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Evaluation of the occurrence of pathogenic free-living amoeba and bacteria in 20 public indoor swimming pool facilities

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Abstract

Recently, indoor swimming pool activities have increased to promote health-enhancing physical activities, which require establishing suitable protocols for disinfection and water quality control. Normally, the assessment of the microbial quality of the water in the pools only considers the presence of different bacteria. However, other less frequent but more resistant pathogens, such as free-living amoebas (FLA), are not contemplated in both existing recommendation and research activities. FLA represent a relevant human health risk, not only due to their pathogenicity but also due to the ability to act as vehicles of other pathogens, such as bacteria. Therefore, this work aimed to study the physicochemical characteristics and the occurrence of potentially pathogenic FLA and bacteria in water samples from 20 public indoor swimming facilities in Northern Portugal. Our results showed that some swimming pools presented levels of pH, free chlorine, and conductivity out of the recommended limits. Pathogenic FLA species were detected in two of the facilities under study, where we also report the presence of both, FLA and pathogenic bacteria. Our findings evidence the need to assess the occurrence of FLA and their existence in the same environmental niche as pathogenic bacteria in swimming pool facilities worldwide and to establish recommendations to safeguard the health of the users.

KEYWORDS
free-living amoeba, indoor swimming pools, Portugal, public health, water quality

1 | INTRODUCTION

Currently, to relieve the effects of a sedentary lifestyle and following World Health Organization (WHO) instructions, the 28 EU countries have adopted policies to promote health-enhancing physical activity (WHO, 2006; Breda et al., 2018; EuropeActive, 2019). As a result of such initiatives, swimming, and other activities in indoor swimming pools are among the practices that have significantly increased in popularity. Vulnerable populations such as children and the elderly are among the groups that typically attend such facilities all year-round. This intense use justifies the need for establishing more restrictive protocols to ensure proper hygiene and safety conditions in swimming pools. Especially, the quality of the water in the pool and of the indoor air in the facilities should deserve special attention.

The prevention of the proliferation of harmful microorganisms in water is typically based on the use of disinfection solutions, as the most commonly employed chlorine-based products (Gabriel et al., 2019; Teo et al., 2015). To ensure that the microbial and physiological inputs, typically added to the water by the bathers, are effectively neutralized health authorities require a constant disinfectant residual concentration in the water (Hwang et al., 2012; Mi et al., 2015; Moradi et al., 2017). However, since the disinfectants can react with the physiological inputs, potentially hazardous compounds, denominated disinfection byproducts (DBP), can be generated and constitute a relevant collateral chemical hazard, if not managed properly (Gabriel et al., 2019; Manasfi et al., 2017; Weisel et al., 2009).

In most of the European countries, health authorities have strived to control water quality in public swimming pools, focusing on physicochemical characteristics, residual disinfectant content, and the presence of different microorganisms, following recommendations from the European Bathing Water Directive (EBWD) (2006) (WHO, 2006). The risk of disease or infection associated with indoor swimming pools is mainly associated with water fecal contamination, resultant from feces released by the bathers, or from contaminated source water (Barna & Kádár, 2012). Human shedding (e.g., vomit, mucus, saliva, skin) into the pool water or surrounding area can also constitute a relevant non-fecal source of pathogenic organisms. Most swimming pool-related outbreaks result from the lack of proper disinfection protocols. A frequent control of the physicochemical quality of water (including turbidity, residual disinfectant, and pH levels) is thus pivotal to guarantee effective water disinfection and proper management of water quality. Outbreaks of recreational water-illness are moderately frequent and have been linked to infection induced by viruses, bacteria, protozoa, or fungi.

The microbial quality of pools and similar environments is typically controlled by the assessment of thermotolerant coliforms, *E. coli*, *Pseudomonas aeruginosa*, among others. Nevertheless, other less frequent but potentially more resistant pathogens are being neglected in routine water quality control. Even though free-living protozoa such as *Naegleria fowleri* and *Acanthamoeba* spp. are mentioned in the EBWD (2006) (WHO, 2006) as non-fecal protozoa, there is no control action established for the presence of these opportunistic protozoa. Free-living amoebae (FLA) present high resistance to chlorines, mainly due to the transformation into the cyst phase (Greub & Raoult, 2003; Marciano-Cabral & Cabral, 2003), and thus the possibility of FLA presence in pools should not be excluded. *Acanthamoeba* spp., *Balamuthia mandrillaria*, *Naegleria fowleri*, *Sappinia* spp., *Valhkampfia* spp. and *Vermamoeba vermiformis* are opportunistic parasites belonging to the FLA group (Niyyati et al., 2010; Qvarnstrom et al., 2009; Scheid et al., 2019; Visvesvara et al., 2007). Since these can exist both as free-living organisms and within a host, they are classified as amphizoic protozoa, which have been isolated from numerous environmental matrices, including water, soils, and dust (Khan, 2006; Shuster & Visvesvara, 2004; Teixeira et al., 2009). FLA present two stages in their life cycle: the trophozoite or vegetative form, through which they feed or multiply, and, as it has been mentioned above, the cyst stage, more resistant to harsh conditions (Lloyd et al., 2001). The most common pathologies induced by these protozoa in humans are encephalitis, keratitis, or other epithelial disorders (Qvarnstrom et al., 2009; Scheid et al., 2019; Visvesvara et al., 2007).

