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Additional Information

Microbiological contamination of conventional and reclaimed irrigation water: evaluation and management measures.

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Abstract

The wide diversity of irrigation water sources (i.e., drinking water, groundwater, reservoir water, river water) includes reclaimed water as a requested measure for increasing water availability, but it is also a challenge as pathogen exposure may increase. This study evaluates the level of microbial contamination in different irrigation waters to improve the knowledge and analyses management measures for safety irrigation. Over a one-year period, the occurrence of a set of viruses, bacteria and protozoa, was quantified and the performance of a wetland system, producing reclaimed water intended for irrigation, was characterized.

Human fecal pollution (HAdV) was found in most of the irrigation water types analysed. Hepatitis E virus (HEV), an emerging zoonotic pathogen, was present in groundwater where porcine contamination was identified (PAdV). The skin-carcinoma associated Merkel cell polyomavirus

(MCPyV), was found occasionally in river water. Noroviruses were detected, as expected, in winter, in river water and reclaimed water. Groundwater, river water and reservoir water also harboured potential bacterial pathogens, like Helicobacter pylori, Legionella spp. and Aeromonas spp. that could be internalized and viable inside amoebas like Acanthamoeba castellanii, which was also detected. Neither Giardia cysts, nor any Cryptosporidium oocysts were detected.

The wetland system removed 3 Log_{10} of viruses and 5 Log_{10} of bacteria, which resembled the river water quality. Irrigation waters were prone to variable contamination levels and according to the European guidance documents, the E. coli (EC) levels were not always acceptable. Sporadic detection of viral pathogens as NoV GII and HAdV was identified in water samples presenting lower EC than the established limit (100MNP/100ml). When dealing with reclaimed water as a source of irrigation the analysis of some viral parameters, like HAdV during the peak irrigation period (summer and spring) or NoV during the coldest months, could complement existing water management tools based on bacterial indicators..

1. Introduction

Surface water and groundwater are considered the main sources for irrigation, worldwide (Gleick, 2009). Those freshwater supplies are becoming insufficient for supporting rapid population growth, a situation exacerbated by inadequate water quality management or water scarcity due to climate change (IPCC, 2019). As recycled water is increasingly accepted as a source of irrigation, pathogen exposure and outbreaks are changing their traditional patterns. In fact, between 2008 and 2011, the European Food Safety Authority reported increases in the numbers of outbreaks, cases, hospitalizations and deaths associated with food of non-animal origin (EFSA, 2013). Consumption of leafy green vegetables irrigated with unsafe water is considered the most common cause of human gastroenteritis illness, due to the presence of bacterial and viral pathogens in the water used for irrigation (FAO, 2013).

Pathogen contamination of ready-to-eat fruits and vegetables can occur at any of the multiple steps from crop to fork. The source of irrigation water and the irrigation method applied play an important

53 role in microbial contamination (Uyttendaele et al., 2015), but quality criteria for irrigation water have
54 only been established where reuse of treated wastewater is common practice. In 2006, the World
55 Health Organization established recommendations for wastewater reuse, based on health risk
56 considerations (WHO, 2006). Some countries developed specific standards on microbial quality for
57 surface water or recycled water used for irrigation, based on Log_{10} removals or maximum allowable
58 concentrations of specific microorganisms. The United Stated of America, Australia and New Zealand
59 established the first guidelines (EPA, 2004; EPHC, NRMMC, 2006), and each state specified water
60 quality standards using different maximum allowable concentrations. Portugal (NP 4434, 2005) and
61 Spain (RD 1620, 2007) also set maximum allowable values per sample, whereas Israel used monthly
62 averages (Inbar, 2007). Cyprus, Greece and Italy set stricter maximum limits, for crops eaten raw,
63 than those legislated for by the other European states (Agrafioti and Diamadopoulos, 2012; Angelakis
64 and Durham, 2008; Kalavrouziotis et al., 2015). On the other hand, different Canadian states as well
65 as France, established minimum Log_{10} reductions in reclaimed water production for irrigation
66 purposes (JORF 0153.29, 2014; Steele and Odumeru, 2004). The European Commission has recently
67 set down the minimum quality requirements for water reuse for agricultural irrigation and produced a
68 guidance document addressing microbiological risks related to agricultural water in the primary
69 production of fresh fruits and vegetables (Alcalde-Sanz and Gawlik, 2017; EU C163, 2017).
70
71 There is no consensus on the best indicator, nor on the optimal sampling frequency for irrigation
72 water management. *E. coli* (*EC*) and Intestinal Enterococci (IE) are used as Fecal Indicator Bacteria
73 (FIB), as detection methods are inexpensive and their presence relates to fecal (animal or human)
74 pollution. But, it is well known that they do not always correlate with important waterborne
75 pathogens that may be present in the diverse irrigation water sources (Girones et al., 2010). As
76 specific screening of every single pathogen is not feasible, a commonly accepted practice is to use
77 multiple indicators. It is also important to settle indicator values for different irrigation water
78 purposes, which may include viral, bacterial or protozoan pathogens.
79

