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

Marta Rusiñol[a], Ayalkibet Hundesa[a], Yexenia Cárdenas-Youngs[a], Ana Fernández-Bravo[b], Alba Pérez-Cataluña[b], Laura Moreno-Mesonero[c], Yolanda Moreno[c], Miquel Calvo[d], Jose Luis Alonso[c], Maria José Figueras[b], Rosa Araujo[a], Sílvia Bofill-Mas[a] and Rosina Girones[a].

[a] Section of Microbiology, Virology and Biotechnology, Department of Genetics, Microbiology and Statistics. Faculty of Biology, University of Barcelona, Catalonia, Spain

[b] Microbiology Unit, Faculty of Medicine and Health Sciences, IISPV, University Rovira and Virgili. Reus, Catalonia, Spain

[c] Instituto de Ingeniería del Agua y Medio Ambiente, Universitat Politècnica de València. Valencia, Spain.

[d] Section of Statistics, Department of Genetics, Microbiology and Statistics, Faculty of Biology, University of Barcelona, Barcelona, Catalonia, Spain

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

role in microbial contamination (Uyttendaele et al., 2015), but quality criteria for irrigation water have only been established where reuse of treated wastewater is common practice. In 2006, the World Health Organization established recommendations for wastewater reuse, based on health risk considerations (WHO, 2006). Some countries developed specific standards on microbial quality for surface water or recycled water used for irrigation, based on Log_{10} removals or maximum allowable concentrations of specific microorganisms. The United Stated of America, Australia and New Zealand established the first guidelines (EPA, 2004; EPHC, NRMMC, 2006), and each state specified water quality standards using different maximum allowable concentrations. Portugal (NP 4434, 2005) and Spain (RD 1620, 2007) also set maximum allowable values per sample, whereas Israel used monthly averages (Inbar, 2007). Cyprus, Greece and Italy set stricter maximum limits, for crops eaten raw, than those legislated for by the other European states (Agrafioti and Diamadopoulos, 2012; Angelakis and Durham, 2008; Kalavrouziotis et al., 2015). On the other hand, different Canadian states as well as France, established minimum Log_{10} reductions in reclaimed water production for irrigation purposes (JORF 0153.29, 2014; Steele and Odumeru, 2004). The European Commission has recently set down the minimum quality requirements for water reuse for agricultural irrigation and produced a guidance document addressing microbiological risks related to agricultural water in the primary production of fresh fruits and vegetables (Alcalde-Sanz and Gawlik, 2017; EU C163, 2017).

There is no consensus on the best indicator, nor on the optimal sampling frequency for irrigation water management. *E. coli* (*EC*) and Intestinal Enterococci (IE) are used as Fecal Indicator Bacteria (FIB), as detection methods are inexpensive and their presence relates to fecal (animal or human) pollution. But, it is well known that they do not always correlate with important waterborne pathogens that may be present in the diverse irrigation water sources (Girones et al., 2010). As specific screening of every single pathogen is not feasible, a commonly accepted practice is to use multiple indicators. It is also important to settle indicator values for different irrigation water purposes, which may include viral, bacterial or protozoan pathogens.

Several waterborne pathogens are relatively resistant to conventional water treatment methodologies and can easily appear in irrigation water sources (Adefisoye et al., 2016; Rodriguez-Manzano et al., 2012; Rusiñol and Girones, 2017). Human adenoviruses (HAdV), widely used as fecal indicators (Albinana-Gimenez et al., 2009; Hewitt et al., 2013; Verani et al., 2018), also pose a threat to public health since they may cause gastrointestinal and respiratory diseases. It is well known that HAdV, stable under many environmental conditions and disinfection treatments, are shed in high concentrations and do not show seasonality (Allard and Vantarakis, 2017). Other enteric viruses, like noroviruses (NoV) or enteroviruses (EV), are excreted in greater concentrations from infected individuals during clinical infections in the seasons with high incidence and then decrease over time (Atmar et al., 2008). Whereas NoV are the leading worldwide cause of gastroenteritis and may be the most important etiologic agent with respect to recycled water, EV go beyond gastroenteritis and cause a diversity of diseases, such as meningitis, paralysis or myocarditis (Kocwa-Haluch, 2001; Koo et al., 2013; Soller et al., 2018; WHO, 2013). Human polyomaviruses are also prevalent in fecally contaminated water bodies. JC polyomavirus (JCPyV) is persistently excreted over a lifetime and has been shown to be human specific, which is not the case with fecal indicator bacteria (Bofill-Mas et al., 2000; McQuaig et al., 2009). Merkel cell polyomavirus (MCPyV) was the first virus detected in environmental samples to have been described as having carcinogenic potential (Bofill-Mas et al., 2010; Rusiñol et al., 2015). In 2008, MCPyV was first related to neuroendocrine tumors in elderly and/or immunosuppressed people (Feng et al., 2008). Finally, hepatitis E virus (HEV), causing acute hepatitis in humans, is mainly transmitted through waterborne, foodborne and zoonotic routes and has been closely related to irrigation water contamination (Kokkinos et al., 2017; Yugo and Meng, 2013).

Bacteria such as *Legionella* spp., *Aeromonas* spp., *Arcobacter* spp., *Campylobacter* and *Helicobacter pylori* have been recognized as emerging pathogens in water, and have been also identified in wastewater and reclaimed water sources (Collado and Figueras, 2011; Fernandez-Cassi et al., 2016; Figueras and Borrego, 2010). Many of these pathogens are able to adhere to biofilms, but in addition, they may be associated with free-living protozoa, including amoebae. Both situations provide acting reservoirs for these pathogens and protect them from the effects of disinfection treatments. Also, *Giardia* cysts and *Cryptosporidium* oocysts, common waterborne parasites infecting humans and

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

Constructed wetlands, with surface flow, are being considered as low-cost technologies for reclaimed water production. These wetlands are used as an additional step (tertiary treatment systems) after secondary treatments and have proved to be efficient at reducing nitrogen and removing organic micropollutants (Llorens et al., 2009; Matamoros et al., 2008). The positive environmental values of these passive treatment systems for wastewater reclamation have been extensively reviewed (Ghermandi et al., 2010). Compared to other advanced treatment systems (e.g., reverse osmosis or membrane bioreactors), the price of the water that flows through the wetland cells is relatively low and has been calculated to range from €0.71 to €0.75 per m^{-3} (Alfranca et al., 2011). Moreover, the seasonal water demand for agriculture, which is a challenge for advanced reclaimed water facilities (NCR, 2012), can be solved using these sustainable systems.

