



# Experimental Evidence for Abiotic Sulfurization of Marine Dissolved Organic Matter

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### Specialty section:

This article was submitted to  
Marine Biogeochemistry,  
a section of the journal  
Frontiers in Marine Science

**Received:** 15 June 2017

**Accepted:** 30 October 2017

**Published:** 15 November 2017

### Citation:

Pohlabein AM, Gomez-Saez GV,  
Noriega-Ortega BE and Dittmar T  
(2017) Experimental Evidence for  
Abiotic Sulfurization of Marine  
Dissolved Organic Matter.  
*Front. Mar. Sci.* 4:364.  
doi: 10.3389/fmars.2017.00364

Dissolved organic sulfur (DOS) is the largest pool of organic sulfur in the oceans, and as such it is an important component of the global sulfur cycle. DOS in the ocean is resistant against microbial degradation and turns over on a millennium time scale. However, sources and mechanisms behind its stability are largely unknown. Here, we hypothesize that in sulfate-reducing sediments sulfur is abiotically incorporated into dissolved organic matter (DOM) and released to the ocean. We exposed natural seawater and the filtrate of a plankton culture to sulfidic conditions. Already after 1-h at 20°C, DOS concentrations had increased 4-fold in these experiments, and 14-fold after 4 weeks at 50°C, indicating that organic matter does not need long residence times in natural sulfidic environments to be affected by sulfurization. Molecular analysis via ultrahigh-resolution mass spectrometry showed that sulfur was covalently and unselectively bound to DOM. Experimentally produced and natural DOS from sediments were highly similar on a molecular and structural level. By combining our data with published benthic DOC fluxes we estimate that 30–200 Tg DOS are annually transported from anaerobic and sulfate reducing sediments to the oceans. Uncertainties in this first speculative assessment are large. However, this first attempt illustrates that benthic DOS flux is potentially one order of magnitude larger than that via rivers indicating that this could balance the estimated global net removal of refractory DOS.

**Keywords:** dissolved organic matter (DOM), dissolved organic sulfur (DOS), sulfurization, structural analysis, sulfidic sediments

## INTRODUCTION

Marine dissolved organic matter (DOM) is among the most complex molecular mixture known on Earth, consisting of at least hundreds of thousands of organic compounds (Zark et al., 2017). Many compounds within the DOM mixture contain sulfur (dissolved organic sulfur, DOS), and in sum they make up the largest reservoir of organic sulfur in the ocean (global inventory of >6.7 Pg S, Ksionzek et al., 2016). Despite the relevance of DOS in global biogeochemical cycles, knowledge on its sources and turnover, and its molecular composition is scarce (Lechtenfeld et al., 2011). Marine primary production is considered a major source of organic sulfur, explaining elevated DOS concentrations in surface waters (Ksionzek et al., 2016). A part of the marine organic sulfur is rapidly cycled and thus labile (Ksionzek et al., 2016), and may contribute little to refractory DOS

in the oceans' interior (Dittmar et al., 2017). The source of the large refractory DOS pool which is evenly distributed in the water column is unknown (Levine, 2016).

In sulfidic sediments, organic matter abiotically reacts with inorganic reduced sulfur species during early diagenesis forming organic sulfur compounds (Sinninghe Damsté et al., 1989; Vairavamurthy et al., 1995; Schmidt et al., 2009). Sulfur can thereby be incorporated into different types of organic compounds, including alkenes, aldehydes (de Graaf et al., 1992; Krein and Aizenshtat, 1994; Schouten et al., 1994), carbohydrates and complex algal material (Kok et al., 2000; van Dongen et al., 2003). The incorporation of sulfur likely protects labile organic matter from microbial alterations (Anderson and Pratt, 1995). There are also indications that natural DOM may be sulfurized in sulfidic marine systems because DOS concentrations are distinctly higher in sulfidic environments than in oxic ones (Sleighter et al., 2014; Gomez-Saez et al., 2016). Benthic fluxes from anoxic marine sediments, where carbon oxidation rates are high, contribute major amounts of iron (Dale et al., 2015) and DOM (Burdige and Komada, 2014) to the ocean. If abiotic sulfurization of DOM takes place in these sediments, major benthic fluxes of DOS may also occur.

Here, we hypothesize that abiotic sulfurization of DOM occurs in marine sulfidic environments, making sediments with high carbon oxidation rates a potential formation site for DOS in the ocean. To test this hypothesis, we performed sulfurization experiments with natural DOM. DOS formation was quantitatively assessed, and sedimentary and experimental DOS were compared on a detailed molecular level. The molecular composition of the sulfurized samples was compared to DOM from sulfidic sediments in the German Wadden Sea (Janssand pore water) that is likely an area where natural sulfurization occurs (for details on the sample and sampling see Seidel et al., 2014). DOM comprises a largely inseparable mixture of compounds and is as such only partially accessible to conventional chromatographic analytical techniques. For the molecular analysis of DOM prior and after the sulfurization experiment we used ultrahigh-resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). FT-ICR-MS coupled to soft electrospray ionization enables the analysis of individual, intact molecules in the otherwise inseparable DOM mixture. The high mass accuracy of FT-ICR-MS allows the assignment of molecular formulae to the detected masses (Hertkorn et al., 2013). Additionally, we performed a series of analytical experiments prior to mass spectrometry analysis and within the FT-ICR-MS to determine the molecular structure of S-containing functional groups (Pohlabein and Dittmar, 2015). Molecular analyses were accompanied by quantitative element analysis of dissolved organic carbon (DOC) and DOS. Because of the high concentrations of inorganic sulfur in all our samples, the samples were desalted via solid phase extraction (SPE) prior to molecular and elemental analysis. Thus, we refer here to the solid phase extractable fraction of DOM, i.e., SPE-DOC and SPE-DOS. For sample preparation and analysis, samples were exposed to atmospheric oxygen. Therefore, compounds that are easily oxidized and that would not survive for long in the oxic water column of the ocean were not targeted by our analysis.

