2.1. Introduction

In natural environments nitrate is present in minimal amount and a high competition exists for its utilization among organisms including plants (Kaye and Hart, 1997). In contrast effluents of certain industries producing chemicals, fertilizers, explosives, etc. contain very high amounts of nitrate with more than 1000 mg L^{-1} (Constantin & Fick 1997; Glass & Silverstein 1999; Zala et al. 1999; Fernandez-Nava et al. 2008]. According to the idea proposed by Becking, which states "everything is everywhere but the environment selects" (Fuhrman, 2009), it is interesting to investigate the kind of bacteria selected in these anthropogenically created environments. Phillipot and Hallin, (2005) proposed denitrifying bacteria as the model microbial community to understand the link between diversity and activity. Also, in order to remove the high concentration of nitrogenous oxides efficiently, it is necessary to understand the abundance, structure and activity of the denitrifying bacteria present in the sludge.

The denitrifying apparatus is distributed widely among the bacteria (Zumft, 1997). Most of the culture independent studies on abundance and diversity of denitrifying bacteria in activated sludge is reported using only FISH probe-designed populations (Ginige et al., 2007; Morgan-Sagastume et al., 2008; Thomsen et al., 2004). However, some denitrifying bacteria are truncated in mostly nitrate and nitrous oxide reduction and are unable to denitrify completely (Focht, 1982; Zumft, 1997). The reduction rate of nitrogenous oxides during denitrification process varies in different species and even strains (Betlach & Tiedje 1981; Carlson & Ingraham 1983). Environmental factors are known to significantly affect the denitrification process (Glass & Silverstein 1998; Thomsen et al. 1994; Tiedje 1994). However, the prominent limiting conditions for efficient denitrification to occur in reactors are high nitrate concentrations in wastewaters (Clarkson et al. 1991; Dhamole et al. 2007; Francis & Mankin 1977) and the accumulation of nitrite (Glass & Silverstein 1998; Rijn et al. 1996). Francis & Mankin (1977) observed nitrate concentrations above 6 kg L^{-1} to inhibit nitrate reduction. Almeida et al. (1995b) observed the dependency of nitrite reduction on the nitrate concentrations in *Pseudomonas fluorescens*. Low nitrite reduction than nitrate reduction causes build-up of nitrite and the accumulated nitrite limits denitrification and growth (Almeida et al. 1995a). Almeida et al. (1995b) also showed that nitrite concentration above 130 mg N L^{-1} limit growth of *P. fluorescens*. High nitrate concentrations possibly limit denitrification because of their chaotropic effect and also the denitrification intermediates,

nitrite and nitric oxide generate reactive nitrogen species (RNS) which are harmful to cells (Poole 2005). Hence, bacteria which can tolerate and denitrify efficiently even at high nitrogenous oxide concentrations are a necessity in nitrate removing reactors.

This chapter assess the denitrifying bacterial composition in different activated sludge samples by both culture dependent and independent means. The functional genes *nir*S and *nos*Z are targeted in this study to analyse the abundance, and *nos*Z gene library constructed to study the diversity of denitrifying bacteria by culture independent method. Further denitrification activity of selected isolates was studied quantitatively.

2.2. Materials and Methods

2.2.1. Activated sludge samples.

The different activated sludge samples designated as, DaS, GS, WL and NL were used in this study. Their sources and the characteristics are listed in Table 2.1. Total organic carbon (TOC) in the sludge was measured in TOC analyser (Shimadzu corp., Japan). Suspended and dissolved solids were measured by standard methods, and different cations (Table 2.1) were measured by inductively coupled plasma spectrometer (ICP) (Optima 3300 RL, Perkin Elmer). All these characteristics of sludge samples were analysed at Sophisticated Instumentation Centre for Applied Research and Testing (SICART, Gujarat).

2.2.2. Most Probable number (MPN) of Denitrifiers

To estimate the density of culturable denitrifiers in activated sludge by MPN method, a 10 fold dilution series of each sludge samples were prepared in the N-saline. 0.5 ml of each dilution was inoculated in respective peptone nitrate broth (PNB) tubes. After 72 hours of incubation, the 2nd highest 10 fold dilutions which showed bubble formation in Durham's tube was selected as a starting point for 6 replicate 10 fold dilution series. The MPN was calculated by the MPN calculator version 4.04 developed by USEPA (http://www.epa.gov/nerlcwww/other.html). Presence of denitrifiers was assessed by nitrate reduction test, where bubble formation in the Durham's tube indicates presence of denitrifier, and formation of reddish pink colour after addition of α-naphthylamine and sulphanilic acid indicates the presence of nitrite. If no colour develops, zinc dust is added to confirm the presence of nitrate which is indicated by the formation of pink colour determining the absence of nitrate utilization.

Table 2.1. Characteristics of the activated sludge samples

Sample Designation	DaS	GS	WL	NL
Source	Denitrifying reactor of a Fertilizer factory	Aeration tank of fertilizer factory	Aeration tank of municipal Effluent treatment plant	Aeration tank of Common effluent treatment plant in an industrial area
TOC (mg L^{-1})	1020.4	1055.6	687.6	910.8
Suspended solids (mg L^{-1})	64	10	151	248
Total dissolved solids (mg L^{-1})	1330	2350	2500	8570
Calcium (ppm)	2810	59.086	285.63	98.904
Magnesium (ppm)	32.266	14.036	71.509	45.292
Iron (ppm)	16.572	5.084	10.729	26.625
Copper (ppm)	BDL	BDL	0.536	0.333
Nitrogenous compounds * (ppm)	400-600 (NO_3-N)	-	-	30-45 (NH_3-N)

BDL = Below detection limit

* = As provided by the plant operators

2.2.3. Isolation, screening and identification of denitrifying bacteria

Isolation of denitrifiers from sludge samples was done using three different media, peptone nitrate medium (PNB), G2M11 and G4M3 (Heylen et al. 2006) (Refer the appendix in section 2.6 for composition of the media). For each sludge sample, serial dilutions were made up to $10^{-4} - 10^{-6}$ and from each dilution 0.1 ml was plated on respective medium, which was incubated at 30°C for 7 days. Isolated colonies with different colony morphologies were picked and inoculated in PNB, which was further screened by nitrate reduction test. Identification of the isolates was done by partial sequencing the 16S rRNA gene amplicon. The 16S rRNA gene was amplified using the primers 27F and 1541R (Table 2.2) from the genomic DNA of the isolated denitrifying bacteria. Sequencing of the PCR amplicon was done in ABI *3730xl* DNA Analyzer at services provided by XcelrisLabs. Primers used for sequencing of the PCR product were 27f, 1541r and also 341f, 534r and 1107r (Table 2.2). Overlapping sequences were analyzed in Mega 4.1 software (Kumar et al. 2008) and corrected to obtain a larger fragment of the 16S rRNA gene.

2.2.4. Amplified Ribosomal DNA Restriction analysis (ARDRA)

A nearly 1500 bp amplicon of the 16S rRNA gene was generated by PCR from the genomic DNA by using the primers, 27f and 1541r (Table 2.2). ARDRA was performed using the restriction enzymes *Alu*I and *Rsa*I (Fermentas) separately. The digested amplicons were run on 8% polyacrylamide gels and silver stained for further analysis. ARDRA profiles were manually analysed by the binary data for presence or absence of bands were computed. The dendrogram was plotted using NTSys software (Exeter software, NY) with UPGMA method.

2.2.5. Denitrification Experiments

2.2.5.1. The isolates were grown in PNB for 18 or 24 hours and centrifuged at 10000 rpm for 7 minutes. The cell pellet was washed twice with phosphate buffered saline (PBS) and resuspended in PBS with absorbance of 0.5 set at 600 nm for all the isolates and 2 ml of this was inoculated in 250 ml Erlenmeyer flasks containing 100 ml MM2 medium. Denitrification experiments were performed in MM2 medium consisting of Sodium succinate 7.9 g, $MgSO_4.7\ H_2O$ 0.2 g, K_2HPO_4 0.2 g, $FeSO_4.7\ H_2O$ 0.05 g, $CaCl_2.2\ H_2O$

0.02 g, $MnCl_2.4\ H_2O$ 0.002 g, $NaMoO_4.2\ H_2O$ 0.001 g, KNO_3 1.0 g, Yeast extract 1.0 g, pH 7.0, Distilled Water 1000 ml. Incubation of the cultures was done at 30° C in static conditions for maintenance of anoxia.

2.2.5.2. Nitrate reduction, nitrite formation and the relative rates (RR) of nitrate and nitrite reduction was calculated according to Dhamole et al. (2008), where relative rate is described as

$$RR = \frac{K_{NO_3}}{K_{NO_3} - k_{NO_2}}$$

Where, K_{NO_3} = rate of nitrate reduction, k_{NO_2} = rate of nitrite formation in presence of nitrate

2.2.5.3. Nitrate and nitrite reduction rates by resting cell suspension were performed as follows. Cells grown for 24 hours in peptone nitrate broth were harvested by centrifugation at 10000 rpm for 5 minutes, washed twice with phosphate buffered saline (pH 7.4) and resuspended in PBS. Succinate and nitrate were added as electron donor and acceptor respectively and the reduction of nitrate and nitrite were estimated for 20 minutes.

2.2.5.4. Denitrification experiments at high nitrates were done in 24 well microtiter plates using 3 ml MM2 medium with C/N ratio of 5.0 maintained at appropriate nitrate concentration. High nitrate concentrations were used in this experiment from 0.1 M to 2 M. 10^8 cells ml^{-1} was used as an inoculum and incubated for 12 hours after which CFU, nitrate and nitrite was measured.

2.2.6. Analytical methods

2.2.6.1. The brucine sulfate method was used to determine nitrate according to Jenkins and Medsker, (1964).

2.2.6.2. Nitrite was estimated according to APHA, (1995).

2.2.6.3. Lowry et al. (1951) method was used for protein estimation, with bovine serum albumin as a standard.

2.2.6.4. Production of nitrous oxide by the culture was analysed by withdrawing the sample from the headspace of the test tube in which the isolate was grown for 18 hours in

PNB medium, and N_2O was measured by gas chromatography (Perkin Elmer, Auto system XL) with an electron capture detector.

Table 2.2. PCR Primers used in the study

Primer name	Sequence 5'-3'	Reference
*nos*ZF1 (1184-1203)	WCSYTGTTCMTCGACAGCCAG	Henry et al. 2006
*nos*ZF2 (1617-1640)	CGCRACGGCAASAAGGTSMSSGT	Henry et al. 2006
*nos*ZR2 (1864-1884)	CAKRTGCAKSGCRTGGCAGAA	Henry et al. 2006
*nir*Scd3aF (916-935)	GTS AAC GTS AAG GAR ACS GG	Throback et al. 2004
*nir*SR3cd (1322-1341)	GAS TTC GGR TGS GTC TTG A	Throback et al. 2004
16S 27F (27-48)	GAGAGTTTGATCCTGGCTCAG	Pillai et al. 2008
16S 340F (340-357)	CCTACGGGAGGCAGCAGA	Pillai et al. 2008
16S 534R (518-534)	ATTACCGCGGCTGCTGG	Pillai et al. 2008
16S 1541R (1522-1541)	AAGGAGGTGATCCAGCCGC	Pillai et al. 2008

* Parenthesis = position in the respective genes

2.2.7. DNA extraction from sludge samples

Extraction of genomic DNA from sludge was done according to Zhou et al. (1996). Extraction buffer consisted of 100mM 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 and 1% polyvinylpyrrolidone (PVP). 15 ml of activated sludge sample was mixed with extraction buffer and incubated at 30°C for 30 minutes in shaking condition. Sodium dodecyl sulphate (SDS) was added to this mixture at 2% final volume and incubated for two

hours at 65°C with intermittent shaking. Centrifugation was done for 10 minutes and phenol-chloroform extraction of the DNA was carried out. The aqueous phase was precipitated with 0.6 volume of isopropanol at room temperature and the crude nucleic acids pellet obtained by centrifugation was washed with cold 70% ethanol, and resuspended in Tris EDTA buffer. The crude DNA was purified by running in a 8% agarose gel containing 2% polyvinylpyrrolidone (Young et al. 1993) and the elution was performed according to the freeze-squeeze method of Gaastra and Jorgensen (1984).

2.2.8. Quantitative real time PCR

The genes, *nosZ*, *nir*S and 16S were quantified with SYBR green master mix (Applied Biosystems) in a 20µl reaction system in StepOne real time PCR system (Applied Biosystem) by the standard curve method. Primer sequences used for *nosZ* (*nosZF2* and *nosZR2*), *nir*S and 16S rRNA(16S 340F and 16S 534R) gene quantitation and their references are listed in Table 2.2. Standard curves were constructed with the plasmid containing insert of respective gene. *nir*S insert was kindly provided by Prof. Nico Boon, Ghent University. *nosZ* and 16S rRNA gene inserts were constructed in the lab. PCR conditions for *nosZ* were according to Henry et al. (2006) with a touchdown PCR. Conditions for PCR of 16S rRNA gene were, an initial denaturation temperature of 95°C for 10 mins, 40 cycles of PCR at 95°C for 15s, 30s at 62°C, and 72°C for 30s. The same conditions were followed for *nir*S, with annealing temperature at 58°C. No Template Controls (NTC) was run for each reaction in triplicates. The samples were spiked with plasmid DNA and quantified to check the potential presence of inhibitors.

