Document downloaded from:

http://hdl.handle.net/10251/101908

This paper must be cited as:

The final publication is available at

https://doi.org/10.1111/1462-2920.13856

Copyright Blackwell Publishing

Additional Information

1 Detection of viable *Helicobacter pylori* inside free-living amoebae in wastewater and

2 drinking water samples from Eastern Spain

3 Moreno-Mesonero, Laura[1*]; Moreno, Yolanda[1*]; Alonso José Luis [1], Ferrús, Mª Antonia[2]

4

5 [1]Research Institute of Water and Environmental Ingeneering (IIAMA), Universitat

6 Politècnica de València, 46022 Valencia, Spain

7 [2]Biotechnology Department, Universitat Politècnica de València, 46022 Valencia, Spain

8 *These authors contributed equally to this work

9

10 For correspondence: Yolanda Moreno. E-mail: ymoren@upv.es ; Tel (+34) 963879797;

11 Research Institute of Water and Environmental Engineering (IIAMA), Universitat

12 Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain.

13

14 **Running title:** Detection of viable *H. pylori* inside aquatic FLA

15 **Summary**

16 *Helicobacter pylori* is one of the most concerning emerging waterborne pathogens. It
17 has been suggested that it could survive in water inside free-living amoebae (FLA), but
18 nobody has studied this relationship in the environment yet. Thus, we aimed to detect
19 viable *H. pylori* cells from inside FLA in water samples.

20 Sixty-nine wastewater and 31 drinking water samples were collected. FLA were
21 purified and identified by PCR and sequencing. For exclusively detecting *H. pylori* inside
22 FLA, samples were exposed to sodium hypochlorite and assayed by specific PMA-qPCR,
23 DVC-FISH and culture.

24 FLA were detected in 38.7% of drinking water and 79.7% of wastewater samples, even
25 after disinfection. In wastewater, *Acanthamoeba* spp. and members of the family
26 *Vahlkampfiidae* were identified. In drinking water, *Acanthamoeba* spp. and
27 *Echinamoeba* and/or *Vermamoeba* were present.

28 In 39 (58.2%) FLA-positive samples, *H. pylori* was detected by PMA-qPCR. After DVC-
29 FISH, 21 (31.3%) samples harboured viable *H. pylori* internalized cells. *H. pylori* was
30 cultured from 10 wastewater samples.

31 To our knowledge, this is the first report that demonstrates that *H. pylori* can survive
32 inside FLA in drinking water and wastewater, strongly supporting the hypothesis that
33 FLA could play an important role in the transmission of *H. pylori* to humans.

34

35 **1. Introduction**

36 Among all the emerging waterborne pathogens, *Helicobacter pylori* is one of the most
37 concerning ones, as it causes chronic bacterial infection in humans, closely related to
38 peptic ulcer and gastric cancer, which is the third worldwide leading cause of death by
39 cancer. Human infection by *H. pylori* is considered to be a great Public Health hazard,
40 with an estimated 50% of the world's population colonized by this bacterium (Torres *et*
41 *al.,* 2000). Of those, around 10% will develop peptic ulcer, approximately 1% gastric
42 carcinoma and less than 0.1% MALT (mucosa-associated lymphoid tissue) lymphoma
43 (Leja *et al.*, 2016). In fact, *H. pylori* is the only bacterium included by the WHO
44 International Agency for Research on Cancer (IARC) as a Class I human carcinogen
45 (IARC Working group).

46 However, the real risk of its presence in water is not yet well established, which
47 prevents the implementation of preventive policies. *Helicobacter pylori* is present in
48 human faeces (Queralt, *et al.,* 2005), which supports the possibility of its transmission
49 via the fecal-oral route. There is also epidemiological evidence associating human
50 infection and even the appearance of gastric cancer with contaminated drinking water
51 in developing countries (Bunn *et al*., 2002). Furthermore, the organism has been
52 detected in different aquatic environments, including rivers, wastewater, irrigation
53 water and drinking water systems (Fujimura, *et al.,* 2004; Moreno and Ferrús, 2012;
54 Tirodimos *et al.,* 2014; Santiago *et al.,* 2015; Ranjbar *et al.,* 2016). However, its
55 transmission route remains unclear, because isolation of the bacteria is unlikely.
56 Nowadays, there are an increasing number of studies that confirm the presence of *H.*
57 *pylori* in water, suggesting that this microorganism is waterborne. However, only a few

58 of them have demonstrated its presence in a viable and cultivable state (Al-Sulami *et*
59 *al.*, 2012; Moreno and Ferrús, 2012; Tirodimos *et al.,* 2014; Santiago *et al.,* 2015;
60 Ranjbar *et al.,* 2016).

61 When environmental conditions are unfavorable for *H. pylori*, the bacteria enter the
62 viable but non-culturable (VBNC) state, in which changes in their metabolism and
63 morphology are observed (Nilsson *et al.,* 2002) although they maintain their infective
64 potential (Piqueres *et al.,* 2006). Moreover, under the VBNC state, bacteria lose their
65 ability to grow on synthetic culture media, which could lead to the underestimation of
66 its presence when samples are analyzed by classical culture methods. This is why
67 molecular techniques such as quantitative polymerase chain reaction (qPCR) or
68 fluorescent *in situ* hybridization (FISH) are essential.

