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Transformation of dissolved organic matter by two Indo-Pacific sponges

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Abstract

Dissolved organic matter (DOM) is the largest organic carbon reservoir in the ocean and an integral component of biogeochemical cycles. The role of free-living microbes in DOM transformation has been studied thoroughly, whereas little attention has been directed towards the influence of benthic organisms. Sponges are efficient filter feeders and common inhabitants of many benthic communities circumglobally. Here, we investigated how two tropical coral reef sponges shape marine DOM. We compared bacterial abundance, inorganic and organic nutrients in off reef, sponge inhalant, and sponge exhalant water of *Melophlus sarasinorum* and *Rhabdastrella globostellata*. DOM and bacterial cells were taken up, and dissolved inorganic nitrogen was released by the two Indo-Pacific sponges. Both sponge species utilized a common set of 142 of a total of 3040 compounds detected in DOM on a molecular formula level via ultrahigh-resolution mass spectrometry. In addition, species-specific uptake was observed, likely due to differences in their associated microbial communities. Overall, the sponges removed presumably semi-labile and semi-refractory compounds from the water column, thereby competing with pelagic bacteria. Within minutes, sponge holobionts altered the molecular composition of surface water DOM (inhalant) into a composition similar to deep-sea DOM (exhalent). The apparent radiocarbon age of DOM increased consistently from off reef and inhalant to exhalant by about 900 ^{14}C years for *M. sarasinorum*. In the pelagic, similar transformations require decades to centuries. Our results stress the dependence of DOM lability definition on the respective environment and illustrate that sponges are hotspots of DOM transformation in the ocean.

Most organic carbon is stored in the ocean as dissolved organic matter (DOM) and is of high relevance to biogeochemical cycles. It largely originates from phytoplankton primary production, but depending on the respective ecosystems, other sources such as terrestrial input, marcoalgae and coral sources can be major producers, contributing to the marine DOM pool (Carlson and Hansell 2015; Rix et al. 2016; Medeiros et al. 2017). Hansell (2013) operationally categorized DOM into short lived (labile) DOM, which does not accumulate due to microbial turnover, and recalcitrant DOM, which accumulates and is observable in the ocean. The latter is further classified into four subgroups (semi-labile, semi-refractory, refractory, ultra-refractory), of which the most abundant DOM fraction is refractory in nature, exhibiting low bioavailability (Hansell 2013). Labile DOM, which includes free amino acids, sugars and organic acids, has turnover rates of minutes to days and can be taken up and metabolized by bacteria (Ogawa and Tanoue 2003). After respiration by microbes, DOM is transferred to higher trophic levels as microbial biomass, a process defined as the microbial loop (Azam et al. 1983; Pomeroy et al. 2007). More recently, a new process of how DOM can be made available to higher trophic levels in oligotrophic waters was reported as the so-called sponge loop (de Goeij et al. 2013). It describes the pathway of DOM uptake by sponges, the subsequent conversion to and release of particulate organic matter (POM), and eventual utilization by higher trophic levels, such as detritivores (de Goeij et al. 2013).

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Additional Supporting Information may be found in the online version of this article.

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Sponges (phylum Porifera) are benthic filter feeders, filtering large quantities of water (up to 1000 L h^{-1} kg^{-1} body mass, Vogel 1977) through their elaborate water channels and chambers. Thereby they can alter the composition of the surrounding water by extracting dissolved and particulate matter, such as microorganisms. While most of the acquired microorganisms are used as food, some bacteria are taken up as bacterial symbionts (Taylor et al. 2007; Webster et al. 2010) and form holobionts with the sponge species-specific bacterial communities (Pita et al. 2018). Microbial associates may reach densities of 10^8–10^{10} bacteria per g of sponge wet weight, which is 2–4 orders of magnitude higher than those typically found in seawater (Hentschel et al. 2006; Gloeckner et al. 2014). These sponges are usually referred to as high microbial abundance (HMA) sponges, whereas low microbial abundance (LMA) sponges host sparse populations of bacteria with densities within the range of natural seawater (10^5–10^6 bacteria per g of sponge wet weight, Hentschel et al. 2006). Sponge holobionts influence inorganic nutrient cycles and the cycling of organic matter and therefore are an important component of benthic-pelagic coupling, especially in oligotrophic marine habitats, such as tropical coral reefs (de Goeij et al. 2013; de Goeij et al. 2017).

Previous studies focused on the uptake of DOM by the sponge holobiont, mainly using bulk measurements or isotopic tracer experiments. The findings indicate that DOM uptake in sponges depends on environmental factors like ambient DOM concentration or DOM source. For example, higher concentrated DOM and DOM originating from algae were removed more quickly than the background of recalcitrant DOM (Mueller et al. 2014; Rix et al. 2016; Morganti et al. 2017). General patterns concerning sponge uptake behavior due to sponge functional traits were not found across all examined studies (Mueller et al. 2014; de Goeij et al. 2017; McMurray et al. 2018). Nevertheless, the microbiome of sponges for example, could be an important driver of DOM uptake as some studies show different uptake rates between HMA and LMA sponges (Hoer et al. 2018; McMurray et al. 2018; Olinger et al. 2021). Others, however, could not correlate bulk measurements of dissolved organic carbon (DOC) with the sponge microbiome (Gantt et al. 2019) or concluded that the processing of DOM is possible without mediation of resident bacteria (Achlatis et al. 2019). These studies underline our limited understanding of the connection between sponge physiology, microbial symbiont physiology and the uptake and processing of DOM through filter-feeding of the sponge holobiont.

Whereas most publications focused on the quantitative uptake, Fiore et al. (2017) were the first to explore the differences in DOM composition between exhalant, inhalant and off reef water from two sponge species. The authors concluded that DOM composition of the exhalant water was different to inhalant and off reef water, but did not find differences in DOM composition between the two species. However, Olinger et al. (2021) concluded that only the three investigated HMA sponges were able to utilize certain DOM compounds, specifically halogenated compounds, thereby changing the composition of the seawater DOM. Letourneau et al. (2020) found relatively fewer compounds with low-carbon numbers, low-oxygen content and high-nitrogen content in exhalant water compared to the ambient seawater, suggesting a decrease in lability of DOM due to the filter feeding by the sponge *Spheciospongia vesparium* from Florida Bay. Overall, previous studies showed that the molecular transformation of DOM by sponges differs between sites and species (Fiore et al. 2017; Letourneau et al. 2020; Olinger et al. 2021).

Sponges are not only linked to the water column microbial community by the uptake of bacterial cells, but also through the competition with pelagic heterotrophs for the same DOM food source, expanding their roles as key players in benthic-pelagic coupling. Potentially, sponge holobionts might also be able to take up DOM, which is considered refractory, and not bioavailable to the pelagic microbial community. This hypothesis originates from the dilution hypothesis, which states that dilution limits encounter of molecules and microbes and thus carbon utilization in the deep sea (Arrieta et al. 2015). Applied to sponges, especially HMA sponges, the chance of symbiotic sponge bacteria encountering and consuming DOM, operationally defined as refractory, is much higher than in the water column because of the high density of microbial symbionts in the sponge and the large quantities of water and DOM pumped through them.