80 Several waterborne pathogens are relatively resistant to conventional water treatment methodologies
81 and can easily appear in irrigation water sources (Adefisoye et al., 2016; Rodriguez-Manzano et al.,
82 2012; Rusiñol and Girones, 2017). Human adenoviruses (HAdV), widely used as fecal indicators
83 (Albinana-Gimenez et al., 2009; Hewitt et al., 2013; Verani et al., 2018), also pose a threat to public
84 health since they may cause gastrointestinal and respiratory diseases. It is well known that HAdV,
85 stable under many environmental conditions and disinfection treatments, are shed in high
86 concentrations and do not show seasonality (Allard and Vantarakis, 2017). Other enteric viruses, like
87 noroviruses (NoV) or enteroviruses (EV), are excreted in greater concentrations from infected
88 individuals during clinical infections in the seasons with high incidence and then decrease over time
89 (Atmar et al., 2008). Whereas NoV are the leading worldwide cause of gastroenteritis and may be the
90 most important etiologic agent with respect to recycled water, EV go beyond gastroenteritis and cause
91 a diversity of diseases, such as meningitis, paralysis or myocarditis (Kocwa-Haluch, 2001; Koo et al.,
92 2013; Soller et al., 2018; WHO, 2013). Human polyomaviruses are also prevalent in fecally
93 contaminated water bodies. JC polyomavirus (JCPyV) is persistently excreted over a lifetime and has
94 been shown to be human specific, which is not the case with fecal indicator bacteria (Bofill-Mas et al.,
95 2000; McQuaig et al., 2009). Merkel cell polyomavirus (MCPyV) was the first virus detected in
96 environmental samples to have been described as having carcinogenic potential (Bofill-Mas et al.,
97 2010; Rusiñol et al., 2015). In 2008, MCPyV was first related to neuroendocrine tumors in elderly
98 and/or immunosuppressed people (Feng et al., 2008). Finally, hepatitis E virus (HEV), causing acute
99 hepatitis in humans, is mainly transmitted through waterborne, foodborne and zoonotic routes and has
100 been closely related to irrigation water contamination (Kokkinos et al., 2017; Yugo and Meng, 2013).
101 Bacteria such as *Legionella* spp., *Aeromonas* spp., *Arcobacter* spp., *Campylobacter* and *Helicobacter*
102 *pylori* have been recognized as emerging pathogens in water, and have been also identified in
103 wastewater and reclaimed water sources (Collado and Figueras, 2011; Fernandez-Cassi et al., 2016;
104 Figueras and Borrego, 2010). Many of these pathogens are able to adhere to biofilms, but in addition,
105 they may be associated with free-living protozoa, including amoebae. Both situations provide acting
106 reservoirs for these pathogens and protect them from the effects of disinfection treatments. Also,
107 *Giardia* cysts and *Cryptosporidium* oocysts, common waterborne parasites infecting humans and

108 animals, are ubiquitous in wastewater and they are frequently included in water management as Fecal
109 Indicator Protozoa (FIP). *Blastocystis* are one of the most common single-celled intestinal parasites
110 found in human stool samples and in a wide variety of domestic animals and wildlife (Souppart et al.,
111 2009).

112 Constructed wetlands, with surface flow, are being considered as low-cost technologies for reclaimed
113 water production. These wetlands are used as an additional step (tertiary treatment systems) after
114 secondary treatments and have proved to be efficient at reducing nitrogen and removing organic
115 micropollutants (Llorens et al., 2009; Matamoros et al., 2008). The positive environmental values of
116 these passive treatment systems for wastewater reclamation have been extensively reviewed
117 (Ghermandi et al., 2010). Compared to other advanced treatment systems (e.g., reverse osmosis or
118 membrane bioreactors), the price of the water that flows through the wetland cells is relatively low
119 and has been calculated to range from €0.71 to €0.75 per m^{-3} (Alfranca et al., 2011). Moreover, the
120 seasonal water demand for agriculture, which is a challenge for advanced reclaimed water facilities
121 (NCR, 2012), can be solved using these sustainable systems.
122 This study evaluates the presence and levels of important circulating pathogens and indicators in
123 diverse sources of irrigation water and proposes evaluation and management measures. Here it is also
124 evaluated the performance of a constructed wetland system as a green tertiary treatment system
125 producing reclaimed water intended for irrigation.

126 **2. Methods**

127 ***2.1. Sampling and microbial parameters analysis***

128 Different sources of irrigation water were selected: drinking water, reservoir water, groundwater, river
129 water and reclaimed water produced in a sustainable tertiary treatment (constructed wetland). To
130 enable quantification of the concentration of pathogens in the main source of microbial pollution
131 coming into the irrigation water bodies, raw sewage and secondary treated effluents were also
132 collected. Conductivity, pH and water temperature data were determined in the field for each sample.

133 Drinking water was sampled from distribution water tanks. Reservoir water was selected from a dam
134 created to store water intended for irrigation, as it is a common source of irrigation. When needed, the
135 reservoir water can be released into irrigation water channels for downstream orchard irrigation. River
136 water samples were collected from the Fluvià River. This 100-km long river receives the effluents
137 from 24 small wastewater treatment plants (WWTPs) treating up to 100,000 PE and it is also
138 impacted by intensive farming and agricultural activities. Groundwater sampling sites were located at
139 the final section of the river, hosting intensive pig and poultry farming.

