



## Research Article

## Bacteriological analysis of ballast water and port area: case study in the Baltic Sea port

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**Citation:** Srėbaliėnė G, Griniėnė E, Gyraitė G, Dabuleviėiėnė T (2026) Bacteriological analysis of ballast water and port area: case study in the Baltic Sea port. *Management of Biological Invasions* 17(1): 51–64, <https://doi.org/10.3391/mbi.2026.17.1.03>

**Received:** 23 January 2024**Accepted:** 12 May 2025**Published:** 12 November 2025**Thematic editor:** Katherine Dafforn**Copyright:** © Srėbaliėnė et al.

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### Abstract

This study highlights the role of ballast water management in preventing the global spread of harmful organisms. The entry into force of the International Convention for the Control and Management of Ships' Ballast Water and Sediments has created new challenges for policy and environmental managers. To adapt the most effective prevention strategies and enhance biosafety in the context of ballast water management systems, there is an increasing need for rapid analytical tools to detect microbes and assess the efficacy of ballast water treatment systems. In this study, ballast water was sampled from vessels before and after treatment at the port of Klaipėda, situated on the Baltic Sea coast. Results showed high levels of coliforms and *Escherichia coli* in untreated ballast water, with reduced levels after treatment, although statistical differences were not significant. No toxigenic *Vibrio cholerae* was found, but prior studies confirm the presence of other pathogenic *Vibrio* spp. Findings highlight the importance of ballast water monitoring and the potential for rapid monitoring methods to enhance biosecurity.

**Key words:** human health, vessels, indicator microbes, pollution, biosecurity

### Introduction

Approximately 7,000 different marine species could be potentially transported in ballast water every day (Raaymakers and Hilliard 2002), ranging from fungi and bacteria to larger organisms, some of which may pose a serious threat to marine ecology or human health (Keller et al. 2011; Güney 2022; Katsanevakis et al. 2024). Long-term studies show that ballast water is the primary vector responsible for the transfer of aquatic microbes, including potentially pathogenic bacteria that can cause various diseases. The presence of such pathogens has potential negative impacts on the ecosystem and also on human health (Altug et al. 2012; Lymperopoulou and Dobbs et al. 2017; Dobaradaran et al. 2018).

Microbial contamination in coastal waters is commonly monitored using fecal indicator bacteria (FIB) such as *Escherichia coli* and Enterococci as a quality measure. Studies of FIB have shown that the ports are a hot spot for these pathogens (Field and Samadpour 2007; Lv et al. 2023). This highlights the importance of measuring microbial pollution in ballast water alongside

port areas in order to identify the potential sources of these pathogens (Luna et al. 2019; Gyraite et al. 2019).

Ballast water monitoring is complex, but critical to understand the diversity of organisms, as well as pathogens, that may be present in vessel tanks (Darling and Frederick 2018). Regulation D-2 of the Ballast Water Management Convention (BWMC) requires that after the ballast water treatment, the number of “live” organisms released should be limited. The International Maritime Organization (IMO) and the United States Coast Guard (USCG) have established regulations for the range of concentrations of organisms after ballast water treatment (IMO 2004; 2007). Accordingly, indicator microbes must meet the following standards: *Vibrio cholerae* < 1 CFU/100 mL; *E. coli* < 250 CFU/100 mL; Intestinal Enterococcus < 100 CFU/100 mL (IMO 2018). To reduce the number of viable organisms, various ballast water management systems (BWMS) have been developed. These systems typically include an initial filtration step followed by a chemical or physical treatment (Werschkun et al. 2014; Cha et al. 2015; David 2015; Batista et al. 2017; Petersen et al. 2019).