The aim of our study was to investigate the role of sponges in shaping reef DOM composition. Our approach included eight replicate sponges of *Melophlus sarasinorum* and seven replicates of *Rhabdastrella globostellata*. Both are HMA sponges and common representatives in coral reefs of the Indo-Pacific (Moitinho-Silva et al. 2017). We performed detailed molecular analyses of DOM via Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), bulk quantification of DOC and dissolved organic nitrogen (DON), and radiocarbon dating of DOC. We used different metrics derived from molecular formulas (MFs) describing DOM composition, including elemental ratios, the degradation index or functional molecular diversity. We hypothesized that the sponges are able to take up DOM that would otherwise be considered as (semi-) refractory due to their high abundance of microbial symbionts. We further hypothesized that uptake of specific DOM constituents is largely species-specific because of differences in the sponge-associated microbial communities.

Materials and methods

Sample collection and preservation

We collected seawater samples in July and August 2019 by scuba diving at the dive site Gab Gab in Apra Harbor, Guam, USA (13°26′45.0″N 144°38′28.0″E). This shallow coral reef slope is dominated by corals of the genus Porites and

protected from strong currents and swell due to its location in the natural harbor on the west coast of Guam. Sampling was done for the two common HMA reef demosponge species *R. globostellata* (seven specimens, 1–2 m depth, Fig. 1a,b) and *M. sarasinorum* (eight specimens, 5–10 m depth, Fig. 1a,c). Water samples were collected for specimens with five 60-mL acid-washed polypropylene syringes (300 mL in total per sample) collecting inhalant and exhalant seawater from the sponges and 200 m away from the reef slope in the harbor (off reef samples). We differentiated between off reef water from *R. globostellata* and *M. sarasinorum*, sampled at 1 and 5 m depth, respectively. Inhalant water represents the water surrounding the sponge specimens and was sampled close to the sponge's base or to the side, away from the oscula and approximately 20 cm off from the ground. Exhalant water was the seawater exiting the sponges' osculum and was collected differently according to the sponge species (Fig. 1a). For *R. globostellata*, which has small oscula of maximum 1 cm in one spot, we created a vacuum in the syringes by pulling the plunger while a valve at the top of the syringe was closed, and placing a piece of plastic between finger flange and plunger head of the syringe (Fig. 1b). We then positioned the tip close to an osculum before opening the valve carefully to start the sample collection. Using this method, we were able to control the flow (10 mL min^{-1}) to only sample the exhalant water without mixing it with ambient seawater. For *M. sarasinorum*, we were able to draw the exhalant water into the syringes very slowly by hand because the oscula were big enough to create a strong seawater effluent allowing for a higher collection rate (60 mL min^{-1}, Fig. 1c). We used the valves for all samples to have the same material and limit confounding factors between samples. We checked for the sponges' pumping activity using fluorescein dye after water collection in order not to contaminate our samples with the dye.

Seawater samples were put on ice immediately after surfacing. Upon return to the University of Guam Marine Laboratory approximately 2–5 h later, 20 mL of seawater was filtered into high-density polyethylene (HDPE) bottles through a 0.45-μm GHP filter and frozen at −20°C for nutrient analyses. The remaining seawater was filtered through combusted glass fiber filters (GF/F; 0.7 μm; Whatman, combustion 4 h, 400°C) and acidified to pH = 2 by adding hydrochloric acid (37%). Twenty milliliters of the acidified water were frozen in HDPE bottles at −20°C for DOC and total dissolved nitrogen (TDN) analyses. The remaining 260 mL acidified seawater was stored at 4°C for the solid-phase extraction of DOM the next day. Before use, all glassware was precombusted at 400°C for 4 h and the other material was cleaned with ultrapure water at pH = 2 and thoroughly rinsed with sample.

Bacterial abundance samples were taken 3 weeks after the initial water sampling at the same location (Gab Gab dive site, Apra Harbour). Using sterile syringes, we took 10 mL samples of inhalant and exhalant water from *R. globostellata* and *M. sarasinorum* with five biological replicates per sponge species via scuba diving. For bacterial abundance samples, we did not differentiate between two different off reef sampling depths, but took five replicates at 2 m depth. After surfacing, samples were stored on ice in a cooler until fixation with glutardialdehyde, which was carried out approximately 3 h later in the laboratory. Fixed samples were stored at −20°C for 5 months until analysis back in Germany.

Bacterioplankton abundances

Prokaryotic organisms, in this manuscript referred to as bacteria samples, were thawed on ice, prefiltered through 50 μm Celtrics filters (Sysmex) and subsequently processed according to Giebel et al. (2019). Briefly, we stained the samples with SybrGreenI (10X final concentration, Invitrogen) for 12 min at 20°C in the dark and added MultiColor Polysciences beads as internal standard. Every sample was then measured for 2 min with a flowrate of 14 μL min^{-1} with the Accuri C6 flow cytometer using a threshold of 900 for the FL1 channel and MilliQ water as fluidics. After visual inspection of the cytograms of the green (FL1, 530 ± 15 nm) vs. red fluorescence (FL3, >670 nm), forward and sideward scatters (FSC and SSC) and their histogram plots, we gated the cells manually to detect bacterial abundances. The exhaling sponge samples showed an artifact with a linear signal that was outside of the bacteria gate in FL1/FL3, which was subsequently excluded from the bacterial counts in the FL1/SSC cytogram. We used the BD Accuri C6 C-Flowsoftware (version 1.0.264.21) for data processing.

Quantification of DOC and TDN

The filtered and acidified DOC samples were thawed at the University of Oldenburg (Germany), and DOC and TDN were quantified in duplicates with a Shimadzu TOC-V Analyzer with a TNM-1 module equipped with an ASI-V autosampler. Deep-sea reference material from the University of Miami (Dennis Hansell, University of Miami) was used for quality control. The mean of the duplicate water samples was used for further analyses, when the deviation between duplicates was within reasonable limits (<10%). When the deviation was above 10%, the lower measurement was used for the calculations.

Quantification of inorganic nutrients

Before analyses, nutrient samples were slowly thawed in a fridge overnight. We then measured NO_x photometrically via vanadium(III)chloride reduction (Schnetger and Lehners 2014) and PO_4^{3-} and NH_4^+ via micro-photometric methods (Laskov et al. 2007). The limit of quantification was set at the lowest point of the calibration (NH_4^+: 5.0 μmol L^{-1}; NO_x: 1.0 μmol L^{-1}; PO_4^{3-}: 0.2 μmol L^{-1}). PO_4^{3-} and NH_4^+ values were therefore not quantified for this study because the values were too low. Consequently, dissolved inorganic nitrogen (DIN) equals the NO_x values.

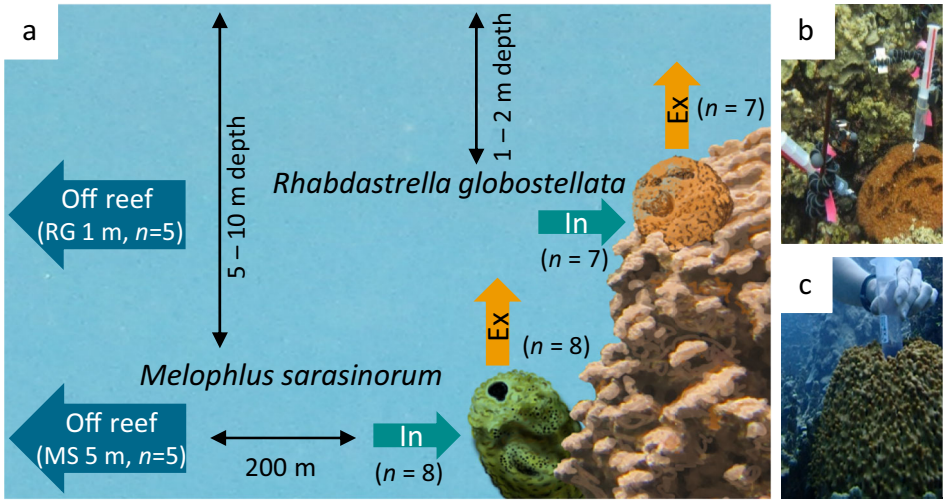

Fig. 1. Sampling design. (**a**) Schematic of sampling design with the three sample types (off reef, inhalant and exhalant), the two sponge species (*Melophlus sarasinorum* and *Rhabdastrella globostellata*), and sampling depth. The number of replicate samples for each sample type are shown (collected from different specimens). Only bacterioplankton abundances had a different sampling design, with $n = 5$ for each sample type (off reef, inhalant, and exhalant), with off reef samples taken at 2 m depth. (**b**) Sampling of inhalant and exhalant water from *R. globostellata* with the vacuum method. (**c**) Sampling of exhalant water by hand from *M. sarasinorum*.

