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Presence and interaction of free-living amoebae and amoeba-resisting bacteria in water from drinking water treatment plants

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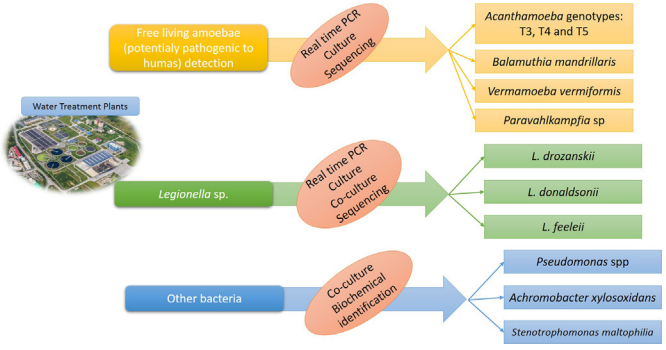
HIGHLIGHTS

- *Acanthamoeba* is distributed throughout DWTPs treatment line, resisting elimination.
- *Acanthamoeba* T5 is reported for the first time in waters from the Castilian Plateau.
- Decrease of free *Legionella* is compensated by an increase in infected *Acanthamoeba*.
- Co-culture allows detection of ARB in treated water, even at low concentration.

GRAPHICAL ABSTRACT



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ABSTRACT

Free-living amoebae (FLA) are ubiquitous and many isolates have been shown to be infected with amoeba-resisting bacteria, as the example of *Acanthamoeba* and *Legionella* interaction. Due to the high environmental prevalence of *Acanthamoeba*. in the Castilian Plateau (Spain), the aims of this work were to investigate the occurrence of *Acanthamoeba* and other FLA in water from several sampling points from four Drinking Water Treatment Plants (DWTP) and to investigate the presence of *Legionella* spp. and other amoeba-resisting bacteria in biofilms in raw and finished water, taking into account that no legislation exists for this protozoa control. *Acanthamoeba* was detected at different sampling points, and sand filters seemed to contribute to amoebic enrichment. After ozonation, a temporary decrease in viable amoebae was observed. The genotypes detected were T3, T4, and T5, revealing the first report of genotype T5 in waters from this region. Moreover, *Balamuthia mandrillaris*, *Vermamoeba vermiformis* and *Paravahlkampfia* sp. were detected. Regarding *Legionella*, PCR detection in raw and finished water was higher than by agar culture, but even higher after *Acanthamoeba* co-culture. Also, *Legionella's* presence was higher in raw water than in finished water. The decrease of free *Legionella* observed from raw (27.5%, by PCR) to finished water (3.4% by PCR) contrasted with the increase of *Legionella*-infected FLA from raw (30.7%) to finished water (52%). At biofilm, free *Legionella* was not detected, and the percentage of infected FLA was low (3.8%). *Legionella* species identified in these samples were *L. drozanskii*, *L. donaldsonii* and *L. feeleii*. Additionally, *Acanthamoeba* co-culture led to the isolation of *Pseudomonas aeruginosa*, *P. stutzeri*,

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P. fluorecens, *Achromobacter xylosoxidans* and *Stenotrophomonas maltophilia*. The highly disseminated presence of *Acanthamoeba* and the detection of amoeba-resisting bacteria inside amoebae highlight the importance of developing methods for controlling FLA in order to limit human pathogenic amoeba-resisting bacteria survival to the water purification processes.

1. Introduction

Free-living amoebae (FLA) are a heterogeneous group of protozoa ubiquitously found in nature, comprising several genera such as *Acanthamoeba*, *Balamuthia*, *Naegleria*, *Vermamoeba*, *Paravahlkampfia*, etc. Among these amoebae, *Acanthamoeba*, *Naegleria fowleri,* and *Balamuthia mandrillaris* appear as the most commonly related to infections in humans and other animals (Dupuy et al., 2014; Magnet et al., 2013b; Visvesvara et al., 2009). Those amoebae present at least two developmental stages, a vegetative feeding form known as trophozoite, and a resistant cyst form that provides protection to harsh environmental conditions, such as changes in temperature, pH or even biocides and disinfectant treatments, such as chlorine (Greub and Raoult, 2004; Thomas et al., 2008; Tsvetkova et al., 2004). Indeed, FLA and other eukaryotes have been isolated from various environmental niches and man-made structures, for example, domestic water networks, hospital water networks and drinking water treatment plants (Inkinen et al., 2019; Marciano-Cabral and Cabral, 2003; Thomas et al., 2008).

In Spain, environmental studies have demonstrated that the presence of *Acanthamoeba* appears to be particularly frequent in both raw and finished water of Drinking Water Treatment Plants (DWTP) and Wastewater Treatment Plants (WWTP), which correlates with the high environmental presence detected in samples from natural pools (Magnet et al., 2012, 2013b, 2015). These data suggested that the water treatment methods employed in the studied plants may not be completely effective in the elimination of those amoebae (Magnet et al., 2012, 2013b, 2015). Although there is no mention of a specific control on FLA presence neither in the Spanish water guidelines (RD 140/2003) nor in the international guidelines; it could be of great value to consider this in order to diminish the chances of human infection, either by direct contact or by contaminating objects used in intimate contacts, such as contact lenses. For example, a study conducted in this region detected a contamination rate of 49.5% in contact lenses from healthy individuals (Gomes et al., 2016).

Moreover, some studies have revealed *Acanthamoeba* colonizing structures inside DWTPs, such as in sand filters and granular activated carbon (GAC) filters. This capacity contributes to the challenge already presented to FLA elimination, in which the purification processes are able to reduce the number of organisms but not to provide an absolute barrier to these amoebae, even in well-operated treatment chains (Thomas et al., 2008).

FLA presence can represent a concern not only for its own pathogenic or opportunistic features but, because many amoebic isolates have been demonstrated to harbor endosymbionts. These endosymbionts could be viruses, yeast, protists, and bacteria of which, some are potential human pathogens (Konig et al., 2019; Siddiqui and Khan, 2012; Tanifuji et al., 2017). Thus, FLA can act as Trojan horses and/or "training grounds" to pathogens such as amoeba-resisting bacteria, protecting, and eventually increasing their pathogenicity (Thomas et al., 2006). Among the amoeba-resisting bacteria, there are obligate intracellular bacteria, facultative intracellular bacteria and bacteria without a previous known eukaryotic cell association. For example, *Pseudomonas* spp. are included in the last category, especially because these bacteria are known for their inhibitory effect on FLA growth in axenic cultures and patients with keratitis, but some *Pseudomonas* spp. apparently evolved to become amoeba-resisting bacteria as was demonstrated by the isolation of *Acanthamoeba* naturally infected with *P. aeruginosa*, an important fact given the role of this bacterium as a causative agent of pneumonia (Greub and Raoult, 2004).

