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Free-living amoebae in sediments from the Lascaux Cave in France

Angela M. Garcia-Sanchez¹, Concepcion Ariza², Jose M. Ubeda²,
Pedro M. Martin-Sanchez¹, Valme Jurado¹, Fabiola Bastian³,
Claude Alabouvette³, and Cesareo Saiz-Jimenez^{1*}

¹Instituto de Recursos Naturales y Agrobiología, IRNAS-CSIC, 41012 Sevilla, Spain

²Universidad de Sevilla, Departamento de Microbiología y Parasitología, Facultad de Farmacia, 41012 Sevilla, Spain

³UMR INRA-Université de Bourgogne, Microbiologie du Sol et de l'Environnement, 21065 Dijon Cedex, France

Abstract: The Lascaux Cave in France is an old karstic channel where the running waters are collected in a pool and pumped to the exterior. It is well-known that water bodies in the vicinity of humans are suspected to be reservoirs of amoebae and associated bacteria. In fact, the free-living amoebae *Acanthamoeba astronyxis*, *Acanthamoeba castellanii*, *Acanthamoeba* sp. and *Hartmannella vermiformis* were identified in the sediments of the cave using phylogenetic analyses and morphological traits. Lascaux Cave sediments and rock walls are wet due to a relative humidity near saturation and water condensation, and this environment and the presence of abundant bacterial communities constitute an ideal habitat for amoebae. The data suggest the need to carry out a detailed survey on all the cave compartments in order to determine the relationship between amoebae and pathogenic bacteria.

Keywords: free living amoebae; *Acanthamoeba*; *Hartmannella*; Lascaux Cave; sediments

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INTRODUCTION

Free-living amoebae (henceforth, amoebae) are ubiquitous and found in a variety of habitats, largely aquatic, but also in soils and sediments. In aquatic environments, the ecological distribution of amoebae encompasses natural and treated water, drinking water treatment plants, swimming pools, sewage, etc. (Khan, 2006). However, amoebae have been scarcely investigated in caves (Coppellotti & Guidolin, 2003; Mulec, 2008; van Hengstum et al., 2009; Walochnik & Mulec, 2009; Mazei et al., 2012). Caves harbour pools, lakes and subterranean rivers, and the sediments and rock walls are constantly wet due to a relative humidity near saturation and water condensation. This environment and the presence of abundant bacterial communities (Bastian et al., 2009; Saiz-Jimenez et al., 2011) constitute an ideal habitat for amoebae.

Lascaux is an uncommon cave which experienced a strong anthropogenic impact. In fact, after opening in 1948, the Lascaux Cave was highly impacted by massive tourism, and suffered several microbial outbreaks, the first one of the Chlorophyta *Bracteacoccus minor* in 1963 due to the effect of an

artificial lighting system (Lefèvre, 1974). Further, fungal outbreaks of *Fusarium solani* and melanised fungi were evidenced in 2001 (Bastian et al., 2010; Martin-Sanchez et al., 2012). Lascaux Cave is different from all other studied caves because was treated for years with benzalkonium chloride to control the fungal outbreaks.

A climate regulation system was installed inside the cave in 1957, with several replacements, the last one in 2001. Therefore, it was not surprising that Bastian et al. (2009) found in many sediment samples the simultaneous presence of sequences related to *Legionella*, *Apifia*, *Aquicella*, and bacterial endosymbionts of amoebae, common in climate systems. The finding of these bacteria motivated us to carry out a survey on the cave sediments in a search for amoebae.

MATERIAL AND METHODS

Four samples were collected from Lascaux Cave sediments (Fig. 1); on 17 February 2010 from a black stain located in the Apse (M6), on 21 September 2010 from the Painted Gallery (S1 and S2) and on 29 September 2010 from the Chamber of Felines (DF). These sites corresponded to the areas in which

abundant bacterial endosymbionts were previously detected (Bastian et al., 2009). Cave environmental data were reported elsewhere (Lastennet et al., 2011).

Samples were maintained at 5°C for 48 hours, during the transport to the laboratory. Upon arrival, the samples were incubated and subjected to cloning and axenisation following standard methods (Page, 1967a, b). Samples M6 and S2 showed an intense fungal contamination, which required continual reseeded, in addition to antifungal treatments with 0.5% nystatin.

For taxonomical identification of amoebae standard methods described by Pussard & Pons (1977, 1979) were used. Trophozoites (feeding stage of amoebae) and cysts (surviving stage during adverse environmental periods) were observed in a Zeiss stereomicroscope IVB, and the images captured with an Olympus camera SP-500 UZ.