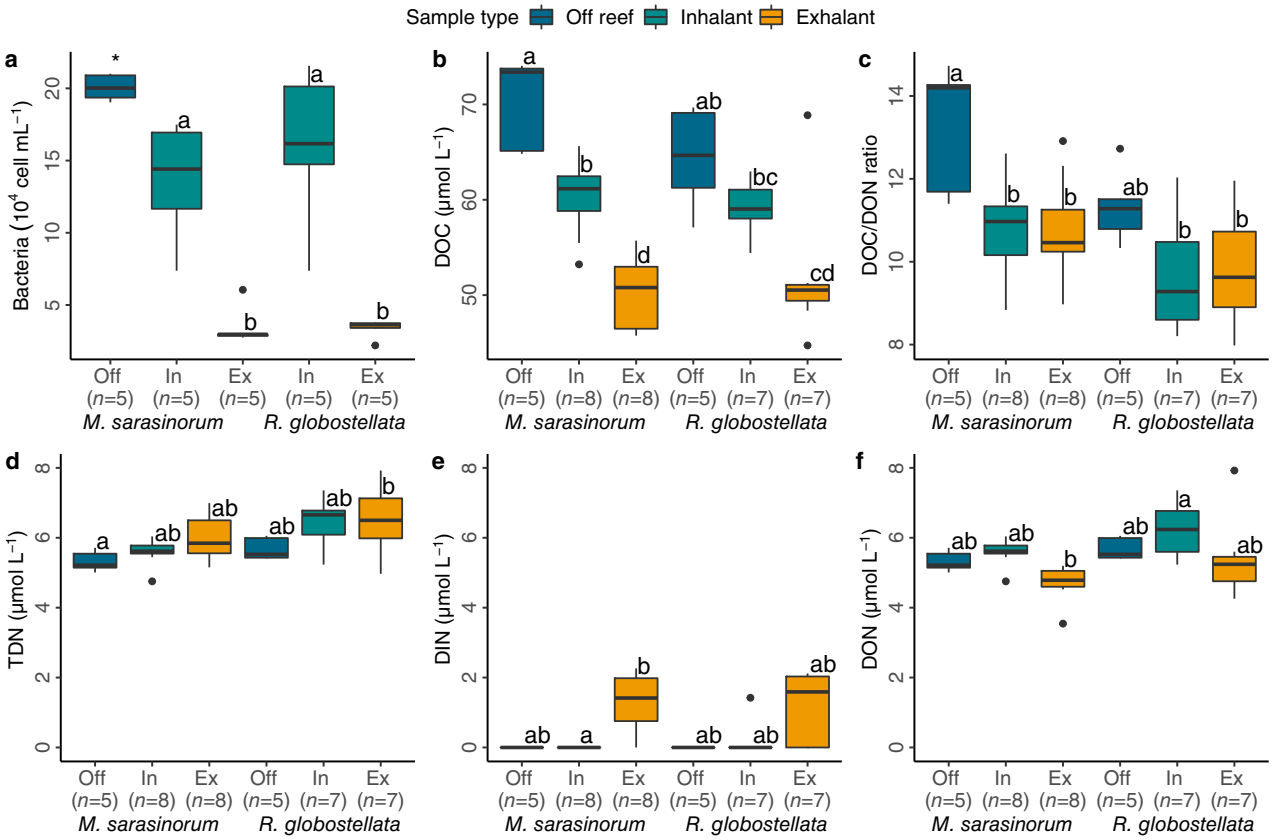

Fig. 2. Concentrations of (**a**) bacterial cells, (**b**) DOC, (**c**) DOC/DON ratio, (**d**) TDN, (**e**) DIN being NO_x, and (**f**) DON from off reef (Off), and inhalant (In) and exhalant (Ex) water from *Melophlus sarasinorum* and *Rhabdastrella globostellata*. Boxplots sharing the same letters are not significantly different from another, while different letters indicate significant differences by the post hoc test at the 5% significance level. Means, standard deviations, and post hoc significance levels are available in Supporting Information Tables S1, S2. *Off reef water for bacterial counts was not included in the methods as there was no differentiation between sponge species.

Composition of DOM

DOM was solid-phase extracted from filtered and acidified samples (250 mL, pH = 2) with commercially available PPL cartridges (500 mg, BondElut, Agilent) according to Dittmar et al. (2008). After loading DOM onto the cartridge, the cartridges were desalted with acidified ultrapure water (0.01 M HCl), remaining water was pushed out with a syringe and the cartridges were frozen at −20°C for about 1 month before complete drying with argon gas and elution of the DOM. The concentration of solid-phase extractable DOC was determined by drying an aliquot of the solid-phase extracts, redissolving it with ultrapure water at pH = 2 (0.01 M HCl) and measuring its concentration with the Shimadzu TOC-V Analyzer as described above to calculate the extraction efficiency. For every set of extractions, ultrapure water was extracted on an additional PPL cartridge to serve as a procedural blank.

For compositional measurements, we diluted the DOM extracts to a final concentration of 1.75 ppm in a 1 : 1 mixture of ultrapure water and methanol (ULC-MS grade, Biosolve, NL), and filtered through 0.2-μm PTFE filter (Carl Roth, Rotilabo-syringe filters). The mass spectra were measured in duplicates with a 15 Tesla Bruker solariX XR FT-ICR-MS (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionization source (Bruker Apollo II) applied in negative ionization mode. Samples were injected with an autosampler (PAL RSI, CTC Analytics, injection rate 0.03 μL s^{-1}). Two hundred scans were accumulated per run in a mass window from 92 to 2000 Da with an ion accumulation time of 0.1 s. An inhouse deep-sea reference sample was measured daily to control for instrument drift (North Equatorial Pacific Intermediate Water [NEqPIW]; Green et al. 2014). We used an internal calibration list for mass calibrating the spectra in the Bruker Daltonics Data Analysis software package and processed the calibrated mass lists using the ICBM-OCEAN software (Merder et al. 2020) to reduce noise and systematic errors, recalibrate and align the mass spectra, and assign the likeliest MFs for further analyses in R (version 3.6.3) to peaks above a method detection limit level of 2.5. Formula assignment was done with a tolerance of < 0.5 ppm and the elemental setting $C_{1–100}$ $H_{2–200}$ $O_{1–70}$ $N_{0–4}$ $S_{0–2}$ $P_{0–1}$.

