KLAIPĖDA UNIVERSITY

Greta KALVAITIENĖ

ACCUMULATION OF FECAL INDICATOR AND POTENTIALLY PATHOGENIC BACTERIA IN MACROPHYTE WRACK ON COASTAL SANDY BEACHES

DOCTORAL DISSERTATION

Natural sciences,
ecology and environmental sciences (N 012)

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Abstract

This thesis presents an extensive study concerning the presence of fecal pollution related bacteria and potentially pathogenic *Vibrio* bacteria in beach wrack, water and sand. The study took place in four recreational beaches located in the southeastern part of the Baltic Sea. In addition to *in situ* investigations, a systematic review of scientific literature, published during the period of 1969–2022, was performed to analyse interactions of potential pathogens and macrophytes or their wrack. The review revealed that different macrophyte groups supported different quantities of fecal indicator bacteria (FIB) and *Vibrio*; red macroalgae supported the highest quantities of potentially pathogenic bacteria, primarily *Vibrio*. The review identified a research gap regarding potentially pathogenic *Vibrio* bacteria in beach wrack. Through *in situ* research, noticeable changes in the structure of the microbial community within the water containing the wrack and within the wrack on the coast were observed, indicating that the presence of the wrack had an impact on microbial dynamics. Beach wrack was found to support significantly higher FIB levels compared to areas with no wrack. Increased FIB levels in water with wrack were related to increased cDOM and turbidity. Moreover, the environment with wrack was found to contain a higher relative abundance of enteric potential human pathogens, as well as a greater abundance and occurrence of ubiquitous potential pathogens, such as *Vibrio vulnificus*, *V. cholerae* and *V. alginolyticus*. Environmental factors, such as relative abundance of *Vertebrata fucoides*, chlorophyll-a, oxygen, temperature and cDOM, significantly influenced the abundance and presence of *V. vulnificus* and *V. cholerae*. The presence of fecal pollution from multiple sources, including humans, birds, ruminant and sewage related to riverine outflow to the sea, was identified using molecular-based methods. The combination of bacteria cultivation, genetic markers and 16S rRNA amplicon sequencing allowed to identify broader range of fecal pollution in recreational beaches.

Key words

Fecal pollution, bathing water quality, microbial source tracking, potential pathogens, 16S rRNA amplicon sequencing

Reziumė

Šiame darbe pristatomas išsamus tyrimas, apimantis su fekaline tarša susijusių bakterijų ir galimai patogeniškų *Vibrio* bakterijų gausumo, įvairovės ir fekalinės taršos šaltinių vertinimą vandenyje ir smėlyje su makrofitų sąnašomis ir pačiose sąnašose ant kranto keturiuose skirtinguose rekreaciniuose paplūdimiuose, esančiuose pietrytinėje Baltijos jūros dalyje. Buvo atlikta sistematinė mokslinės literatūros šaltinių apžvalga, apimanti 1969–2022 m. laikotarpį ir analizuojanti galimai patogeninių bakterijų ir makrofitų ar jų sąnašų sąveiką. Apžvalga atskleidė, kad skirtingi fekalinės taršos ir *Vibrio* bakterijų kiekiai buvo aptikti ant skirtingų grupių makrofitų: ant augančių raudondumblių buvo aptikti didžiausi *Vibrio* bakterijų kiekiai. Apžvalgoje taip pat buvo identifikuotas trūkumas tyrimų, vertinančių *Vibrio* bakterijų gausumą makrofitų sąnašose. Atlikus tyrimus *in situ* buvo nustatyta, kad vandenyje su sąnašomis ir pačiose sąnašose įvyko pastebimi mikroorganizmų bendrijos struktūriniai pokyčiai. Jei aplinkoje stebima fekalinė tarša, reikšmingai didesni fekalinės taršos bakterijų kiekiai aptinkami aplinkoje su sąnašomis. Vandenyje su makrodumblių sąnašomis buvo stebimas cDOM ir drumstumo padidėjimas, kuris nulėmė ir fekalinę taršą rodančių bakterijų pagausėjimą. Taip pat nustatyta, kad aplinkoje su sąnašomis yra didesnis su fekaline tarša susijusių potencialių žmogaus patogenų santykinis gausumas, ir natūraliai vandenyje tarpstančių patogenų (*Vibrio vulnificus*, *V. cholerae* ir *V. alginolyticus*) aptikimo dažnumas. *V. vulnificus* ir *V. cholerae* gausumui ir buvimui didžiausią įtaką turėjo makrodumblio *Vertebrata fucoides* proporcija sąnašose, chlorofilas-a, deguonis, temperatūra ir cDOM. Molekuliniai metodai leido identifikuoti skirtingus fekalinės taršos šaltinius, kurie susiję su upės įtekėjimu į jūrą bei su žmonių, paukščių, galvijų ir nuotekų tarša. Bakterijų kultivavimas, genetinių žymenų ir 16S rRNR sekoskaitos naudojimas leido nustatyti platesnę fekalinės taršos šaltinių įvairovę rekreaciniuose paplūdimiuose.

Reikšmingi žodžiai

Fekalinė tarša, maudyklų vandens kokybė, fekalinės taršos šaltinių identifikavimas, 16S rRNR sekoskaita

Contents

List of original publications

The material of study was presented in four original publications published in peer-reviewed scientific journals, and one manuscript, referred to in the text by their Roman numerals:

I. **Kalvaitienė, G.**, Vaičiūtė, D., Bučas, M., Gyraitė, G., Kataržytė, M., 2023. Macrophytes and their wrack as a habitat for faecal indicator bacteria and *Vibrio* in coastal marine environments. Marine Pollution Bulletin 194, 115325. https://doi.org/10.1016/j.marpolbul.2023.115325

II. **Kalvaitienė, G.**, Bučas, M., Vaičiūtė, D., Balčiūnas, A., Gyraitė, G., Kataržytė, M., 2024. Impact of beach wrack on microorganisms associated with faecal pollution at the Baltic Sea Sandy beaches. Science of The Total Environment 918, 170442. https://doi.org/10.1016/j.scitotenv.2024.170442

III. **Kalvaitienė, G.**, Picazo Espinosa, R., Vaičiūtė, D., Kataržytė, M., 2024. Diverse sources of fecal contamination in macroalgae wrack-affected environment adjacent to river outflow along the Baltic Sea coast. Environmental Pollution 357, 124429. https://doi.org/10.1016/j.envpol.2024.124429

IV. Kataržytė, M., Gyraitė, G., **Kalvaitienė, G.**, Vaičiūtė, D., Budrytė, O., Bučas, M., 2024. Potentially Pathogenic *Vibrio* spp. in Algal Wrack Accumulations on Baltic Sea Sandy Beaches. Microorganisms 12, 2101. https://doi.org/10.3390/microorganisms12102101.

List of additional publications

I. Gyraitė, G., Kataržytė, M., Bučas, M., **Kalvaitienė, G.**, Kube, S., Herlemann, D.P., Pansch, C., Andersson, A.F., Pitkanen, T., Hokajärvi, A.-M., Annus-Urmet, A., Hauk, G., Hippelein, M., Lastauskienė, E., Labrenz, M., 2024. Epidemiological and environmental investigation of the 'big four' *Vibrio* species, 1994 to 2021: a Baltic Sea retrospective study. Eurosurveillance 29, 2400075. https://doi.org/10.2807/1560-7917.ES.2024.29.32.2400075

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Author's contributions

I. G. Kalvaitienė performed data collection and analysis, contributed to the conceptualization, and wrote the manuscript draft.

II. G. Kalvaitienė took part in all samplings, analytical work and data analysis, contributed to sampling strategy design, and wrote the manuscript draft.

III. G. Kalvaitienė performed all sampling campaigns, analytical work and data analysis, contributed to sampling strategy design, and wrote the manuscript draft.

IV. G. Kalvaitienė took part in all sampling campaigns and sample processing, performed a part of the data analysis, and reviewed the manuscript.

Abbreviations

Abbreviation	Explanation
ASV	Amplicon sequence variant
BWD	Bathing Water Directive
BWQ	Bathing water quality
Chl-a	Chlorophyll a
cDOM	Colored dissolved organic matter
EEA	European Environmental Agency
EU	European Union
FIB	Fecal indicator bacteria
GC	Gene copies
MPN	Most probable number
MRF	Multivariate random forest regression
MST	Microbial source tracking
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
RDA	Redundancy analysis
qPCR	Quantitative polymerase chain reaction
SPM	Suspended particulate matter
SPIM	Suspended particulate inorganic matter
SPOM	Suspended particulate organic matter
TC	Total coliforms
YS	Yellow substances

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Introduction

The importance of fecal microbial pollution and other potential pathogens in the southeastern Baltic Sea bathing waters. The Baltic Sea provides many benefits to all nine countries along its coastline, including the recreational use of the sea, non-use related values for individuals, food production and transport. The estimated total annual recreation benefits from the Baltic Sea are close to 15 billion EUR, making sufficient water quality a particularly important aspect in this region (Czajkowski et al., 2015). Bathing water quality is not only essential for bathers' health, but also a strong indication of the overall state of the coastal zones. Coastal bathing waters comprise two-thirds of all bathing sites in Europe. In 1990, 80% of coastal bathing waters in the European Union (EU) reached at least sufficient quality and 70% were rated as having an excellent quality. In the following decades, significant improvements in bathing water quality were achieved, as 88.7% of bathing sites were rated as excellent quality in 2023 (EEA, 2014, 2024).

Water quality in the EU is regulated by the Bathing Water Directive (BWD) (European Parliament, 2006). EU member states must bring into force the laws, regulations, and administrative provisions necessary to comply with the BWD. The requirements of the BWD are implemented in the Lithuanian hygiene norm HN 92:2018 by the Ministry of Health of the Republic of Lithuania (LRSAM, 2018). According to the BWD and Lithuanian hygiene norm HN 92:2018, the coastal bathing water quality must be evaluated based on the thresholds of two microbiological parameters – *Escherichia coli* and *Enterococcus* spp. Together, *E. coli* and *Enterococcus* spp. are referred to as fecal indicator bacteria (FIB). *Enterococcus* spp. and *E. coli* are considered as commensal bacteria exclusively inhabiting the gastrointestinal tract of warm-blooded animals. However, FIB contain multiple pathotypes that were implicated as etiological agents of significant diarrheal and extraintestinal diseases (Croxen et al., 2013; Staley et al., 2014). Human and non-human fecal pollution can introduce multiple etiological agents into recreational bathing water, such as *Campylobacter* spp., *Salmonella* spp., *E. coli* O157: H7 and *E. coli* O111, *Shigella* spp., adenovirus, norovirus, poliovirus, coxsackievirus, echovirus, *Giardia lamblia*, and *Cryptosporidium parvum*. If bathers are exposed to these etiological agents, they might contract various illnesses, such as diarrhoea, respiratory illnesses, skin rashes, fever, ear and eye infections. Therefore, it is important to implement monitoring of potential human pathogens, because the direct enumeration of microbial pathogens would provide the most certain evidence of their presence in bathing sites (Harwood et al., 2014; Tiwari et al., 2021). However, consistent monitoring of all potential pathogens would not be economically or practically feasible, therefore FIB are used as surrogates for human pathogens in recreational beaches, because they are relatively easy to quantify, are assumed to covary with pathogens.Elevated concentrations of FIB were found to correlate with increased risks of gastro-intestinal symptoms in swimmers in both marine and freshwater environment (Prüss, 1998, Harwood et al., 2014). On the other hand, monitoring bathing water solely for FIB presence does not allow to determine the source of fecal pollution because all warm-blooded animals shed FIB in their feces. Coastal areas are exposed to many different fecal pollution sources, however, human-associated fecal pollution is considered particularly dangerous due to the presence of potential pathogens, especially viruses, which infect only humans. In developed countries, most of the waterborne diseases are caused by viral infections (Bosch et al., 1991; Sinton et al., 1998; Reynolds et al., 2008). Library-independent microbial source tracking (MST) methods employing host-specific markers were developed to overcome this issue. MST methods target humans, house pets, agricultural and wild animals (Harwood et al., 2014). Another alternative approach to bacteria cultivation is the High-Throughput Sequencing (HTS) of environmental DNA, which involves sequencing genetic material of interest to identify bacterial taxonomic units associated with feces, sewer or sewage (Newton et al., 2013; Basili et al., 2023). Although an application of such methods would improve the management of fecal contamination in recreational beaches, MST is currently not included among the appropriate methods in the BWD.

The microbiome of the Baltic Sea water predominantly consists of bacteria, viruses, and protozoa that are naturally ubiquitous in the aquatic environment, though some may also occur because of fecal pollution. Visitors of the Baltic Sea recreational beaches might be impacted by the potentially pathogenic *Vibrio* bacteria, ubiquitous microorganisms inhabiting warm estuarine and low to moderately saline waters. This genus is known to contain at least 12 potentially pathogenic species capable of causing human infections referred to as vibriosis. Most *Vibrio*-related infections in the Baltic Sea region are attributed to several species: nontoxigenic *V. cholerae* (non-O1 or 139), *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus*. Most *Vibrio* spp. can cause various clinical symptoms, with mild gastroenteritis being the most common. However, *V. vulnificus*, an opportunistic pathogen, is an exception that can cause wound infections, leading to septicemia with high mortality rates. Temperature is the primary driver in *Vibrio* spp. abundance in water (Baker-Austin et al., 2013, 2017, 2018). Baltic Sea surface temperature has shown a warming pattern of 0.063–0.078°C/yr^{-1} (6.3–7.8°C per century). It is estimated that with increasing sea surface temperature, the number of infection cases will increase this century, especially in temperate regions (Baker-Austin et al., 2013; Trinanes and Martinez-Urtaza, 2021). A recent retrospective analysis of infections caused by *V. alginolyticus*, nontoxigenic *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in Denmark, Estonia, Finland, Germany, Latvia, Lithuania, Poland and Sweden, which encompassed the period from 1994 to 2021, identified 1553 infection cases. However, no cases were officially reported in Lithuania, although a prediction model shows that at least 12 cases could have been expected there during the 2014–2018. The prediction model also demonstrated the common trend that infection numbers are expected to increase over the years (Gyraitė et al., 2024). Despite the occurrences of potentially pathogenic *Vibrio* species and vibriosis cases in the Baltic Sea region, currently, no monitoring of potentially pathogenic *Vibrio* species and the infection cases related to *Vibrio* is implemented in Lithuania.

The impact of macrophyte wrack on the survival and persistence of FIB and other potential pathogens. Certain naturally occurring phenomena, such as beach wrack accumulations, could impact bathing water quality, especially in the coastal areas. Beach wrack refers to organic material, including macroalgae and seagrasses, accumulated along the coast or in the coastal water. Hydrodynamic factors play an important role in the process of detachment, transport, accumulation and removal of aquatic vegetation. Other organic materials, including microalgae, animal carcasses, shells, wood, plastic and higher plant debris, can be entangled within wrack (Macreadie et al., 2017; Cesarini et al., 2021). Wrack plays an important role in the functioning of the coastal marine ecosystem. It can be re-mineralized and cycled back into the system, providing nutrients to sandy beaches, which are high-stress and low-productivity environments. Wrack is also known to support microbial food web up to the protozoan level and to harbour many invertebrate species that might become an important food source for birds (Orr et al., 2005; Dugan et al., 2011; Schlacher et al., 2017; Prasad et al., 2018).

Despite its ecological importance, wrack has also been indicated as a potential habitat and source of human microbial pathogens. Wrack can act as an insulator, increasing ambient temperature and providing an organic-rich environment that retains moisture, vital protection from harmful UV radiation, and supports osmoprotection in the marine environment. These factors create environmental conditions more conducive to the survival of microorganisms, including pathogenic ones (Ghoul et al., 1995; Anderson et al., 1997; Byappanahalli et al., 2003; Beckinghausen et al., 2014; Abdool-Ghany et al., 2022). As a result, environments with wrack accumulations, including water, sand and wrack itself, were found to harbor potential human pathogens like *Campylobacter*, *Clostridium*, *Shigella*, *Salmonella* and FIB, but these findings have been primarily reported from either freshwater environments (Byappanahalli et al., 2003; Whitman et al., 2003; Ishii et al., 2006; Vanden Heuvel et al., 2010; Chun et al., 2013) or from marine areas that are characterized by relatively higher salinity (Anderson et al., 1997; Imamura et al., 2011; Quilliam et al., 2014; Abdool-Ghany et al., 2022) compared to the Baltic Sea. The presence of wrack can affect the quality of bathing water on recreational beaches, as wrack accumulations have been associated with FIB exceedances (Imamura et al., 2011; Russell et al., 2013). The impact of beach wrack on potentially pathogenic bacteria in the brackish waters of the southeastern part of the Baltic Sea has not been studied yet.

Wrack accumulations can be washed away immediately or remain on the beach for extended periods. When the decay process starts, drastic changes in bacterial consortia and physicochemical conditions occur. Increasing abundance of enteric and pathogenic bacteria is observed, whilst these bacteria are not found or found in very low abundances in fresh wrack accumulations (Chun et al., 2017; Ihua et al., 2019; Wang et al., 2023). Decaying wrack was also shown to enhance the survival of FIB in the freshwater environment (Vanden Heuvel et al., 2010; Imamura et al., 2011). In the western part of the Baltic Sea, wrack is primarily dominated by angiosperms, while the eastern parts are dominated by red and brown algae (*Rhodophyceae* and *Phaeophyceae*) accumulations (Suursaar et al., 2014; Möller et al., 2021). Research on the presence of microorganisms in beach wrack and their interactions with potentially pathogenic bacteria in the Baltic Sea region is lacking, with only a few studies focusing on the wrack microbiome. In a study by Berdan et al. (2023), wrack microbiome was characterized in the southwestern part of the Baltic Sea. Decaying wrack has been shown to support potentially pathogenic *Salmonella enteritidis* (Gubelit and Vainshtein, 2011). Until now, no investigations have been conducted on the interactions of FIB and other pathogenic microorganisms, including potentially pathogenic *Vibrio*, in the beach wrack along the southeastern Baltic Sea coast.

1.1. Aim and objectives

The aim of the present study was to assess whether coastal wrack serves as a reservoir for fecal and other potentially pathogenic microorganisms on the sandy beaches of the southeastern Baltic Sea.

The specific objectives of the thesis were:

1. To assess the role of macrophytes and their wrack on the abundance of potentially pathogenic microorganisms related to fecal pollution and *Vibrio* in coastal marine environments based on literature data.
2. To evaluate the temporal dynamics of microbial communities' composition and relative abundance in beach environments with wrack accumulations.
3. To determine whether wrack serves as a habitat for potentially pathogenic microorganisms associated with fecal pollution and *Vibrio* species on the sandy beaches of the Baltic Sea coast, and to identify the sources of fecal pollution in areas with wrack.
4. To identify the environmental conditions that influence the diversity and abundance of fecal indicator bacteria and potentially pathogenic *Vibrio* bacteria in water with wrack.

1.2. Novelty of the study

This is the first study presenting a systematic literature review of interactions of aquatic macrophytes and their wrack with FIB and potential human pathogens, such as *Campylobacter*, *Salmonella*, *Shigella* and *Vibrio*, encompassing marine coastal areas worldwide. This is also the first study that analysed the presence and dynamics of FIB, *Vibrio*, *Salmonella* and *Campylobacter* and revealed the diverse sources of fecal pollution in recreational beaches with wrack accumulations in the southeastern Baltic Sea. Our investigation revealed for the first time that potentially pathogenic *Vibrio* species, including *V. vulnificus* and *V. cholerae*, were present in the recreational beaches with wrack. During the monitoring of bathing water in Lithuania, only bacterial cultivation on the media is used, which does not allow for the identification of the source of fecal pollution. In this work a combination of methods, such as cultivation, genetic markers and 16S rRNA amplicon sequencing analysis was used to determine the sources of fecal pollution. A higher abundance of feces and ruminant-associated taxa, along with elevated concentrations of enteric pathogens, was observed in the wrack near the river outflow, suggesting the river is a potential source of fecal pollution. The results of this thesis are important for institutions responsible for beach management and the public, as fecal pollution sources in recreational beaches and the effect of beach wrack on potential human pathogens and fecal pollution have not yet been studied in Lithuania.

1.3. Scientific and applied significance of the results

A systematic review of literature data revealed that different macroalgae groups (red, green and brown macroalgae) and seagrasses support contrasting abundances of pathogens in water, sediments and on the surface of macrophytes themselves. An important research gap was identified – only two studies focused on the presence of potentially pathogenic *Vibrio* bacteria in beach wrack, but no studies quantified *Vibrio*. This gap was partially filled by our investigation, which found that higher abundances of *V. vulnificus* and *V. cholerae* in several recreational beaches in the southeastern part of the Baltic Sea were found in water with wrack. The presence of macrophyte wrack accumulations on recreational beaches is often a contentious issue because, while wrack is an important feature in coastal ecosystems, it is also considered an olfactory and visual nuisance by tourists. Therefore, beach managers might be conflicted when it comes to recreational beach management. Our research results demonstrate that beach wrack can trap and harbour fecal pollution-associated and ubiquitous pathogens, such as *Vibrio*, potentially aiding beach managers in making more informed decisions. Additionally, the national bathing water monitoring program could be supplemented by a requirement to monitor recreational beaches, specifically in the areas with wrack accumulations, since significantly higher levels of fecal pollution can be expected in such areas. The results of this thesis are also important in applying alternative methods based on cultivation methods for regular fecal pollution monitoring. This is time and labor consuming and does not allow to determine the source of fecal pollution, nor does it protect the public from exposure during the sampling and analytical process, which takes 48 hours. Often, during fecal pollution episodes, exposure has already occurred before the health authorities can prohibit bathing. Therefore, alternatives are needed to supplement findings based on bacterial cultivation. In our investigation, two methods were used: MST, based on qPCR quantification of molecular markers to determine and quantify human and bird fecal pollution; and 16S rRNA amplicon sequencing, which allowed to identify bacterial taxa associated with fecal, sewage and ruminant-associated pollution. Overall, the findings of this thesis provide evidence that beach wrack acts as a potential reservoir and source of fecal pollution-related pathogens and *Vibrio* and may contribute to improved beach management practices and fecal pollution monitoring.

