Release of toxic sulfur gases from decaying seaweed (Ulva intestinalis and Sargassum muticum) presents a health risk that can be mitigated by freshwater additions

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Article

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Running Title: Health risks associated with seaweed strandings

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Abstract

Seaweed strandings can cover several km^2 with many 1000s of tonnes of biomass. They release malodorous corrosive and toxic volatiles causing respiratory illness and death. The toxic gases hydrogen sulfide and ammonia have been suggested as the main hazardous gases but limited information on the diversity and quantity of volatiles released from decomposing seaweeds is available. Here we use PTR-MS and GC-FPD analyses to quantify sulfur gases released from decaying *Ulva intestinalis* (Chlorophyta) and *Sargassum muticum* (Ochrophyta) in seawater and freshwater under hypoxic conditions for up to 18 d. The sums of sulfur gases in seawater incubations were 13,168 and 6.4 µmol g^{-1} in *U. intestinalis* and *S. muticum*, respectively. Production rates in freshwater were often significantly lower than in seawater, suggesting that freshwater addition to seaweed strandings may mitigate against the release of harmful volatiles. Analysis of the bacterial composition of decaying *U. intestinalis* revealed that seaweed strandings can promote the growth of facultative anaerobic pathogens and these may pose additional health hazards to the public, particularly when transmitted via bioaerosolisation. It is timely to include data on the microbial composition and flux of toxic gases when assessing the substantial health risks associated with seaweed strandings.

Key words: seaweed strandings, health risks, toxic sulfur gases, bacterial pathogens, *Ulva*, *Sargassum*

Introduction

Seaweed nuisance blooms are increasingly frequent and severe [1]. Species of the green seaweed genus *Ulva* (phylum Chlorophyta) and the brown seaweed genus *Sargassum* (phylum Ochrophyta) are recurrently documented in the scientific literature and the wider media for causing 'green tide' and 'golden tide' nuisance blooms, respectively [1-3]. Primarily, their nuisance stems from 'beachings' or 'strandings' of large amounts of biomass over short periods (days to weeks) that can smother coastal environments, deter tourism, affect well-being and use of natural spaces (recreation and fisheries) [4,5].

Although seaweeds are typically not harmful to humans, microbial activity results in the decay of seaweed biomass into an unpleasant mass that releases bad-smelling, corrosive and toxic gases which can affect people and infrastructure near the sea. It is likely that the increased microbial degradation leads to oxygen drawdown (hypoxia or anoxia) in the centre of the strandings and this stimulates the release of gases including methane (CH_4), ammonia (NH_3) and hydrogen sulfide (H_2S). Some of these gases are corrosive, can damage electronic equipment including phone and power-supply networks, or be suffocating or toxic to various organisms including humans [5]. Tragically, two persons and several animals died in separate incidents after inhaling toxic gases from decaying seaweed along beaches of the French coast [6]. Often, citizens suffer eye irritation and respiratory illness and are at higher risk of pregnancy problems during periods of seaweed strandings [7-9]. In the case of *Sargassum* golden tides in the Caribbean, adverse health effects and toxicity relating to H_2S exposure is documented in 1000s of patients [8,9]. Medical practitioners are now warning of the increasing cases of gas poisoning from golden tides in the Caribbean and advocate the closure of affected areas followed by costly removal of seaweed within two days of mass beachings [8,9]. This can be logistically and financially challenging: For example, one of the largest seaweed strandings of *Ulva prolifera* occurred during the Chinese Olympic games in Qingdao in 2008 that covered 15 km^2 with 700,000 tonnes of biomass that had to be removed from coastal environments involving >10,000 workers at a cost exceeding US$100 million [10].

In contrast to such adverse effects, algal biomass can also be utilised as a valuable resource to feed into biotechnological processing, for example to produce sustainable sources of energy (e.g. ethanol, butanol, methane) [11]. Additionally, seaweed blooms can form part of mitigation strategies to avert a climate crisis by increasing the flux of climate-cooling gases such as dimethyl sulfide (DMS) [12] or the capability of the ocean to capture CO_2 [13]. Together,

78 this has led to suggestions of a summer seaweed fishery [1] that may provide ecological and
79 economic benefits to coastal societies and beyond.
80
81 Aquaculture practices likely exacerbate nuisance blooms. The well-documented 2008 *Ulva*
82 green tide during the Chinese Olympic Games was stimulated by extensive long-line
83 mariculture of red seaweeds (*Porphyra yezoensis*) which requires the manual removal of
84 undesired *Ulva* spp. attached to the long-lines to increase the harvest of valuable crop [14].
85 *Ulva* and *Sargassum* are seaweeds that readily grow in the unattached, free-floating
86 (planktonic) form, successfully outcompeting the typically dominating microscopic
87 phytoplankton for resources including light and nutrients. In many cases, such nuisance
88 blooms are the result of eutrophication leading to excessive growth under suitable
89 conditions [15,16]. Although point-source additions of excessive nutrients to coastal waters
90 were identified as the main cause of nuisance blooms in the 1970s and 1980s, this paradigm
91 appears to be shifting with more diffuse sources of nutrients recognised for driving an
92 imbalance of the abundance, relative proportions or chemical forms of nitrogen and
93 phosphorous that support growth of selected opportunistic species [17]. Since diffuse additions
94 of nutrients are more difficult to manage at the point of source, the challenges associated
95 with these blooms are likely going to persist and possibly increase in the foreseeable future.
96
97 Hazardous pathogens typically present in coastal waters near urban sites may be enriched
98 in microbial biofilms that establish on seaweed, pose a threat to human health, marine biota
99 and aquaculture activities, and negatively impact coastal seawater quality. Coasts are high-
100 energy environments with conditions such as wave action and winds facilitating the release
101 of airborne pathogens (i.e. creating bioaerosols). As these conditions are also associated
102 with seaweed strandings, and decomposing seaweed promotes a relatively high biomass of
103 bacteria, it is likely that decomposing seaweed is a significant source of potentially harmful
104 bioaerosols in marine habitats. Of particular concern are so called 'ESKAPE pathogens' that
105 comprise of six highly virulent and antibiotic-resistant bacterial pathogens (*Enterococcus*
106 *faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*,
107 *Pseudomonas aeruginosa*, and *Enterobacter* spp.). Although *S. aureus* has been isolated
108 from public marine beaches, information on the persistence and subsequent health risks
109 posed by the ESKAPE isolates in environmental settings is very limited [18]. Apart from
110 *P. aeruginosa*, ESKAPE pathogens are facultative anaerobes. Hence, it is likely that they
111 would be able to reproduce within seaweed strandings and that this promotes the
112 transmission of infectious diseases. Of additional concern are eukaryotic pathogens such as
113 *Ostreopsis* spp., marine epibenthic dinoflagellates which produce highly toxic palytoxins and

ovatoxins that are recognised as vasoconstrictors and triggers of a range of respiratory and skin irritations after bioaerosolisation [19,20].

The aim of our study was to assess the diversity of sulfur gases in two species of seaweed that are abundant in the hypernutrified River Colne, a well-studied model estuary in the southeast of the United Kingdom [21]. We hypothesised that the relative abundance of various sulfur gases is species-specific and our primary objective was to use short-term laboratory microcosm incubations (18 d) to quantitatively compare the diversity of sulfur gases released by decaying *Ulva intestinalis* (phylum Chlorophyta), and the brown seaweed *Sargassum muticum* (phylum Ochrophyta).

Little information is available on their nutritional composition and sulfur content but seaweeds from the Hawaiian Islands have a higher total sulfur content in *Ulva* (*Enteromorpha*) *flexuosa* and *U. fasciata* (3.20 to 5.51 % dryweight) than *Sargassum echinocarpum* and *S. obtusifolium* (1.16 and 1.41 % dryweight)[22]. *S. natans* and *S. fluitans* from the Turks and Caicos Islands have a lower sulfur content of <0.4% dryweight but season and storage conditions are known to affect the sulfur concentration in *Sargassum* [23]. Further research shows that *Ulva* spp. accumulate relatively high intracellular concentrations of the compatible solute dimethylsulfoniopropionate (DMSP), a direct precursor of volatile dimethyl sulfide (DMS), that range from 7-128 µmol g^{-1} freshweight, whereas Ochrophytes including *Sargassum polycystum* contain less than 1 µmol DMSP g^{-1} freshweight [24,25]. Taken together, this suggests that *U. intestinalis* and *S. muticum* investigated in this study may show different potentials for the release of DMS and other sulfur volatiles. An additional objective was to assess whether a salinity change, for example from rainfall or freshwater pumped onto stranded biomass, would significantly affect the production of sulfur gases and, hence, we quantified the effect of freshwater additions. Whereas seawater contains 28 mM sulfate (SO_4^{2-}), rivers, lakes and groundwater have up to 100 times less SO_4^{2-} [26]. This difference affects the microbial production of H_2S by sulfate-reducing microbes (bacteria and archaea) and freshwater addition may mitigate against microbial H_2S in seaweed strandings. Since microbial activity is likely the main driver of the production of sulfur gases, we also explored the microbial community composition in one experiment with *U. intestinalis* under seawater and freshwater conditions. Our findings provide first insight into the diversity of sulfur gases and bacteria in decaying seaweed and the effect of salinity change and suggest that toxicological assessments of nuisance blooms should include microbial pathogens and potentially harmful gases beyond the typically reported and acutely toxic H_2S.