Radiocarbon age of SPE-DOC

Radiocarbon dating of the SPE-DOC was done for up to five replicate extracts per sponge species, inhalant and exhalant, and the off reef references. Extract volumes containing ~ 80 μg C were transferred into 50 μL liquid tin capsules (ELEMENTAR, S03 951619) kept in a custom-made aluminum tray. The tray was placed on a hot plate at 40°C until samples were dry. To ensure complete removal of methanol, the samples were kept in a desiccator under mild vacuum at 60°C for a minimum of 48 h. Radiocarbon analysis were carried out with the EA-GIS-AMS setup at the AWI MICADAS following established standard operation procedures as outlined in Mollenhauer et al. (2021). Radiocarbon data were normalized and blank corrected against standard (NIST Oxalic Acid II; NIST SRM4990C) and blank (^{14}C free) CO_2 gas. Furthermore, the tin capsule blank was determined and corrected for, following the protocol described by Sun et al. (2020).

Data analyses

Data analyses were conducted in R (version 3.6.3), using mainly the tidyverse language (Wickham et al. 2019). Other R packages for statistical analyses included "vegan" (Oksanen et al. 2020), "rstatix" (Kassambara 2021), "multcompView" (Graves et al. 2019), "FSA" (Ogle et al. 2021), and "broom" (Robinson et al. 2021). R package "ggpubr" (Kassambara 2020) was used as an addition to ggplot2 graphs. DON concentrations were calculated by subtracting DIN from TDN concentrations. The concentrations of bacterial cells, DOC, and DON were compared between the treatments of "sponge species" and "sample type," being off reef, inhalant, and exhalant. We tested whether the assumptions for a two-way ANOVA were met using the Shapiro Wilk test (p-value < 0.05) to test for normal distribution and Levene's test (p-value < 0.05) to test for homogeneity of variances. The two-way ANOVA was followed by a Tukey HSD test for pairwise comparison with adjusted p-values using the Bonferroni correction method. If the assumptions were not met, we used a Scheirer–Ray–Hare test, followed by a Dunn's test for pairwise comparison with adjusted p-values using the Bonferroni correction method.

DOM molecular composition was assessed based on the "Likeliest Match" output of ICBM-OCEAN (Merder et al. 2020). From the data table, we first removed the MFs containing heavy isotopes. Next, contaminants, which were identified according to the signal-to-method detection limit (S-MDL) ratio in the blank measurements with an S-MDL-ratio > 15 were deleted. Furthermore, we only kept those masses, which were detected in both technical replicates of the FT-ICR-MS measurements and averaged the intensities. For a sample set-detection limit, we identified the MF peak of lowest intensity for every sample. From those lowest intensities, we then took the maximum and used it as a threshold, setting all MFs with intensities below this threshold to zero.

Based on the MFs assigned to the measured masses, ICBM-OCEAN also provided the modified aromaticity index (AImod = 1 + C—0.5O—S—0.5[H + N + P])/(C—0.5O—S—N—P); Koch and Dittmar 2006) and the double-bond equivalent (DBE = 1 + 0.5 * [2C—H + N + P], Koch and Dittmar 2006), which provided the base for the descriptive classification, like the saturation status, in addition to the elemental ratios (unsaturated = H/C ≥ 1.5 and H/C ≤ 2; highly unsaturated = AImod < 0.5 and H/C < 1.5; table S2 in Merder et al. 2020). We further calculated the C/N ratios for the MFs and added "Lipid-likes" using the definition by Rivas-Ubach et al. (2018, O/C ≤ 0.6 and H/C ≥ 1.32 and N/C ≤ 0.125 and P/C < 0.35).

As the degradation index gives an estimate of how processed DOM material is (Flerus et al. 2012), we calculated it using the formula by Flerus et al. (2012). A higher value for the index indicates older, more processed DOM.

In addition to the DOM molecular characteristics, we considered three diversity measures: molecular richness, Shannon diversity and functional molecular diversity. Molecular richness is the number of MFs identified in a sample. Shannon diversity was calculated, taking into account richness and evenness of the MFs in a sample. Functional diversity is increasingly used in ecological diversity research and describes functions carried out by an ecological community. This had to be transferred to DOM data by assuming that compounds with similar chemical properties react or "function" in a similar way. Functional molecular diversity, here referred to as functional diversity, was defined and calculated after Mentges et al. (2017), based on Rao's quadratic entropy (Rao, 1982) and has the same unit as the investigated trait. Here, we emphasized three functional traits: (1) molecular mass, as it is related to bioavailability; (2) H/C ratio, as compounds with high H/C ratio tend to be more bioavailable (D'Andrilli et al. 2015) and C/N ratio, as nitrogen-containing compounds are readily used by microbes (Hach et al. 2020).

Using the relative abundances of MFs, we calculated a principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarities and run an analysis of similarities (ANOSIM) to test for differences in the DOM composition between sample types. Furthermore, we computed an intensity-weighted table of DOM molecular characteristics by calculating the weighted means for every chemical attribute under consideration of the intensity for every sample. This provides, for example, a contribution of lipid-like MFs to the total relative intensity. Using the intensity-weighted table of DOM molecular characteristics, we could fit the chemical data as vectors showing which DOM molecular characteristics contribute to the differences between samples. In addition, we used the same table to test for differences of samples, comparing their DOM molecular characteristics and the functional diversity individually, using a two-way ANOVA, coupled with a Tukey honestly significant difference

Table 1. Significance values of tested variables with two factors: Sponge species (*Melophlus sarasinorum* or *Rhabdastrella globostellata*) and sample type (off reef, inhalant or exhalant). We used either a two-way ANOVA (aov) or a Scheirer–Ray–Hare test (SRH), depending on the results of the tested assumptions. For aov, we reported the *F*-value and for SRH the *H*-value. Means, standard deviations, and post hoc significance levels are available in Supporting Information Tables S1, S2.

		Factor 1: "Sponge species"			Factor 2: "Sample type"			Interaction: "Sponge species : sample type"		
Variable	Test used	df	*F*-value (*H* value)	*p*-value	df	*F*-value (*H* value)	*p*-value	df	*F*-value (*H* value)	*p*-value
Bulk water samples										
Bacteria	SRH	1	0.32	0.57	1	14.32	2×10^{-4}***	1	0.00	0.970
DOC	aov	1	0.29	0.593	2	33.26	9.92×10^{-9}***	2	1.86	0.171
DOC/DON	aov	1	8.72	0.006**	2	8.95	0.001***	2	0.44	0.649
TDN	aov	1	8.51	0.006**	2	4.05	0.027*	2	0.43	0.656
DIN	SRH	1	0	0.986	2	18.40	1×10^{-4}***	2	0.45	0.800
DON	aov	1	7.17	0.011*	2	5.71	0.007**	2	0.19	0.828
Solid-phase extracted samples										
Bulk SPE-DOC measurements										
^{14}C age	SRH	1	0.76	0.383	2	7.92	0.019*	2	2.08	0.353
FT-ICR-MS measurements										
Moleular mass	aov	1	0.26	0.613	2	13.15	5.87×10^{-5}***	2	0.46	0.634
H/C ratio	aov	1	0.01	0.922	2	12.18	1×10^{-4}***	2	0.96	0.394
Lipid likes	aov	1	0.04	0.85	2	15.52	9.7×10^{-6}***	2	0.69	0.509
C/N ratio	aov	1	14.32	0.001***	2	19.02	2.86×10^{-6}***	2	1.33	0.279
Degradation index	aov	1	6.35	0.017*	2	112.00	1.09×10^{-15}***	2	3.37	0.046*
FD (Mol. Mass)	aov	1	0.24	0.631	2	55.83	1.81×10^{-11}***	2	1.78	0.184
FD (H/C ratio)	aov	1	5.96	0.020*	2	1.74	0.191	2	1.35	0.273
FD (C/N ratio)	aov	1	6.71	0.014*	2	25.51	1.71×10^{-7}***	2	0.40	0.671
Richness	aov	1	5.11	0.03*	2	0.04	0.958	2	0.43	0.656
Shannon diversity	aov	1	7.14	0.012*	2	1.14	0.331	2	0.79	0.462

DIN, dissolved inorganic nitrogen; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; FD, functional diversity; TDN, total dissolved nitrogen.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(HSD) pairwise test (adjusted *p*-value using Bonferroni correction method), after checking whether the assumptions were met. Last, we did a separate Welch's *t*-test (*p*-value < 0.01) for every MF between inhalant and exhalant water from the relative abundance data of the sponge species. We decided for a *p*-value according to the distribution of significant values after doing Welch's *t*-tests with a randomly calculated matrix having the same dimensions and number of zero values. We then did a Spearman correlation between the significantly changed MFs of *M. sarasinorum* and those of *R. globostellata* using their logarithmic fold change, which was defined as the ratio of exhalant to inhalant relative peak intensities.

