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Microorganisms' communities from ballast water transferred into the Odra River estuary

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Abstract: Ballast water is a significant vector for the transport and introduction of aquatic organisms, microorganisms and chemical pollutants which impacts on ecosystems worldwide. In the study, ballast water from short- and long-range vessels with treatment systems was microbiologically evaluated in spring (April 20th) and summer (July 19th) and compared with waters of the main Police Seaport in the Odra River Estuary, southern Baltic Sea. All collected samples were evaluated for their physicochemical properties by standard methods. The number of individual groups of microorganisms was determined using the indirect culture method, according to the technique of inoculation of serial dilutions of samples of the examined waters. The results showed differences in the microorganisms in analysed samples. The phylogenetic analysis of bacteria recorded from the ballast water of short-range ships in spring showed the presence of six species of the genus *Bordetella*, while in summer four species of the genus *Clostridium*. In the ballast water of long-range ships, proteolytic bacteria pre-dominated in spring and halophilic bacteria in summer. In the summer period, eight species of bacteria were recorded, of which six belonged to the genus *Clostridium*. The ballast water treatment processes used on ships influence the composition of bacterial communities through selective recolonisation of water, which may transform bacterial functions as an important element of the marine food web. On the other hand, the presence of pathogenic bacterial species in the tested samples indicates improvement necessity of ballast water treatment systems used on vessels.

Keywords: ballast water management, microorganisms, ship ballast water, water environment, water transport

INTRODUCTION

Maritime transport is a vector for the transfer of aquatic organisms, microorganisms and an anthropogenic pollution factor, with a significant impact on ecosystems, both at local and global scale [Burtón *et al.* 2011; David, Gollasch 2018; Gollasch 2006]. Associated with it problem of alien species continues to grow, mainly due to the expansion of global trade, transport and tourism [Mooney 2001; Ojaveer *et al.* 2010]. The survival and range expansion of non-indigenous species is also facilitated by global warming [Huang *et al.* 2011]. Especially if the new environment is sufficiently similar to the native environment, the non-indigenous species can survive and reproduce. Without encountering natural enemies or other constraints, the species can become invasive: expanding its range and displacing native species by strong competition for food and habitat [Normant-Saremba *et al.* 2017; Ojaveer *et al.* 2016; Smith *et al.* 2018]. Notably vulnerable to such invasions are semi-closed and closed reservoirs with low biodiversity and high pressures resulting from the rapid globalisation of the maritime industry. One of such reservoirs is the Baltic Sea, which is particularly susceptible and sensitive to the introduction of alien species

[Gollasch, David 2019; Katsanevakis *et al.* 2014; Ojaveer *et al.* 2010].

The same situation applies to the Odra River, which in its estuary section is characterised by complex hydrological conditions. It is largely dependent on river tributaries, but also on the inflow of saline waters of the Baltic Sea (Szczecin's Lagoon) [Ligenza *et al.* (eds.) 2021]. The quality of the Odra's waters depends primarily on its upper and middle reaches, into which municipal and industrial wastewater is discharged. The well-oxygenated waters of the Odra are tested in terms of significant pollution indicators [Szczepański *et al.* 2010]. Salinity values (chlorides, sulphates, sodium, potassium, hardness, etc.), the presence of metals (iron, manganese, zinc, copper, nickel, cadmium, mercury, lead, etc.) and specific pollutants (detergents, phenols, etc.) of the Odra waters are in the 1st class of water quality. However, the concentration of nutrients and the sanitary condition of the Odra River are within the standards of purity class III [Miluch *et al.* 2017]. Such classification results are determined by the indicator related to eutrophication and frequent phytoplankton blooms. Also, the microbiological condition, including indicators *coli*, does not exceed the limits of II–III sanitary cleanliness class here. Unfortunately, above Szczecin, the waters of the Odra are strongly degraded. It is associated with a strong deterioration of oxygen conditions, organic load and excessive bacterial contamination. It is related to the lack of an efficient sewage disposal system of the estuary water of the Odra River as well as the heavy load on Odra Brook and the Szczecin Lagoon [Ligenza *et al.* (eds.) 2021; Miluch *et al.* 2017; Szczepański *et al.* 2010].

Considering ballast water as a significant vector for the transfer of non-indigenous organisms, a particularly worrying issue is unknowingly transported microorganisms [Seebens *et al.* 2013]. This is indirectly confirmed by provisions on the control of the microbiological status of ballast water presented by the International Maritime Organization (IMO). This phenomenon of uncontrolled movement of aquatic organisms and pathogens through ballast water, their adverse effect on native species and overall impact on marine ecosystems has been settled in the International Convention for the Control and Management of Ship Ballast Water and Sediments (BWM) which was adopted in February 2004.

Given the requirement to handle ballast water properly, effective legal and technical instruments are being sought and progressively introduced to address the risks arising from the movement of organisms and the microbiological status of ballast water. Despite this, the problem of transport of non-indigenous organisms via ballast water has still not been effectively addressed [Burtón *et al.* 2011; David, Gollasch 2018; Gollasch, David 2019]. According to the IMO's Guidelines and BWM approval of ballast water management systems the microbiological research of ship ballast waters is limited to the determination of the presence of indicator microorganisms (*Escherichia coli* and enterococci) and pathogenic bacteria such as *Vibrio cholerae*. Those harmful or indicator microorganisms' serotypes should not exceed 100, 250 and 1 colony formatting unit (cfu) per 100 cm^3, respectively [International convention … 2004].