However, the historical and most studied of those amoeba-associated pathogens is *Legionella pneumophila*. *Legionella* species have been associated with several protozoa, including FLA such as *Acanthamoeba* and *Vermamoeba* (Pagnier et al., 2015). *Legionella* spp. are the causative agent of Legionnaires' disease, an atypical severe pneumonia that may be accompanied by systemic symptoms. These bacteria have been associated with up to 4% of nosocomial and community-acquired pneumonia (CAP). In Europe, the last surveillance reported 6943 cases in 30 countries. Among the confirmed cases, *L. pneumophila* serogroup 1 appears as the most commonly identified pathogen, accounting for 81% of cultured-confirmed cases notified in 2014 (ECDC, 2016; Magnet et al., 2015). However, other *L. pneumophila* serogroups and other *Legionella* species have also been reported to be responsible for legionellosis. In fact, other *Legionella* spp. than *L. pneumophila* infections are under-reported due to the lack of specific diagnostic methods in hospitals (Vaccaro et al., 2016). A previous environmental study in Spain demonstrated a variety of *L. non-pneumophila* isolates in waters from DWTPs, many of those detected due to the *Acanthamoeba* co-culture methodology. Additionally, some FLA isolates from this study were demonstrated to harbor *Legionella*. Thus, *Legionella* featuring as amoeba-resisting bacteria may facilitate bacterial survival to the disinfection treatment employed in these plants (Magnet et al., 2015).

Moreover, some amoeba-resisting bacteria are known as *Legionella*-like amoebal pathogens (LLAP), which are primarily obligate intracellular parasites of amoeba with an ability to infect and multiply in amoebae in an identical way to *Legionella* spp. Some LLAPs have been hypothesized to be new potential pathogens as they are being recognized as new and unusual *Legionella* species with some of them being related to human infection (*L. drozanskii* -LLAP1-, *L. lytica* -LLAP 3, 7 and 9-, *L. drancourtii*- LLAP 4 and 12- among others) (Adeleke et al., 1996; Lamoth and Greub, 2009).

Due to the high environmental presence of *Acanthamoeba* in the Castilian Plateau (Spain), the aims of this study were to study 1) the presence of some selected FLA (*Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, *Vermamoeba vermiformis* and *Paravahlkampfia* sp.) in samples collected in different points along the water and residual sludge treatment line in four DWTPs from this region, in order to evaluate the influence of a specific treatment on the appearance or elimination of these amoebae; 2) the presence of *Legionella* spp. in biofilm, and in both raw and treated water from these DWTPs, as free bacteria or infecting FLA; and 3) the presence of *Pseudomonas* spp. in raw and treated water from these DWTPs, employing *Acanthamoeba* co-culture.

2. Materials and methods

2.1. Drinking-water treatment plants (DWTPs)

Four treatment plants from the Castilian Plateau area (Spain) were selected for this study. The selection criterion was defined to include plants with different purification processes, i.e., representing different treatment chains. The main characteristics of each plant are exposed shown and a graphic scheme of their water treatment line can be observed in Fig. S1 (Supplementary information).

2.1.1. DWTP 1

The most important characteristics of this plant are the employment of an Accelerator type decanter, an ozonation process, and a granular activated carbon (GAC) filter.

2.1.2. DWTP 2

This plant presents the classic purification process among the studied plants, its main characteristics are the employment of a lamellar decanter and a single filtration process with a sand filter.

2.1.3. DWTP 3

The third selected plant is presented as the state-of-the-art installation included in this study. Its main characteristics are the replacement of the conventional sand filtration by an ultrafiltration process and reverse osmosis.

2.1.4. DWTP 4

The last plant included in this study presented as its most important characteristic the employment of a Pulsator type decanter, in which the water with concentrated sludge at the bottom was partially purged at constant intervals.

2.2. Sample collection

A total of 378 water samples were collected from different regions inside the treatment plants during 2014 (DWTP 1, 2 and 3) and 2015 (DWTP 4). From three of the selected plants (DWTP 1, DWTP 2 and DWTP 4), each sampling point was sampled two times per season during a period of one year. In DWTP 3, each sampling point was sampled twice in winter and spring, but only once in summer and none in autumn due to technical issues. The detailed list of collection points for all DWTPs are shown in Table 1. For each sampling point, up to 60 L of water was collected. Water samples were concentrated by using the IDEXX® Filta Max system, following the manufacturer's instructions. A total of 7 mL was finally obtained from each concentrated sample and fractioned for various analyses (Fig. 1). Biofilm was also collected with the help of a sterile swab from the walls of the decanter. Later, swabs were immersed in sterile PBS for 24 h and centrifuged to concentrate the samples.

Table 1
List of samples collected in each DWTP, both in water and sludge line.

DWTP 1	DWTP 2	DWTP 3	DWTP 4
Water treatment line			
Raw water	Raw water	Raw water	Raw water
Pre-ozonated water	Pre-oxidized water	Pre-ozonated water	Pre-oxidized water
Decanted water	Decanted water	Decanted water	Decanted water
Decanter biofilm	Decanter biofilm	Decanter biofilm	Decanter biofilm
Sand filter surface water	–	–	Sand filter surface water
Sand-filtered water	Sand-filtered water	Ultrafiltered water	Sand-filtered water
Ozonated water	–	–	Ozonated water
GAC filter surface water	–	–	GAC filter surface water
	–	Inverse osmosis derived water	GAC-filtered water
Finished water	Finished water	Finished water	Finished water
Residual mud treatment line			
–	–	–	Decanter purged water
Sand filter rinsing water	Sand filter rinsing water	–	Sand filter rinsing water
Clarified water	Clarified water	Clarified water	Clarified water
Floater water	–	–	Floater water
–	Centrifugation residual water	Centrifugation residual water	–
Decanted sludge	Decanted sludge	Pre-dehydrated sludge	–
Dehydrated sludge	Dehydrated sludge	Dehydrated sludge	Dehydrated sludge

GAC: granular activated carbon.

2.3. FLA culture

Eighty microliters of concentrated water samples were inoculated onto 2% Neff's saline non-nutrient agar plates seeded with heat shock inactivated *Escherichia coli* and incubated at 28 °C. Initial cultures were monitored daily by microscopy and subcultured by transferring small pieces of agar containing amoebas to a fresh plate until amoebae were completely isolated (Magnet et al., 2012).

2.4. Molecular methods for FLA detection and identification

2.4.1. DNA extraction

i) DNA extraction from concentrated water was performed from 200 μL of each water sample with Fast DNA® Kit (MP Biomedicals, Illkkrich, France) modifying the manufacturer's protocol. In brief, to each Fastprep tube another 1/4 inch ceramic sphere was added and lysing cycles were performed in triplicate. ii) From FLA cultures, amoeba DNA was extracted from amoebae by heat shock (99 °C for 20 min) and purified with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) following the manufacturers' instructions.