Materials and Methods

Three types of experiments were conducted with decaying biomass of *Ulva intestinalis* and *Sargassum muticum*: Experiment 1 (7 July 2023) was an exploratory study using proton-transfer reaction mass spectrometry (PTR-MS) to qualitatively examine the diversity of sulfur-containing and other volatiles, and Experiment 2 (*U. intestinalis*: 2-20 October 2023; *S. muticum*: 19 February-8 March 2024) used gas chromatography with flame-photometric detection (GC-FPD) to quantify a selection of predominant sulfur gases. Experiment 3 quantified the community composition of bacteria in seawater, freshwater and *U. intestinalis* after a 5 d incubation (3-8 October 2024).

Sample collection. Seaweeds were collected from the foreshore (*U. intestinalis*) or submerged structures of a floating pontoon (*S. muticum*) in Brightlingsea, Essex. Seawater was collected from the incoming tide and freshwater was collected from Upper Campus Lake, University of Essex. Samples were transported in a cooler box to the laboratory at the University of Essex within 1h after collection and kept in a cold room (4 °C) for <24h until sample preparation.

Sample preparation. A staggered approach was used to prepare samples for the analysis of volatiles. PTR-MS samples were generated 1, 2, 4, and 8 days before analysis whereas GC-FPD samples were set-up approximately every 30 min on the day of first measurements to account for the time taken for gas-chromatographic quantification that followed on days 1, 2, 4, 7, 10, 14 and 18. Seaweeds were centrifuged with 10 ‘push-spins’ using a salad spinner to remove excessive liquid. Obvious epibionts (e.g. amphipods) were removed before an aliquot of seaweed was washed for approximately 5 s in freshly collected seawater (seawater treatment) or freshwater (freshwater treatment). This was followed by removal of excess water via a second salad-spinner centrifugation. Seaweed biomass was weighed in triplicates to approximately 100 mg (Experiment 1) or 250 mg (Experiment 2; *U. intestinalis* 250 ± 13.6 mg; *S. muticum* 255 ± 35.8 mg using a mixture of biomass from blades, stipe and air bladders) and transferred into a crimp-seal vial of 20 mL volume that was closed gas-tightly with a Teflon-coated butyl rubber septum (Pharma-Fix, Merck Life Science UK Ltd). Addition of 200 µL seawater or lake water ensured sufficient humidity and the desired salinity environments. Triplicate vials without seaweed addition were used as negative controls.

Quantification of intracellular dimethylsulfoniopropionate (DMSP). Following the method described in Kerrison, et al. [27], algal thalli free from obvious epibionts were haphazardously selected on the day of sample collection (approximately 100 mg each; n = 5), transferred into a sample vial (volume 4.92 mL) that contained 3 mL 0.5 M NaOH before immediate gas-tight closure with a Teflon-coated silicone septum. After alkaline hydrolysis of DMSP to DMS at 26 °C for 5 d, headspace was injected directly into the gas chromatograph for quantification. Peak areas were calibrated with a series of known DMSP concentrations [27].

Proton-transfer reaction mass spectrometry. Sample vials were transported and stored in a cool box until analysis at the PTR-MS laboratory (<10h). The temperature range during transport and storage was 24.9 to 25.6 °C with a mean temperature of 25.3 ± 0.21 °C (HOBO MX2202, Onset, Bourne, MA, USA). The instrument used for the PTR-MS analyses was a prototype from which a commercial model (PTR3c, Kore Technology Ltd, Ely, UK) was developed. It uses a time-of-flight (ToF) mass spectrometer with a mass resolution of 5,500 M/δM (Full Width at Half Maximum) and a sensitivity of ~1000 counts s^{-1} ppb^{-1} of analyte. The reagent ion used was hydronium (H_3O^+). Depending on analyte concentration, 100 or 25 µL headspace were injected with a gas-tight syringe (SGE Trajan, Milton Keynes, UK) through a septum into a 100 sccm flow of nitrogen gas going to the reactor. The reactor was operated at a room temperature of 25°C and held at a constant pressure of 1.2 mbar throughout. Data from control treatments (excluding seaweed) with either seawater or freshwater were acquired before collecting data for treatments including seaweed.

Gas chromatography. Repeated gas measurements in the headspace of the vial were conducted for 18 d following the methods described in Franchini and Steinke [28]. Depending on the concentration of measured gases, 5, 10 or 200 µL (*U. intestinalis*) or 200 µL (*S. muticum*) of vial headspace was directly injected into a gas chromatograph (Shimadzu GC 2010, Milton Keynes, UK) using gas-tight syringes (SGE Trajan, Milton Keynes, UK). The GC was equipped with a flame-photometric detector and a 60 m × 0.53 mm × 5 µm analytical column with a nonpolar phase of cross-bond dimethyl polysiloxane (RTX-1; Thames Restek UK Ltd., Saunderton, UK). Injector and detector temperatures were set to 200 and 250 °C, respectively. This instrument configuration is capable of quantifying sulfur gases including, amongst others, hydrogen sulfide (H_2S), carbonyl sulfide (COS), methane thiol (MeSH), dimethyl sulfide (DMS) and dimethyl disulfide (DMDS; Supplementary Table S1, Figure 1). Depending on the concentration of sulfur gases, two injections using different oven-temperature programmes were used. The initial injection (Method 1: 200 µL splitless injection) enabled the separation of H_2S and COS, and quantification of low-concentrated

volatiles whereas a second injection (Method 2: 5 or 10 µL injections with split of 1:10) was used to quantify the often highly abundant H_2S and DMS that sometimes resulted in saturation of the detector when using Method 1. The concentration of COS quantified with Method 1 was subtracted from the concentration calculated from the area of the co-eluting COS and H_2S peak when using Method 2. The oven-temperature programme for Method 1 was 40 °C for 5 min, then increased to 120 ºC at 15 °C min^{-1} and held for further 20 min (total programme time was ~36 min). The oven-temperature for Method 2 and for the quantification of DMSP was isothermal at 120 °C.

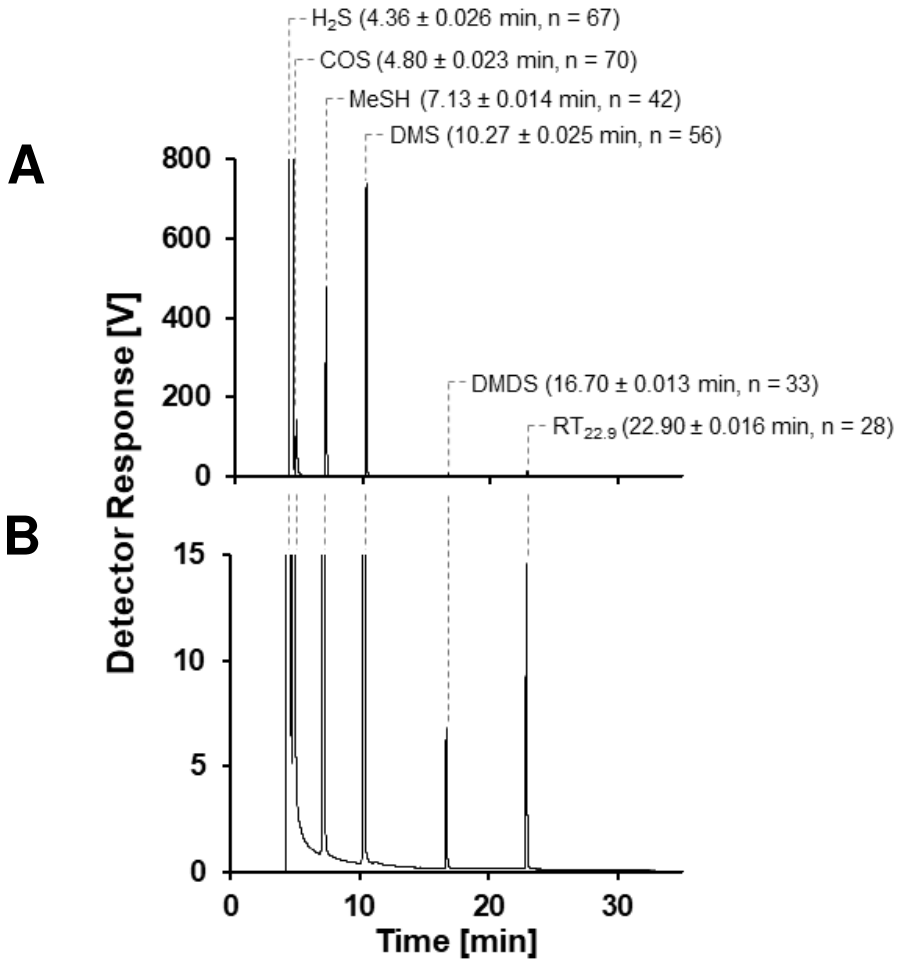


Figure 1. Example chromatogram for day 14 of the seawater incubations with *Ulva intestinalis*. Panels A and B scaled to a detector response of 0-800 V and 0-15 V, respectively. Dashed lines indicate peak identities with retention times and sample sizes shown in brackets.

