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Don E. Canfield[1*], Frank J. Stewart[2], Bo Thamdrup[1], Loreto De Brabandere[1], Tage Dalsgaard[3], Edward F. Delong[2], Niels Peter Revsbech[4], Osvaldo Ulloa[5]

[1] Institute of Biology and Nordic Center for Earth Evolution (NordCEE), University of Southern Denmark, Campusvej 55, 5230 Odense M

[2] Department of Biological Engineering and Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, Cambridge, MA 02138, USA

[3] National Environmental Research Institution (NERI), Aarhus University, Vejlsøvej 25, P. O. Box 314, DK-8600 Silkeborg, Denmark

[4]Section of Microbiology, Department of Biological Sciences, Aarhus University, DK-8000 Aarhus C, Denmark

[5] Departamento de Oceanografía and Centro de Investigación Oceanográfica en el Pacífico Sur-Oriental, Universidad de Concepción, Casilla-160-C, Concepción, Chile

*corresponding author: **dec@biology.sdu.dk**

Nitrogen cycling is normally thought to dominate the biogeochemistry and microbial ecology of oxygen-minimum zones in marine environments. Through a combination of molecular techniques and process rate measurements, we show that both sulfate reduction and sulfide oxidation contribute to energy flux and elemental cycling in oxygen-free waters off the coast of northern Chile. These processes may have been overlooked because the sulfide produced by sulfate reduction immediately oxidizes back to sulfate. This cryptic sulfur cycle is linked to anammox and other nitrogen cycling processes, suggesting it may influence biogeochemical cycling in the global ocean.

Oxygen-minimum zones (OMZs) persist in mid-water depths of the global ocean, where large-scale circulation and the sinking and decomposition of surface-derived organics deplete oxygen compared to higher surface and deep-water oxygen concentrations (*1*). In some regions such as the eastern tropical Pacific, the Arabian Sea, and the Benguela Current upwelling system, water column oxygen concentrations fall below detection (*2-4*), prompting the development of a dynamic nitrogen cycle. In these zones, nitrate is actively reduced to nitrite (*5, 6*). Nitrite further converted to N_2 gas through "classic" heterotrophic denitrification (*7*) and the autotrophic anammox process (*8, 9*), or to NH_4^+ through dissimilative nitrate reduction to ammonium (DNRA). OMZs account for 33% or more of the fixed nitrogen loss from the oceans (*10, 11*), and overall, the nitrogen cycle has been thought to dominate the geochemistry and microbial ecology of these regions.

The recent identification of uncultured *Gammaproteobacteria*, closely affiliated with sulfur-oxidizing symbionts, in OMZ waters off the Chilean coast (*12*) suggests that sulfur

cycling may also play an important role in oxygen-free nitrate-rich OMZs. A similar microbial community with a full compliment of sulfide-oxidizing and nitrate-reducing genes was found in sulfide-free but nitrate-rich portions of the sulfidic Saanich Inlet (*13*), and a sulfate reducer has been isolated from OMZ waters off the coast of Peru (*14*). Direct evidence for large-sclae, active sulfur cycling in OMZs, however, is lacking. When sulfide, the product of sulfate reduction, is observed in OMZs, it originates in rare pockets of nitrate and nitrite-depleted water (*15*) or is released from sediments (*6*).

We explored the dynamics of the sulfur cycle in the upwelling waters off of Iquique, on the northern Chilean coast using a combination of geochemical and metagenomic techniques (*16*). In general, the OMZ is well developed in this region of Chile (*17*). We concentrated our efforts on Station 3 (20°5′9.27′′S; 70°20′8.18′′W ; water depth 1050 m, 23 km from shore), which based on preliminary survey data, was in the most biologically active region of the OMZ in our study area. We expanded our geochemical studies to include Station 5 (20°5′9.69′′S; 70°46′5.78′′W; water depth 1500), located some 44 km further offshore than Station 3. The water chemistry in the northern Chilean OMZ develops within an eastern boundary current, and the chemical profiles are somewhat dynamic (Fig 1). With some variability over time, the redoxcline at Station 5 was located deeper than at Station 3, and while the surface chlorophyll *a* concentrations were higher at Station 3, we frequently observed a pronounced secondary chlorophyll *a* maximum at Station 5. This deep layer consists of novel members of the cyanobacterial genus *Prochlorococcus* (*18*).

