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Nitrogen transformations as inferred from the activities of key enzymes in the Arabian Sea oxygen minimum zone

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Abstract

Vertical distributions of the potential activities of some key enzymes mediating nitrification and denitrification were investigated within the oxygen (O_2) minimum zone of the Arabian Sea at a number of locations between latitudes 17 and 21^oN and longitudes 63 and 68^oE so as to get an insight into the predominant biochemical mode(s) of production and consumption of nitrous oxide (N_2O). Results revealed that the dissimilatory nitrate (NO_3^-) reduction activity was generally very low or absent within the σ_θ range 26.6-26.8, which corresponds to the Persian Gulf Watermass (PGW). Depth profiles of nitrate reductase (NaR), nitrite reductase (NiR) and ammonia monooxygenase (AMO) activities were compared with those of O_2, NO_3^-, nitrite (NO_2^-) and N_2O, and it is concluded that nitrifier denitrification rather than heterotrophic denitrification is active within the core of PGW. The presence of multiple peaks of AMO activity coinciding with distinct maxima in the O_2 profile and with a trend opposite to that of NaR activity indicates that the two processes, *viz*., classical and nitrifier denitrification, occur in discrete layers, probably determined by the variations in the ambient O_2 concentrations at various depths surrounding the PGW core. Further, it appears that at the depths where nitrifier denitrification is active in the absence of heterotrophic denitrification, N_2O builds up as its consumption may be inhibited by O_2. Possible reasons for the occurrence of appreciable nitrate deficit within the core of PGW, where dissimilatory NO_3^- reduction is lacking, are discussed.

Key words: Denitrification, nitrification, enzyme activities, nitrous oxide, oxygen minimum zone, northern Arabian Sea

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Introduction

The global emission of nitrous oxide (N_2O) to the atmosphere is steadily rising, and the current sources of N_2O exceed the sinks (Bouwman *et al*., 1995). This has led to environmental concerns, since N_2O contributes significantly to the greenhouse effect (Lacis *et al*., 1981; Dickinson and Cicerone, 1986) and to the destruction of the stratospheric ozone layer (Crutzen, 1979). Production of N_2O by natural sources is believed to be twice the anthropogenic emission (Prather *et al*.,1995), and oxygen (O_2)-deficient oceanic waters are significant natural sources of N_2O to the atmosphere (Codispoti and Christensen, 1985). The eastern tropical North and South Pacific Ocean and the Arabian Sea are the three major sites in the open ocean where denitrification is the dominant respiratory process in the water column. Denitrification is the anaerobic mode of respiration resorted to by certain bacteria which utilize nitrate (NO_3^-) or nitrite (NO_2^-) as the terminal electron acceptor when dissolved O_2 is limiting or absent. The metabolic pathway involves the stepwise reduction of NO_3^- to dinitrogen (N_2). Several intermediates such as NO_2^-, nitric oxide (NO) and N_2O are formed and can be set free to the environment. The metalloenzymes involved in these conversions are nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS).

The near-total depletion of O_2 at mid-depths (between ~150 m and 1000 m) in the Arabian Sea causes large-scale denitrification, especially in the

upper one-third of the oxygen minimum zone (OMZ), as indicated by the presence of a secondary nitrite maximum (SNM; Naqvi, 1987). The Arabian Sea covers only ~2% of the global oceanic area but accounts for about 20% of the oceanic denitrification and emits 0.5 - 1 Tg N y^{-1} of N_2O to the atmosphere (Bange *et al*., 2000). Additionally, during the SW monsoon, the upwelling centre off the west coast of India is known to become another site of significant N_2O emission (Naqvi *et al*., 1998; 2000). An inherent feature of the Arabian Sea and other pelagic OMZs is the presence of suspended particle maxima in association with the SNM (Naqvi *et al*., 1993). The particle maximum characteristically exhibits enhanced biogeochemical activity as seen from the elevated bacterial biomass (Spinrad *et al*., 1989; Ward et al., 1998) and respiration measured as electron transport system (ETS) activity (Naqvi *et al*., 1993). The composition of the dense bacterial assemblage has not been adequately investigated.

The driving biological process for the unusually large N_2O build-up in oceanic OMZs is mostly unknown. This is because microbes produce N_2O through both nitrification and denitrification, two processes that are regulated by contrasting environmental conditions. Nitrification involves the oxidation of ammonia (NH_3) to NO_3^- via NO_2^- by O_2. Ammonia monooxygenase (AMO) is the key enzyme responsible for the conversion of NH_3 to NO_2^-. However, under certain circumstances, such as low O_2 concentration, nitrifier denitrification, i.e., reduction of NO_2^- to gaseous products by NH_3 oxidizers, can become an important source of N_2O. Studies on the isotopic composition of N_2O in the Arabian Sea OMZ have not yielded clear-cut results in regard to the mechanisms

of N_2O formation (Yoshinari *et al*., 1997; Naqvi *et al*., 1998). Analyses of the interrelationships of O_2, NO_3^- and NO_2^- with biochemical parameters (activities of the electron transport system and enzymes of denitrification, viz., NaR and NiR) have indicated that nitrification could be an important biological process in the core of the denitrifying zone (Shailaja, 2001).

The basic aim of this work was to improve our understanding of the nitrogen dynamics in the Arabian Sea OMZ, especially the mechanisms of N_2O formation. Here we present fine-scale depth profiles of the potential activities of some key enzymes (AMO, NaR and NiR) mediating nitrification and denitrification in relation to relevant chemical parameters (NO_2^-, NO_3^- and N_2O) that provide new insights into vertical distributions of different enzyme activities / microbial processes vis-à-vis physical and chemical gradients within denitrifying waters of the northern Arabian Sea.