Supplementary Table S1. Characteristics of sulfur gases quantified in this study. Shown are their names (with IUPAC name in brackets), chemical formula, odour description and threshold, and hazard and workplace exposure-limit information. TWA = long-term exposure limit (8 h time-weighted average reference period); STEL = short-term exposure limit (15 min reference period); ACGIH = American Conference of Governmental Industrial Hygienists; TLV = Threshold Limit Values

					Exposure Limits				
					TWA		STEL		
Name	**Chemical formula**	**Odour description***	**Odour threshold**	**Relevant Principle Hazards**	**[ppm]**	**[mg/m^3]**	**[ppm]**	**[mg/m^3]**	**Reference**
Hydrogen sulfide (sulfane)	H_2S	Strong odour of rotten eggs	4.7 ppb*	Acute toxicity, serious health hazard, hazardous to the environment 	5	7	10	14	Indicative Exposure Limit Values in Directives 91/322/EEC, 2000/39/EC, 2006/15/EC, 2009/161/EU (12 2009)
Carbonyl sulfide (oxidosulfidocarbon)	COS	Typical sulfide odour except when pure	100 ppb†	Acute toxicity 	23	56	69	170	EPA Acute Exposure Guideline Levels (AEGL 2)
Methane thiol (methanethiol)	CH_3SH	Pungent, decayed cabbage	2.1 ppb*	Serious health hazard, hazardous to the environment 	0.5	1	1.5‡	3‡	UK. EH40 WEL – Workplace Exposure Limits
Dimethyl sulfide (methylsulfanylmethane)	$(CH_3)_2S$	Unpleasant odour of wild radish, cabbage-like	~1 ppb*		10		30‡		ACGIH TLV (United States, 3/2012)
Dimethyl disulfide ((methyldisulfanyl)methane)	$C_2H_6S_2$	Sulfurous, garlic-like	0.029 mg/m^3 *	Acute toxicity, hazardous to the environment 	0.5		1.5‡		ACGIH TLV (United States, 3/2016)

* Data from https://pubchem.ncbi.nlm.nih.gov/; recognition in air in units of mg/m^3 or ppb

† Data from https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=40610

‡ Where no specific short-term exposure limit is available, a figure three times the long-term exposure should be used.

Incubations. Using hypodermic needles connected to incoming and outgoing flow lines, each vial was flushed with nitrogen gas shortly after sample preparation (PTR-MS: 200 mL min^{-1} for 2 min) or immediately after GC measurements of headspace gases (GC-FPD: 80 mL min^{-1} for 2 min). This produced a low-oxygen environment inside the vials that mimicked anaerobic conditions typical for the centre of decaying seaweed strandings. Samples were incubated in the dark (Plant-Growth Chamber MLR-351, Sanyo Gallenkamp, Loughborough, UK) at a temperature of at 26.0 ± 0.04 °C that was monitored using temperature data loggers (HOBO MX2202, Onset, Bourne, MA, USA).

Instrument calibrations, data and statistical analyses. The PTR-MS technique is particularly versatile for real-time analysis, and so for each sample, the data acquisition was begun and the evolution of volatiles of interest was plotted in real time during the gas injections. Afterwards, a mass spectrum was extracted from each data set, usually between 10 to 20 s in duration, according to the appearance of the volatile profiles. Data plots comprise total integrated ion signals of the analytes of interest for any given sample injection. The mean abundance of sulfur volatiles in the controls without seaweed addition (n = 3) were subtracted from treatments with seaweed.

Gas chromatographic data were collected and peak areas integrated using instrument-specific software (GCsolution version 2.10.00 SU1; Shimadzu, Milton Keynes, UK). Peak areas were square-rooted to linearise the detector response. Limits of detection and quantification (LOD and LOQ) were approximately 0.5 and 1.5 pmol sulfur, respectively [28]. The mean of the square-rooted peak areas of sulfur volatiles in the controls without seaweed addition (n = 3) were subtracted from treatments with seaweed before sulfur concentrations were calculated and normalised to seaweed freshweight (SFW) biomass.

The GC-FPD was calibrated with H_2S, COS, DMS and DMDS standard solutions (200 µL) in 20 mL vials. H_2S standard solution was freshly prepared by dissolving sodium sulfite (752 µM Na_2S in milliQ water; Arcos Organics/Thermo Scientific Chemicals, Loughborough, UK) and adding 0, 1, 2, 5, 8 and 11 µL of this to 200 µL of N_2-purged HCl (32%) followed by immediate gas-tight closure. Reaction of Na_2S with HCl inside the vial resulted in the release of equimolar quantities of H_2S. COS standard solution was freshly prepared by dissolving sodium-thiocyanate (740 µM NaSCN in milliQ water; Arcos Organics/Thermo Scientific Chemicals, Loughborough, UK) and adding 0, 2, 5, 8, and 11 µL of this to 200 µL of N_2-purged H_2SO_4 (50%) followed by immediate gas-tight closure and heating to 40 °C for 30 min. Reaction of NaSCN with H_2SO_4 inside the vial resulted in the release of equimolar quantities of COS. DMSP standard solution (75 mM; synthesised from DMS and acrylate as

described in Chambers et al. [29]) was prepared in acidified milliQ water and serially diluted to 75 and 750 µM before using volumes of 0, 5 and 10 µL to add 0, 1.8, 3.6, 5.2, 18.3, 35.7 and 52.5 µM DMSP to 200 µL of NaOH (0.5 M) followed by immediate gas-tight closure. Alkaline hydrolysis of DMSP with NaOH inside the vial resulted in the release of equimolar quantities of DMS [28]. DMDS standard solution (750 µM) was prepared by adding 6.6 µL DMDS (Sigma Aldrich/Merck, Gillingham, UK) to 100 mL milliQ water using a gas tight syringe. Volumes of 0, 2, 5, 8 and 11 µL of this were added to 200 µL artificial seawater followed by immediate gas-tight closure. Vials with standard solutions were vortexed for 20 s, incubated for >1h at 26 °C and vortexed again (20 s) before injection of 200 µL headspace gas for gas chromatographic analysis.

Identification of gas-chromatographic peaks was by retention time (Figure 1) and instrument calibrations provided linear regression data presented in Supplementary Table S2. The retention time of MeSH was verified by injecting headspace of DMDS (Sigma Aldrich/Merck, Gillingham, UK) that contained COS, MeSH and DMS as contaminants. Pooled data from all calibrations were used to quantify methanethiol and an unidentified sulfur compound that eluted at 22.9 min (referred to as $RT_{22.9}$).

Supplementary Table S2. Linear regression data for calibrations with various sulfur gases. Note that DMDS contains two sulfur atoms and data presented here refer to sulfur concentrations.

Compound	Slope	y-intercept	R^2	n
H_2S	223	-156.4	0.9968	12
COS	191	-42.2	0.9983	5
DMS	256	-36.6	0.9998	7
DMDS	266	-307.5	0.9920	5
All	237	-195.0	0.9731	29

Gas chromatographic data were processed and visualised in Microsoft Excel version 2407. Statistical analyses using a Kruskal-Wallace test were dissatisfactory due to the often large differences in data variance between the seawater and freshwater treatments. A statistical analysis using Linear Mixed-Effects Models (family = Poisson) with volatile concentration as the dependent variable and treatment and time as the independent variables proved more successful and were conducted in R-Studio using the R-package '*lme4*' (R version 4.3.3; RStudio 2024.04.2).

