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Bacterial communities on *Fucus* sp. harvested in tidal zones with or without exposure to human sewage in Greenland

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ABSTRACT

Seaweed from Greenland has potential as a food source. However, human sewage is discharged directly to the sea in the vicinity of the communities, a practice which could lead to the seaweed becoming contaminated by human pathogens. The objective of this study was to investigate the effect of wastewater discharge on the bacterial communities of wild populations of *Fucus* sp. sampled in the tidal zones in the vicinity of Sarfannguit, a smaller settlement (~110 inhabitants) with limited discharge, and Sisimiut, Greenland's second biggest town (~5500 inhabitants). Fecal indicator bacteria (coliforms, *Escherichia coli* and the human fecal molecular marker HF183 [*Fucus* sp. only]) were consistently detected on *Fucus* sp. and in seawater from Sisimiut. In contrast, coliforms and *E. coli* were only detected once in samples collected close to the waste dump site in Sarfannguit. Presence of fecal indicator bacteria in seawater and on *Fucus* sp. coincided, indicating the utility of surveying seawater. MALDI-TOF mass spectrometry analysis of bacterial isolates identified fish and human pathogens on seaweed from Sisimiut while >80% of isolates from Sarfannguit could not be identified using existing data bases. Amplicon sequence variants belonging to *Rhodobacteraceae* and *Flavobacteriaceae* were dominant families in all *Fucus* sp. samples. However, wastewater discharge effected major changes in the overall composition of the seaweed microbiota as evidenced by analysis of the beta diversity. In conclusion, the microbiota on *Fucus* sp. harvested in the intertidal zones in a small, relatively unimpacted community, and close to the wastewater discharge of a larger community showed marked differences and the presence of human pathogens on sewage impacted *Fucus* sp. from the large community. It is recommended that microbiological criteria and guidelines regarding suitable seaweed harvest and cultivation sites be established, especially considering potential sources of anthropogenic impact on the local marine environment.

1. Introduction

Seaweeds can become a valuable food source for Greenland for local consumption and export. Greenland has an extended coastline of approximately 44 000 km (Bolatta and Kleemann, 2020), and ample areas that could be suitable for seaweed harvest and/or cultivation. Several seaweed species of interest for human consumption can be found in Greenland waters (Andersen et al., 2019; Kreissig et al., 2021; Whitecloud and Grenoble, 2014). While harvest of some wild seaweed species such as the sublittoral *Saccharina latissima* (sugar kelp) requires a boat, and harvesting or diving gear, other edible species such as *Fucus* sp. grow in the intertidal zone and are accessible directly from the shoreline, also close to settlements.

At present, wastewater treatment is non-existent in Greenland. Wastewater is discharged untreated into recipients such as the sea, bays or fjords from towns and settlements, whose sizes range from 11 to 18 800 inhabitants (Chhetri et al., 2018; Statistikbanken, 2022). Most of the population in the larger communities (>1500 inhabitants) have sewer service, and the sewer outlets are typically placed directly by the shoreline to reduce maintenance issues in the harsh climate. The sewer service in the smaller (<1500 inhabitants) communities is often limited to a few public buildings and local fish processing factories. Sewering of old neighborhoods of the larger communities also is still deficient. Nevertheless, the blackwater is mostly still collected and emptied untreated into adjacent recipients. The environmental

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Table 1
Seaweed and seawater sampling sites in Sarfannguit and Sisimiut, Greenland.

Location	Site	Latitude	Longitude	Location of harvest site	Wastewater load (PE[a])	Sampling events[b]	
						2017	2018
Sarfannguit (control)	C	66.896311	−52.857659	Island East coast	0	1	1
Sarfannguit	A	66.897536	−52.874138	Below waste dump	100[c]	1	1
	B	66.898226	−52.858431	Fish factory	0	1	1
Sisimiut	D	66.928316	−53.673514	Wastewater outlet next to dump site	3000	2	1
	E	66.943028	−53.651677	Wastewater outlet in bay	2000	2	1

[a]PE, person equivalents referring to the wastewater deposition on the sites.
[b]Number of sampling events where multiple samples ($n \geq 3$) of seaweed and seawater were retrieved.
[c]Discharged on land followed by downhill infiltration for approximately 100 m.

impact from nutrients is believed to be low due to the coastal location of communities and low population density (Qeqqata Kommunia, 2020). However, direct discharge of untreated wastewater contributes microbiological, physical and chemical contaminants into the Arctic recipient (Gunnarsdóttir, 2012; Jensen et al., 2013; Neudorf et al., 2017) and can lead to public health concerns (Daley et al., 2019). It is not known how far the microbial pollution from untreated wastewater emissions reaches and how it affects the food safety of seaweeds harvested in the proximity of human settlements.

Seaweeds have an associated epiphytic bacterial community (Egan et al., 2013; Hollants et al., 2013; Singh and Reddy, 2016), which amongst others, often consists of *Gammaproteobacteria* and *Alphaproteobacteria*. This resident microbial biofilm is important for the health and defence of its host (Egan et al., 2013; Hollants et al., 2013; Singh and Reddy, 2016) and may also affect the ability of human pathogenic bacteria to become associated with the algae. An assessment of risks associated with seaweed production led to the identification of four major hazards, namely arsenic, cadmium, iodine and *Salmonella* (Banach et al., 2020). Only iodine was found to be an issue in our recent study, which analyzed the content of microelements in 10 different species of Greenlandic seaweed (Kreissig et al., 2021). *Salmonella* sp. are human enteric pathogenic bacteria and commonly linked to fecal environmental contamination. An outbreak of salmonellosis has been linked to a seaweed farm in Hawaii (Nichols et al., 2017). Other foodborne pathogens have been identified as potential hazards in fresh seaweed, including norovirus (Park et al., 2015; Somura et al., 2017), *Vibrio parahaemolyticus* (Barberi et al., 2020; Mahmud et al., 2007), *Bacillus cereus* (Choi et al., 2014) and *Escherichia coli* (Barberi et al., 2020). Overall, there is a knowledge gap of potential microbial hazards associated with seaweed production.

