Disseminació i traçabilitat de la contaminació viral en conques fluvials

Dissemination and source tracking of viral contamination in river catchments

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DISSEMINACIÓ I TRAÇABILITAT DE LA CONTAMINACIÓ VIRAL EN CONQUES FLUVIALS

DISSEMINATION AND SOURCE TRACKING OF VIRAL CONTAMINATION IN RIVER CATCHMENTS

Per/by

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DISSEMINATION AND SOURCE TRACKING OF VIRAL CONTAMINATION IN RIVER CATCHMENTS

Memòria presentada per
Memory presented by

Marta Rusiñol Arantegui

Per optar al grau de
to optain the degree of

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Tesi realitzada sota la direcció de la Dra. Rosina Girones Llop i la Dra. Sílvia Bofill Mas,

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Rosina Girones Llop, Professor of Microbiology in the University of Barcelona and Sílvia Bofill Mas, Doctor in Microbiology in the University of Barcelona,

DECLARE THAT,

Marta Rusiñol Arantegui has performed the work entitled "Dissemination and source tracking of viral contamination in river catchments" under the direction of the Dr. Rosina Girones Llop and Dr. Sílvia Bofill Mas, in order to obtain the degree of Doctor by the University of Barcelona and that this thesis fulfills the requirements to obtain the mention of "International Doctor".

Signatures,

Rosina Girones Llop

Sílvia Bofill Mas

Barcelona, May 2014th

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CONTINGUTS

ABREVIACIONS

ADN	Àcid desoxiribonucleic
AdV	Adenovirus
AFLP	Polimorfismes a la llargada dels fragments amplificats (de l'anglès" Amplified Fragment Length Polymorphism")
AN	Àcid nucleic
ARA	Anàlisi de resistència a antibiòtic (de l'anglès "Antibiotic Resistance Analysis")
ARN	Àcid ribonucleic
ARP	Patró de resistència a antibiòtics (de l'anglès "Antibiotic Resitance Pattern")
AstV	Astrovirus
BAdV	Adenovirus boví
BKPyV	Poliomavirus BK
bp	Parell de bases (de l'anglès "base pair")
BEV	Enterovirus boví
BFDV	Circovirus aviar causant de la malaltia de bec i plomes en lloros (de l'anglès "Beak and Feather Disease Virus")
BPyV	Poliomavirus boví
BSA	Albúmina de sèrum boví (de l'anglès "Bovine Serum Albumin")
CAV	Gyrovirus de pollastre (de l'anglès "Chicken Anèmia Virus")
CDC	Centres americans de control d'enfermetats (de l'anglès "Centers of Disease Control")
CF	Coliforms fecals
CFU	Unitats formadores de colònies (de l'anglès "colony forming units")
ChPV	Parvovirus de pollastre (de l'anglès "Chicken parvovirus")
CUP	Perfil d'ús de les fonts de carboni (de l'anglès "carbon-source utilization profiling")
DGGR	Electroforesi en gel amb gradient de desnaturalització (de l'anglès "denaturing gradient gel electrophoresis")
dsDNA	ADN bicatenari (de l'anglès "double strand DNA")
dsRNA	ARN bicatenari (de l'anglès "double strand RNA")
E. coli	*Escherichia coli*
EDAR	Estació Depuradora d'Aigües Residuals

EF	Enterococs fecals
EFSA	Autoritat europea de protecció alimentària (de l'anglès "European Food Safety Authority")
EMA	Monoacida d'etidi (de l'anglès " ethidium monoazide")
ENHIS	Agència europea d'informació ambiental i sanitària (de l'anglès "Environment and Health Information System")
EPA	Agència de Protecció Ambiental dels Estats Units d'Amèrica (de l'anglès "Environmental Protection Agency")
et. al	i col·laboradors (del llatí "et alii")
EV	Enterovirus
FAM	Fluoròfor, també anomenat 6-carboxyfluoresceïna
FAME	Metil ester d'àcids grassos (de l'anglès "Fatty Acid Methyl Ester")
FAO	Organització de les nacions unides per l'Alimentació i l'Agricultura (de l'anglès "Food and Agriculture Organization")
FFU	Unitats formadores de fluorescència (de l'anglès "Fluorescence Forming Units")
FST	Traçar l'origen de la contaminació fecal (de l'anglès "Fecal Source Tracking")
FWA	Agents blanquejants fluorescents (de l'anglès "Fluorescent Whitening Agents")
GC	còpies genòmiques (de l'anglès "Genome Copies")
GG	Genogrup
GPV	Parvovirus d'oca (de l'anglès "Goose parvovirus")
ha	hectàrea
HAdV	Adenovirus humà
HAV	Virus de l'hepatitis A
HBoV	Bocavirus humans
HCl	Àcid clorhídric
HEV	Virus de l'hepatitis E
ICC-PCR	PCR integrada amb cultiu cel·lular (de l'anglès "Integrated Cell Culture-PCR")
ICTV	Comitè internacional de taxonomia de virus (de l'anglès " International Comittee on Taxonomy of viruses")
IPCC	Grup intergovernamental sobre canvi climàtic (en anglès "Intergovernmental panel on Climate Change")
ISO	Organització internacional d'estandardització (de l'anglès "International Standard Organization")
JCPyV	Poliomavirus JC humà
Kb	Quilo-bases

KDa	Quilo-daltons
KIPyV	Poliomavirus KI
KgN	Quilograms de nitrogen
LAB	Alquil-benzens lineals (de l'anglès "Linear alkylbenzene")
LOD	Límit detecció
LH-RFLP	Heterogeneïtat en la llargada dels polimorfismes de longitud dels fragments de restricció (de l'anglès " Lenght Heterogeneity- Restriction Fragment Length Polymorphism")
LOQ	Límit quantificació
MAR	Resistència a antibiòtic múltiple (de l'anglès "Multiple Antibiotic Resistance")
MCPyV	Poliomavirus de les cèl·lules de Merkel
MDPV	Parvovirus d'ànec mut (de l'anglès "Muscovy duck parvovirus")
NaOH	Hidròxid de sodi
NS	Regió no estructural del genoma (de l'anglès "non structural")
MR	Mostra representada
MST	Traçar la font de microorganismes (de l'anglès "Microbial Source Tracking")
mS/cm²	Micro-Siemens per centímetre quadrat
MWPyV	Poliomavirus de Malawi, també anomenat MXPyV (poliomavirus de mèxic) i HPyV10 (poliomavirus numero 10)
NoV	Norovirus
OPyV	Poliomavirus oví
PAdV	Adenovirus porcí
PAHs	Hidrocarburs aromàtics policíclics (de l'anglès "Polycyclic Aromatic Hydrocarbon")
PCR	Reacció en cadena de la polimerasa (de l'anglès " Polymerase Chain Reaction")
PEV	Enterovirus porcí
PFGE	Electroforesi de camp polsant (de l'anglès "Pulse-Field Gel Electroforesi")
PFU	Unitats formadores de clapes (de l'anglès "Plaque Forming Units")
pH	Potencial d'hidrogen iònic
PMA	Propidi monoacida (de l'anglès Propidium monoazide)
PTV	Tescovirus porcí
PyV	Poliomavirus
PMMoV	Virus mosaic del pebre (de l'anglès "Pepper mild mottle virus")
qPCR	PCR quantitativa, també anomenada RT-PCR (de l'anglès "real-time PCR")
R	Recuperació del mètode de concentració

RAPD	Polimorfismes d'amplificació d'ADN a l'atzar (de l'anglès " Random Amplified DNA polymorphisms")
RD	Real Decret, norma jurídica espanyola.
Rep-PCR	PCR repetitiva palindròmica i extragènica (de l'anglès "Repetitive extragenic palindromic PCR")
ROS	Espècies reactives d'oxigen (de l'anglès "Reactive Oxygen Species")
RV	Rotavirus
ssDNA	ADN monocatenari (de l'anglès "single strand DNA")
ssRNA	ARN monocatenari
spp.	Espècie
STLPyV	Poliomavirus de Sant Louis
SV40	Virus de simi 40
T-Ag	Antigen tumoral gran
t-Ag	Antígen tumoral petit
$TCID_{50}$	Quantitat de virus que infecten el 50% de les cèl·lules d'un cultiu (de l'anglès "50% tissue culture infective dose")
T-RLFP	Polimorfismes terminals de longitud dels fragments de restricció (de l'anglès " Terminal restriction fragment length polymorphism")
TSPyV	Poliomavirus de la Tricodisplàsia espinulosa
TyPV	Parvovirus de gall d'indi (de l'anglès "Turkey parvovirus")
UE	Unió Europea
UV	Llum ultraviolada
VP	Proteïna de la càpsida viral (de l'anglès "Virion protein")
WFD	Directiva marc de l'aigua (de l'anglès "Water Framework Directive")
WHO	Organització Mundial de la Salut (de l'anglès " World Health Organization")
WUPyV	Poliomavirus WU
x *g*	Unitats de força centrífuga, gravetat

RESUM de la TESI

Aquesta Tesi Doctoral ha estat pensada per avaluar, mitjançant l'estudi de virus contaminants, l'origen de la contaminació fecal i l'avast de la disseminació a l'aigua. Amb aquesta finalitat s'han utilitzat eines moleculars per a la detecció i quantificació de virus especifics humans, bovins i porcins i a més a més s'ha desenvolupat una nova eina per traçar la contaminació ovina a l'ambient. Els principals agents virals amb risc per als humans són d'origen humà però les femtes animals, amb potencials patògens zoonòtics, representen també un risc per a la salut humana. La intensificació de la producció animal o l'increment de plujes torrencials previst amb els escenaris proposats per al canvi climàtic presenten nous reptes per al control de la contaminació fecal a l'aigua.

Bona part de la Tesi, ha anat lligada al projecte europeu VIROCLIME permetent dur a terme estudis a nivell internacional. S'ha caracteritzat la disseminació de virus humans contaminants a l'aigua d'un riu situat en una conca Mediterrània. Mitjançant el registre de variables hidro-climàtiques, la recollida de mostres d'aigua de riu, aigua de mar i aigua residual, i l'anàlisi de FIB i virus indicadors de contaminació fecal humana (HAdV i JCPyV), virus patògens (NoVGII, HEV), i un virus emergent (MCPyV), s'ha estudiat durant un any, l'impacte que l'aigua residual crua o els efluents de les depuradores tenen en la qualitat microbiològica del riu. En un segon estudi realitzat conjuntament amb laboratoris de Suècia, Grècia, Hongría i Brasil, s'ha pogut estandarditzar el mètode de floculació amb llet descremada, per a la concentració de virus, i els assajos de qPCR per identificar i traçar l'origen de la contaminació fecal a l'aigua. Durant 18 mesos de mostrejos a conques de rius situats a les regions Mediterrània, Àrtica, Continental i Tropical, s'han identificat virus humans (HAdV i JCPyV), bovins (BPyV) i porcins (PAdV), demostrant que aquestes eines virals són fiables i útils per a qualsevol àrea geogràfica o matriu d'aigua.

Fins al principi d'aquesta tesi, només es disposava de marcadors virals de contaminació fecal porcina i bovina. Analitzant mostres de femta i orina d'ovella, amb un

assaig de PCR d'ample espectre per a la detecció de poliomavirus, es van obtenir per primera vegada seqüències d'un putatiu nou poliomavirus oví. A partir d'aquí, s'han dissenyat dos assajos de PCR específics per poder traçar la contaminació fecal ovina a l'ambient. Arrel d'aquest treball, durant l'últim any de la tesi, es va plantejar una estada a Nova Zelanda perquè es tracta del país amb més ovelles per càpita i representa bona part de la indústria ramadera del país. Traçar i identificar l'origen de la contaminació fecal animal a l'aigua és doncs bàsic, tant per la gestió dels residus com per l'avaluació dels riscos per a la salut humana. El quart estudi inclòs a la tesi, es va dissenyar per identificar les fonts principals de contaminació fecal i avaluar, al mateix temps, les diferents eines de MST utilitzades a Nova Zelanda i al laboratori de la doctoranda. S'han recollit mostres de riu als territoris ramaders més importants de l'illa del sud, i s'han analitzat *E. coli*, virus específics indicadors de contaminació fecal humana (HAdV, JCPyV) bovina (BPyV) i ovina (OPyV), marcadors bacterians específics d'humans (BacH, BacHum-UCD, BiAdo) i de remugants (BacR) i esterols i estanols indicadors de contaminació fecal humana o de remugant.

D'acord amb els requisits per a obtenir la menció internacional, s'ha realitzat part de la memòria en anglès. El resum de la tesi, els diferents articles publicats i sotmesos, així com la discussió final i les conclusions, s'han traduït a l'anglès, mentre que la resta de seccions estan escrites en català.

SUMMARY of the THESIS

This thesis was designed to evaluate, through the study of the presence of viruses, the source of the fecal contamination and its spread in water matrices. For this purpose, molecular tools have been evaluated for the detection and quantification of specific human porcine and bovine viruses, and a new tool has been developed to trace ovine pollution in the environment. Human waterborne viruses pose the main risk to humans, but animal feces, with potential zoonotic pathogens, also represent a risk to human health. The intensification of animal production and the predicted scenarios for climate change, with increase of torrential rains, represent new challenges for the control of fecal contamination in water.

Much of this work has been linked to the European project VIROCLIME allowing studies conducted worldwide. We characterized the spread of contaminant human viruses in a Mediterranean river basin. By recording hydro-climatic variables, collecting samples of river water, sea water and wastewater and analysing FIB, viruses indicators of human fecal pollution (HAdV and JCPyV), pathogenic viruses (NoVGII, HEV) and an emerging virus (MCPyV), we have studied, thoughouth a year, the impact of raw and treated wastewater the microbiological quality of the river. In a second study, conducted with laboratories from Sweden, Greece, Hungary and Brazil, a viral concentration method, the Skimmed Milk Flocculation, and specific qPCR assays to identify and trace the origin of contamination in water, have been standarized. During 18 sampling months we collected samples in river basins located in the mediterranean, arctic, continental and tropical regions. Human (HAdV and JCPyV), bovine (BPyV) and porcine viruses (PAdV), were identified showing that these tools are reliable and useful for studing water quality in any geographic area or water matrix.

At the beginning of this thesis, only human, porcine and bovine viral fecal indicators were available to trace fecal pollution. Since no specific ovine viral indicator was described before the present work, we designed a study to develop a new tool to trace sheep fecal contamination. Firstly we analyzed urine and stool samples from sheep,

with a broad spectrum PCR assay for the detection of polyomavirus. We obtained sequences of a putative new ovine polyomavirus and designed two specific PCR assays for the new ovine virus which were succesfully applied to detecto vine pollution in environmental samples. During a six months research stage in New Zealand, the country with more sheep per capita, human, bovine and ovine viral indicators were used to trace and identify the source of fecal contamination in water. River samples were collected at major livestock areas in the south island of New Zealand. *E. coli*, human (HAdV, JCPyV), bovine (BPyV) and sheep specific viruses (OPyV), human (BacH, BacHum-UCD, BiAdo) and ruminant bacterial markers (BacR) and sterols/stanols ratios were analyzed.

In accordance with the requirements to obtain the mention of "International Doctor" part of the work has been done in English. The summary of the thesis, the published/submitted articles and the discussion and conclusions have been translated into English, while other sections are written in Catalan.

1. INTRODUCCIÓ

1. INTRODUCCIÓ

1.1. L'aigua i la contaminació fecal

L'aigua és vida, és imprescindible pels éssers vius, essencial per una bona higiene, per generar energia o per al transport; i per tant bàsica per a un bon desenvolupament econòmic. L'aigua és font de vida, però també ha sigut la causa de molts conflictes, fins a 256 arreu del món segons Gleick (2009), i pot ser vector de transmissió de moltes malalties. Per això, respectar el cicle de l'aigua i evitar la contaminació, suposa un gran repte per a la gestió sostenible d'aquest recurs limitat. Es calcula que l'ús de l'aigua durant el segle XX, ha crescut a un ritme superior al doble de la taxa de creixement de la població (FAO, 2013). El creixement demogràfic mundial i el conseqüent increment de superfície dedicada a l'agricultura i la ramaderia, suposen un ús més gran de l'aigua i a la vegada un empobriment de la qualitat. Es calcula que es necessiten d'entre 20 i 40 litres d'aigua, lliure de contaminants i patògens, per persona i dia (FAO, 2013). En molts països però, la ràpida urbanització s'ha "oblidat" de incloure les infraestructures de sanejament necessàries, fet que es tradueix directament en reducció de la quantitat d'aigua disponible o empitjorament de la qualitat.

El capítol 18 de l'Agenda 21, sobre la protecció de la qualitat i el subministrament del recursos d'aigua dolça, és l'únic instrument internacional que proposa criteris per l'aprofitament sostenible d'aquest recurs (United Nations, 1992). No hi ha acords ambientals vinculants a nivell global, que obliguin els estats a protegir els recursos hídrics davant de la contaminació, ja que és una responsabilitat de cada govern nacional. Per la seva banda la Unió Europea (UE), va establir l'any 2000, un marc comunitari d'actuació amb la Directiva marc de l'aigua (WFD 2000/60/CE, 2000). Aquest marc comunitari en política d'aigua, estableix que a l'any 2015 s'ha d'haver complert l'objectiu principal d'aconseguir un "bon estat de l'aigua" en totes les aigües de la UE. Els criteris per determinar l'estat de l'aigua es basen en criteris ben diversos d'abundància de flora i fauna aquàtica, nivells de salinitat o concentració de nutrients. La directiva ressalta la

necessitat de tractar els efluents fecals, especialment humans per evitar la contaminació ambiental però sobretot per controlar la dispersió de malalties.

1.1.1. Patògens d'origen fecal transmesos per l'aigua

La contaminació fecal és la principal font de degradació de l'aigua. La matèria fecal, sigui humana o animal, va associada moltes vegades a microorganismes patògens que comporten un risc potencial per a la població quan arriben a aigües recreacionals, de reg, pesqueres o d'abastament. Les malalties transmeses per l'aigua són ben diverses, tant per l'agent infecciós, com pels seus efectes o la via de transmissió [figura 1]. L'aigua de beguda, els aliments contaminats o les mans, són els vehicles més importants de transmissió de patògens hídrics quan el sanejament i la higiene és escassa.

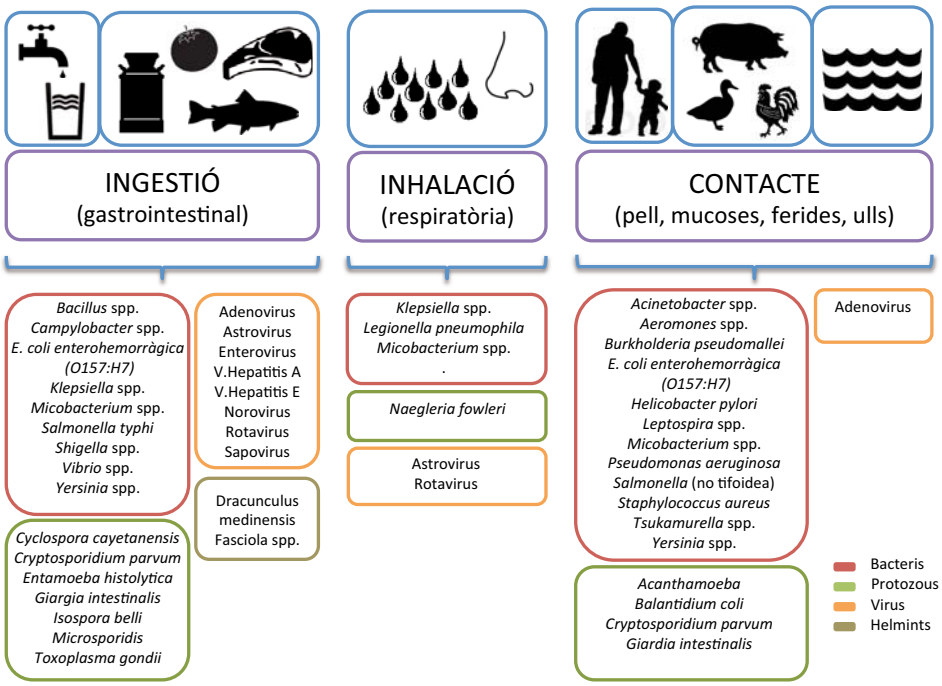

Figura 1. Vies de transmissió dels agents patògens relacionats amb l'aigua.

Les infeccions diarreiques són les més prevalents, entre les malalties d'origen hídric, i son responsables de la mort de fins a 2.2 milions de persones, principalment nens (1.8 milions), 88% dels quals en països de baixa renta (WHO, 2013a). Alguns agents patògens poden estar presents de forma natural a les aigües superficials o subterrànies, a les xarxes de distribució d'aigua o fins i tot al sòl. Quan les condicions de temperatura i disponibilitat de carboni són favorables, o quan les concentracions de clor són baixes, alguns patògens com per exemple: *Acanthamoeba* spp., *Legionella* spp., *Naegleria fowleri o Vibrio cholerae* poden proliferar. Els virus i les formes latents dels paràsits (cists o ous) no es poden multiplicar a l'aigua, depenen d'un hoste per completar el seu cicle replicatiu.

Així doncs, la majoria de patògens vírics, els paràsits i gran part dels patògens bacterians entren al medi mitjançant la contaminació fecal. *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Vibrio cholera* i *Salmonella typhi* són els patògens bacterians més importants en femtes humanes (WHO, 2011). També són excretats pels humans els adenovirus, els virus de la hepatitis A, rotavirus o enterovirus, que produeixen patologies tan variades com cistitis, conjuntivitis, gastroenteritis, hepatitis, pneumònia, o poliomielitis, entre d'altres. En femtes d'animals s'hi troben més sovint *Campylobacter jejuni*, *Escherichia coli* enterohemorràgica o la *Yersinia enterocolítica* i virus com el de la hepatitis E i els rotavirus, que tenen un origen potencial en porcs, vaques i gossos d'entre altres (Cook et al., 2004; Meng, 2013). La transmissió d'aquests patògens zoonòtics pot ser directa des dels animals a les persones, o viceversa, o indirecta, a través dels aliments, de l'aigua o també per el contacte amb cremades o ferides amb estris o mans brutes. Altres virus animals com els norovirus, sapovirus, picobirnavirus o astrovirus també s'han relacionat amb els humans, però la transmissió interespècie no s'ha pogut demostrar (Brugere-Picoux and Tessier, 2010; Scipioni et al., 2008).

1.1.2. El canvi climàtic i la contaminació de l'aigua

L'àrea mediterrània serà una de les zones del món més afectades pel canvi climàtic, caracteritzat per temperatures més altes i disminució de la precipitació (figura 2). Es preveuen menys dies de pluja acumulats en un mateix període de l'any, és a dir que augmentaran tant la freqüència com la intensitat de les pluges extremes o les sequeres (Patz et al., 2008; Semenza and Menne, 2009). L'efecte principal de les pluges torrencials serà el de mobilitzar patògens dispersant-los per l'ambient (Lipp et al., 2001; Martinez-Urtaza et al., 2004). Tant en contextos urbans com agrícoles i ramaders, es pot generar una important escorrentia superficial, que contamini directament punts de captació d'aigua potable o aigües recreacionals, o un increment de la infiltració que arrossegui de manera difusa els microorganismes fins a punts de captació d'aigües subterrànies.

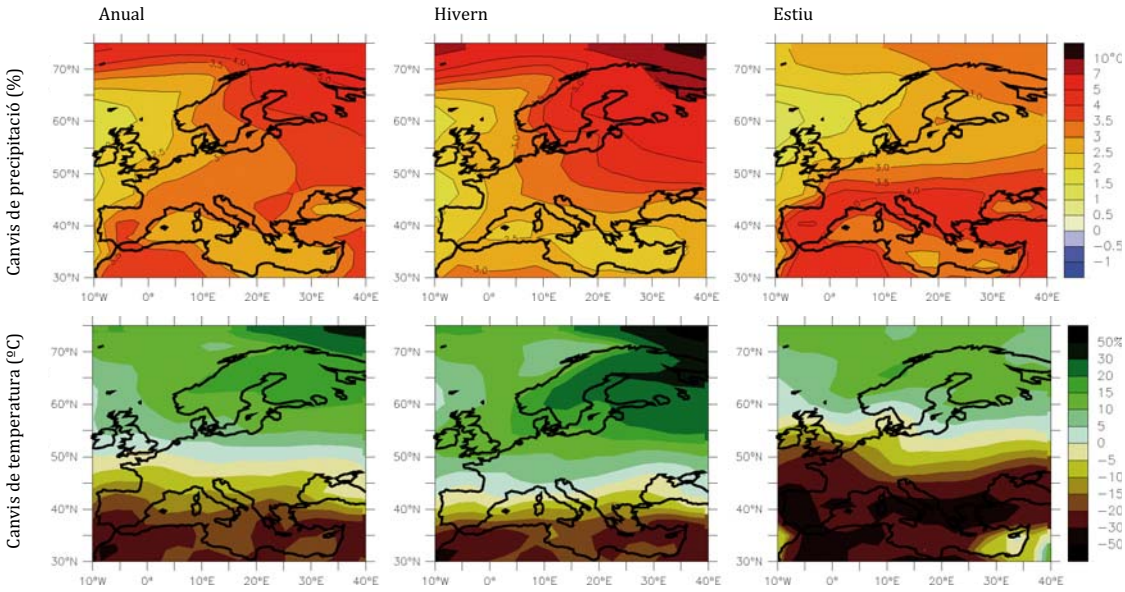

Figura 2. Canvis relatius de temperatura i precipitació, entre els períodes 1980-99 i 2080-99 a Europa, a partir de les simulacions de 21 models globals de canvi climàtic (IPCC, 2007).

Encara avui en dia durant períodes de pluges intenses els sistemes unitaris de clavegueram no poden assumir tot el volum d'aigua i mitjançant els sobreeixidors

presents per tota la xarxa, es facilita la sortida de l'aigua residual directament fora de la xarxa (Hata et al., 2014). Les depuradores per la seva banda, també desvien l'excés d'aigua a l'entrada de la planta depuradora, per tal de protegir el sistema de tractament d'aigües residuals. L'increment d'episodis de forta precipitació altera directament la qualitat de l'aigua i per tant l'accés segur a aquest recurs. De fet, el risc de gastroenteritis o infeccions respiratòries lligades a usos recreacionals és més alt durant els mesos més humits (Brinks et al., 2008). Només a Europa, entre l'any 2000 i el 2007, es van produir 354 brots lligats amb l'aigua de beguda i 70 lligats amb aigües recreacionals, amb 47.617 i 3.132 casos respectivament (ENHIS, 2009).

Un estudi realitzat al Regne unit, va calcular que un 20% dels brots de malalties relacionades amb l'aigua anaven lligades a llargs períodes de sequera (Nichols et al., 2009). Tot i així, les pluges torrencials i les inundacions semblen tenir una relació directe amb l'aparició de brots. Fa ben poc, s'ha publicat una revisió bibliogràfica dels brots identificats després de períodes de pluges fortes a Europa (Cann et al., 2013). Setze brots van relacionar-se amb patògens bacterians (*V. Cholerae, Leptospira, C. Jejuni, E. coli* i *B. Pseudomallei*), 5 amb protozous (*Giardia, Acanthamoeba i Cryptosporidium*) i fins a 10 amb virus patògens (Rotavirus, Norovirus, Enterovirus, Virus de l'Hepatitis A i virus de l'Hepatitis E).

1.1.3. Els virus i la contaminació fecal a l'aigua

Les fonts de contaminació de virus a l'ambient són ben diverses, poden ser puntuals o difoses (figura 3). L'aigua residual, tal i com hem comentat al principi de la tesi, és una de les fonts principals de contaminació, sobretot en països de baixa renta on els sistemes de sanejament són pràcticament inexistents. L'ús i abús de biosòlids o purins com a fertilitzants també pot contaminar el sòl, les aigües subterrànies i fins i tot les aigües superficials. En qualsevol dels casos, un cop els virus són excretats, poden arribar a les aigües recreacionals, aigües de beguda i fins i tot a través de l'aigua de reg a fruites, verdures i mol·luscs bivalves.

Figura 3. Vies de disseminació dels virus humans i animals a l'ambient.

Les malalties i els brots relacionats amb virus sovint no es monitoritzen o simplement no es registren com a tals. Tot i així, s'estima, que un 80% dels casos de gastroenteritis agudes en humans son d'origen víric (Glass, 2013). Segons l'agència europea d'informació ambiental i sanitària, l'ENHIS, entre l'any 2000 i el 2007, 148 dels 424 brots de malalties relacionades amb l'aigua, van ser causats per virus, 136 lligats a l'aigua de beguda i 12 lligats a aigües recreacionals (ENHIS, 2009). A més a més, entre un 45 i 51% dels brots registrats respectivament a Estats Units i a Europa, són causats per virus en aliments (EFSA, 2012; Gould et al., 2013).

Tot i que majoritàriament s'associen a diarrees i gastroenteritis en humans, els virus transmesos per l'aigua poden provocar infeccions respiratòries, conjuntivitis o hepatitis i en alguns casos, en grups de risc com nens, dones embarassades o individus immuno-suprimits, poden provocar meningitis, encefalitis, paràlisi o fins i tot la mort (Griffin et al., 2003). Puntualment, s'han relacionat amb malalties cròniques com miocarditis o diabetis (Griffin et al., 2003). Les infeccions dels virus entèrics en animals normalment són asimptomàtiques, però igual que en el cas dels humans, poden derivar en desordres neurològics, avortament o la mort (Day and Zsak, 2013; Jiménez-Clavero et al., 2003; Ley et al., 2002). Per als humans, les aigües residuals humanes són la principal font de contaminació fecal, i representen un risc per a la salut superior al que representen les aigües residuals d'origen animal (Schoen et al., 2011). No obstant això, alguns agents patògens o zoonòtics poden infectar i causar malaltia tant als humans com als animals (Morse et al., 2012).

La majoria de virus associats a la contaminació fecal de l'aigua pertanyen a 10 famílies (Taula 1): *Adenoviridae, Anelloviridae, Astroviridae, Caliciviridae, Circoviridae, Hepeviridae, Parvoviridae, Picornaviridae, Poliomaviridae* i *Reoviridae*. Les prevalences dels virus inclosos en aquestes famílies són ben diferents. El virus de la hepatitis A (HAV), per exemple, és freqüent en aigües residuals de zones endèmiques i, igual que el virus de la hepatitis E (HEV), és més abundant en zones sense sanejament i baix nivell sanitari (WHO, 2013b, 2013c). La prevalença dels astrovirus (AstV), enterovirus (EV), norovirus (NoV) i rotavirus (RV) és més aviat estacional i depèn de l'època de l'any i de la presència de brots epidèmics a la població (Chen and Chiu, 2012; Ganesh et al., 2012; Reyna-Figueroa et al., 2012; Walter and Mitchell, 2000). I la prevalença dels adenovirus (AdV) i poliomavirus (PyV), que s'excreten durant mesos després de la infecció, és perllongada en el temps i comú en totes les àrees geogràfiques estudiades (Bofill-Mas et al., 2000).

Taula 1. Principals virus humans (H), bovins (B), ovins (O), porcins (P) i aviars (A) relacionats amb malalties de transmissió hídrica.

Familia	Nom (gènere)	Mida, genoma		Malalties associades	Algunes dades epidemiològiques
Adenoviridae	Adenovirus *(Mastadenovirus,* excepte els porcins que pertanyen als Atadenovirus i els aviars que pertanyen als *Aviadenovirus)*	70-90nm, dsDNA	H	Conjuntivitis, gastroenteritis, malalties respiratòries	L'AdV 4, el 7 i el 14 han originat brots esporàdics deguts a l'amuntegament de gent (Huang et al., 2013; O'Shea and Wilson, 2013)
			B	Conjuntivitis, pneumoenteritis, malalties entèriques agudes i mortals	Detectat al 13% (4/23) de femtes, 90% (13/18) d'orines (Sibley et al., 2011)
			O	Gastroenteritis, lesions pulmonars i hepàtiques	46% mortalitat als cabrits (Olson et al., 2004; Wolf et al., 2010)
			P	Gastroenteritis lleus i abortaments	
			A	Enteritis i hemorràgies intestinals	Reducció en la producció d'ous (Cha et al., 2013; Koo et al., 2013)
*Anelloviridae**	Torqueteno virus (Anellovirus, abans dins els Circovirus)	30-32nm, ssDNA	H B O P A	Assimptomàtics, Associats amb hepatitis, malalties pulmonars, miopaties i lupus	Infecten al 90% dels humans i es detecten al 24% (5/21) de les femtes (Jiménez-Melsió et al., 2013) i un 11% (2/16) de l'aigua residual (Vecchia et al., 2012) Presenta infecció persistent (Okamoto, 2009)
Astroviridae	Astrovirus *(Mamastrovirus)*	28-30nm, ssRNA	H	Gastroenteritis	Afecta sobretot nens <2 anys (Chhabra et al., 2013), prevalença superior en països de baixa renta (Papaventsis et al., 2008).
Caliciviridae	Norovirus *(Norovirus)*	27-38nm, ssRNA	H	Gastroenteritis i vòmits explosius	Causa més comuna de gastroenteritis aguda als Estats Units, fins a 800 morts i 71,000 hospitalitzacions l'any (CDC, 2013a)
	Saporovirus *(Saporovirus)*		H	Gastroenteritis (vòmits, diarrea, febre, dolor abdominal)	Principalemt pel consum de marisc contaminat, identificat en el 30% dels brots al Japó (Iritani et al., 2014)
Circoviridae	Circovirus *(Circovirus porcins i aviars i alguns aviars Gyrovirus)*		P	Gastroenteritis, pèrdua de pes, ictericia i engrandiment dels ganglis limfàtics	Arreu del món, importants pèrdues econòmiques a Europa (OIE, 2014)
			A	Anèmia als pollastres (CAV), Malformació i necrosi de les plomes dels lloros (BFDV)	A Europa es vacunen els pollastres, tot i així els tractaments són immunosupresors i afavoreixen patogens oportunistes. La malaltia en lloros acostuma a ser fatal (OIE, 2014)
Hepeviridae	HEV *(Hepevirus)*	25-30nm, ssRNA	H	Hepatitis aguda	20 milions de casos anuals relacionats amb l'aigua i en algún cas, amb la carn de porc crua (WHO, 2013b)
Parvoviridae	Bocavirus *(Bocavirus)*	28-26nm, ssDNA	H B P	Malalties respiratòries i gastroenteritis	Solen coinfectar, del 21-80% de les vegades, amb rotavirus, norovirus i adenovirus (Albuquerque et al., 2007). A més a més, presenten una certa estacionalitat d'excreció en humans (Pozo et al., 2007; Weissbrich et al., 2006)
	Parvovirus *(Parvovirus)*		A	Malalties entèriques a pollastres, gall d'indis i algunes espècies d'ànecs	46-78% de prevalencia en femta de pollastres i galls d'indi sans d'Estats units, Espanya, Croàcia, Hongria i Polònia (Bidin et al., 2011; Murgia et al., 2012; Palade et al., 2011; Tarasiuk et al., 2012)
Picornaviridae	Enterovirus Coxsakievirus Echovirus *(Enterovirus)*	24-30nm, ssRNA	H	Poden causar febre aftosa, gastroenteritis, meningitis, polio, encefalitis i conjuntivitis	10-15 milions d'infeccions l'any només als Estats Units (CDC, 2013b)
			B	Gastroenteritis, avortament (BEV)	Causa grans pèrdues econòmiques i és molt prevalent. Detectats en femtes (78%) i en aigües superficials (Jiménez-Clavero et al., 2005)
			O	Gastroenteritis	Espècie recombinant entre Enterovirus porcí i boví (Boros et al., 2012)
			P	Pneumonia, encefalitis diarrea, dermatitis, malalties vesiculars (PEV)	Grans pèrdues econòmiques (Jiménez-Clavero et al., 2003)

	HAV *(Hepatovirus)*		H	Hepatitis	Verdures fresques i fruites contaminades causen 17.000 casos als Estats units (CDC, 2012), en països de baixa renta 90% dels nens han estat infectats abans dels 10 anys (WHO, 2013c)
	Aichivirus *(Kobuvirus)*		H	Gastroenteritis	25% dels nens entre 7 mesos i 9 anys i aproximadament el 85% d'adults d'entre 30 i 39 anys ténen anticossos contra AiV (Li et al., 2009)
	Klassevirus *(Kobuvirus)*		H	Gastroenteritis	Identificats en el 8.8% de diarrees idiopàtiques en nens als Estats Units, Austràlia i Barcelona (Greninger et al., 2009; Holtz et al., 2009)
	Tescovirus *(Teschovirus)*		P	Encefalomielitis porcina (PTV)	Arreu del món, importants pèrdues econòmiques a Europa (OIE, 2014)
Polyomaviridae	Poliomavirus *(Polyomavirus)*	50-60nm, dsDNA	H	En immunosuprimits causen leucoencefalopatia multifocal progressiva (JCPyV), nefropaties, cistitis renal (BKPyV), càncer de pell (MCPyV). Recentment, HPyV10 i HPyV12 també s'han relacionat amb gastroenteritis	JCPyV l'excreta el 30% de la població amb concentracions de fins a 10^9 partícules víriques per litre. Aquest percetatge augmenta amb l'edat (Liu et al., 2006) Concentracions elevades de JPyV a l'aigua residual, en totes les zones geogràfiques analitzades (Bofill-Mas et al., 2000a)
Reoviridae	Rotavirus *(Rotavirus)*	70-75nm dsRNA	H	Gastroenteritis i en països de baixa renda a vegades mort per deshidratació	Freqüent en nens Abans de la introducció de la vacuna, produïa 527.000 morts l'any (CDC, 2011)

* Virus presents a l'aigua, possible transmissió hídrica.

A continuació s'exposen amb més detall, les característiques d'aquelles famílies objecte d'interès durant el desenvolupament de la tesi.

1.1.3.1. Els adenovirus

Als anys 50, els adenovirus es van aïllar i identificar com a agents etiològics d'enfermetats respiratòries agudes, infeccions gastrointestinals, urinàries i oculars (Hilleman and Werner, 1954; Rowe et al., 1953). Nou anys més tard es va poder demostrar que causaven tumors quan s'inoculaven a rosegadors (Trentin et al., 1962). Els adenovirus infecten a un gran nombre d'espècies: mamífers, rèptils, ocells, amfibis i peixos. De fet, es classifiquen en 4 generes: els Aviadenovirus, que infecten sobretot als ocells, els Atadenovirus, que infecten rèptils, aus i mamífers (inclosos el bestiar boví i oví), els Mastadenovirus, propis dels humans però també s'hi inclouen els adenovirus porcins i els Siadenovirus, que tenen les granotes i els galls d'indi per hostes (ICTV, 2014). Tots presenten una gran variabilitat genètica que dins del mateix gènere oscil·la entre el 48 i el 99% (Lee et al., 1988).

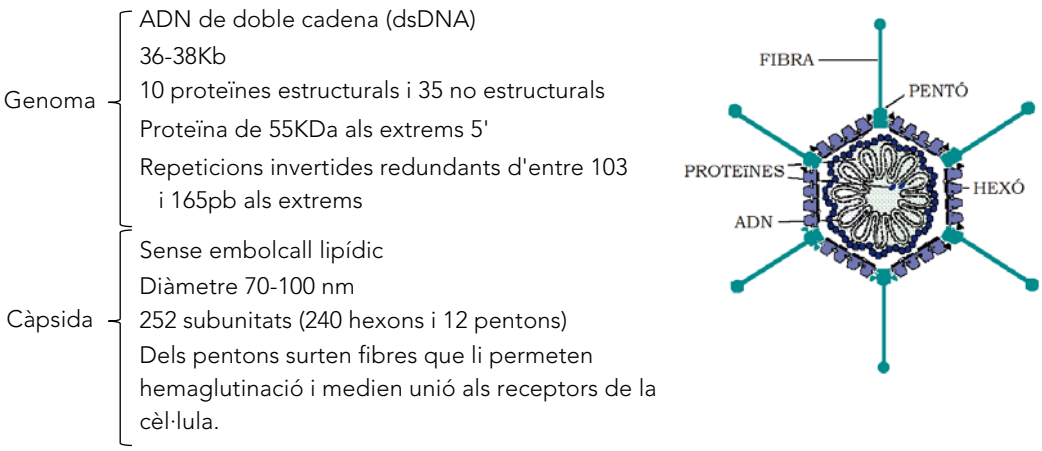

Figura 4. Esquema de la estructura dels adenovirus (ViralZone, 2014; Wold and Horwitz, 2007).

- Els adenovirus humans

Existeixen fins a 57 serotips humans (gènere Mastadenovirus), que s'agrupen en 7 espècies, de la A a la G (figura 5), segons la capacitat d'aglutinar eritròcits humans o la capacitat de generar tumors en rosegadors (Jones et al., 2007; Robinson et al., 2011; Walsh et al., 2011).

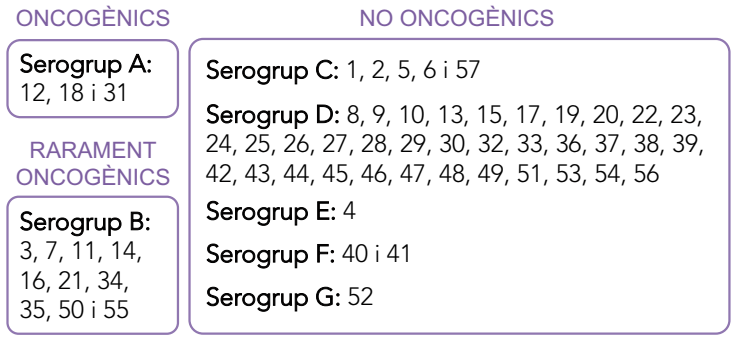

Figura 5. Serogrups i serotips d'adenovirus humans agrupats segons la capacitat de generar tumors a rosegadors.

Les malalties provocades per AdV són ben diverses. La infecció més recurrent causada per els HAdV és la gastroenteritis (espècies F i G). Alguns virus de les espècies B, C i E produeixen malalties respiratòries agudes i causen brots en llocs amb molta gent.

S'han descrit per exemple HAdV14 en brots als reclutes militars americans (Tate et al., 2009). També poden ser la causa de querato-conjuntivitis (espècies B, D i E), pneumònia o febre faringo-conjuntiva (espècies B i D), síndrome pertussis-like (HAdV5, espècie D), cistitis hemorràgica aguda (HAdV-B), meningoencefalitis (espècies A, B i D) i fins i tot hepatitis en nens trasplantats de fetge (HAdV-C) (Wold and Horwitz, 2007). Tot i que el 50% de les infeccions siguin asimptomàtiques, el 60% de les que manifesten símptomes es produeixen en nens menors de 4 anys (Sharp and Wadell, 1995). Els serotips HAdV40 i HAdV41 es transmeten via fecal-oral i són els que es troben més freqüentment a l'ambient.

