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Microbiome changes in a stranding simulation of the holopelagic macroalgae *Sargassum natans* and *Sargassum fluitans*

Inara R. W. Mendonça^{1*}, Tom Theirlynck^{2,3}, Erik R. Zettler², Linda A. Amaral-Zettler^{2,3}, Mariana Cabral Oliveira¹

¹ Department of Botany – Institute of Biosciences – University of Sao Paulo (São Paulo – Brazil).

² NIOZ Royal Netherlands Institute for Sea Research (Texel – The Netherlands).

³ Institute for Biodiversity and Ecosystem Dynamics – University of Amsterdam (The Netherlands).

* Corresponding author: inara.regina@gmail.com

ABSTRACT

Holopelagic *Sargassum* has been causing massive strandings on tropical Atlantic Ocean shorelines. Describing the microbiome associated with *Sargassum* and how it changes after stranding is important to identify potential microbial introductions to coastal environments, as well as sources of potential biotechnological resources. In this study, stranding simulation exploratory experiments were conducted for *S. fluitans* III and *S. natans* VIII on shipboard with minimum external influence. Samples for microbiome identification were collected just after removing healthy *Sargassum* from the seawater (0 hr) and after 24 and 48 hrs of stranding simulation under environmental conditions. The bacterial community was identified by sequencing 16S rRNA gene V3-V4 hypervariable regions, generating a total of 1,565 Amplicon Sequence Variants (ASVs). Of those, 588 were shared between *Sargassum* species and only 25 persisted throughout the stranding. Stranding also changed the dominance of Microtrichales and Rhodobacterales orders at 0 hr to Alteromonadales and Vibrionales after 24 hrs of exposure, the latter representing up to 92% of the relative abundance in the bacterial community. The increase in Vibrionales reinforces the need to monitor stranding sites for any potential pathogenic bacteria. At the functional level, phototrophs were the main group at 0 hr, shifting to chemoheterotrophs and fermentation within the first 24 hrs of *Sargassum* exposure to air conditions. The fermentative groups native to *Sargassum* use stranded biomass as substrate for growth, and therefore constitute the bacteria with higher biotechnological potential.

Keywords: Brown tide, Microbial community, Dysbiosis, High-throughput sequencing, Amplicon Sequence Variants

INTRODUCTION

Sargassum is a genus of brown macroalgae (Sargassaceae, Fucales, Phaeophyceae) comprising more than 350 species (Guiry and Guiry, 2021). Most *Sargassum* species are benthic and grow

attached to a substrate by a structure called holdfast, except for *Sargassum natans* and *S. fluitans*, which are holopelagic (floating throughout their entire life cycle). These species often form floating rafts in open oligotrophic waters and have historically had a geographic range largely confined to the Sargasso Sea. The floating holopelagic *Sargassum* constitutes an ecosystem on its own, with at least 10 endemic species, such as the Angler Fish (*Histrio histrio*), crab species (*Planes minutes*), *Sargassum* shrimp (*Latreutes fucorum*), and *Sargassum*

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pipefish (*Syngnathus pelagicus*) (Coston-Clements et al., 1991; Laffoley et al., 2011). It provides habitat, nursery, and haven for endemic and many other marine organisms in oligotrophic waters with limited floating substrate (Coston-Clements et al., 1991; Godínez-Ortega et al., 2021). For those reasons, it has been named a “Golden Floating Rainforest” by Laffoley et al. (2011).

Floating *Sargassum* is transported by wind and surface currents toward the coastlines in which it strands itself. These stranding events are called “Brown Tides” due to the dark brown color of the accumulated and decaying *Sargassum* biomass (van Tussenbroek et al., 2017). Up until 2011, the stranding events were mostly limited to the Gulf of Mexico and Bermuda, after which massive amounts of both *Sargassum* species started to strand on South American, Caribbean, and African shorelines (Franks et al., 2011; Johnson et al., 2012; Széchy et al., 2012; Milledge and Harvey, 2016), leading to the discovery of a new region of accumulation of *Sargassum* in the tropical Atlantic Ocean. In 2018, Wang et al. (2019) used remote sensing approaches to describe the Great Atlantic *Sargassum* Belt (GASB) in the tropical Atlantic Ocean. It was estimated to be 8,000-km long and to contain more than 20 million metric tons of *Sargassum* biomass. The annual recurrence of *Sargassum* blooms might be the result of changing environmental conditions, including exposure to higher sunlight intensities and seawater temperatures; increased open-ocean upwelling bringing nutrients to the surface; elevated Amazon, Orinoco, and Niger Rivers nutrient inputs; and dust deposition from the Sahara Desert (Oviatt et al., 2019; Wang et al., 2019; Johns et al., 2020).

The reoccurrence of *Sargassum* strandings can threaten coastal environments in cases of massive coastal accumulations. Shortly after stranding, the biomass starts to decompose, turning the water brown, blocking sunlight penetration with subsequent anoxic conditions, release of nutrients, and mass mortality in vulnerable marine communities (van Tussenbroek et al., 2017; Rodríguez-Martínez et al., 2019). After 48 hours onshore, the algae start decaying, releasing toxic gases such as hydrogen sulfide and ammonia, which have been reported to affect the respiratory,

cardiovascular, and neurological systems of humans and other animals roaming around the beach and living in the area (Resiere et al., 2018). Many of the affected regions rely on the removal of *Sargassum* biomass, incurring both monetary and environmental costs. Mexican coastal areas have spent up to 284,000 USD per km on cleaning beaches (Salter et al., 2020). Problems aside, stranded *Sargassum* biomass has also been seen as an opportunity to extract bioproducts such as bioactive compounds, animal feed, fertilizer, and fuel (Milledge and Harvey, 2016).