2.4.2. Triplex real-time PCR for the detection of *Acanthamoeba spp., Balamuthia mandrillaris and Naegleria fowleri*

For concentrated water, a triplex real time-PCR designed to detect these three FLA species simultaneously was used as described previously (Qvarnstrom et al., 2006).

2.4.3. Real-time PCR for *Vermamoeba vermiformis*

For concentrated water, a real time-PCR designed to detect this species, formerly known as *Hartmanella vermiformis*, was used as described previously (Kuiper et al., 2006).

2.4.4. Real time PCR for *Paravahlkampfia sp.*

For concentrated water, a real time-PCR designed to detect *Paravahlkampfia* sp. was used as described previously (Visvesvara et al., 2009).

2.4.5. *Acanthamoeba* genotyping from isolated amoebae

From the FLA cultures identified by morphology as *Acanthamoeba*, the genotypic characterization was performed using the forward and reverse primers JDP1 and JDP2 as described previously, obtaining amplicons of 450–500 bp (Schroeder et al., 2001). Amplicons were purified with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) following the manufacturers' instructions and sequenced at both ends with PCR primers through Macrogen laboratories (Korea) sequencing service. The sequences analyses were performed with Bioedit Sequence Alignment Editor 7.0.5.3 pairwising the forward and reverse sequences to obtain the consensus one (Hall, 1999).

2.5. Legionella culture isolation

One hundred microliters of concentrated water samples were inoculated in Buffered Charcoal Yeast Extract medium (BCYE) (with L-cysteine, glycine, polymyxin B, anisomycin and vancomycin) agar after dilutions and treatments as described below (if not specified, samples were not diluted): i) untreated water (diluted 1/1000 for raw water); ii) heat shock (50 °C/30 min) (diluted 1/10 for raw water); iii) acid shock (HCl 0.2 M–KCl 0.2 M 5 min) and iv) combined heat-acid shock. Water treatments followed before culture were based on ISO 11731:1998 international standard method for *Legionella* detection. Colonies were selected according to their morphology and passed to

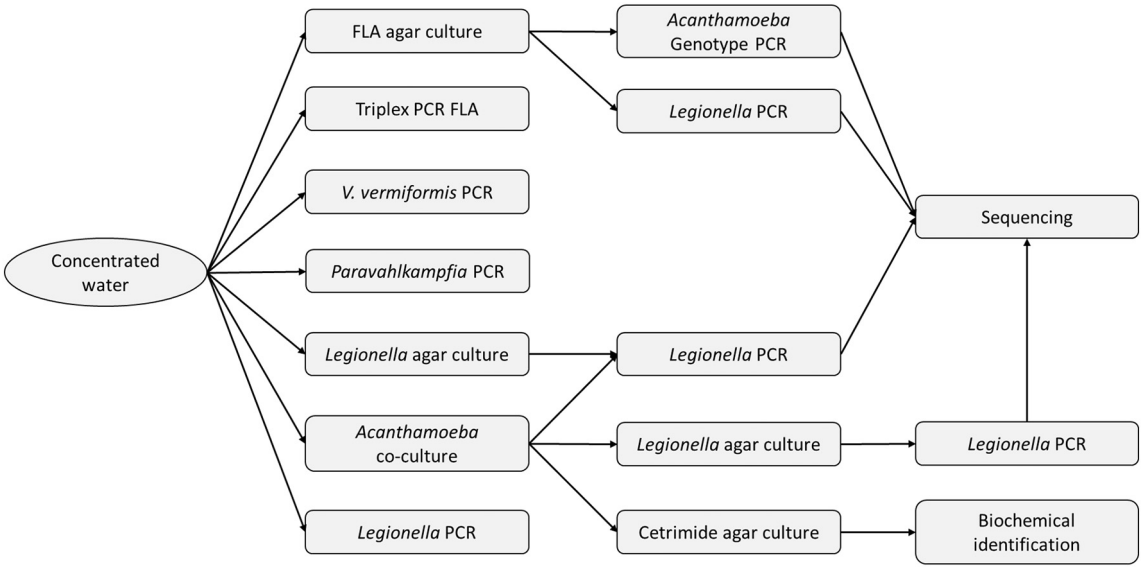

Fig. 1. General scheme of analysis conducted on water samples.

charcoal-yeast extract agar (BCYE) with and without L-cysteine. Colonies that required L-cysteine to grow were considered *Legionella* sp. and these were confirmed by semi-nested PCR reaction as described below (Section 2.8).

2.6. Acanthamoeba co-culture

Co-culture of *Acanthamoeba* and *Legionella* protocol described by La Scola et al. (2001) was used to improve amoeba-resisting bacteria isolation from concentrated water samples. Briefly, the co-culture was conducted using a 24-well culture plate where 3×10^5 *Acanthamoeba* trophozoites were placed in 1.5 mL of Neff's saline per well with 100 μL of the concentrated water and incubated at 33 °C for 8 days. For this purpose, *Acanthamoeba* ATCC 30234 and *Acanthamoeba* USP-CR5-A35, two strains of genotype T4 free of *Legionella* available in our laboratory (Magnet et al., 2012), were used. From each culture well, amoebae and bacteria harvested after co-culture were concentrated by centrifugation (1500 rpm/10 min) and resuspended in 300 μL, separated in 3 aliquots destined for different analyses: i) *Legionella* PCR, ii) agar culture and iii) backup samples.

2.7. Amoeba-resisting bacteria isolation in agar and identification after Acanthamoeba co-culture

A volume of 300 μL of Triton X-100 (Sigma-Aldrich Chemie GmbH, P.O., Germany) 0.05% was added to the 100 μL of concentrated culture to promote amoebic lysis. From this solution, a volume of 100 μL was inoculated in BCYE agar (with L-cysteine), another 100 were inoculated in BCYE agar (without L-cysteine) and finally, 100 μL were inoculated in cetrimide agar. Colonies from BCYE medium were selected according to their morphology and L-cysteine requirement to grow, and confirmed by *Legionella* PCR. Colonies from cetrimide agar were identified using VITEK® 2 instrument (bioMérieux, Marcy l'Etoile, France), with identification cards VITEK® GN 21341 for Gram-negative bacteria.

2.8. Legionella PCR

2.8.1. DNA extraction

i) DNA extraction from water samples was performed using the Fast DNA® Kit (MP Biomedicals, Illkkrich, France). ii) From *Legionella* strains isolated in culture, DNA was extracted from 100 μL of diluted culture using high temperature (99 °C/20 min). DNA was purified with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany). iii) From *Acanthamoeba* co-culture, DNA was extracted from 100 μL of concentrated culture using high temperature and purification, as described for *Legionella* strains isolated in culture. iv) From *Acanthamoeba* isolates, DNA extraction was performed as described before.