- Els adenovirus animals

Els 6 serotips d'adenovirus porcins provoquen malalties lleus. La majoria de porquets presenten símptomes de gastroenteritis com la diarrea, l'anorèxia o la deshidratació, mentre que les truges poden arribar a patir malalties respiratòries multifactorials o avort (Buller and Moxley, 1988). Els adenovirus bovins (BAdV), per la seva banda poden causar conjuntivitis, febre, pneumònia i en alguns casos fins i tot la mort. S'han identificat fins a 10 serotips, agrupats en 2 subgrups (Bartha, 1969). Mentre el subgrup I, (BAdV1, 3 i 10) pertany sobretot al gènere Mastadenovirus, el subgrup II (BAdV4, 6, 7 i 8) forma part dels Atadenovirus, on també s'hi inclouen els adenovirus ovins (OAV).

1.1.3.2. Els poliomavirus

L'any 1953, Ludwig Gross, va descobrir el poliomavirus murí (MPyV) (Gross, 1953). Mentre estudiava el model de transmissió de la leucèmia, va l'inocular cries de ratolí amb homogeneïtzat del fetge i la melsa de ratolins malalts i va observar com s'induïen "poli-oma", es a dir, múltiples-tumors. També indirectament, es va descriure anys més tard el poliomavirus de simi 40 (SV40) (Sweet and Hilleman, 1960). En aquell moment, la vacuna contra la polio es produïa en cèl·lules de ronyó de les mones Rhesus. Tant les vacunes

orals com les injectables estaven fetes amb el virus de la polio inactivat. La inactivació amb formaldehid, però, no eliminava els altres virus que hi poguessin haver al ronyó dels micos, i el SV40, es podria haver transmès fàcilment dels primats als humans. L'any 1971, es van identificar els primers poliomavirus humans: JC del cervell d'un pacient amb Leucoencefalopatia Multifocal Progressiva, una malaltia degenerativa causada per la desmielinització de les neurones, i BK a l'orina d'un pacient trasplantat de ronyó (Gardner et al., 1971; Padgett et al., 1971).

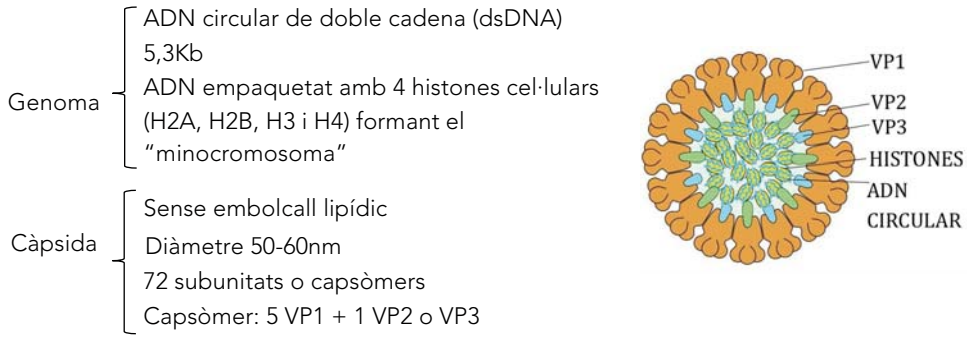

Figura 6. Esquema de la estructura dels poliomavirus (Wold and Horwitz, 2007).

Els poliomavirus infecten a dos grups de vertebrats: ocells i mamífers. Recentment, s'han classificat en tres gèneres: els Wukipolyomavirus i els Orthopolyomavirus, que infecten per exemple a humans i primats, com els orangutans, babuïns i ximpanzés, als cavalls, porcs i vaques, també als dofins o als lleons marins i a rosegadors com els esquirols, ratolins i hàmsters, i els Avipolyomavirus, que infecten ocells (Johne et al., 2011).

- Els poliomavirus humans

Durant gairebé 40 anys, només es coneixien els poliomavirus humans JCPyV i BKPyV, mentre que avui en dia ja se'n coneixen 12 espècies. Tant el poliomavirus KI (Karolinska Institute) com el WU (Washington University) van ser descrits l'any 2007 en mostres de tracte respiratori (Allander et al., 2007; Gaynor et al., 2007). Tot i que no s'ha

descrit un teixit diana específic per aquests dos poliomavirus, s'han pogut detectar a la sang, al cervell, al pulmó i a les amígdales, i els estudis de seroprevalença indiquen que entre un 55 i 90% de la població en presenta anticossos (Dalianis and Hirsch, 2013; Feltkamp et al., 2013; Pinto and Dobson, 2014; Van Ghelue et al., 2012). Tot i que la presència d'aquests dos virus al tracte aerodigestiu suposa una transmissió respiratòria, també s'ha proposat la via fecal-oral; tant KI com WU s'han trobat mitjançant PCR específiques en mostres d'aigua residual i de riu, a Brasil i Barcelona (Bofill-Mas et al., 2010; Calgua et al., 2013a)

Un any després es van identificar seqüències d'un nou poliomavirus, que s'integrava al genoma de les cèl·lules Merkel de la pell (Feng et al., 2008). Aquest treball va incrementar l'interès cap als poliomavirus humans, ja que la seqüència d'aquest poliomavirus de les cèl·lules de Merkel (MCPyV) es trobava integrat al 80% (8/10) de les cèl·lules tumorals, enfront del 8% dels teixits control (5/59). A més a més la integració sembla que segueix un patró idèntic i per tant podria ser una de les causes de l'expansió (Spurgeon and Lambert, 2013) del carcinoma de les cèl·lules de Merkel, un càncer de pell poc freqüent però molt agressiu que afecta normalment a gent gran o a individus immuno-suprimits. Tot i això, fins a un 80% de la població adulta presenta anticossos contra MCPyV (Spurgeon and Lambert, 2013). Spurgeon i Lambert també han suggerit una via de transmissió per contacte. Mitjançant PCR específiques per al virus també s'ha descrit la presència de MC en mostres d'aigua residual i de riu de diferents zones geogràfiques (Bofill-Mas et al., 2010; Calgua et al., 2013a).

Fins a dia d'avui, s'han descobert fins a 7 nous poliomavirus humans: el HPyV6, el HPyV7 i el TSPyV o TSV relacionats amb la pell (Schowalter et al., 2010; van der Meijden et al., 2010), el HPyV9 trobat a la sang i l'orina d'un pacient trasplantat de ronyó (Scuda et al., 2011), el HPyV10 (també anomenat MWPyV o MXPyV) descobert a les berrugues de pacients amb la síndrome de Whim i també a les femtes (Buck et al., 2012; Siebrasse et al., 2012; Yu et al., 2012) i el HPyV11 (també anomenat STLPyV) i el HPyV12 a mostres de femta (Korup et al., 2013; Lim et al., 2013).

- Els poliomavirus animals

Fins avui, s'han descrit poliomavirus de canari, cavall, conill, corb, dofí, elefant, esquirol, hàmster, goril·la, lleó marí, oca, orangutan, pardal, periquito, rata, rat-penat, vaca i ximpanzé. Els poliomavirus més estudiats han sigut aquells que tenen per hoste a un animal domèstic o de granja. Els poliomavirus bovins, van ser descrits per primera vegada l'any 1990 (Schuurman et al., 1990). Infecten al bestiar boví i persisteixen als ronyons fent que la excreció es perllongui en el temps.

1.1.3.3. Els parvovirus

Els parvovirus, virus petits, infecten a vertebrats. Pertanyen a la família *Parvoviridae*, dividida en 2 subfamílies: *Parvovirinae* i *Densovirinae* que a la vegada inclouen 5 gèneres: Parvovirus, Erythrovirus i Dependovirus el primer subgrup i Ambdovirus i Bocavirus al segon. Hi ha diverses espècies descrites que infecten específicament a humans, vaques, porcs, aus, gossos, gats o ratolins.

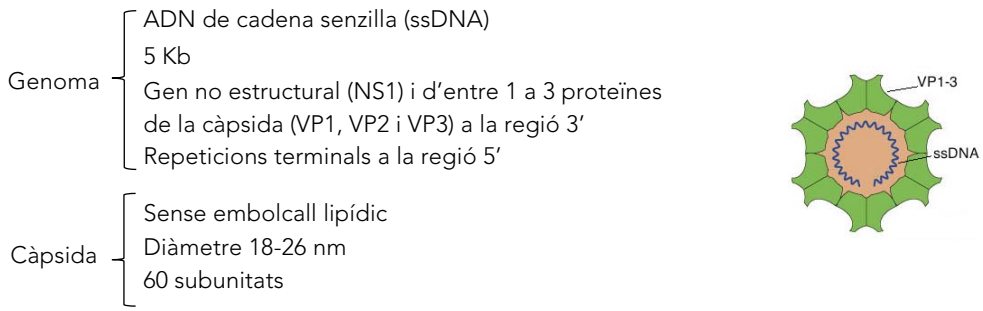

Figura 7. Estructura parvovirus (Day and Zsak, 2010; ViralZone, 2014).

- Els parvovirus humans

El genere Bocavirus (originàriament de BOvine i CAnine), s'ha relacionat durant anys amb malalties respiratòries i actualment també amb gastroenteritis. Els parvovirus

humans, o bocavirus humans (HBoV), van ser descrits per primera vegada en mostres d'aspirats de nasofaringe (Allander et al., 2005). També es troben en femta i en orina (Campe et al., 2008; Pozo et al., 2007; Tozer et al., 2009; Vicente et al, 2007) i s'han detectat en aigua (Hamza et al., 2009a). Es classifiquen en 4 espècies, HBoV1, 2, 3 i 4 (Kapoor et al., 2010) i normalment, coinfecten amb altres virus entèrics, NoV, RoV i AdV (Räsänen et al., 2010).

- Els parvovirus animals

Els parvovirus canins són els que causen un impacte més gran, ja que afecten als gossets d'entre 1 a 6 mesos amb vòmits i diarrees sanguinolentes i poden arribar a provocar la mort per deshidratació o septicèmia. Aquest parvovirus caní es transmet via respiratòria, i per aquest motiu la majoria de cadells es vacunen 45 dies després de néixer (Goddard and Leisewitz, 2010). Els parvovirus d'aus causen malalties entèriques. Els més estudiats infecten pollastres i galls d'indis (Ch/TyPV), ànecs muts (MDPV) i oques (GPV) i tal i com s'ha descrit a la taula 1, presenten una elevada prevalença en individus sans (Day and Zsak, 2013). La càpsida de dimensions reduïdes i sense embolcall lipídic els hi proporciona alta resistència a pH, solvents, i temperatures superiors als 50°C (Mani et al., 2007).

1.2. Control de la contaminació fecal de l'aigua

La detecció de patògens causants de malalties a l'aigua no és una tasca fàcil. Habitualment la seva concentració a l'ambient és baixa i poden no ser-hi presents durant el mostreig. En la majoria de casos, són necessàries línies cel·lulars específiques (pels virus) o medis de cultiu molt específics (per als bacteris, els protozous o els helmints). A més a més, per a tal de detectar el patogen i/o quantificar-lo, són claus: el percentatge de recuperació del mètode de concentració, els límits de detecció de la tècnica o simplement la seva capacitat infectiva després de passar pel medi (Field et al., 2007; Stoeckel and Harwood, 2007).

Els patògens a l'aigua poden ser molt i molt diversos. Detectar-los un per un per controlar la contaminació fecal de l'aigua, seria econòmicament inviable. Però com que identificar la contaminació fecal a l'aigua és essencial per una bona gestió tant de la contaminació com del seu l'origen, és vital poder disposar d'indicadors. Quan més exacte i fiable sigui l'indicador utilitzat, més dirigida i eficaç serà la solució.

1.2.1. Indicadors de contaminació fecal

Un indicador de contaminació fecal és un microorganisme o una substància que presenta un comportament molt semblant al del patogen però que disposa de mètodes d'identificació més senzills, ràpids o econòmics. L'indicador ideal haurà de tenir un origen exclusivament fecal i ser excretat per la majoria dels membres de la població a nivells aproximadament similars en tots els individus (Simpson et al., 2002). Haurà de ser-hi sempre que un patogen sigui present a l'ambient, preferiblement a concentracions iguals o més elevades, i no ser-hi quan el patogen sigui absent; no ha de proliferar a l'aigua; ha de ser estable en el temps, i més resistent que el patogen, enfront als desinfectants o les condicions ambientals (T°, pH, radiació solar..); ha de poder ser detectat, aïllat o cultivat al laboratori de manera fàcil, ràpida i econòmica amb tests

específics, i a ser possible, tenir alguna correlació amb el patogen o representar un risc directe per a la salut (figura 8).

Figura 8. Principals característiques de l'indicadors de contaminació fecal ideal.

Origen fecal, concentracions iguals o superiors al patogen, sense recreixement a l'ambient, estable en el temps i a l'ambient, metodològicament fàcil, ràpid i a ser possible econòmic d'analitzar, sensible, específic d'espècie i prevalent geogràficament.

Des de fa anys, s'han anat proposant diferents microorganismes com a candidats a indicadors de contaminació fecal (figura 5).

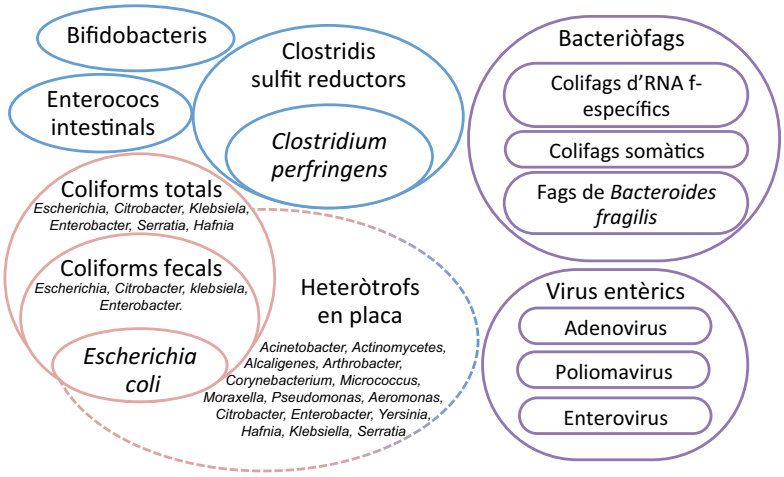

Figura 9. Microorganismes indicadors de la contaminació fecal a l'aigua (Dades extretes de Ashbolt et al., 2001, Bartram et al., 2003 i WHO, 2013).

Tradicionalment, els indicadors de contaminació fecal més emprats han estat els coliforms fecals / *Escherichia coli* i els enterococs intestinals (d'ara en endavant FIB) ja que els mamífers els excreten en grans quantitats. Des de fa anys però, s'ha vist que no tenen un origen exclusivament fecal, que poden créixer a l'ambient i que són més sensibles als tractaments de inactivació, i fins i tot a la radicació solar, que la resta d'indicadors virals o protozous (Hurst et al., 2002; Pote et al., 2009; Sinclair et al., 2009; Solo-Gabriele et al., 2000; Tree et al., 2003; Wéry et al., 2008). A més a més, normalment

no correlacionen ni amb la presència ni amb la concentració de virus i protozous i per tant no són adequats per identificar l'origen de la contaminació fecal (Field et al., 2003; Lipp et al., 2001; Pina et al., 1998a; Savichtcheva and Okabe, 2006; Scott et al., 2002; Simpson et al., 2002).

1.2.2. Regulacions i normatives per a la qualitat de l'aigua

Mitjançant criteris sanitaris, les normatives vigents han seleccionat alguns d'aquests indicadors per poder avaluar la qualitat de l'aigua. Per exemple, un aigua destinada al consum ha de estar lliure d'*E. coli* i d'enterococs per cada 100ml, mentre que si s'envasa també ha d'estar lliure de *Pseudomones aeruginosa* i espores de clostridis sulfit reductors (Directiva Europea 98/83/CE, 1998; RD 140/2003, 2003).

L'aparició de normatives per a l'ús de les aigües regenerades, un recurs no convencional, va suposar un gran repte social i sanitari. Aquesta aigua provinent de la depuració d'aigües residuals, pot ajudar a fer front a la creixent demanda d'aigua o al dèficit de precipitacions. L'article 12 de la Directiva Europea sobre tractament de les aigües residuals, ja assenyalava la importància del reaprofitament d'aquest recurs (Directiva 91/271/CE, 1991), però no va ser fins a la transposició, l'any 2007, quan es van incloure els criteris específics per a cadascun dels usos. Així doncs, es requereix absència d' *E. coli* a les aigües de reg de jardins particulars, per a la recàrrega de sanitaris, per a les torres de refrigeració o per a la recàrrega d'aqüífers, mentre que no es fixa un límit, per exemple, per al reg de boscos.

Per últim, les aigües de bany continentals es consideren de bona qualitat quan no sobrepassen els 400 enterococs intestinals ni les 1000 *E. coli* per 100ml, mentre que per les aigües costeres o de transició aquests llindars baixen fins a 200 i 500 CFU/100ml respectivament (Directiva 2006/7/CE, 2006; RD 1341/2007, 2007). Aquesta normativa, va ser la primera en apuntar la importància de poder determinar l'origen de la contaminació fecal. Discriminar l'origen assegura una millor gestió i control, i alhora permet establir responsabilitats.

1.3. Traçabilitat de la contaminació fecal a l'ambient

1.3.1. Eines de Microbial Source Tracking (MST)

L'any 2002, als Estats Units, es començaven a publicar articles de revisió dels mètodes existents per identificar cadascun d'aquests indicadors de contaminació a l'aigua (Scott et al., 2002; Simpson et al., 2002). Tres anys més tard, apareixia la guia de referència de l'Agència de Protecció Ambiental americana (EPA, 2005), i des de llavors, s'han anat publicant diverses revisions de comparacions de mètodes (Boehm et al., 2013; Harwood et al., 2013; Roslev and As, 2011; Savichtcheva and Okabe, 2006; Stoeckel and Harwood, 2007; Wu et al., 2011). "Fecal Source Tracking" (FST) i en particular el Microbial Source Tracking (MST), són els termes utilitzats per parlar de les diferents metodologies i estratègies per traçar la contaminació fecal humana o animal a l'ambient. Cada una d'aquestes tècniques tenen com a objectiu la identificació de l'origen de la contaminació i poden ser ben diverses: tècniques de cultiu tradicionals, tècniques moleculars, mètodes químics, etc. Els mètodes de FST poden classificar-se segons precisin o no de tècniques de cultiu (Figura 10). Quan el mètode es basa en aïllar, tipificar i posteriorment classificar el microorganisme segons perfils o bases de dades, es diu que és un mètode depenent de base de dades. Dins d'aquest grup, s'han utilitzat tècniques d'anàlisi de caràcters fenotípics, com la resistència a antibiòtics (Carroll et al., 2005) o la utilització per part de la població microbiana d'una o altra font de carboni (Evenson and Strevett, 2006; Moussa and Massengale, 2008).

L'amplificació múltiple (Carlos et al., 2012; Mohapatra et al., 2007), l'anàlisi de polimorfismes en la longitud de fragments amplificats per PCR (Venieri et al., 2004), l'anàlisi de polimorfismes en la longitud de fragments de restricció (Burtscher et al., 2006; Leung et al., 2004), el ribotipatge (Moore et al., 2005; Scott et al., 2003) i les electroforesi de camp pulsant o de gradient desnaturalitzant (D'Elia et al., 2007; Furukawa and Suzuki, 2013) són altres mètodes d'anàlisi de caràcters genotípics. Tots aquests mètodes són laboriosos, necessiten de llibreries o bases de dades prou grans i adequades per a cada

context i de bones eines estadístiques per tal de interpretar els resultats i evitar falsos positius (Stoeckel and Harwood, 2007). A més a més no són facilment generalitzables.

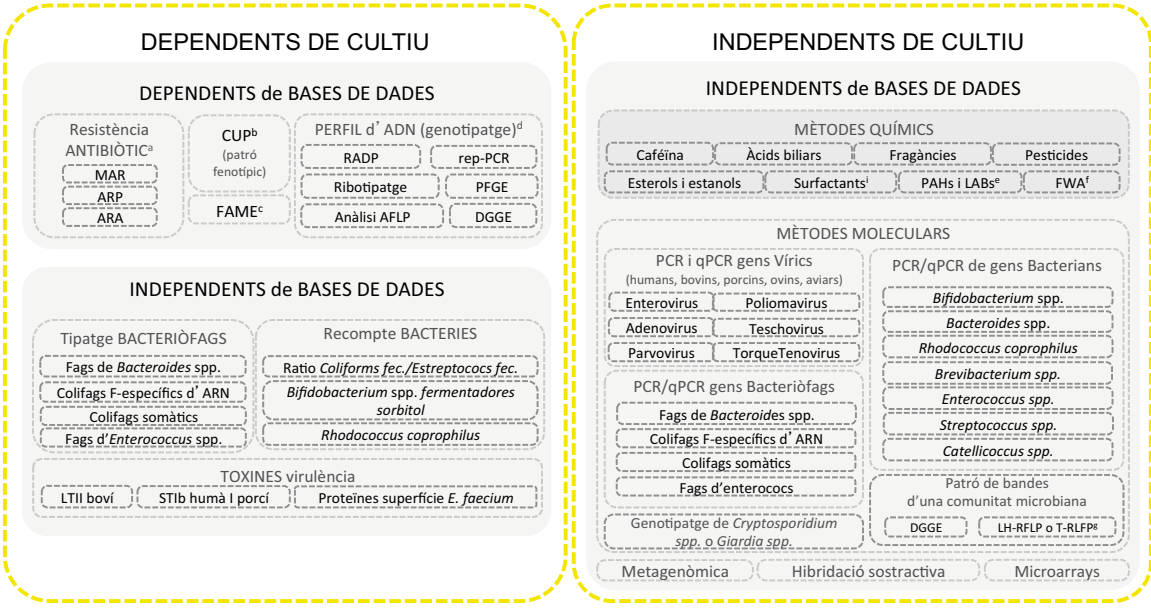

Figura 10. Esquema resum dels mètodes FST disponibles per a traçar la contaminació fecal a l'aigua.

A: MAR, resistència múltiple a antibiòtic, ARP, patró de resistència a antibiòtic, ARA, anàlisi de resistència a antibiòtic; B: CUP, perfil d'ús de la font de carboni; C: FAME, Metil ester d'àcids grassos; D: RAPD, polimorfismes d'amplificació d'ADN aleatoris; Rep-PCR, PCR extragènica palindròmica i repetitiva amb iniciadors REP, BOX o ERIC; PFGE, electroforesi de camp polsant; AFLP, polimorfismes a la llargada dels fragments amplificats; DGGE, electroforesi en gel amb gradient de desnaturalització ; E: PAHs, hidrocarburs aromàtics policíclics i LAB, alquil-benzens lineals; F: FWA, agents blanquejants i fluorescents; G: LH-RLFP, heterogeneïtat en la llargada dels polimorfismes de longitud dels fragments de restricció, T-RLFP, polimorfismes terminals de longitud dels fragments de restricció.

El recompte de bacteris va ser una de les primeres aproximacions de MST. El quocient entre coliformes fecals (CF) i enterococs fecals (EF) és una aproximació per discernir les fonts de contaminació d'origen humà quan CF/EF >4, i les d'origen animal quan CF/EF>0.7 (Edwin et al., 1969). *Rhodococcus coprophilus*, un actinomicet present a les femptes d'herbívors i absent en femptes humanes, també s'ha proposat com indicador (Mara and Oragui, 1981). De manera similar, la detecció de *Bifidobacterium* spp. mitjançant tècniques de cultiu permet diferenciar les espècies específiques

d'humans, fermentadores de sorbitol, de les provinents del tracte digestiu d'animals (Mara and Oragui, 1983; Resnick and Levin, 1981). Anys més tard, es van començar a proposar els fags de *Bacteroides* spp., molt abundants a l'aparell digestiu humà i més resistents a l'ambient aquàtic que el bacteri hoste. La detecció del fag que infecta *Bacteroides fragilis* HSP40, va ser un dels primers fags utilitzats per MST (Tartera and Jofre, 1987). De la mateixa manera, el tipatge de fags F-específics d'ARN, colifags somàtics o fags d'*Enterococcus* spp, que infecten respectivament i de manera específica, als coliforms mitjançant del pili o la paret cel·lular i a diferents espècies d'enterococs, s'han proposat i utilitzat per identificar l'orígen i traçar la contaminació fecal (Bonilla et al., 2010; Havelaar et al., 1986; Santiago-Rodriguez et al., 2012). Tradicionalment els mètodes per la detecció d'aquests fags s'han basat en el recompte de plaques en cultius del bateri hoste, però també existeixen tests ràpids d'aglutinació de làtex o assajos moleculars per distingir, després d'enriquir la mostra, els quatre genogrups de colifags d'ARN F-específics, que permetran identificar contaminació humana quan els genogrups majoritaris siguin el I i el IV, o contaminació animal quan ho siguin els genogrups II i III (Schaper and Jofre, 2000; Schaper et al., 2002). L'amplificació de gens específics després d'un cultiu previ de l'indicador també s'ha utilitzat per a detectar toxines específiques de gens de virulència de l'*E. coli* enterotoxigènica (LTII i STIb) com de gens específics de proteïnes de superfície d'*Enterococcus faecium* (Chern et al., 2004; Khatib et al., 2002; Scott et al., 2005).

Els mètodes químics (Figura 10), tampoc són depenents de bases de dades, es basen en la detecció de molecules o indicadors químics relacionats amb un determinat orígen fecal. Per exemple, la matèria fecal té una composició d'esterols que depèn molt de la dieta, de l'individu però sobretot de la microbiota anaeròbica present al sistema digestiu de la espècie en concret. Els humans, reduïm el colesterol a coprostenol, mentre els herbívors ho fan a 24-etil coprostanol i 24-etil epicoprostanol (Leeming et al., 1996). Les diferents molècules de la via del colesterol es detecten mitjançant cromatografia de gasos d'alta resolució i espectrometría de gasos i a partir d'aquí, es poden calcular els ratios utilitzats en estudis de FST (Derrien et al., 2012; Furtula et al., 2012; Jaffrezic et al., 2011). Mitjançant tècniques com la cromatografia líquida d'alta resolució,

l'espectrometria de masses o la fluorimetria, es poden detectar molècules com la cafeïna, consumida exclusivament pels humans o les fragàncies i els agents blanquejants, utilitzats com a marcadors de contaminació humana (Peeler et al., 2006; Sidhu et al., 2013). Els àcids biliars (Bull et al., 2002), els pesticides (Gourmelon et al., 2010), els surfactants (Eganhouse et al., 1983) i els hidrocarburs aromàtics policíclics (Eganhouse and Sherblom, 2001) també s'han utilitzat per discernir l'origen humà o no-humà de la contaminació.

Els mètodes moleculars identifiquen un marcador genètic específic directament a la mostra d'aigua o a partir d'una extracció d'ADN o ARN, és a dir, sense necessitat d'un cultiu cel·lular previ. En els últims 10 anys, s'han desenvolupat mètodes moleculars ben diversos per traçar l'origen de la contaminació fecal. La identificació del patró de bandes d'una comunitat bacteriana (Cao et al., 2013), el genotipatge de *Cryptosporidium* spp. o *Giardia* spp. (Almeida et al., 2010; Feng et al., 2011), la detecció de seqüències mitocondrials específiques de l'hoste (Caldwell et al., 2007; Kapoor et al., 2013; Martellini et al., 2005), els microarrays i la hibridació sostractiva (Hamilton et al., 2006; Zheng et al., 2009) o la seqüenciació massiva per metagenòmica (Ibekwe et al., 2013; Mokili et al., 2012), han resultat en una millor caracterització i sensibilitat per detectar fonts de contaminació diverses. Tot i que els estudis de metagenòmica s'estan fent molt populars, analitzar acuradament les dades i posteriorment entendre la informació que ens dóna el genoma és el punt més crític a l'hora d'interpretar els resultats. Per això, els mètodes moleculars més utilitzats fins ara es basen en assajos d'amplificació específica mitjançant la reacció en cadena de la polimerasa (PCR) per identificar les fonts de contaminació directament a partir d'extraccions d'ADN de mostres d'aigua. A la taula 2, es resumeixen els marcadors moleculars disponibles per a fer MST.

Taula 2. Llistat de marcadors de PCR disponibles per a traçar la contaminació fecal humana, bovina, porcina i d'altres animals a l'aigua.

ORÍGEN		Tipus de marcador*	Nom del marcador	Referència d'estudis que utilitzen el mètode molecular com a eina de MST
HUMÀ	M	ADN mitocondrial humà	ND5, mtCytb	Caldwell et al., 2007; Schill and Mathes, 2008
	B	*Bifidobacterium* spp.	16sRNA	Gourmelon et al., 2010
		Bacteroides spp.	HF183, HF134, HuBac, BacHum-UCD, Bac-H, HumanBac1, BuniF2, BFragF1, BvulgF1, PcopriF1, BsteriF1, BthetaF2, HumM2, HumM3, Bthetaα, B.fragilis gyr B.	Ahmed et al., 2009b; Haugland et al., 2010; Jenkins et al., 2009; Kildare et al., 2007; Layton et al., 2006; Lee et al., 2010; Okabe and Shimazu, 2007; Ravaliya et al., 2013; Reischer et al., 2007; Seurinck et al., 2005; Shanks et al., 2010b, 2009; Yampara-Iquise et al., 2008
		Rhodococcus coprophilus	16sRNA gene	Savill et al., 2001; Wicki et al., 2012
		Brevibacterium sp.	nifH gene, Mnif	Johnston et al., 2010; Weidhaas et al., 2010
		Enterococcus	esp-1	Ahmed et al., 2008
	F	Colifags F-específics d'ARN	rep	Kirs and Smith, 2007; Wolf et al., 2010
		Fags *bacteroides fragilis*	FRNAPH	Ogorzaly et al., 2009
	V	Adenovirus	HAdV	Pina et al., 1998; Puig et al., 1994
		Poliomavirus	JCPyV, BKPyV	Bofill-Mas et al., 2000a
		Enterovirus	Human EV	Noble et al., 2003
		Tobamovirus	PMMoV	Rosario et al., 2009
BOVÍ	M	ADN mitocondrial boví	Bomito1 and 11, NADH, Bomito-2, cytochrome b, cyt-B Cow	Ballesté et al., 2010; Martellini et al., 2005
	B	*Streptococcus* spp.	16sRNA	Devriese et al., 1999; Lawson et al., 2005
		Bifidobacterium spp.	Bif-CW-Probe	Gómez-Doñate et al., 2012
		Bacteroides spp.	CI125f, Bac708r, 1408r, Cow-Bac1, Cow-Bac2, Cow-Bac3, YCF, BoBac, Bac2, Bac3, CowM2, CowM3,	Jeong et al., 2010; Layton et al., 2006; Reischer et al., 2006; Shanks et al., 2010a, 2006; Stricker et al., 2008
	V	Enterovirus	BEV-2	Jiménez-Clavero et al., 2005; Ley et al., 2002
		Adenovirus	BAdV	Wong and Xagoraraki, 2011, Maluquer de Motes et al., 2004
		Poliomavirus	BPyV	Hundesa et al., 2010, 2006; Wong and Xagoraraki, 2011
PORCÍ	M	ADN mitocondrial porcí	Pomito3 and 11, NADH, Pomito-4, cytochrome b, cyt-B Pig	Ballesté et al., 2010; Caldwell and Levine, 2009; Caldwell et al., 2007; Kortbaoui et al., 2009; Martellini et al., 2005; Schill and Mathes, 2008
	B	*Bacteroides* spp.	PF163, PigBac1, PigBac2, Pig-1-Bac, Pig-2-Bac	Dick et al., 2005; Fremaux et al., 2009; Lamendella et al., 2009; Muniesa et al., 2009
		Bifidobacterium spp.	Bif-PG-Probe, *Bif. Thermacidophilum spp. porcinum*	Gómez-Doñate et al., 2012; Marti et al., 2009
	V	Adenovirus	PAdV	Hundesa et al., 2009; Maluquer de Motes et al., 2004
		Teschovirus	PTV	Jiménez-Clavero et al., 2003; Ley et al., 2002
OVÍ	M	ADN mitocondrial oví	Ovmito-1,2 i 11, cytochrome b, cyt-B-Sheep	Kortbaoui et al., 2009; Martellini et al., 2005; Schill and Mathes, 2008
	B	*Brevibacterium* sp.	LA35	Ryu et al., 2012
AVIAR	M	ADN mitocondrial aviar	NADH i cytochrome b (oca), Ckmito-1 (pollastre)	Caldwell et al., 2007; Kortbaoui et al., 2009; Schill and Mathes, 2008
	B	*Streptococcus* spp.	gull3 (gavina)	Ryu et al., 2012
		Catellicoccus spp.	gull4 (gavina)	Ryu et al., 2012
		Bifidobacterium spp.	Bif-PL-Probe (aus de corral)	Gómez-Doñate et al., 2012
		Bacteroides spp.	CGOF1-Bac i CGOF2-Bac (oca)	Fremaux et al., 2010
	V	Parvovirus	Ch/TyPV (pollastre i gall d'indi)	Carratalà et al., 2012
CANÍ	M	ADN mitocondrial gos	NADH i cytochrome b	Caldwell et al., 2007; Schill and Mathes, 2008

	B	*Bacteroides* spp.	DF475F/Bac708R i BacCan	Dick et al., 2005
FELÍ	M	ADN mitocondrial de gat	NADH (gat)	Caldwell et al., 2007
CÈRVID	M	ADN mitocondrial cèrvol	cytochrome b	Schill and Mathes, 2008
	B	*Bacteroides* spp.	EF447F/990R (ant)	Dick et al., 2005
EQUÍ	M	ADN mitocondrial de cavall	cytochrome b	Dick et al., 2005; Schill and Mathes, 2008
	B	*Bacteroides* spp.	HoF597 i HorseBact	Dick et al., 2005; Silkie and Nelson, 2009
RUMIANT	B	*Bacteroides* spp.	CF128, CF193, BacCow, BacR, Rum-2-Bac	Ahmed et al., 2008; Ballesté et al., 2010; Bernhard and Field, 2000; Kildare et al., 2007; Reischer et al., 2006; Shanks et al., 2010a

* M: ADN mitocondrial, B: bacterià, F: bacteriòfag i V: virus

1.3.2. Els virus com a eines de MST

En general, els virus entèrics són específics d'hoste i no repliquen a l'ambient, però representen un problema de salut pública per diversos motius: la seva dosi infectiva és molt baixa i només calen de 10 a 1000 partícules víriques per infectar (Kundu, et al, 2013; Thebault et al., 2013), l'excreció post infecció es perllonga durant setmanes (figura 11) i són molt estables a l'ambient i als diversos tractaments de desinfecció degut a la manca d'embolcall lipídic (Carratalà et al., 2013; Rzezutka and Cook, 2004).

La infecció per la majoria de virus transmesos per l'aigua consta de tres etapes: un període d'incubació (el temps entre la infecció i l'aparició de símptomes), un període simptomàtic i un període post-infecció, durant el qual ja no es pateixen els símptomes però s'excreten les noves partícules víriques. Els poliomavirus i alguns grups d'adenovirus poden fer infeccions persistents, des de que infecten fins que esdevenen realment patogènics es mantenen latents. En individus immuno-suprimits poden ser la causa de malalties força greus, però molts cops generen infeccions asimptomàtiques i posteriorment s'excreten durant anys en orina (figura 11).

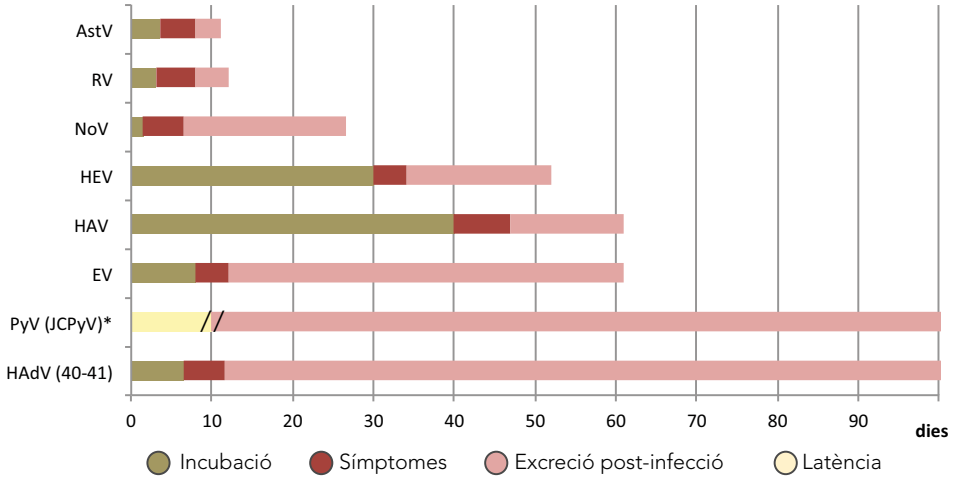

Figura 11. Patrons d'infecció dels principals virus humans presents a l'ambient (dades extretes de Collier and Oxford, 2008; Lee et al., 2013)**.**

Els HAdV igual que els HPyV, produeixen infeccions durant la infància i persisteixen durant mesos i fins i tot anys, fent que la excreció sigui perllongada en el temps. S'excreten via la femta (HAdV) o l'orina (JCPyV) dels individus afectats, són molt resistents a l'ambient i als tractaments de desinfecció, s'han detectat en gran diversitat de mostres durant tot l'any en totes les àrees geogràfiques estudiades (Bofill-Mas et al., 2006, 2000; Hundesa et al., 2006; Pina et al., 1998). Per això i pel fet de que n'hi hagi d'específics d'humans i d'animals, són bons marcadors de MST. Els parvovirus, i en concret els aviars, que s'excreten a la femta de l'aviram de diferents països i s'han descrit entre els virus més resistents a la temperatura, també s'han incorporat recentment a les eines virals per fer MST (Carratalà et al., 2012; Sauerbrei and Wutzler, 2009).

L'estabilitat d'aquests virus humans o animals a l'aigua depèn de diferents factors com per exemple la llum, la temperatura i la humitat, factors naturals d'inactivació, i també dels desinfectants o tractaments químics que es facin a l'aigua. La llum solar, i en concret la franja de l'espectre de llum ultra-violada, causa danys directes als genomes vírics, inhibint la replicació (Wigginton et al., 2012). També les espècies reactives d'oxigen (ROS), produïdes de manera indirecta per l'acció de la llum, generen ambients redox que degraden la càpsida viral (Kohn and Nelson, 2007). Sauerbrei i Wutsler van estudiar la resistència a la temperatura. Mentre els adenovirus resistien fins a 85°C 2h, els poliomavirus ho feien fins a 95°C (Sauerbrei and Wutzler, 2009). Amb calor humida però, el temps i la temperatura d'inactivació son una mica més curts i només calen uns 65°C durant 30 minuts per inactivar poliomavirus (Brodsky et al., 1959). El tractament amb clor, és el més habitual per a desinfectar aigua residual o potabilitzar aigua de beguda. El clor oxida la càpsida del virus i els àcids nucleics (Page et al., 2010). Els HAdV i els JCPyV, resisteixen després de 30 minuts amb clor (2.5mg/L) i la seva concentració baixa fins a 2.7$\log_{10}$ i fins a 1.5$\log_{10}$ respectivament (de Abreu Côrrea et al., 2012).

1.3.3. Adenovirus, poliomavirus i parvovirus, eines de MST.

A Catalunya, la meitat del territori s'utilitza per a conrear o per a les explotacions ramaderes. Amb 16 milions de porcs, 2 milions d'ovelles, mig milió de vaques, 157 milions de pollastres i gallines i 6 milions de galls d'indi i uns 372.000 ànecs, és una de les zones amb més producció animal d'Europa (Generalitat de Catalunya, 2010). Només Bèlgica i Dinamarca superen dins d'Europa la producció Catalana de porcs (Eurostat, 2009). Els residus generats per les explotacions de bestiar porcí a Catalunya, amb més de 16.800 sacrificis l'any 2010, representen un gran repte per la gestió del medi (Generalitat de Catalunya, 2010). Part dels purins s'utilitzen com a adob (Generalitat de Catalunya, 1996) i el volum a aplicar permès dependrà de la concentració (KgN/m^3), del tipus de cultiu, la topografia o la distància a zones vulnerables (Decret 136/2009, 2009; Decret 220/2001, 2001). L'excedent, en qualsevol cas, s'haurà de tractar per evitar l'excés de nitrogen a les aigües superficials i als aqüífers.

Països amb gran tradició ramadera, com Anglaterra, Austràlia o Nova Zelanda, disposen de extenses normatives o guies per reduir l'impacte mediambiental. El tipus d'animal, la mida dels ramats, la gestió de l'explotació o fins i tot el tancament del bestiar als estables durant l'hivern, influencia directament la qualitat de l'aigua. Tot i així, s'ha estimat que el rentat superficial, dels camps de pastura, amb la pluja és l'origen principal de contaminació d'aigües superficials amb microorganismes d'origen fecal animal (Kay et al., 2008; Monaghan et al., 2008).

Per tal de poder fer una gestió sostenible de la terra i de l'aigua, primer cal identificar específicament la font o les fonts de contaminació. Per això, en el grup de recerca on s'ha desenvolupat la tesi, s'han dissenyat eines moleculars de MST basades en la utilització de PCR específiques per a detectar i quantificar contaminació fecal porcina, bovina i d'aviram a l'ambient (Carratalà et al., 2012; Hundesa et al., 2010, 2009; Maluquer de Motes et al., 2004). A les taules 3 i 4, es revisen tots els estudis publicats que utilitzen

qPCRs específiques per detectar contaminació humana mitjançant adenovirus i poliomavirus humans.

Cadascun d'aquests resultats, provenen d'assajos amb diferents matrius d'aigua: aigua residual, aigua de mar, aigua de riu o llac i aigua de beguda o subterrània. Aquestes són taules actualitzades a partir de les publicades a l'article de revisió realitzat durant la tesi (Bofill-Mas et al., 2013) (Annex).

Taula 3. Revisió d'estudis on es quantifiquen adenovirus humans en mostres ambientals.

