Metagenomic analysis of viruses, bacteria and protozoa in irrigation water

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Abstract

Viruses (e.g., noroviruses and hepatitis A and E virus), bacteria (e.g., *Salmonella spp*. and pathogenic *Escherichia coli*) and protozoa (e.g., *Cryptosporidium parvum* and *Giardia intestinalis*) are well-known contributors to food-borne illnesses linked to contaminated fresh produce. As agricultural irrigation increases the total amount of water used annually, reclaimed water is a good alternative to reduce dependency on conventional irrigation water sources. European guidelines have established acceptable concentrations of certain pathogens and/or indicators in irrigation water, depending on the irrigation system used and the irrigated crop. However, the incidences of food-borne infections are known to be underestimated and all the different pathogens contributing to these infections are not known. Next-generation sequencing (NGS) enables the determination of the viral, bacterial and

protozoan populations present in a water sample, providing an opportunity to detect emerging pathogens and develop improved tools for monitoring the quality of irrigation water. This is a descriptive study of the virome, bacteriome and parasitome present in different irrigation water sources. We applied the same concentration method for all the studied samples and specific metagenomic approaches to characterize both DNA and RNA viruses, bacteria and protozoa.

In general, most of the known viral species corresponded to plant viruses and bacteriophages. Viral diversity in river water varied over the year, with higher bacteriophage prevalences during the autumn and winter. Reservoir water contained *Enterobacter cloacae*, an opportunistic human pathogen and an indicator of fecal contamination, as well as *Naegleria australiensis* and *Naegleria clarki*. Hepatitis E virus and *Naegleria fowleri*, emerging human pathogens, were detected in groundwater. Reclaimed water produced in a constructed wetland system presented a virome and bacteriome that resembled those of freshwater samples (river and reservoir water). Viral, bacterial and protozoan pathogens were occasionally detected in the different irrigation water sources included in this study, justifying the use of improved NGS techniques to get a comprehensive evaluation of microbial species and potential environmental health hazards associated to irrigation water.

1. Introduction

Agricultural irrigation accounts for 36% of the total annual water usage in Europe, reaching up to 80% in some parts of the Mediterranean region (European Environment Agency, 2012). In Spain 79% of the total irrigated area (3Mha) is irrigated with surface water and only 21% with groundwater (Drewes et al., 2017). River flow rate fluctuation and the overexploitation of groundwater resources are existing problems in Europe, where integrated water resource plans are currently being implemented. Moreover, climate change is expected to intensify problems of water scarcity and affect irrigation requirements in the Mediterranean region (Collins et al., 2009). To reduce dependency on freshwater, reclaimed water can be used for irrigation, provided that the risk associated with pathogen contamination can be minimised. Reclaimed water can be relatively nutrient rich and may reduce the need for additional applications of inorganic fertilizers (Parsons et al., 2001). In Catalonia, around 300

wastewater treatment plants (WWTP) treat 700 hm^3 of water every year to produce 204 hm^3 of reclaimed water (ACA, 2016; Pérez et al., 2011). The use of reclaimed water sources is regulated by different national regulations and European guidelines, which stipulate the acceptable microbial concentrations in irrigation water based on the irrigation system being used and the irrigated crop (RD 1620, 2007; WHO, 2006). In 2017, the European Commission published the minimum quality requirements for water reuse in agricultural irrigation and aquifer recharge, producing a guidance document addressing microbiological risks in the primary production of fresh fruits and vegetables (Alcalde-Sanz and Gawlik, 2017; EU C163, 2017).

The number of reported illnesses linked to contaminated fresh produce has increased in both developed and developing countries, with the majority of reported outbreaks being in Europe (Chatziprodromidou et al., 2018). Viruses (e.g., noroviruses and hepatitis A and E virus), bacteria (e.g., *Salmonella spp*. and pathogenic *Escherichia coli*) and protozoa (e.g., *Cryptosporidium parvum* and *Giardia intestinalis*) are well-known contributors to these reported food-borne diseases; however, the number of food-borne infections is known to be underestimated and all the different pathogens, including new and emerging strains, contributing to these infections are not known. The risk associated with the consumption of fresh produce irrigated with water containing pathogens depends on many variables, the most important being the concentration of pathogens in the irrigation water (Gonzales-Gustavson et al., 2019). The levels of exposure to solar radiation, the irrigation system, the time from irrigation to consumption and the type of crop are other important factors (Pachepsky et al., 2011; Thebo et al., 2017). If crops are irrigated with reclaimed water, the concentration loads of the pathogens and their specific persistence in the environment are also important factors.

Current regulations and monitoring programs involve the measurements of selected microbial indicators, which do not necessarily correlate with the reference pathogens (Drewes et al., 2017). Therefore, more resistant pathogens, zoonotic microorganisms excreted by livestock or even uncharacterized pathogenic strains may also be present or underestimated in irrigation water.