Large-scale effects of *Sargassum* strandings are an active area of research but we know much less about the contribution of its microbiome to these coastal stranding sites. Recent studies identified *Vibrio* OTUs (Operational Taxonomic Units) that clustered within pathogenic strains in NERR-collected holopelagic *Sargassum* microbiomes (Michotey et al., 2020; Theirlynck et al., 2023), and *Vibrio* pathovars have been identified on different substrates of the Sargasso Sea (Mincer et al., 2023). High abundance of *Vibrio* has also been identified in *Sargassum* stranded in the Caribbean Islands of Martinique and Guadeloupe (Hervé et al., 2021). However, an earlier study in 2010 found no *Vibrio* OTUs among the 20 most abundant bacterial genera associated with the holopelagic *Sargassum* from the Gulf of Mexico (Torralba et al., 2017) indicating that, despite being common in Gulf waters (Johnson et al., 2010; Miller et al., 2021), *Vibrio* was likely present in low abundances in association with *Sargassum*.

The possibility of introducing foreign pathogenic microorganisms imposes yet another threat to coastal regions, alongside possible impacts on the local microbiome, with unknown consequences. The concentration of such opportunistic pathogenic bacteria could increase under global warming conditions (Vezzulli et al., 2013; Archer et al., 2023). However, thermal stress caused seaweed-associated microbiota to undergo dysbiosis, and, even if symbiotic bacteria are present, they are unable to alleviate seaweed stress (Minich et al., 2018; Delva et al., 2023). Holopelagic *Sargassum* microbiome could undergo the same process if stranded. Studies using 16S rRNA gene amplicon sequencing found

that Pseudomonadaceae and Rhodobacteraceae dominated the microbiome of *Sargassum* in the GASB region (Theirylnck et al., 2023). Vibrionaceae and Flavobacteriaceae become dominant once stranded biomass is transferred from the beach to inland storage sites (Hervé et al., 2021). This change in microbial community could result from stranded conditions (e.g., reduced humidity, higher temperature, and decomposition) and the influence of sand, freshwater discharge, and human-related microbiota.

We simulated a stranding event to identify the microbial community changes in *Sargassum* in response to stranding conditions under minimal external microbiota influence. The simulation enabled the identification of the *Sargassum* microbial community from the tropical Atlantic Ocean region, how it changes, and what microbiota is most likely to persist and thrive under stranded conditions in the new stranding sites around the

tropical Atlantic coastlines. We hypothesized that with minimum external influence, the *Sargassum* microbiome undergoes extensive changes in composition, structure, and richness due to reduced humidity and thermal stress caused by stranding.

METHODS

STUDY AREA AND STRANDING SIMULATION

Holopelagic *Sargassum* was collected in the North Equatorial Recirculation Region (NERR) during cruise 64PE455 in the summer of 2019 aboard the NIOZ vessel *RV Pelagia*. The NERR extends from Northern Brazil to the Gulf of Guinea in Western Africa and encompasses the area from approximately 5° S to 10° N. This region is bounded by currents including the South Equatorial Current (SEC), North Equatorial Counter Current (NECC), and North Brazil Current (NBC) (Figure 1) (Franks et al., 2011; Sissini et al., 2017).

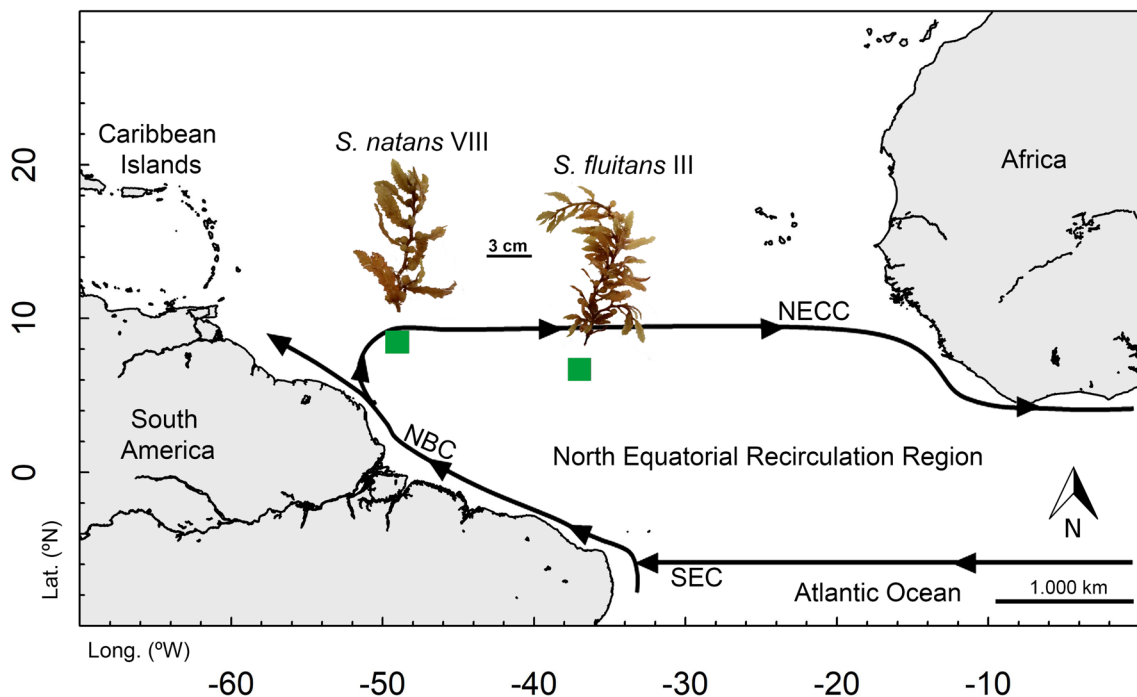


Figure 1. Sampling sites in the tropical Atlantic Ocean. *Sargassum fluitans* III were collected at 6.7400° N -37.0879° W, on 25 July 2019 and *S. natans* VIII were collected at 8.5676° N -49.8546° W, on 4 August 2019 (green squares). The North Equatorial Recirculation Region (NERR) in which holopelagic *Sargassum* accumulates is shown in the center of the North Equatorial Countercurrent (NECC), South Equatorial Current (SEC), and North Brazil Current (NBC). Map source GSHHG, database version 2.3.7, of 2017 (Wessel and Smith, 1996).