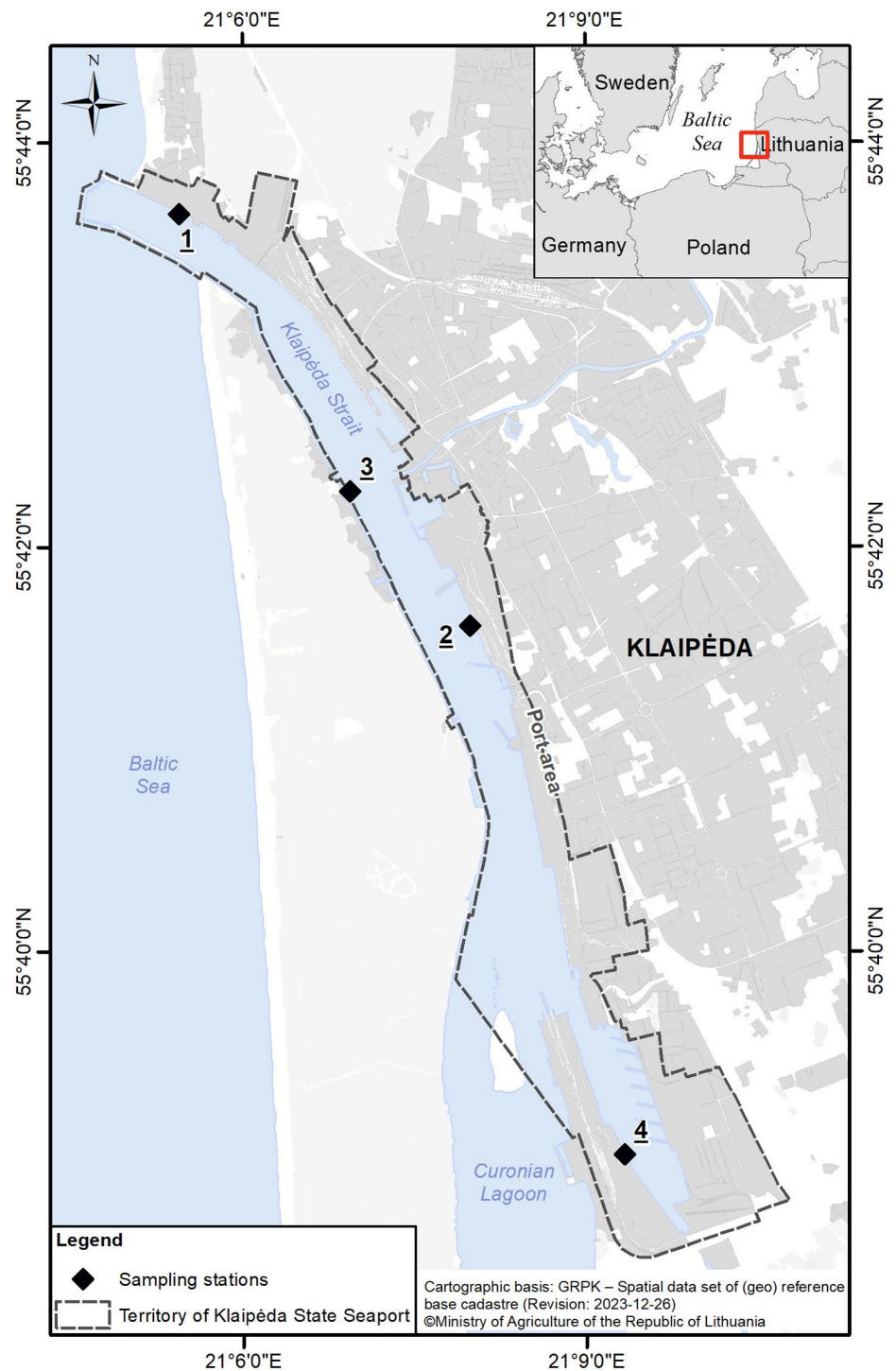
Some studies suggest using the IDEXX method not only for detecting fecal pollution in bathing waters, but also for ballast water testing and monitoring (Paolucci et al. 2015; Ng et al. 2018). The IDEXX method is a standardized, commercially available test, based on defined substrate technology that enables rapid detection and quantification of faecal indicator bacteria (FIB). It is often paired with the most probable number (MPN) statistical approach, which estimates bacterial concentration in a sample by interpreting growth responses across a series of dilutions (Gollasch et al. 2015). In contrast, the standard MEA-BEA method (Marine Enterococcus Agar and Bile Esculin Azide Agar) is a classical culture-based plating technique used to isolate and enumerate *Enterococcus* spp., but it is generally more time-consuming and labor-intensive (Peperzak and van Bleijswijk 2021).

This study aims to quantify microbial contamination levels in the vicinity of port and ballast water, highlighting the detection methods and concentrations of microbial species that might harm marine ecosystems and human health. The measurements of fecal indicator bacteria in ballast water and port waters were performed using the IDEXX (expressed in MPN), and the standard culture (MEA-BEA) method (expressed in CFU), and the total number of bacteria was measured using the flow cytometry method (FCM). Therefore, the objectives of this study were to: i) determine the quantities of microbes present in the port; ii) assess the quantities of microbes before and after ballast water treatment.

## Materials and methods

### *Study location*

Our study location – the Port of Klaipeda is located at the Klaipeda Strait, i.e., the northernmost part of the Curonian Lagoon, which is largest freshwater



**Figure 1.** Map of the study area and sampling sites of Klaipėda Port in the Baltic Sea.

basin in Lithuania and is influenced by the exchange of the freshwater from the Nemunas and other smaller rivers, as well as saline water from the Baltic Sea (Jakimavičius and Kriaučiūnienė 2013). The distance of the Klaipėda Strait is 12 km, and its width is 1.5 km, and a mouth area of 0.4 km (Figure 1) (Stakenienė et al. 2011).

