Evidence of viral dissemination and seasonality in a Mediterranean river catchment: implications for water pollution management.

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Abstract

Conventional wastewater treatment does not completely remove and/or inactive viruses; consequently, viruses excreted by the population can be detected in the environment. This study was undertaken to investigate the distribution and seasonality of human viruses and faecal indicator bacteria (FIB) in a river catchment located in a typical Mediterranean climate region and to discuss future trends in relation to climate change. Sample matrices included river water, untreated and treated wastewater from a wastewater treatment plant within the catchment area, and seawater from potentially impacted bathing water. Five viruses were analysed in the study. Human adenovirus (HAdV) and JC polyomavirus (JCPyV) were analysed as indicators of human faecal contamination of human pathogens; both were reported in urban wastewater (mean values of 10^6 and 10^5 GC/L, respectively), river water (10^3 and 10^2 GC/L) and seawater (10^2 and 10^1 GC/L). Human Merkel Cell polyomavirus (MCPyV), which is associated with Merkel Cell carcinoma, was detected in 75% of the raw wastewater samples (31/37) and quantified by a newly developed quantitative polymerase chain reaction (qPCR) assay with mean concentrations of 10^4 GC/L. This virus is related to skin cancer in susceptible individuals and was found in 29% and 18% of river water and seawater samples, respectively. Seasonality was only observed for norovirus genogroup II (NoV GGII), which was more abundant in cold months with levels up to 10^4 GC/L in river water. Human hepatitis E virus (HEV) was detected in 13.5% of the wastewater samples when analysed by nested PCR (nPCR). Secondary biological treatment (i.e., activated sludge) and tertiary sewage disinfection including chlorination, flocculation and UV radiation removed between 2.22 and 4.52 $\log_{10}$ of the viral concentrations. Climate projections for the Mediterranean climate areas and the selected river catchment estimate general warming and

changes in precipitation distribution. Persistent decreases in precipitation during summer can lead to a higher presence of human viruses because river and sea water present the highest viral concentrations during warmer months. In a global context, wastewater management will be the key to preventing environmental dispersion of human faecal pathogens in future climate change scenarios.

1. Introduction

Understanding the environmental fate of pathogens is useful for minimising the risk to humans. Human viruses are excreted at high concentrations in faeces and urine and can be transmitted through improperly treated wastewater. As part of the EU-FP7-funded VIROCLIME project, the present study developed a surveillance program centred on a typically Mediterranean climate region: the Llobregat River basin (Catalonia, northeast of Spain).

Water microbiological quality is traditionally defined and regulated by faecal indicators that are more sensitive to water treatment and environmental conditions than viral pathogens (de Roda Husman et al., 2009; Figueras and Borrego, 2010). Human adenoviruses (HAdV) and JC polyomavirus (JCPyV) have been proposed as specific human faecal indicators based on their high prevalence in all geographical areas surveyed to date (Bofill-Mas et al., 2000a; Pina et al., 1998). These viruses have been widely used to trace faecal pollution in the environment (Bofill-Mas et al., 2013, Rusiñol et al., 2013). Both viruses are also human pathogens related with enteric and respiratory illness, eye infections and severe disease in immunocompromised patients (Crabtree et al., 1997; Imperiale, 2000). Merkel cell polyomavirus (MCPyV), which has been found integrated in a very high percentage of Merkel cell carcinomas, has also been isolated from urban sewage river water (Bofill-Mas et al., 2010; Calgua et al., 2013a).

Although a sub-cutaneous route seems to be the most likely transmission pathway, the identification of this cancer-related polyomavirus in sewage has been recognised as a significant research question (Spurgeon and Lambert, 2013).

Norovirus genogroup II (NoV GGII) is a single stranded RNA virus that is recognised as the major cause of self-limiting viral gastroenteritis (Craun et al., 2010; Kroneman et al., 2008). Furthermore, NoV GGII is believed to be the most significant etiological agent in documented recreational water-borne outbreaks, followed by adenoviruses (Sinclair et al., 2009). In the wider community, NoV GGII has been associated with the majority of recorded gastroenteritis cases (Lopman et al., 2004). Person-to-person transmission is the most common pathway, but NoV is spread by several routes that include contaminated shellfish, fresh food, processed food and water (Mathijs et al., 2012). NoVs are highly infectious, with a single virus particle having a probability of infection approaching 49% (Teunis et al., 2008).

Hepatitis E virus (HEV) also has a water-borne route of transmission (Orrù et al., 2004). Although HEV is endemic in low-income countries where it produces acute and self-limited hepatitis, it also circulates in industrialised countries (Clemente-Casares et al., 2003; Legrand-Abravanel et al., 2009). In Spain, HEV is found in 30% of urban sewage, which is considered an important source of HEV dissemination (Rodriguez-Manzano et al., 2010).

During the 18-month study period, HAdV, JCPyV, MCPyV, NoV GGII and HEV together with two faecal indicator bacteria (FIB) (*Escherichia coli* (EC) and intestinal enterococci (IE)) were surveyed in a Mediterranean river catchment. Given that the main viral inputs were likely derived from raw or treated effluents, raw and treated water samples were also tested. In this study, we also assessed the repeatability of the skimmed milk flocculation protocol used to concentrate viruses from different water matrices, designed a new qPCR method for the specific detection of MCPyV in water and finally discussed further trends of virus pollution considering climate projections for the Llobregat river basin.

Climate change models continue to predict higher stress on water resources that may contribute to pathogen dispersion, including bacteria, viruses and protozoa (IPCC, 2007). The overall goal of this investigation was to provide empirical data on the spatial and temporal patterns of viral pathogens and indicators in a changing river basin to allow public health managers to assess risks in future scenarios.

2. Materials and methods

2.1. Sample and data collection

The Llobregat River flows approximately 170 km from the Pyrenees Mountains to the Mediterranean Sea, discharging near the city of Barcelona. The 4950-km2 river basin accommodates 5 million people, including more than half of the Catalan population. Treated urban sewage, industrial effluents and agricultural runoff affect the quality of raw river water, which is the main source of treated drinking water for Barcelona and its metropolitan area. In fact, the urban water supply constitutes 65% of the total Llobregat water demand (ACA, 2012). The annual average river discharge volume is 690 cubic hectometers/second (hm^3/s), of which over 40% consists of effluents from the approximately 50 wastewater treatment plants (WWTP) located within the Llobregat river catchment area (annual mean discharges: 300 hm^3/s) (ACA, 2012).