Furthermore, FLA contribute to biofilm formation by establishing close contact with several bacteria (Khan, 2006; Preston et al., 2001; Scheid et al., 2008). From this FLA-bacteria interaction, two major events can occur: i) FLA can use bacteria as a feeding source, and ii) FLA can be infected and colonized by amoeba-resisting bacteria. The last can protect pathogenic bacteria from the common antimicrobial processes (Gomes et al., 2020). The study of the FLA presence has been importantly increased due to these infectious characteristics within water environments and their ability to cause serious infections among humans (Abdul-Majil et al., 2017). *Acanthamoeba* spp., *Dictyostelium discoideum*, *Vermamoeba vermiformis*, and *Naegleria gruberi* are some of the FLA species reported as bacterial hosts (Balczun & Scheid, 2017; Denoncourt et al., 2014; Scheid, 2014; Smirnov et al., 2011). Amoeba-resisting bacteria will gain refuge in these resistant hosts, where they can proliferate and be protected from hostile external conditions (Guimaraes et al., 2016; Strassmann & Shu, 2017). Bacteria such as *Mycobacterium* can resist inside FLA; *Vibrio cholerae* is capable to multiply internally; and *Legionella* or *Listeria* produce an amoeba cell lysis after multiplication (Ben Salah et al., 2009; Moliner et al., 2010; Thomas et al., 2010). Other studies have also demonstrated that the culture of *Campylobacter jejuni*, *Vibrio harveyi*, or *Salmonella typhi* in the presence of *Acanthamoeba* spp. increases bacterial survival (Douesnard-Malo & Daigle, 2011; Reyes-Batlle et al., 2017; Reyes-Batlle et al., 2017).

Although the growing body of evidence on their toxic and disinfection-resistant characteristics and some recent studies reporting a positive detection of FLA in swimming pools (Abdul-Majid et al., 2017; Ghasemi et al., 2019; Papadopoulou et al., 2008; Poor et al., 2018), at the time, the evaluation of the presence of FLA (and internalized bacteria) is not included in the national and international swimming pool sanitary surveillance programs. To be able to determine the need for inclusion of FLA in water quality assessment plans, further studies are needed to better understand the prevalence of FLA in swimming pool environments and its putative impact on health. Thus, this work determined the physicochemical quality and the occurrence and distribution of potentially pathogenic FLA species in water collected from the pools and shower rooms of 20 public indoor swimming pools located in the northern region of Portugal. It is expected that this work will contribute to ascertaining if FLA can represent a health risk to swimming pool users.

2 | MATERIALS AND METHODS

2.1 | Description of the evaluated facilities, study design, and water sampling procedures

Seventeen Municipalities and one sporting entity responsible for managing 20 public indoor swimming pools located in the northern region of Portugal kindly agreed to collaborate in the study. The

details of the location and characteristics of the participating facilities were previously reported by Gabriel et al., 2019. Fieldwork in the 20 facilities under study was conducted in two sampling campaigns—January to March (cold season campaign) and May to July (warm season campaign) 2018. The samples were taken from the biggest pool in each facility twice per day: in the morning (during the first activity of the day) and the afternoon (during the swimming activity, as there is an expected higher number of users in a swimming pool).

Water samples were collected in duplicate from a depth of 10 to 20 cm below the pool water surface and at least 30 cm of the sidewall from one edge of the pool under study, using the following:

(i) A 500-mL container for direct measurement of a set of physicochemical indicators;
(ii) 25-mL amber glass bottles containing traces of sodium thiosulfate (added to neutralize the residual disinfectant) for analysis of trihalomethanes (THM) content; and
(iii) 1000-mL sterile bottles for collecting water to be used for microbiological analyses.

The number of bathers in the pool during sampling procedures was registered.

However, during the warm season, water samples were also collected in one shower room (handwash tap, female or male shower room in accordance with the gender of the researcher who executed the sampling work) in each of the 20 facilities. In facilities where a positive detection of FLA was found, additional sampling (2nd screening) was performed in November 2018 (2 case studies) to evaluate the presence of FLA throughout whole facilities. To go in-depth in the assessment of these 2 "case studies," in addition to the largest pool and handwash tap water from one shower room, water samples from the small swimming pool, toilet tap, female and male shower rooms (hand wash and shower taps), footbath and footbath tap were also analyzed.

After sampling, all bottles were sealed and maintained at 4°C until their processing. In the case of samples for THM analysis, tubes were sealed with plastic screw caps, combined with a Teflon septum, to avoid the volatilization of the compounds in the sample and the samples were analyzed within 24h. For the evaluation of microorganisms, the samples were immediately refrigerated and transported to the *Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias* (Tenerife, Spain), which conducted their analysis as soon as they arrived in the lab (typically 2 to 4 days after sampling).