Materials and methods

The analyses required for this study were carried out on board FORV *Sagar Sampada* (cruise no. 161, January 1998) and *A.A. Sidorenko* (cruise no. 42, February/March 2002) at stations located between latitudes 17 and 21^oN and longitudes 63 and 68^oE in the northern Arabian Sea (Fig. 1). A SeaBird Electronics conductivity-temperature-depth (CTD) profiler SBE-9 equipped with an O_2 probe (SBE-13-02) and a Sea-Tech 25-cm beam transmissometer (129D) and mounted on a General Oceanics rosette fitted with 10L Go-Flo bottles was employed for *in situ* measurements and water sampling. A Skalar analyser was

used to analyse nutrients by standard methods (Grasshoff *et al*., 1983). O_2 values were obtained by the Winkler titration method in discrete samples, without applying any blank corrections.

The subsurface particle maxima are associated most often with the core of the SNM. Water sampling depths were decided with reference to SNM. Samples for analyses of both chemical (NO_3^-, NO_2^- and N_2O) and biochemical (enzyme) parameters were drawn from the same bottles.

Enzyme assays: a) NaR activity. NaR activity was determined by the method of Packard *et al*. (1978). The crude homogenate (total volume ~6.5 ml), prepared immediately after filtration of 5 L seawater by grinding the filter at 0-4^0 C for 2 minutes with 3 ml of 0.2 M phosphate buffer (pH 7.9) containing 9 mg polyvinyl pyrrolidone and 0.5 mg dithiothreitol, was used for both NaR and NiR assays. Incubation of 1 ml of the crude extract with 0.8 ml substrate mixture prepared in 0.2 M phosphate buffer (pH 7.9) and containing 240 μM NADH, 8 mM KNO_3 and 110 μM $MgSO_4$ for NaR assay was done anaerobically in an atmosphere of helium.

b) NiR activity. The reaction mixture for NiR assay was as described earlier (Shailaja, 2001). Briefly, 1 ml of the homogenate (prepared as above) was reacted with substrate mixture consisting of 1.7 ml of 0.2 M phosphate buffer (pH 7.9), 0.1 ml of NADH (2 mM), 0.1 ml of $NaNO_2$ (9 mM in glass-distilled water) and 0.1 ml freshly-prepared sodium dithionite - bicarbonate solution (60 mg each of sodium dithionite and sodium bicarbonate in 2.5 ml glass-distilled water). Incubations were carried out anaerobically for 30 minutes at 24^0 C. The reaction

was terminated with 0.1 ml zinc acetate and 1.9 ml of 95% ethanol. The mixture was clarified by centrifugation (or filtration through a glass fibre filter) and an aliquot of the supernatant (or filtrate) taken for NO_2^- analysis.

c) Ammonia oxidizing activity (AMO). The cell lysate for AMO assay was prepared immediately after filtration of 2.5 L seawater on GF/F filter, essentially following the method described for ETS (Naqvi and Shailaja,1993). 0.5 ml of the crude homogenate was incubated in the dark for 30 minutes with 2.5 ml of a substrate mixture containing 400 µmol of NH_4Cl, 0.5 µmol each of NADP and NADPH, 0.2 µmol ATP in 0.05 M phosphate buffer, pH 7.8. Controls were run without NH_4Cl. Absorbance was measured at 340 nm to estimate the amount of NADPH formed by reverse ETS and the incubation continued up to 60 minutes to measure NO_2^- formed (Grasshoff *et al*., 1983).

All the three enzyme assays involved measurement of NO_2^-, either produced (NaR and AMO) or consumed (NiR). The limit of detection for the assays was 0.048 µM NO_2^- h^{-1}. The enzyme activity measurements were replicated wherever possible, depending on the available sample size. Thus, on the first cruise, NaR and NiR assays were done in duplicate on samples from 4 depths at Stn. 4010 and on only one sample each at the remaining stations (Figs. 2 and 3). On the second cruise, replicate measurements of NaR, NiR and AMO activities were made on six samples (Fig. 6).

Estimation of nitrate deficit. Nitrate deficit was computed from the expected values of the geochemical tracer 'NO' (Broecker, 1974) and the observed O_2, NO_2^- and NO_3^- concentrations using the equation,

$$\Delta N = (\text{'NO'} - O_2) / 9.1 - NO_2^- - NO_3^-$$

The 'NO' values were obtained from the linear relationships with θ observed outside the denitrification zone (Naqvi et al., 1990).

Statistical analyses. Statistical evaluations of correlation were performed with Excel 2000 spreadsheet (Microsoft) software. $P < 0.05$ was considered as statistically significant.

Results and discussion

As stated earlier, denitrification involves an array of unique reductases. Vertical distributions of the activities of NaR and NiR at various stations are shown in Figs. 2 and 3. NaR is the first enzyme in the reduction series and therefore could be considered the initiator enzyme of the denitrification pathway; it is followed by NiR, which catalyses the formation of the first gaseous intermediate of the pathway, NO from NO_2^-. An unexpected feature noted at a number of stations was the absence of NO_3^- reducing activity in waters within the σ_θ range 26.6 and 26.8 (Fig. 2). This density range is distinguished by a salinity maximum corresponding to the Persian Gulf Watermass (PGW) (Ramesh Babu *et al*., 1980; Morrison *et al*., 1998). In the same watermass, activity of NiR was found to be substantial (Fig. 3). These observations support the suggestion that denitrifiers might be active within distinct layers, considering NaR as the diagnostic enzyme for the classical denitrification pathway. However, the potential enzyme activities, expectedly, are different at different times as is true

for the intensity of denitrification in various seasons during the year [indicated by NO_2^- and nitrate deficit values (Naqvi, 1987)]. Even so, the general features of the activity profiles of the denitrifying enzymes have been observed to remain much the same at different times of year, NaR remaining close to zero in the PGW (Shailaja, 2001 and unpublished data).

