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Skip the Dip—Avoid the Risk? Integrated Microbiological Water Quality Assessment in the South-Eastern Baltic Sea Coastal Waters

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Abstract: The bathing water microbiome consists of pathogenic and non-pathogenic microorganisms, such as bacteria, viruses, and protozoa. However, the targets of the Bathing Water Directive (2006/7/EC) focus exclusively on fecal pollution. This study aims to investigate fecal indicator bacteria (FIB), naturally thriving cyanobacteria, and *Vibrio* bacteria in the Lithuanian coastal Baltic Sea and Curonian Lagoon bathing sites, combining information into an integrated microbial risk assessment scheme. The results show that officially monitored indicators, such as FIB, do not exceed the acceptable 'low' risk threshold. Simultaneously, cyanobacteria and *Vibrio cholerae* abundance in the Curonian Lagoon sites reveal a 'high' probability of adverse health effects. In coastal bathing sites, a positive correlation was found between *Escherichia coli*, cyanobacterial harmful algae bloom (cHAB), and *V. cholerae*, indicating that all target microorganisms may occur at the same time, with consequently high risks for the health of bathers. Therefore, implementing new target organisms in national or even regional bathing water monitoring programs is recommended, in order to safeguard the health of beachgoers.

Keywords: fecal indicator organisms; cyanobacterial harmful algae bloom; *Vibrio*; risk assessment; bathing waters; Baltic Sea

1. Introduction

The use of recreational waters poses several potential health risks. The bathing water microbiome consists of fecal- or natural-origin bacteria, viruses, or protozoa. Ingestion of contaminated water while bathing may cause gastrointestinal infections, as well as other common health impacts including diseases of the upper respiratory tract, ears, eyes, nasal cavity, and skin, possibly even including cholera, typhoid, and hepatitis [1–3]. According to the World Health Organization (WHO) [1], 5% of adult bathers are expected to become ill after exposure to lightly polluted recreational waters.

Water-borne diseases caused by pathogens thriving in fecal material [4] pose a significant threat to public health. They contribute to 4% of all deaths worldwide [5]. In 1976, the European Union (EU) adopted the first Bathing Water Directive (BWD) (76/160/EEC) [6], intended to protect human health from potentially pathogenic microorganisms. In 2006, a revised BWD (2006/7/EC) [7] entered into force. It focuses on more specific microorganisms such as the fecal indicator bacteria (FIB) *E. coli* and *Enterococcus* spp., which have been positively correlated to bathing-related illnesses [8]. To date, nearly 90% of all official bathing sites in the EU have been shown to maintain 'good' or 'excellent' water quality status [9]. However, the revised BWD has provoked controversial discussions regarding whether:

(i) FIB are suitable to assess recent fecal pollution, due to their survival in secondary habitats [10]; (ii) FIB show actual threats of viral pathogens and protozoa of fecal origin [11]; and (iii) the methods used to determine bathing water quality are suitable to provide accurate and fast results [12].

Over the past decade, numerous source-specific indicators have been tested and molecular markers have been developed to identify or to quantify the magnitude of fecal pollution [13–15]. Detection of human fecal contamination is mainly based on quantitative polymerase chain reaction (qPCR) detection of human-associated *Bacteroidales* genes [14,16]. *Bacteroidales* are known for their low potential of proliferation in the environment, thus indicating human fecal pollution from sewage, agricultural land, and poor sanitation infrastructure. They are considered a better indicator of so-called microbial or fecal source tracking and tracing (MST) [17]. Therefore, *Bacteroides* could serve as a potential alternative indicator to prevent and predict human-origin contamination, which is known to be the most significant transmitter of potential pathogens into bathing waters [15].

In 2018, the WHO released recommendations for improving bathing water quality parameters [18]. Among scientific, analytical, and epidemiological development suggestions, the WHO has advised the implementation of a new management system for bathing waters at risk of freshwater cyanobacterial blooms and other emerging issues, such as non-gastrointestinal illnesses causing *Vibrio* spp. bacteria. In some countries, great efforts have been made to investigate emerging issues of cyanobacterial blooms [19–21] and wound infections caused by *Vibrio* spp. [22–24]. However, the evidence gathered to date indicates that an assessment approach that integrates several microbial water quality indicators does not exist.

The Baltic Sea is a shallow, semi-enclosed water system having limited water exchange with the North Sea, resulting in low-salinity waters [25]. It is also one of the most eutrophic and fast-warming (seven times the global rate) seas worldwide [22]. The brackish, organic-rich, and fast-warming waters of the Baltic Sea may be linked to emerging infections by marine indigenous pathogens [26]. Moreover, tourism in the Baltic Sea Region generates more than 200 million overnight stays, over half a million jobs [27], and contributes the greatest share to the EU blue economy, in terms of employment, profit, and gross value added [28]. Therefore, recreational water quality requires full attention.

Vibrio are ubiquitous bacteria occurring in warmer (>15 °C) and lower salinity (0–25 PSU) marine and estuarine waters [24]. More than 100 *Vibrio* species have been described, with approximately 12 of them causing infections in humans. Amongst them, *Vibrio vulnificus* has the highest fatality rate (~50%) of any foodborne pathogen [29]. The majority of illnesses occur in immunocompromised and elderly people, acquired through direct contact with recreational water or through consumption of undercooked seafood [30]. Since the very first case of *Vibrio* infection, recorded in 1978 [31], approximately 900 infections have been recorded in the Baltic Sea region [32], which will likely increase with increasing sea surface temperature [22]. Despite the undeniable presence of potentially pathogenic *Vibrio* in the Baltic Sea, these bacteria are not monitored in most south-eastern Baltic Sea countries, with very little attention having been paid throughout Europe [32]. No thresholds exist for interpreting the health risks of *Vibrio vulnificus* and *Vibrio cholerae*. However, the quantitative microbial risk assessment (QMRA) approach has been suggested for estimating health risks posed to swimmers [33]. The QMRA methodology is a structured, systematic, and science-based approach that quantitatively estimates the level of exposure to microbial hazards and the resulting risk to human health [34,35]. We emphasize that such an approach, based on quantitative bacteriological data and dose–response relationship, is quite robust, suitable, and applicable worldwide.

In the coastal waters of the Baltic Sea, recent concern has been raised about cyanobacterial harmful algal blooms (cHAB) [36]. In the case of excessive cHAB formation, exposure to cyanobacterial cells and cyanotoxins may cause adverse health effects in humans [2,37]. cHAB favor the growth of potentially pathogenic, free-living bacteria (e.g., *Vibrio* spp.) [38] by providing dissolved organic matter (DOM) or serve as an attachment surface and vector [39], but may also directly compromise the health of humans physically (e.g., polysaccharides) or chemically (e.g., toxins), thus promoting pathogenic bacterial infections [26]. Several studies in the south-eastern part of the Baltic Sea have reported relatively high

concentrations of cyanotoxins [19–21,40]. However, the BWD only briefly mentions cyanobacteria and the risks associated with their presence. Consequently, bathers rely on bathing sites monitored only for FIB and do not consider sanitary precautions, such as infection of open wounds, ears, or skin irritation after exposure to seawater with high concentrations of the previously mentioned microorganism.

Thus, taking into account naturally occurring and fecal contamination microorganisms (FIB, cyanobacteria, *Vibrio* spp.) that are related to the indication of potential risk to human health, their continuous appearance in the south-eastern part of the Baltic Sea [20,32,41,42] and the lack of more comprehensive assessment of risks in general, this study aims to investigate microorganisms of interest and provide an integrated risk assessment scheme. Moreover, this study seeks to determine whether existing regulations and policies adequately address the health risks for bathers and if such an approach could be used in similar fresh-brackish water systems such as Lithuania's coastal waters and the Curonian Lagoon. Our main objectives are: (a) to assess FIB occurrence and concentrations; (b) to investigate bathing water quality based on cHAB parameters—abundance, biomass, chlorophyll a (Chl *a*), and microcystin (MC) concentration; (c) to enumerate the concentration and assess the community composition of potentially pathogenic *Vibrio* species; (d) to provide an integrated microbial risk assessment scheme; and (e) to apply this to the coastal Baltic Sea and Curonian Lagoon bathing sites.

2. Materials and Methods

2.1. Study Area and Sample Collection

In this study, three officially designated coastal bathing sites (Palanga, Melnrage, and Nida BS) and three Curonian Lagoon sites were chosen (Figure 1; Table S1). Officially designated coastal bathing sites are 1.4–3.2 km long beaches. In summer months, they accommodate approximately 5000 (Nida BS), 10,000 (Melnrage), and 15,000 beachgoers per day (Palanga) [42]. The Nida BS fine sand beach is equipped with facilities for comfortable recreational time and is certified by the international eco-label "Blue flag". Meanwhile, the Melnrage bathing site is located on the north side of the Klaipeda Strait and is often impacted by the outflows of intensively blooming Curonian Lagoon water [20].