140 Reclaimed water was collected from a sustainable wetland system (also known as passive natural
141 treatment system) which receives part of a secondary treated WWTP effluent. The WWTP, treating
142 approximately 112,000 PE, uses a Conventional Activated Sludge (CAS) and chlorinates part of the
143 secondary effluent (70%) before discharging into the river. The remaining part of the WWTP effluent
144 (30%) is conducted to the wetland system to reduce nitrogen and phosphorus, after a retention time of
145 three days. This constructed wetland covers an area of 1 ha and receives a secondary treated effluent
146 flow of between 100 m^3 and 250 m^3 per day. In a single cell, a mixture of *Phragmites australis* and
147 *Typha latifolia* was planted and has proved successful at removing contaminants (Alfranca et al.,
148 2011; Llorens et al., 2009; Matamoros et al., 2008).

149 Drinking and reservoir water samples were collected monthly for 6 months. River water, groundwater
150 and reclaimed water, as well as raw sewage and secondary effluent samples, were collected every
151 month (12 samples each) over a period of one year, from April 2015 to March 2016. A total of 72
152 irrigation water, 12 raw sewage and 12 secondary treated water samples were collected and
153 distributed after each sampling among the partner laboratories for the viral, bacterial and protozoan
154 analyses. FIB and Heterotrophic bacteria counts (HBC) were analysed from 500 mL of each sample
155 within 24 h of collection. All human (HAdV, JCPyV, MCPyV, NoV GGI and GGII, EV and HEV)
156 and animal viruses (bovine polyomavirus (BPyV), porcine adenovirus (PAdV) and avian parvovirus
157 (ChTyPV)), bacteria (*Aeromonas* spp., *Arcobacter* spp., *Helicobacter pylori*, *Legionella* spp.) and
158 protozoa (*Blastocystis* spp., *Acanthamoeba castellanii, Cryptosporidium* spp. *and Giardia* spp.*)* were
159 analysed from a volume of 10 litres of irrigation water or 500 mL of sewage and secondary effluent

160 samples, using molecular based methods after a single Skimmed Milk Flocculation (SMF)
161 concentration protocol (section 2.3.).

162 ***2.2. Fresh water analysis***

163 *2.2.1. Heterotrophic bacteria quantification*

164 Heterotrophic Bacteria were determined and quantified in all the water samples in accordance with
165 ISO 6222:1999 (International Organization for Standardization, 1999), following the standards for
166 water quality (Bartram et al., 2003). Briefly, ten-fold dilution series were prepared in Ringer 1/4
167 (Scharlau Chemie), plated in Plate Count Modified Agar (Scharlau Chemie) and incubated at 22°C for
168 72 h. The limit of detection (LOD) was 50 MPN per 100 mL.

169 *2.2.2. FIB quantification*

170 For FIB detection (*EC* and IE), 100 mL of each sample was collected in parallel from all sites. All
171 samples were kept on ice and processed within 24 h. The enumeration of *EC* and IE was carried out
172 with the 96-well microplate systems (MUG/EC 355-3782 and MUG/EC 355-3783, BioRad®,
173 respectively), according to ISO 9308-2:2012 and ISO 7899-1:1998 (International Organization for
174 Standardization, 2012, 1998), respectively.

175 ***2.3. A single concentration method for viruses, bacteria and protozoa***

176 This study was conducted using Standardized Operational Procedures (SOPs) for viral, bacterial and
177 protozoan concentration, nucleic acid extraction and quantitative detection. All microorganisms were
178 concentrated using the SMF protocol (Gonzales-Gustavson et al., 2017). Irrigation water (10 L) as
179 well as raw sewage and secondary treated effluent samples (500 mL) were acidified to pH 3.5 using 1
180 N HCl. The conductivity was also measured and adjusted with artificial sea salt (Sigma) to achieve a
181 minimum conductivity of 1.5 mS/cm^2. Separately, a Pre-flocculated Skimmed Milk solution (PSM)
182 was prepared by dissolving 10 g of skimmed milk powder (Difco) in 1 L of artificial seawater and
183 adjusting the pH to 3.5. The PSM was added to the previously conditioned samples to obtain a final

184 concentration of 0.01% of skimmed milk. All samples were stirred for 8 h at room temperature and
185 the flocs were allowed to settle by gravity for another 8 h. The supernatants were removed and the
186 sediment was collected and transferred to 500 mL centrifuge containers and centrifuged at 8000 × g
187 for 30 min at 4°C. Pellets were suspended in 5 mL of 0.2 M phosphate buffer at pH 7.5 (1:2, v/v of
188 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4), distributed in refrigerated boxes among partner laboratories
189 and stored at −20°C until the nucleic acid (NA) extractions were performed.