1.4. Scientific approval

1. Jūros ir Krantų tyrimai (poster, 2021.10.21–22, Lithuania) – *Makrofitų sąnašų poveikis mikrobiologinei vandens ir paplūdimio kokybei: pirminis vertinimas*

2. Littoral22 (oral, 2022.09.12–16, Portugal) – *Potentially pathogenic bacteria in beach wrack at the Baltic Sea coast*
3. Microbiology 2022 (poster, 2022.04.22–28, Lithuania) – *Mikroorganizmai ir Lietuvos pajūrio rekreacinių vandenų kokybė*
4. Jūros ir Krantų tyrimai (poster, 2023.04.19–21, Lithuania) – *Fekalinės taršos indikatorių ir kitų bakterijų kaita makrofitų sąnašose Baltijos jūros paplūdimyje*
5. Baltic Sea Science Congress (oral, 2023.08.21–25, Finland) – *The succession of fecal indicator and other potentially pathogenic bacteria in macrophyte wrack on the beach of the Baltic Sea*
6. Final symposium of the project "Pathogenic *Vibrio* bacteria in the current and future Baltic Sea waters: mitigating the problem - BaltVib" (poster, 2024.03.19, Germany) – *Potentially pathogenic Vibrio in the wrack-affected environment along the coastal beaches*
7. Jūros ir Krantų tyrimai (poster, 2024.05.15–17, Lithuania) – *Fekalinės taršos šaltinių įvairovė makrodumblių sąnašose baltijos jūros priekrantėje*

2

Materials and methods

2.1. Study sites

The study sites selected for this thesis are in the southeastern part of the Baltic Sea, within the Lithuanian coastline, where 16 official designated bathing sites are located, and the bathing water quality is monitored according to the BWD requirements. Of those bathing sites, four recreational beaches (Melnragė, Karklė, Palanga and Šventoji) were chosen for sample collection (see *Figure 1*).

Lithuanian coastline stretches for 90.6 km and is characterized by sandy fine-grain beaches and open coast. The coastal waters are shallow (0–25 m), with salinity ranging from mesohaline to oligohaline (5–7.5 PSU) (Snoeijs-Leijonmalm and Andrén, 2017). In 2023, the Lithuanian coastal region attracted up to 680,111 tourists with 2,005,850 overnight stays in the Klaipėda region (Lietuvos Statistikos Departamentas, 2023). This thesis focused on the most visited touristic coastal beaches.

Each of the selected locations differs in terms of wrack accumulation tendencies. Beach wrack tends to accumulate least intensively in Palanga, where wrack accumulations covered up to 39 m^2 of the beach area and, in Melnragė where they reached 315 m^2 in 2021. Meanwhile, in Karklė and Šventoji these accumulations were more intense – on average, beach wrack occupied up to 572 m^2 in Karklė and 3005 m^2 in Šventoji (Tiškus et al., 2023).

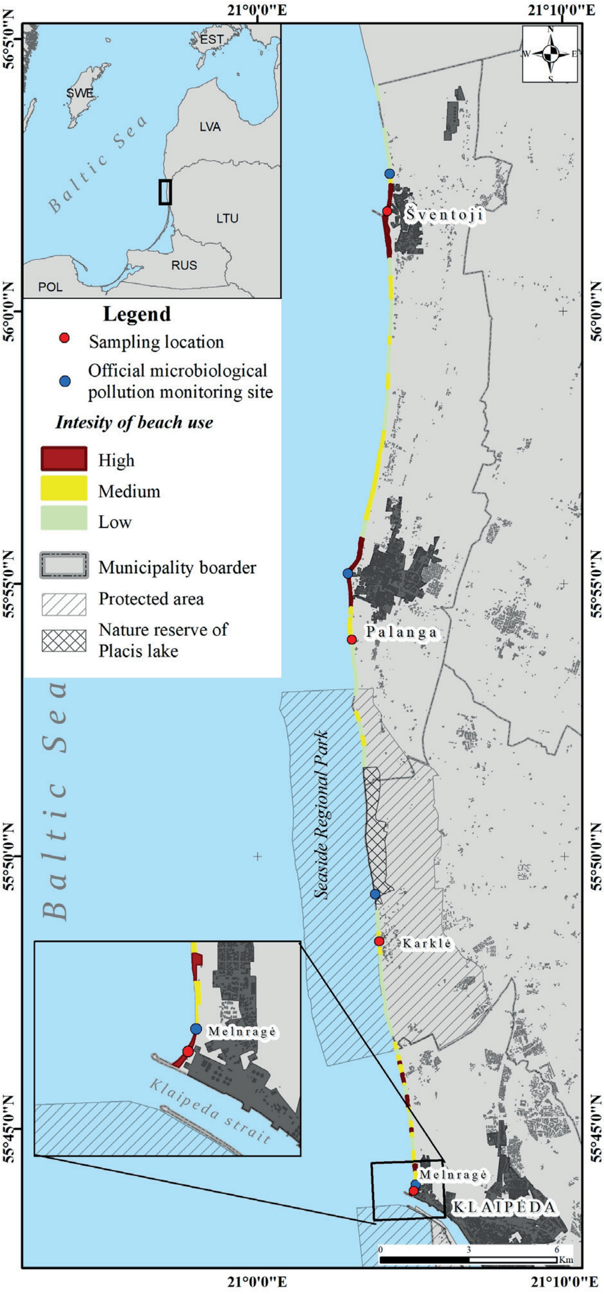

Figure 1. Map of the Lithuanian part of the Baltic Sea coastal area showing the intensity of beach use and sampling locations in Melnragė, Karklė, Palanga and Šventoji (reprinted from *Paper II* with permission)

Some of the selected areas are affected by freshwater outflows: Melnragė is periodically affected by outflows from the Curonian Lagoon (Overlingė et al., 2020). Šventoji and Karklė are affected by the outflows from Šventoji and Cypa rivers, respectively. Since Curonian Lagoon and Šventoji and Cypa rivers flow through densely populated towns, they might be affected by wastewater treatment outflows, especially during summer when the number of tourists increases.

2.2. Literature review methodology

The aim of the systematic review was to evaluate the state of research on FIB and potentially pathogenic microorganisms in live macrophyte habitats and their wrack in coastal marine environments. Particular focus is on *E. coli* and *Enterococcus* spp., which enter the aquatic system via fecal pollution and indicate the presence of fecal pollution-related pathogens, and on *Vibrio* genus, which is naturally present in the water and might cause human infections. Other common pathogens, such as *Campylobacter*, *Salmonella* and *Shigella*, found in water and related to fecal pollution, were also analysed.

The systematic review followed the PSALSAR (Protocol, Search, Appraisal, Synthesis, Analysis, Report) framework (Mengist et al., 2020) and included publications up to the year 2022. Using a combination of several topic-appropriate keywords, ScienceDirect, PubMed and Google Scholar were used as databases to search for scientific publications. The search in those databases was performed by combining multiple keywords based on targeted microorganisms and macrophytes (i.e., seagrass, seaweed, aquatic vegetation, wrack, aquatic plant, macroalgae, pathogen, fecal, faecal, *Vibrio*, *Campylobacter*, *Salmonella*, *Shigella*, *Escherichia coli*, *Enterococcus*) and environment (i.e., bay, coastal, lagoon, estuary, marine, brackish, estuarine). Using specific criteria, each publication was assessed for eligibility by two independent reviewers for inclusion in the systematic review. After appraisal, relevant information was extracted from selected publications (see *Table I*). Keywords, criteria and literature appraisal are explained in greater detail in *Paper I*.

2.3. *In situ* sampling design

During the recreational season of 2021, FIB, *Salmonella*, *Campylobacter* and potentially pathogenic *Vibrio* (*V. cholerae*, *V. vulnificus*, *V. alginolyticus*, *V. parahaemolyticus*) levels were investigated during single-day samplings in areas with beach wrack accumulations (beach sections where wrack covered at least 40 m² of the coast), and also reference areas (without visible wrack). The distance between both areas was at least 100 m. Samples were collected in four recreational beaches: Melnragė, Karklė, Šventoji and Palanga. The abundances of two MST markers (HF183 (human fecal pollution) and GFD (bird fecal pollution)) were investigated to determine the source of fecal pollution. During the recreational season of 2022, three multi-day samplings were performed to investigate FIB and MST marker dynamics in areas with wrack and reference areas in Melnragė and Šventoji. Additionally, 16S rRNA amplicon sequencing was applied to water and wrack DNA samples from Šventoji (2021 and 2022) to determine the changes in microbial community composition, to estimate the abundance and diversity of potential pathogens, including *Vibrio* species, and to evaluate the presence of fecal pollution from multiple sources (fecal, ruminant and sewage) within areas with wrack and reference areas. The main activities are summarized in *Table 1*.

Table 1. Summary of main *in situ* research activities carried out in the coastal area of the Baltic Sea (2021–2022) and literature review investigation (1969–2022)

Systematic literature review				
Investigation period	**Investigation area**	**Assessed parameters**	**Collected literature data**	**Paper**
1969–2022	Coastal marine areas worldwide	*Vibrio*, *Campylobacter*, *Salmonella*, *Shigella*, *E. coli*, *Enterococcus*	Macrophyte species, type (red/green/brown) and state (live/wrack), state of macrophyte community (mixed/only macroalgae/only seagrasses), study design, environment of investigation (water, sand or macrophyte/wrack surface), microorganism identification and quantification methods, number of study site and sampling times per study sites	*Paper I*

***In situ* investigations**							
Sampling period	**Investigation duration**	**Sampled recreational beaches**	**Assessed microbiological parameters**	**Applied methods**	**Assessed environmental parameters in water**	**Type of samples taken**	**Paper**
2021							
2021 (June–September)	Single-day samplings	Melnragė, Karklė, Palanga, Šventoji	FIB	Cultivation on the selective media	Chl-a, phycocyanin, turbidity, temperature, oxygen, salinity, pH YS, SPM, SPIM, SPOM, cDOM, composition of macroalgae species in wrack	Water, sand, wrack	*Paper II, III* and *IV*
			Salmonella, *Campylobacter*, *Vibrio (V. vulnificus, V. cholerae, V. parahaemolyticus, V. alginolyticus)*	PCR			
			HF183 (human pollution marker), GFD (bird pollution marker), *V. vulnificus*	qPCR			
		Šventoji	Ruminant and sewage pollution related microbiota, potential fecal pollution-related pathogens	16S rRNA amplicon sequencing		Water, wrack	
2022							

2022 (29 July–2 August)	5 days	Melnragė	FIB	Cultivation on the selective media	Composition and abundance of phytoplankton groups in water, composition of macroalgae species in wrack, YS, salinity, pH, water temperature	Water, sand, wrack	*Paper III* and *IV*
2022 (1–6 September)	6 days		HF183, GFD	qPCR			
2022 (9–12 August)	4 days	Šventoji	FIB	Cultivation on the selective media,			
			HF183, GFD	qPCR			
			Vibrio diversity, Ruminant and sewage pollution related microbiota, potential fecal pollution-related pathogens	16S rRNA amplicon sequencing		Water, wrack	

2.4. *In situ* data collection and analysis

Two sets of samples were collected: for FIB cultivation water, sand and wrack samples were taken; for molecular analysis (MST markers, 16S rRNA amplicon sequencing), only water and wrack samples were collected. The samples were immediately placed in the cooling box and brought to the laboratory for further processing. In each site, additional wrack samples were collected to determine the species composition of macrophytes. Multiple physical and chemical water parameters were measured *in situ* and later in the laboratory (see *Table 1*). For more detailed information, see *Paper II*, *III* and *IV*.

2.4.1. Quantification of FIB, GFD and HF183 markers and *Vibrio* species, identification of *Salmonella* and *Campylobacter*

FIB cultivation and quantification. Colilert (for *E. coli* quantification) and Enterolert (for *Enterococcus* spp. quantification) tests were used to analyse water, sand and wrack samples according to the manufacturer's instructions. The obtained quantities were recalculated and expressed as MPN/100 mL for water samples and MPN/g for sand and wrack samples. For a more detailed description see *Paper II* and *III*.

GFD and HF183 quantification. Human (HF183 genetic marker) and bird (GFD marker) fecal pollution sources were identified and quantified by applying qPCR, according to the methods described by Haugland et al., 2010 and Green et al., 2012. The quantities were expressed as gene copies (GC) per 100 mL for water and GC/g for sand and wrack. A more detailed description is provided in Paper II and III.

Vibrio quantification and identification. Conventional PCR was used to identify *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus* (Chun et al., 1999; Kim et al., 1999; Kaysner and DePaola, 2001; Luo and Hu, 2008) (see *Table 1*). *V. vulnificus* was also quantified using qPCR (Campbell and Wright, 2003). The quantities were expressed as GC/100 mL for water and GC/g for sand and wrack. For more detailed information see *Paper IV*.

Salmonella spp. and Campylobacter spp. identification. *Salmonella* and *Campylobacter* in water, sand and wrack samples were identified using conventional PCR (Aabo et al., 1993; Linton et al., 1996). A more detailed description is provided in *Paper II*.

2.4.2. Microbial community analysis

16S rRNA amplicon sequencing. Water (with and without wrack) and wrack samples (2021 and 2022) from Šventoji were analysed using a 16S rRNA amplicon sequencing approach. PCR amplification, library preparation and sequencing were performed by Novogene (Cambridge, United Kingdom). A more detailed description of further bioinformatic analysis is provided in *Papers III* and *IV*.

Sample similarity analysis. Bioinformatic analysis of sample similarity between wrack and water (with and without wrack) samples was performed using *qiime2* pipeline. Resulting amplicon sequence variants (ASV) were used to construct phylogenetic clustering trees to compare sample similarity at the phylum level. The clustering method used was the unweighted pair group method with arithmetic mean (UPGMA). The resulting phylogenetic clustering trees are provided in *Paper III*.

Identifying fecal, ruminant and sewage pollution related taxons, potential pathogens and potentially pathogenic Vibrio species. The occurrence of fecal contamination was analysed by identifying operational taxonomic units (OTUs) of bacterial families associated with fecal bacteria, i.e., *Bacteroidaceae*, *Clostridiaceae*, *Enterobacteriaceae, Enterococcaceae*, *Lachnospiraceae*, *Porphyromonadaceae*, *Prevotellaceae*, *Rikenellaceae* and *Ruminococcaceae* and sewage-associated bacteria genera *Acinetobacter*, *Arcobacter* and *Trichococcus* (Basili et al., 2023; Hägglund et al., 2018; Newton et al., 2013). Ruminant-associated genera were also included in the analysis, such as *Fibrobacter*, *Pseudoscardovia*, *Succinimonas*, *Ruminobacter*, *Succinivibrio*, *Selenomonas*, *Acetitomaculum*, *Butyrivibrio*, *Pseudobutyrivibrio* and *Succiniclasticum* (Mizrahi et al., 2021). Potentially pathogenic bacterial taxa were analysed by identifying OTUs of enteric bacteria genera associated with fecal pollution, including *Bacteroides*, *Clostridium*, *Enterococcus*, *Escherichia-Shigella*, *Fusobacterium*, *Helicobacter*, *Lactococcus*, *Prevotella*, *Rickettsia*, *Treponema* and *Vagococcus* (Boukerb et al., 2021). For a more detailed description regarding data preparation and bioinformatic analysis see *Paper III*. OTUs assigned to *Vibrio* genus (≥97% similarity) were identified. A more detailed description regarding *Vibrio*-related data preparation and bioinformatic analysis is provided in *Paper IV*.

2.5. Statistical analyses

The quantities of FIB and fecal pollution markers in water and sand with and without wrack were compared using a non-parametric Mann-Whitney Rank Sum Test. Spearman correlation was used to assess the strength and significance of relationship between: a) FIB and fecal pollution markers (HF183 and GFD), b) FIB, fecal pollution markers, *V. vulnificus* and physical, chemical and biological water parameters, c)

OTU abundances of fecal, ruminant and sewage-associated taxa and fecal pollution markers, d) *Vibrio* OTUs' relative abundance and environmental parameters in water. The significance level of correlation was set at $p<0.05$. Results are described in greater detail in *Papers II*, *III* and *IV*. Redundancy analysis (RDA) was used to assess the importance of environmental variables (physico-chemical and biological) to the patterns of the FIB, total coliform (TC), fecal pollution marker abundances in water. Separately, for areas with wrack accumulations and reference areas in water RDA analyses were performed. RDA application is described in more detail in *Paper II*.

The importance of environmental factors for the variation in the abundance and presence of *V. vulnificus* and presence of *V. cholerae* was assessed with the multivariate random forest regression model (MRF) due to a relatively low amount of data and high multicollinearity among the explanatory variables. A more detailed description of applied statistical tests is provided in *Paper IV*.

3

Results and discussion

3.1. Interactions of potential pathogens and macrophytes in the coastal marine environments worldwide: a retrospective literature review

3.1.1. Spatial and temporal trends

For *Paper I*, an extensive review of scientific papers on *in situ* research was conducted, which analysed the interactions of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus* including the broader *Vibrio* genus (*Vibrio* spp.), *Salmonella*, *Shigella*, *Escherichia coli* and *Enterococcus* with live macrophytes or their wrack in coastal marine areas worldwide. Important information on geographical distribution, temperature and salinity ranges, macrophyte and habitat type and abundances of microorganisms were extracted from analysed publications (see *Table 1*).

Macrophyte-bacteria interactions have been extensively studied for the past 50 years (Chan and McManus, 1969). The first scientific paper on macrophyte wrack was published later in 1985 (Wahbeh and Mahasneh, 1985). A higher interest in interactions between live macrophyte and pathogenic bacteria was also reflected in the number of publications: 44 papers focused on live macrophytes, meanwhile only

12 publications studied macrophyte wrack. *Vibrio* spp. (41.4% of all studies) and FIB (36.2%) were microorganism groups that were researched most intensively (see *Paper I*).

All selected studies were assigned to one of the big marine regions, according to International Hydrographic Organization (IHO) classification of marine regions (IHO, 1953). More studies regarding both potential pathogens (FIB and *Vibrio*) and macrophytes (live and wrack) have been performed in two large marine regions – North Atlantic and North Pacific Oceans (see *Paper I* for full description of results). Most studies were primarily concerned with recreational beach quality or analysed microorganism diversity on macrophytes or in their surrounding environment (Chan and McManus, 1969; Duan et al., 1995; Bonilla et al., 2006; Imai et al., 2006; Dobretsov and Qian, 2010; Imamura et al., 2011; Haley et al., 2012; Izbicki et al., 2012; Gonzalez et al., 2013, 2014; Russell et al., 2014, Quilliam et al., 2014; Martin-Kearley et al., 1994; Crump et al., 2018; Sun et al., 2020; Abdool-Ghany et al., 2022). An additional reason for such interest in research is the development of aquaculture in countries like the People's Republic of China, Japan, the Republic of Korea and the USA, which are adjacent to North Atlantic and North Pacific Oceans. Aquacultures are threatened by *Vibrio*-caused diseases (Ward et al., 2020), therefore pathogens targeting cultivated seaweeds (Martinez and Padilla, 2016; de los Santos et al., 2020; Möller et al., 2020; Tahiluddin et al., 2021) and the suitability of seaweed for human consumption (Mahmud et al., 2006, 2008, 2007; Barberi et al., 2020) are of high relevance.

Salinity and temperature conditions (in macrophyte habitats) were also extracted from analysed publications. Potential pathogens were found in salinity gradients from 0.2 to 37 PSU. However, there was a variation of coverage between different species: *E. coli*, *V. parahaemolyticus* and *V. alginolyticus* were found in higher salinity conditions, *Enterococcus* spp., *Vibrio* spp., *V. vulnificus*, *V. cholerae* and *Salmonella* spp. were found both in lower and higher salinity conditions (see *Paper I*, *Figure 2*). Potential pathogens were also found across a broad temperature gradient ranging from 3.5 to 35.6°C. Almost all potential pathogens, except *Salmonella* and *V. alginolyticus*, were found in higher water temperature conditions reaching 30°C degrees and more (see *Paper I*, *Figure 3*). Overall, temperature gradient coverage was uneven and sporadic, with no clear established relationship between temperature levels and microorganism detection.

3.1.2. Potentially pathogenic bacteria supported by live macrophyte habitats and wrack

For *Paper I*, potentially pathogenic bacteria abundances supported by different macrophyte groups (green, brown, red macroalgae or seagrasses) were analysed. Potentially pathogenic bacteria, including *Vibrio*, *Enterococcus* and *E. coli*, were revealed as one of the notable constituents of macroalgae and seagrass microbiota.

In total, 18 *Vibrio* species, including the ones potentially pathogenic to humans, were associated with macrophyte surface, their rhizosphere and their immediate surrounding environment (see *Figure 2*). Several species pathogenic to marine fish and invertebrates (Prado et al., 2005; Austin and Zhang, 2006; Richards et al., 2015; Bruto et al., 2017; Liu et al., 2018; Li et al., 2021; Song et al., 2021; Feng et al., 2022; Harrison et al., 2022) and non-pathogenic *Vibrio* species (Cerdà-Cuéllar and Blanch, 2002; Beleneva and Kukhlevskii, 2010) were also identified. This systematic review revealed that different macrophyte groups supported varying *Vibrio* abundances: the highest abundances (up to 2.7×10^5 CFU/g) were found on red macroalgae, while lower abundances were observed on brown macroalgae (up to 6.7×10^4 CFU/g). No numbers of *Vibrio* were reported from green macroalgae (see *Paper I*, *Figure 6*). In the seagrass communities, the abundance of *Vibrio* was observed up to 6.9×10^4 CFU/g (see *Paper I*, *Figure 6*). Secondary metabolites play an important role in bacteria settlement and biofilm formation on macrophyte surfaces. Different *Vibrio* species can associate with or feed on exudates from specific macrophyte taxa (Liao et al., 2003; Hall-Stoodley et al., 2004; Ymele-Leki et al., 2013). Previous research shows that the highest rate of attachment to macrophyte surfaces occurred with particular green and red algae (Hood and Winter, 1997). On the other hand, excretion of various secondary metabolites from macrophytes might lead to impairment of *Vibrio* motility, biofilm formation and loss of cultivability, which might prevent precise abundance measurement by cultivation methods (Qiao et al., 2021). *Vibrio* abundances could be affected by various compounds produced by macroalgae, as extracts from green and red macroalgae showed inhibition of *Vibrio* (Liao et al., 2003; Chakraborty et al., 2010). Overall, interactions between potentially pathogenic *Vibrio* species and live macrophytes remain a complex and unexplored topic that should be further investigated in future research.

