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First report of free-living amoebae in sewage treatment plants in Porto Alegre, southern Brazil

Thaisla Cristiane Borella da Silva [a], Daniel Leal dos Santos[b] and Marilise Brittes Rott [a,*]

[a] Department of Microbiology, Immunology and Parasitology, Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[b] Faculty of Geography, Pontifical Catholic University of Rio Grande do Sul, Ipiranga Avenue, N 6681, Porto Alegre, Rio Grande do Sul 90619-900, Brazil
*Corresponding author. E-mail: marilise.rott@ufrgs.br

TCB, 0000-0002-4432-2779; MBR, 0000-0002-5227-4594

ABSTRACT

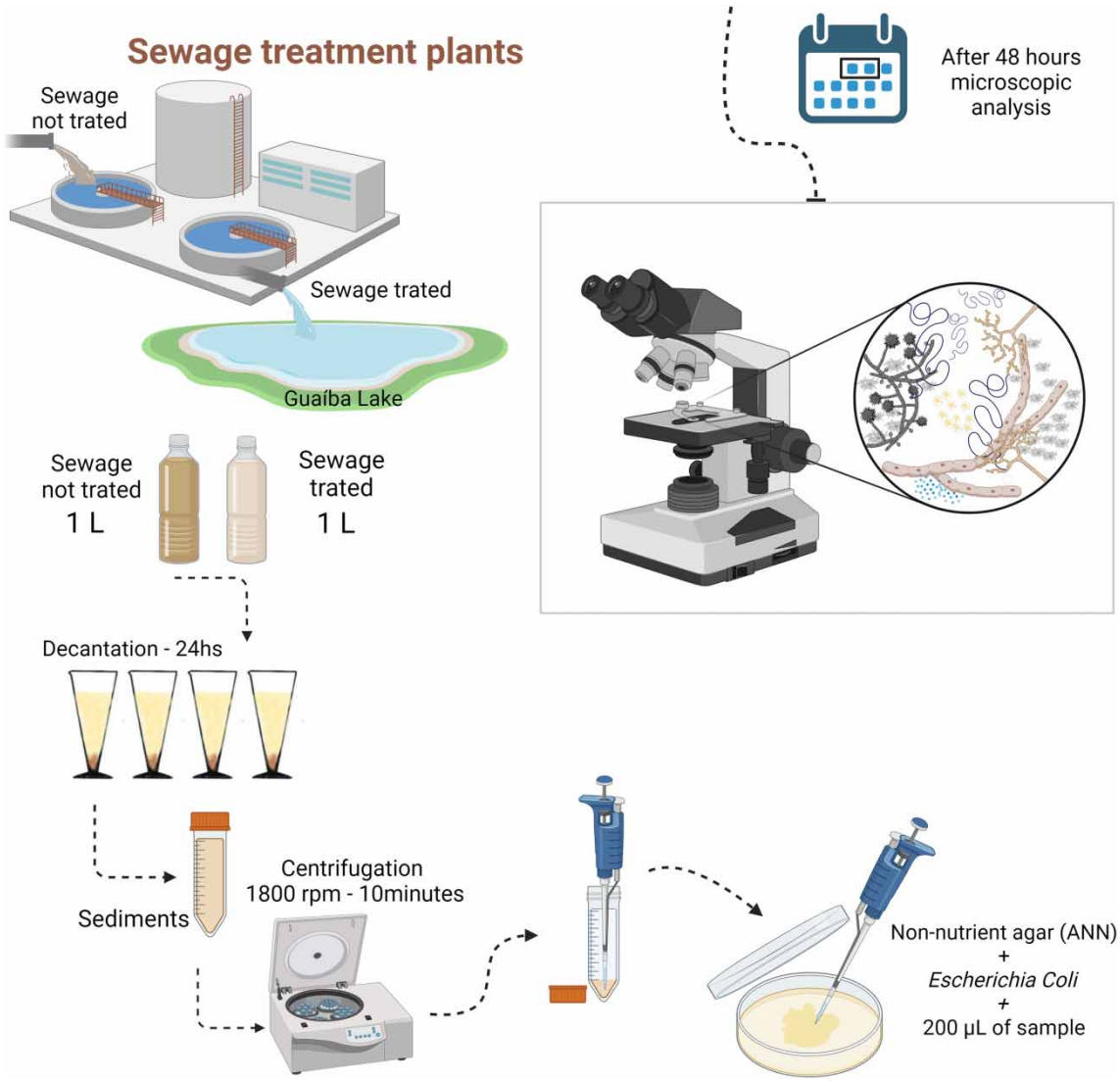
Free-living amoebae (FLA) are amphizoic protozoans with a cosmopolitan distribution. Some strains of species are associated with infections in humans. They feed on microorganisms by phagocytosis; however, some of these can become endocytobionts by resisting this process and taking shelter inside the amoeba. The whole world is experiencing increasing shortage of water, and sewage is being reused, so the study of this environment is important in public health context. The objective of this work was to identify FLA present in sewage treatment plants in Porto Alegre, Brazil. About 1 L samples were collected from eight stations (raw and treated sewage) in January, February, July, and August 2022. The samples were sown in monoxenic culture, and the isolated amoebae were subjected to morphological and molecular identification. Polymerase chain reaction results indicated the presence of the genus Acanthamoeba in 100% of the samples. Gene sequencing showed the presence of Acanthamoeba lenticulata and Acanthamoeba polyphaga - T5 and T4 genotypes - respectively, which are related to pathogenicity. The environment where the sewage is released can be used in recreational activities, exposing individuals to potential interactions with these amoebae and their potential endocytobionts, which may pose risks to public health.