Throughout the manuscript, we provide the means and standard deviations of the variables; FT-ICR-MS derived values represent means ± standard deviation of intensity-weighted data.

Results

Sponge impact on bacterial cells, DOC and DON concentrations

Abundances of bacterial cells decreased slightly from off reef water samples to water samples around the sponges (average decrease of 0.53×10^5 cells mL^{-1}), the average decrease from inhalant to exhalant water was more pronounced with $1.0 \pm 0.3 \times 10^5$ cells mL^{-1} for *M. sarasinorum* and $1.3 \pm 0.5 \times 10^5$ cells mL^{-1} for *R. globostellata* (Fig. 2a; Table 1; Supporting Information Table S2).

Similar to the bacterial cell abundance, there was also a significant decline in DOC concentrations (Fig. 2b) from off reef to inhalant, and from inhalant to exhalant water. When samples of both sponge species were pooled over the sample types, there were significant differences between inhalant and exhalant DOC concentrations (Table 1; Supporting Information S2). Due to an outlier in the replicates of exhalant water from *R. globostellata*, there were no significant differences toward the other sample types regarding DOC concentrations (Fig. 2b; Table 1; Supporting Information Table S2). However, the DOC concentrations in exhalant water samples from *M. sarasinorum* were significantly lower than the DOC concentrations in inhalant and off reef water samples from both sponge species (Fig. 2b; Table 1; Supporting Information Table S2). On average, the difference between inhalant and exhalant DOC concentrations was 10 ± 5.9 and 7.1 ± 7.7 μmol L^{-1} for *M. sarasinorum* and *R. globostellata*, respectively.

TDN concentrations remained the same between sample types, when differentiated between sponge species (Fig. 2d). However, DIN concentrations increased significantly from almost zero in off reef and inhalant seawater to 1.3 ± 0.9 and 1.1 ± 1.1 μmol L^{-1} in exhalant water for *M. sarasinorum* and *R. globostellata*, respectively (Fig. 2e; Table 1). Contrarily, DON decreased from inhalant to exhalant water (Fig. 2f; Table 1; Supporting Information Table S2). Since DOC concentrations also decreased from inhalant to exhalant water, DOC/DON ratios between inhalant and exhalant water from both sponge species were similar. DOC concentrations in off reef water were higher compared to inhalant and exhalant water, but DON concentrations were the same compared to inhalant water. This resulted in a higher DOC/DON ratio in off reef water (Fig. 2c), with a significantly higher DOC/DON ratio for the off reef water sampled at the same depth as *M. sarasinorum* (Table 1; Supporting Information Table S2).

Sponge impact on DOM composition

Extraction efficiencies on a carbon basis were on average 76.75% ± 7.6% and did not significantly differ between sample types (Supporting Information Fig. S1).

Via FT-ICR-MS, we identified 3040 different MFs in a mass range of 101–972 Da in our sample sets in total, of which 2549 MFs were found in *M. sarasinorum* and 2869 MFs in *R. globostellata* samples. MFs were mainly CHO (1485 MFs) compounds, followed by CHON (1060 MFs), CHOS (341 MFs), and CHOP (92 MFs). Roughly a 3rd (1089) of all MFs were present in every sample. About two thirds of the MFs (1832) were exclusively detected in off reef, inhalant and exhalant DOM samples associated with *M. sarasinorum*. Two hundred twelve MFs were exclusive to exhalant samples of *M. sarasinorum*. For the *R. globostellata* dataset, we found 1952 MFs shared by off reef, inhalant and exhalant samples, with 252 unique MFs being only present in exhalant samples.

The ordination (Fig. 3) shows that the DOM composition of the exhalant water replicates was more similar to each other than to off reef and inhalant water samples, regardless of species and depth sampled. For *M. sarasinorum*, there was a

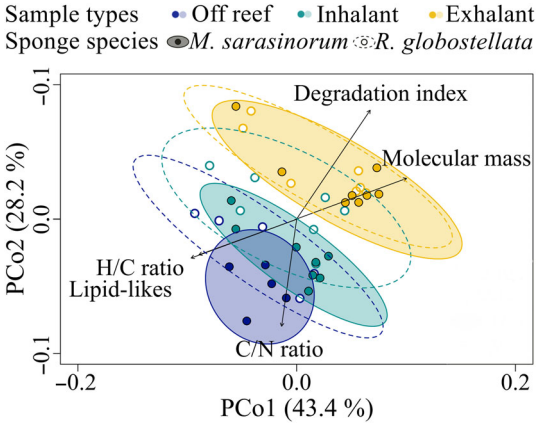

Fig. 3. PCoA of the DOM composition from *Melophlus sarasinorum* (full line, colored background, and filled circles) and *Rhabdastrella globostellata* (dashed line, white background, and empty circles) samples. The PCoA is based on 3040 MFs and their respective FT-ICR-MS signal intensities, fitted parameters are derived from the same data as described in "Materials and methods" section. Colors show the sample types off reef (blue), inhalant (green) and exhalant (orange) water. The axes explain the variation in the input data in percent. Ellipses show confidence limit of 0.95 based on the standard deviation of points (R ordiellipse [kind = "sd," conf = 0.95], ANOSIM, $R = 0.42$, $p = 0.0001$). Length of environmental variable vectors are in relation to the difference of DOM composition explained.

