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Original Article

Population dynamics of *Naegleria fowleri* genotype 2 in natural aquatic environments in Sonora, Mexico, throughout the year

Dinámica poblacional del genotipo 2 de *Naegleria fowleri* en ambientes acuáticos naturales de Sonora, México, a lo largo del año

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ABSTRACT

We carried out monthly samplings to determine the concentration of *Naegleria fowleri* in four natural water bodies of the Yaqui Valley, Sonora, Mexico. We measured the temperature, dissolved oxygen, and pH of the water. The water samples were shaken and processed to determine the concentration using the most probable number (MPN) method and seeded on non-nutritive agar plates with *Escherichia coli*. Each amoeba detected was isolated in a new plate to continue with the identification. MPN tables were used for the amoeba count, and the identity of the genus *Naegleria* and *N. fowleri* was used using specific primers and end-point PCR. We identified *N. fowleri* in all four water bodies during the summer and fall seasons, with 70 MPN L-1 as the highest concentration. We selected seven *N. fowleri* strains for sequencing and genotyping; all belonged to type 2. Through the results, we can affirm that the variations in the number of *N. fowleri* recorded throughout the year at the different sites studied are influenced by different biotic or abiotic factors, in addition to temperature, dissolved oxygen, and pH determined in this study. This makes it difficult to predict their presence in natural aquatic environments with relatively low populations.

Keywords: *Naegleria*; free-living amoebae; environmental distribution; primary amoebic meningoencephalitis; meningitis; thermophilic; *N. fowleri* genotype 2.

RESUMEN

Realizamos muestreos mensuales para determinar la concentración de *Naegleria fowleri* en cuatro cuerpos de agua naturales del Valle del Yaqui, Sonora, México. Medimos la temperatura, el oxígeno disuelto y el pH del agua. Las muestras de agua fueron agitadas y procesadas para determinar la concentración por el método del número más probable (NMP), y sembradas en placas de agar no nutritivo con *Escherichia coli*. Cada amiba detectada se aisló en una nueva placa para continuar con la identificación. Se usaron tablas de NMP para el conteo de amibas, y la identidad de los géneros *Naegleria* y *N. fowleri* fue usando cebadores específicos y PCR de punto final. Identificamos *N. fowleri* en los cuatro cuerpos de agua durante las temporadas de verano y otoño, con 70 NMP L^{-1} como la concentración más alta. Seleccionamos siete cepas de *N. fowleri* para secuenciación y genotipado; todos pertenecían al tipo 2. A través de los resultados podemos afirmar que debido a las variaciones que se dan en la dinámica poblacional y la diversidad ecológica de los sitios estudiados, diferentes factores bióticos o abióticos, además de la temperatura, oxígeno disuelto, y el pH, influyen en la presencia o ausencia de esta especie. Esto dificulta la predicción de su presencia en ambientes acuáticos naturales con poblaciones relativamente bajas.

Palabras clave: *Naegleria*; amibas de vida libre; distribución ambiental; meningoencefalitis amibiana primaria; meningitis; termofílico; *N. fowleri* genotipo 2.

INTRODUCTION

Naegleria fowleri received a new species status in 1970 due to its pathogenicity in humans (De Jonckheere, 2011). This free-living amoeba, also known as "the brain-eating amoeba" (Ruszkiewicz *et al*., 2019), is the causative agent of primary amoebic meningoencephalitis (PAM), an acute and usually fatal disease (Gompf and Garcia, 2019). PAM occurs primarily in children and adolescents with a history of swimming or diving in various aquatic environments. *N. fowleri* can reach the central nervous system (CNS) through water entering the nose (Cope *et al*., 2019). Using devices for washing the nose for medical or religious reasons with water contaminated with *N. fowleri* has been the cause of several deaths from PAM in adults (Yoder *et al*., 2012). This pathogen is a thermophilic ameboflagellate isolated worldwide from freshwater lakes, ponds, rivers, hot springs, thermally polluted water, warm groundwater, inadequately treated swimming pools, sewage, in biofilms of drinking water distribution systems, and soil, where it lives by feeding on bacteria and other microbes

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in the environment. It tolerates temperatures of up to 45 °C and thrives during warmer months when the ambient temperature increases. *Naegleria* is not found in salt water, like the sea (Morgan *et al*., 2016; Puzon *et al*., 2017; CDC, 2024).

Most of the current work is focused on *N. fowleri* in artificial environments, such as drinking water distribution systems rather than natural environments, reporting presence and absence data and how to reduce its presence (Miller *et al*., 2017; Miller *et al*., 2018). Several studies have also focused on improving isolation, culture, and identification procedures by molecular methods, including quantitative determinations of *N. fowleri* from different environments, and how biotic and abiotic factors could explain its presence and distribution (Puzon *et al*., 2009; Streby *et al*., 2015; Miller *et al*., 2015; Moussa *et al*., 2020).

