

Chapter 5

Summary

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5.1 Summary

Denitrification is a microbial process, where nitrate or nitrite is reduced under anaerobic condition to gaseous nitrogen or its intermediary products such as nitric oxide (NO) and nitrous oxide (N₂O) (Zumft, 1997). Bacteria were considered to be the major players in this pathway, but recently the involvement of micro-eukaryotes such as fungi and foraminiferans are also reported (Takaya *et al.*, 2002; Risgaard-Peterson *et al.*, 2006). This is an important pathway in the marine nitrogen cycle as the fixed nitrogen is lost leading to an imbalance in the global nitrogen budget (Codispoti *et al.*, 2003). NO and N₂O produced as intermediates are among the harmful green house gases that influence the earth's climate by the destruction of the ozone in the stratosphere. Denitrification process takes place in enclosed, stagnant water bodies, open ocean oxygen depleted zones and in the coastal waters affected by eutrophication.

Denitrification activity in the Arabian Sea has been reported from the perennial open ocean oxygen minimum zone (OMZ) that contributes to near 50% of the world's oxygen depleted environment and from the coastal regions along the western continental shelf of India (Naqvi *et al.*, 2000). Biological diversity, especially macro organisms are greatly affected by depleting oxygen levels (Levin 2003), but a vast diversity of prokaryotes and micro-eukaryotes have been reported in OMZs from across the globe (Zhou *et al.*, 1997; Giovannoni & Rappé 2000; Dawson & Pace, 2002; Stoeck *et al.*, 2003; Takishita *et al.*, 2005; 2007). Some of the groups described based on environmental DNA analysis are presumably of high relevance for a variety of research disciplines like ecology, evolution, physiology and biogeochemistry (Epstein & Lopez-Garcia, 2007; Lopez-Garcia & Moreira 2008). But there is a lack of knowledge on the role of the specific groups, like fungi present in oxygen-depleted

environments. In this study I have attempted to quantify the abundance of fungi (Chapter-2), estimate the culturable and unculturable diversity (Chapter-3) of fungi and measure the growth and activity of a few selected fungi and in simulated oxygen depleted conditions (microcosm) (Chapter-4).

Abundance of fungi in the sediments of the denitrification zones of the Arabian Sea was estimated from three coastal (CSt stations) and five slope stations (SSt stations) within the OMZ region (Table 2.1).

The CSt stations were located within the coastal, seasonal anoxic region off Goa (Fig 2.). This condition is seen to occur along the coastal waters on the western Indian shelf upto 200 m depth, during June to December, with the maximum intensity during the post-monsoon season in September and October (Naqvi *et al.*, 2000). Periodic sampling was carried out to study the changes in the fungal abundance with the changing dissolved oxygen (DO) levels. Sampling was also carried out in the open ocean, perennial oxygen minimum zone to study the fungal abundance in this region. Fungal mycelia were detected in the sediments by staining with calcoflour, an optical brightener that enhances fluorescence of cellulose and chitin, the latter being signature of the fungal cell wall (Mueller & Sengbusch, 1983). Such staining revealed the presence of hyphae in the sediments, confirming active growth of fungi therein. Occasionally fungal spores were also detected.

Fungal colony forming units (CFUs), fungal biomass C, bacterial biomass C and organic carbon (OC) % was estimated in the sediments collected from the different sampling sites. Statistical analyses of these parameters show that there is a difference in the biological parameters between seasons (Table 2.4). A negative correlation (Table 2.5a) between DO levels and bacterial biomass C in the CSt was observed. This may be due to the ability of bacteria which can adapt to changing DO levels. But in

the open ocean OMZ region there is no evident effect of DO on the microbial abundance or on the fungal CFUs between the different sampling stations (Table 2.5b). The condition in the open ocean OMZ is a perennial condition in comparison to the seasonal anoxic condition. Hence the microbes thriving in this condition could have adapted to the low oxygen levels and a mutual balance between the fungal and bacterial population adapted to this environment is a possibility.