The Government of Greenland has set out some basic rules for licensing of companies that harvest or cultivate seaweed. These rules include the requirement to evaluate the risk of the possible presence of health hazards of endogenous and anthropogenic origin, however, there is currently no specific regulation governing seaweed utilization. Seaweed that is to be exported to the European Union would generally have to comply with the EU regulations, i.e., EC No. 2073/2005 concerning microbiological criteria for foodstuffs (European Commission, 2005). However, this regulation does not contain a food category specifically devoted to seaweed. In Europe, very few rules and guidelines defining suitable hygienic seaweed harvest or cultivation areas exist (Barbier et al., 2020). Barbier et al. (2019) suggested that it would be unnecessary to monitor *Escherichia coli* as part of a future European ecological seaweed regulations, since seaweed does not accumulate bacteria and viruses in the way that filtering bivalves (e.g., blue mussels and oysters) do. In Denmark, the Danish Veterinary and Food Authority studied the presence of *E. coli* and *Salmonella* on different seaweed species in 2017 and concluded that no specific areas were deemed unsafe for seaweed harvest, except for areas located in the vicinity of point pollution sources such as sewage disposal points and harbours (Danish Veterinary and Food Administration, 2018). In Scotland (United Kingdom), the Seaweed Cultivation Policy Statement (SCPS) identified the contamination risk for seaweed from sewage and other effluents, and recommended that seaweed cultivation sites be situated within designated shellfish waters with a monitoring program with regards to water quality (Scottish Government, 2017). Knowledge about the risk and prevalence of enteric pathogens associated with seaweed harvested close to communities in Greenland is lacking. To address this gap in our knowledge, the aim of the present study was to investigate the influence of wastewater emissions on the microbial communities associated with wild *Fucus* sp. in Greenland. We compared the content of fecal indicator bacteria and the microbiota in seaweed and seawater samples, which were collected from four sewage impacted sites located adjacent to Sarfannguit (∼110 inhabitants) and Sisimiut (∼5500 inhabitants) situated within 40 km of each other. Samples were retrieved in August and September 2017, and in August 2018, and included samples from a non-sewage impacted site in Sarfannguit for comparison.

2. Materials and methods

2.1. Samples and sampling locations

Sampling was done at three locations (A, B and C) in Sarfannguit, a smaller settlement situated on an island with 111 inhabitants, and two locations (D and E) in Sisimiut, the second biggest town in Greenland with 5491 inhabitants in 2018 (Statistikbanken, 2022) (see Table 1 and Fig. 1). Both communities lie in Qeqqata municipality on the West Coast of Greenland (Bolatta and Kleemann, 2020). Sampling site C (control) was chosen as this study's control site, as it is located in Sarfannguit at the Eastern coastline of the island in an unpolluted and presumed pristine location with no obvious influence from point pollution sources.

Among Sarfannguit impacted sites A and B, Site A was directly below the discharge site for blackwater in Sarfannguit. The inhabitants in Sarfannguit rely on bag toilets, which are collected and emptied on land, a practice implemented due to the local population's concern for the water quality in the nearby sea, which they rely on for fishing. The idea is for the wastewater to percolate through the soil down the hill and in this way reduce the contamination load to the sea. No evaluation of the efficiency of the method has ever been made. Site B was by the wastewater outlet from the local fish factory, which discharges fish processing wastewater only. All three sites in Sarfannguit are subject to high currents and water exchange. In fact, the name Sarfannguit translates into "The place by the high currents".

The sewage impacted sites in Sisimiut consisted of sampling sites D and E. Site D is located 50 m from a sewage outlet next to

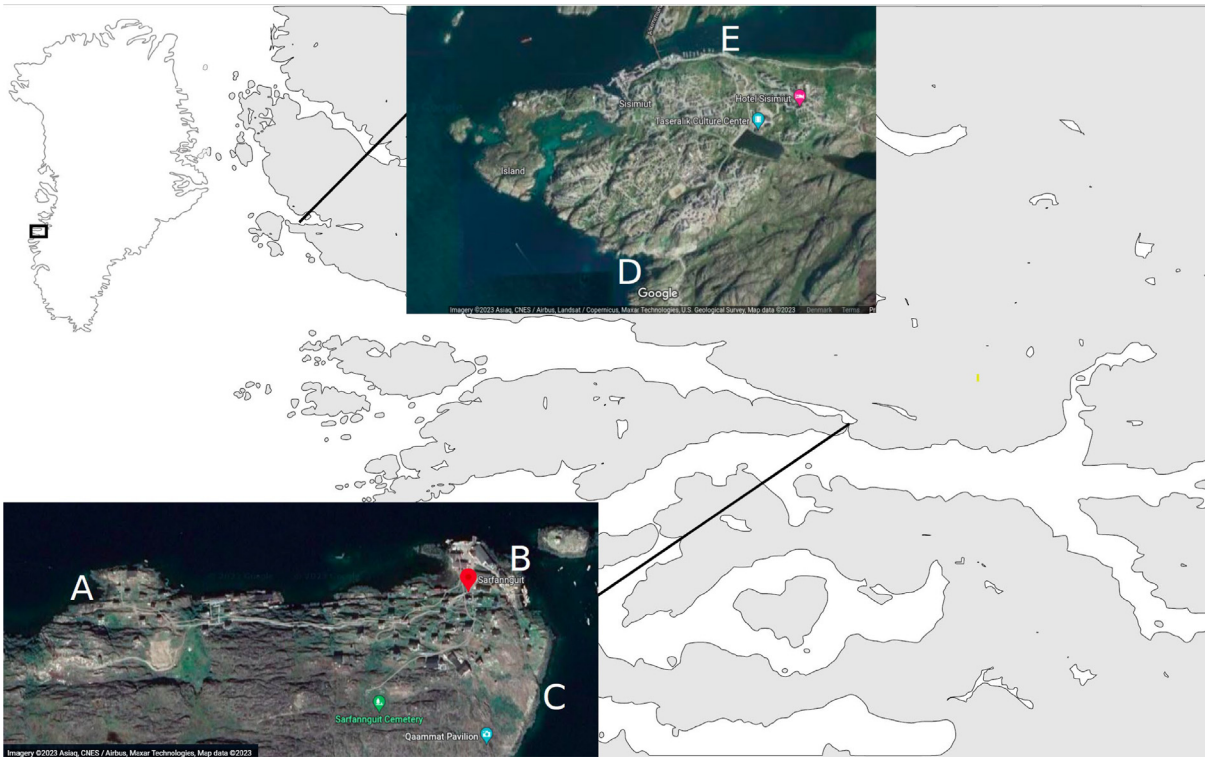

Fig. 1. Location of sampling sites in Sarfannguit (C – control, A – below waste dump, B – fish factory) and Sisimiut (D – next to dump site, E – wastewater outlet in bay). The black box on the large map of Greenland denotes the location of the communities, while the inserts show the individual sample locations in each community. The inserts are reproduced with permission from google maps.

the dump site. In addition to sewage, this site receives blackwater from the 150 households with honey bucket toilets and the 300 households with tank and haul service for a total load equivalent of 3000 person equivalents (PE). Despite the outlet being directly by the open sea at a site with high currents, the recipient is aesthetically impacted and has been evaluated to be in the category III "less good" ecological status according to the Norwegian official categorization (Miljødirektoratet, 1997; Qeqqata Kommunia, 2021). Sisimiut Site E is located about 25 m from a wastewater outlet in Kangerluarsunnguaq Bay, which receives a load of 2000 PE including the wastewater from the community hospital. Both the water exchange and depth are low in this bay, and the status of the site is visually impacted. Like site D, it has been evaluated to be in the category III "less good" ecological status according to the Norwegian official categorization. The sewage from sites D and E has previously been documented to contain 5.4–6.5 log most probably number (MPN) of total coliforms/mL, 4.5–5.5 log MPN of *E. coli*/mL, and 4–5 log MPN of enterococci/mL (Jensen et al., 2013). The presence of bacteria resistant to ampicillin, tetracycline and ceftriaxon has also been observed next to both outlets (Jensen et al., 2013).

