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Additional Information

1 **DEEP-AMPLICON SEQUENCING (DAS) ANALYSIS TO DETERMINE THE PRESENCE OF**
2 **PATHOGENIC *HELICOBACTER* SPECIES IN WASTEWATER REUSED FOR IRRIGATION**

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12 **Abstract**

13 Wastewater has become one of the most important and least expensive water for the
14 agriculture sector, as well as an alternative to the overexploitation of water resources.
15 However, inappropriate treatment before its reuse can result in a negative impact on
16 the environment, such as the presence of pathogens. This poses an increased risk for
17 environmental safety, which can subsequently lead to an increased risk for human
18 health. Among all the emerging wastewater pathogens, bacteria of the genus
19 *Helicobacter* are some of the most disturbing ones, since they are directly related to
20 gastric illness and hepatobiliary and gastric cancer. Therefore, the aim of this study
21 was to determine the presence of potentially pathogenic *Helicobacter* spp. in treated
22 wastewater intended for irrigation. We used a next generation sequencing approach,
23 based on Illumina sequencing in combination with culture and other molecular
24 techniques (qPCR, FISH and DVC-FISH), to analyze 16 wastewater samples, with and
25 without an enrichment step. By culture, one of the direct samples was positive for *H.*
26 *pylori.* FISH and DVC-FISH techniques allowed for detecting viable *Helicobacter* spp.,
27 including *H. pylori*, in seven out of eight samples of wastewater from the tertiary
28 effluents, while qPCR analysis yielded only three positive results. When wastewater
29 microbiome was analyzed, *Helicobacter* genus was detected in 7 samples. The
30 different molecular techniques used in the present study provided evidence, for the
31 first time, of the presence of species belonging to the genus *Helicobacter* such as *H.*

32 *pylori*, *H. hepaticus*, *H. pullorum* and *H. suis* in wastewater samples, even after
33 disinfection treatment.

34 **Keywords**

35 Wastewater, *Helicobacter* spp, molecular techniques, metagenomics, pathogens.

36

37 1. Introduction

38 Water is a scarce resource for a large part of the world population. Around 70% of the
39 world water supplies are used for agriculture (Eslamian, 2016), being the economic
40 sector with the greatest demand of water. The recovery and reuse of treated
41 wastewater is an alternative to the overexploitation of natural water resources. For
42 this reason, wastewater has become one of the most important and less expensive
43 non-conventional water sources for agriculture (Drechsel *et al.,* 2015; Eslamian, 2016).

44 In countries such as France, Italy, Spain, Cyprus, Israel, Jordan or USA, wastewater is a
45 continuous source for agricultural irrigation (Aquarec 2006; Ndèye *et al.,* 2008;
46 Pedrero *et al.,* 2010; EPA 2012; Kalavrouziotis *et al.,* 2013), and has become a valuable
47 resource. The use of treated wastewater for agriculture irrigation brings several
48 advantages, such as less dependence on natural water sources (Parsons *et al.,* 2010),
49 presence of nutrients that reduce the use of artificial fertilizers (Pedrero *et al.,* 2013b;
50 Sánchez *et al.,* 2014; Vivaldi *et al.,* 2015; Lyu *et al.,* 2016) and higher yield in irrigation
51 (Vivaldi *et al.,* 2015).

52 However, poor management prior to water reuse may cause negative impacts on the
53 environment, such as the presence of pathogens, which can reach irrigated foods and
54 affect human health (Lazarova *et al.,* 2013).

55 Despite important progress in wastewater treatment technologies, waterborne human
56 pathogens (viruses, bacteria and protozoa) can be present in the final effluent
57 (Lazarova *et al.,* 2013). Currently, determination of the microbiological quality of water
58 is based on the presence of bacterial indicators, such as coliform bacteria and
59 *Escherichia coli.* However, it has been reported the existence of emerging pathogens

60 that are more resistant to water treatments and disinfection processes than indicators.
61 This is the case of *Helicobacter pylori,* which is more resistant to chlorine than *E. coli*
62 (Johnson *et al.,* 1997), what can explain its survival in wastewater treatment plants
63 (Moreno *et al.,* 2012).

64 Among all the emerging wastewater pathogens, bacteria of the genus *Helicobacter* are
65 some of the most disturbing ones, since they are directly related to gastric illness and
66 hepatobiliary and gastric cancer. A relevant fact is that species belonging to
67 *Helicobacter* genus can coexist with other pathogenic proteobacteria such as
68 *Campylobacter* and *Arcobacter* in the same sample (Petersen *et al.,* 2007).

69 The best-known species of *Helicobacter* genus, *H. pylori* is associated with
70 gastrointestinal disorders which include gastritis, peptic ulcer, duodenal ulcer, gastric
71 cancers and lymphoma associated with mucosal lymphoid tissue (MALT) (FDA 2014).
72 Non-*H. pylori Helicobacter* species (NHPH) such as *H. hepaticus, H. pullorum, H. suis* or
73 *H. bilis* have been related to enteritis, ulcerative colitis, autoimmune hepatitis,
74 hepatocarcinoma, chronic liver disease and autoimmune hepatobiliary disease. They
75 are also associated to hepatobiliary and pancreatic cancers. These types of cancer are
76 highly lethal and difficult to diagnose (Casswasll *et al.,* 2010, Boutin*e et al.,* Mateos-
77 Muñoz *et al.,* 2013).

78 Different strategies, such as growing in biofilms or entering into the Viable But Non-
79 Culturable (VBNC) state, have been suggested to be involved in *H. pylori* long-term
80 survival in the environment (Cellini e*t al.,* 2008; Santiago *et al.,* 2015). Different studies
81 using molecular techniques have confirmed the presence of *H. pylori* in various aquati c
82 environments, suggesting that contaminated water with human feces may be a
83 reservoir for the pathogen (Twing *et al.,* 2011; Moreno *et al.,* 2012; Bahrami *et al.,*
84 2013). It has been also demonstrated its survival in chlorinated water (Moreno *et al.,*
85 2007).

86 Waterborne transmission has also been proposed for other *Helicobacter* species,
87 including *H. mustelae, H. muridarum, H. felis, H. canadensis, H. pullorum, H. canis*
88 (Azevedo *et al.,* 2008) and *H. cetorum* (Goldman *et al.,* 2009). Thus, for accurate
89 determination of their epidemiology, it is crucial to determine the occurrence of

90 *Helicobacter* spp. in water distribution systems, as well as the role of water in its
91 transmission. However, until now, the presence of NHPH species in wastewater has
92 not been studied.

93 Cultivation and isolation of *Helicobacter* spp., especially NHPH, from environmental
94 samples is extremely difficult (Øverby *et al.,* 2016). Molecular techniques such as
95 Polymerase Chain Reaction (PCR), and Fluorescence *in situ* Hybridization (FISH) have
96 been used to detect *Helicobacter* spp. from treated wastewater and drinking, coastal,
97 river, lake, recreational and glacial waters (Cunachi *et al.,* 2016; Fernández-Delgado *et*
98 *al.,* 2016). Despite its high sensitivity, these techniques present a disadvantage, since
99 they are not able to differentiate between viable and nonviable cells, since DNA from
100 living and dead cells as well as extracellular DNA can be amplified (Wang and Levin,
101 2006; Pathak *et al.,* 2012). Currently, some modifications of molecular conventional
102 techniques, such as PMA q-PCR or Direct Viable Count method combined with FISH
103 (DVC-FISH) (Santiago *et al.,* 2015; Moreno-Mesonero *et al.,* 2016) can be used for
104 discriminating between viable and non-viable *Helicobacter* cells.
105 All these molecular assays are based on the detection or quantification of a single
106 pathogen in a sample. Therefore, they do not provide information about the
107 abundance and diversity of significant microorganisms present in the total microbial
108 community (Ye and Zhang 2011). Thus, for knowing the ecology of pathogen in
109 environment, it is very useful to simultaneously study all the microbiota of a sample.
110 Massive sequencing of high performance (Next Generation sequencing, NGS) is the
111 only technique that allows this goal. Through the selection of specific primers, NGS can
112 analyse phylogenetic groups or species (Qichao *et al.,* 2014). It also allows non-
113 cultivable microorganism's detection and typing, since it discriminates even single
114 nucleotide polymorphisms. This technology is currently being used to investigate the
115 microbial diversity in aquatic environments, such a wastewater (Ye and Zhang, 2011;
116 Cai and Zhang 2013; Lu *et al.,* 2015,) drinking water (Vierheilig *et al.,* 2015) and
117 watersheds (Ibekwe *et al.,* 2013).
118 Ilumina high-throughput sequencing technique (Logares *et al.,* 2014) employs surface-
119 attached automated dye sequencing, allowing the identification and characterization
120 of pathogens at genus or even species level. This methodology can be extremely useful

121 for studying the diversity of pathogens in complex environmental samples and,
122 consequently, to detect potential pathogens, which cannot be detected by culture
123 techniques or other molecular techniques.