There are some of works which include transmission this kind of microbes through the ballast water. The modern standard for assessing microbial diversity is high throughput sequencing, an approach based in molecular biology and one that does not involve cultivation, but instead employs DNA sequencing to provide a much more comprehensive view of microbial assemblages [Lymperopoulou, Dobbs 2017]. For instance, Soleimani *et al.* [2021] shows a high bacterial diversity. Authors pointed out the presence of *E. coli* and *Vibrio cholerae* respectively in 19 and 14 simples from all 34 ballast water simples. The positive samples were form ports in Manila – Philippines, Duqm – Oman and Kuwait – Kuwait. In the study, the samples were subjected to the polymerase chain reaction (PCR) technique, the aim of which was to demonstrate the presence of *E. coli*'s Mdh gene and the *Vibrio cholerae*'s OmpW gene [Soleimani *et al.* 2021]. The authors also noted that the PCR technique is high specificity and sensitivity rapid procedure. It allows the identification and immediate detection of specific pathogenic genus of bacteria from different samples which can be used directly. Obtained by them results confirmed that PCR assays could become a faster and more accurate alternative to the conventional culture methods. It can be also more practical in the performance of BWM assessments and strategies for the world's seas and oceans [Soleimani *et al.* 2021]. As well, Ng *et al.* [2015] confirmed the indicator organisms of *Enterococcus*, *E. coli* and *Pseudomonas aeruginosa* in the tested ballast water samples. Another group of researchers managed by Burkholder [2007] also reported that in 48% of surveyed ballast water samples were positive for presence of pathogenic *Vibrio cholerae*, *E. coli*, *P. aeruginosa*, phytoplanktons, *L. monocytogenes*, and *Mycobacterium spp*. Similarly, the study provided by Altug *et al.* [2012] reported the presence of another indicator microorganisms (*Salmonella spp.*) in the samples from the ships form different regions.

Moreover, relatively growing number of literatures also focused on new information on the variety of microorganisms carried in ballast water. Some research studies pointed out that seemingly non-invasive microbial populations can become disproportionately abundant in a new location and begin to threaten the proper functioning of the ecosystem structure in ballast tanks and water bodies [Burtón *et al.* 2011; Hess-Erga *et al.* 2019; Rojas-Tirado *et al.* 2019; Seebens *et al.* 2013]. In addition, the products of ballast water treatment can take the form of increased amounts of dissolved organic matter with high levels of metabolites resulting from the degradation of bacterial cells [Rojas-Tirado *et al.* 2019]. This may lead to enhanced bacterial production associated with the increased availability of perishable organic compounds in treated ballast water and then reduce microbial competition due to the rapid proliferation of opportunistic bacterial species resulting from the elevated concentrations of certain nutrient substrates [Shah *et al.* 2015].

The by-products of disinfection may exert chronic or acute toxic effects on a variety of organisms living in the aquatic environment into which they eventually enter [Burtón *et al.* 2011; Delacroix *et al.* 2013]. Thus, we may be dealing here with the phenomenon of the introduction of apparently non-invasive microorganisms into new environments, which, under favourable conditions for their development, may become an invasive microbiota. The unpredictable interactions of such populations through activities that disrupt local decomposition patterns or naturally formed interspecies interactions in the new environment can pose high risks [Hess-Erga *et al.* 2019; Seebens *et al.* 2013].

Moreover, ballast water can also be a source of chemical contamination [Soleimani *et al.* 2017a]. Especially that there have been no reports on the concentration of ions and metals in the ballast waters around the world. Some scientists got interested and, slowly, disturbing reports of a high concentration of ions and metals such as Pb, Cd, Hg, Cr in ballast water began to appear. Soleimani *et al.* [2017b] decided to focus on the concentration levels of the fluoride (F) and chloride (Cl) ions and its ratio in the ballast water. The presented results showed that high content of F in the examined ballast waters from different ports around the world and there any correlation between F and Cl was found. However, due to high content of F in the examined by ballast waters from different ports around the world, Soleimani *et al.* [2017b] highly suggest more studies on the fluoride level of the sea food harvested form different parts of the sea around the worldwide ports.

Furthermore, the results of the available studies show that the ballast water discharged by tankers in ports can be one of the main sources of metal contamination in discharge areas and in the marine environment and can change the chemical composition in the long term. Mainly that heavy metals discharged and potentially accumulated in the marine ecosystems can affect negatively aquatic organisms and humans [Dobaradaran *et al.* 2018]. It is very important to remember that some of heavy metals even at low concentration level can be very toxic. That's why their existence and concentration in marine environment generate collective health risk harmful not only for humans but also fishes. The pollution of heavy metals in marine organisms has become a significant global concern. That only shows how much important is to report the concentration level of heavy metals in ballast waters [Dobaradaran *et al.* 2018]. In that case the enforcement of international restrictions on ballast water treatment and further research in this direction are necessary to protect the health of aquatic species and the sanitary conditions of port water receivers [Hess-Erga *et al.* 2019; Normant-Saremba *et al.* 2017; Soleimani *et al.* 2017b].

This study aimed to review and determine the seasonal microbiological composition of ballast water from a few of long- and short-range ships with treatment systems in place, entering the southern Baltic Sea.

