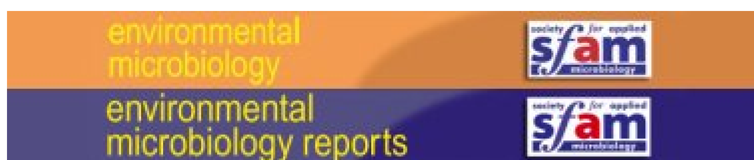

Identification of free-living amoebas and amoeba-resistant bacteria accumulated in Dreissena polymorpha

Journal:	*Environmental Microbiology and Environmental Microbiology Reports*
Manuscript ID	EMI-2020-0675.R1
Journal:	Environmental Microbiology
Manuscript Type:	EMI - Research article
Date Submitted by the Author:	n/a
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Keywords:	Dreissena polymorpha, accumulation, bacteria, free-living amoeba, amoeba-resistant bacteria

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2 **Identification of free-living amoebas and amoeba-resistant bacteria accumulated in *Dreissena***
3 ***polymorpha*.**

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24 Running Title: **Free-living amoebas and bacteria in zebra mussels**

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26 **Abstract**

27 To identify the free-living amoeba (FLA) and amoeba resistant bacteria (ARB) accumulated in zebra
28 mussels and in the water in which they are found, mussels were collected at two locations in the
29 Ebro river basin (North East Spain). Free-living amoebas and bacteria were isolated from mussel
30 extracts and from natural water. PCR techniques were used to identify the FLAs and endosymbiont
31 bacteria (*Legionella*, *Mycobacterium, Pseudomonas* and cyanobacteria), and to detect *Giardia* and
32 *Cryptosporidium*. The most frequently found FLAs were *Naegleria* spp. The presence of *Legionella*,
33 *Mycobacterium,* and *Pseudomonas* inside the FLA was demonstrated, and in some cases both
34 *Legionella* and *Pseudomonas* were found together. Differences between FLAs and ARB identified
35 inside the mussels and in the water were detected. In addition, *Escherichia coli*, *Clostridium*
36 *perfringens, Salmonella* spp. and *Enterococcus* spp. were accumulated in mussels in concentrations
37 unconnected with those found in water. The results show the ability of the zebra mussel to act as a
38 reservoir of potentially pathogenic FLAs, which are associated with potentially pathogenic amoeba-
39 resistant bacteria, although the lack of association between microorganisms inside the mussels and
40 in the water suggests that they are not useful for monitoring microbiological contamination at a
41 specific time.

42 **Keywords:** *Dreissena polymorpha*; accumulation; bacteria; free-living amoeba; amoeba-resistant
43 bacteria.

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45 **1. Introduction**

46 *Dreissena polymorpha*, commonly known as the zebra mussel, is considered one of the 100 most
47 harmful invasive exotic species in the Northern Hemisphere (Karatayev *et al.*, 2015). In North East
48 Spain, *D. polymorpha* bivalves have invaded the Ebro River basin since 2001, including tributaries and
49 areas at the headwaters of the river.

50 Due to their condition of filter feeders, Zebra mussels accumulate and concentrate organic
51 compounds, metals, and microorganisms from the water into their tissues, even when they are
52 present in low concentrations (Mosteo *et al.*, 2016). In consequence, they have been considered a
53 possible tool for monitoring the pollution of the ecosystems in which they live (Carrasco *et al.*, 2008;
54 Parolini and Binelli, 2014; Poma *et al.*, 2014; Mosteo *et al.*, 2016; Benito *et al.*, 2017).

55 The microorganisms accumulated inside the mussels include bacteria, viruses and some pathogenic
56 protozoa (Selegean *et al.*, 2001; Graczyk *et al.*, 2004; Palos Ladeiro *et al.*, 2014; Kerambrun *et al.*,
57 2016; Mezzanotte *et al.*, 2016; Mosteo *et al.*, 2016; Bighiu *et al.*, 2019). Free-living amoebas (FLAs)
58 have been found on the shell of the mussel (Bischoff and Wetmore, 2009), and also in extracts of
59 freshwater bivalves from the Ebro River basin using microscopy techniques, but the genus of the FLA
60 was not identified (Mosteo *et al.*, 2016). FLAs are environmental protozoa, some of which can be
61 potentially pathogenic for humans and animals. *Acanthamoeb*a spp. is the most frequently isolated
62 FLA in the environment (Garcia *et al.*, 2013), followed by *Vermamoeba* spp. (Greub and Raoult,
63 2004). Both of them can cause encephalitis, but are more frequently found as a cause of keratitis
64 associated with the use of contact lenses. *Naegleria fowleri,* the only pathogenic *Naegleria* species
65 which is associated with primary amoebic meningoencephalitis, has not been detected to date in the
66 Ebro River reservoirs (Garcia *et al.*, 2013). The microbial community inside mussels and other
67 bivalves is closely related to the microbiological composition of the environment in which they are
68 attached (Mariné Oliveira *et al*., 2016). FLAs are usually integrated into biofilms, which correspond to
69 complex structures where microorganisms are associated (Castrillon Rivera *et al.*, 2012). The

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70 amoebas released from the biofilms are free in the water and are then accumulated in the mussel,
71 but curiously Bischoff and Horvath (2011) found higher densities of amoebas in water and sediments
72 from American lakes with zebra mussel than in those without mussels.

73 FLAs carry bacteria inside them which are able to survive lysis by amoebas. Some of them such as
74 *Legionella*, even multiply (Goñi *et al.*, 2014). Other intracellular bacteria have limited capacities to
75 grow within FLAs, such as *Chamydia* and *Chlamydia*- related bacteria (Kebbi-Beghdadi and Greub,
76 2014). These bacteria, known as amoeba-resistant bacteria (ARB), are transported from one place to
77 another and protected from external conditions that include extreme temperatures, chemical
78 agents, and disinfectants to which amoebas are resistant (Greub and Raoult, 2004; Goñi *et al.*, 2014;
79 Pagnier *et al.*, 2015). Among the best-known ARBs are species such as *Legionella pneumophila,*
80 *Pseudomonas* spp., toxic cyanobacteria, *Mycobacterium* spp., *Chlamydia* and *Chlamydia*- related
81 bacteria (Calvo *et al.*, 2013; Kebbi-Beghdadi and Greub, 2014). Lately, the presence within FLAs of
82 other bacteria of public health interest, such as *Helicobacter pylori*, *Aeromonas* sp., *Arcobacter* sp.
83 and *Salmonella* has been reported (Moreno-Mesonero et al., 2020). Thus, *D. polymorpha* can
84 accumulate a wide variety of organisms including FLA which, in turn, accumulate bacteria. The
85 possible role of the microorganisms accumulated in the zebra mussel and in the FLA remains
86 unidentified, so symbiotic association cannot be ruled out. The aim of this work was to identify the
87 potentially pathogenic free-living amoebas (FLAs) and their ARB accumulated in zebra mussels from
88 the Ebro river basin, comparing them with those present in the water in which they are found.

