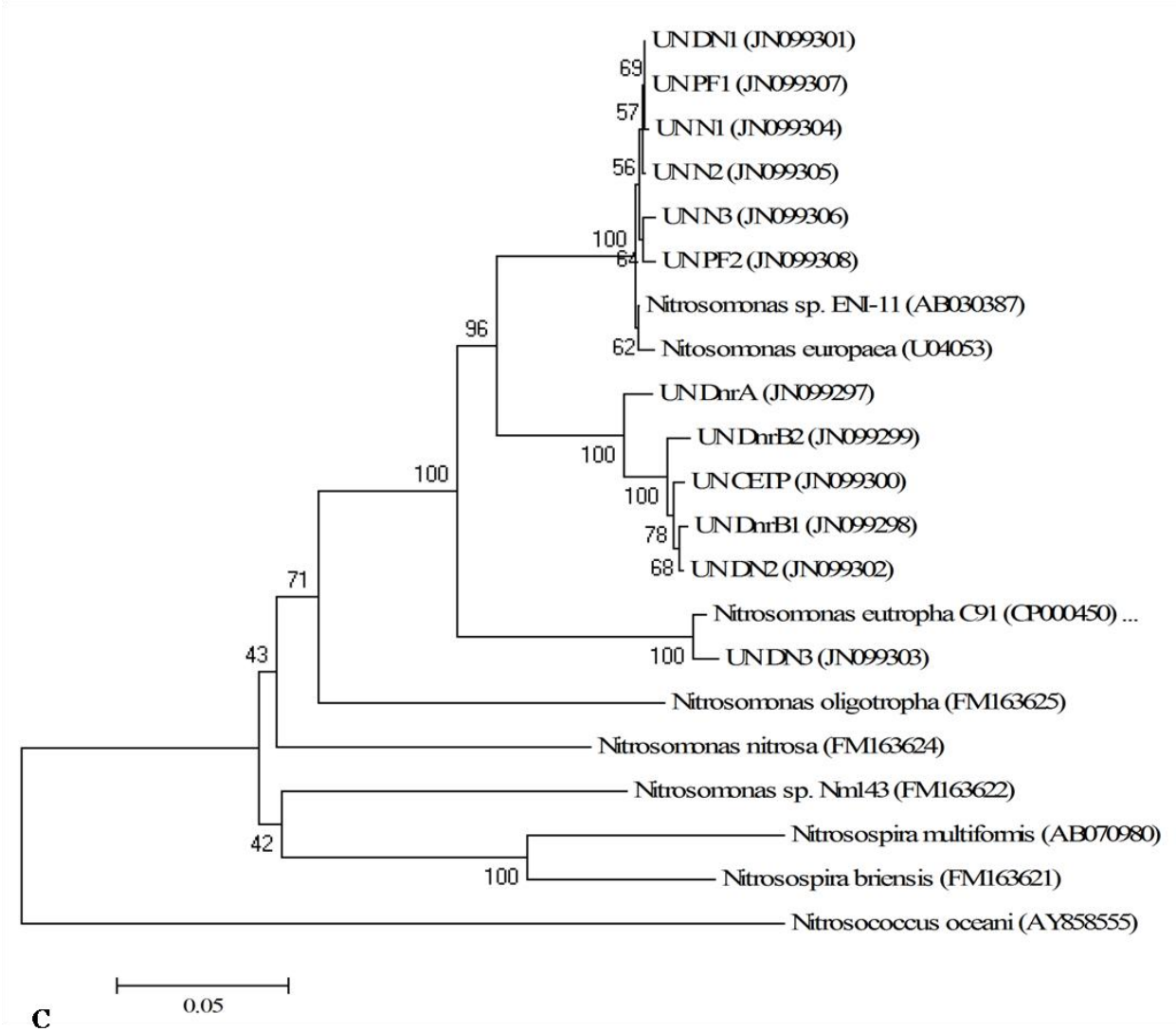


Fig 2b.1 Phylogenetic tree reflecting the relationships of the 21 AOB with respect to the three genes A) 16S rRNA gene B) *amo*A and C) *hao* using the Neighbor-Joining method. The optimal trees based with the sum of branch length = 0.452 for 16S rRNA gene, 1.18 for *amo*A and 1.073 for *hao*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Bar indicates 2% estimated sequence divergence in case of 16S rRNA gene and 5% estimated sequence divergence for *amo*A and *hao* trees.

Nitrosomonas lineage in the present study constituted of three stable branches with 100 bootstrap value. *Nitrosomonas nitrosa* and *Nitrosomonas oligotropha* were not included in *Nitrosomonas* lineage in all the three phylogenies (Fig 2b.1). The first group comprised of UN N2, UN N3, UN PF2,

UN DN1, *Nitrosomonas* sp. ENI 11 and *Nitrosomonas europaea* where UN N1 with 87% 16S rRNA gene sequence similarity with *Nitrosospira sp.* NSP12 formed a separate branch in 16S rRNA gene sequence identity while it clustered in group one in *amo*A (99.8 to 100% sequence identity [Appendix I Table 2]) and *hao* (99.3 to 99.9% sequence identity [Appendix I Table 3]) phylogeny. The second sister branch consisted of UN DnrB1, UN DnrB2, UN CETP, UN DN2 and UN PF1 having 98 to 99% 16S rRNA gene sequence identity (Appendix I Table 1) with *Nitrosomonas* sp. DYS317 and *Nitrosomonas* sp. DYS323. UN PF1 showing higher identity (99.4 to 100%) (Appendix I Table 1) with members of second branch as per 16S rRNA and *amo*A gene, clustered along with branch 1 in the *hao* phylogeny (99.2 to 99.9% sequence identity [Appendix I Table 3]) (Fig 2b.1). Since UN PF1 and UN PF2 had been obtained from the same niche (paddy field), its clustering with branch 1 as per *hao* gene phylogeny is indicative of possible horizontal gene transfer of this gene, which is a common phenomenon observed in closely associated AOB in the natural habitats (Aakra et al., 2001a; Bergmann et al., 2005; Junier et al., 2010). The third close branch consisted of *Nitrosomonas eutropha* C91 and UN DN3. Whereas UN DnrA having 93% identity with *N. eutropha* C91 formed a separate branch in the 16S rRNA gene phylogeny, it clustered in branch 3 (97.3 to 97.8% similarity [Appendix I Table 1]) and in branch 2 (97.1 to 98.1% identity [Appendix I Table 2 and 3]) as per *amo*A and *hao* phylogeny respectively indicating it to be of a novel species of the *Nitrosomonas* genus (Fig 2b.1).

Shannon's index of diversity calculated for 16S rRNA, *amo*A and *hao* gene fragments were 1.7, 1.73 and 1.64 respectively. Evenness in the population calculated were 0.87, 0.88 and 0.85 for 16S rRNA, *amo*A and *hao* genes respectively. These indicated similarities in the diversity of the studied AOB with respect to both richness and evenness in the population. Paired t-tests were applied to the Shannon's index of diversity observed in AOB based on 16S rRNA, *amo*A and *hao* gene fragments. Based on the results a hypothesis was set:

H_0 (null hypothesis): There is no significant difference in the diversity of AOB with respect to the pairs of genes- (16S rRNA and *amo*A; *amo*A and *hao* and 16S rRNA and *hao* genes).

t-tests values calculated for 16S rRNA and *amo*A genes pair was 0.137 which is less than the table value (2.326) (Kothari, 2004) at 0.05 level of significance, similarly t-tests value calculated for *amo*A and *hao* gene pair was 0.531 and for 16S rRNA and *hao* gene pair was 0.163. These calculated t-test values were less than the table value and hence the null hypothesis is true. Moreover, p value of two-tailed test were 0.84, 0.6 and 0.87 for the three pairs of genes mentioned above indicating that there is no significant difference in the diversity observed through 16S rRNA, *amo*A and *hao* gene fragments.

The overall phylogeny of 16S rRNA, *amo*A and *hao* genes showed similarity in tree topology but had distinctly different branch length showing differential deviation from the ancestral genes (Fig 2b.1). The branch lengths were in the following order *amo*A > *hao* > 16S rRNA (Fig 2b.1). Diversity of AOB observed through both sequence dependent (present chapter) and independent (chapter 2a) methods showed that *amo*A gene fragment is a better marker than *hao* gene fragment.

2b.3.2 Magnitude of genetic variation in AOB with respect to 16S rRNA, *amo*A and *hao* genes

Patterns of nucleotide substitutions in the three genes showed higher transitions (Ti) (T↔C or A↔G) than transversions (Tv) (T↔A, T↔G, C↔A, C↔G) as has been observed for most other genes (Gojobori et al., 1982; Wakeley, 1994) (Table 2). Amongst the three genes Ti substitution were more predominant in 16S rRNA than *amo*A and *hao* genes (Table 2b.2). Based on these substitutions Ti/Tv rate bias were calculated.

Table 2b.2 Maximum composite likelihood estimates of the pattern of nucleotide substitution in the 16S rRNA, *amo*A and *hao* genes.

	A			T			C			G		
	16S rRNA	*amo*A	*hao*	16S rRNA	*amo*A	*hao*	16S rRNA	*amo*A	*hao*	16S rRNA	*amo*A	*hao*
A	-	-	-	2.64	6.23	3.72	2.86	4.71	6.54	14.66	10.31	10.16
T	3.39	4.18	6.28	-	-	-	24.5	17.5	21.74	4.03	5.38	5.92
C	3.39	4.18	6.28	22.68	23.17	12.38	-	-	-	4.03	5.38	5.92
G	12.33	8.01	10.79	2.64	6.23	3.72	2.86	4.71	6.54	-	-	-

Estimates of Ti/Tv rate bias are extremely important in studying DNA sequence evolution, distance and phylogeny reconstruction (Wakeley, 1996; Yang and Yoder, 1999). The level of transition bias varies in different microorganisms and also in the different genes in a group of microorganisms; thereby it assists in comprehending the patterns of molecular evolution of the genes (Strandberg and Salter, 2004). It has been observed that high Ti/Tv rate ratios are indicative of low levels of genetic divergence (Yang and Yoder, 1999). 16S rRNA gene showed higher Ti/Tv rates ratios than the functional genes *amo*A and *hao* and overall p-distance calculated for 16S rRNA gene was lower (around 3 folds less) than *amo*A and *hao* (Table 2b.3) indicating lower sequence divergence in 16S rRNA gene than *amo*A and *hao* genes.

Table 2b.3 Estimates of the mean diversity of the entire population with respect to the three genes.

	16S rRNA	*amo*A	*hao*
Ti/Tv rate (Purines) - K1	3.641	1.918	1.717
Ti/Tv rate (Pyrimidines) - K2	8.581	3.717	3.325
Over all Ti/Tv bias – R	2.867	1.508	1.275
Overall average P distance	0.065	0.184	0.169

2b.3.3 Co-evolution *amo*A and *hao* genes and their correlation with 16S rRNA gene

Correlation between the evolution rate of the *amo*A and *hao* genes was, studied by analyzing the sequence similarity resulting from evolutionary distance between pair of species which was then compared with 16S rRNA gene. Percentage sequence similarity values were represented in correlation plots (Fig 2b.2C). A linear regression was applied to the correlation plots for the three genes where a high r^2 value is indicative of co-evolution between pairs of genes and amongst the co-evolving genes, one of the genes evolves faster than the other, can be known from the slope of the curve (Junier et al., 2009). Significant linear correlation was obtained for *amo*A and *hao* pair (r^2=0.9, p value < 0.001) whereas when each of these was compared with 16S rRNA gene, their correlation was found to be lower (*amo*A/16S rRNA gene r^2=0.65 and *hao*/16S rRNA gene r^2=0.57, p value < 0.001) suggesting co-evolution of *amo*A and *hao* genes (Fig 2b.2).

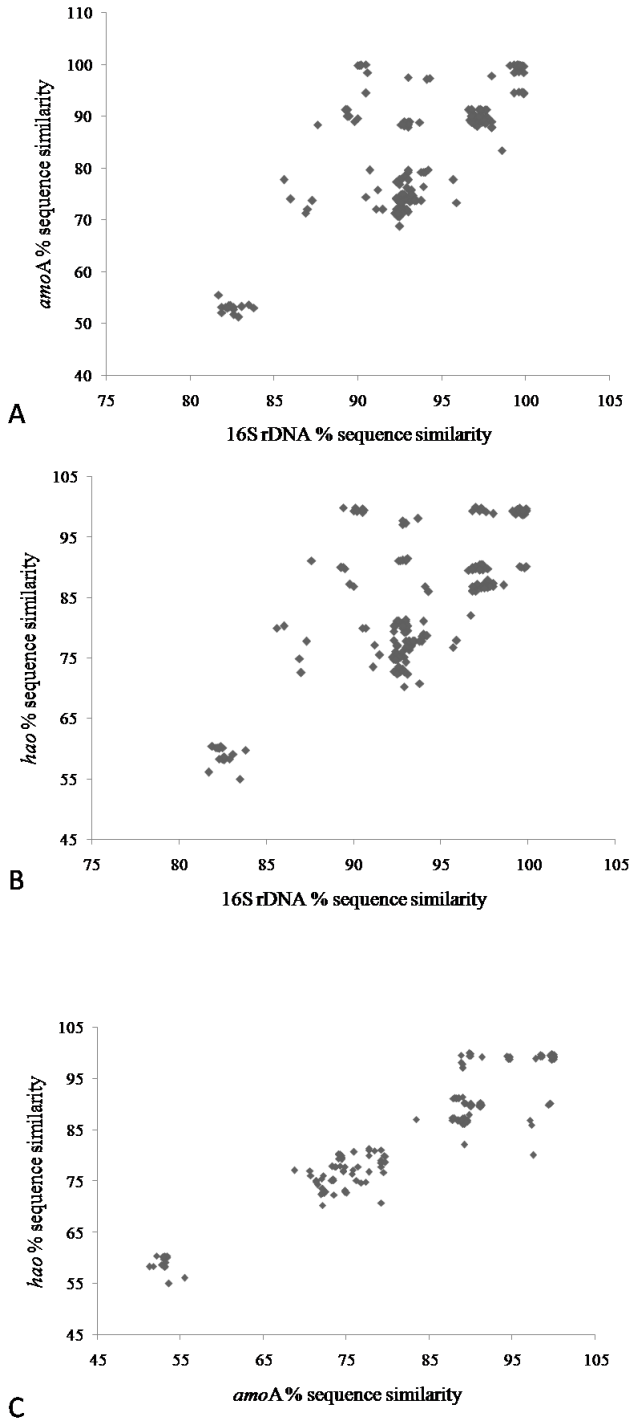


Fig 2b.2 Correlation plots of A) *amo*A and 16S rRNA gene % sequence similarity B) *hao* and 16S rRNA gene % sequence similarity and C) *amo*A and *hao* % sequence similarity.

Evolutionary rates reflected by the slope of the linear regression were highest in *amo*A, followed by *hao* and then by 16S rRNA gene (Fig 2b.2). The differences in the evolutionary rates between the genes have been reported to

correlate with the function of the enzymes coded by the genes (Junier et al., 2009). A different selection pressure would act on *amo*A and *hao* involved in the same pathway of oxidizing ammonia to nitrite, compared to 16S rRNA gene resulting in differences in the evolutionary rates in the genes. AMO can act on a number of substrates including aliphatic, aromatic and halogenated molecules (methane, diethylether, fluoromethane, cloromethane, benzene, toluene, etc.) thereby supporting AOB to endure heterotrophic conditions (Hooper et al., 1997). HAO in AOB can catalyze oxidation of hydroxylamine differentially, producing nitrite under aerobic conditions and N_2O and NO under microaerophilic conditions enabling it to thrive under the said conditions (Hooper et al., 1997). These diversed catalytic activities of both the enzymes involved in the same pathway could have resulted in different selective pressures acting upon them leading to differences in their evolutionary rates. HAO is known to have evolved more than once under different functional pressure based on the catabolic activities of the bacteria harboring them (Klotz et al., 2008) substantiating the differences in the evolutionary rates observed for *hao* and *amo*A gene fragments. The co-evolution of both the gene shown in present study also reflects upon its genetic diversion at par over time.

A significant Pearson's correlation coefficient r = 0.949 (p value <0.001) substantiated co-evolution of *amo*A and *hao* genes observed through linear regression. Pearson's correlation coefficient for 16S rRNA gene with *amo*A and *hao* genes was r = 0.806 and 0.757 respectively with p value < 0.001 indicating lesser correlation of the two genes with 16S rRNA gene compared to one another. Codon based evolutionary relationship between *amo*A and *hao* genes analyzed using Mega 4 software showed that out of the total mutations in *amo*A and *hao* genes, synonymous mutations in both the genes were interestingly 29.6 and 29.5% respectively and nonsynonymous mutations were 70.4 and 70.5% respectively indicating high correlation between *amo*A and *hao* genes. Rates of synonymous (d_S) and nonsynonymous substitutions (d_N) calculated according to Nei and Gojobori (1986) using the MEGA4 software (Table 2b.4) showed higher d_N than d_S in

both *amo*A and *hao* genes. Ratio of d_N:d_S were found to be 1.92 and 1.56 respectively for *amo*A and *hao* genes which is indicative of advantageous selection of new mutants than neutral variants, suggesting adaptive or positive Darwinian selection in both the genes. Similar rates in both synonymous and nonsynonymous mutations in both the genes further confirmed co-evolution of *amo*A and *hao* genes.

Table 2b.4 Estimate of the mean codon-based evolutionary diversity for the entire population.

	*amo*A	*hao*
No. of Synonymous mutations	116.71 (29.6%)	202.41 (29.5%)
No. of Nonsynonymous mutations	277.33 (70.4%)	483.56 (70.5%)
Total mutations	394.043 (100%)	685.04 (100%)
Rate of nonsynonymous mutations (D_N)	0.212	0.180
Rate of synonymous mutations (D_s)	0.110	0.115
D_N/D_S	1.92	1.56

2b.3.4 Structural analysis of hydroxylamine oxidoreductase

Amongst the AMO and HAO crystal structure of only HAO has been studied hence structural analysis in the present study is limited to HAO enzyme only. Of the total mutations observed in *hao* gene 70.5% were nonsynonymous mutations. It was therefore important to know the effect of these mutations on structure of the protein. HAO enzyme amino acid sequences from all were aligned using Clone Manager 7 version 7.11 (Fig 2b.3).

```
DnrA                 ------------------------------------------------
DnrB2                ------------------------------------------------
DnrB1                ------------------------------------------------
DN2                  ------------------------------------------------
CETP                 ------------------------------------------------
N. nitrosa           ------------------------------------------------
N. Nm143             ------------------------------------------------
N. multiformis       ------------------------------------------------
N. briensis          ------------------------------------------------
N. oceanus         1 ----------------------------------lgvdkykaspkefy
N. oligotropha       ------------------------------------------------
DN3                  ------------------------------------------------
N. eutropha        1 mrlgeylkgmllcaglligpvqadistvpdetyealkldrskatpkety
DN1                  ------------------------------------------------
N2                   ------------------------------------------------
N3                   ------------------------------------------------
PF1                  ------------------------------------------------
N1                   ------------------------------------------------
PF2                  ------------------------------------------------
N. europaea        1 mrigewmrglllcaglmmcgvvhadistvpdetydalkldrgkatpkety
N. ENI11          51 mrigewmrglllcaglmmcgvvhadistvpdetydalkldrgkatpkety

DnrA                 ------------------------------------------------
DnrB2                ------------------------------------------------
DnrB1                ------------------------------------------------
DN2                  ------------------------------------------------
CETP                 ------------------------------------------------
N. nitrosa           ------------------------------------------------
N. Nm143             ------------------------------------------------
N. multiformis       ------------------------------------------------
N. briensis          ------------------------------------------------
N. oceanus        15 eaiteryydpaqghgegkyaeyweplpfsqyldpytyysppaqpdkvatr
N. oligotropha       ------------------------------------------------
DN3                  ------------------------------------------------
N. eutropha       51 dalvkrykdpahgagkgtmgdywepialsiymdpntfykppvspkeiaer
DN1                  ------------------------------------------------
N2                   ------------------------------------------------
N3                   ------------------------------------------------
PF1                  ------------------------------------------------
N1                   ------------------------------------------------
PF2                  ------------------------------------------------
N. europaea       51 ealvkrykdpahgagkgtmgdywepiaisiymdpntfykppvspkevaer
N. ENI11          51 ealvkrykdpahgagkgtmgdywepiaisiymdpntfykppvspkeaaer
```

```
DnrA                  ------------------------------------------------
DnrB2                 ------------------------------------------------
DnrB1                 ------------------------------------------------
DN2                   ------------------------------------------------
CETP                  ------------------------------------------------
N. nitrosa            ------------------------------------------------
N. Nm143              ------------------------------------------------
N. multiformis      1 --------delpvwvsackrsthanldkirnlkpedptyykkakledvek
N. briensis         1 ------------------------------------------------k
N. oceanus         65 eeciechedetrgwvhawrksvhanldeirdlpkddsryykkklikevek
N. oligotropha        ------------------------------------------------
DN3                   ------------------------------------------------
N. eutropha       101 kdcvechsdetpvwvrawkrsthanldkirnlkpedplfykkgkleeven
DN1                   ------------------------------------------------
N2                    ------------------------------------------------
N3                    ------------------------------------------------
PF1                   ------------------------------------------------
N1                    ------------------------------------------------
PF2                   ------------------------------------------------
N. europaea       101 kdcvechsdetpvwvrawkrsthanldkirnlksddplyykkgkleeven
N. ENI11          101 kdcvechsdetpvwvrawkrsthanldkirnlksddplyykkgkleevkn


DnrA                1 nlrsmgklgekenlkevgcidchvdinkkdkadhtkdvrmpsadvcgtch
DnrB2               1 nlrsmgtlggketlkklaridchvdinkkdkadhtkdvrmpsadvcgtch
DnrB1               1 nlrsmgklgekenlkklaridchvdinkkdkadhtkdvrmpsadvcgtch
DN2                 1 nlrsmgklgekenlkklaridchvdinkkdkadhtkdvrmpsadvcgtch
CETP                1 nlrsmgklgeketlkklaridchvdinkkdkadhtkdvrmpsadvcgtch
N. nitrosa          1 nlrasgklgadeklkevgcidchvexnakkkadhtkdiimptaeicgtch
N. Nm1431             -------------------idchveinakgkaqhnkdlimptadvcgach
N. multiformis     43 nlrsmgklgandklkevgcidchvdinkkdkadhmkdlrmptadvcgtch
N. briensis         2 nlrsmnrlganeklkevgcidchvdintkgkadhmkdlrmptadvcgvch
N. oceanus        115 nlrsmgkleknenlkevscidchvgvgae-kghhnqdlrlpdaaacgich
N. oligotropha      1 --igvgklgekeslkevgcidchvevnakkkadhtkdlimptadvcgtch
DN3                 1 nlrsmgklgekealkevgcidchvdinakkkadhtkdvrmptadvcgtch
N. eutropha       151 nlrsmgklgekealkevgcidchvdinakkkadhtkdvrmptadvcgtch
DN1                 1 nlrsmgklgeketlkevgcidchvdvnkkdkadhtkdirmptadtcgtch
N2                  1 nlrsmgklgeketlkevgcidchvdvnkkdkadhtkdirmptadtcgtch
N3                  1 nlrsmgklgeketlkevgcidchvdvnkkdkadhtkdirmptadtcgtch
PF1                 1 nlrsmgklgeketlkevgcidchvdvnkkdkadhtkdirmptadtcgtch
N1                  1 nlrsmgklgeketlkevgcidchvdvnkkdkadhtkdirmptadtcgtch
PF2                 1 nlrsmgklgeketlkevgcidchvdvnkkdkadhtkdirmptadtcgtch
N. europaea       151 nlrsmgklgeketlkevgcidchvdvnkkdkadhtkdirmptadtcgtch
N. ENI11          151 nlrsmgklgeketlkevgcidchvdvnkkdkadhtkdirmptadtcgtch
```

```
DnrA              51 lrefaereserdtmiwpngq---------wpagrpshaldytaniettvw
DnrB2             51 lrefaereserdtmiwpngq---------wpagrpshaldytaniettvw
DnrB1             51 lrefaereserdtmiwpngq---------wpagrpshaldytaniettvw
DN2               51 lrefaereserdtmiwpngq---------wpagrpshaldytaniettvw
CETP              51 lrefaereserdtmiwpngq---------wpagrpshaldytaniettvw
N. nitrosa        51 lrefaereserdtmifpngqxr---------egrpshaldwkanvettxw
N. Nm143          32 lqefaereserdtliwpngq---------wpdgrpshaldykanvettvw
N. multiformis    93 lqefaereserdtlvwpnkq---------wpqgrpfhsldwkanvevavf
N. briensis       52 lqefaereserdtliwpnkq---------wpqgrpshaldwkanvevavf
N. oceanus       164 lqqfaereserdtltwpdtdvkgnkidpvwppgrpshaldyqanvdlatw
N. oligotropha    49 lrefaereserdtmiwpngq---------wpdgrpshaldykanvettvw
DN3               51 lrefaereserdtmiwpngq---------wpdgrpshaldytaniettvw
N. eutropha      201 lrefaereserdtmiwpngq---------wpdgrpshaldytaniettvw
DN1               51 lrefaereserdtmvwpngq---------wpagrpshaldytaniettvw
N2                51 lrefaereserdtmvwpngq---------wpagrpshaldytaniettvw
N3                51 lrefaereserdtmvwpngq---------wpagrpshaldytaniettvw
PF1               51 lrefaereserdtmvwpngq---------wpagrpshaldytaniettvw
N1                51 lrefaereserdtmvwpngq---------wpagrpshaldytaniettvw
PF2               51 lrefaereserdtmvwpngq---------wpagrpshaldytaniettvw
N. europaea      201 lrefaereserdtmvwpngq---------wpagrpshaldytaniettvw
N. ENI11         201 lrefaereserdtmvwpngq---------wpagrpshaldytaniettvw

DnrA              92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpearatchsgvd
DnrB2             92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
DnrB1             92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
DN2               92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
CETP              92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
N. nitrosa        92 aampqreiaegcsmchynqnkxdgcxtrhxfsaxxsrkreacatxhsgvd
N. Nm143          73 aampqrevaegcsmchtnqnkcdschtrhefsaaesrkpeacatchsgvd
N. multiformis   134 asmpqreiaegcsmchtnqnkcdschtrhefsaaesrhpeacatchsgvd
N. briensis       93 asmpqreiaegcsmchtnqnkcdschtrhefsaaesrqpetcatchsgvd
N. oceanus       214 aamedrevadgctmchinqnrcdtchtrhqfsavearkpdacgnchngad
N. oligotropha    90 aampqrevaegctmchtnqnkcdschtrhefs----------------
DN3               92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
N. eutropha      242 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
DN1               92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
N2                92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
N3                92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
PF1               92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
N1                92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
PF2               92 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
N. europaea      242 atmpqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
N. ENI11         242 aampqrevaegctmchtnqnkcdnchtrhefsaaesrkpeacatchsgvd
```

```
DnrA              142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
DnrB2             142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
DnrB1             142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
DN2               142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
CETP              142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
N. nitrosa        142 htxxexysxskhxkivxmmgdkwnwdvxxkdaxakgggqsaptcagchmey
N. Nm143          123 hnnweaysmskhgkmvamlgdqwnwdvqlkdayavgggqsaptcagchfey
N. multiformis    184 hnnweaysmskhgktvaimgdkwnwnaplkdaytkgggqtaptcagchfey
N. briensis       143 hnnweaysmskhgktvaimgnkwnwnvplkdayakgggqtaptcqgchfey
N. oceanus        264 hneyenylmskhgttylmlgdtwdlevplkdaiaenggqtaptcafchmey
N. oligotropha        ------------------------------------------------
DN3               142 hnnyeayimskhgklaemnrgnwnwnvrlkdafskgggqtaptcaachmey
N. eutropha       292 hnnyeayimskhgklaemnrgnwnwnvrlkdafskgggqtaptcaachmey
DN1               142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
N2                142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
N3                142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
PF1               142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
N1                142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
PF2               142 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
N. europaea       292 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey
N. ENI11          292 hnnweaytmskhgklaemnrdkwnwevrlkdafskgggqnaptcaachmey

DnrA              192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
DnrB2             192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
DnrB1             192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
DN2               192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
CETP              192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
N. nitrosa        192 egeythnmvrkvrwanypfvpgvaenitsewsearldswvvtctqchser
N. Nm143          173 egeythnvvrkirwanypf--------------------------------
N. multiformis    234 egkyshnvvrkirwanypavpgsaeninsewsearldswvktctschser
N. briensis       193 egkyshnvvrkirwanypavpgiaeninsewsearldswvktctqchfra
N. oceanus        314 kgrfghnvvrkvrwafnpq-ekiannlehewyeyrneawietctnchsat
N. oligotropha        ------------------------------------------------
DN3               192 egeythnitrktrwanypfvpgiaenitsdwsearldpwqltvpsvtse-
N. eutropha       342 egeythnitrktrwanypfvpgiaenitsdwsearldswvvtctqchser
DN1               192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
N2                192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
N3                192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
PF1               192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
N1                192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
PF2               192 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
N. europaea       342 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
N. ENI11          342 egeythnitrktrwanypfvpgiaenitsdwsearldswvltctqchser
```

```
DnrA              242 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktn--------
DnrB2             242 farsyldlmdkgtleglakyqeanaivhkmyeegpltgqktn--------
DnrB1             242 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktn--------
DN2               242 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktn--------
CETP              242 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktn--------
N. nitrosa        242 farsylelmdkgtls-----------------------------------
N. Nm143              ----------------------------------------------------
N. multiformis    284 farsylefmdkgtlhgiakykeahavaeklykeglltgqktnrptplppd
N. briensis       243 fapfyle-------------------------------------------
N. oceanus        363 faesylefvdngiisglkkqleakqivealyedgllpgqktnrpappkpe
N. oligotropha        ----------------------------------------------------
DN3                   ----------------------------------------------------
N. eutropha       392 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktnrpnppape
DN1               242 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktn--------
N2                242 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktn--------
N3                242 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktn--------
PF1               242 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktn--------
N1                242 farsyldlmekgtleglakyqeanaivhkmyedgtltgqktn--------
PF2               242 farsyldlmdkgtleglakyqeanaivhkmyedghltgqktn--------
N. europaea       392 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktnrpnppepe
N. ENI11          392 farsyldlmdkgtleglakyqeanaivhkmyedgtltgqktnrpnppepe

DnrA                  ----------------------------------------------------
DnrB2                 ----------------------------------------------------
DnrB1                 ----------------------------------------------------
DN2                   ----------------------------------------------------
CETP                  ----------------------------------------------------
N. nitrosa            ----------------------------------------------------
N. Nm143              ----------------------------------------------------
N. multiformis    334 kemfagftql----------------------------------------
N. briensis           ----------------------------------------------------
N. oceanus        413 hdapgeffqlfiakgnnptavelqyakmweqdllkhykalahvnpgfwt-
N. oligotropha          ------------------------------------------------------
DN3                   ----------------------------------------------------
N. eutropha       442 kpgfgiftqlfwskgnnpaslelkvlemaennlakmhvglahvnpggwty
DN1                   ----------------------------------------------------
N2                    ----------------------------------------------------
N3                    ----------------------------------------------------
PF1                   ----------------------------------------------------
N1                    ----------------------------------------------------
PF2                   ----------------------------------------------------
N. europaea       442 kpgfgiftqlfwskgnnpaslelkvlemgennlakmhvglahvnpggwty
N. ENI11          442 kpgfgiftqlfwskgnnpaslelkvlemaennlakmhvglahvnpggwty
```

```
DnrA                 ------------------------------------------------
DnrB2                ------------------------------------------------
DnrB1                ------------------------------------------------
DN2                  ------------------------------------------------
CETP                 ------------------------------------------------
N. nitrosa           ------------------------------------------------
N. Nm143             ------------------------------------------------
N. multiformis       ------------------------------------------------
N. briensis          ------------------------------------------------
N. oceanus           ------------------------------------------------
N. oligotropha         ----------------------------------------------
DN3                  ------------------------------------------------
N. eutropha      492 tegwgpmnrayveiqdeytkmqemtalqarvnklegkktslldlkgagek
DN1                  ------------------------------------------------
N2                   ------------------------------------------------
N3                   ------------------------------------------------
PF1                  ------------------------------------------------
N1                   ------------------------------------------------
PF2                  ------------------------------------------------
N. europaea      492 tegwgpmnrayveiqdeytkmqelsalqarvnklegkqtslldlkgtgek
N. ENI11         492 tegwgpmnrayveiqdeytkmqelsalqarvnklegkqtslldlkgtgek

DnrA                 ----------------------------
DnrB2                ----------------------------
DnrB1                ----------------------------
DN2                  ----------------------------
CETP                 ----------------------------
N. nitrosa           ----------------------------
N. Nm143             ----------------------------
N. multiformis       ----------------------------
N. briensis          ----------------------------
N. oceanus           ----------------------------
N. oligotropha         --------------------------
DN3                  ----------------------------
N. eutropha      542 islgglgggmllagaialigwrkrkqtqa
DN1                  ----------------------------
N2                   ----------------------------
N3                   ----------------------------
PF1                  ----------------------------
N1                   ----------------------------
PF2                  ----------------------------
N. europaea      542 islgglgggmllagalaligwrkrkqtra
N. ENI11         542 islgglgggmllagalaligwrkrkqtra
```

Fig 2b.3 Hydroxylamine oxidoreductase amino acid sequence alignment using Clone Manager 7. Sequence indicated in red are the haem binding residues.

Alignment showed that DnrB2 and DN2 showed 100% sequence identity. Similarly, DN1, N2, N3 and PF1 showed 100% sequence identity (Fig 2b.3). Therefore, DnrB2 and DN1, as representatives of each group, were studied further for structural analysis. *Nitrosomonas nitrosa* contained several X (any

amino acid) throughout the protein sequence and hence its structure could not be predicted and was therefore not studied further. The structure of hydroxylamine oxidoreductase was predicted using homology modeling (one of the most commonly used structure prediction method) and compared with *Nitrosomonas europaea* hydroxylamine oxidoreductase (pdb code 1FGJ) as amongst AOB, crystal structure of only *Nitrosomonas europaea* hydroxylamine oxidoreductase has been studied (Igarashi et al., 1997).

Q-MEAN Z-score for the predicted structures was between -2.71 and -5.09 and Q-MEAN4 score was between 0.417 and 0.592 indicating that the quality of the predicted structures was accurate for most AOB (Table 2b.5). Q-MEAN Z –score for DN3, *Nitrosomonas* sp. Nm143, *Nitrosomonas oligotropha* and *Nitrosospira briensis* were between -4.7 to -5.09 and there Q-MEAN4 score was below 0.5 indicating that some part of the protein was not modeled correctly in the structure of HAO of these 4 AOB (Table 2b.5). Structures with Q-MEAN Z-score below -4.0 are not considered good (Cambra et al., 2012). Q-MEAN4 values lie between 0-1 and values towards 1 are considered as good (Benkert et al., 2011).

Model structures were compared with *Nitrosomonas europaea* hydroxylamine oxidoreductase (PDB ID: 1FGJ) (Igarashi et al., 1997) using Accelrys Discovery Studio Visualizer7.0. Root-Mean-Square Deviation (RMSD) values for the predicted structures were between 0.0 to 0.5 (Table 2b.5) indicating that the nonsynonymous mutations in hydroxylamine oxidoreductase did not change its structure considerably (Fig 2b.4). Amongst the hydroxylamine oxidoreductase protein sequences obtained earlier by the author, structures of CETP, DN1, DnrA, DnrB1, N1, PF2 and DnrB2, did not show structural variations with RMSD values (0.01 to 0.03) (Table 2b.5 and Fig 2b.4 A, B, C, D, E, G and H respectively). DN3 showed a variation of RMSD value 0.04Å. Presence of VPSVT (234-238) in DN3 instead of CTQCH (360-364) led to extension of α helix 19 of HAO (Fig 2b.4M). This variation has disrupted the 8th haem binding site in the central domain of the protein which also contains the active site pocket of the enzyme.

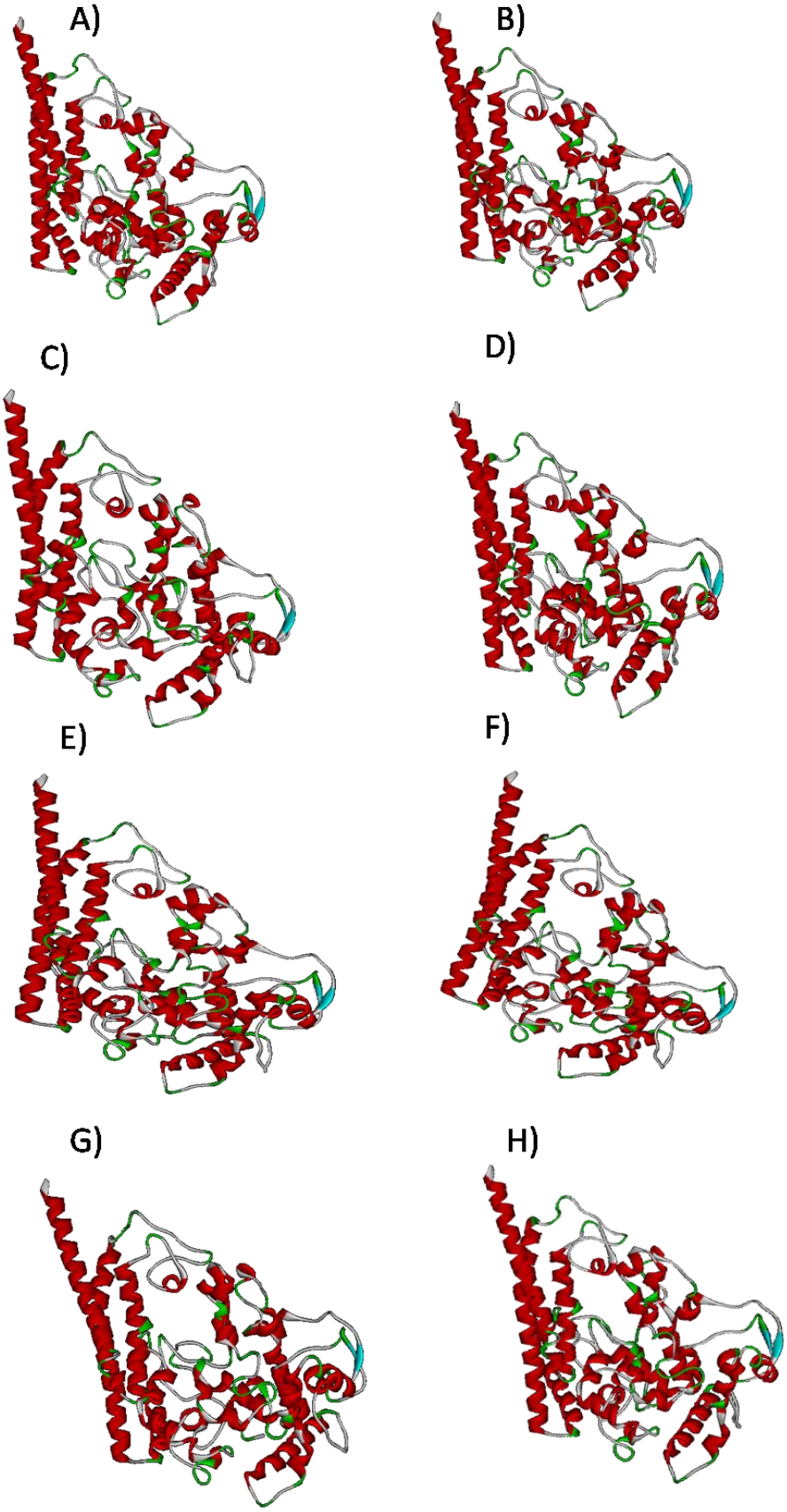
Table 2b.5 Quality of the predicted structures and their comparison with *Nitrosomonas europaea* hydroxylamine oxidoreductase.