Concerning live macroalgae, FIB were quantified in brown and green macroalgae habitats. *E. coli* was found most abundant on macroalgae surface and reached up to 1.8×10^4 CFU/g, *Enterococcus* – in the sediment next to macrophytes and reached up to 3.2×10^3 CFU/g (see *Paper I*, *Figure 6*). In the past, FIB were considered unable to survive outside their host, i.e., the gut of warm-blooded animals. However, it was later discovered that FIB can overcome environmental stressors, such as nutrient deprivations, low temperatures, salinity, exposure to solar radiation, competition with autochthonous microbial communities, and protozoan grazing, allowing them to adapt outside of hosts (Walk et al., 2009; Devane et al., 2020). Association with macrophytes might have been one of the aspects affecting FIB survival and adaptation. Studies have shown that *E. coli* could grow and form a biofilm on macroalgal extracts (Kantachumpoo and Chirapart, 2010; Quero et al., 2015). Macroalgae compounds are important nutritional source for bacteria in the marine aquatic environment, as

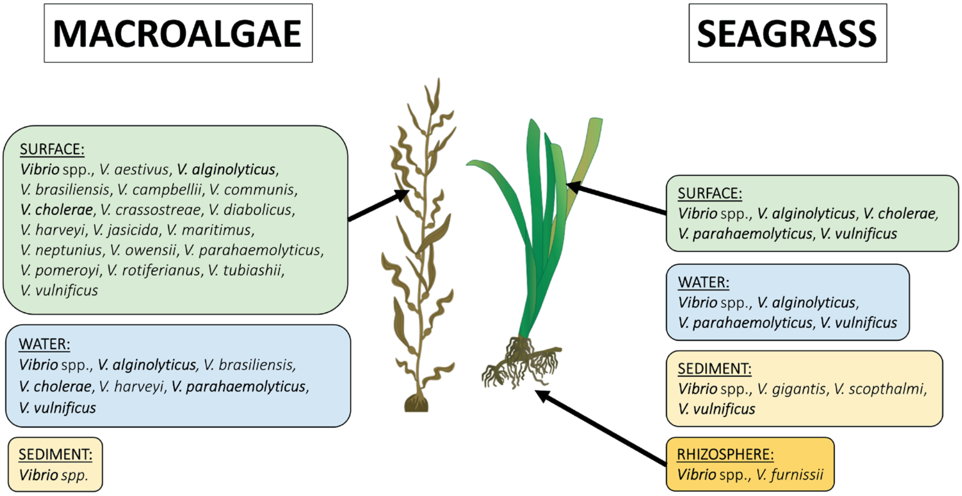

Figure 2. *Vibrio* species detected on the surface and in the rhizosphere of macrophytes, in the water and sediment of their habitats (reprinted from *Paper I* with permission).

they are enriched in various sugars, like fucose (brown macroalgae) or galactose (red macroalgae), which are important for the rapid growth of bacterioplankton (Byappanahalli et al., 2003; Nelson et al., 2013). However, some compounds, such as green macroalgae-produced polysaccharide ulvan, prevent formation of bacteria biofilm on the macroalgae surface (Anisha et al., 2023). Apart from compounds that encourage the proliferation of FIB, bacteria might confer multiple ecological advantages from biofilm formations on the macrophyte surface: higher resistance to predators or UV radiation. Different macroalgae groups display different features potentially influencing bacterial adhesion and survival, including life cycle, light requirement, biochemical composition and surface-to-volume ratio (Quero et al., 2015).

Wrack studies primarily focused on the identification and quantification of FIB, while other potential pathogens were investigated to a much lesser extent. This focus on FIB and wrack interactions might be related to beach management issues: wrack, while considered an important for beach ecosystem functioning, was also proven to be FIB source to adjacent bathing waters (Imamura et al., 2011; Quilliam et al., 2014; Abdool-Ghany et al., 2022). The highest abundances of *E. coli* and *Enterococcus* spp. were detected on the surface of wrack, reaching up to 1.7×10^4 CFU/g and 8.6×10^3 CFU/g, respectively (see *Paper I*, *Figure 8*). The affinity of FIB towards wrack is determined by multiple factors that are favorable for bacteria: FIB are provided with nutrients and a substrate to form a biofilm, protected from UV light and possible predation (Imamura et al., 2011; Egan et al., 2013; Beckinghausen et al., 2014). Another important issue regarding wrack research was identified – only two studies investigated *Vibrio* presence. Since wrack can create a suitable environment for *Vibrio* proliferation and subsequently increase the likelihood of bathers encountering potentially pathogenic *Vibrio* species, its presence, and interactions with beach wrack should be investigated more thoroughly.

3.2. Potentially pathogenic bacteria presence and abundance in the sandy beaches with wrack accumulations

3.2.1. Temporal dynamics of the microbial community at Šventoji beach: comparing areas with and without wrack accumulations

Šventoji beach was selected for a more in-depth analysis of the microbial community due to the higher levels of fecal pollution observed in 2021 and 2022 compared to other studied beaches. Different microbial community dynamics were observed in the beach areas with wrack compared to reference sites (see *Paper III*). During the recreational season of 2021, 68 bacterial phyla were identified, with Proteobacteria (28–31.2% of relative abundance), Cyanobacteria (22.6–30%), Bacteroidota (11.6–14.8%) and Actinobacteriota (10.6–20.2%) being the dominant ones in all samples (see *Figure 3*). Despite these similarities, phylogenetic clustering revealed that microbial communities differed between areas with and without wrack. Water with wrack, across all months (July, August and September) clustered similarly to wrack in July and August, whereas water without wrack showed dissimilarity (see *Figure 4*). Similar tendencies were observed in 2022 during multi-day observations: 50 phyla were identified, Proteobacteria (36.5–40.8%), Cyanobacteria (16.2–18%), Bacteroidota (20.2–21.8%) and Actinobacteriota (11.7–13.4%) were the dominant phyla. The microbial community in water with wrack and the wrack itself also went through more noticeable daily changes compared to water in the reference area, where the microbial composition remained relatively unchanged (see *Figure 5*). Moreover, there was a clear distinction between areas with and without wrack – water from the reference area formed a separate clade, but water with wrack and wrack samples showed remarkable similarity (see *Figure 6*). These results indicated that microbial communities residing within the wrack and in the environment with the wrack are going through significant composition changes. Changes in physical and chemical conditions in water with wrack and wrack itself most likely contribute to dominance of Proteobacteria and Bacteroidota, which can be a part of the intact, actively growing macroalgae microbiota (Berdan et al., 2023; Lu et al., 2023), as well as important algal polysaccharide degraders after they are deposited on the coast (Berdan et al., 2023).

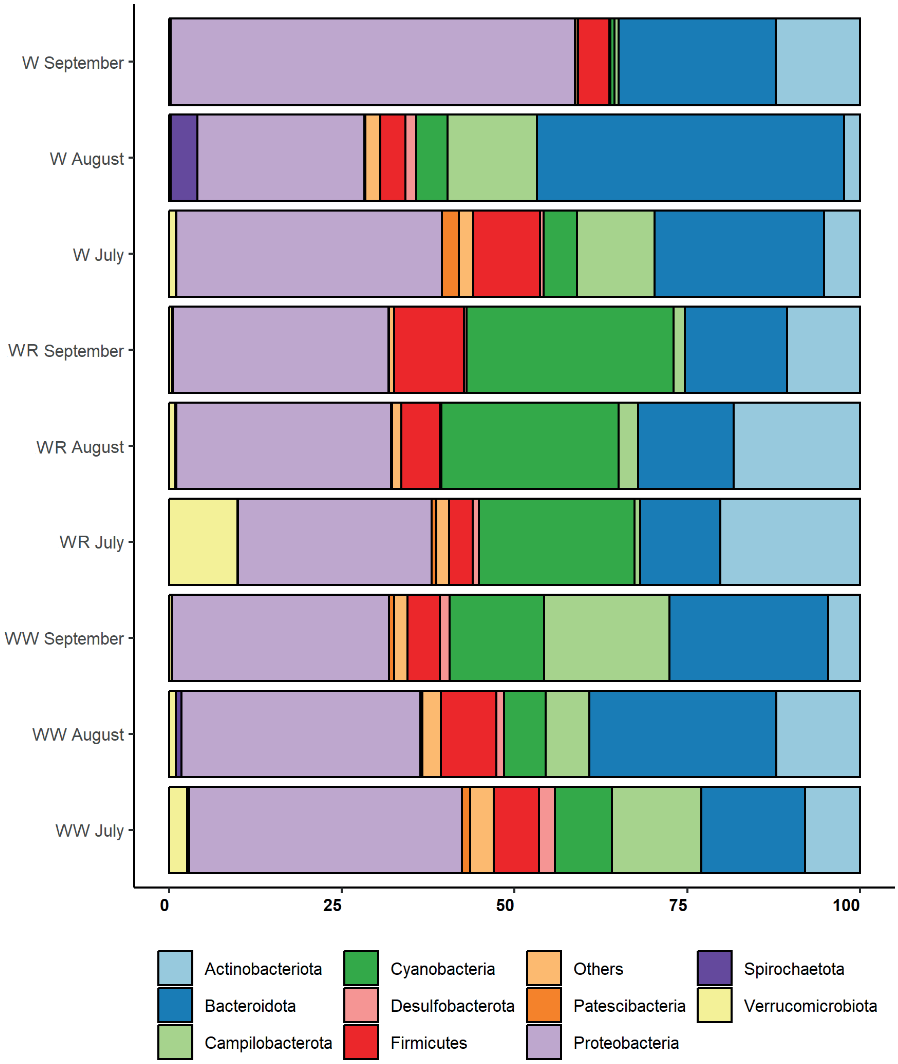

Figure 3. Relative abundance (%) of top 10 phyla during different recreational months in Šventoji (2021); WW – water with wrack, WR – water without wrack (reference area), W – wrack (reprinted from *Paper III* with permission).

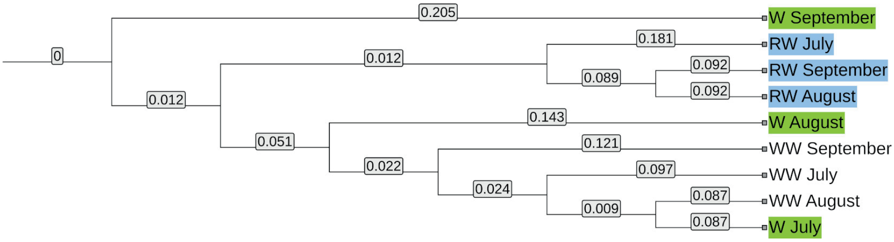

Figure 4. Phylogenetic clustering tree (phylum level) constructed using the UPGMA method showing the relationship between water and wrack samples during different recreational months from Šventoji (2021); WW – water with wrack, RW – water without wrack (reference area), W – wrack (reprinted from *Paper III* with permission).

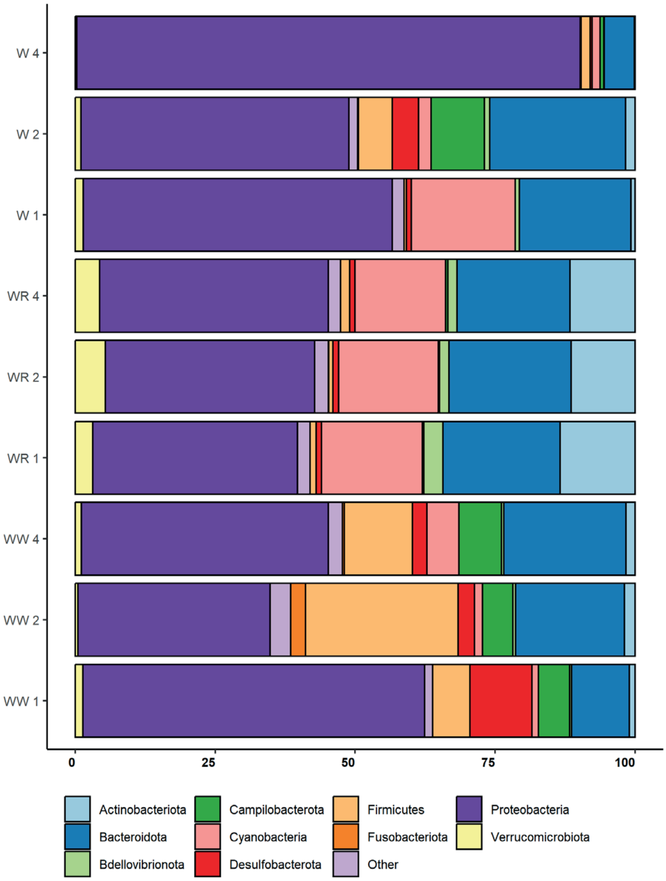

Figure 5. Relative abundance (%) of top 10 phyla in samples during different days (August 9 (1st day), 10 (2nd day) and 12 (4th day)) in Šventoji (2022); WW – water with wrack, WR – water without wrack (reference area), W – wrack (reprinted from *Paper III* with permission).

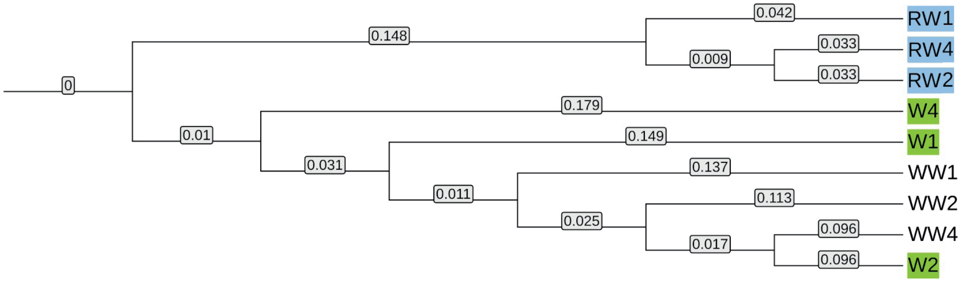

Figure 6. Microbial community similarity constructed using the UPGMA method showing the relationship between water and wrack samples collected on different sampling days (August 9 (1st day), 10 (2nd day) and 12 (4th day)) in Šventoji (2022), based on16S rRNA genes sequences; WW – water with wrack, RW – water without wrack (reference area), W – wrack (reprinted from *Paper III* with permission).

3.2.2. The short and long-term effects of beach wrack accumulations on the presence and abundance of FIB in recreational beaches

FIB levels in water with and without wrack (2021 data) were compared to the thresholds (1000 MPN/100 mL for *E. coli*, 100 MPN/100 mL for *Enterococcus* spp.) set in the hygiene norm 92:2018 (see *Paper II*). FIB abundances above the thresholds indicate that bathing water might be unsafe for swimming. FIB levels were above the thresholds in water with wrack on five occasions, in reference water – on two occasions, while official monitoring data did not identify any exceedances above the thresholds during the recreational season. The differences could arise due to variations in sampling time and techniques, as official monitoring is not linked to the presence of wrack, but follows a predetermined monitoring schedule and site. Meanwhile, our sampling was explicitly conducted when the beach contained wrack accumulations. Results of official monitoring data are provided in *Paper II*.

The wrack degradation process tends to impact physical and chemical water properties. Several water parameters, including turbidity, chlorophyll-a (chl-a), suspended particulate matter (SPM), suspended particulate inorganic and organic matter (SPIM and SPOM) and colored dissolved organic matter (cDOM), measured during the 2021 recreational season, were, on average, significantly higher in water with wrack compared to the reference area. When macroalgae degrade, they are converted into particulate organic or inorganic matter, which may lead to an increase in water parameters such as turbidity or cDOM. Filamentous macroalgae were most likely the source of increased turbidity and cDOM in water, as these macroalgae were positively correlated with turbidity and cDOM (see *Figure 7*). Filamentous macroalgae, i.e., *Cladophora rupestris*, *C. glomerata* and *V. fucoides* were among the dominant macroalgae species found in wrack in 2021. Turbidity and SPM significantly affect FIB presence in the water (Wang et al., 2015; Buer et al., 2018; Kataržytė et al., 2018; Cira et al., 2022) through inactivation of the UV (Sinton et al., 1998; Walters et al., 2013; Kataržytė et al., 2018; Perkins et al., 2022), provision of the surface for attachment and biofilm formation (Jin et al., 2004), and of nutrients. Results of the investigation from the recreational season of 2021 clearly showed that a combination of changes in physical and chemical water conditions had a positive effect on *Enterococcus* spp., as average abundances were significantly higher in water and sand areas with wrack compared to reference areas and were positively correlated with cDOM, turbidity and filamentous macroalgae (see *Figure* 7). In addition to altering the physical and chemical properties of water, wrack can act as a physical barrier, trapping bacteria along the coast. In 2021, he highest levels of fecal pollution were found at Šventoji beach, in water containing wrack (9625.5±688 MPN/100 mL (*E. coli*)), which is located near the outlet of the Šventoji River. FIB was also found most abundant in beach wrack in Šventoji compared to other beaches (2263.04±430.08 MPN/g (*Enterococcus* spp.)). The levels of FIB in sand remained relatively low and showed little variance compared to water and wrack. The results of the 2021 sampling are described in greater detail in *Paper II*.

However, the 2021 study had important limitation – only single-day samplings were conducted, which did not allow us to determine how long the wrack had been deposited along the coast. Physical and chemical water conditions swiftly fluctuate in decaying wrack and environment with wrack and could likely impact the survival of FIB and related potential pathogens and microbial community within. Therefore, three multi-day observations were performed in 2022. Similar to the 2021 season, wrack also had a significant impact on FIB numbers. During the 2022 recreational season, the average *Enterococcus* spp. abundance was significantly higher in water and sand with wrack compared to the reference areas, while *E. coli* abundance was higher in the sand with wrack compared to the reference area. In the sand of Šventoji: the concentrations of *E. coli* (0.26±0.14 MPN/g) and *Enterococcus* spp. (1.06±0.25 MPN/g) on the third day increased to 4.92±0.29 MPN/g and 88.98±122.90 MPN/g, respectively, on the fourth day. On the third day most of the beach wrack biomass was mechanically removed from the beach. Beach wrack tends to be integrated within deeper sand layers; therefore, it is not surprising that FIB concentrations increase, as FIB might be released from deeper layers of sand during beach grooming events. An increase of FIB threshold exceedances were previously associated with beach wrack removal (Russell et al., 2014; Kelly et al., 2018). In the water FIB abundances were relatively low with little variation during different sampling days. FIB tendencies on all sampling events are described in greater detail in *Paper III*.

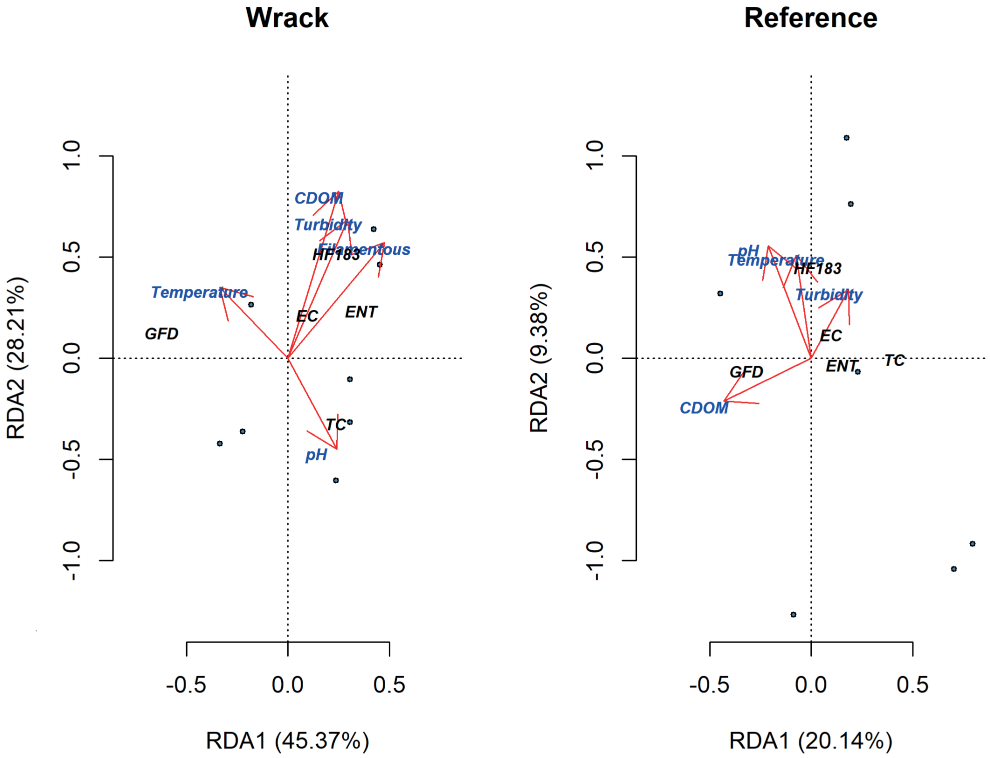

Figure 7. Triplots of redundancy analysis (RDA) showing correlations between FIB (*E. coli* (EC) and *Enterococcus* spp. (ENT)), total coliform (TC), fecal pollution marker (HF183 and GFD) abundances in the wrack accumulation areas (Wrack) and reference areas (Reference) and environmental factors: water temperature, turbidity, pH, CDOM and relative abundance of filamentous macrophytes in water (2021) (reprinted from *Paper II* with permission).

3.2.3. Fecal pollution sources and related potentially pathogenic bacteria within environment with wrack

Conventional bathing water monitoring relies on observations of FIB (*E. coli* and *Enterococcus* spp.) quantities, but these microorganisms cannot be linked to specific hosts. For this reason, we applied human (HF183) and bird-related (GFD) genetic markers both in 2021 and 2022. By applying qPCR, human wastewater-associated *Bacteroides* spp. HF183 (Ahmed et al., 2012) and avian-specific *Helicobacter* spp. GFD markers (Green et al., 2012) were targeted.

During the recreational season of 2021, GFD and HF183 levels were detected in four beaches, and observed abundances in 2021 varied significantly in water, sand, and wrack. Fecal pollution marker HF183 allowed us to partially explain the source of fecal pollution on different beaches. The highest abundance of HF183 was found in the wrack accumulation area in water on 27 August in Šventoji (19,600±70.7 GC/100 mL), in the wrack the highest abundance of HF183 marker was found on 10 August in Melnragė (45,300±1243 GC/g (see *Paper II*, *Figure 3*). GFD and HF183 abundances between wrack accumulation and reference areas in water were not significantly different ($p>0.05$). HF183 demonstrated positive correlations with both *E. coli* and *Enterococcus* spp. abundances (when those abundances were above thresholds set in the HN 92:2018) in all environments (water, sand, and wrack), and was most strongly correlated ($r=0.707$) with *Enterococcus* spp. in the wrack. Elevated FIB levels (above thresholds set in the HN 92:2018) and the presence of HF183 were observed simultaneously in two locations – Šventoji and Palanga. In Palanga, only one sampling was performed and both HF183 and GFD were detected in high abundances. In Šventoji, all samples were collected next to the outflow of the Šventoji River on every occasion. Since Šventoji is located next to one of the most popular resorts in Lithuania, it is likely that fecal pollution was brought with river water from the resort and was subsequently trapped by wrack, leading to threshold exceedances of FIB. Fecal pollution levels in Karklė, Palanga and Melnragė were relatively low and fecal pollution sources in these beaches could not be determined based on two used markers.

No clear correlation tendencies between GFD and FIB were found, as correlations were both positive and negative and varied in strength. Seabirds can be attracted to wrack accumulations to feed and excrete fecal matter, which is known to contain very diverse microbial communities that also include potential human pathogens (Green et al., 2012; Gagnon et al., 2013; Ferreira, 2019). In our case, GFD was quantified most frequently in water with wrack and wrack itself. The highest abundance of GFD was found in the reference area in water on 2 June in Melnragė (22,478±514.8 GC/100 mL), in wrack – on 2 June in Melnragė (7071.2±90.8 MPN/g) (see *Paper II*, *Figure 3*). The only trend regarding the GFD marker was its identification at the beginning of June and in mid-September of 2021, while the human marker was absent. This most likely indicated that birds feeding on the coast experienced less disturbance.

During the recreational season of 2022, when multi-day samplings in Melnragė (two multi-day observations) and Šventoji (one multi-day observation) were performed, different tendencies regarding HF183 and GFD markers were observed. At both locations the GFD marker dominated, while the HF183 marker was detected sporadically. The abundances of GFD in water ranged from absence to the maximum abundance of 7.9×10^3 GC/100 mL, in the sand the abundance peaked at 2.4×10^4 GC/g MPN/g, in wrack GFD marker varied considerably, and the highest concentration reached 3.5×10^5 GC/g. Results are provided in greater detail in *Paper III*.