Short-term inflow events driven by wind cause seawater to enter the northern part of the Curonian Lagoon, resulting in salinity fluctuations from

freshwater to seawater salinity ( $\sim 7\text{‰}$ ) (Zemlys et al. 2013). In turn, such high temporal and spatial dynamics in the water column of the Port of Klaipeda make it challenging to generalize the monitoring results.

### *Sampling activities*

During the period of July to September 2021–2022, samples from ballast water and port waters were collected in Klaipeda port (Figure 1, sampling site 1–4). Four sampling sites were chosen in the port area based on the shipping activity (Figure 1), at each site pH, temperature, salinity and dissolved oxygen (DO) were measured in situ using a YSI 460 multiple probe. Turbidity was measured in the laboratory with a portable turbidity meter, Eutech Instruments TN-100 (Landsmeer, The Netherlands), in Nephelometric Turbidity Units (NTU). The samples were collected in sterile 300 mL plastic bottles and kept in a cool box at a temperature below 10 °C. Within 12 hours of collection, the samples were examined for *E. coli* and Enterococci.

Ballast water samples were collected between June and March 2021–2022. The samples were taken from six vessels with and without ballast water treatment systems (BWTS). Four out of the six vessels were sampled before and after treatment. Ballast water samples were collected following the sampling approach outlined in the IMO sampling guidance, using both in-line (BW1, BW2, BW3, BW4) and in-tank (BW5 and BW6) sampling methods. In-line samples were collected through the ship's sampling points located in the engine room (IMO 2020). In-tank samples were taken directly from the ballast tank. Approximately 1 liter of ballast water sample was collected in sterile bottles from each sampling point and three subsamples were drawn from the sampling point to ensure consistency. Each sample was carefully labeled with key details such as time, location, and environmental conditions. Ship-specific information, including the ship type and the type of Ballast Water Management System (BWMS), was recorded (Supplementary material Table S1).

### *Indicator microbes' enumeration and analysis*

#### Total coliforms, Enterococci, and *E. coli* detection using Idexx (ID)

Port and ballast water samples were analysed using Enterolert-E for Enterococci and Colilert-18 for *E. coli*, obtained from IDEXX (IDEXX Laboratories, Westbrook, ME). During analysis, Enterolert-E and Colilert-18 culture medium were added to 100 mL of each sample. The sample-reagent mixture was poured into an IDEXX quanti-tray/ 2000 with 97 wells, sealed with the IDEXX tray sealer, and then placed into an incubator at 35 °C for 18 hours for *E. coli* enumeration, and at  $41 \pm 0.5$  °C for 24 h for Enterococci (Tsuchioka et al. 2019). Quanti-trays contain 49 large cells and 48 smaller cells, with a total volume of 100 mL.

Yellow fluorescing (positive) wells were counted under UV light, and the concentrations of Enterococci and *E. coli* samples were calculated using the IDEXX MPN table. Wells exhibiting yellow color and blue-white fluorescence under excitation with a 6-W, 365-nm ultraviolet light in a dark environment were considered positive for *E. coli*. The most probable number of microbe colonies was statistically determined from the positive counts in each cell (IDEXX 2006).

#### *E. coli*, Enterococci, and *V. cholerae* detection using standard culture (SC) methods

Based on the recommendation given by IMO Guidelines G2 (IMO 2007, IMO 2008), the culture method was used as the standard method. The analysis was performed by the Lithuanian National Public Health Center Laboratory under the Ministry of Health (LST EN ISO 2006).

