Sulfate reducing bacteria associated to diatoms during a spring phytoplankton bloom

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ABSTRACT

Phytoplankton blooms represent hotspots of primary production and lead to the formation of particulate organic matter composed of living and dead algal cells. These particles are characterized by steep chemical gradients, for instance in oxygen concentration, that provide diverse ecological niches for specifically adapted microbes to thrive. We analyzed cooccurrence networks based on 16S and 18S ribosomal RNA gene amplicon sequences obtained during a spring phytoplankton bloom in the North Sea. Particulate fractions larger than 10 µm size were collected at almost daily intervals between early March and late May in 2018. Metaproteomics was used to asses microbial protein expression at three selected time points during the sampling period. Cooccurrence networks identified two major modules representing bacteria cooccurring with diatoms and with dinoflagellates, respectively. Bacterial communities associated with these two algal lineages differed in composition, with diatoms being distinctly clustered with known sulfate-reducing *Desulfobacterota* as well as with potentially sulfur-oxidizing *Ectothiorhodospiraceae*. Metaproteomics confirmed expression of respective key enzymes in these taxa. Microbial sulfate reduction is known to occur in sinking particles at greater depths (marine snow), but is considered of lesser importance in the upper photic zone during phytoplankton blooms. However, our results suggest presence of sufficiently anoxic niches in diatom-derived particles, which could enable processes akin to cryptic sulfur cycling in oxygen minimum zones to take place also in the largely oxygen-rich photic zone during phytoplankton blooms. This illustrates the need to gain further insights into the functional roles of phytoplankton-associated bacteria.

INTRODUCTION

Phytoplankton blooms are explosions of primary productivity which inject gigatonnes of fresh organic carbon into coastal oceans every year. According to some estimates, over 90% of algal-produced carbon is consumed by heterotrophic bacteria in the immediate vicinity of algal cells, the phycosphere, during typical bloom situations (Seymour et al. 2016). Some of

these bacteria are associated directly to living algal cells, or to sinking aggregates of dead or dying algae, and therefore play an important role in the biological carbon pump. Despite their importance in global carbon cycling, particle-associated (PA) bacterial communities are less well understood than their free-living counterparts. In fact, they are often overlooked due to the common practice of prefiltration to exclude larger organisms prior to community composition analyses.

In temperate coastal waters, a unique succession of phytoplankton species unfolds during the spring bloom every year. Typically, diatoms, dinoflagellates and haptophytes are among the dominant lineages. Several studies have demonstrated that algal cells can have more or less specific microbial communities (microbiomes) associated to them (e.g. Grossart et al. 2005, Barreto Filho et al. 2021), and dying algal cells also attract taxonomically and functionally different bacterial communities which degrade their cellular contents (Buchan et al 2014), impacting carbon export capacity of sinking particles (Vidal-Melgosa et al. 2021). Living and dying algal cells can aggregate during blooms (Thornton 2002), forming larger particles hosting complex bacterial communities. It is challenging to study specific associations between algal taxa and bacteria during natural phytoplankton blooms due to the transient nature of these particles, and the complexity of both algal and bacterial communities present during the bloom. However, temporal cooccurrence analysis offers an indirect way to infer associations, reducing the complexity and thereby enabling hypothesis-generation about specific algal-bacterial interactions and their functional implications.

A defining feature of marine particles are the steep chemical and redox gradients that bacterial communities are exposed to compared to a free-living environment. These gradients have been intensively studied in the context of sinking particles in deeper ocean layers, i.e. marine snow. For example, marine snow particles can feature anoxic microenvironments, enabling microbial niches such as sulfate reduction and denitrification (Bianchi et al. 2018, Raven et al. 2021). In the photic zone, these microenvironments are considered less important, especially associated to living algal cells producing plenty of oxygen during a bloom situation. Rather, aggregates consisting of living algal cells make up a diverse microbiome of heterotrophic bacteria degrading complex polysaccharides excreted by the algal cells, and are selected by factors such as host genotype (Ahern et al. 2021), the surrounding environment (Barreto Filho et al. 2021) and via stochastic processes (Stock et al. 2022).