2.2 | Physicochemical characterization and analysis

First, a set of physicochemical parameters, including pH, temperature (°C), conductivity (µs/cm), total dissolved solids (ppm), and salinity (ppt) were measured in the water using a Eutech Elite PCTS tester (Thermo Fisher Scientific, Singapore). The equipment provides an accuracy of ±0.5°C for temperature, ±0.01 for pH, and ±1% for conductivity, total dissolved solids, and salinity. Free chlorine levels (mg/L in the pools were obtained from the routine internal records of the maintenance staff of the facilities. Water samples for THM determination were transported to and analyzed in the Department of Environmental Health of the National Health Institute Dr. Ricardo Jorge (INSA), located in Porto, Portugal, by gas chromatography coupled to an electron capture detector. The limit of quantification was 5 µg/L for each THM. The calibration curve was linear in the range 5–100 µg/L, presenting a correlation coefficient of at least 0.99 for all THM species. The THM levels in the water were calculated as the sum of chloroform, bromodichloromethane, dibromochloromethane, and bromoform concentration values.

2.3 | Microbiological analysis

2.3.1 | Sample processing

The samples were filtered using a vacuum manifold system and 0.45 µm nitrocellulose filters (Pall, Madrid, Spain), and the filters were seeded inverted onto a 2% non-nutrient agar (NNA) plate and monitored daily to evaluate FLA presence (Lorenzo-Morales et al., 2005; Reyes-Batlle et al., 2015). The plates where FLA growth was detected were subcultured into a clean NNA plate, to extract their DNA as previously described (Reyes-Batlle et al., 2015), by placing 1–2 mL of washed amoeba culture directly into the Maxwell® 16 Tissue DNA Purification Kit sample cartridge (Promega, Madrid, Spain).

2.3.2 | Pathogenic bacteria detection

To evaluate the presence of the most common pathogenic bacteria related to FLA, four selective agar media were used: *E. coli*-Coliforms Chromogenic Medium (BOE) Conda®; TCBS agar (ISO) VWR Chemicals Prolabo®; SS Agar Merck® and Cetrimide Agar (Pseudomonas selective Agar) Scharlau®. One hundred milliliters of water samples were filtered through 0.45 µm nitrocellulose filters (Pall, Madrid, Spain). Individual membranes were placed into a specific selective medium and incubated for 24h at 37°C depending on the microorganism to be detected.

2.3.3 | Free-Living Amoeba molecular characterization

PCR amplification was conducted using 18S rDNA gene primers: FLA-F (5′- CGCGGTAATTCCAGCTCCAATAGC −3) and FLA-R (5′-CAGGTTAAGGTCTCGTTCGTTAAC −3′) (Tsvetkova et al., 2004)

and VAHL- (5´- GTCTTCGTAGGTGAACCTGC- 3´) and VAHL-R (3´- CCGCTTACTGATATGCTTAA- 5´) (De Jonckheere & Brown, 2005). For FLA primers, PCRs, amplification reactions were performed in a 50 µL mixture containing 80 ng DNA, and the PCRs were performed in 40 cycles with denaturation (95° C, 30 s), annealing (50° C, 30 s), and primer extension (72°C, 30 s). For VAHL primers, PCRs, amplification reactions were performed in a 50 µl mixture containing 60 ng DNA, and the PCRs were performed in 35 cycles with denaturation (95° C, 60 s), annealing (55° C, 90 s), and primer extension (72°C, 120 s). After the last cycle, a primer extension was maintained for 7 min at 72° C. Amplification products from all PCRs were analyzed using electrophoresis through a 2% agarose gel, and positive PCR products were sequenced using Macrogen Spain service (Avda. Sur del Aeropuerto, Madrid, Spain).

Sequences were aligned using the Mega X software (Tamura et al. 1998; Kumar et al., 2018). The 18S rRNA molecular phylogenetic analysis was performed using the maximum likelihood method (Tamura & Nei, 1993). The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were 2015 positions in the final dataset. Species identification was based on sequence homology analysis by comparison to the available DNA sequences in the GenBank database.

2.4 | Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 25). Data normality was checked using the Shapiro–Wilk test. Non-parametric tests were applied for variables that did not meet the normality assumptions. The t-test and Mann–Whitney U test were used to assess significant seasonal differences. Regarding the evaluation of the differences among sampling periods, t-test and Wilcoxon tests were performed. The existence of significant correlations was assessed using Pearson and Spearman methods.

3 | RESULTS

3.1 | Physicochemical characterization of the swimming pool water

The average number of bathers engaged in activities in the swimming pool during sample collection was 10 for the period of the first activity of the day and 22 in sampling conducted in the high-attendance period. Concerning the physicochemical water quality indicators, the measured levels were diverse across the 20 swimming pools (Table 1). The pH levels are worth highlighting as 15 of the 20 swimming pools studied presented at least one registry above the recommended national limit (pH 6.9–8.0, DGS, 2009). Most of the pH values out of the recommended ranges were obtained in the samples collected in the warm season (cold season: 1; warm season: 15). Regarding water temperature, 6 swimming pools presented values slightly above the recommended ideal temperature (≤ 30 °C, DGS, 2009), that were consistently obtained in the two seasonal sampling campaigns (cold season: 5; warm season: 6). For free chlorine levels retrieved from the internal records of the facilities, in 8 swimming pools studied, values out of the recommended value ranges (0.5–1.2 mg/L for 6,9> pH ≤7,4 and 1.3–2-0 mg/L for 7,5> pH ≤8,0, DGS, 2009) were found. Also, values exceeding guidelines for water conductivity (1500 µS/cm, DGS, 2009) were detected for 5 swimming pools. The total THM concentrations fulfilled the national recommendations of 100 µg/L, ranging from less than 5 to 99 µg/L in the pool water.