190 ***2.4. Virus quantification***

191 Viral nucleic acids (NA) were extracted from 140 µL of the SMF concentrate using a QIAamp® Viral
192 RNA Mini Kit (Qiagen) and the automated QIACube system (Qiagen), following the manufacturer's
193 instructions. PCR inhibitors were removed by pre-centrifugation of lysate samples before using the
194 automated extraction system. Specific real-time quantification of DNA viruses (HAdV (Bofill-Mas et
195 al., 2006; Hernroth et al., 2002), JCPyV (Pal et al., 2006), MCPyV (Rusiñol et al., 2015), BPyV
196 (Hundesa et al., 2010), PAdV (Hundesa et al., 2009) and Ch/TyPV (Carratalà et al., 2012)) by qPCR
197 or RNA viruses (NoV GGI (da Silva et al., 2007; Hoehne and Schreier, 2006; Svraka et al., 2007) and
198 NoV GGII (Kageyama et al., 2003; Loisy et al., 2005), EV and HEV (Jothikumar et al., 2006)) by
199 quantitative reverse transcription PCR (qRT-PCR), were performed as previously described using
200 TaqMan® Universal PCR Master Mix and the RNA UltraSense™ One-Step qRT-PCR System,
201 respectively (Invitrogen). Quantification was performed with an MX3000P sequence detector system
202 (Stratagene). The standards for viruses were prepared using synthetic gBlocks® Gene Fragments
203 (IDT) (supplementary material) and quantified with a Qubit® fluorometer (Thermo Fisher Scientific).
204 The LOD in 100 mL of water of the (RT)qPCR assays was found to be 21 GC for HAdV, 29 GC for
205 JCPyV, 57 GC for MCPyV, BPyV, PAdV and ChTyPV, 41 GC for NoV GGI and 296 GC for NoV
206 GGII, 81 GC for HEV and 414 GC for EV, following the WHO manual (FAO, 2015). Undiluted and
207 10-fold dilutions of the nucleic acid extracts were analysed in duplicate. The equivalence of 105 mL
208 for DNA viruses and 52.5 mL for the RNA virus were tested from the original irrigation water
209 samples, whereas 5.3 mL and 2.6 mL, respectively, were tested from sewage and secondary effluents.
210 All qPCRs included three non-template control (NTC) to demonstrate that the mix did not produce

211 fluorescence due to contamination.

212 ***2.5. Bacteria analysis***

213 *2.5.1. Legionella* spp. quantification

214 Nucleic acids were extracted from 1 mL of sample concentrates using a Wizard genomic DNA
215 purification kit (Promega). All samples were tested for the presence of *Legionella* spp. using a
216 modified qPCR assay. In summary, a final volume of 25 µL, containing 0.9 µM of each primer
217 (Cervero-Aragó et al., 2015; Herpers et al., 2003), 0.2 µM of the FAM-TAMRA probe with an
218 annealing temperature of 53ºC (Cárdenas Youngs, 2018), 12.5 µL of 1× TaqMan® Universal Master
219 Mix (Invitrogen) and 5 µL of the extracted nucleic acids. The standards for *Legionella* spp. were
220 prepared using DNA extracted from an *L. pneumophila* ATCC 33152 culture and quantified with a
221 Nanodrop. The equivalence of 105 mL was tested from the original irrigation water samples whereas
222 5.3 mL and 2.6 mL, respectively, were tested from sewage and secondary effluents. The LOD was
223 200 GC per 100 mL.

224 *2.5.2. Arcobacter* spp. and *Aeromonas* spp. quantification

225 Bacterial DNA was extracted with the DNeasy PowerSoil kit (Qiagen), following the manufacturer's
226 instructions. The DNA was quantified and checked for quality by using the NanoDrop instrument
227 (NanoDrop Products). A real-time PCR (qPCR) was performed to quantify the *Aeromonas* spp. and
228 *Arcobacter* spp., by using the StepOneplus^{TM} Real-Time PCR System (Applied Biosystems) and DNA
229 Target Species specific dtec-qPCR Test (Genetic PCR Solutions) for each genus. The threshold cycle
230 (Ct) was determined using StepOne software v2.3. The LOD was found to be 5 genome copies of the
231 target.

232 *2.5.3. Helicobacter pylori* quantification

233 DNA was extracted using FastDNA® SPIN Kit for soil (MP Biomedicals), following the
234 manufacturer's instructions. All samples were tested for the presence of *H. pylori,* by means of qPCR.

235 Briefly, the *H. pylori* specific qPCR, based on SYBR Green I fluorescence, was carried out using
236 VacA primers to amplify a 372 bp fragment (Nilsson et al., 2002) in LightCycler® 2.0 Instrument
237 (Roche Applied Science). The final reaction volume was 20 µL, which contained: 2 µL of
238 LightCycler® FastStart DNA SYBR Green I (Roche Applied Science), 1.6 µL of $MgCl_2$ (50 mM), 0.5
239 µL of each primer (20 µM) and 2 µL of DNA template. The amplification consisted of an initial DNA
240 denaturalization at 95°C for 10 min, followed by: 40 cycles each of 95°C for 10 s, 62°C for 5 s and
241 72°C for 16 s; and finally, one cycle at 72°C for 15 s and one at 40°C for 30 s (Santiago et al., 2015).
242 Amplifications were made in triplicate. A positive control with *H. pylori* DNA (reference strain:
243 NCTC 11637) and a control of external contamination, qPCR mix without DNA, were added to the
244 qPCR analysis.

245 ***2.6. Protozoa analysis***

246 A volume of 300 µl of each SMF concentrate was lysed using the FastPrep®-24 instrument (MP
247 Biomedicals). Samples were first homogenized for 60 s. After the bead beating step, samples were
248 placed on ice for 1 min and then homogenized for another 60 s. DNA was extracted with the
249 FastDNA® SPIN Kit (MP Biomedicals) for soil, according to the manufacturer's instructions. The
250 final DNA products were eluted in a final volume of 50 µL. Real-time PCR (qPCR) assays for
251 detection of *Giardia* spp., *Cryptosporidium* spp., *Acanthamoeba* spp. and *Blastocystis* spp. were
252 performed as previously described (Moreno et al., 2018).

