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# Giant Hydrogen Sulfide Plume in the Oxygen Minimum Zone off Peru Supports Chemolithoautotrophy

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## Abstract

In Eastern Boundary Upwelling Systems nutrient-rich waters are transported to the ocean surface, fuelling high photoautotrophic primary production. Subsequent heterotrophic decomposition of the produced biomass increases the oxygen-depletion at intermediate water depths, which can result in the formation of oxygen minimum zones (OMZ). OMZs can sporadically accumulate hydrogen sulfide (H<sub>2</sub>S), which is toxic to most multicellular organisms and has been implicated in massive fish kills. During a cruise to the OMZ off Peru in January 2009 we found a sulfidic plume in continental shelf waters, covering an area >5500 km<sup>2</sup>, which contained ~2.2×10<sup>4</sup> tons of H<sub>2</sub>S. This was the first time that H<sub>2</sub>S was measured in the Peruvian OMZ and with ~440 km<sup>3</sup> the largest plume ever reported for oceanic waters. We assessed the phylogenetic and functional diversity of the inhabiting microbial community by high-throughput sequencing of DNA and RNA, while its metabolic activity was determined with rate measurements of carbon fixation and nitrogen transformation processes. The waters were dominated by several distinct  $\gamma$ -,  $\delta$ - and  $\epsilon$ -proteobacterial taxa associated with either sulfur oxidation or sulfate reduction. Our results suggest that these chemolithoautotrophic bacteria utilized several oxidants (oxygen, nitrate, nitrite, nitric oxide and nitrous oxide) to detoxify the sulfidic waters well below the oxic surface. The chemolithoautotrophic activity at our sampling site led to high rates of dark carbon fixation. Assuming that these chemolithoautotrophic rates were maintained throughout the sulfidic waters, they could be representing as much as ~30% of the photoautotrophic carbon fixation. Postulated changes such as eutrophication and global warming, which lead to an expansion and intensification of OMZs, might also increase the frequency of sulfidic waters. We suggest that the chemolithoautotrophically fixed carbon may be involved in a negative feedback loop that could fuel further sulfate reduction and potentially stabilize the sulfidic OMZ waters.

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## Introduction

Eastern Boundary Upwelling Systems are found along the westward shelves of the continents in both the Atlantic and the Pacific Ocean. They are characterized by high photoautotrophic primary production, which is driven by the upwelling of nutrient-rich waters [1]. The produced biomass supports large fish populations in these regions, underlining the importance of Eastern Boundary Upwelling Systems in providing a source of food for mankind [2–6]. However, a significant proportion of the

produced biomass also sinks through the water column and is remineralized in subsurface waters, contributing to the oxygen (O<sub>2</sub>) depletion in intermediate water depths of these regions. Combined with large-scale ocean circulation patterns and poor ventilation of intermediate waters with the ocean surface, the remineralization of rich organic matter leads to oxygen-depleted zones of varying intensities [7–10]. These oxygen-depleted waters, also referred to as oxygen minimum zones (OMZs), are found in the eastern tropical North and South Pacific, and to a lesser extent in the eastern tropical North and South Atlantic [11]. In addition

to OMZs found in regions of strong upwelling, oxygen-depleted waters are also present in the northern Indian Ocean and in enclosed basins like the Baltic and the Black Sea [12].

The OMZ off Peru, Chile and Ecuador in the South Pacific Ocean is the largest oceanic area where  $O_2$  concentrations are reported to fall below the detection limit of the most sensitive  $O_2$  sensors ( $\sim 10$ – $100$  nM) [13–16]. In the absence of  $O_2$ , organic carbon degradation has been historically attributed to heterotrophic denitrification, the reduction of nitrate ( $NO_3^-$ ) to dinitrogen gas ( $N_2$ ) [17–19]. Some *in situ* experiments have shown active heterotrophic denitrification in OMZ waters [20,21], however numerous studies have demonstrated that anammox, the anaerobic oxidation of ammonium ( $NH_4^+$ ) with nitrite ( $NO_2^-$ ) to  $N_2$ , is responsible for the major loss of fixed nitrogen from the OMZ off Namibia [22], Oman [15], Peru [23–25] and Chile [26]. As anammox is an autotrophic process, its dominance over heterotrophic denitrification questions our understanding of organic matter remineralization in OMZ waters. This is supported by the hypothesis that oxygen-depletion leads to a shift of the microbial community from organoheterotrophs to chemolithotrophs [27,28].

In anoxic sediments, the main process for the degradation of organic carbon is considered to be microbial sulfate ( $SO_4^{2-}$ ) reduction to elemental sulfur ( $S^0$ ) and hydrogen sulfide ( $H_2S$ ) [29]. The release of large quantities of the toxic  $H_2S$  from these underlying sediments can lead to the occasional build-up of high concentrations of  $H_2S$  in bottom waters [30–33]. On the other hand, it has also been suggested that  $H_2S$  build-up in oceanic waters could be caused by  $SO_4^{2-}$  reduction of pelagic microorganisms within the water column [34,35]. A recent study suggested that an active, but cryptic sulfur cycle is present in non-sulfidic subsurface waters in the eastern tropical South Pacific OMZ off northern Chile [14]. According to this hypothesis,  $SO_4^{2-}$  reduction and consequently  $H_2S$  formation would take place in the water column, even when thermodynamically more favourable electron acceptors like  $NO_3^-$  or  $NO_2^-$  are still present. However, the resulting  $H_2S$  would be rapidly re-oxidized to  $S^0$  or  $SO_4^{2-}$ , such that the two processes are in steady-state, thereby preventing an accumulation of  $H_2S$  [31,36].

The presence of chemolithoautotrophic  $\gamma$ -proteobacteria involved in sulfur cycling (e.g. related to the uncultured SUP05 cluster bacterium, referred to henceforth as SUP05) in non-sulfidic OMZ waters supports the hypothesis of a cryptic sulfur cycle [8,14,37–40]. Studies conducted during occurrences of sulfidic events in the Benguela Current upwelling OMZ and in a seasonally anoxic fjord in Canada demonstrated that  $\gamma$ -proteobacteria related to SUP05 are highly abundant in sulfidic waters and that they probably detoxified the waters via the chemolithotrophic oxidation of  $H_2S$  coupled to the reduction of  $NO_3^-$  [31,41], a pathway termed sulfur-driven autotrophic denitrification. In addition, chemolithoautotrophic  $\alpha$ - and especially  $\epsilon$ -proteobacteria detected in the sulfidic waters in the Benguela Current upwelling OMZ as well as in the sulfidic zones of the Baltic and the Black Sea could also be important members of microbial communities in sulfidic plumes [31,42,43].

The initiation, termination and frequency of sulfidic events in oceanic OMZs are so far poorly understood, and  $H_2S$  in the water column has been mostly observed in enclosed basins like the Baltic Sea [43–45], the Black Sea [46–48], the Cariaco trench off Venezuela [49,50] and the Saanich Inlet in Canada [41,51]. With the exception of a few studies mentioning the characteristic odor of  $H_2S$  and anecdotal reports of Peruvian fishermen on ‘black’ fishing gear in relation to the so-called ‘aquajes’ conditions for the OMZ off Peru, sulfidic waters have not been measured in the Pacific Ocean so far [31,34,35,52].

Negative consequences on fish stocks and quality of life along the populated coastal upwelling regions are potentially severe, because  $H_2S$  is highly toxic to animals and humans and has already been invoked as the cause for occasional but massive fish kills in African shelf waters [53–55]. The anticipated decrease in  $O_2$  concentrations and the increase in water column stratification, as predicted from global change [11], as well as local eutrophication [27,35], might lead to more frequent and intense depletion of  $O_2$  and of alternate electron acceptors (e.g.  $NO_3^-$  and  $NO_2^-$ ), favouring the development of sulfidic waters within OMZs [31]. Given that the detoxification of sulfidic water is a microbial process, it is important to assess the phylogenetic structure and the metabolic response of the endemic microbial community to the accumulation of  $H_2S$ .

We explored the microbial community structure and its transcriptional activity in such a sulfidic event off the coast of Peru with high-throughput metagenomic and metatranscriptomic sequencing. We present rate measurements of carbon dioxide ( $CO_2$ ) fixation and nitrogen transformation processes as well as total bacterial cell counts with flow-cytometry. Several of the proteobacterial taxa that were dominant in the sulfidic waters were expressing genes involved in the sulfur cycle, which reflected various metabolic strategies for  $H_2S$  oxidation. Our data further suggests that the microbial communities were responsible for considerable light-independent  $CO_2$  fixation.

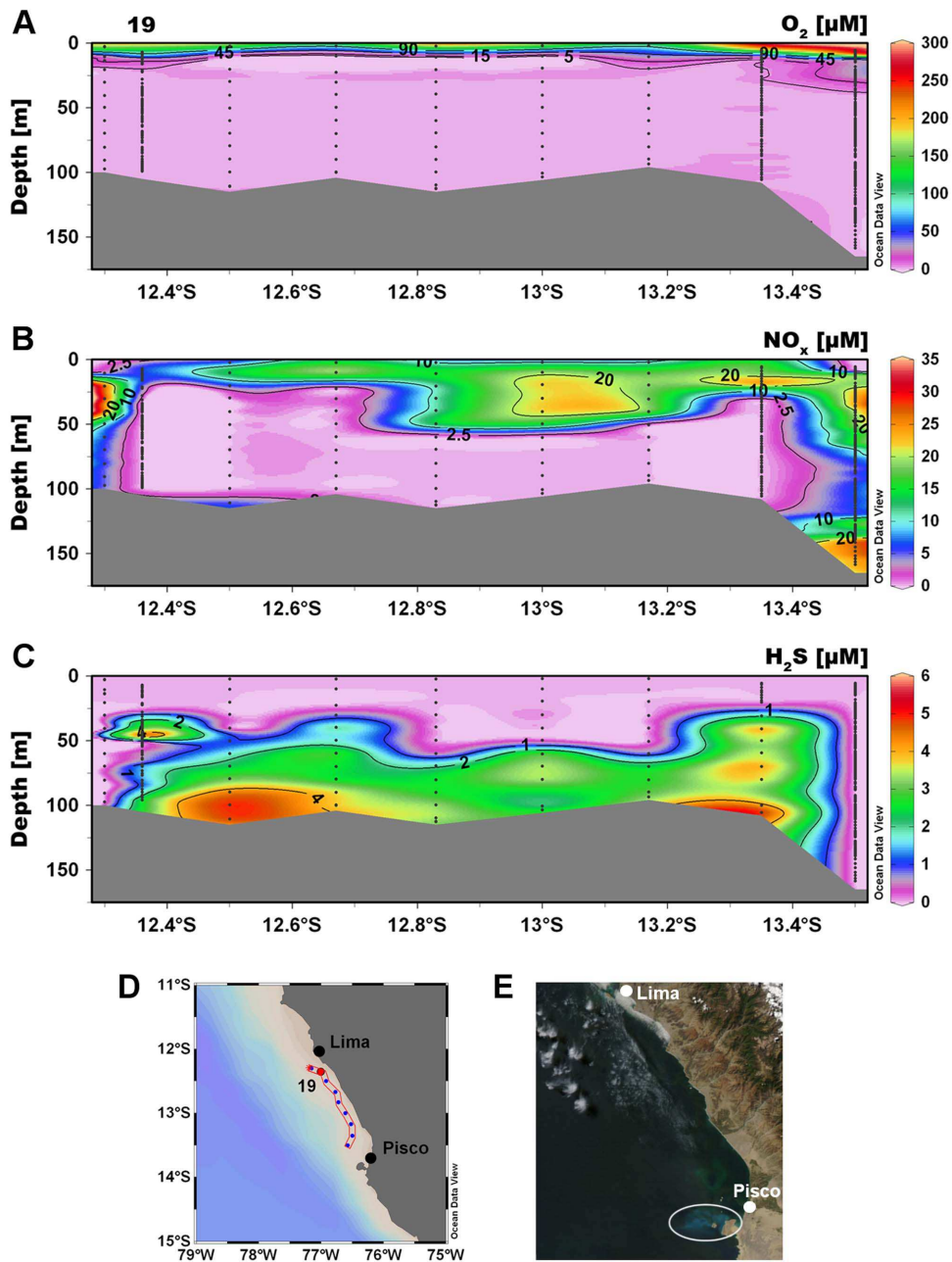
## Results and Discussion

### Description of the sampling site

During RV Meteor cruise M77/3 on the Peruvian shelf in January 2009 we found sulfidic waters stretching from Lima to Paracas National Reserve southwest of Pisco (Figure 1).  $O_2$  concentration in shelf waters in the study area were generally below the detection limit of our microsensor ( $0.5$ – $1$   $\mu M$ ) at water depth below 20 m (from  $12^\circ S$  to  $14^\circ S$ ; Figure 1A), while  $NO_x$  (the sum of  $NO_3^-$  and  $NO_2^-$ ) was heavily depleted in the water column throughout the transect from 20–60 m to the bottom (from  $12^\circ 30' S$  to  $13^\circ 50' S$ ; Figure 1B), mirror-imaging the distribution of  $H_2S$  (Figure 1C). Sulfidic waters were first detected on January 9<sup>th</sup> south of Lima and seemed to have persisted until the end of the cruise, when  $H_2S$ -containing waters covered  $\sim 5500$  km<sup>2</sup> of the shelf. The thickness of the sulfidic layer was about 80 m (Figure 1C), yielding the largest sulfidic plume ( $\sim 440$  km<sup>3</sup>) ever reported for oceanic waters. We calculated a  $H_2S$  content of  $\sim 2.2 \times 10^4$  tons. The total area affected by  $H_2S$  may have been even larger, as we did not map the extent of the sulfidic plume into the protected area of the Paracas National Reserve.

Remote satellite sensing revealed large patches ( $50$ – $150$  km<sup>2</sup>) of turquoise discoloured surface-waters, attributable to the formation of colloidal  $S^0$  upon  $H_2S$  oxidation [56,57] off Lima as well as in Paracas National Reserve during our sampling campaign (Figure S1). The larger extension of  $H_2S$  in deeper waters when compared to the colloidal  $S^0$  in the surface indicated that most of the  $H_2S$  was oxidized in subsurface waters, similar to the observations from the Benguela upwelling system [31]. Colloidal  $S^0$  plumes measuring up to 500 km<sup>2</sup> were observed in the same region again in May 2009, indicating that the occurrence of  $S^0$  plumes detected in January 2009 was not a unique event (Figure 1E). This suggests that sulfidic waters in the OMZ off Peru might be more frequent and persistent than originally thought.