Healthy *Sargassum* were collected using a manta trawl sterilized with 10% (v/v) bleach solution and 70% (v/v) ethanol solution, then immediately transferred with gloved hands to clean buckets sterilized with 10% (v/v) bleach solution, 70% (v/v) ethanol solution, then rinsed 5x and filled with ambient surface sea water. *Sargassum* was sorted by species and morphotype following Parr's (1939) and Winge's (1923) descriptions. Vouchers were pressed on paper (free of fixative) and archived at the SPF herbarium - Universidade de São Paulo (USP) under identification numbers SPF 58583 and SPF 58584 (Index Herbariorum, Herbarium Code: SPF <http://sweetgum.nybg.org/science/ih> or list of SPF records can be accessed through <https://specieslink.net/search/>). Shipboard Restriction Fragment Length Polymorphism (RFLP) of the molecular mitochondrial markers *cox2* and *cox3* (Amaral-Zettler et al., 2017) confirmed our morphology-based species identifications of *S. fluitans* III and *S. natans* VIII morphotypes, hereon referred to as Sf III and Sn VIII. A total of 3 kg of Sf III was collected at 6.74° N -37.09° W, on 25 July 2019, and 0.8 kg of Sn VIII were collected at 8.57° N -49.85° W, on 4 August 2019. The CTD Rosette was deployed at each sampling station to measure temperature, chlorophyll a in-vivo fluorescence, and salinity, whereas seawater was collected in Niskin bottles at a 3-meter depth. Seawater was filtered through a 0.2-µm Acrodisc syringe filter and frozen in 7-mL vials at -20 °C for all nutrients except for silica, which was stored at 4 °C. The samples were taken to the Department of Ocean Systems at NIOZ to measure the concentration of phosphate (PO₄), nitrate (NO₃), nitrite (NO₂), and silica (Si) using a TrAAcs 800 autoanalyzer (Bran+Luebbe, WI, USA) following NSOP 9 guidelines (Hydes et al., 2010).

Immediately following collection, phylloids were cut from the branch tips of three different specimens of each morphotype and preserved in silica gel representing time zero (0 hr) samples. After sampling for the 0 hr time point, *Sargassum* biomass of each species was placed in an individual sterilized plastic tray (70 cm x 70 cm) and covered with a nylon net (3 cm x 3 cm mesh) to avoid biomass removal by wind. The trays were placed on the roof of the ship bridge deck to minimize shading and contamination by activities on lower

decks and were left exposed to environmental conditions (Figure S1a, Supplementary Material). After 24 and 48 hrs of exposure, phylloids were sampled from three different clumps collected from inside the *Sargassum* pile and characterized by humidity and decomposition, whereas the outside layer of the pile seemed dehydrated (Figure S1b). All 18 samples (triplicate samples for 0, 24, and 48 hrs for Sf III and Sn VIII) were cleaned by manually removing most of the fouling fauna and were then preserved in silica gel (Quigley et al., 2018) and stored at -20 °C, later transferred to -80 °C at Laboratório de Algas Marinhas "Edison José de Paula" (USP-Brazil).

Environmental conditions, including air temperature, light intensity, biomass weight changes, and incidence of rain, were monitored during the stranding experiment. Air temperature and light intensity were measured using a sensor data logger (HOBO® Logger Onset USA) placed beside the trays, recording measurements at one-minute intervals (Figure S1b). Complementary air temperature data were obtained from the ship meteorological thermometer. The ship thermometer recorded lower values since it was kept shaded and ventilated, unlike the Hobo Logger, which was directly exposed to sunlight (as was the *Sargassum*) (Table S1b). We estimated total biomass weight with three consecutive measurements by suspending the tray from an electronic scale (WeiHeng® mod 128) before each sampling event. The repetition of measurements was necessary to reduce imprecisions caused by ship movement. The trays had drainage holes to enable rainwater to flow away from the *Sargassum* and despite a rainfall after 24 hrs of Sn VIII experiment, its biomass weight decreased by 58%. Sf III had a total of 70% weight loss (unaffected by rain) (Table S1c).

MICROBIOME – DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

The DNA of the microbial community associated with *Sargassum* was extracted and sequenced as a whole, including the endophytic and remaining epiphytic compartments. DNA extraction, PCR amplification, and high-throughput sequencing were performed at the GoGenetic - Biotechnology

Company (Curitiba, Brazil) using the following steps: the phylloids were pulverized with a bead beater Vortex Genie2 (Scientific Industries, NY-USA) followed by DNA extraction using the Quick-DNA Fecal/Soil Microbe Miniprep kit (Zymo) according to the manufacturer's non-soil samples protocol. The PCR reaction and amplification protocol was based on the Earth Microbiome Project protocol (Caporaso et al., 2018). However, we used universal 341F (5'-CAGCCTACGGGNGGCWGCAG-3') and 805R primers (5'-ACAGGACTACHVGGTATCTAA TCC-3') to amplify the V3-V4 regions of the 16S rRNA gene (Herlemann et al., 2011). PCR amplification using GoTaqG2 Mastermix (Promega) was performed with the following cycle settings: 94 °C for 3 minutes; 18 cycles of 95 °C for 30 seconds, 50 °C for 45 seconds, 72 °C for 30 seconds; final extension of 72 °C for 10 min; and hold at 4 °C. The PCR product was verified with gel electrophoresis, and concentrations were quantified on a Qubit 2.0 Fluorometer (Invitrogen, Life technology, CA, USA). The amplicon sequencing was performed on the Illumina MiSeq platform with the MiSeq Reagent 500 V2 Kit, generating paired-end reads (2 x 250 bp). Raw 16S rRNA sequence data are available on the NCBI Sequence Read Archive (SRA-NCBI) under BioProject accession PRJNA1054507, and further sampling information are given in the MIMARKS Table (Table S2).