This study evaluates the presence and levels of important circulating pathogens and indicators in diverse sources of irrigation water and proposes evaluation and management measures. Here it is also evaluated the performance of a constructed wetland system as a green tertiary treatment system producing reclaimed water intended for irrigation.

2. Methods

2.1. Sampling and microbial parameters analysis

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

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

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

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

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

2.2. Fresh water analysis

2.2.1. Heterotrophic bacteria quantification

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

2.2.2. FIB quantification

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

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

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

concentration of 0.01% of skimmed milk. All samples were stirred for 8 h at room temperature and the flocs were allowed to settle by gravity for another 8 h. The supernatants were removed and the sediment was collected and transferred to 500 mL centrifuge containers and centrifuged at 8000 × g 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 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4), distributed in refrigerated boxes among partner laboratories and stored at −20°C until the nucleic acid (NA) extractions were performed.

2.4. Virus quantification

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

fluorescence due to contamination.

2.5. Bacteria analysis

2.5.1. Legionella spp. quantification

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

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

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

2.5.3. Helicobacter pylori quantification

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

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

2.6. Protozoa analysis

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

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

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

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

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

3. Results

3.1. Microbiological contamination of irrigation water

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

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

Besides the HAdV detection, other viral pathogens were observed in irrigation water. MCPyV and NoV GGII were detected in river water during the coldest months (2/6 in both cases) and HEV was occasionally present in groundwater (1/12). All viral concentrations were near the detection limit of 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

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

3.3. Origin of the fecal contamination

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

4. Discussion

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

reported (Calgua et al., 2013; Rusiñol et al., 2015, 2014), this concentration method is robust and easy to implement for simultaneous concentration of viruses, bacteria and protozoa (Gonzales-Gustavson et al., 2017). The harmonization of the concentration method, for the further detection of indicators and pathogens, may allow water managers to use mathematical approximations when calculating concentrations according to acceptable prediction intervals.

4.1. Irrigation water quality: conventional and reclaimed water sources.

Chlorinated drinking water was the only irrigation water source with no pathogen detection, but in terms of costs, the use of drinking water for irrigation purposes is unaffordable as well as unavailable in many regions. In general, fecal pollution was found in a high percentage of the samples by means of FIB. Occurrences of EC in river water samples were the highest in irrigation water (100%), whereas in reclaimed water both EC and IE were frequently detected (10/12 samples and 11/12 samples respectively) in low concentrations. The fact that IE are distinguished by their ability to survive in more complex matrices, underscores their use as FIB in more complex water matrices. It is also important to state that changes in the WWTP management could explain FIB fluctuations in the treated effluents during spring.

During this one-year surveillance, HAdV was detected in groundwater (17%), river water (17%) and reclaimed water (33%), confirming the human origin of the fecal contamination. This human pathogen is widely detected when water is impacted by sewage (Bofill-Mas et al., 2013; Rusiñol et al., 2014; Rusiñol and Girones, 2017; Vieira et al., 2016). NoV occurrence in river water has been reported when rain events introduce large amounts of pathogens into the receiving water bodies (Hata et al., 2014), during peak infection periods or due to viral outbreaks (Kauppinen et al., 2018). Although we did not detect NoV in groundwater, it has been reported that this highly infectious pathogen remains infective in groundwater for long periods (Seitz et al., 2011). MCPyV was found in 2 of the 12 river water samples, as reported in other studies (Rusiñol et al., 2015). This skin virus is persistently excreted in sewage (Bofill-Mas et al., 2010), so its presence highlights its dissemination into the environment and its resistance to water treatment technologies.

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

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

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

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

4.2. Microbial removals in a sustainable wetland system

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

reduced 3.14 Log_{10} of HAdV a nd 5.17 Log_{10} of *EC*. Comparing Log_{10} removals of HAdV in diverse reclaimed water production systems (Table 4) shows that advanced sewage treatments achieve higher efficiencies (5.20 Log_{10}), but they also have important operational and maintenance costs to be considered (Guo et al., 2014; Hunter et al., 2018; Liu et al., 2013; Prado et al., 2019).

Our treatment process achieved a mean 3.23 Log_{10} removal of HBC, similar to the reported removal when wastewater is treated in conventional wastewater reclamation processes (CAS + chlorination) (Al-Jassim et al., 2015). The analysis of HBC has little value as an indicator of pathogen presence, but can be used in assessing regrowth and presence of biofilms in the reclaimed water system.

Following the health target of $<10^{-6}$ DALY's per person per year for safe drinking-water, the WHO established performance values, or minimum Log_{10} removals, of three reference pathogens: a virus (5.0 Log_{10} of rotavirus), a bacterium (4.0 Log_{10} of *Campylobacter*) and a protozoan (4.9 Log_{10} for *Cryptosporidium*) (WHO, 2017). The European directive does not compel member states to monitor pathogens, and only recommends translating the *EC* monitoring data into treatment performance targets (WHO, 2017). As irrigation water should be free of contamination and, where possible, have of the same quality as drinking water, a similar approach could be used for irrigation water. A recent publication in our group, quantifying the risk of using the wetland effluent to irrigate lettuce, established that the disease burden of NoV GGII and HAdV was higher than 10^{-6} DALYs (Gonzales-Gustavson et al., 2019). Thus, additional disinfection treatment would be required to irrigate these types of crops with reclaimed water produced in the studied wetland system.

4.3. Monitoring irrigation water quality

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

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

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

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

It is assumed that human pathogens are present in low concentrations in irrigation water. However, this will be directly related to the disinfection treatment to which the water has been submitted and its proper storage. *Aeromonas* and *Arcobacter* have been found in lagooning reclaimed water, and the former also in parsley and tomatoes irrigated with water contaminated with these bacteria (Fernandez-Cassi et al., 2016; Latif-Eugenín et al., 2017). The SMF method allowed for the evaluation of a representative volume (10 L) for simultaneous monitoring of waterborne viruses, bacteria and protozoa. This concentration method would reduce costs and facilitate periodic testing of different irrigation water sources. Further investigations are necessary to obtain larger data sets and to assess specific pathogen serotypes.