Composition of Bacterial Community. To characterise the *Ulva intestinalis* holobiont, 250 mg of seaweed was added to a 1.5 mL microcentrifuge tube with 500 µL of ultrapure sterile water. To remove the bacteria from the surface of the seaweed, the samples where sonicated for 30 min and then vortexed at maximum speed using a Vortex-Genie 2 adapter (Qiagen, UK) for 20 min. Then 250 µL of the liquid was transferred to a PowerBead Pro Tube and bacterial DNA was isolated and purified using a DNeasy PowerSoil Pro Kit (Qiagen, UK) according to the manufacturer's instructions, with the following modification: As there was high level of PCR inhibition in the samples, the wash steps were repeated twice, and the samples were further processed with a DNeasy PowerClean Pro Cleanup Kit (Qiagen, UK) according to the manufacturer's instructions. Amplicon sequencing of the full bacterial 16S rRNA genes present in each sample was then completed on MinION (Mk1B, Oxford Nanopore, UK) using the 16S Barcoding Kit 1-24 (SQK-16S024) and a MinION Flow Cell (R10.4.1) according to the manufacturer's instructions. The run time parameters were 72 h run time, with 1.5 h pore-scan frequency, minimum read length of 200, fast model basecalling, using MinKNOW version 24.02.8, Bream 7.9.8, Dorado 7.3.11, MinKNOW Core 5.9.12, yielding a median read length of 1,547 and mean Q-score of 8.5. The basecalled data were then processed with the wf-16s Nextflow (https://github.com/epi2me-labs/wf-16s) workflow on EPI2ME (https://github.com/epi2me-labs) to identify the bacterial amplicons present in each sample with the following parameters: min Q-score of 10, a minimum and maximum read length of 800 and 2000 respectively using the Kraken2 [30] classifier to species level on the NCBI 16/18s database [31]. All statistical analyses were carried out in R 4.2.2 'Innocent and Trusting' [32]. Data were normalised to an even read-depth according to the recommendations of Weiss et al. [33] and McKnight et al. [34]. To test for taxa that were differentially abundant between fresh and seawater incubations, we used multivariate generalised linear models (with a negative binomial error term to account for overdispersion) to explore changes in community composition [35]. Multivariate and univariate p values were obtained by log-likelihood ratio tests and model-free bootstrapping with 10,000 permutations of probability integral transform residuals. The figures were prepared with the R package 'ggplot2' [36], the sequence data were processed with the R package 'Phyloseq' [37], and multivariate generalised linear models were fitted with the R package 'Mvabund' [35].

Assessment of resistant bacterial pathogens in the seaweed holobiont. To determine the presence of extended beta-lactamase (ESBL)-producing bacteria (beta-lactam resistance) and vancomycin-resistant *Enterococcus* (VRE) in the seaweed holobiont, 50 µL of the supernatant from the previous step (before DNA extraction) was spread on VRE ChromoSelect Agar (Merck UK) and incubated at 37ºC for 48 h, with blue or blue-green colonies indicating *E. faecalis*; and a second 50 µL was spread on ESBL ChromoSelect

347 Agar Base (Merck UK) and incubated at 37ºC 48 h, with purple or pink colonies indicating
348 *E. coli*, green colonies members of the *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter*
349 spp. (KESC group), and colourless to brown colonies *Proteus*, *Morganella* and *Providencia*
350 spp. Any negative plates where incubated for a further 2 h hours to confirm lack of growth.

Results

DMSP concentrations

DMSP concentration was approximately 1000-fold higher in *U. intestinalis* (mean ± standard deviation = 15.5 ± 2.65 µmol g^{-1} SFW; n = 4) than in *S. muticum* (17.9 ± 15.44 nmol g^{-1} SFW; n = 5) where the standard deviation around the mean was substantially larger.

PTR-MS qualitative assessment (Experiment 1)

PTR-MS analysis revealed four dominant volatiles (H_2S, COS, Me-thiol and DMS) produced at various rates over an 8 d incubation (Figure 2). The analysis indicated the presence of thioformic acid (O=CH-SH) but DMDS or ammonia (NH_3) were not detected.

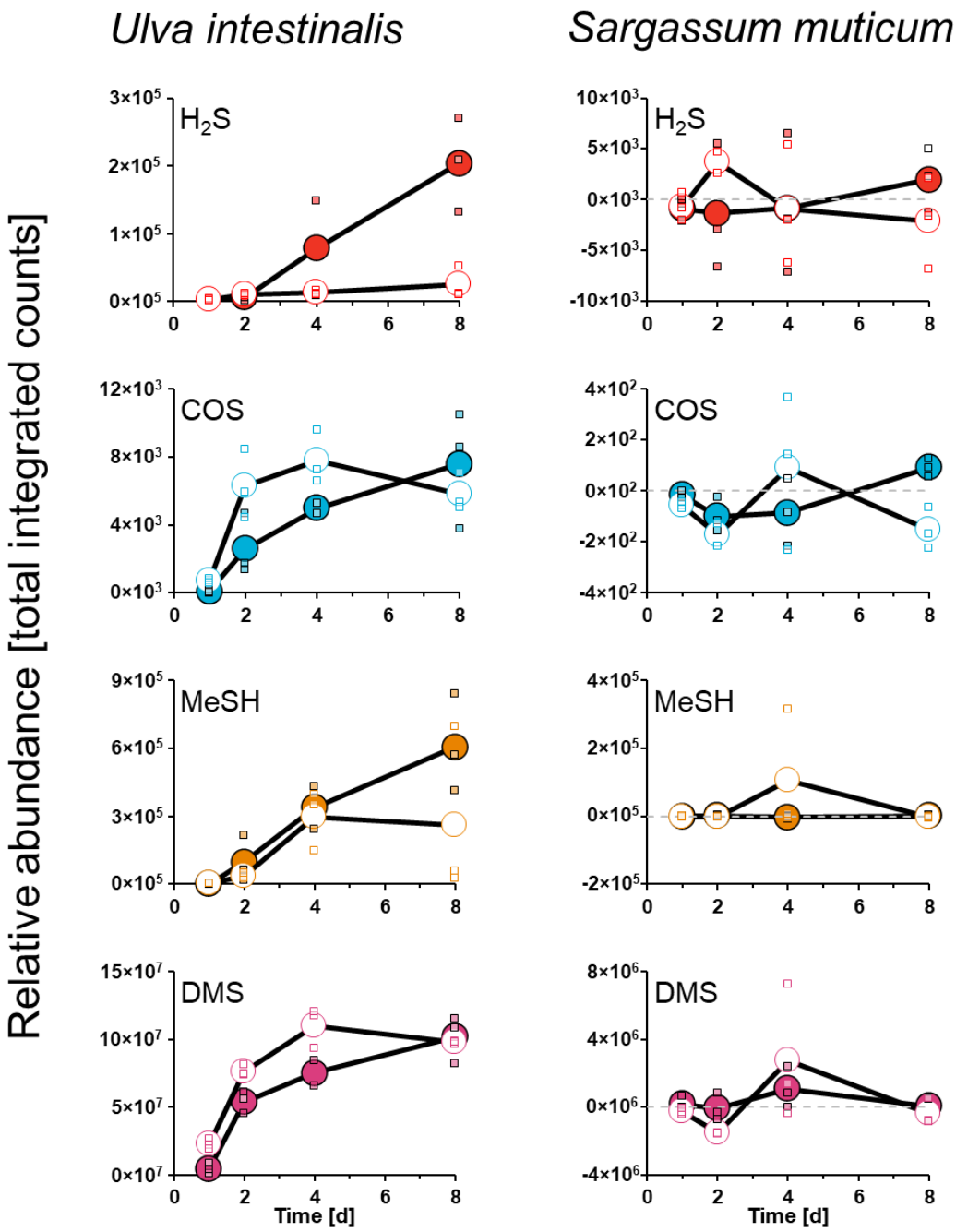


Figure 2. PTR-MS qualitative assessment of sulfur gases produced by decaying seaweed. hypoxic incubations with *Ulva intes*tinalis (left) and *Sargassum muticum* (right) at 26 °C for 8 d. Shown are mean relative abundances after incubation in seawater (SW; closed circles) or freshwater (FW; open circles) with biological triplicates presented using squared symbols (except n = 2 for seawater incubation with *U. intestinalis* on day 4 due to failure of gas-tight seal in one of the vials, and freshwater incubation with *S. muticum* on day 2 due to file corruption). Please note variable scaling along the y-axes with dashed line in *S. muticum* panels indicating zero.

Gas chromatographic quantification of sulfur volatiles (Experiment 2)

A total of 13 different sulfur volatiles produced peak areas above the LOD at different maximum concentrations and depending on the incubation conditions. Of these, six produced peak areas above the LOQ, hence, were dominating the total release of sulfur with five identified by their retention times as: H_2S, COS, MeSH, DMS, and DMDS. Additionally, one unidentified sulfur compound ($RT_{22.9}$) eluted 22.9 min into the chromatography run in the incubations with *U. intestinalis* (Supplementary Figure S1).