Autors	qPCR	País	Matrius analitzades				Principals resultats
			Sub	Riu	Mar	Res	% (mostres positives/mostres analitzades), concentració mitjana
Choi and Jiang., 2005	3	Estats Units					16% (18/114), 1E+02-1E+04 GC/l
Haramoto et al., 2005	3	Japó					45% (29/64)
He and Jiang., 2005	3	Estats Units					Mar: 80% (4/5) 4.3E+04 GC/l; Residual: 100% (11/11), 8.1E+06GC/l
Albinana-Gimenez et al., 2006	1	Catalunya					Riu: 93% (13/14), 4E+02 GC/l; Residual: 100% (10/10),1.4E+07 GC/l
Bofill-Mas et al., 2006	1	Catalunya					100% (6/6), 3,87E+07 GC/l t90 and t99 values of 60.9 and 132.3 days
Albinana-Gimenez et al., 2009b	1	Catalunya					Riu: 90% (102/114), 1E+01-1E+04 GC/l
Dong et al., 2009	2 i 3	Nova Zelanda					Residual: 100% (10/10), 1.87E+03-4.6E+06 GC/l; Mar i riu:83% (5/6), 1. 70E+01-1.19E+03 GC/l
Hamza et al., 2009	2	Alemanya					97.5% (40/41), 1.0E+07-1.7E+08 GC/l
Ogorzaly et al., 2009	1	France					100% (42/42), 1.0E+04G/l
Bofill-Mas et al., 2010	1	Catalunya					Residual: 100% (7/7), 3.2E+06 GC/l; Riu: 100% (7/7), 3E+03 GC/l
Haramoto et al., 2010	3	Japó					61.1% (11/18), 3.6E+03-1.38E+05 GC/l
Jurzic et al., 2010	2	Alemanya					96.3% (193/190), 2.9E+03-7.3E+05GC/l
Rigotto et al., 2010	1	Brasil					64.2% (54/84) 1E+07 GC/l de mitja en totes les matrius, 88.8% d'aquests infecciosos
Schlindwein et al., 2010	1	Brasil					100% (12/12); 5E+04-1.3E+07 GC/l
Aslan et al., 2011	4	Estats Units					36% (31/85), 1E+02-1E+04 GC/l
Calgua et al., 2011	1	Catalunya					100% (7/7), 1E+01-1E+06 GC/l
Guerrero-Latorre et al., 2011	1	Txad					6% (1/16), detecció d'HAdV4
Hamza et al., 2011	2	Alemanya					Riu: 97,3% (108/111), 3E+03 GC/l; Residual: 100% (12/12), 1.0E+07 GC/l
Kokkinos et al., 2011	1	Grècia					45.8% (22/48). Serotips detectats: 8, 40 i 41
Wyn-Jones et al., 2011	1	Europa					Riu/llac: 41% (381/928), 27% (132/482)
Garcia et al., 2012	1	Brasil					100%, 1E+07 GC/l
Fongaro et al., 2012	1	Brasil					96% (46/48)
Rodriguez-Manzano et al., 2012	1	Espanya					100% (44/44), 8.32E+03 GC/l. Reducció 1.03log (89%) post- secundari i 0.13log10 (11%) post-UV
Bradbury et al., 2013	4	Estats Units					Residual: 70% (19/25), Subterrània 69% (18/26)
Fongaro et al., 2013	1	Brasil					69% (25/36) HAdV, 1E+05 GC/l dels quals 52.7% (19/36) infectius
fumian et al., 2013	1	Brasil					100% (24/24), 1E+05-1E+06 GC/l
hewitt et al., 2013	1	Nova Zelanda					61% HAdV(13/21) i 85% HAdV-F(18/21); Riu: 1E+02 GC/l; Mar: 2,8E+02 GC/l; Residual: 1E+05 GC/l
Katukiza et al., 2013	1	Uganda					70% (29/41), 2.65E+04 GC/l
sidhu et al., 2013a	2	Australia					91% (21/23), Aigua de pluja contaminada amb sobreiximent de clavagueram
sidhu et al., 2013b	2	Australia					100% (30/30), 1E+05-1E+06 GC/l
Ye et al., 2012	2	Xina					100% (24/24), 2.28E+04 GC/l
hata et al., 2014	4	Japó					48% (14/29), 5.2E+03 GC/l escorrentia aigua de pluja
Lee et al., 2014	2	Estats Units					40% (26/65), 2,2E+04 GC/l

(1) **Hernroth 2002** [AdF:C(AT)TACATGCACATC(GT)C(CG)GG; AdR:C(AG)CGGGC(GA)AA(CT)TGCACCAG; AdP1 per HAdV-ACDEF:(6FAM)-CCGGGCTCAGGTACTCCGAGGCGTCCT-(TAMRA) i AdP2 per HAdV-B:(6FAM)-CCGGACTCAGGTACTCCGAAGCATCCT-(TAMRA)]; (2)**Heim 2003** [AQ1:GCCACGGTGGGGTTTCTAAACTT; AQ2:GCCCCAGTGGTCTTACATGCACATC; AP:(FAM)-TGCACCAGACCCGGGCTCAGGTACTCCGA-(TAMRA); (3)**He&Jiang 2005** [AD2:CCCTGGTA(GT)CC(GA)AT(GA)TTGTA; AD3:GACTC(CT)TC(AT)GT(GC)AG(CT)GGCC; ADP:(FAM)-AACCAGTC(CT)TTGGTCATGTT(GA)CATTG-(TAMRA)]; (4)**Cromeans 2005** [JHKXF:GGACGCCTCGGAGTACCTGAG; JHKXR:CGCTG(CT)GACC(CT)GTCTGTGG; JHKXP:(FAM)-CACCGATACGTACTTCAGCCTG-(TAMRA)]

En general, els estudis de MST realitzats amb HAdV (Taula 3) demostren que l'indicador es troba en tot tipus d'aigües i a totes les zones geogràfiques analitzades, mentre que els estudis de MST amb JCPyV (Taula 4), que també s'han detectat a tot arreu on s'ha analitzat, demostren que l'indicador és molt específic. Per aquest motiu, s'han proposat els dos virus com a marcadors de contaminació fecal humana a l'ambient.

Taula 4. Revisió d'estudis on es quantifiquen poliomavirus humans en mostres ambientals.

Autors	qPCR	País	Matrius analitzades				Principals resultats
			Sub	Riu	Mar	Res	% (mostres positives/mostres analitzades), concentració mitjana
Albinana-Gimenez et al., 2006	1	Catalunya					Riu: 100% (9/9), 2.7E+04 GC/l; Residual: 100% (5/5), 2.6E+06 GC/l
Bofill-Mas et al., 2006	1	Catalunya					Residual: 100% (6/6), 6.11E+06 GC/l
Albinana-Gimenez et al., 2009a	1	Catalunya					Riu: 75% (18/27), 7.4E+02-1.3E+03 GC/l
McQuaig et al., 2009	2	Estats Units					100% (41/41), 3.07E+07 GC/l
Hamza et al., 2009	3	Alemanya					97% (40/41), 2.4E+04 GC/l
Harwood et al., 2009	2	France					100% (17/17), 1E+07 GC/l
Abdelzaher et al., 2010	2	Estats Units					50% (40/40)
Ahmed et al., 2010	2	Australia					Riu: 25% (5/20), 1E+03GC/l; Residual: 100% (40/40), 1E+05GC/l
Bofill-Mas et al., 2010	1	Catalunya					Residual: 85% (6/7), 1E+05 GC/l; Riu: 100% (7/7), 1E+03 GC/l
Fumian et al., 2010	1	Brasil					96% (6/7), 1.2E+06 GC/l
Haramoto et al., 2010	1	Japó					11% (2/18), 7.91E+02-3.42E+03 GC/l
Jurzic et al., 2010	3	Alemanya					68% (129/188), 1.4E+04 GC/l
Gibson et al., 2011	2	Ghana					Subterrània: 0% (0/4); Residual: 0% (0/9)
Hamza et al., 2011	3	Alemanya					Riu: (73/111) 1E+03 GC/l; Residual: 100% (12/12) 1E+08 GC/l
Hellein et al., 2011	2	Estats Units					Mar: 3% (1/32); Residual: 100% (15/15)
Kokkinos et al., 2011	2	Grècia					68% (33/48)
Chase et al., 2012	2	Estats Units					1% (2/35), 1E+04 GC/l
Fongaro et al., 2012	2	Brasil					21% (10/48)
Gordon et al., 2013	2	Estats Units					12% (90/752)
Rodriguez-Manzano et al., 2012	1	Espanya					100% (6/6), 5.44E+05 GC/l
McQuaig et al., 2012	2	Estats Units					20% (26/130), 5E+02-3,55E+05 GC/l
Staley et al., 2012	2	Estats Units					61% (15/25)
Calgua et al., 2013b	1	Catalunya i Brasil					Riu: 100% (12/12), 9.38E+03; Residual: 100% (12/12), 1,05E4 GC/l
Hewitt et al., 2013	2	Nova Zelanda					Riu: 51% (18/35), 1E+03GC/l; Mar: 67% (7/15), 1E+03GC/l; Residual: (36/37), 1,5E+06GC/l
Fumian et al., 2013	1	Brasil					100% (24/24), 1E+05-1E+06 GC/l
Sidhu et al., 2013a	2	Australia					52% (12/23), Aigua de pluja contaminada amb sobreiximent de clavagueram

(1) **Pal 2006** [JE3F:ATGTTTGCCAGTGATGATGAAAA; JE3R:GGAAAGTCTTTAGGGTCTTCTACCTTT: JE3P:(FAM)-AGGATCCCAACACTCTACCCCACCTAAAAAGA-(BHQ-1)]; (2) **McQuaig 2009** [SM2:AGTCTTTAGGGTCTTCTACCTTT; P6: GGTGCCAACCTATGGAACAG; KGJ3:(FAM)-TCATCACTGGCAAACAT-(MGBNFQ)]; (3) **Biel 2000 i Hamza 2009**; [PV-TMFOR: TCTATTACTAAACACAGCTTGACT; PV-BACK: GGTGCCAACCTATGGAACAG; PV-Prove: (FAM)-TGGAAAGTCTTTAGGGTCTTCTACCTT-(BHQ-1)]

Es detecten concentracions força elevades de poliomavirus tant en aigua residual urbana ($\approx 10^6$-10^7CG/L), com en aigües de riu ($\approx 10^3$-10^4 CG/L) i en aigües de mar ($\approx 10^2$-10^3 CG/L). Cal tenir en compte que, durant brots de gastroenteritis o durant certes èpoques de l'any, es poden detectar concentracions més elevades d'altres virus, com els NoV o els RoV (Miagostovich et al., 2008).

No fa tants anys que s'utilitzen poliomavirus, adenovirus i parvovirus animals pels estudis de MST i per tant els resultats són limitats (Taula 5). Tot i que no produeixen malalties greus, tant els PAdV, els BPyV com els Ch/TyPV, són excretats per bona part del bestiar porcí, boví i l'aviram respectivament (Bidin et al., 2011; Hundesa et al., 2006; Maluquer de Motes et al., 2004; Palade et al., 2011; Tarasiuk et al., 2012). Les eines més utilitzades per MST de contaminació porcina i bovina estan basades en qPCR específiques per a quantificar adenovirus porcins (PAdV) o poliomavirus bovins (BPyV) (Hundesa et al., 2010, 2009). Recentment, també s'ha descrit una nova eina, igualment basada en un assaig de qPCR, per traçar la contaminació fecal provinent de l'aviram (Carratalà et al., 2012) (Annex).

Des de que es van descriure aquests tres assajos MST, al grup de recerca on s'ha desenvolupat la tesi, s'han analitzat mostres de femta i orina de l'hoste i d'altres animals domèstics i salvatges. També s'han testat mostres d'aigua residual d'escorxador (abans i després de tractaments de floculació per poder abocar al clavegueram), mostres de l'aigua d'escorrentia utilitzada per rentar les instal·lacions amb bestiar (tant granges com escorxadors), mostres d'aigua residual urbana on arriben les aigües residuals d'escorxador, i tot tipus de mostres ambientals on es dispersen les fonts de contaminació (riu, mar, pous, reg, beguda). Tots aquests resultats demostren la gran especificitat i efectivitat d'aquests assajos en mostres ambientals.

Taula 5. Revisió d'estudis on es quantifiquen poliomavirus, adenovirus i parvovirus animals en mostres ambientals. PAdV: porcins, BAdV i BPyV: bovins i Ch/TyPV: aviars.

Marcador	qPCR	Autors	Pais	Matrius analitzades				Principals resultats dels estudis
				Sub	Riu	RU	RE	% (mostres positives/mostres analitzades), concentració mitjana
PAdV	1	Hundesa et al., 2009	Catalunya					Riu: 50% (3/6), 3.06E+02 GC/l; RU: 0% (0/8); RE: 91% (10/11), 2.95E+03 GC/l
		Bofill-Mas et al., 2011	Catalunya					Pou clausurat per alta concentració de nitrats: 100% (4/4), 7.74E+02 GC/l
		Viancelli et al., 2012	Brasil					RE: 78% (44/56), 5.25E+05 GC/l
		Viancelli et al., 2013	Brasil					RE: 100% (88/88), 1E+05 GC/l, reducció 1 logaritme després de la planta de tractament d'una granja
	2	Wolf et al., 2010	Nova Zelanda					Riu:50% (3/6), 2.8E+01 GC/l
BAdV	3	Wong & Xagoraki 2010	Estats Units					RE: 75% (12/16), 1E+06-1E+07 GC/l
BPyV	4	Hundesa et al., 2010	Catalunya					Riu: 100% (6/6), 8.38E+02 GC/l; RU: 0% (0/8); RE: 100% (8/8), 1.56E+06 GC/l
	5	Wong & Xagoraki, 2011	Estats Units					RE: 100% (16/16), 1E+05 GC/l
Ch/TyPV	6	Carratalà et al., 2012	Catalunya					RU: 50% (1/2), 2.65E+05GC/l; RE: 81% (2/3), 4.63E+08 GC/l

Sub: aigua subterrània, RU: Residual Urbana, RE: Residual Escorxador

(1) Hundesa 2009 [Q-PAdV-F:AACGGCCGCTACTGCAAG; Q-PAdV-R: AGCAGCAGGCTCTTGAGG; Q-PAdV-P: (FAM)-CACATCCAGGTGCCGC-(BHQ1)]

(2) Wolf 2010 dos Viral Tool Boxes [per a PAdV-3, VTB1-PoAdV3f: CCTCAACAACCTCATTGATACC; VTB1-PoAdV3r: CTTGCAGTAGCGGCCGT; VTB1-PoAdV3probe: (FAM)-TACGGCCTGCGCTACCGCTCCCA-(BHQ1); i per a PAdV-5, VTB2-PoAdV5f: CGGCGCTTCCATCAGAATAG; VTB2-PoAdV5r: ACGWCAGGTAATCAATAAAGGAC; VTB2-PoAdV5probe: (Quasar 705)-CTACTCTGGAGGCCATGTTGAGAAACG-(BHQ-2)]

(3) Wong & Xagoraki 2010 [BAV4-8-F: CRAGGGAATAYYTGTCTGAAAATC; BAV4-8-R: AAGGATCTCTAAATTTYTCTCCAAGA; BAV4-8-P: (FAM)-TTCATCWCTGCCACWCAAAGCTTTTTT-(BHQ-1)]

(4) Hundesa 2010 [QB-F1-1: CTAGATCCTACCCTCAAGGGAAT; QB-R1-1: TTACTTGGATCTGGACACCAAC; QB-P1-2: (FAM)-GACAAAGATGGTGTGTATCCTGTTGA-(BHQ-1)]

(5) Wong & Xagoraki 2011 [BPV-F:TGGCTTTCTGACTCAGCCAAA; BPV-R:TCTCTTCCTGAGAGTCACAGACATG; BPV-P:(FAM)-ACCAACAGCAATTTAGAGGCCTTCCCAG-(BHQ-1)]

(6) Carratalà 2012 [Q-PaV-F: AGTCCACGAGATTGGCAACA; Q-PaV-R: GCAGGTTAAAGATTTTCACG; Q-PaV-Pr: (FAM)-AATTATTCGAGATGGCGCCCACG-(BHQ-1)]

Fins a la realització d'aquesta tesi no es disposava de cap marcador viral de bestiar oví. Alguns estudis han proposat la detecció d'adenovirus ovins (OAdV), norovirus (NoV GII), fags F-específics d'ARN o *Cryptosporidium* com a marcadors potencials (Chalmers et al., 2002; Wolf et al., 2010). Cap d'aquests mètodes s'ha acabat aplicant degut a les inespecificitats existents amb el bestiar boví. El desenvolupament de tècniques de detecció i quantificació de la contaminació fecal ovina a l'ambient, ha estat un dels objectius principals d'aquesta tesi.

1.4. Mètodes per concentrar i detectar els virus a l'ambient

Els virus a l'ambient es troben a concentracions baixes i es distribueixen de manera heterogènia. Per poder-los detectar és imprescindible recollir una mostra prou significativa i per això, sovint cal agafar grans volums d'aigua i concentrar les mostres per després poder aplicar una o altra eina de MST.

1.4.1. Protocols de concentració

S'han descrit multitud de mètodes per recuperar els virus de motres d'aigua (Taula 6). La majoria es basen en processos d'adsorció-elució que juguen amb el punt isoelèctric dels virus i els adsorbeixen a membranes, filtres, o matrius com la llana de vidre (Albinana-Gimenez et al., 2009; Lambertini et al., 2008; Wyn-jones et al., 2010).

Els virus a l'ambient tenen una càrrega neta negativa (Michen and Graule, 2010) i s'uneixen directament a les membranes i filtres electropositius. Els filtres i membranes electronegatives necessitaran sovint d'un pre-acondicionament, ja que la mostra o el suport, que pot estar fet de diversos materials (nitrocel·lulosa, cel·lulosa, fibra de vidre) o combinacions de materials (per exemple, microfibres de vidre amb nano fibres d'alumini (NanoCeram®), han de poder absorbir les partícules virals.

Els mètodes més utilitzats són els VIRADEL (VIRal ADsorption and ELution), que utilitzen un primer pas d'adsorció i elució amb un tampó proteic alcalí, fet a partir d'extracte de carn o llet descremada, i un segon pas de floculació-centrifugació en condicions àcides. Existeixen també altres mètodes basats en la mida i densitat dels virus, que permeten per ultracentrifugació o ultrafiltració concentrar-los des de grans volums d'aigua a volums més petits. Quants més passos o tecnologia es necessiti, més lent, costós i poc estandarditzable serà un mètode. Mitjançant el mètode de floculació amb llet descremada dissenyat al nostre laboratori, es poden concentrar en un sol pas, volums d'entre 50ml a 10l, segons es tracti d'aigua residual o d'aigua de riu, del subsòl o

del mar, i concentrar-los en volums de 1 a 10ml respectivament (Calgua et al., 2013a, 2013b, 2008).

Taula 6. Mètodes disponibles per a concentrar de virus (ordenats relativament de dalt a baix, de més a menys cost per mostra).

Mètode (temps)	Matriu	V_i	Procediment	V_f	Recuperació	Ref.
Ultracentrifugació (4h)	RE,R	42ml	**PAS1**- Ultracentrifugació (110.000 *xg*, 1 h). Pellet en tampó glicina (pH 9.5) 30 min i centrifugació 12.000*xg*, 20 min **PAS2**- Ultracentrifugació (110.000*xg* 1h)	100µl	70% EV	(Pina et al., 1998a)
Ultrafiltració (4h)	M,R,S,B	10l	Pre-tractament filtre amb BSA **PAS1**- Filtració i elució (NaOH) **PAS2**- Neutralització i Ultrafiltració (40 KDa)	300µl	3-6% HAdV 13-33% JCPyV	(Albinana-Gimenez et al., 2009)
Liofilització (24-36h)	R	50ml	Congelar a -80ºC la mostra Liofilitzar durant 24-36 h	500µl	*	(Calgua et al., 2013b)
Mètodes VIRADEL (5h) - Filtres/membranes	R,S B	100l 1000l	Pas previ d'acidificació si filtres/membranes electronegatives o llana de vidre **PAS1**- Filtració i elució amb tampó glicina + extracte carn o llet descremada (pH 9.5) **PAS2**- Floculació (pH 3.5) i centrifugació (7000*xg*, 20 min)	5ml	21-91% EV	(Albinana-Gimenez et al, 2006; Lambertini et al., 2008; Mocé-Llivina et al., 2005; Sobsey and Glass, 1980)
- Llana de vidre	R S,B	10l 50l			8-28% HAdV 16-45% NoV	
Floculació amb llet descremada (10h)	RE M,R,S,B	50ml 10l	Aigua residual (50 ml): Rentat previ 30 min amb gel i tampó glicina (pH 9.5) i centrifugació (12.000*xg*, 20 min) per eliminar matèria orgànica **PAS1**- Floculació (8 h agitació + 8 h sedimentació a pH 3.5 i conductivitat >1.5 mS/cm²) i centrifugació pellet (8000*xg*, 30 min)	1ml 10ml	30–95% HAdV 55–90% JCPyV 45–90% NoV	(Bofill-Mas et al., 2013; Calgua et al., 2013a, 2013b, 2008)

RE: aigua residual, M: aigua de mar, R: aigua de riu, S: aigua subterrània, B: aigua de beguda. * Amb el mètode de liofilització teòricament recuperem tota la mostra, però també concentrem al 100% els inhibidors.

1.4.2. Protocols de detecció i quantificació

Un cop concentrats, és important saber quin percentatge dels virus són infecciosos. Per assegurar que un virus és infectiu, caldria infectar l'hoste, però a nivell pràctic, es considera infecciós quan aconsegueix penetrar la membrana cel·lular i expressar com a mínim un gen viral o replicar el genoma. Existeixen diverses línies cel·lulars disponibles a partir de cèl·lules de ronyó, com les 293, les MA104 o les Vero, cèl·lules epitelials, com les Hep-2, i fins i tot cèl·lules de carcinoma de pulmó com les A549. Així, només quan es disposa d'un cultiu específic, es poden determinar les

concentracions de virus infecciosos a partir del recompte de clapes de lisi (PFU), el càlcul de la dosi necessària per infectar un cultiu al 50% (TCID50) o a través de la observació de proteïnes del virus amb anticossos marcats amb immunofluorescència (IFA) dins de les cèl·lules hoste (FFU). En qualsevol cas, si el virus no fa efecte citopàtic o no es disposa d'una línia cel·lular específica, com en el cas dels NoV, no podrem avaluar el percentatge de partícules víriques infeccioses després de la concentració (Hamza et al., 2011). La ICC-PCR (Integrated Cell Culture-PCR) permet també estimar la quantitat de virus viables quantificant la mostra abans i després d'inocular-la en un cultiu (Balkin and Margolin, 2010; Ballester et al., 2005; Fongaro et al., 2013; Reynolds et al., 2001; Rigotto et al., 2010; Shieh et al., 2008).

Els mètodes de detecció més robustos, sensibles, específics i pràctics, pel que fa a la rapidesa o al preu, són els basats en l'amplificació de seqüencies específiques del genoma dels virus i en concret la PCR quantitativa o a temps real (qPCR o RT-PCR) (Girones et al., 2010). L'extracció dels àcids nucleics vírics es pot fer amb diversos productes comercials. El més utilitzat per a mostres ambientals són les columnes de sílice (QIAgen®) optimitzades per fer tres passos: la lisi de la càpsida vírica, la purificació dels àcids nucleics retinguts a la membrana mitjançant una sèrie de rentats i posteriorment l'elució.

Per determinar amb mètodes moleculars quina part del ADN/ARN detectat correspon a virus infecciosos, s'han descrit diverses aproximacions (Hamza et al., 2011). Alguns autors utilitzen anticossos magnèticament marcats per recuperar virus entèrics específics de l'aigua i posteriorment poder detectar-los via qPCR (Abd El Galil et al., 2004; Haramoto et al., 2010; Yang et al., 2011). Altres, utilitzen pre-tractaments amb colorants com l'EMA (*ethidium monoazide*) i el PMA (*propidium monoazide*) o amb proteases i nucleases, per tenyir o destruir respectivament, abans de l'extracció dels àcids nucleics, els genomes lliures o continguts en partícules virals danyades (Kim et al., 2011; Nuanualsuwan and Cliver, 2002; Parshionikar et al., 2010). Als estudis d'eficàcia d'un procés de desinfecció, enzims com la proteïnasa K i la ribonucleasa (RNasa) degraden les càpsides i genomes dels virus afectats per tractaments de desinfecció com la llum UV, el

clor o la temperatura. Les partícules víriques no danyades, que possiblement encara són infectives, podran ser detectades per qPCR específiques (de Abreu Côrrea et al., 2012; Nuanualsuwan and Cliver, 2002; Pecson et al., 2009). També s'han proposat l'ús de mARN RT-PCR, retro-transcripció de l'ARN missatger seguida d'una qPCR, per estudiar l'efecte de la llum UV sobre l'estabilitat dels HAdV (Ko et al., 2005). Per últim, alguns autors proposen l'ús de qPCR llargues, o específiques de segments molt conservats del genoma viral, per detectar possibles danys que es puguin associar a la infectivitat de la partícula vírica. Li i els seus col·laboradors van poder enumerar posicions concretes del genoma de l'HAV on normalment es produïen la major part de danys després d'un tractament amb clor (Li et al., 2004). El mateix es va descriure per als NoV amb tractaments amb temperatura i ultraviolada (Wolf et al., 2009). Per evitar falsos negatius i donat que petits danys al genoma també poden afectar la viabilitat dels virus, s'han proposat qPCR múltiples de petites regions (Pecson et al., 2011; Shin and Sobsey, 2003).

Tradicionalment, la detecció d'adenovirus amb PCR s'ha fet amplificant un fragment que codifica els hexons de la càpsida dels adenovirus humans (Allard et al., 1990). Desde l'any 2002, la quantificació d'adenovirus a l'ambient, s'ha realitzat amb l'assaig de Hernroth (Hernroth et al., 2002). Paral·lelament, Heim i el seu equip van dissenyar un altre assaig quantitatiu (Heim et al., 2003). La quantificació dels poliomavirus humans JC i BK es duu a terme principalment amb els protocols de Pal o els de McQuaig, dissenyats a les regions del t-Ag i el T-Ag respectivament (McQuaig et al., 2009; Pal et al., 2006). Alguns autors també utilitzen l'assaig de PCR dissenyat per Biel i modificat a qPCR per Hamza (Biel et al., 2000; Hamza et al., 2009b). Degut a la seva rellevància clínica, s'han dissenyat diverses qPCR específiques per a detectar i quantificar MCPyV en mostres clíniques (Foulongne et al., 2010; Loyo et al., 2010; Wieland et al., 2011). En aquesta tesi s'ha descrit i utilitzat un nou assaig, dissenyat per amplificar i quantificar la regió de la proteïna de la càpsida VP1, de MCPyV en mostres ambientals.

Treballs com els d'Hundesa, el de Wolf, el de Wong o el de Carratalà han permès quantificar els adenovirus porcins i bovins, els poliomavirus bovins i els parvovirus de

pollastre i gall d'indi en mostres ambientals d'arreu del món (Carratalà et al., 2012; Hundesa et al., 2010, 2009; Wolf et al., 2010; Wong and Xagoraraki, 2010).

1.4.3. Punts crítics de l'anàlisi de virus a l'ambient

La quantificació amb PCR dels virus utilitzats per MST, requereix doncs de passos previs de concentració i extracció, i dependrà intrínsecament de la recuperació del mètode, dels límits de detecció, dels límits de quantificació de l'assaig i de la quantitat d'inhibidors que hi pugui haver a la mostra. Durant tot el procés d'anàlisi de virus a partir de mostres ambientals, hi ha passos crítics (Figura 12).

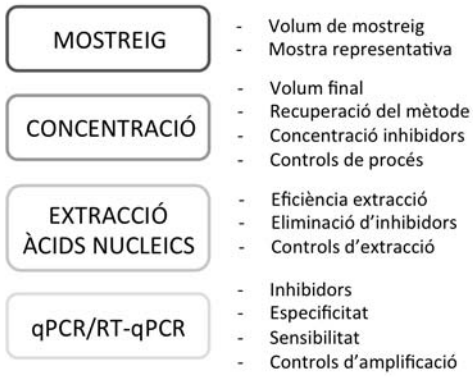

Figura 12. Esquema dels passos necessaris per analitzar virus a partir de mostres ambientals, i llista dels punts crítics a cada pas.

En qualsevol cas, quan analitzem virus a l'ambient, cal recollir una mostra prou representativa i homogènia durant el mostreig. Al procés de concentració cal introduir-hi controls, positius i negatius per així poder localitzar problemes o errors, per exemple, una mostra es pot dopar amb una quantitat coneguda de virus per al control positiu de procés i una mostra d'aigua destil·lada pot servir de control negatiu. A partir d'aquí podrem calcular la recuperació (R) del mètode de concentració. La R és la quantitat de microorganisme indicador que aconseguim concentrar en pocs ml a partir de volums superiors de la mostra inicial. Si tenim un estoc de partícules víriques podem dopar, amb un volum determinat, una mostra i després de concentrar-la calcular la R per aquell virus

en concret. Tal i com s'observa a la Taula 6, la majoria de mètodes presenten una gran variabilitat de recuperació, fent d'aquest pas un dels principals colls d'ampolla per a la detecció de virus a l'ambient. Per al procés d'extracció també s'haurà de disposar de controls positius i negatius, per exemple una suspensió vírica i el mateix tampó d'elució del kit d'extracció. Finalment, per a la detecció amb qPCR haurem d'introduïr controls positius (per exemple una extracció d'un cultiu víric, un gBlock® (DNA sintètic) o un plàsmid) i controls negatius (per exemple, preparar una reacció de qPCR amb un aigua comercial lliure de ARN i ADN al lloc de la mostra).

La sensibilitat i l'especificitat del mètode de detecció, variaran segons el numero de mostres analitzades, però seran claus a l'hora de triar un o altre assaig.

Taula 7. Recull de sensibilitats i especificitats d'alguns dels mètodes moleculars aplicats en diversos estudis de MST (Sargeant et al., 2012).

Marker Detection	Host Category	Sample Type	Sensitivity[1]	Specificity[2]
Bacteroides thetaiotaomicron, PCR	Human	Individual feces Wastewater	0.78, 0.92 (n=9,25) 1.00 (n=20)	0.98,0.76 (n=241,71) NR
Bacteroidales, PCR	Human	Blind samples Individual feces Wastewater	0.70-1.00 (n=10,14) 0.20-0.85 (n=7-25) 1.00 (n=41)	1.00,1.00 (n=6,7) 0.85-1.00 (n=46-73) 1.00 (n=75)
	Ruminants	Blind samples	1.00 (n=7,9)	0.89, 0.92(n=9,12)
	Cattle	Individual feces	1.00, 1.00 (n=19,19)	0.73, 0.70 (n=40,40)
	Ruminants	Individual feces	0.97,1.00 (n=31,20)	1.00,1.00 (n=20,28)
	Ruminants	Wastewater	1.00 (n=75)	0.93 (n=14)
	Dog	Blind samples	0.40 (n=15)	0.86 (n=7)
Bacteroides fragilis phage	Human/ nonhuman	Wastewater Fecal samples	1.00 (n=36) 0.13 (n=90)	0.90 (n=20) 1.00 (n=145)
Bifidobacterium adolescentis PCR	Human	Wastewater	1.00 (n=22)	0.84 (n=60)
Bifidobacterium adolescentis Colony hybridization	Human	Individual Feces Wastewater	0.92 (n=12) 0.67 (n=3)	1.00 (n=85) 1.00 (n=3)
Enterococcus faecium enrichment, PCR	Human	Septic system Wastewater	0.80 (n=10) 1.00 (n=55)	1.00 (n=59) 1.00 (n=43)
Escherichia coli toxin gene	Human Cattle	Blind samples Wastewater	0.75 (n=15) 0.87 (n=31)	0.33 (n=7) 1.00 (n=207)
Escherichia coli Enrichment, PCR	Swine Swine	Wastewater Individual feces	0.90 (n=31) NR	1.00 (n=217) 1.00 (n=224)
Adenovirus Nested, PCR	Human Human Swine Cattle	Wastewater Blind samples Individual feces Individual feces	0.92 (n=12) 0.50 (n=8) 0.74 (n=23) 0.75 (n=8)	1.00 (n=31) 1.00 (n=3) 1.00 (n=20) 1.00 (n=35)
Enterovirus RT-PCR	Human Cattle Cattle, deer	Blind samples Individual feces Individual feces	0.38 (n=8) 0.76 (n=95) 0.63 (n=145)	1.00 (n=4) 0.63 (n=54) 0.75 (n=4)

[1] Sensitivity is the ability to detect a source when it is present (calculated by dividing the number of true-positive results by the number of samples that should contain the target).

[2] Specificity is the ability to detect a source when it is not present (calculated by dividing the number of true-negative results by the number of samples that should not contain the target).

L'especificitat és la probabilitat de detectar veritables negatius (Altman & Bland, 1994). L'indicador de MST ideal s'excreta només per un grup o espècie reduint així els falsos negatius (EPA, 2005). Especificitat = negatius / (negatius + falsos positius) x 100. Marcadors com els poliomavirus són molt específics d'espècie (Hundesa et al., 2006; Harwood et al., 2011; McQuaig et al., 2012), mentre altres marcadors com per exemple la detecció de l'ARN 16s de *Bifidobacterium* spp. no ho son tant (Gourmelon et al., 2010). Per altra banda, la sensibilitat és la capacitat de detectar veritables positius (Altman & Bland, 1994). Representa doncs, el percentatge d'assajos positius necessaris per tal de determinar l'origen de la contaminació fecal. Sensibilitat = positius / (positius + falsos negatius) x 100.

El límit de detecció (LOD) del mètode és la mínima quantitat detectable del marcador en absència d'inhibidors (Santo Domingo et al., 2007). Teòricament els assajos de qPCR han de poder detectar una sola còpia d'un gen per reacció, però quan analitzem quantitats petites i conegudes d'un marcador per tal d'estimar aquest valor, veiem per cada 10 reaccions amb una sola còpia genòmica només en detectem un percentatge. Per això, es construeixen corbes de calibratge i es fan proves repetides amb quantitats conegudes que permeten calcular el LOD.

Límit de quantificació (LOQ) és un valor lleugerament superior al LOD, a partir del qual estem quantificant correctament la mostra. Per sota d'aquest valor, la corba patró de la qPCR no és lineal i per tant, tot i poder detectar el ADN, no el podem quantificar correctament. Per tant sempre serà LOQ > LOD. Inhibició enzimàtica de la PCR és un fet quan analitzem mostres ambientals. Tot i que existeixen Kits de qPCR específics per treballar amb mostres ambientals, es recomana analitzar com a mínim dues dilucions seriades de la mostra, així com introduir controls d'inhibició en els assajos realitzats (Hundesa et al., 2010).

Finalment, la representativitat o mostra representada (MR) també serà important a l'hora de avaluar resultats o triar un o altre mètode de concentració. La MR és la quantitat de mostra inicial que s'analitza realment. Amb el mètode de floculació amb llet

descremada per exemple, som capaços d'analitzar gran volum de la mostra inicial a la qPCR final (Taula 8) i així als 10µl carregats a un assaig qPCR per HAdV, estem quantificant les còpies genòmiques en 17.5mL de la mostra inicial.

Taula 8. Volums dels diferents passos d'anàlisi de virus mitjançant la floculació amb llet descremada. MR: Mostra representada i LOQ: Límit teòric de quantificació.

mostra	volum del concentrat viric	volum extracció d'àcids nucleics	qPCR de virus ADN o ARN	Mostra Representada	LOQ teòric (≈5CG/assaig)
10 L	10mL	140µl ➔ 80µl	ADN 10 µl ARN 5 µl	17,5mL 8,75mL	285 CG/L 571 CG/L
50mL	1mL	140µl ➔ 80µl	ADN 10 µl ARN 5 µl	0,87µL 0,44µL	5 CG/mL 11 CG/mL

2. OBJECTIUS

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Els objectius plantejats en aquesta tesi s'organitzen en dues parts. Una part inicial d'estudis de MST en zones geogràfiques i matrius d'aigües diverses, mitjançant els marcadors moleculars virals per traçar la contaminació fecal humana, bovina i porcina. En segon lloc, la descripció d'un nou marcador de la contaminació fecal d'origen oví com a eina de MST i la seva aplicació en mostres ambientals.

Els objectius específics han estat:

1) Estudiar la disseminació de virus i la contaminació fecal en conques fluvials situades en diferents zones climàtiques, mitjançant marcadors virals humans i animals com a eines de MST.
2) Determinar la repetibilitat i robustesa dels mètodes de concentració i detecció de virus marcadors de contaminació fecal a l'aigua i validar l'aplicació de les eines virals de MST en diferents àrees geogràfiques.
3) Desenvolupar noves eines per a la detecció i quantificació d'un poliomavirus associat a carcinoma de pell: Merkel Cell PyV.
4) Avaluar els possibles efectes del canvi climàtic i l'impacte de l'aigua residual i dispersió de virus segons paràmetres estacionals.
5) Desenvolupar noves eines moleculars per a la detecció i quantificació de la contaminació fecal ovina a l'ambient
6) Avaluar l'ús dels poliomavirus ovins com a indicadors de contaminació fecal ovina en diferents àrees geogràfiques.
7) Analitzar comparativament l'eficiència dels mètodes moleculars de MST basats en la detecció de seqüencies de gens virals, vers l'ús de tècniques moleculars clàssiques i l'ús de mètodes químics.

3. INFORMES dels ARTICLES

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3.1. Llista d'articles inclosos a la tesi

La present tesi està fonamentada en les següents publicacions detallades per ordre de presentació:

- Marta Rusiñol, Xavier Fernandez-Cassi, Natàlia Timoneda, Anna Carratalà, Josep Francesc Abril, Ayalkibet Hundesa, Carolina Silvera, Maria José Figueras, Emiliano Gelati, Xavier Rodó, Sílvia Bofill-Mas and Rosina Girones. Evaluation of viral dissemination in a Mediterranean river catchment and analisis in future climate change scenarios. Manuscrit sotmès al 2014.

- Marta Rusiñol, Xavier Fernandez-Cassi, Ayalkibet Hundesa, Carmen Vieira, Anita Kern, Irene Eriksson, Panos Ziros, David Kay, Marize Miagostovich, Marta Vargha, Annika Allard, Apostolos Vantarakis, Peter Wyn-Jones, Sílvia Bofill-Mas and Rosina Girones. 2014. Application of human and animal viral microbial source tracking tools in fresh and marine waters from five different geographical areas. Water Research, In press. doi: 10.1016/ j.watres.2014.04.013 (Accepted 6th april 2014).

- Marta Rusiñol, Anna Carratalà, Ayalkibet Hundesa, Alex Bach, Anita Kern, Apostolos Vantarakis, Rosina Girones, Sílvia Bofill-Mas. 2013 Description of a novel viral tool to identify and quantify ovine faecal pollution in the environament. Science of the Total Environment 458–460 (2013) 355–360.

- Marta Rusiñol, Elaine Moriarty, Beth Robson, Susan Lin, Margaret Mackenzie, Erin McGill, Rosina Girones, Sílvia Bofill-Mas and Brent Gilpin. Assessing the source of the faecal contamination in fresh water samples from Canterbury and Southland regions in New Zealand. Manuscrit sotmès al 2014.

3.2. Informe de coautoria

Marta Rusiñol, Xavier Fernandez-Cassi, Anna Carratalà, Natàlia Timoneda, Josep Francesc Abril, Ayalkibet Hundesa, Carolina Silvera, Maria José Figueras, Emiliano Gelati, Xavier Rodó, Sílvia Bofill-Mas and Rosina Girones. Evaluation of viral dissemination in a Mediterranean river catchment and analisis in future climate change scenarios. Manuscrit sotmès al 2014.

L'estudi és part del projecte europeu VIROCLIME en el que la doctoranda ha participat activament. La doctoranda ha desenvolupat el disseny experimental i realitzat el treball experimental juntament amb els altres coautors. Addicionalment, ha escrit el manuscrit sota la supervisió de les directores de tesi.

Marta Rusiñol, Xavier Fernandez-Cassi, Ayalkibet Hundesa, Carmen Vieira, Anita Kern, Irene Eriksson, Panos Ziros, David Kay, Marize Miagostovich, Marta Vargha, Annika Allard, Apostolos Vantarakis, Peter Wyn-Jones, Sílvia Bofill-Mas and Rosina Girones. 2014. Application of human and animal viral microbial source tracking tools in fresh and marine waters from five different geographical areas. Water Research In press. doi: 10.1016/ j.watres.2014.04.013 (Accepted 6th april 2014).

L'estudi és part del treball desenvolupat al projecte europeu VIROCLIME en el que la doctoranda ha participat activament. La doctoranda ha realitzat la major part del treball experimental relacionat amb l'estudi a l'area de Catalunya. Ha preparat els SOPs (Standard Operational Procedures), ha format als altres laboratoris participants en les tècniques utilitzades i ha participat en la coordinació del projecte. També ha contribuït en el disseny experimental i juntament amb el segon signant del treball, ha realitzat tots els mostrejos i experiments. Finalment, ha elaborat el manuscrit sota la supervisió de les codirectores de la tesi.

Marta Rusiñol, Anna Carratalà, Ayalkibet Hundesa, Alex Bach, Anita Kern, Apostolos Vantarakis, Rosina Girones, Sílvia Bofill-Mas. 2013. Description of a novel viral tool to identify and quantify ovine faecal pollution in the environment. Science of the Total Environment 458–460 (2013) 355–360.

La doctoranda ha realitzat la major part del treball experimental incloent mostrejos, desenvolupament experimental i anàlisi dels resultats. Finalment, ha redactat el manuscrit sota la supervisió de les codirectores de la tesi.

Marta Rusiñol, Elaine Moriarty, Beth Robson, Susan Lin, Margaret Mackenzie, Erin McGill, Rosina Girones, Sílvia Bofill-Mas and Brent Gilpin. Assessing the source of the faecal contamination in fresh water samples from Canterbury and Southland regions in New Zealand. Manuscrit sotmès al 2014.

La doctoranda ha realitzat la major part del treball experimental durant una estada de sis mesos a Christchurch, Nova Zelanda. Ha realitzat diversos mostrejos i ha dut a terme el desenvolupament dels experiments durant l'estada. Finalment, ha analitzat els resultats i redactat el manuscrit sota la supervisió del Dr. Brent Gilpin i les codirectores de la tesi.

Cap dels coautors dels articles ha utilitzat les dades descrites en aquestes publicacions per a l'elaboració de la seva tesi doctoral.