The investigation of seasonal variations showed statistically significant differences only for THM concentrations (First activity: U = 116.50, z = −2.26, p = 0.023; High-attendance period: U = 114.00, z = −2.33, p = 0.019) and pH (First activity: $t(38)$ = −5.42, p < 0.001; High-attendance period: $t(38)$ = −4.92, p < 0.001). This suggests that the water pH and THM levels are significantly increased in the warm season. Statistically significant differences were observed for pH ($t(39)$ = 2.74, p = 0.009), temperature ($t(39)$ = −3.62, p = 0.001) and

TABLE 1 Descriptive statistics and seasonal differences for the physicochemical parameters measured in the water samples collected in 20 indoor swimming pools.

	Cold season[a]		Warm season[b]		*p*-value	
Parameter	First activity Mean (Range)	High-attendance Mean (Range)	First activity Mean (Range)	High-attendance Mean (Range)	First activity	High-attendance
pH	7.80 (7.28–8.24)	7.78 (7.35–8.24)	8.29 (7.54–8.78)	8.20 (7.54–8.78)	**0.000**	**0.000**
Temperature (°C)	29.0 (26.2–31.1)	29.1 (26.5–31.3)	29.3 (26.6–31.2)	29.4 (26.9–31.3)	0.461	0.460
Conductivity (µs/cm)	1810 (253–>LOQ)	1822 (262–>LOQ)	1812 (298–>LOQ)	1815 (299–>LOQ)	0.941	0.947
TDS (ppm)	1051 (172–>LOQ)	1059 (178–>LOQ)	1050 (203–>LOQ)	1052 (204–>LOQ)	0.947	0.947
Salinity (ppt)	0.89 (0.10–>LOQ)	0.88 (0.10–>LOQ)	0.89 (0.10–>LOQ)	0.89 (0.10–>LOQ)	0.825	0.803
Free Chlorine (mg/L)	1.52 (0.76–2.77)	1.41 (0.68–2.47)	1.61 (0.70–3.08)	1.58 (1.08–3.85)	0.365	0.427
THM (µg/L)	18.2 (<LOQ-67.0)	16.8 (<LOQ-65.0)	29.4 (6.7–98.6)	27.2 (6.3–96.5)	**0.023**	**0.019**

Note: [a]1st sampling campaign conducted from January to March 2018

[b]2nd sampling campaign conducted from May to July 2018 LOQ, the limit of quantification; n.a., not applicable; TDS, total dissolved solids; THM, trihalomethanes. LOQ values: 20000 µs/cm for conductivity; 10000 ppm for TDS, 10 ppt for salinity, and 5 µg/L for THM.

THM concentrations (z = 4.54, p < 0.001). The pH and THM levels were significantly greater during the first activity, while higher water temperature was registered in the afternoon high-attendance period. Considering all datasets, as it is shown in Table 2, pH levels are significantly and negatively associated with the measured water temperature values (r_s= −0.222; p = 0.048). Furthermore, a significant positive correlation was found between the concentrations of THM and the variation of some of the measured parameters, namely pH (r_s = 0.243, p = 0.030), temperature (r_s = 0.357, p = 0.001), conductivity/TDS (r_s = 0.396, p < 0.001) and salinity (r_s = 0.395, p < 0.001) (Table 2).

3.2 | Microbiological content of the collected water samples

3.2.1 | Sampling in the 20 swimming pools

During the two sampling campaigns (cold and warm seasons), 80 samples (60 from swimming pools, 20 from shower rooms) were evaluated (Table 3). No positive detection of FLA was obtained for water collected from swimming pools of the 20 study facilities. However, the presence of FLA was detected and identified in samples collected from the shower room's handwash tap water of the two facilities (2/20; 10%). Specifically, water samples from the shower rooms of the facilities SP01 and SP16 were positive for *V. vermiformis and V. planctonica* growth, respectively. Results from the bacterial growth analysis showed that from the analyzed groups/genera (coliforms, *Pseudomonas* spp., *Vibrio* spp, and *Salmonella* spp.) only *Vibrio* spp. growth was detected in the shower room tap water of SP14 and the swimming pool and the shower room tap samples of SP15 (Table 3).

3.2.2 | Case studies: a broader investigation of FLA-positive facilities

A more comprehensive sampling plan covering 8 water collection points in each facility was implemented in all FLA-positive facilities (SP01 and SP16) (Table 4; Figure 1). In the case of SP01 *V. vermiformis* and *A. griffini* T3 were detected in the women's shower room handwash tap water and the footbath tap water, respectively. *Vannella planctonica* in both the toilet and women's shower room shower tap water, and *Naegleria canariensis* in the footbath water were detected in the SP16 facility (Table 4; Figure 1). In this sampling campaign, also the positive detection of bacterial growth raised for the new 8 sampling sites included. In SP01 *Vibrio* spp. was detected in the male shower room shower tap, while all of the evaluated bacteria were detected in the footbath. In SP16, *P. aeruginosa* was detected in both swimming pools, the toilet tap, the female shower room shower tap, and in the footbath tap. Moreover, Coliforms, *Salmonella* spp., and *Vibrio* spp. were also detected in the footbath.

