UNIVERSITAT DE BARCELONA

Nuevas herramientas para la gestión de los recursos hídricos y los riesgos microbiológicos asociados

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Tesis realizada bajo la dirección del Dr. Francisco Lucena Gutiérrez y la Dra. Cristina García Aljaro en el Departamento de Genética, Microbiología y Estadística de la Facultad de Biología de la Universitat de Barcelona.

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La directora,

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La doctoranda,

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A mi familia

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ÍNDICE

LISTADO DE ABREVIATURAS

16S ARNr	Gen que codifica el ARN ribosomal 16S
ADN	Ácido desoxirribonucleico
ARN	Ácido ribonucleico
ASH	Agua, saneamiento e higiene
ASV	En inglés, *amplicon sequence variant*
ATP	Adenosina trifosfato
DALY	En inglés, *disability-adjusted life years*
DBO	Demanda Biológica de Oxígeno
DEUF	En inglés, *dead-end ultrafiltration method*
DGGE	Electroforesis en gel con gradiente desnaturalizante
DMA	Directiva Marco del Agua
DQO	Demanda Química de Oxígeno
EDAR	Estación Depuradora de Agua Residual
EF	Efluente secundario de la EDAR
ERA	Estación Regeneradora de Agua
ETAP	Estación de Tratamiento de Aguas Potables
FIO	En inglés, *faecal indicator organism*
FISH	Hibridación fluorescente in situ
GA17PH	Bacteriófagos de *Bacteroides thetaiotaomicron* GA17
HMBif	Marcador humano de *Bifidobacterium*
HSPF	En inglés, *hydrological simulation program-Fortran*
k	Constante de decaimiento de los microorganismos indicadores
l	litro
MALDI-TOF MS	En inglés, *matrix assisted laser desorption ionization-time of flight-mass spectrometry*
MARS	Microbiología de Agua Relacionada con la Salud
MST	En inglés, *microbial source tracking*
NGS	En inglés, *next-generation sequencing*
OMS	Organización Mundial de la Salud
P	Valor de probabilidad de significancia estadística

P75	Punto 75 m aguas abajo de la EDAR de la riera de Cànoves
P1000	Punto 1000 m aguas abajo de la EDAR de la riera de Cànoves
PCR	Reacción en cadena de la polimerasa
PSA	Planes sanitarios del agua
QMRA	En inglés, Q*uantitative microbial risk assessment*
qPCR	PCR cuantitativa
r	Coeficiente de correlación de Pearson
SBS	Secuenciación por síntesis
SDD	En inglés, *self-depuration distance*
SOMCPH	Colifagos somáticos
SSRC	Esporas de clostridios reductoras de sulfitos
SWAT	En inglés, *soil and water assessment tool*
T90	Tiempo para reducir el 90% la concentración de microorganismos
TGGE	Electroforesis en gel con gradiente de temperatura
TOC	Carbono orgánico total
Upper	Punto aguas arriba de la EDAR de la riera de Cànoves
USEPA	En inglés, *United States Environmental Protection Agency*
WAM	En inglés, *water assessment model*
°C	Grados Celsius
µm	Micrómetro

ABSTRACT

Water is essential for life and the development of human socio-economic activities, but water resources are not equally distributed across the planet and many areas are affected by water scarcity. During the last years the Mediterranean region has reduced considerably its water resources and it is expected an exacerbation of the current situation during the next years due to the climate change projections. For that reason, water management from One Health and Global Health perspectives is crucial and one of the main strategies is the water quality monitoring, including the microbial risk associated.

The main objective of this PhD thesis has been to develop new tools to perform the microbial water quality monitoring for the improvement of water management. This research has been developed in different studies giving rise to four scientific articles. The dynamics and natural inactivation processes of faecal indicator organisms, pathogens and bacterial communities in the environment and different water treatments and methodologies have been assessed in these studies.

The results obtained provide valuable information about the behaviour of different faecal microbial indicators in the environment, confirming the usefulness of crAssphage as a human source tracking marker. The dynamics of different microbial indicators have been modelled in a Mediterranean stream affected by an input of treated sewage water using two environmental drivers: streamflow and temperature. Changes in the structure and diversity of bacterial communities have also been observed in this river. However, it has been observed that the structure and diversity of the bacterial communities can recover downstream, showing high resilience to the anthropogenic impact. Finally, the detection of low numbers of microbial indicators and pathogens in environmental samples has been improved using a concentration method, confirming its suitability for the microbial risk assessment.

The outcome of this research contributes in the development and assessment of new tools for water management based on One Health and Global Health.

RESUMEN

El agua es esencial para el ser humano y el desarrollo de las actividades socioeconómicas, pero los recursos hídricos no están repartidos de forma equitativa en todo el planeta y la escasez hídrica afecta a muchas regiones. En los últimos años la zona mediterránea ha visto reducidos considerablemente sus recursos hídricos y las proyecciones del cambio climático prevén un agravamiento de la situación. Por esta razón la gestión del agua bajo las perspectivas de *One Health* y *Global Health* es crucial y una de las principales estrategias es la monitorización de la calidad del agua, incluyendo los riesgos microbiológicos asociados.

El principal objetivo de esta tesis doctoral ha sido desarrollar nuevas herramientas para la monitorización de la calidad microbiológica del agua y que permitan mejorar su gestión. Esta investigación se ha desarrollado en diferentes estudios transversales dando lugar a cuatro artículos científicos. En estos estudios se han evaluado las dinámicas y procesos naturales de inactivación de microorganismos indicadores de contaminación fecal, patógenos y comunidades bacterianas en el medio ambiente, además de tratamientos para la eliminación de los microorganismos en plantas potabilizadoras.

Los resultados obtenidos han generado información sobre el comportamiento de diferentes microorganismos indicadores de contaminación fecal en el medio ambiente y de crAssphage, confirmándolo como un marcador útil del origen humano de la contaminación fecal. Por otro lado, se han modelizado las dinámicas de diferentes microorganismos indicadores en un río mediterráneo a partir de dos factores ambientales como son el caudal y la temperatura. En dicho río también se han observado cambios en la estructura y diversidad de las comunidades bacterianas por la entrada de contaminación fecal. No obstante, también se ha observado una recuperación de las mismas aguas abajo. Finalmente, se ha contribuido a la mejora de la detección de los indicadores y patógenos que se encuentran en bajas concentraciones en las muestras ambientales mediante un método de concentración basado en la ultrafiltración, lo que supone una ventaja para la evaluación de riesgo microbiológico y la elaboración de los planes sanitarios del agua.

Los resultados de esta investigación contribuyen con el desarrollo y evaluación de herramientas para una gestión hídrica fundamentada en *One Health* y *Global Health*.

1. INTRODUCCIÓN

1.1 EL AGUA

1.1.1 El ciclo del agua

El agua es un compuesto elemental para el desarrollo de la vida en el planeta Tierra, donde cubre más del 71% de su superficie. A pesar de que la Tierra contiene aproximadamente 1386 millones de km^3 de agua, sólo el 2,5% es agua dulce y el 90% de ésta se encuentra en forma de hielo en los casquetes polares. El agua dulce subterránea y el agua de ríos y lagos constituyen un 0,5% y un 0,01% del total del agua del planeta Tierra, respectivamente. No obstante, el agua dulce tiene una gran relevancia, ya que permite el desarrollo de las actividades socioeconómicas.

El agua es un recurso renovable y como tal presenta un ciclo con el que interaccionan las diferentes actividades humanas (Figura 1).

Figura 1: El ciclo del agua y las interacciones de las actividades humanas con él (Seqwater, 2020).

En la Unión Europea en el año 2017 el 64% del agua extraída para los diferentes usos fue agua de río, mientras que un 24% fue agua subterránea (EEA, 2019). Dependiendo del origen del agua y de sus características, así como también del uso al que está destinada, se requieren unas características específicas para garantizar su salubridad. Además, los usos del agua generan cambios también en las propiedades del agua a nivel físico, químico y microbiológico. Esto hace que existan dos grandes tipos de tratamientos en función de su uso: los tratamientos de potabilización y los tratamientos de depuración de agua residual. Los tratamientos de potabilización son previos al uso del agua y tienen como objetivo eliminar los microorganismos y las sustancias que puedan suponer un peligro para la salud humana. Estos tratamientos se llevan a cabo en las ETAP (Estaciones de Tratamiento de Aguas Potables) y están diseñados como un sistema multibarrera para garantizar la eliminación de los microorganismos y sustancias que pueden poner en riesgo la salud humana. Por otro lado, los tratamientos de depuración se llevan a cabo en las EDAR (Estaciones Depuradoras de Agua Residual) después del uso del agua, ya que ésta puede verse afectada por el aporte de contaminantes que provocan cambios fisicoquímicos y biológicos en las propiedades del agua. Los efluentes secundarios de las EDAR pueden ser vertidos al medio ambiente o pueden ser sometidos a un tratamiento terciario para su uso. Los tratamientos terciarios son realizados en las ERA (Estaciones Regeneradoras de Agua) y el agua resultante es el agua regenerada.

1.1.2 Contexto social

El agua es un recurso imprescindible no sólo para la supervivencia del ser humano sino también para el desarrollo de las actividades socioeconómicas. Actualmente el sector primario (agricultura, acuicultura y ganadería) es el que conlleva con diferencia un mayor consumo de agua (69%), seguido de la industria (19%) y del consumo doméstico (12%). Sin embargo, las proyecciones de futuro sugieren un aumento de la proporción del consumo industrial y doméstico debido al incremento en la demanda de estos sectores en los países con las rentas más bajas (WWAP, 2019).

El incremento de la población mundial, junto con el desarrollo de los países y economías emergentes, ha conducido a un crecimiento de la demanda hídrica en torno al 1% anual desde 1980. Este ritmo de crecimiento se prevé mantener hasta 2050, incrementando así entre el 20 y el 30% la demanda hídrica actual (Burek et al., 2016).

En el año 2010 la Asamblea General de las Naciones Unidas reconoció por primera vez el derecho humano al agua y al saneamiento mediante la Resolución 64/292. Sin embargo, se estima que más de 2000 millones de personas viven en países con estrés hídrico siendo gran parte de ellos países con renta baja. El estrés hídrico hace referencia al déficit de agua, ya sea por la escasez (estrés hídrico físico) o por una calidad deficiente (Figura 2).

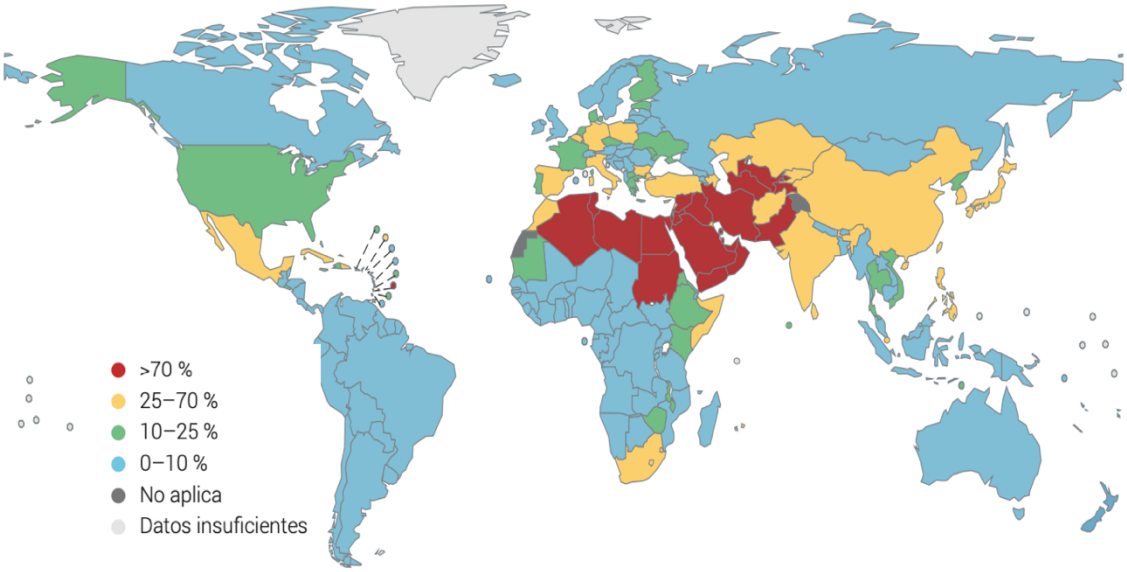

Figura 2: Nivel de estrés hídrico físico causado por escasez de agua (WWAP, 2019). El porcentaje hace referencia al agua dulce extraída respecto al total de recursos de agua dulce.

La escasez hídrica en la mayoría de los casos se produce de forma periódica llegando a afectar a 4000 millones de personas durante al menos un mes al año (Mekonnen and Hoekstra, 2016).

En los próximos años se espera un incremento del estrés hídrico, debido al crecimiento de la población mundial con el consiguiente incremento en la demanda hídrica y a las perspectivas del cambio climático, que auguran un descenso de los recursos hídricos (IPCC, 2013).

A pesar de que el acceso al agua y al saneamiento es un derecho fundamental reconocido, se estima que en el año 2016 un 3,3% de las muertes producidas en todo el planeta y un 4,6% de los DALYs (del inglés *disability-adjusted life years*: años de vida ajustados por discapacidad) fueron causados por acceso inadecuado al agua, saneamiento e higiene (ASH)(WHO, 2019).

El acceso inadecuado al ASH contribuye al desarrollo de diversas enfermedades a través de diferentes vías de transmisión (Prüss et al., 2002):

- Transmisión por ingestión hídrica, siguiendo la vía fecal-oral.

- Transmisión por una higiene personal pobre e inadecuada, doméstica o agrícola, que incluye la transmisión persona-persona, la transmisión fecal-oral por consumo de alimentos contaminados y el uso de agua contaminada para el riego de cultivos y la limpieza.
- Transmisión por contacto con agua contaminada.
- Transmisión de enfermedades a través de vectores que o bien proliferan en el agua o la utilizan como reservorio.
- Transmisión a través de aerosoles originados en agua contaminada.

La diarrea, es la enfermedad más frecuente causada por el acceso inadecuado a ASH, cuya transmisión sigue principalmente la ruta fecal-oral. Las estimaciones atribuyeron a inadecuados accesos a ASH un total de 829.000 muertes por diarrea en el año 2016, siendo el origen también del 8% de la mortalidad infantil en menores de 5 años (Prüss-Ustün et al., 2019) (Figura 3).

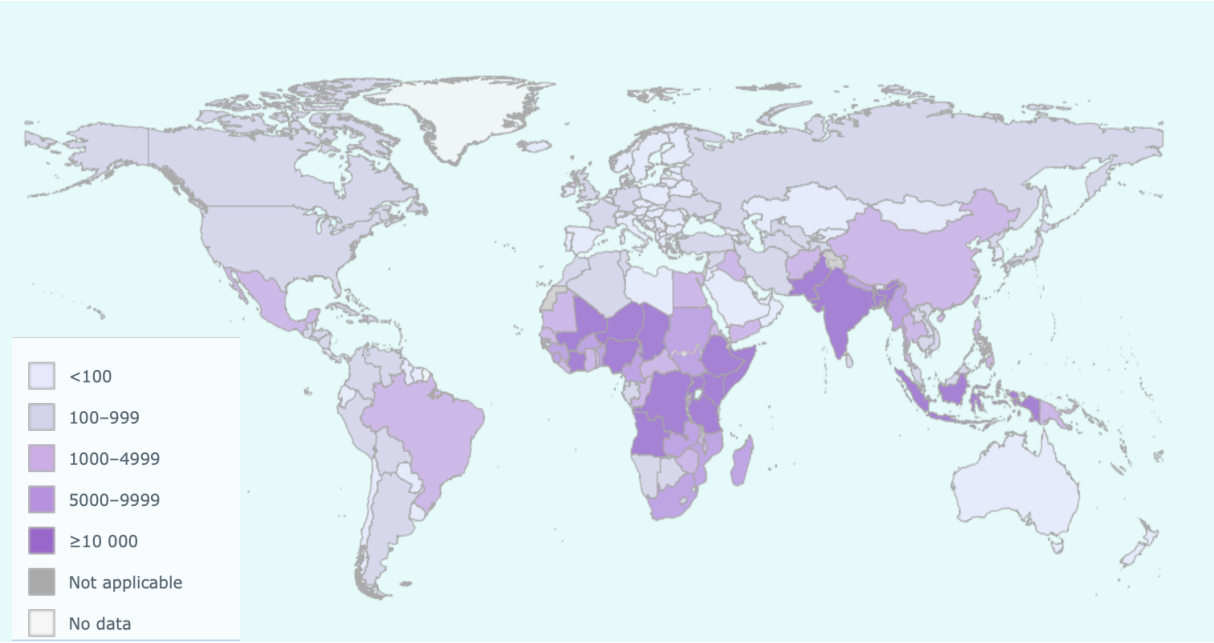

Figura 3: Mortalidad global por diarrea causada por inadecuado acceso a ASH. Adaptado de (WHO, 2019).

Se estima que el 35% de los casos de diarrea tienen su origen en la ingesta de agua contaminada, el 31% en el saneamiento inadecuado y el 12% en una higiene de manos inadecuada (WHO, 2014).

1.1.2.1 La problemática del agua en el clima mediterráneo

El clima mediterráneo se caracteriza por una marcada estacionalidad y por la alternancia de largos e intensos períodos de sequía con episodios de fuertes precipitaciones e inundaciones (Lloyd-Hughes and Saunders, 2002; Martin-Vide et al., 2008). La irregularidad de las precipitaciones es la principal causante de la baja disponibilidad de agua de la cuenca mediterránea, cuyo abastecimiento depende entre un 20 y un 50% del agua superficial llegando a suponer entre el 50 y el 90% de los recursos hídricos de las zonas semiáridas (De Jong et al., 2009).

En Europa durante el período 1990-2017 se produjo un descenso de los recursos hídricos en todas las regiones exceptuando el este del continente. Los mayores descensos se produjeron en España (65%), Malta (54%) y Chipre (32%), evidenciando la problemática hídrica de la zona mediterránea, que puede verse agravada en las próximas décadas (EEA, 2019).

La problemática de la escasez hídrica también está asociada a una demanda creciente de agua, incrementando también la presión antrópica sobre los recursos (García-Ruiz et al., 2011).

Las diferentes proyecciones de cambio climático auguran un descenso de la precipitación de hasta un 20%, lo que podría conllevar hasta una reducción de los recursos hídricos en torno al 30% (Milly et al., 2005) y de la escorrentía superficial en torno a un 50% en el sur de Europa (Arnell, 1999).

La escasez hídrica unida al incremento poblacional y la degradación del medio ambiente pueden tener un efecto sobre los ecosistemas acuáticos reduciendo su resiliencia frente a los cambios naturales y las perturbaciones de origen antrópico (Grantham et al., 2010), haciendo patente más que nunca su necesidad de gestión.

1.1.3 Contexto legislativo

Para garantizar la calidad del agua, el acceso y saneamiento de su población, los países han adoptado diferentes normas. Además, al tratarse en numerosas ocasiones de un recurso compartido por diferentes países, el consenso es necesario para establecer políticas de gestión comunes.

La Unión Europea aprobó en el año 2000 la Directiva 2000/60/CE por la que se establece un marco comunitario de actuación en el ámbito de la política de aguas, más conocida como la Directiva Marco del Agua (DMA) y ésta fue traspuesta al ordenamiento jurídico español mediante la Ley 62/2003.

La DMA se aprobó con la finalidad de alcanzar el buen estado de las diferentes masas de agua europeas (ríos, lagos, aguas subterráneas, continentales y aguas de transición) para el año 2015. Los objetivos de la DMA establecen la gestión integral de las masas de agua, considerándolas no sólo como un recurso, sino como elementos claves para garantizar la conservación del medio ambiente y de la salud animal, fundamentales para mantener la salud humana.

El buen estado de las masas de agua que persigue alcanzar la DMA tiene como objetivos principales:

- La protección de las masas de agua.
- La reducción de la contaminación de las masas de agua.
- La apuesta por el uso responsable y sostenible de los recursos hídricos.
- La regeneración de los ecosistemas acuáticos.

La DMA establece numerosos indicadores para poder evaluar la calidad del agua. Estos indicadores tienen diferente naturaleza y son indicadores biológicos, hidrogeomorfológicos, químicos, fisicoquímicos, así como también contaminantes específicos. A pesar de la gran diversidad de indicadores que establece la DMA, ésta no incluye indicadores microbiológicos.

En general, los valores límites de los diferentes parámetros recogidos en la legislación son variables dependiendo del uso al cual está destinado el agua, siendo siempre los más restrictivos en el caso del agua potable. Las normativas de agua destinadas a los diferentes usos humanos varían en función de cada país según su contexto social y geográfico. Sin embargo, organizaciones como la Organización Mundial de la Salud (OMS) o la *United States Environmental Protection Agency* (USEPA) establecen guías y directrices para el desarrollo de políticas de gestión del agua, saneamiento e higiene (USEPA, 1994; WHO, 2001ª). Las

directrices de la OMS se centran en los diferentes tipos de agua, que según su uso pueden suponer un riesgo para la salud humana y entre ellas destacan:

- Las Directrices sobre la calidad del agua potable.
- Las Directrices para ambientes seguros en aguas recreativas.
- Las Directrices para el uso sin riesgo de aguas residuales.

Además, desde el año 2004 la OMS en las Directrices sobre la calidad del agua potable ha promovido la creación de planes sanitarios del agua (PSA). Éstos consisten en la evaluación del riesgo en todas las etapas de suministro de agua potable desde su captación hasta el consumidor. En el año 2018 el ordenamiento jurídico español estableció mediante Real Decreto 902/2018 la obligatoriedad de la creación y aplicación de PSA en zonas que suministran agua a más de 50.000 habitantes.

El ordenamiento jurídico interno español contiene numerosas normativas relacionadas con la gestión de los recursos hídricos y el control de la calidad del agua. Las normativas de calidad del agua recogen los niveles máximos que puede presentar el agua de los diferentes parámetros físicos, químicos y microbiológicos para los diferentes usos a los que está destinada. Los parámetros microbiológicos recogidos por las normativas son microorganismos indicadores o patógenos de referencia (Tabla 1).

Los límites y los parámetros microbiológicos establecidos en la legislación para algunos tipos de agua dependen del uso final al que está destinada. Por ejemplo, en el caso del agua regenerada se diferencia entre los usos urbanos, agrícolas, industriales, recreativos y ambientales. Además, en cada tipo de uso se establecen calidades diferentes límites en función del riesgo que suponga la actividad para la salud humana.

La legislación española de aguas residuales urbanas no contempla el control de parámetros microbiológicos en el vertido al medio ambiente de los efluentes secundarios. A pesar de ello, la legislación sí establece límites de reducción de la demanda biológica de oxígeno (DBO) y la demanda química de oxígeno (DQO), siendo la primera una medida de la actividad microbiana llevada a cabo en el proceso de degradación de la materia orgánica.

Tabla 1: Principales usos del agua, legislación y parámetros microbiológicos que recoge.

Tipo de agua	Legislación	Parámetros microbiológicos
Aguas residuales urbanas	Real Decreto 817/2015	No establecidos
Aguas regeneradas	Real Decreto 1620/2007	Para todo tipo de usos: ▪ Nematodos intestinales ▪ *Escherichia coli* Dependiendo del uso: ▪ *Legionella* ▪ *Salmonella* ▪ Otros patógenos de referencia
Aguas de baño	Real Decreto 1341/2007	▪ Enterococos intestinales ▪ *Escherichia coli*
Aguas potables	Real Decreto 140/2003	▪ Enterococos intestinales ▪ *Escherichia coli* ▪ *Clostridium perfringens* ▪ *Cryptosporidium* (con turbidez superior a 5 UNT)
Aguas minerales y envasadas	Real Decreto 1799/2010	▪ *Escherichia coli* ▪ Estreptococos fecales ▪ *Pseudomonas aeruginosa* ▪ Recuento de colonias a 22 °C/Incubación 72 horas ▪ Recuento de colonias a 37 °C/Incubación 24 horas ▪ Anaerobios sulfito reductores esporulados

Actualmente se están actualizando las legislaciones europeas de aguas potables y aguas regeneradas. Los borradores de estas normativas incorporan a los bacteriófagos como parámetros microbiológicos a analizar. Cabe remarcar el hecho de que en los últimos años los bacteriófagos ya han sido incorporados en numerosas normativas y guías para el control de la calidad microbiológica del agua y los biosólidos en numerosos países (Tabla 2), por lo que se prevé su implementación próximamente también en la legislación española.

Tabla 2: Los bacteriófagos en las guías y regulaciones de países y organizaciones internacionales.

	Biosólidos	Agua subterránea	Agua recreativa	Agua potable	Agua regenerada	Reutilización directa agua potable
OMS			2017 (Guías)	2017 (recarga de acuíferos y riego)	2017 (Guías)	
EEUU		2006	2015 (Propuesta)	2011 (North Carolina)		
Canadá			2011 (Quebec)			
Colombia	2014					
Australia	2012 (Western Australia)			2011 (Guía nacional)	2005 (Queensland) 2011 (Western Australia)	
Singapur				2017 (Guía OMS)		2017 (Guía OMS)
India				2012		
Unión Europea				2020 (Directiva)	2020 (Directiva)	

Guías/ Recomendaciones	Normativa	Pendiente de aprobación

1.1.4 Los principios de *One Health* y *Global Health*

El concepto de *One Health*, creado en el año 2004, es un concepto que defiende la interconexión de la salud humana, animal y ambiental para llevar a cabo estrategias y políticas de gestión integradoras (WHO, 2017). Esta interconexión engloba numerosas disciplinas y sectores que son claves para el desarrollo de las estrategias de gestión (Figura 4).

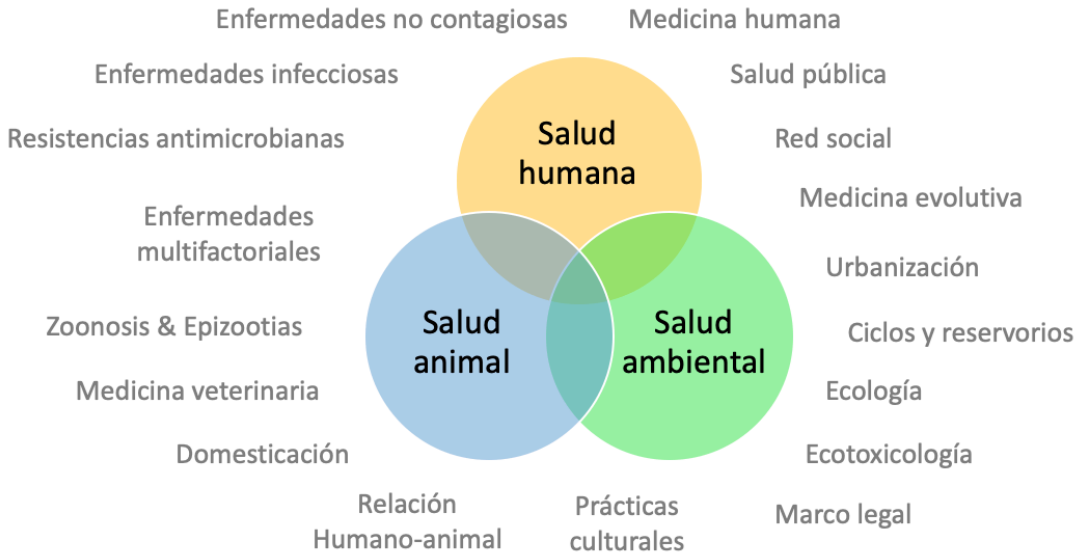

Figura 4: Disciplinas y sectores que engloba el concepto de *One Health*. Adaptado de (Destoumieux-Garzón et al., 2018).

Las relaciones entre la salud humana, animal y ambiental también se hacen patentes en los recursos hídricos, ya que numerosas enfermedades humanas de transmisión hídrica tienen su origen en animales y otros organismos y el estado de los hábitats acuáticos juega un papel fundamental en su diseminación. Por ello, la DMA con su gestión integral de las masas de agua sigue el principio de *One Health*.

Al concepto de *One Health* también hay que sumarle el de *Global Health*. Este concepto se define como el área de estudio, investigación y práctica que tienen como objetivo prioritario mejorar la salud y alcanzar una salud igualitaria para toda la población del planeta (Koplan et al., 2009).

La relación entre *One Health* y *Global Health* es evidente, ya que las conexiones que existen en el planeta también hacen que la salud humana, animal y ambiental de un territorio concreto repercuta sobre la salud de todo el planeta. Estas conexiones pueden ser de diferente índole y no siempre están controladas por la acción humana. El comercio, los movimientos migratorios, los fenómenos meteorológicos y las comunicaciones pueden ser

responsables de que cualquier problemática de salud pueda ser compartida a nivel global en muy poco tiempo (Figura 5).

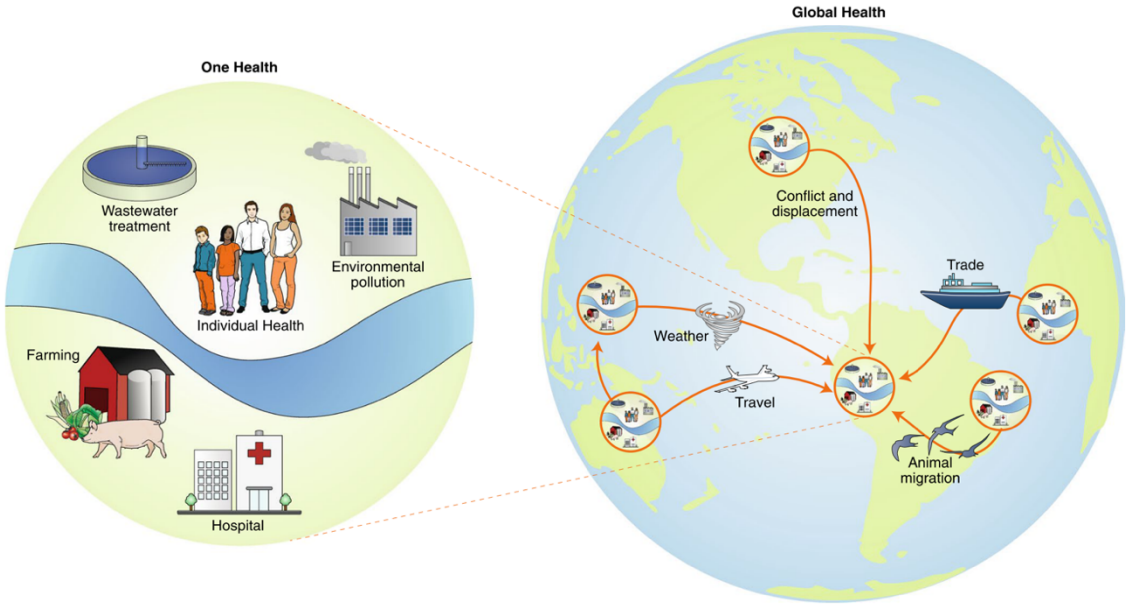

Figura 5: Representación de la conexión entre *One Health* y *Global Health* (Hernando-Amado et al., 2019).

Uno de los ejemplos más recientes de conexión entre *One Health* y *Global Health* es la pandemia causada este 2020 por el virus SARS-CoV-2, un virus de origen zoonótico, que hizo el salto al ser humano en China presumiblemente por malas prácticas de manipulación de animales y que ya se ha expandido en todo el planeta (Zhou et al., 2020).

Otra de las grandes problemáticas de salud global es la dispersión de genes de resistencias a antibióticos. El uso excesivo de antibióticos, por ejemplo, en la industria ganadera, hace que se generen resistencias que llegan al medio ambiente, a los alimentos y al ser humano, donde se encuentran ampliamente distribuidos (Colomer-Lluch et al., 2011; Larrañaga et al., 2018; Perreten et al., 1997). El comercio internacional de alimentos y el transporte de los seres humanos provoca la dispersión de estos genes de resistencia a antibióticos a nivel global, convirtiéndolo en una de las grandes amenazas para la salud global del planeta (WHO, 2018).

1.2 LA GESTIÓN DEL AGUA

Ante las condiciones presentes y las predicciones de futuro es imprescindible la gestión de los recursos hídricos. La gestión actual de los recursos tiene dos estrategias fundamentales: el incremento de los recursos hídricos y la monitorización del estado de los recursos actuales (Iglesias et al., 2007).

El incremento de los recursos hídricos puede llevarse a cabo mediante la aplicación de tecnologías que permiten el aprovechamiento de recursos no convencionales como son las aguas grises (agua residual de uso doméstico), el agua regenerada o el agua de mar.

La monitorización del estado de los recursos hídricos actuales es indispensable para garantizar su conservación y poder solventar y minimizar las perturbaciones naturales del sistema o aquellas derivadas de su uso antrópico.

Entre las líneas de investigación del grupo Microbiología de Aguas Relacionada con la Salud (MARS), donde se ha llevado a cabo esta tesis doctoral, relacionadas directa o indirectamente con la gestión del agua se encuentran: las mejoras metodológicas en la detección de patógenos y microorganismos indicadores de contaminación fecal, donde destaca una amplia trayectoria en investigación sobre los bacteriófagos; la trazabilidad del origen de la contaminación fecal en el agua y el estudio de la diversidad microbiana en ambientes acuáticos.

En esta tesis se han desarrollado y evaluado diferentes herramientas para monitorizar la calidad microbiológica del agua bajo las perspectivas de *One Health* y *Global Health,* profundizando desde las diferentes líneas de investigación del grupo.

En los siguientes apartados se introducen los campos en los que se han desarrollado los diferentes estudios que han sido llevados a cabo en esta tesis:

i. Microorganismos indicadores de contaminación fecal y patógenos.
ii. Trazabilidad de la contaminación fecal.
iii. Modelización de los parámetros microbiológicos.
iv. Las comunidades microbianas del agua.

1.2.1 Microorganismos indicadores de contaminación fecal y patógenos

La principal herramienta de gestión hídrica desde el punto de vista de la salud humana es el control de la calidad del agua. La calidad del agua requerida depende del uso al cual está destinada o del control del impacto sobre el ecosistema que la va a recibir y se puede medir a través de diferentes parámetros físicos, químicos y biológicos.

Entre los parámetros físicos y químicos que determinan la calidad del agua destacan el pH, la conductividad, la turbidez, el oxígeno disuelto, la materia orgánica, los sólidos en suspensión, los nutrientes como el nitrógeno y el fósforo en sus diferentes formas, así como también determinados elementos o compuestos químicos que puedan poner en riesgo la salud humana, animal o ambiental como los sulfatos, el cloro o el flúor, además de los contaminantes emergentes (Schwarzenbach et al., 2010). Los contaminantes emergentes son nuevos productos, químicos o compuestos xenobióticos sin normativa reguladora cuyos efectos sobre el medio ambiente y la salud humana son desconocidos como es el caso de numerosos fármacos (antiinflamatorios, analgésicos, hormonas, etc.), pesticidas, plastificadores, desinfectantes o compuestos clorados (Deblonde et al., 2011; Richardson and Ternes, 2018).

Los parámetros biológicos también son utilizados para evaluar la calidad del agua y abarcan un amplio rango de seres vivos, desde los microorganismos como los virus y las bacterias hasta los animales vertebrados, pasando por los vegetales.

La contaminación fecal es una de las grandes fuentes de contaminación del agua que tiene lugar como consecuencia del vertido al medio ambiente de residuos fecales humanos y animales. Por ello, la evaluación de diferentes parámetros relacionados con la contaminación fecal es una de las principales herramientas utilizadas en el control de la calidad del agua.

Los residuos fecales introducen grandes cantidades de microorganismos, entre los cuales hay patógenos, al ciclo de agua, poniendo así en riesgo la salud humana. Es imposible realizar el análisis de todos los patógenos del agua, por ello se utilizan diferentes aproximaciones. La principal aproximación es el análisis de microorganismos indicadores y de patógenos de referencia. Ambos grupos tienen la función de indicar la presencia de patógenos y por ello deben contar con una serie de características (Tabla 3).

Tabla 3: Características que deben presentar los microorganismos indicadores de contaminación fecal y los patógenos de referencia. Adaptado de WHO (2011).

Microorganismo indicador	**Patógeno de referencia**
▪ Estar presente en elevadas concentraciones en las heces humanas y animales. ▪ No multiplicarse en el medio ambiente. ▪ No ser patógeno. ▪ Tener una persistencia en el medio ambiental similar a la de los patógenos fecales. ▪ Estar en concentraciones más elevadas que los patógenos. ▪ Responder a los tratamientos de forma similar a los patógenos. ▪ Ser detectado mediante técnicas de cultivo que sean rápidas y con bajo coste.	▪ Presentar una vía de transmisión fecal-oral. ▪ Disponer de información suficiente de la dosis-respuesta y epidemiología en humanos para realizar la evaluación de riesgo microbiológico (QMRA). ▪ Estar presente en el origen. ▪ Persistir en el medio ambiente. ▪ Presentar una sensibilidad a los tratamientos de inactivación o eliminación similar a la del grupo que representan. ▪ Presentar una infectividad, severidad e incidencia de la enfermedad similar a la del grupo que representan.

El uso de microorganismos indicadores para el control de la calidad del agua se remonta a casi un siglo atrás (National Research Council US, 2004) y se distingue entre tres tipos de microorganismos indicadores (WHO, 2001):

- Indicadores de proceso: grupo de microorganismos que demuestran la eficacia de tratamientos y procesos.
- Indicadores fecales: indican la presencia de contaminación fecal e infieren la presencia de patógenos.
- Microorganismos índices y modelos: microorganismos indicativos de la presencia de patógenos y con un comportamiento similar al de éstos.

Hasta finales del siglo pasado los microorganismos indicadores utilizados para el control de la calidad microbiológica del agua eran principalmente bacterias entéricas, especialmente *Escherichia coli* y otros coliformes (Pipes et al., 1977). Sin embargo, la comunidad científica

manifestó que determinados grupos de patógenos como los virus o los protozoos, cuya persistencia es superior, podían permanecer en el agua cuando las bacterias no estaban presentes (Gerba et al., 1979; IAWPRC Study Group on Health Related Water Microbiology, 1991; Payment and Franco, 1993). De esta manera, otros microorganismos indicadores como los bacteriófagos o las esporas de clostridios reductores de sulfito han sido ampliamente investigados por su potencial para el control microbiológico de la calidad del agua (Armon and Kott, 1996; Bisson and Cabelli, 1980).

