DVC-FISH to identify potentially pathogenic *Legionella* inside Free-Living Amoebae from water sources

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ABSTRACT

Despite all safety efforts, drinking and wastewater can still be contaminated by *Legionella* and free-living amoebae since these microorganisms are capable of resisting disinfection treatments. An amoebae cyst harboring pathogenic *Legionella* spp. can be a transporter of this organism, protecting it and enhancing its infection abilities. Therefore, the aim of this work is to identify by DVC-FISH viable *Legionella* spp and *Legionella pneumophila* cells inside FLA from water sources in a specific and rapid way with the aim of assessing the real risk of these waters.

A total of 55 water samples were processed, 30 reclaimed wastewater and 25 drinking water). FLA presence was detected in 52.7% of the total processed water samples. When DVC-FISH technique was applied, the presence of viable internalized *Legionella* spp. cells was identified in 69.0% of the total FLA-positive samples, concretely in 70.0 % and 66.7% of wastewater and drinking water samples, respectively. *L. pneumophila* was simultaneously identified in 48.3% of the total FLA-positive samples, specifically in 50.0% and 44.4% of wastewater and drinking water samples, respectively.

By culture, potentially pathogenic *Legionella* cells were recovered in 27.6 % of the total FLA-positive bacteria, particularly in 35.0% and 11.1% of wastewater and drinking water samples, respectively.

These findings demonstrate that FLA may promote resistance of bacteria to the performed disinfection treatments for drinking as well as for wastewater. So, in addition to the risk for the presence of pathogenic FLA in water it is necessary to take into account that these can be transporters of the pathogenic bacteria *Legionella*, which are able to survive inside them. The DVC-FISH method described here has been proved to be a rapid and specific tool to identify pathogenic *Legionella* spp. and *L. penumophila* viable cells harboured by FLA in these water sources, posing particular public health concern.

Keywords: Free-living amoebae, *Legionella* spp., DVC-FISH, wastewater, drinking water

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1. INTRODUCTION

The bacterial genus of *Legionella* includes more than 50 described species, of which at least 20 of them are associated with human disease (Burstein et al., 2016). It is an opportunistic pathogen that mainly affects immunocompromised individuals or people suffering from other diseases, exceptionally producing asymptomatic infections in the healthy population. *Legionella* spp. includes microorganisms which are found in natural aquatic habitats and whose ecological niches are surface water of rivers, lakes, ponds, streams, hot springs, or even the stagnant water near volcanoes or the Antarctica's lakes (Scaturro et al., 2016; Borella et al., 2004; Borella et al., 2005). Therefore, it is possible to find it in any aquatic habitat aside from estuaries and saline water (Graells et al. 2018).

Legionellosis, the disease caused by *Legionella*, has a high media impact when associated with community outbreaks, usually related to hot sanitary water, cooling towers, hot tubs or ornamental fountains (Gomez-Valero et al., 2014; Scaturro et al., 2016). *Legionella pneumophila* specie is included in the water contaminant candidate list 4 (CCL4), from the United States Environmental Protection Agency (US EPA). There are other *Legionella* species considered pathogenic among which *Legionella micdadei*, *Legionella longbeachae*, *Legionella dumoffii* and *Legionella bozemanii* can be found (Bartram et al., 2007; Lau and Ashbolt, 2009).

The significance of the study of *Legionella* spp. does not only focus on its important health implications but also needs to encompass the socio-economic ones, since it affects important sectors such as tourism, trade or petrochemicals (Borella et al., 2004). The circumstances that contribute to the spread of *Legionella* spp. are temperature in the range of 25-45°C, with an ideal temperature of 35-37°C, the existence of stagnant or low velocity of circulation water, the presence of nutrients, a suspended solids deposit, a rough surface with calcified deposits and corrosion and the creation of biofilms in the presence of protozoa, bacteria or algae (Moens, 2002).

