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Occurrence of free-living amoebae in streams of the Mexico Basin

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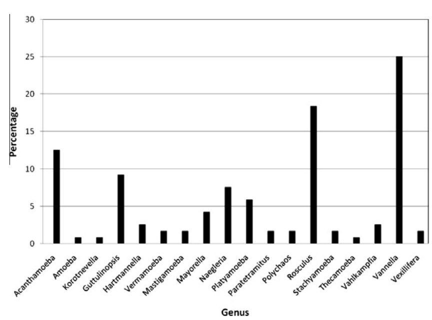
HIGHLIGHTS

- Spatial distribution of FLA in the streams of México Basin was very variable.
- *Vannella*, *Acanthamoeba* and *Rosculus* were the most common genera.
- Only three *Acanthamoeba* isolates had low virulence.
- Water temperature was the most influential variable on amoebae presence.

GRAPHICAL ABSTRACT

Percentage of FLA isolates by genus in streams of the Mexico Basin.



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ABSTRACT

Free-living amoebae (FLA) are protozoa that are widely distributed in nature. They are important in the cycling of nutrients in aquatic food chains, but their distribution in natural aquatic environments is not well known. We conducted a survey to determine the presence and distribution of FLA and their relation to some physicochemical parameters in streams of the Mexico Basin in Central Mexico. Thirty-two sites from 18 streams were sampled. Samples were centrifuged and cultured onto NNA-media to isolate amoebae. Identifications were based on morphology. The pathogenicity of *Acanthamoeba* isolates was tested. Oxygen saturation, temperature, pH, specific conductance, water flow, dissolved reactive phosphorus, nitrites, nitrates, ammonia, dissolved inorganic nitrogen and coliforms were determined. One hundred-and-twenty FLA representing 18 genera were identified. The most frequent genera were *Vannella, Rosculus* and *Acanthamoeba.* The frequency of potentially pathogenic FLA was low and only 3 *Acanthamoeba* isolates were invasive in mice. The highest species richness of FLA was found in streams located into agriculture activity areas and those close to small villages that discharge wastewater into them. Water temperatures were always below 17 °C. Oxygen saturation and pH were within the limits for the growth of most FLA. The presence of *Acanthamoeba* and *Naegleria* was low; nevertheless, they include potentially pathogenic species and can act as vectors and reservoirs for microbial pathogens and can produce human infections.

1. Introduction

Free-living amoebae (FLA) are ubiquitous protozoa that occur in a wide range of habitats throughout the world and include pathogenic and opportunistic organisms found in man and other animals. In nature, FLA ingest organic material, bacteria, yeasts,

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algae and other organisms as a food source (Anderson, 1997; Bradley and Marciano-Cabral, 1996; Fenchel, 1987; Rogerson, 1981) the rate of microbial degradation, and hence of nutrient cycling, is enhanced by the grazing activity of protozoa including amoebae. Clarholm (1981) suggested that their presence stimulates the microbial turnover of phosphorus and nitrogen fixation. However, little is known of their distribution in natural aquatic environments such as streams, or of the influence of physicochemical parameters.

Many aquatic bodies are near urban areas and therefore receive continual discharge of wastewater. This discharge provides organic material and bacteria, which serve as food for protozoa. The tumbling action of water flowing through the streams maintains a high concentration of oxygen, which favors FLA. It has been suggested (Greub and Raoult, 2004; Preston, 2003; Rodriguez-Zaragoza, 1994; Thomas et al., 2010) that amoebae living in the water column must attach to particulate matter in order to feed, that suspended bacteria do not provide particularly favorable conditions for amoebae, and that the proliferation of FLA in water is due mainly to their grazing on biofilms. Thus, they often live on biofilms and at water–soil, water–air, and water–plant interfaces.

