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Free living amoebae isolation in irrigation waters and soils of an insular arid agroecosystem

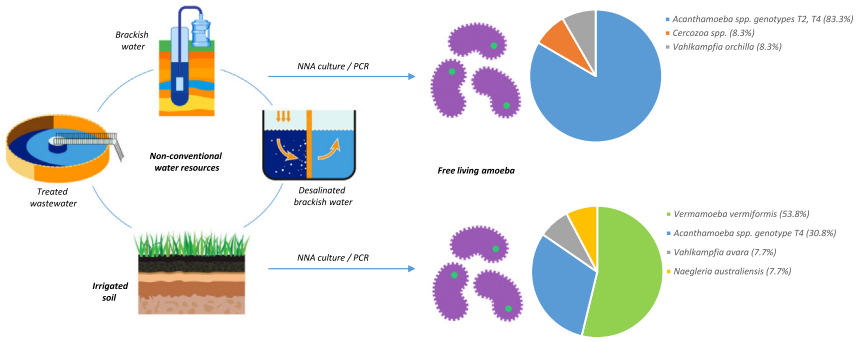
María Reyes-Batlle [a,b,*], Francisco J. Díaz [c], Ines Sifaoui [a,b], Rubén Rodríguez-Expósito [a,b], Aitor Rizo-Liendo [a,b], José E. Piñero [a,b], Jacob Lorenzo-Morales [a,b]

[a] Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, Spain / Departamento de Obstetricia y Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología. Universidad De La Laguna, La Laguna, Tenerife, 38203 Islas Canarias, Spain
[b] Red de Investigación Colaborativa en Enfermedades Tropicales (RICET), Spain
[c] Department of Animal Biology, Soil Science and Geology, University of La Laguna, Spain

HIGHLIGHTS

- Pathogenic FLA are distributed in agricultural soils and irrigation RWW, BW and DBW of an arid agroecosystem.
- *Acanthamoeba* spp. and *Vermamoeba vermiformis* are, respectively, the most common FLA in the evaluated soil and water samples.
- The bacteria load decreases contrary to FLA presence in no irrigated soils.
- The coexistence of pathogenic bacteria and FLA increases the risk of human infection in agroecosystems.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of freshwater in agricultural systems represents a high percentage of total water consumption worldwide. Therefore, alternative sources of water for irrigation will need to be developed, particularly in arid and semi-arid areas, in order to meet the growing demand for food in the future. The use of recycled wastewater (RWW), brackish water (BW) or desalinated brackish water (DBW) are among the different non-conventional water resources proposed. However, it is necessary to evaluate the health risks for humans and animals associated with the microbiological load of these waters. Protozoa such as free-living amoebae (FLA) are considered an emerging group of opportunistic pathogens capable to cause several diseases in humans (e.g. cutaneous and ocular infections, lung, bone or adrenal gland conditions or fatal encephalitis). In the present study we evaluate FLA presence in three different irrigation water qualities (RWW, BW and DBW) and its survival in irrigated agricultural soils of an extremely arid insular ecosystem (Fuerteventura, Canary Islands, Spain). Samples were cultured on 2% Non-Nutrient Agar (NNA) plates covered with a thin layer of heat killed *E. coli* and checked daily for the presence of FLA. According to the prevalence of FLA, *Vermamoeba vermiformis* (53,8%), *Acanthamoeba* spp. (30,8%), *Vahlkampfia avara* (7,7%) and *Naegleria australiensis* (7,7%) were detected in the analysed water samples, while *Acanthamoeba* (83,3%), *Cercozoa* spp. (8,3%) and *Vahlkampfia orchilla* (8,3%) were isolated in irrigated soils. Only *Acanthamoeba* strains were isolated in no irrigated soils used as control, evidencing the

* Corresponding author at: Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, Spain / Departamento de Obstetricia y Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología (Universidad de La Laguna),Spain
E-mail address: mreyesba@ull.edu.es (M. Reyes-Batlle).

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capability of these protozoa to resist environmental harsh conditions. Additionally, all analysed water sources and the irrigated soils presented growth of several pathogenic bacteria. Therefore, the coexistence in water and soils of pathogenic bacteria and FLA, can mean an increased risk of infection in agroecosystems.

1. Introduction

Irrigation plays a fundamental role in crop production and agricultural development in many arid regions despite their limited freshwater sources (Dorta-Santos et al., 2016). Competition for water from agriculture, households, industry and the environment gradually intensifies as the population increases and the effects of climate change becomes more pronounced (Grattan et al., 2015; Jia et al., 2019). In a global context of water scarcity, use of water in agricultural systems account for approximately 87% of total water consumption, a figure that will likely increase to meet the growing demand for food in the future (Ben-Gal et al., 2009). As a result, alternative sources of water for irrigation will need to be developed, particularly in arid and semi-arid areas (Grattan et al., 2015; Monterrey-Viña et al., 2020). Among the different water conservation practices proposed, the use of non-conventional water resources (i.e. treated wastewater, brackish water, agricultural drainage water, desalinated seawater), for agricultural irrigation is becoming common practice in such regions (Díaz et al., 2013, 2018).

Although it has been widely reported that those water quality can alleviate pressure on conventional water resources and expand crop production into marginal lands (6), its use involves several risk factors that must be taken into account (Becerra-Castro et al., 2015; Elgallal et al., 2016). In the mid-long term, the potential salinization and sodification of the soils, the contamination of groundwater by organic or inorganic compounds (e.g., nitrates), the accumulation to toxic levels of trace elements (e.g., boron, selenium), and the presence of other chemical components such as endocrine disruptors and compounds of pharmaceutical activity, may pose a potential risk not fully evaluated for human health and the environment (Grattan et al., 2015; Becerra-Castro et al., 2015; Hong et al., 2018).