The Curonian Lagoon is a transitional water body divided into two parts: the southern part is characterized by an average salinity of 0.08 and the northern part, which experiences intrusions of brackish Baltic Sea water, has an average salinity of 2.45 [43]. The Kintai site is the only officially monitored bathing site within the Curonian Lagoon. Nida CL is a recreational area located on the Curonian Spit side, which has been chosen as a potential new bathing site [44]. The Port area is a non-bathing site that serves as a transitional area between the Curonian Lagoon and the Baltic Sea in this study.

Surface water samples (50–60 cm) were collected monthly during the official bathing season of 2018. Water samples were taken in sterile, darkened plastic bottles, transported in cooling boxes, and processed within 4 h after collection. Water temperature and salinity were recorded in situ using a multi-parameter probe (YSI Professional Series, Yellow Springs, OH, USA). Simultaneously, pH and turbidity were determined from the collected samples in the laboratory using a benchtop pH meter (Thermo ScientificTM Orion Star™ A111, Waltham, MA, USA) and a portable turbidity meter (Eutech Instruments TN-100, Landsmeer, The Netherlands), respectively. Detailed information about the environmental conditions of each sampling site is provided in Table S1.

For further molecular analyses of microbial abundance and diversity, all water samples (0.5–1 L) were filtered through 0.22 µm pore size mixed cellulose ester filters (MontaMil® Membrane Filters, Frisenette ApS, Knebel, Denmark) and kept at −20 °C until DNA extraction. To assess contamination during the laboratory process, 1 L of distilled water was filtered at the same time and used as a negative control in all further analyses.

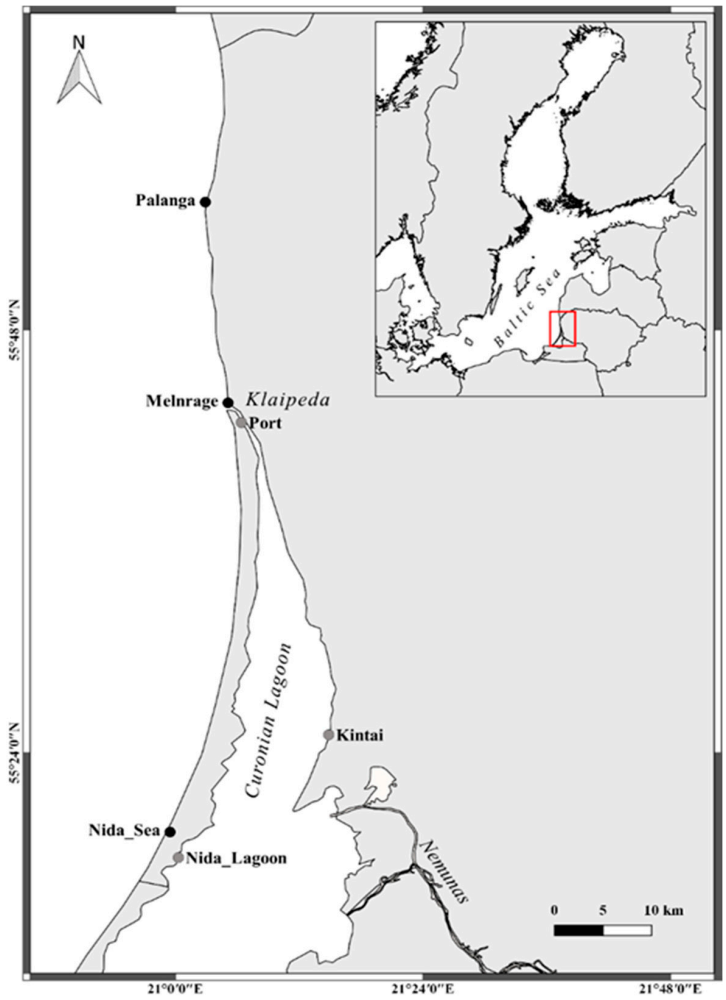

Figure 1. Locations of Lithuanian Baltic Sea coastal bathing sites (black points) and Curonian Lagoon sites (grey points).

Genomic DNA extraction was performed using the PowerWater® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. Aliquots were stored at −20 °C for long-time conservation.

2.2. Identification and Enumeration of Microorganisms

Enumeration of *E. coli*, *Enterococcus*, and *Bacteroidales*. Enumeration of *E. coli* was performed by membrane filtration on TTC Tergitol 7 agar (ISO 9308-1:2000). The cultures were incubated at 37 °C for 24 h. Afterwards, the membranes were transferred to new TBX agar plates and incubated at 44 °C for two hours. All colonies that turned from yellow-orange (on TTC Tergitol 7 agar) to blue-green (on TBX, indicating production of β-D-glucuronidase) were counted as *E. coli* [45,46]. The results were expressed as colony-forming units per 100 mL (CFU 100 mL^{-1}).

The abundance of *Enterococcus* and *Bacteroidales* from water samples were enumerated using TaqMan-based real-time PCR assay, following the methods described by Haugland et al. [16,47]. Species-specific primers and hydrolysis probes targeting species of interest are shown in Table 1. Quantitation was determined from standard curves obtained from six tenfold serial dilutions (10^6–10^1) of gene fragments (gBlocks, Integrated DNA Technologies, Inc., Coralville, IA, USA). The limit of detection (LOD) was set as 10 copies per reaction. The lower limit of quantification (LLOQ) was determined to be Cycle of quantification (Cq) 36 (for H183) and 35 (for Entero1). The efficiency of all qPCR standards was 90%–100%. Reactions were run in triplicate using TaqMan Universal PCR Master

Mix (Applied Biosystems, Carlsbad, CA, USA), 1000 nM of each forward and reverse primer, 80 nM of the probe, and 2 µL of genomic DNA. Amplification was performed using a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA), using the following cycle conditions: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. The results were expressed as gene copies (GC) per 100 mL. The GC values were calculated after taking into account the volume of the processed sample, DNA extraction volume, template volume used in the reaction, and the dilutions used for each sample analyzed.

Identification of *Vibrio* spp. Enumeration and identification of potentially pathogenic *Vibrio* spp. were carried out by cultivation on *Vibrio*-specific culture media TCBS (Figure S1). At least 5 single-cell colonies per sample were picked and suspended in 100 µL of DNase-, RNase-, and Protease-free water (APPLIC-HEM, Darmstadt, Germany), lysed by heating at 95 °C for 10 min, centrifuged, and the supernatant of crude DNA was stored at −20 °C. Isolates identification was performed by bacterial 16S rRNA gene amplification using 341F/907F primers (Table 1).

The PCR reactions and conditions were performed as described in [48]. The PCR products were detected by electrophoresis in 1.5 % agarose gel stained with SYBR® Safe DNA Gel stain (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA).

The PCR products were sequenced by Macrogen Europe BV (Amsterdam, the Netherlands). The sequences obtained were aligned using MEGA 7.0.26. To determine the closest relatives of environmental isolates, the 16S rRNA gene sequences were compared against known taxa present in the GenBank database. The sequences obtained in this study are available from GenBank under the accession numbers MT549191–MT549296 and MT549902–MT549997. Maximum-likelihood bootstrapping (1000 bootstraps) analyses were carried out using MEGA 7.0.26. The tree was visualized using the Tree of Life online tool iTOL v4 (Letunic and Bork, 2019).

The abundance of potentially pathogenic *V. cholerae* and *V. vulnificus* in water samples was enumerated using TaqMan-based real-time PCR assay, following the methods described in Fykse et al. [49,50] and Gyraite et al. [32], respectively. Standard curves for each real-time PCR were plotted from triplicate samples using C_t values of 10-fold dilutions of purified DNA from *V. vulnificus* and *V. cholerae* ranging from 10^8–10^3 gene copies. The efficiency of all qPCR standards was 90%–100%. The results were expressed as GC per 100 mL.

Data of cHAB and microcystin. To investigate multiple risks occurring at the bathing sites, cyanobacterial abundance (cells mL^{-1}), biomass (mm^3 L^{-1}), Chl *a* (µg L^{-1}), and total MC (µg L^{-1}) concentration were assessed. The data was collected and analyzed during the same sampling campaign published by Overlingė et al. [20]. For this study, cHAB abundance was modified from counts mL^{-1} to cells mL^{-1}, as recommended by the Baltic Marine Environment Protection Commission (Helsinki Commision—HELCOM) [51] and the International Council for the Exploration of the Sea (ICES) (http://www.ices.dk/marine-data/Documents/ENV/PEG_BVOL.zip). At the same time, total MC was obtained by summing eight different MC variants and used for integrated microbial risk assessment, following selected guideline values and thresholds (Table 2).