124 Thus, the objective of the present work was to determine the presence of potentially
125 pathogenic *Helicobacter* spp. in treated wastewater intended for irrigation. To reach
126 this goal, Illumina-based NGS approach, specifically directed to *Helicobacter* genus, in
127 combination with culture and molecular techniques, has been used.

128

129 **2. Materials and methods**

130 **2.1. Sampling**

131 Wastewater samples were collected from an activated sludge wastewater treatment
132 plant in a Mediterranean region of Spain from February 2016 to May 2017. The urban
133 wastewater treatment plant serves a total population of 155,674 equivalent
134 inhabitants (36,625 m^3/day). The final effluent is used for irrigation.

135 A total of 16 water samples were aseptically collected, 8 of them after biological
136 secondary treatment ("S" samples), and 8 after disinfection treatment with chlorine
137 and UV treatment ("T" samples). Samples were refrigerated and analyzed within 2
138 hours.

139 **2.2. Wastewater sample analysis.**

140 One liter of each wastewater sample was centrifuged at 3,220 g for 20 min. The
141 supernatant was removed and centrifuged again at 8,000 g for 8 min at 4ºC to ensure
142 the elimination of all residual water. The resulted pellet was suspended in 10 mL of
143 phosphate-buffered saline (PBS 1X: 130 mM sodium chloride, 10 mM sodium
144 phosphate, pH 7.2). From this, 200 μL were taken for *Helicobacter* spp. culture, 1 mL
145 for qPCR analysis, and another 1 mL for DVC-FISH analysis ("D" samples). An additional
146 aliquot of 5 mL from each sample was incubated in 10 mL of Brucella broth enrichment
147 media (BBL™ (Becton Dickinson, USA), 10% (v/v) fetal bovine serum (Fisher, USA) and
148 Dent selective supplement (Oxoid, UK)) under microaerophilic conditions at 37ºC for

149 24 hours. After the incubation period, the presence of *Helicobacter* spp. was also
150 analyzed by culture, qPCR and FISH ("E" samples).

151 For Illumina-based NGS analysis, 1 mL of the 8 samples from tertiary effluent (after
152 disinfection), both directly and after the enrichment step, were processed as described
153 below.

154 **2.3. Culture of *Helicobacter* spp.**

155 Aliquots of 100 µL of all the samples were spread, before and after enrichment, on
156 Dent selective agar (Blood Agar Base (Pronadisa, Spain), 10% (v/v) defibrinated horse
157 blood (Oxoid, UK), 0.025% (v/v) sodium pyruvate (Fisher, USA), DENT supplement,
158 (Oxoid)) and on Pylori® Agar (PA) (bioMérieux SA, Spain). A filter technique previously
159 described by Steele and McDermot (1984) and modified by Moreno and Ferrús (2012)
160 was used. Briefly, a sterile 0.65 µm pore size membrane (Whatman, Maidstone,
161 England) was placed onto agar plates, and then 100 µL of each sample were placed on
162 the filters. Plates were incubated under microaerophilic conditions at 37ºC. After 24 h
163 of incubation, the membranes were removed from the plates, which were incubated
164 for 14 days.

165 After the incubation period, agar plates were examined for the presence of
166 *Helicobacter* spp. Suspicious colonies were preliminary identified by Gram stain and
167 subsequently analyzed by specific *Helicobacter* spp. rDNA PCR and *H. pylori VacA* qPCR
168 as described below.

169 **2.4. Detection of *Helicobacter* spp. and *H. pylori* by FISH and DVC-FISH**

170 FISH and DVC-FISH analysis were performed according to the studies carried out by
171 Moreno *et al.* (2003) and Piqueres *et al.* (2006), respectively.

172 For FISH, one mL of wastewater sample was centrifuged at 8,000 r.p.m at 4 ºC for 8
173 min. The supernatant was removed, and the pellet was resuspended in 1 mL of PBS 1X,
174 washed and fixed with three volumes of 4 % paraformaldehyde for 1.5 h at 4ºC.
175 Afterwards, samples were centrifuged and washed with PBS1X buffer and finally
176 resuspended in 1:1 PBS/ethanol (v/v). Samples were stored at -20ºC until their
177 hybridization.

178 For DVC-FISH analysis, another mL of each sample was added to 9 mL of DVC broth
179 (BBL™ Brucella broth (BAS, Becton Dickinson, USA) supplemented with 5% fetal bovine
180 serum (Fisher, USA) and 0.5 mg/L of novobiocin) and incubated for 24 hours at 37ºC
181 under specific microaerophilic conditions. After incubation, DVC broth was centrifuged
182 at 8,000 rpm at 4ºC for 8 min and fixed with paraformaldehyde as above described for
183 FISH. Samples were stored at -20ºC until their hybridization.

184 In this work, two different probes, one for *Helicobacter* spp. and another for *H. pylori*,
185 were used:

186 A specific 16S rRNA probe (target position 717-737) designed by Chan *et al.,* (2005)
187 (HEL717-FAM-AGGTCGCCTTCGCAATGAGTA) was used to identify *Helicobacter* spp.
188 among the fixed samples. Previously, the specificity of HEL717-FAM was determinate
189 *in silico* by "Probe match" online tool against RDP´S collection of 16S rRNA sequences
190 (https://rdp.cme.msu.edu/probematch/search.jsp). The specificity was also
191 determined *in vitro* by whole-cell hybridization with different reference strains
192 belonging to *Helicobacter* genus (*H. pylori* DSM 10242*, H. hepaticus* DSM 22904, *H.*
193 *fennelliae* DSM 7491, *H. cinaedi* DSM 5359 and *H. suis* DSM 19735) and other
194 phylogenetic related species, belonging to the genus *Arcobacter* (*A. butzleri* NCTC
195 12481*, A. skirrowii* NCTC 12713*, A. cryarophilus* NCTC 11885) and *Campylobacter* (*C.*
196 *jejuni* NCTC 11168). Reference strains were obtained from Leibnuz-Institute DSMZ-
197 German Collection of Microorganisms and Cell Cultures and the National Collection of
198 Types Cultures (NCTC, United Kingdom).

199 We also used a 16S rRNA oligonucleotide probe (HPY-CTGGAGAGACTAAGCCCTCC)
200 which has proven to be specific for *H. pylori* detection in environmental samples
201 (Moreno-Mesonero *et al.,* 2020; Vesga *et al.,* 2018)

202 For both assays, FISH and DVC-FISH, aliquots of 5 µL of each fixed sample were
203 hybridized on diagnostic slides, pretreated with 0.1% gelatin. Slides with fixed samples
204 were dehydrated by serial immersions in 50%, 80% and 100% ethanol for 3 min each.
205 Formamide at a final concentration of 40% and 50 ng of each probe were included in
206 the hybridization buffer (0.9 mL/L NaCl, 0.01 % SDS, 20mm/L Tris-HCl [pH 7.6]). The
207 reaction was completed under dark conditions at 46ºC for 1.5 h. Afterwards, the slides

208 were incubated with 50 mL of washing solution (0.10 M NaCl, 0.02 M HCl-Tris, 0.01 %
209 SDS and 0.005 M EDTA) under dark conditions at 48ºC for 15 min. Finally, slides were
210 washed with distilled water and air-dried.

211 Slides were mounted with VECTASHIELD® Antifade Mounting Medium (VECTOR
212 laboratories, United Kingdom) and visualized using epifluorescence Olympus BX 50
213 with U-MWIB, U-MWIB and U-MIWG exciter filters. Pictures were taken with the
214 camera Olympus DP-12 camera. For all analysis, a fixed pure culture of the *H. pylori*
215 NCTC 11637 strain was used as a positive control.