2.2.9. Clone library construction and RFLP analysis

The primers used for generating amplicons by PCR for cloning was the *nosZF1* and *nosZF2* (Table 2.2) generating a nearly 700bp amplicon. Around four to five separate PCR reactions were pooled and *nosZ* clone library was constructed from the fertilizer factory (samples DaS and GS) activated sludge samples using InsTAclone cloning kit (Fermentas) according to manufacturer's instructions. Plasmid DNA was extracted from the putative clones by the alkaline lysis method (Maniatis et al. 1982) and positive clones were confirmed by PCR. The PCR amplicons were digested with *Alu*I and *Rsa*I enzymes seperately (Fermentas), run on 8% polyacrylamide gel, which was later silver stained to

view the bands. One or two representative *nosZ* clones from each RFLP OTU were randomly chosen and the *nosZ* amplicon was sequenced in ABI *3730xl* DNA Analyzer at services provided by XcelrisLabs. Rarefaction analysis of the RFLP data was done using Analytic Rarefaction version 1.3 software (http://www.uga.edu/strata/software/).

2.2.10. Nucleotide sequence accession numbers

Gen bank accession numbers of the partial 16S rRNA gene sequences are HQ625210, HQ625212 to HQ625213, HQ625218 to HQ62521, HQ625224, and HQ625226 to HQ625228. *nosZ* sequences listed in this study are HQ625230-31, HQ625233, HQ625235- 36, HQ625238- 44, HQ625246-50, and HQ625258.

2.3. Results

2.3.1. Abundance of denitrifying bacteria in the activated sludge samples

2.3.1.1. Abundance of culturable denitrifying bacteria

Most probable number was used to assess the abundance of culturable bacteria in the different activated sludge samples. The formation of bubble in Durham's tube was considered as an evidence for the presence of denitrifiers. Sample DaS showed a density of 2.28 X 10^8 per ml, whereas sample GS showed the highest MPN of 2.8 X 10^9 (Fig. 2.1). The MPN of samples WL and NL was found to be 1.39 X 10^9 and 5.6 X 10^8 respectively (Fig. 2.1).

2.3.1.2. Abundance of total denitrifying bacteria

Abundance of denitrifiers, including culturable and unculturable were assessed by targeting functional genes, *nosZ* and *nir*S of denitrification pathway and 16S ribosomal gene fragment as a target for estimating total eubacterial abundance. The plasmids containing respective gene inserts were used to plot standard curve and PCR efficiency for all three genes was between 90-103% and the slope of the standard curve between -3.2 to -3.6 with R^2 value above 0.97. The melt curve is shown in Fig. A1 in the appendix (Section 2.6). Calculated results after the samples were spiked with plasmid DNA showed the absence of PCR inhibitors. As shown in Fig. 2.2, The total bacterial density varied between 1.8 X 10^9 and 1.4 X 10^{10} copy number 16S rRNA gene per ml of sludge,

in all the samples, with DaS showing a significantly ($p<0.05$) higher copy number. *nosZ* gene copy number per ml sludge varied between 2.5 X 10^4 and 1.5 X 10^6 in all the samples, however the DaS sample showed higher abundance ($p<0.05$) of *nosZ* gene than other samples. *nir*S gene copy number in all the samples was in the range 1.4 X 10^4 to 2.2 X 10^5. Interestingly, the NL sample had higher amount of *nir*S than *nosZ* gene with a *nosZ*/*nir*S ratio of 0.531.

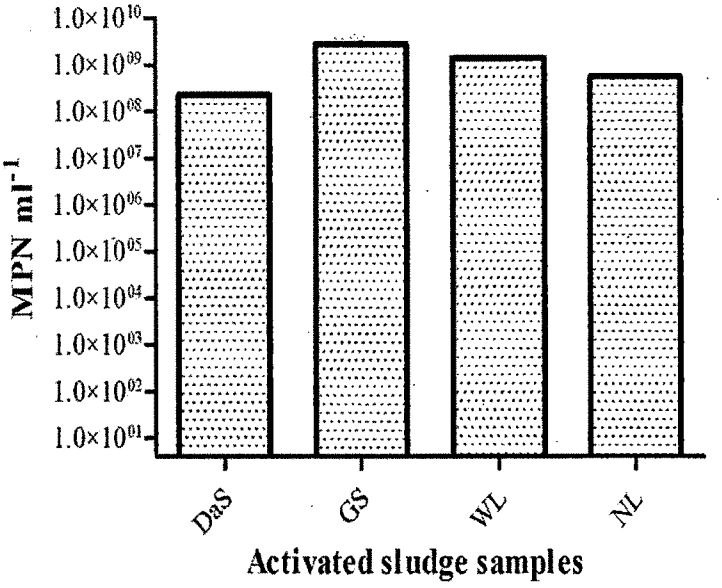

Fig. 2.1. Most probable number of the denitrifying bacteria in different activated sludge samples

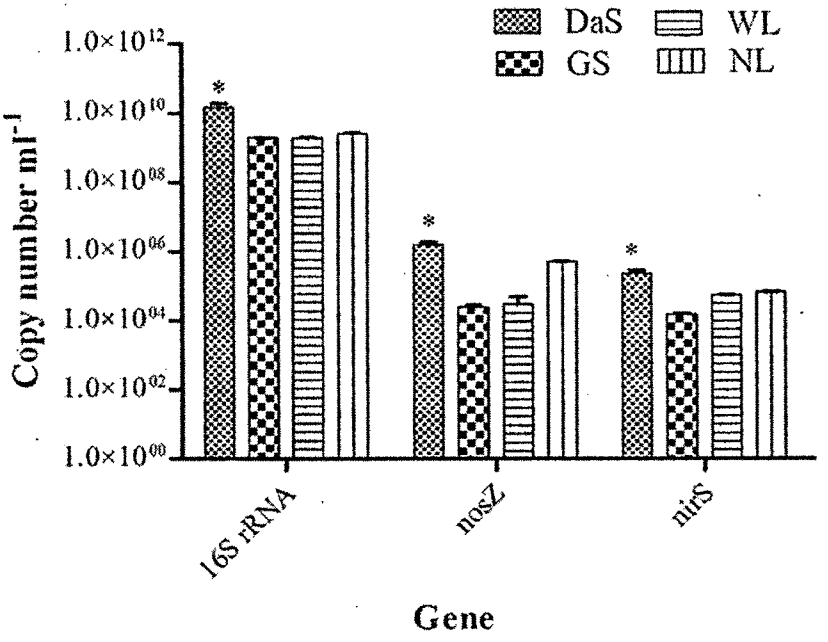

Fig. 2.2. Abundance of 16S rRNA, *nosZ* and *nirS* genes in the different sludge samples determined by quantitative real-time PCR. Error bars represent the standard deviation. One way ANOVA with Tukey test was used to determine significant differences.
* denotes statistical significance with $p<0.05$

Table 2.3. Percentage and ratio of the denitrification genes in the activated sludge samples

Sl. no.	Sample	% *nosZ*	% *nirS*	*nosZ*/*nirS*
1	Das	0.01	0.001	6.850
2	GS	0.001	0.0007	1.716
3	WL	0.001	0.0029	0.531
4	NL	0.02	0.0027	7.560

2.3.2. Diversity of Denitrifying bacteria in different activated sludge samples

2.3.2.1. Cultivation of denitrifying bacteria

A total of 116 cultures with different colony morphology were isolated from the sludge samples using PNB, G2M11 and G4M3 media. The cultures were screened further for nitrate reducers, truncated and complete denitrifiers by the nitrate reduction test (Table 2.4). Out of 51 cultures capable of utilizing nitrogenous oxides, 29 were nitrate reducers, which could reduce nitrate upto nitrite. A total of 22 isolates were found capable of denitrification, out of which 12 cultures could denitrify from nitrate (henceforth called as nitrate denitrifiers), 2 could reduce both nitrate and nitrite and remaining 8 were able to denitrify only from nitrite (henceforth called as nitrite denitrifiers) (Table 2.4). The nitrate denitrifiers were able to denitrify from nitrate, but unable to reduce when nitrite was provided in absence of nitrate.

2.3.2.2. Amplified Ribosomal DNA Restriction Analysis (ARDRA) of the isolated denitrifying bacteria

The diversity of the denitrifying isolates was investigated by performing the ARDRA using *Rsa*I and *Alu*I restriction enzymes which resulted in eight different ARDRA profiles (Fig. 2.2, Fig. A2 & A3 of section 2.6). Among the eight ARDRA OTU patterns, OTU 7 was the most abundant which was also distributed in all the sludge samples, while OTU 4 was unique to the NL sludge sample. Other OTUs contained only one member per ARDRA group. Partial sequencing of 16S rRNA gene from a representative isolate of each ARDRA OTU was aligned with GenBank database using BLAST program (Table 2.5). The OTU 7 aligned with more than 97% similarity with *Alcaligenes faecalis*, OTU 1 had similarity of 100% to *Brevundimonas diminuta*, which belong to alpha proteobacteria and OTU 3 of the ARDRA pattern with the isolate WL3b belonged to *Comamomas* sp with 99% similarity. Remaining OTUs of ARDRA belonged to different species of *Pseudomonas* with similarity ranging from 98-100%.

Table 2.4. Nitrate reduction test results of the isolates

Sample	Nitrate denitrifiers	Nitrite denitrifiers	NO_3 reducers to NO_2
Das	Das12a, Das10b, PDas4	Das1b, Das2b	Das1a, Das5a, Das8a, Das9a, Das11a, Das3b, Das4b, Das8b, Das9b, Das11b, PDas1
GS	PGS8, PGS9	GS1a, GS2a, GS3a, GS4a, GS5a, GS1b	GS6a, GS7a, GS8a, GS9a, GS5b, GS7b, PGS2, PGS4, PGS6
NL	NL2a, NL5a, NL10a, PNL4, PNL6	NL2a, NL5a	NL6a, NL8a, NL2b, PNL5
WL	WL3b, PWL4, PWL9, PWL11	-	WL1a, WL2b, PWL3, PWL6, PWL8

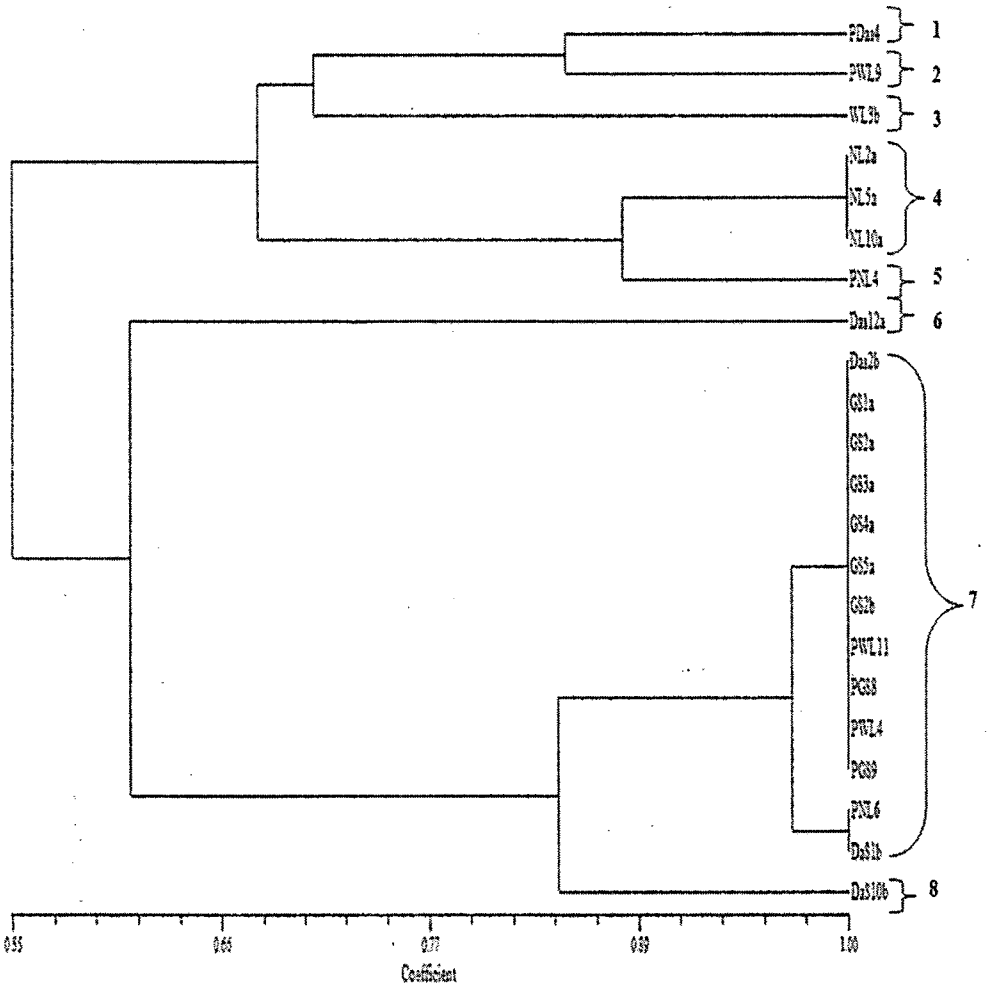

Fig. 2.3. Phylogenetic similarity of the isolated denitrifiers constructed based on the UPGMA method from binary data of ARDRA profiles obtained by digestion with *Rsa*I and *Alu*I enzymes.