We investigated the temporal association (cooccurrence) of PA bacterial taxa with algal taxa during a spring phytoplankton bloom in the North Sea. We hypothesized that bacteria cooccurring with different dominant algal lineages would differ in composition and function. Using 16S and 18S rRNA amplicon data from a well-resolved time series (near daily sampling) from the Helgoland Kabeltonne station collected between early March and late May in 2018, we constructed cooccurrence networks looking exclusively on 16S-18S cooccurrences in particles larger than 10 µm. In addition, we addressed bacterial functional gene expression by analysis of metaproteomes from three selected timepoints during the bloom.

MATERIALS AND METHODS

Sampling and filtration

Sampling was performed as previously described (Teeling *et al.* 2012). Subsurface seawater (1 m depth) was sampled at near daily intervals between early March and late May in 2018 at the station "Kabeltonne" (50° 11.3' N, 7° 54.0' E) near Helgoland in the south-eastern North Sea. Bacterial biomass was filtered with peristaltic pumps using 10 µm pore-sized filters (47 mm for 16S and 18S rRNA gene amplicon sequencing, 142 mm diameter for metaproteomics) to separate PA microbes from smaller bacterial fractions.

rRNA gene amplicon sequencing

For amplicon sequencing, 51 samples were collected from 01.03.2018 to 29.5.2018. DNA was extracted from the filters using the Qiagen Dneasy Power soil Pro kit according to the manufacturer's instructions. Dislocation of microbial cells from the filters and mechanical lysis was achieved by bead beating in a FastPrep 24 5G (MP Biomedicals). DNA concentrations were measured at a Qubit 3.0 fluorometer (Invitrogen). Extracted DNA was amplified with primer pairs targeting the V4 region of the 16S rRNA gene [515f: 5 -GTGYCAGCMGCCGCGGTAA-3 , 806r: 5 -GGACTACNVGGGTWTC
TAAT-3' (Walters et al., 2016)] and the V7 region of the 18S rRNA gene [F-1183mod: 5'-AATTTGACTCAACRCGGG-3', R-1443mod: 5'-GRGCATCACAGACCTG-3'] (Ray et al. 2016) coupled to custom adaptor-barcode constructs. PCR amplification and Illumina MiSeq library preparation and sequencing (V3 chemistry) was carried out by LGC Genomics in Berlin.

Sequence reads free of adaptor and primer sequence remains were processed using the DADA2 package in R (version 1.2.0, Callahan et al., 2016). In summary, forward and reverse Illumina reads were truncated to 200 bp, filtered (maxEE = 2, truncQ = 2, minLen = 175), dereplicated and error rates were estimated using the maximum possible error estimate from the data as an initial guess. Sample sequences were inferred, paired forward and reverse reads were merged and chimeric sequences were removed using the removeBimeraDenovo function. The resulting amplicon sequence variants (ASVs) were taxonomically classified using the Silva database (nr 99 v 138.1) for 16S rRNA and the PR2 database (version 4.13, minboot: 50) for 18S rRNA reads using the build-in rdp classifier.16S rRNA amplicon reads classified as chloroplasts and mitochondria, as well as 18S rRNA reads classified as *Metazoa* (Zooplankton) were removed prior to the downstream analyses.

Cooccurrence networks were generated in R using Spearman rank correlation, as described previosly (Bengtsson et al. 2017, correlation coefficient>0.7, adjusted p<0.01). We considered exclusively correlations between 18S and 16S ASVs and the final network was plotted using the igraph package, including only diatom and dinoflagellate 18S ASVs.

Metaproteomics

For metaproteomic analysis, proteins were extracted from the filters of 17.04., 08.05. and 24.05.2018 as described previously (Schultz et al. 2020) and analysed with liquid chromatography-tandem mass spectrometry in triplicates. Briefly, proteins were extracted from the filters via bead-beating followed by aceton precipitation. The extracts were separated and fractionated by 1D SDS-PAGE and in-gel trypsin digested. After desalting and concentration of the peptides using C18 Millipore® ZipTip columns, samples were measured

with an Orbitrap Velos™ mass spectrometer (ThermoFisher Scientific). After conversion into mgf file format using MS convert (ProteoWizard), spectra were matched against a metagenome-based database containing 14,764,755 entries. Mascot (Matrix Science, London, UK) and Scaffold (Proteome Software, Portland, US) were used for peptide-spectrum-matching, protein identification and protein grouping. Identified protein groups were annotated via Prophane (version 6.2.3, Schiebenhoefer et al. 2020), using the Uniprot TrEMBL and NCBI nr databases for taxonomic and EggNog (v5) and Tigrfams databases for functional annotation with default settings. Relative abundances were calculated by Prophane as NSAF values based on exclusive unique spectrum counts.