In the short term, the health risks for humans and animals associated with the microbiological load of these waters, particularly in recycled wastewater (RWW), constitute the most relevant problem and to which greater attention has been given in the scientific literature (Muñoz et al., 2010; Rusiñol et al., 2020). Because a significant part of the RWW comes from the treatment of sewage, it is common to find fecal microorganisms, but also opportunistic microorganisms of free life, which constitute a potential cause of infection (Toze, 2006). Although to a lesser extent than with RWW, microbial contamination of irrigation groundwater has also been reported (De Giglio et al., 2017). Microbial pathogens are considered one of the globally leading causes of water quality impairment in agricultural watersheds (Díaz et al., 2010). Within a watershed, pathogenic bacteria and protozoa can be delivered to groundwater bodies from a variety of vectors including humans, many species of livestock, wildlife, and pets (Knox et al., 2007). Although discharge from point sources such as wastewater treatment facilities has traditionally represented a potential source of pathogens, regulation and enhanced treatment technologies have decreased its importance as a pollution source. Instead, non-regulated, non-point sources have become the main source of microbial pollution in waterways, with agricultural activities, including crop production, animal operations, pastures, and rangeland, being the single largest contributor (Díaz et al., 2010).

The risk of infection associated with the use of marginal quality waters depends on many factors such as infectious dose, the survival capacity of pathogens, dispersion capacity in water and soil, irrigation management, and the susceptibility of the exposed population (Toze, 2006). The most common pathogens founded in marginal quality waters are bacteria, although other organisms such as protozoa, helminths and viruses are also present (Helmecke et al., 2020). Within protozoa, free-living amoeba (FLA), which do not require a host organism to be able to survive (Cateau et al., 2014) are considered as an emerging group of opportunistic pathogens (Lorenzo-Morales et al., 2015). A great diversity of these ubiquitous organisms have been isolated from natural and artificial environments, including fresh water, brackish water, seawater, water treatment plants, and various types of soils (Scheikl et al., 2014). FLAs are capable of causing several diseases in humans and other animals, from cutaneous and ocular infections to lung, bone or adrenal gland conditions and fatal encephalitis (Visvesvara et al., 2007). Among the most common pathogenic AFLs in humans there are four genera/species: *Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris* and *Sappinia pedata* (Schuster and Visvesvara, 2004). Other genera such as *Vahlkampfia*, *Paravahlkampfia* and *Hartmannella* (*Vermamoeba*) also appear to be associated with human infections (Scheid et al., 2019). Additionally, FLAs act as vehicles for bacteria potentially pathogenic, which have acquired resistance mechanisms to the FLAs digestive enzymes (Todd et al., 2015). Therefore, the resistance of the amoeba in a cyst state can favor the survival of bacteria (Siddiqui and Khan, 2012).

Due to increasing incidence of FLAs human and animal diseases, some of them with high mortality rate, an assessment of its fate in agroecosystems using non-conventional water resources is urgently needed in order to know the relevance of this activity as potential dispersion vector of these microorganisms. Since the use of marginal quality waters, in a global climate change framework that could further limiting the availability of water resources for agriculture, constitutes a key factor for agricultural production in arid regions, its application in irrigation must be associated with studies that evaluate and allow minimize the associated risks mentioned above. In this study we focus on Fuerteventura Island (Canary Islands, Spain), one of the most arid territories in the European Union (mean annual precipitation ≈150 mm; evaporation rates ≈1800–2000 mm yr^{-1}), where agricultural production mainly relies on the use of recycled urban wastewater (RWW) and brackish water (BW). FLA where isolated and identified by molecular techniques from irrigation water and soil samples with the general objective of evaluating the clinical risk associated with the use of this type of water quality.

2. Material and methods

2.1. Study site

The study was conducted between January 2017 and July 2018 on the island of Fuerteventura (28°45′N, 13°49′W; Canary Island, Spain), specifically on the "Pozo Negro" experimental farm owned by the local government (Cabildo Insular). The climatic conditions during the study period were as follows: average annual rainfall of 81 mm, evapotranspiration 1706 mm yr^{-1}, average annual temperature 21 °C, average wind speed 3.2 m s^{-1}, average radiation 19.2 M J m^{-2} day^{-1} and average sunshine of 11 h day^{-1}. In this farm several soil plots have been dedicated since November 2016 to alfalfa (*Medicago sativa* L.) production to supply forage to the local market with a high demand for livestock feed, one of the main economic activities in the island. Alfalfa is grown in soils with texture ranging from clay loam to sandy loam, and a depth of 60–110 cm. Water qualities used for irrigation are: *i*) recycled waste water (RWW) from a nearby treatment plant, which

receives urban wastewater from a coastal touristic village. The treatment process consists of pre-treatment (removal of solids using filters), primary treatment (decantation) and secondary treatment (biological digestion); *ii*) brackish water (BW) without any treatment drawn from a depth of 45–60 m at the farm; and *iii*) desalinated brackish water (DBW) generated from that saline groundwater and treated at a desalination plant in the same farm applying a pretreatment by filtering with filter cartridges prior to dual membrane reverse osmosis. Water is storage in top open tanks for variable period of times until its use for crops irrigation.

2.2. Soil and water sampling

A total of 16 plots (9 m^2 in size) were chosen in the farm for soil sampling (4 irrigated with RWW, 4 with BW, 4 with DBW, and 4 without irrigation nor crop used as control). In each plot one soil sample (as a combination of three subsamples; $n = 16$) was taken in February of 2018 from top soil (0–10 cm). Soils were collected in 50 mL polyethylene sterile tubes and kept at 4 °C until microbiological analysis in the laboratory. An additional 500 g of soil for each sample were collected in plastic bags for physicochemical characterization. Water samples (RWW, BW and DBW) were taken approximately each 45 days ($n = 33$) during the study period at the stop-valves of the fields. The water was collected in 50 mL polyethylene sterile tubes and kept at 4 °C until the seeding in the laboratory. An additional 1 L of water was collected in plastic bottles for physicochemical characterization.