5. Conclusions

Considering the current guidelines at the EU, with the single recommendation of *EC* testing, most of the sources of irrigation evaluated here would meet the EU requirements. However sporadic detection of viral pathogens was found in water samples with *EC* values lower than 100 MPN/100ml. It is assumed that groundwater is less vulnerable to fecal pollution than reservoir or river water, but the detection of porcine fecal pollution (PAdV) and an emergent pathogen as HEV, would confirm that pigs act as a reservoir of this viruses and enhances the importance of having a good characterization of this irrigation source.

Compared to other microorganisms evaluated, HAdV presented low reduction values in the wetland system, demonstrating its high resistance to treatment. Due to the higher demand for reclaimed water for agriculture during the warm season, when noroviruses where not detected, we would recommend evaluating the presence of HAdV as a complementary management measure of the performance of the water reclamation process. A viral pathogen like NoV might be considered during the coldest months.

Neither Giardia cysts, nor any Cryptosporidium oocyst where detected in the analysed water samples, showing a low prevalence of these protozoa in the irrigation water sources studied.

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. The detection of ubiquitous

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

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References

Adefisoye, M.A., Nwodo, U.U., Green, E., Okoh, A.I., 2016. Quantitative PCR Detection and Characterisation of Human Adenovirus, Rotavirus and Hepatitis A Virus in Discharged Effluents of Two Wastewater Treatment Facilities in the Eastern Cape, South Africa. Food Environ. Virol. 8, 262–274. https://doi.org/10.1007/s12560-016-9246-4

Agrafioti, E., Diamadopoulos, E., 2012. A strategic plan for reuse of treated municipal wastewater for crop irrigation on the Island of Crete. Agric. Water Manag. 105, 57–64. https://doi.org/10.1016/j.agwat.2012.01.002

Al-Jassim, N., Ansari, M.I., Harb, M., Hong, P.-Y., 2015. Removal of bacterial contaminants and antibiotic resistance genes by conventional wastewater treatment processes in Saudi Arabia: Is the treated wastewater safe to reuse for agricultural irrigation? Water Res. 73, 277–290. https://doi.org/10.1016/j.watres.2015.01.036

Albinana-Gimenez, N., Miagostovich, M.P., Calgua, B., Huguet, J.M., Matia, L., Girones, R., 2009. Analysis of adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants. Water Res. 43, 2011–2019.

https://doi.org/10.1016/j.watres.2009.01.025

Alcalde-Sanz, L., Gawlik, B.M., 2017. Minimum quality requirements for water reuse in agricultural irrigation and aquifer recharge Towards a legal instrument on water reuse at EU level. https://doi.org/10.2760/887727

Alfranca, O., García, J., Varela, H., 2011. Economic valuation of a created wetland fed with treated wastewater located in a peri-urban park in Catalonia, Spain. Water Sci. Technol. 63, 891–898. https://doi.org/10.2166/wst.2011.267

Allard, A., Vantarakis, A., 2017. Adenoviruses, in: Rose, J., Jiménez-Cisneros, B. (Eds.), Global Water Pathogens Project. UNESCO, Michigan.

Angelakis, A.N., Durham, B., 2008. Water recycling and reuse in EUREAU countries: Trends and challenges. Desalination 218, 3–12. https://doi.org/10.1016/j.desal.2006.07.015

Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Graham, D.Y., 2008. Norwalk virus shedding after experimental human infection. Emerg. Infect. Dis. 14, 1553–1557. https://doi.org/10.3201/eid1410.080117

Banting, G., Figueras, M.J., 2018. Arcobacter, in: Pruden, A., Ashbolt, N., Miller, J. (Eds.), Global Water Pathogen Project. Michigan State University. https://doi.org/10.14321/waterpathogens.22

Banting, G.S., Braithwaite, S., Scott, C., Kim, J., Jeon, B., Ashbolt, N., Ruecker, N., Tymensen, L., Charest, J., Pintar, K., Checkley, S., Neumann, N.F., 2016. Evaluation of Various Campylobacter-Specific Quantitative PCR (qPCR) Assays for Detection and Enumeration of Campylobacteraceae in Irrigation Water and Wastewater via a Miniaturized Most-Probable-Number–qPCR Assay. Appl. Environ. Microbiol. 82, 4743–4756. https://doi.org/10.1128/AEM.00077-16

Bartram, J., Cotruvo, J., Exner, M., Fricker, C., Glasmacher, A., 2003. Heterotrophic Plate Counts and Drinking-water Safety.

Bellack, N.R., Koehoorn, M.W., Macnab, Y.C., Morshed, M.G., 2006. A conceptual model of water's role as a reservoir in Helicobacter pylori transmission: a review of the evidence. Epidemiol. Infect. 134, 439–449. https://doi.org/10.1017/S0950268806006005

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J.,

Allard, A., Calvo, M., Girones, R., 2006. Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. Appl. Environ. Microbiol. 72, 7894–7896. https://doi.org/10.1128/AEM.00965-06

Bofill-Mas, S., Pina, S., Girones, R., 2000. Documenting the epidemiologic patterns of polyomaviruses in human populations by studying their presence in urban sewage. Appl. Environ. Microbiol. 66, 238–245.

Bofill-Mas, S., Rodriguez-Manzano, J., Calgua, B., Carratala, A., Girones, R., 2010. Newly described human polyomaviruses Merkel cell, KI and WU are present in urban sewage and may represent potential environmental contaminants. Virol. J. 7, 141. https://doi.org/10.1186/1743-422X-7-141

Bofill-Mas, S., Rusiñol, M., Fernandez-Cassi, X., Carratalà, A., Hundesa, A., Girones, R., 2013. Quantification of human and animal viruses to differentiate the origin of the fecal contamination present in environmental samples. Biomed Res. Int. 2013, 192089. https://doi.org/10.1155/2013/192089

Bourrouet, A., García, J., Mujeriego, R., Peñuelas, G., 2001. Faecal bacteria and bacteriophage inactivation in a full-scale UV disinfection system used for wastewater reclamation. Water Sci. Technol. 43, 187–94.

Calgua, B., Fumian, T., Rusiñol, M., Rodriguez-Manzano, J., Mbayed, V.V.A., Bofill-Mas, S., Miagostovich, M., Girones, R., 2013. Detection and quantification of classic and emerging viruses by skimmed-milk flocculation and PCR in river water from two geographical areas. Water Res. 47, 2797–810. https://doi.org/10.1016/j.watres.2013.02.043

Cárdenas Youngs, Y.I., 2018. Determinación de la contaminación microbiológica del agua de riego aplicando nuevas estrategias de análisis. TDX (Tesis Dr. en Xarxa). Universitat de Barcelona.