89 **2. Results and Discussion**

90 **2.1. FLA isolation and identification in natural water and zebra mussel**

91 Natural water and zebra mussels were taken from two sampling points along the Ebro river basin,
92 located in the North East (NE) of Spain: the Sobrón reservoir in Santa María de Garoña (Burgos,
93 Spain) and the Rimer irrigation channel downstream from Caspe (Zaragoza, Spain) (Figure 1). Both
94 places are described in section 3.1. During the maintenance of the zebra mussels from the Sobrón

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95 Reservoir in a laboratory scale aquarium, the death of a large number of specimens was observed.
96 They were analysed as a different sample and treated as described in section 3.1.

97 FLAs were isolated in the 100 % of the mussel and water samples. Their identification is shown in
98 table 1. Together with two other amoebas, a flagellate was observed and captured when the extract
99 obtained from the dead mussels from Sobrón was visualized by microscopy (Fig. 2). This was
100 identified by PCR techniques as *Cercomonadida*, a biflagellate bacterivorous protozoon abundant in
101 soil and freshwater. Its morphological differentiation from *Naegleria clarki* grown in NN Agar is
102 shown in Figure 2.

103 As Bischoff and Hovarth (2011) demonstrated, it is reasonable to predict that zebra mussels have an
104 impact on the heterotrophic communities of the aquatic ecosystems they invade. This is why, it is
105 important to know if zebra mussels accumulate FLAs and to identify them. The variety of amoeba
106 species isolated in the two sampling sites could be due to the greater agricultural and livestock
107 activity associated with a greater richness of FLA species and higher concentrations of bacteria
108 (Rodríguez-Zaragoza *et al.*, 1994; Bonilla-Lemus *et al.*, 2014).

109 Overall, stands out the high presence of the genus *Naegleria* spp., and the low isolation of
110 *Acanthamoeba* spp. (Table 1). Also, different genera of FLA were found in one sample (Table 1).

111 In 2013, Garcia *et al.*, ,found *Acanthamoeba* spp. and *Naegleria* spp. in 37.45 % and 15.66 %
112 respectively, of the samples collected in reservoirs and water treatment plants in Aragón, Spain. The
113 high proportion of samples with *Naegleria* [37.5 % (3/8)] in the present study could be associated
114 with the low precipitation rate in this area during the years prior to the sampling for the study which
115 would have favored their concentration (Sente et al, 2016). Two of the 3 FLA identified as *Naegleria*
116 spp. correspond to the species *Naegleria clarki*, and the other has 99 % homology with *Naegleria*
117 *clarki* (KC527832.1) and with *Naegleria gruberi* (GU320598.1). *Naegleria clarki* has been described as
118 a nonpathogenic FLA; however, it is able to host other microorganisms inside it.

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119 *Acanthamoeba* was found only in the sample corresponding to natural water from the Sobrón
120 reservoir, in contrast with the high proportion of samples with this genus found by García *et al.*,
121 2013. The genus *Acanthamoeba* spp. has been reported as the most common FLA in the
122 environment and is often associated with bacteria-rich water (Bonilla-Lemus *et al*., 2014).

123 *Vexillifera bacillipedes* is a non-encysting amoeba, morphologically very similar to *Acanthamoeba*,
124 which causes disease in fish (Dyková *et al*., 1998). Its prevalence in the present work [25.0 % (2/8)]
125 was similar to the frequency observed in streams in the Mexico Basin in Central Mexico in 2014
126 (Bonilla-Lemus *et al*., 2014).

127 The composition of FLA populations depends on the characteristics of each ecosystem and the
128 moment of sampling (Bonilla-Lemus *et al*., 2014; Sente *et al*., 2016). Amoebas are usually integrated
129 into biofilms (Castrillon Rivera and Palma-Ramos, 2012). When their populations in water are
130 analyzed, only those amoebas that emerge from biofilms and are present in water in high
131 concentrations can be detected. Climate change and the increase in temperatures produces changes
132 in FLA populations favoring the growth of some of them such as those of the *Naegleria* genus which
133 survive and multiply better at high temperatures (Lam *et al.*, 2019). The mussels accumulate those
134 amoebas which are more abundant in the water, and they accumulate FLAs from juveniles to adult
135 whereas the FLAs found in water samples were the dominant amoebas at the sampling moment. In
136 addition, the mussel might prefer some specific genus of FLA. The release of accumulated amoebas
137 upon the death of the mussel can have an impact on the microbial communities of the ecosystem by
138 changing the dominant genus and by increasing the FLA number. When these amoebas are
139 potentially pathogenic for humans or animals, the increase in their concentration in water could pose
140 a health threat..

141 **2.2. ARB identification**

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142 A multiplex PCR was performed in order to detect *Legionella pneumophila*, *Mycobacterium* spp.,
143 *Pseudomonas* spp and toxic cyanobacteria inside the FLAs (ARB) and in water (Calvo et al., 2013).
144 Thirteen DNA samples were analysed, 8 of them corresponding to FLA cultures, 4 to water and 1 to
145 the flagellate captured. Six (46.2 %) of them were positive, four of them from cultures of FLA,
146 representing 50 % of the FLA analysed (Table 2). This proportion of ARB positive FLAs was similar to
147 that found by Garcia *et al.* in 2013 in FLAs from reservoirs of the Ebro River (41.9 %).

148 Of the analysed bacteria, *Pseudomonas* spp. was found inside *Naegleria clarki, Vexillifera bacillipedes*
149 and *Protacanthamoeba bohemica* whereas *L. pneumophila* was also found inside *Acanthamoeba*
150 *pustulosa*. Of the amoebas isolated, 44 % (4/9) hosted *Legionella* spp. and 33 % (3/9) *Pseudomonas*
151 spp. It should be highlighted that 75.0 % of the amoebas that harbored bacteria simultaneously
152 hosted *Pseudomonas* spp. and *L. pneumophila* while the other 25 % only contained *L. pneumophila.*
153 As an exception, only *Legionella* spp. was found inside *Acanthamoeba* spp. isolated from the natural
154 water of the Sobrón reservoir. *Mycobacterium* spp., and toxic cyanobacterium were not found inside
155 the amoebas.

156 FLAs have an important role in the spread of several pathogens, such as *Legionella pneumophila*,
157 *Mycobacterium*, *Chlamydia*-related bacteria, viruses, and fungi (Goñi *et al.*, 2014; Kebbi-Beghdadi
158 and Greub, 2014). This is one of the most important risks associated with the presence of FLAs, since
159 disinfection treatments do not affect the bacteria within the FLA when the amoebas are in their
160 cystic stage (Cateau *et al.*, 2014; Delafont *et al.*, 2014).

161 It is believed that FLAs are the main niche for replication of *Legionella* spp. in the environment which
162 in addition, increases their pathogenicity inside the protozoa. When hosted in FLA, *Legionella* can
163 reach artificial water systems such as ornamental fountains, sprinkler systems, or cooling towers,
164 where the bacterium colonizes ducts and may be released producing *Legionella* outbreaks if their
165 concentration is high, creating a major public health problem (Thomas *et al.*, 2010).