2.8.2. PCR and sequencing

A semi-nested PCR described by Miyamoto et al. (1997) was used for partial amplification of the 16S rRNA gene of *Legionella*, with some modifications performed by Magnet et al. (2015). The amplified products (650 bp) from positive samples in the first PCR were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany) and sequenced by Macrogen laboratories sequencing service (Seoul, Korea). The sequences analyses were performed with Bioedit Sequence Alignment Editor 7.0.5.3 pairwising the forward and reverse sequences to obtain the consensus one (Hall, 1999).

The second PCR was performed with samples that resulted in negative in the first PCR and reported *Legionella* spp.

3. Results

3.1. Acanthamoeba in water samples

Acanthamoeba was detected in several samples, from different collection points of all studied DWTPs, by culture and/or real-time PCR. The detailed results of *Acanthamoeba* detection in these DWTPs are shown in Tables 2.1 and 2.2. In raw water, these amoebae were detected in all samples (100%) in DWTPs 1 and 3 while the detection in this type of sample in DWTPs 2 and 4 was approximately 60%. In finished water, these results are maintained in DWTPs 1 and 3. For DWTP 2, while the frequency of *Acanthamoeba* in finished water was observed to be higher than it was in raw water samples. For DWTP 4, the frequency of these amoebae varied along the water treatment line but finally, the results obtained in finished water demonstrated a lower frequency than the one observed in raw water.

Separately, some specific treatments employed in these water purification lines indicated a decrease in the presence of *Acanthamoeba* in some water treatment plants, even though these effects were not observed for every plant that uses these methods. In DWTP 1, the pre-ozonation step decreased the amoebic frequency, but at the subsequent steps, *Acanthamoeba* presence was demonstrated to increase and keep increasing until the final process, with a single slight decrease before GAC filtration, which represents a water sample derived from ozonation

Table 2.1
Detection of *Acanthamoeba* spp. in samples collected in different points of water and sludge treatment lines of DWTPs 1 and 2. Results are presented for both samplings carried out in each season, sampling 1/sampling 2.

Collected samples	Winter			Spring			Summer			Autumn			AF	
	Cult	PCR	Gt	Cult	PCR	Gt	Cult	PCR	Gt	Cult	PCR	Gt	Cult	PCR
DWTP 1														
Water treatment line														
Raw water	+/+	+/+	T4/7	+/+	−/+	T4	+/+	+/+		+/+	−/+		**100%**	**75%**
Agua pre-ozonated	−/+	−/−		−/−	−/−		+/−	−/+		−/−	−/−		25%	12.5%
Decanted water	+/+	+/+	T4/16	−/+	−/+		+/+	+/+		−/−	+/−		62.5%	75%
Decanter biofilm	NA/+	NA/−		+/+	−/−	T4/16	+/NA	−/NA	T4/16	−/+	−/−		83.3%	0%
Sand filter surface water	+/NA	+/NA	T4/22	−/+	+/+		+/+	+/+	T4/16	+/+	+/−	T4/16	85.7%	85.7%
Sand-filtered water	+/+	−/+	T4/7	−/+	−/−		+/+	+/+	T4/8	+/+	−/−	T4	87.5%	37.5%
Ozonated water	−/+	−/+		−/+	−/−	T4/8	+/+	+/+	T4/8	+/NC	−/NC		71.4%	42.8%
GAC filter surface water	+/+	−/+	T4/8	−/+	+/−	T4/12	+/+	+/+		+/−	+/−	T4	75%	62.5%
Finished water	+/+	+/+	T4/36	+/+	+/+		+/+	+/+	T4/28	+/+	+/+		**100%**	**100%**
Mud treatment line														
Sand filter rinsing water	+/+	+/+	T4/16	−/+	+/+	T4/12	+/+	+/+		+/+	−/−	T4	87.5%	75%
Clarified water	+/−	−/+		−/−	−/−		+/−	+/−		−/−	+/−		25%	37.5%
Floater water	−/−	−/−		−/−	−/−		−/−	+/−		−/−	+/+		0%	37.5%
Decanted sludge	−/+	−/+	T4/16	−/−	−/−		−/−	−/+		+/−	+/−	T4	25%	37.5%
Dehydrated sludge	+/+	NA/−	T4/16	−/+	−/−		+/+	−/−	T4/16	+/+	−/−		87.5%	0%
DWTP 2														
Water treatment line														
Raw water	+/−	−/−	T4	−/+	+/−		+/+	−/+		+/−	−/−	T4	**62.5%**	**25%**
Pre-oxidized water	+/+	−/+	T4/7	−/−	+/+		+/+	+/+	T4/16	+/−	−/−		62.5%	62.5%
Decanted water	+/−	+/+	T4	+/+	−/+	T4/7	−/+	+/+		+/+	−/−	T4/13	75%	87.5%
Decanter biofilm	NA/+	−/−		+/+	+/−		−/+	−/+		−/−	−/−		57.1%	25%
Sand-filtered water	−/−	−/−		−/+	+/−	T4	+/+	−/+	T4/1,T4/8	+/−	−/+	T4/16	50%	37.5%
Finished water	−/+	+/−	T4	+/+	+/−		+/+	+/+	T4/1,T4/8	+/+	+/+	T4/7	**87.5%**	**75%**
Mud treatment line														
Sand filter rinsing water	+/+	−/−	T4/16	−/−	+/+		+/+	−/+	T4/8,T4/22	NC/−	NC/−		57.1%	42.8%
Clarified water	−/+	−/+	T4/7	−/+	+/+	T4/16	+/+	+/−	T4/30	−/−	−/−		50%	50%
Centrifugation residual water	+/NC	−/NC	T4/16	−/+	+/+	T4/8	+/+	+/+	T4/16	−/NC	+/NC		66.6%	66.6%
Decanted sludge	−/+	−/−	T4/7	−/−	−/−		−/−	−/−		+/−	−/−	T4/16	25%	0%
Dehydrated sludge	+/+	−/−	T4/7,T4/28	−/+	−/−	T4/27	+/−	−/−		+/−	−/−		62.5%	0%

Cult: analysis by culture; PCR: DNA amplification detection; Gt: Genotype; AF: Annual frequency; NA: not analyzed; NC: not collected; GAC: granular activated carbon.

treatment. In DWTP 2, *Acanthamoeba* presence was observed to increase in decanter water, followed by a gradual decrease at the other points. However, these amoebae frequency again increased in finished water, after sand filtration, suggesting this step could be contributing to amoebic enrichment. In DWTP 3, the frequency of *Acanthamoeba* was shown to be elevated at the initial points, with a slight temporal decrease in viable amoebae after pre-ozonation treatment, but the amoebic presence continued to vary in the following samples, and finally, an amoebic increase was observed after inverse osmosis treatment. In DWTP 4, the results obtained revealed an initial decrease in the presence of *Acanthamoeba* after pre-oxidation treatment, followed by a variable amoebic presence at the other treatment points, decreasing once again after the ozonation step and, finally, the variable amoebic frequency reached the finished water indicating to be slightly lower than the frequency observed at raw water.

