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Application of human and animal viral microbial source tracking tools in fresh and marine waters from five different geographical areas

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ABSTRACT

Integrated river basin management planning to mitigate the impacts of economic, demographic and climate change is an important issue for the future protection of water resources. Identifying sources of microbial contamination via the emerging science of Microbial Source Tracking (MST) plays a key role in risk assessment and the design of remediation strategies. Following an 18-month surveillance program within the EU-FP7-funded VIROCLIME project, specific MST tools were used to assess human markers such as adenoviruses (HAdV) and JC polyomaviruses (JCPyV) and porcine and bovine markers such as porcine adenoviruses (PAdV) and bovine polyomaviruses (BPyV) via quantification with real-time PCR to analyze surface water collected from five sites within different climatic zones: the Negro River (Brazil), Glafkos River (Greece), Tisza River (Hungary), Llobregat River (Spain) and Umeälven River (Sweden). The utility of the viral MST tools and the prevalence and abundance of specific human and animal viruses in the five river catchments and adjacent seawater, which is impacted by riverine contributions from the upstream catchments, were examined. In areas where no sanitation systems have been implemented, sewage can directly enter surface waters, and river water exhibited high viral loads; HAdV and JCPyV could be detected at mean concentrations of 10^5 and 10^4 Genome Copies/Liter (GC/L), respectively. In general, river water samples upstream of urban discharges presented lower human viral loads than downstream sampling sites, and those differences appeared to increase with urban populations but decrease in response to high river flow, as the elevated river water volume dilutes microbial loads. During dry

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seasons, river water flow decreases dramatically, and secondary effluents can represent the bulk of the riverine discharge. We also observed that ice cover that formed over the river during the winter in the studied areas in North Europe could preserve viral stability due to the low temperatures and/or the lack of solar inactivation. Porcine and bovine markers were detected where intensive livestock and agricultural activities were present; mean concentration values of 10^3 GC/L indicated that farms were sometimes unexpected and important sources of fecal contamination in water. During spring and summer, when livestock is outdoors and river flows are low, animal pollution increases due to diffuse contamination and direct voiding of feces onto the catchment surface. The field studies described here demonstrate the dynamics of fecal contamination in all catchments studied, and the data obtained is currently being used to develop dissemination models of fecal contamination in water with respect to future climate change scenarios. The results concerning human and animal targets presented in this study demonstrate the specificity and applicability of the viral quantitative parameters developed to widely divergent geographical areas and their high interest as new indicators of human and animal fecal contamination in water and as MST tools.

1. Introduction

The use of integrated river basin management planning is necessary to mitigate the impacts of climate change and to protect water resources. In Europe, this is being implemented through the 'Programs of Measures' as outlined in Article 11 of Directive 2000/60/EC, the Water Framework Directive. Climate change will undoubtedly influence water quality in rivers, lakes and marine waters used for drinking water abstraction, recreational activities, shellfish harvesting and assimilation of point and diffuse fluxes of human and livestock effluents. Thus, the risk profile and treatment interventions necessary for sewage and potable waters must be changed. The fourth assessment report of the Intergovernmental Panel on Climate Change (IPCC) noted that higher temperatures, changes in precipitation regimes and more frequent weather-related disasters are primary changes that represent risks for agriculture, food, and particularly water supplies. Moreover, increasing urban expansion and associated intensification of farm production are increasing microbial loads, which are then discharged into receiving waters worldwide. Achieving legal water quality criteria will, therefore, require intelligent and integrated application of sustainable treatment and urban drainage technologies (SUDs), upgrade of wastewater treatment plants (WWTP), enhanced storm water retention facilities, and application of agricultural best management practices (BMPs) designed to reduce diffuse pollution from livestock farming.

There is increasing concern regarding the levels of fecal pollution in surface waters due to point-source discharges from community sewage treatment plants and livestock concentrations derived from slaughterhouse discharges (Collins et al., 2005; Jamieson et al., 2004). Also, non-point diffuse microbial pollution may originate from direct fecal voiding by grazing livestock, manure spreading or urban surface water runoff, which can derive from roof and road surfaces contaminated with avian, domestic and wild animal feces and also from cross-connections to urban sewerage system, all of which can exhibit high microbial loads (Llopart-Mascaró et al., 2010; Brownell et al., 2007; Bercu et al., 2011). The identification of fecal contamination sources in water using specific markers is a key step for good management and remediation protocols.

Microbial Source Tracking (MST) encompasses a group of methodologies that aim to identify and, in some cases, quantify the dominant sources of fecal contamination in the environment, particularly in water resources (Field et al., 2004; Fong and Lipp, 2005). MST plays a very important role in informing remediation strategies directed against specific pollutant sources. Molecular markers used for MST are target sequences in host-associated microorganisms or sequences derived directly from the host. These can come from prokaryotes, eukaryotes and viruses. Widely-used human and animal-associated markers have been identified in the order *Bacteroidales* (Bernhard and Field, 2000a, b) and other bacteria. Polymerase chain reaction (PCR) assays have been developed and validated for markers of fecal contamination from humans and a diversity of animals (reviewed in Roslev and Bukh, 2011). Although these molecular markers represent interesting methods for MST, they do have significant limitations. For example, (i) there is a lack of absolute host specificity among human- and animal-associated microbial markers; (ii) there is a lack of temporal stability of some host-associated microbial markers in different host groups and (iii) non-fecal sources of markers potentially exist (Roslev and Bukh, 2011; Stapleton et al., 2009). The use of highly host specific, ubiquitous and stable MST markers that produce persistent excretions in their human or animal hosts could overcome these limitations.