In *U. intestinalis*, the order of maximum mean sulfur concentrations (highest to lowest = 3982 to 4.3 µmol g^{-1} SFW) were H_2S > DMS > MeSH > COS > DMDS > $RT_{22.9}$ and samples from freshwater treatments sometimes showed less H_2S, COS and MeSH in comparison to the samples from seawater treatments (Supplementary Figure S1). It is important to note that DMDS contains two sulfur atoms per molecule and the number of sulfur atoms for $RT_{22.9}$ are unknown, hence, the concentration of DMDS can be calculated as half of the DMDS sulfur concentration but cannot be estimated for $RT_{22.9}$. Mean sulfur concentrations in samples with *S. muticum* were substantially lower than samples from *U. intestinalis* and showed an order of DMS > MeSH > H_2S > COS > DMDS (range = 3.8 to 0.1 µmol g^{-1} SFW). There was no clear difference between the seawater and freshwater treatments with *S. muticum*.

Sums of volatiles released. Since each concentration measurement was followed by the removal of the sample headspace and exchange with nitrogen gas to promote hypoxic conditions, we also calculated the sum of sulfur volatiles released over the incubation period to facilitate comparison with PTR-MS data (Figures 2 and 3). This confirmed that the release of sulfur volatiles was species specific and often quasi-linear for a few days after starting the incubation with the total sum of sulfur volatiles in seawater and freshwater treatments of 13,168 and 2,778 µmol g^{-1} SFW in *U. intestinalis* and 6.4 and 6.0 µmol g^{-1} SFW in *S. muticum*, respectively. Furthermore, the summed data demonstrated that treatment with FW significantly decreased the release of H_2S, COS and MeSH in *U. intestinalis* (General Linear-Effect Model; $P<0.001$) and DMS in *S. muticum* ($P<0.01$). In contrast to this, the FW treatment increased the release of MeSH in *S. muticum* and DMDS in both species (significant difference for *S. muticum*; $P<0.001$).

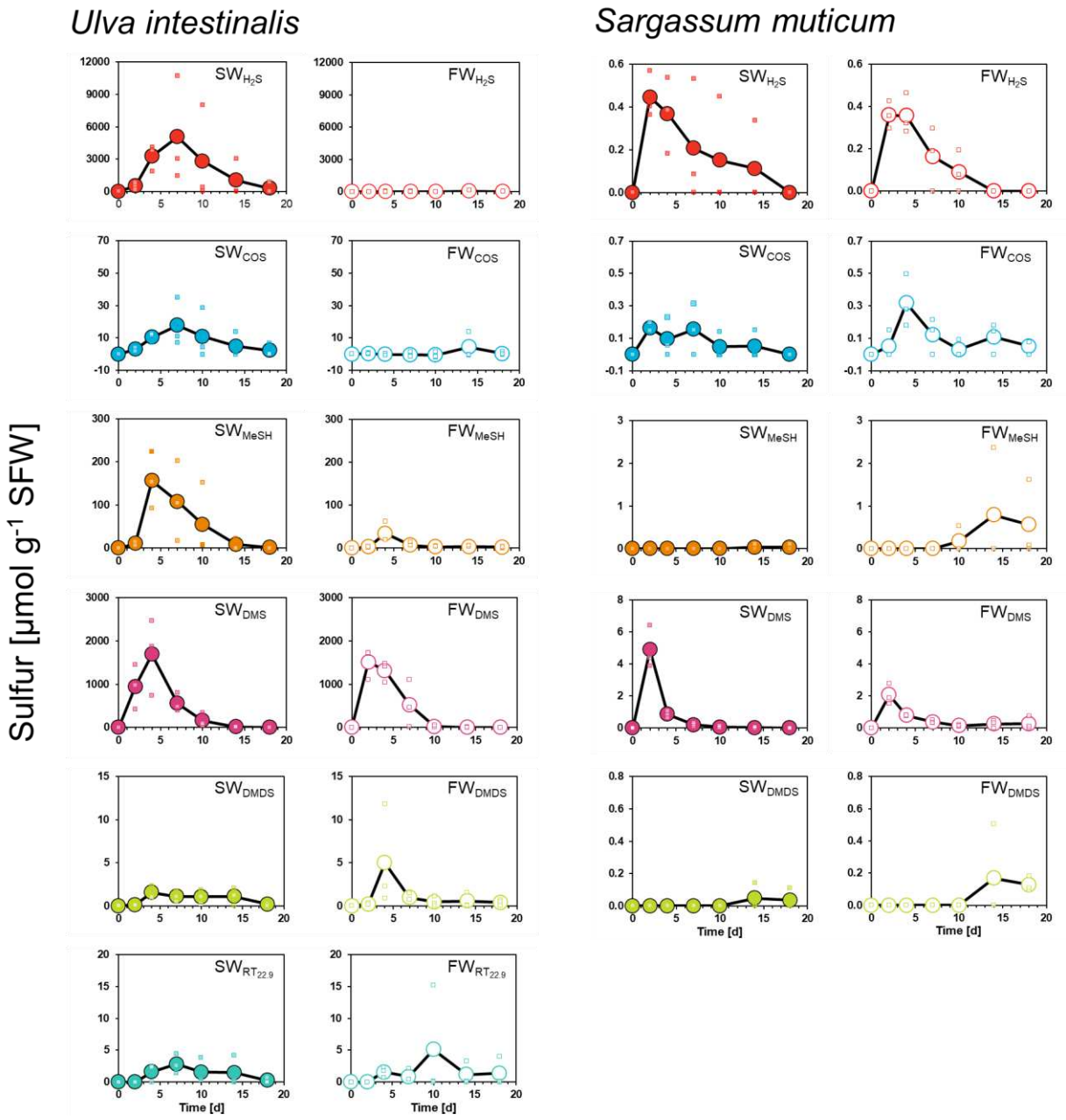


Figure S1. Gas-chromatographic quantification of volatile sulfur released from decaying seaweed. Hypoxic incubations with *Ulva intestinalis* (left) and *Sargassum muticum* (right) at 26 °C for 18 d. Shown are mean values after incubation in seawater (SW; closed circles) or freshwater (FW; open circles). Data were normalised to seaweed freshweight (SFW) biomass with biological triplicates presented using squared symbols ($RT_{22.9}$ = unidentified sulfur volatile). Please note variable scaling along the y-axes.

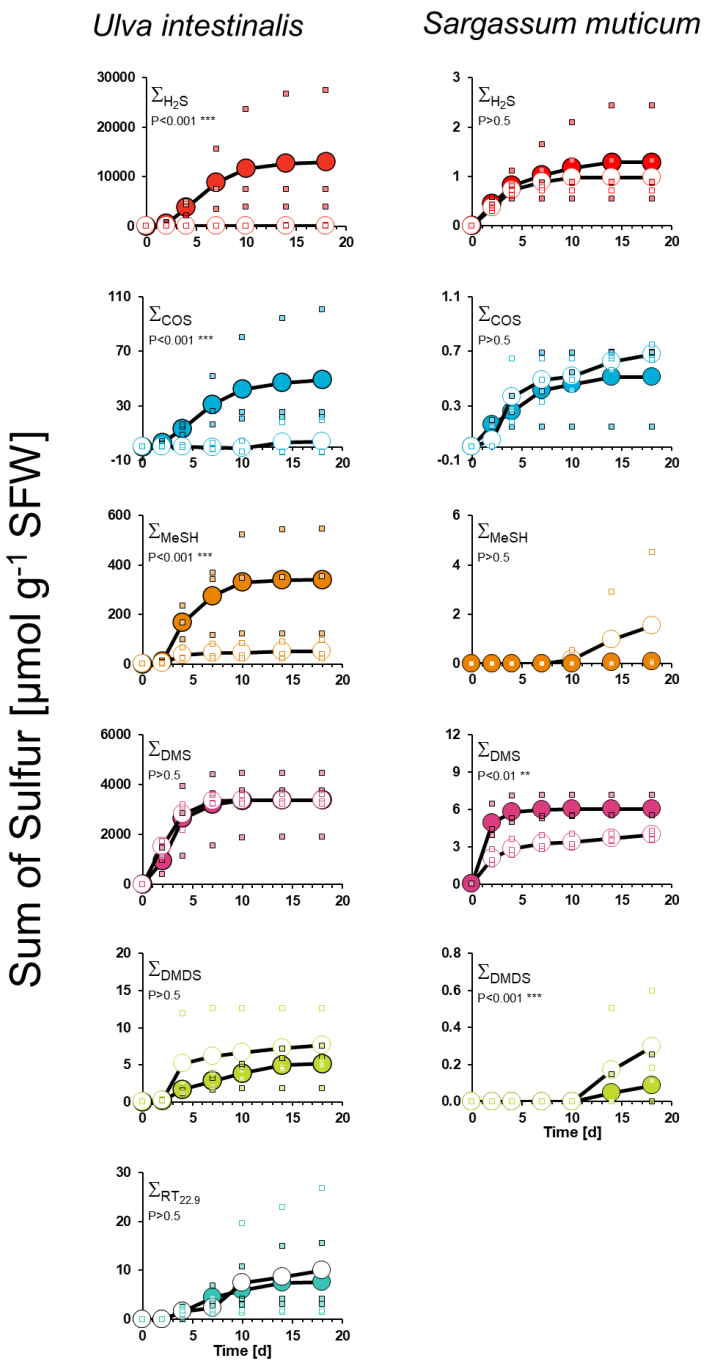


Figure 3. Sum of volatile sulfur released from decaying seaweed. Hypoxic incubations with *Ulva intestinalis* (left) and *Sargassum muticum* (right) at 26 °C for 18 d. Shown are mean values after incubation in seawater (SW; closed circles) or freshwater (FW; open circles). Data were normalised to seaweed freshweight (SFW) biomass with biological triplicates presented using squared symbols ($RT_{22.9}$ = unidentified sulfur volatile). Please note variable scaling along the y-axes. Asterisks and associated P values indicate the level of statistical difference between SW and FW treatments.