The current study involved a seasonal search of *N. fowleri* in three natural surface bodies of water and one thermal spring from Yaqui Valley, the cradle of the green revolution, in northwestern Mexico. The objective was to determine the number and distribution of amoebas, and assess whether the presence of *N. fowleri* correlates with seasonal factors or other environmental factors and which type of *N. fowleri* predominates in this region of the eight genotypes recorded worldwide (De Jonckheere, 2011). It is still unclear how often *N. fowleri* is present in natural surface waters and at what concentration its presence becomes a human health hazard.

MATERIAL AND METHODS

Sampling sites

In the study, we included four natural water bodies with recreational activities. La Isleta (27°45′21.46″ N, 109°54′04.79″ O), a lagoon called Las Palmas (27°43′58.42″ N, 109°52′28.56″ O), a thermal spring called Aguacaliente (27°43′54.87″ N, 109°50′12.69″ O) and, the Laguna del Náinari (27°29′42.73″ N, 109°58′02.81″ O). The climate in the area is sweltering, with an average annual precipitation of 410 mm (INAFED, 2020).

Sampling

Samples were taken monthly from each site from May 2017 to April 2018. The sampling point was previously established, referring to the presence of organic matter and inert matter as support. Once we selected the sampling spot, rubbed and mixed the waters, we removed any amoeba adhering to the substrate, and we collected 1 L per site in sterile containers. Likewise, the temperature of the water and the environment were measured in situ with a BRANNAN mercury thermometer, dissolved oxygen with a YSI oximeter, salinity with a VITAL SINE refractometer and, in the laboratory, the pH with a potentiometer HANNA (Pernin *et al*., 1998; Lares-Villa and Hernández-Peña, 2010). We performed all statistical analyses using the STATGRAPHICS Plus software (version 5.1, Statistical Graphics Corp., USA).

Culture on non-nutritive agar with *Escherichia coli* (NNE)

Bacteriological agar MCD-LAB at 5% was prepared, heated until complete dissolution, and autoclaved at 121 °C for 15 minutes. We then emptied the medium into disposable KLINICUS Petri dishes. After solidifying, we applied 200 µL of live *E. coli* suspension to each plate and spread it with a Drigalski spatula.

Sample processing to determine the most probable number (MPN)

After stirring to homogenize, we filtered the water samples through 1.2 µm Merck Millipore cellulose membranes using the Micro Filtration Systems filter kit, where five volumes of 100 mL and five volumes of 10 mL were filtered separately (Pernin *et al*., 1998; Lares-Villa and Hernández-Peña, 2010; Blodgett, 2024). We cut the filters in half and placed them inverted on the same NNE plate to allow amoebae to exit quickly after incubation. Likewise, we planted directly in NNE plates in quintuplicate, 1 and 0.1 mL per sample, leaving them at rest for 20 minutes before incubating the Petri dishes with inverted NNE and in plastic bags to prevent them from drying out. We obtained 80 plaques per month from the four sites and incubated them at 45 °C. The reason for planting four volumes of water samples, 100, 10, 1, and 0.1 mL, is that we did not know the number of amoebae that could exist in the different bodies of water (Pernin *et al*., 1998; Lares-Villa and Hernández-Peña, 2010).

Isolation and counting of thermophilic amoebae

We examined each culture daily under an inverted Axiovert 135 ZEISS microscope (Carl Zeiss, Gottingen, Germany) for five days and registered the amoebae presence or absence from each NNE plate. After we compared the results with NMP tables to determine the MPN of amoebae per liter from each sampling site, the MPN/L obtained by filtration had a correction multiplied by two, due to loss of amoebae with this concentration method. All plates that did not show growth after five days were considered negative. We collected each amoeba growth that emerged along the two halves of the filter for isolation and subculture. Similarly, each amoeba "colony" was grown separately on seeded plates with 1 and 0.1 mL of the water sample, and transferred to a new NNE plate to prevent further cloning of the amoeba. For this last part, the first observation was before 24 hours of incubation. For counting thermophilic amoebae (TA), only the positive or negative growth of each seeded plate and the corresponding dilution registered were compared with MPN tables to obtain the final count (Pernin *et al*., 1998; Lares-Villa and Hernández-Peña, 2010; Blodgett, 2024).