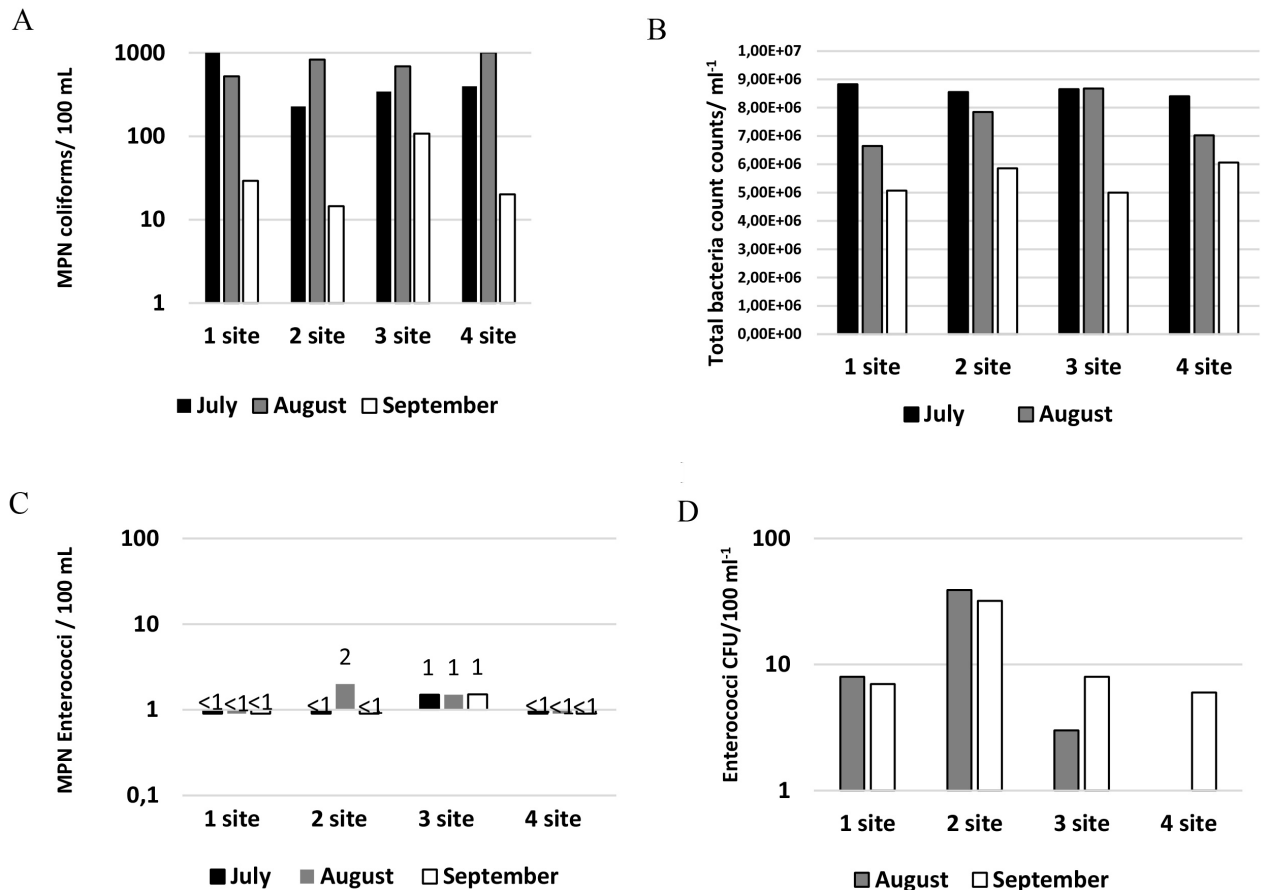
Accordingly, *E. coli* was analyzed following the national standard MT-SVP 5.4-03:2015. The detection of Enterococci was done according to ISO 7899 (LST EN ISO 7899-2:2001), while *V. cholerae* was analyzed using ISO 21872 (LST EN ISO 21872-1:2017). The concentration of total viable *V. cholerae* in ballast and port water samples was determined using a plate counting method with thiosulfate-citrate-bile salts-sucrose agar (TCBS, Sigma-Aldrich, USA). These analytical approaches were implemented exclusively from 2022 onwards, due to post-pandemic regulations that limited their application during the 2021 sample period.

#### Total bacteria detection using flow cytometry (FCM) method

The total bacteria were measured using the flow cytometry method, and the abundance of heterotrophic bacteria was determined from filtered samples through a 50 µm mesh size net and preserved in 0.25% glutaraldehyde and stored at -80 °C (Marie et al. 2005; Hoell et al. 2017). Determination of heterotrophic bacteria biomass were stained with SYBR Green I (Invitrogen) till final concentration of 1: 10 000 (Marie et al. 2005), diluted with Milli-Q water, and analyzed with a flow cytometer (BD Accuri™ C6, DB Biosciences) at a medium flow rate (35 µL min<sup>-1</sup>) for 2 min. (Zilius et al. 2021).

#### *Statistical analysis*

Descriptive statistics (mean, standard deviation, minimum, maximum) were used to assess the values of microbes and environmental parameters. The software used for analysis were Brodgar (v. 2.5) and R Development Core Team (2010). Brodgar (2.7.5.) and R (3.3.3) (Highland Statistics Ltd.) packages were used for redundancy analysis (RDA). The Wilcoxon Test was used to determine any significant relationships between indicator bacteria values in ballast water before and after treatment. The significance level for all analyses was set at  $p < 0.05$ .



**Figure 2.** Detected (A) total coliforms using ID, (B) total bacteria counts using FCM method, (E) *E. coli* using ID method; (F) *E. coli* using SC method (only in 2022), (C) *Enterococci* using ID method; (D) *Enterococci* using SC method (only in 2022) in Klaipeda port area.