We observed extremely low O_2 concentrations of < 13 nM, starting from between 60 and 85 meters depth (depending on station and time of sampling) and continuing to >

180 m (Fif. S1). Similar low values were found off the southern Peruvian coast in an earlier study (*19*), suggesting that essentially anoxic waters define this region of the eastern tropical South Pacific OMZ. There is an upper nitrite maximum related to aerobic processes, but nitrite accumulated as oxygen disappeared in the anoxic core of the OMZ. Nitrate reduction was the most likely source for this nitrite. We measured with ^{15}N-enriched nitrite the rates and pathways of N_2 formation, and similar to an early study (*8*), anammox was the dominant pathway (Table 1), considerably outpacing denitrification (*16*). The overall rates of N_2 formation are similar to previous measurements in this region (*8*).

Pyrosequencing of community DNA from below the oxycline (70-80 m) and in the core of the anoxic zone (150-200 m) at Station 3 suggested a substantial role for sulfur-based metabolic pathways in the Chilean OMZ (Fig. 2; Figs. S4-7). Gene sequences matching diverse sulfide-oxidizing and sulfate-reducing taxa (Table S3) constituted 6.3-16.2% and 2.1-2.4% of all sequencing reads with matches to protein-coding genes in the NCBI-nr database, consistent with percentages based on 16S rRNA gene-encoding reads (Figs. S4-5). In contrast, sulfur-oxidizer and sulfate-reducer sequences represented only 0.5% and 0.3% of the total protein coding sequences recovered in an aerobic community from another coastal site (Monterey Bay, 10 m; Fig. S5). The Chilean OMZ metagenomes were particularly enriched in sequences matching the genomes of sulfur-oxidizing endosymbionts of deep-sea clams [*Candidatus* Ruthia magnifica (Rm) and *Candidatus* Vesicomyosocius okutanii (Vo) (*20, 21*)] and the endosymbiont-related SUP05 pelagic lineage from Saanich Inlet (*13*) (Fig. 2A). These taxa increased in abundance in January 2010 (austral summer), relative to samples

collected from the same site in August 2009 (late winter). Notably, in the January-2010 samples, SUP05-like sequences dominated the identifiable protein-coding gene pool (up to 7.5% of all hits to NCBI-nr). The SUP05 metagenome (*13*) was represented at high coverage, matching 80% of all SUP05 genes (1169 of 1456) with relatively uniform abundances, and an average amino acid similarity of 70% (Fig. S7). The sulfate-reducing population contributed to a lower, but appreciable proportion of sequencing reads, and was represented by a diverse population that included *Desulfatibacillum*, *Desulfobacterium*, *Desulfococcus*, *Syntrophobacter* and *Desulfovibrio* species (Figs. S4-6).

The prevalence of sulfur metabolizing taxa was paralleled by a strong representation of sulfur energy metabolism genes. These genes occur in various combinations across diverse sulfur-utilizing taxa (*22*). Here, genes of the dissimilatory sulfite reductase enzyme (dsr), the sulfur oxidation (sox) gene complex mediating thiosulfate oxidation, and the adenosine 5′-phosphosulfate (APS) reductase (apr) were present throughout the OMZ (Fig. 2B). Several of the proteins encoded by these genes, including dsr and apr enzymes, function in both oxidative and reductive pathways *(23, 24)*. Here, the majority of the sequences recovered in the OMZ matched known sulfide oxidizers, consistent with the high abundance of the SUP05 group. Putative sulfide-oxidizing and sulfate-reducing taxa constituted 62.0% and 2.2% of top hits to *aprA* sequences, respectively, with the remainder matching *aprA* genes of the alphaproteobacterial genus *Pelagibacter*, whose function in sulfide oxidation is not yet clear (*25*) (Fig. S6). Overall, the metagenomic data suggest a prevalent summer OMZ community of both oxidative and reductive sulfur cycling bacteria.

