PATHOGENIC MICROORGANISMS IN SEAWATER SAMPLES AT THE PORT IN RIO GRANDE, RS, BRAZIL: A PUBLIC HEALTH ISSUE

RAÚL ANDRÉS MENDOZA SASSI[*]
CESAR FRANCISCO SILVA DA COSTA[**]
CARLOS JAMES SCAINI[***]
PEDRO EDUARDO SILVA DE ALMEIDA[****]
FABIANE AGUIAR DOS ANJOS GATTI[*****]
ERICA S SILVEIRA[******]
MÁRCIA MATA[*******]
EDUARDA HALLAL[********]
CAROLINA COCH GIÓIA[*********]
DANIELA FERNANDES RAMOS[**********]
RICARDO LUIZ RICCI FALCHI[**********]
RALF KERSANACH[**********]
FERNANDA GUIMARÃES MARQUES[**********]
TATIANA RAMOS[**********]
DIEGO SOUZA[**********]
NEUSIANE CHAVES DE SOUZA[**********]
RUBENS LOBATO[**********]
ANA MARIA BARRAL DE MARTÍNEZ[**********]

RESUMO

Objetivo: Identificar microrganismos patogênicos na água do porto sul-brasileiro de Rio Grande, incluindo bactérias, protozoários e fungos. **Metodologia**: Foram obtidas amostras entre Junho e Novembro de 2006, em pontos representativos ao longo do Porto e analisadas para a presença de bactérias, protozoários e fungos. Foi realizada uma análise qualitativa e quantitativa dos microrganismos. **Resultados**: Todos os pontos tiveram contagens elevadas de coliformes totais e fecais. Protozoários foram encontrados em 61,9% das amostras representados por *Acanthamoeba* spp. *(*28,6%), *Giardia* spp (16,7%), *Criptosporidum* spp. (9,5%) e *Naegleria* spp. (7,1%). Os fungos foram positivos em 88,3% dos casos.

[*] Faculdade de Medicina – FAMED. E-mail: dmiraul@furg.br
[**] Escola de Enfermagem. E-mail: enf.cesar@ibest.com.br
[***] Laboratório de Parasitologia- FAMED. E-mail: dpacjs@furg.br
[****] Laboratório de Microbiologia – FAMED. E-mail: pedrefurg@gmail.com
[*****] E-mail: dmiraul@furg.br
[******] Laboratório de Micologia – FAMED. E-mail: erisil_67@yahoo.com.br
[*******] Laboratório de Micologia – FAMED. E-mail: marcinha.sul@terra.com.br
[********] Laboratório de Microbiologia – FAMED. E-mail: eduardahd@hotmail.com
[*********] Laboratório de Microbiologia – FAMED. E-mail: dpacccg@furg.br
[**********] Laboratório de Microbiologia – FAMED. E-mail: daniferamos@gmail.com
[**********] Laboratório de Patologia – FAMED. E-mail: rfalchi@pop.com.br
[**********] Instituto de Oceanologia. E-mail: dmbralf@furg.br
[**********] Laboratório de Parasitologia – FAMED. E-mail: nandabiopok@gmail.com
[**********] Laboratório de Parasitologia – FAMED. E-mail: tsrfarma@hotmail.com
[**********] Biologia. E-mail: dmsfurg@yahoo.com.br
[**********] Laboratório de Microbiologia – FAMED. E-mail: neusisouza@hotmail.com
[**********] Laboratório de Micologia – FAMED. E-mail: rubenslobatobio@yahoo.com.br
[**********] Laboratório de Microbiologia – FAMED. E-mail: nani60@gmail.com

Os mais frequentes foram *Aspergillus* spp. (33,1 %), *Penicillium* spp.(23,8%) *Cladosporium* spp. (21,4%) e *Rodothorula* spp. (11,9%). **Conclusões:** A água portuária contém não só bactérias patogênicas, mas também protozoários e fungos. Esta situação exige uma vigilância apropriada de forma a reduzir o risco em virtude das atividades de lastreamento e deslastreamento de navios.

PALAVRAS CHAVES: Bactéria; Ambiente e Saúde pública; Fungo; Protozoário; Microbiologia; Porto.