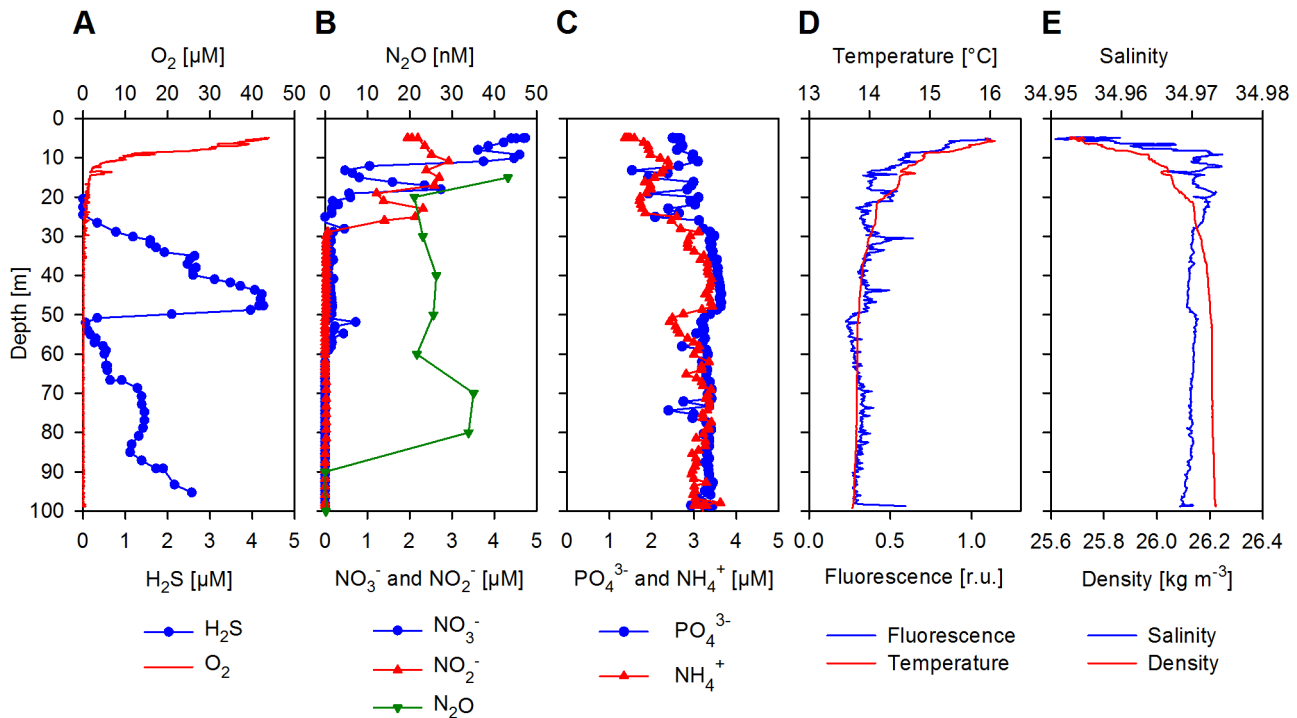
A vertical profile of the sulfidic water column (station 19), sampled during the upcast with a pump-CTD on January 9<sup>th</sup>, 2009 at a site located  $\sim 15$  km offshore Lima ( $12^\circ 21.88' S$ ,



**Figure 1. Extent of the sulfidic plume off the Peruvian coast.** (A) Vertical distribution of  $O_2$  concentrations. (B) Vertical distribution of  $NO_x$  (the sum of  $NO_3^-$  and  $NO_2^-$ ) concentrations. (C) Vertical distribution of  $H_2S$  concentrations. (D) Areal view of stations sampled along the transect off the Peruvian coast between Lima and Pisco. The station (19) that was analyzed in detail is marked with a red dot. (E) Satellite image (MODIS) showing a colloidal  $S^0$  plume (white circle) on May 8<sup>th</sup>, 2009. doi:10.1371/journal.pone.0068661.g001

77°00'W, ~100 m water depth; Figure 1D) was the target of a detailed analysis. The surface mixed layer was shallow with the thermocline at about 10 m water depth (Figure 2D). The surface temperature ( $>16^\circ\text{C}$ ) was only  $\sim 2^\circ\text{C}$  warmer than the bottom waters and the salinity (34.95–34.97) changed merely slightly with depths, which indicated an active upwelling of subsurface waters. Even the surface waters were characterized by low  $O_2$  conditions down to 40  $\mu\text{M}$  (or  $\sim 15\%$  saturation; Figure 2A). During the downcast,  $O_2$  decreased at the thermocline and dropped to about the detection limit of our amperometric  $O_2$  microsensors (0.5–1  $\mu\text{M}$ ) at around 20 m. Nevertheless, trace amounts of  $O_2$

( $<1 \mu\text{M}$ ) were still detected with some variability down to  $\sim 40$  m water depth. These low concentrations of  $O_2$  were close to detection limit of our sensor and we cannot rule out whether this was due to water advection caused by the CTD rosette or to a memory effect of the sensor. However, using a highly-sensitive self-calibrating Switchable Trace amount Oxygen (STOX) sensor with a detection limit of  $\sim 50$  nM [13,16] during the upcast of the CTD rosette,  $O_2$  was undetectable below 20 m, and therefore we defined this zone as anoxic. We detected large vertical movement in the oxycline from 5–18 m due to internal waves, which may



**Figure 2. Vertical distribution of physical, chemical and biological water properties.** (A) Concentrations of H<sub>2</sub>S and O<sub>2</sub>. (B) Concentrations of NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> and N<sub>2</sub>O. (C) Concentrations of PO<sub>4</sub><sup>3-</sup> and NH<sub>4</sub><sup>+</sup>. (D) *In situ* fluorescence (chlorophyll, relative units as measured with the pump-CTD) and temperature. (E) Salinity and density.  
doi:10.1371/journal.pone.0068661.g002

have caused non steady state conditions and induced a flux of O<sub>2</sub> down into the anoxic waters.

Phosphate (PO<sub>4</sub><sup>3-</sup>) and NH<sub>4</sub><sup>+</sup> concentrations remained high (both around 3 μM) and stable throughout the water column, with NH<sub>4</sub><sup>+</sup> only having a minor drop in concentrations near 50 m (Figure 2C). NO<sub>3</sub><sup>-</sup> concentrations (detection limit ~0.1 μM) were lower than those measured in the southern part of the study area and lower than expected from strong upwelling regions in general. Highest NO<sub>3</sub><sup>-</sup> concentrations with ~5 μM were found in surface waters, but dropped rapidly below 1 μM, just beneath the oxycline at ~19 m (Figure 2B). Detectable concentrations of NO<sub>3</sub><sup>-</sup> (ranging from 0.1–0.2 μM) were measured down to 59 m with the exception of 52–55 m, where a small increase up to 0.7 μM was observed. NO<sub>2</sub><sup>-</sup> (detection limit ~0.01 μM) was high (1.5–3 μM) in surface waters down to 26 m. Trace concentrations (~25 nM) were measurable down to 50 m and again from 67–81 m. Nitrous oxide (N<sub>2</sub>O) concentrations ranged between 20–40 nM from 15–80 m and dropped below the detection limit closer to the bottom of the water column (Figure 2B). H<sub>2</sub>S was first detected (with both microsensor and wet chemistry) at 26 m and increased steadily, reaching a concentration of 4.2 μM at 48 m (Figure 2A). This maximum was followed by a rapid drop in concentrations to below 0.1 μM at 52–53 m, before increasing again to about 2.6 μM at 95 m, approximately 5 m above the sediment. H<sub>2</sub>S concentrations directly at the sediment-water interface were probably even greater, but were not measured in this study.

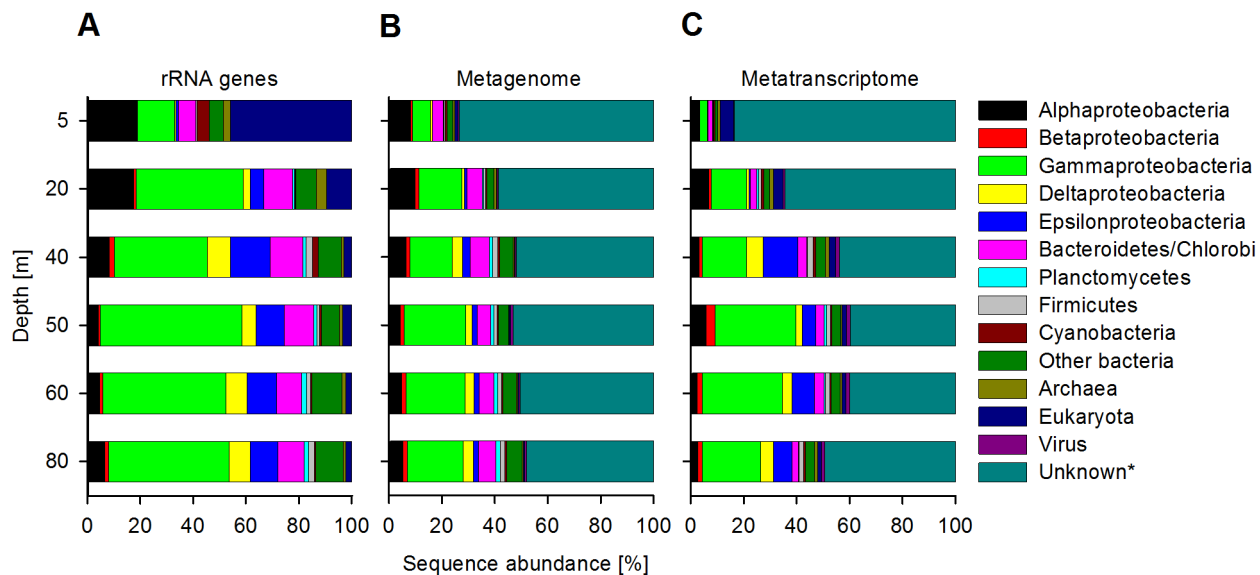
### Phylogenetic diversity of the microbial community

Based on the monitoring of O<sub>2</sub> and H<sub>2</sub>S during the upcast, we defined three zones within the water column, where we carried out a detailed sampling: the oxic surface (5 m sample, where sampling

was stopped when internal waves decreased O<sub>2</sub> concentrations to below 30 μM), the upper boundary of the anoxic zone (15 and 20 m samples) and the sulfidic zone (30, 40, 50, 60, 80 and 100 m samples). Both a hierarchical clustering approach with a statistical analysis of taxonomic assignments and a non-parametric Multi-dimensional Scaling indicated that the selected sample groups were justified (an ANOSIM test using a Bray-Curtis distance measure showed a Global R value of 0.83 and a significance level of 0.1%, Figure S2).

Using a 98% similarity cut off, the metagenomes and metatranscriptomes accounted for an average of 263,606 (DNA) and 98,785 (RNA) unique sequences (clusters) per depths (Table S1). A total of 4809 (DNA) and 3872 (RNA) different taxa were identified using BLAST-searches, revealing a highly diverse microbial community at all depths (Table S2). The taxonomic composition based on all rRNA genes (5,923 sequences) using BLASTn-searches against the SILVA database is shown in Figure 3A and based on the metagenomes (1,882,842 sequences, excluding all rRNA genes) and the metatranscriptome (421,528 sequences, excluding all rRNAs) using BLASTx-searches against the non-redundant database of NCBI in Figure 3B and 3C. A large percentage of the sequences found in both the metagenomes and metatranscriptomes had no significant match against the non-redundant database of NCBI. On average, 49% of the sequences remained unidentified, which is comparable to other studies that utilized high-throughput sequencing technologies in marine habitats [37,40,58–60].

The community structure presented a stable and uniform distribution at the phylum-level, especially within the sulfidic zone (Figure 3). The metagenome data suggested that the microbial community was overall dominated by proteobacteria (16.6–34.1% of all DNA sequences, including all unidentified sequences); while



**Figure 3. Vertical distribution of taxonomic assignments.** Shown on either domain, phylum or class level. (A) rRNA genes in percent of all rRNA genes (5,923 sequences). (B) Metagenomic sequences in percent of all metagenomic sequences (1,882,842 sequences, excluding rRNA genes). (C) Metatranscriptomic sequences in percent of all metatranscriptomic sequences (421,528 sequences, excluding rRNAs). ‘Other Bacteria’ include Acidobacteria, Actinobacteria, Aquificae, Chlamydiae, Chloroflexi, Deferribacteres, Deinococcus-Thermus, Dictyoglomi, Elusimicrobia, Fibrobacteres, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Nitrospirae, Spirochaetes, Synergistetes, Tenericutes, Thermotogae and Verrucomicrobia. \*Sequences with no significant match against the non-redundant database of NCBI or sequences with a match that lacks taxonomic information. doi:10.1371/journal.pone.0068661.g003

the Bacteroidetes/Chlorobi-group was the second largest group we could identify (3.9–7.4%). In oxic and anoxic waters, both  $\alpha$ - and  $\gamma$ -proteobacterial sequences were abundant (6.9–16.4%), similar to previous findings from the OMZs off northern Chile [38] and the Arabian Sea [61]. In sulfidic waters,  $\gamma$ -proteobacteria were clearly dominating (up to 23.2%) and we further found a significant increase in the frequencies of  $\delta$ - and  $\epsilon$ -proteobacterial sequences (1.9–4.0%), which were much less abundant in the 5 and 20 m samples. The  $\delta$ - and  $\epsilon$ -proteobacterial sequences were even more abundant within the rRNA gene dataset (Figure 3A) when compared to the metagenomes (Figure 3B), probably due to the lack of representatives genomes for these groups.

