

## Nitrate and sulfate reducers-retrievable number of bacteria and their activities in Indian waters

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**Abstract**-Culturable heterotrophic, nitrate reducing and sulfate reducing bacteria (HB, NRB and SRB) were enumerated from 25, 50, 100 and 200 m depths at 15 stations and their potential activities viz. Nitrate reducing (NRA) and Sulfate reducing (SRA) were determined. NRB and SRB were observed throughout these depths and did not relate to HB numbers. HB and NRB were recorded at 3 orders per ml and SRB at 2. High numbers of reducers were encountered in shallow depths as frequently as in deeper waters and did not correlate to potential activities. Average NRA showed decreasing trend with depth from 60  $\mu\text{g}$  at / ml/hr. at 25 m to 2.4  $\mu\text{g}$  at / ml/hr. at 100m. However the activity was 2.6 times higher at 200m when compared to that at 25 m. SRA showed a steady decreasing trend from 0.16  $\mu\text{g}$  at/ml/hr at 25m to 0.03  $\mu\text{g}$  at/ml/hr at 200m. It is suggested that while activities of SRB and shallow water NRB are dependant more on the carbon regime of their source, NRA of the deeper waters are more dependant on the nitrate regime.

### INTRODUCTION

While nitrate reduction a facultatively anaerobic process is known to occur both in aerobic and anaerobic realms, sulfate reduction a strictly anaerobic process has also been shown to occur in aerobic regions (JORGENSEN,1977). The two processes are known to proceed concurrently in the same regions (SORENSEN *et al*, 1979) and most of these studies have been restricted to sediments. Bacteriological studies of the Arabian Sea has shown that SRB are distributed quite abundantly in aerobic surface waters (LOKA BHARATHI and CHANDRAMOHAN, 1990). Colorless Sulfur oxidising bacteria present in these waters cannot only bring about the oxidation of sulfide produced by SRB but also simultaneously reduce nitrate either autotrophically or organotrophically (LOKA BHARATHI, 1989 ). The present study aims at studying the distribution of organotrophic nitrate reducers and their activity in relation to the sulfate reducers and their potential activity in a water column where there is a gradient from oxic to nearly anoxic conditions.

### MATERIAL AND METHODS

Water samples were collected from 15 stations (Fig. 1) at 25, 50, 100 and 200 m depths using the JZ samplers and analysed on board.

The total population was estimated in Zobell 2215E medium. The heterotrophic nitrate reducers were estimated using the medium described by SUGAHARA *et al* (1974) by MPN methods. The sulfate reducers were enumerated on shake agar using HATCHIKIAN's medium (1972). All the media were prepared in artificial sea water (SIEBURTH 1979). In the case of SRB the sea water was modified by eliminating  $\text{MgSO}_4$  and  $\text{Na}_2\text{SO}_4$  and reducing  $\text{K}_2\text{HPO}_4$  to 0.2g/L. The nitrate reduction was determined by measuring the amount of nitrite - N formed by 1 ml of the sea water sample in 50 ml quantities of the liquid medium after 24 hrs of incubation and expressed as percentage of reduction (100% concentration of nitrate in the medium = 5g/l). Nitrite was measured using the method described by PARSONS *et al* (1984). Sulfate reduction was estimated

by the amount of sulfide formed by 1 ml of sea water sample after 24 hrs of incubation in 15 ml of the liquid medium in screw capped tubes filled to the brim. Whenever the growth in the tubes were delayed the values were extrapolated after incubating the tubes longer. Sulfide estimation was carried out by PACHMAYR's method cited by TRUPER and SCHLEGAL (1964). Suitable controls were also incubated simultaneously for both nitrate and sulfate reduction. The reducing capacity was expressed as percentage of nitrate reduced by 1 ml of sea water in 24 hrs in the case of nitrate reduction and  $\mu\text{g}$  at  $\text{S}^{2-}$  formed per ml sea water in 24 hrs in the case of sulfate reduction.