## Results

### *Environmental parameters*

Physical chemical parameters such as temperature, salinity, pH, turbidity, and dissolved oxygen were measured at all sampling stations of the Klaipeda Port (Table S1). During the sampling activities, the temperature ranged from 12.8 °C to 24.7 °C, salinity ranged from 0.46 to 6.93‰, pH ranged from 8.38 to 9.06, dissolved oxygen ranged from 8.18 to 11.5 mg/L, and turbidity ranged from 2.8 to 15.1 NTU.

For the ballast water samples, based on availability, salinity, turbidity were measured. The ballast water sample salinity ranged from 0.22 to 36 PSU. Turbidity varied in a range of 0.63–76.84 NTU (Table S2).

### *Detection of indicator microbes in the port area*

The number of total bacteria, *E. coli*, *Vibrio* spp., Enterococci, and total coliform in the port water samples was measured using IDEXX (ID), standard culture method (SC), and flow cytometry method (FCM), and the numbers are shown in Figure 2 (Table S3).

*Escherichia coli* measured using the ID method ranged from < 1 to 8.7 MPN 100 ml<sup>-1</sup>, with an average of 2.1 MPN 100 ml<sup>-1</sup>, while using the SC method *E. coli* values ranged from 1 to 65.7 CFU 100 ml<sup>-1</sup>, with an average of 21.7 CFU 100 ml<sup>-1</sup>. The standard culture method was used only for samples from the year 2022.

For the port water samples, the Enterococci levels measured using the ID method ranged from < 1 to 7.5 MPN 100 ml<sup>-1</sup>, with an average of 2.3 MPN 100 ml<sup>-1</sup>. The SC method showed levels ranging from < 1 to 39 CFU 100 ml<sup>-1</sup>, with an average of 14.7 CFU 100 ml<sup>-1</sup>.

The levels of total coliforms, as detected by the ID method, ranged from 19.7 to 941.6 MPN 100 ml<sup>-1</sup>, with an average of 303.2 MPN 100 ml<sup>-1</sup>, and total bacteria counts using FCM ranged from  $4.9 \times 10^6$  to  $9 \times 10^6$  cells/ml<sup>-1</sup>, with an average of  $7 \times 10^6$  cells ml<sup>-1</sup>. Pathogenic bacteria *V. cholerae* was not detected during the sampling events in the port area.

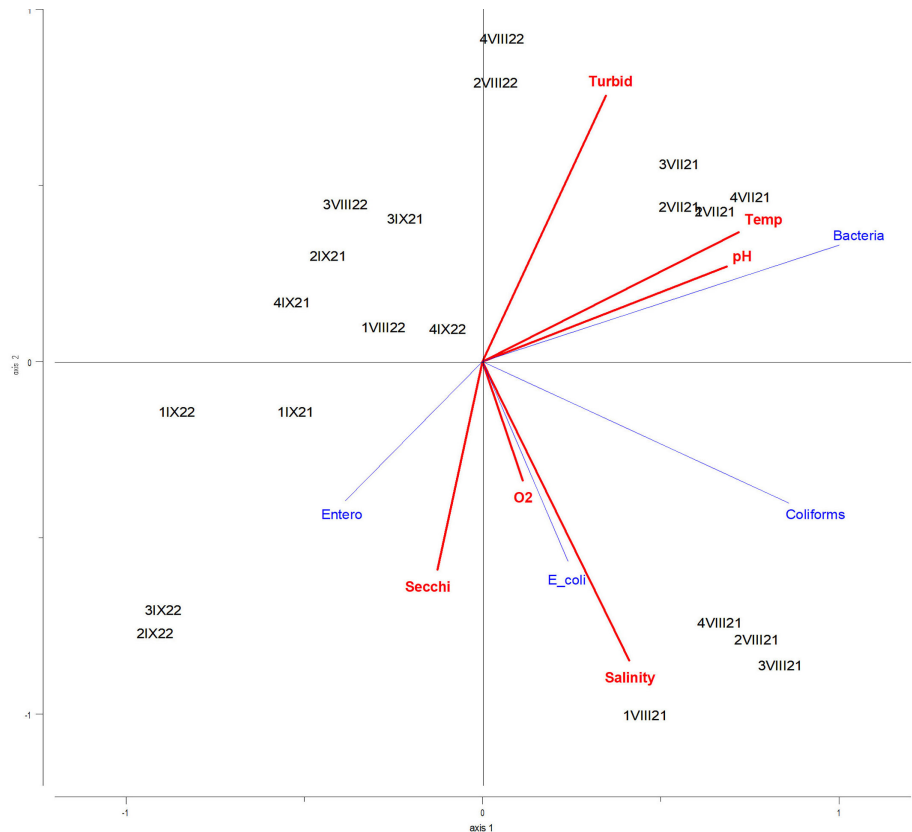
#### *Relationships between the environmental parameters and indicator microbes in port water*