Discrepancies in the detection tendencies of HF183 and GFD markers observed during 2021 and 2022 demonstrated that these markers, while useful for source identification, may not always align with general fecal pollution levels indicated by FIB cultivation. The absence of significant correlations might be due to several reasons, such as FIB cultivation yielding viable *E. coli* and *Enterococcus* spp. counts associated with most warm-blooded animals, while MST markers originate from specific hosts and may detect non-viable environmental DNA (Ahmed et al., 2016). The occurrence of MST markers might also differ geographically (Green et al., 2012). These results also indicate that fecal pollution sources could have originated not only from humans and birds.

16S rRNA amplicon sequencing was applied to identify bacterial families associated with fecal pollution in Šventoji. Comparing feces-associated families from both years (2021 and 2022), a greater variety and abundance were observed in 2021 (see *Paper III*, *Supplementary Fig. 9*). In 2021, feces-associated families were present most abundantly in the wrack in July (5.2% of total abundance) and in the water with wrack in August (5.1%). Dominant feces-associated families were *Clostridiaceae* and *Lachnospiraceae* (see *Figure 8, C*). During multi-day sampling in Šventoji (2022), the abundance of feces-associated families varied greatly between different sampling days, but was primarily dominated by *Clostridiaceae* and reached the maximum abundance (17.8%) on the second day in water with wrack, but decreased on the fourth day to below 5%, which might be attributed to the removal of wrack from Šventoji beach on the third day (see *Figure 9, C*). *Ruminococcaceae* was also observed in higher abundance (0.06-1.48%), especially in water with wrack in July and August of 2021 (see *Figure 8, C*). *Clostridiaceae*, which was a dominant family during both recreational seasons, is considered to comprise a cosmopolitan diversity of microorganisms, related to both mammals and birds. Meanwhile *Lachnospiraceae* and *Ruminococcaceae* are closely linked to mammalian gut environments (Biddle et al., 2013; Boukerb et al., 2021). The fecal pollution in Šventoji could have originated from human sources, as human fecal pollution marker HF183 was positively and significantly correlated with *Lachnospiraceae*, *Prevotellaceae* and *Rickenellacea* abundances, on the other hand the GFD marker was correlated negatively with fecal pollution-associated families (see *Paper III*, *Supplementary Table S6*).

16S rRNA amplicon sequencing also helped to reveal the presence of specific fecal pollution sources, associated with ruminant and sewage pollution in Šventoji. Ruminant-associated fecal pollution microbiota was more frequently detected in 2021 compared to 2022. The highest abundances were detected in wrack samples in July and August of 2021. While the 2021 season was marked by a high variety of ruminant-associated microorganisms, only *Fibrobacter* and *Fibrobacteriaceae* were detected in 2022 (see *Paper III*, *Supplementary Fig. S10*). Sewage-associated pollution microbiota was also detected in both recreational seasons in low abundances, except for wrack in July (2021), when the abundance reached 3% of the total abundance (see *Figure 8,* *D*). The levels of sewage pollution microorganisms remained relatively low in 2022 (see *Figure 9, D*). In both seasons *Acinetobacter* was the dominant genus. This type of pollution could have resulted from untreated sewage from summer households and seasonal residences in Šventoji, as *Acinetobacter* was previously associated with urban sewage infrastructure (Vandewalle et al., 2012).

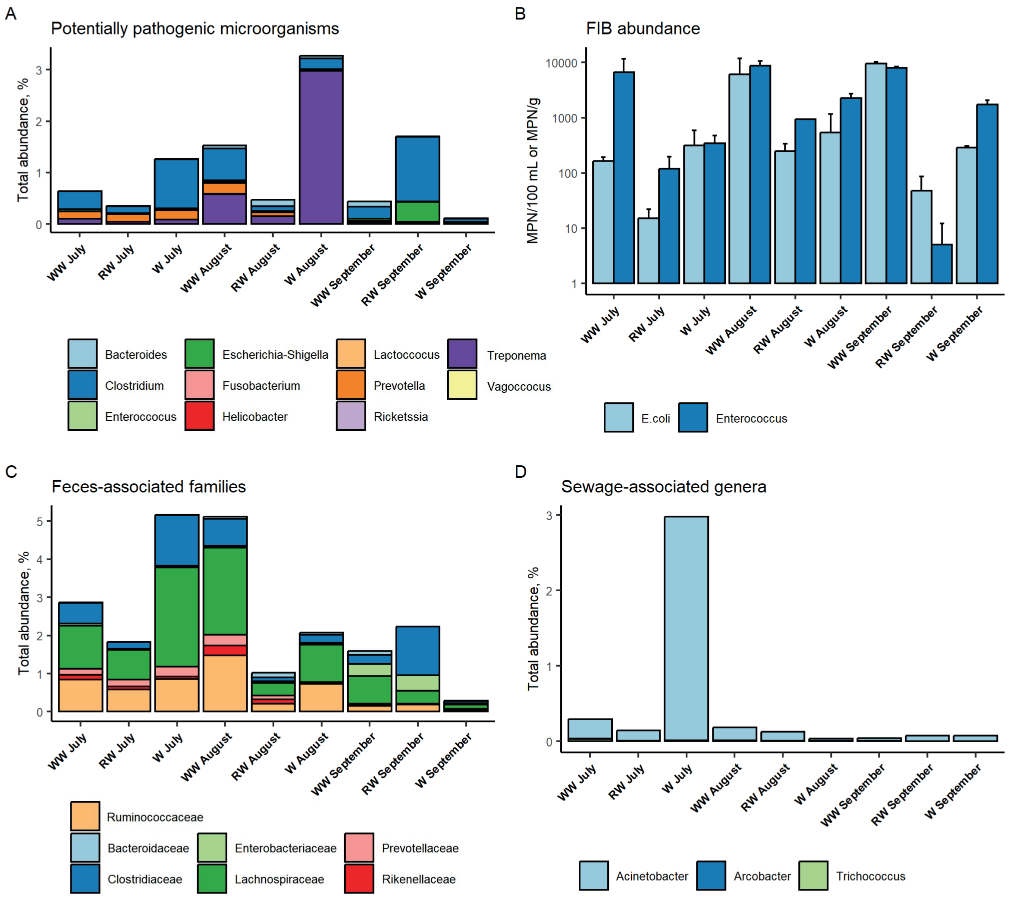

Figure 8. Relative abundance (% of total abundance) of potentially pathogenic microorganism genera (A), quantities of FIB (B), relative abundance (% of total abundance) of feces-associated families (C) and relative abundance (% of total abundance) of sewage-associated genera (D) during different sampling months in Šventoji (2021); WW – water with wrack, RW – water without wrack (reference area), W – wrack (reprinted from *Paper III* with permission).

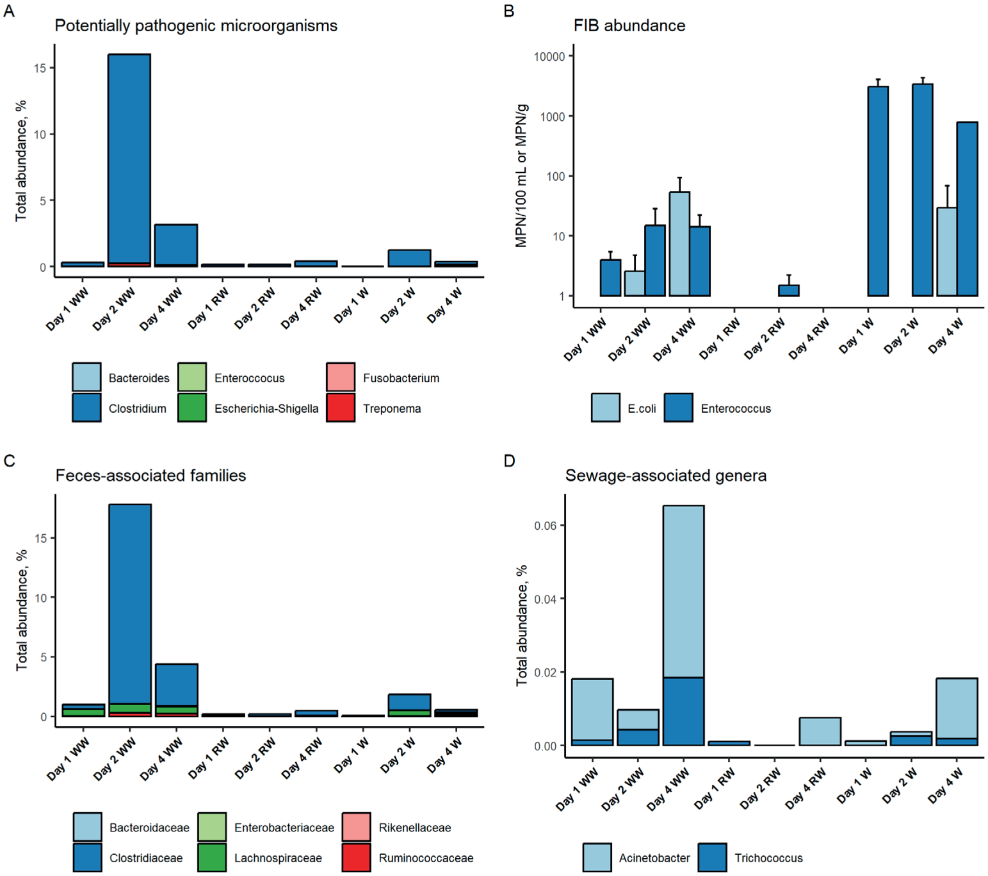

Figure 9. Relative abundance (% of total abundance) of potentially pathogenic microorganism genera (A), quantities of FIB (B), relative abundance (% of total abundance) of feces-associated families (C) and relative abundance (% of total abundance) of sewage-associated genera (D) during different sampling days (August 9 (1st day), 10 (2nd day) and 12 (4th day)) in Šventoji (2022); WW – water with wrack, RW – water without wrack (reference area), W – wrack (reprinted from *Paper III* with permission).

Wrack accumulations often serve as a food source for birds and their fecal pollution can become one of the primary sources of potential human pathogens, such as *Campylobacter* spp. and *Salmonella* spp. (Lévesque et al., 2000; Broman et al., 2002). Therefore, water, sand and wrack samples (2021 recreational season) were tested for the presence of *Salmonella* spp. and *Campylobacter* spp. using the PCR method. *Campylobacter* spp. was detected only in water and sand with wrack and wrack itself, only with one exception, when it was found in a reference area of water in Šventoji (see *Paper II*, *Table 1*). This indicates that beach wrack could serve as a potential habitat for these pathogens and could expose beach visitors to pathogenic bacteria, who often look for amber entangled within washed-up macroalgae. Another targeted pathogen (*Salmonella* spp.) was not detected in any of the samples. However, more research is needed to identify potential sources, quantities, and tendencies of these pathogens in areas with wrack.

By applying 16S rRNA sequencing, multiple enteric pathogens, mostly attributed to fecal pollution (Boukerb et al., 2021), were found in water and wrack samples from Šventoji in both studied seasons (2021 and 2022): *Bacteroides*, *Clostridium*, *Enterococcus*, *Escherichia-Shigella*, *Fusobacterium*, *Helicobacter*, *Lactococcus*, *Prevotella*, *Rickettsia*, *Treponema* and *Vagococcus*. The 16S rRNA amplicon sequencing approach is useful for providing a more comprehensive picture of microbial diversity and the presence of potential pathogens compared to conventional FIB monitoring. In the recreational season of 2021, the highest abundance of potentially pathogenic bacteria was found in wrack in August (3.3% of total abundance) and was dominated by *Treponema* genus (3%). On the remaining occasions the samples were dominated by *Clostridium* (0.06–1.3%) and *Prevotella* (from absence to 0.2%) (see *Figure 8, A*). On a multi-day observation in Šventoji during 2022, potentially pathogenic bacteria were found in the highest abundance on the second day in water with wrack (16%) and were entirely dominated by *Clostridium* (15.72%), but decreased significantly to 3.02% on the fourth day, which can be attributed to beach cleaning which took place on the third day of observation (see *Figure 9, A*). A high abundance of potential pathogens is expected in decaying wrack, as previous research has shown that the abundance of potential pathogens, including *Clostridium*, becomes more predominant in wrack with increasing decay time (Chun et al., 2017). However, the visible decrease in potential pathogens in water with wrack suggests that beach wrack removal could be a viable management option in recreational beaches as the abundance of potential pathogens decreases after its removal.

3.2.4. Potentially pathogenic *Vibrio* bacteria diversity and abundance found within environment with wrack

Currently, in the Baltic Sea, several *Vibrio* species are considered as potential human pathogens of concern: *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* (non-O1/non-O139) (Gyraitė et al., 2024). Multiple *Vibrio* species, including potential human pathogens, are important constituents of the live macrophyte microbiome, however, little is known about their presence or diversity in the beach wrack or what environmental conditions might affect their survival (see *Paper I*). Considering the favorable environmental conditions in the Baltic Sea (low salinity and high eutrophication) (Riedinger et al., 2024), it is important to investigate the potential of beach wrack to harbor potentially pathogenic *Vibrio* species.

Using the PCR approach, targeted *Vibrio* species (*V. cholerae* (non-O1/non-O139) and *V. vulnificus*) were detected more frequently in the environment with wrack accumulations than in reference sites. *V. vulnificus* was most frequently detected in water with wrack accumulations (88.9% of all water samples with wrack), and in wrack itself (70% of wrack samples), *V. cholerae* was detected in 55.6% of samples of water with wrack and 60% of wrack samples. Meanwhile, *V. alginolyticus* was detected in the Lithuanian part of the Baltic Sea for the first time, only once in July in the wrack accumulation site, and *V. parahaemolyticus* was not detected at all (see *Paper IV*, *Figure 2*).

Temperature has been identified as the main driving factor of *Vibrio* abundance in the Baltic Sea, especially the pathogenic species (Fleischmann et al., 2022; Riedinger et al., 2024). Our investigation revealed that other environmental parameters might be important for the survival of potentially pathogenic *Vibrio* bacteria, too. The most important factor for the *V. vulnificus* abundance was the relative abundance of *V. fucoides* in wrack, chl-a and oxygen, while for the presence of *V. vulnificus* – chl-a, cDOM, for the presence of *V. cholerae* – temperature, chl-a and oxygen (see *Paper IV*, *Supplementary Table S5*).

Applying the 16S rRNA sequencing approach, eight *Vibrio* OTUs were identified in water with wrack and 10 – in wrack (2021 season). Ten different OTUs were identified in 2022, both in water with wrack and wrack (see *Figure 10*). In both cases water with wrack and wrack supported a higher relative abundance of *Vibrio* compared to reference water (see *Paper IV*, *Figure 6*), indicating that wrack could harbor an extensive variety of *Vibrio* species. Some OTUs showed high similarity to specific *Vibrio* taxa, such as *V. ostreae*, *V. cholerae*, *V. anguillarum*, *V. pommerensis*, *V. rumoiensis*, *Vibrio sp.* MI-15 and *Vibrio* sp. F74 (see *Paper IV*, *Supplementary Figure 1*).

Of the potentially pathogenic species only *V. cholerae* was identified by 16S rRNA amplicon sequencing and PCR. Four different OTUs (OTU 7618 and 8435 in 2021, OTU 326 and 451 (*Vibrio* sp. F74) in 2022) were identified only in water with wrack and wrack itself, indicating that they might be a part of live macroalgae microbiome or participate in the decomposition of macrophyte wrack. For example, OTU 326, which was found only in water with wrack and wrack was positively and significantly correlated with the relative abundance of *V. fucoides* and *Ulva intestinalis*. Some *Vibrio* species might possess alga-specific polysaccharidases alongside very specific carbohydrate-active enzymes, which might participate in macroalgae degradation (Martin et al., 2014). However, when macrophytes decompose after being cast on the coast, multiple nutrients are released (Egan et al., 2013), which could support the growth of microorganisms that feed on these nutrients, including opportunistic pathogens.

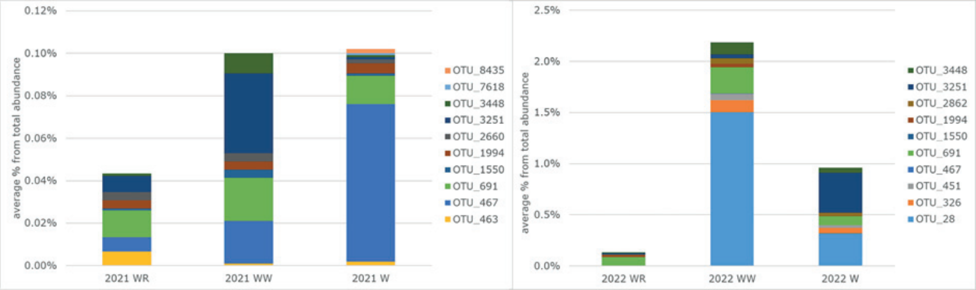

Figure 10. The average relative abundances of OTUs assigned to *Vibrio* in different conditions during single-day sampling events in 2021 and multiple-day sampling in 2022; WR – water without wrack, WW – water with wrack, W – wrack (reprinted from *Paper IV* with permission).

4

Recommendations

1. *Focusing on potentially pathogenic Vibrio identification and quantification in wrack (Paper I* and *IV)*. Currently there exists a research gap concerning potentially pathogenic *Vibrio* and wrack interactions in coastal marine areas. Considering the growing threat of potential *Vibrio* infections in the future with increasing sea surface temperatures, it is important to address this issue and take sufficient actions concerning wrack management.
2. *Wrack management in recreational beaches (Paper II* and *III)*. Results from *Paper II* and *III* indicated that wrack in recreational beaches could act as a trap for fecal pollution and associated pathogenic microorganisms, especially when a recreational beach is located close to the potential fecal pollution source. It is recommended to remove beach wrack from recreational beaches when it accumulates near river outflows on the Lithuanian coast or to relocate beach wrack to a coastal area not used by visitors to preserve the natural ecosystem and autochthonous fauna. However, other wrack-related aspects, such as residence time and the size of wrack accumulations, should be evaluated before coming to the decision to remove the beach wrack.
3. *Detection of fecal pollution sources (Paper II* and *III)*. Our results demonstrate that recreational beaches along the Lithuanian coast of the Baltic Sea are affected by multiple sources of fecal pollution, including humans, birds, rumi-

nants and sewage. To track the origin of fecal pollution, regular cultivation-based monitoring could be supplemented with molecular-based methods, such as 16S rRNA amplicon sequencing and MST markers.

4. *Public education about the presence of potential pathogens in beach wrack.* Although beach wrack plays an important role in the functioning of the beach ecosystem, it can also be a reservoir and secondary source of potential human pathogens; therefore, it is important to inform beach visitors about potential dangers related to beach wrack on recreational beaches.

5

Conclusions

1. Macrophyte-potentially pathogenic bacteria interactions in coastal marine environments have been studied for the past 50 years, primarily focusing on interactions of live macrophytes with FIB and *Vibrio* bacteria. Macrophytes serve as a habitat for FIB and potentially pathogenic *Vibrio*. Systematic review of the literature revealed that red macroalgae supported the highest quantities of potentially pathogenic bacteria, lower quantities were reported from green and brown macroalgae and seagrasses. Wrack studies mostly focused on FIB quantification; however, the abundance of *Vibrio* was not assessed.
2. Beach wrack presence significantly influences the microbial community in coastal waters. Phyla, such as Proteobacteria, Cyanobacteria, Bacteroidota and Actinobacteriota were dominant in environments with and without wrack. However, microbial community similarity analysis showed that the microbiota from water with wrack was clustered similarly to microbiota from wrack itself, while microbiota from reference water showed dissimilarity. The microbial community in the wrack-affected environment underwent more rapid temporal composition shifts throughout the wrack accumulation process compared to reference areas. These shifts are likely driven by changes in the physical and chemical properties of water with wrack.

3. Beach wrack accumulations dominated by red macroalgae are important habitat for fecal pollution-related bacteria and *Vibri*o on sandy beaches of the Baltic Sea. Significantly higher FIB quantities were observed in areas with wrack compared to reference areas, with concentrations often exceeding the thresholds set in the hygiene norm 92:2018. While beach wrack plays an important ecological role in the coastal ecosystem, it also presents potential health risks to humans by harbouring human pathogens in recreational areas. Higher relative abundances of enteric pathogens (*Campylobacter*, *Bacteroides*, *Clostridium*, *Enterococcus*, *Escherichia-Shigella*, *Fusobacterium*, *Helicobacter*, *Lactococcus*, *Prevotella*, *Rickettsia*, *Treponema* and *Vagococcus*) and ubiquitous *Vibrio* bacteria (*V. vulnificus* and *V. cholerae*) were detected in the environment with wrack compared to reference areas.
4. Application of genetic markers HF183 and GFD allowed to identify the presence of fecal pollution related to humans and birds to a different degree. While it is a quick and reliable method to detect fecal pollution sources, it does not reveal all possible sources. 16S rRNA sequencing additionally revealed the presence of fecal pollution related to ruminants and sewage in Šventoji. These findings emphasize the need for more comprehensive monitoring strategies that account for various sources of fecal pollution, as well as the dynamic nature of wrack and its potential impact on public health risks and the importance of multi-day observations to understand better long-term fecal pollution trends in coastal areas with wrack accumulations.
5. The increased relative abundance of filamentous macroalgae (*C. glomerata*, *C. rupestris*, *V. fucoides*) in wrack was associated with higher cDOM and turbidity, which contributed to increased FIB levels. Chl-a, oxygen, cDOM, temperature and relative abundance of *V. fucoides* significantly influenced the presence and abundance of *V. vulnificus* and *V. cholerae*. Wrack also contributed to the trapping or release of bacteria along the coast, especially during beach grooming events.

6

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7

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8

Summary in Lithuanian

ĮVADAS

Pakrančių maudyklų vandens kokybė Europos sąjungoje (ES) nuolat gerėja, ir 2023 metais 88,7% iš jų vandens kokybė buvo įvertinta puikiai (EEA, 2014, 2024).

Pagal ES maudyklų vandens kokybę reglamentuojančią Maudyklų direktyvą (European Parliament, 2006), kurios nuostatos įtrauktos į Lietuvos higienos normą HN 92:2018 (LRSAM, 2018), pajūrio maudyklų vandens kokybė turi būti vertinama remiantis dviejų mikrobiologinių parametrų – *Escherichia coli* ir *Enterococcus* spp. – ribinėmis vertėmis. *E. coli* ir *Enterococcus* spp. bakterijos yra fekalinę taršą (FIB) rodantys indikatoriai. Kartu su fekaline tarša į maudyklų vandenį gali patekti įvairių žmogaus ligų sukėlėjų, todėl rekomenduojama vertinti ir kitų potencialių žmogaus patogenų kiekį (Harwood et al., 2014; Tiwari et al., 2021). Visų patogenų monitoringas nėra nei tvarus ekonomiškai, nei praktiškai įmanomas, todėl FIB naudojamos kaip alternatyva (Prüss, 1998; Harwood et al., 2014). Kita vertus, FIB nerodo fekalinės taršos šaltinio, nes šios bakterijos aptinkamos visų šiltakraujų gyvūnų virškinamajame trakte. Fekalinės taršos šaltiniai gali būti įvairūs, tačiau su žmonėmis susijusi fekalinė tarša laikoma pavojingiausia (Bosch et al., 1991; Sinton et al., 1998; Reynolds et al., 2008). Siekiant identifikuoti fekalinės taršos šaltinius, buvo sukurti molekuliniai metodai, kuriuose naudojami konkrečiam organizmui priskiriami genetiniai žymenys (Harwood et al., 2014). Kitas alternatyvus taršos identifikavimo metodas yra eDNR didelio našumo sekoskaita, kuri padeda nustatyti su fekaline ir nuotekų tarša susijusius bakterijų taksonominius vienetus (Newton et al., 2013; Basili et al., 2023).