La monitorización de la calidad microbiológica del agua para el análisis de riesgo se basa en la detección de microorganismos por métodos de cultivo. El uso de bacterias como microorganismos indicadores presenta el inconveniente de que las bacterias pueden tener diferentes estados metabólicos dificultando su análisis, mientras que en los bacteriófagos sólo existen dos estados: infeccioso y no infeccioso. Por ello, la utilización de los bacteriófagos como microrganismos indicadores de la contaminación fecal vírica en el agua, los biosólidos e incluso los alimentos está ampliamente aceptada y extendida (Lucena and Jofre, 2010) como se hace patente en su inclusión en las guías y normativas de calidad de agua, como se ha indicado anteriormente. Hay tres grupos principales de bacteriófagos utilizados para el control de la calidad del agua:

- Colifagos somáticos: son bacteriófagos que infectan *E. coli* por unión a su pared celular y que son excretados tanto por el ser humano como por los animales homeotermos. Son principalmente utilizados como indicadores de la contaminación viral y de proceso, aunque también se han propuesto en numerosas ocasiones como sustitutos de virus entéricos (Araujo et al., 1997; Gantzer et al., 1998; Moce et al., 2005).
- Bacteriófagos F-específicos: son bacteriófagos que infectan a través del pilus sexual. Los bacteriófagos FRNA a menudo son utilizados para la monitorización de los procesos y la calidad del agua. Además, están formados por diferentes genogrupos que tienen cierta especificidad de huésped (Ogorzaly and Gantzer, 2006).
- Bacteriófagos de *Bacteroides*: presentan un estrecho rango de huéspedes. No obstante, al tratarse *Bacteroides* de un género estrictamente anaerobio, los métodos de cultivo para la detección de estos bacteriófagos a pesar de estar estandarizados requieren más tiempo y tienen un coste más elevado (Lucena and Jofre, 2010).

Los patógenos de referencia pueden ser bacterias (*Salmonella* spp., *Campylobacter* spp., *Legionella* spp.), virus (enterovirus, norovirus), protozoos (*Giardia* spp., *Cryptosporidium* spp.)

y helmintos. Como se ha descrito anteriormente, los patógenos de referencia lo son en parte por haber sido ampliamente estudiados y contar con métodos de detección estandarizados. Sin embargo, estos métodos suelen ser más largos y costosos comparados con los de los microorganismos indicadores.

Otro de los principales inconvenientes que puede presentar el análisis de microorganismos indicadores y de patógenos de referencia generales es que no permiten discriminar entre el origen humano y animal de la contaminación fecal, por lo que se aborda más adelante en esta introducción.

1.2.1.1 Métodos de concentración de microorganismos indicadores y patógenos

Las bajas concentraciones de microorganismos indicadores y de patógenos que presentan algunos tipos de agua, requieren la concentración de grandes volúmenes. La concentración del agua puede ser primaria, donde se concentran unos cuantos litros hasta un volumen máximo de 1 l, o secundaria, donde el volumen obtenido de la concentración primaria se concentra hasta unos pocos mililitros. Las técnicas de concentración primaria de agua son muy diversas (Bridle, 2014; National Research Council US, 1999):

- Centrifugación: los microorganismos son concentrados por sedimentación al someter al agua a una fuerza centrífuga. Los principales inconvenientes de esta técnica de concentración son la dificultad de concentrar grandes volúmenes y que los microorganismos más pequeños como los virus necesitan elevadas velocidades (ultracentrifugación) para su sedimentación. La centrifugación puede ser empleada como método de concentración secundaria.
- Floculación-precipitación: técnica basada en la utilización de sustancias para formar flóculos con los microorganismos y su precipitación. Su principal inconveniente es que depende de las propiedades de los microorganismos para formar flóculos y dificulta la concentración de grandes volúmenes (superiores a los 10 l). También puede ser empleada como método de concentración secundaria.
- Adsorción-elución: técnica basada en la retención de los microorganismos en membranas o filtros por sus propiedades electrostáticas e hidrofóbicas, lo que puede ser un inconveniente para la concentración de algunos tipos de microorganismos. Suele combinarse con la filtración.

- Filtración: técnica basada en la retención de los microorganismos por su medida, por lo que pueden concentrarse todos los tipos de microorganismos.

Los protocolos estandarizados tanto para el análisis de los microorganismos indicadores como los de los patógenos de referencia suelen estar optimizados para un tipo de microorganismo, dificultando así el análisis del resto.

Los protocolos de concentración de bacterias y protozoos en grandes volúmenes de agua (superiores a los 10 l) establecen la filtración por membranas como la técnica más adecuada para su concentración primaria (Efstratiou et al., 2017; Henry et al., 2015). Algunos protocolos estandarizados para la concentración de protozoos utilizan la centrifugación de flujo continuo para concentrar volúmenes de hasta 50 l (USEPA, 2005).

En el caso de los virus, las técnicas de concentración empleadas son muy variadas, pero la mayoría de ellas son técnicas basadas en la adsorción-elución aprovechando las cargas que presentan las cápsides víricas. Estas técnicas pueden llevarse a cabo mediante filtros y cartuchos que pueden ser electropositivos o electronegativos, pero también mediante materiales como la lana de vidrio o NanoCeram® entre otros. Los principales inconvenientes para la mayoría de las técnicas de adsorción-elución es que necesitan preacondicionamiento de la muestra (ajuste de pH, adición de cationes, etc). Además, la presencia de materia orgánica y/o partículas puede dificultar la adsorción de los virus o su posterior elución (Costán-Longares, 2008).

La diversidad de técnicas existente hace patente la necesidad de métodos unitarios que permitan la concentración de virus, bacterias y protozoos de forma simultánea. De todos estos métodos, la filtración, al estar basada en el tamaño de partícula y no en sus propiedades, puede dar lugar a la mejor recuperación del conjunto de microorganismos (Liu et al., 2012). Existen numerosos estudios que tratan de discernir sobre cuál es el mejor material filtrante y la mejor técnica para llevar a cabo la concentración de los diferentes tipos de microorganismos en su conjunto. La ultrafiltración es la técnica que parece proporcionar los mejores resultados, con recuperaciones superiores al 50% para virus, bacterias y protozoos (Bridle, 2014; Liu et al., 2012; USEPA and CDC, 2011). A pesar de que otros métodos como la filtración mediante lana de vidrio o los cartuchos de NanoCeram® también permiten la concentración de bacterias, virus y protozoos en volúmenes superiores a los 100 l, éstos proporcionan mejores recuperaciones para los virus (de hasta un 80%), siendo menos efectivos para la concentración de bacterias y protozoos (0-12%) (Francy et al., 2013). Las

características fisicoquímicas de la muestra como pH y turbidez condicionan la recuperación de los microorganismos en los métodos de adsorción-elución (Bridle, 2014; Lee et al., 2011).

Para la ultrafiltración se emplean filtros con diámetros de poros de 0,02 a 0,1 µm y de materiales sintéticos no celulósicos como el poliacrilonitrilo o la polisulfona. Existen dos principales tipos de ultrafiltración que permiten la concentración simultánea de los diferentes microorganismos en grandes volúmenes de agua: la ultrafiltración de flujo tangencial y la ultrafiltración mediante fibras huecas (Olszewski et al., 2005).

En la ultrafiltración de flujo tangencial el flujo del agua es paralelo a las membranas y permite la recirculación de la muestra hasta su completa concentración. En cambio, la ultrafiltración mediante fibras huecas se basa en un entramado de fibras con microporos que permiten el paso del agua, pero que retienen los microorganismos en el exterior del entramado (Figura 6).

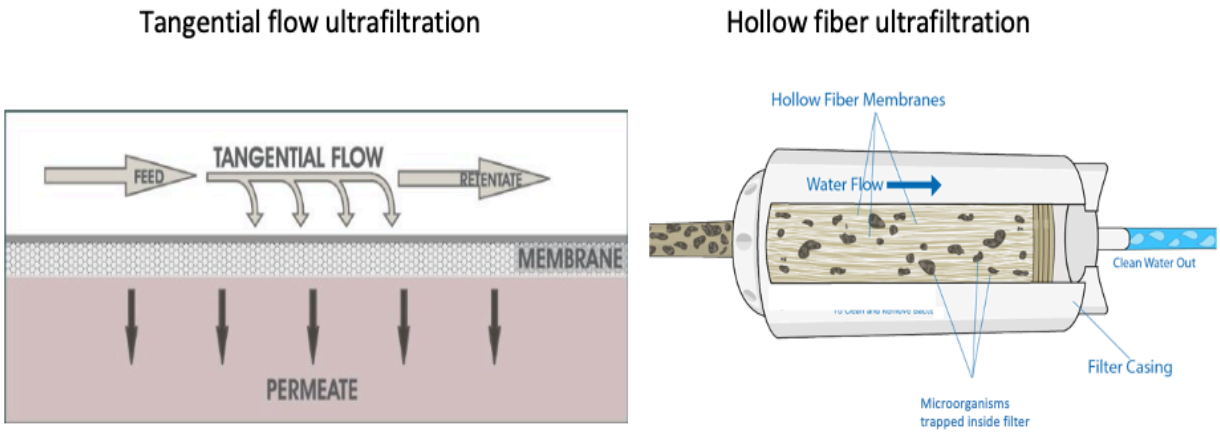

Figura 6: Esquema de la ultrafiltración de flujo tangencial y la ultrafiltración mediante fibras huecas. Adaptado de (Filter of hope, 2020; Taherian et al., 2015).

Existen dos principales variantes de ultrafiltración con fibras huecas: la que permite la recirculación del agua y la ultrafiltración sin salida (DEUF, del inglés: *dead-end ultrafiltration*). La recirculación de la muestra dificulta trabajar con grandes volúmenes, mientras que el método DEUF permite concentrar grandes volúmenes de agua (superiores a los 100 l) y se puede llevar a cabo *in situ*.

Cabe destacar que la mayoría de los estudios que emplean el método DEUF se han desarrollado y empleado en aguas potables o bajo condiciones controladas de laboratorio. Sin embargo, la aplicación de esta metodología en muestras ambientales, con características más complejas, no ha sido estudiada en profundidad. El desarrollo de métodos robustos para la

concentración simultánea de diferentes tipos de microorganismos en grandes volúmenes de agua es fundamental para el análisis de riesgo de los planes sanitarios del agua. Por ello, en esta tesis se ha evaluado el método DEUF para la concentración de microorganismos indicadores de contaminación fecal y patógenos de referencia en muestras ambientales y muestras de las diferentes etapas del tratamiento de potabilización del agua. El filtro utilizado es de fibras de polisulfona con una superficie efectiva de 2,5 m^2. Es un filtro creado para la diálisis de sangre, por lo que es capaz de retener compuestos tóxicos con un tamaño inferior al de los virus, los microorganismos de menor tamaño (Bosch et al., 2016).

1.2.2 Trazabilidad del origen de la contaminación fecal

Detectar la presencia de contaminación fecal es importante para evaluar la calidad del agua. Sin embargo, determinar el origen de la contaminación fecal es crucial para la gestión. A lo largo de las últimas décadas se han desarrollado diferentes técnicas de *faecal source tracking* (en inglés, trazabilidad del origen de la contaminación fecal). Existen diferentes parámetros para determinar el origen de la contaminación fecal:

- Métodos químicos: determinadas moléculas pueden ser utilizadas como indicadoras de la presencia de la contaminación fecal de origen humano como la cafeína, esteroles fecales, agentes blanqueadores o productos farmacéuticos.
- Métodos microbiológicos: conocidos como trazabilidad microbiológica de la contaminación fecal (del inglés *microbial source tracking* (MST)), consisten en la detección de microorganismos, moléculas o actividades llevadas a cabo por ellos.

Existen diferentes clasificaciones de los métodos de MST, pero las más comunes son las clasificaciones en función de la dependencia de bibliotecas y en función de la dependencia de cultivo (Tabla 4). A pesar del amplio abanico de métodos de MST, las técnicas más utilizadas son las técnicas independientes de bibliotecas o bases de datos.

Tabla 4: Métodos de MST más comunes. Adaptado de USEPA (2011).

Dependiente de bibliotecas		Independiente de bibliotecas	
Dependiente de cultivo			Independiente de cultivo
Fenotípicos	Genotípicos	Fenotípicos	Genotípicos
▪ Resistencia a antibióticos ▪ Uso de fuentes de carbono	▪ PCR múltiple ▪ PFGE: electroforesis en campo pulsado ▪ Ribotipado	▪ Bacteriófagos ▪ Cultivo bacteriano	▪ PCR de bacterias específicas de huésped ▪ PCR de virus específicos de huésped ▪ qPCR de bacterias específicas de huésped

Los géneros bacterianos *Bacteroides* y *Bifidobacterium* son dos géneros mayoritarios de la microbiota humana y de otros animales homeotermos. Además, numerosos estudios han encontrado especificidades de huésped para algunas de las especies de estos géneros por lo que se han utilizado ampliamente como marcadores del origen de la contaminación fecal (Ballesté et al., 2010; Casanovas-Massana et al., 2015; Gómez-Doñate et al., 2012). La

descripción de *Bacteroides* spp. y *Bifidobacterium* spp. específicas de huésped ha permitido el desarrollo de técnicas para su detección (Ballesté, 2009; Gómez-Doñate, 2014).

La detección de bacteriófagos específicos de huésped es otra de las técnicas empleadas en MST. Los bacteriófagos de *Bacteroides* o los genogrupos de los bacteriófagos FRNA permiten discriminar entre el origen humano y animal de la contaminación fecal, por lo que su potencial como marcadores de MST también ha sido ampliamente estudiado (Araujo et al., 1997; Jofre et al., 2014; Ogorzaly et al., 2009).

Los bacteriófagos específicos de ciertas cepas de especies de *Bacteroides*, principalmente *B. fragilis*, *B. thetaiotaomicron* y *B. ovatus* han sido utilizados en numerosos estudios de MST. No obstante, su utilización viene condicionada por la situación geográfica (Hagedorn et al., 2011). En el caso del sur de Europa, la cepa de *B. thetaiotaomicron* GA17 es la que permite discriminar mejor el origen humano de la contaminación fecal, frente a otras cepas como las de *B. fragilis* RYC2056 o HB13 (Hagedorn et al., 2011; Payan et al., 2005). También los fagos de *Bacteroides thetaiotaomicron* CW18, *B. fragilis* PG76 y *B. fragilis* PL122 han sido empleados para la detección de los orígenes bovino, porcino y aviar de la contaminación fecal (Gómez-Doñate et al., 2011).

Se han descrito cuatro genogrupos de fagos F-específicos: los genogrupos II y III, que se relacionan con muestras de origen humano y los genogrupos I y IV, que se relacionan con un origen animal de las muestras (Schaper et al., 2002). Los diferentes genogrupos pueden ser detectados mediante técnicas moleculares como la RTqPCR, pero los métodos de cultivo estandarizados para la detección de fagos FRNA no permiten diferenciar los genogrupos (Friedman et al., 2009).

Se han establecido una serie de requisitos que deberían cumplir los marcadores de MST ideales y aquellos que deberían cumplir los marcadores que pueden ser útiles para determinar el origen de la contaminación fecal (Tabla 5).

Tabla 5: Comparación de las características del marcador de MST ideal y el marcador de MST útil. Adaptado de Hagedorn et al. (2011).

Característica	**Marcador MST ideal**	**Marcador MST útil**
Especificidad	Marcador sólo encontrado en la especie huésped diana	Marcador distribuido de forma diferente en las especies huésped
Distribución en la población de huéspedes	Encontrado en todos los miembros de las especies huéspedes diana; contribuye a la sensibilidad del método	Encontrado consistentemente en las especies huésped cuyas heces impactan los lugares diana
Regularidad	La cantidad en las heces de los individuos es similar	Cantidad en orígenes combinados (agua residual, poblaciones animales) es similar
Estabilidad temporal en el huésped	La frecuencia y concentración en los individuos y poblaciones huéspedes no cambian a lo largo del tiempo	Aunque haya variaciones en la frecuencia y concentraciones en individuos los niveles en poblaciones son estables
Rango geográfico/ estabilidad	La frecuencia y concentración en poblaciones de huéspedes separadas geográficamente son similares	Puede ser consistentemente detectado y cuantificado a lo largo de un área geográfica para ser estudiado
Persistencia ambiental	Tasa de decaimiento constante en varias matrices y hábitats; no incrementa bajo ninguna condición; responde a tratamientos y procesos y las concentraciones son similares a las de los patógenos	Tasa de decaimiento predecible en varias matrices y hábitats; no incrementa en condiciones ambientales; responde a tratamientos y procesos y las concentraciones están caracterizadas
Evaluación cuantitativa	Puede ser cuantificado con precisión	Indica con precisión la presencia/ ausencia del origen de contaminación
Relevancia para los parámetros regulatorios	El marcador deriva de un organismo que aparece en las normativas	El marcador está correlacionado con un organismo que aparece en las normativas
Relevancia para el riesgo sanitario	El marcador está fuertemente correlacionado con el riesgo de todos los tipos de enfermedades de transmisión hídrica (gastroenteritis, dermatitis, etc.)	El marcador constituye un riesgo sanitario o está correlacionado con un subconjunto de enfermedades de transmisión hídrica (por ejemplo, gastroenteritis viral)

La microbiota intestinal está formada por grandes cantidades de microorganismos y varía entre especies y entre individuos de la misma especie porque está condicionada por factores como la edad, la dieta, el ambiente y la salud del individuo (Lozupone et al., 2012). Esta variabilidad entre individuos de la misma especie dificulta la obtención de marcadores de MST. Otro de los principales inconvenientes que presentan los diferentes marcadores de MST es la variabilidad geográfica que presentan, ya que el hecho de que la microbiota varíe en función de la dieta y de las condiciones del entorno, condiciona la obtención de marcadores que funcionen a lo largo de todo el planeta.

1.2.2.1 CrAssphage y su potencial como marcador viral de origen humano

Los bacteriófagos son los microorganismos más abundantes del planeta (Suttle, 2005) y los estudios metagenómicos han confirmado a los bacteriófagos como los grandes dominantes del viroma humano en abundancia y ubicuidad (Navarro and Muniesa, 2017). No obstante, debido a la gran variedad de bacteriófagos que existen, se hace difícil el desarrollo de técnicas moleculares para la detección de los grandes grupos utilizados como indicadores de contaminación fecal como los colifagos somáticos, los bacteriófagos F-específicos o los bacteriófagos de *Bacteroides*. Su análisis se realiza mediante técnicas de cultivo estandarizadas y las técnicas moleculares que existen se limitan a especies concretas de bacteriófagos como el fago F-específico MS2 (Lodder et al., 2013) o a genogrupos (Ogorzaly and Gantzer, 2006).

En el año 2014 la aplicación de técnicas de secuenciación masiva dio lugar a la descripción del grupo de bacteriófagos más abundante del viroma fecal, crAssphage (Dutilh et al., 2014). CrAssphage, cuyo nombre es un acrónimo del inglés cross-assembly phage, fue descubierto mediante el estudio de las secuencias metagenómicas fecales que estaban disponibles en las bases de datos. Su gran abundancia, ya que ha sido detectado en aproximadamente el 90% del viroma intestinal humano, y su amplia distribución geográfica lo han convertido en un candidato idóneo como microorganismo indicador de contaminación fecal vírica.

Los primeros estudios de crAssphage llevados a cabo ya apuntaban la posibilidad de que se tratara por su secuencia de un bacteriófago de *Bacteroides* con una morfología Podoviridae, caracterizada por su cola corta no contráctil (Dutilh et al., 2014; Yutin et al., 2018).

Estudios posteriores han conseguido el aislamiento de crAssphage a través de métodos de cultivo propagando en *Bacteroides intestinalis*. Además, este primer crAssphage aislado,

denominado ΦcrAss001, ha sido observado mediante microscopía electrónica confirmando su morfología compatible con la de la familia Podoviridae (Figura 7) (Shkoporov et al., 2018).

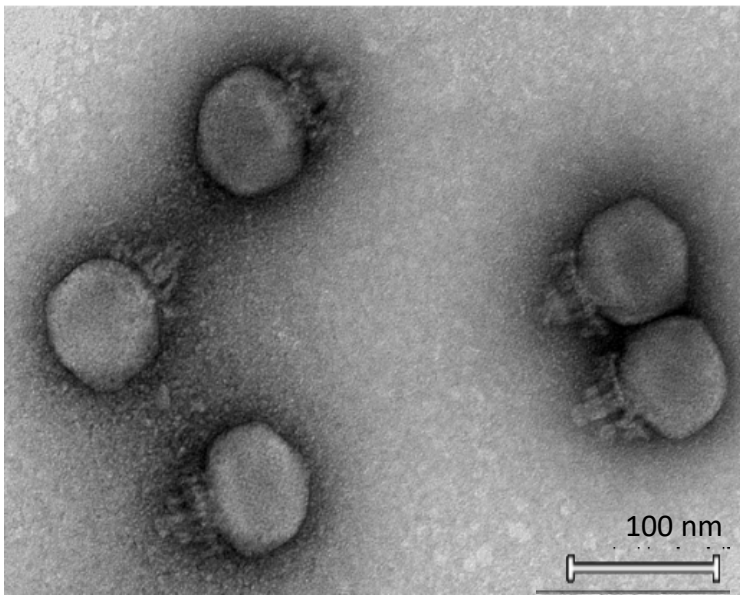

Figura 7: Imagen de ΦcrAss001 obtenida mediante microscopía electrónica de transmisión. Adaptado de Shkoporov et al. (2018).

Una de las principales características que presenta ΦcrAss001 es la peculiaridad de su ciclo replicativo. A pesar de que ΦcrAss001 es un bacteriófago virulento, es decir, que lleva a cabo el ciclo lítico, es capaz de coexistir con la bacteria huésped sin causar su lisis en una especie de equilibrio, que podría ser beneficioso para la bacteria y para el virus en un contexto de elevada competencia como es el intestino. Además, hasta ahora los intentos de conseguir lisógenos de ΦcrAss001 han fallado, pero otros crAssphage cuentan en su genoma con integrasas compatibles con el ciclo lisogénico (Koonin and Yutin, 2020). La ampliación del conocimiento del ciclo podría promover su uso como indicador de contaminación fecal mediante técnicas de cultivo.

En los últimos años se han puesto a punto diferentes qPCR para la detección y cuantificación de crAssphage como indicador de la presencia de contaminación fecal en aguas y muestras ambientales, analizando también su potencial como marcador de MST por su elevada especificidad con el origen humano (García-Aljaro et al., 2017a; Stachler et al., 2017). En esta tesis doctoral se profundiza en el conocimiento del comportamiento de crAssphage en el medio ambiente

1.2.3 Modelización de los parámetros microbiológicos

La modelización de los contaminantes y de los procesos que tienen lugar en las masas de agua pueden ser una herramienta muy útil para la gestión de los recursos hídricos. Los modelos permiten monitorizar la calidad del agua, ya que permiten integrar toda la información de la masa de agua, incluyendo la información relativa a la calidad microbiológica. La modelización de los microorganismos indicadores de contaminación fecal y de los patógenos presenta varias finalidades (Figura 8):

Figura 8: Esquema de los objetivos de la modelización microbiológica como herramienta de gestión de la calidad del agua. Adaptado de De Brauwere et al. (2014).

Los microorganismos indicadores de contaminación fecal y patógenos son microorganismos alóctonos en el medio ambiente, es decir, son microorganismos que forman parte de la microbiota humana o animal. Es por ello, que cuando llegan al medio ambiente están sometidos a diferentes procesos propios del agua y a diferentes factores que condicionan su persistencia. La modelización persigue dilucidar todos los procesos y factores que determinan la presencia de microorganismos indicadores y patógenos de contaminación fecal.

Los modelos pueden presentar mayor o menor complejidad en función de las características de la masa de agua. Otro de los pilares de la modelización es identificar cuáles son los *inputs* o entradas de contaminación fecal, que se describen más adelante. La

identificación de los *inputs* es complicada cuando se trata de contaminación fecal difusa, puesto que al no haber una fuente concreta de contaminación fecal su modelización resulta más complicada. Sin embargo, la identificación de las fuentes de contaminación adquiere gran relevancia no sólo para la gestión de los recursos hídricos sino también la gestión del territorio y de las actividades que se llevan a cabo en él (Collins and Rutherford, 2004).

La presión antrópica sobre los recursos hídricos por su extracción o por el vertido de contaminantes supone un impacto sobre las masas de agua modificando la calidad de éstas. La calidad de las masas de agua también puede verse modificada por eventos naturales como son los eventos de precipitación, inundaciones o sequías. La inclusión de estos eventos y de la presión antrópica en los modelos permite predecir las consecuencias que pueden tener sobre la calidad microbiológica del agua.

La modelización tiene como finalidad principal integrar toda la información de la masa de agua para ayudar a predecir los procesos con mayor relevancia para la calidad de agua y ayudar a la toma de decisiones por parte de los gestores. Por esta razón, es una herramienta empleada en esta tesis doctoral.

Existen dos tipos principales de modelos: los modelos basados en regresiones o de caja negra y los modelos basados en procesos o modelos mecánicos (De Brauwere et al., 2014).

1.2.3.1 Modelos basados en regresiones o de caja negra

Los modelos basados en regresiones o modelos de caja negra consisten en relacionar una serie de variables con otras. Las variables que se utilizan como *inputs* del modelo son variables explicativas y sirven para poder modelizar las variables *output*. En el caso de los modelos de microorganismos indicadores y patógenos de contaminación fecal se pueden utilizar gran cantidad de variables como *input*: variables hidrológicas, usos del suelo, variables meteorológicas y variables geomorfológicas. También pueden emplearse datos de calidad microbiológica previos como *input* del modelo. En cambio, las variables *output* del modelo son los microorganismos indicadores de contaminación fecal y de patógenos.

Este tipo de modelos establecen correlaciones entre las variables explicativas y los microorganismos, que son las variables *output*. Estas correlaciones acostumbran a ser regresiones lineales múltiples y permiten llevar a cabo predicciones de la calidad microbiológica del agua de forma rápida y eficaz.

Se han descrito numerosos modelos basados en regresiones para el control de la calidad microbiológica de diferentes tipos de agua, aunque gran parte de ellos están destinados a predecir la calidad del agua de baño (Crowther et al., 2003; David and Haggard, 2011; Stidson et al., 2012).

1.2.3.2 Modelos basados en procesos o modelos mecánicos

Los modelos basados en procesos o mecánicos son los más utilizados en ecología. Estos modelos describen matemáticamente las fuentes de contaminación fecal, así como todos los procesos a los que están sometidos los microorganismos. Estos modelos requieren la identificación de aquellos procesos y parámetros que explican los microorganismos (Cuddington et al., 2013).

Para desarrollar modelos basados en procesos se utilizan ecuaciones que describen los procesos e incluyen los parámetros que explican la presencia, concentración y comportamiento de los microorganismos en el agua. Estas ecuaciones cumplen el balance de masas teniendo en cuenta los inputs, los procesos que se llevan a cabo en la masa de agua y el transporte de los microorganismos.

Uno de los inconvenientes que presentan este tipo de modelos es que suelen funcionar correctamente donde han sido descritos pero los procesos y los parámetros que explican los microorganismos cambian de una masa de agua a otra, por lo que requieren un esfuerzo de adaptación y una nueva validación (De Brauwere et al., 2014).

A diferencia de los modelos de caja negra que sólo permiten una predicción de la calidad microbiológica del agua, los modelos mecánicos además permiten actuar para poder realizar una gestión de la calidad microbiológica de la masa de agua. Esta actuación puede llevarse a cabo o bien controlando las entradas de contaminación fecal en el sistema, o mediante la modificación de los procesos que se llevan a cabo.

Los modelos basados en procesos se han ayudado también de programas informáticos y de los sistemas de información geográfica capaces de integrar toda la información y predecir la calidad del agua en cuencas hidrológicas. Existe una gran variedad de programas informáticos de modelización como SWAT (soil and water assessment tool), WAM (watershed assessment model) o HSPF (hydrological simulation program Fortran) (Benham et al., 2006; Bottcher et al., 2012; Cho et al., 2016). En el caso del software SWAT para la predicción de la contaminación fecal difusa y desarrollado por el Departamento de Agricultura de los Estados

Unidos, ha sido utilizado en un gran número de estudios (Bougeard et al., 2011; Coffey et al., 2013; Liu et al., 2015).

1.2.3.3 Procesos y parámetros incluidos en los modelos

Los procesos y parámetros que se incluyen en los modelos son muy diversos varían en función de su relevancia según las características de la masa de agua (Figura 9).

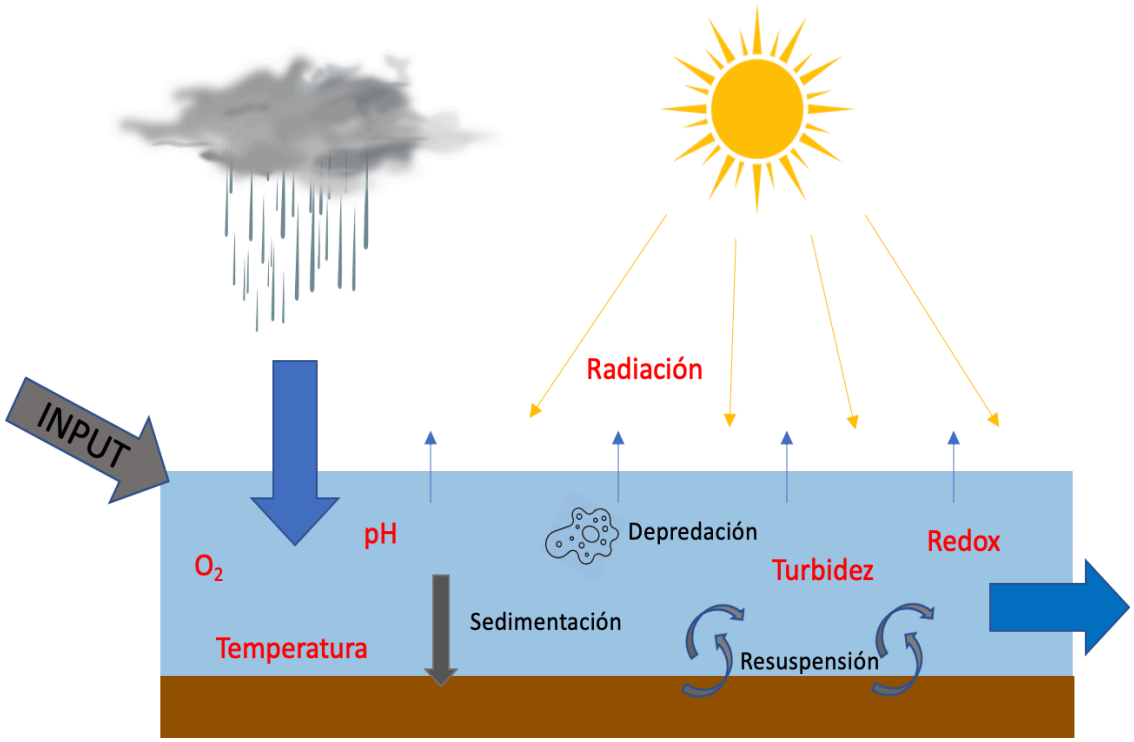

Figura 9: Esquema de los procesos y factores que condicionan la presencia y persistencia de los microorganismos de contaminación fecal en el agua.

La contaminación fecal llega a la masa de agua ya sea a través de una fuente concreta como por ejemplo el efluente de una depuradora, o bien puede tener un origen difuso y remoto en el tiempo y en el espacio. Una vez en la masa de agua hay numerosos procesos que afectan a los microorganismos y al tratarse de microorganismos alóctonos tienen tendencia a inactivarlos o a disminuir sus concentraciones. Estos procesos pueden ser de 2 tipos:

- Procesos bióticos: aquellos en que los seres vivos intervienen, principalmente la depredación y el recrecimiento, aunque éste último no debería darse en los microorganismos indicadores, puesto que es un requisito indispensable para serlo.
- Procesos abióticos: aquellos en los que no intervienen directamente los seres vivos. Son procesos abióticos la sedimentación, la resuspensión y la dilución.

También hay factores abióticos que condicionan la presencia y persistencia de los microorganismos de contaminación fecal en el medio ambiente. Estos factores abióticos son tan diversos como la temperatura, la radiación solar, el oxígeno, el potencial rédox o el pH entre otros.

Las entradas y salidas de agua del sistema pueden dar lugar a aumentos o descensos de la concentración de microorganismos por efectos de dilución o concentración de éstos. Estas entradas así como los aportes de contaminación fecal también están condicionados a las características hidrogeomorfológicas de la masa de agua (Cho et al., 2016).

1.2.4 Las comunidades bacterianas del agua

El agua contiene una microbiota propia que puede verse alterada por la entrada de microorganismos derivada de los diferentes usos, como los microorganismos de contaminación fecal.

Las comunidades bacterianas de las aguas superficiales de ríos, lagos y océanos han sido largamente estudiadas, así como también las de aguas residuales y las aguas de consumo humano. El agua dulce de los diferentes orígenes se ha mostrado entre los hábitats más ricos en diversidad bacteriana (Tamames et al., 2010). La microbiota del agua varía según su origen y los procesos y factores naturales a los que está sometida (Staley et al., 2015). Sin embargo, a pesar de las variaciones en los diferentes tipos de agua se han podido determinar o establecer algunos patrones, siendo Proteobacteria, Bacteroidetes, Actinobacteria y Firmicutes los filos bacterianos más frecuentes de los diferentes orígenes (Vaz-Moreira et al., 2014).

1.2.4.1 Las técnicas de análisis de las comunidades bacterianas

Las técnicas utilizadas en los estudios de las comunidades bacterianas han ido evolucionando conforme se han ido desarrollado las diferentes tecnologías. Se distinguen 3 tipos de técnicas utilizadas en el estudios de las comunidades microbianas (Douterelo et al., 2014) (Tabla 6):

Tabla 6: Esquema de las técnicas disponibles para el análisis de las comunidades en el agua. Adaptado de Douterelo et al. (2014).

Detección-Enumeración		Composición/ caracterización		Actividad microbiana/ genes funcionales
Dependientes de cultivo	Independientes de cultivo	Dependientes de cultivo	Independientes de cultivo	Independientes de cultivo
- Siembra y recuento en placa - Reacciones enzimáticas	- Citometría - Métodos basados en PCR - Contajes mediante microscopía (FISH, etc.)	- Baterías bioquímicas - MALDI-TOF MS	- Huella genética (DGGE/TGGE, etc.) - Metagenómica - NGS	- Biomasa y actividad total (ATP) - Proteómica - Metatranscriptómica (Microarrays) - Actividad enzimática específica

- Técnicas basadas en la detección y/o enumeración: consisten en la cuantificación de determinadas bacterias como las indicadoras de contaminación fecal o las patógenas.
- Técnicas de identificación y caracterización: estas técnicas se basan en la diferenciación de las comunidades bacterianas a través de sus características.
- Técnicas basadas en la actividad microbiológica o genes funcionales: son técnicas independientes de cultivo y consisten en la detección de moléculas o genes relacionados con la actividad bacteriana.

Existe un gran número de técnicas para el estudio de las comunidades bacterianas del agua. Aún así, teniendo en cuenta que el porcentaje de bacterias cultivables es muy pequeño (<1%) (Amann et al., 1995), las técnicas independientes de cultivo y más concretamente la secuenciación masiva (NGS, del inglés next-generation sequencing), ha sido la que más datos sobre las comunidades bacterianas ha generado en los últimos años (Simon and Daniel, 2011).

1.2.4.2 Nuevas tecnologías para el estudio de las comunidades

Mucho han evolucionado las técnicas desde que en 1977 Sanger y Coulson revolucionaran la biología con el descubrimiento de una herramienta para secuenciar primero genes y después genomas completos (Sanger et al., 1977). El desarrollo y mejora de numerosas tecnologías de secuenciación ha permitido que el coste de ésta se reduzca de tal manera que ya es prácticamente asequible para la mayoría de los laboratorios de investigación, no sólo para la secuenciación de genes sino para estudios de metagenómica (Schuster, 2008).

La metagenómica consiste en el análisis genético completo de las comunidades y organismos de una muestra, a menudo de origen ambiental (Thomas et al., 2012). Ya los primeros estudios de "genómica ambiental" llevados a cabo por Stein y colaboradores y los de Handelsman y colaboradores, que utilizaron por primera vez el término metagenómica, pusieron de manifiesto el potencial de la secuenciación en el estudio de las comunidades microbianas, no sólo de las bacterias cultivables sino también de las que hasta entonces no habían podido ser cultivadas (Handelsman et al., 1998; Stein et al., 1996). Desde entonces, la secuenciación masiva se ha aplicado al estudio de metagenomas en ecosistemas tan diversos como pueden ser el ser humano, los animales, los vegetales e incluso los rincones más inhóspitos del planeta como los fondos oceánicos o las piscinas volcánicas entre otros.

Los estudios de muestras ambientales mediante NGS presentan un gran potencial no sólo por la cantidad de información que pueden arrojar sobre el microbioma de las muestras

ambientales sino también sobre las funciones que las comunidades microbianas ejercen en los sistemas acuáticos (Figura 10).

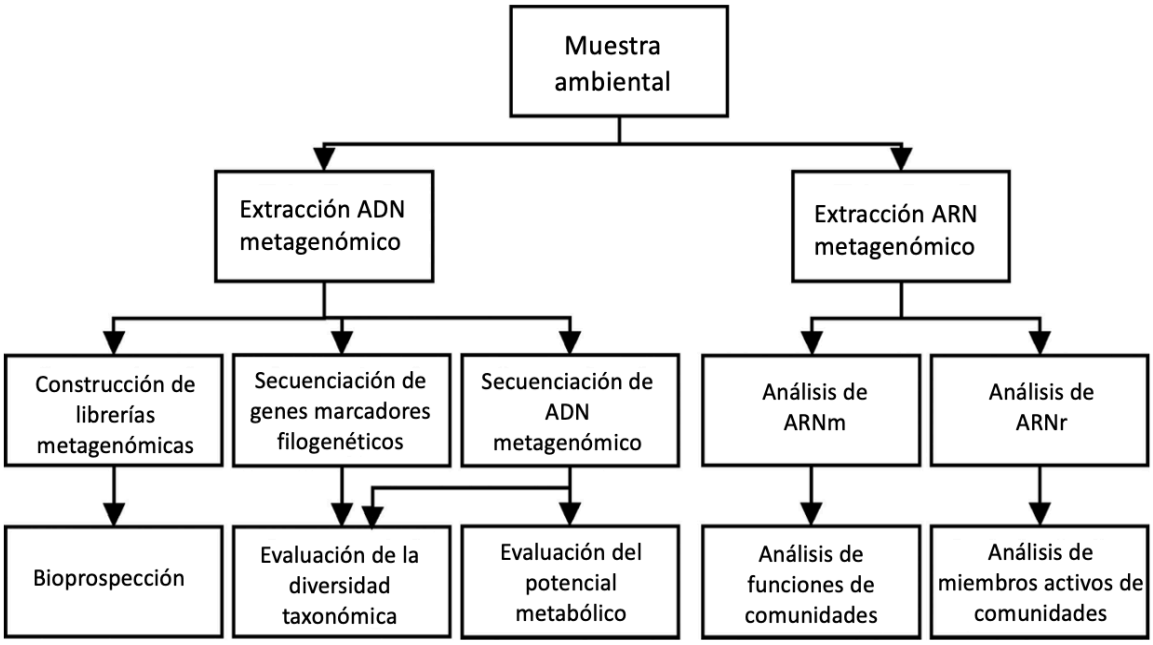

Figura 10: Esquema de los análisis metagenómicos de las comunidades microbianas en muestras ambientales. Adaptado de Simon and Daniel (2011).