Fungal CFUs are not affected by changing DO levels in both the coastal, seasonal and perennial, oceanic station. This shows that fungal cultures though are affected by the changing DO levels are able to thrive and play a role in the denitrifying environments, in contrast to the existing reports which show that fungi have no ecological role in the oxygen depleted environments (Dighton, 2003; Mansfield & Barlocher, 1993). In this study I have for the first time quantified the fungal biomass C and have shown that fungi thrive in oxygen depleted environments (Jebaraj & Raghukumar, 2009).

Diversity of fungal cultures was studied from the CSt, SSt and LSt stations (Table 2.1) using cultivation based and cultivation-independent approach. Shannon's diversity index and Simpson's diversity index shows that the diversity was less compared to the mangrove ecosystem and deep sea sediments (Table 3.3 & 3.4). The cultures identified based on the 18S rDNA sequence could be assigned to Basidiomycetes and Ascomycetes (Table 3.5). Eleven isolates belonged to Basidiomycetes, predominantly Pucciniomycotina; twenty-one of them belonged to Ascomycetes, a majority of it belonged to Eurotiomycetes. The 18S rDNA sequence analysis of the isolates shows that two of the cultures isolated # 11 and # 87 are very divergent from previously described and sequenced fungi (Table 3.5).

Uncultured fungal diversity was studied from the five clone libraries constructed from a CSt, SSt and LSt station (Table 3.1). UPGMA analysis of the fungal communities obtained from each of the libraries shows that there are no differences in the community of fungi between the anoxic and oxic seasons (Fig. 3.3). This may be due to the physiological adaptation of the fungal community to the changing oxygen concentrations. But there was a marked spatial difference between the CSt, SSt and LSt station (Fig. 3.3).

Phylogenetic analysis of the environmental sequences obtained from this study shows that the phylotypes can be categorized to at least five unique clusters of environmental sequences. These clusters have sequences obtained from this study locations and groups with sequences obtained from similar anoxic and extreme environments. Of the five unique environmental clusters identified, one cluster with six phylotypes from this study groups within the Eurotiomycetes of Ascomycota (Fig.3.4). This cluster has sequences obtained from the anoxic Mediterranean basin, anoxic Meromictic Lake sediment and marine anoxic Bolinas Lagoon DNA and a few common culturable forms such as *Penicillium* and *Aspergillus* sp. Three clusters were seen within the basidiomycota belonging to Cystobasidiomycetes, Exobasidiomycetes, and Ustilagomycetes (Fig.3.5). The cluster within the Cystobasidiomycetes groups with environmental sequences of uncultured clones from anoxic Gotland Deep of Baltic Sea. The second environmental cluster within the basidiomycota grouped along with environmental sequences closely related to Exobasidiomycetes. The sequences clustered with phylotypes obtained from anoxic Norwegian fjords, anoxic Mediterranean basin, anoxic Gotland Deep of Baltic Sea and a fungal clone obtained from detritus community. The only known named species was *Exobasidium gracile* which branched out separately from this environmental cluster which is supported with a high bootstrap support. The third cluster belonging to Ustilagomycotina is within a clade

that has been recognized as “hydrothermal and/or anaerobic fungal group” (Lopez *et al.*, 2007). This cluster has representatives from mud volcanoes, hydrothermal ecosystems, anoxic Mediterranean basin and anoxic Berkeley Aquatic Park sediment, deep oceans and a leaf epiphyte (Fig. 3.5). One phylotype belonging to zygomycete was obtained from this study and this OTU grouped with CCW35 from the anoxic Cariaco Basin (Fig. 3.6). These unique clusters of uncultured environmental sequences show that there is a vast diversity of fungi which are yet to be isolated and studied.