In recent years, many studies have examined human adenovirus (HAdV) and JC polyomavirus (JCPyV) as human fecal indicators, as they are persistently excreted by infected

humans both with and without clinical symptoms in the feces or urine (Bofill-Mas et al., 2001). Thus, they are commonly detected in urban wastewater in all geographical areas throughout the annual cycle (Koralnik et al., 1999; Bofill-Mas el al., 2000, 2006; Schlindwein et al., 2010; Kokkinos et al., 2011; Rodríguez-Manzano et al., 2012; Bofill-Mas et al., 2013). Traditionally, standard fecal indicator bacteria (FIB) are used to indicate the presence of human or animal fecal contamination. However, FIB counts cannot discriminate between animal or human contamination, whereas AdV and PyV are host-specific and derive from the gastrointestinal and urinary tracts (Bofill-Mas et al., 2000; Maluquer de Motes et al., 2004). In a study using PCR, Harwood et al. (2009) suggested that human polyomaviruses were the most specific human marker for MST among various tools analyzed. Importantly, candidate viral MST indicators do not multiply in the environment and are more resistant to environmental stressors, such as UV irradiance from sunlight and water treatment processes, than FIBs (Bofill-Mas et al., 2013). They may, therefore, represent a better index for viral pathogens such as Hepatitis A and E viruses and noroviruses (NoV) than do the common FIBs used as regulatory parameters world-wide, e.g. *Escherichia coli* and intestinal enterococci. A large diversity of concentration protocols for viruses in water have been described (Albinana-Gimenez et al., 2009). Viral detection consists of several steps: concentration of viruses from the environmental water sample into a suitable volume, extraction of the DNA or RNA and detection or quantification of the viral segment with molecular techniques. Low viral concentration and viral viability are the main handicaps of these PCR techniques.

Efficient and cost-effective techniques for virus preconcentration in water have been developed using skimmed milk direct flocculation procedures (Calgua et al., 2008; 2013a), which have potential for the routine analysis of viruses in water samples. Moreover, sensitive and reliable molecular detection techniques based on real-time PCR designed for specific DNA viruses, such as HAdV and JCPyV, porcine adenoviruses (PAdV) and bovine polyomaviruses (BPyV), have

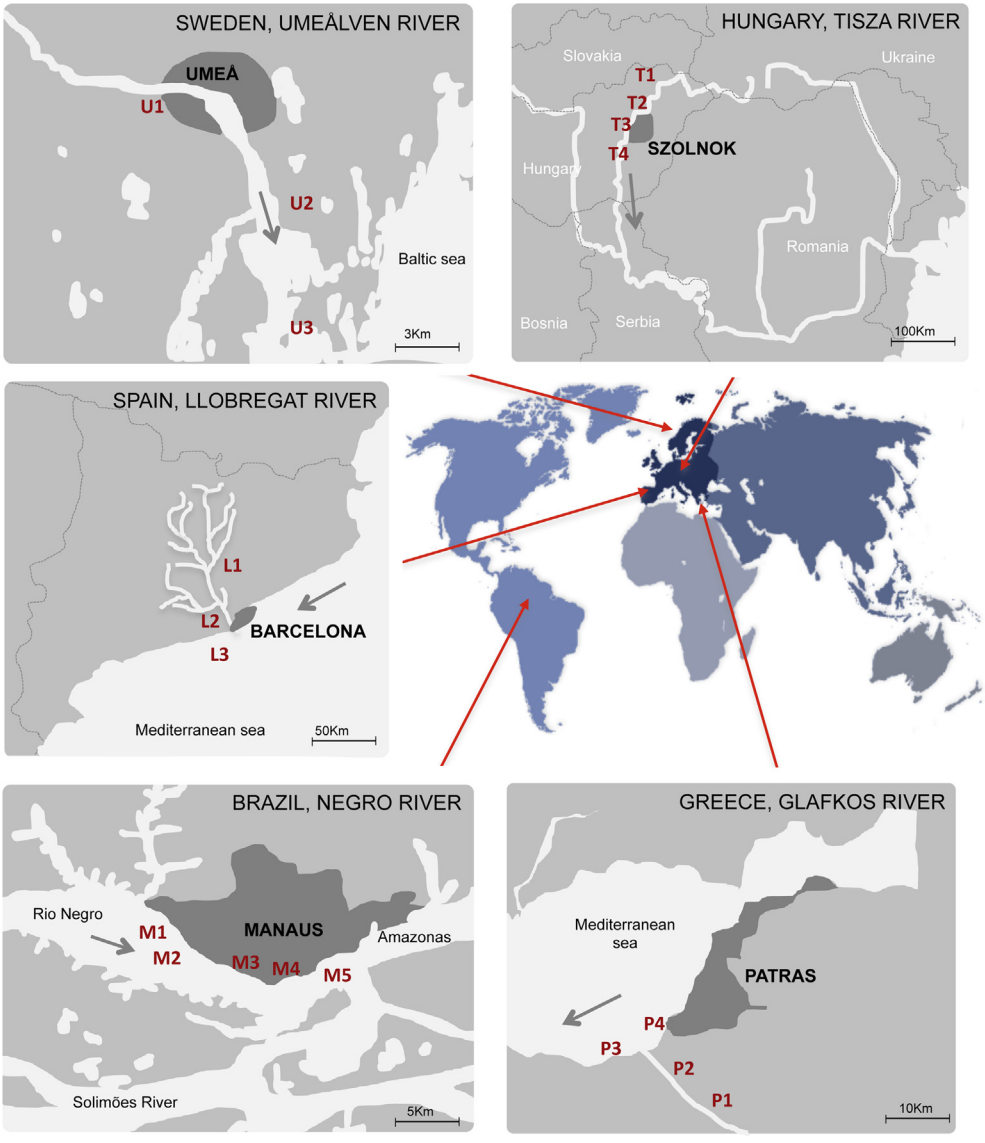

Fig. 1 – Geographic localization and sampling sites of the five case studies analyzed.