69 Some authors have also suggested that *H. pylori* could survive adverse environmental
70 conditions by being attached to biofilms or associated to free-living amoebae (FLA)
71 (Winiecka-Krusnell *et al.,* 2002; Watson *et al.,* 2004; Percival *et al.,* 2009). FLA, such as
72 *Acanthamoeba*, *Naegleria*, *Vermamoeba* or *Balamuthia*, are ubiquitous protozoa
73 commonly found in water (Sheehan *et al.,* 2003; Magnet *et al.,* 2012; Retana-Moreira
74 *et al.,* 2014; Niyyati *et al.,* 2015; Sente *et al.,* 2016). They have two life cycle stages: the
75 trophozoite, a metabolic active form, and the cyst, a resting form. It has been
76 previously shown that FLA can act as hosts for some bacterial pathogens which are
77 able to resist amoebal digestion (amoeba resisting bacteria, ARB). Different pathogens
78 such as *Legionella*, *Mycobacterium*, *Aeromonas*, *Listeria*, *Arcobacter* or *Campylobacter*
79 have been isolated from inside FLA in co-culture assays (Steinert *et al.,* 1998; García *et*
80 *al.,* 2007; Rahman *et al.,* 2008; Snelling *et al.,* 2008; Akya *et al.,* 2009; Bui *et al.,* 2012;

81 Villanueva *et al.,* 2016). Inside them, these bacteria survive and are more resistant to
82 harsh environmental conditions that would normally kill them, such as chlorination or
83 presence of biocides (Thomas *et al*., 2010; Dupuy *et al.,* 2014). Therefore, FLA act as
84 “Trojan horses” for these ARB (Barker and Brown, 1994), thus allowing bacterial
85 survival and/or transmission to susceptible hosts (Siddiqui and Khan, 2012).

86 Recently, an *in vitro* study has demonstrated that *H. pylori* is able to survive inside
87 *Acanthamoeba castellanii* after resisting a chlorination disinfection treatment
88 (Moreno-Mesonero *et al.,* 2016). Viable *H. pylori* cells from inside *A. castellanii* were
89 successfully detected by the molecular technique DVC-FISH. This technique primarily
90 consists of an incubation of samples in the presence of Novobiocin antibiotic, which is
91 an inhibitor of DNA gyrase that leads to elongated and swollen cells. Cells are then
92 observed under an epifluorescence microscope, and viable and non-viable cells can be
93 discriminated by differences in size (Piqueres *et al.,* 2006). Along with this study,
94 Winiecka-Krusnell *et al.,* 2002 and Smith and Ashbolt, 2012 also suggested that FLA
95 provide shelter for *H. pylori*, enabling it to survive under different treatments and
96 making its transmission to humans possible. However, this relationship has not
97 previously been studied in environmental samples.

98 Some FLA are opportunistic pathogens for humans with *Acanthamoeba, Naegleria*,
99 *Balamuthia* and *Sappinia* genera containing species of FLA known to cause disease in
100 humans (Gelman *et al.,* 2001; Visvesvara *et al.,* 2007; Qvarnstrom *et al.,* 2009; Thomas
101 *et al*., 2010). The aim of this study was to investigate the presence of viable *H. pylori*
102 cells from inside FLA in wastewater and drinking water samples originating in Spain

103 and to isolate and identify FLA from the same samples. Results of this work support
104 that *H. pylori* belongs to the ARB group, surviving inside FLA in water samples.

105

106 **2. Results**

107 Chlorine measurement indicated that the free residual chlorine in drinking water
108 samples ranged between 0.50 and 0.90 mg/l, which are in the range of potable
109 drinking water according to the World Health Organization (WHO).

110 A total of 67 (67.0%) of the analyzed samples were positive for the presence of FLA
111 after up to one month of incubation period. Wastewater samples yielded 55 (79.7% of
112 wastewater samples) FLA positive cultures. Among these, 65.2% of the samples were
113 taken after secondary biological treatment and 34.8% of the samples after applying
114 disinfection treatment. Drinking water samples yielded 12 (38.7% of water samples)
115 FLA positive cultures (Table 1).

116 A total of 39 FLA were purified using a micromanipulator, 31 from wastewater and 8
117 from drinking water samples. By means of multiplex PCR, 23 of the FLA cultures were
118 identified (Table 2) (Figure 2). In wastewater samples, several bands appeared in some
119 amplification products. In all of these type of samples, *Acanthamoeba* spp. and
120 members of the family Vahlkampfiidae (i.e. *Naegleria* and *Vahlkampfia*) were
121 identified. There was one wastewater sample which could not be identified because it
122 showed no amplification. In another sample, an unidentified fragment of 100 bp was
123 also detected. In drinking water samples, *Acanthamoeba* spp. and *Echinamoeba*
124 and/or *Vermamoeba* were identified. By means of conventional PCR followed by

125 sequencing, 14 of the purified FLA were identified (GenBank accession numbers
126 MF399028 - MF399037). In wastewater samples, *Acanthamoeba* spp., *A. castellanii*, *A.*
127 *tubiashi*, *A. polyphaga*, *Naegleria* spp., *Vannellidae* spp. and *Cercozoa* spp. were
128 identified. In drinking water samples, *Acanthamoeba* spp and *A. mauritaniensis* were
129 the identified FLA (Table 2). Morphologies of the isolated FLA were observed under the
130 inverted microscope (Figure 3).

131 Overall, in 39 (58.2%) samples out of the 67 FLA-positive ones it was possible to detect
132 *H. pylori* DNA by means of PMA-qPCR, which indicated that it was harboured within
133 FLA. In wastewater, 28 (50.9%) samples out of the 55 FLA-positive ones, including
134 samples both after secondary and tertiary treatment, contained *H. pylori* inside them
135 at quantifiable concentrations from $3.41 \cdot 10^1$ to $2.77 \cdot 10^3$ genomic units per sample.
136 In the case of drinking water samples, 11 (91.7%) out of the FLA-positive ones had
137 internalized *H. pylori* (Table 3) at quantifiable concentrations from $1.48 \cdot 10^1$ to $1.36 \cdot$
138 10^4 genomic units per sample. In both types of water, some samples were positive but
139 out of the range of quantification.

140 When DVC-FISH was applied, a total of 21 (31.3%) samples out of the 67 FLA-positive
141 ones were positive for viable (elongated and/or swollen) *H. pylori* internalized cells
142 (Figure 4). In the case of wastewater, 16 (29.1%) samples out of the 55 FLA-positive
143 ones had viable *H. pylori*. In drinking water, 5 (41.7%) samples out of the 12 FLA-
144 positive ones contained *H. pylori* in its viable state. (Table 3).