Flagellation test

All those strains that showed morphological characteristics suspected of belonging to the genus *Naegleria* underwent the flagellation test. This test consisted of adding 2 mL of sterile distilled water to each plate and incubating at 37 °C for four hours. Then, we examined each dish by inverted microscopy at two, three, and four hours, and in the presence of flagellated bodies, 0.1 mL of the liquid was transferred to a new plate with NNE to incubate again for 24 h at 45 °C and prepare them for DNA extraction (De Jonckheere, 1977).

DNA extraction

Once the pure cultures were obtained and had sufficient growth, we collected the amoebae for DNA extraction using the commercial kit DNeasy® Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. Subsequently, we verified its integrity by electrophoresis in 1% agarose gel, stained with ethidium bromide, at 50 V for one hour. Finally, we quantify DNA using a NanoDrop 2000c spectrophotometer (Zysset-Burri *et al*., 2014).

PCR endpoint and counting of thermophilic *Naegleria* (TN) and *Naegleria fowleri* (Nf)

We performed PCR amplification according to the manufacturer's instructions using the GoTaq Flexi DNA Polymerase Kit (Promega). Primers ITS1 5'-GAACCTGCGTAGGGATCATTT-3' and ITS2 5'-TTTCTTTTCCTCCCCTTATTA-3' were used for the identification of the genus *Naegleria* and for the confirmation of *N. fowleri* the primers used were FW1 5'-GTGAAAACCTTTTTTCCATTTACA-3', RV1 5'-AAATAAAAGATTGACCATTTGAAA-3', with an expected amplicon length of 410 bp and 310 bp respectively (Zhang *et al*., 2018). The thermal cycler conditions were as follows: initial denaturation of 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Then, we visualized the PCR products in a UV transilluminator at a wavelength of 260 nm after electrophoretic separation in a 2% agarose gel stained with ethidium bromide (Panda *et al*., 2015). From the thermophilic isolates positive to *Naegleria* genus and *N. fowleri*, through PCR test, the number of plates where they grew, of each dilution, was registered to obtain the MPN of thermophilic *Naegleria*, and the MPN of *N. fowleri* per liter (Lares-Villa and Hernández-Peña, 2010; Blodgett, 2024).

Sanger sequencing and bioinformatic analysis to identify genotypes

For strains confirmed as *N. fowleri*, the ITS1, 5.8S, and ITS2 regions were amplified with the ITS primers. Using the Sanger technique, we purified and sequenced the PCR products in both directions. Subsequently, we edited the sequences, aligned them, and analyzed them with the CLC Genomics Workbench v.20 programs (https://digitalinsights.qiagen.com/) for genotype characterization. We used the representative sequences for each genotype deposited in the GenBank in comparing alignments: AY376149, X96564, X96562, AJ132030, AJ132028, FR875287, X96563 and FR875288 (Zhang *et al*., 2018).

RESULTS AND DISCUSSION

Sampling sites

Except for Aguacaliente, which is a source of thermal water, the water from the other study sites, La Isleta (where water flows continuously through a canal), Las Palmas (a natural lake), and Laguna del Nainari (an artificial lake), is supplied by water from the Alvaro Obregon dam. It is important to note that in certain areas and times of the year, this water is supplemented with groundwater to meet the specific agricultural needs of the region. While the water in the Yaqui Valley is predominantly used for irrigation, the four sites we investigated are also popular recreational spots for families, offering opportunities for picnics and swimming. However, if *N. fowleri* is present, the water could be a source of significant health risks. This underscores the gravity of the situation and the importance of our study's findings for public health.

MPN of thermophilic amoebae (TA)

The data in Table 1 reveal that thermophilic amoebae were present throughout the year. The term 'thermophilic' is used for organisms that grow above 40 °C, according to De Jonckheere (2002). In our study, we focused on the growth of *N. fowleri*, selecting 45 °C as the main isolation temperature to eliminate organisms that cannot grow at this temperature. The data in Table 1 reveal the intriguing complexity of our findings, with thermophilic amoebae being present throughout the year. Still, their figures vary significantly depending on the month and sampling site, ranging from 4 NMP TA/L to 2398 NMP TA/L.

Table 1 presents a clear pattern: Aguacaliente consistently recorded the highest TA concentrations, a trend directly linked to favorable climatic conditions. These conditions, characterized by temperatures above 40 °C, provide an ideal environment for the growth of microorganisms. Among the thermophilic or thermotolerant amoebas reported in Aguacaliente in previous studies are the genera *Naegleria*, *Acanthamoeba*, *Balamuthia*, *Vermamoeba*, and *Stenamoeba* (Lares-Jiménez *et al*., 2018).