Baltijos jūros paplūdimiuose poilsiautojus taip pat gali infekuoti potencialiai patogeniškos, bet su fekaline tarša nesusijusios *Vibrio* bakterijos (Gyraitė et al., 2024). Dauguma su *Vibrio* susijusių infekcijų Baltijos jūros regione priskiriamos šioms rūšims: *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* ir *V. alginolyticus*. Minėtos rūšys gali sukelti įvairius klinikinius simptomus, dažniausiai – lengvą gastroenteritą, tačiau *V. vulnificus* gali sukelti septicemiją. Temperatūra yra pagrindinis *Vibrio* spp. gausumą vandenyje lemiantis veiksnys (Baker-Austin et al., 2013, 2017, 2018). Baltijos jūros paviršiaus temperatūra ateityje kils 0,063–0,078 °C/metus, todėl tikėtina, kad infekcijų atvejų skaičius ateityje didės (Baker-Austin et al., 2013; Trinanes and Martinez-Urtaza, 2021). Nepaisant to, kad Baltijos jūroje aptinkama potencialiai patogeniškų *Vibrio* rūšių ir nustatomi vibriozės atvejai, šiuo metu ne visame regione, įskaitant ir Lietuvą, vykdoma potencialiai patogeniškų *Vibrio* bakterijų ir su *Vibrio* susijusių infekcijos atvejų stebėsena.

Makrofitų sąnašos – organinių medžiagų, sudarytų iš makrodumblių ar jūržolių, sankaupos pakrantėje ir priekrantės vandenyje – taip pat gali turėti įtakos maudyklų vandens kokybei (Macreadie et al., 2017; Cesarini et al., 2021). Sąnašos yra svarbus paplūdimių ekosistemos maistinių medžiagų šaltinis ir bestuburių buveinė (Orr et al., 2005; Dugan et al., 2011; Schlacher et al., 2017; Prasad et al., 2018). Tačiau jos gali sudaryti palankias sąlygas potencialiai patogeniškų bakterijų išgyvenimui (Ghoul et al., 1995; Anderson et al., 1997; Byappanahalli et al., 2003; Beckinghausen et al., 2014; Abdool-Ghany et al., 2022). Potencialių žmogaus patogenų buvo rasta vandenyje ir smėlyje su sąnašomis ir pačiose sąnašose tiek gėluose (Byappanahalli et al., 2003; Whitman et al., 2003; Ishii et al., 2006; Vanden Heuvel et al., 2010; Chun et al., 2013), tiek didelio druskingumo vandens telkiniuose (Anderson et al., 1997; Imamura et al., 2011; Quilliam et al., 2014; Abdool-Ghany et al., 2022). Paplūdimio sąnašų poveikis potencialiai patogeninėms bakterijoms Baltijos jūroje praktiškai nėra tirtas, išskyrus Gubelit and Vainshtein (2011) tyrimą, kuriame buvo analizuojamas potencialiai patogeniškų *Salmonella enteritidis* bakterijų augimas irstančių makrodumblių sąnašose rytinėje Suomijos įlankos dalyje.

Tyrimo tikslas ir pagrindiniai uždaviniai

Pagrindinis disertacijos tikslas – įvertinti ar makrofitų sąnašos gali turėti poveikį su fekaline tarša susijusių ir kitų potencialiai patogeniškų mikroorganizmų gausumui ir įvairovei smėlio paplūdimiuose pietrytinėje Baltijos jūros pakrantėje.

Šios disertacijos uždaviniai:

1. Remiantis literatūros duomenimis įvertinti makrofitų ir jų sąnašų poveikį su fekaline tarša susijusių ir potencialiai patogeniškų *Vibrio* gausumui jūrų pakrančių aplinkose.

2. Įvertinti mikroorganizmų bendrijų įvairovės ir santykinio gausumo dinamiką laike paplūdimio aplinkoje su sąnašų sankaupomis.
3. Nustatyti, ar Baltijos jūros pakrantės smėlėtuose paplūdimiuose besikaupiančios sąnašos yra su fekaline tarša susijusių potencialiai patogeninių mikroorganizmų ir *Vibrio* rūšių buveinė ir nustatyti fekalinės taršos šaltinius aplinkoje su makrofitų sąnašų sankaupomis.
4. Nustatyti aplinkos sąlygas, kurios lemia fekalinę taršą rodančių bakterijų ir potencialiai patogeninių *Vibrio* bakterijų įvairovę ir gausumą vandenyje su makrofitų sąnašų sankaupomis.

Darbo naujumas

Parengta pirmoji sistematinė literatūros apžvalga, įvertinanti makrofitų ir jų sąnašų sąveikas su FIB ir potencialiais žmogaus patogenais, *Campylobacter*, *Salmonella*, *Shigella* ir *Vibrio*, jūrų pakrančių aplinkoje. Be to, tai pirmasis tyrimas, kuriame analizuota FIB, *Vibrio*, *Salmonella*, *Campylobacter* ir kitų potencialių patogenų dinamika ir atskleisti skirtingi fekalinės taršos šaltiniai rekreaciniuose paplūdimiuose su makrofitų sąnašų sankaupomis pietrytinėje Baltijos jūros dalyje. Pirmą kartą nustatyta, kad potencialiai patogeniškos *Vibrio* bakterijos, įskaitant *V. vulnificus* ir *V. cholerae*, yra randamos pietrytinės Baltijos jūros dalyje esančiuose rekreaciniuose paplūdimiuose kuriuose kaupiasi makrofitų sąnašos. Šiuo metu Lietuvos maudyklų vandens kokybės monitoringo metu naudojamas tik bakterijų kultivavimas ant terpių, neparodantis fekalinės taršos šaltinių. Tuo tarpu šiame darbe fekalinės taršos šaltinių nustatymui rekreaciniuose paplūdimiuose naudota kultivavimo, genetinių žymenų ir 16S rRNR sekoskaitos metodų kombinacija, padėjusi atskleisti fekalinės taršos šaltinių bei su fekaline tarša susijusių potencialių patogenų gausumą ir įvairovę sąnašose arti upės žiočių. Šie rezultatai yra itin svarbūs už paplūdimių tvarkymą atsakingoms institucijoms bei visuomenei, kadangi iki šiol fekalinės taršos šaltinių įvairovė ir pačių sąnašų poveikis potencialiems patogenams bei fekalinei taršai Lietuvos rekreaciniuose paplūdimiuose nebuvo tiriami.

Rezultatų mokslinė ir praktinė reikšmė

Sistematinė literatūros apžvalga atskleidė, kad skirtingos makrofitų grupės (žaliadumbliai, rudadumbliai ir raudondumbliai bei jūržolės) palaikė skirtingus potencialiai žmogui patogeniškų bakterijų kiekius vandenyje, nuosėdose ir ant pačių makrofitų paviršiaus. Taip pat buvo identifikuota tyrimų spraga – tik dvi publikacijos analizavo potencialiai patogeniškų *Vibrio* bakterijų ir makrofitų sąnašų sąveikas, ir nė viena iš jų nevertino *Vibrio* kiekių jose. Ši spraga buvo iš dalies užpildyta mūsų tyrimu, kuris parodė, kad potencialiai patogeniškų *V. vulnificus* ir *V. cholerae* bakterijų yra paplūdimiuose su raudondumblių sąnašomis, taip pat didesni *Vibrio* bakterijų kiekiai rasti vandenyje su sąnašomis. Mūsų tyrimai taip pat rodo, kad raudondumblių sąnašos gali sulaikyti ir sukoncentruoti su fekaline tarša susijusius patogenus bei natūraliai vandenyje esančias *Vibrio* bakterijas. Kadangi tyrimų rezultatai rodo, kad makrofitų sąnašos gali sukurti tinkamas sąlygas potencialiai patogeniškoms bakterijoms, šios žinios gali padėti už paplūdimių tvarkymą atsakingoms institucijomis priimti tinkamus sąnašų tvarkymo sprendimus. Taip pat Lietuvos nacionalinėje maudyklų monitoringo programoje būtų tikslinga įtraukti reikalavimą papildomai vykdyti monitoringą tose vietose, kuriose yra sąnašų sankaupų, nes būtent ten gali būti aptinkamos didesnės fekalinės taršos koncentracijos. Šiuo metu atliekant maudyklų vandens kokybės monitoringą naudojami tik bakterijų kultivavimo metodai, kurie nenustato galimo fekalinės taršos šaltinio ir neapsaugo visuomenės nuo galimo fekalinės taršos poveikio, kadangi mėginių surinkimas bei jų analizė užtrunka ne mažiau nei 48 valandas. Todėl reikalingi alternatyvūs metodai, kurie papildytų informaciją, surinktą bakterijų kultivavimo metu. Todėl šio darbo rengimo metu fekalinės taršos ir jos šaltinių vertinimas buvo atliktas tiek bakterijų kultivavimo ant terpių metodu, tiek papildant juos alternatyviais vertinimo metodais: fekalinės taršos šaltinio identifikavimu naudojant genetinius žymenis, kurie leido nustatyti fekalinę taršą susijusią su žmonėmis bei paukščiais, bei 16S rRNR sekoskaita, kuri leido papildomai identifikuoti bakterijų taksonus, susijusius su fekaline, nuotekų bei galvijų tarša. Šio darbo rezultatai parodė, jog raudondumblių sąnašos gali būti svarbus antrinis šaltinis bakterijoms, susijusioms su įvairios kilmės fekaline tarša, todėl būtų galima rekomenduoti fekalinės taršos šaltinių stebėjimą įtraukti į Lietuvos nacionalinę maudyklų monitoringo programą.

TYRIMŲ MEDŽIAGA IR METODAI

Tyrimų rajonas

Tyrimai buvo atlikti keturiuose intensyviausiai rekreacijai naudojamuose paplūdimiuose Lietuvos priekrantėje – Melnragėje, Karklėje, Palangoje ir Šventojoje. Visiems paplūdimiams būdingos skirtingos makrofitų sąnašų kaupimosi ir druskingumo tendencijos.

Literatūros apžvalga

Pagrindinis apžvalgos tikslas – įvertinti dabartinę potencialiai patogeniškų bakterijų (*Vibrio*, *Campylobacter*, *Salmonella*, *Shigella*, *Escherichia coli*, *Enterococcus*) ir gyvų makrofitų bei jų sąnašų sąveikas jūrų priekrančių aplinkoje. Mokslinių straipsnių paieškai naudotos atitinkamų raktažodžių kombinacijos ScienceDirect, PubMed ir Google Scholar duomenų bazėse. Analizei reikalinga informacija paimta iš straipsnių, atrinktų pagal specifinius kriterijus.

In situ mėginių rinkimas ir analizė

2021 metais Melnragės, Karklės, Šventosios ir Palangos paplūdimiuose buvo analizuojami FIB, *Salmonella*, *Campylobacter* ir potencialiai patogeniškų *Vibrio* rūšių (*V. cholerae*, *V. vulnificus*, *V. alginolyticus*, *V. parahaemolyticus*) buvimas ir kiekiai, taip pat genetinių žymenų GFD (paukščių fekalinė tarša) ir HF183 (žmonių fekalinė tarša) kiekiai.

Tyrimams pasirinktos vietos su makrofitų sąnašomis, kuriose jos pilnai padengė ne mažesnį nei 40 m^2 plotą, ir referentinės vietos, kuriose vizualiai matomų sąnašų nebuvo. Vietos su sąnašomis buvo nutolusios nuo referentinių vietų mažiausiai 100 metrų atstumu. 2022 metais trys daugiadienės analizės buvo atliktos Melnragės (2022 liepos 29 – rugpjūčio 2 ir rugsėjo 1 – 6 dienomis) ir Šventosios (2022 rugpjūčio 9 – 12 dienomis) paplūdimiuose siekiant nustatyti FIB ir genetinių žymenų dinamiką vietose su sąnašomis ir referentinėse vietose. Iš Šventosios 2021 ir 2022 metais vandens ir sąnašų mėginių buvo išskirta DNR ir atlikta 16S rRNR sekoskaita siekiant išsiaiškinti potencialių patogenų ir fekalinės taršos šaltinių įvairovę vietose su sąnašomis ir referentinėse vietose.

FIB kultivavimui ir molekulinei analizei surinkti vandens, smėlio ir sąnašų mėginiai buvo įdėti į šaltkrepšį su šaldymo elementais ir pargabenti į laboratoriją tolimesniam apdorojimui bei analizei. Paimti papildomi mėginiai nustatyti makrofitų rūšinę sudėtį, bei cheminius ir fizinius vandens parametrus.

FIB, GFD ir HF183 žymenų, *Vibrio* bakterijų kiekio nustatymas, *Salmonella* ir *Campylobacter* identifikacija

E. coli ir *Enterococcus* spp. kiekiui vandenyje, smėlyje ir sąnašose nustatyti naudoti atitinkamai Colilert ir Enterolert metodai. HF183 (žmonių fekalinės tarša) ir GFD (paukščių fekalinė tarša) žymenų kiekiui nustatyti naudota tikralaikės polimerazės grandininės reakcijos (PGR) metodas pagal Haugland et al., 2010 ir Green et al., 2012 (II ir III publikacijos). Įprastinė PGR naudota identifikuoti *V. cholerae, V. parahaemolyticus, V. vulnificus* ir *V. alginolyticus* (Chun et al., 1999; Kim et al., 1999; Kaysner and DePaola, 2001; Luo and Hu, 2008) (IV publikacija), bei *Salmonella* ir *Campylo-*

bacter buvimą (Aabo et al., 1993; Linton et al.,1996) (II publikacija). Tikralaikė PGR naudota nustatyti *V. vulnificus* kiekį (Campbell, Wright, 2003) (IV publikacija).

Mikroorganizmų bendrijų analizė

Iš Šventosios vandens ir sąnašų mėginių 2021 ir 2022 metais buvo išskirta DNR ir atlikta 16S rRNR sekoskaita (Novogene, Cambridge, Jungtinė Karalystė). Remiantis filogenetine klasterine analize buvo lyginama mikroorganizmų sudėties mėginiuose panašumas (III publikacija). Remiantis 16S rRNR sekoskaitos duomenimis buvo identifikuoti operaciniai taksonominiai vienetai (OTV), rodantys fekalinę, nuotekų (Newton et al., 2013; Hägglund et al., 2018; Basili et al., 2023) bei galvijų (Mizrahi et al., 2021) (III publikacija) taršą. Atskirai buvo identifikuotos su fekaline tarša susijusių patogeninių bakterijų gentys (Boukerb et al., 2021) (III publikacija), bei OTV priklausantys *Vibrio* genčiai (≥97% panašumas) (IV publikacija).

Statistinė analizė

Neparametrinis Mann-Whitney testas buvo naudotas palyginti FIB ir genetinių žymenų kiekius vandenyje ir smėlyje su sąnašomis ir be jų. Spearman‘o koreliacija buvo naudojama vertinant ryšio stiprumą ir reikšmingumą tarp: a) FIB ir fekalinės taršos žymenų, b) FIB, fekalinės taršos žymenų ir *V. vulnificus* bei fizinių, cheminių ir biologinių vandens parametrų, c) fekalinę, galvijų ir nuotekų taršą identifikuojančių OTV bei fekalinės taršos žymenų gausumo, d) *Vibrio* santykinio gausumo (pagal 16S rRNR sekoskaitos duomenis) ir aplinkos parametrų vandenyje. Koreliacijos reikšmingumo lygis buvo nustatytas $p<0,05$ (II, III ir IV publikacijos).

Perteklinė analizė (angl. *redundancy analysis* (RDA)) buvo naudojama siekiant įvertinti fizikinių-cheminių ir biologinių aplinkos kintamųjų svarbą FIB, bendrųjų koliforminių bakterijų, fekalinės taršos žymenų gausumui vandenyje (II publikacija).

Aplinkos faktorių svarbai *V. vulnificus* gausumui ir buvimui bei *V. cholerae* buvimui nustatyti naudotas atsitiktinių medžių (angl., *multivariate random forest* (MRF)) modelis (IV publikacija).

REZULTATAI IR DISKUSIJA

Potencialių patogeninių bakterijų ir makrofitų sąveika jūrų pakrančių aplinkoje: retrospektyvi literatūros šaltinių analizė

Tendencijos erdvėje ir laike

Išsamioje 55 mokslinius straipsnius apėmusioje literatūros apžvalgoje analizuojama *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* ir *V. alginolyticus* (įskaitant *Vibrio* spp.), *Salmonella*, *Shigella*, *Escherichia coli* ir *Enterococcus* spp. sąveika su makrofitais arba jų sąnašomis jūrų pakrantėse visame pasaulyje. Išrinkta informacija apie potencialiai patogeniškų mikroorganizmų bei makrofitų geografinį pasiskirstymą, temperatūros ir druskingumo diapazonus, makrofitų tipus bei mikroorganizmų gausą.

Makrofitų ir potencialiai patogeninių bakterijų sąveikų tyrimai pradėti nuo 1969 m. (Chan and McManus, 1969), sąnašos pradėtos tyrinėti vėliau – 1985 m. (Wahbeh and Mahasneh, 1985). 44 darbai nagrinėjo bakterijas ant augančių makrofitų, tik 12 – makrofitų sąnašose. Labiausiai ištirtos bakterijos priklausė *Vibrio* spp. (41,4%) ir FIB (36,2%). Dauguma tyrimų buvo vykdyti Šiaurės Atlanto ir Šiaurės Ramiajame vandenynuose, daugiausia dėmesio skiriant paplūdimių kokybės vertinimui ir mikroorganizmų įvairovei nustatyti (Chan and McManus, 1969; Martin-Kearley et al., 1994; Duan et al., 1995; Bonilla et al., 2006; Imai et al., 2006; Dobretsov and Qian, 2010; Imamura et al., 2011; Izbicki et al., 2012; Haley et al., 2012; Gonzalez et al., 2014; Quilliam et al., 2014; Russell et al., 2013, 2014; Crump et al., 2018; Sun et al., 2020; Abdool-Ghany et al., 2022).

Tirtos bakterijos buvo rastos druskingumo gradiente nuo 0,2 iki 37 PSU. *E. coli*, *V. parahaemolyticus* ir *V. alginolyticus* buvo randami didesnio druskingumo sąlygomis, o *Enterococcus* spp., *Vibrio* spp., *V. vulnificus*, *V. cholerae* ir *Salmonella* spp. buvo nustatytos platesniame druskingumo diapazone. Bakterijos taip pat rastos plačiame temperatūrų gradiente (nuo 3,5 iki 35,6°C), o dauguma, išskyrus *Salmonella* ir *V. alginolyticus*, buvo aptinkami esant aukštesnei nei 30°C temperatūrai. Ryšys tarp temperatūros ir mikroorganizmų aptikimo buvo nenuoseklus, nebuvo nustatytas aiškus ryšys tarp temperatūros lygio ir mikroorganizmų aptikimo.

Potencialiai patogeniškos bakterijos randamos augančių makrofitų buveinėse bei jų sąnašose

Vibrio, *Enterococcus* ir *E. coli* yra labai svarbūs makrofitų mikrobiotos komponentai. Iš viso 18 *Vibrio* rūšių, įskaitant potencialiai patogeniškas *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus* ir *V. cholerae,* buvo rasta ant makrofitų paviršiaus, jų rizosferoje ir juos supančioje aplinkoje. Didžiausi *Vibrio* spp. kiekiai rasti ant raudondumblių ($2{,}7\times10^5$ CFU/g), mažesni kiekiai rasti ant rudadumblių ($6{,}7\times10^4$ CFU/g). Skirtingos *Vibrio* rūšys gali skaidyti skirtingus makrofitų išskiriamus antrinius metabolitus, taip pat metabolitai yra svarbūs bioplėvelių susidarymui makrofitų paviršiuje (Liao et al., 2003; Hall-Stoodley et al., 2004; Ymele-Leki et al., 2013). Tačiau antriniai metabolitai gali ir slopinti kai kurių *Vibrio* rūšių prisitvirtinimą prie makrofitų paviršiaus (Liao et al., 2003; Chakraborty et al., 2010;). Potencialiai patogeniškų *Vibrio* rūšių ir gyvų makrofitų sąveika tebėra mažai ištirta tema, kuriai ateityje turėtų būti skiriama daugiau dėmesio.

Didžiausi *E. coli* ir *Enterococcus* kiekiai buvo rasti gyvų rudadumblių ir žaliadumblių bendrijose. *E. coli* gausiausiai rasta ant makrodumblių paviršiaus ($1{,}8\times10^4$ CFU/g), *Enterococcus* – nuosėdose, esančiose makrofitų aplinkoje ($3{,}2\times10^3$ CFU/g). Šiuo metu žinoma, kad FIB gali išgyventi ne tik šiltakraujų gyvūnų žarnyne, bet ir aplinkoje (Walk et al., 2009; Devane et al., 2020). Makrofitų antriniai metabolitai gali būti laikomi šių bakterijų mitybos šaltiniu, todėl bakterijos ant makrofitų paviršiaus gali formuoti bioplėvelę (Kantachumpoo and Chirapart, 2010; Quero et al., 2015).

Publikacijose, skirtose bakterijoms makrofitų sąnašose, daugiausiai nagrinėjamas FIB identifikavimas bei jų kiekio nustatymas. Tai susiję su sąnašų sankaupų paplūdimiuose tvarkymo aspektu, nes sąnašos laikomos FIB šaltiniu maudyklų vandenyje (Imamura et al., 2011; Quilliam et al., 2014; Abdool-Ghany et al., 2022). Didesni *E. coli* ($1{,}7\times10^4$ CFU/g) ir *Enterococcus* ($8{,}6\times10^3$ CFU/g) kiekiai aptikti pačios sąnašose, negu vandenyje ar smėlyje su sąnašomis. Nustatyta tyrimų spraga, susijusi su *Vibrio* – tik dvejose publikacijose (Wahbeh and Mahasneh, 1985; Trevathan-Tackett et al., 2020;) įvertintas *Vibrio* buvimas sąnašose, tačiau netirti kiekiai.