En una gran parte de los estudios de comunidades microbianas de muestras ambientales se han empleado amplicones del gen que codifica el ARN ribosomal 16S (16S ARNr), siendo utilizado también en los estudios realizados mediante secuenciación masiva. El gen 16S ARNr se encuentra en todos los organismos procariotas y contiene regiones hipervariables, que han evolucionado permitiendo la clasificación de los procariotas en los diferentes niveles taxonómicos y regiones conservadas que son utilizadas para el diseño de cebadores que permiten la amplificación de las regiones hipervariables (Sambo et al., 2018).

El estudio de la microbiota de las muestras ambientales mediante la secuenciación masiva del 16S ARNr comprende cuatro etapas (Tringe and Rubin, 2005):

- Concentración de la muestra. En el caso de las muestras de agua se puede realizar una concentración mediante la filtración de la muestra. La etapa de filtración es de especial relevancia, ya que tanto el tamaño de poro como el método de filtración permiten escoger qué microorganismos se concentran. Las muestras sólidas como las de suelos o sedimentos no se pueden concentrar tan fácilmente y a menudo deben

ser tratadas para eliminar ácidos húmicos y otras sustancias inhibidoras de las reacciones enzimáticas necesarias para la secuenciación.

- Extracción de ADN: se emplean los protocolos que mejores resultados ofrezcan en función de la concentración y pureza de ADN requerida.
- Secuenciación de las muestras: el proceso se explica detalladamente más adelante.
- Análisis bioinformático de los resultados.

Aunque existen diferentes tecnologías de secuenciación, en la actualidad es la secuenciación por síntesis (SBS) de la plataforma Illumina la responsable de entre el 70 y el 90% de los datos de secuenciación generados a nivel mundial (Illumina, 2016; Lu et al., 2016). El proceso de secuenciación por síntesis, que es el más utilizado, consta de cuatro etapas principales (Illumina, 2016) (Figura 11):

1. Preparación de las librerías: las librerías de secuenciación son preparadas por fragmentación aleatoria del ADN de la muestra, seguido de una ligación de los adaptadores añadidos a los fragmentos generados. Alternativamente, este proceso puede incrementar su eficiencia mediante la tagmentación, que es el nombre que recibe la fragmentación y ligación en un solo paso.
2. Generación de clústeres: las librerías son cargadas en unas celdas de flujo donde se encuentran los cebadores complementarios a los adaptadores. Cada fragmento es amplificado generando mediante la técnica de PCR bridge fragmentos de ADN idénticos también denominados clústeres clonales. Cuando la generación de clústeres clonales termina, éstos ya están listos para su secuenciación.
3. Secuenciación: la secuenciación por síntesis utiliza nucleótidos terminadores marcados con diferentes fluoróforos, que son incorporados en la primera base. La celda de flujo es monitorizada y las emisiones de cada clúster son registradas, permitiendo la identificación de las bases en función de la longitud de onda y la intensidad de emisión. El ciclo es repetido “n” veces hasta crear un *read* de longitud de “n” bases. Dependiendo de la tecnología utilizada la longitud óptima de los *reads* varía, en el caso de Illumina MiSeq, la tecnología utilizada en esta tesis, la longitud óptima de los *reads* es de 250 pares de bases.
4. Análisis de datos: las secuencias generadas o *reads* son alineadas y mediante programas bioinformáticos son analizadas la cantidad y calidad de las secuencias obtenidas.

Finalmente, se realiza una comparación con las secuencias existentes en las bases de datos para su identificación.

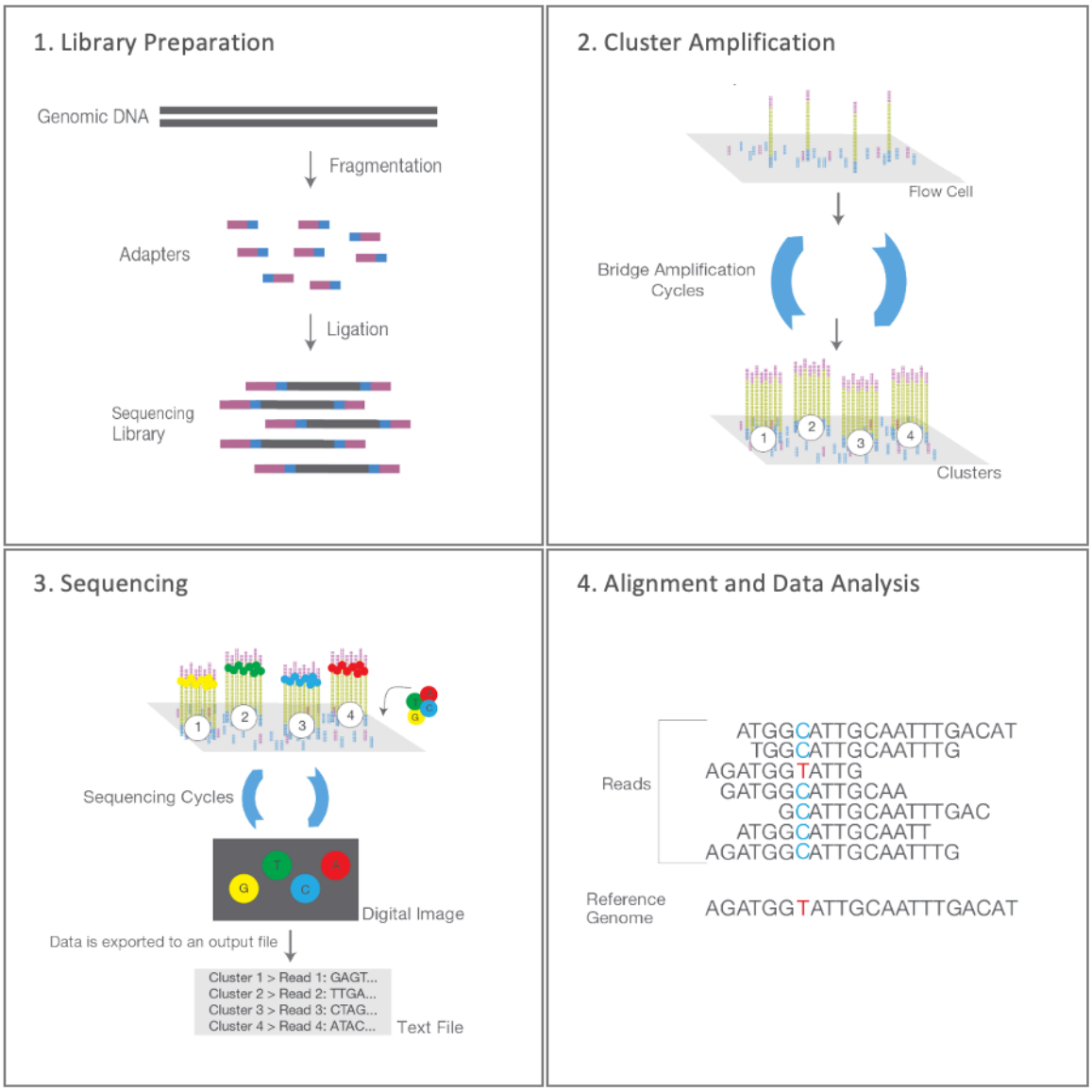

Figura 11: Esquema del proceso de SBS de Illumina. Adaptado de (Illumina, 2016).

Los estudios que utilizan el 16S ARNr han permitido ahondar enormemente en el conocimiento de la biodiversidad de las comunidades bacterianas. Por otro lado, su uso presenta una serie de limitaciones como la obtención de longitudes de *reads* cortas, las diferencias en función de la región del gen escogida o la baja efectividad para definir especies bacterianas muy próximas (Poretsky et al., 2014). En el estudio llevado a cabo en esta tesis, se ha utilizado la región hipervariable V4 del 16S ARNr, la cual está recomendada por diferentes autores para el estudio de la diversidad de las comunidades bacterianas (Nelson et al., 2014; Youssef et al., 2009).

1.2.4.3 El rol de las comunidades bacterianas en los sistemas acuáticos

Las comunidades bacterianas juegan un papel importante en numerosos procesos bioquímicos que tienen lugar en los ecosistemas acuáticos. Los principales procesos bioquímicos están relacionados con los ciclos de los nutrientes (carbono, nitrógeno, fósforo, azufre), el metabolismo de los metales pesados o metales traza y la degradación de compuestos químicos más complejos (Rousk and Bengtson, 2014). Los ciclos de los diferentes nutrientes están ligados entre sí por relaciones estequiométricas de las reacciones en las que están implicados, las cuales son mayoritariamente reacciones de oxidación y reducción.

Los microorganismos utilizan los nutrientes o compuestos como sustrato para obtener energía y los factores ambientales condicionan estas reacciones debido a que la actividad bacteriana viene determinada también, entre otros factores, por la temperatura, el pH y la conductividad (Madsen, 2011). Por tanto, las bacterias llevan a cabo reacciones que depuran de manera natural el agua y las entradas de contaminación fecal pueden alterar estas reacciones porque suponen una entrada de microorganismos, nutrientes y contaminantes que modifican la estructura de las comunidades bacterianas existentes lo que puede también alterar la resiliencia de éstas frente a los contaminantes.

Además, la entrada de microorganismos de contaminación fecal produce también cambios en la diversidad y la riqueza de especies del agua. Numerosos estudios han reportado una relación inversa entre la diversidad y la contaminación fecal, siendo mayor la diversidad bacteriana y la riqueza de especies cuando los niveles de contaminación fecal son más bajos (Halliday et al., 2014; Mansfeldt et al., 2019; Paruch et al., 2019). En cambio, otros autores han reportado la tendencia inversa, es decir, que la contaminación fecal aumenta la diversidad (Wakelin et al., 2008). Sin embargo, sí que parece haber un consenso en que la entrada de contaminación fecal al medio produce un impacto sobre las comunidades bacterianas. Por tanto, la entrada de contaminantes y de microorganismos de contaminación fecal a los ecosistemas acuáticos, así como los factores ambientales pueden generar cambios en la estructura y la diversidad de las comunidades bacterianas y también alterar las funciones metabólicas que llevan a cabo (Abia et al., 2018; Oh et al., 2011).

El conocimiento del impacto de la contaminación fecal sobre las comunidades bacterianas en los sistemas acuáticos, así como también su interacción con los factores ambientales pueden ser de gran relevancia para la gestión de la calidad del agua y de los recursos hídricos, razón por la que han sido abordados en esta tesis.

2. OBJETIVOS

Esta tesis doctoral tiene como objetivo principal generar conocimiento sobre la dinámica y el comportamiento de diferentes microorganismos indicadores, patógenos y comunidades microbianas en el medio ambiente y en diferentes tratamientos del agua. El fin de este conocimiento es desarrollar herramientas para mejorar la monitorización de la calidad microbiológica del agua que puedan ser de utilidad para la gestión de los recursos hídricos bajo las perspectivas de *One Health* y *Global Health*.

Los objetivos específicos para el estudio de las muestras ambientales han sido los siguientes:

1) Evaluar la abundancia de crAssphage en entornos acuáticos con diferentes niveles de contaminación fecal.
2) Analizar la dinámica de inactivación de crAssphage frente a diferentes factores ambientales respecto a la de otros microorganismos indicadores de contaminación fecal y marcadores humanos de MST.
3) Estudiar y comparar las dinámicas estacionales de cinco microorganismos indicadores en un río mediterráneo afectado por el efluente secundario de una EDAR.
4) Modelizar la autodepuración del río para cada uno de los indicadores en relación con los diferentes factores ambientales.
5) Adaptar la técnica de secuenciación masiva de 16S ARNr para el estudio de la estructura y diversidad de las comunidades bacterianas aguas abajo de la EDAR.
6) Evaluar las diferencias estacionales en la estructura y diversidad de las comunidades bacterianas en el río tras el impacto del efluente.
7) Evaluar la resiliencia de las comunidades bacterianas del río frente al impacto que supone el vertido del efluente de la EDAR.

La consecución de los objetivos 1 y 2 ha permitido obtener el **artículo 1** "Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments". Los objetivos 3 y 4 constituyen el contenido del **artículo 2** "Modelling the seasonal impacts of a wastewater treatment plant on water quality in a Mediterranean stream using microbial indicators". El trabajo experimental realizado para alcanzar los objetivos 5, 6 y 7 ha dado lugar al **artículo 3** "Impact of treated sewage effluent on the bacterial community composition in an intermittent Mediterranean stream".

Los objetivos planteados respecto al estudio de la dinámica de patógenos e indicadores a lo largo del tratamiento del agua potable son los siguientes:

8) Adaptar y evaluar la capacidad de un método de ultrafiltración por membrana para concentrar microorganismos indicadores y patógenos de referencia en grandes volúmenes de agua con diferentes características fisicoquímicas para valorar su utilidad en la elaboración de PSA en las ETAP.
9) Evaluar la eliminación de microorganismos indicadores y patógenos de referencia en las diferentes etapas de tratamiento de dos ETAP.

Los objetivos 8 y 9 han permitido la obtención del **artículo 4** "Assessment of dead-end ultrafiltration for the detection and quantification of microbial indicators and pathogens in the drinking water treatment processes".

Además de los cuatro estudios incluidos en esta tesis doctoral, la participación y colaboración de la doctoranda en el grupo de investigación ha dado lugar a otros tres estudios que se recogen en el Anexo 1 de esta tesis y que están relacionados con la detección de indicadores de contaminación fecal y patógenos.

3. INFORMES

3.1 INFORME SOBRE EL FACTOR DE IMPACTO

Los artículos que constituyen la memoria de esta de tesis doctoral han sido publicados en revistas internacionales indexadas en *Journal Citation Reports* o están sometidos para su publicación en ellas.

El artículo **"Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments"** fue publicado en el año 2019 en la revista *Water Research* (DOI: 10.1016/j.watres.2019.02.042). Dicha revista se encuentra en el primer cuartil (Q1) en todas sus categorías y tiene un factor de impacto de 7,913.

El artículo **"Modelling the seasonal impacts of a wastewater treatment plant on water quality in a Mediterranean stream using microbial indicators"** ha sido publicado en 2020 en la revista *Journal of Environmental Management* (DOI: 10.1016/j.jenvman.2020.110220). Dicha revista se encuentra en el primer cuartil (Q1) en todas sus categorías y presenta un factor de impacto de 4,865.

El artículo **"Impact of treated sewage effluent on the bacterial community composition in an intermittent Mediterranean stream"** está actualmente en fase de revisión en la revista *Environmental Pollution*. Dicha revista se encuentra en el primer cuartil (Q1) en todas sus categorías y presenta un factor de impacto de 5,714.

El artículo **"Assessment of dead-end ultrafiltration for the detection and quantification of microbial indicators and pathogens in the drinking water treatment processes"** está actualmente en fase de revisión en la revista *International Journal of Hygiene and Environmental Health*. Esta revista presenta un factor de impacto de 4,379 y se encuentra en el primer cuartil (Q1) en todas sus categorías.

Dr. Francisco Lucena Gutiérrez Dra. Cristina García Aljaro

Barcelona, junio de 2020

3.2 INFORME DE PARTICIPACIÓN EN LAS PUBLICACIONES

La doctoranda Miriam Pascual Benito ha participado en todas las publicaciones que forman parte de su tesis de la manera que se detalla a continuación. Ninguno de los coautores de los artículos ha utilizado los datos y las publicaciones para la elaboración de su tesis doctoral.

Elisenda Ballesté, **Miriam Pascual-Benito**, Julia Martín-Díaz, Anicet R. Blanch, Francisco Lucena, Maite Muniesa, Juan Jofre y Cristina García-Aljaro. Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments. *Water Research* (2019), Vol. 155; 233-244, (DOI: 10.1016/j.watres.2019.02.042).

La doctoranda ha contribuido en el diseño experimental del estudio y ha realizado la recogida de muestras de la riera de Cànoves y el análisis de los indicadores de contaminación fecal de dichas muestras por método de cultivo. También ha participado activamente en el análisis de los resultados obtenidos y ha colaborado en la redacción del artículo.

Miriam Pascual-Benito, Daniel Nadal-Sala, Marta Tobella, Elisenda Ballesté, Cristina García-Aljaro, Santi Sabaté, Francesc Sabater, Eugènia Martí, Carles A. Gracia, Anicet R. Blanch y Francisco Lucena. Modelling the seasonal impacts of a wastewater treatment plant on water quality in a Mediterranean stream using microbial indicators. *Journal of Environmental Management* (2020), Vol. 261, (DOI: 10.1016/j.jenvman.2020.110220).

La doctoranda ha intervenido en el diseño experimental del estudio y ha llevado a cabo la recogida de las muestras y el análisis de los indicadores de contaminación fecal. Finalmente ha participado activamente en el análisis de los resultados obtenidos, su organización y discusión liderando la redacción del artículo.

Miriam Pascual-Benito, Elisenda Ballesté, Toni Monelón-Getino, Jordi Urmeneta, Anicet R. Blanch, Cristina García-Aljaro y Francisco Lucena. Impact of treated sewage effluent on the bacterial community composition in an intermittent Mediterranean stream (sometido en *Environmental Pollution*).

La doctoranda ha colaborado en el diseño experimental del estudio, realizando la recogida de muestras, concentración y extracción de ADN de éstas. También ha participado activamente en la organización y discusión de los resultados, así como también en la redacción del artículo.

Miriam Pascual-Benito, Pere Emiliano, Raquel Casas-Mangas, Cristina Dacal-Rodríguez, Mercedes Gracenea, Rosa Araujo, Fernando Valero, Cristina García-Aljaro y Francisco Lucena. Assessment of dead-end ultrafiltration for the detection and quantification of microbial indicators and pathogens in the drinking water treatment processes (sometido en *International Journal of Hygiene and Environmental Health*).

La doctoranda ha participado en el diseño experimental del estudio llevando a cabo el procesamiento de las muestras, el análisis de los indicadores de contaminación fecal y de enterovirus. La doctoranda ha realizado el análisis, la organización y discusión de los resultados liderando la redacción del artículo.

Dr. Francisco Lucena Gutiérrez Dra. Cristina García Aljaro

Barcelona, junio de 2020

4. ARTÍCULOS

4.1 ARTÍCULO 1

Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments

Elisenda Ballesté, **Miriam Pascual-Benito**, Julia Martín-Díaz, Anicet R. Blanch, Francisco Lucena, Maite Muniesa, Juan Jofre y Cristina García-Aljaro

Water Research (2019); 155: 233-244; DOI: 10.1016/j.watres.2019.02.042

Durante los últimos años, el uso de técnicas de secuenciación masiva ha permitido la descripción del genoma de crAssphage, un grupo de bacteriófagos del que se estima que es el más abundante en el tracto intestinal humano. Desde su descubrimiento, han sido varias las propuestas de su uso como indicador de la presencia de contaminación fecal y se han desarrollado varias qPCR para su detección y cuantificación. El hecho de que su origen sea humano y que se haya mostrado ampliamente distribuido geográficamente, ha permitido también su postulación como potencial marcador humano de MST. Sin embargo, para su uso en la gestión y monitorización de la calidad de agua, es necesario ampliar el conocimiento de su comportamiento y distribución en el medio ambiente. Por ello, este estudio tiene como principal objetivo ampliar el conocimiento de la distribución y el comportamiento de crAssphage en el medio ambiente respecto a otros microorganismos indicadores y marcadores de MST.

Para llevar a cabo el estudio se analizaron diferentes tipos de muestras: efluentes secundarios de diferentes EDAR; agua de dos ríos con diferentes niveles de contaminación fecal; el río Llobregat y la riera de Cànoves, incluyendo un transecto de este último; sedimentos del río Llobregat y mesocosmos para evaluar su dinámica de inactivación.

En las diferentes muestras se analizaron *E. coli* y colifagos somáticos (SOMCPH) por métodos de cultivo como indicadores de contaminación fecal. También fueron analizados como marcadores de MST de origen humano los bacteriófagos de *Bacteroides thetaiotaomicron* GA17 (GA17PH) por métodos de cultivo y los marcadores moleculares de *Bifidobacterium* (HMBif) y *Bacteroides* (HF183) además de crAssphage mediante análisis por qPCR con sondas específicas que habían sido descritas previamente.

Los resultados mostraron elevadas concentraciones de crAssphage en las muestras de efluente de diferentes EDAR y con elevadas correlaciones con *E. coli* y SOMCPH. Las EDAR con

tratamiento de eliminación de nutrientes presentaron menores concentraciones de los diferentes indicadores, pero sin poderse establecer un patrón concreto. Asimismo, las concentraciones de crAssphage del efluente de una misma EDAR fueron estables a lo largo del estudio, correlacionándose bien con los marcadores moleculares de MST y *E. coli,* pero sin mostrar ningún tipo de estacionalidad.

Las concentraciones de crAssphage fueron elevadas en el río Llobregat, como era de esperar por tratarse de un río con elevados niveles de contaminación. La concentración de crAssphage mostró una correlación positiva con la precipitación debido a diferentes factores, entre ellos la resuspensión de sedimentos. Este hecho viene corroborado por las elevadas concentraciones de crAssphage obtenidas en los sedimentos, así como también del resto de indicadores analizados, mostrándose los sedimentos como un reservorio de microorganismos. Por otro lado, en el transecto estudiado de la riera de Cànoves se observaron concentraciones decrecientes de crAssphage y del resto de indicadores a medida que aumenta la distancia con la EDAR. Esta reducción en las concentraciones podría ser debida a la inactivación de los microorganismos o también a los procesos de sedimentación. Las concentraciones de los diferentes indicadores se correlacionan fuertemente, de forma positiva con la precipitación y de forma negativa con la temperatura. Además, crAssphage presentó una persistencia mayor que los marcadores moleculares bacterianos pero menor que los indicadores cultivables, especialmente los bacteriófagos (SOMCPH y GA17PH).

Los resultados de los experimentos en los mesocosmos mostraron una inactivación mayor en verano que en invierno para todos los indicadores analizados debido a que en verano los factores como la temperatura, la radiación o la depredación aumentan la tasa de decaimiento de los microorganismos, mientras que en invierno son las características intrínsecas de cada microorganismo las que determinan esta inactivación. La T90 de crAssphage resultó ser superior a la del resto de marcadores de MST y *E. coli* indicando una mayor resistencia de éste a los parámetros ambientales. El decaimiento de crAssphage fue muy similar al de los SOMCPH en invierno, mientras que en verano éstos últimos presentaron una mayor persistencia en el medio. La mayor similitud en el comportamiento de crAssphage con los indicadores bacterianos o con los indicadores víricos puede deberse a la singularidad de la replicación de crAssphage que ha sido descrita para el único crAssphage aislado por métodos de cultivo.

De este estudio se pueden extraer las siguientes conclusiones:

- CrAssphage muestra una elevada correlación con los otros indicadores de contaminación fecal analizados (*E. coli*, SOMCPH, GA17PH, HMBif y HF183).
- CrAssphage es detectado en concentraciones similares a las de los otros marcadores moleculares bacterianos de contaminación fecal (HMBif y HF183) y en concentraciones superiores a las de *E. coli*, SOMCPH y GA17PH en muestras de efluentes de EDAR.
- Las concentraciones de crAssphage detectadas en el río Llobregat fueron en torno a 2 logaritmos superiores a las de la riera de Cànoves. El resto de los microorganismos indicadores analizados mostraron diferencias similares para ambos ríos.
- Los sedimentos suponen un reservorio de microorganismos, incluido crAssphage, que pueden ser movilizados y pasar a la columna de agua.
- La inactivación de crAssphage se correlaciona positivamente con la temperatura, siendo su persistencia muy similar a la de SOMCPH (T90~10 días) cuando las temperaturas son bajas.
- En verano, la persistencia de crAssphage en el agua (T90= 2,41 días) es muy inferior a la de los SOMCPH (T90=4,8 días) y similar a la de otros marcadores moleculares de MST (HMBif y HF183) y *E. coli*, mostrándose así muy dependiente de las condiciones ambientales, principalmente la temperatura.
- Las fuertes correlaciones de crAssphage con los indicadores de origen humano sugieren que crAssphage puede ser utilizado como marcador humano de MST, complementando la información de otros marcadores e indicadores de contaminación fecal.

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Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments

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Faecal indicators

ABSTRACT

Recent studies have shown that crAssphage is abundant in human faecal samples worldwide. It has thus been postulated as a potential microbial source tracking (MST) marker to detect human faecal pollution in water. However, an effective implementation of crAssphage in water management strategies will depend on an understanding of its environmental dynamics. In this work, the abundance and temporal distribution of crAssphage was analysed in the effluent of wastewater treatment plants using different sewage treatments, and in two rivers (water and sediments) that differ in pollution impact and flow regime. Additionally, the influence of environmental conditions (temperature and rainfall) on the removal of the marker was studied along a river section, and natural inactivation was assessed by a mesocosms approach. Molecular and culture-based tools were used to compare crAssphage abundance and dynamics with those of bacteria and bacteriophages currently applied as global indicators (*E. coli*, somatic coliphages, *Bacteroides* GA17 bacteriophages, and the human-associated MST markers HF183 and HMBif). CrAssphage concentrations in sewage effluent and river samples were similar to those of HF183 and HMBif and higher than other general and/or culture-based indicators (by 2–3 orders of magnitude). Measurement of crAssphage abundance revealed no temporal variability in the effluent, although rainfall events affected the dynamics, possibly through the mobilisation of sediments, where the marker was detected in high concentrations, and an increase in diffuse and point pollution. Another factor affecting crAssphage inactivation was temperature. Its persistence was longer compared with other bacterial markers analysed by qPCR but lower than culturable markers. The results of this study support the use of crAssphage as a human source tracking marker of faecal pollution in water, since it has similar abundances to other molecular human MST markers, yet with a longer persistence in the environment. Nevertheless, its use in combination with infectious bacteriophages is probably advisable.

1. Introduction

Over the last decades there has been a worldwide effort to develop and improve indicators to detect the risk of faecal pollution in water and avoid the transmission of waterborne diseases. Faecal indicator bacteria (FIB), mainly *E. coli* and enterococci, have been widely used to assess water quality and have played a key role in reducing the number of waterborne diseases and improving public health (Edberg et al., 2000; WHO, 2001). Methods developed to detect these indicators have been largely based on culture-based techniques, and are sensitive, specific, easy-to-use and inexpensive, thus permitting a global use. An alternative approach is offered by molecular methods based on qPCR detection (Lavender and Kinzelman, 2009; USEPA, 2012; Wade et al., 2006). However, research shows that FIB may not provide sufficient public health protection given that viruses have different persistence, survival and transport rates in water bodies (Edberg et al., 2000). Thus, water management protocols are beginning to include somatic coliphages as viral faecal indicators, because they are more persistent than *E. coli* and enterococci and can be used as a surrogate to indirectly measure enteric viruses (Jofre et al., 2016; McMinn et al., 2017; NHMRC, 2011; USEPA, 2016, 2006). A new type of markers have been introduced in the last decades to determine the source of faecal pollution in water, namely the microbial source

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tracking (MST) markers (Bernhard and Field, 2000; Gómez-Doñate et al., 2012; Green et al., 2014; Mieszkin et al., 2009; Shanks et al., 2008). MST markers rely in the detection of host-associated bacteria or viruses (bacteriophages or host pathogens) or directly mitochondrial DNA of the target host (Roslev and Bukh, 2011). They allow a better management of polluted water areas as they can determine the source of faecal pollution, allowing effective restoration measures on the origin. Thus, the strategy for water quality improvement is changing from a Public Health to a One Health approach.

MST markers targeting host-associated bacteria or bacteriophages can be determined by culturable methods or by quantitative PCR (qPCR) (Jofre et al., 2014; McMinn et al., 2017). The use of phages for MST purposes involves measuring the different proportions of the four genogroups of F-specific RNA bacteriophages (Hsu et al., 1995; Ogorzaly et al., 2009; Schaper et al., 2002). More recently, the enumeration of bacteriophages infecting *Bacteroides* strains isolated from humans or animals has been described (Gómez-Doñate et al., 2011; Jofre et al., 1986; Payan et al., 2005; Tartera et al., 1989). However, the considerable geographical variability in the sensitivity of the different *Bacteroides* strains used for detecting phages of different faecal origin requires the isolation of a suitable strain from every area (Ebdon et al., 2007; Payan et al., 2005).

Human viruses like poliomaviruses and adenoviruses have been proposed as alternative human source tracking markers (Ahmed et al., 2015; Liang et al., 2015), but the prevalence, infection process and shedding of these pathogens also vary considerably according to region, thereby limiting their sensitivity (Gerba et al., 2017; Harwood et al., 2013) and use as indicators.

Next-generation sequencing has represented a giant step towards unravelling viral "dark matter" (Hurwitz et al., 2016). These techniques allow viral metagenomes of yet-to-be cultured organisms to be assembled and new viruses identified. This has been the case for a highly abundant group of human gut bacteriophages described after cross-assembling the viral metagenomes of faeces from 12 people (Dutilh et al., 2014). The assembled phage genome obtained, named crAssphage (for "cross assembly"), was detected in all the studied individuals, and *Bacteroides* species have been identified as the putative host (Dutilh et al., 2014; Shkoporov et al., 2018). Mapping the genome to already published metagenomes showed a high abundance of crAssphage in human samples (faeces and sewage) from different geographical areas (including USA, Europe, Africa and Asia) (Cinek et al., 2018; Dutilh et al., 2014; Stachler and Bibby, 2014), outnumbering other known human-associated viruses such as noroviruses and adenoviruses (Stachler and Bibby, 2014).

Prompted by the great abundance of crAssphage in the human gut (Dutilh et al., 2014) compared to other potential viral source tracking markers, two different methods have already been described for its application as an MST marker. One approach was developed in the USA (CPQ_056 and CPQ_064) (Stachler et al., 2017) and the other in Europe, adapting the target to the sequence of the crAssphage variant detected there, which was different from the one found in USA (García-Aljaro et al., 2017). Both markers showed similar abundance in human samples and were absent in animal samples or found in lower amounts than in human samples (Ahmed et al., 2018; García-Aljaro et al., 2017a; Stachler et al., 2017).

The evaluation of the abundance, persistence, inactivation, and resuspension of FIB and MST in the environment requires an understanding of their behaviour and the availability of data for modelling purposes, as shown in previous studies (Ballesté et al., 2018; Bell et al., 2009; Blaustein et al., 2013; Drummond et al., 2014; Garcia-Aljaro et al., 2017b; Jamieson et al., 2005). To assess the potential of crAssphage as a human MST marker in the environment, the aim of the present work was to measure its dynamics in potentially human-polluted water samples. Thus, the abundance of crAssphage was determined in sewage effluents and rivers with different pollution impacts, including water and sediments. CrAssphage removal in a river with human point pollution was monitored to determine the effect of environmental parameters. Additionally, inactivation was assessed *in situ* using previously developed mesocosms experimental approaches (Ballesté et al., 2018). The dynamics of crAssphage were compared with those of other bacterial and viral faecal indicators and MST markers detected by culture-based and molecular techniques: *E. coli*, somatic coliphages (SOMCPH), bacteriophages infecting *Bacteroides* GA17 strain (GA17PH) (Gómez-Doñate et al., 2011; Payan et al., 2005), the human-associated *Bifidobacterium* marker HMBif (Gómez-Doñate et al., 2012) which has been designed and applied in the area of study (Casanovas-Massana et al., 2015; Yahya et al., 2017) and the human-associated *Bacteroides* marker HF183 (Haugland et al., 2010) which is being used as MST in a wide geographical area (Ahmed et al., 2018; Cao et al., 2018; Mayer et al., 2018).

2. Material and methods

2.1. Intra and inter wastewater effluent comparison

Treated wastewater was obtained from 18 wastewater treatment plants (WWTPs) in Catalonia, north-eastern Spain, which serve a population equivalent (PE) ranging between 1,877 and 358,333 with a median of 5,546 PE. Six of the WWTPs use a biological treatment, 6 biological treatment and nutrient removal (nitrogen and phosphorus), 3 biological treatment with nitrogen removal, 1 biological treatment with phosphorus removal, 1 primary treatment, and 1 biological nutrient removal and a tertiary treatment with UV disinfection.

The effluent of another WWTP was subjected to annual sampling, with 11 samples collected over 2 years, to evaluate the intra-effluent variation (Jan, Feb, May, Jun, July, Oct, Nov 2016, Feb, June 2017, Feb 2018). This WWTP treated sewage for 12,500 PE with a biological and nutrient removal system.

2.2. CrAssphage evaluation in river samples

2.2.1. River with a high pollution impact

A total of 11 water samples were collected at the lower transect of the River Llobregat during one year. This Mediterranean river has an annual mean flow of 12 $m^3 s^{-1}$, ranging from 3 $m^3 s^{-1}$ to 1,200 $m^3 s^{-1}$ after a heavy rainfall event and/or during snowmelt. The river supplies water to the city of Barcelona and is subjected to high human pollution pressure due to the effluent of several WWTPs located upstream (Casanovas-Massana et al., 2015). In addition, 20 samples of sediment (1 cm deep) were also collected.

2.2.2. River with a low pollution impact

A total of 10 water samples were collected from the "Riera de Cànoves" during 2 years. This temporal Mediterranean river has a low annual mean flow of 0.005 $m^3 s^{-1}$, ranging from 0 $m^3 s^{-1}$ to 0.012 $m^3 s^{-1}$ during a heavy rainfall event. Upstream land use is mainly forestry, some households that may have septic tanks, and agricultural areas (Fig. 1).

2.3. CrAssphage reduction in the environment

2.3.1. In situ, downstream in the low-polluted river

CrAssphage dynamics were evaluated in the "Riera de Cànoves". This water system was chosen because it has a small catchment area (12 Km^2), the flow is controlled by a dam, and the main

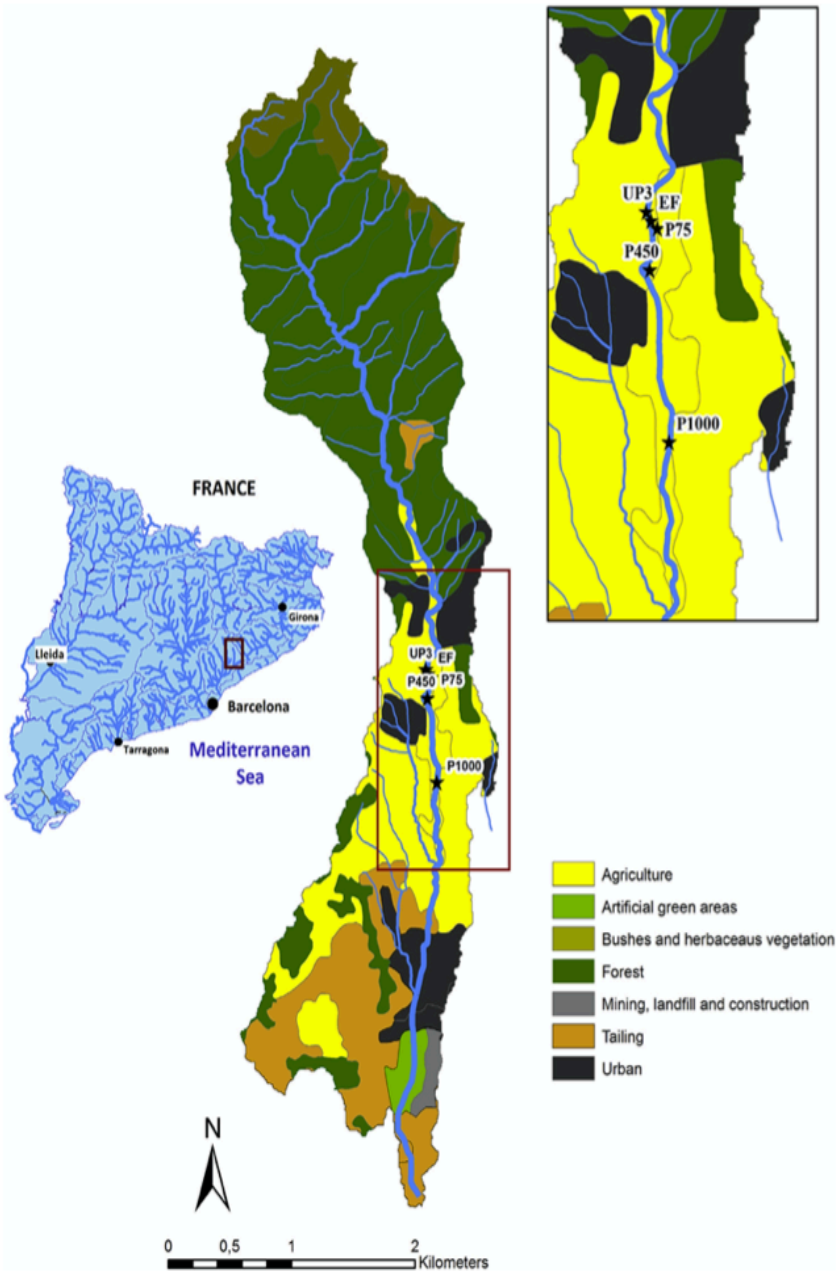

Fig. 1. Sampling sites and land use in the "Riera de Cànoves" catchment area.

pollution pressure is the effluent of a WWTP, which should be the main source of crAssphage. Before the discharge, diffuse pollution of mainly human origin was detected, but at a few orders of magnitude lower than after the discharge. Thus, before the discharge it was considered a low-polluted river. The effluent accounts for 33% of the flow on wet days to 100% on dry days. Samples were collected 75 m after the discharge of the WWTP effluent (P75) to allow it to mix completely with the river water, as well as at 450 m and 1000 m downstream (P450 and P1000) (Fig. 1). The concentration of the different faecal indicators (*E. coli* and SOMCPH) and MST markers (crAssphage, GA17PH, HMBif and HF183) were measured to monitor their removal (due to inactivation and/or sedimentation). A total of 11 sampling campaigns were performed in two years, including 6 samples taken at low temperature (maximum temperature (Tmax) < 13 °C), 3 at an intermediate temperature (Tmax between 13 and 25 °C), and 2 at a high temperature (Tmax > 25 °C) (Table 1). Environmental data (temperature and rainfall) were obtained from the closest automatic meteorological station (Catalan Meteorological Service: http://www.meteo.cat/) (Table 1). Water samples were collected using sterile containers, transferred to the laboratory at 4 °C and analysed within 6 h.