The behavior of FLA in nature is not well known but environmental and ecological factors may contribute to the epidemiology of the infections they cause. Of the many FLA that are found in nature, only four genera, namely *Acanthamoeba, Naegleria*, *Balamuthia* and *Sappinia* are responsible for opportunistic and non-opportunistic infections in humans and other animals. They can infect the central nervous system and *Acanthamoeba* can also cause cutaneous lesions and amoebic keratitis, an infection of the cornea (Khan, 2006; Marciano-Cabral and Cabral, 2003; Qvarnstrom et al., 2009; Visvesvara et al., 2007). FLA have been isolated from freshwater lakes, thermally polluted waters, sediment, hot springs, swimming pools, soil, dust, air conditioning vents, sewage and the domestic water supply, contact lenses, and dialysis units (Corsaro et al., 2010; Marciano-Cabral and Cabral, 2003; Martinez and Visvesvara, 1997; Rivera et al., 1987; Rivera et al., 1993; Trabelsi et al., 2012; Visvesvara et al., 2007). In addition, FLA can also act as vectors and reservoirs for several viruses such as *Mimivirus* (La Scola et al., 2003), enterovirus (Greub and Raoult, 2004), and adenoviruses (Scheid and Schwarzenberger, 2012), and for pathogenic bacteria such as *Legionella pneumophila*, *Helicobacter pylori, Chlamydia* sp., *Mycobacterium avium, Escherichia coli* O157:H7, *Vibrio cholerae,* and *Pseudomonas aeruginosa* (Barker and Brown, 1994; Cirillo et al., 1997; Marciano-Cabral, 2004; Thomas et al., 2010; Visvesvara et al., 2007). Furthermore, it has been shown that the growth of bacteria in FLA increases bacterial resistance to antibiotics and biocides, and increases bacterial virulence (King et al., 1988), consequently representing an important reservoir of human pathogens.

The aim of this study is (i) to characterize the distribution and species richness of free-living amoebae in streams of the Mexico Basin through the morphological identification of free-living amoebae, (ii) to investigate the relationship between distribution and the physicochemical water composition, and (iii) to determine the potential risk to human health in the region.

1.1. Study area

The Mexico Basin lies at 19–20° N and 98–100° W (Fig. 1) and is surrounded by the Trans-Mexican Volcanic Belt which includes Popocatépetl, Iztaccíhuatl and Ajusco volcanoes and a chain of mountains down which run 45 streams (Legorreta, 2009). These are high mountain streams (higher than 2000 m a.s.l.) at subtropical latitude. The basin covers ~9600 km^2 and includes the territories of five central states. Of the total area, 5136 km^2 is flat land, while the remaining 4464 km^2 is mountainous, some of which is a protected natural area. The streams of the basin are subject to continuous use for agriculture, fish farming, tourism and urban activities. In spite of their biological and social importance, there are few studies related to the streams and their communities.

2. Material and methods

In total 32 water samples were collected from 18 streams during the warm dry ($n = 13$), warm rainy ($n = 16$) and cool dry ($n = 3$) seasons from March 2012 to June 2013.

2.1. Water sampling

For FLA, samples of 100 ml were collected from the surface of water bodies in sterile plastic bags (Nasco, Whirl-Pak, USA) and transported to the laboratory at ambient temperature. At the same time, samples of 150 ml were taken for bacteriological analyses in sterile plastic bags and were transported to the laboratory at 4 °C in the dark (APHA, 2000).

2.2. Isolation and culture

From each sample a volume of 50 ml were centrifuged at 1840*g* for 15 min and the pellet was seeded onto a non-nutrient agar plate with a heat-killed suspension of *Enterobacter aerogenes* as a food source for the amoebae (NNA medium) (Page, 1988). The plates were incubated at 25 °C and observed daily under an inverted light microscope at 10× and 20× to detect the growth of amoebae. *Acanthamoeba* isolates were brought into axenic culture in phosphate-biotryptase-serum-glucose medium (Rivera et al., 1987) and 2% (w/v) Bacto™ Casitone medium (Becton Dickinson Co. USA) supplemented with 10% (v/v) fetal bovine serum (Cerva, 1969).