Key words: *Acanthamoeba lenticulata*, *Acanthamoeba polyphaga*, genotype T4, genotype T5, Gravataí River, Guaíba Lake

HIGHLIGHTS

- Free-living amoebae were isolated for the first time in sewage treatment plants (STPs) in Brazil.
- In the STPs of Porto Alegre, RS, Brazil, only the genus *Acanthamoeba* was isolated, and genotypes associated with pathogenicity (T4 and T5) were identified.
- The Guaíba lake and the Gravataí river receive the city's sewage and, consequently, can disperse *Acanthamoeba spp.*
- Further research on the prevalence and identification of free-living amoebae in sewage is still needed in Brazil.

GRAPHICAL ABSTRACT



INTRODUCTION

Free-living amoebae (FLA) are protozoan, which can be found in water for human consumption, water courses, sewage, hot springs, lakes, swimming pools, and rivers (Khan 2006; Carlesso *et al.* 2007; De Souza *et al.* 2017; Scheid 2018), which are known as amphizoics, as they can be in the environment or parasitizing humans and animals. Among some strains of potentially pathogenic species and/or genera we have: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, *Paravahlkampfia*, *Sappinia*, and *Vermamoeba vermiformis*, that can cause diseases such as keratitis, granulomatous amoebic encephalitis (GAE), skin lesions, lung lesions and primary amoebic meningoencephalitis (PAM), in association or alone (Rodríguez-Zaragoza 1994; Visvesvara *et al.* 2009; Trabelsi *et al.* 2012; José Maschio *et al.* 2015; Delafont *et al.* 2018; Scheid 2019).

These amoebae have two morphological stages, the trophozoite (active form) and the cyst (resistance form), except *N. fowleri* which has an intermediate flagellate stage (Visvesvara *et al.* 2007; Delafont *et al.* 2018). Nutrition is carried out by amoebic phagocytosis of algae, bacteria, fungi, or protozoa. However, some microorganisms can survive this process, shelter inside, and protect themselves from chlorination and biocidal processes; these microorganisms are called endocytobionts (Lorenzo-Morales *et al.* 2007; De Souza *et al.* 2017; Scheid 2019; Gomes *et al.* 2020). In this way, some amoebae can serve as

a 'Trojan horse', carrying and disseminating some microorganisms such as *Bacillus anthracis*, *Cryptococcus neoformans*, *Escherichia coli O157*, *Legionella pneumophila*, *Staphylococcus aureus* methicillin-resistant and others (Balczun & Scheid 2017; De Souza *et al.* 2017; Soares *et al.* 2017; Scheid 2019).

Data from the World Health Organization (WHO) revealed that about 85% of known diseases are associated with or transmitted by water (Monteiro *et al.* 2016). Wastewater (sewage) is composed of several microorganisms, which vary depending on the location and seasonality, and the indicator of the quality of the water that is released into the environment can be done with the microbiological analysis of these waters (Maritz *et al.* 2019).

In Brazil, 55% of sewage is treated, with 43% of the population having sewage collected and treated and 12% using a septic tank (individual solution). However, 45% of the sewage that is generated in the country is still not treated; this problem is associated with deficits in sewage collected and in the treatment capacity of sewage treatment plants (STPs) (ANA 2017). The most used sewage treatments in Brazil are activated sludge, stabilization ponds, and anaerobic sewage treatments (INCT 2019).

The treatment of sewage by activated sludge is considered an aerobic treatment, where the activated sludge is composed of a mass of algae, bacteria, fungi, and protozoa, which make the degradation of the organic matter present in the sewage in the presence of oxygen. The effluent is directed to a tank for aeration, where the organic matter is consumed by the aerobic microorganisms and after that, the effluent is sent to the decanter where the solid part (sludge) is separated from the treated sewage. This is one of the most used treatments in Brazil (Manaia *et al.* 2018; INCT 2019).

Stabilization ponds are straightforward treatment systems, offering various configurations based on the available conditions. These configurations include aerobic, anaerobic, facultative, maturation and polishing ponds. Within these ponds, the breakdown of organic matter can take place within an aerobic, anaerobic, or combined environment, contingent upon the specific type of pond. Warmer regions accelerate the metabolic rate of microorganisms, although this can be detrimental to the overall efficiency of the ponds (INCT 2019).

The anaerobic sewage treatment takes place using anaerobic microorganisms that break down organic matter. In this process, the affluent passes through a sealed reactor, and the organic matter undergoes degradation. There are two primary types of sealed reactors: the Upflow Anaerobic Reactor (UAR) and the Upflow Anaerobic Sludge Blanket (UASB). The UASB treatment is like the UAR treatment; however, it can capture the biogas generated during the degradation of organic matter, which can then be utilized for energy production. This approach is the most widely employed treatment method in Brazil (INCT 2019).

Sewage is released into the aquatic environment, a medium that can be used for aquatic and recreational activities, such as swimming and diving, but these environments can present a potential risk of the transmission of pathogenic microorganisms to individuals in these places (Soller *et al.* 2010; Oliveira & Buss 2018). The scarcity of drinking water in the world is increasing (Jacobi *et al.* 2016), and with this, solutions to reuse water have been used, such as the use of sewage as a source of water for irrigation of plantations, which has been a practice adopted for years (Bastos 1999; Simões *et al.* 2013). However, one concern is that sewage can contain an abundance of biological contaminants, and these can end up accumulating in soil and plants (Al-Lahham *et al.* 2003).

The present study aimed to isolate potentially pathogenic FLA in eight STPs in Porto Alegre, RS, Brazil. The treated sewage from these stations is released into Lake Guaíba and/or the Gravataí River, which are easily accessible to the population and commonly utilized for recreational purposes.

METHODS

Study area and sample collection

The study was carried out in eight STPs, which were located in the city of Porto Alegre, southern Rio Grande do Sul, Brazil. The STPs in the study were STP Belém Novo, STP Navegantes, STP Serraria, STP Sarandi, STP Rubem Berta, STP Lami, STP do Bosque, and STP Parque do Arvoredo, which were identified on the map as shown in Figure 1.