421 *Production rates of sulfur volatiles*. Data for the sum of sulfur volatiles were used to calculate
422 the daily net-production rates of sulfur volatiles (Figure 4). Highest production rates in
423 *U. intestinalis* occurred on days 4 (MeSH, DMS and DMDS) and 7 (H_2S and COS). In
424 *S. muticum*, highest rates of production for H_2S and DMS were on Day 2 and for MeSH and
425 DMDS on Day 14. The general patterns for *U. intestinalis* suggest that the production of
426 MeSH, DMS and DMDS is at maximum rates in the first five days after stranding with rates
427 decreasing quickly after this period. In contrast, production rates for H_2S and COS were at
428 maximum on day 7 of the incubation and remained relatively high until at least day 10.
429 Overall, the production rates were much lower in *S. muticum* and either showed maximum
430 rates before day 5 (H_2S, COS, DMS) or only started increasing after day 10 (MeSH, DMDS).
431

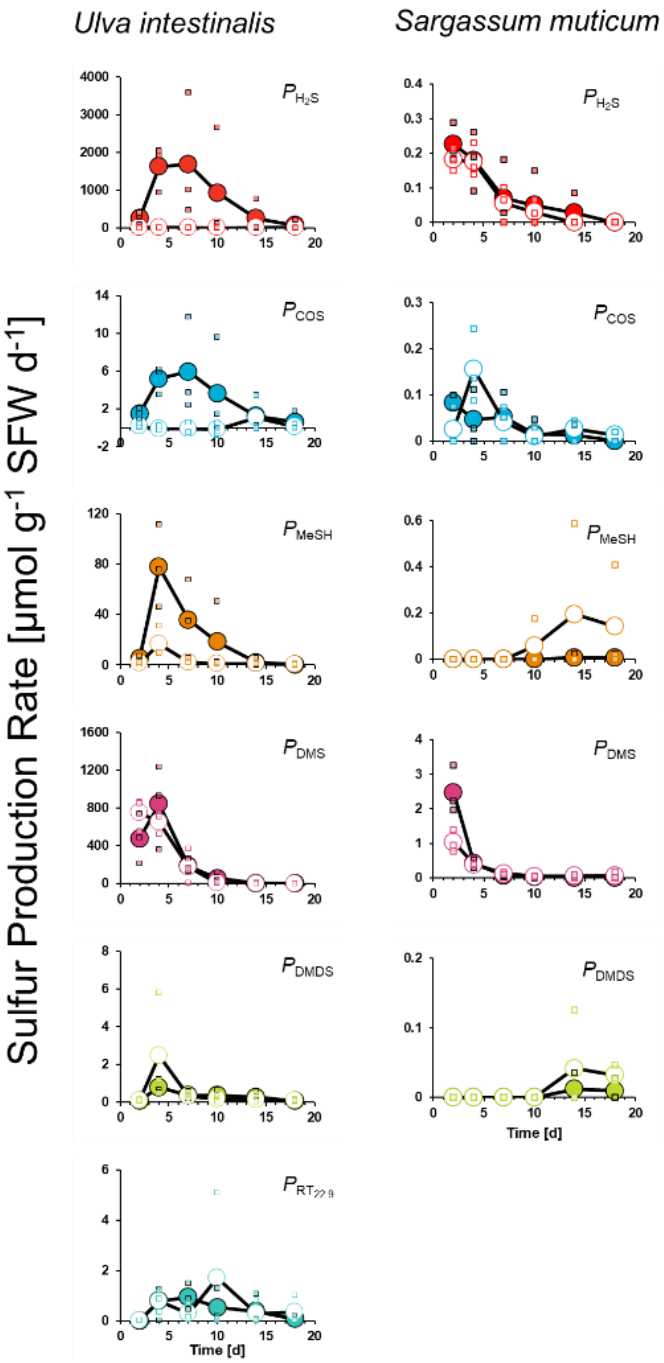


Figure 4. Net-production rate of volatile sulfur released from decaying seaweed. Hypoxic incubations with *Ulva intestinalis* (left) and *Sargassum muticum* (right) at 26 °C for 18 d. Shown are mean values after incubation in seawater (SW; closed circles) or freshwater (FW; open circles). Data were normalised to seaweed freshweight (SFW) biomass with biological triplicates presented using squared symbols ($RT_{22.9}$ = unidentified sulfur volatile).

Bacterial diversity

The bacterial assemblage present during the decomposition of *U. intestinalis* was dominated by anaerobes such as Firmicutes in both treatments. In particular, the freshwater incubations where Firmicutes (specifically *Clostridium* pathogens) made up 80 ± 32% of the community, but also in seawater where Firmicutes contributed 46 ± 22%. Seawater incubations also had high relative abundance of Bacteroidota (25 ± 11%) and Spirochaetes (19 ± 17%), while freshwater incubations had high relative abundance of Proteobacteria (12 ± 3%). However, while both treatments were dominated by Firmicutes, there were significant differences in the specific taxa present in each treatment. Seventy-eight taxa showed significantly different abundances between the treatments (Figure 5) with almost all the taxa significantly associated with freshwater being Clostridia (32 of the 49), notably *Terrisporobacter petrolearius, Proteiniclasticum aestuarii, C. thiosulfatireducens*). In contrast the taxa with the largest increase in the seawater treatment were species from the phylum Spirochaetota: *Pleomorphochaeta caudata* and *P. naphthae* along with *Fusobacterium perfoetens*. There was also a significant increase in the abundance of the Bacteroidota taxa *Dysgonomonas termitidis, Draconibacterium mangrove* and *D. halophilum, Paludibacter propionicigenes, and Ancylomarina longa*. Although no specific pathogen was of particularly high abundance, diverse pathogens were detected at low levels (<50 16S rRNA reads) including *Clostridium tetani*, *Clostridioides difficile*, *Clostridium botulinum, Clostridium haemolyticum*, *Peptostreptococcus anaerobius*, *Staphylococcus aureus* and *S. haemolyticus*, along with various *Enterococcus* including *Enterococcus faecalis*.