Carratalà, A., Rusiñol, M., Hundesa, A., Biarnes, M., Rodriguez-Manzano, J., Vantarakis, A., Kern, A., Sunen, E., Girones, R., Bofill-Mas, S., 2012. A Novel Tool for Specific Detection and Quantification of Chicken/Turkey Parvoviruses To Trace Poultry Fecal Contamination in the Environment. Appl. Environ. Microbiol. 78, 7496–7499. https://doi.org/10.1128/AEM.01283-12

Cervero-Aragó, S., Rodríguez-Martínez, S., Puertas-Bennasar, A., Araujo, R.M., 2015. Effect of Common Drinking Water Disinfectants, Chlorine and Heat, on Free Legionella and Amoebae-

Associated Legionella. PLoS One 10, e0134726. https://doi.org/10.1371/JOURNAL.PONE.0134726

Collado, L., Figueras, M.J., 2011. Taxonomy, epidemiology, and clinical relevance of the genus Arcobacter. Clin. Microbiol. Rev. 24, 174–192. https://doi.org/10.1128/CMR.00034-10

da Silva, A.K., Le Saux, J.-C.C., Parnaudeau, S., Pommepuy, M., Elimelech, M., Le Guyader, F.S., 2007. Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II. Appl. Environ. Microbiol. 73, 7891–7. https://doi.org/10.1128/AEM.01428-07

EFSA, 2013. Scientific Opinion on the risk posed by pathogens in food of non-animal origin . Part 1 (outbreak data analysis and risk ranking of food/pathogen combinations), EFSA Journal. https://doi.org/10.2903/j.efsa.2013.3025.

EFSA BIOHAZ Panel, 2017. Scientific Opinion on the guidance on the requirements for the development of microbiological criteria. EFSA J. 15, 60. https://doi.org/10.2903/j.efsa.2017.5052

EPA, U.S., 2004. Guidelines for Water reuse.

EPHC, NRMMC, A., 2006. National Guidelines for Water Recycling: Managing Health and Environmental Risks.

EU C163, 2017. Guidance document on addressing microbiological risks in fresh fruits and vegetables at primary production through good hygiene.

FAO, 2019. AQUASTAT - FAO's Information System on Water and Agriculture [WWW Document]. URL http://www.fao.org/aquastat/en/ (accessed 5.30.19).

FAO, 2015. Codex Alimentarius Commission Codex Alimentarius Commission.

FAO, 2013. FAOWater - Water scarcity [WWW Document]. URL http://www.fao.org/nr/water/topics_scarc_agri.html

Feng, H., Shuda, Ma., Chang, Y., Moore, P.S., 2008. Clonal integration of a polyomavirus in human merkel cell carcinoma. Science (80-.). 319, 1096–1100. https://doi.org/10.1126/science.1152586.Clonal

Fernandez-Cassi, X., Silvera, C., Cervero-Aragó, S., Rusiñol, M., Latif-Eugeni, F., Bruguera-

Casamada, C., Civit, S., Araujo, R.M., Figueras, M.J., Girones, R., Bofill-Mas, S., 2016. Evaluation of the microbiological quality of reclaimed water produced from a lagooning system. Environ. Sci. Pollut. Res. 23. https://doi.org/10.1007/s11356-016-6812-0

Figueras, M.J., Ashbolt, N., 2019. Aeromonas, in: Pruden, A., Ashbolt, N., Miller, J. (Eds.), Global Water Pathogen Project. Michigan State University. https://doi.org/10.14321/waterpathogens.21

Figueras, M.J., Borrego, J.J., 2010. New Perspectives in Monitoring Drinking Water Microbial Quality. Int. J. Environ. Res. Public Health 7, 4179–4202. https://doi.org/10.3390/ijerph7124179

Ghermandi, A., van den Bergh, J.C.J.M., Brander, L.M., de Groot, H.L.F., Nunes, P.A.L.D., 2010. Values of natural and human-made wetlands: A meta-analysis. Water Resour. Res. 46. https://doi.org/10.1029/2010WR009071

Girones, R., Ferrús, M., Alonso, J., Rodriguez-Manzano, J., Calgua, B., Corrêa, A., Hundesa, A., Carratala, A., Bofill-Mas, S., 2010. Molecular detection of pathogens in water--the pros and cons of molecular techniques. Water Res. 44, 4325–4339. https://doi.org/10.1016/j.watres.2010.06.030

Gleick, P., 2009. Water Conflict Chronology Timeline List [WWW Document]. The world's water. URL http://www.worldwater.org/conflict/list/

Gonzales-Gustavson, E., Cárdenas-Youngs, Y., Calvo, M., da Silva, M.F.M., Hundesa, A., Amorós, I., Moreno, Y., Moreno-Mesonero, L., Rosell, R., Ganges, L., Araujo, R., Girones, R., 2017. Characterization of the efficiency and uncertainty of skimmed milk flocculation for the simultaneous concentration and quantification of water-borne viruses, bacteria and protozoa. J. Microbiol. Methods 134, 46–53. https://doi.org/10.1016/j.mimet.2017.01.006

Gonzales-Gustavson, E., Rusiñol, M., Medema, G., Calvo, M., Girones, R., 2019. Quantitative risk assessment of norovirus and adenovirus for the use of reclaimed water to irrigate lettuce in Catalonia. Water Res. 153, 91–99. https://doi.org/10.1016/j.watres.2018.12.070

Guo, T., Englehardt, J., Wu, T., 2014. Review of cost versus scale: water and wastewater treatment and reuse processes. https://doi.org/10.2166/wst.2013.734

Hata, A., Katayama, H., Kojima, K., Sano, S., Kasuga, I., Kitajima, M., Furumai, H., 2014. Effects of rainfall events on the occurrence and detection efficiency of viruses in river water impacted by

combined sewer overflows. Sci. Total Environ. 468–469, 757–63. https://doi.org/10.1016/j.scitotenv.2013.08.093

Hernroth, B., Conden-Hansson, A., Rehnstam-Holm, A., Girones, R., Allard, A., 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, Mytilus edulis: the first Scandinavian report. Appl. Environ. Microbiol. 68, 4523–4533. https://doi.org/10.1128/AEM.68.9.4523