145 By means of cultivation of an aliquot of the hypochlorite treated samples, *H. pylori* was
146 only recovered from wastewater samples. Out of the 55 wastewater FLA-positive
147 samples, growing of *H. pylori* was achieved in 10 (18.2%) of them (Table 3). All

148 presumptive *H. pylori* colonies from drinking water samples yielded negative results
149 after qPCR identification.

150 In positive *H. pylori* qPCR and/or DVC-FISH wastewater samples, both *Acanthamoeba*
151 genera and members of the family Vahlkampfiidae were identified by means of the
152 multiplex PCR or the 18S PCR plus sequencing. Equally, both *Acanthamoeba* and
153 *Vermamoeba* and/or *Echinamoeba* genera were identified by the previously cited PCRs
154 in *H. pylori* positive drinking water samples.

155 **3. Discussion**

156 It has been suggested that *H. pylori* could survive adverse environmental conditions by
157 association with FLA (Winiecka-Krusnell *et al.,* 2002; Watson *et al.,* 2004; Percival *et al.,*
158 2009). Taking into account the increasing evidence for the role of water in *H. pylori*
159 transmission and the difficulties in culturing the bacterium from these samples, this
160 becomes a question of great public health concern which could explain how *H. pylori* is
161 able to survive in the environment and reach humans through consumption of
162 contaminated water or vegetables. Thus, our work aimed to detect the presence of
163 FLA in drinking water and wastewater intended for irrigation, and demonstrate the
164 presence of viable *H. pylori* cells inside them.

165 FLA were detected in a large number of samples, both drinking and wastewater (Table
166 1). Purified FLA identification was performed by multiplex PCR (Le Calvez *et al.,* 2012)
167 and by conventional PCR followed by sequencing (Thomas *et al.,* 2006) (Table 2). Some
168 species of the detected FLA are known to be opportunistic pathogens for humans. As
169 expected, *Acanthamoeba* species were the most frequently identified genera in both
170 types of analyzed samples. In Spain, Magnet *et al.,* 2012 identified *Acanthamoeba* by

171 qPCR in 87.5% of the environmental samples studied, which included wastewater,
172 drinking water and locations of influence on river basins. Moreover, Magnet *et al.,*
173 2013 carried out a more extensive study in the same type of samples and identified
174 *Acanthamoeba* by qPCR in 94.6% of these samples. In our study, members of the
175 *Vahlkampfiidae* family, which includes the genus *Naegleria* among others, were
176 present in a significant amount of wastewater samples. In drinking water samples,
177 *Echinamoeba* and/or *Vermamoeba* were also identified by means of multiplex PCR in
178 this work. *V. vermiformis* had been previously identified in wastewater (García *et al.,*
179 2011), in recreational water (Reyes Batlle *et al.,* 2016a) and even in soil (Reyes-Batlle *et*
180 *al.,* 2016b). Thomas *et al.,* 2008 also identified *V. vermiformis* and *Echinamoeba*
181 *exudans* in river water and at various stages of a drinking water plant fed with this river
182 water in France.

183 In order to exclusively detect *H. pylori* which had been internalized by FLA and survived
184 inside them, a two-step protocol was applied to FLA-positive samples (Moreno-
185 Mesonero *et al*., 2016). First, sodium hypochlorite treatment was used to kill external
186 cells and then a PMA treatment was applied to remove exogenous DNA and DNA from
187 non-internalized *H. pylori* dead cells, allowing for the exclusive detection of *H. pylori*
188 DNA from the inside of FLA. Other authors, such as García *et al*., 2013, used a washing
189 step with an acidified saline serum (pH 2) prior to DNA extraction for this aim.
190 However, this approach is not useful for ARB resistant to acid conditions like *H. pylori*.
191 In another study carried out by Thomas *et al.,* 2008, several steps of subcultivation of
192 positive FLA water samples were used to get rid of exogenous bacteria; then FLA were
193 lysed and the content added to *A. castellanii* cultures to recover ARB. However, in this
194 case one cannot be sure that external bacteria are completely eliminated and that

195 exogenous DNA is not detected in subsequent PCRs. Therefore, some FLA-associated
196 bacteria could be overrated.

197 qPCR analysis of the treated samples showed the presence of intracellular *H. pylori*
198 DNA in 28 (50.9%) of wastewater samples and in 11 (91.7%) of drinking water samples.
199 The high percentage of internalized *H. pylori* cells in drinking water samples could be
200 due to the relatively low number of FLA-positive samples analyzed. Maybe, this could
201 also be due to the fact that when FLA are present in drinking water samples it is highly
202 likely that *H. pylori* could be found.

203 Quantification of each sample was only possible when the amplification occurred prior
204 to Cq 35.00. The non-quantifiable samples were considered positive, but we could not
205 provide a quantification value. Quantification values ranged between $3.41 \cdot 10^1$ to 2.77
206 $\cdot 10^3$ genomic units per sample in wastewater samples and between $1.48 \cdot 10^1$ to $1.36 \cdot$
207 10^4 genomic units per sample. Although *H. pylori* concentration in a drinking water
208 sample was greater than those obtained in wastewater, this quantification value was
209 within the range described by Santiago *et al.,* 2015 in drinking water samples. By
210 means of DVC-FISH analysis, viable elongated *H. pylori* cells were observed in 16
211 (29.1%) wastewater and in 5 (41.7%) drinking water samples. For both types of samples,
212 the percentage of positive DVC-FISH results was lower than the qPCR positive ones.
213 The differences in results obtained by the two molecular techniques were not
214 surprising, because qPCR is unable to distinguish between viable and non viable
215 internalized cells (Moreno-Mesonero *et al.,* 2016). Thus, those differences are likely
216 due to the presence of non-viable, dead cells inside some FLA, which are not able to

217 elongate under DVC conditions (metabolically inactive cells) but whose DNA is
218 amplified by qPCR.