2.3.2.3. *nosZ* gene diversity in the fertilizer factory activated sludge samples

The DaS and GS activated sludge samples from fertilizer factories were further selected for construction of the *nosZ* gene fragment library. The *nosZ* gene amplified from the gDNA of the fertilizer factory sludge samples was cloned and the amplicons obtained from these clones were analyzed by RFLP technique (See section 2.6 appendix Fig. A4 & A5). Rarefaction analysis showed that the sample DaS was nearly reaching the asymptote, whereas increasing the clone number would have shown more diversity in the GS sample (Fig. 2.4). *nosZ* gene diversity, as analysed by Simpson's reciprocal and S-W index was also higher in GS sample than DaS (Table 2.6). The translated protein sequence of OTU 3, which was abundant in DaS sample (Fig. 2.5), clustered with nitrous oxide reductase sequence of *Acidovorax* sp. in the phylogenetic tree (Fig. 2.6), and OTU 11 found to be abundant in the clones of sample GS (Fig. 2.5) was also positioned in cluster I (Fig. 2.6). The OTUs 3, 4, 6 and 9 were distributed in both the samples (Fig. 2.5), but the OTUs 1, 2, 5, 7, 8 and 10 were unique to DaS sample, while the OTUs 12 to 18 were unique to the GS sample. The OTUs 7 and 10 clustered with *Thiobacillus denitrificans*. The cluster I had most of the OTUs, but the cluster II contained only OTU 5 with *Pseudomonas stutzeri* and other bacteria from gamma proteobacteria. Bacteria belonging to alpha proteobacteria were positioned in cluster III with the remaining 6 OTUs namely 9, 12, 13, 14 15 and 18. The GS sample had clones evenly distributed between alpha and betaproteobacteria without a single clone from gammaproteobacteria. However the numerically dominant was found to belong to the betaproteobacterial group (Fig. 2.5 & 2.6).

Table 2.5. Determination of the denitrifying bacterial isolates by partial 16S rRNA gene sequence similarity

Isolate name	Sequence length (bp)	Similar species	Percent similarity	Accession no. of the similar organism
DaS1b	699	*Alcaligenes faecalis*	97	AY54838
DaS10b	681	*Pseudomonas pseudoalcaligenes*	98	AB276371
DaS12a	967	*Pseudomonas mendocina*	100	DQ178224
PDaS4	729	*Brevundimonas diminuta*	100	AB167225
PGS8	600	*Alcaligenes faecalis*	99	AF155147
NL5a	748	*Pseudomonas stutzeri*	99	AJ312165
PNL4	538	*Pseudomonas fluorescens*	99	FJ896114
PWL9	762	*Pseudomonas sp.*	99	GQ241352
WL3b	732	*Comamonas sp.*	99	DQ851179

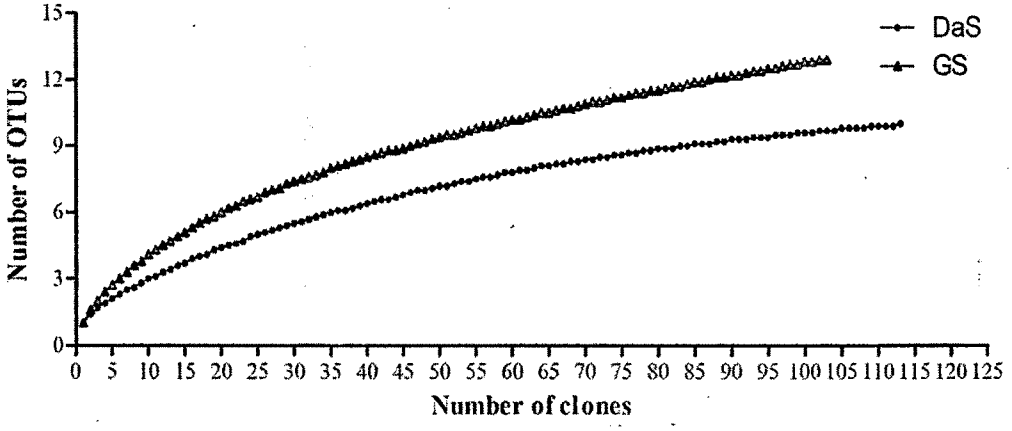

Fig. 2.4. Rarefaction analysis of the *nosZ* clones from DaS and GS activated sludge samples

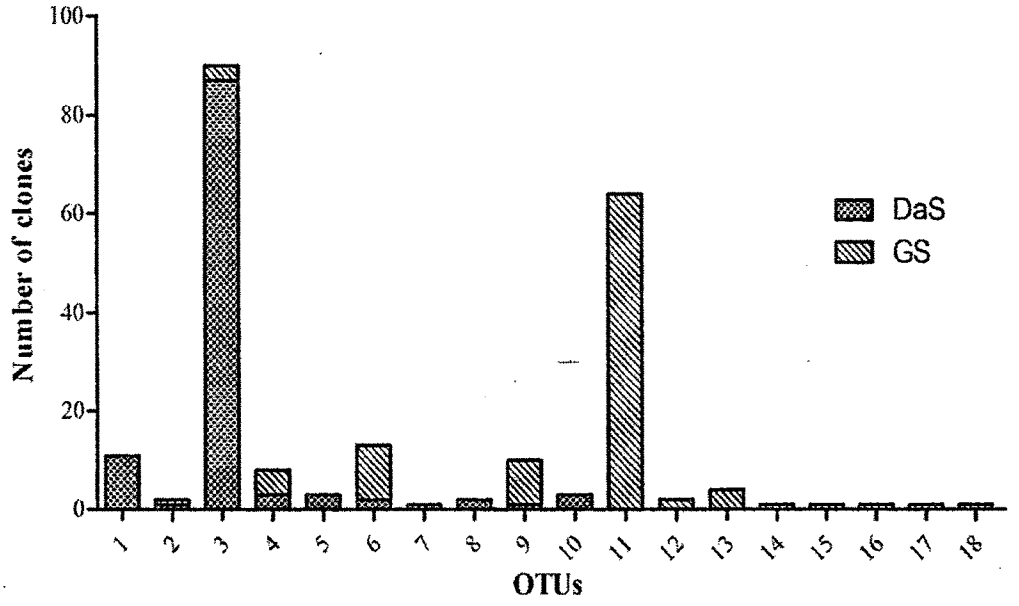

Fig. 2.5. Distribution of *nosZ* OTU's in DaS and GS activated sludge samples

2.3.3. Denitrification pattern of the denitrifying bacterial isolates

2.3.3.1. Denitrification between *Paracoccus* sp.W1b and *Diaphorobacter* sp. D1

Two cultures, W1b and D1 previously isolated in PNB from denitrifying reactor sludge sample (DaS) of a different batch than the one listed in table 2.1 were characterized for denitrification. The presence of nitrous oxide in the headspace of the growth tube confirmed them to be denitrifiers (Fig. 2.7). Isolate W1b was observed to be a Gram negative cocci and BLAST results of 1094 bp partial 16S rRNA gene showed 99% identity with *Paracoccus* species and isolate D1, a gram negative rod, showed 98% similarity with *Diaphorobacter* species with a 1437 bp partial 16S rRNA gene sequence. Phylogenetic positions of both the isolates are shown in Fig. 2.8, where the isolates W1b and D1 clustered with *Paracoccus* sp. and *Diaphorobacter* sp. respectively.

The nitrate reduction and nitrite accumulation of both isolates were monitored in batch mode upto 36 hours in 250ml Erlenmeyer flasks under static condition. Nitrate was reduced to 1.65 mM from 12.0 mM in 36 hours by *Paracoccus* sp. W1b with negligible amount of nitrite accumulation at the 6^{th} hour interval (Fig. 2.9). *Diaphorobacter* sp. D1 could reduce nitrate from 12.0 mM to 0.89 mM in 36 hours, whereas a substantial amount of nitrite was accumulated in the medium (Fig. 2.9). Accumulation of nitrite

increased to around 7.8 mM in 24 hours and decreased to 2.1 mM at 36th hour. The calculated relative rates (RR) was higher in *Diaphorobacter* sp. D1 than *Paracoccus* sp. W1b (Table 2.7) suggesting that the rate differences between nitrate and nitrite reduction is the possible reason for nitrite accumulation.

Reduction rates of nitrogenous oxides were further estimated in the resting-state of these two cultures. The nitrate reduction rate was 1.5 folds higher than the nitrite reduction rate in *Diaphorobacter* sp. D1, whereas the nitrate to nitrite reduction rate ratio of *Paracoccus* sp. W1b was found to be 1.0 (Table 2.8), suggesting similar reduction rates of nitrate and nitrite. However, influence of increasing nitrate concentrations on reduction of nitrite showed increased accumulation of nitrite in *Diaphorobacter* sp. D1 whereas nitrite build-up was not observed in *Paracoccus* sp. W1b with increase in nitrate concentration (Fig. 2.10). The nitrate reduction rate at increasing nitrate concentrations is shown in Table 2.9, where the nitrate reduction rate increased substantially with 10 mM nitrate.

2.3.3.2. Influence of carbon to nitrogen (C/N) ratio on denitrification by the isolates

Amount of carbon source plays a crucial role in reducing nitrates in denitrifying reactors, so the optimum C/N ratio was tested for efficient denitrification in the cultures. Nitrate was provided as both electron acceptor and nitrogen source in the medium to the bacteria. Growth and nitrate reduction were significantly affected in both the isolates at C/N ratio of less than 1, but much differences were not found at increasing C/N ratios more than 1.0 (Fig.2.11 a & d). Increase in nitrite accumulation was seen at decreasing C/N ratio in *Paracoccus* sp. W1b, though significant nitrite accumulation changes were not observed in *Diaphorobacter* sp. D1 with changes in the C/N ratio (Fig. 2.11 b & e). A twenty seven fold increase in nitrite was seen in C/N ratio of 0.25 than 1.0, and C/N ratio of 0.5 had nine fold increase in nitrite than C/N ratio of 1.0 in *Paracoccus* sp. W1b (Fig. 2.11 b). Nitrite accumulation was in the range 3.5 to 6.9 mM in *Diaphorobacter* sp. D1 at all C/N ratios (Fig. 2.11 e).

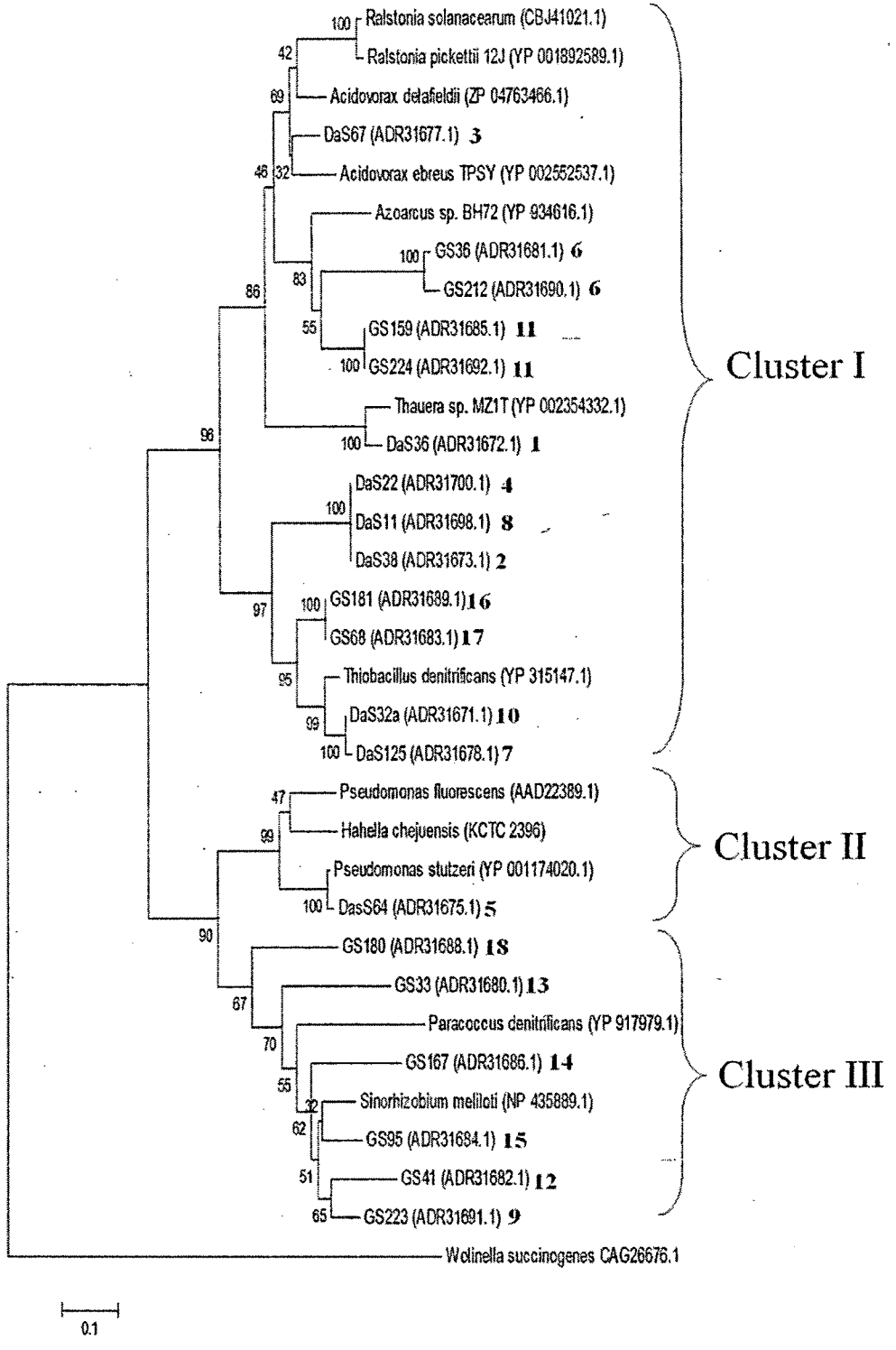

Fig. 2.6. Phylogenetic tree of translated *nosZ* sequences determined by neighbour joining method with 1000 bootstrap iterations. Parenthesis contain the GenBank accession numbers.