The ability of four cultures (# An-2, # 11, # 31, # 31(a)) isolated from the anoxic coastal marine sediments was studied for their growth under anaerobic conditions. While all the cultures grew much better under aerobic conditions, one culture (# 11) produced almost the same amount of biomass under both aerobic and anaerobic conditions (Table 4.2). The denitrification ability of the cultures was studied by growing them in medium containing nitrate and estimating the nitrite and ammonia accumulation in the culture supernatant (Fig 4.2 & 4.3). Their activity in anaerobic condition was shown by studying the ETS activity in the presence of the nitrate in the medium (Fig. 4.4). The cultures screened varied widely in their denitrification ability (Table 4.3, 4.5 & 4.6). The activity of fungi under simulated *in-situ* condition (microcosm) was studied based on their Electron transport system (ETS) enzyme activity (Fig. 4.5). The screening studies were a preliminary attempt to study the denitrification activity of marine fungal isolates. Detailed analysis of all the denitrification products and their enzymes or the genes coding of the enzymes could give us better insights in to the contribution by fungi to the marine denitrification process.

5.2 Conclusions

Major inferences that could be drawn from this study are:

- The microbial abundance as fungal CFUs, fungal biomass C and bacterial biomass C were quantified from the oxygen depleted sediments, collected from the denitrification zones of the Arabian Sea (Jebaraj & Raghukumar, 2009).
- Fungal CFUs, fungal biomass C and bacterial biomass C significantly differ between seasons in the coastal station sediments, showing that the changing DO levels has an impact on the total microbial abundance.
- The fungal CFU numbers, fungal biomass C and total organic carbon estimated were significantly different between the coastal and open ocean sampling stations.
- Culturable fungi could be isolated from all the sampling locations irrespective of the DO levels, but the diversity index shows that the overall diversity was low in comparison with the mangrove or deep sea regions. The indices from the slope stations within the open ocean OMZ region is also lower in comparison with the coastal station.
- Fungal cultures isolated belonged to the two major phylums Ascomycota or Basidiomycota. Analysis based on the 18S rDNA sequence shows that two of the cultures (# 11 and # 87) were very divergent from any of the cultures described so far.
- Phylogenetic analysis of the environmental fungal phylotypes groups many of the uncultured sequences obtained from this study into clusters with environmental sequences obtained from similar anoxic regions from across the globe (Jebaraj CS, Raghukumar C, Behnke A, Stoeck T, MS in review process. Targeted environmental sequencing

combined with cultivation reveals high fungal diversity in oxygen-depleted regions of the Arabian Sea).

- Screening of a few fungal cultures isolated from the denitrification zones shows that they are able to grow under anaerobic condition. Their ability to denitrify was also demonstrated under suboxic and anoxic conditions. The contribution by fungi in simulated *in-situ* conditions (microcosms) shows that fungi could be playing an important role as denitrifiers in the oxygen depleted regions.

Though the abundance and diversity of fungi has been detailed from this study, the actual contribution of fungi to the denitrification activity in the marine ecosystem is yet to be estimated. Some of the studies that are being carried out as a continuation of the present study are as follows:

- a) Using *nor* gene sequence as a marker to study the fungal denitrification in cultures and in the environmental samples.
- b) Description of the fungus # 11 using multi-gene phylogeny

a) Using *nor* gene sequence as a marker to study the fungal denitrification in cultures and in the environmental samples.

In fungal denitrification system nitric oxide (NO) is reduced to nitrous oxide (N₂O) by an enzyme cytochrome P450*nor*. The catalytic properties of *nor* in fungal system is very distinct in their physiological properties and differ significantly from the bacterial denitrification system (Takaya & Shoun, 2000). The cDNA of the P-450_{dNIR} was sequenced by immunoscreening technique and it came to be the first soluble P-450 of eukaryote origin (Kizawa *et al.*, 1991). Deeper insight into the evolutionary and physiological aspects of P450*nor* came from studying the gene sequence. A cDNA sequence revealed that the polypeptide was about 403 amino