253 ***2.7. Log reduction values and analysis of season and water type effects***

254 Following analysis of the recovered microorganisms the Log_{10} reduction values (LRV) were
255 calculated according to the formula: LRV = - Log_{10} (concentration in effluent / concentration in
256 influent). Where the resultant effluent concentration was a none detected, the LOD values were
257 assumed for the calculation. In order to assess the significance of season and water type we adjusted a
258 linear model for the Log_{10} value of the counts of every organism. The model included the four
259 physical-chemical variables measured as covariates. For organisms detected in two or more types of
260 water at least in two samples per season the equation was:

261 $$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma\, p_{ijk} + \delta\, c_{ijk} + \eta\, u_{ijk} + \lambda\, t_{ijk} + \varepsilon_{ijk}$$

262 Where y_{ijk} was the Log_{10} of the counts, μ the overall mean, α_i the effect of the *i*-th season, β_j the effect
263 of the *j*-th water type and $(\alpha\beta)_{ij}$ the interaction of both effects. The continuous covariates were pH
264 (p_{ijk}), conductivity (c_{ijk}), turbidity (u_{ijk}) and temperature (t_{ijk}) with γ, δ, η and λ standing for their
265 respective regression coefficients. Finally, ε_{ijk} was the random error of the *k*-th replicate. Several
266 viruses were detected in raw sewage, but were mostly undetected in some, or all, the periods studied
267 for the rest of water types. For these cases, we considered a simplified version of the model without
268 the season factor. All models were analysed using the *lm* method of the R software, version 3.6.1 (R
269 Core Team, 2019).

270 **3. Results**

271 ***3.1. Microbiological contamination of irrigation water***

272 *3.1.1. Conventional irrigation water sources: drinking water, groundwater, reservoir and*
273 *riverwater.*

274 Results obtained for fecal indicator organisms and specific pathogens are summarized in Table 1.
275 Drinking water was the only source of water in which no microorganisms were detected. HAdV were
276 occasionally found in 2/12 samples from groundwater and river water. The FIB were persistently
277 found over the sampling year. *EC* was more prevalent in river water (12/12) than in reclaimed water
278 (10/12), whereas IE was more commonly found in reclaimed water (11/12) than in river water (7/12).
279 Groundwater and reservoir water sources always presented lower FIB concentration levels and
280 prevalence, but in contrast, *Aeromonas* and *Arcobacter* showed higher concentrations. No FIP were
281 detected in any of the irrigation water sources analysed.

282 Besides the HAdV detection, other viral pathogens were observed in irrigation water. MCPyV and
283 NoV GGII were detected in river water during the coldest months (2/6 in both cases) and HEV was
284 occasionally present in groundwater (1/12). All viral concentrations were near the detection limit of
285 the technique (Table 1). Heterotrophic bacteria were present in all irrigation water samples (except

drinking water) at mean Log_{10} concentrations in a range between 2.42 and 5.55 Log CFU/100 mL. *Aeromonas* spp. and *Arcobacter* spp. prevalence was higher in river water than in groundwater, but concentrations showed higher fluctuations in groundwater samples. *Legionella* spp. was found in 7/12 of groundwater samples, 5 of the positive results being observed during the warmest seasons. *Helicobacter pylori* was detected in the groundwater and river water samples tested. *Acanthamoeba castellanii* was found in all types of conventional irrigation water (except drinking water).

3.1.2. Reclaimed water.

Viral, bacterial and protozoan concentrations in reclaimed water (wetland effluents) are shown in Table 1. Fecal contamination was very prevalent although detected in low concentrations: 2.02 and 1.54 Log_{10} MPN/100 mL of EC and IE, respectively. Heterotrophic bacteria, *Helicobacter pylori* and *Acanthamoeba castellanii,* were detected in all reclaimed water samples analyzed whereas *Legionella* spp. was found in 33% of the tested water. *Blastocystis* sp., *Cryptosporidum* spp. and *Giardia* spp. were not present in wetland water after treatment.

HAdV was detectable throughout the sampling year in all raw and secondary effluents, but only one third of the reclaimed water samples tested positive for this virus (Table 2). Mean concentrations decreased significantly (see figure 2 and supplementary material 2) throughout the treatment, being 4.52, 3.04 and 2.11 Log_{10} GC/100 mL in raw, secondary and reclaimed water, respectively. Viral pathogens, like polyomaviruses (JCPyV and MCPyV) and noroviruses (NoV GGI and GGII), were also detected in 100% of raw sewage, but after Conventional Activated Sludge (CAS) treatment and the duration of retention in the wetland system, their prevalence dropped to under 25% positive sampling (Table 2).