Free-living amoebae (FLA) are environmental protozoa that have been isolated from almost all environmental sources (Trabelsi et al., 2012). Members of the species of *Acanthamoeba* are the most common FLA found in water, soil and air

environments (Khan, 2006). Typically, FLA have two stages in their life cycle: the trophozoite, which is the metabolically active form, and the cyst, which is the dormant form and is acquired under unfavourable circumstances. While FLA are in favourable environmental conditions (trophozoite stage), one type of food they feed on is bacteria, some of which are able to avoid phagocytation and the ones phagocytised can even replicate inside FLA, becoming either commensal symbionts or even parasites. Thus, these bacteria would be sheltered and kept safe from stressed environmental conditions. For this reason, FLA have been considered as "Trojan Horses" (Barker and Brown, 1994) for these amoeba-resistant bacteria (ARB) (Greub and Raoult, 2004). Indeed, it has been extensively studied that human pathogens, such as *Legionella*, *Listeria* and *Campylobacter* can be potentially transmitted through FLA (Akya et al., 2009; Bui et al., 2012; Conza et al., 2013).

As it has been previously stated, some bacteria are able to escape protozoan phagocytation due to their inherent characteristics, such as virulence factors, toxin production or simply their size. However, some other bacteria can be ingested but have evolved strategies not only to evade digestion but also to multiply within protozoa, being the most studied example *L. pneumophila* which have used its interaction with FLA as learning to be able to infect macrophages (Cirillo et al., 1999; Adiba et al., 2010; Bui et al., 2012).

Up to now, little number of studies have investigated the interaction between FLA and *Legionella* spp. in environmental samples (Conza et al., 2013). However, *Legionella* have been found in aquatic environments such as drinking water or reclaimed wastewater systems, either in planktonic form, attached to a biofilm or related to protozoa (Declerck et al., 2007). Thus, FLA may play a role in the proliferation and the protection of *Legionella* against disinfection processes, which could serve as a carrier in the transmission of the Legionnaires' disease. *Legionella* spp. are highly pleomorphic bacteria which can actively replicate or be found in their stationary phase form in an extracellular nutrient-rich environment or a biofilm (Byrne and Swanson, 1998; Robertson et al., 2014). Similarly, when *Legionella* spp. are inside FLA or human macrophages they can acquire the replicative phase form until nutrients are depleted, at which time they differentiate into the infectious form inside FLA or macrophages (Garduño et al., 2002; Abdelhady and Garduño, 2013).

The official and internationally accepted method for *Legionella* spp. detection in water samples (ISO 11371) is based on cultivation. The main drawbacks of cultivation method are considerable time required for the assay (10 days) and that it only allows the detection of colony-forming cells. Nowadays, this delay is unacceptably long, and a sensitive, specific and fast screening method is of much importance. (Füchslin et al., 2010)

The exposure to stress can induce gram-negative bacteria in the water environment to enter the viable but non-culturable (VBNC) state as a survival strategy (Oliver, 2010), which can be induced by different stressors. Some of these stressors are heat, starvation or chemical treatments such as chlorination (Steinert et al., 1997; García et al., 2007; Allegra et al., 2008). Due to all this, the evaluation of the viability of the pathogen by culture is an arduous task.

The combination of Direct Viable Count technique with FISH (DVC-FISH) has been reported as an efficient method to successfully detect viable and VBNC cells of some pathogenic microorganisms such as *Helicobacer pylori* (Tirodimos et al., 2014; Moreno and Ferrús, 2012). However, this technique has never been developed and applied to *Legionella* spp. identification. The advantages of DVC-FISH are that it does not require DNA extraction and there are non-specific binding problems or inhibitory substances hindering hybridization. Moreover, FISH combines the visual information of microscopy to the accuracy of molecular genetics, allowing the visualization and identification of individual microbial cells in their native microenvironment. Nevertheless, FISH technique cannot differentiate between viable and dead cells without the combination with DVC incubation. DVC is a method based on samples' incubation in a nutrient-rich broth medium with the presence of an antimicrobial agent (quinolones antibiotics) (Kogure et al., 1979). This antimicrobial agent acts as a specific inhibitor of DNA synthesis, thus preventing cell division without affecting other metabolic activities. This way, viable cells start the replication process but cell division is prevented. As a result, they appear elongated and/or thicker after incubation.

It has been shown that *Legionella* spp. VBNC cells potentially resuscitate, recovering the replicative and infective capacity, through passage through FLA (Garcia et al., 2007; Ducret et al., 2014; Dietersdorfer et al., 2018). Consequently, the foremost objective of this research was to optimize and apply DVC-FISH

technique for the specific detection of viable and in all likelihood potentially infectious *Legionella* spp. and *L. pneumophila* cells harboured by FLA from wastewater and drinking water samples. The viability of the bacteria within FLA was also investigated by standard culture methods.