Although our metagenomic libraries suggest an active sulfur cycle, it is cryptic with no obvious in situ chemical expression. To explore the geochemical importance of the sulfur cycle and any possible to nitrogen cycling, we measured rates of sulfate reduction with $^{35}SO_4^{2-}$ (e.g. (*26*)). We subdued the immediate reoxidation of sulfide produced during sulfate reduction by adding 10 to 13 μM of unlabeled sulfide to trap any radiolabeled sulfide from sulfate reduction (*16*). Radiolabeled sulfate was added within 10 hours of sample collection. In some cases, our added unlabelled sulfide was substantially oxidized during the incubations (*16*), implying radiolabeled sulfide must also have been oxidized and lost as a result. After estimating the loss of radiolabeled sulfide due to sulfide oxidation, we corrected the rates to obtain estimates of the gross sulfate reduction rates (*16*) (Fig.3). Our findings contrast with the current consensus that sulfate reduction in OMZs will only be active when other more thermodynamically favorable electron acceptors, like nitrate and nitrite, are fully utilized (*27*). Although not the most favorable, our calculations show that sulfate reduction is still a thermodynamically favorable process in these OMZ waters (*16*). Previous observations of pure cultures of sulfate-reducing bacteria that actively reduce sulfate in the presence of nitrate (*28, 29*) also support our observations of active sulfate reduction.

Rates of sulfate reduction were much higher at Station 3 compared to Station 5. Indeed, corrected rates at Station 3, match and even exceed rates of denitrification and anammox (Table 1), implying that sulfate reduction is an important pathway of organic carbon mineralization at this site. Depth-integrated corrected rates of sulfate reduction at Station 3 are equivalent to about 2 mmoles C oxidized m^{-2} d^{-1} assuming 2 moles organic carbon oxidized per mole sulfate reduced. Sediment traps studies at coastal and off-shore

stations about 200 km south of our study site (*17*) reveal about 5.50 mmol m^{-2} d^{-1} of carbon mineralization within the OMZ waters from between 65 and 300 depth. If these rates apply to Station 3, then sulfate reduction would account for about 33% of the total organic carbon mineralization in the OMZ waters.

Sulfate reduction may also contribute to the ammonium requirements of other indigenous bacteria participating in the anammox process. Indeed, the source of ammonium for anammox has proven elusive because insufficient ammonium is liberated during organic matter decomposition by denitrification to drive measured anammox rates in many OMZ waters (*8, 9*). In a partial resolution to this dilemma, the dissimilatory reduction of nitrate to ammonium and the heterotrophic reduction of nitrate to nitrite have been identified as significant ammonium sources in OMZ waters off the Peruvian coast (*9*) (the later due to the ammonium liberated during heterotrophic organic matter mineralization). But even these extra sources do not account for all of the ammonium demand. From our sulfate reduction rates at Station 3, sulfate reduction produces a total of about 0.30 mmoles m^{-2} d^{-1} assuming a 6.6/1 ratio between carbon oxidation and ammonium liberation (*30*). This would contribute 22% of the ammonium needs for anammox at Station 3 (Table 1). At Station 5, sulfate reduction would only contribute about 8% of the ammonium needs for anammox, underlining the complexity of the nitrogen cycle and the variability of ammonium sources for anammox (*9*).

We also explored the dynamics of sulfide oxidation in these waters, and the relationship between sulfide oxidation and the nitrogen cycle (*16*). In parallel with our sulfate reduction rate determinations, we incubated OMZ water from two depths at both Stations 3 and 5 with and without added sulfide. Sulfide oxidation was strongly coupled

to nitrate reduction to nitrite, and at Station 5, nitrate reduction to nitrous oxide was also enhanced with sulfide addition (Fig. S8). At Station 3, N_2 production from both nitrite and nitrate (at 75 m depth) increased, and in general, rates of sulfide oxidation and subsequent rates of nitrogen turnover were much higher at Station 3 than Station 5. This is consistent with the higher rates of sulfate reduction at Station 3 and a more active sulfur cycle.

Admittedly, our added levels of sulfide and subsequent rates of sulfide oxidation exceed in situ levels. Never the less, our results demonstrate the inherent capacity for active in situ coupling between the sulfur and nitrogen cycles in OMZ zones of the marine water column. This cycling is analogous to that observed at the sulfide/nitrate interface in other strongly redox stratified marine systems (*13, 31, 32*), and demonstrates that nitrite, N_2 and N_2O may all be products of this coupling. We speculate other nitrate-rich oxygen-free OMZs may also house actively coupled sulfur and nitrogen cycles.