Su fekaline tarša susijusios ir potencialiai patogeniškos *Vibrio* bakterijos pietryčių Baltijos paplūdimiuose su raudondumblių sąnašomis

Vienadienis ir daugiadienis bakterijų bendrijų pokyčių vertinimas Šventosios paplūdimyje

2021 m. vienadienių tyrimų metu buvo identifikuotos 68 skyriams priklausančios bakterijos. Vandenyje su sąnašomis dominavo Proteobacteria (28-31,2% santykinis gausumas), Cyanobacteria (22,6-30%), Bacteroidota (11,6-14,8%) ir Actinobacteriota (10,6-20%). Klasterinė analizė parodė, kad bakterijų bendrijos vandenyje su sąnašomis bei pačiose sąnašose labiau skyrėsi tarpusavyje, nei bakterijų bendrijos vandenyje iš referentinių vietų. Tai gali būti susiję su tuo, kad mėginiai buvo surinkti skirtingose sąnašų kaupimosi stadijose. 2022 m. daugiadienių mėginių rinkimo metu aptikta bakterijų, priklausančių 50 skirtingų skyrių, dominavo Proteobacteria (36,5-40,8%), Cyanobacteria (16,2-18%), Bacteroidota (20,2-21,8%) ir Actinobacteriota (11,7-13,4%). Mikroorganizmų bendrijos vandenyje su sąnašomis ir pačiose sąnašose patyrė intensyvesnius struktūrinius pokyčius palyginus su vandeniu be sąnašų. Taip pat remiantis klasterine analize nustatyta, kad mikroorganizmų bendrijos vandenyje be sąnašų suformavo atskirą medžio atšaką rodančią šių mėginių tarpusavio panašumą, bet vandens su sąnašomis bei pačių sąnašų mėginiai tarpusavyje buvo mažiau panašūs. Tai rodo, kad tiek vandenyje su sąnašomis, tiek pačiose sąnašose esančios bakterijų bendrijos patiria reikšmingus struktūrinius pokyčius.

Vienadienis ir daugiadienis paplūdimių sąnašų poveikio vertinimas FIB buvimui ir gausai rekreaciniuose paplūdimiuose

FIB koncentracija vandenyje su sąnašomis ir be jų (2021 m. duomenys) buvo palyginta su Higienos normos 92:2018 ribinėmis vertėmis (1000 MPN/100 ml – *E. coli*, 100 MPN/100 ml – *Enterococcus* spp.). Ribinių verčių viršijimai rodo, kad maudykla yra nesaugi maudymuisi. Mūsų tyrimo metu FIB kiekis penkis kartus viršijo ribines vertes vandenyje su sąnašomis ir du kartus referentiniame vandenyje, o oficialaus savivaldybių monitoringo duomenimis tų pačių paplūdimių vandenyje FIB kiekiai neviršiojo ribinių verčių. Rezultatai galėjo skirtis dėl nevienodo mėginių rinkimo laiko ir vietos – oficialaus savivaldybių monitoringo metu mėginiai buvo imami ne tuo pačiu kaip šio tyrimo metu, be to, mėginiai buvo paimti skirtingose maudyklų vietose.

Sąnašų irimo procesas turi įtakos vandens aplinkai, vidutinis vandens drumstumo, chlorofilo-a, suspenduotos dalelinės medžiagos (SDM) ir ištirpusios spalvotosios organinės medžiagos (ISOM) kiekis buvo reikšmingai didesnis vandenyje su sąnašomis, negu referentinėse vietose. Labai tikėtina, kad siūlinių dumblių (*V. fucoides, C. rupestris and C. glomerata*) irimas prisidėjo prie padidėjusio vandens drumstumo ir ISOM kiekio. Vidutinės *Enterococcus* spp. koncentracijos buvo reikšmingai didesnės tiek vandenyje su sąnašomis, tiek smėlyje, ir teigiamai koreliavo su ISOM, drumstumu ir siūlinių makrodumblių proporcija sąnašose. Sąnašos taip pat gali veikti kaip fizinis barjeras, sulaikantis bakterijas – didžiausi FIB kiekiai aptikti Šventosios paplūdimyje, šalia Šventosios upės ištakų, vandenyje kuriame kaupėsi sąnašos (9625.5±688 MPN/100 mL (*E. coli*)). FIB kiekis smėlyje išliko žemas palyginus su vandeniu bei sąnašomis. Tačiau vienadienių tyrimų trūkumas yra tas, kad nėra aišku, kurioje kaupimosi ant kranto stadijoje yra sąnašos.

2022 metais buvo atlikti trys daugiadieniai stebėjimai, kurie apėmė stebėsenos laikotarpį nuo keturių iki šešių dienų – iki tol, kol sąnašos buvo nuplautos bangų, arba pašalintos atsakingų institucijų. Panašiai kaip ir 2021 metais, vandenyje ir smėlyje su sąnašų sankaupomis vidutinės *Enterococcus* koncentracijos buvo reikšmingai didesnės, negu referentinėse vietose, o *E. coli* –smėlyje su sąnašomis. Sąnašų pašalinimas iš Šventosios trečiąją mėginių rinkimo dieną nulėmė pastebimą FIB padidėjimą smėlyje sekančią dieną. Tikėtina, kad sąnašų pašalinimas leido FIB patekti iš gilesnių į paviršinius smėlio sluoksnius.

Fekalinės taršos šaltiniai ir su fekaline tarša susijusios bakterijos aplinkoje su sąnašomis

Fekalinės taršos šaltinių nustatymui buvo panaudoti žmogaus (HF183 (*Bacteroides* spp.)) (Ahmed et al., 2012) ir paukščių fekalinę taršą (GFD (*Helicobacter* spp.)) (Green et al., 2012) identifikuojantys genetiniai žymenys.

2021 metų stebėjimų metu GFD ir HF183 koncentracijos vandenyje, smėlyje ir sąnašose labai kito. HF183 tik iš dalies paaiškino FIB kiekių padidėjimą aplinkoje. Rugpjūčio 27 d. didžiausia HF183 koncentracija buvo aptikta Šventojoje, vandenyje su sąnašomis (19 600±70.7 GC/100 ml), o rugpjūčio 10 d. – Melnragėje, pačiose sąnašose (45 300±1243 GC/g). HF183 teigiamai koreliavo su *E. coli* ir *Enterococcus* spp. (kai FIB koncentracijos viršijo HN 92:2018 nustatytas ribines vertes), ypač stipriai koreliavo su *Enterococcus* koncentracijomis sąnašose (r=0,707). Tuo pačiu metu padidėjusios FIB koncentracijos (didesnės už HN 92:2018 nustatytas ribines vertes) ir HF183 buvo rasti Šventojoje ir Palangoje. Tikėtina, kad fekalinę taršą Šventojoje atnešė Šventosios upės vanduo, o sąnašos veikė kaip fizinis barjeras, sulaikantis su fekaline tarša susijusias bakterijas. Remiantis šiais dviem genetiniais žymenimis, kitose tyrimų vietose fekalinės taršos šaltinis nebuvo nustatytas.

Aiškių koreliacijos tendencijų tarp GFD ir FIB nerasta, įvairaus stiprumo koreliacijos buvo tiek teigiamos, tiek neigiamos. Prie fekalinės taršos prisidedančius paukščius sąnašos gali pritraukti kaip mitybos šaltinis (Green et al., 2012; Gagnon et al., 2013; Ferreira, 2019). Mūsų tyrime GFD žymuo dažniausiai buvo nustatytas vandenyje su sąnašomis. Didžiausias GFD gausumas birželio 2 d. nustatytas referentinėje zonoje vandenyje Melnragėje (22 478±514,8 GC/100 ml), birželio 2 d. – Melnragėje pačiose sąnašose (7071,2±90.8 MPN/g).

2022 metais pastebėtos skirtingos HF183 ir GFD aptikimo tendencijos. Tiek Melnragėje, tiek Šventojoje dominavo GFD, HF183 buvo aptinkamas sporadiškai. GFD gausumas vandenyje svyravo nuo 0 iki $7,9 \times 10^3$ GC/100 mL, smėlyje didžiausias gausumas siekė $2,4 \times 10^4$ GC/g MPN/g, sąnašose GFD kiekis varijavo, didžiausia koncentracija siekė $3,5 \times 10^5$ GC/g.

2021 m. HF183 buvo dažnai aptinkamas vandenyje ir sąnašose, bet 2022 m. buvo aptinkamas retai visuose mėginiuose. 2021 m. abu žymenys buvo retai aptinkami smėlyje, tačiau GFD žymuo buvo gausiai aptinkamas smėlyje 2022 metais. Šie du žymenys reikšmingai nekoreliavo su FIB. Reikšmingų koreliacijų nebuvimas gali atsirasti dėl daugelio priežasčių, viena pagrindinių – kultivavimo metu užauginamos tik gyvybingos FIB, tuo tarpu žymenys gali identifikuoti ir DNR (Ahmed et al., 2016). Žymenų efektyvumas taip pat gali geografiškai skirtis (Green et al., 2012). Taip pat fekalinės taršos šaltiniai gali būti ir kiti.

Su fekaline tarša susijusių bakterijų įvairovei Šventojoje nustatyti buvo išanalizuoti 16S rRNR sekoskaitos duomenys. 2021 m. pastebėta didesnė negu 2022 m. su fekaline tarša susijusių bakterijų šeimų įvairovė ir santykinis gausumas. 2021 m. didžiausias gausumas buvo nustatytas liepą sąnašose (5,2% santykinis gausumas) ir rugpjūtį vandenyje su sąnašomis (5,1%); dominavo *Clostridiaceae* ir *Lachnospiraceae*. 2022 m. didžiausias gausumas (17,8%) nustatytas vandenyje su sąnašomis antrą mėginių rinkimo dieną; dominavo *Clostridiaceae* šeima. Tuo tarpu ketvirtą dieną bakterijų gausumas sumažėjo iki 5%. HF183 teigiamai koreliavo su *Lachnospiraceae*, *Prevotellaceae* ir *Rickenellaceae* šeimoms priklausančių bakterijų gausumu.

Pagal 16S rRNR sekoskaitos rezultatus identifikuota galvijų ir nuotekų tarša. 2021 m. liepą ir rugpjūtį sąnašose aptiktas didžiausias su galvijų tarša susijusių bakterijų santykinis gausumas. 2022 metais šių bakterijų gausumas buvo mažesnis, rasta tik *Fibrobacter* genčiai ir *Fibrobacteriaceae* šeimai priklausančios bakterijos. 2021 metais sąnašos kaupėsi arčiau Šventosios upės žiočių, negu 2022 metais, taip dar kartą patvirtinant prielaidą, kad didesnė fekalinė tarša susijusi su upės atnešimu. Aplinkoje su raudondumblių sąnašomis taip pat aptikta įvairių žmogaus patogeninių bakterijų. Naudojant PCR metodą, *Campylobacter* genties bakterijų buvo rasta vandenyje ir smėlyje su sąnašomis bei pačiose sąnašose, išskyrus vieną kartą, kai šių bakterijų buvo aptikta vandenyje su sąnašomis Šventojoje. *Salmonella* spp. nebuvo rasta. Remiantis 16S rRNR sekoskaitos duomenimis, Šventojoje vandens ir sąnašų mėginiuose 2021 ir 2022 metais rastos šios su fekaline tarša susijusios potencialiai patogeniškos bakterijos: *Bacteroides*, *Clostridium*, *Enterococcus*, *Escherichia-Shigella*, *Fusobacterium*, *Helicobacter*, *Lactococcus*, *Prevotella*, *Rickettsia*, *Treponema* ir *Vagococcus*. 2021 m. didžiausias santykinis potencialiai patogeniškų bakterijų gausumas aptiktas sąnašose liepos mėn. (3,3%), dominavo *Treponema* gentis. 2022 m. Šventojoje atlikus daugiadienius stebėjimus, didžiausias galimai patogeniškų bakterijų santykinis gausumas aptiktas antrą dieną vandenyje su sąnašomis (16%); dominavo *Clostridium* gentis. Santykinio gausumo sumažėjimą iki 3,02% ketvirtą dieną galima sieti su paplūdimio išvalymu dieną prieš tai.

Potencialiai patogeniškų *Vibrio* bakterijų įvairovė ir gausumas aplinkoje su sąnašomis

Naudojantis PGR metodu potencialiai patogeniškos *V. cholerae* (nepriklausančios O1/O139 serogrupėms) ir *V. vulnificus* buvo dažniau aptinkamos aplinkoje su sąnašomis, negu referentinėje aplinkoje. *V. vulnificus* buvo dažniausiai aptinkama vandenyje su sąnašomis (88,9% vandens su sąnašomis mėginiuose) ir sąnašose (70% sąnašų mėginių). *V. cholerae* buvo aptikta 55,6% vandens su sąnašomis mėginiuose ir 60% sąnašų mėginių. *V. alginolyticus* buvo aptikta tik vieną kartą per visą mėginių rinkimo laikotarpį, aplinkoje su sąnašomis.

Mūsų tyrimas atskleidė, kad tokie aplinkos veiksniai kaip chlorofilas-a, deguonis, SOM, temperatūra ir makrodumblio *Vertebrata fucoides* proporcija sąnašose buvo reikšmingi potencialiai patogeniškų rūšių *V. vulnificus* ir *V. cholerae* gausumui ir buvimui.

Vandenyje su sąnašomis 2021 m. buvo identifikuoti aštuoni skirtingi OTV, sąnašose – dešimt. Dešimt skirtingų OTV identifikuota 2022 vandenyje su sąnašomis ir sąnašose. Tiek 2021, tiek 2022 metais vandenyje su sąnašomis bei pačiose sąnašose rastas didesnis *Vibrio* santykinis gausumas palyginus su referentine vieta vandenyje. Aplinkoje su sąnašomis rasta gausi *Vibrio* įvairovė, įskaitant *V. ostreae*, *V. cholerae*, *V. anguillarum*, *V. pommerensis*, *V. rumoiensis*, *Vibrio* sp. MI-15 ir *Vibrio* sp. F74. *Vibrio* bakterijos gali būti gyvų makrofitų mikrobiotos dalis arba dalyvauti sąnašų irimo procese. Kai kurios *Vibrio* rūšys gali produkuoti polisacharidazes ir aktyvius angliavandenių fermentus, kurie gali dalyvauti makrodumblių skaidyme (Martin et al., 2014). Tačiau, kai makrofitai pradeda irti, išsiskiria daugybė maistinių medžiagų (Egan et al., 2013), todėl gali pagausėti mikroorganizmų, galinčių maitintis išsiskyrusiomis maistinėmis medžiagomis, įskaitant patogenines *Vibrio* bakterijas.

REKOMENDACIJOS

1. *Potencialiai patogeniškų Vibrio rūšių identifikavimas ir kiekio įvertinimas makrofitų sąnašose* (*Publikacija I ir IV*). Šiuo metu trūksta tyrimų, analizuojančių potencialiai patogeniškų *Vibrio* ir makrofitų sąnašų sąveikas jūrų pakrantėse. Dėl kylančios vandens paviršiaus temperatūros ateityje gali didėti potencialiai patogeniškų *Vibrio* infekcijų atvejų skaičius, todėl svarbu priimti tinkamus sprendimus dėl sąnašų tvarkymo.
2. *Makrofitų sąnašų tvarkymas rekreaciniuose paplūdimiuose* (*Publikacija II ir III*). Makrofitų sąnašos rekreaciniuose paplūdimiuose gali veikti kaip fizinis barjeras, sulaikantis fekalinę taršą ir su ja asocijuotus potencialiai patogeniškus mikroorganizmus, ypač kai rekreacinis paplūdimys yra šalia potencialaus fekalinės taršos šaltinio. Rekomenduojama pašalinti makrofitų sąnašas, kai jos yra

susikaupusios šalia upių ištakų Lietuvos pajūrio paplūdimiuose, arba sąnašas perkelti į lankytojų nenaudojamą paplūdimio vietą, kad būtų galima išsaugoti natūralią ekosistemą bei vietinę fauną. Prieš priimant sprendimą sąnašas pašalinti, turi būti įvertinta sąnašų buvimo ant kranto trukmė bei sankaupos dydis.
3. *Fekalinės taršos šaltinių aptikimas* (*Publikacija II ir III*). Lietuvos pakrantėje esančius rekreacinius paplūdimius veikia įvairūs fekalinės taršos šaltiniai: žmonės, paukščiai, galvijai ir nuotekos. Įprasto monitoringo metu naudojami bakterijų kultivavimo metodai galėtų būti naudojami kartu su molekuliniais metodais – 16S rRNR sekoskaita bei fekalinės taršos žymenimis, kurie padėtų nustatyti fekalinės taršos šaltinį.
4. *Visuomenės švietimas apie potencialius patogenus makrofitų sąnašose.* Nors sąnašos vaidina svarbų vaidmenį paplūdimio ekosistemos funkcionavime, tačiau jos taip pat gali būti potencialių žmogaus patogenų šaltinis, todėl svarbu informuoti paplūdimių lankytojus apie makrofitų sąnašų keliamas grėsmes.

IŠVADOS

1. Makrofitų ir potencialiai patogeninių bakterijų sąveika jūrų pakrančių aplinkoje buvo tiriama pastaruosius 50 metų, daugiausia dėmesio skiriant augančių makrofitų sąveikai su FIB ir *Vibrio* bakterijomis. Makrofitai gali tapti buveine FIB bei potencialiai patogeniškoms *Vibrio* bakterijoms. Sistematinė literatūros apžvalga atskleidė, kad didžiausi potencialiai patogeniškų bakterijų gausumai nustatyti ant augančių raudondumblių, mažesni – ant žaliadumblių, rudadumblių ir jūržolių. Makrofitų sąnašų tyrimuose daugiausia dėmesio buvo skiriama kiekybiniam FIB įvertinimui, tuo tarpu potencialiai patogeniškų *Vibrio* gausumas nebuvo vertintas.
2. Raudondumblių sąnašos daro reikšmingą įtaką bakterijų bendrijoms pakrančių vandenyje. Tiek vietose su sąnašomis, tiek be sąnašų dominavo Proteobacteria, Cyanobacteria, Bacteroidota ir Actinobacteriota skyriams priklausančios bakterijos, bet mikroorganizmų bendrijų panašumo analizė parodė, kad bakterijų bendrijos vandenyje su sąnašomis bei pačiose sąnašose labiau skyrėsi tarpusavyje, nei bakterijų bendrijos vandenyje iš referentinių vietų. Bakterijų bendrija aplinkoje su sąnašomis nuo kaupimosi pradžios iki pašalinimo patyrė didesnius struktūrinius pokyčius, negu vandens aplinkoje be sąnašų. Tikėtina, kad šiuos pokyčius nulėmė fizinių ir cheminių charakteristikų kaita vandenyje su sąnašomis.
3. Iš raudondumblių sudarytos paplūdimio sąnašos yra svarbi buveinė su fekaline tarša susijusioms ir *Vibrio* bakterijoms pietrytinėje Baltijos jūros pakrantėje. Aplinkoje su sąnašomis stebimi FIB kiekiai buvo reikšmingai didesni, negu

referentinėse vietose, o FIB koncentracijos vandenyje dažnai viršijo Higienos normoje 92:2018 nustatytas ribines vertes. Nors makrofitų sąnašos yra svarbi paplūdimio ekosistemos dalis, jos taip pat gali kelti grėsmę žmogaus sveikatai, nes rekreaciniuose paplūdimiuose esančiose sąnašose aptinkamos potencialiai patogeniškos bakterijos. Palyginus su referentinėmis vietomis, aplinkoje su sąnašomis nustatyti santykinai didesni su fekaline tarša susijusių patogeninių bakterijų (*Campylobacter*, *Bacteroides*, *Clostridium*, *Enterococcus*, *Escherichia-Shigella*, *Fusobacterium*, *Helicobacter*, *Lactococcus*, *Prevotella*, *Rickettsia*, *Treponema* ir *Vagococcus*) ir potencialiai patogeniškų *Vibrio* bakterijų (*V. vulnificus* ir *V. cholerae*) kiekiai.

4. Fekalinės taršos šaltinius leido identifikuoti žmogaus (HF183) ir paukščių (GFD) genetiniai žymenys. Šis metodas yra greitas ir pakankamai tikslus, tačiau neparodo pilnos fekalinės taršos šaltinių įvairovės. 16S rRNR sekoskaitos pagalba Šventosios paplūdimyje buvo nustatyti papildomi taršos šaltiniai, susiję su valymo įrenginių nuotekomis ir galvijais. Todėl aplinkos monitoringo programose turėtų būti atsižvelgiama į galimą fekalinės taršos šaltinių įvairovę, dinamišką sąnašų prigimtį ir jų poveikį visuomenės sveikatai. Tuo tarpu daugiadieniai stebėjimai leidžia geriau suprasti ilgalaikes fekalinės taršos tendencijas potencialiose sąnašų kaupimosi zonose.
5. Vandenyje su didesne siūlinių makrodumblių (*C. glomerata*, *C. rupestris*, *V. fucoides*) proporcija sąnašose FIB gausumą labiausiai paveikė vandens drumstumas bei spalvotojų ištirpusių organinių medžiagų kiekis. Chlorofilas-a, deguonis, spalvota organinė ištirpusi medžiaga, temperatūra ir makrodumblio *V. fucoides* proporcija sąnašose buvo reikšmingi potencialiai patogeniškų rūšių *V. vulnificus* ir *V. cholerae* gausumui ir buvimui. Sąnašos taip pat veikia kaip fizinis barjeras, kuriame gali būti sulaikomos ar paplūdimių valymo metu atpalaiduojamos su fekaline tarša susijusios bakterijos.

CURRICULUM VITAE

Biography. Greta Kalvaitienė was born on 1992 May 1 in Klaipėda, Lithuania. In 2015, she graduated with Bachelor's in Biology from Klaipėda University in Lithuania. In 2015 she started her Master's in Life Sciences at Klaipėda University in Lithuania (Supervisor: Prof. dr. Mindaugas Žilius). After her Master's degree, she started working as an Environmental Engineer in UAB "Ekosistema", Lithuania. She began her PhD position at the Marine research institute of Klaipeda University in 2020 (Lithuanian state funded scholarship) (Supervisor: Dr. Marija Kataržytė).
During her PhD time Greta participated in several projects: in "Estimation, monitoring and reduction of plastic pollutants in Latvian-Lithuanian coastal area via innovative tools and awareness raising" (ESMIC) from 2020 to 2022 and BaltVib "Pathogenic *Vibrio* bacteria in the current and future Baltic Sea waters: mitigating the problem" from 2021 to 2024.

Research interests: Water fecal pollution, fecal pollution sources, innovative methods of fecal pollution detection, bathing water quality.

Biografija. Greta Kalvaitienė gimė 1992 m. gegužės 1 d. Klaipėdoje. 2015 m. baigė biologijos bakalauro studijas Klaipėdos universitete, Lietuvoje. 2015 m. pradėjo gyvybės mokslų magistro studijas Klaipėdos universitete, Lietuvoje (vadovas prof. dr. Mindaugas Žilius). Įgijusi magistro laipsnį, pradėjo dirbti aplinkos inžiniere UAB "Ekosistema" įmonėje. Doktorantūros studijas Klaipėdos universiteto Jūros tyrimų institute ji pradėjo 2020 m. (Lietuvos valstybės finansuojama stipendija) (vadovė dr. Marija Kataržytė).
Doktorantūros studijų metu Greta dalyvavo keliuose projektuose: "Plastiko teršalų įvertinimas, stebėsena ir mažinimas Latvijos ir Lietuvos pajūrio zonoje inovatyviomis priemonėmis ir informuotumo didinimu" (ESMIC) nuo 2020 iki 2022 ir "BaltVib" "Patogeninės vibrio bakterijos esamuose ir būsimuose Baltijos jūros vandenyse: problemos mažinimas" nuo 2021 iki 2024.