219 To recover *H. pylori* by culture, FLA-positive treated samples were cultivated on Agar
220 Dent for 7 days, so that FLA could excrete pellets containing the pathogen (Marciano-
221 Cabral and Cabral, 2003; Moreno-Mesonero *et al.,* 2016). In an *in vitro* predation assay
222 of *Salmonella* and *A. polyphaga* it was checked that the amoeba excreted the bacteria
223 within 2-4 hours (Gaze *et al.,* 2003). However, the egestion period may depend on
224 environmental conditions, FLA species and bacterial species excreted. This is the
225 reason why cultures were maintained under *H. pylori* specific conditions up to 7 days,
226 so that they could be excreted and able to grow in the culture media.

227 The suspected colonies obtained from 10 (18.2%) wastewater samples were
228 unequivocally identified as *H. pylori* by qPCR. These 10 samples had also shown
229 positive results for *H. pylori* inside FLA by qPCR and/or viability by means of DVC-FISH.
230 The fact that *H. pylori* colonies were able to grow on a plate means that these samples
231 contained potentially infective *H. pylori*. In the work conducted by Moreno-Mesonero
232 *et al.,* 2016; in which a co-cultulture of *A. castellanii* and *H. pylori* was carried out, no
233 colonies of the bacteria were recovered by culture. The fact that we were able to
234 culture *H. pylori* in ten of the wastewater FLA-positive samples may indicate that other
235 environmental FLA different from *A. castellanii* were present in these samples or that
236 environmental FLA, including *A. castellanii*, could be suitably protecting *H. pylori* from
237 environmental conditions. We were not able to recover colonies by culture from some
238 wastewater FLA-positive samples and from any of the drinking water positive samples.
239 This could indicate, according to Moreno-Mesonero *et al.,* 2016, that the bacteria

240 could have acquired the VBNC state, since its viability after the hypochlorite treatment
241 was observed by DVC-FISH.

242 To our knowledge, this is the first report that demonstrates that *H. pylori* can survive
243 inside FLA in environmental water, including drinking water and wastewater, even
244 after disinfection treatment. It strongly supports the hypothesis that FLA could act as
245 Trojan Horses for this pathogen and could play an important role in the transmission of
246 *H. pylori* to humans through the consumption of water.

247 **4. Experimental Procedures**

248 **4.1. Samples**

249 A total of 100 samples (69 wastewater and 31 drinking water) were collected from
250 February 2013 to July 2016. Drinking water samples were collected from different
251 public fountains from Eastern Spain. Wastewater samples were obtained from two
252 wastewater treatment plants located in Valencia, Spain, at two different treatment
253 points: after applying secondary biological treatment and after UV or chlorine tertiary
254 disinfection treatment. All of these wastewater effluents are intended for irrigation
255 purposes.

256 All samples were placed into sterile bottles, refrigerated and processed within a few
257 hours. After the collection of each drinking water sample, the measurement of residual
258 free chlorine was performed using the Spectroquant® Nova 60 system (Merck,
259 Germany) and its specific chlorine test kit (Cl_2 range: 0.010-6.00 mg/l) (ref.
260 1.00599.0001, Merck, Germany).

261 Five litres of drinking water and 1 l of irrigation water samples were filtered through
262 nitrocellulose filters with 3 µm of pore size (Whatman, Maidstone, England).
263 Membranes were placed upside-down on Non-Nutrient-Agar (NNA, bacteriological
264 agar dissolved in Page's Amebic Saline (PAS) solution (Loveno *et al.,* 2010)) and
265 incubated at 28°C for 24 h. In the case of drinking water samples, NNA plates were
266 seeded with 100 µl of dead *Escherichia coli* (submitting a suspension of *E. coli* to 100°C
267 for 5 minutes) to serve as nutrients for the amoeba (Badirzadeh *et al.,* 2011).

268 After 24 h, filters were removed and plates were kept at 28°C. Then, NNA plates were
269 monitored daily up to 30 days for the presence of FLA by inverted and phase contrast
270 microscopy.

271 When FLA growth was observed, PAS was added to NNA plates in order to collect their
272 content using a sterile cell scraper. Content was concentrated in 500 µl of PAS solution
273 by centrifugation at 500 g for 3 min. Then, bacteria outside FLA were killed using
274 sodium hypochlorite for 1 hour at a final concentration of 104 ppm as previously
275 described (Moreno-Mesonero *et al.,* 2016). Sodium hypochlorite was removed from
276 samples by washing them three times (500 g for 3 min) and finally resuspended in 1 ml
277 of PBS 1X. After this treatment, 1 ml aliquots were analyzed by means of PMA-qPCR,
278 DVC-FISH and culture (Figure 1).

279 **4.2. Identification of *H. pylori* inside FLA.**

280 **4.2.1. PMA-qPCR**

281 In order to detect only bacterial DNA from inside FLA, 500 µl of sodium hypochlorite
282 treated samples were exposed to propidium monoazide (PMA) to avoid amplification

283 signals from external *H. pylori* dead cells DNA (Moreno-Mesonero *et al.,* 2016). Briefly,
284 PMA (GenIUL, Spain) was dissolved in 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich®,
285 St. Louis, MO, USA) to create a stock concentration of 2 mM. 12.5 µl of PMA were
286 added to the samples, to reach a final concentration of 50 µM. Then, samples were
287 incubated for 10 min under darkness with occasional mixing to allow better reagent
288 penetration. Then, samples were exposed to blue LED light for 15 min at the photo
289 activation system PhAST Blue (GenIUL, Spain). Afterwards, samples were centrifuged
290 at 14000 rpm for 5 min and resuspended in 200 µl of PBS 1X (Agustí *et al.,* 2010).

291 After PMA treatment, DNA was extracted from samples by using the GeneJet™
292 genomic DNA purification kit (ThermoScientific, Germany) following the mammalian
293 tissue protocol instructions, with the exception of the incubation time at 56°C, which
294 was increased from 10 min to 30 min (Moreno-Mesonero *et al.,* 2016).