2.3. Soil and water characterization

All the soil samples were air-dried and passed through a 2 mm sieve prior to analysis. The following parameters were analysed in the samples: pH (pH_e) and electrical conductivity (EC_e) in saturated paste extract; calcium carbonate equivalent ($CaCO_3$); organic matter (OM); available phosphorous (P-Olsen); and total nitrogen (TN). All the soil analyses followed Standard Methods (Soil Survey Staff, 1996). The following parameters were examined in the water samples: pH, EC, cations (Ca^{2+}, Mg^{2+}, Na^+, K^+, NH_4^+), anions (Cl^-, SO_4^{2-}, PO_4^{3-}, NO_3^-), Kjeldahl nitrogen (Ntotal), total phosphorous (Ptotal), chemical oxygen demand (COD), biological oxygen demand (BOD), total suspended solids (TSS), and turbidity. All the analyses followed standard methods for the examination of water and wastewater (APHA, 1998).

In order to evaluate the presence of the most common pathogenic bacteria related to FLA, four selective agar mediums were used: *E. coli*-coliforms Chromogenic Medium (BOE) Conda® (Ushiyama and Iwasaki, 2010); TCBS agar (ISO) VWR Chemicals Prolabo® (Pfeffer and Oliver, 2003); SS Agar Merck® (Zhang and Lampel, 2010) and Cetrimide Agar (Pseudomonas selective Agar) Scharlau® (Brown and Lowbury, 1965). One hundred millilitres of water sample were filtrated through 0.45 μm nitrocellulose filters (Pall, Madrid, Spain). Individual membranes were placed into the specific selective medium and incubated for 24 h or 48 h at 37 °C depending on the microorganism to be detected and following the instructions of the manufacturer of each culture media.

2.4. Culture of FLA

The samples were kept at 4 °C until their processing. Once in the laboratory, 1 L of each water sample was filtered using a vacuum multiple system and 0.45 μm nitrocellulose filters (Pall, Madrid, Spain). Then, filters were cultured inverted onto 2% non-nutrient agar (NNA) plates with a layer of heat killed *E. coli* at room temperature. To process soil samples, 0.5 g of each soil sample was seeded onto 2% NNA plates. All these plates were monitored daily for the presence of FLA. The plates suspicious for FLA growth following the morphological features using the Page key (Page, 1988), were cloned by dilution in NNA until a monoxenic culture was obtained (Lorenzo-Morales et al., 2005a; Reyes-Batlle et al., 2015).

2.5. DNA extraction

DNA from the positive samples was extracted using the Maxwell® 16 System and the Maxwell® 16 Tissue DNA purification kit sample cartridge (Promega, Madrid, Spain) as it has been previously described (Reyes-Batlle et al., 2019a, 2019b). Amoebic genomic DNA yield and purity were determined using the DS-11 Spectrophotometer (DeNovix®, USA).

2.6. PCR and molecular characterization of isolates

To carry out the molecular identification of the positive samples, PCR analysis was performed using 18S rRNA specific primers P-FLA F 5′-CGCG GTAATTCCAGCTCCAATAGC-3′/P-FLA R 5′-CAGGTTAAGGTCTCGTTCGTT AAC-3′ (Tsvetkova et al., 2004) and VAHL-1 5′-GTCTTCGTAGGTGAAC CTGC-3′/VAHL-2 3′-CCGCTTACTGATATGCTTAA-5′ (De Jonckheere and Brown, 2005a, 2005b) (Tm = 55 °C). 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 (62 °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 analysed by 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 Mega 5.0 software program (Tamura and Nei, 1993; Kumar et al., 2018). Species identification was based on sequence homology analysis by comparison to the available DNA sequences in the Genbank database.

2.7. Phylogenetic analyses

Sequences were aligned using Mega 5.0 software program (Tamura and Nei, 1993; Kumar et al., 2018). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 34 and 12 nucleotides respectively sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair. Species identification was based on sequence homology analysis by comparison to the available DNA sequences in the Genbank database.

2.8. Statistical analyses

Statistical methods were implemented using Statistical Package for the Social Sciences (SPSS; version 25.0). The level of significance for all tests was set to $p < 0.05$. Differences between water quality parameters, and between soil properties under different treatments were assessed using an ANOVA and post-hoc Tukey's test. A Kuskal–Wallis test and a non-parametric Tukey-type multiple comparisons test were used when parameters did not conform to a normal distribution (Kolmogorov Smirnov test) and homogeneity of variance (Levene test).

3. Results and discussion

3.1. Irrigation water quality

Mean values of the physico-chemical and microbiological variables analysed in the irrigation waters are given in Table 1. Average TSS and turbidity were statistically significant greater in RWW than in BW and DBW ($p < 0.05$). The highest pH values were observed in BW (range = 8.1–8.6), while RWW and DBW showed similar pH

level (average ≈7.2–7.6). Salinity levels were also significantly higher in BW ($p < 0.05$), with an average EC ≈ 5 dS m^{-1}, twice and twenty five times greater than in RWW and DBW, respectively. With regard to cations and anions composition, all irrigation water qualities were sodic chloride-dominated, reaching maximum levels in BW where Na^+ and Cl^- content averaged 1054 mg L^{-1} and 1004 mg L^{-1}, respectively, and minimum levels in DBW (44 mg L^{-1} and 43 mg L^{-1}, respectively). BW also displayed the largest content in sulfate and bicarbonate (approximately 400 and 500 mg L^{-1}, respectively). Nutrients content (K, N and P), COD and BOD in RWW widely exceeded those in BW (with the exception of N) and DBW, indicating that RWW could be considered a potential source of organic matter and essential nutrients for plant and microorganisms. Nitrogen level (TN and nitrate) found in BW point out to a potential contamination of groundwater by nitrate. As it was expected DBW presented residual load of most nutrients and salt (Table 1).