## MATERIALS AND METHODS

### Mesocosm Experiments and Samplings

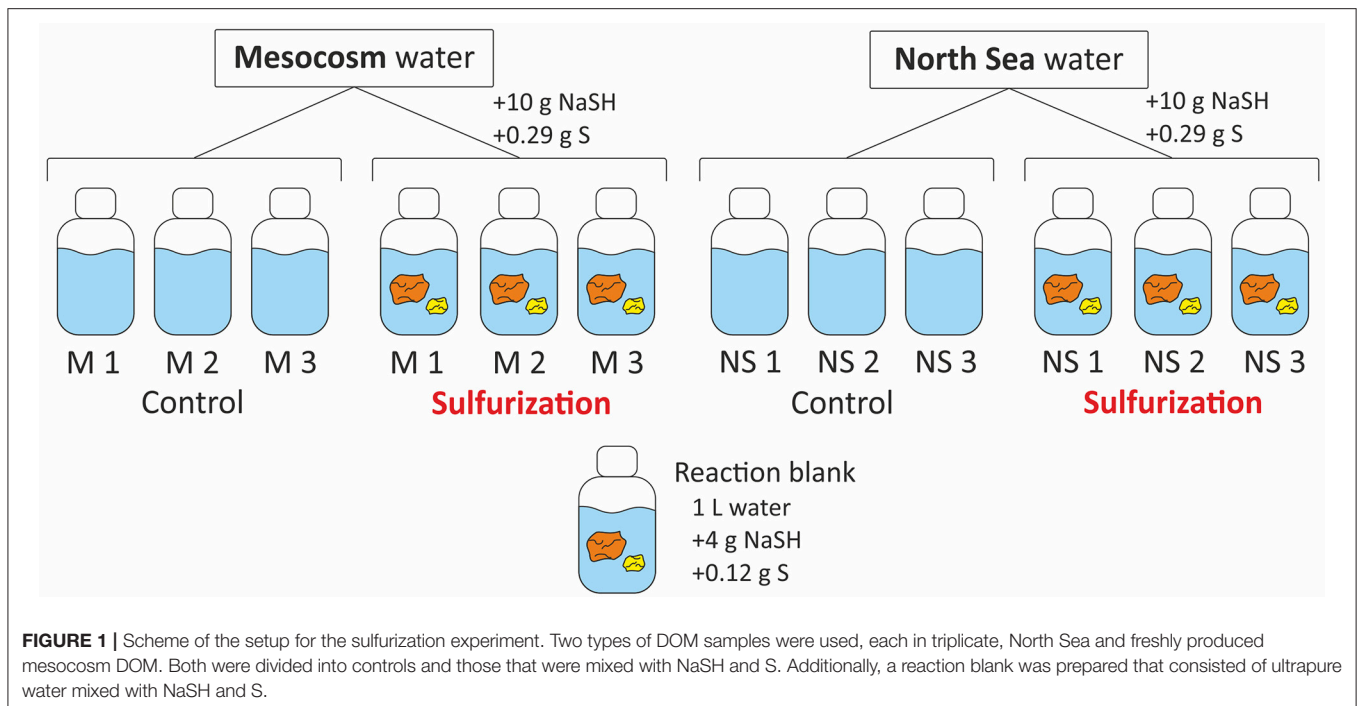
For the sulfurization experiment, a freshly produced DOM sample from in-house mesocosms inoculated with a natural marine planktonic community was compared to a natural DOM sample from a coastal site in the North Sea (Germany). The planktonic mesocosm experiments were conducted as in Osterholz et al. (2015). The mesocosms were set up in triplicates (M1, M2, M3) each consisting of 4.95 L artificial nutrient-enriched seawater (DOC 18  $\mu\text{mol C L}^{-1}$ ) (Osterholz et al., 2015) mixed with 0.05 L prefiltered (poresize: 100  $\mu\text{m}$ ) coastal North Sea water containing the natural communities of phyto- and bacterioplankton as inoculum (Spiekeroog, Germany, March 18th 2015, 53°01.30' N, 8°27.10'E, low tide, DOC 157  $\mu\text{mol C L}^{-1}$ ) in acid-rinsed 5 L glass bottles (final DOC concentration of artificial seawater plus inoculum 19–20  $\mu\text{mol C L}^{-1}$ ). The mesocosms were incubated at approximately 17°C and illuminated for 12-h per day (400–700 nm) while the water was constantly stirred using magnetic stirrers. After 18 days the algae that were dispersed in the water before had clustered together and the DOC concentration of the mesocosm water ranged between 100 and 150  $\mu\text{mol C L}^{-1}$ . At this time point the incubation was stopped and the mesocosm water was filtered sequentially through glass microfiber filters (2  $\mu\text{m}$ , GME, Whatman, USA) and glass fiber filters (0.7  $\mu\text{m}$ , GFE, Whatman, USA) and acidified to pH 2 with hydrochloric acid (p.a., Merck, Germany) to stop any microbial activity.

The North Sea sample for the long-term sulfurization experiment was taken on March 3rd 2015 at low tide and the sample for the 1-h incubation on September 15th 2015 at high tide, both at the same location as the mesocosm inoculum near Spiekeroog Island, Germany. Samples were filtered and acidified as described above and stored at 4°C until the experiment.

### Sulfurization Experiments

The sulfurization experiment was adapted from Kok et al. (2000). We chose this original setup to facilitate the comparison with published results for particulate organic matter. Sulfurization was tested by addition of NaSH and S to the samples as done previously for particulate organic matter (Kok et al., 2000). This experimental procedure was chosen because sulfide and S are present in sulfidic seawater and together form polysulfides (Adam et al., 1998). Polysulfides are the most nucleophilic species of reduced sulfur and likely the most important sulfur species for diagenetic sulfur incorporation into organic matter (Krein and Aizenshtat, 1994; Amrani et al., 2007). Long-term (4 weeks) sulfurization experiments were complemented by a very short (1-h) incubation. For the long-term incubation a temperature of 50°C was set in order to cope for the longer residence time that can be up to decades in coastal tidal flats (e.g., Janssand; Røy et al., 2008), in only 4 weeks of incubation. In addition, we conducted the short-term experiment at room temperature.

The filtered and acidified mesocosm and North Sea samples were transferred to 2.5 L amber glass bottles (Figure 1). The pH of all samples was adjusted to pH 8 with NaOH (p.a. Roth, Germany) to simulate the natural seawater pH and left overnight.



The pH was checked at the next day and each sample was bubbled with argon for 20 min to expel all  $O_2$  from the bottles. No further treatments were conducted with the “control” samples. For the sulfurization approach, inorganic sulfur compounds (10 g NaSH and 0.29 g elemental sulfur) (Kok et al., 2000) were added to the samples that we refer to as “Sulf” samples in the following. Sulfur reagents were analytical grade (p.a. Sigma Aldrich). A reaction blank consisting of one liter of ultrapure water was bubbled with argon and 4 g NaSH and 0.12 g elemental sulfur were added. All bottles—controls, Sulfs and blank—were placed in ovens at  $50^\circ C$  for 4 weeks (Kok et al., 2000). The “Sulf” samples were shaken daily to disperse the inorganic sulfur compounds as the elemental sulfur does not dissolve in water but NaSH and elemental sulfur together form water soluble polysulfides over time. After 4 weeks at  $50^\circ C$  the samples were acidified and filtered as described above.

