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SUMMARY AND CONCLUSION

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This thesis entitled “Studies on Nitrifying Microorganisms in Cochin Estuary and Adjacent Coastal Waters” reports for the first time the spatial and temporal variations in the abundance and activity of nitrifiers (Ammonia oxidizing bacteria-AOB; Nitrite oxidizing bacteria- NOB and Ammonia oxidizing archaea- AOA) from the Cochin Estuary (CE), a monsoon driven, nutrient rich tropical estuary along the southwest coast of India. It also form the first study of similar nature carried out from any estuary in the Indian region.

Estuaries are the transition zones between fresh- and marine-waters, and are greatly influenced by near shore and anthropogenic activities. Being ecologically sensitive, any change in this buffer zone will have severe impact on the biogeochemical cycles especially the complex biogeochemical cycle of nitrogen. Among the various processes of nitrogen cycle in the estuaries, nitrification has attracted considerable research interest among both chemical and biological oceanographers due to various reasons, from its environmental importance in eutrophication and nitrous oxide emission to the intricacies of chemical transformations and diversity of microorganisms involved. Nitrification is a microbiologically mediated two-step process involving the conversion or ammonia
Studies on nitrifying microorganisms in Cochin estuary and adjacent coastal waters

1. Distribution pattern of nitrifying bacteria, their response to environmental changes in the CE and adjacent coastal waters.
3. Nitrification activity in the CE and adjacent coastal waters and the differential contribution of ammonia oxidizers towards the process.

To fulfil the above objectives, field observations were carried out for a period of one year (2011) in the CE. Surface (1 m below surface) and near-bottom water samples were collected from four locations (stations 1 to 3 in estuary and 4 in coastal region), covering pre-monsoon, monsoon and post-monsoon seasons. Station 1 is a low saline station (salinity range 0-10) with high freshwater influx while stations 2 and 3 are intermediately saline stations (salinity range 10-25). Station 4 is located ~20 km away from station 3 with least influence of fresh water and is considered as high saline (salinity range 25-35) station. Ambient physico-chemical parameters like temperature, pH, salinity, dissolved oxygen (DO), ammonium, nitrite, nitrate, phosphate and silicate of surface and bottom waters were measured using standard techniques. Abundance of Eubacteria, total Archaea and ammonia and nitrite oxidizing bacteria (AOB and NOB) were quantified using Fluorescent in situ Hybridization (FISH) with oligonucleotide probes labeled with...
Cy3. Community structure of AOB and AOA was studied using PCR Denaturing Gradient Gel Electrophoresis (DGGE) technique. PCR products were cloned and sequenced to determine approximate phylogenetic affiliations. Nitrification rate in the water samples were analyzed using chemical NaClO₃ (inhibitor of nitrite oxidation), and ATU (inhibitor of ammonium oxidation). Contribution of AOA and AOB in ammonia oxidation process was measured based on the recovered ammonia oxidation rate. The contribution of AOB and AOA were analyzed after inhibiting the activities of AOB and AOA separately using specific protein inhibitors. To understand the factors influencing or controlling nitrification, various statistical tools were used viz. Karl Pearson’s correlation (to find out the relationship between environmental parameters, bacterial abundance and activity), three-way ANOVA (to find out the significant variation between observations), Canonical Discriminant Analysis (CDA) (for the discrimination of stations based on observations), Multivariate statistics, Principal components analysis (PCA) and Step up multiple regression model (SMRM) (First order interaction effects were applied to determine the significantly contributing biological and environmental parameters to the numerical abundance of nitrifiers).

6.1 Salient Results

Environmental Parameters

- A marked gradient in salinity (0 to 35) was observed in the CE during the study period. The average salinity was 3.8 ± 4.3 in the low saline station, 15.5 ± 9.8 in the intermediate saline stations and 24.3 ± 8.1 in the high saline coastal station. Salinity decreased with the onset of monsoon, (the lowest observed in this study was in July), slowly increased during post-monsoon and reached the maximum during pre-monsoon at all the stations.

- Water column in the study area was well oxygenated throughout the year except for few sampling occasions (average 5.04 ± 1.4 mg L⁻¹). Low level of DO observed in the intermediate saline station. DO in the CE did not follow any seasonality.
Suspended particulate matter (SPM) levels in the CE varied from 3.2 to 155 mg L\(^{-1}\), with higher concentration during monsoon. High turbidity in the CE may enhance the nitrification rate.

Seasonal and spatial variations of nutrient levels were observed at all the stations. Ammonia was the major component of dissolved inorganic nitrogen and was significantly lower during the peak monsoon period and maximum at the end of pre-monsoon. Ammonia varied from 5.65 to 47.72 μM in the low saline station, 1.32 to 49.0 μM in intermediate saline stations and 0.17 to 28.35 μM in the high saline station. Unlike ammonia, nitrite levels were relatively high during monsoon and low during the pre-monsoon period. While the nitrate concentration exhibited the vice versa. Nitrite concentration varied from 0.04 to 0.84 μM, whereas nitrate varied from 0.34 to 28.35 μM. Phosphate concentration ranged from 0.1 to 2.81 μM with higher values during monsoon season. The N / P ratio was higher during the pre-monsoon (average 72.0 ± 62.4) and post-monsoon (average 44.1 ± 32.5) seasons, but relatively lower (average 11.5 ± 8.7) during the monsoon season. Silicate concentration ranged from 0.01 to 83.53 μM with higher values during monsoon and lower values during the pre-monsoon months.