All the FLA sequences obtained present >90% of homology with the DNA sequences available in the GenBank database.

4 | DISCUSSION

Public swimming pools must implement strict water quality control plans to guide all maintenance actions needed for a proper level of disinfection to avoid any water-related microbiological and chemical risks for users. In this work, the assessment of physicochemical indicators across 20 chlorine-disinfected indoor swimming pools produced the following results: i) detected THM concentrations were consistent in compliance with the limit value recommended in Portugal and in most European countries (<100 µg/L), and ii) in some facilities levels of free chlorine, pH and conductivity were out of the recommended ranges. The first result shows that the levels of hazardous volatile DBP formed from chlorination processes are maintained at safe levels for users in the pool water. In contrast, the noncompliance with recommendations that were observed for other parameters may indicate a potentially compromised efficiency of inactivation and proliferation of pathogens.

Indeed, an important number of different pathogenic microorganisms can be inadvertently introduced in swimming pools by numerous environmental and human-related sources. Free-living

TABLE 2 Spearman and Pearson correlation coefficients for physicochemical parameters assessed in the 20 swimming pools.

	1.	2.	3.	4.	5.	6.	7.
1. pH	1						
2. Temperature	−0.222[a,b]	1					
3. Conductivity/TDS	−0.019	0.064	1				
4. Salinity	−0.036	.061	.989[d]	1			
5. Free Chlorine	0.081	.096	−.111	−.052	1		
6. N.º bathers	−0.153	.142	.172	.161	−.223	1	
7. THM	0.243[b]	.357[c]	.396[d]	.395[d]	.104	.106	1

TDS, total dissolved solids; THM, trihalomethanes.

[a]Pearson correlation *r* values; Remaining cases: Spearman correlation *rho* values.

[b]Correlation is significant at .05 level (2-tailed)

[c]Correlation is significant at .01 level (2-tailed)

[d]Correlation is significant at .001 level (2-tailed)

TABLE 3 Results from the microbiological evaluations of the water samples collected in the 20 indoor swimming pools and shower room taps (–: negative result; n.a.: not applicable).

		Sampling location/time		
		Swimming pool		
Season	Pool ID	Fist activity	High-attendance period	Shower Room tap
COLD[a]	SP01	–	–	n.a.
	SP02	–	–	n.a.
	SP03	–	–	n.a.
	SP04	–	–	n.a.
	SP05	–	–	n.a.
	SP06	–	–	n.a.
	SP07	–	–	n.a.
	SP08	–	–	n.a.
	SP09	–	–	n.a.
	SP10	–	–	n.a.
	SP11	–	–	n.a.
	SP12	–	–	n.a.
	SP13	–	–	n.a.
	SP14	–	–	n.a.
	SP15	–	–	n.a.
	SP16	–	–	n.a.
	SP17	–	–	n.a.
	SP18	–	–	n.a.
	SP19	–	–	n.a.
	SP20	–	–	n.a.
Warm[b]	SP01	n.a.	–	*Vermamoeba vermiformis*
	SP02	n.a.	–	-
	SP03	n.a.	–	-
	SP04	n.a.	–	-
	SP05	n.a.	–	–
	SP06	n.a.	–	–
	SP07	n.a.	–	–
	SP08	n.a.	–	–
	SP09	n.a.	–	–
	SP10	n.a.	–	–
	SP11	n.a.	–	–
	SP12	n.a.	–	–
	SP13	n.a.	–	–
	SP14	n.a.	–	+ *Vibrio* spp
	SP15	n.a.	+ *Vibrio* spp	+ *Vibrio* spp
	SP16	n.a.	–	*Vannella planctonica*
	SP17	n.a.	–	–
	SP18	n.a.	–	–
	SP19	n.a.	–	–
	SP20	n.a.	–	–

[a]1st sampling campaign conducted from January to March 2018

[b]2nd sampling campaign conducted from May to July 2018

TABLE 4 Results from the microbiological analysis of the water samples collected in the two FLA-positive case study facilities, SP01 and SP16, and respective identification of the isolated FLA species. The additional sampling for the 2 case studies was carried out in November of 2018.

Sample ID	Site	Coliforms	*P. Aeruginosa*	*Salmonella* spp.	*Vibrio* spp.	FLA species
SP01A	LP*	–	–	–	–	–
SP01B	SP	–	–	–	–	–
SP01C	TT	–	–	–	–	–
SP01D	FSR/HT*	–	–	–	–	*Vermamoeba vermiformis*
SP01E	FSR/ST	–	–	–	–	–
SP01F	MSR/ST	–	–	–	+	–
SP01G	FT	–	–	–	–	*Acanthamoeba griffini* T3
SP01H	F	>100	+	+	+	–
SP16A	LP*	–	+	–	–	–
SP16B	SP	-	+	–	–	–
SP16C	TT	–	+	–	–	*Vannella planctonica*
SP16D	FSR/HT*	–	–	–	–	*Vannella planctonica*
SP16E	FSR/ST	–	+	–	–	–
SP16F	MSR/HT	–	–	–	–	–
SP16G	FT	–	+	–	–	–
SP16H	F	50	–	+	+	*Naegleria canariensis*

Largest pool (LP)*; Smallest Pool (SP); Toilet Tap (TT); Female Shower Room Handwash Tap (FSR/HT)*; Female Shower Room Shower Tap (FSR/ST); Male Shower Room Handwash Tap (MSR/HT); Footbath Tap (FT); Footbath (F). *water sampling points that are common to those included in the previous warm season sampling campaign.

