

UNIVERSITAT DE BARCELONA

El mostreig passiu per a la detecció i caracterització de virus en aigües: aplicacions en vigilància epidemiològica i gestió de recursos hídrics

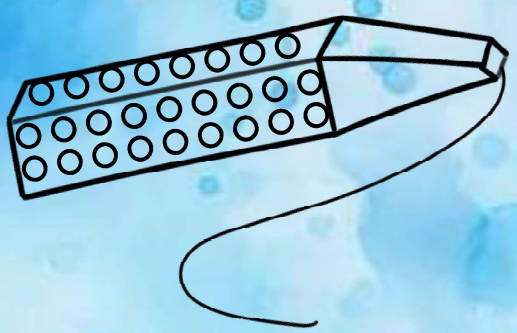
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Tesi doctoral

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Facultat de Biologia
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El mostreig passiu per a la detecció i caracterització de virus en aigües: aplicacions en vigilància epidemiològica i gestió de recursos hídrics

Memòria presentada per

Cristina Mejías Molina

per a optar al grau de

Doctora per la Universitat de Barcelona.

Doctoranda:



Cristina Mejías Molina

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Directora i tutora

Sílvia Bofill-Mas

Directora

Marta Rusiñol Arantegui

A mis padres,

Aquesta tesi ha estat finançada amb una beca *Ajuts per al recolzament a departament i unitats de investigació universitaris per a la contractació de personal investigador predoctoral en formació (FI SDUR 2022)*

Agraïments

Ara mateix deu fer prop de tres mesos que dic que estic a punt d'acabar la tesi. "Acabar d'acabar-la" ha estat igual o més llarg que el procés d'escriptura. Però ara, que el tribunal està avisat, vaig per la versió 5 del PDF i no queda ningú a qui demanar-li que s'ho miri, sembla que arriba el final d'aquest procés i, amb aquest, el final de la meva etapa de doctorat. Amb això arriba el moment dels agraïments, un dels moments més emotius de la tesi, per qui l'escriu i per a qui la llegeix, perquè aquesta etapa ha sigut una època de creixement personal i científic. I la persona i científica que sóc ara és producte, en bona part, de la gent que m'ha acompanyat en aquest procés i que ha fet que aquesta tesi en si sigui com és, així que no puc fer una altra cosa que agrair-ho.

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taula on em vas ensenyar que un bon excel és la base de tot. Tant de bo tot el que m'has ensenyat em faci ser una mica com tu.

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Lab 11

Desembre de 2025

RESUM

Molts dels virus que infecten humans són excretats al medi a través de la femta o de l'orina, independentment que l'individu infectat presenti o no símptomes. L'estudi dels virus presents en l'aigua residual permet, per tant, conèixer la circulació d'aquests dins d'una comunitat concreta.

En aquesta tesi s'ha avaluat un sistema de mostreig passiu per a la detecció i caracterització per metagenòmica de virus en aigua residual amb l'objectiu principal d'aplicar-lo en contextos poblacionals petits i/o de difícil accés amb als sistemes tradicionals de mostreig, com ara edificis. Un cop validat com a mètode sensible per a la caracterització dels virus excretats, s'ha utilitzat per analitzar el viroma d'aigües residuals provinents d'edificis amb perfils demogràfics diferenciats, amb la finalitat d'identificar les diferències existents entre aquests grups poblacionals.

Els virus presents a les aigües residuals poden convertir-se en una font de contaminació del medi aquàtic a través d'abocaments d'aigua residual no tractada derivats de fuites de clavegueram o de fosses sèptiques, però també a través dels efluents de les estacions depuradores d'aigua residual (EDARs) que tot i els diferents passos de tractament, alliberen contínuament virus al medi per mitjà dels efluents. Paral·lelament, el context actual marcat per una creixent escassetat de recursos hídrics fa necessari avançar cap a una gestió més eficient i sostenible dels recursos, això implica tenir en compte l'estat de fonts d'aigua potencialment contaminades i de l'aigua regenerada potencialment reutilitzada.

Per aquest motiu, en aquesta tesi s'ha aplicat per primera vegada el mostreig passiu per al monitoratge de virus en aigües regenerades i aigües subterrànies, entorns on els virus es troben habitualment en concentracions molt baixes. Els resultats obtinguts a l'aqüífer demostren que aquesta metodologia és una alternativa viable per detectar contaminació fecal d'origen humà i millorar la gestió d'aquesta font d'aigua mitjançant la

detecció de patògens vírics La seqüenciació massiva ha permès per primera vegada en aquest compartiment la descripció del viroma de l'aigua subterrània. Per altra banda, l'ús de mostrejadors passius per al monitoratge de l'aigua regenerada, produïda en sistemes basats en la natura com els aiguamolls construïts, fa possible monitoritzar de manera senzilla la presència de patògens víric d'importància per a la salut pública.

En conjunt, els resultats d'aquesta tesi posen de manifest que el mostreig passiu és una metodologia versàtil per a la detecció i caracterització de virus en aigües amb diferents graus de contaminació i que amplia la capacitat de vigilància en aquells entorns on les tècniques convencionals presenten limitacions o són difícils d'implementar.

PARAULES CLAU

Mostreig passiu, aigua residual, aigua subterrània, metagenòmica, PCR, aigua regenerada, contaminació vírica.

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ABREVIACIONS

AAV	Virus Adeno-associat
ADN	Àcid desoxiribonucleic
ADNdc	ADN bicatenari
ADNmc	ADN monocatenari
ADS	Seqüenciació massiva d'amplicons (de l'anglès *Amplicon Deep Sequencing*)
AiV	Aichivirus
ARN	Àcid ribonucleic
ARNdc	ARN bicatenari
ARNmc	ARN monocatenari (de l'anglès *single strand RNA*)
BKPyV	Poliomavirus BK
CDC	Centres per al Control i la Prevenció de Malalties (*Centers for Disease Control and Prevention)*
GC	Còpies genòmiques (de l'anglès *genome copies*)
CW-BC	Aiguamoll contruït amb carbó actiu (de l'anglès *Constructed wetland with biochar)*
CW-RM	Aiguamoll construït amb un sistema amb catifes d'arrels (de l'anglès *Constructed wetland with floating root mat*)
CMV	Citomegalovirus
dPCR	PCR digital
ddPCR	PCR digital basada en gotes
EDAR	Estació Depuradora d'Aigües Residuals

EV	Enterovirus
GC	Còpies genòmiques
HAdV	Adenovirus humà
HAstV	Astrovirus humà
HAV	Virus de l'hepatitis A
HBoV	Bocavirus humà
HBuV	Bufavirus
HEV	Virus de l'hepatitis E
HMPV	metapneumovirus humà
HPV	Virus del papil·loma humà
HTS	Seqüenciació d'alt rendiment (de l'anglès *High Troughtput Sequencing*)
HuACV	Circovirus humà
HuACyV	Ciclovirus humà
IAV	Virus de la influença A
IBV	Virus de la influença B
JCPyV	Poliomavirus JC
JRC	Joint Research Centre
KIPyV	Poliomavirus KI
MCPyV	Poliomavirus de les cèl·lules de Merkel
MDA	Amplificació de desplaçament múltiple (de l'anglès *Multiple Displacement Amplification*)
ml	Mil·lilitre

Mpox	Virus de la verola del mico
MST	Traçar la font de microorganismes (de l'anglès *Microbial Source Tracking*)
NBS	Solucions basades en la natura (de l'anglès *Nature based solutions)*
NoV	Norovirus
nPCR	PCR niuada (o *nested* PCR)
OMS	Organització Mundial de la Salut
PaV	Parvovirus
PCR	Reacció en cadena de la polimerasa (de l'anglès *Polymerase Chain Reaction*)
PMMoV	Virus del motejat suau del pebrot (de l'anglès *Pepper mild mottle virus*)
PeV	Parecovirus
qPCR	PCR quantitativa
RoV	Rotavirus
RSV	Virus respiratori sincitial
SARS-CoV-2	Coronavirus 2 del síndrome respiratori agut greu (de l'anglès *Severe acute respiratory syndrome-related coronavirus 2*)
SaV	Sapovirus
SISPA	Amplificació independent de seqüència (de l'anglès *Sequence-Independent single-primer amplification*)
TES	Seqüenciació amb enriquiment de dianes (de l'anglès *target enrichment sequencing*)
TTV	Torque teno virus

WBE	Epidemiologia d'aigües residuals (de l'anglès *Wastewater Based Epidemiology*)
WUPyV	Poliomavirus WU

INTRODUCCIÓ

1. Els virus i la contaminació fecal de l'aigua

S'han descrit centenars de virus que infecten humans, la majoria dels quals són excretats al medi mitjançant femta, orina, altres secrecions de fluids corporals com la saliva, o per la descamació de la pell. Els virus que infecten les cèl·lules del tracte gastrointestinal són excretats en quantitats molt elevades a les femtes (10^7–10^9 CG/g) per part d'individus infectats, tant si presenten símptomes com si no, i sovint fins i tot després d'haver superat el període de malaltia. Aquests virus poden arribar a contaminar diferents tipus de masses d'aigua per diferents vies, que s'expliquen més endavant en aquesta introducció, convertint a l'aigua i els aliments en vehicles per a la seva transmissió (Rusiñol & Girones, 2019).

Els principals virus patògens que s'associen a la transmissió per l'aigua i els aliments pertanyen a sis famílies: *Picornaviridae, Caliciviridae, Hepeviridae, Reoviridae, Astroviridae* i *Adenoviridae* (Fig. 1). Poden provocar des d'infeccions asimptomàtiques fins a brots i casos clínics amb una gran diversitat de manifestacions, que inclouen gastroenteritis, meningitis, malalties respiratòries, conjuntivitis, miocarditis, paràlisi o hepatitis.

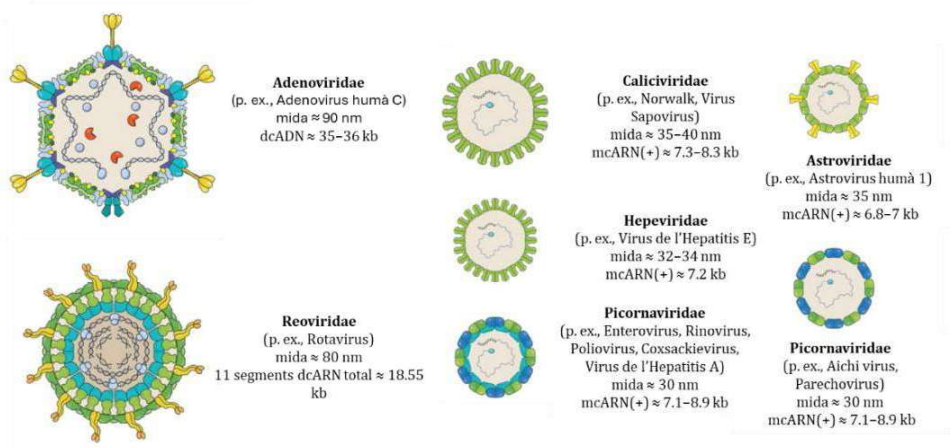


Figura 1. *Representació de les 6 principals famílies de virus transmesos per l'aigua. Adaptació de Armanious & Mezzenga, 2022.*

D'altra banda, a més d'aquestes famílies, n'hi ha d'altres que es poden detectar en l'aigua residual i que la seva excreció o bé no està associada a malaltia i són excretats de manera persistent o bé no es coneix la malaltia que ocasionen. Entre aquestes famílies destaquen *Anelloviridae, Circoviridae Parvoviridae, Papillomaviridae, Picobirnaviridae* i *Polyomaviridae.*

A la taula 1 s'indiquen les principals famílies víriques que s'excreten i es troben a l'aigua residual, els principals virus patògens humans que en formen part, per quina via s'excreten i les possibles malalties relacionades amb la seva infecció en humans.

Taula 1. *Principals famílies de virus que s'excreten a través de femta, orina o pell i les seves característiques. Adaptada de Rusiñol i Girones, 2017.*

Família (genoma, mida)	**Gènere**	**Espècies que infecten humans**	**Excreció**	**Malalties relacionades**
Adenoviridae (ADNdc, 70- 90nm)	*Mastadenovirus*	Adenovirus humans (HAdV) A-G	Persistent	Gastroenteritis, infeccions respiratòries, conjuntivitis, cistitis
Anelloviridae (ADNmc, 30-32nm)	*Alphatorquevirus*	Torquetenovirus (TTV)	Persistent	Possible associació amb diverses malalties com hepatitis o malalties pulmonars
Astroviridae (ARNmc, 28-30nm)	*Mamastrovirus*	Astrovirus (HAstV) 1-9	Associada a malaltia	Gastroenteritis
Caliciviridae (ARNmc, 27- 40nm)	*Norovirus*	Norovirus (NoV) GI, GII, GIV	Associada a malaltia	Gastroenteritis
	Sapovirus	Sapovirus (SaV) GI, GII, GIV, GV	Associada a malaltia	Gastroenteritis

Circoviridae (ADNmc, 15- 20nm)	*Circovirus*	Circovirus humà (HuACV) 1	Associada a malaltia / Persistent	Possible associació amb infeccions sistèmiques
	Cyclovirus	Ciclovirus humà (HuACyV) 1-12	Associada a malaltia / Persistent	Possible associació amb infeccions sistèmiques
Coronaviridae (ARNmc, 80-220nm)	*Betacoronavirus*	SARS-CoV-2	Associada a malaltia	Síndrome respiratòria aguda greu
		Coronavirus humans(HCoV)	Associada a malaltia	Infeccions respiratòries
Hepeviridae (ARNmc, 27- 34nm)	*Orthohepevirus*	Virus de l'hepatitis E G1,2,3,4,7 (HEV)	Associada a malaltia	Hepatitis aguda
Picornaviridae (ARNmc, 24-30nm)	*Enterovirus*	Enterovirus A-D, Rhinovirus A-C,	Associada a malaltia	Paràlisis, meningitis, malaltia boca-mà-peu, anomalies cardíaques, erupció cutània, poliomielitis
	Hepatovirus	Virus de l'hepatitis A GI-III (HAV)	Associada a malaltia	Hepatitis aguda
	Kobuvirus	Aichivirus A-C (AiV)	Associada a malaltia	Gastroenteritis
	Parechovirus	Parechovirus 1-16 (PeV)	Associada a malaltia	Gastroenteritis, infeccions respiratòries, encefalitis, meningitis, hepatitis
Reoviridae (ARNdc, 70- 75nm)	Rotavirus	Rotavirus A-G (RoV)	Associada a malaltia	Gastroenteritis
Parvoviridae (ADNmc, 18-26nm)	*Erythroparvovirus*	Parvovirus B19 (PaV)	Associada a malaltia	Eritema infecciós en nens, artropatia, hepatitis
	Bocaparvovirus	Human bocavirus 1-4 (HBoV)	Associada a malaltia	Gastroenteritis, relacionats amb infeccions respiratòries
	Protoparvovirus	Bufavirus (HBuV)	Associada a malaltia	Gastroenteritis
	Dependovirus	Virus Adeno-associats (AAV)	Associada a malaltia?	Desconegut
Papillomaviridae (ADNdc, 50- 60nm)	*Alphapapillomavirus*	Papil·loma virus humans (HPV) 16, 18, 6, 11, entre d'altres	Associada a malaltia / Persistent	Càncer de cèrvix, penis, anus, vulva i orofaringe. També associats a berrugues genitals, infeccions respiratòries
	Betapapillomavirus	HPV 20, 80, 110, 120, entre d'altres	Persistent	Poden estar associats a berrugues genitals, anals i perianals

Polyomaviridae (ADNdc, 50 -60nm)	*Betapolyomavirus*	Poliomavirus JC (JCPyV)	Persistent	Leucoencefalopatia multifocal progressiva (LMP)
		Poliomavirus BK (BKPyV)	Persistent	Infeccions respiratòries lleus, nefropatia, cistitis hemorràgica
		Poliomavirus WU (WUPyV)	Persistent	Possible associació amb infeccions respiratòries
		Poliomavirus KI (KIPyV)	Persistent	Possible associació amb infeccions respiratòries
	Alphapolyomavirus	Poliomavirus de les cèl·lules de Merkel (MCPyV)	Persistent	Associats al carcinoma de cèl·lules Merkel

La presència d'algunes d'aquestes famílies en aigües residuals i el paper de l'aigua en la seva transmissió s'han documentat i caracteritzat des dels inicis de la virologia ambiental. La presència d'altres famílies víriques ha estat descrita gràcies als avenços fets en biologia molecular, en una primera etapa gràcies als mètodes basats en PCR i més endavant gracies a la metagenòmica.

En els darrers anys s'ha evidenciat que també alguns virus respiratoris, com el virus de la influenza A i B (IAV i IBV), el citomegalovirus (CMV), el virus d'Epstein-Barr, els rinovirus, el virus respiratori sincitial (RSV) i el SARS-CoV-2, poden ser excretats i detectats en aigües residuals encara que no es transmetin per la via fecal-oral .

Paral·lelament, l'expansió de les tècniques de seqüenciació massiva ha revelat l'existència d'una gran quantitat de seqüències que corresponen potencialment a virus desconeguts que infecten humans i que també poden ser excretats de manera persistent. Segons el tipus de mostra, aquestes seqüències no identificades poden representar entre el 40 i el 90% de totes les seqüencies (Wang, 2020). Tot plegat, amplia enormement les possibilitats de la virologia ambiental com a disciplina.

2. L'epidemiologia basada en aigües residuals

L'epidemiologia basada en aigües residuals (de l' anglès WBE, wastewater-based epidemiology) es defineix com una eina per avaluar la salut comunitària mitjançant l'anàlisi de mostres d'aigües residuals per detectar una varietat d'agents químics i biològics, incloent-hi drogues i patògens, en temps gairebé real (Kasprzyk-Hordern et al., 2024). Aquesta aproximació (Fig. 2) proporciona informació valuosa sobre l'estat de salut de la població d'una comunitat determinada (Mao et al., 2020). Aquests agents poden incloure drogues, fàrmacs o productes de cura personal, marcadors poblacionals, indicadors d'exposició a contaminants industrials, marcadors d'estrès, dieta i alimentació, així com agents microbians (Choi et al., 2018).

Quan s'aplica per al monitoratge de virus o altres microorganismes, aquesta eina permet identificar brots, seguir tendències temporals de la infecció a la població, recollir dades epidemiològiques en regions específiques i obtenir informació en temps real sobre la situació sanitària (Prado et al., 2023). La WBE es va aplicar per primer cop al segle XX per a detectar l'agent causant de la febre tifoide (Gray, 1929; Wilson, 1928) i, posteriorment, per monitorar la circulació del virus causant de la poliomelitis (Paul et al., 1939; Poyry et al., 1988). En aquest últim cas, la vigilància ambiental del poliovirus, coordinada per l'Organització Mundial de la Salut (OMS) dins la Iniciativa Global per a l'Erradicació de la Poliomielitis, ha permès identificar els nivells de circulació i avaluar-ne els nivells d'immunització a la població (Hovi et al., 2012; Nakamura et al., 2015; Ndiaye et al., 2014; Tiwari & Dhole, 2018).

Des de llavors, la seva aplicació ha permès conèixer quins virus estan presents en aigües residuals i en quines concentracions (Corpuz et al., 2020; Haramoto et al., 2018). Estudis de WBE han permès saber; que els poliomavirus humans eren excretats en altíssims percentatges i podien ser emprats com a indicadors de contaminació fecal d'origen humà (Bofill-Mas

et al., 2000), descriure la circulació del virus de l'hepatitis E (HEV) en països industrialitzats (Clemente-Casares et al., 2003) o avaluar l'efecte de la vacunació en la circulació de soques de virus de l'hepatitis A (HAV) o rotavirus (RoV) (Matthijnssens et al., 2012; Pintó et al., 2007).

Atès que el SARS-CoV-2 també és un virus que s'excreta en femta (Medema et al., 2020) i que la seva concentració en aigües residuals es pot correlacionar amb el nombre de persones infectades (Huisman et al., 2022), el seu monitoratge va ser clau durant la pandèmia de COVID-19 l'any 2020 per fer el seguiment de l'evolució de la malaltia i per recolzar la presa de decisions en salut pública (Randazzo et al., 2020).

Com a resultat, nombroses administracions a nivell mundial van establir xarxes de vigilància basades en l'anàlisi de dianes genòmiques del virus a les aigües residuals. Avui en dia, la plataforma web COVIDPopps19, encara recull un llistat de més de 4.000 punts de vigilància actius en un total de 72 països. (UC Merced, n.d.). A Catalunya, actualment es fa una anàlisi mensual de la concentració de dianes genòmiques del virus SARS-CoV-2, així com el tipatge de les variants circulants (*Sarsaigua*, n.d.). A nivell estatal, també es va desenvolupar al 2020 una xarxa de vigilància mitjançant aigües residuals, tot i que des de 2024 no es troba en actiu(Ministerio de Sanidad, n.d.) .

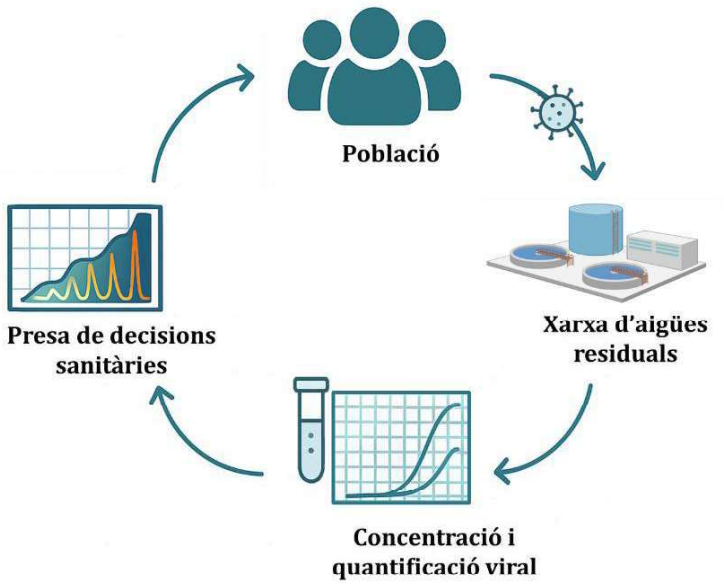


Figura 2. *Esquema de flux de procediments i decisions en els que es basa l'epidemiologia basada en aigües residuals.*

La crisi provocada per la COVID-19 va reforçar la visibilitat de la WBE i va impulsar-ne el desenvolupament com a eina clau per la salut pública. De fet, l'any 2020 es va produir un 89% d'augment en el nombre d'estudis sobre epidemiologia de dianes víriques basada en les aigües residuals respecte el 2019 (Barcellos et al., 2023). Molts d'aquests estudis s'han centrat en la presència de SARS-CoV-2 (Bivins et al., 2020; Itarte, Bofill-Mas, et al., 2021; Kitajima et al., 2020; Martínez-Puchol et al., 2021a; Rimoldi et al., 2020; Rosa et al., 2020; Rusiñol et al., 2021) però també han aparegut investigacions sobre altres virus presents en aigües residuals, com per exemple norovirus (NoV) i enterovirus (EV) (Boehm et al., 2023; Guo et al., 2022).

A conseqüència d'això no només han aparegut estudis sinó també iniciatives a nivell governamental amb l'objectiu de crear sistemes de detecció precoç, prevenció, monitoratge a temps real d'amenaces pandèmiques i brots, més enllà de les que ja existien pel SARS-CoV-2.

A nivell europeu ha sorgit el EU Sewage Sentinel System que dins del marc del *European Health Union*, actualment coordina la vigilància de SARS-CoV-2, IAV i RSV en les aigües residuals de 14 països europeus (ECDC, n.d.)). També ha sorgit un consorci internacional anomenat GLOWOACON (*Global Consortium for Wastewater and Environmental Surveillance for Public Health)* amb l'objectiu d'establir sistemes sentinella per a la detecció precoç, prevenció i monitoreig a temps real de potencials epidèmies i brots (European Comission, 2024.).

A Estats Units ha estat implementat des del *Centers for Disease Control and Prevention* (CDC) el *National Wastewater Surveillance System* (NWSS) per a la detecció de SARS-CoV-2, IAV, RSV, virus de la verola del mico (Mpox), i el poliovirus i que cobreix més del 45% de la població (CDC, 2023), A més, també ha aparegut el programa nacional WastewaterSCAN amb l'objectiu de fer el seguiment de EV D68, el metapneumovirus humà (HMPV), el RSV, IAV i IBV, SARS-CoV-2, NoV Genogrup II (NoV GII), *Candida auris*, HAV, Mpox, el virus del xarampió, RoV, parvovirus i els HAdV del grup F (principalment HAdV 40 i 41). Aquestes dades també alimenten el sistema NWSS, però, a diferència d'aquest, WastewaterSCAN es finança exclusivament amb fons filantròpics (WastewaterSCAN, n.d.).

Tanmateix, no és l'aigua residual l'única font amb potencial per a dur a terme estudis epidemiològics, qualsevol matriu que presenti contaminació vírica i aquest provingui d'un nombre gran d'individus és susceptible a poder ser emprada per aquest supòsit en el marc de que s'anomena vigilància epidemiològica ambiental, que en els recents anys ha guanyat protagonisme com a estratègia de salut pública i gestió de riscos.

3. L'aigua residual com a font de contaminació de l'aigua

Com s'ha comentat al principi d'aquesta introducció, els humans, i també els animals, excretem virus i altres patògens que poden arribar a les masses d'aigua tant per vies directes, com la defecació dins de cursos fluvials, com per vies indirectes, com ara les fuites dels sistemes de clavegueram i fosses sèptiques, l'escorrentia urbana o agrícola, o l'ús de fems animals com a fertilitzants en pràctiques agrícoles (Farkas et al., 2020; Hata et al., 2014; Jurado et al., 2019).

La contaminació fecal constitueix la principal font de contaminació microbiològica de l'aigua (WHO, 2017) i les infeccions gastrointestinals associades al consum o ús de l'aigua contaminada suposen uns 4.000 milions de casos anuals, amb una mortalitat estimada de 2 milions de persones a l'any, sent la majoria d'infants menors de cinc anys i en països de baixa renda (WHO, 2010). Una part significativa d'aquests casos s'atribueix directament a virus transmesos per via fecal-oral, essent el contacte directe amb individus infectats la via principal de transmissió (Katayama & Vinjé, 2019; Ramani & Kang, 2009). Aquests virus són persistents en entorns afectats per les descàrregues d'aigües residuals urbanes i sovint s'associen a brots relacionats amb l'aigua (Gibson, 2014; Kauppinen et al., 2019; Sekwadi et al., 2018).

La contaminació de l'aigua (Fig. 3) també constitueix una via important d'entrada dels virus als aliments: en el cas de fruites i verdures, mitjançant aigua de reg contaminada, o bé per manipulació durant el cultiu, la collita, el transport o el processament (Koopmans & Duizer, 2004; Pachepsky et al., 2011). A més, l'exposició a virus per parts dels treballadors de les EDARs pot suposar un risc per la seva salut ja sigui per inhalació d'aerosol o la ingestió oral a través de superfícies contaminades i el contacte mà-boca (Itarte et al.,

2024). Els mol·luscs bivalves, com musclos i ostres, filtren grans volums d'aigua i poden acumular patògens presents en zones costaneres afectades per abocaments residuals, ja sigui per fuites de clavegueres i escorrenties durant períodes de pluja, o bé per aigües residuals tractades de manera deficient, (Koopmans & Duizer, 2004; Landrigan et al., 2020).

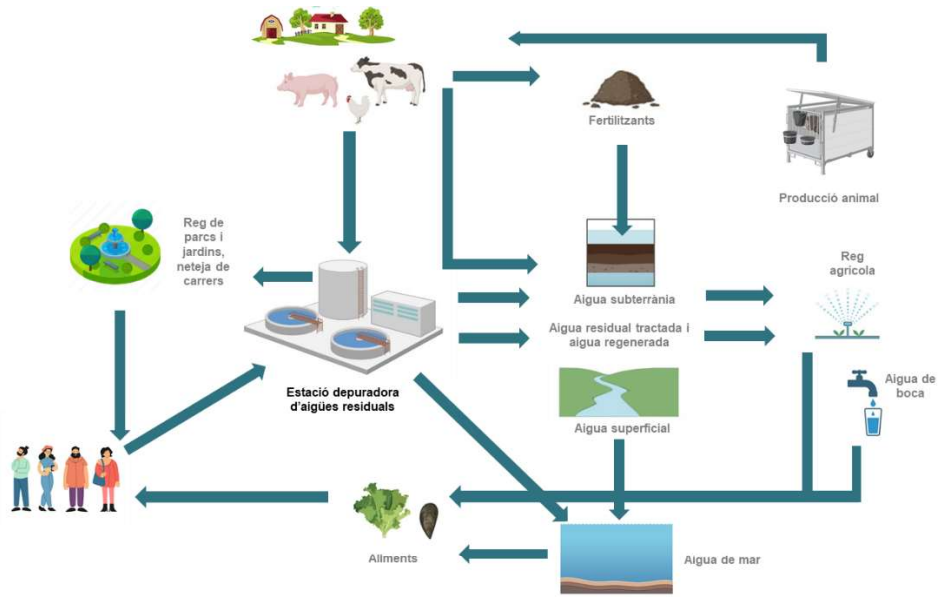


Figura 3. *Esquema que recull les principals vies de contaminació fecal de l'aigua.*

4. Riscos sanitaris emergents en un context de canvi climàtic i escassetat d'aigua

La presència i transmissió de patògens per l'aigua s'inscriu dins del paradigma One Health, que entén la salut humana, animal i ambiental com a interdependents i que promou estratègies ben coordinades per fer front a riscos sanitaris emergents (Brown et al., 2024). Quan s'afegeix la situació actual de canvi climàtic i estrès hídric que afecta per igual a la salut de les persones, dels animals i dels ecosistemes es posa de manifest la necessitat de promoure estratègies coordinades per fer front als riscos sanitaris emergents.

L'àrea mediterrània és un dels punts del planeta més vulnerables al canvi climàtic, amb projeccions que indiquen un augment de la temperatura mitjana superior a 2ºC i una reducció de les precipitacions anuals de fins al 20-30% cap a finals de segle XXI (IPCC, 2023). Com a conseqüència, la freqüència i la intensitat dels fenòmens extrems com les sequeres prolongades i les pluges intenses concentrades en períodes curts, incrementaran els episodis d'escorrentia i la càrrega de contaminants als sistemes hídrics. La combinació d'aquestes condicions afavoreix la mobilització de patògens i la seva dispersió a l'ambient tant en zones agrícoles i ramaderes com en zones urbanes, fins a punts de captació d'aigua potable o fins a zones recreatives, afectant diferents masses d'aigua ja sigui per escorrentia superficial o per infiltració a l'aigua subterrània (UNEP, 2021; Jung et al., 2023).

Tot això suposa un risc creixent de contaminació de l'aigua i afegeix nous riscos per a la salut pública, especialment en escenaris de menor disponibilitat d'aigua que obligaran a reutilitzar aigües regenerades o aprofitar recursos d'aigua impactats directament per aquesta contaminació.

5. Fonts alternatives d'aigua microbiològicament segura

Els virus que es troben presents en l'aigua són un motiu de preocupació per la salut pública, tant per la seva capacitat de causar malalties agudes com per la seva baixa dosi infecciosa, que facilita la transmissió fins i tot a concentracions molt petites (Haas et al., 1993). La concentració en l'aigua pot variar de manera significativa en temps i espai i si la font de contaminació és contínua o el resultat d'una entrada sobtada de contaminació fecal (La Rosa et al., 2012). Els virus no poden replicar-se fora de les cèl·lules del seu hoste i, per tant, no poden multiplicar-se en l'entorn. Tot i això, poden sobreviure al medi ambient durant períodes prolongats, més que la majoria de bacteris intestinals, cosa que fa que no sigui segur basar-se només en els estàndards bacteriològics per a determinar la qualitat de l'aigua. S'ha observat que alguns adenovirus poden sobreviure i mantenir la seva infectivitat fins a 130 dies en aigua marina, fins a 120 dies en aigua dolça i fins a 100 dies en sòl a 20-30 °C (Fong & Lipp, 2005). Les comparacions entre diferents virus també mostren variabilitat, per exemple els AdV que poden sobreviure més temps a l'aigua que altres virus, com per exemple el HAV i el poliovirus (La Rosa et al., 2012).

5.1 L'aigua residual tractada

El tractament de les aigües residuals a EDARs inclou filtres de sorra (tractament primari) i clarificadors amb fangs activats (tractament secundari). Tot això és insuficient per l'eliminació completa dels patògens presents (Gerba, 2008). Els virus indicadors de la contaminació fecal humana com els HAdV i els poliomavirus JC (JCPyV) han estat detectats en efluents de tractaments secundaris i terciaris de les EDARS i són alliberats al medi constantment (Bofill-Mas et al., 2006; Rusiñol et al., 2015). Altres virus

entèrics com els NoV, RoV i els EV també han estat detectats als efluents de les EDAR (Sano et al., 2016).

La presència d'aquests virus a l'aigua residual tractada, segons el destí final d'aquesta, pot tenir implicacions importants sobre la qualitat del medi receptor i la salut pública, sobretot si aquesta aigua s'aboca a masses d'aigua superficial com rius o llacs amb usos recreatius o bé abastament o reg. Llavors, pot existir un risc d'exposició directa o indirecta a aquests patògens.

Tal i com s'ha comentat a la secció anterior, trobar nous usos per a la reutilització d'aigua residual tractada o regenerada és essencial en un context de canvi climàtic. L'ús d'aquesta font d'aigua regenerada en agricultura és una pràctica cada vegada més estesa per fer front a l'escassetat hídrica. De fet, al juny de 2023 va entrar en vigor a la Unió Europea el reglament (UE), 2020/741 transposat a la llei espanyola al reial decret 1085/2024, que estableix els diferents usos i els requisits mínims per a la reutilització de les aigües residuals tractades (Regulation (EU) 2020/741, 2020). Aquesta normativa fixa estàndards microbiològics, protocols de monitoratge i l'obligació d'implementar plans de gestió de risc en tota la cadena d'ús de l'aigua regenerada. Segons la Comissió Europea, l'aplicació d'aquest marc legal pot multiplicar per sis la reutilització d'aigua residual tractada a la UE, que actualment només representa un 2,4 % del total disponible (European Commission, 2022). Per altra banda, el *Joint Research Centre* (JRC), el servei científic i tècnic intern de la UE, ha elaborat una guia tècnica per facilitar-ne la implementació, amb exemples de bones pràctiques aplicades a diversos estats membres, com Espanya, pioner en l'ús d'aigua regenerada per a l'agricultura (European Commission, 2022). Aquesta iniciativa normativa s'emmarca en un context de creixent vulnerabilitat hídrica: al voltant del 10% de la població europea viu en zones amb estrès hídric crític (UN-Water & FAO, 2024), i gairebé la meitat de les conques fluvials europees podrien patir escassetat d'aigua abans de 2030 (UNESCO-WWAP, 2022).

Aquesta normativa centra la qualitat microbiològica d'aquestes aigües en la presència de dos indicadors bacterians de la contaminació fecal i un índex viral per validar sistemes de tractament segons l'ús final que en tingui aquesta que són: *Eschericchia coli* , legionel·la i els colifags somàtics. Malgrat això, diferents estudis han demostrat la presència de virus patògens e indicadors en aigües recreatives que complien amb la normativa (Schets et al., 2008) arribant a suposar un risc elevat d'infecció (Purnell et al., 2020).

La reutilització de l'aigua residual és una eina clau per a l'adaptació al canvi climàtic, especialment en regions mediterrànies afectades per sequeres recurrents.

5.2 L'aigua subterrània

Malgrat que l'aigua és essencial per a la vida, una part significativa de la població mundial encara no té accés garantit a aigua potable en condicions segures. Segons l'informe conjunt de la OMS i UNICEF (Joint Monitoring Programme, 2023), al voltant del 27 % de la població mundial (aproximadament 2.200 milions de persones) no disposen d'un servei d'aigua potable gestionat de manera segura, és a dir, disponible al domicili, quan es necessita i lliure de contaminació. A més, 703 milions de persones continuen sense accés tan sols a serveis bàsics d'aigua potable. La demanda global d'aigua dolça ha crescut aproximadament un 1 % anual des dels anys vuitanta i es preveu que augmenti entre un 20 % a 30 % cap al 2050, arribant a valors entre 5.500 i 6.000 km^3 anuals (WRI, 2025; WWDR, 2019). Simultàniament, més de la meitat de la població mundial (52 %) viurà en zones afectades per escassetat hídrica com a mínim un mes cada any, augmentant fins a un 57 % el 2050 .

Aquesta situació es veu agreujada pels efectes del canvi climàtic, l'increment demogràfic, factors que accentuen la pressió sobre els recursos hídrics i evidencien la necessitat urgent de diversificar i ampliar les fonts d'abastament, així com garantir-ne la qualitat i sostenibilitat. Davant d'aquest

escenari, estratègies com la reutilització d'aigua regenerada o la protecció dels aqüífers com a fonts i reservoris d'aigua de beguda, emergeixen per garantir l'accés universal a l'aigua en el futur.

L'aigua subterrània és una font imprescindible per a l'abastament d'aigua potable a escala global. Proveeix aproximadament el 50 % de l'aigua domèstica mundial, incloent-hi milions de persones sense accés a sistemes de subministrament. També representa el 25 % de l'aigua utilitzada en regadiu, impactant llavors en el camp de la seguretat alimentària (UNESCO-WWAP, 2022)

En un context de canvi climàtic i pressions sobre les masses d'aigua superficials, la seva resiliència hidrològica (la seva estabilitat temporal, menor exposició a l'evaporació i capacitat de funcionar com a reserva natural durant períodes de sequera) fa que sigui especialment valuosa (UNESCO-WWAP, 2022). A més, la seva qualitat microbiològica sovint superior respecte de les aigües superficials i la capacitat de tamponament davant les fluctuacions hidrològiques han impulsat el seu ús en àrees urbanes i rurals amb escassetat hídrica creixent (MacDonald et al., 2012; Taylor et al., 2013).

En els països desenvolupats, l'aigua subterrània guanya protagonisme en les estratègies de gestió hídrica. A la Unió Europea, per exemple, l'extracció d'aigua subterrània ha augmentat un 6 % entre 2000 i 2022, mentre que l'ús d'aigua superficial s'ha reduït un 25 %. Actualment, aquesta font representa el 62 % de l'aigua potable subministrada per a consum públic i el 33 % de l'aigua emprada en agricultura (European Environment Agency, 2024). Als Estats Units, l'aigua subterrània proporciona el 39 % del subministrament públic i gairebé un terç del total d'aigua dolça utilitzada (Lovelace et al., 2020), i en regions de l'Àfrica subsahariana, s'està promovent el desenvolupament d'aquest recurs com a mesura d'adaptació climàtica, malgrat les limitacions en infraestructura i vigilància (MacDonald et al., 2012).

6. Control de la presència de virus en l'aigua

La detecció de la contaminació vírica a l'aigua és essencial per garantir-ne la seguretat. No obstant això, actualment no es disposa de mètodes estandarditzats que, alhora, siguin fàcils d'implementar, econòmics i eficaços. En les diferents matrius d'aigua, els virus es troben distribuïts de manera heterogènia i, atès que sovint es presenten en concentracions molt baixes, la seva detecció ambiental resulta especialment complexa. Per això, sovint cal recollir grans volums d'aigua i concentrar les mostres.

6.1 Mètodes de mostreig i processament de mostres ambientals

6.1.1 Mostreig

Els procediments de mostreig i processament de mostres ambientals varien segons la matriu i el tipus de virus que es vol analitzar, així com en funció de les anàlisis a realitzar (Taula 2). En termes generals, un cop recollida la mostra de manera específica segons el seu tipus, és necessari separar les partícules víriques de la matriu i, si cal, concentrar-les en un volum adequat per possibilitar-ne la detecció. En cas que el mètode de detecció es basi en àcids nucleics, és imprescindible dur a terme una extracció dels àcids nucleics prèvia abans de l'anàlisi (Pepper & Gerba, 2015).

Un monitoratge efectiu depèn tant de la recollida de mostres representatives com de la detecció del virus en qüestió. Una estratègia de mostreig inadequada pot conduir tant a falsos positius com a falsos negatius, independentment de la qualitat del procés analític posterior. Això és especialment rellevant en situacions de baixa prevalença, on la variabilitat en la càrrega viral i la composició de l'aigua residual pot fer que mostres puntuals no reflecteixin l'estat real de la contaminació. A més, és essencial controlar factors que poden afectar la integritat del material genètic, com el temps de transport, la temperatura d'emmagatzematge i l'eficiència dels

processos de concentració i extracció, ja que qualsevol pèrdua en aquestes etapes pot comprometre la detecció (Ahmed et al., 2022). Els mètodes tradicionals de mostreig, principalment basats en mostres puntuals, han estat àmpliament emprats per la seva facilitat d'ús, baix cost i comoditat. No obstant això, presenten limitacions importants, especialment la incapacitat de captar variacions temporals, fet que sovint condueix a una infraestimació dels nivells de contaminació. Per compensar-ho, és important ubicar el punt de mostreig de manera estratègica, augmentar-ne la freqüència o bé utilitzar mostres compostes (Farkas et al., 2023). Aquestes s'obtenen combinant diverses submostres recollides en intervals regulars de temps o en funció del cabal, durant un període determinat (normalment hores o un dia), mitjançant mostrejadors automàtics.

Tot i que el mostreig compost ofereix una solució parcial a les limitacions del mostreig puntual, i s'ha utilitzat àmpliament en estudis en EDARs urbanes, els mostrejadors automàtics comporten costos elevats i desafiaments logístics, especialment en localitzacions de difícil accés, com trams de clavegueram secundaris, edificis o hospitals. A més, requereixen subministrament elèctric, fet que en dificulta l'aplicació en matrius d'aigua diferents de l'aigua residual, com aigües superficials o subterrànies.

6.1.2 Concentració de virus

A l'hora de detectar virus a l'aigua, cal tenir en compte que la seva recuperació, especialment en mostres amb contaminació fecal baixa o moderada, requereix concentrar els virus present en diversos litres fins a obtenir un volum molt menor, adequat per a la seva posterior detecció (Bofill-Mas & Rusiñol, 2020). Des dels anys seixanta, s'han desenvolupat i aplicat diversos procediments de concentració de virus en diferents tipus d'aigua, exercint un paper rellevant en el progrés de la virologia ambiental com a disciplina (Cashdollar & Wymer, 2013; Ikner et al., 2012). Entre els mètodes actualment disponibles, el tipus i el volum de mostra a processar,

així com la seva terbolesa, són factors clau per seleccionar l'estratègia més adequada, ja que no existeix un únic procediment vàlid per a totes les situacions.

A les aigües residuals, els virus solen estar presents en concentracions elevades, permetent la detecció amb volums de mostreig relativament reduïts (<100 ml). En canvi, en aigües superficials o subterrànies, on la concentració vírica és molt més baixa, cal recollir volums considerablement superiors (entre 10 i 100 litres) per aconseguir una detecció eficaç (Bofill-Mas & Rusiñol, 2020). Tot i això, aquesta concentració pot augmentar puntualment en situacions concretes com fuites del clavegueram o episodis de pluja torrencial (Rusiñol et al., 2015).

Aquests mètodes, en la majoria dels casos, són laboriosos i comporten la pèrdua d'un percentatge relativament elevat de les partícules víriques presents durant el procés de concentració. Per aquest motiu, sovint es combinen un o més mètodes en sèrie per tal d'aconseguir una concentració eficaç dels virus presents en les mostres. Actualment, hi ha una gran varietat de tècniques basades en diferents processos fisicoquímics, que es fonamenten en l'adsorció de les partícules víriques a diverses matrius o bé en la seva retenció en funció de la mida (Bofill-Mas & Rusiñol, 2020).

Atès que no totes les metodologies actuals han estat validades per a tots els tipus de virus ni per a totes les matrius ambientals, cal aplicar el criteri més adequat segons l'objectiu de l'estudi i els recursos disponibles. Encara que cada mètode pot resultar útil, la seva aplicabilitat sovint és limitada per la complexitat, el cost o el temps requerit. Per aquest motiu, la recerca en aquest àmbit és un camp en continu moviment orientada al desenvolupament de nous sistemes de mostreig i concentració viral, així com en la millora dels ja existents, amb l'objectiu d'obtenir tècniques més ràpides, senzilles, econòmiques i eficients.

Taula 2. Avantatges i desavantatges dels principals mètodes de concentració de virus en aigua. SMF, floculació amb llet descremada.