For the 1-h sulfurization experiment, the pH of the sample was also adjusted to pH 8 and the sample was split (540 ml each) in two controls and two “Sulf” samples. The samples were purged with Argon gas and 2 g NaSH and 60 mg sulfur were added to the “Sulf” samples. The solutions were kept in the dark at room temperature for 1-h with two intervals for shaking the samples, then they were filtered and acidified as described above.

An adduct test was conducted to ensure that the observed sulfurization products originate from covalent incorporation of inorganic sulfur into organic molecules and not only from hydrogen sulfide ion adducts. For this, the DOM methanol extract (see section Sample Preparation and Elemental and Molecular Formula Analyses) of sample “North Sea Control 1” was mixed with an aqueous solution of NaSH (DOC-to-sulfur ratio: 10:1, final DOC concentration: 1.25 mM) and immediately analyzed with the FT-ICR-MS. Potential sulfur-rich adducts of elemental sulfur or polysulfides could be ruled out in our sulfurization experiments because all detected DOS

compounds contained not more than two sulfur atoms in their molecular formula (on the basis of our used molecular formulae assignment). A broad band mass spectrum was recorded after the fast addition of NaSH and additionally two nominal masses ( $m/z = 377$  and  $389$ ) were fragmented. For comparison, the same FT-ICR-MS analysis was done with the “North Sea Control 1” extract without addition of inorganic sulfur.

## Sample Preparation and Elemental and Molecular Formula Analyses

All samples including the blank were extracted using SPE on styrene divinyl benzene polymer filled cartridges (1 g, Agilent Bond Elut PPL, USA) (Dittmar et al., 2008). Bulk DOC concentrations of the samples prior to and after incubation were determined by high-temperature catalytic oxidation on a Shimadzu TOC-VCPH analyzer. Accuracy of the DOC determination was validated by analyzing the deep sea reference sample (Batch 10, Lot# 05–10), from the Consensus Reference Material (CRM) project, provided by D. Hansell and colleagues (University of Miami, USA). Accuracy was within 5%. DOC concentrations of all methanol extracts obtained by SPE were determined by taking an aliquot of the extract, removing the methanol by evaporation and dissolving the residue in 0.01 M HCl which was then analyzed on the same Shimadzu TOC-VCPH analyzer. From those values the extraction efficiencies were calculated. DOS concentrations of the SPE-extracts were measured on an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, iCAP 6000, Thermo Fisher Scientific GmbH, Bremen, Germany) and bulk DOS concentrations were estimated based on the extraction efficiency for DOC as done previously (Pohlabein and Dittmar, 2015; Gomez-Saez et al., 2016; Ksionzek et al., 2016).

FT-ICR-MS measurements were performed with a solarix Fourier-transform ion cyclotron resonance mass spectrometer with a 15 T magnet system (Bruker Daltonik GmbH, Bremen, Germany). A Bruker Daltonik Apollo II atmospheric pressure electrospray ionization unit (ESI) was used as the external ion source in negative ionization mode. All samples were analyzed in a 1:1 volumetric mixture of methanol (or acetonitrile for derivatization experiment, see section Molecular Analysis of Sulfur Functional Groups) and ultrapure water (Pohlabein and Dittmar, 2015). The DOC concentration was adjusted to 15 mg C L<sup>-1</sup>. The samples were directly infused into the ESI source at a flow rate of 120  $\mu$ L h<sup>-1</sup>. For fragmentation experiments, the DOC concentration was adjusted to 100 mg C L<sup>-1</sup>, and a flow rate of 360  $\mu$ L h<sup>-1</sup> was used for ESI. Reproducibility was monitored by analyzing an in-house reference sample from North Equatorial Pacific Intermediate Water (Green et al., 2014) every morning and evening. Five hundred transient scans in broadband mode were accumulated for each run, covering the mass range of 150–2,000 Da. A method detection limit was applied to remove noise peaks from the data set (Riedel and Dittmar, 2014). All detected ions were singly charged. After internal calibration, the mass error was <100 ppb. At this high mass accuracy, molecular formulae were assigned with very high certainty to all compounds containing the elements C, H, O, S, N and P. Formula assignment for each detected mass was done following established procedures (Rossel et al., 2013) but the allowed number of nitrogen atoms was increased to four. Molecular formulae detected in the reaction blanks of the sulfurization experiments were disregarded from further consideration. The identified molecular formulae were tentatively assigned to compound groups based on their molar ratios, aromaticity index, and heteroatom content (Seidel et al., 2014; Stubbins et al., 2014). These compound groups were polycyclic aromatics, polyphenols, sugars, and peptides. Because of the multitude of possible isomers behind a given molecular formula, these assignments are not unambiguous but they provide a reasonable overview of possibly structures behind the cocktail of detected molecular formulae.

For statistical analysis, the detected masses were normalized to the sum of all mass intensities of the corresponding sample. For presence-absence analysis only masses were considered when present in two out of three triplicates or in both of duplicates. Further multivariate statistical analysis was done on the normalized data (Bray-Curtis dissimilarity). For the analysis of the derivatization and hydrolysis alteration reactions a variance test was performed as described in the following. To evaluate whether sulfurization significantly increased the sulfur content of the samples, Student's *t*-tests were performed for the relevant parameters (Table 1, one-tailed *t*-test, assuming equal variance).

## Molecular Analysis of Sulfur Functional Groups

Each sample from the sulfurization experiment, controls and "Sulfs," were analyzed for their S-containing functional groups. For this purpose, functional group selective wet-chemical alteration reactions were conducted (Pohlabein and Dittmar, 2015), and samples were analyzed in broadband mode on the FT-ICR-MS before and after the alteration reaction as described

above. Each sample was analyzed at least in duplicate. In brief, to test for thiols SPE-DOM samples were dried and dissolved in an acetonitrile solution of the thiol-selective reagent 2-bromo-1,4-naphthoquinone for 30 min at 20°C (derivatization experiment). To test for thioesters, sulfonic acid esters and alkylsulfates, samples were hydrolyzed by dissolving the dried SPE-DOM samples in hydrochloric acid (25%, p.a., Merck, Germany) and heated to 110°C for 24-h. To test for non-aromatic thioethers and sulfoxides oxidation and deoxygenation experiments were done. For this purpose, DOM samples were dissolved in acetonitrile and cyanuric chloride, and hydrogen peroxide or potassium iodide, respectively, were added. Then, the mixture was allowed to stand at 20°C for 2-h.