RESULTS AND DISCUSSION

Distinct network modules around Diatoms and Dinoflagellates

Cooccurrence analysis based on significant positive correlations between eukaryotic amplicon sequence variants (ASVs, 18S rRNA gene) and prokaryotic ASVs (16S rRNA gene) including all timepoints of the bloom (n= 51) resulted in a network with three distinct modules. Two of these modules were dominated by 18S ASVs belonging to diatoms and dinoflagellates, respectively, while the third module mostly contained diatom ASVs and was linked to the dinoflagellate-dominated network module (Fig. 2). These network modules roughly corresponded to the phytoplankton taxa prevalent in the early stages of the bloom (mainly diatoms), around peak chlorophyll (see Fig. 1), and during the late stages of the bloom (mainly dinoflagellates).

Bacteria involved in sulfur cycling were temporally associated to Diatoms

Our cooccurrence network analysis identified several ASVs belonging to the *Desulfobacterota* within the main diatom network module (Fig. 2). *Desulfobacterota* do not appear to be detected as frequent members of diatom microbiomes in culture or in the field (Helliwell et al. 2022). However, a recent global survey of the diatom interactome detected positive correlations between diatoms and sulfate-reducing bacteria in samples from the Tara Oceans expedition (Vincent & Bowler 2020). By comparing the bacterial ASVs cooccurring with Diatoms and those cooccurring with Dinoflagellates, it can be seen that *Desulfobacterota* ASVs were only positively correlated with Diatoms. In addition, ASVs classified as the *Ectothiorhodospiraceae*, which are purple sulfur bacteria also preferring anaerobic conditions (Imhoff 2005), were also positively associated with Diatoms. These bacteria can oxidize H_2S, which is produced via dissimilatory sulfate reduction by bacteria within the *Desulfobacterota*. This underlines the potential for anaerobic sulfur cycling in association to diatoms during this phytoplankton bloom.

Metaproteomics of *Desulfobacterota* revealed key enzymes of dissimilatory sulfate reduction

Metaproteomics of the >10 µm filter fraction from three timepoints (see Fig. 1) during the bloom resulted in the detection of 4584 protein groups. Among these, 22 protein groups

were taxonomically classified as belonging to different orders of the *Desulfobacterota*, using the TrEMBL and NCBI nr databases (see Materials and Methods for details). The relative abundance of *Desulfobacterota*-affiliated proteins in the metaproteome, expressed as NSAF, was 1.8 permille. This value is rather similar to the relative abundance of *Desulfobacterota* phylotypes in the microbiome (see above).

Functional analysis of the *Desulfobacterota* proteins revealed the functional category 'energy production and conversion' as most abundant (Fig. 4). Other categories were 'translation, ribosomal structure and biogenesis' as well as 'amino acid transport and metabolism' and 'carbohydrate and metabolism', as expected for actively metabolizing cells. Remarkably, key-enzymes of dissimilatory sulfate reduction, the hallmark metabolic pathway of sulfate reducing bacteria, were comprising 18% of all proteins (Fig. 4). Both the alpha and the beta subunits of dissimilatory sulfite reductase (DSR) were detected. This enzyme catalyses the six-electron reduction of sulfite to (hydrogen) sulfide, the terminal step in dissimilatory sulfate reduction, where most free energy is being conserved. Furthermore, adenylyl-sulfate reductase, catalyzing the conversion of adenosyl-phosphosulfate (APS) to sulfite, was also detected. Only ATP sulfurylase, the third enzyme of the dissimilatory sulfate reduction pathway, was not detected.