Table 1. Oligonucleotide primers used for multiplex polymerase chain reaction (PCR) and real-time PCR.

Target Organism	Primer Sequences	Base Pairs (Bp)	T_m (°C)	Reference
	Conventional PCR			
Bacterial 16S rRNA	341F: 5′-GCCTACGGGAGGCAGCAG-3′ 907R: 5′-CCGTCAATTCMTTTGAGTTT-3′	630	59	Romero and Navarrete [52]
	Real-time PCR			
V. vulnificus	vvha_F: 5′-GTTTATGGTGAGAACGGTGACA-3′ vvha_R: 5′-TTCTTTATCTAGGCCCCAAACTTG-3′ vvha_P: (FAM)-CCGTTAACCGAACCACCCGCAA-(TAMRA)	-	60	Campbell et al. [53]
V. cholerae	groEL_F: 5′-GGTTATCGCTGCGGTAGAAG-3′ groEL_R: 5′-ATGATGTTGCCCACGCTAGA-3′ groEL_P: (FAM)-CTGTCTGTACCTTGTGCCGATACTAAAGC-BBQ)	117	58	Fykse et al. [50]
Enterococcus	ENT_F: 5′-GAGAAATTCCAAACGAACTTG-′3 ENT_R: 5′-CAGTGCTCTACCTCCATCATT-′3 ENT_P: (FAM)-GGTTCTCTCCGAAATAGCTTTAGGGCTA-(TAMRA)	92	60	Haugland et al. [47]
Bacteroides	HF183_F: 5′-ATCATGAGTTCACATGTCCG-′3 HF183_R: 5′-CGTAGGAGTTTGGACCGTGT-′3 Probe: (FAM)-CTGAGAGGAAGGTCCCCCACATTGGA-(MGB)	167	60	Haugland et al. [16]

2.3. Integrated Microbial Risk Assessment of Swimming-Associated Illnesses

A set of indicator organisms was chosen to assess swimming-related illnesses risks in coastal and transitional water bathing sites. A table of risk levels and thresholds of potentially pathogenic microorganisms was constructed (Table 2) and used for interpreting multiple risks occurring at the investigated sites during the summer of 2018:

(a) bathing water suitability for recreational use was defined by established limits of fecal indicator bacteria (FIB) such as *E. coli* and *Enterococcus* (BWD, 2006/7/EC) [7];
(b) rapid molecular measures for *Bacteroides* established by the U.S. EPA and their benchmark values were applied in this study [8,16,47,54,55] (Table 2);
(c) for safe practice in managing bathing waters, WHO guideline values classifying severity and probability of cyanobacteria impact on health at three levels of risk ('low', 'moderate', and 'high'; see Table 2) were applied. Total cells of cyanobacteria (mL^{-1}), Chl *a* ($\mu g\ L^{-1}$), cyanobacterial biomass ($mm^3\ L^{-1}$), and the total concentration of MCs ($\mu g\ L^{-1}$) were used as indicators of potential risk for adverse health outcome from exposure to cyanobacteria blooms [1,56,57]; and
(d) due to a lack of existing thresholds for water-based potentially pathogenic *Vibrio* spp. bacteria, minimum infectious dose (10^4 cells mL^{-1} or 10^6 cells 100 mL^{-1}), as defined by Hornick et al. [58], was used for *V. cholerae* risk assessment. In this study, we assume that GC corresponds to a cell number. For potentially pathogenic *V. vulnificus*, QMRA was applied.

Quantitative Microbial Risk Assessment (QMRA). The exposure of swimmers to species of interest was estimated using 100 mL [59] of water volume ingested (V_{ing}) during the recreational activity and concentration of pathogenic bacteria (P_{conc}) observed at the time of exposure in different bathing waters (Equation (1)). The abundance of *V. vulnificus* was obtained by real-time PCR and used as pathogen concentration input for the calculations of P_{inf}.

$$D_{exp} = V_{ing} \times P_{conc} \quad (1)$$

$$P_{inf} = 1 - \left[1 + \frac{D_{exp}}{\beta}\right]^{-\alpha} \quad (2)$$

Table 2. Guideline values and thresholds of fecal indicator bacteria (FIB), *V. cholerae*, *V. vulnificus*, cyanobacteria, and microcystins (MCs) for integrated and safe practice in managing bathing waters.

Indicator	Unit	Risk Level			Reference
FIB		Low	Moderate	High	
Guidance level		0–3% probability of gastrointestinal illness	3–5% probability of gastrointestinal illness	5–8.4% probability of gastrointestinal illness	
E. coli	CFU 100 mL^{-1}	<250	250–500	>500	BWD [7]
Enterococcus	GC 100 mL^{-1}	<100	101–200	>200	
Bacteroides	GC 100 mL^{-1}	<860		>860	Ashbolt et al. [17]
QMRA		Acceptable benchmark		Not acceptable	
V. vulnificus	Probability (Infection) (%)	<19 infections per 1000 bathers		>19	U.S. EPA [60]
V. cholerae	Minimum infectious dose	<10^6 GC 100 mL^{-1}		>10^6 cells 100 mL^{-1}	Mouriño-Pérez et al. [61]
cHABs		Low	Moderate	High	
Guidance level		Relatively low probability of adverse health effects	Moderate probability of adverse health effects	High probability of adverse health effects	
Cyanobacteria	Cells mL^{-1}	<20,000	20,000–100,000	>100,000	Churro et al. [57] WHO [1]
	Chl *a* µg L^{-1}	<10	10–50	>50	
	Biomass mm^3 L^{-1}	<0.2	0.2–10	>10	
MC	µg L^{-1}	2–4	10–20	>1000	Codd et al. [56]

To calculate the probability of infection (P_{inf}) from a single-time exposure to *V. vulnificus*, species-specific $\alpha = 9.3 \times 10^{-6}$ and $\beta = 1.1 \times 10^5$ parameters, defined by the dose–response model, were used (Equation (2)) [32,33]. A benchmark of less than 19 infections per 1000 bathers [60] was considered as low-to-moderate risk level, while above 19 infections was considered as 'high' risk level (Table 2).

For interpretation of the integrated microbial risk assessment of bathing waters, a decision scheme has been proposed, based on the 'one-out, all-out' principle [62].

2.4. Statistical Analysis

Descriptive and statistical analyses were performed using the R Studio (Rcmdr commander) and XLSTAT software [63]. Before the analysis, the normality of variables was tested using the Kolmogorov–Smirnov test. The data deviated from the normal probability distribution. Therefore, the non-parametric Kruskal–Wallis test was applied to compare spatial and temporal differences of the studied microorganisms. In contrast, the Mann–Whitney test for pair-wise comparison of two samples was applied to compare microorganism parameters between coastal and lagoon sites. Spearman correlation (r_s) was used to define a statistically significant relationship between potentially harmful organisms. A log-linear (Poisson) regression model was used to assess the significance of *E. coli* and Chl *a* concentration, together with environmental parameters such as water temperature, salinity, pH, and turbidity, in explaining the log-transformed *V. cholerae* abundance. Sampling sites were indicated as an offset. Results were considered significant at a *p*-value less than 0.05.

3. Results

3.1. Spatial and Temporal Variability of FIB

During the study period, the concentration of *E. coli* varied from 0 to 450 CFU 100 mL^{-1} (Figure 2). *E. coli* concentrations ranged from 1–185, 0–87, and 0–85 CFU 100 mL^{-1} in Palanga, Melnrage, and Nida BS, respectively. The lowest average concentration of *E. coli* in 2018 was observed at the Nida BS site. The threshold for 'moderate' risk was exceeded only on one occasion, in Nida CL, when the *E. coli* concentration reached 450 CFU 100 mL^{-1}. The *E. coli* concentration varied from 6–111 and 6–70 CFU

100 mL^{-1} at the Port and Kintai sites, respectively. Average *E. coli* concentration observed in the coastal sampling sites (Palanga, Melnrage, and Nida BS) were significantly lower ($p < 0.05$, N = 36) than in the Curonian Lagoon sites (Port area, Kintai, and Nida CL).