MATERIALS AND METHODS

SAMPLING

Ballast water samples were obtained from four vessels (two short-distance routes containerships with size 89.95 m and 89.99 m; two long-distance routes bulk carriers with size 189.98 m and 199.90 m) berthing in town Police Seaport of the southern Baltic Sea in the Republic of Poland (Odra River estuary of the southern Baltic Sea, 53°33'44.8" N 14°35'15.2" E). Samples were collected by trained sailor who took them around 15 min after the ballast tanks were reopened when the vessel was stationed in the dock. To keep all samples representative, they were prepared in accordance with harmonised international IMO Guidelines for Ballast Water Sampling [IMO 2013]. The sampling process consisted of pumping the water through the ballast water pump until it lost suction. Sample of port water was collected approximately 10–20 cm below the water surface. All samples were collected on two dates: April 20th for spring samples and July 19th for summer samples in 2019. Due to the fact that the samples were taken directly from the ballast tanks, they constituted a heterogeneous mixture of water and sediment. Shortly after the samples were taken, they were transported by car straight to the laboratory and tested. Before starting separation and analysis of the samples, each of them was thoroughly mixed so as to form a representative, homogeneous mixture. The sample volume was divided equally for each performed analysis. The vessels from which the samples were taken have installed ballast water treatment systems and were divided into short-range (European routes) and long-range vessels (transatlantic routes). One single sample of water taken from the Police Seaport was used as a reference for local waters. All vessels from which samples were collected had ballast water treatment systems in place. All samples were labeled according to the vessel's course, taken into 20l containers and secured to avoid contamination.

PHYSICOCHEMICAL AND MICROBIOLOGICAL ANALYSIS

All collected samples were evaluated for their physicochemical properties, namely pH (determined by using pH meter), salinity determined by using conductivity meter – in practical salinity units (PSU) – based on the Venice system for the classification of marine waters, electrochemical potential (mV) determined by using conductivity meter, electrolytic conductivity (*EC*) determined by using conductivity meter, total hardness (°N) by titration method with the addition of ammonium buffer with disodium edetate solution (complexometric titration) of known concentration against Eriochrome black based on PN-ISO 6059:1999, presence of biogenic compounds according to norms PN-ISO 7150-1:2002, PN-EN ISO 10304-1:2009, and PN-EN ISO 6878:2006.

The number of individual groups of microorganisms was determined using the indirect culture method, according to the technique of inoculation of serial dilutions of samples of the examined waters. This is a standard method of determining the number of colony-forming units (cfu) using inoculation on an agar plate (PN-EN ISO 6222). The groups of microorganisms were cultured following the aforementioned standard on substrates modified for specific requirements of the target groups of microorganisms [Altug *et al.* 2012; Bianchi *et al.* 1992; Feng *et al.* 2011; Tomaru *et al.* 2014]. Enriched agar, BioMaxima S.A., was used to culture heterotrophic bacteria (composition, in g·dm^{-3}: meat extract – 3; enzymatic animal tissue hydrolysate – 5.0, NaCl – 5.0, agar – 15). To isolate the group of halophilic bacteria, the agar was additionally enriched with 10% sodium chloride. The fungi were incubated on rose bengal, BioMaxima S.A. agar (composition, in g·dm^{-3}: agar – 15, glucose – 10, peptone – 5, KH_2PO_4 – 1, $MgSO_4 \cdot 7H_2O$ – 0.5, rose bengal – 0.035, streptomycin – 0.03). Bacteria of the genus *Pseudomonas* were incubated in BD *Pseudomonas* Agar P medium (composition, in g·dm^{-3}: peptone – 20, MgC_{l2} – 1.4, K_2SO_4 – 10, glycerol – 10, agar – 15). To detect the presence and establish the counts of amylolytically active bacteria a Waksman medium with composition, in g·dm^{-3} of: K_2HPO_4 – 0.5, KH_2PO_4 – 0.5, $MgSO_4$ – 0.2, $(NH_4)_2SO_4$ – 0.2, starch – 10, agar – 15 was used [Burbianka *et al.* 1983]. The substrate for lipolytic microorganisms contained (in g·dm^{-3}) peptone – 5, yeast extract – 3, tributryn – 10, agar – 15 and distilled water – 1 g·dm^{-3} (substrate with triworksite)

[Burbianka *et al.* 1983]. The substrate for proteolytic microorganisms contained 50 g·dm^{-3}, powdered milk, 20 g·dm^{-3} agar and distilled water 1 dm^3 (substrate of Frazier and Rupp) [Burbianka *et al.* 1983]. Microbial incubation was carried out under conditions suitable for the specific groups of microorganisms [Błaszczyk 2010; Burbianka *et al.* 1983]. Growing colonies were counted using a LKB 2002 colony counter.

STATISTICAL METHODS

To determine whether the distribution of the results obtained was normal and characterised by a constant variance, a uniformity of variance test and Leven test were used. Analysis of variance was carried out and a Tukey test was used as a post-hoc test [Sokal, Rohlf 2012]. All statistical analyses were carried out using Statistica v13.3 software.