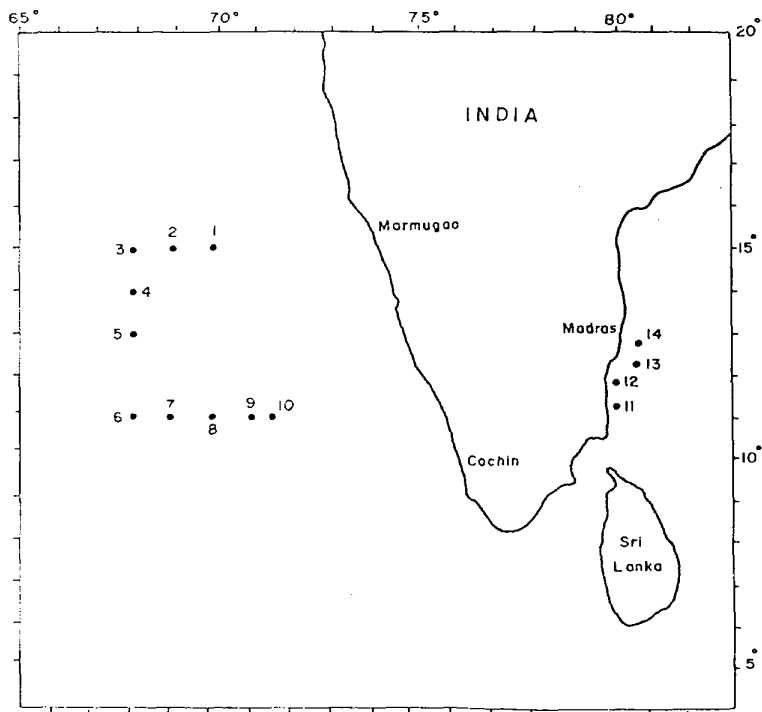


Fig. 1. Sampling locations.

Cultures isolated from the surface and deeper waters (300M) have been used to test nitrate reduction under different concentration of glucose 0.005 to 0.05% and nitrate (0.004 to 0.1 % ) in sea water amended with 0.001% peptone.

## RESULTS

The variations in physical and chemical parameters are given in Table 1. The oxygen levels ranged from 0.08 at 200m to 4.7 ml/l at 25m. There was a significant variation of  $\text{NO}_3\text{-N}$  with depth and ranged from 15.59 to 27.93  $\mu\text{g}$  at N/l at 200m depth.  $\text{NO}_2\text{-N}$  was absent in most of the stations at 25 and 50m depth but present at 100 and 200m. Salinity varied from 33.8 to 36.5. As sulfate:chlorine (a conservative element) ratio is constant 0.14+ 0.4% (WILSON, 1975) it was determined at a few

offshore stations and the values ranged from 2.46-3.06g/l (ie from 18.6mM to 23.18mM) in the surface waters. Sulfide was not detected in any of the stations.