While pathogen-specific tests for water quality assessment are appropriate for estimating irrigation water risks, next-generation sequencing (NGS) techniques can detect a wide range of microbial populations in a given sample. Using NGS technologies, most studies have focused on bacterial communities instead of viral or protozoan populations in different environments. Several studies have demonstrated that sewage reflects the bacteriome (Newton et al., 2015), virome (Cantalupo et al., 2011; Fernandez-Cassi et al., 2018; Ng et al., 2012) and parasitome (Bradley et al., 2016) of human populations. Different studies have also characterized virus, bacteria or protozoa diversity in groundwater (D'Auria et al., 2018), river water (Uyaguari-Diaz et al., 2016), and reclaimed water (Rosario et al., 2009). Metagenomic methods have been used to tackle viruses and bacteria found in fresh fruits and vegetables surfaces, highlighting the potential role of irrigation water as vector of waterborne pathogens (Aw et al., 2016; Fernandez-Cassi et al., 2017; Jackson et al., 2013; Leff and Fierer, 2013). Viruses lack a common marker for their taxonomical classification and identification, making difficult to monitor the entire population in a single assay. Uyaguari-Diaz and colleagues selected the gene 23 (encoding the major capsid protein of T4 bacteriophages) and the gene encoding the RNA-dependent RNA polymerase (present in most RNA viruses) as the target amplicons for viral metagenomics (Shi et al., 2016; Uyaguari-Diaz et al., 2016). As with the use of 16S or 18S ribosomal RNA analysis to identify bacteria or protozoa, respectively, the use of a common marker for identifying viruses might result in an imprecise taxonomical classification due to the poor resolution caused by using a single gene (Poretsky et al., 2014). Viruses show great diversity in their genome structure and organization. Thus, important waterborne pathogenic DNA viruses (e.g. adenovirus) lacking an RNA-dependent RNA polymerase might be overlooked when using this marker to identify viruses. In this study, we applied a metagenomic approach to characterize the viral populations in different water sources used for irrigation, including both DNA and RNA viruses. To our knowledge, this is the first study to assess the virome, bacteriome and parasitome of the same irrigation water samples using metagenomics after a single concentration method for all the studied microorganisms.

2. Materials and methods

2.1. Water samples and concentration of microorganisms using the skimmed milk flocculation method (SMF).

Four conventional irrigation water sources (drinking water, reservoir water, groundwater and river water) and one reclaimed water source, currently used for irrigation, were selected for the study. Raw sewage was included as a reference of the microbial contamination circulating within the studied area. Samples were collected every month over a period of one year, from April 2016 to March 2017.

Drinking water was sampled directly from water tanks across the water distribution network of Barcelona. This water source is frequently used in urban community orchards. Reservoir water, a common source of irrigation water in the Catalan territory, was collected from the Foix river reservoir. Groundwater, which is a conventional irrigation water source in the Mediterranean region, was sampled from the north-east of Catalonia, a region with intensive farming and agricultural activities. River water samples were collected from the Fluvià river, which receives effluents from 24 small WWTPs treating up to 100,000 Hab/Eq. The orchard sites surrounding the river are supplied directly with water diverted or pumped from the river.

The outlet water from a sustainable wetland system treating the urban WWTP effluents, was selected as the reclaimed water source. The WWTP uses mechanical treatment to remove sand and large particles and a conventional activated sludge to digest the organic matter. About 70% of the secondary effluent is chlorinated and directly discharged into the River Congost, while the remaining 30% is discharged into the constructed wetland system where its nitrogen and phosphorus contents are reduced by natural plant absorption. This system produces 120,000 m^3 of recycled water every year, which nowadays is mainly used to irrigate green areas. Raw sewage was directly collected from the urban WWTP.

All microorganisms were concentrated using a standardized SMF protocol (Calgua et al., 2013a, 2013b; Gonzales-Gustavson et al., 2017). This protocol achieves high recovery efficiencies, with low variability for selected fecal indicators and a wide range of bacterial, viral and parasitic pathogens

(Gonzales-Gustavson et al., 2017). Briefly, irrigation water samples (10L), as well as raw sewage (500ml), were acidified to a pH 3.5, using 1 N HCl. Conductivity was also measured and adjusted with artificial sea salt (Sigma, Aldrich Chemie GMBH, Steinheim, Germany) to achieve a minimum conductivity of 1.5 mS/cm^2. A pre-flocculated skimmed milk solution (PSM) was prepared by dissolving 10 g of skimmed milk powder (Difco-France) in 1 L of artificial seawater (33,33 gr of sea salts), adjusting the pH to 3.5. The PSM was then added to the samples to obtain a final concentration of 0.01 % of skimmed milk. The 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 then removed, and the sediment was collected and transferred to 500-mL centrifuge containers and centrifuged at 8,000 *g* for 30 min at 4 °C. Pellets were suspended in 5 mL of 0.2 M phosphate buffer, pH 7.5 (1:2, v/v of 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4). The samples were pooled according to the season they were collected in, distributed in refrigerated boxes among the collaborating laboratories and stored at −20 °C.

2.2. Viral DNA and RNA pre-NGS amplification and bioinformatics analysis

Season-pooled concentrates (900 µL) were filtered through 0.45 µm Sterivex filters (Millipore). Free viral DNA and non-viral DNA was removed using TURBO DNA-free Kit (Ambion). The nucleic acids (NA) of viral origin were then extracted with the Viral RNA Mini Kit (Qiagen), without the carrier RNA. Sequence-independent, single-primer amplification (SISPA) was applied, as previously described (Fernandez-Cassi et al., 2018). Briefly, NAs were retrotranscribed using SuperScript III (Life Technologies) and tagged with random Primer A for the detection of both RNA and DNA viruses (Table 1). The second cDNA strand was constructed using Sequenase version 2.0 Kit (USB/Affymetrix). PCR amplification (10 min at 95°C, 25 cycles of 30 s at 94°C, 30 s at 40°C, and 30 s at 50 °C, and a final step of 1 min at 70ºC) was performed using the AmpliTaq Gold Master Mix (Life Technologies) and Primer B to obtain enough dsDNA for library preparation (Table 1). PCR products (100 µL) were cleaned and concentrated into smaller volumes (15 µL) with the DNA Clean & Concentrator Kit (Zymo research). Amplified DNA samples were quantified using Qubit 2.0 fluorometer (Life Technologies, Oregon, USA) and libraries were constructed using a Nextera XT DNA Sample Preparation kit (Illumina Inc.) following the manufacturer's instructions. Samples were

sequenced on an Illumina MiSeq platform, 2 × 300 bp, producing paired-end reads.