Seaweed and water samples were collected using aseptic techniques at low tide in August 2017, September 2017 (only Sisimiut) and September 2018. The seawater (1 L) was collected into presterilized Nalgene bottles (VWR, Denmark), and the seaweed (approximately 500 g, 3 to 4 individuals) was collected into sterile stomacher bags (Seward, Fisher Scientific, Denmark) after cutting the algae with sterile (70% v/v ethanol) scissors or a knife. Samples were kept in a cooler (maximum 5 °C) and transported to the DTU Sisimiut Campus laboratory and processed for microbiological analyses (see Sections 2.2–2.3) within 24 h of sampling (Sisimiut samples) or 24 to 36 h of sampling (Sarfannguit samples). Fresh seaweed samples were in September 2018 transported cooled (3 °C, approximately 12 h) back to the DTU's campus in Kgs. Lyngby, Denmark for identification of bacterial isolates.

2.2. Preparation of seaweed samples for culture and culture-independent microbiological analyses

To remove microorganisms, 20 g seaweed was mixed with sterile aliquots of 20-mL phosphate buffered saline (PBS, for 2017 samples) or 20-mL peptone saline (PS, 0.1% (w/v) peptone (Oxoid, Denmark), 0.85% (w/v) NaCl (Sigma, Denmark) for 2018 samples) in a stomacher bag (Seward) and massaged by hand for one minute. This method was tested in preliminary trials and shown not to result in significantly ($p > 0.05$) different counts when compared to stomaching seaweed samples for one min (data not shown).

2.3. Enumeration of total aerobic count, coliforms, and E. coli

The total bacterial load and fecal bacterial load in the seaweed and seawater samples were enumerated on Petri films. Briefly, for each site, one mL of the dilution liquid (PBS or PS), and subsequent suitable tenfold dilutions from the stomacher bag, or one mL of seawater was inoculated on 3M™ Petrifilm™ Aerobic Count Plates (AC) (3M, St. Paul, Minnesota, USA) and 3M™ Petrifilm™ E. coli/Coliform Count Plate (EC) according to manufacturer's instructions. In 2017, counts of psychrophilic bacteria were obtained for selected samples by incubation of the AC plates at 3 °C for 7 days. Mesophilic counts on AC plates were obtained for all seaweed samples and most seawater samples after incubation at 35 °C for 48 h. The mesophilic count indicates recent fecal contamination, i.e., microorganisms growing at temperatures closer to that of the mammalian body temperature. EC plates were incubated at 35 °C for 24 h followed by the counting of typical blue and/or red colonies with gas bubbles (EC plates). Counts were log-transformed and reported as log colony forming units (CFU) per g seaweed or mL seawater.

Table 2
List of culture-independent methods, gene targets, and primers ('5–3') used to analyze DNA extracted from microbial communities present on *Fucus* sp. harvested in Greenland.

Method	Target	Purpose	Primer and probe	Reference
qPCR	16S rRNA	Total bacterial count	Forward: CGGTGAATACGTTCYCGG, Reverse: GGWTACCTTGTTACGACTT	Suzuki et al. (2000)
	HF183	Human fecal indicator	Forward: ATCATGAGTTCACATGTCCG, Reverse: CTTCCTCTCAGAACCCCTATCC, Probe: FAMCTAATGGAACGCATCCC-BHQ1	Seurinck et al. (2005) and Layton et al. (2013)
Amplicon sequencing	V1-V3 regions of 16S rRNA	Bacterial community	Forward: AGAGTTTGATCATGGCTCAG, Reverse: GTATTACCGCGGCTGCTG	Leser et al. (2002) and Weisburg et al. (1991)

2.4. Identification of bacterial isolates using matrix-assisted laser desorption/ionization - time-of-flight (MALDI-TOF)

In 2018, bacterial colonies were picked from AC plates used for enumeration of mesophiles on seaweed samples and subcultured on marine agar (MA, PanReac AppliChem ITW Reagents 414 680.1210, Darmstadt, Germany). Single colonies (up to thirty per seaweed sample location) were isolated, re-streaked on MA to cultivate and check purity (15 °C, 3 days) and preserved by scraping colony mass into cryopreservation beads (Microorganism Preservation System – Protect, Technical Service Consultants Ltd, Lancashire, UK) for long-term storage at −80 °C. Prior to identification by MALDI-TOF, isolates were thawed and subcultured on MA at 15 °C for 3 days. Proteins from colonies from fresh plates were extracted with the ethanol/formic acid/acetonitrile protocol used by Nonnemann et al. (2019), as described by Bizzini et al. (2010). Mass spectra were produced with an Autoflex Speed instrument (Bruker Daltonics, Billerica, Massachusetts, USA) and identified using the Main Spectrum Database (DTU-Vet MSP Database) housed at the National Veterinary Institute, Technical University of Denmark, as described in Nonnemann et al. (2019). Log scores between 0 and 3 were calculated by the Biotyper algorithm (Bruker Daltonics, Bremen, Germany). Log scores <1.7 do not lead to identification. Log scores $1.7 \leq x < 2.0$ provide genus identification, while log scores ≥ 2.0 provide species identification.

2.5. Culture-independent microbiology

Deoxyribonucleic acid (DNA) was extracted from two mL of the seaweed-diluent mix in the stomacher bags. Briefly, a cell pellet was obtained by centrifugation at 9614 × relative centrifugal force (rcf) (Hettich Mikro 185, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) for 10 min. The supernatant was carefully removed, and the pellet resuspended in 200 μL of the remaining liquid in the tube. These 200 μL were then transferred to the DNEasy PowerBead tube of the Qiagen DNEasy PowerSoil Kit (Qiagen, Hilden, Germany). DNA extraction was carried out following the manufacturer's protocol. DNA was also extracted from a field control sample consisting of the diluent alone. All DNA extracts were stored at −20 °C until analysis.

Quantitative PCR (qPCR) was used to enumerate the 16S rRNA gene copy numbers as a measure of the total bacterial count (Neudorf et al., 2017) and gene copy numbers of the human-specific fecal indicator marker, HF183, which detects the 16S rRNA copies from human fecal *Bacteroidales* species (Seurinck et al., 2005).

For qualitative analyses of the seaweed bacterial communities, 16S rRNA amplicon sequencing was carried out on the Illumina Miseq platform. Table 2 lists the different primer sets.

2.6. Enumeration of the culture-independent total bacterial community and presence of human fecal bacteria by qPCR

Quantification of 16S rRNA and HF183 gene copy numbers were performed in optical tubes and caps (Agilent Technologies, Santa Clara, California, USA) on a Stratagene Mx3005P™ qPCR System (Agilent Technologies). Each sample run included positive plasmid standards (see below), non-template controls and water-only wells. All samples were determined in technical duplicates.