Multivariate redundancy analysis (RDA) was used to identify relationships between environmental factors (explanatory variables: temperature, oxygen concentration, salinity, and turbidity) and concentrations of different bacteria (only for ID and FCM methods) as response variables, including total bacteria concentration, *E. coli*, Enterococci, and coliforms. The statistical procedure followed recommendations described by Zuur et al. (2007). According to the RDA, environmental parameters included in the analysis explain 43% of the variation of bacteria different group abundances (Figure 3). Permutation tests revealed that temperature ( $F = 8.27$ ,  $p = 0.001$ ), salinity ( $F = 8.75$ ,  $p = 0.001$ ), dissolved oxygen ( $F = 3.0$ ,  $p = 0.023$ ) and pH ( $F = 3.39$ ,  $p = 0.07$ ) were significant explanatory factors.

Strong positive correlation between the total abundance of bacteria and temperature and pH was observed during the summer of 2021 (July), while these variables were negatively correlated with *Enterococcus* numbers. *E. coli* numbers were positively correlated with salinity and dissolved oxygen concentration in August 2021 at all stations.

#### *Detection of indicator microbes in ballast water*

Detection of indicator microbes *E. coli*, *Vibrio* spp., Enterococci, and total coliforms in ballast water samples is shown in Figure 4 (Table S4). For the ballast water samples, *E. coli* levels measured using the ID method ranged from < 1 to 601.5 MPN 100 ml<sup>-1</sup>, with an average of 143.5 MPN 100 ml<sup>-1</sup>, using while the SC method *E. coli* values ranged from 6.3 to 2419.6 CFU 100 ml<sup>-1</sup>, with an average of 896.2 CFU 100 ml<sup>-1</sup>. All ballast water samples met the regulation D-2, with less than 1 CFU/100 mL for pathogenic bacteria *V. cholerae*.



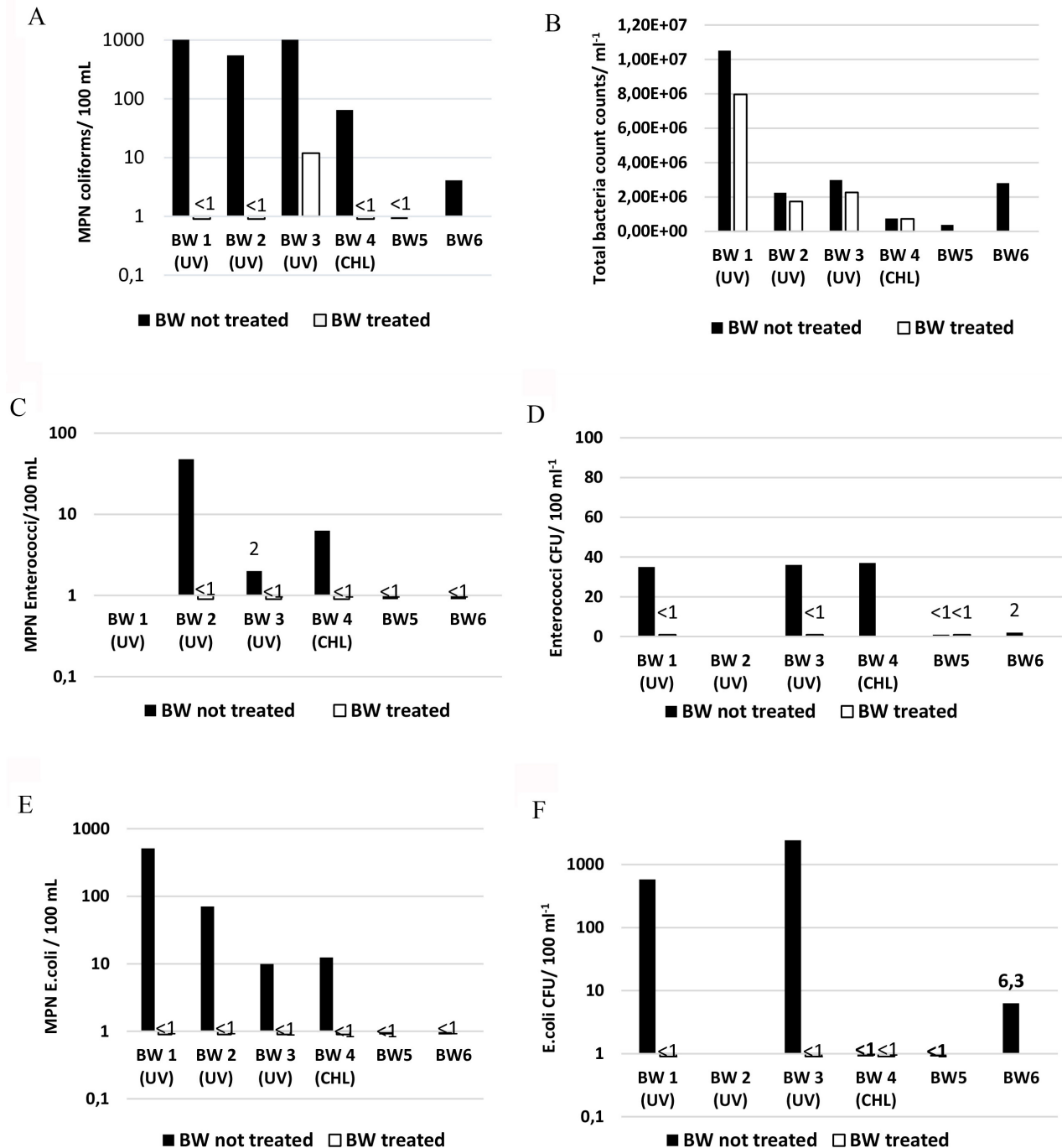
**Figure 3.** Redundancy analysis (RDA) triplot showing relationships between response variables (total bacteria abundance - Bacteria, *Enterococci* - Entero, *E. coli* - E\_coli ) and explanatory variables (Temp – temperature; O2 – dissolved oxygen concentration) and stations (1 – station; VIII – August; 21 – 2021 coding correspond 1VIII21; 2VIII21(2 – station; VIII – August; 21 – 2021); 3VIII21 (3 – station; VIII – August; 21 – 2021); 4VIII21 (4 – station; VIII – August; 21 – 2021); 1IX21 (1 – station; IX – September; 21 – 2021); 2IX21 (2 – station; IX – September; 21 – 2021); 3IX21 (3 – station; IX – September; 21 – 2021); 4IX21 (4 – station; IX – September; 21 – 2021)); and etc.