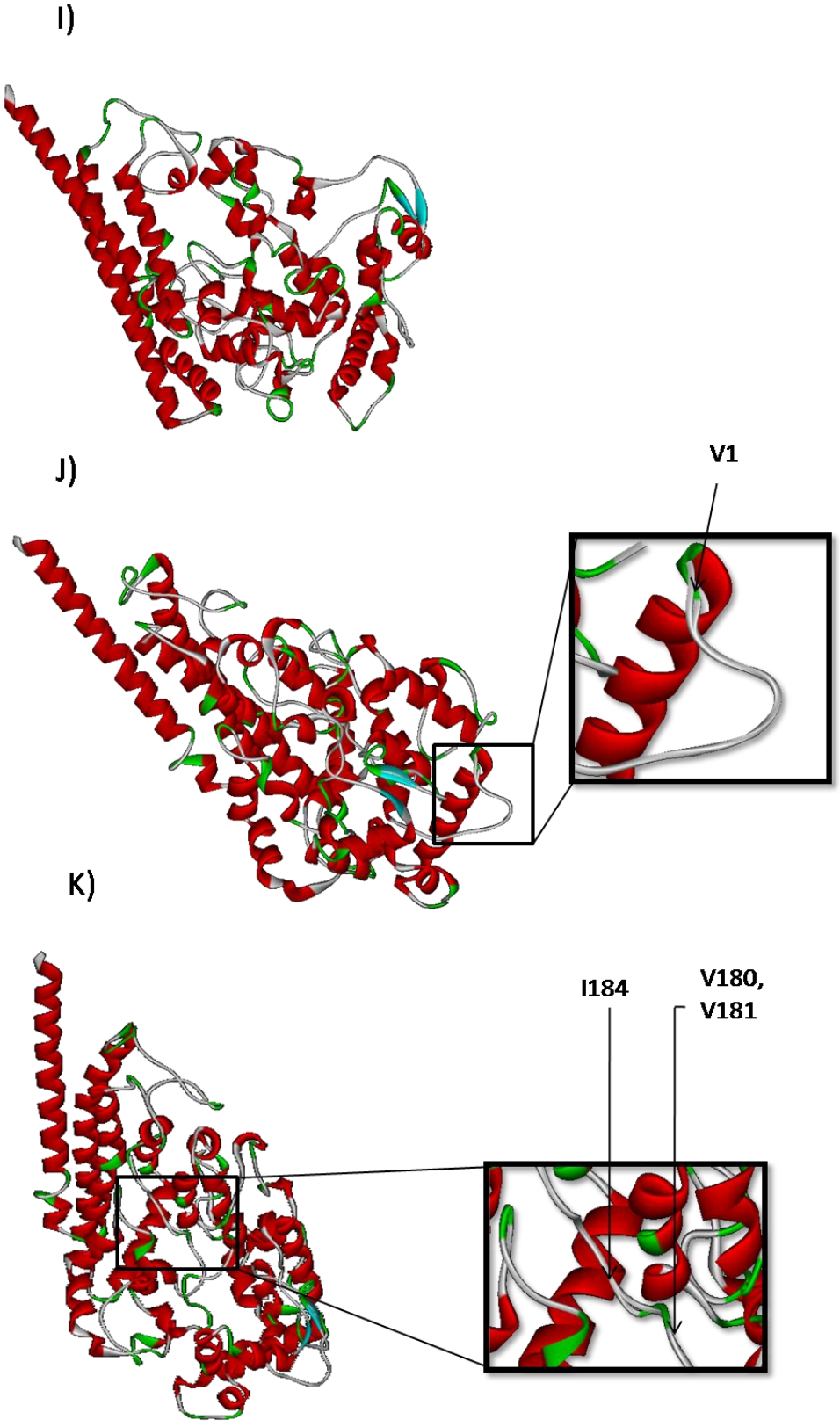
AOB	Modeled residue range	Based on template (A^0)	Sequence identity (%)	Quality analysis of the predicted homology model			RMSD (A^0)	No. of aligned residues
				E value	Z-score	Q MEAN4 score		
Nitrosomonas europaea	25-523	2.8	100	0	-2.73	0.591	-	-
DnrA	1-283	2.8	97.17	9.1e-161	-4.04	0.516	0.01	283
DnrB1	1-283	2.8	96.11	1.89e-159	-4.03	0.516	0.01	283
DnrB2	1-283	2.8	95.05	1.3e-157	-4.18	0.507	0.03	283
CETP	1-283	2.8	96.47	5.8e-160	-3.99	0.519	0.01	283
DN1	1-283	2.8	99.65	2.04e-163	-3.98	0.519	0.01	283
DN3	1-240	2.8	90.83	1.8e-126	-4.79	0.481	0.04	240
N1	1-283	2.8	99.23	7.9e-163	-3.95	0.521	0.01	283
PF2	1-283	2.8	99.3	1.01e-162	-4.02	0.517	0.01	283
Nitrosomonas eutropha C91	25-523	2.8	94.4	0	-2.72	0.592	0.02	499
Nitrosomonas sp. ENI11	25-523	2.8	99.2	0	-2.71	0.592	0.0	499
Nitrosomonas sp. Nm143	1-191	2.8	80.63	4.2e-94	-5.09	0.417	0.03	169
Nitrosomonas oligotropha	3-121	2.8	88.24	3.6e-60	-4.7	0.448	0.02	119
Nitrosospira briensis	2-249	2.8	75.8	1.3e-114	-5.23	0.46	0.04	248
Nitrosospira multiformis	1-343	2.8	76.4	2.6e-157	-3.56	0.534	0.05	343
Nitrosococcus oceani	1-461	2.8	56.59	0	-3.37	0.566	0.5	452

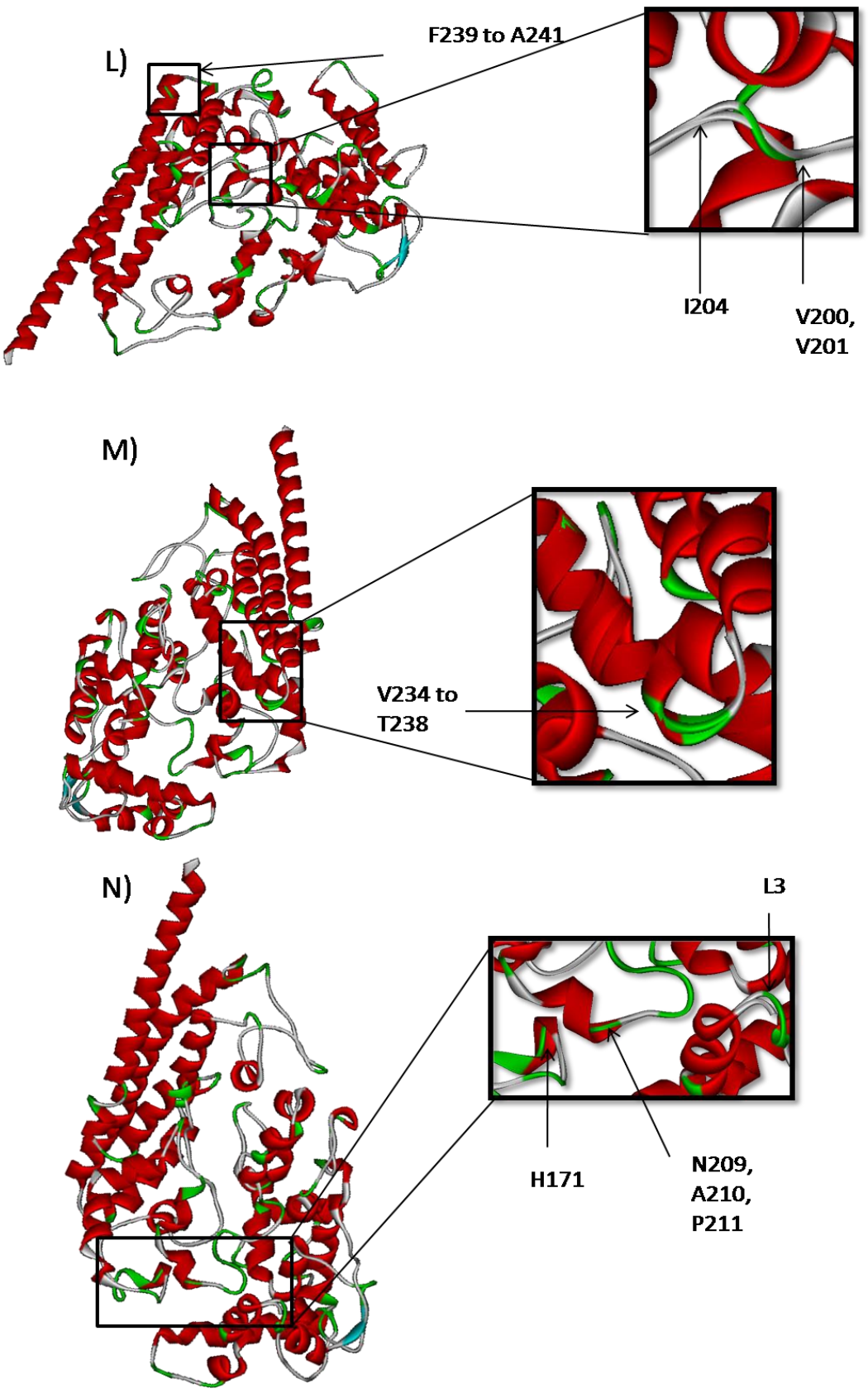
Disruption of haem 8 did not lead to loss in the activity of the enzyme, as the activity of this protein was observed through its activity staining in Chapter 2a. This justifies earlier report that DN3 HAO enzyme was different from other reported HAO, observed through HAO enzyme activity staining (Chapter 2a). Variation in the structure of HAO was also not observed in *Nitrosomonas* sp. ENI11 and *Nitrosomonas eutropha* (Fig 2b.4F and I). In

Nitrosomonas oligotropha change of V instead of M caused variation in loop between α helix 7 and α helix 8 (Fig 2b.4J). In *Nitrosomonas* sp. Nm143 variation was observed between V180 to I184, leading to variation in loop structure between α helix 17 and α helix 18 (Fig 2b.4K). Variation between *Nitrosomonas sp.* was between 0.0 to 0.04A°, which did not cause major changes in the structure of the protein (Fig 2b.4).

Maximum variation was observed in *Nitrosococcus oceani* (RMSD 0.5A°) Table 2b.5). Insertion of 9 amino acid residues (D206VKGNKIDP214) *in Nitrosococcus oceani* HAO between G195 and Q196 (with respect to *Nitrosomonas europaea* HAO) led to extension of loop between α helix 10 and 11 (Fig 2b.4Oii). Deletion of V152 and F336 also led to variation in the loop structures between α helix 8 and β sheet 2 and α helix 17 and 18 respectively (Fig 2b.4Oii and iii). *Nitrosococcus oceani* showed major structural variation in its hydroxylamine oxidoreductase structure justifying it to be separated from other AOB belonging to betaproteobacteria. *Nitrosospira briensis* and *Nitrosospira multiformis* showed a variation of 0.04 and 0.05A° respectively (Table 2b.5 and Fig 2b.4L and M). These changes caused variation in α helix 20 (longest helix in hydroxylamine oxidoreductase structure) position F239 to A241 and in the loop structures position V200 to I204 between α helix 17 and 18 in *Nitrosospira briensis* (Fig 2b.4L) whereas in *Nitrosospira multiformis* change in the loop structure was observed with change of T to L3 between α helix 4 and 5 and in the loop at position N209 to P211 and H171 (Fig 2b.4N).


A)
B)
C)
D)
E)
F)
G)
H)


I)
J)
V1
K)
I184
V180,
V181


L)
F239 to A241
I204
V200,
V201
M)
V234 to
T238
N)
L3
H171
N209,
A210,
P211

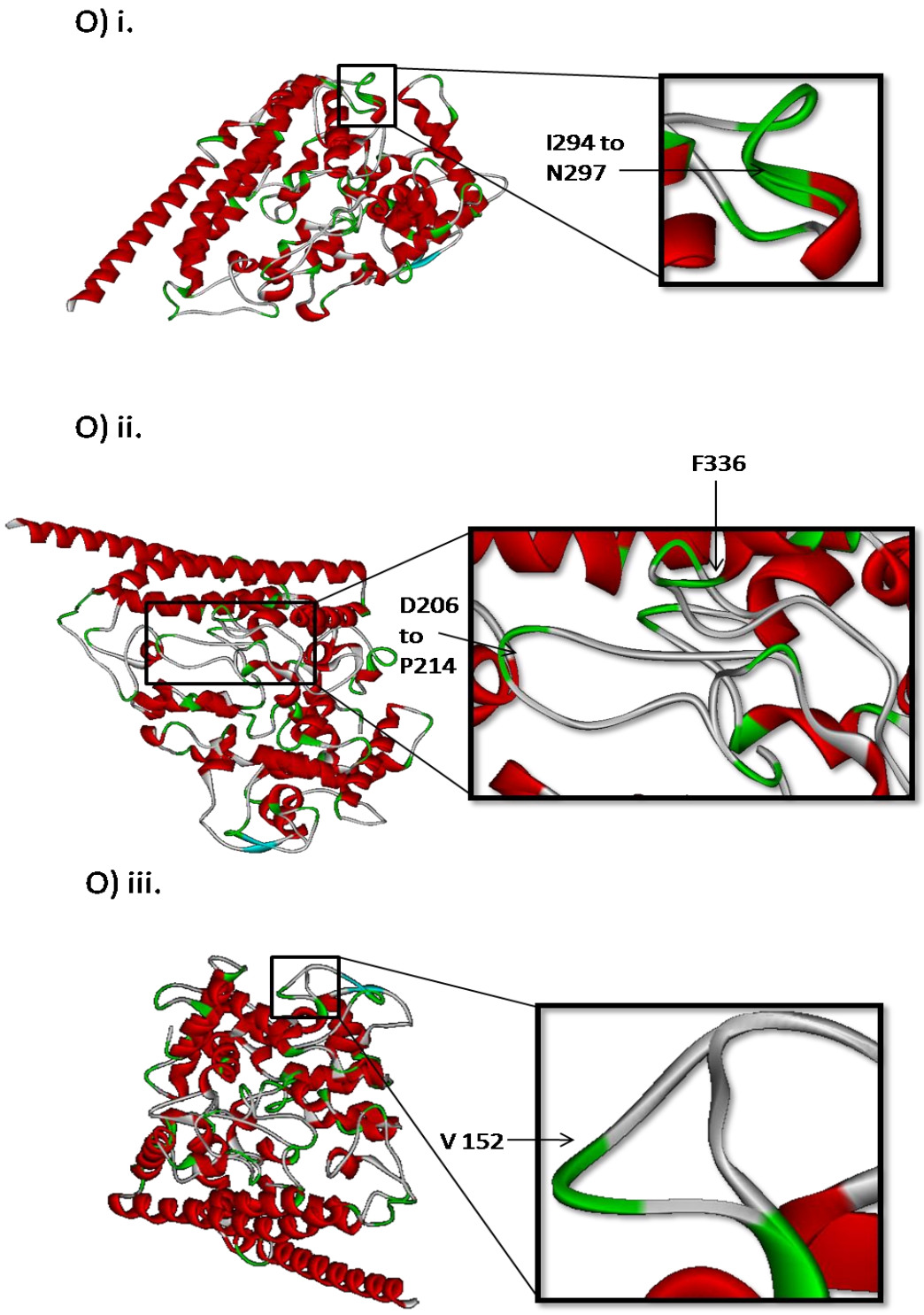


Fig 2b.4 Hydroxylamine oxidoreductase structure alignment of all AOB with hydroxylamine oxidoreductase of *N. europaea* through Accelrys Discovery Studio Visualizer v2.0.1.7347 A)CETP B)DN1 C)DnrA D)DnrB1) E)N1 F)*Nitrosomonas* sp. ENI11 G)PF2 H)DnrB2 I)*Nitrosomonas eutropha*

J)*Nitrosomonas oligotropha* K)*Nitrosomonas* sp. Nm143 L)*Nitrosospira briensis* M)DN3 N)*Nitrosospira multiformis* O)*Nitrosococcus oceani* i) ii) iii). Insets have been made to show the variations observed in protein structure.

In conclusion, phylogenetic trees constructed using the three genes *amo*A, *hao* and 16S rRNA genes showed statistically significant similar topologies. Mutational analysis of AOB carried out for the first time revealed that AOB genes show bias towards transitions over transversions. It was also deduced that sequence divergence was higher in *amo*A and *hao* gene fragments compared to 16S rRNA gene and therefore these genes have higher evolutionary rates compared to 16S rRNA gene. Synonymous and nonsynonymous substitutions were exceedingly similar in *amo*A and *hao* gene fragments. Variation in amino acid sequence did not cause major variation in HAO structure. The following properties of *hao* made it an excellent alternative marker for the detection of AOB i) It was highly sensitive in detecting AOB (present study; Schmid et al. 2008). ii) It was larger in size than *amo*A gene (present study) iii) *hao* based phylogeny of AOB show higher diversity compared with *amo*A and 16S rRNA gene based phylogeny with better separation of *Nitrosomonas* sp. (present study) iv) Co-evolution of *amo*A and *hao* genes involved in the oxidation of ammonia to nitrite supported that *hao* can also be used at par with *amo*A as an alternative phylogenetic marker in studying diversity and evolution of AOB (present study) v) It had both conserved and variable regions (Schmid et al., 2008). vi) HAO zymogram (involving whole protein structure) could resolve AOB of the *Nitrosomonas* genus (Chapter 2a). All these put together, substantiate high potential of *hao* as an alternative phylogenetic marker in studying diversity and evolution of AOB.

2b.4 Appendix

Table 2b.6 16S rDNA sequence similarity amongst the studied AOB

% 16S rDNA sequence similarity

Strains	*Nitrosomonas* sp. ENI 11	*N. europaea*	*N. nitrosa*	*N. oligotropha*	*Nitrosomonas* sp. Nm143	*Nitrosococcus oceanus*	*N. eutropha* C91	*Nitrosospira multiformis*	*Nitrosospira briensis*	UN DnrA	UN DnrB1	UN DnrB2	UN CETP	UN DN1	UN DN2	UN DN3	UN N1	UN N2	UN N3	UN PF1
Nitrosomonas sp. ENI 11																				
N. europaea	99.9																			
N. nitrosa	93	93																		
N. Oligotropha	92.9	92.8	93																	
Nitrosomonas sp. Nm143	93.4	93.9	92.8	94.2																
Nitrosococcus oceanus	82.3	82.3	81.9	81.7	83.8															
N. eutropha C91	97.5	98	93	92.3	93.2	82.6														
Nitrosospira multiformis	92.6	93.1	92.5	92.5	95.7	83.5	92.9													
Nitrosospira briensis	92.4	92.8	92.5	92.3	95.9	83.1	93	98.6												
UN DnrA	93.1	93	90.7	90.5	91.2	82.9	94.1	91.1	91.5											
UN DnrB1	97..5	97.7	94	93.1	93.3	82.6	97.6	92.8	92.7	93.7										
UN DnrB2	97.3	97.4	94	93.	93.2	82.3	97	92.7	92.5	92.8	99.7									
UN CETP	97.4	97.6	93.9	93	93.2	82.5	97.1	92.7	92.6	93	99.8	99.6								
UN DN1	99.4	99.6	92.6	92.4	93.5	81.9	97.5	92.5	92.3	92.8	97	96.8	97							
UN DN2	97.2	97.4	93.8	92.9	93.1	82.5	97	92.6	92.5	92.8	99.5	99.3	99.5	97						
UN DN3	97.7	98	93	92.3	92.9	82.6	98	92.5	92.6	94.3	97	96.7	96.8	97.4	96.8					
UN N1	90.6	90.5	85.6	86	87.3	82.5	89.8	87	86.9	87.6	89.5	89.3	89.4	90.1	89.3	90				
UN N2	99.9	99.9	93	92.9	93.8	82.3	98	92.9	92.7	93	97.4	97.2	97.3	99.5	97.3	97.8	90.5			
UN N3	99.3	99.3	92.5	92.3	93.2	82.1	97.5	92.3	92.2	92.6	96.9	96.9	96.6	96.8	90	96.8	97.3	99.5		
UN PF1	97.4	97.6	94	93.1	93.2	82.4	97.1	92.7	92.6	93	99.8	99.8	99.6	99.9	97	99.5	96.8	97.3	96.8	
UN PF2	99.6	99.8	93	92.8	93.4	82.2	97.7	92.5	92.4	92.7	97.2	97.2	97	97.2	99.4	97.2	97.5	99.7	99.1	97.2

Table 2b.7 *amo*A sequence similarity amongst the studied AOB

	% *amo*A sequence similarity																						
Strains	*Nitrosomonas* sp. ENI 11	*N. europaea*	*N. nitrosa*	*N. oligotropha*	*Nitrosomonas* sp. Nm143	*Nitrosococcus oceanus*	*N. eutropha* C91	*Nitrosospira multiformis*	*Nitrosospira briensis*	UN DnrA	UN DnrB1	UN DnrB2	UN CETP	UN DN1	UN DN2	UN DN3	UN N1	UN N2	UN N3	UN PF1
Nitrosomonas sp. ENI 11																				
N. europaea	94.3																			
N. nitrosa	97.5	79.7																		
N. Oligotropha	74	74.2	75.9																	
Nitrosomonas sp. Nm143	73.7	76.4	78.4	79.7																
Nitrosococcus oceanus	53.1	53.4	52.1	55.5	53															
N. eutropha C91	89.2	87.8	79.2	74.2	75.7	51.7														
Nitrosospira multiformis	72.3	73.5	70.6	76.8	77.7	53.6	72.1													
Nitrosospira briensis	70.7	72.2	68.7	77.3	73.3	53.2	71.5	83.4												
UN DnrA	89	87.9	79.6	74.4	75.8	51.2	97.1	72.1	72											
UN DnrB1	89.4	91.3	79.2	74.4	74.6	53.1	88.6	74.8	73.4	88.8										
UN DnrB2	89.2	91.1	79.2	74.4	74.6	53.1	88.4	74.8	73.4	89	99.8									
UN CETP	89.2	91.1	79.2	74.4	74.6	53.1	88.4	74.8	73.4	89	99.8	100								
UN DN1	98.6	94.7	77.7	74	73.7	53.1	89.2	72.1	71.3	88.1	90.2	90	90							
UN DN2	89.2	91.1	79.2	74.4	74.6	53.1	88.4	74.8	73.4	89	99.8	100	100	90						
UN DN3	89.8	87.8	79.5	74	76.2	52.7	97.8	72.5	72	97.3	89	89.2	89.2	89..4	89.2					
UN N1	98.4	94.5	77.7	74	73.7	53.1	89	72.1	71.3	86.3	90	91.2	91.2	99.8	91.2	89.6				
UN N2	98.4	94.5	77.7	74	73.7	53.1	89	72.1	71.3	88.3	90	91.2	91.2	99.8	91.2	89.6	100			
UN N3	98.4	94.5	77.7	74	73.7	53.1	89	72.1	71.3	88.3	90	91.2	91.2	99.8	91.2	89.6	100	100		
UN PF1	88.8	91.3	79.2	74.2	74.8	53.4	88	75	73.2	88.5	99.4	99.6	99.6	89.8	99.6	88.8	90	90	90	
UN PF2	98.6	94.7	77.7	74	73.7	52.9	88.8	71.9	71.3	88.5	89.8	90	90	99.6	90	89.4	99.8	99.8	99.8	89.8

Table 2b.8 *hao* sequence similarity amongst the studied AOB

	% *hao* sequence similarity																						
Strains	*Nitrosomonas* sp. ENI 11	*N. europaea*	*N. nitrosa*	*N. oligotropha*	*Nitrosomonas* sp. Nm143	*Nitrosococcus oceanus*	*N. eutropha* C91	*Nitrospira multiformis*	*Nitrospira briensis*	UN DnrA	UN DnrB1	UN DnrB2	UN CETP	UN DN1	UN DN2	UN DN3	UN N1	UN N2	UN N3	UN PF1
Nitrosomonas sp. ENI 11																				
N. europaea	99.5																			
N. nitrosa	80.1	79.9																		
N. Oligotropha	80.3	80	80.7																	
Nitrosomonas sp. Nm143	77.8	77.8	80.9	78.7																
Nitrosococcus oceanus	60.1	60.1	60.4	56.1	59.8															
N. eutropha C91	87.4	87.4	77.8	78	76.4	58.4														
Nitrosospira multiformis	72.6	72.3	77	74.7	76.8	55	70.3													
Nitrosospira briensis	76.1	76.1	77.1	74.8	77.9	59.1	74.3	87.1												
UN DnrA	91.5	91.1	80	80	77.2	58.3	86.9	73.5	75.5											
UN DnrB1	90.2	89.8	79	79.5	77	58.2	86.8	73.1	75.2	98.1										
UN DnrB2	90.1	89.6	78.6	79.3	76.9	58.3	86.7	72.9	75.2	97.1	98.7									
UN CETP	90.4	89.9	78.6	79.6	77	58.3	87.1	72.9	75.1	97.3	99.2	98.8								
UN DN1	99.6	99.2	81.1	80.3	77.8	60.4	87.3	72.7	75.1	91.2	89.9	90.1	90.2							
UN DN2	90.4	89.9	78.8	79.3	77	58.3	86.9	73.3	75.5	97.8	99.6	99.1	99.5	90.2						
UN DN3	87.9.2	86.9	76.7	79.4	75	58.7	98.9	73	73.6	86	86.1	82.1	86.2	86.9	86.1					
UN N1	99.5	99.1	80	80.3	77.8	60.2	87.2	72.6	74.9	91..1	89.8	90	90.1	99.9	90.1	86.9				
UN N2	99.8	99.3	81.1	80.3	77.8	60.2	87.4	72.7	75.1	91.3	90	90.1	90.4	99.9	90.4	86.9	99.8			
UN N3	99.3	98.8	81.1	80.3	77.8	60.2	86.9	72.8	75.1	91.1	89.8	89.5	89.9	99.4	89.6	86.6	99.3	99.3		
UN PF1	99.6	99.2	81.1	80.3	77.8	60.4	87.3	72.7	75.1	91.2	89.9	90.1	90.2	100	90.2	86.9	99.9	99.9	99.4	
UN PF2	99.3	98.8	81.4	80.3	77.8	60.2	86.7	72.4	748	91.1	89.8	89.8	89.6	99.6	89.6	86.6	99.3	99.3	99.3	99.4

Chapter 3

Identifying physiological significance of heterotrophs co-existing with autotrophic ammonia oxidizing bacteria in an ammonia oxidizing colony

Chapter 3

Identifying physiological significance of heterotrophs co-existing with autotrophic Ammonia Oxidizing Bacteria in an ammonia oxidizing colony

ABSTRACT

Coexistence of autotrophic Ammonia Oxidizing Bacteria (AOB) and heterotrophic bacteria was consistently observed when cultured in an inorganic medium without an external supply of organic carbon. Present study was undertaken to understand the nature of association between *Nitrosomonas* sp. RA and the associated heterotrophs. Hg^{2+} selectively suppressed the growth of heterotrophs and thereby a system, containing active *Nitrosomonas* sp. RA and dormant heterotrophs, was developed and was used to delineate the interactions between them. The study revealed interdependence of heterotrophs and *Nitrosomonas* sp. RA for growth under iron limited condition. Increased growth of *Nitrosomonas* sp. RA was observed in the presence of exogenously supplied partially purified siderophore from *Pusillimonas* sp. (one of the heterotrophs) whereas survival of the heterotrophs depended on soluble microbial products released by the autotroph in the inorganic medium. Nitrite produced by the autotrophs could also be utilized by the heterotrophs relieving AOB from nitrite inhibition caused by high nitrite concentration. The nature of mutual interaction established between heterotrophs and *Nitrosomonas* sp. RA plays a significant role in stabilizing ammonia oxidizing system involved in bioremediation of ammonia.

3.1 Introduction

Autotrophic Ammonia Oxidizing Bacteria (AOB) are ubiquitously present in environments (aerobic and/or anoxic) where ammonia is made available either naturally or through anthropogenic activities (Kowalchuk and Stephen, 2001). AOB are exceedingly slow growing with petite growth yield that makes their isolation difficult and time consuming. Moreover, heterotrophs tend to build up rapidly associated with AOB even without an external supply of organic carbon (Okabe et al., 1996; Kowalchuk and Stephen, 2001; Kindaichi et al., 2004). Interestingly, aerobic ammonia oxidation is reported to proceed more rapidly in the presence of the contaminating heterotrophs (Clark and Schmidt, 1966; Jones and Hood, 1980). However, till date little is known about the functional significance of such association.

AOB are chemolithoautotroph and use ammonia and carbon dioxide for their growth (Chain et al., 2003). Iron is one of the important nutrients in the physiology of these organisms as Fe-containing cytochromes and proteins are involved in ammonia-oxidizing metabolism (Upadhyay et al., 2003). In natural niche of AOB, iron exists in ferric state as an insoluble hydroxide and therefore its biological availability is low (Andrews et al., 2003). Microorganisms produce low molecular weight iron specific chelator, 'siderophores', to scavenge iron from the environment. The siderophores form soluble Fe^{3+}–siderophore- complexes that are internalized by outer membrane siderophore receptor protein mediated active transport mechanism (Byers and Arceneaux, 1998). Reports on siderophore synthesis by AOB are scanty. Amongst AOB, only *Nitrosococcus oceani* has a complete set of siderophore (aerobactin, a hydroxymate type siderophore) synthesizing genes, whereas the same are either absent or incomplete in other AOB (Klotz et al., 2006). All AOB rather encode genes for iron acquisition (siderophore receptors and transporters), which allows efficient scavenging and uptake of multiple forms of iron from iron-limited environments to meet their high iron requirement (Chain et al., 2003; Klotz et al., 2006; Stein et al., 2007; Norton et al., 2008).

AOB release soluble microbial products (SMP) by reducing inorganic carbon to organic carbon and cell mass (Rittmann et al., 1994; Ohashi et al., 1995). In a nitrifying consortium SMP released by AOB and nitrite oxidizing bacteria have been shown to be utilized by the associated heterotrophs thus preventing build up of the waste metabolites in the biofilm (Kindaichi et al., 2004; Nogueria et al., 2005). Distribution of heterotrophs in a nitrifying biofilm is correlated with the diversity and distribution of SMP in a particular niche (Kindaichi et al., 2004). Though occurrence of competitive interactions between heterotrophs and AOB for dissolved oxygen and space has been reported (Okabe et al., 1996; Nogueria et al., 2005), not much is known about the significance of such association and a detailed investigation is required to understand and improve aerobic ammonia oxidation process.

Present investigation reports for the first time a systematic study on the interactions between AOB and heterotrophs found closely associated in an ammonia oxidizing colony. Association of these organisms was studied by developing a system of AOB devoid of heterotrophs. The study revealed dependence of *Nitrosomonas* sp. RA, on the siderophore produced by associated heterotrophs for complementing their iron requirement. This is further reiterated by the demonstration of TonB dependent siderophore receptor gene in *Nitrosomonas* sp. RA. The heterotrophs in turn survive at the expense of SMP released by *Nitrosomonas* sp. RA during its chemolithotrophic metabolism.

3.2 Materials and Methods

Twelve kinds of isolated colonies of AOB were obtained on Nylon66 membrane filters as mentioned in Chapter 2a. Purity of the isolated colonies was checked by streaking on LA plate, which showed presence of heterotrophs in the red colored isolated AOB colonies. DnrA was used as a case study to understand the mechanism of interactions between autotrophs and heterotrophs coexisting in inorganic media without external organic carbon supply.

3.2.1 DnrA sample description

Enrichment of AOB from sludge of a denitrifying reactor of a fertilizer industry was carried out for one month at 30˚C in dark in standard inorganic medium with sodium carbonate and ammonia sulphate as the sole source of carbon and nitrogen (Hyman and Arp, 1992) as mentioned in chapter 2a. Enriched sample was streaked on Nylon66 membrane filters kept on the same inorganic medium containing 1% agar to get purified AOB colony. Isolated colony of AOB was inoculated in 50 ml inorganic medium and its ammonia oxidizing activity was measured in terms of ammonia consumed as per the method by Scheiner (1976) and nitrite released as described by Griess-Romijn (1996). The picked colony was restreaked on fresh plates several times and its purity was checked by streaking on Luria Bertani plates. Heterotrophs coexisting with *Nitrosomonas* sp. RA in inorganic media were enumerated as a function of time by spreading on LA plates.

3.2.2 Heterotrophic ammonia utilization by the heterotrophs

Acidovorax sp., *Janibacter* sp. and *Pusillimonas* sp. were grown in the presence of ammonia as the nitrogen and acetate and pyruvate as the carbon sources. Growth of the heterotrophs was measured after 72 h. Ammonia oxidizing activity by the heterotrophs was measured from the acetate containing media as mentioned earlier.

3.2.3 Identification of microorganisms

Genomic DNA from the isolated colony was extracted according to Schmidt et al., (1991). 16S rDNA was amplified from the extracted genomic DNA using universal primer 27F and 1541R and purified using PCR Clean-up gel extraction NucleoSpin Extract II Kit (Macherey – Nagel GmbH and Co.KG, Germany) according to the manufacturer's instructions. These were cloned in pTZ57R/T vector using INSTA cloning kit (Fermentas, Inc). Amplified ribosomal DNA restriction analysis was carried out using *Alu*I restriction enzyme to differentiate the clones. 16S rRNA genes were commercially

sequenced by single pass analysis from Bangalore Genei, India. 16S rRNA gene was also amplified from pure heterotrophs obtained on LA plates and digested with *Alu*I restriction enzyme.

3.2.4 Effect of mercury (Hg^{2+}) on growth of *Nitrosomonas* sp. RA and coexistent heterotrophs

Nitrosomonas sp. RA was cultured in standard inorganic medium (where it was observed to coexist with heterotrophs) in the presence of different Hg^{2+} concentrations (0, 2, 4, 6, 8, 10, 20, and 40 ppm). Growth was measured by optical density (O.D.) at 600 nm and ammonia oxidizing activity was measured as mentioned earlier. Growth of heterotrophs was measured by spreading different dilutions on Luria Bertani agar medium (LA). Lack of growth of coexistent heterotrophs at 20 ppm Hg^{2+} on LA was confirmed by spreading the culture on several other media like Nutrient Agar, Yeast Extract Agar, Potato Dextrose Agar, Meat Extract Agar, Tryptone Broth and filter sterilized culture supernatant obtained from growth of *Nitrosomonas* sp. RA in inorganic medium.

3.2.5 Scanning electron microscopy (SEM)

Isolated colony of *Nitrosomonas* sp. RA on standard inorganic media containing 20 ppm Hg^{2+} was picked and smear was made on a grease free glass slide. SEM was carried out by fixing with glutaraldehyde and dehydrated in a series of increasing acetone concentrations according to Holger et al., (1999). Preparations were dried sputter-coated with silver and examined in a Joel Scanning Electron Microscope with Oxford EDS system model No. JSM-5610LV (Patel et al., 2011).

3.2.6 Revival of the dormant heterotrophs

Mercury exposed cells were further exposed to 45°C for 1 min and immediately cooled on ice for 5 min. These heat shocked cells were spread on LA plates and incubated at 30°C till growth appeared.

3.2.7 Effect of Fe^{2+} on growth of *Nitrosomonas* sp. RA

Iron is an important cofactor in the transport of electron in the cytochromes and several enzymes like hydroxylamine oxidoreductase in AOB hence effect of iron was checked on *Nitrosomonas* sp. RA by growing it in the presence of 20 ppm Hg^{2+} at different Fe^{2+} concentrations (0, 0.01, 0.1, 1.0 and 10 µM) and growth of AOB was monitored by measuring O.D. at 600 nm. Medium without Fe^{2+} and Hg^{2+} was used as control to see the effect of heterotroph on the growth of *Nitrosomonas* sp. RA.

3.2.8 Siderophore detection, quantification and extraction

Siderophore production was detected by the Chrome Azurol-S (CAS) plates as described by Schwyn and Neilands (1987). *Pusillimonas* sp. was grown in deferrated Luria Bertani medium for 48 h and centrifuged at 7500 X g for 20 min. Deferration of the medium with 0.25% 8-hydroxyquinoline and chloroform was achieved as per the method described by Schwyn and Neiland (1987). Culture supernatant was used to detect the type of siderophore produced and to quantify it. Siderophore was extracted from the culture supernatant according to Jadhav and Desai (1992). Catecholate type siderophore was detected and estimated using 2, 3-dihydroxybenzoic acid (2, 3-DHBA) as a standard as per the method described by Arnow (1937). Hydroxamate type siderophore was detected and estimated using hydroxylamine hydrochloride as the standard as per Gibson and Magrath (1969).

3.2.9 Minimum inhibitory concentration of EDTA for *Nitrosomonas* sp. RA

Nitrosomonas sp. RA was spreaded on Nylon66 membrane filter kept on inorganic medium without $FeSO_4$ in the presence of various concentration of EDTA (0.0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) in standard inorganic media containing agar. These were subcultured 2 times at an interval of 2 days during which growth was observed on filter paper and MIC-EDTA concentration could be found out. To

measure growth, filters were washed in N-saline and O. D. was measured at 600 nm.

3.2.10 Siderophore bioassay

Nitrosomonas sp. RA, as the test organism, was surface spread on inorganic medium without $FeSO_4$ containing minimum inhibitory concentration of EDTA (0.8 mM) and 20 ppm Hg^{2+}. This is considered as iron deplete condition. A filter disc (6 mm diameter) soaked with partially purified siderophores (1 mg/ml) from *Pusillimonas* sp. was placed over it. Positive and negative controls were filter disc soaked with $FeSO_4$ (2 mg/ml) and inorganic medium respectively. Standard hydroxamate type siderophores used were desferrioxamine (1 mg/ml) and ferrichrome (1 mg/ml) (Sigma Aldrich).

3.2.11 Effect of varied concentration of exogenously supplied siderophores on the growth of *Nitrosomonas* sp. RA in the absence of heterotrophs.

Effect of siderophore on the growth of *Nitrosomonas* sp. RA was monitored by exogenously supplementing partially purified siderophores from *Pusillimonas* sp. at varied concentrations (100-500 µg/ml) in inorganic medium without $FeSO_4$ in the presence of mercury. Growth of *Nitrosomonas* sp. RA was monitored by measuring O.D. at 600 nm.

3.2.12 Effect of exogenous addition of siderophore (200 µg/ml), on growth of *Nitrosomonas* sp. RA in presence (-Hg^{2+}) and absence (+Hg^{2+}) of heterotrophs.

Following set of experiments were used to see the effect of exogenous addition of siderophores on the growth of *Nitrosomonas* sp. RA in the presence (-Hg^{2+}) and absence (+Hg^{2+}) of heterotrophs. 200 µg/ml siderophore and 20 ppm Hg^{2+} were used in the experiment.

a) without siderophore with Hg^{2+} b) with siderophore with Hg^{2+}, c) without siderophore without Hg^{2+} and d) with siderophore without Hg^{2+} were used. Growth under these conditions were compared with the growth in normal

inorganic medium with $FeSO_4$ (10 µM) without Hg^{2+}. Growth was monitored by measuring the O.D. at 600 nm.

3.2.13 Amplification of TonB-dependent siderophore receptor gene fragment

Primers were designed for the amplification of TonB-dependent siderophore receptor gene from *Nitrosomonas* sp. RA grown in the presence of Hg^{2+}. F (forward primer) - 5'GCCGACAACATCAACGTGCG3' and R (reverse primer) - 5'TGCATGCGATCAATTTGGGTA3' having binding position 17-36 and 999-1019 respectively relative to *Nitrosomonas eutropha* C91 TonB-dependent siderophore receptor gene (Accession No. - NC008344, position 1839391-1841520). Amplification was carried out in a 30 µl reaction system containing 1 µl of DNA (about 50 $ng\mu l^{-1}$), 3 µl of 10X Taq buffer E, 1.5 µl of dNTP (2.5 mM), 1 µl of each forward and reverse primer (10 $pmol\mu l^{-1}$) (all reagents were purchase from Bangalore Genei, India). Conditions for amplification were, an initial denaturation 94°C for 5 min, followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 45 s and elongation at 72°C for 1 min this was followed by final elongation at 72°C for 10 min. The amplified fragment was cloned in pTZ57R/T using INSTA cloning kit as mentioned earlier. Confirmed clone was commercially sequenced using single pass analysis from Bangalore Genei, India. The obtained sequence was submitted to NCBI.

3.2.14 Measurement of Soluble Microbial Products (SMP)

Nitrosomonas sp. RA was grown in inorganic medium in the presence and absence of Hg^{2+}. After 4 days of incubation, SMP was measured in terms of Chemical Oxygen Demand (COD), titrable acidity, total polysaccharides and pyruvate released from the culture supernatant. COD, titrable acidity, and polysaccharides were measured according to Tomar (1999), Eisenman (1998) and Scott and Melvin (1953) respectively. Organic acids were extracted by diethylether according to Vega et al., (1970) and pyruvic acid was measured colorimetrically with dinitrophenyl hydrazine according to Anthon and Barrett (2003).

Growth (O.D. at 600 nm) of heterotrophs was measured after 3 days of incubation in standard inorganic medium containing with 40 mg% sodium pyruvate and sodium acetate in place of sodium carbonate to check their capabilities to utilize organic acids known to be present in SMP.

3.2.15 Effect of nitrite on the growth of *Nitrosomonas* sp. RA

The effect of various concentrations of nitrite (0, 2, 4, 8, and 12 mg/ml) was checked on the growth of *Nitrosomonas* sp. RA in the presence (-Hg^{2+}) and absence (+Hg^{2+}) heterotrophs.

3.2.16 Nitrite utilization of by heterotrophs

Acidovorax sp. *Janibacter* sp. and *Pusillimonas* sp. were grown individually in media (50 ml) containing nitrite as the nitrogen source and acetate as the carbon source under aerobic conditions. Nitrite removal was checked after 72 h.

3.2.17 Nucleotide accession number

The GenBank accession number for the 16S rRNA gene sequence of *Nitrosomonas* sp. RA is JN099273, of *Janibacte*r sp. is JX143799, of *Acidovorax* sp. is JX143800, of *Pusillimonas* sp. is JX143801 and for TonB-dependent siderophore receptor gene fragment is JX262377.

3.3 Results and Discussion

3.3.1 Enrichment of AOB and identification of bacteria present in the isolated colonies

AOB were enriched from soil and sludge samples from various industries and when these enriched cultures were streaked on Nylon 66 membrane kept on inorganic medium, red colored colonies appeared within 14 days of incubation as shown in Chapter 2a (Fig 2a.2). AOB do not grow on heterotrophic media like LA and hence their purity was checked by streaking them on heterotrophic media. Surprisingly, different kinds of heterotrophic

colonies appeared within 3 days of incubation which suggested coexistence of heterotrophs with autotrophs in inorganic media (Fig 3.1).

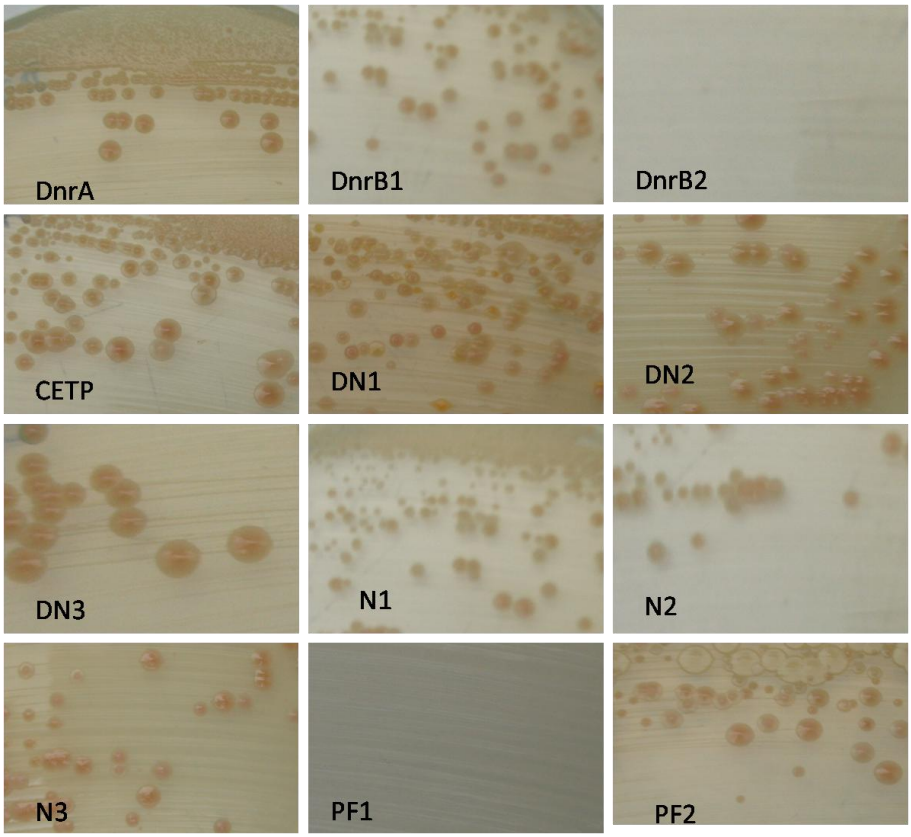


Fig 3.1 Growth of heterotrophs on Luria Bertani plates from twelve AOB isolates.