Principi de concentració	Avantatge	Desavantatge
Precipitació/ floculació	Útil per mostres molt tèrboles Baix cost Pot arribar a ser d'un sol pas i arribar a processar 40L de mostra	No es poden processar grans volums d'aigua Requereix pre-condicionament o centrifuga
Centrifugació	Bones recuperacions Útil per mostres tèrboles	Equipament car No es poden processar grans volums d'aigua
Ultrafiltració	Útil per grans volums Segons el tipus de mostra, pot ser d'un sol pas Adaptable a terreny Ràpid i reproduïble	Pot requerir pre-condicionament del filtre No és adequat per mostres tèrboles Material car
Ultrafiltració centrífuga	Mètode d'un sol pas Ràpid i simple	Equipament i materials cars No es poden processar grans volums d'aigua
Filtració electronegativa	De baix cost L'extracció es pot realitzar directament des de la membrana Bones recuperacions	No és adequat per mostres tèrboles Requereix de pre-condicionament
Filtració electropositiva	Útil per grans volums Adaptable al terreny No requereix pre-acondicionament Pot ser automatitzat	No és adequat per mostres tèrboles Equipament car Recuperacions variables
Mostreig passiu	Adaptable a terreny Independent d'electricitat Baix cost Ràpid i simple	No és quantificable

6.1.3 El mostreig passiu

El mostreig passiu es pot definir, en termes generals, com la tècnica en que es col·loca un material adsorbent o absorbent al medi durant un període determinat perquè acumuli de manera natural els microorganismes presents, sense necessitat d'una força activa (com bombes peristàltiques) per impulsar el procés de recollida (Górecki & Namiésnik, 2002; Matrajt et al., 2018; Salim & Górecki, 2019).

El mostreig passiu no és una metodologia exclusiva per a la detecció de patògens sinó que també ha estat, i és, emprada per a la detecció de contaminants químics. Ara bé, es basen en principis diferents: en el cas de contaminants químics, sovint implica la captura de substàncies dissoltes mitjançant interaccions químiques específiques (Charriau et al., 2016); en canvi, la captura de patògens es basa, en adsorbents que atrapen matèria orgànica la qual els patògens estan adherits en solució (Armanious et al., 2016; Jones et al., 2022). L'eficàcia dels diferents materials per adsorbir contaminants pot veure's fàcilment influïda per la química de l'aigua de l'entorn (Kot-Wasik et al., 2007). Les dinàmiques d'adsorció de contaminants, competitives o de co-adsorció (Fig. 4) mitjançant mostrejadors passius és crucial per entendre la seva eficàcia (Salim & Górecki, 2019).

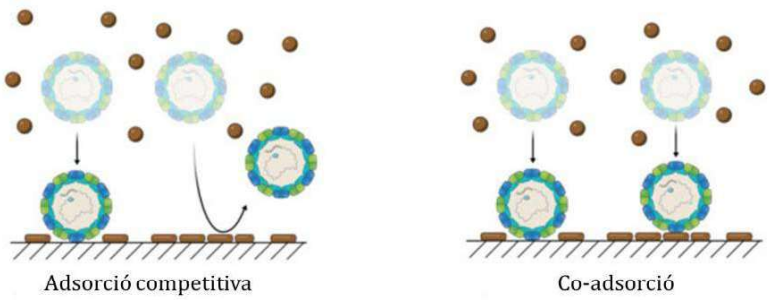


Figura 4. *Esquema que mostra dos escenaris diferents d'adsorció de virus en presència de co-adsorbents.*

Per tant, entendre aquestes dinàmiques és essencial per a la correcta interpretació de les dades i per estimar les concentració dels analits durant el període de mostreig.

El primer ús documentat de mostrejadors passius (Fig. 5) per a la detecció de patògens data de 1916, per identificar el bacil de Koch, causant de la tuberculosi (L. Brown et al., 1916). El seu ús es va començar a popularitzar a partir de 1948 amb la descripció de les gases de Moore (de l'anglès *Moore swab*) per a la detecció de *S. Paratyphi* (Moore, 1948) i s'han fet servir per a la detecció de diferents bacteris i virus com coxsackievirus, *Vibrio cholera*, *E. coli* O157:H7, NoV i poliovirus (Barrett et al., 1980; De Melo Cassemiro et al., 2016; Isaäcson et al., 1974; Kelly S. et al., 1957; Kelly et al., 1955; Matrajt et al., 2018; Pazzaglia et al., 1993; Sattar & Westwood, 1977; Sbodio et al., 2013; Tian et al., 2018).

Més de mig segle després, la pandèmia de la COVID-19, va tornar a situar els mostrejadors passius en primer pla. Entre el 2019 i el 2023 es van publicar 54 articles on s'empra aquesta tècnica, reflectint un patró de resposta ràpida per part de la comunitat científica davant l'amenaça de noves malalties infeccioses (Hayes & Gagnon, 2024).

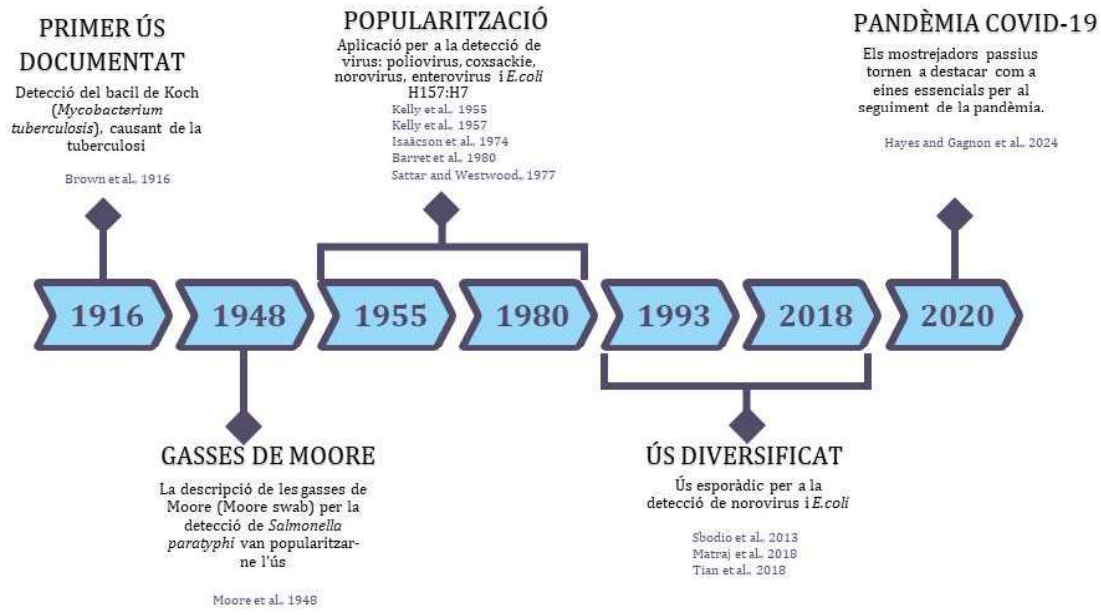


Figura 5. *Cronologia de l'ús de mostrejadors passius per a la detecció de patògens*

Com ja s'ha explicat anteriorment en aquesta tesi, la crisi provocada per la COVID-19 va augmentar la visibilitat i va impulsar l'ús de la WBE com a eina clau per a la salut pública. En aquest context, els mostrejadors passius van tornar a destacar com a eines essencials per al seguiment de la pandèmia (Schang et al., 2021). Gràcies a la seva aplicabilitat, van permetre superar algunes limitacions dels estudis tradicionals de WBE, que es basaven en l'anàlisi de mostres d'aigües residuals a l'entrada de EDARs que donen servei a poblacions molt grans, i van començar a ser aplicats en àmbits més reduïts, com ara edificis (Sellers et al., 2022).

El funcionament bàsic consisteix en instal·lar un dispositiu en un punt de captació d'aigua durant un període determinat, permetent que els virus presents a l'aigua hi interactuïn. A l'interior del dispositiu, que pot adoptar diverses configuracions (com ara colador, recipient, caixa de mistos o torpede), s'hi col·loca un material capaç d'adsorbir els virus. Un cop finalitzat el temps de mostreig, aquestes material adsorbent es retira i s'analitzen mitjançant tècniques moleculars.

Segons la revisió publicada al 2024 per Hayes i col·laboradors, la majoria dels estudis publicats es centren en aigua residual, i amb diversitat de materials com membranes electronegatives, turundes de cotó, carbó activat granular, membranes de niló... però també hi ha alguns articles aplicats en altres matrius com l'aigua de mar, l'aigua de irrigació o aigua superficial (Hayes & Gagnon, 2024).

Un dels principals inconvenients d'aquests sistemes és la impossibilitat de quantificar els resultats, atès que no es pot conèixer amb exactitud el cabal d'aigua que ha estat en contacte amb el mostrejador (Schang et al., 2021; M. Wilson et al., 2022). Tot i així, la seva aplicabilitat i accessibilitat converteix el mostreig passiu en un recurs valuós per a la detecció de patògens, especialment en regions on la vigilància clínica és limitada o en altres contextos ambientals per monitorar l'aparició o reaparició de patògens de rellevància. La seva expansió a països de renda mitjana i baixa podria millorar de manera significativa els esforços globals de vigilància de patògens.

6.2 Indicadors de contaminació fecal

Els patògens a l'aigua poden ser molt diversos i la seva detecció no sempre és fàcil. Per tant, detectar-los tots un per un per a controlar la contaminació de l'aigua seria econòmicament inviable. No obstant, la detecció de la contaminació fecal de l'aigua és essencial per una bona gestió de la contaminació i del seu origen. És aquí on radica la importància de disposar d'indicadors de la contaminació fecal.

Un indicador de la contaminació fecal és un microorganisme que per les seves característiques presenta un comportament similar als del patogen d'interès però pel qual els mètodes d'identificació són més senzills, ràpids o econòmics. L'indicador ideal ha 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). Segons

els criteris establerts per l'Organització Mundial de la Salut, un bon indicador ha de complir diversos requisits: ser fàcilment detectable mitjançant mètodes senzills, ràpids i econòmics; estar present universalment en excrements en nombres elevats; no ser patogen; trobar-se en concentracions més altes que els patògens fecals; no multiplicar-se a l'aigua sota condicions naturals; presentar una persistència o inactivació similar a la dels patògens fecals, i respondre als tractaments de desinfecció de manera comparable (WHO, 2017).

Diversos microorganismes han estat proposats com a indicadors de la contaminació fecal. Els coliforms fecals / *E. coli* i els enterococs intestinals han estat els més utilitzats ja que els mamífers els excreten en grans quantitats. Tanmateix, els bacteris indicadors de la contaminació fecal presenten diverses limitacions: no tenen un origen estrictament fecal, manquen d'especificitat d'hoste, poden proliferar en l'ambient i resulten més sensibles als tractaments de desinfecció. A més, la seva presència no mostra una correlació consistent amb la detecció ni amb la concentració de virus o protozous (Cabral, 2010; Sano et al., 2016).

En aquest context, els bacteriòfags han estat àmpliament utilitzats com a indicadors alternatius de contaminació fecal i de la presència potencial de virus entèrics humans. Entre els més emprats hi ha els colifags somàtics i els fags-F-específics (ARN-fags), que repliquen en *E. coli* i serveixen com a indicadors de contaminació fecal (IAWPRC, 1991; ISO 10705-2). També destaquen els fags de *Bacteroides fragilis*, més específics d'origen humà o animal, que han estat utilitzats en estudis de MST (Puig et al., 1997; Puig & Gironés, 1999). Més recentment, els crAssphage, uns bacteriòfags que infecten *Bacteroidetes* intestinals humans i que és altament abundant i persistent a les femtes i aigües residuals; s'ha proposat com un indicador d'origen humà (Dutilh et al., 2014; García-Aljaro et al., 2017).

Tot i que els virus excretats al medi ambient no poden replicar-se fora de l'hoste, constitueixen un problema rellevant de salut pública, ja que la seva dosi infectiva és molt baixa (Kundu et al., 2013; Thebault et al., 2013). L'excreció vírica pot prolongar-se durant setmanes i, en alguns casos, de manera persistent. A més, molts virus entèrics presenten una elevada estabilitat tant en el medi ambient com davant de diversos tractaments de desinfecció (Carratalà et al., 2013; Rzezutka & Cook, 2004).

És per això que algunes famílies virals com els adenovirus i els poliomavirus humans han estat proposats com a indicadors de la contaminació fecal Aquests poden causar infeccions persistents, habitualment asimptomàtiques, i ser excretats en femta (HAdV) o en orina (JCPyV) pels individus infectats, tot i que ho fan en concentracions més baixes que, per exemple *E. coli*. Aquests virus es caracteritzen per la seva elevada resistència ambiental i a diversos tractaments de desinfecció, i s'han detectat de manera constant al llarg de tot l'any en una àmplia varietat de mostres i en diferents regions geogràfiques (Ahmed et al., 2016; Bofill-Mas et al., 2006; Hundesa et al., 2006; Pina et al., 1998). A més, el fet que existeixin soques específiques d'humans i d'animals els converteix en eines valuoses per traçar la contaminació ambiental mitjançant tècniques de *Microbial Source Tracking* (MST).

Més recentment, el virus del motejat suau del pebrot (*Pepper mild mottle virus*, PMMoV) ha despertat un gran interès com a indicador de contaminació fecal humana. Es tracta del virus més abundantment excretat per una elevada proporció d'humans sans (Kitajima et al., 2018; Zhang et al., 2005). A més, la seva presència s'ha confirmat arreu del món, mostrant una àmplia distribució geogràfica (Colson et al., 2010; Hamza et al., 2011; Zhang et al., 2005). Pel que fa als animals, només s'ha detectat en femtes de pollastres i gavines, i sempre en concentracions molt més baixes que en humans (Rosario et al., 2009).

6.3 Detecció molecular i caracterització genètica

En l'actualitat, i des del desenvolupament de la PCR, la detecció de virus es fa majoritàriament amb mètodes moleculars. La PCR és un mètode d'alta sensibilitat que implica cicles d' augment de temperatura i refredament de la mescla de reacció per tal de facilitar l'amplificació de l'ADN diana (Garibyan & Avashia, 2013; Mullis, 1990). La detecció de genomes vírics es basa en l'ús d'encebadors específics que permeten amplificar les regions d'interès del genoma viral. Hi ha variants d'aquesta tècnica que incrementen la sensibilitat quan la concentració de virus és baixa, com la PCR niuada (nested PCR o nPCR), o que permeten quantificar el nombre de còpies dels genomes diana mitjançant l'ús de rectes patró, com la PCR quantitativa (qPCR). La qPCR és la tècnica més àmpliament utilitzada i es el protocol de referència per a la quantificació d'àcids nucleics malgrat la seva baixa reproductibilitat (Bustin et al., 2013; Dijkstra et al., 2014; Hayden et al., 2008; Zhang et al., 2007). La PCR digital (dPCR) permet la quantificació absoluta dels àcids nucleics diana presents en una mostra i resol algunes limitacions de la qPCR fent que en els últims anys l'interès per aquesta tècnica hagi anat en augment. En la dPCR, la mostra es divideix primer en moltes subreaccions independents de PCR, de manera que cada partició conté pocs o cap gen diana. Després de la PCR, la fracció de particions amb amplificació positiva s'utilitza per quantificar la concentració de la seqüència diana amb una precisió definida estadísticament mitjançant les estadístiques de Poisson (Dube et al., 2008; Whale et al., 2013). Aquesta partició de la mostra concentra les seqüències diana dins dels microreactors aïllats (Fig.6).

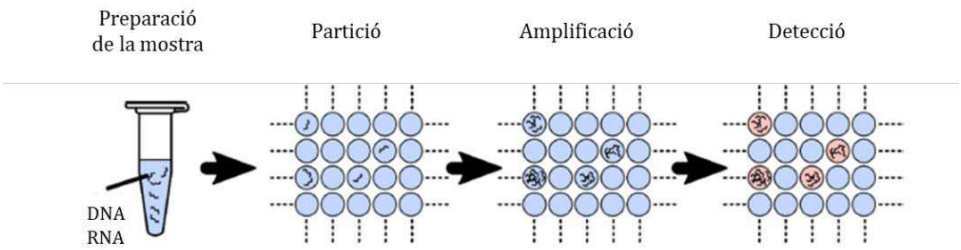


Figura 6. *Principis de la PCR digital. La mostra es divideix en moltes particions independents, de manera que cadascuna conté unes poques o cap seqüència diana (Quan, 2018).*

La partició de la mostra divideix de manera eficient les seqüències diana dins dels microreactors aïllats. Aquest efecte pot augmentar la tolerància als inhibidors presents en una mostra. S'han desenvolupat diferents plataformes de dPCR, com per exemple la dPCR basada en gotes (ddPCR), basada en l'emulsió de gotes d'aigua-oli o les cambres de microfluídica (Quan et al., 2018).

Tot i això, els mètodes basats en PCR no aporten informació sobre la infectivitat dels virus analitzats, llevat que es combinin amb tractaments destinats a eliminar genomes no encapsidats o amb compostos que només s'uneixen a partícules víriques íntegres, potencialment infeccioses, l'ús d'intercalants i la captura imunomagnètica (Bofill-Mas et al., 2006; Haramoto et al., 2018; Quijada et al., 2016; Randazzo et al., 2018).

6.3.1 Seqüenciació per a la detecció de virus

Els mètodes tradicionals per a la detecció de virus sovint no permeten captar la diversitat vírica real present en una mostra, ja que solen ser molt específics o estar dirigits a un grup concret de virus, la qual cosa requereix conèixer prèviament les característiques del virus objecte d'estudi (Kumar et al., 2017). En canvi, l'aparició i desenvolupament de les tecnologies de seqüenciació massiva ha suposat un avenç important, permetent una anàlisi més complet i exhaustiu de les poblacions víriques tant en mostres clíniques com ambientals. Aquestes tècniques, en constant evolució, són essencials per

a la descripció precisa dels virus circulants, sent eines clau per a estudis epidemiològics, de salut pública i per al descobriment de virus nous (Cantalupo et al., 2011; Fernandez-Cassi et al., 2018; D. Wang, 2020).

La seqüenciació massiva ha permès la descripció de comunitat víriques, el viroma, en tot tipus de mostres, , sense tenir un coneixement preexistent de la seva composició (Barzon et al., 2013; Kapgate et al., 2015). També ha permès la caracterització i identificació dels virus que constitueixen el viroma humà (Bai et al., 2022). La seva aplicació, però, està limitada en general però especialment en mostres ambientals com poden ser les mostres d'aigua, ja que els genomes virals son molt petits i es troben en menor proporció respecte als genomes bacterians o de l'hoste (en el cas de les mostres clíniques).

Al no tenir una quantitat de ADN/ARN viral suficient procedent de l'extracció d'àcids nucleics, sovint s'han de realitzar passos addicionals per augmentar-ne la quantitat a l'inici del protocol de seqüenciació. Aquests passos inclouen pre-tractaments tant del concentrat viral com de l'extracció d'àcids nucleics. Per exemple, és habitual l'ús d'enzims que degradin l'ADN i ARN no encapsidat per augmentar la ratio entre àcids nucleics virals i ADN bacterià o de l'hoste. Posteriorment a l'extracció, és necessari, també, una pre-amplificació dels àcids nucleics aleatòria, ja sigui per desplaçament múltiple (mètode MDA) o independents de seqüència (mètodes SISPA) (Allander et al., 2001; Cantalupo et al., 2011; Kohl et al., 2015) per augmentar la quantitat de material genètic viral del qual fer la seqüenciació.

6.3.2 Seqüenciació massiva per metagenòmica

La seqüenciació massiva per metagenòmica o d'alt rendiment (High Troughput Sequencing, HTS) té com a objectiu la seqüenciació de tots els genomes que es troben en una mostra.

Un cop feta la pre-amplificació per passar el ARN a ADN i per incrementar la concentració d'ADN viral, es constitueixen les llibreries de seqüenciació (Fig. 7). El protocol per a l'elaboració d'aquestes, dependrà de la plataforma de seqüenciació escollida o de la seva automatització (Head et al., 2014). En general, es parteix d'ADN de doble cadena que és fragmentat per medis mecànics o enzimàtics, a excepció de que la plataforma de seqüenciació permeti la seqüenciació de fragments llargs com són les desenvolupades per Oxford Nanopore. Seguidament es reparen els extrems generats i es lliguen els adaptadors que s'uniran a la plataforma de seqüenciació. En el cas de voler multiplexar diverses mostres, és a dir, seqüenciar a la vegada diverses mostres, s'han de lligar també els índexs, que són marcadors específics que etiquetaran les seqüències obtingudes segons la mostra que provinguin. Amb els adaptadors i índexs lligats, es pot dur a terme una selecció de fragments mitjançant boles magnètiques, amb l'objectiu d'eliminar aquells que són massa curts o massa llargs, ja que podrien afectar negativament la qualitat de la seqüenciació (Guan & Sung, 2016). Depenent del protocol emprat, les llibreries poden ser amplificades per PCR i posteriorment purificades abans de ser carregades al seqüenciador (Martínez-Puchol, 2020).

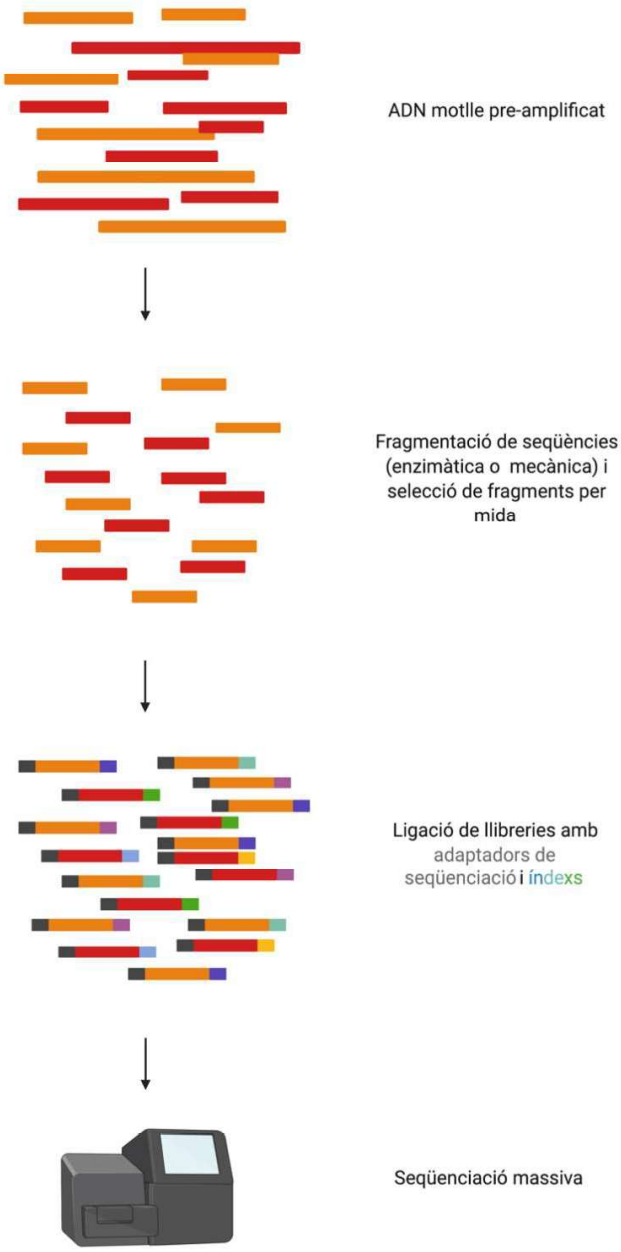


Figura 7. *Protocol de seqüenciació massiva per metagenòmica (HTS). Producció de llibreries. Extret de Martínez-Puchol, 2022.*

La HTS ha estat utilitzada en una gran varietat de mostres d'aigua , com aigües residuals (Cantalupo et al., 2011; Fernandez-Cassi et al., 2018; Martínez-Puchol et al., 2020), aigua de mar (Brum et al., 2015; Hurwitz &

Sullivan, 2013), llacs (Djikeng et al., 2009), aigües subterrànies (Djikeng et al., 2009; Kallies et al., 2019) i aigües de reg (Fernandez-Cassi et al., 2017; Rusiñol et al., 2020).

La HST permet l'estudi del viroma complet sense dirigir-la cap a un grup taxonòmic complet. No obstant, és important tenir en compte que, en mostres complexes, les seqüències víriques poden arribar ser només un 1% del total de dades generades pels seqüenciadors, fet que pot provocar que es perdin seqüències víriques que es trobin en una proporció menor (Martínez-Puchol, 2020.). Per aquest motiu, s'han desenvolupat tècniques de seqüenciació massiva amb enriquiment de dianes o mitjançant la generació d'amplicons.

6.3.4 Seqüenciació amb enriquiment de dianes

La seqüenciació amb enriquiment de dianes té com a objectiu incrementar la sensibilitat i millorar la seqüenciació cap a determinats grups vírics. Aquesta metodologia consisteix en incorporar un biaix durant la producció de llibreries que permet dirigir la seqüenciació cap als virus d'interès sense perdre rendiment amb reads de virus i altres organismes que no són el focus de l'estudi. Aquest protocol (Fig. 8) es basa en la hibridació de sondes amb les llibreries de seqüenciació. El pas d'enriquiment de dianes és introduït un cop finalitzat el protocol d'elaboració de llibreries. Aquestes són hibridades amb unes sondes, que estan conjugades amb biotina, el que permetrà la seva posterior recuperació amb boles magnètiques cobertes d'estreptavidina. Així es recuperen els híbrids i s'eliminen les seqüències que no siguin diana i només seran seqüenciades les seqüències d'interès. Per acabar, s'amplifica la llibreria capturada per obtenir una major profunditat de seqüenciació i diversitat vírica (Pogka et al., 2022). Aquesta aproximació s'ha aplicat amb èxit en nombroses mostres ambientals (Itarte et al., 2024; Itarte et al., 2021, Martínez-Puchol, et al., 2021; Martínez-Puchol et al., 2020, 2021, 2024).

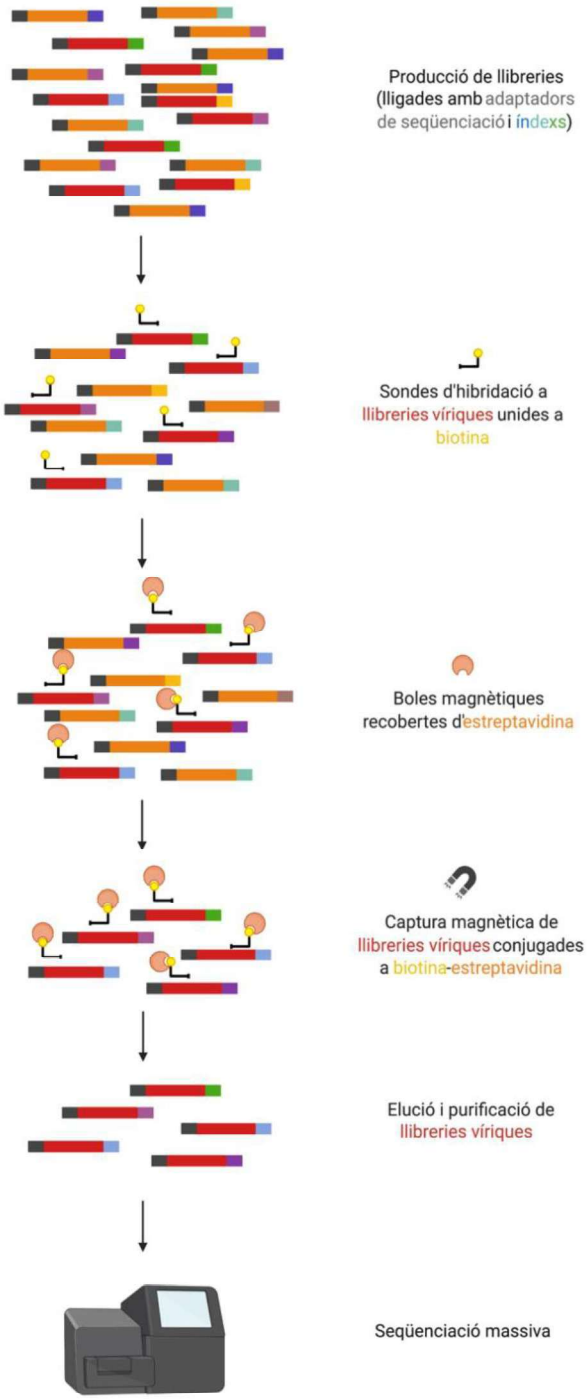


Figura 8. *Protocol de seqüenciació amb enriquiment de dianes mitjançant sondes conjugades amb biotina. . Extret de Martínez-Puchol, 2022.*

La primera aplicació de la seqüenciació amb enriquiment de dianes per a virus va ser al 2015 amb el panell VirCapSeq-VERT® (Roche) dissenyat per a incrementar la sensibilitat de la seqüenciació cap a virus que infecten vertebrats. Posteriorment han aparegut altres panells de captura de diferents cases comercials com pot ser Illumina Inc. o Twist Bioscience, dirigits a l'enriquiment de grups concrets com poden ser virus respiratoris o SARS-CoV-2 (Ceballos-Garzon et al., 2024). També poden ser dissenyats de manera personalitzada i dirigir així la seqüenciació de manera molt concreta i específica cap a aquells grups de virus concrets que es vulguin estudiar (Martínez-Puchol et al., 2024).

Aquestes tècniques han millorat notablement els resultats obtinguts en l'estudi de virus mitjançant seqüenciació massiva, i la seva aplicació evoluciona i es perfecciona constantment. Tot i això, presenten algunes limitacions. La principal és el biaix inherent a l'enriquiment dirigit a virus coneguts. No obstant això, com ja s'ha observat amb alguns kits disponibles, és possible reduir la restricció de la hibridació per tal d'ampliar les possibilitats de detectar virus desconeguts.

Una altra limitació de la hibridació amb sondes és la cobertura incompleta del genoma en estudis de variants víriques. Si alguna regió del virus no hibrida amb les sondes dissenyades (ja sigui per la presència de noves variants o per la pèrdua de fragments), aquestes zones quedarien sense cobrir. Una alternativa que permet superar aquest inconvenient és la seqüenciació massiva d'amplicons.

6.3.5 Seqüenciació massiva d'amplicons

Tradicionalment, la seqüenciació d'amplicons de PCR s'ha realitzat amb la tècnica de Sanger (Sanger et al., 1977). El principal inconvenient d'aquest mètode és que, en mostres amb diverses variants d'un mateix virus, la seqüència obtinguda correspon únicament a la variant predominant. Per caracteritzar les variants minoritàries és necessari dur a terme el clonatge de poblacions, un procés laboriós i limitant. La introducció de la seqüenciació massiva d'amplicons ha permès superar aquesta limitació, ja que possibilita obtenir un gran nombre de seqüències en un sol assaig i analitzar múltiples mostres de manera simultània. Aquest avenç proporciona informació rellevant sobre mutacions de baixa freqüència i ofereix una visió més precisa de l'estructura poblacional dels virus (Beerenwinkel & Zagordi, 2011).

El protocol per a la seqüenciació massiva d'amplicons (Fig. 9) es basa en la purificació i lligació d'adaptadors de seqüenciació a partir del producte que s'obté d'una o vàries PCR o PCR niuades. Els adaptadors també es poden incorporar a la hora de fer les PCR incorporant aquestes seqüències al encebadors. Igual que en la seqüenciació massiva HTS, per a la seqüenciació simultània de diverses mostres es poden lligar índex per identificar cada mostra amb una seqüència única.

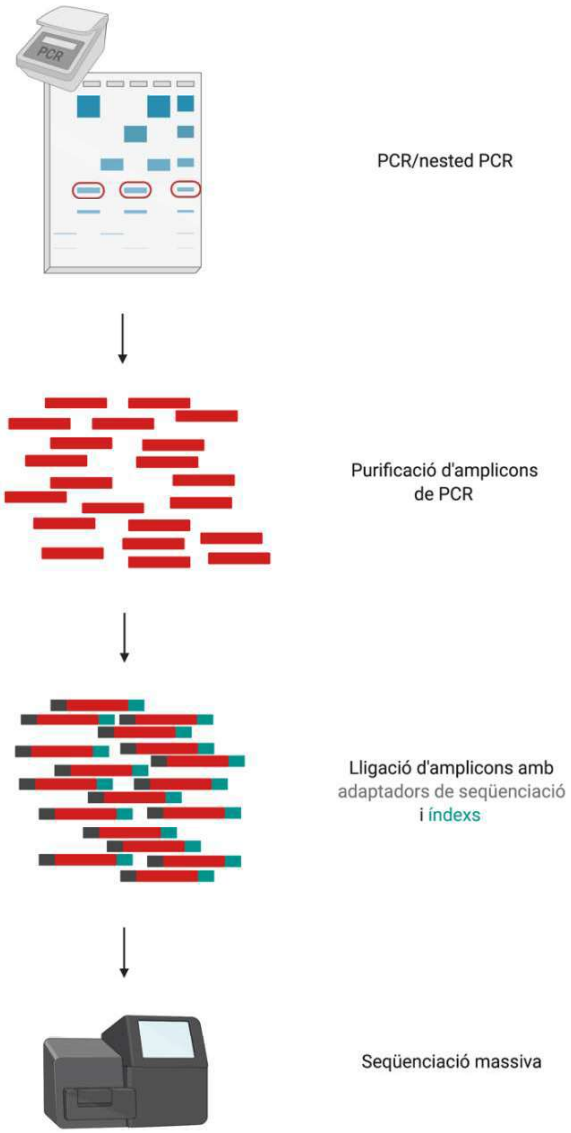


***Figura 9**. Protocol de seqüenciació massiva d'amplicons. Extret de Martínez-Puchol, 2022.*

Aquesta aproximació és relativament senzilla i sensible, però presenta certes limitacions, com la mida màxima determinada per la reacció de PCR i la necessitat de disposar de regions adequades per a la unió dels encebadors, assegurant al mateix temps que la regió amplificada sigui prou específica per distingir diferents membres dins d'un mateix grup víric (Kuchinski et al., 2022)

OBJECTIUS

L'objectiu global d'aquesta tesi és avaluar el potencial del mostreig passiu com a eina per a la detecció i caracterització de virus humans en diferents tipus d'aigua en el marc de la vigilància epidemiològica ambiental.

Els objectius específics són:

1. Avaluar un mètode de mostreig passiu per a la detecció i caracterització de virus en aigües residuals.
2. Aplicar l'epidemiologia basada en aigües residuals emprant el mostreig passiu en aigua residual d'edificis per a la caracterització del viroma en poblacions de diferents edats.
3. Aplicar el mostreig passiu per a la detecció i caracterització de virus en aigües subterrànies urbanes.
4. Avaluar l'ús dels mostrejadors passius per al monitoratge de virus humans en aigua residual tractada mitjançant sistemes basats en la natura (NBS).

INFORMES DELS ARTICLES

1. Llista d'articles inclosos a la tesi

Aquesta tesi està basada en les següents publicacions, enumerades segons l'ordre de presentació.

- **Mejías-Molina, C.,** Pico-Tomàs. A., Beltran-Rubinat, A., Martínez-Puchol, S., Corominas, L., Rusiñol, M., Bofill-Mas, S. (2023). Effectiveness of passive sampling for the detection and genetic characterization of human viruses in wastewater. *Enviromental Water Researc & Technology* *9*(4), 1195-1204; DOI: 10.1039/d2ew00867j

  (Aquest article forma part dels Outstanding Papers of 2023 from RSC's *Environmental Science journals*).
- **Mejías-Molina, C.,** Pico-Tomàs, A., Martínez-Puchol, S., Itarte, M., Torrell, H., Canela, N., Borrego, C. M., Corominas, L., Rusiñol, M., Bofill-Mas, S. (2024) Wastewater-based epidemiology applied at the building-level reveals distinct viroma profiles based on the age of the contributing individuals. *Human Genomics*. 2024; 18: 10; DOI: 10.1186/s40246-024-00580-1
- **Mejías-Molina, C.,** Estarlich-Landajo, I., Martínez-Puchol, S., Bofill-Mas, S., Rusiñol, M. (2024). Exploring waterborne viruses in groundwater: Quantification and Virome characterization via passive sampling and targeted enrichment sequencing. *Water Research.,* Volume 26 ,122305. https://doi.org/10.1016/j.watres.2024.122305
- **Mejías-Molina, C.**, Valdivia-Carrera, C.A., Laguna-Marin, C., Hudesa, A., Bofill-Mas, S., Rusiñol, M., (2025). Constructed wetlans as a Nature-Based Solution for viral removal in treated wastewater. Article en procés de revisió en la revista *Journal of Environmental Management.*

2. Informe de coautoria

- **Mejías-Molina, C.,** Pico-Tomàs. A., Beltran-Rubinat, A., Martínez-Puchol, S., Corominas, L., Rusiñol, M., Bofill-Mas, S. (2023). Effectiveness of passive sampling for the detection and genètic characterization of human viruses in wastewater. *Enviromental Water Research & Technology* *9*(4), 1195-1204; DOI: 10.1039/d2ew00867j
- **Mejías-Molina, C.,** Pico-Tomàs, A., Martínez-Puchol, S., Itarte, M., Torrell, H., Canela, N., Borrego, C. M., Corominas, L., Rusiñol, M., Bofill-Mas, S. (2024) Wastewater-based epidemiology applied at the building-level reveals distinct viroma profiles based on the age of the contributing individuals. *Human Genomics*. 2024; 18: 10; DOI: 10.1186/s40246-024-00580-1

Aquest estudis s'engloben dins el projecte VirWASTE: *Wastewater as a source of knowledge on SARS-CoV-2 and other potentially pandemic Viruses: a One Health approach* (2020PANDE00044; IP: Sílvia Bofill Mas), finançat en el marc de la convocatòria PANDÈMIES 2020 de l'Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR), i del projecte EPISARS: *Tracking the spread of COVID-19 through wastewater-based epidemiology: typing, community testing and occupational risk assessment* (544/C/2021; IP: Sílvia Bofill Mas), finançat per la fundació La Marató de TV3. La doctoranda va participar activament en l'estudi descrit, incloent el disseny experimental, el processament de les mostres, l'obtenció de les dades i l'anàlisi dels resultats**.** També va participar de la planificació i les reunions del projecte. Finalment, ha redactat dos dels articles que se'n deriven sota la supervisió de les seves directores de tesi.

- **Mejías-Molina, C.,** Estarlich-Landajo, I., Martínez-Puchol, S., Bofill-Mas, S., Rusiñol, M. (2024). Exploring waterborne viruses in groundwater: Quantification and Virome characterization via passive sampling and targeted enrichment sequencing. *Water Research.,* Volume 26 ,122305. https://doi.org/10.1016/j.watres.2024.122305

- **Mejías-Molina, C.,** Valdivia-Carrera, C.A., Laguna-Marin, C., Hundesa, A., Bofill-Mas, S., Rusiñol, M., (2025) Passive sampling for viral surveillance in Nature-Based wastewater treatment systems.. Article en procés de revisió en la revista *Journal of Environmental Management.*

Aquests estudis han format part del projecte UPWATER: *Understanding groundwater Pollution to protect and enhance WATER quality* (10108107; IP: Enric Vázquez), finançat per la convocatòria HORIZON (Horizon-CL6-2022-ZEROPOLLUTION-01). La doctoranda va participar activament en aquest estudi, incloent el disseny experimental, els mostrejos, l'optimització del mètodes, el processament de les mostres, l'obtenció de les dades i l'anàlisi dels resultats. També va participar de les reunions i *workshops* del projecte Finalment, ha redactat els dos articles que se'n deriven sota la supervisió de les seves directores de tesi.

Signat,





Dra. Sílvia Bofill Mas

Barcelona, desembre de 2025

Dra. Marta Rusiñol Arantegui

Barcelona, desembre de 2025

3. Informe sobre el factor d'impacte dels articles publicats

Els estudis que integren aquesta tesi doctoral han estat publicats o es troben en procés de revisió en revistes científiques rellevants dins de la línia de recerca en la qual ha estat treballant la doctoranda durant els anys de tesi.

L'article "Effectiveness of passive sampling for the detection and genetic characterization of human viruses in wastewater" va ser publicat l'any 2023 a la revista *Enviromental Science: Water Research & Technology***,** la qual es situava dins del Q1 i del D1 de l'àrea "Water Science and technology" amb un factor d'impacte 5.2 segons JCR™**.**

L'article "Wastewater-based epidemiology applied at the building-level reveals distinct virome profiles based on the age of the contributing individuals" va ser publicat l'any 2024 a la revista *Human Genomics***,** la qual es situava dins del Q1 i del D2 de l'àrea "Biochemistry, Genetics and Molecular Biology" amb un factor d'impacte 4.5 segons JCR™**.**

L'article "Exploring waterborne viruses in groundwater: quantification and viromechracterization via passive sampling and targeted enrichment sequencing" va ser publicat l'any 2024 a la revista *Water Research***,** la qual es situava dins del Q1 i del D1 de l'àrea "Environmental Science" amb un factor d'impacte 11.4 segons JCR™**.**

L'article "Constructed wetlans as a Nature-Based Solution for viral removal in treated wastewater" es troba actualment en procés de revisió (setembre 2025) a la revista *Journal of Environmental Management*.

Signat,





Dra. Sílvia Bofill Mas

Barcelona,desembre de 2025

Dra. Marta Rusiñol Arantegui

Barcelona, desembre de 2025

CAPÍTOL 1: Ús de mostrejadors passius en l'epidemiologia basada en aigües residuals i l'estudi del viroma

Article 1. Efectivitat del mostreig passiu per a la detecció i caracterització genètica de virus humans en aigua residual.

"Effectiveness of passive sampling for the detection and genetic characterization of human viruses in wastewater"

Mejías-Molina. C., Pico-Tomàs. A., Beltran-Rubinat. A., Martínez-Puchol. S., Corominas. L., Rusiñol. M., Bofill-Mas. S

Enviromental Water Researc & Technology *9*(4), 1195-1204;

DOI: 10.1039/D2EW00867J

L'epidemiologia basada en aigües residuals és una eina clau per a la detecció de virus circulants en una població sense la necessitat d'analitzar mostres clíniques individuals. Recentment, s'ha demostrat que aquesta tècnica és aplicable no només a escala de ciutat, sinó també en àmbits poblacionals més petits com edificis.

Aquest treball avalua l'ús de mostrejadors passius, concretament mostrejadors tipus torpede amb membranes electronegatives a l'interior, com a alternativa als mètodes tradicionals de mostreig d'aigua residual, en dos contextos diferents: una planta de tractament d'aigües residuals (EDAR) i una residència de gent gran. També s'avalua l'ús d'una o més membranes en l'interior del mostrejador, el seu potencial per a la semi-quantificació i la seva potencial aplicació en estudis de viroma.

Els resultats obtinguts van indicar que l'anàlisi de dues membranes permetia l'obtenció de resultats més robustos. La comparació en la freqüència de detecció de 8 dianes virals diferents, va demostrar una sensibilitat equivalent entre mostres composades i mostreig passiu en l'aigua residual mostrejada a la residència, però més baixa en la EDAR per a la detecció de virus patògens. Malgrat que els resultats obtinguts amb aquesta metodologia reporten resultats qualitatius, en aquest estudi s'han obtingut correlacions positives

amb les quantificacions obtingudes per les mostres composades, indicant el potencial dels mostrejadors tipus torpede emprats per a semiquantificació de dianes virals

En aquest estudi, es va fer servir la seqüenciació massiva amb enriquiment de dianes per caracteritzar el viroma tant en la EDAR com en la residència d'avis. Es van detectar menys seqüències víriques a les mostres obtingudes amb mostrejadors tipus torpede en comparació a les observades en les mostres composades, mentre que no es van observar diferències en el nombre de famílies víriques detectades a les mostres d'aigua residual obtingudes a la residència mitjançant les dues aproximacions.

Els resultats suggereixen que el mostreig passiu amb torpedes i dues membranes electronegatives és una eina adequada per a la detecció i caracterització de virus i que funciona de manera equivalent o millor que el mostreig composat en el cas de l'aigua residual de un edifici petit tipus residència de gent gran mentre que l'ús d'aquesta estratègia en EDARs grans és encara millorable.