Table 1 also shown the bacteria detection results as intervals (minimum and maximum number of colony-forming units; c.f.u) throughout the study period (11 samplings). The three evaluated water sources have presented coliforms and *Pseudomonas* growth. However, *Salmonella* and *Shigella* were not detected in any of them, while *Vibrio* presented growth in RWW and BW. *E. coli* was also detected in all water qualities, although to levels that not exceeded the Spanish legislation limit to type 2.1 RWW irrigational use (RD 1620/2007).

3.2. Impact of irrigation on soil properties

Chemical and microbiological soil characterization after approximately 16 months of irrigation with different water qualities is shown in Table 2. Soil water content was similar in all irrigated soils (≈20%) and exceeded by a factor of 2.5 the water content in control (non-irrigated) soils. Irrigation with all types of water led to a significant decreased in soil salinity with regard to control soil, likely as a consequence of relatively low salinity in those water qualities and the high leaching fractions applied (≈25%). Soils irrigated with DBW showed the lowest salinity (EC ≈ 1.2 dS m^{-1}), while those irrigated with BW had the greatest one (EC ≈ 3.4 dS m^{-1}). Soil reaction was alkaline with pH levels close to 8. Irrigation with BW significantly increased those values up to 8.5, in accordance with the highest pH in that type of water (pH of BW ≈ 8.3; Table 1). Irrigation with BW and DBW induced a significant decrease in carbonate soil contents. In spite of the relatively high load in organic matter present in some water qualities, particularly in the RWW (Table 1), irrigation did not promote a significant increase in organic matter and N contents with regard to control soil ($p < 0.05$). These results are similar to those reported by other authors following variable periods of irrigation with RWW (Díaz et al., 2013). The incorporation of easily degradable organic substances as a result of RWW use can increase both the microbial population and microbial activity and, consequently, can increase organic-matter mineralisation. In the case of total nitrogen, the addition represented by RWW and BW use (Table 1) can be offset by nitrogen leaching below the root zone, losses to volatilisation and crop removal (Bar-Tal, 2011). However, irrigation with RWW did increase significantly soil available phosphorous content compared to non-irrigated soil, which could be related to the high TP content in that type of water (Table 1).

Regarding the bacteria content, the irrigated soils with the three different water qualities have presented bacteria growth, while the no irrigated soils were negative for all tested pathogenic bacteria (Table 2). *Salmonella*, *Shigella* and *E. coli* were not detected in soil samples. However, coliforms, *Pseudomonas* and *Vibrio* grew in irrigated soils, generally with a greater load in those under RWW irrigation (Table 2). This presence is likely due to direct bacteria transfer from the water to the irrigated soils. For example, the soils irrigated with RWW and BW (which showed *Vibrio* contamination), have also presented *Vibrio* growth. However, the soils irrigated with DBW (without *Vibrio*) did not show growing of that bacteria (Table 2).

Table 1
Irrigation water characterization (RWW, recycled wastewater; BW, brackish water; DBW, desalinated brackish water) through the study period; mean ± standard deviation; minimum – maximum; $n = 11$; different letters indicate significant differences ($p < 0.05$) between water qualities.

Parameter	RWW	BW	DBW
TSS mg L^{-1}	13.6 ± 9.4 a	5.3 ± 3.9 b	1.2 ± 2.9 b
Turbidity UTN	12.0 ± 6.9 a	2.4 ± 1.1 b	3.5 ± 4.7 b
pH	7.6 ± 0.4 a	8.3 ± 0.2 b	7.2 ± 0.5 a
EC dS m^{-1}	2.5 ± 1.0 a	5.0 ± 0.7 b	0.2 ± 0.2 c
Ca^{2+} mg L^{-1}	27.4 ± 7.9 a	29.3 ± 6.6 a	2.3 ± 1.7 b
Mg^{2+} mg L^{-1}	30.6 ± 17.0 a	42.2 ± 8.9 a	1.0 ± 1.4 b
Na^+ mg L^{-1}	424.9 ± 183.1 a	1053.8 ± 139.7 b	44.2 ± 33.4 c
K^+ mg L^{-1}	29.0 ± 11.8 a	12.2 ± 1.5 b	0.7 ± 0.5 c
NH_4^+ mg L^{-1}	2.0 ± 4.7 a	0.0 ± 0.0 a	0.4 ± 0.5 a
Cl^- mg L^{-1}	603.3 ± 273.9 a	1004.1 ± 190.6 b	42.6 ± 30.6 c
HCO_3^- mg L^{-1}	165.7 ± 80.9 a	544.3 ± 68.9 b	36.1 ± 17.7 c
SO_4^{2-} mg L^{-1}	94.9 ± 46.1 a	403.2 ± 147.3 b	9.8 ± 10.9 c
PO_4^{3-} mg L^{-1}	14.1 ± 15.0 a	0.4 ± 0.8 b	0.1 ± 0.0 b
NO_3^- mg L^{-1}	9.2 ± 11.5 a	31.6 ± 9.7 b	4.4 ± 0.8 a
TN mg L^{-1}	13.7 ± 10.9 a	11.0 ± 5.6 a	1.3 ± 1.2 b
TP mg L^{-1}	5.2 ± 3.3 a	0.5 ± 0.2 b	0.2 ± 0.1 b
COD mg L^{-1}	52.9 ± 29.3 a	8.3 ± 9.6 b	2.9 ± 4.2 b
BOD_5 mg L^{-1}	14.9 ± 14.5 a	1.4 ± 3.9 b	1.1 ± 3.1 b
Total Coliforms cfu 100 mL^{-1}	>100	>100–0	>100–0
E. coli cfu 100 mL^{-1}	10–0	30–0	10–0
Pseudomonas spp. cfu 100 mL^{-1}	>100–0	>100–0	>100–0
Vibrio spp. cfu 100 mL^{-1}	>100–20	>100–0	0
Salmonella spp. cfu 100 mL^{-1}	0	0	0
Shigella spp. cfu 100 mL^{-1}	0	0	0