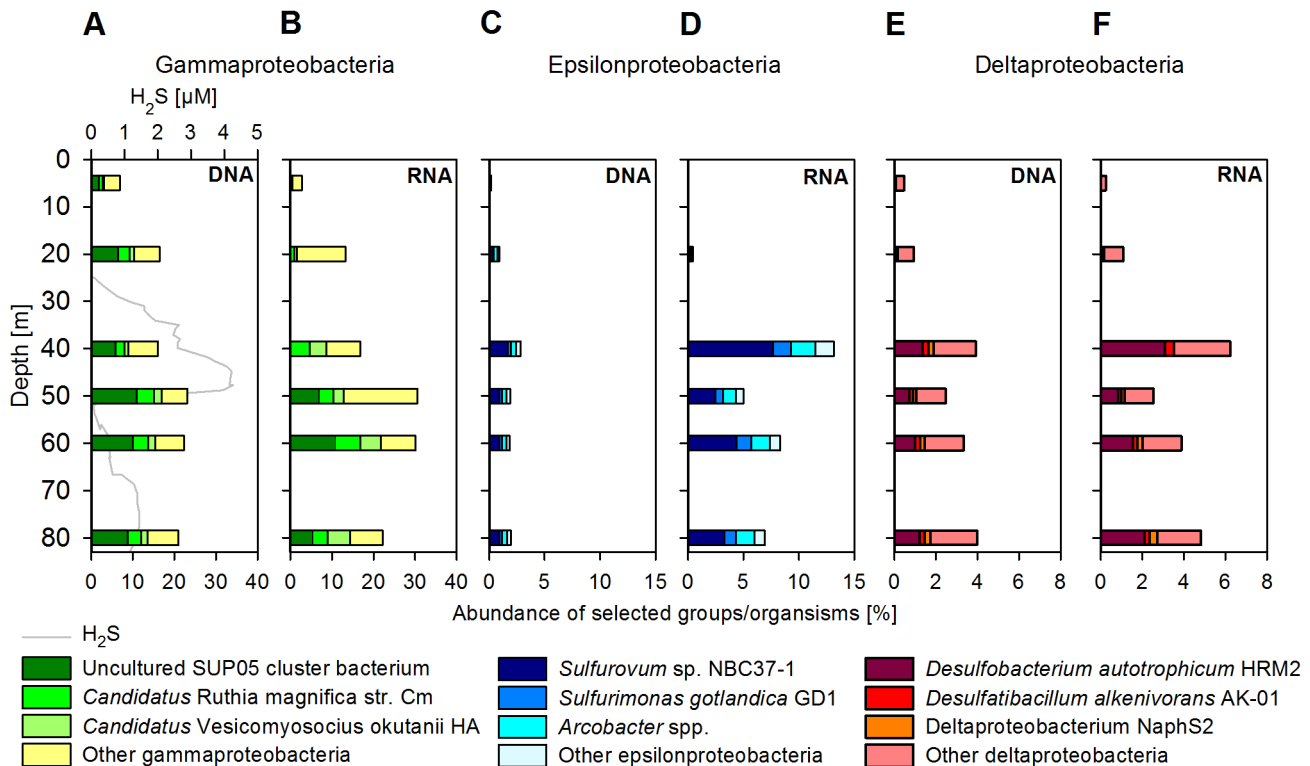
The metatranscriptomes showed a more variable picture of the microbial community (Figure 3C). In surface waters eukaryotic sequences formed the largest identifiable group (5%), while at all other depths  $\gamma$ -proteobacteria were dominating (13.3–30.5%). In sulfidic waters  $\epsilon$ -proteobacterial transcripts were further identified in relatively high, but also more variable proportions (5.0–13.2%), when compared to the metagenomes. Notably, ‘other bacteria’, summarizing 19 bacterial phyla, were present at all depth, but never exceeded 5.8% of the sequences, in both the metagenome and the metatranscriptome datasets.

A more detailed analysis of the metagenomes revealed that the oxic surface waters harboured several different photoheterotrophic organisms (Figure S3). Prokaryotes similar to *Candidatus Pelagibacter* sp. HTCC7211 accounted for 1.6% and relatives of *Candidatus Pelagibacter* sp. HTCC1002 made up 0.4% of all DNA sequences, which is in agreement with other studies conducted in OMZs [14,37,40,61]. Additionally, photosynthetic *Synechococcus* spp., which are also known to be present in OMZ waters [62] accounted for about 0.4% of the sequences. However, the most abundant single taxon identified in the oxic surface metagenome had high similarity to the uncultured SUP05 cluster bacterium (1.9%), a chemolithoautotrophic sulfur oxidizer, which has been detected previously in oxygen-depleted [14,37,38,40] and sulfidic

waters [31,41,42,63]. In the metagenome sample from the anoxic zone (20 m), SUP05 was also the dominant taxon with 6.6% of all DNA sequences (Figure 4A and S3). At 20 m and below,  $\gamma$ -proteobacterial sulfur oxidizers (GSO) related to gill symbionts of deep-sea hydrothermal-vent clams, *Candidatus Ruthia magnifica* str. Cm (2.6%) and *Candidatus Vesicomysocius okutanii* HA (1.4%) [64,65] became increasingly abundant. This indicated that the GSO-community at our sampling site was composed of at least three separate taxa. In the sulfidic zone, the dominance of the GSO-group was even higher, reaching a maximum of 17% of all DNA sequences at 50 m (Figure 4 and S3). Other common microorganisms in the metagenome were similar to the  $\epsilon$ -proteobacterium *Sulfurovum* sp. NBC37-1 (up to 1.7%) and to the  $\delta$ -proteobacterium *Desulfobacterium autotrophicum* HRM2 (up to 1.4%) [66–68]. In all sulfidic depths, organisms related to SUP05, *R. magnifica*, *V. okutanii*, *Sulfurovum* and *D. autotrophicum* were the five most abundant organisms that we identified with BLAST-searches.

In contrast to the metagenomes, the metatranscriptomes reflect the suite of genes that were expressed in the microbial community at the time of sampling and therefore displayed a more variable picture of the microbial community, showing to some extent its actual metabolic activity. In the oxic surface waters, we found RNA sequences similar to the archaeal ammonia-oxidizer *Nitrosopumilus maritimus* SCM1 and the  $\alpha$ -proteobacterium *Magnetspirillum gryphiswaldense* to comprise the largest single taxa (0.7 and 0.4%). *N. maritimus* is considered a ‘classical’ inhabitant of the oxycline in OMZ waters [14,37,39,40]. At 20 m, RNA sequences similar to the  $\gamma$ -proteobacteria *Marinomonas* sp. MWYL1 and *Neptuniibacter caesariensis* were most abundant (1.3 and 1.2%).

Similar to the metagenome assignments, the GSO-group was predominant in the metatranscriptomes throughout the sulfidic zone. However, the composition of the GSO-group was variable and changed with depths. While SUP05 was virtually undetectable using BLAST-searches in the 5, 20 and 40 m metatranscriptomes



**Figure 4. Vertical distribution of dominant proteobacterial taxa.** Shown in percent of all sequences (excluding rRNA genes and rRNAs). (A) DNA and (B) RNA sequence abundances for  $\gamma$ -proteobacteria. (C) DNA and (D) RNA sequence abundances for  $\epsilon$ -proteobacteria. (E) DNA and (F) RNA sequence abundances for  $\delta$ -proteobacteria. doi:10.1371/journal.pone.0068661.g004

(0–0.1%; Figure 4B and S3), GSO-related RNA sequences at these depths could be almost exclusively assigned to the relatives of *R. magnifica* and *V. okutanii*. In all other metatranscriptomes from sulfidic waters (50, 60 and 80 m), sequences similar to SUP05 represented the most abundant identifiable taxon (5.5–11%).

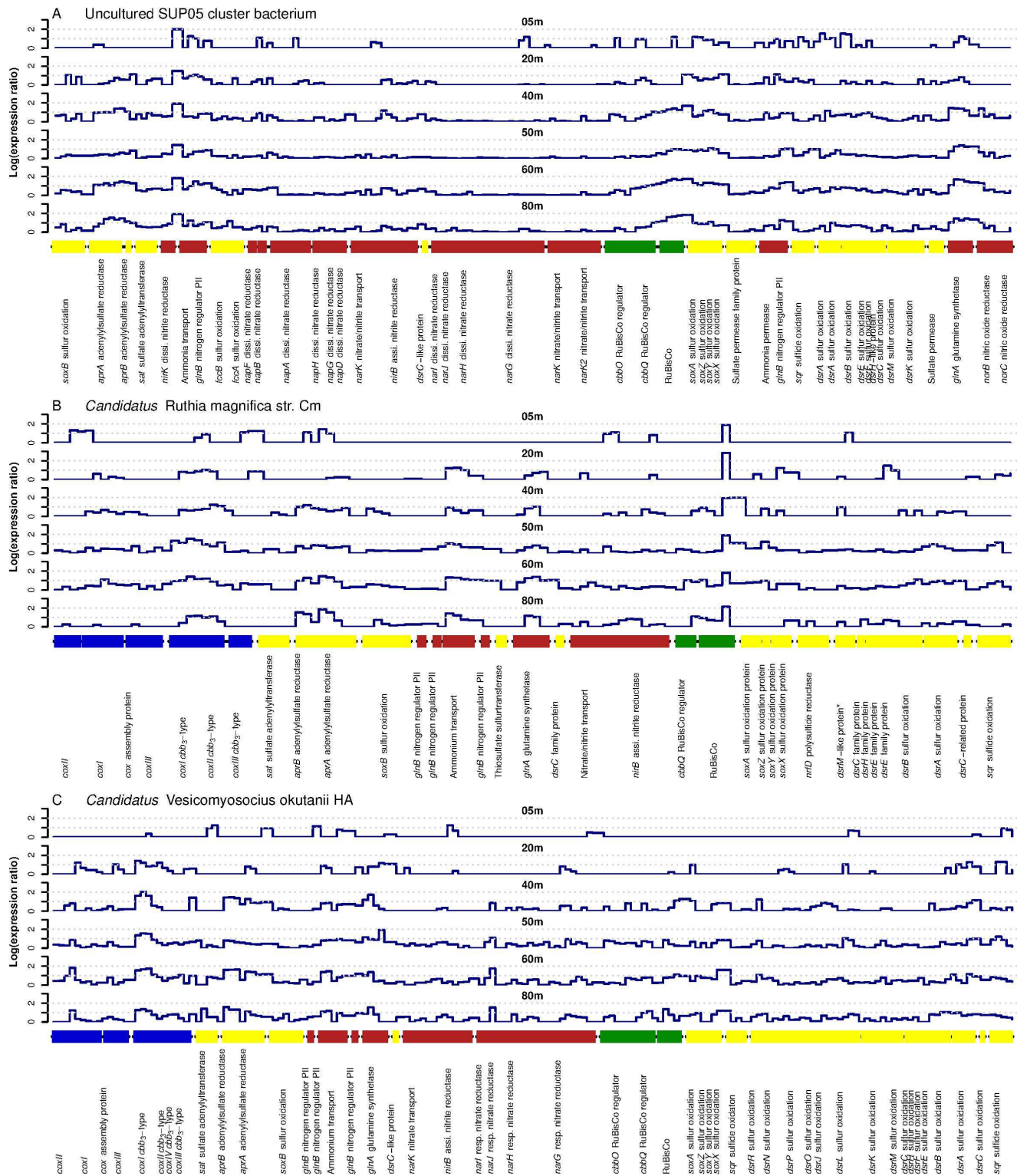
Also similar to the metagenomes, the five most abundant organisms detected in the metatranscriptomes within sulfidic waters (except at 40 m) were related to SUP05, *R. magnifica*, *V. okutanii*, *Sulfurovum* and *D. autotrophicum*. We also identified several other taxa in the metatranscriptomes that were poorly represented in the metagenomes, some of which were closely related to members of hydrothermal vent communities. At 40 m, organisms related to the  $\epsilon$ -proteobacterium *Arcobacter butzleri* RM4018 accounted for 2% of all RNA sequences and at 50 m the  $\gamma$ -proteobacterium *Colwellia psychrerythraea* 34H accounted for 3%. In deeper parts of the water column (60 and 80 m), organisms similar to the  $\epsilon$ -proteobacteria *Sulfurimonas gotlandica* GDI (1.1–1.3%) and *Arcobacter nitrofigilis* DSM 7299 (0.9–1%) were detected. Although sequences similar to the anammox-planctomycete *Candidatus Kuenenia stuttgartiensis* have been found in high abundances in OMZ waters [14,37,40], they were relatively rare within our samples, never exceeding 0.7% of all DNA and 0.8% of all RNA sequences.

### Metabolic activity and functional diversity of the microbial community

**General activity patterns.** To assess the functional diversity of the microbial community in detail, we used three different approaches to investigate our sequence data. The BLAST-searches were supplemented by scans of our sequences with

profile hidden Markov models of the ModEnZA Enzyme Commission (EC) groups [69] and of the Pfam protein families [70]. Furthermore, we recruited the DNA and RNA sequences onto the genomes of the five organisms most often recognized by our BLAST-searches, SUP05, *R. magnifica*, *V. okutanii*, *Sulfurovum* and *D. autotrophicum* [41,64–67]. For these genome recruitments, we calculated the expression-ratio, a measure of the enrichment of selected transcripts over the corresponding genes, normalized to the total pool of all protein-coding sequences (Figure 5 and S4). In general, the genome recruitment plots showed high expression-ratios for ribosomal proteins, DNA and RNA polymerases, cell division proteins and transcription and translation factors indicating a growing microbial community (data not shown). Similarly, the collection of all abundant EC numbers also suggested an overall active microbial community (Figure S5). Sequences encoding for ubiquitous proteins related to general metabolic activity like DNA and RNA polymerases, DNA topoisomerases and adenosinetriphosphatases (a general ATP-binding and hydrolyzing motif in sequences which lack further specific functional information) were among the most abundant at all depths.