216 **2.5. Detection of *Helicobacter pylori* by qPCR**

217 DNA extraction from samples was carried out using the GeneJet™ genomic DNA
218 purification kit (ThermoScientific, Germany). Specific *H. pylori* qPCR based on SYBR
219 green I fluorescence was conducted using VacA primers to amplify a 372 bp fragment
220 according to Vesga *et a*l. (2018). Reactions were performed in a final volume of 20 µl
221 containing 2 µl of Light-Cycler® FastStart DNA SYBR Green I (Roche Applied Science,
222 Spain), 1.6 µl of $MgCl_2$ (50 mM), 0.5 µl of each primer (20 mM) and 2 µl of DNA
223 template. The amplification consisted of an initial DNA denaturation step at 95 °C for
224 10 min, followed by 40 cycles of: 95 °C for 10 s, 62 °C for 5 s and 72 °C for 16 s; and
225 finally, one cycle at 72 °C for 15 s and another at 40 °C for 30 s. Amplifications were
226 conducted by duplicate. DNA from *H. pylori* strain NCTC 11637 was used as a positive
227 control and qPCR mix without DNA served as negative control in all the qPCR analysis.

228

229 **2.6. Amplicon-based metagenomics analysis for detection of *Helicobacter* spp.**

230 **Evaluation of primers**

231 The primers HS-Forward (5´-CTAATACATGCAAGTCGAACGA-3) and HS2-Reverse (5´-
232 GTGCTTATTCGTTAGATACCGTCAT-3´) were selected to amplify the V3-V4 region of the
233 16S rRNA gene of Helicobacteraceae family. The primer HS was designed in this study
234 and HS2 was previously used by Huang et al., (2002).

235 The Oligo Analyzer 3.1 tool (Integrated DNA Technology) was used to determine the HS
236 and HS2 oligonucleotide properties. TestPrime tool (https://.arb-
237 silva.de/search/testprime) was used to calculate the coverage of the primer pairs,
238 using Silva SSUr132 database; and the sequence collection RefNR, allowing 0, 1 and 2
239 mismatches in the union of primer pairs (Klindworth *et al.,* 2013).

240 Specificity was also evaluated *in vitro* by conventional PCR as described below. DNA
241 from *H. pylori* DSM 10242*, H. hepaticus* DSM 22904, *H. fennelliae* DSM 7491, *H. cinaedi*
242 DSM 5359 and *H. suis* DSM 19735 , as well as reference strains of *Arcobacter* (*A.*
243 *butzleri* NCTC 12481*, A. skirrowii* NCTC 12713*, A. cryarophilus* NCTC 11885, *A. cibarius*
244 (environmental isolate)) and *Campylobacter* (*C. jejuni* NCTC 11168) were used to
245 determinate the specificity of the pair of primers.

246 **PCR reaction**

247 DNA extraction from samples was carried out using the GeneJet™ genomic DNA
248 purification kit (ThermoScientific, Germany). The amplification was performed in a
249 final reaction volume of 25 µL which contained 2.5 µL 10X Buffer, 1.30 µL of $MgCl_2$
250 (50Mm), 0.75 µL of each deoxynucleotide (10mM), 0.5 µL of each primer (10 mM), 5 U
251 of *Taq* polymerase (IBIAN TECHNOLOGIES S.L, Zaragoza, Spain) and 4 µL of DNA
252 template. Cycling conditions were 95ºC for 5 min, followed by a 28-cycle of
253 amplification (denaturation at 95ºC for 45s; annealing at an optimum temperature of
254 59ºC for 45s and extension at 72ºC for 45 s) and additional extension step for 5 min at
255 72ºC. Annealing temperature range between 55ºC to 66ºC was tested.

256 The amplified products were analyzed by 1% agarose gel containing RedSafe™ and
257 observed under UV light.

258 **High-throughput 16S rRNA Sequencing**

259 A mock sample was created in order to evaluate both the optimal conditions of the
260 *Helicobacter* spp. 16S rRNA fragment amplification and the bioinformatics pipeline
261 after Illumina sequencing. The mock solution was formed by the mix of different
262 volumes of DNA of each reference strain listed above, in order to give a concentration
263 of 2 ng each into a single tube.

264 In order to test the suitability of the amplicon-sequencing conditions in the complex
265 sample community, 3 µl of a water sample from the effluent after tertiary treatment
266 (sample W1TD) was inoculated with 7 µl of the mock solution.
267 Finally, DNA from all the direct and enriched samples taken after disinfection
268 treatment was processed by Illumina amplicon-based sequencing.
269 All sequencing analysis (mock, sample W1TD inoculated with the mock and
270 wastewater samples) were carried out by using Illumina MiSeq platform through a
271 2 × 300bp paired-end run at FISABIO sequencing service (Valencia, Spain). The
272 amplicon sequencing protocol targets V3-V4 region of the 16S rRNA gene, using the
273 primers previously described. DNA amplicon libraries were created as explained by
274 Illumina guide (http://www.illumina.com/
275 content/dam/illumina-support/documents/documentation/chemistry_
276 documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.
277 pdf) using the enzyme KAPA HiFi HotStart (KAPABIOSYSTEMS, USA). PCR conditions
278 were: 95ºC for 3 min, following by 28-cycles of amplification (denaturation at 95ºC for
279 30 s; annealing at 55ºC for 30 s and extension at 72ºC for 30 s) and an additional
280 extension step for 5 min at 72ºC.

281 **Bioinformatics analysis.**

282 QIIME 1.9.1 (Caporaso *et al.*, 2010) was used to analyze the raw sequences obtained
283 from the Illumina sequencing platform. For that, the corresponding scripts used in
284 Microbiome Helper virtual box (Comeau *et al.,* 2017) were applied. Initially, PEAR
285 v0.9.19 (Zhang *et al.*, 2014) was used to join forward and reverse sequences.
286 Moreover, to prove that reads were rightly stitched, FastQC (Andrews, 2010) tool was
287 employed. Afterward, FASTX-Toolkit v0.014 (Gordon 2009) was applied to filter the
288 stitched reads by length and quality score (minimum of Q30 over at least 90% of the
289 read or less than 200 bp and reads with any ambiguous base ("N") were removed).
290 Following this, FastQC tool was used again to remove the merged sequences with low-
291 quality tails.

292 The tool VSEARCH v1.11.1 (Rognes *et al.,* 2016) was used to filter the potential
293 chimeric sequences. SortMeRNA v2.0 (Kopylova *et al.,* 2012) and SUMACLUST v1.0.00

294 (Mercier *et al.,* 2013) methods were used to process the remaining sequences to form
295 clusters using the open QIIME sequences. Operational Taxonomic Units (OUTs) were
296 defined at the 97% genetic similarity cut-off. The Silva v132 high quality ribosomal RNA
297 database was used as the reference (Quast *et al.,* 2013).

298 OTUs' sequences which were not identified at species level by applying Silva v132
299 rRNA database were aligned against NCBI database by using the BLAST online tool
300 (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

301 **3. Results and Discussion**

302 Various routes of transmission for *Helicobacter* spp. have been proposed, including the
303 oral-oral and fecal-oral route, since this microorganism has been isolated from saliva
304 and feces (Anand *et al.,* 2014; Safaei *et al.,* 2011). Additionally, many studies have
305 confirmed the presence of *Helicobacter* spp. by molecular techniques in different
306 aquatic environments. In this report, the presence of *Helicobacter* species was
307 evidenced in wastewater samples after secondary and tertiary treatment by molecular
308 techniques, including NGS, and culture.

309 **3.1. Culture of *Helicobacter* spp. and *H. pylori***

310 All the enriched samples were negative for *Helicobacter* growth in any of the culture
311 media used. However, characteristic colonies were observed in three plates from
312 direct samples, although it was not possible to isolate the colonies, due to the
313 presence of a mass of non-specific bacterial growth. Thus, to demonstrate the
314 presence of culturable *Helicobacter* cells, all the growth from these plates where
315 suspicious colonies were observed was recovered, and DNA was extracted and
316 analyzed by *Helicobacter* spp. PCR and *H. pylori* qPCR amplicons were subsequently
317 sequenced.