One has to note that a conservative threshold for protein identification was applied in this study: at least two peptides were needed to consider a protein to be validly detected. The abundant detection of key enzymes of dissimilatory sulfate reduction thus provide strong support that the *Desulfobacterota* detected in this study are indeed capable of sulfate reduction and that they likely are gaining energy through this type of anaerobic respiration. Furthermore, the study shows once more the power of metaproteomics for functional characterization of even rare taxa, such as the *Desulfobacterota* in our study, that comprise much less than 1% of the microbiome.

New roles for diatom-associated bacterial communities?

Sulfate reduction is a process that is well known and described for various marine habitats where oxygen is limited (Ugarelli et al. 2017, D'Hondt et al. 2002), including sinking particles (e.g. Bianchi et al. 2018, Raven et al. 2021). Our study focused on the upper layers of the photic zone, during an active spring phytoplankton bloom, a scenario typically assumed to be completely saturated in oxygen due to the activities of photosynthetic phytoplankton. In this environment we detected key enzymes involved in sulfate reduction, expressed by bacteria belonging to the *Desulfobacterota*. Further, we observed that *Desulfobacterota* were temporally associated to abundant diatom taxa during the bloom, whereas this was not the case for abundant dinoflagellate taxa.

Temporal associations such as detected by our cooccurrence network analysis have to be interpreted with caution as other temporally variable factors not measured may underlie such correlations. Nevertheless, cooccurrence network analysis provides a tool for formulating hypotheses about interactions between taxa that can be tested experimentally. Our observations could indicate that living diatom-derived particles harbour sufficiently

anoxic niches, at least during some parts of the day, to sustain anaerobic sulfur cycling in the photic zone. Diatoms such as *Thallassiosira* spp. and *Skeletonema* spp. are known to form aggregates (Thornton 2002) held together by exopolymeric substance (EPS) produced by the algal cells, stimulated by associated bacteria (Gärdes et al. 2011). Such aggregates consisting of living, photosynthesizing, algal cells produce ample oxygen during the day, yet respiration during night, both by heterotrophic bacteria and the algal cells themselves, may deplete oxygen concentrations during the night, enabling anaerobic microniches and associated metabolic functions. This would add a dimension of complexity to algal-bacterial interactions in particles during algal blooms and underlines the need to study these as spatially heterogeneous entities. Diatom microbiomes may thus be as complex as those of animals and other multicellular organisms, featuring distinct sub-microbiomes that depend on the local chemical environment. Analogous to animal skin and gut microbiomes, the diatom phycosphere may select for bacteria that carry out drastically different functions in the periphery and center of diatom-derived aggregates. Anoxic niches in diatom-derived aggregates may also affect the biological carbon pump, as sulfate reduction may alter the chemical properties of organic material in particles, making them resistant to microbial degradation (Raven et al. 2021).

We showed that there was a temporal association between Diatom taxa and bacterial taxa belonging to the *Desulfobacterota* during a North Sea phytoplankton spring bloom and that key enzymes involved in sulfate reduction by these bacteria were expressed during the same bloom. However, a physical association between these bacteria and diatoms was not proven by our approach and would require microscopic investigation using specific phylogenetic probes, for example. Likewise, it has yet to be investigated whether such temporal and physical associations are recurrent between different years, and in that case under what specific conditions they occur. The presence of anaerobic niches inside aggregates of living diatoms would change the premises for algal-bacterial-organic matter interactions in the photic zone, with important implications for carbon export from phytoplankton blooms.