Dra. Rosina Girones Llop

Dra. Sílvia Bofill Mas

Barcelona, Maig del 2014

3.3. Informe sobre el factor d'impacte

Els treballs que formen part de la present tesi doctoral s'han publicat o sotmès a publicació en revistes científiques rellevants per a la línia d'investigació en la qual la doctoranda ha participat durant els 4 anys de tesi.

L'article "**Description of a novel viral tool to identify and quantify ovine faecal pollution in the environament**" s'ha publicat a la revista *Science of the Total Environment* l'any 2013. L'index d'impacte d'aquesta revista a l'any 2012 era de **3.258**. L'article "**Application of human and animal viral microbial source tracking tools in fresh and marine waters from five different geographical areas**" ha estat publicat a la revista *Water Research.* L'any 2012, l'índex d'impacte era de **4.655**.

Els articles "**Evaluation of viral dissemination in a Mediterranean river catchment and analisis in future climate change scenarios**" i "**Assessing the source of the faecal contamination in fresh water samples from Canterbury and Southland regions in New Zealand**" s'han sotmès a la revista *Water Research* i a Applied and Environmental Microbiology respectivament (els índex d'impacte l'any 2012 éren: **4.655 i 3.678**)

Signat,

Dra. Rosina Girones Llop

Dra. Sílvia Bofill Mas

Barcelona, Maig del 2014

ARTICLE I

Avaluació de la disseminació de virus en una conca Mediterrània i anàlisi en futurs escenaris de canvi climàtic

4. ARTICLES

4.1. Avaluació de la disseminació de virus en una conca Mediterrània i anàlisi en futurs escenaris de canvi climàtic.

"Evaluation of viral dissemination in a Mediterranean river catchment and analisis in future climate change scenarios"

Marta Rusiñol, Xavier Fernandez-Cassi, Natàlia Timoneda, Anna Carratalà, Josep Francesc Abril, Ayalkibet Hundesa, Carolina Silvera, Maria José Figueras, Emiliano Gelati, Xavier Rodó, Sílvia Bofill-Mas i Rosina Girones.

Sotmès al 2014

L'increment dels episodis extrems de precipitació i de sequera a l'àrea Mediterrània és una de les previsions més consistents per als nous escenaris de canvi climàtic. En aquest nou context, tant l'aigua residual crua o l'escorrentia superficial urbana, poden afectar greument la qualitat de l'aigua i el que és més important, poden incrementar el nombre de brots derivats de microorganismes patògens transmesos per l'aigua.

Aquest estudi va ser dissenyat per investigar l'efecte que la precipitació, el cabal i la temperatura poden tenir en la dispersió de la contaminació fecal a una conca Mediterrània com la del riu Llobregat i a l'aigua de mar on desemboca. Dos virus indicadors de contaminació fecal humana, adenovirus humans (HAdV) i JC poliomavirus (JCPyV)), dos virus patògens com els norovirus (NoV GII) i els poliomavirus de les cèl·lules de Merkel (MCPyV) i també dos bacteris indicadors de contaminació fecal (*Escherichia coli* (EC) i Enterococs intestinals (EI)) van ser analitzats en mostres d'aigua de riu, aigua residual i aigua de mar recollides dos cops al mes durant divuit mesos en la conca del riu Llobregat.

Tant els HAdV com els JCPyV van ser detectats durant tot l'any a les mostres d'aigua del riu mentre que els NoV GII van presentar patrons típicament estacionals i relacionats amb les èpoques on hi ha més brots a la població. MCPyV es va detectar, mitjançant un assaig de PCR quantitativa dissenyat en aquest estudi, intermitentment a les mostres de riu i, per primera vegada, també a l'aigua de mar. La presència de patògens vírics i bacterians a l'aigua de mar, afectada per la descàrrega del riu al mar, va resultar molt més variable. Excepte els NoV GII, que presenten un patró d'excreció durant els mesos freds, la resta de patògens vírics es detectaven sovint a l'aigua de mar durant els mesos més calorosos, coincidint amb les èpoques de bany. Per contra, els patògens bacterians van ser més freqüentment detectats durant la primavera o la tardor.

Tot i el tractament de l'aigua residual, els genomes vírics segueixen detectant-se a l'aigua de sortida de la depuradora, es detecten durant tot el recorregut del riu i fins i tot a l'aigua de mar, indicant que la contaminació fecal es difon ràpidament en el medi aquàtic. Per tal de trobar alguna correlació entre els diferents patògens vírics i/o bacterians, o entre paràmetres climàtics i els patògens, es van sotmetre les dades a testos estadístics. No es van identificar correlacions significatives entre els patògens i paràmetres com la temperatura, la precipitació o el cabal. Tot i així, durant els períodes més plujosos s'observava una disminució de la prevalença, possiblement deguda a l'efecte de dilució de les pluges sobre les masses d'aigua analitzades.

Donat un nou escenari de canvi climàtic la gestió de l'aigua residual i dels efluents de les depuradores serà el punt clau per a la disseminació de la contaminació fecal a rius Mediterranis com el riu Llobregat.

Evaluation of viral dissemination in a Mediterranean river catchment and analisis in future climate change scenarios.

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Keywords: Microbial Source Tracking (MST), adenovirus, polyomavirus, norovirus, water, climate change.

Abstract

Climate change is expected to increment the intensity and frequency of floods and droughts in the Mediterranean region. Then, fecal contamination may pose higher risks to human health if natural dilution and inactivation processes are altered by extreme climate events. Within the EU-FP7-funded VIROCLIME project, the presence of human viruses and fecal indicator bacteria (*Escherichia coli* (EC) and intestinal enterococci (IE)) has been assessed in the Llobregat river basin (Catalonia, NE of Spain) from January 2011 to June 2012. Sample types included river water, untreated and treated wastewater from a wastewater treatment plant within the catchment area and seawater from an influenced

beach. High genome copy numbers (GC) of human adenovirus (HAdV) and JC polyomavirus (JCPyV) were reported in urban wastewater (Mean values of 10^3 GC/L and 10^5 GC/L respectively). Human Merkel Cell polyomavirus (MCPyV) was detected in 75% of the raw wastewater samples (28/37) and quantified by a new developed quantitative PCR assay (qPCR) with mean concentrations of 10^3 GC/L. This virus was found in 29% and 18% of river water and seawater samples, respectively. Detection and quantitation of this skin cancer related virus in seawater, demonstrated its ubiquitous dissemination in the environment. A seasonal distribution in the norovirus genogroup II (NoV GGII) occurrence was observed with cold period levels presenting up to 10^4 GC/L in river and 10^6 GC/L in untreated wastewater. Presence of human hepatitis E virus (HEV) in wastewater samples was 9.8% when analyzed by nested PCR (nPCR). After a secondary treatment (activated sludge) and a tertiary treatment (including chlorination, flocculation and UV radiation) of raw wastewater, the biggest viral concentration reduction was observed for the NoV GGII, with 2.50 $\log_{10}$ post-secondary treatment and 1.12 $\log_{10}$ post-tertiary. The river receives wastewater treated effluents throughout the catchment. Then, dispersion of fecal contamination occurred and seemed to be significantly lower when tertiary treatments were used. Significant correlations were observed between HAdV and JCPyV, and between EC and IE, with the highest values in the wastewater matrices (p-values <0.01, Rho: 0.75 and 0.94 respectively). Although correlations between viral and bacterial pathogens in wastewater were also significant, the p-values were between 0.57 and 0.69. Data obtained indicates that human fecal contamination is widely dispersed in the environment despite sanitation. In climate change scenarios for the Mediterranean region, wastewater management would be the key to prevent environmental dispersion of human fecal pathogens. Acceptable water quality levels may be guaranteed only if wastewater containment and treatment are fully operational when floods or extreme rainfalls occur. Projections for the Llobregat River catchment, estimate general warming and an increase of total precipitation amounts during the winter months and persistent decreases from May to October. Thus, river- and sea-water quality appears vulnerable when considering climate scenarios for the Mediterranean region.

1. Introduction

Improperly treated wastewater may lead to the transmission of human viruses that are excreted in feces and urine at high concentrations. Climate change models predict excessive temperature waves as well as more frequent and intense flood and drought events (IPCC, 2007). Moreover, distribution and burden of several infectious diseases may shift and human exposure may differ under the predicted climate change scenarios (Morse et al., 2012). Thus, assessing the impacts of climatic change on waterborne viral transmission is needed. Integrated river basin management is a key tool to mitigate the possible impacts of future climate change on the quality of water resources.

Recent advances in the concentration methods and new molecular approaches, as metagenomics, provide sensitive and exhaustive analytical tools (Cantalupo et al., 2011; Girones et al., 2010). Such developments allow a better understanding of the role of viral infections in diverse organisms and of their dissemination as fecal contaminants in aquatic environments.

Following a surveillance program within the EU-FP7-funded VIROCLIME project, we selected the Llobregat river basin because of its very high vulnerability to water scarcity typical of Mediterranean catchments (Barceló and Sabater, 2010). The Llobregat River (Catalonia, northeast of Spain) flows for approximately 170 km from the Pyrenees Mountains to the Mediterranean Sea near the city of Barcelona. The river basin, whose area is 4950 km^2, is inhabited by 5 million people, which constitute more than half of the Catalan population. Treated urban sewage, industrial activities and agricultural runoff affect the quality of river water, which is the main source of water abstraction to produce drinking water for Barcelona and its metropolitan area. In fact, urban water supply constitutes 65% of the total water demand (ACA, 2012). The annual average river discharge volume is 690 hm^3, of which over 40% consists of effluents from the approximately 50 wastewater treatment plants (WWTP) located along the basin (annual mean discharges: 300 hm3) (ACA, 2012). Climate change scenarios for the Mediterranean region predict higher frequencies of floods and summer droughts, as well as higher temperatures (Calbo, 2010). Extreme rainfall events have been globally linked with drinking-water related outbreaks (Figueras and Borrego, 2010; Cann et al., 2013; Semenza

et al., 2012). Thus outbreak frequency may increase unless improved water management strategies are built by considering the proposed climate change scenarios.

Water microbiological quality is evaluated based on bacterial fecal 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 fecal indicators based on their high prevalence in any of the geographical areas analyzed (Bofill-Mas et al., 2000a; Pina et al., 1998). Indeed, they have been widely used to survey the water quality and trace fecal pollution in the environment (reviewed in detail in Bofill-Mas et al., 2013).

Among all waterborne viral pathogens, noroviruses (NoV), single stranded RNA viruses, are recognized as one of the major common causes of illness (Craun et al., 2010; Kroneman et al., 2008). In fact, noroviruses are believed to be the single largest cause of disease in documented recreational water-borne outbreaks followed by adenoviruses (Sinclair et al., 2009). Norovirus GGII has been associated with the majority of the reported gastroenteritis cases (Lopman et al., 2004). While person-to-person is the most common transmission route, noroviruses are spread by several routes including shellfish, fresh food, processed food and water (Mathijs et al., 2012). Remarkably, noroviruses are highly infectious: a single virus particle carries a probability of infection of near 49% (Teunis et al., 2008).

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

The Merkel cell PyV (MCPyV), found integrated in a very high percentage of Merkel cell carcinomas, has been also described in urban sewage and in river water (Bofill-Mas et al., 2010; Calgua et al., 2013a). Although a cutaneous route seems to be the most likely way of transmission, the identification of this cancer-related polyomavirus in sewage has risen its interest (Spurgeon and Lambert, 2013).

In this study, we present a picture of the dissemination of HAdV, JCPyV, MCPyV, NoV GGII and HEV together with two Fecal Indicator Bacteria (FIB), *Escherichia coli* (*EC*) and

Intestinal Enterococci (IE), in a Mediterranean river catchment and an influenced beach. During an 18-month surveillance period, river samples from the Llobregat River and seawater samples were collected, processed and analyzed. Considering that the main viral inputs may come from raw or treated wastewater, several WWTP influent and effluent water samples were also analyzed to study the dissemination as fecal contaminants. Meteorological and hydrological data were collected in order to evaluate possible effects of climate change in viral dissemination. Here, the repeatability of the skimmed milk flocculation protocol used to concentrate viruses from all the water matrices was also evaluated.

2. Materials and methods

2.1. Data collection

Llobregat river discharge was recorded by two gauges located in Balsareny, site A in Figure1, and Sant Joan Despí, site B. Daily stream flow measurements were provided by the Catalan Water Agency (ACA, 2012). We assumed wastewater treatment plant (WWTP) effluents were equivalent to the WWTP inflows. Water temperatures were recorded during samplings, while precipitation data were delivered by the Catalan Meteorological Institute (Meteocat, 2014). Data from river flow, precipitation and temperature of all seasons are shown in Table 1.

2.2. Samples and sites

A total of 195 water samples were collected, from January 2011 to June 2012. All viral and bacterial pathogens were quantified bimonthly in samples collected at 2 sampling points in the Llobregat river catchment (named A and B hereafter), and 1 beach affected by the Llobregat river plume (site D). Additionally, raw wastewater and two differently treated effluents from a WWTP were simultaneously collected and tested along the sampling period (site C). The specific location of each sampling site is shown in (Figure 1). All WWTP samples were also tested for the presence of HEV by nPCR. Conductivity, pH and temperature were determined for each water sample.

Site A is located 80 km upstream (number of samples: n= 36) of the river mouth. The volume rate of water flow or mean river discharge volume at site A is 0.5 m^3/s, corresponding to 14hm^3 of mean annual discharge. Ten WWTP are located upstream of the sampling site and present a mean annual effluent discharge of 6 hm^3.

Site B (n=37) is located 70 km downstream from site A and 10 km from the river mounth and close to the city of Barcelona. The Llobregat in site B has 255 hm^3 of annual discharge (mean flow 8.1m^3/s), of which 154 hm^3 come from the fifty upstream WWTP effluents.

Most of the sewage generated in the metropolitan area of Barcelona is treated in site C, which is a WWTP designed to handle around 153 hm^3 of water per year using secondary and eventually tertiary treatment. Ninety-one samples were collected from the WWTP collected at: the entry point (C1, n=37), the outlet of secondary treatment, consisting of a biological reactor and a sedimentation (C2, n=32) and at the exit point from the tertiary treatment (C3, n=22) after chlorination, Actiflo® filtration and flocculation, and UV disinfection.taken when the complete treatment was running. The site D, in the seashore, was a beach site affected by the river plume that is modulated by the Mediterranean seawater currents (n=32). Water samples of 10 L were collected from the river, the sea and the tertiary treatment effluents were used to quantify viral genome copies and 50 mL in the case of wastewater and secondary treatment effluents.

2.3. Viral concentration and nucleic acid extraction

Detection of viruses in the environment requires the concentration of viruses into small volumes. The study was conducted using optimized Standardized Operational Procedures (SOPs) for virus concentration, nucleic acid extraction and quantitative PCR (qPCR) detection,this latter including several process controls and standard plasmid preparation. First, samples with high levels of organic matter or sand were permitted to sediment for 1 hour in order to avoid any interference with the concentration method. Then, clarified water was transferred to a new container without disturbing the sediment. Seawater samples were collected avoiding their contamination with sand and suspended macro algae.

Water samples were concentrated as previously described by Calgua and coworkers (Calgua et al., 2013a, 2013b, 2008). Briefly, a Pre-flocculated 1% (w/v) Skimmed Milk solution (PSM) was prepared by dissolving 10g skimmed milk powder (Difco, Detroit, MI, USA) in 1L of artificial seawater at pH 3.5 (Sigma, Aldrich Chemie GMBH, Steinheim, Germany). All samples were carefully acidified to pH 3.5 using HCl 1N and the conductivity was adjusted to 1.5 mS/cm^2. Then, the PSM solution was added to each of the previously conditioned sample to obtain a final concentration of skimmed milk in the sample of 0.01%. Samples were stirred for 8h at RT and flocks were allowed to sediment by gravity for 8h. Supernatants were carefully removed using a vacuum pump without disturbing the sediment. Pellets were suspended using 10mL of phosphate buffer at pH 7.5, except for raw sewage samples and secondary treated effluents that were suspended in 1mL. 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, respectively, as positive and negative control of the nucleic acid (NA) extraction experiment. Finally, NA elutes were stored at -20°C until use.

2.4. Quantitative and nested PCR assays

Specific real-time quantitation of DNA viruses (HAdV, JCPyV) by qPCR or RNA viruses (NoV GGII) by quantitative reverse transcription PCR (qRT-PCR) was performed as previously described using TaqMan® Universal PCR Master Mix and the RNA UltraSense™ One-Step qRT-PCR System respectively (Invitrogen, Carlsbad, CA, USA) (Hernroth et al., 2002; Kageyama et al., 2003; Loisy et al., 2005; Pal et al., 2006). Undiluted and $\log_{10}$ dilutions of the nucleic acid extracts were analyzed in duplicate. All qPCRs included more than one non-template control (NTC) to demonstrate that the mix did not produce fluorescence due to contamination. Quantitations were 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 as described elsewhere (Erker et al., 1999). 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 carried out with AmpliTaqTM Gold DNA

polymerase (Applied Biosystems Foster City, CA, USA). All primers and probes used in this study are described in Table 2. Detection limits when using qPCR or nRT-PCR techniques have been quantified between 1-10 genome copies (GC) per reaction.

2.5. MCPyV qPCR assay

A new qPCR assay was designed for MCPyV detection in water matrices. A fragment of the VP1 gene (132bp), obtained by applying a specific PCR (Bofill-Mas et al., 2010), was cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). The standard curves were generated transferring the plasmid construction into *E. coli* DH5α cells (Invitrogen, Carlsbad, CA, USA). After checking by conventional PCR, the transformed colonies holding the target sequence and purifying them with the QIAGEN Plasmid Midi kit (QIAGEB GmbH Inc., Hilden, Germany), the constructions were linearized with Sal I restriction enzyme (Promega, Madison, WI). Then, two primers and a fluorogenic probe were designed, based in a TaqMan® assay, to amplify the specific VP1 fragment of the viral genome (Table 2). Annealing temperatures as well as primer and probe concentrations for the novel MCPyV qPCR were optimized 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 of DNA and 15 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.9 µM of each primer (MCF and MCR) and 0.225 µM of fluorogenic probe (MCP). Following activation the AmpliTaq Gold DNA polymerase for 10 min at 95°C, 40 cycles (15 s at 95°C and 1 min at 60°C) were performed with an MX3000P detector system (Stratagene, LaJolla, CA, USA). Serial dilutions of the confirmed standard, ranging from 10^0 to 10^5 molecules per 10µL, were performed with TE buffer and stored at -80°C until use.

2.6. 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, kindly donated by Dr. A. Allard of the University of Umeå (Sweden), 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, including flocculation, NA extraction and DNA quantification. NoV GGII.13 genome was also extracted, on each

sampling, as positive control of the nucleic acid extraction and RNA quantification (10^4 Genome Copies/reaction).

Plasmid DNA was used as a positive control and as a quantitative standard. For the HAdV and JCPyV, the hexon region (8961bp) and the whole genome (5130bp) of HAdV41 and JCPyV Mad1 respectively, were cloned in pBR322. To reduce the possibility of DNA contamination in the laboratory, 10µg of plasmid DNA was linearized 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 pTrueBlue®-Pvu II vector (Kindly donated by Dr. J. Vinjé of the CDC, Atlanta), was used as a qRT-PCR standard. Here: 10 µg of this construct was linearized with XhoI (Promega) to prevent contamination and serially diluted from 10^0 to 10^5 molecules per 5 or 10 µL (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 fluorogenic Taqman probes are described in Table 2. UltraPure™ DNase/RNase-Free distilled water was used as negative control of the NA extraction and qPCR assays.

2.7. FIB detection

For the FIB detection, 100 mL of each sample were collected in parallel in all sites. All samples were kept on ice and processed within 24h. Determination of *E. coli* was carried out in a 96 well microplate MUG/EC 355-3782 (BioRad, Barcelona, Spain ®) according to ISO 9308-2:2012 which uses the most probable number (MPN) procedure (Donovan et al., 1998) to detect *E. coli* based on the expression of the enzyme β-D-glucuronidase present in most of the *E. coli* 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 that is based on the detection of the expresion of β-glucosidase enzyme characteristic of enterococci.

2.8. Assay of repeatability of the concentration procedure

An intra-laboratory assay was performed to test repeatability of the concentration method. This involved mixing 200L of seawater, river water or mineral water spiked with HAdV35 and NoV GGII.13 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, which included mineral water as a control. Viral particles were concentrated as described in 2.3. HAdV and NoV GGII.13 were quantified by qPCR and qRT-PCR after nucleic acid extraction. Additionally, sample inherent JCPyV were also quantified in the 20 seawater replicates.

2.9. Statistical analyses

The statistical analysis was performed using R software version 2.15.1 (Verzani, 2004). Data distribution was tested with Shapiro-Wilk and Kolmogorov-Smirnov analyses. After proving that data was not normally distributed, correlation analysis of the transformed (log_{10}) data was done with parametric (Pearson correlation) and non-parametric (Spearman correlation) analyses. Results were considered to be significant with a p of <0.01 and values less than the detection limit were considered as 1. Correlations between the temperature, flow, precipitation and microbial concentration were analized. The potential utility of quantitative viral concentrations in river to predict the putative viral concentrations in new scenarios was also evaluated with a linear regression model.

Repeatability of the concentration procedure was also analyzed using R software version 2.15.1 (Verzani, 2004). Both raw (GC/ml) 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 was calculated.

2.10. Temperature and precipitation projections, methods and datasets

Historical temperature and precipitation data were extracted from ERA-interim reanalysis database (http://data-portal.ecmwf.int). As data can contain gaps due to several reasons, we established the criteria for a gridpoint to be eligible to contain a minimal fraction of 30 valid points for each gridpoint comprised in the box 0 to 5°E and 40°N-45°N. Precipitation is in mm/day and temperature in degrees Kelvin (°K). A minimal fraction of 30 valid points is mandatory for each gridpoint also to be considered in the analysis. Both precipitation and average, minimum and maximum temperature simulations come 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-H, GISS-E2-H, GISS-E2-R, GISS-E2-R, 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.

3. Results

3.1. 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. Intrinsec JCPyV presented the most repeatable results with CV of 12.4%. The smaller variation in HAdV results was 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 variability was calculated for NoV GGII.13 in seawater (CV: 36.3%).

3.2 Occurrence of viruses and FIB along the Llobregat river basin: river water and seawater samples.

The median genomic copies (log_{10} GC/L) or MPN/L and concentration ranges of HAdV, JCPyV, MCPyV, NoV GGII, EC and IE found in each sampling location and season are shown in Figure 2. The HAdV concentrations were found stable during the year with mean values of $8.1x10^2$ GC/L in site A, $1.4x10^3$ GC/L in site B and $1.2x10^3$ GC/L in site C (Figure 2). Overall, 100% of river and seawater samples were positive for HAdV in summer and mean values were eventually as high as those quantified in effluent water samples from tertiary treatments. Despite JCPyV was not detected in spring over site A, the prevalence was still high and mean concentrations were similar from the reported for HAdV. No JCPyV was detected near the seashore in wintertime. MCPyV were punctually detected along the River sampling sites (Figure 2). Higher values were found in summer when positive samples were also found in the sea. Indeed, during the summer season the

highest measured concentration was $6.9x10^2$ GC/L. A seasonal distribution of NoV occurrence in river water and sewage was observed. With exception of the summer months, NoV GGII was found in river and seawater samples all over the year (Figure 2). 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). As far as FIB concentrations are concerned, EC and IE were detected during all seasons in both river sites (A and B), at mean values of 10^3 MPN/L, whereas in seawater FIB were only detected in spring (EC) and autumn (EC and IE). All pathogen box plots organized per season and location are summarized as supplementary material.

3.2. Viruses and FIB in wastewater samples and its treatment removal efficiencies

HAdV were detected in all 26 influent wastewater samples, showing similar mean values during the year. High viral titers were observed in raw wastewater ($8.4x10^5$ GC/L), secondary treatment ($1.2x10^5$ GC/L) and tertiary treatment effluents ($1.9x10^3$ GC/L). Also JCPyV was highly prevalent in raw wastewater and secondary treatment effluents at mean concentrations of $7.5x10^5$ GC/L and $1.6x10^5$ GC/L, rather than and tertiary effluents with only 7 positive out of 22 tested samples. MCPyV was detected at mean concentrations $1.6x10^5$ GC/L in 73% of the raw wastewater. Although prevalence in secondary and tertiary effluents was very variable, MCPyV occurred to be present mostly in summer and winter. In the WWTP, the highest NoV GGII mean values were quantified in winter: $2.5x10^8$ GC/L in raw sewage, $2.1x10^7$ GC/L in secondary treated wastewater and $2.2x10^5$ GC/L after the 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). The wastewater results are in box plots as supplementary material.

Reduction of viral and bacterial concentrations was calculated for all treatment steps. All tested pathogens and fecal indicators were detected in the samples taken at different levels of treatment, with exeption of HEV that was not detected in any tertiary treatment sample. Removal of the quantified pathogens was studied by calculating the $\log_{10}$

reduction through the treatment process, after the secondary treatment (C2) and after the tertiary treatment (C3). Results are summarized in Figure 3. The combination of the two processes (C2+C3) came to 3.16 log_{10}, 3.41 log_{10} and 3.61 log_{10} reduction values for EC, IE and NoV GGII respectively. Human DNA viruses presented lower total removal efficiencies (1.63 log_{10} for MCPyV, 2.29 log_{10} for HAdV and 2.49 log_{10} for JCPyV).

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

The calculated correlations between climate parameters and pathogens Showed, as expected, that in general terms EC and IE correlated (corr: 0.93, *p*-value ≈0) along the river basin locations and seasons (Table 3). In turn, every viral pathogen presented a significant (*p*-value <0.01) but low correlations with the other viral pathogens (Table 3). Air and river water temperatures correlated well and significantly with each other (*p*-value <0.01 and corr: 0.93) (Data not shown). No significant correlation was found between viral pathogens and water and air temperatures or stream flows. Only in seawater samples, HAdV and FIB presented significant correlations with accumulated precipitation (EC corr: 0.75 and IE corr: 0.95) and river flow (EC corr: 0.60 and IE rho: 0.58).

3.5. Climate projections

Climate projections by the end of the XXIst century were generated with the suite of climate models contained in CMIP5+ project database (see Methods). Due to the large uncertainty associated with highly detailed spatial resolution simulations, a grid of 5x5 degrees lat lon was preferred. Results for the change 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 reanalysis (ERA) (http://data-portal.ecmwf.int/data/d/interim_daily/). A bias between current climate conditions 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 the regional

precipitation yield an estimate of a slight increase of total amounts between the simulated winter months of 2070-2100 and 1980-2010 (on average around 1-3mm/month) and persistent decreases between May and October of around 2.5mm/month on average (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 localized maximum increases of around 30% in the main winter months).

Temperature projections for the region appear in Figure 4B. In this case, systematic differences betwen observations and simulations for the common 1980-2010 interval are much lower than for rainfall above. The overall change in temperature between the two intervals (2070-2100 vs 1980-2010) indicates a general warming throughout the year with an increase in the minimum winter temperatures, that is even positive when compared to observations. Increases are of above 13% in summer months and around 11% in winter.

4. Discussion

Here, we evaluate the effects of meteorological parameters on dissemination of fecal contamination from WWTP effluents to river water and finally to the receiving seawater by analyzing HAdV, JCPyV, MCPyV, NoV GGII, HEV, and FIB. This study reports, throughout one and a half year of sampling activity, the presence and relationships between the viral and bacterial indicators and, to our knowledge, it is the first description of MCPyV presence in seawater samples, which could be a putative route of transmission through bathing water.

HAdV and JCPyV were prevalently detected in the river water samples, 80% and 59% respectively, at similar concentrations to those previously described (Albinana-Gimenez et al., 2009). The lower frequency of HAdV and JCPyV in the seawater was consistent with other studies that pointed at sea currents dilution or disinfection by the high salinity as the factors that justify this low frequency (Hawley and Garver, 2008; Wyn-jones et al., 2010). The high prevalence and concentrations of HAdV and JCPyV observed in influent wastewater and water effluents from the secondary and tertiary treatments 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).

The frequent detection of MCPyV in urban sewage samples (31/37) suggested a persistent excretion of this virus by human populations. Prevalence and concentrations of MCPyV in sewage and river water were similar to those previously reported (Bofill-Mas et al., 2010; Calgua et al., 2013a). The most relevant finding was the detection of MCPyV in seawater during summer which may represent a risk of transmission considering the summer recreational activities. The presence of this polyomavirus, which has been described to have a skin tropism, may also be linked to the recreational activities rather than exclusively to sewage.

NoV GGII prevalence showed a remarked seasonality: the highest concentrations were measured during winter months in agreement with colder temperatures. Winter peaks of noroviruses in the environment are well known (Kitajima et al., 2012, 2010; Nordgren et al., 2009) and in the northern hemisphere have been associated with cold temperatures and low air humidity, low population immunity and the emergence of novel genogroups (Lopman et al., 2009). Recently other authors performed a norovirus survey in the same river catchment finding similar results as those decribed in our work (Collado et al., 2010; Pérez-Sautu et al., 2012).

HAdV, JCPyV, NoV GGII and MCPyV were widely detected in raw sewage samples, whereas HEV were found in low percentages (9.8%). Although previous studies reported HEV to be present in a 43.5% of the raw sewage from Barcelona (Clemente-Casares et al., 2003), the low occurrence could be related with a short excretion period or the efficiency of the concentration method with that particular virus. Nevertheless, this is the first study that analyses 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 standards included in current Spanish legislation that determines the water quality required for irrigation of non-processed food i,e, less than 100 colony-forming units (cfu) in 100mL (Spanish Government, 2007a). 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, all FIB levels in seawater samples complied with the bathing water regulations and exhibited an "excellent" water quality with less than 100 cfu/100mL of EI and less than 250 cfu/100mL of EC (Spanish Government, 2007b).

The WWTP secondary treatment and its activated sludge play an important role in reducing pathogens since microorganisms are mostly separated from the water through sedimentation, either by themselves or attached to activated sludge. The results (Figure 3) show that this step was the most important for removal of pathogens, with the maximum reductions of 69% for NoV GGII (2.50 log_{10}) and 52% for IE (1.79 log_{10}), from the total reduction process. Similar findings have been described in the literature (Rodriguez-Manzano et al., 2012). Despite achieving a maximum decay of 3-log when compared to the initial concentrations in raw sewage, viruses were also detected in the majority of analyzed reclaimed water samples that accomplished the current legislation based on traditional bacterial indicators. Conventional WWTP secondary effluents in the river (mean annual discharge of 153 hm^3) and only tertiary treatment effluents from the WWTP near the seashore (site C) are discharged into the sea through long sea submarine outfalls (mean annual discharge of 154 hm^3). Considering that the average viral load of a secondary treatment effluent is 10^5 GC/L, we could assume that every year a total amount of $3x10^{16}$ potentially infectious viruses are discharged into the river. From our results, we can infer that sewage effluents discharged in the vicinity of the studied locations have a direct impact on the presence of human viruses in the river water or in the beach seawater. Treating water with only a secondary or with a complete tertiary treatment will affect river and sea water quality and consequently the drinking water treatment as well as the recreational seawater uses.

The repeatability assays suggested the quantitative robustness of the protocols used because only between 12.4 and 36.3% CV was found in the intra-laboratory trials. When taking all samples into account, HAdV significantly correlated with JCPyV (corr >0.8, p-value <0.01), indicating that HAdV may be used to predict the concentration of JCPyV. Significant but lower correlations were also found between HAdV and the other tested pathogens, being HAdV the best predictor of the majority of pathogen concentrations (correlation values ranged from 0.53 and 0.70). As recently discussed by other authors (Payment and Locas, 2011) direct correlation between pathogens and indicators of fecal contamination becomes improbable in water bodies receiving sewage discharges because of different dilution, transport 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 robust tool to identify and manage fecal contamination in order to improve water quality in a wide variety of new climate scenarios.

Data obtained demonstrate that despite the performed treatment at the WWTP there exist a dissemination of human adenoviruses, polyomaviruses and noroviruses from sewage to the downstream aquatic environments. Our results are consistent with other studies about the shedding of enteric viruses by sewage treatment plants (Simmons and Xagoraraki, 2011). These findings make very evident the inefficacy of current interventions to eliminate viral pathogens from sewage and indicate the need of new legislation including viral quality standards. Where climate change scenarios predict more frequent floods, the pollution events caused by sewage overflows will pose a big challenge for water managers. It is likely that predicted reduction in the number of rainfall events in the Mediterranean area will produce more frequent low river flows and likely higher variability in fecal contamination levels (Figueras and Borrego, 2010; Cann et al., 2013). Then drought events and eventually powerful rainfalls could cause severe water quality deterioration due to sewage concentration or storm water runoff (Figueras and Borrego, 2010). The results observed in this study show high concentration of viruses in river and seawater during the dry months. Projections for the regional precipitation yield estimated persistent decreases between May and October anticipating an increase in the viral loads in the absence of waste water treatment improvement. Due to the nature of the simulations, it is beyond the scope of this study to determine whether increase of temperatures would be felt in the form of more severe local rainfall events or throughout a general increase of the mean daily rainfall values. The form in which the predicted excess heat will be entering the region (e.g. increase heatwaves or milder winters) will be further investigated.

5. Conclusions

1. The concentration of HAdV was stable in raw sewage all over the year, with mean values of 8.38×10^5GC/L, while other viral and bacterial pathogens exhibited seasonally variable concentrations.

2. Secondary treatments reduce pathogen concentrations between 1.5 and 2.5 log10. As conventional WWTPs discharge secondary effluents into river, the Llobregat River is persistently impacted by human fecal pollution as evidenced by the presence of human adenoviruses, JC polyomaviruses and FIB in the river water samples.

3. Seasonal NoV GGII excretion patterns are observed throughout the wastewater and environmental samples. From the most upstream sampling site to the seawater impacted by the river discharge, NoV GGII are detected during spring, autumn and 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 the Merkel cell carcinoma in water matrices.

5. This is the first study reporting detection and quantification of MCPyV in seawater samples (with 12% of prevalence and mean concentration of $1.18x10^2$GC/L). The high prevalence of MCPyV in sewage (83%) suggests that the human population commonly sheds these viruses in skin or urine.

6. In general, the highest virus concentrations were detected in summer when the minimum precipitation levels were measured (0.35 mm). Temperature and precipitation predictions, for the Llobregat River catchment, estimate a persistent increase of temperatures, increase in the precipitation yield during winter and decrease between May and October. Viral and bacterial pathogens may be washed down the river during the first runoff, but diluted away with the increased flows.

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Figures and Tables

Table 1: Flow, precipitation and water and air temperature measurements by location. Season mean values (M) and standard deviations (SD).

W: winter, P: spring, S: summer and A: autumn.

		River (site A)				River (site B)				Sea (site D)				Wastewater (site C1)			
		W	P	S	A	W	P	S	A	W	P	S	A	W	P	S	A
Flow (m^3/s)	M	1.48	1.19	0.45	0.74	6.38	13.04	8.69	10.46								
	SD	3.57	0.70	0.73	0.66	2.53	2.93	5.63	3.86								
Precipitation (mm)*	M	2.54	0.23	0.51	1.86	3.36	1.77	0.35	11.83								
	SD	9.11	0.21	1.12	2.21	11.99	1.91	0.78	15.93								
Water temperature (ºC)	M	5.18	13.32	23.03	12.49	9.78	15.11	23.58	15.91	8.99	16.78	23.39	15.68	8.94	15.68	23.55	15.40
	SD	3.14	3.68	1.22	4.75	2.52	3.37	1.27	3.61	3.19	3.59	0.99	4.01	3.29	3.51	1.08	3.75
Air temperature (ºC)	M	7.14	12.61	20.25	14.84	9.19	15.00	24.12	17.39	12.77	20.89	25.60	20.95	11.42	14.57	24.33	20.51
	SD	2.19	3.77	1.64	3.50	2.29	3.73	0.37	6.18	4.98	1.84	1.54	3.78	2.97	0.55	2.23	4.35

* Precipitation was calculated as three days accumulated precipitation. Flow and temperature measurements were done during the sampling day.

Figure 1: Sampling point locations in the Llobregat river catchment (Catalonia, Spain). Site A: upstream river water, site B: downstream river water, site C: raw sewage (C!, secondary (C2) and tertiary (C3) efluents from a WWTP near the river end, site D: seawater.

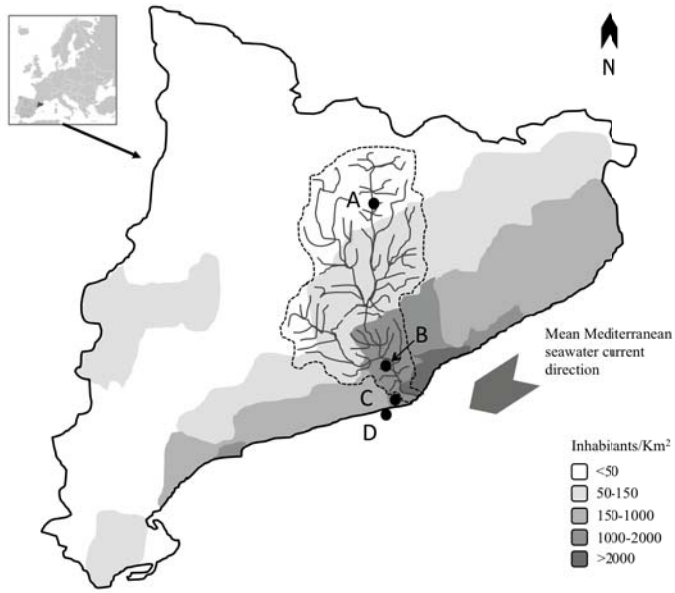
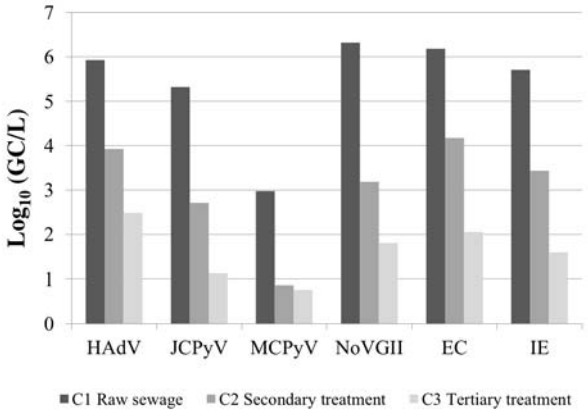


Table 2: Oligonucleotide primers and probes used for the detection and quantification of viral pathogens.

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 3: Summary of WWTP Log_{10} concentrations and treatment Log_{10} reduction values for each viral and bacterial pathogen.



	HAdV	JCPyV	MCPyV	NoVGII	EC	IE
Secondary (C2)	1.42	1.60	1.57	2.50	1.43	1.79
Tertiary (C3)	0.87	0.89	0.06	1.12	1.73	1.62
TOTAL(C2+C3)	2.29	2.49	1.63	3.61	3.16	3.41

Figure 2: Boxplots representin pathogen concentrations (GC or MPN/L) by matrix (A: upstream river water, B: downstream river water and D: seawater) and season (P:spring, S: summer, A: autumn and W: winter).

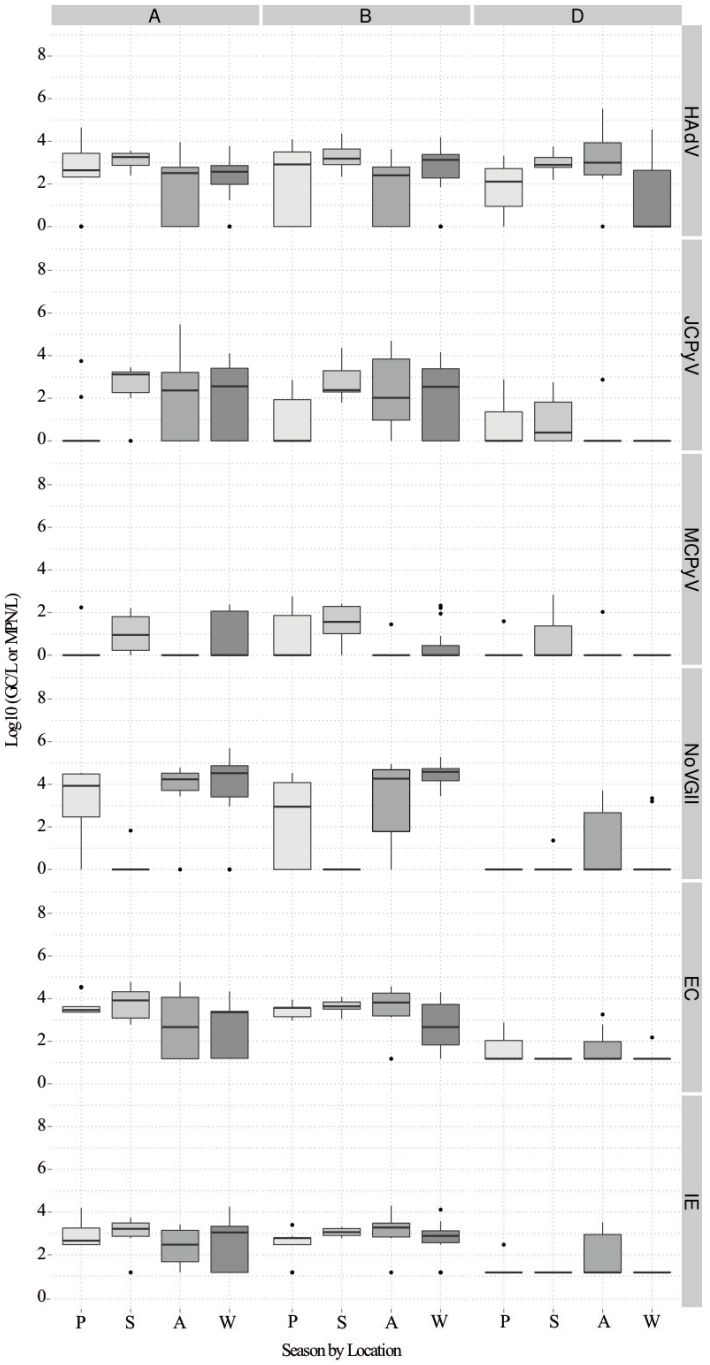
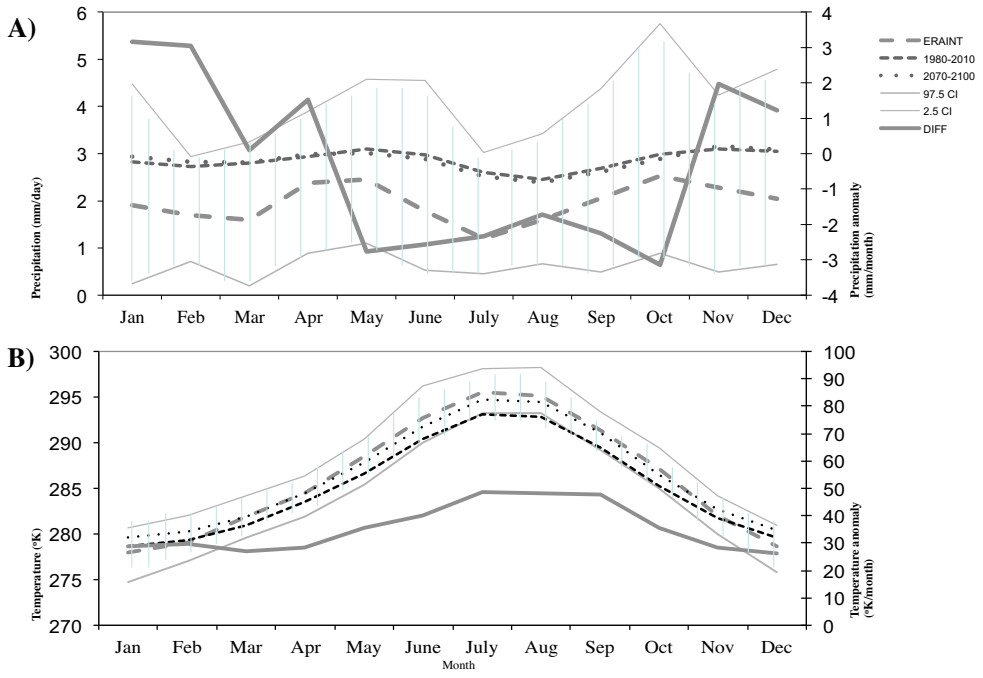


Table 3: Significant correlations between pathogens in raw wastewater matrices.