SEQUENCE ANALYSIS

Microbial community analysis was performed using the Quantitative Insights into Microbial Ecology (QIIME2 version 2022.2.0) bioinformatics platform (Bolyen et al., 2019). The reads were merged, denoised, and chimera checked, and the sequences were clustered into Amplicon Sequence Variants (ASVs) with the DADA2 pipeline (Callahan et al., 2016; 2017). Low frequency ASVs were removed (<10) across samples. All samples were sequenced deeply enough to reach saturation (Figure S2), and rarefaction for alpha-diversity analyses was performed at a sampling depth of 29,050 reads per sample. The ASVs were assigned taxonomy using a pre-trained Silva 138 database (Quast et al., 2013) and naïve Bayes machine learning classifier (Bokulich et al., 2018;

Pedregosa et al., 2011) (Table S3). ASVs matching mitochondria and chloroplasts were removed. The putative function of the microbial community was obtained using Annotation of Prokaryotic Taxa (FAPROTAX v1.1) (Louca et al., 2016). The frequency of each functional annotation was converted into percentage per sampling time, and results were plotted into a heat map using ggplot2 on R (Wickham et al., 2016).

All statistical analyses and construction of graphs were performed on ASV- and order-level data. Statistical analysis were performed in R, version 4.0.2 (R Core Team, 2021). The rarefied ASV table was used to calculate Shannon index and Shannon values applied to a nested one-way Analysis of Variance (ANOVA) with the Time factor (0, 24, and 48 hrs) nested in the top factor *Sargassum* (Sf III and Sn VIII) using the GAD package for nested factors (Sandrini-Neto and Camargo, 2020) after verifying homoscedasticity (Bartlett's test) and normality (Shapiro-Wilk's test) of the data. Significant results were compared with Tukey HSD post-hoc pairwise tests. ASV richness was used to build Venn diagrams using the VennDiagram package (Chen, 2018). ASVs shown as shared between Sf III and Sn VIII underwent further analyses to compare their abundances (number of reads) over time and between *Sargassum* species using a Permutational Multivariate ANOVA (PERMANOVA) (Bray Curtis distance matrix and 9999 permutations).

To describe beta diversity changes in the microbial community, community structure was analyzed by applying total ASV relative abundance data in a PERMANOVA (Bray Curtis distance matrix and 9999 permutations). Total ASV relative abundances were then transformed into presence/absence data to perform a community composition PERMANOVA (Jaccard distance matrix and 9999). A Principal Coordinate Analysis (PCoA) was performed to show the differences between *Sargassum* species and the effect of the stranding simulation. A PERMANOVA statistical test was also used to evaluate microbiome order abundance and presence/absence variations. PCoAs and PERMANOVAs were generated in R statistics using the "vegan" package (Oksanen et

al., 2012) after checking for homogeneity of group dispersions using Betadisper.

RESULTS

The most abundant bacterial orders associated with Sf III 0 hr samples (before the stranding simulation) were Rhodobacterales (23% ± 2.2 sd), Microtrichales (17% ± 1.1 sd), and Caldilineales (11% ± 0.8 sd). A similar distribution occurred in Sn VIII 0 hr samples, with the dominance of Microtrichales (43% ± 3 sd), Rhodobacterales (10% ± 0.8 sd), and Caulobacterales (7% ± 3.6 sd) (Figure 2). After 24 hrs of simulation, the microbiome underwent dysbiosis, causing the drastic reduction in the abundance of most associated bacteria. In contrast, some orders increased in abundance, such as Alteromonadales (Sf III: 9% ± 3 sd; Sn: VIII 4.6% ± 1.5 sd), but nothing compares to

Vibrionales, which reached up to 92% (± 2.4 sd) of the bacterial relative abundance in 24 hrs in Sn VIII. After 48 hrs, Vibrionales was still the most abundant order (Sf III: 68% ± 9.5 sd; Sn: VIII 65% ± 16 sd), and Alteromonadales kept increasing in relative abundance (Sf III: 22% ± 7.9 sd; Sn: VIII 10% ± 1.5 sd). Abundance of orders was overall more similar between *Sargassum* species (PERMANOVA: $p = 0.056$, Table S4) than at different time points during the stranding simulation (PERMANOVA: $p < 0.001$, Table S4). The presence/absence of orders differed between *Sargassum* species (PERMANOVA: $p = 0.007$, Table S4) due to differential frequencies of orders; for example, Synechococcales and Phormidiales are present in all Sn VIII samples and four and three Sf III samples, respectively; on the other hand, Pseudomonadales is present in all Sf III samples and only in five Sn VIII.