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Effectiveness of passive sampling for the detection and genetic characterization of human viruses in wastewater†

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Wastewater-based epidemiology is a promising tool to anticipate potential viral outbreaks not only at city-scale but also at precincts-scale or even buildings. Passive samplers (PS) and, particularly, torpedo-shaped devices containing electronegative membranes, are cost-effective alternatives to traditional sampling methods to trace the circulation of pathogens amongst a community through wastewater. Here we evaluated the effectiveness of torpedo devices to detect eight different viral targets in wastewater from an urban WWTP and from a nursing home (NH), in comparison to 24 h composite active sampling (AS). The use of one or two membranes within the torpedo devices, their potential use for semi-quantification analysis and the performance of torpedoes to conduct studies of the wastewater virome was examined. Two membranes analysis provided more certain results than one single membrane. Considering PS as a semi-quantitative approach, it produced equivalent sensitivity to AS at the NH, but lower sensitivities at the WWTP. Less viral sequences were detected with torpedoes as compared to AS when analyzing the WWTP samples while no differences at the level of viral families detected were obtained at NH when applying both approaches. Our results suggest that PS is a powerful tool for viral detection and characterization at a building scale.

Water impact

Passive sampling approaches are becoming promising tools in wastewater-based epidemiology. Torpedo devices fitted with electronegative membranes are useful, affordable and practical tools to monitor viral pathogens in small scale scenarios (*e.g.* nursing homes). They can be used to study the presence of a diversity of viruses as well as to characterize the wastewater virome.

1. Introduction

Viruses that infect humans are shed through multiple body secretions. Wastewater surveillance is advantageous because it can be used to estimate disease burden without having to collect individual clinical specimens. This approach is simple, cost-efficient, non-invasive and allows us to obtain rapid results. Wastewater-based epidemiology (WBE) for virus surveillance has been used since the 1980s to determine the spread of poliovirus, the etiological agent for poliomyelitis.[1–3] It has recently reemerged as an important tool for public health monitoring to trace the communal circulation of SARS-CoV-2 and to anticipate potential COVID-19 outbreaks caused by pre-symptomatic or asymptomatic infected individuals.[4]

Most WBE studies focus on wastewater collected from wastewater treatment plants (WWTPs) and provide useful city-scale or suburban-scale information.[5] Most studies conducted at WWTPs rely on automatic samplers that are deployed at the inlet of the WWTP. Autosamplers collect a composite sample proportional to the daily flow and/or proportional to a given time interval[6] and give a representative view of the spread of a virus in the population served. Studies focusing on small sewerage systems such as nursing homes (NH), schools, hospitals or university campuses are important for early warning to target early actions in vulnerable communities. The use of autosamplers

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† Electronic supplementary information (ESI) available. See DOI: **https://doi.org/10.1039/d2ew00867j**

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at smaller settings are limited by their cost, the need of a power supply and, in some cases, the difficulty to deploy and operate such complex devices in small drainage systems.[7] Passive samplers have thus been proposed as affordable and easy-to-use alternative for virus surveillance in these smaller scale scenarios.[8] Their deployment and further collection are easy and fast, they operate without electricity, so they can be used in any point of the sewer system.

The first passive sampler described was a folded gauze with a string, known as the "Moore swab" that was immersed for 48 h in a sewer for the detection of *Salmonella typhi*.[9] Moore swabs have been used to detect several bacterial and viral pathogens such as coxsackieviruses, *Vibrio cholera*, *E. coli* O157:H7, norovirus, and poliovirus.[10–18] During the COVID-19 pandemic, Moore swabs and other passive samplers were used for wastewater surveillance in both large and small catchments.[19] Schang and coworkers developed a 3D-printed sampler unit that resembles a torpedo.[5] Among gauzes, electronegative membranes, and cottons swabs, electronegative membranes were reported to be the best collecting material to be placed within the torpedoes.[4,5] According to the available literature at the time of writing, a deployment time of 24 hours was recommended to allow viruses to concentrate into the membranes.[20] Since the beginning of the COVID-19 pandemics, passive samplers have been proposed as affordable and easy-to-use alternative for virus surveillance in small scale scenarios.[8]

Next generation sequencing (NGS) techniques are excellent tools to monitor and identify viral pathogens circulating among the population. Sewage contains a high number of viruses and viral metagenomics (*i.e.*, viromics) is a very promising tool that could be paired to WBE for a more complete description of viruses circulating within a population as well as for the study of viral (re)emergences and discovery.[21,22]

The hypothesis of this study was that passive sampling (PS) could complement and even replace data obtained from the analysis of composite samples actively collected (AS) for the presence of human viruses in wastewater. To do that, a comparison between 24 h wastewater samples from a WWTP and a NH were collected using AS and PS. The variability and semi quantification of replicate samples was evaluated. The second aim of this study was to evaluate the potential of the torpedo devices to study the sewage virome.

2. Materials and methods

2.1. Study site, passive and active wastewater sampling

An urban WWTP serving 1.5 million inhabitants and the sewer from a NH, hosting 300 residents and 200 workers were selected to validate the use of passive samplers for the recovery of a diversity of viruses (see next section). From January 10th till March 22nd, 2022, a total of 30 samples from the inlet of the WWTP were collected using the two sampling methodologies. Fifteen AS using an ISCO 6712FR autosampler (Teledyne, Lincoln, NE, USA), that collects 24 h influent time-proportional samples (100 ml per hour over a 24 h period), and 15 PS devices, also known as torpedo sampling units (kindly donated by David McCarthy), containing 3 electronegative membranes. Torpedoes were deployed and retrieved 24 h later. Over the same time period, 30 samples from the residence building were also collected using both methods (a HACH-Bühler 2000 autosampler (HACH-LANGE GmbH, Germany) was used for AS (at the rate of 100 ml per hour over a 24 h period)). Passive and active samples (in a final volume of 250 ml of the total 2.4 litres of wastewater collected by the autosamplers) were handled using protective equipment (lab coats, glasses, gloves) in sterile containers (sealable plastic bags or 250 ml bottles respectively) and transported into a cooler to the laboratory for processing in less than 2 h.

2.2. Concentration of viruses, extraction of nucleic acids and analysis using quantitative PCR (qPCR)

Concentration of viral particles from composite wastewater samples (hereafter active sampling, AS), was performed by first removing debris by centrifuging 100 mL of the total 250 ml of sample at 4750 × *g* for 30 min. The resulting supernatant (80 mL) was then ultrafiltered using the automatic Concentration Pipette (CP-Select™) with 150 KDa tips (Innovaprep) into a final volume of 300 μL as previously described.[23] All water samples were spiked with the bacteriophage MS2 as process control, at a final concentration of 1×10^5 GC ml^{-1}, before any processing was carried out. Viral nucleic acids (NA) were extracted with the QIAamp Viral RNA mini kit using the QIAcube automatic system (Qiagen). The sample concentrates (140 μL) were used for the extraction in an elution volume of 70 μL. A negative control of the viral nucleic acid extraction, using PBS was added per batch of samples. PS units were dismantled on the day of retrieval. Two electronegative membranes fitted into the PS were collected and placed in a Petri dish with 0.5 ml of RNA preservative (RNA shield, Zymo research) and maintained at 4 °C until extraction on the next day. The third membrane was stored at −80 °C as a counter sample. Viral RNA extract was carried out using the RNeasy Power Microbiome Kit (Qiagen) into a final volume of 50 μL following manufacturer instructions and adding a step of bead-beating for 30 s at 4 m s^{-1} using FastPrep-24™ (MP Bio, USA).

Specific qPCR and RT-qPCR assays were used to quantify MS2 bacteriophage,[24] human adenovirus (HAdV)[25,26] JC polyomavirus (JCPyV),[27] human enterovirus (EV),[28] rotavirus (RoV),[29] norovirus genogroup I (NoV GI),[30–32] and norovirus genogroup II (NoV GII)[33,34] as previously described. For SARS-CoV-2, the N1 and N2 assays[35] targeting the gene encoding for the viral nucleocapsid protein were selected. The TaqMan® Environmental Master Mix 2.0 (Thermofisher Scientific) and the RNA Ultrasense™ One-Step RT-qPCR System (Invitrogen) were used for DNA and RNA viruses, respectively. In exception of the synthetic SARS-CoV-2 control (control 51 from Twist Biosciences), all the qPCR standards were prepared (as described by Rusiñol *et al.*, 2020 (ref. 36))

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using synthetic gBlocks Gene fragments (IDT), quantified with a Qubit fluorometer (Thermofisher Scientific), and diluted serially from 10^0 to 10^7 copies per reaction. Quantification was performed in a QuantStudio™ Real-Time PCR System from ThermoFisher Scientific. Undiluted and 10-fold dilutions of the NA extracts were analysed, and MS2 was also assessed in wastewater samples collected by AS, to test for inhibition. Non-template controls were included in each qPCR plate. All qPCR preparations were performed in a clean laboratory and template addition was done inside a PCR cabinet. Standard qPCR curves were accepted under the following parameters: mean slope, between −3.1 and −3.5; r^2 = 0.999; and mean efficiency between 85 and 110%. The limit of detection (LoD) of the qPCR method was calculated by running six replicate tenfold dilutions of target SARS-CoV-2, JCPyV, HAdV suspensions around the detection end point (2.5, 5, 25 and 50 GC per well). The concentration that produced at least 95% positive replicates was assumed to be the LoD of the assay. The limit of quantification (LoQ) was estimated using the procedure described previously.[37]

2.3. Target enrichment sequencing (TES)

2.3.1. Sequence-independent, single-primer amplification (SISPA). A total of 24 samples (6 AS and 6 PS from the NS and 6 AS and 6 PS from the WWTP) were prepared prior to the library construction following the protocol previously described.[38] To analyse both RNA and DNA viruses, NA were retrotranscribed using in this study the SuperScript IV enzyme (Invitrogen) and random nonamer primers. The second cDNA strand was obtained using Sequenase 2.0 (Applied Biosystems) and then amplified following 25 PCR cycles using AmpliTaq Gold DNA polymerase (Applied Biosystems) to obtain enough dsDNA for downstream analyses. The PCR products were further purified with Zymo DNA Clean & Concentration kit (Zymo Research) and the resulting cDNA quantified using Qubit 2.0 (Life technologies) and the Qubit dsDNA HS Assay Kit (Invitrogen).

2.3.2. Library construction. Libraries were constructed using KAPA HyperPlus Library Preparation Kit (KAPA Biosystems, Roche). Following the manufacturer's instructions, enzymatic fragmentation was conducted from a starting quantity of 100 ng. After fragmentation, an end-repair, an A-tailing reaction and an adapter's ligation was performed following manufacturer instructions. Each sample was paired with the desired index using the KAPA UDI Primer mixes (KAPA Biosystems, Roche). A post-ligation clean-up was followed with the magnetic selection using KAPA HyperPure Beads (KAPA Biosystems, Roche). Libraries were then amplified using a 7 cycles LM-PCR and purified. The concentration of the resulting libraries was measured using Qubit 2.0 (Life technologies).

2.3.3. Capture of viral sequences by VirCapSeq-VERT capture panel. The VirCapSeqVERT Capture Panel (Roche) consisting in sequences from vertebrate viral pathogens was used to enrich the sample with vertebrate virus sequences. The libraries were carefully mixed to obtain a pool of 1 μg. Using the HyperCap Target Enrichment Kit (Roche) and the HyperCap Bead Kit (Roche), the pool was hybridised for 20 h at 47 °C and recovered with the Capture Beads (HyperCap Bead Kit, Roche). The captured DNA was amplified with 14 cycles LM-PCR and purified using HyperPure Beads (Roche). The concentration was measured using Qubit 2.0 (Life technologies) and sequenced on an Illumina NextSeq platform (400 M reads).

2.3.4. TES bioinformatic processing. Pair-end FASTQ files generated from the sequencing were analysed using IDseq portal, a cloud-based, open source bioinformatic platform.[39] First, short-read sequencing data was validated performing a subtraction of host sequences *via* STAR (spliced transcripts alignment to a reference) of a raw read to a host-specific database (STAR, RRID: SGR_015899).[40] Illumina adapters were trimmed *via* Trimmomatic[41] and low-quality reads, duplicates, and low complexity reads were removed using the Paired-Read Interactive Contig Extension (PRICE) computational package (PRICE, RRID:SCR 013063),[42] the CD-HITDUP tool v4.6.8 (CD-HIT, RRID:SCR 007105),[43] and a filter based on the Lempel–Ziv–Welch (LZW) compression score, respectively. Viral reads were taxonomically classified using an assembly-based alignment to the NCBI nucleotide (nt) and non-redundant protein (nr) databases[44] using GSNAPL[45] and RAPsearch2.[46] Only viral reads with a 70% identity and >100 nt length were selected.

3. Results and discussion

3.1. Determination of the number of membranes needed for a representative passive sampling

To our knowledge and since the first use of the torpedo-shaped passive samplers for SARS-CoV-2 surveillance in wastewater, only Habtewold and coworkers used more than one electronegative membrane from each passive sampler to test for the presence of viral pathogens and indicators.[4] To our knowledge our study is the first one testing the rationality of using two membranes from the same sampling device to give reliable results.[5,7,47,48] In the current work, two electronegative membranes were tested per torpedo device to evaluate the presence of 3 different viral targets using qPCR: two viral indicators of human faecal contamination (HAdV and JCPyV) and SARS-CoV-2 (N1). A total of 15 PS, each containing 2 membranes, were collected in parallel to two 24 h-AS replicates. Fig. 1 shows the concentration (in genome copies per litre) of the target viral indicators and SARS-CoV-2 (N1 assay) obtained from AS replicates while Fig. 2 shows the values obtained when analysing the torpedo membranes.

As shown in Fig. 1, whereas no noticeable differences were observed comparing viral GC values obtained from different AS replicates, (Pearson's correlation coefficient test 0.76, 0.61 and 0.96 for JCPyV, HAdV and SARS-CoV-2 (N1 assay) respectively) a lower correlation was obtained between the PS membranes (Pearson's correlation coefficient test below

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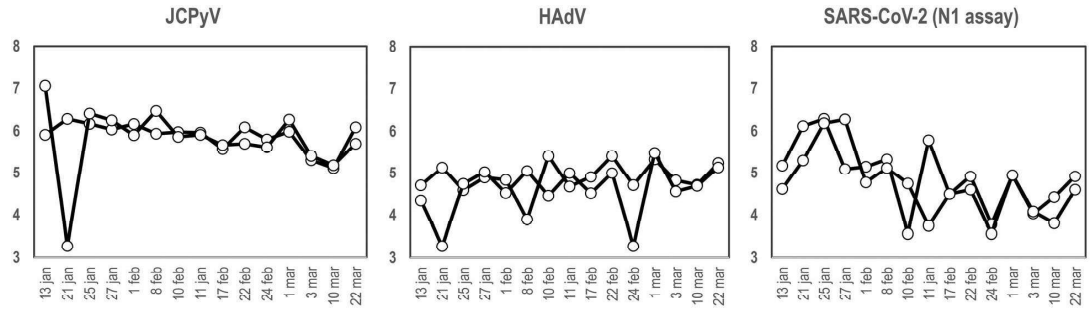


Fig. 1 Quantification (in $\log_{10}$ GC L^{-1}), of JCPyV, HAdV and SARS-CoV-2 (N1 assay), from two different wastewater replicates collected at different dates (*X*-axis) using AS in a WWTP.

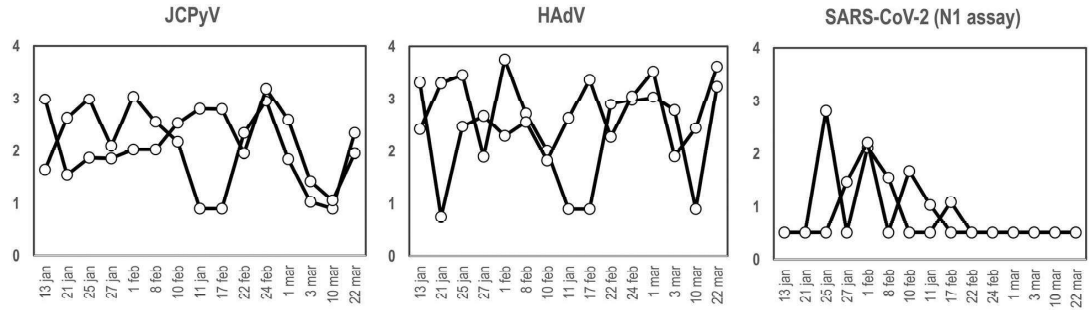


Fig. 2 Quantification (in $\log_{10}$ GC/reaction), of JCPyV, HAdV and SARS-CoV-2 (N1 assay), from two different membranes from a PS at different dates (*X*-axis) at the inlet of a WWTP.

0.33). Note that for PS membranes results are expressed in copies per reaction since the exact volume of wastewater passing through the torpedo is unknown and the calculation of viral concentrations is, thus, unattainable. Exceptional differences were detected on January 21st in JCPyV and HAdV quantifications, which may arise from errors in the analytical procedure (*e.g.* ultrafiltration tips, pipetting errors).

Membrane replicates showed greater variability (Fig. 2) probably due to irregular distribution of the wastewater flowing inside the torpedo. In that case a positive detection was considered when qPCR result was above the limit of detection (LoD) (10, 8 and 3 genome copies (GC) for JCPyV, HAdV and SARS-CoV-2 respectively). Considering positive and negative results, membrane replicates matched 12 out of 15 days for JCPyV and HAdV and 11 out of 15 days for SARS-CoV-2. For the further analysis in this study, it was decided to include in the analysis at least 2 membranes to obtain a representative result from each sample. A result was considered positive when at least one of the membranes resulted positive in virus detection.

3.2. Detection of human viruses at a WWTP and a NH by applying two different sampling/detection strategies

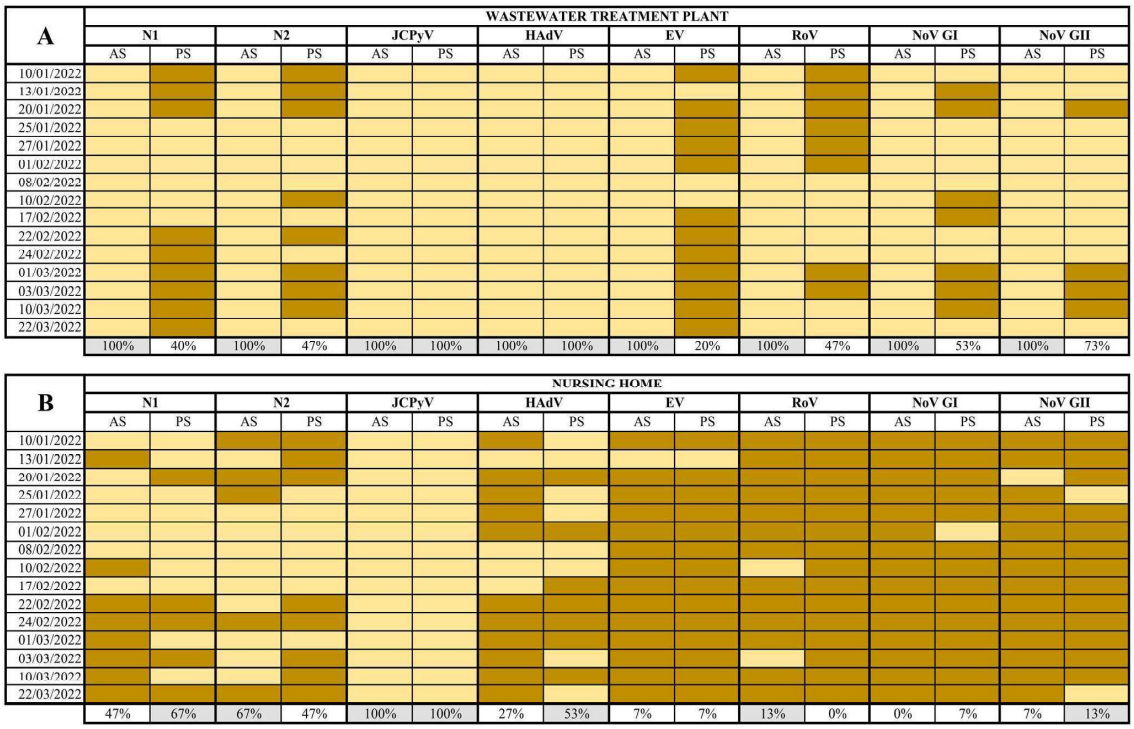
SARS-CoV-2 (N1 and N2 assays), JCPyV, HAdV, EV, RoV, NoV GI and GII, were analysed in a total of 60 samples collected using AS and PS at both WWTP and the NH from January 10th to March 22nd, 2022.

At the WWTP, all composite samples collected tested positive for all viruses analysed while the percentages of detection using PS ranged from 20% to 100% as summarized in Fig. 3. The full dataset of quantification/semi quantification values is shown in the ESI† Tables S1–S3. Inhibition was observed by quantifying JCPyV, as internal process control in 5 out of 120 total samples (Table S4†). The calculated MS2 recovery in the WW samples resulted in 40% of recovery of the seeded material (data not shown).

While human faecal viral indicators, at the WWTP, showed 100% positivity for both AS and PS, the other viral pathogens analysed resulted in 100% positivity by AS between 20% and 73% when using PS. Regarding SARS-CoV-2, between 40–47% positives were reported in PS samples, in contrast to 100% positive samples from AS. This comparison is based on the different performances between PS and AS over a 24 h period while some studies have reported higher PS sensitivities compared PS samples with grab samples.[5,7,19,47] High levels of suspended solids in wastewater may affect the adsorption of viruses to the electronegative membranes. Hayes and coworkers[49] demonstrated that adsorption of the virus to the sampling material is highly dependent of the characteristics of the water. Retention of solids can improve RNA recovery but and excessive retention can inhibit de RNA extraction process.

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A — WASTEWATER TREATMENT PLANT

	N1		N2		JCPyV		HAdV		EV		RoV		NoV GI		NoV GII	
	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS
10/01/2022																
13/01/2022																
20/01/2022																
25/01/2022																
27/01/2022																
01/02/2022																
08/02/2022																
10/02/2022																
17/02/2022																
22/02/2022																
24/02/2022																
01/03/2022																
03/03/2022																
10/03/2022																
22/03/2022																
	100%	40%	100%	47%	100%	100%	100%	100%	100%	20%	100%	47%	100%	53%	100%	73%

B — NURSING HOME

	N1		N2		JCPyV		HAdV		EV		RoV		NoV GI		NoV GII	
	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS
10/01/2022																
13/01/2022																
20/01/2022																
25/01/2022																
27/01/2022																
01/02/2022																
08/02/2022																
10/02/2022																
17/02/2022																
22/02/2022																
24/02/2022																
01/03/2022																
03/03/2022																
10/03/2022																
22/03/2022																
	47%	67%	67%	47%	100%	100%	27%	53%	7%	7%	13%	0%	0%	7%	7%	13%

Fig. 3 Detection of SARS-CoV-2 (N1 and N2 assay), JCPyV, HAdV, EV, RoV, NoV GI and GII in wastewater samples collected using PS and AS from a WWTP (A) and wastewater samples collected using PS and AS from a NH (B). Yellow cells indicate detection and brown cells no detection.

It should be noticed that nucleic acids analysed in the current study were extracted using kits designed to provide highly pure RNA isolation by using inhibitor removal. PS performance might also have been altered by temperature, pH, or dissolved solids concentration.[50] It is also expected that different viruses show different presence patterns despite being adsorbed and concentrated with the same method, since they present different physical properties. Li and coworkers[51] suggested that the different structure between non-enveloped viruses (EV, HAdV, RoV, NoV) and enveloped ones (SARS-CoV-2) could vary their behavior in the affinity for membranes or in the recovery process. All in all, our experimental design did not allow us to differentiate whether the virus adsorption or sample processing were the critical steps in the process. According to recent studies, a low viral recovery could also have been caused by a short deployment time.[50,52] Li and coworkers recently demonstrated that the maximum viral accumulation using electronegative membranes was obtained after 48 h of deployment.[50] Wilson and coworkers[7] also concluded that sensitivities of PS could overcome grab sampling when more than 24 h of wastewater flow were considered.[7] Since the concentration of viruses from AS and PS was performed using different methods for viral recovery, the direct comparison of results is risky if not impossible.

On the other hand, at the NH, the percentage of virus detection using AS ranged from 0% for RoV to 100% for JCPyV and from 0 to 100% for the same viruses when using PS (Fig. 3, the complete data set is available in ESI† Table S3). In general, nearly equivalent results were observed when applying both methods: the more frequently detected virus was JCPyV followed by SARS-CoV-2 (N1 and N2) and HAdV while EV, NoVs and RoV were rarely detected. Negative detection for SARS-CoV-2 by PS corresponded to samples collected during a period of low prevalence of infections in the assisted community[53] suggesting lower sensitivity (in terms of the capacity to produce a positive detection of viral nucleic acid) than traditional detection using AS.

It is remarkable to notice that JCPyV and HAdV showed 100% positivity in both AS and PS in the WWTP and JCPyV also showed 100% positivity in the NH samples. These viruses are used as process controls and fecal indicators because of their abundance in wastewater and their correlation with fecal pollution.[54] It is clear that all NH samples analyzed presented fecal contamination and that JCPyV is more appropriate as fecal indicator at small-scale sewage systems as previously proposed.[55] This is a relevant output of our work since it may be used as a tool to rule out false negative samples. Unlike the WWTP, in this specific site, the presence and spread of a symptomatic viral infection within a small community is more infrequent. Consequently, the likelihood of detection of viral pathogens in wastewater is very low despite the sampling protocol. This is sustained by

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the high number of negative samples obtained by both methods for RoV, EV, NoV GI, and NoV GII. The obtained results confirm that PS is a very good wastewater sampling method for viral studies in small-scale scenarios where AS is not feasible.

3.3. Evaluation of the PS as semiquantitative approach

The PS method here evaluated is based on the free flow of viral particles through the membranes and since we could not assume that all the flow contacts the PS membrane, results cannot be expressed per unit volume (*e.g.*, CG mL^{-1}). Although most studies using this methodology report qualitative results, some authors have shown a significant correlation between the measured SARS-CoV-2 RNA concentrations in samples collected using AS and PS methods.[5,20] These results suggest that PS can express semi-quantitative results for virus detection in wastewater, meaning that a higher quantity of virus in wastewater correlates with a higher accumulation of viruses in membranes and, therefore, a higher GC quantification. To perform a semiquantitative analysis, and by using all the GC values obtained in this study for the different viral species determined, we considered half of the LoD when a result was below the LoD was considered. The semiquantitative detection of JCPyV in both sampling sites presented the highest Pearson's correlation observed, being $R^2 = 0.42$ and $R^2 = 0.38$ at the WWTP and the NH respectively. The results indicate low correlation for SARS-CoV-2 (N1 assay) in both sites (0.13 at the WWTP and 0.18 at the NH). In other words, fluctuations in the genome copy detection at the qPCR would indicate increases/decreases in the genome copies per ml of wastewater.

For SARS-CoV-2, correlations improve when considering results above the LoD, being 0.78 at the WWTP and 0.8 at the NH. Other authors reported correlations between 0.27 and 0.76, in PS collected from WWTP inlets or manholes.[5,20] All in all, when viral concentrations are above the LOD, PS results correlates positively with qualitative results obtained after AS.

3.4. Virome characterization from samples obtained using PS

A secondary aim of this study was to evaluate the performance of torpedoes to study the sewage virome. With this aim, and to increase the sensitivity of the mass sequencing on-wards viruses that infect vertebrates, a target enrichment sequencing was conducted using Vir-CapSeq-VERT panel.

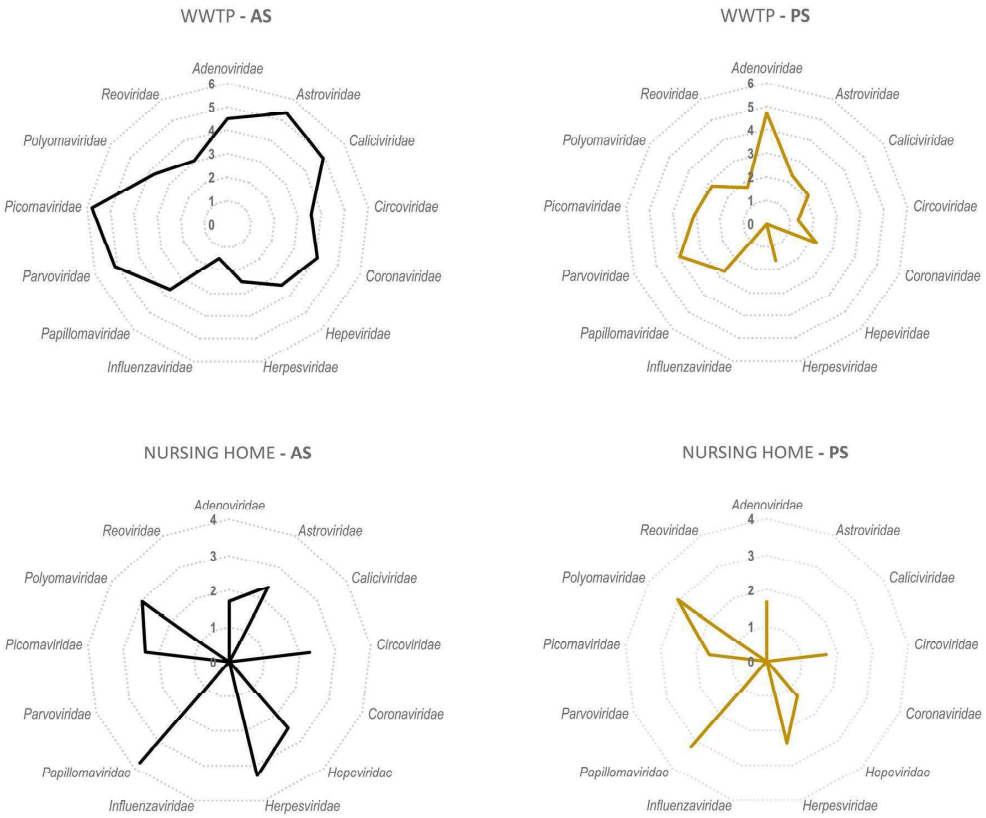


Fig. 4 Comparison of the number of $\log_{10}$ viral reads for each viral family obtained from 6 different wastewater samples when using a AS *versus* PS in both a WWTP and a NH. Numbers 1 to 6 indicate $\log_{10}$ of number of reads.

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At the WWTP, viral families observed were those expected and reported in other studies where the same viral enrichment method was applied.[21] Results showed a higher diversity of viral families and genus present at wastewater samples when collected by AS (Fig. 4 and 5). The number of reads and the number of samples presenting viruses of specific viral groups were higher in samples collected using AS rather than PS. However equivalent viral groups were detected.

The most importantly excreted human pathogens are members of nine families: *Adenoviridae*, *Astroviridae*, *Caliciviridae*, *Hepeviridae*, *Parvoviridae*, *Papillomaviridae*, *Picornaviridae*, *Polyomaviridae*, and *Reoviridae*.[56] All these families have been also described as members of the sewage virome[21,57] and more recently also *Coronaviridae* and *Influenzaviridae* have been included in this list.[58] Aligned with the bibliography,[21] the *Picornaviridae* family, including important pathogens like enterovirus, poliovirus or hepatitis A virus, were the most abundant in raw wastewater. *Reoviridae* and *Hepeviridae* were found to be the least abundant when using AS. Although lower number of reads were obtained using PS at the WWTP (2 log fold), most viral families representing the sewage virome were also found using this passive approach. Viruses belonging to the *Reoviridae* family were not represented when using this sampling methodology probably because of a lower sensitivity of the methodology.

Pathogenic viruses belonging to *Adenoviridae*, *Astroviridae*, *Hepeviridae*, *Picornaviridae* and *Polyomaviridae* were present at the NH. Both methodologies provided similar abundances except for *Astroviridae* for which the AS provided higher number of viral sequences. Interestingly, a high diversity of *Herpesviridae* and *Papillomaviridae* reads were detected in the building WW. Among those viruses herpesviruses-2 and -3 as well as human papillomaviruses β and γ, highly prevalent in older people compared to young adults,[59] were found at the NH facility. The absence of *Coronaviridae* or *Picornaviridae* at NH over the sampling period could be explained because the virus was not circulating over the period of sampling in the tested community.

Passive sampling seems to be less sensitive in terms of detection and number of viral families detected by massive sequencing. Despite providing a lower number of sequences, equal diversity of viral families was obtained using both sampling methodologies. Shannon index (H') when using AS indicates higher diversity of viruses, at the WWTP (H' = 4.8), than at the NH (H' = 4.1). Nevertheless, when applying NGS at building level, the diversity of viruses obtained using PS was higher than when using AS.

To our knowledge, this is the first study applying passive sampling to the successful genetic characterization of a wastewater virome, which could be of relevance in terms of viral emergence surveillance and key information for the corresponding targeted actions. The purpose of this study was to provide information that support the use of torpedo passive sampler as an useful affordable and practical tool to provide information on viral presence in the frame of

		WWTP		NURSING HOME	
FAMILY	**GENUS**	**AS**	**PS**	**AS**	**PS**
Adenoviridae	Mastadenovirus	31645	53138	50	46
Astroviridae	Mamastrovirus	231300	214	232	0
Caliciviridae	Norovirus	72606	138	0	0
	Sapovirus	11070	0	0	0
Circoviridae	Cyclovirus	3746	22	188	48
Coronaviridae	Alphacoronavirus	11359	181	0	0
	Betacoronavirus	390	0	0	0
Hepeviridae	Orthohepevirus	2805	0	316	20
Herpesviridae	Lymphocryptovirus	314	41	1852	232
	Simplexvirus	0	0	130	0
Influenzaviridae	Gammainfluenzavirus	32	0	0	0
Papillomaviridae	Alphapapillomavirus	0	210	0	116
	Betapapillomavirus	3813	286	5380	1506
	Gammapapillomavirus	567	0	720	0
	Mupapillomavirus	404	0	0	0
Parvoviridae	Bocaparvovirus	128080	8492	0	0
Picobirnaviridae	Picobirnavirus	206841	58	238	42
Picornaviridae	Cosavirus	2072	0	0	0
	Enterovirus	56633	422	0	0
	Hepatovirus	68	542	0	0
	Kobuvirus	109925	198	0	0
	Salivirus	241430	46	0	0
Polyomaviridae	Alphapolyomavirus	106	80	302	346
	Betapolyomavirus	5446	575	624	742
	Deltapolyomavirus	168	0	0	0
Reoviridae	Rotavirus	1121	56	0	0

1/6 positive samples
2/6 positive samples
3/6 positive samples
4/6 positive samples
5/6 positive samples
6/6 positive samples

Fig. 5 Total number of reads obtained for each viral family after mass sequencing using a target enrichment approach to test a pool of 6 wastewater samples for each category. Color gradation indicates the number of samples (out of 6 analyzed) presenting viral reads within each viral group.

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wastewater-based epidemiology at building scale level. To obtain more detailed information about viral species some improvements in the protocol of the sequencing methodology should be conducted and will be the aim of further studies.

4. Conclusions

Passive sampling presents a low-cost, safe and power-free solution for smaller sewer catchments. Torpedo shaped devices fitted with electronegative membranes can be applied as an efficient method that is sensitive to a few infection cases residing in the same building. In the event of new epidemic outbreaks, the use of passive samplers can be used for targeted actions or to track back the excreted virome of specific facilities where reside or travel vulnerable population (*e.g.* schools, universities, or aged care facilities).

Conflicts of interest

There are no conflicts to declare.

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Article 2. L'epidemiologia basada en aigües residuals aplicada a nivell d'edifici revela perfils diferents en el viroma basat en l'edat dels individus que contribueixen.

"Wastewater-based epidemiology applied at the building-level reveals distinct viroma profiles based on the age of the contributing individuals"

Mejías-Molina. C., Pico-Tomàs. A., Martínez-Puchol. S., Itarte. M., Torrell. H., Canela. N., Borrego. C. M., Corominas. L., Rusiñol. M., Bofill-Mas. S.

Human Genomics. 2024; 18: 10 DOI: 10.1186/s40246-024-00580-1

Els virus humans excretats al medi i presents en aigües residuals poden, a més de ser detectats i quantificats mitjançant assajos basats en PCR pels que ens cal conèixer quins virus volem detectar, ser caracteritzats genèticament. La metagenòmica permet seqüenciar els àcids nucleics de virus sense conèixer la seva presència en la mostra prèviament. L'estudi del viroma d'aigües residuals ofereix una perspectiva general sobre la circulació de virus en una població i ja són força nombrosos els estudis duts a terme que descriuen la presència d'unes famílies víriques comunes a totes les aigües residuals arreu del món.

Donat que l'ocurrència i la gravetat de les infeccions virals poden variar al llarg de la vida d'una persona, estudiar el viroma en aigües residuals, on han contribuït segments demogràfics concrets, pot proporcionar informació valuosa sobre la prevalença d'infeccions virals dins d'aquests grups. En aquest estudi, es va utilitzar la seqüenciació amb enriquiment de dianes per a caracteritzar el viroma humà en aigües residuals d'edificis de diferents característiques. Això es va aconseguir mitjançant el mostreig passiu d'aigües residuals en escoles, universitats i residències de gent gran en dues ciutats de Catalunya. L'aigua residual d'una EDAR urbana es va analitzar com a referència del viroma humà excretat pel conjunt de les franges d'edat que

conformen la població. En aquestes mostres es van observar astrovirus i bocavirus humans com a virus més prevalents, seguits pels adenovirus humans, poliomavirus i papil·lomavirus.

Es van observar variacions significatives en els perfils vírics en diferents tipus d'edificis estudiats. El mamastrovirus 1 era predominant en mostres obtingudes en escoles, salivirus i poliomavirus humans JC i BK en universitats mentre que a les residències de gent gran s'observava un major equilibri en la distribució de famílies víriques representades per papil·lomavirus, picornavirus i alguns virus relacionats amb la immunosupressió.

Aquest estudi mostra la potencialitat de l'epidemiologia basada en aigües residuals a nivell d'edificis com una eina eficaç per al seguiment de la presència de virus que circulen en grups d'edat específics. A la vegada, proporciona informació valuosa en termes de salut pública i per a estudis epidemiològics.

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Human Genomics

RESEARCH

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Wastewater-based epidemiology applied at the building-level reveals distinct virome profiles based on the age of the contributing individuals

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Abstract

Background Human viruses released into the environment can be detected and characterized in wastewater. The study of wastewater virome offers a consolidated perspective on the circulation of viruses within a population. Because the occurrence and severity of viral infections can vary across a person's lifetime, studying the virome in wastewater samples contributed by various demographic segments can provide valuable insights into the prevalence of viral infections within these segments. In our study, targeted enrichment sequencing was employed to characterize the human virome in wastewater at a building-level scale. This was accomplished through passive sampling of wastewater in schools, university settings, and nursing homes in two cities in Catalonia. Additionally, sewage from a large urban wastewater treatment plant was analysed to serve as a reference for examining the collective excreted human virome.

Results The virome obtained from influent wastewater treatment plant samples showcased the combined viral presence from individuals of varying ages, with astroviruses and human bocaviruses being the most prevalent, followed by human adenoviruses, polyomaviruses, and papillomaviruses. Significant variations in the viral profiles were observed among the different types of buildings studied. Mamastrovirus 1 was predominant in school samples, salivirus and human polyomaviruses JC and BK in the university settings while nursing homes showed a more balanced distribution of viral families presenting papillomavirus and picornaviruses and, interestingly, some viruses linked to immunosuppression.

Conclusions This study shows the utility of building-level wastewater-based epidemiology as an effective tool for monitoring the presence of viruses circulating within specific age groups. It provides valuable insights for public health monitoring and epidemiological studies.

Keywords Human age-related virome, Building-level, Passive samplers, Wastewater-based epidemiology, Targeted enrichment sequencing

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Background

Human viruses can be released into the environment through faeces, urine, saliva or desquamation of skin cells, [1] and, as other excreted substances, they can be identified and detected in wastewater. Analysing the sewage virome offers a significant understanding into the viruses that are actively circulating at a given population and has also the potential to elucidate the introduction of emerging viruses and their modes of transmission [2]. From the initial polio surveillance efforts in the early 1930s to the present-day COVID-19 pandemic, wastewater-based epidemiology (WBE) has been widely employed to monitor vaccine-derived viruses and evaluate population immunity in areas where inactivated virus vaccines have been administered [3].

Numerous studies have documented the virome of urban wastewater, revealing the coexistence of viral families associated with persistent infections (*Anelloviridae, Parvoviridae, Adenoviridae, Papillomaviridae, Polyomaviridae, Herpesviridae*) alongside commonly viral families that contribute more directly to viral disease burdens (including *Astroviridae, Picornaviridae, Coronaviridae*) [4, 5].

It should be noted that the occurrence and severity of viral infections can vary across different stages of an individual's life. School-age children generally experience milder symptoms compared to other age groups, while the severity of viral infections tends to increase well before reaching old age [6].

To the best of our knowledge, various studies have explored the application of WBE at the building level with a focus on SARS-CoV-2 occurrence [7–14]. However, the building scale has been hardly considered for virome studies. Notably, McCall et al. [15] recently reported distinct viral profiles in wastewater collected from different types of buildings. Obtaining representative wastewater samples at a building-scale is challenging, but passive sampling has recently reemerged as an effective solution to address this issue [13, 16].

Next-generation sequencing (NGS) techniques are evolving as valuable complements to traditional molecular methods for analysing environmental samples. NGS provides insights into viral sequences present in these samples without the need for prior information. In recent years, advancements in sample multiplexing, streamlined library preparation protocols and bioinformatic tools have made NGS a more practical and accessible approach for viral detection [17]. Although viral detection by NGS in complex samples such as wastewater is still challenging, targeted enrichment sequencing (TES) strategies have shown to be a promising tool for viral detection and discovery in wastewater [2, 18]

The objective of this work was to characterize the virome present in wastewater at building-level, specifically in schools, university settings and nursing homes hosting residents of different age's ranges, social habits and levels of vulnerability. The goal was to assess how different age groups contribute with different viral profiles to wastewater. To achieve this, passive samplers were utilized to collect wastewater samples from schools, university settings and nursing homes in two different cities. These samples were then subjected to virome characterization using a TES approach. Obtained results were compared with the virome profile of a large urban wastewater treatment plant (WWTP), which served as a reference.

Results

Abundance and diversity of viral reads in different types of wastewater samples

Bioinformatic analysis of the raw data generated from TES applied to the four distinct wastewater types analysed yielded a total of 3,019,032 reads. These reads were taxonomically ascribed to 11 vertebrate viral families, 9 of which included families containing human viruses, encompassing 21 genera. Within the comprehensive analysis of wastewater samples, we identified a total of 48 distinct viral species, and in certain instances, we were able to further classify them into serotypes, serogroups, or genotypes. After manually trimming, a total of 142,643 human viral reads were obtained from the WWTP influent and the wastewater collected from schools, university settings, and nursery homes yielded 1,495,358, 50,520, and 142,643 human viral reads, respectively. Shannon indexes were calculated in the sites presenting a higher number of reads being of 1.77, 1.69, 1.48 and 1.31 in the WWTP, School (B), University (A) and Nursing Home (A), respectively. The relative abundances of different viral families comprising human viruses detected at each sampling site are depicted in Fig. 1, while the number of different viral species identified for each viral family is illustrated in Fig. 2.

Wastewater treatment plant

Urban wastewater encompassed a wide range of viral families and viral species, displaying remarkable diversity (Fig. 3 and Additional file 1: Table S1). The higher number of reads were associated with the family *Astroviridae*, particularly with Mamastrovirus 1 (AstV-1). Notably, reads matching other *Astroviridae* species like AstV-6, AstV-8, and HMO-AstV-A were exclusively detected in urban wastewater, distinguishing it from the other wastewater samples analysed from the buildings.