**Hydrogen sulfide sources.**  $\text{H}_2\text{S}$  formation through microbial  $\text{SO}_4^{2-}$  reduction commonly occurs in anoxic marine sediments, where it is considered to be the main heterotrophic process for the degradation of organic carbon [29]. The sedimentary flux has been shown to be the main source of  $\text{H}_2\text{S}$  in the water column during sulfidic events in the Benguela Current upwelling system [30,31]. However, at times water column  $\text{SO}_4^{2-}$  reduction could also contribute significantly to the  $\text{H}_2\text{S}$  accumulation in oceanic waters [34,35], as demonstrated in the ~2000 m thick anoxic water column in the Black Sea, where pelagic  $\text{SO}_4^{2-}$  reduction rates ranged between 0.01–3.5  $\text{nmol l}^{-1} \text{d}^{-1}$  [71].



**Figure 5. Vertical distribution of sequences recruited onto the genomes of three  $\gamma$ -proteobacterial sulfur oxidizers.** Shown are selected genes encoding for enzymes involved in oxygen (blue), sulfur (yellow), nitrogen (red) and carbon metabolism (green) in the corresponding order of the genomes. The y-axis depicts the log of the expression-ratio, a measure for the selective enrichment of transcripts over the corresponding gene, normalized to the total pool of protein-coding sequences. A list of the start and end position of all genes and the full names of the corresponding enzymes are shown in Table S3. (A) Uncultured SUP05 cluster bacterium. (B) *Candidatus Ruthia magna* str. Cm. (C) *Candidatus Vesicomysocius okutanii* HA. \*This DsrM-like protein has also high similarity to a *narG* respiratory nitrate reductase. doi:10.1371/journal.pone.0068661.g005

Moreover, H<sub>2</sub>S formation from SO<sub>4</sub><sup>2-</sup> has been measured even in the presence of more favourable electron acceptors (NO<sub>x</sub>) in the OMZ waters off northern Chile after preincubations with H<sub>2</sub>S [14].

We investigated the sulfur cycling by identifying genes and transcripts indicative of specific metabolic functions related to sulfur transformation processes in our collection of sequences and used flux calculations to estimate the sedimentary source of H<sub>2</sub>S. *D. autotrophicum*, one of the most abundant organism we identified in our sequence dataset, is a metabolically versatile SO<sub>4</sub><sup>2-</sup>-reducing marine  $\delta$ -proteobacterium, which can completely oxidize organic carbon compounds to CO<sub>2</sub>, but is also capable of growing autotrophically on hydrogen (H<sub>2</sub>) [67]. The genome recruitment plots of *D. autotrophicum* (Figure S4B) show regions of the genome mostly related to energy metabolism and nutrient cycling. High expression-ratios for key sulfur metabolizing enzymes like the dissimilatory sulfite reductase (*dsrABD*), the adenylylsulfate reductase (*aprAB*) and the sulfate adenylyltransferase (*sat2*) suggest that *D. autotrophicum* could have been reducing SO<sub>4</sub><sup>2-</sup> and thus may have contributed to the formation of H<sub>2</sub>S at our sampling site. However, since many sulfur cycling proteins (e.g. *dsr* and *apr*) can function in both the oxidation and reduction of sulfur species [72,73], other chemolithoautotrophic metabolic processes (e.g. the disproportionation of sulfur compounds resulting in the simultaneous formation of H<sub>2</sub>S and SO<sub>4</sub><sup>2-</sup>) could have been catalyzed by these enzymes at the time of sampling [74]. The presence of large plumes of colloidal S<sup>0</sup> (Figure S1) in the study area would have supported this chemolithoautotrophic reaction.

A numerical compilation of all sequences affiliated to known SO<sub>4</sub><sup>2-</sup> reducers and sulfur oxidizers is shown in Figure 6 [14,72,73]. Although the abundance of SO<sub>4</sub><sup>2-</sup> reducing organisms throughout sulfidic waters was not correlated with the H<sub>2</sub>S concentrations, it is likely that they contributed to SO<sub>4</sub><sup>2-</sup> reduction and H<sub>2</sub>S formation in the water column at the time of sampling. However, assuming the maximum SO<sub>4</sub><sup>2-</sup> reduction rates (1.3–12 nmol l<sup>-1</sup> d<sup>-1</sup>) measured in OMZ waters off northern Chile [14], it would take more than one year (in the absence of any oxidation) to accumulate the H<sub>2</sub>S concentrations reported in our study. In contrast, SO<sub>4</sub><sup>2-</sup> reduction in sediments underlying the eastern tropical South Pacific OMZ is generally very high (10–30 mmol m<sup>-2</sup> d<sup>-1</sup>) [75] and thus the most likely source of the H<sub>2</sub>S we measured in the water column.

Assuming steady state conditions, we used the density structure and the H<sub>2</sub>S concentration gradient (Figure 2A) in the bottom water to estimate a turbulent diffusion of  $\sim 10^{-4}$  m<sup>2</sup> s<sup>-1</sup> and, subsequently, a sedimentary efflux of  $\sim 2$  mmol H<sub>2</sub>S m<sup>-2</sup> d<sup>-1</sup>. This is well within the estimates based on sedimentary flux calculations and SO<sub>4</sub><sup>2-</sup> reduction rate measurements (1–11 mmol m<sup>-2</sup> d<sup>-1</sup>) from sediments directly underlying sulfidic events [76]. Moreover, the repeated observations of the H<sub>2</sub>S maxima in bottom waters along the transect (Figure 1C) point towards the sediment as the main H<sub>2</sub>S source.

We observed a second distinct H<sub>2</sub>S maximum in the water column at  $\sim 48$  m with no direct contact to the sediment. However, the salinity and the corresponding PO<sub>4</sub><sup>3-</sup> and NH<sub>4</sub><sup>+</sup> concentrations (Figure 2C and 2E) indicated that the upper H<sub>2</sub>S layer was most probably created by lateral advection of nearby bottom waters that had recently been in contact with sulfidic sediments rather than by production of H<sub>2</sub>S within the water column.

**Sulfur oxidation.** The largest functional group of microorganisms detected in sulfidic waters were  $\gamma$ -proteobacterial sulfur oxidizers. Figure 5 shows the expression-ratio for selected genome regions of the three most abundant GSO-representatives. The

recruitment of the sequences onto separate genomes supported the presence of at least three distinct taxa within the GSO-community (similar to SUP05, *R. magnifica* and *V. okutanii*) at our study site, which were actively growing and metabolizing. Figure S4A further depicts a genome recruitment plot for  $\epsilon$ -proteobacterial *Sulfurovum*. The recruitment of sequences onto the genome of *Sulfurovum* was much less extensive than for the GSO-group, and showed low coverage especially in the oxic and anoxic depths (5 and 20 m).

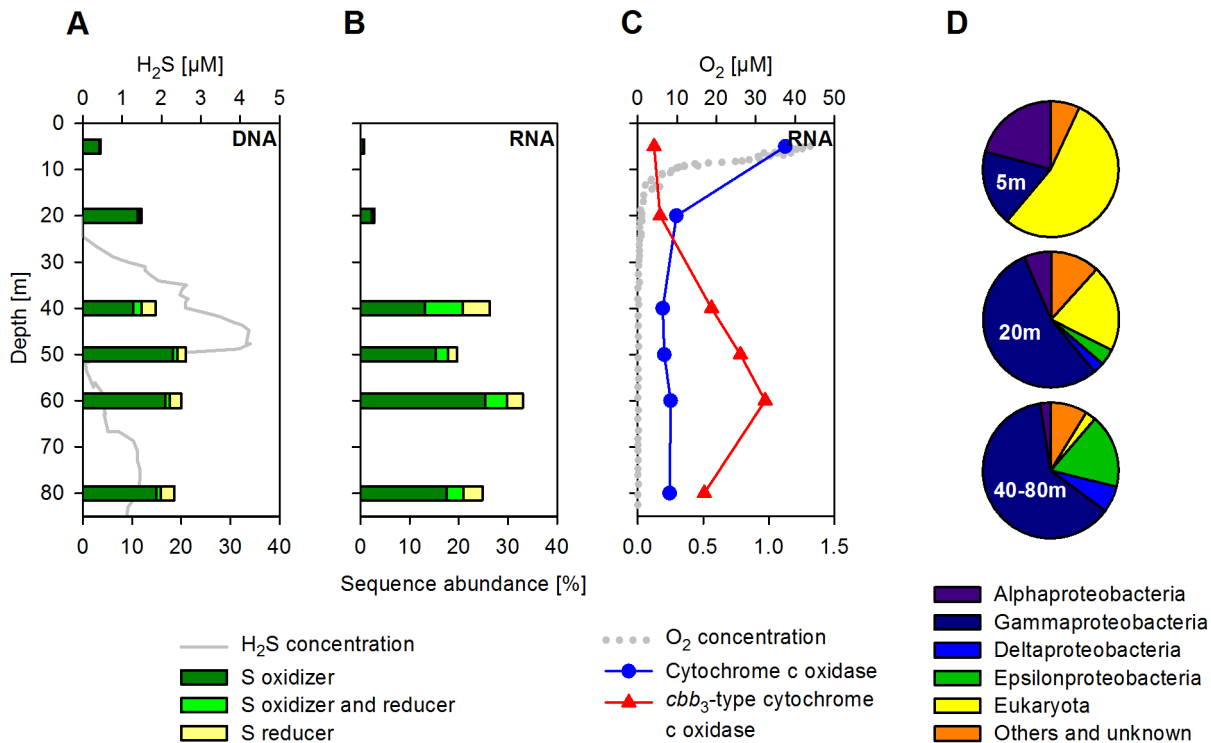
A large number of transcripts were recruited onto genes (if present in the genomes) of the reverse dissimilatory sulfite reduction (*dsr* - oxidation of intracellular S<sup>0</sup>) and the periplasmic sulfur oxidation (*sox* - S<sub>2</sub>O<sub>3</sub><sup>2-</sup> oxidation) pathways, both encoding for enzymes involved in the oxidation of reduced sulfur compounds. Additionally, we found high expression-ratios for the sulfide:quinone oxidoreductase (*sqr* - H<sub>2</sub>S oxidation to S<sup>0</sup>), the adenylylsulfate reductase (*apr*) and the sulfate adenylyltransferase (*sat* - latter both SO<sub>3</sub><sup>2-</sup> oxidation to SO<sub>4</sub><sup>2-</sup>). The abundance and coverage of transcripts matching these clusters for the GSO-group and *Sulfurovum* increased generally with depth, delivering the highest expression-ratios of genes encoding for sulfur oxidizing proteins within sulfidic waters. For *Sulfurovum*, which is thought to be capable of both sulfur oxidation and SO<sub>4</sub><sup>2-</sup> reduction [66,77,78], the transcript coverage for its *sox*- and *sqr*-genes suggested that it was likely acting as a sulfur oxidizer at the time of sampling.

*R. magnifica*, *V. okutanii* and *Sulfurovum* genomes harbour genes for different cytochrome c oxidases (*cox*) which are used in oxic respiration and are absent in the currently annotated version of the SUP05 genome [41,64–66]. Of the transcripts recruiting to the *R. magnifica* genome, the cytochrome c oxidase was among the most abundant in the oxic surface. In anoxic and sulfidic waters, however, expression patterns changed and transcripts for a *cbb*<sub>3</sub>-type cytochrome c oxidase dominated instead. Transcripts for the *cbb*<sub>3</sub>-type cytochrome c oxidase recruiting to the genomes of *V. okutanii* and *Sulfurovum* were also among the most highly expressed in sulfidic waters.

The *cbb*<sub>3</sub>-type cytochrome c oxidase is thought to be involved in a specialized microaerobic respiration. This enzyme has an extremely high affinity to O<sub>2</sub>, with a *K<sub>M</sub>* value as low as 7 nM, allowing certain proteobacteria to colonize oxygen-limited or even presumed anoxic environments [79,80], well below the detection limit of the microsensors (0.5–1  $\mu$ M) and the STOX sensor ( $\sim 50$  nM) we used in this study. Furthermore, it was shown that *Escherichia coli* cultures actively grew and respired O<sub>2</sub> even below the detection limit of a highly sensitive STOX sensor ( $\leq 3$  nM), probably using a high-affinity cytochrome *bd* oxidase [81].

A clear separation of the cytochrome c oxidase expression was visible between the oxic and the anoxic/sulfidic zone, directly reflecting the availability of O<sub>2</sub> (Figure 6C). In oxic surface waters, the low-affinity cytochrome c oxidase was dominant and assigned mainly to eukaryotic sequences, as well as to diverse bacterial groups belonging to  $\alpha$ - and  $\gamma$ -proteobacteria (Figure 6D). In contrast, in sulfidic waters where the high-affinity *cbb*<sub>3</sub>-type cytochrome c oxidase prevailed, as much as 80% of the transcripts could be assigned to either  $\gamma$ - and  $\epsilon$ -proteobacteria. For the *R. magnifica*-like organism, which possesses both types of cytochrome oxidases, the switch in the expression from the low-affinity type in oxic surface waters (5 m) to the high-affinity type in anoxic and sulfidic waters (20–80 m) can be observed in the genome recruitment plots (Figure 5B).