318 One of the direct samples taken after the tertiary (disinfection) treatment (W6TD)
319 yielded the expected 16S rRNA fragment and *Vac*A gene amplicons by PCR and, after
320 alignment against NCBI database by BLAST tool, the sequence corresponded to the
321 specie *Helicobacter pylori*, with a sequence identity of 100%.

322 Currently, no optimal culture medium is known to allow the isolation of *Helicobacter*
323 spp. from feces and other environments such as food and water (Janet *et al.,* 2003).
324 Conventional culture analysis is extremely limited for recovering these bacteria from
325 environmental samples, because of rapid overgrowth of accompanying microbiota. In
326 this work, however, we have observed the occurrence of cultivable *H. pylori* in
327 wastewater after disinfection treatment, what poses some questions about the actual
328 risk for the consumption of vegetables irrigated with this type of water.

329

330 **3.2. *Helicobacter* spp. and *H. pylori* detection by FISH and DVC-FISH.**

331 *In silico* alignment by "Probe match" online tool against RDP's collection of 16S rRNA
332 sequences showed the specificity of HEL717-FAM probe for *Helicobacter* spp. under
333 the specific conditions described above.

334 When *in vitro* assays were conducted, all *Helicobacter* strains included in this work
335 showed positive hybridization with the probe. Species belonging to the
336 Campylobacterales order, such as *Campylobacter* and *Arcobacter,* were tested under
337 the same conditions, yielding negative results.

338 Under rigorous conditions, the HEL717-FAM probe was able to demonstrate the
339 presence of *Helicobacter* spp. in 11 out of the 16 (69%) enriched samples, 5 from the
340 secondary effluent and 6 after tertiary treatment (Table 1). When DVC-FISH was used
341 for detecting viable cells of *Helicobacter* spp. in direct samples with the same probe,
342 positive results were obtained in 13 out of the 16 (81%) direct samples, 6 of them
343 taken from secondary treatment and 7 after tertiary treatment.

344 When *H. pylori*-specific HPY probe was used, the organism was evidenced to be
345 present in 10 out of the 16 (69%) enriched samples, 5 from the secondary effluent and
346 5 after tertiary treatment. DVC-FISH yielded viable elongated (viable) cells in 10 out of
347 the 16 (63%) direct samples, 4 of them coming from secondary treatment and 6 after
348 tertiary treatment.

349 *Helicobacter* spp. FISH assays yielded two negative samples after secondary treatment
350 (W1SE and W4SE) which became positive after tertiary treatment (W1TE, W4TE). For

351 DVC-FISH, this fact was observed also in one sample (W1TD). With the use of specific
352 *H. pylori* HPY probe, again, two FISH negative (W1SE and W4SE) and three DVC-FISH
353 negative (W1SD, W4SD and W6SD) secondary samples were positive after tertiary
354 treatment. This could be due, either because the massive presence of high amounts of
355 microbiota in samples from the secondary, what hides the presence of fluorescent
356 *Helicobacter* cells to the microscope operator, or to the fact that the samples were
357 contaminated after going outside the secondary tank, during their passing through the
358 tertiary equipment. In fact, *H. pylori* can maintain in equipment and tubes in biofilms,
359 which may act as a reservoir and contamination source for this microorganism (García
360 *et al.,* 2014).

361 Overall, FISH technique was able to detect *Helicobacter* spp., including *H. pylori*, in 7
362 out of 8 samples of wastewater from the tertiary effluents, all of them presenting
363 viable, and thus, potentially infective cells. Our findings support that these techniques
364 allow a rapid analysis to investigate the presence and epidemiology of *Helicobacter*
365 spp., improving the safety and quality of reuse wastewater for agriculture purposes.

366 **3.3. Detection of *Helicobacter pylori* by qPCR**

367 Six samplings (75%) were positive for *H. pylori* by qPCR (Table 1), 2 of them both after
368 secondary and tertiary treatments, two of them after secondary but not after tertiary
369 and the other two only from tertiary treatment. These findings, in agreement with
370 FISH results, show that the disinfection treatment of wastewater does not eliminate *H.*
371 *pylori*.

372 In five samples in which hybridization or NGS showed the presence of *H. pylori* (W1TE,
373 W2TD, W2TE, W4TE, W6TD), qPCR was negative, probably due to the presence of
374 organic compounds in wastewater acting as PCR inhibitors (Li *et al.,* 2014). This is
375 reinforced by the fact that in two of these samples (W1T and W4T) the qPCR was
376 positive prior to enrichment and became negative after this step, which increases the
377 amount of organic material in the template. Thus, although qPCR is a very specific
378 technique, and has proven to be very sensitive for other types of samples (Botes *et al.,*
379 2013; Clavel *et al.,* 2016) it seems that it can yield false negative results when applied
380 to complex matrices like wastewater.

381

382 ### 3.4. Detection of *Helicobacter* spp. by Illumina sequencing

383 Amplicon-based sequencing is a culture-independent method which allows the
384 simultaneous identification of microorganisms present in a sample, even at species
385 level, and could be an accurate technique in order to detect low represented bacteria,
386 such as pathogenic species, in complex environments (Cao *et al.,* 2017). Moreover, the
387 genus-specific NGS approach opens new perspectives in massive quantitative, specific
388 and sensitive diagnostics (Pereira et al., 2017).

389 In this work, a region of the V3-V4 segment of the 16S rRNA gene of *Helicobacter* spp.
390 was selected for amplification and deep sequencing through Illumina MiSeq.

391 **Evaluation of primers**

392 The primers were analyzed *in silico*, to know their coverage, through the TestPrime
393 program using the Silva SSUr132 database.

394 In all the cases*, in silico* analysis with the databank sequences demonstrated that the
395 primers allowed for recovering mostly amplified sequences belonging to *Helicobacter*
396 spp.. When no mismatch was allowed, primers aligned against *Helicobacter* spp. with
397 64.6% coverage. However, when 1 mismatch was allowed in the annealing of the
398 primers, *Helicobacter* spp. showed a coverage of 90.1% and *Campylobacter* sequence
399 coverage was 22,7%, while Epsilonproteobacteraota yielded a coverage of 14,1%.
400 When 2 mismatches were allowed, sequences coverage increased to 96.2% for
401 *Helicobacter*, 31.3% for Campylobacteraceae, 2.6% for Arcobacteraceae and 19,1% for
402 total Epsilonproteobacteraota. According to this *in silico* analysis, it was not possible to
403 establish an optimal PCR reaction for the only detection of *Helicobacter* spp.

404 Amplification conditions were then tested *in vitro* by amplification of the DNA of all the
405 reference strains included in the study. At an annealing temperature of 59ºC all
406 Helicobacter DNAs were specifically amplified. Nevertheless, the standard annealing
407 temperature of 55ºC recommended by Illumina protocol was used for DAS in
408 environmental samples.

409 **Optimization of 16S r RNA amplicon-based sequencing**

410 The potential bias of the primers due to the sample properties and microbial
411 composition was evaluated by comparing the sequences obtained after the amplicon-
412 based sequencing analysis of a mock community, an environmental sample (W1TE)
413 and the same environmental sample inoculated with the mock.

414 After quality filtering, trimming and elimination of chimeras, the analysis of the three
415 samples yielded 19614 high-quality reads which were clustered in 233 prokaryotic
416 OTUs with assigned taxonomy, assigned at 97% similarity against Silva v132 database.

417 All species included in the mock community were recovered after sequencing analysis.
418 As expected, a bias towards *Helicobacter* amplification was detected, since the
419 sequences of the other species were underrepresented (Table 2).

420 In the water sample inoculated with the mock (W1TD+mock), only *Helicobacter*
421 sequences were recovered after sequencing. Amplicon-based sequencing does not
422 seem to be affected by possible inhibitor substances present in the sample, since
423 almost the same number of sequences of *Helicobacter* were recovered from both the
424 inoculated sample and the mock (Table 2).

425 Some authors have proposed the use of mock communities when NGS is performed in
426 environmental samples. Their inclusion can directly help post-sequencing analyses
427 (Bokulich *et al.,* 2013; Brooks *et al.,* 2015; Pereira et al., 2017). It can also be used to
428 calculate the chimerism rate, the sequencing error rate and the drift in the
429 representation of a community structure (Schloss *et al.,* 2011). In this work, despite
430 the mock was inoculated with the same amount of DNA of each reference strain, the
431 relative abundance of detected *Helicobacter* sequences was much higher than the
432 other species. This fact is even more evident in the results obtained from the sample
433 inoculated with the mock, in which only *Helicobacter* sequences were recovered after
434 amplicon-based sequencing, and shows the important bias of the sequencing
435 procedure with the used primers, designed for favoring *Helicobacter* detection.