The quality of the raw and clean sequences was assessed using the FASTX-Toolkit version 0.0.14 software (Hannon Lab; http://www. hannonlab.org). The sequences were cleaned using Trimmomatic version 0.32 (Bolger et al., 2014), with the trimming of low-quality reads presenting a Phred score above Q15 over a running window of 4 nucleotides. Duplicated reads were removed in a subsequent step to accelerate the downstream assembly. Viral reads were assembled into contigs, based on 90% identity over a minimum of 50% of the read length (coverage), using CLC Genomics Workbench 4.4 (CLC bio USA). The resulting contigs were queried for sequence similarity, using NCBI-BLASTN against the NCBI Viral Genomes database (Brister et al., 2015) and the viral sequences from the GenBank database (Benson et al., 2015). On the other hand, NCBI-BLASTX was also run to compare the translated nucleotide contig sequences against the viral protein sequences from the UniProt database (UniProt Consortium, 2015). Based on the best BLAST result from those three comparisons on each contig, considering a minimum 90% identity cut-off and an E-value lower than 10^{-5}, each sequence was classified into its likely taxonomic group.

2.3. NGS of bacterial populations

Total DNA was extracted from 300 µl of the SMF concentrates using the DNeasy PowerSoil kit (Qiagen), following the manufacturer's instructions and was measured using Qubit 2.0 (Life Technologies, Invitrogen, Oregon, USA). The metagenomic analysis targeted the V3-V4 region of the 16S rRNA gene with previously described primers (Claesson et al., 2009; Herlemann et al., 2011) and the overhang adaptor sequences from Illumina were added to the locus-specific primer for the targeted region (Table 1). Paired-end libraries were built using the Nextera XT DNA sample preparation kit (Illumina Inc.) and sequenced on an Illumina MiSeq platform, 2 x 250 bp. End reads with a quality Phred score above Q30 were trimmed with QIIME 1.9.1. (Caporaso et al., 2010) and the cleaned reads were merged with the FLASH software (Magoč and Salzberg, 2011). All sequences with ambiguous base calls were also discarded. Taxonomic classification was performed using the SILVA database (Quast et al., 2012), with a 95% cut-off for sequence identity.

188 *2.4. NGS of protozoan populations*

189 A volume of 300 µl of each SMF concentrate was lysed using the FastPrep®-24 instrument (MP
190 Biomedicals). DNA was extracted with the FastDNA® SPIN Kit (MP Biomedicals) for soil, according
191 to the manufacturer's instructions. A modified protocol for the lysing step was followed, as described
192 previously (Shields et al., 2013). Samples were first homogenized for 60 s. After the bead beating
193 step, samples were placed on ice for 1 min and then homogenized for another 60 s. The final DNA
194 products were eluted in a final volume of 50 µL. All the DNA extractions were performed in
195 duplicate. Afterwards, the duplicate samples were mixed and purified with the OneStep™ PCR
196 Inhibitor Removal Kit (Zymo Research) to remove inhibitors that could have affected downstream
197 enzymatic reactions such as the PCR. Genomic DNA concentrations were measured with the Qubit®
198 dsDNA BR Assay Kit (Thermo Fisher Scientific). The 260:280 ratio was measured using the
199 NanoDrop ND-1000 UV/Vis spectrophotometer (Thermo Fisher Scientific). The amplicon sequencing
200 protocol for protozoa, targeting the hypervariable V4 region of the 18S rRNA gene, was performed as
201 previously described (Moreno et al., 2018), using the primers described in Table 1. Briefly, DNA
202 amplicon libraries were built using the Nextera XT DNA Sample Preparation Kit (Illumina Inc.) and
203 sequenced on an Illumina MiSeq platform. The obtained reads were analyzed using QIIME 1.9.1
204 (Caporaso et al., 2010), applying additional scripts that are available in the Microbiome Helper
205 Virtual Box (Comeau et al., 2017). OTUs were defined at the 97% similarity cut-off. The Protist
206 Ribosomal Reference database pr2_gb203_version_4.5 fasta (Guillou et al., 2013) was used as the
207 reference.

208 *2.5. Statistics and heatmaps*

209 The richness and diversity indices were calculated using the Catchall version 4.0 software (Bunge et
210 al., 2012), which included the species richness estimate (Chao1) and the Shannon diversity index as
211 measures of the number of different species detected and the relative abundances of the species
212 present in a sample, respectively. Heatmaps were generated using the R library ggplot2 graphics
213 (Kolde, 2015).