In addition, collision-induced fragmentation experiments were performed on selected nominal masses (Pohlabein and Dittmar, 2015): 12 nominal masses (for M2 Sulf only 6 masses due to shortage of sample) consisting of three CH<sub>2</sub>-homologous series were analyzed. For each of these 12 nominal masses, 4–7 sulfur-containing molecular formulae were fragmented.

For the statistical interpretation of the functional group selective alteration reactions a variance test was performed (Pohlabein and Dittmar, 2015). With this approach, it was tested whether discrepancies between the mass spectra of the derivatization and hydrolysis experiments were actually based on the reaction processes or just due to measurement variations. Briefly, the spectra prior to and after reaction were screened for trends in the FT-ICR-MS signal intensities of the *m/z*-ratios (decreasing or increasing trend after reaction). The same analysis was done for the spectra of the reference sample that was measured every morning and evening, representing the instrument variability. The detected variance in the reference material was set as threshold. Only variance between treated and untreated DOM sample that were above the threshold (higher than instrument variability) were considered as reaction-induced. Compared to the derivatization and hydrolysis, the oxidation and deoxygenation experiments do not lead to a clear separation of reaction products. While there is a characteristic molecule addition in the thiol derivatization and molecule cleavage in hydrolysis, there is only a small (one or two oxygen atoms) increase or decrease in oxygen content for a S-containing molecule in the oxidation and deoxygenation experiment. To determine and visualize even slight differences between the spectra of the oxidation and deoxygenation experiments, the mass spectra were interpreted via multivariate statistical analysis (Bray Curtis dissimilarity; Figure S2).

## Microbial Analysis

To verify the lack of microbial activity during sulfurization, samples for cell counts were taken prior to the experiments and afterwards. The samples were fixed with glutaraldehyde (1% final concentration, Carl Roth, Germany) and cells were counted with a BD accuri C6 Flow Cytometer (BD Biosciences, USA) using SYBRGreen (Invitrogen, United Kingdom) following Gasol and Del Giorgio (2000). Fluorescent microscopy was also used to verify the negative results from the flow cytometer. Samples were filtered (0.2  $\mu$ m polycarbonate) and SYBRGreen was added. After a 30 min dark incubation, the filters were analyzed under the

**TABLE 1** | Element concentrations and molar ratios for all samples obtained from elemental analysis and molecular formulae (from FT-ICR-MS): intensity weighted = number of carbon or sulfur atoms, respectively, for a detected molecular formula multiplied by its mass intensity divided by the sum of all mass intensities, the *p*-value represents the significance of Student's *t*-test of Sulf > Con (one-tailed *t*-test, assuming equal variance).

Sample	Bulk DOC [μM]	SPE-DOC [μM]	Extraction efficiency (%)	Bulk DOS [μM]	SPE-DOS [μM]	DOS/DOC ratio	No. all formulae (FT-ICR-MS)	No. S-formulae (FT-ICR-MS)	No. S-formulae/ No. all formulae (FT-ICR-MS) [%]	intensity-weighted S/C (FT-ICR-MS) x1000
M1 Con	138	39	28	1.5	0.4	0.011	7,658	1,995	26.0	6.8
M2 Con	132	42	32	1.0	0.3	0.008	7,949	2,045	25.7	5.8
M3 Con	179	51	28	1.2	0.4	0.007	7,177	1,736	24.2	6.1
M1 Sulf	150	32	21	10.3	2.2	0.069	12,086	5,438	45.0	32.4
M2 Sulf	143	15	10	21.3	2.2	0.149	13,948	6,122	43.9	32.2
M3 Sulf	206	24	12	19.7	2.3	0.096	11,757	5,268	44.8	34.3
<i>p</i> -value M	–	–	–	<0.01	<0.001	<0.01	=0.001	<0.001	<0.001	<0.001
NS1 Con	188	98	52	1.4	0.8	0.008	12,514	3,645	29.1	7.0
NS2 Con	191	88	46	1.6	0.7	0.008	12,180	3,550	29.1	7.0
NS3 Con	192	89	46	1.5	0.7	0.008	11,165	3,107	27.8	6.7
NS1 Sulf	217	37	17	16.2	2.8	0.075	15,970	6,003	37.6	12.2
NS2 Sulf	209	29	14	22.8	3.2	0.109	16,467	6,280	38.1	12.4
NS3 Sulf	228	31	14	23.2	3.2	0.102	16,472	6,295	38.2	12.7
<i>p</i> -value NS	–	–	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
1h Con A	181	61	34	3.1	1.0	0.017	13,724	4,376	31.9	7.7
1h Con B	180	65	36	2.8	1.0	0.016	14,191	4,608	32.5	7.8
1h Sulf A	203	40	20	10.2	2.0	0.050	15,735	5,675	36.1	10.1
1h Sulf B	205	32	16	13.4	2.1	0.065	16,236	5,956	36.7	10.3
Pore water	1276	873	68	47.3	32.3	0.037	14,285	5,713	40.0	17.3

epifluorescence microscope AxioImager.Z2m (Carl Zeiss, Jena, Germany).