2. MATERIALS AND METHODS

2.1 Sample collection

A total of 55 samples (30 reclaimed wastewater and 25 drinking water) were collected. Wastewater samples were taken from the final disinfected (UV, chlorine) effluent of two wastewater treatment plants in a Mediterranean area from Spain. All of these reclaimed wastewater samples are available mainly for agriculture. Drinking water samples were collected from both public drinking water sources and different domestic units located in the East of Spain.

2.2. Isolation of free-living amoebae

Samples were collected into sterile bottles and processed within a maximum time of 2 hours. The residual free chlorine measurement of each drinking water sample was carried out by Spectroquant Nova 60 system (Merck, Germany).

One litre of wastewater and five litres of drinking water samples were filtered in duplicate through 3 μm pore size nitrocellulose filters (Whatman, Maidstone, England). Membranes were placed face down on Non-Nutrient-Agar (Iovieno et al., 2010)] and incubated at 28°C for 24 h. For drinking water samples, NNA plates were added with 0.1 ml of a suspension of *Escherichia coli* heated at 100°C for 15 min. Filters were removed after 24 h and plates continued the incubation at 28°C. NNA plates were controlled in order to observe FLA growth by phase contrast and inverted microscopy for 4 weeks. Then, NNA plates' content was collected by adding PAS solution and using a sterile cell scraper only if FLA growth was observed. One of the duplicate NNA plates was used to detect intra-amoebic *Legionella* spp. cells (section 2.3) and the other to detect two of the considered most widespread FLA: *Acanthamoeba* spp. and *Vermamoeba vermiformis* (section 2.4).

2.3. Detection of intra-amoebic *Legionella* spp.

Once NNA plates' content was collected, it was concentrated by centrifugation (500g, 3 min) and the sediment was resuspended in 500 μl of PAS solution Thereafter, FLA-external bacteria were treated using sodium hypochlorite at a concentration of 104 ppm for 1 h (Moreno-Mesonero et al., 2016) in order to kill them. Finally, hypochlorite was removed performing 3 washes with phosphate-

buffered saline (PBS) and finally resuspended in 1.1 ml of the same buffer. Aliquots of 1.1 ml were analysed by DVC-FISH method and culture as explained in 2.3.1 and 2.3.2 sections, respectively, in order to detect intra-amoebic *Legionella* spp. and *L. pneumophila* cells.

2.3.1. DVC-FISH

Optimization

Optimization of DVC incubation was previously performed with pure cultures of the reference *Legionella pneumophila* CECT 7109 strain (Spanish Type Culture Collection) and an environmental *Legionella* spp. strain which belonged to our own collection. DVC optimization was performed by testing different antibiotics which belonged to the quinolones group (novobiocine and ciprofloxacin), at concentrations ranging from 0.5 to 8 mg/l and at different incubation times: 3, 6 and 24 h. Briefly, 24 h *Legionella* spp. and *L. pneumophila* BCYE (buffered charcoal yeast extract; Oxoid, United Kingdom) cultures were inoculated in PBS buffer to reach a concentration of 10^6 cells/ml. One ml of the suspension was inoculated into 9 ml of the different DVC media tubes (Brucella broth supplemented with 5% fetal bovine serum (FBS) (PAA Laboratories, Austria) and piruvate (0.025%)), each containing the different antibiotic concentrations above cited and incubated at 37°C for the different specified times. A DVC tube without antibiotic was also inoculated with 1 ml of *Legionella* spp. and *L. pneumophila* suspension as a control assay.

DVC tubes were centrifuged at 8000 rpm for 10 min and the pellet was resuspended in 250 µl of PBS. Then, 750 µl of 4% paraformaldehyde (PFA) were added to each one and they were incubated at 4°C for at least 2.5 h in order to fix them. Afterwards, cells were washed twice using PBS to remove the remaining PFA and resuspended in ethanol and PBS (1:1). Thereafter, hybridization was carried out by depositing 10 µl of each trial on the wells of hybridization slides and hybridized as previously described by Moreno et al. (2003). To achieve the double identification of both *Legionella* spp. and *L. pneumophila,* two different probes were used: LEG705: 5'-CTG GTG TTC CTT CCG ATC-3' which is complementary to a region of the 16S rRNA gene of members of the Legionellaceae family (Manz et al., 1995) and LEGPNE1: 5'-ATC TGA CCG TCC CAG GTT-3', an specific probe for the

detection of *L. pneumophila*, which is complementary to a variable domain of the 16S rRNA gene of this bacteria (Grimm et al., 1998). Probes were synthesized and labelled with 6-FAM (6-Carboxyfluorescein) and CY3 respectively, by TIB Molbiol (Berlin, Germany). In order to avoid unspecific hybridization of the probes, the hybridization buffer contained a 35% of formamide.