Despite the presence of H<sub>2</sub>S, the oxygen microsensors showed trace amounts of O<sub>2</sub> ( $< 1$   $\mu$ M) down to 40 m water depth during the downcast, which might be an artefact from water advection caused by the CTD rosette (see section 'description of the sampling



**Figure 6. Vertical distribution of organisms involved in the sulfur cycle and abundance and taxonomic affiliation of transcripts encoding for cytochrome c oxidases.** (A) H<sub>2</sub>S concentration and abundance of DNA sequences affiliated to organisms either capable of oxidizing or reducing inorganic sulfur species. Shown in percent of all DNA sequences (excluding rRNA genes) and summed according to their metabolic potentials. (B) Abundance of RNA sequences affiliated to organisms either capable of oxidizing or reducing inorganic sulfur species. Shown in percent of all RNA sequences (excluding rRNAs) and summed according to their metabolic potentials. (C) O<sub>2</sub> concentrations and transcript abundance of the (low-affinity) cytochrome c oxidase and the (high-affinity) *cbb*<sub>3</sub>-type cytochrome c oxidase (both EC 1.9.3.1). Shown in percent of all protein-coding RNA sequences. (D) Phylogenetic affiliation of the transcripts encoding for both types of the cytochrome c oxidase. doi:10.1371/journal.pone.0068661.g006

site<sup>3</sup>). Assuming that the measured O<sub>2</sub> concentrations were not an artifact, we calculated a vertical down flux of 0.07 mmol O<sub>2</sub> m<sup>-2</sup> d<sup>-1</sup> between 17 and 27 m. Although this O<sub>2</sub> flux could only account for the oxidation of ~6% of the sedimentary H<sub>2</sub>S flux of 2 mmol m<sup>-2</sup> d<sup>-1</sup>, it could partly sustain microaerobic activity in the sulfidic waters as suggested by the presence of the *cbb*<sub>3</sub>-type cytochrome c oxidase transcripts. Lateral advection of oxic waters and water exchange induced by internal waves may also have supported microaerobic activity at O<sub>2</sub> concentrations that would remain below the detection limit of our STOX sensor (~50 nM). However, mixing due to internal waves would have influenced only the upper part of the water column and the widespread anoxic conditions below 30 m gave little indication of lateral advection of oxic waters from 30–100 m, where the *cbb*<sub>3</sub>-type cytochrome c oxidase was nevertheless strongly expressed. Either, the O<sub>2</sub> was consumed shortly before our sampling campaign or advective transport of oxic waters supplied O<sub>2</sub> in concentrations below the detection limit of our STOX sensor. An alternative explanation for the high *cbb*<sub>3</sub>-type cytochrome c oxidase expression in the sulfidic waters could also be the use of nitric oxide (NO) as an alternate substrate instead of O<sub>2</sub>, as hypothesized for a  $\gamma$ -proteobacterial species [82] and supported by structural similarities of *cbb*<sub>3</sub>-type cytochrome c oxidases to bacterial nitric oxide reductases [83]. Although we did not measure NO in our study, a high expression-ratio of the genes encoding for nitric oxide reductases (*norBC*) were found in the genome recruitments for SUP05 (these genes are absent from currently available versions of the *R. magnifica* and *V. okutanii* genomes) in sulfidic depths, and at

60 m also for *Sulfurovum*. Furthermore, transcripts for the dissimilatory nitrite reductase (*nirK*) were highly expressed in SUP05-like organisms throughout the water column. Since this enzyme reduces NO<sub>2</sub><sup>-</sup> to NO, minor concentrations of NO might have been available for respiration processes linked to sulfur oxidation.

Alternatively to O<sub>2</sub> (and NO) utilization, SUP05, *V. okutanii* and *Sulfurovum* and several other  $\epsilon$ -proteobacteria [84,85] are also capable of coupling the oxidation of H<sub>2</sub>S to the reduction of NO<sub>x</sub>. Low but persistent concentrations of NO<sub>x</sub> were measurable at most depths down to 60 m, and nitrate as well as nitrite reductase (*nar*-, *nap*- and *nir*-genes) transcripts were expressed in all anoxic and sulfidic depths (Figure 5 and S4). The genome recruitment plots for SUP05 (the genome contains genes from all three clusters) show that *nap*- and especially *nir*-genes were expressed, while relatives of *V. okutanii* and *Sulfurovum* showed high expression-ratios mostly for *nar*- and *nap*-genes, respectively. In contrast, most of the genes mentioned above are absent from the currently available version of the *R. magnifica* genome and it is thought that *R. magnifica* reduces NO<sub>x</sub> for assimilatory reasons only [65].

We could not calculate fluxes from the trace amounts of NO<sub>x</sub> below 30 m. However, at the upper boundary of the second H<sub>2</sub>S peak (25–29 m) the NO<sub>2</sub><sup>-</sup> and H<sub>2</sub>S profiles overlap. The ratio of the opposing concentration gradients of NO<sub>2</sub><sup>-</sup> (-0.53  $\mu$ M m<sup>-1</sup>) and H<sub>2</sub>S (0.22  $\mu$ M m<sup>-1</sup>) between 25 and 29 m depth indicates that the NO<sub>2</sub><sup>-</sup> flux was sufficient to oxidize the upward flux of H<sub>2</sub>S. A diffusion coefficient of  $\sim 2 \times 10^{-5}$  m<sup>2</sup> s<sup>-1</sup> can be estimated from the density gradient at this depth, resulting in an upward flux

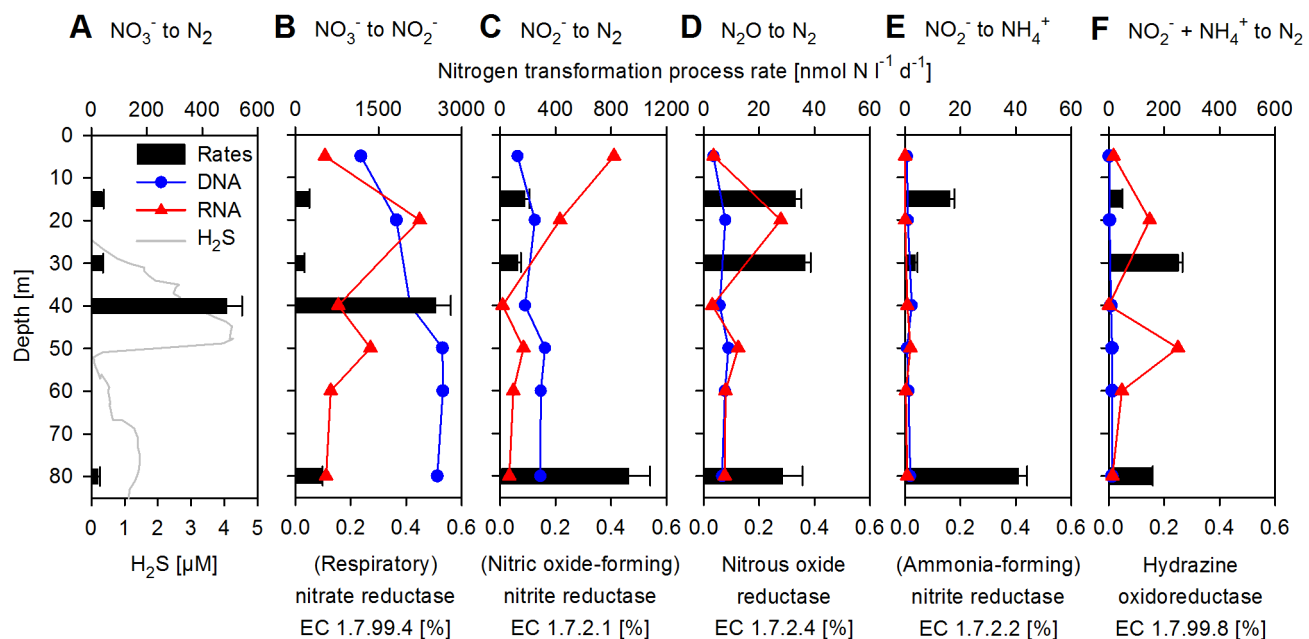
of  $0.38 \text{ mmol H}_2\text{S m}^{-2} \text{ d}^{-1}$  and an average oxidation rate of  $\sim 100 \text{ nmol H}_2\text{S l}^{-1} \text{ d}^{-1}$  within the  $\sim 4 \text{ m}$  thick overlapping layer. This is in the same range as the experimentally measured reduction of  $\text{NO}_2^-$  to  $\text{N}_2$  ( $126 \text{ nmol N l}^{-1} \text{ d}^{-1}$ ) at  $30 \text{ m}$  depth (see section 'nitrogen cycling' and Figure 7), suggesting that  $\text{H}_2\text{S}$  oxidation was carried out partly via sulfur-driven autotrophic denitrification. The removal rate of  $\text{NO}_2^-$  calculated from the downward flux of  $\text{NO}_2^-$  was  $230 \text{ nmol N l}^{-1} \text{ d}^{-1}$  and matched the experimentally measured  $\text{NO}_2^-$  removal of  $255 \text{ nmol N l}^{-1} \text{ d}^{-1}$  from combined denitrification, anammox and dissimilatory nitrate reduction to ammonia (DNRA).

Although our results suggest that  $\text{H}_2\text{S}$  was oxidized by both microaerobic activity and sulfur-driven autotrophic denitrification well below the oxic zone ( $>1 \mu\text{M O}_2$ ), our flux calculations indicate that the larger part of the  $\text{H}_2\text{S}$  was probably oxidized anaerobically with  $\text{NO}_x$ .

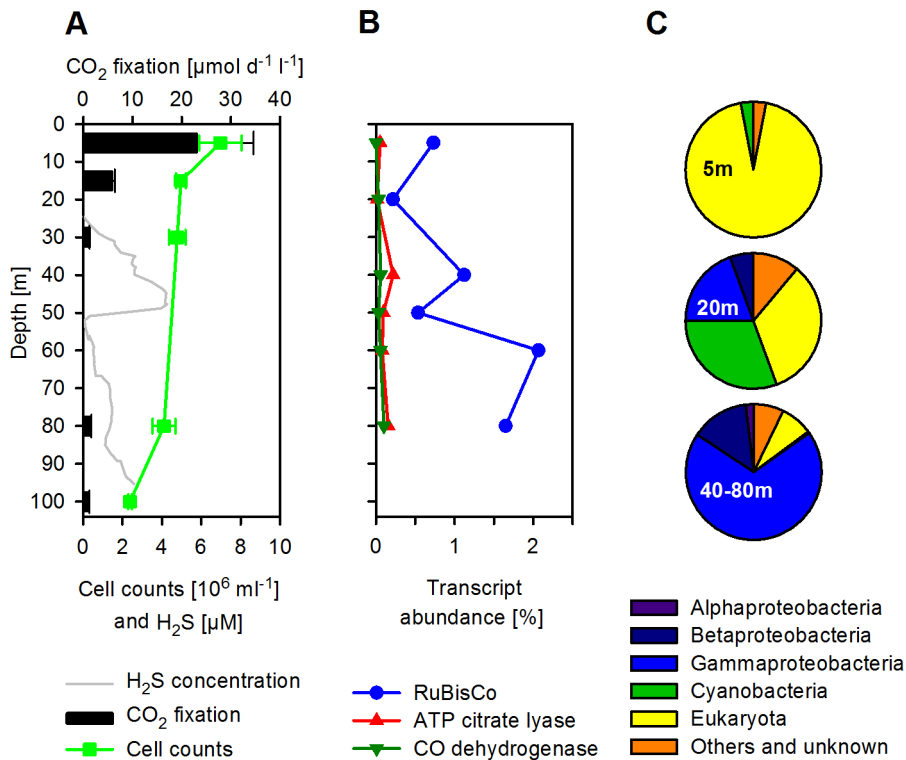
**Nitrogen cycling.** To shed light on the nitrogen cycling carried out by the microbial community, we measured potential rates of various nitrogen transformation processes using  $^{15}\text{NO}_3^-$ ,  $^{15}\text{NO}_2^-$ ,  $^{15}\text{N}_2\text{O}$  or  $^{15}\text{NH}_4^+$  incubations and compared them with corresponding abundances of functional genes and transcripts (the sum of BLAST-hits, EC number- and Pfam-assignments) involved in their turnover (Figure 7). Reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  was active throughout the anoxic and sulfidic zones, with the highest rates measured at  $40 \text{ m}$  ( $2500 \text{ nmol N l}^{-1} \text{ d}^{-1}$ ; Figure 7B). In comparison, the much lower rate measured at  $30 \text{ m}$  ( $150 \text{ nmol N l}^{-1} \text{ d}^{-1}$ ) might have been caused by limitations in reductants, as suggested by the much lower  $\text{H}_2\text{S}$  concentrations at that depth. The transcript abundance for respiratory nitrate reductase (EC 1.7.99.4) peaked in the anoxic zone at  $20 \text{ m}$  (0.35% of all protein-coding sequences) and then dropped within the sulfidic waters following the decrease in  $\text{NO}_3^-$  concentrations. Gene abundances however, increased with depth ( $\sim 0.5\%$ ) and it is possible that the

addition of  $\text{NO}_3^-$  necessary for rate measurements stimulated a fast response of the microbial community yielding the actual potential for  $\text{NO}_3^-$  reduction to  $\text{NO}_2^-$  rather than the *in situ* rate. The majority of the genes and transcripts encoding for respiratory nitrate reductase at  $5$  and  $20 \text{ m}$  were similar to *K. stuttgartiensis*, while transcripts in sulfidic waters belonged to diverse groups of  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ - and  $\epsilon$ -proteobacteria, indicating a clear taxonomic separation with depth.