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166 *Pseudomonas* is an environmental bacterium with high intrinsic resistance to several antibiotics and
167 is responsible for a wide range of nosocomial infections, especially in immunocompromised patients
168 (Cateau *et al*., 2014). Also, *Pseudomonas* spp. infection is most frequently associated with amoebic
169 keratitis (Dini *et al.*, 2000; Sharma *et al.*, 2013). The simultaneous presence of *Pseudomonas* and
170 *Legionella* is surprising due to the ability of many *Pseudomonas* species to produce bacteriocin and
171 bacteriocin-like substances which are active against *Legionella* (Guerrieri *et al.*, 2008). The high
172 specificity of these bacteriocins against certain species of *Legionella* suggests that it is possible that
173 these substances have not been produced or are not active against the *Legionella* with which
174 *Pseudomonas* share their habitat. It is also possible that *Legionella* and *Pseudomonas* do not come
175 into contact inside the amoebas. Future studies are necessary to clarify these interactions. On the
176 other hand, there are differences between the bacteria identified in the water and inside the
177 amoebas, suggesting that the amoebas come from another point upstream or they were the majority
178 population long ago. Only a small number of bacteria has been analyzed, which are those included in
179 the multiplex PCR used. Given the results obtained, the analysis of other bacteria such as *Chlamydia*-
180 related bacteria, *Salmonella* or *Aeromonas*, and their quantification for the determination of relative
181 amounts of ARB using quantitative PCR, will be of interest in future research to increase our
182 knowledge of FLA-ARB interactions (Lienard et al., 2011; Moreno-Mesonero et al., 2020.

183 **2.3. Presence of pathogenic bacteria in natural water and zebra mussel**

184 To confirm differences between microorganisms in the water and in the mussel, other bacteria were
185 studied. These were the bacteria found in greater quantities and which can be grown easily and
186 quickly for quantification. The selected bacteria were fecal contamination indicator bacteria and
187 *Salmonella* which in addition provide information about the influence of human activities on aquatic
188 environments. Furthermore, the counting methods guaranteed the viability of the bacteria studied.
189 The quantification of the selected bacteria in the water samples and the mussel extract are shown in
190 Table 3. The concentrations of bacteria in both water and mussels from the Sobrón Reservoir are

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191 higher than those from the Rimer Channel, which explains the greater variety of amoebas found at
192 this point. An index was calculated in order to compare the results obtained at the two sampling
193 points. This index shows how many times a gram of mussel tissue accumulates the number of
194 bacteria contained in the same amount of water. A high index indicates a high accumulation of
195 *Salmonella* and *Clostridium* in mussels (Table 3), suggesting a high capacity of *D. polymorpha* to
196 accumulate these bacteria or a singular discharge. Stands out the low accumulation index for *E. coli*
197 which indicates a low accumulation in the mussels from both sampling points despite the high
198 concentration of this bacterium in water. This could be due to many factors, such as the use of the
199 bacteria as a primary carbon source (Silverman *et al*., 1995; Vathanodorn and Parsons, 1996), a
200 preferential uptake of bacteria other than *E. coli*, or the capacity of these bacteria to multiply in
201 water.
202 The low index for *Enterococcus* in mussels from the Sobrón with a high concentration in water
203 contrasts with the high value of this index in the Rimer Channel with a low concentration in water
204 reinforcing the hypothesis that there was a discharge time ago. However, the CFUs in the Rimer
205 Irrigation channel are very low, making it difficult to draw conclusions regarding the possible use of
206 zebra mussels in monitoring *Enterococcus* contamination.

207 Multiple factors, including climatic factors such as temperature and rainfall, the amounts of water
208 that enter a river along its course, and human activity (livestock and agriculture) could affect the
209 survival of bacteria. The counts of indicator bacteria and *Salmonella* found in the Rimer Channel in
210 this work are similar to those found in the samples taken two years previously (Mosteo *et al.*, 2016),
211 indicating stability in the bacterial community of both the waters and mussels. The main reservoir of
212 *Salmonella* spp. and *Clostridium perfringens* is the digestive tract of animals and humans. Considering
213 that animal husbandry and agriculture are the most important activities in the areas under study, the
214 way natural fertilizers are used in agricultural activities and the proximity of population centers could
215 explain the differences in the bacterial counts (www.chebro.es).

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216 The correlation analysis between total bacterial accumulation in mussels and water showed a
217 moderate correlation ($r= 0.74$, $p<0.01$) for the Sobrón Reservoir and a low correlation ($r=0.40$,
218 $p=0.06$) for the Rimer Channel. This contrasts with the one found by other authors (Mosteo *et al*.,
219 2016; Bighiu *et al.*, 2019), questioning the possible use of the mollusc as an indicator of water
220 pollution, but it reinforces the option of its possible use in the detection of past discharges. On the
221 other hand, it is possible that some of the bacteria identified as mussel-accumulated bacteria are
222 located inside the amoebas. If they have been transported into the amoebas, they could not be
223 related to discharges at this point but upstream. This could partly explain the lack of correlation
224 between the bacteria concentration found in the mussel extracts and in the environmental water.
225 However, it should be borne in mind that few sampling points have been taken into account for the
226 correlation study, so future studies are needed to obtain more information, including a study of all
227 the bacteria accumulated in FLAs.

228 Three morphologically different colonies grew In the culture of water suspension of dead mussel
229 from Sobrón reservoir, which were identified as *Citrobacter braakii, Stenotrophomonas maltophilia*
230 and *Pseudomonas* spp. These bacteria accumulated inside of the zebra mussels may have caused
231 their death in the aquarium, as has been observed by other authors for one species of *Pseudomonas*
232 (Molloy *et al*., 2013).

233 **2.4. Intestinal protozoa analysis.**

234 *Giardia duodenalis* and *Cryptosporidium* spp. were not detected by observation under microscope,
235 either by direct observation, after modified Zielh-Neelsen staining or by PCR techniques, either in
236 zebra mussels or in natural water from both sample sites. Many studies have demonstrated the
237 capacity of zebra mussels to accumulate *Giardia duodenalis*, *Cryptosporidium* spp., and *Toxoplasma*
238 *gondii* in amounts proportional to their environmental concentration (Graczyk *et al.*, 2004; Palos

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239 Ladeiro *et al.*, 2014; Kerambrun *et al.*, 2016). Although these protozoa are commonly present in
240 water, this was not the case in this study..
241 As a conclusion, the results of the study show that *Dreissena polymorpha* accumulates bacteria and
242 FLAs, while FLAs accumulate bacteria. When these microorganisms are potentially pathogenic, the
243 death of mussels can increase their concentration in waters and pose a risk to health and the
244 environment. Further studies should be performed to determine if the relationship between *D.*
245 *polymorpha*, FLAs and ARB corresponds to an endosymbiosis model in which microorganisms are
246 inside one another like a matryoshka doll, as described by Woyke and Schulz (2019)

247 **3. Experimental procedures**

248 **3.1. Site description**

249 Natural water and zebra mussels were collected at two sampling points along the Ebro river basin,
250 located in the North East (NE) of Spain. With an area of 85,362 km^2, the river discharges into the
251 Mediterranean Sea and supplies water to a population of around three million people along its
252 course The sampling site areas selected for this study are described as follows (Fig. 1):

253 - Point 1: Sobrón reservoir (N 42 ˚ 45 ’ 42.7 ’’, W 3 ˚ 09 ’ 56.2 ’’) located in Santa María de
254 Garoña (Burgos, Spain), in the upper area of the river course, between the Alava and
255 Burgos provinces. The reservoir is used as a water supply and for the generation of
256 hydro-electrical power. Agricultural and livestock activities (mainly poultry and pig
257 farms) occur in the area around and recreational fishing activities are carried out in the
258 reservoir.
259 - Point 2: The Rimer irrigation channel (N 41 ˚ 13 ’ 44 ’’, E 0 ˚ 00 ’ 32 ’’) downstream from
260 Caspe (Zaragoza, Spain), comes from the Guadalope river (a tributary of the Ebro river),
261 at a point near the river mouth. Agricultural and livestock activities (mainly poultry, pig
262 and rabbit farms) are present in the area.