Table 1. The most probable number of thermophilic amoebae (TA) from La Isleta, Las Palmas, Aguacaliente, and Laguna del Nainari in 2017-2018.
Tabla 1. Número más probable de amibas termófilas (TA) de La Isleta, Las Palmas, Aguacaliente y Laguna del Náinari en 2017-2018.

Sampling	MPN of thermophilic amoebae			
	La Isleta	Las Palmas	Aguacaliente	Laguna del Nainari
	MPN TA/L	MPN TA/L	MPN TA/L	MPN TA/L
01/05/2017	788 (± 458)	1724 (± 860)	2398 (± 1209)	1299 (± 669)
20/06/2017	1299 (± 669)	14* (± 4)	201 (± 200)	10* (± 3)
10/07/2017	788 (± 458)	48* (± 12)	2398 (± 1209)	8* (± 3)
14/08/2017	201 (± 200)	201 (± 200)	22* (± 6)	10* (± 3)
13/09/2017	<200	70* (± 19)	70* (± 19)	201 (± 200)
18/10/2017	184* (± 54)	322* (± 89)	184* (± 54)	4* (± 2)
15/11/2017	452 (± 319)	322* (± 89)	201 (± 200)	108* (± 33)
11/12/2017	452 (± 319)	<200	1299 (± 669)	16* (± 5)
15/01/2018	108* (± 33)	10* (± 3)	184* (± 54)	10* (± 3)
12/02/2018	26* (± 7)	18* (± 5)	<200	4* (± 2)
12/03/2018	26* (± 7)	14* (± 4)	184* (± 54)	48* (± 12)
16/04/2018	322* (± 89)	10* (± 3)	322* (± 89)	28* (± 7)

- Not detected; *Corrected number; ( ) Standard deviation organisms /L.

MPN of thermophilic *Naegleria* spp.

Table 2 clearly shows the differences between the studied water bodies. In Aguacaliente hot springs, the genus *Naegleria* was present throughout the sampling year. Compared to the Pearson analysis, we did not observe a correlation between climatic parameters and the presence of *Naegleria* spp. The number of *Naegleria* spp. identified by flagellar transformation and confirmed by PCR varied between 4 NMP/L and 788 NMP/L in the different sites sampled, with La Isleta, Las Palmas, and Laguna del Nainari having the lowest densities and frequencies of monthly isolates compared with Aguacaliente. Of the 47 species of *Naegleria* described worldwide by different methods, including molecular ones, 20 species grow between 40 and 45 or more degrees Celsius, but mainly only seven species grow at 45 °C (Guzmán-Fierros *et al*., 2008). Of the few genetic studies on *Naegleria* spp. conducted in different bodies of water in this region, including the four sites of this study, only the presence of thermophilic *N. fowleri*, *N. thiangensis*, and *N. lovaniensis* has been reported. The latter is the most abundant thermophilic *Naegleria* species (Guzmán-Fierros *et al*., 2008; Lares-Villa and Hernández-Peña, 2010; Lares-Jiménez *et al*., 2018).

MPN of *Naegleria fowleri*

The results shown in Table 3 confirm the presence of *N. fowleri* in all four study sites and establish a connection with the findings of Lares-Villa and Hernández Peña (2010). The levels of *N. fowleri* varied depending on the month and location of sampling, ranging between 4 NMP Nf/L and 70 NMP Nf/L. Notably, *N. fowleri* was detected during August, September, and October, which aligns with the months reported in the study mentioned above and with data ranging from 4 to 18 NMP Nf/L. This correlation enhances the reliability of our research and contributes to the existing knowledge about *N. fowleri* in natural water bodies.

This report marks the first documented presence of *N. fowleri* in Laguna del Nainari and Aguacaliente. Our findings suggest that the lagoon's connection to La Isleta, which supplies its water, may have facilitated the amoeba's presence in September. Similarly, the onset of the rainy season in October could have introduced the pathogenic amoeba into the thermal water source in Aguacaliente, challenging the notion that temperatures above 40 °C alone are responsible (Stahl and Olson, 2021).

Table 4 presents the results of identifying free-living amoebae, which were isolated and grown at the precise temperature of 45 °C at the four specific sampling points under study. Our research involved a meticulously designed and executed process of isolation and identification of a total of 655 thermophilic AVL strains. Among these, 260 showed flagellated bodies. Through PCR analysis, we identified 238 as *Naegleria* spp. and 22 as *N. fowleri*, providing a comprehensi-

Table 2. The most probable number of thermophilic *Naegleria* (TN) from La Isleta, Las Palmas, Aguacaliente, and Laguna del Nainari in 2017-2018.
Tabla 2. Número más probable de *Naegleria* termófilas (TN) de La Isleta, Las Palmas, Aguacaliente y Laguna del Náinari en 2017-2018.