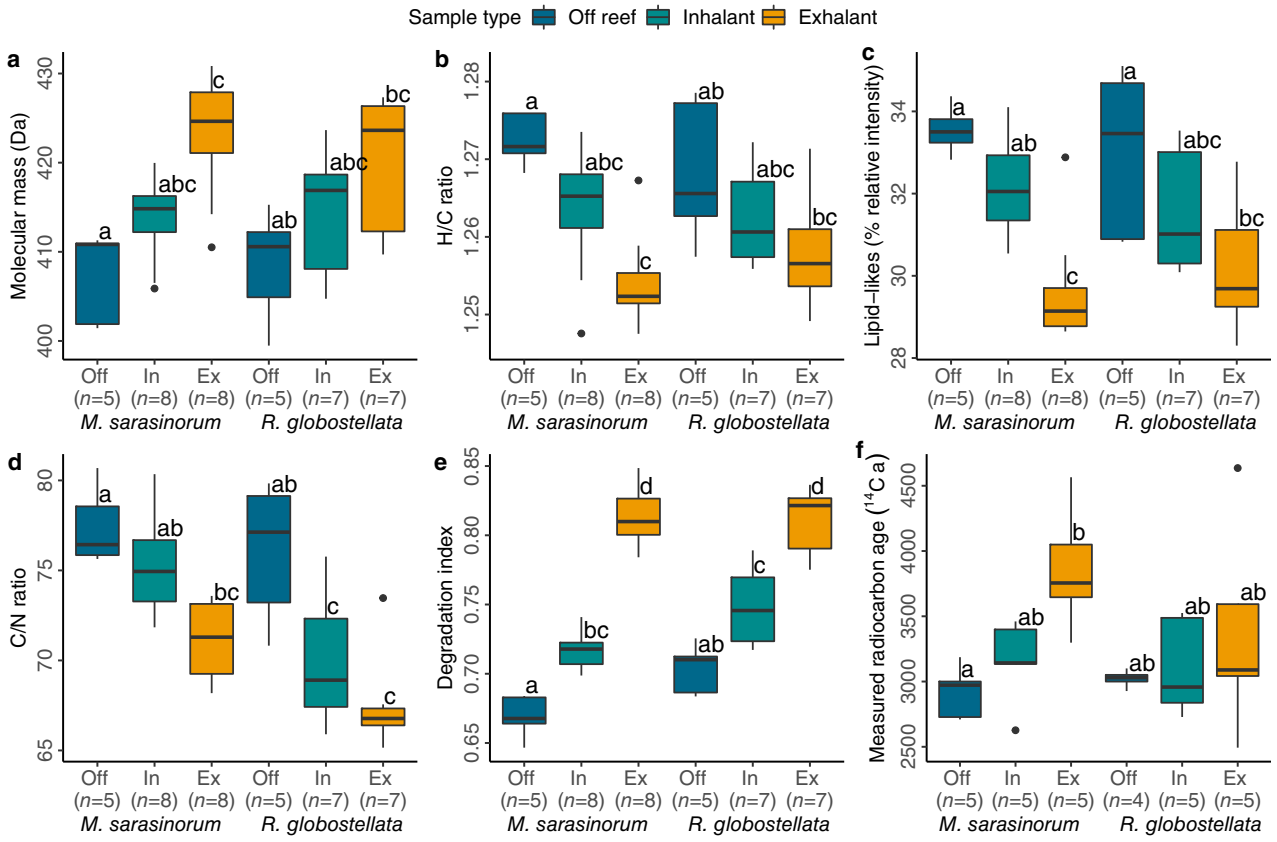

Fig. 4. Boxplots of the DOM characteristics (**a**) molecular mass, (**b**) H/C ratio, (**c**) relative amount of lipid-like MFs, (**d**) C/N ratio in MFs, (**e**) degradation index based on MF assignments from FT-ICR-MS, and (**f**) measured radiocarbon age from bulk SPE-DOC between off reef (Off), inhalant (In) and exhalant (Ex) water from *Melophlus sarasinorum* and *Rhabdastrella globostellata*. Boxplots sharing the same letters are not significantly different from another, while different letters indicate significant differences by the post hoc test at the 5% significance level. Means, standard deviations, and post hoc significance levels are available in Supporting Information Tables S1, S2.

significant difference between off reef, inhalant and exhalant DOM compositions (ANOSIM, $R = 0.54$, $p = 0.0001$), but the 95% confidence intervals of off reef and inhalant water samples overlapped, whereas the one from exhalant samples separated from the rest. These differences between sample types were also significant in the samples of *R. globostellata*

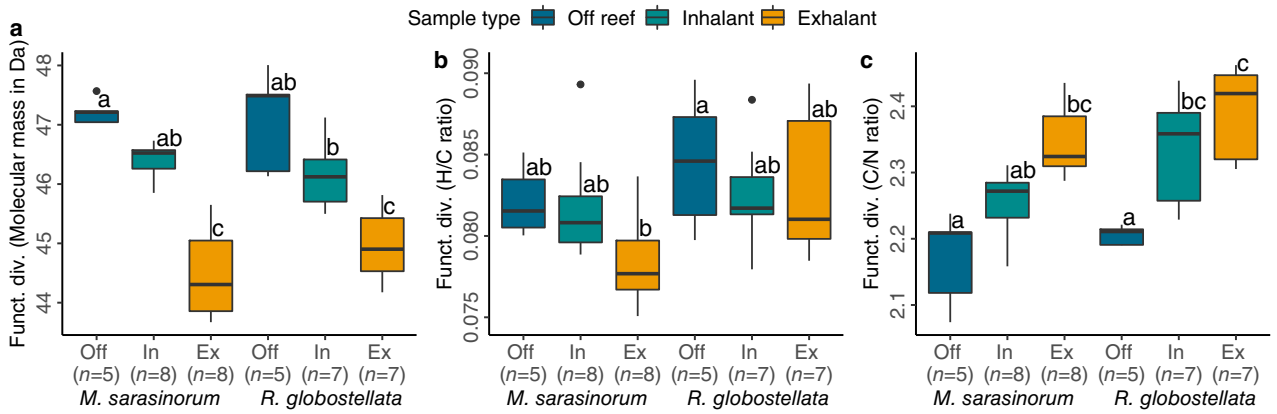

Fig. 5. Functional molecular diversity (Funct. div.) of DOM based on MFs and their FT-ICR-MS signal intensities of (**a**) molecular mass, (**b**) H/C ratio, and (**c**) C/N ratio between of reef (Off), inhalant (In), and exhalant (Ex) DOM from *Melophlus sarasinorum* and *Rhabdastrella globostellata*. Boxplots sharing the same letters are not significantly different from another, while different letters indicate significant differences by the post hoc test at the 5% significance level. Means, standard deviations, and post hoc significance levels are available in Supporting Information Tables S1, S2.

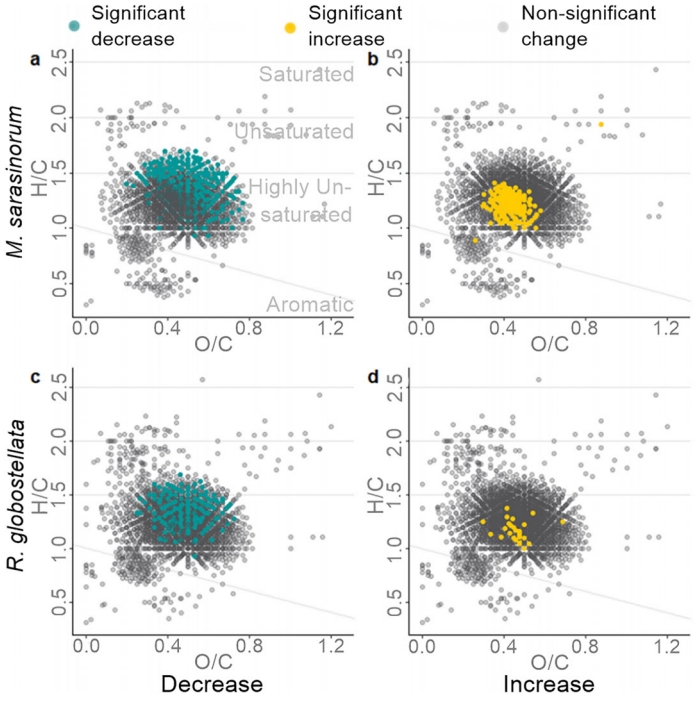

Fig. 6. Van Krevelen diagrams of the statistically significant decreased and increased MFs in *Melophlus sarasinorum* and *Rhabdastrella globostellata* from inhalant to exhalant DOM. Gray lines in the background indicate the saturation status of MFs. Green dots show MFs, which significantly decreased in their relative abundance (p-value < 0.01) and orange dots show significant increases (p-value < 0.01). Gray dots are MFs found in sponge species without significant changes.