Table 2.6. Calculated diversity estimates of *nos*Z gene in the fertilizer factory activated sludge samples

Sample	Number of clones	Number of OTU's	Simpson's reciprocal index (1/D)	Shannon-Weiner index (H)
DaS	114	10	1.691	0.946
GS	104	13	2.518	1.506

Shannon index (H') and Simpson's index (D) were calculated according to Hill et al. (2003).

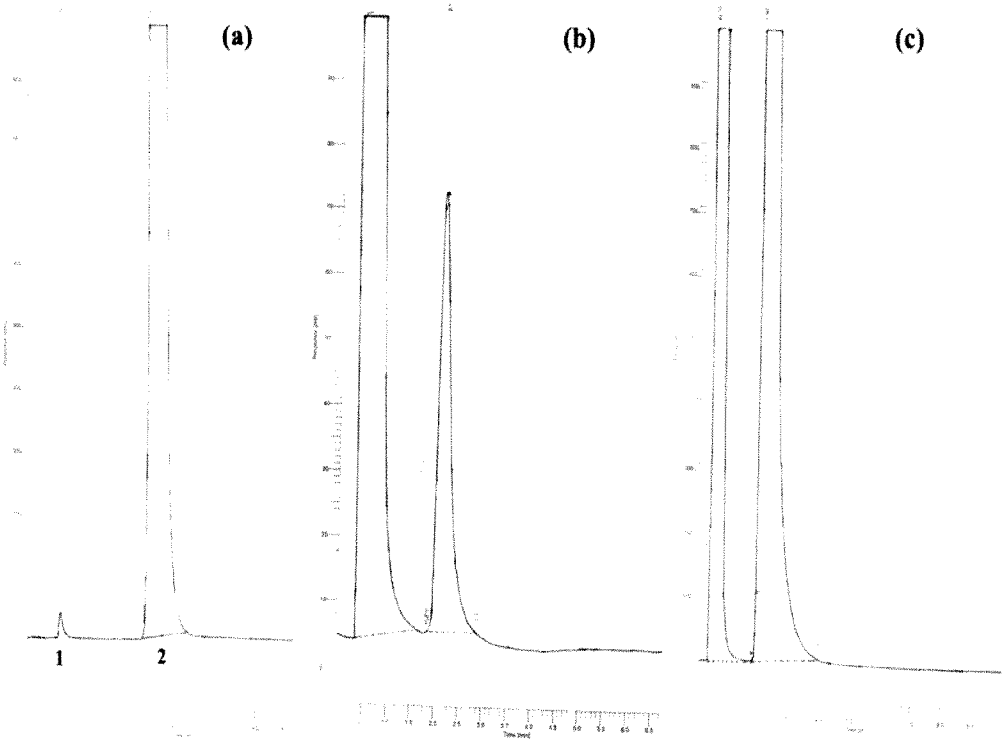

Fig.2.7. Nitrous oxide production analysed for confirmation of denitrification ability (a) Standard (b) *Paracoccus* sp. W1b (c) *Diaphorobacter* sp. D1

1 = Nitrogen peak

2 = Nitrous oxide peak

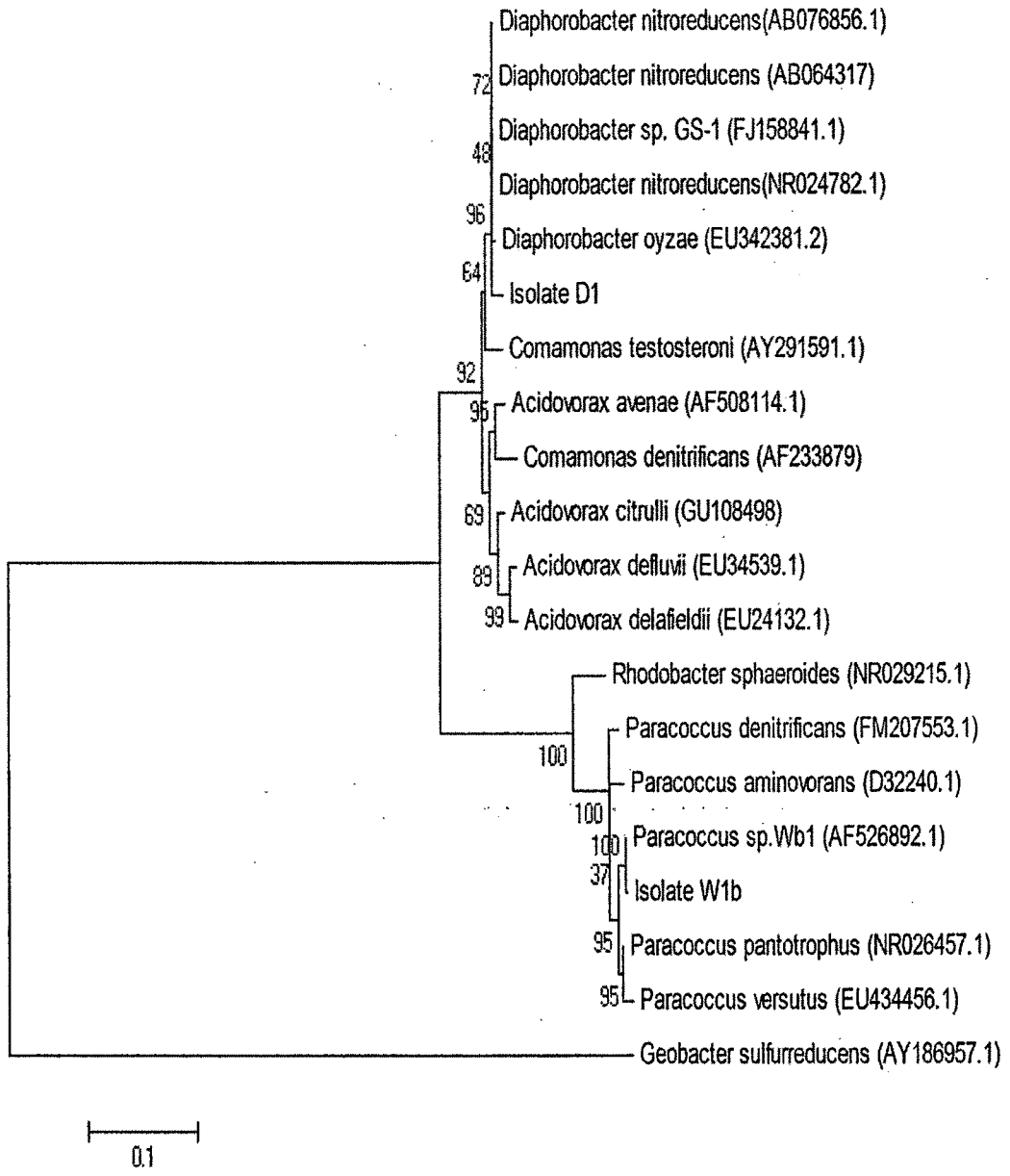

Fig. 2.8. Phylogenetic tree constructed by neighbor-joining method showing position of *Paracoccus* sp. W1b and *Diaphorobacter* sp. D1 with other related cultures. Bootstrap analysis of 1000 resampling by maximum-likelihood method was used to construct tree. Parenthesis contains the accession number of the cultures.

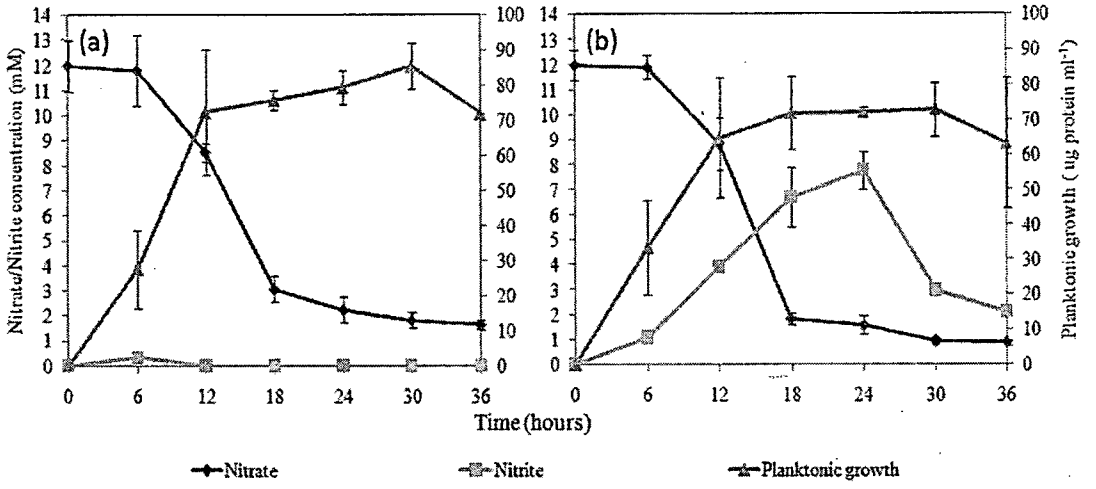

Fig. 2.9. Denitrification pattern of the isolates (a) *Paracoccus* sp. W1b and (b) *Diaphorobacter* sp. D1. Error bars represent the standard deviation.

Table 2.7. Calculated rates during denitrification deduced from data shown in Fig. 2.9

Isolates	μM NO_3 reduction min^{-1}	μM NO_2 formation min^{-1}	RR
Paracoccus sp. W1b	7.22	0.9	1.14
Diaphorobacter sp.D1	9.72	6.1	2.68

RR = Relative rate of nitrate and nitrite reduction (Rates calculated according to Dhamole et al. (2008). RR = 1 implies no nitrite build-up, whereas RR > 1 signifies nitrite accumulation

* Rates determined by linear regression. R^2 value > 0.87

Table 2.8. Nitrate and nitrite reduction rates by resting-state cells

Isolates	μM NO_3 reduced mg^{-1} protein min^{-1}	μM NO_2 reduced mg^{-1} protein min^{-1}	Ratio of nitrate to nitrite reduction
Paracoccus sp. W1b	159.0 ± 85.0	147.0 ± 15.0	1.08
Diaphorobacter sp. D1	117.0 ± 13.0	78.0 ± 14.0	1.50

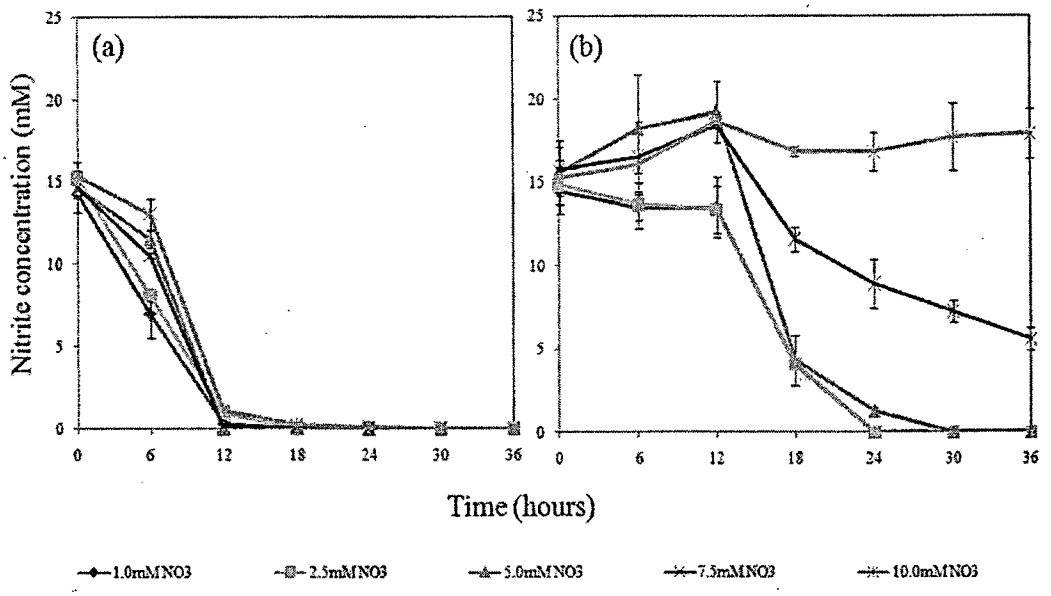

Fig. 2.10. Nitrite accumulation at increasing nitrate concentration in the medium (a) *Paracoccus* sp. W1b (b) *Diaphorobacter* sp. D1. Error bars represent the standard deviation.