At the sludge treatment line, an enrichment of the presence of *Acanthamoeba* was observed in the samples analyzed belonging to a direct line of treatment and dehydration of this resultant sludge. Specifically, the samples from sand filtration rinsing water (employed in the washing of sand filters) and dehydrated sludge (final sludge product) demonstrated some of the most elevated amoebic frequency, respectively 87.5% and 87.5% in DWPT 1, 57.1% and 62.5% in DWPT 2, 62.5% and 75% in DWPT 4. In DWTP 3, there was no sample of sand filter rinsing water because this plant did not have a sand filter, yet the most elevated frequency was observed in this plant: 80% in clarified water, 50% in centrifuge residual water and 100% for both pre-dehydrated and dehydrated sludge.

Regarding *Acanthamoeba* genotypes, most identified isolates were shown to belong to genotype T4. However, a variable amoebic population was demonstrated by applying the nomenclature of Booton et al. (2002) grouping those isolates in genotypes T4/1, T4/7, T4/8, T4/12, T4/13, T4/16, T4/22, T4/27, T4/28, T4/30, T4/34, T4/36, and T4/37. Genotype T4/16 was the most prevalent in these samples. Additionally, two isolates obtained during spring at DWTP 3 were identified as belonging to genotypes T3 and T5. In fact, this is the first report of genotype T5 isolation in water samples from the Castilian Plateau area (Spain).

3.2. Other FLA in water samples

By using the triplex real-time PCR, the presence of *N. fowleri* was not detected in the analyzed samples. However, one sample of decanter biofilm collected during winter at the DWTP 3 demonstrated the amplification of *B. mandrillaris* DNA.

Regarding *V. vermiformis*, the detection of this amoeba was observed by real-time PCR in samples collected from several points, however, it occurred with higher frequency in samples derived from decanter biofilm or from sludge treatment line. On the other hand, *Paravahlkampfia* sp. was rarely detected, presenting DNA amplification in a few samples derived from decanter biofilm (water treatment line) and in samples derived from clarified water and dehydrated sludge (residual sludge treatment line). The detailed data can be observed in Tables S1.1 and S1.2 (Supplementary information).

3.3. Legionella in water samples

In the present study, water samples from raw and finished water were analyzed by four different methods: BCYE agar culture, water PCR, *Acanthamoeba* co-culture and *Legionella* specific PCR in isolated amoebae from those samples. Additional biofilm samples from decanter were also analyzed, but *Acanthamoeba* co-culture methodology was not performed due to the high organic concentration in these samples. The isolation of *Legionella* was obtained by agar

Table 2.2
Detection of *Acanthamoeba* spp. in samples collected in different points of water and sludge treatment lines of DWTPs 3 and 4. Results are presented for both samplings carried out in each season, sampling 1/sampling 2.

Collected samples	Winter			Spring			Summer			Autumn			AF	
	Cult	PCR	Gt	Cult	PCR	Gt	Cult	PCR	Gt	Cult	PCR	Gt	Cult	PCR
DWTP 3														
Water treatment line														
Raw water	+/+	+/+	T4/7, T4/16	+/+	+/+	T4/8	+/NC	+/NC		NC/NC	NC/NC		**100%**	**100%**
Pre-ozonated	+/−	+/+		+/+	+/+	T5	+/NC	+/NC	T4	NC/NC	NC/NC		80%	100%
Homogenized water	+/+	+/+	T4	+/+	+/+	T4	+/NC	+/NC	T4	NC/NC	NC/NC		100%	100%
Decanted water	+/−	+/+	T4/7	+/+	+/+		+/NC	+/NC		NC/NC	NC/NC		80%	100%
Decanter biofilm	NA/−	−/+		+/+	+/+	T3	+/NC	+/NC		NC/NC	NC/NC		75%	80%
Ultrafiltered water	−/+	−/+		+/+	+/−		+/NC	−/NC		NC/NC	NC/NC		80%	40%
Inverse osmosis derived water	+/+	+/+	T4	+/+	+/−		+/NC	−/NC	T4	NC/NC	NC/NC		100%	60%
Finished water	+/+	+/+	T4/36	+/+	+/−	T4/22	+/NC	+/NC	T4	NC/NC	NC/NC		**100%**	**80%**
Mud treatment line														
Clarified water	+/−	NA/+	T4/30	+/+	+/−	T4/8, T4/13	+/NC	+/NC		NC/NC	NC/NC		80%	75%
Centrifugation residual water	NC/NC	NC/NC		+/−	−/−	T4/37	NC/NC	NC/NC		NC/NC	NC/NC		50%	0%
Pre-dehydrated sludge	NC/NC	NC/NC		+/+	+/+	T4/34	NC/NC	NC/NC		NC/NC	NC/NC		100%	100%
Dehydrated sludge	NC/NC	NC/NC		+/+	−/−		NC/NC	NC/NC		NC/NC	NC/NC		100%	0%
DWTP 4														
Water treatment line														
Raw water	+/+	−/+		−/−	+/+		+/+	−/+	T4	−/+	−/−		**62.5%**	**50%**
Pre-oxidized water	−/−	−/−		+/−	+/+		+/−	−/−		−/−	−/−		25%	25%
Decanted water	−/−	−/−		+/−	−/−		−/−	−/−		+/−	−/−		25%	0%
Decanter biofilm	−/+	+/+		+/+	+/−		+/+	−/+		+/+	−/−		87.5%	50%
Sand filter surface water	−/+	+/+		−/−	−/−		+/+	−/−		−/−	−/−		37.5%	25%
Sand-filtered water	+/+	+/−		+/+	−/−		−/+	−/−		+/−	−/−		75%	12.5%
Ozonated water	+/+	−/−	T4/8	−/−	−/−		+/−	−/−		+/−	−/−		50%	0%
GAC filter surface water	+/+	+/+	T4/13	−/−	−/−		−/−	−/−		−/−	−/+		25%	37.5%
GAC-filtered water	+/−	+/−	T4/8	+/−	−/−		+/+	−/−		+/−	−/−		62.5%	12.5%
Finished water	−/+	+/+	T4/8	−/−	+/−		+/+	−/−	T4/8	+/−	−/−		**50%**	**37.5%**
Mud treatment line														
Decanter purged water	−/+	−/−		+/−	−/−		−/−	−/−		−/−	−/−		25%	0%
Sand filter rinsing water	−/+	−/−		−/+	−/−		+/−	−/+		+/+	−/−		62.5%	12.5%
Clarified water	+/−	−/+		−/−	+/+		−/+	−/+		−/+	−/−		37.5%	50%
Floater water	−/−	+/+		+/−	−/−		+/−	−/−		+/−	−/−		37.5%	25%
Dehydrated sludge	+/−	−/−		+/+	−/−		+/+	−/−		+/−	−/−		75%	0%

Cult: analysis by culture; PCR: DNA amplification detection; Gt: Genotype; AF: Annual frequency; NA: not analyzed; NC: not collected; GAC: granular activated carbon.

culture in one sample of water collected during summer in DWTP 3, in which two species, *L. donaldsonii* and *L. feeleii*, were identified. Nevertheless, there was no isolation of *Legionella* by agar culture at the other water samples, nor on the *Acanthamoeba* co-culture derived samples.