been suggested in previous studies for the quantification of these specific markers, offering the potential to delineate human and/or animal fecal contributions in environmental water matrices (Bofill-Mas et al., 2006, 2013; Hundesa et al., 2006, 2009 and 2010).

This study aimed to test the applicability of human and animal adenoviruses and polyomaviruses in widely diverse river catchments by applying skimmed milk flocculation and qPCR to define sources of fecal contamination in all areas and scenarios.

2. Materials and methods

2.1. Control viruses and plasmids

Human adenovirus type 35 (ATCC, LGC Standards AB, Borås, Sweden) stocks were produced by infecting A549 cells cultured in Earl's minimum essential medium (EMEM) supplemented with 1% glutamine, 20 μg of streptomycin and 20U of penicillin per mL and 10% (growth medium) or 2% (maintenance medium) of heat-inactivated fetal bovine serum (FBS). Viruses were released from cells by freezing and thawing the cultures 3 times. Then, a centrifugation step at 3000 *g* for 20 min was applied to eliminate cell debris. Finally, viral suspensions were quantified, distributed among the participant laboratories and stored in 3.5-mL aliquots at −80 °C until used.

Plasmid DNA has been used as a positive control and as a quantitative standard. For BPyV, a 416-bp amplicon corresponding to a fragment of the VP1-coding gene was cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). The same vector containing a 612-bp sequence of the PAdV-3 hexon was used for PAdV. Finally, the hexon region (8961 bp) and the whole genome (5130 bp) of HAdV41 and JCPyV Mad1 respectively, were cloned in pBR322 and used as standards for human markers.

2.2. Studied areas

River water and seawater were collected based on ISO 19458 (2006) from five different geographical areas: (i) Llobregat river catchment in Catalonia (Spain), (ii) Glafkos River in Patras (Greece), (iii) Umeälven River in Umeå (Sweden), (iv) Tisza River in Szolnok (Hungary) and (v) the Negro River in the urban area of Manaus (Brazil) (Fig. 1).

The Glafkos River flows into the Gulf of Patras (Ionian Sea) in Patras, a city of 250,000 inhabitants. The river does not dry completely during the summer, but stream flow decreases dramatically. All Glafkos samples were collected bi-monthly from four sampling sites (Fig. 1): P1 and P2 in the river and two marine sampling points, P3 and P4, at the beach in the Gulf of Patras.

The Llobregat river catchment is located in Catalonia, northeast Spain. It flows for 170 km from the Pyrenees to the Mediterranean Sea. The river basin contains more than half of the Catalan population (approximately 5 million people out of approximately 8 million). The river transports urban sewage and agricultural runoff into the stream, and it is used as a source of drinking water after appropriate treatment. The Llobregat River was sampled bi-monthly at three locations: 80 km upstream of the coast (site L1), downstream near the city of Barcelona (L2), which has 3 million people, and from seawater receiving the riverine discharge (L3) (Fig. 1).

The Umeälven River in northern Sweden flows in a southeasterly direction from its origin at Lake Överuman. The

Table 1 – Summary of flow, length, basin areas, year accumulated rainfall and inhabitants (approximatively values). Glossary of the sampling sites, number of samples and analyses included in the study.

Sampling sites	Mean flow (m^3/s)	Length (Km)	Basin area (Km^2)	Average annual rainfall (mm)	Inhabitants (thousands)	Sites	Water matrix	Sample number		Total number of analyses[a]
Glafkos river	5	98	340	1100	250	P1	Riverwater	40	140	560
						P2	Riverwater	40		
						P3	Seawater	30		
						P4	Seawater	30		
Llobregat river	17	170	5000	900	5000	L1	Riverwater	30	89	356
						L2	Riverwater	31		
						L3	Seawater	28		
Umeå river	450	470	27000	650	80	U1	Riverwater	54	162	648
						U2	Riverwater	54		
						U3	Seawater	54		
Tisza river[b]	472	965 (584 in Hungary)	55000 (47000 in Hungary)	495	75 (in Szolnok)	T1	Riverwater	33	129	516
						T2	Riverwater	32		
						T3	Riverwater	32		
						T4	Riverwater	32		
Negro river	28000	2250	691000	2500	1400 (in Manaus)	M1	Riverwater	56	272	1088
						M2	Riverwater	56		
						M3	Riverwater	56		
						M4	Riverwater	56		
						M5	Riverwater	48		

[a] All samples were analyzed by specific qPCR assays for HAdV, JCPyV, BPyV and PAdV.
[b] Raw sewage ($n = 33$) and secondary treatment ($n = 32$) samples were also collected from Szolnok city WWTP.

river is covered by ice from January to the middle of April. Water samples (Fig. 1) were collected from two river water sampling sites upstream and downstream of the city of Umeå (U1 and U2), which has approximately 80,000 inhabitants. In addition, seawater from Ljumviken (U3) was sampled bi-monthly.