For enumeration of the 16S rRNA gene copy numbers, each qPCR reaction consisted of 9.5 μL nuclease free water, 12.5 μL EvaGreen® master mix (Type-it HMR, Qiagen, Hilden, Germany), 1 μL of each of 0.4 μM, forward and reverse primers and 1 μL of sample DNA for a total reaction volume of 25 μL. The temperature program consisted of initial denaturation for 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 50 °C, and 30 s at 68 °C, then 5 min at 68 °C. During the melting curve analysis, the temperature was increased from 45 to 95 °C. Gene copies per gram seaweed were calculated based on a standard curve that was constructed using 10-fold dilutions of a plasmid DNA extracted from an *E. coli* DH5α culture containing a positive control plasmid (pCR2.1, TOPO TA PCR 2.1) with the inserted gene of interest (gift from Dr. C. Yost, University of Regina, Canada). The standard curve was determined from triplicate measurements of 1 to 1×10^9 gene copies per reaction. The qPCR efficiency was 113%, with R^2 value of 0.993. The limit of quantification (LOQ) and limit of detection (LOD) were 10^4 and 10^3 gene copies per reaction, respectively. In the seaweed samples this corresponded to a LOD of 10^5 gene copies/g.

For the quantification of the HF183 marker of human fecal contamination, each qPCR reaction (25 μL) contained 6.875 μL nuclease free water, 12.5 μL TaqMan® master mix (SsoAdvanced Universal Probes Supermix, Bio-Rad, Hercules, California, USA), 1.5 μL each of 0.6 μM forward and reverse primers, 0.625 μL Taqman probe specific for human *Bacteroidales* 16S rRNA (Layton et al., 2013) and 2 μL of sample DNA. The conditions for the PCR were initial denaturation for 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C and 30 s at 58 °C, then 5 min at 68 °C. The positive control consisted of a plasmid DNA standard (see Stea et al., 2015). The standard curve was built as described above for the 16S rRNA protocol and had an efficiency of 105%, with an R^2 value of 0.991. Both the LOQ and LOD were 3.9 gene copies

Table 3
Content of aerobic bacteria (log CFU/g or log CFU/mL), total bacteria (16S rRNA log gene copies/g) and fecal indicator bacteria (coliforms, *E. coli* log CFU/g or log CFU/mL and the human fecal *Bacteroides* molecular HF183 indicator, log gene copies/g) in *Fucus* sp. and seawater samples from Greenland.

Sample	Site	Bacterial counts			Fecal bacteria indicators			Month	Year
		Psychrophilic aerobes 3 °C	Mesophilic aerobes 35 °C	16S rRNA total bacteria	Coliforms	*E. coli*	HF183		
Seaweed	C (Control)	10.3	5.3	8.9	<LOD[b]	<LOD	<LOD[c]	August	2017
		–[a]	5.7	9.2	<LOD	<LOD	<LOD	September	2018
	A	9.3	6.4	9.0	0.3	0.3	<LOD	August	2017
		–	7.5	9.3	<LOD	<LOD	<LOD	September	2018
	B	9.5	3.3	9.0	0.3	<LOD	<LOD	August	2017
		–	4.3	9.3	<LOD	<LOD	<LOD	September	2018
	D	–	7.4	9.3	7.3	6.0	3.5	August	2017
		–	12.5	8.5	8.4	5.3	3.1	September	2017
			12.4	10.1	7.5	4.6	4.0	September	2018
	E	–	10.3	9.1	8.0	3.9	4.0	August	2017
		–	8.7	9.0	4.8	4.2	3.5	September	2017
			10.1	9.1	4.9	3.5	3.4	September	2018
Seawater	C (Control)	8.3	–	–	<LOD	<LOD	–	August	2017
		–	3.1	–	<LOD	<LOD	–	September	2018
	A	7.5	–	–	5.7	5.7	–	August	2017
		–	4.4	–	<LOD	<LOD	–	September	2018
	B	6.1	–	–	<LOD	<LOD	–	August	2017
		–	3.1	–	<LOD	<LOD	–	September	2018
	D	5.7	3.6	–	5.0	3.9	–	August	2017
		–	9.6	-	4.8	2.1	–	September	2017
		–	6.2	–	2.3	2.3	–	September	2018
	E	5.4	8.0	–	8.6	7.1	–	August	2017
		–	9.7	-	6.4	5.8	–	September	2017
		–	12.2	–	6.5	6.1	–	September	2018

[a]Not performed.
[b]Limit of detection (LOD) was 0.3 log CFU/g in seaweed and 0 log CFU/mL in seawater.
[c]LOD for HF183 was 2 log CFU/g in seaweed.

per reaction, respectively. This resulted in an LOD in the seaweed samples 10^2 gene copies/g.

2.7. 16S rRNA amplicon sequencing of the seaweed microbiota (bacterial communities)

Libraries were prepared from aliquots of 20 μL sample DNA using the pipeline of Eurofins Genomics (Konstanz, Germany), followed by sequencing of a target region of the V1-V3 part of the 16S rRNA gene (Table 2) on an Illumina MiSeq platform (Illumina, San Diego, California, United States). Along with the samples, the negative field control sample (see above) and a mock community (ATCC® MSA-1000™) were sequenced. Eurofins performed a quality control check and trimmed the primer sequence region from the reads. Paired-end reads were deposited at the NIH NCBI Sequence Read Archive with the accession number PRJNA699133.

Quantitative Insights Into Microbial Ecology 2 (QIIME2) (Bolyen et al., 2019), the DADA2 pipeline (Callahan et al., 2016) and the standard operating procedure Amplicon SOP v2 (Douglas, 2020) were used to assign Amplicon Sequence Variants (ASV) from the assembled paired-end reads. To minimize sequencing carry-over contamination between MiSeq runs, ASVs with an abundance of less than 0.1% of the total observations were filtered out. The minimum sampling depth of the analysis was set to 38,000 reads, based on the number of reads in the sample with fewest reads, aside from the negative field control, which had less than 3000 reads. To assign a taxonomical classification to the ASVs, a Native Bayers trained classifier was obtained from the SILVA 138 SSU Ref NR 99 database (Bokulich et al., 2020; Quast et al., 2013). Alpha diversity was investigated using the diversity plugin within QIIME2 and visualized with the "taxa barplot" function in ggplot 2 (Wickham, 2016) to create a stacked barplot. Beta diversity was investigated using the QIIME2 diversity plugin to calculate the Jaccard similarity index "–p-metric: jaccard", subsequently translated to Jaccard distance and unweighted UniFrac with "–p-metric: unweighted_unifrac" (based on the qualitative abundance of ASVs in samples) and visualized using ggplot2.

2.8. Statistical analysis

The Student's T-test (Excel, two-tailed and paired) was used to compare log-transformed microbial counts between sites, sample locations and matrix (seawater versus seaweed). Concentrations of *E. coli* and coliforms in seawater and seaweed samples were subjected to linear regression to determine if concentrations in the seawater could predict concentrations in the seaweed.