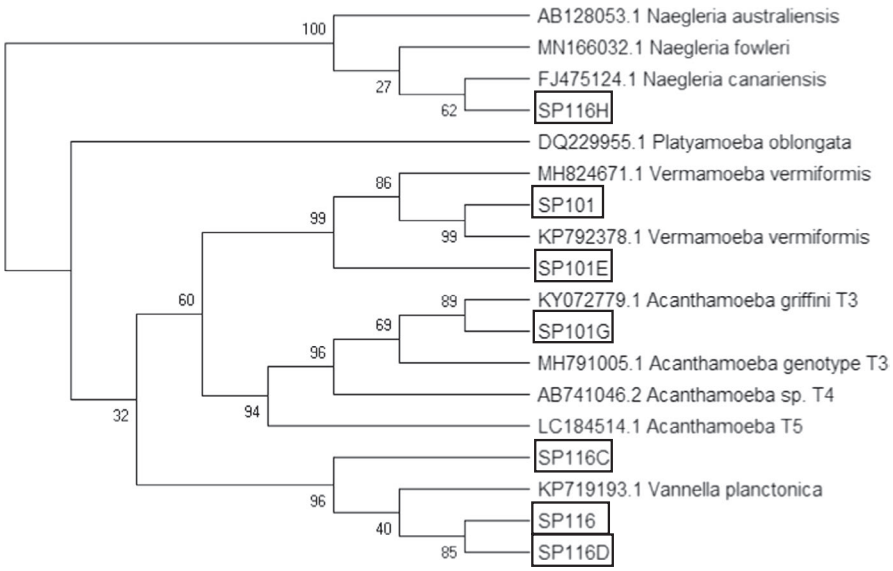

FIGURE 1 Relationship between the characterized FLA strains isolated in the present study. The 18S rRNA molecular phylogenetic analysis was performed using the maximum likelihood method (Tamura & Nei, 1993). The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2015 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The isolates obtained in this study are specified in boxes. The type sequences were taken from GenBank.

aquatic bacteria and amoeba can survive in pool water, components, or on wet surfaces in swimming pool facilities (Papadopuolou et al., 2008). In the survey conducted in this work, none of the samples collected in the 20 swimming pools, which were investigated in two seasonal sampling campaigns, presented positive results for the presence of FLA. Nevertheless, the growth of FLA was detected only in water samples collected from the handwash tap water of the shower rooms of two of the 20 facilities surveyed. At this stage,

Vermamoeba vermiformis and *Vannella planctonica* were the amoeba species isolated. FLA-positive facilities were selected to be part of a second sampling plan, in which the assessment of amoeba was extended to a higher number of water sampling points. From this activity, we could confirm the presence of *V. vermiformis* and *A. griffini* at the handwash tap water of the female shower rooms from SP01 and SP16, respectively, in accordance with the results from the first screening (sampling campaign covering the 20 facilities). As the number of sampling points increased, in addition to the two FLA species detected in the first survey, *Acanthamoeba griffini* genotype T3 and *Naegleria canariensis* were also identified, mainly in samples collected from shower rooms and footbaths. *Acanthamoeba*, *Naegleria*, and *Vermamoeba* genus are among the most common FLA genera/species isolated from water bodies (Fouque et al., 2015; Lorenzo-Morales et al., 2015; Reyes-Batlle et al., 2016a, b; Reyes-Batlle et al., 2019; Reyes-Batlle, Wagner, et al., 2017). *Acanthamoeba* spp., *Vermamoeba* spp. and *Vannella* spp. are included in the Lobosea class (Smirnov et al., 2011), which is demonstrated in figure 1, where the sequences from isolated Lobosea strains present a higher homology grade with the Lobosea type sequences used in this study. However, the isolated *N. canariensis* has been linked to other type sequences of *Naegleria* spp., which belong to the Heterolobosea class (De Jonckheere, 2011).

N. fowleri, *V. vermiformis*, *Acanthamoeba* spp. have been described as causal agents of fatal encephalitis (Centeno et al., 1996; De Jonckheere, 2011; Visvesvara et al., 2007). There is a previous study related to *Acanthamoeba* presence in Portugal: *Acanthamoeba* spp. was detected in corneal scrapes from patients diagnosed with infectious keratitis by Oliveira-Ferreira and colleagues in 2019. However, this is the first report of *Acanthamoeba* in swimming pools in Portugal. Additionally, this work constitutes the first report of *Vermamoeba vermiformis*, *Vannella planctonica*, and *Naegleria canariensis* in Portugal.