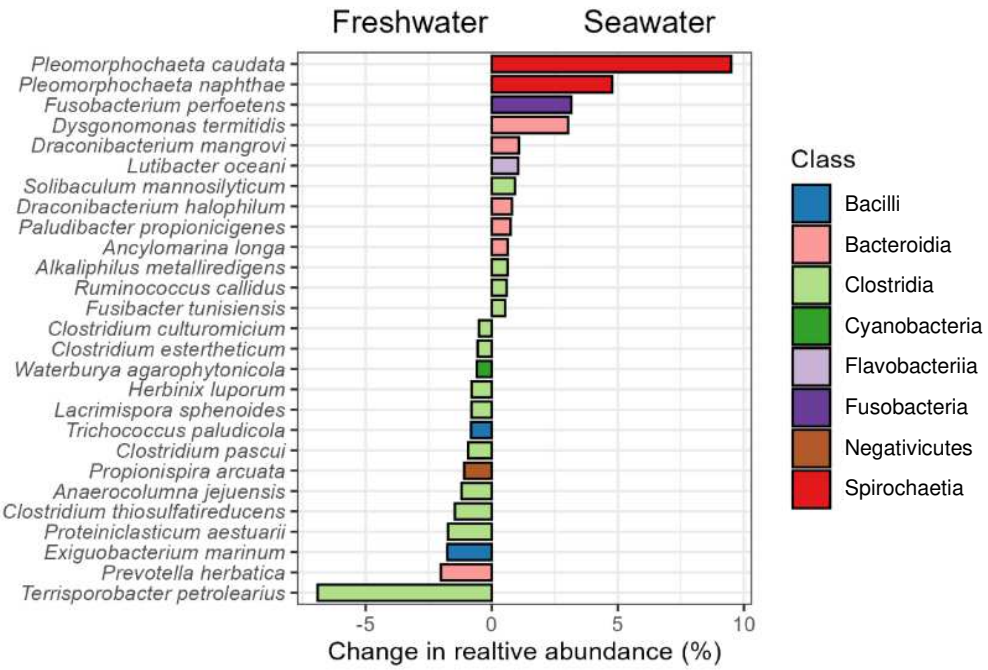


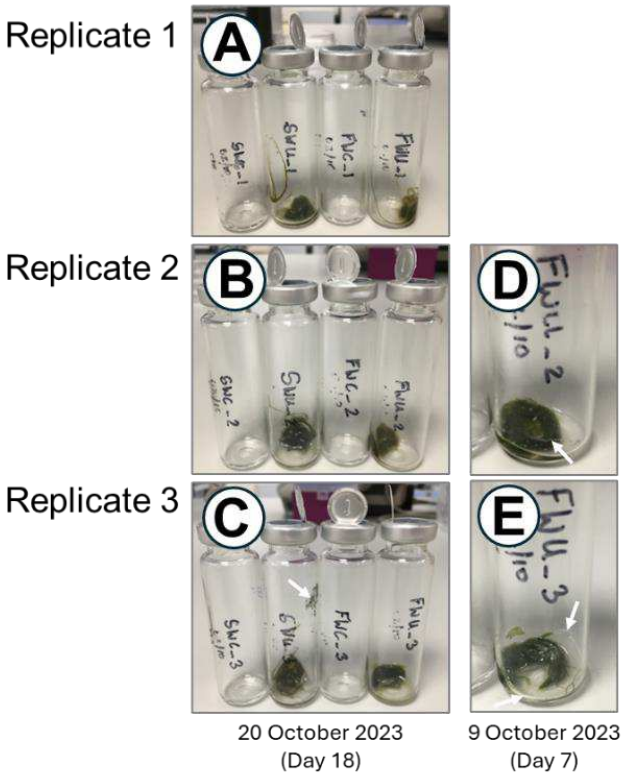
Figure 5. Change in relative abundance of bacteria identified on decomposing *Ulva intestinalis*. A positive value indicates an increase in taxa associated with seawater, a negative value an increase in taxa associated with freshwater. Only the top 25 most-abundant species with a significant association are shown (adjusted p-value < 0.05, multivariate GLM with negative binomial error distribution).

Resistant bacteria

No resistant bacteria could be cultured from the control (day 0) samples (Table 1). The most-abundant resistant bacteria species isolated from the decomposing seaweed was vancomycin-resistant *Enterococcus faecalis* (VRE), which showed high abundance in seawater (2000 ± 3455 colony-forming units (CFU) g^{-1} SFW) and freshwater (1297 ± 1028 CFU g^{-1} SFW) incubations with *U. intestinalis*, contrasting with *S. muticum* where VRE was only found in the freshwater incubations (264 ± 285 CFU g^{-1} SFW). There were also vancomycin-resistant colonies that could not be identified based on colony morphology in the seawater treatments for both seaweeds (19.4 ± 34 and 10 ± CFU g^{-1} SFW for *S. muticum* and *U. intestinalis*, respectively). ESBL-producing bacteria were detected on *U. intestinalis* including *E. coli* (8 ± 14 CFU g^{-1} SFW) and the KESC group (2 ± 3 CFU g^{-1} SFW) in freshwater only, and unidentified biofilm-type colonies on seaweed decomposing in fresh and seawater conditions (10 ± 17 and 15 ± 26 CFU g^{-1} SFW for fresh and seawater respectively; Table 1).

Table 1. Assessment of resistant bacterial pathogens in the seaweed holobionts. Number of colony-forming units (CFU) per g of seaweed freshweight (SFW) for vancomycin-resistant *Enterococcus* (VRE) and extended beta-lactamase (ESBL)-producing bacteria. Seaweed was incubated for 5 d. Data show mean ± standard deviation rounded to nearest CFU (n = 3). 'Unknown' = putative identification impossible.

		Resistant taxa identified (CFU g^{-1} SFW)				
Resistance		Vancomycin		ESBL-producing bacteria		
Putative identification		*E. faecalis*	unknown	*E. coli*	KESC group	biofilm
S. muticum	Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Freshwater	264 ± 285	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Seawater	0 ± 0	19 ± 34	0 ± 0	0 ± 0	0 ± 0
U. intestinalis	Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Freshwater	1297 ± 1028	0 ± 0	8 ± 14	2 ± 3	10 ± 17
	Seawater	2000 ± 3455	10 ± 17	0 ± 0	0 ± 0	15 ± 26



Supplementary Figure S2. Photographs of incubation vials in the experiment with *Ulva intestinalis* (2-20 October 2023). Shown are three replicates on Day 18 of (left to right) seawater control, seawater *Ulva*, freshwater control and freshwater *Ulva* (A-C), and freshwater *Ulva* replicates 2 and 3 on Day 7 (D and E). Noticeable microbial biomass is indicated by white arrows in panels C (black growth on inside glass surface), D and E (white growth).

Discussion

The ocean and coastal wetlands are recognised as substantial sources of volatile sulfur compounds that are produced by diverse abiotic and biotic processes [38,39]. GC-FPD analysis revealed the production of five and six abundant sulfur volatiles in *S. muticum* and *U. intestinalis*, respectively. Another seven sulfur volatiles with minor abundance were detected in *U. intestinalis* but not further considered here since their concentrations were often below the LOQ of the GC-FPD method. The GC-FPD data broadly matched results from the exploratory PTR-MS experiment with H_2S and DMS being dominant volatiles in *U. intestinalis* followed by COS and MeSH. Interestingly, there was no evidence of the production of ammonia in our samples. Volatile production in *S. muticum* was substantially lower with seaweed treatments often below the seawater and freshwater controls. Taken together, our results suggest that seaweed strandings can be a substantial source of malodorous and toxic sulfur volatiles within 2 weeks after the initial stranding but that their diversity and quantity are species-specific. Since our sample size was small (two species from two different phyla), it is premature to infer whether certain taxonomic groups produce more of the sulfur volatiles investigated than others. A recent review identifies 15 genera that caused nuisance blooms between 1976 and 2018 [40] and similar studies could address volatile diversity and quantity by screening more seaweed genera. This may include, for example, species from the most-frequently recorded blooming genus *Ulva*, such as *U. prolifera U. compressa* and *U. rigida* [10], *Sargassum* seaweeds *S. natans* and *S. fluitans* [1], and selected red seaweeds, for example, *Gracilaria* spp. (phylum Rhodophyta)[40].

Contribution of microbial processes to volatile profiles. Abiotic production of organosulfur compounds via photochemistry [39] is possible at the illuminated surface of seaweed strandings but was inhibited in our dark incubations. Hence, it is likely that the biological production from the development and succession of microbial consortia during the seaweed-decay process substantially added to the observed patterns in sulfur-volatile production. This may also explain the sometimes-large variance in our results since the differential biological succession of specific microbial taxa could have resulted in various outcomes in volatile production when temperature and light conditions were relatively stable. Microbial growth resembling bacterial colonies or fungal hyphae was evident in some replicates but not in others by day 7 (Supplementary Figure S2) suggesting that the profiles of volatile release at the end of the incubation may differ greatly between replicates despite the relatively homogenous starting and incubation conditions. Temperature will likely affect the diversity and succession of microbial consortia. Whereas we provided a constant 26 °C in our

incubations, natural seaweed strandings will receive variable internal heating from the microbial degradation processes and variable external heating from climate-zone dependent solar radiation (e.g. tropical, subtropical or temperate zones). It is likely that the outcomes would differ between samples when incubating them at a range of temperatures.

Uncovering the details on the microbial processes resulting in the transformation of sulfur compounds were beyond the scope of this research but reviewed elsewhere [41,42]. Here, we focus on selected examples to illustrate the diversity in their production and related processes contributing to the overall release of sulfur volatiles from various environments (e.g. ocean, coastal wetlands, soil) [38]. Anaerobic sulfur/sulfate-reduction by microorganisms (bacteria and archaea) or sulfate-independent processes including the bacterial degradation of sulfur-containing amino acids substantially contribute to H_2S production in marine sediments [43]. Similar biotic processes in seaweed strandings will likely drive the release of H_2S, the most-abundant sulfur volatile in our experiments. For example, anaerobic digestion of seaweed typically produces 50-70% methane, 30-45% carbon dioxide, <2% hydrogen and <3.5% hydrogen sulphide [44]. The seawater incubations contained at least ten-times more sulfate (28 mM in seawater) than our freshwater incubations (maximum in lakes = 2.6 mM) [26]. Hence, growth of sulfate-reducing microbes was likely limited in the incubations with freshwater and promoted with seawater. The seawater incubations showed a significant increase of bacteria in Spirochaetota and Bacteroidota – two phyla typically not associated with sulfate-reduction. However, novel lineages in these phyla have recently been demonstrated to perform sulfate reduction and are known to reduce elemental sulfur to H_2S [45]. Furthermore, *Fusobacterium* spp. are recognised for their production of H_2S from the enzymatic desulfhydration of cysteine [46], suggesting that diverse microbial processes will have contributed to the significant production of H_2S in the seawater incubations with *U. intestinalis*. Fermentative and hydrolytic bacteria such as Clostridia were abundant in the freshwater incubations and have been demonstrated as versatile producers of H_2S using various metabolic routes [46]. For example, *C. thiosulfatireducens* (Figure 5), a species that reduces thiosulfate and elemental sulfur to sulfide in the presence of casamino acids [47], was significantly associated with the freshwater incubation of *U. intestinalis*.