To investigate the role played by PGW in the discrete distribution of the denitrifying activity, we made observations over several days aboard *A.A. Sidorenko* at 21°N, 64°E (station 19, Fig.1). The salinity profile at this site showed PGW to be prominent within the depth range 170-380 m, corresponding to a σ_θ range of ~25.7-26.86 (Fig. 4). The vertical profile of O_2 within this watermass displayed several peaks closely matching those in salinity, as shown by the CTD O_2 sensor data (Fig. 4). The SNM was located in the upper part of the PGW, at around σ_θ =26.13, while the highest nitrate deficit was found just below this watermass, i.e., at σ_θ =26.9 (Fig. 5). Interestingly, the lower part of PGW was associated with appreciably high nitrate deficits.

The vertical profile of NaR activity exhibited several peaks in close association with the minima in the O_2 profile (Fig. 6). In contrast, at many depths within the PGW, NaR activity was absent, and these depths generally corresponded to peaks in the O_2 profile (based on discrete Winkler measurements). It is known that the ambient O_2 levels and the nature of nitrogen oxides available for respiration together control expression of the denitrification enzymes, NaR, NiR and NOS (Körner and Zumft, 1989). In facultative anaerobes, synthesis of membrane-bound NaR is induced by NO_3^- under low O_2

tension (Ferguson, 1994). An additional respiratory pathway is also known in which NO_3^- is reduced by a widely distributed, periplasmic NaR, synthesized and active in the presence of O_2 (Bedzyk and Ye, 1999; Siddiqui *et al*., 1993). The latter type of NaR was, arguably, not present in our samples, since the presence of O_2 (>5 μM) in samples obtained at 250 m (σ_θ = 26.439) and 300 m (σ_θ = 26.658) was found to abolish the NaR activity totally.

On the other hand, the NiR activity, unlike NaR, was never completely absent. The depths where crests occurred in the NiR activity profile were practically the same as those of NaR activity maxima (Fig. 6). However, the two profiles differed in respect of the depths where NaR activity was zero but NO_2^- reduction by NiR continued to occur (though not maximally) in the presence of O_2. The system evidently is quite complex, and the various enzymes probably are regulated at different levels due to biochemical constraints of the cell and bacterial diversity. The observed differences in the magnitude of NaR and NiR activities (Figs. 2, 3 and 6) seem to reflect a temporal decoupling in the intensification/decline of the two activities: values shown in Figs. 2 and 3 were obtained in early January, i.e., during mid-NE monsoon season, whereas those shown in Fig. 6 were obtained towards the end of the same season, i.e., from February end to early March. This may well be a constant feature, especially in the PGW-affected waters of the Arabian Sea OMZ, as similar observations have been made even during early NE monsoon, i.e., mid-November (Shailaja, 2001).

The synthesis of each denitrifying enzyme is differentially inhibited by O_2 concentration, the enzymes being induced sequentially as the environment

moves towards anaerobiosis (Otte et al., 1996). The threshold value of O_2 for synthesis of NiR (cytochrome cd_1), which is under particularly stringent control by O_2, is about half that of NaR (Körner and Zumft, 1989), which is not consistent with our present observations. In our study, the O_2 profile exhibited maxima at the depths where NaR activity was nil but NiR could still be measured, thereby ruling out contribution from regular denitrifiers to the NiR activity at those depths. Furthermore, studies with a mutant of *Pseudomonas fluorescens* whose narG gene had been disrupted to cause abolition of the NaR activity have demonstrated a regulatory link between NO_3^- and NO_2^- respiration in the denitrification pathway (Ghiglione *et al*., 1999). Thus, based on the above observations and the possibility that there was no substrate (NO_2^-) available for NiR induction in the heterotrophic denitrifiers owing to the lack of NaR activity, it is our hypothesis that the NiR enzyme occurring in the absence of NaR is derived from a source other than the classical denitrifiers.

Source(s) of NiR

Apart from the denitrifying bacteria, the ability to produce NO and N_2O is widespread among autotrophic ammonia oxidizers (Hooper, 1968; Goreau et al., 1980; Poth and Focht, 1985). The reduction of NO_2^- to the gaseous products (NO and N_2O) during nitrifier denitrification is aided by a Cu-dependant NiR (Hooper, 1968; Ritchie and Nicholas, 1974; Miller and Nicholas, 1985), whereas in the heterotrophic denitrifying bacteria two distinct types of NiR are found: one containing a heme cd1 as the redox-active centre, and the other containing Cu as the redox-active transition metal. The two forms are functionally equivalent but

structurally different. Genetic evidence shows that the Cu-containing NiR of *Nitrosomonas marina* is homologous with its counterpart in classical denitrifiers, but preliminary gene sequence data of NiR of *Nitrosomonas europaea* have shown a rather low similarity (Casciotti and Ward, 2001). In contrast to denitrifying bacteria, the expression of NiR in *N. europaea* is not repressed by O_2 (Whittaker *et al*., 2000). However, the enzyme, while present, does not transform NO_2^- when O_2 concentration is high but reacts only when it is diminished (Goreau *et al*., 1980). Reasonably, therefore, the NiR activity observed in the absence of NaR in our study could have been due to nitrifying bacteria. The enzyme assay procedures normally followed for NiR, which are based on the measurement of consumption of the substrate (NO_2^-), do not make a distinction between nitrifier and denitrifier NiRs. Hence, to ascertain the presence of ammonia oxidizers within the OMZ, we estimated ammonia oxidising activity at several depths including those where NaR was absent.

In the nitrification process, oxidation of NH_4^+ to NO_2^- is carried out sequentially by ammonia monooxygenase, which oxidizes NH_4^+ to hydroxylamine using NADH as electron donor, while NO_2^- is formed from hydroxylamine by the enzyme hydroxylamine oxidoreductase (Wood, 1986). This oxidation produces a proton gradient which forces the electrons all the way back to NAD(P) through an energy-consuming reverse ETS, to generate reducing power, viz., NAD(P)H (Wood, 1986), whereas the normal or respiratory ETS oxidizes reduced NAD(P). Reverse ETS is a distinctive feature of nitrification.