In raw water samples, the direct PCR on water demonstrated a variable presence of *Legionella* spp., ranging from 0 to 60%. However, this PCR detection was demonstrated to be greatly increased after *Acanthamoeba* co-culture, demonstrating a higher frequency in all plants studied, with a general detection rate rising from 27.5% in direct water PCR to 86.3% after *Acanthamoeba* co-culture. Also, *Legionella* PCR conducted on the amoebae isolated by culture from the samples demonstrated a significant presence of *Legionella* inside these amoebae, with the exception of DWTP 4. The detection rate of *Legionella* as amoebae endosymbionts ranged from 20 to 50% in FLA isolated from raw water.

In biofilm samples from the decanters, no detection was observed with agar culture or direct water PCR. However, FLA culture from DWTP 1, spring sampling, demonstrated specific amplification of *Legionella* DNA inside the isolated amoebae.

In finished water samples, direct PCR on the water showed the presence of *Legionella* spp. in only one sample collected in winter in DWTP 3. On the other hand, PCR analysis in samples from *Acanthamoeba* co-culture detected *Legionella* in several samples, presenting a general detection rate as high as 75.8%. Additionally, *Legionella* PCR conducted on isolated amoebae demonstrated a significant presence of *Legionella* inside these amoebae, in all plants studied, with a detection rate ranging from 37.5% to 62.5%. The detailed results obtained by employing these techniques in raw water, decanter biofilm and finished water are shown in Table 3.

3.4. Isolation of other bacteria after Acanthamoeba co-culture

Using cetrimide agar plates, some of the bacteria harvested from *Acanthamoeba* co-culture were isolated in this specific medium commonly employed for *Pseudomonas* sp. isolation. Indeed, *P. aeruginosa* was detected twice in raw water samples and once in finished water, in a sample collected in DWTP 3 during summer. Besides *P. aeruginosa*, other *Pseudomonas* species were detected, such as *P. stutzeri* (once in raw water) and *P. fluorescens* (once in finished water). Additionally, colonies from other bacteria were also identified: *Stenotrophomonas maltophilia*, in raw and finished water, and *Achromobacter xylosoxidans*, once in raw water and in 4 samples of finished water. The detailed data about these other amoeba-resisting bacteria detected are shown in Table S2 (Supplementary information).

4. Discussion

FLA are ubiquitous organisms that can be isolated from different natural environments, such as lakes, ponds, and recreational water, but also from domestic water supplies. These amoebae have been associated with infections of the central nervous system and cornea in humans. Besides their own pathogenicity, these amoebae also can act as Trojan horses, being infected with potentially pathogenic amoeba-resisting bacteria (Marciano-Cabral et al., 2010; Thomas et al., 2006). Previous environmental studies have demonstrated the environmental presence of *Acanthamoeba* and *Legionella* in raw and finished water from DWTPs from this region of Spain (Magnet et al., 2015). In the present study, water samples were collected from several points inside the treatment line employed in four DWTPs that were selected according to the

Table 3
Detection of *Legionella* spp. in collected samples by different techniques. Results are presented for both samplings carried out in each season, sampling 1/sampling 2.

DWTPs/seasons	Samples and analysis										
	Raw water				Decanter biofilm			Finished water			
	BCYE agar	Water PCR	*Acanthamoeba* co-culture	PCR in isolated amoebae	BCYE agar	Water PCR	PCR in isolated amoebae	BCYE agar	Water PCR	*Acanthamoeba* co-culture	PCR in isolated amoebae
DWTP 1											
Winter	−/−	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−	−/+	+/−
Spring	−/−	−/+	+[a]/+	+/+	−/−	−/−	+/−	−/−	−/−	+[b]/+	+/−
Summer	−/−	−/−	+/+	+/+	−/−	−/−	−/NA	−/−	−/−	−/+	−/+
Autumn	−/−	−/−	+[b]/+[b]	−/−	−/−	−/−	NA/−	−/−	−/−	+/+	+/+
Total	0%	37.5%	100%	50%	0%	0%	16.7%	0%	0%	75%	62.5%
DWTP 2											
Winter	−/−	−/−	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Spring	−/−	−/−	+/+	−/+	−/−	−/−	−/−	−/−	−/−	+/+	−/+
Summer	−/−	−/−	+/−	+/+	−/−	−/−	−/−	−/−	−/−	+/−	+/+
Autumn	−/−	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−	+/+	−/−
Total	0%	0%	75%	37.5%	0%	0%	0%	0%	0%	62.5%	37.5%
DWTP 3											
Winter	−/−	+/+	−/+	−/−	−/−	−/−	NI/−	−/−	−/+	−/+	−/+
Spring	−/−	+/−	+[a]/+[b]	−/−	−/−	−/−	−/−	−/−	−/−	+/+	+/+
Summer	+[c]/NC	+[b]/NC	+[b]/NC	+/NC	−/NC	−/NC	−/NC	−/NC	−/NC	+[b]/NC	−/NC
Autumn	NC/NC	NC/NC	NC/NC	NC/NC	NC/NC	NC/NC	NC/NC	NC/NC	NC/NC	NC/NC	NC/NC
Total	20%	60%	80%	20%	0%	0%	0%	0%	20%	80%	60%
DWTP 4											
Winter	−/−	−/+	+/+[a]	−/−	−/−	−/−	−/−	−/−	−/−	+/−	NI/+
Spring	−/−	−/−	+/+[a]	NI/NI	−/−	−/−	−/−	−/−	−/−	+/+	NI/NI
Summer	−/−	−/−	+/+	−/−	−/−	−/−	−/−	−/−	−/−	+/+	+/−
Autumn	−/−	−/−	+/+	NI/−	−/−	−/−	−/−	−/−	−/−	+/+	−/NI
Total	0%	12.5%	100%	0%	0%	0%	0%	0%	0%	87,5%	50%
Total detection	3.4%	27.5%	86.3%	30.7%	0%	0%	3.8%	0%	3.4%	75.8%	52%

PCR: DNA amplification detection; NA: not analyzed; NC: not collected; NI: No isolation of amoebae.
[a] *L. drozanskii.*
[b] *L. donaldsonii.*
[c] Both *L. donaldsonii* and *L. feeleii.*

differences in their water purification processes, in order to compare the efficacy of these treatments.