acid residues, corresponding to a molecular weight of 44,371 Daltons (Kizawa *et al.*, 1991). The full length sequence of the p450nor was designated p450norA and the isolated protein was shown to contain an additional threonine at the N-terminus, but a second isoform was also detected with threonine and the adjacent methionine lacking (Nakahara & Shoun, 1996). This P450norB contained the N-terminus blocked by an acetylated alanine, which usually is seen after post- or co-translational elimination of a methionine residue. This latter isoform was mainly found in the cytosol, whereas the longer form (P450norA) was located to the particulate and mainly mitochondrial fraction. It was assumed that P450norA was synthesized with a presequence of 26 amino acids that targets it to mitochondria. This localization of the two isoforms was shown in the fungus *Cylindrocarpon tonkinense*, but in this fungus two separate genes (*CYP55A2* and *CYP55A3*) were found to code for the mitochondrial and cytosolic enzymes, respectively (Kudo *et al.*, 1996). A fourth gene of the P450nor family was isolated from the yeast *Trichosporon cutaneum*, a basidiomycete (*CYP55A4*) and this showed only 65% similarity to the other three genes (zhang *et al.*, 2001). This gene has been isolated from *Aspergillus oryzae* (*CYP55A5*) and the sequence has striking difference with previously described *nor* gene sequences (Kaya *et al.*, 2004). The sequence similarity with the other sequences is only 60%, and the fungal whole genome project of many candidate isolates such as *Neurospora crassa* has increased the database of the fungal *nor* gene sequence available for study. The fungal whole genome project has added to the available database of the p450 *nor* gene sequence obtained from fungi.

In my study, I tried to design degenerate primers based on the sequence data available on the NCBI genebank deposits. The list of primers designed is (Table 5.1) based on the Clustal W alignment of the protein and DNA sequence and identifying a conserved region. As the

homology between the sequence is less than 60%, PCR amplification using the primers designed was very difficult. With the preliminary results obtained and increasing whole genome sequencing of the fungal cultures, refined primers are being designed. Designing of primers to amplify the *nor* gene from fungal cultures and environmental samples could prove to be a power tool for studying the fungal denitrification from various oxygen depleted environments.

Table 5.1 List of primers for the *nor* gene amplification

Primer ^a	Position ^b (nt)	Primer sequence (5'-3')
nor F1	307- 323	ACD TTY GTB GAY ATG GA
nor R1	769-789	CGA ATC ATG YTB ACS AYR GT
nor F2	559-575	GCS AYB CGV ASN AAY GG
nor R2	1087-1103	TCN GCR ATR CAD CKR TG

^a forward and reverse primers are indicated by F and R as the last letter respectively,

^b Positions correspond to the *nor* gene of *Trichosporon cutaneum* (gi:14456619;dbj|AB052733.1 CYP55A4 gene) and nt, nucleotide.

b) Description of the fungus # 11 using multi-gene phylogeny

The fungus # 11 was isolated from the CSt-26 station during the anoxic season (Table 3.). The 18S rDNA sequence analysis of this culture showed only a 92% similarity with the known sequences and the culture grouped closely to Pucciniomycotina of phylum Basidiomycota. Based on the morphological identification, this culture resembled an Ascomycete *Tritirachium* sp. The identification was also confirmed by experts from the Centraalbureau voor Schimmelcultures (CBS) Utrecht, The Netherland. Hence to resolve the taxonomic position of this culture we are presently carrying out a multi-gene phylogeny. For this sequences of the (internal transcribed spacer) ITS region, the large subunit nuclear rDNA gene

sequence (nLSU), largest subunit (RPB1) and the second largest subunit (RPB2) of RNA polymerase II gene sequence and the elongation factor (EF-a) gene sequence was amplified and aligned with the sequences available in the NCBI database (Table 5.2). Results obtained from this study will not only give us the taxonomic position of this culture, but may also give many insights on fungal phylogeny. More detailed description of the morphological features based on SEM and TEM images and physiological analysis of the culture is also being carried out.

Table 5.2 List of primers used for the multi-gene phylogeny.