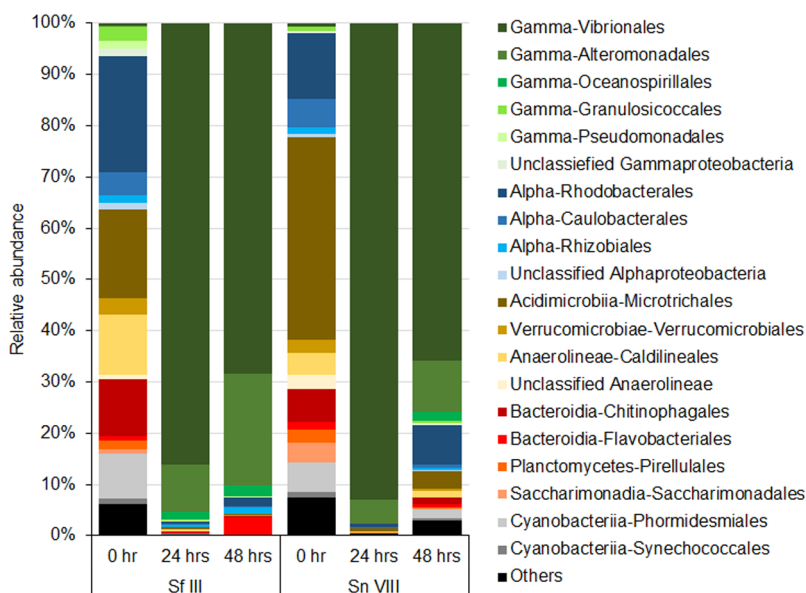


Figure 2. Distribution of bacterial orders associated with *S. fluitans* III and *S. natans* VIII throughout the stranding simulation. Relative abundance (% of reads) of orders associated with *S. fluitans* III and *S. natans* VIII within the stranding simulation sampling times (0, 24, and 48 hrs) ($n = 3$ per sampling time). Only orders with overall abundance of reads $> 1\%$ are shown, the remaining orders are shown under “Others.”

Sequencing generated 2,360,794 raw paired-end reads. This resulted in 1,074,882 high-quality sequences with a mean length of 414 bp (± 17 sd) corresponding to 1,565 ASVs. The PCoA plot in Figure 3 shows the effect of the stranding simulation at the ASV level, revealing that, at 0 hr, the community structure was

very similar in both species, shifting after the beginning of the stranding simulation (Figure 3a). Community composition (based on presence/absence data), on the other hand, differed between *Sargassum* species at 0 hr, and these differences increased throughout the stranding simulation (Figure 3b). PCoA patterns agreed with

the PERMANOVA results, showing significant changes in microbial community structure and composition within the stranding simulation sampling times (PERMANOVA: $p < 0.001$ for both community structure and composition, Table S5). The microbiome also differed when comparing *Sargassum* species because, out of

1,565 ASVs, only 588 occurred in Sf III and Sn VIII (Figure 4, bottom) (PERMANOVA: community structure $p = 0.002$ and community composition $p < 0.001$, Table S5). Despite such sharing, these 588 ASVs had different relative abundances in each *Sargassum* species (PERMANOVA, $p = 0.012$, Table S5).

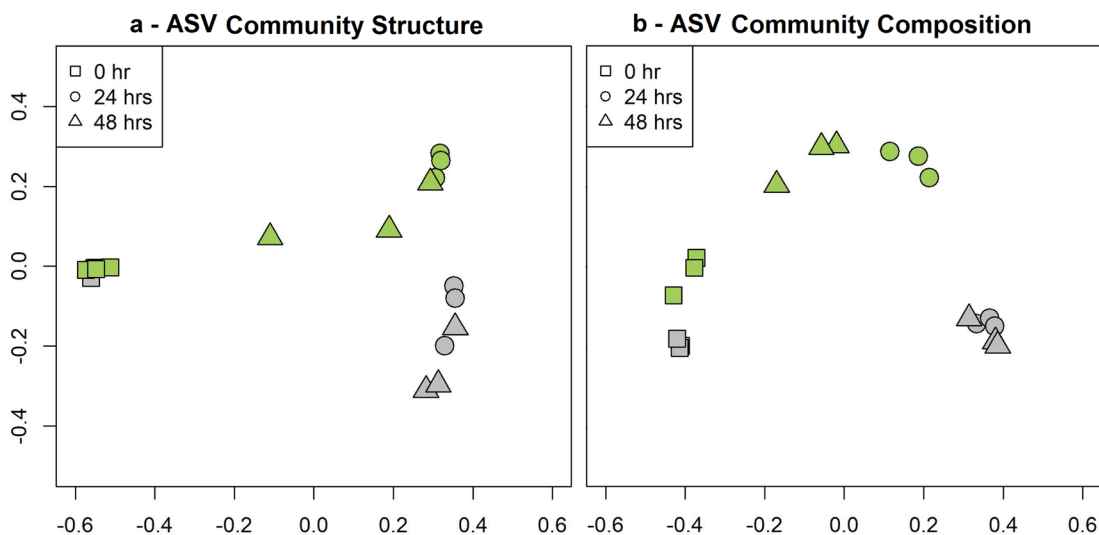


Figure 3. Structure and composition of the *Sargassum* microbiome throughout the stranding simulation. Principal Coordinate Analysis (PCoA) of the bacterial communities associated with *S. fluitans* (Sf III) and *S. natans* (Sn VIII) throughout the stranding simulation. PCoA of community structure based on abundance data (a) and community composition based on presence/absence data and (b) of Amplicon Sequencing Variants (ASVs) associated with *Sargassum*. Grey symbols – Sf III; Green symbols – Sn VIII; Squares 0 hr; Circles 24 hrs; Triangles 48 hrs (18 samples are plotted).

The Shannon diversity index, based on rarefied ASV data, showed similar diversity between *Sargassum* species and a significantly higher microbiome diversity in 0-hr samples than in those after 24 hrs of exposure. Shannon's Index values at 24 and 48 hrs were similar for Sf III (Tukey HSD: $p = 0.99$) (Figure S3; Table S6). The same did not occur for Sn VIII, which showed a higher diversity after 48 hrs than after 24 hrs (Tukey HSD: $p = 0.009$). The increase in diversity is related to richness increasing from 241 ASVs at 24 hrs to 629 ASVs at 48 hrs (Figure 4, side Venn).