Regarding *Acanthamoeba*, its presence was detected in several sampling points, by agar culture, by real-time PCR or both. In some samples, the results obtained using these two methods might differ. Although real-time PCR is recognized as a more sensitive technique, it can give false-negative results when used with environmental samples due to the presence of inhibitors. Therefore, these two methods work as complementary techniques for *Acanthamoeba* detection in environmental samples (Magnet et al., 2012).

In DWTPs 1 and 3, amoebic detection reached 100% by culture both in raw and finished water, indicating a limited or ineffective elimination of these amoebae. In DWTP 2, *Acanthamoeba* frequency increased in finished water if compared to the samples collected at the entrance of the plant, suggesting these amoebae may be encountering favorable growing conditions inside this plant. Nevertheless, the last disinfection step in all DWTPs includes chloramine that has a time-dependent biocide effect, because of this, a higher amoebicidal effect is expected to occur during the distribution time (Dupuy et al., 2014).On the other hand, the presence of *Acanthamoeba* in DWTP 4 was shown to be diminished from the entrance to the exit of the water treatment line. DWTP 4 and DWTP 1 are highly similar so, a possible answer which could explain these contradictory results is the different type of decanter or differences in the maintenance strategies of each DWTP.

Regarding the efficiency of treatments employed, the sand filters seem to be associated with amoebic enrichment and this feature was observed both in the passage from sand filter surface to sand-filtered water and in those samples derived from sand filter rinsing water. In DWTPs 1 and 4, it was possible to observe a general decrease in *Acanthamoeba* isolation by culture after ozonation treatment, an additional treatment employed before GAC filtration. However, the amoebic presence increased again in the following treatments in those DWTPs, possibly due to amoebic presence at the GAC filters. Amoebic multiplication in sand and GAC filters has been previously reported and the optimization of filter backwash procedures was suggested as a possibility to better control these FLA and the risk associated with their intracellular hosts (Thomas et al., 2008). Some new alternative strategies in water purification processes could arise as solutions to prevent this amoebic persistence; however, the use of new technologies such as ultrafiltration and inverse osmosis were not shown to solve this challenge in this study. The recirculation of the clarified water recovered from sludge treatment at the general water treatment line could also be a feature that contributes to amoebic persistence in these DWTPs. Thus, it is complicated to establish processes that might work all together to eliminate these amoebae. In addition, the identification of different genotypes throughout the water treatment line indicated the appearance of new amoebic population at several points, also suggesting the possibility of amoebic colonization occurring inside these plants, and the different populations detected in these water samples may reflect an occasional FLA release from colonized surfaces, as previously suggested (Thomas et al., 2008). To study if there is variation in FLA presence in relation to the climate, the sampling scheme was carried out in different seasons of the year. However, no difference was observed in the presence of FLA.

Similar results have been reported in studies conducted in other regions of Spain (Corsaro et al., 2009, 2010; Garcia et al., 2013; Magnet et al., 2013a) as well as in France (Thomas et al., 2008). In a study carried out in two DWTP in Malaysia more than 90% of samples were positive for the presence of *Acanthamoeba* (Richard et al., 2016), while some studies in Germany (Hoffmann and Michel, 2001) and Japan (Edagawa et al., 2009) showed a high presence (over 60%) of positive tap water sources samples for FLA.

The highest frequency of genotype T4 population among these amoebic isolates might be related to the higher environmental distribution of this genotype worldwide. Among these samples, the presence of genotypes T3 and T5 was also observed. In fact, this is the first report of

isolation of genotype T5 in waters from this region of Spain, an important datum to consider due to its clinical relevance. Genotype T5, as well as genotypes T3 and T4, has been associated with pathology (Magnet et al., 2014). Moreover, previous studies have demonstrated that this genotype presents higher resistance to amoebicidal substances if compared to genotype T4 (Carnt and Stapleton, 2016; Ledee et al., 2009; Spanakos et al., 2006).

In the present study, real-time PCRs were also employed to detect the presence of some other selected FLA: *N. fowleri*, *B. mandrillaris*, *V. vermiformis,* and *Paravahlkampfia* sp. The results obtained with these protocols demonstrated no detection of *Naegleria fowleri* in the analyzed samples while *B. mandrillaris* was detected in only one biofilm sample. *V. vermiformis* and *Paravahlkampfia* sp. were detected in samples from several collection points, though their presence was higher in biofilm samples and from samples of the sludge treatment line. These types of samples concentrate more organic material, which favors the contact between amoebae and bacteria and thus, their presence. In fact, biofilm formation over surfaces inside the treatment plants is believed to contribute to the colonization of these plants. Because of this, establishing measures to limit biofilm formation could be a strategy against FLA presence in treated water (Thomas et al., 2008).

Also, FLA presence in these biofilms in close contact with several bacteria not only provides a feeding source for those amoebae but facilitates their infection by amoeba-resisting bacteria, protecting those bacteria from the antimicrobial processes employed later in these DWTPs. An example of these bacteria is *Legionella* spp. (Pagnier et al., 2015; Thomas et al., 2008). Aware of this, *Legionella* detection was attempted in this study in 3 types of samples (raw water, decanter biofilm and finished water) using different techniques. Our results demonstrated that PCR detection was higher than detection by agar culture, but even higher after *Acanthamoeba* co-culture, confirming the usefulness of this methodology as a tool to detect amoeba-resisting bacteria like *Legionella* spp., as proposed in the literature (Magnet et al., 2015; Rowbotham, 1983). Additionally, the presence of *Legionella* was observed as being higher in raw water than in finished water, according to the three techniques used. The use of *Legionella* PCR in amoebae isolated from these water samples also demonstrated the presence of *Legionella* infecting these amoebae both in raw water and finished water (30.7% and 52%, respectively). These results confirm the capacity of these bacteria to infect FLA in order to receive protection from harsh environmental conditions. Indeed, the decrease of free *Legionella* observed in finished water compared to raw water was accompanied by an increase in the frequency of infected FLA from raw to finished water. In biofilm samples, however, free *Legionella* was not detected, only a low percentage of infected FLA being detected. Perhaps, the biofilm surface providing a favorable niche to microbial growth to a diverse variety of microorganisms also creates a highly competitive environment, limiting the presence of *Legionella*. A similar situation is observed with complex environmental samples in which the abundant microbial growth hampers Legionellae isolation by culture methods (Yáñez et al., 2005).