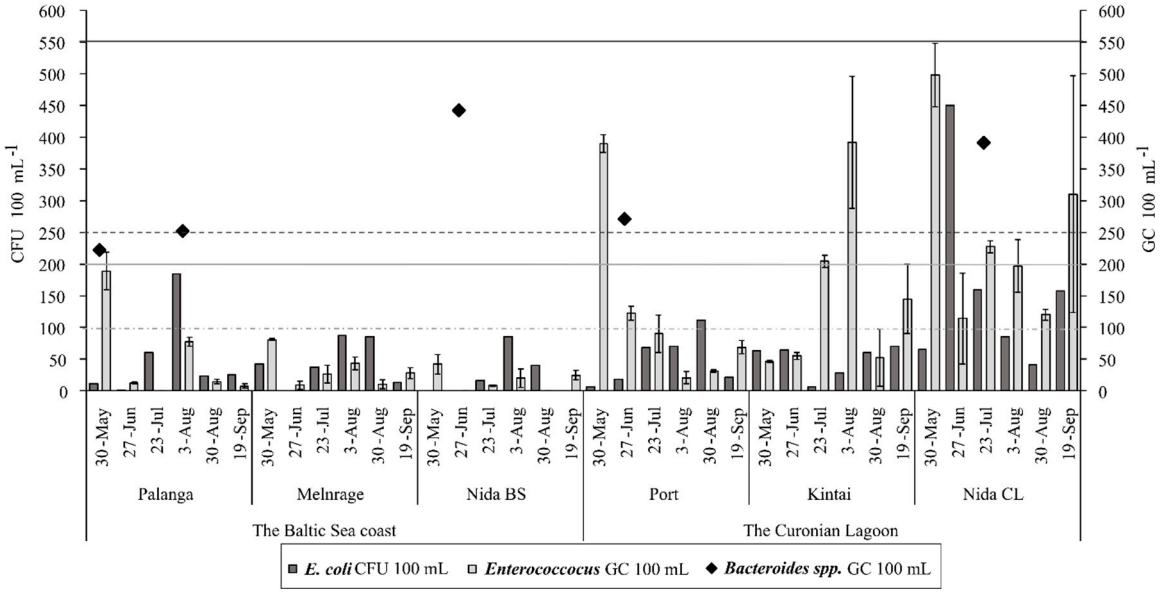

Figure 2. FIB concentration obtained in May–September 2018 along the coast of Lithuania and the Curonian Lagoon. Black horizontal lines represent lower (250 colony-forming units (CFU) 100mL^{-1}) and upper (500 CFU 100 mL^{-1}) thresholds of *E. coli*; grey lines represent *Enterococcus* lower (100 GC 100 mL^{-1}) and upper (200 GC 100 mL^{-1}) thresholds. For *Bacteroides* sp. (♦), 860 GC 100 mL^{-1} was applied as a threshold level. Error bars represent the SD (N = 3).

The abundance of *Enterococcus* in the Palanga site varied from 0 to 189 GC in 100 mL^{-1}, exceeding the 'moderate' risk benchmark only once (Figure 2). During the study period, *Enterococcus* concentrations in Melnrage and Nida BS varied from 0–81 and 0–42 GC per 100 mL, respectively, thus not surpassing the recommended 'low' risk benchmark. The abundance of *Enterococcus* was observed to be significantly higher ($p < 0.05$, N = 36) in the Curonian Lagoon sites, compared to the coastal bathing sites. *Enterococcus* abundance at the Port varied from 21–389 GC per 100 mL and the 'high' and 'moderate' risk thresholds were exceeded in May and June, respectively. In the Kintai site, *Enterococcus* abundance was 52–392 GC per 100 mL, surpassing 'moderate' risk once and the 'high' risk threshold twice over the study period. The highest average abundance of *Enterococcus* was observed at Nida CL. At Nida CL, the *Enterococcus* abundance varied from 114–498 GC in 100 mL^{-1}, thus exceeding the 'moderate' and 'high' risk benchmarks over the study period.

Bacteroides were detected in only five samples. Thus, the abundance varied from 0 to 442 GC per 100 mL, not surpassing the recommended benchmark (Figure 2).

3.2. Spatial and Temporal Variability of cHAB

Over the study period, the 'moderate' (20–100 thousand cells mL^{-1}) and 'high' (>100 thousand cells mL^{-1}) risk thresholds of cyanobacteria abundance were always surpassed at the Palanga bathing site, with the exception of 30 August. The highest risk was observed on 3 August when biomass and Chl *a* 'moderate' risk thresholds were exceeded. Total MCs concentration at the Palanga site did not exceed the 'low' risk benchmark of 2–4 µg L^{-1} (Figure 3).

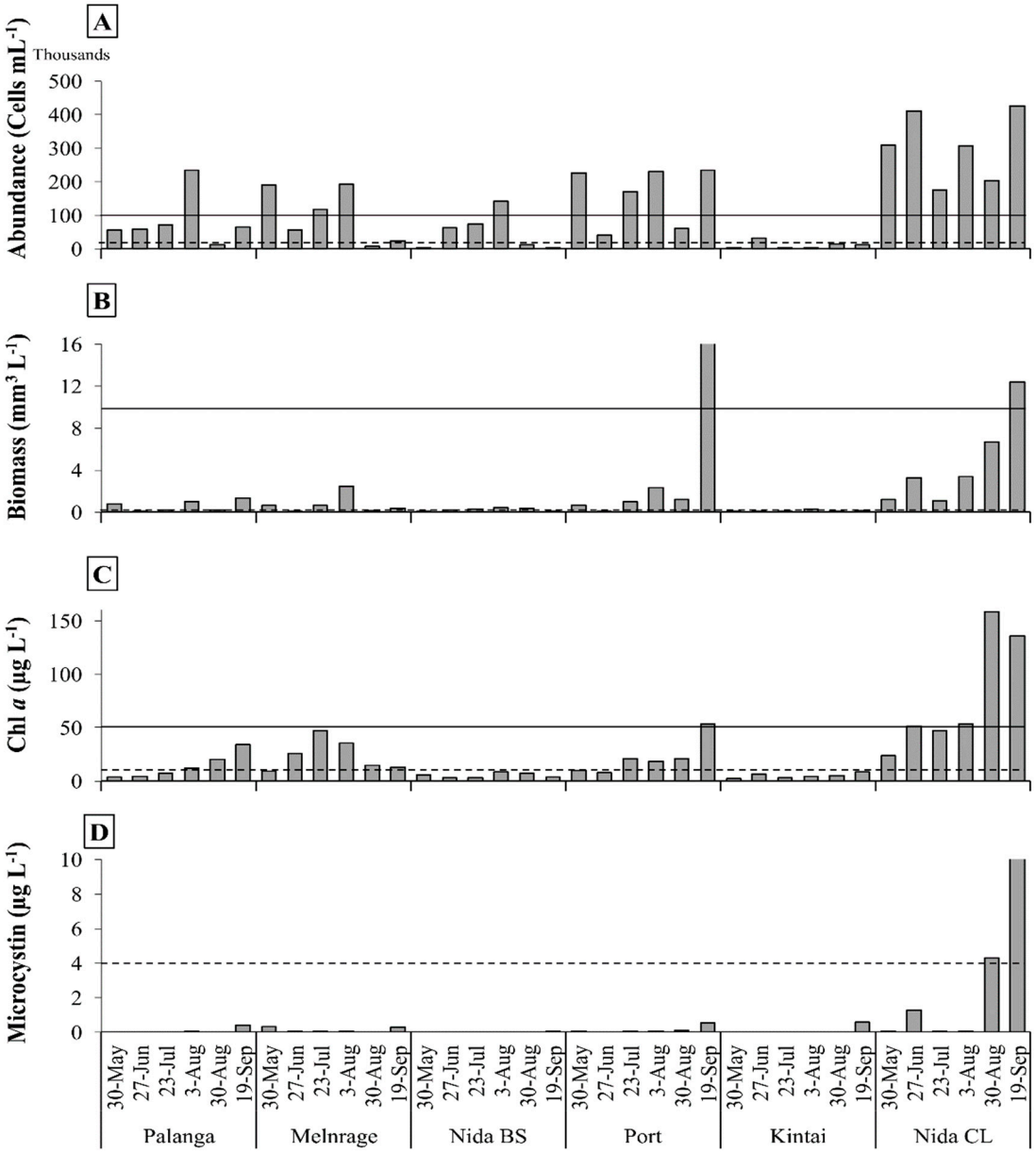

Figure 3. Cyanobacterial abundance (cells mL^{-1}) (**A**) cyanobacterial biomass (mm^3 L^{-1}); (**B**) chlorophyll (Chl) *a* (µg L^{-1}); (**C**) and microcystin (MC) concentration (µg L^{-1}); (**D**) obtained in May–September 2018 along the coast of Lithuania and the Curonian Lagoon. Black horizontal lines represent 'high' risk alert level, while the dotted lines indicate 'low' risk level thresholds.