ABSTRACT

PATHOGENIC MICROORGANISMS IN SEAWATER SAMPLES AT THE BRAZILIAN PORT OF RIO GRANDE: A PUBLIC HEALTH PROBLEM

The study aimed to identify microorganisms in the water of a Brazilian Port, including protozoa and fungus, due to the risk of increasing ballast water activities. Water samples were obtained between June and November 2006, from representative sites of the Port, and analyzed for the presence of bacteria, protozoa and fungus. Qualitative and quantitative counts of microorganisms were performed. Higher counts of total and fecal coliforms were identified in all stations. Protozoa were found in 61.9% of the samples: *Acanthamoeba spp. (*28.6%), *Giardia spp* (16.7%), *Criptosporidum spp.* (9.5%) and *Naegleria spp.* (7.1%). Fungus were positive in 88.3% of the samples and the most frequent were *Aspergillus spp*. (33.1%), *Penicillium spp*.(23.8%) *Cladosporium spp*. (21.4%), *Rodothorula spp*. (11.9%). Samples from Brazilian port water contains not only pathogenic bacteria but also protozoa and fungus, which demands appropriate surveillance and measures to reduce the potential hazard in view of deballasting and ballasting ship activities.

KEY WORDS: Bacteria; Environment and Public Health; Fungi; Protozoa; Microbiology; harbor

INTRODUCTION

Ballast water is added to or discharged into separate tanks in order to stabilize and guarantee the structural integrity of ships. The water is obtained from the environment where the ship operates. Studies of its biological content have been intensified in recent years because of the worldwide increase in ship traffic and the possibility of transporting species from one environment to another[1]. Emphasis has been placed on species of marine and estuarine bacteria and viruses[1,2]. Nevertheless, there is evidence that human pathogens can also be transported among ports via ballast water[3,4]. After the most recent cholera epidemic in South America, the bacterium *Vibrio cholerae* got special attention. Research has demonstrated the potential hazards posed by this bacterium when transported via ballast water among shores[3,4] However, very few studies have investigated other pathogenic microorganisms (e.g., fungi and protozoa) because the IMO guidelines[5] do not directly mention them.

The Port of Rio Grande, the southernmost Brazilian seaport, is located in the *Lagoa dos Patos* estuary. It is the third most active Brazilian port in terms of container movement and one of the busiest ports in several products, such as

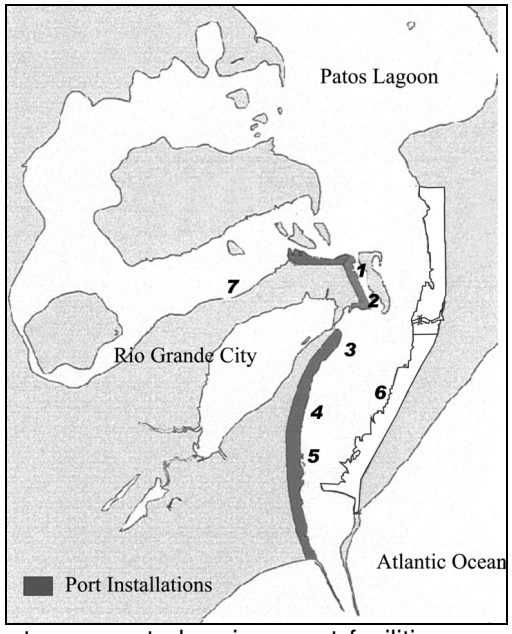
fertilizers and grains. Ships that may or may not have followed the IMO ballast water regulations arrive from and depart for African ports and others on the Atlantic Ocean.

Resolution A.868, issued by the IMO, recommends that signatory countries promote research in this area to study the normal biota of the ports and to monitor the export and import of species by ballasting and deballasting operations, so as to minimize the risk of health and environmental problems[5]. This study, which is in accordance with this recommendation, aimed at testing the port water for human pathogens, such as protozoa and fungi, and at assessing risk posed by possibly hazardous pathogenic organisms.

METHODS

Study Site

The study was carried out at the Port of Rio Grande, located 32° 7' 20" south and 52° 5' 36" west, in Rio Grande, Rio Grande do Sul state, in southern Brazil. The Port of Rio Grande, the southernmost Brazilian seaport, is located on the western shore of the *Canal do Rio Grande*, which connects the *Lagoa dos Patos* with the South Atlantic Ocean. It is an important port for bulk cargo vessels, especially those coming from Africa and South America; in 2006 alone, it received 2,783 ships that transported 19 million tons of goods.