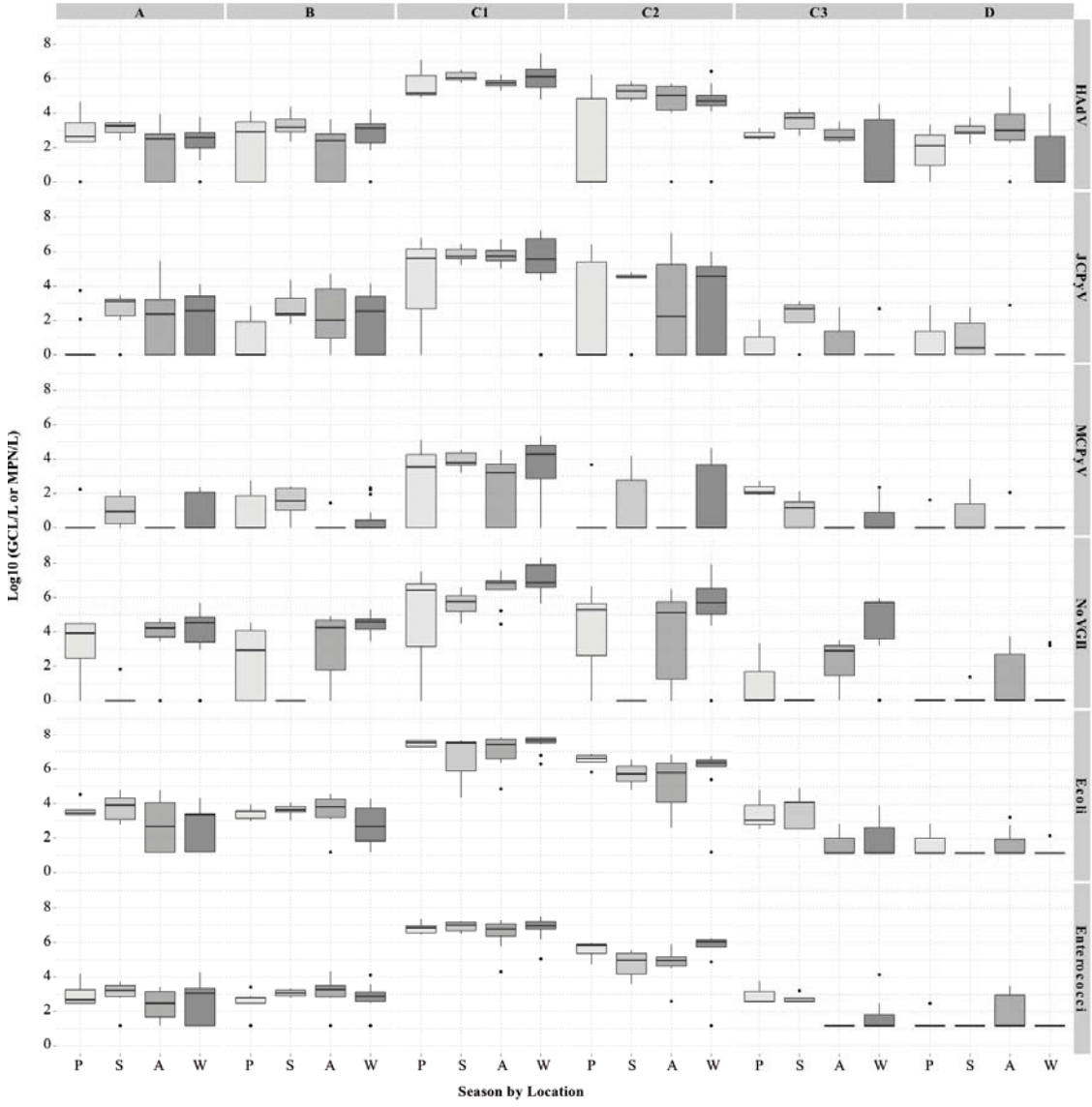
p-values / correlations	Wastewater matrix HAdV	JCPyV	MCPyV	NoVGII	EC	IE
HAdV		<0.01	<0.01	<0.01	<0.01	<0.01
JCPyV	0.65		<0.01	<0.01	<0.01	<0.01
MCPyV	0.63	0.56		<0.01	<0.01	<0.01
NoVGII	0.53	0.47	0.37		<0.01	<0.01
EC	0.60	0.52	0.34	0.50		<0.01
IE	0.70	0.64	0.51	0.57	0.92	

Table 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.



Supplementary information:

S1: Summary of boxplots representin pathogen concentrations (GC/ or MPN/L) by matrix (A: upstream river water, B: downstream river water C1: raw sewage, C2: secondary treatment C3: tertiary treatment, and D: seawater) and season (P: spring, S: summer, A: autumn and W: winter).



S2: Modelling groups and model names

Modeling Center (or Group)	Institute ID	Model Name
Commonwealth Scientific and Industrial Research Organization (CSIRO) and Bureau of Meteorology (BOM), Australia	CSIRO-BOM	ACCESS1.0 ACCESS1.3
Beijing Climate Center, China Meteorological Administration	BCC	BCC-CSM1.1 BCC-CSM1.1(m)
Instituto Nacional de Pesquisas Espaciais (National Institute for Space Research)	INPE	BESM OA 2.3
College of Global Change and Earth System Science, Beijing Normal University	GCESS	BNU-ESM
Canadian Centre for Climate Modelling and Analysis	CCCMA	CanESM2 CanCM4 CanAM4
University of Miami - RSMAS	RSMAS	CCSM4(RSMAS)*
National Center for Atmospheric Research	NCAR	CCSM4
Community Earth System Model Contributors	NSF-DOE-NCAR	CESM1(BGC) CESM1(CAM5) CESM1(CAM5.1,FV2) CESM1(FASTCHEM) CESM1(WACCM)
Center for Ocean-Land-Atmosphere Studies and National Centers for Environmental Prediction	COLA and NCEP	CFSv2-2011
Centro Euro-Mediterraneo per I Cambiamenti Climatici	CMCC	CMCC-CESM CMCC-CM CMCC-CMS
Centre National de Recherches Météorologiques / Centre Européen de Recherche et Formation Avancée en Calcul Scientifique	CNRM-CERFACS	CNRM-CM5 CNRM-CM5-2
Commonwealth Scientific and Industrial Research Organization in collaboration with Queensland Climate Change Centre of Excellence	CSIRO-QCCCE	CSIRO-Mk3.6.0
EC-EARTH consortium	EC-EARTH	EC-EARTH
LASG, Institute of Atmospheric Physics, Chinese Academy of Sciences and CESS,Tsinghua University	LASG-CESS	FGOALS-g2
LASG, Institute of Atmospheric Physics, Chinese Academy of Sciences	LASG-IAP	FGOALS-gl FGOALS-s2
The First Institute of Oceanography, SOA, China	FIO	FIO-ESM
NASA Global Modeling and Assimilation Office	NASA GMAO	GEOS-5
NOAA Geophysical Fluid Dynamics Laboratory	NOAA GFDL	GFDL-CM2.1 GFDL-CM3 GFDL-ESM2G GFDL-ESM2M GFDL-HIRAM-C180 GFDL-HIRAM-C360
NASA Goddard Institute for Space Studies	NASA GISS	GISS-E2-H GISS-E2-H-CC GISS-E2-R GISS-E2-R-CC
National Institute of Meteorological Research/Korea Meteorological Administration	NIMR/KMA	HadGEM2-AO
Met Office Hadley Centre (additional HadGEM2-ES realizations contributed by Instituto Nacional de Pesquisas Espaciais)	MOHC (additional realizations by INPE)	HadCM3 HadGEM2-CC HadGEM2-ES HadGEM2-A
Institute for Numerical Mathematics	INM	INM-CM4
Institut Pierre-Simon Laplace	IPSL	IPSL-CM5A-LR IPSL-CM5A-MR IPSL-CM5B-LR
Japan Agency for Marine-Earth Science and Technology, Atmosphere and Ocean Research Institute (The University of Tokyo), and National Institute for Environmental Studies	MIROC	MIROC-ESM MIROC-ESM-CHEM
Atmosphere and Ocean Research Institute (The University of Tokyo), National Institute for Environmental Studies, and Japan Agency for Marine-Earth Science and Technology	MIROC	MIROC4h MIROC5
Max-Planck-Institut für Meteorologie (Max Planck Institute for Meteorology)	MPI-M	MPI-ESM-MR MPI-ESM-LR MPI-ESM-P
Meteorological Research Institute	MRI	MRI-AGCM3.2H MRI-AGCM3.2S MRI-CGCM3 MRI-ESM1
Nonhydrostatic Icosahedral Atmospheric Model Group	NICAM	NICAM.09
Norwegian Climate Centre	NCC	NorESM1-M NorESM1-ME

ARTICLE II

Traçabilitat de la contaminació fecal al medi mitjançant la quantificació de virus específics

4.2. Traçabilitat de la contaminació fecal al medi mitjançant la quantificació de virus específics.

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

Marta Rusiñol, Xavier Fernandez-Cassi, Ayalkibet Hundesa, Carmen Vieira, Anita Kern, Irene Eriksson, Panos Ziros, David Kay, Marize Miagostovich, Marta Vargha, Annika Allard, Apostolos Vantarakis, Peter Wyn-Jones, Sílvia Bofill-Mas i Rosina Girones.

Publicat a Water Research, 2014

La utilització d'eines que permetin traçar la contaminació fecal humana i animal a l'ambient serà, en un futur, la clau per mitigar els possibles efectes del canvi climàtic, del constant creixement de la població i de la conseqüent intensificació de la producció animal. Mitjançant virus específics humans i animals es pot identificar l'origen i la magnitud de la contaminació fecal per tal de poder gestionar la qualitat de l'aigua. En aquest context, durant el transcurs del projecte VIROCLIME "Impact of climate change on the transport, fate and risk management of viral pathogens in water", finançat per la Unió Europea, es van quantificar virus específics per humans (HAdV i JCPyV), bestiar porcí (PAdV) i bestiar boví (BPyV), per traçar l'origen de la contaminació fecal en cinc conques de zones climàtiques ben diferents: el Río Negro a Brasil, el riu Glafkos a Grècia el riu Tisza a Hongria, el riu Llobregat i el riu Umeälven a Suècia. Durant els 18 mesos de mostreig es van recollir quasi 800 mostres d'aigües superficials. Les partícules víriques present a cada mostra, van ser concentrades, pel mètode de floculació amb llet descremada, i quantificades mitjançant qPCR específiques per a cada un dels marcadors.

De forma general, a tots els punts de mostreig aigües avall de grans poblacions, la contaminació fecal humana és més alta que als punts de mostreig aigües amunt, la concentració mitjana dels HAdV passa de 10^3 a 10^4 CG/L. Tant la prevalença com la concentració disminueixen durant les èpoques de pluges o durant els mesos del desgel.

Els cabals dels rius situats a la regió Mediterrània són molt variables (Rius Llobregat i Glafkos), i fan que la qualitat de l'aigua d'aquests rius sigui molt vulnerable a qualsevol descàrrega puntual o episodi de pluja. En canvi, en el cas del Río Negro, afluent de l'Amazones, l'efecte dilució és molt important tot i no disposar de cap sistema de sanejament. Als punts de mostreig més propers a la població, la contaminació fecal impacta directament sobre la qualitat de l'aigua del riu, amb concentracions que arriben a ser de 10^5 CG/L pels HAdV i de 10^4 CG/L pels JCPyV, i aigües avall les concentracions disminueixen fins a 2 logaritmes.

La contaminació fecal animal es detecta consistentment a les conques fluvials amb més activitat ramadera, per exemple a la conca del Tisza. A les conques com la del Llobregat o el riu Glafkos es detecta contaminació animal, majoritàriament porcina, de manera puntual mentre que a la conca de l'Umeälven s'hi detecta contaminació fecal bovina durant la primavera i l'estiu, quan els ramats pasturen a l'aire lliure i també hi ha escorrentia superficial deguda al desgel.

Els protocols i els marcadors virals aplicats en aquest estudi multi-laboratori han demostrat ser robustos, cost-efectius i aplicables als anàlisis de MST de rutina. Els virus humans i animals analitzats en aquest treball han permès identificar les fonts de contaminació fecal a totes les àrees geogràfiques analitzades.

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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

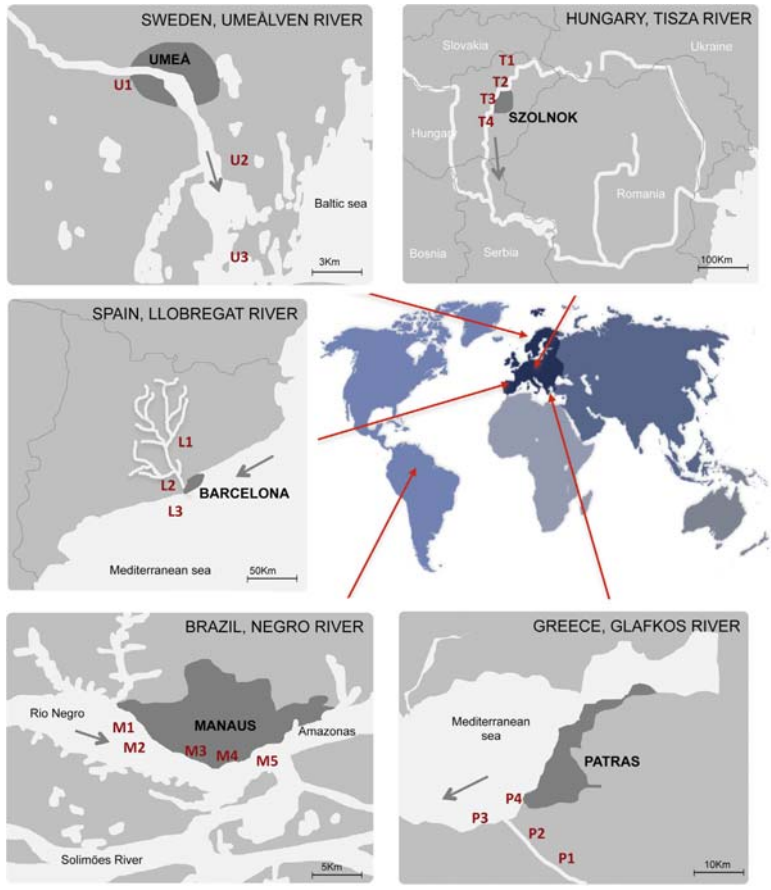

Fig. 1 – Geographic localization and sampling sites of the five case studies analyzed.

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

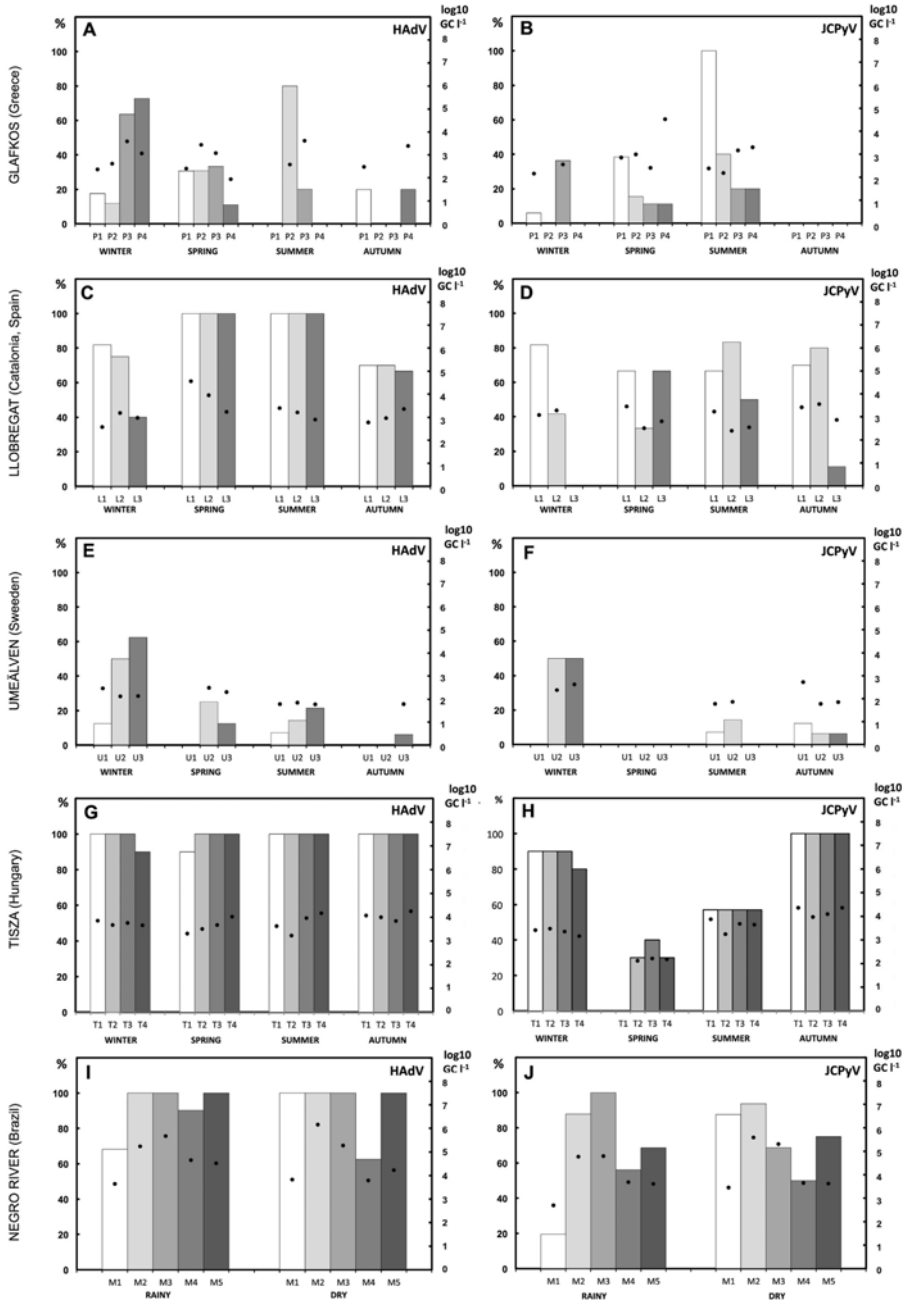

Fig. 2 – Quantitative results of human (HAdV and JCPyV) viral fecal markers in different water matrices from Glafkos river, potted in A, B, Llobregat river, C, D, Umeålven river, E, F, Tisza river, G, H, and Negro River I and J. In columns the prevalences (% of positive samples) in each sampling site.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

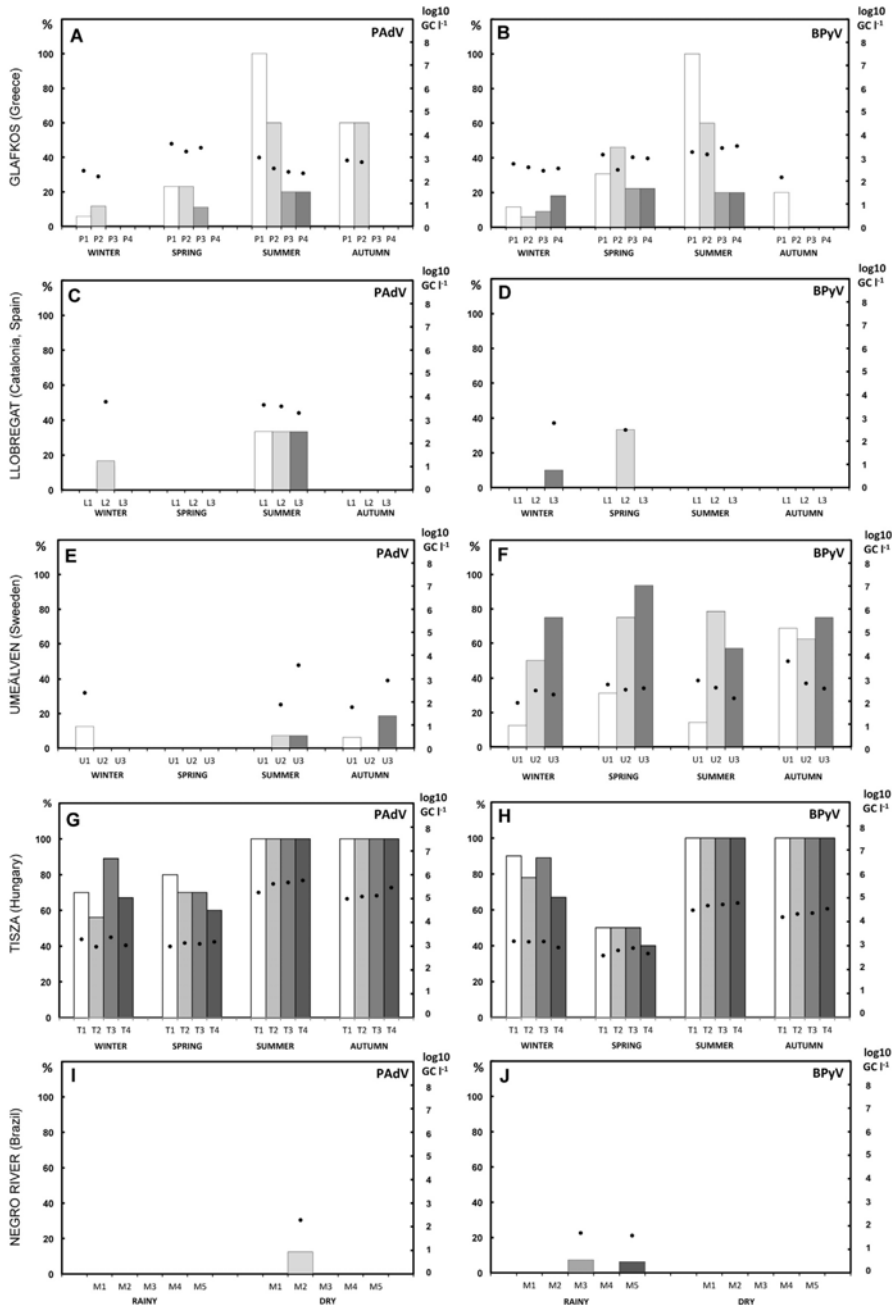

Fig. 3 – Quantitative results of the porcine (PAdV) and bovine (BPyV) viral fecal markers in different water matrices from Glafkos river, potted in A, B, Llobregat river, C, D, Umeålven river, E, F, Tisza river, G, H, and Negro River I, and J. In columns the prevalences (% of positive samples) in each 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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ARTICLE III

Desenvolupament d'una nova eina per detectar i traçar la contaminació d'origen oví a l'ambient

4.3. Desenvolupament d'una nova eina per detectar i traçar la contaminació d'origen oví a l'ambient

"Description of a novel viral tool to identify and quantify ovine faecal pollution in the environment"

Marta Rusiñol, Anna Carratalà, Ayalkibet Hundesa, Alex Bach, Anita Kern, Apostolos Vantarakis, Rosina Girones i Sílvia Bofill-Mas.

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El bestiar, i en concret els animals domèstics, contribueixen significativament en el deteriorament de la qualitat de l'aigua. Aporten gran quantitat de microorganismes a l'ambient i poden actuar de reservori de patògens humans. A nivell global, les poblacions més grans de bestiar són les de porcs, vaques pollastres i ovelles. Per tal de disposar d'eines que permetin fer una gestió integral de la contaminació fecal animal a l'ambient, durant els últims anys s'han dissenyat indicadors virals específics per poder traçar la contaminació fecal porcina, bovina i d'aviram a l'aigua. Tot i la importància del bestiar oví en moltes àrees geogràfiques, els indicadors disponibles són limitats i la seva especificitat és, en alguns casos, dubtosa.

Partint del fet que els poliomavirus són molt específics d'espècie, i que encara avui en dia se'n continuen identificant a diferents espècies de vertebrats, es va treballar sobre la hipòtesi de l'existència d'un poliomavirus específic d'ovella. Inicialment, es van testar orines d'ovella amb un assaig de PCR d'ample espectre que amplifica la regió VP1 dels poliomavirus. A partir de l'obtenció de seqüències de la regió VP1 d'un putatiu i nou poliomavirus oví, s'han desenvolupat i avaluat dos mètodes moleculars per a la detecció i quantificació de poliomavirus ovins en mostres ambientals. L'especificitat d'aquests assajos s'ha estudiat analitzant mostres femtes i orines de cabres, vaques, pollastres i porcs, així com d'aigua residual d'escorxador i aigua residual d'hospital i aigua residual

urbana. En tots els casos, es van obtenir resultats negatius. La sensibilitat dels mètodes s'ha avaluat analitzant repetidament, fins a 10 vegades, quantitats conegudes de ADN.

Amb aquestes noves eines s'ha detectat el nou marcador oví en un 69% de les mostres d'orina d'ovella a concentracions de fins a $1.6x10^5$ CG/L. Mostres d'efluents d'escorxadors d'ovelles, mostres d'aigua residual urbana on arriba l'aigua dels escorxadors i mostres d'aigua de dos rius diferents, amb presència de bestiar oví a les conques fluvials, també han resultat positives per al nou marcador. Així doncs, els dos assajos desenvolupats són una bona eina per detectar, quantificar de manera sensible i específica i caracteritzar genèticament la contaminació fecal ovina a l'ambient.

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Description of a novel viral tool to identify and quantify ovine faecal pollution in the environment

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HIGHLIGHTS

- A new ovine specific polyomavirus (OpyV) was identified.
- A qPCR specific and sensitive assay for the quantification of OPyV was developed.
- The new ovine marker was detected in environmental samples from different European regions.

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ABSTRACT

Farmed animals such as sheep, cattle, swine and poultry play an important role in microbial contamination of water, crops and food, and introduce large quantities of pathogens into the environment. The ability to determine the origin of faecal pollution in water resources is essential when establishing a robust and efficient water management system. Animal-specific viruses have previously been suggested as microbial source tracking tools, but specific ovine viral markers have not been reported before now. Previous studies have shown that polyomaviruses are host-specific, highly prevalent and are commonly excreted in urine. Furthermore, they have been reported to infect several vertebrate species but not sheep. That situation encouraged the study of a new putative ovine polyomavirus (OPyV) and its use to determine whether faecal pollution originates from ovine faecal/urine contamination.

Putative OPyV DNA was amplified from ovine urine and faecal samples using a broad-spectrum nested PCR (nPCR). Specific nested PCR and quantitative PCR assays were developed and applied to faecal and environmental samples, including sheep slurries, slaughterhouse wastewater effluents, urban sewage and river water samples. Successful amplification by PCR was achieved in sheep urine samples, sheep slaughterhouse wastewater and downstream sewage effluents. The assay was specific and was negative in samples of human, bovine, goat, swine and chicken origin. Ovine faecal pollution was detected in river water samples by applying the designed methods. These results provide a quantitative tool for the analysis of OPyV as a suitable viral indicator of sheep faecal contamination that may be present in the environment.

1. Introduction

Most environmental waters, soil crops and foods are susceptible to faecal contamination of human or animal origin, representing significant impacts on human/animal health and environmental management. The ability to determine whether microbial indicators or pathogens present in the environment originate from human or animal sources would enable better management of water pollution problems. Human faeces are more likely to contain human-specific enteric pathogens, but animals can also serve as reservoirs of standard bacterial faecal indicators and human pathogens. Thus, faecal contamination source tracking is essential to ensure its elimination, to minimise its impact or to identify uncontrolled spills (Scott et al., 2002). Current environmental microbial quality assessment is based on combining rapid screening methods and detailed source tracking techniques (Roslev and Bukh, 2011). The most commonly used microbial source tracking (MST) tools are bacteria such as coliforms, coliphages such as F-RNA phages, *Bacteroides* spp., *Rhodococcus coprophilus* or bifidobacteria, phages

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such as *Bacteroides fragilis*, and more recently, human and animal viruses such as human adenoviruses (HAdV) and polyomaviruses (JCPyV), porcine adenovirus (PAdV), bovine polyomavirus (BPyV) and chicken/turkey parvovirus (Ch/TuPV) (Carratalà et al., 2012; Hundesa et al., 2006; Fong et al., 2005; Maluquer de Motes et al., 2004; Bofill-Mas et al., 2000; Leclerc et al., 2000; Pina et al., 1998).

Viruses are excreted in high concentrations not only by infected human or animal individuals but also by healthy populations (Girones and Bofill-Mas, 2012). HAdV and JCPyV have been reported to be widely disseminated and persistent throughout the year (Bofill-Mas et al., 2000). Their high stability, host-specificity and high prevalence in different geographical areas support the use of HAdV and JCPyV for the identification and quantification of faecal contamination in the environment (Bofill-Mas et al., 2011a,b; Girones et al., 2010; Albinana-Gimenez et al., 2009).

Livestock contribute large quantities of faecal pollution in the environment. There is a need to ensure proper management of livestock manure, and of all animal-based organic fertilisers in general, in order to avoid contamination of ground water or water streams that may later be used as drinking water sources or for crop irrigation (Regulation (EC) no, 1774/2002). In fact, recent studies have related the presence of high levels of nitrates in groundwater with the presence of pig slurries in the surroundings of the sampled wells (Bofill-Mas et al., 2011a,b). Despite the widespread perception that human faecal pollution presents greater risks than animal pollution, in Catalonia alone (7.5 million people), over half the land area is used for livestock farming, with approximately sixteen million swine, half a million cattle, about one hundred and sixty million fowl and nearly two million sheep (Health Protection Agency of the Catalonian Government, 2010).

Among the potential sources of animal viral pathogens, cattle, swine, poultry and ovine slurries, slaughterhouses or faeces/urine deposited on grazing land may represent the main sources of animal faeces in the environment. *Campylobacter* spp. *Brucella melitensis*, and *Listeria monocytogenes* are some of the suspected pathogens having zoonotic infection routes from sheep to humans (Gilpin et al., 2008; Ramos et al., 2008; Czuprynski, 2005).

Depending on the soil structure, viral indicators can be rapidly transported below the surface and leach from the soil following rainfall (Aislabie et al., 2011). Viral indicators have been proposed and described in previous studies as tools to survey animal faecal contamination in water and food. PAdV, BPyV and Ch/TuPV have been proposed as specific faecal markers of swine, bovine and poultry populations, respectively (Carratalà et al., 2012; Hundesa et al., 2010; Hundesa et al., 2009). However, no specific ovine viral faecal indicator has been described until now.

Several studies have reported potential ovine faecal indicators, including adenovirus (OAdV), noroviruses (NoV GIII), F + RNA bacteriophages or *Cryptosporidium* (Wolf et al., 2010; Chalmers et al., 2002), but the described assays have been reported to be prone to detecting bovine faecal pollution. Recent studies have suggested that each vertebrate species could host its own set of PyV (Orba et al., 2011; Wellehan et al., 2011), so we employed a nested broad-spectrum PCR (Johne et al., 2005) that uses degenerated primer pairs to screen sheep urine samples from five different domestic sheep breeds. Once we detected polyomavirus-related sequences, we designed a specific nested PCR (nPCR) and a quantitative PCR assay (qPCR) for the specific detection and quantification of a new putative ovine polyomavirus (OPyV) in the environment.

2. Material and methods

2.1. Faecal, urine and water samples analysed

A total of thirty-two ovine urine samples together with fifty straw-bed and eighteen sheep stool pooled samples were collected from farm soil in nine different ovine farming areas in three different countries over a two-year period (2010–2011). Sheep urine samples were collected directly from individuals, and wet straw-beds and wet stools (pooled samples) were collected from the soils of sheep farms in nine different locations: Catalonia (six farms in the north east of Spain), the Basque country (one farm in the north of Spain), Szolnok (one farm in central Hungary) and Patras (one farm in the south of Greece). The mean herd size was 40 animals, but herd sizes ranged from 10 to 200 animals. Samples (Table 1) were collected from breeding and fattening animals at different ages. Numbering over one billion animals, domestic sheep (*Ovis aries*) are the most numerous species of sheep in the world. For this study, different breeds were selected, located in different geographical areas. *Ripollesa* and *Xisqueta* are autochthonous breeds located in the north of Catalonia (Spain). These breeds originated by crossing native sheep from the central Pyrenees with merino-type individuals. *Latxa* is a breed of domestic sheep native to the Basque Country of Spain. *Merino* is the main breed in Hungary and *Serraika* and *Boutsko* are the native breeds of Greece.

Raw and effluent wastewater samples and biosolids were collected during summer 2010 from a slaughterhouse and from a downstream sewage treatment plant (STP) which processes 100,000 inhabitant equivalents. During the same time period, raw sewage samples were collected from a hospital with more than 1000 beds located in Catalonia, on the assumption that these samples would not contain any animal effluents.

River water samples were collected from two river basins: the Llobregat River in Catalonia and the Glafkos River in Greece. The Llobregat River crosses Catalonia from the Pyrenees to the Mediterranean Sea and receives several STP effluents along its course. In the case of the Glafkos River, large sheep herds live outdoors beside the river and may pollute the natural waterway.

Cow urine, pooled porcine faeces, pooled chicken faeces, raw wastewater from bovine, swine and avian slaughterhouses which do not sacrifice sheep and raw sewage from a STP with no ovine-related effluents were collected to perform the PCR specificity assays. A set of sixteen faecal samples from domestic goats (*Capra aegagrus*) was collected directly from individuals and six wet straw-beds from goat farms (pooled samples) were collected from four different locations in Catalonia and Greece (Table 2). All urine, stool, sewage, effluent and biosolid samples were kept on ice and processed within 24 h.

2.2. Sample processing and DNA extraction

Viruses were concentrated from 14 ml of bovine, goat and sheep urine samples by ultracentrifugation (110,000 ×*g* for 1 h at 4 °C).

Table 1
Detection and quantification of ovine polyomavirus in urine/wet-straw-beds/stool and environmental samples by nPCR and qPCR at the VP1 region.

Sample	Positive/tested samples (mean values in genome copies/l or g)	
	Ovine VP1 nPCR	Ovine VP1 qPCR
Sheep urine	9/13	5/5 ($1{,}56 \times 10^2$)[a]
Sheep wet straw-beds	21/42	5/5 ($1{,}34 \times 10^2$)[a]
Sheep stool	7/13	5/5 ($7{,}60 \times 10^1$)[a]
Ovine slaughterhouse raw sewage	3/4	3/4 ($9{,}81 \times 10^2$)
Ovine slaughterhouse treated effluent	1/2	1/2 ($4{,}85 \times 10^1$)
STP influent (downstream ovine slaughterhouses)	3/5	3/5 ($6{,}51 \times 10^1$)
STP effluent (downstream ovine slaughterhouses)	2/5	2/5 ($6{,}06 \times 10^0$)
STP biosolids (downstream ovine slaughterhouses)	1/1	1/1 ($2{,}36 \times 10^1$)
Llobregat river water (Catalonia)	NT	1/8 ($1{,}05 \times 10^2$)
Glafkos river water (Greece)	5/8	3/4 ($1{,}14 \times 10^1$)

NT: not tested.
[a] All quantified samples were previously positive for the specific nPCR.

Table 2
Specificity of the ovine polyomavirus nPCR and qPCR assays. Positive samples vs tested samples.

Sample origin	Sample type	N	Ovine VP1 nPCR	Ovine VP1 qPCR	Other polyomaviruses (genome copies/ml)
Bovine	Individual urine samples	5	0/5	0/5	BPyV1 5/5 (8.34 × 101)
	Slaughterhouse sewage	4	0/4	0/4	BPyV1 5/5 (7.75 × 102)
Chicken	Pooled faeces representing less than 5 individuals	5	0/5	0/5	
	Slaughterhouse sewage	2	0/2	0/2	
Goat	Pooled faeces representing less than 5 individuals	6	0/6	0/6	
	Individual urine samples	10	0/10	0/10	
	Wet straw-beds	6	0/6	0/6	
Human	Hospital raw sewage	10	0/10	0/10	JCPyV2 10/10 (1.61 × 102)
	Urban sewage without ovine slaughterhouse effluents	4	NT	0/4	JCPyV2 4/4 (3.33 × 103)
Porcine	Pooled faeces representing less than 5 individuals	5	0/5	0/5	
	Slaughterhouse sewage	4	0/4	0/4	

NT: not tested. 1 bovine polyomavirus, 2 human JC polyomavirus.

The pellet was resuspended in 100 μl of PBS, and stored at −80 °C. Forty grammes of wet straw-bed samples was concentrated by elution in 20 ml of 0.25 N glycine buffer (pH 9.5), kept on ice and shaken for 30 min before centrifugation (9200 ×*g* for 15 min). Lastly, the supernatant was concentrated by ultracentrifugation (110,000 ×*g* for 1 h at 4 °C) and viral particles were resuspended in 100 μl of PBS and stored at −80 °C.

Viral particles from faeces were concentrated as previously described (Maluquer de Motes et al., 2004). Briefly, 1 g of each sheep, goat, swine or chicken sample was eluted in 3.5 ml of 0.25 N glycine buffer (pH 9.5), kept on ice for 30 min and then centrifuged (9200 ×*g* for 15 min). Finally, the supernatants were concentrated by ultracentrifugation (110,000 ×*g* for 1 h at 4 °C) and viral particles were resuspended in 140 μl of PBS and stored at −80 °C.

Sewage and effluent samples were concentrated as previously detailed (Pina et al., 1998). Briefly, 42 ml of wastewater was subjected to ultracentrifugation at 110,000 ×*g* for 1 h at 4 °C to pellet all the viral particles with other suspended solid materials. The pellet was eluted with 4 ml of glycine 0.25 N at pH 9.5 and shaken for 20 min at 4 °C. Suspended solids were separated by centrifugation at 12,000 ×*g* for 15 min. Finally, viruses were concentrated by ultracentrifugation at 110,000 ×*g* for 1 h at 4 °C and resuspended in 140 μl of phosphate-buffered saline and stored at −80 °C until nucleic acid extraction was performed.

River water samples were concentrated as recently described (Calgua et al., 2013). Briefly, 10 l samples were carefully acidified to pH 3.5 using HCl 1 N and conductivity was adjusted to 1.5 mS. Then, a pre-flocculated skimmed milk solution was prepared and 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 RT and flocks allowed to sediment by gravity for 8 h. Supernatants were carefully removed and the final volume, about 500 ml, containing the sediment, was centrifuged at 7000 ×*g* for 30 min at 12 °C. Pellets were dissolved with 10 ml phosphate buffer at pH 7.5 and stored at −80 °C until nucleic acid extraction was performed.

Viral DNA was extracted from all samples using the QIAmp Viral RNA kit (Qiagen, Inc., Valencia, CA) with the QIAcube automated platform. Positive and negative controls were included in all the nucleic acid extraction procedures. Finally, NA eluates were stored at −20 °C until used.

2.3. Broad spectrum PCR

In order to identify a new polyomavirus infecting ovine, we screened seventy-four sheep samples using a broad-spectrum PCR assay targeted specifically to the VP1 gene (Johne et al., 2005). DNA samples obtained from thirty-two urine and thirty-two wet straw-bed samples and from ten faecal pools were analysed. Putative PyV-like sequences of approximately 240 bp in the VP1-encoding region were detected. PCR fragments were purified using a QIAquick purification kit (QIAGEN, Inc.) and the purified amplicons were directly sequenced using the ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

2.4. Specific nested-PCR amplification

A set of specific PCR primers was designed and tested in duplicate in urine and wet straw-bed and stool samples (Table 3). Amplification reactions were established in a volume of 50 μl, containing 1× Gold buffer at 50 mM, $MgCl_2$ 25 mM, 25 mM of each deoxynucleotide, primers at 0.25 μM, 1 U of the AmpliTaq Gold DNA polymerase (Applied Biosystems, Inc.) with 10 μl in the direct or 1 μl in the 1-fold dilution of the extracted DNA. The first denaturation cycle was performed for 10 min at 95 °C. The conditions for the 35-cycle amplification were as follows: denaturing at 95 °C for 1 min, annealing at the corresponding annealing temperature (Table 3) for 1 min and extension at 72 °C for 1 min. Amplifications were followed by a final 10-min incubation at 72 °C. PCR products were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide.

2.5. Quantitative-PCR (qPCR) primer and probe set design

Sequences obtained applying the nPCR described above were aligned using the ClustalX2 programme (European Bioinformatics Institute, UK) in order to choose the most conservative region and design a specific primer and probe set to perform a qPCR assay. Five urine, five wet straw-bed and five stool samples which had previously

Table 3
Oligonucleotide primers and probe used for the detection and quantification of ovine polyomavirus.