Hybridization slides were washed under darkness in washing solution (0.10 M NaCl, 0.02 HCl‑Tris, 0.01% SDS and 0.005 M EDTA) at 48°C for 15 min. Finally, washing solution was removed from slides using distilled water and they were allowed to air-dry. Then, they were mounted in Vectashield® mounting medium (Vector Laboratories, UK) between the coverslip and the slide and were visualized using an Olympus BX 50 fluorescence microscope with the filters U-MWB, U-MWIB and U-MWIG.

Samples

One ml of the sodium hypochlorite-treated samples was added to 9 ml of the optimized DVC broth, i.e. with 0.5 mg/l of novobiocine antibiotic. Samples were incubated at the optimal tested conditions, i.e. 37°C under aerobic conditions for 24 h. After incubation, DVC tubes were centrifuged and resuspended in PBS as above described. Then, samples were fixed and hybridized as aforementioned. A pure culture of fixed *L. pneumophila* cells incubated for 24 h under DVC conditions was used as a positive control of the FISH reaction.

2.3.2. Culture

The existence of culturable intra-amoebic *Legionella* spp. cells was detected by cultivating a portion of 100 µl of the sodium hypochlorite-treated samples on BCYE agar. Samples were incubated for 2‑10 days at 37°C under aerobic conditions. Suspicious *Legionella* spp. colonies were picked and subsequently identified by FISH analysis as described in section 2.3.1.

2.4. Detection of *Acanthamoeba* spp. and *Vermamoeba vermiformis*

In order to detect the FLA *Acanthamoeba* spp. and *V. vermiformis*, each plate content was collected as explained above and concentrated in 500 µl of PBS. DNA was subsequently extracted using GeneJET™ Genomic DNA Purification Kit

(Thermo Scientific, Germany) following the cultured mammalian cells DNA extraction protocol increasing from 10 to 30 minutes the incubation time at 56°C for cell lysis (Moreno-Mesonero et al., 2016). DNA was eluted in 50 µl of the provided elution buffer and stored at -20°C until use.

Afterwards, specific *Acanthamoeba* spp. and *V. vermiformis* TaqMan and SYBR Green-based qPCRs, respectively, were performed.

For the specific detection of *Acanthamoeba* spp., AcantF900: 5'-CCC AGA TCG TTT ACC GTG AA-3' and AcantR1100: 5'-TAA ATA TTA ATG CCC CCA ACT ATC C-3' primers were used to amplify a 180 bp fragment of *Acanthamoeba* spp. 18S rRNA gene and the TaqMan probe AcantP1000: 5'-Cy5-CT GCC ACC GAA TAC ATT AGC ATG G-BHQ3-3' for its detection. The reaction was carried out in LightCycler® 2.0 Instrument (Roche Applied Science, Spain) in a final volume of 20 µl, which contained 4 µl of LightCycler® TaqMan® Master Reaction mix (Roche Applied Science, Spain), 0.8 µl of each primer (10 µM stock solution), 0.4 µl of the probe (10 µM stock solution), 0.4 µl of BSA (1 mg/ml stock solution) and 3 µl of DNA template. The amplification consisted of an initial denaturalization step at 95°C for 10 min followed by 40 cycles of: 95°C for 10 s, 63°C for 8 s and 72°C for 7 s; and finally, one cycle at 40°C for 30 s (Qvarnstrom et al., 2006).

For the specific detection of *V. vermiformis*, Hv1227F: 5'-TTA CGA GGT CAG GAC ACT GT-3' and Hv1728R: 5'-GAC CAT CCG GAG TTC TCG-3' primers were used for the amplification of a 502 bp fragment of *V. vermiformis* 18S rRNA gene. The reaction was performed in a final volume of 20 µl, which contained 2 µl of LightCycler® FastStart DNA Master SYBR Green I (Roche Applied Science, Spain), 0.4 µl of each primer (10 µM stock solution), 2,4 µl of $MgCl_2$ (25 mM stock solution), 2 µl of BSA (4 mg/ml stock solution) and 4 µl of DNA template. The amplification consisted of an initial denaturalization step at 95°C for 10 min followed by 40 cycles of: 95°C for 10 s, 56°C for 10 s and 72°C for 25 s; and finally, one cycle at 40°C for 30 s. At the end of each run, a melting curve analysis was performed (Kuiper et al., 2006).