Sequencing data was analyzed and visualized with RStudio version 1.1.463 (RStudio Team, 2016) with R version 3.4.4 (2018-03-15) (R Core Team, 2018), transformed using dplyr (Wickham et al., 2020), statistically analyzed (ANOVA) with the stats package (R Core Team, 2018), and visualized with ggplot2 (Wickham, 2016). Statistical significance was denoted at the 5% level.

3. Results and discussion

3.1. Content of aerobic bacteria, total bacteria (16S rRNA gene copies) and fecal bacterial indicators in Fucus sp. and seawater

Counts of aerobic psychrophiles and mesophiles at the control site C were 9.3 and 6.4–7.5 log CFU/g, respectively (Table 3). Seaweed samples from anthropogenically impacted sites in Sisimiut had significantly ($p < 0.05$) higher mesophilic aerobic counts (median 10.2 log CFU/g, standard deviation 1.4 log CFU/g) than those from Sarfannguit (median 5.4 CFU/g, standard deviation 1.7 log CFU/g) (Table 3), indicating that seaweed from sites receiving the higher PE load were more dominated by a mesophile population. However, mesophilic aerobic counts varied considerably for seaweed sampled at the same site in two consecutive months in 2017 or between years, indicating a highly variable microbial population. Lytou et al. (2021) examined the mesophilic aerobic count on previously frozen brown kelps (*Alaria esculenta* and *Saccharina latissima*) from Scotland and similarly reported marked differences in the microbial loads in seaweed from two different harvest seasons.

Table 4
MALDI-TOF identification of bacterial isolates from wild *Fucus* sp. harvested in Greenland in 2018.

Site	Number (N) of isolates	Identified N (% of isolates)	Database identification[a] (n from that genus or species)
C (control)	30	2 (6.7)	*Vibrio splendidus* (2)
A	27	2 (7.4)	*Vibrio splendidus* (2)
B	29	8 (27.6)	*Acinetobacter ursingii* (1), *Aeromonas* sp. (1), *Enterobacter* sp. (1), *Pseudomonas* sp. (1), *Serratia proteamaculans/liquefaciens* (1), *Stentrophomonas* sp. (1), *Vibrio* sp. (2).
D	25	10 (40.0)	*Aeromonas eucrenophila* (1) *Klebsiella* sp. (2), *Photobacterium* sp. (1), *Serratia fonticola* (1) *Shewanella frigidimarina* (5)
E	29	13 (44.8)	*Aeromonas eucrenophila* (1), *Citrobacter freundii* (1), *Enterobacter cloaceae* (2) *Flavobacterium glaciei* (1), *Klebsiella oxytoca* (2), *Klebsiella variicola* (1), *Serratia proteamaculans/liquefaciens* (1), *Shewanella frigidimarina* (3), *Yersinia intermedia* (1)

[a]DTU database, genus and species if log score ≥2.0, genus only if log score ≥1.7 but <2.0.

The content of bacterial 16S rRNA genes varied from 8.9 to 10.1 log gene copies/g seaweed for all samples (Table 3). For seaweed from less impacted Sarfannguit sites (A, B) and the control site C, the content of 16S rRNA genes were in the same order of magnitude as the psychrophilic aerobic counts obtained in August 2017, which were significantly higher ($p < 0.05$) than the mesophilic (35 °C) aerobic counts (Table 3). This indicated that bacterial communities on seaweed from Sarfannguit was dominated by psychrophiles. Lytou et al. (2021) also reported isolating mostly psychrophilic bacteria in their study of winged and sugar kelp from Scotland. Therefore, enumeration of psychrophilic and mesophilic aerobic counts in seaweed from Greenland and other cold climate areas could be used to assess the size of the community and indicate the level of anthropogenic impacts.

Counts of coliforms and *E. coli* remained below detection limit (0.3 log CFU/g or 0 log CFU/mL for seaweed and seawater, respectively) for the control site C (Table 3). Impacted Sarfannguit sites tested positive for coliforms in seaweed in August 2017, where both seawater and seaweed samples from site A also tested positive for *E. coli*. None of these sites tested positive in September 2018 indicating the transient nature of contamination at these less impacted sites. In Sisimiut, *E. coli* were consistently detected on seaweed samples with concentrations being significantly ($p < 0.05$) higher at site D (5.3 ± 1.0 log CFU/g) than at site E (3.9 ± 0.5 log CFU/g). The inverse situation was the case for the seawater samples, with significantly ($p < 0.05$) lower *E. coli* counts were found at site D (2.3 ± 0.3 log CFU/mL) compared to site E (6.1 ± 0.5 log CFU/mL). This may be due to the higher water exchange rates at the exposed site D versus site E, which is in a sheltered bay. The marker of human fecal contamination, HF183, was not detected in any of the samples from Sarfannguit (below the detection limit of 2 log genes/g seaweed). In contrast, HF183 concentrations in seaweed samples from Sisimiut ranged from 3.4 to 4.0 log gene copies/g seaweed. Since HF183 originates from mesophilic anaerobic bacteria from human *Bacteroides* spp. (Layton et al., 2013), detection of this marker pointed to the contamination with human fecal bacteria at these heavily impacted sites.

Presence of fecal indicator bacteria on seaweed indicates the risk of possible presence of health hazards of anthropogenic origin, which would render the seaweed inappropriate for human consumption. The results showed that seaweed harvested nearby wastewater outlets become contaminated with *E. coli*, however, this is dependent on the scale of wastewater emissions. This was exemplified by *E. coli* being detected in one sampling event at site A receiving 100 PE of percolated wastewater as opposed to the consistent detection for sites D and E, which both receive 2500–3000 PE of untreated wastewater. Other factors possibly affecting the contamination include water exchange rates, weather events, and temperature. Barberi et al. (2020) compared *E. coli* counts in seaweed (*Saccharina latissima*) harvested from three different cultivation sites and found that contents differed among sites, and sampling events over a 4-month period. Interestingly, they reported that *E. coli* numbers on seaweed differed from those of the surrounding water. This aligns with observations by Quigley et al. (2020), who noted differences in the microbiomes between *Fucus* spp. and the surrounding water. However, our results showed that presence of coliforms or *E. coli* in seawater with one exception coincided with detection of the fecal indicator organisms in seaweed samples (Table 3), indicating that for monitoring purposes analysis of seawater or seaweed would both be suitable. The relationship between concentrations of fecal indicator bacteria in the seawater and seaweed was only weakly quantitative, with linear regression coefficients (R^2) of 0.330 ($p = 0.051$, NS) and 0.493 ($p = 0.011$) for *E. coli* and coliforms, respectively (see supplemental material, Figures S1 and S2). In a paper discussing future European guidelines for cultured seaweed, Barbier et al. (2019) recommended that monitoring of *E. coli* be considered as a measure for hygiene as per current microbiological criteria for other foods (European Commission, 2005). Our results show that detection of *E. coli* in seawater would be a good predictor of the presence of *E. coli* in seaweed but not the quantity. Reasons for this was not examined in the present study but may be due to multiple factors such as localized currents and wave activity, competition between the endogenous microbiota and the fecal contaminants, seaweed health, etc. Detection of HF183 could serve as an alternative molecular indicator of potential human health hazards in seaweed, similarly to previous reports for environmental waters (Layton et al., 2013; Stea et al., 2015). Future research should look at whether detection of HF183 in seaweed coincides with presence in seawater.