Primer	Sequence (5′-3′)	Annealing temperature (°C)	Product size (bp)
Ov_FI	AGATGGCCTCTTTCTCACA	52	227
Ov_RI	TTTATCTCCAGTCATGGCC		
Ov_nFI	AGACATTGTGGGCATGATTA	54	162
Ov_nRI	TTCCAATCTGGGCATAAGAT		
qOv_F	CAGCTGYAGACATTGTGG	60	168
qOv_R	TCCAATCTGGGCATAAGATT		
qOv_P	5′FAM-ATGATTACCAAGCCAGACAGTGGG-3′BHQ-1		

tested positive in the specific nPCR assay were selected to run the designed qPCR, which used two primers and a fluorogenic TaqMan probe to amplify a 168 bp fragment of the VP1 gene of the putative OPyV.

Standard curves were generated by transferring pGEM-T Easy plasmid (Promega, Madison, WI, USA) containing the 168 bp fragment of the VP1 gene into *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA, USA). PCR was used to check that the transformed colonies contained the target sequence, and after purification with the QIAGEN Plasmid Midi kit (QIAGEN, GmbH Inc., Hilden, Germany), elutes were linearised with restriction enzyme EcoRI (Promega), and serial dilutions were performed with TE buffer to obtain standard dilutions ranging from 10^0 to 10^5 plasmid DNA molecules per 10 μl of sample in the qPCR reaction.

The designed probe was tagged with FAM (6-carboxyfluorescein) as the reporter dye at the 5′ end and BHQ-1 (Black-Hole Quencher 1) as the quenching dye at the 3′ end. The sequences of the primer/probe set are given in Table 3. Annealing temperatures as well as primers and probe concentrations were optimised by assaying primer concentrations ranging from 0.4 to 0.9 μM and probe concentrations ranging from 0.225 to 0.9 μM for each reaction.

PCR master mix reagents were prepared in a DNA-free working area, samples were loaded in a pre-PCR working area and standards were finally added in a separate laboratory. Amplifications were performed in a mixture containing 10 μl of DNA and 15 μl of TaqMan® Universal PCR Master Mix, 0.4 μM of each primer (qOv_F and qOv_R) and 0.225 μM of fluorogenic probe (qOv_P).

TaqMan® Universal PCR Master Mix is supplied in a 2× concentration and contains AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, passive reference, optimised buffer components and AmpErase® uracil-N-glycosylase. Following activation of the uracil-N-glycosylase (2 min, 50 °C) and activation of the AmpliTaq Gold for 10 min at 95 °C, 45 cycles (15 s at 95 °C, 20 s at 55 °C and 20 s at 58 °C) were performed with an MX3000P detector system (Stratagene, La Jolla, CA, USA).

Direct and one ten-fold dilution (1:10) of the extracted DNA was run in duplicate (4 runs/sample) for analysing environmental samples, while ten-fold serial dilutions of the qPCR standard were run in triplicate when quantifying viral genome copies (GC). In all the qPCRs carried out, the amount of DNA was defined as the mean of the data obtained. A non-template control was added to each assay. The faecal or urine samples tested by qPCR assays were quantified using duplicated undiluted and 10-fold dilution of the extracted DNA, producing more reliable results and minimising the effect of the potential inhibitors in the samples.

Depending on the starting volumes of water or the amounts of faecal matter analysed, and taking into account the several methods utilised, the quantity of sample analysed in one qPCR assay corresponded to 0.1 g of stools, 1 ml of urine, 3 ml of sewage or effluent and 17.5 ml of river water.

2.6. Specificity and sensitivity analysis

The specificity of the designed nPCR and qPCR assays was verified with samples collected from areas where no faecal contamination from ovine origin was expected to occur: bovine urine samples (n = 5) and bovine slaughterhouse wastewater (n = 4), chicken or turkey faecal pooled samples (n = 5), goat faeces (n = 6) and goat urine (n = 10) and wet straw-bed (n = 6) samples, hospital sewage (n = 10) and urban sewage (n = 4), porcine faeces (n = 5) and porcine slaughterhouse wastewater (n = 4). All samples suspected to be potentially positive for other animal-specific polyomaviruses were tested by the previously designed qPCRs. More specifically, bovine-related samples were tested for the presence of BPyV and human-related samples were tested for the presence of human JCPyV, both of which are already used as MST tools (Hundesa et al., 2010; Albinana-Gimenez et al., 2009). Known amounts of standard DNA containing 10^3, 10^2, 10^1, 10^0 and 10^{-1} OPyV GC/reaction were analysed ten times to determine the sensitivities of both specific nPCR and qPCR assays.

2.7. Sequence analysis

The amplicons obtained by nested PCR were purified using a QIAquick purification kit (QIAGEN, Inc.) following the manufacturer's instructions. After purification of the amplicons, both strains were sequenced using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS (Applied Biosystems), according to the manufacturer's instructions.

The obtained sequences were compared with the nucleotide sequences available in the Genbank using the BLAST algorithm from NCBI (National Center for Biotechnology Information), and were aligned with the ClustalX2 programme. The sequences reported in this paper have been submitted in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/index.html).

3. Results

3.1. Identification of a novel OPyV in ovine samples by a broad-spectrum PCR

The broad-spectrum PCR developed by Johne et al. (2005) amplified the genome of a putative new polyomavirus in sheep urine, wet straw-bed and stool samples. After analysing several *O. aries* breeds of different origin (*Ripollesa* and *Xisqueta* from Catalonia, *Latxa* from the Basque country, *Merino* from Hungary and *Serraika* and *Boutsko* from Greece), polyomavirus-related sequences were detected by the broad-spectrum PCR in 31/64 (48.4%) of the urine and wet straw-bed samples and in 1/10 (10%) of the analysed stools.

3.2. Sensitivity and specificity of the nPCR and qPCR assays

Sensitivity and specificity were analysed in the nPCR and qPCR assays. One DNA copy was detected by nPCR in 7 of the 10 assays but only in 3 with the qPCR assay. Ten DNA genome copies were detected in 100% of the performed nPCR and qPCR reactions. 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. The qPCR assay developed for the quantification of OPyV was shown to be specific both by the sequence analysis “in silico” of primers and probes considering nucleotide sequence databases (NCBI BLAST) and by experimental assays.

No positive samples were detected in any of the tested goat-related samples, urine, wet straw-bed or faeces (Table 2). All the hospital raw sewage samples also tested negative for the nPCR specific assay. No false positive results due to cross-reactivity with non-target DNA from the viruses infecting the various hosts analysed (bovine, chicken, goat, human and porcine) were detected with these assays.

3.3. Detection of the OPyV in sheep samples by the specific nPCR assay

An nPCR assay was developed in the polyomavirus VP1 region based on the sequences obtained by the broad-spectrum PCR. The developed nPCR assay proved to be specific for the new polyomavirus, which was detected in 9/13 (69.2%) of the urine and 21/42 (50%) of the wet straw-bed samples, and in 7/13 (53.8%) of the stool samples tested. The novel ovine polyomavirus was prevalent in all the geographical areas tested: 45% of the samples collected in Catalonia, 50% in the Basque country, 50% in Budapest and 62% in Greece tested positive.

3.4. Detection of OPyV in environmental samples by the specific nPCR assay

The nPCR was positive in three out of four sheep slaughterhouse wastewater samples and in one out of two tested samples of the same slaughterhouse effluent after a standard inorganic flocculation. When analysing downstream urban sewage treatment plant influents, 3/5 (60%) of the raw sewage samples and 2/5 (40%) of the treated effluents tested positive (Table 1). A very low level of inhibitors was observed in the undiluted samples.

3.5. Quantification OPyV from ovine and environmental samples

The qPCR was designed in the polyomavirus VP1 region based on the sequences obtained by the specific nPCR. The quantitative method was first used to quantify OPyV present in urine, wet straw-bed and stool samples which had previously tested positive in the nPCR. OPyV was also quantified in the slaughterhouse wastewater and effluents, as well as in urban sewage and secondary treatment effluents from the downstream STP. The qPCR assay amplified OPyV sequences in urine/faecal and slaughterhouse wastewater, STP sewage and effluent water samples. All mean concentrations and positive percentages are presented in Table 1. The analysis of environmental samples resulted in one positive result from the eight Catalan river water samples and five positive results from the eight Greek river water samples tested.

3.6. Nucleotide sequences and accession numbers

Eleven samples that tested positive in the Broad Spectrum nPCR assay were further studied by sequencing the obtained amplicons. No correlation was observed between either of the two sequences and the type, breed or geographical origin of the samples analysed. All the urine, wet straw-bed and stool sequences, obtained using the specific nPCR, from the Catalonian, Basque, Hungarian and Greek samples, as well as the sequences obtained from slaughterhouse wastewater (raw and effluent water), raw urban sewage with ovine effluents and the secondary effluent from the sewage treatment plant, resulted in a group of sequences with a shared identity of 99.5%. The positive river water samples were also sequenced and showed a 100% homology with the urine-related sequences.

A total of 23 samples were sequenced in this study, resulting in two groups of sequences differing by 4/215 nucleotides between them (98.1% similarity). The two sequences reported in this paper *Xisqueta* C1 and *Ripollesa* B5 presented high identities with other PyV. *Xisqueta* showed an identity of 74% with Bat PyV and 71% with goose haemorrhagic PyV and *Ripollesa* B5 81% identity with Bat PyV and 73% with the Chimpanzee PyV (*Pan troglodyte verus*). Both sequences have been deposited in the GenBank database under accession no. KC145150 for *Xisqueta* C1 and KC145151 for *Ripollesa* B5.

4. Discussion

A putative novel polyomavirus has been detected in sheep urine, wet straw-bed and stool samples by a broad-spectrum PCR assay. Samples tested in the study were collected from healthy animals, suggesting the asymptomatic presence and excretion of this virus in the sheep breeds studied (Hundesa et al., 2010; Bofill-Mas et al, 2000). The ICTV defines different polyomavirus species based on sequence identity; whole-genome nucleotide sequences with less than 81% identity can be classified as different species (Reimar et al., 2011). Therefore, further studies focused on genetic characterisation of the whole genome of the putative OPyV are being conducted at the moment to confirm that the detected virus constitutes a novel species within the Polyomaviridae family.

After developing specific and highly sensitive nPCR and qPCR, the putative novel OPyV was detected and quantified in sheep urine/faecal samples as well as in environmental samples. Both conventional and quantitative PCR assays were specific and able to distinguish ovine contamination but not other animal-originated contamination. Negative results were obtained when analysing slaughterhouse wastewater from bovine, porcine or chicken slurries, hospital sewage and urban sewage without any known ovine slaughterhouse effluent.

The DNA primer and probe sequences designed, in the polyomavirus VP1 gene region, appear to be highly specific and sensitive for OPyV. A high prevalence of OPyV was observed on the farms in all the geographical areas studied.

As expected, OPyV was detected in higher percentages in urine than in pooled stool samples, since the pattern of excretion identified in other polyomaviruses, such as the human JCPyV or the bovine BPyV, is persistently excreted in urine (Hundesa et al., 2010; Bofill-Mas et al, 2000).

With the developed assay, it was possible to trace ovine contamination from slaughterhouse raw wastewater (mean values of 9.81×10^5 GC/l) in the urban sewage treatment plant located downstream (mean values of 6.51×10^4 GC/l in raw sewage and mean value of 6.06×10^3 GC/l in the secondary effluent). Quantification of OPyV in slaughterhouse wastewater samples showed values, which were similar to human JCPyV concentrations detected in urban sewage in the same area (Bofill-Mas et al., 2011a,b). Similarly, the assay was able to detect ovine faecal contamination in the river water samples from an agricultural area in Greece receiving rainfall run-off and other discharges directly into the river basin (mean values of 1.14×10^4 GC/l). Depending on the recovery efficiency of the viral concentration method used for concentrating river water (Calgua et al., 2013) and the sensitivity of the detection methods designed, an amount of viral particles, ranging from 50 to 500 GC/l, should be present in river water samples in order to obtain a positive assay. On the Llobregat River, slaughterhouses are mostly located at the river mouth, so effluents are treated at STP located near the coast and the final effluent is discharged via an outfall to the sea. Most of the Llobregat River water samples tested negative since they were collected before receiving most of slaughterhouse discharges. Nevertheless, when sampling after Eid al-adha (feast of the sheep sacrifice) in which million sheep were slaughtered, OPyV was detected into the river water (1.05×10^2 GC/l).

5. Conclusions

The developed OPyV qPCR assay consistently detected the new viral marker in sheep urine samples, slaughterhouse wastewater and urban sewage, as well as in river water samples concentrated by applying a low cost and easy procedure (Calgua et al., 2013). The methods reported here have been proven to be specific and sensitive to detection of the specific ovine virus in environmental samples, being the first tools able to distinguish between ovine and bovine pollution. The sequences detected in different geographical areas were highly similar in their nucleotide sequence. Thus, the virus seems to be highly conserved. All these features indicate the potential use of the putative novel OPyV towards efficient microbial source tracking in countries where sheep is an important livestock.

The bovine polyomavirus (BPyV) is excreted in urine at mean concentrations of 2.21×10^4 GC/l and can be detected where intensive livestock and agricultural activities are present at concentration values of 3.06×10^2 GC/l (Hundesa et al., 2010). As the ovine marker is just as excreted in urine at mean concentrations of 1.56×10^5, we can presume that from the studied environmental samples it is a source tracking practical and effective marker for determining ovine faecal contamination.

This new MST tool, together with the previously described human and animal specific viral quantification tools, targeting human and porcine adenoviruses (HAdV and PAdV) and human and bovine polyomaviruses (JCPyV and BPyV) (Carratalà et al., 2012; Hundesa

et al., 2010; Hundesa et al., 2009; Bofill-Mas et al., 2000), represents a suitable toolbox to quantify human and animal livestock (porcine, bovine, poultry and ovine) faecal contamination in the environment.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.04.028.

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Supplementary table: Summary of the Broad Spectrum nPCR, OPyV VP1 specific nPCR and qPCR assays realized in the study.

Sample origin	Sample type	Nº samples tested	Broad spectrum nPCR	VP1 specific nPCR	VP1 specific qPCR
Sheep	urine	**32**	32	13	5[1]
	wet straw-beds	**50**	32	42	5[1]
	feces	**18**	10	13	5[1]
	raw slaughterhouse sewage	**4**	NT	4	4
	slaughterhouse treated effluent	**2**	NT	2	3
	STP influent (downstream ovine slaughterhouses)	**5**	NT	5	5
	STP effluent (downstream ovine slaughterhouses)	**5**	NT	5	5
	STP biosolids (downstream ovine slaughterhouses)	**1**	NT	1	1
Goat	Pooled feces representing less than 5 individues	**6**	NT	6	6
	Individual urine samples	**10**	NT	10	10
	Wet straw-beds	**6**	NT	6	6
Bovine	Individual urine samples	**5**	NT	5	5
	Slaughterhouse sewage	**4**	NT	4	4
Porcine	Pooled feces representing less than 5 individues	**5**	NT	5	5
	Slaughterhouse sewage	**4**	NT	4	4
Chicken	Pooled feces representing less than 5 individues	**5**	NT	5	5
	Slaughterhouse sewage	**2**	NT	2	2
Human	Hospital raw sewage	**10**	NT	10	10
	Urban sewage without ovine slaughterhouse effluents	**4**	NT	NT	4
Llobregat river water (Catalunya)		**8**	NT	NT	8
Glafkos river water (Greece)		**8**	NT	8	4

NT: not tested, [1] All quantified samples were previously positive for the specific nPCR.

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Corrigendum

Corrigendum to "Description of a novel viral tool to identify and quantify ovine fecal pollution in the environment" [Sci. Total Environ. 458–460(2013) 355–360]

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The authors regret that some of the concentrations in Table 1 are expressed in mistaken units although in the conclusions all values are correct. The correct table is:

The authors would like to apologize for any inconvenience caused.

Table 1
Detection and quantification of ovine polyomavirus in urine/wet-straw-beds/stool and environmental samples by nPCR and qPCR at the VP1 region.

Sample	Positive/tested samples (mean values in genome copies/l or g)	
	Ovine VP1 nPCR	Ovine VP1 qPCR
Sheep urine	9/13	5/5 (1.56×10^5)[a]
Sheep wet straw-beds	21/42	5/5 (1.34×10^5)[a]
Sheep stool	7/13	5/5 (7.60×10^4)[a]
Ovine slaughterhouse raw sewage	3/4	3/4 (9.81×10^5)
Ovine slaughterhouse treated effluent	1/2	1/2 (4.85×10^4)
STP influent (downstream ovine slaughterhouses)	3/5	3/5 (6.51×10^4)
STP effluent (downstream ovine slaughterhouses)	2/5	2/5 (6.06×10^3)
STP biosolids (downstream ovine slaughterhouses)	1/1	1/1 (2.36×10^4)
Llobregat river water (Catalonia)	NT	1/8 (1.05×10^2)
Glafkos river water (Greece)	5/8	3/4 (1.14×10^1)

NT: not tested.
[a] All quantified samples were previously positive for the specific nPCR.

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ARTICLE IV

Poliomavirus bovins i ovins per traçar respectivament la contaminació especifica de vaques i ovelles al medi

4.4. Poliomavirus bovins i ovins per traçar respectivament la contaminació específica de vaques i ovelles al medi.

"Assessing the source of the faecal contamination in fresh water samples from Canterbury and Southland regions in New Zealand"

Marta Rusiñol, Elaine Moriarty, Beth Robson, Susan Lin, Margaret Mackenzie, Erin McGill, Rosina Girones, Sílvia Bofill-Mas i Brent Gilpin.

Sotmès al 2014

Nova Zelanda és el país amb el nombre més alt d'ovelles (9) i vaques (2) per càpita. Aproximadament, hi ha 148 ovelles i 35 vaques per quilòmetre quadrat que pasturen lliurement durant tot l'any. Aquest números fan que el país, amb només 4.4 milions de persones s'enfronti a grans reptes per gestionar els residus animals. De fet a les regions amb més presència de bestiar s'ha detectat un increment de les malalties zoonòtiques transmeses per l'aigua i causades per *Cryptosporidium* spp. *Campylobacter* spp. o *Salmonella* spp (Moriarty, 2013).

Amb l'objectiu d'identificar les principals fonts d'origen de la contaminació fecal ovina i dotar als gestors d'eines per traçar la contaminació a l'ambient, en aquest treball, es van recollir 42 mostres d'aigua de riu de dues regions ramaderes, Canterbury i Southland, i es van analitzar tres tipus d'indicadors fecals relacionats amb el bestiar oví: virus específics d'ovella (OPyV), indicadors bacterians de rumiants (BacR) i ratios d'esterols/estanols fecals típics dels hervíbors. Com que les dues industries ramaderes més potents, en aquestes regions de l'illa del sud de Nova Zelanda, son la indústria bovina i ovina, també es va utilitzar el marcador viral de contaminació fecal bovina (BPyV). A més a més, es van analitzar indicadors virals, bacterians i químics específics de contaminació fecal humana a totes les mostres de riu i a 9 mostres d'aigua del riu que travessa la ciutat de Christchurch que, encara avui en dia, té una xarxa de clavegueram molt afectada pels forts terratrèmols del 2011 i 2012.

La contaminació fecal humana és la més prevalent a la regió de Canterbury (74% de les 31 mostres), mentre que al sud, la regió de Southland, presenta principalment contaminació fecal majoritàriament ovina (detectada al 100% de 11 mostres). Els HAdV han demostrat ser significativament més sensibles que la resta d'eines per traçar la contaminació fecal humana. Pel que fa la contaminació d'origen animal, el marcador bacterià BacR acompanyat d'alguna altre eina és la convinació més sensible. Tot i així, només quan s'utilitzen els indicadors virals específics d'ovella o vaca, s'aconsegueix identificar el tipus de contaminació animal.

Els indicadors de contaminació fecal humana han permès identificar contaminació d'origen humà a les zones més urbanitzades amb les concentracions més elevades durant els sobreeiximents del clavegueram en episodis de pluja. En qualsevol cas la qualitat de les aigües superficials en ambdues zones es veu fortament afectada per la gran quantitat d'explotacions ramaderes distribuïdes per tot el territori.

Assessing the source of the fecal contamination in fresh water samples from farming and urban regions in New Zealand.

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Keywords: Fecal source tracking (FST), river water, animal viruses, fecal sterols, bacteria molecular markers.

ABSTRACT

This study investigated the sources of fecal pollution in different rivers across two regions in New Zealand. Bovine and ovine livestock are considered to be important sources of fecal contamination to New Zealand's freshwaters. River water samples were collected along different river catchments within two important farming regions in New Zealand: Canterbury and Southland. Fecal pollution was initially measured by testing *E. coli*, then, human and animal specific fecal pollution sources were assessed with previously reported quantitative PCR assays targeting human-, bovine-, or ovine-specific viruses (Human adenoviruses (HAdV), human JC polyomaviruses (JCPyV), bovine polyomaviruses (BPyV) and ovine polyomaviruses (OPyV)) and human-, and ruminant-associated Bacteroidales DNA markers (BiAdo, BacHum-UCD, BacH and BacR). Fecal sterols and stanols were also measured and the human and ruminant indicative ratios were calculated. The human fecal viral marker (HAdV) was widely prevalent in urban and farming areas, and was the most frequently observed human marker. However animal fecal sources, when present,

were detected at higher concentrations. In contrast the bacterial ruminant marker (BacR), was the most frequently observed ruminant marker, but only OPyV and BPyV, were able to identify and discriminate between ovine and bovine fecal sources. The toolbox approach of *E. coli* measurement coupled with analysis of several FST markers targeting animal and human pathogens allowed identification of pollution sources, and therefore prioritization of interventions to be undertaken.

INTRODUCTION

Water quality is a much-debated issue in New Zealand, with grazing livestock considered to be the dominant source of fecal contamination in New Zealand's freshwaters (1). There are estimated to be 31 million sheep and 10 million cattle, with smaller numbers of other ruminants (2). Moriarty and co-workers, demonstrated that *Escherichia coli*, and *Enterococci* spp are able to survive in both sheep and cattle feces for extended periods of time when deposited on pasture (3). Thus, long survival periods, rainfall, farming practices, land topology and temperature will determine the pathways of pathogens to the waterways (4–7).

In order to manage the pollution, information is required on the sources of fecal pollution. There are increasingly large numbers of methods available, which can be used to identify the possible sources of fecal pollution (8–10). Among these, one approach is to extract total DNA from a water sample and examine the sample using the polymerase chain reaction (PCR) for DNA from source-specific anaerobic bacteria as *Bacteroides* spp. and *Bifidobacterium* spp. Human-specific PCR assays such as BiAdo (11), BacHum-UCD (12) and BacH (13) or the ruminant PCR marker, BacR (14), have been extensively used to identify fecal pollution (15–17). Cross amplifications have been observed among closely associated animal species, for example human with cats and dogs, and among animals with close digestive physiologies, such as human and swine (18). Use of each assay should take account of the sensitivity and specificity of each assay. For example BacH, has

demonstrated sensitivities up to 77% but specificities not higher than 53%, due to cross reactivity with diverse non-human fecal sources (19).

An alternative PCR approach is to evaluate the presence of specific human and animal viruses (20). Human adenoviruses (HAdV) and JC polyomaviruses (JCPyV) are the most commonly used human viral fecal indicators, as they are excreted by a high percentage of the human population and are highly prevalent all over the world in different environmental water matrices (21, 22). There are also animal viral fecal indicators that can be used to trace bovine, ovine, porcine and avian specific fecal pollution (23–26). Bovine (BPyV) and ovine polyomaviruses (OPyV) have a wide dissemination among livestock, although they do not produce clinically severe diseases (23, 24). A recent review by Bofill-Mas and co-workers summarized human and animal viral quantitative tools applied by the science community in different countries and diverse water matrices, and underlined the high host-specificity and high sensitivities of these viral tools (27).

Chemical FST methods can also be used to detect fecal pollution. Fecal sterols are a group of C27-, C28- and C29- cholestane based sterols found mainly in animal feces. The analysis of sterol composition has a quite distinctive fingerprint from one species to another and can also indicate relatively fresh fecal pollution. The sterol profile of feces depends on the interaction of three factors being the animal's diet; secondly, whether an animal synthesizes sterols and thirdly the types of anaerobic bacteria present in the animal gut which biohydrogenate sterols to stanols. Different fecal sterols/stanols ratios can be used to identify the main source of pollution in the water. The use of those chemical fecal markers assists the identification of fecal contamination sources when the contamination levels are low to moderate (28).

The aim of this study was to assess the source/s of fecal contamination using a variety of FST tools. We examined human FST markers in river water from an urban catchment and human and animal FST markers in two farming regions of New Zealand, characterized by dairy and sheep farming. By testing a range of human, bovine and ovine viral fecal indicators, human and ruminant bacterial markers and analyzing the fecal sterol ratios, we

point out the use of a complete toolbox, or combination of FST methods in order to facilitate appropriate management strategies.

MATERIALS AND METHODS

Sampling sites and samples

Water samples were chosen to represent pastoral streams, urban streams, rural bush, and urban streams including samples impacted by sewage overflow. We selected eight river catchments flowing through farmland around the Canterbury and Southland regions in New Zealand (Figure1). A total of 42 river water samples were collected from five Canterbury (Waimakariri, Selwyn-Waikirikiri, Ashley-Rakahuri, Hurunui, Waiau) and three Southland river catchments (Oreti-Hokonui, Mataura and Waituna), over the sampling period from May to July 2013. All sampling sites were characterized by intensive grazing (Figure 1).

The selected urban catchment was the Avon River that traverses the city of Christchurch with approximately 375,000 inhabitants. It is susceptible to intermittent impacts from industrial and human effluents as well as from animals including dogs and wildfowl. Three water samples from three sites along the Avon River (Figure 2): Boat Sheds (BS) in the city centre, Kerrs Reach (KR) beside the Rowing clubs and at Owles Terrace (OT) where it flows into the sea, were collected during two low flow conditions (March 11th and 25th) and one rainfall impacted flow (May 17th). Wastewater overflows into the Avon were reported during the last sampling event. Table 1 summarizes the number of samples collected in each sampling location.

Fecal Source Tracking (FST) tools

Microbial water quality was primarily measured by testing for the indicator bacteria *E. coli*. The identification of the specific human and animal fecal sources was done with human and animal viruses, bacterial DNA markers and fecal sterols. A summary of the FST tools applied in each sampling region is presented in Table 1.

Escherichia coli

One-litre samples were collected and serial dilutions were prepared using peptone water (0.1%; Fort Richards Laboratories, Otahuhu, New Zealand). *E. coli* was enumerated by standard pour-plate method (31) using selective agar. Duplicate samples (1ml) from appropriate dilutions were added to a sterile agar plate and molten BrillianceTM *E. coli*/Coliform Selective Medium (Oxoid, UK) was added (15 ml). *E. coli* plates were incubated at 30°C for 4hrs followed by 37°C for 20hrs, and then blue-violet colonies were counted.

Bacterial DNA markers

DNA was extracted from 100 mL river water samples filtered through a Supor 200, 0.2 µM polyethersulfone (PES) filter (Pall Corp. Washington Port, NY, USA). The filter was transferred to a 50 mL falcon tube, and 1 mL of guanidine isothiocyanate (GITC) buffer (5 M guanidine thiocyanate, 0.1M EDTA, 0.5% sarcosyl) was added, after which the filter was frozen at -20°C. After thawing and repeated vortexing of the filter DNA extraction was performed on the filter using the Qiagen DNeasy Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. During each sample extraction procedure, a blank of purified water was extracted to monitor for DNA contamination. Four different qPCR assays were selected. Three of them human-specific: BiAdo (11), BacHum-UCD (12) and BacH (13) were analysed in 9 urban and 28 farming regions samples, while the BacR (14), specific for ruminants, was tested in the 28 farming regions samples (Table 1). All PCR amplifications were performed with 2xLightCycler 480 Probes Master mix (Roche Diagnostics Ltd) in a LightCycler 480® (Roche Diagnostics Ltd) according manufacturer instructions.

Viral fecal indicators

Specific human and animal viruses were used to test all environmental water samples. The human (HAdV), bovine (BPyV) and ovine (OPyV) viral fecal indicators were analyzed in all the farming sites (n=42) and urban locations (n=9) where the human JC poliomavirus (JCPyV) was also included in the analyses (Table 1). Ten-liter river water samples were collected in duplicate, and concentrated by the Skimmed Milk flocculation protocol (30).

Briefly, the samples were adjusted to a pH of 3.5 and conductivity of 1.5 mS/cm2. Pre-flocculated skimmed milk solution (PMS) was added to each of the previously conditioned samples to a final concentration of 0.01% (w/v). Samples were stirred for 8 h and flocks were allowed to sediment by gravity for 8 h. Supernatants were removed leaving a volume of approximately 50 mL of liquid and sediment, which was centrifuged at 7,000 x g for 30 min. Pellets were dissolved in 10 ml phosphate buffer at pH 7.5. The viral DNA extractions were performed with the Viral Nucleic Acid Extraction Kit II (Geneaid Biotech Ltd., Taiwan). A negative control (Gibco UltraPure water (Invitrogen)) was included in all the nucleic acid extractions.

Specific real-time qPCR assays were used to detect and quantify HAdV, JCPyV, BPyV and OPyV (23, 24, 31, 32). Plasmid DNA was used 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 168-bp sequence of the VP1 gene was used for OPyV. 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. All reactions were performed in a 25µL volume of PerfeCta® qPCR ToughMixTM (Quanta BioSciences Inc., Forest City, CA). Undiluted and a 10-fold dilution of the nucleic acid extract were analyzed in duplicate and ten-fold serial dilutions of the related qPCR standard were run in duplicate when quantifying viral genome copies (GC). More than one non-template control (NTC) was included to prove that the qPCR mix did not produce fluorescence. Dilutions of the standard DNA were used to spike selected representative samples in order to evaluate potential enzymatic inhibition due to inhibitors present in the studied samples as well as Gibco UltraPure water (Invitrogen) as negative process control in order to prove no cross contamination occurred.

Fecal sterols and stanols

The analysis of fecal sterols and stanols included: coprostanol (5β-cholestan-3β-ol), cholestanol (5α- cholestan-3β-ol), 24-ethylcholesterol (24-ethyl cholest-5en-3β-ol) and its biohydrogenated product, the 24-ethylcoprostanol (24-ethyl 5β-cholestan-3β-ol). Fecal sterols and stanols were analyzed in 28 farming sites and in the 3 sites along the Avon

River, a total of 37 samples. Sterol extraction from water included two stages: liquid-liquid extraction and derivitization. Four liter river water samples were filtered through Whatman® glass microfiber filters (grade GF/F 47mm) (Sigma-Aldrich, St. Louis, MO, USA). Filters were stored frozen until they were analyzed using the extraction procedure of Mudge and Norris (33). Filter paper were spiked with a deuterated internal standard and refluxed with 6% methanolic KOH for 4 hours. The supernatant containing the hydrolyzed sterols was cleaned by centrifugation, and evaporated to dryness. Each sample was derivatized, a system-monitoring compound added, and analyzed by gas chromatography with mass spectrometric detection (Perkin Elmer Clarus 500 (USA)). Interpretation of the sterol is based on comparisons of ratios of key sterols. The ratios used in this study include: four indicators of human fecal sources (H1: coprostanol / (coprostanol and cholestanol), H2: coprostanol / (24-ethylcoprostanol and 24-ethylcoprostanol), H3: % coprostanol/ total sterols and H4: % coprostanol / coprostanol) and three herbivore fecal ratios (R1: % 24-ethylcoprostanol, R2: coprostanol / (coprostanol and 24-ethylcoprostanol) and R3: 24-ethylcholesterol / 24-ethylcoprostanol).

Statistical analysis method

Most quantitative statistical methods require normally distributed data. However, as with most environmental data, there is a range of values below the limit of detection or samples which values are too high, resulting in non-normal distribution. Quantitative values were obtained by the viral specific qPCR assays, whereas different qualitative magnitudes were reported with the bacterial PCR assays and the fecal sterol ratios. Thus, to overcome those differences, two approximations were undertaken. First of all, data was expressed in ranks from 0, when non-detected results were obtained, to 3 when the viral concentration, bacterial PCR signal or fecal sterol ratio was in the maximum values. In a second test, we considered a 0 when non-detected results and 1 when detected, in order to find possible association across the assays. Twenty-eight of the forty-two river water samples from the farming regions in Canterbury and Southland were tested for all the assays. The ranks and the results for each assay are specified in table 4. The statistical analysis was performed using R software version 2.15.1 (34). The data was subjected to a Goodman-Kruskal gamma test that measures the rank of correlation between data when

ranked by quantities. This test, extracts more information from the data through the use of multivariate statistical methods in a single analysis. Results were considered to be significant with a p of <0.01 and values less than the detection limit were considered as 0. Correlation values (τ) ranged from negative association, -1, to positive association, 1. A value of zero indicated the absence of association.

RESULTS

Ocurrences of viruses and *E. coli*

Farming areas: Canterbury and Southland

Mean *E. coli* counts and the human and animal viral concentrations are summarized in table 2, with the raw data presented in the supplementary information. Ten of the forty-two samples exceeded the regulatory thresholds (>550 *E. coli*/100ml) for freshwater recreational purposes (35). Specific-human fecal pollution (HAdV) was detected in all samples from the Waiau, Hurunui and Ashley-Rakahuri river catchments, and frequently in the other two rural Canterbury river catchments (23/31 samples). In the Southland region, only one of the Oreti river water samples was positive for the HAdV, with the sample taken where the river meets the city of Invercargill. The animal fecal pollution in the Canterbury plans was scattered across the region sampling sites. All river catchments presented both bovine and/or ovine fecal pollution and only 4/31 samples had both sources at the same time. The highest OPyV concentration (151 GC/L) was detected in the Waiau River whereas the highest levels of BPyV were found in the Selwyn district, the Waikirikiri River catchment (81 GC/L). The ovine-specific fecal marker was detected in all Southland samples at concentration levels ranging from 43 to 3500 GC/L. Pollution from bovine livestock was less prevalent, 3/11 samples, but in the Mataura catchment BPyV concentration rose up to 1200 GC/L.

Urban area, the Avon River

The Avon River rises in the suburb of Avonhead and flows across Hagley Park and the central district of Christchurch till the eastern suburbs. After the 2010/2011 Christchurch

earthquakes direct sewage discharges occurred in the Avon River and since then a fragile wastewater system results in intermittent sewage leakages. In this urban environment, the *E. coli* counts exceeded the present legislation in all the water samples (36). Kerrs Reach (KR) had the highest *E. coli* per 100 ml on each sampling occasion (Table 3). Specific fecal pollution from human origin was detected in all the sampling sites along the river. HAdV was highly prevalent (6/9 samples), being detected in all the KR samplings. JCPyV was less prevalent (3/9 samples), but as it is highly human-specific it was used to confirm the human contamination. Owles Terrace presented intermittent human fecal pollution but, when present, both viral concentrations were higher than in other sites. During high flow conditions, third sampling day, HAdV were detected in all sampling sites: BS, KR and OT.

Viral indicators, bacterial markers and fecal sterol ratios to assess the fecal source origin

A total of 28 of the 42 water samples from farming areas, were tested for three human- and animal-specific methods: viruses, bacterial markers and fecal sterols (Table 4). Mean *E. coli* counts and qualitative results of the MST methods are summarized in table 4, with the raw data presented as supplementary information. Animal fecal sources were detected in a 96% of the tested samples (27/28) and only 4 of the 28 water samples did not tested positive in any human-specific assay. Nevertheless, human fecal pollution was frequently detected with HAdV that were identified in 75% of the samples. Also BiAdo, BacHum-UCD and BacH were found in 42%, 11% and 31% of the water samples whereas human fecal sterol ratios were only found in 3 water samples. In general, 11 of the 21 human polluted samples could be identified only with the HAdV assay. Viral and bacterial markers were able to detect human pollution in the same water samples (n=7) and only 3 of the samples were confirmed by the three methodologies. Nearly 85% of all surface water analyzed in Canterbury presented ruminant pollution by the BacR assay. Fecal sterol ratios allowed the identification of ruminant fecal pollution in 21 of the 28 samples analyzed with this methodology. Animal specific-viruses were able to identify bovine and/or ovine fecal sources in a 57% of the 28 samples. Here, most of the samples could be confirmed by more than one methodology. All three methods were able to identify animal pollution in 11 of the 28 samples.

When sewerage overflows, the three methods were able to identify human fecal pollution in the more downstream sites (KR and OT) (Table 3). In general, high ratios of coprostanol: 24-ethylcoprostanol suggested human fecal contamination, identified in 78% (7/9) of the samples.

Analyses and correlations between different FST tools

The number of positive samples by one, two or three methodologies is summarized in Table 5. In general terms, animal fecal pollution was highly prevalent in all the water samples. It was detected with at least one of the FST tools in 93% of the samples whereas human fecal pollution was detected in the 86%. Viral and bacterial fecal indicators were able to identify the same origin of the fecal pollution in 50% of the analyses. Using the qualitative results in the supplementary table, data was expressed in ranks (Table 4), from 0 for the non-detected to 3 for the strongly detected assays and correlations between the FST tools were calculated. Significant correlations (p-value<0.01) were found between two bacteria PCR markers, BiAdo and HumBac-UCD (γ: 0.87 and standard error: 0.32), and between one bacteria marker and the fecal sterol ratios of human pollution, BacH and human fecal steriol ratios (γ: 0.89 and standard error: 0.31). The same correlations were observed when analyzing qualitative data: detected (1) and non-detected (0). No other significant association was found across data.

DISCUSSION

The primary sources of water quality degradation seem to be highly dependent on the land use. Although human fecal pollution was persistently detected in more than the half of the country farmland water samples, animal fecal pollution was present more frequently. The recently described ovine-specific fecal indicator, OPyV, together with the BPyV, were found to be efficient tools for discriminating the two major ruminant pollution sources in New Zealand. Both, ovine and bovine fecal sources were highly prevalent in both farming regions, particularly in Southland where 100% of the tested samples were positive for the ovine fecal marker. Different livestock management practices climate, rainfall or soil type could explain different prevalence between both regions. For

example, *E. coli* levels can be lower where livestock graze within fenced parcels, because it excludes livestock from stream (37). Collins also stated that on hill country farmland, where animals are spread out, the topography contribute to significant surface runoff and groundwater convergence delivering microbes to waterways (7). What is clear is that ubiquitous farming activities strongly affect the quality of water.

Human sewage is also partly responsible for the degradation of water quality in urban and even in rural catchments. In the urbanized Avon river catchment, intermittent human fecal pollution was present in the river water. Rainfall resulted in significant degradation of the microbial water quality of the river due to occasional sewage inputs from the sewerage overflows. The water quality in KR was also unsatisfactory, in terms of public health risk, with average *E. coli* concentrations close to those seen during the active discharges post-earthquakes (36). The infrastructure work post-earthquakes (build repair/demolition and wastewater pipe repair) could explain the presence of human fecal sources in the Avon. Bacterial PCR markers and viral fecal indicators can persist more extended periods than *E. coli* (20, 38). Thus, they are suitable to predict the sources fecal contamination. Human or/and animal fecal sources were identified in all the samples, except one from Selwyn-Waikirikiri, with *E. coli* levels near the limit of detection (15 cfu/100mL). Individually, fecal sterols and bacterial PCR markers are unlikely to give a complete picture of fecal pollution (39). Where sterols indicated a human source, bacterial and viral markers also indicated a human source supporting the validity of this tool. The same conclusion can be made for human bacterial markers relative to viral markers in 10/13 samples with the three samples positive for human bacterial markers, but not human viral markers, were only very weakly positive. The eleven samples only positive with human viral markers may indicate a greater sensitivity of viral markers, and/or be a consequence of larger volume of water analysed with viral markers. In contrast the bacterial ruminant BacR marker was the most prevalent ruminant marker detected (24 samples), with twenty of these confirmed by sterol analysis, and 14 with the bovine or ovine viral markers. This would suggest ruminants other than cattle and sheep, as likely source in samples positive with BacR and/or fecal sterol ratios but not viral

markers (10/21). When searching for significant correlations, only BacHum-UDC with BiAdo and BacH with the human fecal sterol ratios presented significant *p*-values.

Several FST field studies applied multiple assays to evaluate fecal pollution sources in order to find the "perfect" combination. Usually, the most sensitive method is not always the most specific, and moreover when performed in the field it might not be the most rapid and cost-effective. Most of the studies in literature have only compared performances between human associated markers (15, 40). Here we examined by chemical and quantitative methods, both human and animal fecal pollution. The use of multiple methods to identify the animal sources is valuable because each assay may have particular strengths and weaknesses. For example, BacR, is ruminant specific, but can also detect horse or opossum samples (15) which are also frequent in New Zealand. Nevertheless, the use of multiple markers has been helpful in interpreting the origin of the fecal pollution, but only the two assays targeting bovine and ovine polyomaviruses were able to distinguish between both fecal sources in water.

While other countries may have larger sheep and dairy farming operations, New Zealand has the highest ratio of animals per capita in the world, with 9.1 sheep and 2.3 cattle per person (2). Agriculture intensification and an expansion of intensive dairying may make it challenging for rural communities to access safe drinking water, and maintain clean streams. Sensitive, specific and reliable FST techniques are important tools that will contribute to overcoming this key challenge.

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Figure 1: Sampling areas, farming sites across three Southland river catchments: (O) Oreti-Hokonui, (N) Waituna, (M) Mataura; and five Canterbury river catchments: (S) Selwyn-Waikirikiri, (W) Waimakariri, (A) Ashley-Rakahuri, (H) Hukunui and (U) Waiau.

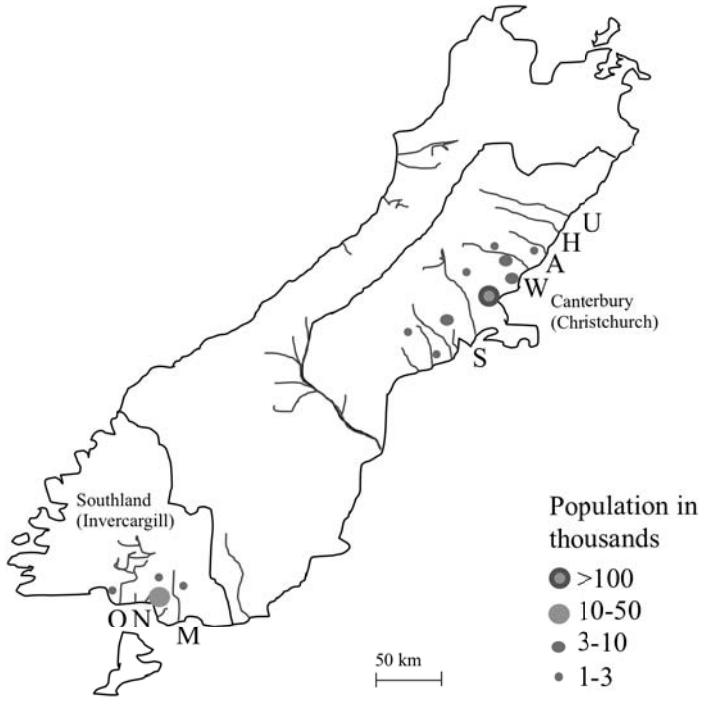

Figure 2: Christchurch city, Avon river sampling sites: (BS) Boad sheds (KR) Kerrs reach and (OT) Owles terrace near the sea.