The second most abundant sulfur volatile in our experiments was DMS which showed particularly high concentrations in the incubations with *U. intestinalis*. In marine environments, DMS is produced enzymatically from its biological precursor DMSP, a secondary metabolite accumulated to relatively high concentrations in chlorophyte seaweeds including *U. intestinalis* [25]. It is likely that bacterial degradation of DMSP provided major pathways for the release of DMS and MeSH [48-50]. However, microbial production may not be

572 solely responsible for the release of sulfur volatiles. For example, *U. intestinalis* contains at
573 least six DMSP-lyase homologs encoding for putative enzymes involved in the direct
574 cleavage of DMSP to DMS [51]. Since the enzyme is likely constitutively expressed but
575 intracellularly segregated from the substrate DMSP [52,53], it is possible that loss of cell
576 integrity during degradation results in the mixing of substrate and enzyme that releases DMS
577 directly from the seaweed. The microbial biomethylation of highly abundant H_2S was a
578 possible additional source of DMS and MeSH [54,55]. Hence, the high abundance of specific
579 sulfur compounds may result in the production of others. For example, MeSH may have
580 been converted to COS and/or DMDS [56] which could explain the sequential maxima in
581 MeSH and COS production rates on days 4 and 7, respectively. Further processes resulting
582 in COS include the microbial consumption of cystine, cysteine and thiocyanate [57] whereas
583 methanethiol can react to DMDS and dimethyl trisulfide (DMTS) in the presence of
584 ascorbate, Fe(III) ions and H_2S [58]. It is possible that DMTS was the source of the peak
585 observed at a retention time of 22.9 min ($RT_{22.9}$), but initial checks of its retention time
586 remained inconclusive so that the identity of $RT_{22.9}$ is unresolved. Furthermore, the PTR-MS
587 analysis suggested the presence of thioformic acid (O=CH-SH), another candidate for the
588 identity of $RT_{22.9}$ in the GC-FPD measurements. DMS has been suggested to decompose to
589 COS but a recent theoretical study suggests that the production of
590 hydroperoxymethylthioformate from DMS can limit COS release and result in
591 thioperhemiacetals that lead to formic and thioformic acids [59].
592
593 *Health effects of sulfur volatiles*. Due to its high concentration, reactivity and acute toxicity
594 (Supplementary Table S1), H_2S is likely a major contributor to the reported adverse effects
595 including corrosion of metallic objects and ill health or death. The acute health effects of this
596 gas are well researched [60] since it presents a lethal occupational and environmental hazard,
597 and is the second most-often reported fatal gas inhalation in the workplace (7.7% – after
598 carbon monoxide at 36%). Chronic effects of H_2S materialise at exposure of less than 10
599 ppm when inhalation triggers a transition from aerobic to anaerobic cellular metabolism.
600 Less is known about the toxicology of the other sulfur volatiles but the mode of action of
601 DMS in humans is similar to that of H_2S [61] and the 8-hour time-weighted average and short-
602 term exposure limits for MeSH and DMDS are substantially lower than that for H_2S
603 (Supplementary Table S1). This suggest that even low concentrations of MeSH and DMDS
604 produced in seaweed strandings may contribute to the overall ill health effects experienced
605 in their vicinity. Since COS and DMDS also carry the risk of acute toxicity, monitoring their
606 concentrations during episodes of ill health is advisable. It is premature to speculate about
607 local concentrations surrounding seaweed strandings since these will be highly dependent
608 on environmental conditions, for example, the wind speed and resulting mixing of toxic

gases in the overlying atmosphere. Repeated field measurements conducted at stranding sites could quantify gas-flux rates in the undisturbed seaweed and during removal efforts – important information that can feed into atmospheric dispersal models to predict the effects of seaweed strandings on public health.

Health effects of bacteria during seaweed decomposition. Many of the bacterial taxa identified on decomposing seaweed are associated with the gut microbiome/faeces of humans and animals (including *Clostridium* spp., *Enterococcus* spp. *Fusobacterium perfoetens, Dysgonomonas termitidis*, various *Ruminococcus* spp.). Decomposing seaweed may therefore provide a microenvironment that can support regrowth of faecal anaerobic bacteria (including potential pathogens) removed in sewage treatment. Ordinarily the aim of sewage treatment is to reduce microbial load to a point at which that dilution – when released into receiving waters – will mitigate any health risk. Given the right conditions, however, and even when discharging microorganisms at low abundances, they can quicky proliferate and acquire antimicrobial resistance (AMR) in the environment [62-65]. Preventing regrowth in the environment is therefore a core challenge in managing wastewater treatment and supporting a healthy environment [66,67]. As coastal waters receive high levels of treated (and untreated) effluents there is a risk that decomposing seaweeds could provide an environment supporting the regrowth, and subsequently new source (to water, air, or humans and animals) of faecal microorganisms, which may include pathogens and AMR microorganisms. For example, in this study faecal microorganisms where not isolated at the start of incubations but were able to proliferate on *U. intestinalis* to >1000 CFU g^{-1} *E. faecalis* over 5 days. Furthermore, we were able to isolate bacteria resistant to beta-lactam antibiotics, and significantly the aminoglycoside vancomycin which is reserved for the treatment of complicated and otherwise resistant infections (e.g. methicillin-resistant *Staphylococcus aureus* (MRSA)). The most abundant resistant bacterium detected was VRE (Vancomycin-resistant *Enterococcus faecalis*). Enterococci live commensally in the human (and animal) gastrointestinal tract, but VRE are an emerging and significant challenge in healthcare [68,69]. For example, AMR *E. faecalis* was responsible for >100,000 deaths in 2019, the 12th highest of any bacteria [70]. They can cause a variety of infections (commonly urinary tract infection), with *E. faecalis* (observed in this study) the most common cause of infections, while *E. faecium* is generally less virulent but more commonly resistant and is on the 2024 WHO bacterial priority pathogens list [71]. The presence of VRE in decomposing seaweed is therefore clearly a health concern, either as a risk of direct infection, a source for onwards proliferation of AMR bacteria or genes via horizontal gene transfer across the One-Health (human-animal-environment) Continuum [72].

Effect of freshwater addition. A study by Milledge et al. [73] shows that washing of *S. muticum* in freshwater before anaerobic digestion delays the production of methane. This suggest that at locations where freshwater is readily available, its addition to seaweed strandings may alter the production of volatiles, knowledge that could be used to mitigate the release of toxic and malodorous gases and lessen deleterious health effects. Our data suggest significant decreases in the production of H_2S, COS and MeSH with freshwater addition in *U. intestinalis*. However, potential decrease in toxic gases must be balanced with possible stimulation of growth of pathogens including *Clostridium* spp. in freshwater that may impact public health.

To summarise, seaweed strandings release diverse sulfur gases at quantities that may produce health risks to coastal users. Acutely toxic volatiles (e.g. H_2S, COS and DMDS) are of particular concern but the sometimes highly concentrated DMS and MeSH may add to the prevalence of respiratory illnesses experienced in the vicinity of recent strandings. Additionally, decaying seaweed promotes the growth of bacterial pathogens that may result in infection when people come in contact with stranded material or after transmission of bioaerosolised particles. Where readily available, freshwater addition may offer a cost-effective strategy to mitigate the release of volatiles. This may provide local stakeholders with extra time to organise the removal of seaweed biomass and could create benefits through the sustainable use of such raw materials.

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Competing Interests Statement

The authors declare no competing interests.

Author Contributions Statement

Corresponding author M.S. provided conceptualization, data acquisition, formal analysis, investigation, methodology, resources, supervision, visualisation, writing of the original draft, review, and editing. L.G., S.T., G.L. provided conceptualization and developed the gas chromatography methodology. R.M.W.F. and M.M.C. collected microbial diversity data. R.M.W.F. provided methodology, resources, supervision, visualisation and writing of the original draft. F.R. collected PTR-MS data and provided data analysis and writing of the original draft. All authors discussed the results and contributed to the final manuscript.

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