Table 2.9. Nitrate reduction rates during denitrification at increasing nitrate concentrations

Initial nitrate concentration (mM)	µM Nitrate reduction min^{-1}	
	Paracoccus sp. W1b	*Diaphorobacter* sp. D1
1.0	3.08	5.25
2.5	4.16	5.00
5.0	4.16	4.16
7.5	5.27	5.27
10.0	8.88	9.44

* Rates determined by linear regression. R^2 value > 0.87

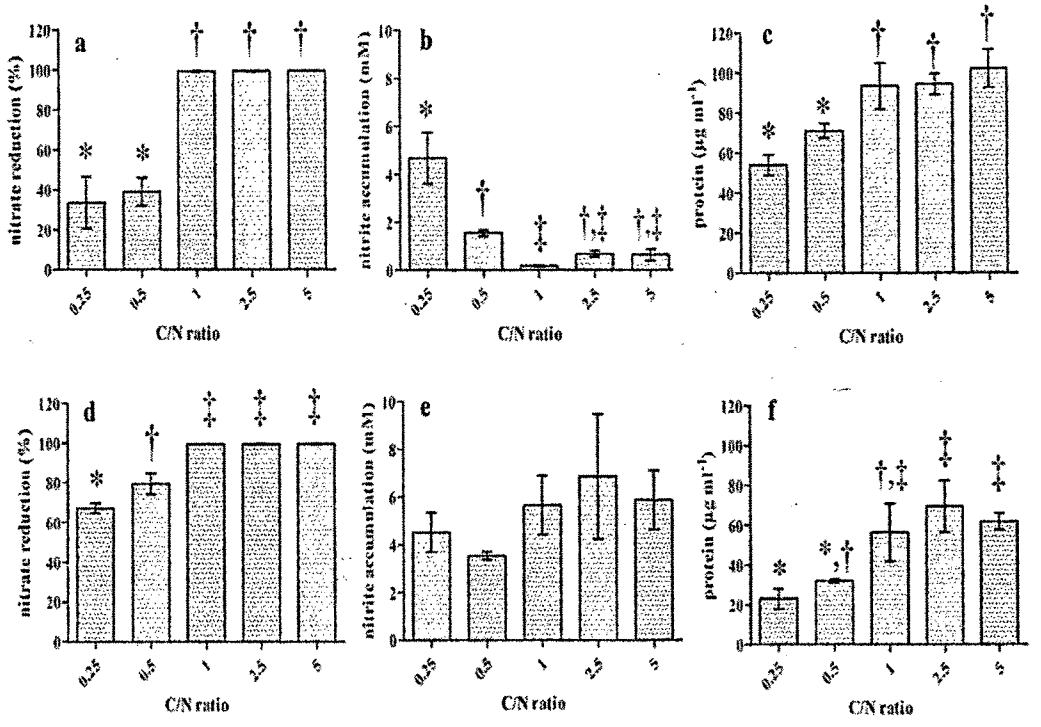

Fig. 2.11. Influence of C/N ratio on nitrate reduction, nitrite accumulation and growth of (a, b, c) *Paracoccus* sp. W1b and (d, e, f) *Diaphorobacter* sp. D1 Bars represent average values of at least three independent experiments and error bars represent the standard deviation. Same symbols above the bars indicate no significant difference. One way ANOVA with Tukey test was used to determine significant differences ($p < 0.05$).

2.3.3.3. High nitrate denitrification by *Paracoccus* sp.W1b and *Diaphorobacter* sp. D1

Nitrate reduction and accumulation of nitrite was investigated in both the isolates at high nitrate concentrations, results of which are shown in Table 2.10. A C/N ratio of 5.0 was maintained at all nitrate concentration tested. *Paracoccus* sp. W1b reduced nitrate in the range 54% - 76.5% in twelve hours for all the nitrate concentrations tested. Nitrite accumulation was in the range 1.15 – 3.4 mM although 6.5 mM nitrite accumulated with 0.5 M initial nitrate. An average CFU of 10^9 ml^{-1} was maintained at all tested nitrate concentrations (Table 2.10). Nitrate reduction was high in *Diaphorobacter* sp. D1 in the range 70% - 80% upto 0.5 M initial nitrate concentration whereas a drastic decrease in nitrate reduction with 17.5 and 15.7 percent and a two log decrease in CFU with 10^7 cells

were observed when the nitrate was increased to 1 M and 2 M respectively. Nitrite accumulation in *Diaphorobacter* sp. D1 was higher than *Paracoccus* sp. W1b with a range of 8.1 -13.6 mM.

2.3.3.4. Denitrification pattern of the isolates cultivated from the DaS, GS, WL and NL sludge samples

Denitrification pattern in some of the isolates which could denitrify from nitrate was checked (Fig. 2.12). The isolate *Pseudomonas mendocina* DaS12a and *Brevundimonas diminuta* PDaS4 reduced 96.16% and 96.46% of nitrate in 17 hours with 2.59 and 1.84 mM of nitrite accumulated respectively at 14 and 17 hour interval (Fig. 2.12 a & d). The isolate *Pseudomonas stutzeri* NL5a reduced nitrate only uptil 0.62 mM or 79.3% in 17 hours with a significant amount of nitrite accumulation (Fig. 2.12 b). Isolate *Pseudomonas fluorescens* PNL4 and *Comamomas* sp WL3b denitrified efficiently as compared to other isolates by reducing nitrate to an undetectable quantity in 17 hours (Fig. 2.12 c & e). Nitrite accumulation was transient in *Comamomas* sp WL3b, but was completely reduced to insignificant amount in 17 hours. The isolate *P.fluorescens* PNL4 showed no significant amount of nitrite accumulated in the media. However, *Paracoccus* sp. W1b reduced nitrate completely at 11 hours with only an insignificant transient accumulation of nitrite at the 8^{th} hour interval (Fig. 2.12 f).

Table. 2.10. Denitrification and growth of the isolates at high nitrate concentrations.

Initial	Nitrate concentration (M)				Nitrite accumulation (mM)		CFU* (x 10^8)	
	Paracoccus		*Diaphorobacter*					
	Final	% reduced	Final	% reduced	*Paracoccus*	*Diaphorobacter*	*Paracoccus*	*Diaphorobacter*
0.1	0.046±0.004	54.0	0.029±0.002	71.0	1.55 ± 0.40	11.3 ± 1.55	10.0	30.0
0.2	0.047±0.0005	76.5	0.040±0.003	80.0	1.15 ± 0.15	13.6 ± 1.45	10.0	50.0
0.5	0.198±0.003	60.4	0.150±0.002	70.0	6.50 ± 2.15	11.8 ± 0.85	10.0	27.0
1.0	0.404±0.077	59.6	0.825±0.246	17.5	2.00 ± 0.45	8.1 ± 4.1	15.0	0.30
2.0	0.693±0.127	65.3	1.686±0.332	15.7	3.40 ± 0.20	9.05 ± 2.05	13.5	0.56

* Initial CFU = 10^8.

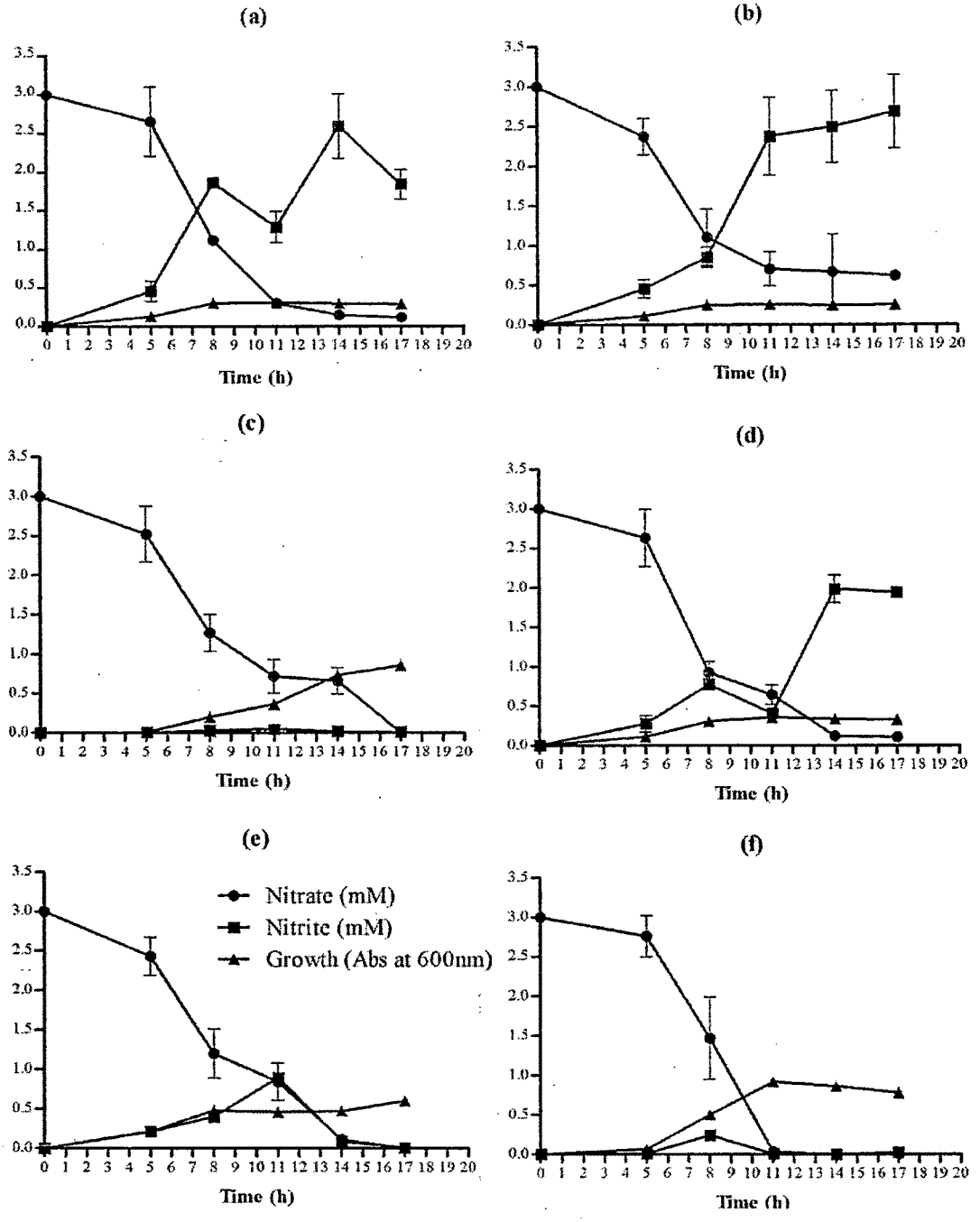

Fig. 2.12. Denitrification pattern of the isolates obtained from the activated sludge samples. (a) *Pseudomonas mendocina* DaS12a (b) *Pseudomonas stutzeri* NL5a (c) *Pseudomonas fluorescens* PNL4 (d) *Brevundimonas diminuta* PDaS4 (e) *Comamonas sp.*WL3b (f) *Paracoccus* sp. W1b. Error bars indicates the standard deviation of three independent experiments.

2.4. Discussion

2.4.1. Abundance and diversity of the denitrifying bacteria in different activated sludge samples

Abundance and diversity of denitrifying bacteria in activated sludge samples by both culturable and unculturable methods were investigated in this study. MPN of denitrifiers showed DaS sludge sample, which is from a denitrifying reactor to have low count, whereas GS sludge sample, which is from the aeration tank of a fertilizer factory with a high count (Fig.2.1). However contrasting results were found by realtime PCR, where the *nosZ* and *nir*S copy numbers were higher in DaS sample, but low in GS (Fig. 2.2). The results suggest that the sample DaS possibly contains numerically dominant denitrifiers which are unculturable by the methods we used. DaS sample also showed a high copy number ($p<0.05$) of 16S rRNA, *nosZ* and *nir*S genes than other samples (Fig. 2.2), but the percentage of *nosZ* gene to 16S rRNA was high in the NL sludge sample, which is from a common effluent treatment plant and percentage *nir*S was high in WL (sludge sample from an aeration tank of domestic wastewater treatment plant) and NL samples (Table 2.3). Two kinds of nitrite reductases are found in denitrifiers, NirK and NirS, containing copper and cytochrome d_1 respectively with either of them is present in an organism, but never the both (Zumft, 1997). To get an idea about the abundance of total denitrifying bacteria in an environment, it would be appropriate to target denitrification genes other than *nir*S or *nir*K to overcome this redundancy. However, not all denitrifying bacteria are equipped with all the reductases involved in denitrification pathway. Some bacteria are truncated in any of the reductase gene, thus disabling them in carrying out complete denitrification (Focht, 1982; Zumft, 1997). The WL sample possibly consists of numerically dominant denitrifying bacteria truncated in *nosZ* gene because it showed *nosZ* to *nir*S ratio of 0.531 (Table 2.3). Similar kind of results, where *nir*K was higher than *nosZ* was obtained by Henry et al., (2006). Truncation of *nosZ* genes has an important implication on environment because nitrous oxide is considered a potential greenhouse gas (Lashof and Ahuja, 1990).