A total of 196 water samples were collected from January 2011 to June 2012 from 4 different sampling sites (Figure 1). Site A is located 80 km upstream of the river mouth (n= 36 samples). The water flow rate or mean river discharge volume at site A was 0.5 m^3/s, corresponding to 14 hm^3/s of mean annual discharge. Ten WWTP are located upstream of the sampling site and present a mean annual effluent discharge of 6 hm^3/s. Site B (n=37) is located approximately 70 km downstream from site A and 10 km from the river mouth close

to the city of Barcelona. The Llobregat River at site B has 255 hm^3/s of annual discharge, of which 154 hm^3/s come from fifty upstream WWTP effluents.

Most of the sewage generated in the metropolitan area of Barcelona is treated at site C, a WWTP designed to handle approximately 153 hm^3/s of water using secondary and tertiary treatment. Ninety-one samples were collected from the WWTP, first at the inflow (C_1, n=37), at the outlet of secondary treatment consisting of a biological reactor and sedimentation (C_2, n=32) and at the outflow from the tertiary treatment (C_3, n=22) that comprised chlorination, Actiflo® filtration and flocculation and UV disinfection. These samples were collected when the complete treatment system was operating. Site D consists of a marine bathing water site affected by the river plume diluted by Mediterranean seawater (n=32). Water samples of 10 L were collected from the river, the sea and the tertiary effluent, whereas only 50 mL were collected from the secondary effluent and raw wastewater.

All viral (HAdV, JCPyV, MCPyV and NoV GGII) and bacterial (EC and IE) parameters were quantified bimonthly in all river water samples (sites A and B) and in all seawater samples from the beach affected by the Llobregat river plume (site D). Raw wastewater and two differently treated effluents from a WWTP (site C) were simultaneously collected and also tested for the presence of HEV by nPCR.

Stream flow measurements were recorded in two gauges located at sites A and B by the Catalan Water Agency (ACA, 2012). We assumed wastewater treatment plant (WWTP) effluents were equivalent to the recorded WWTP inflows. Conductivity, pH and water temperature data were determined in the field for each sample. Precipitation data were acquired from the Catalan Meteorological Institute (Meteocat, 2014). Data from seasonal river flow, precipitation and temperature is shown in the supplementary material (S1).

2.2. Virus analyses

2.2.1. Concentration and nucleic acid extraction

The detection of viruses in the environment requires the concentration of the viral particles into small volumes. This study was conducted using optimised Standardised Operational Procedures (SOPs) for virus concentration, nucleic acid extraction and quantitative PCR (qPCR and RT-qPCR) detection. The SOPs incorporated process controls and standard plasmid preparation. First, samples with high levels of organic matter or sand were permitted to sediment for 1 hour to avoid any interference with the concentration method. Then, clarified water was transferred to a new container without disturbing the sediment. As established in ISO 5667-9:1992, seawater samples were collected from one meter below sea level to avoid contamination with sand and suspended macro algae.

Water samples were concentrated as previously described by Calgua and co-workers (Calgua et al., 2013a, 2013b, 2008). Briefly, a pre-flocculated 1% (w/v) skimmed milk solution (PSM) was prepared by dissolving 10 g skimmed milk powder (Difco, Detroit, MI, USA) in 1 L of artificial seawater at pH 3.5 (Sigma-Aldrich Chemie GMBH, Steinheim, Germany). All samples were carefully acidified to pH 3.5 using 1N HCl and the conductivity was adjusted to 1.5 mS/cm^2. Then, the PSM solution was added to each of the previously conditioned samples to obtain a final concentration of skimmed milk in the sample of 0.01%. Samples were stirred for 8 h at room temperature and flocks were allowed to sediment by gravity for 8 h. Supernatants were carefully removed using a vacuum pump without disturbing the sediment. The sediment was resuspended using 10 mL of phosphate buffer at pH 7.5, with the exception of the raw sewage samples and secondary treated effluents that were suspended in 1 mL. On the same day, viral DNA was extracted from all samples using the QIAamp Viral RNA kit (Qiagen, Inc., Valencia, CA). Adenovirus type 35 and UltraPure™ DNase/RNase-Free distilled water were used as positive and negative controls of the nucleic acid (NA) extraction experiment, respectively. Finally, NA elutants were stored at -20°C until use.

2.2.2. Quantitative and nested PCR assays

Specific real-time quantitation of DNA viruses (HAdV and JCPyV) by qPCR or an RNA virus (NoV GGII) by quantitative reverse transcription PCR (qRT-PCR) was performed as previously described (Table 1) using TaqMan® Universal PCR Master Mix and the RNA UltraSense™ One-Step qRT-PCR System, respectively (Invitrogen, Carlsbad, CA, USA) (Hernroth et al., 2002; Bofill-Mas et al, 2006; Kageyama et al., 2003; Loisy et al., 2005; Pal et al., 2006). Undiluted and log10 dilutions of the nucleic acid extracts were analysed in duplicate. The equivalence of 35 mL for DNA viruses and 17.5 mL for the RNA virus were tested from river and seawater, whereas 1.7 mL and 0.9 mL, respectively, were tested from wastewater. All qPCRs included more than one non-template control (NTC) to demonstrate that the mix did not produce fluorescence due to contamination. Quantitation was performed with an MX3000P sequence detector system (Stratagene, La Jolla, CA, USA).

The amplification conditions of the HEV nested RT-PCR (nRT-PCR) methods used for qualitative detection were described elsewhere (Erker et al., 1999). Primers are described in Table 1. The reverse transcription of the extracted RNA was performed with a one-step RT-PCR Kit (Qiagen, Valencia, CA, USA) and the nRT-PCR was performed with AmpliTaq^{TM} Gold DNA polymerase (Applied Biosystems Foster City, CA, USA).