In the Melnrage bathing site, the average abundance of cyanobacteria varied from 6.7 to 191 thousand cells mL^{-1}. The 'high' risk threshold was exceeded three times and the 'moderate' risk was exceeded twice over the study period. Biomass and Chl *a* 'high' risk thresholds were not exceeded; however, 'moderate' risks occurred four and five times, respectively. The MCs concentration at the Melnrage site was below the 'low' risk level at all sampling times (Figure 3).

The lowest abundance (90 cells mL^{-1}) of cyanobacteria was observed at the end of May in the Nida BS bathing site. From 27 June to 23 July, we observed 'moderate' risk threshold exceedances. Later, on 3 August, cyanobacteria abundance reached 141 thousand cells mL^{-1}, thus exceeding the 'high' risk level. No Chl *a* or MC concentration 'low' risk level exceedances were observed over the study period (Figure 3).

At the Kintai site, the threshold of 'moderate' risk level for cyanobacteria abundance was surpassed just once (31 thousand cells mL^{-1}). Biomass, Chl *a*, and MC concentrations were observed below the 'low' risk threshold (Figure 3).

Cyanobacteria abundance in the Port area varied from 39 to 234 thousand cells mL^{-1}, meaning that 'moderate' and 'high' risk levels occurred over the study period. The highest cyanobacteria biomass of all the sampling sites occurred in the Port area, on 19 September. On that day, Chl *a* concentration was detected as higher than 50 μg L^{-1} (i.e., at 'high' risk level). The lowest biomass and Chl *a* concentration (<'low' risk) was obtained on 27 June (Figure 3).

At Nida CL, cyanobacteria abundance exceeded the WHO recommendations for 'high' risk threshold (>100,000 cells mL^{-1}) [1] between May and September 2018. The average abundance of cyanobacteria over the study period in Nida CL was 304 thousand cells mL^{-1}. The highest Chl *a* concentration was detected to be 158.5 μg L^{-1}. MC 'moderate' risk level was exceeded twice (30 August and 19 September; Figure 3).

Cyanobacteria abundance ($p = 0.001$, N = 36), biomass ($p = 0.001$, N = 36), and Chl *a* concentration ($p = 0.0001$, N = 36) differed significantly among sampling sites, but not among the sampling months ($p > 0.05$, N = 36). Significant spatial ($p = 0.01$, N = 36) and temporal ($p = 0.03$, N = 36) differences were observed for MC concentrations.

3.3. *V. vulnificus* and *V. cholerae* Abundance and the Risk of Infection

In the summer season of 2018, both *V. vulnificus* and *V. cholera* were found at the studied sites (Figure 4). *V. vulnificus vvhA* gene was obtained only in the coastal bathing sites of Palanga, Melnrage, and Nida BS. Therein, *vvhA* GC varied from 0 to 6.74 log GC 100 mL^{-1}. The earliest observation of *V. vulnificus* (4.94 *log* GC 100 mL^{-1}) was recorded on 23 July at the Nida BS bathing site; while, in Melnrage, *V. vulnificus* was observed with an abundance of 6.01 *log* GC 100 mL^{-1} only at the end of August, when the sea surface salinity was 6.9. The abundance of *V. vulnificus vvhA* gene in Palanga varied from 5.21 to 6.74 *log* GC 100 mL^{-1}. According to the average abundance of *V. vulnificus vvhA* gene, the risk of *V. vulnificus* infection per single exposure at an ingested water volume of 100 mL varied from 0 to 3.67×10^{-5} at the coastal bathing sites (Palanga, Melnrage, and Nida BS). The highest risk of infection occurred on 3 August in Palanga; however, it did not exceed the acceptable illness benchmark of 19 per 1000 bathers.

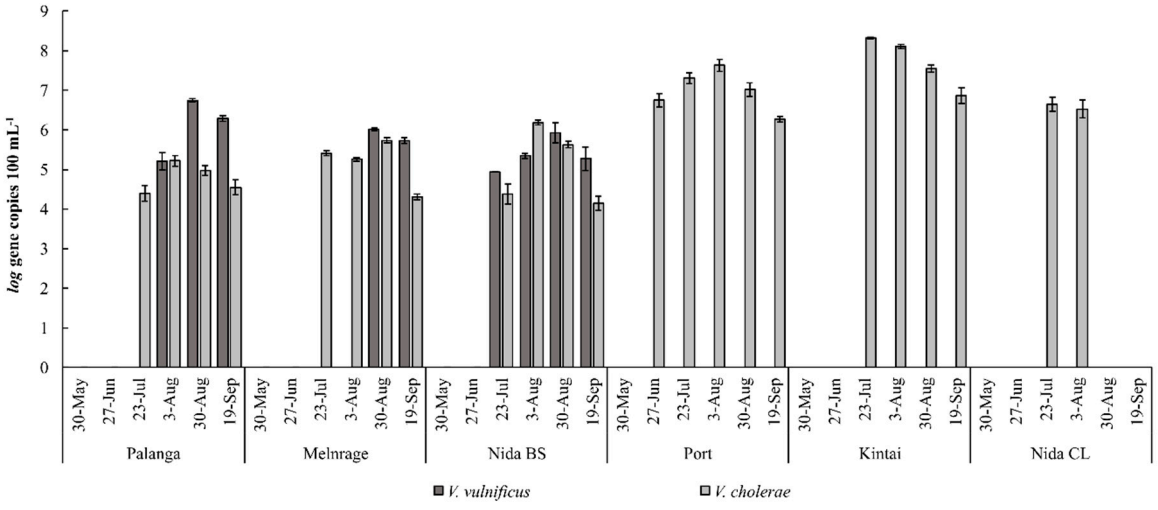

Figure 4. The abundance of *V. vulnificus vvhA* and *V. cholerae groEL log* GC 100 mL^{-1} obtained in May–September 2018, at six sampling sites along the coast of Lithuania and the Curonian Lagoon. Error bars represent the SD (N = 3).

V. cholerae groEl gene was observed in all sampling sites. However, *groEL gene* abundance in the coastal bathing sites was significantly lower ($p < 0.05$, N = 36) than in sampling sites within the Curonian Lagoon. In the coastal bathing sites, the *groEL* gene varied in the ranges 0–5.22, 0–5.73, and 0–6.18 log GC 100 mL^{-1} in Palanga, Melnrage, and Nida BS, respectively. Meanwhile, in the lagoon waters, it varied from 0–7.63, 0–8.32, and 0–6.65 log GC 100 mL^{-1} in Port, Kintai, and Nida CL, respectively. The earliest record of the *V. cholerae groEL* gene in 2018 was observed on 27 June in the Port area. The minimum infectious dose of 10^6 GC per 100 mL was exceeded once in the Nida BS bathing site on 3 August. Meanwhile, in the Curonian Lagoon sampling sites, this threshold was exceeded repeatedly over the study period.

3.4. Diversity of Potentially Pathogenic Bacteria

Overall, 203 isolates were amplified with bacterial 16S rRNA primers and sequenced. A total of 52.47% (N = 106) of all isolates could be assigned to bacteria of the *Vibrio* genus and were 99–100% similar to already-known strains in the GenBank database. A total of 14.85% (N = 30) of isolates were designated as *V. vulnificus*, 33.66% (N = 67) as *V. cholerae*, 2.47% (N = 5) as *V. anguillarum*, and 1.48% (N = 3) as *V. fluvialis* (Figure 5). *V. anguillarum* was recorded only in May and June at two coastal bathing sites (Palanga and Melnrage).