Table 1. Physico-chemical parameters

Stn	Depth (m)	Sal. ( $\times 10^{-3}$ )	Temp. ( $^{\circ}\text{C}$ )	PO <sub>4</sub> /l ( $\mu\text{g.at.}$ )	NH <sub>3</sub> /L ( $\mu\text{g.at.}$ )	NO <sub>3</sub> -N/L ( $\mu\text{g.at.}$ )	NO <sub>2</sub> -N/L ( $\mu\text{g.at.}$ )	Oxygen (ml/L <sup>-1</sup> )
1517	25	36.4	29.9	0.24	0.22	0.65	0.0	4.44
	100	36.3	24.7	1.08	0.66	10.07	0.0	2.55
1519	25	36.1	30.3	0.12	0.44	0.0	0.0	4.17
	50	36.1	30.6	0.06	0.44	0.45	0.0	3.97
	100	36.5	27.0	0.42	0.0	1.95	0.0	3.75
	200	35.5	17.2	2.1	0.22	27.93	4.59	0.43
1521	25	36.0	30.4	0.06	0.0	0.0	0.0	4.17
	200	35.6	17.1	2.04	0.22	23.38	2.82	0.08
1523	25	36.0	29.4	0.24	0.0	0.0	0.0	4.05
	50	36.6	28.6	0.30	0.0	0.33	0.0	4.02
	100	36.3	25.6	0.90	0.44	5.52	0.18	2.04
	200	35.3	16.4	2.22	0.44	20.14	0.08	3.04
1525	25	36.0	30.9	0.30	0.0	0.0	0.0	4.20
	50	36.0	29.0	0.06	0.0	0.33	0.0	4.66
	100	36.1	25.0	1.08	0.36	8.44	0.36	2.43
	200	35.5	16.6	2.22	3.06	21.11	3.06	0.20
1529	25	35.7	29.2	0.18	0.44	0.0	0.0	4.70
	50	35.8	28.2	0.78	0.66	0.0	0.0	4.68
	100	35.2	25.4	0.96	0.88	5.52	0.14	3.36
1531	25	35.9	31.0	0.36	0.22	0.0	0.0	4.21
	50	36.4	28.3	0.36	0.22	0.0	0.22	4.26
	100	36.4	26.1	0.78	0.00	15.3	0.03	3.18
	200	35.6	19.7	2.16	0.44	17.54	0.0	0.72
1533	25	35.9	30.5	0.24	0.0	0.0	0.0	4.2
	50	36.0	28.6	0.36	0.88	0.0	0.0	4.07
	100	36.0	24.1	1.56	0.66	11.37	0.06	1.10
	200	35.3	15.2	2.28	0.66	18.36	0.0	0.18
1535	25	34.9	29.7	0.06	0.0	0.0	0.0	4.22
	50	35.1	28.5	0.0	0.44	0.0	0.02	3.94
	100	36.0	22.8	2.04	0.44	16.24	0.02	0.85
	200	35.4	15.0	1.98	0.22	18.19	0.04	0.38
1536	200	35.4	15.0	1.92	0.88	20.78	0.02	0.10
1554	25	34.1	28.6	0.30	0.0	0.0	0.0	4.22
	100	34.7	23.3	1.44	0.0	8.12	0.14	1.20
	200	34.9	15.6	2.11	0.0	15.91	0.04	0.23
1555	25	33.9	28.5	0.42	0.0	0.0	0.0	4.14
	50	34.0	28.7	0.48	0.0	0.0	0.06	4.17
	100	34.6	24.8	1.38	0.22	7.15	0.14	1.66
	200	34.9	14.6	2.16	0.0	15.59	0.08	0.33
1556	25	33.8	28.7	0.42	0.0	0.00	0.06	4.28
	50	34.0	28.7	0.36	0.0	0.0	0.02	4.17
	100	34.6	24.8	1.62	0.0	4.22	0.18	1.82
	200	34.9	14.6	2.76	0.0	15.59	0.06	0.21
1557	25	33.5	29.8	0.30	0.22	0.0	0.06	4.34
	50	34.2	30.2	0.36	0.0	0.0	0.04	4.41
	100	34.5	27.3	0.96	0.0	3.9	0.48	2.36
	200	34.8	17.5	2.40	0.0	15.59	0.06	0.13

CTB - Close to the bottom

HB did not show any significant variation with depth and ranged from  $0.01 \times 10^2$ /ml to  $14.4 \times 10^2$ /ml (Fig. 2).

NRB were detected at almost all stations and did not correlate with NRA measured in the samples (fig 3). Average potential NRA showed decreasing trend with depth from  $6.0 \mu\text{g at/ml/hr}$  at 25m to  $2.4 \mu\text{g at/ml/hr}$  at 100m. However the activity at 200m was 2.6 times higher than at 25m (Table 2).

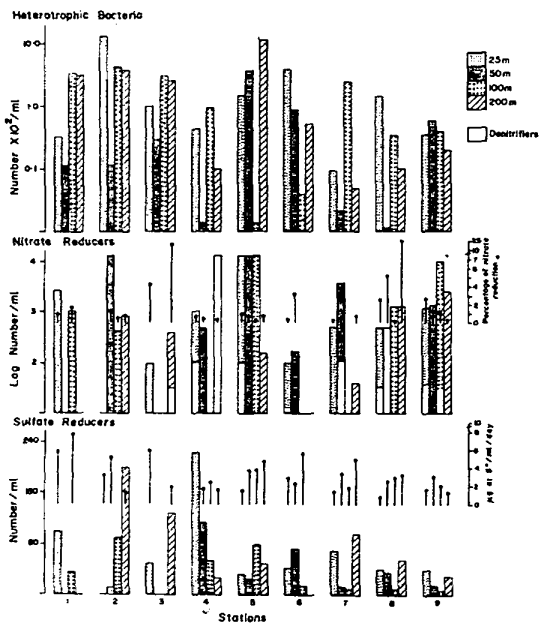


Fig. 2. Heterotrophic bacteria; Nitrate and Sulfate Reducers and their activities in the offshore stations.