3.3.2 Identification of microorganisms present in the red colored AOB colonies

Genomic DNA was extracted from these and used for the molecular analysis of the microorganisms present in the colonies. 16S rRNA gene was cloned from all the 12 isolates and differentiated by Amplified ribosomal DNA restriction analysis (ARDRA) which revealed several different patterns (Fig 3.2).

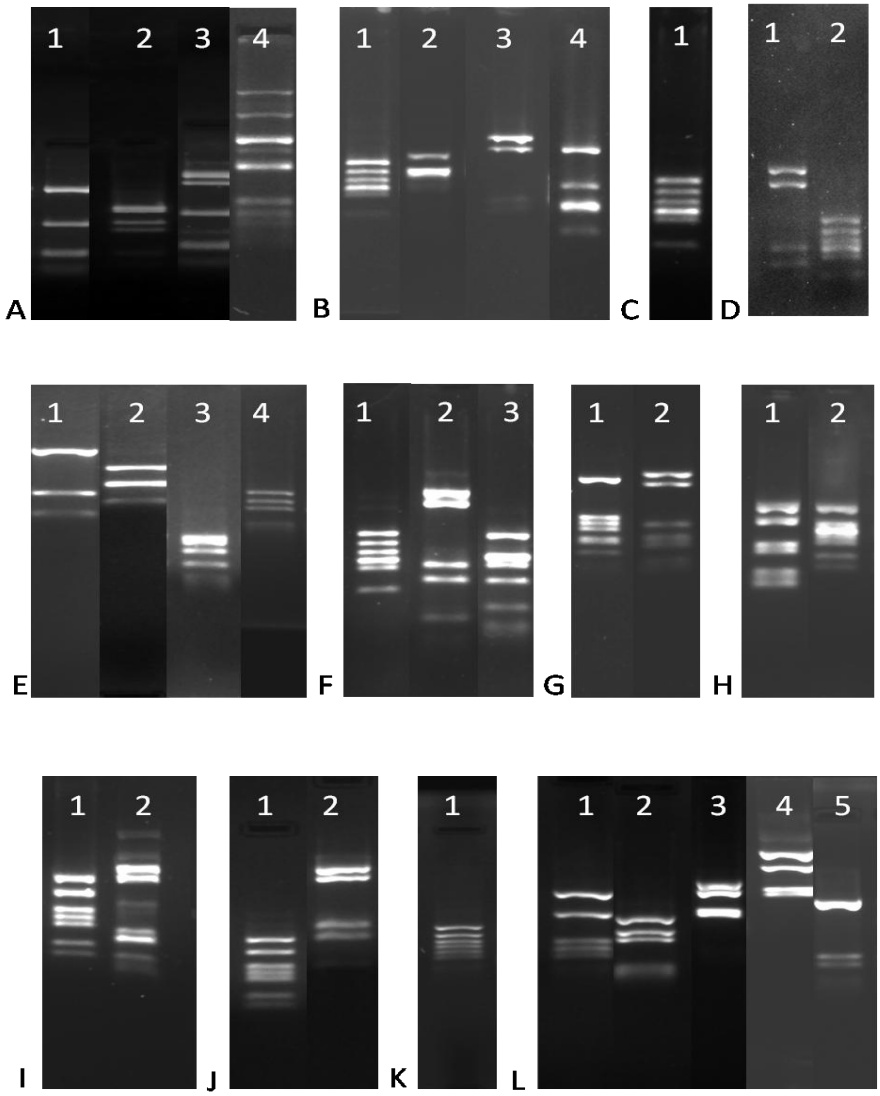


Fig 3.2 Representative ARDRA pattern observed by digesting cloned 16S rRNA gene with *Alu*I restriction enzyme from each colony. A) DnrA B) DnrB1 C) DnrB2 D) CETP E) DN1 F) DN2 G) DN3 H) N1 I) N2 J) N3 K) PF1 L) PF2

Five patterns were observed in PF2, 4 patterns were observed in DnrA, DnrB1 and DN1, 3 patterns were observed in DN2, 2 patterns were observed in CETP, DN3, N1, N2, N3 and 1 pattern was observed in DnrB2 and PF1 (Fig 3.2). These results also confirmed that more than one kind of bacteria were present in most colonies.

Eleven of the twelve colonies contained *Nitrosomonas* spp. whereas N1 contained AOB having 87% similarity with *Nitrosospira* sp. (as mentioned in Chapter 2a). 16S rDNA also revealed presence of heterotrophs like *Pusillimonas sp., Acidovorax sp., Acromobacter sp., Janibacter sp., Alcaligenes*

sp., Sphingopyxis granuli, Mezorhizoium sp. R2, *Thermomonas sp.* and *Castellaniela defragnans* coexisting along with AOB which was confirmed by spreading the isolated colonies on Luria Bertani plates.

DnrA, in which this kind of association was observed repeatedly for more than one year, was used as a representative to unravel the mechanism of coexistence between them.

Upon streaking the isolated red colony (Fig 3.3A) from the inorganic medium on LA plate, three kinds of heterotrophs differentiated based on colony morphology (Fig 3.3B). This colony was maintained for more than one year wherein the presence of the heterotrophs regularly monitored by spreading on LA plate which were easily distinguishable based on their colony morphology (Fig 3.3B). In a single colony, four different kinds of cells were observed in the gram staining using light microscopy (Fig 3.3 C).

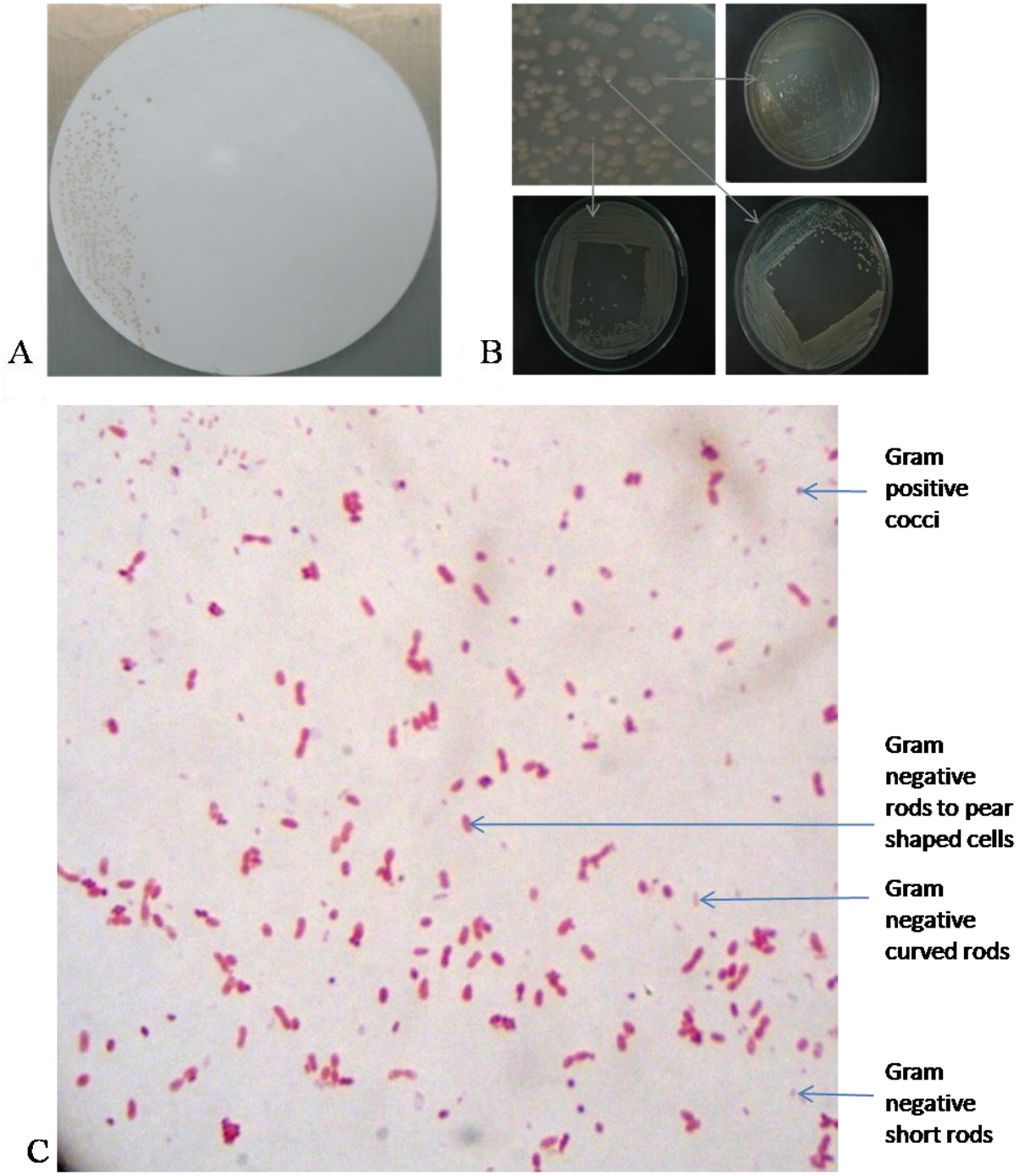


Fig 3.3 A) Red colored isolated colonies of enriched AOB sample obtained on filters within two weeks of incubation B) presence of heterotrophs on Luria Bertani media C) gram staining from isolated colony showing four different kinds of cells.

More than 200 clones were obtained on cloning 16S rDNA in *E. coli* DH5α. Four distinct patterns were obtained when ARDRA was performed using *Alu*I restriction enzyme (Fig 3.4A)

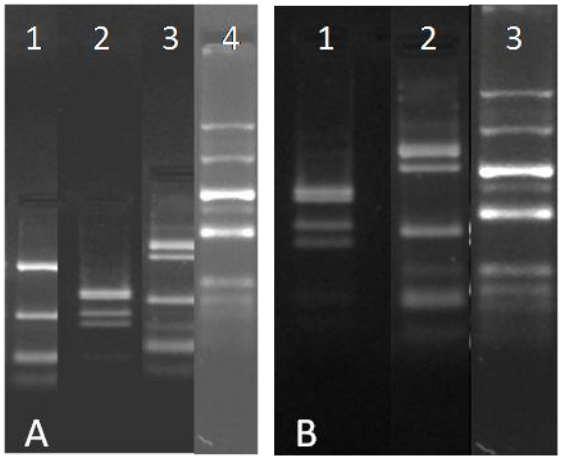


Fig 3.4 A) Representative ARDRA gel showing 4 distinct patterns of 16S rRNA gene obtained by digestion with *Alu*I restriction enzyme from the isolated colony. B) RFLP of 16S rRNA gene obtained from pure heterotrophs 1) *Janibacter* sp. 2) *Pusillimonas* sp. 3) *Acidovorax* sp.

Sequencing of 16S rRNA gene from these clones revealed presence of a single AOB having 93% similarity with *Nitrosomonas eutropha* C91 (accession No. JN099273), designated henceforth as *Nitrosomonas* sp. RA and three heterotrophs, *Pusillimonas* sp. Mn5-9 (99% identity; accession No. JX143801), *Janibacter* sp. BQN4P5-02d (100% identity; accession No. JX143799) and *Acidovorax* sp. Ic3 (99% identity; accession No. JX143800). 16S rRNA gene form pure heterotrophs obtained on LA plate was digested with *Alu*I restriction enzyme and compared it with ARDRA gel obtained by cloning 16S rRNA gene from *Nitrosomonas* sp. RA colony and showed similarity in the banding patterns (Fig 3.4 A and B).

3.3.3 Growth of *Nitrosomonas* sp. RA and associated heterotrophs

Growth of *Nitrosomonas* sp. RA in inorganic medium reached stationary phase by the third day while that of the heterotrophs in the same experiment, monitored by plating on heterotrophic medium, reached the stationary phase by the second day (Fig 3.5).

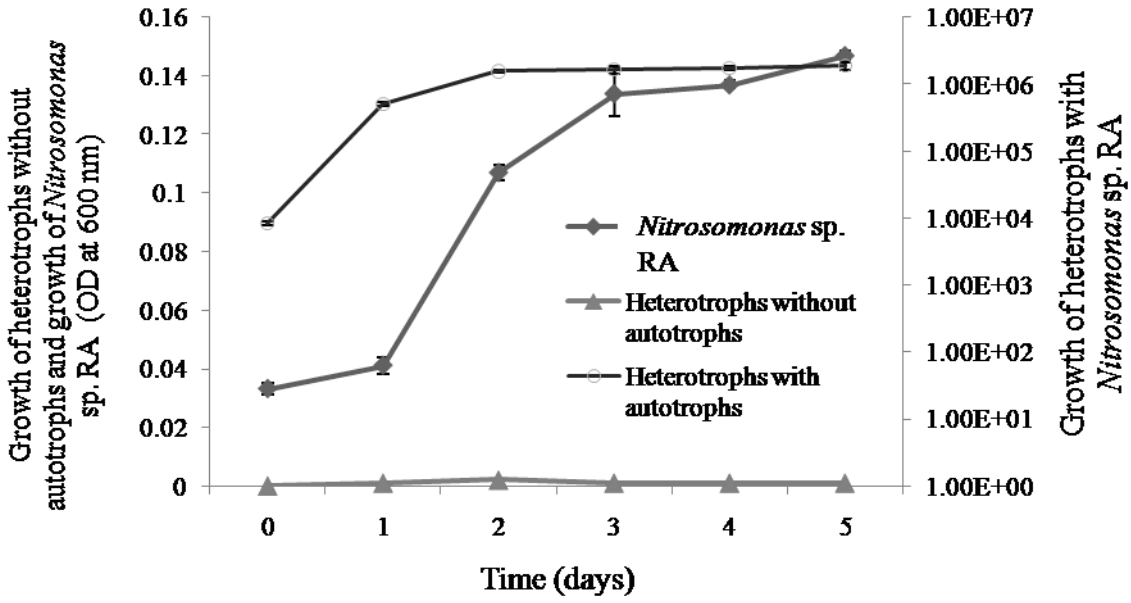


Fig 3.5 Growth of *Nitrosomonas* sp. RA and heterotrophs in inorganic media in the presence of *Nitrosomonas* sp. RA and as pure cultures.

Pure heterotrophs failed to grow alone in inorganic medium, however, they showed growth only in the presence of *Nitrosomonas* sp. RA in the same medium (Fig 3.5), implying their dependence on the *Nitrosomonas* sp. RA for growth in the said medium.

Aerobic ammonia oxidizing activity of *Nitrosomonas* sp. RA, measured in terms of ammonia consumed and nitrite produced, increased linearly with time reaching plateau at around fourth day (Fig 3.6).

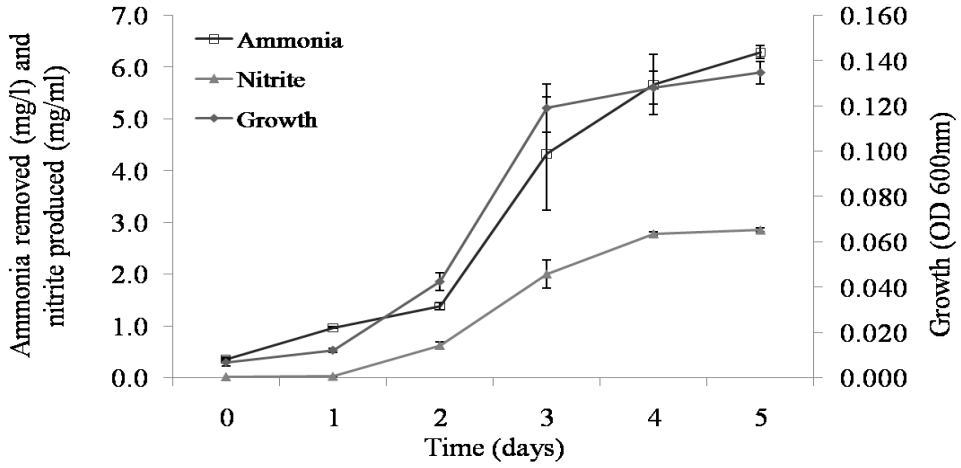


Fig 3.6 Growth and ammonia oxidizing activity of *Nitrosomonas* sp. RA in inorganic media.

At the end of five days 6.3 ± 0.124 mg/ml NH_4^+-N was oxidized to produce 2.9 ± 0.035 mg/ml NO_2^--N. Ammonia oxidizing activity of *Nitrosomonas* sp. RA clearly correlated with the growth of microorganisms observed by measuring O.D. at 600 nm (Fig 3.6). The stoichiometry of ammonia removal to nitrite formation for *Nitrosomonas* sp. RA was 0.15 mM: 0.03 mM) less than the required stoichiometry (1 mM : 0.98 mM) (Ahn, 2006) indicated higher utilization of ammonia from the system.

Ammonia is the sole source of nitrogen in the standard inorganic media for the growth of the microorganisms. AOB utilize ammonia for nitrite formation as well as for biomass formation. Similarly, heterotrophic bacteria present along with *Nitrosomonas* sp. RA also utilize it for their growth. Presence of glutamate dehydrogenase and glutamine synthetase are reported in all the three heterotrophs (*Janibacter hoylei* PVAS-1 whole genome shotgun sequencing project Accession no. NZ_ALWX01000000; *Acidovorax* sp. JS42 chromosome complete genome accession no. NC_008782; and *Pusillimonas* sp. T7-7 chromosome, complete genome Accession No., NC_015458, Cao et al., 2011). Hence, ammonia utilization by the heterotrophs was checked in the presence of acetate as the carbon source. Pyruvate was detected as one of the major organic acid in the released SMP in this study whereas acetate has been reported as representative organic carbon constituent of SMP by Kindaichi et al., (2004) hence pyruvate and acetate were used to check growth of heterotrophs and ammonia assimilation by heterotrophs.

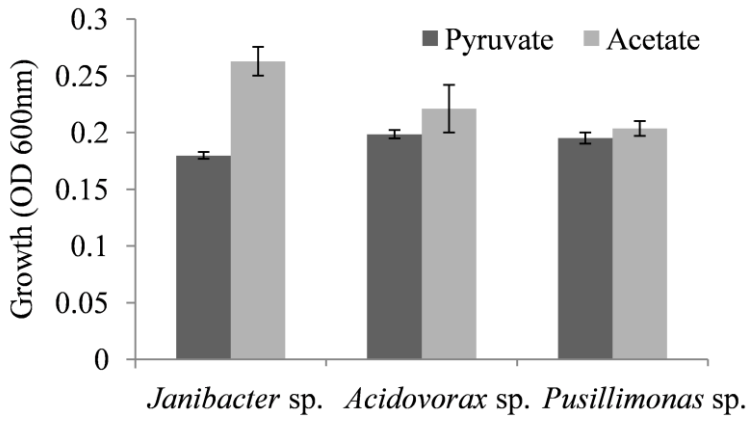


Fig 3.7 Growth of heterotrophs in the presence of acetate and pyruvate as carbon source.

Growth of all three heterotrophs was observed with ammonia as the sole nitrogen source in presence of both pyruvate and acetate and growth was more in the presence of acetate than pyruvate suggesting that acetate is the preferred carbon source for these heterotrophs (Fig 3.7).

The results justified higher ammonia utilization observed in the system containing the autotrophs and the heterotrophs. Heterotrophic nitrification by the three heterotrophs when checked, nitrite formation was observed by all the three heterotrophs and was highest in *Pusillimonas* sp. but the nitrite formed was 1000 times less compared to nitrite produced by *Nitrosomonas* sp. RA (Fig 3.8).

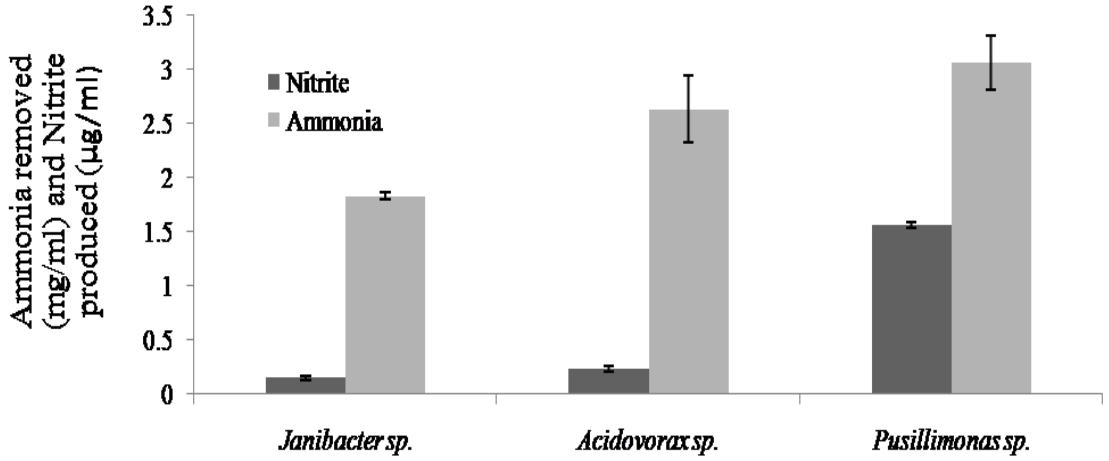


Fig 3.8 Ammonia oxidizing activity of the heterotrophs in the presence of acetate as the carbon source.

Thus, ammonia, apart from getting utilized through assimilation by the heterotrophs, it may also get utilized by heterotrophic nitrification (Fig 3.8). This further supports higher utilization of ammonia by the system.

3.3.4 Purification of *Nitrosomonas* sp. RA.

As the objective of the present study was to understand the functional co-existence of *Nitrosomonas* sp. RA and heterotrophs, it was necessary to develop a system where growth of heterotrophs in the colony could be inhibited without affecting that of *Nitrosomonas* sp. RA. Serial dilution carried out to get purified AOB failed to separate it from the heterotrophs.

Growth of *Nitrosomonas* sp. RA was observed till 10^{-9} dilution but heterotrophs still appeared on LA plate during purity check (Fig 3.9).

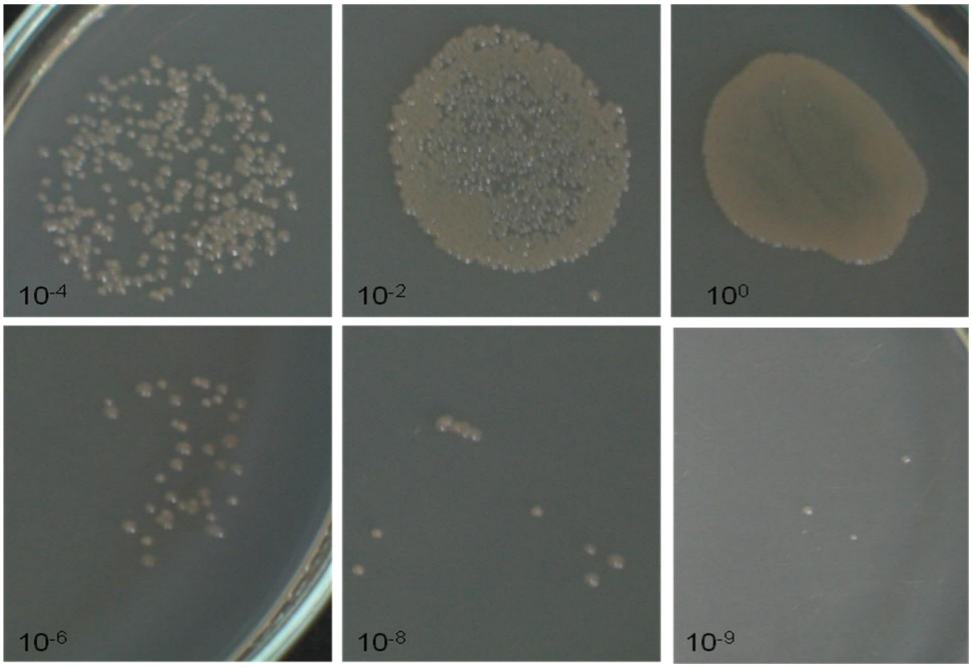


Fig 3.9 Growth of heterotrophs observed in the various dilutions of inorganic media.

Since it was not possible to physically separate both groups of organisms, a different strategy of inhibiting growth of one organism without affecting that of the other was attempted. Published genome sequence of *N. eutropha* C91 showed presence of a 117Kbp genomic island encoding multiple genes for heavy metal resistance, including clusters for copper and mercury (Hg^{2+}) transport (Stein et al., 2007) implying organism's resistance to these heavy metals. Effect of copper and mercury therefore was checked on the growth of *Nitrosomonas* sp. RA and the heterotrophs. Copper, up to 100 µg/ml concentration, failed to inhibit growth of either organisms and beyond this concentration growth of *Nitrosomonas* sp. RA was inhibited (Fig 3.10).

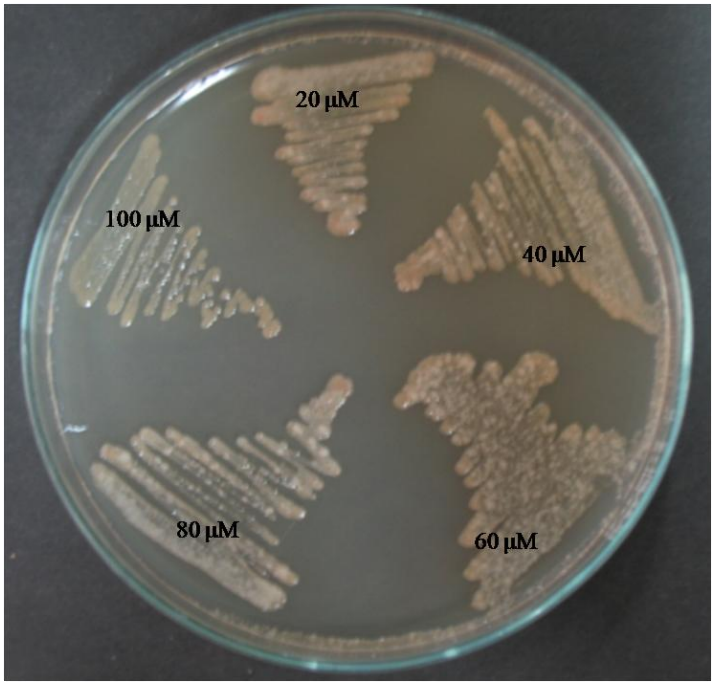


Fig 3.10 Growth of heterotrophs on Luria Bertani plate after exposure to different copper (20-100 µM) concentrations.

When effect of Hg^{2+} was checked on the growth of the organisms, heterotrophs survived up to 8 ppm Hg^{2+}, and showed complete inhibition at and beyond 10 ppm Hg^{2+} (Fig 3.11A). *Nitrosomonas* sp. RA on the other hand survived till 20 ppm Hg^{2+} (Fig 3.11B) but with a growth inhibition of approximately 27% and a decrease of 21.2% in nitrite production (Fig 3.11C). Longer lag period however was observed in growth of AOB in presence of 20 ppm Hg^{2+} (Fig 3.11B and C).

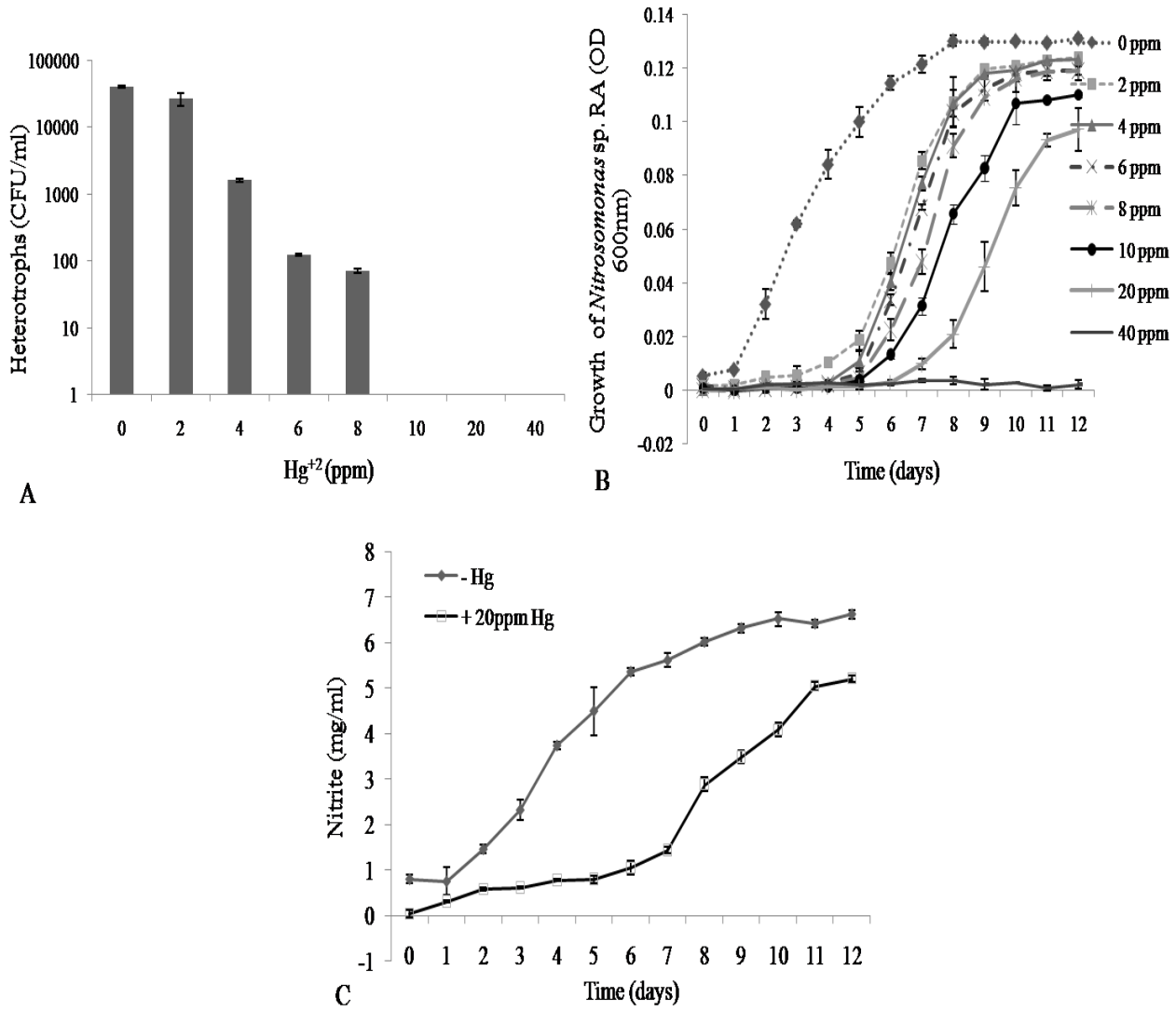


Fig 3.11 Effect of mercury on the growth of A) Heterotrophs B) *Nitrosomonas* sp. RA. C) Ammonia oxidizing activity (in terms of nitrite produced) by *Nitrosomonas* sp. RA in presence and absence of Hg^{2+}.

Hg^{2+} (20 ppm) treated *Nitrosomonas* sp. RA culture did not show growth on six different heterotrophic media even after 15 days of incubation confirming inhibition of associated heterotrophs. However, SEM of this culture still showed presence of two type of cells (cocci closely associated with rods) (Fig 3.12A).

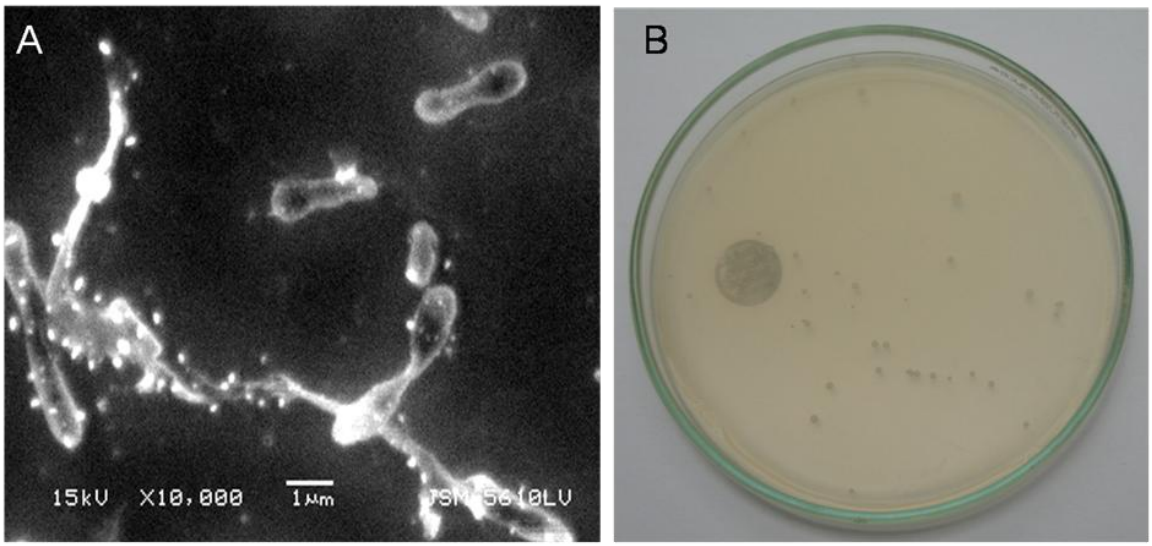


Fig 3.12 A) SEM image of *Nitrosomonas* sp. RA after exposure to 20 ppm mercury. B) Growth of heterotrophs on Luria Bertani plate after resuscitation.

The heterotrophs therefore were thought to be present in the dormant state. Heat shocked cells grew on LA within 48 h of incubation at 30°C (Fig 3.12B). The heterotroph that survived was identified to be *Pusillimonas* sp. through 16S rRNA gene sequencing. A system containing active *Nitrosomonas* sp. RA devoid of physiologically active heterotrophs was thus developed. This system was used to delineate interdependence of growth between *Nitrosomonas sp.* RA and the associated heterotrophs. Accordingly, growth with/without Hg^{2+} corresponded to a system without/with heterotrophs respectively.

3.3.5 Effect of iron on the growth of *Nitrosomonas* sp. RA.

Ammonia oxidizers are known for their tremendous iron (Fe^{2+}) requirement due to involvement of cytochromes and haem-containing enzymes in ammonia oxidizing metabolism (Upadhyay et al., 2003). *Nitrosomonas europaea,* has been reported to show high iron requirement and concentrations ranging from 10 to 250 µM Fe^{2+} resulted in normal growth of *N. europaea* (Wei et al., 2006). Iron concentration 10 µM present in the standard inorganic media supported normal growth of *Nitrosomonas* sp. RA, hence, it was grown in medium containing varied Fe^{2+} concentrations (0 to

10 µM) in the presence of Hg^{2+}. Lower Fe^{2+} concentrations (0, 0.01, 0. 1 and 1.0 µM) failed to support growth whereas about 80% of the growth, compared to its growth in standard inorganic medium without Hg^{2+}, was observed at 10 µM Fe^{2+}. The results indicated that *Nitrosomonas* sp. RA showed higher iron requirement compared to *Nitrosomonas europaea* which has been reported to show 30% and 60% growth at 0.1 and 0.2 µM iron concentration by Wei et al., (2006). However, about 90% growth, compared to its growth in the standard inorganic medium, was observed without external supply of iron in absence of Hg^{2+} (Fig 3.13). This observation clearly indicated role of associated heterotrophs in iron nutrition of *Nitrosomonas* sp. RA.

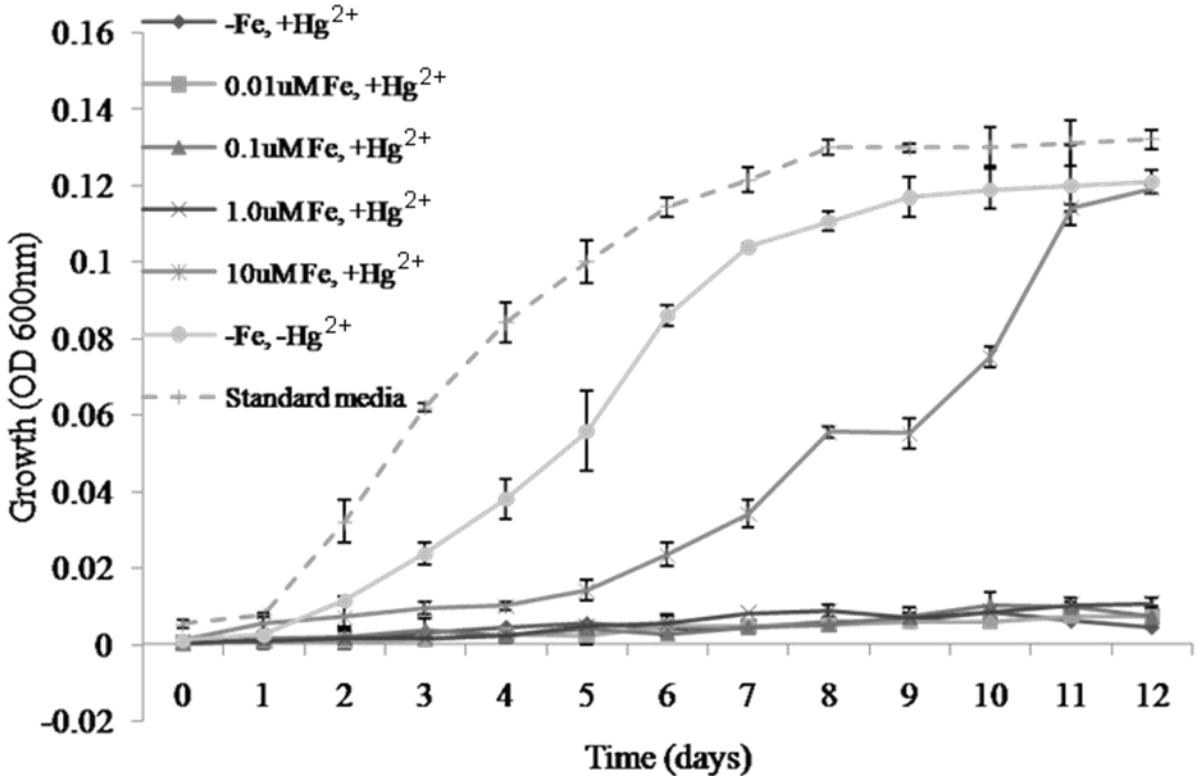


Fig 3.13 Growth of *Nitrosomonas* sp. RA at varied iron ($FeSO_4$) concentrations (0 to 10 µM) in the presence and absence of Hg^{2+}.

3.3.6 Siderophore production by heterotrophs

Most prokaryotes use siderophore mediated iron uptake system to meet their iron requirement. Amongst AOB only *N. europaea* has been studied for its iron and siderophore requirement in detail. Amid the sequenced AOB, *N. europaea* shows absence of siderophore biosynthesis genes (Chain et al., 2003), *N. eutropha* C91 and *Nitrosospira multiformis* show presence of incomplete siderophore biosynthesis genes (Stein et al., 2007; Norton et al., 2008) whereas only *N. oceani* shows a complete set of siderophore biosynthesis genes (Klotz et al., 2006) but reports on the functionality of these genes are lacking. Inability of *Nitrosomonas* sp. RA to grow under iron limiting conditions in the absence of hetetrotrophs implied its inability to produce siderophores. The heterotrophs were therefore checked for siderophore production by CAS assay. All the three heterotrophs showed siderophore production but *Pusillimonas* sp. showed higher production and comparable to *Pseudomonas fluorescence* (positive standard strain control) (Fig 3.14).

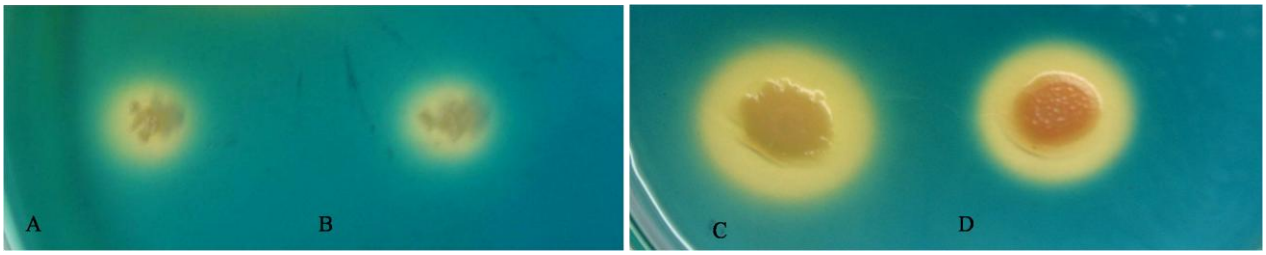


Fig 3.14 Siderophore production by heterotrophs under deferrated conditions A) *Acidovorax* sp. B) *Janibacter* sp. C) *Pusillimonas* sp. and D) *Pseudomonas fluorescence.*

The siderophore produced (17 mg/ml in 72 h) by *Pusillimonas* sp. was identified to be hydroxamate type. The siderophore was extracted and partially purified according to Jadhav and Desai (1992) through which 1.01 mg/ml siderophore could be extracted with extraction efficiency 5.99%.

3.3.7 MIC of EDTA concentration for *Nitrosomonas* sp. RA

In order to develop siderophore bioassay to demonstrate utilization of exogenously supplied siderophore towards growth of *Nitrosomonas* sp. RA, use was made of EDTA known to be chelating utilizable iron leading to iron deficient conditions (Joshi et al., 2008). *Nitrosomonas* sp. RA was grown at various EDTA concentrations to find out the EDTA concentration at which iron would be completely chelated from the system and the test organism would not grow. MIC of EDTA for *Nitrosomonas* sp. RA was found to be 0.8 mM (Fig 3.15). This is lower than those reported earlier for rhizhospheric isolates (3.5 mM to 8 mM) (Joshi et al., 2006) indicating higher sensitivity of *Nitrosomonas* sp. RA to iron limited conditions.