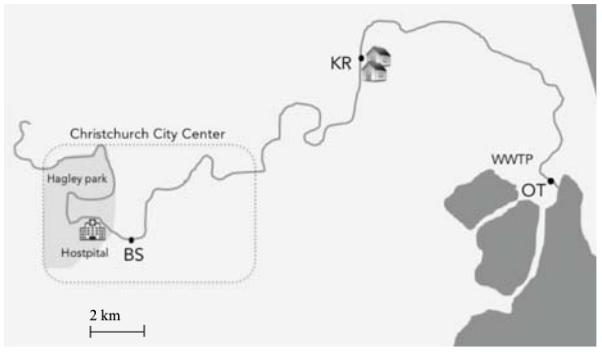

Table 1: Number of analyses per sampling site and FST tool

		River catchments	Southland			Canterbury								analyses/ tool
			Oreti - Hokonui	Mataura - Piano flat	Waituna - Waikawa	Waimakariri[1]	Selwyn - Waikirikiri[1]	Ashley - Rakahuri	Hurunui	Waiau	Avon River (Christchurch city) Boat sheds[2]	Kerrs Reach	Owles terrace	
		Sites	4	4	3	8	8	8	5	2	1	1	1	
		Samples	4	4	3	8	8	8	5	2	3	3	3	
Viral qPCR fecal indicators	human	HAdV	4	4	3	8	8	8	5	2	3	3	3	51
		JCPyV	NT	NT	NT	NT	NT	NT	NT	NT	3	3	3	9
	bovine	BPyV	4	4	3	8	8	8	5	2	NT	NT	NT	42
	ovine	OPyV	4	4	3	8	8	8	5	2	3	3	3	51
Bacteria FST PCR markers	human	BiAdo	2	NT	NT	5	6	8	5	2	3	3	3	37
		HumBac	2	NT	NT	5	6	8	5	2	3	3	3	37
		BacH	2	NT	NT	5	6	8	5	2	3	3	3	37
	rumiant	BacR	2	NT	NT	5	6	8	5	2	NT	NT	NT	28
Chemical FST markers	faecal sterol ratios		2	NT	NT	5	6	8	5	2	3	3	3	37
FIB	*E.coli*		4	4	3	8	8	8	5	2	3	3	3	51
Analyses/sampling location			26	16	12	57	62	72	45	18	24	24	24	380

NT: non tested.

[1] Additionaly, Waimakariri and Selwyn-Waikirikiri catchments were tested for the porcine viral indicator (PAdV)

[2] Additionaly, Avon river samples were also tested for the chicken and turkey viral markers (Ch/TyPV)

Table 2: Summary of *E. coli* counts, viral quantifications and percentatges of positives amples in river catchments from farming aresa within Southland and Canterbury regions.

Farming region and river catchments		n	*E.coli* cfu/100mL % of positive	Specific viral fecal markers Genome Copies /L and % of positive		
				human HAdV	bovine BPyV	ovine OPyV
Canterbury	Waiau	2	77 100%	47 100%	9 50%	151 50%
	Hurunui	5	141 100%	388 100%	6 20%	43 60%
	Ashley - Rakahuri	8	146 100%	147 100%	31 25%	64 38%
	Waimakariri	8	59 100%	106 50%	6 25%	96 13%
	Selwyn - Waikirikiri	8	94 100%	222 50%	22 38%	12 25%
Southland	Mataura	4	175 100%	ND 0%	1202 25%	486 100%
	Waituna	3	180 100%	ND 0%	37 33%	2203 100%
	Oreti - Hokonui	4	1060 100%	1264 25%	87 25%	169 100%

ND: non detected

Table 3: Human FST data on the unrbanized Avon River water samples.

Sample ID			FIB (cfu/100mL)	Viral FST qPCR markers (Genome Copies /L)		Bacteria qPCR Markers (+ very weak, ++ weak, +++ positive)			Faecal Sterols*			
			E.coli	HAdV	JCPyV	BiAdo	BacHum-UCD	BacH	H3>1	H1>5	H2>0.7	H4>75
Christchurch urban area	Boat sheds	11-Mar	1055	ND	ND	ND	ND	+	1.3	3.9	0.35	56.8
		25-Mar	900	ND	ND	+++	ND	+	0.9	3.2	0.48	47.4
		17-May	1150	59	ND	+++	ND	+	0.7	4.3	0.52	41.0
	Kerrs Reach	11-Mar	5000	54	ND	ND	ND	ND	1.0	1.3	0.20	50.8
		25-Mar	1070	36	ND	+++	+	+	1.4	3.1	0.41	58.9
		17-May	4500	33	40	+++	ND	+	1.0	3.4	0.22	50.8
	Owles terrace	11-Mar	650	109	3	ND	ND	ND	1.7	1.9	0.27	63.6
		25-Mar	750	ND	ND	+	ND	ND	1.1	1.2	0.29	53.3
		17-May	2400	634	611	+++	ND	ND	1.6	4.5	0.54	61.5

ND: non detected.

*Shading indicates that the sample meets the criteria for the ratio, H3: % coprostanol/total sterols, H1: coprostanol /coprostanol and cholestanol, H2: coprostanol / 24-ethylcoprostanol, H4: %coprostanol /coprostanol and 24-ethylcoprostanol.

Table 4: FST results in diferent river river catchments from farming areas within Southland and Canterbury regions. Blank spaces are tested samples below the limit of detection. V: viral[1], B: bacterial[2] ,FS: fecal sterols/stanols[3].

ID	V	B	FS	V	V	B	FS
	HUMAN			BOVINE	OVINE	RUMINANT	
1	++	++	+	+	+++		
2	++					+	
3	+++	++		+	++	++	++
4	+++	+				+	+
5	+++	+++			++	+	+
6	+++	+++			++	++	++
7	++	++	+			+	+
8	+++	++			+++	++	
9	+++					++	+++
10	++					+	+
11	+++					+	+++
12	++					+	+++
13	++	+++	+++		++	+	
14	+++			++		+	+
15	++			++	++	+	
19		+		+		+	+
20	++						
21	+++	++		+		+++	++
22						++	++
23						++	++
26	+++				++		+
27							
28	++					+	++
29	+++			++		++	+++
30				+		++	+
31	+++	+++		++	+	+++	++
40		+			+++	++	++
41		+		++	++	++	++

[1] Quantitative results of the viral specific qPCR assays: + (1-10GC/L), ++ (10-100GC/L), +++ (100-1000GC/L).

[2] Results of the qPCR assays: + (very weak), ++ (weak), +++ (positive). Human bacteroidales results are the summary of the three qPCR markers (BiAdo, HumBac and BacH).

[3] Summary of human (H1, H2, H3, H4) and ruminant (R1,R2, R3) fecal sterol/stanol ratios: + (very weak), ++ (positive), +++ (strong positive).

*Shading indicates human- or animal- specific fecal sources were identified in the water sample with the three different methodologies.

Table 5: Summary of positive samples by one, two or three MST methodologies. V: viral markers, B: bacterial markers, FS: fecal sterol ratios.

		Summary of positive samples			
		1 method	2 methods	3 methods	Total
Human	V	11	7	3	21
	B	3	7	3	13
	FS	0	0	3	3
Animal	V	1	4	11	16
	B	1	11	12	24
	FS	0	10	11	21

Supplementary information: Summary of human and animal FST results in all farming sites within Canterbury and Southland regions.

Region and river catchments		Samples	FIB (cfu/100mL)	Specific viral fecal markers (Genome Copies /L)			Molecular markers (+ very weak, ++ weak, +++ positive)				Faecal Sterols and stanols						
				human	bovine	ovine		human		ruminant	human %				ruminant %		
			E. coli	HAdV	BPyV	OPyV	BiAdo	BaHum-UCD	BacH	BacR	H3 >1	H1 >5	H2 >0.7	H4 >75	R3 <1	R1 >5	R2 <30
Canterbury	Waiau	1	45	39	9	151	+	ND	+	ND	0,8	2%	1,0	43,5%	7,3	2,0%	43,5%
		2	132	56	ND	ND	ND	ND	ND	+	1,0	3%	0,6	48,7%	6,7	3,0%	48,7%
	Hurunui	3	79	367	6	47	+	ND	+	++	0,3	2%	0,6	22,6%	4,5	7,1%	22,6%
		4	43	838	ND	ND	+	ND	ND	+	0,3	0%	0,3	25,0%	8,3	1,4%	25,0%
		5	77	905	ND	28	++	ND	+	+	0,3	2%	0,4	20,4%	4,2	6,3%	20,4%
		6	1795	643	ND	61	+	ND	++	++	0,2	4%	0,7	16,6%	1,1	22,2%	16,6%
		7	120	49	ND	ND	+	ND	+	+	0,1	0%	1,0	7,6%	14,8	3,3%	7,6%
	Ashley - Rakahuri	8	181	538	ND	124	+	ND	+	++	0,5	1%	0,4	31,8%	16,2	2,2%	31,8%
		9	92	688	ND	ND	ND	ND	ND	++	0,3	2%	0,5	21,0%	0,7	8,7%	21,0%
		10	72	33	ND	ND	ND	ND	ND	+	0,1	1%	0,3	12,2%	11,5	5,3%	12,2%
		11	42	488	ND	ND	ND	ND	ND	+	0,2	2%	0,4	14,1%	0,7	10,5%	14,1%
		12	850	55	ND	ND	ND	ND	ND	+	0,3	2%	0,4	23,2%	2,3	7,3%	23,2%
		13	515	50	ND	60	+	+	+	+	3,3	3%	0,7	76,5%	55,2	0,8%	76,5%
		14	48	417	43	ND	ND	ND	ND	+	0,2	1%	0,5	19,1%	10,1	4,9%	19,1%
		15	199	32	22	36	ND	ND	ND	+	0,7	3%	0,4	42,1%	6,8	3,8%	42,1%
	Waimakariri	16	30	ND	ND	ND	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		17	117	30	ND	96	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		18	92	194	ND	ND	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		19	45	ND	3	ND	ND	ND	+	+	0,2	1%	0,4	18,4%	8,0	4,5%	18,4%
		20	23	45	ND	ND	ND	ND	ND	ND	0,9	0%	0,1	48,0%	237,2	0,2%	48,0%
		21	175	489	10	ND	+	ND	+	+++	0,2	2%	0,4	18,9%	4,3	7,4%	18,9%
		22	28	ND	ND	ND	ND	ND	ND	++	0,3	2%	0,5	23,5%	4,6	6,1%	23,5%
		23	88	ND	ND	ND	ND	ND	ND	++	0,3	4%	0,7	25,0%	2,0	10,7%	25,0%
	Selwyn - Waikirikiri	24	73	ND	ND	ND	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		25	84	ND	ND	ND	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		26	70	286	ND	77	ND	ND	ND	ND	0,5	3%	0,5	34,6%	3,0	5,3%	34,6%
		27	32	ND	ND	ND	ND	ND	ND	ND	0,6	1%	0,5	37,5%	21,7	2,2%	37,5%
		28	104	52	ND	ND	ND	ND	ND	+	0,3	3%	0,5	24,3%	2,5	8,2%	24,3%
		29	83	314	81	ND	ND	ND	ND	++	0,2	4%	0,5	18,4%	0,6	18,0%	18,4%
		30	156	ND	6	ND	ND	ND	ND	+	0,3	1%	0,4	24,3%	6,7	4,3%	24,3%
		31	325	518	21	2	+	+	+	+++	0,2	3%	0,5	18,9%	1,4	13,3%	18,9%
Southland	Mataura	32	160	ND	ND	139	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		33	140	ND	ND	384	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		34	350	ND	1202	976	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		35	120	ND	ND	1076	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	Waituna	36	255	ND	37	3419	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		37	28	ND	ND	895	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		38	815	ND	ND	3495	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	Oreti - Hokonui	39	495	ND	ND	2693	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
		40	1250	ND	ND	157	ND	ND	+	-+	0,41	2,5	0,62	29,0%	6,73	6,0%	29,0%
		41	1200	ND	87	43	ND	ND	+	-+	0,2	1,8	0,5	16,0%	5,2	9.2%	16,0%
		42	1700	1264	ND	44	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

NT:non tested. ND: non detected

5. DISCUSSIÓ

5. DISCUSSIÓ/DISCUSSION

- Discussió (català)

La contaminació fecal de l'aigua va estretament lligada amb la salut de la població. Els microorganismes patògens provinents del tracte intestinal van a parar a l'aigua pel contacte directe amb persones, amb el bestiar o els animals salvatges, però sobretot mitjançant l'aigua residual que amb sobreiximent del clavegueram, els efluents de les depuradores, l'escorrentia superficial o simplement per abocament directe, arriba al riu. Desde principis del segle XX existeixen regulacions per garantir la qualitat de l'aigua de beguda i les aigües recreacionals, però encara avui en dia, més de dos bilions de persones, arreu del món, careixen de sistemes de sanejament i més d'un bilió no ténen ni accés a l'aigua potable (WHO, 2013a).

D'entre els microrganismes patògens, els virus s'identifiquen, en bona part, amb malalties entèriques tot i que el rapid transcurs dels símptomes fa que moltes vegades no es registrin els casos (Glass, 2013). Tot i això, els tractaments de desinfecció habituals de l'aigua residual, només estan basats en la eliminació de bacteris, i no aconsegueixen eliminar els virus (Rodriguez-Manzano et al., 2012). Els principals agents virals amb risc pels humans són d'origen humà però les femtes animals, amb potencials patògens zoonòtics, igualment representen un risc per a la salut humana. En aquest context, la intensificació de la producció animal o l'increment de plujes torrencials previst amb els escenaris proposats per al canvi climàtic presenten nous reptes per al control de la contaminació fecal a l'aigua (Hata et al., 2014).

Aquesta tesi doctoral es centra en l'estudi de la disseminació de virus i la traçabilitat de la contaminació fecal a l'aigua. Des del punt de vista legislatiu és

important identificar els tipus de disseminació de la contaminació fecal, puntual o difusa, i entendre la magnitud de la dispersió dels virus al medi.

Disseminació de virus indicadors de contaminació fecal a l'aigua

El capítol I d'aquesta tesi, inclou un estudi on es caracteritza l'impacte de la disseminació dels virus a la conca Mediterrània del riu Llobregat. El riu des del naixement, a Castellar de N'Hug, fins al mar Mediterrani, rep els efluents de fins a 51 plantes de tractament d'aigues residuals, la majoria de les quals aboquen l'aigua just després del tractament secundari (decantador amb fangs activats) quan només s'han reduït entre un i dos logaritmes les concentracions de virus (Rodriguez-Manzano et al., 2012). Aquest estudi s'ha enfocat a avaluar l'impacte i la dispersió de la contaminació humana al riu, i per això, durant 18 mesos, s'han recollit mostres d'aigua residual a diferents punts del tractament, aigua de riu i aigua del mar on desemboca el riu. S'han analitzat dos indicadors bacterians de contaminació fecal: *E. coli* i enterococs intestinals, dos virus indicadors de contaminació fecal humana: adenovirus humans (HAdV), poliomavirus JC (JCPyV), dos patògens: norovirus GGII (NoVGII) i el virus de la hepatitis E (HEV), i un virus emergent: el poliomavirus de les cèl·lules de Merkel (MCPyV) pel qual s'ha dissenyat també una qPCR específica.

L'alta prevalença observada dels HAdV i els JCPyV demostra que l'aigua residual juga un paper molt important en la transmissió dels virus. Els HAdV es detecten durant tot l'any a totes les mostres d'aigua residual a concentracions de 10^6CG/L. Amb el tractament secundari de les depuradores i en alguns casos de la desinfecció amb el terciari, s'aconsegueixen reduccions de fins a 3 Log_{10}. Tal i com ja s'ha descrit en altres estudis, la contaminació viral humana arriba al riu i juga un paper important en la transmissió de patògens (Bofill-Mas et al., 2006a, 2000a). Un altre exemple, son els norovirus GGII, que es detecten a l'aigua residual, al riu i en algunes mostres d'aigua de mar, durant la tardor, l'hivern i la primavera, que és quan s'identifiquen més brots a la

població (Butlletí Epidemiològic de Catalunya, 2013). El nou assaig de qPCR específic per a quantificar MCPyV, ha permès detectar per primera vegada aquest virus considerat oncogènic a l'aigua de mar. El patró d'excreció ha resultat molt semblant al de JCPyV, tot i que les concentracions en les que MCPyV es detecta a l'aigua residual crua són aproximadament un logaritme menors i en alguns casos segurament per sota del límit de detecció de la tècnica (285 CG/L). Després d'analitzar estadísticament les dades, només es detecten correlacions significatives entre els diferents patògens a l'aigua residual. Els patògens virals i els bacterians no correlacionen a l'aigua de riu ni a l'aigua de mar, pel que és especialment rellevant poder incloure els paràmetres virals a les anàlisis de rutina de la qualitat microbiològica d'aquestes aigües.

En general, la dispersió viral és especialment important just al principi dels episodis de pluja però més imporant és encara, l'efecte de dilució del riu. Per això, és precisament durant l'estiu, i durant l'època de bany, quan es detecten més virus particularment a l'aigua de mar. En els escenaris de canvi climàtic, previstos per la regió Mediterrània, els llargs periodes de sequera possiblement seran les èpoques amb més contaminació, mentre que les plujes torrencials impactaran puntualment a la conca del riu.

Traçabilitat de la contaminació fecal mitjançant adenovirus i poliomavirus humans i animals.

Des d'un punt de vista sanitari i ambiental, és igualment important conèixer l'orígen humà o animal d'aquesta contaminació. Poder traçar la font de contaminació permetrà, en la majoria dels casos, implementar protocols de remediació. L'article II, es va dissenyar per estandaritzar un mètode de concentració i uns assajos per identificar l'origen de la contaminació fecal a l'aigua, d'ara en endavant MST (Microbial Source Tracking). El treball inclòs en aquest apartat forma part d'un projecte de la Unió Europea: Viroclime "Impact of climate change on the transport, fate and risk management of viral

pathogens in water". L'estudi multi-laboratori es va realitzar juntament amb grups d'Hongría, Suècia, Grècia, Brasil i Catalunya. Mitjançant el mètode de floculació amb llet descremada i tècniques moleculars específiques per a la identificació de virus humans, bovins i porcins s'han fet "fotografies" de l'origen i l'impacte de la contaminació fecal a cada context.

La conca del Tisza a Hongría, és la més impactada amb contaminació fecal tant humana com animal. En aquest estudi s'han detectat concentracions d'entre 10^3 i 10^4 CG/L de virus humans (JCPyV i HAdV), i d'entre 10^4 i 10^5 GC/L de poliomavirus bovins (BPyV) i adenovirus porcins (PAdV) amb prevalences que, durant l'estiu i la tardor, arriben al 100%. Hongria disposa de plantes de tractament de l'aigua residual, però el recorregut transfronterer del riu, que neix a Ucraïna i atravessa Romania abans de arribar a Hongria, condiciona molt la qualitat de l'aigua. A Romania per exemple, només es tracten les aigües residuals del 57% de la població (United Nations, 2013). A més a més, només el 4% de la poblacio rural (45%) tracta les aigües residuals. A part d'això, Hongria disposa d'una important indústria ramadera amb 0.75 milions de vaques i 3 milions de porcs (Hungarian Central Statistical Office, 2013). Pel que fa als resultats obtinguts per als virus animals, s'observa que la contaminació fecal afecta de manera difusa la qualitat del riu als punts de mostreig Hongaresos.

A la conca del Río Negro, afluent de l'Amazones a Manaus (Brasil), la disseminació de la contaminació fecal és més aviat puntual i d'origen humà. En aquest cas, l'aigua residual de la ciutat Manaus va directament al riu i la contaminació fecal d'origen humà, en algunes zones, s'ha vist que és semblant a l'aigua residual crua. Als punts de mostreig més propers a la ciutat es detecten valors de fins a 10^5 i 10^4 CG/L d'HAdV i de JCPyV respectivament, mentre que només uns quilòmetres riu avall, el gran cabal de l'afluent de l'Amazones i la localització de la ciutat, aïllada al mig de la selva, sembla que permet diluir la càrrega viral en quasi dos logaritmes. En aquesta zona, no s'ha detectat contaminació bovina ni porcina significativa.

Justament al contrari succeeix a la conca del riu Umeälven a Umea (Suècia). En aquest context rural en un clima àrtic, s'ha vist que la principal font de degradació de l'aigua és la contaminació fecal bovina. A l'hivern, quan els animals són dins les granjes, es redueix el nivell i la prevalença de poliomavirus bovins. Durant l'època del desgel, a la primavera, les concentracions més baixes de virus (humans i porcins) es troben segurament per sota del límit de detecció. La temperatura i la irradiació solar són els principals factors d'inactivació de virus (Carratalà et al., 2013). La capa de gel que es forma sobre del riu Umeälven durant gairebé 5 mesos a l'any, actuaria protegint els virus de la inactivació.

Les conques mediterrànies estudiades, del riu Glafkos i del riu Llobregat, presenten concentracions de virus a l'aigua molt variables. Quan més baix és el cabal més vulnerable és la qualitat de l'aigua del riu. De forma general, durant les èpoques de pluja a la primavera i la tardor, els nivells de contaminació es dilueixen. En ambdós casos, l'origen principal de contaminació fecal són els efluents de les plantes de tractament d'aigua residual i en algun cas sobreeiximent de clavegueram durant episodis de pluges torrencials. A la conca del riu Llobregat la contaminació fecal és bàsicament d'origen humà, mentre que a la del riu Glafkos, sobretot durant l'estiu, els animals domèstics pasturen lliurement per la llera del riu i automàticament s'observa un increment de la prevalença de virus animals (PAdV i BPyV) a l'aigua. Tal i com s'ha vist en el primer estudi d'aquesta tesi, és important destacar, que és durant l'estiu quan els indicadors i alguns patògens com MCPyV presenten nivells més alts a l'aigua de mar, representant un risc important per a la població.

Amb aquest estudi, es demostra que l'aplicació d'eines virals de MST permet identificar l'origen i l'impacte de la població i del bestiar a qualsevol de les àrees geogràfiques analitzades i durant qualsevol època de l'any. Aquesta aproximació facilita molt la gestió del risc i permet definir les accions més cost-efectives sobre el territori.

Una nova eina viral per identificar i traçar la contaminació fecal ovina a l'aigua

A nivell global, han canviat molt els règims alimentaris i cada any creix la demanda de carn animal. La intensificació de la producció de bestiar significa també un increment de la quantitat de residus que cal gestionar. Catalunya, amb una població de aproximadament 7,5 milions de persones, té una industria càrnica rellevant, amb 6,8 milions de porcs, 0,5 milions de vaques i fins a 0,6 milions d'ovelles (Idescat, 2012). La gestió de les dejeccions ramaderes contempla l'espargiment al sòl, segons el tipus de cultiu, quantitat de nitrògen aplicat per hectàrea i any, i sobretot la distància a zones vulnerables per així evitar la contaminació de rius o aigües subterrànies (Decret 136/2009, 2009; Decret 220/2001, 2001). Traçar i identificar el tipus de contaminació fecal animal a l'aigua és doncs bàsic, tant per la gestió d'aquests residus, com per l'avaluació dels riscos per a la salut humana.

Fins al principi d'aquesta tesi, només es disposava de marcadors virals de contaminació fecal porcina i bovina. Tot i que alguns treballs assenyalen els adenovirus ovins (OAdV), els norovirus GIII o bacteriòfags F-específics d'ARN (Chalmers et al., 2002; Wolf et al., 2010) com a marcadors de la contaminació fecal ovina, aquests microorganismes no son prou específics i també es detecten en mostres de bestiar boví. L'article 3 és fruit d'un treball enfocat a dissenyar un nou marcador de contaminació fecal ovina. Sabent la gran espeficitat i estabilitat dels poliomavirus a l'ambient es va decidir testar mostres fecals ovines (orines, femtes i llits de palla mullats) amb una PCR d'ample espectre dissenyada per amplificar una regió de la proteïna VP1 de la càpsida dels poliomavirus (Johne et al., 2005). A partir de les seqüències obtingudes, es van dissenyar dos assajos de PCR específics per al nou virus oví i marcador de contaminació fecal ovina. L'especificitat dels assajos desenvolupats es va analitzar en un ampli conjunt de mostres que incloïa aigüa residual d'origen exclusivament humà (recollida en dos hospitals

diferents), aigua d'escorxador de aviar, boví, oví i porcí i mostres de femptes o orina dels animals respectius. En tots els casos van ser negatius.

El 69% de les mostres d'orina d'ovella (9/13) van ser positives per el nou marcador oví. També es va detectar el poliomavirus oví al 50% dels llits de palla humits (21/42) i al 53% de les femtes (7/13). La prevalença d'aquest virus es va analitzar en mostres de Catalunya, del País Basc, d'Hongria i de Grècia, i va ser al voltant del 48% en totes les àrees geogràfiques analitzades. Mitjançant la seqüenciació de 23 mostres positives amb l'assaig de nPCR específic, s'han pogut identificar dos grups de seqüències amb un 98.1% de similitud, és a dir, només 4/215 nucleòtids diferents. Segons l'ICTV (Johne et al., 2011) els poliomavirus són de la mateixa espècie quan sobrepassen el 81-84% d'identitat. Aquestes noves seqüències, identificades segons el nom de les races d'ovella on es van detectar, *Xisqueta C1* i *Ripollesa B5*, presentaven gran similitud amb els poliomavirus de rat penat (74%), de ximpanzé (73%) o d'oca (71%). Ambdós seqüències s'han introduït a la base de dades del *GenBank* com a putatius fragments d'un nou poliomavirus oví.

Amb el disseny del nou assaig de qPCR, amb bons resultats de sensibilitat i especificitat, s'han quantificat les mostres positives per nPCR. Tant a l'orina com a l'aigua residual d'escorxador els valors mitjans van ser de 10^5 CG/L. Amb el tractament de les aigües a l'escorxador, segons el reglament de domini públic hidràulic (RD 849/1986, modificat pels RD 1315/1992, RD 995/2000, i RD 606/2003), s'aconsegueix reduir la concentració de OPyV en 1 logaritme. Els efluents dels escorxadors estudiats, juntament amb aigües residuals urbanes, es tracten aigües avall a la estació municipal de tractament d'aigües residuals. Un 40% de les mostres d'efluent de la depuradora (2/5) van donar un resultat positiu, obtenint un valor mitjà de 10^3 CG/L. Tot i que és necessari l'anàlisi d'un nombre de mostres més elevat està clar que als efluents que s'aboquen al medi encara s'hi poden detectar partícules víriques del nou marcador oví.

Per tal de demostrar l'aplicabilitat del nou indicador en mostres ambientals, es van recollir mostres d'aigua de riu d'una conca altament urbanitzada, el riu Llobregat a Sant Joan Despí (Catalunya) i d'una conca agrícola, el riu Glafkos a Patras (Grècia). Cinc de les 8 mostres analitzades del riu Glafkos van donar positiu per nPCR i es van quantificar per qPCR obtenint un valor mitjà al voltant del límit de quantificació $1.14x10^{1}$CG/L. Tot i la gran quantitat d'escorxadors i plantes de tractament d'aigües residuals urbanes situades dins la conca del riu Llobregat només una mostra es va poder quantificar amb l'assaig de qPCR en $1.05x10^{2}$CG/L. Aquesta mostra va ser recollida durant un període en el que es sacrifiquen i consumeixen grans quantitats de xai: la festa musulmana del sacrifici "*Eid-al adha*".

Amb l'objectiu d'avaluar aquesta nova eina per traçar, de manera específica, la contaminació fecal ovina a l'ambient, es va plantejar un estudi amb l'equip del centre de recerca de ciences ambientals de la ciutat de Christchurch, Nova Zelanda (Environmental Science Research center, ESR). En aquest país, el bestiar boví i oví són considerats la font principal de contaminació de l'aigua (Wilcock et al., 2007). Amb 2 vaques i 9 ovelles per persona, Nova Zelanda és el país amb el ratio per càpita més elevat (Statistics New Zealand, 2012). L'ús d'eines per traçar de manera específica la contaminació fecal humana, però sobretot la animal és clau per a poder analitzar i gestionar possibles riscos.

En aquest estudi, descrit a l'article IV, es van recollir mostres de riu de zones ramaderes, a les regions de Canterbury i Southland, i mostres del riu urbà Avon, a la ciutat de Christchurch. Amb l'objectiu de identificar les fonts principals de contaminació fecal i avaluar diferents eines de MST, les mostres es van analitzar amb quatre mètodes diferents: recompte d'*E. coli*, quantificació de virus específics indicadors de contaminació fecal humana (HAdV, JCPyV) bovina (BPyV) i ovina (OPyV), quantificació de marcadors bacterians específics d'humans (BacH, BacHum, BiAdo) i de remugants (BacR específic per bovins, ovins giràfids i camèlids), i càlcul dels ratios esterol/estanol indicadors de contaminació fecal humana o de remugant.

Al 86% de les mostres d'aigua de zones ramaderes analitzades amb els tres mètodes (n =28), es va detectar contaminació fecal humana. Deu de les mostres amb contaminació humana es van identificar amb més d'un mètode i només 3 mostres es van confirmar amb els tres mètodes de MST. Els HAdV van ser els que més fàcilment van identificar aquesta font de contaminació (21/28), el segon va ser el marcador bacterià BiAdo (11/28).

Arrel dels terratrèmols del 2010 i 2011, la ciutat de Christchurch presenta encara danys importants al sistema de clavegueram. El 100% de les mostres del riu Avon, es va detectar contaminació fecal humana amb un o altre mètode. L'ús de HAdV i JCPyV va permetre identificar i confirmar que Kerrs Reach, amb els recomptes d'*E. coli* més alts en tots els mostrejos, era també el punt al centre de la ciutat amb més contaminació fecal humana. Tanmateix es va poder confirmar que durant episodis de pluja on es produeixen sobreeiximents del clavegueram, la contaminació fecal humana augmenta i tots els marcadors son capaços d'identificar-la.

La contaminació fecal animal es va poder detectar en 27 de les 28 mostres analitzades amb algun dels tres mètodes de MST i confirmar amb dos i tres mètodes a 13 i 11 mostres respectivament. Mentre el marcador bacterià (BacR) i esterols fecals només van detectar contaminació de remugant, el nou marcador oví (OPyV) i el boví (BPyV) van permetre la quantificació de l'origen específic. Es va identificar contaminació bovina i ovina a les dues regions, amb prevalences al voltant del 29 i 32% a Canterbury, on hi predomina el bestiar boví, i de 27 i 100% a Southland, on predomina el bestiar oví. Així doncs, les prevalences i concentracions de la contaminació fecal bovina i ovina, detectades a la regió de Canterbury, serien semblants tot i el predomini d'explotacions bovines. Per altra banda, la topografia també afecta a l'impacte de la contaminació a l'aigua perquè en terrenys de pastura inclinats la pluja arrossega fàcilment els microorganismes patògens (Collins et al., 2005). A les dues regions es practica la ramaderia extensiva, però mentre les ovelles de la regió de Canterbury pasturen en camps plans i ballats, el bestiar de la regió de Southland pastura pels turons sense límits. L'accés lliure als torrents, rierols i rius així com la topografia, són dos dels factors que

possiblement afecten a les prevalences i les concentracions detectades de poliomavirus oví que, a la conca del Waituna (Southland), arriben a ser de $2.0x10^3$CG/L.

Els patògens entèrics, i en concret els virus d'animals, poden sobreviure llargs períodes de temps fora de l'hoste i causar malalties zoonòtiques als humans via transmissió fecal-oral. La presència de virus indicadors a l'aigua genera, si més no, inquietud per la possible presència d'altres patògens. Aquest estudi, ha permès evidenciar el fort impacte generat particularment pel bestiar boví i oví a Nova Zelanda. L'aplicació de mètodes que permeten determinar la font concreta de contaminació fecal poden facilitar la gestió de purins, la gestió dels espais dedicats a la ramaderia o fins i tot planificar un model productiu més sostenible.

La contaminació de virus humans i animals suposa un risc per als països de baixa renta, però també per als països, que amb sistemes complexos de sanejament, no aconsegueixen eliminar la totalitat dels virus presents a l'aigua. Es tracta d'un problema global, que causa un gran impacte a la salut i importants pèrdues econòmiques. La capacitat de monitoritzar aquesta contaminació ha anat incrementant els últims anys, juntament amb l'aparició de nous mètodes de concentració, detecció i quantificació. En aquest sentit, els mètodes moleculars utilitzats per a quantificar virus a l'aigua, són sensibles i específics i permeten estudiar la disseminació dels virus i traçar-ne l'origen. Identificar i entendre el comportament tant dels virus humans, com dels animals, a l'ambient és important per poder triar les millors mesures protectores cost-efectives, així com per establir mètodes de remediació.

- Discussion (english)

Fecal contamination of water is closely linked to human health. Pathogens from the intestinal tract arrive to water by direct contact with people, livestock or wildlife, but especially by sewerage overflows, wastewater treatment plant effluents, surface runoff and direct discharges into the river. Since the twentieth century water regulations are available to ensure the drinking and recreational water quality, but still nowadays, more than two billion people worldwide lack sewerage systems and more than a billion have no access to safe drinking water (WHO, 2013a).

Among the pathogenic microorganisms, viruses have been identified with enteric diseases although the rapid course of symptoms and the available methodolgies are not always able to identify the viral pathogen and, therefore, cases are not registered (Glass, 2013). However, common wastewater disinfection treatments are only based on the elimination of bacteria, and allow the discharge of viruses in the environment (Rodriguez-Manzano et al. , 2012).

This thesis focuses on the study of the spread of viruses and the trackability of fecal contamination in water. From the legislative point of view, it is important to identify the type of fecal dissemination (puntual or diffuse) and understand the magnitude of the spread of viruses in the environment.

Dissemination of virus fecal pollution indicators in water

The first chapter of this thesis includes a study that characterizes the impact of viral spread in the Mediterranean Llobregat river basin. The river flows from Castellar de N'Hug, in the Pirenees, to the Mediterranean Sea and receives the secondary effluents (activated sludge) of 51 wastewater treatment plants which only reduces viral loads between one and two logarithms (Rodriguez-Manzano et al., 2012). This study was aimed at evaluating the impact and spread of human contamination in the river. During an 18-month period, samples were collected at two different wastewater treatment steps (after

the secondary and after the tertiary treatment) and three different sites from the river and the beach influenced by the riverine discharge. Two bacterial indicators of fecal contamination (*E. coli* and intestinal Enterococci), human adenoviruses (HAdV) and polyomavirus JC (JCPyV)), two pathogens (norovirus GGII (NoVGII) and hepatitis E virus (HEV)) and an emergint and pathogenic virus (Merkel Cell polyomavirus (MCPyV)) were detected and quantified. A specific qPCR assay for the detection of MCPyV was designed in this study.

High prevalence of HAdV and JCPyV was observed, suggesting that wastewater plays an important role in the transmission of viruses. HAdV were detected all year long in wastewater at concentrations up to 10^6 CG/L. With the secondary treatment and in some cases disinfection with tertiary viral concentrations, achieved reductions up to 3 $\log_{10}$. As previously described, human viral contamination reaches the river and plays an important role in the transmission of pathogens (Bofill-Mas et al., 2006a, 2000a). Also norovirus GGII were detected in sewage, river and seawater samples collected in autumn, winter and spring, when most outbreaks are identified in the population (Butlletí Epidemiològic de Catalunya, 2013). The new specific qPCR assay to quantify MCPyV has allowed the first identification of this oncogenic virus in seawater. The excretion pattern was similar to JCPyV, although the concentrations in which MCPyV was detected in the raw wastewater are about one logarithm lower and in some cases probably below the detection limit of the technique (285 CG/L). After statistical data analysis, viral and bacterial pathogens did not show any correlation in river water nor in seawater. It is then especially important to include viral parameters in routine analysis of the microbiological quality of these waters.

Human and animal adenoviruses and poliomaviruses to trace the origin of the fecal contamination

From an environmental and public health point of view, it is equally important to know the origin of both human and animal contamination. Tracing the pollution source allows in most cases the implementation of corrective measures. The second study included in the thesis, was designed to standardize a concentration method and the PCR

assays to identify the source of fecal contamination in water, hereafter MST (Microbial Source Tracking). The work included in this section is part of a European Union project: VIROCLIME "Impact of climate change on the transport, fate and risk management of viral pathogens in water". This interlaboratory study was conducted by groups from Hungary, Sweden, Greece, Brazil and Catalunya. By means of a skim milk flocculation method and specific molecular tools for the identification of human, bovine and porcine viruses we have pictured the origin and studied the impact of fecal contamination in each context.

The basin of the Tisza in Hungary, is the most impacted with both human and animal fecal contamination. Human viruses (JCPyV and HAdV) were detected at concentrations between 10^3 and 10^4 CG/L, bovine polyomaviruses (BPyV) and porcine adenovirus (PAdV), with values up to 10^4 and 10^5 GC/L, presented higher prevalences, reaching 100% during the summer and autumn. Although Hungary treats most of wastewater, the river transboundery through Ukraine and Romania where wastewater is not always treated, affect the water quality. In Romania, for example, only 57% of the wastewater is treated (United Nations, 2013). Furthermore, rural population (45%) has the lowest ratios 4%. Hungary has a significant cattle industry with 0.75 million cows and three million pigs (Hungarian Central Statistical Office, 2013). The animal viruses results demonstrate that animal fecal contamination is diffuse and present along the hungarian river sampling points.

In the Rio Negro basin, tributary of the Amazon river in Manaus (Brazil), dissemination of fecal contamination is quite punctual and from human origin. In this case, the wastewater of Manaus city is directly discharged into the river and human fecal contamination, in some cases, becoming similar to raw wastewater quality. Concentrations up to 10^5 and 10^4 CG/L of HAdV and JCPyV, respectively, were detected at the nearest sampling points to the city. Few kilometers downstream, the large Amazon flow and the isolation of the city in the middle of the jungle, seem to help in diluting the viral load in almost two logarithms. In this area neither bovine nor porcine pollution was detected.

Exactly the opposite happens in the basin of the Umeälven in Umea (Sweden). In that rural context within an Arctic climate, the main source of water degradation is from bovine livestock. In winter, when the animals are in stalls, levels and prevalence of bovine polyomavirus are reduced. During the snowmelt, in spring, virus concentrations are lower and possibly under the limit of detection. Temperature and solar radiation are the main factors of viral inactivation (Carratalà et al., 2013). The ice cover formed over the river Umeälven for over 5 months, may protect virus inactivation.

The Glafkos and Llobregat Mediterranean river basins presented a wide variability in virus concentrations. The lower the flow volume is, the more vulnerable the quality of the river water is. In general terms, during the rainy seasons, spring and autumn, pollution levels are diluted. In both cases, the main source of fecal pollution are wastewater treatment plants effluents and ocasionally the sewerage overflows that occur during torrential rains. In the Llobregat river basin, fecal contamination is basically from human origin, while in the river Glafkos, especially during the summer, domestic animals graze without limits in the riverbed increasing the prevalence of animal viruses (PAdV and BPyV) in water. As seen in the first study of this thesis, is during the summer when indicators and some pathogens as MCPyV present higher values in seawater, posing higher risk to the population.

This study demonstrates that the application of MST tools allows to identify the origin and impact of viral contaminants in all the geographical areas analyzed during any time of the year. With this approach feasible risk management and cost-effective actions can be carried on the territory.

A new viral tool to identify and trace the sheep fecal contamination in water

Globally, human diet has changed a lot and year by year there is an increasing demand for animal products. The intensification of livestock production also means an

increase in the amount of animal waste to be managed. Catalonia, with a population of approximately 7.5 million people, has an excellent meat industry, with 6.8 million pigs, 0.5 million cattle, 0.6 million sheep (IDESCAT, 2012). Most of livestock manure is disposed to the ground considering crop type, nitrogen amounts applied per hectare and year, and especially according to the distance to vulnerable areas in order to avoid contamination of groundwater or rivers (Decret 136/2009, 2009, Decret 220/2001, 2001). Tracing and identifing the type of animal fecal contamination in water is therefore essential, both for the waste management and for the assessment of human health risks.

Since the beginning of this thesis, only porcine and bovine viral markers were available to trace animal fecal pollution. Although some studies indicate ovine adenovirus (OAdV), noroviruses GIII and F-specific RNA bacteriophages (Chalmers et al., 2002; Wolf et al., 2010) as markers of ovine fecal contamination, these microorganisms are not specific enough and are also detected in bovine samples. The third article of the thesis is the result of the work focused on designing a new marker of sheep fecal contamination. Knowing the polyomavirus large specificity and stability in the environament, we decided to test ovine fecal samples (urine , feces and wet straw beds) with a broad spectrum PCR designed to amplify a region of the capsid protein VP1 of polyomavirus (Johne et al., 2005). From the obtained sequences (belonging to a putative ovine polyomavirus), two specific PCR assays were designed to detect and trace the ovine fecal contamination. The specificity of the tests was tested by a wide range of samples including human wastewater (collected from two different hospitals), slaughterhouse wastewater and feces/urine from poultry, cattle, sheep and pigs. In all cases all samples were negative.

69% of the urine samples from healthy sheep (9/13) were positive for the new marker. Also 50% of the wet straw beds (21/42) and 53% of the feces (7/13) were positive for the new ovine virus. The prevalence of this virus was analyzed in samples of Catalonia, Basque Country, Hungary and Greece. 23 positive samples were amplified and sequenced using a nPCR specific assay, and two different groups of sequences were identified with 98.1% of similarity, which means that only 4/215 nucleotides were different. According to the ICTV (Johne et al., 2011) polyomavirus are from the same species when

exceed 81-84% identity. These new sequences identified as the sheep breeds, Xisqueta Ripollesa C1 and B5, showed great similarities with bat polyomavirus (74%), chimpanzee (73%) and goose (71%). Both sequences were introduced in the GenBank database as putative fragments of a new ovine polyomavirus.