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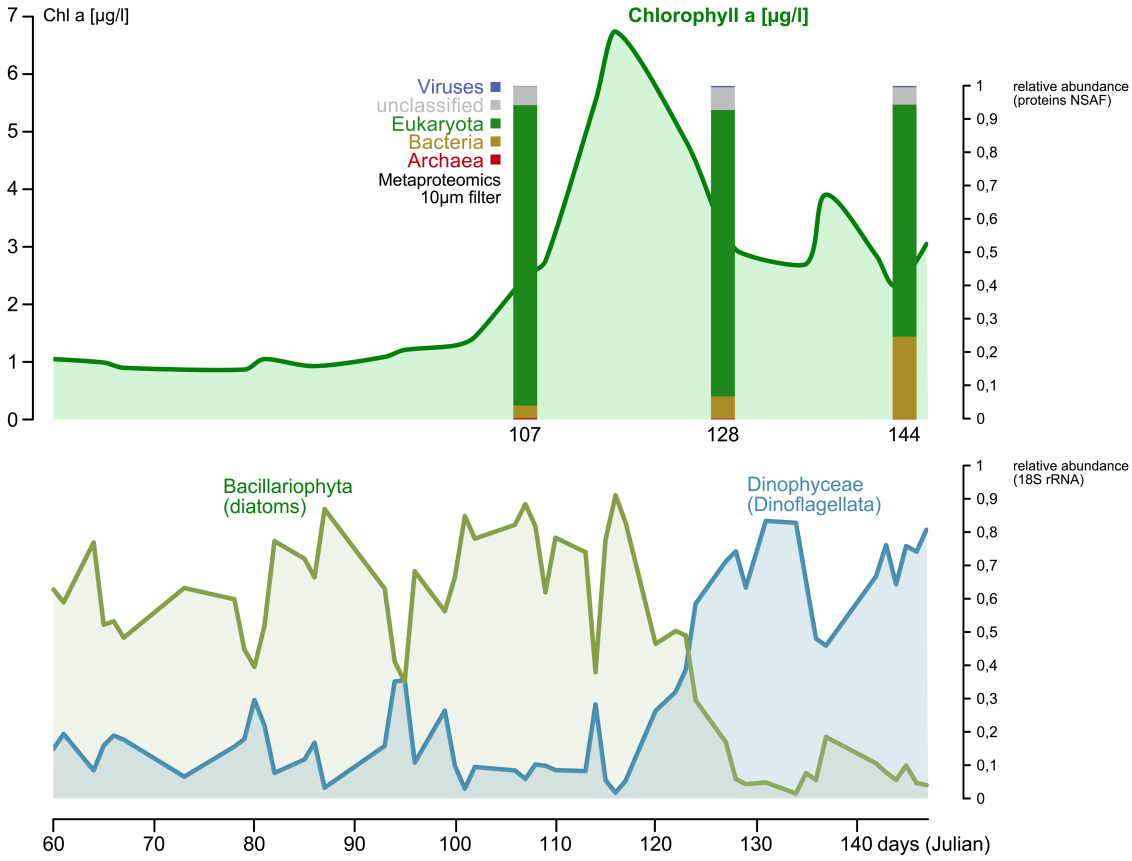

Figure 1: In the phytoplankton spring bloom of 2018 (01 March - 29 May), particles larger than 10 µm were collected via filtration and microbial communities were analyzed using 16S and 18S rRNA gene amplicon sequencing and metaproteomics. A) algal biomass (Chlorophyll a concentration) peaked around 26th of April (Julian day 116). Metaproteomics data from three selected timepoints displayed a dominance of eukaryotic proteins. B) Diatoms and dinoflagellates were the most abundant phytoplankton lineages as detected via 18S rRNA gene amplicon sequences and varied along the course of the bloom with dinoflagellates being more abundant after the Chlorophyll peak.