Moksliniai interesai: Vandens fekalinė tarša, fekalinės taršos taršos šaltiniai, naujoviški fekalinės taršos aptikimo metodai, maudyklų kokybė.

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Researchgate: https://www.researchgate.net/profile/Greta-Kalvaitiene

Publications

PAPER I

Kalvaitienė, G., Vaičiūtė, D., Bučas, M., Gyraitė, G., Kataržytė, M., 2023. Macrophytes and their wrack as a habitat for faecal indicator bacteria and *Vibrio* in coastal marine environments. Marine Pollution Bulletin 194, 115325. https://doi.org/10.1016/j.marpolbul.2023.115325

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PAPER II

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PAPER III

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PAPER IV

microorganisms

MDPI

Article

Potentially Pathogenic *Vibrio* spp. in Algal Wrack Accumulations on Baltic Sea Sandy Beaches

Marija Kataržytė [1,*], Greta Gyraitė [1], Greta Kalvaitienė [1], Diana Vaičiūtė [1], Otilija Budrytė [1,2] and Martynas Bučas [1]

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Abstract: The *Vibrio* bacteria known to cause infections to humans and wildlife have been largely overlooked in coastal environments affected by beach wrack accumulations from seaweed or seagrasses. This study presents findings on the presence and distribution of potentially pathogenic *Vibrio* species on coastal beaches that are used for recreation and are affected by red-algae-dominated wrack. Using species-specific primers and 16S rRNA gene amplicon sequencing, we identified *V. vulnificus*, *V. cholerae* (non-toxigenic), and *V. alginolyticus*, along with 14 operational taxonomic units (OTUs) belonging to the *Vibrio* genus in such an environment. *V. vulnificus* and *V. cholerae* were most frequently found in water at wrack accumulation sites and within the wrack itself compared to sites without wrack. Several OTUs were exclusive to wrack accumulation sites. For the abundance and presence of *V. vulnificus* and the presence of *V. cholerae*, the most important factors in the water were the proportion of *V. fucoides* in the wrack, chl-a, and CDOM. Specific *Vibrio* OTUs correlated with salinity, water temperature, cryptophyte, and blue-green algae concentrations. To better understand the role of wrack accumulations in *Vibrio* abundance and community composition, future research should include different degradation stages of wrack, evaluate the link with nutrient release, and investigate microbial food-web interactions within such ecosystems, focusing on potentially pathogenic *Vibrio* species that could be harmful both for humans and wildlife.

Keywords: algae wrack; recreational waters; *Vibrio vulnificus*; *Vibrio cholerae*; One Health

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1. Introduction

The Bathing Water Directive (BWD), which regulates bathing water quality monitoring in the European Union (EU) [1], includes only thresholds for *Escherichia coli* and *Enterococcus* spp., which are associated with fecal pollution risks. However, other microorganisms unrelated to fecal pollution can simultaneously be found in recreational coastal bathing areas, such as *Vibrio* bacteria [2]. Unlike fecal indicator bacteria, *Vibrio* spp. are common autochthonous bacteria in water. Several *Vibrio* species are potentially pathogenic and widely distributed across the globe [3].

Vibrio-related infections are increasing worldwide in humans and aquatic animals. This rise has been associated with the global increase in sea surface temperatures, the primary physical consequence of global warming [4]. *Vibrio* infections, associated with heat waves, have also been observed in the Baltic Sea in the last decade [5–7].

Several *Vibrio* species are considered of concern in the Baltic Sea: *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* [7]. Serogroups designated as *V. cholerae* non-O1/non-O139 are prevalent in the Baltic Sea and might cause wound or ear infections and gastroenteritis. *V. vulnificus* can lead to severe wound infections, particularly in immunocompromised people [3]. Given the low salinity and highly eutrophied water conditions typical for the Baltic Sea, which are preferred by pathogenic *Vibrio* bacteria [8,9], it is crucial to consider the trend of rapidly increasing sea surface temperature in this area since these conditions are expected to become more favorable for these pathogens' growth [6,7,10].

Vibrio spp. can be considered an essential constituent of the macrophyte microbiome [11–13], contributing to the biogeochemical cycling of nutrients and potentially controlling pathogens in seawater [14]. Considering that the surface of live macrophytes can be a reservoir for *V. cholerae* [15] and *V. vulnificus* [16], after detachment and by accumulating on the coastal beaches as wrack, they also might serve as a reservoir for these *Vibrio* species. Beach wrack, an accumulation of macroalgae or seagrasses, including microalgae, animal carcasses, shells, wood, and higher plant debris, has an important ecological value for coastal ecosystems. In the western part of the Baltic Sea, wrack is primarily dominated by angiosperms, while the eastern part is dominated by accumulations of red and brown algae (*Rhodophyceae* and *Phaeophyceae*) [17,18]. Wrack might also play a role in the entanglement of plastic, which is known to act as a vector for transporting *Vibrio* bacteria [19]. *Vibrio* bacteria can form biofilms on the surfaces of plastic debris, thus potentially increasing their persistence and acting as the source of potential pathogens and horizontal gene transfer [20]. Moreover, wrack itself can support the survival of fecal bacteria [13,21,22] and potentially pathogenic microorganisms such as *Shigella*, *Salmonella*, and *Campylobacter* [23] due to the release of dissolved organic compounds into the aquatic environment [24], its surface for forming biofilms, and protection from harmful UV light and predation [25]. However, research on *Vibrio* bacteria in beach wrack worldwide is scarce or nonexistent [13].

Considering the abovementioned aspects, we aimed to assess the presence, abundance, and diversity of potentially pathogenic *Vibrio* bacteria in the recreational sandy beach areas of the southeastern Baltic Sea coast and the relation with wrack accumulation. This knowledge might have important implications for safeguarding public health on coastal beaches.

2. Materials and Methods

2.1. Study Area and Sampling Strategy

Samples were taken along the Lithuanian Baltic Sea coastline on four beaches—Melnragė, Karklė, Palanga, and Šventoji, covering the coastal stretch from the Curonian Lagoon outflow toward the Latvian coast with a salinity gradient affected by the lagoon outflow (Figure 1).

Environmental samples (wrack, water, and sand) were collected in 2021 (in June, July, August, and September) based on beach wrack accumulation events (nine sampling campaigns). In 2022, a multi-day sampling campaign was performed in Šventoji (one sampling event). Sampling campaigns were performed during the bathing season.

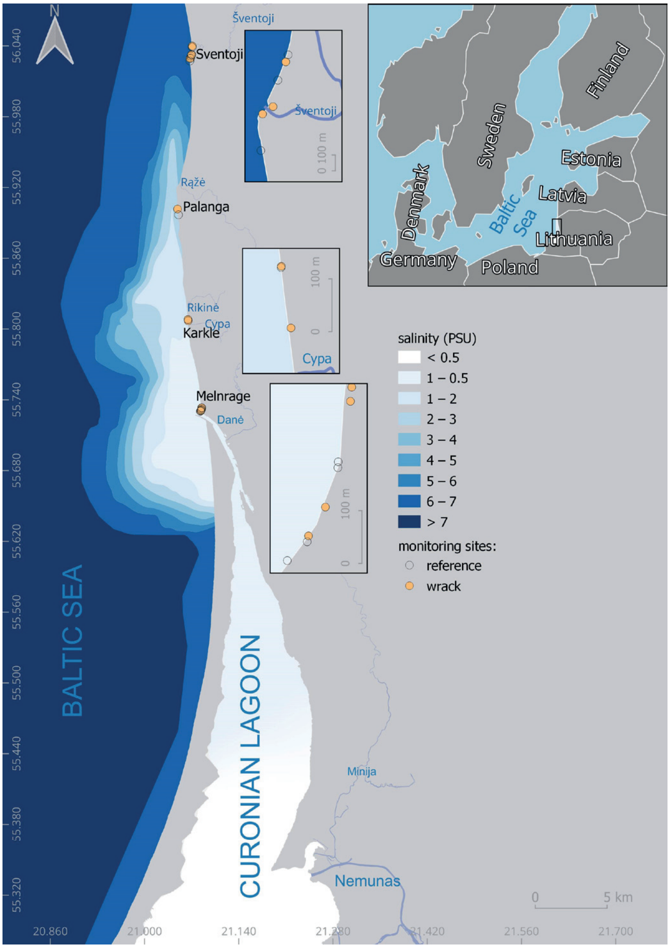


Figure 1. Sampling sites on Lithuania's Baltic Sea coast along the salinity gradient. The map was created using salinity data from August 2022 from the Copernicus Marine Service product [26].

Two subsites were selected for each sampling campaign—an area with accumulated wrack and that without (reference site). Three separate samples of water, sand, and/or wrack (only from the wrack site) were collected from each site at places at a distance of 1 m (more in [21]). Before DNA extraction, replicates of each sample were pooled to reduce the costs [27]. Besides environmental samples, plastic items (n = 30) were collected from sites with wrack accumulation (n = 21) and sand in sites without accumulations (n = 9) [28].

Water was collected using sterile 200 mL Nalgene bottles to assess environmental parameters. Sand samples were gathered in conical 50 mL tubes (VWR), and wrack samples were placed in Whirl-Pak bags (VWR). All samples were immediately stored in a cooling box, transported to the laboratory, and processed within four hours. Environmental parameters, including chlorophyll-a (chl-a) (mg/m^3) and phycocyanin (mg/m^3), were measured on-site using an AlgaeTorch (bbe Moldaenke GmbH, Schwentinental, Germany)—a fluorescence measurement device. Turbidity (NTU), temperature (°C), oxygen (mg/L), salinity (PSU), and pH were measured on-site with a YSI Professional Plus Environmental multimeter probe (Xylem Analytics, Yellow Springs, OH, USA) (more in [21]).

2.2. Sample Processing in the Laboratory

Water samples were analyzed in the laboratory for chl-a and colored dissolved organic matter (aCDOM) using spectrophotometry [29–31]. Suspended particulate matter (SPM) was analyzed gravimetrically [32], and organic and inorganic fractions were determined after filters were combusted at 550 °C for four hours. Detailed information on how environmental parameters were analyzed is provided in [21,22].

Sand and wrack samples were diluted with 110 mL of sterile MiliQ water and ultrasonicated for 15 s with 30 s breaks, with an intensity of 1 W/cm^2. This was repeated eight times using an ultrasonic bath (Bandelin Sonorex Digiplus, Berlin, Germany) to detach bacterial biofilm from sand and macrophytes.

For molecular analysis, water was filtered (Advantec Membrane Filter, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and kept in a −80 °C freezer before DNA extraction. Genomic DNA was extracted using DNeasy® PowerWater® Kit (QIAGEN, Hilden, Germany) and kept in a −20 °C freezer for further molecular analysis.

The dry weight of wrack samples was assessed by drying (at 60 °C) the samples until a constant weight. The algal species were identified using a Nikon SMZ800N stereomicroscope (Nikon, Tokyo, Japan). The species composition of the macrophyte community in the wrack, along with the environmental parameter data, is provided in [21].

2.3. Identification and Quantification of Vibrio Bacteria Using Molecular Methods

Four species of potentially pathogenic bacteria belonging to the *Vibrio* genus were targeted in DNA samples (both environmental and plastic)—*V. vulnificus*, *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus*. Conventional PCR was used to identify the presence of these potential pathogens using species-specific primers in the water, sediment, and wrack samples (Supplementary Table S1).

Reaction mixtures for PCR contained 12.5 µL Platinum™ Green Hot Start PCR Master Mix (2X) (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA), 7.5 µL water for molecular biology (Sigma-Aldrich by Merck, Merck KGaA, Darmstadt, Germany), 0.2 µM of each primer, 3 µL of DNA. The final volume of the reaction mixture was 25 µL. The PCR reaction was performed in a ProFlex PCR thermocycler (Thermo Fisher Scientific, Waltham, MA, USA); the conditions for the amplification were as follows: denaturation of 1 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 53 °C for 1 min, and extension at 72 °C for 1.5 min, and final extension at 72 °C for 10 min [33].

PCR products were visualized after electrophoresis on a 1.5% agarose gel, stained with SYBR® Safe DNA Gel stain (Invitrogen, Thermo Fisher Scientific, Invitrogen, Waltham, MA, USA), and observed under UV light. *Vibrio vulnificus* was quantified using TaqMan-based qPCR assays. Standard curves were generated from triplicate samples of 10-fold serial dilutions of purified *V. vulnificus* DNA, with concentrations ranging from 10^8 to 10^3 gene copies.

Each sample was analyzed using TaqMan Universal PCR Master Mix (Applied Biosystems by Thermo Fisher Scientific, Foster City, CA, USA). Each qPCR run included a positive control, a negative extraction, and a non-template control. qPCR amplification was performed using the StepOnePlus™ real-time PCR system (Applied Biosystems by Thermo Fisher Scientific).

The extracted DNA samples were also shipped for Illumina NovaSeq 6000 16s rRNA gene amplicon sequencing at Novogen Inc., Cambridge, UK. The forward (CCTAYGGGR-BGCASCAG) and reverse (GGACTACNNGGGTATCTAAT) primers were used to target the V3-V4 hypervariable regions of the 16S rRNA gene. Sequencing libraries were prepared using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). Paired-end reads were merged using FLASH (V1.2.11, http://ccb.jhu.edu/software/FLASH/, accessed on 15 June 2023). Data Filtration Quality filtering on the raw tags was performed using fastp (Version 0.23.1) software to obtain high-quality clean tags [34]. The tags were compared with the reference database (Silva database (16S), https://www.arb-silva.de/, accessed on 15 June 2023) using the UCHIME algorithm to detect chimera sequences, and then the chimera sequences were removed [35]. Sequence analyses were performed by Uparse software (Uparse v7.0. 1001, http://drive5.com/uparse/, accessed on 15 June 2023). Sequences with ≥97% similarity were assigned to the same operational taxonomic unit (OTU). Taxonomic information for each representative sequence was annotated using the Silva Database (http://www.arb-silva.de/, accessed on 10 August 2024) [36], based on the Mothur algorithm. The representative sequences of *Vibrio* OTUs were further identified by a blast search against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 16 August 2024). The sequence data were uploaded to NCBI BioProject under the accession number PRJNA1067868.

Phylogenetic analysis was performed by using neighbor-joining in mega 11 [37]. Bootstrap analysis [38] was performed for 1000 replications.

2.4. Data and Statistical Analysis

A systematic literature review was performed to discover similar studies that analyzed *Vibrio* presence in beach wrack worldwide, both in freshwater and marine environments. We selected several databases to search for publications: Google Scholar, PubMed, Web of Science (WoS), and Scopus. A search was performed by combining several keywords with Boolean operators "AND" and "OR": "Vibrio" AND "cast" OR "wrack" OR "beachcast" OR "debris" OR "detritus". The search was performed on 26 August 2024 and resulted in only one publication that analyzed *Vibrio* bacteria presence in beach wrack in the Mediterranean. Information extracted from this publication is provided in Supplementary Table S6.

Statistical tests were performed using the R software (version 4.4.1) environment (R Core Team, 2023). Figures were composed using the 'ggplot2' (3.4.4) package [39] in R software and Microsoft Excel 2019.

Spearman correlation was used to estimate the strength and significance of the relationships between *V. vulnificus* abundance and environmental parameters in water and OTUs' relative abundance and environmental parameters in water.

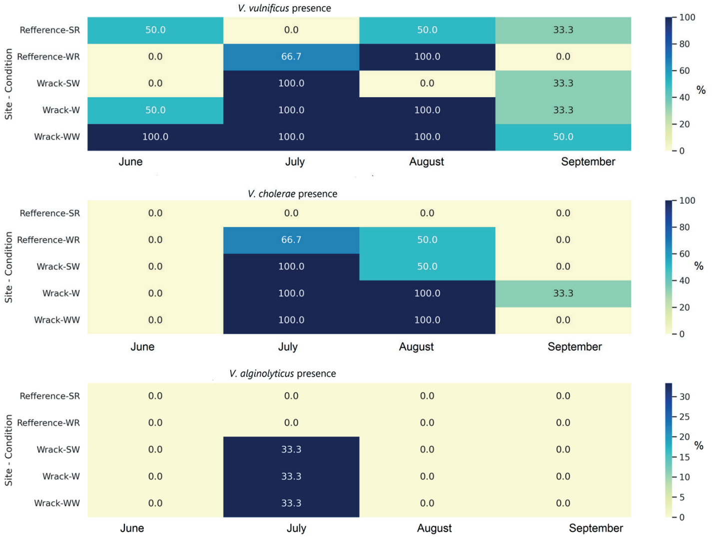
The Kruskal–Wallis test was used to assess differences between environmental parameters. Comparisons between categorical variables (presence/absence) were made using the chi-square test. The importance of environmental factors in explaining the variation in the abundance and presence of *V. vulnificus* and the presence of *V. cholerae* was assessed using a multivariate Random Forest regression model (MRF), due to the relatively small dataset and high multicollinearity among the explanatory variables; e.g., chl-a was highly ($r > 0.8$) correlated with CDOM and *V. fucoides*. The MRF was performed using the "randomForestSRC" package [40] in R. The number of trees (250) was selected based on the significant decrease in the error rate. Statistical significance was set at $p < 0.05$ in the used tests.

3. Results

3.1. V. vulnificus, V. cholerae, and V. alginolyticus on Beaches

The *vvhA* gene of *V. vulnificus* was found in 54.2% of tested samples and was present at all investigated beaches during different months, except for Melnragė beach in September. The prVC gene (*V. cholerae*) was found on all beaches (in 37.5% of samples). In July, three targeted *Vibrio* species were detected (Supplementary Table S2).

In water at wrack accumulation sites and within the wrack itself, *V. vulnificus* was present in 88.9% and 70% of the samples, respectively. *V. vulnificus* appeared in only 30% of the sand samples from reference sites. *V. cholerae* in water at wrack accumulation sites and in the wrack was found in 55.6% and 60% of samples, respectively, and it was not detected in sand from the reference site. *V. alginolyticus* was detected only once in July and at the wrack accumulation site (Figure 2). *V. parahemolyticus* was not detected during this research.



V. vulnificus presence

Site - Condition	June	July	August	September
Refference-SR	50.0	0.0	50.0	33.3
Refference-WR	0.0	66.7	100.0	0.0
Wrack-SW	0.0	100.0	0.0	33.3
Wrack-W	50.0	100.0	100.0	33.3
Wrack-WW	100.0	100.0	100.0	50.0

V. cholerae presence

Site - Condition	June	July	August	September
Reference-SR	0.0	0.0	0.0	0.0
Refference-WR	0.0	66.7	50.0	0.0
Wrack-SW	0.0	100.0	50.0	0.0
Wrack-W	0.0	100.0	100.0	33.3
Wrack-WW	0.0	100.0	100.0	0.0

V. alginolyticus presence

Site - Condition	June	July	August	September
Refference-SR	0.0	0.0	0.0	0.0
Refference-WR	0.0	0.0	0.0	0.0
Wrack-SW	0.0	33.3	0.0	0.0
Wrack-W	0.0	33.3	0.0	0.0
Wrack-WW	0.0	33.3	0.0	0.0

Figure 2. The identification frequency of *Vibrio* species (% from analyzed samples) by PCR during different months in environmental samples (SR—sand in reference; WR—water in reference; SW—sand in wrack; W—wrack; WW—water in wrack).

V. vulnificus and *V*. *cholerae* were found in 53.3% and 40% of the analyzed plastic samples, respectively. *V. vulnificus* was found in 50% of the plastic samples from the wrack, 66.7% of the samples from the water with wrack, and 44.4% of the samples from the reference water. The highest detection rates for *V. vulnificus* and *V. cholerae* on plastic were in July (73%) and August (70% and 60%, respectively). *V. cholerae* was found in 30% of the plastic samples from the wrack, in 44.4% of the samples from water in the wrack, and from the reference site (Figure 3).

The chi-square test revealed that both *Vibrio* species in the environment and on plastic were significantly associated with the month. The environmental parameters, such as temperature, oxygen, and turbidity, significantly differed among months (Supplementary Table S4). Additionally, *V. cholerae* was significantly linked to the subsite (wrack or reference), while the same bacteria attached to plastics were associated with the beach location (Supplementary Table S3).

The highest concentrations of the *V. vulnificus vvhA* gene were detected in Karklė and Palanga in July, specifically in water containing wrack (2.6×10^7 and 2.3×10^7 GC/100 mL, respectively). In the same month, the highest concentration of *V. vulnificus* in reference water (1.0×10^6 GC/100 mL) was found in Palanga (Supplementary Table S2).

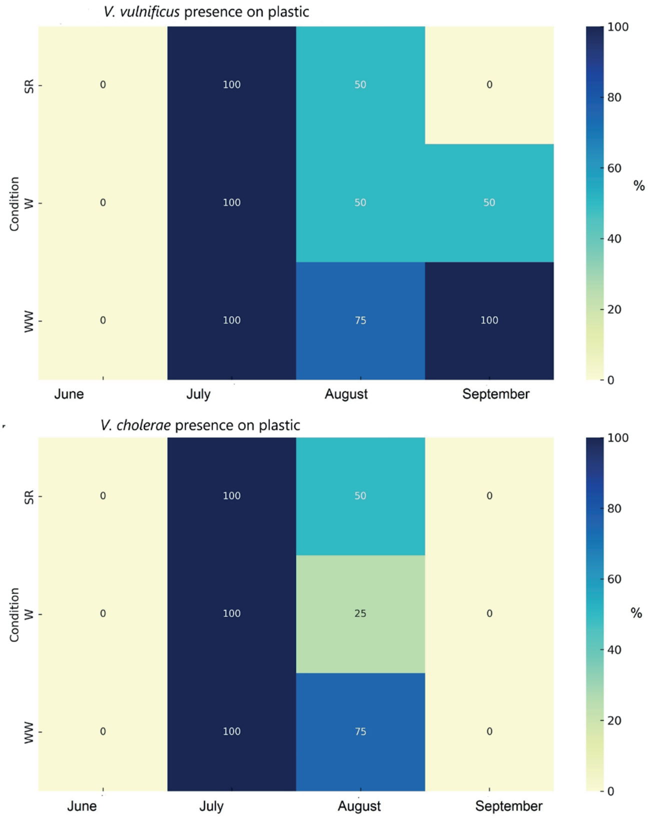


Figure 3. The identification frequency of *Vibrio* species (% from analyzed plastic samples) by PCR on plastic samples during different months (SR—sand in reference; W—wrack; WW—water in wrack).