The second-highest number of reads matched Human Bocavirus (*Parvoviridae).* The third-highest number of reads corresponded to three different subgroups within

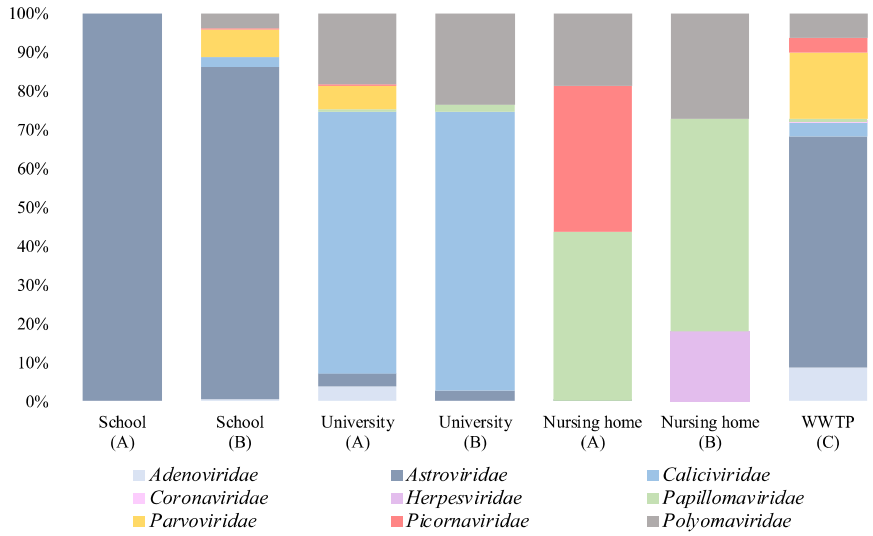


Fig. 1 Relative abundance (% reads matching a viral family/total reads) of the detected viral families at building and WWTP-level representing different age-group wastewater contributors. **A**–**C** represents the different cities studied

	School	University	Nursing home	WWTP
Adenoviridae	1	1	0	3
Astroviridae	4	1	1	5
Caliciviridae	5	5	0	6
Coronaviridae	0	0	0	2
Herpesviridae	0	0	1	0
Papillomaviridae	0	3	4	12
Parvoviridae	1	1	0	1
Picornaviridae	5	3	1	10
Polyomaviridae	2	2	3	3

Fig. 2 Heatmap profile showing the abundance of viral species detected in building and WWTP-level wastewater. Numbers indicate the quantity of different species that has, at least, one sequence with a positive BLAST hit that passed all the selection criteria. Data spanned from yellow (not detected) to red (high abundance)

the *Adenoviridae* family (AdV). Among these, AdV-40 and -41 (group F) exhibited the highest relative number of classified AdVs reads.

Less abundantly, we also detected sequences matching the persistently excreted viruses JC, BK and MC polyomaviruses (JCPyV, BKPyV and MCPyV) and 12 different species of human papillomaviruses, that together with identified members of the family *Picornaviridae* displayed the greatest diversity observed in these samples (Fig. 2). The majority of the *Picornaviridae* reads matched Aichivirus A and Enterovirus-C (EV-C) species. Among the observed *Caliciviridae* sequences, those corresponding to Norwalk Virus, Salivirus A (Sal-A), Salivirus FHB (Sal-FHB), Human Salivirus (HSalV) and Sapovirus (SapV) were the most prevalent. Finally, reads corresponding to the *Coronaviridae* family, including SARS-CoV-2 and Human Coronavirus HKU1, were also detected being WWTP the only site where this viral family was detected along this study.

Schools

School wastewater primarily contained viruses associated with gastroenteritis. Among the viral reads detected in school samples, sequences matching members of the *Astroviridae* family were found to be predominant resulting in over 6 logs of reads. In the site A school, *Astroviridae* was the sole viral family with only a few reads identified. More in detail, a high number of reads matching AstV-1 were consistently found in schools, especially in samples collected in January. Sequences matching other AstV such as AstV-3 and AstV-MLB1 were also identified.

Caliciviridae and *Picornaviridae* were the two families presenting the highest number of viral species (Fig. 2). Members of the family *Caliciviridae* in these buildings included HSalV, Norwalk Virus, Sal-FHB, and Sapporo virus. *Picornaviridae* species detected were Aichivirus A, Enterovirus C, and Parechovirus A (HPeV-A). AdV-40 and -41 (group F) and Human Bocavirus (HBoV), belonging to the *Parvoviridae* family, and Human

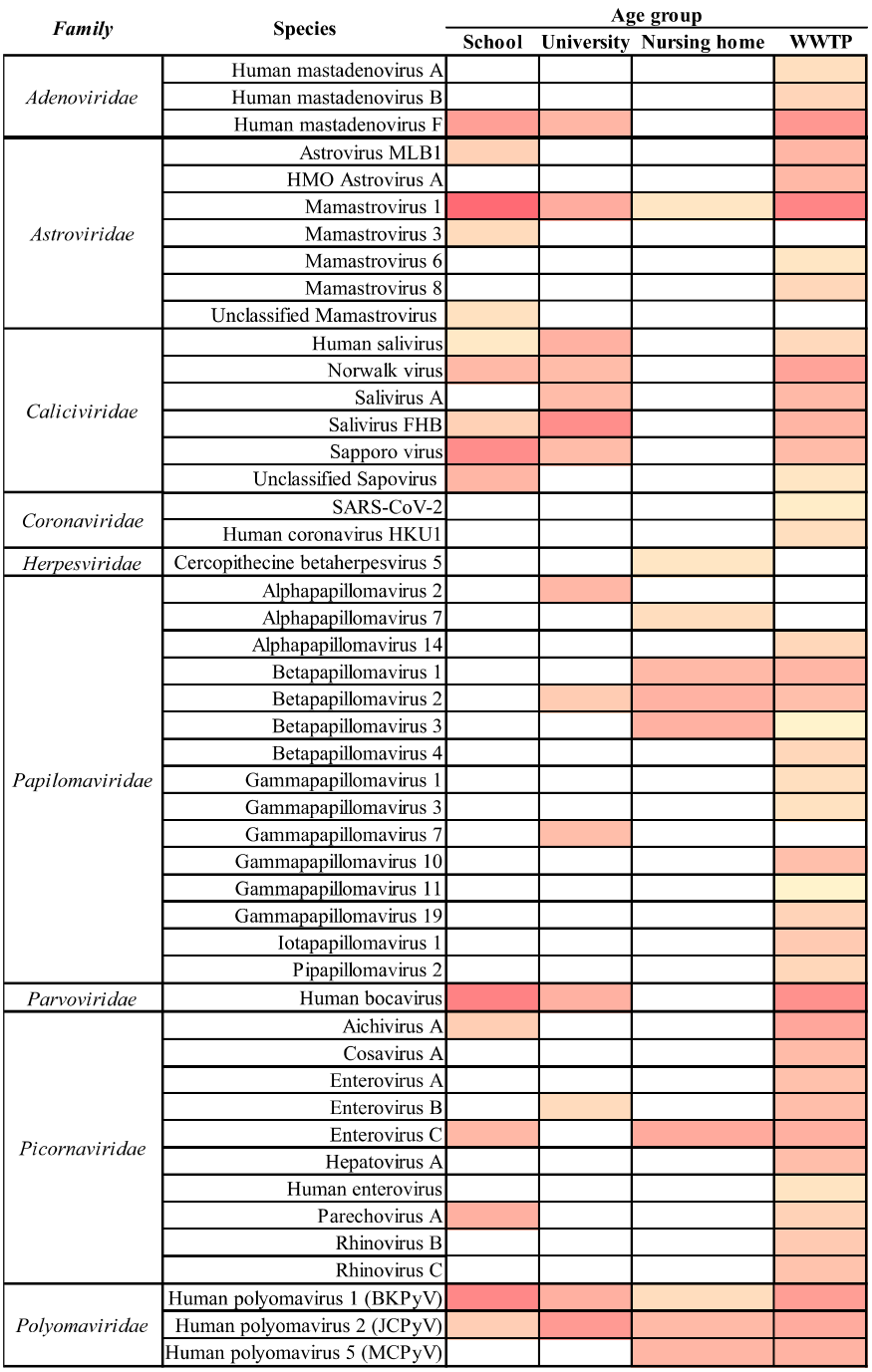


Fig. 3 Relative viral species abundance (Log10 number of reads) detected at building and WWTP-level

polyomavirus 1 (HPyV-1 or BKPyV) and Human polyomavirus 2 (HPyV-2 or JCPyV) were also observed in school samples.

University settings

Viral signatures identified in wastewater collected at the university settings in both cities showed remarkably similar patterns of viral excretion in their sewer outlets, with 60–70% of viral reads matching members of the family *Caliciviridae,* which also encompassed the highest diversity of viral species (HSalV, Sal-A, Sal-FHB and SapV). Additionally, reads matching members of the *Polyomaviridae,* JCPyV and BKPyV (15–20%), and the *Astroviridae* (AstV-1), (< 10%), were also consistently detected in both sites. Moreover, in site A university setting, there were reads belonging to families *Parvoviridae* (HBoV) (6%) and *Picornaviridae* (EV-B) (0,13%). Viral reads assigned to Alphapapillomavirus 2 (α-PV-2), Betapapillomavirus 2 (β-PV-2) and Gammapapillomavirus 7 (γPV-7) were also detected.

Nursing homes

The viromes of the wastewater collected at the nursing homes were less diverse than those obtained from the other wastewater types. In nursing homes from both cities, the relative abundances of the viral families detected ranged between 15 and 30%. Sequences assigned to families *Polyomaviridae* (JCPyV, BKPyV and MCPyV) and *Papillomaviridae* (PV-7, β-PV-1, β-PV-2, and β-PV-3) were found in both locations, with *Papillomaviridae* having the highest diversity of viral species. In site A, viral reads matching the *Picornaviridae* family were identified (EV-C), while site B also presented few reads matching the Cercopithecine betaherpesvirus 5, also known as human cytomegalovirus (CMV), were unique to this type of setting.

Discussion

In this study, WBE has been conducted at building-scale to study the human virome excreted by different aged populations. This has been achieved by analysing different types of wastewater using torpedo passive samplers previously reported as useful tools for viral detection and characterization in wastewater, especially at sites where composite samples are not always easy to obtain [13, 19]. Moreover, the use of TES allowed the detection of a high number of reads matching viral families that specifically infect humans.

Schools, university settings and nursing homes from two different cities were sampled and analysed for virome determination. The results, in terms of relative viral abundance, demonstrated similar viral excretion patterns for the same types of buildings in two different cities. It should be noted that some differences among buildings from the same type but from different cities arose, as well as variations within the same building sampled at different times. These differences were less pronounced when analysing results from the WWTP at different times (data not shown). Despite being derived from a restricted sample size, these results align with a recent study conducted by McCall and co-workers [15], who described higher variations among viral groups observed when comparing different buildings of the same type, as opposed to the variations observed among WWTPs. This observation appears reasonable when we consider the smaller number of individuals contributing to the wastewater virome at the building level. It also considers the possibility of small outbreaks being limited to very small communities, which are typically observed at the WWTP level, where the excretions of a significant percentage of the population are consolidated.

The wastewater virome obtained from the largest urban WWTP in Catalonia, analysed as the most representative sample of the complete human excreted virome in the studied geographical area, revealed a similar viral profile than reported in previous studies using the same TES approach [2, 19]. Astrovirus were the most prevalent viral family, followed by Human Bocavirus, Adenovirus F and Human Polyomaviruses. Interestingly, *Papillomaviridae* was the family with the highest number of different viral species. These findings align with the results reported by McCall and co-workers [15] who also reported *Astroviridae* and *Parvoviridae* as the predominant viral families detected in WWTP after applying a TES approach. Of the 37 distinct viral species detected in the WWTP, 18 were exclusively found in these WWTP samples and not in the building-scale wastewater. This probably indicates these viruses are excreted at low concentrations or by a small number of people and are detected when analysing samples integrating the shedding of a high number of contributors. On the other side, there were species belonging to the *Astroviridae* family which were solely identified in school wastewater, while some members of the *Papillomaviridae* family were exclusively detected in university settings and nursing homes but not in the WWTP. Furthermore, the relative abundances of certain viral species that were present at both building and WWTP-level were different, emphasizing the potential of this type of analysis to provide insights into the viral burden within different demographic groups. Given this finding, the significance of the abundance and observed occurrence trends of the detected viral species in the various samples analysed in this study will be further explored in the subsequent sections.

Wastewater virome associated with persistent infections

Different families of viruses known to persistently infect humans have been found in the different wastewater samples analysed. A discernible trend reveals a higher excretion of these viral families among the elderly, followed by adults, and finally, by children. It is essential to emphasize that persistent infection does not necessarily culminate in clinical manifestations, although viruses that persistently infect humans can sporadically lead to pathological symptomatology.

Human adenoviruses

With more than 110 types described, different organ tropisms and a wide variety of clinical manifestations (revised in Rusiñol and Girones [1]), Human AdV are persistently excreted and make a significant contribution to the viral community present in sewage [2] being proposed as a human viral faecal indicator. The urban wastewater analysed contained reads that matched AdV-F, -A and -B. Among those AdV types identified (Additional file 1: Table S1), AdV-7, -21 and −B3 (Human mastadenovirus B), first isolated from young children, are associated with several respiratory diseases [20], while types A and F have been linked to gastrointestinal infections. Wastewater from the schools and the university settings wastewater only presented AdV-F (Adv-70 and -41)[21].

While both, elderly individuals and young children, are at a heightened risk of AdV infection [22], AdVs reads were not detected in the wastewater from nursing homes. The detection of human AdVs in wastewater have been correlated with the size of the contributing population being sometimes not detected in small size sewer systems [19, 23].

Human polyomaviruses

Human polyomaviruses, with 14 different members, are known to be transmitted among humans during the first years of live through close contact and are excreted by a significant percentage of the population in their urine, typically without recognizable clinical symptoms. Clinical manifestations of these viruses are primarily linked to deep immunosuppression. Among these, Merkel Cell polyomavirus (MCPyV) was discovered to cause Merkel Cell Skin Carcinoma linked to immunosuppression in elderly people and that ageing is a risk factor to develop it.

In this study, sequences matching BK, JC and MCPyV were detected in the WWTP influent. BK and JCPyV were detected in all building samples, whereas MCPyV reads were only observed in the nursing home. An observed seroprevalence increasing with age for JCPyV and decreasing for BKPyV has been previously described [24] and results here obtained are almost in fully agreement with this. The nursing homes studied are contributed to by a small number of people, which would explain the low number of JCPyV reads found. Its presence, along with the absence of human AdV, corroborates previously reported observations describing JCPyV as a faecal viral indicator [25] useful in small sewers [19, 23].

Human papillomaviruses

Within the *Papillomaviridae* family, β-PV and α-PV infect non-genital mucosa and skin. Their excretion has been linked to epithelial shedding in urine, and the presence of these viruses and their high diversity in the environment could be considered emerging [26, 27]. Members of the genus α-PV primarily infect oral and genital mucosa, as well as external genitals, and are associated with mucosal tumour development in humans [28]. Members of this genus are differentiated by their potential to induce cancer, classified as either low-risk Human Papillomavirus (LR-HPV) or high-risk Human Papillomavirus (HR-HPV), and are known to be causing persistent infections [29]. Their occurrence has been widely documented within the environment [30] reinforcing that investigating these viruses in these matrices is highly valuable for describing their prevalence in specific population groups.

Mucosal HPV infections have traditionally been considered sexually transmitted diseases, although could infect children through horizontal transmission, primarily from mother to child [31]. In this study, HPV reads were observed at WWTPs, university settings and nursing homes but not in school's wastewater. In fact, this family was the more diverse found in urban wastewater with sequences matching 12 different viral species being HPV-90, a rare emergent LR-HPV, one of the more prevalent types found [32]. Out of the 12 different HPV species detected in urban sewage, 9 were not found at building-level wastewaters. β-PV 1 and 2 species (HPV-22, HPV-100 and HPV-75) and α-PV 7 (HPV-70) were described exclusively in nursing homes wastewater. HPV-70 has been linked to the development and progression of cervical intraepithelial neoplasia and other squamous intraepithelial lesions [33, 34] and to scrotal calcinosis development [34] and showed high prevalence among some unvaccinated male groups [35]. Lastly, α-PV s 7 (HPV-149) and α-PV 2 (HPV-28) have been detected exclusively in university settings, they are known to cause skin warts due to the cutaneous tropism of this species, unlike most α-PV which exhibits mucosal tropism [36, 37].

Herpesviruses

Regarding members of *Herpesviridae* family, which establish latency and persist for the life of the individual

[38], human cytomegalovirus (CMV) is the only member of the family present in one of the nursing homes tested. CMV is the most common opportunistic pathogen in immunocompromised patients [39] but it can also infect immunocompetent individuals causing self-limiting illness resembling mononucleosis [40, 41]. Interestingly, this virus was also detected in a building wastewater by McCall [15].

Wastewater virome associated with acute infections

Additionally, sequences ascribed to viruses belonging to families commonly associated with acute infections have been identified in the different wastewaters analysed. The overall observed trend suggests a higher excretion of these viruses by children and lower excretion by adults and the elderly. It is important to highlight that the presence of these viruses in wastewater indicates viral replication but does not necessarily translate to clinical manifestations, as these viruses can often cause subclinical infections, particularly in children.

Astroviruses

Human Astrovirus (HAstV) is one of the most important causes of viral gastroenteritis worldwide. Although described in faecal samples from adults, they are responsible for the 10% of acute viral gastroenteritis in children [42]. In this study, the only species common to all wastewater types analysed was Mamastrovirus-1, which decreased in reads number in agreement with the age of the contributor individuals (Fig. 3). HAstV sequences were consistently more prevalently detected in January samples (data not shown), aligning with their known seasonal pattern [43]. A deeper analysis of the sequences of Mamastrovirus-1, allowed a further classification into serotypes in some of the pooled samples (Additional file 1). Serotype HAstV-1 was identified in all samples which aligns with reported data indicating its high prevalence within the population [43]. These results agree with a prior study conducted in Spain, specifically targeting children under the age of 5 who suffered from acute gastroenteritis, and that study reported a 11.5% prevalence rate [44]. The abundance of reads corresponding to *Astroviridae* (AstV), and, in particular to AstV-1, were higher in school samples compared to other studied locations. AstV reads account for more than 80% of reads resulting from school wastewater analysis. This explains why the total numbers of reads from schools was higher than those obtained from the WWTP possibly indicating a higher viral load in the school environment due to elevated AstV excretion. AstV-6, AstV-8 and HMO Astrovirus A were only detected at WWTP-level. HAstV-2 and HAstV-3 reads have also been detected in school and university settings whereas HAstV-4 reads were only found in nursing home wastewater.

Caliciviruses

Norwalk virus is the only species belonging to the Norovirus genus (NoV). NoV infections are the leading cause of gastroenteritis outbreaks [45], are usually self-limiting in healthy patients although in immunocompromised individuals, elderly people and young children can cause severe complications [46, 47]. Norwalk species are divided into genogroups, that are divided into 30 genotypes. In this study, NoV was detected in schools, university settings and at the urban WWTP. A deeper analysis of the sequences obtained allowed a classification into genogroups in some samples (Additional file 1). Despite, genogroups GI, GII and GIV are prevalent human pathogens, only GI and II were found in this study. In particular, the GII.17 genotype, that was identified in the school from city B and at the WWTP, has gained prominence in recent years, overtaking GII.4 as the dominant strain in Asia [48] and has also been described in Europe [49–51].

Sapovirus is an important cause of diarrhoea in children, especially in countries where vaccines for rotavirus are available [52, 53]. They also have been identified as pathogen in adults, especially in immunocompromised individuals or those who live in nursing homes [54–56]. The results obtained showed Sapovirus reads in the wastewater collected from schools and university settings, which is consistent with previous reports indicating that it represents one of the highest burdens of disease in young children [57] as well as its association with sporadic and limited outbreaks [58].

All in all, results obtained suggest children and young adults are the main shedders of members of the *Caliciviridae* family while these viruses were not detected in nursing home wastewater suggesting no active infections or low excretion levels of these viruses at the time these analyses were conducted.

Coronaviruses

To date, seven human coronaviruses (HCoVs) have been described. In this study, only Human coronaviruses HKU1 (HCOV-HKU1) and SARS-CoV-2 were reported in the urban wastewater collected at the studied WWTP. HCOV-HKU1 has been associated with severe complications in vulnerable populations presenting cold-like symptoms [59] and SARS-CoV-2 with the Covid-19 [60]. These results suggest that if HCoV were excreted at building-level, which is likely, excretion levels were low and under the detection limits of techniques employed. It should be said that SARS-CoV2 was detected in all the buildings by applying qPCR [13] which is a more sensitive technique than NGS [18]. Recently, a specific

probe-based capture panel designed for the specific sequencing of CoV has been successfully applied to the detection of a high diversity of human and animal CoV in wastewater (Martinez-Puchol et al., submitted to publication).

Parvoviruses

Human bocavirus (HBoV) is associated with respiratory symptoms and gastroenteritis in children [61] although its role as a causative agent has been not described yet [62]. HBoV is usually attributed to infectious in infants although it can affect children older than 5 years and adults with higher incidences in winter and spring [63, 64]. In this study, HBoV was present in school, university, and WWTP samples with the highest number of reads obtained from school samples in agreement with excretion patterns described for this virus and with previous studies regarding its presence in sewage [65–67]

Picornaviruses

Picornavirus sequences were detected in all the wastewater types analysed although WWTP samples presented the highest diversity of viral species suggesting excretion related to small outbreaks within small communities.

Aichivirus (AiV) is associated with childhood infections [68] responsible of a low proportion of gastroenteritis outbreaks [69], it has been detected in the stool of patients with gastroenteritis among different age groups in Spain [70] and in this study has been found in WWTP samples collected from schools and nursing homes.

The genus Cosavirus include five species of Human Cosavirus (HCoSV-A, B, D, E, F). It was first detected in stool samples of healthy children and children with non-polio acute flaccid paralysis [71, 72] and, later, in children with or without diarrhoea [73–76]. HCoSVs have also been found in sewage and rivers [67, 77, 78]. Despite their presence and diversity in sewage has been described [79], only few clinical studies have been published, showing a very low detection rate if any [80–83] that could explain why sequences matching HCoSV-A were only detected at the WWTP level but not at building-level.

The same happened with Hepatitis A virus (HAV) which usually cause self-limited infections being transmitted by faecal-oral route but also be spread by person-to-person transmission causing large epidemics [84]. The prevalence of HAV correlates with the socioeconomic status of the studied area [85]. In developed countries, where vaccination programs against HAV exist, the prevalence of HAV has decreased [86]. In this study, HAV was only detected at the WWTP-level. In Spain, specifically in Catalonia, vaccination against HAV is systemic and included in the vaccination schedule for children at 15 months old. Although it is not technically mandatory, it is widely practiced. We hypothesize that there was no detection at schools and university settings as children and young people are heavily immunised but people travelling to endemic areas, men having sex with men, prisoners, homeless and other susceptible collectives may contribute to the HAV observed at WWTP-level [87].

Both enterovirus (EV) and Human Parechovirus (HPeV) are picornaviruses causing a common infection in children. HPeV causes mainly respiratory and gastrointestinal symptoms [88] although in children have been associated with central nervous system infections [89]. EV have been associated with wide range of illness: from mild febrile illness, respiratory infection, gastroenteritis, meningitis, encephalitis, feet-hand-mouth disease and other pathologies [90]. However, EV is usually seen in older children and adults while HPeV is not frequent at these ages [91]. This aligns with our results since HPeV reads were only present at school and WWTP while EV reads were observed in all samples studied with differences in the distribution of the three species (A, B and C).

Human rhinoviruses (HRV) are the most prevalent human respiratory viruses and responsible for more than a half of cold-like illnesses each year. In this study, Rhinovirus B and C were only found in the WWTP and was no detected in building's wastewater probably because the sampling was conducted in winter when influenza and respiratory syncytial viruses are predominant over HRV [92–94].

Salivirus (SaV) was first described from stool samples from children with gastroenteritis [95]. Its relationship with gastroenteritis remains still unclear [96] However, SaV has been detected worldwide in clinical samples and urban sewage [97, 98]. In our study, two species of SaV were identified: SaV-A at university settings and at the WWTP and SaV-FHB in schools, universities, and at the WWTP. These results agree with previous publications describing its association with infection in patients always under 20-year-old and its presence in children's stool [99, 100]. SaV reads were the more abundant reads at the university settings even when comparing to WWTP, suggesting young adults as the SaV target population.

In summary, it can be hypothesized that the virome in human wastewater comprises a mosaic of viruses originating from a variety of sources. Some viruses may originate from individuals of various age groups, while others might be specific to certain populations. Additionally, our findings indicate that viruses associated with acute infections causing limited outbreaks are more frequently excreted by children than by the older population, and these viruses are typically detectable in WWTPs.

These viruses can also be detected at the building level during an ongoing outbreak at the time of sampling, or when asymptomatic individuals contribute to increased viral load in wastewater. On the other hand, certain viral species that are restricted to specific individuals or demographic groups, such as certain HPV or astrovirus species, may be more readily detectable at the building level and could become diluted to undetectable levels in the influent of larger wastewater treatment systems. Furthermore, the observed virome profiles can be valuable for understanding the epidemiology of these viruses, including their excretion patterns and transmission pathways.

Conclusions

- Passive sampling, in the form of torpedo devices, was applied as a straightforward and efficient method for the wastewater virome characterization at a building-scale. TES provided a comprehensive list of viruses present at WWTP and building-level scale.
- The virome profiles observed in the two cities studied for the same type of building linked to a specific demographic group were similar suggesting different viral excretion profiles for each sewer system analysed.
- WWTP virome, analysed as a reference, presented astrovirus, particularly MAstV-1, as the more abundant viruses followed by HBoV, adenoviruses, polyomaviruses, picornaviruses and caliciviruses.
- Astrovirus (particularly AstV-1) predominated in school samples, while salivirus and human polyomaviruses JC and BK dominated in university settings. Nursing homes showed a more balanced distribution of viral families presenting papillomavirus and picornaviruses. Notably, viruses associated with immunosuppression, such as MCPyV and CMV.
- A discernible trend emerged: viruses associated with acute infections were more frequently detected in schools but less so in elderly residences, while viruses linked to persistent infections exhibit an inverse trend, being more commonly detected in elderly residences and less frequently in schools.
- Overall, the study provides insights into the diversity and distribution of human viruses in different types of wastewaters sources. The results highlight the potential of building-scale WBE as a tool for tracking communal infectious diseases. Such information can be useful in gaining insights into these infections and in designing targeted public health intervention strategies for specific populations.

Methods

Sample collection

While numerous WBE studies predominantly utilize composite samples gathered by automatic samplers at WWTP inlets, the advent of the COVID-19 pandemic has spurred the adoption of flexible passive sampling techniques [14, 16]. These approaches facilitate the monitoring and viral profiling of various waterborne pathogens within compact sewer catchments [16, 19].

Our study focussed on three distinct building types—schools, university settings, and nursery homes—in two cities in Catalonia, Northeast Spain, as outlined in Table 1. School buildings mainly accommodate students aged 3 to 12 but also a diverse range of users, including teachers, administrative staff, parents, guardians, maintenance, and support personnel, as well as participants in various after-school programs. Buildings at the university campus primarily cater to individuals aged 18 to 30, but given the dynamic nature of campuses, these spaces also host researchers, administrators, and other faculty members throughout the day.

The studied nursing homes, accommodating 300 predominantly elderly residents as full-time inhabitants, represent facilities with comparatively lower user capacity. These residents constitute their primary users.

Additionally, wastewater from a third city (C), with an urban population of about 1.5 million, was obtained from the main WWTP inlet. This wastewater served as a reference for a comprehensive analysis of the excreted virome.

Buildings were sampled two/three times during January and March 2022 except for the nursery home at city A where only samples in March were collected (detailed in Additional file 1). The sampling was conducted using 3D printed torpedo-shaped passive sampling units (kindly donated by Prof. McCarthy) fitted with 2 electronegative membranes (EZ-Pak filters 0.45 µm, Merck Millipore). Torpedoes were deployed for 24 h at the outlet of each building sewer and at the inlet of the

Table 1 Sampling sites and building users description

Age group	City	Sampling sites	Population represented
3–12	A	School sewer outlet	500
18–30	A	University campus sewer outlet	100
>65	A	Nursing home sewer outlet	300
3–12	B	School sewer outlet	500
18–30	B	University campus sewer outlet	400–1000
>65	B	Nursing home sewer outlet	500
All ages	C	Inlet urban wastewater treatment plant	1.5 M

WWTP. They were retrieved and transported inside a plastic bag to the laboratory in a portable icebox.

Elution of the viral particles, nucleic acid extraction and DNase treatment

Prior to nucleic acids (NA) extraction, the elution of the viruses adsorbed to the electronegative membranes was performed. Electronegative membranes were carefully introduced, using sterile tweezers, inside Power Bead Tubes (glass 0.1 mm Qiagen), 700 µl of glycine (0.25 N, pH = 8) were added and then bead-beating was applied for 30 s at 4 m/s using FastPrep-24™ (MP Bio, USA). Sample tubes were centrifuged for 1 min at 20.000×*g* and the resulting 60 µl supernatant was treated with Turbo DNase (Invitrogen, Carlsbad, CA, USA) for 1 h at 37 °C. The QIAamp Viral RNA mini kit and the Qiacube Automatic system (Qiagen) were used for the NA extraction into a final volume of 50 µl.

Sequence-independent, single-primer amplification (SISPA) and target enrichment sequencing (TES)

Nucleic acid extractions analysed in this study were pooled according to Additional file 2 and amplified before the sequencing library was constructed as previously described [101]. For this reason, viral sequences identified in each sampling site are the result of the viral sequences present in 2–3 different samples collected in January and March and are presented together to provide a more robust composition of a particular virome.

Briefly, NA were retrotranscribed and tagged using SuperScript IV enzyme (Invitrogen), random nonamer primers and Sequenase 2.0 (Applied Biosystems). The cDNA was then amplified to obtain enough cDNA for the next steps by following 25 PCR cycles using AmpliTaq Gold DNA polymerase (Applied Biosystems). The resulting product of the PCR was purified using Zymo DNA Clean & Concentration kit (Zymo research) and quantified using Qubit 2.0 and the Qubit dsDNA HS Assay Kit (Invitrogen).

Libraries were prepared following manufacture's instruction using the KAPA HyperPlus Library Preparation Kit (KAPA Biosystems, Roche). Starting with 100 ng of amplified cDNA, enzymatic fragmentation, A-tailing and adapter's ligation were conducted. Each sample was ligated with KAPA UDI Primer mixes (KAPA Biosystems Roche) and a clean-up was performed with the KAPA HyperPure Beads (KAPA Biosystems, Roche). The libraries were amplified with a LM-PCR of 7 cycles, purified and then quantified using Qubit 2.00 and the Qubit dsDNA HS Assay Kit (Invitrogen).

Capture of viral sequences by VirCapSeq-VERT™ capture panel (Roche)

Libraries were pooled to obtain a final quantity of 1 µg. Then it was hybridised for 20 h with probes of the VirCapSeqVERT™ capture panel (Roche) that contains sequences of viruses infecting vertebrates using the HyperCap Target Enrichment Kit (Roche). The captured DNA was recovered using the HyperCap Bead Kit (Roche), amplified with 14 cycles of LM-PCR, purified (HyperPure Beads, Roche) and quantified with Qubit 2.0. Sequencing of the captured libraries was performed on an Illumina NextSeq2000 platform achieving up to 1000 M 150×2 pb pair-end reads.

The generated Pair-end FASTQ files were analysed using ID-seq, an open source and cloud-base bioinformatic tool [102]. Briefly, Illumina adapters, duplicates, low quality and complexity reads were cleaned using Trimmomatic [103] and CD-HITDUP tool v4.6.8 (CD-HIT, PRID:SCR 007105) [104] Reads were paired using the Paired-Reads Interactive Contig Extension (PRICE) computational package (PRICE, RRID:SCR 013063) [105] and the Lempel–Ziv–Wech (LZW) compression score. GSNAPL [106] and RAPsearch2 [107] were used for an assembly-based alignment to the NCBI nucleotide (nt) and non-redundant protein (nr) databases [108]. A minimum of 70% identity and >100 nt length were considered for analysis. However, for human viruses, all species were assigned with alignments to the NCBI above 90% of identity.

Viral reads of *Adenoviridae* and *Papillomaviridae* longer than 100 bp were queried for similarity using BLASTN against the NCBI GenBank nucleotide collection database [108]. For specific characterization, Calicivirus and Enterovirus the Typing Tool developed by RIVM were used (version 2.0 and 0.1, respectively) [109].

Statistical and diversity index calculations

The Shannon diversity indexes were calculated using Excel, as measures of the relative abundances of the species present in a sample. The formula to calculate the Sannon índex (H) is:

$$H = -\sum_{i=1}^{S} p_i \cdot In\ p_i.$$

where S is the total number of species in the community and p^i the proportion of reads belonging to the species.

Abbreviations

Adv	Adenovirus
AiV	Aichivirus
α-PV	Alphapapillomavirus

β-PV	Betapapillomavirus
BKPyV	BK polyomavirus
COVID-19	Coronavirus infectious disease
DNA	Deoxyribonucleic acid
EV	Enterovirus
EV-C	Enterovirus-C
γ-PV	Gammapapillomavirus
GI	Genogrup 1
HAV	Hepatitis A virus
HR-HPV	High-risk Human Papillomavirus
HAstV	Human Astrovirus
HMO-AstV-A	Human astrovirus A
HBoV	Human Bocavirus
HCoV	Human coronaviruses
HCoSV	Human Cosavirus
CMV	Human cytomegalovirus
HPV	Human Papillomavirus
HPyV	Human polyomavirus
HSalV	Human Salivirus
JCPyV	JC polyomavirus
LR-HPV	Low-risk Human Papillomavirus
AstV	Mamastrovirus
MCPyV	MC polyomavirus
NGS	Next-generation sequencing
NoV	Norovirus
NA	Nucleic acids
HPV	Human Papillomavius
HPeV-A	Paraechovirus A
qPCR	Quantitative Polymerase Chain Reaction
SaV	Salivirus
Sal-A	Salivirus A
Sal-FHB	Salivirus FHB
SapV	Sapovirus
SISPA	Sequence-independent, single-primer amplification
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TES	Target Enrichment approach
WWTP	Wastewater treatment plant
WBE	Wastewater-based epidemiology

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40246-024-00580-1.

Additional file 1. List of Viral species and serotypes, serogroups or genotypes identified in each building type. Mean ID%: average percentage identity of alignments; Mean CV: the per cent of the reference accession that is covered by at least one contig; Coverage depth: indicates the average read depth across the length of the accession. Metagenomic metrics are mean values obtained at the different samples analysed per each building type and at the WWTP.

Additional file 2. Sample dates and pools of the nucleic acid extractions analysed. Each column corresponds to one pool.

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Author contributions
The investigation, data analysis, and the first original draft were conducted by CMM. APT performed the sampling campaign. HT and NC performed the metagenomic analysis. SMP and MI collaborated in the data analysis. LC, CMB, MR and SBM conceptualized this study. Funding acquisition and project administration were conducted by CMB, NC, LC, and SBM. All the authors commented and revised previous versions of the manuscript. All the authors approved the final manuscript.

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Availability of data and materials
The datasets generated during the current study are available in zenodo under the DOI numbers: https://doi.org/10.5281/zenodo.8178269, https://doi.org/10.5281/zenodo.10077991, https://doi.org/10.5281/zenodo.10078619, https://doi.org/10.5281/zenodo.10149180, https://doi.org/10.5281/zenodo.10158645.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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Capítol 2: Noves aplicacions dels mostrejadors passius: detecció i caracterització de virus en aigües subterrànies i regenerades

Article 3. Explorant virus en aigua subterrània: quantificació i caracterització del viroma mitjançant mostrejadors passius i seqüenciació amb enriquiment de dianes.

"Exploring waterborne viruses in groundwater: Quantification and Virome characterization via passive sampling and targeted enrichment sequencing"

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Els aqüífers proporcionen aigua potable a gairebé la meitat de la població mundial i s'enfronten a reptes importants pel que fa a la contaminació microbiana, en particular de virus transmesos per l'aigua.

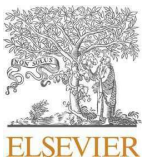
Aquest estudi investiga la contaminació vírica present en aigües subterrànies urbanes mitjançant mostreig passiu, quantificació per qPCR i seqüenciació amb enriquiment de dianes. Es va avaluar l'abundància de patògens virals (HAdV, EV, genogrup I i II de NoV, RoV, IVA, HEV i SARS-CoV-2) i es va investigar el viroma de l'aigua a l'aqüífer del Delta del Besòs a Catalunya.

Durant un període de 7 mesos, es van recollir 114 mostres de l'aqüífer utilitzant membranes de niló i nitrocel·lulosa contingudes dins uns mostrejador passiu de tipus torpede per adsorbir virus submergits durant un període de 10 dies. La contaminació fecal humana es va detectar en gairebé el 50% de les mostres d'aigua subterrània analitzades, amb concentracions de HAdV que anaven de 1.23E+02 fins a 3.66E+03 GC/mbr, i deteccions puntuals d'EV i GI i GII NoV.

A més, la seqüenciació va revelar un viroma divers a l'aqüífer, amb detecció de patògens humans, incloent les famílies virals *Adenoviridae, Astroviridae, Calicivirida i Herpesviridae.*

D’acord amb els resultats obtinguts, el mostreig mitjançant mostrejadors passius augmenta la probabilitat de detecció de virus en les aigües subterrànies analitzades i, quan es combina amb seqüenciació massiva amb enriquiment de dianes, pot proporcionar una comprensió més profunda de la prevalença de virus en aquest compartiment. Aquest enfoc pot permetre racionalitzar els esforços de seguiment a llarg termini i permetre a les comunitats petites o als gestors d'aigua amb recursos limitats gestionar eficaçment aquest recurs.

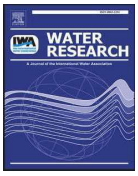
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Exploring waterborne viruses in groundwater: Quantification and Virome characterization via passive sampling and targeted enrichment sequencing

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ABSTRACT

Aquifers, which provide drinking water for nearly half the world's population, face significant challenges from microbial contamination, particularly from waterborne viruses such as human adenovirus (HAdV), norovirus (NoV) and enterovirus (EV). This study, conducted as part of the UPWATER project, investigates the sources of urban groundwater contamination using viral passive sampling (VPS) and target enrichment sequencing (TES). We assessed the abundance of eight viral pathogens (HAdV, EV, NoV genogroup I and II, rotavirus, influenza A virus, hepatitis E virus and SARS-CoV-2) and investigated the virome diversity of groundwater in the aquifer of the Besòs River Delta in Catalonia. Over a period of 7 months, we collected 114 samples from the aquifer using nylon and nitrocellulose membranes to adsorb viruses over a 10-day period. Human faecal contamination was detected in nearly 50 % of the groundwater samples, with mean HAdV total counts ranging from 1.23E+02 to 3.66E+03 GC, and occasional detections of EV and NoV GI and GII. In addition, deep sequencing revealed a diverse virome in the aquifer, with detection of human pathogens, including adenovirus, astrovirus, calicivirus, enterovirus, herpesvirus, papillomavirus and rotavirus. Time-integrated sampling using VPS increases the likelihood of virus detection and, when combined with TES, can provide a deeper understanding of virus prevalence in this important water compartment. This approach is expected to streamline long-term monitoring efforts and enable small communities or water managers with limited resources to effectively manage their groundwater reservoirs.

1. Introduction

Aquifer serve as a freshwater resource for approximately 50 % of the world's population (WHO\UNICEF, 2021). Groundwater, sustaining fast urban population growth, faces challenges as viruses are reported as the main cause of drinking water-related outbreaks (WHO, 2019), yet comprehensive monitoring of its microbial content remains rather poor. A recent revision on the fate of waterborne viruses in urban groundwater environments, listed human adenoviruses (HAdV), norovirus (NoV), and enteroviruses (EV) as the most frequently detected pathogens at the groundwater sources (Rusiñol, 2023) stating a lack of systematic monitoring as well considering sample processing and virome assessment. Domestic animals, wildlife, and humans are major sources of this viruses in groundwater (Pandey et al., 2014) and recent advancements in next generation sequencing (NGS) technologies have enabled a more comprehensive understanding of the groundwater virome, including bacteria, vertebrates, invertebrates, and plants among other viral hosts (Itarte et al., 2021 and 2024; Martínez-Puchol et al., 2020; Rusiñol et al., 2020b). These approaches have unveiled a diverse array of viruses, shedding light on potential human, animal and environmental risks associated with groundwater contamination.

Large amounts of pathogens may be introduced into urban aquifers through multiple sources, primarily from sewer leakage, sewer overflows, urban runoff, and wastewater treatment plant effluents. Despite treatment efforts, treated urban wastewaters continue to introduce viruses to the environment due to incomplete viral removal during wastewater treatment processes (Fernandez-Cassi et al., 2017; Rodriguez-Manzano et al., 2012). Initiatives addressing water scarcity issues,

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such as the EU's focus on groundwater replenishment through indirect water reuse, highlight the growing importance of understanding the complete virome of groundwater sources (European Commission, 2012). Climate variability or increasing groundwater extraction for irrigation purposes is already inducing changes in water quality (García-Ruiz et al., 2011; Scanlon et al., 2023). The impacts of disinfected effluent discharges into the microbial loads are evident in freshwater environments, where changes in community compositions have also been monitored (Munné et al., 2023; Múrria et al., 2024). To date, and considering the new strategies for groundwater protection, it is important to develop effective monitoring strategies for these waters, specifically addressing the presence of a selection of waterborne viruses and studies on the changing groundwater virome.

This research, as part of the UPWATER project (www.UpWater.eu), aims to enhance our understanding of the pollution sources impacting the quality of urban groundwater. The study site, located within the aquifer of the Besòs River Delta (Catalonia, NE Spain), represents a highly impacted system as evidenced by the presence of illicit drugs, pharmaceuticals, and personal care products (Jurado et al., 2012; López-Serna et al., 2013; Serra-Roig et al., 2016). It is well characterized that approximately 22 % of the groundwater recharge originates from rainfall recharge and runoff infiltration (37 %), followed by the sewer network exfiltration (30 %), the water supply network losses (22 %), and the Besòs river (11 %) (Vazquez-Suñé et al., 2010). The Besòs river has a mean annual flow rate of 2,9m^3/s. However, it is notable that during dry winters and hot summers, almost the entire flow derives from the effluents discharged by the 27 wastewater treatment plants situated within the river basin. This phenomenon significantly impacts the quality of groundwater in the area (Mas-Ponce et al., 2021). The aquifer's groundwater, currently used for garden irrigation and street cleaning, may see expanded applications pending thorough characterization of pollution sources.

The primary objective of this study was to assess the abundance of 8 viral pathogens (human adenovirus (HAdV), enterovirus (EV), norovirus genogroup I and II (NoV GI and GII), rotavirus (RoV), influenza A virus (IAV), SARS-CoV-2 and hepatitis E virus (HEV)) and explore the diversity of the groundwater virome using a cost-effective viral passive sampler (VPS). Pepper mild mottle virus (PMMoV), CrAssphage and Escherichia coli (*E. coli*) were tested as process controls and indicators of fecal pollution in the water samples. Herein, we present the results of a 7-month surveillance in the Besòs River aquifer (including 114 samples) and investigate the minimum requirements and characteristics necessary to achieve reliable, sensitive, and cost-effective surveillance of viruses in groundwater sources. We present the first description of the use of viral passive samplers (VPS), as a time-integrated approach for the detection of low virus occurrence in groundwater, relying on the fact that viruses attach onto the charged surface of the sampling material.

2. Methology

2.1. Materials and reagents

2.1.1. Passive samplers (VPS) and adsorbing materials

Virus sampling was conducted using the 3D printed passive samplers described by Schang et al. (2021). The samplers, designed to resemble a torpedo, were directly deployed in boreholes and fixed using a nylon rope onto the well cap. Each sampler had an internal metal weight to ensure that it was submerged vertically at a depth of 3 m in piezometer SAP-2b and 11 m at piezometer SAP1. The position, below the water table in the piezometric boreholes, was measured using the same nylon rope used to fix the VPS. Each 3D printed device had multiple entry points for groundwater to interact with the different adsorbing materials.

Each VPS was charged with different materials: nitrocellulose membranes (NC) (EZ-Pak filters 0.45 mm, Merk Millipore), nylon membranes (NY) (0,47 mm, Merk), Cotton-tampons (CTN) (Tampax® Compak Super) and Granular Activated Carbon (GAC) (FILTRASORB 300, Calgon Carbon Corporation). Prior to use, 1 gr of GAC was sealed into nylon sealable teabags (4 cm x 5 cm) and then each bag was washed with distilled water to remove any impurities and minimize interferences by soluble organic residues, then let to dry overnight. Each VPS housing, with torpedo shape design, was filled with a maximum of three materials. After retrieval of the samplers and processing of the absorbing materials, each VPS housing was submerged in 1 L of distilled water at 0.5 % of sodium hypochlorite for 20 min, and rised with distilled water before reuse.