The pathogenic potential of *V. vermiformis* is determined by its capability to produce an infection by itself (Centeno et al., 1996; Scheid et al., 2019) and by its numerous reported relationships with pathogenic bacteria such as *Stenotrophomonas maltophilia* and *Legionella* spp. (Pagnier et al., 2015). *V. vermiformis* has been isolated from natural and artificial pools (Javanmard et al., 2017) but, until now, there are no clinical cases related to its presence in these environments. Similarly, *N. fowleri* has been isolated from indoor swimming pools in different countries including Mexico (Rivera et al., 1983), Czech Republic (Kadlec et al., 1980) or New Zealand (Cursons et al., 1979). Recently, several cases of *N. fowleri*-induced fatal encephalitis (Primary Amoebic Encephalitis, PAM) among pool bathers have been reported worldwide (Abrahams-Sandí et al., 2015; Mavridou et al., 2018; Wagner et al., 2017). In this investigation, the less common *Naegleria* species, *N. canariensis*, was isolated in the footbath of one facility. *N. canariensis* was first reported and named by De Jonckheere (2006) for an isolate from the Canary Islands, Spain. It has been isolated in recreational waters from Taiwan (Huang & Hsu, 2010) and in river water samples from Brazil (Bellini et al., 2020) but currently, there is no report of *N. canariensis* pathogenicity.

Acanthamoeba spp. Is the most abundant FLA genus isolated in environmental and clinical samples worldwide (Lorenzo-Morales et al., 2015; Siddiqui & Khan, 2012) and natural and artificial pools (Al-Herrawy et al., 2014; Bunsuwansakul et al., 2019; Rivera et al., 1983; Teixeira et al., 2009; Visvesvara, 2013). The most common pathology produced by *Acanthamoeba* spp. is *Acanthamoeba* keratitis (AK), which consists of a severe sight-threatening ocular infection (Lorenzo-Morales et al., 2015). The number of AK cases has increased recently not only due to the rise of contact lens wearers but also due to significant research in the field and consequent improvements of diagnostic techniques (Lorenzo-Morales et al., 2015). The Granulomatous Amoebic Encephalitis (GAE) case, associated with swimming in different artificial and non-artificial pools of Lima, Perú, is the most threatening disease induced by *Acanthamoeba* reported so far (Cabello-Vílchez et al., 2020). Twenty-two *Acanthamoeba* genotypes (T1–T22) have been described, all with pathogenic potential (Corsaro et al., 2015; Tice et al., 2016) being the T4 genotype the most frequently identified in human infection cases (Castro-Artavia et al., 2017; Omaña-Molina et al., 2016). T3 genotype has been described as responsible for several amoeba-related clinical endpoints (Omaña-Molina et al., 2016). Thus, *Acanthamoeba griffini* genotype T3, isolated in the water from the footbath tap, is likely to represent an important health risk to users. Although water should not fall directly in the eyes, contaminated water drops could signify an important risk, particularly in contact lens wearers.

In this study, the *Vannella planctonica* strain was isolated in both the toilet tap and the female shower room handwash tap of one facility. *Vannella* spp. was previously isolated from freshwater, soil, brackish water, and others (Nazar et al., 2012; Smirnov et al., 2011) and corneal scrapings of a keratitis patient (Michel et al., 2000; Scheid 2007). However, few studies are investigating the link between environmental *Vannella* isolates and clinical cases. Importantly, this genus can constitute a relevant risk for humans due to its documented capability of acting as a Trojan horse for microbial organisms, such as microsporidian parasites, supporting their endosymbiont proliferation (Hofmann et al., 1998; Michel et al., 2000).

Also, although with a small prevalence, pathogenic bacteria, including *Vibrio* spp., *Salmonella* spp. and Coliforms, were detected in water samples collected in the 20 indoor swimming pool facilities. In the cross-sectional study conducted in this work, *Vibrio* spp. was detected in water samples collected from one swimming pool (1 of 60 samples) and tap water from shower rooms (2 of 20 samples) (Table 3). In the two FLA-positive case studies *Vibrio* spp. was also found in the shower tap of one shower room (SP01F) and both footbaths analyzed (SP01H and SP16H) (Table 4). Several Vibrio species are pathogenic to humans producing several gastrointestinal disorders (Kunkle et al., 2020). *Salmonella* spp. and Coliforms were detected only in footbaths (2 out of two) (Table 4). These bacterial groups are causal agents of several gastrointestinal disorders and are markers of the inefficacy of water disinfection methods (Barna & Kádár et al., 2012). Although all water samples collected from the cross-sectional study in the 20 swimming pools produced negative results for the presence of *Pseudomonas* spp, in the two case studies, this bacterial agent was detected in a footbath