For both qPCRs, a positive control with DNA of either *A. castellanii* or *V. vermiformis* and a negative control in which DNA was substituted for ultrapure water were added to each qPCR run.

3. RESULTS AND DISCUSION

Legionella is the causative agent of legionellosis, which is a severe form of pneumonia and can be acquired through aerosols inhalation. Although different disinfection processes are applied to eliminate *Legionella* from water, it has been detected in different water sources. Since this pathogenic bacterium should be sensitive to all these treatments, it is plausible that it can be protected by FLA in these aquatic environments, where FLA are frequently found. Furthermore, these FLA probably act as a vector or, in another words, "Trojan Horse" for the bacteria, thus facilitating human infection. This way, the goal of this work is to identify these viable and potentially pathogenic cells of *Legionella* spp. and *L. pneumophila* internalized inside FLA isolated from reclaimed and drinking water.

In a total of 29 (52.7%) out of the 55 processed samples it was possible to observe FLA growth. Specifically, wastewater samples, which were taken after tertiary disinfection treatment, showed FLA positive cultures in 20 (66.7%) out of the 30 processed samples, while drinking water samples yielded FLA positive cultures in 9 (36.0%) out of the 25 processed samples (Table 1).

Morphological characterization of the different isolated FLA by microscopy is an arduous task, since many of them have similar morphologies and, therefore, cannot be unequivocally identified by just observing them. This is why in this work two of the most common FLA found in water were molecularly detected by means of qPCR, although many different FLA morphologies were observed under the phase contrast microscope, thus indicating the presence of FLA different from *Acanthamoeba* spp. and *V. vermiformis*. *Acanthamoeba* spp., which comprises many different species indistinguishable by the used technique, was globally detected in 25 (86.2%) out of the 29 water FLA-positive samples. Concretely, *Acanthamoeba* spp. was detected in 17 (85.0%) out of the 20 wastewater FLA-positive samples and in 8 (88.9%) out of the 9 drinking water FLA-positive samples (Table 1). In the case of *V. vermiformis*, this FLA was successfully detected in 7 (24.1%) out of the 29 water FLA-positive samples. In wastewater, this FLA was detected in 5 (25.0%) out of the 20 FLA-positive samples, and in drinking water, *V. vermiformis* was detected in 2 (22.2%) out of the 9 FLA-positive samples (Table 1).

These results are in line with other studies also carried out in Spain in which *Acanthamoeba* spp. was detected by qPCR in almost all of the processed samples,

322 exactly in 87.5% and 93.7% of wastewater and drinking water samples,
323 respectively (Magnet et al., 2012; Magnet et al., 2013). *V. vermiformis* distribution
324 has not been as extensively studied as that of *Acanthamoeba* spp., although it is
325 well known that the former FLA is a frequent component of fresh surface water
326 (Kuiper et al., 2006). It has been detected in different aquatic environments, in
327 which wastewater (García et al., 2013; Ramírez et al., 2014) and drinking water
328 distribution systems (Nazar et al., 2012; Montalbano Di Filippo et al., 2015) are
329 included.
330 The fact that FLA have been found in reclaimed wastewater after tertiary
331 disinfection treatment and drinking water in this study matches with the results
332 derived from Magnet et al. (2013) work. They detected *Acanthamoeba* spp. in
333 wastewater and drinking water in both raw and after treatment, thus indicating
334 that this treatment was not effective for the removal of FLA. This seems to suggest
335 that the presence of FLA in water treatment plant effluents could be due to FLA
336 resistance to the carried out treatments as well as to the fact that they could
337 survive and grow in biofilms present in the distribution system (Thomas and
338 Ashbolt, 2011).
339 In this work, the presence of *Legionella* spp. and *L. pneumophila* was only tested
340 in those samples which showed positive FLA growth in order to exclusively detect
341 FLA-internalized bacteria. This way, all FLA-positive cultures were collected and
342 treated with a very high concentration of sodium hypochlorite so that all bacteria
343 outside FLA were killed. The same treatment was previously applied by our group
344 in order to identify FLA-internalized *H. pylori* cells from both wastewater and
345 drinking water origins (Moreno-Mesonero et al., 2017).
346 The interaction of *Legionella* spp. with FLA has been widely studied. García et
347 al. (2007) showed reciprocal benefits in the interaction of the FLA *Acanthamoeba*
348 *polyphaga* with *L. penumophila* in their resistance to water disinfection. Magnet et
349 al. (2015) carried out a study in Spain in which water from different sources such
350 as drinking water and wastewater treatment plants and natural ponds were
351 analysed. This study concluded that environmental *Acanthamoeba* seems to be
352 widely infected by *Legionella* spp. Also, *Legionella* spp. was confirmed as part of the
353 FLA microbiome when drinking water samples containing FLA were analysed by
354 means of 16S pyrosequencing (Delafont et al., 2013). However, although it has been