295 Thereafter, specific *H. pylori* qPCR based on SYBR®Green I fluorescence dye was
296 performed using VacA primers to amplify a 372 bp fragment (Nilsson *et al.,* 2002) in
297 LightCycler® 2.0 Instrument (Roche Applied Science, Spain). The final reaction volume
298 of 20 µl contained: 2 µl of LightCycler® FastStart DNA Master SYBR Green I (Roche
299 Applied Science, Spain), 1.6 µl of $MgCl_2$ (25 mM stock solution), 0.5 µl of each primer
300 (20 µM stock solution) and 2 µl of DNA template. The amplification consisted of an
301 initial DNA denaturalization step at 95°C for 10 min, followed by 40 cycles of: 95°C for
302 10 s, 62°C for 5 s and 72°C for 16 s; and finally, one cycle at 72°C for 15 s and one at
303 40°C for 30 s (Piqueres *et al.,* 2006). A positive control with *H. pylori* DNA and a control
304 of external contamination (qPCR mix without DNA) were added to the qPCR analysis.
305 The quantification cycle (Cq) value along with a standard curve were used to calculate

306 the quantification of the number of DNA copies (genomic units, GU) of *H. pylori,* as
307 previously described by Santiago *et al.,* 2015. PCR products were checked in 1%
308 agarose gel electrophoresis prepared with 0.001% of GelRed™ (Biotium, USA)
309 visualized under ultraviolet light.

310 **4.2.2. DVC-FISH**

311 DVC-FISH analysis was performed according to Piqueres *et al.,* 2006. Briefly, 400 µl of
312 sodium hypochlorite treated samples were incubated in 3.6 ml of DVC broth (BBL™
313 Brucella broth supplemented with 5% fetal bovine serum (FBS) and 0.5 mg/l of
314 Novobiocin antibiotic) for 24 hours at 37°C under *H. pylori* specific microaerophilic
315 conditions (5% O_2, 10% CO_2 and 85% N_2). After incubation, DVC tubes were centrifuged
316 at 8000 rpm for 8 min and resuspended in 1 ml of PBS 1X.

317 Afterwards, samples were fixed in 4% paraformaldehyde for 3 h at 4°C and washed
318 with PBS 1X. Then, samples were hybridized on hybridization slides. Once samples
319 were deposited in each well and allowed to dry, slides were dehydrated by successive
320 immersions in 50%, 80% and 100% ethanol for 3 min each. Thereafter, each well was
321 covered with 10 µl of hybridization buffer (0.9 M NaCl, 20 mM HCl-Tris, 0.01% SDS and
322 40% formamide, pH 7.5) containing 50 ng of each probe. The reaction was carried out
323 at 46°C for 1.5 h (Moreno *et al.,* 2003). A combination of three EUB338 probes,
324 complementary to a region of the Eubacteria domain 16S rRNA was used as a positive
325 control. For the specific detection of *H. pylori*, a previously designed probe (Moreno *et*
326 *al.,* 2003) with LNA modifications to increase its specificity (Piqueres *et al.,* 2006) was
327 used: LNA-HPY: 5’- CTG GAG AGA C+ TA AGC CC+ T CC-3’.

328 Subsequently, slides were washed under darkness at 48°C for 15 min in 50 ml of
329 washing solution (0.10 M NaCl, 0.02 M HCl-Tris, 0.01% SDS and 0.005 M EDTA). Finally,
330 they were washed with distilled water and air-dried under darkness. Slides were
331 mounted with FluoroGuard Antifade Reagent (Bio-Rad, Spain) between the coverslip
332 and the slide. They were visualized using an Olympus BX 50 fluorescence microscope
333 with the filters U-MWB, U-MWIB and U-MWIG. Photographs were taken with an
334 Olympus DP-12 camera. A pure culture of DVC-incubated *H. pylori* cells was used as a
335 positive control of the reaction.

336 **4.2.3. Culture**

337 The presence of living culturable *H. pylori* cells inside the amoeba was investigated by
338 spreading aliquots of 100 µl of the sodium hypochlorite treated samples on Agar Dent
339 plates (ADent: Campylobacter selective agar (Merck, Spain) containing 10% (v/v)
340 defibrinated horse blood (Oxoid, United Kingdom) and *Helicobacter pylori* selective
341 supplement Dent (Oxoid, United Kingdom)). Samples were incubated for 5-7 days at
342 37°C under *H. pylori* specific microaerophilic conditions detailed above.

343 Suspicious *H. pylori* colonies were picked and suspended in 200 µl PBS 1X.
344 Subsequently, DNA was extracted using the GeneJet™ genomic DNA purification kit
345 (ThermoScientific, Germany) following the Gram-negative bacteria protocol and qPCR
346 was performed as explained in section 4.2.1.

347 **4.3. Identification of FLA isolated from samples**

348 When water samples were positive for FLA growth, they were individually isolated
349 using a micromanipulator (Narishige, Japan). Each isolated amoeba was incubated on

350 NNA at 28°C for 3-5 days. Once growth of a single FLA was checked under the phase
351 contrast microscope, NNA content was collected by adding PAS and using a sterile cell
352 scraper.

353 Once in PAS, tubes were centrifuged at 500 g for 3 min and resuspended in 200 µl of
354 PBS 1X. Then, DNA was extracted using the GeneJet™ genomic DNA purification kit
355 (ThermoScientific, Germany) as explained in 4.2.1.

356 Identification of FLA by multiplex PCR was performed by amplification of 150 bp, 130
357 bp and 50 bp 18S rDNA gene fragments corresponding to Vahlkampfiidae (i.e.
358 *Naegleria* and *Vahlkampfia*), *Acanthamoeba* spp., *Naegleria* spp. and *Vermamoeba* sp
359 and *Echinamoeba* spp, respectively (Le Calvez *et al.,* 2012).