Three different kinds of media were used in this study to isolate denitrifying bacteria from sludge samples. A conventional method was adopted by using Peptone nitrate medium (PNB) (Tiedje, 1994). G2M11 and G4M3 media was also used to isolate

denitrifiers because it was reported to be best for isolating diverse kind of denitrifying bacteria (Heylen et al., 2006). G2M11 contains nitrite and G4M3 contains nitrate, as sole electron acceptors. With this media we could isolate *Comamonas* sp, *Pseudomonas* sp. and *Alcaligenes faecalis*, though none of alpha proteobacteria was isolated (Table 2.5). Cultures incapable of denitrification from nitrate, whereas having denitrifying ability from nitrite was isolated, which were possibly truncated in the first step of denitrification. Some isolates were able to completely denitrify from nitrate, but unable to reduce when nitrite was provided in absence of nitrate (Table 2.4). Interestingly, these isolates did not carry out denitrification in presence of nitrite, but did so in presence of nitrate. Presence of nitrate activates the other reductases of the denitrification pathway in some organisms (Zumft, 1997), but nitrite not activating its own reductase or other denitrifying enzymes in absence of nitrate, as observed in this study, adds another dimension to the complex regulation of denitrification.

Culture independent diversity of denitrifying bacteria in the fertilizer factory wastewater sludge (DaS & GS samples) was assessed by cloning the partial *nosZ* gene and its RFLP analysis. The DaS sample reached near saturation with 120 clones as analysed by rarefaction, whereas the GS sample was still unsaturated (Fig. 2.4). Industrial wastewaters are a habitat with high concentrations of some nutrient or recalcitrant compounds. The DaS sample is denitrifying reactor sludge where fusel oil which is a mixture of hydrocarbons with major content as methanol (Zala et al., 1999) is supplied as a carbon source. Also calcium and manganese concentrations were in excess in the sample (Table 2.1) which has naturally selected the organisms with higher fitness to that habitat, thus making it less diverse. The *nosZ* protein sequence of the OTU 3 with highest number of clones, clustered around *Acidovorax* sp. and OTU 11 which also had high number of clones clustered peripherally with *Azoarcus* sp. (Fig. 2.5 & 2.6). However, OTU 3 was abundant in DaS sample and OTU 11 consisted of clones from only GS sample. *Acidovorax* sp. belongs to the family Comamonadaceae and Ginige et al., (2005) observed clones of the family Comamonadaceae to dominate the acetate-fed denitrifying reactor sludge. OTU 1 and 6 clustered with *Thauera* sp and *Azoarcus* sp. respectively which belong to betaproteobacteria which are reported to be abundant in activated sludges of denitrifying reactors (Lee et al., 2002; Ginige et al., 2005). *Thauera* sp and *Azoarcus* have also been implicated to have a dominant functional role of denitrification

in activated sludge processes (Morgan-Sagastume et al., 2008). The DaS sample consisted of almost betaproteobacterial *nosZ* clones with few clones related to alpha and gammaproteobacteria clustered with *Sinorhizobium* sp. and *Pseudomonas stutzeri* respectively (Fig. 2.6). The GS sample had clones evenly distributed between alpha and betaproteobacteria without a single clone from gammaproteobacteria. However the numerically dominant was found to belong to the betaproteobacterial group (Fig. 2.5 & 2.6).

The numerically dominant cultured bacteria from DaS and GS samples belonged to the genus *Pseudomonas* and *Alcaligenes,* but the culture independent study did not reflect this numerical abundance. A similar observation was obtained by Lee et al., (2002), where they isolated different species of *Pseudomonas*, while the culture independent results indicated betaproteobacteria to be abundant. This shows that the dominant players in the sludge habitats are not the ones usually obtained by conventional culture methods. The numerically dominant clones of *nosZ* sequences clustered with betaproteobacteria in DaS sample (Fig. 2.6), whereas the cultured bacteria from the same sample were from Pseudomonadaceae family in gammaproteobacteria. This result was consistent with the quantitative results, where the MPN was low in DaS sample as compared to GS (Fig. 2.1) while the real time quantitative PCR showed a high abundance of denitrification genes (Fig. 2.2). The media used in this study were G4M3, G2M11 (Heylen et al., 2006) and the traditional peptone nitrate agar, but none of the dominant betaproteobacteria related to the cloned *nosZ* sequences were isolated with these. Heylen et al., (2006) had recommended G4M3 and G2M11 media for cultivation of denitrifiers after optimization procedures involving an evolutionary algorithm. But both these media did not support the growth of betaproteobacteria in this study. However, *Acidovorax* sp., *Thauera* sp., *Azoarcus* sp. and *Thiobacillus denitrificans*, which were dominant in the sludge, were also not isolated by Heylen et al., (2006) by these two media.

Among the isolates obtained in this study, truncated denitrifiers in nitrate reduction were also cultivated (Table 2.4), and also quantitative results suggested the possible presence of denitrifiers truncated in the nitrous oxide reductase gene (Table 2.3). One of the reasons for inefficient nitrate removal and accumulation of intermediates of denitrification in wastewater treatment reactors could be the presence of truncated

denitrifiers in high numbers, which has serious implications ecologically. This study also substantiates the earlier reports (Figuerola and Erijman, 2007; Lashof and Ahuja, 1990; Thomsen et al., 2007) of betaproteobacteria being numerically dominant denitrifiers in activated sludge habitats. Future studies can aim at culturing the predominant denitrifying bacteria in sludge habitats by analysing their denitrifying potential, and understanding the environmental determinants capable of selecting the community that can efficiently denitrify without accumulation of the intermediates.

2.4.2. Denitrification activity in the cultures isolated from activated sludge

Though similar denitrification apparatus is present in diverse groups of bacteria, the denitrifying activity of each step differs in different organisms. In this study, we also compared the denitrification pattern of two cultures W1b and D1, isolated from the activated sludge of a denitrifying reactor (DaS) and identified as *Paracoccus* sp. W1b and *Diaphorobacter* sp. D1 respectively (Fig. 2.8). The nitrate reduction rate is higher in *Diaphorobacter* sp. D1, but *Paracoccus* sp. W1b reduced nitrate without much accumulation of nitrite transiently unlike *Diaphorobacter* sp. D1 which accumulated nitrite significantly (Fig. 2.9). *Diaphorobacter* sp. D1 species are reported to also nitrify ammonia to nitrite (Khardenavis et al. 2007), however ammonia was not provided in the MM2 medium (refer to materials & methods for composition), suggesting that the nitrite accumulation was due to nitrate reduction. Accumulation of nitrogenous oxide intermediates during denitrification was explained by Betlach and Tiedje (1981), where they showed nitrite accumulation in *Alcaligenes* sp. and *Pseudomonas fluorescens*, was due to differences in the reduction rates of nitrate and nitrite.

Investigating nitrate and nitrite reduction rates in resting-cell suspensions provides insight into the overall reduction rate including the flux of nitrate/nitrite into and outside the cell. It was observed that *Paracoccus* sp. W1b have a nitrate to nitrite reduction ratio of 1.08, suggesting nearly equal rates in the reduction of these two nitrogenous oxides, but *Diaphorobacter* sp. D1 showed a ratio of 1.5 (Table 2.8). High nitrate reduction rate than nitrite reduction was the possible reason for accumulation of nitrite in *Diaphorobacter* sp. D1. Increasing nitrate concentrations in the medium increased the nitrite build-up in *Diaphorobacter* sp. D1, whereas, nitrite accumulation was not observed in *Paracoccus* sp. W1b (Fig. 2.10). The low nitrite reduction than nitrate could

be the possible reason for the nitrite build-up in *Diaphorobacter* sp. D1, but ratio of 1.08 (Table 2.8) for nitrate to nitrite reduction found in *Paracoccus* sp. W1b could not explain the increased reduction rate of nitrite when nitrate concentrations were increased in the medium. Branched electron flow to the nitrogenous oxides in *P. denitrificans* has been reported by Kucera et al. (1983). Similarly, the increased nitrite reduction with increase in nitrate concentration observed for *Paracoccus* sp. W1b suggests 'inhibition by product via respiratory chain' to be the possible phenomenon, as suggested by Kucera et al. 1983.

C/N ratio significantly influenced nitrate reduction and growth in both the isolates (Fig. 2.11). Low C/N ratio also affected nitrite accumulation significantly in *Paracoccus* sp. W1b. Carbon source acts as an electron donor; hence higher amounts of carbon source than nitrate is required to completely reduce nitrates. C/N ratio of 5.0 was provided in the further studies of high nitrate reduction. High nitrate concentrations tested did not affect the nitrate reduction and growth significantly in *Paracoccus* sp. W1b, but a substantial drop in nitrate reduction and growth was observed in *Diaphorobacter* sp. D1 from 1.0 M nitrate concentration (Table 2.10). Nitrates in excess can be harmful to the cell because of their chaotropic effect. A *Klebsiella oxytoca* strain was isolated by Pinar et al. (1997), which could tolerate nitrate upto 1.0 M, but *Klebsiella* species are also reported to have the property of dissimilatory nitrate reduction to ammonium (DNRA) (Tiedje et al. 1982). However, *Paracoccus* sp. W1b could denitrify efficiently even at 2.0 M nitrate concentration and tolerate upto 4.0 M nitrate concentrations (data not shown). The denitrification intermediates, nitrite and nitric oxide generate reactive nitrogen species (RNS) which are more toxic to the cells (Poole 2005). Efficient denitrification by branched electron transfer (Kucera et al. 1983) in *Paracoccus* sp. W1b is possibly the mechanism to detoxify its microenvironment, whereas the accumulation of nitrite in *Diaphorobacter* sp. D1 possibly lowered the fitness of the cell at high nitrate concentration.

The denitrification pattern of the cultures isolated from the four sludge samples (Table 2.1) and capable of denitrification from nitrate, were compared with the *Paracoccus* sp. W1b (which showed to be an efficient denitrifier). The denitrification varied among the isolates tested, where the nitrite accumulation was low and transient in isolates *P. fluorescens* PNL4 and *Comamonas* sp. WL3b, but high in others including

Brevundimonas diminuta PDaS4 (Fig. 2.12 c & e). Nitrite accumulation in *Paracoccus* sp. W1b was undetectable although an insignificant amount was found only at the 8th hour interval (Fig. 2.12 f). Substantial differences in nitrite accumulation could be observed in different species of Pseudomonads, with *P.fluorescens* PNL4 accumulating insignificant amounts of nitrite compared to *P.mendocina* DaS12a and *P.stutzeri* NL5a, which accumulated higher amounts of nitrite (Fig. 2.12 b & c). *P. stutzeri* and *P. mendocina* belong to the same phylogenetic branch of 16S rRNA (Lalucat et al., 2006). Carlson and Ingraham, (1983) also observed different patterns of denitrification between *Pseudomonas aeruginosa* and *P. stutzeri.* Korner and Zumft, (1989) observed higher nitrate concentrations to inhibit the reduction rate of nitrite in *P. stutzeri.* Later on, Almeida et al., (1995b) showed competitive inhibition of nitrite reduction by nitrate to be independent of nitrate to nitrite reduction ratio in *P. fluorescens.* Betlach and Tiedje, (1981), observed transient nitrite accumulation in the resting state of *P. fluorescens*, however undetectable amounts of nitrite was seen in *P. fluorescens* PNL4 isolate in the batch culture (Fig. 2.12 c). It could thus be noted that the regulation of denitrification in different species and even strains of *Pseudomonas* varies.

The *Paracoccus* sp. W1b was shown to be efficient in denitrification than the other cultures isolated from various sludge samples. The branched electron transfer strategy of *Paracoccus* sp. W1b possibly makes the organism efficient in denitrification without accumulation of the intermediate nitrite and might help the organism adapt to environments containing high nitrogenous oxide concentrations like the industrial wastewaters. Further pure culture studies on the biofilm formation were carried out with this *Paracoccus* sp. W1b isolate.