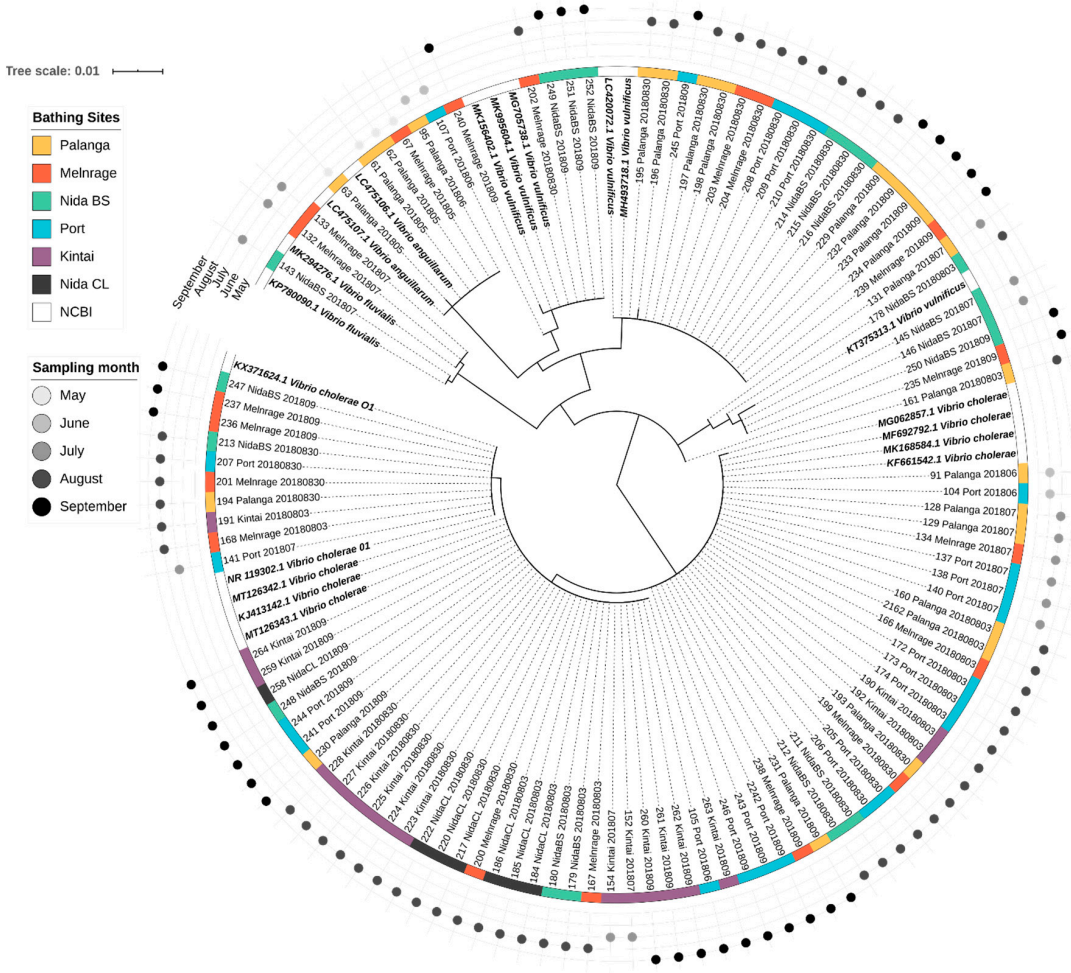

Figure 5. Phylogenetic tree of members of the genus *Vibrio* based on 16S rRNA sequences. Strains indicated in bold are from the National Center for Biotechnology Information (NCBI) database.

V. fluvialis was detected in July in the Melnrage and Nida BS bathing sites. In total, 41 *V. cholerae* isolates were obtained from the Curonian Lagoon sites (Kintai, Nida CL, and the Port area). At the 16S rRNA level, ten isolates showed high phylogenetic similarity to the pandemic strain *V. cholerae* 01 (accession number KX371624.1 and NR119302.1). A total of 31.18% (N = 63) of isolates were identified as *Aeromonas veronii* and 7.42% as *Aeromonas* spp. Bacteria of the *Aeromonas* genus were obtained from all sampling sites and sampling periods. The rest 9.40% (N = 18) included other aquatic bacteria species, such as *Morganella morganii*, *Plesiomonas shigelloides*, *Staphylococcus warneri*, *Schewanella upenei*, *Citrobacter* spp., *Providencia* spp., and *Exiguobacterium* spp.

3.5. Decision Scheme for Integrated Microbial Risk Assessment

The decision tree for interpretation of an integrated microbial risk assessment is a stepwise process to be followed when monitoring recreational waters. This process starts by taking water samples and testing them for microorganisms of interest. The minimum annual sample number per bathing site should be no less than four, as indicated in the BWD [7]. This scheme recommends actions needed to address FIB, cHAB, and *Vibrio* bacterial potential risks, based on the thresholds shown in Table 2. In principle, if none of the microbial indicators exceeds the 'low' risk threshold, no actions are required by the local health authorities and the monitoring is continued (Figure 6A). A 'moderate' alert level must be announced to the public when at least two microbial indicators exceed 'moderate' risk thresholds. Suppose at least one microbial indicator exceeds the 'high' risk threshold. In that case, the quality of bathing water on that particular day is interpreted as not suitable for water-related activities and should be highlighted as being at a 'high' alert level (Figure 6B). Water-related activities are prohibited until the repeated measurements of potentially harmful organisms fall back to 'moderate' or 'low' risk thresholds. In the case of 'moderate' and 'high' risk alert levels, public announcements should include information about potential symptoms after exposure to the occurring microorganisms.

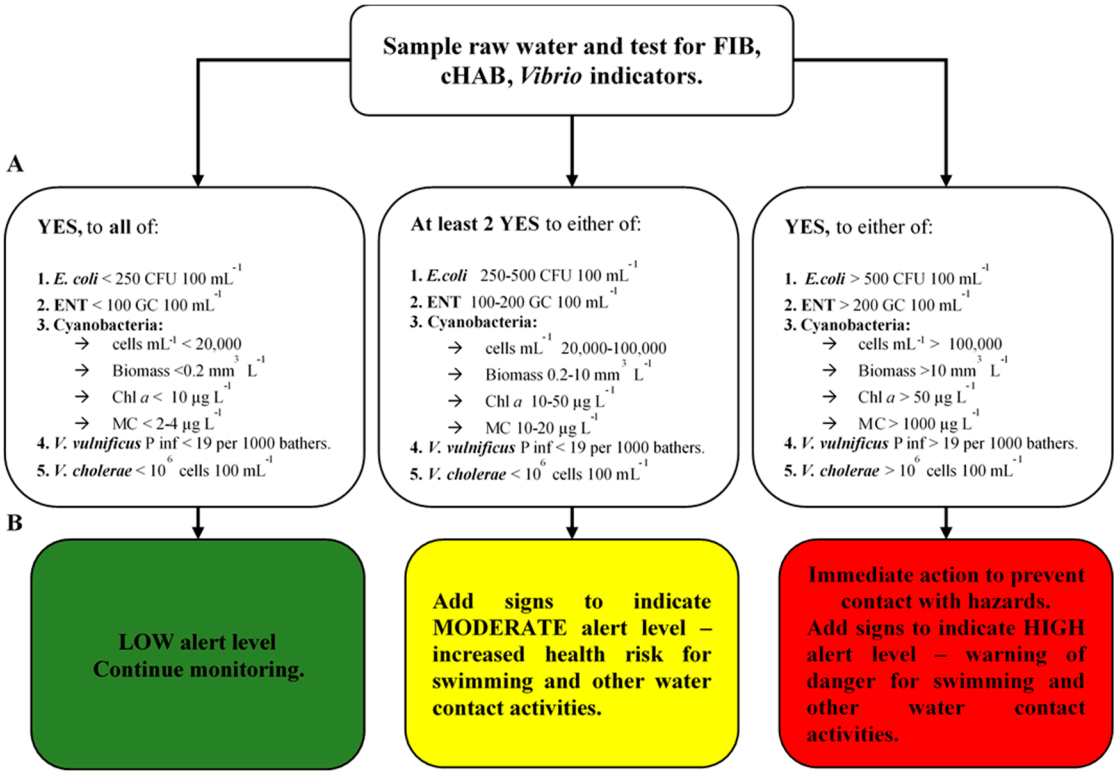

Figure 6. Decision tree for interpretation of integrated microbial risk assessment in recreational waters: (**A**) microbial indicators of water quality and their thresholds (from Table 2); and (**B**) decision-making and recommendations for authorities.

3.6. Integrated Microbial Risk Assessment of the Recreational Waters

For the assessment of bathing water quality along the Lithuanian Baltic Sea and the Curonian Lagoon bathing sites, several microbial indicators were evaluated (based on the threshold exceedances) and interpreted (based on the decision tree shown in Figure 6). Table S2 summarizes the microbial water quality indicators and integrated risks per sampling period of 2018 and study sites along the Lithuanian coast of the Baltic Sea and the Curonian Lagoon.