2.1.2. Virus concentration using ultrafiltration (UF)

On specific sampling dates (refer to Section 2.2.3), grab samples were concurrently collected alongside the VPS. Viruses were concentrated from 100 L groundwater samples on-site, employing the ultrafiltration protocol described by Forés and coworkers (Forés et al., 2022). The Large Volume Concentration kit (LVC kit) by InnovaPrep® integrates a Rexeed 25-A polysulfone hollow-fiber ultrafilter with the Tris-PBS wet foam elution, enabling the concentration of groundwater viruses from large-volume water samples into a minimal eluate volume (30–50 mL). A Hunday HY900Si inverter generator was used to feed the Solinst® peristaltic pump (Model 410) set at 350 rpm. Eluates refrigerated at 4 °C during transport to the laboratory for subsequent concentration using the ultrafiltration device CP-Select™ 150 KDa (InnovaPrep, Drexel, MO, USA). Viral particles were finally retrieved in 300 µL and stored at −80 °C until further analysis.

2.1.3. Nucleic acid extraction and qPCR

NC and NY membranes were processed following the protocol described by Mejías-Molina et al., (Mejías-Molina et al., 2024). Briefly, membranes were carefully introduced inside Power Bead Tubes (glass 0.1 mm Qiagen) and 700 µl of glycine (0.25 N, pH 9.5) were added. After 30 s at 4 m s^{-1} of bead-beating using a Fast-prep homogenizer, the tube was centrifuged at 14.000*xg* for 1 min and 280 µl were used in the NA extraction using the Viral RNA Mini Kit (Qiagen) and the Qiacube Automatic system (Qiagen) into a final volume of 70 µl. CTN-tampons and GAC were placed into 10 ml falcon tubes. Ten millilitres of glycine (0.25 N, pH 9.5) were added and placed into a lab rotator for 30 min at 30 rpm. After that, CTN-tampons, and the remaining glycine, were placed into a syringe. A volume of around 10 ml was recovered and further ultrafiltered using ultrafiltration tips (150 KDa) and PBS elution buffer with the CP-Select™ (Innovaprep). NA extraction was performed as described above into a final volume of 70 µl.

Specific real-time q(RT)PCR assays were used to quantify 8 viral pathogens (HAdV, EV, NoV GI and GII, RoV, IAV, SARS-CoV-2, HEV), one viral process control (PMMoV) and two fecal indicators (ChrAssphage and *E.coli*) following the specifications previously described (Da Silva et al., 2007; García-Aljaro et al., 2017; Hernroth et al., 2002; Hoehne and Schreier, 2006; Jothikumar et al., 2006; Kageyama et al., 2003; Khan et al., 2007; Loisy et al., 2005; Rusiñol et al., 2020a; Svraka et al., 2007; Zeng et al., 2008). For DNA viruses and *E. coli*, TaqMan® Environmental Master Mix 2.0 (Thermofisher Scientific) was utilized, while RNA Ultrasense™ One-Step RT-qPCR (Invitrogen) was employed for RNA viruses. The standard suspensions for viral quantification were prepared using synthetic gBlocks® Gene Fragments (IDT) and quantified with a Qubit® fluorometer (Thermo Fisher Scientific). For bacteria, DNA was extracted from cultures of known concentration and ten-fold dilutions were prepared from 10° to 10^{7} copies per reaction. All the q(RT) PCR assays included four non-template controls and all qPCR tests were performed in triplicate. Results were reported as average genome copies per membrane or liter.

The limits of detection (LoD) for all qPCR and RT-qPCR were determined by running six replicates of 10-fold dilutions of target DNA suspensions near the detection threshold (2.5, 5, 25 and 50 Genome Copies/reaction) for each virus examined. Utilizing the method outlined by Forootan et al., the concentration yielding at least 95 % positive

replicates was considered the LoD of the qPCR assay (Forootan et al., 2017). LoD was transformed into the LoD of the entire method using the NA extraction volume tested and assuming 100 % efficiency of the membrane extraction method (stated in Fig. 3).

2.1.4. Spiking materials: virus stocks and wastewater

Human adenovirus type 35 (HAdV-35) (ATCC® VR-718TM) were produced by infecting A549 (ATCC® CCL-185TM) cells, respectively. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies) containing 2 × (maintenance medium) or 10 × (growth medium) of heat-inactivated fetal bovine serum (FBS). DMEM was supplemented with 2 % glutamine and 1 % penicillin-streptomycin (Gibco, Life Technologies). All viruses were released from cells by freezing and thawing the cultures three times. A centrifugation step at 3.000 × *g* for 10 min was then applied to eliminate cell debris. The supernatant obtained was ultracentrifuged for 1 h at 34.500 × *g* resuspended in PBS and stored in 10-mL aliquots at −80 °C until use. One-hundred-millilitre wastewater samples were collected from the inlet of an urban wastewater treatment plant (that collects wastewater from over 2 million inhabitants) and employed herein to mimic a natural source of fecal contamination.

2.2. Basics of the passive sampling in groundwater

2.2.1. Evaluation of the different adsorbing materials under laboratory conditions

Two 50-litre groundwater samples with turbidity levels of 0.35 ± 0.25 NTU (nephelometric turbidity units) were used to characterise the recovery of the method. One sample was spiked with 1 ml of 10^6 Genome Copies/ml (GC/ml) of HAdV and the other one with raw wastewater (1:100). The spiked groundwater samples were stirred for 1 h prior to the adsorption experiments and during the experiment the water was recirculated using a peristaltic pump to simulate groundwater flow. Five replicate samples of each passive sampler material were placed into the VPS, with a maximum of three materials per VPS. Samples were gently recirculated using a peristaltic pump to maintain water movement. Half of the VPSs were retrieved after 24 h and the other half after 72 h. HAdV and PMMoV, highly prevalent viruses and indicators of fecal pollution, were used to evaluate the recovery of spiked HAdV and naturally occurring ones (present in wastewater used for spiking).

2.2.2. Temporal dynamics of virus adsorption

Ten VPS units, each containing 3 NY or 3 NC membranes (the two most effective adsorbing materials observed in this study), were deployed in two 50-litre groundwater samples spiked with wastewater (1:100) (see Section 2.1.3.). As indicated in Section 2.2.1. samples were stirred before VPS deployment and the water was recirculated to simulate the groundwater flow. Three replicates of both NC and NY membranes were exposed for 0 h, 24 h, 72 h, 7d or 10d to ensure sufficient virus interaction with the adsorbent. Again, HAdV and PMMoV quantifications over time were used to evaluate the temporal dynamics of adsorption.

2.2.3. Validation of vps compared to ultrafiltration devices

VPS containing NY and NC membranes were compared to the ultrafiltration (UF) method for the detection of two commonly used human fecal indicators (HAdV and CrAssphage), the internal process control (PMMoV) and *E. coli*. Five 100 L groundwater samples were collected from the study site during the samplings in September and October 2023. UF was conducted on-site, and eluates were transported for further processing as indicated in Section 2.1.2. Five VPS charged with 3 replicates of NC membranes and 5 with 3 replicates of NY were deployed in the aquifer groundwater. VPS retrieval was performed after 10 days and membranes were sent to the lab for further NA extraction and qPCR detection.

2.3. Monitoring of viruses in the river Besòs swallow aquifer

A total of 10 sampling campaigns were carried out between September 2023 and May 2024. Fifty-four membrane samples were collected from piezometer SAP-2b (upper screened borehole, depth of the VPS at 3 m), and 60 from piezometer SAP1 (medium screened borehole, depth of the VPS at 11 m). Each VPS, charged with 3 membranes/samples, was deployed for 10 days. A list of samples included in the surveillance can be found in Supplementary information 1. At the day of recover, viral NA from NC and NY membranes were extracted (see Section 2.1.3) and PMMoV and HAdV specific qPCR were performed. RT-qPCR for the detection of EV, HEV, IAV, NoV GI and GII, RoV-A and SARS-CoV-2 were performed in samples collected during the colder months (October, December, and January) where higher incidence NoV, RoV and respiratory virus outbreaks are reported in Catalonia (SIVIC, 2024).

Accumulated precipitation data, registered by the Automatic Weather Stations (AWS) (XEMA,Meteocat), was reported and summed after each 10-day sampling event. Groundwater temperature, pH and electrical conductivity measurements were done during the first sampling campaign because previous work in the aquifer, and at the same sampling wells, reported no variations in general hydrochemistry between winter and summer (Jurado et al., 2019). Groundwater samples were collected after pumping (with a Solinst® peristaltic pump (Model 410) set at 500 rpm) a volume of equal to at least three times the borehole volume (147 liters). Electrical conductivity was measured using a Hanna Groline HI98318 probe and temperature and pH were measured using a waterproof tester Hanna Combo HI98121.

2.4. The groundwater virome

Eight VPS (half charged with NC and the other half with NY) and 2 water samples (previously concentrated using UF), were used to conduct target enrichment sequencing (TES). To assess the efficacy of sequencing replicates in enhancing output yield, viral nucleic acid extractions from 4 VPS collected in October 2023 underwent the entire library and capture process in 3 replicates each. A list of the specific samples included in TES analysis can be found in Supplementary information 1. The selection of samples was done according to the presence of fecal contamination. A negative control was added to the Illumina sequencing.

2.4.1. Sequence-Independent, single-primer amplification (SISPA)

NA were retrotranscribed and tagged using SuperScript IV enzyme (Invitrogen), random nonamer primers and Sequenase 2.0 (Applied Biosystems). The cDNA was then amplified to obtain enough cDNA for the next steps by following 25 PCR cycles using AmpliTaq Gold DNA polymerase (Applied Biosystems). The resulting product of the PCR was purified and quantified using Zymo DNA Clean & Concentration kit (Zymo research) and Qubit dsDNA HS Assay Kit (Invitrogen) respectively.

2.4.2. Library construction

Libraries were pooled to obtain a final quantity of 1 μg. Enzymatic fragmentation was conducted at 37 °C for 40 min, followed by end repair and dA-tailing utilizing the Twist EF Library Prep 2.0 kit and following the manufacturer's instructions. Ligation of adapters with distinct dual barcodes (Twist (HT) Universal Adapter System) was performed using the Twist library Preparation EF Kit 2.0. Subsequent purification using DNA beads (Twist Library Preparation Kit 2) and PCR amplification, consisting of 7 cycles, was performed to increase the sequencing yield.

2.4.3. Capture of viral sequences using twist panel

The amplified libraries were purified again and aggregated in groups of 8 samples for their hybridization with the Twist Comprehensive Virus probe panel. This commercial panel contains approximately 1 million

probes targeting 15,488 diverse viral strains infecting both humans and animals. After 16-hour of incubation period at 70 °C and several washing steps (Twist Dry Down beads and Twist Wash buffers), the captured fragments underwent further 14 cycles amplification using Equinox Library Amp Mix (2x). Purification of the final libraries was done utilizing AmpureXP Beads, prior quantification and fragment size analysis using Qubit (Thermo Fisher, Waltham, MA, USA) and Fragment Analyser (Agilent, Santa Clara, CA, USA), respectively. Sequencing of the captured libraries was performed on an Illumina NextSeq500, and approximately 1 million 150 bp paired-end reads were generated per sample in accordance with the manufacturer's protocols (Illumina Inc.).

2.4.4. TES bioinformatic processing

The generated pair-end FASTQ files were analysed using ID-seq, an open source and cloud-base bioinformatic tool (IDseq Portal). Briefly, Illumina adapters, duplicates, low quality, and complexity reads were cleaned using Trimmomatic (Bolger et al., 2014) and CD-HITDUP tool v4.6.8 (CD- HIT, PRID:SCR 007105) (Li et al., 2006). Reads were paired using the Paired-Reads Interactive Contig Extension (PRICE) computational package (PRICE, RRID:SCR 013063) (Ruby et al., 2013) and the Lempel–Ziv–Wech (LZW) compression score. GSNAPL (Wu et al., 2010) and RAPsearch2 (Ye at al., 2011) were used for an assembly-based alignment to the NCBI nucleotide and non-redundant protein databases (NCBI 2024). A minimum of 70 % identity and >100 nt length were considered for analysis. However, for human viruses, all species were assigned with alignments to the NCBI above 90 % of identity.

2.5. Statistical analyses

R version 4.1.2 (R Core Team, 2013) was used to perform all correlations. Normality was assessed using the Shapiro-Wilk test before selecting the appropriate statistical tests. We run Pearson's correlation coefficient test to evaluate potential associations between viral recovery and VPS materials. In Section 2.2.3, for each virus and method, Wilcoxon signed rank tests for paired data were used to evaluate whether there were statistically significant differences between VPS-NC, VPS-NY and UF methods. Spearman's non-parametrical correlation was used to evaluate differences between sequencing replicates.

3. Results

3.1. Selection of adsorbing materials, deployment times and validation compared to ultrafiltration

The recovery of PMMoV and HAdV from groundwater after 24 and 72 h of deployment using the four different adsorbing materials is presented in Fig. 1A. Examining the HAdV spiked recoveries, significant differences in viral recovery between different materials were observed ($p < 0.05$), both after 24 h and after 72 h of deployment. The highest mean recoveries were achieved after 72 h using NY or NC membranes.

When considering naturally occurring HAdV recoveries (using groundwater spiked with wastewater), differences were rather low ($p > 0.05$). However, it was observed that NC and NY membranes adsorbed increasing amounts of viruses over time, while GAC recoveries were only detectable above the LOD after 72 h, and CTN HAdV recoveries decreased over time. The recovery of the internal process control, PMMoV, which naturally occurs in groundwater, exhibited consistency across the four different materials, with mean values ranging from 43 % to 64 % (Fig. 1A).

Fig. 1B, depicts the temporal dynamics of HAdV (blue) and PMMoV (green) adsorption onto VPS-NC and VPS-NY over time. For both adsorption materials and viruses, maximum virus adsorption occurred between 7 and 10 days after deployment.

When comparing detection rates of virus using VPS (passive sampling over 10-day period) or traditional large-volume sampling, VPS were twice as sensitive as ultrafiltration (Fig. 2). Although the sampling approaches and sample volumes differ, with VPS being a semi-quantitative method and ultrafiltration being a quantitative method, the higher detection rates observed with VPS highlight its greater sensitivity. After 10 days of deployment, HAdV were detected using both NY and NC, in 5 out of the 5 membranes analyzed, while only 3 water samples concentrated by ultrafiltration (100 L) detected HAdV.

3.2. Waterborne virus surveillance in the Besòs aquifer groundwater

A total of 54 membranes were tested from SAP-2b and 60 from SAP-1 respectively, with pathogens and human fecal pollution noted at the two selected wells with different sampling depths: 3 m for SAP-2b and 11 m for SAP-1. Temperature, pH and electrical conductivity of the groundwater was 19.6 °C, 7.15 and 1324mS/cm. Samples were validated for pathogen detection when PMMoV was detected. Fig. 3 summarizes virus detection and the reported accumulated precipitation (in millimeters) during the 7-month campaign (September 2023 to May 2024). No correlation was observed between precipitation (mm) and virus detection ($p > 0.3$).

Nearly 50 % of all groundwater samples analyzed presented human fecal pollution using the HAdV qPCR assay (with mean concentrations between 1.23E+02 and 3.66E+03 GC per membrane). EV and NoV GI and GII were occasionally detected. EV were consistently detected during December and January 2024 at both sampling depths and using

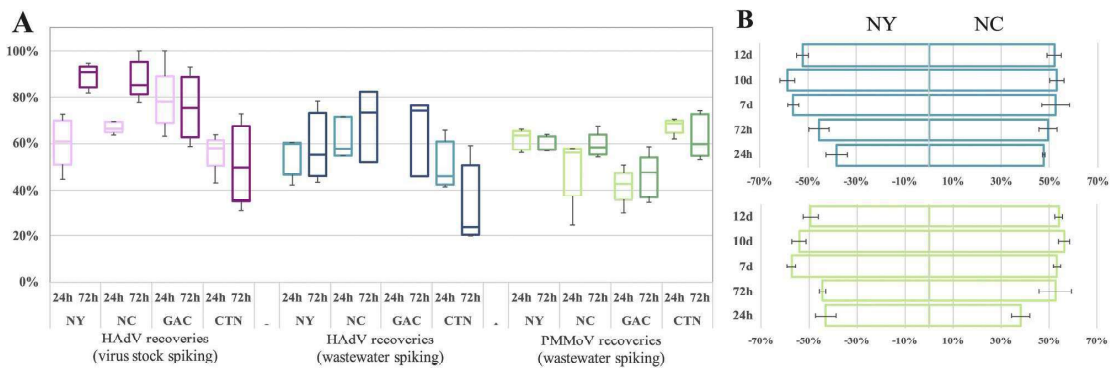


Fig. 1. Summary of the VPS lab-scale validation. (A) Box plots representing HAdV and PMMoV recoveries over time (24 and 72 h) using four different sampling materials (NY: nylon, NC: nitrocellulose, GAC: Granular Activated Carbon, CTN: Cotton tampons). (B) Temporal dynamics of virus adsorption using groundwater spiked with urban wastewater. Mean HAdV (blue) and PMMoV (green) recoveries calculated over time (24 and 72 h, 7, 10 and 12 days) using VPS-NY or VPS-NC. Error bars on each data point indicate standard deviation ($n = 5$).

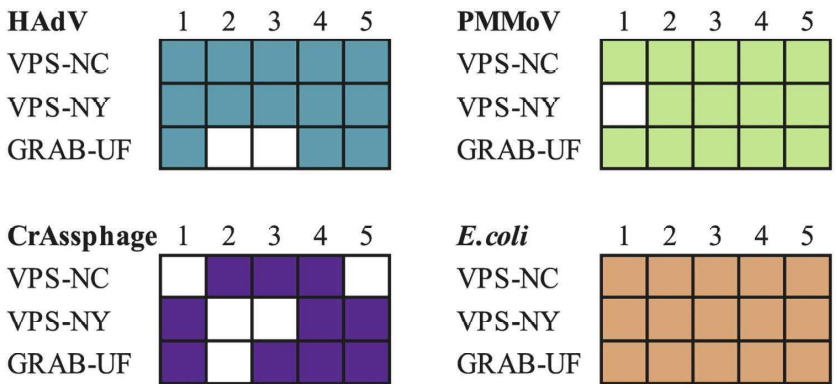


Fig. 2. Detection of fecal indicators in field samples (n:5) collected using passive samplers (VPS charged with NC or NY) or grab sampling and UF.

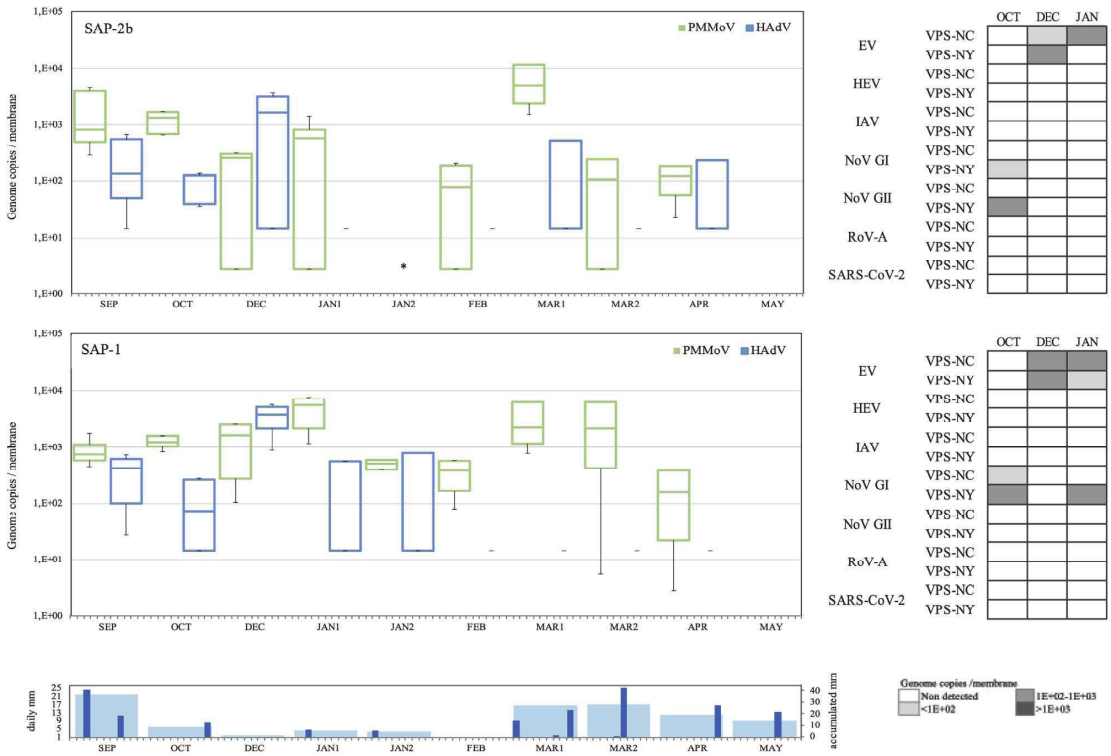


Fig. 3. Boxplots and heatmaps representing virus concentrations in genome copies per membrane (GC/mbr) detected during the surveillance program in SAP-2b (top charts) and SAP-1 (bottom charts). Each boxplots includes the results of 3 NC and 3 NY membranes. *SAP-2b was not monitored in JAN2. LoD: HAdV 1.45E+01 GC/mbr, PMMoV 2.77E+00, EV, HEV, IAV, RoV: 3.00E+01, NoV GI: 4.0E+01, NoV GII: 1.55E+02 and SARS-CoV-2: 9.7E+01 GC/mbr. Bar chart below the boxplots indicates accumulated precipitation (mm) over each sampling event.

both VPS-NY and -NC. Norovirus GIs were detected in the VPS from October at both wells, but only in SAP-2b did the VPS-NY adsorb enough NoV GII to be detected and quantifiable.

3.3. *Insights into the groundwater virome*

The viral assignments and the relative abundance of sequenced reads (Fig. 4) represent the sum of the viral sequences present in the various samples (Supplementary information 1). The samples were collected using passive samplers, VPS charged with NC or NY, or using grab samples (100 L) ultrafiltered using the Large Volume Concentration method (UF) described in Section 2.1.2. The capture-probe approach applied in this study, designed against 3000 human and animal viruses, enabled the identification of a total of 21 viral families, 12 of which include species infecting humans. Specifically, only *Adenoviridae, Herpesviridae* and *Reoviridae* were detected using the three sampling methodologies. Using VPS, we identified significant human pathogens from various viral families, including *Adenoviridae, Astroviridae, Caliciviridae,*

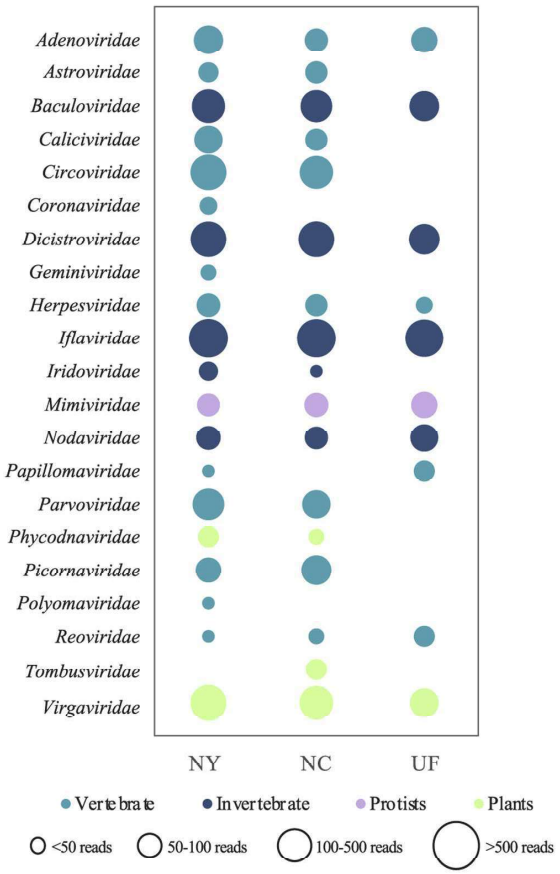


Fig. 4. Viral assignments and reads sequenced using VPS with nylon (NY) or nitrocellulose (NC) membranes or Ultrafiltration devices (UF). Bubble size represent the relative abundance of read counts (Log10).

Herpesviridae, Papillomaviridae, Picornaviridae, Polyomaviridae and *Reoviridae.* Although a small number of reads were ultimately trimmed from groundwater samples, the mean percentage of identities was 96,07 %, with a mean coverage of 238pb (Supplementary Table 2). Several assignments of human mastadenovirus-F were obtained from nearly all sequenced samples. Furthermore, human adenoviruses were detected in both wells via qPCR, suggesting persistent injection of human fecal sources into the aquifer. Reads attributed to aichivirus-A, cosavirus-A, mamastrovirus-1, NoV, and rotavirus-C species, known to cause enteric disease across Multiple species, were identified within the examined urban aquifer. Notably, samples obtained through VPS yielded over 50 reads each for norovirus and aichivirus-A, whereas no such reads were obtained from ultrafiltered (UF) groundwater samples. Concurrently, both NoV and aichivirus-A were detected and quantified via qPCR during the same sampling event (Fig. 3).

A diverse number of ssDNA viruses belonging to the *Circoviridae and Parvoviridae* families were detected among the different pools analyzed. The highest number of assignments belonged to viruses infecting animals. Circoviruses infecting bats, birds and cattle, and parvovirus sequences related to a diversity of animals (e.g. rabbit, cats, dogs and pigs) were commonly found in all the groundwater samples analyzed. Animal *Adenoviridae, Astroviridae,* and *Herpesviridae* also appeared in the sequencing output. Avian coronavirus or porcine associated gemykibivirus-1 reads, belonging to *Coronaviridae* and *Geminiviridae* were identified. While herpesvirus (alfaHV2, betaHV7) were successfully detected using all sampling approaches, members of the *Papillomaviridae* family (alfaPV4, betaPV2 gammaPV12) were occasionally detected using VPS-NC and UF (Fig. 4).

Many viruses infecting insects, including members of the *Baculoviridae, Dicistroviridae, Iflaviridae, Iridoviridae* (dsDNA) and *Nodaviridae* (ssDNA) families that infect aphids, cockroaches, butterflies, flies, vespa and mosquitoes, were detected in the groundwater samples.

To study differences between NGS sequencing replicates, here we evaluated quantitative (number of reads/total reads *100) and qualitative differences (number of membranes in which specific viral reads were detected) among the viral family's assignments. Heatmap profiles (Fig. 5 and Supplementary information 3) showed the specific relative abundances of each viral family identified. Human pathogens with a low relative abundance of reads belonging to *Adenoviridae, Caliciviridae, Herpesviridae, Picornaviridae, Polyomaviridae* and *Reoviridae* were only detected in one of the replicates analysed, regardless the sampling well and VPS adsorbing material. Viral reads belonging to the *Astroviridae, Iridoviridae,* and *Poliomaviridae* were only detected in SAP-2b, and reads belonging to *Coronaviridae, Geminiviridae* and *Papillomaviridae* were only detected in SAP-1. Conversely, *Circoviridae, Parvoviridae,* and *Picornaviridae* assignments, in some cases with similar relative abundances as the viruses stated above, were frequently detected in all sequencing replicates analysed. Nevertheless, strong positive correlations (mean spearman's correlation coefficient 0.74, $p < 0.01$) were observed between relative abundances of each viral family, and particularly between sequencing replicates (Supplementary information 3).

Plant viruses belonging to *Virgaviridae, Tombusviridae or Phycodnaviridae*, which are ubiquitous and can be found in human fecal samples due to their presence in our diet, contribute to a reduction in the sequencing output allocated to animal and human viruses. Sequencing reads associated with plant viruses were shown in Fig. 4 to highlight that there is still room for further development of the capture panels.

4. Discussion

Since 2020, literature regarding passive samplers for virus surveillance has increased substantially (Bivins et al., 2022; Shakallis et al., 2022) However, only few authors have evaluated performance of NC membranes (Mejías-Molina et al., 2023), NY membranes (Jones et al., 2022; Vincent-Hubert et al., 2021) CTN (Jones et al., 2022) and GAC (Hayes et al., 2022) for recovery of viruses other than SARS-CoV-2 from seawater or wastewater. To our knowledge there is currently no available literature on passive samplers for virus surveillance in groundwater compartments (Hayes et al., 2024). When considering viral capture, electropositive membranes and CTN-based samplers have previously demonstrated superior performance in capturing SARS-CoV-2 from wastewater (Jones et al., 2022). Electronegative membranes, with combined glycine elution and bead-beating before the NA extraction, also demonstrated to be a powerful tool for viral detection and characterization (Mejías-Molina et al., 2024, 2023). Here, for groundwater virus surveillance, both NY and NC demonstrated similar virus capture pattern and are proposed to be included in combination in the VPS.

The maximum adsorption capacity of NC and NY materials has only been studied for SARS-CoV-2 in wastewater (Habtewold et al., 2022; Hayes et al., 2021; Li et al., 2021). This capacity to attach to the materials depends on the size, isolectric point, and abundance of the virus. With the typical pH range of 6 to 8.5 in groundwater, viruses carry a negative charge, rendering them prone to adsorption onto positively charged soils (Rusiñol, 2023). It has been reported that sand and gravel aquifers, such as the Besòs River aquifer under study, are particularly susceptible to retain viral contamination compared to other aquifer types (Abbaszadegan et al., 2003). Consequently, it is plausible to expect that the adsorption of viral particles present in groundwater onto membranes would be enhanced by the presence of suspended solids, which in turn adhere to VPS materials. Further research should include trials to evaluate the composition of groundwater suspended solids

	SAP-2b		SAP1		SAP-2b		SAP1	
	NY	NC	NY	NC	NY	NC	NY	NC
Adenoviridae	0,66	0,10	2,56	2,69	1	1	2	2
Astroviridae	1,00	0,88	0,00	0,00	2	2	0	0
Baculoviridae	3,98	1,56	5,63	6,28	3	3	3	3
Caliciviridae	0,00	0,68	2,90	0,30	0	3	2	1
Circoviridae	6,47	20,37	11,69	17,94	3	3	3	3
Coronaviridae	0,00	0,00	0,34	0,00	0	0	1	0
Dicistroviridae	9,95	4,58	16,72	35,43	3	3	3	3
Geminiviridae	0,00	0,00	0,26	0,00	0	0	2	0
Herpesviridae	0,17	0,10	0,09	0,15	1	1	1	1
Iflaviridae	49,09	53,51	39,68	14,35	3	3	3	3
Iridoviridae	0,50	0,29	0,00	0,00	1	1	0	0
Mimiviridae	0,66	0,49	0,51	0,90	3	3	3	3
Nodaviridae	1,00	0,97	0,77	0,60	3	3	3	3
Papillomaviridae	0,00	0,00	0,17	0,00	0	0	1	0
Parvoviridae	3,15	1,17	2,82	4,48	3	3	3	3
Phycodnaviridae	0,17	0,10	0,51	0,15	1	1	2	1
Picornaviridae	1,33	8,77	0,85	2,09	2	2	3	3
Polyomaviridae	0,33	0,00	0,00	0,00	1	0	0	0
Reoviridae	0,33	0,19	0,17	0,30	1	1	1	1
Tombusviridae	0,00	0,39	0,00	0,90	0	2	0	2
Virgaviridae	21,23	5,85	14,33	13,45	3	3	3	3

Fig. 5. Sequencing read analysis in VPS membrane replicates: Abundance of reads in percentages (heatmap in blue) and number of membranes with confirmed assignations to specific viral family reads (heatmap in yellow).

adsorbed to the membranes.

Suspended solids and sand from the saturated zone of the aquifer are expected to bind viruses and subsequently help in their attachment to the membranes (Gerba et al., 1984). However, the presence of humic substances, which are commonly found in groundwater systems, may inhibit virus detection by interfering with the binding process (Sidstedt et al., 2020). This phenomenon was previously studied for EV (Sobsey and Glass, 1984) and is likely to vary based on the physical and chemical nature of the particulate solids.

Recent trends in groundwater surveillance indicate a preference for ultrafiltration over electropositive filtration for virus concentration, leading to the incorporation of ultrafiltration devices, considered the gold standard, in this study. Compared to grab sampling, VPS provides a more representative method for monitoring viruses that travel with the groundwater flow. VPSs for groundwater virus surveillance could potentially replace cumbersome large-volume grab sampling and even ultrafiltration in the field, due to their time-integrated nature that improves the likelihood of detecting viruses in groundwater. VPS can be especially beneficial for accessing restricted sampling areas, simultaneously sampling multiple groundwater wells, or sampling locations where automatic samplers are impractical. Fecal pollution, and particularly excreted viruses, will copies can be detected even in very low concentrations in groundwater. This makes VPS particularly effective for capturing transient events or fluctuations in contaminant levels over time, offering a more comprehensive understanding into specific contamination events, though this may not always represent the average groundwater quality. Nevertheless, membrane replicates exhibited variability (Fig. 1A), likely attributed to the irregular distribution of groundwater flowing inside the VPS and the low occurrence of viruses in this type of samples. Thus, to ensure reliable results during the surveillance of viruses, three membranes were further analyzed from each VPS. This approach, which increases the effective surface area of the membranes, enhances the likelihood of detecting viruses. Future research may consider utilizing multiple VPS at varying borehole depths to obtain more comprehensive data on virus presence and distribution.

The prevalence of waterborne viruses obtained after the surveillance campaign in the urban aquifer studied here, corresponds to the prevalence reported for these three viruses in literature, as reviewed by (Rusiñol, 2023). In the surveillance studies published over the last 5 years in urban groundwaters, and considering only the virus included here, the most frequently detected virus has been HAdV (17–48 %), followed by RoV (1.5–44 %), NoV (0.5–6.7 %), EV (0.9 %) and HEV (0.8 %) (de Lambert et al., 2021; Pang et al., 2021; Race et al., 2024; Rusiñol et al., 2020a; Sorensen et al., 2021; Stokdyk et al., 2020). Beside the different LoD of their respective qPCR assays, their prevalences are high in urban groundwater environments, where very often sewer exfiltration occurs. Nevertheless, the current enteric virus detection frequency is highly variable. Mediterranean climate change projections, considering scenarios with changes in precipitation distribution over the year, indicate that virus dissemination may increase due to increase in floods and prolonged periods of heat and drought (Dupke et al., 2023; Rusiñol et al., 2015). Under a short study time of study (10 sampling campaigns in 7 months), no correlation was observed between precipitation (mm) and virus detection ($p > 0.3$). Extended surveillance periods should be considered in future studies.

To our knowledge this is the first study using passive sampling for virus monitoring in groundwater compartments which may give a new perspective on virus monitoring, increasing the chances of detection and

enabling realistic microbial assessment studies. It is true, that quantifying concentrations of viruses using qPCR could result in higher risk estimates, because not all genome copies will be part of infectious viral particles, but it is still important to understand the vulnerability of an aquifer to potentially persistent viral strains.

The virome analysis detected notable pathogens from diverse viral families, encompassing *Adenoviridae, Astroviridae, Caliciviridae, Herpesviridae, Papillomaviridae, Picornaviridae, Polyomaviridae*, and *Reoviridae*. Despite the small number of reads obtained from groundwater, high percentage of genetic similarities and coverage were reported. Several assignments of human mastadenovirus-F were obtained using TES, being confirmed through qPCR detection of HAdV. NoV and/or EV, as well as HAdV, are commonly used to investigate human fecal pollution originating viral outbreaks. NoV and EV can be identified in 89 % (25/28) and 72 % (18/25) of the studied outbreaks after the intrusion of wastewater into the drinking water sources (Rusiñol, 2023) and their qPCR analysis are included simultaneously in 55 % of the published studies (20/36). Given the challenges in culturing norovirus and the cumbersome methods applied for the analysis of large volumes of water, deep sequencing for viral genome detection stands as a viable alternative.

Under a one-health approach, and because most emerging infectious diseases that affect humans originate from livestock animals and wildlife, it is important to mention the detection of a wide diversity of avian, bovine, cervid, equid, porcine, and rabbit viruses belonging to *Adenoviridae, Astroviridae, Circoviridae* and *Herpesviridae* in the groundwater of the studied aquifer. Unravelling new virus environmental reservoirs will help us understand virus transmission, evolution, and emergence. These findings align with existing literature, wherein a notable abundance of circovirus was identified through alternative TES applied to groundwater samples (Itarte et al., 2021 and 2024). Human associated cycloviruses, previously found in stool samples from gastroenteritis-related studies were also identified in (Fernandez-Cassi et al., 2020; Leitão et al., 2020; Martínez-Puchol et al., 2020). Moreover, river water and wastewater, the main contributors to groundwater pollution in the studied aquifer, contain a wide diversity of papillomavirus and herpesvirus (Itarte et al., 2021; Martínez-Puchol et al., 2020).

The highest number of sequencing reads, belonged to *Baculoviridae, Dicistroviridae, Iflaviridae* and *Nodoviridae* used as bio-insectivide for control of pests in multiple cultivated crops (e.g. citrus, potatoes, maize and greenhouse crops), ornamental crops and honeybee colonies (Carballo et al., 2017; Cuartas-Otálora et al., 2020; Fenibo et al., 2022; Jiang et al., 2023; Yang et al., 2019; Zhou et al., 2023). Those viruses infecting insects have previously been reported in river and reservoir water samples (Rusiñol et al., 2020b). The strategies of biological control utilizing entomopathogenic viruses can diminish the reliance on non-specific and toxic chemical insecticides. Moreover, their environmental distribution, along with their prevalent detection in the groundwater samples tested here, will require future consideration.

All in all, NGS facilitates the detection of low concentration of viruses in groundwater samples, offering a comprehensive insight into the groundwater virome, plenty of plant viruses and bacteriophages. By using viral probe capture sets, targeting thousands of viral species, the sequencing increase target virus relative abundance by orders of magnitude (Kantor and Jiang, 2024; Martínez-Puchol et al., 2020; Tisza et al., 2023). This TES approach applied to groundwater samples seems to be compulsory to study the groundwater virome. The little available literature describing the groundwater virome, has used the VirCap-SeqVERT Capture Panel (Roche, Basel, Switzerland) (Briese et al., 2015), designed to capture sequences from 207 vertebrate viral pathogens (Itarte et al., 2021 and 2024). Few human and animal viruses belonging to *Circoviridae, Papillomaviridae* and *Picornaviridae* viral families were identified using this panel designed in 2015. The panel applied here, and described in 2019, was designed to target 356 viral species known to infect humans (Metsky et al., 2019). Both the database updates, and the increased number of unique probes may have allowed increased virus identification.

Sequencing replicates showed strong positive correlations between relative abundances of each viral family, and particularly between sequencing replicates indicating no need of doing replicate analysis to study the virome. Nevertheless, different viruses may require different strategies for improved capture sequencing and new targeted panels specifically designed for a selection of viral families of interest may increase sequencing depth.

Looking for waterborne viruses is a challenging issue even in sewage samples (Schaeffer et al., 2023). Scientists are proposing customized panels designed to capture a wide diversity of species to increase sequencing depth and facilitating viral discovery (Martínez-Puchol et al., 2024). Groundwater analysis, with expected very low concentrations of viruses, can result in very different results depending on the sample analysed. Using VPS to adsorb the viruses travelling in the groundwater flow during the 10-day sampling period, we expect to reduce differences between samplings. Nevertheless, the few differences observed, considering the target relative abundances, can be attributable to limit of detection of the TES approach. As Kantor and Jiang have recently suggested, the human and animal virus read detection limit may also be due to the presence of off-target sequences that are simultaneously concentrated and/or captured (e.g., plant viruses, insect viruses) (Kantor and Jiang, 2024). While certain authors propose a comparable association between NGS counts and qPCR cycle threshold values (Park et al., 2023), this remains a research gap that requires further exploration in samples of this nature.

Groundwater sequencing is clearly a topic for further investigation. Although groundwater from the Besòs aquifer is currently not utilized for drinking water purposes, its potential inclusion as a future source of water during rain shortage periods hinges upon the mitigation of associated risks. The Metropolitan area of Barcelona, already incorporates sustainable management practices in its plans for urban aquifers because it has been calculated that the Besòs aquifer (annually serving 5.6 hm3), will suffer a 25 % average decrease in recharge during the period 2071–2100 due to reduced precipitation, increased evapotranspiration (due to temperature increases), and surface runoff (AMB 2023). TES applied to understand the changes in groundwater quality over the year and under drought periods, may have implications for local public health practices.

5. Conclusions

To our knowledge this is the first study using passive sampling for virus monitoring and virome characterization of groundwater. Before performing a sampling campaign, it is recommended to characterize the groundwater sampling site and perform a preliminary test of materials and deployment times to determine the best-suited for each location because no single material will necessarily perform well in all scenarios for all viruses of interest. Further research is needed to understand how saturation of VPS membranes with contaminants from isolated pollution events could hinder their ability to capture additional viruses. At the Besòs River aquifer, NY and NC were the best-performing materials with a 10-day deployment period enabling effective virus detection and characterization, comparable to traditional grab sampling and concentration of viral particles using ultrafilter devices. The qPCR and TES approaches applied in this study are suitable for recording the contamination levels of several key viruses of concern, including adenovirus, enterovirus, and norovirus. However, future capture panel approaches applied to the study of the groundwater virome should be tailored to target viruses of particular interest and increase sequencing depth.

Future studies should consider longer periods of groundwater monitoring or multiple VPS at varying borehole depths to better understand the relationships between rainfall and virus spread. If continuous sampling over time is not feasible due to time or budget constraints, a single sampling event may not detect the presence of

viruses. Given the low infectious dose and low concentration of viruses in groundwater, it is crucial to improve time-integrated methods, such as the VPS proposed here. Passive samplers are more cost-effective, easy-to-use, and have a shorter processing time before testing than large-volume sampling of groundwater. This method should facilitate long-term monitoring campaigns and enable small communities or water managers with limited resources to effectively manage their groundwater sources.

CRediT authorship contribution statement

Cristina Mejías-Molina: Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Ignasi Estarlich-Landajo:** Writing – review & editing, Methodology. **Sandra Martínez-Puchol:** Writing – review & editing, Methodology. **Sílvia Bofill-Mas:** Writing – review & editing, Validation, Funding acquisition. **Marta Rusiñol:** Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marta Rusinol reports financial support was provided by European Commission. Marta Rusinol reports administrative support was provided by Government of Catalonia Agency for Administration of University and Research Grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw sequencing data generated during the current study are available in Zenodo under the DOI number 10.5281/zenodo.11444847 and 10.5281/zenodo.11470648.

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Supplementary materials

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Article 4. Mostreig passiu per la vigilància vírica en sistemes de tractament d'aigües residuals basats en la natura

"Passive sampling for viral surveillance in Nature-Based wastewater treatment systems"

Mejías-Molina. C., Valdivia-Carrera C.A., Laguna-Marin, C., Hundesa, A., Bofill-Mas, S., Rusiñol, M.,

Article en procés de revisió en la revista *Journal of environmental Managment.*

Les solucions basades en la natura (NBS), com ara els aiguamolls construïts, s'estan promovent com a alternatives sostenibles i de baix cost als tractaments terciaris convencionals. Tanmateix, la seva eficàcia en l'eliminació de virus és encara poc coneguda. El seguiment de la contaminació vírica és essencial per garantir la qualitat de l'aigua i la salut pública, especialment davant la necessitat creixent de reutilitzar aigües residuals tractades i protegir els aqüífers com a recursos hídrics estratègics per al futur.

En aquest estudi s'ha avaluat l'ús de mostrejadors passius com a eina complementària per al monitoratge de la contaminació vírica en aigües regenerades. Aquesta metodologia temps-integrat, augmenta la sensibilitat de detecció respecte al mostreig puntual. Per validar-ne l'aplicabilitat, es van comparar dues metodologies de mostreig (puntual i passiva) i dues configuracions de NBS: una catifa d'arrels flotant (CW-RM) i un sistema amb carbó actiu o biochar (CW-BC), tots dos tractant l'efluent secundari d'una EDAR urbana.

En conjunt, les NBS van assolir reduccions mitjanes de 2–3 $\log_{10}$ per a HAdV i NoV GII, i superiors a 5 $\log_{10}$ per *a Escherichia coli,* demostrant que aquests sistemes, especialment els que incorporen biochar, poden reduir de manera efectiva la contaminació vírica i contribuir a una gestió més sostenible de l'aigua. Els mostrejadors passius van permetre detectar virus mitjançant PCR digital fins i tot en dies en què els mostrejos puntuals eren negatius, posant de manifest la

seva alta sensibilitat i capacitat d'integració temporal. L'HEV només es va detectar mitjançant mostreig passiu, indicant la seva eficàcia per detectar concentracions víriques baixes.

Passive sampling for viral surveillance in Nature-Based wastewater treatment systems.