The characteristics of the STPs can be obtained from the Municipal Basic Sanitation Plan (2013) of Porto Alegre, where the STPs such as Belém Novo and Lami use treatment by a stabilization pond and release treated sewage into Lake Guaíba; the STPs such as Sarandi and Bosque use treatment by the UASB and release the treated sewage into the Gravataí River; STP Serraria uses treatment by the UASB and release treated sewage into Lake Guaíba, and STPs Navegantes, Rubem Berta and Parque do Arvoredo use activated sludge treatment and release treated sewage into the Gravataí River.

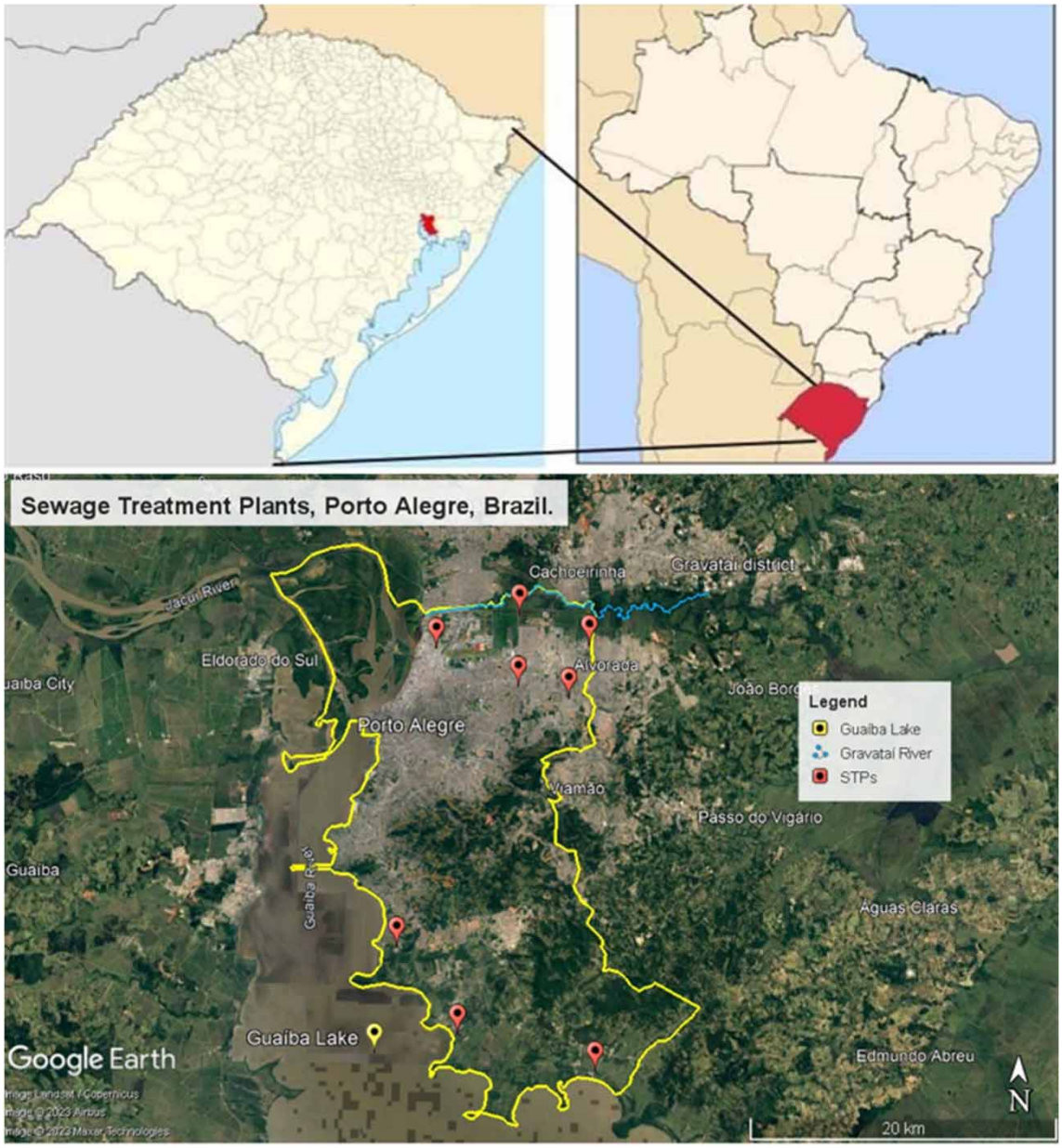


Figure 1 | Map of Porto Alegre with STPs.

Seventeen samples were collected in the summer period (January and February) and 17 samples in the winter period (July and August), with two samples from each point: affluent (1) and effluent (1) from each station, except for STP Rubem Berta which has two different modules in operation, each one of them in a treatment phase and, therefore, three samples were collected: affluent (1) and effluent (2), totaling 34 samples. Samples were collected in 1 L sterilized glass bottles.

Sample processing

All water samples were sedimented in a sedimentation cup for 24 h. Afterwards, the sediment obtained was centrifuged at 2,500 rpm for 10 min. After centrifugation, 200 μL of the sedimented material was transferred to a Petri dish containing 1.5% non-nutrient agar previously coated with heat-inactivated *E. coli* (ATCC 10536), and this process was performed in quintuplicate. The plates were inoculated at 30 °C (ideal FLA growth temperature) (Schuster 2002) for up to 15 days, examined

daily, and reinoculated to eliminate any contamination by other microorganisms. Cellular cloning was performed by using the methodology of Diehl *et al.* (2021) to obtain different FLA isolates.