The Tisza River is a vital artery of Central Europe. It rises in Ukraine and flows roughly along the Romanian border with Hungary until it meets the Danube in northern Serbia. Floods in spring and summer characterize the Tisza's flow regime. Bimonthly samples were collected in the river from four sites in the vicinity of the city of Szolnok (Fig. 1). Szolnok is a city of 75,000 inhabitants. Raw sewage and secondary (biological) treated effluent (i.e. using the activated sludge process) were also collected at the wastewater treatment plant on each sampling date to characterize the potential fecal input of the viral markers into the river.

The Negro River joins the Amazon River at Manaus. The city of Manaus has 1.4 million inhabitants within an area of 11.5 km^2, and it is located 1450 km inland from the Atlantic coast in the heart of the Amazon rain forest. The urban area is strongly impacted by untreated sewage and garbage in these river waters. Five different shoreline sampling locations were selected (Fig. 1): upstream of the city (M1), in two small tributaries crossing the city that flow directly into the main course (M2 and M3) and two sites in southern Manaus (M4 and M5).

2.3. Collection of samples

The sampling and water analysis phase was conducted from January 2011 to June 2012. Eighteen months of sample collection was completed at each site. A description of the characteristics of each river catchment studied, the mean flow and yearly rainfall during the study period and the numbers of samples are described in Table 1. Within the Mediterranean areas (Glafkos and Llobregat Rivers) the impact of rainfall was limited to spring and autumn. In the continental climate, Tisza catchment, regular rain was observed over the sampling months but flooding occurred in early spring. The Umeälven subarctic climates had low precipitation due to the low moisture content on air, although moderate rainfall occurred in summer. Finally, the Negro river in Manaus had the typical features of a tropical climate: twelve months at temperatures not lower than 18 °C and a monthly average precipitation of 250 mm during the rainy season and 100 mm during the dry period.

A total of 792 samples were analyzed across the 5 groups of sampling sites. Samples with high levels of organic matter or sand were permitted to sediment for 1 h, and the clarified water was transferred to a new container. Seawater samples were collected so as to avoid contamination with sand and suspended macro algae. Turbidity, conductivity, pH and temperature were determined for each water sample. On each sampling day, an extra sample was collected and spiked with adenovirus type 35 (10^5 viral particles/mL) as a process control, including concentration, NA extraction and DNA quantification.

2.4. Viral concentration and nucleic acid extraction

Detection of viruses in the environment requires the concentration of viruses into small volumes. Common standardized operational procedures (SOPs) were utilized for all protocols employed in this study, including sampling, virus concentration, nucleic acid extraction, quantitative PCR (qPCR) assays and process controls and standard plasmid preparation.

All water samples were collected and concentrated using the skimmed milk flocculation (SMF) protocol developed in previous studies (Calgua et al., 2008, 2013b). When analyzing river or seawater samples, 10 L were collected and analyzed to ensure representative volumes for virus detection, whereas 50 mL of wastewater were enough to quantify viral genome copies. When necessary, conductivity was adjusted to a minimum of 1.5 mS/cm^2 in fresh water samples by adding sea salts (Sigma, Aldrich Chemie GMBH, Steinheim, Germany), and then both river water and seawater samples were acidified to pH 3.5 using 1N HCl. A pre-flocculated skimmed milk solution 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) and adding this to each sample to a final concentration of

Table 2 – Oligonucleotide primers and probes used for the detection and quantification of viral indicators.

Virus	Primers and probes	Position[a]	Sequence (5′–3′)	Reference
Human adenovirus (HAdV)	ADF	18869–18887	CWTACATGCACATCKCSGG	Hernroth et al., 2002
	ADR	18919–18937	CRCGGGCRAAYTGCACCAG	
	ADP1	18889–18916	FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ1	
JC Polyomavirus (JCPyV)	JE3F	4317–4339	ATGTTTGCCAGTGATGATGAAAA	Pal et al., 2006
	JE3R	4251–4277	GGAAAGTCTTTAGGGTCTTCTACCTTT	
	JE3P	4313–4482	FAM-AGGATCCCAACACTCTACCCCACCTAAAAAGA-BHQ1	
Bovine polyomavirus (BPyV)	QB-F1-1	2122–2144	CTAGATCCTACCCTCAAGGGAAT	Hundesa et al., 2010
	QB-R1-1	2177–2198	TTACTTGGATCTGGACACCAAC	
	QB-P1-2	2149–2174	FAM-GACAAAGATGGTGTGTATCCTGTTGA-BHQ1	
Porcine adenovirus (PAdV)	Q-PAdV-F	20701–20718	AACGGCCGCTACTGCAAG	Hundesa et al., 2009
	Q-PAdV-R	20751–20768	AGCAGCAGGCTCTTGAGG	
	Q-PAdV-P	20722–20737	FAM-CACATCCAGGTGCCGC-BHQ1	

[a] The sequence positions are referred to strains J01917.1 (HAdV), NC_001699.1 (JCPyV), D13942 (BPyV) and AJ237815 (PAdV).

0.01%. Samples were then stirred for 8 h at room temperature, and aggregates were permitted to sediment by gravity for 8 h. Supernatants were carefully removed, and the final volume containing the sediment was centrifuged at 8000*g* for 30 min at 4 °C. Pellets were suspended using 10 mL of phosphate buffer at pH 7.5 and stored at −80 °C until nucleic acid (NA) extraction was performed. Sewage samples were also analyzed for human and animal viruses in a wastewater treatment plant in the Hungarian case study using a protocol described by Calgua et al. (2013a). Briefly, sewage samples were mixed with 0.25N glycine buffer (pH 9.5) to elute viruses from the organic matter and were then shaken for 30 min on ice followed by centrifugation. The resulting supernatant was processed following the SMF protocol.