(ANOSIM, $R = 0.2$, $p = 0.03$), but contrary to *M. sarasinorum* the 95% confidence interval of the inhalant water samples overlapped with the confidence intervals of both, off reef and exhalant, samples. When all samples from both sponge species were pooled, molecular mass (Fig. 4a) and degradation index (Fig. 4e) increased from off reef via inhalant to exhalant water, whereas H/C ratio, relative amount of lipid-like MFs and C/N ratio in the MFs decreased significantly (Fig. 4b–d, Table 1; Supporting Information Table S2). The apparent radiocarbon age of solid-phase extracted DOC was comparable with ~3000 ^{14}C years in off reef and slightly, although within analytical uncertainty, older in the inhalant fraction with ~3100 ^{14}C years (Fig. 4f; Supporting Information Table S1). On average the exhalent DOM fraction increased in radiocarbon age slightly for *R. globostellata* (3339 ± 746 ^{14}C years) and distinctly for *M. sarasinorum* (3852 ± 457 ^{14}C years). These ages are "apparent" because DOM is a mixture of fractions of various ages and isotopically diverse, with a broad range of potential sources and cycling timescales. While the analytical precision for all replicates was comparable ($2\sigma \sim 200$ ^{14}C years), the variance between replicate analysis seemed to increase from off reef to inhalant to exhalent fractions, shown by the increase in standard deviations of the respective means (Supporting Information Table S3).

There were no trends in richness or Shannon diversity between sample types (Table 1; Supporting Information Table S2). However, functional diversity decreased for the traits "molecular mass" significantly and "H/C ratio" insignificantly from off reef to inhalant, and from inhalant to exhalant water (Table 1; Supporting Information Table S2). Functional diversity of the trait "C/N ratio" in MFs increased significantly from off reef to inhalant, and from inhalant to exhalant water (Fig. 5; Table 1; Supporting Information Table S2).

The intensities of 435 MFs (out of 2450 MFs) and 167 MFs (out of 2748 MFs) changed significantly from inhalant to exhalant DOM, for *M. sarasinorum* and *R. globostellata*, respectively (Fig. 6). In detail, 200 MFs and 23 MFs, defined as highly unsaturated, increased significantly from inhalant to exhalant water for *M. sarasinorum* and *R. globostellata*, respectively. Generally, more MFs decreased significantly in intensities from inhalant to exhalant water, these comprised 157 highly unsaturated MFs and 76 unsaturated MFs for *M. sarasinorum*, and 110 highly unsaturated MFs and 34 unsaturated MFs for *R. globostellata*.

We found 142 significantly decreased or increased MFs, which were shared between the two sample sets of *M. sarasinorum* and *R. globostellata*. Comparing these in relative magnitude, we obtained a positive correlation (Fig. 7). For *M. sarasinorum*, an additional 311 MFs changed significantly in their relative abundance, whereas this number was much lower for *R. globostellata* (30 MFs).

Discussion

Sponges take up bacteria from seawater

Both sponge species removed bacteria from the surrounding water with bacterial cell concentrations decreasing from off reef to inhalant to exhalant water. Cell counts from off reef and inhalant water were slightly lower than the average found for coral reefs worldwide with 9.2×10^5 bacterial cells mL^{-1} (Tanaka and Nakajima 2018), but still in the reported range of $0.57–21 \times 10^5$ bacterial cells mL^{-1} (table 2.4 in Tanaka and Nakajima 2018). While there have been similar reports for other sponge species, we showed for the first time that also the two HMA sponges *M. sarasinorum* and *R. globostellata* filter bacterial cells from the seawater. These bacterial cells are likely a food source (Reiswig 1971, 1975; Pile et al. 1997; Ribes et al. 1999) or can be incorporated into the sponge's microbiome as microbial symbionts (Wilkinson et al. 1984; Wehrl et al. 2007). Future studies should further investigate the microbial communities in inhalant and exhalant waters as well as in the sponges' microbiome to delineate which bacteria are taken up by the sponge, are released, or are incorporated into the microbiome. This will enable linking the different bacterial communities and potential functions within the sponges to differences in DOM composition to gain a better understanding of the processes involving the

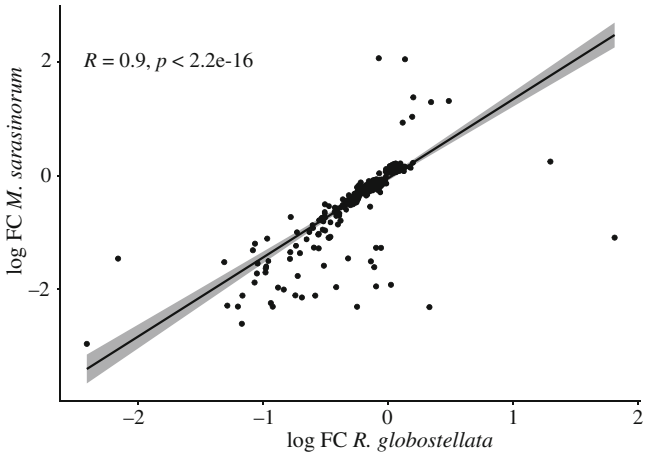

Fig. 7. Significant correlation ($R = 0.91$, $p < 0.001$) of 142 common MFs, which significantly increased or decreased between inhalant and exhalant DOM from *Melophlus sarasinorum* (MS) and *Rhabdastrella globostellata* (RG) on the basis of the logarithmic fold change (Welchs test, *p*-value < 0.01).

sponge holobiont and their role in shaping the biochemical conditions in the reef.

DOM is a food source for Indo-Pacific sponges

Both sponge species removed DOM from the surrounding seawater. The measured reef inhalant DOC concentrations (61.9–110 μmol L^{-1}) were similar to those reported by other studies for ambient seawater taken close to the sponges (Mueller et al. 2014; Morganti et al. 2017; Hoer et al. 2018).

Uptake of DOM is the first step in the sponge loop and has been observed in many different sponges (reviewed in de Goeij et al. 2017). *M. sarasinorum* and *R. globostellata* removed bacterial cells and also took up DOM from the surrounding seawater, as seen with other sponges, where DOC withdrawal accounted for up to 90% of total organic carbon removal (including DOC and particles) in an in situ incubation study (de Goeij et al. 2008). Differences between inhalant and exhalant DOC concentrations of different sponge species were usually between 1.4 and 27 μmol L^{-1} (Morganti et al. 2017; Hoer et al. 2018; McMurray et al. 2018). In our study, the difference was within this range ($\Delta DOC_{M.sarasinorum} = 10.0 \pm 6.3$ μmol L^{-1}, $\Delta DOC_{R. globostellata} = 7.1 \pm 8.3$ μmol L^{-1}). For an accurate determination of rates, the water volume cycled through the sponge needs to be measured precisely, which was not possible in our field study.

DOC/DON ratios were similar between inhalant and exhalant water, suggesting that the sponges take up DOC and DON at similar rates. Off reef DOC/DON ratios were slightly higher due to higher DOC concentrations in off reef water samples. Previous studies also observed only small net differences (−1.8 to 1.4 μmol L^{-1}) between inhalant and exhalant DON concentrations for five common Mediterranean sponge species of which some sponges released and others took up DON (Ribes et al. 2012; Morganti et al. 2017). DIN release by sponges in our study was also similar to previous findings, where values of 0.02–1.86 μmol L^{-1} were reported, often in the form of NO_x as the major nitrogenous waste product (Ribes et al. 2012; Morganti et al. 2017; Gantt et al. 2019). However, a few reports of sponges consuming NO_x also exist (Ribes et al. 2012; Fiore et al. 2013).