2.2.3. Development of the MCPyV qPCR assay

A new qPCR assay was designed for MCPyV detection in water matrices. A fragment of the VP1 gene (132 bp) was obtained by applying a specific PCR (Bofill-Mas et al., 2010) and cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). Standard curves were generated by transferring the plasmid construct into *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA, USA). After verifying the presence of transformed colonies containing the target sequence by conventional PCR and purifying them with the Qiagen Plasmid Midi kit

(Qiagen GmbH Inc., Hilden, Germany), the constructions were linearised with the Sal I restriction enzyme (Promega, Madison, WI). Then, two primers and a hydrolysis probe were designed based on the TaqMan® assay to amplify the specific VP1 fragment of the viral genome (Table 1). Annealing temperatures and primer and probe concentrations for the novel MCPyV qPCR were optimised by assaying primer concentrations ranging from 0.4 to 0.9 μM and probe concentrations ranging from 0.225 to 0.4 μM for each reaction. Amplifications were performed in a mixture containing 10 μl DNA, 15 μl TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.9 μM of each primer (MCF and MCR) and 0.225 μM of the probe (MCP). Following activation with AmpliTaq^{TM} Gold DNA polymerase for 10 min at 95ºC, 40 cycles (15 s at 95°C and 1 min at 60°C) were performed with an MX3000P detector system (Stratagene, La Jolla, CA, USA). Serial dilutions of the confirmed standard ranging from 10^0 to 10^5 molecules per 10 μL were performed with TE buffer and stored at -80ºC until use. Known amounts of standard DNA containing 10^0, $2x10^0$, $5x10^0$, 10^1, $2x10^1$, $5x10^1$ and 10^2 GC/reaction were analysed according to MIQE guidelines using 6 replicates to determine the sensitivity of the qPCR assay (Bustin et al., 2009). The specificity was verified with available standard plasmids from other human polyomaviruses (JCPyV, BKPyV, KIPyV and WUPyV) and animal polyomaviruses (ovine PyV and bovine PyV).

2.2.4. Control viruses and plasmid DNA for the viral qPCR assays

Human adenovirus type 35 (HAdV35) and norovirus genogroup II type 13 (NoV GGII.13) stocks were kindly donated by Dr. A. Allard of the University of Umeå (Sweden) and were used as positive process controls. On each sampling day, an extra sample was collected and spiked with HAdV35 (10^5 viral particles/mL) as a process control for flocculation, NA extraction and DNA quantification. The NoV GGII.13 genome was also extracted from each sample as a positive control for nucleic acid extraction and RNA quantification (10^4 genome copies in each reaction of 5 μL).

Plasmid DNA was used as a positive control and as a quantitative standard. The hexon region (8961 bp) and the whole genome (5130 bp) of HAdV41 and JCPyV Mad1, respectively, were cloned into pBR322. To reduce the possibility of DNA contamination in the laboratory, 10 µg of plasmid DNA was linearised with BamHI for HAdV and NruI for JCPyV (Promega, Madison, WI) and the reaction products were purified and quantified. The capsid protein region of NoV GGII.13 cloned into the pTrueBlue®-Pvu II vector (kindly donated by Dr. J. Vinjé, CDC, Atlanta) was used as a qRT-PCR standard. A total of 10 µg of this construct was linearised with XhoI (Promega) to prevent contamination. Serial dilutions ranging from 10^0 to 10^5 molecules per 5 or 10 µL for RNA or DNA virus qPCR, respectively, were performed with TE buffer. Standard dilutions were distributed into tubes and stored at -80°C until use. Specific primers and hydrolysis probes are described in Table 1. UltraPure™ DNase/RNase-Free distilled water was used as a negative control for the NA extraction and qPCR assays.

2.2.5. Recovery efficiency and repeatability of the concentration procedure

An intra-laboratory assay was performed to test the repeatability of the concentration method. Briefly, 200 L of seawater, river water or mineral water spiked with HAdV35 and NoV GGII.13 was mixed in a large plastic container. Water was mixed by manual stirring, acidified and then distributed into twenty 10-litre sample containers. This procedure was repeated for each matrix and included mineral water as a control. Viral particles were concentrated as described in 2.2.1. HAdV and NoV GGII.13 were quantified by qPCR and qRT-PCR after nucleic acid extraction, respectively. Additionally, indigenous JCPyV was quantified in the 20 seawater replicates.

2.3. Faecal Indicator Bacteria (FIB) detection

For FIB detection, 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 was carried out in a 96-

well microplate (MUG/EC 355-3782, BioRad, Barcelona, Spain ®) according to ISO 9308-2:2012 using the most probable number (MPN) procedure (Donovan et al., 1998) to detect EC based on the expression of the enzyme β-D-glucuronidase present in most EC strains. Intestinal enterococci were also quantified by MPN in a 96-well microplate (MUG/EC 355-3783, BioRad ®) following the ISO 7899-1:1998 procedure based on the detection of the expression of the β-glycosidase enzyme characteristic of enterococci.

2.5. Statistical analyses

Statistical analysis was performed using R software version 2.15.1 (Verzani, 2004). The data distribution was tested with the Shapiro-Wilk and Kolmogorov-Smirnov tests. After proving that the raw data were not normally distributed, the viral and bacterial raw data were $\log_{10}$ transformed to improve normality and facilitate parametric analyses. Correlation analysis of the transformed ($\log_{10}$) data was completed using both parametric (Pearson correlation) and non-parametric (Spearman correlation) approaches. The results were considered to be significant when p was <0.01. Values less than the detection limit were given the value of 1. Correlations between the temperature, flow, precipitation and microbial concentration were calculated. Coefficients of variability of the concentration procedure calculated for each water matrix with raw (GC/L), and $\log_{10}$ transformed data were tested for normality and variability (Coefficient of Variation (CV), where CV= standard deviation / mean value x 100) among the replicate concentrations.

2.6. Climate projections for a Mediterranean river catchment: methods and datasets

Temperature and precipitation records were extracted from the ERA-interim reanalysis database (http://data-portal.ecmwf.int). Because data can contain gaps, we established the criteria that for a grid point to be eligible it must contain a minimal fraction of 30 valid points for each grid point comprised in the box 0 to 5ºE and 40ºN-45ºN. Precipitation is reported in

mm/day and temperature in degrees Kelvin (ºK). Both precipitation and average, minimum and maximum temperature simulations were downloaded from the Climate Model Intercomparison Project version 5 (CMIP5) at http://cmip-pcmdi.llnl.gov /cmip5/data_portal.html. The data for each point is the monthly multi-model mean of historical +rcp26 experiments for the interval 1860-2100 of the following group of climate models: BCC-CSM1-1, BCC-CSM1-1-m, BNU-ESM, CanESM2, CCSM4, CESM1-CAM5, CNRM-CM5, CSIRO-Mk3-6-0, EC-EARTH, FGOALS-g2, FIO-ESM, GFDL-CM3, GFDL-ESM2G, GFDL-ESM2M, GISS-E2-H, GISS-E2-R, HadGEM2-AO, HadGEM2-ES, IPSL-CM5A-LR, IPSL-CM5A-MR, MIROC5, MIROC-ESM, MIROC-ESM-CHEM, MPI-ESM-LR, MPI-ESM-MR, MRI-CGCM3, NorESM1-M and NorESM1-ME (see supplementary information S2).