436 However, even with this bias, after the amplicon sequencing analysis of the mock
437 community and water sample, sequences belonging to other families and even other

438 phyla were also recovered, especially other phylogenetically close genus belonging to
439 Campylobacterales order, such as *Arcobacter* or *Campylobacter*. This fact was
440 expected, according to the previous *in silico* and *in vitro* evaluation of the primers, and
441 taking into account the low stringent conditions of the amplification reaction, as
442 described by Illumina, which can allow amplification of more abundant sequences.

443 ***Helicobacter* spp. microbiome in treated wastewater samples**

444 Wastewater samples after tertiary treatment were analyzed by amplicon-based
445 Illumina MiSeq sequencing, both direct and after enrichment procedure. After NGS
446 analysis, as expected by *in silico* and *in vitro* evaluation, it was observed that the
447 primers also allow the identification of some sequences belonging to other phyla
448 different to Proteobacteria.

449 A total of 746,198 raw sequences were obtained. After filtering by quality, joining
450 paired–end- reads and eliminating chimeras, a total of 673,902 high quality sequences
451 remained, which were grouped into 3,078 OTUs (3% cutoff level)

452 With regard to relative abundance, Proteobacteria phylum was the most dominant in
453 all analyzed samples (31,214% of the total sequences recovered), as expected,
454 followed by Bacteroidetes (23.7%) and Firmicutes (15.01%). This result is according to
455 previous studies that demonstrated that this phylum is the most abundant in
456 municipal wastewater treatment plants (Wagner and Loy, 2002; Wang *et al.,* 2012;
457 Qiao *et al.,* 2015). These results are also consistent with other investigations in which
458 wastewater samples were analyzed by high throughput pyrosequencing and Illumina
459 analysis (Ye and Zhang 2013; Kumaraswamy *et al.,* 2014; Xie *et al.,* 2018).The
460 Epsilonbacteraeota phylum (28.04%) was the most abundant in 5 out of 8 wastewater
461 direct samples. All bacteria belonging to the Epsilonbacteraeota in the analyzed
462 wastewater samples were assigned to the Campylobacterales order. Regarding
463 bacterial genera, *Arcobacter* and *Helicobacter* represented 27.06% of the total reads.

464 *Helicobacter* spp. sequences were present in 7 out of 8 water samples (Table 3). As the
465 assigned taxonomy at 97% was unable to discriminate between species, the sequences

466 of each OTU of *Helicobacter* spp., were aligned against NCBI database by the BLAST
467 online tool.

468 According to NCBI database*, Helicobacter* spp. taxonomy was assigned at species level
469 in 20 OTUs. Eight OTUs, corresponding to *H. hepaticus*, with a sequence identity higher
470 than 97%, were recovered in all samples, except for WT1 and WT8 (Table 3).
471 Sequences identified as *H. pylori,* were present in samplings W2, W4, W5, W6 and W7.

472 Furthermore, 2 OTUs identified as *H. pullorum* with a sequence identity of 98% and
473 99%, were present in samples W4TDE and W7DE, while an only OTU of *H. suis* was
474 present in sample W5TD. The remaining OTUs, recovered from samplings W1, W4, W5
475 and W6, were identified only at genus level with a sequence identity higher than 97%.
476 In two direct samples which were positive for *Helicobacter* spp. / *H. pylori* by other
477 molecular methods (W1TD and W3TD), NGS yielded negative results.

478 Results showed that an enrichment step enhanced *H. hepaticus* sequences
479 identification, since in 3 negative direct samples (W2TD, W3TD and W7TD)*,* sequences
480 assigned to this genus were recovered after enrichment (Table 3). On the contrary,
481 enrichment seems to be a disadvantage for *H. pylori* detection by NGS, as observed in
482 samples W6TE and W7TE

483 Even though *H. pylori* is the most known pathogen belonging to the genus *Helicobacter*
484 and there are numerous recent studies about its transmission and presence in
485 wastewater (Bai et al., 2016;; Vesga *et al.,* 2018; Farhadkhani *et al.,* 2019) in this work,
486 the dominant *Helicobacter* species was *H. hepaticus* (6.16%), followed by *H. pylori*
487 (0.19%). A research carried out by Hamada et al. (2009) suggested that viable *H.*
488 *hepaticus* can infect the liver, the gallbladder epithelium or the intestine of humans. To
489 our knowledge this is the first time that *H. hepaticus* is detected in environmental
490 samples. Currently there is no investigation that reports the coexistence of both
491 pathogens in aquatic environments.

492 Additionally, low relative abundances of the species *H. pullorum* and *H. suis* were
493 found. *H. pullorum* has been associated with Crohn's disease and cholelithiasis (Fukuda
494 *et al.,* 2002; Bohr *et al.,* 2004; Chen *et al.,* 2007; Karagin *et al.,* 2010). *H. suis* is the

most predominant gastric NHPH in humans and involves 13.9% to 78.5% of human NHPH infections (Groote *et al.,* 2005; Haesebrouck *et al.,* 2009). The main reservoirs of those two potentially pathogenic NHPH species are pigs and chickens, respectively (Ménard and Smet, 2019). Thus, they could reach water through animal faces. However, our results are too preliminary to obtain conclusions about this subject. There is only a previous study completed by Fernandez-Delgado *et al.* (2016), in which the presence of both *Helicobacter* species in freshwater was detected.

Since the aim of this work was to optimize the detection of *Helicobacter* spp., in the present work we included an enrichment step with the purpose to increase sensitivity and selectivity in the detection of *Helicobacter* spp. in wastewater samples. Other authors reported the effectiveness of an enrichment process to increase the sensitivity of detection techniques based on PCR (Ahmed *et al.,* 2009; Santiago *et al.,* 2015). However, results have only showed the effectiveness of this procedure for *H. hepaticus* NGS detection, while for the other techniques there were no significant differences between enriched and direct sample. As a limited number of samples have been examined, these results should be confirmed by more wide studies.

4. Conclusion

To our knowledge this is the first study which reports the presence of Helicobacter spp. in wastewater samples from Spain, comprising disinfection effluents used for irrigation purposes, carried out by different molecular techniques, including 16S rRNA deep sequencing. These results demonstrate the presence of species belonging to the genus Helicobacter such as *H. pylori*, *H. hepaticus*, *H. pullorum* and *H. suis* in wastewater samples, even after disinfection treatment. This suggests the possible role of wastewater as a vehicle of transmission of pathogenic Helicobacter species to humans through irrigation. The amplicon-sequencing method optimized here showed to be a specific and sensitive method for the simultaneous detection of pathogenic Helicobacter species in environmental samples.

523 **Bibliography:**

524 Ahmed, A., Younis, E., Ishida, Y., Shimamoto, T., 2009. Genetic basis of multidrug resistance in
525 Salmonella enterica serovars Enteritidis and Typhimurium isolated from diarrheic calves in
526 Egypt. Acta Tropica 111, 144-149. https://doi.org/10.1016/j.actatropica.2009.04.004.

527 Aquarec Project 2006. Work Package 2: Guideline for Quality Standards for Water Reuse in
528 Europe. EVK1-CT-2002-00130.

529 Anand, P., Kamath,P., Anil, S., 2014. Role of dental plaque, saliva and periodontal disease in
530 Helicobacter pylori infection. World Journal of Gastroenterology 20, 5639–5653.
531 https://doi.org/10.3748/wjg. v20.i19.5639.

532 Andrews, S., 2010. FastGC: A Quality-control Tool for High-throughput Sequence Data.
533 Babraham Institute, Cambrige, United Kingdom.
534 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

535 Azevedo, N.F., Almeida, C., Fernandes, I., Cerqueira, L., Dias, S., Keevil, C.W., Vieira, M.J., 2008.
536 Survival of Gastric and Enterohepatic Helicobacter spp. in Water: Implications for
537 Transmission. Applied and Environmental Microbiology 74, 1805–1811.
538 https://doi.org/10.1128/AEM.02241-07.