Table 2
Soil characterization (RWW, irrigated with recycled wastewater; BW, irrigated with brackish water; DBW, irrigated with desalinated brackish water; NI, no irrigation and no crop); mean ± standard deviation; minimum – maximum; $n = 4$; different letters indicate significant differences ($p < 0.05$) between soil treatments.

Parameter	RWW	BW	DBW	NI
Water content %	20.1 ± 2.0 a	21.3 ± 5.0 a	19.6 ± 6.4 a	8.0 ± 1.8 b
EC_e dS m^{-1}	2.7 ± 0.7 a	3.4 ± 0.3 a	1.2 ± 0.0 b	10.6 ± 4.6 c
pH_e	8.1 ± 0.1 a	8.5 ± 0.1 b	8.0 ± 0.1 a	8.1 ± 0.3 a
$CaCO_3$ g kg^{-1}	146.2 ± 23.3 a	86.3 ± 4.2 b	117.7 ± 7.0 ab	152.3 ± 37.2 a
Organic C g kg^{-1}	10.0 ± 0.9 a	10.8 ± 2.6 a	6.7 ± 1.2 a	9.5 ± 1.4 a
Active Org C mg kg^{-1}	588.9 ± 80.0 a	611.7 ± 33.8 a	534.4 ± 34.6 a	522.7 ± 56.4 a
TN g kg^{-1}	1.3 ± 0.7 a	1.4 ± 0.8 a	0.8 ± 0.6 a	0.7 ± 0.2 a
Olsen-P mg kg^{-1}	12.6 ± 2.8 a	8.3 ± 1.5 ab	5.4 ± 2.9 b	4.1 ± 2.2 b
Total Coliforms cfu g^{-1}	>100	0	>100–0	0
E. coli cfu g^{-1}	0	0	0	0
Pseudomonas spp. cfu g^{-1}	>100–0	0	>100–0	0
Vibrio spp. cfu g^{-1}	>100	>100–0	0	0
Salmonella spp. cfu g^{-1}	0	0	0	0
Shigella spp. cfu g^{-1}	0	0	0	0

3.3. FLA presence detection

From the 33 analysed water samples, 12 of them (12/33; 36,36%) were positive for the presence of FLA (Fig. 1, Table 3). The RWW was the source where the highest number of FLA strains (8/13; *V. vermiformis* (6), *A. hatchetti* (1/8) and *V. avara* (1/8)) was isolated, followed by the BW with 3 *Acanthamoeba* spp. strains (*Acanthamoeba sp.* T4 (1/3), *A. polyphaga* (1/3) and *A. hatchetti* (1/3)), and the DBW with only 2 different species (*N. australiensis* (1/2) and *V. vermiformis* (1/2)). *V. vermiformis* has been the most common species from the total of FLA species found (7/13; 53,8%), followed by *Acanthamoeba* genus (4/13; 30,8%). *V. avara* and