Measured rates for the subsequent steps in denitrification, the reduction of  $\text{NO}_2^-$  to  $\text{N}_2$  were highest close to bottom waters at  $80 \text{ m}$  ( $900 \text{ nmol N l}^{-1} \text{ d}^{-1}$ ; Figure 7C), whereas the transcript abundance for NO-forming cd-cytochrome nitrite reductase (EC 1.7.2.1) was highest in the oxic and anoxic zones at  $5$  and  $20 \text{ m}$  (0.4% and 0.2%), mirroring the availability of  $\text{NO}_2^-$ . The majority of the transcripts at  $5$  and  $20 \text{ m}$  were similar to the gene from *N. maritimus*, while those recovered from sulfidic depths were affiliated with diverse groups of proteobacteria. We also found high expression of ammonia monooxygenase (EC 1.14.99.39) transcripts related to *N. maritimus* (1.5%, data not shown) in the oxic surface ( $5 \text{ m}$ ), which may be partly responsible for the high  $\text{NO}_2^-$  concentrations in the surface waters. Some of the  $\text{NO}_2^-$  reduction ( $\text{N}_2$  production) could be attributed to anammox activity, with the highest rates measured at  $30 \text{ m}$  ( $250 \text{ nmol N l}^{-1} \text{ d}^{-1}$  based on  $^{15}\text{NO}_2^-$  addition, Figure 7F and  $96 \text{ nmol N l}^{-1} \text{ d}^{-1}$  based on  $^{15}\text{NH}_4^+$  addition, data not shown). At  $80 \text{ m}$  anammox activity could only be detected by  $^{15}\text{NO}_2^-$  addition ( $152 \text{ nmol N l}^{-1} \text{ d}^{-1}$ ); an incubation with added  $^{15}\text{NH}_4^+$  did not stimulate any  $\text{N}_2$  production, most likely due to limitations in  $\text{NO}_x$ . The abundance of genes encoding for hydrazine oxidoreductase (EC 1.7.99.8) was generally very low at all depths (less than 0.015%; Figure 7F) while transcripts, mostly annotated as similar to *K. stuttgartiensis*, peaked in abundance at  $20$  and at  $50 \text{ m}$  (0.15 and 0.25%, respectively). The  $50 \text{ m}$  transcript



**Figure 7. Vertical distribution of nitrogen transformation process rates and abundances of sequences encoding for involved enzymes.** Shown in percent of all protein-coding DNA and RNA sequences, respectively. (A)  $\text{NO}_3^-$  reduction to  $\text{N}_2$  (denitrification). (B)  $\text{NO}_3^-$  reduction to  $\text{NO}_2^-$ , respiratory nitrate reductase (EC 1.7.99.4). (C)  $\text{NO}_2^-$  reduction to  $\text{N}_2$ , (NO forming) nitrite reductase (EC 1.7.2.1). (D)  $\text{N}_2\text{O}$  reduction to  $\text{N}_2$ , nitrous-oxide reductase (EC 1.7.2.4). (E)  $\text{NO}_2^-$  reduction to  $\text{NH}_4^+$  (DNRA), ( $\text{NH}_4^+$  forming) nitrite reductase (EC 1.7.2.2). (F)  $\text{NO}_2^- + \text{NH}_4^+$  to  $\text{N}_2$  (anammox, based on the sole addition of  $\text{NO}_2^-$ ), hydrazine oxidoreductase (EC 1.7.99.8). Please note that at  $40 \text{ m}$  only  $\text{NO}_3^-$  reduction to  $\text{N}_2$  (A) and  $\text{NO}_3^-$  reduction to  $\text{NO}_2^-$  (B) were measured. doi:10.1371/journal.pone.0068661.g007



**Figure 8. Vertical distribution of CO<sub>2</sub> fixation rates, microbial cell counts and abundance of transcript encoding for key carbon-fixing enzymes.** (A) CO<sub>2</sub> fixation rates and total microbial cell counts. (B) Abundances of transcripts encoding for carbon-fixing enzymes (in percent of all protein-coding RNA sequences): Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo, EC 4.1.1.39, Calvin-Benson-Bassham cycle), ATP citrate lyase (EC 2.3.3.8, Arnon-Buchanan cycle) and CO-dehydrogenase (EC 1.2.99.2, Wood-Ljungdahl pathway). (C) Phylogenetic affiliation of the transcripts encoding for RuBisCo. doi:10.1371/journal.pone.0068661.g008

maximum of hydrazine oxidoreductase (we did not measure rates at this depth) is in good agreement with a small peak in  $\text{NO}_3^-$  concentrations (to 0.7  $\mu\text{M}$ ) and a minor dip in  $\text{NH}_4^+$  concentrations (Figure 2C). The transcript maximum might also be influenced by the much lower  $\text{H}_2\text{S}$  concentrations at this depth, as anammox activity was shown to be inhibited by  $\text{H}_2\text{S}$  [86].

$\text{N}_2\text{O}$  is an intermediate in denitrification ( $\text{NO}_x$  to  $\text{N}_2$ ) and we measured the reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$ , which turned out to be of smaller magnitude (30  $\text{nmol N l}^{-1} \text{d}^{-1}$ ) than the  $\text{NO}_2^-$  reduction to  $\text{N}_2$  (Figure 7D). The  $\text{N}_2\text{O}$  concentrations, which ranged between 20 and 40 nM from surface to ~80 m, dropped below detection limit at 80 m (Figure 2B), indicating either a complete reduction of  $\text{N}_2\text{O}$  as previously observed for this area [87] or a lack of production due to the limitation in  $\text{NO}_x$ . Gene and transcript abundance for nitrous oxide reductase (EC 1.7.2.4) were highest in anoxic waters (20 m) reaching 0.3% and comparable in magnitude to the abundances of sequences encoding for nitrate and nitrite reductases from the same depths.

We also conducted rate measurements of complete denitrification ( $\text{NO}_3^-$  to  $\text{N}_2$ ; Figure 7A). The highest rate (490  $\text{nmol N l}^{-1} \text{d}^{-1}$ ) was found at 40 m within the first  $\text{H}_2\text{S}$  maximum. Much lower rates were observed for the other depths (26–41  $\text{nmol N l}^{-1} \text{d}^{-1}$ ), which might be attributed to incomplete denitrification (e.g.  $\text{NO}_3^-$  reduction to  $\text{NO}_2^-$ ,  $\text{NO}$  or  $\text{N}_2\text{O}$ ) and potentially also to limitations in concentrations of the reductant ( $\text{H}_2\text{S}$ ).

Rates for DNRA were the lowest of the nitrogen transformations processes we measured, not exceeding 40  $\text{nmol N l}^{-1} \text{d}^{-1}$  (Figure 7E). Gene abundance for cytochrome c nitrite reductase (EC 1.7.2.2) was also lower than genes implicated in the other

nitrogen transformation processes. Except for 50 m depth, the transcripts for this gene were even rarer than the gene abundances (less than 0.01%). This suggests only a minor role of DNRA in the microbial community metabolism at the time of sampling.

Although rate measurements do not provide information on the phylogenetic affiliation of organisms carrying out the nitrogen transformations, the predominance of sulfur oxidizing proteobacteria found in our sequence data suggests that the sulfur-driven autotrophic denitrification was most likely the dominant pathway for N-loss during our sampling campaign.

**Carbon assimilation.** Eastern Boundary Upwelling Systems are characterized by high primary productivity due to photosynthetic (photoautotrophic) growth in surface waters, which in turn stimulates heterotrophic respiration processes in underlying waters. However, autotrophic lifestyles have also been found in these underlying waters, e.g. by organisms responsible for nitrification [28,88], anammox [22–24,26] and sulfur-driven autotrophic denitrification [31,44,84]. To investigate the magnitude of inorganic carbon assimilation of the microbial community, we conducted rate measurements at selected depths with <sup>13</sup>C-bicarbonate incubations and compared them to the relative abundance of transcripts encoding for key carbon-fixing enzymes (Figure 8). High abundances of transcripts for ribulose-bisphosphate carboxylase/oxygenase (RuBisCo, EC 4.1.1.39) were found at all depths, but especially in sulfidic waters. At 40, 60 and 80 m transcripts encoding for RuBisCo were the most abundant to be identified in the metatranscriptomes (1.1, 2.1 and 1.6% of all protein-coding sequences, respectively; Figure 8B and Figure S5). The phylogenetic diversity of the RuBisCo transcripts varied

throughout the water column. Transcripts from photosynthetic organisms such as algae and diatoms (e.g. similar to *Heterosigma akashiwo*, *Odontella sinensis* and *Thalassiosira* spp.) as well as cyanobacteria dominated the 5 and 20 m samples. In the sulfidic zone (40–80 m),  $\beta$ - and especially  $\gamma$ -proteobacterial transcripts were most abundant (Figure 8C). Approximately 25% of all RuBisCo-transcripts were most similar to a bacterial artificial chromosome-clone of unknown bacterium 560, which also appears to belong to a SUP05 genome [89]. Altogether, transcripts from  $\gamma$ -proteobacteria contributed about 70% of all RuBisCo transcripts in sulfidic waters.

While the high proportions of RuBisCo transcripts were indicative of an active Calvin-Benson-Bassham cycle, other CO<sub>2</sub> fixation pathways were also represented by the presence of transcripts for key enzymes of the Arnon-Buchanan cycle (ATP citrate lyase, EC 2.3.3.8) and the Wood-Ljungdahl pathway (CO-dehydrogenase, EC 1.2.99.2). Transcripts encoding for these two enzymes accounted for up to 0.3% of all protein-coding sequences at 40 m depth (Figure 8B) and were also recruited onto the *Sulfurovum* and *D. autotrophicum* genomes, respectively (Figure S4).

CO<sub>2</sub> fixation rates measured at selected depths with <sup>13</sup>C-bicarbonate incubations (Figure 8A) were highest in the nutrient-rich oxic surface waters (23  $\mu\text{mol C l}^{-1} \text{d}^{-1}$ ), reflecting the dominance of large-sized eukaryotic phytoplankton. However, dark incubations with <sup>13</sup>C-bicarbonate yielded chemolithoautotrophic CO<sub>2</sub> fixation rates ranging from 0.9 to 1.4  $\mu\text{mol C l}^{-1} \text{d}^{-1}$  at depths of 30, 80 and 100 m. These measured rates are comparable to chemolithoautotrophic activity found in the redoxclines and the sulfidic zones of the Baltic and the Black Sea [42,43,90–93]. In comparison, carbon assimilation rates of 0.3  $\mu\text{mol C l}^{-1} \text{d}^{-1}$  by heterotrophic bacteria, after the addition of <sup>13</sup>C-glucose (data not shown), was only about one third when compared with the chemolithoautotrophic CO<sub>2</sub> fixation. Based on total microbial cell counts, each cell fixed  $\sim 0.3 \text{ fmol C d}^{-1}$ , a magnitude that was also observed in the sulfidic zone of the Baltic Sea [92].

Integrating the CO<sub>2</sub> fixation rates over the predicted predominantly photic zone (0–20 m) we estimated  $\sim 288 \text{ mmol C m}^{-2} \text{d}^{-1}$  being fixed. This is in good agreement with the highest primary production rates modelled for the study area during the same period of time (250  $\text{mmol C m}^{-2} \text{d}^{-1}$ ) [25]. On the other hand, the integrated light-independent CO<sub>2</sub> fixation rates in the predicted predominantly aphotic zone (20–100 m) reached  $\sim 96 \text{ mmol C m}^{-2} \text{d}^{-1}$ . Consequently,  $\sim 25\%$  of the total CO<sub>2</sub> fixation at our study site was carried out by chemolithoautotrophic microorganisms. This is similar to the dark CO<sub>2</sub> fixation in the redoxcline of the Baltic Sea, which was shown to contribute  $\sim 30\%$  of the surface productivity and to the dark CO<sub>2</sub> fixation rates as reported for Chilean waters, which accounted for an average of  $\sim 20\%$  of total CO<sub>2</sub> fixation [88,94]. The chemolithoautotrophic CO<sub>2</sub> fixation at our study site could represent as much as 33–53% of the estimated average CO<sub>2</sub> fixation rate per square meter for the Humboldt Current System (182–290  $\text{mmol C m}^{-2} \text{d}^{-1}$ ) [2,4]. Assuming the measured rates were maintained throughout the entire sulfidic plume ( $\sim 5500 \text{ km}^2$ ), the CO<sub>2</sub> fixed through chemolithoautotrophy would have been  $\sim 6.3 \times 10^3$  tons C per day. In comparison, in this area an estimate of the total primary production by remote sensing is  $\sim 5.5 \times 10^5$  tons C per day [2]. Consequently, the chemolithoautotrophic activity during this sulfidic event would have contributed  $\sim 1.2\%$  of total CO<sub>2</sub> fixed in the Humboldt Current System off the Peruvian coast at the given time. This is intriguing, since the Humboldt Current System is one of the most productive marine systems world-wide and supports the production of more fish per unit area than anywhere

else in the world [2–4,95]. Moreover, since the sulfidic plume may have been considerably larger than the extent we recorded and was either recurrent or prevailed for several months, the chemolithoautotrophic growth is significant in terms of carbon retention. Considering that only 15–30% of the photosynthetic surface production is actually exported to OMZ waters [25] the chemolithoautotrophic growth may act as an important, but up to now neglected factor promoting SO<sub>4</sub><sup>2-</sup> reduction and thus stabilizing sulfidic conditions in OMZ waters.

## Conclusions

The Eastern Boundary Upwelling System off the Peruvian coast is one of the world's most productive oceanic regions and comprises one of the largest OMZs world-wide [2,4,11]. We reported here the detection of a sulfidic plume within continental shelf waters of the Peruvian OMZ in January 2009. The sulfidic plume covered an area  $>5500 \text{ km}^2$  and contained  $\sim 2.2 \times 10^4$  tons of toxic H<sub>2</sub>S, representing with  $\sim 440 \text{ km}^3$  the largest sulfidic plume ever observed in oceanic waters and the first time that H<sub>2</sub>S was measured in Peruvian OMZ waters.

The microbial community was largely dominated by several distinct  $\gamma$ - and  $\epsilon$ -proteobacteria related to SUP05, *R. magnifica*, *V. okutanii* and *Sulfurovum*, which transcribed a broad range of genes involved in sulfur (H<sub>2</sub>S, S<sub>2</sub>O<sub>3</sub><sup>2-</sup>, S<sup>0</sup> and SO<sub>3</sub><sup>2-</sup>) oxidation. Our data suggested that these sulfur oxidizing proteobacteria probably utilized several different oxidants ranging from O<sub>2</sub>, NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, NO to N<sub>2</sub>O to oxidize the H<sub>2</sub>S well below the oxic surface. While sequences related to SUP05 indicated that genes involved in the reduction of NO<sub>x</sub> and NO were being expressed, *R. magnifica*-, *V. okutanii*- and *Sulfurovum*-like transcripts related to a microaerophilic *cbb<sub>3</sub>*-type cytochrome c oxidase also pointed to the use of O<sub>2</sub> for H<sub>2</sub>S oxidation.

High-throughput sequencing data further showed a high abundance and transcriptional activity of  $\delta$ -proteobacterial SO<sub>4</sub><sup>2-</sup> reducing *D. autotrophicum*. Transcripts recruiting to the SO<sub>4</sub><sup>2-</sup> reduction genes of *D. autotrophicum* were found suggesting that SO<sub>4</sub><sup>2-</sup> reduction may have occurred in the water column. However, our flux calculation indicated that the main source for H<sub>2</sub>S in the water column was sedimentary SO<sub>4</sub><sup>2-</sup> reduction. Given the fact that many sulfur cycling enzymes can both oxidize and reduce sulfur species [72,73] and the presence of colloidal S<sup>0</sup> plumes within the sampling area as observed with remote satellite sensing, the disproportionation of sulfur compounds could also be a way of energy acquisition for some of the proposed SO<sub>4</sub><sup>2-</sup> reducers [74].