2.3. Morphological diagnosis

The identification of free-living amoebae was based on the morphological features of trophozoites and cysts, temperature tolerance and the flagellate transformation test to differentiate isolates of *Naegleria* from *Vahlkampfia* (Page, 1988; Pussard and Pons, 1977).

2.4. Physicochemical analysis

Water temperature, pH and specific conductance (25 °C) were measured with a PC-18 conductivity meter (Conductronic, Puebla, Mexico). Oxygen saturation percentage with the oxygen meter YSI-85 (Yellow Springs Instruments, Ohio). Water flow was calculated using data of the cross-sectional area of the stream measured using a rule, and the mean water velocity with a current meter Swoffer 2100 (Swoffer Instruments, Seattle). Dissolved nutrients were sampled by filtering 1 L of stream water through 0.45 and 0.22 μm pore filter membranes (*in situ*) and were preserved frozen until measured in the laboratory with a DR 3900 laboratory Spectrophotometer (Hach Company, Loveland, Colorado). These analyses are adapted from Standard Methods for the Examination of Water and Wastewater (APHA, 1995). All measurements were made in duplicate.

2.5. Bacteriological analyses

The samples were analyzed according to standard procedures for the enumeration of total coliforms and fecal coliforms (APHA, 1995). The results were expressed as most probable number per 100 ml (MPN/100 ml).