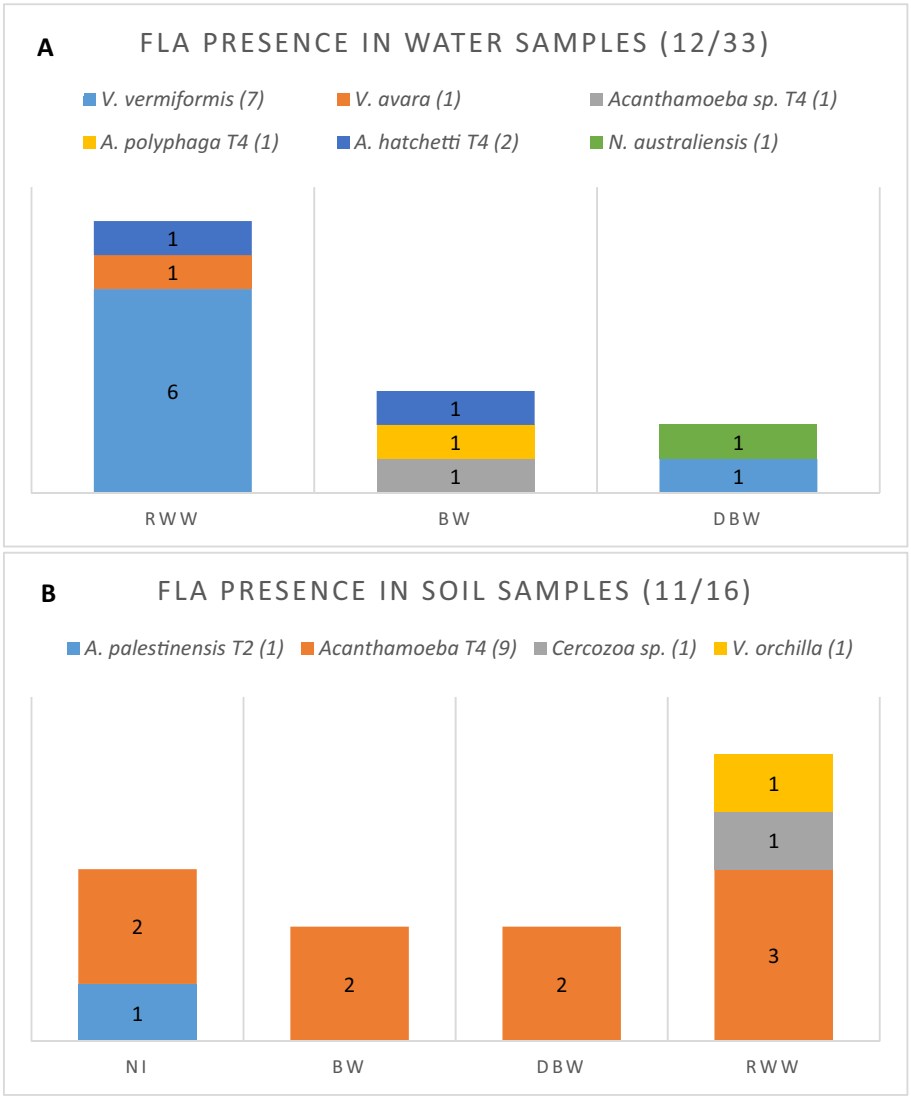

Fig. 1. A: FLA genera/species identified in each water sample categories (RWW, recycled wastewater; BW, brackish water; DBW, desalinated brackish water); B: FLA genera/species identified in each soil sample categories (NI, no irrigation and no crop; BW, irrigated with brackish water; DBW, irrigated with desalinated brackish water; RWW, irrigated with recycled wastewater).

N. australiensis were isolated in two water samples (1/13; 7,7%). While in RWW and DBW samples it can be appreciated a FLA genus diversity, in the BW source only could be isolated *Acanthamoeba* strains.

Table 3
FLA species isolated from irrigational use water of Fuerteventura Island (RWW, recycled wastewater; BW, brackish water; DBW, desalinated brackish water) (NNA: FLA growth in non-nutrient agar culture; PCR: FLA detection by PCR).

Sample code	NNA	PCR	Species	Homology (%)[a]
RWW01/2017	+	+	*Vermamoeba vermiformis*	98%
RWW03/2017	+	+	*Vermamoeba vermiformis*	95%
RWW04/2017	+	+	*Vermamoeba vermiformis*	98%
RWW06/2017	+	+	*Acanthamoeba hatchetti* T4	95%
BW06/2017	+	+	*Acanthamoeba sp.* T4	97%
RWW07/2017	+	+	*Vermamoeba vermiformis*	98%
BW02/2018	+	+	*Acanthamoeba polyphaga* T4	96%
DBW02/2018	+	+	*Vermamoeba vermiformis*	98%
RWW05/2018	+	+	*Vermamoeba vermiformis*	98%
DBW05/2018	+	+	*Naegleria australiensis*	99%
BW05/2018	+	+	*Acanthamoeba hatchetti* T4	98%
RWW07/2018	+	+	*Vahlkampfia avara*	94%
		+	*Vermamoeba vermiformis*	98%

[a] Homology (%) related to NCBI Data Base sequence.

A total of 16 soil samples, 12 irrigated with three types of water (RWW, BW, and DBW), and 4 samples from non-irrigated soil (NI) used as controls were evaluated to the presence of FLA. From the analysed samples, 11 of them (11/16; 75%) were positive for the presence of FLA, and 12 FLAs strains were isolated (Table 4). *Acanthamoeba* spp. has been the most common genera from the total of FLA species found (10/12; 83,3%), being the T4 genotype the most abundant (9/10; 90%), followed by the T2 genotype (1/10; 10%). *Cercozoa* spp. (1/12; 8,3%) and *Vahlkampfia* orchilla (1/12; 8,3%) were isolated in RWW irrigated soils. On the other hand, we can observe that the highest FLA diversity is present in the soils irrigated with RWW, where it could be isolated 5 strains from *Acanthamoeba* spp. (3/5), *V. orchilla* (1) and *Cercozoa* spp. (1).

All the FLA sequences obtained have been deposited in the Genbank database under the accession numbers MT319991-MT320014 and present >90% of homology with the available DNA sequences in this database (Tables 3 and 4). The optimal tree for the *Amoebozoa* phylogenetic relationship with the sum of branch length = 3.73691589 is shown in Fig. 2A. The optimal tree for the *Heterolobosea* phylogenetic relationship with the sum of branch length = 6.62249916 is shown in Fig. 2B. The isolates obtained in the present study are identified in boxes.

Table 4
FLA species isolated from agriculture use soils of Fuerteventura Island (NI, no irrigation and no crop; BW, irrigated with brackish water; DBW, irrigated with desalinated brackish water; RWW, irrigated with recycled wastewater) (NNA: FLA growth in non-nutrient agar culture; PCR: FLA detection by PCR).

Soil	Sample	NNA	PCR	Species	Homology (%)[a]
NI	1	+	+	*Acanthamoeba palestinensis*. T2	96%
	2	+	+	*Acanthamoeba sp.* T4	98%
	3	+	+	*Acanthamoeba sp.* T4	97%
	4	−	−	−	−
BW	1	+	+	*Acanthamoeba sp.* T4	95%
	2	+	+	*Acanthamoeba sp.* T4	95%
	3	−	−	−	−
	4	−	−	−	−
DBW	1	−	−	−	−
	2	−	−	−	−
	3	+	+	*Acanthamoeba sp.* T4	95%
	4	+	+	*Acanthamoeba sp.* T4	95%
RWW	1	+	+	*Acanthamoeba sp.* T4	95%
			+	*Cercozoa sp.*	90%
	2	+	+	*Acanthamoeba sp.* T4	95%
	3	+	+	*Acanthamoeba sp.* T4	95%
	4	+	+	*Vahlkampfia orchilla*	94%