We determined ammonia oxidising activity (termed here as AMO activity) both from the measurement of reverse ETS (i.e., the amount of NADPH formed) and formation of NO_2^- from NH_4^+ in crude cell homogenates. The enzyme activity assayed by the former method was linear up to 60 minutes. Also, the reverse ETS activity (determined after 30 minutes of incubation) and the amount of NO_2^- formed (determined between 90 and 120 minutes of incubation) showed very similar trends (Fig. 7). However, the former could not be calibrated with reference to the amount of NO_2^- formed, which could be measured only after ~90 minutes of incubation. Multiple activity peaks of AMO were observed, generally coinciding with maxima in the O_2 profile. Mostly, AMO and NaR activities displayed opposing trends although no significant correlation was found between them or between any of the enzyme activities.

The samples with high AMO activity obtained from the σ_θ range of 26.4 - 26.8 had Winkler O_2 concentrations ranging from ~4.8 to 7.2 μM (Fig. 6). As stated earlier, the Winkler O_2 data were not corrected for blanks. The minimum O_2 concentration observed by us within the SNM was ~3 μM. Colorimetric measurements have shown that for active denitrification to take place, as reflected by the appearance of secondary NO_2^-, the O_2 concentration must fall below a threshold value of < 1 μM (Morrison et al., 1999). Thus, while our Winkler O_2 values are probably systematically higher by ~2 μM than the actual values, the O_2 concentrations within the PGW core are certainly above the denitrification threshold as evident from the O_2 data. These concentrations are also above the experimentally observed threshold (2 μM) required for the

synthesis of NiR in the heterotrophic denitrifiers (e.g., *Achromobacter cycloclastes;* Coyne and Tiedje, 1990). Thus, it is highly unlikely that the NiR activity observed within the PGW core was due to classical denitrifiers. Although no significant correlation could be made out between AMO and NiR activities ($p>0.5$), a maximum in the latter activity was observed at depths where peak NaR and AMO activities were also present. Maximum accumulation of NO_2^- occurred when NaR activity was high and simultaneously AMO activity was also present, that is, in waters of σ_θ = 26.131 (Figs. 5 and 6). On the other hand, in the near-absence of AMO as seen at σ_θ = 26.314, NO_2^- accumulation was much less in spite of NaR activity being substantial. We could not find any significant correlation between the NO_2-consuming (NiR) activity and the NO_2-producing NaR and AMO activities probably because both denitrifiers and NH_3 oxidizers contribute to the pool of NiR, the individual contributions being subject to control by in situ O_2 levels. Also, a lack of correspondence between AMO and NiR activities would arise, particularly in the absence of NaR, if the NO_2^- formed by AMO were to be consumed by more than one process.

A comparison of the depth profiles of AMO activity and N_2O showed that while the peaks in AMO corresponded to high N_2O concentrations, no significant correlation was seen between the two; which was true for other enzyme- and chemical parameters as well. The depth where N_2O was consumed, as indicated by a decrease in its concentration, was the same as that of NaR maximum (Figs. 5 and 6). Considering the facts that (a) NO_3^- reduction is the initiator reaction for denitrification, and (b) the gene encoding for NaR is not present in the nitrifiers

such as *N. europaea* (Whittaker et al., 2000), it seems reasonable to infer that heterotrophic denitrification and nitrifier denitrification occur in close proximity to each other and, possibly, in discrete layers within the Arabian Sea OMZ. The separation of the processes into distinct layers appears to be largely on account of the differences in O_2 concentration brought about by the inflow of PGW having only modest O_2 that leads to the creation of a mosaic of low O_2 concentrations suitable for the two processes to flourish at different depths immediately surrounding its core.

On the other hand, nitrifier denitrification, associated with peak AMO activity but moderate NiR activity and completely devoid of NaR activity, stops short of reducing N_2O, thus leading to its accumulation. This seems reasonable, since only a few species of autotrophic ammonia oxidizers have been shown so far to produce N_2, while production of NO and N_2O is more widespread (Schmidt and Bock, 1997; Schmidt et al., 2004). Where nitrifiers are actively denitrifying, heterotrophic denitrifiers, if present, do not reduce N_2O because NOS is reportedly the most O_2-sensitive among the denitrifying enzymes and its activity is inhibited by O_2 (Otte *et al*., 1996), thereby leading to N_2O accumulation.

Nitrifier denitrification and nitrate deficit

Nitrate deficit (ΔN) is a quantity integrated over long periods of time and also affected by mixing processes. Therefore, although it indicates the extent of denitrification a parcel of water has been subjected to, it does not necessarily reflect ambient denitrifying conditions. The occurrence of appreciable ΔN (Fig.

5e) within layers of PGW in the Arabian Sea OMZ that apparently did not experience heterotrophic denitrification is intriguing, but there are several possible ways by which these deficits could be produced: (1) The water-column O_2 concentrations in the Persian Gulf never reach suboxic levels (Brewer and Dyrssen, 1985), but N:P ratio in the PGW is lower than in the water it mixes with after flowing out of the Hormuz Strait (Deuser et al., 1978). This shallow, warm, productive marginal sea is expected to experience vigorous sedimentary denitrification that may create ΔN in the outflowing water; (2) Lateral mixing causes an "export" of ΔN from the core of the denitrification zone located to the east of the sampling site (Naqvi, 1991); (3) While the bulk of the medium may not be anaerobic, prevalence of such conditions within micro-sites may support loss of combined nitrogen through anaerobic ammonium oxidation (Anammox; Jetten *et al*., 1998; Kuypers et al., 2005); and (4) Nitrifier denitrification may lead to the creation of ΔN due to the reductive loss of NO_2^- formed through the oxidation of NH_4^+. Li et al. (in review) have recently found evidence for appreciable loss of NO_3^- within the lower oxycline of the Arabian Sea where the O_2 concentrations are well above the denitrification threshold. These anomalies were attributed to "partial nitrification," whereby a part of the NH_4^+ oxidized is lost as either N_2 or N_2O, or both. Which of the above processes, either individually or in unison, and to what extent, contributes to the production of the computed ΔN remains to be investigated.