PHYLOGENETIC RELATIONSHIPS

Following macroscopic analysis, dominant colonies were selected and DNA was isolated according to Genomic Mini AX protocol (A&A Biotechnology) using lyticase, lysozyme and mutanolysis (Sigma – Aldrich). For amplification of extracted DNA, universal 338G starters were used: 5'-CGC CCG GGG CGC GGG CGC GCC GGC GGC GGG GCG GCG GCG GCG CGG GGG GCG GCG CGG GCG GCG GCC GCT GG-3' and RP534: 5′-ATT ACC GCG GCT GCT GG-3′ [Mrazek *et al.* 2008]. The polymerase chain reaction (PCR) reaction was carried out in a final volume of 0.025 cm^3 containing 0.001 cm^3 $MgCl_2$, 0.0025 cm^3 dNTPs, 0.002 cm^3 of each starter, 1U Taq polymerase DNA (Eppendorf), 0.0025 cm^3 polymerase buffer and 0.003 cm^3 DNA matrix. The PCR reaction was carried out in an thermocycler (Mastercycler gradient Eppendorf) with the following thermal profile conditions: initial denaturation for 180 s at 94°C followed by 35 cycles of 60 s at 94°C, 30 s at 55°C, 60 s at 72°C and a final elongation step of 10 min at 72°C [Muyzer *et al.* 1993]. PCR products were electrophoretically separated according to generally accepted principles of the Denaturing Gradient Gel Electrophoresis (DGGE) method in 8% polyacrylamide gel stained with ethidium bromide [Hesham *et. al.* 2011] and visualised using a GelDoc instrument (Bio-Rad). PCR and its products of the dominant strains were obtained in a reaction carried out in a 0.05 cm^3 solution of 0.005 cm^3 DNA, 0.025 cm^3 PCR Master Mix Plus High GC, 2·0.002 cm^3 starters: B-all For (GAG TTT GAT CCT GGC TCA G) and B-all Rev (ACG GCT ACC TTA CGA CTT). The obtained sequencing DNA profiles (Macrogen) were subjected to phylogenetic analysis by using Genebank BLAST. UPGMA method was used to analyse the similarity of the obtained amplification profiles.

RESULTS AND DISCUSSION

The results of physiochemical analyses of the ballast waters showed differences between short-range and long-range vessels (Tab. 1). The pH of all tested water samples was within the neutral range (pH = 7.16–7.32). Partially analogous pH level of ballast water samples was obtained by Soleimani *et al.* [2017a], which oscillated in range 7.30–8.44. The salinity in the ballast water of the short-range vessels ranged from 1.90 to 4.67 PSU, while for long-range vessels salinity ranged from 29.20 to 32.60 PSU, depending on the season. The higher salinity of the samples, the lower the oxidoreductive potential of the ballast water. Moreover, the increase in electrolytic conductivity was accompanied by increased total hardness. The highest results of these parameters (*EC* = 45.60 mS·cm^{-1} and total hardness of 271.10 °N) were obtained for ballast water from long-range vessels taken in spring. On the other hand, the lowest values of these parameters were characteristic for the ballast water samples from the short-range vessels. The ballast water samples from the short-range vessels were characterised by an oxygen content above 2 mg·dm^{-3} and significantly less for the long-range vessels. Among the biogenic compounds, the highest concentrations in the ballast water of all vessels were found for NO_3 in spring and slightly lower for NH_4 for short-range (summer) and long-range (spring) vessels (Tab. 1).

Table 1. Physicochemical properties of the port water and ballast water samples.

Property		Sample				
		P	S1	S2	L1	L2
pH		7.19	7.16	7.32	7.23	7.32
PSU		0.45	1.90	4.67	32.60	29.20
Venice system for the classification of water salinity		limnetic	oligoha-line	oligoha-line	euha-line	polyha-line
Oxidoreductive potential (mV)		10.50	12.10	3.60	8.80	4.10
Electrolytic conductivity (mS·cm^{-1})		3.08	1.77	2.42	45.60	29.80
Total hardness (°N)		21.50	13.70	17.90	271.10	167.10
Dissolved oxygen (ppm)		0.43	6.03	2.21	0.00	0.15
Levels of biogenic elements (mg·dm^{-3})	NO_2	<0.005	<0.005	<0.005	0.006	<0.005
	NO_3	0.25	0.78	0.36	0.60	<0.08
	NH_4	5.22	<0.005	2.75	1.41	0.98
	PO_4	0.53	0.13	0.29	0.01	0.02

Explanations: PSU = practical salinity of tested water samples, P = port waters, S = short-range vessels: S1 = spring, S2 = summer, L = long-range vessels: L1 = spring, L2 = summer.

Microbiological analysis showed a large variety of microorganism counts depending on the sampling season and vessel range (Fig. 1). The greatest differences were recorded in yeast cell count between short- and long-range vessels. Yeast cells were statistically significantly more abundant in short-range vessels compared to the long-range vessels (Tukey's test $p > 0.05$, Fig. 2a). A similar yeast count was recorded between Police Seaport waters and long-range vessels' ballast waters taken in spring (P: 11·10^{-3} cfu; L1: 16·10^{-3} cfu, respectively).

The total abundance of lipolytic bacteria in the ballast water also varied between sampling season and vessel range. The lowest levels of lipolytic bacteria were observed in spring at 25·10^{-2} cfu

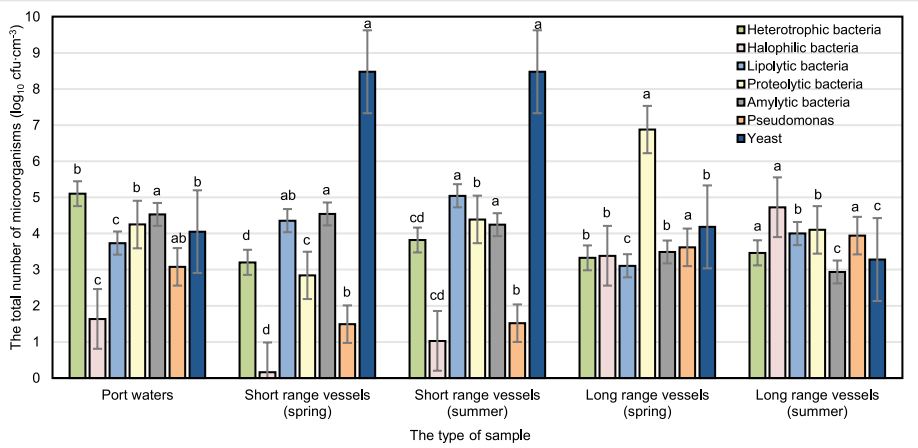


Fig. 1. The counts of the studied groups of microorganisms from port waters (Odra River water) and spring and summer samples of ballast water from ships operating in European seas and ocean waters; a, ab, b, bc, c, cd, d = statistical significance of individual groups of microorganisms between samples; source: own study