[a] Homology (%) related to NCBI Data Base sequence.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 593 and 2082 positions respectively in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

4. Discussion

FLA species such as *Acanthamoeba* spp., *N. fowleri*, *B. mandrillaris*, *S. pedata*, *Vahlkampfia* spp., *Paravahlkampfia* spp. and *Vermamoeba* spp. (Schuster and Visvesvara, 2004) have been described as causal agents of several opportunistic diseases including epithelial disorders or fatal encephalitis (Scheid et al., 2019). Different studies have reported that conventional wastewater treatment commonly eliminates *E. coli*, other coliforms or intestinal enterococci. However, other bacteria, viruses, protozoa, and helminths could be still present in recycled wastewater (Helmecke et al., 2020). In that case, the microorganisms can contaminate natural resources and food chains, with major potential effects on the health of humans and animals. FLA are ubiquitous protozoa which have been reported in different sources such as soils, water, dust and air among others (Schuster and Visvesvara, 2004), contributing to the microbiological contamination of the environment (Guimaraes et al., 2016). FLA morphology and physiology are strongly dependent on the niche where they are developing. The environment parameters such as humidity or temperature, affects directly to the amoeba morphology and its physiology. Therefore, a molecular analysis such as the DNA genotyping is necessary to characterize the found FLAs. In this work, we have reported the presence of different FLA species and also demonstrating their relationship from a phylogenetic point of view (Fig. 2). These phylogenetic links show the homology of the *Acanthamoeba* and *Vermamoeba* strains (Fig. 2A), which belong to *Amoebozoa* class, and *Naegleria*, *Vahlkampfia* and *Cercozoa* strains (Fig. 2B), which belong to *Heterolobosea* class, isolated in this study with the sequences present in the NCBI Data Base.

The presence of FLAs species in all types of both managed water and soils (irrigated and non-irrigated) demonstrated the capability of these protozoa to resist to environmental harsh conditions (Trabelsi et al., 2012). *Acanthamoeba* spp. have been reported in several water related

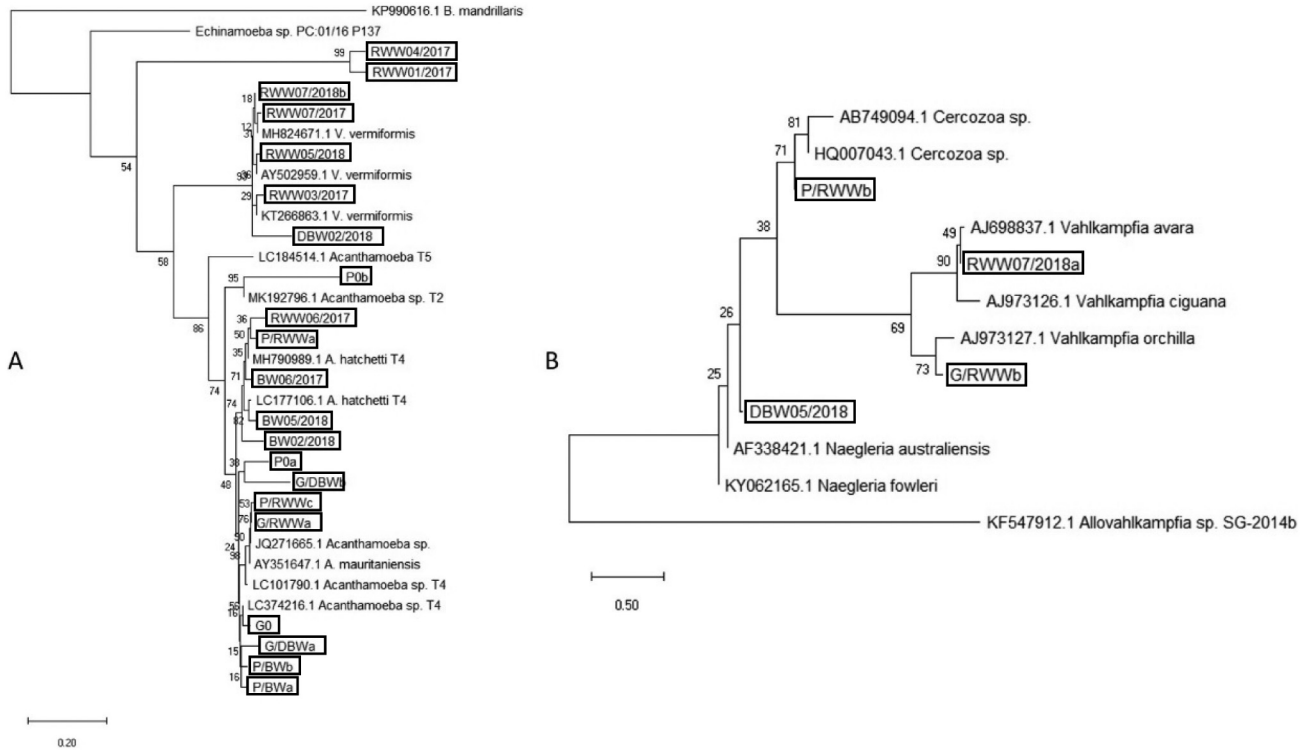

Fig. 2. Evolutionary relationships of taxa. A: phylogenetic relationship of the *Amoebozoa* strains isolated in the present study; *Echinamoeba* sp. and *B. mandrillaris* were used as outgroups for the *Acanthamoeba* and *Vemamoeba* clades. B: phylogenetic relationship of the *Heterolobosea* strains isolated in the present study. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The isolates obtained in the present study are identified in boxes.

environments (Koyun et al., 2020; Milanez et al., 2020), and this genus is usually the most common FLA in the environment, being the genotype T4 the most abundant in soil samples (Geisen et al., 2016). We have corroborated this fact in the current study, being able to survive in highly saline and very dry soils (control soil). This capability is due to the acquisition of a resistance phase or cyst stage common to the most of the FLAs species. This stage plays an important role in the evaluation of the risk that these microorganisms means to the human health.