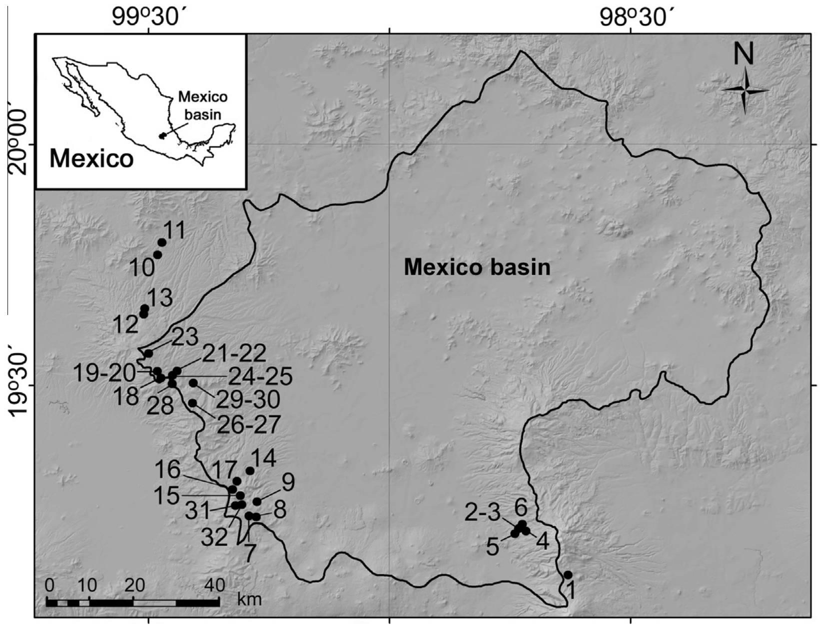

Fig. 1. Sampling sites in streams of the Mexican Basin. 1. Apatlaco (ApWd12). 2. San Rafael Vereda (RvWd12). 3. San Rafael Canal (RcWd12). 4. Cosa mala I (CcWd12). 5. Cosa mala II (CcWd12). 6. La Castañeda (CtWd12). 7. Monte Alegre I (Mo1Wd12). 8. Monte Alegre II (Mo2Wd12). 9. Eslava (EsCd13). 10. La Cabañita (CaWr12). 11. La Planta (PlWr12). 12. El Llano I (Tl1Wr12). 13. El Llano II (Tl2Wr12). 14. Santa Rosa (SrWr12). 15. Santa Rosa Alto (SaWd13). 16. La Capilla (CpWd13). 17. Arroyo Desierto (RdWr12). 18. Organillos (OrWr12). 19. Presa Iturbide I (PiWr12). 20. Presa Iturbide II (PiWr13). 21. Truchero Don Alvaro (TaWr12). 22. Truchero Don Alvaro (TaWr13). 23. Manantial las Palomas (MlpWr13). 24. Manantial Capoxi (McWr12). 25. Rio Capoxi (RxWr12). 26. Manantial San Pedro I (MpWd12). 27. Manantial San Pedro II (MpWr13). 28. La Caldera (LcWd12). 29. Xopachi II (XoWd12). 30. Xopachi I (XoWr13). 31. Chautitle Alto (ChCd13). 32. Chautitle Cañada (ChcCd13). The first 2 letters represent the sampling site and warm dry (Wd), warm rainy (Wr) and cool dry (Cd) the next acronyms. The number represents the year of the sampling, 2012 or 2013. It must be noticed that five sampling sites are outside of the basin. But they are very close and can be also included as high altitude streams.

2.6. Pathogenicity test

To determine the pathogenic potential of *Acanthamoeba* isolates, 3-week-old white mice were inoculated intranasally with 0.02 ml of culture containing from 10^4 to 10^6 amoebic cells. When each mouse died, or was killed after 30 days, the brain, lungs, kidneys and liver were seeded onto NNA plates to recover the amoebae (Cerva et al., 1973; De Jonckheere, 1980).

2.7. Statistical methods

The Spearman Correlation Coefficient (Rho) was applied to look for correlations between environmental variables and amoeba species isolates. Significance was chosen at 0.1 level that is adequate for experiments were scarce control of variables is exerted, as was the case in the present research.

3. Results

3.1. Isolation of FLA

One-hundred-and-twenty isolates belonging to 18 genera of FLA were found (Fig. 2). Fifty-five amoeba isolates were found in the warm dry season, 11 in the cool dry season and 54 in warm rainy season. It must be considered that the cool dry season had the lowest number of samples (Table 1).

3.2. Morphological diagnosis

Twenty-six species belonging to 18 genera of FLA were identified (Table 1).

3.3. Physicochemical parameters

All the FLA were found at relatively low temperatures (4.9–16.9 °C), in slow to fast flowing waters (0.001–1.09 $m^3\ s^{-1}$) that were moderately acidic to alkaline (pH 6.0–8.0) and with relatively low ionic content (30.6–150 $\mu S\ cm^{-1}$). Oxygen saturation was high (80–127%). Conditions varied according to trophic level: DRP 0.19–0.8 $mg\ L^{-1}$, nitrites 0.002–0.8 $mg\ L^{-1}$, nitrates 0.002–0.8 $mg\ L^{-1}$ and ammonia from 0.0 to 0.225 $mg\ L^{-1}$.

3.4. Bacteriological analysis

With the exception of two samples, CaWr12 (2200 MPN/100 ml) and PiWr12 (1700 MPN/100 ml), the total coliform bacteria were below 1100 MPN/100 ml. In ApWd12 and RvWd12 no coliform bacteria were found.

3.5. Pathogenicity test

The frequency of *Naegleria* and *Acanthamoeba* was low; *Naegleria* with 9 of 120 isolates (7.5%) and *Acanthamoeba* 15 of 120 (12.5%), and only three *Acanthamoeba* isolates showed low virulence; these, recovered from the brains of mice, originated from Tl1Wr12, SaWd13 and ChCd13 sampling sites.

3.6. Statistical analysis

Of the environmental variables measured (Table 2), water temperature had the most influence on the presence of amoebae.