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ABSTRACT

The growing scarcity of water, driven by rising demand and climate change, has intensified the need for sustainable and alternative water sources. Water reuse is therefore a vital strategy for enhancing water security and reducing pressure on freshwater supplies. Although nature-based solutions (NBS), such as constructed wetlands, are increasingly being promoted as environmentally friendly alternatives for tertiary treatment, their effectiveness in removing viruses remains poorly understood. Monitoring viral contamination is crucial for safeguarding water quality and public health, especially as the importance of reclaimed wastewater and aquifers as managed water sources increases.

Passive samplers have emerged as a sustainable tool for viral monitoring, offering sensitive, time-integrative detection. This study aimed to assess their applicability as a complementary surveillance tool in reclaimed wastewater. To this end, two sampling approaches (grab sampling and passive sampling) were evaluated across two NBS configurations: a floating root mat (CW-RM) and a system combining root mats with biochar (CW-BC). Both configurations treated secondary effluent from an urban wastewater treatment plant. Viral pathogens (human adenovirus (HAdV), norovirus genogroup II (NoV GII) and hepatitis E virus (HEV)) and *Escherichia coli* (*E. coli*) were monitored to evaluate the $\log_{10}$ reduction values.

Passive samplers detected viruses on days when grab samples were negative, demonstrating their higher sensitivity and time-integrative capacity. Notably, HEV was only detected via passive sampling, demonstrating the ability to detect low concentrations of viruses. Overall, the NBS achieved average reductions of 2–3 $\log_{10}$ for HAdV and NoV GII, and of over 5 $\log_{10}$ for *E. coli*. Biochar-based wetlands showed the highest viral removal efficiency. These results suggest that NBS can effectively reduce viral contamination while providing an environmentally sustainable form of tertiary treatment. Passive samplers proved to be a cost-effective and practical monitoring tool.

1. INTRODUCTION

Growing water scarcity, exacerbated by climate change and rising demand, poses a threat to water security worldwide. Nearly one-third of the global population currently lacks access to clean water. In Europe, extreme droughts affecting up to 90% of irrigated areas in severe years highlight the importance of finding alternative sources of water. 2024 was the warmest year on record for the continent, with severe droughts reported in the eastern and Mediterranean regions (WMO & C3S, 2025). Between 2000 and 2023, an average of 68% of EU irrigated areas were affected by drought, reaching 90% in extreme years (EEA, 2025). These trends highlight the importance of diversifying water sources while ensuring their sustainability and quality.

Reclaimed water, derived from domestic, industrial and agricultural activities, is increasingly being promoted as a solution alongside the protection of aquifers as managed reservoirs. However, as water reuse expands, ensuring microbiological safety becomes paramount, necessitating robust monitoring systems that can identify potential health risks and emerging pathogens, as well as evaluate treatment performance.

Reclaimed water contains complex mixtures of chemical and biological contaminants, including pathogenic viruses. Viral indicators such as human adenoviruses (HAdV) and JC polyomavirus (JCPyV) persist in treated effluents, even after secondary and tertiary treatment, and are continuously released into the environment (Rusiñol et al., 2015). Human pathogenic viruses, including noroviruses (NoV), rotaviruses (RoV) and enteroviruses (EV), are of particular concern due to their low infectious dose and ability to persist in the environment (Espinosa et al., 2008; Girón-Guzmán et al., 2025; Seitz et al., 2011). This emphasises the importance of having sensitive monitoring tools that can detect viral pathogens at low concentrations, since conventional microbiological indicators may not accurately reflect viral contamination.

To minimise microbiological risk, wastewater treatment plants (WWTPs) should implement advanced tertiary processes, such as microfiltration and ultrafiltration, to target the removal of

viruses. Enhanced surveillance is also necessary to detect emerging pathogens. However, these technologies require substantial technical expertise and strict operation, as well as a high initial investment, posing challenges in low-resource settings. Against this backdrop, nature-based solutions (NBS), such as constructed wetlands, have gained renewed attention as sustainable, energy-efficient alternatives to conventional tertiary treatments (G. Chen et al., 2024).

CWs are widely used as an additional treatment step for treated effluent due to their low energy requirements, ease of operation and cost-effectiveness (Beharrell, 2004). They are commonly used in low- and middle-income countries where conventional infrastructure is limited (Mahmood et al., 2013; Makopondo et al., 2020). The mechanisms operating in CWs, including adsorption, sedimentation, generation of reactive oxygen species, and predation by the microbial community, have been linked to the removal of faecal indicator bacteria(Kadlec & Wallace, 2008). However, evidence for viral removal is comparatively limited and inconsistent, raising questions about the ability of CWs alone to ensure virus-safe reclaimed water (Gerba et al., 2013). Beyond assessing removal efficiency, it is crucial to determine whether enteric viruses persist in reclaimed water, as their presence is one of the most significant public health concerns associated with water reuse (ATSE, 2013). Therefore, understanding the occurrence and behaviour of these viruses within CWs is crucial to ensure that such systems provide effective treatment and microbiologically safe effluents.

Effective water reuse policies require improved treatment strategies and reliable pathogen surveillance for their safe implementation. Current regulatory frameworks often rely on bacterial or viral surrogate indicators yet fail to set explicit limits for many human viral pathogens. Spain's discharge rules, for example, set limits for conventional parameters such as BOD, COD and TSS, but not for microbiological parameters (Royal Decree 509/1996, transposing Directive 91/271/EEC). Current regulatory frameworks in Spain set limits for conventional indicators such as BOD, COD, and *E. coli*, yet human viral pathogens are not explicitly regulated, underscoring the need for scalable viral surveillance (Directive (EU) 2020/741).

In this context, monitoring tools such as passive samplers (PS) are emerging as an essential complement to treatment processes, offering a practical, cost-effective and time-efficient approach to addressing knowledge gaps. PSs continuously capture pathogens throughout the sampling period, concentrating low or transient viral signals that are often missed by grab samples (Bivins et al., 2022; Mejías-Molina et al., 2023). PS can also be deployed at multiple points along a water sanitation system or in hard-to-reach locations (Mejías-Molina et al., 2024; Yapi et al., 2025). Recent reviews and field studies have demonstrated that PSs made from materials such as gauze, tampons, electronegative membranes, polymer membranes and granular activated carbon can achieve detection sensitivities that are comparable to or higher than those of grab and

composite sampling. This is particularly true in locations with low or variable viral loads (Hayes & Gagnon, 2024; Karamati N. et al., 2024). However, translating sampler load into traditional volumetric concentrations and determining optimal deployment duration remains challenging.

This study evaluates passive samplers for detecting human viral pathogens in treated wastewater, assesses the viral removal efficiency of a constructed wetland, and compares nature-based and conventional tertiary treatment strategies in terms of their compatibility with viral monitoring.

2. MATERIALS AND METHODS

2.1. Experimental set-up

The NBS evaluated in this study was supplied with water from the secondary treatment outlet of a WWTP that treats approximately 72.600 m3/day of raw sewage from around 248.261 inhabitants. Two NBS configurations constructed wetlands with floating root mat (CW-RM) and CW with RM and biochar (CW-BC) were tested. The design of these systems was adapted from the work of (Laguna-Marín et al., 2025) who demonstrated in bench-scale experiments that the integration of vegetation and biochar significantly enhances the removal of contaminants of emerging concern. Moreover, it is well studied that floating root mats provide extensive root surface area that promotes microbial activity and pollutant uptake, while biochar enhances adsorption capacity and microbial colonization due to its high porosity and surface area (Ahmad et al., 2014; Headley & Tanner, 2012). Each NBS had a surface area of 12 m^2 and a water depth of 0.3 m and operated at a flow rate of 400 mm/day, receiving effluent from the secondary treatment on a continuous basis.

2.2. Sampling strategy

A total of 60 samples were collected, using two sampling strategies, during five sampling campaigns conducted between May and July 2025. For each sampling campaign, 500 ml grab samples were collected from the CW inlet (secondary treatment effluent from the WWTP), and from the two CW outlets (see Section 2.1). Additionally, 500 ml sample of raw wastewater were collected in each sampling event from the WWTP inlet.

In parallel, virus monitoring was also performed using PS, according to the design outlined by (Schang et al., 2021). These 3D-printed devices contained three electronegative membranes each and were deployed for seven consecutive days at the NBS inlet and outlets. At the WWTP inlet, PS were deployed for 24 hours, following the protocol of Mejías-Molina 2023. Grab samples were collected in parallel on the same day that the PS were retrieved, thereby ensuring direct comparability between the two sampling approaches.

2.3. Concentration and elution of viruses and extraction of nucleic acids

Grab and PS samples were handled using protective equipment (lab coats, goggles and gloves), collected in sterile containers (sealable plastic bags or 500 ml bottles respectively) and transported in a cooler to the laboratory for processing within 2 hours.

Viral particle concentration from water (grab samples), was performed using ultrafiltration. For the raw wastewater samples, debris was first removed by centrifuging 100 ml of the 250 ml total sample at 4,750 *g* for 30 minutes. The resulting 80 ml supernatant were ultrafiltered using an automatic concentration pipette (CP-Select™) with 150 kDa tips (Innovaprep) to a final volume of 300 µl as previously described (Mejías-Molina et al., 2023). Samples collected from the NBS inlet and outlets (250 ml) were concentrated using the same concentration pipette system into a final volume of 300 µl. Viral nucleic acids (NA) were extracted with a QIAamp Viral RNA Mini Kit on a QIAcube automated system (Qiagen). A 140 µl aliquot of the concentrated samples was processed, yielding an elution volume of 70 µl.

Nitrocellulose membranes from the PS were processed following the Mejías-Molina et al. (Mejías-Molina et al., 2024) protocol, with slight modifications. Each membrane was placed in a PowerBead Tube containing 0.1 mm glass beads (Qiagen) and 700 µl of 0.25 N glycine buffer (pH 9.5). Bead beating was performed at 4 m s^{-1} for 30 s using a FastPrep-24™ homogeniser, followed by centrifugation at 14,000 *g* for 1 min. For each PS, the supernatants from the three membranes were collected and pooled (420 µl in total per PS) and used for NA extraction with the QIAamp Viral RNA Mini Kit (Qiagen), following the manufacturer's instructions, with a final elution volume of 70 µl.

2.4. Digital PCR-Based Quantification of Viruses, *E. coli,* and *Cryptosporidium spp.*

Specific digital PCR (dPCR) assays were employed to quantify three viral pathogens: human adenoviruses (HAdV), norovirus genogroup II (NoV GII), and hepatitis E virus (HEV), as well as *E.coli* and *Cryptosporidium* spp., using primer and probe sets described previously (Chern et al., 2011; Garson et al., 2012; Hernroth et al., 2002; Hundesa et al., 2006; Jothikumar et al., 2006; Kageyama et al., 2003; Loisy et al., 2005; Mary et al., 2013) A complete list of primers and probes and their final concentrations at the dPCR is provided in the supplementary information 1.

Reactions were performed on the Absolute Q™ Digital PCR System (Thermo Fisher Scientific). The dPCR 16-well nanoplates with a partition capacity of 22,000 partitions were used for dPCR tests. For DNA targets (HAdV, *E.coli, Cryptosporidium spp.*), reactions were prepared using Absolute Q™ Universal DNA dPCR Master Mix (5X) whereas RNA virus reactions (NoV GII and HEV) were performed with Absolute Q™ 1-step RT-dPCR Master Mix (4X).

Thermal cycling conditions were as follows: an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s, with an additional reverse transcription step at 50 °C for 10 min. The annealing/extension temperatures were optimized for each target: 57 °C for HAdV and NoV GII, 60 °C for HEV, and 55 °C for *E. coli* and *Cryptosporidium*, based on experimental calibration. For HEV and NoV GII a step of retrotranscription during 50 °C for 10 min was added. Each assay included a negative control (nuclease-free water) and a positive control consisting of an artificial sample spiked with a synthetic gBlock gene fragment (Integrated DNA Technologies, IDT) containing the corresponding target sequence. Data were analysed using the Absolute Q™ Analysis Software (Thermo Fisher Scientific). In accordance with the WHO manual (FAO, 2015), the limits of detection (LODs) for the passive samplers were calculated as 53 GC, 604 GC, and 88 GC per sampler for HEV, NoV GII, and HAdV, respectively.

2.5. E.coli quantification using MUG plates

One hundred millilitres of each grab sample collected as described in Section 2.3 were used for the enumeration of *E. coli* using the 96-well microplate system (MUG/EC 355-3782, Bio-Rad®) in accordance with ISO 9308-2:2012 (International Organization for Standardization, 2012). This method allows quantitative detection of *E. coli* based on the hydrolysis of the MUG substrate by the enzyme β-D-glucuronidase, producing a fluorescent signal that can be measured for identification.

2.6. Statistical analyses

To assess the semi-quantitative performance of the passive samplers, viral concentrations obtained from PS (expressed as GC/passive sampler) were compared with those from grab samples collected on the same sampling days (expressed as GC/L). All data were $\log_{10}$-transformed prior to analysis Linear regression analysis was performed using Microsoft Excel and the coefficient of determination (R^2) was calculated to evaluate the strength of the relationship between both sampling approaches for each target virus.

2.7. Literature review

A literature review was conducted to identify studies reporting the reduction of human pathogenic viruses in wastewater treatment systems, with a particular focus on nature-based and sustainable tertiary treatments. The bibliographic search was carried out using scientific databases and search platforms (Web of Science, Google Scholar, Connected papers) and included combinations of the following keywords using booleans AND and OR for “nature-based solutions”, “viruses”,

"reclaimed water", "water reuse", "filtration", ultrafiltration", "reverse osmosis", "wetland", "lagooning", "reduction", "tertiary treatment", "removal" and NOT for "lab-scale", "spiked", "bacteriophages".

The initial search results were screened by title and abstract to identify potentially relevant studies. Full-text articles were then evaluated according to predefined inclusion and exclusion criteria. Only studies providing quantitative data on the removal or log reduction values (LRV) of human pathogenic viruses were included. Publications that reported solely on bacteriophages or other viral surrogates were excluded. Likewise, studies lacking quantitative results or those performed exclusively under laboratory-scale conditions (i.e., not at pilot or full scale) were not considered.

3. RESULTS AND DISCUSSION

3.1. Insights from the literature on viral pathogen removal in sustainable treatment systems

A total of 20 studies addressing the removal of human viruses in tertiary wastewater treatment systems were initially identified through a literature search. Of these, only six were selected for inclusion as they were the only ones with comparable methodology (PCR-based assays) and reporting of viral concentrations before and after treatment (Bailey et al., 2018; Rachmadi et al., 2016; Rusiñol et al., 2015, 2020; Simmons et al., 2011; Zhou et al., 2015) . It is worth noting that very few studies provide actual virus concentrations, which limits the possibility of directly comparing viral removal efficiencies across systems.

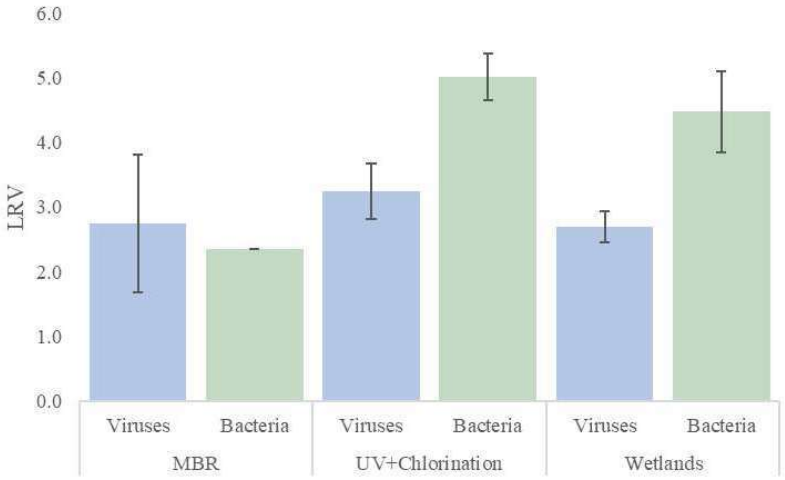


Figure 1. Summary of $\log_{10}$ reduction values (LRVs) for viruses and bacteria across three treatment approaches: membrane bioreactor (MBR), UV combined with chlorination, and constructed wetlands. Values represent aggregated data from multiple published studies.

The selected studies focused on three treatment technologies with comparable objectives and analytical approaches: constructed wetlands (CWs), ultraviolet (UV) disinfection combined with chlorination, and membrane bioreactors (MBRs) (see **Fig. 1**). To facilitate comparison, viral and bacterial data were aggregated within each treatment category, as differences in the microorganisms studied across publications precluded pathogen-specific analyses. Rather than evaluating disinfection efficiency alone, this comparative analysis aimed to assess the performance of each treatment strategy in terms of microbial and viral removal and monitoring capability.

The compiled data reveals clear differences in log reduction values (LRVs) between bacteria and viruses across the three treatment approaches. UV combined with chlorination achieves the highest bacterial removal, with average reductions of around 5 log units. In contrast, MBR and wetlands achieve lower, albeit comparable, reductions. LRVs for viruses are consistently lower across all technologies, ranging between approximately 2 and 3 log units. This pattern aligns with previous evidence indicating that viruses are generally more resistant to inactivation processes than bacteria (Gerba et al., 2013; Kong et al., 2021).

This variability also reflects differences in detection methods: bacterial indicators such as E. coli are typically quantified via culture-based MPN methods, whereas viral concentrations are mostly determined by qPCR, which detects total viral genomes without distinguishing infectious from non-infectious particles. Consequently, viral LRVs may underestimate actual removal of infectious viruses. Differences in operational conditions (e.g., retention time, dose, environmental factors) and intrinsic microbial characteristics (capsid stability, size, resistance) further contribute to heterogeneity in performance within the same treatment type (Kadoya et al., 2022; Lin et al., 2020; Oishi et al., 2022; Rachmadi et al., 2018). In contrast, bacterial indicators tend to respond more uniformly, leading to higher and less variable LRVs (Worley-Morse et al., 2019).

When compared to the complied literature, the system evaluated in this study achieved similar LRVs for HAdV and NoV GII but higher for *E.coli*. This confirms the robust bacterial removal and stable, moderate viral reduction achieved by wetlands. When the system is compared to systems that combined UV with chlorination, it shows slightly lower LRVs, particularly for viral targets. UV combined with chlorination is one of the most common advanced disinfection processes, and it has a high disinfection efficacy specially for bacterial indicators, where $\log_{10}$ reductions around 5 are frequently reported (X. Chen et al., 2022; H. Wang et al., 2020; L. Wang et al., 2021; Wu et al., 2021). However, chlorination processes can lead to undesirable side effects, including the formation of disinfection by-products (DBPs), increased energy demands, and higher maintenance costs (Ghernaout & Elboughdiri, 2020; Jasper et al., 2017; Khajouei et al., 2022). On the other hand, wetlands, and particularly NBS offer a passive, low-energy, and environmentally sustainable alternative, capable of reducing microbial contaminants without generating harmful by-products.

Research on virus reduction across tertiary treatment systems reveals significant variability in system design, analytical approaches, and reporting practices (see supplementary material). CWs are known to reduce microorganisms, primarily fecal indicator bacteria, through combined physicochemical and biological processes (Kadlec & Wallace, 2008). However, evidence for virus removal is limited and largely focuses on coliphages (Gerba et al., 2013).

Overall, this synthesis underscores the potential of CWs as a sustainable solution for improving microbial quality in WWTP effluents. Despite variability, LRVs for bacterial and viral indicators indicate effective pathogen reduction while avoiding the environmental and operational drawbacks of energy-intensive technologies. The lack of standardization in target microorganisms, analytical methods, and reporting formats remains a major limitation for establishing regulatory benchmarks. Integrating CWs with systematic monitoring of reclaimed water could enhance risk management by enabling early viral detection and improving water reuse safety. Harmonizing monitoring protocols, particularly for viral pathogens, will be critical to fully assess, optimize, and integrate CWs into future water reuse strategies.

3.2. Efficiency of Constructed Wetlands in Reducing Viral and Bacterial Contaminants

Figure 2 summarises the $\log_{10}$ reduction values (LRVs) obtained for the three microbial targets in the two constructed wetland configurations (CW-RM and CW-BC). Overall, the patterns observed reflect the broader trends identified in the literature: viruses consistently show lower and more variable removal than bacterial indicators

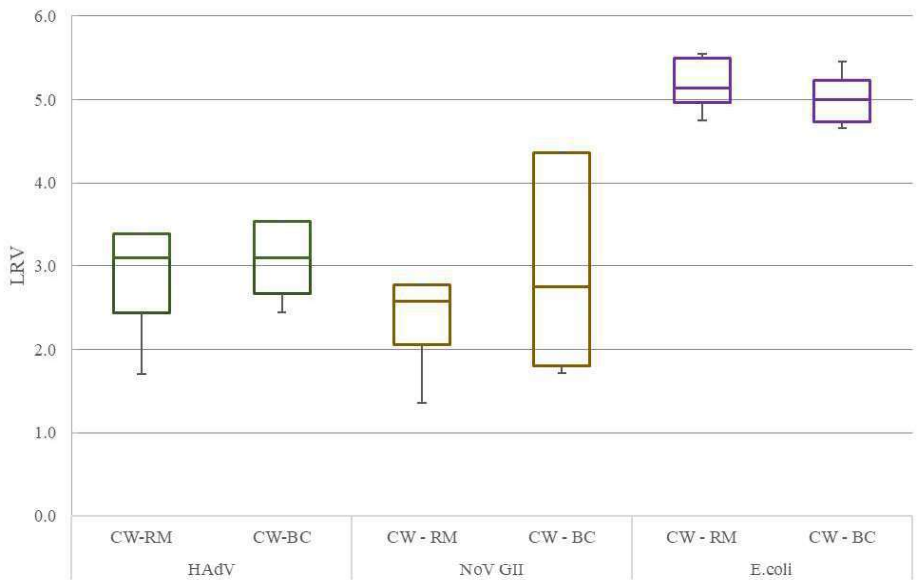


Figure 2. $\log_{10}$ reduction values of human adenovirus (HAdV), norovirus GII and *Escherichia coli* in two constructed wetland configurations: CW-RM (constructed wetlands with reactive media) and CW-BC (constructed wetlands with biochar).

For HAdV, both configurations exhibited stable and comparable removal, with LRVs between 2 and 3.5 $\log_{10}$. The similar medians and interquartile ranges suggest that the addition of biochar did not substantially influence HAdV reduction, indicating a robust and configuration-independent performance for this virus.

In contrast, NoV GII showed greater variability, particularly in CW-BC, where LRVs ranged widely. This heterogeneity suggests that NoV removal may be more sensitive to operational conditions or environmental factors a behaviour consistent with previous observations that different viruses respond differently under identical treatment conditions (Kitajima et al., 2014). Temperature and biological activity, for example, can strongly influence viral degradation, yet certain viruses remain highly resistant to environmental fluctuations (Rachmadi et al., 2016a). HAdV is known for its persistence and resistance to UV radiation and for exhibiting long survival in water (Bofill-Mas et al., 2006; Linden et al., 2007; Rigotto et al., 2011; Thurston-Enriquez et al., 2003)

As expected, *E. coli* showed the highest LRVs, with median removals above 5 $\log_{10}$ and low variability across configurations. This strong reduction reflects the well-established greater susceptibility of bacterial indicators to removal processes compared with enteric viruses (Haas et al., 2014). However, this also highlights a critical limitation of relying solely on E. coli as a regulatory indicator: its behaviour does not necessarily mirror that of viral pathogens. While viruses in this study achieved only 2–3 $\log_{10}$ reductions, E. coli removals exceeded regulatory demands, potentially creating a misleading impression of overall microbiological safety. These observations reinforce the need to incorporate viral indicators into monitoring frameworks for nature-based systems.

Reported removal efficiencies in CWs generally align with our findings, typically achieving reductions of 2–4 $\log_{10}$ for enteric viruses and 3–5 $\log_{10}$ for bacterial indicators, depending on the configuration and hydraulic loading (Sim(Bailey et al., 2018; Rachmadi et al., 2016b; Rusiñol et al., 2015; Simmons et al., 2011; Zhou et al., 2015)mons et al., 2011; Rachmadi et al., 2016; Rusiñol et al., 2015; Bailey et al., 2018; Zou et al., 2015). The observed virus LRVs (2–4 $\log_{10}$) remain below the recommended reductions of 6–7 $\log_{10}$ in WHO and EU risk-based frameworks for unrestricted irrigation. While this level of performance may be acceptable for restricted irrigation, it is insufficient for high-exposure pathways. This discrepancy highlights the importance of integrating viral indicators into NBS monitoring frameworks, given that bacterial indicators tend to overestimate microbiological safety in relation to viral pathogens.

3.3. Passive sampling (PS) for virus monitoring in reclaimed water systems.

To complement the evaluation of treatment performance, this section examines the suitability of passive sampling (PS) as a tool for monitoring viruses in reclaimed water systems. Figure 3 summarises the detection frequencies of HAdV, NoV GII and HEV obtained using grab sampling and PS across the different sampling points, showing where each method has monitoring advantages.

Cryptosporidium spp. and *E. coli* remained undetected in PS using dPCR. This is consistent with previous studies demonstrating that bacterial recovery from PS membranes is generally poor due to their larger size, lower diffusivity, and greater susceptibility to hydrodynamic drag, all of which reduce the likelihood of cell-surface interactions (Liu et al., 2025). In contrast, viruses, smaller and more diffusive, are more efficiently transported to and adsorbed onto membrane surfaces (Margalit et al., 2013; Miller, 1924).

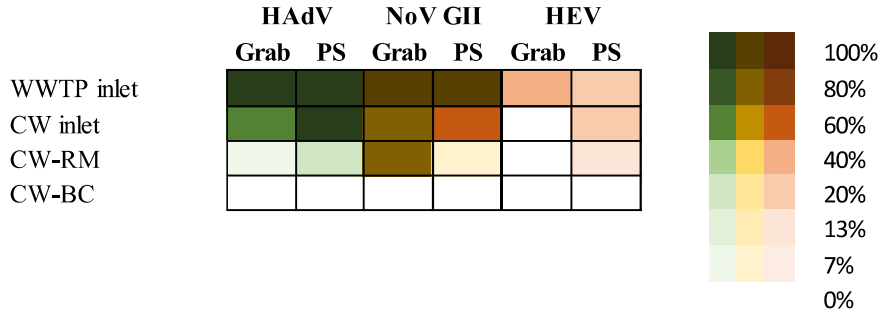


Figure 3. Heatmap representing detection frequencies for HAdV, NoV GII, HEV when using the two sampling methodologies: Grab and passive sampling (PS) at the different sampling points (WWTP inlet, CW: constructed wetland, RM: root mat, BC: biochar)

At the WWTP inlet, both grab sampling and PS achieved 100% detection of HAdV and NoV GII, reflecting the high viral loads typically found in untreated wastewater. HEV detection was higher with grab sampling than PS (40% versus 20%). This is consistent with reports that passive samplers underperform in highly turbid or particle-rich waters. In such conditions, viruses remain strongly adsorbed to suspended solids, making them less available for membrane (Mejías-Molina et al., 2023; Simmons et al., 2011). Conversely, downstream of the pretreatment stage, PS outperformed grab sampling, particularly with regard to HAdV and HEV. This is in line with observations in other tertiary systems, such as those involving UV-chlorination and MBRs, where PS enhances the detection of low-level or intermittent viral contamination (Bailey et al., 2018; Zhou et al., 2015).

Downstream of the pretreatment stage (CW inlet and outlets), PS outperformed grab sampling for HAdV and HEV. This was most evident at the CW-RM outlet, where HEV was detected exclusively by PS. This behaviour reflects the intrinsic time-integrative nature of PS, which accumulates viruses continuously and thus enhances the detection of low-level or intermittent contamination that would be missed by instantaneous grab samples (Li et al., 2024;Kostoglou et al., 2022) or the co-concentration of PCR inhibitors during PS elution (Hayes et al., 2023), cannot

be excluded. Controlled spiking and inhibition assays may be necessary to distinguish true removal from artefacts of the analysis.

As PS does not permit estimation of the sampled water volume, viral concentrations cannot be expressed as gc/mL; therefore, LRVs were calculated exclusively from grab samples. However, paired measurements (**Fig. 4**) showed that genome copy numbers quantified by dPCR from PS extracts tracked relative changes in waterborne viral loads. HAdV showed a strong correlation (R^2 = 0.79), while NoV GII showed a moderate correlation (R^2 = 0.56). These findings are supported by multiple studies reporting agreement between PS and composite (Bivins et al., 2022b; Mejías-Molina et al., 2023) and confirm that PS can provide semi-quantitative insights into viral trends.

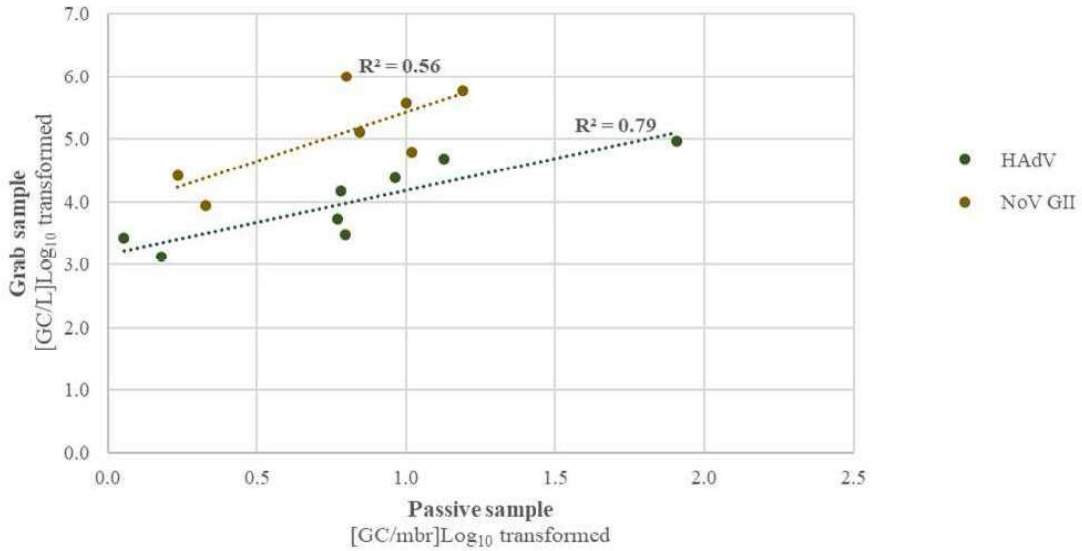


Figure 4. Comparison of HAdV and NoV genome copies measured in composite samples versus passive samplers (PS). Both axes are log_{10}-transformed. Dotted lines show linear regression with corresponding R^2 values.

PS's ability to detect viruses on days when grab samples test negative further demonstrates its value as a complementary monitoring tool. This is particularly advantageous in settings with limited laboratory capacity where the use of ultrafiltration equipment and routine grab sample processing may be impractical. This reinforces the importance of PS in supporting the validation and long-term monitoring of nature-based treatment systems.

4. CONCLUSIONS

This study demonstrates that passive samplers (PS) can function as both qualitative detectors and robust semi-quantitative tools for monitoring viral pathogens in nature-based solutions (NBS). The strong correlation observed between PS and grab sampling for human adenoviruses (HAdV) suggests that the accumulation of viruses on sampler membranes accurately reflects changes in the number of viruses in the water over time. Furthermore, the exclusive detection of hepatitis E virus (HEV) in treated effluents using PS highlights the ability of time-integrated sampling to identify low or intermittent viral signals, which are frequently overlooked by conventional grab sampling.

Constructed wetlands, particularly those incorporating biochar, showed consistent viral and bacterial removal, achieving reductions in pathogens comparable to those of more technologically intensive and costly tertiary treatments. These results reinforce the potential of PS as a cost-effective, scalable, and operationally simple surveillance strategy for reclaimed water systems. Integrating digital PCR (dPCR) further enhances analytical sensitivity and quantification reliability, supporting the early detection of pathogens and enabling informed decision-making to protect public health in water reuse applications.

HIGHLIGHTS:

- PS enables semi-quantitative viral monitoring in NBS.
- HAdV shows a strong correlation between PS and grab sampling.
- HEV is only detected with PS in treated effluents.
- Biochar-CWs achieve pathogen removal comparable to that of costly systems.
- PS combined with dPCR can detect viruses in reclaimed water at very low levels.

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DISCUSSIÓ

Els quatre articles que integren aquesta tesi han permès avaluar el mostreig passiu com a eina per millorar la detecció i caracterització de virus en diferents tipus d'aigua en el marc de la vigilància epidemiològica ambiental.

Validació i aplicació del mostreig passiu en aigües residuals

Com ja s'ha comentat a la introducció, existeixen 3 grans tipus de mostreig a realitzar quan es vol detectar virus en mostres d'aigua: mostreig puntual (en el temps i en el volum de mostra), mostreig compost (segons el temps o segons el flux d'aigua) i mostreig passiu. En aquesta tesi, tal i com s'explica a l'article *"Efectivitat del mostreig passiu per a la detecció i caracterització genètica de virus humans en aigua residual*", es va avaluar l'estratègia del mostreig passiu en comparació amb el mostreig compost per a la detecció i caracterització genètica de virus en aigua residual. En primer lloc es va avaluar la necessitat d'utilitzar una o dues membranes d'adsorció dins d'un mateix mostrejador passiu per detectar dos indicadors vírics de contaminació fecal humana (JCPyV i HAdV) i un patògen (SARS-CoV-2). Es van comparar els resultats obtinguts a partir de rèpliques de mostres compostes d'una EDAR urbana amb els resultats de cada membrana de dins d'un mostrejador. Mentre que els resultats van mostrar una alta correlació entre rèpliques de mostres compostes (coeficient de Pearson de 0,76, 0,61 i 0,96 per JCPyV, HAdV i SARS-CoV-2, respectivament), la correlació entre membranes va ser molt baixa (<0,33), amb més variabilitat, probablement causada per la distribució irregular de l'aigua dins del torpede, per la presència de materials interferents o per la disposició irregular de les membranes. Per aquest motiu, es va optar per analitzar almenys dues membranes (2 rèpliques) per a cada mostrejador/mostra.

Al comparar vuit dianes víriques (N1, N2, JCPyV, HAdV, EV, RoV, NoV GI i NoV GII) en mostres compostes i en mostreig passiu en dos escenaris: una EDAR urbana gran (2,8 milions d'habitants equivalents) i una residència d'avis (amb 300 residents i 200 persones treballadores), en un total de 60 mostres

analitzades (gener–març 2022), es va observar que mentre que a l'EDAR, les mostres compostes van ser positives per a tots els virus, els mostrejadors passius van mostrar percentatges de detecció d'entre el 20% i el 73%. Els dos indicadors de contaminació fecal humana (HAdV i JCPyV) van presentar un 100% de positivitat amb ambdós mètodes, però la detecció de les altres dianes víriques va ser notablement inferior amb mostreig passiu en comparació amb el 100% de les mostres composades. Una possible explicació és que els nivells elevats de sòlids en suspensió a les aigües residuals urbanes poden afectar l'adsorció dels virus a les membranes electronegatives. De fet, l'any 2021, Hayes i col·laboradors ja van demostrar que l'adsorció dels virus al material de mostreig depèn en gran mesura de les característiques de l'aigua (Hayes et al., 2021), i, en concret, que l'adsorció de sòlids en suspensió pot millorar la recuperació d'ARN. Tanmateix, una retenció excessiva de sòlids podria també inhibir el procés d'extracció. Uns altres fenòmens que explicarien les diferències de rendiment del mostreig passiu vers al mostreig composat, son la temperatura, el pH o la concentració de sòlids dissolts (Li et al., 2021). A més, diferents virus poden presentar patrons de detecció diferenciat tot i ser adsorbits i concentrats amb el mateix mètode ja que presenten propietats físiques diferents. Li i col·laboradors (Li et al., 2022) van suggerir que les diferències estructurals entre virus no embolcallats (EV, HAdV, RoV, NoV) i embolcallats (SARS-CoV-2) podrien influir en el seu comportament pel que fa a l'afinitat dels virus per les membranes o en el procés de recuperació. Altres estudis també plantegen la hipòtesi que hi ha una baixa recuperació viral quan el temps d'exposició dels mostrejadors a l'aigua d'estudi és massa curt (Kevill et al., 2022; Li et al., 2021) i que una exposició de més de 24h pot superar la recuperació viral en comparació al mostreig tradicional (Wilson et al., 2022).

El mostreig passiu, però, ofereix resultats sensibles i consistents quan s'analitza l'aigua residual produïda per poblacions petites, com la residència de gent gran, on els resultats obtinguts emprant aquest tipus de mostreig i el

mostreig compost son equivalents: el virus detectat amb més freqüència va ser JCPyV, seguit de SARS-CoV-2 (N1 i N2) i HAdV, mentre que EV, NoVs i RoV van ser detectats en menor freqüència. Les deteccions negatives per a SARS-CoV-2 mitjançant mostreig passiu van correspondre a mostres recollides durant un període de baixa prevalença d'infeccions a la comunitat assistida (Pico-Tomàs et al., 2023), fet que suggereix una capacitat de detecció lleugerament inferior a la del mostreig compost en condicions de baixa circulació vírica.

A més de la detecció per qPCR, es va explorar l'ús d'aquest tipus de mostrejadors de baix cost per caracteritzar el viroma d'aigües residuals mitjançant seqüenciació massiva amb previ enriquiment de certes famílies de virus (Vir-CapSeq-VERT). Aquesta aproximació va permetre seqüenciar els principals patògens humans excretats: *Adenoviridae*, *Astroviridae*, *Calciviridae*, *Hepeviridae, Parvoviridae, Papillomaviridae*, *Picornaviridae*, *Polyomaviridae* i *Reoviridae* (Rusiñol & Girones, 2019). Aquestes famílies virals formen part del viroma de l'aigua residual descrit abans de la pandèmia del 2020 (Adriaenssens et al., 2021; Martínez-Puchol et al., 2020). Més recentment, també s'han descrit com a habituals en aigües residuals virus respiratoris com els pertanyents a les famílies *Coronaviridae* i *Orthomyxoviridae* (Martínez-Puchol et al., 2021a). En concordància amb aquests estudis, les espècies virals membres de la família *Picornaviridae,* de les més abundants en l'aigua residual, es van detectar tant amb el mostreig compost com amb els mostrejadors passius. Altres famílies menys abundants a les mostres compostes, com *Reoviridae* i *Hepeviridae,* es van poder seqüenciar fàcilment amb el mostreig passiu. Encara que el nombre de lectures de seqüenciació obtingudes amb mostreig passiu va ser més baix que amb mostres compostes, la majoria de les famílies virals presents en el viroma de l'aigua residual també van ser trobades amb aquest tipus de mostreig.

Aquesta estratègia permet detectar i caracteritzar de manera fiable les principals famílies virals presents en l'aigua residual. Això és útil especialment en aquells contextos on les metodologies convencionals són difícils d'implementar per limitacions logístiques o econòmiques. És en aquests contextos on l'ús de mostrejadors passius és rellevant i pot ser una eina útil per entendre la circulació de virus en comunitats concretes i detectar la introducció de virus emergents i possibles nous patrons de transmissió de virus SARS-CoV-2 i Influenza (Gouthro et al., 2025; Yapi et al., 2025).

A partir d'aquesta base metodològica, es va aplicar el mostreig passiu a escala d'edifici per caracteritzar el viroma en escoles, universitats i residències d'avis, entorns amb perfils demogràfics i nivells de vulnerabilitat diferenciats. Els resultats d'aquest estudi es troben recollits a *l'article "L'epidemiologia basada en aigües residuals aplicada a nivell edifici revela perfils diferents en el viroma basat en l'edat dels individus que contribueixen."* El viroma obtingut a l'EDAR, representativa del conjunt de la població, va mostrar un perfil víric coherent amb el descrit en estudis previs que empraven la mateixa metodologia de seqüenciació dirigida però a partir de mostres compostes (Martínez-Puchol et al., 2021b). El nombre més elevat de lectures va correspondre a la família *Astroviridae,* seguida de bocavirus humà (*Parvoviridae*), adenovirus espècie F i poliomavirus humans, mentre que la família *Papillomaviridae* va presentar la major diversitat d'espècies. En total, es van detectar 37 espècies víriques, 18 de les quals només a l'EDAR i no a escala d'edifici, probablement per la seva baixa concentració o circulació restringida en un petit nombre d'individus.

La variació en l'abundància relativa de determinades espècies virals en funció del tipus d'edifici evidencia el potencial d'aquesta estratègia de mostreig per identificar patrons de circulació vírica diferenciats, reflectint les característiques epidemiològiques pròpies de cada grup demogràfic. Així, els

astrovirus, especialment MAstV-1, van ser predominants a les escoles; a les universitats destacaven salivirus i poliomavirus humans JC i BK; mentre que a les residències d'avis hi havia una major presència de papil·lomavirus, picornavirus i alguns virus associats a immunosupressió, com MCPyV i CMV. Aquestes resultats són coherents amb el coneixement actual sobre la prevalença d'infeccions agudes en poblacions infantils i la major presència de virus persistents en persones grans o immunocompromeses (Greninger et al., 2009; Kamminga et al., 2018; Ng et al., 2016; Quiroga-Garza et al., 2013; Vu et al., 2019; Yu et al., 2015), i suggereixen que el viroma present a les aigües residuals humanes constitueix un mosaic de virus provinents de diverses fonts. Alguns virus poden provenir-ne en individus de diferents grups d'edat, mentre que d'altres poden ser específics de determinades poblacions. Per exemple, els virus associats a infeccions agudes que provoquen brots limitats, són excretats amb més freqüència per infants que per la població adulta, i aquests virus són habitualment detectables a les EDAR. Considerant que l'ocurrència i la severitat de les infeccions víriques varien al llarg de la vida (Glynn & Moss, 2020), aquest estudi va permetre explorar com diferents grups d'edat contribueixen a perfils virals diferenciats en les aigües residuals. Aquests resultats, tot i provenir d'una mida de mostra limitada, són coherents amb l'estudi publicat al 2023 per McCall i col·laboradors (McCall et al., 2023), que va descriure variacions més grans entre grups vírics en comparar diferents edificis d'un mateix tipus, en contraposició a les variacions observades entre EDARs. També es va detectar CMV en l'aigua residual d'un edifici i es va reportar que les famílies víriques més predominants en una EDAR quan s'aplicava TES eren *Astroviridae* i *Parvoviridae.* L'obtenció de mostres d'aigües residuals representatives a escala d'edifici pot ser complexa, però el mostreig passiu ha reemergit recentment com una solució efectiva per afrontar aquest (Ahmed et al., 2022; Pico-Tomàs et al., 2023; Schang et al., 2021). L'estudi publicat per McCall i col·laboradors, però, es basa en l'ús de mostres puntuals, i és, juntament amb

els dos articles inclosos a la tesi, l'únic que ha explorat la caracterització del viroma a nivell edifici.

Fins ara, només uns pocs estudis han combinat l'ús de mostrejadors passius amb tècniques de seqüenciació massiva. Un d'aquest és l'estudi de Cha et al., 2024. En aquest treball, els autors van aplicar mostrejadors passius per caracteritzar comunitats bacterianes presents en aigües residuals, avaluant-ne la composició i la diversitat microbiana mitjançant tècniques de seqüenciació d'alta resolució. Els resultats van mostrar que els mostrejadors passius permeten recuperar una diversitat microbiana comparable a la de mostres composades tot i que amb diferències en l'abundància relativa d'alguns grups bacterians. A més, es va observar que factors com el temps d'exposició i les condicions ambientals poden influir en el perfil microbià obtingut. Cal destacar, però així mateix, l'estudi d'Alamin i col·laboradors, que va combinar mostrejadors passius amb tècniques de seqüenciació d'amplicons per a la detecció de variants de SARS-CoV-2 en aigües residuals (Alamin et al., 2024).

Junts, aquests dos primers treballs, que composen el primer capítol de la tesi, consoliden el mostreig passiu com una alternativa sensible, assequible i adaptable per a la vigilància vírica en aigües residuals. Aquesta capacitat s'ha corroborat igualment en estudis externs, com el de de Jong et al. (2024), en què l'aplicació de la vigilància basada en aigües residuals mitjançant mostreig passiu als Països Baixos va permetre detectar HAV en una escola després d'un brot, inclús en un centre on no s'havien notificat casos clínics. Aquest resultat posa de manifest la capacitat del mostreig passiu per detectar també circulació vírica en absència de casos simptomàtics i reforça el seu potencial com a eina de vigilància sensible i de detecció primerenca d'esdeveniments epidemiològics (de Jong et al., 2024).