DNA extraction from the cultures was performed using the FastDNA SPIN Kit for Soil (MP Biomedicals, OH, USA) according to the manufacturer's instructions. The specific detection of *Acanthamoeba* spp. was performed by PCR using the primers set JDP1 (5'-GGCCCAGATCGTTTACCGTGAA-3') and JDP2 (5'-TCTACAAGCTGCTAGGGGAGTCA-3'), which specifically amplify a 450 bp fragment of the 18S rDNA gene in the genus *Acanthamoeba* (Schroeder et al., 2001). The amplification products were sequenced using the same primers and the obtained sequences were compared with those deposited in GenBank using the NCBI BLASTn algorithm. Phylogenetic relationships between the *Acanthamoeba* spp. isolated from Lascaux Cave and the closest related sequences from GenBank were conducted using MEGA 5.0 with the Maximum-Likelihood method. Gaps were treated as missing data and bootstrap values were generated using 1000 replicates. The resulting topology was compared with results from other treeing algorithms, including the Neighbor-Joining and Maximum-Parsimony methods.

In order to identify other genera of amoebae, a region of 18S rDNA was amplified using the primers set EukA (5'-AACCTGGTTGATCCTGCCAGT-3') and

EukB (5'-TGATCCTTCTGCAGGTTACCTAC-3') as described by Diez et al. (2001). Clone library was constructed using this purified PCR product, using standard methods.

RESULTS AND DISCUSSION

Direct microscopy observations allowed the identification of trophozoites and cysts of the genera *Acanthamoeba* and *Hartmannella* in sample S1 (Table 1). In sample DF were also present species of the genera *Acanthamoeba* and *Hartmannella*, but the species of *Acanthamoeba* was different to that found in sample S1 (Fig. 2). In sample S2 only a species of *Acanthamoeba*, similar to that found in sample DF, was observed. However, trophozoites or cysts of *Hartmannella*, unlike the other two samples, were not detected. In sample M6 only trophozoites of *Acanthamoeba* but no cysts were detected, therefore its morphology could not be defined.

The genus *Acanthamoeba* could be easily recognized in sample S1 because of the morphological characteristics of the trophozoites and cysts (Fig. 2A, B). Based on the size and morphological features of cysts the *Acanthamoeba* was identified as *A. astronyxis*, characterized by large trophozoites and cysts ($\leq 18 \mu\text{m}$), with smooth ectocyst and stellate endocyst (Page, 1967b; Pussard & Pons, 1977). The 18S gene sequence showed a 99% similarity with the type strain *A. astronyxis* (ATCC30137), isolated from water, and genotype T7 according to Stothard et al. (1998). In the phylogenetic analysis these two strains also formed a homogenous cluster with 81% of bootstrap value confirming the species identification (Fig. 3).

The *Acanthamoeba* sp. found in sample DF corresponded to a different species. The strain is close related to *A. mauritaniensis* because some endocysts had fewer than 6-rays and the cysts showed reticulation even after fixation with formaldehyde in the heat impregnation technique (Fig. 2C) (Pussard & Pons, 1977). However, analysis of the 18S gene sequence from this strain showed a 99% similarity

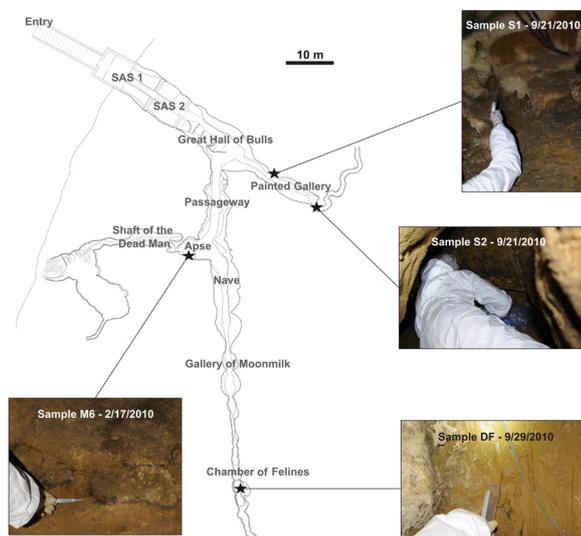


Fig. 1. Map of Lascaux Cave and sampling location.