360 When the multiplex PCR failed to identify the isolates because amplicons' molecular
361 weight were not described by Le Calvez *et al.,* 2012, 18S rRNA conventional PCR and
362 subsequent sequencing (Thomas *et al.,* 2006) was performed from purified FLA.

363 Products were visualized in 1.5% agarose gel electrophoresis prepared with 0.001% of
364 GelRed™ (Biotium, USA). Sequences were analyzed using the online analysis tool
365 BLAST(https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the ones with homologies greater
366 than 96% deposited in GenBank.

367 **5. Acknowledgments**

368 This study has been supported by the Spanish Ministry of Economy and
369 Competitiveness AGL2014/53875-R grant and by the Spanish Ministry of Economy and
370 Competitiveness Program International Joint Programming Actions (JPIW2013-095-
371 C03-02).

372 **6. References**

373 Agustí, G., Codony, F., Fittipaldi, M., Adrados, B., and Morato, J. (2010) Viability
374 determination of *Helicobacter pylori* using propidium monoazide quantitative PCR.
375 *Helicobacter* **15**: 473-476.

376 Akya, A., Pointon, A., and Thomas, C. (2009) Viability of *Listeria monocytogenes* in co-
377 culture with *Acanthamoeba* spp. *FEMS Microbiol Ecol* **70**: 20-29.

378 Al-Sulami, A.A., Al-Edani, T.A.A., and Al-Abdula, A.A. (2012) Culture method and PCR
379 for the detection of *Helicobacter pylori* in drinking water in Basrah Governorate Iraq.
380 *Gastroenterol Res Pract* **2012:** 5 pages. Article ID 245167.

381 Badirzadeh, A., Niyyati, M., Babaei, Z., Amini, H., Badirzadeh, H., and Rezaeian, M.
382 (2011) Isolation of free-living amoebae from Sarein hot springs in Ardebil Province,
383 Iran. *Iran J Parasitol* **6**: 1-8.

384 Barker, J., and Brown, M.R.W. (1994) Trojan-horses of the microbial world - protozoa
385 and the survival of bacterial pathogens in the environment. *Microbiol-Uk* **140**: 1253-
386 1259.

387 Bui, X.T., Winding, A., Qvortrup, K., Wolff, A., Bang, D.D., and Creuzenet, C. (2012)
388 Survival of *Campylobacter jejuni* in co-culture with *Acanthamoeba castellanii*: role of
389 amoeba-mediated depletion of dissolved oxygen. *Environ Microbiol* **14**: 2034-2047.

390 Bunn, J.E.G., MacKay, W.G., Thomas, J.E., Reid, D.C., and Weaver, L.T. (2002) Detection
391 of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in
392 early life. *Lett Appl Microbiol* **34**: 450-454.

393 Corsaro, D., Pages, G.S., Catalan, V., Loret, J.F., and Greub, G. (2010) Biodiversity of
394 amoebae and amoeba-associated bacteria in water treatment plants. *Int J Hyg Environ*
395 *Health* **213**: 158-166.

396 Crespo, E.P., Mallen, M.M., Ares, M., Fernández, M.C.A., and Combarro, M.P.C. (1990)
397 Isolation of amebas of the genera *Naegleria and Acanthamoeba* from public fountains
398 in Galicia (N.W. Spain). *Water Air Soil Pollut* **53**: 103-111.

399 Dupuy, M., Mazoua, S., Berne, F., Bodet, C., Garrec, N., Herbelin, P. *et al.* (2011)
400 Efficiency of water disinfectants against *Legionella pneumophila* and *Acanthamoeba*.
401 *Water Res* **45**: 1087-1094.

402 Fujimura, S., Kato, S., and Kawamura, T. (2004) *Helicobacter pylori* in Japanese river
403 water and its prevalence in Japanese children. *Lett Appl Microbiol* **38**: 517-521.

404 García, A., Goñi, P., Clavel, A., Lobez, S., Fernández, M.T., and Ormad, M.P. (2011)
405 Potentially pathogenic free-living amoebae (FLA) isolated in Spanish wastewater
406 treatment plants. *Environ Microbiol Rep* **3**: 622-626.

407 García, A., Goñi, P., Cieloszyk, J., Fernández, M.T., Calvo-Beguería, L., Rubio, E. *et al.*
408 (2013) Identification of free-living amoebae and amoeba-associated bacteria from
409 reservoirs and water treatment plants by molecular techniques. *Environ Sci Technol* **47**:
410 3132-3140.

411 García, M.T., Jones, S., Pelaz, C., Millar, R.D., and Abu Kwaik, Y. (2007) *Acanthamoeba*
412 *polyphaga* resuscitates viable non-culturable *Legionella pneumophila* after
413 disinfection. *Environ Microbiol* **9**: 1267-1277.

414 Gaze, W.H., Burroughs, N., Gallagher, M.P., and Wellington, E.M.H. (2003) Interactions
415 between *Salmonella typhimurium* and *Acanthamoeba polyphaga*, and observation of a
416 new mode of intracellular growth within contractile vacuoles. *Microbial Ecol* **46**: 358-
417 369.

418 Gelman, B.B., Rauf, S.J., Nader, R., Popov, V., Borkowski, J., Chaljub, G. *et al.* (2001)
419 Amoebic encephalitis due to *Sappinia diploidea*. *JAMA-J Am Med Assoc* **285**: 2450-
420 2451.

421 IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Schistosomes,
422 liver flukes and *Helicobacter pylori*. (1994) Lyon, 7-14 June 1994. *IARC monographs on*
423 *the evaluation of carcinogenic risks to humans* **61**: 1-241.

424 Iovieno, A., Ledee, D.R., Miller, D., and Alfonso, E.C. (2010) Detection of bacterial
425 endosymbionts in clinical *Acanthamoeba* isolates. *Ophthalmology* **117**: 445-452.