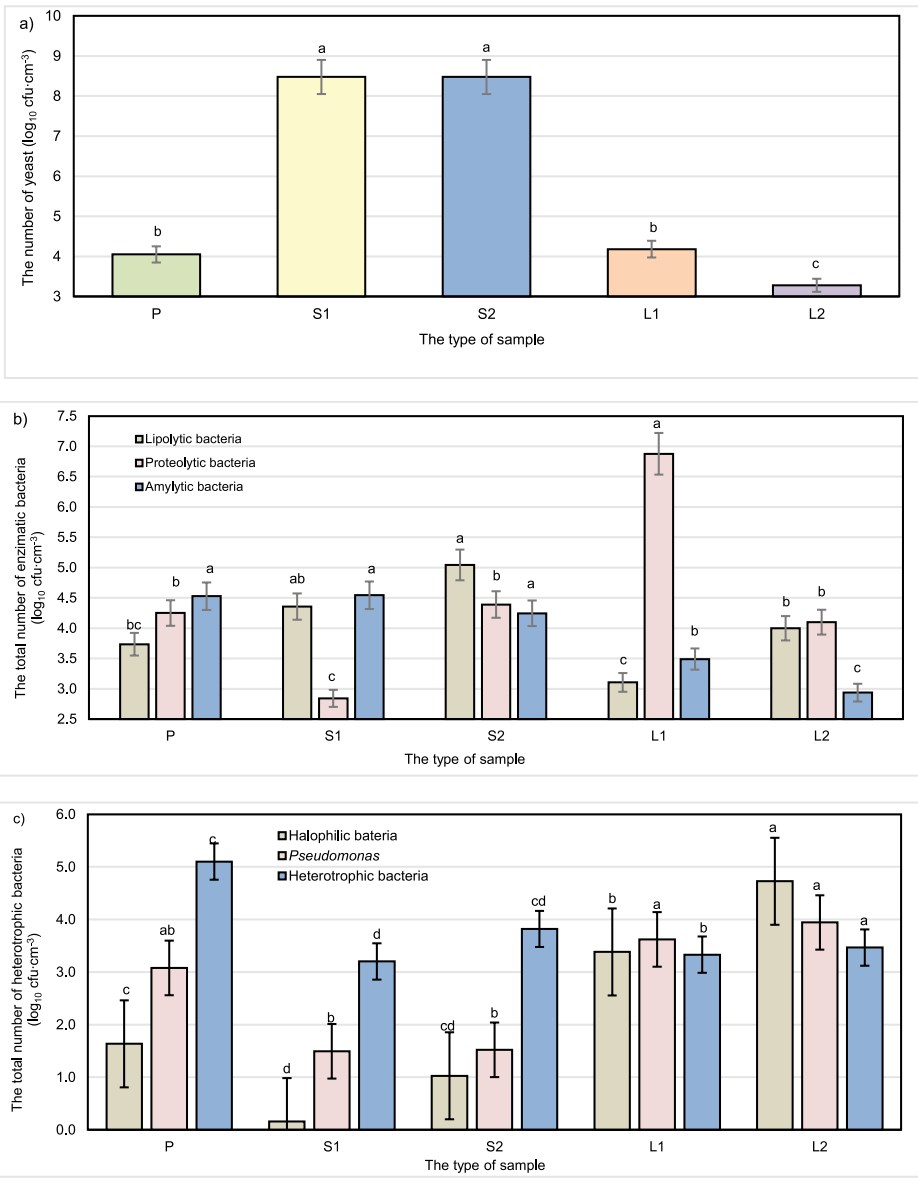
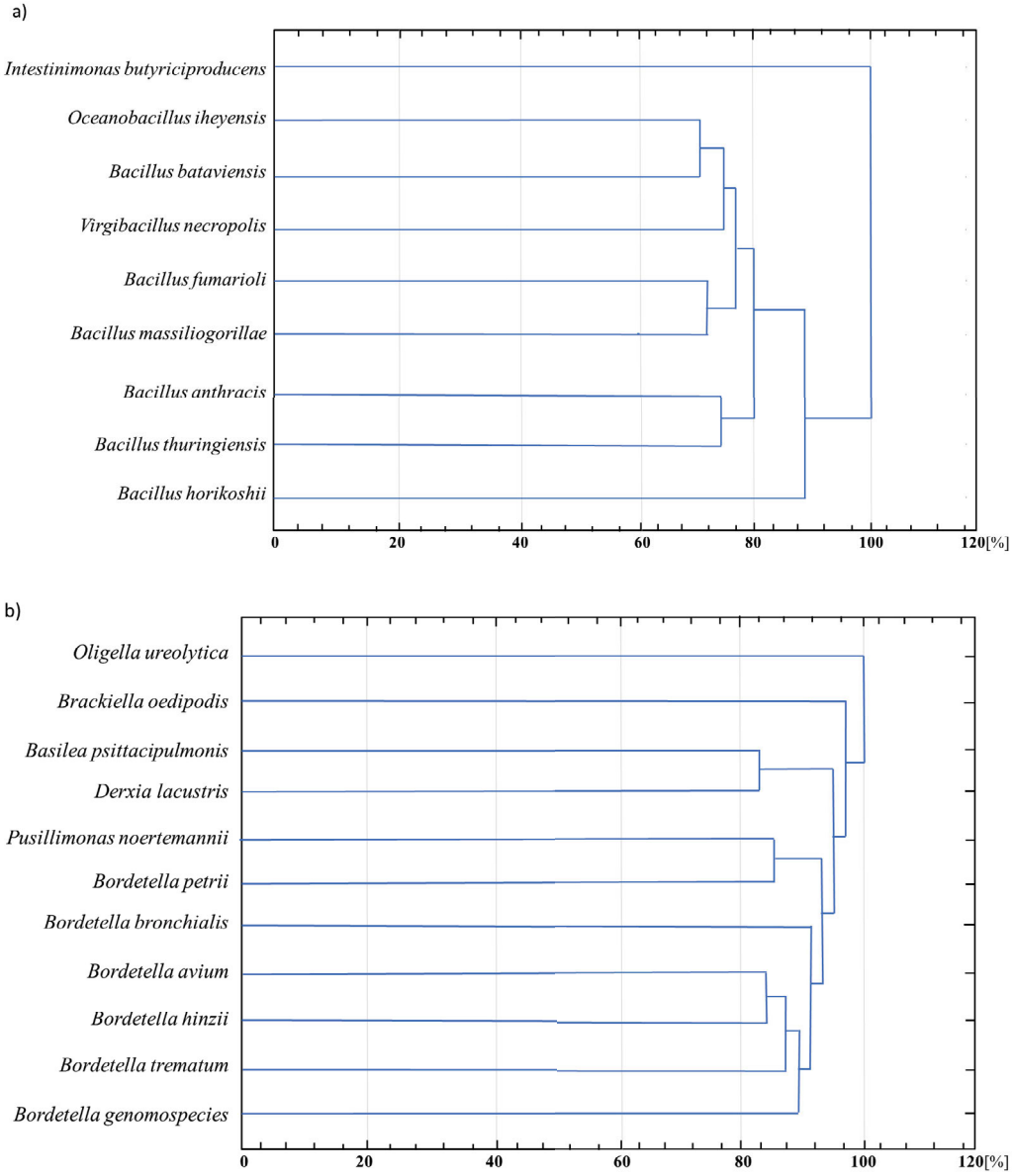