3. Results and discussion

3.1. Performance of the assays

3.1.1. Recovery efficiency and repeatability of the Skimmed Milk Flocculation protocol

Based on the outcome of the normality test, CV values were calculated with the $\log_{10}$-transformed data generated from the replicate enumerations for each determinant. Indigenous JCPyV presented the most repeatable results with a CV of 12.4%. Smaller variations in HAdV results were found among the 20 river water replicates (CV: 14.8%), whereas NoV GGII.13 measurements were more repeatable in mineral water (CV: 20.3%). The highest CV was calculated for NoV GGII.13 in seawater (CV: 36.3%). The concentration protocol by means of skimmed milk flocculation proved to be repeatable for use in the study.

3.1.2. Sensitivity and specificity of the new MCPyV quantitative assay

The qPCR assay developed for the quantification of MCPyV was shown to be specific both by "in silico" sequence analysis of the primers and the probe considering nucleotide sequence databases (NCBI BLAST) and by experimental assays. No false positive results were detected due to cross-reactivity with non-target DNA from the viruses contained in the different plasmid constructs assayed (human polyomaviruses JCPyV, BKPyV, KIPyV and WUPyV and animal polyomaviruses OPyV and BPyV). A total of 20 and 10 DNA genome copies were detected in 100% and 80% of the performed qPCR reactions, respectively. Sensitivity did not vary even when high levels of exogenous but related viral DNA (samples with high levels of JCPyV) were added to the test tubes.

3.2. Occurrence of viral pathogens and indicator microorganisms in river and seawater

The geometric mean genomic copies (log_{10} GC/L or MPN/100 mL) and concentration ranges of HAdV, JCPyV, MCPyV, NoV GGII, EC and IE found in each sampling location and season are shown in Figure 2. HAdV and JCPyV were commonly detected in river water samples with prevalences of 80% and 59%, respectively, and at similar concentrations to those previously described (Albinana-Gimenez et al., 2009). These concentrations were stable throughout the year, with mean values of $8.1x10^2$ GC/L in site A and $1.4x10^3$ GC/L in site B. Overall, 100% of river and seawater samples were positive for HAdV in the summer and mean values were at times as high as those quantified in treated sewage effluent from tertiary treatment plants. Despite the lack of detection of JCPyV in the spring from site A, the prevalence was still high and the mean concentration was similar to that reported for HAdV. No JCPyV was detected in marine samples collected in winter. The lower frequency of HAdV and JCPyV in seawater was consistent with other studies that suggested marine dispersion, dilution and disinfection due to the high salinity may be the cause of the low concentrations reported (Hawley and Garver, 2008; Wyn-Jones et al., 2010).

MCPyV was principally detected among the river sampling sites, with a higher prevalence in seawater during the summer ($6.9x10^2$ GC/L) (Figure 2). Prevalence and concentrations of MCPyV in river water were similar to those previously reported (Calgua et al., 2013a). The most significant new finding was the detection of MCPyV in seawater during the summer bathing season, which may indicate a risk of transmission for recreators. The presence of this polyomavirus that has been described to have a skin tropism may also be linked to the recreational activities themselves rather than being exclusively derived from sewage effluent inputs into the bathing zone. This is the first description of the presence of MCPyV in seawater samples and bathing water, suggesting a putative transmission pathway for this pathogen.

NoV GGII prevalence showed a marked seasonality: the highest concentrations were measured during winter months with colder temperatures. A high prevalence of NoV GGII was detected in the studied river in winter (83% and 100% of positive samples in sampling points A and B, respectively) and autumn (77% and 88% in sampling points A and B, respectively) (Figure 2). Outbreaks of winter vomiting syndrome caused by noroviruses in the environment are well reported (Kitajima et al., 2012, 2010; Nordgren et al., 2009; Lopman et al., 2009). Recently, other authors performed a norovirus survey in the same river catchment and reported similar results to those confirmed in the present investigation (Collado et al., 2010; Pérez-Sautu et al., 2012).

EC and IE were detected during all seasons at both river sites (A and B) with mean values of 10^3 MPN/100 mL, whereas in seawater FIB were under the limit of detection of the technique (15 MPN/100 mL) and were only detected in spring (EC) and autumn (EC and IE). Occasionally after spring and early summer rain events FIB levels exceeded those limits, but no significant FIB and rain correlations were observed (p-value>0.01). Moreover, most FIB

levels in seawater samples complied with the 'good' EU bathing water criteria, with less than 100 cfu/100 mL of EI and less than 250 cfu/100 mL of EC (Spanish Government, 2007b).

3.3. Viruses and FIB in wastewater samples and treatment removal efficiencies

HAdV were detected in all 37 influent raw sewage samples with similar geometric mean values during the year (data not shown). High viral titers were observed in untreated wastewater ($8.4x10^5$ GC/L), secondary treated effluents ($1.2x10^5$ GC/L) and tertiary treated effluents ($1.9x10^3$ GC/L). Moreover, JCPyV was consistently prevalent in raw wastewater and secondary treated effluents with mean concentrations of $7.5x10^5$ GC/L and $1.6x10^5$ GC/L, respectively. Tertiary treated effluents had only 7 positive samples for JCPyV out of 22 tested samples. The high prevalence and concentrations of HAdV and JCPyV observed in raw sewage inputs to the treatment plant and treated effluents from the secondary and tertiary treatment stages were similar to published data (Bofill-Mas et al., 2000; Fong et al., 2010; Hewitt et al., 2013; McQuaig et al., 2009; Rodriguez-Manzano et al., 2012).

MCPyV was detected at median concentrations of $1.6x10^4$ GC/L in 75% of the raw wastewater. Although its prevalence in secondary and tertiary effluents was extremely variable, MCPyV was mostly present in summer and winter. The frequent detection of MCPyV in urban sewage samples (31/37) suggested a persistent excretion of this virus by the contributing human population. Prevalence and concentrations of MCPyV in sewage were similar to those previously reported (Bofill-Mas et al., 2010).

The highest NoV GGII mean values from the WWTP were observed in winter, with $2.5x10^8$ GC/L in raw sewage, $2.1x10^7$ GC/L in secondary treated effluent and $2.2x10^5$ GC/L after tertiary treatment. Positive HEV samples were detected in 9 of the 91 samples collected from the influent raw sewage (5/37) and the secondary treatment effluent (4/32).