There were significant differences in *V. vulnificus* abundance among months, both in water and sand with wrack, when, on average, the highest quantity was found in July (Kruskal–Wallis = 8.824 and 7.875, respectively; $p < 0.05$, n = 9) (Figure 4). There were no significant differences in abundance between months in other conditions. In July, a higher average abundance was observed in the water with wrack ($1.7 \pm 1.34 \times 10^7$ GC/100 mL) compared with the reference conditions ($4.6 \pm 5.05 \times 10^5$ GC/100 mL). However, it was not statistically significant ($p > 0.05$).

Spearman correlation coefficients calculated between *V. vulnificus* and water parameters were significant: positive with temperature, SPM, chl-a, and turbidity and negative with oxygen (Table 1). In water with wrack, *V. vulnificus* significantly correlated with diatoms + dinoflagellates, chl-a concentration, and the proportion of *Vertebrate fucoides* in the wrack, and negatively with oxygen.

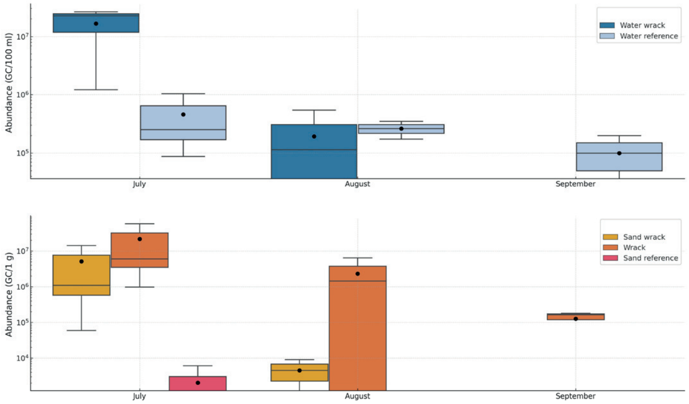


Figure 4. The average abundances of *V. vulnificus* (*vvhA* gene) under various conditions in July, August, and September are shown, with whiskers indicating the maximum and minimum values. The line within each box plot represents the median, and the circles denote the average quantities.

Table 1. Spearman correlation of *V. vulnificus* with environmental parameters in water samples based on qPCR results (statistically significant correlation coefficients are marked by * ($p < 0.05$) or ** ($p < 0.01$)).

	***V. vulnificus*, Gene Copies/100 mL in All Sites (N = 18)**	***V. vulnificus*, Gene Copies/100 mL in Wrack Sites (N = 9)**	***V. vulnificus*, Gene Copies/100 mL in Reference Sites (N = 9)**
Temperature,°C	0.549 *	0.58	0.43
Oxygen, mg L^{-1}	−0.651 **	−0.74 *	−0.68 *
Salinity, PSU	−0.083	−0.026	0.40
pH	−0.332	−0.65	−0.31
SPM, g m^{-3}	0.491 *	0.43	0.57
SPIM, g m^{-3}	0.523	0.49	0.63
SPOM, g m^{-3}	0.368	0.38	0.22
CDOM, m^{-1}	0.363	0.56	0.31
Blue-green algae, mg chl-a m^{-3}	0.099	0.13	−0.20
Diatoms + dinoflagellates, mg chl-a m^{-3}	0.371	0.76 *	0.30
Green algae, mg chl-a m^{-3}	−0.033	−0.08	−0.17
Cryptophytes, mg chl-a m^{-3}	0.0159	0.313	−0.62
Chl-a concentration, mg m^{-3}	0.481 *	0.78 *	0.68 *
Turbidity, NTU	0.549 *	0.58	0.43
Furcellaria lumbricalis, %	−0.070	−0.64	
Vertebrata fucoides, %	0.379	0.79 *	
Cladophora rupestris, %	0.372	0.61	
Cladophora glomerata, %	−0.152	−0.13	

The MRF model with the environmental factors explained 21% of the variation in the abundance and presence of *V. vulnificus* and the presence of *V. cholerae* (Figure 5).

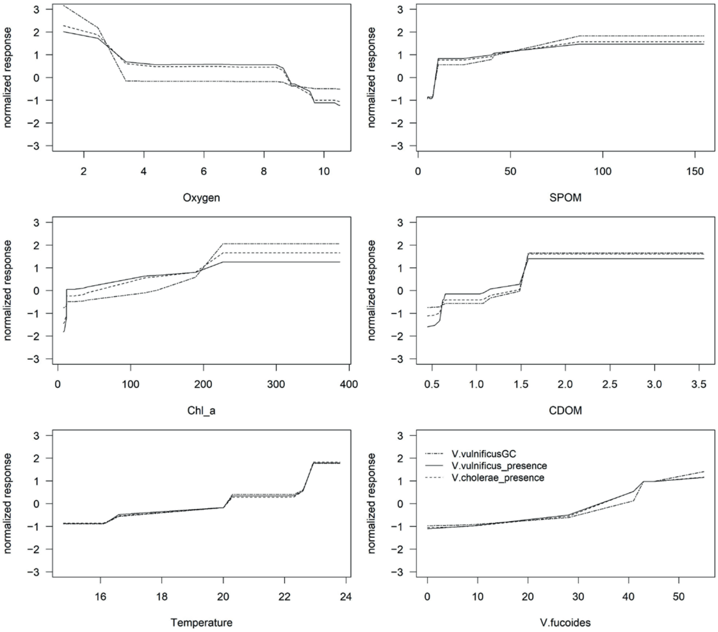


Figure 5. Partial dependence plots of environmental parameters on *V. vulnificus* abundance and presence and *V. cholerae* presence using MRF.

For *V. vulnificus* abundance, the most important factors were the coverage of *V. fucoides*, chl-a, and oxygen; for *V. vulnificus* presence, they were chl-a and CDOM; and for the presence of *V. cholerae*, they were temperature, chl-a, and oxygen (Supplementary Table S5).

3.2. Vibrio Diversity Based on 16S rRNA Sequencing on Šventoji Beach During Single- and Multiple-Day Sampling

During the overall studied period, 14 different OTUs belonging to the *Vibrio* genus were identified (11 in 2021 and 9 in 2022). Some OTUs showed high similarity (≥97%) to specific *Vibrio* taxa, such as *V. ostreae*, *V. cholerae*, *V. anguillarum*, *V. pommerensis*, *V. rumoiensis*, *Vibrio sp. MI-15*, and *Vibrio sp. F74* (Supplementary Figure S1).

During the single-day sampling in 2021, OTU 691 (*V. anguillarum*) was found in all samples. A higher relative abundance of *Vibrio* OTUs was observed in wrack sampled in July, dominated by OTU 467 (*V. cholerae*), and in water with wrack in September, dominated by OTU 3251 (*V. ostreae*) (Figure 6). In other environments during June, July, and August, the relative abundance of *Vibrio* OTUs was similar.

In 2021, 8 *Vibrio* OTUs in water without and with wrack were identified, while 10 were identified in wrack. On average, a higher abundance of *Vibrio* OTUs was observed in wrack or water with wrack (Figure 7). In wrack, the highest abundance was of OTU 467 (*V. cholerae*), while in water with wrack, it was of OTU 3251 (*V. ostreae*).

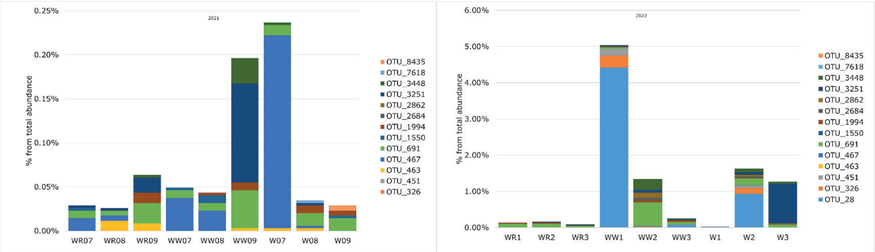


Figure 6. The relative abundances of OTUs assigned to *Vibrio* in different conditions (WR—water in reference site; WW—water in wrack site; W—wrack) during single-day sampling events in 2021 (7—July; 8—August; 9—September) and during multiple-day events in 2022 (1—first day of sampling; 2—second day; 3—fourth day).

During multi-day sampling in 2022, 8 *Vibrio* OTUs in water without wrack and 10 OTUs in wrack and water with wrack were identified. OTU 3448 (*V. pommarensis*) was found in all samples. High abundance (up to 5% of total abundance) was observed in water with wrack on the first accumulation day, dominated by OTU 28. Comparably higher abundance (>1%) was identified in water with wrack (dominated by OTU 691 (*V. anguillarum*)) on the second day and in wrack on the second (OTU 28) and fourth (OTU 3251) days. The abundance of *Vibrio* OTUs was similar in water from the reference site during the studied period. On average, higher abundance was observed in water with wrack accumulation, and the lowest abundance was observed in reference water (Figure 7).

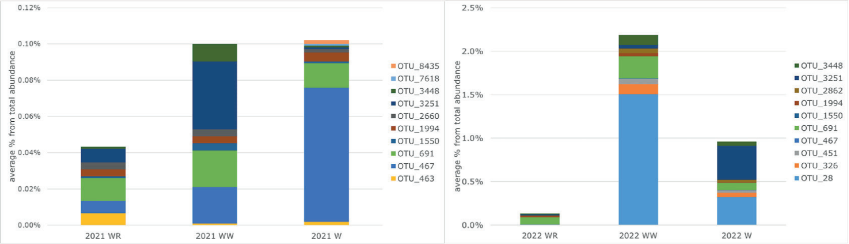


Figure 7. The average relative abundances of OTUs assigned to *Vibrio* in different conditions (WR—water in reference site; WW—water in wrack site; W—wrack) during single-day sampling events in 2021 and multiple-day sampling in 2022.

Some OTUs were found only in wrack and water with wrack: in 2021, these were OTU 7618 and OTU 8435, and in 2022, they were OTU 326 and OTU 451 (*Vibrio* sp. F74).

Hierarchical cluster analysis based on the Bray–Curtis dissimilarity group did not show a statistically significant difference between the sites (ANOSIM Global R = -0.02634, $p = 0.571$). However, samples from July had a similar composition of *Vibrio* OTUs (Supplementary Figure S2).

Several environmental parameters showed significant correlations ($p < 0.05$) with the relative abundance of specific OTUs. Temperature was negatively correlated with the abundance of OTU 463, while salinity was negatively correlated with OTU 467 (*V. cholerae*). Cryptophytes exhibited significant positive or negative correlations with nearly all OTUs, with varying degrees (Table 2). Additionally, a higher proportion of *Vertebrata fucoides* and *Ulva intestinalis* in the wrack was positively correlated with OTUs 28 and 326.

Table 2. Spearman correlation heatmap between OTU relative abundances and environmental parameters in the water from Šventoji beach. Only significantly important correlation coefficients ($p > 0.05$) are provided. The color gradient indicates the strength and direction of the correlations: blue cells indicate negative correlations, and red cells represent positive correlations.

	OTU_28	**OTU_326**	**OTU_451**	**OTU_463**	**OTU_467**	**OTU_691**	**OTU_1550**	**OTU_1994**	**OTU_2684**	**OTU_2862**	**OTU_3251**	**OTU_3448**
Temperature				−0.66								
Salinity					−0.74						0.58	
Chl-a												
Green algae												
Blue-green algae	0.69	0.67		−0.59						0.73		
Diatoms + dino algae												
Cryptophytes	−0.76	−0.64	−0.18	0.32	0.40	−0.62	0.66	−0.64		−0.86		−0.61
Furcelaria lumbricalis												
Vertebrata fucoides		0.70										
Ulva intestinalis	0.63	0.72										
Cladophora sp.												

4. Discussion

The presence of Vibrio species such as *V. vulnificus*, *V. fluvialis*, *V. anguillarum*, *V. cholerae*, *V. alginolyticus*, *V. cincinnatiensis*, *V. furnissi*, *V. navarrensis*, *V. harveyi*, and *V. mentschinkowi* that are known for causing infections [41–46] has recently been found in coastal waters of the SE Baltic Sea [7,33]. Moreover, the best predictors of *V*. *vulnificus* along the Baltic Sea's salinity gradients were eutrophication-related parameters, such as particulate organic carbon, nitrogen, phosphate, and the occurrence of potential phytoplankton blooms [8].

Along with phytoplankton blooms, macroalgae wrack accumulations are observed after stormy conditions on coastal beaches of the Baltic Sea, negatively affecting water quality [47] and enriching the coastal ecosystems with nutrients. Due to eutrophication, the contribution of ephemeral and nutrient-opportunistic seaweeds is increasing in the wrack of the Baltic Sea, thus increasing nuisance compared to late successional macrophytes [48]. The wrack accumulated on the coast favors the survival and proliferation of bacteria and pathogens related to fecal pollution [13,24]. However, based on our systematic review analysis, we found only one study on *Vibrio* species from the polluted Mediterranean Sea [49], where the microbiome of *Posidonia* wrack was analyzed. In our case, the wrack consisted of perennial macroalgae such as *Furcellaria lumbricalis* and *Vertebrata fucoides* (relative abundance varied from 48 to 81%) and ephemeral algae such as *Cladophora* (relative abundance varied from 3 to 28%) [21].

Our study revealed that during the recreational period, at least three species of *Vibrio* were identified or quantified using species-specific primers in the wrack accumulation sites along the Baltic Sea: *V*. *vulnificus*, *V*. *cholerae*, and *V. alginolyticus*. Up to 14 OTUs belonging to the *Vibrio* genus were identified in a wrack-affected environment based on 16s rRNA gene amplicon sequencing. *V. cholerae* was identified in samples using both approaches. However, caution should be exercised when identifying species within the genus *Vibrio* based only on a fragment of the 16S rRNA gene, as this marker lacks the necessary phylogenetic resolution for precise species determination [50,51].

V. *vulnificus* and *V*. *cholerae* were more frequently identified in wrack environments than in sites without wrack accumulation. *V. alginolyticus*, identified on the Lithuanian coast for the first time, was found only in environments with wrack accumulation. Some OTUs (OTU 7618, 8435, OTU 326, and OTU 451 (*Vibrio* sp. F74)) were found only in wrack accumulation sites, indicating that they might be either constituents of live macroalgae or taking part in the degradation process after macroalgae accumulate. Kolda et al. [49] found that *Vibrio* spp. in *Posidonia oceanica*-dominated wrack contributed not only to fermentation and aerobic chemoheterotrophy but also to nitrate reduction and associations with animal parasites. For example, in our study, the relative abundance of OTU 326 significantly correlated with the presence of *Vertebrata fucoides* or *Ulva intestinalis.* Some *Vibrio* species are known for possessing algae-specific polysaccharidases (e.g., agarases, carrageenases, and alginate lyases) [52] and participating in macroalgae degradation. For example, *V. alginolyticus* can degrade agar, cellulose, sodium alginate, xylan, laminarin, and carrageenan [53]. On the other hand, macroalgae polysaccharides or live-macroalgae-associated bacteria are known to inhibit the growth of potentially pathogenic bacteria such as *V. anguillarum*, *V. cholerae*, and others [54,55]. However, as macroalgae degrade, associated microorganisms may lose the ability to defend against pathogenic microorganisms. The degradation of tissue leads to the release of nutrients [25], which could support an increase in microorganisms capable of feeding on the released nutrients. Some of them might be opportunistic pathogens. Our study limitation was that we did not analyze the nutrients (such as nitrogen or phosphorus); thus, we can only hypothesize about their significance, as demonstrated in other studies [8,9]. Particulate organic carbon and nitrogen were closely associated with chl-a in research on *V. vulnificus* along the salinity gradient in the Baltic Sea [8]. In our case, chl-a concentrations were significantly higher in water with wrack accumulations than in the reference. The nutrients released from the degrading wrack might affect the growth of phytoplankton, including algae that produce chlorophyll-a. Chl-a was an important environmental parameter explaining the abundance and occurrence of *V. vulnificus* and *V. cholerae* in our case. The importance of chl-a for *V. vulnificus* abundance was demonstrated in other studies as well. Genetic markers of *V. vulnificus* were observed when chl-a concentrations ranged from 5 to 25 µg/L in the Chesapeake Bay [56], and a correlation with chl-a was also found in the Baltic Sea [8].

As another important variable, the water temperature was related to the higher detection frequency and abundance of *V. vulnificus* in July, when it reached 23.8 °C [21]. Numerous authors have shown temperature to be the main driving factor of *Vibrio* abundance in the Baltic Sea, especially for potentially pathogenic *Vibrio* species [8,9]. Salinity, as one of the environmental factors that influence the geographic distribution of *Vibrio* species and affect the concentration of certain *Vibrio* species [7,8], was not significant for *V. vulnificus* abundance or presence in our case. However, the highest abundances of *V. vulnificus* were observed on the Palanga and Karklė beaches, where wrack accumulation occurred under salinity conditions of around 6 PSU [21]. Based on 16S rRNA gene amplicon sequencing at Šventoji beach, where salinity ranged from 0.2 PSU to 6.6 PSU during our sampling, we found that the relative abundance of OTU 467 (clade *V. cholerae*) increased as salinity decreased. On this beach, wrack accumulated near the Šventoji river, whose outflow diluted the saline conditions [22], potentially contributing to the shift in *Vibrio* composition. *V. cholerae* in the Baltic Sea was found across a salinity gradient ranging from 0.24 to 29.4 PSU [7]. However, previous studies on the Lithuanian coast reported higher abundances of *V. cholerae* in the low-salinity or freshwater conditions of the Curonian Lagoon [2], compared to the higher-salinity conditions of the Baltic Sea.

Vibrio is an early colonizer of plastics, and higher nutrients could enhance the faster establishment of *Vibrio* populations in the Baltic Sea [50]. In our case, *V. vulnificus* and *V. cholerae* were identified on all studied plastic samples from July, with a higher frequency on plastic from water with wrack accumulations; however, they were also found on plastic from the coast without wrack accumulations. In the reference sites, the higher frequency of *Vibrio* on plastic in the sand, compared to its presence in the sand, was most probably due to biofilm formation that develops in the aquatic environment on the plastic and later, when the plastic is deposited on the coast, can protect *V. vulnificus* from environmental stressors and enhance its survival [19,57]. However, more studies or simulations could be performed to understand this process.

OTU 691 (clade *V. anguillarum*) was found in all samples and increased in abundance during multi-day sampling on the second day of wrack accumulation in water. That day, higher average chlorophyll and blue-green algae abundances were observed compared to other sampling days (67.3 and 37.15 mg m^{-3}, respectively) [21]. *V. anguillarum* is abundant in the brackish waters of the Baltic Sea and is known to correlate with chlorophyll and cyanobacterial abundance [58]. Also, it is known as a fish pathogen, capable of infecting other marine animals and, in some rare cases, humans [41,59]. On the other hand, this species is a seaweed-associated microorganism that positively affects the settlement of zoospores of *Enteromorpha* or *Ulva* [12]. OTU 3448 found in both years showed high similarity to *Vibrio navarrensis* biotype *pommerensis* that was first isolated from the Baltic Sea [60] and is known for causing human infections [46] and able to utilize lactose as a sole carbon source, which is also specific to *V. vulnificus*. In our study, there was a higher relative abundance of this OTU in water in wrack both in September of 2021 and on the second day of sampling in 2022.

Grazing pressures on cyanobacteria in the environment could explain different OTUs' correlation with the abundance of cryptophytes. Riedinger et al. [8] found that when there is a higher abundance of cryptophyte *Teleaulax*, there is a decrease in certain cyanobacteria, nutrients, and *V. vulnificus* abundance. This may be attributed to the potential role of cryptophytes due to the grazing of cyanobacteria, limiting *Vibrio* access to the related organic nutrients [8]. The inconsistent composition of *Vibrio* during the studied period, related to the presence or absence of accumulated wrack, could be attributed to the sampling strategy based on the single-day sampling campaigns (except in 2022) when the samples were taken at different degradation stages. For example, following the allocation of samples from September 2021 with samples of the fourth sampling day in 2022 in a dendrogram, we can assume that the wrack was at a similar succession stage. However, a different research strategy should be used to prove this, considering all wrack succession stages and using more frequent sampling. Using only molecular methodologies can also be considered as our study limitation. 16s gene amplicon sequencing allows the detection of *Vibrio* present in a non-culturable state and less-characterized *Vibrio* species compared to traditional culturing methods. However, this method cannot differentiate between live and dead cells, limiting its ability to assess the viability of potentially pathogenic *Vibrio*; moreover, depending on the sequencing depth, it can limit the detection of low-abundance species. In contrast, culturing on selective media can provide information on viable *Vibrio*, and isolates can be used for further characterization. Recent advancements in qPCR can supplement the assessment of live cells and non-viable cell quantification of dead cells [61].

5. Conclusions

Accumulated algal wrack on coastal sandy beaches plays an important role due to habitat and nutrient provision; however, it might be an issue for beaches used for recreational activities. As our study revealed, in such wrack accumulations, there are potentially pathogenic *Vibrio* present, with their abundances increasing during periods of higher water temperature, which is usually related to increased beachgoer numbers. *Vibrio* presence should be considered when managing wrack accumulations on recreational beaches to prevent people from being exposed to potential pathogens. From the ecological perspective, more research should be conducted to analyze how, in such ecosystems, the microbial food web changes during wrack degradation stages, including the nutrient release and grazing effect, and what implications it has on the abundance and presence of potentially pathogenic *Vibrio* species.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microorganisms12102101/s1: Table S1: Targeted bacterial groups and oligonucleotide primers used for qPCR and PCR; Table S2: Average quantities of *V. vulnificus* and presence of *Vibrio* based on PCR results (W—wrack accumulation area; R—reference area with no wrack); Table S3: The presence of *V. vulnificus*, *V. cholerae*, and *V. alginolyticus* in environmental samples and on plastic. Numbers in bold refer to significant values; Table S4: Kruskal–Wallis test results for water environmental parameter differences by month, site, and condition. Statistically significant ($p < 0.05$) differences are marked by *; Table S5: The relative importance of environmental factors in water for response variables: *V. vulnificus* abundance, *V. vulnificus*, and *V. cholerae* presence based on the MRF model. Table S6. The information related to systematic literature review data. Figure S1: Phylogenetic tree of all *Vibrio* OTUs from the water of the Šventoji beach. Two species, *Photobacterium* and *Aeromonas*, were used as outgroups. The tree was reconstructed by using the neighbor-joining method. Bootstrap values (>50%) are shown at the nodes; Figure S2. Cluster diagram of Bray–Curtis similarities calculated from square-root-transformed relative OTU abundances for each sample (06—June; 07—July; 08—August; 09—September; 1—first day; 2—second day; 3—the fourth day; SR—sand in reference; WR—water in reference; SW—sand in the wrack; W—wrack; WW—water in water wrack). References [62–65] are cited in the Supplementary Materials file.

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ACCUMULATION OF FECAL INDICATOR AND POTENTIALLY PATHOGENIC BACTERIA IN MACROPHYTE WRACK ON COASTAL SANDY BEACHES
Doctoral dissertation

FEKALINĖS TARŠOS INDIKATORINIŲ IR POTENCIALIAI PATOGENINIŲ BAKTERIJŲ KAUPIMASIS MAKROFITŲ SĄNAŠOSE JŪROS PAKRANČIŲ SMĖLIO PAPLŪDIMIUOSE
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