Finally, the obtained data were used to calculate the distance in which the initial population was reduced by 90% (D_{90} values [Km]):

$$\frac{N_d}{N_0} = 10^{-k_s \cdot d}$$

$$D_{90} = \frac{1}{k_s}$$

where N_d is the concentration of the microbial markers (gene

Table 1
Meteorological data obtained from an automatic station close to "Riera de Cànoves" stream during the sampling campaigns. Season: winter (W), summer (S), medium (M). Tmax: Maximum temperature (°C). Tmin: Minimum temperature (°C). R: Rainfall of the sampling day (mm). RR: Rainfall of the sampling day and 1 day before (mm). RRR: Rainfall of the sampling day and 2 days before (mm). Flow rate ($m^3\ s^{-1}$).

Sampling	Date	Season	Tmax	Tmin	R	RR	RRR	Flow rate
1	18/01/2016	W	6.4	2.3	0.0	0.0	0.0	0.007
2	08/02/2016	W	9.8	5.7	0.0	7.2	7.2	0.009
3	29/02/2016	W	11.9	5.8	0.0	1.5	33.8	0.018
4	02/05/2016	M	18.8	10.8	0.0	0.0	3.5	0.013
5	06/06/2016	S	17.5	17	0.0	0.0	1.3	0.009
6	18/07/2016	S	25.4	20.4	0.0	0.0	0.0	0.006
7	17/10/2016	M	16.5	13.3	0.0	0.0	0.1	0.009
8	29/11/2016	W	9.4	5.2	0.4	0.0	2.0	0.015
9	27/02/2017	W	12.9	5.3	0.0	0.0	0.1	0.011
10	20/06/2017	S	27.4	17.4	0.0	0.0	0.0	0.008
11	13/02/2018	W	−0.2	−7.4	0.0	13.9	13.9	na

na: Not available data.

copies (gc) 100 ml^{-1}, the plaque-forming units (PFU) 100 ml^{-1} or the colony-forming units (CFU) 100 ml^{-1}) at distance *d* (in Km), and N_0 the concentration of the microbial markers at the initial point (Km = 0).

2.3.2. In mesocosms

The persistence of FIB and MST markers was studied by a mesocosms approach, and crAssphage persistence was determined as previously described (Ballesté et al., 2018). Briefly, different dialysis bags were filled with a 1:10 dilution of sewage in groundwater and kept in a 60 m^3 concrete deposit fed by well water located in the grounds of the university and used for experimental purposes. Experiments were performed in two different seasons: summer (water temperature of around 25 °C) and winter (water temperature of around 14.5 °C). Sewage was collected from a WWTP that serves a population equivalent of 300,000. Dialysis bags were collected regularly in order to evaluate the abundance and decay of crAssphage. Finally, the obtained data were used to calculate the time required to achieve a 90% reduction in the initial population (T_{90} values [days]). A detailed description of the experiments and conditions can be obtained in Ballesté et al. (2018).

2.4. Enumeration of faecal indicator bacteria and bacteriophages by culture media

The concentrations of *E. coli* in treated sewage were analysed using Chromocult® Coliform Agar (Merck, Darmstadt, Germany) at 44.5 °C for 24 h (ISO, 2000a). Results were expressed as CFU 100 ml^{-1}. Somatic coliphages were enumerated according to ISO10705-2 (ISO, 2000b) using Modified Scholtens' Agar (MSA), and GA17PH using the double layer plaque assay reported in ISO10705-4 with the corresponding host strain as previously described (Muniesa et al., 2012). Briefly, $MgCl_2$ was added to 100 ml of water to a final concentration of 0.05 M, and concentrated by filtration using a 0.22 μm pore size mixed cellulose ester membrane (Merck Millipore, Cork, Ireland). Membranes were transferred into flasks with 6 ml Elution Buffer (1% Beef Extract, 0.5 M NaCl and 3% Tween 80) and viruses were eluted using an ultrasound bath for 4 min (Méndez et al., 2004). The elution solution was brought to a pH of 7 and filtered through a low protein-binding 0.2-μm pore size PES syringe filter (Merck Millipore) to remove any remaining bacterial cells. One ml of the solution was titred in triplicate with the corresponding host strain. Additionally, for water with a high concentration of SOMCPH (highly polluted surface water and treated sewage), 1 ml of the sample was also directly analysed in triplicate. Results were expressed as PFU 100 ml^{-1}.

2.5. Enumeration of microorganisms by molecular methods

2.5.1. Nucleic acid extraction

Different volumes of water were used for microbial source tracking analysis according to the origin of the sample and the suspended particles able to fill the filter. Each sample was concentrated using a 0.22 μm pore size cellulose ester membrane (SO-PAK, Millipore, Darmstadt, Germany), from which DNA was extracted. Membranes were placed in 0.5 ml of GITC buffer (5 M guanidine thiocyanate, 100 mM EDTA [pH 8.0], 0.5% sarkosyl) and frozen at −20 °C in lysis buffer until DNA extraction. The DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) with some modifications, as previously reported (Gourmelon et al., 2007). The same DNA extraction was used to quantify the different molecular MST markers tested. Filtration and DNA extraction controls were run together with the samples.

2.5.2. Total E. coli quantification by real-time quantitative PCR

Total *E. coli* were enumerated by quantification of a fragment of 16S rRNA gene by a qPCR TaqMan® assay using the TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Barcelona, Spain) as previously described (Huijsdens et al., 2002).

2.5.3. MST marker quantification by real-time quantitative PCR

Different MST markers linked to human faecal sources were analysed by real-time qPCR: crAssphage (García-Aljaro et al., 2017), the human-associated *Bifidobacterium* marker HMBif (Gómez-Doñate et al., 2012) and the HF183 marker (Haugland et al., 2010), using the primers, probes and protocols previously described. Amplification was performed in a 20 μl reaction mixture using TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA) in a StepOne Real-Time PCR System (Applied Biosystems). Each mixture contained the corresponding concentration of each primer and probe (García-Aljaro et al., 2017a; Gómez-Doñate et al., 2012; Haugland et al., 2010) and 5 μL of the DNA sample. The thermal-cycler conditions were 10 min at 95 °C, 40 denaturation cycles of 15 s at 95 °C, and 1 min of annealing and extension at 60 °C.

All samples, negative controls, and extraction and filtration blanks were run in duplicate, and analyses were repeated when discordance between duplicates was detected. Results were expressed as gc 100 ml^{-1}. Five points of the standard curves were included in duplicate for each run and were generated from different 10-fold serial dilutions of the linearized plasmid containing the target gene. pGEM®-T Easy Vector (Promega, Madison, WI, USA) was used for HMBif and crAssphage linearized using ScaI restriction digestion. pBluescript II SK (Agilent Tech) was used for HF183 linearized using NotI restriction digestion. The quantification limit was 6, 45 and 3 gc per reaction for crAssphage, HMBif, and HF183, respectively.

2.6. Data analysis

Microbial abundances were log-transformed and analysed for descriptive statistics. Data were analysed and plotted using the statistical software R version 3.5.1 (R Core Team, 2016) through the RStudio interface (Cahn et al., 2011) including packages "car" 3.0–2, "ggplot2" v. 3.0.1 (Wickham, 2016) and "reshape2" (Wickham, 2007). The average and standard deviation values were reported when data were normally distributed. Otherwise, the median and median absolute deviation values were used. Correlation analysis was performed among the different markers and indicators, and environmental parameters using Pearson linear correlation (Pearson coefficient, r). Values with *P* values lower than 0.05 and 0.10 are shown.

3. Results

3.1. Intra and inter comparison of crAssphage in wastewater effluents

The abundance of crAssphage was analysed in 18 WWTP effluents (inter-effluent comparison) and compared with that of *E. coli*, SOMCPH, GA17PH and HMBif. All 18 samples were positive for all the markers with the exception of GA17PH, which was below the limit of detection in 3 samples. The average concentrations of crAssphage in treated sewage were 5.28 $\log_{10}$ gc 100 ml^{-1} (with a range from 4.43 to 6.39 $\log_{10}$ gc 100 ml^{-1}), similar to those obtained with HMBif (5.38 gc 100 ml^{-1}), slightly higher than for *E. coli* (4.54 $\log_{10}$ CFU 100 ml^{-1}) and SOMCPH (4.45 PFU $\log_{10}$ 100 ml^{-1}), and around 3 $\log_{10}$ higher than for GA17PH (2.01 $\log_{10}$ PFU 100 ml^{-1}) (Table 2, Fig. 2a). Since the WWTPs sampled used different sewage treatments, which could affect the relative abundance of each microbial marker in the effluent, the correlation between different indicators was evaluated. A positive correlation was observed between crAssphage and HMBif, *E. coli* and SOMCPH but not with GA17PH. The low abundance of GA17PH may have hampered any possible correlation (Table 3, Table S1). Lower concentrations of all the markers were detected in the WWTP using nutrient removal, but otherwise no clear pattern associated with the sewage treatment was observed (Fig. S1).

After monitoring the crAssphage in the effluent of the same WWTP for two years to assess temporal variability (intra-effluent comparison), an average abundance of 5.34 $\log_{10}$ gc 100 ml^{-1} of crAssphage was found (with a range from 4.65 to 6.72 $\log_{10}$ gc 100 ml^{-1}). No seasonality was detected: the minimum and maximum concentration were observed in January and February 2016, respectively, with a return to average levels after 15 days (Fig. S2). An abundance similar to crAssphage was observed for HMBif (5.53 $\log_{10}$ gc 100 ml^{-1}) and HF183 (5.32 $\log_{10}$ gc 100 ml^{-1}), a 1 $\log_{10}$ lower concentration for *E. coli* (4.42 $\log_{10}$ CFU 100 ml^{-1}) and SOMCPH (4.26 $\log_{10}$ PFU 100 ml^{-1}), and almost 4 logs lower for GA17PH (1.69 $\log_{10}$ PFU 100 ml^{-1}) (Fig. 2b and Table 2). CrAssphage correlated best with HF183, and moderately well with HMBif and *E. coli* (Table 3, Table S1).

3.2. CrAssphage evaluation in a river highly impacted by pollution

CrAssphage abundance were measured in water samples collected in the Llobregat River over one year (n = 11). All the samples were positive for crAssphage, with an average abundance of 5.42 $\log_{10}$ units gc 100 ml^{-1} (Table 2, Fig. 2c). These values were similar to those already obtained for the molecular markers HMBif and *E. coli* (detected by qPCR), 1 $\log_{10}$ unit higher than for culture-detected *E. coli* and SOMCPH, and 3 $\log_{10}$ units higher than GA17PH (Garcia-Aljaro et al., 2017a,b). A significant correlation was observed with GA17PH and *E. coli* by qPCR (Table 3, Table S1).

All 20 samples of river sediments analysed for crAssphage were positive, with an average of 6.51 $\log_{10}$ units gc gr dry weight $(dw)^{-1}$, a minimum value of 5.17 $\log_{10}$ units gc gr dw^{-1} dry weight and maximum of 8.29 $\log_{10}$ units gc gr dw^{-1} (Table 2). Similar values were obtained for *E. coli* by qPCR, whereas the concentration of the culture-based markers *E. coli* and SOMCPH were 2 $\log_{10}$ units lower and GA17PH 5 $\log_{10}$ units lower. In this case, crAssphage correlated strongly with all the markers and indicators analysed: *E. coli* (by qPCR and culture), SOMCPH and GA17PH (Table 3, Table S1).

3.3. CrAssphage evaluation in a river with a low pollution impact

All 10 samples from the "Riera de Cànoves" (UP3) were positive

Table 2
Descriptive statistics of faecal indicators: *E. coli*, SOMCPH and human microbial source tracking markers: GA17PH, HMBif, HF183 and crAssphage from: inter WWTPs effluents comparison, intra WWTP effluent comparison for 2 years, in Llobregat River (water and sediments), and "Riera de Cànoves" stream. Data is given in $\log_{10}$ gc (*E. coli*, HMBif, HF183 and crAssphage), PFU (SOMCPH and GA17PH), CFU (*E. coli*) per 100 ml for water samples or per gr of dry weight for sediment samples. The data for the Llobregat River of *E. coli* (culture and qPCR), SOMCPH, GA17PH and HMBif is already published in García-Aljaro et al. (2017b). na: Not available.

Sample	values	*E. coli*	*E. coli* qPCR	SOMCPH	GA17PH	HMBif	HF183	crAssphage
Inter	n	18/18	Na	18/18	15/18	18/18	na	18/18
Effluent	mean	4.54	Na	4.45	2.01	5.38	na	5.28
comparison	sd	0.94	Na	0.88	0.75	0.56	na	0.54
	min	2.92	Na	3.10	0.60	4.23	na	4.43
	max	6.56	Na	6.28	2.95	5.99	na	6.39
Intra	n	11/11	Na	11/11	11/11	10/10	10/10	11/11
Effluent	mean	4.42	Na	4.26	1.69	5.53	5.32	5.34
comparison	sd	0.48	Na	0.18	0.50	0.63	0.89	0.59
	min	3.50	Na	4.03	0.78	4.30	3.37	4.65
	max	4.97	Na	4.59	2.27	6.54	6.44	6.72
Llobregat	n	11/11	11/11	11/11	11/11	11/11	na	11/11
River	mean	4.49	5.08	4.46	2.26	6.08	na	5.42
Water	sd	0.45	0.88	0.23	0.68	0.55	na	0.60
	min	3.94	4.04	4.12	0.60	5.05	na	4.53
	max	5.21	6.33	4.78	3.26	6.76	na	6.47
Llobregat	n	18/20	20/20	18/20	18/20	na	na	20/20
River	mean	4.10	6.18	4.31	1.04	na	na	6.51
Sediments	sd	1.20	0.67	1.28	1.10	na	na	0.76
	min	1.12	5.05	1.61	−0.38	na	na	5.17
	max	5.71	7.55	5.81	2.62	na	na	8.29
Riera de	n	10/10	Na	9/10	3/10	8/10	7/10	10/10
Cànoves	mean	2.86	Na	1.94	0.93	3.56	3.46	3.46
Stream	sd	0.53	Na	0.55	0.64	0.66	0.87	1.29
	min	2.03	Na	0.97	0.30	2.68	2.35	0.95
	max	3.49	Na	2.67	1.58	4.77	4.41	5.28

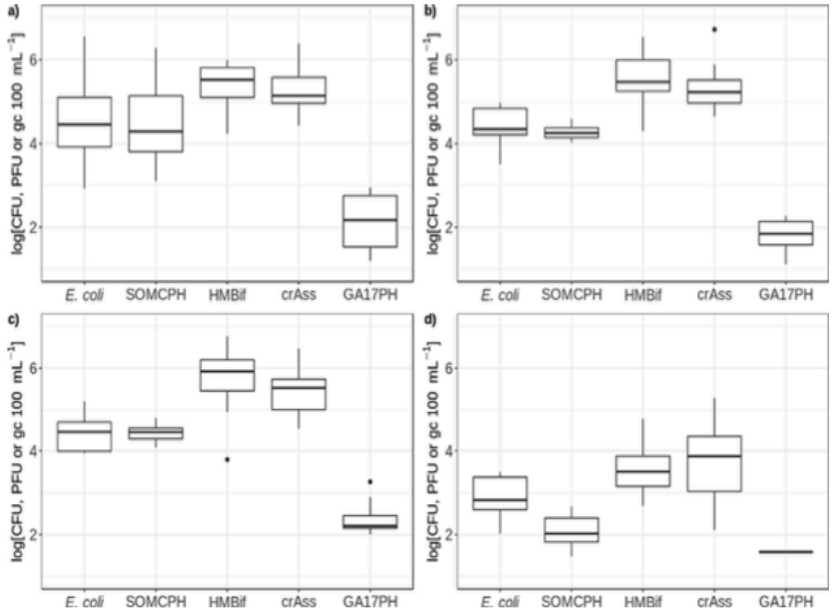

Fig. 2. Boxplot comparing the concentrations of the faecal indicators *E. coli* and SOMCPH, and human microbial source tracking markers HMBif, crAssphage and GA17PH in the: a) inter-effluent comparison (18 WWTPs), b) intra-effluent comparison (1 WWTP during 11 samplings), c) Llobregat River (11 samples) and d) "Riera de Cànoves" (10 samples).

Table 3
Significant Pearson correlation coefficients between crAssphage and other markers in the different environments tested in this study.

	E. coli	*E. coli* qPCR	SOMCPH	GA17PH	HMBif	HF183
Inter effluent comparison	0.729[a]	na	0.665[a]	nsc	0.757[a]	na
Intra effluent comparison	0.585[b]	na	nsc	nsc	0.603[b]	0.642[a]
Llobregat River Water	0.589[b]	nsc	nsc	0.708[a]	nsc	na
Llobregat River Sediments	0.804[a]	0.878[a]	0.843[a]	0.755[a]	na	na
Riera de Cànoves Stream	0.589[b]	na	nsc	nsc	0.794[a]	0.877[a]
Reduction along the stream	nsc	na	0.875[a]	nsc	0.802[a]	0.759[a]

nsc: Not significant correlation.
na: Not available.
[a] Significant at $p \leq 0.05$.
[b] Significant at $p \leq 0.1$ and ≥ 0.05.

for crAssphage and *E. coli*, whereas 9 were positive for SOMCPH, 8 for HMBif, 7 for HF183 and 3 for GA17PH. The crAssphage concentration at this sampling point were highly variable (within a range of 0.95–5.28 $\log_{10}$ units gc 100 ml^{-1}), with an average concentration of 3.46 $\log_{10}$ units gc 100 ml^{-1} (Table 2 and Fig. 2d). CrAssphage also showed a high variability when compared with other markers, with a range of 4.33 $\log_{10}$ units, and a high positive correlation was found with HF183, HMBif, and *E. coli* (Table 3, Table S1). CrAssphage concentrations correlated positively with the accumulated rainfall data from the sampling day and 3 days before (r_s: 0.656, *P* value: 0.039). Downstream of this sampling point, crAssphage concentration in the effluent discharged by the WWTP was 5.34 ± 0.59 $\log_{10}$ units gc 100 ml^{-1}. The highest crAssphage values for UP3 and the effluent were observed on the same sampling date (February 8th, 2016) after a rainfall event (rain the day before: 7.2 m^3).

3.4. CrAssphage reduction in the environment: in situ downstream

The reduction of crAssphage was measured in the "Riera de Cànoves" together with that of *E. coli*, SOMCPH, GA17PH, HMBif and HF183. Samples were first collected at 75 m downstream of the effluent discharge to allow the mixing of river and effluent waters, and then at 450 m and 1000 m. The concentrations of the markers usually decreased downstream at different rates (Fig. 3). A shorter D_{90} (distance in which a 90% reduction of the population was observed) represents a higher reduction, whereas a longer D_{90} represents a higher persistence in the water flow and a longer diffusion along the water body. The D_{90} of crAssphage oscillated between 0.23 and 1.06 Km, with a median of 0.62 Km (Table 4, Fig. 4), with an outlier value of 10.62 Km in sample 11 after a heavy rainfall event (the day before the sampling: 13.9 m^3). Although there was a big rainfall event before sampling 3, the D_{90} was within the range. This could be because the rain fell two days before the sampling (33.8 m^3) and the system had already recovered, although other factors may have contributed. The longest D_{90}s were observed for the infectious phages SOMCPH and GA17PH (Table 4), followed by *E. coli* with similar values to crAssphage, and finally the shortest D_{90}s were obtained for HF183 and HMBif. For the latter, no reduction could be calculated during the high temperature samplings (6 and 10) since the concentrations were under the limit of detection at P450 and P1000. For these samples the smallest D_{90} value obtained was used (sampling 1). The reduction in crAssphage strongly correlated with that of SOMCPH, HMBif and HF183, and a moderate correlation was observed with *E. coli* and GA17PH (Table 3, Table S1).

Records of maximum and minimum temperature and rainfall were used to assess correlations between the indicators and environmental parameters (Table 1). Occasionally, the concentration of the different markers did not decrease downstream or even

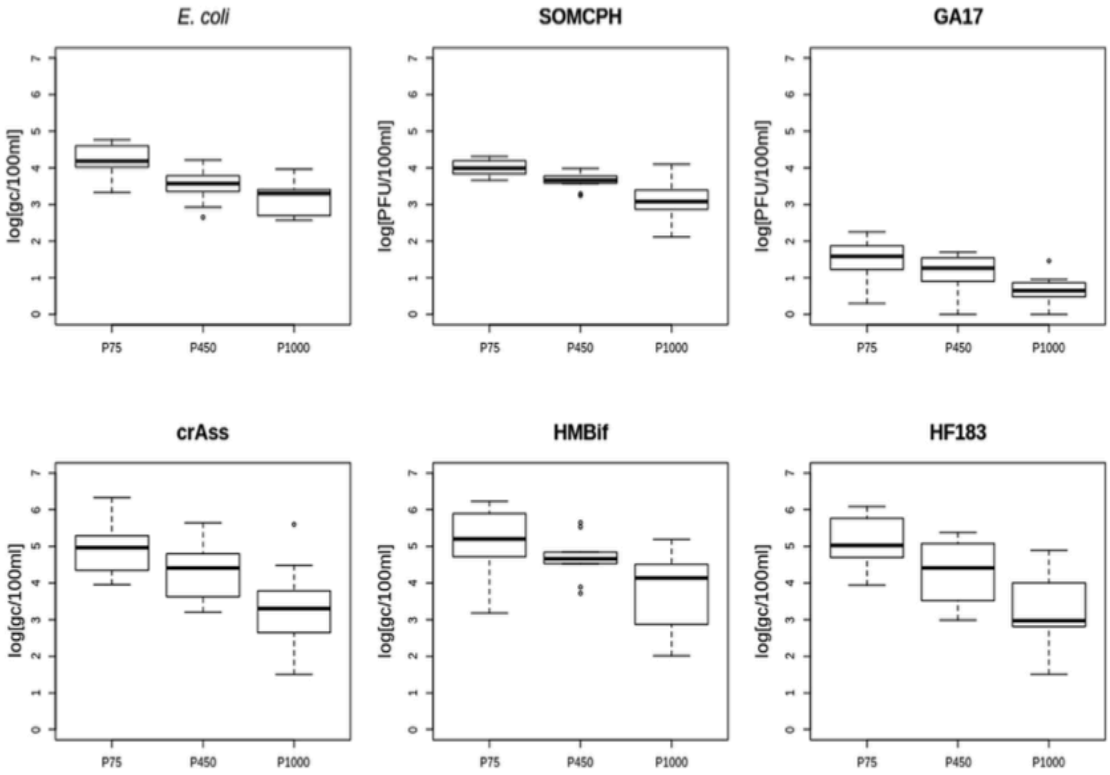

Fig. 3. Concentration of *E. coli*, SOMCPH, GA17PH, crAssphage, HMBif and HF183 in different sampling points along "Riera de Cànoves" stream. P75 – 75 m downstream the WWTP discharge; P450 – 450 m downstream the discharge; P1000 – 1000 m after the discharge.

Table 4
Distance (km) with a 90% reduction of the initial bacterial or phage concentration (D_{90}) obtained in "Riera de Cànoves" catchment for *E. coli* culture-based, SOMCPH, GA17PH, HMBif, HF183 and crAssphage. The distance sampled to calculate the D_{90} was 925 m.

Sampling	*E. coli*	SOMCPH	GA17PH	HMBif	HF183	crAssphage
1	0.53	0.56	0.78	0.28	0.30	0.49
2	1.05	1.20	3.61	0.82	0.72	1.06
3	1.70	1.65	−2.56	1.92	1.03	0.82
4	0.47	1.18	0.93	1.37	0.59	0.43
5	0.63	0.71	1.72	0.55	0.40	0.62
6	0.52	0.42	0.23	<0.28	0.20	0.23
7	0.67	1.00	1.27	0.33	0.49	0.58
8	0.71	1.23	1.59	0.40	0.58	0.73
9	1.49	1.88	1.97	1.01	0.87	0.79
10	1.49	2.57	0.63	<0.28	0.38	0.37
11	1.42	4.86	NA	2.95	NA	10.62
Median	**0.71**	**1.20**	**1.10**	**0.55**	**0.54**	**0.62**
Mad[a]	**0.36**	**0.72**	**0.82**	**0.40**	**0.25**	**0.28**

[a] Mad: Median absolute deviation.

increased slightly after rainfall events (Fig. S3, Fig. 4). CrAssphage showed a very strong positive correlation with the rainfall data (the accumulated rainfall from the sampling day and the day before) (r: 0.900, df: 9, *P* value < 0.001), indicating that a longer distance was needed for the initial population to decrease after rainfall. Moreover, a strong negative correlation with temperature was observed, with the population decreasing more rapidly at higher temperatures (T_{max} −0.631, *P* value: 0.037 and r T_{min} −0.700, df: 9, *P* value: 0.017). CrAssphage was the indicator with the strongest correlation with environmental parameters, although SOMCPH and HMBif also correlated highly with rainfall (r: 0.768, df: 9, *P* value: 0.006 and r: 0.739, df: 7, *P* value: 0.023), but less significantly with the minimum temperature (r: −0.601, df: 9, *P* value: 0.051 and r: −0.635, df: 7, *P* value: 0.066). However, a low correlation was observed between crAssphage and flow rate, whereas the correlation was high for HF183 and HMBif (r: 0.788, df: 8, *P* value: 0.007 and r: 0.770, df: 8, *P* value: 0.009).

3.5. CrAssphage reduction in the environment: mesocosms

The inactivation of crAssphage was evaluated using mesocosms during seasons of low and high temperature. CrAssphage was detected during 6 days in the summer experiment, when water temperatures ranged from 22 to 28 °C (mean of 25 °C) and the maximum global solar radiation from 784 W m^{-2} to 1,100 W m^{-2}. In winter, with ranges of water temperature of 8.5–11 °C and maximum global solar radiation of 273–871 W m^{-2}, the marker was detected for up to 21 days (the duration of the experiment) (Fig. 5). The average obtained T_{90}s were 2.41 days in summer and 10.21 days in winter. These values were similar to or slightly higher than for *E. coli* and other MST markers in summer (T_{90} of 1.05 days for HF183, 2.20 days for HMBif, 1.52 days for *E. coli* culture and 2.62 days for *E. coli* qPCR) and much higher in winter (T_{90} of 3.26 days for HF183, 6.12 days for HMBif, 2.06 days for *E. coli* culture and 4.26 days for *E. coli* qPCR) (Table S2) (Ballesté et al., 2018). T_{90} values for SOMCPH were similar to crAssphage in winter (9.90 ± 1.58 days) and higher in summer (4.84 ± 0.30 days).

4. Discussion

The recently described *Bacteroides* bacteriophage crAssphage is reported to be one of the most abundant viruses in sewage and human faecal samples (Dutilh et al., 2014; Shkoporov et al., 2018; Stachler and Bibby, 2014). Due to its prevalence, it has been postulated as a potential human MST marker and two different methods have already been developed for its application. The assay

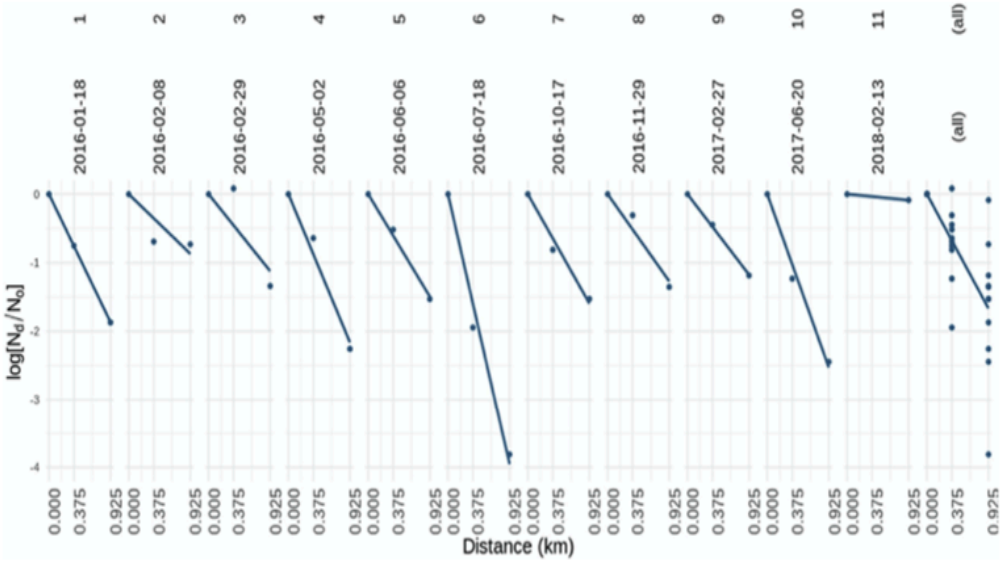

Fig. 4. CrAssphage reduction along 1 Km in the "Riera de Cànoves" during 11 different sampling campaigns over 2 years. Logarithmic reduction is calculated as the difference of the logarithm of gc 100 ml^{-1} at each distance (N_d) and Km 0 (N_o). The last facet includes the accumulation of all the reductions.

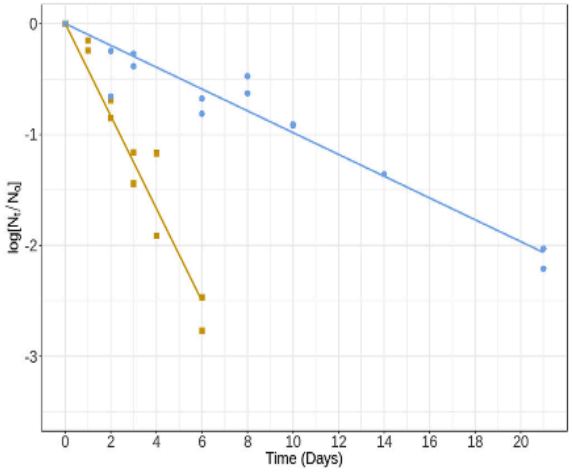

Fig. 5. CrAssphage inactivation assessed by an *in situ* mesocosms approach in two seasons: summer (squares) and winter (dots). Logarithmic reduction is calculated as the difference of the logarithm of gc 100 ml^{-1} at each time (N_t) and the beginning of the experiment (N_o).

designed in the USA (CPQ_056 and CPQ_064) showed the marker was highly abundant in raw sewage (9.1–10.0 $\log_{10}$ gc L^{-1}), with a high specificity and sensitivity, although cross-reactivity has been observed, mainly with poultry samples (Ahmed et al., 2018; Stachler et al., 2017). The other method developed in Europe, was adapted and validated to the crAssphage sequence detected in the area, that shows few differences with the crAssphage sequence in USA (García-Aljaro et al., 2017). This assay revealed a similar marker abundance to that of CPQ_056 and CPQ_064 in raw sewage (8.4–9.9 $\log_{10}$ gc L^{-1}) and high sensitivity, although with some occurrence in animal samples. However, the concentration in non-targeted samples was found to be 2–4 orders of magnitude lower than in untreated sewage samples (García-Aljaro et al., 2017a; Stachler et al., 2017). Given that this marker targets a virus, and its concentration is at least two orders of magnitude higher than enteric viruses (Ahmed et al., 2018), it could be a more effective pathogen indicator than the classical faecal indicators *E. coli* and enterococci. Another advantage is that it is a molecular method and the same DNA extraction can be used to analyse complementary faecal indicators to improve the source identification (Casanovas-Massana et al., 2015). However, to date no studies have determined crAssphage dynamics in the environment, which is fundamental for its application for water management purposes.

In our study, crAssphage was detected in WWTP effluents at higher concentrations (1 order of magnitude) than the general culture-based indicators SOMCPH and *E. coli*, whereas its abundance in raw sewage was similar to that of other human molecular MST markers like HMBif and HF183 (García-Aljaro et al., 2017a; Stachler et al., 2017). In addition, crAssphage showed a concentration 3–4 logs higher than infectious *Bacteroides* strain GA17 bacteriophages (GA17PH). GA17PH has proved to be a good human-associated marker in the studied area and has been used as a culturable indicator of human enteric viruses (Jofre et al., 2014; Muniesa et al., 2012). The intra-effluent comparison showed that crAssphage abundance was quite stable along the year, with variations possibly related to rainfall events, which overwhelmed WWTP capabilities by increasing the flow. Comparing crAssphage concentrations in the WWTP effluents in the current study with those previously reported in untreated sewage (García-Aljaro et al., 2017) (mean of 8.24 $\log_{10}$ units gc 100 ml^{-1}) reveals a decrease of around 3 $\log_{10}$ after the wastewater treatment. However, the crAssphage concentration in the effluents discharged to the environment was similar to that of bacterial molecular markers and higher than cultured bacterial and infectious viral markers. Similar values for faecal indicators and human source tracking markers other than crAssphage were observed in treated wastewaters from Europe and Northern Africa than in those of this work (i.e. *E. coli* and SOMCPH around 4 $\log_{10}$ CFU and PFU 100 ml^{-1}, GA17PH between 0.1 and 3 $\log_{10}$ PFU 100 ml^{-1} and HF183 between 4 and 6 $\log_{10}$ gc 100 ml^{-1}) (Mayer et al., 2018, 2016; Yahya et al., 2015; Wery et al., 2008). Meanwhile values of around 2.5 $\log_{10}$ and 1–2 $\log_{10}$ gc 100 mL^{-1} were observed for human adenoviruses and polyomaviruses in effluents in Europe (Bofill-Mas et al., 2006; Mayer et al., 2016), 3–4 $\log_{10}$ lower concentration than crAssphage.

The abundance of crAssphage was measured in two different kinds of rivers: the Llobregat River, which has a medium-high flow and a high human pollution impact, and the temporal "Riera de

Cànoves", which has a low flow and is impacted by diffuse pollution (upstream of the WWTP effluent discharge). The same pattern of abundances was observed in the two rivers and also in the WWTP effluents. The molecular markers detected by qPCR (crAssphage and HMBif) were 2 orders of magnitude more abundant than *E. coli* and SOMCPH detected by culture-based methods, and almost 3 orders of magnitude higher than GA17PH. The concentration of crAssphage in the Llobregat River remained stable throughout the 11 sampling campaigns, with values similar to those of the WWTP effluents, thereby confirming the low water quality of this river at this site (Casanovas-Massana et al., 2015). A high concentration of crAssphage was also found in the sediments in the lower transect of the river, similar to other qPCR markers and indicators (Garcia-Aljaro et al., 2017). The lower concentration of cultured indicators confirms that DNA markers can still be detected when viability has decreased. The high concentration of MST markers indicate that microorganisms accumulate in the sediments and that these may act as a reservoir of microorganisms, including indicators, which can be mobilized after a rainfall event (Calero-Cáceres et al., 2017; Cho et al., 2016; Fauvel et al., 2016; Garcia-Aljaro et al., 2017b; Jamieson et al., 2005; Wu et al., 2009). Although it cannot be completely excluded, growth of the MST markers is not expected to take place in the sediments, firstly because most of them are indicators, hence unable to propagate in the environment (Martín-Díaz et al., 2017). In addition, for CrAssphage, the anaerobic metabolism of its bacterial host, *Bacteroides*, precludes its propagation.

A high variability in crAssphage abundance was observed in the "Riera de Cànoves", with a strong correlation with two human markers, suggesting that the diffuse human pollution in the area may be due to septic tank leakages in households without a sewer connection. This supposition is supported by a moderate correlation of crAssphage with 3–4 days of accumulated rainfall, which may have increased the leakage (Peed et al., 2011).

The concentration of crAssphage in the "Riera de Cànoves" downstream of the WWTP effluent discharge was normally lower, unless there was a big rainfall event (as in sampling campaign 11). CrAssphage persisted longer than other bacterial markers analysed by qPCR but less than the culturable markers, especially the viral indicators SOMCPH and GA17PH, for which the D_{90} was almost double. The inactivation results obtained for the infectious bacteriophages analysed in this study are in accordance with the study of Fauvel and co-workers, who obtained a D_{90} between 2.8 and 9.5 km for infectious FRNA phages in a river (Fauvel et al., 2017). These results show that although viral indicators generally persist longer in the environment than bacterial indicators, crAssphage had a lower persistence than the infectious bacteriophages, even when detected by molecular methods. Despite these differences, a strong correlation was observed between crAssphage and SOMCPH. This may happen when the pollution is still recent, then correlation can be maintained and may disappear when the pollution becomes aged. Besides, it has to be considered that *E. coli* and SOMCPH are general indicators and their source in the catchment maybe attributed to other animal sources, although the presence of animal-associated MST markers was not relevant at the catchment (data not shown).

The reduction of crAssphage in the "Riera de Cànoves" cannot be attributed only to natural inactivation, but to removal from the water column by sedimentation since viruses and bacteria may become attached to particles and sediment in the waterbed (Cho et al., 2016; Jamieson et al., 2005). The correlation of higher crAssphage concentration with rainfall events may be associated with an increase in diffuse pollution originating from septic tanks, an increased effluent flow from the WWTPs, and/or the mobilisation of sediments which, as already suggested may become a reservoir of faecal indicators (Calero-Cáceres et al., 2017; Garcia-Aljaro et al., 2017). However, some other reports indicate that viruses are mainly free in water bodies (Peduzzi and Luef, 2008), if this is the case, natural inactivation factors can play a major role in the reduction of crAssphage in this site.

The persistence of crAssphage was measured using an *in situ* mesocosms approach performed previously to analyse the die-off of different MST markers (Ballesté et al., 2018). Thus, DNA extractions stored at −80 °C were used to evaluate the crAssphage inactivation during high and low temperature seasons and it was compared with that of other human MST markers like HMBif and HF183 and faecal indicators like *E. coli* and SOMCPH. A higher inactivation was observed in summer, as already found for bacterial indicators, but the T_{90}s of crAssphage were higher than those of the other MST markers and *E. coli* during both seasons, suggesting a greater resistance to environmental parameters. This effect was particularly significant in winter, when crAssphage was detected on more than 21 days compared to 10 days for the other MST markers, which had between 1.66 and 5-fold lower T_{90}. In winter the decay of crAssphage was similar to that of SOMCPH (10.21 and 9.90 days, respectively), although in summer SOMCPH persisted for longer. Other studies have reported similar results for infectious SOMCPH and *Bacteroides* phages with T_{90}s of 3–4 days in summer and 14–16 days in winter (Casanovas-Massana and Blanch, 2013) and 1.72 days in summer and 13.54 days in winter (Wu et al., 2016). Thus, crAssphage has similar persistence to SOMCPH, the slight variation being possibly due to the different methodological and environmental parameters of each study.