It was positively correlated with *Acanthamoeba* spp., *H. cantabrigiensis* and *Naegleria gruberi*, and inversely correlated with *Mayorella cultura*. K_{25} values favored the presence of *Acanthamoeba* spp. and *Platyamoeba placida*. *Acanthamoeba* spp. and *Vannella platypodia* were negatively affected by the increase in altitude, and

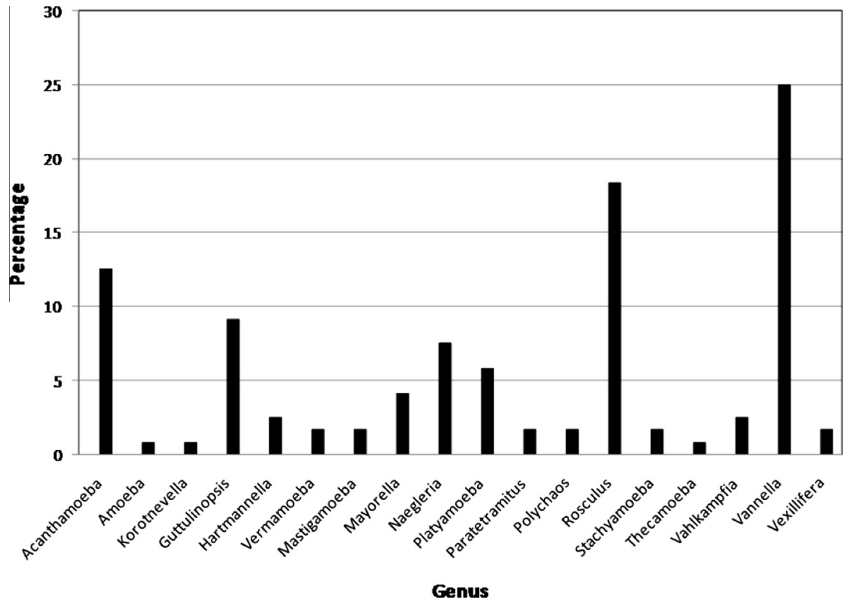

Fig. 2. Percentage of FLA isolates by genus.

Table 1
Distribution of FLA species in warm dry, cool dry and warm rainy seasons in streams of the Mexico Basin.

FLA species	Warm dry season $n = 13$	Cool dry season $n = 3$	Warm rainy season $n = 16$	Total $n = 32$
Acanthamoeba spp. Volkonsky, 1931	7	1	7	15
Amoeba diminutiva Bovee, 1972	1	0	0	1
Korotnevella stella (Schaeffer, 1926) Goodkov, 1988	1	0	0	1
Guttulinopsis nivea Raper, Worley and Kessler, 1977	1	2	1	4
G. vulgaris E.W. Olive, 1901	6	0	1	7
Hartmannella cantabrigiensis Page,1974	0	0	3	3
Vermamoeba vermiformis Page, 1967 emend. Smirnov et al., 2005	0	0	2	2
Mastigamoeba sp. Schulze, 1875	2	0	0	2
Mayorella cultura Bovee, 1961	2	1	0	3
M. penardi Page, 1972	0	0	1	1
M. vespertilioides Page, 1983	0	0	1	1
Naegleria gruberi Schardinger, 1899	4	0	5	9
Paratetramitus jugosus Page, 1967	1	0	1	2
Platyamoeba placida Page, 1968	4	0	3	7
Polychaos timidum Bovee, 1972	2	0	0	2
Rosculus ithacus Hawes, 1963	8	3	11	22
Stachyamoeba lipophora Page, 1975	0	1	1	2
Thecamoeba similis Greeff, 1891	0	0	1	1
Vahlkampfia avara Page, 1967	1	0	0	1
V. enterica Page, 1974	1	0	0	1
V. lobospinosa Craig, 1912	0	1	0	1
Vannella cirrifera Frenzel, 1892	1	0	0	1
V. lata Page, 1988	2	0	1	3
V. platypodia Gläser, 1912	9	2	14	25
V. simplex Wohlfarth-Bottermann, 1969	1	0	0	1
Vexillifera bacillipedes Page, 1969	1	0	1	2
Total	55	11	54	120

Vannella (25%), *Rosculus* (18.3%) and *Acanthamoeba* (12.5%) were the most frequently isolated genera and *Amoeba, Korotnevella* and *Thecamoeba* were isolated only once (Fig. 2).