214

3. Results

3.1. Output of the metagenomic libraries

In this study, the viral, bacterial and protozoan communities present in the different irrigation water sources were evaluated using a metagenomic approach. The total number of reads obtained as well as the richness and diversity indices are summarized in Table 2. In total, 95.13% of the Illumina viral reads were assembled into contigs. The highest viral richness and diversity were observed in raw sewage samples, particularly those collected in winter. Drinking water samples presented the lowest number of viral reads compared to the other water sources. Regarding bacterial populations, reservoir water samples as well as river water samples collected in the summer and spring showed the highest richness and diversity values. For the reservoir, groundwater and river water samples, the highest Chao values and Shannon indices for bacteria were obtained in the samples collected during summer (Table 2). After quality filtering, trimming and detection of PCR-chimeras, the highest number of raw reads (110.766) obtained from the analysis of protozoa mock community was obtained for the pooled samples of reclaimed water collected in the summer season. Due to the wide range of raw reads, obtained from the 18S rRNA amplicon-based analysis, and the relatively low abundances of pathogenic protozoa, it was not possible to calculate richness and diversity values.

3.2. Virome of the different irrigation water sources and raw sewage

The diversity of the viral species detected is graphically represented in Figure 1 according to the viral family and water source. Human viral pathogens were detected in all the different types of samples analyzed, except for drinking and reservoir water (Table 3). Over 67% of the viral metagenomic sequences showed significant similarity to the viral sequences stored in one or more of the three databases used (NCBI Viral Genomes database, GenBank and UniProt). Most of the known viral species (> 77%) detected in the conventional irrigation water sources were plant viruses or bacteriophages. Bacteriophages belonging to the *Siphoviridae* (dsDNA), *Myoviridae* (dsDNA), *Podoviridae* (dsDNA) and *Microviridae* (ssDNA) families were always the most represented in all the irrigation water sources.

242 The 95% of the viral reads from the drinking water samples corresponded to phages infecting
243 different bacteria, including *Arthrobacter* spp., *Propionibacterium* spp. and *Pseudomonas* spp. Up to
244 39 different species belonging to 6 phage families were detected in this type of water. NGS did not
245 detect any waterborne pathogens in drinking water (Table 3), with only a few contigs identified that
246 corresponded to the *Myoviridae* and *Microviridae* phage families and *Phycodnaviridae*.
247 *Phycodnaviridae* (dsDNA), which includes viruses that infect freshwater eukaryotic algae and
248 phytoplankton, was very prevalent in the reservoir, groundwater and river water samples. Viruses
249 belonging to *Virgaviridae* (ssRNA+) and *Tombusviridae* (ssRNA+) were highly abundant in the river
250 water samples collected in the autumn and winter, whereas a greater diversity of viruses from the
251 *Phycodnaviridae* family were detected in groundwater in summer and river water in spring. A large
252 number of viruses affecting insects, including members of the *Iridoviridae* (dsDNA) and *Parvoviridae*
253 (ssDNA) families that infect cockroaches, flies and mosquitoes, were mainly detected in the river and
254 reservoir water samples. The drinking water samples had higher proportions of known viral sequences
255 (77%) than river (68%) or reclaimed water (67%) samples; however, a large fraction of the viral reads
256 could not be assigned. Reclaimed water samples presented a mixture of bacteriophages, plant viruses
257 and viruses infecting invertebrates (e.g., aphids and flies), but no human pathogens. Less than 1% of
258 the viral reads corresponded to non-pathogenic viruses that infect humans. More than 30 different
259 viral families were detected in the urban sewage samples, with the majority of the contigs belonging
260 to the *Siphoviridae* and *Myoviridae* families, which showed the highest diversity in the samples
261 collected during the summer (167 and 103 different species, respectively) and winter (172 and 144
262 species, respectively). When analyzing the data obtained from the raw sewage samples pooled
263 according to season (Figure 1), pathogenic viruses belonging to the *Caliciviridae* and *Astroviridae*
264 families were present throughout the year, but the number of contigs showed seasonality, being higher
265 during the autumn and winter (Table 3). A high diversity of human astrovirus contigs were detected in
266 the raw sewage samples collected in the winter and autumn (maximum length, 2,483 bp). Human
267 adenovirus 41 was detected in raw sewage, as well as in groundwater samples collected during the
268 autumn. Norovirus GI and GII as well as sapovirus GI, GIV and GV, which are important human
269 pathogens belonging to the *Caliciviridae* family, were found throughout the year in raw sewage

samples. However, the number of species, as well as the number of detected reads, was higher during the winter and autumn. Norovirus GII was also detected in the river water samples collected during the autumn, corresponding with the peak excretion season. A 396-bp contig belonging to the beta human papillomavirus 104 was found in the raw sewage sample collected during spring. Within the *Picornaviridae* family, sequences belonging to human enteroviruses A, B and C were detected in the raw sewage samples throughout the year. Remarkably, a 653- and an 867-bp contig were detected that corresponded to EV-71, showing 86% and 94.8% identity over 99% of the sequence coverage, respectively. Among the viruses belonging to the *Picornaviridae* family that could cause gastroenteritis, the Aichi virus presented greater abundance during the summer, whereas salivirus was more prevalent during the winter. Interestingly, hepatitis E virus (HEV) was detected in the raw sewage and groundwater samples collected in the summer.

3.3. Bacteriome of the different irrigation water sources and raw sewage

The diversity of the bacteriome varied among the samples. Drinking water as well as samples of groundwater, river water and reclaimed water collected in the winter and autumn presented the lowest diversity values (Table 2). Raw sewage diversities did not vary across seasons. Figure 2 shows the relative abundances of several bacteria that could be related with human infections in each water sample.

The most abundant bacterial genus in drinking water, accounting for 33.2% of the bacterial reads, was *Pseudomonas* (Figure 2). The genera *Cellvibrio* (27.0%), *Anoxybacillus* (5%) and *Caulobacter* (5%), which are associated with plant rhizospheres, streams and fresh water, were also prevalent in drinking water.