539 Bahrami, A.R., Rahimi, E.,Safaei, H.G., 2013. Detection of Helicobacter pylori in city water,
540 dental unit´s water, and bottled mineral water in Isfahan, Iran. The Scientific World Jurnal
541 2013, 5. https://doi.org/10.1155/2013/280510.

542 Bai, X., Xi, C., Wu, J., 2016. Survival of Helicobacter pylori in the wastewater treatment proce ss
543 and the receiving river in Michigan, USA. Journal of Water and Health 14, 692-698.
544 https://doi.org/10.2166/wh.2016.259.

545 Bokulich, N.,Subramanian, S., Faith,J., Gevers,D., Gordon, J.,Knight,R.,Mills,D.,Caporaso,J.,
546 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing.
547 Nature Methods 10, 57-59.

548 Bohr, U.,Glasbrenner,B., Primus, A., Zagoura, A., Wex,T., Malfertheiner,P., 2004. Identification
549 of Enterohepatic Helicobacter Species in Patients Suffering from Inflammatory Bowel Disease.
550 Journal of Clinical Microbiology 72, 2766–2768. https://doi.org/10.1128/JCM.42.6.2766–
551 2768.2004.

552 Botes, M., Kwaadsteniet, M.,Cloete, T.E., 2013. Application of quantitative PCR for the
553 detection of microorganisms in water. Analytical and Bioanalytical Chemistry 405, 91–108.
554 https://doi.org/10.1007/s00216-012-6399-3.

555 Boutin, S.R., 2005. Molecular pathogenesis of Helicobacter hepaticus induced liver disease,
556 Massachusetts Institute of Technology. htt p://biblioteca.universia.net/html_bura/fi
557 cha/params/ id/34665877.html.

558 Brooks,J.., Edwards, D.., Harwich, M..,Rivera, M.,Fettweis,J., Serrano, M.,Reris,R.,Sheth,N.,
559 Huang,B.,Girerd,P.,Consortion, V., Strauss, J., Jefferson, K.,Buck., G., 2015. The thruth about
560 metagenomics: quantifying and counteracting bias in 16S r RN studies. BMC Microbiology
561 15,66. https://doi.org/10.1186/s12866-015-0351-6.

562 Cai, L., Zhang, T., 2013. Detecting human bacterial pathogens in wastewater treatment plants
563 by a High-Throughput Shotgun Sequencing Technique. Environmental Science and Technology
564 47, 5433–5441. https://doi.org/10.1021/es400275r.

565 Caporaso, J. ,Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, F.K., Fierer, N.,
566 Gonzalez Peña, A., Goodrich, J., Gordon, J., Huttley, G., Kelley, S., Knights, D., Koenig, J., Ley, R.,
567 Lozupone, C., McDonald ,D., Muegge, B., Pirrung, M., Reeder,J.,Sevinsky, J., Turnbaugh,
568 P.,Walters, W., Widmann, J., Yatsunenko, T., Zaneveld, J.,Knight,R.,2010. QIIME allows analysis
569 of high-throughput community sequencing data. Nature Methods 7, 335-336.
570 https://doi.org/10.1038/nmeth.f.303

571 Casswall, T.H., Németh, A., Nilsson, I., Wadström, T., Nilsson, H.O., 2010. Helicobacter species
572 DNA in liver and gastric tissues in children and adolescents with chronic liver disease.
573 Scandinavian Journal of Gastroenterology 45, 160–167.

574 Cao, Y., Fanning, S., Proos, S., Jordan,K., Srikumar, S., 2017. A review on the applications of
575 Next Generation Sequencing Technologies as Applied to Food-Related Microbiome Studies.
576 Journal Frontiers in Microbiology. https://doi.org/10.3389/fmicb.2017.01829

577 Cellini, L., Grande, R., Di Campli, E., Di Bartolomeo, S.,Traini, T., Trubiani, O., 2008.
578 Characterization of a Helicobacter pylori environmental strain. Journal of Applied Microbiology
579 105, 761–769. https://doi.org/10.1111/j.1365-2672.2008.03808.x

580 Chan, V., Crocetti, G., Grehan, M., Zhang, L., Danon, S., Lee, A., Mitchell, H., 2005. Blackwell
581 Publishing, Ltd. Visualization of Helicobacter Species Within the Murine Cecal Mucosa Using

582 Specific Fluorescence In Situ Hybridization. Helicobacter 10, 114–124.
583 https://doi.org/10.1111/j.1523-5378.2005.00298.x

584 Chen, D., Hu, L., Yi, P., Liu, W.W., Fang, D.C., Cao, H., 2007. H. pylori are associated with
585 chronic cholecystitis. World Journal of Gastroenterology 13, 119-1122.
586 https://doi.org/10.3748/wjg.v13.i7.1119

587 Clavel, M., Barraud, O., Moucadel, V., Meynier, F., Karam, E., Ploy, M., François, B., Pichon, N.,
588 Vignon, P., Droual, R., Duchiron, C., Vignaud, J., Chainier, D., Mattei, M., Sommabere, A.,
589 Mercier, E., Le Brun, C., Desachy, A., Garandeau, C., Rodrigue,M., Lacroix, M., Prudent,S.,
590 Jestin, M.A., Yugueros-Marcos, J., 2013. Molecular quantification of bacteria from respiratory
591 samples in patients with suspected ventilator-associated pneumonia. Clinical Microbiology and
592 Infection 22, 812.e1-812.e7. https://doi.org/10.1016/j.cmi.2016.06.013.

593 Comeau, A.M., Douglas, G.M., Langille, M.G.I., 2017. Microbiome helper: a custom and
594 streamlined workflow for microbiome research. Methods and Protocols Novel Systems Biology
595 Techniques 2 , e00227-16. http://dx.doi.org/10.1128/mSystems.00127-16.

596 Corry, J., Atabay, H., Forsythe, S., Mansfield, L., 2003. Culture media for the isolation of
597 campylobacters, helicobacters and arcobacters. Progres in Industrial Microbiology 37, 271-316.
598 https://doi.org/10.1016/S0079-6352(03)80021-8.

599 Cunachi, A.M., Fernández-Delgado, M., Suárez, P., Contreras, M., Michelangeli, F., García-
600 Amado, M.A., 2016. Detection of Helicobacter DNA in different water sources and penguin
601 feces from Greenwich, Dee and Barrientos Islands, Antarctica. Polar Biology 39,1539-1546.

602 Drechsel, P., Qadir, M., Wichelns, D., 2015. Wastewater: Economic Asset in an Urbanizing
603 World. Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-94-017-9545-6.

604 EPA., 2012. Guidelines for Water Reuse. Environmental Protection Agency (EPA). Washington
605 DC (EPA/600/R-12/618).

606 Eslamian, S., Sayahi, M., Khosravi, B., 2016. Urban Water Reuse Handbook. Chapter B2.
607 Conjunctive Use of Water Reuse and Urban Water.

608 Farhadkhani, M., Nikaeen, M., Hassanzadeh, A., Nikmanesh, B., 2019. Potential transmission
609 sources of Helicobacter pylori infection: detection of H. pylori in various environmental
610 samples. Journal of Environmental Health Science and Engineering 17, 129-134.
611 https://doi.org/10.1007/s40201-018-00333-y.

612 Fernández-Delgado, M., Giarrizzo, J.G., García-Amado, M.A., Contreras, M., Salazar, V., Barton,
613 H., Suárez, P., 2016. Evidence of Helicobacter spp. in freshwaters from Roraima Tepui,
614 Guayana Shield, South America. Antonie van Leeuwenhock 109,529-542.

615 Food and Drug Administration (FDA). 2014. Establishing a List of Qualifying Pathogens Under
616 the Food and Drug. Act Federal Register: 79,108. https://federalregister.gov/a/2014-13023.

617 Fukuda, K., Kuroki, T., Tajima, Y., Tsuneoka, N., Kitajima, T., Matsuzaki, S., Furui, J., Kanematsu,
618 T., 2002. Comparative analysis of Helicobacter DNAs and biliary pathology in patients with and
619 without hepatobiliary cancer. Carcinogenesis vol 23, 1927–1931.
620 https://doi.org/10.1093/carcin/23.11.1927

621 García, A., Salas-Jara, M.J., Herrera, C., González, C., 2014. Biofilm and Helicobacter pylori:
622 from environment to human host. World Journal of Gastroenterology 20, 5632–5638.
623 https://doi.org/10.3748/wjg.v20.i19.5632.