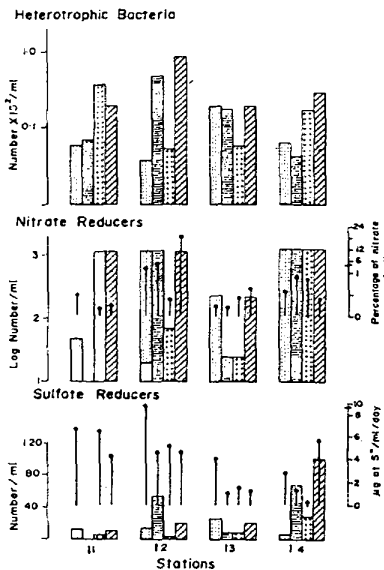


Fig. 3. Heterotrophic bacteria; Nitrate and Sulfate Reducers and their activities in the nearshore stations.

**Table 2. Average number and activity of bacteria**

Depth(m)	NRB ( $\times 10^3$ /ml)				NRA ( $\mu\text{g at NO}_2\text{-N/ml/hr}$ )			
	25	50	100	200	25	50	100	200
Offshore	2.2	3.7	3.1	2.1	5.1	4.2	3.0	15.3
Nearshore	0.8	0.7	0.7	1.1	7.5	5.7	1.5	16.2
Total	1.6	2.8	2.2	1.9	6.0	4.8	2.4	15.6

	SRB ( $\times 10^3$ /ml)				SRA ( $\mu\text{g at S}^{2-}\text{/ml/hr}$ )			
	25	50	100	200	25	50	100	200
Offshore	5.9	2.9	2.9	6.5	0.15	0.10	0.11	0.05
Nearshore	1.5	3.2	1.2	3.8	0.18	0.13	0.08	0.01
Total	4.7	3.0	2.4	5.7	0.16	0.11	0.10	0.03

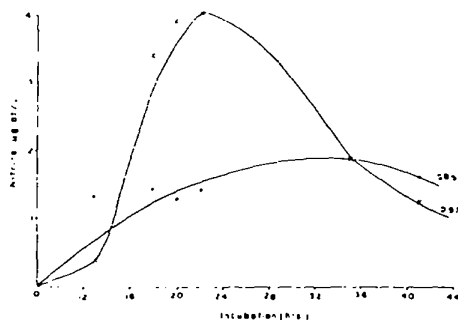
**Table 3.  $\mu\text{g at NO}_2\text{-N}$  produced /ml at 500mg /l and 10  $\mu\text{g at NO}_3\text{-N/l}$**

Incubation (hrs)	SB9	S71	Av	D97	D29	Av
12	23.8	33.1	18.5	14.3	15.9	15.1
14	25.2	28.3	26.8	15.1	17.8	16.5
20	51.5	51.5	51.5	47.6	47.6	47.6

**Table 4. Nitrate reduction at the end of 42 hrs.**

	% Nitrate used	% remaining as nitrite
IICIN	81.8	21.2
D97	100.0	9.3
SB9	43.3	27.2
IIICIN	43.9	7.7
SB9	100.0	1.3
IIICIN	100.0	6.3

I C = 100mg/l; N = 4  $\mu\text{g at NO}_3\text{-N/l}$   
 II C = 100mg/l; N = 20  $\mu\text{g at NO}_3\text{-N/l}$   
 III C = 500mg/l; N = 4  $\mu\text{g at NO}_3\text{-N/l}$



**Fig. 4. Nitrate reduction by surface and deeper water strains under controlled conditions (glucose=1.1g/l; Nitrate=4  $\mu\text{g at/l}$ )**

Denitrifiers were also not restricted to any specific depth. High numbers were encountered at 100 and 200 m depth (Fig. 3).

SRB too did not show any trend with depth and did not correlate to the activity measured. Their number ranged from 3-223/ml. SRA showed a steady decreasing trend from 0.16  $\mu\text{g}$  at/ml/hr at 25m to 0.03  $\mu\text{g}$ /ml/hr at 200m (Table 2).

Testing of shallow water and deep water cultures under different carbon and nitrate regime showed that increased carbon always showed increased activity in all the cultures but surface culture SB9 and S71 showed better NRA than deeper isolates. Results of one of the experiments is shown in Table 3.