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2.6. Appendix

I) Media composition

Basal composition of G2M11 and G4M3

Solution A:
KH_2PO_4 13.6 g, Na_2HPO_4 54.2 g, Distilled water 100 ml
10ml solution in 1L medium
Solution B:
$(NH_4)_2SO_4$ 10 g, Distilled water 100 ml
3ml solution in 1L medium
Solution C:
$MgSO_4.7H_2O$ 1.97 g, Distilled water 100 ml
5ml solution in 1L medium
Solution D:
$CaCl_2.2H_2O$ 110.5 g, Distilled water 100 ml
5ml solution in 1L medium
Solution E:
EDTA 160 mg, $FeSO_4.7H_2O$ 137.5 mg, $ZnSO_4.7H_2O$ 57.5 mg, MnSO4.4H2O 85 mg, CuSO4.$5H_2O$ 18.75 mg, $Co(NO_3)_2.6H_2O$ 11.75 mg, $(NH_4)_6MoO_2.24H_2O$ 6.25 mg, Distilled water - 250 ml
Add 5 ml solution E to 1L medium
Vitamin solution:
4–amino benzoic acid 4 mg, D(+) biotin 2 mg, Nicotinic acid 10 mg, Calcium D-(+)-pentothenate 5 mg, Pyridoxine hydrochloride 15 mg, Folic acid 4 mg, Lipoic acid 1 mg, Dissolve in 100 ml of 10 mM NaH_2PO_4 at pH 7.1
Add 1ml vitamin solution to 1L medium
Thiamine solution:
Thiamine hydrochloride 10mg, 25 mM NaH_2PO_4 100 ml, pH 3.4
Add 2 ml to 1L medium
Cobalamine solution:
Cyanocobalamine 50 mg, Distilled water 1L
Add 2 ml to 1L medium
Carbon source: Sodium succinate
Nitrogen source: C/N ratio – 20
pH – 7.0

3mM $NaNO_2$ is used in G2M11 medium and 3mM KNO_3 is used in G4M3

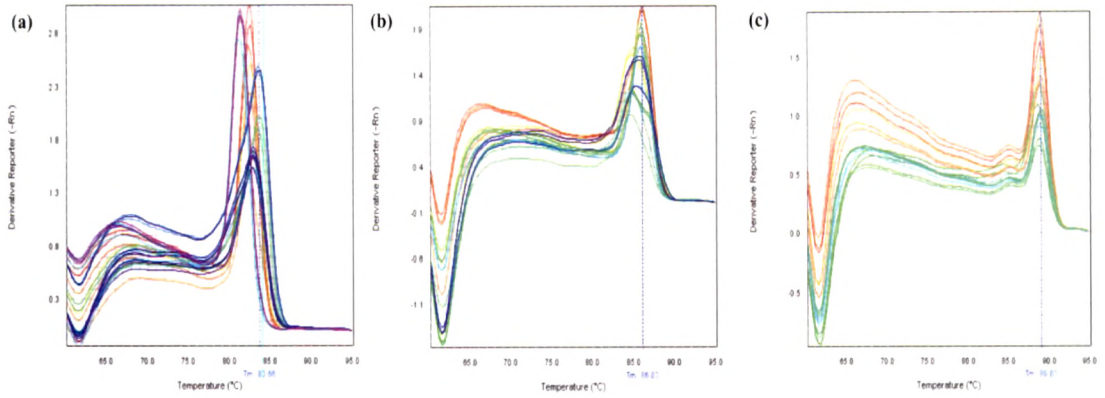


Fig. A1. Melt curve of the (a) 16S rRNA gene (b) *nos*Z gene and the (c) *nir*S gene. The acquisition temperature was 80°C.

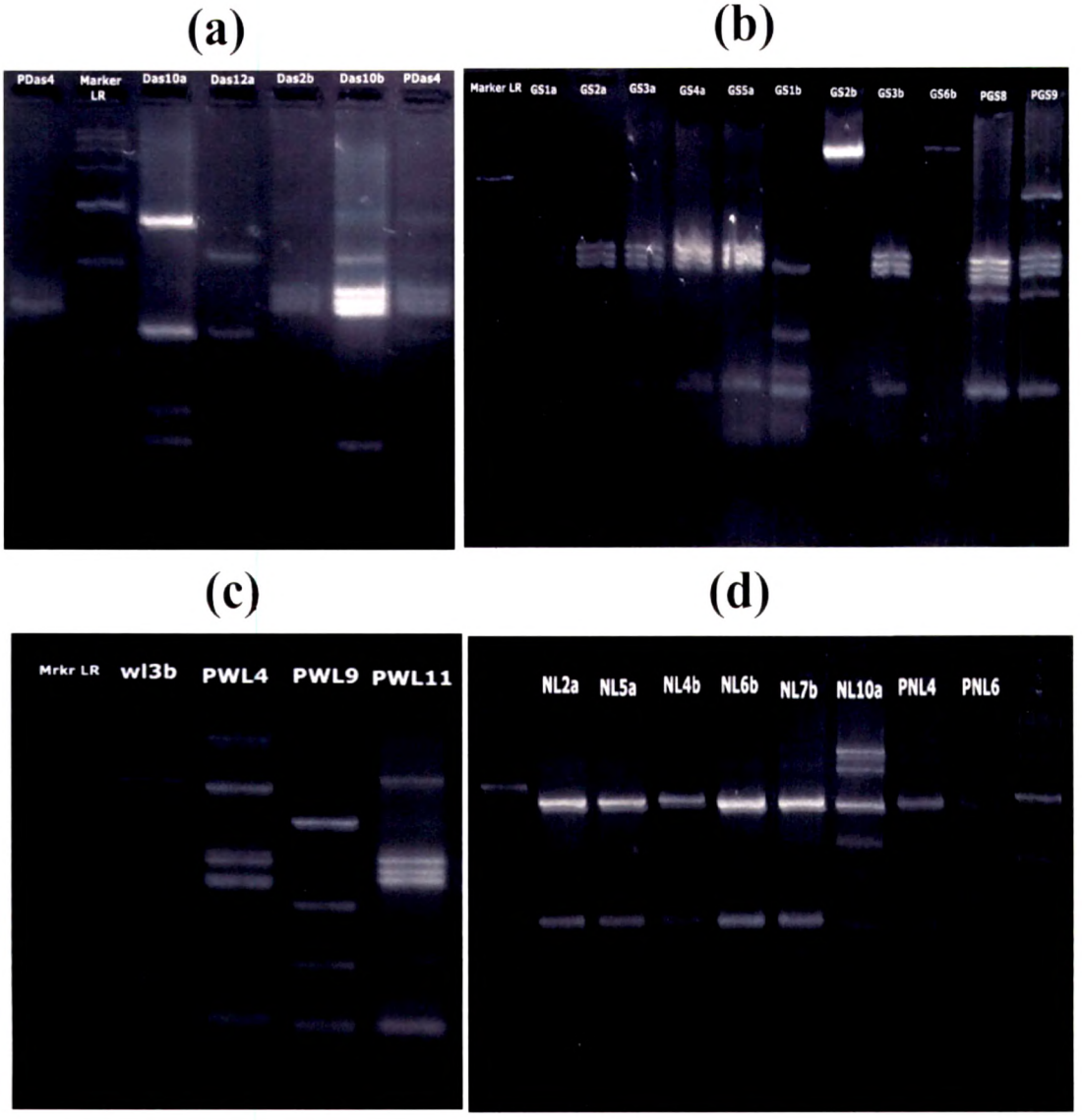


Fig. A2. ARDRA of the 16S rRNA gene of the isolates digested by the enzyme *Rsa*I

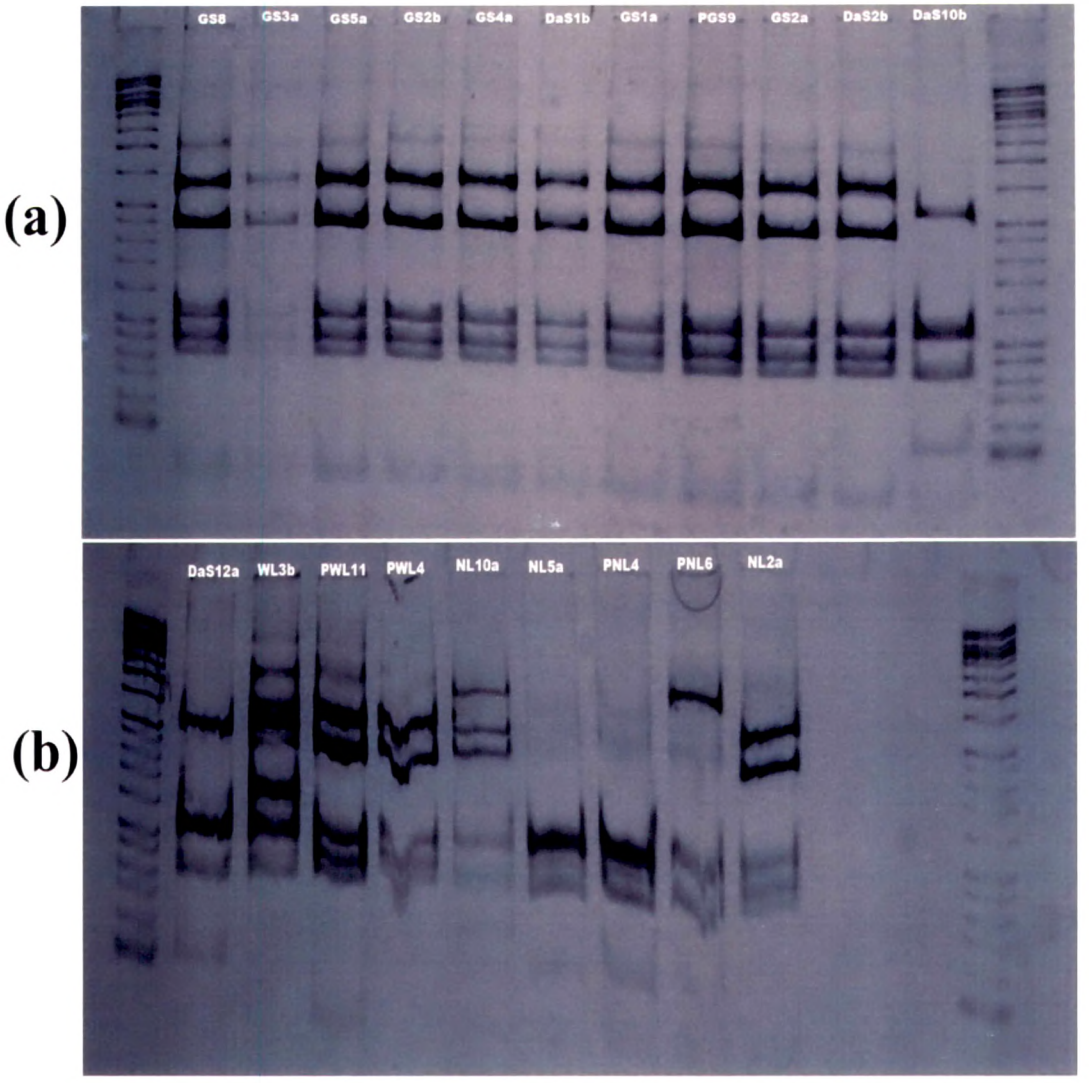
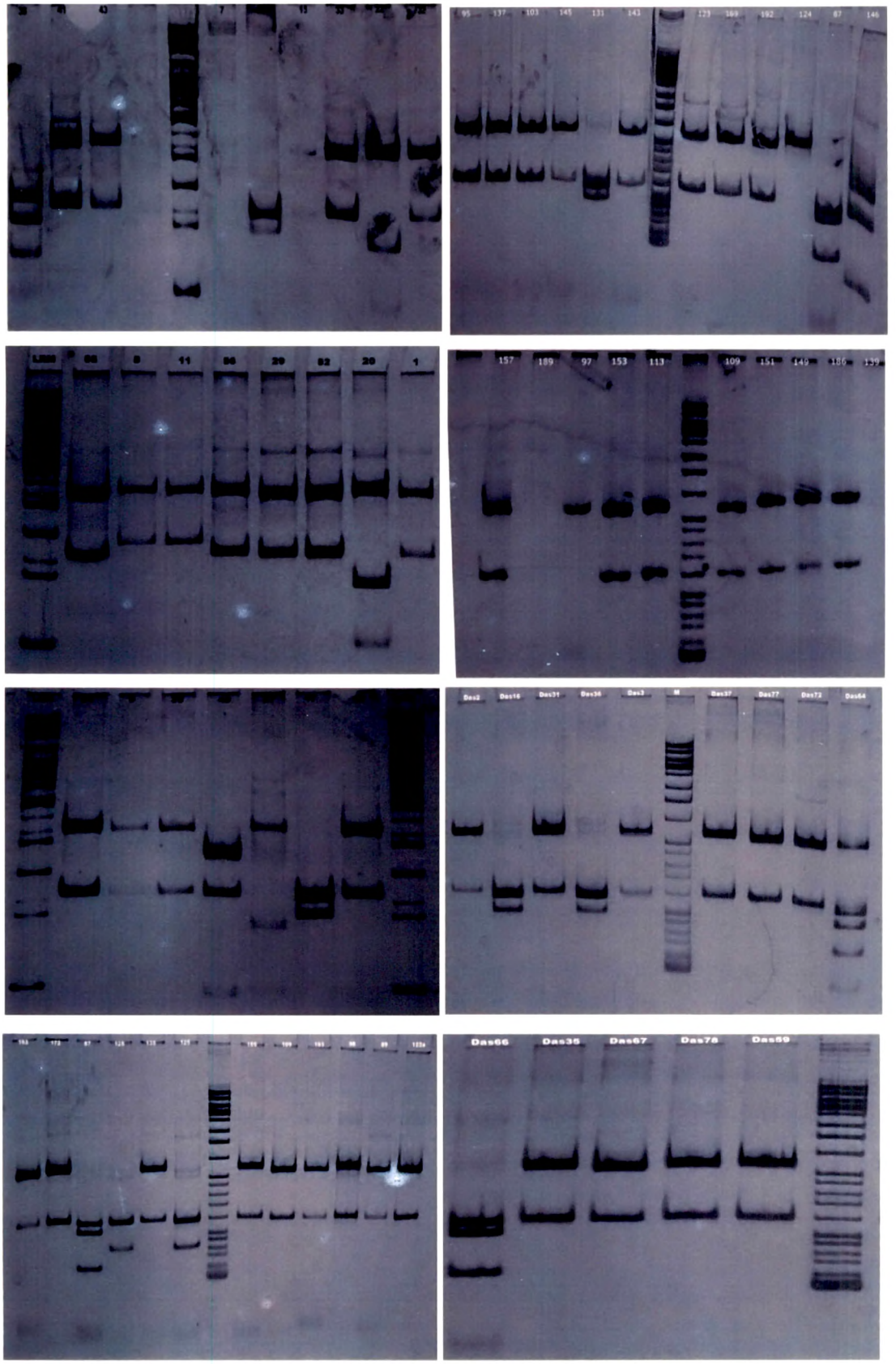