Before the experiment began, Sf III and Sn VIII shared 35% of 0-hr ASVs (420 ASVs shared out of the 1,199 ASVs identified at 0 hr) (Figure 4, top). The first 24 hrs of exposure to environmental conditions saw a five-fold decrease in the ASV observed richness for Sf III (0 hr = 941; 24 hrs = 200) and three-fold decrease in that of Sn VIII (0 hr = 678; 24 hrs = 241). Despite drastic

changes in richness and diversity, persistent ASVs were identified throughout the stranding simulation of Sf III (44 ASVs) and Sn VIII (111 ASVs) (Figure 4, center). Among the persistent ASVs, 25 occurred in both Sn VIII and Sf III at the end of the simulation. Those ASVs belonged to the orders Vibrionales (six ASVs), Microtrichales (five ASVs), Rhodobacterales (three ASVs), Caulobacterales (three ASVs), Alteromonadales (two ASVs), Caldilineales (two ASVs), Chitinophagales, Propionibacteriales, Rhizobiales, and Staphylococcales. Among the persistent ASVs, some occurred in low abundances at 0 hr, such as Vibrionales and Alteromonadales (Figure 5a), whose abundance increased after exposure to air from less than 300 reads to more than 5000 reads on average. On the other hand, Microtrichales and Caldilineales showed reduced abundance, from more than 2000 reads to less than 700 reads on average.

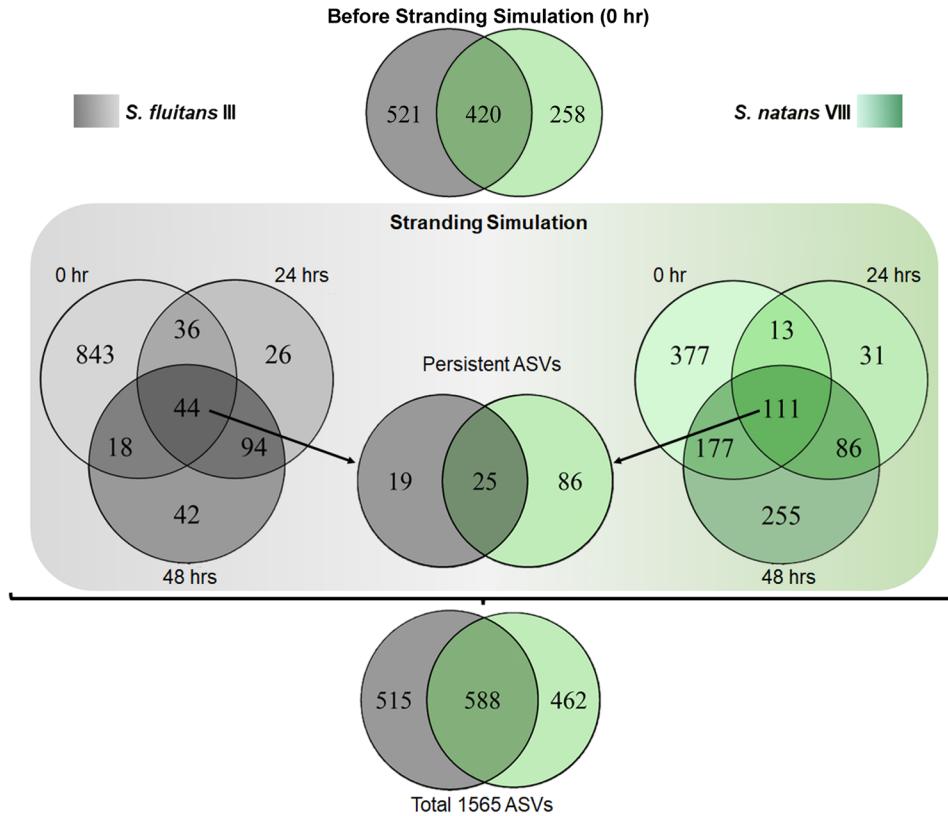


Figure 4. Venn diagram representing amplicon sequencing variant (ASVs) distribution along the stranding simulation in *S. fluitans* (Sf III - left, shades of grey) and *S. natans* (Sn VIII - right, shades of green). At the top center, Venn diagram representing ASVs present at 0 hr, showing that 420 were shared between species before the stranding simulation. The side Venn diagrams show the changes in ASVs in all three sampling times per species and, in the center, the ASVs that persisted throughout the stranding simulation. Among the persistent ASVs, 25 were commonly identified in both Sf III and Sn VIII simulations. The bottom Venn diagram shows the total recovered 1,565 ASVs, 588 of which were identified in both *Sargassum* species at some point during the stranding.

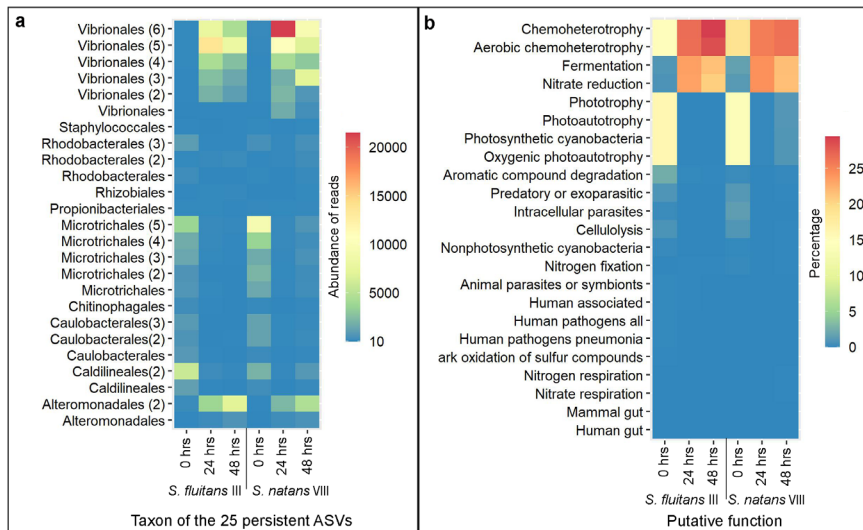


Figure 5. Heatmaps – a) abundance of the 25 persistent Amplicon Sequence Variants (ASVs) at the order level. Numbers within parenthesis indicate different ASVs belonging to a same order. b) Representation of putative functional groups identified throughout the stranding simulation in percentage. Only putative functions with occurrence above 0.02% are shown.