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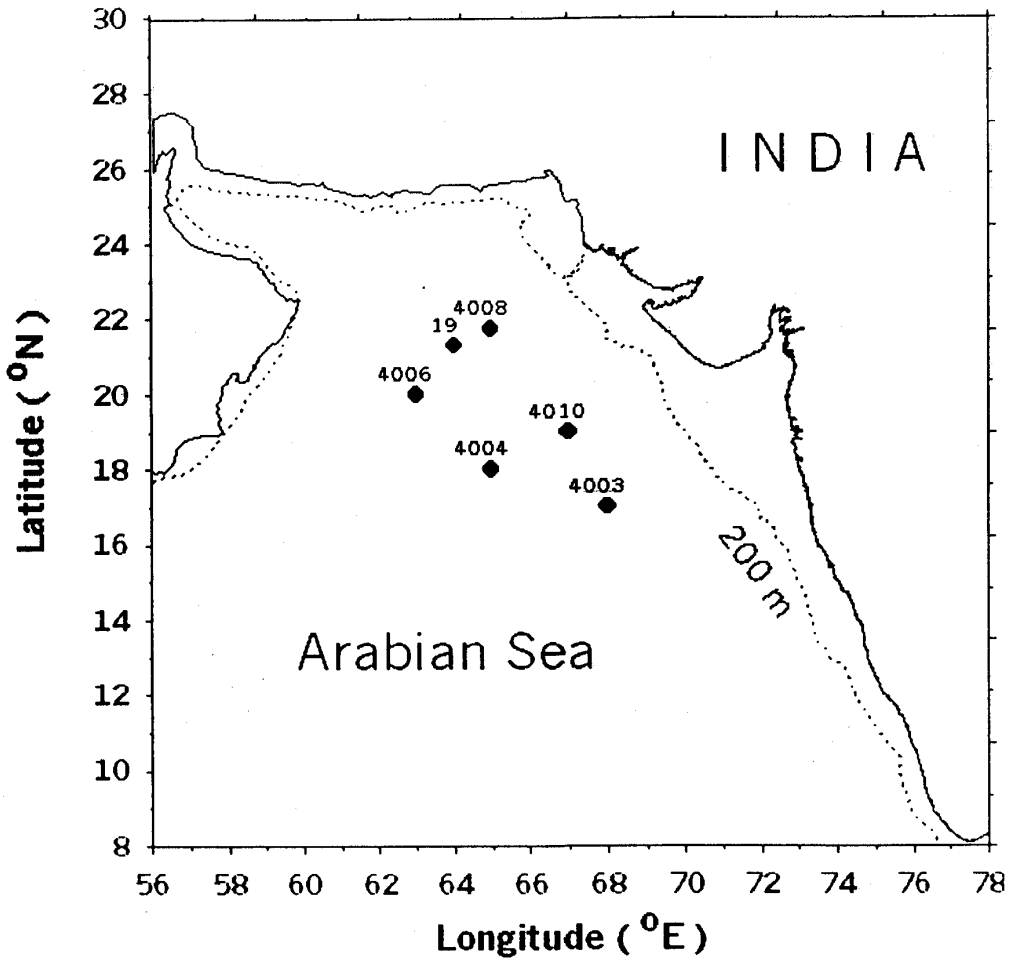

Fig.1. Location of the stations where the present study was carried out. Stations 4003 (17°N, 68°E); 4004 (18°N, 65°E); 4006 (20°N, 63°E); 4008 (21°43'N, 65°E) and 4010 (19°N, 67°E) were occupied on *Sagar Sampada* cruise161 while station 19 (21°N, 64°E) was sampled during *A.A. Sidorenko cruise 42*.