The design of the new qPCR assay presented good sensitivity and specificity. Both urine and slaughterhouse wastewater average values were 10^5 CG/L. With slaughterhouse water treatment, according to public water regulations (RD 849/1986, as amended by RD 1315/1992, RD 995/2000 and RD 606/2003), it is possible to reduce the concentration of OPyV in 1 logarithm. Slaughterhouse effluents, along with urban sewage, are treated in the downstream municipal treatment facilities. After the waste water treatment plant, still a 40% of the effluent samples were positive for the detection of OPyV (2/5), with mean values of 10^3 CG/L.

In order to demonstrate the applicability of the new indicator in environmental samples, water samples were collected from a highly urbanized river in Catalonia, the Llobregat river, and from the river Glafkos in Patras (Greece). Five of the eight tested samples from Glafkos were positive by nPCR and quantified by qPCR obtaining an average value around the limit of quantification $1.14x10^1$CG/L. Despite the large number of slaughterhouses and sewage treatment plants located within the urban Llobregat river basin only one positive sample could be quantified with the qPCR assay obtaining $1.05x10^2$CG/L. This sample was collected during a period in which lamb are sacrified and consumed in large quantities: the Muslim festival of sacrifice "Eid-al-Adha".

In order to evaluate this new tool to trace, specifically, sheep fecal contamination in the environment, a study was designed with the team in Environmental Science Research center in the Christchurch city, New Zealand (ESR). In this country, the bovine and ovine livestock are considered the main source of water pollution (Wilcock et al., 2007). With 2 cows and 9 sheep per inhabitant, New Zealand is the country with the highest ratio per capita (Statistics New Zealand, 2012) . The use of tools to specifically

trace human fecal contamination, and also the animal pollution, is especially important to be able to analyze and manage risks.

In this study, described in article IV, river water samples were collected from the two main grazing areas of the country (Canterbury and Southland) and samples from the urban river Avon in Christchurch. With the aim to identify the main sources of fecal contamination and evaluate the various MST tools samples were tested by four different methods: *E. coli* counts, quantification of specific human (HAdV, JCPyV), bovine (BPyV) and ovine (OPyV) viruses, quantification of human (BacH, BacHum, BiAdo) and ruminants (BacR) specific bacterial markers, and sterol/stanol fecal ratios. In farming areas, 86% of the water samples analyzed were positive for human pollution with at least one of the three methods (n=28). Ten of those samples were identified positive with one method and only three samples were confirmed with the three methodologies. The HAdV was the tool that easily identified the human source (21/28) and BiAdo was also highly prevalent (11/28).

Since the earthquakes of 2010 and 2011, the city of Christchurch present important damages in its sewer system. 100% of the samples of the Avon river were positive for human fecal contamination with either method. The use of HAdV and JCPyV allowed the identification and confirmation of the human pollution source with Kerrs Reach presenting the highest *E . coli* counts in all samplings. This site, in the center of the city, was the most human impacted. In case of sewerage overflows, caused by rainfall events, human fecal contamination increased and all methods were able to identify the fecal pollution.

Animal fecal contamination could be detected in 27 of the 28 samples with at least one of the MST tools and confirmed by two or three methods in 13 and 11 samples respectively. While the bacterial marker (BacR) and fecal sterols only detect contamination from ruminants, the ovine (OPyV) and the bovine (BPyV) viral markers could distinguish the specific fecal contamination origin. Both sources of contamination were idenfied in both regions, with OPyV prevalences up to 32% in Canterbury and 100%

in Southland, where sheep are predominant. Thus, the prevalence and concentrations of ovine and bovine fecal contamination detected in the region of Canterbury would be similar despite the predominance of cattle farms. Several factors may influence the microbial quality of waterways, including the type of farming undertaken, the intensity of farming, on-farm practices and regulations that govern waste disposal (Collins et al., 2005). Microbes from animal feces can enter water via several pathways, including direct deposition, discharge of effluent collected on-farm and overland flow of fecal material during rainfall. While in the Canterbury plains animal graze within fenced fields, in the Southland hills, animals grace without limits. Free access to streams, creeks and rivers as well as the topography, are two of the factors that may affect the prevalence and concentrations of the detected ovine polyomavirus in the basin of Waituna river (Southland) that reaches $2.0x10^3$CG/L.

Enteric pathogens, in particular animal viruses can survive long periods outside their host and cause zoonotic diseases to humans via fecal-oral transmission. The presence of viral indicators in water warns us about the presence of other pathogens. This study evidences the strong impact generated by bovine and ovine livestock in New Zealand. The application of both methods to determine the specific source of fecal contamination from manure can facilitate the management of livestock manure and can help in planning a more sustainable production model.

Both human and animal contaminant viruses pose a risk in low income countries, but also in areas with complex sanitation systems that can not completely eliminate the viruses present in water. This is a global problem causing big health impacts and important economic losses. The pollution monitoring capacity has increased in recent years together with the outcome of new concentration, detection and quantification methods. In this sense, the molecular methods to detect and quantify viruses in water are sensitive and specific for the study of viral dissemination and allow to track the origin of the pollution. Understanding the behaviour of both human and animal viruses in the environment is important when choosing the best and the more cost-effective measures as well as when establishing remediation methods.

6. CONCLUSIONS

6. CONCLUSIONS

- Conclusions (català)

- L'aigua residual urbana és la principal font de degradació de la qualitat de l'aigua de la conca del Llobregat, amb concentracions d'HAdV de fins a 10^2 i 10^3 CG/L segons el punt de mostreig del riu i de fins a 10^2CG/L a l'aigua de mar on desemboca el riu.

- Els HAdV, els JCPyV i els MCPyV, es detecten durant tot l'any a l'aigua residual amb concentracions mitjanes i prevalences de $8.4x10^5$ (100%), $7.5x10^5$ (89%) i $1.6x10^5$CG/L (73%) respectivament. NoV GGII són més prevalents durant els mesos freds, quan es detecten concentracions que arriben fins a $2.5x10^8$ CG/L. Els HEV es detecten només al 9.8% de l'aigua residual. Els tractaments primari i secundari de les depuradores redueixen les concentracions de virus entre 1.4 i 2.5Log_{10}.

- S'ha dissenyat un assaig de qPCR specific per MCPyV, que ha permès quantificar i identificar aquest virus per primera vegada a l'aigua de mar. L'aigua de bany pot ser una via de transmissió d'aquest virus. L'alta prevalença observada en aigua residual (83%) també suggereix que aquest virus, relacionat amb cancer de pell, s'excreta persistentment a la pell i/o a l'orina.

- Durant les èpoques més seques les concentracions de virus al riu Llobregat són en general més elevades. Els escenaris previstos de canvi climàtic a la zona Mediterrània preveuen un increment de freqüència i intensitat de les plujes torrencials així com de les sequeres, pel que la contaminació viral a l'aigua podria augmentar.

- La detecció i quantificació dels virus humans (HAdV i JCPyV) i animals (BPyV i PAdV) utilitzats com a eines de MST, han demostrat ser específiques i sensibles i han permès descriure l'impacte de les diferents fonts de contaminació a les cinc regions climàtiques estudiades: Brasil, Catalunya, Grècia, Hongria i Suècia.

- Tant el mètode de concentració de virus mitjançant floculació amb llet descremada, com la quantificació dels genomes vírics mitjançant qPCR específicques per cada un dels paràmetres vírics (HAdV, JCPyV, BPyV i PAdV), s'han validat en diferents laboratoris i han demostrat ser efectius i robustos, i per tant aptes pels anàlisis rutinaris de MST. L'ús d'eines virals de MST permet entendre com es dissemina la contaminació fecal a cada periode de l'any, i també definir accions de remediació específiques per a cada context.

- S'ha identificat el primer poliomavirus oví (OPyV). Els assajos de PCR dissenyats per detector i quantificar el nou OPyV han estat aplicats per al control i identificació de la contaminació fecal d'origen oví a l'aigua i han demostrat ser sensibles i específics.

- El marcador oví dissenyat s'ha utilitzat amb èxit per traçar la contaminació fecal del bestiar oví a Catalunya, Grècia i Nova Zelanda, aquest últim el país amb més ovelles per càpita del món.

- S'han analitzat comparativament diferents paràmetres virals (HAdV, BPyV i OPyV), bacterians (BacH, BacHum-UCD, BiAdo, BacR) i químics (ratios esterols/estanols) en mostres de d'aigua de riu. La quantificació mitjançant PCR d'HAdV ha demostrat ser l'eina més sensible per a identificar la contaminació fecal humana al medi.

- L'ús de poliomavirus ovins (OPyV) i bovins (BPyV) per separat, permet identificar específicament la font de contaminació de remugant. Ambdós mètodes quantitatius poden ser de gran utilitat per a la gestió dels residus de la indústria ramadera.

- Conclusions (english)

- Sewage is the main source of water quality degradation, in the Llobregat river catchment, with concentrations of HAdV between 10^2 and 10^3 GC/L in the river water and up to 10^2GC/L in the seawater receiving the riverine discharge.

- The HAdV, JCPyV and MCPyV are detected in raw sewage, all over the year, with mean values and prevalences of $8.4x10^5$ (100%), $7.5x10^5$ (89%) and $1.6x10^5$ GC/L (73%) respectively. NoV GGII exhibit seasonal variability with concentrations during colder months around $2.5x10^8$ GC/L and HEV are only detected in the 9.8% of the samples. The wastewater primary and secondary treatments reduce viral concentrations between 1.4 and 2.5Log_{10}.

- We have designed a new MCPyV-specific qPCR assay that allowed for the first time, the identificacion and quantification of this virus in seawater and elucidates bathing water as a new transmission pathway. The high prevalence observed in wastewater (83 %) suggests that this virus, related to skin cancer, is persistently excreted in the urine and/or skin.

- During dought periods, concentration of viruses in the Llobregat River is generally higher. The predicted increment of frequency and intensity of extreme hydrological events due to climate change may increase viral contamination on that Mediterranean river catchment.

- The detection and quantification of human (HAdV and JCPyV) and animal viruses (BPyV and PAdV) as MST tools, have proven to be sensitive and specific in all geographic areas and all types of water matrices. Those tools enable identifying the impact of the specific sources of pollution in all climatic region estudied: Brazil, Catalunya, Greece, Hungary and Sweeden

- The viral concentration method, by means of Skimmed Milk Flocculation, and the HAdV-, JCPyV-, BPyV- and PAdV- specific qPCR assays have been validated in different laboratories and demonstrated its repetitiveness and robustness and therefore have proved to be suitable protocols for MST routine analysis.

- The application of viral MST tools to river catchments reflects exploitative pressures in the 5 geographical areas analyzed (Brazil, Catalunya, Greece, Hungary and Sweeden) and can help to define remediation actions specific to each context. By using the viral MST tools we are able to understand the dissemination of fecal pollution in any period of the year and defining specific remediation strategies in each context.

- We have identified the first ovine polyomavirus (OPyV). The two PCR assys designed to control and identify the ovine fecal contamination proved to be sensitive and specific.

- The new molecular marker, OPyV, has been successfully applied to trace fecal ovine contamination in Catalunya, Greece and New Zealand (the country with most sheep per capita in the world).

- A comparative analisis has been done between viral (HAdV, BPyV, OPyV), bacterial (BacH, BacHum-UCD, BiAdo, BacR) and chemical MST tools (sterol/stanol ratios) in river water samples. The HAdV quantification proved to be the most sensitive tool for human fecal source identification.

- The use of the new ovine polyomavirus (OPyV) together with the bovine (BPyV) permitted to identify the specific ruminant contamination source. Both quantitative methods can be very usefull when managing waste from livestock industry.

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8. ANNEXOS

8. ANNEXOS

8.1. Informació sobre les PCR i qPCR utilitzades durant la tesi

Virus	Nom primer/sonda	Posició	Sequència (5'-3')	Temperatures anellament qPCR/PCR	Referència
qPCR HAdV	ADF	Hexó 18869-937	CWTACATGCACATCKCSGG	60	Hernroth et al. 2002
	ADR		CRCGGGCRAAYTGCACCAG		
	ADP1		FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ1		
qPCR PAdV	QPAdV-F	Hexó 20701-768	AACGGCCGCTACTGCAAG	55	Hundesa et al., 2009
	QPAdV-R		AGCAGCAGGCTCTTGAGG		
	QPAdV-P		FAM-CACATCCAGGTGCCGC-BHQ1		
qPCR JCPyV	JE3F	T-antigen 4251-482	ATGTTTGCCAGTGATGATGAAAA	60	Pal et al., 2006
	JE3R		GGAAAGTCTTTAGGGTCTTCTACCTTT		
	JE3P		FAM-AGGATCCCAACACTCTACCCCACCTAAAAAGA-BHQ1		
qPCR MCPyV	MCF	VP1 1232-93	ATTTGGGTAATGCTATCTTCTC	60	Rusiñol et al., 2014
	MCR		CTAATGTTGCCTCAGTTCCAA		
	MCP		FAM- AACCACAGATAATACTTCCACTCCT-BHQ1		
qPCR BPyV	QB-F1-1	VP1 2122-2198	CTAGATCCTACCCTCAAGGGAAT	60	Hundesa et al., 2010
	QB-R1-1		TTACTTGGATCTGGACACCAAC		
	QB-P1-2		FAM-GACAAAGATGGTGTGTATCCTGTTGA-BHQ1		
qPCR OPyV	qOv-F	VP1 no descrit	CAGCTGYAGACATTGTGG	58	Rusiñol et al., 2013
	qOv-R		TCCAATCTGGGCATAAGATT		
	qOv-P		FAM-ATGATTACCAAGCCAGACAGTGGG -BHQ1		
qPCR Ch/TyPV	Q-PaV-F	VP2 3326-407	AGTCCACGAGATTGGCAACA	60	Carratalà et al., 2013
	Q-PaV-R		GCAGGTTAAAGATTTTCACG		
	Q-PaV-Pr		FAM-AATTATTCGAGATGGCGCCCACG-BHQ1		
qPCR NoV GGII	QNIF2d	Proteina càpsida 5012-100	ATGTTCAGRTGGATGAGRTTCTCWGA	60	Primers: Loisy et al., 2005 Sonda: Kageyama et al., 2003
	COG2R		TCGACGCCATCTTCATTCACA		
	QNIFS		FAM-AGCACGTGGGAGGCGATCG-TAMRA		
nPCR OPyV	Ov-FI	VP1 no descrit	CAGCTGYAGACATTGTGG	52	Rusiñol et al., 2013
	Ov-RI		TTTATCTCCAGTCATGGCC		
	Ov-nFI		AGACATTGTGGGCATGATTA	54	
	Ov-nRI		TTCCAATCTGGGCATAAGAT		
nPCR Ch/TyPV	Par1	Regió no estructural 661-1073 682-1043	GGTACAAGATATGCTAGATTTG	53	Carratalà et al., 2013
	Par2		CGGATGGCTAAATTATCATCT		
	Par3		CCATCGCAGGAATTAACTCCAG	53	
	Par4		GTGTCAACATCTCCATGTATTG		
nPCR Ch/TyPV	VP2- Par1	VP2 3119-3492 3173-3422	TGGAATTGTGATACTATATGGG	56	Carratalà et al., 2013
	VP2-Par2		TCYTGATCTGCAAATATTTG		
	VP2-Par3		CATTGTGTCTGTCTWATGCGTGAC	64	
	VP2-Par4		GTTTTCTGGATGACTTGCA		
HEV**	HEVORF2con-a1	ORF2 6283-479	GACAGAATTRATTTCGTCGGCTGG	55	Erker et al,1999
	HEVORF2con-s1		CTTGTTCRTGYTGGTTRTCATAATC		
	HEVORF2con-s2		GTYGTCTCRGCCAATGGCGAGC	55	

**seminested PCR

[a] Positions de referència segons les soques: J01917.1 (HAdV), AJ237815 (PAdV), NC001699.1 (JCPyV), HM011557.1 (MCPyV), D13942 (BPyV), KC145150 (OPyV), GU214706 (Ch/TyPV), AF145896 (NoVGII) i AF060668 (HEV).

8.2. Altres publicacions

A continuació es detallen per ordre cronològic altres publicacions no incloses en aquesta tesi però realitzades durant el doctorat.

- Bofill-Mas S, Hundesa H, Calgua B, Rusiñol M, Maluquer de Motes C, Girones R. 2011. Cost-effective method for Microbial Source Tracking using specific human and animal viruses. Journal of Visual Experiments. (58), e2820.
La doctoranda va realitzar bona part del treball experimental, incloent els mostrejos i l'anàlisi de totes les mostres amb qPCR específiques per HAdV i JCPyV.

- Carratalà A, Rusiñol M, Hundesa A, Biarnes M, Rodriguez-Manzano J, Vantarakis A, Kern A, Suñen E, Girones R, Bofill-Mas S. 2012. A novel tool for specific detection and quantification of chicken/turkey parvoviruses to trace poultry fecal contamination in the environment. Appl Environ Microbiol. 78 (20): 7496-7499.
La doctoranda va participar en el disseny experimental i la recolecció de les mostres de depuradora i escorxador, així com en l'anàlisi per qPCR específiques de HAdV i JCPyV.

- Rodriguez-Manzano J, Alonso JL, Ferrús MA, Moreno Y, Amorós I, Calgua B, Hundesa A, Guerrero-Latorre L, Carratala A, Rusiñol M, Girones R. 2012. Standard and new faecal indicators and pathogens in sewage treatment plants, microbiological parameters for improving the control of reclaimed water. Water Sci Technol. 66 (12): 2517- 2523.
La doctoranda va participar en el processament de les mostres amb ultracentrifugació i en el desenvolupament d'alguns anàlisi de qPCR per JCPyV.

- Calgua B, Fumian T, Rusiñol M, Rodriguez-Manzano J, Mbayed VA, Bofill-Mas S, Miagostovich M, Girones R. 2013. Detection and quantification of classic and emerging viruses by skimmed-milk flocculation and PCR in river water from two geographical areas. Water Res. 47: 2797-2810.

La doctoranda va realitzar l'anàlisi dels virus nous i emergents amb nPCR específiques per als Asfar-like virus ASFLV i els poliomavirus KIPyV, WUPyV i MCPyV a les mostres de Bacelona. També va sequenciar totes les mostres positives d'aquesta àrea geogràfica..

- Carratalà A, Rodriguez-Manzano J, Hundesa A, Rusiñol M, Fresno S, Cook N, Girones R. 2013. Effect of temperature and sunlight on the stability of human adenoviruses and MS2 as fecal contaminants on fresh produce surfaces. Int J Food Microbiol. 164: 128-134.

La doctoranda va participar en el recompte en placa dels bacteriòfags MS2.

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

La doctoranda va realitzar la revisió bibliogràfica dels estudis de MST i va participar en la redacció de l'article de revisió.

- Carratalà A, Rusiñol M, Rodriguez-Manzano J, Guerrero-Latorre L, Sommer R, Girones R. 2013. Environmental effectors on the inactivation of human adenoviruses in water. Food Environ Virol. 5: 203-214.

La doctoranda va participar en el disseny experimental i als assajos d'immunofluorescència i recompte en placa del bacteriòfag MS2.

- Rodriguez-Manzano J, Hundesa A, Calgua B, Carratala A, Maluquer de Motes C, Rusiñol M, Moresco V, Ramos AP, Martínez-Marca F, Calvo M, Monte Barardi CR, Girones R, Bofill-Mas S. 2013. Adenovirus and norovirus contaminants in commercially distributed shellfish. Food Environ Virol. 6: 31-41.

La doctoranda va participar en els mostrejos d'aigua i musclos, així com en el processament de les mostres.

Video Article

Cost-effective Method for Microbial Source Tracking Using Specific Human and Animal Viruses

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URL: http://www.jove.com/details.php?id=2820
DOI: 10.3791/2820

Keywords: Immunology, Issue 58, Quantitative PCR, qPCR, flocculation, virus, adenovirus, polyomavirus, water, Microbial Source Tracking, bovine, human, porcine, contamination,

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Abstract

Microbial contamination of the environment represents a significant health risk. Classical bacterial fecal indicators have shown to have significant limitations, viruses are more resistant to many inactivation processes and standard fecal indicators do not inform on the source of contamination. The development of cost-effective methods for the concentration of viruses from water and molecular assays facilitates the applicability of viruses as indicators of fecal contamination and as microbial source tracking (MST) tools. Adenoviruses and polyomaviruses are DNA viruses infecting specific vertebrate species including humans and are persistently excreted in feces and/or urine in all geographical areas studied. In previous studies, we suggested the quantification of human adenoviruses (HAdV) and JC polyomaviruses (JCPyV) by quantitative PCR (qPCR) as an index of human fecal contamination. Recently, we have developed qPCR assays for the specific quantification of porcine adenoviruses (PAdV) and bovine polyomaviruses (BPyV) as animal fecal markers of contamination with sensitivities of 1-10 genome copies per test tube. In this study, we present the procedure to be followed to identify the source of contamination in water samples using these tools. As example of representative results, analysis of viruses in ground water presenting high levels of nitrates is shown.

Detection of viruses in low or moderately polluted waters requires the concentration of the viruses from at least several liters of water into a much smaller volume, a procedure that usually includes two concentration steps in series. This somewhat cumbersome procedure and the variability observed in viral recoveries significantly hamper the simultaneous processing of a large number of water samples.

In order to eliminate the bottleneck caused by the two-step procedures we have applied a one-step protocol developed in previous studies and applicable to a diversity of water matrices. The procedure includes: acidification of ten-liter water samples, flocculation by skimmed milk, gravity sedimentation of the flocculated materials, collection of the precipitate and centrifugation, resuspension of the precipitate in 10 ml phosphate buffer. The viral concentrate is used for the extraction of viral nucleic acids and the specific adenoviruses and polyomaviruses of interest are quantified by qPCR. High number of samples may be simultaneously analyzed using this low-cost concentration method.

The procedure has been applied to the analysis of bathing waters, seawater and river water and in this study, we present results analyzing groundwater samples. This high-throughput quantitative method is reliable, straightforward, and cost-effective.

Video Link

The video component of this article can be found at http://www.jove.com/details.php?id=2820

Protocol

1. Concentration of the viral particles present in water samples

1. Collection and conditioning of water samples
    1. Collect a minimum of 2 replicates of 10 L per sample in plastic containers with flat bottom and one extra sample as a process control. This last sample will be spiked with a known amount of viral particles and used as a control.

       Note: It is recommended to have special separate material (bottles, tube, etc.) for spiked samples.
    2. Check the calibration of the conductimeter and recalibrate if necessary. Prepare a negative control by using tap water previously adjusted to the adequate conductivity (see below 1.6) in one extra plastic 10 L container.
    3. For spiked samples: Add the standard volume of the control virus (approximately 10^5 genome copies per 10 L of water) to the sample. Mix by stirring avoiding splashing and aerosols. Positive controls could consist in an uncommon strain of adenovirus such as HAdV-35 or a bacteriophage such as MS2.
    4. If the sample presents high quantity of suspended material (sand or other materials), let it sediment for 15 minutes. Transfer the water into a new container.
    5. Adjust the pH of the water sample to 3.5 (± 0.1) by the addition of 1 N HCl. This step is important for the concentration of the viruses, so make sure the pH has been properly adjusted. Mix the water thoroughly by vigorous stirring while adding the HCl. (Note: If the pH is lower than 3.5 add 1 M NaOH).

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A Novel Tool for Specific Detection and Quantification of Chicken/Turkey Parvoviruses To Trace Poultry Fecal Contamination in the Environment

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Poultry farming may introduce pathogens into the environment and food chains. High concentrations of chicken/turkey parvoviruses were detected in chicken stools and slaughterhouse and downstream urban wastewaters by applying new PCR-based specific detection and quantification techniques. Our results confirm that chicken/turkey parvoviruses may be useful viral indicators of poultry fecal contamination.

Animal populations can serve as reservoirs for human pathogens and may facilitate transmission of those crossing the species barrier. Therefore, the origin of animal fecal contamination must be identified and tracked to monitor water quality, assess potential health risks, and determine optimal remediation strategies. In particular, poultry farming is an industry that produces a large volume of different by-products occasionally used as manure to fertilize crops, which can introduce pathogens into the surrounding environment and into the food chain (1, 5, 8). However, until recently, there had been little effort to develop suitable techniques to characterize the origin of avian fecal contamination (2, 13, 17).

Bacterial fecal indicators often fail to predict the presence of pathogenic microorganisms in water and food (7). Thus, viruses have emerged as a promising tool to increase water quality standards, due to their high host specificity and stability in different environments (9, 11, 14, 20, 21).

The high levels of prevalence of parvovirus in chickens (ChPV) and turkeys (TuPV) in different countries (3, 18, 19, 23, 24) and the high level of stability of animal parvovirus (15, 22) have been described. Here, the potential role of ChPV/TuPV as a new tool for microbial source tracking was evaluated by developing nested and also quantitative PCR-based assays for the detection and quantification of ChPV/TuPV in environmental samples.

All sequences available in GenBank for ChPV and TuPV were aligned, and two nested PCR (nPCR) assays, targeting the nonstructural and VP1/VP2 regions, and a quantitative PCR (qPCR), targeting the VP1/VP2 regions, were optimized (Table 1).

A total of 30 chicken fecal pools were collected from different farms in Catalonia (coastal Northeast Spain), the Basque Country (Northern Spain), Patras (Greece), and Budapest (Hungary) between February and December 2010. Three turkey, 2 partridge, and 7 hen pooled fecal samples collected from farms in Catalonia were also tested. All samples were collected from the ground and distributed into sterile 50-ml polyethylene containers that were kept at 4°C for less than 24 h prior to the analysis. Viral particles were concentrated from 250 mg of fecal material that was homogenized by vortexing with 2.5 ml of phosphate-buffered saline (PBS) during 2 min and centrifuged at 3,000 × *g* for 15 min, after

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TABLE 1 Oligonucleotide primers used for the detection and quantification of chicken/turkey parvoviruses

Primers	Genome region[a]	Position[b]	Amplification reaction	Amplicon size (bp)	Annealing temp (°C)	Sequence (5′–3′)
Par1	NS	661–682	First	412	53	GGTACAAGATATGCTAGATTTG
Par2		1053–1073				CGGATGGCTAAATTATCATCT
Par3		718–739	Nested	325	53	CCATCGCAGGAATTAACTCCAG
Par4		1022–1043				GTGTCAACATCTCCATGTATTG
VP-Par1	VP1/VP2	3119–3140	First	373	56	TGGAATTGTGATACTATATGGG
VP-Par2		3473–3492				TCYTGATCTGCAAATATTTG
VP-Par3		3173–3196	Nested	249	64	CATTGTGTCTGTCTWATGCGTGAC
VP-Par4		3405–3422				GTTTTCTGGATGACTTGCA
Q-PaV-F	VP1/VP2	3326–3345	qPCR	81	60	AGTCCACGAGATTGGCAACA
Q-PaV-R		3388–3407				GCAGGTTAAAGATTTTCACG
Q-PaV-Pr		3356–3378				6FAM-AATTATTCGAGATGGCGCCCACG-BHQ1

[a] NS, nonstructural protein 1; VP1, virion protein 1; VP2, virion protein 2.
[b] The sequence positions are with reference to accession number GU214706 from GenBank.

Standard and new faecal indicators and pathogens in sewage treatment plants, microbiological parameters for improving the control of reclaimed water

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ABSTRACT

This study involved collaboration between three centres with expertise in viruses, bacteria and protozoa. The focus of the research was the study of the dissemination and removal of pathogens and faecal indicators in two sewage treatment plants (STP1 and STP2) using tertiary treatments. Samples were collected over a period of five months through the sewage treatment processes. Analysis of the samples revealed that the plants were not efficient at removing the faecal indicators and pathogens tested during the study. From entry point (raw sewage) to effluent level (tertiary treatment effluent water), the experimental results showed that the reduction ratios of human adenoviruses were 1.2 $\log_{10}$ in STP1 and 1.9 $\log_{10}$ in STP2. Whereas for *Giardia* spp. and *Cryptosporidium* spp. the reduction ratios were 2.3 $\log_{10}$ for both pathogens in STP1, and 3.0 and 1.7 $\log_{10}$ in STP2, respectively. Furthermore, the presence of faecal indicators and pathogens at different sampling points was evaluated revealing that the tested pathogens were present in reclaimed water. Human adenovirus and *Arcobacter* spp. showed positive results in infectivity assays for most of the tertiary effluent water samples that comply with current legislation in Spain. The pathogens detected must be evaluated using a risk assessment model, which will be essential for the development of improved guidelines for the re-use of reclaimed water.

Key words | efficiency removal, enteric pathogens, faecal indicators, sewage, tertiary treatment, treatment plant

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INTRODUCTION

Numerous studies have documented the presence of enteric pathogens in raw and treated sewage and, by using new molecular methods, new pathogens and potential indicators have also been described (Girones *et al.* 2010). Most pathogens found in environmental waters (rivers, lakes, seawater and groundwater) originate from contamination with sewage or directly with human or animal excreta. Although most pathogens can be removed by sewage treatment, many are discharged into the effluent and enter receiving waters, allowing their transmission through contaminated water. Many regions face water supply challenges due to water scarcity, resulting in an increased need for water re-use and for ways to solve water resource issues or create new sources of good quality water supplies.

Classic microbiological indicators such as faecal coliforms, *Escherichia coli* and enterococci are the most commonly analysed indicators used to evaluate the level of faecal contamination. However, whether these bacteria are suitable indicators of the occurrence and concentration of human viruses and protozoa cysts has been questioned (Lipp *et al.* 2001; Tree *et al.* 2003; Wéry *et al.* 2008). Compared with viral or protozoan pathogens, indicator bacteria are more sensitive to inactivation through treatment processes and exposure to sunlight (Hurst *et al.* 2002; Sinclair *et al.* 2009). Other limitations have been associated with their application: short survival compared with pathogens (McFeters *et al.* 1974), non-exclusive faecal source (Scott *et al.* 2002; Simpson *et al.* 2002), ability to multiply in some environments

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Detection and quantification of classic and emerging viruses by skimmed-milk flocculation and PCR in river water from two geographical areas

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ABSTRACT

Molecular techniques and virus concentration methods have shown that previously unknown viruses are shed by humans and animals, and may be transmitted by sewage-contaminated water. In the present study, 10-L river-water samples from urban areas in Barcelona, Spain and Rio Janeiro, Brazil, have been analyzed to evaluate the viral dissemination of human viruses, validating also a low-cost concentration method for virus quantification in fresh water. Three viral groups were analyzed: (i) recently reported viruses, klassevirus (KV), asfarvirus-like virus (ASFLV), and the polyomaviruses Merkel cell (MCPyV), KI (KIPyV) and WU (WUPyV); (ii) the gastroenteritis agents noroviruses (NoV) and rotaviruses (RV); and (iii) the human fecal viral indicators in water, human adenoviruses (HAdV) and JC polyomaviruses (JCPyV). Virus detection was based on nested and quantitative PCR assays. For KV and ASFLV, nested PCR assays were developed for the present study. The method applied for virus concentration in fresh water samples is a one-step procedure based on a skimmed-milk flocculation procedure described previously for seawater. Using spiked river water samples, inter- and intra-laboratory assays showed a viral recovery rate of about 50% (20–95%) for HAdV, JCPyV, NoV and RV with a coefficient of variation ≤50%. HAdV and JCPyV were detected in 100% (12/12) of the river samples from Barcelona and Rio de Janeiro. Moreover, NoV GGII was detected in 83% (5/6) and MCPyV in 50% (3/6) of the samples from Barcelona, whereas none of the other viruses tested were detected. NoV GGII was detected in 33% (2/6), KV in 33% (2/6), ASFLV in 17% (1/6) and MCPyV in 50% (3/6) of the samples from Rio de Janeiro, whereas KIPyV and WUPyV were not detected. RV were only analyzed in Rio de Janeiro and resulted positive in 67% (4/6) of the samples. The procedure applied here to river water represents a useful, straightforward and cost-effective method that could be applied in routine water quality testing. The results of the assays expand our understanding of the global distribution of the viral pathogens studied here and their persistence in the environment.

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Effect of temperature and sunlight on the stability of human adenoviruses and MS2 as fecal contaminants on fresh produce surfaces

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ABSTRACT

Determining the stability, or persistence in an infectious state, of foodborne viral pathogens attached to surfaces of soft fruits and salad vegetables is essential to underpin risk assessment studies in food safety. Here, we evaluate the effect of temperature and sunlight on the stability of infectious human adenoviruses type 2 and MS2 bacteriophages on lettuce and strawberry surfaces as representative fresh products. Human adenoviruses have been selected because of their double role as viral pathogens and viral indicators of human fecal contamination. Stability assays were performed with artificially contaminated fresh samples kept in the dark or under sunlight exposure at 4 and 30 °C over 24 h. The results indicate that temperature is the major factor affecting HAdV stability in fresh produce surfaces, effecting decay between 3 and 4 log after 24 h at 30 °C. The inactivation times to achieve a reduction between 1 and 4-log are calculated for each experimental condition. This work provides useful information to be considered for improving food safety regarding the transmission of foodborne viruses through supply chains.

1. Introduction

Symptomatic and asymptomatic individuals excrete a wide diversity of viruses in urine or feces that are collected in urban wastewater (Cantalupo et al., 2011). Even in areas with implemented sanitation programs it represents the main vehicle for the dissemination of viral pathogens through the environment (Koopmans and Duizer, 2004; Laverick et al., 2004; Rodriguez-Manzano et al., 2010; Albinana-Gimenez et al., 2009). Despite regulations intended to assure food safety, many viral foodborne and waterborne outbreaks occur each year in developed countries. Recent foodborne outbreaks in Europe have been caused by Noroviruses present in lettuce (Ethelberg et al., 2010) or HAV in semidried tomatoes (Gallot et al., 2011).

Pre-harvest contamination of produce by foodborne viruses can occur through a variety of agents including irrigation water (Cheong et al., 2009) and natural fertilizers. In addition, a further route for viral transmission to food products occurs during harvest or processing by infected food handlers or by using polluted water and utensils at some point of the food production chain (Barrabeig et al., 2010; Carter, 2005). The pathogens associated with environmental transmission routes, including water and food, encompass a wide diversity of bacteria, protozoa and viruses such as Norovirus (NoV), hepatitis A virus (HAV), hepatitis E virus (HEV) and adenoviruses. Thus, the use of indicators is essential for investigating water quality, food safety and industrial microbiology. Some concerns have been stated regarding current regulations, mainly based on bacteriologic parameters. Fecal-derived coliforms, thermo-tolerant coliforms, *Escherichia coli* and enterococci, have several drawbacks and limitations in their role as indicators of fecal pollution. Various authors have concluded that these indicators could fail to predict the risk for water and food-borne pathogens including viruses (Gerba et al., 1979; Lipp et al., 2001). Moreover, the levels of bacterial indicators do not always correlate with the concentrations of viruses, especially when these indicators are present at low concentrations (Pina et al., 1998).

Several studies have focused on describing viruses that may be used as indicators of fecal contamination in the environment and food. Human adenoviruses have been proposed as an alternative indicator and as a viral index to improve the control of the microbiological quality of water and food and to reduce the microbiological risk associated with medium and low levels of fecal contamination in water (Puig et al., 1994; Pina et al., 1998; Formiga-Cruz et al., 2003; Rodríguez-Lázaro et al., 2012). Data showing high year-round prevalence in urban sewage and contaminated river water of diverse geographical areas has been accumulating (Pina et al., 1998; Bofill-Mas et al., 2006; Fong et al., 2010; Kokkinos et al., 2011; Rigotto et al., 2010; Simmons and Xagoraraki, 2011). HAdV have also been detected as the most common

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Review Article

Quantification of Human and Animal Viruses to Differentiate the Origin of the Fecal Contamination Present in Environmental Samples

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Many different viruses are excreted by humans and animals and are frequently detected in fecal contaminated waters causing public health concerns. Classical bacterial indicator such as *E. coli* and enterococci could fail to predict the risk for waterborne pathogens such as viruses. Moreover, the presence and levels of bacterial indicators do not always correlate with the presence and concentration of viruses, especially when these indicators are present in low concentrations. Our research group has proposed new viral indicators and methodologies for determining the presence of fecal pollution in environmental samples as well as for tracing the origin of this fecal contamination (microbial source tracking). In this paper, we examine to what extent have these indicators been applied by the scientific community. Recently, quantitative assays for quantification of poultry and ovine viruses have also been described. Overall, quantification by qPCR of human adenoviruses and human polyomavirus JC, porcine adenoviruses, bovine polyomaviruses, chicken/turkey parvoviruses, and ovine polyomaviruses is suggested as a toolbox for the identification of human, porcine, bovine, poultry, and ovine fecal pollution in environmental samples.

1. Fecal Contamination of the Environment

Significant numbers of human microbial pathogens are present in urban sewage and may be considered environmental contaminants. Viruses, along with bacteria and protozoa in the intestine or in urine, are shed and transported through the sewer system. Although most pathogens can be removed by sewage treatment, many are discharged in the effluent and enter receiving waters. Point-source pollution enters the environment at distinct locations, through a direct route of discharge of treated or untreated sewage. Nonpoint sources of contamination are of significant concern with respect to the dissemination of pathogens and their indicators in the water systems. They are generally diffuse and intermittent and may be attributable to the run-off from urban and agricultural areas, leakage from sewers and septic systems, storm water, and sewer overflows [1–3].

Even in highly industrialized countries, viruses that infect humans prevail throughout the environment, causing public health concerns and leading to substantial economic losses. Many orally transmitted viruses produce subclinical infection and symptoms in only a small proportion of the population. However, some viruses may give rise to life-threatening conditions, such as acute hepatitis in adults, as well as severe gastroenteritis in small children and the elderly. The development of disease is related to the infective dose of the viral agent, the age, health, immunological and nutritional status of the infected individual (pregnancy, presence of other infections or diseases), and the availability of health care. Human pathogenic viruses in urban wastewater may potentially include human adenoviruses (HAdVs) and human polyomaviruses (HPyVs), which are detected in all geographical areas and throughout the year, and enteroviruses, noroviruses, rotaviruses, astroviruses,

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ORIGINAL PAPER

Environmental Effectors on the Inactivation of Human Adenoviruses in Water

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Abstract Environmental factors are highly relevant to the global dissemination of viral pathogens. However, the specific contribution of major effectors such as temperature and sunlight on the inactivation of waterborne viruses is not well characterized. In this study, the effect of temperature (7, 20, and 37 °C), UVB and UVA radiation on viral inactivation was evaluated in phosphate buffered saline (PBS), mineral water, wastewater, 1,000-fold diluted wastewater and seawater. The stability of human adenoviruses infectivity, known as human pathogens and indicators of fecal contamination, was monitored during 24 h, both in the dark and exposed to UV radiation by immunofluorescence assays. In the dark, no Human adenovirus (HAdV) inactivation was observed in PBS and mineral water at any of the temperatures studied, whereas at 37 °C in reactors with higher microbial concentration (wastewater, diluted wastewater, and seawater), decays between 2.5 and 5 log were recorded. UVB radiation showed a dramatic effect on HAdV inactivation and 6-log were achieved in all reactors by the end of the experiments. The effect of UVA showed to be dependent on the water matrix analyzed. At 20 °C, HAdV showed a 2-log decay in all reactors radiation while at 37 °C, results in wastewater, diluted wastewater, and seawater reactors were equivalent to those observed in the dark. These results suggest UVB radiation as the major environmental factor challenging viral inactivation, followed by biotic activity indirectly associated to higher temperatures and finally, by UVA radiation.

Keywords UVA · UVB · Temperature · Microorganisms · Inactivation · Viruses · Water · Infectivity

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Introduction

Wastewater and contaminated water used for irrigating, drinking, and recreational purposes has been widely linked with the transmission of infectious viral diseases among human populations (McKinney et al. 2006; ter Waarbeek et al. 2010; Riera-Montes et al. 2011; Nenonen et al. 2012). Viral pathogens are frequently released in stool and urine in high numbers, and may contaminate surface and coastal waters establishing an important route for their transmission and a public health concern.

It is widely accepted that the stability of a virus is key to its capability to remain infectious over time in the environment or after disinfection treatments and consequently, to spread and cause disease. In particular, a high stability has been reported for certain enteric viruses in a range of environmental conditions and after various disinfection treatments (Carter 2005). However, it is known that viral pathogens may be naturally inactivated, by both abiotic and biotic factors (Gordon and Toze 2003; Bertrand et al. 2012).

Sunlight is known as a challenge to the persistence of viruses (Viau et al. 2011; Boehm et al. 2009) and it has been

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ORIGINAL PAPER

Adenovirus and Norovirus Contaminants in Commercially Distributed Shellfish

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Abstract Shellfish complying with European Regulations based on quantification of fecal bacterial indicators (FIB) are introduced into markets; however, information on viruses, more stable than FIB, is not available in the literature. To assess the presence of noroviruses (NoVs) GI and GII and human adenoviruses (HAdV) in domestic and imported mussels and clams ($n = 151$) their presence was analyzed during winter seasons (2004–2008) in north-west Spanish markets through a routine surveillance system. All samples tested negative for NoV GI and 13 % were positive for NoV GII. The role of HAdV as viral indicator was evaluated in 20 negative and 10 positive NoV GII samples showing an estimated sensitivity and specificity of HAdV to predict the presence of NoV GII of 100 and 74 % (cut-off 0.5). The levels of HAdV and NoVs and the efficiency of decontamination in shellfish depuration plants (SDP) were evaluated analyzing pre- and post-depurated mussels collected in May–June 2010 from three different SDP. There were no statistically significant differences in the prevalence and quantification of HAdV between pre- and post-depurated shellfish and between seawater entering and leaving the depuration systems. Moreover, infectious HAdV were detected in depurated mussels. These results confirm previous studies showing that current controls and depuration treatments limiting the number of FIB do not guarantee the absence of viruses in shellfish.

Keywords Food safety · Norovirus · Human adenovirus · Food-borne · Depuration systems · Shellfish

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Introduction

Noroviruses (NoV) are known as the major cause of outbreaks of acute gastroenteritis and have been reported in hospitals, cruise ships, military and holiday camps, nursing homes, nurseries, and hotels (Tuan Zainazor et al. 2010; Rodríguez-Lázaro et al. 2012). Epidemiological studies have estimated NoVs to be responsible for 60–80 % of all food-borne outbreaks of gastroenteritis worldwide (Koopmans et al. 2001; Reuter et al. 2005). Consumption of contaminated shellfish is known to be one of the main transmission routes for NoVs infection (Loisy et al. 2005). Shellfish consumption can lead to NoV outbreaks because shellfish is frequently consumed raw or insufficiently cooked around the world, which increases the risk of viral infection (Murchie et al. 2005).

All European Union (EU) Member States are subjected to the requirements of the Shellfish Hygiene Directive, which sets out the need for depuration or treatment before shellfish from Class B or C designated waters are put on the market. These standards rely exclusively on *E. coli*