High CO<sub>2</sub> fixation rates were measured in photic surface waters (5.8–23  $\mu\text{mol C l}^{-1} \text{d}^{-1}$ ), but also in the dark sulfidic zone, ranging from 0.9 to 1.4  $\mu\text{mol C l}^{-1} \text{d}^{-1}$ . Many identified microorganisms, both sulfur oxidizers and SO<sub>4</sub><sup>2-</sup> reducers were expressing transcripts encoding for key carbon-fixing enzymes. The light-independent, chemolithoautotrophic CO<sub>2</sub> fixation is similar to observations made in permanently stratified systems like the Baltic and the Black Sea [42,43,90–93]. If the rates prevailed throughout the entire sulfidic zone, they would have represented as much as  $\sim 30\%$  of the photoautotrophic CO<sub>2</sub> fixation at that site and would have been of similar magnitude as the photosynthetic surface production that is exported to OMZ waters [25].

The presence of colloidal S<sup>0</sup> plumes in the study area again in May 2009 indicated that sulfidic waters in the OMZ off Peru might be more frequent and persistent than originally thought. Although the frequency and duration of H<sub>2</sub>S accumulations cannot be estimated from direct observations, their occurrence might increase in future resulting from eutrophication and global warming [27,31,35]. In addition, the carbon retention due to the

chemolithoautotrophic activity presented in this study may enhance  $\text{SO}_4^{2-}$  reduction and consequently  $\text{H}_2\text{S}$  formation and thus could act as an important mechanism to stabilize sulfidic conditions in OMZ waters, potentially reducing the liveable habitat of many higher organisms.

## Materials and Methods

### Sample Collection

All waters samples were collected during RV Meteor cruise M77/3 (December 27<sup>th</sup>, 2008 to January 24<sup>th</sup>, 2009) on the Peruvian continental shelf. Station 19 (January 9<sup>th</sup>, 2009; 12°21.88'S, 77°00.00'W), located approximately 15 km off the coast of Lima was selected for detailed analysis. During the upcast, water was pumped from depth directly on board using a pump-conductivity-temperature-depth (pump-CTD) water sampler. We monitored density as well as  $\text{O}_2$  and  $\text{H}_2\text{S}$  to account for internal waves and moved the pump-CTD accordingly.

Samples for nucleic acid extraction were filled (oxygen-free) in 4.5 l polycarbonate bottles. For each sample, 1.5–2 l of the water was prefiltered through 10  $\mu\text{m}$  pore size filters (Whatman Nuclepore Track-Etch) and then collected upon 0.22  $\mu\text{m}$  pore size filters (Millipore Durapore Membrane) using a vacuum pump. Less than 18 minutes elapsed from the time when the water was pumped on board until the filters were put in microcentrifuge reaction tubes and flash frozen in liquid nitrogen. Samples for incubation experiments and nutrient analysis were taken in parallel (see description below).

### RNA extraction and cDNA synthesis

DNA and RNA were extracted using the DNA/RNA-Allprep kit (Qiaagen) with minor modifications in the protocol for the lyses step: The frozen filters were crushed using a disposable pestle and incubated with 200  $\mu\text{l}$  lysozyme (10  $\mu\text{g}/\mu\text{l}$ ) and 1 mM EDTA at ambient temperatures for 5 minutes. Subsequently, 40  $\mu\text{l}$  of Proteinase K (10  $\mu\text{g}/\mu\text{l}$ ) were added, followed by another incubation of 5 minutes at ambient temperatures. After adding 500  $\mu\text{l}$  buffer RLT-Plus (containing 10  $\mu\text{l}/\text{ml}$   $\beta$ -mercaptoethanol) the manufacturer's instructions were followed. DNA was eluted in 150  $\mu\text{l}$  elution buffer; RNA in 50  $\mu\text{l}$  nuclease-free water, followed by a subsequent step of DNA digestion (Turbo DNA-free kit, Ambion). Prokaryotic rRNA was removed with *mRNA only* prokaryotic mRNA isolation kit (Epicentre). Further depletion of bacterial rRNA was achieved by using the Ambion MicrobExpress kit (see also section 'sequencing statistics'). Cleaned and rRNA-depleted mRNA was subjected to an *in vitro* transcription (amplification) step using Ambion MessageAmp. Finally, cDNA was synthesised using the Invitrogen superscript III cDNA synthesis kit with random hexameric primers (Qiaagen). Throughout the procedure all DNA and RNA samples were subsequently quantified with a NanoDrop spectrophotometer and checked for degradation with a BioRad Experion Automated Electrophoresis System. Leftover reactants and reagents were removed using the PCR Mini Elute Kit (Qiaagen). DNA and RNA was stored at  $-80^\circ\text{C}$  until pyrosequencing. Throughout the whole procedure nuclease-free plastic consumables and nuclease-free water and reagents were used to hinder any possible degradation of DNA or RNA.

### Sequencing

For both DNA and RNA (cDNA), 50  $\mu\text{l}$  were sequenced with the GS-FLX (Roche) pyrosequencer at the Institute of Clinical Molecular Biology in Kiel. Each sample was loaded on one quarter of a PicoTiter plate (except the 5 m RNA sample, which

was loaded on two quarters of a PicoTiter plate). This resulted in 1,888,768 (DNA) and 1,560,959 (RNA) sequences with an average length of 392 base pairs, accounting for 757,439,211 and 599,103,110 base pairs of sequence information, respectively (Table S1).

### Sequence annotation pipeline

The sequence data was organized and analyzed with the Meta2Pro annotation pipeline [96]. All raw sequences were clustered using Cd-hit [97] with a sequence identity threshold of 98% and a word length of 8, delivering about 1,581,637 (DNA) and 592,711 (RNA) cluster representative sequences in total. The rRNA genes and rRNAs in these cluster representatives were identified by a BLASTn-search [98] against the SILVA database [99] including both prokaryotic and eukaryotic small and large ribosomal subunit sequences with a bit score cut off of 86. The bit score cut off of 86 as described earlier [100] was validated using a simulation exercise. All sequences deposited in the SILVA database (477,749 sequences) were randomly fragmented into one million sequences to simulate a pyrosequencing run. The generated fragments had a normal length distribution with mean and standard deviation values similar to those from our own dataset (mean: 420 base pairs and standard deviation: 150 base pairs). This simulated dataset was Cd-hit-clustered (as described above) and BLAST-searches against the SILVA database itself were carried out. All queries which hit themselves in the database (e.g. the sequence of origin of the query) or hit a sequence belonging to the same taxonomic lineage (allowing a mismatch of up to two taxonomic levels) were considered true positives, while those fragments that hit sequences from other taxonomic lineages were false positives. The bit score distributions for the true and false positives were binned and a threshold sweep was used to calculate the sensitivity and specificity value at each bit score threshold. A bit score value of 86 in the resulting plot of the sensitivity and specificity distributions for this threshold sweep gave a specificity of 99.35% and a sensitivity of 99.85% respectively. Hence, this cut off was used for all further analysis of the sequences against the SILVA database and also in MEGAN to make taxonomic assignments (using a minimum support of 5 and a 10% score range for its LCA algorithm) [101]. The cluster representative sequences without a hit in the SILVA database were compared against the non-redundant database from NCBI using BLASTx with a bit score cut off of 35. The top hit of each BLASTx-search was used for the functional assignment of the cluster representatives.

The cluster representative sequences without a hit in the SILVA database were further scanned with profile hidden Markov models of the ModEnzA EC groups [69] and the Pfam protein families [70]. The Pfam-hits were converted to EC numbers and along with the ModEnzA EC-hits mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) reference pathways using the FROMP pathway mapping and visualization tool [96]. For ease of data analysis all cluster representative sequences, including clustering information from Cd-hit (cluster identification number, cluster size and all clustered nucleotide sequences), results from BLAST-, Pfam- and EC-searches as well as the taxonomic assignment from MEGAN were added to a MySQL database [96].

### Sequencing statistics

Combining all DNA sequences, 0.3% of them were of rRNA gene origin. For the RNA sequences, the percentage of rRNA sequences varied between 58 and 76% with the exception of the surface sample collected at 5 m, which was dominated by

eukaryotic rRNA sequences (>80%). The high count of eukaryotic rRNA sequences in the surface indicates that a further depletion of eukaryotic rRNAs supplementing the depletion of prokaryotic rRNAs would have been worthwhile. Nevertheless, also the depletion of prokaryotic rRNA still resulted in relatively high counts of bacterial rRNAs (7–55%), whereas the number of archaeal rRNAs was mostly negligible (2–11%). The rRNAs and rRNA genes were excluded from further analysis (except for Figure 3A), leaving 1,882,842 DNA and 421,528 RNA sequences. From this pool, 54% (DNA) and 53% (RNA) of the sequences could be identified as protein-coding (matching either the non-redundant database of NCBI or profile hidden Markov model scans of the ModEnZA EC groups or the Pfam protein families); the remainder could not be assigned. Of all protein-coding sequences, 82% of the DNA and 99% of the RNA sequences could be taxonomically identified (matched the non-redundant database of NCBI).

### Hierarchical clustering of sequences using the taxonomic profiles

The taxonomic profiles of the metagenomes and the metatranscriptomes (the occurrence of the microbial taxa in the samples as a percentage of the total number of sequences having a BLASTx-hit) as presented in Figure 3 were used for a hierarchical clustering with the PRIMER 6 program [102]. The samples were grouped into 6 categories according to the depths and a multivariate statistical test (ANOSIM) was used to determine if the groupings were distinct from each other in terms of their microbial communities. The relationships between the depth groups were visualized in a non-parametric Multidimensional Scaling plot using PRIMER 6.

### Metabolic and taxonomic diversity measures

The EC activity matrix (with sample sizes equalized to the smallest sample) was exported from the FROMP pathway mapping tool [96] and the EC counts for each sample were used to calculate the inverse of Simpson's index ( $1/D$ ) where  $D = \sum P_i^2$  and  $P_i$  representing the proportional abundance of species  $i$ , and the Evenness  $E = (1/D)/S$  with  $S$  being the number of unique species. Similar calculations were also performed for the taxonomic assignments (as presented in Figure 3) from the BLASTx-searches normalized to total number of sequences having a BLASTx-hit.

### Sequence recruitment onto reference genomes

The sequence data was recruited onto the reference genomes of the five most abundant organisms (as detected with BLAST-searches) SUP05, *R. magnifica*, *V. okutanii*, *Sulfurovum* and *D. autotrophicum* using the MUMmer program [103]. For SUP05, the draft genome (the metagenome with an ordered assembly of the contigs) as provided by Walsh and colleagues [41] was used and treated like the other genomes. The recruited sequences were re-assessed using a BLAST-search against the reference genomes. Sequences that hit more than one genome with a bit score difference of less than 5% between the first and second hits were discarded, giving rise to a non-overlapping set of sequences for each genome. These sequences were then recruited onto the genomes again to calculate the average coverage over non-overlapping windows of 300 base pairs. The coverage of RNA sequences in every reference genome window was normalized by the total number of BLASTx-hits for that metatranscriptome and divided by the coverage of DNA sequences (which have also been normalized by the total number of BLASTx-hits in that

metagenome) for the same window. This value, the expression-ratio, was subsequently corrected for differences in sizes of the metatranscriptome and metagenome and selected regions of the reference genomes were plotted using customized R and PERL scripts.

### Flow-cytometry cell counts

Samples for flow-cytometry were fixed with a final concentration of 1% paraformaldehyde and stored at  $-80^\circ\text{C}$  until analysis. Total microbial cell counts were performed on a FACSCalibur flow cytometer (BD Biosciences). After 20 minutes staining of the samples with SybrGreen (Qiagen) at  $4^\circ\text{C}$ , cells were counted for 2 minutes or until a total count of 50,000 was reached. Sample flow rate was calibrated with standard beads (Trucount, BD Biosciences) and cell numbers were calculated via the time of measurement.

### Chemical analysis and microsensor measurements

Our pump-CTD was equipped with a custom-built amperometric  $\text{O}_2$  microsensor to obtain vertical profiles of dissolved  $\text{O}_2$  (detection limit 0.5–1  $\mu\text{M}$ ). In addition, the recently developed self-calibrating Switchable Trace amount Oxygen (STOX) sensor was deployed, which allows high-accuracy  $\text{O}_2$  measurements in near anoxic environments (detection limit  $\sim 50$  nM during our measurements) [13,16]. After a minimum of ten minute sensor equilibration at a given sampling depth, at least five measuring cycles were used to calculate  $\text{O}_2$  concentrations.

Water samples for nutrient analysis were taken with a depth resolution of 1 to 2 m.  $\text{NH}_4^+$  was measured fluorometrically [104] and  $\text{NO}_2^-$  was analyzed spectrophotometrically [105] on board. Water samples for  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  were stored frozen until spectrophotometric determination [105] with an autoanalyzer (TRAACS 800, Bran & Lubbe). Detection limits for  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  were 10, 10, 100 and 100  $\text{nmol l}^{-1}$ , respectively. Dissolved  $\text{N}_2\text{O}$  concentrations were determined on board in triplicates measurements using the GC headspace equilibration method as described elsewhere [106].

$\text{H}_2\text{S}$  concentrations were measured continuously on water sampled from the pump-CTD using a custom-built microsensor with a detection limit of  $\sim 0.5$   $\mu\text{M}$  [107]. Chemically determined  $\text{H}_2\text{S}$  concentration (both  $\text{H}_2\text{S}$  and  $\text{HS}^-$ ; detection limit  $\sim 0.5$   $\mu\text{M}$ ) on discrete water samples were used to calibrate the microsensor [108]. Although the sulfidic waters contained a composite of  $\text{H}_2\text{S}$  and  $\text{HS}^-$ , we use the term  $\text{H}_2\text{S}$  throughout the manuscript to avoid unnecessary complexity.