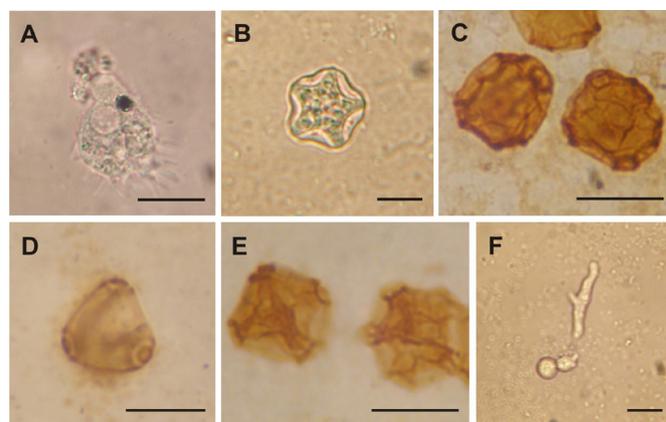


Fig. 2. Morphology of amoebae. a) *In vivo* trophozoite with acanthopodia of *Acanthamoeba astronyxis* S1. b) Five ray-cyst of *Acanthamoeba astronyxis* S1. c) Cysts of *Acanthamoeba* sp. DF under heat impregnation. d) Cysts of *Acanthamoeba castellanii* S2 under PATAg-r staining. e) Cysts of *Acanthamoeba castellanii* S2 under heat impregnation. f) Trophozoite and cyst of *Hartmannella vermiformis*. The bar represents 10 μm .

Table 1. Identification of free-living amoebae in Lascaux Cave.

Samples	Morphological identification ^a	Molecular identification ^b	Genbank Accession No.
S1	<i>Acanthamoeba astronyxis</i>	<i>Acanthamoeba astronyxis</i> (99%), genotype T7 ^c	HE653911
	<i>Hartmannella vermiformis</i>	<i>Hartmannella vermiformis</i> (100%)	FR832469
DF	<i>Acanthamoeba</i> sp., group II ^d	<i>Acanthamoeba</i> sp. (99%), genotype T14 ^e	HE653914
	<i>Hartmannella vermiformis</i>	Not determined	
S2	<i>Acanthamoeba</i> sp., group II ^d	<i>Acanthamoeba castellanii</i> (99%), genotype T4 ^e	HE653912
M6	<i>Acanthamoeba</i> sp.	<i>Acanthamoeba castellanii</i> (99%), genotype T4 ^e	HE653913

^aMorphological identification based on microscopic studies of trophozoites and cysts.

^bMolecular identification based on phylogenetic study of 18S gene, and comparison with GenBank by BLAST algorithm (NCBI); similarity percentages are included.

^cClassification according to Stothard et al. (1998).

^dClassification according to Pussard and Pons (1977)

^eGenotype described by Horn et al. (1999)

with *Acanthamoeba* sp. strain UWET, used by Horn et al. (1999) to describe the genotype T14. In the phylogenetic analysis these two strains form a homogenous cluster with 94% of bootstrap value, clearly separated from *A. mauritaniensis* (Fig. 3).

In the *Acanthamoeba* sp. S2 strain, the morphological classification leads to a similar result to that obtained for the sample DF as denotes the similarity in size of the cysts. However, periodic acid thiocarbohydrazide silver reduced staining (PATAg-r) (Pussard & Pons, 1979) shows a somewhat different feature. In this case, the opercular complexes are stained more markedly, showing very characteristic concentric circles (Fig. 2D). Heat impregnation shows clearly the cysts reticulation, similar to that obtained in the sample DF (Fig. 2C, E). In sample M6 only trophozoites of *Acanthamoeba* sp. were observed, but not the presence of cysts in the culture plates, thus preventing a morphological characterization.

The 18S gene sequences from S2 and M6 strains were identical, confirming that they are the same species. These *Acanthamoeba* showed a 99% of similarity with different strains of *A. castellanii* (genotype T4) including the type strain Castellani