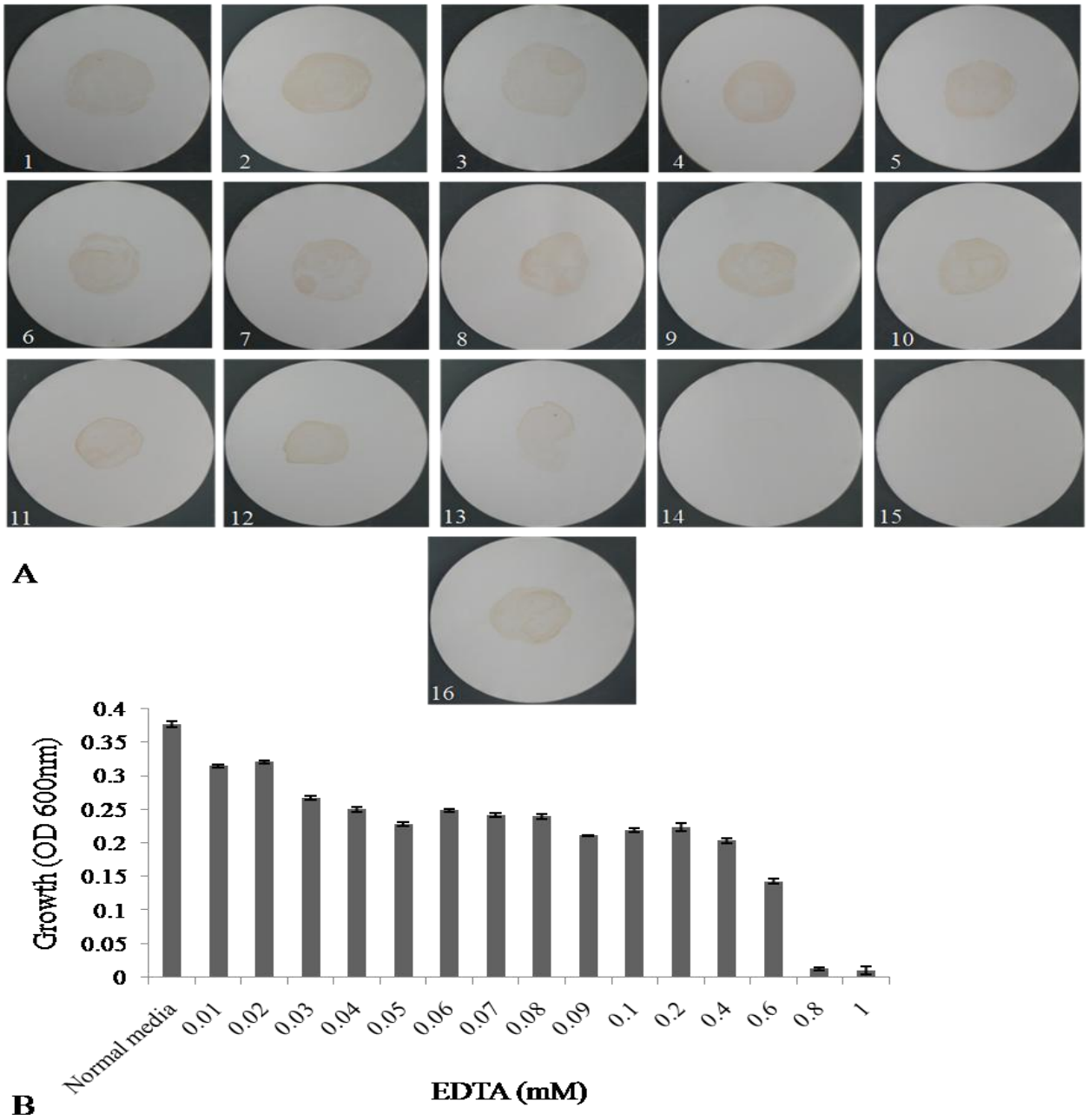


Fig 3.15 Effect of varied EDTA concentration (mM) on the growth of *Nitrosomonas* sp. RA 1) 0.01 2) 0.02 3) 0.03 4) 0.04 5) 0.05 6) 0.06 7) 0.07 8) 0.08 9) 0.09 10) 0.1 11) 0.2 12) 0.4 13) 0.6 14) 0.8 15) 1.0 16) Normal media A) visual growth on filter B) OD at 600 nm

3.3.8 Utilization of externally supplied partially purified siderophores by *Nitrosomonas* sp. RA

A novel bioassay was developed to assess the ability of *Nitrosomonas* sp. RA to take up externally supplied siderophore on a plate containing 20 ppm Hg^{2+} under iron deplete condition. *Nitrosomonas* sp. RA, as the test organism, was surface spread on inorganic medium and a filter disc containing partially purified siderophores (1 mg/ml) from *Pusillimonas* sp. was placed over it. *Nitrosomonas* sp. RA showed zone of growth exhibition around the filter disc demonstrating utilization of this ferri-siderophores by *Nitrosomonas* sp. RA for its growth (Fig 3.16A). The experiment was validated by taking proper controls. Zone of growth exhibition was observed around filter disc containing $FeSO_4$ (2 mg/ml) (positive control) and no growth was observed in the presence of deferrated medium (negative control) (Fig 3.16E and C). *Nitrosomonas* sp. RA could also utilize iron complexed with ferrichrome (1 mg/ml) whereas Fe-desferrioxamine (1 mg/ml) was weakly utilized (Fig 3.16B and D). Increase in growth of *Nitrosomonas* sp. RA in the presence of externally supplied siderophores in the siderophore bioassay suggests that it has the potential of utilizing externally supplied siderophores. Similarly, *N. europaea* has also been reported to utilize externally supplied desferal, ferrichrome and enterobactin siderophores for iron acquisition (Wei et al., 2006).

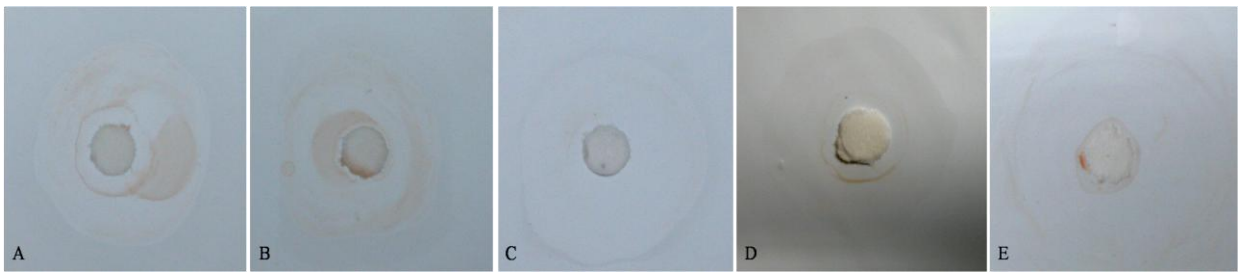


Fig 3.16 Siderophore Bioassay showing utilization of exogenously supplied siderophores by *Nitrosomonas* sp. RA. A) Partially purified siderophores from *Pusillimonas* sp. B) Ferrichrome (standard hydroxamate siderophore) C) Deferrated medium (negative control) D) Fe-desferroxamine (standard hydroxamate type siderophore) E) $FeSO_4$ (positive control).

Effect of exogenous addition of partially purified siderophore from *Pusillimonas* sp. on the growth *Nitrosomonas* sp. RA was also checked in the deferrated liquid medium in presence of Hg^{2+} to validate our earlier observation. Increase in growth was observed in the presence of externally supplied siderophore at varied concentration (100-500 µg/ml) (Fig 3.17). Growth of *Nitrosomonas* sp. RA was marginally higher at 200 µg/ml concentration compared to the other siderophores concentrations used.

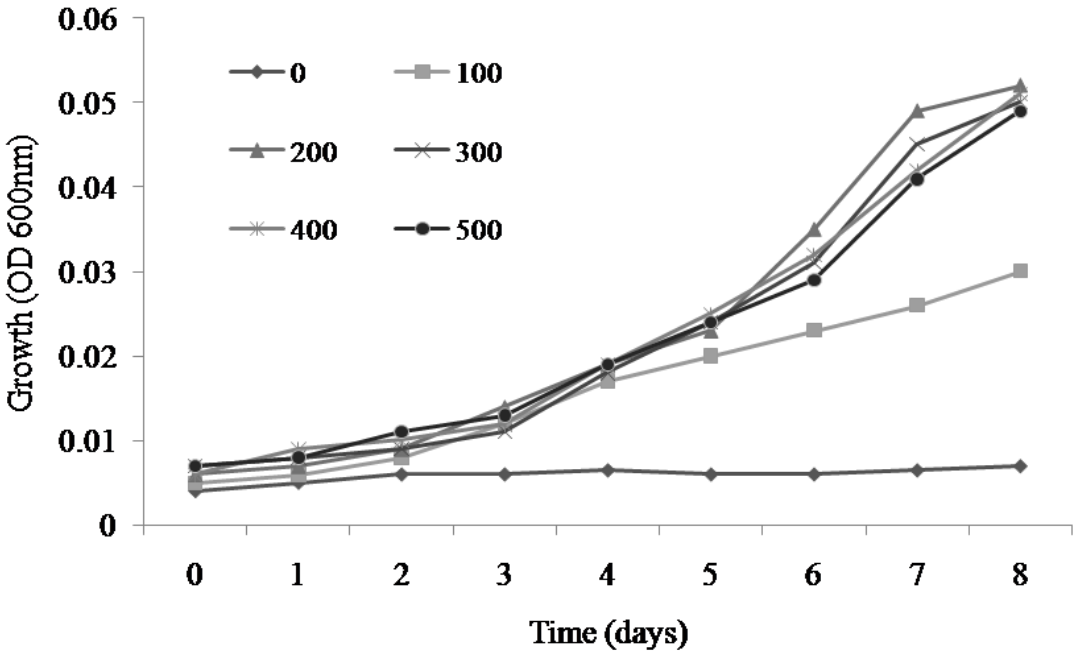


Fig 3.17 Effect of different siderophore concentrations (0-500 µg/ml) on the growth of *Nitrosomonas* sp. RA in the absence of heterotrophs.

As there was not much difference between growth observed in the presence of 200-500 µg/ml siderophore concentration, 200 µg/ml siderophore concentration (being least) was used in the subsequent study to check the effect of heterotrophs. In the absence of heterotrophs (+Hg^{2+}), 47% increase in the growth of *Nitrosomonas* sp. RA was observed in presence of siderophore in the iron deplete medium but with an initial lag phase of 3 days as compared to its growth in the normal media. Increased growth observed in the presence of externally supplied siderophores validated our siderophore bioassay observations. However, practically no difference in growth of *Nitrosomonas* sp. RA was observed with and without siderophore

in the presence of heterotrophs (–Hg^{2+}) (Fig 3.18). The growth of *Nitrosomonas* sp. RA with and without siderophore in presence of heterotrophs was almost same, indicating that sufficient siderophores were being provided by heterotrophs towards meeting its iron requirement. This suggests dependence of *Nitrosomonas* sp. RA on heterotrophs for their growth in iron limited inorganic media.

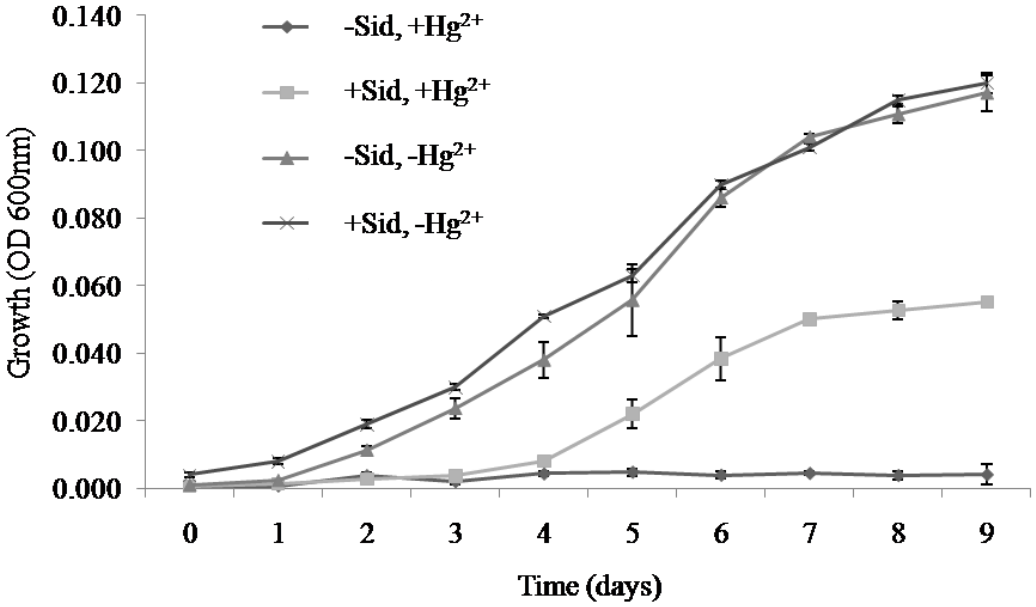


Fig 3.18 Growth of *Nitrosomonas* sp. RA with and without exogenously supplied siderophore (200 µg/ml) in absence (+Hg^{+2}) and presence (-Hg^{+2}) of heterotrophs.

Genomic DNA extracted from *Nitrosomonas* sp. RA grown in presence of Hg^{2+} showed amplification of siderophore receptor gene fragment (1002bp) (Fig 3.19).

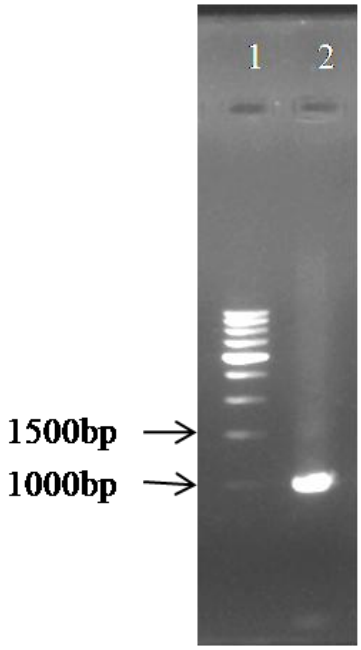


Fig 3.19 Amplification of TonB-dependent siderophore receptor gene fragment (1002bp). Lane 1. Marker Lane 2. TonB-dependent siderophore receptor gene fragment from *Nitrosomonas* sp. RA with Hg^{2+}.

The amplified TonB-dependent siderophore receptor gene fragment was sequenced and submitted in NCBI. The sequence showed 94% identity to the *N. eutropha* C91 TonB-dependent siderophore receptor gene. Genome sequence of *N. eutropha* C91 showed presence of two ferrichrome receptor genes for hydroxamate type siderophore (Stein et al., 2007). The utilization of hydroxamate type siderophore produced by *Pusillimonas* sp. and ferrichrome, a standard hydroxamate type siderophore (Fig 3.14A and B), found to be abundantly present in natural environment, confirmed functionality of the TonB-dependent siderophore receptor gene in *Nitrosomonas* sp. RA. Ferrichrome is the main source of iron in the rhizospheric soil (Powell et al., 1983). Previous studies conducted in authors' lab have shown that cloning of ferrichrome siderophore receptor gene in *Rhizobium* spp. led to increase in its competitive survival in the rhizosphere which has been attributed to the strain's ability to acquire iron from the environment (Joshi et al., 2008; Geetha et al., 2009). In a similar fashion *Nitrosomonas* sp. RA could make use of siderophores produced by the

associated heterotrophs towards acquiring iron from the natural iron stressed environment to fulfill its iron requirement.

3.3.9 Release of soluble microbial products by AOB

Organic metabolites, collectively denoted as soluble microbial products (SMP), inevitably produced by AOB during its autotrophic growth, supports the growth of heterotrophs in the inorganic medium (Kindaichi et al. 2004). SMP was measured in terms of COD, titrable acidity, total polysaccharides and pyruvate released in the medium by AOB in the presence and absence of heterotrophs. SMP produced were approximately 0.7 to 0.8 folds higher in the absence of heterotrophs ($+Hg^{2+}$) than in its presence ($-Hg^{2+}$) in all the cases (Table 3.1). Higher amount of SMP produced by *Nitrosomonas* sp. RA in absence of heterotrophs than in its presence indicated utilization of SMP by associated heterotrophs. In the presence of mercury *Acidovorax* sp. and *Janibacter* sp. got eliminated as they were sensitive to mercury and *Pusillimonas* sp. went into the dormant state but partial respiration may occur. Hence, requirement of organic carbon reduced but a sharp increase in the organic carbon levels was not observed.

Table 3.1 SMP produced by *Nitrosomonas* sp. RA in the presence and absence of heterotrophs.

	COD (mg/L)	Titratable acidity (mg/L)	Polysaccharides (mg/ml)	Pyruvic acid (µM)
Nitrosomonas sp. RA with heterotrophs ($-Hg^{2+}$)	0.94±0.015	23.59±0.34	0.16±0.07	0.81±0.088
Nitrosomonas sp. RA without heterotrophs ($+Hg^{2+}$)	1.19±0.02	29.84±0.52	0.2±0.032	1.12±0.029

3.3.10 Effect of nitrite on the growth of AOB

Nitrite, the end product of ammonia oxidation carried out by AOB, at higher concentrations is known to inhibit ammonia monooxygenase and thereby inhibit the growth of AOB (Stein and Arp, 1998). It was therefore important to check the effect of nitrite on the growth of *Nitrosomonas* sp. RA.

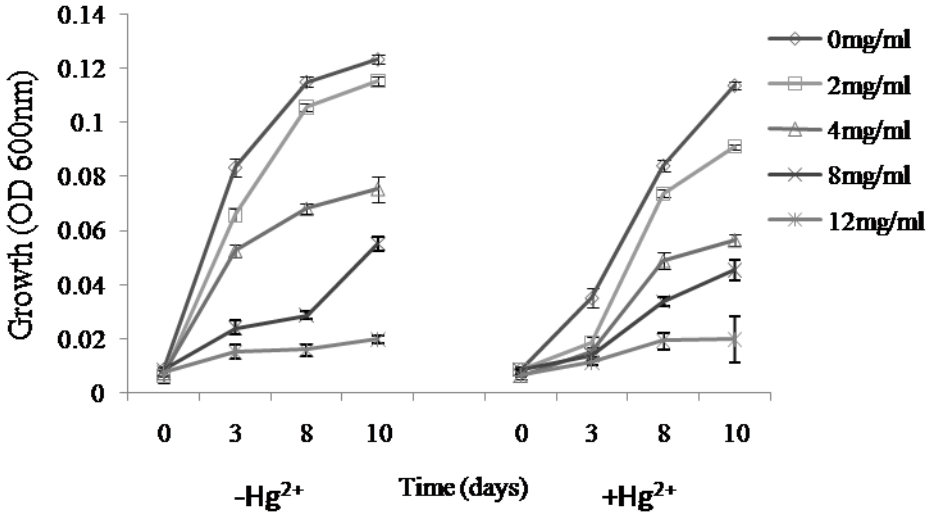


Fig 3.20 Effect of nitrite on the growth of *Nitrosomonas* sp. RA A) without heterotrophs ($+Hg^{2+}$) and B) with heterotrophs ($-Hg^{2+}$).

Growth of *Nitrosomonas* sp. RA decreased in the presence of nitrite but the effect was less in the presence of heterotrophs ($-Hg^{2+}$) (Fig 3.20).

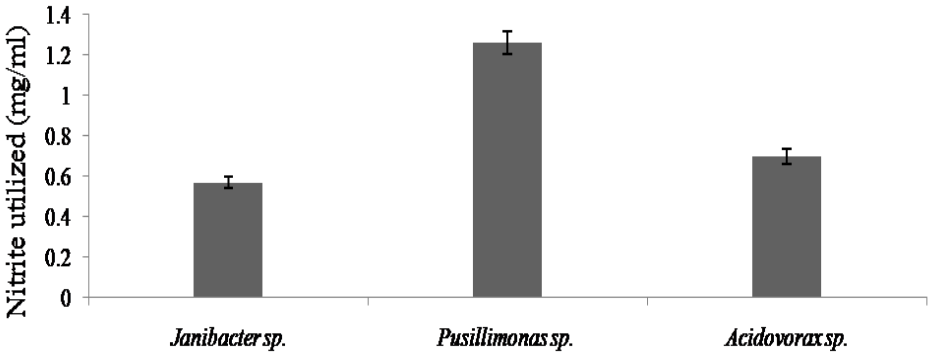


Fig 3.21 Nitrite utilization by heterotrophs.

Nitrite was utilized as the nitrogen source by all the three heterotrophs and *Pusillimonas* sp. utilized it maximally (Fig. 3.21). The results justified the

observation of decreased inhibition in growth of *Nitrosomonas* sp. RA observed in the presence of heterotrophs (Fig 3.20). The associated heterotrophs by way of utilizing nitrite would rescue *Nitrosomonas* sp. RA of growth inhibition by nitrite, the end product of ammonia oxidation. Removal of nitrite aerobically from the system also disturbed the stoichiometry of ammonia to nitrite conversion as observed earlier in the study.

Higher growth and ammonia removal by AOB in the presence of heterotrophs than pure cultures has also been reported earlier (Clark and Schmidt, 1966; Jones and Hood, 1980).

Inability of heterotrophs to survive alone in the inorganic medium with carbonate as the carbon source and their growth along with *Nitrosomonas* sp. RA in the inorganic medium suggested its obligatory dependence on *Nitrosomonas* sp. RA to suffice their organic carbon requirement for growth in the said medium.

In conclusion, *Nitrosomonas* sp. RA and heterotrophs present in a single colony were dependent mutually on each other for growth demonstrating a functional association. Mercury induced dormant state in the heterotrophs whereas, *Nitrosomonas* sp. RA was found to be resistant to mercury and hence a system containing *Nitrosomonas* sp. RA without physiologically active heterotrophs was obtained. This is the first report where *Nitrosomonas* sp. RA has been demonstrated, through bioassay, to utilize siderophores produced by associated heterotrophs towards meeting its iron requirement. Heterotrophs in turn survived at the expense of the SMP produced by *Nitrosomonas* sp. RA. In addition, organic carbon sequestration by heterotrophs could facilitate the growth of *Nitrosomonas* sp. RA as organic carbon is reported to inhibit growth of autotrophs (Hockenbury and Grady, 1977). Nitrite utilization by the heterotrophs improved growth of autotrophs by removing nitrite inhibition. A mutual interdependence amongst the two groups of organisms for growth thus could be established. Such interactions are often observed in natural environment and are extremely important for the proper stabilization and functioning of the microbial community. The

mechanism of co-existence of both groups of organisms as derived from the study has been summarized in the schematic (Fig 3.22).

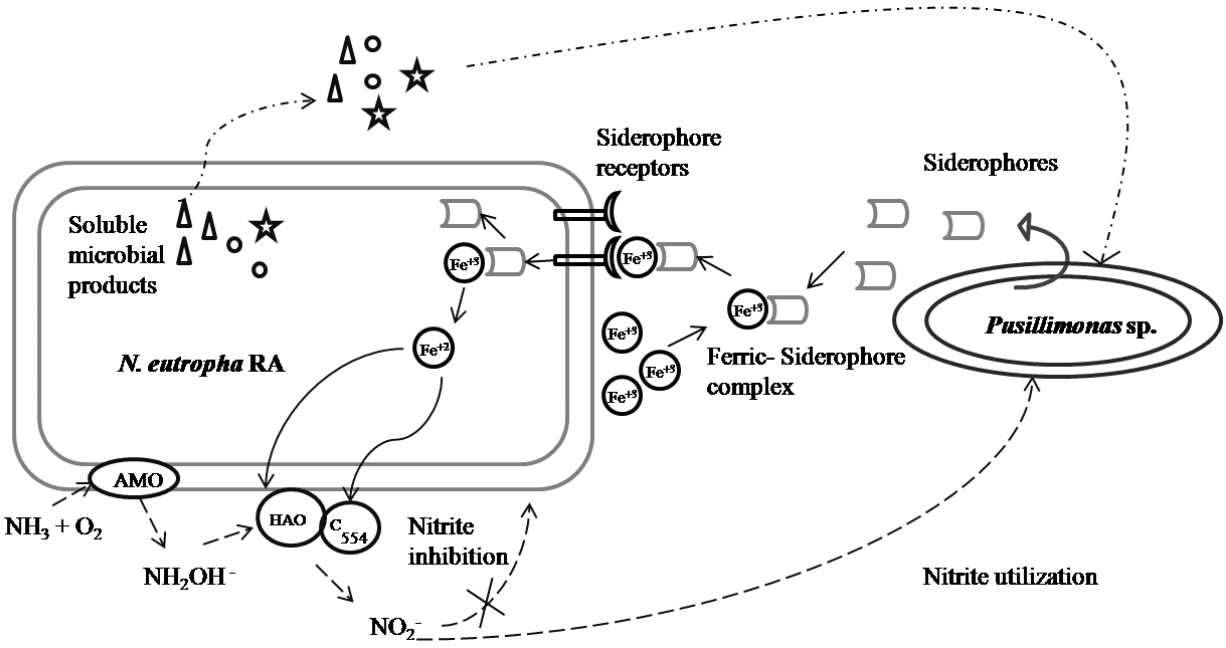


Fig 3.22 Schematic diagram showing mechanism of co-existence of *Nitrosomonas sp.* RA and heterotrophs.

Chapter 4

Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for removal of ammonia from effluent of a fertilizer industry

Chapter 4

Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for removal of ammonia from effluent of a fertilizer industry

ABSTRACT

A simultaneous partial nitrification, anaerobic ammonia oxidation (anammox) and denitrification (SNAD) process was developed for the treatment of ammonia laden effluent of a fertilizer industry. Autotrophic anaerobic ammonia oxidizing (anammox) biomass was enriched which removed ammonia and nitrite in the ratio similar to the expected stoichiometry of anammox reaction carried out under anaerobic conditions. Anoxic removal of ammonia by the enriched anammox biomass using nitrite produced by autotrophic Ammonia Oxidizing Bacteria (AOB) was confirmed with synthetic effluent system. A seed consortium was developed by mixing two of the enriched anammox biomass and AOB biomass (as per chapter 2) having maximum activity in 1:1 proportion. This was applied in the treatment of ammonia laden effluent of a fertilizer industry in an oxygen limited bench scale SNAD type reactor (1 L) run at ambient temperature (~30°C). Around 98.9% ammonia removal was achieved with ammonia loading rate of 0.35 kgNH_4^+-N/m^3day in the presence of 46.6 mg/L COD with 2.31 days hydraulic retention time. Qualitative and quantitative analysis of the biomass from upper (oxic) and lower (anoxic) zone of the reactor revealed presence of AOB, Planctomycetes and denitrifiers as the dominant bacteria carrying out anoxic oxidation of ammonia in the reactor. Physiological and molecular studies strongly indicated presence of anammox bacteria in the anoxic zone of the SNAD reactor.

4.1 Introduction

Ammonia released in the effluent of agriculture based industries has become a prime concern with the increasing awareness of pollution in water bodies leading to eutrophication and acidification of the aquatic systems. Conventional ammonia removal from wastewater accomplished through the combination of nitrification (aerobic) and denitrification (anaerobic) is expensive, energy and space requiring process that generates high amount of sludge (Bagchi et al., 2012). Attention therefore has been focused on the development of novel cost-effective processes for the treatment of high ammonia containing wastewater with no/low level of organic carbon.

New technologies developed over the time for efficient removal of ammonia from wastewaters towards meeting the rising demand of environment protection include, SHARON (single reactor system for high activity ammonia removal over nitrite), SND (simultaneous nitrification denitrification), SNAP (single stage nitrogen removal using anammox and partial nitritation), CANON (complete autotrophic nitrogen removal over nitrite), OLAND (oxygen limited autotrophic nitrification and denitrification), DEMON (DEamMONification), and BABE (Bio-Augmentation Batch Enhanced), each having its own advantages and disadvantages (Bagchi et al., (2012). These processes involve partial nitrification (oxidation of ammonia to nitrite) followed by anoxic oxidation of the remaining ammonia by the anammox bacteria in presence of nitrite as electron acceptor. These processes are operated in a single reactor unit with reduced aeration (1 kWh/kgN) and without external organic load requirements; saving 90% of the operation costs (Wang et al., 2010). Although significant nitrification is not expected at DO below 0.3 mg/L, treatment processes that promote simultaneous nitrification-denitrification can reach up to 80% of the total nitrification under anoxic conditions (Stenstrom and Poduska, 1980). In agreement with this, nitrifiers, denitrifiers and anammox bacteria (having optimum activities under completely different conditions) are reported to coexist in the same environment (Xiao et al., 2008).

Of the methods mentioned for ammonia removal, OLAND and CANON are more suitable for the treatment of wastewaters with high ammonia concentration and without organic carbon, but have limited application for wastewaters containing organic carbon (Lan et al., 2011). **S**imultaneous partial **N**itrification, **A**nammox and **D**enitrification (SNAD) described by Chen et al., (2009) was developed to solve the problem of organic carbon in CANON process. This recently developed process has been used efficiently at the full scale land fill leachate treatment plant for nitrogen removal (Wang et al., 2010).

Present study reports application of SNAD for the treatment of high ammonia containing effluent of a fertilizer industry with low levels of COD (46.6 mg/L). The process involved partial nitrification of ammonia by aerobic autotrophic Ammonia Oxidizing Bacteria (AOB) that convert ammonia to nitrite, the remaining ammonia and nitrite so formed are converted to molecular nitrogen by anammox bacteria which in turn release low levels of nitrate in the process. The nitrates so formed could be reduced to N_2 by denitrifiers at the expense of organic carbon in the effluents. Levels of COD and nitrate in the system would tend to limit the growth of denitrifiers such that, less sludge is developed during the process. Activity and growth of one kind of microorganism seems to provide substrate for the next establishing a succession of bacteria that maintain harmony and cooperation in the reactor to effectively remove ammonia. The kinetics of biological ammonia removal from the ammonia laden wastewater is discussed together with the characterization and quantification (by RT-PCR) of AOB, anammox and denitrifiers developed in the reactor during the process.

4.2 Materials and Methods

4.2.1 Enrichment of anaerobic ammonia oxidizers (anammox) biomass

Several soil and activated sludge samples from municipal wastewater treatment plant and fertilizer industries were used for the enrichment of anammox biomass (Table 4.1). Enrichment for anammox biomass was carried out with samples (5%) inoculated in inorganic medium (100 ml)

containing ammonium sulphate (3 mM) as the nitrogen source, sodium nitrite (6 mM) as the electron acceptor, potassium bicarbonate (25 mM) as the carbon source and other trace metals as described by Egli et al., (2001). Anaerobic conditions were created by flushing helium (He) gas (99.99% purity) through the medium. Anammox activity of the enriched biomass was measured by monitoring the amount of ammonia and nitrite removed. Gas produced during the process was confirmed to be nitrogen through gas chromatography (GC) analysis (Model:Varian 3600) using He as the carrier gas. Molecular sieve column made of stainless steel with 5 A° diameter and 3 m length was used. Gas tight syringe (SGE, Australia) was used to inject sample. To avoid air contamination the tubes were kept in a closed beaker in which He atmosphere was created. Two of the enriched biomass so developed showing higher anammox activity were used in the further study and were designated as PF-anammox and N4-anammox. Effect of hydrazine was checked on its anammox activity by adding of 0.25 mg/ml hydrazine. Amplification of Planctomycetes specific and anammox specific regions in the 16S rRNA gene was carried out as per following: Primers - Pla46F (forward primer) 5'GACTTGCATGCCTAATCC 3' and 1392R (reverse primer) 5'GACGGGCGGTGTGACAA 3' were used for Planctomycetes specific amplification and Pla46F (forward primer) and Amx820 (reverse primer) 5'CCTTTCGGGCATTGCGAA3' for anammox specific amplification according to Tal et al., (2006).

4.2.2 Maintenance of the enriched anammox biomass (PF-anammox and N4-anammox)

The enriched biomass was inoculated into small rubber tube with 3 mm diameter and 4 ft length containing inorganic medium (composition as described in 4.2.1) for growth and maintenance. Both ends of the tube were sealed and kept at 37°C for one month. Care was taken to exclude air intrusion by removing all air bubbles from the rubber tube. The rubber tubes being transparent facilitated monitoring of biomass development and generation of gas.

4.2.3 Development of seed consortium for SNAD bioreactor

Closed systems (500 ml) having synthetic effluent were used in this study to check the ability of PF-AOB and N4-AOB (developed as per chapter 2a) to survive and oxidize ammonia under anoxic conditions and to check the ability of PF-anammox and N4-anammox to utilize nitrite produced by AOB. The composition of synthetic effluent was same as that of the inorganic medium used for the enrichment of AOB and anammox biomass respectively without and with nitrite as mentioned below. The different reactor conditions and controls, used were as follows: i) AOB control- this constituted uninnoculated synthetic effluent containing ammonium sulphate as the nitrogen source without sodium nitrite ii) Anammox control – this constituted uninnoculated synthetic effluent containing ammonium sulphate and sodium nitrite and was made anaerobic by flushing He gas. iii) PF-AOB and N4-AOB (experimental sets) constituted of synthetic effluent containing only ammonium sulphate under anoxic condition inoculated with enriched PF-AOB and N4-AOB biomass (5% inoculum size) respectively. iv) PF-anammox and N4-anammox (experimental sets) constituted of synthetic effluent containing both ammonium sulphate and sodium nitrite under anaerobic conditions created by flushing He gas and inoculated with enriched PF-anammox and N4-anammox biomass (5% inoculum size) V) PF-AOB+PF-anammox and N4-AOB+N4-anammox (experimental sets) contained synthetic effluent with only ammonium sulphate under anoxic condition inoculated with mixture of PF-AOB and PF-anammox (ratio 1:5) and N4-AOB and N4-anammox (ratio 1:5). The reactors in which anoxic condition was to be created were not flushed with He, but were tightly closed such that external air could not enter. All the reactors were incubated at 30°C for three months. Concentration of ammonia, nitrite and nitrogen were measured after 3 months of incubation.

Final seed culture for the 1 L SNAD type reactor comprised of N4-AOB + N4-anammox and PF-AOB + PF-anammox mixed in 1:1 ratio.

4.2.4 Operating conditions for removal of ammonia from effluent of a fertilizer industry in a 1 L SNAD type reactor

A cylindrical reactor with 1 L effective volume was designed with 45 cm height, internal diameter of 7 cm and outlet at 35 cm height. Schematic diagram of the reactor is shown in Fig 4.1. The reactor was operated in the up-flow mode as the influent was introduced from the bottom. Operating temperature was 30°C and was run in the batch mode for initial 15 days. Inlet and outlet ammonia, nitrite and nitrate were measured continuously every two days for 125 days while pH and dissolved oxygen was measured (YSI200 portable DO meter, USA) in the upper and lower zone of the reactor every 15 days. Total solids (TS), total dissolved solids (TDS), total suspended solids (TSS), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and sludge volume index (SVI) were estimated at the commencement and termination of the reactor. Flow rate of the reactor was maintained at 0.3 ml/min and hydraulic retention time (HRT) was 2.31 days.

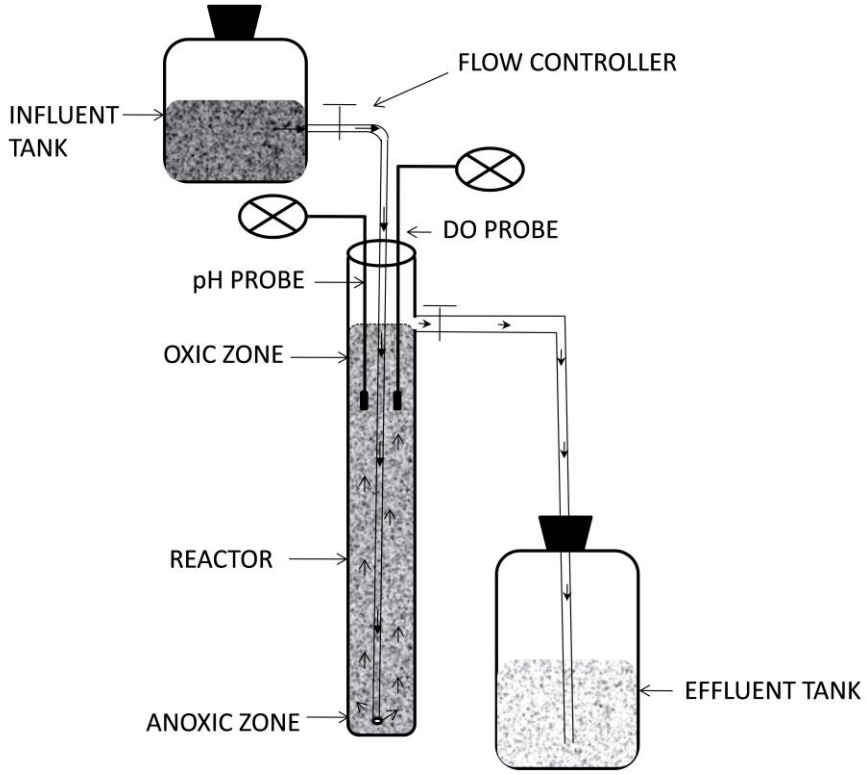


Fig 4.1 Schematic diagram of the SNAD type reactor. ⟶ Indicates direction of inflow of effluent ⟶ Indicates direction of flow of effluent in the reactor and ⟶ Indicates outflow of effluent.

4.2.5 Ammonia conversion efficiency

In the SNAD process, AOB would partly convert ammonia to nitrite. The remaining ammonia would be anaerobically oxidized with the help of nitrite to form molecular nitrogen along with nitrate. This conversion of ammonia was calculated throughout the run according to Davery et al., (2012) by the equation

$$Y_{(NO_2^- + NO_3^-) / NH_4^+} = \frac{\text{Eff. } \{(NO_2^- - N) + (NO_3^- - N)\}}{\text{Inf. } (NH_4^+ - N) - \text{Eff. } (NH_4^+ - N)} \times 100\%$$

Theoretically, 100% ammonia has been reported to produce 88% nitrogen and 11% nitrate (Davery et al., 2012). However, presence of heterotrophic denitrifiers have been reported to reduce ammonia conversion efficiency from its theoretical value (11%) because of the reduction of nitrate to N_2 by the heterotrophic denitrifiers. Presence of nitrite oxidizing bacteria would

increase it due to oxidation of nitrite to nitrate, inhibiting anammox activity in the presence of high DO concentration (Davery et al., 2012).

4.2.6 Analytical methods

Ammonia consumed and nitrite released was measured according to Scheiner (1976) and Griess-Romijn (1996) respectively. Brucine sulfate method was used to determine nitrate according to Jenkins and Medsker (1964). Hydrazine and hydroxylamine were estimated from the seed cultures according to Watt (1952) and Frear and Burrel (1955) respectively. TS, TDS, TSS, MLSS, MLVSS and SVI were measured according to standard protocols (APHA, 1995).

Calculation for N_2% formed by the enriched anammox biomass (carried out in triplicate): For calculating N_2 produced in the sample, contaminating N_2 from air was subtracted from the sample N_2. For this area of N_2 and O_2 in air calculated from the GC chromatogram were 2297852 and 569121 respectively for two. Ratio of O_2 to N_2 in air is 1:3.7. Percentage of O_2 in the sample =

[area of sample O_2 * 21]/569121.

Percentage of contaminating N_2 in the sample =

[Percentage of sample O_2*3.7].

Therefore, percentage of N_2 produced in the sample = [{area of sample N_2 * 78.08} / 2297852 (area of N_2 in air)] – percentage of contaminating N_2 in the sample.

4.2.7 Scanning Electron Microscopy (SEM) from the reactor

Morphology of the bacteria present in the lower anoxic zone of the reactor was studied using Joel Scanning Electron Microscope with Oxford EDS system model No. JSM-5610LV. Biomass from the lower zone was taken and fixed on a grease free glass slide using gluteraldehyde and dehydrated in a

series of increasing acetone concentration as mentioned in Chapter 3. Preparations were dried sputter-coated with silver and examined in SEM.

4.2.8 Qualitative analysis of the biomass generated in reactor

Effluent and sludge (1 ml) from the upper oxic zone and lower anoxic zone of the reactor respectively were used to extract genomic DNA. The effluent and sludge were centrifuged and washed with N-saline. These were mixed with 450 µl of extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM sodium EDTA pH 8.0, 100 mM sodium phosphate pH 8.0, 1.5 M NaCl, 1% CTAB) for half an hour at 37°C and were passed through French press at 1000 psi pressure. Extraction of genomic DNA was carried out according to Schmidt et al. 1991. Amplification of *amo*A (Rotthauwe et al., 1997), *nir*S (Throback et al., 2004), *nos*Z (Henry et al., 2006), genes and planctomycetes and anammox specific 16S rRNA (Tal et al., 2006) gene regions were carried out using respective primers to demonstrate the presence of AOB, denitrifiers and anammox bacteria. 16S rRNA gene was amplified using 27F and 1541R (universal primers) with following PCR program: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation 95°C for 30 s, annealing 58°C for 45 s and elongation at 72°C for 1.5 min and a final elongation at 72°C for 10 min. The amplified fragment was cloned in pTZ57R/T vector using INSTA cloning kit (Fermentas, Inc.). Amplified ribosomal DNA restriction analysis (ARDRA) was carried out using *Alu*I restriction enzyme and the distinct patterns obtained were sent for commercial sequencing through single pass analysis from Xcelris Labs (Ahmedabad, India). The sequences determined in the study submitted in GenBank under accession numbers JX143764 – JX143801.

4.2.9 Quantitation of biomass generated in the reactor by Real-time PCR analysis

Absolute quantification of the genes was carried out by Real-time PCR analysis in Step One Real-Time PCR system (Applied Biosystems, USA) by the standard curve method. Bacterial 16S rRNA [amplified according to Quan et al. (2008)], ammonia monooxygenase (*amo*A), nitrite reductase

(*nir*S) and nitrous oxide reductase (*nos*Z) genes were quantified using SYBR Green master mix (Applied Biosystems, USA) in 10 µl reaction system. Standard curves for all the genes were constructed with plasmids containing individual genes (16S rRNA, *amo*A, *nir*S and *nos*Z) prepared by cloning the genes in pTZ57R/T vector using INSTA cloning kit (Fermentas Inc.). The specificity of the PCR amplification was determined by the melt curve analysis and R^2 values obtained were greater than 0.98 for all the curves. PCR protocol used for 16S rRNA gene was: an initial denaturation temperature of 95°C for 10 min, 40 cycles of PCR with denaturation at 95°C for 15 s, annealing at 43°C for 45 s, and extension at 72°C for 30 s. Same protocol was followed for *amo*A and *nir*S genes, with annealing temperature 54°C and extension time 1min for *amo*A and 45 s for *nir*S whereas for *nos*Z touchdown PCR protocol used was according to Henry et al. (2006).

4.2.10 Phylogenetic analysis

Phylogenetic analysis of the microorganisms present in the reactor was carried out using 16S rRNA gene sequences by MEGA version 4.0 software (Tamura et al., 2007). 16S rRNA gene sequence obtained by universal primers 27F and 1541R and anammox specific primes Pla46F and Amx820 were used to find the types of the microorganisms present in the reactor. Phylogenetic tree was constructed using the Neighbor-Joining method. All positions containing gaps and missing data were eliminated from the data set by complete deletion option. There were a total of 520 positions in the final data set.