Functional Annotation of Prokaryotic Taxa (FAPROTAX) identified representatives of 40 functional groups (Figure 5b). Members of a taxonomic group can be classified into different functional groups, as with 22 cyanobacteria assigned to four functional groups (phototrophy, photosynthetic cyanobacteria, photoautotrophy, and oxygenic photoautotrophy). These 22 cyanobacteria were common at 0 hr but less common after the beginning of the stranding simulation. The aerobic chemoheterotrophy and chemoheterotrophic taxa present at 0 hr such as *Vibrio* sp., *Alteromonas* sp., and *Pseudoalteromonas* sp., increased in abundance during the stranding simulation; for that reason, various chemoheterotrophic taxa also occurred within the 25 persistent ASVs. Lastly, fermentation and nitrate reduction functional groups comprised 18 and 14 reported taxa, respectively. However, only two were abundant and they both belong to the *Vibrio* genus. The remaining 32 functional groups had an overall abundance below 3%.

DISCUSSION

This study characterized the dysbiosis in the microbial community associated with holopelagic *Sargassum fluitans* III and *S. natans* VIII under a simulated stranding event with minimum external influence. The dysbiosis of the microbial community of Sf III and Sn VIII happened in just 24 hrs of exposure to air conditions. The major outcome for both species was a drop in diversity of bacterial orders and a shift of dominant and functional groups. The results were similar between Sf III and Sn VIII even though they were collected approximately 12° of longitude apart and experiments took place 10 days apart from each other.

Many microorganisms identified before the simulation (0 hr) were not recorded or had a drastic reduction in abundance after the stranding simulation, except for Vibrionales, Alteromonadales, and Oceanospirillales, whose relative abundances increased. *Vibrio* quickly became the dominant genus after just 24 hrs. Mean temperatures of 30 °C recorded at the site favored *Vibrio* proliferation, a genus known to grow in higher sea surface temperatures up to 40 °C

(Oliver et al., 2012; Percival and Williams, 2013). Even though we were often unable to classify to the species level using V3-V4 16S rRNA, potential pathogenic strains of *Vibrio* have been reported associated with holopelagic *Sargassum* (Michotey et al., 2020; Hervé et al., 2021; Theirlynck et al., 2023). A more recent study sequenced the genome of 16 *Vibrio* spp. isolated from Sargasso Sea substrates (*Sargassum* sp., leptocephalus eel larvae, and plastic marine debris) and found the genomes were closely related to the pathogens *V. alginolyticus*, *V. campbellii*, *V. fortis*, and *V. parahaemolyticus* (Mincer et al., 2023). They identified pathogenic genes, including adhesion, toxin, hemolysis, and phospholipases, that together make *Vibrio* a potent opportunistic pathogen. Furthermore, all 16 isolates had alginate lyase genes, enhancing the probability of *Vibrio* using *Sargassum* as a source of carbon. In addition to Vibrionales, Alteromonadales is known for its pathogenic genera such as *Pseudoalteromonas* and *Alteromonas* (Sawabe et al., 2000; Wang et al., 2008). It is necessary to thoroughly investigate *Vibrio* at stranding sites to ascertain the possible increase in Vibrionales and Alteromonadales (as in this study) and determine their potential pathogenicity (Mincer et al. 2023). This is very important given efforts to harvest stranded *Sargassum* biomass under climate change scenarios, which will likely lead to increasing reports of *Vibrio*-related illnesses (Vezzulli et al., 2013; Baker-Austin et al., 2018; Deeb et al., 2018, Archer et al., 2023).

Our results provide a baseline for newly stranded *Sargassum*-associated microbiome because we controlled for some variables such as morphotype, aging, and coastal influences (sand, freshwater discharge, and human activity-associated microbes) thus reducing exposure to external sources of microbes, except open ocean airborne and sea spray microbiome (Uetake et al., 2020). A previous study by Hervé et al. (2021) collected *Sargassum* samples near the shore ($n = 30$) and stranded on the beach ($n = 9$) in the Caribbean Islands with unknown stranding dates. They found a predominance of Vibrionales but in lower proportion (18%) than in our results (>60%). They also collected nine samples from four inland

Sargassum storage sites (Martinique) both from the top, as well as the middle of *Sargassum* piles, and the material was partially or completely dried, making the separation of morphotypes and aging difficult. In this mix of inland *Sargassum*, they identified a predominance of Flavobacteriales, an order that only started to show some minor increase after 48 hours in our experiment. Therefore, time and condition of storage are important factors to be included in future research and experiments. At the functional level, Herve's and our results showed similar pattern, with phototrophy and chemoheterotrophy dominating in fresh *Sargassum*. Phototrophic individuals were less representative after 24 hrs of the stranding simulation, as well as in the inland storage of *Sargassum* biomass in Martinique, probably due to desiccation and diminished light penetration in the interior of the pile.