of SP01 facility, and the two pools and tap water collected from a toilet, shower room and footbath of the SP16. Although *P. aeruginosa* was isolated from the tap water of the SP16 footbath (SP16G), it was not isolated directly from the footbath receptacle (SP16H). This observation can be related to: i) the existence of residual disinfectant in the receptacle that is regularly used (at least 2 times per day) to clean the footbath surfaces, and ii) the ability of *Pseudomonas* spp. to form biofilms, and water sampling did not scrape the surface of the footbath. Bacterial species such as *Vibrio* spp. (Espinoza-Vergara et al., 2020) or *Pseudomonas* spp. (Ilk et al., 2020) can form biofilms involving an infection hazard. Biofilms are microbial-derived sessile communities, which can be formed in aqueous environments and offer a protective environment for bacteria, preventing the action of antimicrobials and evading host defense mechanisms (Sethupathy et al., 2016; Wang et al., 2012). In swimming pool-related environments, biofilms can be formed in the "air–water" interface or water in the surroundings of the pools (Preston et al., 2001), increasing significantly the cell attachment (Davis et al., 1981). Moreover, biofilm formation contributes to the establishment of FLA cultures in the environment (Khan, 2006; Preston et al., 2001; Scheid et al., 2008) and, mutually, FLA could act as a protective host for some bacterial species. FLA are opportunistic and pathogenic protozoa, but also are capable of acting as vehicles of other pathogens such as bacteria and viruses (Balczun & Scheid, 2017; Pagnier et al., 2015; Scheid, 2014; Thomas et al., 2010). Bacterial species capable of avoiding amoeba digestion will gain a shelter where they could be able to proliferate protected from hostile external conditions (Guimaraes et al., 2016; Strassmann & Shu, 2017). Although *Acanthamoeba* have been linked to biofilm formation and have been reported *to* exhibit a high binding affinity to *Pseudomonas* colonized biofilm (Lorenzo-Morales et al., 2015), in this study this co-existence setting was not verified. However, in this study, we have demonstrated the existence of pathogenic bacteria and free-living protozoa in the same two environmental niches from the SP16 facility (Toilet tap water, SP16C, and footbath water, SP16H). While in the toilet tap water (SP16C) *P. aeruginosa* and *Vannella planctonica* were jointly isolated, we could isolate *N. canariensis* together with Coliform species, *Salmonella* spp. and *Vibrio* spp. from the footbath water sample (SP16H). It has been reported that different *Naegleria* strains present permissiveness with some pathogenic bacterial species such as *Pseudomonas* spp. (Hoffmann & Michel, 2001; Muchesa et al., 2017) and *Vibrio* spp. (Thom et al., 1992). Even though *N. canariensis* has not been established as a human pathogen, the possibility of health risks to humans, namely, due to the ability to act as a vehicle for pathogenic bacteria should not be disregarded. These results agree with previous reports on FLA and bacteria reciprocal relation, corroborating the co-existence in water collected from public swimming pools and highlighting the importance of establishing control procedures for bacteria and amoeba in water-related environments. Intracellular survival of *Vibrio* species has been reported in various eukaryotic cells, including in *Acanthamoeba* spp. and *Naegleria gruberi* (Abd et al., 2007; Thom et al., 1992). Moreover, in this study we have reported the existence in the same water sample (SP16H) of *Vibrio* spp. and the free-living protozoa *N.canariensis*. Bacteria are considered the main food source of FLA species (Scheid et al., 2014). Nevertheless, some of these microorganisms have gained adaptive strategies to survive the amoeba intracellular conditions, avoiding harsh external conditions, being protected from negative environmental influences, and taking advantage of the dispersal in the environment by their amoebic host (Scheid et al., 2014). Therefore, the presence of FLA species and pathogenic bacteria in the same environmental niche always means a remarkable health risk.

5 | CONCLUSIONS

Overall, the findings of this work support that:

The maintenance staff should assess the physicochemical properties of the pool water more frequently and act to ensure strict compliance with the recommended range of values and to maintain proper water quality conditions. An elevated conductivity was found in 25% of swimming pools, in relation to recommendations. Since this is a recognized indicator of water aging and improper bather load, tighter control of this parameter is particularly advised to ensure an adequate water renovation rate.

Although FLA was not identified as a priority microbiological parameter to be included in chlorine-disinfected swimming pool water quality assessment plans, FLA should be monitored in other water sources within swimming pool facilities such as heated water taps and footbaths.

Footbaths are particularly important reservoirs of pathogens, including FLA and bacteria, and thus, the establishment of stricter recommendations for the disinfection of footbaths is pivotal in the mitigation of FLA and bacterial-related health risks. Since the water of footbaths was analyzed only in the two FLA-positive case facilities in this study, further investigations with a larger sample size are strongly recommended.

Besides, there is a need to keep the investigation related to the control of amoebic presence in swimming pool facilities worldwide, as well as to create new strategies to improve the elimination of these pathogenic protozoa. Likewise, it is crucial to limit their sources of contamination and those niches that could favor their establishment, such as water and air–water biofilms.

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CONFLICT OF INTERESTS

None declared.

ETHICS STATEMENT

None required

AUTHOR CONTRIBUTION

María Reyes-Batlle: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Software (equal); Validation (equal); Visualization (equal); Writing-original draft (equal); Writing-review & editing (equal). **Marta F Gabriel:** Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing-original draft (equal); Writing-review & editing (equal). **Rubén Leocadio Rodríguez-Expósito:** Investigation (equal); Methodology (equal). **Fátima Felgueiras:** Investigation (equal); Methodology (equal). **Ines Sifaoui:** Investigation (equal); Methodology (equal); Writing-review & editing (equal). **Zenaida Mourão:** Software (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing-review & editing (equal). **Eduardo de Oliveira-Fernandes:** Project administration (equal); Resources (equal); Software (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing-review & editing (equal). **José E Piñero:** Project administration (equal); Resources (equal); Software (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing-review & editing (equal). **Jacob Lorenzo-Morales:** Project administration (equal); Resources (equal); Software (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

All the FLA sequences obtained were deposited in the GenBank database under the accession numbers MT274371 - MT274376 and MW377585: https://www.ncbi.nlm.nih.gov/nuccore/MT274371,MT274372,MT274373,MT274374,MT274375,MT274376,MW377585

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