Nevertheless examination of the nitrate reduced (Table 4) showed that D97 has marginally better utilised (ie 0.5%) with only 7.7% remaining as nitrite as compared to 27.2% of SB9.

Under low carbon and nitrate regime (100 mg/L and 4  $\mu\text{g}$  at  $\text{NO}_3\text{-N/L}$  respectively). D97 fared better (Fig. 4).

## DISCUSSION

Retrievable number of bacteria by plate counts and MPN may give underestimates but they reflect a population characteristic and could be useful for comparing data from different areas (VAN Es and MEYER-REIL, 1982). Direct counts from these waters carried out during other studies (unpublished) show that they are 2-3 orders more than the retrievable numbers. Likewise, estimation of their activities in media with elevated concentrations of the substrates could give an overestimate but meaningful results could be obtained for spatial and temporal comparison.

While HB, NRB and SRB did not show any trend in distribution, NRA and SRA had definite decreasing trend with depth. The present work however does not distinguish between assimilative and dissimilative nitrate reduction/reducers. It measures only the nitrite which is a definite intermediate in many types of nitrate reduction. The number of bacteria did not correlate with the activity measured. Similar lack of correlation between activity and number has been indirectly indicated by ESTEVES *et al* (1986). NRA in non polluted environment was dependant on nitrate concentration but were independant of the weight of the sediment. The absence of correlation between nitrate reducers and activity may suggest that the measured activity was not entirely due to the bacteria enumerated. Perhaps free enzymes in the milieu could play a role.

As nitrate reducers are facultative anaerobes and the persistence of denitrification capacity under aerobic condition being a rule rather than an exception (LLOYD *et al*, 1987), NRA studies were carried out under aerobic conditions. NRA i.e. only upto the formation of nitrite has been discussed in the present study. The dissimilatory nitrate reduction is more fermentative than respiratory where nitrate may be considered as an incidental electron acceptor (FENCHEL and BLACKBURN, 1979).

It is interesting to note that the average NRA of these depths showed decreasing trends from 25 - 100m depth but increased 2.6 fold at 200m depth. It is now shown that nitrate reducing bacteria are active not only at varying levels of oxygen concentrations (ROBERTSEN and KUENEN, 1984) but also in a whole spectrum of carbon regime from autotrophy  $\rightarrow$  mixotrophy. (TUTTLE *et al* 1974, KUENEN 1975, and LOKA BHARATHI, 1989). In the Arabian sea waters the PCHO (particular carbohydrates) ranged from 22-125  $\mu\text{g}/\text{l}$  at the surface and from 4.06 - 40.5  $\mu\text{g}/\text{l}$  at 300m (BHONSALE and WAGH, 1989). This decrease in carbon content of the waters could be responsible for the average decrease in heterotrophic nitrate reducing capacity with depth. The

concentration of nitrate in the milieu also evokes certain responses in the organisms in the bacterial communities from those regions with greater nitrate concentrations exhibiting faster rates of nitrate reduction. Similar adaptation of sediment bacterial communities to greater nitrate concentrations and exhibiting faster rates of nitrate reduction have been recorded by KING and NEDWELL (1987).

The nitrate content in the deeper waters (table 1) remains high and could be due to multiplicity of reasons. One main reason could be that the nitrate generation in these systems being faster than the reducing rates. However this aspect has not been looked into. With the discovery that the same organisms could contribute to both oxidative and reductive cycles (ROBERTSEN and KUENEN, 1989) the complexity of the process still increases. There is considerable indirect evidence that for many environments, the vast majority of nitrate reduced in denitrification is provided by bacterial nitrification in the same systems (VANDERBORGT *et al*, 1977, KOIKE and HATTORI, 1978, SEITZINGER *et al*, 1984). Similarly, aerobic denitrifiers are also heterotrophic nitrifiers. (CASTIGNETTI and HALLOCHER, 1984). Thus, while NRA at shallow water depths are influenced by higher temperature and carbon content and are carried out by NRB that can use two electron acceptors simultaneously, NRA in the lower layers could be conditioned by nitrate content. While instant generation and utilisation could explain the lack of nitrate in the shallow waters, the higher oxidation vis a vis reduction could explain the higher content of nitrate in these deeper waters. As a number of different bacteria can denitrify even when oxygen approach or exceed air saturation values the aerobic bacterial denitrification with products of  $N_2$  and  $N_2O$  may contrary to the beliefs of many be as widespread and ecologically important as its anaerobic counterparts (LLOYD *et al*, 1987). Besides, it is now accepted that diauxy may be an exception rather than the rule and mixotrophy may be an important metabolic parameter in determining the competitive success of a species (BEUDEKER *et al*, 1982, ROBERTSON KUENEN, 1990). Mixotrophy where two electron donors are used simultaneously (here,  $O_2$  and  $NO_3$ ) could be prevalent in most of the aerobic marine environment where resources are limited.