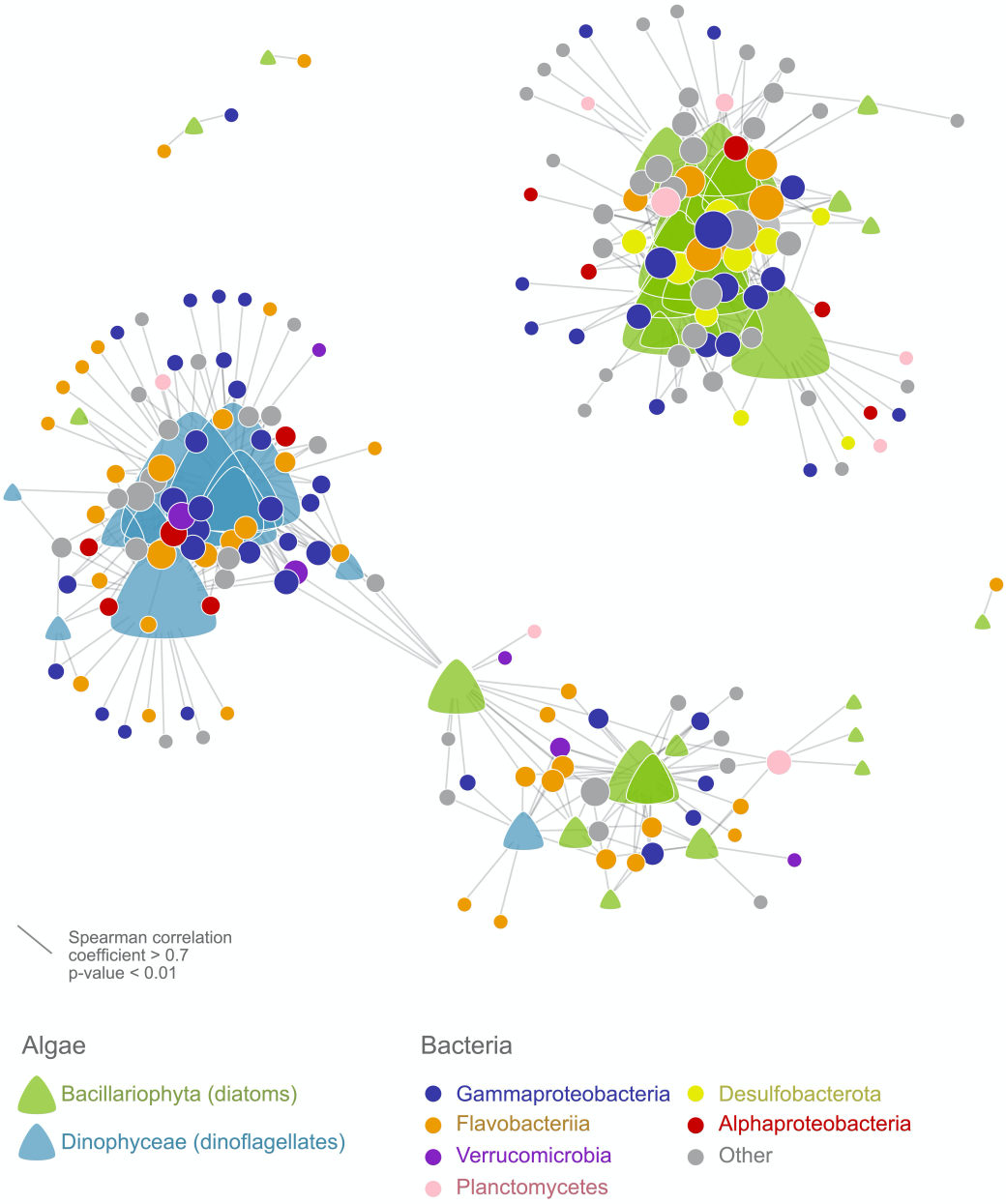

Figure 2: A network analysis of 18S rRNA gene ASV and 16S rRNA ASV cooccurrences identified two major network modules containing diatom and dinoflagellate 18S ASVs respectively as well as one mixed module. The network was calculated based on Spearman correlations (correlation coefficient >0.7, $p < 0.01$) between 18S ASVs and 16S ASVs exclusively. For clarity, only Diatom- and Dinoflagellate 18S ASVs (triangles) are shown in the plot. Several bacterial groups (circles) were associated to all modules, yet *Desulfobacterota* (yellow) were only associated to the diatom module. The size of the symbols correspond to the degree (number of significant correlations) of the nodes.