For the ballast water samples, Enterococci measured using the ID method ranged from  $< 1$  to  $49.6 \text{ MPN } 100 \text{ ml}^{-1}$ , with an average of  $17.9 \text{ MPN } 100 \text{ ml}^{-1}$ , the SC method ranged from  $< 1$  to  $37 \text{ CFU } 100 \text{ ml}^{-1}$ , with an average of  $29.0 \text{ CFU } 100 \text{ ml}^{-1}$ .

Total coliforms using the ID method ranged  $< 1$  to  $1011.2 \text{ MPN } 100 \text{ ml}^{-1}$ , with an average of  $448.3 \text{ MPN } 100 \text{ ml}^{-1}$ , and total bacteria counts using FCM method ranged from  $1.7 \times 10^6$  to  $10.6 \times 10^6 \text{ cells ml}^{-1}$ , with an average  $3.9 \times 10^6 \text{ cells ml}^{-1}$ .

### *Microbes quantities before and after ballast water treatment*

When considering the indicator microbe values in the ballast water before and after treatment, it was observed that the values of *E. coli* in two vessels (BW1 and BW3) before treatment exceeded the IMO limits. However, all ballast water samples (BW1–BW4) reached the limit of non-detection ( $< 1 \text{ MPN}/100 \text{ mL}$ ) after treatment. It was also observed that in vessels without ballast water treatment, the values of *E. coli* reached the limit of non-detection ( $< 1 \text{ MPN}/100\text{mL}$ ).



**Figure 4.** Detected (A) total coliforms using ID, (B) total bacteria counts using FCM method, (E) *E.coli* using ID method; (F) *E.coli* using SC method, (C) *Enterococci* using ID method; (D) - *Enterococci* using SC method vessel ballast water before and after treatment. BW – ballast water UV- ballast water system type using filtration and UV treatment, CHL - ballast water system type using filtration and chlorination treatment. For graph the average of subsamples are shown.

Furthermore, as shown in Table 1, the total bacterial counts in ballast water were significantly lower ( $p < 0.05$ ) after ballast water treatment. Although the number of *E. coli*, *Enterococcus*, and coliforms decreased sharply after the treatment, the differences in the detection rates between untreated and treated ballast water were not statistically significant (Table 1).

**Table 1.** Wilcoxon matched-pairs test for differences in counts of total bacteria (FCM), *E.coli* (ID), *Enterococcus* (ID), coliforms (ID) between ballast water samples before treatment and after treatment.