3.2. Performance of the sustainable wetland as a water reclamation system

Figure 1 summarizes in boxplots the most prevalent viruses and FIB concentrations across the water reclamation process, including CAS and the sustainable wetland system. Although concentrations of NoV GGI and GGII in raw sewage were higher than HAdV, with respective mean and maximum

311 values of 2.26 and 1.61 Log_{10} GC/100 mL for NoV GGI and 1.51 and 1.52 Log_{10} GC/100 mL for
312 NoV GGII, they were less prevalent than HAdV in the wetland effluent (Table 2). The water
313 reclamation system reached means of 3.42 and 2.97 total LRV for NoV GGI and NoV GGII,
314 respectively. FIB showed a similarly high removal behavior, but the percentage of positive samples at
315 the end of the process was still persistent. EV and HEV were occasionally detected in sewage and
316 secondary effluents. A seasonal distribution of HAdV, EC and IE was not clearly observed in raw
317 sewage (Figure 2), but a different behavior was observed in the secondary effluents. While both virus
318 levels were relatively constant in the treated effluents, showing no significant effects of season nor
319 interaction water type-season, FIB concentrations exhibited peaks in the spring samplings, showing
320 significant effect of season (supplementary material). Important viral pathogens, like NoV, presented
321 higher median concentration during winter and spring. After the activated sludge process, NoV GGI
322 was not detected during autumn or summer. In general, NoV GGI and GGII mean concentrations
323 were higher than HAdV, but HAdV was the most stable over the year, both in secondary effluents and
324 after passing through the wetland system.

325 ***3.3. Origin of the fecal contamination***

326 Table 3 summarizes the concentrations and percentage rates of detection of MST markers in different
327 irrigation water samples and raw sewage. Human fecal contamination (HAdV) was detected in 17%
328 of the groundwater and river water samples, and 33% of the reclaimed water samples, at similar
329 concentrations. Porcine fecal pollution (PAdV) was very prevalent (44%) in the groundwater samples.
330 Mean concentrations of porcine fecal pollution reached 2.47 Log_{10} GC/100 mL in groundwater.
331 Bovine (BPyV) and avian (Ch/TyPV) fecal indicators were only detected when there were cow and
332 chicken farms near the extraction well. It is also interesting to note the detection of the emergent
333 zoonotic virus HEV in the sample from November, with a value of 2.83 Log_{10} PAdV GC/100 mL.

334 **4. Discussion**

335 The SMF method proved to be useful for the concentration of microorganisms after monitoring the
336 microbial quality of different types of irrigation water applying molecular methods. As previously

337 reported (Calgua et al., 2013; Rusiñol et al., 2015, 2014), this concentration method is robust and easy
338 to implement for simultaneous concentration of viruses, bacteria and protozoa (Gonzales-Gustavson
339 et al., 2017). The harmonization of the concentration method, for the further detection of indicators
340 and pathogens, may allow water managers to use mathematical approximations when calculating
341 concentrations according to acceptable prediction intervals.
342
343 ***4.1. Irrigation water quality: conventional and reclaimed water sources.***
344
345 Chlorinated drinking water was the only irrigation water source with no pathogen detection, but in
346 terms of costs, the use of drinking water for irrigation purposes is unaffordable as well as unavailable
347 in many regions. In general, fecal pollution was found in a high percentage of the samples by means
348 of FIB. Occurrences of EC in river water samples were the highest in irrigation water (100%),
349 whereas in reclaimed water both EC and IE were frequently detected (10/12 samples and 11/12
350 samples respectively) in low concentrations. The fact that IE are distinguished by their ability to
351 survive in more complex matrices, underscores their use as FIB in more complex water matrices. It is
352 also important to state that changes in the WWTP management could explain FIB fluctuations in the
353 treated effluents during spring.
354 During this one-year surveillance, HAdV was detected in groundwater (17%), river water (17%) and
355 reclaimed water (33%), confirming the human origin of the fecal contamination. This human
356 pathogen is widely detected when water is impacted by sewage (Bofill-Mas et al., 2013; Rusiñol et
357 al., 2014; Rusiñol and Girones, 2017; Vieira et al., 2016). NoV occurrence in river water has been
358 reported when rain events introduce large amounts of pathogens into the receiving water bodies (Hata
359 et al., 2014), during peak infection periods or due to viral outbreaks (Kauppinen et al., 2018).
360 Although we did not detect NoV in groundwater, it has been reported that this highly infectious
361 pathogen remains infective in groundwater for long periods (Seitz et al., 2011). MCPyV was found in
362 2 of the 12 river water samples, as reported in other studies (Rusiñol et al., 2015). This skin virus is
363 persistently excreted in sewage (Bofill-Mas et al., 2010), so its presence highlights its dissemination
364 into the environment and its resistance to water treatment technologies.