Most of the bacterial reads (18%) for reservoir water samples corresponded to genera representing less than 1% abundance in the samples, thus demonstrating high bacterial diversity and richness. Only the *Bacillus* (17%) and *Enterobacter* (6%) genera presented relatively high abundances in this type of irrigation water source (Figure 2). The other taxa represented by 4 to 6% of the reads were *Raoultella*, *Actinobacter* and non-culturable bacteria.

297 Regarding waterborne human pathogens, groundwater samples collected in the autumn presented the
298 highest abundance of *Enterobacter* (34.9%) (Figure 2), while the genera *Geobacillus* (20%) and
299 *Thermus* (18.1%) were abundant in the samples collected in the spring. The genus *Pantoea* showed a
300 relative abundance of 38.1% in the samples collected in winter, but less than 1% abundance in the
301 samples from the other seasons. Different bacterial genera with relative abundances ranging from 9%
302 to 14% were observed in the samples collected in summer, which showed the highest values for
303 bacterial richness and diversity compared to the samples collected in the other seasons (Table 2). The
304 percentage of non-culturable bacteria ranged from 0.65% in winter to 4.8% in spring.
305 River water samples also presented variations in bacterial richness and diversity between the seasons.
306 *Limnohabitans,* common planktonic bacteria occurring in river streams, was detected in low
307 percentages across the seasons (3.9%), but showed increased prevalence in the autumn, with over a
308 third of the total number of reads corresponding to this bacterial genus (32.5%). The human-
309 associated *Bacillus, Pseudomonas* and *Yersinia* genera showed low abundances throughout the year,
310 but increased prevalence in the winter (maximum abundances of 23.9%, 21.6% and 12.4%,
311 respectively) (Figure 2). Similarly, *Flavobacterium* presented increased relative abundance during the
312 autumn (21.1%). *Enterobacter* and *Serratia* were detected in all the river water samples, but at lower
313 percentages (Figure 2).
314 The most prevalent bacteria in reclaimed water was *Actinobacteria* (10.8% in spring to 2.5% in
315 autumn), with bacteria belonging to the *Limnohabitans* genus being relatively abundant throughout
316 the year. The number of reads corresponding to Rickettsiales bacteria increased only in the winter, but
317 none of these were associated with pathogenic species. The most prevalent human pathogens were
318 *Flavobacterium* and *Serratia* bacteria, showing peak abundances during the winter (Figure 2).
319 The number of human-associated bacterial genera ranged from 10 in the winter to 6-7 in the other
320 seasons for raw sewage samples. Among these genera, *Arcobacter* was the most abundant, with
321 percentages ranging from 14.9% in the autumn to 34.9% in the spring. However, *Arcobacter* showed
322 a generally high prevalence throughout the year, being more prevalent in the spring and summer and
323 showing a small decrease in the colder seasons. *Pseudomonas* was more prevalent in the summer and
324 winter, decreasing to 1% abundance in the spring. The other less abundant genera were *Acinetobacter*,

Aeromonas, *Bacteroides* and *Streptococcus*, with some of these containing bacteria that are pathogenic to humans. Differences within a genus were observed between the seasons. For example, the genus *Yersinia* showed an abundance of 7.4% in the winter, but an abundance of less than 0.1% in the other seasons (Figure 2).

3.4. Parasitome of the different irrigation water sources

The waterborne protozoan pathogens detected in raw sewage and the different irrigation water sources are summarized in Table 4. *Blastocystis* sp. was the most prevalent protozoa, while different free-living amoeba (FLA) such as *Acanthamoeba* spp, *Entamoeba coli*, *E. dispar*, *E. moshkovskii*, *Naegleria fowleri*, *N. australiensis*, *N. clarki* and members of the *Hartmannellidae* family (including *Vermamaoeba vermiformis*) were also detected. *Naegleria* spp. were most frequently detected in reservoir water, groundwater, river water and reclaimed water samples collected during the summer. *N. fowleri*, the causative agent of primary amoebic meningoencephalitis (PAM), was detected in groundwater. *Cryptosporidium* spp. was detected in reclaimed water samples collected during the summer at a low relative abundance (< 0.05%). *Giardia* was not detected in any of the samples analyzed.

4. Discussion

The metagenomic approach enables the description of viral, bacterial and protozoan populations in a water sample, providing an opportunity to detect emerging pathogens and develop improved tools for monitoring the quality of irrigation water. However, it is not an exhaustive detection method, since pathogens detected by other molecular tools (e.g., q(RT)-PCR) can be missed (Fernandez-Cassi et al., 2018). Moreover, the detection of pathogens using metagenomics may be hampered by low target abundances and/or by higher microbial concentrations in the sample. The quantification of bacterial, protozoan or viral abundances based on the number of reads is in fact a relative number because the resulting data only indicate values in relation to the total number of microorganisms in a specific sample. Since metagenomics assays are not suitable for quantification, these results are intended to

indicate the global distribution of families/species and the relationship between diversity and seasonality.

4.1. Raw sewage, the main source of microbial contamination

Season-pooled samples of raw sewage were analyzed as sewage is the most important source of environmental contamination and has been demonstrated to be a powerful tool for epidemiological surveillance and risk evaluation (Fernandez-Cassi et al., 2018; Newton et al., 2015). As previously reported, the majority of viruses identified in the raw sewage virome were bacteriophages (Bibby and Peccia, 2013; Cantalupo et al., 2011). The low abundance of known human pathogenic viral families (less than 1%) in raw sewage has also been reported in the literature (Cantalupo et al., 2011; Fernandez-Cassi et al., 2018).