Morphological study of isolates

Each selected clone was analyzed for movement, size, and morphological aspects of trophozoites and cysts, presence of acanthopods, and contraction time of contractile vacuoles using the criteria described by Page (1988). The diameter of the trophozoites and cysts was randomly measured by selecting 10 cells from each isolate; and the contraction time of the pulsatile vacuole of five cells randomly selected was also noted. For the identification of groups of the genus *Acanthamoeba*, the morphology of the cysts was analyzed as proposed by Pussard & Pons (1977) 3 days after the encystment process. For the species *N. fowleri* identification, which has the flagellar phase, the exflagellation technique was performed (Silva & Rosa 2003).

DNA extraction

An Na-adapted protocol from Embrapa (Oliveira *et al.* 2007) was used to extract DNA. Briefly, the plate containing amoebae was placed in contact with ice, 3 mL of 1× phosphate-buffered saline solution (PBS) was added, and gentle movements were made to detach the amoebae from the agar. The liquid from the plates was placed in a Falcon tube and centrifuged at 1,800 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 500 μL of 1× PBS and homogenized. About 300 μL of the homogenate was transferred to a 2 mL microtube, and 2.2 μL of proteinase K and 500 μL of 20% sodium dodecyl sulfate were added, homogenized by vortexing, and incubated in a water bath for 1.5 h at 60 °C. Subsequently, 800 μL of chloroform was added and vortexed, and 350 μL of protein precipitation solution was added (3 M potassium acetate with 6.6 M glacial acetic acid) and stirred three times by hand. Afterwards, the microtube was centrifuged for 15 min at 11,000 rpm. Approximately 1 mL of the supernatant was transferred to a new 2 mL tube in which 1 mL of cold absolute ethanol was added and homogenized by inversion for 2 min, after which it was centrifuged at 11,000 rpm for 15 min. Discarding the supernatant, 1 mL of ice-cold 70% ethanol was added and centrifuged at 11,000 rpm for 5 min. After discarding the supernatant, the open microtube was placed on a flat surface for 10 min. After drying, 30 μL of Tris–EDTA buffer and 3 μL of RNAse were added and incubated for 1 h at 37 °C. All extracted DNA was stored at −14 °C. The DNA was quantified with a nanospectrophotometer (Kasvi® K23-0002, version 01/13).

Molecular identification and sequencing

To perform the polymerase chain reaction (PCR) amplification technique, gene-specific oligonucleotides were used (Table 1). PCRs were performed with 10 pmol of each primer, 2.5 mM deoxynucleoside triphosphate (DNTP), 50 mM $MgCl_2$, 2.5 μL of 10× buffer, and 1 U of Taq polymerase (Invitrogen®) to a final volume of 25 μL.

Thermal cycling conditions common to *Acanthamoeba* spp., *Naegleria* spp., and *Vermamoeba* spp. were initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 45 s, extension at 72 °C for 1 min and 15 s for *Acanthamoeba*

Table 1 | Specific oligonucleotides used

Organism	Gene	Sequence 5′–3′	Anelament	Amplicon size (pb)	Bibliography
Acanthamoeba	Região ASA.S1	JDP1: GGCCCAGATCGTTTACCGTGAA JDP2: TCTCACAAGCTGCTAGGGGATA	60 °C for 40 s	450–500	Booton *et al.* (2005)
Balamuthia	16S rRNA	5′Balspec16S: CGCATGTATGAAGAAGACCA 3′Balspec16S: TTACCTATATAATTGTCGATACCA	48 °C for 40 s	1,075	Booton *et al.* (2003)
Bacterial domain	16S rDNA	27F: AGAGTTTGATCCTGGCTCAG 1492R: GGTTACCTTGTTACGACTT	55 °C for 60 s	1,200–1,600	Silva *et al.* (2015)
Naegleria	Region ITS	ITS1: GAACCRGCGTAGGGATCATTT ITS2: TTTCTTTTCCTCCCCTTATTA	55 °C for 40 s	400–453	Pélandakis *et al.* (2000)
Vermamoeba	18S rDNA	Hv1227F: TTACGAGGTCAGGACACTGT Hv1728R: GACCATCCGGAGTTCTCG	58 °C for 40 s	505	Kuiper *et al.* (2006)

spp. and *Naegleria* spp. and 72 °C for 30 s for *Vermamoeba* spp., and, for Domain Bacteria, thermal cycling conditions were initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 60 s and extension at 72 °C for 2 min. The annealing conditions and sequence are in Supplementary Table S1.

The negative control was made with DNA-free, and the positive control was made with the clinical isolate of *Acanthamoeba* spp. (Dos Santos *et al.* 2022), Domain Bacteria (Dos Santos *et al.* 2022), and *N. fowleri* (Henker *et al.* 2021), and an environmental isolate of *V. vermiformis* (Soares *et al.* 2017). After the PCR, the generated amplicons were submitted to electrophoresis and analyzed in 1.2% agarose gel. Sequencing was performed using the same specific primers by the company ACTGene Molecular analyses using an ABI Prism 3500 Genetic Analyzer sequencer (Genetic Analyzer – Applied Biosystems®). The identified forward and reverse sequences were analyzed and submitted to homology analysis in BLAST®, aligned by Clustal W 2.1, and deposited in the GenBank database.

Construction of the phylogenetic tree of the samples

The phylogenetic analysis was based on the results obtained in sequencing. Evolutionary analyses were performed on MEGA11 to build a phylogenetic tree based on the Neighbor-Joining method using forward and reverse sequences. To determine the statistical reliability of each node, 500 bootstrap replicates were performed. This analysis involved 42 nucleotide sequences.

RESULTS

Morphological identification

Of the 34 samples that were collected from the STPs, all (100%) were positive for the presence of FLA. Using the cell cloning technique, a total of 37 clones were selected.