426 Le Calvez, T., Trouilhe, M.C., Humeau, P., Moletta-Denat, M., Frere, J., and Hechard, Y.
427 (2012) Detection of free-living amoebae by using multiplex quantitative PCR. *Mol Cell*
428 *Probe* **26**: 116-120.

429 Leja, M., Axon, A., and Brenner, H. (2016) Epidemiology of *Helicobacter pylori*
430 infection. *Helicobacter* **21**: 3-7.

431 Lorenzo-Morales, J., Ortega-Rivas, A., Foronda, P., Martínez, E., and Valladares, B.
432 (2005) Isolation and identification of pathogenic *Acanthamoeba* strains in Tenerife,
433 Canary Islands, Spain from water sources. *Parasitol Res* **95**: 273-277.

434 Magnet, A., Fenoy, S., Galván, A.L., Izquierdo, F., Rueda, C., Vadillo, C.F., and del Águila,
435 C. (2013) A year long study of the presence of free living amoeba in Spain. *Water Res*
436 **47**: 6966-6972.

437 Magnet, A., Galván, A.L., Fenoy, S., Izquierdo, F., Rueda, C., Vadillo, C.F. *et al.* (2012)
438 Molecular characterization of *Acanthamoeba* isolated in water treatment plants and
439 comparison with clinical isolates. *Parasitol Res* **111**: 383-392.

440 Marciano-Cabral, F., and Cabral, G. (2003) *Acanthamoeba* spp. as agents of disease in
441 humans. *Clin Microbiol Rev* **16**: 273-307.

442 Moreno, Y., and Ferrús, M.A. (2012) Specific detection of cultivable *Helicobacter pylori*
443 cells from wastewater treatment plants. *Helicobacter* **17**: 327-332.

444 Moreno, Y., Ferrús, M.A., Alonso, J.L., Jiménez, A., and Hernández, J. (2003) Use of
445 fluorescent *in situ* hybridization to evidence the presence of *Helicobacter pylori* in
446 water. *Water Res* **37**: 2251-2256.

447 Moreno-Mesonero, L., Moreno, Y., Alonso, J.L., and Ferrús, M.A. (2016) DVC-FISH and
448 PMA-qPCR techniques to assess the survival of *Helicobacter pylori* inside
449 *Acanthamoeba castellanii. Res Microbiol* **167**: 29-34.

450 Nilsson, H.O., Blom, J., Abu Al-Soud, W., Ljungh, A., Andersen, L.P., and Wadstrom, T.
451 (2002) Effect of cold starvation, acid stress, and nutrients on metabolic activity of
452 *Helicobacter pylori*. *Appl Environ Microbiol* **68**: 11-19.

453 Niyyati, M., Lasgerdi, Z., and Lorenzo-Morales, J. (2015) Detection and molecular
454 characterization of potentially pathogenic free-living amoebae from water sources in
455 Kish Island, Southern Iran. *Microbiol insights* **8**: 1-6.

456 Percival, S.L., and Thomas, J.G. (2009) Transmission of *Helicobacter pylori* and the role
457 of water and biofilms. *J Water Health* **7**: 469-477.

458 Piqueres, P., Moreno, Y., Alonso, J.L., and Ferrús, M.A. (2006) A combination of direct
459 viable count and fluorescent *in situ* hybridization for estimating *Helicobacter pylori* cell
460 viability. *Res Microbiol* **157**: 345-349.

461 Queralt, N., Bartolomé, R., and Araujo, R. (2005) Detection of *Helicobacter pylori* DNA
462 in human faeces and water with different levels of faecal pollution in the north-east of
463 Spain. *J Appl Microbiol* **98**: 889-895.

464 Qvarnstrom, Y., da Silva, A.J., Schuster, F.L., Gelman, B.B., and Visvesvara, G.S. (2009)
465 Molecular confirmation of *Sappinia pedata* as a causative agent of amoebic
466 ancephalitis. *J Infect Dis* **199**: 1139-1142.

467 Ranjbar, R., Khamesipour, F., Jonaidi-Jafari, N., and Rahimi, E. (2016) *Helicobacter*
468 *pylori* isolated from Iranian drinking water: vacA, cagA, iceA, oipA and babA2 genotype
469 status and antimicrobial resistance properties. *Febs Open Bio* **6**: 433-441.

470 Retana-Moreira, L., Abrahams-Sandi, E., Cabello-Vilchez, A.M., Reyes-Batlle, M.,
471 Valladares, B., Martinez-Carretero, E. *et al.* (2014) Isolation and molecular
472 characterization of *Acanthamoeba* and *Balamuthia mandrillaris* from combination
473 shower units in Costa Rica. *Parasitol Res* **113**: 4117-4122.

474 Reyes-Batlle, M., Wagner, C., Zamora-Herrera, J., Vargas-Mesa, A., Sifaoui, I., Gonzalez,
475 A.C. *et al.* (2016a) Isolation of thermotolerant *Vermamoeba vermiformis* strains from
476 water sources in Lanzarote Island, Canary Islands, Spain. *Acta Parasit* **61**: 650-653.

477 Reyes-Batlle, M., Wagner, C., Zamora-Herrera, J., Vargas-Mesa, A., Sifaoui, I., González,
478 A.C. *et al.* (2016b). Isolation and molecular identification of *Vermamoeba vermiformis*
479 Strains from Soil Sources in El Hierro Island, Canary Islands, Spain. *Curr Microbiol* **73:**
480 104-107

481 Reyes-Batlle, M., Wagner, C., López-Arencibia, A., Sifaoui, I., Martínez-Carretero, E.,
482 Valladares, B. *et al.* (2017) Isolation and molecular characterization of a *Naegleria*
483 strain from a recreational water fountain in Tenerife, Canary Islands, Spain. *Acta*
484 *Parasit* **62:** 265-268.

485 Santiago, P., Moreno, Y., and Ferrús, M.A. (2015) Identification of viable *Helicobacter*
486 *pylori* in drinking water supplies by cultural and molecular techniques. *Helicobacter* **20**:
487 252-259.