Based on the integrated microbial risk assessment, the two sampling sites considered in this study were classified as 'high' alert level sites. These were Nida CL and the Port, which were located within the Curonian Lagoon, over the study period (May–September; Figure 7 and Table S1). Within those sites, cyanobacterial indicators, such as abundance, biomass, Chl *a*, and *V. cholerae* minimum infectious dose thresholds, were exceeded. At the Kintai bathing site, water quality at the beginning of the bathing season (May–June) was determined as 'low' risk level. However, in July, the *V. cholerae* minimum infectious dose (10^6 cells 100 mL^{-1}) and *Enterococcus*' high' risk level thresholds were exceeded, and the bathing site was noted as being at 'high' alert level until September. Within the Curonian Lagoon sampling sites, a positive correlation (rS = 0.48, $p < 0.05$) was found between *E. coli* and Chl *a* concentrations.

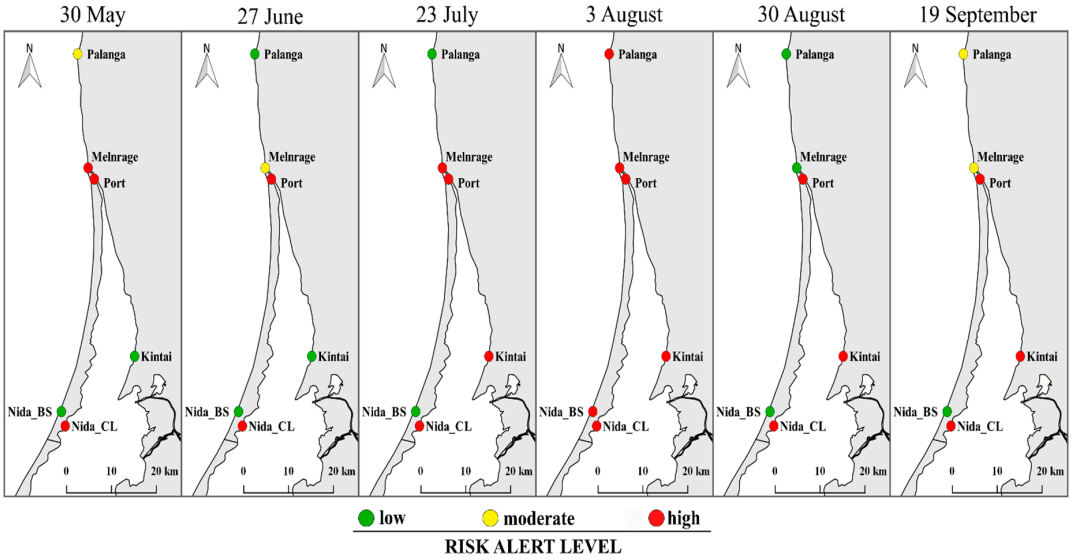

Figure 7. Summary of integrated microbial risk assessment per sampling period of 2018 and study sites along the coast of Lithuania and the Curonian Lagoon. Green dots—'low', yellow dots—'moderate', and red dots—'high' alert level.

The Nida BS coastal bathing site was identified as being a 'low' alert level bathing site throughout the sampling period, except on one occasion—3 August—when abundances of two microbial indicators (cyanobacteria and *V. cholerae*) exceeded 'high' risk thresholds. On the same day, when the water surface temperature reached 26 °C, all coastal and Curonian Lagoon sampling sites were classified as 'high' alert level bathing waters. The Melnrage bathing site, located next to the Klaipeda strait, was assessed three times as a 'high' alert level site and only once (on 30 August) as a 'low' risk bathing site over the study period. The 'high' alert level in the Melnrage and Palanga bathing sites was mainly caused by the exceedance of cyanobacteria abundance threshold values. Palanga mostly fell into 'moderate' and 'low' alert level categories (except on the 3 of August). Within the coastal bathing sites of Lithuania, positive correlations between *E. coli* concentration and cHAB abundance (rS = 0.60, $p < 0.05$) and biomass (rS = 0.64, $p < 0.05$), as well as with *V. cholerae* (rS = 0.75, $p < 0.05$), were determined. In the meantime, *V. cholerae* correlated significantly positively with Chl *a* (rS = 0.47, $p < 0.05$) and *V. vulnificus* (rS = 0.58, $p < 0.05$).

For log *V. cholerae* abundance, log-linear regression was calculated with microbial and environmental parameters as independent variables. The parameters log Chl a, salinity, temperature, pH, and turbidity were significantly ($p < 0.0001$) related to *V. cholerae* abundance. The overall fit of the log-linear regression was statistically significant ($R^2 = 0.55$, $p < 0.0001$; see Table S3).

4. Discussion

The implementation of the BWD (2006/7/EC) [7] has been considered a success story by the EU. Based on FIB assessment, the water quality of more than 87.4% of EU and 81% of Lithuanian coastal bathing sites have been classified as 'excellent' [9]. In agreement, in this study, the bathing water quality based on *E. coli* data in all sampling sites was assessed as being at a 'low' risk alert level, with the exception of Nida CL. Moreover, in the summer season of 2018, *E. coli* concentrations observed at the Kintai bathing site were lower, when compared to the same period in 2011–2016 [41]. However, a recent study in the Curonian Lagoon has revealed that *E. coli* carrying virulence genes consisted of up to 5% of all isolates and high percentages of virulent isolates were found with low *E. coli* concentrations [41]. Following fecal pollution indication, more *Enterococcus* GC threshold exceedances were observed during this research, more often within the Curonian Lagoon compared to the coastal bathing waters. Yet, the literature suggests that *Enterococcus* correlates best to gastroenteritis in marine waters [64].

For the first time in the south-eastern Baltic Sea recreational waters, *Bacteroides* was assessed as a human-derived fecal contamination marker. *Bacteroides* were found only occasionally and did not exceed the thresholds suggested by Ashbolt et al. [17]. Usually, the presence of *Bacteroides* is expected to correlate with the presence of *E. coli* and *Enterococcus.* However, in this study, *Bacteroides* were not detected at relatively high FIB abundance sites (e.g., Port and Nida CL); this could be due to different fecal pollution sources than humans or due to the resuspension of sediments. Numerous studies have shown that FIB can be associated with various secondary habitats, such as aquatic vegetation, beach sand, fresh, and marine water sediments [10]. Water birds, such as cormorants or gulls, may play a significant role in fecal pollution [41,65,66]. Therefore, this may confound accurate recreational water quality assessments.

However, the recent concerns regarding cHAB in coastal and transitional waters [36,67] are not covered by the BWD. The lack of proper attention in the BWD to cyanobacteria is explained by the fact that the proliferation of cHAB does not affect all EU Member States or does not happen on a large scale [9]. The monitoring and water quality assessment based on cyanobacteria is left for each country's implementation.

Toxic cyanobacteria blooms are phenomena of the lacustrine environment; however, severa studies have shown their importance in coastal or transitional waters [20,67]. A 'moderate' risk level of MCs (4 to 10.32 μg L^{-1}) was observed in Nida CL. It is known that 48.6 μg L^{-1} of microcystin-LR can cause an acute case of intoxication [68]. In the Curonian Lagoon, MC has also been detected in much higher quantities; up to 134.25 μg L^{-1} [40] and 153.60 μg L^{-1} [19].

cHABs may also play an essential role as a reservoir or vector for potentially pathogenic *Vibrio* [69]. Allen [70] reported exceedances of the minimum infectious dose of *V. cholerae* combined with increased *Nodularia spumigena*-derived DOM levels within the Baltic Sea. In this study, a significant correlation between Chl *a* and abundance of *V. cholerae groEL* gene was found in the coastal bathing sites. Furthermore, in other studies, satellite-based estimates of Chl *a* have been used as a surrogate variable for forecasting *V. cholerae* outbreaks in endemic regions [71–73]. The log-linear regression model performed in this study indicated that Chl *a*, together with temperature, salinity, pH, and turbidity parameters, were significant in explaining the abundance of *V. cholerae*.

Even though the first cases of *Vibrio* infections in the Baltic Sea region were recorded almost four decades ago [31], the first data on potentially pathogenic *Vibrio* presence in the south-eastern Baltic Sea was reported only in 2017 [32]. In 2018, the Baltic Sea surface temperature reached extreme values (up to 26 °C) lasting for several weeks. In the same year, the Centre for Communicable Diseases and AIDS of Lithuania released a *Vibrio* risk warning [74] for the first time and the abundance of

Vibrio vulnificus vvhA gene in Lithuanian coastal bathing sites was detected to be higher than in 2017 (10^7 GC L^{-1} compared to 10^4 GC L^{-1}) [32]. However, the acceptable illness benchmark of 19 per 1000 bathers [60] was not exceeded.