MPN of thermophilic *Naegleria*				
Sampling	**La Isleta**	**Las Palmas**	**Aguacaliente**	**Laguna del Nainari**
	MPN TN/L	MPN TN/L	MPN TN/L	MPN TN/L
01/05/2017	-	4* (± 2)	322* (± 89)	-
20/06/2017	-	-	201 (± 200)	-
10/07/2017	22* (± 6)	4* (± 2)	788 (± 458)	-
14/08/2017	4* (± 2)	70* (± 19)	10* (± 3)	-
13/09/2017	4* (± 2)	16* (± 5)	34* (± 9)	70* (± 19)
18/10/2017	-	10* (± 3)	184* (± 54)	-
15/11/2017	4* (± 2)	4* (± 2)	34* (± 9)	4* (± 2)
11/12/2017	-	16* (± 5)	201 (± 200)	-
15/01/2018	-	-	184* (± 54)	-
12/02/2018	-	-	8* (± 3)	-
12/03/2018	-	-	34* (± 9)	-
16/04/2018	-	-	22* (± 6)	-

- Not detected; *Corrected number; ( ) Standard deviation organisms /L.

Table 3. The most probable number of *N. fowleri* (Nf) from La Isleta, Las Palmas, Aguacaliente, and Laguna del Nainari in 2017-2018.
Tabla 3. Número más probable de *N. fowleri* (Nf) de La Isleta, Las Palmas, Aguacaliente y Laguna del Náinari en 2017-2018.

MPN of *Naegleria fowleri*				
Sampling	**La Isleta**	**Las Palmas**	**Aguacaliente**	**Laguna del Nainari**
	MPN Nf/L	MPN Nf/L	MPN Nf/L	MPN Nf/L
01/05/2017	-	-	-	-
20/06/2017	-	-	-	-
10/07/2017	-	-	-	-
14/08/2017	-	22* (± 6)	-	-
13/09/2017	4* (± 2)	16* (± 5)	-	70* (± 19)
18/10/2017	-	-	18* (± 5)	-
15/11/2017	-	-	-	-
11/12/2017	-	-	-	-
15/01/2018	-	-	-	-
12/02/2018	-	-	-	-
12/03/2018	-	-	-	-
16/04/2018	-	-	-	-

- Not detected; *Corrected number; ( ) Standard deviation organisms /L.

Table 4. Free-living amoebae isolated and grown at 45 °C, from La Isleta, Las Palmas, Aguacaliente, and Laguna del Nainari, Sonora, from May 2017 to April 2018.
Tabla 4. Amibas de vida libre aisladas y cultivadas a 45 °C, de La Isleta, Las Palmas, Aguacaliente y Laguna del Náinari, Sonora, de mayo de 2017 a abril de 2018.

Free living amoebae	La Isleta	Las Palmas	Aguacaliente	Laguna del Náinari	Total
Thermophilic amoebae	273	104	225	53	655
Positive flagellation test	16	36	198	10	260
Thermophilic *Naegleria* spp.	8	29	192	9	238
Naegleria fowleri	1	10	4	7	22

ve and reliable understanding of the presence of these strains in the studied areas. It was found that 91.5% of amoeboid organisms with flagella tested positive in the endpoint PCR for *Naegleria* species. This suggests that another genus with flagellated organisms in its life cycle was identified in the study, potentially including different amoebae belonging to the Heterolobosea class (Page, 1988; Robinson *et al*., 1989). The isolation of *N. fowleri*, representing approximately 10% of the isolates, is a significant finding. However, when we compare it with the total thermophilic AVL, the number of *N. fowleri* would barely represent approximately 3.4%. This means that *N. fowleri*, the only strain of *Naegleria* pathogenic for humans reported so far, is found in meager proportions in natural environments.

Regarding the strains positive for *N. fowleri*, they represent only 9.2% of *Naegleria* spp. It has been found that there is a more significant proliferation of other species, such as *N. lovaniensis*, which has been previously demonstrated (Guzmán-Fierros *et al*., 2008; Lares-Jiménez *et al*., 2018), in addition to the fact that the pathogen represents only 3.4% of the total, results that agree with those obtained by Lares-Villa and Hernández-Peña (2010), where *N. fowleri* only represented 1.8% of the total isolated thermophilic amoebas. This reiteration of the agreement of our results with previous studies should instill confidence in the scientific community. Although none of the sites reported levels of *N. fowleri* greater than 100 MPN L-1, a value established by Cabanes *et al*. (2001), where they mention that values higher than this number mean a risk of infection for the population, or if the standard applied by the Australian government were taken as a reference, which implies two thermophilic *Naegleria* per liter (De Jonckheere, 2014), it is must prevent the population from accessing all the sampling locations studied, particularly during August, September and October.