HAdV, JCPyV, NoV GGII and MCPyV were widely detected in raw sewage samples, whereas HEV was found in low percentages (13.5%). Previous studies reported HEV to be present in 43.5% of the raw sewage from Barcelona (Clemente-Casares et al., 2003); the low occurrence detected in this study could be related to a short excretion period or the small volume of sample analysed using the concentration method. Nevertheless, this is the first study that has analysed HEV in wastewater after concentrating the viral particles with the skimmed milk flocculation method. In the majority of the river water samples, the EC levels complied with the Spanish standards for irrigation of non-processed food (i.e., less than 100 colony-forming units (cfu) in 100 mL) (Spanish Government, 2007a).

The reduction of viral and bacterial concentrations was calculated for all treatment steps (Figure 3). All tested pathogens and faecal indicators were detected in the samples collected to characterise different levels of treatment with exception of HEV, which was not detected in any tertiary treatment samples. Pathogen removal was quantified by $\log_{10}$ reduction throughout the treatment process, after the secondary treatment (C2) and after the tertiary treatment (C3). The results showed that the activated sludge process was the most important step for the removal of pathogens, with maximum reductions of 3.14 $\log_{10}$ for NoVGGII. Similar findings have been described in the literature (Rodriguez-Manzano et al., 2012). The combination of the two processes (C2+C3) resulted in 4.13 $\log_{10}$, 4.11 $\log_{10}$ and 4.52 $\log_{10}$ reduction values for EC, IE and NoV GGII, respectively. Human DNA viruses exhibited variable total removal efficiencies (2.22 $\log_{10}$ for MCPyV, 3.44 $\log_{10}$ for HAdV and 4.19 $\log_{10}$ for JCPyV). Despite achieving a maximum decay of 4-$\log_{10}$ when compared to the initial concentrations in raw sewage, viruses were also detected in the majority of treated final effluent, including those in compliance with bacterial indicator standards (Spanish Government, 2007b). Although infectivity should be highly reduced after tertiary treatment, previous studies detected infectious HAdV using immunofluorescence assays after UV disinfection at the same WWTP (Rodriguez-Manzano et al., 2012).

Considering that the average viral load of secondary treatment effluent is 10^5 GC/L, we can assume that every year a total amount of $3x10^{16}$ potentially infectious viruses are discharged into the river from WWTPs. These have a direct impact on the presence of human viruses in the river water and in the seawater bathing area studied. Treating sewage with only a secondary or tertiary process will not prevent impairment of riverine and marine receiving waters or guarantee satisfactory quality of raw surface waters used for the drinking water supply. The extent to which the concentrations (as GC number) identified after disinfection treatment with UV reflect viable infective organisms is of course debatable. However, our results are consistent with studies reporting viral loading from sewage treatment plants (Simmons and Xagoraraki, 2011). These findings provide evidence of the inefficacy of current interventions in removing qPCR signals indicating viral pathogens. New legislation including viral quality standards may be required as viability and infectivity information become increasingly available, especially after disinfection treatments.

3.4. Correlations among pathogens, river flow, precipitation, and water and air temperatures.

The calculated Pearson correlations between microbial parameters showed that $\log_{10}$ EC and $\log_{10}$ IE correlated (r=0.92, $p\approx0$) along the river basin locations and seasons. In wastewater, the $\log_{10}$ transformed viral data exhibited significant correlations between viruses (p <0.01), but with lower correlation coefficients compared to the bacterial indicators (Table S3 in the supplementary information). The concentrations of NoV GGII correlated with both river and sea water and air temperatures, highlighting the seasonality of the viral infection (r=-0.64 for both river water temperature and sea water temperature; $p\approx0$). HAdV, NoV GGII and FIB presented significant correlations with accumulated precipitation only in seawater samples (r=0.75, 0.95, 0.75, and 0.78 for EC, IE, HAdV and NoV GGII, respectively). When taking all samples into account, HAdV significantly correlated with JCPyV (p<0.01), indicating that

HAdV may potentially be used to predict the concentration of JCPyV. Statistically significant but lower correlations were also found between HAdV and the other tested pathogens. HAdV was the best predictor of the majority of pathogen concentrations (correlation values ranged from 0.53 and 0.70). As recently noted by other authors (Payment and Locas, 2011), direct correlation between pathogens and indicators of faecal contamination becomes improbable in water bodies receiving sewage discharges because of different dilution, transportation and inactivation rates. Nevertheless, the high prevalence of HAdV in all type of water matrices, seasons and river locations strongly supports their applicability as a viral indicator with potential for indexing the fate of viral pathogens and faecal contamination.

3.5. Future trends in virus concentrations considering climate projections for the Mediterranean river catchment.

The risk associated with waterborne viral infections would be influenced by the exposure (concentration of virus in water) and stability of the viruses in water. Thus, the temporal pattern and population burden of several infectious diseases may shift and human exposure may differ under future scenarios (Morse et al., 2012). To study future trends in viral concentrations in the selected river catchment, we generated climate projections to 2100 with the suite of climate models contained in the CMIP5+ project database (see Methods). Due to the large uncertainty associated with detailed spatial resolution simulations, a grid of 5x5 degrees was preferred and applied. The results for the trend in the seasonality of temperature and precipitation are presented in Figure 4 together with the observed values for the same region derived from the European Centre for Medium-Range Weather Forecasts (ECMWF) interim re-analysis (ERA) (http://data-portal.ecmwf.int/data/d/interim_daily/). A bias between current climate conditions compared to those simulated with the ensemble of climate models is clearly evident when compared to the observations (derived from the ERA interim reanalysis). While it is true that there may be a noticeable difference between station data and

reanalysis fields for a small region, we thought it convenient to highlight these disparities to better define the scope of our study. Projections for regional precipitation yield an estimate of a slight increase in total amounts between the simulated winter months of 2070-2100 and 1980-2010 (on average approximately 1-3 mm/month) and persistent decreases between May and October (on average 2.5 mm/month) (Figure 4A). While these differences might be deemed low, values rise considerably in relative terms when compared to current observations (a general increase throughout the year with localised maximum increases of approximately 30% in the main winter months). Temperature projections for the region are presented in Figure 4B. In this case, systematic differences between observations and simulations for the common 1980-2010 interval are much lower than for the rainfall described above. The overall change in temperature between the two intervals (2070-2100 vs 1980-2010) indicates a general warming trend throughout the year, with an increase in the minimum winter temperatures that is positive when compared to observations. Increases are above 13% in summer months and approximately 11% in winter.