4.3 Results and Discussion

4.3.1 Biomass development of anammox bacteria

Amongst the samples enriched for anammox biomass (Table 4.1), PF-anammox and N4-anammox biomass showed higher anammox activity under anaerobic conditions.

Ratio of ammonia:nitrite removed was 1:1.89 for PF-anammox and 1:1.08 for N4-anammox, indicating that higher nitrite was utilized by the enriched

biomass. The observation was as per the expectation as nitrite in anammox reaction was used not just as an electron acceptor for oxidizing ammonia but also was used as electron donor for biomass formation in CO_2 fixation as per that reported by Kuenen and Jetten (2001). Stoichiometry obtained in the present study was almost similar to the reported stoichiometry for anammox bacteria (1:1.33) by Strous et al., (1999). Gas formed in the process was identified to be nitrogen through gas chromatography (44% by PF-anammox and 39% by N4-anammox) (Fig 4.2A and B).

Table 4.1 Anammox activity by enriched samples under anaerobic conditions.

Sample	Nitrite removed (mM)	Ammonia removed (mM)
N1 (CEPT, Nandesari)	0.64	1.08
A1 (Alembic sludge)	0.50	1.2
N4 (Municipal waste water treatment plant, Nandesari)	**1.18**	**1.09**
D1(effluent, DNR A reactor, GNFC, Bharuch)	0.8	2.85
D2(effluent, DNR B reactor, GNFC, Bharuch)	0.10	2.64
DnrA (Solid sludge, DNR A reactor, GNFC, Bharuch)	0.46	0.26
GS (Garden soil, Vadodara)	0.03	6.35
CP (Model farm soil, growing cereals and pulses in rotation, Vadodara)	0.31	6.38
PF (Paddy field soil, Mandya)	**2.27**	**1.2**

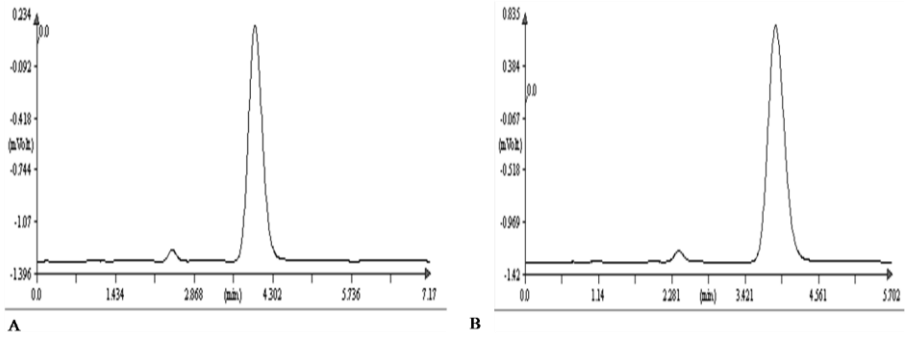


Fig 4.2 Gas chromatography confirming production of N_2 by A) N4-anammox and B) PF-anammox biomass.

Hydrazine as an intermediate has been reported to strongly stimulate anammox activity (Strous et al., 1999). Presence of hydrazine and hydroxylamine were not detected during the enrichment process. Being intermediates in the anammox process, hydrazine and hydroxylamine would be used as substrates in the further reaction and hence they presumably do not accumulate to detectable levels. However, addition of hydrazine in the system led to 1.5 and 1.7 fold increase in ammonia removal, 1.09 and 1.23 fold increase in nitrite removal and 0.98 and 1.1 fold increase in nitrogen formation for N4-anammox and PF-anammox respectively. The enriched biomass also showed remarkable increase in red biomass in the presence of hydrazine in case of PF-anammox (Fig 4.3).

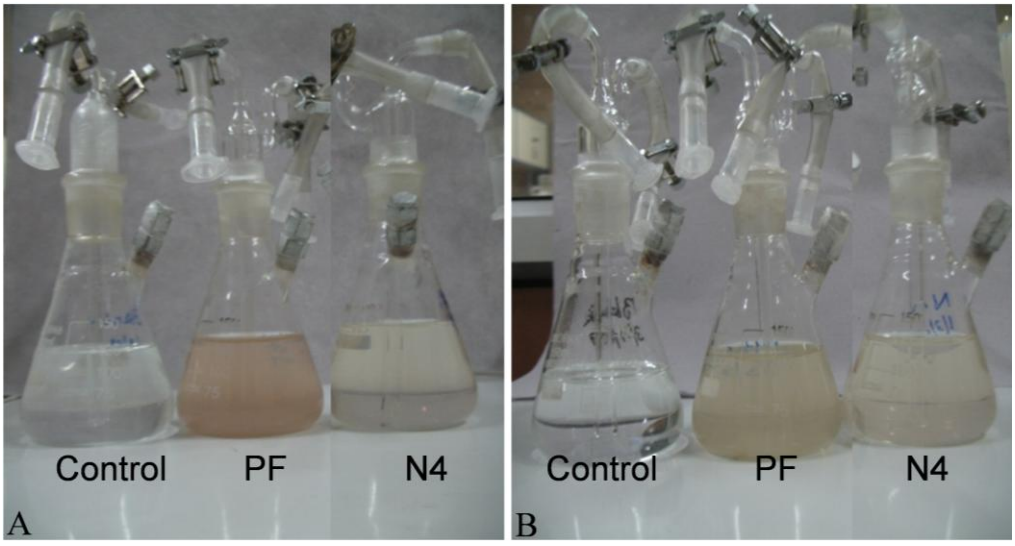


Fig 4.3 Effect of hydrazine on the growth of enriched anammox biomass. A) with hydrazine and B) without hydrazine

Amplification of planctomycetes specific and anammox specific regions of the 16S rRNA gene confirmed presence of anammox bacteria in the enriched biomass (Fig 4.4).

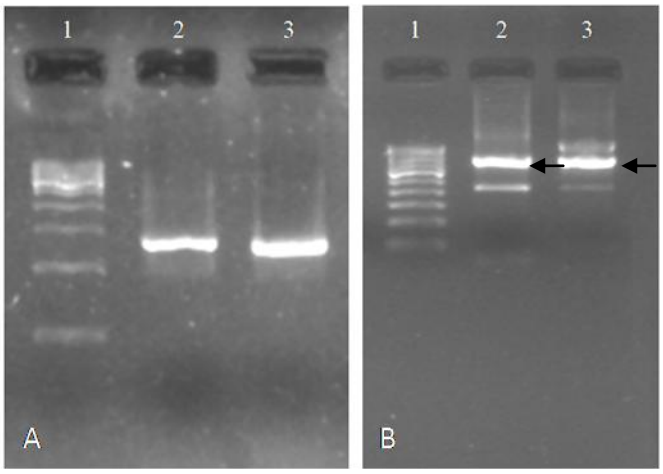


Fig 4.4 Confirmation of anammox bacteria in the enriched biomass A) Planctomycetes specific amplification (1350bp); Lane 1. 500bp ladder, Lane 2. PF-anammox, Lane 3. N4-anammox B) Anammox specific amplification (750bp) Lane 1. 100bp ladder, Lane 2. PF-anammox, Lane 3. N4-anammox.

4.3.2 Growth and maintenance of anammox biomass in rubber tubes

Both the enriched anammox biomass (PF-anammox and N4-anammox) were maintained in rubber tubes with very small diameter (3 mm) but 4 ft in length where in increase in growth and gas formed in the process could be visually monitored. Within 14 days of incubation in dark at 37°C temperature, bright red colored biomass developed (Fig 4.5C and D) indicating that optimum conditions were being provided for the growth of the anammox bacteria. Ammonia and nitrite removal by the enriched biomass together with generation of N_2 gas (Fig 4.5E) showed consistency in anammox activity by the enriched biomass. Gas produced could be visually observed as bubble formed in the tube and was confirmed to be nitrogen through gas chromatography (Fig 4.5A and B). Consistency in the detection of anammox activity substantiated earlier observation of Planctomycetes/anammox specific amplification and validated presence of anammox group of bacteria in the enriched anammox biomass.

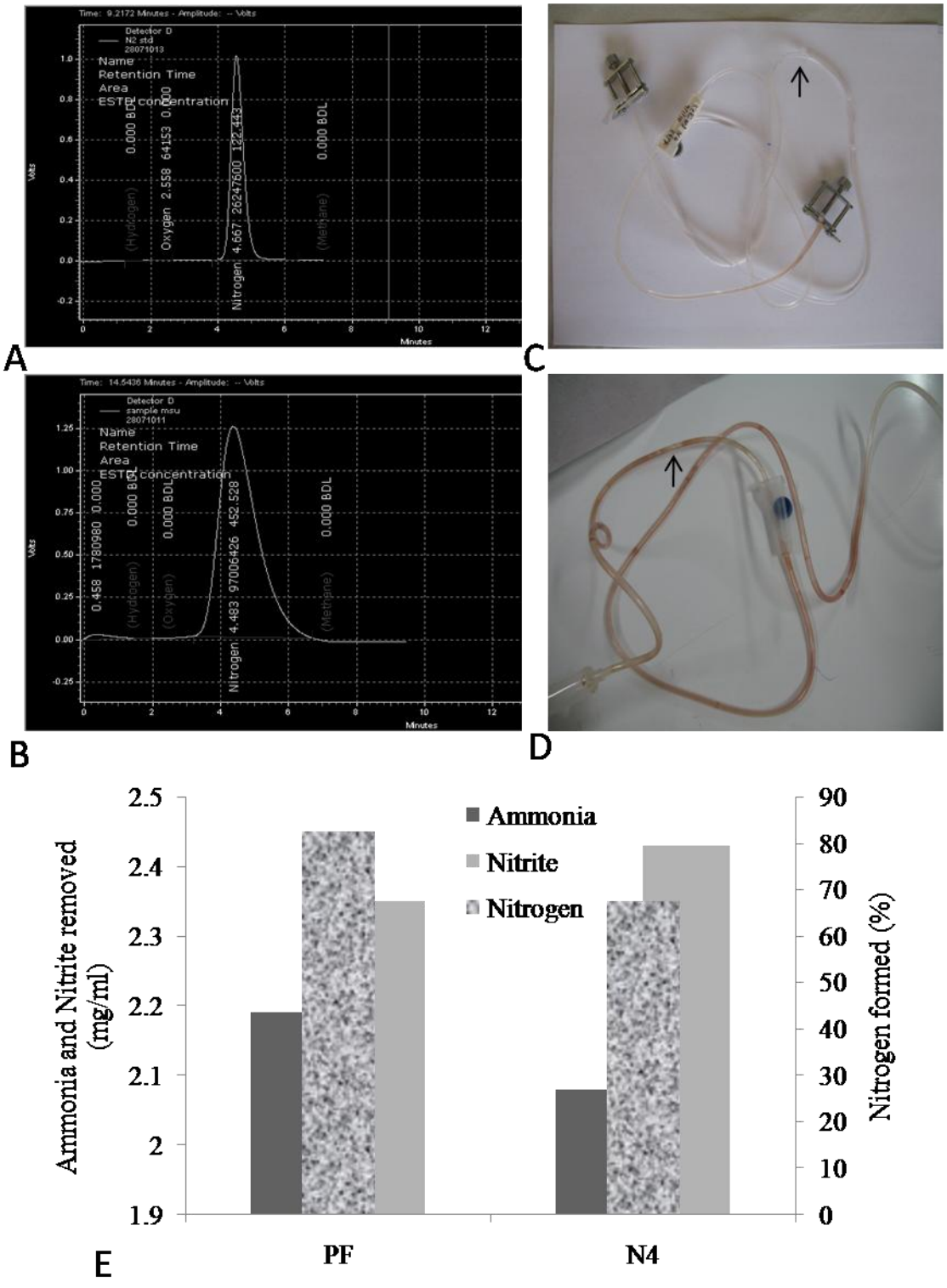


Fig 4.5 Growth and maintenance of anammox bacteria in rubber tubes A) Nitrogen formed by N4-anammox B) Nitrogen formed by PF-anammox C) growth observed in N4-anammox D) growth observed in PF-anammox E) anammox activity observed in PF and N4-anammox enriched biomass.

4.3.3 Development of AOB-Anammox seed consortium for SNAD type bench scale (1 L) laboratory bioreactor

Anoxic removal of ammonia requires mainly two groups of the organisms, AOB, carrying out partial nitrification reaction and anammox bacteria carrying out anammox reaction leading ultimately to the removal of ammonia in the form of molecular nitrogen. Both groups of organisms are slow growers, therefore, for effective anoxic ammonia removal from ammonia laden effluent at a bench scale reactor would require sufficient biomass to be generated of both groups of the organisms to be used as seed in the reactor. Small reactors (500ml) were designed so as to make it run under both anoxic and anaerobic conditions for optimizing growth and nitrifying activity of AOB and growth and anammox activity of anammox bacteria. PF-AOB + PF-anammox (1:5) and N4-AOB + N4-anammox (1:5) were seeded in these reactors containing synthetic effluent. Red colored growth appeared in the reactors after 90 days of incubation as shown in Fig 4.6.

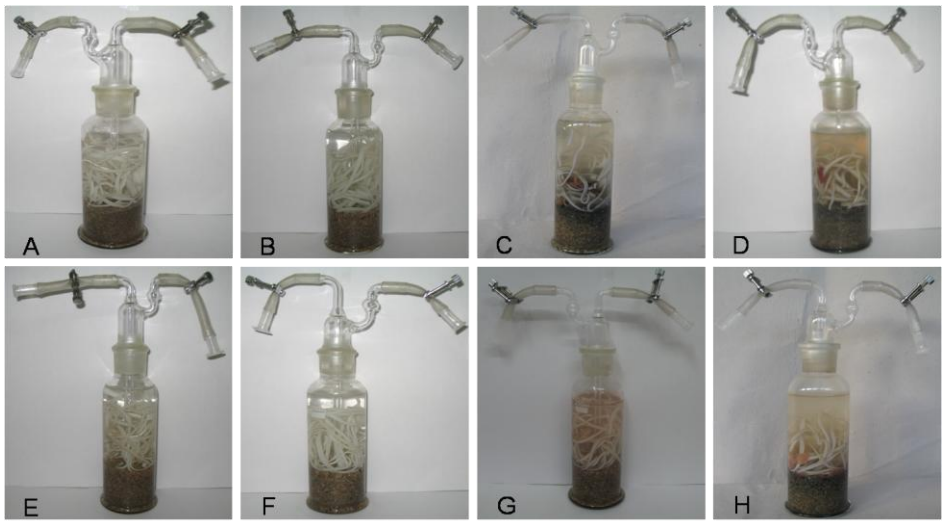


Fig 4.6 Bioreactor design for monitoring anammox activity of enriched biomass using synthetic effluent A) AOB control B) Anammox control C) PF-anammox D) N4-anammox E) PF-AOB F) N4-AOB G) PF-AOB + PF-anammox H) N4-AOB + N4-anammox.

Ammonia removing ability of the consortium was checked under anoxic conditions by keeping appropriate control as described in methods sec

4.2.2. As expected, decrease in ammonia and nitrite concentration was not observed in AOB and anammox control reactors (Table 4.2). PF-AOB and N4-AOB showed 79.8 (60.7%) and 81.8 (66.7%) mg/L utilization of ammonia with marginal increase in nitrite concentration, without accumulation of nitrate, with 69.78% and 66.79% nitrogen gas formation implying that simultaneous nitrification and denitrification activity was being carried out by the enriched AOB biomass. AOB belonging to *Nitrosomonas* genus has been reported to show this activity under anoxic conditions (Bock et al., 1995; Schmidt and Bock, 1997). PF-anammox and N4-anammox biomass showed decrease in both ammonia and nitrite under anaerobic conditions, with increased nitrogen gas formation (1.06 to 1.1 times) compared to PF-AOB and N4-AOB biomass (Table 4.2). The PF-AOB + PF-anammox and N4-AOB + N4-anammox system showed 97.6 (88.73%) and 108.4 (92.5%) decrease in ammonia without accumulating nitrite and nitrate (Table 4.2). Removal of ammonia by the combined AOB-anammox biomass was marginally less than anammox bacteria (Table 4.2). Nitrite removed by the system cannot be measured as the nitrite produced by the AOB would be simultaneously utilized by anammox bacteria present in the system (Table 4.2). Gas produced in all the system was confirmed to nitrogen through gas chromatography (Fig 4.7).

Gas formed by PF-AOB + PF-anammox consortium was 1.09 times higher than only PF-AOB but 0.98 times less than only PF-anammox (Table 4.2). Similar results were also obtained for N4-AOB + N4-anammox consortium (Table 4.2). Results emphatically confirmed ammonia removing ability of PF-AOB + PF-anammox and also N4-AOB + N4-anammox consortia under anoxic conditions. N4-AOB + N4-anammox biomass was mixed in 1:1 proportion with PF-AOB + PF-anammox and this PF-N4 AOB-anammox bacterial biomass was used as a seed consortium for the 1 L SNAD type reactor study.

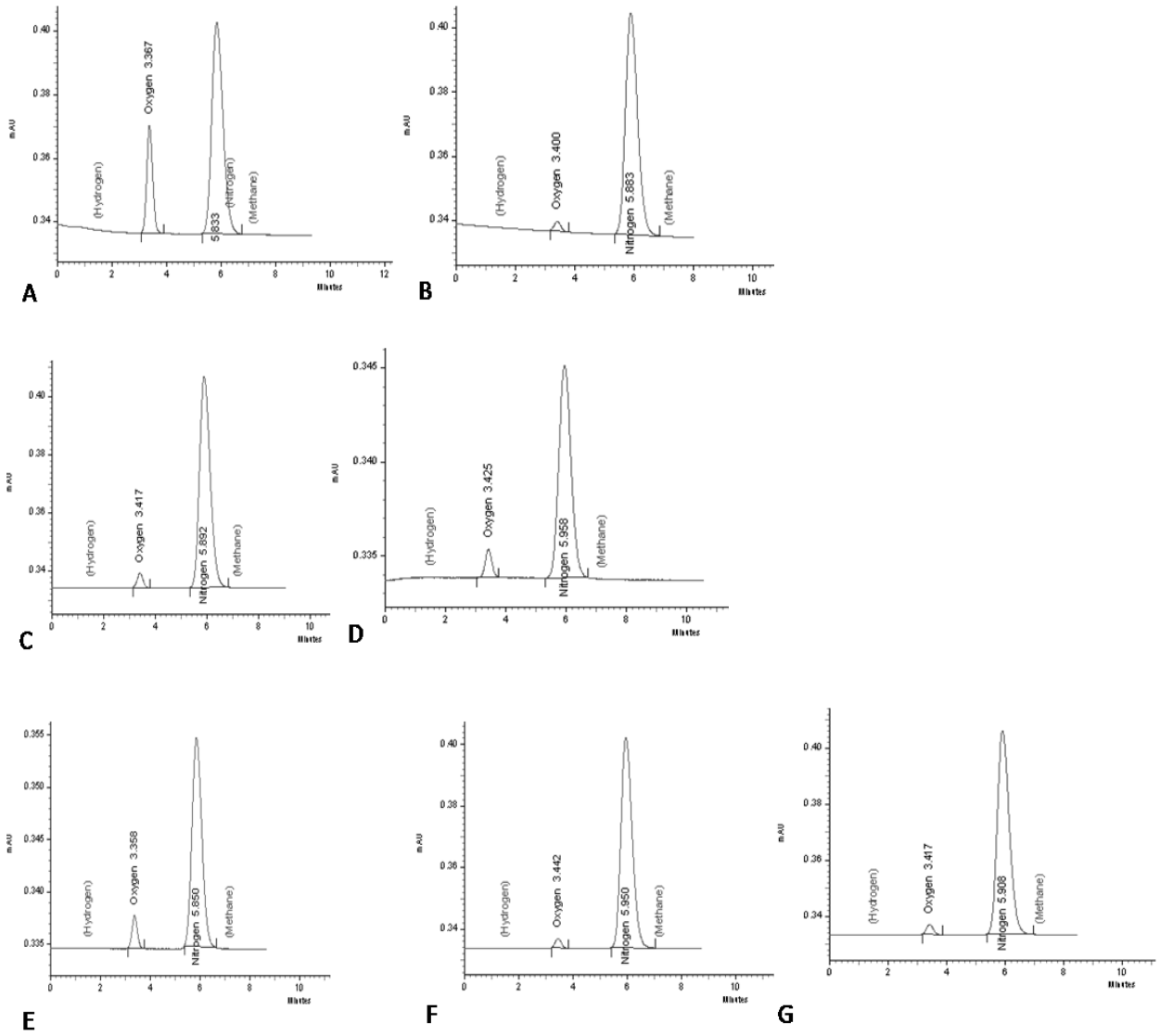


Fig 4.7 Gas chromatography showing N_2 in all the system A) AOB control B) N4-anammox C) PF-anammox D) N4-AOB E) PF-AOB F) N4-AOB+N4-anammox G) PF-AOB+PF-anammox

Table 4.2 Removal of ammonia from synthetic effluent under anoxic conditions.

	Ammonia Removed (mg/L)	Nitrite Removed (mg/L)	Gas formed (%)
AOB control	-	-	-
Anammox control	-	-	-
PF AOB	79.8±8.4	-1.55±1.39	69.78±3.5
N4 AOB	81.8±5.7	-1.08±2.7	66.78±0.9
PF anammox	107.8±7.7	133.5±2.67	74.1±1.2
N4 anammox	109.5±4.5	130.1±1.8	74.4±3.1
PF AOB + anammox	97.6±4.4	*	72.36±1.8
N4 AOB + anammox	108.4±3.4	*	72.92±3.7

*cannot be measured.

4.3.4 Ammonia removal performance of the SNAD type bioreactor from the effluent of a fertilizer company

An up flow, cylindrical SNAD type reactor with a working volume of 1 L was used for the removal of ammonia from the effluent of a fertilizer industry, seeded with 100 ml PF-N4 AOB-anammox consortium (1.0 g/L VSS) developed as described above. Proximate composition of the effluent to be treated is depicted in Table 4.3 for the 0^{th} day.

It had high NH_4^+-N concentration with COD / NH_4^+-N ratio 0.066 whereas the same in the SNAD processes reported earlier ranged from 0.2 to 0.87 (Chen et al., 2009; Wang et al., 2010; Lan et al., 2011; Davery et al., 2012). The reactor was run in the batch mode for the first 15 days during which DO in the lower zone of the reactor reduced to 0.37 ppm and thereafter fluctuated between 0.1 to 0.4 ppm (conditions favorable for the growth of anammox bacteria) whereas in the upper region of the reactor, DO fluctuated between 2.9 to 3.9 ppm (Fig 4.8C). Nitrite present in the reactor, at any time was < 100 mg/L, in the range that does not inhibit the anammox activity according to Strous et al., (1999). During the batch mode, VSS reached to 41 mg/L with 52.84% removal in the ammonia concentration

(Fig. 4.8A and D). A significant reduction in the nitrite concentration was also observed during the batch mode (Fig 4.8B). pH of the reactor reduced from 9.2 to 7.5 and thereafter fluctuated between pH 7.5 to 8.1 (Fig 4.8D). The reactor was made continuous after 15 days with flow rate of 0.3 ml/min and HRT of 2.31 days. Inlet ammonia concentration varied from 700 to 800 ppm (Fig 4.8A). The concentration of ammonia in the wastewater reduced to permissible limits (32.38 ppm) within 64 days and was stably maintained at that level for the next 60 days with a development of 11.5 g/L VSS. The concentration of inlet ammonia (725-760 ppm) and ammonia removed (700-750 ppm) became identical (Fig 4.8A), leading to development of steady state in the reactor. COD concentrations in the reactor reduced to 24 mg/L at the end of the run. Ammonia conversion efficiency at the start of the reactor was higher than the theoretical value (Fig 4.8E). On the 14^{th} day it reached 11.8%, indicating optimum anammox activity in the reactor (Fig 4.8E). The ammonia conversion efficiency fluctuated between 0.4 to 9.0% during the continuous mode (Fig 4.8E). Between 52 - 74^{th} day, the process was run with ammonia conversion efficiency reaching near 0.4% to 0.02% suggestive of higher denitrifying activity than anammox activity (Fig 4.8E).

Table 4.3 Measurement of the effluent parameters at the start and end of process.

Days	COD	NH_4^+-N (ppm)	NO_2^--N (ppm)	NO_3^--N (ppm)	DO (ppm)	Total Solid (mg/L)	Total Suspended Solids (mg/L)	Total Dissolved solids (mg/L)	MLSS (mg/L)	MLVSS (mg/L)	SVI (ml/g)	pH	MLVSS/ MLSS
0	46.66	700-800	20-60	Not detectable	5.8	4300	300	4000	1.65	1.46	0	9.2	0.88
125	24	26	5.05	Not detectable	0.39	4750	700	4050	212	114	14	7.5	0.536

Beyond this period, the efficiency steadily increased reaching 4.9% by the end of the reactor, indicating anammox activity along with denitrifying activity in the reactor (Fig 4.8E). The overall efficiency of the process was similar to reactors designed earlier for the removal of ammonia using the SNAD process (Chen et al., 2009; Lan et al., 2011; Daverey et al., 2012). A highly efficient and stable system was thus developed for the treatment of ammonia laden effluent from a fertilizer industry without addition of external carbon or nitrite source and was operated at ambient temperature (30°C).

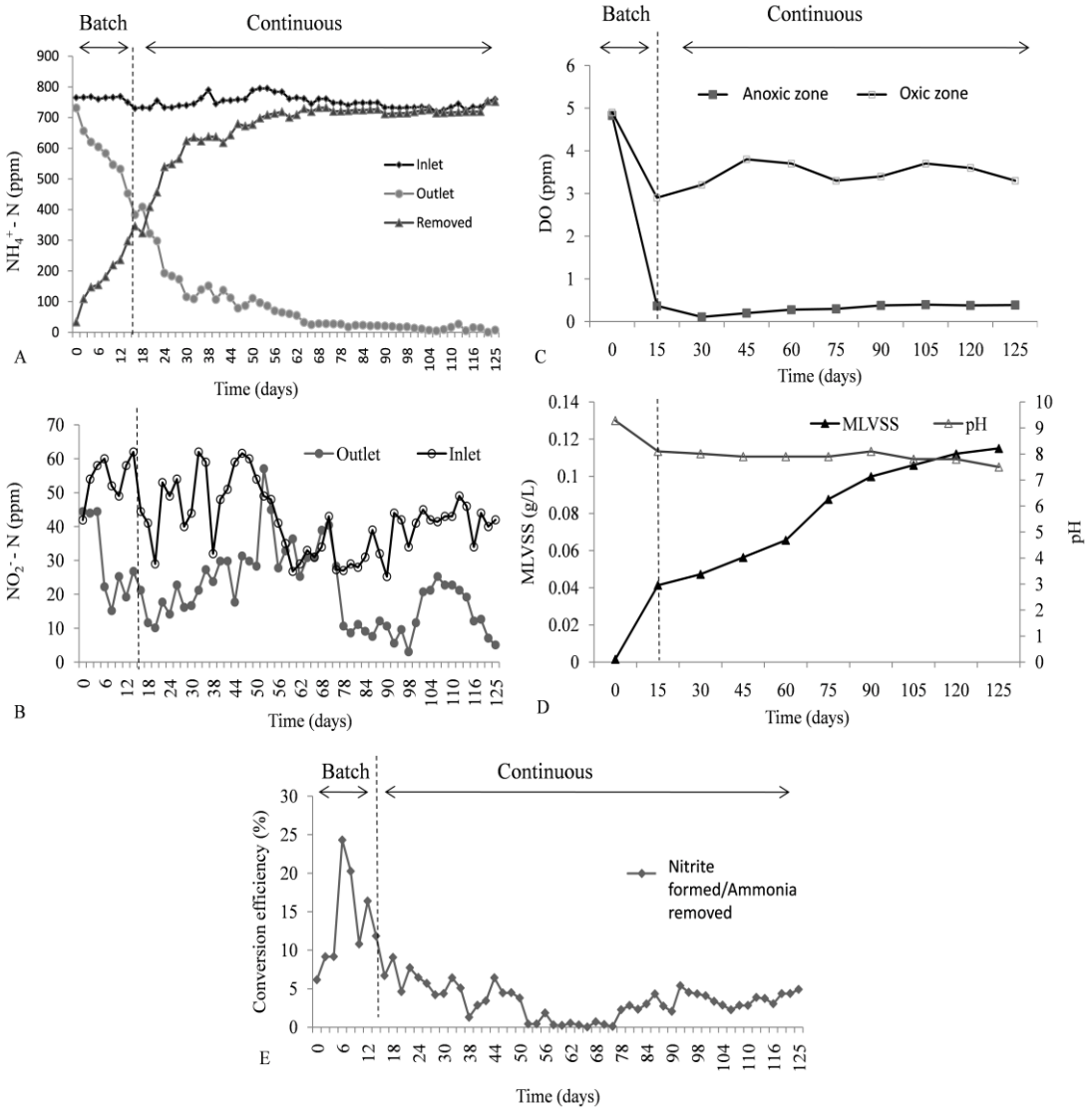


Fig 4.8 Nitrogen removal performance and other parameters in the reactor during the process A) Ammonia B) Nitrite C) Dissolved oxygen D) pH and MLVSS E) Ammonia conversion efficiency.

4.3.5 Molecular analysis of the biomass developed in the reactor

Visually red colored biomass developed at the base of the reactor and red colored film developed on the upper wall of the reactor by the end of the run. SEM of the biomass from the anoxic zone showed dominance of coccoidal shaped cells forming aggregates with pear shaped cells and flagellated microorganisms (Fig 4.9A, B and C). Short rods were also observed through SEM. Budding pear and coccoidal cells in the anoxic zone of the reactor could be Planctomycetes (Fig 4.9B, C, D marked by arrow).

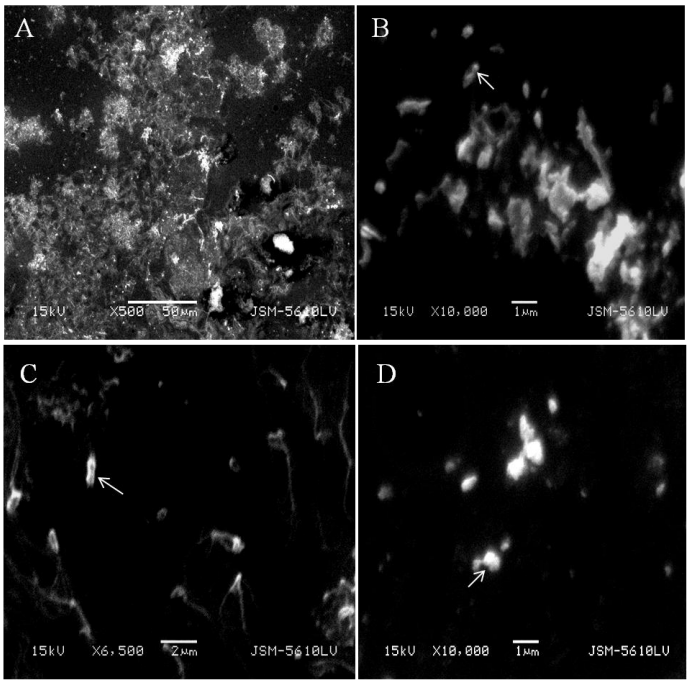


Fig 4.9 SEM images of biomass taken from lower anoxic zone of reactor showing presence of A) cell aggregates formed, B, C and D are diluted biomass. Budding cells are marked by arrow in B, C and D. D) different shaped cells: tear shaped cells, flagellated cells, coccoidal cells, short rods.

The observation was supported by the amplification of Planctomycetes specific (1350bp) and anammox specific (750bp) regions of 16S rRNA gene from the biomass obtained from lower anoxic zone of the reactor (Fig 4.10 A and B). Cloning and sequencing of the anammox specific gene revealed clones having similarity with Planctomycetes (uncultured-93% similarity).

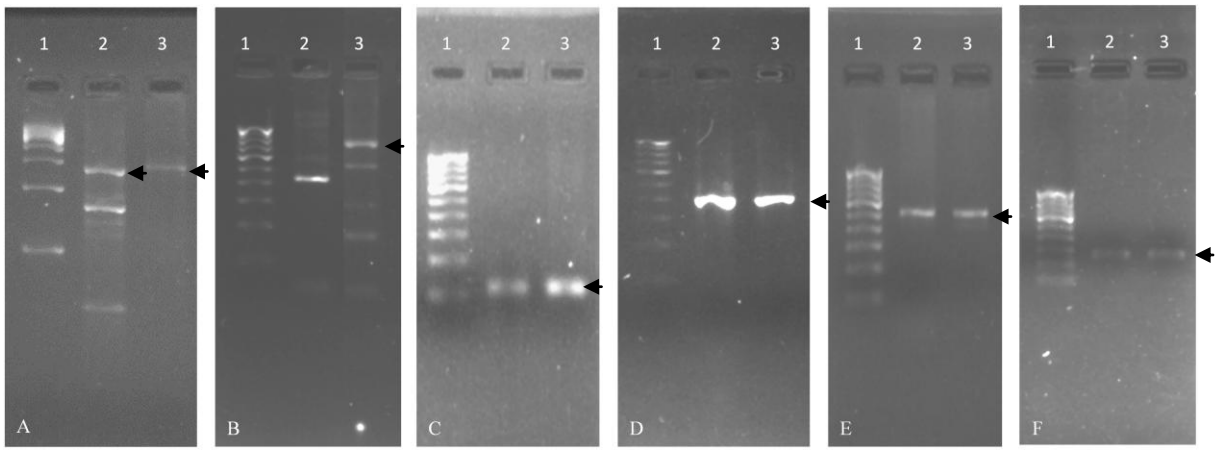


Fig 4.10 Amplification of anammox, nitrifiers and denitrifiers specific gene fragment from upper and lower zone of the reactor. A) Planctomycetes specific 16S rRNA gene (1350bp) B) anammox specific 16S rRNA gene (750bp) C) 16S rRNA gene (102bp) for quantification of total bacteria D) *nir*S gene (425 bp) E) *amo*A gene (491bp) F) *nos*Z gene (267bp) for quantification of respective group of organisms. Lane 1 A 500bp ladder, B-F 100bp ladder. Lane 2 A-F amplification of the respective genes from the upper zone of the reactor. Lane 3 A-F amplification of the respective gene from lower zone of the reactor.

Twenty eight distinct sequences were obtained by cloning 16S rRNA gene from both oxic and anoxic zones of the reactor (Fig 4.11). Phylogenetic tree was constructed by Neighbor-Joining method using MEGA version 4.0 software (Fig 4.12) representing relationship between the nitrifiers, denitrifiers and Planctomycetes present in the reactor. Mainly these could be grouped as aerobic and anaerobic ammonia oxidizers, aerobic and anaerobic denitrifiers and bacteria capable of simultaneous nitrification and denitrification (Fig 4.12). Specific enrichment for denitrifiers was not addressed in the study however; they must have got enriched due to anoxic conditions and presence of SMP in the reactor. Coexistence of denitrifiers along with anammox and AOB sharing nutrient metabolites is well documented (Kindaichi et al., 2004; Xiao et al., 2008), hence detection of denitrifiers with AOB and anammox bacteria was not unusual.

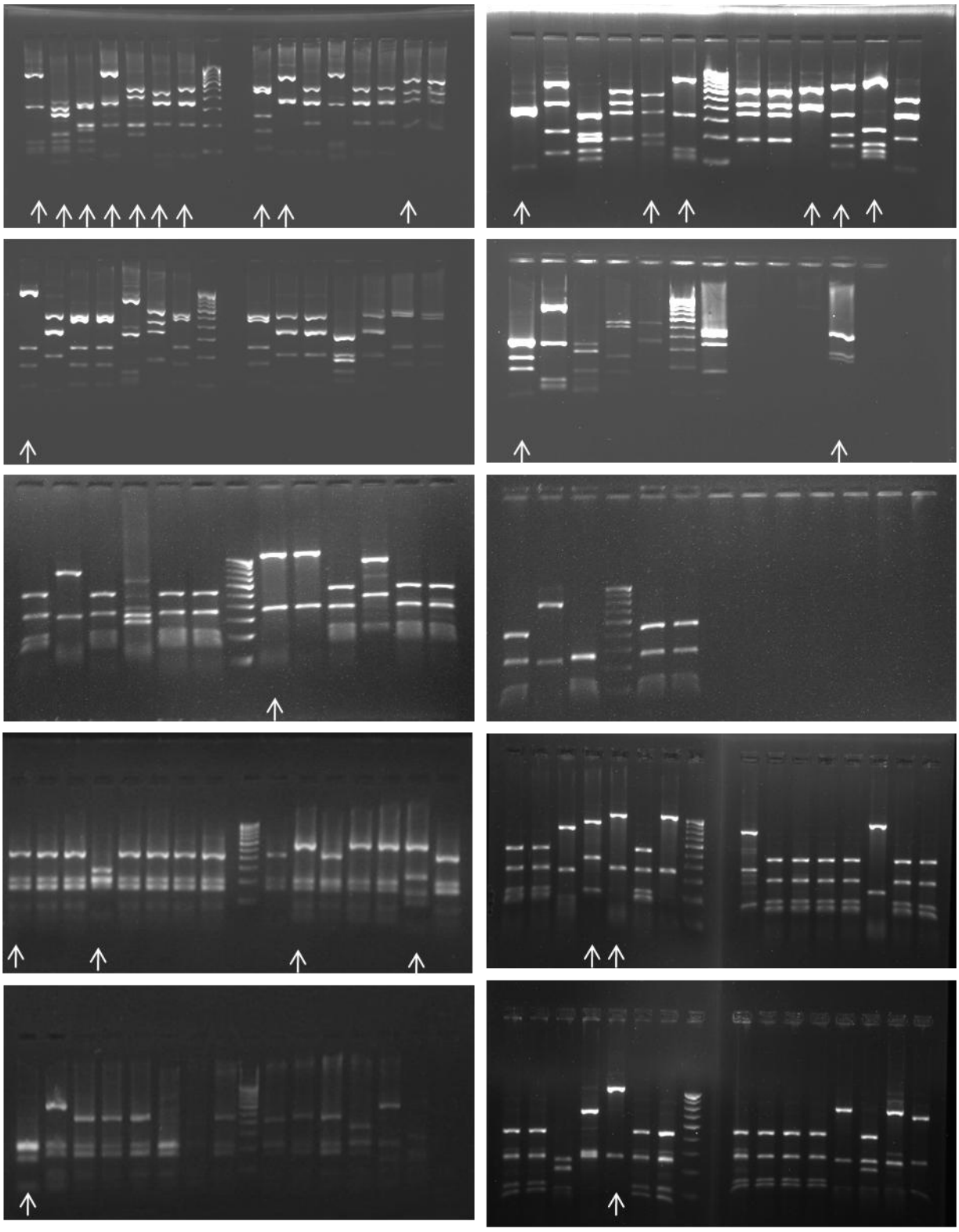


Fig 4.11 Representative ARDRA gel showing different patterns of 16S rRNA gene differentiating the different clones. The different patterns are marked by arrow.

Eight distinct species of AOB showing similarity to *Nitrosomonas* genus were identified. Amongst the microorganisms identified, AOB exhibited maximum diversity as they were the sole providers of nitrite to denitrifiers and anammox bacteria. This observation was in congruence with the earlier report by Xiao et al., (2008), who showed higher diversity of AOB amongst coexisting nitrosifiers, denitrifiers and anammox bacteria in sequencing batch biofilms reactor treating ammonia rich landfill leachate. Denitrifiers identified belonged to alpha (*Rhodopseudomonas* sp.), beta (*Thauera* sp., *Pusillimonas* sp., *Acidovorax* sp., *Comammonas* sp.) and gamma (*Thermomonas fusca*, *Xanthomonas* sp.), proteobacteria. Other heterotrophic key players possibly involved in the treatment process in the present study were *Rhodospesudomonas* sp., *Diaphorobacter* sp., *Acidovorax* sp. and *Comamonas* sp. reported to carry out simultaneous heterotrophic nitrification-denitrification (Satoh et al., 2006; Khardenavis et al., 2007; Heylen et al., 2008; Xiao et al., 2008). High temperature (30°C), high flow rate and low dissolved oxygen concentration prevalent in the reactor were not favorable for the growth of nitrite oxidizing bacteria (NOB) as reported by Jianlong and Ning (2004) and therefore they could have got washed out and hence not detected.

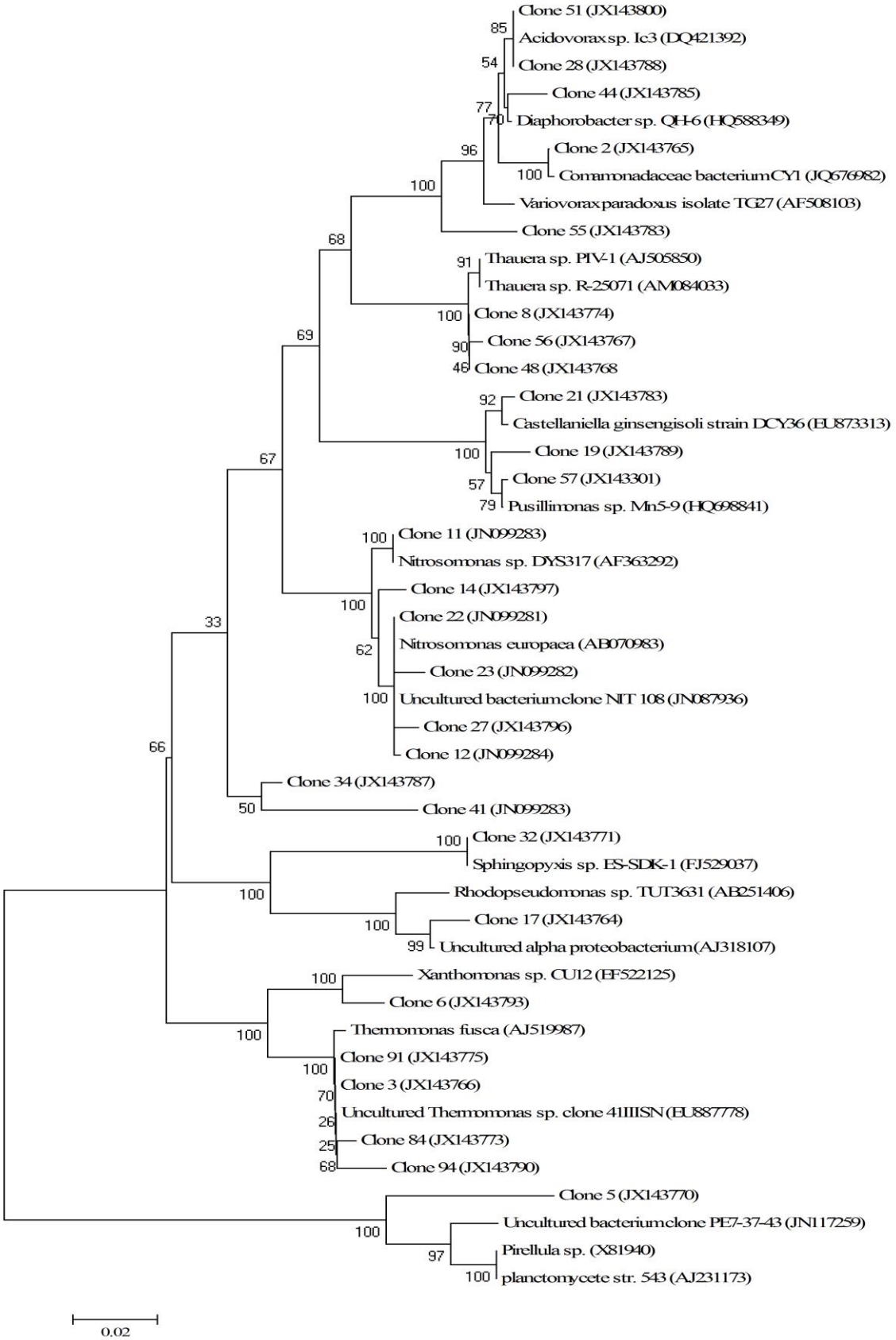


Fig 4.12 Phylogenetic Neighbor-Joining tree showing relationship between the microorganisms present in the reactor based on the 16S rRNA gene

sequences obtained using universal primers and anammox specific primers. Bar indicates 2% sequence divergence. Values shown next to the branches indicate bootstrap values. Accession numbers of the sequences are given in parenthesis.