Genotyping of *Naegleria fowleri*

Once the nucleotide sequences were obtained, assembled, edited, aligned, and analyzed with the CLC Genomics program for genotype characterization, we found that the seven *N. fowleri* strains belong to type 2, which have a length of 42 bp in their ITS1 (Figure 1) and nucleotide T at position 31 in its 5.8S rDNA (Figure 2). As described by De Jonckheere (2011), there are eight genotypes distributed around the world that have been characterized based on the number of repetitions in the ITS1 region, which can vary in size depending on the characteristics of each genotype, having a range of 42-142 bp and a C / T transition at position 31 in the 5.8S region. The ITS2 region is identical among all *N. fowleri* strains and, therefore, is not analyzed in these characterizations (De Jonckheere, 2004). Based on this and the analysis carried out in this work, it was observed that the seven *N. fowleri* strains belong to type 2, which have a length of 42 bp in their ITS1 (Figure 1) and nucleotide T at position 31 in its 5.8S rDNA (Figure 2). The discovery of type 2 in the Yaqui Valley aligns with the findings of De Jonckheere (2011), who reported that type 1, 2, and 3 genotypes have been found in America. Additionally, Vargas-Zepeda *et al*. (2005) conducted a molecular analysis of *N. fowleri* isolated from a CSF sample in Sonora and identified type 2. This suggests the need to expand the analysis in Mexico to confirm the prevalence of genotype 2 in both the region and the country. Genotype 2 has been found in environmental samples as well as in clinical cases (Pelandakis *et al*., 2000; Zhou *et al*., 2003; Cogo *et al*., 2004; Nicolas *et al*., 2010). In Taiwan, the first case of PAM was reported in November 2011, and subsequent sampling of recreational hot springs visited by the patient revealed the presence of *N. fowleri* type 2, which matched the type found in the patient. This molecular characterization method was used as an epidemiological tracing tool (Tung *et al*., 2013). More recently, Zhang *et al*. (2018) conducted a molecular typing study of this amoeba from a patient's sample in the Zhejiang province of China and once again found type 2.

Water temperature, environmental temperature, pH, and dissolved oxygen at the sampling sites

Figures 3-6 show the mean and standard deviation of the monthly measured parameters (water temperature, ambient temperature, pH, and dissolved oxygen) grouped by seasons of the year and sampling sites. In general, samples collected during the winter (December to February) had the lowest average water and ambient temperatures, while the highest temperatures were obtained in the summer (June to August),

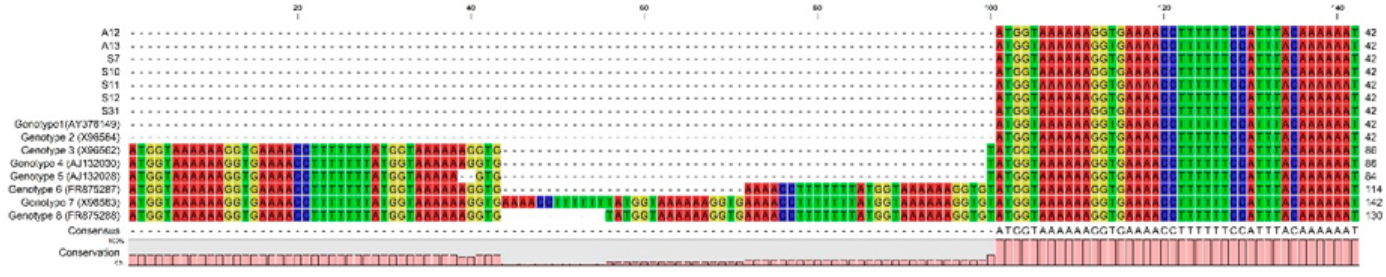


Figure 1. Alignment of the ITS1 regions of *Naegleria fowleri*. DNA sequence alignments were performed using QIAGEN CLC Genomics Workbench 21.0. The length of the ITS1 region and the base at position 31 of the 5.8S rRNA sequences, were compared using genotypes 1–8 deposited in the National Center for Biotechnology Information (NCBI) database.

Figura 1. Alineación de las regiones ITS1 de *Naegleria fowleri*. Las alineaciones de secuencias de ADN se realizaron utilizando QIAGEN CLC Genomics Workbench 21.0. La longitud de la región ITS1 y la base en la posición 31 de las secuencias de ARNr 5.8S se compararon utilizando los genotipos 1 a 8 depositados en la base de datos del Centro Nacional de Información Biotecnológica (NCBI).