Consistently, *M. sarasinorum* and *R. globostellata* also released largely NO_x, possibly as a by-product (reviewed in Maldonado et al. 2012; Zhang et al. 2019). A slight decrease from inhalant to exhalant DON was also observed, indicating that both sponges consumed DON. Most likely, they fulfill their nitrogen demand from the uptake of bacteria in the form of POM or through their symbionts, such as nitrogen-fixing cyanobacteria, as other studies have reported (Freeman and Thacker 2011). However, it is also possible that DOM, including DON, was taken up by the sponge directly and not with the help of microbial symbionts (Achlatis et al. 2019).

Marine sponges target specific DOM constituents

In our study, DOM composition differed between off reef, inhalant and exhalant water for both sponge species (Fig. 3), indicating the uptake or release of specific compounds by the sponge specimens. More parameters were significantly different according to the sample type than according to sponge species, indicating that the two investigated sponge species were more similar to each other than their impact on the DOM composition in exhalant water. The trends were mostly the same, but more pronounced in samples associated with *M. sarasinorum*, which could potentially be explained by the larger osculum of this species, making the sampling of exhalant water easier and possibly more precise. Fiore et al. (2017) obtained similar results with off reef and inhalant DOM compositions being molecularly more similar to each other and more dissimilar to exhalant DOM from two different sponge species indicating a unique signature of the sponge exhalant DOM composition. However, they concluded that the influence of sponges on DOM molecular composition is localized and mainly restricted to the exhalent plume. Furthermore, consistent with Fiore et al. (2017), we found a similar trend in the DOM molecular composition in the exhalant water of both sponge species, which was significantly different to inhalant and off reef DOM molecular compositions (Fig. 3).

As the majority of the MFs were found in all sample types, distinctions between DOM molecular composition were mainly caused by the differences in the relative abundances of certain MFs in our study, similar to previous work (Fiore et al. 2017; Letourneau et al. 2020; Olinger et al. 2021). DOM processing through the sponges was selective to a small degree, as MFs that changed in relative abundance had similar H/C and O/C ratios. However, the two sponge species also targeted a wide spectrum of different MFs with similar characteristics (Fig. 6), making their feeding not only selective towards certain compounds, but also broad towards their preferred MF properties. The relative decrease of unsaturated and

highly unsaturated compounds, in combination with decreasing DOC concentration, is likely caused by a selective uptake of these compounds. The lower H/C ratio in the exhalant water (Fig. 4b) is an indicator of compound saturation, underlining our hypothesis that (highly) unsaturated compounds are likely consumed.

One explanation of the slight decrease in C/N ratios of MFs (Fig. 4d) could be that lipid-like compounds are consumed (Fig. 4c) and nitrogen-containing compounds might be left behind. However, Letourneau et al. (2020) and Olinger et al. (2021) reported exhalant DOM having fewer compounds with a high nitrogen content compared to the ambient DOM, suggesting an uptake of nitrogen-containing compounds by the sponges, and found no lipid-likes in the excurrent-depleted DOM (Olinger et al. 2021). Possibly, *M. sarasinorum* and *R. globostellata*, both containing cyanobacteria with nitrogen fixation capabilities (Steinert et al. 2016; Mohanty et al. 2020), gain their nitrogen demand from their symbionts, thereby reducing the need to take up nitrogen-containing compounds from DOM. Cyanobacteria and other microbial symbionts should further be investigated in regards of their role in DON cycling.

Additionally, sponges seem to target low molecular mass compounds, resulting in a higher mean molecular mass of exhalant MFs (Fig. 4a), which was also reported from *S. vesparium* (Letourneau et al. 2020). Thus, sponges take up fresh material and release DOM, which has a similar molecular mass distribution as refractory deep-sea DOM. The mean molecular mass is lower in the upper ocean, where freshly produced DOM from primary producers prevails and photodegradation takes place, than in the deep sea, where refractory, aged DOM abounds (Martínez-Pérez et al. 2017). This is in line with other studies showing that also bacteria in the water column preferentially take up fresh material (Pontiller et al. 2020).

In our samples, the degradation index increased from off reef, via inhalant to exhalant DOM, indicating that the material was more degraded and resembled in composition a deep-sea reference DOM (NEqPIW, Green et al. 2014) after passing through the sponge holobiont (Supporting Information Fig. S2). The apparent "aging" of the DOM, inferred by the change from a typical fresh surface ocean to refractory deep-sea DOM molecular profile, by the sponge was further supported by changes in the functional molecular diversity of DOM (Fig. 5). According to Mentges et al. (2017), older DOM has lower functional diversity than younger DOM. Functional diversity based on molecular mass and the H/C ratio decreased, meaning that the DOM became molecularly more homogeneous. By pumping water through their tissue and processing the associated DOM, sponges change the molecular composition leading to an apparent "aging" of DOM within minutes, a process that would otherwise take decades to hundreds of years in the surface ocean and during deep water transport.

In the case of *M. sarasinorum*, the systematic uptake of apparently semi-labile to semi-refractory compounds was also age selective. We classified the removed DOM into these subgroups based on the changes in DOM molecular composition and apparent radiocarbon age that resembled the differences between surface and deep ocean, as described more in detail above. *M. sarasinorum* appears to selectively target compounds with elevated radiocarbon content leading to increasing apparent radiocarbon ages of exhalent DOM (Fig. 4f). This observation supports the hypothesis that the fraction of DOM that is considered semi-refractory in the ocean water column may be labile under different environmental settings, such as in sponge holobionts. This was different in the case of *R. globostellata*: the radiocarbon age did not change significantly despite clear changes in molecular DOM composition, which might be largely due to the high variability of $\Delta^{14}C$ within each set of samples. How DOM degradation processes in the oceanic water column and the sponge holobiont differ to result in incongruent age and molecular composition patterns remains to be understood.

Overall, the results demonstrate that sponges play a pivotal role for the transformation of DOM composition in reefs by "aging" the DOM and possibly making it less bioavailable to bacterial communities in the water column.

Conclusions

This study examined the transformation of DOM composition by two sponge species common in the reefs of Guam. First, we confirmed processes that have been previously observed in other sponge species: the uptake of bacterial cells and DOM, and the release of inorganic nitrogen. Second, we studied the DOM transformation through the sponge holobiont on a molecular level. Most likely, DOM processing in sponges is a combination of the sponge cells and the microbial symbionts of the sponge, both providing different physiological functions and should therefore be examined in more detail to disentangle their respective contributions. The two sponge species targeted specific DOM constituents, with a preference for the removal of low-molecular mass compounds with high H/C and high C/N ratios. The increase from inhalant to exhalant DOM in molecular mass and degradation index, and the decrease in the functional diversity of molecular mass and H/C ratio indicate an "aging" of the DOM on MF level. These molecular trends were partially mirrored in changes of apparent radiocarbon ages of SPE-DOC. The apparent radiocarbon age increased consistently from off reef and inhalant to exhalant DOM by about 900 ^{14}C years for *M. sarasinorum*. For *R. globostellata*, the variability of $\Delta^{14}C$ was relatively higher without a significant trend. The rapid apparent molecular "aging" of the DOM through the sponge holobiont challenges the definition of semi-refractory DOM in the water column, because it is cycled like labile material under certain environmental conditions.

Our results emphasize that the DOM lability definition depends on the investigated environments and show that sponges are hotspots of DOM transformation in the ocean.

Data availability statement

The data that support the findings of this study are openly available in Dryad at Hildebrand et al. (2022).

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Conflict of Interest

None declared.

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