Five sampling stations that represented various port facilities were selected (Figure 1): the depot for liquid and solid bulk cargo (1), the “Roll on–Roll off” facility (2), the oil depot (3), the grain terminal (4) and the container terminal (5). The sixth station, the ferryboat dock, was selected to be the control station because it is situated in a cargo-free area of the port, far from the urban sewage outlets. The seventh station, designated as the urban station, is located in an urban area in Rio Grande, separated from, but in communication with, the port area. The quality of the city sewage treatment was poor at the time of the study since most water underwent no treatment. Thus, the discharge was a potential source of biological contaminants. For this reason, it was selected to be a positive control.

WATER SAMPLING AND ANALYSIS

Sampling was carried out monthly between June and November 2006. At each station, two 500 ml samples were collected in sterilized bottles from depths between 30 to 100 centimeters following standard methods[6]. The samples were collected following the usual precautions for microbiological analysis, stored on ice and filtered by 0.45 µm and 0.22 µm acetate cellulose membranes. Filtration took place in a class II biological safety cabinet.

International Maritime Organization[7] criteria were followed to identify *Vibrio cholerae, Streptococcus fecalis* and total and fecal coliforms. The diagnostic criteria were the following: Toxigenic O1 and O139 *Vibrio cholerae* were considered positive if there was at least one colony formation unit (cfu) per 100 milliliters or fewer than 1 cfu per gram (wet weight) of sample; *Escherichia coli* was considered positive if there were at least 250 cfu/100 ml. *Streptococcus fecalis* was considered positive if there were at least 100 cfu/100 ml.

The multiple-tube fermentation technique was used to identify total and fecal coliforms. The confirmatory mediums were EC and Brilliant Green. Atypical colonies were selected and identified by biochemical tests: LIA (Lysin Iron Agar), TSI (Triple Sugar Iron), Simmons citrate and urea, to identify *Salmonella* spp.; and LIA, SIM, Simmons citrate and lactose, to identify *E. coli*.

Molecular identification was carried out by extracting DNA with the phenol-chloroform method. The chosen molecular marker was 16S rDNA, and the gene was amplified by the primers -FD1 5`AGAGTTTGATC YTGGYTYAG 3` and -rP2 5`ACGGCTACCTTGTTAC GACTT 3`. PCR was carried out by a Mini Cycler – MJ Research, with the profile denaturation at 95ºC for 30 s, annealing at 55ºC for 0:30 min and extension at 72ºC for 1:50 min. Sequencing took place at the Biotechnology Center of the Universidade Federal de Pelotas, RS, Brazil, by a MegaBACE TH 1000 (GE HEALTHCARE) automatic DNA sequencer and the DYEnamic ET DYE Terminator Cycle (GE HEALTHCARE) sequencing kit. A PCR reaction was carried out on a 96 PCR plate, using 100 and 500 ng of the purified PCR product, 5 pmol of each of the oligonucleotids for rRNA amplification of 16S rRNA (FD1, RP2 and S33 -533 5`GTGCCAGCAGCC GCGGTAA 3`)[8] and 2 µl of the sequencing mixture DYEnamic ET terminator cycle premix; its final volume was 5 µl. Amplification was carried out by an automatic thermal cycler (Eppendorf) with 25 cycles at 95^0C for 20 s, 50^0C for 15 s and 60^0C for one minute. Products of the reactions were purified with ethanol (0.5 µl ammonia acetate and 13.7 µl absolute ethanol) and washed with 70% ethanol. After drying, the purified material was resuspended with a loading buffer and inserted into the DNA Automatic Sequencer. Samples were injected into a capillary matrix,

with 1 to 2 KV voltage for 12 to 75 s and run at 9 KV for approximately 130 min. Sequencing results were analyzed by the Vector NI program and compared to the 16S rRNA gene sequence of the GeneBank database. The comparison was made by means of sequence alignment by the BLASTIN program of the National Center for Biotechnology Information (NCBI).

Free-living amoebae were studied by incubating the samples in a Petri dish with 1.5% agar and inactivated *Escherichia coli*, for 4 days at 28°C. A flagellation test for *Naegleria* spp. was carried out in positive cultures. Material was concentrated by a filtration and centrifugation-sedimentation technique[9]. Testing for protozoan cysts was carried out by the Faust and the Ritchie techniques. Cysts and trophozoites were identified by using trichrome stain. For *Cryptosporidium* spp., plates were prepared with the sediment obtained from the centrifugation-sedimentation method. Samples were stained by the Kinyoun acid-resistant method. *Giardia* spp. was investigated by the enzyme-linked immunosorbent assay (ELISA)[10].