It is important to highlight that the lowest FLA diversity correspond to the BW source, which presents the highest pH value (Table 1). *Acanthamoeba* is a FLA genus capable to resist harsh environmental conditions such as the high pH level (Khan, 2009; Siddiqui and Khan, 2012). Moreover, *Acanthamoeba* spp. was the only genus isolated from the BW irrigated soil samples (Fig. 1, Table 4). The highest number of strains and FLA diversity was isolated in the soils irrigated with RWW, which possesses the greatest organic matter load and bacteria content among the three analysed water qualities. As it is well known, bacteria are part of the main FLA diet, favouring FLA proliferation in the environment (Samba-Louaka et al., 2019; Gomes et al., 2020).

In general terms a greater FLA diversity was found in water than in soil samples. For example, *Naegleria* spp., a widely distributed pathogenic protozoa in Europe, North America, Asia and Oceania (De Jonckheere, 2014) was isolated from DBW samples but it was not detected in the soils irrigated with that water. The same can be applied for *Vermamoeba* spp., isolated in water but not in soil. The soil water content, lower than 20% (Table 2) could mean a disadvantage in the FLA metabolism as these species are mainly isolated from aquatic environments (Scaglia et al., 1983; Montalbano Di Filippo et al., 2017; Reyes-Batlle et al., 2019a, 2019b; Bellini et al., 2020; Milanez et al., 2019; Pazoki et al., 2020). Thus, this could evidence the difficulties to tolerate the edaphic conditions (i.e. salinity, soil desiccation, predation by other soil organisms).

Vahlkampfia spp., has been characterized in the soils under RWW irrigation. Some *Vahlkampia* species have acquired an interest because of their possible implication in keratitis (Niyyati et al., 2010). Despite Amoebic keratitis is mainly due to *Acanthamoeba* spp. (Lorenzo-Morales et al., 2015), there are several reports of mixed infections of *Acanthamoeba* and *Vahlkampfia* (Niyyati et al., 2010; Arnalich-Montiel et al., 2013) or a corneal damage due to *Vahlkampfia* (Aitken et al., 1996).

Cercozoa is a protist phylum belonging to *Percolozoa* (*Heterolobosea*) class (Fig. 2B) and Rhizaria subgroup (Cavalier-Smith et al., 2018), and dominant in terrestrial systems (Urich et al., 2008; Dumack et al., 2017). Rhizaria are mostly heterotrophic flagellates, amoebae or amoeba-flagellates and they comprise predominantly flagellate phylum Cercozoa (Cavalier-Smith et al., 2018). Grazing experiments carried out in previous studies indicate that leaf-associated Cercozoa could impact on the composition and function of bacterial phyllosphere communities (Dumack et al., 2017; Flues et al., 2017; Xiong et al., 2020). In the present research, *Cercozoa* sp. was isolated in RWW irrigated soils, but not in water, which could indicate other potential sources such as plant material. As in the present study no specific *Cercozoa* primers were used, further analyses are required to evaluate this protozoa ecology and physiology. Even though *Cercozoa* spp. it does not present any associated pathology, these free living protozoa could contribute to other pathogens communities (Bass et al., 2009).

The FLA diet includes microorganisms such as fungi, protozoa and bacteria, as well as organic particles (Cateau et al., 2014; Glücksman et al., 2010; Schulz-Bohm et al., 2017). FLAs have been shown to act as reservoirs and transmission vectors for pathogenic bacteria capable of living within trophozoites and even cysts (Lorenzo-Morales et al., 2015; Mella et al., 2016). Several bacteria species have acquired resistance mechanisms to the FLAs digestive enzymes (Amoeba-Resistant Bacteria; ARB), using these protozoa as vehicles. Moreover, the cyst stage can favor the intracellular survival of bacteria, avoiding the common water disinfection systems, non-effective against FLAs cysts (Lorenzo-Morales et al., 2015). It has been demonstrated that the only presence of FLA can favor bacteria growth in harsh conditions (Reyes-Batlle et al., 2017a, 2017b), increasing their potential for transmission and virulence, since they hide from the host's immune system inside amoebas and can accumulate within vesicles (Mella et al., 2016). ARBs include important pathogens such as *Legionella* sp., *Pseudomonas aeruginosa*, *Cryptococcus neoformans*, *Chlamydophila pneumoniae*, *Listeria monocytogenes*, *Mycobacterium leprae*, *Helicobacter pylori and Escherichia coli* serovar O157, among others (Greub and Raoult, 2004). Therefore, the coexistence in the soils of pathogenic bacteria and FLA, can mean an increased risk of infection in agroecosystems.

Although FLAs pose a direct threat as infectious agents and as transmitters of pathogenic bacteria, their presence in soils, far from being harmful to them, is of special ecological importance by improving the nutrient availability and modify bacterial populations, with positive effects on plant growth (Guimaraes et al., 2016). Our results confirm the presence of pathogenic protozoa in irrigation water and soils destined to agricultural production in an extremely arid ecosystem, where agricultural production mainly relies on non-conventional water resources. The further use of these kind of resources needs to evaluate the associated human health risk.

CRediT authorship contribution statement

Francisco J. Díaz: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Ines Sifaoui:** Investigation, Methodology, Writing - review & editing. **Rubén Rodríguez-Expósito:** Investigation, Methodology. **Aitor Rizo-Liendo:** Investigation, Methodology. **José E. Piñero:** Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing. **Jacob Lorenzo-Morales:** Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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