355 largely shown *in vitro* that *Legionella* spp. is able to multiply within FLA, to our
356 knowledge no study has shown FLA-internalized *Legionella* spp. viability by means
357 of molecular techniques in reclaimed and drinking water samples.

358 FISH technique has been reported as an efficient method, better than culture, to
359 detect *Legionella* in biofilms and cooling towers (Zeybek et al., 2017). This
360 technique is not influenced by the inhibitor substances present in the environment,
361 is able to identify VBNC cells, which have lost their ability to grow on synthetic
362 media, and it allows to observe the morphology and even count *in situ* the cells
363 present in the sample. The combination of DVC, a procedure which allows viable
364 bacteria to elongate in presence of nutrients and whose division are inhibited due
365 to a gyrase blocker, with FISH, which is performed on rRNA-targeted sequences,
366 has been proved effective in detecting and identifying viable cells in mixed
367 microbial communities such as rivers (Tirodimos et al., 2014), wastewater
368 (Moreno and Ferrús, 2012), water distribution systems (Mezule et al., 2013;
369 Santiago et al., 2015) and even inside FLA isolated from wastewater and drinking
370 water (Moreno-Mesonero et al., 2017). Moreover, *Legionella* DVC-FISH technique
371 has the advantage of its rapidity compared to the classical standard culture method
372 of these bacteria. While a complete *Legionella* DVC-FISH analysis could be
373 performed in less than 30 hours, its standard cultivation method and identification
374 requires up to 10 days. As far as we are aware, this is the first study in which viable
375 *Legionella* spp. and *L. pneumophila* have been detected simultaneously using this
376 molecular technique in water systems. Specific probes used in this assay have been
377 previously published (Manz et al., 1995; Grimm et al., 1998). Despite their
378 specificity has also been assayed in those reports, in this work, the possible match
379 of the probes with a rRNA region of other microorganism different from *Legionella*
380 has been tested *in silico* by the alignment analysis of the probes through NCBI
381 database. Although both probes resulted specific for *Legionella* spp. (LEG705) and
382 *L. pneumophila*(LEGPNE1) *in silico*, hybridization reaction was performed under
383 restrictive conditions (35% formamide).

384 The previous incubation of cells in DVC broth, which has a DNA gyrase inhibitor
385 agent (novobiocine, a quinolone antibiotic) allows differentiating viable and non-
386 viable cells after FISH detection. Novobiocine antibiotic at a concentration of 0.5
387 mg/l and after 24 h incubation time yielded the best results, since *Legionella* cells