## Potential Sulfurization Reactions Analysis

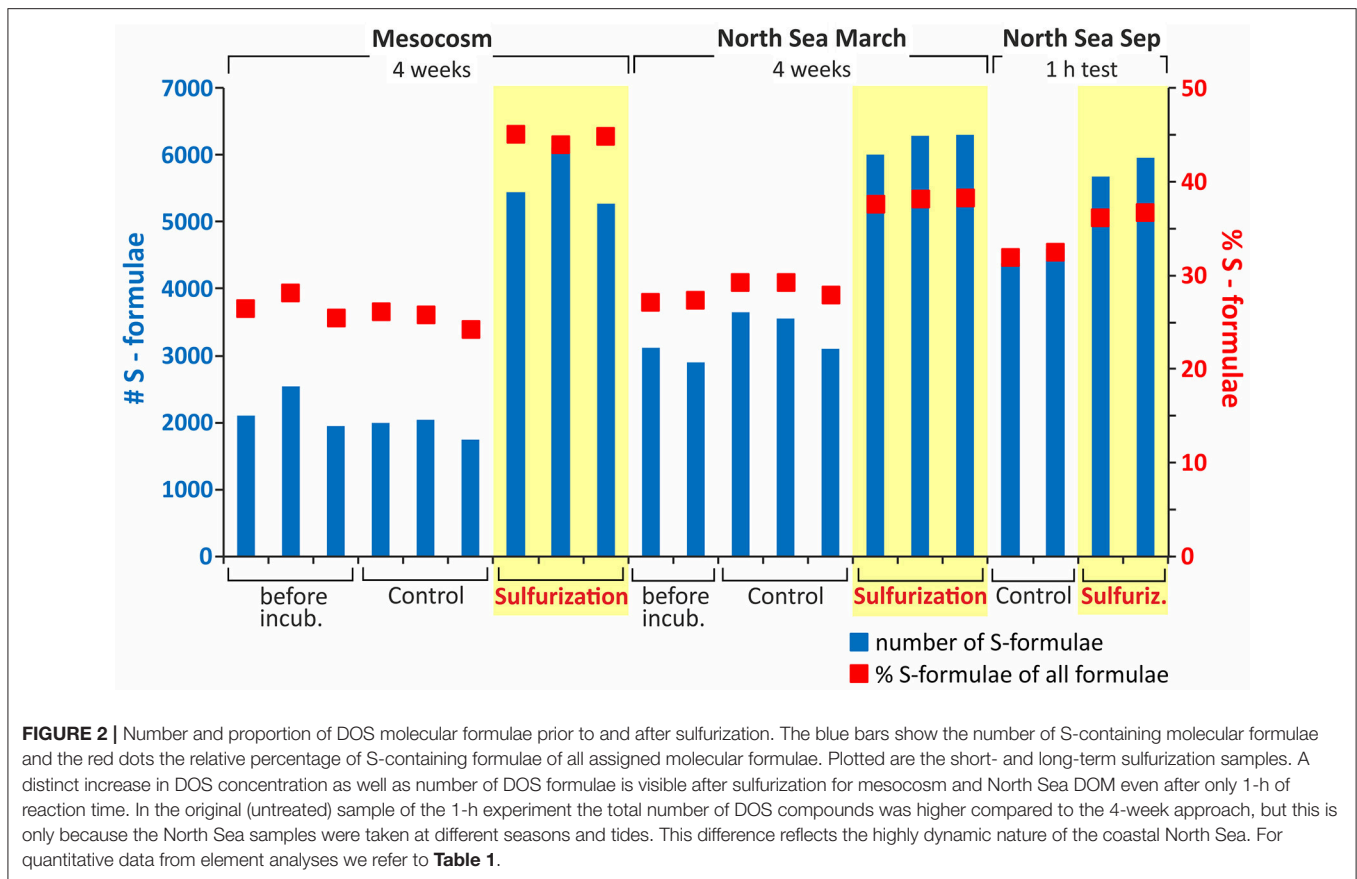
In this study, 20 potential sulfurization reactions were chosen (Gomez-Saez et al., 2016), following nine possibilities of S addition while adding/removing H and/or O:  $+S_1$ ;  $+S_1 - H_n$ ;  $+H_nS_1O_n$ ;  $+S_1O_n$ ;  $+S_1O_n - H_n$ ;  $+H_nS_1$ ;  $+S_1 - H_nO_n$ ;  $+S_1 - O_n$ ;  $+H_nS_1 - O_n$ . The corresponding potential reactions of S addition were proposed as the equivalent  $+H_2S$  reaction. They were exchanging  $H_2O$ ,  $H_2$ , and/or  $O_2$  by a  $H_2S$  molecule and accordingly compounds with  $S_1$  were obtained (Table S1 and Figure S1). The effectiveness of the potential reactions was considered as a percentage of  $S_1$  formulae present in the sulfurized samples with one potential precursor following the corresponding reaction (Gomez-Saez et al., 2016). Additionally, two different groups of sulfur compounds were targeted: DOS formulae produced by sulfurization (Table S1A) and those DOS formulae already present in the samples before incubation (Table S1B). In this second case, two criteria were applied: 1) The intensity of the DOS compound's mass peaks increased after sulfurization and 2) the mass peaks of the corresponding CHO precursor of the DOS compounds decreased in intensity.

## RESULTS AND DISCUSSION

### Experimental Sulfurization of DOM

A distinct increase of bulk DOS concentrations determined by ICP-OES occurred in the 4 week sulfurization experiments

with planktonic and North Sea DOM (increase by factor 14, on average, **Table 1**). The molar DOS/DOC ratio also increased strongly due to sulfurization. Consistently, the total number of non-S-containing compounds detected by FT-ICR-MS also increased (mesocosm: +16%; North Sea: +24%; **Table 1**). Up to 6000 sulfur-containing molecular formulae were identified in the sulfurized samples by FT-ICR-MS, about double than in the original and control samples (**Figures 2, 3**). A decrease of DOC extraction efficiency after sulfurization was observed (**Table 1**). As the bulk DOC concentrations did not decrease after sulfurization, this decrease in extraction efficiency cannot be due to coagulation with inorganic sulfur particles. It is possible though, that during sulfurization or due to second step rearrangement reactions like cyclisation reactions (Eglinton et al., 1994), small non-solid-phase-extractable organic compounds were formed. Nevertheless, the FT-ICR-MS signal intensity-weighted sulfur-to-carbon ratio (S/C) was higher after sulfurization (factor 5.3 for planktonic DOM, factor 1.8 for North Sea DOM). The sulfurization led to a higher number of shared DOS compounds between planktonic and North Sea DOM (**Figures 4A,B**), indicating a high similarity of DOS on a molecular level. These detected changes in DOS content must result from abiotic reactions as cell counts in the sulfurization experiment revealed no bacterial growth in any of the experiments (data not shown). Furthermore, we excluded the possibility of simple sulfur adducts formed in solution or during ionization by an adduct test where we added sulfide (NaSH) to the control samples immediately prior to FT-ICR-MS analysis. In this test no new DOS compounds were observed verifying the