3.2. Identity of bacterial isolates from Fucus sp. samples as determined by MALDI-TOF

A total of 140 bacteria were isolated from *Fucus* sp. samples collected in September 2018, with 30 from the control site C, 54 derived from samples from Sisimiut (sites D and E) and 56 from impacted Sarfannguit (sites A and B, Table 4).

While 40.0–44.8% of the seaweed isolates could be identified from sites D and E in Sisimiut with more intensive pollution, the database-dependent MALDI-TOF mass spectrometry (MS) method was far less successful for identification of organisms from the less polluted sites (A, and B) and the unimpacted control site C in Sarfannguit, where 6.7% to 27.6% of the isolates were successfully identified (Table 4). The influence of the fish processing factory

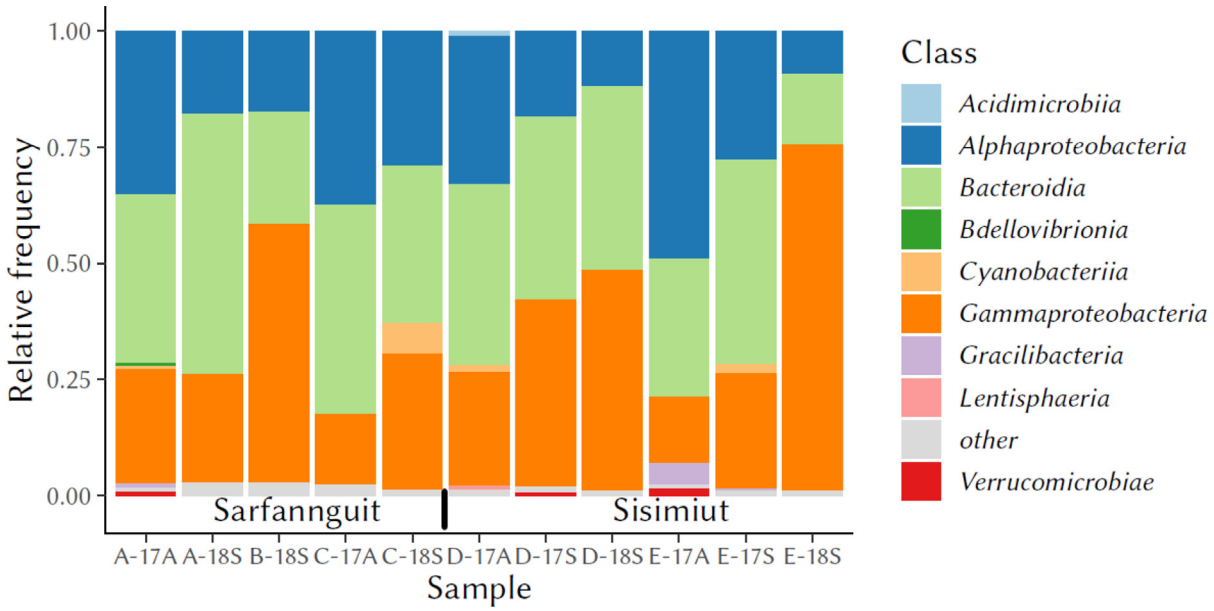

Fig. 2. Relative abundance of bacterial classes on wild *Fucus* sp. harvested in Greenland. Samples were collected from the impacted sites (A, B) and the unimpacted control site (C) in Sarfannguit and impacted sites in Sisimiut (D, E) in 2017 (17) and 2018 (18) in August (A) and September (S). The category "other" contains classes with less than 300 reads.

in Sarfannguit seemed to increase the proportion of identifiable bacteria (site B, 27.6%).

The identified bacteria belonged exclusively to Gram-negative families, which are mostly psychrotrophilic/psychrotrophs and known to be associated with the marine environment and/or food spoilage, e.g., *Shewanella frigidimarina* (Frolova et al., 2011), *Photobacterium* spp. (Sørensen et al., 2020; Fuertes-Perez et al., 2019), *Serratia proteamaculans/liquefaciens* (Nychas and Drosinos, 2014) and *Flavobacterium glaciei* (Zhang et al., 2006). Several human pathogens, such as *Acinetobacter urginsii* (Yakut et al., 2016), *Aeromonoas eucrenophila* (Pessoa et al., 2022), *Citrobacter freundii* (Whalen et al., 2007), *Enterobacter cloaceae*, *Klebsiella oxytoca* (Leitner et al., 2015) or *Yersinia intermedia* (Brenner et al., 1980) could be identified (Table 4). The prevalence of these human pathogens was notably higher for seaweed harvested at the impacted sites D and E in Sisimiut. It should be noted that isolation from countable Petrifilm plates would indicate high pathogen quantities on the sewage impacted *Fucus* sp. samples. Fish pathogens, i.e., *Aeromonas* spp. (Kozinska and Guz, 2004; Schubert and Hegazi, 1988) and *Vibrio splendidus* (Le Roux et al., 2007) were isolated from seaweed from both Sisimiut and Sarfannguit. The finding of low levels of human pathogens such as *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Salmonella enterica* ser. Typhimurium and *E. coli* O157:H7 on cultivated *S. latissima* from presumed pristine locations in Maine, USA was recently reported by Barberi et al. (2020). As reviewed by Banach et al. (2020), there is limited data on the prevalence of human pathogens (e.g., *Salmonella* spp., norovirus) in wild and cultivated seaweed, an observation that highlights the issue and need for more studies to support regulatory guidelines for suitable cultivation sites.

Although MALDI-TOF MS is a promising method to study the bacteria associated with macroalgae (Singh and Reddy, 2016), its usefulness may be limited by the available MALDI-TOF database, which was primarily built for veterinary and medical purposes including foodborne pathogens and not geared to identify bacteria from the arctic marine environment. For full exploitation of the MALDI-TOF MS identification methodology, future work should expand the database to include environmental microorganisms from the Arctic.

3.3. Composition of the microbiota on Fucus sp.

The low number of reads in the field process control sample (DNA extracted from peptone saline used for bacteria removal) confirmed the absence of contamination during the DNA extraction protocol. The average species richness (number of ASVs) in seaweed samples was 345 $\pm$ 58 with no significant ($p > 0.05$) differences among sampling locations. Analysis of the alpha diversity showed the presence of three major bacterial classes in all samples, namely *Alphaproteobacteria*, *Bacteroidia* and *Gammaproteobacteria* (Fig. 2).