Among the chemoheterotrophic lineages, we identified Verrucomicrobiales, a known producer of fucoïdan-degrading enzymes (Sichert et al., 2020). Fucoïdians are common brown algal cell wall polysaccharides with bioactive effects with therapeutic, anti-inflammatory, antioxidant, and anticoagulant properties (Hou et al., 2012; Ale and Meyer, 2013). Verrucomicrobiales decreased in relative abundance when the stranding simulation began but still occurred at the end of the experiment. The Verrucomicrobiales remaining after 48 hrs of stranding simulation tolerated drying conditions and could be an option for *Sargassum* fucoïdan degradation. Not only fucoïdians, but also bacterial fermenting alginates are of growing interest to produce low molecular weight prebiotics and third-generation bioethanol (Takeda et al., 2011; Ramnani et al., 2012; Orozco-González et al. 2022). Some target bacteria for alginate degradation include *Pantaea* sp. (Zhang and Zhang, 2018), *Bacteroides* sp. (Li et al., 2016), and various marine *Vibrio* spp. (Wargacki et al., 2012; Doi et al., 2017; Zhuang et al., 2018). Even though we found no *Pantaea* and *Bacteroides* among the generated sequences, we identified *Vibrio* and Alteromonadales as the main fermentation representatives.

A concern related to *Sargassum* degradation is the production of hydrogen sulfide, which is a toxic gas (Thompson et al., 2020). We identified only

three taxa related to the sulfur cycle and always in low abundance (*Sulfitobacter*, *Thiothrix*, and *Citricella*), indicating that more efficient hydrogen sulfide-producing bacteria are likely found in coastal waters and not associated with *Sargassum* from the open ocean. Hervé et al.'s work (2021) supported this hypothesis with the identification of sulfur-respiring microorganisms in nearshore water samples, as well as in stranded *Sargassum*.

Contrary to expectations, the richness of ASVs associated with Sn VIII increased after 48 hrs of exposure to the air, driven by 255 ASVs that appeared only in the 48-hr samples. The effect of richness increase is visible in the PCoA plot (Figure 3), in which 48-hr samples were placed between 0 and 24 hr. It is important to point out that these exclusive Sn VIII 48-hr ASVs showed low abundance. Therefore, this increase in richness could have originated from airborne microbiota introduced with the rain that occurred after 24 hrs. Another possibility is associated epifauna introducing another source of variability. Sn VIII and Sf III are encrusted by different species and quantities of hydrozoans and bryozoans likely harboring different microbiomes (Mendoza-Becerril et al., 2020; Salter et al., 2020). Even though most of the associated fauna was manually removed, any remaining faunal microbiomes would have been sequenced. In summary, the richness increase could have stemmed from the variation within the *Sargassum* clumps, and our sampling design prohibited the identification of the variation source.

Sargassum fluitans III and *S. natans* VIII before the beginning of the simulation (0 hr) showed a predominance of Proteobacteria, Actinobacteriota, Chloroflexi, Bacteroidota, and Cyanobacteria. Similar phylum-level dominance patterns have been identified in holopelagic *Sargassum* spp. collected in 2018 in stranding sites at the Caribbean Islands (Hervé et al., 2021), open ocean collected in 2017 and 2019 (Michotey et al., 2020; Theirlynyck et al., 2023) and in the Gulf of Mexico in 2010 (Torralba et al., 2017). This pattern has been identified in benthic *Sargassum muticum* sampled in Portugal (Serebryakova et al., 2018) and *S. hystrix* and *S. furcatum* sampled in Martinique Island (Michotey et al., 2020).

Despite phylum-level similarities, a 70% divergence at the ASV level occurred between Sf III and Sn VIII before any manipulation (0 hr). The *Sargassum* species were collected at different sites (a distance of 12° of longitude), so that could account for some of the observed inter-species differences. Previous studies have reported species, morphology, and biogeography as sources of microbial variations in holopelagic *Sargassum* species (Michotey et al., 2020; Li et al., 2022; Theirlynck et al., 2023). However, it is unlikely that the water column microbiome itself could affect our results since water and macroalgal microbiomes greatly differ (Minich et al., 2018; Michotey et al., 2020; Quigley et al., 2020). Other less explored sources of microbial variation could be related to the competitive lottery model, in which *Sargassum* microbial associations are functionally related and not necessarily taxonomically similar (Burke et al., 2011; Ghaderiardakani et al., 2017). Morphologically different structures can also harbor unique strains of bacteria (Serebryakova et al., 2018; Quigley et al., 2020). In summary, even though *Sargassum* morphotypes become intertwined in open ocean waters, their microbiomes are different, but respond similarly to stranding conditions.

CONCLUSION

Our study presents a baseline of the microbial composition in decaying *Sargassum* before it hits the coastal areas, with reduced exposure to external sources of microbes. The stranding simulation caused microbiome dysbiosis, reducing richness and drastically changing the dominant bacterial groups in the first 24 hrs. Due to the increase in Vibrionales abundance, we emphasize the need of stranding event monitoring to verify the presence of potentially pathogenic bacteria. The change in dominance is likely caused by bacteria with alginate-degrading capacity and resistance to the new set of environmental conditions (e.g., Verrucomicrobiales, Vibrionales, and Alteromonadales). Isolation of these associated bacteria may lead to identification of useful fucoidan degrading bacteria and the possible use of *Sargassum* stranded biomass as a source of bioactive compounds.

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AUTHOR CONTRIBUTIONS

I.R.W.M.: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – Original Draft.
 T.T.: Investigation, Writing – Review & Editing.
 E.R.Z.: Conceptualization, Investigation, Methodology, Writing – Review & Editing.
 L.A.A.Z; M.C.O.: Conceptualization, Funding Acquisition, Project Administration, Resources, Supervision, Writing – Review & Editing.

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