Expansió de l'ús dels mostrejadors passius a noves matrius ambientals

Un cop validada l'estratègia del mostreig passiu en aigües residuals, es va ampliar el seu ús a nous escenaris ambientals: a aigües subterrànies, un entorn on aquesta metodologia no s'havia aplicat prèviament; i per altra, per a l'avaluació de la qualitat microbiològica d'aigües residuals tractades o regenerades amb sistemes naturals.

Es va aplicar el mostreig passiu a aigües subterrànies urbanes, amb l'objectiu d'avaluar la seva utilitat per a detectar i caracteritzar virus humans en aquest tipus de matriu ambiental. Els resultats d'aquest estudi van ser publicats a l'article "*Explorant virus en aigua subterrània: quantificació i caracterització del viroma mitjançant mostrejadors passius i seqüenciació amb enriquiment de dianes*". Gairebé el 50% de totes les mostres analitzades van presentar contaminació fecal humana (amb concentracions mitjanes d'entre $1{,}23 \times 10^2$ i $3{,}66 \times 10^3$ GC per membrana). EV i NoV GI i GII es van detectar de manera esporàdica. Els EV es van detectar de manera consistent durant els mesos de desembre i gener de 2024 a totes dues profunditats de mostreig i ambdós tipus de membrana. Aquesta prevalença és coherent amb les xifres reportades a la bibliografia per aquests tres virus (Rusiñol, 2023). En estudis de vigilància publicats en els darrers 5 anys en aigües subterrànies urbanes (considerant només els virus inclosos en aquest treball), el virus més detectat ha estat HAdV (17–48%), seguit de RoV (1,5–44%), NoV (0,5–6,7%), EV (0,9%) i HEV (0,8%) (de Lambert et al., 2021; Pang et al., 2021; Race et al., 2024; Rusiñol, Hundesa, et al., 2020; Sorensen et al., 2021; Stokdyk et al., 2020). Malgrat les diferències en el límit de detecció dels respectius assajos de qPCR, les seves prevalences són elevades en entorns d'aigua subterrània urbana, on sovint es produeixen fuites al sistema de clavegueram i en un context on l'aigua del riu, principal font d'aigua de l'aqüífer, està format

principalment per efluents 27 d'EDARs situades aigües amunt del riu. Tot i així, la freqüència de detecció de vírica és molt variable.

De manera similar al que ocorre en l'aigua residual, la capacitat d'adsorció dels virus a les membranes depèn de la seva mida, punt isoelèctric i abundància. El pH de l'aigua subterrània, que habitualment oscil·la entre 6 i 8,5, afavoreix que els virus, carregats negativament, s'adsorbeixin sobre superfícies o sòls amb càrrega positiva (Rusiñol, 2023). En conseqüència, és plausible esperar que la presència de sòlids en suspensió afavoreixi l'adsorció de partícules víriques sobre les membranes, atès que aquests sòlids tendeixen a adherir-se als materials dels mostrejadors passius. No obstant això, la presència de substàncies húmiques, habituals en sistemes d'aigües subterrànies, pot inhibir la detecció vírica en interferir en el procés d'unió (Sidstedt et al., 2020). Això pot explicar les freqüències de detecció baixes i variables. És per això, que és important realitzar test preliminars dels materials i temps de mostreig per determinar el material òptim per a la detecció eficient de virus, especialment en matrius ambientals on es troben en tan baixes concentracions.

Les projeccions de canvi climàtic per a la regió mediterrània, que contemplen canvis en la distribució de les precipitacions al llarg de l'any, indiquen que la disseminació de virus podria augmentar com a conseqüència d'un increment d'episodis d'inundacions i de períodes prolongats de calor i sequera (Dupke et al., 2023; Rusiñol et al., 2015a). En aquest estudi de curta durada (10 campanyes de mostreig en 7 mesos), no es va observar cap correlació significativa entre la precipitació (mm) i la detecció de virus ($p > 0,3$). Per aquest motiu, seria recomanable plantejar períodes de vigilància més prolongats en futurs estudis.

L'anàlisi del viroma va detectar patògens destacats procedents de diverses famílies víriques, incloent-hi *Adenoviridae, Astroviridae, Caliciviridae, Herpesviridae, Papillomaviridae, Picornaviridae, Polyomaviridae i Reoviridae.*

Tot i el baix nombre de lectures obtingudes en les mostres d'aigua subterrània, es van observar alts percentatges de similitud genètica i de cobertura. Es van obtenir diverses assignacions de adenovirus espècie F mitjançant seqüenciació amb enriquiment de dianes i es van confirmar posteriorment per detecció de HAdV per qPCR. Els HAdV, són marcadors habitualment utilitzats per investigar contaminació fecal humana d'origen víric, mentre que NoV i/o EV estan associat a brots. Concretament, NoV i EV han estat identificats en el 89% (25/28) i 72% (18/25) dels brots analitzats després d'intrusions d'aigües residuals en fonts d'aigua potable (Rusiñol, 2023), i la seva anàlisi mitjançant qPCR s'inclou simultàniament en el 55% dels estudis publicats (20/36). Donades les dificultats per al cultiu de NoV i la complexitat dels mètodes utilitzats per analitzar grans volums d'aigua, la seqüenciació massiva per a la detecció de genomes virals representa una alternativa viable.

El nombre més elevat de lectures de seqüenciació va correspondre a les famílies *Baculoviridae, Dicistroviridae, Iflaviridae i Nodaviridae.* Aquests virus entomopatògens ja s'havien reportat prèviament en mostres d'aigua de rius i embassaments (Rusiñol, Martínez-Puchol, et al., 2020b). Curiosament, aquestes famílies utilitzades com a bioinsecticides per al control de plagues en nombrosos cultius (per exemple, cítrics, patates, blat de moro i cultius d'hivernacle), així com en cultius ornamentals i colònies d'abelles (Carballo et al., 2017; Cuartas-Otálora et al., 2020; Fenibo et al., 2022; Jiang et al., 2023; Yang et al., 2019; Zhou et al., 2023). Les estratègies de control biològic de plagues, basades en aquests virus redueixen la dependència d'insecticides químics no específics i tòxics. No obstant això, la seva distribució ambiental i la seva detecció freqüent en aigües subterrànies plantegen la necessitat d'una futura consideració en el monitoratge de la qualitat de l'aigua.

El coneixement previ del viroma de l'aigua subterrània era molt limitat (Rusiñol, 2023), principalment perquè els mètodes de concentració emprats

eren costosos (p.ex. ultrafiltració) o poc sensibles (p.ex. floculació) (Forés et al., 2022; Kallies et al., 2019; Smith et al., 2013; van de Vossenberg et al., 2021; Zaouri et al., 2020). La sequenciació massiva a partir dels mateixos mostrejadors passius facilita doncs la detecció de virus en concentracions baixes en mostres d'aigua subterrània, oferint una visió integral del viroma d'aquest entorn, incloent-hi nombrosos virus vegetals, virus que infecten insectes i bacteriòfags. L'ús de sondes dirigides a milers d'espècies virals permet incrementar l'abundància relativa dels virus diana en diversos ordres de magnitud (Kantor & Jiang, 2024; Martínez-Puchol et al., 2020; Tisza et al., 2023). Aquest enfocament de seqüenciació amb enriquiment de dianes aplicat a mostres d'aigua subterrània es perfila com una eina essencial per a l'estudi del viroma dels aqüífers.

Tenint en compte l'aigua subterrània és una font d'aigua dolça que guanya cada vegada més importància, especialment com a conseqüència del canvi climàtic (Joint Monitorin Programme, 2023; MIT Joint Program on the Science and Policy of Global Change., 2023; UN-Water, 2020). Els períodes perllongats i recurrents de sequera i l'augment de les extraccions per a usos agrícoles ja estan provocant alteracions en la qualitat d'aquest recurs globalment (García-Ruiz et al., 2011; Scanlon et al., 2023). A més, els abocaments d'efluents desinfectats tenen un impacte notable al medi on s'aboquen, on també s'han observat modificacions en la composició de les comunitats microbianes (Munné et al., 2023; Múrria et al., 2024). En aquest context, i considerant la necessitat de protegir i gestionar de manera sostenible els aqüífers, és essencial desenvolupar estratègies de monitoratge més eficients per a aquest tipus d'aigua, especialment per a la detecció de virus transmesos per l'aigua. Atès que els virus presents en l'aigua subterrània es troben habitualment en dosis infeccioses molt baixes, és fonamental aplicar mètodes temps-integrat com el mostreig passiu emprat en aquest treball. Els resultats obtinguts posen de manifest que el mostreig passiu constitueix una alternativa viable per facilitar campanyes de vigilància

més prolongades ja que permetrien mostrejos simultanis a molts punts de mostreig alhora i a millorar la gestió d'aquesta font d'aigua tan valuosa.

D'altra banda, més enllà de la vigilància vírica, és imprescindible diversificar i ampliar les fonts de subministrament d'aigua garantint-ne la qualitat i sostenibilitat. En aquest sentit, la reutilització d'aigües residuals tractades i la protecció dels recursos subterranis representen vies complementàries per reforçar la seguretat hídrica. En el context d'aquesta tesi, les dues vies convergeixen en l'aqüífer del Besòs, impactat directament per les descàrregues de les EDARS situades a la conca del riu Besòs (Mas-Ponce et al., 2021; Vàzquez-Suñé et al., 2010).

Partint d'aquesta realitat i després d'haver avaluat la presència de contaminació vírica a l'aqüífer i la utilitat dels mostrejadors passius amb aquest propòsit, el darrer article inclòs en aquesta tesi *"Mostreig passiu per la vigilància vírica en sistemes de tractaments d'aigües residuals basats en solucions naturals*" es va centrar en l'avaluació del potencial del mostreig passiu com a eina de vigilància vírica en aigües regenerades i la seva aplicació en sistemes de tractament terciari basats en solucions naturals. Es va comparar l'ús de mostrejadors passius i el mostreig puntual d'aigua acoblat a una concentració de les partícules víriques mitjançant ultrafiltració, per avaluar el mostreig passiu com a eina de monitoratge de virus en aigües regenerades i en la seva aplicació en sistemes de tractament terciari d'aigua residual basats en solucions naturals com els aiguamolls. El sistema avaluat va ser un aiguamoll construït de flux superficial, específicament els basats en mantells flotants d'arrels (de l'anglès: floating root mats, FMR), formats per plantes hidropòniques flotants les arrels de les quals queden suspeses dins la columna d'aigua (Vymaza, 2022) i els quals la seva eficiència ha quedat provada per l'eliminació de contaminants, nutrients i metalls (Colares et al., 2020; J. Mao et al., 2024; Reyes-Contreras et al., 2012). Diversos estudis *in silico* han explorat la possibilitat de combinar els FMR amb certes barreres o

materials reactius com el biochar, que permetria potenciar la degradació de contaminants químics (Parihar et al., 2022). En comparació a altres materials reactius, com els òxids minerals o el carbó actiu, el biochar presenta avantatges en termes de cost, capacitat d'adsorció i sostenibilitat ambiental (Mohan et al., 2014), però la seva utilitat en sistemes d'eliminació de virus no havia estat fins al moment estudiada.

Pel que fa l'aigua d'entrada de l'EDAR, ambdós mètodes de mostreig mostren un comportament similar per a HAdV i NoV GII, assolint un 100% de deteccions en tots dos casos. En canvi, per a l'HEV, el mostreig puntual va presentar una taxa de detecció més alta (40% vs 20%). Resultats que coincideixen amb els del primer estudi d'aquesta tesi, que indiquen que els mostrejadors passius poden ser menys eficients en aigües residuals d'una EDAR urbana, on l'abundància de sòlids en suspensió pot reduir la disponibilitat dels virus per a la seva adsorció a la membrana, reduint així la sensibilitat.

En canvi, en els altres punts de mostreig (entrada i sortida dels aiguamolls construïts), la detecció amb mostrejadors passius supera el mostreig puntual per a HAdV i HEV, amb freqüències de detecció més altes, especialment a la sortida del CW-RM, on l'HEV només es detecta amb mostrejadors passius. Aquesta major detecció s'explica per la seva capacitat d'enriquiment acumulatiu, que permet capturar virus presents en concentracions molt baixes o variables al llarg del temps (Hayes et al., 2023). Però, aquesta tendència no es va s'observa amb NoV GII, on les freqüències de detecció són més baixes amb mostrejadors passius que amb mostres puntuals suggerint que l'eliminació del NoV als aiguamolls, o la seva menor persistència ambiental, podria haver-ne limitat la disponibilitat per a la seva adsorció a les membranes i posterior detecció. En conjunt, aquests resultats confirmen que l'eficiència del mostreig passiu depèn tant del tipus de virus com de les característiques de la matriu d'aigua, essent menys efectiu en aigües amb alta

càrrega de sòlids però més sensible per a la detecció de virus en efluents tractats (Aguayo-Acosta et al., 2023).

El fet que el mostreig passiu permeti la detecció vírica quan no ho fan les mostres puntuals recollides en paral·lel, posa de manifest la seva utilitat com a eina complementària de vigilància. Aquest enfocament pot donar suport al monitoratge i validació de sistemes basats en solucions naturals, especialment en regions on la infraestructura de laboratori és limitada i no es disposa de la capacitat d'ultrafiltració ni dels recursos analítics necessaris per al processament de mostres puntuals.

Els sistemes de tractament natural avaluats, especialment el que incorpora biochar, van assolir reduccions de 2–3 $\log_{10}$ per a HAdV i NoV GII i superiors a 5 $\log_{10}$ per a *E. coli*. Aquests valors són comparables als de tractaments convencionals més costosos (Simmon, 2011; Zou, 2015), però amb un impacte ambiental i uns requeriments operatius inferiors (Ghernaout and Elboughdiri, 2020; Jasper, 2017; Khajouei, 2022; Han, 2024). Tot i la poca literatura disponible sobre la reducció de microorganismes en tractaments terciaris, els resultats coincideixen amb altres estudis que mostren una elevada eliminació bacteriana i una reducció vírica més moderada. En comparació amb sistemes basats en llum ultraviolada combinada amb cloració, les reduccions víriques són lleugerament inferiors (Chen et al., 2022; H. Wang et al., 2020; L. Wang et al., 2021; Wu et al., 2021), tot i que aquests tractaments poden generar subproductes tòxics i comportar una major demanda energètica i costos de manteniment (Ghernaout i Elboughdiri, 2020; Jasper, 2017; Khajouei, 2022; Han, 2024).

Malgrat aquests resultats, les reduccions observades se situen per sota dels nivells necessaris per garantir la seguretat virològica de l'aigua regenerada en usos no restringits, que requereixen reduccions d'entre 6 i 7 $\log_{10}$ segons les directrius internacionals (Sano et al., 2016; WHO, 2006). Això indica que, fins i tot si es compleixen els criteris basats en indicadors bacterians, els virus

podrien persistir a concentracions que representin un risc per a la salut. D'aquí la necessitat de reforçar el monitoratge de l'aigua regenerada incorporant la detecció directa de patògens vírics. En aquest context, els mostrejadors passius emergeixen com una eina sensible, rendible i fàcil d'implementar per a la vigilància de la presència de virus en efluents tractats.

Per tant, la gestió segura de l'aigua regenerada requereix un enfocament integrat que combini la millora dels tractaments amb una vigilància microbiològica contínua i eficient. Els mostrejadors passius es presenten com una alternativa rendible, de baix cost i fàcil implementació, capaços d'integrar la presència de virus al llarg del temps i de detectar-los fins i tot quan es troben en concentracions molt baixes. D'altra banda, els aiguamolls, i especialment les solucions basades en la natura, ofereixen una alternativa de baix consum energètic i sostenible, capaç d'eliminar contaminants microbians sense generar subproductes nocius i amb una complexitat i uns costos operatius més baixos. La combinació d'aquests dos enfocaments, representa un pas clau per garantir la seguretat sanitària i ambiental de l'aigua regenerada i avançar cap a una gestió més sostenible dels recursos hídrics.

En conjunt, els quatre estudis ofereixen una visió complementària: des de la validació metodològica inicial, l'adaptació a diferents nivells de contaminació (aigua residual, aigua regenerada i aigua subterrània), fins a la seva aplicació pràctica en diversos contextos ambientals i demogràfics. Els resultats obtinguts evidencien la versatilitat del mostreig passiu i la robustesa dels resultats obtinguts fa d'aquesta eina una oportunitat per ampliar el camp d'aplicació de l'epidemiologia basada en aigües residuals, millorant la capacitat de detecció, caracterització i vigilància de virus en entorns on les estratègies convencionals són difícils d'aplicar. També s'evidencien les limitacions i els aspectes que encara cal estudiar i optimitzar.

Aportacions més significatives d'aquesta tesi

Més enllà dels resultats específics, les aportacions més significatives d'aquesta tesi han estat sobretot rellevants pel que fa a les potencials aplicacions del mostreig passiu:

- L'avaluació sistemàtica de l'ús d'una o dues membranes dins d'un mateix mostrejador ha permès millorar la sensibilitat del mètode a partir de l'anàlisi de dues membranes, i no només per a la detecció de virus mitjançant qPCR, sinó també com a eina per a la seqüenciació massiva, ampliant així el seu potencial analític. A més, al llarg del desenvolupament de la tesi s'han optimitzat progressivament els protocols d'extracció per augmentar la sensibilitat en matrius amb concentracions víriques baixes, i s'ha incorporat la dPCR com a eina complementària per millorar els límits de detecció i quantificació. Aquesta tesi introdueix per primera vegada, l'aplicació del mostreig passiu per a la detecció de virus en aigües subterrànies i aigües residuals tractades o regenerades.

- El mostreig passiu, per la seva practicitat, baix cost i versatilitat, pot ser implementat en entorns on fins ara la vigilància ambiental presentava limitacions, com ara clavegueram o canalitzacions petites dels sistemes de clavegueram o aigües subterrànies. Això permet detectar brots de manera precoç, orientar intervencions de salut pública més focalitzades i millorar el control de la qualitat de l'aigua.

- El treball desenvolupat demostra que el mostreig passiu és eficaç en contextos on el mostreig tradicional presenta limitacions logístiques o econòmiques i ha demostrat que en alguns casos pot oferir una sensibilitat igual o superior a la d'altres mètodes convencionals.

El fet d'haver demostrat l'eficàcia del mostreig passiu en escenaris petits i específics evidencia que l'epidemiologia basada en aigües residuals pot evolucionar cap a una eina de vigilància més precisa, focalitzada i adaptable a diferents contextos. La seva aplicació en aigües subterrànies i regenerades obre noves vies per millorar els plans de monitoratge i seguiment de la qualitat de l'aigua, ampliant així l'abast de la vigilància mediambiental més enllà de les grans infraestructures urbanes. Aquest enfocament permet no només una detecció primerenca i localitzada d'esdeveniments epidemiològics, sinó també una caracterització detallada de les dinàmiques de circulació vírica en diferents entorns, reforçant així les capacitats tant de recerca com de gestió i prevenció en salut pública.

Reptes i perspectives futures

Tot i els avenços assolits en aquesta tesi, encara hi ha diversos reptes i línies de recerca futures que cal abordar per consolidar i optimitzar l'ús del mostreig passiu com a eina de vigilància vírica ambiental:

- Un dels aspectes clau és la optimització dels processos d'extracció dels àcids nucleics, ja que al llarg d'aquest treball s'ha evidenciat que hi ha un marge de millora important pel que fa a la sensibilitat i l'eficiència en la recuperació de virus. En els darrers anys s'han publicat nombrosos estudis centrats en aquesta qüestió, fet que obre la porta a avaluar de manera sistemàtica els factors que condicionen l'adsorció dels virus a la membrana i l'extracció (com el pH, la matriu d'aigua o el tipus de membrana) per identificar combinacions òptimes que maximitzin la detecció. Així mateix, l'estandardització de protocols esdevé essencial per garantir la comparabilitat dels resultats entre laboratoris i estudis, un pas imprescindible per a la consolidació del mostreig passiu com a eina de vigilància integrada a escala global.

- D'altra banda, un dels principals punts febles de la metodologia actual és la seva limitada capacitat per transformar els resultats en valors quantitatius robustos. En aquest sentit, és necessari desenvolupar models que permetin extrapolar els resultats obtinguts a estimacions quantitatives, fet que augmentaria de manera substancial el seu valor com a eina de monitoratge epidemiològic i ambiental. Abordar aquests reptes no només permetrà millorar la sensibilitat i la precisió de la metodologia, sinó que també contribuirà a consolidar-la com una estratègia fiable i estandarditzada per a la vigilància vírica en un ampli ventall de contextos.

CONCLUSIONS

- L'aplicació del mostreig passiu en aigua residual, aigua residual tractada, aigua regenerada i aigua subterrània ha permès detectar i caracteritzar virus humans, demostrant que és una alternativa viable per al monitoratge de virus en diferents matrius ambientals.

- Els mostrejadors passius han demostrat ser una metodologia rendible, de baix cost i de fàcil implementació, capaç d'integrar la presència de virus al llarg del temps i de detectar-los fins i tot quan es troben en concentracions molt baixes.

- La comparació entre el mostreig passiu i el mostreig composat en aigües residuals ha mostrat que el mostreig passiu ofereix resultats sensibles i consistents en poblacions petites, on ambdues estratègies són equivalents quan la circulació vírica és moderada o elevada.

- L'ús de mostrejadors passius ha permès descriure el viroma en poblacions petites, obtenint resultats equivalents als del mostreig composat i evidenciant el potencial de l'epidemiologia basada en aigües residuals a escala d'edifici per monitorar malalties infeccioses.

- La combinació de mostreig passiu i seqüenciació dirigida ha facilitat la caracterització del viroma en entorns amb perfils demogràfics diferenciats (escoles, universitats i residències d'avis), mostrant patrons vírics coherents amb les característiques de cada grup. Els virus associats a infeccions agudes (p. ex., astrovirus) es detecten més freqüentment en escoles, mentre que els virus associats a infeccions persistents (poliomavirus, papil·lomavirus o CMV) són més prevalents en residències d'avis, en coherència amb els perfils immunològics i demogràfics de les poblacions estudiades.

- En aigües subterrànies, el mostreig passiu ha demostrat ser una eina sensible per detectar contaminació fecal humana i per caracteritzar el viroma en condicions de concentració vírica baixa, facilitant campanyes

de mostreig prolongades i millorant el coneixement sobre la seva qualitat microbiològica.

- En aigües regenerades, els mostrejadors passius han mostrat una sensibilitat superior a les mostres puntuals per a la detecció d'HEV i HAdV, especialment en efluents amb càrrega vírica baixa o variable, posant de manifest la seva utilitat com a eina de detecció de virus en aigua regenerada.

- L'aplicació del mostreig passiu presenta reptes tècnics que cal abordar, especialment pel que fa a l'optimització de l'extracció d'àcids nucleics, l'estandardització de protocols i la transformació dels resultats en valors quantitatius robustos. Aquests aspectes són essencials per millorar la comparabilitat entre estudis i consolidar el mostreig passiu com una eina fiable per a la vigilància vírica i l'epidemiologia ambiental en un ampli ventall de contextos.

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ANNEXOS

MATERIAL SUPLEMENTARI DE L'ARTICLE 1

Supplementary table 1. Quantification of SARS-CoV-2, JCPyV and HAdV in GC/L at the waste water treatment plant (WWTP) and the nursing home (NH).
AS: Active sampling, PS: passive sampling.

		SARS-CoV-2 N1				JCPyV				HAdV			
		WWTP		NH		WWTP		NH		WWTP		NH	
Sampling date	Replicate	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS
10/1/2022	1	6.01E+05	ND	ND	1.08E+01	9.07E+05	ND	9.09E+05	4.81E+02	4.70E+04	ND	ND	ND
	2	4.06E+04	ND	1.08E+04	ND	1.13E+07	9.75E+02	6.67E+05	ND	2.18E+04	2.09E+03	ND	3.93E+01
13/1/2022	1	1.49E+05	ND	ND	5.71E+00	8.08E+05	4.38E+01	1.71E+05	6.25E+02	5.02E+04	2.63E+02	ND	5.39E-01
	2	1.98E+05	ND	ND	5.44E+01	ND	3.49E+01	6.72E+02	1.37E+03	ND	5.68E+00	3.24E+02	3.42E+00
20/1/2022	1	1.30E+06	ND	1.07E+04	ND	1.91E+06	4.24E+02	1.48E+05	1.77E+02	1.34E+05	2.02E+03	ND	ND
	2	1.52E+06	ND	ND	ND	2.56E+06	7.37E+01	1.87E+05	2.34E+02	5.42E+04	2.96E+02	ND	ND
25/1/2022	1	1.92E+06	6.47E+02	2.45E+05	3.83E+02	1.47E+06	9.71E+02	3.16E+04	3.82E+02	3.85E+04	2.88E+03	ND	2.84E+00
	2	1.87E+06	2.97E+01	ND	2.66E+02	1.78E+06	7.12E+01	1.46E+05	1.78E+02	1.05E+05	4.73E+02	ND	1.24E+00
27/1/2022	1	1.23E+05	ND	2.97E+05	2.45E+02	1.08E+06	1.23E+02	2.09E+05	1.03E+02	7.69E+04	7.84E+01	ND	2.65E+00
	2	5.84E+04	1.61E+02	ND	3.48E+01	7.92E+05	1.05E+02	1.32E+05	1.01E+02	3.28E+04	1.96E+02	ND	ND
1/2/2022	1	1.40E+05	1.29E+02	1.37E+05	4.49E+02	1.45E+06	1.10E+03	1.32E+05	2.56E+02	6.74E+04	5.61E+03	ND	ND
	2	1.30E+05	ND	9.05E+04	2.42E+02	2.94E+06	1.05E+02	1.38E+05	4.89E+02	1.11E+05	3.59E+02	ND	ND
8/2/2022	1	2.10E+05	3.55E+01	4.28E+05	2.54E+02	8.57E+05	3.57E+02	1.49E+05	2.05E+02	7.76E+03	5.25E+02	2.21E+02	ND
	2	5.51E+04	4.72E+01	7.40E+04	3.95E+02	7.22E+05	3.40E+02	1.11E+05	2.13E+02	2.83E+04	6.64E+01	ND	4.83E+00
10/2/2022	1	ND	ND	ND	4.57E+01	9.35E+05	1.48E+02	8.57E+04	1.70E+02	2.56E+05	1.02E+02	ND	6.60E+00
	2	5.53E+03	1.09E+01	ND	ND	8.04E+05	6.31E+02	1.45E+05	6.32E+01	9.91E+04	4.31E+02	3.14E+02	2.57E+00
17/2/2022	1	3.08E+04	1.23E+01	6.81E+04	8.19E-01	3.71E+05	ND	5.89E+04	ND	7.99E+04	ND	ND	ND
	2	3.08E+04	ND	5.10E+04	ND	4.62E+05	6.23E+02	3.79E+04	1.17E+02	3.24E+04	2.34E+03	1.87E+03	ND
22/2/2022	1	8.19E+04	ND	ND	ND	1.21E+06	2.19E+02	3.12E+04	1.07E+02	2.56E+05	8.01E+02	ND	ND
	2	3.91E+04	ND	ND	ND	4.98E+05	8.93E+01	3.75E+04	5.77E+01	9.92E+04	1.86E+02	ND	ND
24/2/2022	1	5.95E+03	ND	ND	ND	6.31E+05	9.01E+02	6.31E+05	3.95E+02	5.05E+04	9.46E+02	ND	ND
	2	ND	ND	ND	ND	4.11E+05	1.55E+03	4.11E+05	ND	1.34E+02	1.14E+03	ND	ND
1/3/2022	1	8.58E+04	ND	ND	ND	9.54E+05	6.92E+01	1.18E+04	2.82E+02	2.13E+05	1.08E+03	ND	ND
	2	8.76E+04	ND	ND	5.74E+02	1.84E+06	3.93E+02	3.79E+04	4.49E+02	3.05E+05	3.34E+03	ND	ND
3/3/2022	1	1.06E+04	ND	ND	ND	2.00E+05	1.07E+01	3.18E+04	ND	6.59E+04	6.08E+02	ND	1.30E+00
	2	1.19E+04	ND	ND	ND	2.56E+05	2.63E+01	2.89E+04	ND	3.59E+04	7.98E+01	ND	9.39E-01
10/3/2022	1	2.61E+04	ND	ND	ND	1.30E+05	ND	1.87E+04	1.58E+01	5.15E+04	ND	ND	ND
	2	6.35E+03	ND	ND	3.53E+01	1.52E+05	1.14E+01	1.35E+04	1.69E+01	4.86E+04	2.75E+02	ND	ND
22/3/2022	1	8.19E+04	ND	ND	ND	1.21E+06	2.19E+02	3.12E+04	1.07+02	1.74E+05	1.73E+03	ND	2.13E+00
	2	3.91E+04	ND	ND	ND	4.98E+05	8.93E+01	3.75E+04	5.77+01	1.36E+05	4.13E+03	ND	2.27E+00

Supplementary table 2. Quantification and semi-quantification of SARS-CoV-2 (N1 and N2 assays), JCPyV, HAdV, EV, RoV and NoV GI and GII in GC/L at the waste water treatment plant (WWTP) .
AS: Active sampling, PS: passive sampling.

		WWTP															
		SARS-CoV-2 N1		SARS-CoV-2 N2		JCPyV		HAdV		EV		RoV		NoV GI		NoV GII	
Sampling date	Replicate	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS
10/1/2022	1	6.01E+05	ND	3.50E+05	ND	9.07E+05	ND	4.70E+04	ND	8.49E+06	ND	1.85E+06	ND	3.33E+05	ND	2.72E+05	ND
	2	4.06E+04	ND	1.22E+04	ND	1.13E+07	9.75E+02	2.18E+04	2.09E+03	4.21E+06	ND	1.91E+06	ND	2.39E+05	6.75E+00	2.03E+05	2.19E+01
13/1/2022	1	1.49E+05	ND	6.23E+05	ND	8.08E+05	4.38E+01	5.02E+04	2.63E+02	3.53E+06	2.71E+02	4.56E+06	ND	2.01E+05	ND	2.65E+03	8.11E+00
	2	1.98E+05	ND	ND	ND	ND	3.49E+01	ND	5.68E+00	ND	1.01E+02	ND	ND	ND	ND	ND	ND
20/1/2022	1	1.30E+06	ND	5.59E+05	ND	1.91E+06	4.24E+02	1.34E+05	2.02E+03	2.53E+06	ND	2.53E+06	ND	2.46E+05	ND	3.12E+05	ND
	2	1.52E+06	ND	5.45E+05	ND	2.56E+06	7.37E+01	5.42E+04	2.96E+02	1.79E+06	ND	3.80E+06	ND	2.66E+05	ND	3.53E+05	ND
25/1/2022	1	1.92E+06	6.47E+02		7.21E+01	1.47E+06	9.71E+02	3.85E+04	2.88E+03	1.35E+05	ND	ND	ND	7.77E+04	7.18E+01	5.95E+04	2.01E+00
	2	1.87E+06	2.97E+01	4.94E+02	ND	1.78E+06	7.12E+01	1.05E+05	4.73E+02	1.69E+06	ND	2.82E+06	ND	2.02E+05	ND	3.30E+05	ND
27/1/2022	1	1.23E+05	ND	2.47E+02	ND	1.08E+06	1.23E+02	7.69E+04	7.84E+01	2.57E+06	ND	4.01E+06	ND	1.22E+05	1.87E+01	1.29E+05	1.67E+01
	2	5.84E+04	1.61E+02	4.80E+01	3.35E+01	7.92E+05	1.05E+02	3.28E+04	1.96E+02	3.99E+06	ND	1.73E+07	ND	1.74E+05	ND	2.75E+05	7.72E+01
1/2/2022	1	1.40E+05	1.29E+02	7.10E+01	5.09E-01	1.45E+06	1.10E+03	6.74E+04	5.61E+03	3.24E+06	ND	3.54E+06	ND	2.39E+05	1.91E+01	1.90E+05	1.20E+01
	2	1.30E+05	ND	5.96E+02	2.17E+02	2.94E+06	1.05E+02	1.11E+05	3.59E+02	3.25E+06	ND	5.31E+06	ND	4.09E+05	1.13E-01	2.63E+05	ND
8/2/2022	1	2.10E+05	3.55E+01	4.15E+04	1.76E+00	8.57E+05	3.57E+02	7.76E+03	5.25E+02	3.40E+05	6.81E+03	ND	8.27E+03	2.69E+05	1.94E+02	5.85E+04	2.66E+02
	2	5.51E+04	4.72E+01	3.45E+04	2.02E+01	7.22E+05	3.40E+02	2.83E+04	6.64E+01	8.38E+06	ND	7.43E+06	ND	2.42E+05	3.30E+01	1.66E+05	3.92E+01
10/2/2022	1	ND	ND	8.83E+04	ND	9.35E+05	1.48E+02	2.56E+05	1.02E+02	7.90E+06	ND	ND	ND	2.58E+05	ND	3.95E+05	1.20E+01
	2	5.53E+03	1.09E+01	1.88E+05	ND	8.04E+05	6.31E+02	9.91E+04	4.31E+02	1.16E+07	1.64E+03	6.82E+07	9.01E+02	2.14E+05	ND	1.95E+05	ND
17/2/2022	1	3.08E+04	1.23E+01	2.37E+04	ND	3.71E+05	ND	7.99E+04	ND	9.52E+06	ND	9.06E+06	ND	2.78E+05	ND	2.62E+05	ND
	2	3.08E+04	ND	4.30E+04	1.62E+01	4.62E+05	6.23E+02	3.24E+04	2.34E+03	1.32E+07	ND	4.98E+06	2.82E+03	3.47E+05	ND	2.90E+05	5.68E+00
22/2/2022	1	8.19E+04	ND	1.33E+05	ND	1.21E+06	2.19E+02	2.56E+05	8.01E+02	9.39E+06	ND	2.22E+07	1.81E+03	9.12E+05	4.45E+00	7.36E+05	1.12E+00
	2	3.91E+04	ND	3.81E+04	ND	4.98E+05	8.93E+01	9.92E+04	1.86E+02	5.09E+06	ND	1.23E+07	1.46E+03	3.47E+05	8.13E+00	2.10E+05	1.13E+00
24/2/2022	1	5.95E+03	ND	4.74E+01	9.28E+00	6.31E+05	9.01E+02	5.05E+04	9.46E+02	8.59E+06	ND	7.56E+06	1.33E+04	4.40E+05	ND	3.43E+05	1.12E+00
	2	ND	ND	ND	ND	4.11E+05	1.55E+03	1.34E+02	1.14E+03	ND	ND	ND	ND	2.05E+04	3.57E+00	4.15E+02	4.51E+00
1/3/2022	1	8.58E+04	ND	1.35E+05	ND	9.54E+05	6.92E+01	2.13E+05	1.08E+03	2.40E+07	ND	4.08E+07	ND	8.44E+05	ND	3.33E+05	ND
	2	8.76E+04	ND	2.70E+05	ND	1.84E+06	3.93E+02	3.05E+05	3.34E+03	2.17E+07	ND	6.54E+07	ND	1.13E+06	ND	5.36E+05	ND
3/3/2022	1	1.06E+04	ND	8.16E+03	ND	2.00E+05	1.07E+01	6.59E+04	6.08E+02	6.14E+06	ND	2.45E+07	ND	7.32E+05	ND	3.27E+05	ND
	2	1.19E+04	ND	1.17E+04	ND	2.56E+05	2.63E+01	3.59E+04	7.98E+01	8.78E+06	ND	1.42E+07	ND	6.21E+05	ND	2.90E+05	ND
10/3/2022	1	2.61E+04	ND	3.56E+02	ND	1.30E+05	ND	5.15E+04	ND	3.80E+06	ND	1.60E+07	5.67E+04	5.00E+05	ND	2.08E+05	ND
	2	6.35E+03	ND	2.56E+04	ND	1.52E+05	1.14E+01	4.86E+04	2.75E+02	7.14E+06	ND	2.95E+07	ND	6.95E+05	ND	2.90E+05	ND
22/3/2022	1	8.19E+04	ND	4.29E+04	3.91E+01	1.21E+06	2.19E+02	1.74E+05	1.73E+03	4.12E+06	ND	6.00E+06	2.11E+04	2.83E+05	8.18E+01	1.47E+05	4.06E+01
	2	3.91E+04	ND	4.23E+04	1.23E+02	4.98E+05	8.93E+01	1.36E+05	4.13E+03	5.66E+06	ND	9.68E+06	ND	3.57E+05	6.59E+01	1.52E+05	8.17E+01

Supplementary table 3. Quantification and semi-quantification of SARS-CoV-2 (N1 and N2 assays), JCPyV, HAdV, EV, RoV and NoV GI and GII in GC/L at the nursing home (NH) . AS: Composite sampling, PS: passive sampling.

		NURSING HOME															
		SARS-CoV-2 N1		SARS-CoV-2 N2		JCPyV		HAdV		EV		RoV		NoV GI		NoV GII	
Samplig date	Replicate	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS	AS	PS
10/1/2022	1	ND	1.08E+01	ND	ND	9.09E+05	4.81E+02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	1.08E+04	ND	ND	ND	6.67E+05	ND	ND	3.93E+01	ND	ND	ND	ND	ND	ND	ND	ND
13/1/2022	1	ND	5.71E+00	6.42E+03	ND	1.71E+05	6.25E+02	ND	5.39E-01	ND	1.23E+04	ND	ND	ND	ND	ND	ND
	2	ND	5.44E+01	ND	ND	6.72E+02	1.37E+03	3.24E+02	3.42E+00	2.80E+06	3.73E+04	ND	ND	ND	ND	ND	ND
20/1/2022	1	1.07E+04	ND	ND	ND	1.48E+05	1.77E+02	ND	ND	ND	ND	ND	ND	ND	ND	2.13E+02	ND
	2	ND	ND	ND	ND	1.87E+05	2.34E+02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
25/1/2022	1	2.45E+05	3.83E+02	ND	1.06E+02	3.16E+04	3.82E+02	ND	2.84E+00	ND	ND	ND	ND	ND	ND	ND	ND
	2	ND	2.66E+02	ND	8.30E+01	1.46E+05	1.78E+02	ND	1.24E+00	ND	ND	ND	ND	ND	ND	ND	7.20E+00
27/1/2022	1	2.97E+05	2.45E+02	4.81E+01	2.55E+02	2.09E+05	1.03E+02	ND	2.65E+00	ND	ND	ND	ND	ND	ND	ND	ND
	2	ND	3.48E+01	ND	1.57E+02	1.32E+05	1.01E+02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1/2/2022	1	1.37E+05	4.49E+02	ND	7.69E+01	1.32E+05	2.56E+02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	9.05E+04	2.42E+02	4.10E+02	3.25E+02	1.38E+05	4.89E+02	ND	ND	ND	ND	ND	ND	ND	8.68E+00	ND	ND
8/2/2022	1	4.28E+05	2.54E+02	2.88E+05	6.22E+00	1.49E+05	2.05E+02	2.21E+02	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	7.40E+04	3.95E+02	2.99E+05	1.72E+02	1.11E+05	2.13E+02	ND	4.83E+00	ND	ND	ND	ND	ND	ND	ND	ND
10/2/2022	1	ND	4.57E+01	1.04E+05	ND	8.57E+04	1.70E+02	ND	6.60E+00	ND	ND	ND	ND	ND	ND	ND	ND
	2	ND	ND	1.56E+03	8.28E+01	1.45E+05	6.32E+01	3.14E+02	2.57E+00	ND	ND	3.09E+06	ND	ND	ND	ND	ND
17/2/2022	1	6.81E+04	8.19E-01	7.85E+04	ND	5.89E+04	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	5.10E+04	ND	1.09E+04	3.41E+01	3.79E+04	1.17E+02	1.87E+03	ND	ND	ND	ND	ND	ND	ND	ND	ND
22/2/2022	1	ND	ND	1.09E+04	ND	3.12E+04	1.07E+02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	3.75E+04	5.77E+01	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24/2/2022	1	ND	ND	ND	ND	6.31E+05	3.95E+02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	4.11E+05	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1/3/2022	1	ND	ND	ND	4.14E+01	1.18E+04	2.82E+02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	ND	5.74E+02	9.16E+03	ND	3.79E+04	4.49E+02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3/3/2022	1	ND	ND	9.54E+02	ND	3.18E+04	ND	ND	1.30E+00	ND	ND	ND	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	2.89E+04	ND	ND	9.39E-01	ND	ND	4.64E+05	ND	ND	ND	ND	ND
10/3/2022	1	ND	ND	6.17E+04	ND	1.87E+04	1.58E+01	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	ND	3.53E+01	4.34E+04	ND	1.35E+04	1.69E+01	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
22/3/2022	1	ND	ND	ND	ND	3.12E+04	1.07+02	ND	2.13E+00	ND	ND	ND	ND	ND	ND	ND	3.15E+00
	2	ND	ND	ND	ND	3.75E+04	5.77+01	ND	2.27E+00	ND	ND	ND	ND	ND	ND	ND	6.70E-02

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Supplementary information 1: List of Viral species and serotypes, serogroups or genotypes identified in each building type. Mean ID%: avarage percentatge identity of alignments; Mean CV: the percent of the reference accession that is covered by at least one contig; Coverage depth: indicates the average read depth across the length of the accession. Metagenomic metrics are mean values obtained at the different samples analysed per each building type and at the WWTP.