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, respectively, as positive and negative control of the NA extraction experiment. Finally, NA elutes were stored at −20 °C until use.

2.5. Quantitative PCR assays

Specific real-time qPCR assays were used to quantify HAdV, JCPyV, PAdV and BPyV. Each qPCR assay contained a set of specific primers and a TaqMan®-fluorogenic probe. Both human and animal viral markers were quantified using methods previously described (Table 2). Undiluted and 10-fold dilutions of the nucleic acid extracts were analyzed in duplicate. All qPCR assays included more than one non-template control (NTC) to demonstrate that the mix did not produce fluorescence. HAdV type 35 was the control process in the study and dilutions of the standard DNA were run in order to evaluate potential enzymatic inhibition due to inhibitors present in the studied samples. Again, UltraPure™ DNase/RNase-Free distilled water was used as negative process control to demonstrate that no cross-contamination occurred.

3. Results

3.1. Quantification of viral markers in five case studies

In this study, samples collected over eighteen months in five river catchments with different land uses and climatological conditions were analyzed for four well-known viral fecal markers (HAdV, JCPyV, PAdV and BPyV). Common SOPs were used in all laboratories for the analysis of the samples. Positive and negative control process produced the expected positive and negative results in all the assays. Inhibition was observed when high levels of organic matter or sand occurred in water samples. In that case, water was allowed to sediment for 1 h, the clarified water was transferred to a new container and 1 more log10 dilution was included into the qPCR analysis. The resulting viral marker loads are plotted separately per virus, sampling site and season, indicating percentages of positive samples and geometric mean values (Figs. 2 and 3). Data is presented for each sampling site by season: winter, spring, summer and autumn, except for the equatorial Negro River catchment, where the climate is split into the rainy and dry periods.

3.1.1. Glafkos river catchment

Human fecal contamination, tested by analyzing HAdV and JCPyV, was detected throughout the year but with low prevalence during autumn. During this season positive samples were only identified in P1 and P4 (20%), and no JCPyV was detected at any of the sampling points. PAdV and BPyV were detected throughout the year in the river at both sampling points; during summer, all river water samples upstream the city of Patras (P1) exhibited positive values for both porcine and bovine viral markers. HAdV and JCPyV viruses were detected in the Glafkos River at concentrations up to 10^5 GC/L, whereas the animal viruses BPyV and PAdV were detected at concentrations up to 10^4 GC/L. The results for each season are represented in Figs. 2 and 3 panels A and B.

3.1.2. Llobregat river catchment

Human fecal contamination was the most important source of contamination in this catchment. HAdV was the most prevalent marker detected at all times and locations within the Llobregat river catchment, with 100% of samples testing positive in spring and summer. HAdV viruses were detected in the Llobregat River at concentrations ranging 10^3–10^5 GC/L. JCPyV was present at concentrations up to 10^4 GC/L and with prevalence of about 80%. JCPyV was found to be present throughout spring, summer and autumn in many of the seawater samples analyzed. Porcine fecal pollution was detected in 30% of the summer samples at the three sampling sites at concentrations up to 10^3 GC/L (Fig. 3C), while bovine was occasionally detected in spring and winter at concentrations up to 10^3 GC/L (Fig. 3D).

3.1.3. Umeå river catchment

The results are summarized in Figs. 2 and 3 panels C and D. Bovine contamination was found to be present with a high prevalence throughout the year with a mean value of 3.9×10^2 GC/L and maximum and minimum values of 5.2×10^3 GC/L and 8.5×10^1 GC/L. The highest BPyV concentration, 5.2×10^4 GC/L, was detected at site U1 located upstream of the Umeå urban center (Fig. 1) during autumn when livestock were grazing adjacent fields, but the highest percentage of positives for BPyV were found during spring at the downstream site U3 (Fig. 3F). During winter, when ice covers the river, the prevalence of both human markers was higher than for the rest of the year: up to 60% for HAdV and 50% for JCPyV with concentrations between 10^2 and 10^3 GC/L (Fig. 2E and F). Porcine fecal pollution was detected occasionally in winter (1/24 samples), summer (5/48 samples) and autumn (1/42 samples) (Fig. 3E).

3.1.4. Tisza river catchment

A high level of fecal contamination, from both human and animal origin, was observed in the Tisza River. The results are described in Figs. 2 and 3 panels G and H. A prevalence of 100% for HAdV (human) was found at all sample sites and seasons, and concentrations were as large as 10^5 GC/L. JCPyV concentrations ranged from 10^3–10^4 GC/L in winter and spring, increasing to 10^5–10^6 GC/L during summer and autumn. The