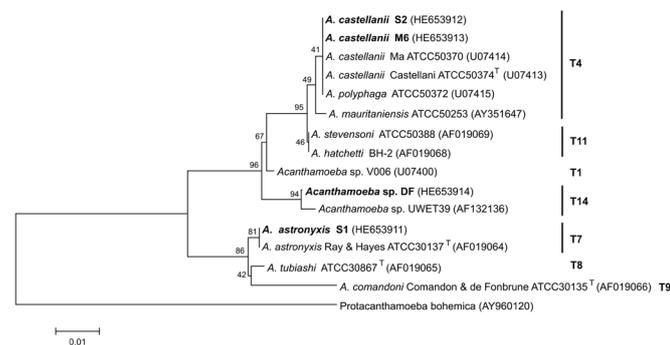


Fig. 3. Phylogenetic tree derived from 18S rDNA sequences corresponding to the specific amplicon ASA.S1 showing the relationships between the *Acanthamoeba* strains isolated from Lascaux Cave (in bold) and their close relative sequences from GenBank. The accession numbers are in parentheses. T1, T4, T7, T8, T9, T11 and T14 are recognised *Acanthamoeba* genotypes. The tree was constructed using the Maximum Likelihood method applying Hasegawa-Kishino-Yano model, based on a comparison of 317 nucleotides. The tree with the highest log likelihood (-739.4) is shown. It was rooted with *Protacanthamoeba bohemica* (*Acanthamoebida*) as outgroup. Bootstrap values are expressed as percentages of 1,000 replications. All nodes of the tree were also recovered using Neighbour Joining and Maximum Parsimony treeing algorithms. Bar, 0.01 substitutions per nucleotide position.

ATCC 50374 isolated from a yeast culture and the strain Ma ATCC 50370 from a keratitis patient. Also showed 99% of similarity with strains of *A. polyphaga*, and 96% similarity with *A. mauritaniensis*, both species included in the genotype T4. This genotype was reported as the one most commonly found in the environment (Booton et al., 2002) and was associated to a high percentage of amoebic keratitis (Stothard et al., 1998). The phylogenetic analysis (Fig. 3) and the morphological traits (Pussard & Pons, 1977) suggested that S2 and M6 strains would be *A. castellanii*.

Strains of the genus *Hartmannella* was found in sample S1 and DF. The description of Page (1976) for the genus *Hartmannella* is coincident with our strains, and they were identified as *H. vermiformis* (Fig. 2F). Molecular data confirmed the identification of the strain isolated from sample S1 (Table 1). *Acanthamoeba castellanii* genotype T4, and *H. vermiformis* were previously found in Slovenian caves (Mulec, 2008; Walochnik & Mulec, 2009).

To understand the presence of amoebae it must be taken into account that the Lascaux Cave is an old karstic channel with many water percolation points and a main emergence point at the entrance air-lock. The running waters are collected in a pool under the machinery room (a thermic and hygrometric regulation system is installed inside, near the cave entrance) and pumped to the exterior (Lastennet et al., 2011). It is well-known that water bodies in the vicinity of humans are a reservoir of amoebae and associated bacteria (Atlas, 1999).

In a previous paper (Bastian et al., 2009), 696 bacterial clones were retrieved from different Lascaux Cave halls and galleries. Phylotypes related with pathogenic bacteria amounted to 45%, from which the presence of *Afipia* spp., *Aquicella* spp. and *Legionella* spp. were remarkable. In addition, other bacteria identified in the cave, such as *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia* and *Pseudomonas fluorescens* were reported to be responsible for complete lysis of amoebae or found as intracellular pathogens (Pagnier et al., 2008). Hence, the simultaneous presence of sequences of *Afipia*, *Aquicella* and *Legionella* in some cave halls, and the identification of *Acanthamoeba* spp. and *H. vermiformis* suggest a close relationship among intracellular pathogenic bacteria and amoebae in the cave.

Many protozoan species have been found to harbour intracellular *Legionella* spp. These include *H. vermiformis* and among other *Acanthamoeba*, *A. castellanii*. For some authors it was clear that *A. castellanii* provided an intracellular niche in which environmental strains of *Legionella* can proliferate. In addition, growth of *Legionella pneumophila* in potable water occurs only in the presence of amoebae and *L. pneumophila* may remain culturable for up to 6 months in a medium containing *A. castellanii*, whereas free-living *Legionella* within biofilms may be inactivated within a few weeks (Lau & Ashbolt, 2009).

Srikanth & Berk (1994) reported that amoebae from cooling tower, containing *Legionella*, may adapt to biocides and may even be stimulated by them. The

biocides tested by these authors included quaternary ammonium compounds (QAC) and isothiazoline derivatives. In Lascaux Cave benzalkonium chloride was in use from 2001 till 2004 and isothiazolines were employed in 2008 (Bastian et al., 2010), therefore it is not surprising that amoebae living in the cave were not killed, taking into account their adaptation, the formation of resistant cysts and the relative rapid inactivation of these biocides by biotic and abiotic processes. Berk et al. (1998) reported that QAC and isothiazolines biocides were found useless for eliminating *Legionella* because these bacteria within *Acanthamoeba* vesicles were viable after biocide exposure.