Experiments conducted with different concentrations of glucose and nitrate showed that surface cultures reduce nitrate faster under high carbon regime. Similarly, earlier studies on CSOB which oxidise thiosulfate and reduce nitrate simultaneously indicated that strains isolated from near shore and surface waters were more active nitrate reducers than their counterparts in off shore and deeper waters. (LOKA BHARATHI *et al*, 1988). Though deep isolates only marginally outscore the surface isolates under increased nitrate and reduced carbon, they outdo them under low carbon and low nitrate regime.

SRB did not show any trend with depth or distance from the shore. They did not relate to number of heterotrophic bacteria.

SRB are stricter anaerobes but their distribution is not inversely related to the oxygen content of the water column. The distribution and activity clearly point out that they are not associated with a negative Eh. The absence of a negative correlation between the oxygen level and the reducing capacity of the water could be due to various factors- the total activity being dependant not entirely on the total number of bacteria but on the reducing capacity of each individual that forms the habitat. In addition, like in NRA sulfate reducing enzymes of the water could also influence ie the activity being independant on the living bacteria once again suggesting that the macroenvironment does not fall within the limits of Eh and pH of sulfate reducing bacteria set by BASS BECKING and KAPLAN (1956). Their distribution is more related to the carbon content of the waters which tend to decrease with depth. Their retrieval is in three orders of magnitude but it is possible that there are higher numbers of

these organisms (GAYLARDE and COOK, 1987). SRA shows a definite decreasing trend with depth. It is possible that the activity is governed by the organic carbon content of the source. As sulfate is not limiting in the marine environment the activity is, often limited by the availability of their substrate (SORENSEN *et al.*, 1979). The activity showed a decreasing trend with depth from 0.16  $\mu\text{g}$  at 25m to 0.03  $\mu\text{g}$  at/ml/hr at 200m depth (Table 2). Most SRA studies have been carried out with sediments. SRA in waters and sediments have been measured using radio tracer techniques and values upto 100  $\text{nm SO}_4^{2-}/\text{cm}/\text{day}$  have been recorded in summer months (JORGENSEN, 1977). JORGENSEN, (1979) has also measured upto 9.8- 15.1  $\text{mM}/\text{m}^2/\text{day}$  by radio tracer technique and upto 1200 $\text{nm}/\text{cm}^3/\text{day}$  (JORGENSEN,1989) in sediment lagoon. HINES *et al.* (1989) measured 2.5  $\mu\text{mol}/\text{day}$  in sediment pore water.

Sulfide was not detected in the water samples. However, the presence of SRB and their reducing capacity in these waters suggest that sulfide however is produced but is readily utilised. The presence of colorless sulfur-oxidising bacteria capable of oxidising sulfide, in the Arabian Sea has been discussed (LOKA BHARATHI and CHANDRAMOHAN, 1988, LOKA BHARATHI, 1989) and they have been found to be significantly correlated to SRB ( $r= 0.6760$  P 0.001, LOKA BHARATHI and CHANDRAMOHAN, 1990). Besides it has also been shown that colourless sulfur oxidising bacteria assimilate isotopically light sulfur produced via sulfate reduction (PETERSON *et al.*1986)

Our observations indicate that nitrate reduction and denitrification take place in the whole column from oxygenated to the hardly oxygenated layers. Though the highest numbers were not recorded at any particular depth always, the highest NRA were measured at 200m. Likewise SRB and SRA were recorded in all depths with higher activities being at shallower depths. It has been shown that oxygen and nitrate respiration are not exclusive (SMITH *et al.*, 1976, FENCHEL and BLACKBURN, 1976). Like in sediments nitrate and sulfate respiration are not mutually exclusive in water columns; they are perhaps more temporally rather than spatially isolated.

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