Herpers, B.L., de Jongh, B.M., van der Zwaluw, K., van Hannen, E.J., 2003. Real-time PCR assay targets the 23S-5S spacer for direct detection and differentiation of Legionella spp. and Legionella pneumophila. J. Clin. Microbiol. 41, 4815–6. https://doi.org/10.1128/jcm.41.10.4815-4816.2003

Hewitt, J., Greening, G.E., Leonard, M., Lewis, G.D., 2013. Evaluation of human adenovirus and human polyomavirus as indicators of human sewage contamination in the aquatic environment. Water Res. 47, 6750–61. https://doi.org/10.1016/j.watres.2013.09.001

Hoehne, M., Schreier, E., 2006. Detection of norovirus genogroup I and II by multiplex real-time RT-PCR using a 3???-minor groove binder-DNA probe. BMC Infect. Dis. 6, 1–6. https://doi.org/10.1186/1471-2334-6-69

Hundesa, A., Bofill-Mas, S., Maluquer de Motes, C., Rodriguez-Manzano, J., Bach, A., Casas, M., Girones, R., 2010. Development of a quantitative PCR assay for the quantitation of bovine polyomavirus as a microbial source-tracking tool. J. Virol. Methods 163, 385–389. https://doi.org/10.1016/j.jviromet.2009.10.029

Hundesa, A., Maluquer de Motes, C., Albinana-Gimenez, N., Rodriguez-Manzano, J., Bofill-Mas, S., Suñen, E., Rosina Girones, R., 2009. Development of a qPCR assay for the quantification of porcine adenoviruses as an MST tool for swine fecal contamination in the environment. J. Virol. Methods 158, 130–135. https://doi.org/10.1016/j.jviromet.2009.02.006

Hunter, R.G., Day, J.W., Wiegman, A.R., Lane, R.R., 2018. Municipal wastewater treatment costs with an emphasis on assimilation wetlands in the Louisiana coastal zone. https://doi.org/10.1016/j.ecoleng.2018.09.020

Inbar, Y., 2007. New standards for treated wastewater reuse in Israel, in: Zaidi, M. (Ed.), Wastewater

Reuse-Risk Assessment, Decision-Making and Environmental Security. Springer, Dordrecht, pp. 291–296.

International Organization for Standardization, 2012. ISO 9308-2:2012 Water quality — Enumeration of Escherichia coli and coliform bacteria — Part 2: Most probable number method.

International Organization for Standardization, 1999. ISO 6222:1999 - Water quality — Enumeration of culturable micro-organisms — Colony count by inoculation in a nutrient agar culture medium.

International Organization for Standardization, 1998. ISO 7899-1:1998 Water quality — Detection and enumeration of intestinal enterococci — Part 1: Miniaturized method (Most Probable Number) for surface and waste water.

IPCC, 2019. Climate Change and Land.

JORF 0153.29, 2014. Utilisation d'eaux issues du traitement d'épuration des eaux résiduaires urbaines pour l'irrigation de cultures ou d'espaces verts.

Jothikumar, N., Cromeans, T.L., Robertson, B.H., Meng, X.J., Hill, V.R., 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J. Virol. Methods 131, 65–71. https://doi.org/10.1016/j.jviromet.2005.07.004

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F.B., Takeda, N., Katayama, K., 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J. Clin. Microbiol. 41, 1548–57.

Kalavrouziotis, I.K., Kokkinos, P., Oron, G., Fatone, F., Bolzonella, D., Vatyliotou, M., Fatta-Kassinos, D., Koukoulakis, P.H., Varnavas, S.P., 2015. Current status in wastewater treatment, reuse and research in some mediterranean countries. Desalin. Water Treat. 53, 2015–2030. https://doi.org/10.1080/19443994.2013.860632

Kauppinen, A., Pitkänen, T., Miettinen, I.T., 2018. Persistent Norovirus Contamination of Groundwater Supplies in Two Waterborne Outbreaks. Food Environ. Virol. 10, 39–50. https://doi.org/10.1007/s12560-017-9320-6

Kitajima, M., Iker, B.C., Pepper, I.L., Gerba, C.P., 2014. Relative abundance and treatment reduction of viruses during wastewater treatment processes — Identification of potential viral indicators.

Sci. Total Environ. 488–489, 290–296. https://doi.org/10.1016/j.scitotenv.2014.04.087

Kocwa-Haluch, R., 2001. Waterborne Enteroviruses as a Hazard for Human Health. Polish J. Environ. Stud. 10, 485–487.

Kokkinos, P., Kozyra, I., Lazic, S., Söderberg, K., Vasickova, P., Bouwknegt, M., Rutjes, S., Willems, K., Moloney, R., de Roda Husman, A.M., Kaupke, A., Legaki, E., D'Agostino, M., Cook, N., von Bonsdorff, C.H., Rzeżutka, A., Petrovic, T., Maunula, L., Pavlik, I., Vantarakis, A., 2017. Virological Quality of Irrigation Water in Leafy Green Vegetables and Berry Fruits Production Chains. Food Environ. Virol. 9, 72–78. https://doi.org/10.1007/s12560-016-9264-2

Koo, B.S., Lee, H.R., Jeon, E.O., Han, M.S., Min, K.C., Lee, S.B., Mo, I.P., 2013. Molecular survey of enteric viruses in commercial chicken farms in Korea with a history of enteritis. Poult. Sci. 92, 2876–85. https://doi.org/10.3382/ps.2013-03280

Latif-Eugenín, F., Beaz-Hidalgo, R., Silvera-Simón, C., Fernandez-Cassi, X., Figueras, M.J., 2017. Chlorinated and ultraviolet radiation -treated reclaimed irrigation water is the source of Aeromonas found in vegetables used for human consumption. Environ. Res. 154, 190–195. https://doi.org/10.1016/j.envres.2016.12.026

Liu, P., Herzegh, O., Fernandez, M., Hooper, S., Shu, W., Sobolik, J., Porter, R., Spivey, N., Moe, C., 2013. Assessment of human adenovirus removal by qPCR in an advanced water reclamation plant in Georgia, USA. J. Appl. Microbiol. 115, 310–8. https://doi.org/10.1111/jam.12237

Llorens, E., Matamoros, V., Domingo, V., Bayona, J.M., García, J., 2009. Water quality improvement in a full-scale tertiary constructed wetland: effects on conventional and specific organic contaminants. Sci. Total Environ. 407, 2517–24. https://doi.org/10.1016/j.scitotenv.2008.12.042