From the samples collected in the summer (Figure 2), a total of 19 clones were obtained, one clone from the affluent samples and one clone from the effluent, in the summer period for the STPs Belém Novo, Navegantes, Sarandi, Rubem Berta, do Bosque, and Parque do Arvoredo. In the STPs Serraria and Lami, one clone of the affluent and two clones of the effluent were obtained from each STP. It is noteworthy that additional clones were derived; however, the selection process was based on their morphology. While it is acknowledged that various other amoebae could potentially exist within this environment, employing this methodology allows us to deduce that the most dominant strains were isolated.

As for the winter samples (Figure 3), a total of 18 clones were obtained, one clone from the affluent sample and one clone from the effluent, in the summer period for the STPs Belém Novo, Navegantes, Sarandi, Rubem Berta, Lami, do Bosque, and Parque do Arvoredo. From STP Serraria, one clone of the affluent and two clones of the effluent were obtained.

The clones had their morphological characteristics analyzed, and the contraction time of the pulsatile vacuoles timed (Supplementary Tables S2 and S3). Some amoebae had their dimensions compatible with species of the genera *Acanthamoeba* and *Naegleria*. None of the samples showed dimensions that coincided with species of the genera *Balamuthia*, *Vermamoeba*, *Paravahlkampfia*, or *Sappinia*.

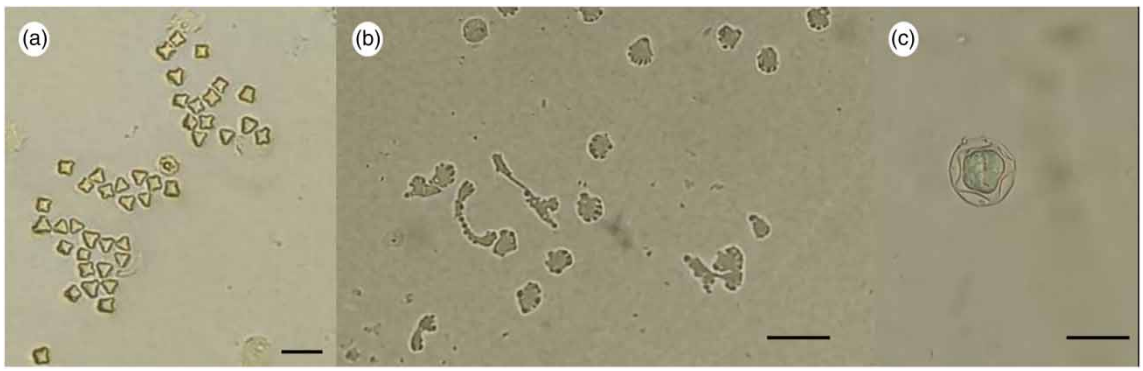


Figure 2 | Free-living amoeba from summer samples. (a) STP Sarandi effluent cysts, bar = 37 μm; (b) trophozoites from the STP Lami affluent, bar = 36 μm; (c) cysts from the Parque do Arvoredo STP affluent, bar = 18.5 μm. 40× magnification (images a and b) and 400× magnification (image c).

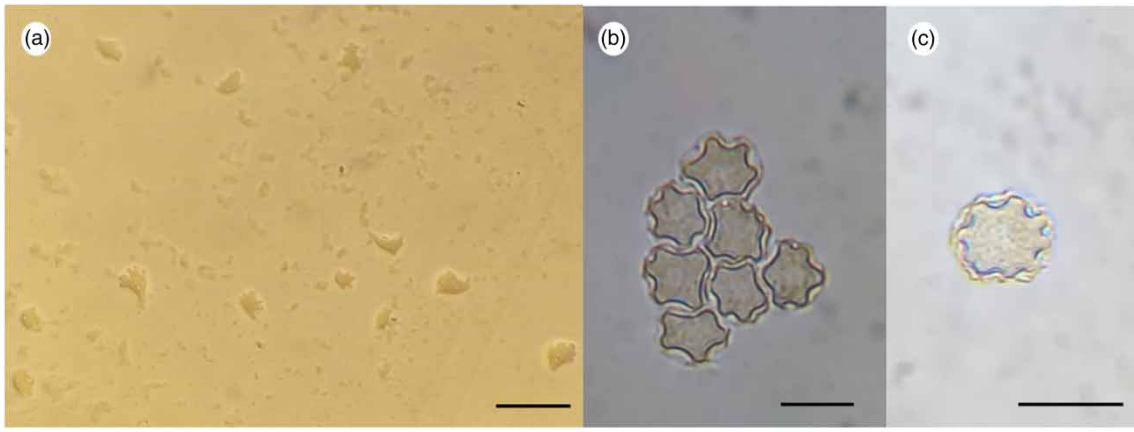


Figure 3 | Free-living amoeba from winter samples. (a) Trophozoites from the tributary of STP Belém Novo, bar = 36 μm; (b) cysts from the STP Rubem Berta tributary, bar = 18.5 μm; (c) cyst from the STP do Bosque effluent, bar = 18.5 μm. 40× magnification (image a) and 400× magnification (images b and c).

Pussard & Pons's (1977) classification was used for all samples, since they presented morphological characteristics compatible with *Acanthamoeba,* such as slow locomotion, cytoplasm with well-defined endoplasm and ectoplasm, presence of acanthopods, and presence of a nucleus with a perinuclear halo.

Cysts from 35 samples were compatible with Group II (Figure 4), which present rounded, polygonal, quadrangular, and triangular endocysts, which were characteristics of Group II in the classification (Visvesvara & Schuster 2008). Cysts from two samples were compatible with Group III (Figure 4), which presents a rounded or slightly angular endocyst (Visvesvara & Schuster 2008).

Molecular identification of FLA

After DNA extraction, the PCR was used as a molecular tool for identification. The reaction was performed for the 37 clones obtained and the results indicated positivity for the genus *Acanthamoeba* in 100% of the samples (Supplementary Table S4). The exflagellation test was performed for all samples, and the result was negative.