365

366 Emerging pathogens, like HEV, *Arcobacter* spp. and *Helicobacter pylori*, were also detected. HEV
367 presence in groundwater may be attributed directly to the presence of livestock in the aquifer recharge
368 area, as porcine fecal pollution (PAdV) was also detected and no human viruses were found in that
369 sample. Previous studies have evidenced the impact of the presence of livestock and agricultural
370 practices on the microbial quality of river water (Rusiñol et al., 2014). Considering that groundwater
371 provides half of all drinking water worldwide or that 70% of groundwater withdrawal is used for
372 agriculture (FAO, 2019), it is important to consider the potentially infective pathogens that are found
373 in this type of matrix. From a one-health perspective, the putative risks to farm animals should also be
374 considered when engineering the irrigation of feeding crops. *Arcobacter* spp. is highly resistant to
375 sanitation and disinfection treatments, as well as showing tenacious survivability in the environment
376 (Banting and Figueras, 2018). Canadian researchers showed that it is frequently detected in irrigation
377 water, where it is often underestimated due to the cross-amplification with *Campylobacter* (Banting et
378 al., 2016).
379

380 In this study, groundwater, river water and reservoir water all harbored potential bacterial pathogens,
381 like *Helicobacter pylori*, *Legionella* spp. and *Aeromonas* spp. The association of these bacteria with
382 biofilms can act as a reservoir in irrigation waters (Richards et al., 2018). In fact, *Helicobacter pylori,*
383 as previously stated for *Legionella* spp., can be internalized and viable inside *Acanthamoeba*
384 *castellanii* (Moreno-Mesonero et al., 2016), which could also be detected in all samples tested. The
385 presence of *Aeromonas* has been related to stagnant water with low/no levels of chlorine and presence
386 of organic matter (Figueras and Ashbolt, 2019). Our persistent detection of *Helicobacter pylori* in the
387 untreated irrigation water sources has been related to the exposure to sewage (Bellack et al., 2006).
388 According to the Spanish regulation for water reuse (RD 1620, 2007), the occurrence of *Legionella*
389 spp. in this study would restrict the use of reclaimed water for drop irrigation of produce intended for
390 raw consumption.
391

392 Reclaimed water and river water presented similar HAdV and NoV concentrations, although viral
393 occurrences were higher in the wetland effluents. Human-specific JC polyomavirus was only detected
394 in reclaimed water in November. This virus is very prevalent in wastewater worldwide and low
395 reductions have been reported after CAS (Mayer et al., 2016; Rusiñol et al., 2015). When tertiary
396 treatments are applied, different reductions are observed but JCPyV is still frequently detected. In
397 accord with our results, Rachmadi and collaborators reported removals below the LOD in subsurface
398 wetlands (Rachmadi et al., 2016). Both the LOD of the technique (29GC in 100 mL) and the low
399 volume of the original sample represented in the analysis (35 mL) may explain the absence of positive
400 results.

401

402 If we check the minimum quality criteria set down by the EU for reclaimed water used as class A
403 irrigation water (Alcalde-Sanz and Gawlik, 2017), only drinking water could be used for crops where
404 the edible portion is in direct contact with the irrigation water (class A), because only there were the
405 *EC* levels below the LOD. Groundwater, reservoir and reclaimed water would be in class B (*EC* ≤100
406 cfu/100 mL) and could be used for raw consumption crops only where the edible part is produced
407 above ground and is not in direct contact with the irrigation water. According to our results, river
408 water would be in class C (*EC* ≤1000 cfu/100 mL) and the irrigation method for edible vegetables
409 should be limited to drip systems.

410

411 ***4.2. Microbial removals in a sustainable wetland system***

412

413 There is an increasing amount of evidence regarding the presence of viral pathogens in reclaimed
414 water used for irrigation (López-Gálvez et al., 2016; Randazzo et al., 2016). HAdV are being used as
415 wastewater reclamation indicators, together with FIB, because they are more resistant to removal than
416 other viruses (Kitajima et al., 2014; Prado et al., 2019; Sidhu et al., 2018). In our study, their numbers
417 varied from 1.12 to 2.92 Log_{10} GC/100 mL, which is comparable to the reported numbers in other
418 constructed wetlands (Rachmadi et al., 2016). In total, the wetland fed with secondary effluent

419 reduced 3.14 Log_{10} of HAdV a nd 5.17 Log_{10} of *EC*. Comparing Log_{10} removals of HAdV in diverse
420 reclaimed water production systems (Table 4) shows that advanced sewage treatments achieve higher
421 efficiencies (5.20 Log_{10}), but they also have important operational and maintenance costs to be
422 considered (Guo et al., 2014; Hunter et al., 2018; Liu et al., 2013; Prado et al., 2019).
423 Our treatment process achieved a mean 3.23 Log_{10} removal of HBC, similar to the reported removal
424 when wastewater is treated in conventional wastewater reclamation processes (CAS + chlorination)
425 (Al-Jassim et al., 2015). The analysis of HBC has little value as an indicator of pathogen presence,
426 but can be used in assessing regrowth and presence of biofilms in the reclaimed water system.
427
428 Following the health target of $<10^{-6}$ DALY's per person per year for safe drinking-water, the WHO
429 established performance values, or minimum Log_{10} removals, of three reference pathogens: a virus
430 (5.0 Log_{10} of rotavirus), a bacterium (4.0 Log_{10} of *Campylobacter*) and a protozoan (4.9 Log_{10} for
431 *Cryptosporidium*) (WHO, 2017). The European directive does not compel member states to monitor
432 pathogens, and only recommends translating the *EC* monitoring data into treatment performance
433 targets (WHO, 2017). As irrigation water should be free of contamination and, where possible, have
434 of the same quality as drinking water, a similar approach could be used for irrigation water. A recent
435 publication in our group, quantifying the risk of using the wetland effluent to irrigate lettuce,
436 established that the disease burden of NoV GGII and HAdV was higher than 10^{-6} DALYs (Gonzales-
437 Gustavson et al., 2019). Thus, additional disinfection treatment would be required to irrigate these
438 types of crops with reclaimed water produced in the studied wetland system.
439