The identification to species level in *Legionella* positive samples was not always possible. However, the presence of *L. drozanskii*, *L. donaldsonii,* and *L. feeleii* was confirmed at different points. The pathogenic species *L. pneumophila* was not detected among the samples studied, but the presence of these *L.* non-*pneumophila* species should not be ignored. *L. drozanskii* (LLAP-1) was never reported as a causative agent of human infection, but the association of LLAPs and human infections has been proved for *L. lytica* (LLAP-3), previously reported as the etiological agent of pneumonia (Adeleke et al., 1996, 2001). The detection of these bacteria exclusively after *Acanthamoeba* co-culture amplification of concentrated raw water is consistent with its general description as a LLAP. The species *L. donaldsonii* and *L. feeleii*, on the other hand, were reported as agents of human infection in previous studies, more frequently associated with immunocompromised individuals (Han et al., 2015; Lee et al., 2009). Both species were detected and identified in agar culture from a sample of raw water, *L. donaldsonii* was also detected in direct water PCR of the same sample. At finished water, *Legionella* was detected in one sample when analyzed by direct water PCR but not identified, after co-culture. However, *L. donaldsonii* was identified twice among the samples in which *Legionella* was detected. In a previous study conducted in this region, *L. feeleii* was reported to be the most frequently isolated *Legionella* species (Magnet et al., 2015). However, in the present study, this species was detected in only one sample. This difference could be due to certain aspects: although both studies were conducted in the same region, they did not evaluate the same DWTPs; also, the present study assessed water samples from all seasons during an entire year at each treatment plant (except for DWTP 3) while the previous study only analyzed samples from autumn and winter. Additionally, these studies were conducted with a time interval of 2 to 3 years, and climatic conditions could have affected the microbial population. For instance, data from 2011 (collection time of the previous study) and 2014–2015 (present study) indicates some of the highest annual temperatures. However, the humidity was higher in 2014 if compared to 2011 (Agencia Estatal de Meteorología, 2011, 2014).

The presence of *Pseudomonas* spp. in these water samples was also evaluated using the *Acanthamoeba* co-culture methodology. For this purpose, an aliquot of the co-culture was harvested in cetrimide agar, and further identification of the isolated colonies demonstrated the presence of *Pseudomonas aeruginosa*, *P. fluorescens*, *P. stutzeri*, *Achromobacter xylosoxidans* and *Stenotrophomonas maltophilia* in these samples, proving the usefulness of co-culture for the amplification of these bacteria. The isolation of bacteria other than *Pseudomonas* species in this selective agar might be related to their similar growth requirements and colony appearance, so that these species are easily misidentified as *Pseudomonas* sp. (Abbott and Peleg, 2015). *P. aeruginosa* is one of the most prevalent Gram-negative bacteria associated with nosocomial pneumonia, especially in individuals with some type of immunosuppression. On the other hand, its prevalence in community-acquired pneumonia (CAP) is low, 0.05% of CAP patients (Ding et al., 2016). Other species, such as *P. fluorescens* and *P. stutzeri*, are less frequently associated with human infections, which might be related to lower pathogenicity. Even though, they also depend on the host conditions, these infections have been reported in immunocompromised individuals (Pérez-Miravete et al., 2001). As *Pseudomonas* sp., the species *S. maltophilia* and *A. xylosoxidans* are more commonly associated with respiratory nosocomial infections, presenting a higher risk to immunocompromised individuals, even though they are also able to produce severe community-acquired infections. These bacteria are not considered highly pathogenic, but they are gaining attention as emerging pathogens due to the report of multidrug resistance patterns in these species, both intrinsic and acquired (Brooke, 2012; Swenson and Sadikot, 2015).

The detection of these bacteria in water, especially in samples of finished water, might appear as alarming, but it is important to note that these samples passed through several concentration steps before each analysis and that most of these detections were obtained due to the employment of *Acanthamoeba* co-culture methodology. Moreover, all finished water analyzed had successfully complied with the Spanish water guidelines. It is important to report the presence of live potential pathogens in water since viable microorganisms could multiply to important infective concentrations if the environmental conditions become favorable. For this purpose, the *Acanthamoeba* co-culture is confirmed as an effective methodology for the detection of low concentrations of *Legionella* and other amoeba-resisting bacteria in environmental samples.

As well as *Acanthamoeba* co-culture appearing to be an effective methodology for the recovery and amplification of amoeba-resisting bacteria from environmental samples, the existence of this interaction in nature is a warning that the presence of FLA at these DWTPs might increase amoeba-resisting bacteria presence. The detection of *Legionella* inside some of the isolated amoebae confirms the interaction of these microorganisms inside these plants and the eventual vectorial role that *Acanthamoeba* assume in the transmission of *Legionella*, and probably of other amoeba-resisting bacteria. Thus, it is important to keep investigating strategies to control the amoebic presence inside the plants. Due to the lack of confirmed methods for the complete elimination of these amoebae, it might be useful to follow some of the suggested preventive actions, such as limiting biofilm formation, thus diminishing the occurrence of favorable niches for the colonization and multiplication of both bacteria and amoebae.

5. Conclusions

- *Acanthamoeba* spp. is largely distributed throughout the water and sludge treatment line from these DWTPs, and the presence of different genotypes suggests the possibility of amoebic colonization occurring inside these plants.
- This study reports the first isolation of *Acanthamoeba* genotype T5 in waters from the Castilian Plateau area (Spain).
- The formation of biofilms inside the DWTPs seems to allow a higher and more diverse presence of FLA, including from species not so highly disseminated, such as *Balamuthia mandrillaris*, *Vermamoeba vermiformis*, and *Paravahlkampfia* spp.
- The effectivity of the water treatment for *Legionella* is reflected in the decrease of free *Legionella* throughout the purification chain. On the other hand, an increase in *Legionella*-infected *Acanthamoeba* is observed.
- *Acanthamoeba* co-culture confirms its effectivity in increasing the detection of important amoeba-resisting bacteria in waters, denoting the presence of potentially pathogenic bacteria in water, especially treated water.

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CRediT authorship contribution statement

Thiago Santos Gomes: Methodology, Investigation, Writing - original draft, Visualization. **Lucianna Vaccaro:** Methodology, Investigation. **Angela Magnet:** Methodology, Investigation, Writing - review & editing, Visualization. **Fernando Izquierdo:** Methodology, Investigation. **Dolores Ollero:** Investigation. **Carmen Martínez-Fernández:** Investigation. **Laura Mayo:** Investigation. **María Moran:** Investigation. **María José Pozuelo:** Methodology. **Soledad Fenoy:** Conceptualization, Writing - review & editing. **Carolina Hurtado:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Carmen del Águila:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

All authors declare no actual or potential conflict of interest including any financial, personal or other relationships with founding organizations.

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