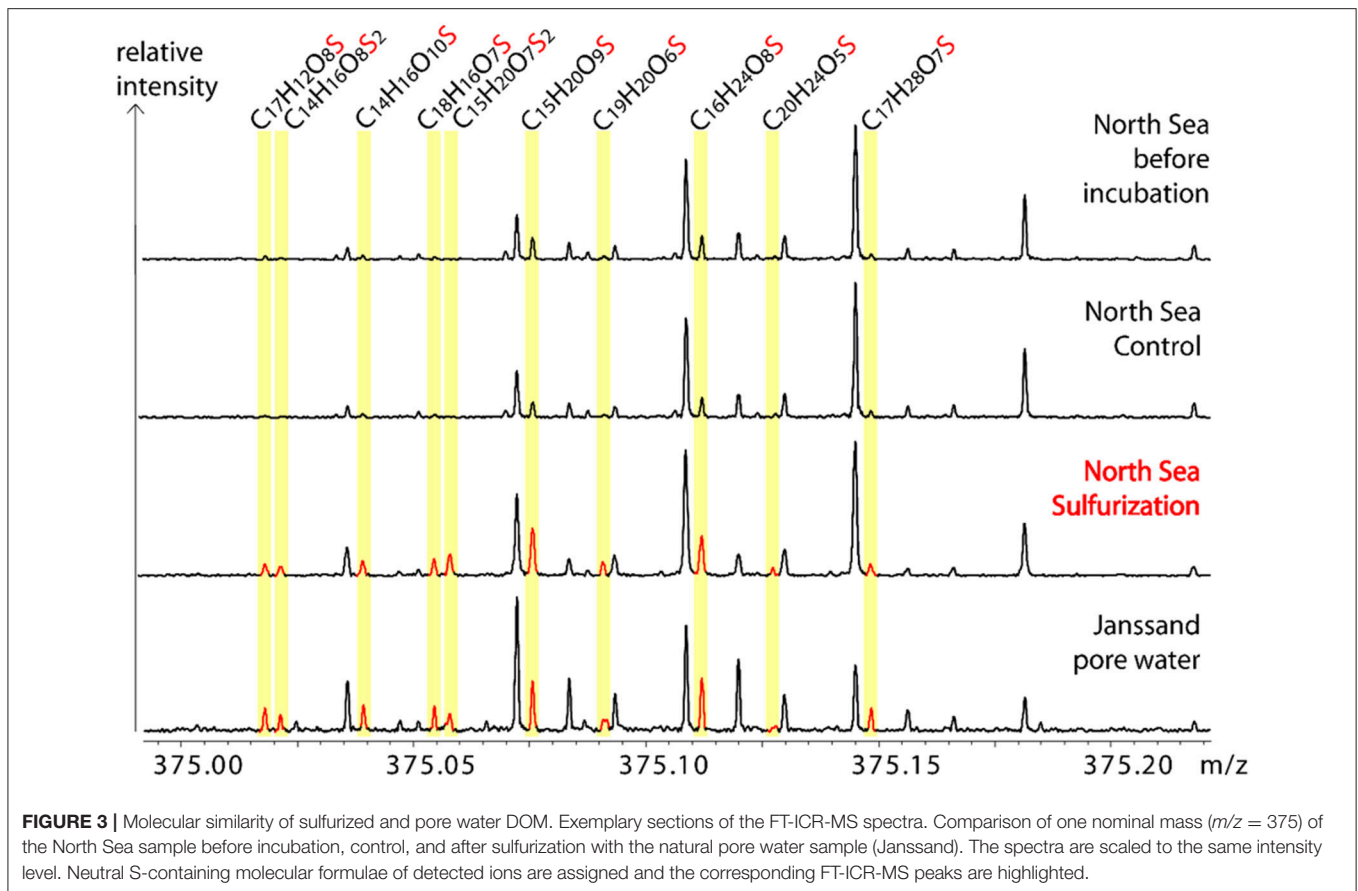


covalent incorporation of inorganic sulfur into organic matter in our sulfurization experiment as the presence of adducts could be ruled out.

In natural sulfidic environments as well as in our experiments, sulfide concentrations are in excess compared to the susceptible organic molecules and reach up to mM levels (e.g., Jansen et al., 2009; Seidel et al., 2014). In our experiments, we chose a higher reaction temperature than in nature to enhance the reaction rate. We did this to simulate the longer residence time of DOM in natural sulfidic environments, that can be up to decades in coastal tidal flats (e.g., Janssand; Røy et al., 2008), in only 4 weeks of incubation. To obtain general information on the speed of the sulfurization reaction of DOM, we incubated a North Sea water sample for only 1-h at 20°C in comparison to the 4 week incubation at 50°C. Even for the short-term sulfurization experiment an increase in bulk DOS concentration by factor 4 was detected (**Table 1**). Also the number of sulfur-containing molecular formulae increased by factor 1.3 (**Figure 2**), and the sulfurization products after 1-h of incubation were molecularly very similar to those after 4 weeks of incubation (**Figure 4C**). Thus, sulfur incorporation into DOM happens fast, indicating that organic matter does not need long residence times in natural sulfidic environments to be affected by sulfurization. A similar timescale was observed by Raven et al. (2016) for the sulfurization of sinking particles.

## Molecular Similarity of Natural and Artificial DOS

Our experimental setup mimicked an aquatic, anoxic, and highly sulfidic environment. The molar DOS/DOC ratios of the sulfurized samples (mesocosms: 0.105, North Sea: 0.095) were higher than in the pore water (0.037) (**Table 1**). This is possibly due to the high sulfide concentration and the stable reaction conditions in the artificial sulfurization experiment. In nature, H<sub>2</sub>S concentrations in pore waters are dynamic (Jansen et al., 2009), and advective pore water transport across redox gradients or sediment reworking may temporarily interrupt sulfurization. Despite these higher reaction yields in our experiments, the artificial and natural DOS were very similar in their molecular composition. Sulfurization led to an almost undistinguishable molecular pattern compared to the pore water (**Figures 3, 4F**). Comprehensive, presence-absence analysis showed an increase in the number of shared DOS compounds of our samples and the pore water due to sulfurization (**Figures 4D,E**). In addition, a statistical Bray Curtis dissimilarity analysis was performed, taking into account also semi-quantitative FT-ICR-MS signal intensities. The sulfurized samples were more similar to the pore water than the controls (Figure S2A) (Bray Curtis dissimilarity indices: pore water vs. mesocosm control: 0.65; pore water vs. mesocosm sulfurized: 0.58; pore water vs. North Sea control: 0.52; pore water vs. North Sea sulfurized: 0.36).