388 became the most elongated and the concentration of cells remained the same. The
389 number of cells in DVC control tube increased and cells size remained the same, not
390 being elongated.
391 By means of DVC-FISH, the presence of viable internalized *Legionella* spp. cells
392 (elongated more than twice the original size) was observed in a total of 20 (69.0%)
393 samples out of the 29 FLA-positive ones (Figure 1). Some FLA were also observed
394 as presenting a low auto-fluorescence, mainly with U-MWIB filter. However, that
395 background fluorescence did not interfere with *Legionella* spp. or *L. pneumophila*
396 hybridized cells visualization. Specifically, metabolically activated *Legionella* spp.
397 cells were detected in 14 (70.0%) out of the 20 FLA-positive reclaimed wastewater
398 samples and in 6 (66.7%) out of the 9 FLA-positive drinking water samples. When
399 hypochlorite-treated FLA-positive samples were hybridized with *L. pneumophila,* a
400 total of 14 (48.3 %) samples contained viable *L. pneumophila* cells. Concretely, 10
401 (50.0%) out of the 20 FLA-positive wastewater samples and 4 (44.4%) out of the 9
402 FLA-positive drinking water samples exhibited viable internalized *L. pneumophila*
403 cells (Table 1).
404 Another useful technique which could have been used apart from DVC-FISH is
405 PCR (Polymerase Chain Reaction) due to its sensitivity and rapidity. However, the
406 latter cannot detect living cells. To do so, it has to be combined with viability dyes
407 such as propidium monoazide (PMA) or ethidium monoazide (EMA) which
408 irreversibly bind to external DNA or to DNA from membrane-damaged cells and
409 block these in any subsequent PCR amplification (Nocker et al., 2006).
410 Nevertheless, in this study samples have been treated with a high concentration of
411 sodium hypochlorite in order to kill FLA-external bacteria. Thus, PMA or EMA will
412 only prevent amplification of non-internalized cells because they would not enter
413 inside FLA, not being able to distinguish between live and dead FLA-internalized
414 cells (Moreno-Mesonero et al., 2016). This is the reason why DVC-FISH technique
415 was the chosen molecular technique to study the viability of *Legionella* spp. and *L.*
416 *pneumophila* FLA-internalized cells in water samples.
417 The high percentage of viable *Legionella* positive samples confirm, as other
418 authors suggested (Jjemba et al., 2010) that this bacteria is able to survive inside
419 protozoa, where it is protected against chlorine. Therefore, both the exposure to
420 reclaimed wastewater and drinking water could be a risk of *Legionella* infection. As

concluded by Jjemba et al. (2010) even though no *Legionella* outbreaks have been directly related to reclaimed water systems, effective measures should be stabilised in order to control *Legionella* and protozoa in these systems to avoid future public health problems, since some of them are human pathogens.

As results indicate, although the pathogenic bacteria *L. pneumophila* has been identified inside FLA in the processed samples, other species than *L. pneumophila* were present in the cultured FLA, some of which may not be pathogenic. As Muder and Yu (2002) reported, potentially pathogenic *Legionella* species other than *L. pneumophila* could be present inside FLA, since it has been previously shown that they are able to survive and even multiply inside them. Thus, future studies designing specific probes for FISH detection of other non-*L. pneumophila* species should be addressed in order to know the real exposure to pathogenic and non-pathogenic *Legionella* species in water systems. Moreover, it has been previously proved that growth and multiplication of *Legionella* spp. are both improved when they are within FLA or in nutrient-rich biofilms (Brown and Barker 1999; Thomas et al. 2004; Lau and Ashbolt 2009). For this reason, the identification of these bacteria, some of which could be human pathogens, inside FLA in water systems should be exhaustively performed, since FLA could help bacteria to reach humans.

When the classical culture technique was used to cultivate an aliquot of the samples, typical *Legionella* colonies were retrieved from both reclaimed wastewater and potable water samples in a total of 8 (27.6%) samples out of the 29 FLA-positive ones. Concretely, 7 (35.0%) out of the 20 FLA-positive wastewater samples and only one (11.1%) out of the 9 FLA-positive drinking water samples showed the presence of viable cultivable *Legionella* spp. cells (Table 1). Presumptive colonies were fixed and hybridized using the previously specified probes and conditions and all of them were identified as *L. pneumophila*.

Besides the fact that the standard culture method needs up to 10 days for its analysis, the presence of viable and internalized *Legionella* spp. cells could be unnoticed because of inhibition due to competitive microbiota or the presence of VBNC cells (Kirschner, 2016). *Legionella* spp. can acquire the VBNC state under unfavourable conditions. In this state, cells are unable to form colonies on synthetic media as they do in normal conditions. This way, in these cases, standard methods are of no use, since they would yield false negative results. Moreover, culture

454 methods are neither useful for some pathogenic species, such as *L. fallonii*, *L.*
455 *rowbothamii* or *L. drozanskii*, previously known as "Legionella-like amebal
456 pathogens" (LLAP), which can be preferably detected by molecular methods
457 because they are non-cuturable and obligate intracellular species found in water
458 environments which grow very low on agar media (Huang et al., 2011).
459 In this study, DVC-FISH technique allowed to identify in a rapid and specific way
460 the presence of viable *Legionella* spp and *L. pneumophila* cells inside the different
461 isolated FLA from drinking water and wastewater samples, among which
462 *Acanthamoeba* spp. and *V. vermiformis* were included. This showed that this
463 technique is a powerful tool when detecting metabolically active intra-amoebic
464 *Legionella* spp. cells in water systems which could pose a risk for human health.