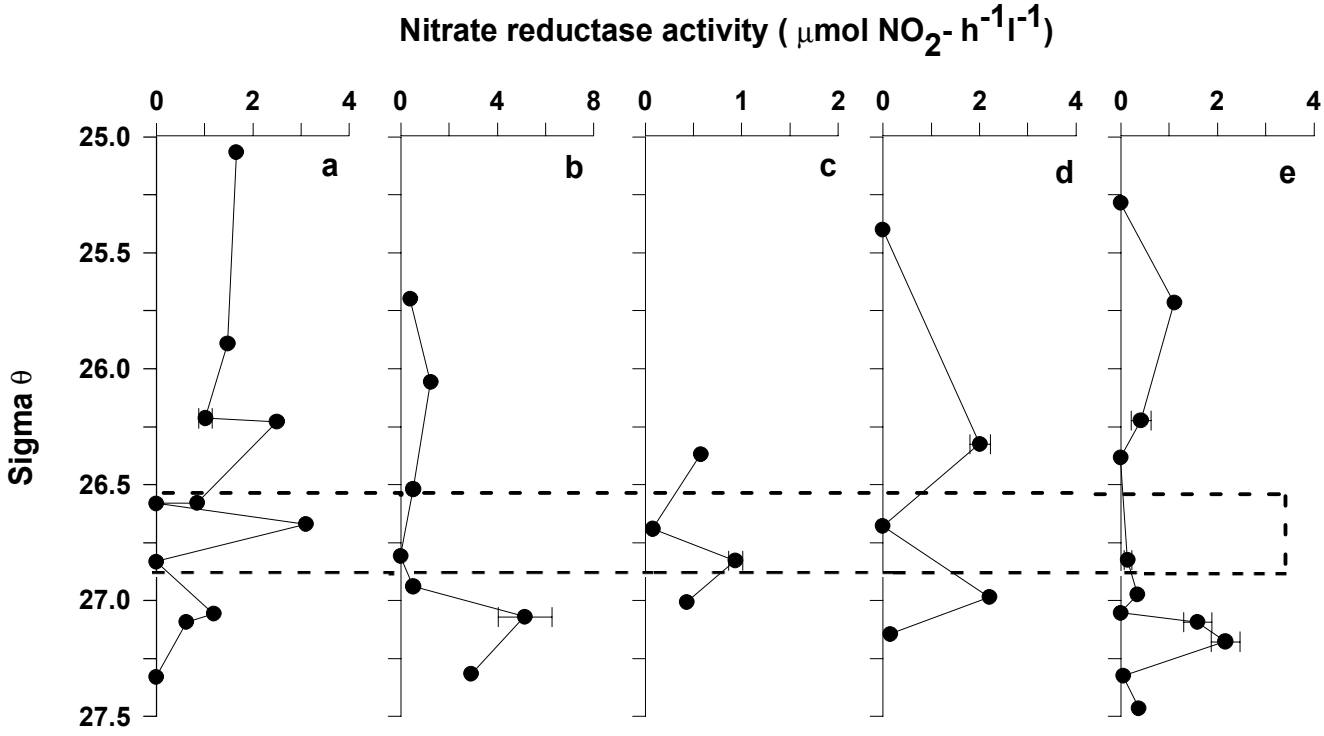

Fig. 2. Distribution of potential activities of nitrate reductase versus Sigma θ at station (a) 4003 ; (b) 4004 ; (c) 4006; (d) 4008 and (e) 4010. The marked areas in the figure denote the density range of PGW.

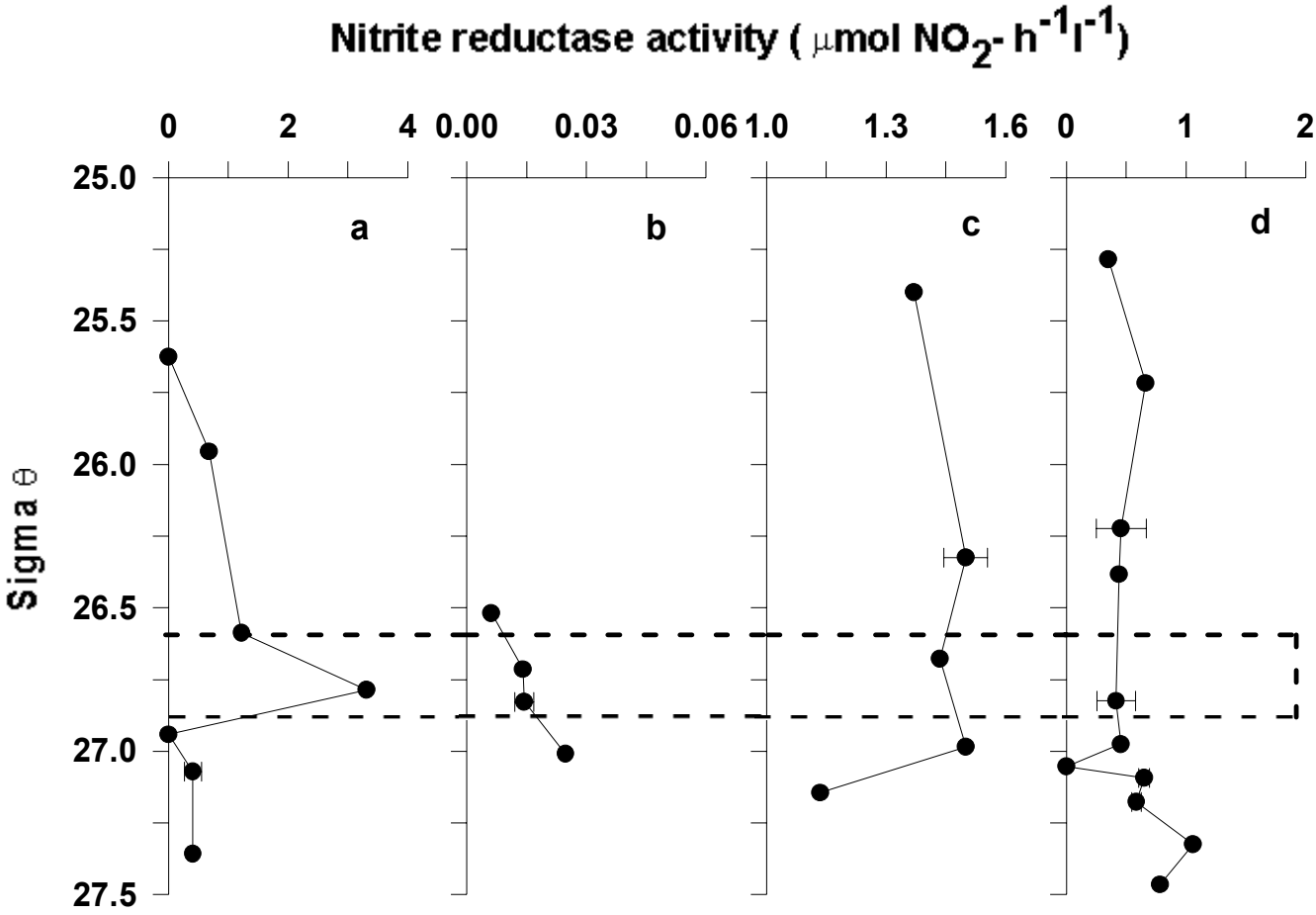

Fig.3. Distribution of potential activities of nitrite reductase versus Sigma θ at station (a) 4004 ; (b) 4006 ; (c) 4008 and (d) 4010. Other details as for Fig. 2.