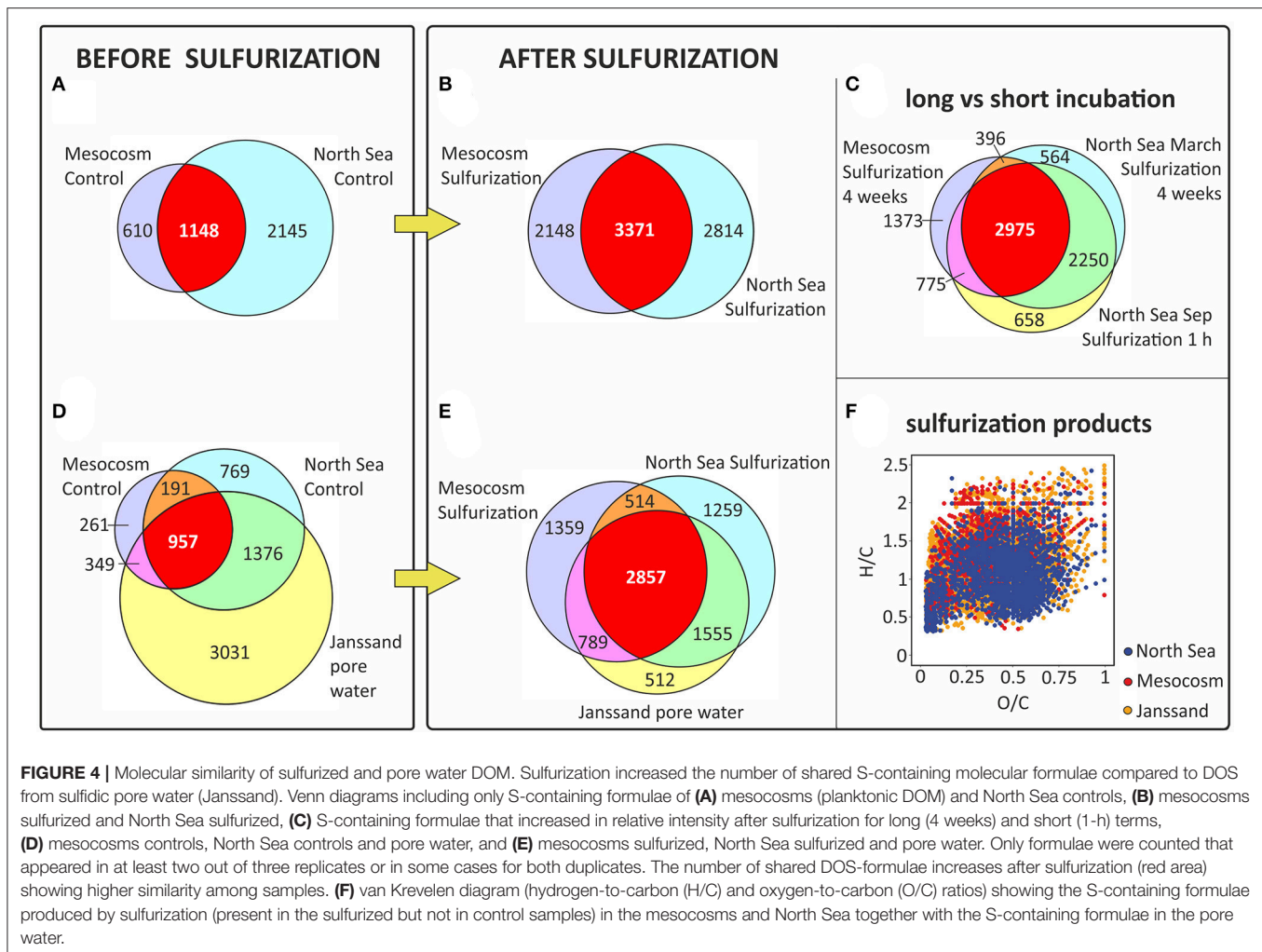
For a comparison on a structural molecular level, we performed extensive analysis of the sulfur-containing functional groups (Pohlabein and Dittmar, 2015). Even on a structural basis, the sulfurized samples and the pore water DOS showed a high level of similarity: sulfonic acids were the predominant functionality in all analyzed experimental and natural samples (Tables S2, S3), and none of the samples contained detectable or only minor amounts of thiols, thioesters, sulfonic acid esters, alkylsulfates, or sulfoxides. The samples differed in the fragmentation experiments by the occurrence of the neutral loss of  $H_2S$ . These  $H_2S$  fragments occurred in all sulfurized and the pore water samples, but not in any of the controls (Table S3). The loss of  $H_2S$  is not indicative for an explicit functional group (Pretsch et al., 2009) but it is an indicator for reduced sulfur compounds (sulfur oxidation state  $\leq 0$ ). This trend is reasonable as reduced inorganic sulfur is incorporated into DOM during sulfurization.

Similar to the statistical comparison on a molecular formula level, we used the molecular fragmentation pattern as structural fingerprints for Bray Curtis dissimilarity analyses. The dissimilarity analysis was done with the FT-ICR-MS signal intensities of those fragments that lost  $SO_3$  and  $H_2SO_3$  (from sulfonic acids) which were normalized to the intensity of the respective precursor-ion. Again, planktonic and North Sea DOS were more similar to the pore water after sulfurization than the controls (Figure S2B). This further confirms the structural

similarity of DOS produced by artificial sulfurization compared to the DOS in natural pore water. The similarity of the artificially sulfurized DOM to the naturally sulfurized pore water supports the authenticity of our experimental setup.

## Potential Reaction Pathways of Sulfur Incorporation

The detailed mechanism of sulfur incorporation in reduced sediments is unknown, but the most discussed possible mechanism for the sulfur incorporation is the Michael addition that would result in thiols (Krein and Aizenshtat, 1994; Movassagh and Shaygan, 2006; Amrani et al., 2007). As thiols were not detected here, it is likely that the thiols which are reactive nucleophiles themselves (Movassagh and Shaygan, 2006; Nair et al., 2014) incur a second Michael addition intra- or intermolecularly to form thioethers. However, thioethers were also not abundant in the sulfurization products which indicates additional reactions after the incorporation of sulfur. Possibly, sulfurized molecules react in an intramolecular fashion producing thiophenes which we are not able to unambiguously detect because thiophenes are too unreactive for selective alteration reactions and do not show characteristic neutral losses in fragmentation experiments. Potentially, this production of thiophenes resembles processes in later diagenesis or even catagenesis of organic material (Sinninghe Damsté and de Leeuw, 1990; Krein and Aizenshtat, 1994; Aizenshtat et al., 1995).



Furthermore, the abundance of the sulfonic acid group and the essential lack of chemically oxidizable sulfur functionalities in all samples indicate almost complete secondary oxidation after sulfurization. Thiols and thioethers are in general unstable under the presence of oxygen (Dupont et al., 2006) and had likely been oxidized prior to analysis. In analogy, reduced sulfur-containing compounds are potentially quickly oxidized once they escape into the oxic open ocean (Gomez-Saez et al., 2016). The main oxidation products are apparently sulfonic acids which we found in all our so far analyzed environments even in the deep sea (Pohlabein and Dittmar, 2015). Sulfonic acids are very stable compounds and it has been suggested that sulfur incorporation preserves organic matter from microbial degradation (Sinninghe Damsté and de Leeuw, 1990; Hansell, 2013).