Primer	Sequence (5' to 3')	Gene amplified	Reference
ITS1	TCCGTAGGTGAACCTGCGG	ITS	Mitchell <i>et al.</i> , 1994
ITS4	TCCTCCGCTTATTGATATGC	ITS	Mitchell <i>et al.</i> , 1994
983F	GCYCCYGGHCAYCGTGAYTTYAT	EF1-a	Rehner & Buckley, 2005
2218R	ATGACACCRACRGCACRGTGTG	EF1-a	Rehner & Buckley, 2005
RPB1-Ac	GARTGYCCDGGDCAYTTYGG	RPB1	Stiller & Hall, 1997
RPB1-Df	TACAATGCY GAYTTYGAYGG	RPB1	Stiller & Hall, 1997
RPB1-G2R	GTCATYTG DGT DGC DGGYTC DCC	RPB1	Stiller & Hall, 1997
fRPB2-5F	GAYGAYMGWGATCAYTTYGG	RPB2	Liu <i>et al.</i> , 1999
RPB2-11bR	CAATCWCGYTCCATYTCWCC	RPB2	Liu <i>et al.</i> , 1999
LR0R	GTACCCGCTGAACTTAAGC	LSU	Vilgalys <i>et al.</i> , 1990
LR7	TACTACCACCAAGATCT	LSU	Vilgalys <i>et al.</i> , 1990

5.3 Future directions

- To develop probes to specifically study the unique fungal clusters obtained from the environmental clone libraries.
- Improve isolation technique to isolate fungal cultures from oxygen depleted environments in anaerobic condition with the use of anaerobic chamber.
- Screen an increased number of cultures obtained from the anoxic regions for their denitrification ability.
- Use mRNA approach to quantify the denitrification ability in the cultures.
- *In-situ* experiments using labeled nitrates should be carried out to precisely study the fungal contribution in the marine denitrification zones.
- Substrate induced respiratory inhibition (SIRIN) studies to quantify the role of fungi in marine denitrification zones.

APPENDIX

1. Inorganic mineral medium

KH ₂ PO ₄	1.36 g
Glucose	30 g
Peptone	2 g
MgSO ₄	0.2 g
Trace element solution	1 ml
NaNO ₃	10 mM
Artificial Sea Water	1000 ml

2. Trace element solution

FeSO ₄ . 7H ₂ O	0.2 g
CoCl ₂ . 6H ₂ O	1.0 g
CuSO ₄ . 6H ₂ O	0.38 g
Na ₂ MoO ₄ . 7H ₂ O	8.6 mg
CaCl ₂	0.2 g
De-ionized water	1000 ml

3. Artificial sea water (pH 7.5)

NaCl	24 g
KCl	0.7 g
MgCl ₂	7 g
De-ionized water	1000 ml

4. Magnesium Chloride 3 M solution for dissolved oxygen estimation

MgCl ₂	6 g
De-ionized water	100 ml

5. Sodium hydroxide solution for dissolved oxygen estimation

Sodium hydroxide pellets	3.2 g
Sodium Iodide	6 g
De-ionized water	100 ml

6. Sulphuric acid 10 N for dissolved oxygen estimation

7. Standard thiosulphate solution (0.01 N) for dissolved oxygen estimation

Sodium thiosulphate	2.9 g
Na ₂ CO ₃	0.1 g
De-ionized water	1000 ml

8. Sulfanilamide (SFN) solution for nitrate and nitrite estimation

SFN	5 g
HCl (conc.)	50 ml
De-ionized water	500 ml

9. N- (1-naphthyl) – ethylenediamine dihydrochloride (NED) solution for nitrate and nitrite estimation

NED	0.5 g
De-ionized water	500 ml

10. Concentrate ammonium chloride solution for nitrate estimation

NH ₄ Cl	125 g
De-ionized water	500 ml

11. Dilute ammonium chloride solution for nitrate estimation

NH ₄ Cl (conc)	50 ml
De-ionized water	2000 ml

12. Phenol solution for nitrate estimation

Phenol crystals	20 g
95 % Ethanol	200 ml

13. Sodium nitroprusside solution for ammonia estimation

Sodium nitroprusside	1 g
95 % Ethanol	200 ml

14. Alkaline reagent for ammonia estimation

Sodium citrate	100 g
Sodium hydroxide	5 g
De-ionized water	500 ml

15. Sodium hypochlorite solution for ammonia estimation

Commercially available Hypochlorite (Chlorox) about 1.5 N

16. Oxidizing solution for ammonia estimation

Mix 100 ml of alkaline reagent (14) and 25 ml of Sodium hypochlorite solution (15)

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ANNEXURE

List of publications from this study:

Cathrine Sumathi Jebaraj and Chandralata Raghukumar (2009)
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