Loisy, F., Atmar, R.L., Guillon, P., Le Cann, P., Pommepuy, M., Le Guyader, F.S., 2005. Real-time RT-PCR for norovirus screening in shellfish. J. Virol. Methods 123, 1–7. https://doi.org/10.1016/j.jviromet.2004.08.023

López-Gálvez, F., Truchado, P., Sánchez, G., Aznar, R., Gil, M.I., Allende, A., 2016. Occurrence of enteric viruses in reclaimed and surface irrigation water: relationship with microbiological and physicochemical indicators. J. Appl. Microbiol. 121, 1180–1188. https://doi.org/10.1111/jam.13224

Matamoros, V., García, J., Bayona, J.M., 2008. Organic micropollutant removal in a full-scale surface flow constructed wetland fed with secondary effluent. Water Res. 42, 653–660. https://doi.org/10.1016/j.watres.2007.08.016

Mayer, R.E., Bofill-Mas, S., Egle, L., Reischer, G.H., Schade, M., Fernandez-Cassi, X., Fuchs, W., Mach, R.L., Lindner, G., Kirschner, A., Gaisbauer, M., Piringer, H., Blaschke, A.P., Girones, R., Zessner, M., Sommer, R., Farnleitner, A.H., 2016. Occurrence of human-associated Bacteroidetes genetic source tracking markers in raw and treated wastewater of municipal and domestic origin and comparison to standard and alternative indicators of faecal pollution. Water Res. 90, 265–276. https://doi.org/10.1016/j.watres.2015.12.031

McQuaig, S.M., Scott, T.M., Lukasik, J.O., Paul, J.H., Harwood, V.J., 2009. Quantification of human polyomaviruses JC virus and BK Virus by TaqMan quantitative PCR and comparison to other water quality indicators in water and fecal samples. Appl. Environ. Microbiol. 75, 3379–3388. https://doi.org/10.1128/AEM.02302-08

Moreno-Mesonero, L., Moreno, Y., Alonso, J.L., Ferrús, M.A., 2016. DVC-FISH and PMA-qPCR techniques to assess the survival of Helicobacter pylori inside Acanthamoeba castellanii. Res. Microbiol. 167, 29–34. https://doi.org/10.1016/j.resmic.2015.08.002

Moreno, Y., Moreno-Mesonero, L., Amorós, I., Pérez, R., Morillo, J.A., Alonso, J.L., 2018. Multiple identification of most important waterborne protozoa in surface water used for irrigation purposes by 18S rRNA amplicon-based metagenomics. Int. J. Hyg. Environ. Health 221, 102–111.

NCR, 2012. Water Reuse : Potential for Expanding the Nation ' s Water Supply Through Reuse of Municipal Wastewater. Washington DC, USA.

Nilsson, H.-O., Blom, J., Al-Soud, W.A., Ljungh, A., Andersen, L.P., Wadstrom, T., 2002. Effect of Cold Starvation, Acid Stress, and Nutrients on Metabolic Activity of Helicobacter pylori. Appl. Environ. Microbiol. 68, 11–19. https://doi.org/10.1128/AEM.68.1.11-19.2002

NP 4434, 2005. Reutilização de Águas Residuais na Rega,.

Pal, A., Sirota, L., Maudru, T., Peden, K., Lewis, A.M., 2006. Real-time, quantitative PCR assays for the detection of virus-specific DNA in samples with mixed populations of polyomaviruses. J.

Virol. Methods 135, 32–42. https://doi.org/10.1016/j.jviromet.2006.01.018

Prado, T., De Castro Bruni, A., Renata, M., Barbosa, F., Garcia, S.C., Maria De, A., Melo, J., Inês, M., Sato, Z., 2019. Performance of wastewater reclamation systems in enteric virus removal. Sci. Total Environ. 678, 33–42. https://doi.org/10.1016/j.scitotenv.2019.04.435

R Core Team, 2019. R A language and environament for statistical computing. [WWW Document]. R Found. Stat. Comput. Vienna, Austria. URL https://www.r-project.org/ (accessed 12.16.19).

Rachmadi, A.T., Kitajima, M., Pepper, I.L., Gerba, C.P., 2016. Enteric and indicator virus removal by surface flow wetlands. Sci. Total Environ. 542, 976–982. https://doi.org/10.1016/j.scitotenv.2015.11.001

Randazzo, W., López-Gálvez, F., Allende, A., Aznar, R., Sánchez, G., 2016. Evaluation of viability PCR performance for assessing norovirus infectivity in fresh-cut vegetables and irrigation water. Int. J. Food Microbiol. 229, 1–6. https://doi.org/10.1016/j.ijfoodmicro.2016.04.010

RD 1620, 2007. Real Decreto 1620/2007. Régimen jurídico de la reutilización de las aguas regeneradas, 2007. Boletín Oficial del Estado 294-21092.

Richards, C.L., Broadaway, S.C., Eggers, M.J., Doyle, J., Pyle, B.H., Camper, A.K., Ford, T.E., 2018. Detection of Pathogenic and Non-pathogenic Bacteria in Drinking Water and Associated Biofilms on the Crow Reservation, Montana, USA. Microb. Ecol. 76, 52–63. https://doi.org/10.1007/s00248-015-0595-6

Rodriguez-Manzano, J., Alonso, J.L.L., Ferrús, M.A.A., Moreno, Y., Amorós, I., Calgua, B., Hundesa, A., Guerrero-Latorre, L., Carratala, A., Rusiñol, M., Girones, R., 2012. Standard and new faecal indicators and pathogens in sewage treatment plants, microbiological parameters for improving the control of reclaimed water. Water Sci. Technol. 66, 2517–23. https://doi.org/10.2166/wst.2012.233

Rusiñol, M., Fernandez-Cassi, X., Hundesa, A., Vieira, C., Kern, A., Eriksson, I., Ziros, P., Kay, D., Miagostovich, M., Vargha, M., Allard, A., Vantarakis, A., Wyn-Jones, P., Bofill-Mas, S., Girones, R., 2014. Application of human and animal viral microbial source tracking tools in fresh and marine waters from five different geographical areas. Water Res. 59, 119–29. https://doi.org/10.1016/j.watres.2014.04.013

Rusiñol, M., Fernandez-Cassi, X., Timoneda, N., Carratalà, A., Abril, J.F.J.F., Silvera, C., Figueras, M.J.M.J., Gelati, E., Rodó, X., Kay, D., Wyn-Jones, P., Bofill-Mas, S., Girones, R., 2015. Evidence of viral dissemination and seasonality in a Mediterranean river catchment: Implications for water pollution management. J. Environ. Manage. 159, 58–67. https://doi.org/10.1016/j.jenvman.2015.05.019

Rusiñol, M., Girones, R., 2017. Summary of Excreted and Waterborne Viruses, in: Rose, J.B., Jiménez-Cisneros, B. (Eds.), Global Water Pathogens Project. Michigan State University, E. Lansing, MI, UNESCO, Michigan.