These results confirm that in summer there are several inactivation parameters, including higher temperature, higher irradiation and more active predators like grazing protozoa and bacteria (Barcina et al., 1997; Menon et al., 2003; Wanjugi and Harwood, 2013), which exert a similar effect on the different MST markers and indicators. In contrast, in winter the intrinsic characteristics of the marker become more significant (Ballesté et al., 2018), in this case being the higher resistance of a viral particle. It should be noted that ΦCrAss001, the only bacteriophage isolated from the crAssphage family, has an unusual way of replication; it can generate free virions, and although not a temperate phage it interacts with *Bacteroides intestinalis* without causing lysis in a sort of carrier state life cycle (Shkoporov et al., 2018). Thus, phage virions should be able to coexist with bacterial cells carrying the non-integrated phage. The proportion of each state in natural samples is unknown and may be related to both intrinsic and extrinsic parameters. Therefore, the persistence would vary depending on the crAssphage state (bacterial or virion). Likewise, the virion state of crAssphage can maintain good correlation with SOMCPH despite the differences in persistence shown by the bacterial state. In this work, crAssphage was analysed by molecular methods from the total DNA isolated from samples, hence it was impossible to discern whether the detected phage was in the virion state or inside the bacterial cell. Differences in the phage state in different environmental conditions (after inactivation or seasonal) could explain the variable correlations with bacterial molecular markers or infectious phages found in this study.

In general, crAssphage correlated highly with other human-associated markers like HMBif and HF183 and with *E. coli* in the wastewater effluents and in the "Riera de Cànoves". In the highly polluted Llobregat River correlation was found only with GA17PH and *E. coli* measured with molecular methods. The high level of pollution in this river, with some sources far upstream, may alter the correlation between parameters. On the other hand, crAssphage correlated with SOMCPH in the inter-effluent comparison and also in sediments, where crAssphage correlated with all the markers tested. The reduction of crAssphage also correlated with all the observed indicators. Ahmed et al. (2018) observed similar

trends in crAssphage and HF183 in polluted waters during dry and wet weather, although the presence and absence did not always coincide, a discrepancy was attributed to different rates of decay. The present study confirms the variable decay rates of crAssphage and human MST markers, including HF183, with the differences being notably higher in the low temperature season.

Based on these observations, crAssphage can been postulated as a useful alternative to be included in the global MST toolbox (Dutilh et al., 2014; García-Aljaro et al., 2017a; Stachler et al., 2017). However, as already mentioned, crAssphage does not show 100% specificity and has been detected in animal samples, albeit at low concentration (Ahmed et al., 2018; García-Aljaro et al., 2017a; Stachler et al., 2017), which means it cannot be used as a human MST marker on its own. Nevertheless, since it is detected at a low concentration in non-targeted samples (around 3 $\log_{10}$ lower), it may not be problematic in the analysis of diluted environmental samples unless a big volume of sample is concentrated. Besides, some methodological pitfalls have to be considered, in this case crAssphage was evaluated as the bacterial MST markers. However, the concentration method used (filtration through 0.22 μm cellulose ester membrane) might not be the optimal for the recovery of free bacteriophages that might have been lost through the filter, then crAssphage abundance could have been underestimated. However, it has already been observed that the viral fraction of sewage holds 1 $\log_{10}$ lower crAssphage concentration than the total DNA fraction, what suggests that a big part is in the bacterial cell (García-Aljaro et al., 2017). Moreover, molecular techniques may show differences depending on the DNA extraction method used, and here the same DNA extraction method was used for all the markers to avoid the bias caused by the different efficiencies during the extraction step.

Some controversy appears when proposing molecular or culture-based methods for MST. Molecular MST markers do not depend on the use of a right host (for phages or viruses) or on the viable-but not culturable state of the marker, allowing the detection of the markers regardless their physiological state. In contrast, molecular detection does not provide information of the infectivity of the marker, although this should not pose a serious drawback for MST that aims to know the faecal source of the pollution. Nevertheless, it is highly advisable to use different MST markers that, in combination with other strategies such as conditional probabilities or machine learning approaches, can provide the maximum accuracy in determining the source of faecal pollution (Ballesté et al., 2010; Kildare et al., 2007; Mayer et al., 2018; Sánchez et al., 2011).

5. Conclusions

- CrAssphage is highly sensitive, abundant, and shows high correlation with other faecal indicators (viral and bacterial, cultured and molecular). Thus, crAssphage enlarges the toolbox of potential MST markers and can be used for water management and monitoring.
- CrAssphage is highly abundant and stable in WWTP effluents, showing similar abundances to and high correlation with human bacterial source tracking markers and higher abundance than *E. coli* and other viral indicators.
- CrAssphage was detected in human-impacted rivers at similar abundance to other human MST markers and at higher concentration than faecal indicators for both point and diffuse pollution. In the latter case, abundance was related with rainfall events.
- CrAssphage inactivated more rapidly at high than at low temperatures. During the low temperature season it showed a T_{90} of around 10 days, which is similar to SOMCPH and much higher than other bacterial human source tracking markers.
- Sediments can become a reservoir of crAssphage together with other molecular markers, which can be mobilized after rainfall events.
- CrAssphage persisted longer in waterbodies than other molecular MST markers like HMBif and HF183 but less than culture viral markers SOMCPH and GA17PH. Thus, factors other than natural inactivation, including particle adsorption, sedimentation or the bacteriophage state (virion or inside the cell), may affect crAssphage reduction.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.02.042.

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Supplementary material

Figure S1. Comparison between the levels of *E. coli*, SOMCPH and different human MST markers GA17PH, HMBif and crAssphage in treated sewage from 18 WWTP with different depuration treatments (inter effluent comparison). PT – primary treatment; B – Biological treatment, B+N – Biological and Nitrogen removal; B+P– Biological and Phosphorus removal; B+N+P– Biological, Nitrogen and Phosphorus removal; B+N+P+T – Biological, Nitrogen and Phosphorus removal and tertiary treatment.

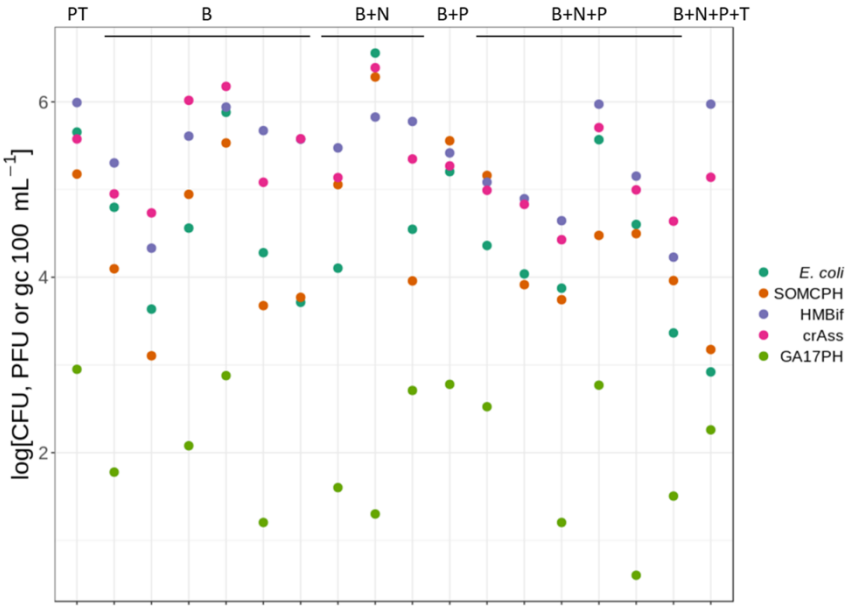

Figure S2. Temporal comparison between the levels of E. coli SOMCPH and different human MST markers GA17PH, HMBif and crAssphage in treated sewage from a WWTP along 2 years (11 samplings) (intra effluent comparison).

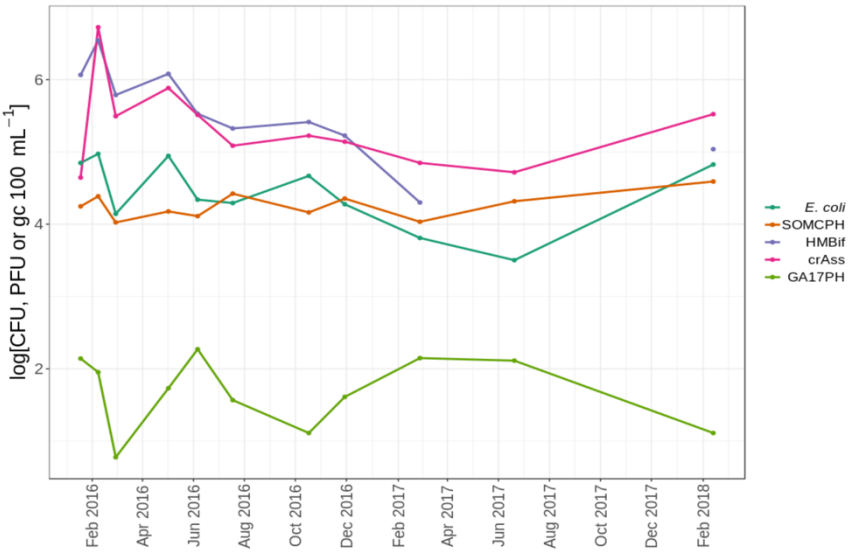

Figure S3. Decrease of the levels of E. coli, SOMCPH, GA17PH, HMBif, HF183 and crAssphage along the "Riera de Cànoves" stream in the 11 sampling campaigns. The last facet shows an integrated graph for all the 11 samplings.

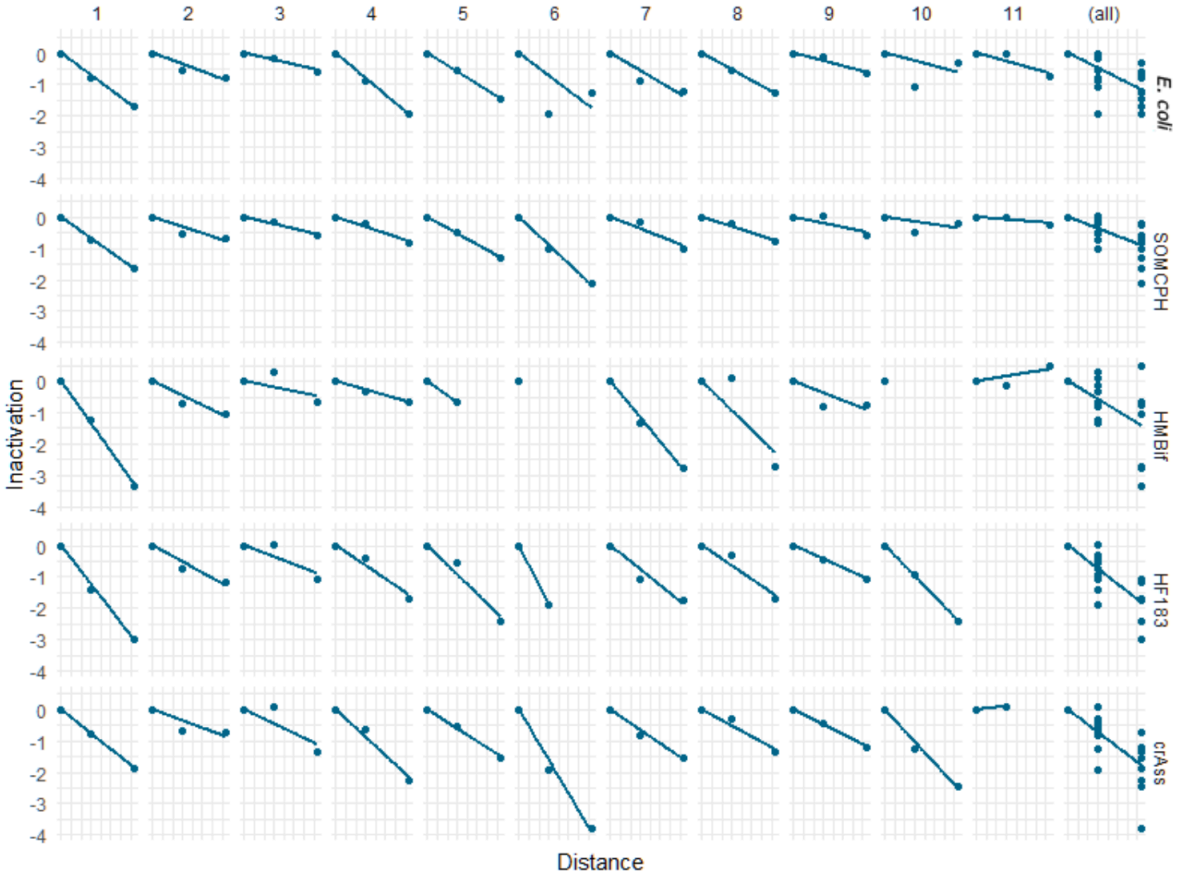

Table S1. Pearson Correlation coefficent between E. coli, SOMCPH and human microbial source tracking markers: GA17PH, HMBif, HF183 and crAssphage from: inter WWTPs effluents comparison, intra WWTP effluent comparison for 2 years, in Llobregat River (water and sediments), "Riera de Cànoves" stream, and between reductions in "Riera de Cànoves" stream. Entries above the diagonal and labelled in bold are the P-value associated to the test and values below de diagonal are the Pearson coefficient.

Inter WWTPs effluent	*E. coli*	SOMCPH	GA17PH	HMBif	crAssphage
E. coli		**0.00**	**0.29**	**0.02**	**0.00**
SOMCPH	0.817		**0.39**	**0.10**	**0.00**
GA17PH	0.291	0.237		**0.06**	**0.16**
HMBif	0.535	0.399	0.504		**0.00**
crAssphage	0.729	0.665	0.378	0.757	

Intra WWTPs effluent	*E. coli*	SOMCPH	GA17PH	HMBif	HF183	crAssphage
E. coli		**0.36**	**0.58**	**0.02**	**0.00**	**0.06**
SOMCPH	0.305		**0.77**	**0.84**	**0.97**	**0.65**
GA17PH	-0.187	-0.101		**0.90**	**0.70**	**0.66**
HMBif	0.714	0.075	0.046		**0.00**	**0.06**
HF183	0.888	0.013	-0.141	0.950		**0.05**
crAssphage	0.585	0.156	-0.150	0.603	0.642	

Llobregat river	*E. coli*	*E. coli* qPCR	SOMCPH	GA17PH	HMBif	crAssphage
E. coli		**0.72**	**1.00**	**0.07**	**0.34**	**0.06**
E. coli qPCR	-0.107		**0.00**	**0.31**	**0.64**	**0.19**
SOMCPH	-0.001	0.712		**0.27**	**0.85**	**0.28**
GA17PH	0.473	0.294	0.306		**0.17**	**0.01**
HMBif	0.318	0.167	0.065	0.447		**0.74**
crAssphage	0.589	0.428	0.359	0.708	0.154	

Riera de Cànoves stream	*E. coli*	SOMCPH	GA17PH	HMBif	HF183	crAssphage
E. coli		**0.79**	**0.27**	**0.25**	**0.01**	**0.07**
SOMCPH	0.103		**0.73**	**0.55**	**0.18**	**0.16**
GA17PH	0.911	0.417		**0.17**	**0.11**	**0.74**
HMBif	0.460	0.252	-0.966		**0.06**	**0.02**
HF183	0.862	0.572	0.984	0.742		**0.01**
crAssphage	0.589	0.510	-0.340	0.794	0.877	

Reduction in Riera de Cànoves	*E. coli*	SOMCPH	GA17PH	HMBif	HF183	crAssphage
E. coli		**0.03**	**0.38**	**0.10**	**0.03**	**0.30**
SOMCPH	0.660		**0.74**	**0.07**	**0.17**	**<0.01**
GA17PH	-0.310	-0.120		**0.19**	**0.60**	**0.38**
HMBif	0.527	0.766	-0.454		**<0.01**	**<0.01**
HF183	0.692	0.473	-0.188	0.831		**0.01**
crAssphage	0.347	0.874	0.310	0.803	0.759	

Table S2. Inactivation data (Ks, R , and T90) of *E. coli* measured by culture and qPCR methods (culture-EC, qPCR-EC), somatic coliphages (SOMCPH) and the molecular human source tracking markers: HMBif, HF183 and crAssphage performed during two seasons (summer and winter).

Season		**culture-EC**		**qPCR-EC**		**SOMCPH**	
		1	**2**	**1**	**2**	**1**	**2**
Summer	K_s	-0.68	-0.64	-0.40	-0.37	-0.22	-0.20
	R^2	0.94	0.84	0.78	0.75	0.93	0.98
	T_{90}	1.46	1.57	2.51	2.73	4.63	5.05
Winter	K_s	-0.49	-0.48	-0.25	-0.22	-0.09	-0.11
	R^2	0.97	0.98	0.93	0.86	0.976	0.97
	T_{90}	2.06	2.07	3.98	4.53	11.02	8.78
		HMBif		**HF183**		**crAssphage**	
		1	**2**	**1**	**2**	**1**	**2**
Summer	K_s	-0.50	-0.42	-1.09	-0.85	-0.38	-0.46
	R^2	0.97	0.93	0.87	0.89	0.98	0.99
	T_{90}	2.01	2.39	0.93	1.18	2.64	2.19
Winter	K_s	-0.16	-0.16	-0.29	-0.32	-0.10	-0.96
	R^2	0.80	0.84	0.97	0.97	0.98	0.96
	T_{90}	6.15	6.10	3.42	3.10	10.06	10.36

4.2 ARTÍCULO 2

Modelling the seasonal impacts of a wastewater treatment plant on water quality in a Mediterranean stream using microbial indicators

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El análisis de indicadores de contaminación fecal es una de las técnicas más utilizadas para la evaluación de la calidad del agua y su gestión. No obstante, en las últimas décadas, en un contexto donde muchas regiones del planeta se ven afectadas por la escasez hídrica y una elevada presión antrópica sobre los recursos, se han buscado otras estrategias para la gestión hídrica. La modelización de la contaminación ha adquirido gran relevancia como estrategia de gestión de los recursos hídricos.

Esta investigación se planteó evaluar las dinámicas estacionales de diferentes microorganismos indicadores aguas abajo de una EDAR, analizar las distancias de autodepuración de cada indicador y modelizarlas en función de factores ambientales para poder comparar el comportamiento de los diferentes indicadores.

La zona de estudio fue la riera de Cànoves, un río intermitente, en un tramo afectado por el vertido del efluente secundario de una EDAR. Los puntos de muestreo fueron un punto previo al vertido de la EDAR (Upper), el efluente secundario de la EDAR (EF), un tramo aguas abajo de la EDAR muestreando cada 75 m hasta los 450 m y finalmente, un punto situado a 1 km aguas abajo de la EDAR. En los nueve puntos de muestreo se analizaron cinco microorganismos indicadores de contaminación fecal: *E. coli* (como indicador bacteriano), colifagos somáticos (SOMCPH, como indicador vírico), esporas de clostridios reductores de sulfito (SSRC, como indicador de formas de resistencia), bacteriófagos de *Bacteroides thetaiotaomicron* GA17 (GA17PH, como indicador vírico de origen humano) y el marcador humano de *Bifidobacterium* (HMBif como marcador humano de MST). El estudio se llevó a cabo en un período de dos años (2016-2018).

Los resultados mostraron un caudal con una gran temporalidad, diferenciando dos temporadas: la temporada seca y la húmeda. La temporada seca, principalmente el verano, presentó un caudal inferior a 0,005 m^3/s y el efluente de la depuradora llegó a constituir un 100% del caudal aguas abajo de la EDAR. Mientras que en la temporada húmeda constituyó un 50-55% del caudal.

Los indicadores de contaminación fecal se detectaron en el río antes del vertido de la EDAR en concentraciones bajas, pero de forma recurrente (en torno a 2 log_{10}/100ml). Sin embargo, el efluente de la EDAR provocó un aumento significativo en las concentraciones de todos los indicadores analizados en el río 75 m aguas abajo de la EDAR (en torno a 4 log_{10}/100ml). Asimismo, aguas abajo de la EDAR se observó una reducción de las concentraciones de los indicadores a medida que aumentaba la distancia con el lugar del vertido del efluente, lo que permitió calcular las constantes de decaimiento (*k*) para cada indicador. Además, la *k* obtenida se mostró dependiente de las características intrínsecas del microorganismo indicador. Es decir, la *k* fue mayor para *E. coli* como indicador no conservativo, mientras que la *k* menor se obtuvo para SSRC como indicador no conservativo. Los indicadores víricos, así como HMBif se comportaron como parámetros semiconservativos, mostrando una *k* intermedia. Aunque los resultados no mostraron una estacionalidad en las concentraciones, sí que se observó una mayor *k* en la época seca, cuando el caudal fue menor y la temperatura mayor.

A partir de los datos observados se realizó la modelización de los microorganismos indicadores. El modelo estadístico confirmó el efecto de dos factores ambientales, el caudal y la temperatura como los parámetros más relevantes para explicar el comportamiento de los indicadores. La temperatura fue el factor que adquirió más importancia en la temporada húmeda, mientras en la temporada seca, a pesar de las elevadas temperaturas, el bajo caudal explicó mejor el decaimiento de los microorganismos indicadores. Con las series de datos registrados se modelizó en la cuenca de estudio para los años 2016 y 2017 la *k* y la distancia de autodepuración (SDD), es decir, la distancia necesaria para volver a las concentraciones previas al vertido de la EDAR. El modelo obtenido reflejó la estacionalidad en la *k* y la SDD de los diferentes indicadores, dependiendo de las características intrínsecas del microorganismo indicador, siendo más acusada esta estacionalidad en los indicadores no conservativos y los semiconservativos. De esta manera y teniendo en cuenta todos los indicadores analizados, la SDD modelizada, tuvo su máximo en la temporada húmeda con 15 km, determinada por

HMBif, mientras que en la temporada seca fue tan sólo 3 km y vino determinada por SSRC y HMBif.

De este estudio se pueden extraer las siguientes conclusiones:

- La EDAR supone una fuente de contaminación fecal que incrementa de forma significativa las concentraciones de los microorganismos de contaminación fecal aguas abajo, las cuales se van reduciendo a medida que se alejan de la EDAR. Esta reducción depende de las características de cada indicador y de los factores ambientales.
- Según su inactivación, las SSRC presentan un comportamiento conservativo, mientras que SOMCPH, GA17PH y HMBif son indicadores semiconservativos y *E. coli* se muestra como un parámetro no conservativo.
- La SDD, permite monitorizar la inactivación de los diferentes indicadores a través de la distancia al punto de vertido.
- La temperatura y el caudal son los factores ambientales que explican tanto la *k* como la SDD. En la temporada seca, con elevada temperatura y bajo caudal, el decaimiento de los indicadores es mayor, reduciendo las SDD y, por tanto, aumentando la capacidad de autodepuración del río.
- Se ha desarrollado un modelo que es capaz de reproducir la *k* y la SSD para los diferentes microrganismos indicadores a lo largo del período de estudio.
- El modelo permite proyectar las diferencias estacionales en la *k* y la SDD de los diferentes microorganismos indicadores siendo aplicable también a otros contaminantes, por lo que podría resultar una herramienta muy útil para la monitorización de la calidad del agua en función de parámetros fácilmente medibles como el caudal y la temperatura.

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Research article

Modelling the seasonal impacts of a wastewater treatment plant on water quality in a Mediterranean stream using microbial indicators

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ABSTRACT

Faecal pollution modelling is a valuable tool to evaluate and improve water management strategies, especially in a context of water scarcity. The reduction dynamics of five faecal indicator organisms (*E. coli*, spores of sulphite-reducing clostridia, somatic coliphages, GA17 bacteriophages and a human-specific *Bifidobacterium* molecular marker) were assessed in an intermittent Mediterranean stream affected by a wastewater treatment plant (WWTP). Using Bayesian inverse modelling, the decay rates of each indicator were correlated with two environmental drivers (temperature and streamflow downstream of the WWTP) and the generated model was used to evaluate the self-depuration distance (SDD) of the stream. A consistent increase of 1–2 $\log_{10}$ in the concentration of all indicators was detected after the discharge of the WWTP effluent. The decay rates showed seasonal variation, reaching a maximum in the dry season, when SDDs were also shorter and the stream had a higher capacity to self-depurate. High seasonality was observed for all faecal indicators except for the spores of sulphite-reducing clostridia. The maximum SDD ranged from 3 km for the spores of sulphite-reducing clostridia during the dry season and 15 km for the human-specific *Bifidobacterium* molecular marker during the wet season. The SDD provides a single standardized metric that integrates and compares different contamination indicators. It could be extended to other Mediterranean drainage basins and has the potential to integrate changes in land use and catchment water balance, a feature that will be especially useful in the transient climate conditions expected in the coming years.

1. Introduction

Water scarcity is currently threatening many areas of the planet, with severe implications both for ecosystems and the well-being of human societies. One such area is the Mediterranean basin, where already 80 million people are living below the water poverty threshold of 500 $m^3 \cdot person^{-1} \cdot year^{-1}$ (Milano et al., 2013), and ca. 60% of the renewable freshwater resources are currently being used by the population (Thivet and Blinda, 2011).

The Mediterranean climate is characterized by a strong seasonality in precipitation, with most of it concentrated in early spring and late autumn (Lionello et al., 2006). Recurrent summer drought stress drastically reduces the flow of Mediterranean rivers (Otero et al., 2011; Bonada and Resh, 2013). Moreover, climate projections for the 21st century predict a sharp increase in global temperature, a 10–15% decrease in precipitation in the Mediterranean by the year 2050, a concentration of precipitation in fewer but more intense events, and an increase in rain seasonality, thus reducing not only summer but also winter and spring precipitation (Lionello et al., 2014; Lionello and Scarascia, 2018). The higher temperatures will also reduce water availability due to higher levels of evapotranspiration (IPCC, 2013; Mariotti et al., 2015; Serrano-Notivoli et al., 2018). Under such circumstances, a reduction in streamflow during summer is expected, as well as longer zero-flow periods. In addition, anthropogenic pressure,

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understood as an increase in water demand and faecal pollution discharge into rivers, is predicted to increase in Mediterranean ecosystems in the next decades, thus amplifying the impacts of climate change (Bonada and Resh, 2013; IPCC, 2013; Stella et al., 2013).

Wastewater treatment plants (WWTP) are designed to reduce pollutant concentration and to avoid the direct discharge of wastewater into rivers. However, their effluents are still an important source of pollutants and faecal microorganisms, including pathogens. In the Mediterranean summer, reductions in water flow lead to higher concentrations of these pollutants (Merseburger et al., 2005; Mosley, 2015), and WWTP effluents may constitute most of the flow in intermittent streams (Muñoz et al., 2009). Extreme rainfall events are also associated with a higher concentration of waterborne pathogens, caused by the re-mobilization of river sediments to which they are attached (García-Aljaro et al., 2017; Jamieson et al., 2005; Martín-Díaz et al., 2017). Intense precipitation may also lead to an oversaturation and disruption of WWTP functionality (Curriero et al., 2001), as well as a reduction of the decay rates of faecal microorganisms due to decreased river bio-reactivity (Jonsson and Agerberg, 2015; Merseburger et al., 2005). Increases in pollutant concentrations may result in human health risks due to pathogen exposure (Auld et al., 2004; Curriero et al., 2001; Rose et al., 2010; Super et al., 1981), thereby compromising water usability (WHO, 2017).

Faecal microorganisms, including pathogens, are released by WWTP effluents into rivers and subjected to inactivation while being transported downstream (Agulló-Barceló et al., 2013; Jonsson and Agerberg, 2015). The assessment of the entire range of pathogen microorganisms would be difficult and expensive, so microbial indicators are frequently used in water quality management (García-Aljaro et al., 2018; Saxena et al., 2015; WHO, 2009, 2001). However, each microbial indicator may respond differently to exogenous factors such as water temperature, solar irradiance, dilution, predation, sedimentation or re-suspension (Auer and Niehaus, 1993; Ballesté et al., 2018; Martín-Díaz et al., 2017). Self-depuration distance (SDD) is proposed here as a standardized metric (in km) integrating all available indicator information to assess the distance needed to recover water quality downstream of the WWTP.

Five faecal indicator organisms (FIO) were selected and their in-stream decay rates were monitored downstream of the WWTP. The SDD, defined as the distance needed to return to the indicator concentrations upstream of the WWTP, was assessed as a measure to provide information about the spread of faecal pollution in water. Previous studies of FIOs in rivers have focused on inactivation time rather than distance (Dankovich et al., 2016; Fiorentino et al., 2018; Jonsson and Agerberg, 2015; Muirhead et al., 2004; Vinten et al., 2004). However, the pollutant travel distance per unit of time for a given river is dependent on river discharge (Runkel, 1998). The purpose of the SDD metric is to evaluate how far downstream the WWTP may negatively impact the water quality, taking into account the impact of seasonal variations on river discharge. This impact is implicit in the metric, rather than being added *a posteriori*, as it is based on inactivation distance rather than inactivation time.

The aims of this research were to: i) study and compare the seasonal dynamics of different faecal indicator organisms in a low-order Mediterranean stream affected by a WWTP effluent; ii) assess the SDD considering seasonal variations; iii) model in-stream pollutant SDD dynamics according to different environmental drivers, and iv) use the SDD metric to integrate and compare modelled pollutant dynamics.

The initial hypotheses of the study were: i) the inactivation of microbial indicators, and consequently the SDD, presents a seasonal behaviour, assuming that ii) the main factors explaining the SDD are streamflow and temperature (Ballesté and Blanch, 2010; Burkhardt et al., 2000; García-Aljaro et al., 2018), and iii) the decay rates of most conservative microbial indicators are less dependent on environmental conditions (Martín-Díaz et al., 2017).

2. Material and methods

2.1. Study site

The *Riera de Cànoves* is a third-order stream ca. 50 km north-east from Barcelona (NE Spain). Its source is located in the Natural Park and Biosphere Reserve of the Montseny mountain range and it has a catchment of 16.4 km^2 until the Cànoves-Samalús WWTP. The catchment is dominated by a siliceous substrate of granite and schist and it has smooth slopes (2%) (Catalan Cartographic Institute, 2018). Forest cover of the catchment is 77% and land uses include irrigated agriculture of cereals and legumes (15%) and a small cattle ranching industry (~0.1%). Although the urbanized fraction of the area is small (~5%), it is disseminated throughout the catchment in residential zones, thus implying concomitant basal human pollution. Climate characteristics correspond to sub-humid Mediterranean, with mild winters, wet springs, and dry summers. In the 1996–2017 period, the mean annual temperature averaged 12.0 °C (Catalan Meteorological Service) and the annual precipitation averaged 780.8 mm, with values ranging from 600 to 1000 mm $year^{-1}$. Located in the first 4 km of the stream, the Vallforners reservoir, with a 2.1 hm^3 maximum storage capacity and a consistent output flow of about 0.005 $m^3\ s^{-1}$ throughout the year, strongly regulates the streamflow dynamics downstream. As a result of water demand, evapotranspiration and lack of rainfall, waterflow between the reservoir and the WWTP is sometimes zero.

The WWTP of *Cànoves-Samalús* treats the water of 9200 inhabitant-equivalents. The plant consists of a pre-treatment and biological treatment system using activated sludge, with a complete mixture and two concentric reactor-decanter lines. Daily discharge of the WWTP ranges from 0.008 to 0.02 $m^3\ s^{-1}$, with slightly higher values in spring and winter than in summer and autumn (Fig. 1). The riverbed downstream of the WWTP is a mixture of rock and stones (5%), gravel (40%), sand (40%) and silt and clay (15%).

Twelve sampling campaigns were performed during 2016–2017. Water samples were collected at 9 different points of the *Riera de Cànoves*: i) a site located 150 m upstream of the WWTP, ii) the WWTP effluent, iii) a 450 m-long stretch downstream of the WWTP where 6 samples were collected every 75 m (75 m, 150 m, 225 m, 275 m, 350 m and 450 m downstream of the WWTP) and iv) a point located 1000 m downstream of the WWTP. Water samples were collected from the surface of the stream in sterile containers and transported to the laboratory at 4 °C. Analyses were performed within 8 h of collection.

2.2. Microbial detection and enumeration

Culturable *Escherichia coli* and spores of sulphite-reducing clostridia (SSRC) were selected as bacterial indicators, as they show different behaviour: *E. coli* is a non-conservative microbial indicator mostly used to detect faecal pollution, whereas the highly resistant SSRC is a conservative indicator that proxies the presence of protozoa oocysts and helminth ova (Agulló-Barceló et al., 2013).

Culturable *E. coli* were enumerated using a pour plate method in Chromocult® agar (Merck, Darmstadt, Germany). Dark blue and/or purple colonies were counted after an overnight incubation at 44 °C (Astals et al., 2012).

To enumerate SSRC, samples were subjected to a thermal shock at 80 °C for 10 min, anaerobically cultured by mass inoculation in *Clostridium perfringens* selective agar (Scharlab, Barcelona, Spain) and incubated overnight at 44 °C, as previously described (Ruiz-Hernando et al., 2014).

Two bacteriophages were used as viral indicators: somatic coliphages (SOMCPH), related to general faecal pollution, and bacteriophages infecting *Bacteroides thetaiotaomicron* strain GA17 (GA17PH), associated with human pollution and used as microbial source tracking (MST) markers to determine the origin of pollution in water (Jofre et al., 2014). SOMCPH and GA17PH were enumerated by the double agar layer technique as indicated in the ISO standards 10705–2 and 10705–4

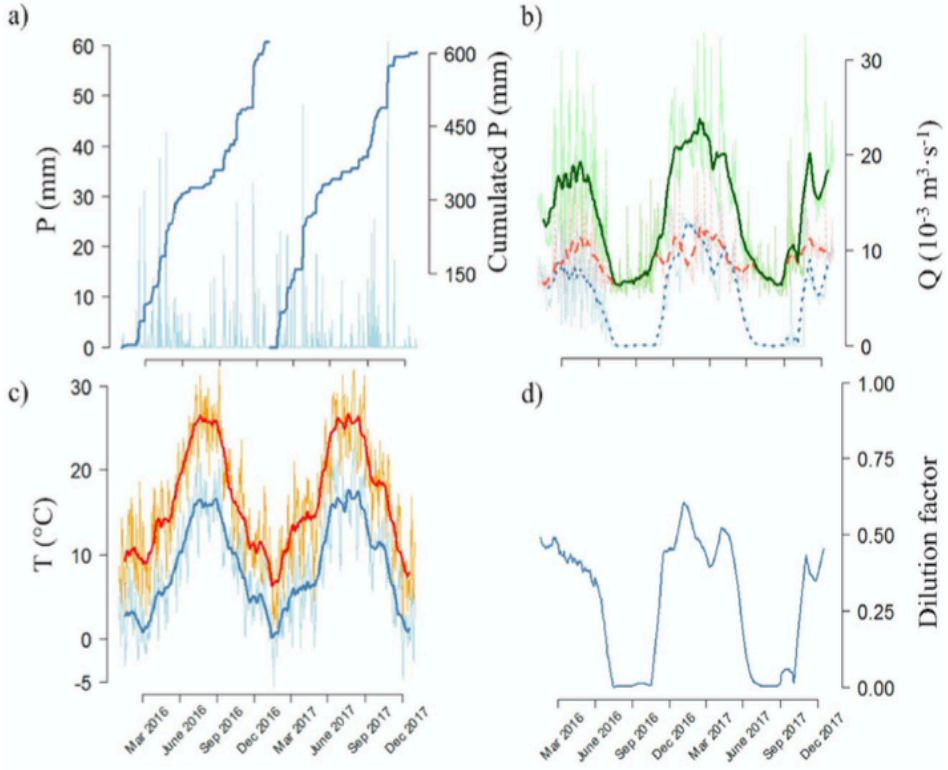

Fig. 1. Evolution of: a) precipitation (light blue) and cumulative precipitation (dark blue), in mm; b) Qstream (blue line), Qeffluent (red line) and Qdownstream (green line) in 10–3·m3·s-1; c) maximum (red line) and minimum temperature (blue line), in °C and d) contribution of the Qstream to the Qdownstream (dilution factor) during 2016–2017. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(ISO, 2001, 2000), respectively. In order to detect human-specific bacteriophages, the ISO standard 10705–4 was modified by using *Bacteroides thetaiotaomicron* strain GA17 (Muniesa et al., 2012).

A molecular marker targeting human-specific *Bifidobacterium* (HMBif) was also analysed by qPCR as in previous studies (Gómez-Doñate et al., 2012). For this, DNA was extracted from different sample volumes (from 0.2 to 0.5 l) according to the amount of suspended particles able to saturate the membranes. Samples were concentrated by filtration through a polycarbonate membrane with a pore size of 0.22 μm (SO-PAK, Millipore, Darmstadt, Germany). Membranes were then placed in 0.5 ml of GITC buffer (5 M guanidine thiocyanate, 100 mM EDTA [pH 8.0], 0.5% sarkosyl) and frozen at −20 °C in lysis buffer until DNA extraction. The DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) with some modifications (Gourmelon et al., 2007). Samples, negative controls, DNA extraction controls and five points on the standard curve were analysed for two replicates by qPCR, as previously described (Gómez-Doñate et al., 2012).

2.3. Streamflow calculation

In order to calculate the streamflow above the WWTP, twelve additions of NaCl, a conservative tracer, were performed (Gordon et al., 1992). Briefly, this method estimates the streamflow from a known concentration of a conservative tracer, whose signal records in-stream conductivity. In each addition, 1 l of solution of known conductivity was added to the stream, and the streamflow was estimated by the integration of the in-stream conductivity breakthrough curve corrected by basal conductivity. To obtain the conservative-tracer breakthrough curves, electrical conductivity (EC, $\mu S \cdot cm^{-1}$) was measured with a portable conductivity meter (WTW, Weilheim, Germany) at the bottom of the reach every 5 s during the solute injection.

Additionally, one piezometer was placed in the riverbank 150 m upstream of the WWTP at a depth of 50 cm. A pressure sensor (HOBO® U20-001-04 Water Level Logger) was placed inside the piezometer to record changes in pressure corresponding to changes in streamflow. In order to differentiate between pressure changes due to increases in water level and atmospheric pressure, another sensor was placed near the stream but outside the water. A continuous daily discharge time-series was obtained by non-linear regression between the daily averaged water level record against the twelve discrete streamflow measurements by conservative tracer addition. Gaps in the atmospheric pressure register were filled with observations from a nearby meteorological station.