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683 **TABLES**

684 Table 1. Obtained results of wastewater and drinking water samples

685

	SAMPLES	SAMPLE ID	FLA	*Acanthamoeba* spp.	*V. vermiformis*	DVC-FISH spp	DVC-FISH pn	Culture
Wastewater	1	WW1	+	+	-	+	+	+
	2	WW2	+	+	-	+	-	-
	3	WW3	-	N/A	N/A	N/A	N/A	N/A
	4	WW4	+	+	-	+	+	+
	5	WW5	+	-	+	-	-	-
	6	WW6	-	N/A	N/A	N/A	N/A	N/A
	7	WW7	+	+	+	+	+	+
	8	WW8	+	+	-	+	-	-
	9	WW9	+	+	+	+	+	+
	10	WW10	-	N/A	N/A	N/A	N/A	N/A
	11	WW11	-	N/A	N/A	N/A	N/A	N/A
	12	WW12	+	+	-	-	-	-
	13	WW13	-	N/A	N/A	N/A	N/A	N/A
	14	WW14	+	+	-	-	-	-
	15	WW15	+	+	-	+	-	-
	16	WW16	+	+	+	+	+	+
	17	WW17	+	+	-	-	-	-
	18	WW18	-	N/A	N/A	N/A	N/A	N/A
	19	WW19	+	+	-	+	+	-
	20	WW20	-	N/A	N/A	N/A	N/A	N/A
	21	WW21	+	-	+	+	-	-
	22	WW22	+	+	-	+	+	+
	23	WW23	+	-	-	-	-	-
	24	WW24	+	+	-	+	+	-
	25	WW25	+	+	-	+	+	-
	26	WW26	-	N/A	N/A	N/A	N/A	N/A
	27	WW27	-	N/A	N/A	N/A	N/A	N/A
	28	WW28	+	+	-	+	+	+
	29	WW29	+	+	-	-	-	-
	30	WW30	-	N/A	N/A	N/A	N/A	N/A
Drinking water	31	DW1	-	N/A	N/A	N/A	N/A	N/A
	32	DW2	+	+	-	+	-	-
	33	DW3	-	N/A	N/A	N/A	N/A	N/A
	34	DW4	-	N/A	N/A	N/A	N/A	N/A
	35	DW5	-	N/A	N/A	N/A	N/A	N/A
	36	DW6	+	-	+	+	+	+
	37	DW7	+	+	-	-	-	-
	38	DW8	-	N/A	N/A	N/A	N/A	N/A

SAMPLES	SAMPLE ID	FLA	*Acanthamoeba* spp.	*V. vermiformis*	DVC-FISH spp	DVC-FISH pn	Culture
39	DW9	-	N/A	N/A	N/A	N/A	N/A
40	DW10	-	N/A	N/A	N/A	N/A	N/A
41	DW11	-	N/A	N/A	N/A	N/A	N/A
42	DW12	+	+	+	+	+	-
43	DW13	-	N/A	N/A	N/A	N/A	N/A
44	DW14	-	N/A	N/A	N/A	N/A	N/A
45	DW15	+	+	-	-	-	-
46	DW16	-	N/A	N/A	N/A	N/A	N/A
47	DW17	+	+	-	-	-	-
48	DW18	-	N/A	N/A	N/A	N/A	N/A
49	DW19	-	N/A	N/A	N/A	N/A	N/A
50	DW20	+	+	-	+	-	-
51	DW21	+	+	-	+	+	-
52	DW22	-	N/A	N/A	N/A	N/A	N/A
53	DW23	-	N/A	N/A	N/A	N/A	N/A
54	DW24	-	N/A	N/A	N/A	N/A	N/A
55	DW25	+	+	-	+	+	-

686 **+**: positive result; **-**: negative result, **N/A**: not applicable

687 **Legend to figures**

688 Fig.1. Simultaneous identification of both *Legionella* spp. and *L. pneumophila* by FISH hybridization
689 with the probes LEG705-FAM (green fluorescence) and LEGPNE1-CY3 (red fluorescence) observed
690 by double filter U-MWB (red and green fluorescence). (A) Reclaimed wastewater sample; (B)
691 drinking water sample.