488 Sente, C., Erume, J., Naigaga, I., Mulindwa, J., Ochwo, S., Magambo, P.K. *et al.* (2016)
489 Prevalence of pathogenic free-living amoeba and other protozoa in natural and
490 communal piped tap water from Queen Elizabeth protected area, Uganda. *Infect Dis*
491 *Poverty* **5**: 68. DOI 10.1186/s40249-016-0162-5

492 Sheehan, K.B., Fagg, J.A., Ferris, M.J., and Henson, J.M. (2003) PCR detection and
493 analysis of the free-living amoeba *Naegleria* in hot springs in Yellowstone and Grand
494 Teton National Parks. *Appl Environ Microbiol* **69**: 5914-5918.

495 Siddiqui, R., and Khan, N.A. (2012) War of the microbial worlds: Who is the beneficiary
496 in *Acanthamoeba*-bacterial interactions? *Exp Parasitol* **130**: 311-313.

497 Smith, C.D., and Ashbolt, N.J. (2012) The Fate of *Helicobacter pylori* phagocytized by
498 *Acanthamoeba polyphaga* demonstrated by fluorescent *in situ* hybridization and
499 quantitative qolymerization chain reaction tests. *Curr Microbiol* **65**: 805-812.

500 Snelling, W.J., Stern, N.J., Lowery, C.J., Moore, J.E., Gibbons, E., Baker, C., and Dooley,
501 J.S.G. (2008) Colonization of broilers by *Campylobacter jejuni* internalized within
502 *Acanthamoeba castellanii*. *Arch Microbiol* **189**: 175-179.

503 Steinert, M., Birkness, K., White, E., Fields, B., and Quinn, F. (1998) *Mycobacterium*
504 *avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and
505 survive within cyst walls. *Appl Environ Microbiol* **64**: 2256-2261.

506 Storey, M.V., Winiecka-Krusnell, J., Ashbolt, N.J., and Stenstrom, T.A. (2004) The
507 efficacy of heat and chlorine treatment against thermotolerant Acanthamoebae and
508 Legionellae. *Scand J Infect Dis* **36**: 656-662.

509 Thomas, V., Herrera-Rimann, K., Blanc, D.S., and Greub, G. (2006) Biodiversity of
510 amoebae and amoeba-resisting bacteria in a hospital water network. *Appl Environ*
511 *Microb* **72**: 2428-2438.

512 Thomas, V., Loret, J.F., Jousset, M., and Greub, G. (2008) Biodiversity of amoebae and
513 amoebae-resisting bacteria in a drinking water treatment plant. *Environ Microbiol* **10**:
514 2728-2745.

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

518 Tirodimos, I., Bobos, M., Kazakos, E., Haidich, A.B., Dardavessis, T., Kostopoulos, I., and
519 Arvanitidou, M. (2014) Molecular detection of *Helicobacter pylori* in a large
520 Mediterranean river, by direct viable count fluorescent *in situ* hybridization (DVC-
521 FISH). *J Water Health* **12**: 868-873.

522 Torres, J., Pérez-Pérez, G., Goodman, K.J., Atherton, J.C., Gold, B.D., Harris, P.R. *et al.*
523 (2000) A comprehensive review of the natural history of *Helicobacter pylori* infection in
524 children. *Arch Med Res* **31**: 431-469.

525 Villanueva, M.P., Medina, G., and Fernández, H. (2016) *Arcobacter butzleri* survives
526 within trophozoite of *Acanthamoeba castellanii*. *Rev Argent Microbiol* **48**: 105-109.

527 Visvesvara, G.S., Moura, H., and Schuster, F.L. (2007) Pathogenic and opportunistic
528 free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*,
529 and *Sappinia diploidea*. *Fems Immunol Med Mic* **50**: 1-26.

530 Watson, C.L., Owen, R.J., Said, B., Lai, S., Lee, J.V., Surman-Lee, S., and Nichols, G.
531 (2004) Detection of *Helicobacter pylori* by PCR but not culture in water and biofilm
532 samples from drinking water distribution systems in England. *J Appl Microbiol* **97**: 690-
533 698.

534 Winiecka-Krusnell, J., Wreiber, K., Von Euler, A., Engstrand, L., and Linder, E. (2002)
535 Free-living amoebae promote growth and survival of *Helicobacter pylori*. *Scand J Infect*
536 *Dis* **34**: 253-256.

537 **6. Table and figure legends**

538 Table 1. Number and percentage of analyzed samples contaminated with FLA

539 Table 2. Identification of isolated FLA in wastewater and drinking water samples by means of
540 multiplex PCR or conventional PCR plus sequencing. Purified FLA sampling point: [1]: after
541 secondary treatment. [2]: after tertiary treatment.

542 Table 3. Summary of number of FLA-positive samples and qPCR, DVC-FISH and culture positive
543 *H. pylori* results

544 Figure 1. Workflow overview

545 Figure 2. Gel electrophoresis of the purified FLA identified by means of multiplex PCR. Lane 1:
546 *A. castellanii* positive control. Lane 2: Purified amoeba (PA) 4. Lane 3: PA 5. Lane 4: PA 11. Lane
547 5: PA 12. Lane 6: PA 22. Lane 7: PA 23. Lane 8: PA 25. Lane 9: PA 36. Lane 10: PA 39. Lane 11:
548 PA 17. Lane 12: PA 20. Lane 13: negative control. M: 100 bp ladder

549 Figure 3. Phase contrast microphotographs of purified FLA (400x). (a) Purified FLA number 12
550 identified as *Acanthamoeba* spp. (b) Purified FLA number 8 identified as *Naegleria* spp.

551 Figure 4. Viable *H. pylori* cells from inside environmental FLA from wastewater (a, b) and
552 drinking water (c, d) samples identified by DVC-FISH (1000x). (a, c) Hybridization with EUB-338
553 probe. (b, d) Hybridization with HPY-LNA probe.