Daily effluent discharge ($Q_{effluent}$) values were obtained from the WWTP register during the same period. During heavy rainfall or maintenance operations, the WWTP allowed a bypass of non-treated water, thus exponentially increasing its discharge into the stream. The $Q_{effluent}$ time-series has been corrected to avoid these anomalous flow peaks by assuming a $Q_{effluent}$ equal to the monthly median $Q_{effluent}$ when $Q_{effluent}$ was higher than 95% monthly values or lower than 5% monthly values. This correction was applied to 21 registers, which corresponded to less than 3% of the daily values. No seasonal trend was observed.

The streamflow was classified to study its seasonality. The dry season was defined as the period when the streamflow upstream of the WWTP was lower than 0.005 $m^3 s^{-1}$ and the dilution factor was lower than 0.1, which corresponded to summer. The wet season was the period when the streamflow was higher than 0.005 $m^3 s^{-1}$ and the dilution factor was higher than 0.1, which corresponded to the other seasons (Fig. 1d).

Meteorological data (i.e. daily mean air temperature and atmospheric pressure) was supplied by MeteoCat (Catalan Meteorological Service) from Tagamanent meteorological station, located ca. 9.5 km northwest of the WWTP.

2.4. Data analysis and modelling approach

2.4.1. Data analysis

A two-sample T-test was performed to analyse seasonal differences for each FIO concentration before and after the WWTP. Normality of log-transformed FIO concentrations was confirmed by a Shapiro-Wilk test.

2.4.2. Measured self-depuration distance

The concentration of each individual indicator was obtained after the WWTP effluent (I_0) for each sampling campaign [in log(cfu·l^{-1}), log (pfu·l^{-1}) or log(GC·l^{-1})].

$$I_0 = \frac{I_{stream} \cdot Q_{stream} + I_{effluent} \cdot Q_{effluent}}{Q_{stream} + Q_{effluent}} \quad \text{[Eq.1]}$$

where I_{stream} is the indicator concentration in the stream before the WWTP effluent [(log(cfu·l^{-1}), log(pfu·l^{-1}) or log(GC·l^{-1})], Q_{stream} is the flow upstream of the WWTP effluent (m^3·s^{-1}), $I_{effluent}$ is the indicator concentration in the WWTP effluent [(log(cfu·l^{-1}), log(pfu·l^{-1}) or log (GC·l^{-1})] and $Q_{effluent}$ is the discharge of the WWTP effluent (m^3·s^{-1}).

For each sampling campaign and studied indicator, the natural logarithm of the concentration obtained at sampling points downstream of the WWTP was related to the distance to the WWTP effluent by a linear least squares approach, the decay rate (k, in km^{-1}) thus being the negative slope of the linear relationship between the concentration of a given indicator and the distance.

Each indicator concentration at d distance after the WWTP effluent (I_d) was modelled by an exponential decay rate depending on an indicator-specific decay rate (k) and the distance to I_0, according to the logarithm form of Chick's equation (Chick, 1908)

$$I_d = I_0 e^{(-kd)} \quad \text{[Eq.2]}$$

where I_d is the indicator concentration [(log(cfu·l^{-1}), log(pfu·l^{-1}) or log (GC·l^{-1})] at a given distance (d) from the WWTP effluent (d, in km) and k the decay rate, which varies between each sampling campaign and indicator.

From "*in situ*" I_{stream}, I_0, and k measurements, and assuming no changes in streamflow downstream of the WWTP, equation (2) was rearranged in order to calculate the SDD (in km) for each microbial indicator.

$$SDD = \frac{\ln(I_{stream}) - \ln(I_0)}{k} \quad \text{[Eq.3]}$$

2.4.3. Modelling k from streamflow and temperature

In order to model how changes in temperature and streamflow affected the SDD, the relationship of the k coefficient with measured streamflow and air temperature was modelled for each sampling campaign according to

$$k_i = f(T_i) + f(D_i) + \varepsilon_i \quad \text{[Eq.4]}$$

where k_i is the decay rate (k) for a given FIO and campaign, T_i is the mean daily air temperature during the i campaign, D_i is the mean daily flow during the i campaign and ε_i is the error. Air temperature was used instead of water temperature due to the reliability of the meteorological data and the fact that air temperature and water temperature are highly correlated in low-discharge rivers on a daily basis (Morrill et al., 2005; Pilgrim et al., 1998). Thus, it was assumed that k responses to air temperature were reproducing k responses to water temperature.

Theoretically, it was expected that a higher temperature would accelerate the decay rate due to enhanced biological, physical and chemical processes, while an increasing streamflow would reduce it (Jonsson and Agerberg, 2015). Thus, equation (5) dependencies on temperature and streamflow may be expressed as:

$$f(T_i) = \frac{a}{b + \exp^{(-T_i * c)}} \quad \text{[Eq.5]}$$

$$f(Q_{downstream}) = d^* Q_{downstream}^{\ e} \quad \text{[Eq.6]}$$

where a, b, c, d and e are empirically determined unitless coefficients, T_i is the daily mean temperature in °C, and $Q_{downstream}$ is the mean daily streamflow after WWTP discharge (i.e. $Q_{downstream} = Q_{stream} + Q_{effluent}$).

A likelihood-based inverse Bayesian model calibration (Hartig et al., 2012) was used. This robust approach has proved to be a very useful tool when data is scarce, or when using models with a high number of parameters (Hartig et al., 2014; Lagarrigues et al., 2015; O'Hara et al., 2002; Purves et al., 2007). However, as complete Bayesian calibration may be computationally expensive, only the set of parameters providing the optimal fit of the model to observations was considered (i.e. a maximum "*a posteriori*" estimation approach). A double-exponential (Laplace) error function was selected, as it makes the likelihood function less sensitive to outliers compared to the Gaussian error distribution function (Augustynczik et al., 2017). Bayesian approaches were also needed for prior parameter distributions. A flat, wide, non-informative uniform prior distribution for all parameters was assumed with boundaries determined by expert judgment. After building the likelihood function and establishing the prior distribution, Bayesian optimizations were run using the "DEOptim" R package (Ardia et al., 2011; Mullen et al., 2011), which performs a Bayesian parameter optimization using a Differential-Evolution MCMC with a memory and snooker update sampler (Ter Braak and Vrugt, 2008).

2.4.4. Obtaining monthly k_i and SDD

Daily k_i values were calculated from equations (4)–(6) with the empirical coefficients obtained for each FIO and daily observed Q and T. Then, I_0 was calculated daily following equation (1), and according to daily measured Q_{stream} and $Q_{effluent}$. As no significant seasonal trend in I_{stream} and $I_{effluent}$ was observed throughout the experiment, the uncertainty of SDD related to unknown FIO concentrations was evaluated as follows: for each FIO and for I_{stream} and $I_{effluent}$, mean ± SD were obtained, as well as their 95% CI. Then, the sensitivity of model outputs to I_{stream} and $I_{effluent}$ was assessed by obtaining 1000 random samples of daily I_{stream} and $I_{effluent}$ for each FIO, according to a truncated normal N ($\bar{x}$ = mean, σ^2 = sd, min = 5%CI, max = 95%CI). No temporal autocorrelation was accounted for in daily random sample generation. For a given I_0, I_{stream} and k_i, the daily SDD was calculated for the 2016–2017 period according to equation (3). Finally, SDD values were integrated as median daily ±95% CI values from the 1000 random samples. Daily k_i and SDD values were reported as median monthly values (±95CI in the case of SDD to account for I_{stream} and $I_{effluent}$ uncertainty in model projections), to make the results more easily understandable.

3. Results and discussion

3.1. Observed flow data

Even considering the constant output from the Vallforners reservoir, the flow of the *Riera de Cànoves* was strongly seasonal above the WWTP due to fluctuating precipitation, evapotranspiration and water extraction for agricultural purposes. The flow data upstream of the WWTP obtained during 2016–2017 ranged from 0 m^3 s^{-1} to 0.015 m^3 s^{-1}, with dry season values of zero or close to zero. WWTP contributions to streamflow were also slightly seasonal, with values ranging from 0.015 m^3 s^{-1} during the wet season to 0.007 m^3 s^{-1} during the dry season. Downstream of the WWTP, streamflow ranged from about 0.007 m^3 s^{-1} during the summer to an observed peak of 0.03 m^3 s^{-1} in the spring of 2017 (Fig. 1). The dilution factor ranged from 0 in the dry season to 0.5 in the wet season, thus reflecting the high impact of WWTP water input on the *Riera de Cànoves*. Continuous Q records were used to calculate k

and SDD for the different FIOs as a model input.

3.2. Seasonal faecal indicator dynamics

A basal faecal pollution was consistently and repeatedly detected above the WWTP due to human-origin diffuse pollution from isolated houses with septic systems, and the presence of wildlife and farming activities in the surrounding area. The concentrations observed were in accordance with reports for similar streams (Ishii and Sadowsky, 2008; Nguyen et al., 2018) (Table 1). Nonetheless, a statistically significant increase of 1–2 $\log_{10}$ was observed in the concentration of each FIO downstream of the WWTP compared to the values obtained upstream ($p < 0.05$), indicating that the WWTP effluent constituted an input of faecal pollution into the stream. No statistically significant differences were found in seasonal FIO concentrations downstream of the WWTP, with within-season variability higher than between-season, which suggested that most of the FIOs belonged to the WWTP effluent (Table 1). Moreover, no differences in FIO concentrations were observed below the WWTP, even considering that during the wet season Q_{stream} provided ca. 45–50% of the downstream streamflow, while during the dry season $Q_{downstream}$ consisted almost entirely of $Q_{effluent}$, indicating that dilution was not a crucial factor.

A gradual reduction in concentration was observed for all the studied FIOs downstream of the WWTP, with indicator-specific decay rates (Table 2). Irrespective of season, the decay rates were higher for *E coli*, the non-conservative indicator, than for SSRC, the conservative indicator, whereas the viral and MST markers, as semi-conservative indicators, presented intermediate values. Additionally, decay rates for all FIOs presented seasonal differences, being higher in the dry season, though statistically significant differences were only observed for SOMCPH ($p < 0.05$).

Also showing seasonality, the measured SDDs for all target FIOs were higher in the wet than the dry season (Table 2). Nevertheless, those differences were only statistically significant for *E. coli* ($p < 0.01$), which presented an SDD of 0.6 km during the dry season and 3.1 km during the wet season. In this study, the dry season, when the flow upstream of the WWTP was nearly 0 $m^3 s^{-1}$, coincided with the highest temperatures. These results are in agreement with previous studies (Ballesté et al., 2018; Ballesté and Blanch, 2010; Bonjoch et al., 2009; Fauvel et al., 2017; Wu et al., 2016) where the seasonality of decay rates for different FIOs was strongly correlated to changes in temperature. Concurrently, SDDs were related with decay rates, indicating that in the wet season the stream capacity to self-depurate decreased, as longer transport distances were needed to return to the concentrations upstream of the WWTP. In contrast, in the dry season, increased evapotranspiration due to higher temperatures and lower precipitation reduced the streamflow, increasing the water residence time and therefore the decay rates. The higher decay rates may be due to enhanced in-stream biotic processes such as predation (Romo et al., 2013), but also to abiotic processes such as increased sedimentation (Yakirevich et al., 2013) or longer exposure to sunlight (Sinton et al., 2002).

Table 1
Mean concentrations and standard deviation of faecal indicator organisms (FIO) before and after the wastewater treatment plant (WWTP) for the wet (n = 7) and dry season (n = 3). Data is given in $\log_{10}$ CFU per 100 ml for *E. coli* and spores of sulphite-reducing clostridia (SSRC), in $\log_{10}$ PFU per 100 ml for SOMCPH and GA17PH and $\log_{10}$ GC per 100 ml for HMBif. Statistically significant differences after a *t*-test between Before (upstream of the WWTP) and After (75 m downstream of the WWTP) concentrations for each season (bold font) and between After concentrations during wet and dry seasons (font) are also noted ($p < 0.05$, n (Wet season) = 7, n (Dry season) = 3).

Indicator	Wet season concentration		Dry season concentration	
	Before WWTP	After WWTP	Before WWTP	After WWTP
E. coli	2.64 ± 0.5	4.25 ± 0.5	3.46 ± 0.0	4.21 ± 0.3
SSRC	2.23 ± 0.4	3.36 ± 0.4	2.22 ± 0.9	3.42 ± 0.2
SOMCPH	1.80 ± 0.7	3.93 ± 0.2	1.75 ± 0.4	4.21 ± 0.1
GA17PH	0.17 ± 0.3	1.29 ± 0.6	0.79 ± 1.1	1.83 ± 0.4
HMBif	3.60 ± 0.8	5.52 ± 0.6	3.67 ± 0.0	4.19 ± 1.4

E. coli; SSRC: spores of sulphite-reducing clostridia; SOMCPH: somatic coliphages; GA17PH: GA17 bacteriophages; HMBif: human-specific *Bifidobacterium* molecular marker.

Table 2
Mean decay rates (*k*, in km^{-1}) and self-depuration distances (SDD, in km) with the standard deviation for the five faecal indicator organisms (FIO). Decay rate (unit less) is given in $k \cdot 10^{-3}$, while SDD is given in km. Statistically significant differences after a *t*-test between Dry and Wet seasons are noted in bold font ($p < 0.05$, n (Wet) = 7, n (Dry) = 3).

k (km^{-1})			SDD (km)	
Indicator	Dry season	Wet season	Dry season	Wet season
E. coli	−3.6 ± 2.0	−1.2 ± 0.5	**0.6 ± 0.4**	**3.1 ± 0.7**
SSRC	−1.2 ± 0.0	−0.8 ± 0.4	2.1 ± 1.8	4.4 ± 3.8
SOMCPH	**−1.6 ± 0.5**	**−0.9 ± 0.4**	4.0 ± 0.5	5.5 ± 1.6
GA17PH	−2.5 ± 1.0	−0.7 ± 0.5	2.7 ± 1.9	4.3 ± 3.4
HMBif	−1.8 ± 0.0	−1.2 ± 0.8	2.0 ± 0.0	5.0 ± 3.5

E. coli; SSRC: spores of sulphite-reducing clostridia; SOMCPH: somatic coliphages; GA17PH: GA17 bacteriophages; HMBif: human-specific *Bifidobacterium* molecular marker.

3.3. Modelling environmental drivers for faecal indicator organisms

After calibration, the statistical model successfully captured the effect of environmental drivers [i.e. daily mean air temperature (T, in °C) and daily mean flow after the WWTP effluent ($Q_{downstream}$, in $m^3 \cdot s^{-1}$)] upon *k* coefficients (Fig. 2, Supplementary material 1). Regarding the FIOs, the R^2 between the observed and modelled *k* ranged from 0.6 for SSRC to 0.96 for GA17PH, with a root mean square error (RMSE) ranging from $7 \cdot 10^{-4}$ in *E. coli* to $1 \cdot 10^{-4}$ in SSRC. Among the bacterial indicators, *E. coli* presented the best model fit ($R^2 = 0.77$, RMSE = $7 \cdot 10^{-4}$) to measured *k*, whilst SSRC presented the worst ($R^2 = 0.6$, RMSE = $1 \cdot 10^{-4}$). On the other hand, the model reproduced well the observed *k* values for the viral and MST indicators, with R^2 scores of 0.85–0.96, and RMSE values of roughly $2 \cdot 10^{-4}$ for each of the three FIOs. The poorer predictive capacity for SSRC may be attributable to the low correlation between SSRC decay rates and environmental factors. Some authors have reported that SSRC decay rates are less related to climate than to other aspects not taken into account in the current study, such as predation, sedimentation and resuspension (Galfi et al., 2016; García-Aljaro et al., 2017). Moreover, previous studies have reported similar responses of non-conservative *E. coli* and semi-conservative viral and MST indicators to environmental factors (Ahmed et al., 2014; Bonjoch et al., 2009; Davies et al., 1995; Jonsson and Agerberg, 2015; Sinton et al., 2002), with temperature and solar irradiance being the most important parameters explaining their behaviour. Other environmental determinants (e.g. oxygen, redox potential and particle re-suspension) were indirectly taken into account in our study, due to their correlation with the seasonality of the streamflow (Capello et al., 2016). Solar radiation, which can play an important role in bacterial inactivation (Sinton et al., 2002), was implicitly included in air temperature changes, as it is difficult to discriminate between the effect of these two highly correlated parameters (Spearman's r = 0.75) (Hassan et al., 2016; Li et al., 2014; Prieto et al., 2009).

All FIOs responded similarly to the two environmental drivers considered, with maximum decay rates at higher temperatures and lower streamflow. Conversely, the decay rates were lower during conditions of low flow and lower temperatures (Fig. 3). All FIOs responded similarly to changes in flow, except for SSRC, which were practically unaffected by flow increases (Fig. 3a). This trend could be explained by the higher velocity of a stronger streamflow, which implies a shorter water residence time. No dilution effect on inactivation constants was observed, suggesting it is negligible compared to other factors, including

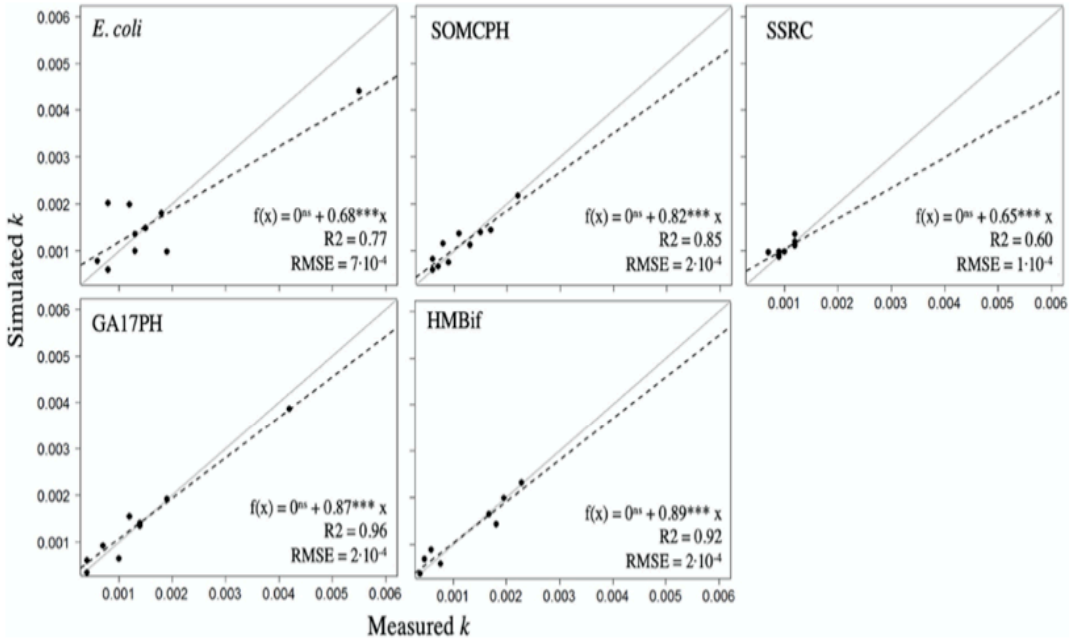

Fig. 2. Agreement between observed and modelled *k* values for all sampling campaigns and each faecal indicator organism (FIO). Regression (dashed line) compared to 1:1 (solid line), RMSE and R^2 are noted for each FIO.

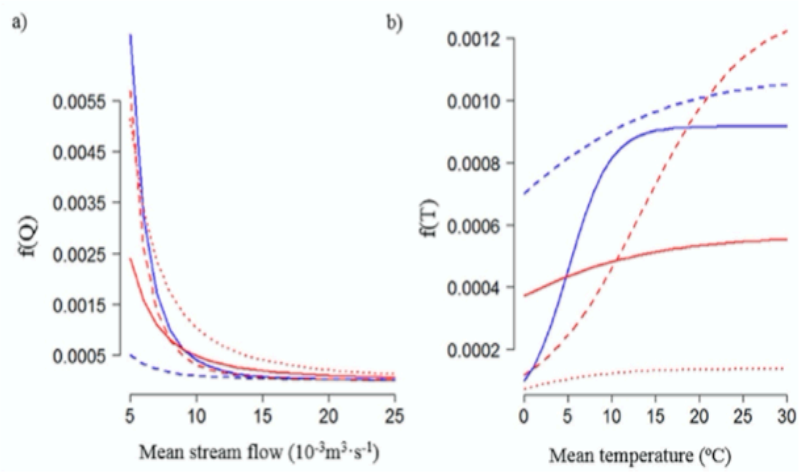

Fig. 3. Response of faecal indicator organisms (FIO) to streamflow and temperature according to equations (5) and (6). *E. coli* (solid blue line); SSRC: spores of sulphite-reducing clostridia (dashed blue line); SOMCPH: somatic coliphages (solid red line); GA17PH: GA17 bacteriophages (dashed red line); HMBif: human-specific *Bifidobacterium* marker (dotted red line). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

those accounted for in the model, i.e. total river discharge and temperature.

FIO decay rates differed in response to temperature increments (Fig. 3b); for instance, *E. coli* and GA17PH were more affected by environmental factors and temperature increases compared to SOMCPH and HMBif. These results confirm the non-conservative behaviour of *E. coli* (Bonjoch et al., 2009; Davies et al., 1995), and the semi-conservative behaviour of SOMCPH and HMBif (Ballesté et al., 2018; García-Aljaro et al., 2018; Sinton et al., 1999). Although SSRC are resistant and conservative indicators (Agulló-Barceló et al., 2013; Galfi et al., 2016; Pascual-Benito et al., 2015), they may have been affected by the stimulatory effect of high temperatures on biological processes such as predation (Beveridge et al., 2018). The low concentrations of the semi-conservative viral indicator GA17PH could also explain its strong response to temperature increases.

Finally, to shed light on the contribution of temperature and streamflow to the decay rates, the fraction of the total decay rate caused by temperature was calculated for the whole study period (Fig. 4). The contribution of temperature was strongly seasonal, increasing in autumn, peaking in winter and decreasing in spring to reach the lowest values in the summer months. This trend was repeated for each FIO, albeit with some differences. The SSRC decay rate could be explained by temperature throughout the period, the contribution ranging from 80% in summer to 100% in autumn. In contrast, the contribution of temperature to the HMBif decay rate ranged from 5% in summer to 40% in winter; and for *E. coli*, SOMCPH and GA17PH decay rates showed similar variations, the contribution ranging between 20% in summer and 90% in winter. Although the highest contributions of temperature to the total decay rates were expected in summer, the results showed otherwise. This may be explained by the very low summer streamflow, which increased water residence time and led to streamflow replacing temperature as the most important factor in the decay rate.

3.4. Modelling seasonal k and SDD for faecal indicator organisms

The decay rates of the studied FIOs were modelled for the 2016–2017

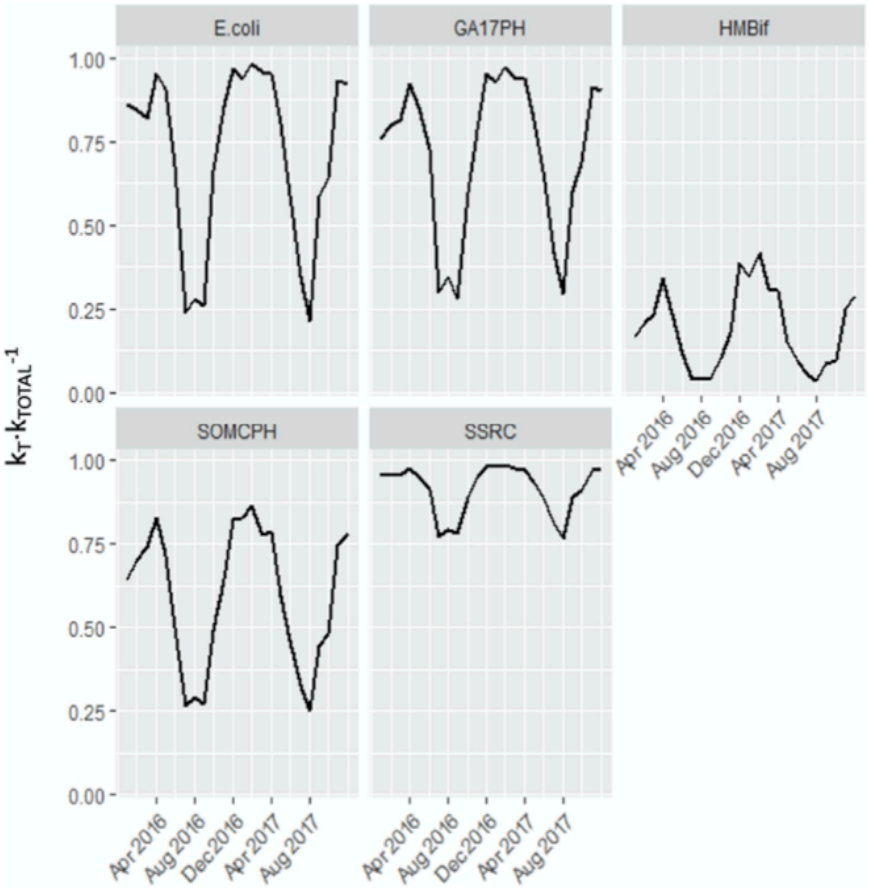

Fig. 4. Monthly median contribution of temperature (T) to the total decay rate (*k*). *E. coli*; SSRC: spores of sulphite-reducing clostridia; SOMCPH: somatic coliphages; GA17PH: GA17 bacteriophages; HMBif: human-specific *Bifidobacterium* molecular marker.

period with environmental data (i.e. T and Q) (Fig. 5). Seasonal variations in modelled *k* were observed for all FIOs and the general trend was an increase of *k* from May to September followed by a sharp decline in autumn and winter in both years. Those variations were more or less robust depending on the indicator behaviour. However, very low seasonal variations in SSRC *k* were observed, which indicates a high decoupling of the decay rates from environmental drivers. This is in accordance with what is expected from a resistant microbe and confirms its conservative indicator behaviour. SSRC are therefore of great value for assessing the impact of a WWTP in rivers using SDD measurements.

The modelled SDD also showed quite pronounced seasonal variations according to the studied FIO (Fig. 6). The highest SDD was found for HMBif in the winter of 2017, when ca. 15 km were required to decrease its concentration to the levels observed upstream of the WWTP. When all FIOs were considered together, the minimum modelled distance needed for the stream to self-depurate was just under 3 km; SSRC and HMBif had the most impact during the dry season, whereas the maximum SSD was found in winter, driven by HMBif. However, the strong seasonal changes may be attributable to the particularly high concentrations of HMBif upstream of the WWTP, and the fact that it is detected in both active and inactive forms. Regarding SSRC, constant SDD values reflect that these indicators were practically unaffected by environmental conditions. It should be noted that the minimum modelled SDDs were lower than 1 km for *E. coli* and GA17PH in August, when WWTP dilution was null, indicating the stream had a high capacity for self-depuration.

A combined maximum SDD could be particularly useful for water management practices, since it would allow a distance threshold to be established, below which river water would be considered unsuitable for human use due to the health risks associated with WWTP water pouring. During the wet season, the combined SDD was 5-fold higher than during the dry season. This extremely marked seasonal behaviour indicates the need for season-specific water management practices, especially in summer, when water availability in the Mediterranean area is expected to fall in the future (Cook et al., 2014; Orlowsky and Seneviratne, 2013).

Many models have been developed to describe the origin, transport, fate and processes related to faecal microbial pollution as well as to predict the faecal microbial load in catchments, using different tools and techniques such as Geographic Information Systems and simulations (Cho et al., 2016). Moreover, inactivation distances have been used previously by researchers to provide valuable information for water management (Fauvel et al., 2017; Jonsson and Agerberg, 2015). The model presented here, based on multiple FIOs and their environmental drivers, which are easy to measure in the field, constitutes a new tool to determine the spread of faecal pollution and predict the impact of a WWTP on water quality. Furthermore, the SDD provides a metric capable of integrating all types of water quality indicators when assessing WWTP impacts, not only FIOs but also ecological and chemical factors. Thus, the developed model could provide cross-cutting knowledge for water management that may be crucial in the coming years. Climate change, leading to higher temperatures and lower streamflow, is expected to reduce the SDD for all FIOs. However, land use changes together with growing human pressure may increase $Q_{effluent}$ and FIO load, thus increasing faecal microbial concentration downstream of the WWTP. Under such circumstances, non-linear responses of SDD should be expected, as SDD is dependent on *k*, but also sensitive to FIO concentration (Fig. 6). Likewise, the clear relationship found between the SDD for FIOs and easily measurable environmental drivers opens an interesting field of research focused on anticipating how global change

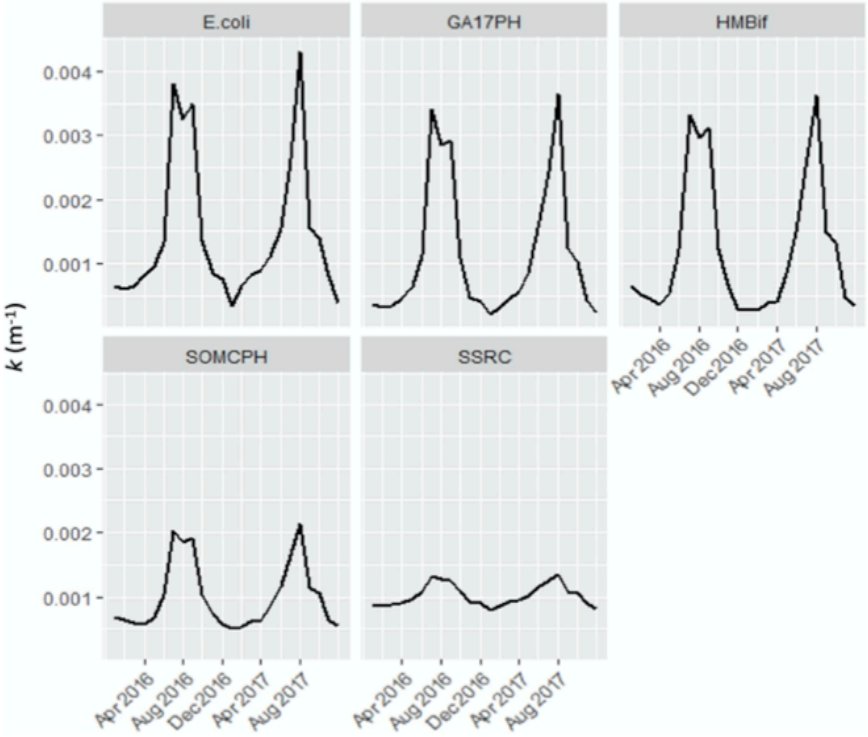

Fig. 5. Modelled decay rate (k, in m^{-1}) for the studied faecal indicator organisms. *E. coli*; SSRC: spores of sulphite-reducing clostridia; SOMCPH: somatic coliphages; GA17PH: GA17 bacteriophages; HMBif: human-specific *Bifidobacterium* molecular marker.

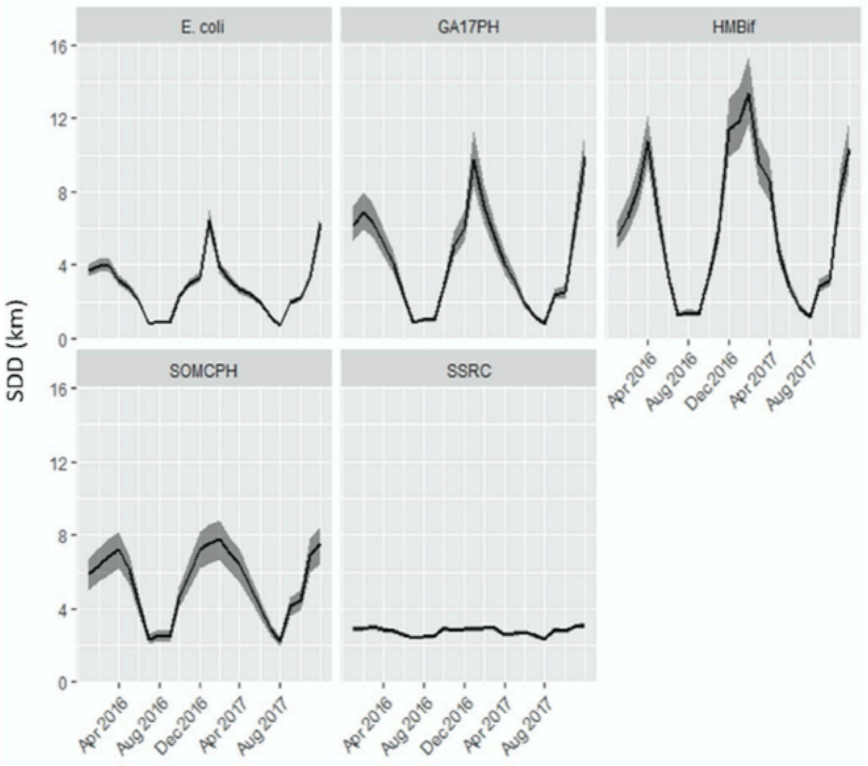

Fig. 6. Modelled self-depuration distance (SDD) for the studied faecal indicator organisms. *E. coli*; SSRC: spores of sulphite-reducing clostridia; SOMCPH: somatic coliphages; GA17PH: GA17 bacteriophages; HMBif: human-specific *Bifidobacterium* molecular marker.

will affect water quality in the near future, and how in-stream self-depuration processes will interact with ever-increasing human pressure.

Further research should be directed to obtaining a broader range of "*in-situ*" decay rate estimates in order to increase the predicative power of the model, as well as to include in the "k" coefficient other processes found to affect FIO inactivation rates, such as sedimentation, sediment resuspension or predation. Adding them to the model might help to differentiate their effect from that of temperature and streamflow, although the inclusion of highly correlated covariates has been observed to hinder model performance (Andrade et al., 1999; Zhao and Yu, 2006). Finally, implementing the model in other contrasting catchments is essential to test its strengths. If the SDD metric demonstrates its robustness when applied to other study cases under different climate conditions, it might become a crucial tool for assessing WWTP impacts on water quality in future climate conditions, and therefore for evaluating the optimum water management practices in a drier and warmer Mediterranean region.

4. Conclusions

- The WWTP effluent significantly increased the concentration of all faecal microbial indicators downstream of the WWTP. While being transported downstream, the FIOs were reduced to a greater or lesser degree according to their inherent characteristics and the environmental drivers, although no seasonality was observed in their concentrations.
- The lowest SDDs were observed during the dry season, indicating this is when the capacity of the stream to recover from the WWTP impact is highest.
- Temperature and streamflow successfully explained decay rates and SDDs. Temperature contribution was minimal in summer, when the contribution of a low flow was more relevant.
- Seasonal differences in the SDD of a range of FIOs were captured by the developed SDD metric. This approach allows different faecal pollutants to be integrated in a single standardized metric.
- If validated in other Mediterranean water courses, the SSD metric has the potential to help water managers to anticipate the effects of climate change on water quality depending on a few environmental drivers, thus improving their ability to adapt to future climate conditions.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2020.110220.

The intercept was always not statistically different from zero after a Student t-test. RMSE measures the error for each individual k estimate. R^2 is the variability within the data explained by the model. All modes were visually checked for homoskedasticity and normality of their residuals. *E. coli*; SSRC: spores of sulphite-reducing clostridia; SOMCPH: somatic coliphages; GA17PH: GA17 bacteriophages; HMBif: human-specific *Bifidobacterium* molecular marker.

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Supplementary Material 1. Coefficients to calculate the dependences on streamflow (Q) and temperature (T) according to equations 5 and 6, and R^2 and RMSE between observed and simulated *k*.

E. coli; SSRC: spores of sulphite-reducing clostridia; SOMCPH: somatic coliphages; GA17PH: GA17 bacteriophages; HMBif: human-specific *Bifidobacterium* molecular marker.

	$f(Q_{downstream})$		$f(T_{air})$				
Indicator	*a*	*b*	*c*	*d*	*e*	R^2	RMSE
E. coli	4.91	-4.09	$1.1 \cdot 10^{-4}$	0.42	0.12	0.77	$7 \cdot 10^{-4}$
SOMCPH	0.097	-2.3	$1.1 \cdot 10^{-3}$	0.11	1.95	0.84	$2 \cdot 10^{-4}$
SSRC	0.024	-2.4	$2 \cdot 10^{-3}$	0.1	1.85	0.6	$1 \cdot 10^{-4}$
GA17PH	4.99	-4.21	$1.3 \cdot 10^{-4}$	0.17	0.1	0.96	$2 \cdot 10^{-4}$
HMBif	0.212	-2.31	$1.6 \cdot 10^{-4}$	0.2	1.16	0.92	$2 \cdot 10^{-4}$

4.3 ARTÍCULO 3

Impact of treated sewage effluent on the bacterial community composition in an intermittent Mediterranean stream

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Artículo sometido en Environmental Pollution

Desde el punto de vista de la salud pública, la gestión hídrica se ha realizado tradicionalmente mediante la monitorización de la calidad microbiológica del agua a través del análisis de indicadores y de patógenos. No obstante, la implantación de la Directiva Marco del Agua establece la gestión de los recursos hídricos no sólo para garantizar la salud humana sino que también se debe alcanzar la salud ambiental fomentando la autodepuración natural de los ríos, en la que las bacterias juegan un papel importante. Es importante conocer cómo el impacto antrópico afecta a la composición y diversidad de las comunidades bacterianas del agua y para ello las nuevas tecnologías de secuenciación masiva ofrecen gran cantidad de información llegando ser muy útiles para su estudio.

El objetivo de este estudio fue analizar el impacto del efluente de una EDAR en la estructura y las comunidades del río receptor. También se pretendió evaluar la estacionalidad en la diversidad y la estructura de las comunidades bacterianas y su resiliencia frente al impacto de la EDAR.

El tramo de estudio comprendió un punto previo al vertido del efluente secundario de la EDAR (Upper), el propio efluente secundario (EF), un punto 75 m aguas abajo de la descarga del efluente (P75) y finalmente un punto 1 km aguas abajo (P1000) de la EDAR. De cada muestra se analizaron cuatro microorganismos indicadores de contaminación fecal (*E. coli*, SSRC, SOMCPH, GA17PH) y un patógeno de referencia (*Salmonella* spp.). Por otro lado, para el análisis de las comunidades bacterianas se recuperaron los microorganismos con un tamaño comprendido entre 0,22 µm y 3,0 µm mediante la filtración de 1 l de muestra y se realizó la extracción de su ADN. Se secuenció la región hipervariable V4 del gen 16S del ARNr mediante la plataforma Illumina Miseq y las secuencias obtenidas fueron procesadas bioinformáticamente para su análisis.

En el Upper se observó la presencia de indicadores de contaminación fecal con una concentración basal incrementada significativamente en el P75 por el vertido del efluente. En cambio, en el P1000 no se observaron diferencias significativas con las concentraciones del Upper para la mayor parte de indicadores analizados, excepto para los bacteriófagos.