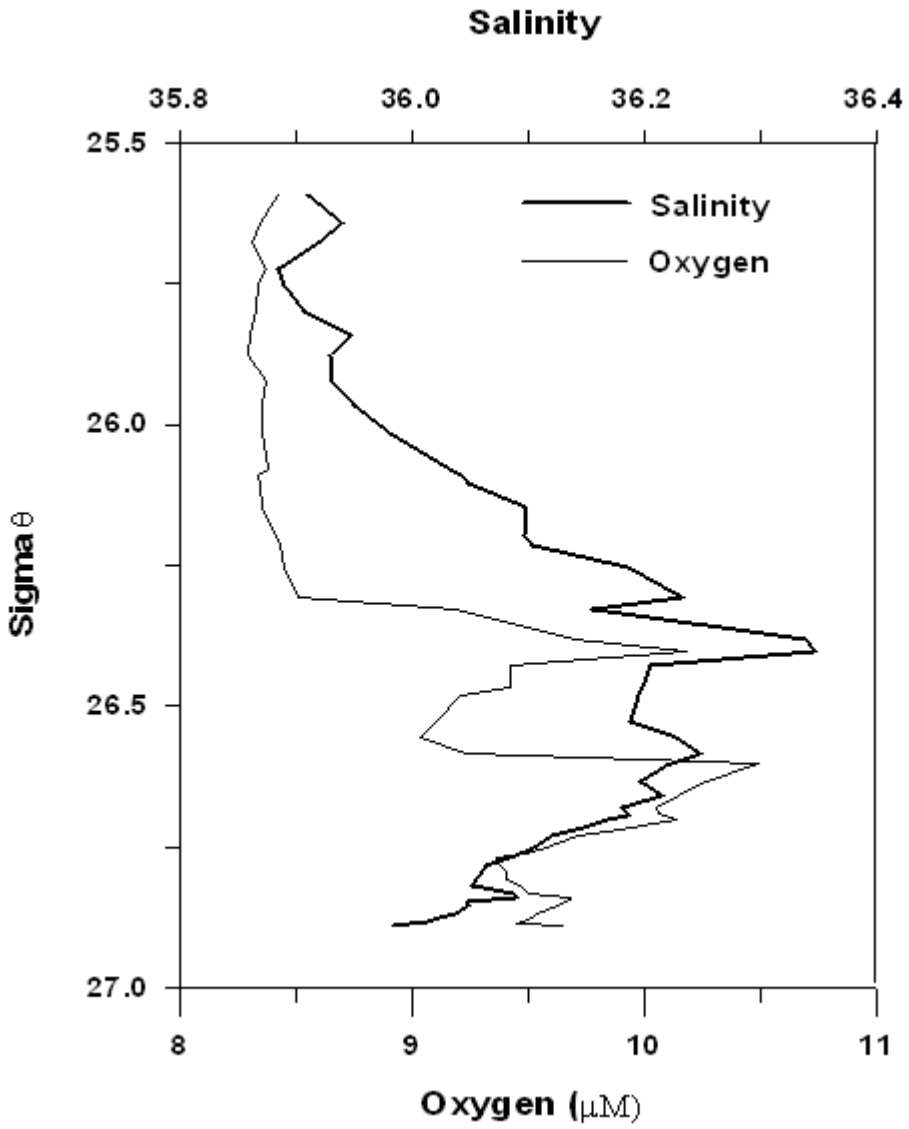

Fig.4. Continuous vertical profiles of salinity and oxygen at station 19. (The CTD oxygen sensor data shown have not been corrected with reference to the titrimetric Winkler concentrations.)

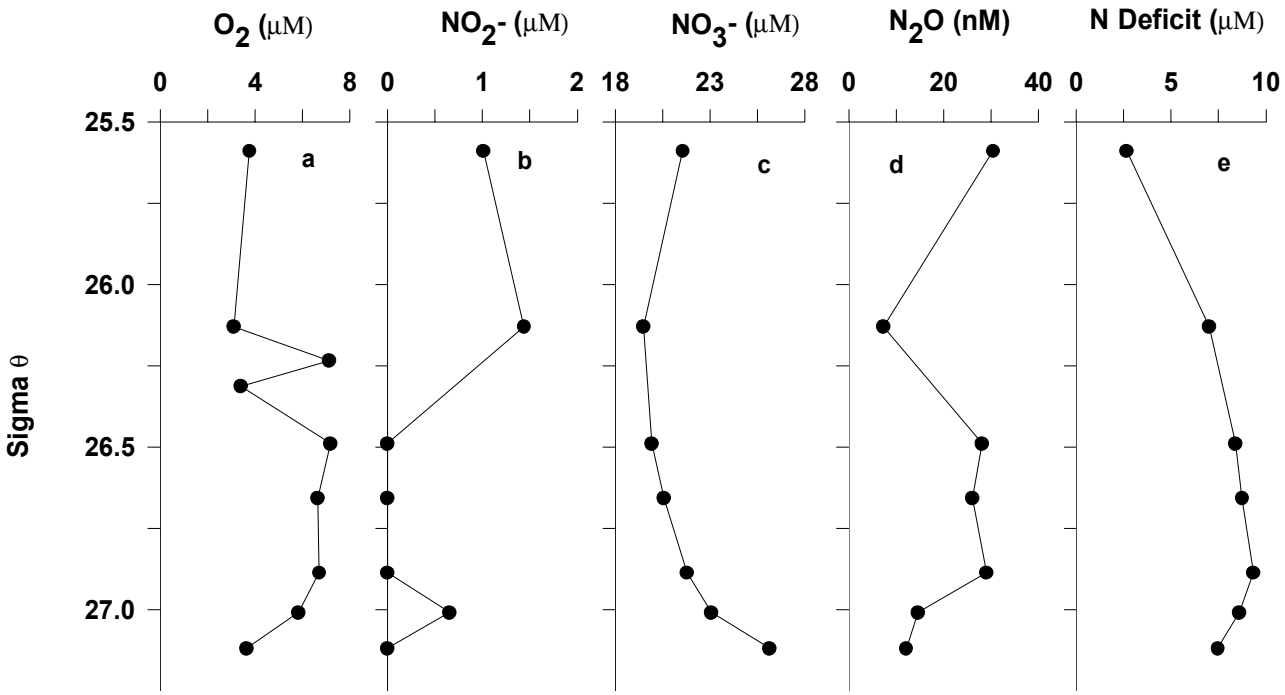

Fig.5.Depth profiles of (a) O_2, (b) NO_3^-, (c) NO_2^-, (d) N_2O and (e) nitrate deficit at station 19.