440 ### ***4.3. Monitoring irrigation water quality***

441

442 The first microorganism included in the monitoring of water quality and water reuse legislation was
443 *EC* (RD 1620, 2007; WHO, 2017). It is prevalent through seasons in different irrigation water
444 sources, but as stated before, it does not always correlate with the presence of other pathogens. The
445 European Food Safety Authority identified *Salmonella, Yersinia, Shigella* and noroviruses as the most
446 important risks within food of non-animal origin, but the guidance document for irrigation water only

447 fixes *EC* maximum thresholds as an indicator of fecal contamination (EFSA BIOHAZ Panel, 2017;
448 EU C163, 2017). With the single recommendation of *EC* testing, most of the results of this study,
449 including different sources of irrigation water, would meet the EU requirements for irrigation of
450 ready-to-eat vegetables and fruits. Nevertheless, in some particular cases (e.g., groundwater), where
451 fecal pollution is occasional and viruses can survive longer periods, it is necessary to consider human
452 and animal specific MST indicators when evaluating microbial water quality.

453 When agricultural water comes into direct contact with the edible portion of a crop, or the source of
454 irrigation water is vulnerable to contamination, the introduction of viral parameters would
455 complement the information used by water managers. Regarding public health, it is necessary to
456 include direct indicators of risk. *Bacteriodes* spp., *Bifidobacterium* spp., bacteriophages, *Clostridium*
457 *perfringens* and HAdV analyses have been proposed to evaluate reclaimed water quality (Bofill-Mas
458 et al., 2013; Bourrouet et al., 2001; Verani et al., 2018), but there are no compelling data about their
459 utility for irrigation water monitoring. Our study of this type of water confirms the prevalence of
460 HAdV through seasons and its low removal during treatment, supporting the argument for use of this
461 waterborne pathogen together with FIB for characterization of irrigation water quality. The risk
462 associated with the presence of viral pathogens supports the use of qPCR for irrigation water
463 management, even if some degree of overestimation of risk has been suggested (Symonds and
464 Breitbart, 2015). Although direct pathogen screening is not feasible, when water is used to irrigate
465 ready-to-eat fruits and vegetables, we recommend including NoV testing in peak concentration
466 months, to validate and complement existing management strategies.

467 Besides FIB and HAdV, *Legionella* spp. analysis should also be considered, depending on the crop
468 and the irrigation system. In fact, the Spanish legislation includes maximum acceptable values for
469 *Legionella* when there is aerosolization and/or potential regrowth. Values (100 or 1000 cfu/mL) and
470 minimum analytical frequencies (every two weeks and once a month) will depend on the usage of the
471 reclaimed water for irrigation.

472

473 It is assumed that human pathogens are present in low concentrations in irrigation water. However,
474 this will be directly related to the disinfection treatment to which the water has been submitted and its
475 proper storage. *Aeromonas* and *Arcobacter* have been found in lagooning reclaimed water, and the
476 former also in parsley and tomatoes irrigated with water contaminated with these bacteria (Fernandez-
477 Cassi et al., 2016; Latif-Eugenín et al., 2017). The SMF method allowed for the evaluation of a
478 representative volume (10 L) for simultaneous monitoring of waterborne viruses, bacteria and
479 protozoa. This concentration method would reduce costs and facilitate periodic testing of different
480 irrigation water sources. Further investigations are necessary to obtain larger data sets and to assess
481 specific pathogen serotypes.
482
483 **5. Conclusions**
484 Considering the current guidelines at the EU, with the single recommendation of *EC* testing, most of
485 the sources of irrigation evaluated here would meet the EU requirements. However sporadic detection
486 of viral pathogens was found in water samples with *EC* values lower than 100 MPN/100ml. It is
487 assumed that groundwater is less vulnerable to fecal pollution than reservoir or river water, but the
488 detection of porcine fecal pollution (PAdV) and an emergent pathogen as HEV, would confirm that
489 pigs act as a reservoir of this viruses and enhances the importance of having a good characterization
490 of this irrigation source.
491 Compared to other microorganisms evaluated, HAdV presented low reduction values in the wetland
492 system, demonstrating its high resistance to treatment. Due to the higher demand for reclaimed water
493 for agriculture during the warm season, when noroviruses where not detected, we would recommend
494 evaluating the presence of HAdV as a complementary management measure of the performance of the
495 water reclamation process. A viral pathogen like NoV might be considered during the coldest months.
496 Neither Giardia cysts, nor any Cryptosporidium oocyst where detected in the analysed water samples,
497 showing a low prevalence of these protozoa in the irrigation water sources studied.
498 Groundwater, river water and reservoir water also harboured potential bacterial pathogens, like
499 *Helicobacter pylori*, *Legionella* spp. and *Aeromonas* spp. that could be internalized and viable inside
500 amoebas like *Acanthamoeba castellanii,* which was also detected. The detection of ubiquitous

501 potential bacterial pathogens and free-living amoebae should be also considered when evaluating the
502 role that irrigation water could play in the transmission of bacterial pathogens, been internalized
503 bacteria more resistant to disinfection processes.
504

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513

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