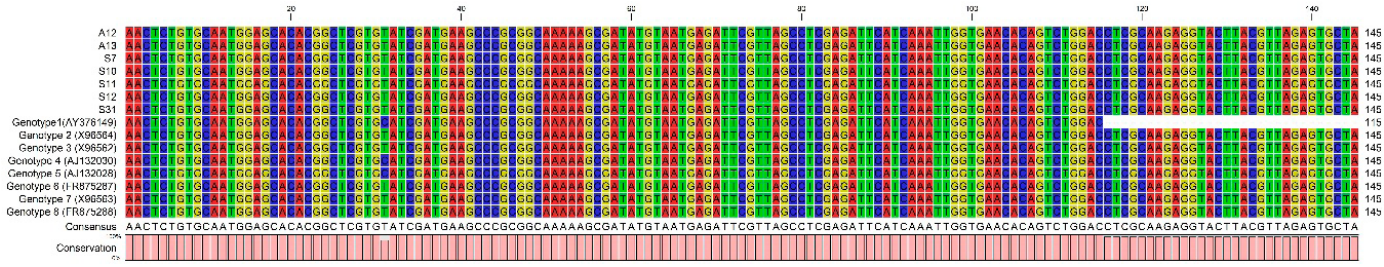
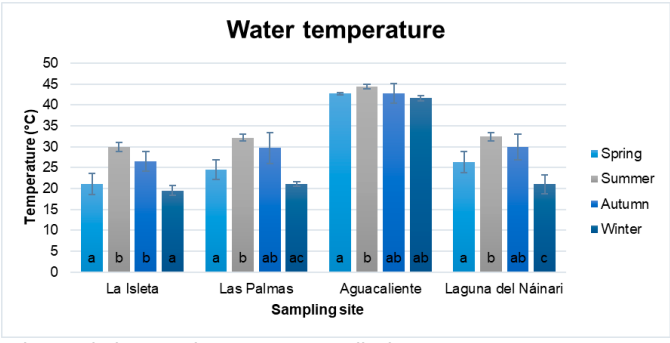
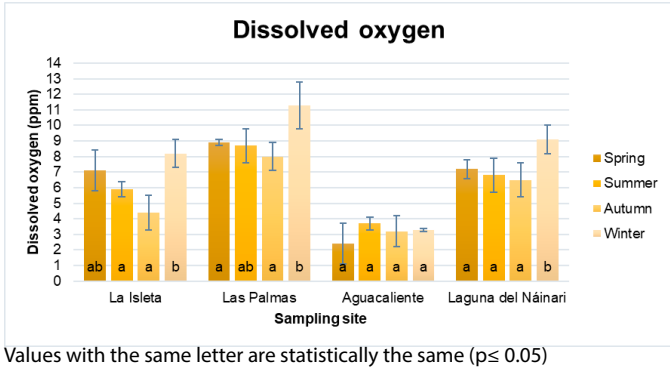


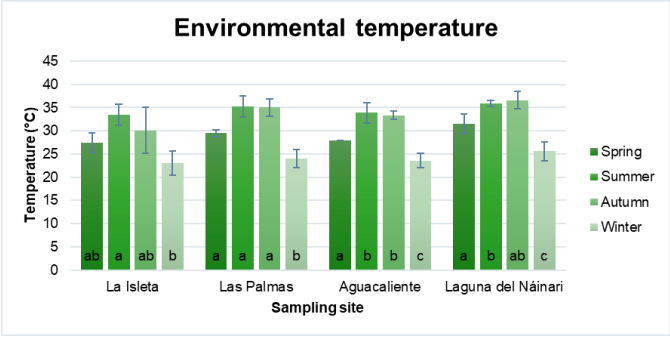
Figure 2. Alignment of the 5.8S region (145 bp) showing the C / T transition at position 31.
Figura 2. Alineación de la región 5.8S (145 pb) que muestra la transición C/T en la posición 31.



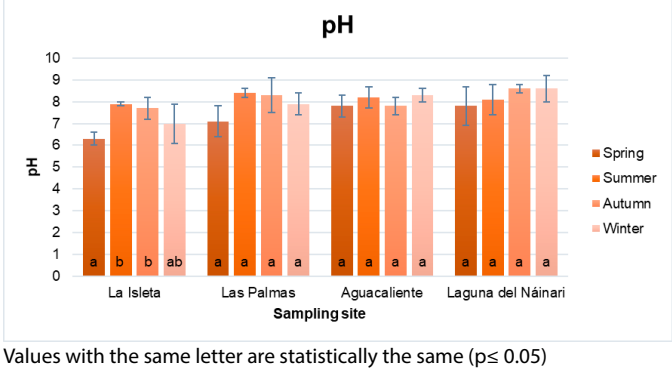
Values with the same letter are statistically the same (p≤ 0.05)
Figure 3. Annual behavior of water temperature at sampling sites.
Figura 3. Comportamiento anual de la temperatura del agua en los sitios de muestreo.