Our metagenomic approach identified important human pathogens in raw sewage that are transmitted via the fecal-oral route. Human enteroviruses A, B and C, adenoviruses, astroviruses, caliciviruses and picornaviruses, which mainly cause gastroenteritis, were detected in raw sewage throughout the year. The higher abundance and diversity of astroviruses and caliciviruses during the colder seasons are consistent with their well-documented seasonal peak that has been demonstrated by other molecular tools (Bosch et al., 2014; Haramoto et al., 2005). HEV, an emerging zoonotic virus that causes acute hepatitis, was detected in raw sewage samples collected during the summer. Although it was occasionally detected, it is still important to report its presence as there is an increasing number of foodborne HEV cases being reported in Europe (EFSA BIOHAZ Panel et al., 2017).

The *Arcobacter* and *Pseudomonas* genera were the most abundant bacterial populations observed in raw sewage (Figure 2). Whereas *Pseudomonas* was more prevalent in the summer and winter, *Arcobacter* showed a higher prevalence throughout the year, as has been stated by other studies (Tang et al., 2016). Most of the bacterial reads corresponded to *A. cryaerophilus*, which is associated with human and animal disease and has been previously reported to be the predominant *Arcobacter* species occurring in raw sewage (Ferreira et al., 2015; Figueras et al., 2014; Fisher et al., 2014; Levican et al., 2016; Salas-Massó et al., 2018).

Other pathogenic bacteria such as *Bacteroides* and *Aeromonas* also occurred at high percentages in all the raw sewage samples analyzed, being more prevalent in those collected in the spring. Interestigly Aeromonas and Arcobacter have been found predominating in metagenomics analysis of wastewater and it have been indicated that this is due to the capacity of this bacteria to multiply in the sewerage system (McLellan et al., 2010; McLellan and Roguet, 2019).

Blastocystis spp. were the most prevalent protozoa detected in raw sewage although it should be noted that the rRNA copies of 18S used to identify protozoa vary in different types of waterborne protozoa (Moreno et al., 2018). The effects of *Blastocystis* on human health remain controversial (Greige et al., 2018). *Blastocystis* is a common protozoan parasite found in the human digestive tract and is the most common eukaryotic parasite of the human intestine (Greige et al., 2018; Nieves-Ramírez et al., 2018; Souppart et al., 2009). It has been linked to gastrointestinal symptoms, but has also been described as an asymptomatic colonizer of the normal intestinal microbiota (Nieves-Ramírez et al., 2018).

FLA are ubiquitous protozoa that can cause opportunistic and non-opportunistic infections in humans (Plutzer and Karanis, 2016; Serrano-Luna et al., 2013). FLA such as *Naegleria*, *Acanthamoeba* and *Vermamoeba* species are ubiquitously distributed worldwide in various warm aquatic environments (Panda et al., 2015). Waterborne transmission of pathogenic strains of *Acanthamoeba* spp. and *Naegleria* spp. is of great relevance (Plutzer and Karanis, 2016). In our study, *N. clarki* was the most frequently detected species in raw sewage.

Three *Entamoeba* spp. were identified in our raw sewage samples: *E. moshkovskii*, *E. coli* and *E. dispar*. *E. moshkovskii* frequently occurs in regions where amebiasis is highly prevalent and has been isolated from wastewater, rivers, lakes and brackish water (Serrano-Luna et al., 2013). *E. moshkovskii* is primarily considered an FLA, but it can infect humans (Ali et al., 2003; Mahmoudi, 2015). *E. dispar* has traditionally been considered non-pathogenic, but this view is being re-evaluated (Caliari et al., 2015). Current evidence suggests that the risk factors for acquiring *E. moshkovskii* infections are similar to those described for *E. histolytica* and. *E. dispar* (Serrano-Luna et al., 2013).

4.2. Diversity of microorganisms in different irrigation water sources

4.2.1.Drinking water

Viruses infecting bacteria and algae were detected in drinking water. Previous studies with potable water have described latent forms of bacteriophages to be the most abundant viruses in this type of water source (Rosario et al., 2009). Although our results confirmed that bacteriophages were detectable after chlorination, there were no human viral pathogens in drinking water.

Pseudomonas (33.2%) and *Cellvibrio* (27.0%) bacteria were highly abundant in drinking water, as has been previously reported (Bertelli et al., 2018; El-Chakhtoura et al., 2018). Despite the low abundance of this species, it is still higher than that reported in other studies (Bertelli et al., 2018). Among the protozoa, we detected *Acanthamoeba* at low abundances in drinking water. This free-living opportunistic protozoan parasite has been detected in drinking water used for irrigation and has been also reported to be present in bottled water (Khan, 2003). Pathogenic protozoa have been detected in municipal drinking water systems, even in countries with highly regulated water infrastructures, causing waterborne and food-borne outbreaks (Kulinkina et al., 2016). This might be particularly alarming in countries where no residual disinfectant is applied to the water (Braeye et al., 2015). The low abundances of the two pathogenic microorganisms detected in the drinking water samples need to be analyzed further, given that NGS data present important limitations and that the detected microorganisms might be inactive.

4.2.2.Reservoir water and river water

Reservoir water and river water showed very similar distributions of the viral families, sharing 97 of the complete viral genomes. Although the two types of water sources were sampled from different river basins, it is well known that microbial communities are similar in these two ecosystems (Vaz-Moreira et al., 2014). The reduced runoff within the Mediterranean region that might be caused by climate change will affect the circulation patterns of river water into reservoirs and consequently the proportions of nutrients and organic carbon in these ecosystems (Imek et al., 2011). Thus, viral diversity may vary between intense rainfall events and long dry periods, when the demand for irrigation water increases and the limited water circulating into reservoirs increases anoxic conditions.