Sampling site	Family	Viral specie	metagenomics metrics			Serotypes, serogroups or genotypes identified
			Mean ID%	Mean CV%	Coverage depth	
School	*Adenoviridae*	*Human mastadenovirus F*	99.9	7	2.2x	AdV-40, AdV-41
	Astroviridae	*Astrovirus MLB1*	97.5	5.9	0.33x	
		Mamastrovirus 1	99.35	97.5	2490x	HAstV-1, HAstV-3
		Mamastrovirus 3	98.9	-	-	
		Unclassified Mamastrovirus	93.1	-	-	
	Caliciviridae	*Human salivirus*	95.8	-	-	
		Salivirus FHB	98	-	-	
		Sapporo virus	97.55	25.6	22.5x	SapV-GI, -GII, -GIV
		Unclassified Sapovirus	97.8	-	-	
	Parvoviridae	*Human bocavirus*	99.8	52.6	96.1x	
	Picornaviridae	*Parechovirus A*	91.9	9.3	2.6x	
		Aichivirus A	97.3	-	-	
		Cardiovirus B	93	4.1	0.24x	
		Enterovirus C	93.2	4.8	0.44x	
		Norwalk virus	99.7	7.3	0.69x	NoVGII.17
	Polyomaviridae	*Human polyomavirus 1*	99.9	59.6	178.2x	
		Human polyomavirus 2	99.4	-	-	
University	*Adenoviridae*	*Human mastadenovirus F*	99.93	4.3	0.31x	
	Astroviridae	*Mamastrovirus 1*	98	5.4	0.48x	HAstV-1, HAstV-3
	Caliciviridae	*Human salivirus*	95.6	25	3.9x	
		Norwalk virus	99.7	23.6	3.5x	
		Salivirus A	94.1	-	-	
		Salivirus FHB	96.8	45	52x	
		Sapporo virus	96.4	5.6	0.72x	
	Papillomaviridae	*Alphapapillomavirus 2*	99.1	5.2	1.1x	HPV-28
		Betapapillomavirus 2	99.3	4.9	0.32x	HPV-9, HPV-110
		Gammapapillomavirus 7	98.6	28	2.2x	HPV-149
	Parvoviridae	*Human bocavirus*	99.3	10.9	4x	
	Picornaviridae	*Cardiovirus B*	92.35	-	-	
		Enterovirus B	94.6	-	-	
		Human cosavirus SH4	92.8	-	-	
	Polyomaviridae	*Human polyomavirus 1*	99.8	25	5.4x	
		Human polyomavirus 2	99.9	34.8	20x	
Nursing home	*Astroviridae*	*Mamastrovirus 1*	99.5	-	-	
	Herpesviridae	*Cercopithecine betaherpesvirus 5*	97.5	-	-	
	Papillomaviridae	*Alphapapillomavirus 7*	99.9	-	-	HPV-70
		Betapapillomavirus 1	98.8	3.1	0.39x	HPV-105
		Betapapillomavirus 2	99.3	-	-	HPV-22, HPV-100
		Betapapillomavirus 3	96.6	-	-	HPV-75
	Picornaviridae	*Cardiovirus B*	94.6	7.5	1x	
		Enterovirus C	94.2	39	6.5x	Coxsakie-A22, -A1
	Polyomaviridae	*Human polyomavirus 1*	98.8	-	-	
		Human polyomavirus 2	99.5	9.5	1.6x	
		Human polyomavirus 5	99.8	8.9	0.75x	
WWTP	*Adenoviridae*	*Human mastadenovirus A*	99.7	-	-	
		Human mastadenovirus B	99.7	-	-	AdV-7, -21 -B3
		Human mastadenovirus F	98.1	35.1	5.7x	
	Astroviridae	*Astrovirus MLB1*	96.8	15.7	2x	
		Mamastrovirus 1	98.6	88.1	182.8x	HAstV-1, HAstV-3, HAstV-4
		Mamastrovirus 6	98.2	-	-	
		Mamastrovirus 8	96.7	5.2	0.2x	
		HMO Astrovirus A	93.6	13.8	2.9x	
	Caliciviridae	*Human salivirus*	97.1	-	-	
		Norwalk virus	99.7	44.6	1.8x	NV-GI.P10, NV-GII.P7
		Salivirus A	96.05	-	-	
		Salivirus FHB	93.5	21	2.1x	
		Sapporo virus	99.3	63.3	5.1x	
		Unclassified Sapovirus	97.8	-	-	
	Coronaviridae	*SARS-CoV-2*	96.2	-	-	
		Human coronavirus HKU1	100	-	-	
	Papillomaviridae	*Alphapapillomavirus 14*	99.9	-	-	HPV-90
		Betapapillomavirus 1	97.1	14.2	2x	HPV-21, HPV-36
		Betapapillomavirus 2	100	8.8	0.8x	HVP-110
		Betapapillomavirus 3	97.9	-	-	HPV-76
		Betapapillomavirus 4	97.2	-	-	HPV-92
		Gammapapillomavirus 1	100	-	-	HPV-65
		Gammapapillomavirus 3	96.9	-	-	HPV-50
		Gammapapillomavirus 10	100	3.1	0.35x	HPV-133
		Gammapapillomavirus 11	95.3	-	-	HPV-169
		Gammapapillomavirus 19	94.3	4.8	0.29x	HPV-169
		Iotapapillomavirus 1	99.3	-	-	
		Pipapillomavirus 2	100	-	-	
	Parvoviridae	*Human bocavirus*	99.8	48.4	85.9x	
	Picornaviridae	*Aichivirus A*	98	16.3	3.8x	
		Aichivirus E	88.5	-	-	
		Cardiovirus B	95.65	-	-	
		Cardiovirus C	93.6	7.3	0.77x	
		Cosavirus A	93.5	6	0.49x	
		Enterovirus B	93.7	3.5	0.21x	
		Enterovirus C	91.5	6.7	1.2x	Coxsackie-A19
		Hepatovirus A	98.5	8.4	2.2x	
		Human cosavirus	94.1	-	-	
		Human enterovirus	94.2	-	-	
		Parechovirus A	97.2	-	-	
		Rhinovirus B	97.6	-	-	
		Rosavirus B	98.9	3	0.1x	
	Polyomaviridae	*Human polyomavirus 1*	97.4	55.6	28.4x	
		Human polyomavirus 2	99.8	26.5	6.1x	
		Human polyomavirus 5	99.8	9.7	2.6x	

Supplementry information 2: Sample dates and pools of the nucleic acid extractions. Each column correspond to one pool

School				University				Nursing home			WWTP	
A		B		A		B		A		B	C	
Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Pool 7	Pool 8	Pool 9	Pool 10	Pool 11	Pool 12	Pool 13
10/1/2022	10/3/2022	13/1/2022	17/3/2022	10/1/2022	10/3/2022	10/1/2022	17/3/2022	13/1/2022	10/3/2022	22/3/2022	18/1/2022	10/3/2022
13/1/2022	17/3/2022	20/1/2022	22/3/2022	18/1/2022	17/3/2022	13/1/2022	22/3/2022	20/1/2022	17/3/2022	10/3/2022	20/1/2022	22/3/2022
20/1/2022	22/3/2022					20/1/2022			22/3/2022	17/3/2022		

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Supplementary 1: List of samples included in the surveillance study. Viral NA extractions from membranes corresponding to samples highlighted in blue were pooled and the virome was further analysed using TES. * indicates that samples underwent the entire library and capture process in 3 replicates each. Samples highlighted in yellow, positive of EV qPCR detection, were selected for further ADS to study the diversity of EV.

Sampling site	VPS-material	SEP	OCT	DEC	JAN1	JAN2	FEB	MAR1	MAR2	APR	MAY
		13-26 Sep	4-16 Oct	25-4 Dec	7-17 Jan	17-29 Jan	29-08 Feb	23-4 Mar	4-14 Mar	10-23 Apr	8-19 May
SAP-2b (3m)	VPS-NC	3	3*	3	3	-	3	3	3	3	3
	VPS-NY	3	3*	3	3	-	3	3	3	3	3
	UF	-	1	-	-	-	-	-	-	-	-
SAP-1 (11m)	VPS-NC	3	3*	3	3	3	3	3	3	3	3
	VPS-NY	3	3*	3	3	3	3	3	3	3	3
	UF	-	1	-	-	-	-	-	-	-	-

Supplementary table 2. Viral assignments obtained in each sample using VPS qith NC or NY and UF. R: number of sequencing reads, ID: percentage of identity, C: coverage

			SAP-2b															SAP-1											
			SEP VPS-NC			OCT VPS-NC			SEP VPS-NY			OCT VPS-NY			OCT UF			SEP VPS-NC			OCT VPS-NC			OCT VPS-NY			OCT UF		
Family	**Genus**	**Specie**	R	ID	C	R	ID	C	R	ID	C	R	ID	C	R	ID	C	R	ID	C	R	ID	C	R	ID	C	R	ID	C
Adenoviridae	Aviadenovirus	Pigeon aviadenovirus B										1	95	119							12	99.5	326.5	1	100	151			
		Pigeon adenovirus 2a										1	96.6	148										1	100	151			
		Fowl aviadenovirus C				1	92.9	42																					
	Mastadenovirus	Human mastadenovirus D										2	98.5	261															
		Human mastadenovirus F	2	99.7	151				10	99.5	144.3				6	99.7	136.3	6	99.8	225	5	100	271	28	100	197.4			
		Human mastadenovirus A							2	95.4	151																		
Astroviridae	Mamastrovirus	Rabbit astrovirus				8	93.6	151.3				6	91.4	430															
		Mamastrovirus 1				1	88.9	99																					
Baculoviridae	Alphabaculovirus	Spodoptera exigua multiple nucleopolyhedrovirus	28	99.5	159.3	8	100	142.5	38	98.3	160.9	22	99	132.1				14	99.2	148.3	42	99.3	141	66	97.9	128.4	14	99	172
		Troides aeacus nucleopolyhedrovirus										1	92.7	41															
	Betabaculovirus	Spodoptera frugiperda granulovirus										1	1	97.3															
Caliciviridae	Norovirus	Norovirus				7	100	40										2	96	149.5				34	99.1	404.8			
Circoviridae	Cyclovirus	Cyclovirus TN9										6	97.5	243							1	98.5	136						
		Cyclovirus TN8																			3	100	107						
		Cyclovirus TN10																			2	100	151	2	100	103			
		Cyclovirus TN15										2	100	93				8	99.4	340	4	99.3	141.7	11	99.7	366			
		Cyclovirus TN16																			1	98	151						
		Human associated cyclovirus 4	4	96.4	151				174	95.8	998.1	16	97.7	314.3				20	95.4	263.8	88	97.3	881.5	52	98.3	327.9			
		Human associated cyclovirus 6										1	95	121							4	94.9	138	10	92.1	151			
		Human associated cyclovirus 10																			2	96.5	173	4	96	140			
		Squirrel associated cyclovirus 1																			1	89.6	125						
		Bat associated cyclovirus 2																											
	Circovirus	Pigeon circovirus				1	78.7	61	7	93.8	200.4										4	87.1	151						
		Bat circovirus																											
		Starling circovirus	22	98.6	291.9																1	89.1	128						
		Chicken circovirus							1	96	151										2	98.1	132						
	Gemycircularvirus	Chicken genomovirus																									2	99	151
		Cattle blood-associated gemycircularvirus	2	94.7	141.5							2	99	147.5				2	94.3	229	5	94.4	151	7	96.7	151	1	95	113
		Mosquito associated gemycircularvirus 1																			2	90.8	119						
Coronaviridae	Gammacoronavirus	Avian coronavirus																						4	99.5	151			
Dicistroviridae	Aparavirus	Kashmir bee virus																			6	97.7	140.5						
	Cripavirus	Aphis gossypii virus	10	98.9	200				16	99	227.9	18	98.5	255.8	2	99	141.5	12	98.9	198.3	52	99.2	171.6	110	98.7	330.4			
		Aphid lethal paralysis virus	50	98.3	253.1	2	94.4	151	8	98.2	143	21	98.5	144.4	4	99	147.8	54	97.5	227.5	176	97	374	72	98.2	295.4	10	98	147
		Rhopalosiphum padi virus	2	99.7	143	11	98.4	190							2	98.7	230				3	95.7	151	4	99.3	147			
		Aphis glycines virus 3																						3	97	137			
Geminiviridae	Gemykibivirus	Porcine associated gemykibivirus 1																						3	92.1	76			
Herpesviridae	Varicellovirus	Bovine alphaherpesvirus 5							1	100	36	1	100	53															
		Equid alphaherpesvirus 1							1	97.2	36										6	86.9	160	7	97.9	47			
		Cervid alphaherpesvirus 1	2	97.3	37																								
	Simplexvirus	Human alphaherpesvirus 2										1	97.6	41													1	98	41
Iflaviridae	Iflavirus	Spodoptera exigua iflavirus 1	72	91.3	364	549	90.6	1135.3	38	92	281.4	152	92.3	686.6	42	91.6	234.9	40	92.9	300.9	96	92	383.9	465	91.4	364.9	120	91	770
Iridoviridae	Chloriridovirus	Invertebrate iridovirus	2	91.9	60							1	88.4	86															
	Iridovirus	Invertebrate iridescent virus	1	98.1	104							2	97.4	135															
Mimiviridae	Mimivirus	Megavirus chiliensis				1	88.6	70				1	100	38	1	88.7	53				2	97.6	42	3	92.1	75.5			
		Mimivirus	3	90.2	61				1	87.3	79	1	79.6	108	3	89	65				1	89.7	58	2	88.8	58			
		Acanthamoeba castellanii mamavirus										1	93.3	45							1	96.1	51						
		Megavirus vitis	2	90.6	53										1	77	74							1	92.3	39			
		Acanthamoeba polyphaga mimivirus	2	97.2	36																2	95.3	43						
Nodaviridae	Alphanodavirus	Flock House virus				7	90.1	151				2	91.2	97	2	91.7	72				3	90.3	62	1	91.1	79	1	88	125
		Nodamura virus										2	91.9	148	4	90	175.3							6	95.2	124	2	89,5	258
		Black beetle virus				2	90.8	147																1	89.7	126			

Papillomaviridae	Alphapapillomavirus	Human papillomavirus 4																											2	100	151
	Betapapillomavirus	Betapapillomavirus 2																													
Parvoviridae	Dependoparvovirus	Rhinolophus sinicus adeno-associated virus 1																													
		Rodent dependoparvovirus 2																													
		Avian dependoparvovirus 1																													
	Iteradensovirus	Lepidopteran iteradensovirus 2																													
		Iteravirus sp.																													
		Danaus plexippus plexippus iteravirus																													
		Iteradensovirus sp.																													
		Hordeum marinum Itera-like densovirus																													
	Protoparvovirus	Carnivore protoparvovirus 1																													
		Rodent protoparvovirus 1																													
	Bocaparvovirus	Lupine bocavirus																													
		Rabbit bocavirus																													
		Porcine bocavirus																													
		Rodent bocavirus																													
		Ungulate bocaparvovirus 5																													
Phycodnaviridae	Chlorovirus	Acanthocystis turfacea Chlorella virus				1	92.1	38				1	76.2	63																	
		Paramecium bursaria Chlorella virus	2	90.2	41																										
Picornaviridae	Kobuvirus	Aichivirus A	46	97.2	569.8				6	99.9	151	2	94.6	129				2	98.9	275	12	96.6	147.3	8	98.6	119					
	Cosavirus	Cosavirus A																							1	99.3	151				
		Human cosavirus																							1	98	151				
Polyomaviridae	Betapolyomavirus	Human polyomavirus 1										2	100	133.5																	
Reoviridae	Rotavirus	Rotavirus C	1	86.8	38	2	84.6	39							1	97.9	146				2	89.2	37	2	82.5	40	1	85	39		
Tombusviridae	Tombusvirus	Erysiphales associated tombus-like virus 1				1	96.4	56																							
		Citrullus lanatus tombusvirus				1	81	116																							
		Eggplant mottled crinkle virus	2	95.7	151																										
		Ginkgo biloba tombusvirus																			1	87.7	57								
	Aureusvirus	Pothos latent virus																			2	96.4	151								
Virgaviridae	Tobamovirus	Tomato brown rugose fruit virus	52	99.8	610.9				6	99.6	148.3	47	99.5	312.5	6	99.7	357.2	8	99.7	195.8	67	99.8	307.4	89	99.3	341.1					
		Tomato mosaic virus	10	99.6	147.8							13	98.2	215.8	2	98.2	139	4	99.4	166.5	11	99.5	153.7	57	99.2	223					
		Tobacco mosaic virus							2	99.7	151	2	98.6	147.5																	
		Cucumber green mottle mosaic virus	4	98.9	119	2	100	269	2	98.9	143	2	99.7	151							6	99.7	151	10	99.8	102.3					
		Ribgrass mosaic virus				1	95.8	143																							
		Turnip vein-clearing virus				1	97.2	143																4	91.6	94	2	93	142		
		Pepper mild mottle virus																			2	100	141.5	4	100	142.5					
		Tobacco mild green mosaic virus	2	99.3	140.5																2	99.3	139.5				1	92,2	128		

Supplementary 3. Relative abundance of viral reads in each sample (SAP-2b or SAP1 with VPS-NY or -NC) and replicate (1,2,3). Spearman's Rank Test coefficients between replicates (bold), wells and VPS adsorbent materials.

	SAP-2b OCT			SAP-2b OCT			SAP1 OCT			SAP1 OCT		
	NY1	NY2	NY3	NC1	NC2	NC3	NY1	NY2	NY3	NC1	NC2	NC3
Adenoviridae	1,67	0	0	0	0	0,42	4,16	2,70	0	0	1,50	6,43
Astroviridae	0	2,08	0	0	0,79	2,50	0	0	0	0	0	0
Baculoviridae	4,18	2,78	7,89	0,99	2,11	1,67	4,57	6,47	6,25	7,67	3,01	6,43
Caliciviridae	0	0	0	0,25	0,26	2,08	3,74	0	5,00	0,70	0	0
Circoviridae	3,77	8,68	6,58	24,63	21,05	12,08	6,65	18,60	11,25	20,91	13,53	16,87
Coronaviridae	0	0	0	0	0	0	0	1,08	0	0	0	0
Dicistroviridae	4,18	13,54	14,47	3,94	4,74	5,42	14,76	17,79	18,44	35,89	33,08	36,14
Genomoviridae	0	0	0	0	0	0	0,42	0,27	0	0	0	0
Herpesviridae	0	0,42	0	0,42	0	0	0	0	0,42	0,42	0	0
Iflaviridae	63,60	44,44	21,05	51,97	49,74	62,08	43,04	35,58	39,38	16,38	15,04	11,65
Iridoviridae	1,26	0	0	0	0,79	0	0	0	0	0	0	0
Mimiviridae	0,42	0,35	2,63	0,74	0,26	0,42	0,62	0,27	0,63	0,70	1,50	0,80
Nodaviridae	0,84	0,69	2,63	0,25	0,26	3,33	0,21	1,08	1,25	0,35	1,50	0,40
Papillomaviridae	0	0	0	0	0	0	0,42	0	0	0	0	0
Parvoviridae	4,18	2,08	3,95	0,49	2,11	0,83	3,53	3,77	0,63	4,18	3,01	5,62
Phycodnaviridae	0	0	1,32	0,25	0	0	0,83	0,54	0	0	0	0,40
Picornaviridae	0,84	2,08	0	11,33	11,58	0	0,83	1,08	0,63	2,09	1,50	2,41
Polyomaviridae	0	0,69	0	0	0	0	0	0	0	0	0	0
Reoviridae	0,84	0	0	0,49	0	0	0,42	0	0	0	0	0,80
Tombusviridae	0	0	0	0	0,53	0,83	0	0	0	1,05	2,26	0
Virgaviridae	14,23	22,22	39,47	4,43	5,79	8,33	15,80	10,78	16,25	9,76	24,06	12,05

	SAP-2b NY1	SAP-2b NY2	SAP-2b NY3	SAP-2b NC1	SAP-2b NC2	SAP-2b NC3	SAP-1 NY1	SAP-1 NY2	SAP-1 NY3	SAP-1 NC1	SAP-1 NC2	SAP-1 NC3
SAP-2b NY1												
SAP-2b NY2	**0,7**											
SAP-2b NY3	**0,77**	**0,67**										
SAP-2b NC1	0,70	0,75	0,75									
SAP-2b NC2	0,72	0,80	0,66	**0,71**								
SAP-2b NC3	0,60	0,70	0,73	**0,53**	**0,72**							
SAP-1 NY1	0,75	0,56	0,75	0,74	0,58	0,62						
SAP-1 NY2	0,79	0,68	0,82	0,68	0,62	0,61	**0,80**					
SAP-1 NY3	0,70	0,79	0,82	0,85	0,75	0,77	**0,76**	**0,71**				
SAP-1 NC1	0,67	0,77	0,77	0,83	0,83	0,73	0,70	0,69	0,90			
SAP-1 NC2	0,82	0,75	0,83	0,70	0,78	0,76	0,73	0,82	0,78	**0,89**		
SAP-1 NC3	0,88	0,69	0,80	0,80	0,64	0,61	0,86	0,87	0,72	**0,72**	**0,85**	

MATERIAL SUPLEMENTARI DE L'ARTICLE 4

List of primers and probes used for the dPCR assays

Virus	Primer/probe	Sequence	Final PCR conc*
HAdV	AdF	CWTACATGCACATCKCSGG	0,9 µM
	AdR	CRCGGGCRAAYTGCACCAG	0,9 µM
	AdP1	6-JUN-CCGGGCTCAGGTACTCCGAGGCGTCCT-QSY	0,225 µM
HEV	JVHEVF	GGTGGTTTCTGGGGTGAC	0,4 µM
	JVHEVR	AGGGGTTGGTTGGATGAA	0,4 µM
	JVHEVP	6-FAM-TGATTCTCAGCCCTTCGC-QSY	0,2 µM
NoV GII	QNIF2d	ATGTTCAGRTGGATGAGRTTCTCWGA	0.5 µM
	COG2R	TCGACGCCATCTTCATTCACA	0.9 µM
	QNIFS	6-ABY-AGCACGTGGGAGGGCGATCG-QSY	0.25 µM
E.coli	EC_F	GGTAGAGCACTGTTTTGGCA	0.4 µM
	EC_R	TGTCTCCCGTGATAACTTTCTC	0.4 µM
	EC_PChern	FAM-TCATCCCGACTTACCAACCCG-QSY	0.2 µM
Cryptosporidium spp.	Pan-cryptoF	CATGGATAACCGTGGTAAT	0.4 µM
	Pan-cryptoR	TACCCTACCGTCTAAAGCTG	0.4 µM
	Pan-cryptoP	FAM- CTAGAGCTAATACATGCGAAAAAA-QSY	0.2 µM

Cycling conditions for each dPCR

Virus	PCR cycling programe
HAdV	96°C 10 min, 40 x (95°C 15s, 60°C 1 min)
HEV	50ºC 10 min, 96ºC 10 min, 45 x (95ºC 15s, 60ºC 1 min)
NoV GII	50ºC 10 min, 96ºC 10 min, 45 x (95ºC 15s, 60ºC 1 min)
E.coli	96°C 10 min, 40 x (95°C 15s, 55°C 1 min)
Cryptosporidium spp.	96°C 10 min, 40 x (95°C 15s, 55°C 1 min)

Summary of the studies included in the literature review used to generate Figure 3. The table details the tertiary treatment system design, sampling approach, and analytical methods applied for virus and bacteria concentration and detection. Abbreviations: SMF – skimmed milk flocculation; PEG – polyethylene glycol precipitation; qPCR – quantitative polymerase chain reaction; MPN – most probable number; NA – not applicable.

Reference	Tertiary system design	Sampling method	Virus concentration and detection method	Bacteria concentration and detection method
Simmons et al., 2011	MBR	Grab sampling	Positive filtration + flocculation	NA
			qPCR	
ZHou et al., 2015	MBR	Grab sampling	PEG	Filtration-membrane
			qPCR	Culture and plate count
Bailey et al., 2018	UV+Chlorination	Grab sampling	Ultrafiltration + PEG	
			qPCR	MPN method
Rusiñol et al., 2015	UV+Chlorination	Grab sampling	SMF	
			qPCR	MPN method
Rusiñol et al., 2020	Wetlands	Grab sampling	SMF	
			qPCR	MPN method
Rachmandi et al., 2016	Wetlands	Grab sampling	Electronegative filtration	NA
			qPCR	

ALTRES PUBLICACIONS

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

- Martínez-Puchol, S., Itarte, M., Rusiñol, M., Forés, E., **Mejías-Molina, C.,** Andrés, C., Antón, A., Quer, J., Abril, J. F., Girones, R., Bofill-Mas, S,. (2021). *Exploring the diversity of coronavirus in sewage during COVID-19 pandemic: Don't miss the forest for the trees.* Science of the Total Environment, 800, 149562. IF: 7.137 (Q1)

- Guerrero-Latorre, L., Collado, N., Abasolo, N., Anzaldi, G., Bofill-Mas, S., Bosch, A., Bosch, L., Busquets, S., Caimari, A., Canela, N., Carcereny, A., Chacón, C., Ciruela, P., Corbella, I., Domingo, X., Escoté, X., Espiñeira, Y., Forés, E., Gandullo-Sarró, I., Garcia-Pedemonte, D., Girones, R., Guix, S., Hundesa, A., Itarte, M., Mariné-Casadó, R., Martínez, A., Martínez-Puchol, S., Mas-Capdevila, A., **Mejías-Molina, C.**, Moliner i Rafa, M., Munné, A., Pintó, R. M., Pueyo-Ros, J., Robusté-Cartró, J., Rusiñol, M., Sanfeliu, R., Teichenné, J., Torrell, H., Corominas, L., Borrego, C. M. (2022). *The Catalan Surveillance Network of SARS-CoV-2 in sewage: design, implementation, and performance.* Scientific Reports, 12(1), 1–10. IF: 4.996 (Q1)

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- Zammit, I., Badia, S., **Mejías-Molina, C.**, Rusiñol, M., Bofill-Mas, S., Borrego, C. M., Corominas, L. (2024). *Zooming in to the neighborhood level: A year-long wastewater-based epidemiology monitoring campaign for COVID-19 in small intraurban catchments.* Science of the Total Environment, 907, 167811. IF: 8.2 (Q1)

- Itarte, M., Calvo, M., Martínez-Frago, L., **Mejías-Molina, C.**, Martínez-Puchol, S., Gironés, R., Medema, G., Bofill-Mas, S., Rusiñol, M. (2024). *Assessing environmental exposure to viruses in wastewater treatment*

plant and swine farm scenarios with next-generation sequencing and occupational risk approaches. International Journal of Hygiene and Environmental Health, 259, 114360. IF: 1.51 (Q1)

- Tarradas-Alemany, M., Martínez-Puchol, S., **Mejías-Molina, C.**, Itarte, M., Rusiñol, M., Bofill-Mas, S., Abril, J. F. (2024). *CAPTVRED: an automated pipeline for viral tracking and discovery from capture-based metagenomics samples.* Bioinformatic Advances, 4(1), vbae150. IF: 2.4 (Q1)

- Martínez-Puchol, S., Tarradas-Alemany, M., **Mejías-Molina, C.**, Itarte, M., Rusiñol, M., Baliellas, J., Abasolo, N., Canela, N., Monastiri, A., López-Roig, M., Serra-Cobo, J., Abril, J. F., Bofill-Mas, S. (2024). *Target enrichment metaviromics enables comprehensive surveillance of coronaviruses in environmental and animal samples.* Heliyon, 10(11), e31556. IF: 3.4 (Q1)

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Exploring the diversity of coronavirus in sewage during COVID-19 pandemic: Don't miss the forest for the trees

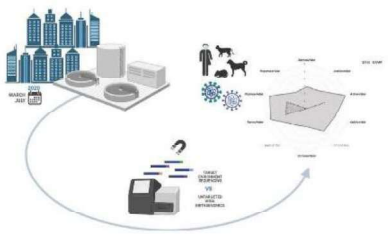
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HIGHLIGHTS

- NGS methods have been applied to study the sewage virome during COVID-19 pandemic.
- *Coronaviridae* sequences were not detected in sewage using untargeted metagenomics.
- Target Enrichment provided with SARS-CoV-2 sequences as part of the sewage virome.
- Human and animal CoV co-circulation in sewage only detected with Target Enrichment.

GRAPHICAL ABSTRACT



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ABSTRACT

In the wake of the COVID-19 pandemic, the use of next generation sequencing (NGS) has proved to be an important tool for the genetic characterization of SARS-CoV-2 from clinical samples. The use of different available NGS tools applied to wastewater samples could be the key for an in-depth study of the excreted virome, not only focusing on SARS-CoV-2 circulation and typing, but also to detect other potentially pandemic viruses within the same family. With this aim, 24-hours composite wastewater samples from March and July 2020 were sequenced by applying specific viral NGS as well as target enrichment NGS. The full virome of the analyzed samples was obtained, with human *Coronaviridae* members (CoV) present in one of those samples after applying the enrichment. One contig was identified as HCoV-OC43 and 8 contigs as SARS-CoV-2. CoVs from other animal hosts were also detected when applying this technique. These contigs were compared with those obtained from contemporary clinical specimens by applying the same target enrichment approach. The results showed that there is a co-circulation in urban areas of human and animal coronaviruses infecting domestic animals and rodents. NGS enrichment-based protocols might be crucial to describe the occurrence and genetic characteristics of SARS-CoV-2 and other *Coronaviridae* family members within the excreted virome present in wastewater.

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The Catalan Surveillance Network of SARS-CoV-2 in Sewage: design, implementation, and performance

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Wastewater-based epidemiology has shown to be an efficient tool to track the circulation of SARS-CoV-2 in communities assisted by wastewater treatment plants (WWTPs). The challenge comes when this approach is employed to help Health authorities in their decision-making. Here, we describe the roadmap for the design and deployment of SARSAIGUA, the Catalan Surveillance Network of SARS-CoV-2 in Sewage. The network monitors, weekly or biweekly, 56 WWTPs evenly distributed across the territory and serving 6 M inhabitants (80% of the Catalan population). Each week, samples from 45 WWTPs are collected, analyzed, results reported to Health authorities, and finally published within less than 72 h in an online dashboard (https://sarsaigua.icra.cat). After 20 months of monitoring (July 20–March 22), the standardized viral load (gene copies/day) in all the WWTPs monitored fairly matched the cumulative number of COVID-19 cases along the successive pandemic waves, showing a good fit with the diagnosed cases in the served municipalities (Spearman Rho = 0.69). Here we describe the roadmap of the design and deployment of SARSAIGUA while providing several open-access tools for the management and visualization of the surveillance data.

Shortly after the onset of the outbreak of the current COVID-19 pandemic, scientists renewed their interest in the application of wastewater-based epidemiology (WBE)[1] to track the communal circulation of SARS-CoV-2 through the quantification of its genetic traces in sewage[2]. This resulted in an overwhelming number of

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Evaluation of pathogen disinfection efficiency of electrochemical advanced oxidation to become a sustainable technology for water reuse

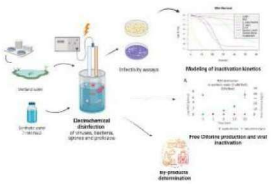
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HIGHLIGHTS

- Electrochemical disinfection for safe water production with low energy consumption.
- Disinfection of bacteria, bacteria spores, bacteriophages, virus, and protozoa.
- Description of inactivation kinetics using logistic, exponential, and linear models.
- Disinfection efficiency is limited for virus, spores and amoebas.
- Bacteria and bacteriophages are not a suitable model for viral disinfection.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Electrochemical disinfection
Microbial pathogens
Microbial indicators
Inactivation kinetics
Viruses
Water reuse

ABSTRACT

Water treatment and reuse is gaining acceptance as a strategy to fight against water contamination and scarcity, but it usually requires complex treatments to ensure safety. Consequently, the electrochemical advanced processes have emerged as an effective alternative for water remediation. The main objective here is to perform a systematic study that quantifies the efficiency of a laboratory-scale electrochemical system to inactivate bacteria, bacterial spores, protozoa, bacteriophages and viruses in synthetic water, as well as in urban wastewater once treated in a wetland for reuse in irrigation. A Ti|RuO_2-based plate and Si|BDD thin-film were comparatively employed as the anode, which was combined with a stainless-steel cathode in an undivided cell operating at 12 V. Despite the low resulting current density (<15 mA/cm^2), both anodes demonstrated the production of oxidants in wetland effluent water. The disinfection efficiency was high for the bacteriophage MS2 (T99 in less than 7.1 min) and bacteria (T99 in about 30 min as maximum), but limited for CBV5 and TuV, spores and amoebas (T99 in more than 300 min). MS2 presented a rapid exponential inactivation regardless of the anode and bacteria

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Surveillance of SARS-CoV-2 in sewage from buildings housing residents with different vulnerability levels

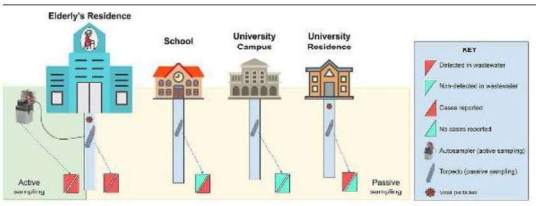
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HIGHLIGHTS

- The COVID-19 prevalence detected *via* wastewater was 0.4 % using active sampling and 2.2 % using passive sampling.
- In non-residential buildings, infrequent toilet use causes a decoupling between cases and sewage viral load.
- Passive sampling is an affordable tool to monitor COVID-19 prevalence in buildings.
- Sewage surveillance using passive samplers is complementary to clinical testing.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Warish Ahmed

Keywords:
COVID-19
Wastewater-based epidemiology
Wastewater surveillance, passive sampling

ABSTRACT

During the last three years, various restrictions have been set up to limit the transmission of the Coronavirus Disease (COVID-19). While these rules apply at a large scale (*e.g.*, country-wide level) human-to-human transmission of the virus that causes COVID-19, the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), occurs at a small scale. Different preventive policies and testing protocols were implemented in buildings where COVID-19 poses a threat (e.g., elderly residences) or constitutes a disruptive force (e.g., schools). In this study, we sampled sewage from different buildings (a school, a university campus, a university residence, and an elderly residence) that host residents of different levels of vulnerability. Our main goal was to assess the agreement between the SARS-CoV-2 concentration in wastewater and the policies applied in these buildings. All buildings were sampled using passive samplers while 24 h composite samples were also collected from the elderly residence. Results showed that passive samplers performed comparably well to composite samples while being cost-effective to keep track of COVID-19 prevalence. In the elderly residence, the comparison of sampling protocols (passive *vs.* active) combined with the strict clinical testing allowed us to compare the sensitivities of the two methods. Active sampling was more sensitive than passive sampling, as the former was able to detect a COVID-19 prevalence of 0.4 %, compared to a prevalence of 2.2 % for passive sampling. The number of COVID-19-positive individuals was tracked clinically in all the monitored buildings. More frequent detection of SARS-CoV-2 in wastewater was observed in residential buildings than in non-residential buildings using passive samplers. In all buildings, sewage surveillance can be used to complement COVID-19 clinical testing regimes, as the detection of SARS-CoV-2 in wastewater remained positive even when no COVID-19-positive individuals were reported. Passive sampling is useful for building managers to adapt their COVID-19 mitigation policies.

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Zooming in to the neighborhood level: A year-long wastewater-based epidemiology monitoring campaign for COVID-19 in small intraurban catchments

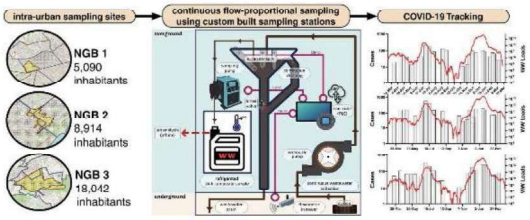
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HIGHLIGHTS

- SARS-CoV-2 surveillance in sewage at the neighborhood level was compared to the WWTP level.
- Correlations were higher at the neighborhood level as compared to the WWTP.
- Lower loads per clinical case were found during the Omicron wave.
- No differences were found across neighborhoods differing in sociocconomic status.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Kyle Bibby

Keywords:
Sewage surveillance
COVID-19
Small catchment
Intracity
Neighborhood scale

ABSTRACT

In recent years, wastewater-based epidemiology (WBE) has emerged as a valuable and cost-effective tool for monitoring the prevalence of COVID-19. Large-scale monitoring efforts have been implemented in numerous countries, primarily focusing on sampling at the entrance of wastewater treatment plants (WWTPs) to cover a large population. However, sampling at a finer spatial scale, such as at the neighborhood level (NGBs), pose new challenges, including the absence of composite sampling infrastructure and increased uncertainty due to the dynamics of small catchments. This study aims to investigate the feasibility and accuracy of WBE when deployed at the neighborhood level (sampling in sewers) compared to the city level (sampling at the entrance of a WWTP). To achieve this, we deployed specific WBE sampling stations at the intraurban scale within three NGBs in Barcelona, Spain. The study period covers the 5th and the 6th waves of COVID-19 in Spain, spanning from March 2021 to March 2022, along with the WWTP downstream from the NGBs. The results showed a strong correlation

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Assessing environmental exposure to viruses in wastewater treatment plant and swine farm scenarios with next-generation sequencing and occupational risk approaches

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ARTICLE INFO

Keywords:
Occupational exposure
Aerosol sampling
Sticker-based surface sampling
Target enrichment sequencing
Viral metagenomics
Quantitative microbial risk assessment

ABSTRACT

Occupational exposure to pathogens can pose health risks. This study investigates the viral exposure of workers in a wastewater treatment plant (WWTP) and a swine farm by analyzing aerosol and surfaces samples. Viral contamination was evaluated using quantitative polymerase chain reaction (qPCR) assays, and target enrichment sequencing (TES) was performed to identify the vertebrate viruses to which workers might be exposed. Additionally, Quantitative Microbial Risk Assessment (QMRA) was conducted to estimate the occupational risk associated with viral exposure for WWTP workers, choosing Human Adenovirus (HAdV) as the reference pathogen. In the swine farm, QMRA was performed as an extrapolation, considering a hypothetical zoonotic virus with characteristics similar to Porcine Adenovirus (PAdV). The modelled exposure routes included aerosol inhalation and oral ingestion through contaminated surfaces and hand-to-mouth contact.

HAdV and PAdV were widespread viruses in the WWTP and the swine farm, respectively, by qPCR assays. TES identified human and other vertebrate viruses WWTP samples, including viruses from families such as *Adenoviridae*, *Circoviridae*, *Orthoherpesviridae*, *Papillomaviridae*, and *Parvoviridae*. In the swine farm, most of the identified vertebrate viruses were porcine viruses belonging to *Adenoviridae*, *Astroviridae*, *Circoviridae*, *Herpesviridae*, *Papillomaviridae*, *Parvoviridae*, *Picornaviridae*, and *Retroviridae*.

QMRA analysis revealed noteworthy risks of viral infections for WWTP workers if safety measures are not taken. The probability of illness due to HAdV inhalation was higher in summer compared to winter, while the greatest risk from oral ingestion was observed in workspaces during winter. Swine farm QMRA simulation suggested a potential occupational risk in the case of exposure to a hypothetical zoonotic virus.

This study provides valuable insights into WWTP and swine farm worker's occupational exposure to human and other vertebrate viruses. QMRA and NGS analyses conducted in this study will assist managers in making evidence-based decisions, facilitating the implementation of protection measures, and risk mitigation practices for workers.

1. Introduction

People can be exposed to various risk factors in workplaces, with biological factors being of particular importance. Exposure to bioaerosols in the occupational environment has garnered attention due to its association with various health effects, including infectious diseases, acute toxic effects, allergies, and cancer (Douwes et al., 2003). Bioaerosols are composed of small particles containing microorganisms

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Application Note

OXFORD

Metagenomics

CAPTVRED: an automated pipeline for viral tracking and discovery from capture-based metagenomics samples

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Abstract

Summary: Target Enrichment Sequencing or Capture-based metagenomics has emerged as an approach of interest for viral metagenomics in complex samples. However, these datasets are usually analyzed with standard downstream Bioinformatics analyses. CAPTVRED (*Capture-based metagenomics Analysis Pipeline for tracking ViRal species from Environmental Datasets*), has been designed to assess the virome present in complex samples, specially focused on those obtained by Target Enrichment Sequencing approach. This work aims to provide a user-friendly tool that complements this sequencing approach for the total or partial virome description, especially from environmental matrices. It includes a setup module which allows preparation and adjustment of the pipeline to any capture panel directed to a set of species of interest. The tool also aims to reduce time and computational cost, as well as to provide comprehensive, reproducible, and accessible results while being easy to costume, set up, and install.

Availability and implementation: Source code and test datasets are freely available at `github` repository: https://github.com/CompGenLabUB/CAPTVRED.git

1 Introduction

Over the past 3 years, the benefits of virome analyses from environmental samples to monitor the species (or even strains) present in each geographical region have become clear for the scientific community. Despite the recent advancements in the detection, concentration, and subsequent bioinformatic analyses of these samples (Mastriani *et al.* 2022), several major challenges remain to be addressed. Virome studies from environmental samples pose particular difficulties due to the high presence of contaminants, the low concentration of viral particles, and the substantial proportion of phages and bacteria, making it challenging to obtain sufficient high-quality genomic material to accurately represent the entire eukaryotic virome. Consequently, concentration methods are required, together with amplification or enrichment approaches, to obtain a comprehensive representation of the virome of interest in the environment under study (Rusiñol *et al.* 2020, McClary-Gutierrez *et al.* 2021). These challenges, as well as the impact on health and economy of the recent pandemic outbreak, have led to the launch of several projects aimed at improving our understanding of potential pandemic viruses through the analyses of environmental samples from a One-Health perspective (Sinclair 2019).

Metagenomics approaches based on high-throughput sequencing to assess viral diversity have been shown to provide more comprehensive information than previous analyses using conventional molecular protocols (Donaldson *et al.* 2010, Hjelmsø *et al.* 2017, Martínez-Puchol *et al.* 2020). Several approaches are available for processing the samples depending on the goal of the analysis. The amplicon sequencing approach—such as ARTIC (Quick 2020)—is widely used for describing sequence variability and polymorphisms in a full or partial genome. In contrast, the whole-genome sequencing (WGS) approach enables the characterization of all the genomic material present in a sample (Garner *et al.* 2021). Halfway, when the interest is on a set of species or families that constitute a small fraction of the entire virome, the capture-based approach provides higher sensitivity. This method is based on the design of capture probes selected from a set of genomic sequences of the species of interest (e.g. `VirCapSeq-VERT`; Briese *et al.* 2015). By using these species-specific probes, the samples are enriched with the targeted sequences by positive selection. In clinical samples, the capture-based metagenomics approach has been proved to achieve a 100- to 1000-fold increase in sequenced reads of interest, reducing the background noise (such as host DNA), and increasing coverage up to 95% (Briese *et al.* 2015).

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Research article

Target enrichment metaviromics enables comprehensive surveillance of coronaviruses in environmental and animal samples

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A B S T R A C T

The COVID-19 pandemic has underscored the importance of understanding the role of animals in the transmission of coronaviruses (CoVs) and their impact on human health. A One Health approach, integrating human, animal, and environmental health, is essential for effective CoVs control. Next-generation sequencing has played a pivotal role in identifying and monitoring the evolution of novel CoVs strains, like SARS-CoV-2. However, viral occurrence and diversity studies in environmental and animal samples are challenging because of the complexity of viral communities and low abundance of viruses in these samples.

Target enrichment sequencing (TES) has emerged as a valuable tool for investigating viral families in challenging samples. This approach involves the specific capture and enrichment of viral genomes using sequence-specific probes, thereby enhancing the efficiency of detection and characterization.

In this study, we aimed to develop and validate a TES panel to study CoVs in various complex environmental and animal derived samples. The results demonstrated the panel's effectiveness in capturing and sequencing a wide diversity of CoVs providing valuable insights into their abundance and host diversity in urban wastewater, farm animal corpses lixiviates and bat guano samples. In sewage samples, CoVs were detected solely when TES was employed while in guano samples, sequencing of CoVs species was achieved in 2 out of 4 samples showing an almost three-logarithmic increase in the number of reads obtained in comparison with the untargeted approach. For animal lixiviates, only the TES application enabled the acquisition of CoVs reads. The information obtained can significantly contribute to early detection, surveillance, and control measures for CoVs, including viral discovery and potential spillover events. Additionally, this sequencing panel shows potential for studying other significant viral families and monitoring viral diversity in different animal populations.

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Article

Tracking Age-Linked Antibiotic Resistance Patterns through Building-Level Wastewater Analysis

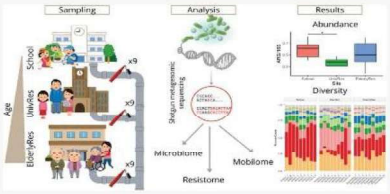
Anna Pico-Tomàs,* Alejandro Sanchís, Cristina Mejías-Molina, Marc Comas-Cufí, José Luis Balcázar, Sílvia Bofill-Mas, Helena Torrell, Núria Canela, Carles M. Borrego, and Lluís Corominas

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ABSTRACT: Antimicrobial resistance (AMR) is a global health challenge, and monitoring different demographic populations can improve our understanding of its spread and prevalence in urban settlements. This study applies building-level wastewater-based epidemiology (WBE) to analyze the resistome and mobilome of age-segregated populations from an elementary school (School), a university residence (UnivRes), and an elderly care facility (ElderlyRes) all located in Girona (Catalonia, Spain). Metagenomic analyses were subsequently conducted to investigate differences in bacterial communities, antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs). The results revealed age-linked variations in the relative abundance and diversity of ARGs. The wastewater collected at the School exhibited the highest abundance of ARGs, while the ElderlyRes showed the highest diversity. Furthermore, sequences affiliated with bacterial pathogens were more prevalent in samples from both the School and the ElderlyRes, emphasizing potential public health implications. Among the 12 bacterial genera most strongly correlated with ARGs (Pearson $R > 0.7$), 11 were identified as members of the gut microbiota, underscoring their predominant role as reservoirs of resistance compared to bacteria of environmental origin. By integrating localized wastewater sampling with metagenomics, our study uncovers demographic-specific resistome patterns, delivering actionable evidence to strengthen AMR surveillance and intervention strategies in urban populations.



KEYWORDS: *antimicrobial resistance (AMR), wastewater-based epidemiology (WBE), resistome, mobilome, age-groups, sewers, passive sampling*

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1. INTRODUCTION

The human gut serves as a reservoir for antimicrobial resistance genes (ARGs), collectively known as the resistome.[1] Exposure to antibiotics either through therapeutic use or environmental sources exerts selective pressure on gut bacteria, fostering the proliferation and exchange of resistance genes. These resistant bacteria, along with their genetic material, are shed into wastewater systems, where they can disseminate further into the environment, contributing to the global antimicrobial resistance (AMR) burden.

Understanding how AMR varies across human populations is essential for developing targeted interventions to mitigate its spread. Age modulates a series of factors such as the immune system maturity, antibiotic usage patterns, dietary habits, and healthcare practices, which shape distinct gut microbiomes,[2−5] and thus, different resistome profiles within communities.[6] For instance, elderly individuals often exhibit a unique gut microbiota composition compared to children or young adults, influencing the abundance and diversity of resistant bacteria they host.[7] Recognizing these population-specific differences is crucial for moving beyond one-size-fits-all approaches to AMR management, enabling more effective strategies such as age-specific antibiotic stewardship or tailored infection control measures.

Despite the variations in the carriage of antibiotic-resistant bacteria in the individual's gut microbiota is well-known in relation to antibiotic usage,[8] little is known about how the resistome varies across age groups or how these variations could inform AMR management strategies. Historically, research on AMR in humans has relied on clinical samples, predominantly blood[9] or stool,[7,10,11] collected from symptomatic patients with limited ability to capture population-wide trends. To overcome these limitations, wastewater-based epidemiology (WBE) has emerged as a powerful approach for monitoring AMR at the community level.[12−14] WBE

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