263 **3.2. Natural water and zebra mussel collection and preservation**

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264 Groups of around 50 zebra mussels of similar size adhered on hard substrates at a depth of less than
265 1 metre, were randomly selected and manually collected from each of the sampling points. In
266 addition, 1 L of water was taken at the same depth, following the sampling protocol developed by
267 the Ebro Hydrographic Confederation (CHE, 2006). The samples were taken in spring, between 20th
268 March and 3rd June, 2015. The analysis of the mussels was carried out within 48 hours after
269 collection, but some additional specimens were kept until the work was finished in anticipation of
270 possible repetitions or extraordinary analysis. These mussels were preserved in a laboratory-scale
271 aquarium (volume: 100 L) filled with water from the sampling point, under optimal temperature (18-
272 20 ºC) and conditions (calcium content, total hardness, pH, dissolved oxygen and conductivity)
273 previously described (Claudi and Mackie, 1994; O'Neill, 1996; Mosteo *et al.*, 2016).
274 During the maintenance of the zebra mussels from the Sobrón Reservoir in the laboratory scale
275 aquarium, the death of a large number of specimens was observed. To study the cause, five of the
276 dead specimens were removed from the shells and the whole soft tissue was suspended in sterile
277 water and incubated at 30 ˚C for 24 hours. An aliquot of this suspension was cultured on Muller-
278 Hilton agar (Scharlau®) supplemented with 5 % of ram blood and incubated at 37 ˚C for 24 hours. The
279 colonies that grew in this media were identified by the API system. The suspension of dead mussels
280 in water was included as an additional sample in subsequent trials.
281 A homogeneous extract was obtained with the zebra mussel soft tissue and interstitial water.
282 Individuals with a similar valve size, between 1.8 and 2.1 cm, were randomly selected among the
283 specimens collected. The valves and the byssus were removed and approximately 2 g (corresponding
284 to 5 specimens) of wet soft tissue were weighed, pooled, ground, and homogenized with 10 mL of
285 phosphate-buffered saline (PBS). This extract was divided into aliquots for the bacteriological analysis
286 and protozoa identification.
287 Samples of natural water, the extract of zebra mussel and water suspension of dead mussels were
288 cultured with non-nutritive agar (NNA) plates seeded with preheated *E. coli*, incubated at 30 ºC

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289 during 15 days, and microscopically observed daily, searching forthe growth of FLAs. Those plates in
290 which no growth of FLAs was observed were considered negative. When FLA growth was observed,
291 subcultures were performed on fresh NNA to prevent fungal contamination and to isolate the
292 different FLA present. The morphologically distinct FLAs were subcultured in different fresh NNA
293 plates at least 4 times, to ensure that the ARBs to be identified were located inside the amoeba
294 (Calvo *et al.*, 2013). Morphological criteria documented by Smirnov and Goodkov (1999) were used
295 and complemented with Page (1988) identification keys.

296 **3.3. DNA Extraction, PCR Techniques, and Nucleotide Sequencing**

297 DNA extraction from natural water, amoeba cultures, and zebra mussel samples was performed
298 using a commercial kit (Norgen Biotek Corp Stool DNA Isolation Kit, Ontario, Canada).
299 Generic and specific PCRs were used to identify the genera and species of FLA present in the zebra
300 mussel and water with the primers and protocols shown in Table 4.
301 Amoeba resistant bacteria were studied by a pentaplex-nested-PCR described by Calvo *et al.* (2013)
302 using as a template the DNA of FLAs isolated from natural water samples and zebra mussel extract
303 (Table 4). In addition, the analysis was performed with DNA extracted directly from the water sample
304 and mussel extract, to determine the possible relationship between the bacteria detected in the
305 watercourse, the zebra mussel and the FLA isolated in both samples. This PCR identified the presence
306 of *Mycobacterium*, *Legionella*, *Pseudomonas* and toxic cyanobacteria.
307 The amplicons obtained were purified using the commercial kit GFX™ PCR ADN and the Gel Band
308 Purification Kit (GE Healthcare) and subjected to direct sequencing in the two directions. The
309 sequences obtained were analysed using the BioEdit® alignment program and a data-processing tool
310 (Basic Local Alignment Search Tool of the National Centre for Biotechnology Information, NCBI) and
311 compared with sequences deposited in the database GenBank under accession numbers MH678804-
312 MH678815 and MH675517.

313 **3.4. Bacteriological and intestinal protozoa analysis**

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314 To analyze the accumulation of bacteria in the zebra mussel, bacteria indicators of fecal
315 contamination, *Salmonella* and *Pseudomonas* were selected.
316 Detection of *Clostridium perfringens*, *Enterococcus* spp., *Escherichia coli,* and *Salmonella* spp., was
317 carried out from water and mussel extracts by the spread plate standard method 9215C or by the
318 membrane filtration method. A filtration ramp, the Microfil® Filtration Funnels kit with S-pack™ and
319 0.45 µm Millipore® sterile filters were used for *Enterococcus* spp., *Escherichia coli,* and *Salmonella*
320 spp. detection, while 0.22 µm Millipore® sterile filters were used for *Clostridium perfringens*
321 detection. The assays were undertaken in triplicate. In the case of *Clostridium perfringens* analysis,
322 only the membrane filtration method was performed.
323 The culture and enumeration of the bacteria were performed by following the procedures specified:
324 - *C. perfringens*: UNE-EN ISO 26461-2:1986. Using SPS agar (Scharlau®).
325 - *Enterococcus* spp.: UNE-EN ISO 7899-2:2000. Slanetz & Bartley agar (Scharlau®).
326 - *E. coli:* UNE-EN ISO 9308-1:2000. Using McConkey agar (Scharlau®)
327 - *Salmonella* spp.: UNE-EN ISO 19250:2010. Using Salmonella-Shigella agar (Scharlau®).
328 Colony enumeration was undertaken in terms of colony-forming units (CFUs) per 100 mL in natural
329 water samples and CFUs per wet gram of zebra mussel soft tissue. These concentrations were
330 transformed into log10 for correlation studies.
331 Intestinal protozoa were investigated by direct observation of the mussel extract by optic microscopy
332 (Nikon Eclipse 80i) at 10 X, 40 X, and 100 X magnifications. To identify *Cryptosporidium* oocysts and
333 other coccidians, modified Zielh-Neelsen staining was performed.
334 The sterile water suspension that was visualized by microscopy was subjected to the same analysis as
335 the extracts obtained from the mussels.

336 **3.5. Statistical analysis**

337 To assess the mussel´s ability to concentrate each bacterium, an index was defined as the resulting
338 value of dividing CFU·g mussel^{-1}/ CFU·g water^{-1}, considering the water density as 1 gram per mL,

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339 giving an idea of the bacteria accumulated in the mussel compared to those in the water. This index
340 is an approximation that allows a comparison of the results obtained at the two sampling points.

341 The relationship between the total concentration of bacteria accumulated in the mussel and in the
342 water was evaluated by the Spearman correlation coefficient. The significance threshold was $p<0.05$.