**Microbiology Parameters**

- Total microbial abundance estimated by DAPI staining ranged between 7.53 × 10^5 and 1.91 × 10^6, cells ml\(^{-1}\). Eubacteria and Archaea enumerated using FISH ranged between 3.3 and 6.9 × 10^5 and 1.9 and 5.48 × 10^5 cells ml\(^{-1}\), respectively. Maximum abundance of Eubacteria and Archaea was observed during the pre-monsoon season and minimum during the monsoon season with higher abundance at low and intermediate saline stations. Irrespective of the stations or seasons bottom waters recorded higher population of both Eubacteria and Archaea than the surface waters.

- Abundance of β AOB and *N. mobilis* ranged from 3.15 to 9.31 × 10^4 and 1.01 to 4 × 10^4 cells ml\(^{-1}\), respectively. Among the NOB, *Nitrobacter* sp. and *Nitrospira* sp. abundance ranged from 2.69 to 7.63 × 10^4 and 2.51 to 6.17 ×
10^4 cells ml^{-1}, respectively. Higher abundance of nitrifiers were observed during the pre-monsoon months.

- Abundance of AOB and NOB showed heterogeneity between the sampling sites. Higher abundance of AOB and NOB was recorded during the pre-monsoon and the lowest during the monsoon, indicating significant seasonal variation (p <0.01). Significant difference in the abundance was also observed between surface and bottom waters (p < 0.05).

- Diversity of AOA was higher than AOB. Community structure of AOB did not show spatial and temporal changes whereas AOA showed spatial and temporal changes. Phylogenetic analysis of DGGE bands showed major affiliation of AOB to β proteobacteria.

**Nitrification**

- Nitrification rate varied from 0.05 to 10.22 μM N day^{-1} in the CE with comparatively higher activity in estuarine stations than the coastal station. A 10 to 40 fold increase in the nitrification rate was observed during the pre-monsoon season compared to the monsoon season (0.05- 0.26 μM N day^{-1}).

- The recovered ammonia oxidation rate of AOB was in the range of 45-65%, whereas for AOA, it was 15-45 %, indicating that AOB were mostly responsible for the ammonia oxidation in the CE.

**Inter-relationship**

- The sampling stations in the CE evolved from a low ammonia; low AOB- low nitrification in the monsoon season to high ammonia; high AOB high nitrification rate in the pre-monsoon with post monsoon season as a transition period where the ratio gradually increased.

- Ammonia concentrations modulate the nitrification in the CE and intermediate salinity was the most preferred environmental condition.

- AOB were the major players in modulating ammonia oxidation compared to AOA.
The overall assessment is that, though the CE was under the influence of high anthropogenic load, frequent eutrophication was not observed in these waters due to nitrification. Most importantly, it regulated the nutrient flux into coastal waters as the concentration of ammonia at the coastal station was much less than that observed in the estuarine stations.

The study highlights the trophic nature of the nitrifiers prevailing in the CE waters, which will enable the efficient management of this estuary.

6.2 Conclusions

In the CE, nitrification is modulated by the complex interplay between different nitrifiers and environmental variables which in turn is dictated by various hydrodynamic characteristics like fresh water discharge and seawater influx brought in by river water discharge and flushing. AOB in the CE are more adapted to varying environmental conditions compared to AOA though the diversity of AOA is higher than AOB. The abundance and seasonality of AOB and NOB is influenced by the concentration of ammonia in the water column. AOB are the major players in modulating ammonia oxidation process in the water column of CE. The distribution pattern and seasonality of AOB and NOB in the CE suggest that these organisms coexist, and are responsible for modulating the entire nitrification process in the estuary. This process is fuelled by the cross feeding among different nitrifiers, which in turn is dictated by nutrient levels especially ammonia. Though nitrification modulates the increasing anthropogenic ammonia concentration the anthropogenic inputs have to be controlled to prevent eutrophication and associated environmental changes.

6.3 Future research plan

In this study, I could demonstrate the presence of AOA and its contribution to nitrification. There are still lots of questions to be answered while considering AOAs’ ecology. The main reason for our poor knowledge on physiology of AOA is the fastidious nature which makes it difficult to culture these organisms in the laboratory. Another ammonia oxidizing pathway that has recently been established is Anammox, it is anaerobic oxidation of ammonia using nitrite as
the electron accepter and nitrogen gas as the end product. It occurs mostly in suboxic regions and is the final stage of nitrogen removal in the estuary especially in the sediments. My future plan of work will be to understand the diversity, physiology, and underlying biochemistry of estuarine AOA and Anammox and the contribution of Anammox in the maintenance of homeostatic of the estuary.

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