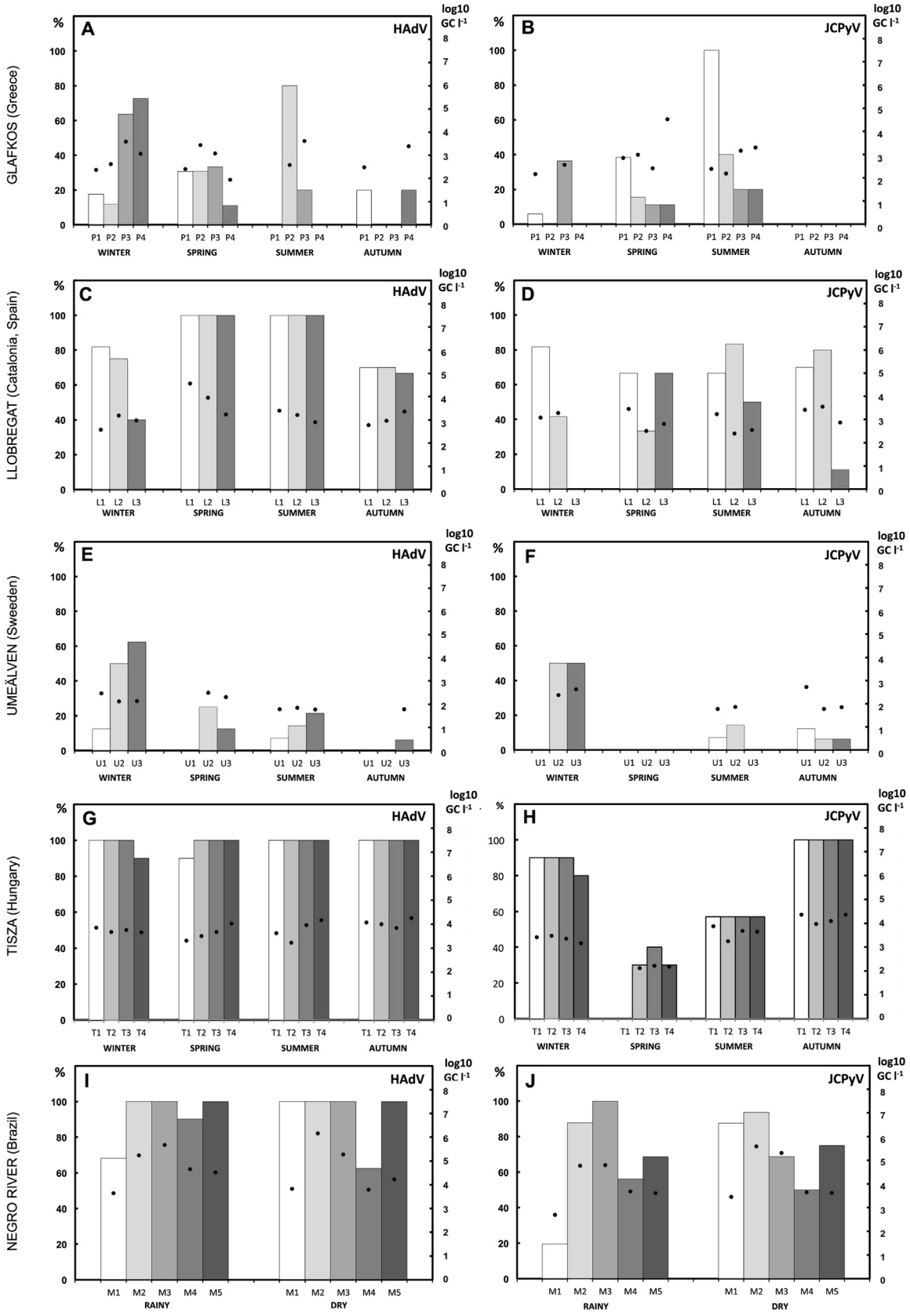

Fig. 2 – Quantitative results of human (HAdV and JCPyV) viral fecal markers in different water matrices from Glafkos river, potted in A, B, Llobregat river, C, D, Umeålven river, E, F, Tisza river, G, H, and Negro River I and J. In columns the prevalences (% of positive samples) in each sampling site. Dots represent geometric mean concentration values, viral genome copies per litre (GC/L).