Globally, climate change is expected to shrink potable water availability. In the Mediterranean climate regions it is predicted to increase the intensity and/or frequency of floods and droughts. Faecal contamination by means of viral concentrations may increase, but at the same time environmental factors producing viral inactivation could also increase in intensity. For example, UVB radiation and the biotic activity indirectly associated with higher temperatures (Carratalà et al., 2013) could reduce viral viability, compensating for the increasing viral loads. Where climate change scenarios predict more frequent floods, the pollution events caused by sewage overflows will increase, posing a challenge for water managers. It is likely that the predicted reduction in the number of summer rainfall events in the Mediterranean climate regions will produce more frequent low river flows with greater proportions of treated effluents entering surface water bodies, possibly increasing viral pathogen concentrations in river water and the impacted beaches (Figueras and Borrego,

2010; Cann et al., 2013).

4. Conclusions

1. The concentration of HAdV is stable in raw sewage throughout the year, with mean values of $8.38x10^5$ GC/L.

2. Secondary biological treatment reduces pathogen concentrations between 2.0 and 3.1 log_{10} (as GC/L). Because conventional WWTPs discharge secondary effluents into rivers, the Llobregat River is persistently impacted by human faecal pollution as evidenced by the presence of HAdV, JCPyV and FIB in river water samples.

3. Seasonal NoV GGII patterns are observed at all sampling sites including wastewater and environmental samples. From the most upstream sampling site to the seawater impacted by the river discharge, NoV GGII was detected during spring, autumn and especially in winter when more outbreaks are identified in the population.

4. A new quantitative PCR method has been developed for the detection of the emergent MCPyV related to Merkel cell carcinoma in water matrices. This is the first study reporting detection and quantification of MCPyV in seawater samples (with 18% prevalence and mean concentration of $1.18x10^2$ GC/L). The high prevalence of MCPyV in sewage (75%) suggests that the human population commonly sheds this virus.

5. Temperature and precipitation predictions to 2100 for the selected Mediterranean river catchment suggest an increase in temperatures throughout the year and increased precipitation during winter with a decrease in precipitation between May and October. The highest virus concentrations are detected in summer when minimum

precipitation and river flow occurs. The reduced dilution of treated effluents by rivers will result in elevated summer pathogen concentrations, with the potential for increased concentrations in winter resulting from intermittent combined sewage overflows discharging during the more frequent storm events.

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TABLE 1. Oligonucleotide primers and probes used for the detection and quantification of viral pathogens.

Virus	Primers and probes	Position[a]	Sequence (5'-3')	References
Human adenovirus (HAdV)	ADF	Hexon gene 18869-18937	CWTACATGCACATCKCSGG	Hernroth et al., 2002
	ADR		CRCGGGCRAAYTGCACCAG	
	ADP1		FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ1	
JC Polyomavirus (JCPyV)	JE3F	T-antigen gene 4251-4482	ATGTTTGCCAGTGATGATGAAAA	Pal et al., 2006
	JE3R		GGAAAGTCTTTAGGGTCTTCTACCTTT	
	JE3P		FAM-AGGATCCCAACACTCTACCCCACCTAAAAAGA-BHQ1	
Merkel Cell Polyomavirus (MCPyV)	MCF	VP1 gene 1232-1293	ATTTGGGTAATGCTATCTTCTC	This study
	MCR		CTAATGTTGCCTCAGTTCCAA	
	MCP		FAM- AACCACAGATAATACTTCCACTCCT-BHQ1	
Norovirus genogroup II (NoV GGII)	QNIF2d	Capsid protein gene 5012-5100	ATGTTCAGRTGGATGAGRTTCTCWGA	Primers: Loisy et al., 2005 Probe: Kageyama et al., 2003
	COG2R		TCGACGCCATCTTCATTCACA	
	QNIFS		FAM-AGCACGTGGGAGGCGATCG-TAMRA	
Hepatitis E* virus (HEV)	HEVORF2con-a1	ORF2 region 6283-6479	GACAGAATTRATTTCGTCGGCTGG	Erker et al.,1999
	HEVORF2con-s1		CTTGTTCRTGYTGGTTRTCATAATC	
	HEVORF2con-s2		GTYGTCTCRGCCAATGGCGAGC	

*seminested PCR

[a] The sequence positions are referred to strains J01917.1 (HAdV), NC001699.1 (JCPyV), HM011557.1 (MCPyV), AF145896 (NoVGII) and AF060668 (HEV).