Sequences obtained were submitted for analysis using the BLAST (Basic Local Alignment Search Tool) program of the U.S. National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST) in order to classify the *Acanthamoeba* isolates. All isolates obtained identity greater than 95.4% reaching 99.76%, and the sequences were deposited in GenBank (Tables 2 and 3).

Phylogenetic analysis of *Acanthamoeba* spp.

The 37 clones were used with five reference sequences (Table 4) for the construction of the phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 42 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 485 positions in the final dataset. Evolutionary analyses were conducted in MEGA11. The resulting tree can be seen in Figure 5.

DISCUSSION

FLA feed on microorganisms such as algae, bacteria, fungi, protozoa, and organic matter, which is important in the maintenance of the ecosystem (Pickup *et al.* 2007). Despite not having been identified, with the oligonucleotides used, endocytobionts in the samples of this study, the capacity of *Acanthamoeba* spp. harboring endocytobionts is a risk to human health, as the amoeba protects chlorination processes for pathogenic microorganisms, processes that occur in STPs, and protection against

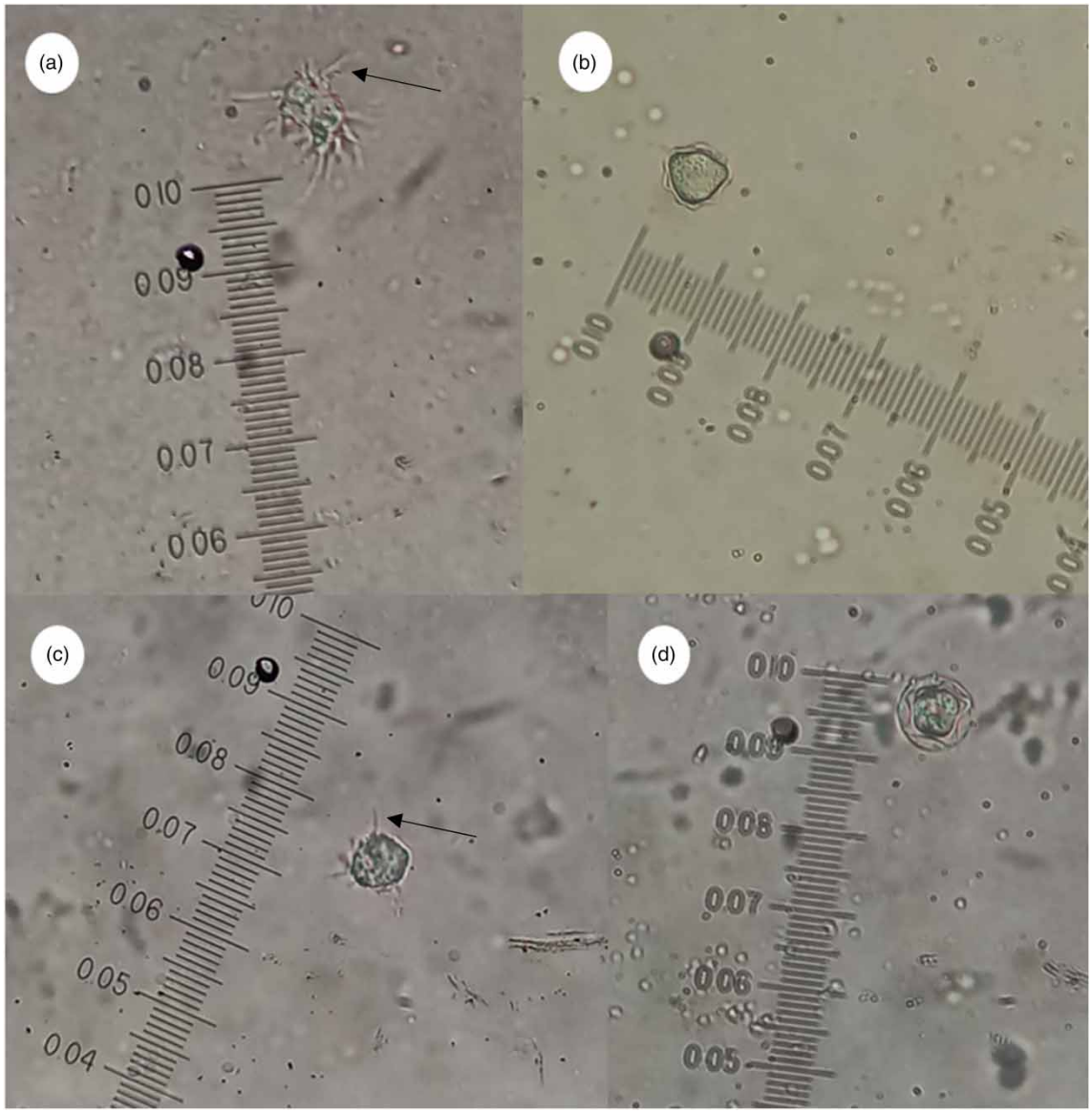


Figure 4 | Summer sample of the effluent from the Belém Novo STP. (a) Trophozoite with acanthopodium shown by the arrow, bar = 18 μm and (b) polygonal cyst, bar = 18.5 μm. Winter sample of the STP Navegantes effluent. (c) Trophozoite with acanthopodium shown by the arrow, bar = 18 μm, and (d) polygonal cyst, bar = 18.5 μm. 400× magnification.

biocides, which are used to combat microorganisms. Thus, *Acanthamoeba* spp. acts as a reservoir of microorganisms and can transmit them to the host (De Souza *et al.* 2017; Delafont *et al.* 2018; Scheid 2018, 2019).