De la secuenciación masiva de las muestras se obtuvieron un total de 5.593.724 *reads* dando lugar a un total de 20.650 variantes de secuencias de amplicón (ASV), con una representatividad de la biodiversidad de entre el 92% y el 97,5%. En general, para todos los puntos de muestreo, los phyla más abundantes fueron Proteobacteria, Bacteroidetes, Patescibacteria y Actinobacteria. Mientras que a nivel de género *Rhodoferax*, género ampliamente distribuido en ríos a lo largo del planeta, fue el más abundante en las muestras de Upper y P1000. En cambio, los géneros más abundantes en el EF y el P75 han sido asociados con ambientes de elevada presencia de contaminación fecal. *Flavobacterium* fue el género más abundante en el EF y *Polynucleobacter* y *Arcobacter* fueron los más abundantes en el P75.

A pesar de que cada uno de los puntos de muestreo mostró una distribución taxonómica diferente, los resultados no mostraron diferencias significativas entre Upper y P1000, de la misma manera que tampoco se observaron entre EF y P75. Los resultados también muestran una contribución del efluente en las comunidades del río decreciente aguas abajo. Este hecho sugiere que las comunidades podrían verse recuperadas del impacto del efluente en 1 km. Esta tendencia también ser observó en los análisis de diversidad. El Upper mostró los mayores niveles de diversidad alfa para todos índices analizados, mientras que el EF mostró los menores niveles provocando la reducción de la diversidad alfa en el P75. No obstante, los valores de diversidad alfa se vieron incrementados de nuevo en el P1000, demostrando que el efluente secundario de la EDAR tiene un impacto en las comunidades bacterianas reduciendo su biodiversidad. Además, la diversidad alfa también mostró estacionalidad, corroborada a través de la correlación moderada pero estadísticamente significativa ($r=-0,68$, $P<0,01$) obtenida entre el índice de Shannon y la temperatura del agua, viéndose reducida la diversidad alfa cuanto mayor es la temperatura.

De este estudio se pueden extraer las siguientes conclusiones:

- Los géneros bacterianos detectados por métodos de cultivo (*Escherichia* y *Salmonella*) no son detectados en las muestras o lo son, pero en porcentajes muy bajos (inferiores al 0,01%). De la misma manera, *Bifidobacterium* y *Bacteroides*, los géneros

mayoritarios en muestras fecales son detectados también en porcentajes muy bajos (0,01%-2%).

- La estructura de las comunidades se ve alterada por la descarga del efluente de la EDAR.
- La estructura de las comunidades del P75 muestra una gran similitud con la del EF, mientras que la del Upper es muy similar a la del P1000. Se observan diferencias estacionales en lo que respecta a la estructura de las comunidades del río.
- La diferencia en las proporciones observada en los 20 ASV más abundantes es suficiente para clasificar mediante un análisis discriminante los puntos Upper y P1000 tanto en invierno como en verano, mientras que los puntos EF y P75 se confunden entre sí, especialmente en verano.
- La contribución del efluente a las comunidades bacterianas del río es mayor en verano que en invierno y disminuye aguas abajo de la EDAR.
- El efluente de la EDAR impacta reduciendo la diversidad alfa aguas abajo, aunque ésta se recupera en el P1000. Las comunidades bacterianas muestran una disminución de su riqueza de especies, con el aumento de temperatura, obteniendo los niveles de diversidad más bajos en verano.
- Las técnicas de secuenciación masiva generan información sobre el impacto antrópico en las comunidades bacterianas que puede ser muy útil para la gestión integral de los recursos hídricos.

Impact of treated sewage effluent on the bacterial community composition in an intermittent Mediterranean stream

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ABSTRACT

Water quality monitoring is essential to safeguard human and environmental health. The advent of next-generation sequencing techniques in the last years, which allow a more in-depth study of environmental microbial communities in the environment, could broaden the perspective of water quality monitoring to include impact of faecal pollution on ecosystem. In this study, 16S rRNA amplicon sequencing was used to evaluate the impact of wastewater treatment plant (WWTP) effluent on autochthonous microbial communities of a temporary Mediterranean stream characterized by high flow seasonality.

Seven sampling campaigns were performed under different temperatures and streamflow conditions (winter and summer). Water samples were collected upstream (Upper) of the WWTP, and 75 m (P75) and 1000 m (P1000) downstream of the WWTP. The secondary effluent (EF) of the WWTP was also analysed.

A total of 5,593,724 sequences were obtained, giving rise to 20,650 amplicon sequence variants (ASV), which were further analysed and classified into phylum, class, family and genus. Each sample presented different distribution and abundance of taxa. Although taxon distribution and abundance differed in each sample, the microbial community structure of P75 resembled that of EF samples, and Upper and P1000 samples mostly clustered together. Alpha diversity showed the highest values for Upper and P1000 samples and presented seasonal differences, being higher in winter conditions of high streamflow and low temperature.

Our results suggest that some of the autochthonous bacterial communities were able to recover from the impact of the WWTP effluent in 1 km. Alpha diversity results indicates a possible influence of environmental factors on the bacterial community structure.

This study shows the potential of next-generation sequencing techniques as useful tools in water quality monitoring and management.

Keywords: 16S rRNA sequencing, river, faecal pollution, biodiversity, Illumina

INTRODUCTION

Water quality monitoring is essential to ensure public health and protect the environment. Increasing anthropogenic pollution, which is related to the population growth or urban concentration in certain areas, implies a high pressure on water bodies. The development and construction of wastewater treatment plants (WWTPs) has greatly contributed to the improvement of water ecological status in Europe by reducing the concentration of contaminants reaching water bodies (Brion et al., 2015). However, WWTPs effluents still discharge organic matter, nutrients, pollutants and pathogens, which leads to oxygen deficiencies, eutrophication and disruption of biotic communities. This is of particular concern in areas with high population density and water stress, such as the Mediterranean region, which frequently suffers from water shortages. The Mediterranean climate is marked by irregular precipitation, concentrated in spring and autumn, and recurrent episodes of drought and extreme rainfall events (Bonada and Resh, 2013). As a result, strategies are being developed to improve water management, which will be crucial in the coming years, considering that the Mediterranean is one of the areas most vulnerable to climate change (IPCC, 2013).

From a public health point of view, water management has mainly focused on monitoring water quality through the analysis of faecal indicator organisms (FIO) and reference pathogens (WHO, 2001b). However, as this approach can only provide a snapshot of water quality at a given moment, modelling faecal pollution dynamics has attracted increasing interest, as it integrates information about the parameters and processes affecting faecal microorganism persistence in the environment.

Water management has also gained importance from an ecological perspective, with the implementation of the Water Framework Directive (EC, 2000) and the adoption of the One Health strategy, which advocates a holistic approach to tackling global health challenges. In this context, a “healthy” river ecosystem is able to restore water status after different impacts through its riparian zone, fauna, and microbiota (Grizzetti et al., 2017; Merlo et al., 2014). Prokaryotes in particular are key players in biogeochemical cycles and ecosystem processes crucial in river self-depuration. However, the resilience of the ecosystem depends on the type and pressure of a given impact, which may bring about significant changes in biodiversity and weaken the self-depuration capacity. Consequently, studying changes in biodiversity can

provide valuable insights into water quality deterioration and help develop suitable water management policies.

Next-generation sequencing (NGS) techniques are being increasingly used in water environmental microbiology and have been proposed as water quality monitoring tools (Chen et al., 2018; Savio et al., 2015; Staley et al., 2013). However, to date their application has been mainly to describe the biodiversity of water ecosystems, including seas, lakes (Llirós et al., 2014) and, less frequently, rivers (Read et al., 2015). The presence of microorganisms able to persist in a dormant state until conditions turn more favourable have been detected in these environments (Caporaso et al., 2011; Gibbons et al., 2013). Accordingly, predictable seasonal variation in bacterial biodiversity has been reported in rivers in polar regions (Crump et al., 2009). Additionally, biotic and abiotic factors such as temperature, radiation, stream flow, sedimentation and predation have been observed to alter the bacterial community structure (Zeglin, 2015). However, to the best of our knowledge, no studies have applied NGS techniques in Mediterranean rivers, which are characterised by continually changing factors.

In a previous work, the presence, dynamics and inactivation of different FIOs along a temporary Mediterranean stream (*Riera de Cànoves*) were assessed, giving rise to the development of a model to assess the microbial water quality along the stream (Ballesté et al., 2019; Pascual-Benito et al., 2020). The stream was affected by the secondary effluent of a WWTP that could constitute up to 100% of the streamflow during the summer period. Such streamflow variation is typical in the Mediterranean regions and, therefore, this catchment is ideal to study the effect of these disturbances on the microbial populations in relation not just from a health-related point of view but also from an ecological perspective.

The aims of this research were to: i) assess the seasonal differences in the bacterial diversity and community structure along the stream; ii) study the spatial impact of the effluent on the bacterial diversity and community structure downstream of the WWTP, and iii) evaluate the resilience of the autochthonous populations to overcome the impact of the WWTP effluent.

MATERIAL AND METHODS

Sampling site and sample collection

The study site was located in a 1 km transect along the *Riera de Cànoves*, a temporary Mediterranean water course in Catalonia (Figure 1). The 16.4 km^2 catchment area is mainly forest (77%) and agricultural land (15%).

The stream is characterized by extreme changes in flow between the seasons, ranging from 0.02 m^3/s in winter to 0.006 m^3/s in summer. The studied transect is affected by the effluent of a WWTP serving 9,200 population equivalents. The WWTP treats municipal wastewater, performing a biological treatment to remove nitrogen and phosphorous. Its secondary effluent discharges mainly organic matter and faecal pollutants into the stream (with DBO <25 mgO_2/l and suspended solids <35 mg/l). The contribution of the effluent to the total discharge ranged from 32% in winter to up to 100% in summer, when the stream was completely dry upstream of the WWTP. The streamflow and temperature at the different sampling campaigns are found in (Supplementary Table 1).

Samples were collected in 2016-2018. Seven sampling campaigns were performed in two different periods of the year: 3 in summer and 4 in winter (mean water temperatures of 19.6 °C and 10.1 °C, respectively) to account for differences in temperature and streamflow, which in a previous work were identified as the main environmental drivers for microbial faecal indicators dynamics in this site (Pascual-Benito et al., 2020).

Samples were collected from four different sites: i) 150 m upstream of the WWTP (Upper) (6 samples); ii) directly from the WWTP effluent (EF) (7 samples); iii) 75 m downstream of the WWTP (P75) (6 samples) and iv) 1 km downstream of the WWTP (P1000) (7 samples).

Samples were collected in 2 L sterile flasks and transported refrigerated to the laboratory where they were processed in the following 4 hours.

Enumeration of microbial indicators and *Salmonella* spp.

The enumeration of culturable *E. coli* was performed by the pour plate method in Chromocult® agar, as previously described (Astals et al., 2012). Plates were incubated overnight at 44°C and dark blue/purple colonies were counted.

Spores of sulphite reducing clostridia (SSRC) were analysed after submitting the samples to a thermal shock at 80°C for 10 minutes. The samples were then cultured by mass inoculation in *Clostridium perfringens* selective agar and incubated overnight at 44°C (Ruiz-Hernando et al., 2014a).

Somatic coliphages (SOMCPH) and bacteriophages of *Bacteroides thetaiotaomicron* GA17 (GA17PH) were enumerated by the double agar layer method according to ISO 10705-2 and ISO 10705-4, respectively (ISO, 2001, 2000a).

In order to enumerate *Salmonella* spp., we adapted the ISO protocol (ISO, 2017) to a most probable number method. Briefly, 500, 50 and 5 ml of each water sample were filtered through a 0.45 µm pore-size nitrocellulose membrane. Filters were placed in 10 ml of Buffered Peptone Water (BPW) pH 7 and incubated at 37°C for 24 h. A 0.1 ml volume of the pre-enriched BPW was inoculated in 10 ml of Rappaport-Vassiliadis *Salmonella* enrichment broth and incubated at 42°C for 24 h. Then, 0.01 ml was inoculated in SMS® agar in triplicate and incubated at 42°C for 24 h. The presence of *Salmonella* spp. was confirmed by seeding on Hektoen agar, incubating at 37°C for 24 h, followed by incubation in TSA at 37°C for 24 h. Finally, the presence of *Salmonella* spp. was confirmed using the oxidase and API-20E test kits.

DNA extraction

One litre of each sample was filtered by vacuum filtration through a 3 µm pore- size mixed ester cellulose membrane. The filtrate, which corresponded to microorganisms with a size less than 3 µm, was collected in a sterile glass bottle and subsequently filtered through a 0.22 µm pore-size polycarbonate membrane. Filters were then placed in a 2 ml screw vial containing glass beads and stored at -80 °C until DNA extraction, which was performed according to previously described methods (Sala-Comorera et al., 2019; Walters et al., 2014) with some modifications. Briefly, 400 µl of phenol, 400 µl of CTAB buffer and 400 µl of chloroform/isoamyl alcohol (24:1 v/v) were added to the screw vial containing the filter and glass beads, mixed by vortex for 15 min and chilled on ice for 1 min. Samples were centrifuged at 13000 x g for 5 min at room temperature (RT) and the supernatant was transferred to a vial containing 500 µl of chloroform/isoamyl alcohol (24:1 v/v), mixed by vortex followed by a centrifugation (13000 x g, 5 min, RT). The supernatant was transferred to a vial containing 270 µl of isopropanol and stored overnight at RT. Tubes were mixed by inversion and centrifuged (13000 x g, 15 min, RT). The supernatant was discarded by decanting and 1 ml of ethanol (70%, ice-cold) was added, followed by centrifugation (13800 x g, 5 min, 4°C). The supernatant was removed, and the pellet was dried at 60°C for 30 min. Finally, the pellet was recovered in 100 µl of Elution buffer (Invitrogen, USA). A negative control, which included the reagents used in the DNA extraction, was performed in parallel. Samples were quantified by Qubit (Invitrogen) in order to determine the concentration of DNA obtained from the extraction and stored at -80°C until analysis.

Illumina 16S rRNA amplicon sequencing

Before sequencing, the presence of bacterial DNA in the samples was confirmed by conventional PCR. The V4 hypervariable region from the 16S rRNA gene of the samples was amplified using the degenerated primers 515f (5'- GTGCCAGCMGCCGCGGTAA-3') and 806r (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011).

A negative control (blank reagents) as well as a positive control (commercial mock microbial community Zymmobiomics, Zymmo Research) was included for the 16S rRNA amplicon sequencing. Illumina sequencing of samples was performed in a single run using the Illumina MiSeq platform at the Research Technology Support Facility of Michigan State University (Michigan, USA). Amplicon libraries of the V4 hypervariable region of the 16S rRNA gene were prepared using the previously described primers 515f and 806r with corresponding adaptors, following a reported protocol (Kozich et al., 2013). Sequencing was performed in 2 x 250 bp paired end format using a MiSeq v2 reagent cartridge following the manufacturer's instructions (Illumina MiSeq, USA). Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.

16S rRNA gene amplicon data analysis

Sequences were processed to amplicon sequence variants (ASV) using the default parameters of the Dada2 workflow (Callahan et al., 2016). Reverse reads were trimmed to 160 bp as recommended to improve downstream processing of the reads (Callahan et al., 2016) and a maximum of 2 errors per read were allowed (maxEE=2). This parameter has been shown to be a better filter than simply averaging quality scores (Edgar and Flyvbjerg, 2015). Taxonomic classifications were assigned to the ASV using the reference SILVA database v132.

Biodiversity analysis

Alpha and beta diversity were analysed using the Phyloseq R package (McMurdie and Holmes, 2013). For alpha diversity analysis, the Chao, Shannon and Inverse Simpson indices were calculated after rarefying the ASV table. For beta diversity analysis, samples were previously transformed to relative proportions, the Bray Curtis index was calculated, and samples were clustered accordingly.

Differential abundance analysis of ASV proportions

A binomial test of proportions with adjustment of the p-value ("FDR" method) for multiple hypothesis testing was used to assess differential ASV proportions among the different groups.

Determination of the contribution of the secondary effluent to the bacterial community downstream

SourceTracker2 was used to determine the contribution of the secondary effluent to the communities downstream of the WWTP impact, as previously described (Knights et al., 2011).

FISH analysis

FISH analysis was performed to validate the Illumina results and to estimate the number of total bacteria in the samples. For this, formaldehyde was added to 100 ml of sample to a final concentration of 2-4% and it was fixed for 1 h at RT. Samples were then filtered through 0.22 µm pore-size polycarbonate membrane filters, which were washed with 20 ml of sterile water. Filters were stored at -20°C in a Petri dish until their analysis according to a described protocol (Pernthaler et al., 2001). Briefly, the filter was cut in sections, each section was placed on a 10 µl drop of probe solution on a microscope slide and covered with another 10 µl of probe solution. The slide was stored in a dark humid chamber, coated with the hybridization buffer and incubated at 46°C for 3 hours. The filter section was then washed with buffer for 10 min at 48°C and with distilled water for 2 min in the dark. The filter section was dried and mounted in Vectashield (Vector Laboratories). Cells were stained with 1.5 $\mu g \cdot ml^{-1}$ DAPI (Sigma, USA) for counting with a Leica TCS SP2 confocal microscope.

Different probes were used to quantify the different phyla and classes (Alm et al., 1996): i) Alphaproteobacteria (5'-GGTAAGGTTCTGCGCGTT-3'), ii) Betaproteobacteria (5'-GCCTCCCCACTTCGTTT-3'), iii) Actinobacteria (5'-TATAGTTACCACCGCCGT-3'), iv) Firmicutes (5'-CCGAAGATTCCCTACTGC-3'), v) Bacteroidetes (5'-GGACCCTTTAAACCCAAT-3'), and vi) Gammaproteobacteria (5'-GCCTCCCCACATCGTTT-3'). All probes were labelled with Cy5 fluorochrome.

Statistical analyses

Statistical analyses were performed using different R functions and libraries (R Core Team, 2016). The BDbiost3 library for R (Monleón-Getino et al., 2017), was used to assess the coverage of the sequenced reads, and for discriminant and exploratory data analysis.

The coverage of the sequenced reads was analysed to assess the representativeness of the obtained ASV, as previously described (Monleón-Getino et al., 2017). For this purpose, the PILI3() function of the BDbiost3 library function was used. PILI3() allowed the computation of the rarefaction curve between the number of reads and the amount of ASV obtained. This function was projected to an infinite rarefaction curve in order to verify its saturation or if it still had a margin to saturate. For exploratory analysis, contingency tables (ASV abundance tables) were obtained separately for the different studied sample groups. These data followed a multinomial distribution (Monleón-Getino and Frías-López, 2020) and allowed us to apply an exploratory dimension reduction technique using the non-metric multidimensional scaling (nMDS). Discriminant analysis was computed using the 20 most abundant ASV, and made possible by the function MDSdbhatta.PAM.Metagen1() of the BDbiost3 library, which allowed the evaluation of 5 different discriminant methods: linear discriminant analysis (LDA), support vector machine (SVM), xboosting (Xboost), kernel discrimination (kernel) and artificial neural nets (ANN). The results obtained also offered final classification accuracy and a confusion matrix as a result of the different discrimination methods performed.

Spearman's correlation coefficients were calculated to assess the significance of the biodiversity changes in relation to the different environmental variables.

RESULTS

Faecal indicator organisms and *Salmonella* spp.

The faecal pollution in the *Riera de Cànoves* was characterized through the analysis of faecal indicator organisms (FIO) and *Salmonella* spp. (Table 1). Upper samples revealed low concentrations of faecal indicators associated with diffuse human faecal pollution and no *Salmonella* spp. were detected. EF samples contained high FIO concentrations, constituting a source of human pollution downstream of the WTTP, and *Salmonella* spp. were detected in some samples but at low concentrations (0.3 $\log_{10}$ (MPN/100ml). The WWTP discharge significantly increased FIO and *Salmonella* spp. concentrations in P75 (P<0.05), where FIO concentrations were about 1-2 logarithms higher than in Upper samples and not significantly

different compared to the EF (*P*>0.05); *Salmonella* spp. were detected and quantified in low concentrations, as in EF samples. Compared to Upper samples, the concentrations of all faecal indicator bacteria in P1000 were not significantly different (P>0.05), whereas those of SOMCPH and GA17PH were significantly higher (*P*<0.05). *Salmonella* spp. were only detected in one P1000 sample.

General description of the sequencing results

A total of 5,593,724 reads were generated for a total of 26 samples, ranging between 140,897 and 311,146 reads per sample. A total of 4,639,854 reads were selected after quality processing and chimera removal. These reads yielded 23,372 ASV after DADA2 algorithm processing, 20,650 of which were affiliated to Bacteria and kept for further analysis. It should be noted the 2,460 ASV affiliated to Archaea were discarded, because the used primers were targeting the bacterial 16S rRNA gene, in accordance with the study aim. A total of 16,854 ASV were represented by more than 10 reads (>80% of the ASV). The representativeness of the reads with respect to ASV biodiversity was very high, ranging from 92% to 97.5% (Supplementary Fig. 1). The ASV coverage obtained for Upper and P1000 samples was close to 97.5%, indicating that the obtained reads reflected the expected diversity in these samples. In the case of EF and P75, a slightly lower value was obtained (around 92%), showing a lower ASV coverage in these samples compared to others, although the majority of ASV were still represented in the study.

Bacterial communities in the sampling sites

Bacterial communities in Upper

The bacterial communities of the Upper site were represented by 15,791 ASV. A minor number of ASV was shared by all the samples (144 ASV), whereas five times higher number of ASV (760 ASV) were detected in 5 out of 6 samples. At phylum level (Figure 2), 67% of the reads affiliated to Proteobacteria, 11% Bacteroidetes, 8% Epsilonbacteraeota, 7 % Patescibacteria, 1% Actinobacteria, 1% Firmicutes followed by phyla each one with abundance lower than 1% (Dependentiae, Chlamydiae, Fibrobacteres, Cyanobacteria, Verrucomicrobia, Fusobacteria, Synergistetes, Omnitrophicaeota, Elusimicrobia, Nitrospirae, Planctomycetes, Acidobacteria, Spirochaetes, Chloroflexi, Rokubacteria). One percent of the reads were

unclassified phyla. Class, order and family affiliation is shown in Supplementary material file (Supplementary Fig. 2 to Fig. 4)

Bacteroides and *Bifidobacterium*, two of the most abundant genera in human faeces, constituted less than 1% of the Upper genera, whereas pathogenic bacteria with faecal-oral transmission, such as *Campylobacter* or *Salmonella*, were not detected. *Rhodoferax* (16%) was the most abundant genus in both seasons (Figure 3). These results should be interpreted with caution, as each genome is known to carry different copies of the 16S rRNA gene, even in species of the same genus (Větrovský and Baldrian, 2013).

The quantification of the Upper samples by FISH analysis resulted in 7.8 $\log_{10}$ (cells/100 ml) and indicated that 89.3% of the bacteria belonged to 4 phyla: Proteobacteria (50.5%), Firmicutes (27.6%), Actinobacteria (8.3%) and Bacteroidetes (2.9%).

Bacterial communities in EF

The bacterial communities of the 7 EF samples were represented by 8,062 ASV, only 227 of which were present in all the samples, whereas 500 ASV were present in 6 out of 7 samples. At the phylum level (Figure 2), reads corresponded to Proteobacteria (35%), Patescibacteria (31%), Bacteroidetes (17%), Epsilonbacteraeota (6%), Actinobacteria (2%), Firmicutes and Dependentiae (1%), and other phyla with an abundance lower than 1% (Chlamydiae, Fibrobacteres, Cyanobacteria, Verrucomicrobia, Fusobacteria, Synergistetes, Tenericutes, Omnitrophicaeota, Elusimicrobia, Nitrospirae, Lentisphaerae , Planctomycetes). Two percent of the reads were unclassified.

Flavobacterium (11%) was the most abundant genus in EF samples in both seasons (Figure 3). Despite being the most abundant genera in faeces, *Bacteroides* and *Bifidobacterium* constituted only 2% and 0.01%, respectively, of the total ASV.

Bacterial communities in P75

In P75 (Figure 2), the reads indicated a predominant affiliation to the phylum Proteobacteria (42%), followed by Patescibacteria (26%), Bacteroidetes (16%), Epsilobacteraeota (6%), Firmicutes (2%), Actinobacteria (1%) and Fibrobacteres (1%), and other phyla with abundances lower than 1% (Chlamydiales, Cyanobacteria, Verrucomicrobiae, Fusobacteriales, Synergistaceae, Omnitrophicaeota, Elusimicrobia, Nitrospiraceae,

Lentisphaerae, Planctomycetes, Acidobacteria, Spirochaetes and Chloroflexi). Two percent of the reads were unclassified.

Polynucleobacter (6%) and *Arcobacter* (6%) were the most abundant genera in both seasons (Figure 3), while *Bacteroides* and *Bifidobacterium* represented 2% and 0.03% of the total ASV, respectively.

The quantification of the total bacteria by FISH showed a total of 8.4 $\log_{10}$ (cells/100 ml). The analysis performed in p75 samples showed that 4 phyla accounted for 86.7% of the bacteria: Proteobacteria (71.2%), Bacteroidetes (10.7%), Firmicutes (2.7%) and Actinobacteria (2.1%).

Bacterial communities in P1000

In P1000, the distribution of the reads at phylum level (Figure 2) was mainly in Proteobacteria (62%), followed by Patescibacteria (13%), Bacteroidetes (6%), Epsilobacteraeota (7%), Actinobacteria (3%) and other phyla with abundances lower than 1% (Firmicutes, Chlamydiales, Fibrobacteres, Cyanobacteria, Verrucomicrobiae, Fusobacteriales, Synergistaceae, Omnitrophicaeota, Elusimicrobia, Nitrospiraceae, Lentisphaerae, Planctomycetes, Acidobacteria, Spirochaetes, Gemmatimonadetes, Chloroflexi). Two percent of the reads were unclassified.

Rhodoferax (9%) was the most abundant genus followed by *Arcobacter* (7%) and *Polynucleobacter* (7%) (Figure 3), while *Bacteroides* and *Bifidobacterium* represented 0.4% and 0.0%, respectively.

The FISH analyses of P1000 samples showed that 95.7% of the bacteria belonged to the 4 analysed phyla: Proteobacteria (42.7%), Firmicutes (20.4%), Actinobacteria (18.3%) and Bacteroidetes (14.3%). The total amount of bacteria quantified in P1000 was 7.9 $\log_{10}$ (cells/100 ml).

Tracking the origin of the ASV after the anthropogenic impact

Using Kernel discriminant analysis (Supplementary Table 2 and Supplementary Fig. 5), it was possible to separate samples belonging to the different sampling points with an accuracy of 0.692 (CI95%: 0.4821, 0.8567) taking into consideration the 20 most abundant ASV. This result reflected that the discriminant analysis had a limited ability to separate P1000 from Upper samples and P75 from EF samples. However, the accuracy improved if the samples

were separated by season (accuracy 0.818 [CI95%:0.4822, 0.9772] and 0.8667 [CI95%:0.5954, 0.9834] in summer and winter, respectively), supporting a seasonal difference of the community structure.

Further analysis of the effect of the sewage effluent on the communities downstream of the WWTP (Supplementary Table 3) showed that the EF contribution to the communities in P75 was higher in samples taken in summer (mean contribution of 74.7%) than in winter (mean contribution of 56.8%). The EF had a lower contribution to the P1000 than the P75 communities, but it was also higher in summer (47.6%) than in winter (38.1%). In contrast, the Upper contribution to P75 communities was higher in winter (8.1%) than in summer (3.2%). Moreover, Upper had a greater impact on P1000 than P75, being higher in summer (20.5%) than in winter (16.5%). Certain percentages were of unknown origin (22.1-45.4%).

Impact of the sewage effluent on community structure

We analysed the differences in ASV abundances between the samples upstream and downstream of the WWTP according to the season (Supplementary Table 4). Comparing abundance in Upper with downstream (P75 and P1000) sampling points, significant differences were found in 25 ASV in winter and 32 in summer. Among these, 15 ASV (60%) of the winter samples and 11 ASV (34%) of the summer samples showed significant differences between Upper and P75, but not between Upper and P1000, suggesting a recovery of the bacteria from the WWTP impact.

The ASV with significant differences between Upper and P75 showed two types of behaviour, increasing or decreasing their proportion. Compared to the Upper site, the proportion of some ASV was significantly higher in P75: 22 of the 25 ASV (88%) in winter and 20 of the 32 (62.5%) ASV in summer. Significant differences in proportion were also observed between Upper and P1000 sites for 5 ASV in winter and 4 ASV in summer. Only 3 ASV (ASV1 ASV6 and ASV22) were significantly lower in P75 compared to Upper samples and showed a recovery in P1000 in both seasons; they corresponded to *Flavobacterium*, an unclassified genus of the order_Absconditabacteriales, and *Rhodoferax*. In contrast, the differences between Upper and P75 samples for ASV10 and ASV14 were maintained in P1000 in both seasons; they belonged to C39 (*Rhodocyclaceae*) and *Flavobacterium*, respectively.

Impact of the sewage effluent on the bacterioplankton diversity

In order to assess the impact of the sewage effluent on the bacterioplankton diversity, alpha and beta diversity indices, which are indicative of species richness and overall bacterial community structure, were calculated. Three different indices of alpha diversity were analysed: Chao 1, Shannon and Inverse Simpson (Figure 4). Bacterioplankton alpha diversity was highest in Upper and P1000 samples and lowest in the sewage effluent, indicating that the effluent discharge reduced the alpha diversity in P75. Similar results were observed for the three analysed indices. Additionally, the alpha diversity values were higher in winter than in summer, suggesting an association between diversity and abiotic factors. To shed further light on this relationship, the possible correlation between the alpha diversity measured by the Shannon index and water temperature, one of the main environmental factors (Supplementary Fig. 6), was studied. Results showed a negative statistically significant correlation ($r=-0.68$, $P<0.01$) between water temperature and alpha diversity, i.e., the lowest alpha diversity values corresponded to the highest temperatures.

Beta diversity, which quantifies the bacterial communities considering the river space, was also analysed through Bray-Curtis dissimilarity (Figure 5). This measure divided the samples in two main clusters: one was constituted mainly by Upper and P1000 samples and the other mainly by EF and P75 samples, which were subclustered according to the season. Clustering of the samples was also observed in the multidimensional scaling of the dissimilarity (Supplementary Fig. 7).

DISCUSSION

Access to high quality water, already a major global problem, is expected to worsen due to urban demographic growth and a concomitant increase in water demand. These pressures can also lead to the functional deterioration of water ecosystems, especially in areas highly vulnerable to the impact of climate change, such as the Mediterranean (IPCC, 2013). Focusing on pollution with a point source rather than diffuse origin, the aim of this study was to analyse the impact of the secondary effluent discharged from a WWTP in a Mediterranean stream with a low and intermittent flow regime.

Although faecal pollution was observed in the *Riera de Cànoves* upstream of the WWTP, the sewage effluent significantly increased the downstream concentration of faecal indicators and pathogens such as *Salmonella* spp. Faecal pollution levels subsequently returned to those

of Upper samples (after a distance of 3-15 km) due to the *Riera de Cànoves* self-depuration capacity, previously described and modelled (Ballesté et al., 2019; Pascual-Benito et al., 2020). Similar behaviour has been reported in other streams (Price et al., 2018).

In the *Riera de Cànoves*, 84% of the bacterioplankton community structure consisted of four phyla (Proteobacteria, Bacteroidetes, Patescibacteria and Actinobacteria), with a variable distribution according to the sampling point. Proteobacteria was predominant throughout the studied river transect, whereas Bacteroidetes and Patescibacteria increased after the WWTP effluent discharge, subsequently decreasing in the sampling points downstream. Although with different percentages, the trends for Proteobacteria and Bacteroidetes were confirmed by FISH analysis. NGS techniques have been used to describe bacterioplankton community structure in rivers of different characteristics and geographical areas. Studies on the Danube (Europe), Mississippi (USA), Tama (Japan) and Apies (South Africa) rivers also report Proteobacteria, Bacteroidetes and Actinobacteria as the most abundant phyla, although with varying proportions (Abia et al., 2018; Reza et al., 2018; Savio et al., 2015; Staley et al., 2013).

Proteobacteria, one of the most abundant phyla in water ecosystems worldwide (Newton et al., 2011), is divided into different classes such as Alphaproteobacteria and Gammaproteobacteria, whose diverse characteristics drive its ubiquity. Bacteroidetes, a phylum widely distributed in marine and freshwater ecosystems, is highly specialized in organic matter degradation (Traving et al., 2017), which explains its high percentages in the sewage effluent and samples downstream of the WWTP. The phylum Actinobacteria has been proposed as a water quality indicator due to its sensitivity to the conditions that cause cyanobacterial blooms (Ghai et al., 2014). Patescibacteria, which was observed in high percentages in the *Riera de Cànoves*, especially after the sewage effluent discharge, has been reported in other rivers but in lower abundances (Zemskaya et al., 2019). Given the anaerobic nature of Patescibacteria, a likely source is sewage water (Castelle et al., 2017), although another source could be the streambed, as the phylum is associated with mobilizable sediments (Herrmann et al., 2019).

Species sorting, a concept that refers to community selection by environmental factors (Logue and Lindström, 2010), could explain the decrease of phyla such as Bacteroidetes and Patescibacteria from P75 to P1000 samples. Most of these bacteria are commensal or related with faecal pollution, and in a freshwater ecosystem selective pressure favours the recovery of autochthonous river communities as they are transported downstream. Although

Bacteroides and *Bifidobacterium* are described as the most abundant genera in human faeces, they were found in low frequencies in EF samples and decreased from P75 to P1000 samples. Another factor in the reduction of these anaerobic genera is the aerobic conditions found in both the WWTP and the stream.

The freshwater *Rhodoferax*, reported in rivers worldwide (Cottrell et al., 2005; Galand et al., 2008), was the most abundant genus found in Upper and P1000 samples. In P75 the most abundant genera were *Polynucleobacter* and *Arcobacter*, also freshwater bacteria. However, *Arcobacter* has been associated with human faeces and sewage water (Lerner et al., 1994), with high abundances reported in WWTP influents, due to a capacity to proliferate in infrastructures such as sewage pipes (Assanta et al., 2002). This behaviour suggests the WWTP effluent is the most plausible source of this genus. *Arcobacter* is important in water quality monitoring, as some species are human pathogens and have been reported to cause waterborne outbreaks (Collado et al., 2010; Prouzet-Mauléon et al., 2006). The most predominant genus in EF samples was *Flavobacterium*, in agreement with previous studies that detected high abundances in eutrophic water and faecally polluted urban streams (Eiler and Bertilsson, 2007). Overall, the abundance of genera with pathogenic species was higher in EF, P75 and P1000 than in Upper samples. For instance, higher abundances of *Escherichia*, *Shigella*, *Klebsiella* and *Enterobacter* were found in P75 and P1000 compared to Upper samples. This result confirms the relevance of secondary effluents in the spread not only of faecal pollutants but also of human pathogens.

The impact of the WWTP effluent on the community structure along the stream transect was also observed in proportional changes of ASV in the different testing sites. In P75, most of the ASV whose proportion had increased compared to Upper samples belonged to sewage water-related genera, such as *Flavobacterium, Arcobacter* or *Polynucleobacter*, whereas some of the ASV whose proportions had declined belonged to freshwater genera, such as *Rhodoferax*, *Rhizobacter*, *Limnohabitans*, *Novosphingobium* and *Pseudarcicella*. Changes in ASV behaviour and relative abundance along the transect could help to determine the impact of the WWTP effluent and to identify potential water quality indicator genera.

ASV from the Upper reaches and sewage effluent were the source of 71.5% of P75 ASV and 60.4% of P1000 ASV, indicating that the bacterial community structure and distribution downstream of the WWTP were mainly determined by the bacterial composition of both Upper and EF. This prediction is lower than the 95% of Mansfeldt and co-workers (Mansfeldt

et al., 2019), who also described that the contributions of the WWTP effluents were higher than 50% downstream of the discharge point, similar to the EF contribution obtained here. The EF contributed more to P75 than to P1000, suggesting the sewage effluent lost influence on the community composition with distance. A percentage of sequences of unknown origin (ranging from 16% to 39% in P75 and 19% to 62% in P1000) was determined, possibly related to communities in the streambed sediments, which could constitute a reservoir of mobilizable bacteria. This phenomenon has been widely explored in studies of faecal indicators and pathogens (García-Aljaro et al., 2017; Jamieson et al., 2005) and could play an important role in the diversity of the water column.

The high contribution of ASV from EF to P75 reflects the degree of impact of the sewage effluent on P75. The impact was also reflected by the decrease in alpha diversity. The sewage effluent had the lowest alpha diversity values of the study and decreased the biodiversity of the stream immediately after the effluent impact. These results are in agreement with previous studies (Drury et al., 2013; Mansfeldt et al., 2019), where wastewater reduced the alpha diversity of the receiving water body, although an increase in diversity downstream of the WWTP has also been reported by other authors (Marti and Balcázar, 2014; Wakelin et al., 2008). Such contradictory results could be due to differences in the environmental context and the techniques used, including improved next-generation sequencing. The increase in alpha diversity in P1000 suggests a partial recovery from the effluent impact.

In addition, a significant negative correlation was found between temperature and alpha diversity, the lowest temperature being related with the highest alpha diversity and vice versa. This trend has been reported previously (Kent et al., 2004; Rubin and Leff, 2007), indicating that environmental factors play an important role in the bacterial community structure and diversity in water ecosystems. In a previous study on faecal pollution in the *Riera de Cànoves*, temperature and streamflow were found to be crucial in the self-depuration capacity downstream of the WWTP, which increased at high temperatures and low streamflow (Pascual-Benito et al., 2020). The results obtained here support the important role of the streamflow in the community structure downstream of the WWTP. During summer, when the streamflow is low and the sewage effluent barely diluted, the contribution of EF bacterial sequences to P75 and P1000 was highest. However, although the temperature reduced the self-depuration distances, it also reduced the alpha diversity. Therefore, from an ecological

point of view, the increase in temperature in a climate change scenario would be detrimental for the *Riera de Cànoves* biodiversity.

García-Armisen and collaborators have described the seasonal resilience of bacterial communities, reporting their recovery from the WWTP impact in the driest season (García-Armisen et al., 2014), although the river they studied is not comparable with the *Riera de Cànoves*, in terms of dimensions, WWTPs and climate. Overall, the bacterial communities from the *Riera de Cànoves* showed a high resilience to the impact of the sewage effluent, which consisted mainly of organic matter. This was demonstrated by analysing the FIO and pathogen concentrations and the bacterial communities and diversity, all of which showed recovery in only 1 km.