343 **3.6. Ethical and legal aspects**

344 Legal permission was obtained for the sampling, handling, and storage of zebra mussel specimens in
345 this study. Once the research was finalized, the bivalves were destroyed as described by the Ebro
346 Hydrographic Confederation (CHE, 2011) protocol, with an adequate dosage of NaClO.

347 **Acknowledgements**: This work was financed by the Gobierno de Aragón (Spain) (Research Reference
348 Team Water and Environmental Health B43_20R) and co-financed by Feder 2014-2020 “Building
349 Europe from Aragón”.

350 **Conflict of interest**: None to declare

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352 **References**

353 Benito, M., Mosteo, R., Rubio, E., LaPlante, D., Ormad, M.P., and Goñi, P. (2017) Bioaccumulation of
354 Inorganic Elements in *Dreissena polymorpha* from the Ebro River, Spain: Could Zebra Mussels Be
355 Used as a Bioindicator of the Impact of Human Activities?. *River Res Appl* **33**: 718-728.

356 Bighiu, M.A., Norman Haldén, A., Goedkoop, W., and Ottoson, J. (2019) Assessing microbial
357 contamination and antibiotic resistant bacteria using zebra mussels (*Dreissena polymorpha*). *Sci Total*
358 *Environ* **650:** 2141-2149.

359 Bischoff P.J., and Horvath T.G. (2011) Abundances of naked amoebae and macroflagellates in central
360 New York lakes: Possible effects by zebra mussels. *Acta Protozool* **50:** 23–31.

361 Bischoff P.J., and Wetmore S. (2009) Seasonal abundances of naked amoebae in biofilms on shells of
362 zebra mussels (*Dreissena polymorpha*) with comparative data from rock scrapings. *J. Eukaryot.*
363 *Microbiol* **56:** 397–399.

364 Bonilla-Lemus, P., Caballero Villegas, A.S., Carmona Jiménez, J, and Lugo Vázquez, A. (2014)
365 Occurrence of free-living amoebae in streams of the Mexico Basin. *Exp Parasitol* **145**: S28-33.

366 Calvo, L., Gregorio, I., García, A., Fernández, M.T., Goñi, P., Clavel, A., Peleato, M.L., and Fillat, M.F.
367 (2013) A new pentaplex-nested PCR to detect five pathogenic bacteria in free living amoebae. *Water*
368 *Res* **47:** 493-502.

369 Carrasco, L., Díez, S., Soto, D.X., Catalan, J., and Bayona, J.M. (2008) Assessment of mercury and
370 methylmercury pollution with zebra mussel (*Dreissena polymorpha*) in the Ebro River (NE Spain)
371 impacted by industrial hazardous dumps. *Sci Total Environ* **407:** 178-184.

372 Castrillon Rivera, L.E., and Palma-Ramos, A. (2012) Biofilms: a survival and resistance mechanism of
373 microorganisms. In: Antibiotic Resistant Bacteria – A Continuous Challenge in the New Millennium.
374 Ed Dr. Marina Pana (Ed). InTech, [www document] URL

For Peer Review Only

375 http://www.intechopen.com/books/antibiotic-resistant-bacteria-a-continuous-challenge-in-the-
376 newmillennium/biofilms-a-survival-and-resistance-mechanism-of-microorganisms.

377 Cateau, E., Maisonneuve, E., Peguilhan, S., Quellard, N., Hechard, Y., and Rodier, M.H. (2014)
378 *Stenotrophomonas maltophilia* and *Vermamoeba vermiformis* relationships: bacterial multiplication
379 and protection in amoebal-derived structures. *Res Microbiol* **165:** 847-851.

380 CHE (2011) Disinfection and cleaning protocol to avoid the spread of the plague of the zebra mussel
381 (*Dreissena polymorpha*). [www document] URL
382 www.chebro.es/contenido.streamFichero.do?idBinario=5049

383 CHE (2006) Sampling and identification of larvae of zebra mussel, internal document, [www
384 document] URL www.chebro.es/contenido.streamFichero.do?idBinario=10377

385 Claudi, R., and Mackie, G.L. (1994) Practical Manual for Zebra Mussel Monitoring and Control. Boca
386 Raton (FL): CRC Press Inc., Lewis Publishers.

387 Delafont, V., Mougari, F., Cambau, E., Joyeux, M., Bouchon, D., Héchard, Y., and Moulin, L. (2014)
388 First evidence of amoebae-mycobacteria association in drinking water network. *Environ Sci Tech* **48**:
389 11872-11882.

390 Dini, L.A., Cockinos, C., Frean, J.A., Niszl, I.A., and Markus, M.B. (2000) Unusual case of
391 *Acanthamoeba polyphaga* and *Pseudomonas aeruginosa* keratitis in a contact lens wearer from
392 Gauteng, South Africa. *J Clin Microbiol* **9:** 826-829.

393 Dyková, I., Lom, J., Machácková, B., and Pecková, H. (1998) *Vexillifera expectata* sp. n. and other non-
394 encysting amoebae isolated from organs of freshwater fish. *Folia Parasitol* **45:** 17-26.

395 Garcia, A., Goñi, P., Cieloszyk, J., Fernandez, M.T, Calvo-Beguería, L., Rubio, E., Fillat, M.F., Peleato,
396 M.L., and Clavel, A. (2013) Identification of free-living amoebae and amoeba-associated bacteria
397 from reservoirs and water treatment plants by molecular techniques. *Environ Sci Tech* **47:** 3132-3140.

For Peer Review Only

398 Goñi, P., Fernández, M.T., and Rubio, E. (2014) Identifying endosymbiont bacteria associated with
399 free-living amoebae. *Environ Microbiol* **16:** 339-349.

400 Graczyk, T.K., Conn, D.B., Lucy, F., Minchin, D., Tamang, L., Moura, L.N., DaSilva, A.J. (2004) Human
401 waterborne parasites in zebra mussels (*Dreissena polymorpha*) from the Shannon River drainage
402 area, Ireland. *Parasitol Res* **93:** 385-391.

403 Greub, G. and Raoult, D. (2004) Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev*
404 **17:** 413-433.

405 Guerrieri, E., Bondi, M., Sabia, C., de Niederhäusern, S., Borella, P., and Messi, P. (2008) Effect of
406 bacterial interference on biofilm development by *Legionella pneumophila. Curr Microbiol* **57:** 532-
407 536.

408 Hirt, R.P., Healy, B., Vossbrinck, C.R., Canning, E.U., and Embley, T.M. (1997) A mitochondrial Hsp70
409 orthologue in *Vairimorpha necatrix*: molecular evidence that microsporidia once contained
410 mitochondria. *Curr Biol* **7**: 995-998.

411 Karatayev, A.Y., Burlakova, L.E., Mastitsky, S.E., and Padilla, D.K. (2015) Predicting the spread of
412 aquatic invaders: insight from 200 years of invasion by zebra mussels. *Ecol Appl* **25:** 430-440.

413 Kebbi-Beghdadi C, Greub G. (2014) Importance of amoebae as a tool to isolate amoeba-resisting
414 microorganisms and for their ecology and evolution: the *Chlamydia* paradigm.
415 *Environ Microbiol Rep* **6**:309-324.