624 Goldman, C.G., Loureiro, J.D., Matteo, M.J., Catalano, M., Gonzalez, A.B., Heredia, S.R.,
625 Zubillaga, M.B., Solnick, J.V., Cremaschi, G.A-. 2009. Helicobacter spp. from gastric biopsies of
626 stranded South American fur seals (Arctocephalus australis). Research in Veterinary Science
627 86, 18-21. https://doi.org/10.1016/j.rvsc.2008.04.001.

628 Gordon, A., 2009. FASTX-Toolkit: FASTQ/A Short-reads Pre-processing Tools. Cold Spring
629 Harbor Laboratory, Cold Spring Harbor, NY. http://hannonlab.cshl.edu/fastx_toolkit/.

630 Groote, D., Doorn, L.J.,Bulck, K., Vandamme, P., Vieth, M.,Stolte, M., Debongnie, J.C., Burette,
631 A., Haesebrouck, F., Ducatelle, R.,2005. Detection of Non-pylori Helicobacter Species in
632 “Helicobacter heilmannii”-Infected Humans. Helicobacter 10, 398–406.
633 https://doi.org/10.1111/j.1523-5378.2005.00347.x

634 Haesebrouck, F., Pasmans, F., Flahou, B., Chiers, B., Baele, M.,Meyns, T., Decostere, A.,
635 Ducatelle, R., 2009. Gastric Helicobacters in Domestic Animals and Nonhuman Primates and
636 Their Significance for Human Health. Clinical Microbiology 22, 202–
637 223. https://doi.org/10.1128/CMR.00041-08.

638 Huang, Y., Fan, X.G., Chen, Y.P., Li, N., Tang, L.J., 2002. Detection of Helicobacter species 16S r
639 RNA gene in paraffin-embedded hepatocellular carcinoma tissues. Shijie Huaren Xiaohua Zazhi
640 10, 877-882.

641 Ibekwe, A.M., Ma, J., Murinda, S.E., 2016. Bacterial community composition and structure in
642 an Urban River impacted by different pollutant sources. Science of the Total Environment 566–
643 567, 1176–1185. https://doi.org/10.1016/j.scitotenv.2016.05.168.

644 Johnson, C.H., Rice, E.W., Reasoner, D.J., 1997. Inactivation of Helicobacter pylori by
645 chlorination. Applied and Environmental Microbiology 63, 4969-4970.

646 Kalavrouziotis, I.K., Kokkinos, P., Oron, G., Fatone, F., Bolzonella, D., Vatyliotou, M., Fatta-
647 Kassinos, D., Koukoulakis, P.H., Varnavas, S.P., 2013. Current status in wastewater treatment
648 reuse and research in some Mediterranean countries. Desalination and Water Treatment 53,
649 1-16.

650 Karagin, P., Stenram, U., Wadström, T., Ljungh, A., 2010. Helicobacter species and common gut
651 bacterial DNA in gallbladder with cholecystitis. World Journal of Gastroenterology 16, 4817-
652 4822. https://doi.org/10.3748/wjg.v16.i38.4817.

653 Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013.
654 Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation
655 sequencing-based diversity studies. Nucleic Acids Research 41e1
656 https://doi.org/10.1093/nar/gks808.

657 Kopylova, E., Noé, L., Touzet, H.,2012. SortMeRNA: fast and accurate filtering of ribosomal
658 RNAs in metatranscriptomic data. Bioinformatics 28: 3.211-3.217.

659 Kumaraswamy, R., Amha, Y., Anwar, M., Henschel, A., Rodríguez, J., Ahmad, F., 2014.
660 Molecular Analysis for Screening Human Bacterial Pathogens in Municipal Wastewater
661 Treatment and Reuse. Environmental. Science and Technology 48, 1161-11619.
662 https://doi.org/10.1021/es502546t

663 Lazarova, V., Asano, T., Bahri, A., Anderson, J., 2013. Milestone in Water Reuse: The Best
664 Success Stories. IWA Publishing https://doi.org/10.2166/9781780400716.

665 Li, D., Tong, T., Zeng, S., Lin, Y., Wu, S., He, M., 2014. Quantification of viable bacteria in
666 wastewater treatment plants by using propidium monoazide combined with quantitative PCR
667 (PMA-qPCR). Journal of Environmental Sciences 26, 299–306. https://doi.org/10.1016/S1001-
668 0742(13)60425-8.

669 Logares, R., Sunagawa, S., Salazar, G., Cornejo-Castillo, F.M., Ferrera, I., Sarmento, H.,
670 Hingamp, P., Ogata, H., Vargas, C., Lima-Mendez, G., Raes, J., Poulain, J., Jaillon, O., Wincker,

671 P., Kandels-Lewis, S., Karsenti, E., Bork, P., Acinas, S.G. 2014. Metagenomic 16S rDNA 24ioinfor
672 tags are a powerful alternative to amplicon sequencing to explore diversity and structure of
673 microbial communities. Environmental Microbiology 16, 2659-2671.
674 https://doi.org/10.1111/1462-2920.12250

675 Lu, X., Zhang, X.X., Wang, Z., Huang, K., Wang, Y., Liang, W., Tan, Y., Liu, B., Tang, J., 2015.
676 Bacterial Pathogens and Community Composition in Advanced Sewage Treatment Systems
677 Revealed by Metagenomics Analysis Based on High-Throughput Sequencing. PLOS ONE 10, 5.
678 https://doi.org/10.1371/journal.pone.0125549.

679 Lyu, S., Chen, W., Zhang, W., Fan, Y., Jiao, W., 2016. Wastewater reclamation and reuse in
680 China: opportunities and challenges. Journal of Environmental Science 39, 86-96.
681 https://doi.org/10.106/j.jes.2015.11.012.

682 Ménard, A., Smet, A., 2019. Review: Other Helicobacter species. Helicobacter 24 e12645.
683 https://doi.org/10.1111/hel.12645.

684 Mercier, C., Boyer, F., Bonin, A., Coissac, E., 2013. SUMATRA and SUMACLUST: Fast and Exact
685 Comparison and Clustering of Sequences. http://metabarcoding.org/sumatra/

686 Moreno, Y., Ferrús, M.A., Alonso, J.L., Jiménez, A., Hernández, J., 2003. Use of fluorescent in
687 situ hybridization to evidence the presence of Helicobacter pylori in water. Water Research 37,
688 2251-2256. https://doi.org/10.1016/S0043-1354(02)00624-3.

689 Moreno, Y., Piqueres, P., Alonso, J.L., Jiménez, A., González, A., Ferrús, M.A., 2007. Survival and
690 viability of Helicobacter pylori after inoculation into chlorinated drinking water. Water
691 Research 41, 3490-3496. https://doi.org/10.1016/j.watres.2007.05.020.

692 Moreno, Y., Ferrús, M.A., 2012. Specific detection of cultivable Helicobacter pylori cells from
693 wastewater treatment plants. Helicobacter 17,327-332. https://doi.org/10.1111/j.1523-
694 5378.2012.00961.x.

695 Moreno-Mesonero, L., Moreno, Y., Alonso, J.L., Ferrús, M.A., 2016. DVC-FISH and PMA-q PCR
696 techniques to assess the survival of Helicobacter pylori inside Acanthamoeba castellanii.
697 Research in Microbiology 167, 29-34. https://doi.org/10.1016/j.resmic.2015.08.002.

698 Moreno-Mesonero, L., Hortelano, I., Moreno, Y., Ferrús, M.A., 2020. Evidence of viable
699 Helicobacter pylori and other bacteria of public health interest associated with free-living
700 amoebae in lettuce samples by next generation sequencing and other molecular techniques.

701 International Journal of Food Microbiology 318, 108477.
702 https://doi.org/10.1016/j.ijfoodmicro.2019.108477

703 Mateos-Muñoz, B., Pérez-de-la-Serna, J., Ruiz-de-León, A., Serrano-Falcón, B., Casabona-
704 Francés, S., Velasco-Cerrudo, A., Rey-Díaz-Rubio, E., 2013. Enterohepatic Helicobacter other to
705 Helicobacter pylori. Revista Española de Enfermedades Digestivas 105, 477-485.