References:

1. K. Wyrtki, *Deep-Sea Res.* **9**, 11 (1962).
2. J. D. Cline, F. A. Richards, *Limnol. Oceanogr.* **17**, 885 (1972).
3. D. B. Olson, G. L. Hitchcock, R. A. Fine, B. A. Warren, *Deep-Sea Res. II* **40**, 673 (1993).
4. S. E. Calvert, N. B. Price, *Deep-Sea Res.* **18**, 505 (1971).
5. J. M. Morrison *et al.*, *Deep-sea Res. II* **46**, 1903 (1999).
6. V. Brüchert *et al.*, *Geochim. Cosmochim. Acta* **67**, 4505 (2003).
7. S. E. Bulow, J. J. Rich, H. S. Naik, A. K. Pratihary, B. B. Ward, *Deep-Sea Res. Part I-Oceanogr. Res. Pap.* **57**, 384 (2010).
8. B. Thamdrup *et al.*, *Limnol. Oceanogr.* **51**, 2145 (2006).
9. P. Lam *et al.*, *Proc. Natl. Acad. Sci. U. S. A.* **106**, 4752 (Mar, 2009).
10. L. A. Codispoti *et al.*, *Scientia Marina* **65**, 85 (2001).
11. J. N. Galloway *et al.*, *Biogeochemistry* **70**, 153 (2004).
12. H. Stevens, O. Ulloa, *Environ. Microbiol.* **10**, 1244 (2008).
13. D. A. Walsh *et al.*, *Science* **326**, 578 (2009).
14. K. W. Finster, K. U. Kjeldsen, *Antonie Van Leeuwenhoek* **97**, 221 (2010).

15. R. C. Dugdale, J. J. Goering, R. T. Barber, R. L. Smith, T. T. Packard, *Deep-Sea Res.* **24**, 601 (1977).
16. see supporting online material (SOM)
17. R. Escribano *et al.*, *Deep-Sea Res. II* **51**, 2389 (2004).
18. P. Lavin, B. González, J. F. Santibáñez, D. J. Scanlan, O. Ulloa, *Envir. Microbiol. Reports*, (2010).
19. N. P. Revsbech *et al.*, *Limnol. Oceanogr.: Methods* **7**, 371 (2009).
20. I. L. G. Newton *et al.*, *Science* **315**, 998 (2007).
21. H. Kuwahara *et al.*, *Current Biology* **17**, 881 (2007).
22. C. Dahl, C. G. Friedrich, Eds., *Microbial Sulfur Metabolism*, (Springer-Verlag, Heidelberg, 2008), pp. 308.
23. B. Meyer, J. Kuever, *Microbiology-Sgm* **153**, 3478 (2007).
24. B. Meyer, J. Kuever, *Microbiology-Sgm* **153**, 2026 (2007).
25. B. Meyer, J. Kuever, *Appl. Envir. Microbiol.* **73**, 7664 (2007).
26. D. B. Albert, C. Taylor, C. S. Martens, *Deep-Sea Res.* **42**, 1239 (1995).
27. P. N. Froelich *et al.*, *Geochim. Cosmochim. Acta* **43**, 1075 (1979).
28. T. Dalsgaard, F. Bak, *Appl. Environ. Microbiol.* **60**, 291 (1994).
29. R. G. L. McCready, W. D. Gould, F. D. Cook, *Arch. Microbiol.* **135**, 182 (1983).
30. A. C. Redfield, B. H. Ketchum, F. A. Richards, in *The Sea,* N. M. Hill, Ed. (Academic Press, London, 1963), vol. 2, pp. 26-77.
31. M. M. Jensen, J. Petersen, T. Dalsgaard, B. Thamdrup, *Mar. Chem.* **113**, 102 (2009).
32. G. Lavik *et al.*, *Nature* **457**, 581 (2009).

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Table 1. Summary of process rate averages

	nmol l^{-1} h^{-1}	
Process	Sta 3	Sta 5
anammox	0.43 ± 0.21[b]	0.29 ± 0.10[c]
denitrification[a]	0.079 ± 0.04[b]	0.042 ± 0.029[c]
Sulfate reduction	0.51 ± 0.21[d]	0.055 ± 0.023[e]
Depth-integrated rates	mmol m^{-2} d^{-1}	
anammox	1.21 ± 0.45	0.70 ± 0.24
denitrification	0.22 ± 0.11	0.10 ± 0.069
sulfate reduction	1.00 ± 0.40[f]	0.28 ± 0.12
carbon oxidation in OMZ[g]	5.50	5.50

[a]measured as nitrite reduction to N_2
[b]from 65 to 183 meters depth (n=17)
[c]from 73.5 to 173 meters depth (n=16)
[d]from 85 to 150 meters depth (n=8)
[e]from 85 to 300 meters depth (n=14)
[f]assuming sulfate reduction stops first at 200 meters
[g]estimated from data in ref (*17*)

Figure captions

Figure 1. Representative nutrient, oxygen and chlorophyll *a* profiles from the OMZ off the northern Chilean coast at Station 3 (left) and Station 5 (right).