416 Kerambrun, E., Palos Ladeiro, M., Bigot-Clivot, A., Dedourge-Geffard, O., Dupuis, E., Villena, I., *et al.*
417 (2016) A. Zebra mussel as a new tool to show evidence of freshwater contamination by waterborne
418 *Toxoplasma gondii. J Appl Microbiol* **120:** 498-508.

419 Lam, C., He, L., and Marciano-Cabral, F. (2019) The effect of different environmental conditions on
420 the viability of *Naegleria fowleri* amoebae. *J Euk Microbiol* **66**: 752-756.

For Peer Revi

421 Lienard J., Croxatto A., Aeby S., Jaton K., Posfay-Barbe K., Gervaix A. et al. (2011) Development of a
422 new chlamydiales-specific real-time PCR and its application to respiratory clinical samples. *J Clin*
423 *Microbiol* 49:2637-2642.

424 Mariné Oliveira, G.F., do Couto, M.C., de Freitas Lima, M., and do Bomfim, T.C. (2016) Mussels (*Perna*
425 *perna*) as bioindicator of environmental contamination by *Cryptosporidium* species with zoonotic
426 potential. *Int J Parasitol: Parasites Wild.* **5:** 28-33.

427 Mezzanotte, V., Marazzi, F., Bissa, M., Pacchioni, S., Binelli, A., Parolini, M., *et al.* (2016) Removal of
428 enteric viruses and *Escherichia coli* from municipal treated effluent by zebra mussels. *Sci Total*
429 *Environ* **539**: 395-400.

430 Molloy, D.P., Mayer, D.A., Gaylo, M.J., Morse, J.T., Presti, K.T., Sawyko, P.M., *et al.* (2013)
431 *Pseudomonas fluorescens* strain CL145A - a biopesticide for the control of zebra and quagga mussels
432 (*Bivalvia: Dreissenidae*). *J Inv Pathol* **113:** 104-114.

433 Moreno-Mesonero L., Hortelano I., Moreno Y., Ferrús M.A. (2020) Evidence of viable Helicobacter
434 pylori and other bacteria of public health interest associated with free-living amoebae in lettuce
435 samples by next generation sequencing and other molecular techniques.
436 *Int J Food Microbiol* **318**:108477.

437 Mosteo, R., Goñi, P., Miguel, N., Abadías, J., Valero, P., and Ormad, M.P. (2016) Bioaccumulation of
438 pathogenic bacteria and amoeba by zebra mussels and their presence in watercourses. *Environ Sci*
439 *Poll Res* **23:** 1833-1840.

440 O'Neill, C.R. Jr. (1996) The Zebra Mussel. Impacts and control. Cornell Cooperative Extension,
441 Information Bulletin, 238; New York Sea Grant. Cornell University. State University of New York**.**

442 Page, F.C. (1988) A new key to freshwater and soil *Gymnamoebae* with instruction for culture.
443 *Freshwater Biological Association,* Ambleside**,**

For Peer Review Only

444 Pagnier, I., Valles, C., Raoult, D., and La Scola, B. (2015) Isolation of *Vermamoeba vermiformis* and
445 associated bacteria in hospital water. *Microb Path* **80**: 14-20.

446 Palos Ladeiro, M., Aubert, D., Villena, I., Geffard, A., and Bigot, A. (2014) Bioaccumulation of human
447 waterborne protozoa by zebra mussel (*Dreissena polymorpha*): interest for water biomonitoring.
448 *Water Res* **48**: 148-155.

449 Parolini, M., and Binelli, A. (2014) Temporal trends of polycyclic aromatic hydrocarbons (PAHs) in
450 *Dreissena polymorpha* specimens from Lake Maggiore (Northern Italy). *Environ Sci Poll Res* **21:** 7006-
451 7023.

452 Pèlandakis, M., and Pernin, P. (2002) Use of multiplex PCR and PCR restriction enzyme analysis for
453 detection and exploration of the variability in the free-living amoeba *Naegleria* in the environment.
454 *App Environ Microbiol* **68:** 2061-2065.

455 Poma, G., Binelli, A., Volta, P., Roscioli, C., and Guzzella, L. (2014) Evaluation of spatial distribution
456 and accumulation of novel brominated flame retardants, HBCD and PBDEs in an Italian subalpine lake
457 using zebra mussel (*Dreissena polymorpha*). *Environ Sci Poll Res* **21:** 9655-9664.

458 Roch, N., and Maurin, M. (2005) Antibiotic susceptibilities of *Legionellla pneumophila* strain Paris in
459 THP-1 cells as determined by real-time PCR assay. *J Antimicrob Chemother* **55:** 866-871.

460 Rodríguez-Zaragoza, S. (1994) Ecology of free-living amoebae. *Crit Rev Microbiol* **20:** 225-41.

461 Schroeder, J.M., Booton, G.C., Hay, J., Niszl, I.A., Seal, D.V., Markus, M.B., *et al.* (2001) Use of
462 subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of
463 *Acanthamoebae* from humans with keratitis and from sewage sludge. *J Clin Microbiol* **39***:* 1903-1911.

464 Selegean, J.P., Kusserow, R., Patel, R., Heidtke, T.M., and Ram, J.L. (2001) Using zebra mussels to
465 monitor *Escherichia coli* in environmental waters. *J Environ Qual* **30:** 171-179.

For Peer Review Only

466 Sente, C., Erume, J., Naigaga, I., Mulindwa, J., Ochwo, S., Magambo, P.K., *et al.* (2016) Prevalence of
467 pathogenic free-living amoeba and other protozoa in natural and communal piped tap water from
468 Queen Elizabeth protected area, Uganda. *Infect Dis Pov* **5**: 68.

469 Sharma, R., Jhanji, V., Satpathy, G., Sharna, N, Khokhar, S., and Agarwar, T. (2013) Coinfection with
470 *Acanthamoeba* and *Pseudomonas* in contact lens-associated keratitis. *Opt Vis Sci* **90:** e53-5.

471 Silverman, H., Achberger, E.C., Lynn, J.W., and Dietz, T.H. (1995) Filtration and utilization of
472 laboratory-cultured bacteria by *Dreissena polymorpha, Corbicula fluminea,* and *Carunculina*
473 *texasensis. Biol Bull* **189:** 308-319.

474 Smirnov, A., and Goodkov, A. (1999) An illustrated list of basis morphotypes of *Gymnamoebia*
475 (Rhizopoda, Lobosea). *Protistol* **1**: 20-29.

476 Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M. *et al.* (2003)
477 Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia*
478 *doudenalis*. *Emerg Infect Dis* **9**:1444-1452.

479 Thomas, V., McDonnell, G., Denyer, S.P., and Maillard, J.Y. (2010) Free-living amoebae and their
480 intracellular pathogenic microorganisms: risks for water quality. *FEMS Microbiol Rev* **34**: 231-259.

481 Tsvetkova, N., Schild, M., Panaiotov, S., Kurdova-Mintcheva, R., Gottstein, B., Walochnik, J., et al.
482 (2004) The identification of free-living environmental isolates of amoebae from Bulgaria. *Parasitol*
483 *Res* **92:** 405-413.

484 UNE-EN ISO 19250:2010 Standard. Water quality. Detection of *Salmonella* spp.

485 UNE-EN ISO 26461-2:1986 Standard. Water quality. Detection and count of spores of anaerobic
486 sulfite reducing microorganisms (clostridia). Part 2: membrane filtration method.