Fig. 2. Total counts recorded of microorganisms in port waters and balast water samples from ships operating in European and ocean waters: a) yeasts, b) enzymatic bacteria (lipolytic, proteolytic, amolytic), c) halophilic bateria, *Pseudomonas*, heterotrophic bacteria; a, b, c, d, ab, bc, cd = statistical significance of individual groups of microorganisms between samples; source: own study

in the short-range vessels and $16{\cdot}10^{-2}$ cfu in the long-range vessels, while in the summer season the short-range vessels contained $16{\cdot}10^{-4}$ cfu and the long-range vessels contained $1{\cdot}10^{-4}$ cfu. The opposite trend was recorded for amylolytic bacteria. Their levels in the short-range vessel ballast water in spring and summer seasons were $36{\cdot}10^{-3}$ cfu and $18{\cdot}10^{-3}$ cfu, and in the long-range vessel ballast water, $32{\cdot}10^{-2}$ cfu and $9{\cdot}10^{-2}$ cfu respectively (Fig. 2b). The total abundance of halophilic bacteria in the particular seasons in the ballast waters of the short- and long-distance vessels is shown in Figure 2c. Due to the higher salinity of the ballast waters of the long-distance vessels, the abundance of halophilic bacteria in both the spring and summer seasons (Tukey's test $p > 0.05$) was statistically significantly higher compared to the Police Seaport and the ballast waters of the short-distance ships. Interestingly, both *Pseudomonas* and heterotrophic bacteria highest numbers were recorded in the waters from Police Seaport (Fig. 2c). Additionally, these two groups of bacteria showed higher numbers in the summer period than in spring samples, both in short- and long-distance ships.

Each of the samples was characterised by a large variety of microorganisms with a predominance of different types of bacteria. The reference sample from the Police Seaport was characterised by a microbiota in which bacteria of the *Bacillus* genus were predominant, with genetic similarity ranging between 74 and 76% (Fig. 3a). The sample from a short-range ship in the spring (S1) was a microbiological group with 84–89% genetic similarity of bacteria from the genus *Bordetella* (Fig. 3b). The greatest richness of species diversity of the microbiota was found in the spring ballast water samples from a long-range vessel. In this numerous groups of microorganisms, the *Acholeplasma* genus with a similarity of 75–77% prevailed (Fig. 3c). On the other hand, both samples of summer waters were predominantly inhabited by *Clostridium* bacteria within a genetic similarity range of 78–84% for the S2 sample from a short-range ship (Fig. 3d), and 80–90% for the L2 sample from a long-range ship (Fig. 3e).