The known resistance of encysted amoebae and the endosymbionts to disinfection and biocides (Srikanth & Berk, 1994; Berk et al., 1998), which were tested and applied in Lascaux between 2001 and 2008, and the limited field of action in a cave with rock art result in a pessimistic scenario regarding elimination of these microorganisms. This study also suggests the need to control show caves with massive visits from a sanitary point of view, particularly those with small lakes, artificial pools constructed for aesthetical reasons or with water flows accessible to visitors.

The contamination of Lascaux Cave was a consequence of the increasing number of visitors in the early years, which required a climate regulation system. The subsequent fungal outbreaks were treated with biocides, which profoundly altered the microbial ecosystem of the cave. It was intended that the climate system installed in the year 1957 could mitigate visitor's ravages by controlling carbon dioxide, wall condensation and relative humidity. Unfortunately, with the perspective of time, the decision adopted was wrong and likely resulted in the origin of the problems that the cave has suffered in its history. Instead, the adoption of a protocol of preventive conservation with adequate control and regulation of visits would have been more advantageous and less harmful to the cave.

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REFERENCES

- Atlas R.M., 1999 - *Legionella: from environmental habitats to disease pathology, detection and control*. Environmental Microbiology, **1**: 283-293. <http://dx.doi.org/10.1046/j.1462-2920.1999.00046.x>
- Bastian F., Alabouvette C. & Saiz-Jimenez C., 2009 - *Bacteria and free-living amoeba in Lascaux Cave*. Research in Microbiology, **160**: 38-40. <http://dx.doi.org/10.1016/j.resmic.2008.10.001>
- Bastian F., Jurado V., Nováková A., Alabouvette C. & Saiz-Jimenez C., 2010 - *The microbiology of Lascaux Cave*. Microbiology, **156**: 644-652. <http://dx.doi.org/10.1099/mic.0.036160-0>
- Berk S.G., Ting R.S., Turner G.W. & Ashburn R.J., 1998 - *Production of respirable vesicles containing live Legionella pneumophila cells by two Acanthamoeba spp.* Applied and Environmental Microbiology, **64**: 279-286.
- Boon G.C., Kelly D.J., Chu Y.W., Seal D.V., Houang E., Lam D.S., Byers T.J. & Fuerst P.A., 2002 - *18S ribosomal DNA typing and tracking of Acanthamoeba species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of Acanthamoeba keratitis patients in Hong Kong*. Journal of Clinical Microbiology, **40**:1621-1625. <http://dx.doi.org/10.1128/JCM.40.5.1621-1625.2002>
- Coppellotti O. & Guidolin L., 2003 - *Taxonomy and ecology of ciliate fauna (Protozoa, Ciliophora) from karst caves in North-East Italy*. Subterranean Biology, **1**: 3-11.
- Díez B., Pedrós-Alió C., Marsh T.L. & Massana R., 2001 - *Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques*. Applied and Environmental Microbiology, **67**: 2942-2951. <http://dx.doi.org/10.1128/AEM.67.7.2942-2951.2001>
- Horn M., Fritsche T.R., Gautom R.K., Schleifer K.H. & Wagner M., 1999 - *Novel bacterial endosymbionts of Acanthamoeba spp. related to the Paramecium caudatum symbiont Caedibacter caryophilus*. Environmental Microbiology, **1**: 357-367. <http://dx.doi.org/10.1046/j.1462-2920.1999.00045.x>
- Khan N.A., 2006 - *Acanthamoeba: biology and increasing importance in human health*. FEMS Microbiology Reviews, **30**: 564-595. <http://dx.doi.org/10.1111/j.1574-6976.2006.00023.x>
- Lastennet R., Lopez B., Denis A. & Mertz J.D., 2011 - *Hydrogeologie, caracterisation des eaux d'infiltration et proprietes du support a Lascaux*. In: Coye N. (Ed.) *Lascaux et la Conservation en Milieu Souterrain*. Paris: Editions de la Maison des sciences de l'homme: 93-120.
- Lau H.Y. & Ashbolt N.J., 2009 - *The role of biofilms and protozoa in Legionella pathogenesis: implications for drinking water*. Journal of Applied Microbiology, **107**: 368-378. <http://dx.doi.org/10.1111/j.1365-2672.2009.04208.x>
- Lefèvre M., 1974. *La maladie verte de Lascaux*. Studies in Conservation, **19**: 126-156. <http://dx.doi.org/10.2307/1505660>
- Mazei, Y., Belyakova, O., Trulova, A., Guidolin, L. & Coppellotti, O., 2012 - *Testate amoebae communities from caves of some territories in European Russia and North-Eastern Italy*. Protistology, **7**: 42-50.
- Martin-Sanchez P.M., Nováková A., Bastian F., Alabouvette C. & Saiz-Jimenez C., 2012 - *The use of biocides for the control of fungal outbreaks in subterranean environments: The case of the Lascaux Cave in France*. Environmental Science & Technology, **46**: 3762-3770. <http://dx.doi.org/10.1021/es2040625>
- Mulec J., 2008 - *Microorganisms in hypogean*. Acta Carsologica, **37**: 153-160.
- Page F.C., 1967a - *Taxonomic criteria for limax amoebae, with descriptions of three new species of Hartmannella and three of Vahlkampfia*. Journal of Protozoology, **14**: 499-521.
- Page F.C., 1967b - *Re-definition of the genus Acanthamoeba with descriptions of three species*. Journal of Protozoology, **14**: 709-724.
- Page F.C., 1976 - *An illustrated key to freshwater and soil amoeba with notes on cultivation and ecology*. Freshwater Biological Association of Science, Cumbria Publication N° 34, 155 p.
- Pagnier I., Raoult D. & La Scola B., 2008 - *Isolation and identification of amoeba-resisting bacteria from water in human environment by using an Acanthamoeba polyphaga co-culture procedure*. Environmental Microbiology, **10**: 1135-1144. <http://dx.doi.org/10.1111/j.1462-2920.2007.01530.x>