487 UNE-EN ISO 7899-2:2000 Standard. Water quality. Detection and count of intestinal enterococci. Part
488 2: membrane filtration method.

For Peer Review Only

489 UNE-EN ISO 9308-1:2000. Water Quality. Detection and count of *Escherichia coli* and coliform
490 bacteria. Part 1: membrane filtration method.

491 Vathanodorn, K., and Parsons, R.H. (1996) Bacteria as a direct foot source of zebra mussels. Section
492 III: 19pp. In W.C. Nieder and J.R. Waldman (eds.) Final Reports of the tibor T. Polgar Fellowship
493 Program, Hudson River Foundation, New York.

494 Woyke T., and Schulz F. (2019). Entities inside one another - a matryoshka doll in biology?. Environ
495 Microbiol Rep **11:** 26–28

496 Xiao, L., Escalane, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., *et al.* (1999) Phylogenetic
497 analysis of C*ryptosporidium* parasites base on the small-subunit rRNA gene locus. *Appl Environ*
498 *Microbiol* **65**: 1578-1583.

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502 **Figure 1.** Sampling Points

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506 **Figure 2**.- Microscope visualization of the extract obtained from dead mussels: 1-5
507 *Cercomonadida*. Picture 6- *Cercomonadida* and *Naegleria* spp. Flagellate. Picture 7- *Naegleria*
508 spp. cyst. Picture8- *Naegleria* spp. trophozoite. Magnifications: Pictures 4 and 7, 100 X; Pictures
509 1-2: 400 X; Pictures 5, 6 and 8: 1000 X and Picture 3, 5: 2000 X. The pictures 2.1 to 2.5
510 correspond to images of *Cercomonadida*. In picture 2.6 *Naegleria* spp. appears together with
511 *Cercomonadida* in its characteristic flagellate development stage, the main difference between
512 them is the position of the base of the flagella, separated in *Cercomonadida*. The images 2.7 and
513 2.8 show *Naegleria* spp. cysts and a trophozoite, respectively.

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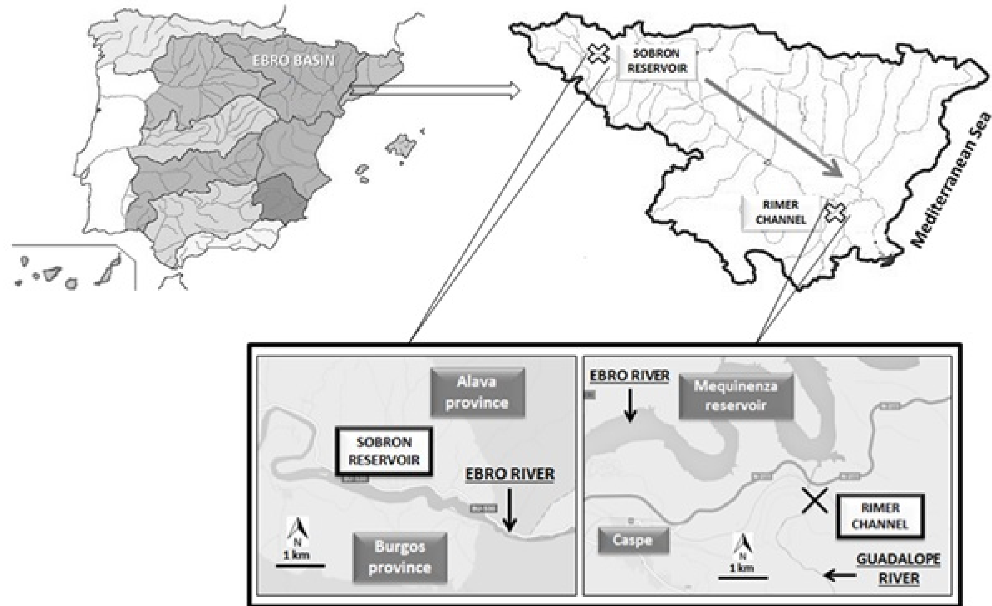

Figure 1. Sampling Points

47x30mm (323 x 323 DPI)

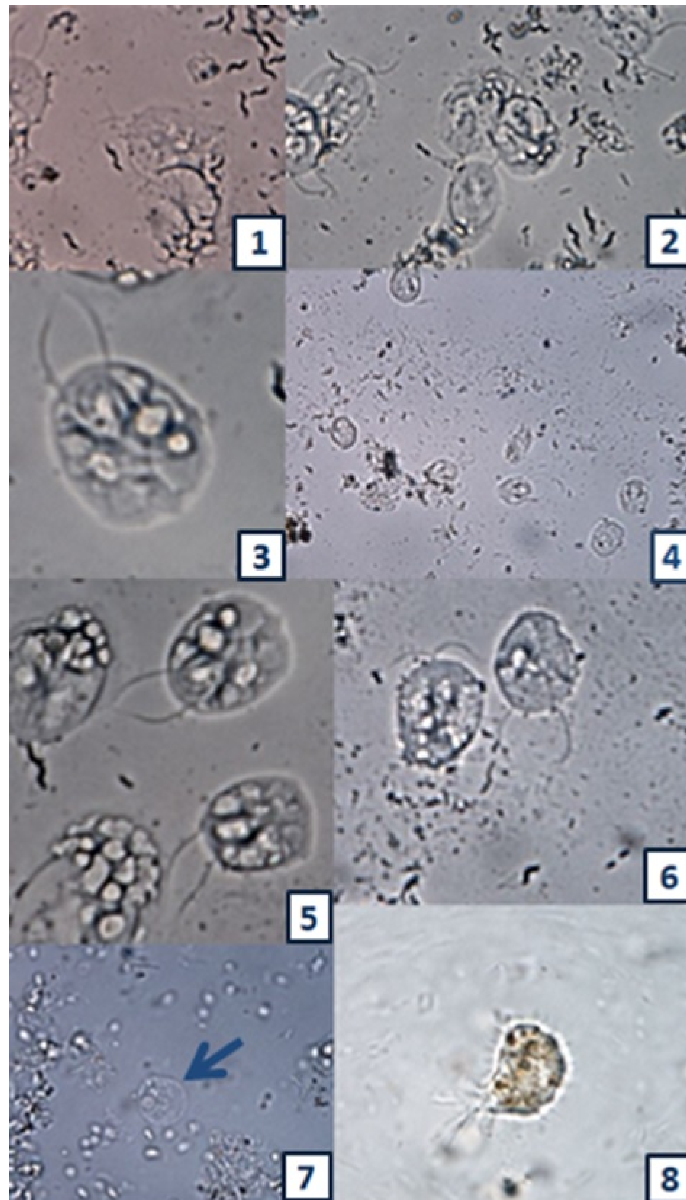

Figure 2.- Microscope visualization of the extract obtained from dead mussels: 1-5 Cercomonadida. Picture 6- Cercomonadida and Naegleria spp. Flagellate. Picture 7- Naegleria spp. cyst. Picture8- Naegleria spp. trophozoite. Magnifications: Pictures 4 and 7, 100 X; Pictures 1-2: 400 X; Pictures 5, 6 and 8: 1000 X and Picture 3, 5: 2000 X. The pictures 2.1 to 2.5 correspond to images of Cercomonadida. In picture 2.6 Naegleria spp. appears together with Cercomonadida in its characteristic flagellate development stage, the main difference between them is the position of the base of the flagella, separated in Cercomonadida. The images 2.7 and 2.8 show Naegleria spp. cysts and a trophozoite respectively.