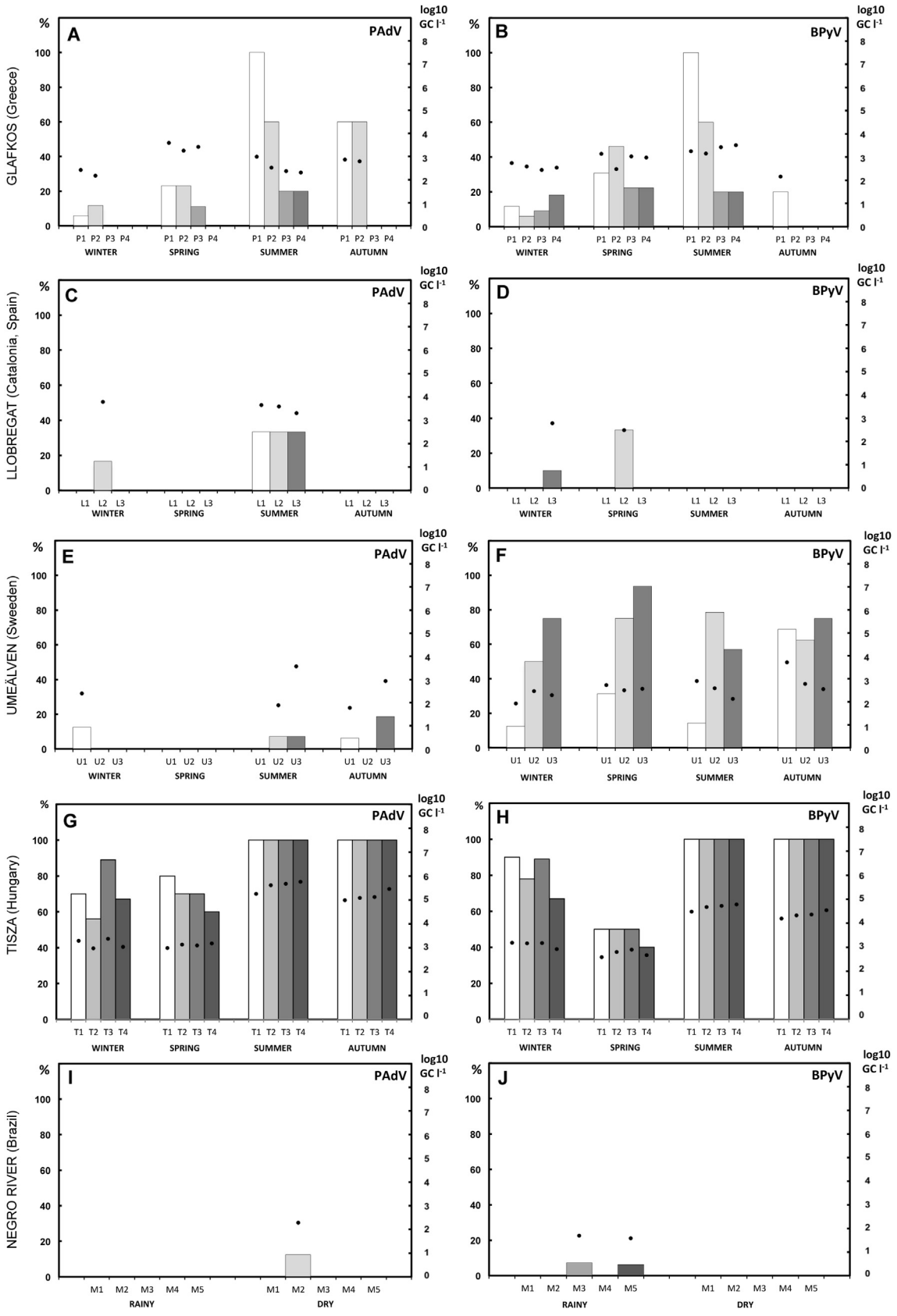

Fig. 3 – Quantitative results of the porcine (PAdV) and bovine (BPyV) viral fecal markers in different water matrices from Glafkos river, potted in A, B, Llobregat river, C, D, Umeålven river, E, F, Tisza river, G, H, and Negro River I, and J. In columns the prevalences (% of positive samples) in each sampling site. Dots represent geometric mean concentration values, viral genome copies per litre (GC/L).

animal viral markers exhibited high prevalence at all sampling sites throughout the year. For example, PAdV (porcine) prevalence in winter ranged between 55 and 90% among river water sites and rose to 100% during spring and autumn, with up to 100% in summer. The viral markers were also quantified in untreated sewage and secondary effluent after activated sludge treatment. Human viruses were between 2 and 2.5 $\log_{10}$ higher in raw sewage than in fresh water. A high prevalence of all human and animal viruses was observed in the wastewater samples, although human viruses were more abundant. As expected, all raw sewage samples ($n = 33$) were positive for both human markers at mean concentrations of 8.7 and 9.2×10^6 GC/L. Secondary effluents ($n = 32$) were also 100% positive for HAdV, and approximately 80% were positive for JCPyV with concentrations between 1.3 and 1.2×10^5 GC/L, respectively. Animal viruses were also detected in sewage samples; porcine fecal pollution rose to 2.7 and 1.4×10^7 GC/L in raw and secondary effluents, respectively (with more than 95% positive samples), and bovine fecal pollution was detected at mean concentrations of 3.1×10^6 GC/L in 90% of raw sewage samples and 2.4×10^6 GC/L in secondary effluents (88% positive). In winter mean values were reduced by approximately 1 log10.

3.1.5. *Negro river catchment*

The prevalence of HAdV (human) in the Negro River was 100% at all sample sites except for site M1, which was located upstream of the city, and during the rainy season at site M4, which was situated downstream the city of Manaus. HAdV concentrations were present in increments of up to 2 log10 throughout the city, with a concentration of up to 6.6×10^3 GC/L upstream (M1) and up to 1.4×10^6 GC/L when sampling downstream of the city (M2). JCPyV was prevalent throughout the year at lower concentrations than HAdV, specifically 8.2×10^3 versus 5.6×10^4 GC/L during the rainy season and 2.1×10^4 versus 4.5×10^4 GC/L during the dry season. The number of positive samples was also lower during the rainy periods: 58 and 73% for JCPyV versus 90 and 91% for HAdV for the rainy and dry periods, respectively. Both human markers were more prevalent during the dry season. The animal markers were rarely detected, with only two positive samples for PAdV (porcine) during the dry season and five positive samples for BPyV (bovine) during the rainy period at concentrations between 10^2 and 10^1 GC/L for both. The results are presented in Figs. 2 and 3 panels I and J.

4. Discussion

In this study, novel parameters were investigated as microbial source-tracking tools designed to map the origins of fecal contamination to human (HAdV and JCPyV), porcine or bovine (PAdV and BPyV) viruses. DNA-based viral tools were applied in five different river scenarios in five countries: Greece, Spain, Sweden, Hungary and Brazil.