Phylogenetic analysis of isolates revealed a number of potentially pathogenic *Vibrio* species, such as *V. fluvialis* and *V. anguillarum*, which are adapted to cold temperatures and brackish waters [38] and are often associated with aquaculture [75–77]. Ten isolates were clustered close to a pandemic *V. cholerae* O1 strain. *V. cholerae* O1 and O139 have been identified among the most important *Vibrio* infection-causing bacteria in the freshwater system [29] and have been related to several hospitalized cases in the Baltic Sea [23]. *V. cholerae* non-O1, as well as O1, can be transferred to cormorants from their fish prey [78] and have been found in water bird cloacal swabs, feces, and intestine samples. In this way, *V. cholerae* may be transferred in a short time across and between continents [79]. Furthermore, chironomids are natural reservoirs of *V. cholerae* and copepods may act as a vector [70,79]. In the last few decades, *V. cholerae* has been identified as a notorious multidrug resistant enteric pathogen that can withstand most of the antibiotics used for infection treatments [80].

A wide variety of physical and chemical hazards to human health can occur in bathing waters. However, the core of bathing water policy and science is based on microbial pollution assessment [9]. However, the current BWD does not consider naturally occurring potentially pathogenic or toxic microorganisms. This study aimed to integrate FIB, cHAB, and *Vibrio*, as suggested by the WHO [18], and to classify water quality at the bathing site using the 'one-out, all-out' principle. The proposed scheme of integrated microbial risk assessment caused relatively more exceedances of the 'high' risk alert level, in comparison with the assessment only based on FIB. In fact, while FIB thresholds remained below 'low' risk level, cHAB parameters in the coastal bathing sites of Melnrage and Palanga indicated 'moderate' and 'high' risk levels, deriving from the Curonian Lagoon water outflow and its impact zone in the coastal Baltic sea. One of the cHAB parameters—cyanobacteria abundance—exceeded the recommended thresholds in almost all investigated sites. Counting cells per mL may yield inaccuracies due to filamentous (e.g., *Aphanizomenon flosaquae*, *Planktothrix agardhii*, etc.) and picocyanobacteria (e.g., *Aphanocapsa* spp., *Aphanothece* spp.) dominance. Filamentous cyanobacteria cells are difficult to discern microscopically, while picocyanobacteria can be analyzed only with limited certainty [51,81]. Overlingė et al. [20] recommended cyanotoxins as the parameter that should be monitored in bathing waters for the south-eastern Baltic Sea. At the same time, cHAB abundance and biomass should be measured depending on dominating cyanobacteria species. Furthermore, semi-quantitative field screening kits for MC could be used for fast decisions in beach management situations [82].

Current monitoring of bathing water quality relies on culture-based enumeration, which does not reflect viable but non-cultivable (VBNC) microorganisms [10]. *Vibrio* bacteria may persist in the VBNC state under unfavorable conditions and revert to a cultivable state with consequent disease outbreaks in susceptible coastal areas when the conditions become optimal [83]. In the case of *Vibrio* spp. cultivation on TCBS media, only 60% of isolates are identified as *Vibrio* spp., which may misrepresent the results [84]. Therefore, in this study, we used culture-independent methods such as real-time PCR for some of the potentially pathogenic bacteria. These methods can tackle bacteria in the VBNC state [85] and are of priority for further development, according to the U.S. EPA [86]. One of the major limitations of real-time PCR is its inability to discriminate between live and dead bacteria due to DNA persistence after cell death [87]. Therefore, the amplification of environmental DNA can lead to an overestimation of health risks posed by environmental water, resulting in questionable management decisions [88]. However, using a culture-independent method which combines ethidium mono-azide (EMA) with real-time PCR, which can distinguish between viable and dead cells [89], local authorities can make decisions with regards to recreational water quality on the day of sampling, based on more accurate data.

Furthermore, in this study using phylogenetic analysis of bacterial 16S rRNA, we discovered multiple other potentially human pathogenic bacteria, such as *Aeromonas veronii*, *Morganella morganii*, *Plesiomonas shigelloides*, *Staphylococcus warneri*, and *Schewanella upenei* [26]. Some of these have been

found in the intestinal tracts of fish from the Baltic Sea [90]. In similar systems, the risks arising from naturally occurring microorganisms (e.g., *Vibrio* or cyanobacteria) could be of higher importance than fecal pollution. Thus, more attention should be paid to their monitoring and the implementation of preventative measures.

Finally, the risks for bathers reported in this study are not unusual and should be considered relevant to all coastal brackish water bathing sites. Every year, we observe intensive cyanobacteria harmful algal blooms [19,20,40] or heat wave-associated vibriosis cases around the Baltic Sea [23,24,91], as well as in other European coastal waters (e.g., the British Channel [92], the Netherlands [93]) and the USA [94]. Countries such as Germany, Sweden, and Finland had already recognized the problem of wound infections caused by *Vibrio* decades ago. Therefore, these bacteria are monitored during the recreational season, infection cases are investigated, and risk warnings are announced by public health agencies [32]. In the meantime, information about *Vibrio* infection cases in the south-eastern Baltic Sea countries is impossible to find. Warnings about the potential risks while having contact with recreational waters are released based on the ECDC Vibrio Map Viewer [95], without any real-time measurements or epidemiological surveillance afterwards. In fact, the lack of epidemiological studies, which can explain the relationships between the density of microbial indicators and the occurrence of adverse health effects among bathers is a major shortcoming in setting guidelines and standards for recreational waters [96]. Therefore, to further develop the integrated microbial risk assessment, we recommend performing initial epidemiological studies in the investigated region. Epidemiological evidence may provide clear answers regarding whether having contact with waters containing various amounts of microbial hazards makes a difference in terms of illness incidence. Results from epidemiological studies can provide a solid baseline for deciding: (i) the monitoring frequency (current annual sample number of 4 or 20, as suggested by the WHO [18]); (ii) cost-efficient methods to use (e.g., only screening for cyanotoxins or monitoring all the parameters of cHAB); and (iii) the possible linkage of monitoring programs and implementation of integrated microbial risk assessment at the European scale. It is also important to highlight that, by standardizing the risk assessment scheme, sampling strategies could be adjusted for each country independently. For instance, sampling could be performed weekly instead of monthly if the observed (incl. Satellite images) physical and chemical parameters of bathing waters are favorable to the faster proliferation and spread of pathogenic microorganisms.

5. Conclusions

- This is the first study presenting an integrated microbial risk analysis in the Lithuanian coastal and Curonian Lagoon bathing sites.
- The *E. coli* concentration in all sampling sites over the study period was below the 'moderate' risk alert level, except in June in Nida CL. Exceedances of 'moderate' and 'high' *Enterococcus* risk thresholds were observed for the Curonian Lagoon sites.
- cHAB abundance, biomass, and Chl *a* exceeded 'moderate' and 'high' risk thresholds in more than 50% of all samples at all the sampling sites, except Kintai. The concentration of MC reached the 'moderate' alert level threshold only in Nida CL.
- In 2018, the potential infection risk from *V. vulnificus* was below the acceptable illness benchmark (19 out of 1000 bathers), although its abundance at some sampling sites was two times higher than in 2017 [32]. In the Curonian Lagoon sites, the minimum infectious dose of *V. cholerae* was exceeded over the study period, except at the beginning of the bathing season in Kintai.
- A new approach for bathing water profiling based on more target indicators for water quality, such as cHAB and *Vibrio*, revealed more bathing sites of 'high' and 'moderate' risk alert level. In contrast, the FIB indicators showed an excellent water quality.
- The integrated microbial risk assessment presented in this study covers a wider spectrum of risks caused by potentially harmful organisms and could be applied in similar fresh–brackish water systems.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/11/3146/s1, Figure S1: log Vibrio spp. concentration in CFU 100 mL^{-1} obtained in May–September 2018 along the coast of Lithuania and the Curonian Lagoon. Error bars represent the SD (N = 3), Table S1: Environmental conditions at the sampling sites (mean ± standard deviation (SD), minimum (Min) and maximum (Max), extracted from Overlingė et al. (2020) [20], Table S2: Summary of microbial indicators of water quality per sampling period of 2018 and study sites along Lithuanian coast of the Baltic sea and the Curonian Lagoon, Table S3: Results of log-linear regression.

Author Contributions: Conceptualization, G.G., M.K., G.S.; methodology, G.G., M.K., D.O., D.V., G.S.; formal analysis, G.G.; investigation, G.G., M.K., D.O., D.V., E.J.; resources, G.G., M.K., D.O.; writing—original draft preparation, G.G.; writing—review and editing, M.K., D.O., D.V., G.S.; visualization, G.G.; funding acquisition, G.G., D.V., G.S. All authors have read and agreed to the published version of the manuscript.

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