Quantification of biomass carried out from upper (oxic) and lower (anoxic) regions of the bioreactor using RT-PCR with group specific genes revealed predominance of AOB (carried out by *amoA* gene amplification) (Fig 4.10E) in the upper oxic region (61.2%) whereas just 6% of the total population belonged to AOB in the lower anoxic region (Table 3). Denitrifiers (quantified using *nir*S and *nos*Z genes) constituted 10% of the total population in the upper oxic zone and 22% in the lower anoxic layer of the reactor (Table 3). The results suggested dominance of AOB in the upper region of the reactor where as that in the lower zone of the reactor, showed dominance of neither AOB (6%) nor denitrifiers (22%). Anammox may constitute major population in this part of the reactor as evidenced by the detection of uncultured Planctomycetes (93% similarity) in the biomass amplified using Planctomycetes and anammox specific primers (Fig 4.10A and B). The bacteria identified in the system showed less similarity with the other reported members of this group and hence may be a novel organism belonging to Planctomycetes. However, this group of bacteria could not be quantified due to the presence of nonspecific amplicons obtained along with the required 750bp amplicon. Another proof for the presence of Planctomycetes in the lower zone of the reactor was given by SEM showing presence of budding coccoidal cells (Fig 4.9D), a characteristic for this group of organisms.

The bacterial community developed in the reactor could remove ammonia with high efficiency during the process. Oxidation of ammonia to nitrite in the upper oxic portion of the reactor could mainly be contributed by AOB followed by aerobic heterotrophic nitrifiers as the former being predominant population in this part of the reactor. Nitrite in the lower anoxic zone of the reactor would be utilized either by anaerobic denitrifiers or anammox bacteria. Competition for nitrite would always be there between the

anammox bacteria and denitrifiers. Owing to the low COD content in the reactor, denitrifiers would also need to depend on SMP released by AOB for fulfilling their organic carbon requirement. Dependence of denitrifiers on SMP released by AOB has been reported by Kindaichi et al., (2004). Conditions in the lower region of the reactor therefore favored growth of anammox bacteria capable of autotrophic growth as compared to denitrifiers. The observation justified Real-Time PCR results showing only 22% population in the lower layer constituted of denitrifiers (Table 4.4). However, lack of quantitation data for anammox bacteria failed to conclude this result. Bacteria capable of anammox activity in the reactor were mainly *Planctomycetes* sp. and *Nitrosomonas* sp. (Fig 4.12) as these organisms have been shown to exhibit anammox activity (Schmidt and Bock 1997; Strous et al., 1999). The molecular phylogenetic analysis of the biomass from the reactor revealed coexistence of AOB, heterotrophic nitrifiers, denitrifiers and anammox bacteria, also reported earlier by Xiao et al., (2008) and Kumar and Lin (2010).

Table 4.4 Quantification of the organisms present in the upper oxic and lower anoxic zones of the reactor by Real-Time PCR.

Target gene	Size (bp)	Upper oxic zone of the SNAD Type Reactor		Lower anoxic zone of the SNAD Type Reactor	
		Gene copy No. per gram VSS	Ratio of gene copy No./16S rRNA	Gene copy No. per gram VSS	Ratio of gene copy No./16S rRNA
16S rRNA	102	$6.46 \times 10^9 \pm 6.15 \times 10^8$	1.0	$1.31 \times 10^{11} \pm 2.5 \times 10^9$	1.0
amoA	491	$3.96 \times 10^9 \pm 2.52 \times 10^9$	0.612	$7.92 \times 10^8 \pm 3.97 \times 10^7$	0.006
nirS	425	$6.39 \times 10^8 \pm 1.82 \times 10^8$	0.099	$3.19 \times 10^{10} \pm 2.15 \times 10^9$	0.244
nosZ	267	$3.35 \times 10^8 \pm 6.39 \times 10^7$	0.052	$2.1 \times 10^{10} \pm 6.65 \times 10^9$	0.161

An adequate balance between the different types of bacteria is required in activated sludge systems to have good settling ability of the sludge, such that low suspended solids level prevails in the effluent. Sludge volume index (SVI) is commonly used in wastewater treatment plants to analyze the separation of solids in the effluent and for characterizing the sludge settling ability. SVI provides just macroscopic evaluation of the activated sludge; therefore, microscopic characteristic of the sludge has been recently used where filamentous bacterial content in microbial aggregates is measured (Mesquita et al., 2009). Most of the denitrifiers detected in the present study (Fig 4.9) are reported to be flagellated (Hougardy et al., 2000; Shen et al., 2001; Mergaert et al., 2003; Heylen et al., 2008). These flagellated bacteria assist in forming microbial aggregates (Sjoblad et al., 1985). In congruence with this, in the present study too, filamentous bacteria observed formed aggregates (Fig. 4.9A) which improved settling ability of the sludge in the reactor and prevented entangled cells from getting washed off from the reactor. Food to microbe ratio calculated was found to be 0.62 day^{-1} indicating endogenous growth of microorganisms which was reported to have better settling ability and is more stable in nature (Rao and Datta, 1987).

To conclude, a SNAD type bioreactor was developed for efficient removal of ammonia from effluent of a fertilizer industry and was run continuously for 125 days wherein 98.9% ammonia removal was achieved. Coexistence of nitrosifiers, anammox bacteria and denitrifiers was confirmed in the reactor without supplementation of external organic carbon and without accumulation of nitrite or nitrate. Molecular phylogenetic analysis of the biomass generated revealed dominance of AOB in the upper oxic zone of the reactor while that of anammox followed by denitrifiers dominated in the lower anoxic zone of the reactor.

Chapter 5

Kinetics of ammonia removal in a 5.3 L open reactor: An aerobic solution to high strength ammonia containing wastewater of a fertilizer industry

Chapter 5

Kinetics of ammonia removal in a 5.3 L open reactor: An aerobic solution to high strength ammonia containing wastewater of a fertilizer industry

ABSTRACT

A novel microbial system was developed to remove ammonia from wastewater of a fertilizer company with extremely low COD. Autotrophic Ammonia and Nitrite Oxidizing Bacteria (AOB and NOB) were enriched from paddy field soil sample. Identification of microorganisms in the AOB and NOB enriched biomass was carried out by cloning and sequencing 16S rRNA gene. These were employed for the removal of ammonia in a 5.3 L open aerobic reactor. The reactor was run in the batch mode for the first 30 days during which the biomass was acclimatized to the effluent by using various dilutions of the effluent at an increasing effluent concentration. 90% reduction in the ammonia levels was achieved by the end of batch mode with 82.68 mg/L biomass accumulation. The reactor was made continuous with 6.75 days retention time. More than 99% removal of ammonia was achieved by the end of the run (75 days) with inlet ammonia concentrations fluctuating between 0.6 to 0.75 g/L and pH maintained near neutral. Aeration and agitation was intermittently switched on and off at 24 hr interval. During the aeration period high dissolved oxygen concentration (7.6 to 7.9 ppm) was maintained by external aerators and it dropped to 4.8 to 5.3 ppm during the off mode. Stability of the reactor was achieved after 58 days of the run. Nitrite concentrations steadily reduced to 7 ppm by the end of the run and nitrate levels reached below detectable limit after 64 days of the run. The present method is highly stable as linear correlation was observed in the amount of ammonia oxidized to the amount of ammonia loaded. The investigation thus reports kinetics of ammonia removal achieved by the developed system under aerobic condition without accumulation of nitrite

and nitrate from wastewater of a fertilizer industry containing high strength ammonia.

5.1 Introduction

Nitrification and denitrification are the two main steps in the global nitrogen cycle and it is known since long that they are carried out under aerobic and anaerobic conditions without and with organic carbon respectively. Recently, unusual behavior of nitrifiers and denitrifiers has been reported. Nitrous oxide is reported to be produced by nitrifiers through nitrifier denitrification (Wrage et al., 2001; Schmidt et al., 2004; Hayatsu et al., 2008). Moreover, presence of denitrifying enzymes, nitrite reductase and nitric oxide reductase has also been reported in the genome of *Nitrosomonas europaea* ATCC 19718 (Chain et al., 2003). Denitrifying enzymes are sensitive to oxygen, was a long established fact but recently *Paracoccus denitrificans, Thiosphaera pantotropha, Mesorhizobium sp., Burkholderia cepacia* and others are know*n* to denitrify under aerobic conditions (Okada et al., 2005; Matsuzaka et al., 2003; Su et al., 2004). This indicates that conversion of ammonia to molecular nitrogen can be carried out either by a single kind of bacteria e.g. AOB, or nitrification and denitrification can occur concurrently in a single reactor under identical operating conditions and this new process was called simultaneous nitrification denitrification (Diagger and Littleton, 2000).

Full scale reactors with staged, closed-loop bioreactor process known as 'Orbal' designed to remove ammonia from industrial effluents do not entirely provide uniform environments in the reactor (Diagger and Littleton, 2000). There are pockets in the reactor that are anoxic in nature and some others that are completely aerobic (Diagger and Littleton, 2000). It is therefore important to study the mechanism and reactions going on in the reactor with varying oxygen conditions. The objective of the present study was to investigate the potential of a lab scale reactor for autotrophic removal of ammonia with a nitrifying biomass as the active component with intermittent aeration and agitation. The developed single reactor system

removed ammonia from wastewater to the permissible limit without nitrite and nitrate accumulation and without addition of organic carbon externally.

5.2 Materials and methods

5.2.1 Enrichment of autotrophic ammonia and nitrite oxidizing bacteria (AOB and NOB)

Enrichment of AOB was carried out from soil and sludge samples (Chapter 2a) and paddy field soil sample, showing maximum ammonia oxidizing activity was selected (Chapter 2a). Enrichment of NOB was carried out from different soil and sludge sample (Table 5.1) by inoculating 5% sample in inorganic medium containing sodium nitrite (30 mM) and sodium carbonate (4 mM) as the sole nitrogen and carbon source respectively as described by Starkenburg et al., (2007) with modifications. Since NOB are slow growing with doubling time in the range of few hours to days (Bock et al., 1990; Watson et al., 1986) their enrichment was carried out in dark for one month. The enriched biomass was inoculated in fresh media and their nitrite oxidizing activity was measured by monitoring nitrite removed and nitrate formed.

5.2.2 Identification of microorganisms present in the NOB enriched biomass

Microorganisms present in the NOB enriched biomass were identified by 16S rRNA gene amplification, cloning and sequencing. Enriched biomass from log phase (50 ml) were centrifuged at 19,200 X g for 15 min and DNA was extracted according to Schmidt et al., 1991. 16S rRNA gene was amplified using universal primer 27F and 1541R and cloned in pTZ57R/T vector using INSTA cloning kit (Fermentas). Amplified ribosomal DNA restriction analysis (ARDRA) of 16S rDNA clones was carried out using *Alu*I restriction enzyme to differentiate the clones. Representatives of the different patterns were sequenced commercially by Single Pass Analysis (Bangalore Genei, India).

5.2.3 Reactor design and operating conditions

A 5.3 L open aerobic reactor (16 x 26 x 39 cm) was operated continuously for the removal of ammonia from effluent of a fertilizer company. A settler tank was attached to the reactor at 45° angle (Fig 5.1). The reactor was kept on a magnetic stirrer for agitation at 240 rpm. Dissolved oxygen (DO) in the reactor, measured by YSI DO meter (YSI200 portable DO meter, USA), was maintained using an external aerator. Aeration and agitation were switched on and off intermittently at 24 h interval. AOB and NOB enriched biomass were mixed in 1:4 proportions to prepare seed consortium such that MLVSS in the reactor was 8.2 g/L. The reactor was run in the batch mode for first 30 days and was made continuous with hydrolytic retention time of 6.75 days.

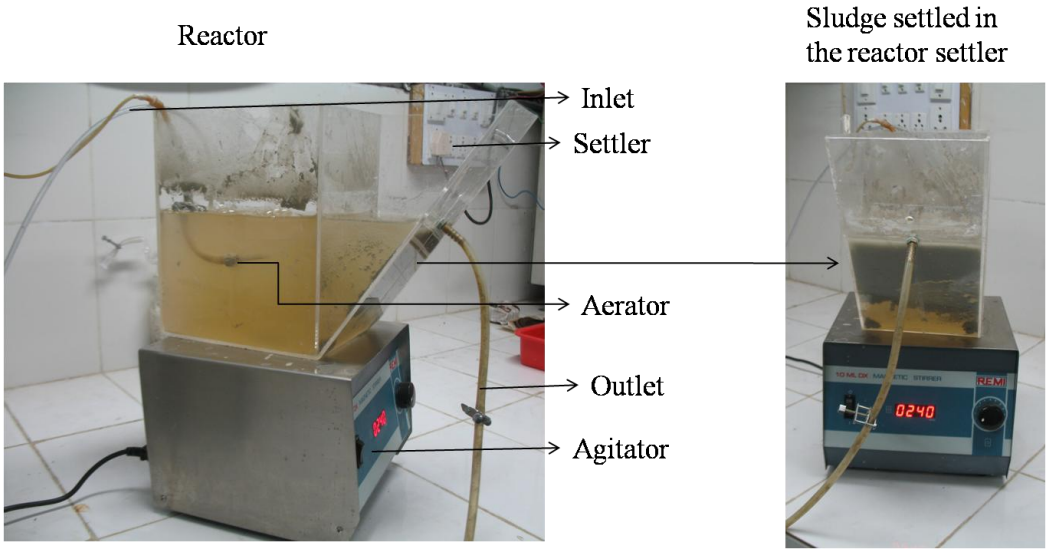


Fig 5.1 Open reactor used in the study and the sludge retained in the settler.

5.2.4 Ammonia conversion efficiency

In the SND process, ammonia is oxidized to nitrate by the nitrifying bacteria which gets further reduced to molecular nitrogen by the aerobic denitrifiers. The ammonia conversion efficiency of the reactor system was measured according to Davery et al., (2012)

$$Y_{(NO_2^- + NO_3^-)\,/\,NH_4^+} = \frac{\text{Eff. } \{(NO_2^- - N) + (NO_3^- - N)\}}{\text{Inf. } (NH_4^+ - N) - \text{Eff. } (NH_4^+ - N)} \times 100\%$$

5.2.5 Analytical measurement

Levels of ammonia, nitrite, nitrate, pH, DO, MLVSS, TS, TSS, TDS, MLSS, SVI, COD were measured as per the methods described in chapter 4. Ammonia, nitrite, nitrate, pH, DO, MLVSS were estimated every second day. All the other parameters were measured at the start of the reactor, end of the batch mode and end of the continuous reactor.

5.3 Results and Discussion

The anammox bacteria are extremely slow growers and essentially require nitrite to carry out the reaction. Main limitation of the process include maintenance of anaerobic conditions and elimination of the nitrite oxidizers from the system as they compete with anammox bacteria for nitrite and reduce their growth (Egli et al., 2003). Therefore, removal of ammonia from effluent of a fertilizer industry was treated by the SND process.

5.3.1 Enrichment of AOB and NOB

Enrichment of AOB in inorganic media for 30 days substantial increased their biomass as mentioned in chapter 2a. NOB were also enriched for one month in inorganic medium containing nitrite as the nitrogen and energy source and sodium bicarbonate as the carbon source. The enriched biomass showed 50-70% utilization of nitrite in all consortia except N3, N4 and GSFC which did not show increase in nitrate concentration, indicating absence of NOB in these samples. PF-NOB showed maximum growth and nitrite oxidizing activity (Table 5.1).

Table 5.1 Growth and nitrite oxidizing activity of NOB measured after 1 week of incubation enriched from varied sources.

Sr. No.	Sample	Description	Nitrite utilized (mg/ml)	Nitrate formed (mg/ml)	Growth (OD 600 nm)
1	N1	Common effluent treatment plant, Nandesari	1.092±0.05	0.53±0.08	0.024±0.009
2	N2	Sludge from Nandesari (Deepak nitrite)	0.22±0.03	0.31±0.06	0.056±0.003
3	N4	(Municipal waste water treatment plant, Nandesari)	0.27±0.09	0.2±0.023	0.006±0.002
4	GSFC	Sludge sample Vadodara,	0.0±0.0.02	0.12±0.09	0.042±0.003
5	DNRB	(DNR-B reactor), Sludge sample GNFC, Bharuch,	1.092±0.14	0.45±0.06	0.022±0.01
6	WC	Winogradsky's column	1.092±0.54	0.62±0.103	0.019±0.008
7	NW	Narol, wheat bulk soil	1.093±0.2	0.54±0.014	0.015±0.003
8	**PF**	**Mandya, paddy field soil**	**1.094±0.22**	**0.67±0.054**	**0.065±0.009**
9	ECP	Jowar soil (Effluent channel project) Ahemdabad,	1.093±0.9	0.61±0.043	0.023±0.01
10	VW	Wheat soil, Vinjal	1.092±0.19	0.55±0.108	0.041±0.015

5.3.2 Identification of microorganisms present in the PF AOB and NOB enriched biomass and development of seed consortium for simultaneous nitrification- denitrification (SND)

Cloning of 16S rRNA gene showed three patterns when digested with *Alu*I restriction enzymes indicating three kinds of microorganisms present in PF-NOB enriched biomass (Fig 5.2).

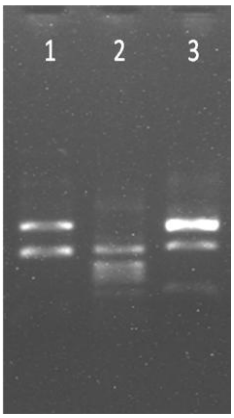


Fig 5.2 Representative ARDRA patterns observed by digesting 16S rRNA gene with *Alu*I restriction enzyme from PF-NOB.

Amongst NOB, *Nitrobacter* spp. are known to be easily cultivable. Conditions present during enrichment of NOB (high nitrite and oxygen concentration) favored growth of *Nitrobacter* which are reported to have higher growth rate and affinity for nitrite and oxygen (Schramm et al., 1999) compared to *Nitrospira* which are known to thrive under nutrient deprived conditions (Nogueira and Melo, 2006). Microorganisms identified in PF-AOB enriched biomass were *Nitrosomonas* sp. ENI11; *Sphingopyxis macrogoltabida*; *Alcaligenes* sp.; *Acromobacter* sp.; *Pusillimonas* sp., and *Nitrosomonas* sp. DYS317.

Growth and ammonia oxidizing activity of PF-AOB biomass was higher than growth and nitrite oxidizing activity of PF-NOB enriched biomass (Fig 5.3). A longer lag period was observed in the nitrite oxidizing activity of PF-NOB whereas PF-AOB showed increase in ammonia oxidizing activity almost parallel to its growth (Fig 5.3).

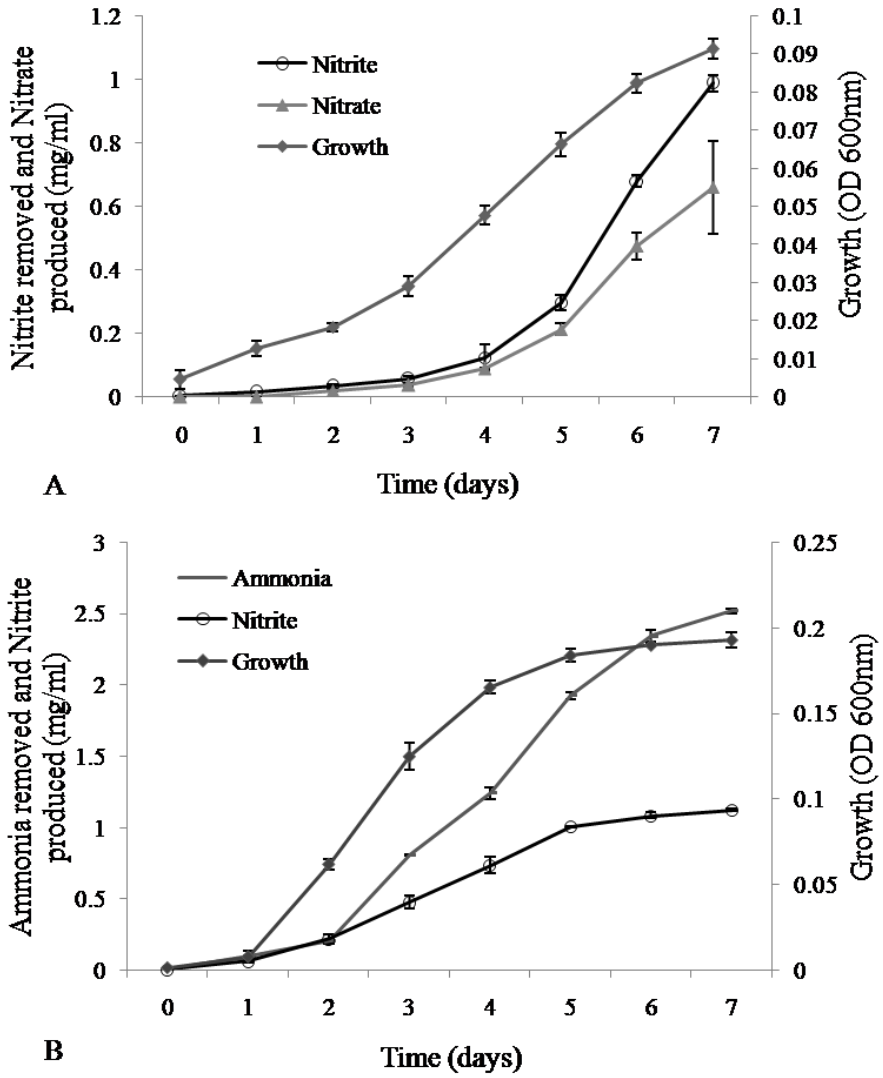


Fig 5.3 Growth and nitrifying activity of enriched biomass A) PF-NOB B) PF-AOB.

Specific growth rate constants for the PF-NOB and PF-AOB enriched biomass were 0.384 and 1.24 with doubling time of 1.8 and 0.56 days respectively. The growth of PF-AOB was 3.214 times faster than that of PF-NOB. PF-AOB and PF-NOB enriched biomass were mixed in 1:4 ratio (AOB:NOB) for effective consortium formation so as to get maximum removal of ammonia. NOB are reported to coexist with AOB in biofilms or in aggregates in wastewater treatment plants (Schramm et al., 1999). As reviewed by Kowalchuk and Stephen (2001), a syntrophic association exists between these two groups of microorganisms. AOB are present in the outer regions of the biofilms or aggregates and provide nitrite (nitrogen source) to

NOB residing in their close vicinity in the inner portion of the biofilms or aggregates (Schramm et al., 1996).

5.3.3 Ammonia removal performance in a SND type reactor treating high strength ammonia rich effluent of a fertilizer industry

A 5.3 L open reactor was designed for the aerobic autotrophic removal of ammonia from effluent of a fertilizer industry. Characteristics of the effluent to be treated are as mentioned in table 5.2.

Table 5.2. Characteristics of the industrial effluent.

Parameters	Concentration
pH	9.6
BOD	29 ppm
COD	46.66 ppm
Dissolved Oxygen	16 ppm
Ammonical nitrogen	700-800 ppm
Nitrite nitrogen	20-60 ppm
Nitrate nitrogen	Below detectable limit
Nickel (Ni)	1.16 ppm
Chromium (Cr)	Below detectable limit
Vanadium (V)	Below detectable limit
Iron (Fe)	0.07 ppm

Treatment of the effluent was divided into two main processes. The reactor was first run in the batch mode for 30 days and then in the continuous mode till the end of the reactor. Agitation and aeration in the reactor was kept on and off alternately for 24 h throughout the run period.

Batch reactor was run with increasing ammonia load and linear regression was applied to show that ammonia removing efficiency was not affected by the increased loading rate. Four dilutions (1:5, 1:3, 1:1 and 1:0.5) of the effluent were used to increase the ammonia load in the influent. A steady increase in the percentage of the ammonia removed with increase in ammonia load was observed (Fig 5.4A). Biomass in the reactor was acclimatized to higher ammonia concentration leading to 90% ammonia removal with 82.68 mg/L biomass accumulation by the end of the run (Fig 5.4A and C). A distinct drop in the pH of the reactor, during the batch mode, was indicative of the nitrifying activity in the reactor (Fig 5.4C). A continuous reduction in the nitrite concentration was suggestive of the growth of nitrite oxidizing bacteria during the batch culture (Fig 5.4B). Reduction of COD from 46 ppm to below detectable limits without increase in nitrate concentration was indicative of active denitrifying activity in the reactor. Nitrogen formed in the system could not be monitored as it was an open reactor.

During the start of the continuous mode some amount of biomass was washed away and the MLVSS value dropped to 31.56 mg/L which then steadily increased to around 116 mg/L by 55 days, during which a steady state was achieved in the reactor (Fig 5.4C). More than 90% ammonia removal was observed beyond 56th day, which steadily increased and reached 99% by the end of the run (Fig 5.4A). Nitrite levels also gradually decreased and reached within the permissible limit (0.7 ppm) by the end with no detectable nitrate (Fig 5.4B).

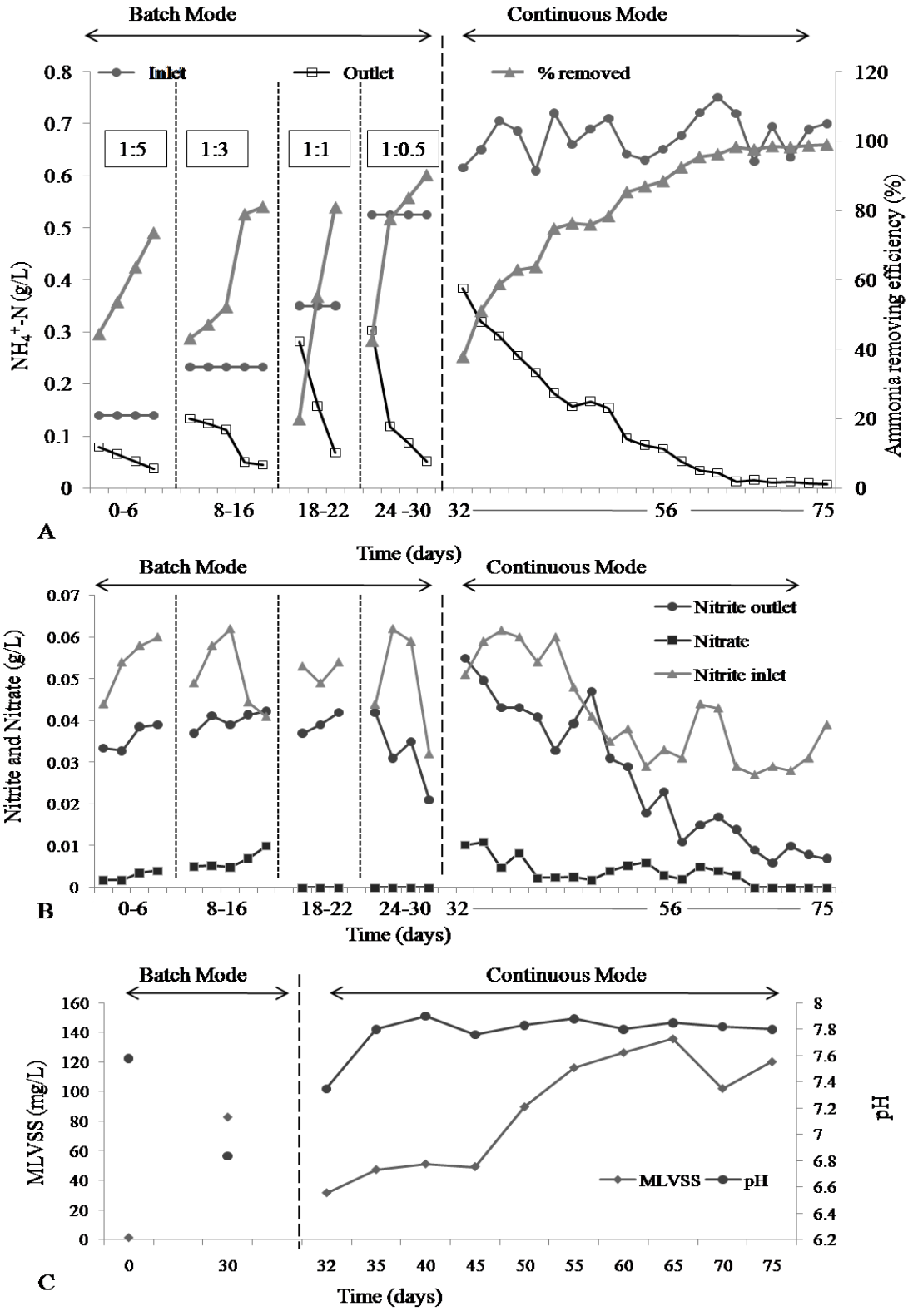


Fig 5.4 Nitrogen removal kinetics observed in the reactor. Change in the levels of A) Ammonia and Ammonia removing efficiency of the reactor B) Nitrite and nitrate C) pH and MLVSS. Values mentioned in the box are the ratio of effluent dilution with inorganic media without nitrite or ammonia.

The ratio of MLVSS:MLSS was less than 1 (0.45) indicating that the amount of food was less than the microorganisms which is considered to be signifying good conditions in the bioreactor. pH reduced from 8.0 to 6.8 indicative of nitrifying activity in the reactor. Settler played an important role in retaining microorganisms. Flocs formed in the reactor settled on the walls of the settler (Fig 5.1). SVI was less (22.4 ml/g) representing less sludge formation hence sludge removal may not be a problem using the present process for ammonia removal.

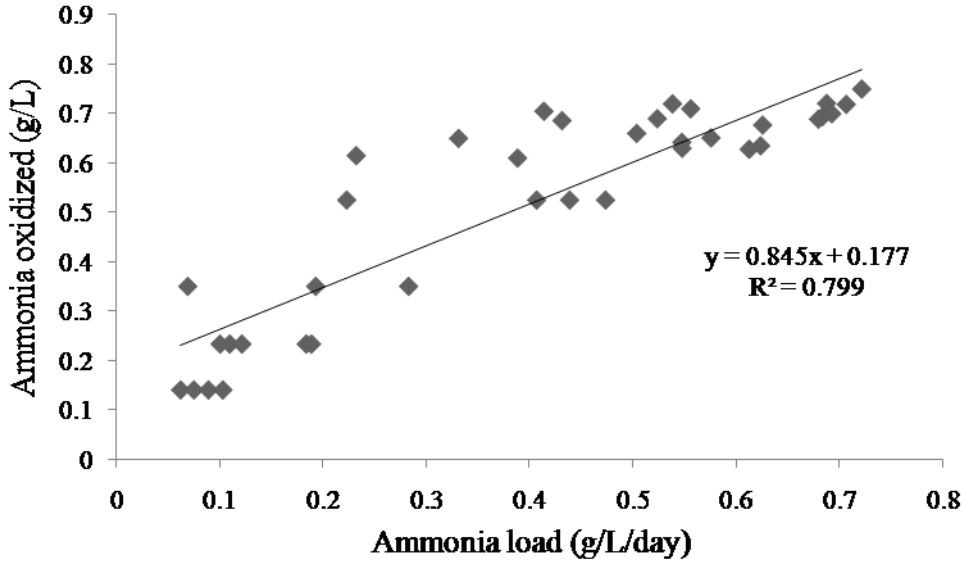


Fig 5.5 Simple linear regression of the concentration of ammonia oxidized (g/L) to the applied ammonia load (g/L/day).

The reactor was fed with increasing ammonia load till 30 days after which undiluted effluent was used. A linear correlation (R^2 = 0.799) existed between the ammonia load and the amount of ammonia removed indicating that the ammonia added to the reactor could be stably converted to molecular nitrogen (Fig 5.5). The ammonia conversion efficiency in the reactor was calculated throughout its run according to Davery et al., (2012)

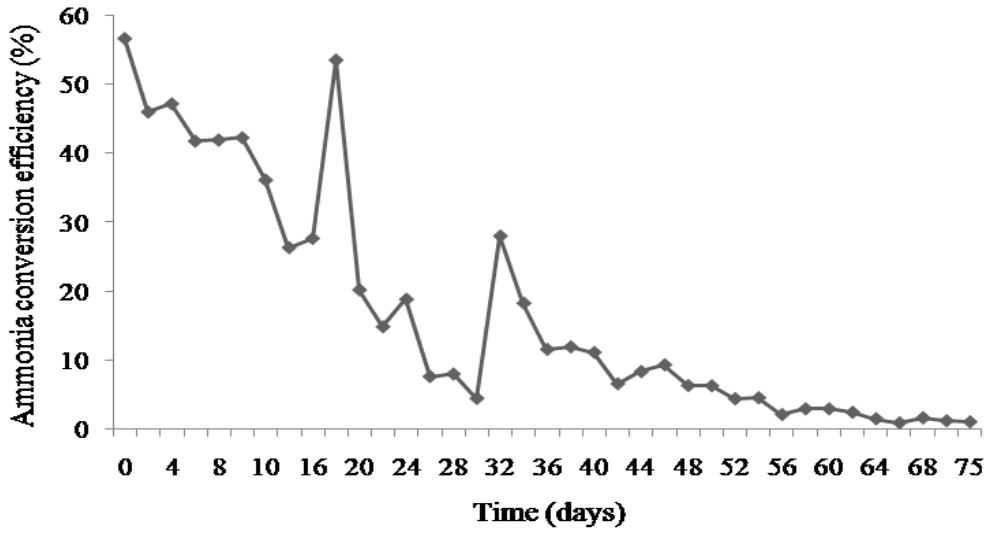


Fig 5.6 Ammonia conversion efficiency $Y_{(NO_2^- + NO_3^-)/NH_4^+} = \frac{\text{Eff. } \{(NO_2^- - N) + (NO_3^- - N)\}}{\text{Inf. } (NH_4^+ - N) - \text{Eff. } (NH_4^+ - N)} \times 100\%$ in the SND reactor.

Ammonia conversion efficiency was very low during the steady state (56-75th day) was indicative of higher denitrifying activity during the run period (Fig 5.6). Hence, ammonia removal could be achieved possibly with the growth and activity AOB, NOB and denitrifiers in the reactor.

5.3.4 Possible microbial mechanism of ammonia removal from the effluent through SND

The seed consortium had autotrophs - two kinds of AOB and one NOB, and heterotrophs most of which are identified to be aerobic denitrifiers. Ammonia would get oxidized to nitrate with concerted action of AOB and NOB. Heterotrophic bacteria which were co-enriched with AOB and NOB helped removal of nitrate from the system. Main key role players included *Achromobacter* sp., [aerobic nitrite denitrifier, which is reported to denitrify at DO levels between 3 to 10 ppm (Zhu et al., 2012)]. Other aerobic denitrifiers included *Sphingomonas* sp., *Pusillimonas* sp. and *Alcaligenes* sp. (Patureau et al., 2000; Joo et al., 2005; Srinivasan et al., 2010). These heterotrophs are commonly found in wastewater treatment plants. Some heterotrophic aerobic denitrifiers also carry out heterotrophic nitrification oxidizing ammonia to nitrite (Joo et al., 2005). The COD present in the effluent supported the growth of the heterotrophic denitrifiers justifying

removal of COD from the effluent (Table 5.2). Very few studies have focused on the use of heterotrophic nitrification and aerobic denitrification for the treatment of high strength ammonia containing wastewater (Gupta and Gupta 2001). Dissolved oxygen concentration played an important role in stabilizing the system. DO concentration of 7.6 to 7.9 ppm was maintained in the reactor by the help of external aerators during the aeration and agitation period whereas it dropped to 4.8 to 5.3 ppm during the off mode. High dissolved oxygen would favor nitrification and low DO would favor aerobic denitrification, thus maintaining favorable conditions for both the reactions to occur simultaneously in the same reactor. High HRT provided sufficient time for both nitrification and aerobic denitrification to occur effectively. Higher sludge retention resulted in larger flocs size and density which was highly suitable for the SND process to proceed steadily (Zajzon, 2012). These results will also help in inferring the reactions occurring in full scale reactors at varying oxygen concentrations.

To conclude, the present study demonstrated significance of nitrification and aerobic denitrification occurring simultaneously in a single reactor without addition of organic carbon. A stable bench scale reactor system could be developed with nitrification and denitrification rate such that nitrite and nitrate did not accumulate in the system and 99% ammonia removal was achieved. This system has the benefit of occurring in a single reactor without addition of organic carbon but the only limiting factor for its application in full scale reactor system would be the cost for aeration.

Summary

SUMMARY

Chapter 2a

Comparison of polymorphism in ammonia monooxygenase and hydroxylamine oxidoreductase genes for analyzing *Nitrosomonas* spp. diversity

- Amongst the 22 samples, 6 samples DnrA, DnrB, CETP, DN, N and PF showed higher nitrite production.
- Presence of AOB in the 6 enriched samples was confirmed by amplifying *amo*A gene.
- Twelve kinds of isolated red colored colonies were obtained. These contained heterotrophs but only one kind of autotroph per colony.
- *Nitrosomonas* spp. were observed to be present in 11 of the 12 colonies based on 16S rRNA gene cloning and sequencing.
- *amo*A gene fragment digested with *Hha*I and *Hae*III restriction enzyme showed 4 distinct patterns with resolution similar to 16S rRNA gene fragments from the same AOB.
- *hao* gene fragment when digested with *Msp*I restriction enzyme showed only 2 patterns and therefore a more sensitive method was required to get proper resolution with *hao* gene.
- Through SSCP analysis *amo*A and *hao* gene fragments were resolved into six and four groups respectively which were statistically analyzed and showed significant difference with P value < 0.001.
- Overall 53% resemblance was observed between both the genes in fingerprinting the enriched AOB.
- HAO activity staining was used for the first time for differentiating AOB.
- Validation experiments proved that the obtained bands were of hydroxylamine oxidoreductase.
- Zymogram pattern HAO enzyme was analyzed and compared with *amo*A and *hao* gene fragment SSCP and showed 61.5% and 46.1%

similarity with *amo*A and *hao* gene fragments SSCP respectively in resolving AOB.

- Simpson's index of diversity was calculated for the three methods and was found to be 0.64, 0.85 and 0.68 for HAO zymogram, *amo*A gene fragment SSCP and *hao* gene fragment SSCP respectively whereas the concerted use of the three methods could differentiate the 13 AOB with simpson's index of diversity 0.95.
- The three methods used to differentiate *Nitrosomonas* sp. followed the following order: *amo*A gene fragment SSCP > *hao* gene fragment SSCP > HAO activity staining.
- Better primers are required to be designed for *hao* to be used analogous to *amo*A gene fragment as a molecular marker for identifying AOB.

Chapter 2b

Assessing *hao* as a molecular and phylogenetic marker in comparison with *amo*A and 16S rRNA genes for analyzing autotrophic Ammonia Oxidizing Bacteria

- 16S rRNA, *amo*A and *hao* gene fragments considered in the present study, used to test *hao* as a phylogenetic and functional marker, were such that all three genes were obtained from the same AOB. These were obtained from NCBI as well as those obtained earlier in Chapter 2a.
- Phylogenetic trees constructed using the three genes showed similar tree topologies which were statistically analyzed and paired t-test was applied to the Shannon's index of diversity. As the values obtained by the t-test were less than the table value and p-value for the two-tailed test were high null-hypothesis that there is no significant difference in the diversity of AOB with respect to the three gene pairs, was considered true.

- Magnitude of the genetic variation in AOB was studied with respect to the three genes and it was found that rates of transition substitutions were more than transversion in 16S rRNA than *amo*A and *hao* genes. Ti/Tv rate ratios and p-distances calculated for the genes indicated lower sequence divergence in 16S rRNA gene than *amo*A and *hao* genes.
- Correlation plots based on sequence similarity showed co-evolution of *amo*A and *hao* genes with linear regression r^2 value 0.9. This was further confirmed by Pearson's correlation coefficient r 0.949 with p value <0.001 for the two genes.
- Patterns of mutations were also similar for *amo*A and *hao* genes dN:dS ratio were found to be 1.92 and 1.56 respectively for *amo*A and *hao* genes indicating positive Darwinian selection was going on in both the genes.
- 70.5% mutations observed in the *hao* gene were nonsynonymous mutations therefore effect of these mutations was checked on the structure of the protein by comparing all the structures with HAO of *Nitrosomonas europaea* pdb ID: 1FGJ
- Their RMSD values were between 0 to 0.04 indicating no significant variation in the structure of the proteins amongst *Nitrosomonas* spp. but distinct differences were observed in the structure of *Nitrosococcus oceani* HAO.

Chapter 3

Identifying physiological significance of heterotrophs co-existing with autotrophic Ammonia Oxidizing Bacteria in an ammonia oxidizing colony

- Heterotrophs were found to be coexisting with autotrophs in isolated colonies.
- These were found to be *Pusillimonas sp., Acidovorax sp., Acromobacter sp., Janibacter sp., Alcaligenes sp., Sphingopyxis granuli, Mezorhizoium*

sp. R2, *Thermomonas sp.* and *Castellaniela defragnans* through 16S rRNA gene cloning and sequencing.