In the present study, *Acanthamoeba* spp. were isolated in 100% of the isolates both in morphological identification and in the PCR technique. This data is similar to those found in the literature since studies carried out in aquatic environments demonstrate the genus *Acanthamoeba* spp. as the most isolated worldwide, and also the one with the highest number of species and pathogenic genotypes (Carlesso *et al.* 2007; Bellini *et al.* 2022; Milanez *et al.* 2023). Of the 37 isolates, two species with the T5 genotype and 35 with the T4 genotype were identified. These genotypes are known to be related to infections in humans (Siddiqui & Khan 2012; Fabres *et al.* 2016; Hajialilo *et al.* 2016).

Marín *et al.* (2015) collected samples from a domestic STP for 1 year and found *Acanthamoeba* in both raw and treated sewage, commenting that the treatment that was used in that plant was not effective in eliminating *Acanthamoeba*. Ramirez *et al.* (2014), in a textile STP, found species of the genera *Acanthamoeba*, *Naegleria*, and *Vermamoeba*, a worrying fact since the textile effluent is rich in residues of dyes, fixatives, and surfactants, evidencing the resistance of FLA in different environments.

Table 2 | Analysis of the identity of summer clones

Clone	Scientific name/genotype	Sequence compared in GenBank and identity (%)	Identity GenBank record
BN_AFLU_B_F	*Acanthamoeba lenticulata*/T5	MF076633.1 (95.40%)	OR243669
BN_EFLU_B_F	*Acanthamoeba lenticulata*/T5	MF076633.1 (95.40%)	OR233173
NA_AFLU_C_R	*Acanthamoeba polyphaga*/T4	KT892910.1 (99.27%)	OR288588
NA_EFLU_C_R	*Acanthamoeba polyphaga*/T4	KY934458.1 (99.30%)	OR288650
SE_AFLU_C_F	*Acanthamoeba polyphaga*/T4	KY934458.1 (99.30%)	OR289670
SE_EFLU_A	*Acanthamoeba polyphaga*/T4	KR780551.1 (99.06%)	OR364831
SE_EFLU_C_F	*Acanthamoeba polyphaga*/T4	HQ833414.1 (99.49%)	OR241303
SA_AFLU_B_F	*Acanthamoeba polyphaga*/T4	KT892910.1 (99.76%)	OR288579
SA_EFLU_A_F	*Acanthamoeba polyphaga*/T4	HQ833414.1 (99.50%)	OR241300
RB_AFLU_A_F	*Acanthamoeba polyphaga*/T4	KT892910.1 (99.53%)	OR288523
RB_EFLU1_B_F	*Acanthamoeba polyphaga*/T4	KT892907.1 (97.52%)	OR241297
RB_EFLU2_A_R	*Acanthamoeba polyphaga*/T4	MF576062.1 (99.49%)	OR241299
LA_AFLU_B_F	*Acanthamoeba polyphaga*/T4	KT892907.1 (99.25%)	OR241295
LA_AFLU_A_F	*Acanthamoeba polyphaga*/T4	KT894164.1 (97.86%)	OR241292
LA_AFLU_B_F	*Acanthamoeba polyphaga*/T4	KY934458.1 (99.28%)	OR241289
BO_AFLU_C_F	*Acanthamoeba polyphaga*/T4	KT892907.1 (99.28%)	OR241293
BO_EFLU_C_F	*Acanthamoeba polyphaga*/T4	HQ833414.1 (99.49%)	OR241286
AR_AFLU_A_F	*Acanthamoeba polyphaga*/T4	KT892907.1 (99.51%)	OR241283
AR_EFLU_B_F	*Acanthamoeba polyphaga*/T4	KT892907.1 (99.75%)	OR241284

AFLU, affluent; EFLU, effluent; F, forward; R, reverse.

Table 3 | Analysis of the identity of winter clones

Clone	Scientific name/genotype	Sequence compared in GenBank and identity (%)	Identity GenBank record
BN_AFLU_A_F	*Acanthamoeba polyphaga*/T4	JQ408989.1 (98.51%)	OR241290
BN_EFLU_A_F	*Acanthamoeba polyphaga*/T4	KX688025.1 (98.24%)	OR241302
NA_AFLU_A_F	*Acanthamoeba polyphaga*/T4	KT892907.1 (99.75%)	OR241282
NA_EFLU_A_F	*Acanthamoeba polyphaga*/T4	KX688025.1 (95.44%)	OR241281
SE_AFLU_A_F	*Acanthamoeba polyphaga*/T4	KX688025.1 (99.24%)	OR241287
SE_EFLU_A_F	*Acanthamoeba polyphaga*/T4	KR780551.1 (98.25%)	OR241291
SE_EFLU_B_F	*Acanthamoeba polyphaga*/T4	KT892907.1 (99.28%)	OR241285
SA_AFLU_A_F	*Acanthamoeba polyphaga*/T4	HQ833414.1 (99.75%)	OR241280
SA_EFLU_A_F	*Acanthamoeba polyphaga*/T4	HQ833414.1 (99.75%)	OR241301
RB_AFLU_A_F	*Acanthamoeba polyphaga*/T4	KR780551.1 (97.75%)	OR241294
RB_EFLU1_A_F	*Acanthamoeba polyphaga*/T4	KR780551.1 (98.00%)	OR241298
RB_EFLU2_B_F	*Acanthamoeba polyphaga*/T4	KR780551.1 (98.25%)	OR241288
LA_AFLU_A_F	*Acanthamoeba polyphaga*/T4	KX688025.1 (98.73%)	OR241296
LA_AFLU_A_F	*Acanthamoeba polyphaga*/T4	KR780551.1 (97.75%)	OR233179
BO_AFLU_A_F	*Acanthamoeba polyphaga*/T4	HQ833414.1 (98.49%)	OR233178
BO_EFLU_A_F	*Acanthamoeba polyphaga*/T4	KT892907.1 (99.49%)	OR233175
AR_AFLU_A_F	*Acanthamoeba polyphaga*/T4	KR780551.1 (98.00%)	OR233176
AR_EFLU_A_F	*Acanthamoeba polyphaga*/T4	KX688025.1 (98.48%)	OR233174

AFLU, affluent; EFLU, effluent; F, forward; R, reverse.