We observed higher viral diversity in the river water samples collected during autumn and winter. Except in the pooled samples collected in the summer, the most abundant *Microviridae* phage family detected was the recently described Eel River Basin Microviridae (ERBM), most frequently found in sediments (Bryson et al., 2015). It has been reported that the microbial community structure and associated metagenomes, especially for bacteriophage families, can change after rainfall events (Tseng et al., 2013). Thus, the seasonality in viral diversity for river water samples may be due to the fact that the river flow decreases during the summer months (from 8 m^3/s to 1 m^3/s) and bacteriophages are not resuspended from river sediments.

Regarding plant viruses, it is not clear how these viruses found in irrigation water could infect the plants being irrigated. Detailed studies are required to elucidate their potential impact on agriculture (Balique et al., 2015; Colson et al., 2010).

While pathogenic *Bacillus* species were not detected in reservoir water, 30% of the *Enterobacter* reads corresponded to *Enterobacter cloacae*, which is an opportunistic human pathogen and an indicator of fecal contamination (Chen et al., 2014). Thus, the presence of this bacterial species could indicate the existence of a source of human fecal contamination. Contigs corresponding to common fecal indicators, as crAssphage (1 in autumn) and Pepper mild mottle virus (PMMoV) (23 in winter and 29 in autumn), were detected in riverwater.

As stated above, *N. australiensis* and *N. clarki* were detected in reservoir water (0.13%) and river water (0.008%). *Naegleria* isolation has been reported to be higher during the warmer seasons in diverse aquatic habitats worldwide (De Jonckheere, 2004; Panda et al., 2015; Pernin et al., 1998). *N. australiensis* can be pathogenic to mice and could affect human health (Kao et al., 2012).

It is interesting to note that *Pseudomonas* and *Bacillus* bacteria were predominant in the pooled samples from winter and summer. Similar results of the relative abundances of the *Flavobacteriaceae* and *Comamonadaceae* families were also obtained from the nearby river Ter (Lekunberri et al., 2018).

4.2.3.Groundwater

459 It has been reported that viruses can survive for a long time in groundwater and can pass through the
460 aquifer pores that physically filter out larger pathogens (Hunt et al., 2010; Ogorzaly et al., 2010). In
461 our study, waterborne excreted pathogens were not frequently detected in groundwater samples,
462 whereas plant viruses and bacteriophages were widely prevalent. However, we did detect HEV in the
463 pooled sample of groundwater collected in the summer and human adenovirus (HAdV) in the sample
464 collected during autumn. HAdV, which indicates human fecal contamination, has been associated
465 with precipitation events and can persist in the environment for a long time (up to 1,300 days)
466 (Bradbury et al., 2013; Kauppinen et al., 2018). These results were confirmed by running specific RT-
467 qPCR (Jothikumar et al., 2006) and sequencing (data not shown). Interestingly, we detected porcine
468 adenovirus in the groundwater sample collected during the summer (at a concentration of 10E+03
469 GC/L), confirming porcine fecal pollution (data not shown). HEV, a zoonotic pathogen that can cause
470 self-limiting or fulminant hepatitis in humans, was detected in groundwater despite its low prevalence
471 in the studied area. Intensive pig farming occurs in the surrounding areas from where the groundwater
472 was extracted, suggesting that leakage from the slurry tanks could have contaminated the groundwater
473 with the zoonotic pathogen.
474 Pooled samples of groundwater showed different distributions of bacterial populations between the
475 seasons. *Geobacillus* (20.0% in the spring to 0.9% in the summer), followed by *Thermus* and
476 *Anoxybacillus,* all showed seasonal peaks and were frequently distributed in water springs. The high
477 abundance of proteobacteria in groundwater has been also reported previously (Hong et al., 2013).
478 It is interesting to point out the relative abundance of *Vermamoeba vermiformis* (2.63%) in the
479 summer groundwater sample. *V. vermiformis* colonizes water systems and is a reservoir of pathogenic
480 bacteria such as *Legionella pneumophila* (Fouque et al., 2014). The detection of *N. fowleri* in
481 groundwater is of particular concern for human health as it is the principal cause of PAM, a rare and
482 severe disease that causes inflammation and destruction of the brain. The risk of infection depends on
483 the relatively high concentrations of *N. fowleri* in water (Cabanes et al., 2001). The relative
484 abundance of *N. fowleri* in the summer groundwater sample was 0.63%, but we were not able to
485 determine the concentration of the amoeba with our method. *Naegleria* spp. present an additional

threat to human health because they can act as vehicles for the multiplication and dispersal of pathogenic bacteria (Huang and Hsu, 2010).

4.2.4.Reclaimed water

Plant viruses, including *Virgaviridae*, *Tombusviridae* and *Phycodnaviridae*, and bacteriophages, such as *Siphoviridae*, *Myoviridae*, *Podoviridae* and *Microviridae*, accounted for more than 90% of the known viral reads obtained with our reclaimed water samples, representing over 80% of the known viral species detected (Figure 1). The wetland from where the reclaimed water originated appeared to restore the naturally occurring microbial communities, as the distribution of the viral families detected in the reclaimed water samples resembled that of freshwater samples (river and reservoir water). We identified different circoviruses and parvoviruses in reclaimed water, which agrees with the findings of other metagenomic studies performed on raw sewage and reclaimed water produced after the chlorination of treated secondary effluents (Blinkova et al., 2009; Rosario et al., 2009). Viruses belonging to these two families are highly stable in the environment and have a broad host range that includes mammals and invertebrates. Given the huge biodiversity in complex ecosystems such as wetlands, these viruses might not be of concern for human health. CrAssphage (1 in summer) and PMMoV contigs (5, 21 and 23 in summer, winter and autumn respectively), were detected at the wetland effluent samples.