M. cultura and *Vannella lata* were positively correlated with DRP concentration. Ammonium concentration was a positive factor for *P. placida* and negative for *M. cultura* (Table 3).

4. Discussion

4.1. Distribution and diversity of FLA

Spatial distribution of FLA in the streams studied was very variable. *V. platypodia*, *Rosculus ithacus* and *Acanthamoeba* spp. were the most prevalent amoebae throughout the study area. The comparatively high prevalence of *Vannella* species, isolated from 26 of 32 samples (81%), is similar to results from a stream in Virginia, USA (Ettinger et al., 2003). Their prevalence may reflect their ability to move into the water when the trophozoites change to the radiate floating form (pelagic form). Cysts have not been found in any species in this genus (Page, 1988). Baldock et al. (1983) found *Vannella* to be a common genus in chalk streams in southern England and suggested that the flat shape of this genus is an adaptation to resist high current velocity. Preston (2003) reported that *Acanthamoeba, Vannella* and *Naegleria* are found in the aquatic surface microlayers where they feed on bacteria.

Table 2
Environmental variables for each season. Values are mean ± S.D.

Season	OS (%)	Temp. (°C)	pH	K_{25} (μS cm^{-1})	Q (m^3 s^{-1})	DRP mg l^{-1}	NO_2 mg l^{-1}	NO_3 mg l^{-1}	NH_4 mg l^{-1}	DIN mg l^{-1}	TC MPN/ 100 ml	FC MPN/ 100 ml
Warm dry (*n* = 13)	93.9 ± 7.5	10.1 ± 4	7.0 ± 0.5	75 ± 42	0.07 ± 0.11	0.41 ± 0.18	0.21 ± 0.35*	0.18 ± 0.28	0.05 ± 0.06	0.43 ± 0.39	258 ± 316	219 ± 226
Warm rainy (*n* = 16)	99.3 ± 11.9	11.9 ± 2.2	7.0 ± 0.5	63 ± 7	0.20 ± 0.34	0.38 ± 0.12	0.005 ± 0.002	0.17 ± 0.24	0.06 ± 0.05	0.24 ± 0.25	2200 ± 40*	438 ± 538
Cool dry (*n* = 3)	90.8 ± 8.1	7.1 ± 3.6*	6.9 ± 0.1	76 ± 29	0.1 ± 0.1	0.32 ± 0.12	0.004 ± 0.002	0.16 ± 0.25	0.04 ± 0.04	0.20 ± 0.23	95 ± 69	95 ± 69*

OS, oxygen saturation; $K_{25,}$ specific conductance (at 25 °C); Q, flow; DRP dissolved reactive phosphorus; DIN dissolved inorganic nitrogen; TC, total coliforms; FC, fecal coliforms.
* Indicates a significant difference ($P < 0.05$).

R. ithacus was found in 22 of 32 sampling sites (69%). There is little information about this species. Page (1988) describes it as voracious, quickly consuming bacteria. These amoebae are very small and non-pathogenic; they have been found in fresh water, forest litter, groundwater, and rivers (Page, 1988; Ettinger et al., 2003; Bonilla et al., 2009; Ramírez et al., 2009).

The genus *Acanthamoeba* has been reported as the most common FLA in the environment (Page, 1988) and is often associated with contaminated, bacteria-rich water (Bonilla et al., 2009). In this study, this genus was found in 14 of 32 sampling sites (41%).

Naegleria isolates were recovered from 9 of 32 (28%) sampling sites. In general these amoebae are more sensitive to environmental conditions such as drying, pH extremes and low temperature. According to De Jonckheere (2012), the pathogenic species *N. fowleri* can grow in the environment only when the temperature is higher than 30 °C, which explains its absence from this study area.

The other FLA genera identified in this study have been reported from several types of water such as freshwater ponds (Anderson, 1997), streams (Ettinger et al., 2003; Bonilla et al., 2009) and groundwater (Ramírez et al., 2009).