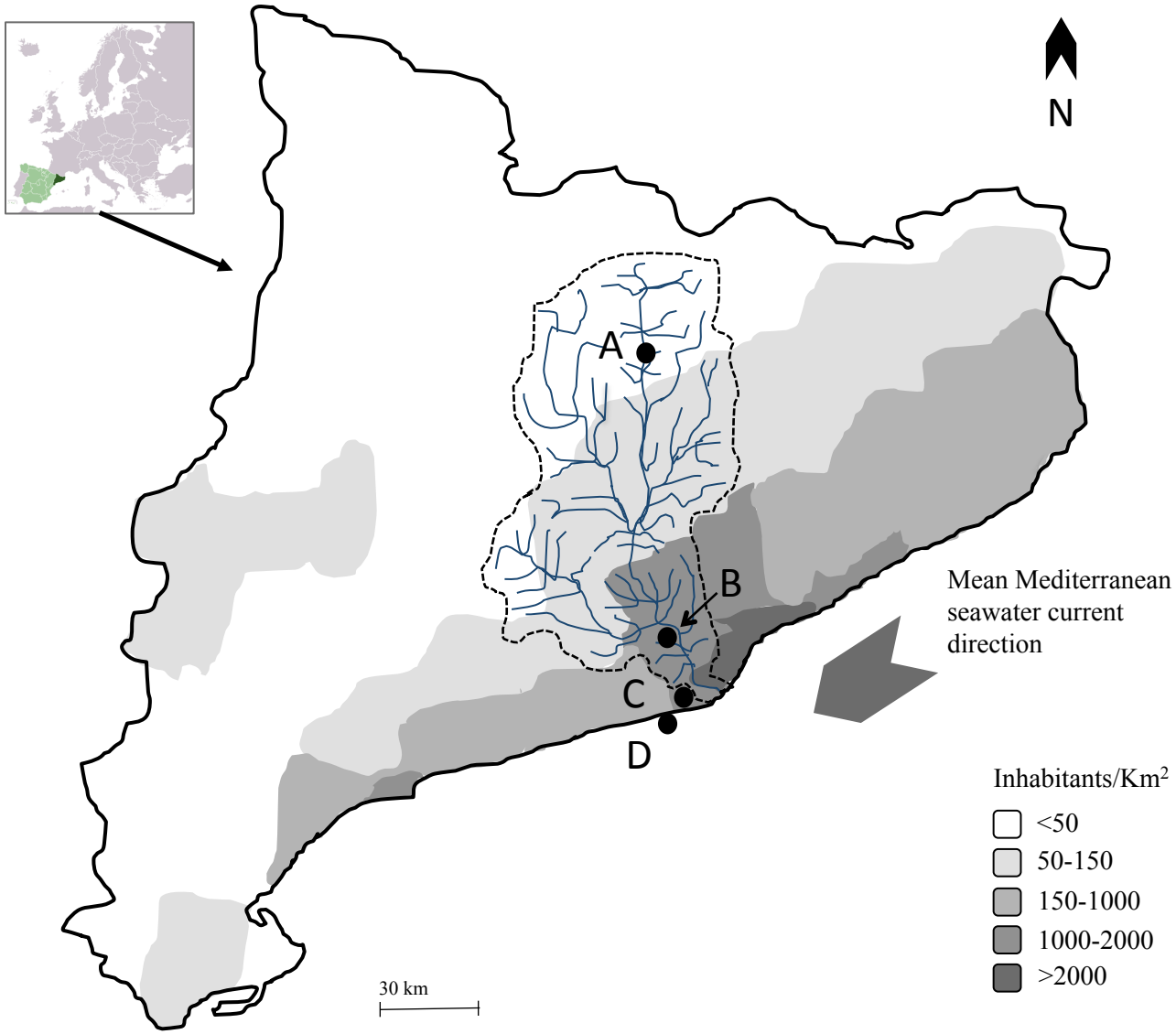


FIGURE 1, Sampling sites location in the Llobregat river catchment (Catalonia, Spain). Site A: upstream river water; Site B: downstream river water; Site C: raw sewage (C_1), secondary (C_2) and terciary (C_3) effluents from a WWTP; Site D: seawater.

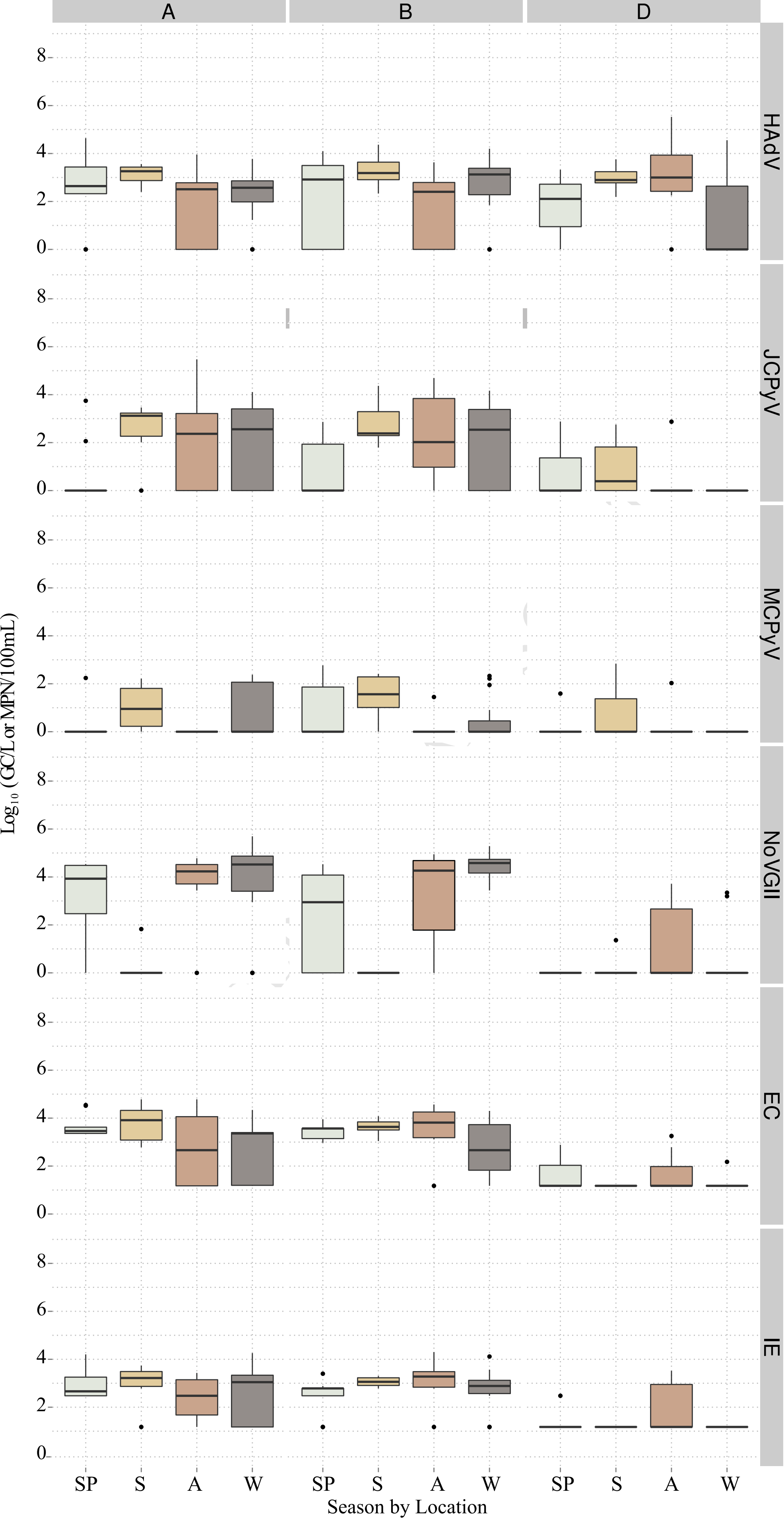
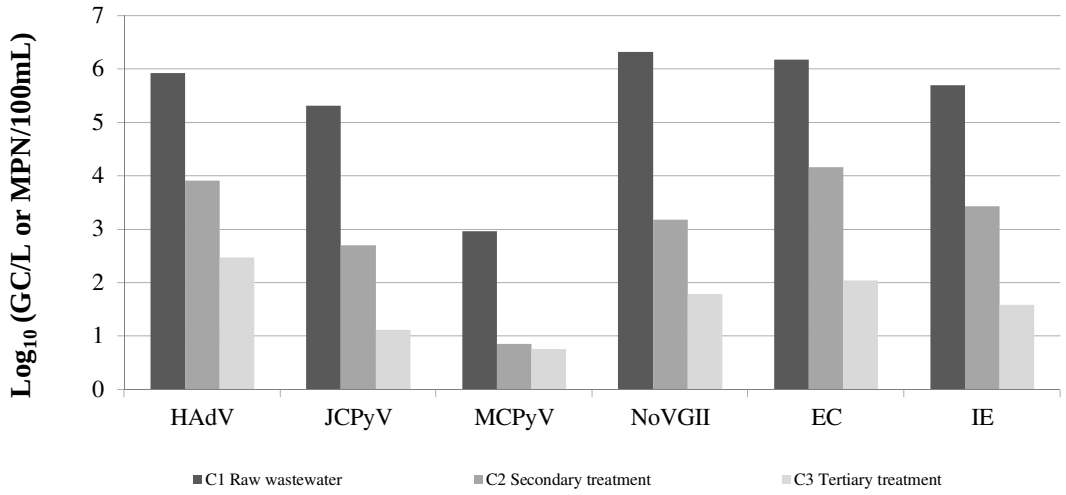