Fungi were identified by the membrane filtration technique with 0.45 µm membranes - Millipore[6]. Afterwards, filtration membranes were spread on plates with Sabouraud dextrose agar (SDA) and Sabouraud agar supplemented with cycloheximide, in duplicate. In addition, serial decimal dilutions made from 1 mL of the original sample were transferred to a tube containing 9 mL PBS, up to a final dilution of 10^{-8}. One milliliter of each dilution was added by the "pour plate" technique to 25 ml Sabouraud agar and Sabouraud agar/cycloheximide in duplicate. The material was incubated at 37°C for 7 days and assessed on a daily basis. The identification of fungus isolates was based on the observed growth velocity, the superficial aspect of the mycelium and the pigmentation, and the recognition of macromorphological characteristics. Micromorphological characteristics were obtained by the microculture technique[11].

Absolute and relative frequencies of pathogens were calculated for each sampling station and for each month when data was collected. The mean, standard deviation and range of water salinity, pH and temperature parameters were also assessed.

RESULTS

In all, 42 samples were collected during the above-mentioned period, six at each station. Water parameters for the sample collection period were the following: mean salinity 0.45 % (SD 0.33, lowest value 0.3, highest value 1); pH 7.03 (SD 0.24, lowest value 6.5, highest value 7.5); and mean temperature 17° C (SD 3.03, lowest value 13, highest value 23).

Tables 1 and **2** show the total and fecal coliform colony counts. The highest total coliform counts were found at stations 1 and 3, located at the port, and at station 7 (the urban one). Fecal coliform counts were higher at stations 3 (oil

depot) and 7 (urban station). The highest counts of *E. coli* were observed at the Roll on–Roll off pier and at the oil depot. For *Salmonella* spp., the highest cfu values were obtained at the oil depot and at the grain terminal. A *Vibrio* spp. identified by traditional methods was not confirmed by the sequencing results. A high count was also observed at the urban station.

Table 1 – Total coliform distribution at the sampling stations per month. Port of Rio Grande, RS, Brazil. 2006 (no. cells/100 ml)

Sampling station	June	July	August	September	October	November
1- Solid bulk depot	1.1×10^3	1.5×10^3	2.9×10^3	1.5×10^3	1.1×10^4	4,.6 x 10^3
2- "Roll on-Roll off" pier	1.5×10^2	9.3×10^2	4.6×10^3	1.5×10^3	2.4×10^3	1.1×10^4
3 - Oil terminal	4.3×10^1	> 1.1x 104	2.4×10^3	1.1×10^4	$>1.1 \times 10^4$	2,.1 x 10^3
4 – Container terminal	2.10×10^2	4.6×10^3	1.5×10^3	4.6×10^3	2.4×10^3	4.6×10^3
5 – Grain terminal	4.3×10^1	1.1×10^4	$> 1.1 \times 10^4$	4.6×10^3	9.3×10^2	1.5×10^3
6 – Ferryboat dock	1.1×10^1	4.3×10^2	1.1×10^4	1.5×10^3	7.5×10^2	1.5×10^3
7 – Urban station	Absence	1.1×10^4	4.6×10^3	$>1.1 \times 10^4$	$>1.1 \times 10^4$	2.1×10^3

Table 2 – Fecal coliform distribution at the sampling stations per month. Port of Rio Grande, RS, Brazil. 2006. (no. cells/100 ml)

Sampling station	June	July	August	September	October	November
1- Solid bulk depot	9.2×10^1	2.3×10^2	3.5×10^2	9.2×10^1	4.6×10^3	1.5×10^3
2- "Roll on-Roll off" pier	3.6×10^2	3.6×10^2	1.5×10^3	1.5×10^3	1.5×10^2	2.3×10^2
3 – Oil terminal	4.3×10^1	7.5×10^2	7.4×10^2	9.2×10^1	2.3×10^2	2.4×10^3
4 - Container terminal	Absence	3.6×10^1	9.2×10^2	1.5×10^2	9.3×10^2	9.2×10^2
5 – Grain terminal	4.3×10^1	1.5×10^2	4.6×10^3	4.3×10^2	9.3×10^2	9.3×10^2
6 – Ferryboat dock	Absence	Absence	3.6×10^1	2.3×10^2	9.2×10^1	Absence
7 – Urban station	Absence	4.6×10^3	4.3×10^2	2.4×10^3	$>1.1 \times 10^4$	1.5×10^3

One of the microorganisms, initially identified biochemically as *Escherichia coli*, was found to be an enteroinvasive *E. coli* (EIEC) 53638 strain when 16S rDNA sequence analysis was carried out.