- Pussard M. & Pons R., 1977 - *Morphologie de la paroi kystique et taxonomie du genre Acanthamoeba (Protozoa, Amoebida)*. Protistologica, **13**: 557-598.
- Pussard M. & Pons R., 1979 - *Étude des pores kystiques de Naegleria (Vahlkampfiidae - Amoebida)*. Protistologica, **15**: 163-175.
- Saiz-Jimenez C., Cuezva S., Jurado V., Fernandez-Cortes A., Porca E., Benevante D., Cañaveras J.C. & Sanchez-Moral S., 2011 - *Paleolithic art in peril: Policy and science collide at Altamira Cave*. Science, **334**: 42-43. <http://dx.doi.org/10.1126/science.1206788>
- Schroeder J.M., Booton G.C., Hay J., Niszl I.A., Seal D.V., Markus M.B., Fuerst P.A. & Byers T.J., 2001 - *Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of Acanthamoebae from humans with keratitis and from sewage sludge*. Journal of Clinical Microbiology, **39**:1903-1911. <http://dx.doi.org/10.1128/JCM.39.5.1903-1911.2001>
- Srikanth S. & Berk S.G. 1994 - *Adaptation of amoebae to cooling tower biocides*. Microbial Ecology, **27**: 293-301. <http://dx.doi.org/10.1007/BF00182412>
- Stothard D.R., Schroeder-Diedrich J.M., Awwad M.H., Gast R.J., Ledee D.R., Rodriguez-Zaragoza S., Dean C.L., Fuerst P.A. & Byers T.J. 1998 - *The evolutionary history of the genus Acanthamoeba and the identification of eight new 18S rRNA gene sequence types*. Journal of Eukaryotic Microbiology, **45**: 45-54. <http://dx.doi.org/10.1111/j.1550-7408.1998.tb05068.x>
- van Hengstum P.J., Reinhardt E.G., Beddows P.A., Schwarcz H.P. & Gabriel J.J., 2009 - *Foraminifera and testate amoebae (thecamoebians) in an anchialine cave: Surface distributions from Aktun Ha (Carwash) cave system, Mexico*. Limnology and Oceanography, **54**: 391-396. <http://dx.doi.org/10.4319/lo.2009.54.1.0391>
- Walochnik J. & Mulec J., 2009 - *Free-living amoebae in carbonate precipitating microhabitats of karst caves and a new vahlkampfiid amoeba, Allovahlkampfia spelaea gen. nov., sp. nov.* Acta Protozoologica **48**: 25-33.