- DnrA in which such association was observed for more than one year was used to unravel the mechanism of coexistence between the autotrophs and the heterotrophs.
- Three kinds of heterotrophs *Pusillimonas* sp., *Acidovorax* sp., and *Janibacter* sp., and an autotroph *Nitrosomonas eutropha* (designated as *Nitrosomonas* sp. RA) were found in an isolated red colored colony.
- Pure heterotrophs failed to grow alone in the inorganic media which showed their dependence on *Nitrosomonas* sp. RA in the said media.
- Pure heterotrophs could grow and utilize ammonia as nitrogen source in the presence of organic carbon like acetate. Thus, higher ammonia removal was observed in the system.
- Serial dilution was carried to separate AOB and heterotrophs. Growth of autotrophs was observed till 10^{-9} dilution but with the associated heterotrophs.
- Heterotrophs were not eliminated even in the presence of copper upto 100 μM concentration.
- Growth of autotrophs was observed upto 20 ppm mercury where as growth of heterotrophs was not observed beyond 8 ppm mercury concentration.
- Heterotrophs were observed in SEM image of *Nitrosomonas* sp. RA exposed to 20ppm merucury, suggesting it to be in the dormant state in the presence of mercury. The heterotroph could be resuscitated by giving heat shock and was found to be *Pusillimonas* sp.
- Longer lag phase was observed in the growth of *Nitrosomonas* sp. RA with reduction in 21.2% in nitrite production.
- In the absence of heterotrophs growth of autotrophs did not occur till iron concentration reached 10 μM Fe^{2+}, whereas in the presence of heterotrophs, growth of autotrophs occurred even without providing an external iron source.
- *Pusillimonas* sp. showed highest siderophore production amongst the heterotrophs.

- The siderophore produced by *Pusillimonas* sp. was found to be hydroxymate type and supplied exogenously at 1 mg/ml concentration to the autotrophs where increase in growth was observed in the presence of the externally supplied siderophores.
- In the absence of heterotrophs, 200 µg/ml siderophore concentration maximally supported the growth of autotrophs.
- Further, the growth and uptake of exogenously supplied siderophores were checked in the presence of heterotrophs. Growth of autotrophs was more in the presence of heterotrophs even in the absence of exogenously supplied siderophores implying sufficient siderophores were been provided by the heterotrophs to support the growth of autotrophs.
- Amplification of TonB dependent siderophore receptor gene showed presence of siderophore uptake system in *Nitrosomonas* sp. RA and increase in the growth of the autotrophs in the presence exogenously supplied siderophores suggested functionality of the siderophore receptors in *Nitrosomonas* sp. RA.
- Increase in SMP levels released by the autotrophs in the presence of mercury suggest dependence of heterotrophs on autotrophs for their organic carbon requirement.
- Nitrite at higher concentration was shown to inhibit the growth of autotrophs.
- Nitrite could be utilized by the heterotrophs in the presence of acetate as carbon source, this would increase the growth of autotrophs as they would be relived from nitrite inhibition.
- Thus, a mutual interdependence amongst the two groups of organisms was established.

Chapter 4

Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for removal of ammonia from effluent of a fertilizer industry

- PF-anammox obtained from the paddy-field soil sample and N4-anammox obtained from municipal wastewater treatment plants showed highest anammox activity with ammonia and nitrite removal in the ratio similar to the reported stoichiometry for anammox bacteria (1:1.33). Gas formed by the system was also identified to be nitrogen through gas chromatography.
- Addition of hydrazine in the system led to increase in anammox activity of both N4 and PF-anammox.
- Amplification of Planctomycetes and anammox specific amplification confirmed presence of anammox bacteria in both the system.
- These were maintained in small rubber tubes.
- Ammonia removing ability was checked under anoxic conditions by mixing enriched AOB and anammox biomass in 1:5 ratio using synthetic effluent. Gas formed in the system was confirmed to be nitrogen through GC. Further, seed culture was developed by mixing PF and N4 AOB-anammox bacterial biomass in 1:1 ratio.
- An upflow SNAD type bioreactor was run continuously for 125 days. 98.9% removal of ammonia from effluent of a fertilizer company was achieved using the developed system.
- Molecular analysis of the biomass carrying out the anammox activity showed presence of nitrifiers, denitrifiers in the upper part of the reactor where presence of anammox bacteria and denitrifiers was also observed in the lower part of the reactor.
- AOB were dominant in the upper part of the reactor whereas denitrifiers and anammox bacteria were majorly found in the lower anoxic region of the reactor.
- Presence of budding coccoidal shaped cells also suggested presence of anammox bacteria through SEM.
- Twenty eight distinct sequences suggesting 28 different kinds of bacteria mainly nitrifiers, denitrifiers and Planctomycetes were found to be coexisting in the reactor.

- Presence of flagellated cells observed through SEM would help in forming microbial aggregates and increase settling ability of the sludge in the reactor and thus prevent the entangled cells from getting washed off from the reactor.
- Food to microbe ratio calculated was found to be 0.62 day^{-1} which was indicative of endogenous growth of microorganisms and have better settling ability.
- A microbial system was thus developed converting ammonia to molecular nitrogen from effluent of a fertilizer company.

Chapter 5

Kinetics of ammonia removal in a 5.3 L open reactor: An aerobic solution to high strength ammonia containing wastewater of a fertilizer industry

- PF-NOB showed highest nitrite oxidizing activity amongst the enriched NOB.
- Microorganisms identified in the NOB enrichment culture were *Nitrobacter winogradsky* Nb-255 (95% identity), Uncultured *Sphingomonas* sp. clone Plot18-2H12 (96%), Uncultured *Acidobacteri*a bacterium clone 34 (95%).
- Specific growth rate constants for the PF-NOB and PF-AOB enriched biomass were 0.384 and 1.24 with doubling time of 1.8 and 0.56 days respectively.
- PF-AOB and PF-NOB were mixed in 1:4 proportion to make the seed culture for the reactor.
- The reactor was run in the batch mode for the first 30 days with increasing effluent concentration.
- Biomass in the reactor got acclimatized to high ammonia concentration, with reduction of around 90% ammonia and 82.68 mg/L biomass getting accumulated by the end of the run in the batch mode.

- Beyond 56th day steady state was achieved and 99% reduction in ammonia was achieved by the end of the run.
- Nitrite levels reached permissible limits (0.7 ppm) with no detectable nitrate.
- Settler efficiently retained the microorganisms in the reactor.
- A linear correlation existed between the ammonia added and removed from the reactor with high ammonia conversion efficiency.
- Heterotrophic aerobic denitrifiers were co-enriched with AOB and NOB hence the seed consortium mainly consisted of AOB, NOB and denitrifiers. These would play a major role in the removal of ammonia from the industrial effluent aerobically.
- COD present in the reactor would support the growth of denitrifiers in the reactor.
- Aeration and agitation were kept in the on and off mode for 24 h alternately. Thus, high dissolved oxygen in the on mode would favor nitrification whereas the off mode would favor aerobic denitrification.
- High HRT in the system provided sufficient time for both nitrification and aerobic denitrification to occur simultaneously.
- Higher sludge retention time resulted in larger floc size and density which is suitable for simultaneous nitrification and denitrification to proceed steadily.

Conclusion

CONCLUSION

Ammonia is released in high concentrations in effluent discharged from agriculture based industries and food processing industries. Ammonia at higher concentrations causes eutrophication and oxygen depletion in the receiving water bodies affecting entire aquatic life and causing numerous health hazards. Several reactors have been developed for the treatment of ammonia from industrial effluent. Design of reactors has been given great importance since a long time in the treatment of ammonia, but performance of the reactor greatly depends on the microbial community carrying out the reactions. Hence, knowledge regarding the microbial community composition would help in improving the stability and performance of the reactor. The study aimed, in developing a microbial process for the treatment of ammonia containing effluent of a fertilizer industry, identifying types of microorganisms involved and their diversity and also understanding the ecophysiological significance of the specific groups of organisms found to be coexisting in the process.

Most of the enriched autotrophic Ammonia Oxidizing Bacteria (AOB) were found to have identity with *Nitrosomonas* spp. Diversity studies of these isolated AOB was carried out in two parts a) sequence independent study b) sequencing based study. Hydroxylamine oxidoreductase was explored as a molecular and functional marker in comparison with *amo*A gene in differentiating the obtained AOB through SSCP analysis for the first time. The novel use hydroxylamine oxidoreductase (HAO) zymogram showed variation in the studied AOB. These three methods had differentiated *Nitrosomonas* spp. with resolution in the following order; *amo*A gene > *hao* gene > HAO enzyme zymogram. Hence, amongst the three novel techniques used in the present study to differentiate AOB belonging to a single genus, *amo*A gene fragment SSCP exhibited highest potential and for *hao* gene to be used at par with *amo*A gene SSCP in resolving AOB, better primers are required to be designed such that *hao* can be amplified from all AOB. HAO enzyme zymogram technique being simple to perform can be used as a preliminary method to study diversity. The concerted use of these polyphasic

approaches provided a better understanding of their pivotal role in metabolic and functional diversity of the organisms involved in the process.

Sequence based analysis of *hao* gene as a molecular and functional marker was carried out in comparison with *amo*A gene (a reported functional marker) and 16S rRNA gene (a conventional phylogenetic marker). Phylogenetic trees that were constructed using the three *amo*A, *hao* and 16S rRNA gene sequences were analyzed statistically and were found to have significantly similar topologies. Analysis of AOB genes carried out for the first time showed bias towards transitions over transversions. Higher sequence divergence in functional genes like *amo*A and *hao* compared to 16S rRNA gene was indicative of higher evolutionary rate of the genes compared to 16S rRNA. *amo*A and *hao* gene fragments showed similarity in synonymous and nonsynonymous substitutions pattern. HAO structural analysis carried out revealed that variation in amino acid sequence caused by the nonsynonymous substitutions did not cause major variation in its structure. Co-evolution of *amo*A and *hao* genes involved in the oxidation of ammonia to nitrite and their correlation with 16S rRNA gene were examined for the first time in the present study. Co-evolution of *amo*A and *hao* genes supported that *hao* can also be used at par with *amo*A as an alternative phylogenetic marker in studying diversity and evolution of AOB.

AOB are extremely slow growing with very low growth yield which make their isolation not just difficult but also time consuming. Contaminating heterotrophs tend to build up rapidly in association with AOB, even without an external supply of organic carbon. Intriguingly, it has been reported that aerobic ammonia oxidation proceeds more rapidly along with the contaminating heterotrophs. Knowledge regarding the functional significance of such association is far from complete. Present study reports for the first time a systematic analysis of the interaction between AOB and heterotrophs found closely associated in an ammonia oxidizing colony. *Nitrosomonas* sp. RA and 3 heterotrophs present in a single colony were dependent mutually on each other for growth. A system was developed where in growth of heterotrophs was inhibited by mercury without significantly affecting that of

Nitrosomonas sp. RA thereby a system containing *Nitrosomonas* sp. RA without physiologically active heterotrophs was obtained. This is the first report where *Nitrosomonas* sp. RA has been demonstrated, through bioassay, to utilize siderophores produced by associated heterotrophs towards meeting its iron requirement. Presence of siderophore receptor gene in *Nitrosomonas* sp. RA was shown by the amplification of TonB-dependent siderophore receptor gene fragment and growth of AOB in the presence of exogenously supplied siderophore confirmed it to be functional in the AOB. SMP produced by *Nitrosomonas* sp. RA supported growth of heterotrophs in the inorganic media. Organic carbon sequestration by heterotrophs would consequently facilitate the growth of *Nitrosomonas* sp. RA as organic carbon is reported to inhibit growth of autotrophs. Nitrite produced by the AOB could be utilized by the heterotrophs which in turn would increase the growth of AOB by removing nitrite inhibition. A mutual interdependence amongst the two groups of organisms for growth thus could be established. Mutual interactions and interdependence between different groups of microorganisms as analyzed in the present study are often observed in natural environment and are extremely important for the proper stabilization and functioning of the microbial community.

With the rising demand in environment protection various new technologies have been designed for the treatment of high ammonia containing effluent from different industries. Some of these high ammonia containing effluent also contain low COD like the one in the present study where Simultaneous Nitrification, Anammox and Denitrification (SNAD), which has not been explored in the treatment of effluent from fertilizer industry so far, was applied. The SNAD type bioreactor developed could efficiently remove ammonia from effluent with C:N ratio - 0.066 (much less than that used in the SNAD processes reported till date) and was run continuously for 125 days. 98.9% ammonia removal from the effluent was achieved. Coexisting nitrosifiers, anammox bacteria and denitrifiers were confirmed to be the major microorganisms that were responsible to carry out the reaction in the reactor without supplementation of external organic carbon and without

accumulation of nitrite or nitrate. Qualitative and quantitative analysis of the biomass generated revealed dominance of AOB in the upper oxic zone of the reactor while anammox followed by denitrifiers dominated in the lower anoxic zone of the reactor. Physiological and molecular studies strongly indicated presence of anammox bacteria in the anoxic zone of the SNAD reactor.

Full scale reactors do not entirely provide uniform environments in the reactor. There are pockets in the reactor that are anoxic in nature and some others that are completely aerobic. It is therefore important to study the mechanism of ammonia removal by the microorganisms and reactions going on in the reactor with varying oxygen conditions. A bench scale reactor with intermittent aeration and agitation was developed and was run for 75 day continuously. Present study demonstrated significance of nitrification and aerobic denitrification occurring simultaneously in a single reactor without addition of organic carbon. The system developed could run with similar nitrification and denitrification rate such that nitrite and nitrate did not accumulate in the system and 99% reduction in ammonia levels was achieved. This system has the benefit of occurring in a single reactor without addition of organic carbon but the only limiting factor for its application in full scale reactor system would be the aeration costs.

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Publications and Presentations

LIST OF PUBLICATIONS

1. Evaluation of hydroxylamine oxidoreductase as a functional and phylogenetic marker to differentiate *Nitrosomonas* spp. **Accepted in Journal of Basic Microbiology.**

2. Mutualism between autotrophic ammonia oxidizing bacteria (AOB) and heterotrophs inhabitant in an ammonia oxidizing colony. **Under revision in Archives of Microbiology.**

3. Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for removal of ammonia from effluent of a fertilizer industry. **Under Review in Bioresource Technology.**

4. Diversity assessment of hydroxylamine oxidoreductase gene and its comparison with 16S rRNA and ammonia monooxygenase genes as a molecular and phylogenetic marker for analyzing autotrophic Ammonia Oxidizing Bacteria. **Manuscript in preparation.**

5. Kinetics of ammonia removal in a 5.3 L open reactor: An aerobic solution to high strength ammonia containing wastewater of a fertilizer industry. **Manuscript in preparation.**

LIST OF PRESENTATIONS

1. Poster presentation: "Enrichment And Characterization Of Anaerobic Ammonia Oxidizing Bacteria From Industrial Sludge Samples"at 49th Annual conference of Association of Microbiologists of India (AMI) Delhi, India. (2008).

   Awarded - American Society for Microbiology Best Poster Award.

2. Poster presentation: "Diversity Analysis Of Autotrophic Ammonia Oxidizing Bacteria From Enriched Nitrifying Consortia Using a*mo*A And *hao* Genes As Markers "at 51st Annual conference of Association of Microbiologists of India (AMI) Ranchi, India. (2010).

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Research Paper

Evaluation of hydroxylamine oxidoreductase as a functional and phylogenetic marker to differentiate *Nitrosomonas* spp.

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Nitrosomonas genus belongs to beta-subclass of *Proteobacteria* and encompasses closely related species. Sequence independent techniques like single strand confirmation polymorphism (SSCP) was attempted in the present study to resolve AOB using ammonia monooxygenase (*amo*A) and hydroxylamine oxidoreductase (*hao*) gene fragments, unique to AOB. Variation in hydroxylamine oxidoreductase (HAO) enzyme zymogram of isolates observed in the study was also explored as an additional sequence independent method to substantiate the observations. *Nitrosomonas europaea* (standard strain) and 12 isolates, obtained by enriching environmental samples, were differentiated into six and four groups by SSCP analyses of *amo*A and *hao* gene fragments, respectively, whereas they could be resolved into six distinct groups through activity staining of HAO enzyme. *amo*A gene fragment was therefore found to be better than *hao* gene fragment in resolving the studied AOB based on richness and evenness with Simpson's index of diversity – 0.85. However, the ensembled use of these molecular methods (SSCP of *amo*A and *hao* gene fragments) and HAO enzyme zymogram in fingerprinting AOB provide better resolution and evenness, contributing significantly in AOB diversity studies. Grouping of AOB isolates by *hao* gene SSCP analysis followed almost the same pattern as that by 16S rRNA gene based sequence analysis, hence it is suitable as a phylogenetic marker.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Introduction

Autotrophic ammonia oxidizing bacteria (AOB) play an important role in oxidizing ammonia to nitrite aerobically in the global nitrogen cycle [1]. This oxidation occurs in two steps: ammonia is first oxidized to hydroxylamine by ammonia monooxygenase that is subsequently oxidized to nitrite by hydroxylamine oxidoreductase enzyme (HAO). 16S rRNA gene based phylogenetic analysis has been the golden standard method for fingerprint microorganisms [2]. However, use of genes unique to specific group of organisms has always been preferred as they target the particular group of organisms making fingerprinting analysis more focused. Of the two gene unique to AOB, ammonia monooxygenase (*amo*A) gene fragment have been widely used to fingerprint AOB [3] but very few studies have applied hydroxylamine oxidoreductase gene (*hao*) as a marker to study AOB diversity. The use of *hao* gene as a molecular marker for detecting AOB was first suggested by Shinozaki and Fukui [4] and recently Schmid *et al.* [5], developed primers for the amplification of *hao* gene fragment. However, *hao* gene fragment (850 bp) being larger size than *amoA* gene fragment (491 bp), it was important to compare its efficiency as a marker with *amo*A and 16S rRNA genes, in differentiating AOB.

Single-strand conformation polymorphism (SSCP) is a simple, reliable, nonradioactive technique to identify variability in a gene within and between various groups

Nucleotide sequence data are available at Genetic sequence database at the National Centre for Biotechnical Information, under GenBank accession numbers JN099273 to JN099310.

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of organisms [6, 7]. Differences in nucleotide sequence of PCR products leads to variation in electrophoretic mobility of single-stranded conformers developed during SSCP analysis. The technique is extremely useful in the study of evolutionary biology and molecular ecology and has been reported not only to match the resolution of sequencing but also over follows an order of magnitude more efficient in terms of fastness, labor, and resources [8]. It has been used to differentiate species of a single genus [9] and can therefore be applied to study the diversity of AOB. Since the technique is highly sensitive and able to detect even a single base substitution [7], it has been used, in the present study, as a tool to analyze variations in *hao* and *amo*A gene fragments towards differentiating AOB.

HAO enzyme is a complex hemoprotein of α2 or α3 oligomer containing eight heme units per 63 kDa subunit and molecular mass of 125–175 kDa has been reported for the holoenzyme [10]. Gene sequence based diversity studies applying molecular tools generally rely on primers designed to amplify the gene of interest from all microorganisms under study. Mutations at the primer binding site would inhibit binding of the primers and therefore would fail to amplify the gene. Consequently, some microorganisms in spite of being present will not to be detected from the system. The use of enzyme zymogram based fingerprinting methods to differentiate microorganisms can overcome this problem as it depends on enzyme activity [11]. This novel approach was therefore used to differentiate *Nitrosomonas* spp. (members of a single genus). The aim of the present study therefore was to evaluate *hao* gene as a functional and phylogenetic marker in comparison to *amo*A gene, a reported functional marker and 16S rRNA gene, a reported phylogenetic marker. *Nitrosomonas* spp. isolated from varied natural niche were differentiated by (i) employing SSCP analysis of *amo*A gene and its comparison with that of *hao* gene, (ii) evaluating the resolution obtained for *amo*A and *hao* gene fragments SSCP with 16S rRNA gene sequence based analysis, and (iii) studying HAO zymogram based resolution of AOB and its comparison with the gene based techniques. The concerted use of all these three methods not only enabled better resolution of AOB being studied in the present investigation, but also resolved them with higher evenness.

Materials and methods

Enrichment of AOB and growth conditions

Soil and sludge samples (5 g) were inoculated in 100 ml inorganic medium containing ammonium sulfate as nitrogen source and sodium carbonate as carbon source [12] and were incubated for 1 month at 30°C and pH 8.0 in dark (Table 1). The enriched samples were streaked on Nylon 66 membrane filter (45 mm diameter, 0.2 µm pore size) placed on the same medium containing 1% agar to get isolated colonies. Filters were transferred on fresh plates every third day for six transfers consecutively till visibly large red colored, isolated colonies appeared on the membrane.

DNA extraction, PCR amplification, cloning, and sequencing

Isolates were identified by 16S rRNA gene amplification, cloning, and sequencing. Log phase cultures, with

Table 1. Identification of AOB isolated from varied habitats based on 16S rDNA analysis.

Sr. No	Isolates used in the study	Origin	Blast result with 16S rDNA (%) identity
1	*Nitrosomonas europaea ATCC*19178 standard strain (NE)	Dr. Daniel Arp. Corvallis	–
2	DnrA	Solid sludge from (Denitrifying reactor) DnrA reactor, GNFC, Bharuch	*Nitrosomonas eutropha* C91 (94)
3	DnrB1	Solid sludge from (Denitrfying reactor 2) DnrB reactor, GNFC, Bharuch	*Nitrosomonas* sp. DYS317 (99)
4	DnrB2		*Nitrosomonas* sp. DYS317 (98)
5	CETP	Biofilm from Central Effluent Treatment Plant, GNFC, Bharuch	*Nitrosomonas* sp. DYS317 (99)
6	DN1	Effluent from Deepak Nitrite, Nandesari	*Nitrosomonas* sp. ENI-11 (99)
7	DN2		*Nitrosomonas* sp. DYS317 (98)
8	DN3		*Nitrosomonas eutropha* C91 (99)
9	N1	Activated sludge from Municipal waste water, Nandesari	*Nitrosospira* sp. Nsp12 (87)
10	N2		*Nitrosomonas* sp. ENI-11 (99)
11	N3		*Nitrosomonas* sp. ENI-11 (99)
12	PF1	Surface soil (2 cm from the surface) paddy field, Mandya	*Nitrosomonas* sp. DYS317 (99)
13	PF2		*Nitrosomonas* sp. ENI-11 (99)

$O.D_{600\ nm}$ in the range of 0.120–0.134 (50 ml), were centrifuged at 19,200 *g* for 15 min and DNA was extracted according to Schmidt *et al*. [13]. Amplification of 16S rRNA gene was carried out with *Taq* polymerase using universal primer 27F and 1541R. PCR protocol included an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1.5 min and a final extension at 72°C for 10 min. *amo*A gene fragment was amplified using modified amoA1F (5′-TGGGGTTTCTACTGGTGGT-3′) and reported amoA2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) primers designed by Rotthauwe *et al*. [14], targeting 331–349 and 802–822 positions, respectively of *Nitrosomonas europaea amo*A gene open reading frame [14]. *hao* gene fragment was amplified as described by Schmid *et al*. [5] using haoF4 and haoR2 primers (binding positions 451–467 and 1283–1299, respectively relative to *N. europaea hao* gene) and protocol. Primer haoR3 (binding position 1174–1189 relative to *N. europaea hao* gene) was used as reverse primer to amplify *hao* gene fragment from DN3, which failed to give amplification with haoR2 as the reverse primer [5]. Purification of the amplified 16S rRNA gene was carried out using PCR clean-up gel extraction NucleoSpin Extract II Kit (Macherey – Nagel GmbH and Co.KG, Germany) according to the manufacturer's instructions. These were then cloned in pTZ57R/T vector using INSTA cloning kit (Fermentas). Clones of 16S rRNA gene were differentiated based on amplified ribosomal DNA restriction analysis using *Alu*I restriction enzyme. Representatives of the different patterns were sequenced commercially by Single Pass Analysis (Bangalore Genei). *amo*A and *hao* genes from all the isolates were similarly cloned, sequenced, and submitted in NCBI.

Single strand conformation polymorphism

SSCP of *amo*A and *hao* genes was carried out, to fingerprint the enriched AOB, according to Sambrook and Russel [15] with modifications. Purified PCR product (2 μl) was denatured by mixing in 18 μl of a solution containing 98% formamide, 0.01 M ethylenediaminetetraacetic acid (EDTA) and bromophenol blue. The mixture was incubated at 95°C for 10 min and immediately cooled on ice. Single stranded DNA molecules (conformers) were resolved in (DCode Universal Mutation Detection System, Bio-Rad) 15 cm gels with 0.75 mm thickness. The mixture was loaded onto 8% nondenaturing gel containing 30% acrylamide–0.8% bisacrylamide and was run in 1× TBE (90 mM Tris-borate at pH 8.3, 4 mM EDTA). The run was carried out at constant 70 V and 25°C temperature. SSCP was carried out for 18 h in case of *amo*A gene and 20 h for *hao* gene. Silver staining technique was used to visualize the bands. Electrophoretic mobility of the single strand conformers was calculated by the AlphaEaseFC version 4.0 software.

Preparation of cell free extract

Cell free extracts were prepared to get a crude enzyme lysate for HAO enzyme polymorphism study. Cells (100 ml) grown to log phase were harvested by centrifugation (19,200 *g* for 10 min), washed once with phosphate-buffered saline (PBS) pH 7.4 and resuspended in sterile distilled water. The cell free extract was prepared by passing the cells through French press at 1000 psi followed by centrifugation at 19,200 *g* for 10 min at 4 °C. Protein concentration was determined as per Bradford's method [16].

HAO enzyme activity staining

HAO enzyme activity on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), under non-reducing condition was detected by formazan formation as described by Nejidat *et al*. [10]. Protein (10 μg) was mixed with loading buffer containing SDS but without beta-mercaptoethanol and loaded onto 7.5% SDS–PAGE gel. The run was carried out for 6 h at 25°C at 70 V (1 h after the dye front starts coming out) after which the gel was immersed into a solution containing 50 mM Tris–HCl, pH 8.0, 0.1 mM phenazine methosulfate, 0.2 mg ml^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide and 5 mM hydroxylamine (prepared fresh and mix hydroxylamine just before immersing the gel). Dark blue to black colored formazan formed over the HAO enzyme activity band just within 15 min of incubation in dark. The protein marker lane was cut and stained separately with Coomassie brilliant blue G250 stain. Electrophoretic mobility of HAO enzyme activity staining band was calculated using AlphaEaseFC version 4.0 software. Absence of hydroxylamine in the staining solution was used as substrate control. Addition of 2-mercaptoethanol (reducing agent) was used as a control to check the specificity of HAO enzyme. Cell free extract from *Escherichia coli* DH5α was used as a negative control.

Statistical analysis

All experiments were carried out in triplicate to check their reproducibility. The electrophoretic mobility of *amo*A and *hao* gene fragment single stranded conformers and HAO enzyme activity stained bands were statistically analyzed using SigmaStat version 3.5 software.

Calculations for diversity

Simpson's diversity index [17] was used as a measure to study the diversity of AOB as it takes into account both

richness (the number of species detected per technique and in combination) and evenness (relative abundance of the different species). For this Simpson's index "D" was initially calculated by:

$$D = \frac{[\Sigma n(n-1)]}{[N(N-1)]}$$

where n = total number of organisms belonging to a particular species and N = total number of organisms of all the species analyzed.

Simpson's index of diversity = $1 - D$ where D was Simpson's index.

Further, evenness in the population was calculated according to Pielou index according to Pommier *et al.* [18].

Phylogenetic analysis of AOB based on 16S rRNA gene

Multiple sequence alignment of 16S rRNA gene sequences from the different AOB was carried. Evolutionary distances were calculated by Jukes–Cantor method and are in the units of base substitutions per site. Phylogenetic tree was constructed using Neighbor-Joining method and in order to validate the inferred tree bootstrap analysis consisting of 100 resampling of the data was performed. All phylogenetic analysis was carried using *MEGA* version 4.0 software [19].

Results

AOB were enriched from industrial sludge and paddy field soil samples (Table 1). Owing to the slow growth rates of AOB, they were initially enriched for 1 month in inorganic medium that led to substantial increase in their number. Twelve different kinds of isolated colonies (based on colony morphology) appeared within 2 weeks of incubation (Supplementary Fig. S1). Identification of all the isolates based on 16S rRNA gene partial sequence (Table 1). Identification of all the isolates based on 16S rRNA gene partial sequence analysis revealed presence of *Nitrosomonas* species in 11 of the 12 isolates whereas N1 showed identity with *Nitrosospira* sp. Nsp 12 (Table 1).

Through SSCP analyses, *amo*A and *hao* gene fragments were resolved into six and four groups, respectively (Fig. 1A and B). Statistical analysis showed significant differences in the SSCP patterns amongst samples with p values of <0.001.

According to the electrophoretic mobility of the single strand conformers of *amo*A gene fragment, *N. europaea*, DnrA and DN1 formed one group; DnrB1, DnrB2, CETP, and DN2 formed a second group; and N1, N2, and PF2 formed third group; whereas DN3, N3, and PF1 showed distinct patterns (Fig. 1A). N1 grouped with N2 and PF2 as per *amo*A gene analysis but according to 16S rRNA gene sequence analysis N1 showed 87% identity with *Nitrosospira* sp. Nsp 12 whereas N2 and PF2 showed 99% identity to *Nitrosomonas* sp. ENI-11 (Table 1), indicating N1 to be phylogenetically very different from the other member of this group. Similar results were also obtained by Böttcher [20] and Juretschko *et al.* [21] where unexpected high similarity in *amo*A gene observed between different genera resulted from lateral *amo*A gene transfer events between species of ammonia oxidizers of different genera. A similar phenomenon may be occurring with N1, residing in close vicinity with N2 in the same niche.

SSCP analysis of *hao* gene fragment grouped *N. europaea*, DN1, DN2, N2, N3, and PF1 together; DnrB1, DnrB2, CETP, and PF1 formed another group; and DnrA and N1 showed distinct patterns (Fig. 1B). Some nitrosomonads that failed to get resolved through *amo*A gene fragment SSCP, like DnrA from *N. europaea*; DN2 from DnrB1, DnrB2, and CETP; and N1 from N2 and PF2; were easily distinguished through SSCP analysis of *hao* gene fragment (Fig. 1A and B). Similarly, PF2 and N3 that could not be resolved through SSCP analysis of *hao* gene fragment were distinguished through *amo*A gene fragment SSCP (Fig. 1A and B). DN3 *hao* gene fragment that could not be amplified using haoF4 and haoR2 primer set was subsequently amplified using reverse primer haoR3. However, owing to the difference in the size of *hao* amplicon obtained from DN3, it could not be included in the *hao* gene SSCP analysis. Overall 53.8% resemblance was observed between both the genes in fingerprinting of enriched soil and sludge samples for AOB diversity studies.

HAO enzyme activity staining was validated by using appropriate controls. HAO activity bands were not observed in substrate control (without hydroxylamine) and *E. coli* cell free extract (Supplementary Fig. S2). Loss of *N. europaea* HAO activity was observed upon addition of 2-mercaptoethanol (Supplementary Fig. S2; being a reducing agent, it cleaved disulfide bonds at the active site of the enzyme [22]). These controls confirmed that the activity bands were specific to HAO enzyme and were not mere artifacts.

Although *hao* gene from DN3 failed to get amplified using the reported primers (haoF4 and haoR2), its HAO enzyme activity band appeared in the zymogram. DN1 that could not be differentiated from *N. europaea* by SSCP analysis of both genes was resolved through HAO enzyme zymogram (Fig. 1C). *amo*A gene fragment SSCP showed 61.5% similarity with HAO enzyme zymogram while *hao*

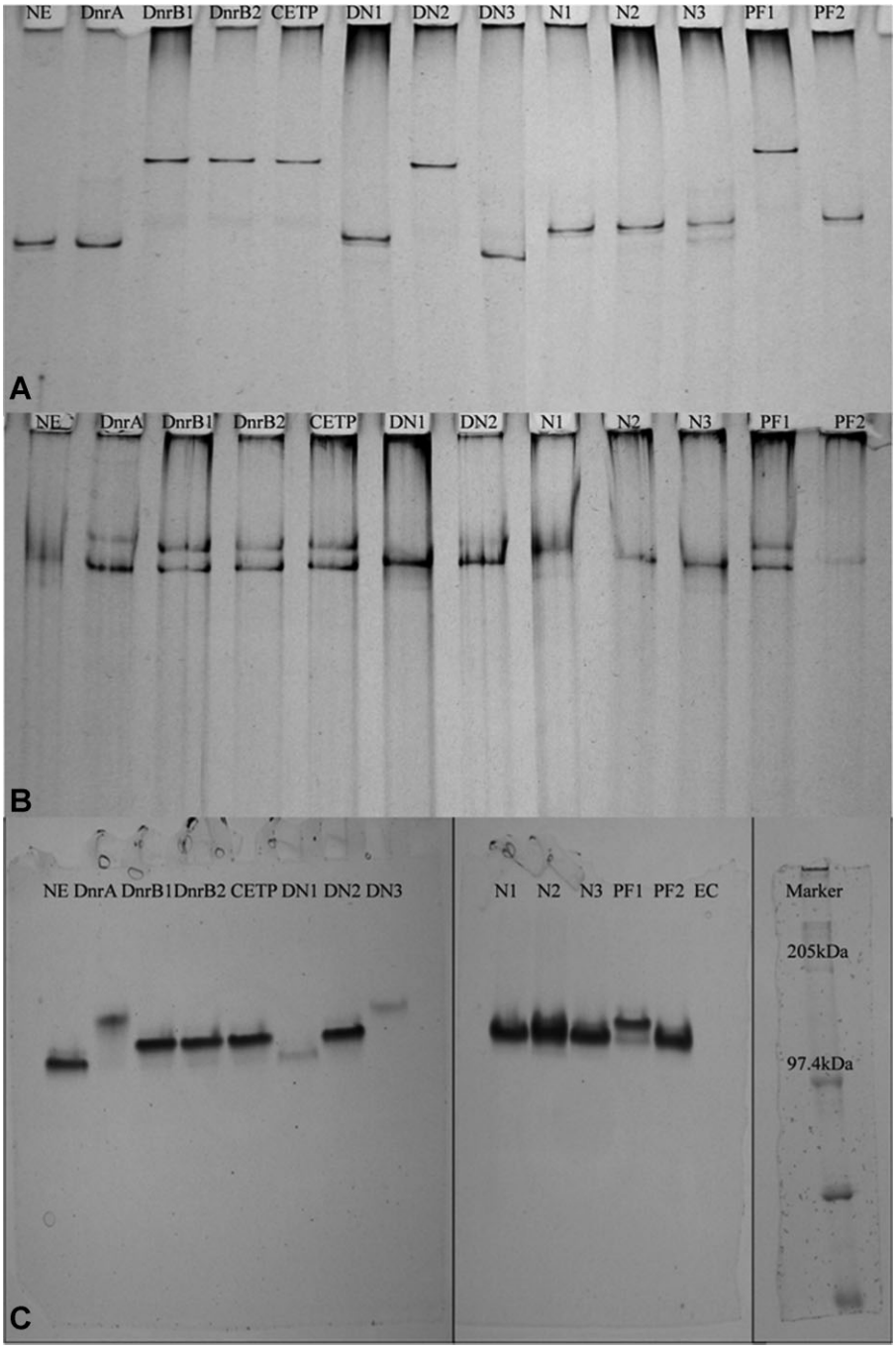

Figure 1. Diversity of *Nitrosomonas* spp. (A) *amo*A gene fragment (492bp) SSCP profiles. (B) *hao* gene fragment (850 bp) SSCP profiles. (C) Hydroxylamine oxidoreductase (HAO) zymogram pattern of *Nitrosomonas* spp. (NE-*N. europaea* and EC – *E. coli* DH 5α).

gene fragment SSCP showed 46.1% similarity with HAO enzyme zymogram in differentiating AOB.

Simpson's index of diversity [17] calculated for all the three methods was found to be 0.64 for HAO enzyme zymogram, 0.85 for *amo*A gene fragment SSCP, and 0.68 for *hao* gene fragment SSCP.

Richness and evenness in the population as depicted by the SSCP analysis of *amo*A and *hao* gene fragments and HAO enzyme zymogram are shown in Fig. 2. Higher AOB species richness (six groups) and evenness (Pielou's index of evenness 0.91) was observed in SSCP analysis of *amo*A gene fragment, compared to *hao* gene fragment (four

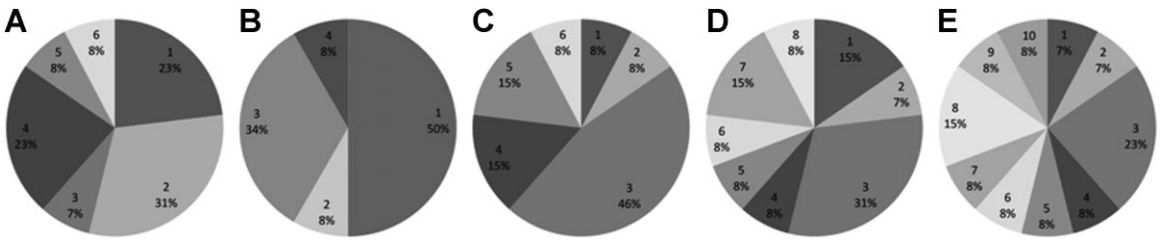

Figure 2. Richness and evenness in the diversity of *Nitrosomonas* spp. (A) *amo*A gene fragment SSCP. (B) *hao* gene fragment SSCP. (C) HAO activity staining. (D) concerted analysis of *amo*A and *hao* gene fragment SSCP. (E) concerted analysis of *amo*A and *hao* gene fragment SSCP together with HAO activity staining zymogram (R – richness and E – evenness).

groups and Pielou's index of evenness 0.81) (Fig. 2A and B). Some AOB that were not differentiated through SSCP analysis of *amo*A gene fragment, got resolved through *hao* gene fragment SSCP and vice versa. Therefore, when *amo*A and *hao* gene fragments SSCP patterns were analyzed together, the 13 AOB got differentiated into eight groups (Simpson's diversity index – 0.9) with not more than 31% population belonging to a single group, thereby indicating a higher richness and evenness (Pielou's index of evenness 0.93) in the diversity of AOB compared to their individual analysis (Fig. 2D).

AOB were required to be resolved based on 16S rRNA sequence to evaluate *hao* gene as a phylogenetic marker. According to 16S rRNA gene sequence analysis, the studied AOB could be grouped into five (Fig. 3). First group comprised of *N. europaea*, N2, N3, DN1, and PF2; second groups included DN2, DnrB1, DnrB2, CETP, and PF1; whereas DN3, DnrA, and N1 formed individual groups (Fig. 3). Simpson's index of diversity for 16S rRNA gene in differentiating the studied AOB was 0.74 and Pielou's index of evenness for the same was 0.825.

Discussion

The objective of the study was mainly to evaluate *hao* gene fragment and HAO zymogram technique, as a functional and molecular marker in comparison with *amo*A gene, a reported functional and molecular marker, towards studying the diversity of AOB, the study was focused on isolated AOB rather than the environmental samples so as to get clear and irrefutable results on the set objectives. *Nitrosomonas* spp. were present in 11 of the 12 isolates obtained from the enriched samples (Table 1). *Nitrosomonas* spp. amongst AOB have been reported and found to be dominant and primarily present in waste water treatment plants and enriched soil and ground water samples [23, 24].

Higher diversity in AOB species observed through *amo*A gene fragment SSCP analysis (Simpson's index of DIVERSITY – 0.85) compared to *hao* gene fragment SSCP (Simpson's index of diversity – 0.68; Fig. 2–C) suggested *amo*A gene fragment as a better marker compared to *hao* gene fragment. Though the studied AOB could be

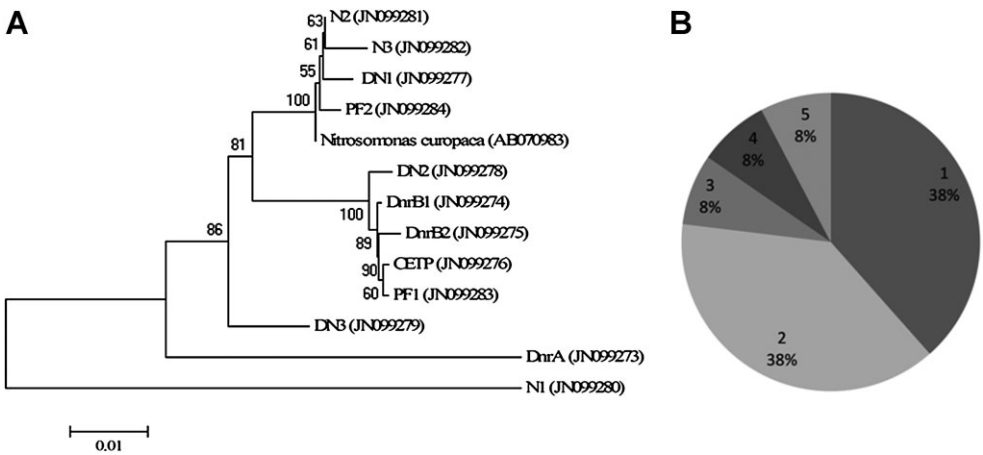

Figure 3. (A) Phylogenetic tree of AOB based on 16S rRNA gene sequences, constructed by Neighbor-Joining method. The scale bar indicates 0.01 substitutions per nucleotide position. Bootstrap values are given at the nodes for 100 replicates. (B) Richness and evenness in the diversity of *Nitrosomonas* spp. observed through 16S rRNA gene sequences.

differentiated into four groups through *hao* gene fragment SSCP, higher diversity could be obtained of the same if shorter fragment of the gene was used (as shorter fragments give better resolution).

HAO enzyme activity staining was used for the first time to study the diversity of the enriched *Nitrosomonas* species. Variation in the electrophoretic mobility of the enzyme was observed on the SDS gel (Fig. 1C) contradicting to the results of Nejidat *et al*. [10] where no difference was observed in HAO of *N. europaea* ATCC 19718, *N. europaea* LMD 86.25, *Nitrosomonas eutropha* N904, *Nitrosomonas* spp. and *Nitrosolobus* spp. The resolution of enzymes in the present study could be attributed to the extended electrophoresis time (1 h after the removal of the dye front from the gel, at 70 V), resulting in significant variation in the HAO enzyme activity staining bands of *Nitrosomonas* spp. (p values <0.001). The technique is independent of the sequence variations hence HAO enzyme activity, could also be observed from DN3 isolate that was distinctly different from the other HAO enzyme of the same genus (Fig. 1C). The method thus takes into consideration more diverged species of AOB that may not be considered by general PCR based fingerprinting methods, thus providing additional information in the diversity study of enriched AOB however, the method cannot be applied directly to environmental samples.