At least one protozoan species was found in all stations (**Table 3**).

Table 3 – Protozoan distribution at the sampling stations (one sample per month). June-November 2006, Port of Rio Grande, RS, Brazil.

Sampling station	Acanthamoeba	Giardia	Naegleria	Criptosporidum
1- Solid bulk depot	1/6 (16.7%)	2/6 (33.3%)	Absence	Absence
2- "Roll on-Roll off" pier	Absence	4/6 (66.7%)	Absence	1/6 (16.7%)
3 – Oil terminal	3/6 (50.0%)	Absence	2/6 (33.3%)	1/6 (16.7%)
4 - Container terminal	1/6 (16.7%)	Absence	1/6 (16.7%)	Absence
5 – Grain terminal	3/6 (50.0%)	Absence	Absence	Absence
6 – Ferryboat dock	1/6 (16.7%)	1/6 (16.67%)	Absence	Absence
7 – Urban station	3/6 (50.0%)	Absence	1/6 (16.7%)	1/6 (16.7%)
Total	12/42 (28.6%)	7/42 (16.67%)	4/42 (9.5%)	3/42 (7.1%)

Pathogenic protozoa were found in 61.9% of samples. The most frequent one at any sampling station was *Acanthamoeba* spp., isolated in 28.6% of samples, followed by *Giardia* spp. (16.7%). Other pathogenic protozoa were *Naegleria* spp. (9.5%) and *Cryptosporidium* spp. (7.1%). The urban station also presented positive results for most protozoa.

At least one species of fungus was found in 35 out of 42 samples (88.3%). **Table 4** shows the frequency of isolated fungi at each station. As can be seen, most sampling locations had at least three or more different types of fungus: two of them were also identified at the urban station.

Table 4 – Most frequently isolated fungi at the sampling stations (one sample per month). June-November 2006, Port of Rio Grande, RS, Brazil.

Sampling station	*Aspergillus*	*Penicillium*	*Cladosporium*	*Rodothorula*	*Mucor*	*Madurella*	*Trichophyton*	*Scullariopsis*
1- Solid bulk depot	2/6 (33.3%)	2/6 (33.3%)	1/6 (16.7%)	1/6 (16.7%)	Absence	Absence	Absence	Absence
2- "Roll on-Roll-off" pier	1/6 (16.7%)	Absence	1/6 (16.7%)	1/6 (16.7%)	Absence	Absence	1/6 (16.7%)	Absence
3 - Oil terminal	2/6 (33.3%)	2/6 (33.3%)	1/6 (16.7%)	Absence	Absence	Absence	1/6 (16.7%)	1/6 (16.7%)
4 - Container terminal	2/6 (33.3%)	4/6 (66.7%)	2/6 (33.3%)	1/6 (16.7%)	1/6 (16.7%)	2/6 (33.3%)	Absence	Absence
5 – Grain terminal	4/6 (66.7%)	2/6 (33.3%)	3/6 (50.0%)	Absence	2/6 (33.3%)	Absence	Absence	2/6 (33.3%)
6 – Ferryboat dock	3/6 (50.0%)	Absence	1/6 (16.7%)	Absence	Absence	1/6 (16.7%)	Absence	Absence
7 – Urban station	Absence	Absence	Absence	2/6 (33,3%)	Absence	Absence	1/6 (16.7%)	Absence
Total	14/42 (33.3%)	10/42 (23.8%)	9/42 (21.4%)	5/42 (11.9%)	3/42 (7.1%)	3/42 (7.1%)	3/42 (7.1%)	3/42 (7.1%)

The most frequent species was *Aspergillus* spp. (33.3%), followed by *Penicillium* spp. (23.8%), *Cladosporium* spp. (21.4%), *Rodothorula* spp. (11.9%) and *Mucor* spp., *Madurella* spp., *Trichophyton spp.* and *Sculariopsis* spp. (7.1% each). Other species, such as *Candida* spp., *Streptomyces* spp., *Geotrichum* spp., *Cryptococcocus* spp. and *Geocladium* spp. were less frequent.

DISCUSSION

This study assessed the risk posed by pathogenic agents at the Port of Rio Grande. Results demonstrated the presence not only of bacteria but also of pathogenic protozoa and fungi. The source of these agents may have been the sewage water, judging from the levels found at the port stations and from the positive control.