Pair of variables	n	T	Z	p-Value
Total bacteria counts before treatment and total bacteria after treatment	n = 15	16.00	2.061	0.039244*
Coliform counts before treatment and coliform after treatment	n = 15	25.00	1.096	0.272096
<i>Enterococcus</i> counts before treatment and <i>Enterococcus</i> counts after treatment	n = 15	21.00	1.066	0.286004
<i>E. coli</i> (ID method) counts before treatment and <i>E. coli</i> counts after	n = 15	28.00	0.862	0.388187

## Discussion

The intense shipping traffic and industrial activities that occur in ports and harbors make them typical hotspots for chemical and biological pollution and sites with potentially high microbial contamination (Kataržytė et al. 2018). To reduce the spread of hazardous aquatic organisms and pathogens (HAOPs) through ballast water, the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWMC) (IMO 2007) was enacted. Following ballast water treatment, the number of “live” organisms released from each category, including bacteria, zooplankton, and phytoplankton, should be limited in accordance with the D-2 standard. To enhance biosafety in ballast water management systems (Whittle et al. 2013; Endresen et al. 2004), it is necessary to conduct research on specific and reliable analytical instruments and procedures for control, and assure that monitoring methods are accurate to assess the efficacy of treatment methods. (Khandeparker and Anil 2017; Soleimani et al. 2016, 2017).

Most previous studies in the Baltic region have typically sampled ballast water (Dheenana et al. 2016), sediments (Wong et al. 1995), or fecal pollution in coastal and port areas on an infrequent basis. The key findings of bacteriological analysis in the Baltic Sea port are consistent with those of other studies that have demonstrated that the utilization of ballast water treatment systems immediately prior to the discharge of water into the port area has the potential to considerably reduce indicator microbes present in ballast water (Cohen et al. 2012; Salleh et al. 2021). Sampling ballast water is inherently complex due to factors such as the large volume of water, the shape and size of ballast tanks, and the heterogeneous distribution of organisms within these tanks. These variables can lead to difficulties in obtaining representative samples, which is crucial for accurate assessment of treatment efficacy.

This study focused on the initial efforts to monitor and quantify indicator microorganisms in ballast water and the port of Klaipėda. The measurements of microbial organisms in ballast and port waters were performed using both standard and novel methods, such as the IDEXX kit (IDEXX Laboratories, Inc.). This method is fast and the accuracy of the analysis is high. The port sampling data revealed the presence of fecal indicator bacteria at all research sites within the port.

In this study, ballast water sampled before treatment showed high values of total coliforms, *E. coli*, while quantities after treatment showed that BWMS was working effectively, and that the microorganisms targeted by the BWMC reached the regulatory threshold, however statistical analysis revealed that these changes were not significant for *E. coli*, *Eterococcus* and coliforms.

Although no toxigenic *V. cholerae* strain was recovered from the sampled ballast water in this study, Altug et al. (2012) show that other potentially pathogenic *Vibrio* spp. have been isolated from the ballast water (Ng et al. 2015, 2018).

## Conclusions

This study on microbial contamination in the Port of Klaipeda, Lithuania, shows that ballast water treatment systems effectively reduced indicator microorganisms to regulatory levels, as confirmed by bacteriological analysis of both ballast water and the port area. We acknowledge that the data provided for this study has been limited, which prevented us from directly comparing standard and novel methods for FIB quantification or ballast water treatment methods. We anticipate that our study will provide a basis for subsequent research in this area and will highlight the importance of monitoring to test the efficacy of ballast water management systems.

Additionally, the study revealed that monitoring methods that are rapid and quick have the potential to significantly increase the sampling throughout for environmental assessments.

Future research should focus on addressing existing knowledge gaps and exploring the application of new tools, such as environmental DNA (eDNA) and machine learning, to further enhance ballast water and port environmental management practices. These advancements could lead to more effective and sustainable solutions for managing ballast water risks and support the long-term protection of marine ecosystems.

## Author's contributions

GS: conceptualization, data curation, methodology, supervision, visualization, writing – original draft, writing – review and editing. EG: data curation, investigation, methodology, writing – review and editing. GG: methodology, writing – review and editing. TD: writing – review and editing.

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## Acknowledgements

The authors would like to thank Prof. Sergej Olenin for providing helpful insights to the study, and Greta Kilmonaitė for visual design of map. Also, the authors wish to express their sincere appreciation to the anonymous reviewers for their insightful comments and constructive feedback, which significantly enhanced the clarity and quality of the manuscript.

## Funding declaration

Greta Srėbaliėnė was supported by the European Social Fund, under project no 09.3.3- LMT-K-712, the “Development of Competences of Scientists, other Researchers and Students through Practical Research Activities” measure, grant agreement no. 09.3.3-LMT-K-712–19-0083. This research for GS was supported by the Klaipėda University Postdoctoral Fellowship Program. Publication of this study was supported by the European Union’s Horizon Europe HORIZON-CL6-2024-BIODIV-01 project “GuardIAS - Guarding European Waters from IAS”, under grant agreement no. 101181413 (Katsanevakis et al. 2024).

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## Supplementary material

The following supplementary material is available for this article:

**Table S1.** Port water measurements of temperature, salinity, pH, oxygen, turbidity, and microbial indicators.

**Table S2.** Ballast water measurements of salinity, pH, turbidity, detection of microbes using different methods.

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