Alphaproteobacteria and *Gammaproteobacteria* have previously been reported to represent the most frequent operational taxonomic unit (OTUs) on Baltic *F. vesiculosus* collected in Kiel, Germany (Lachnit et al., 2011; Stratil et al., 2013, 2014; Mensch et al., 2020). Another study from the same city found *Alphaproteobacteria* to be the dominant class (Parrot et al., 2019).

The major families identified in all samples of this study were *Flavobacteriaceae* and *Rhodobacteraceae* (Fig. 3). *Thiotrichaceae* were exclusively found in samples from Sisimiut. Samples from the same sites exhibited differences between years or even months. For example, *Thiotrichaceae* were found at the Sisimiut site E in August 2017 but not in September 2017 or September 2018. Lachnit et al. (2011) similarly found that bacterial communities on *Fucus vesiculosus* Linnaeus 1753 changed with seasons in the Kieler Fjord, in the Baltic Sea.

Two of the most common families (*Flavobacteriaceae* and *Rhodobacteraceae*) in this study were also identified as the three most common families on another macroalgae, *Ascophyllum nodosum*, which is also in the *Fucaceae* family (Martin et al., 2015), and have previously been described in *Fucus spiralis* (Dogs et al., 2017) and *F. vesiculosus* harvested on both sides of the North Atlantic (Quigley et al., 2020; Capistrant-Fossa et al., 2021). *Flavobacteriaceae* contains over 90 genera and hundreds of species, which include fish pathogens such as *Flavobacterium psychrophilum* and human pathogens such as *F. glaciei* (McBride, 2014; Zhang et al., 2006). Isolates belonging to *Rhodobacteraceae* have been found on German North Sea *F. spiralis* and other macroalgae, where the bacteria likely lead an epiphytic lifestyle, participate in the biogeochemical cycles of sulphur and carbon and contribute with

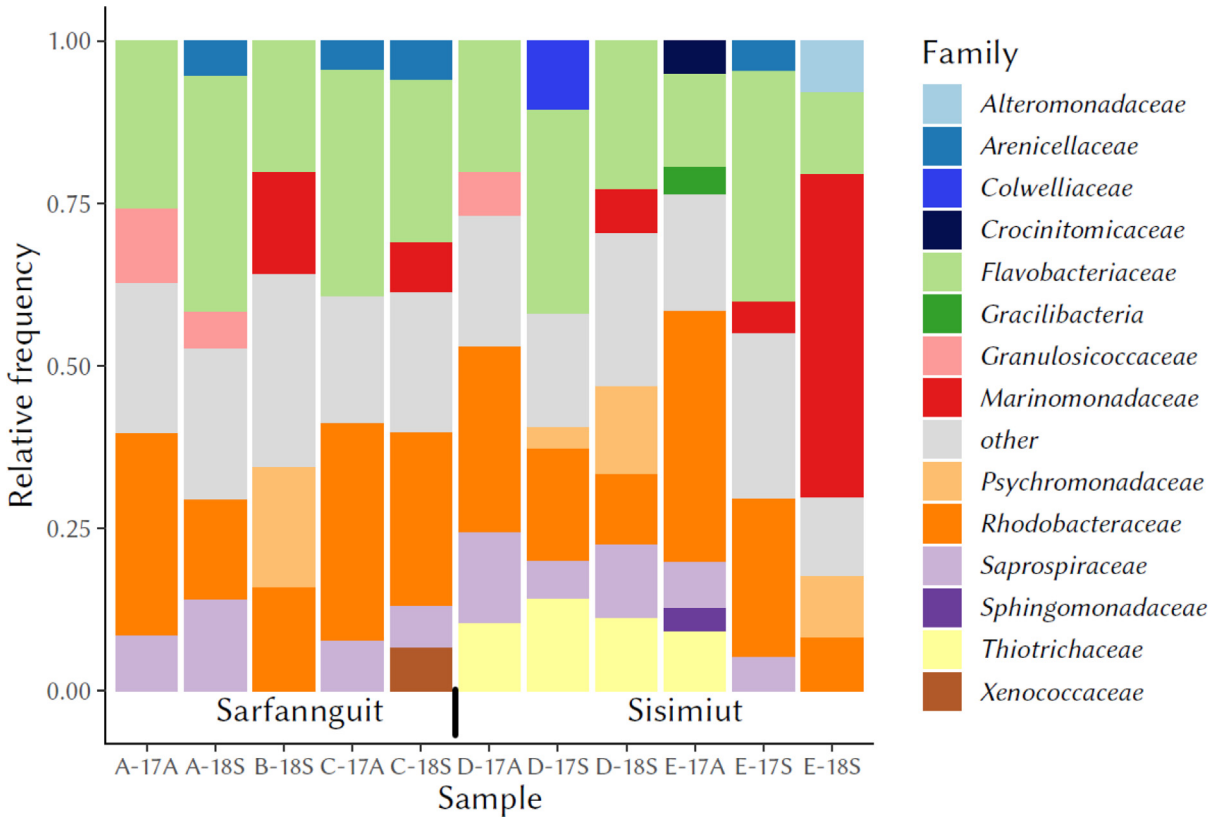

Fig. 3. Relative abundance of bacterial families on wild *Fucus* sp. harvested in Greenland. Samples were collected from the impacted sites (A,B) and the unimpacted control site (C) in Sarfannguit and the impacted sites in Sisimiut (D, E) in 2017 (17) and 2018 (18) in August (A) and September (S). The category "other" contains classes with less than 2000 reads for the individual samples, which was also the case for all unassigned reads.

B12 synthesis and sequestering of iron for the benefit of the *Fucus* sp. (Dogs et al., 2017; Pujalte et al., 2014). Members of the family *Thiotrichaceae* were identified on *Fucus distichus* from British Columbia, Canada (Davis et al., 2021) and are associated with aquatic environments and conversion of sulphur (Garrity et al., 2005). *Thiotrix*, a genus in the family of *Thiotrichaceae*, is commonly associated with domestic wastewater, e.g., activated sludge systems for the treatment of septic and sulphide-containing wastewaters (Garrity et al., 2005), emphasizing the prevalence of this group of organisms in wastewater impacted sites, i.e., Sisimiut in this study. A trans-Atlantic study of bacteria on *F. vesiculosus* samples revealed *Granulosicoccus* from the *Granulosicoccaceae* family being a dominant group on samples from the Northern study area (55–70°N, Capistrant-Fossa et al., 2021). In the present study, however, members of this family were only detected at sites A and D indicating that the *Fucus* sp. microbiomes are site specific as also reported by Davis et al. (2021). As an example of site specificity, it can be noted that *Psychromonadaceae,* and *Saprospiraceae* were detected on *Fucus* sp. from both Sarfannguit, Sisimiut and Maine, USA (Quigley et al., 2020), while *Marinomonadaceae* and *Granulosicoccaceae* were detected on Greenland *Fucus* sp. but not on the American *Fucus* sp.