Santiago, P., Moreno, Y., Ferrús, M.A., 2015. Identification of Viable *Helicobacter pylori* in Drinking Water Supplies by Cultural and Molecular Techniques. Helicobacter 20, 252–259. https://doi.org/10.1111/hel.12205

Seitz, S.R., Leon, J.S., Schwab, K.J., Lyon, G.M., Dowd, M., McDaniels, M., Abdulhafid, G., Fernandez, M.L., Lindesmith, L.C., Baric, R.S., Moe, C.L., 2011. Norovirus infectivity in humans and persistence in water. Appl. Environ. Microbiol. 77, 6884–8. https://doi.org/10.1128/AEM.05806-11

Sidhu, J.P.S., Sena, K., Hodgers, L., Palmer, A., Toze, S., 2018. Comparative enteric viruses and coliphage removal during wastewater treatment processes in a sub-tropical environment. Sci. Total Environ. 616–617, 669–677. https://doi.org/10.1016/j.scitotenv.2017.10.265

Soller, J.A., Eftim, S.E., Nappier, S.P., 2018. Direct potable reuse microbial risk assessment methodology: Sensitivity analysis and application to State log credit allocations. Water Res. 128, 286–292. https://doi.org/10.1016/j.watres.2017.10.034

Souppart, L., Sanciu, G., Cian, A., Wawrzyniak, I., Delbac, F., Capron, M., Dei-Cas, E., Boorom, K., Delhaes, L., Viscogliosi, E., 2009. Molecular epidemiology of human Blastocystis isolates in France. Parasitol. Res. 105, 413–421. https://doi.org/10.1007/s00436-009-1398-9

Steele, M., Odumeru, J., 2004. Irrigation water as source of foodborne pathogens on fruit and vegetables. J Food Prot 67, 2839–49.

Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteijn, B., Koopmans, M., 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from

1994 through 2005. J. Clin. Microbiol. 45, 1389–94. https://doi.org/10.1128/JCM.02305-06

Symonds, E.M., Breitbart, M., 2015. Affordable Enteric Virus Detection Techniques Are Needed to Support Changing Paradigms in Water Quality Management. CLEAN - Soil, Air, Water 43, 8–12. https://doi.org/10.1002/clen.201400235

Uyttendaele, M., Jaykus, L.-A.A., Amoah, P., Chiodini, A., Cunliffe, D., Jacxsens, L., Holvoet, K., Korsten, L., Lau, M., McClure, P., Medema, G., Sampers, I., Rao Jasti, P., 2015. Microbial Hazards in Irrigation Water: Standards, Norms, and Testing to Manage Use of Water in Fresh Produce Primary Production. Compr. Rev. Food Sci. Food Saf. 14, 336–356. https://doi.org/10.1111/1541-4337.12133

Verani, M., Federigi, I., Donzelli, G., Cioni, L., Carducci, A., 2018. Human adenoviruses as waterborne index pathogens and their use for Quantitative Microbial Risk Assessment. Sci. Total Environ. 651, 1469–1475. https://doi.org/10.1016/j.scitotenv.2018.09.295

Vieira, C.B., de Abreu Corrêa, A., de Jesus, M.S., Luz, S.L.B., Wyn-Jones, P., Kay, D., Vargha, M., Miagostovich, M.P., 2016. Viruses Surveillance Under Different Season Scenarios of the Negro River Basin, Amazonia, Brazil. Food Environ. Virol. 8, 57–69. https://doi.org/10.1007/s12560-016-9226-8

WHO, 2017. Drinking Water Parameter Cooperation Project. Support to the revision of Annex I Council Directive 98/83/EC on the Quality of Water Intended for Human Consumption (Drinking Water Directive).

WHO, 2013. The world health report 2013. World Heal. Organ. Press 146.

WHO, 2006. GUIDELINES FOR THE SAFE USE OF WASTEWATER , EXCRETA AND GREYWATER Volume 2 Wastewater use in agriculture.

Yugo, D.M., Meng, X.J., 2013. Hepatitis E virus: Foodborne, waterborne and zoonotic transmission. Int. J. Environ. Res. Public Health 10, 4507–4533. https://doi.org/10.3390/ijerph10104507

Table 1: Viruses, bacteria and protozoa detected in different irrigation water samples. Mean Log_{10} concentrations (standard deviations, percentatges of positive samples). NA: Non analysed ND: Non detected.

	Units	**Drinking water**	**Groundwater**	**Reservoir water**	**River water**	**Reclaimed water**
Human Adenovirus	GC/100mL	ND	1.44 (0.42, 17%)	ND	1.41 (0.22, 17%)	1.49 (0.47, 33%)
Human JC polyomavirus	GC/100mL	ND	ND	ND	ND	1.44 (8%)
Merkel cell polyomavirus	GC/100mL	ND	ND	ND	1.74 (0.20, 17%)	ND
Norovirus Genogroup I	GC/100mL	ND	ND	ND	ND	1.68 (8%)
Norovirus Genogroup II	GC/100mL	ND	ND	ND	2.42(0.24, 17%)	2.45 (0.22, 25%)
Human Enterovirus	GC/100mL	ND	ND	ND	ND	ND
Hepatitis E Virus	GC/100mL	ND	2.13 (8%)	ND	ND	ND
Escherichia coli	MPN/100 mL	ND	2.03 (0.42, 33%)	2.20 (0.21, 25%)	2.09 (0.84, 100%)	2.02 (0.51, 83%)
Intestinal enterococci	MPN/100 mL	ND	2.22 (0.24, 17%)	2.17 (0.26, 25%)	2.07 (0.81, 58%)	1.54. (0.36, 92%)
Heterotrophic Bacteria	MPN/100 mL	ND	3.85 (1.15, 75%)	2.42 (0.24, 100%)	4.85 (0.59, 100%)	5.55 (0.58, 100%)
***Aeromonas* spp.**	GC/100mL	ND	5.05 (1.13, 78%)	NA	4.83 (1.19, 100%)	NA
***Arcobacter* spp.**	GC/100mL	ND	3.97 (1.33, 33%)	NA	6.72 (1.64, 50%)	NA
***Legionella* spp.**	GC/100mL	ND	3.52 (1.40, 61%)	ND	ND	3.37 (1.68, 33%)
Helicobacter pylori	-	ND	detected (100%)	detected (50%)	detected (100%)	detected (100%)
***Blastocysts* sp.**	-	ND	ND	ND	ND	ND
Acanthamoeba castellanii	-	ND	detected (100%)	detected (100%)	detected (100%)	detected (100%)
Cryptosporidium	-	ND	ND	ND	ND	ND