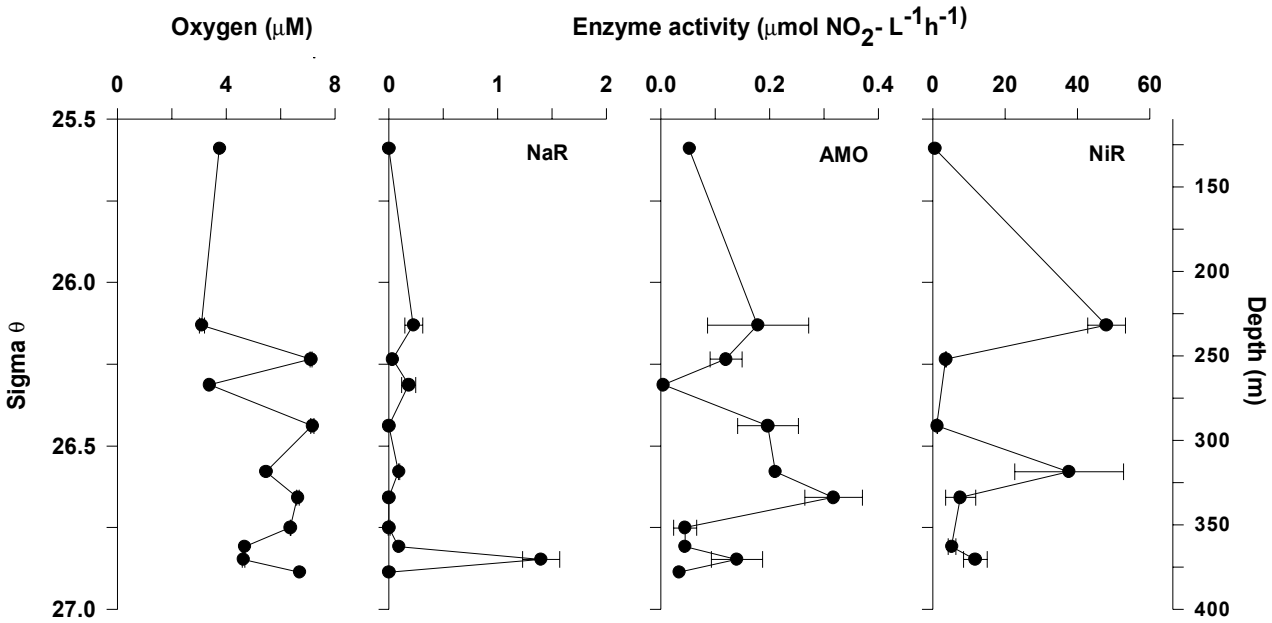

Fig.6. Depthwise distribution of potential activities of nitrate reductase, ammonia oxidizing activity and nitrite reductase in comparison with O_2 at station 19.

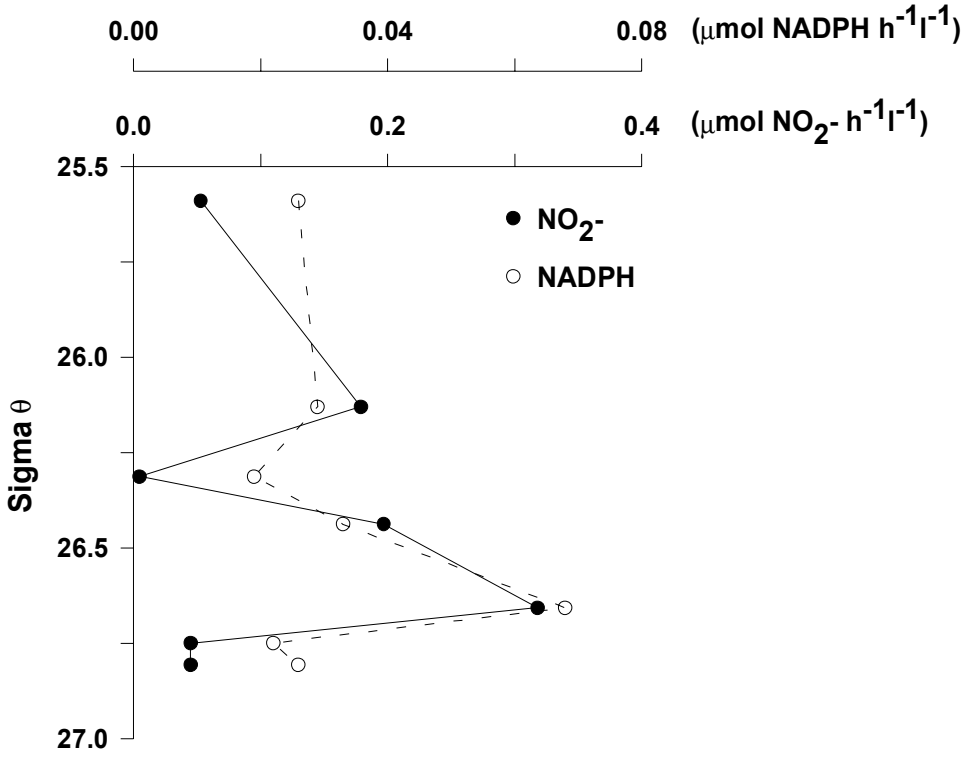

Fig. 7. Ammonia oxidizing activity measured by reverse ETS (NADPH formation) and NO_2^- formation.