FIGURE 2: Boxplots representing interquartile pathogen concentrations (Log_{10}GC/L or MPN/100mL) by season (SP: spring, S: summer, A: autumn and W: winter) and matrices. Lines extending vertically from the boxes indicate variability outside the upper and lower quartiles, and the outlier values are plotted as individual points. A: upstream river site, B: dowstram river site and D: seawater site.



	HAdV	JCPyV	MCPyV	NoVGII	EC	IE
Secondary (C2)	2.00	2.61	2.11	3.14	2.01	2.27
Tertiary (C3)	1.44	1.58	0.11	1.38	2.12	1.84
TOTAL(C2+C3)	3.44	4.19	2.22	4.52	4.13	4.11

FIGURE 3. Summary of WWTP log10 concentrations and log10 reduction values by treatment for each viral and bacterial pathogen.

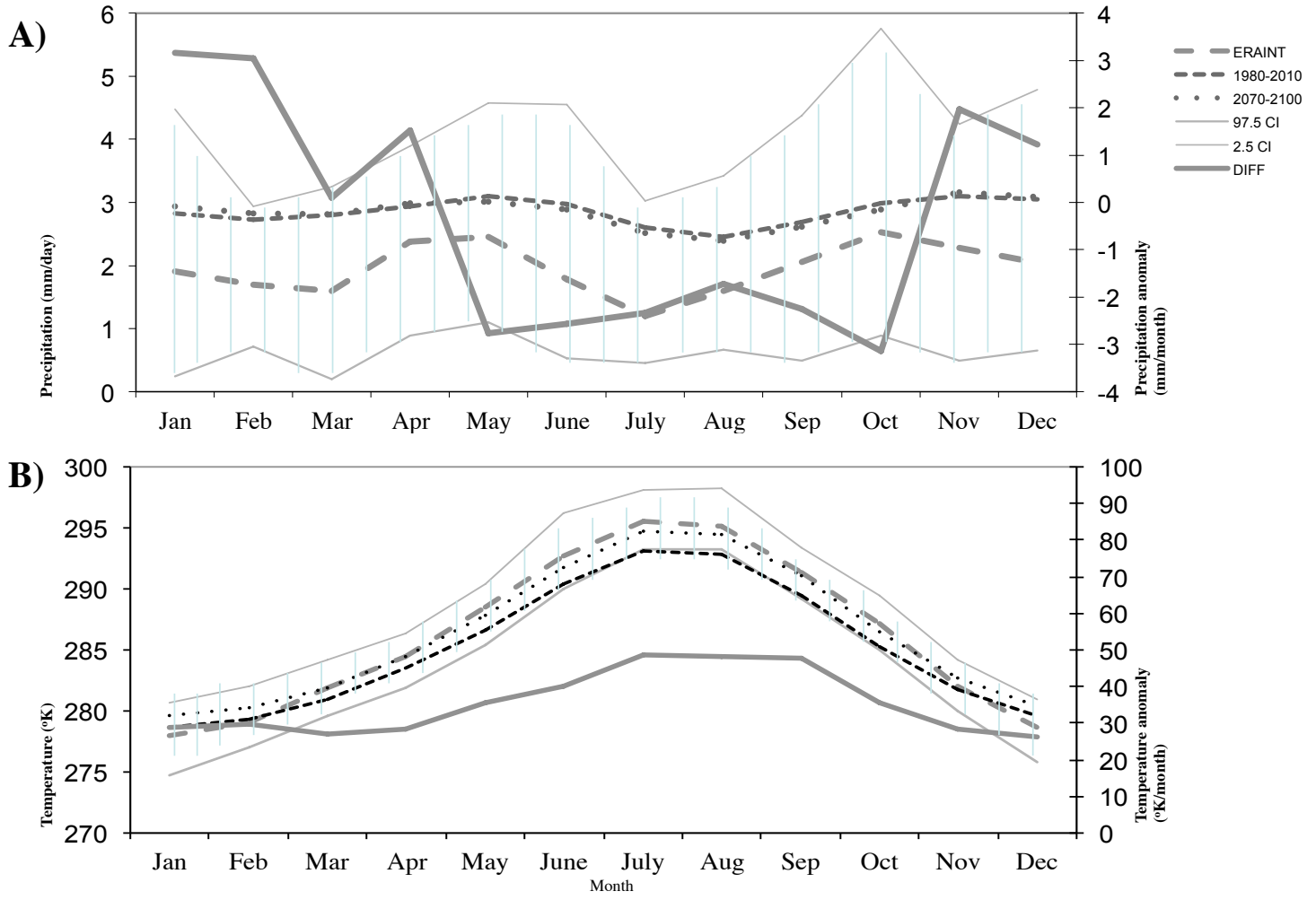


Figure 4: A) Precipitation and B) mean temperature changes between the intervals 2070-2100 and 1980-2010 according to the suite of climate models contained in the rcp26 experiment of CMIP5+ (see Methods for details). 95% confidence interval is denoted by hatched area plots. DIFF denotes the difference in values for each variable obtained from 2100-2070 minus 1980-2010 and are referred to the secondary Y axis. ERA interim line yields values for the observational reanalysis of the region's temperature and precipitation values in the historical period (1980-2010). Temperature is in degrees Kelvin (°K) and precipitation in mm/day. Secondary Y axes have units referred to monthly totals.