The protocols used in the study have been shown to be easily applicable in routine analysis of a wide diversity of water matrices. The estimated recovery efficiencies of the concentration method, evaluated in previous studies, are between 30 and 95% for adenovirus and between 55 and 90% for JC polyomavirus (Calgua et al., 2013b). At all case study sites, human markers were detected in river and seawater samples. The theoretical limit of detection of the quantification protocol was 60 GC/L. The analyzed human viruses have been described and quantified in previous studies in sewage and river water (Bofill-Mas et al., 2000, 2006; Miagostovich et al., 2008; Fumian et al., 2010; Staley et al., 2012), and the results obtained in this study confirm that human fecal contamination is widespread and also that viral tools are applicable as fecal indicators in all geographical areas studied.

The results describe five different locations with specific characteristics and contamination patterns in each studied area. In the city of Manaus, raw sewage is discharged directly into the Negro River, and the receiving water exhibits high viral loads, with HAdV and JCPyV detected at mean concentrations of 10^5 GC/L and 10^4 GC/L, respectively. The absence of significant bovine or porcine markers is in agreement with available information describing the absence of any significant farming activity in the area.

The Tisza River area exhibited very high numbers of positive samples both for human and animal viruses, with increasing downstream levels reflecting the urban discharges in the area. Intensive livestock farming is practiced throughout the basin, specifically around Szolnok, where 13 pig farms and 20 dairy farms are located immediately upstream of the sampling sites. All the farms and slaughterhouse effluents are treated in the urban WWTP. Both porcine and bovine contaminations were highly abundant and exhibited lower concentrations in winter, while higher values for both human and animal contamination were present in summer and autumn. The Tisza is one of the main rivers of Central Europe, with seasonal flooding in early spring and early summer. Although these flows will dilute fecal contamination, may represent a significant microbiological risk for the population, considering the high level of human and animal contamination observed all over the sampling periods.

Two rivers were studied in the Mediterranean area: the Llobregat River (17 m^3/s), in Catalonia, which is heavily impacted by more than 50 urban sewage treatment plant secondary effluents, and the Glafkos River in the European East Mediterranean, a smaller river (5 m^3/s) with a flow that is also highly dependent on rainfall and that drastically decreases in summer up to 1 m^3/s. The Llobregat River exhibited human fecal contamination as the most significant source of pollution, which reflects the high amount of secondary effluent discharged into the river basin (from more than fifty plants). The Glafkos River presented a different profile, with variable concentrations over the year from both human and farm animals. Human settlements and animals drinking directly in the river were observed during the summer period posing a risk of direct voiding of urine/feces and uncontrolled discharges. Rain events in the Glafkos River may rapidly affect water quality. Rainy periods with higher river flows represent higher dilution levels of fecal contamination and viral markers in river and seawater. In agreement with this, lower numbers of HAdV and JCPyV were observed in autumn (the most common rainy season in Mediterranean areas) in Greece and Spain. The Patras River samples that were tested presented high levels of animal pollution, while seawater

samples primarily indicated human pollution; this suggests (as expected) that other fecal urban discharges may influence the microbiological quality of seawater in the area. This information has been confirmed, and several treated sewage discharges were also identified in the studied beach area. It is important to note the high levels of viral contamination observed in seawater samples during the summer period. Reduction in river flow levels results in a lower dilution of viral input and therefore increased viral contamination when discharging into the sea, posing a public health risk.

The predicted reduction in total rainfall and the number of summer rainfall events as a result of climate change would produce lower river flows and likely higher variability in fecal contamination levels, particularly where treated or untreated wastewater represents a significant proportion of the river water flow (Kay et al., 2011). Therefore, drought events could represent a reduction in water quality due to sewage concentration and animal effluents, although environmental die-off of microbial pollutants would also be enhanced under such low-flow conditions. Although most communities in the Glafkos, Llobregat, Umeälven and Tisza catchments treat their wastewater, present sewage treatment systems are not designed to specifically reduce virus concentrations, and high viral loads were observed in the receiving water bodies.

In the more rural Swedish catchment, the main source of fecal contamination was identified as being produced by bovine cattle. High concentrations of bovine viral markers were observed in all seasons, with the lower contamination levels during winter months, probably related to the fact that livestock is indoors and diffuse contamination is reduced. The strong ice cover formed on the river surface and the low temperatures could protect viruses against inactivation. Low levels of human fecal viral MST markers were detected at this site, with a low number of positives and concentrations 1 $\log_{10}$ lower than those observed for BPyV.

In order to standardize the qPCR assay in different laboratories, reproducibility tests, reference materials and common standard DNA suspensions could be needed. In this study the viral concentration and quantification was robust and reproducible when analyzing twenty repeated water matrices, specifically river or seawater. Coefficients of variance were 0.64 for HAdV in river water and 0.41 for JCPyV in seawater respectively (Data not shown). Simple cost-effective protocols are available for the quantification of AdV and PyV in routine laboratories with responsibilities for water quality. The results of this study probe the feasibility of using the proposed protocols and viral markers for quantification of the levels and sources of fecal contamination in river catchments in any geographical area.

5. Conclusions

1. The novel MST tools described in this paper have been shown to be specific, sensitive and provide quantitative data describing source-specific fecal impact in river catchments in different geographical areas.
2. The protocols and viral markers applied in this multi-laboratory study have proven to be robust, cost-effective and applicable for routine MST analysis in all types of water matrices and geographical areas.
3. The human (HAdV and JCPyV) and animal (PAdV and BPyV) viruses analyzed in this study identified the sources of fecal contamination in all river catchment areas analyzed in Europe and in South America.
4. The application of viral MST tools to river catchments clearly reflects the exploitative pressures in these respective areas, either from human or animal sources, and will contribute to risk assessment analysis and define remediation actions.

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