Values with the same letter are statistically the same (p≤ 0.05)
Figure 6. Annual behavior of dissolved oxygen at sampling sites.
Figura 6. Comportamiento anual del oxígeno disuelto en los sitios de muestreo.



Values with the same letter are statistically the same (p≤ 0.05)
Figure 4. Annual behavior of environmental temperature at sampling sites.
Figura 4. Comportamiento anual de la temperatura ambiental en los sitios de muestreo.



Values with the same letter are statistically the same (p≤ 0.05)
Figure 5. Annual water pH behavior at sampling sites.
Figura 5. Comportamiento anual del pH del agua en los sitios de muestreo.

showing significant differences in both seasons. Therefore, values with the same letter are statistically equal (p≤ 0.05) (Figures 3 and 4).

In the case of water temperatures, the effect of the different seasons of the year is observed in the three reservoirs of natural water coming from the dam, and this is not the case in Aguacaliente, which is a source of natural thermal water (Figure 3). At this site, the water temperature was above 40 °C all year round. Although slight variations in environmental temperature are observed in the four sampling sites, we can practically observe two climates, summer and autumn, with the highest temperatures, and winter and spring, with the lowest temperatures (Figure 4). As seen in Table 1, the thermal body of Aguacaliente presented the highest concentrations of thermophilic amoebas throughout the year, a situation attributed to the primary favorable climatic condition for the growth of this type of microorganisms, which is water temperatures above 40 °C throughout the year. When performing the Pearson analysis, we did not observe a correlation between the behaviors of the number of thermophilic amoebae and the presented climatic conditions.

The pH averages obtained were similar at the four sampling sites throughout the year, except for La Isleta, which showed a statistically significant decrease in the spring with a pH R-value of -0.9020. This decrease in pH was particular because the waters of La Isleta, Las Palmas, and the Nainari Lagoon are interconnected, and the pH of the water did not vary significantly. The decrease in pH was probably due to the discharge of some contaminant into the water flow

from upstream on the day of sampling. Regarding dissolved oxygen, except in Aguacaliente, the other sites showed significant differences, mainly between the autumn and winter seasons. In the Pearson analysis carried out to observe correlations between the variables, it was shown that there is a statistically significant relationship between the water temperature and the ambient temperature for La Isleta, Las Palmas and Laguna del Náinari, with an R value of 0.9563, 0.9711 and 0.9647, respectively. In addition, in the case of the Laguna del Náinari, a significant relationship was found between dissolved oxygen and water temperature, as well as statistically significant for environmental temperature, with an R-value of -0.9170 and -0.9717, respectively. On the other hand, for Las Palmas, only a meaningful relationship was presented, with an R-value of -0.9226 between dissolved oxygen and environmental temperature.

According to Marín-Galvín (2019), dissolved oxygen is a very relevant gas in the dynamics of water, where its solubility is a function of several factors: temperature, pressure, coefficient of solubility, vapor pressure, salinity, and physicochemical composition of water. In this way, we can see that the amount of dissolved oxygen varies inversely proportional to temperature, results that have already been observed in various studies. This is associated with the fact that at higher temperatures, aquatic organisms require more oxygen, which tends to decrease (Rosenfeld, 2017). Although the effect of some abiotic factors remains inconclusive for the presence of *N. fowleri* in natural surface waters, including the increase in water temperature due to climate change (Navarro-Estupiñan *et al*., 2018), it is necessary to continue studying not only abiotic factors, but also biotic and nutritional factors to know whether or not these determine the increase or control of the population of this pathogenic amoeba (Stahl and Olson, 2021).

CONCLUSIONS

Having determined the presence of this pathogen in the four sites studied demonstrates its wide ecological diversity because each of them shows different characteristics, and they also have great distances between them, so it would be interesting to focus future studies on the transport routes that can influence the movement of this pathogen, together with implementing greater parameters that allow a better understanding of the behavior of the species. Likewise, it is suggested that the four sites continue to be monitored because, with the current climate change and environmental contamination, the population densities of this pathogen may increase, posing a great risk of infection for those who visit these sites. Specifically, constant monitoring of the Laguna del Náinari is suggested since it was the site that showed the highest concentration and is located within the city, in addition to its supply being made through the low channel to which the population has access and is used to irrigate crops, generating contamination risks in nearby towns.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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