Table 4 | Reference sequences used to build the phylogenetic tree

BLASTn/accession	Source	Strain/isolation source	Size (pb)
MF076633.1	*Acanthamoeba lenticulata* T5	Hospital environment	364
KT892910.1	*Acanthamoeba polyphaga* T4	Environmental	452
KT892907.1	*Acanthamoeba polyphaga* T4	Environmental	451
KX688025.1	*Acanthamoeba polyphaga* T4	Hospital environment	405
HQ833414.1	*Acanthamoeba polyphaga* T4	Hospital environment	399

Note: The sequences used for the construction of the phylogenetic tree were based on their similarity and/or similar isolation conditions to this work.

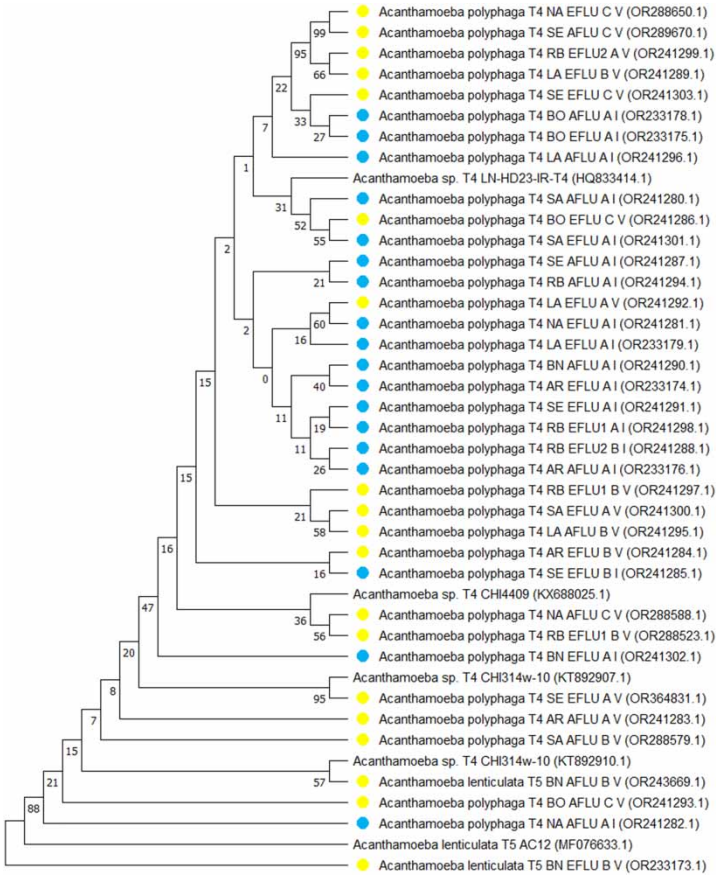


Figure 5 | The evolutionary relationship of *Acanthamoeba* spp. was based on the sequence of Region ASA.S1. Summer clones are identified with yellow circles and winter clones are identified with blue circles.

Another study analyzed an STP at an oil refinery, an environment that is known to have pH variations and high loads of toxic components in the water; the study found species of *Acanthamoeba* with genotypes T4 and T11 (Saeid *et al.* 2022). These genotypes are known to cause infections in humans (Hajialilo *et al.* 2016).

Acanthamoeba polyphaga species is known to cause keratitis in humans (Marciano-Cabral & Cabral 2003; Schuster & Visvesvara 2004; Caumo *et al.* 2009), and the species *A. lenticulata* is associated with cases of keratitis and granulomatous amoebic encephalitis (Cabello-Vílchez 2015). Therefore, the presence of these species in the treated and untreated sewage in our work is worrying, as they may present a risk to individuals who use Lake Guaíba and River Gravataí for aquatic activities.

The presence of *Acanthamoeba* spp. in treated and untreated sewage has already been studied in some parts of the world such as South Africa, China, Egypt, Spain, the United States of America (USA), Iran, Mexico, the United Kingdom, and Taiwan (Hsu *et al.* 2009; Gaze *et al.* 2011; Muchesa *et al.* 2014; Ramirez *et al.* 2014; Garner *et al.* 2017; Lass *et al.* 2017;

Moreno-Mesonero *et al.* 2017; Salahuldeen *et al.* 2021; Saeid *et al.* 2022). However, so far in Brazil, no study has been carried out investigating potentially pathogenic FLA in STPs, with the present study being the first carried out in our country.

CONCLUSION

The cultivation of FLA was successful, showing the isolation of amoebae in all samples. Using morphological identification and the PCR technique, it was possible to identify the genus *Acanthamoeba* in all analyzed samples, being the species *A. polyphaga* and *A. lenticulata* with genotypes T4 and T5, respectively, identified both genotypes associated with human diseases.

Our study presents environmental and public health importance, since the FLA genus found is known to cause keratitis, granulomatous amoebic encephalitis, and skin and lung infections in humans, in addition to being known to carry pathogenic or non-pathogenic endocytobionts.

The aquatic environment, where treated sewage is discharged, is used for recreation by immunocompetent individuals or not, presenting a risk to these individuals, either by infections caused by the FLA themselves, or by diseases caused by possible endocytobionts carried by them. As it was the first study carried out on STPs in Brazil, it is suggested that further research be carried out in this environmental field to collect more data on these natural and anthropogenic environments.

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AUTHORS' CONTRIBUTIONS

T.C.B.S. conceived and wrote the project, collected and analyzed the data, and wrote the manuscript. D.L.d.S. performed data verification and revised the manuscript. M.B.R. managed the project and revised the manuscript. All authors approved the publication of this version of the manuscript.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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