Another interesting result of our sulfurization experiment was the non-selectivity of sulfur incorporation. Sulfurization occurred irrespective of saturation, aromaticity, degree of oxidation or heteroelement content (e.g., nitrogen) of the precursor compounds (e.g., **Figure 4F**). This makes sulfurization fundamentally different from other abiotic transformations

of DOM like photodegradation (selective toward aromatic compounds, Kujawinski et al., 2004) or adsorption onto iron minerals (selective toward carboxylic-rich aromates, Riedel et al., 2013). The complexity of the sulfurization process became also apparent when analyzing potential precursor-product-relationships among the molecular formulae (Table S1). In general, the same potential reaction patterns were observed between the planktonic and North Sea samples. The most effective sulfurization reactions (precursors for over 80% of DOS formulae) were those exchanging one or two  $H_2$  by a  $H_2S$  molecule (Table S1) which would not represent the Michael addition mechanism. However, we also found strong indication for the Michael pathway ( $+H_2S$ , ~70%, Table S1) and potential reactions supporting the high abundance of the sulfonic acid groups ( $+O_2$  or  $+H_2O$  reactions, ~50–80%, Table S1).

## Global Relevance of Sedimentary DOS Flux

Our experiments showed that natural marine and planktonic DOM is efficiently sulfurized under sulfidic conditions (e.g., Kok et al., 2000). Sulfurized DOM and DOM from sedimentary pore



water were very similar on a molecular formula and molecular structural level (Figures 3, 4). Motivated by these consistent results we attempt a speculative assessment of the potential global relevance of sulfurization in marine sediments. Benthic fluxes of trace elements and DOM from reduced marine sediments are globally significant, and are related to carbon oxidation rates in these sediments. As a result, anaerobic sediments release more DOC to the ocean than more oxidized ones (Burdige and Komada, 2014). Intertidal sediments that are largely covered by salt marshes, mangroves, seagrass and macroalgae deliver 106–416 Tg DOC a<sup>-1</sup> to the global ocean (Maher and Eyre, 2010). This estimates includes large uncertainties and may be an underestimate because it does not include all forms of advective transport through permeable sediments (Santos et al., 2012). Coastal and continental margin sediments deliver an additional 121–233 Tg C a<sup>-1</sup> (Burdige et al., 1999; Burdige and Komada, 2014), and sediments >2,000 m water depth deliver ~100 Tg DOC a<sup>-1</sup> (Dunne et al., 2007; Burdige and Komada, 2014). The latter estimate hinged on the assumption of a constant DOC release of 36% of the total organic carbon reaching the sediments, but this percentage varies widely (from 6 to 32%; Otto and Balzer, 1998). Furthermore, all these estimates are based on a very limited number of studies. Therefore, the uncertainties are inherently large, but taken together, the integrated benthic flux from marine sediments (Burdige and Komada, 2014) may exceed the annual flux of DOC delivered by rivers to the ocean (Hedges et al., 1997). The actual impact these fluxes have on the oceanic carbon cycle ultimately depends on the extent to which sediment-derived DOM is reactive in the water.

To derive DOS fluxes from these estimates, we make two reasonable, yet untested, assumptions. First, we assume that the stoichiometry and efficiency of the sulfurization reaction is globally similar and that the sedimentary DOS compounds are stable over extended time periods. The consistency of our experimental results and field observations is supportive for these assumptions. Second, we assume that benthic fluxes largely originate from sulfidic pore waters, for most intertidal (Dittmar et al., 2006; Roy et al., 2012; Seidel et al., 2014) and marine sediments (Burdige and Komada, 2014).

Based on these two assumptions we estimate the global sedimentary DOS flux by multiplying benthic DOC fluxes with measured DOS/DOC concentration ratios. On the one hand, the minimum DOS flux results from the lower estimate of benthic fluxes (327 Tg C a<sup>-1</sup>, which is the sum of values cited above) and the DOS/DOC ratio in Janssand pore water (0.037; molar ratio, Table 1). To the best of our knowledge, this is the only quantitative number available for S/C ratios in sedimentary DOM and is comparable to the few reported S/C ratios in sedimentary solid organic matter, at the Peru margin (0.038 ± 0.026) (Mossmann et al., 1991), at the Cariaco Basin (0.048 ± 0.011) (Quijada et al., 2016), at the coast of British Columbia

(0.041 ± 0.011) (Francois, 1987), or the Delaware salt marsh (0.038 ± 0.020) (Ferdelman et al., 1991), and the DOS/DOC ratio of sulfidic hydrothermal fluids in Milos Island (0.035 ± 0.029) (Gomez-Saez et al., 2016). On the other hand, our upper flux estimate results from the upper estimate of benthic fluxes (749 Tg C a<sup>-1</sup>, which is the sum of values cited above) and the average DOS/DOC ratio obtained in our sulfurization experiments (planktonic DOM: 0.105, North Sea DOM: 0.095; molar ratios, Table 1). This results in an approximate global benthic DOS flux from marine sediments of 30–200 Tg DOS a<sup>-1</sup>.

The uncertainties in this first speculative assessment are large, mainly due to the lack of global data on benthic DOC fluxes, the element stoichiometry of sedimentary DOM and direct measurements of benthic DOS fluxes. However, this first attempt illustrates that benthic DOS flux is potentially one order of magnitude larger than the riverine organic sulfur input to the ocean (8 Tg S a<sup>-1</sup>, Ksionzek et al., 2016) and may be able to balance the estimated global net removal of refractory DOS (1.1 Tg S a<sup>-1</sup>, Ksionzek et al., 2016). Based on our estimate, we suggest that sulfurization in sulfidic environments is possibly an important source mechanism of refractory DOS to the oceans. This pathway is not considered in current models of the global sulfur cycle (Ksionzek et al., 2016). A significant lack of knowledge still exists with respect to the reactivity and stability of the various DOS fractions in sulfidic and open ocean waters, which should be target of future studies.

## AUTHOR CONTRIBUTIONS

AP and TD conceived the study. AP performed laboratory work. AP, GG-S, BN-O, and TD analyzed data. AP, GG-S, and TD wrote the manuscript with input from BN-O.

## ACKNOWLEDGMENTS

We are grateful to K. Klapproth for support in FT-ICR-MS analyses, B. Schnetger and E. Gründken for ICP-OES analyses, and to M. Friebe and I. Ulber for help with the experiments and DOC measurements. We thank M. Seidel for sharing the Janssand pore water sample, A. Braun for taking North Sea samples, A. Mentges for MATLAB help and M. Wolterink for support in cell counts. We also thank the editor and three reviewers whose comments helped to improve an earlier version of this manuscript. This study was financially supported by the Deutsche Forschungsgemeinschaft (DFG, DI 842/6-1).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2017.00364/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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