706 Ndour, N.Y.B., Budoin, E., Guissé, A., Seck, M., Khouma, M., Brauman, A., 2008. Impact of
707 irrigation water quality on soli nitrifying and total bacterial communities. Biol. Fertil. Soils 44,
708 797-803

709 Øverby, A., Yamagata Murayama, S., Matsui, H., Nakamura, M., 2016. In the Aftermath of
710 Helicobacter pylori: Other Helicobacters Rising Up to Become the Next Gastric Epidemic?
711 Digestion 93,260-5. https://doi.org/10.1159/000445399.

712 Ma, Q., Qu, Y., Shen, W., Zhang, Z., Wang, J., Liu, Z., Li, D., Li, H., Zhou, J., 2015. Bacterial
713 community compositions of coking wastewater treatment plants in steel industry revealed by
714 Illumina high-throughput sequencing. Bioresource Technology 179,436–443.
715 https://doi.org/10.1016/j.biortech.2014.12.041.

716 Quast, C., Pruedde, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P, Peplies, J., Glöckner, F.O.,
717 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-
718 based tools. Nucleic Acids Res, 41 (Database issue): D590-D596.

719 Parsons, L.R., Sheikh, B., Holden, R., York, D.W. 2010. Reclaimed water as an alternative water
720 source for crop irrigation. HortScience 45, 1620-1690.
721 https://doi.org/10.21273/HORTSCI.45.11.1626.

722 Pathak, E., El-Borai, F.E., Campos-Herrera, R., Johnson, E.G., Stuart, R.J., Graham, J.H., Duncan,
723 L.W.,2012. Use of real-time PCR to discriminate parasitic and saprophagous behavior by
724 nematophagous fungi. Fungal Biology 116, 563-573.
725 https://doi.org/10.1016/j.funbio.2012.02.005.

726 Pedrero, F., Kalavrouziotis, I., Alarcón, J.J., Koukoulakis, P., Asano, T., 2010. Use of treated
727 municipal wastewater in irrigated agriculture review of some practices in Spain and Greece.
728 Agricultural Water Management 97, 1233-1241.

729 Pedrero, F., Moinzer, O., Alarcón, J.J., Bayona, J.M., Nicolás, E., 2013. The viability of irrigating
730 mandarin trees with saline reclaimed water in a semi-arid Mediterranean region: a preliminary
731 assessment. Irrigation Science 31, 759-768.

732 Pereira, R.P.A., Peplies, J., Brettar, I., Höfle, M., 2017. Development of a genus-specific next
733 generation sequencing approach for sensitive and quantitative determination of the Legionella
734 microbiome in freshwater systems. BMC Microbiol 17, 79. https://doi.org/10.1186/s12866-
735 017-0987-5.

736 Petersen, R.F., Harrington, C.S., Kortegaard, H.E., On, S.L.W.,2007. A PCR-DGGE method for
737 detection and identification of Campylobacter, Helicobacter, Arcobacter and related
738 Epsilobacteria and its application to saliva samples from humans and domestic pets. Journal of
739 Applied Microbiology 103, 2601-2615.

740 Piqueres, P., Moreno, Y., Alonso, J.L., Ferrúa, M.A., 2006. A combination of direct viable count
741 and Fluorescent in situ hybridization for estimating Helicobacter pylori cell viability. Research
742 Microbiology 157 345-349. https://doi.org/10.1016/j.resmic.2005.09.003.

743 Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source
744 tool for metagenomics. PeerJ, 4: e2584.

745 Safaei, H.G., Rahimi, E., Zandi, A., Rashidipour, A., 2011. Helicobacter pylori as a zoonotic
746 infection: the detection of H. pylori antigens in the milk and feces of cows. Journal of Research
747 in Medical Sciences 16, 184–187.

748 Sánchez, J.V., Nicolás, E., Pedrero, F., Alarcón, J.J., Maestre-Valero, J.F., Fernández, F., 2014.
749 Arbuscular mycorrhizal symbiosis alleviates detrimental effects of saline reclaimed water in
750 lettuce plants. Mycorrhiza 4,339–348.

751 Santiago, P., Moreno, Y., Ferrús, M.A., 2015. Identification of viable Helicobacter pylori in
752 Drinking Supplies by Cultural and Molecular Techniques. Helicobacter 20.
753 https://doi.org/10.1111/hel.12205.

754 Schloss, P.,Gevers, D., Westcott, S., 2011. Reducing the Effects of PCR Amplification and
755 Sequencing Artifacts on 16S Rrna-Based Studies. Plos One 6,e2731
756 https://doi.org/10.1371/journal.pone.0027310.

757 Steele, T.W., McDermott, S.N., 1984. Technical note: the use of membrane filters applied
758 directly to the surface of agar plates for the isolation of Camplylobacter jejuni from faeces.
759 Pathology 6, 263-5. https://doi.org/10.3109/00313028409068535

760 Tu, Q., He, Z., Zhou, J., 2014. Strain/species identification in metagenomes using genome-
761 specific markers. Nucleic Acids Research 42e67. https://doi.org/10.1093/nar/gku138

762 Twing, K.I., Kirchman, D.L., Campbell, B.J., 2011. Temporal study of Helicobacter pylori
763 presence in coastal freshwater, estuary and marine waters. Water Research 45, 1897-1905
764 https://doi.org/10.1016/j.watres.2010.12.013.

765 Vesga, F.J., Moreno, Y., Ferrús, M.A., Campos, C., Trespalacios, A.A., 2018. Detection of
766 Helicobacter pylori in drinking water treatment plants in Bogotá, Colombia, using cultural and
767 molecular techniques. International Journal of Hygiene and Environmental Health 221, 595–
768 601. https://doi.org/10.1016/j.ijheh.2018.04.010.

769 Vierheilig , J., Savio, D., Ley, R.E., Mach, R.L., Farnleitner, A.H., Reischer, G.H,.2015. Potential
770 applications of next generation DNA sequencing of 16S rRNA gene amplicons in microbial
771 water quality monitoring. Water Science and Technology 72, 1962-72.
772 https://doi.org/10.2166/wst.2015.407.

773 Vivaldi, G.A., Camposeo, S., Mastro, M.A., Lacolla, G., Lonigro, A., Rubino, P., 2015. Effect of
774 irrigation with different municipal wastewaters on ripening indexes and chemical components
775 of nectarine fruits. Acta Horticulture 1084,401-408
776 https://doi.org/10.17660/ActaHortic.2015.1084.57.

777 Wagner, M., Loy, A., 2002. Bacterial community composition and function in sewage treatment
778 systems. Current Opinion Biotechnology 13,218–227. https://doi.org/10.1016/S0958-
779 1669(02)00315-4.

780 Wang, S., Levin, R.E.,2006. Discrimination of viable Vibrio vulnificus cells from dead cells in
781 real-time PCR. Journal of Microbiological Methods 64, 1-8.
782 https://doi.org/10.1016/j.mimet.2005.04.023.

783 Wang, X., Hu, M., Xia,Y., Wen, X., Ding, K., 2012. Pyrosequencing analysis of bacterial diversity
784 in 14 wastewater treatment systems in China. Applied and Environmental Microbiology
785 78,7042–7047. https://doi.org/10.1128/AEM.01617-12.

786 Xie, X., Liu, N., Ping, J., Zhang, Q., Zheng, X., Liu, J., 2018. Illumina MiSeq sequencing reveals
787 microbial community in HA process for dyeing wastewater treatment fed with different co-
788 substrates. Chemosphere 201, 578e585. https://doi.org/10.1016/j.chemosphere.2018.03.025

789 Ye, Lin., Zhang, T., 2011. Pathogenic Bacteria in Sewage Treatment Plants as Revealed by 454
790 Pyrosequencing. Environmental Science and Technology 45, 7173-7179.
791 https://doi.org/10.1021/es201045e

792 Ye, Lin., Zhang, T., 2013. Bacterial communities in different sections of a municipal wastewater
793 treatment plan revealed by 16S rDNA 454 pyrosequencing. Applied Microbiology and
794 Biotechnology 97,2681–2690. https://doi.org/ 10.1007/s00253-012-4082-4

795 Zhanf, J., Kobert, k., Flouri, T., Stamatakis, A., 2014a. PERA: a fast and accurate Illumina Paired-
796 End 28ioi merge R. Bioinformatics 30, 614-620.

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798