Figure 2. Taxonomic representation of protein-coding genes and relative abundances of sulfur energy metabolism genes in OMZ metagenomic data. **(A)** Most abundant taxa identified from annotations of protein-coding genes (in the NCBI-nr database) in pyrosequencing reads from genomic DNA. Reads matching multiple putative sulfate-reducer reference taxa (Fig. S1, Table S2) are binned in a single category (black bar). **(B)** Abundances (hit counts per gene) of dissimilatory sulfur metabolism genes, shown relative to the putative single copy per organism RNA polymerase subunit B (*rpoB*). Abundances per gene are normalized to gene length but not to copy number variation. *dsr* = dissimilatory sulfite reductase gene cluster, *sox* = sulfur oxidation gene cluster; *aprBA* = adenosine 5′-phosphosulfate (APS) reductase; *aprM*, APS reductase membrane anchor; FCSD, flavocytochrome *c* sulfide dehydrogenase; SQR, sulfide-quinone reductase.

Figure 3. Sulfide-oxidation corrected and uncorrected rates of sulfate reduction at Staions 3 and 5. Standard deviations represent variability during scintillation counting (*16*).

Figure 1.

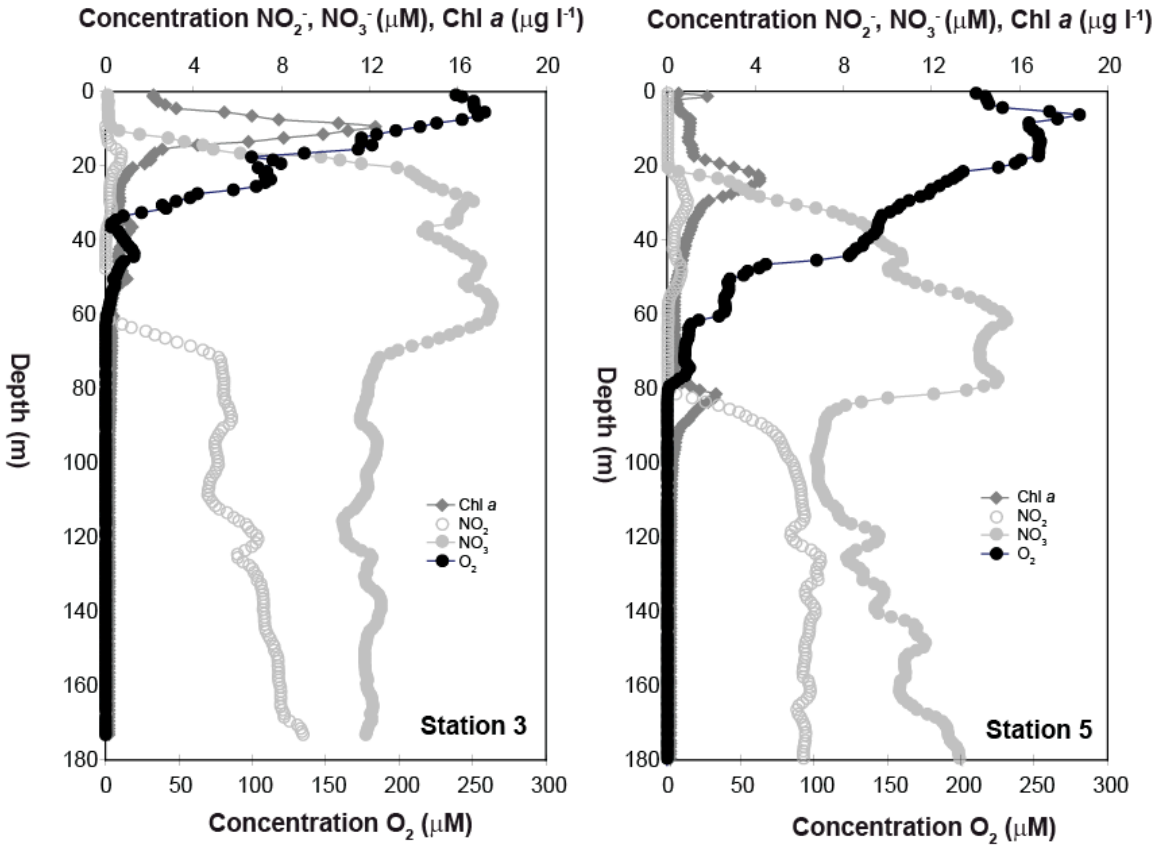

Concentration NO_2^-, NO_3^- (μM), Chl *a* (μg l^{-1})
Depth (m)
Concentration O_2 (μM)
Chl *a*
NO_2
NO_3
O_2
Station 3
Station 5

Figure 2

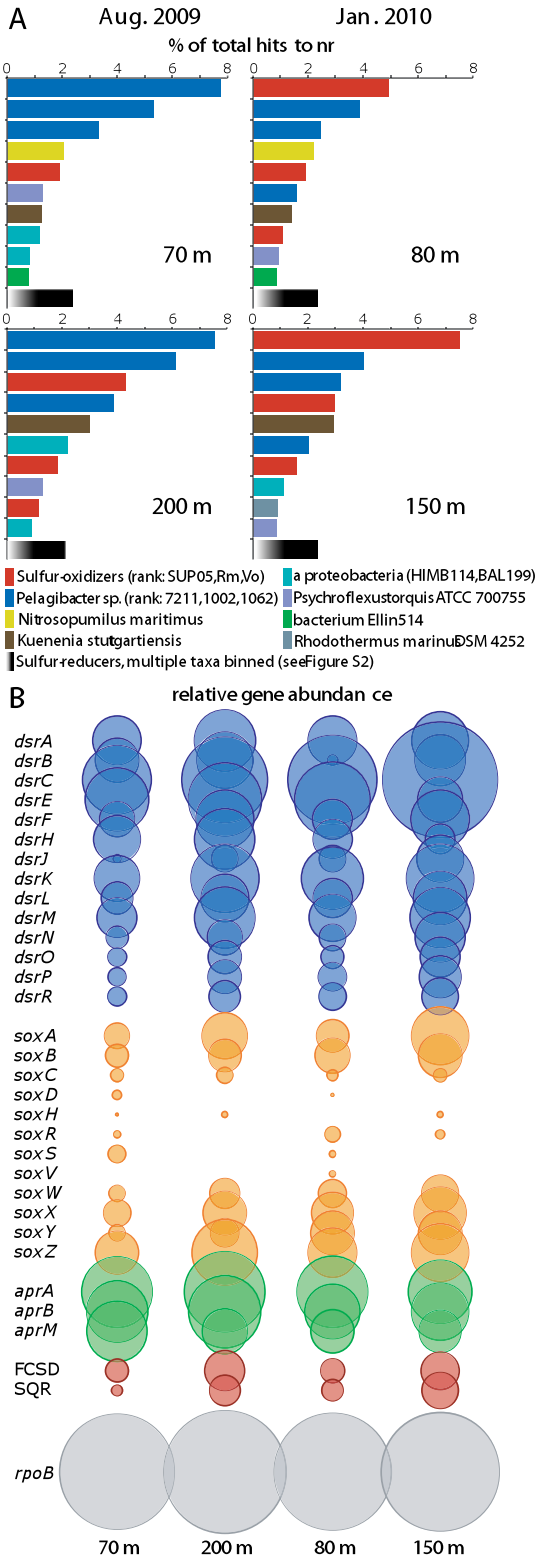
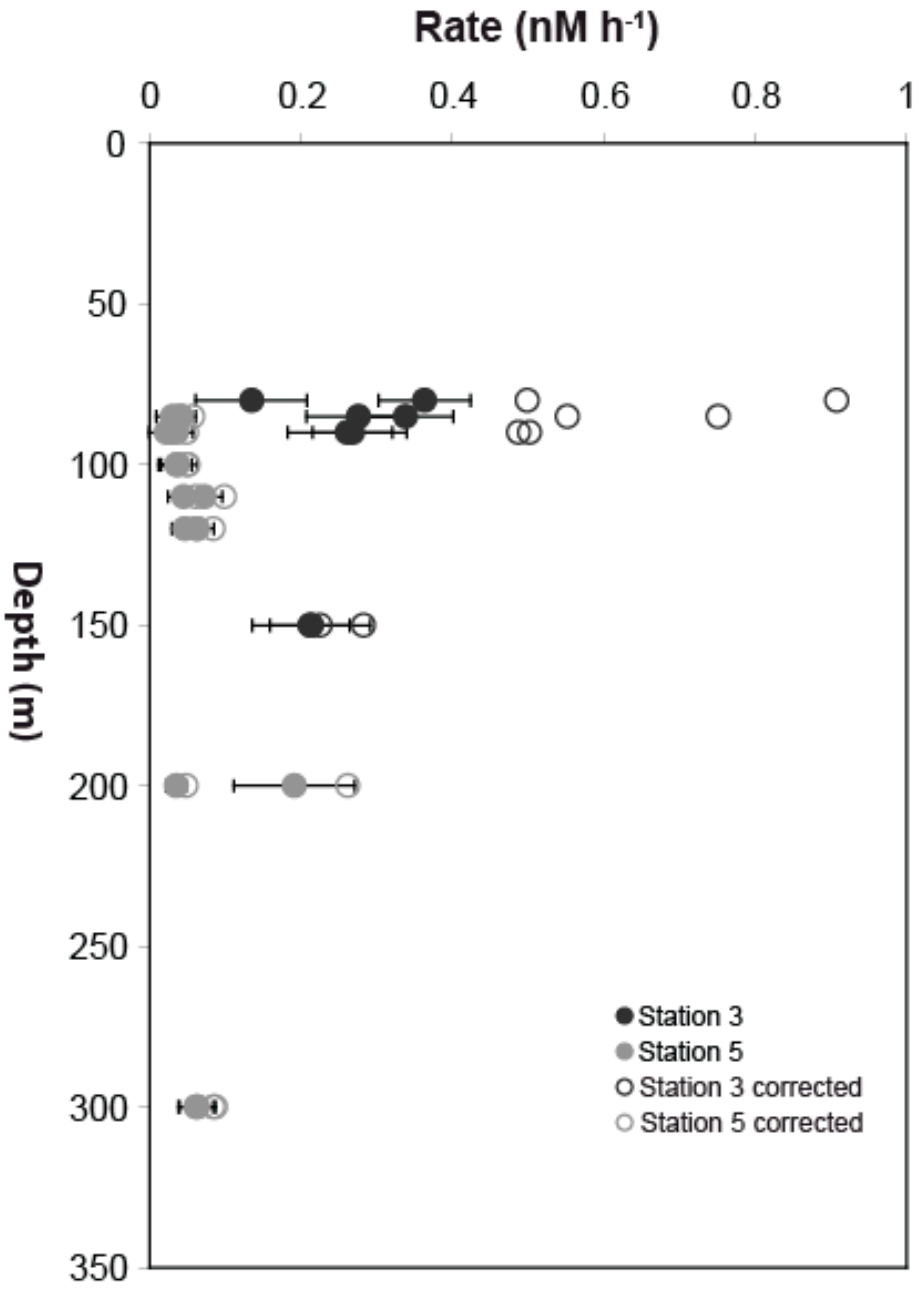

A
Aug. 2009
Jan. 2010
% of total hits to nr
70 m
80 m
200 m
150 m
Sulfur-oxidizers (rank: SUP05,Rm,Vo)
Pelagibacter sp. (rank: 7211,1002,1062)
Nitrosopumilus maritimus
Kuenenia stutgartiensis
Sulfur-reducers, multiple taxa binned (seeFigure S2)
a proteobacteria (HIMB114,BAL199)
Psychroflexustorquis ATCC 700755
bacterium Ellin514
Rhodothermus marinus DSM 4252
B
relative gene abundan ce
dsrA
dsrB
dsrC
dsrE
dsrF
dsrH
dsrJ
dsrK
dsrL
dsrM
dsrN
dsrO
dsrP
dsrR
soxA
soxB
soxC
soxD
soxH
soxR
soxS
soxV
soxW
soxX
soxY
soxZ
aprA
aprB
aprM
FCSD
SQR
rpoB
70 m
200 m
80 m
150 m

Figure 3


Rate (nM h-1)
0
0.2
0.4
0.6
0.8
1
Depth (m)
0
50
100
150
200
250
300
350
Station 3
Station 5
Station 3 corrected
Station 5 corrected