Using a 125 m depth cut off and a grid resolution of 1' (bathymetrical data was obtained from the National Geophysical Data Center) along the  $\sim 200$  km cruise track on the Peruvian shelf where  $\text{H}_2\text{S}$  was detected in the water column, a total area of  $\sim 5500$   $\text{km}^2$  was calculated to be affected by the sulfidic plume. The shelf contained mean  $\text{H}_2\text{S}$ -maxima close to the bottom of the water column of 3.4  $\mu\text{M}$ .  $\text{H}_2\text{S}$  was extending vertically over the water column with an averaged depth of  $\sim 80$  m, yielding  $\sim 440$   $\text{km}^3$  of  $\text{H}_2\text{S}$ -containing waters. Based on an average  $\text{H}_2\text{S}$  concentration of 1.5  $\mu\text{M}$ , we estimated a total content of  $\sim 2.2 \times 10^4$  tons  $\text{H}_2\text{S}$  within the plume.

### Flux calculations

The density was calculated using the data processing program SeaSoft (Sea-Bird Electronics). The stability of the water column was expressed using the Brunt-Väisälä frequency  $N$ , defined as:  $N^2 = -(g/\rho) \times (\partial\rho/\partial z)$  where  $g$  is the gravitational acceleration,  $\rho$  the water density and  $z$  the water depth. The density gradient was calculated over 4 m bins. The turbulent diffusivity  $E_z$  was

calculated as described earlier [109] from the Brunt-Väisälä frequency and the dissipation rate of turbulent kinetic energy  $\epsilon$ :  $Ez = \gamma\epsilon/N^2$  with the mixing coefficient  $\gamma = 0.2$ . We applied a mean  $\epsilon$  of  $1.85 \times 10^{-9} \text{ W kg}^{-1}$  [110]. This value was measured for the open-ocean thermocline [110] and was applied in several rate diffusion models [111,112]. Vertical concentration gradients for  $\text{O}_2$ ,  $\text{H}_2\text{S}$ , and  $\text{NO}_3^-$  were calculated over 4 m bins. Fluxes of  $\text{O}_2$ ,  $\text{H}_2\text{S}$ , and  $\text{NO}_3^-$  at respective depths were calculated according to Fick's law:  $J_i = Ez \times (\partial C / \partial z)$ .

### Satellite images

Data of the sensors MODIS (Moderate Resolution Imaging Spectroradiometer) aboard the satellites Aqua and Terra (NASA) as well as MERIS (Medium Resolution Imaging Spectrometer) aboard the satellite Envisat (ESA) were implemented to study milky turquoise discoloration in waters off the Peruvian coast. Cloudy weather north of Pisco during most of our cruise made remote sensing difficult, but turquoise discolorations were observed off Lima on January 20–21<sup>st</sup> (Figure S1A) and 27–28<sup>th</sup> (Figure S1B). The estimation of the extent of the plumes requires data of higher spatial resolution; the algorithm for the identification of colloidal  $\text{S}^0$  plumes is based on the high spectral resolution of MERIS data [57]. Since full resolution MERIS data is not available for this region we present a high resolution MODIS true colour image in our main figure (Figure 1E).

However, the reflectance spectra derived from reduced resolution MERIS data (Figure S1C), revealed that the turquoise plume southwest of Pisco conformed the criteria for  $\text{S}^0$  in the identification algorithm distinguishable from optically similar coccolithophore blooms [113]. Differences in the shape and appearance of the colloidal  $\text{S}^0$  plume on the MERIS image (Figure S1C) and the MODIS images (Figure 1E) from May 7<sup>th</sup> and 8<sup>th</sup> demonstrate the temporal variability, which is also visible in changes in the cloud structure between the two images.

### Rate measurements of nitrogen transformation processes

Rates of microbial nitrogen transformations were measured at three or four depths in  $^{15}\text{N}$ -labeling experiments as described previously [114,115]. Briefly, nitrogen-loss via anammox and denitrification as well as dissimilatory  $\text{NO}_3^-$  reduction to  $\text{NO}_2^-$  and  $\text{NH}_4^+$  were measured in short-term incubation experiments amended with either  $^{15}\text{NO}_3^-$ ,  $^{15}\text{NO}_2^-$ ,  $^{15}\text{N}_2\text{O}$  or  $^{15}\text{NH}_4^+$  (20, 10, 1 and  $5 \mu\text{mol l}^{-1}$ , respectively; isotopes: Campro Scientific). Time-series incubations were carried out in 12 ml Exetainers (Labco) and biological activity was stopped in one replicate Exetainer at each time interval (0, 6, 12, 24 and 48 h) by the addition of saturated mercuric chloride. Anammox and denitrification was measured as the production of  $^{15}\text{N}$ -labeled  $\text{N}_2$  in  $^{15}\text{NO}_2^-$  and  $^{15}\text{NO}_3^-$ ,  $^{15}\text{NO}_2^-$  and  $^{15}\text{N}_2\text{O}$  incubations, respectively. The N-isotopic composition of  $\text{N}_2$  gas produced in these experiments was determined by gas chromatography isotope-ratio mass spectrometry (GC/IRMS, Fisons VG Optima) [115].  $\text{NO}_2^-$  produced from  $^{15}\text{NO}_3^-$  and  $\text{NH}_4^+$  produced from  $^{15}\text{NO}_2^-$  was determined by GC/IRMS after conversion of  $\text{NO}_2^-$  and  $\text{NH}_4^+$  to  $\text{N}_2$  by sulfamic acid [114] and alkaline hypobromite [24], respectively. Production rates were calculated from the increase of  $^{15}\text{N}$ -concentrations over time and only significant and linear productions of  $^{15}\text{N}$ -species without a lag-phase were considered ( $t$ -tests,  $p < 0.05$ ;  $R^2 > 0.8$ ). Rates are presented as net production rates corrected for the mole fractions of  $^{14}\text{N}$  in the original substrate pools.

### Rate measurements of carbon fixation

Triplicate incubations of 4.5 l seawater were set up with water from 5, 15, 30, 80 and 100 m. To each bottle 4.5 ml of  $^{13}\text{C}$  bicarbonate solution (1 g  $^{13}\text{C}$  bicarbonate in 50 ml water) was added and bottles were incubated in on-deck incubators shaded to 25% surface irradiance with blue lagoon light foil (Lee Filters) and continuously cooled with surface seawater (5 and 15 m) or incubated at  $12^\circ\text{C}$  in the dark (30, 80 and 100 m) for 24 hours. At the end of the incubation 1–2 l were filtered on precombusted ( $450^\circ\text{C}$  for 6 hours) Whatman GF/F filters (as these filters have an average pore size of  $0.7 \mu\text{m}$  and small microorganisms may have passed through, the calculated  $\text{CO}_2$  fixation rates we present here have to be considered as minimal rates). The filters were oven dried ( $50^\circ\text{C}$  for 24 hours) and stored for later analysis at room temperature. Filters were smoked overnight with 37% HCl to remove inorganic carbon retained on the filters, dried for 2 hours at  $50^\circ\text{C}$  and then analyzed in a CHN analyzer coupled to an isotope ratio monitoring mass spectrometer. The  $\text{CO}_2$  fixation rate was calculated according to the enrichment of  $^{13}\text{C}$  in the samples relative to unlabeled background values:  $C_{\text{fix}} = (\text{At}\%_{\text{sample}} - \text{tAt}\%_{\text{background}}) / (\text{At}\%_{\text{label}} - \text{At}\%_{\text{background}}) \times (\text{POC} / \text{time})$  where  $\text{At}\%_{\text{sample}}$  is the ratio of  $^{13}\text{C}/^{12}\text{C}$  times 100 in the particulate organic carbon pool (POC),  $\text{At}\%_{\text{background}}$  is the same ratio in unlabeled POC and  $\text{At}\%_{\text{label}}$  is the final ratio of  $^{13}\text{C}/^{12}\text{C}$  in the incubation bottle after label addition. The resulting ratio is multiplied with the concentration of POC and divided by the incubation time in days. Since we did not perform killed controls, we cannot exclude or estimate the contribution of anapleurotic carbon fixation in our samples. We averaged the  $\text{CO}_2$  fixation over the predicted predominantly photic (0–20 m,  $\sim 14.4 \mu\text{mol C l}^{-1} \text{d}^{-1}$ ) and aphotic (20–100 m,  $\sim 1.2 \mu\text{mol C l}^{-1} \text{d}^{-1}$ , dark incubations) zones by multiplying the rates with the respective water depths. A photic zone  $\text{CO}_2$  fixation of  $\sim 288 \text{ mmol C m}^{-2} \text{d}^{-1}$  and a light-independent  $\text{CO}_2$  fixation of  $\sim 96 \text{ mmol C m}^{-2} \text{d}^{-1}$  was estimated.

When compared to the overall mean  $\text{CO}_2$  fixation rate of the Humboldt Current System off Peru ( $2.18 \text{ g C m}^{-2} \text{d}^{-1}$  or  $182 \text{ mmol C m}^{-2} \text{d}^{-1}$  [2] and  $3.5 \text{ g C m}^{-2} \text{d}^{-1}$  or  $292 \text{ mmol C m}^{-2} \text{d}^{-1}$  [4]), our measured dark  $\text{CO}_2$  fixation (over an 80 m deep aphotic zone) contributed 33–53% of the total  $\text{CO}_2$  fixation. Extrapolating the dark  $\text{CO}_2$  fixation rates over the entire sulfidic plume ( $\sim 5500 \text{ km}^2$ ), we calculated a total  $\text{CO}_2$  fixation of  $\sim 5.3 \times 10^8 \text{ mol C d}^{-1}$  or  $\sim 6.3 \times 10^3 \text{ tons C d}^{-1}$ . This  $\text{CO}_2$  fixation estimate would contribute 1.2% of the total primary production of the Humboldt Current System off the Peruvian coast as presented by Carr, 2002 ( $\sim 2 \times 10^8 \text{ tons C y}^{-1}$  or  $\sim 5.5 \times 10^5 \text{ tons C d}^{-1}$ ). The average  $\text{CO}_2$  fixation rates per cell were calculated from total microbial cell counts as obtained from flow-cytometry.

### Accession numbers of sequence data

Metagenomic and metatranscriptomic sequences have been deposited in the metagenomics analysis server (MG-RAST) under accession numbers 4460677.3, 4450892.3, 4450891.3, 4460736.3, 4461588.3, 4460676.3, 4452038.3, 4460734.3, 4452039.3, 4452042.3, 4460735.3, 4460734.3 and 4452043.3.

### Supporting Information

**Figure S1 Satellite images of the Peruvian coast.** The red circles mark colloidal  $\text{S}^0$  plumes. (A) Satellite image (MODIS) of the area around Lima on January, 29<sup>th</sup>, 2009. (B) Satellite image

(MODIS) of the area around Pisco on January, 27<sup>th</sup>, 2009. (C) Satellite image (MERIS) of the area around Pisco on May 7<sup>th</sup>, 2009. (TIF)

**Figure S2 Multivariate statistical analysis and clustering of all protein-coding sequences based on shared taxonomic categories.** Taxonomic categories are chosen according to Figure 3. (A) Hierarchical clustering. (B) Non-parametric Multidimensional Scaling. Plot is labelled by prior groupings of the samples. The solid green circles mark the hierarchical clusters obtained using a similarity cut off of 86%. (C) ANOSIM test for significance of difference between the prior groupings. (TIFF)

**Figure S3 Vertical distribution of the most abundant taxa.** Shown are the eight most abundant organisms (on the highest taxonomic level possible) in percent of all sequences in the DNA and RNA datasets (excluding rRNA genes and rRNAs); ordered descending according to DNA counts and supplemented with the remainder of the top eight organisms from the RNA dataset if not already present in the DNA dataset. Please note that no RNA sequences were identified as similar to the SUP05 cluster bacterium at 20 and 40 m with BLASTx-searches against the non-redundant database of NCBI. (PDF)

**Figure S4 Vertical distribution of sequences recruited onto the genomes of (A) *Sulfurovum* sp. NBC37-1 and (B) *Desulfobacterium autotrophicum* HRM2.** Shown are selected genes encoding for enzymes involved in oxygen- (blue), sulfur- (yellow), nitrogen- (red), carbon- (green) and hydrogen-metabolism (purple) in the corresponding order of the genomes. The y-axis depicts the log of the expression-ratio, a measure for the selective enrichment of transcripts over the corresponding gene, normalized to the total pool of protein-coding sequences. A list of the start and end position of each gene and the full name of the corresponding enzyme are shown in Table S3. (TIFF)

**Figure S5 Vertical distribution of the most abundant functional assignments.** Shown are the top five most abundant EC numbers in percent of all protein-coding sequences in the DNA and RNA datasets; ordered descending according to the DNA counts and supplemented with the remainder of the top five EC numbers from the RNA dataset if not already present in the DNA dataset. Please note that the data presented here is based

only on EC number- and Pfam-assignments; BLAST-hits are not included. (PDF)

**Table S1 Sequencing statistics.** <sup>a</sup>as obtained with Cd-hit. <sup>b</sup>as obtained by BLASTn-searches against the SILVA database. <sup>c</sup>as obtained by BLASTx-searches against the non-redundant database of NCBI and by scans with profile hidden Markov models of the ModEnzA EC groups and of the Pfam protein families. <sup>d</sup>average. (DOC)

**Table S2 Metabolic and taxonomic evenness and diversity in all protein-coding sequences.** A collection of all EC number- and Pfam-assignments was used to determine the metabolic diversity, while the taxonomic diversity was calculated using all hits from BLASTx-searches. Shown are the evenness and the diversity (inverse of the Simpson's index) for both the metagenomic and metatranscriptomic datasets. (DOC)

**Table S3 Genomic regions of the sequences recruited onto proteobacterial genomes as plotted in Figures 5 and S4.** Shown are the start and end position of each gene and the corresponding enzyme name for the uncultured SUP05 cluster bacterium, *Candidatus* Ruthia magnifica str. Cm, *Candidatus* Vesicomysocius okutanii HA, *Sulfurovum* sp. NBC37-1 and *Desulfobacterium autotrophicum* HRM2. (DOC)

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## Author Contributions

Conceived and designed the experiments: H. Schunck GL JL. Performed the experiments: H. Schunck GL DKD TG TK CRL AP SC JL. Analyzed the data: H. Schunck GL DKD TG TK CRL AP MH H. Siegel JL. Contributed reagents/materials/analysis tools: PR MBS MG RAS MMMK. Wrote the paper: H. Schunck GL DKD JL. Authors contributed equally to the work: H. Schunck GL DKD.

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