Different levels of total and fecal coliforms were identified at most port sampling stations. Some counts are above the Brazilian standards defined by CONAMA, the Brazilian National Environmental Council for swimming waters. Depending on the station and month, bacterial counts were as high as 10^4 cells/100 ml. This level of total and fecal coliforms is close to that reported in Hong Kong[12] but lower than the one found at Mumbai Harbor, India[4]. This finding is related to the potential presence of pathogenic human bacteria[13]. *Escherichia coli* was more prevalent than *Salmonella* spp., a result that is consistent with the findings of the Mumbai study[4]. The *E. coli* 53638 strain identified by our study belongs to the enteroinvasive (EIEC) group and produces inflammatory diarrhea. This agent is responsible for a form of human dysentery that is similar to, but less severe than, that produced by *Shigella* spp.[14].

Four pathogenic species of protozoa were identified. *Acanthamoeba* spp. was the most common one, followed by *Giardia* spp., *Naegleria* spp. and *Cryptosporidium* spp. Every station presented at least one type. *Naegleria* spp. and *Acanthamoeba* spp., two free-living amoebae that have been found in various types of water sources, are responsible for encephalitis and keratitis in humans[15,16]. *Cryptosporidium* spp. is a pathogen associated with immunodeficient[17,] but it also occurs in immunocompetent subjects, causing a diarrheal disease[18]. Epidemics involving *Cryptosporodium* spp. have been described worldwide since 1984. Relations between this protozoan and fecal coliforms have been reported in other studies[19].

This study identified different genera of fungi. The most common ones were *Aspergillus* spp. and *Penicillium* spp. A study carried out in swimming areas at a different geographical location also identified both genera as the most common ones[20]. Other fungi found in this study, such as *Candida* spp., *Cryptococcus* spp. and *Rhodotorula* spp., were also recognized at different geographical locations, which is evidence of the ubiquity of these yeasts[21,22]. Some of these

fungi may cause diseases in non-immunecompromised individuals, as well as in patients who have underlying diseases or compromised immune response[23].

It is relevant to make some considerations regarding port ballasting/deballasting activities and their consequences for human health. Studies have shown that microorganisms, e.g., *E. coli* and *Vibrio cholerae* O1, can survive in seawater and ballast water for several weeks[24]. Little information can be found about the survival of fungi and protozoa in this environment.

Another concern is that prevalent pathogenic local biota could be affected by foreign species introduction via ballast water. When a pathogenic microorganism is introduced into the human and animal biota, even if it has already been in this new location, it can carry intrinsic modifications that were acquired at its source, such as drug resistance and changes in virulence and pathogenic profiles[25].

Although fungi, bacteria and protozoa in port water are less likely to infect humans than agents introduced through other forms of transmission (such as contaminated drinking water), some studies have described the use of contaminated sea or freshwater food associated with bacterial[26] or protozoan infection[27]. Isolates of the fungus *Aspergillus fumigatus* collected in water were found to be genetically related to samples of isolates obtained in clinical samples[28]. The well-known relation between *V. cholerae* and ballast water is even more remarkable. Some studies have found that this bacterium can be transported through ballast water from epidemic areas to epidemic-free ones[3]. It has been hypothesized that it is one of the mechanisms that could have been responsible for the introduction of the bacterium at the beginning of the South American cholera outbreak in 1991.

This study has fulfilled the objective of highlighting the contamination of human pathogens in the port water in Rio Grande. The most likely source of these pathogenic species is sewage contamination. This finding emphasizes the problem of transporting pathogenic species from one body of water to others when ships take ballast water from the shore rather than from deep waters, away from the port, as required by IMO rules.

These findings also justify the need to expand future research on pathogens, such as fungi and protozoa, and to include this issue in the health sector agenda as a public health problem, owing to its potentially negative impact on human health.

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Conflicts of Interest

The authors declare no potential conflict of interest.

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Participation

RAMS, CFSC, CJS, PSA participated in the project elaboration, data analysis and interpretation, manuscript writing and final version review. ESS, MMM and EH participated in the data analysis and interpretation, manuscript writing and final version review. DFR, TR, FMG, DS, RL, RLRF and NCS participated in the data analysis and interpretation, manuscript writing and final version review. CCG, RK and AMBM participated in the project elaboration, data analysis, manuscript writing and final version review.