Comparing the data obtained in our study, a variable correlation was noticed between the microbiological profile and the sampling date (spring–summer). Each of the samples was characterised by a large variety of microorganisms with



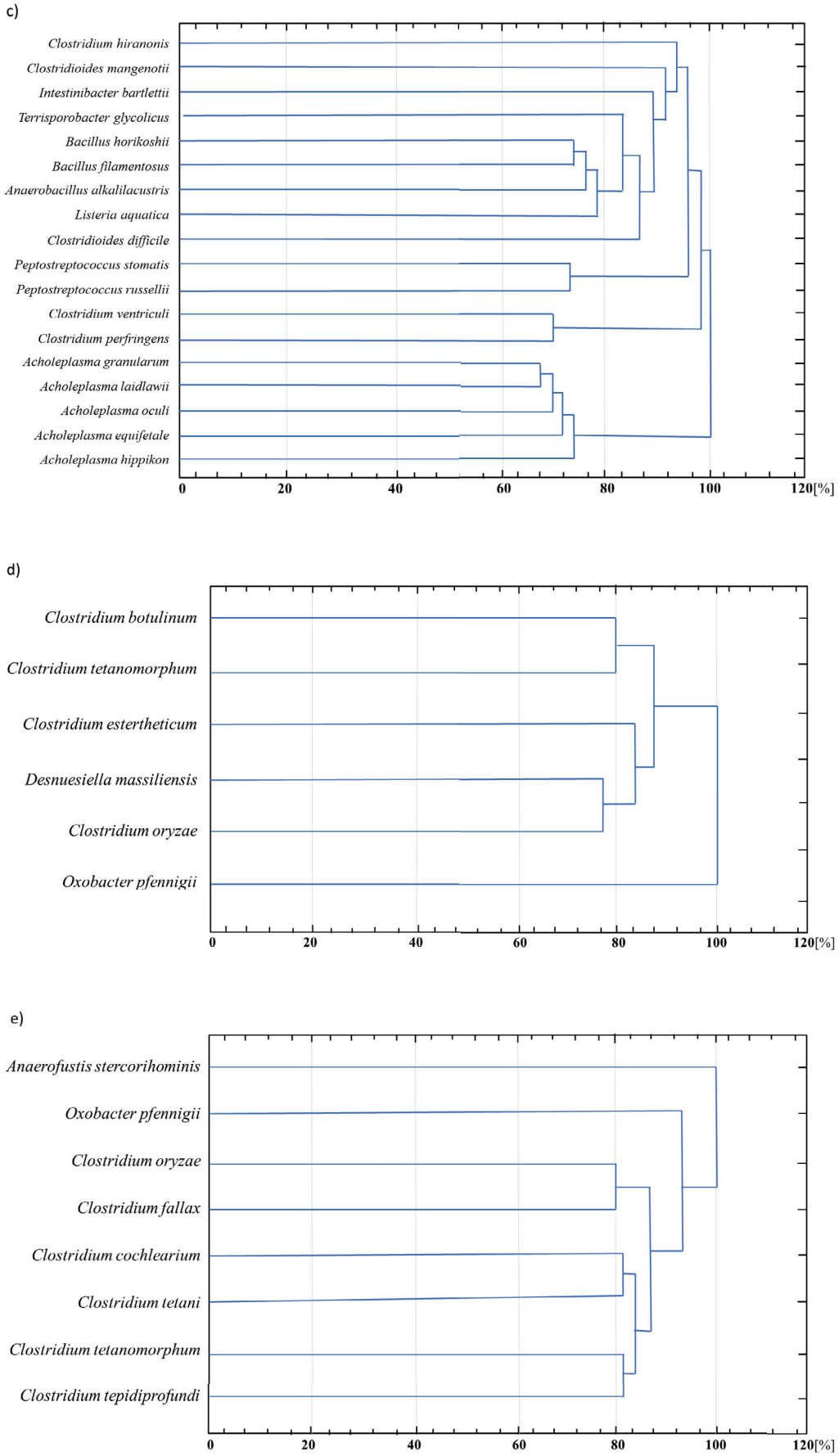


Fig. 3. Cluster analysis (UPGMA) of the relations between organisms isolated from different samples: a) P, b) S1, c) L1, d) S2, e) L2; source: own study

a predominance of the genus *Bordetella* (S1) and *Acholeplasma* (L1) in springtime and *Clostridium* bacteria in the summer (S2 and L2). Our results also indicate the presence of some pathogenic microorganisms in the ballast water of ships operating both in European and transoceanic waters. For example, we detected representatives of the genus *Bacillus*, which belongs to the phylum *Firmicutes*. This genus is quite common in natural environments and a very wide group with the ability to form endospores with extraordinary resistance to adverse environmental conditions, including disinfectants [Logan, de Vos 2015]. Among the representatives of this genus in ballast water, we recorded the presence of *Bacillus anthracis*, a known etiological agent of anthrax – an infectious disease harmless to aquatic organisms, but harmful among humans and livestock [Koehler 2009]. We also found in our samples another human pathogenic Gram-negative bacteria from the genus *Bordetella*. The species *Bordetella avium*, *B. hinzii* and *B. trematum* found in our genetic analysis of ship ballast water can cause respiratory diseases in poultry and mammals, including humans [Shah *et al.* 2013]. Another significant but also naturally common in environment group found in our ballast water samples were Gram-positive bacteria of the genus *Clostridium*, which presence was also demonstrated in ballast waters from Chinese and Japanese seas [Buzoleva *et al.* 2012]. This genus includes a form of an important indicator of the sanitary status of natural waters (presence of *Clostridium perfringens* indicates long-established faecal water pollution) as some *Clostridium* species are known as pathogenic microorganisms. This genus includes several human pathogens, which, among others, were identified in the tested samples taken in summer from the ballast water of the long- and short-range ships. Three of the species present in the samples – *Clostridium botulinum, C. perfringens* and *C. difficile* – are food spoilage associated bacteria, and infection would lead to gastrointestinal diseases in both humans and animals [Di Bella *et al.* 2016; Kiu, Hall 2018]. The species *Clostridium tetani*, on the other hand, is an indicator of tetanus. Also, compounds produced by these pathogenic microorganisms not only pose a direct threat to health but may antagonistically affect the formation of environmental microbiota in new areas [Poulain, Popoff 2019].