The highest diversity of FLA was found at XoWd12 and CaWr12 (Fig. 1), probably because these streams are close to small villages that discharge wastewater into them, and there is agriculture in these areas.

The lowest diversity of FLA was found at PiWr12 and MpWr13 springs (Fig. 1), which may be due to the distance of those sites from human influence.

4.2. Physicochemical parameters

It has been reported that temperature, pH, dissolved oxygen and the availability of bacteria may all influence the presence of FLA (Ettinger et al., 2003). However, it has been difficult to demonstrate a clear association between any of these values and the presence of FLA. In this study, temperature was the environmental variable with the greatest influence on the presence of amoebae. Because of the altitude, the water temperature in these Mexican streams is low (<17 °C) and this could influence the presence of amoeba species. In contrast, oxygen saturation and pH values were constant across seasons and they range between optimal values (close to saturation for oxygen and around neutrality for pH). Several environmental variables associated with waste water pollution showed a relationship with amoebae isolation. These variables included total coliforms and DRP and ammonium concentration that favored *Acanthamoeba* spp. and *V. lata* presence. Both species could be considered as indicators of poor water quality. *M. cultura* was favored by DRP concentration but affected by higher levels of ammonium. DRP is an important nutrient for algal growth, and algae are a significant food resource for this amoeba. Finally, the second most common species found in the present study, *R. ithacus*, was positively related with flow velocity. It is a small size amoeba (5–17 μm) with a flat form. These traits can be very useful to stay in place when the current velocity is high.

4.3. Pathogenicity

The presence of *Acanthamoeba* and *Naegleria* is important because these genera include pathogenic species, although they grow better at higher water temperatures (Visvesvara et al., 2007; De Jonckheere, 2012) than were registered in the streams studied here. Warm water appears to be an important factor in the distribution of pathogenic amoebae, but the precise mechanisms remain unknown. In this study, most of the isolates belonging to these genera were non-pathogenic and only three *Acanthamoeba* isolates were invasive in mice.

Pernin and Pélandakis (2001) suggested that the pathogenicity of FLA is related more to significant changes in the water temperature than to a constant elevated temperature. This occurs where inter-seasonal differences in temperature are marked, but this does not occur in the area studied, where water temperature showed no significant differences between seasons.

5. Conclusions

This work demonstrates the diversity of FLA present in streams of the Mexico Basin. Spatial distribution of FLA was very variable but *Vannella*, *Acanthamoeba* and *Rosculus* were the most common

Table 3
Spearman Correlation Coefficient (Rho) between environmental variables and isolations of amoeba species. All values shown are significant at $P < 0.1$.

Species/variables	*Acanthamoeba* spp.	*Hartmannella cantabrigiensis*	*Mayorella cultura*	*Naegleria gruberi*	*Platyamoeba placida*	*Rosculus ithacus*	*Vannella lata*	*Vannella platypodia*
Altitude m a.s.l.	−0.552				−0.414			−0.360
Temperature °C	0.352	0.314	−0.447	0.301				
K_{25} μS cm^{-1}	0.518				0.393		−0.424	
DRP mg l^{-1}					0.324		0.302	
NH_4 mg l^{-1}			−0.483		0.419			
DIN mg l^{-1} as N					0.438			
Current velocity m s^{-1}						0.329		
NO_2 mg l^{-1}				−0.325				

genera. Only three *Acanthamoeba* isolates had low virulence which seems to be related to low water temperatures. Physicochemical factors were within the limits for the growth of most free-living amoebae. Water temperature was the environmental variable with the strongest influence on amoebal presence.

Although most FLA isolated in this study were non-pathogenic, their presence in streams represents a potential hazard since some species of these genera are able to cause disease in humans and can act as reservoirs for the presence and transmission of other human pathogens. Further investigations should be conducted to better understand their ecology and their possible impact on human and animal health.

Acknowledgments

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