According to the minimum quality requirements for water reuse in agricultural irrigation, wetland effluents may be optimal for crop irrigation as class B recycled water, given that it is rich in macronutrients like nitrogen and phosphorus (reported mean values over the year of sampling: 63 mg/l for N and 10.7 mg/l for P (data not shown)) and no human pathogens were detected.

The *Actinobacteria* and *Limnohabitans* genera were predominant in all the reclaimed water samples. These bacteria inhabit a broad range of freshwater habitats and can constitute up to 30% of the free-living bacteria in freshwater systems (Zwart et al., 2002). As previously stated for viruses, the bacteriome of reclaimed water was similar to that of river water, demonstrating that the treatment used to produce the reclaimed water restores the bacterial populations observed in river water.

512 It should be noted that the prevalence of *Cryptosporidium* spp. and *Giardia intestinalis* might have
513 been underestimated by our metagenomic approach. These parasites are the two most commonly
514 occurring protozoa associated with waterborne disease outbreaks (Plutzer and Karanis, 2016) and are
515 highly prevalent in sewage and reclaimed water. We detected *Cryptosporidium* spp. in the samples
516 collected during summer. Although the SMF method has demonstrated to be usefull to recover
517 Giardia (Gonzales-Gustavson et al., 2017), this protozoan pathogen was not found in any of the
518 samples analyzed. The high microbial concentrations could have masked the presence of Giardia. It is
519 also interesting to note that *Entamoeba* was detected in reclaimed water. Although the pathogenicity
520 of the detected *E. moshkovskii* remains unknown, the presence of this protozoa confirms previous
521 claims that its prevalence might be underestimated (Shimokawa et al., 2012).

522 *4.3. Metagenomic approach as a public health tool*

523 Our results give a global picture of the microbial communities present in different types of irrigation
524 water sources produced by using a single concentration method and a sequencing platform. This
525 approach has huge potential given that NGS techniques are expected to become more affordable and
526 automatized. However, the use of metagenomics to assess water quality will not replace current water
527 quality tests in the near future. Increasing the sampling locations and the number of samples
528 sequenced will contribute to the monitoring of microbial trends and diversity shifts. Furthermore, the
529 metagenomics approach can be used to detect viable but non-culturable bacteria/protozoa as well as
530 emerging and known viral pathogens. As these new tools are still in development, several
531 improvements are needed to avoid possible biases and uncertainties associated with the pre-
532 amplification step and the loss of information during the filtering and processing of the huge amounts
533 of data. Further improvements in NGS technologies might enable the study of all 16S/18S rRNA gene
534 sequences to provide greater resolution for better taxonomical classification. The earlier we identify
535 the origin of fecal contamination, the better our chances are to find and eliminate the source of
536 microbial pathogens. Thus, it is critical to have a comprehensive understanding of all the viruses,
537 bacteria and protozoa (including potential pathogens) circulating in different irrigation water sources,
538 as this information helps assess water quality.

We detected only a few human pathogens in both conventional irrigation water sources (drinking, reservoir, groundwater and river water) and reclaimed water. Microbial abundances and diversities in the reclaimed water samples were very similar to those in the river water and reservoir water samples. In the Mediterranean region, increased temperatures and decreased precipitation will cause a general decrease in water availability. This may result in pathogens occurring at higher concentrations in rivers as the river flow rate will not be sufficient to dilute the effluents from WWTPs (Rusiñol et al., 2015). Seasonal effects have to be carefully evaluated, as one pooled sample per season might result in overestimation of the seasonal effects. As sequencing costs of the high-throughput platforms are being reduced, researchers may develop studies with large number of samples per season, which we consider as one caveat of this study.

The Urban Waste Water Treatment Directive (91/271/CEE, 1991) does not require disinfection prior to discharge despite the fact that WWTP effluents contain elevated levels of pathogens (Naughton and Rousselot, 2017; Rusiñol and Girones, 2017). Thus, sustainable and green water treatments to produce reclaimed water are not only a way of producing new irrigation water sources, but also a way of reducing pathogen levels in the receiving water bodies. Water reuse for irrigation purposes is consistent with population growth plans and is a chance to close the human water cycle.

5. Conclusions

- Viral diversity in river water varied between seasons, with bacteriophages becoming more prevalent during autumn and winter, when norovirus GII strains also become detectable.
- Reservoir water contained *Enterobacter cloacae*, an opportunistic human pathogen and indicator of fecal contamination, *Naegleria australiensis* and *Naegleria clarki*.
- The metagenomic approach revealed the presence of the human pathogens HEV, HAdV and *Naegleria fowleri* in groundwater samples collected during the summer.
- A constructed wetland used as a sustainable system to treat secondary effluents from an urban WWTP seemed to restore naturally-occurring microbial communities as the virome and bacteriome of the reclaimed water resembled those of freshwater (river and reservoir water).

Data availability statement

Raw data can be found in zenodo under the DOI number 10.5281/zenodo.2620427.

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