28x49mm (421 x 421 DPI)

Table 1. Free living amoeba and flagellate identification.

Sample point	Sample (nº of isolates)	Identified FLA species
Sobrón reservoir	Natural water (1)	*Acanthamoeba* spp.
	Zebra mussel extract (1)	*Naegleria clarki*
	Dead mussels extract (3)	*Naegleria clarki*. *Vexillifera bacillipedes* *Cercomonadida*
Rimer irrigation channel	Natural water (2)	*Naegleria* spp. Not identified FLA
	Zebra mussel extract (2)	*Vexillifera bacillipedes* *Protacanthamoeba bohemica*

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Table 2. Endosymbiont bacteria identified in FLA and in water.

	ARB from FLA analysis			Bacteria from direct analysis	
Sample point	**Sample**	**FLA**	**Amoeba Resistant Bacteria (ARB)**	**In Zebra mussel extract**	**In Natural water**
Sobrón reservoir	Natural water	*Acanthamoeba pustulosa*	*Legionella* spp.	-	*Mycobacterium* spp.
	Dead mussel	*Naegleria clarki*	*Pseudomonas* spp. *Legionella* spp.		
		Vexillifera bacillipedes	*Pseudomonas* spp. *Legionella* spp		
Rimer irrigation channel	Zebra mussel extract	*Protacanthamoeba bohemica*	*Pseudomonas* spp. *Legionella* spp	*Mycobacterium* spp. *Legionella* spp.	-

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Table 3. Bacterial analysis of natural water and Zebra mussel extracts.

Bacteria	Rimer irrigation channel			Sobrón reservoir		
	Natural water ($CFU\ 100mL^{-1}$) N=3	Zebra mussel ($CFU\ g^{-1}$ mussel) N=3	Index	Natural water ($CFU\ 100mL^{-1}$) N=3	Zebra mussel ($CFU\ g^{-1}$ mussel) N=3	Index
C. perfringens	$5.6 \pm 1.4 \cdot 10^2$	$6.4 \pm 1.5 \cdot 10^2$	114.3	$1.9 \pm 0.0 \cdot 10^4$	$2.6 \pm 0.0 \cdot 10^4$	136.8
E. coli	$5.9 \pm 1.2 \cdot 10^3$	$2.3 \pm 0.0 \cdot 10^2$	3.9	$2.3 \pm 0.1 \cdot 10^4$	$8.3 \pm 2.3 \cdot 10^2$	3.6
Salmonella spp.	88 ± 12	$4.0 \pm 0.9 \cdot 10^2$	454.5	$2.9 \pm 0.1 \cdot 10^4$	$5.1 \pm 0.3 \cdot 10^4$	175.9
Enterococcus spp.	34 ± 5	70 ± 42	205.9	$9.0 \pm 1.4 \cdot 10^2$	$2.3 \pm 0.0 \cdot 10^2$	25.5

Data shown are mean ± standard deviation; N: times that each analysis has been repeated

The index was defined as the resulting value of dividing $CFU \cdot g\ mussel^{-1} / CFU \cdot g\ water^{-1}$, considering the water density as 1 gram per mL

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Table 4. Protocols of the PCRs used in the study

Specificity	Gene	Primers	Sequence 5'→3'	Size (pb)	Reference
Acanthamoeba	ASA.S1 (DF3)	JDP 1	GCCCAGATCGTTTACCGTGAA	500	Schroeder et al., 2001 [32]
		JDP 2	TCTCACAAGCTCTAGGGAGTCA		
Hartmannella v *Naegleria fowleri* *Vanella spp.* *Vahlkampfia ovis* *A. castellanii* *A. poluphaga* *A. lenticulaa* *A. hatchetti* *A. comandonni* *A. astronyxis*	*18S rDNA*	FLA F	CGCGGTAATTCCAGCTCCAATAGC	800-1500	Tsvekova et al. 2004[33]
		FLA R	CAGGTTAAGGTCTCGTTCGTTAAC		
Naegleria spp	*ITS*	ITS 1	GAACCTGCGTAGGGATCATTT	400-453	Pelandakis et al. 2002 [34]
		ITS 2	TTTCTTTTCCTCCCCTTATTA		
Naegleria fowleri	*5.8S rRNA*	Fw 1	GTGAAAACCTTTTTTCCATTTACA		
		Fw 2	AAATAAAAGATTGACCATTTGAAA		
Eukariota	*18S rDNA*	EUK F	AACCTGGTTGATCCTGCCAGTAGTCAT	Variable	Hirt et al., 1997 [35]
		EUK R	GCTTGATCCTTCTGCAGGTTCACCTAC		
Cryptosporidium spp.	*SSU rRNA*	SSU 1	TTCTAGAGTAATACATGCG	1325	Xiao et al., 1999 [36]
		SSU 2	CCCTAATCCTTCGAAACAGGA		
		SSU 3	GGAAGGGTTGTATTTATTAGATAAAG	826-864	
		SSU 4	AAGGAGTAAGGAACAACCTCCA		
Giarda duodenalis	*tpi*	AL3543	AAATTATGCCTGCTCGTCG	605	Sulaiman et al., 2003 [37]
		AL3546	CAAACCTTTTCCGCAAACC		
		AL3544	CCCTTCATCGGTGGTAACTT	530	
		AL3545	GTGGCCACCACTCCCGTGCC		
Legionella pneumophila	*mip*	Mip-Leg-F 186	GCATTGGTGCCGATTTGG	186	Roch and Maurin, 2005 [38]
		Mip-Leg-R 186	GTTTTGCATCAAATCTTTCTG		
		N-Mip-Leg-F 186	GAAGCAATGGCTAAAGGCATGCAA	112	Calvo L. et al., 2013 [23]
		SN-Mip-Leg-R186	GCTTTGCCATCAAATCTTCTGAAACTTG		
Toxic Cyanobacteria	*mcyD*	McyD-dir	GAGCATTAAGGGCTAAATCG	282	Calvo L. et al., 2013 [23]
		McyD-rev	CTTGGTGCTTCATCAACTC		
		N-McyD-dir	TCATAGCCCCATATCCTTTAGCGGC	194	
		N-McyD-rev	CTGCTGTATCTTTAATTGGCTCGGC		
Mycobacterium spp.	*hsp65*	Hsp65-dir	CCCGTACGAGAAGATCGG	354	Calvo L. et al., 2013 [23]
		Hsp65-rev	GACTCCTCGACGGTGATG		
		N-Hsp65-dir	GAGCTGGTCAAGGAAGTCGCC	300	
		N-Hsp65-rev	GTTGCCGACCTTGTCCATCGA		
Pseudomonas spp.	*16S rDNA*	R16S-dir	GGTCTGAGAGGATGATCAGT	962	Calvo L. et al., 2013 [23]
		R16S-rev	TCTGTACCGACCATTGTAGC		
		N-R16S-dir	GACGTTACCGACAGAATAAGCACCG	476	
		N-R16S-rev	ACCCACATGCTCCACCGCTTGTG		

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