The results presented in this paper show that despite the use of IMO-approved ballast water treatment processes, ballast waters are quite numerously inhabited by different types of microorganisms. Although the total count of heterotrophic bacteria and *Pseudomonas* was quite low in all samples (S1, S2, L1, L2) compared to the sample from Police Seaport waters. The presence of microorganisms with the ability to break down fat, protein and sugar molecules was already much higher, and strongly varied depending on the vessel range. This type of bacteria was also demonstrated by Burkholder *et al.* [2007] who analysed ballast water from nine ports on the U.S. West Coast and four ports on the U.S. East Coast. The *Pseudomonas* microbiota in ballast water has also been recorded in 34 ports worldwide [Soleimani *et al.* 2017a] and in a study in Japan and China [Buzoleva *et al.* 2012].

Comparing obtained results with other scientific studies on microbial aggregations in ballast water, we can meet not only pathogenic microbiota but also those with unknown potential [Hess-Erga *et al.* 2019]. Takahashi *et al.* [2008] mentioned studies on marine bioinvasions in the transfer of nonindigenous microorganisms but also largely focus on pathogenic bacteria such as *Vibrio cholerae*, *Clostridium perfringens* and indicator microorganisms such as coliforms and enterococci.

In study of Lymperopoulou and Doobs [2017] concentrations of faecal-indicator (*E. coli*, enterococci) and pathogenic (*Vibrio cholerae*) bacteria, results indicated few such organisms among 17 ships. Whether through DNA sequence analysis or qPCR amplification of extracted DNA, these organisms were found, if at all, in miniscule numbers. Of other potentially pathogenic *Vibrio* species tested for with qPCR, only *V. parahaemolyticus* was detected in several ballast tanks. In a regression approach, salinity explained about one-quarter of species richness; neither ballast water age nor temperature on collection were significant in this regard. Ng *et al.* [2015], in an extension of the study of bacteriological assessment of ballast waters, mention that apart from the detection of *Vibrio spp.* and *Salmonella spp.* the microbial diversity of ballast and harbour waters is also characterised by the presence of groups such as *Proteobacteria, Cyanobacteria, Bacteroidetes*, *Planctomyces*, *Chloroflexi*, *Actinobacteria* and *Aquificae*. The author also indicates the significance of the resistance of potentially pathogenic microorganisms and suggests the use of different molecular assays and conventional microbiological methods to provide detailed microbial measurements to determine the threat to microbiological safety in ballast waters [Ng *et al.* 2015].

Dobbs [2008] notes that although microorganisms are ubiquitous and it is difficult to define which of them are non-indigenous, some do have strong biogeographic affiliations. In addition, the author also notes that an organism transferred in the ballast water of ships does not necessarily become an invasive species and does not have to settle in a new environment and interact with native species. This is a very significant aspect as sea and ocean waters are, in contrast to freshwater and terrestrial ecosystems, more open to the unnoticed spread of organisms over long distances [Hess-Erga *et al.* 2019]. Although free-living microorganisms are considered not to be invasive but can significantly disrupt the environmental diversity of the area [Seebens *et al.* 2013]. For example, obtained in our research bacterial species such as: *Bacillus bataviensis*, *Pusilimonas noertemannii*, *Oligella ureotylica*, *Bordetella petrii*, *Derxia lacustris*, and *Clostridium hiranonis* shows the ability to decompose various substances and at the same time not having a defined impact on human and animal health or the aquatic environment [Logan, de Vos 2015].

This study has some limitations. The biggest limitation in our study was process of collecting samples. As unauthorised persons and with the lack of appropriate training, we were unable to take the samples ourselves. In addition, the COVID-19 pandemic and the related safety restrictions against the spread of the virus prevented us from direct sampling and supervision of the process (we were not allowed to board the ship). We were reliant on the help of trained sailors. Also, the research design could be more specific. Unfortunately, despite the ambitious plans to thoroughly analyse the obtained ballast water samples, our research design depended on the equipment available to us. That is why we are aware of many shortcomings, which we plan to correct in the future research. The limit of our work was also the team's inexperience in work on ballast water. This work is only our beginning in this field of research. When developing our action plan, we made use of the available literature. We now know that we should streamline and specify each stage of the work

more precisely, starting with creating a very detailed methodology. We hope that with the experience gained in this study, improved methodology and wider access to equipment, we will be able to conduct richer and better organised research.

CONCLUSIONS

The openness of marine ecosystems and the potential for long-distance dispersal of organisms via ballast water has been reported to be a major mechanism for the introduction of organisms of different sizes into new environments. Newly introduced microorganisms can affect a given ecosystem by disrupting naturally occurring interspecific interactions at the very lowest level, which may contribute to increased susceptibility to invasion by them, for example through the resulting eutrophication that may cause blooms and reduce the diversity of a given environment. Consequently, it may increase or decrease the invasive potential of ballast water organisms. Therefore, it is important to determine the microbiological status of port waters and compare it to the microbiota of ballast waters collected from individual ships. Setting such a goal for this study allowed us to determine the effectiveness of ballast water treatment systems used on the studied ships and the ability of these systems to perform elimination of undesirable bacteria and, in particular, pathogenic ones. Unfortunately, data on the presence of microorganisms in ballast water from numerous scientific literatures indicate the unreliability of currently used ballast water treatment systems. It is worth emphasising that the main area of further research should be the assessment of the importance of microorganisms in terms of interactions with higher trophic levels in new aquatic environments, the impact of these microorganisms on native aquatic organisms and above all, economically important fish species.

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