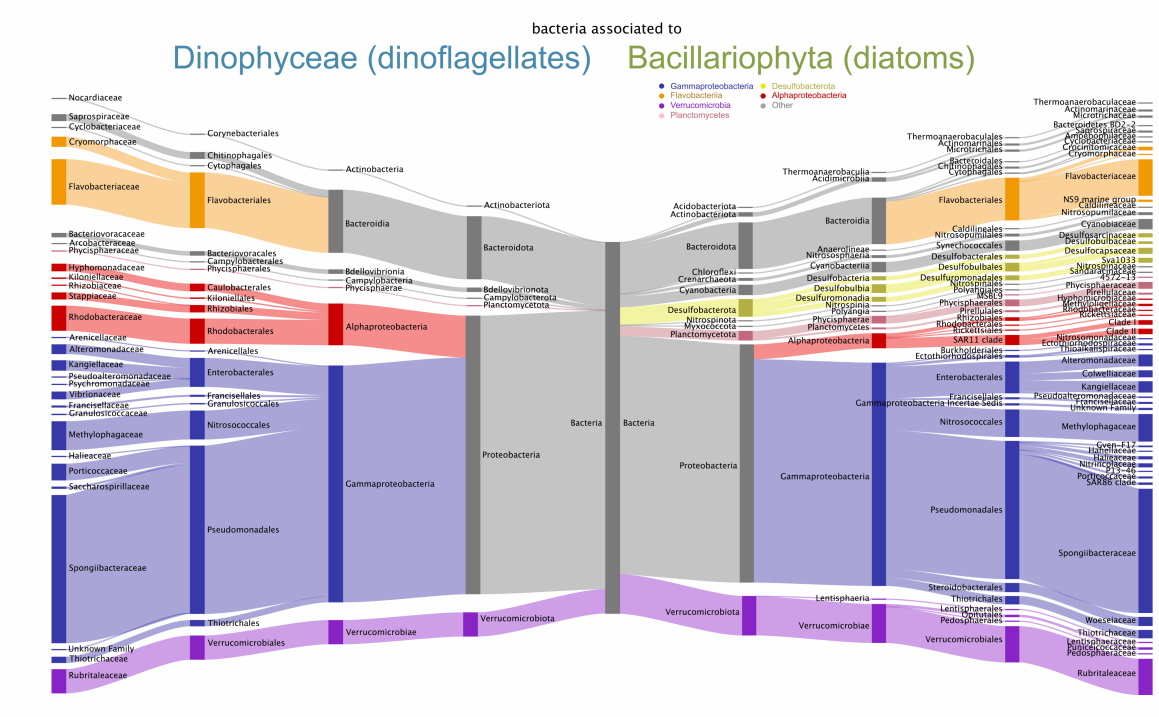

Figure 3: The bacterial ASVs temporally associated to diatoms and dinoflagellates were taxonomically distinct. *Desulfobacterota* were positively associated with diatoms and not dinoflagellates.

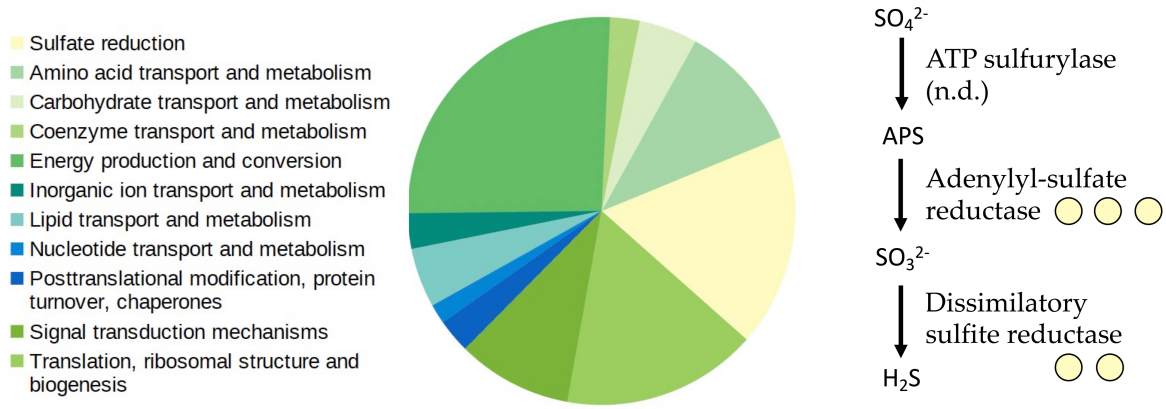

Figure 4: Key enzymes from *Desulfobacterota* involved in the sulfur cycle were detected in metaproteomes from a spring phytoplankton bloom in the North Sea. The pie chart displays relative abundances for all 22 protein groups classified as belonging to *Desulfobacterota* during all three time points sampled for metaproteomics. Of these, 5 protein groups represent enzymes involved in dissimilatory sulfate reduction (yellow circles). n.d. not detected.