Beta diversity comparative analysis of the microbiota of samples via principal coordinate analysis (PCoA, Fig. 4) supported the existence of site-specific microbial communities as both the Unweighted Unifrac (Panel A) and Jaccard Similarity Index (Panel B) methods for comparing community structure revealed three clusters, which were comprised of samples from (1) Sarfannguit (control site C and the impacted A and B sites, except for site A in August 2017), (2) Sisimiut site D and (3) Sisimiut site E. The PC1 and PC2 derived from the PCoA of the distance matrices explained a total of 60 and 53% of the variation observed with the Unweighted Unifrac and Jaccard Similarity Index, respectively, indicating phylogenic and special differences between the sampling sites (Fig. 4).

The August 2017 sample from Sarfannguit site A (A-17A) is a clear outlier from the other Sarfannguit samples, with detection of *E. coli* (Table 3) and a bacterial community that resembled bacterial community in seaweed from the Sisimiut site E (Fig. 4). Factors influencing the different bacterial communities on seaweed from the different sites could be the number of households discharging to each site (Table 1), type of sewage deposits, exchange of water and weather conditions. Notably, the weather was unusually warm during the sampling period in August 2017, with daily air temperatures up to 20 °C, which may have contributed to the differences.

Temperatures have previously been shown to drive differences in the bacterial communities of *F. vesiculosus* (Capistrant-Fossa et al., 2021). To the best of our knowledge, it is the first time that the marked role of sewage disposal on the seaweed bacterial community has been shown. How these changes affect the health of the macroalgae is unknown. Also, since this study employed "worst case" samples obtained close to wastewater outlets, future studies should examine the role of distance, emitted sewage volumes, rate of water exchange, seasonal water temperatures, etc., on the colonization of human pathogens on to the seaweed to help establish safe distance guidelines.

3.4. Legislation regarding microbial food safety of fresh seaweed

While seaweed is not explicitly named in the European consolidated commission regulation No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, European Commission (2005) or Danish legislation concerning microbial criteria,

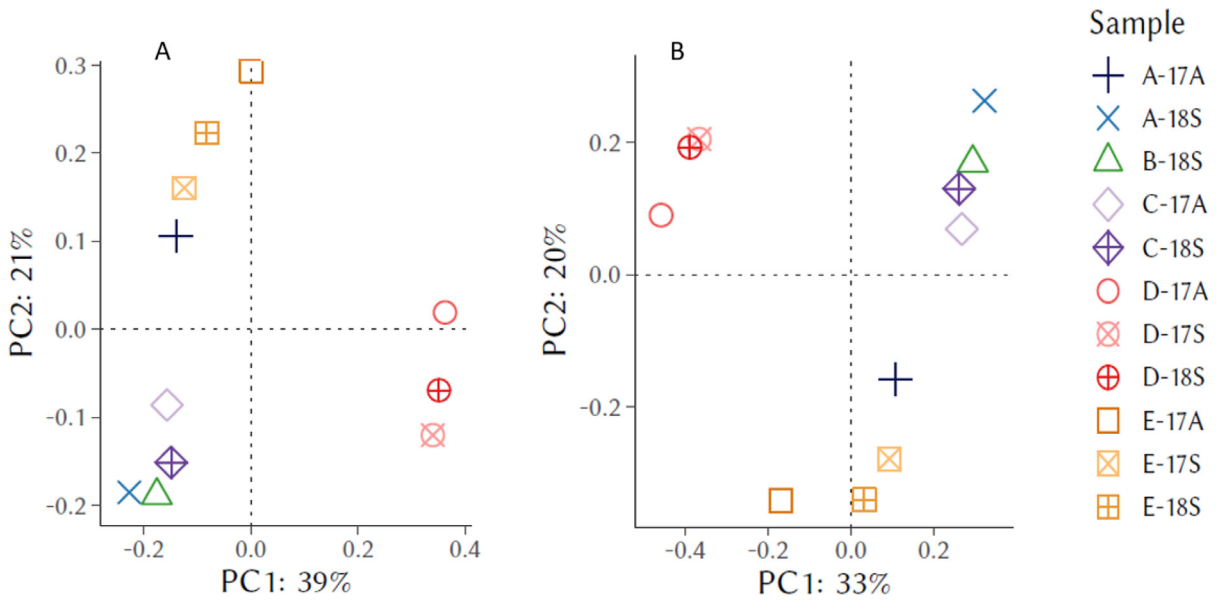

Fig. 4. Unweighted Unifrac (Panel A) and Jaccard Similarity Index (Panel B) based PCoA plots of the beta diversity of bacterial communities associated with wild *Fucus* sp. harvested in Greenland. Samples were collected from impacted sites (A, B) and an unimpacted control site (C) in Sarfannguit and impacted sites in Sisimiut (D, E) in 2017 (17) and 2018 (18) in August (A) and September (S).

France has set out the following rules which apply for dried seaweed products: mesophilic aerobic bacteria <100 000/g, fecal coliforms <10/g and *Salmonella* absence in 25 g of dried product (French Agency for Food, Environmental and Occupational Health and Safety, 2018). If the same rules were to apply to fresh seaweed, all samples from Sisimiut would fail these microbial criteria due to the presence of *E. coli*. The European Union is currently collecting information on the dietary exposure to some elements of concern in seaweed (European Commission, 2018). However, to the best of our knowledge no work is being done to develop microbiological criteria that are specific for seaweed.

4. Conclusion

Results from this study showed that wastewater emissions affect the microbial community of wild *Fucus* sp., however, the contamination with human bacterial pathogens and indicators of human fecal contamination depended on the size of the community. Detection of fecal indicator bacteria in the surrounding seawater coincided with their presence on the seaweed, raising the possibility of testing the microbial quality of seawater to monitor harvest or cultivation sites. Bacterial communities on seaweed from the sewage impacted Sisimiut samples and the unimpacted Sarfannguit samples showed marked differences in the beta diversity. Two major bacterial families, *Flavobacteriaceae* and *Rhodobacteraceae*, appeared in seaweed from both locations while *Thiotrichaceae* was only found in *Fucus* sp. from Sisimiut. Future implementation of site assessment protocols and microbiological criteria would facilitate the use of science-based criteria for harvest and cultivation site approvals and safeguard the quality of seaweed for human consumption. Also, to aid the establishment of criteria for safe distance between sewage outlets and seaweed cultivation and harvesting sites, future research should investigate factors such as the size of the community, distance between outlet and seaweed, prevailing currents, etc.

CRediT authorship contribution statement

Katharina J. Kreissig: Conceptualisation, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Visualisation, Writing – original draft, Review & editing. **Jonas Steenholdt Sørensen:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Pernille Erland Jensen:** Conceptualisation, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Lisbeth Truelstrup Hansen:** Conceptualisation, Funding acquisition, Project administration, Resources, Supervision, Formal analysis, Writing – original draft, Review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.rsma.2023.102928.

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