Through HAO enzyme activity staining, AOB could be differentiated into six groups (Fig. 2C), with one of the groups predominantly representing 46% of the total AOB studied (Fig. 2C). Species richness observed through HAO enzyme activity staining was comparable to *amo*A gene fragment SSCP analysis and higher than *hao* gene fragment SSCP but evenness in the population was less (Pielou's index of evenness 0.75), (Fig. 1B and C). *hao* gene fragment under study constitutes just 39.1% of the hydroxylamine oxidoreductase complete coding DNA sequence (CDS) [5, 25]. Therefore, the diversity of AOB represented here is just a small fraction of the total diversity that can be represented by hydroxylamine oxidoreductase complete CDS. This justifies the higher diversity observed in the AOB population by HAO enzyme activity staining (involving complete enzyme) than SSCP analysis of *hao* gene fragment. Interestingly, the concerted use of these three techniques (*amo*A gene fragment and *hao* gene fragment SSCP and HAO enzyme zymogram) differentiated the 13 AOB into ten groups (Simpson's index of diversity – 0.95; Fig. 2E), projecting higher richness and evenness (Pielou's index of evenness 0.96) in the studied population.

These sequence independent methods were compared with 16S rRNA gene based diversity analysis of the studied AOB. Simpson's index of diversity (0.85) for *amo*A gene fragment SSCP which resolved AOB into six groups was higher than the Simpson's index of diversity (0.74) for 16S rRNA gene that resolved AOB into five groups (Fig. 3), indicating *amo*A gene fragment SSCP to be a better marker compared to 16S rRNA gene. Using HAO enzyme zymogram the studied AOB could be differentiated into six groups but showed lower evenness in the population and therefore its Simpson's index of diversity was 0.64 indicating lower efficiency of HAO enzyme zymogram than 16S rRNA gene in resolving AOB. Though the studied AOB could be differentiated into four groups through *hao* gene fragment SSCP and its Simpson's index of diversity (0.68) was lower than 16S rRNA gene, it showed highest similarity (91.6%) with 16S rRNA in the grouping the studied AOB, indicating it as a potential phylogenetic marker comparable to 16S rRNA (provided better primers could be designed so that *hao* gene fragment could be amplified from all AOB; Supplementary Table S1).

In conclusion, these methods differentiated *Nitrosomonas* spp. with resolution in the following order: *amo*A gene fragment SSCP > 16S rRNA sequence polymorphism > *hao* gene fragment SSCP > HAO enzyme zymogram. Hence, amongst all the techniques used in the present study to resolve AOB based on single genus, *amo*A gene fragment SSCP exhibited highest potential and therefore is a superior functional marker than *hao* gene fragment. Better primers are required to be designed for *hao* gene to be used as an analog to *amo*A gene SSCP and HAO enzyme activity staining, so that *hao* can be amplified from all AOB. As *hao* gene fragment SSCP analysis showed high congruence with 16S RNA gene in the AOB grouping pattern, therefore it may be used as a phylogenetic marker. HAO enzyme zymogram technique being simple to perform can be used as a preliminary method to study diversity. The consorted use of these polyphasic approaches provides a better understanding of their pivotal role in metabolic and functional diversity in the biogeochemical cycle of varied ecosystem.

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Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for removal of ammonia from effluent of a fertilizer industry

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HIGHLIGHTS

- SNAD process was developed to treat ammonia laden effluent of fertilizer industry.
- Enriched biomass developed stably treated effluent with C/N ratio 0.066.
- 98.9% ammonia removal was achieved in a single reactor.
- Sequencing 16S rDNA showed presence of nitrifiers, denitrifiers & anammox bacteria.
- Quantitative analysis of bacteria revealed their spatial arrangement in reactor.

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ABSTRACT

A simultaneous partial nitrification, anammox and denitrification (SNAD) process was developed for the treatment of ammonia laden effluent of a fertilizer industry. Autotrophic aerobic and anaerobic ammonia oxidizing biomass was enriched and their ammonia removal ability was confirmed in synthetic effluent system. Seed consortium developed from these was applied in the treatment of effluent in an oxygen limited bench scale SNAD type (1L) reactor run at ambient temperature (~30 °C). Around 98.9% ammonia removal was achieved with ammonia loading rate 0.35kgNH_4^+-N/m^3 day in the presence of 46.6 mg/L COD at 2.31 days hydraulic retention time. Qualitative and quantitative analysis of the biomass from upper and lower zone of the reactor revealed presence of autotrophic ammonia oxidizing bacteria (AOB), Planctomycetes and denitrifiers as the dominant bacteria carrying out anoxic oxidation of ammonia in the reactor. Physiological and molecular studies strongly indicate presence of anammox bacteria in the anoxic zone of the SNAD reactor.

1. Introduction

Ammonia released in the effluent of agriculture based industries has become a prime concern with the increasing awareness of pollution in the water bodies leading to eutrophication and acidification of the aquatic systems. Conventional ammonia removal from wastewater accomplished through the combination of nitrification (aerobic) and denitrification (anaerobic) is expensive, energy and space requiring process generating high amount of sludge (Bagchi et al., 2012). Development of a novel cost-effective process for the treatment of high ammonia and low or no level of COD, therefore has been the need of the day.

New technologies developed over the time for efficient removal of ammonia from wastewaters include, SHARON (single reactor system for high activity ammonia removal over nitrite), SND (simultaneous nitrification denitrification), SNAP (Single stage nitrogen removal using anammox and partial nitritation), CANON (complete autotrophic nitrogen removal over nitrite), OLAND (oxygen limited autotrophic nitrification and denitrification), DEMON (DEamMONification), and BABE (Bio-Augmentation Batch Enhanced), as reviewed by Bagchi et al. (2012) involve partial nitrification (oxidation of ammonia to nitrite) followed by anoxic oxidation of the remaining ammonia by the anammox bacteria in the presence of nitrite as electron acceptor. These processes are operated in a single reactor unit with reduced aeration (1kWh/kgN) and external organic load requirements, saving 90% of the operation costs (Wang et al., 2010). Although significant nitrification is not expected at DO below 0.3 mg/L, treatment processes that promote simultaneous nitrification–denitrification can reach

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up to 80% of the total nitrification under anoxic conditions (Stenstrom and Poduska, 1980). In agreement with this, nitrifiers, denitrifiers and anammox bacteria (having optimum activities under completely different conditions) are reported to coexist in the same environment (Xiao et al., 2008).

OLAND and CANON processes are more suitable for the treatment of wastewaters with high ammonia concentration and without organic carbon (Lan et al., 2011). Simultaneous partial nitrification, anammox and denitrification (SNAD) described by Chen et al. (2009) was developed to solve the problem of organic carbon in CANON process. This recently developed process has been used efficiently at the full scale land fill leachate treatment plant for nitrogen removal (Wang et al., 2010).

Present study reports application of SNAD for the treatment of high ammonia containing effluent of a fertilizer industry with low levels of COD (46.6 mg/L). The process involved partial nitrification of ammonia by aerobic autotrophic ammonia oxidizing bacteria (AOB), the remaining ammonia and nitrite so formed get converted to molecular nitrogen by anammox bacteria releasing low levels of nitrate in the process. The nitrates so formed get reduced to N_2 by denitrifiers at the expense of organic carbon in the effluents. Activity and growth of one kind of microorganism provided substrate for the next establishing a succession of bacteria maintaining harmony and cooperation in the reactor leading to effective removal of ammonia. The kinetics of biological ammonia removal from the ammonia laden waste water is discussed together with the characterization and quantification by RT-PCR, of AOB, anammox and denitrifiers developed in the reactor during the process.

2. Methods

2.1. Enrichment of aerobic and anaerobic ammonia oxidizers to develop biomass

Several soil and activated sludge samples from municipal wastewater treatment plant and fertilizer industries were used for the enrichment of aerobic and anaerobic ammonia oxidizers (data not shown). For the enrichment of AOB, the samples (5%) were enriched for one month under aerobic conditions with sodium carbonate and ammonium sulphate as the sole source of carbon and nitrogen respectively in the medium (100 ml) described by Hyman and Arp (1992). Ammonia oxidizing activity of the enriched AOB was monitored by the levels of ammonia removed and nitrite produced at the end of incubation. An enriched biomass so developed showing high aerobic ammonia oxidizing activity was used in the further study and was designated as PF-AOB. Amplification of *amo*A gene was carried out using primers (*amo*A-1F 5′TGGGGTTTCTACTGGTGG 3′ and *amo*A-2R 5′CCCCTCKGSAAAGCCTTCTTC 3′) and protocol as described by Rotthauwe et al. (1997) to confirm the presence of AOB in the enriched biomass. Enrichment for anaerobic ammonia oxidizing (anammox) biomass, was carried out with samples (5%) inoculated in inorganic medium (100 ml) containing ammonium sulphate (3 mM) as the nitrogen source, sodium nitrite (6 mM) as the electron acceptor, potassium bicarbonate (25 mM) as the carbon source and other trace metals as described by Egli et al. (2001). Anaerobic conditions were created by flushing helium (He) gas (99.99% purity) through the medium. Anammox activity of the enriched biomass was measured by monitoring the amount of ammonia and nitrite removed. Gas produced during the process was confirmed to be nitrogen through gas chromatography (GC) analysis (Model:Varian 3600) using He as the carrier gas. An enriched biomass so developed showing high anammox activity was used in the further study and was designated as PF-anammox. Effect of hydrazine was checked on its anammox activity by adding of 0.25 mg/ml hydrazine. Amplification of Planctomycetes specific and anammox specific regions in the 16S rRNA gene was carried out to confirm the presence of anammox bacteria. Primers Pla46F (forward primer) 5′GACTTGCATGCCTAATCC 3′ and 1392R (reverse primer) 5′GACGGGCGGTGTGACAA 3′ were used for Planctomycetes specific amplification and Pla46F (forward primer) and Amx820 (reverse primer) 5′CCTTTCGGGCATTGCGAA3′ for anammox specific amplification according to Tal et al. (2006).

2.2. Development of seed consortium for SNAD bioreactor

Closed systems (500 ml) were used in this study to check the ability of PF-AOB to survive and oxidize ammonia under anoxic conditions and the ability of PF-anammox to utilize nitrite produced by the aerobes using synthetic medium. The composition of synthetic medium was same as that of the inorganic medium used for enrichment of AOB and anammox biomass respectively. Different reactor conditions and controls, used were as follows: AOB control (i) and experimental (ii) sets - constituted respectively of uninoculated and PF-AOB inoculated synthetic effluent containing ammonium sulphate as the nitrogen source and kept under anoxic condition. Anammox control (iii) and experimental (iv) sets - constituted respectively of uninoculated and PF-anammox inoculated synthetic medium containing ammonium sulphate and sodium nitrate and was made anaerobic by flushing He gas. (v) Mixture of PF-AOB + PF-anammox (1:5 ratios) was inoculated in synthetic effluent containing only ammonium sulphate under anoxic condition. The reactors in which anoxic condition was to be created were not flushed with He, but were tightly closed such that external air could not enter. Concentration of ammonia, nitrite and nitrogen were measured after 3 months of incubation. Another consortium prepared from the sample N4 (obtained from activated sludge of municipal wastewater treatment plant, Nandesari), by enriching for AOB (N4-AOB) and anammox (N4-anammox) showing almost similar ammonia removing potential was N4-AOB + N4-anammox. Final seed culture used in the reactor comprised of N4-AOB + N4-anammox and PF-AOB + PF-anammox mixed in 1:1 ratio.

2.3. Operating conditions for removal of ammonia from effluent of a fertilizer industry in a 1L SNAD type reactor

A cylindrical reactor with 1L effective volume was designed with 45 cm height, internal diameter of 7 cm and outlet at 35 cm height (Fig 1). The reactor was operated in the up-flow mode as the influent was introduced from the bottom. Operating temperature was 30 °C and was run in the batch mode for initial 15 days. Inlet and outlet ammonia, nitrite and nitrate were measured continuously every two days for 125 days while pH and dissolved oxygen (YSI200 portable DO meter, USA) were measured in the upper and lower zone of the reactor every 15 days. Total solids (TS), total dissolved solids (TDS), total suspended solids (TSS), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and sludge volume index (SVI) were estimated initially and finally at the commencement and termination of the reactor respectively. Flow rate of the reactor was maintained at 0.3 ml/min and hydraulic retention time (HRT) was 2.31 days.

2.4. Ammonia conversion efficiency

The anoxic conversion of ammonia to molecular nitrogen was calculated throughout the run according to Daverey et al. (2012) by the equation

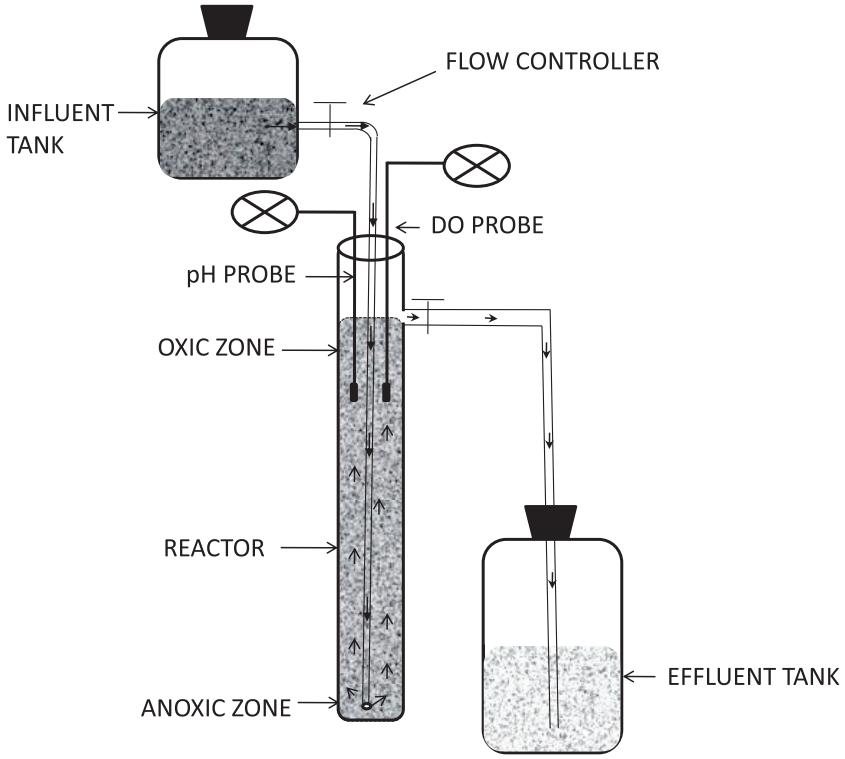

Fig. 1. Schematic diagram of the SNAD reactor.→ Indicates direction of inflow of effluent → Indicates direction of flow of effluent in the reactor and → Indicates outflow of effluent.

$$Y_{(NO_2^- + NO_3^-)/NH_4^+} = \frac{\text{Eff.}\{(NO_2^- - N) + (NO_3^- - N)\}}{\text{Inf.}(NH_4^+ - N) - \text{Eff.}(NH_4^+ - N)} \times 100\%$$

2.5. Analytical methods

Ammonia consumed and nitrite released were measured according to Scheiner (1976) and Griess-Romijn et al. (1996) respectively. Brucine sulfate method was used to determine nitrate according to Jenkins and Medsker (1964). Hydrazine and hydroxylamine were estimated from the seed cultures according to Watt (1952) and Frear and Burrell, 1955) respectively. TS, TDS, TSS, MLSS, MLVSS and SVI were measured according to standard protocols (APHA, 1995).

2.6. Scanning electron microscopy (SEM) from the reactor

Morphology of the bacteria present in the lower anoxic zone of the reactor were observed using Joel Scanning Electron Microscope with Oxford EDS system model No. JSM-5610LV. Biomass from the lower zone was fixed on a grease free glass slide using gluteraldehyde and dehydrated in a series of increasing acetone concentration according to Rheims et al. (1999). Preparations were dried sputter-coated with silver and examined in SEM.

2.7. Qualitative analysis of the biomass generated in reactor

Effluent (1 ml) from the upper layer of the reactor and 1 ml sludge from the base of the reactor were used to extract genomic DNA. The effluent and sludge were centrifuged and washed with N-saline. These were mixed with 450 µl of extraction buffer (100 mM Tris–HCl pH 8.0, 100 mM sodium EDTA pH 8.0, 100 mM sodium phosphate pH 8.0, 1.5 M NaCl, 1% CTAB) for half an hour and were passed through French press at 1000 psi pressure. The extraction of genomic DNA was carried out according to Schmidt et al. (1991). Amplification of *amo*A gene (Rotthauwe et al., 1997), *nir*S (Throback et al., 2004), *nos*Z (Henry et al., 2006), and planctomycetes and anammox specific 16S rRNA (Tal et al., 2006) gene region was carried out from both upper and lower zone of the reactor to demonstrate the presence of AOB, denitrifiers and anammox bacteria. 16S rRNA gene was amplified using 27F and 1541R (universal primers) with following PCR program: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation 95 °C for 30 s, annealing 58 °C for 45 s and elongation at 72 °C for 1.5 min and a final elongation at 72 °C for 10 min. The amplified fragment was cloned in pTZ57R/T vector using INSTA cloning kit (Fermentas, Inc.). Amplified ribosomal DNA restriction analysis (ARDRA) was carried out using *Alu*I restriction enzyme and the distinct patterns obtained were sent for commercial sequencing through single pass analysis from Xcelris Labs (Ahmedabad, India). The sequences determined in the study submitted in GenBank under accession numbers JX143764 – JX143801.

2.8. Quantitating biomass generated in the reactor by real-time PCR analysis

Absolute quantification of the genes was carried out by Real-time PCR analysis in Step One Real-Time PCR system (Applied Biosystems, USA) by the standard curve method. Bacterial 16S rDNA [amplified according to Quan et al. (2008)], ammonia monooxygenase (*amo*A), nitrite reductase (*nir*S) and nitrous oxide reductase (*nos*Z) genes were quantified using SYBR Green master mix (Applied Biosystems, USA) in 10 µl reaction system. Standard curves for all the genes were constructed with plasmids containing individual genes (16S rDNA, *amo*A, *nir*S and *nos*Z) prepared by cloning the genes in pTZ57R/T vector using INSTA cloning kit (Fermentas Inc.). The specificity of the PCR amplification was determined by the melt curve analysis and R^2 values obtained were greater than

0.98 for all the curves. PCR protocol used for 16S rRNA gene was: an initial denaturation temperature of 95 °C for 10 min, 40 cycles of PCR with denaturation at 95 °C for 15 s, annealing at 43 °C for 45 s, and extension at 72 °C for 30 s. Same protocol was followed for *amo*A and *nir*S, with annealing temperature 54 °C and extension time 1 min for *amo*A and 45 s for *nir*S whereas for *nos*Z touchdown PCR protocol used was according to Henry et al. (2006).

2.9. Phylogenetic analysis

Phylogenetic analysis of the microorganisms present in the reactor was carried out using 16S rRNA gene sequences by MEGA4.0 software (Tamura et al., 2007). 16S rRNA gene sequence obtained by universal primers (27F and 1541R) and anammox specific primes (Pla46F and Amx820) were used to find the types of the microorganisms present in the reactor. Tree was constructed using the Neighbor-Joining method. All positions containing gaps and missing data were eliminated from the data set by complete deletion option. There were a total of 520 positions in the final data set.

3. Results and discussion

3.1. Biomass development of AOB and anammox bacteria

PF-AOB biomass that was enriched for AOB bacteria showed highest ammonia oxidizing activity amounting to removal of 20 mM ammonia with production of 0.15 mM of nitrite. Presence of AOB in this enriched biomass was confirmed by the amplification of *amo*A gene fragment (Fig. S1). Similarly, PF-anammox biomass showed highest anammox activity under anaerobic conditions amongst the samples enriched for anammox bacteria (data not shown). Its anammox activity was expressed as decrease in ammonia and nitrite concentration, 1.2 mM, and 2.27 mM respectively. Ratio of ammonia:nitrite removed was 1:1.89. The stoichiometry obtained was almost similar to the stoichiometry reported for anammox bacteria (1:1.32) by Strous et al. (1999). Gas formed in the process was identified to be nitrogen through gas chromatography (44% nitrogen was produced by the enriched biomass). Hydrazine as an intermediate has been reported to strongly stimulate anammox activity (Strous et al., 1999). Addition of hydrazine in the system led to 1.7-fold increase in ammonia removal, 1.23-fold increase in nitrite removal and 1.1-fold increase in nitrogen formation. The enriched biomass also showed remarkable increase in red biomass in the presence of hydrazine (Fig. S2). Amplification of planctomycetes specific and anammox specific regions of the 16S rRNA gene confirmed presence of anammox bacteria in the enriched biomass (Fig. S1).

3.2. Development of AOB-Anammox seed consortium for 1L SNAD type laboratory bioreactor

Inoculum prepared by mixing PF-AOB + PF- anammox enriched biomass in 1:5 ratio was seeded in 500 ml closed reactors containing synthetic effluent. Red colored growth appeared in the reactors after 90 days of incubation as shown in Fig. S3. Ammonia removing ability of the consortium was checked under anoxic conditions by keeping appropriate control as described in methods sec 2.2. As expected, decrease in ammonia and nitrite concentration was not observed in AOB and anammox control reactors (Table 1). PF-AOB experimental showed 66.9% utilization of ammonia, without accumulation of nitrite and nitrate, with 66.78% nitrogen gas formation implying that simultaneous nitrification and denitrification activity was being carried out by the enriched AOB biomass. AOB belonging to *Nitrosomonas* genus has been reported to show this activity under anoxic conditions (Bock et al., 1995; Schmidt and Bock, 1997). PF-anammox experimental biomass showed decrease in both ammonia and nitrite under anaerobic conditions, with higher nitrogen gas formation (1.06 times) compared to PF-AOB biomass (Table 1). The PF-AOB + PF-anammox system showed 88.73% decrease in ammonia without accumulating nitrite and nitrate (Table 1). Gas formed by PF-AOB + PF-anammox consortium was 1.09 times higher than PF-AOB but 0.98 times less than PF-anammox (Table 1). The results emphatically confirmed ammonia removing ability of PF-AOB + PF-anammox under anoxic conditions. Another sample N4, obtained from municipal wastewater treatment plant was also enriched similarly under aerobic and anaerobic conditions and tested for its ability to remove ammonia under anoxic conditions, showed results comparable to PF sample (data not shown). This N4-AOB + N4-anammox biomass was mixed in 1:1 proportion with PF-AOB + PF-anammox and PF-N4 AOB-anammox bacterial biomass was used as a seed for the 1L SNAD type reactor study.

Table 1
Anammox activity in different reactors using synthetic effluent.

Reactors	Ammonia Removed (mg/L)	Nitrite Removed (mg/L)	N_2 formed (%)
AOB control	–	–	–
Anammox control	–	–	–
PF AOB	79.8 ± 8.4	*	69.78 ± 3.5
PF anammox	107.8 ± 7.7	133.5 ± 2.67	74.1 ± 1.2
PF AOB + anammox	97.6 ± 4.39	*	72.36 ± 1.8

*Nitrite removed could not be determined accurately as nitrite was not added externally and nitrite produced by the AOB may be utilized by anammox bacteria and denitrifiers.

3.3. Ammonia removal performance of the SNAD type bioreactor from the effluent of a fertilizer company

An up flow, cylindrical SNAD type reactor with a working volume of 1L was used for the removal of ammonia from the effluent of a fertilizer industry, seeded with 100 ml PF-N4 AOB-anammox consortium (1.0 g/L VSS) developed as described above. Proximate composition of the effluent to be treated is depicted in Table 2. It had high NH_4^+-N concentration with COD/NH_4^+-N ratio 0.066 whereas the same in the SNAD processes reported earlier ranged from 0.2 to 0.87 (Chen et al., 2009; Wang et al., 2010; Lan et al., 2011; Daverey et al., 2012). The reactor was run in the batch mode for the first 15 days during which DO in the lower anoxic zone of the reactor reduced to 0.37 ppm and thereafter fluctuated between 0.1 and 0.4 ppm (conditions favorable for the growth of anammox bacteria) whereas in the upper oxic region of the reactor, DO fluctuated between 2.9 and 3.9 ppm (Fig. 2C). Nitrite present in the reactor, at any time was <100 mg/L, in the range that does not inhibit the anammox activity according to Strous et al. (1999). During the batch mode, VSS reached to 41 mg/L with 52.84% removal in the ammonia concentration (Fig. 2A and D). A significant reduction in the nitrite concentration was also observed during the batch mode (Fig. 2B). pH of the reactor reduced from 9.2 to 7.5 and thereafter fluctuated between pH 7.5 and 8.1 (Fig. 2D). The reactor was made continuous after 15 days with flow rate of 0.3 ml/min and HRT of 2.31 days. Inlet ammonia concentration varied from 700 to 800 ppm (Fig. 2A).The concentration of ammonia in the wastewater reduced to permissible limits (32.38 ppm) within 64 days and was stably maintained at that level for the next 60 days with a development of 11.5 g/L VSS. The concentration of inlet ammonia (725–760 ppm) and ammonia removed (700–750 ppm) became identical (Fig. 2A), leading to development of steady state in the reactor. COD concentrations in the reactor

Table 2
Measurement of the effluent parameters at the start and end of process.

Sr. No	Days	COD	NH_4^+-N (ppm)	NO_2^- -N (ppm)	NO_3^- -N (ppm)	DO (ppm)	Total Solid (mg/L)	Total Suspended Solids (mg/L)	Total Dissolved Solids (mg/L)	MLSS (mg/L)	MLVSS (mg/L)	SVI (ml/g)	pH	MLVSS: MLSS
1	0th	46.66	700–800	20–60	Not detectable	5.8	4300	300	4000	1.65	1.46	0	9.2	0.88
2	125th	24	26	5.05	Not detectable	0.39	4750	700	4050	212	114	14	7.5	0.536

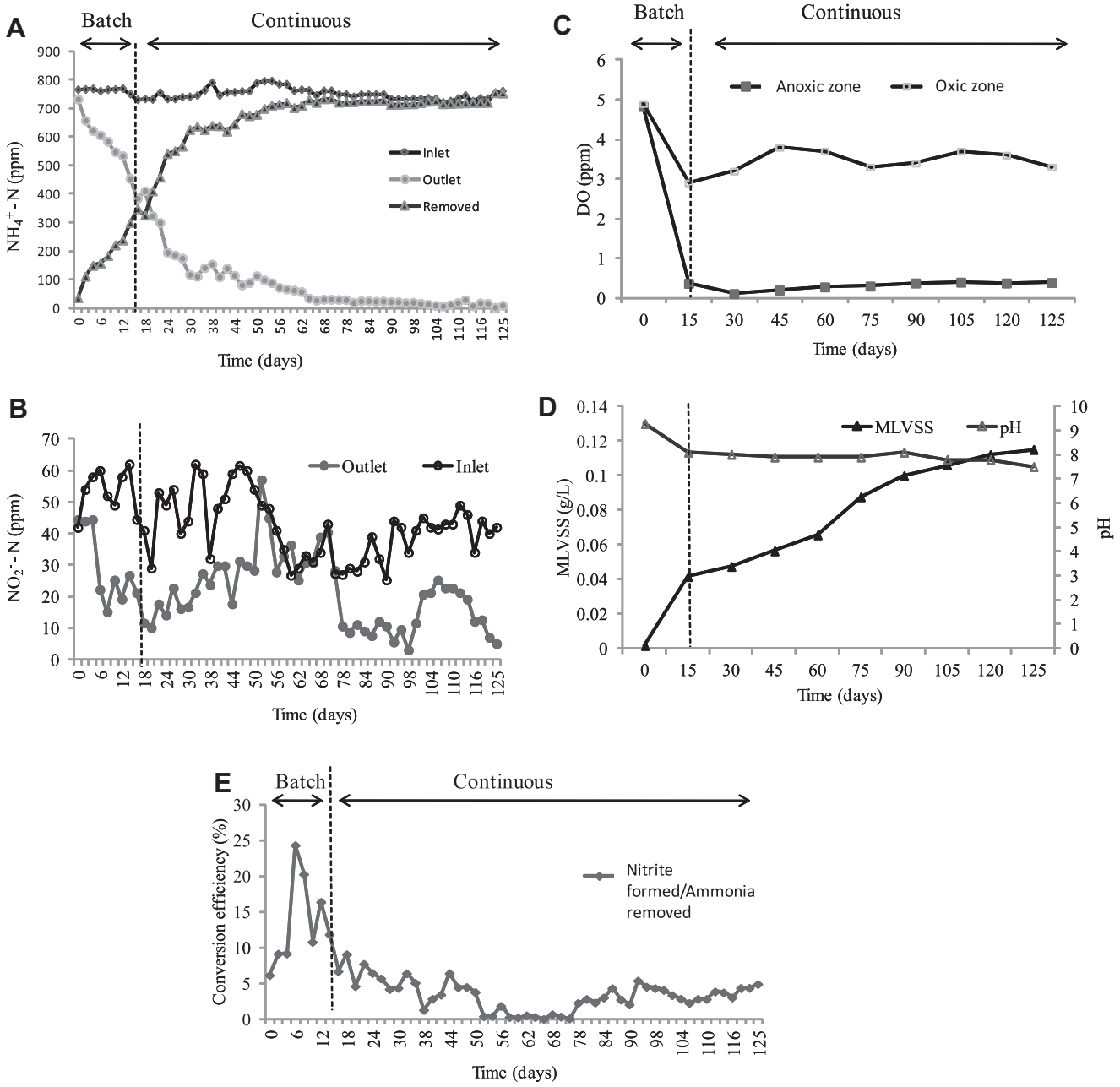

Fig. 2. Nitrogen removal performance and other parameters in the reactor during the process (A) Ammonia (B) Nitrite (C) Dissolved oxygen (D) pH and MLVSS (E) Ammonia conversion efficiency.

reduced to 24 mg/L at the end of the run. Ammonia conversion efficiency at the start of the reactor was higher than the theoretical value (Fig. 2E). Theoretically ammonia conversion efficiency has been reported to be 11% and presence of heterotrophic denitrifiers tend to reduce it from its theoretical value because of reduction of nitrate to N_2 (Daverey et al., 2012). On the 14th day it reached 11.8%, indicating optimum anammox activity in the reactor (Fig. 2E). The value of ammonia conversion efficiency fluctuated between 0.4 and 9.0% during the continuous mode (Fig. 2E). Between 52 and 74th day period, the process was run with ammonia conversion efficiency reaching near 0.4% to 0.02% suggestive of higher denitrifying activity than anammox activity (Fig. 2E).

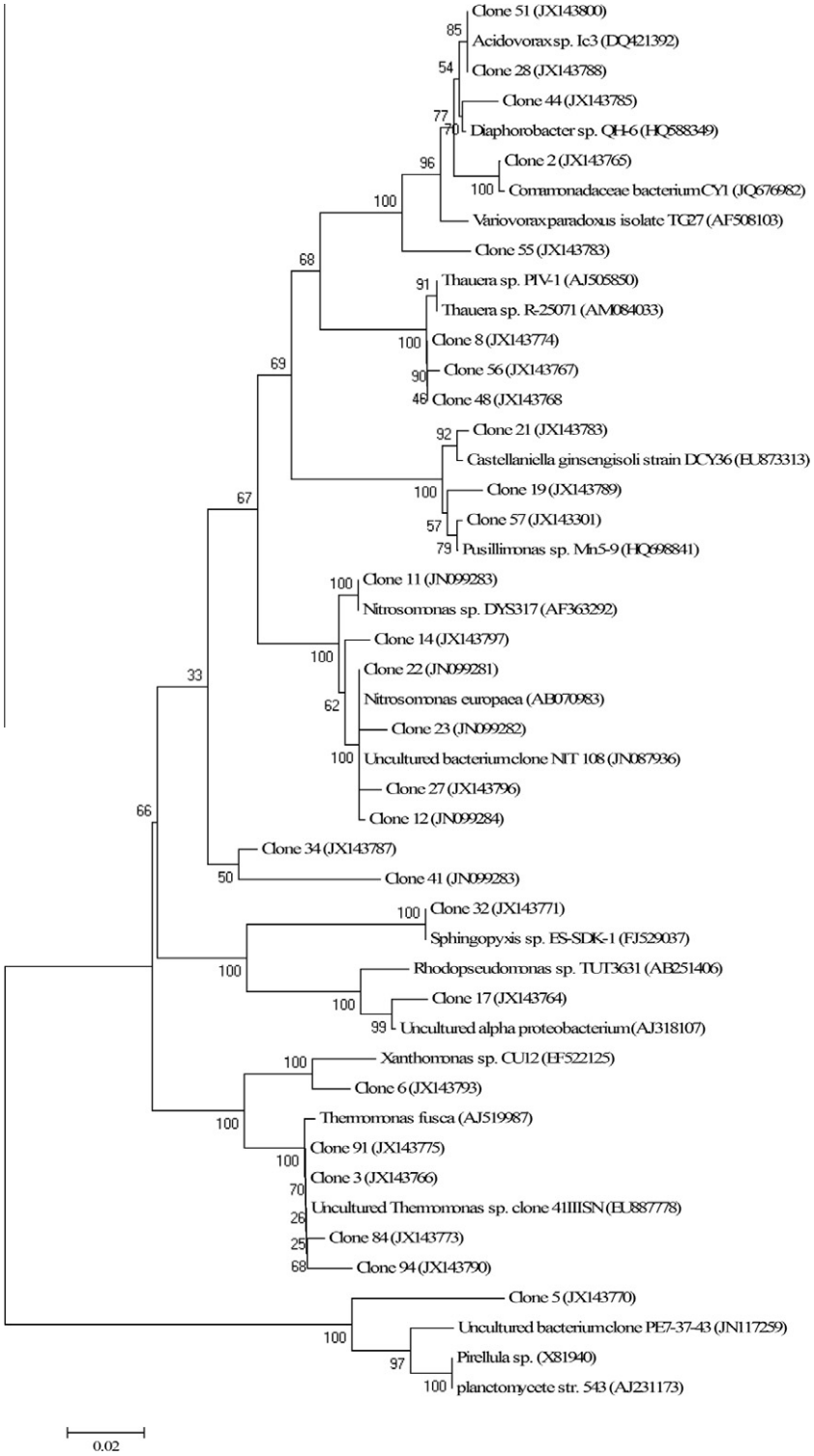

Fig. 3. Phylogenetic Neighbor-Joining tree showing relationship between the microorganisms present in the reactor based on the 16S rRNA gene sequences obtained using universal primers and anammox specific primers. Bar indicates 2% sequence divergence. Values shown next to the branches indicate bootstrap values. Acession numbers of the sequences are given in parenthesis.

Beyond this period, the efficiency steadily increased reaching 4.9% by the end of the reactor, indicating anammox activity along with denitrifying activity in the reactor (Fig. 2E). The overall efficiency of the process was similar to reactors designed earlier for the removal of ammonia using the SNAD process (Chen et al., 2009; Lan et al., 2011; Daverey et al., 2012).

3.4. Molecular analysis of the microorganisms carrying out ammonia oxidation

Visually red colored biomass developed at the base of the reactor and red colored film developed on the upper wall of the reactor by the end of the run. SEM of the biomass from the anoxic zone

Table 3
Quantification of the organisms present in the upper oxic and lower oxic zones of the reactor by Real-Time PCR.

Target gene	Size (bp)	Upper oxic zone of the SNAD Reactor		Lower anoxic zone of the SNAD Reactor	
		Gene copy No. per gram VSS	Ratio of gene copy No./16S rRNA	Gene copy No. per gram VSS	Ratio of gene copy No./16S rRNA
16S rRNA	102	$6.46 \times 10^9 \pm 6.15$ X 10^8	1.0	$1.31 \times 10^{11} \pm 2.5$ X 10^9	1.0
amoA	491	$3.96 \times 10^9 \pm 2.52$ X 10^9	0.612	$7.92 \times 10^8 \pm 3.97$ X 10^7	0.006
nirS	425	$6.39 \times 10^8 \pm 1.82$ X 10^8	0.099	$3.19 \times 10^{10} \pm 2.15$ X 10^9	0.244
nosZ	267	$3.35 \times 10^8 \pm 6.39$ X 10^7	0.052	$2.1 \times 10^{10} \pm 6.65$ X 10^9	0.161

showed dominance of coccoidal shaped cells forming aggregates with pear shaped cells and flagellated microorganisms (Fig. S4A, B and C). Budding pear and coccoidal cells in the anoxic zone of the reactor could be Planctomycetes (Fig. S4B, C, D marked by arrow). The observation was supported by the amplification of Planctomycetes specific (1350 bp) and anammox specific (750 bp) regions of 16S rRNA gene from the biomass obtained from lower zone (anoxic region) of the reactor (Fig. S5 A and B). Cloning and sequencing of the anammox specific gene revealed clones having similarity with Planctomycetes (93% identity).

28 distinct sequences were obtained by cloning 16S rRNA gene from both aerobic and anoxic zones of the reactor. Phylogenetic tree constructed by Neighbor-joining method using MEGA4.0 software (Fig. 3) which represents relationship between the nitrifiers, denitrifiers and Planctomycetes present in the reactor. Mainly these could be grouped as aerobic and anaerobic ammonia oxidizers, aerobic and anaerobic denitrifiers and bacteria capable of simultaneous nitrification and denitrification (Fig. 3). Specific enrichment for denitrifiers was not addressed in the study however they must have got enriched due to anoxic conditions and presence of SMP in the reactor. Coexistence of denitrifiers along with anammox and AOB sharing nutrient metabolites is well documented (Kindaichi et al., 2004; Xiao et al., 2008), hence presence of denitrifiers with AOB and anammox bacteria was expected.

Amongst the microorganisms identified, AOB exhibited maximum diversity and eight distinct species of AOB showing similarity to *Nitrosomonas* genus were identified. This observation was in congruence with the earlier report of Xiao et al. (2008), who showed higher diversity of AOB amongst coexisting nitrosifiers, denitrifiers and anammox bacteria in sequencing batch biofilms reactor treating ammonia rich landfill leachate. Denitrifiers identified belonged to alpha (*Rhodopseudomonas* sp.), beta (*Thauera* sp., *Pusillimonas* sp., *Acidovorax* sp., *Comammonas* sp.) and gamma (*Thermomonas fusca*, *Xanthomonas* sp.), proteobacteria. Key players in the treatment process observed in the present study were *Rhodospesudomonas* sp., *Diaphorobacter* sp., *Acidovorax* sp. and *Comamonas* sp. reported to carry out simultaneous heterotrophic nitrification–denitrification (Satoh et al., 2006; Khardenavis et al., 2007; Heylen et al., 2008; Xiao et al., 2008). High temperature (30 °C), high flow rate and low dissolved oxygen concentration prevalent in the reactor were not favorable for the growth of nitrite oxidizing bacteria (NOB) as reported by Jianlong and Ning (2004) and therefore led to the washout of NOB.

Quantification of biomass carried out from upper (oxic) and lower (anoxic) regions of the bioreactor using RT-PCR with group specific genes revealed predominance of AOB (carried out by *amoA* gene amplification) (Fig. S5E) in the upper region (61.2%) whereas just 6% of the total population belonged to AOB in the anoxic lower region (Table 3). Denitrifiers (quantified using *nir*S and *nos*Z genes) constituted 10% of the total population in the upper oxic layer and 22% in the lower anoxic layer of the reactor (Table 3). The results suggested dominance of AOB in the upper oxic region of the reactor where as lower anoxic zone of the reactor, showed dominance of neither AOB (6%) nor denitrifiers (22%). Anammox may constitute major population in this part of the reactor as evidenced by the detection of uncultured Planctomycetes (93% identity) in the biomass amplified using Planctomycetes and anammox specific primers (Fig. S5A and B). The bacteria identified in the system showed less similarity with the other reported members of this group and hence may be a novel organism belonging to Planctomycetes. However, this group of bacteria were not quantified due to the presence of nonspecific amplicons obtained along with the required 750 bp amplicon. Another proof for the presence of Planctomycetes in the lower zone of the reactor was given by SEM showing presence of budding coccoidal cells (Fig. S4D), a characteristic for this group of organisms.

The bacterial community developed in the reactor could remove ammonia with high efficiency during the process. Oxidation of ammonia to nitrite in the upper portion of the reactor could mainly be contributed by AOB followed by aerobic heterotrophic nitrifier. Nitrite in the lower anoxic zone of the reactor would be utilized either by anaerobic denitrifiers or anammox bacteria. Low COD content in the reactor would favor growth of anammox bacteria capable of autotrophic growth as compared to denitrifiers. The observation justified Real-Time PCR results showing only 22% population in the lower layer belonged to denitrifiers (Table 3). However, lack of quantitation data for anammox bacteria failed to conclude this result. Bacteria capable of anammox activity in the reactor were mainly *Planctomycetes* sp. and *Nitrosomonas* sp. (Fig. 3) as these organisms have been shown to exhibit anammox activity (Schmidt and Bock 1997; Strous et al., 1999). The molecular phylogenetic analysis of the biomass from the reactor revealed coexistence of AOB, heterotrophic nitrifiers, denitrifiers and anammox bacteria, also reported earlier by Xiao et al. (2008) and Kumar and Lin (2010).

An adequate balance between the different types of bacteria is required in activated sludge systems to have good settling ability of the sludge, such that low suspended solids level prevails in the effluent. Sludge volume index (SVI), provides just macroscopic evaluation of the activated sludge; therefore, microscopic characteristic of the sludge has been recently used where filamentous bacterial content in microbial aggregates is measured (Mesquita et al., 2009). Most of the denitrifiers detected in the present study (Fig. S4) are reported to be flagellated (Heylen et al., 2008). As flagellated bacteria are reported to assist in forming microbial aggregates (Sjoblad et al., 1985) the filamentous bacteria observed in the study may have facilitated formation of aggregates thereby preventing the biomass from getting washed out from the reactor. Food to microbe ratio was found to be 0.62 day^{-1} indicating endogenous growth of microorganisms which is reported to have better settling ability and is more stable in nature (Rao and Datta, 1987).

4. Conclusion

A highly efficient and stable system was thus developed for the treatment of ammonia laden effluent from a fertilizer industry without addition of external carbon or nitrite source and was operated continuously for 125 days at 30 °C where in 98.9% ammonia removal as achieved without accumulation of detectable levels of

nitrite or nitrate. Coexistence of nitrosifiers, anammox bacteria and denitrifiers was confirmed in the reactor. Molecular phylogenetic analysis of the biomass generated revealed dominance of AOB in the upper oxic zone of the reactor while that of anammox followed by denitrifiers dominated in the lower anoxic zone of the reactor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.12.066.

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