parvum						
Giardia Duodenalis	-	ND	ND	ND	ND	ND

Table 2: Percentage of samples with detectable viral genome copies and Fecal Indicator Bacteria. CAS: conventional activated sludge

	Raw sewage	Secondary (CAS)	Reclaimed (wetland)
Number of samples	12	12	12
Human Adenovirus	100%	100%	33%
Human JC polyomavirus	100%	42%	8%
Merkel cell polyomavirus	100%	8%	0%
Norovirus Genogroup I	100%	42%	8%
Norovirus Genogroup II	100%	75%	25%
Human Enterovirus	8%	8%	0%
Hepatitis E Virus	8%	0%	0%
Escherichia coli	100%	100%	83%
Intestinal enterococci	100%	100%	92%

Table 3: Microbial Source Tracking markers in different irrigation water samples and raw sewage. Mean Log_{10} concentrations and percentage of detection of viral, bacterial (FIB) and protozoan (FIP) markers. NA: not analysed, ND: non detected

	Human	**Porcine**	**Bovine**	**Avian**	**FIB**		**FIP**	
	HAdV	PAdV	BPyV	Ch/TyPV	*EC*	IE	*CP*	*GD*
Groundwater	1.44 (17%)	2.47 (44%)	ND	3.06 (33%)	2.03 (33%)	2.22 (17%)	ND	ND
River water	1.41 (17%)	1.36 (8%)	1.51 (17%)	2.04 (17%)	2.09 (100%)	2.07 (58%)	ND	ND
Reclaimed water	1.49 (33%)	2.74 (17%)	ND	ND	2.02 (83%)	1.54 (92%)	ND	ND
Raw sewage	4.63 (100%)	4.58 (17%)	3.29 (25%)	ND	7.18 (100%)	6.56 (100%)	NA	NA

HAdV: Human Adenovirus, PAdV: Porcine Adenovirus, BPyV: Bovine Polyomavirus, *EC: Escherichia Coli,* IE: Intestinal enterococi, *CP: Cryptosporidium Parvum, GD: Giardia Duodenalis.*

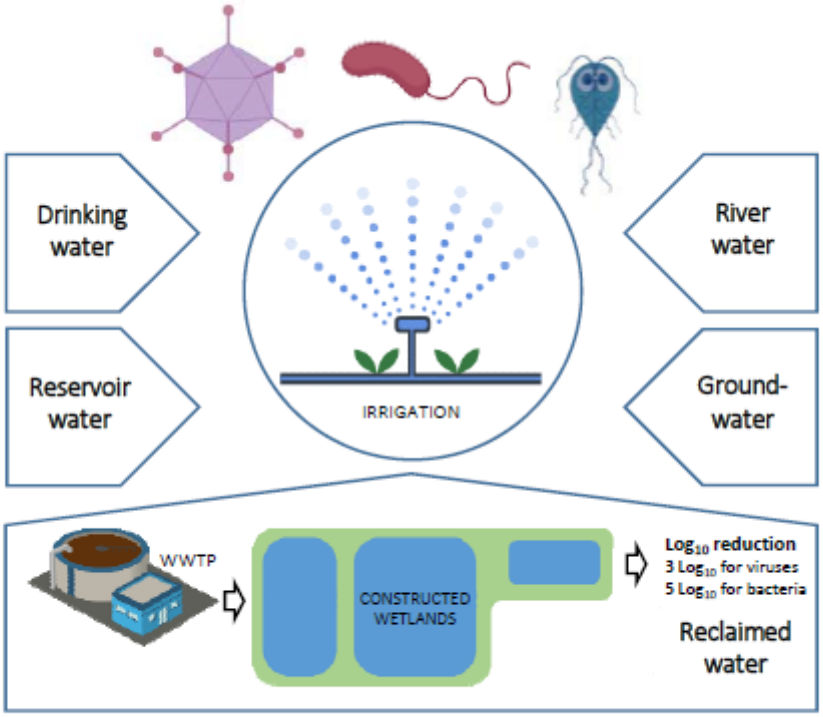
Table 4: Log_{10} reduction values (LRV) of Human Adenovirus (HAdV) in production of reclaimed water. CAS: conventional activated sludge.

	HAdV LRV	Reference
CAS + lagooning	2.01	Fernandez-Cassi et al., 2016
CAS + coagulation and flocculation + Chlorine + UV	2.64	Rusiñol et al., 2015
CAS + sandanthracite filters + zeolite	2.92	Prado et al., 2019
CAS + sustainable wetland system	**3.14**	**this study**
CAS + granular activated carbon	3.20	Liu et al., 2013
CAS + membrane bioreactor (MBR) + reverse osmosis	3.54	Prado et al., 2019
CAS + ultrafiltration	5.20	Liu et al., 2013

Figure 1: Barplots of index virus/viral pathogens and fecal indicator bacteria concentrations in Raw, Secondary (Sec) and Wetland (Wet) effluents.
Mean and maximum Log_{10} reduction values (LRV) along the treatment process. CAS: conventional activated sludge treatment.

Figure 2: Barplots representing seasonal concentrations of viruses and fecal indicator bacteria in raw sewage, secondary treated effluents and
wetland effluents (reclaimed water).

Graphical abstract



Highlights

Virus, bacteria and protozoa contamination was evaluated in irrigation water
HEV was present in groundwater with porcine fecal pollution but low levels of *EC*
HAdV analysis in summer, and NoV in winter, would complement water monitoring
Internalization of pathogenic bacteria should be considered to be in irrigation water