Fig. A3. ARDRA of the 16S rRNA gene of the isolates digested by the enzyme *Alu*I



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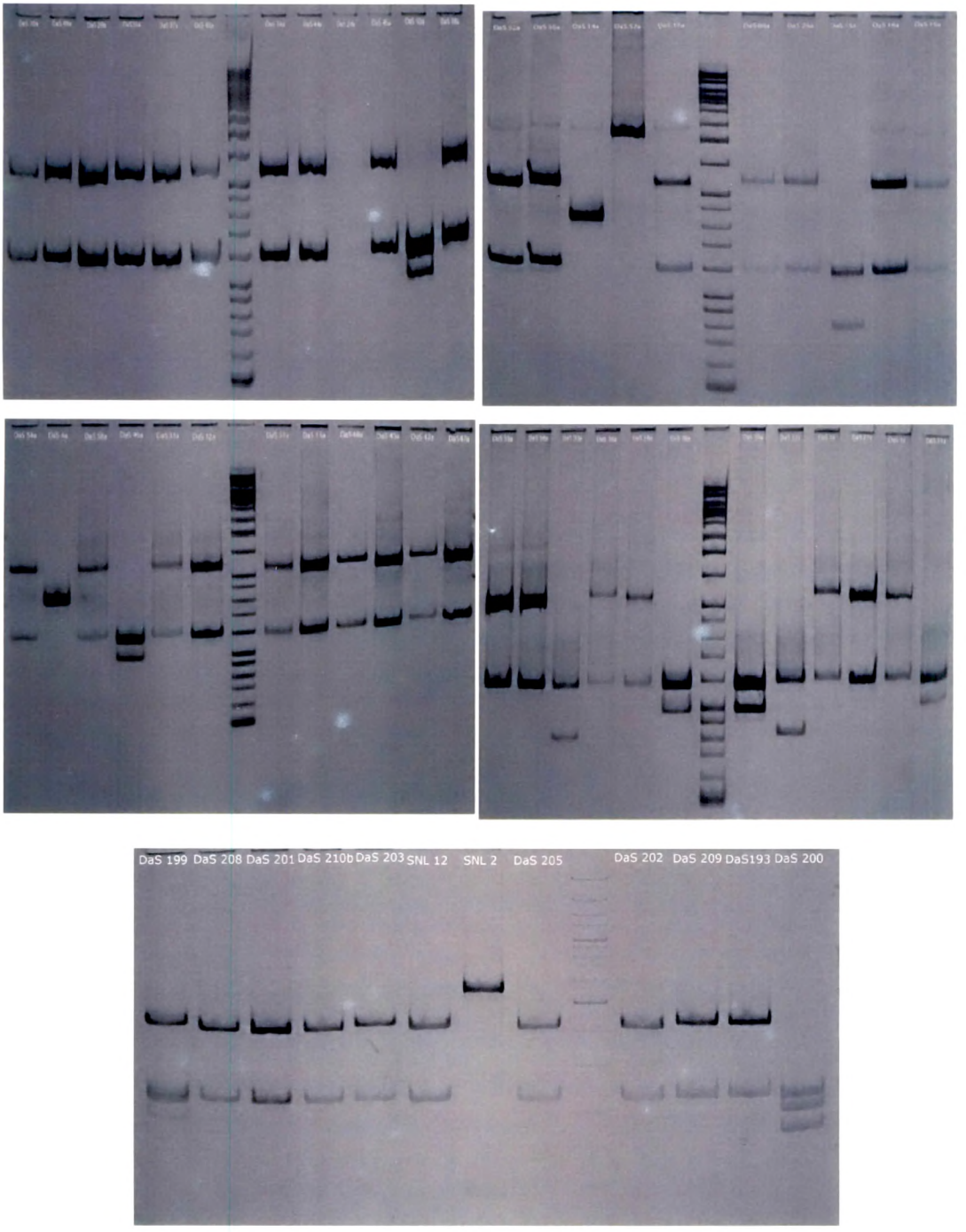
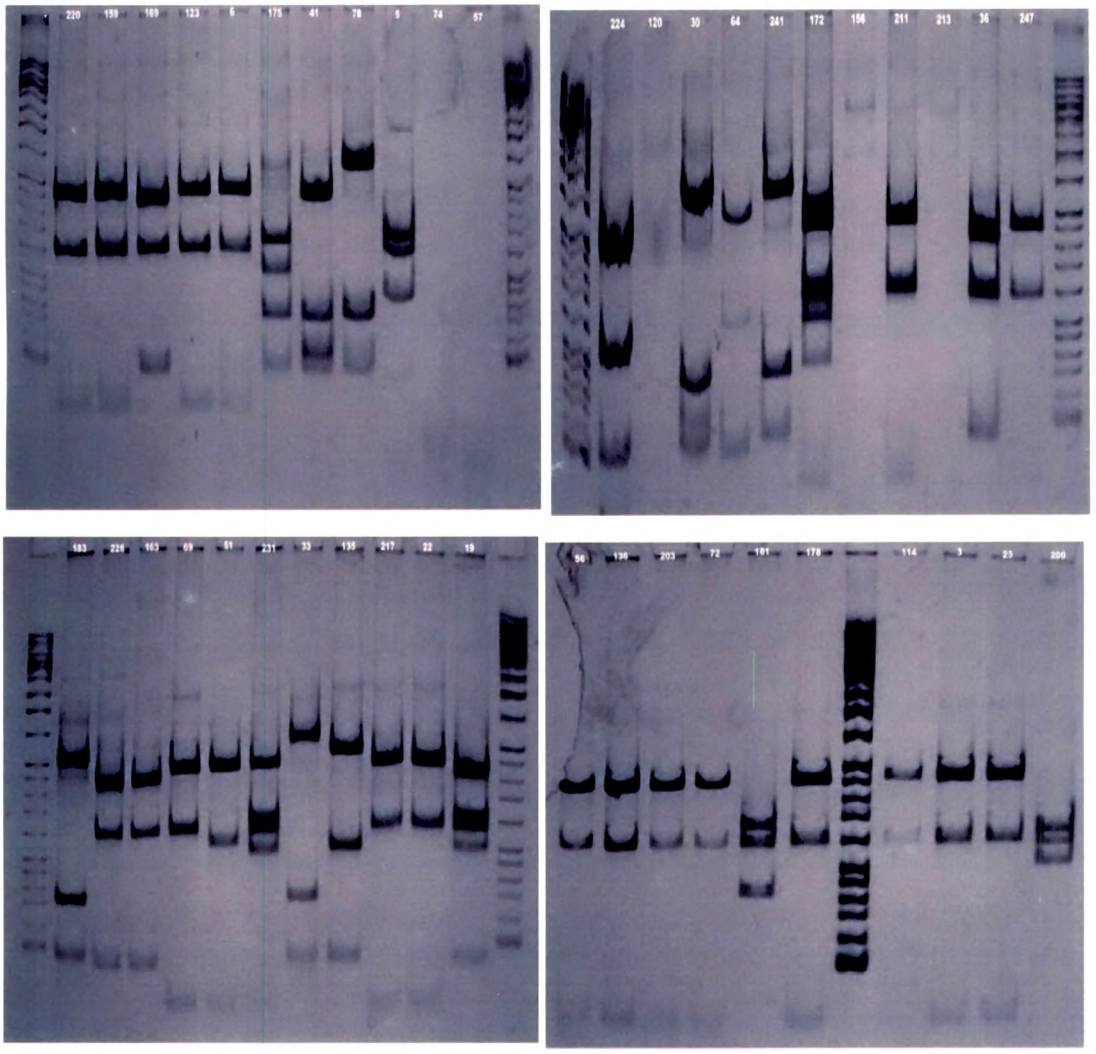


Fig. A4. RFLP images of the*nos*Z gene clones from theDaS sludge sample



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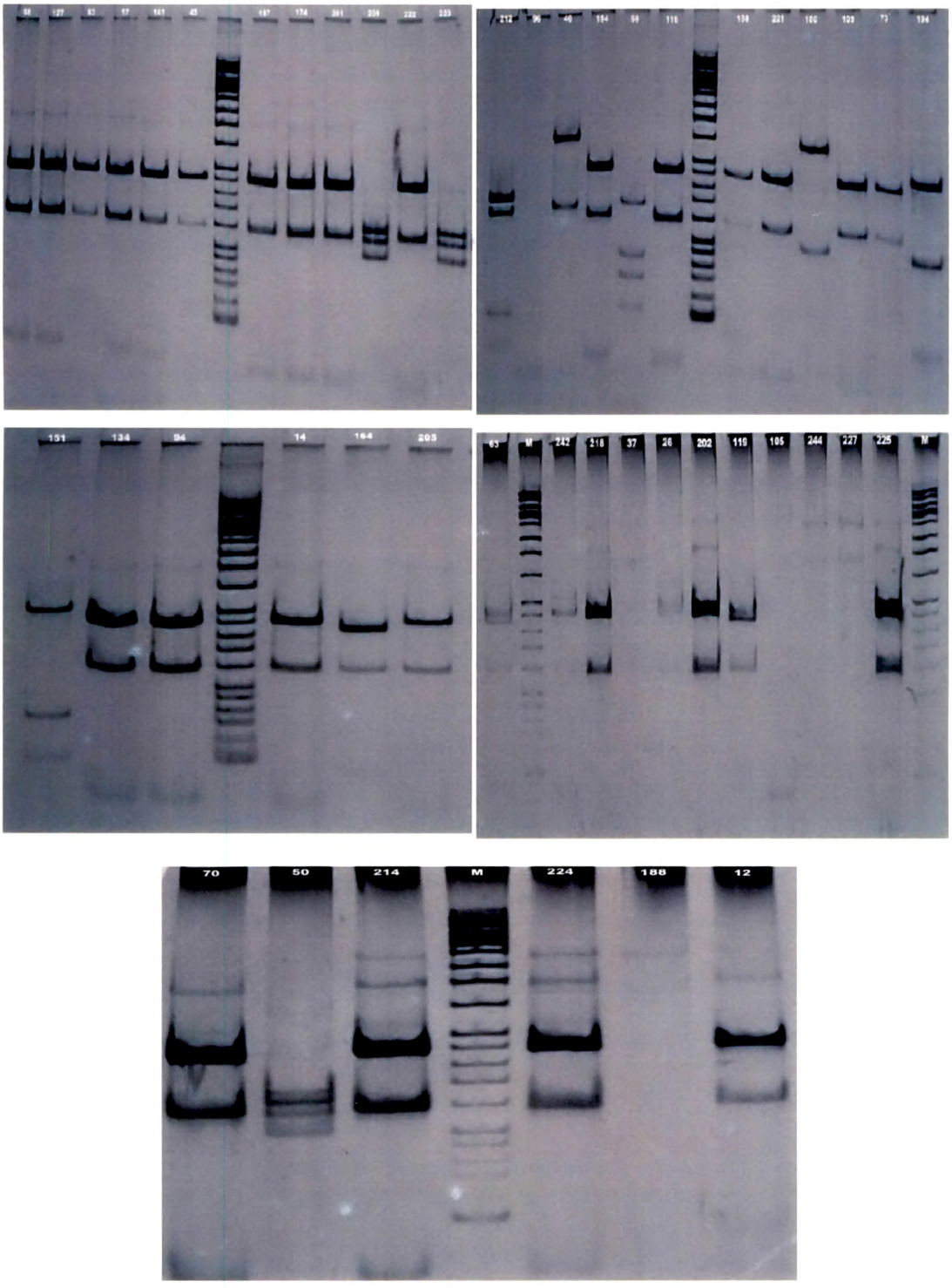

Fig. A5. RFLP images of the *nos*Z gene clones from the GSsludge sample