CONCLUSIONS

The anthropogenic impact of the WWTP secondary effluent in the *Riera de Cànoves* caused an alteration of the community structure and reduced the alpha diversity at the P75 sampling point. However, the results suggest that the autochthonous communities of the *Riera de Cànoves* had partially recovered 1 km downstream of the WWTP effluent, showing a high resilience that was dependant on environmental factors such as temperature. Within the climate change scenario, which predicts an increase in temperature and decrease in streamflow, the resilience of the bacterial communities and diversity may be negatively affected. Modelling the stream resilience under different environmental conditions may therefore be crucial for water management and quality monitoring in the future. This study shows that next-generation sequencing techniques can be useful to monitor bacterial community dynamics and identify water samples with different levels of anthropogenic impact. They therefore have potential application as practical tools in water management to assess the ecological status of rivers.

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DATA AVAILABILITY

The research data are available at: https://data.mendeley.com/datasets/gvs398nfzb/draft?a=29e1bcab-8ae2-4824-8f88-5560e2a6b19a

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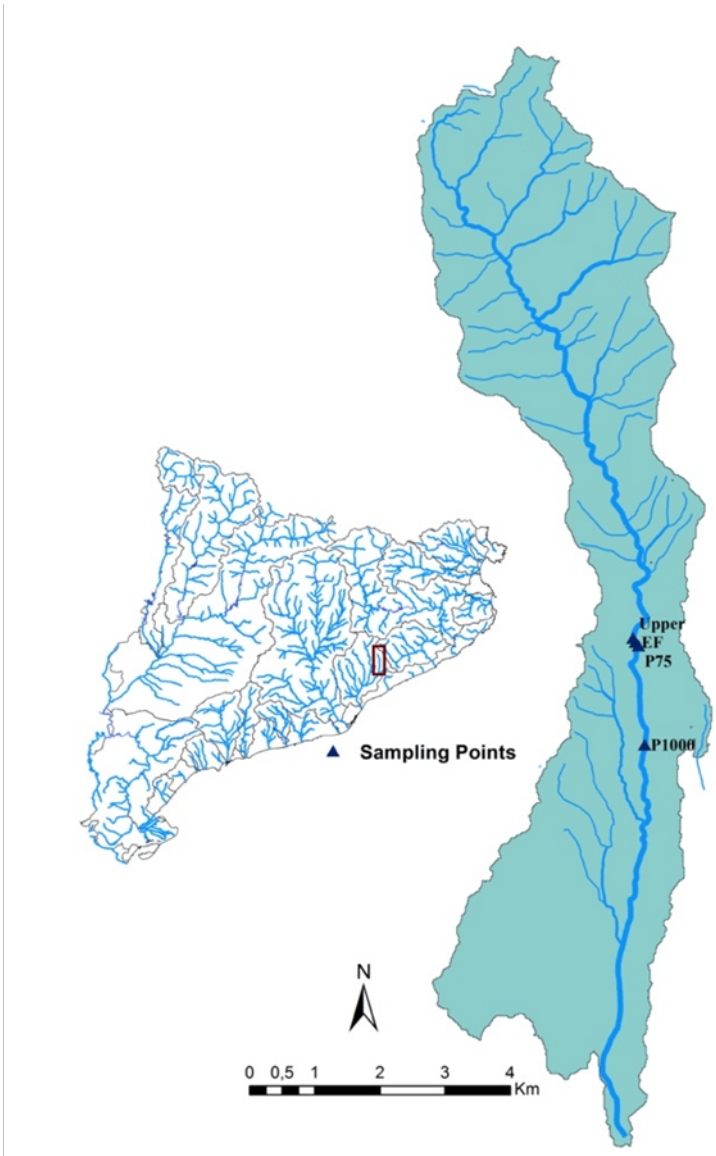
Tables

Table 1: Mean concentration and standard deviation of *E. coli*, spores of sulphite reducing clostridia (SSRC) (in log_{10} (cfu/100ml), somatic coliphages (SOMCPH), bacteriophages of *Bacteroides thetaiotaomicron* GA17 (GA17PH) (in log_{10} (pfu/100ml) and *Salmonella* spp. (log_{10} (MPN/100ml)).

	E. coli	SSRC	SOMCPH	GA17PH	*Salmonella* spp.
Upper	3.4 ± 0.8	2.3 ± 0.4	2.2 ± 0.4	0.3 ± 0.6	<-0.7
EF	4.2 ± 0.4	3.7 ± 0.2	4.3 ± 0.2	1.7 ± 0.6	0.3 ± 0.6
P75	4.1 ± 0.3	3.4 ± 0.2	4.1 ± 0.2	1.6 ± 0.7	0.2 ± 0.6
P1000	3.1 ± 0.4	2.6 ± 0.6	3.2 ± 0.7	0.9 ± 0.4	<-0.7

Figures

Figure 1: Study site.



Site	Point_X	Point_Y	Latitude	Longitude
UPPER	2.355749	41.682615	N41° 40' 57,414"	E2° 21' 20,696"
EF	2.35532	41.682778	N41° 40' 58,001"	E2° 21' 19,152"
P75	2.355918	41.682294	N41° 40' 56,435"	E2° 21' 21,316"
P1000	2.357199	41.66862	N41° 40' 7,032"	E2° 21' 25,916"

Figure 2: Distribution of phyla in each sampling point. Rare taxa grouped the phyla with proportions <1%.

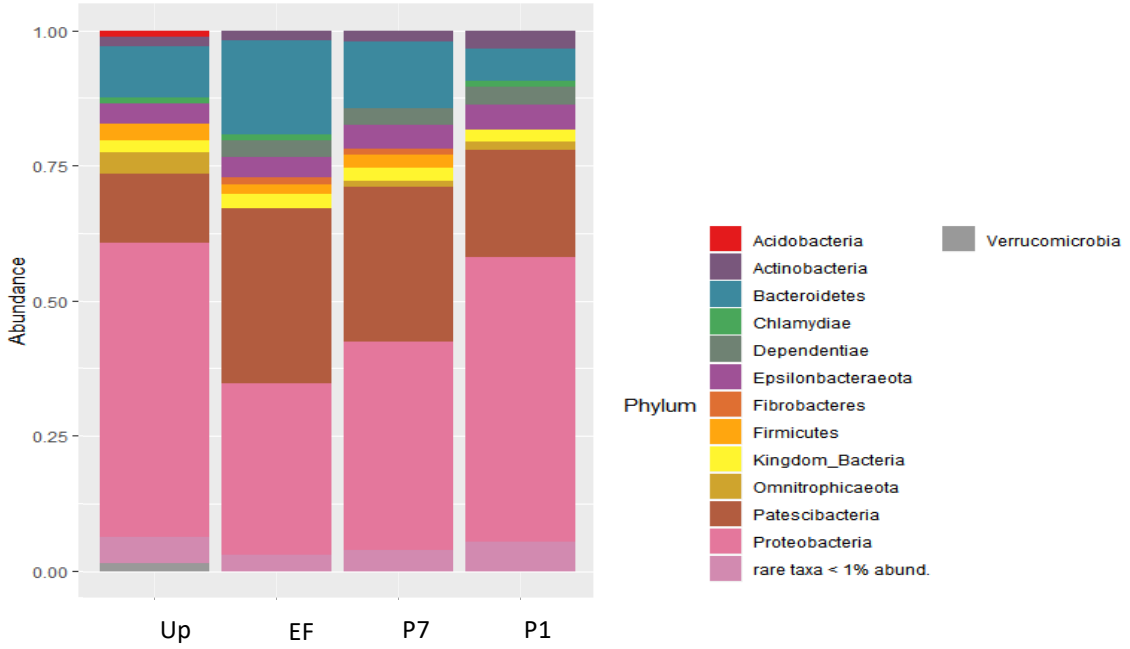


Figure 3: Distribution of genera in each sampling point and separated by season (summer and winter). Rare taxa grouped the genera with proportions <1%.

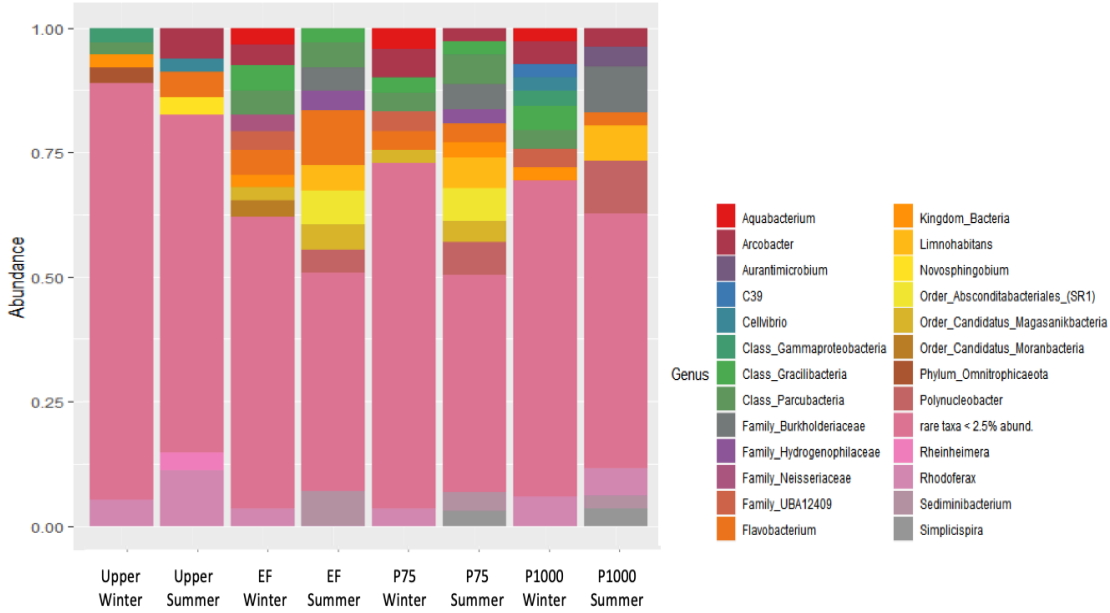


Figure 4: Alpha diversity in each sampling point expressed by Chao 1, Shannon and inverse Simpson indices.

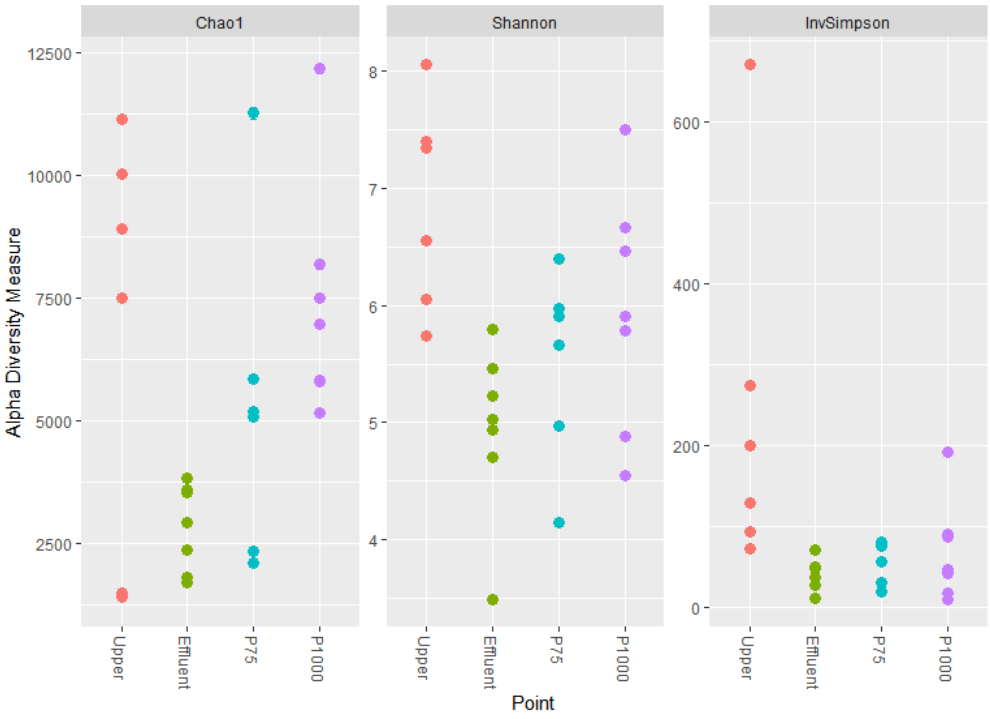
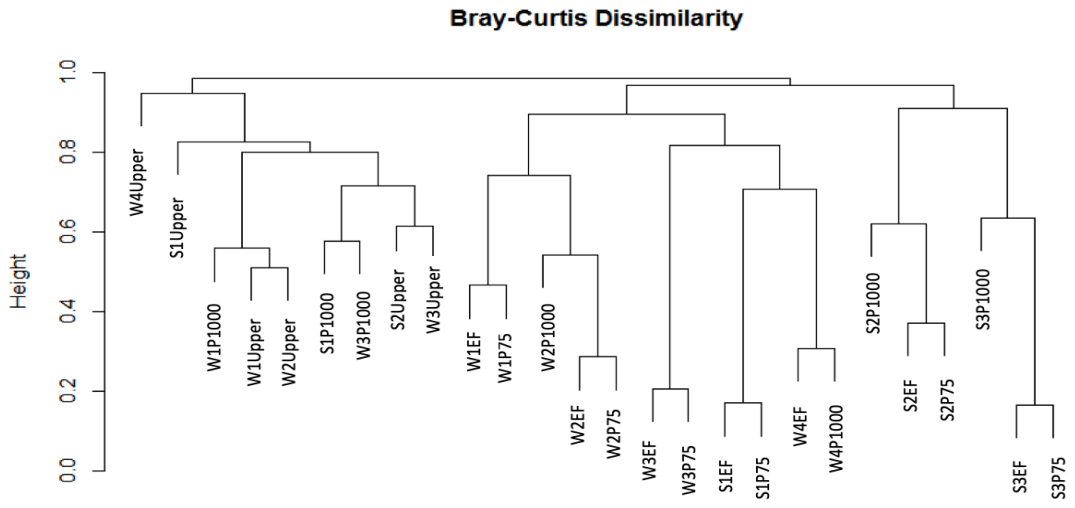


Figure 5: Clustering of samples according to Bray-Curtis Dissimilarity. Seasonality is indicated in each sample with W or S, corresponding to winter or summer, respectively. Sampling campaign is indicated with a number from 1 to 4 depending on the season, before the sampling site name.



Supplementary material

Supplementary Table 1: Streamflow (Q, in m^3/s) in Upper, EF and downstream of the WWTP (P75) and main water temperature (T, in ºC) in each sampling campaign. Seasonality is indicated in each sample with W or S, corresponding to winter or summer, respectively and sampling campaign is indicated with a number from 1 to 4 depending on the season.

Sampling campaign	Q_{Upper}	Q_{EF}	Q_{P75}	T
S1	0.003	0.006	0.009	17.1
S2	0.001	0.007	0.008	20.7
S3	0.000	0.006	0.006	21.1
W1	0.001	0.006	0.007	8.6
W2	0.003	0.006	0.009	9.7
W3	0.004	0.010	0.014	12.5
W4	0.008	0.001	0.018	9.5

Supplementary Table 2: Statistical descriptors (sensitivity analysis and confusion matrix) of Kernel discriminant analysis used to separate samples according to the distribution of the most abundant 20 ASVs and confusion matrix.

a) All samples together

	Upper	Effluent	P75	P1000
Sensitivity	1.0000	0.8571	0.5000	0.4286
Specificity	0.9000	0.7895	0.9000	1.0000
Positive Prediction Value	0.7500	0.6000	0.6000	1.0000
Negative Prediction Value	1.0000	0.9375	0.8571	0.8261

Prediction/Reference	Upper	Effluent	P75	P1000
Upper	6	0	0	2
Effluent		6	3	1
P75		1	3	1
P1000				3

b) Summer samples

	Upper	Effluent	P75	P1000
Sensitivity	1.0000	0.6667	0.6667	1.0000
Specificity	1.0000	0.8750	0.8750	1.0000
Positive Prediction Value	1.0000	0.6667	0.6667	1.0000
Negative Prediction Value	1.0000	0.8750	0.8750	1.0000

Prediction/Reference	Upper	Effluent	P75	P1000
Upper	2	0	0	0
Effluent		2	1	0
P75		1	2	0
P1000				3

c) Winter samples

	Upper	Effluent	P75	P1000
Sensitivity	1.0000	0.9091	0.6667	0.7500
Specificity	1.0000	0.8750	0.9167	1.0000
Positive Prediction Value	1.0000	0.8000	0.6667	1.0000
Negative Prediction Value	1.0000	1.0000	0.9167	0.9167

Prediction/Reference	Upper	Effluent	P75	P1000
Upper	4	0	0	0
Effluent		4	1	0
P75			2	1
P1000			0	3

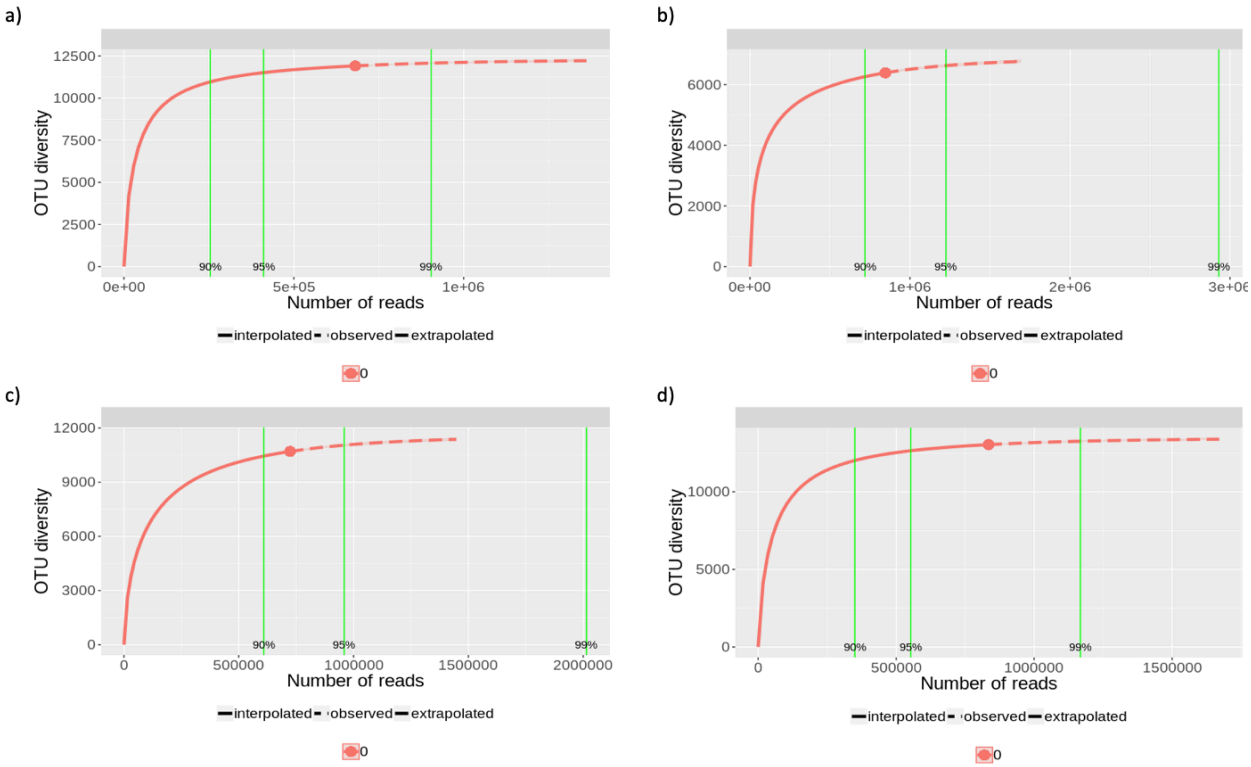
Supplementary Table 3: Contribution (%) of the river (Upper), sewage effluent (EF) and unknown origin to the reads in P75 and P1000 in winter (W) and summer (S).

	Upper	EF	Unknown
WP75	8.2	56.8	35.0
SP75	3.2	74.7	22.1
WP1000	16.5	38.1	45.4
SP1000	20.5	47.6	31.9

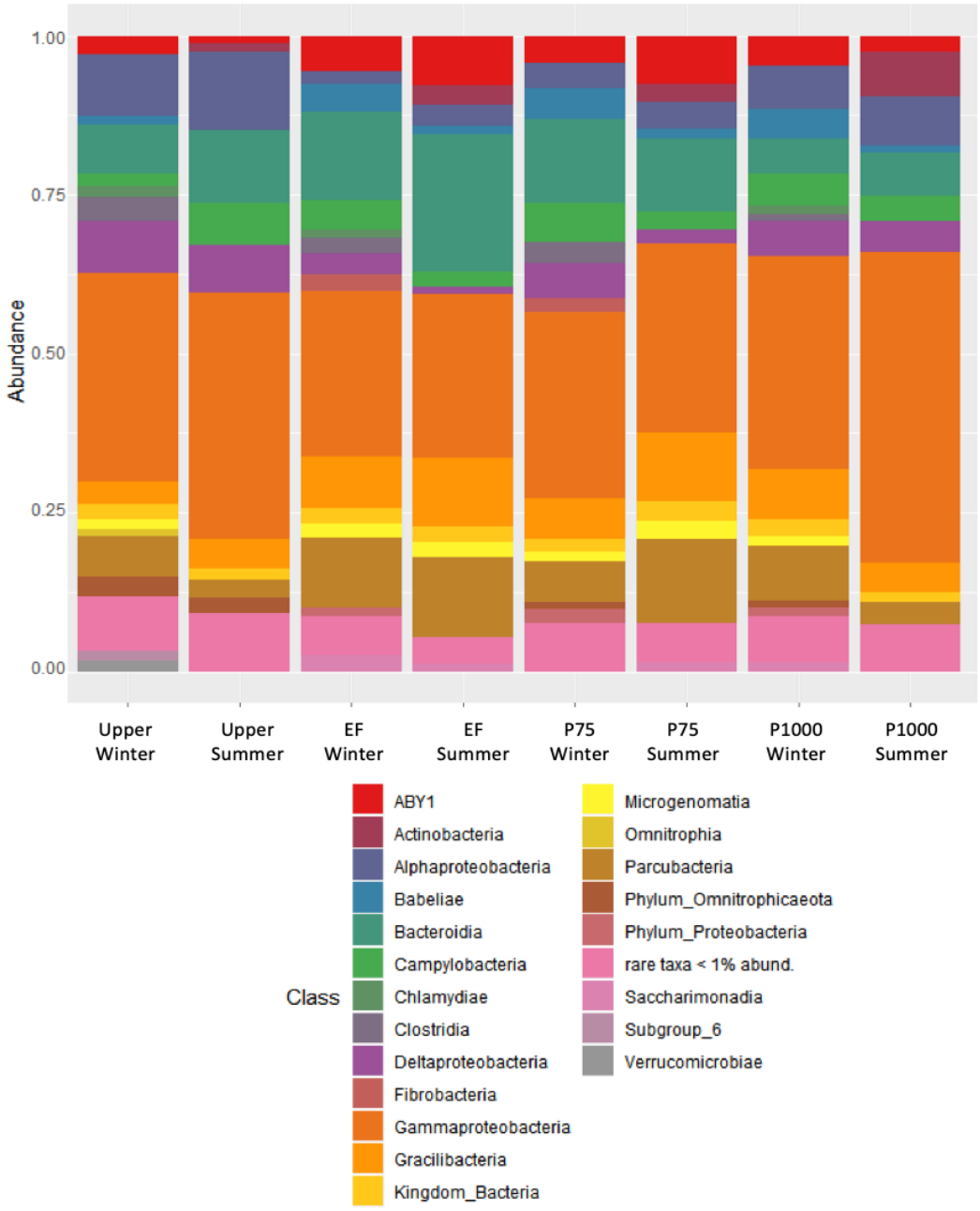
Supplementary Table 4: Statistically significant differences in ASV relative abundance in the different sampling sites. In red, ASV showing an increase with respect to Upper, and in white there is no difference.

	Winter		Summer		
	P75	**P1000**	**P75**	**P1000**	**Genus**
ASV1					*Flavobacterium*
ASV2					*Rhodoferax*
ASV3/ASV27					*Limnohabitans*
ASV4/ASV34					*Polynucleobacter*
ASV5					Family_Burkholderiaceae
ASV6					Order_Absconditabacteriales_(SR1)
ASV7/ASV18					*Rhodoferax*
ASV8					*Sediminibacterium*
ASV9					Family_Neisseriaceae
ASV10					C39
ASV11					*Arcobacter*
ASV12					*Simplicispira*
ASV13					*Arcobacter*
ASV14					*Flavobacterium*
ASV15					Class_Gracilibacteria
ASV16					Family_UBA12409
ASV17					*Aquabacterium*
ASV19					Family_Hydrogenophilaceae
ASV20					Order_Candidatus_Magasanikbacteria
ASV21					*Cellvibrio*
ASV22					*Rhodoferax*
ASV24					Order_Candidatus_Moranbacteria
ASV26					*Aurantimicrobium*
ASV28/ASV53					Order_Absconditabacteriales_(SR1)
ASV29					*Rhodoferax*
ASV30					Class_Gracilibacteria
ASV32					Class_ABY1
ASV35					*Flavobacterium*
ASV36					Class_Gammaproteobacteria
ASV37					*Cavicella*
ASV39					Family_UBA12409
ASV42					*Polynucleobacter*
ASV43					*hgcI_clade*
ASV44					*Acidovorax*
ASV45					*Arcobacter*
ASV46/ASV84					*Novosphingobium*
ASV47					Class_Parcubacteria
ASV50					*Sediminibacterium*
ASV54					*Prevotella_9*
ASV55					*Rheinheimera*
ASV57					Class_Gracilibacteria
ASV58					Phylum_Proteobacteria
ASV60					Family_Fibrobacteraceae
ASV68					*Rhizobacter*
ASV93					*Pseudarcicella*

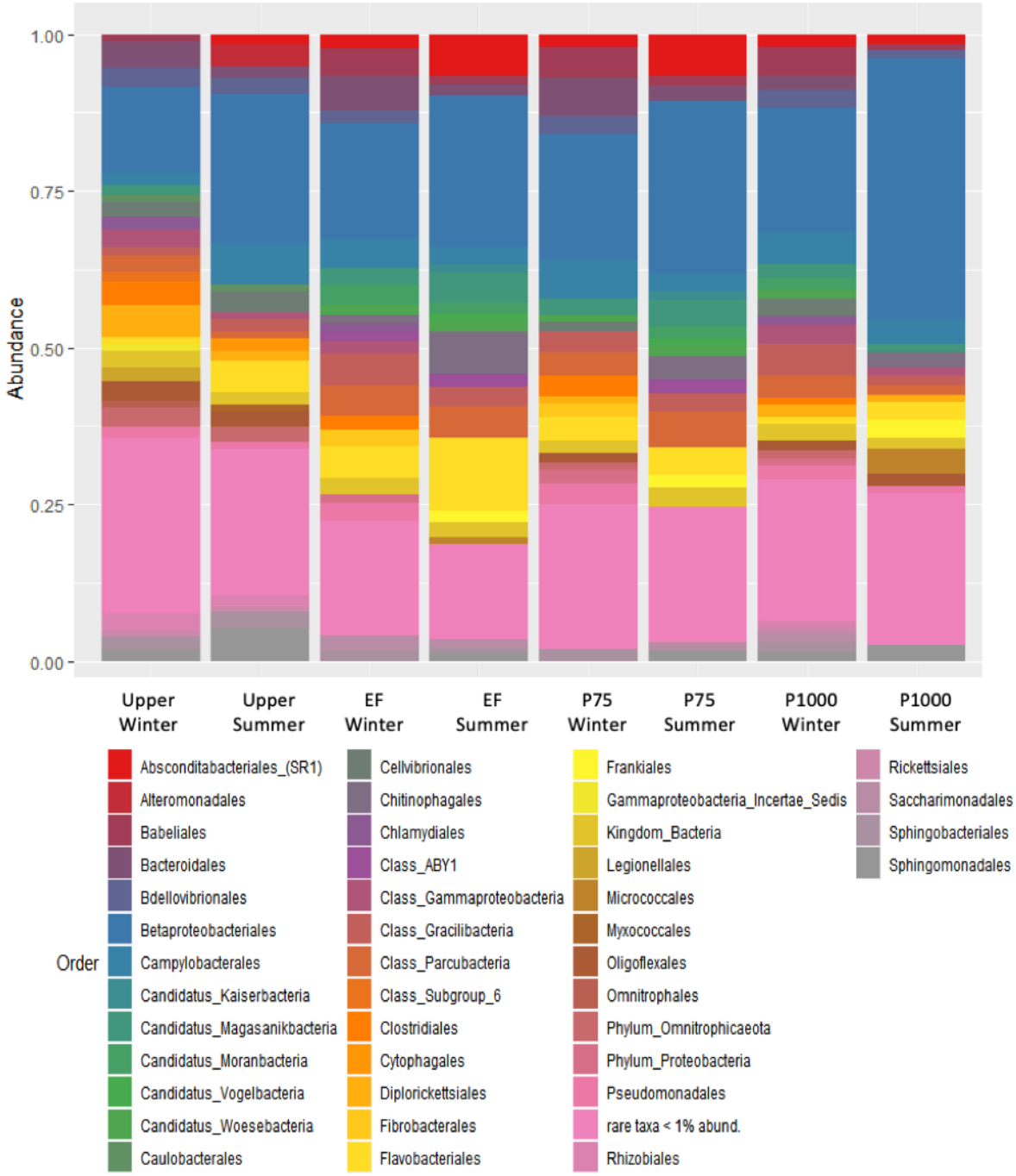
Supplementary Fig. 1: Representability of the sequencing results using rarefaction plots. The graphical representation of the calculated diversity with respect to the expected diversity in relation to the obtained sequencing reads is shown: a) Upper point; b) Effluent point; c) P75 point and d) P1000 point.



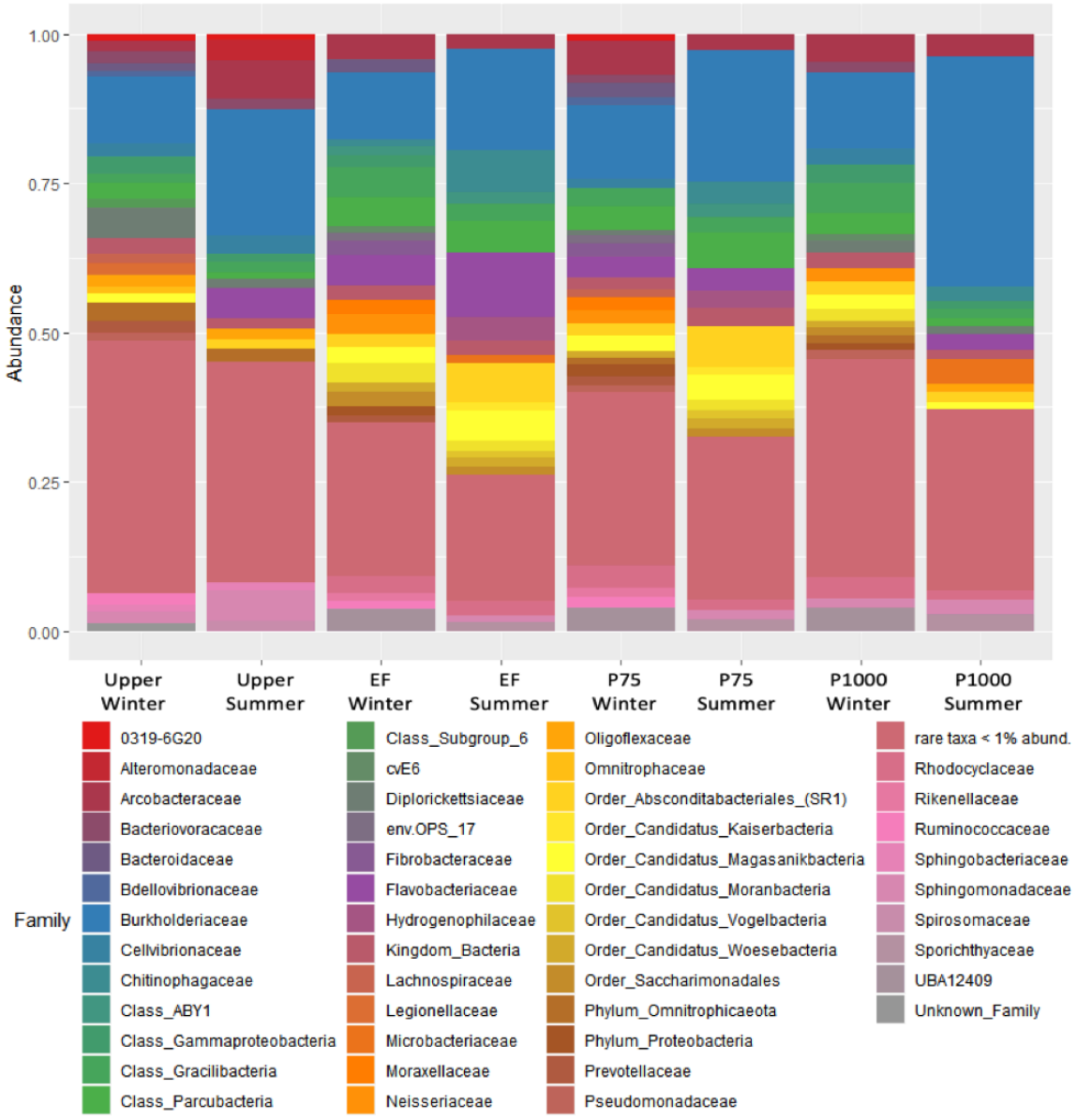
Supplementary Fig. 2: Distribution of classes in each sampling point and separated by season (summer and winter). Rare taxa grouped the genera with proportions <1%.



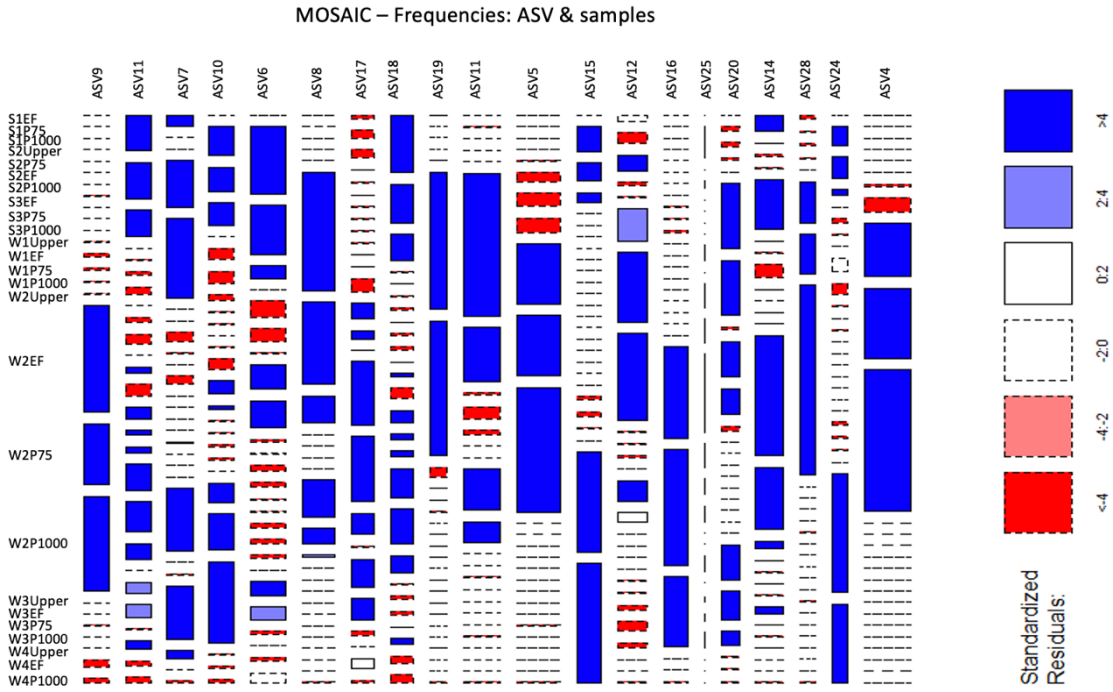
Supplementary Fig. 3: Distribution of orders in each sampling point and separated by season (summer and winter). Rare taxa grouped the genera with proportions <1%.



Supplementary Fig. 4: Distribution of families in each sampling point and separated by season (summer and winter). Rare taxa grouped the genera with proportions <1%.

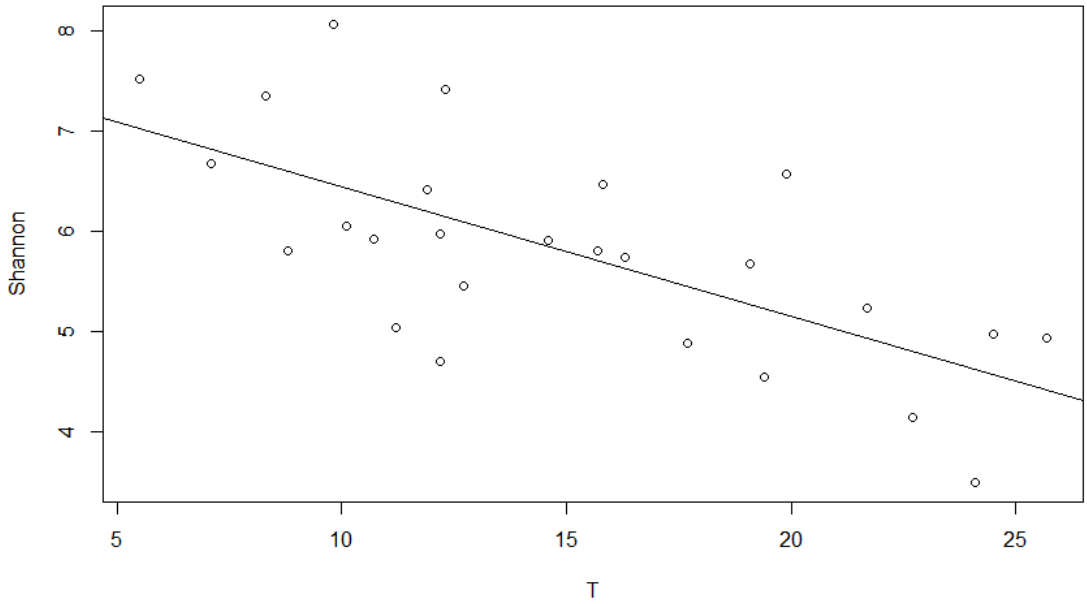


Supplementary Fig. 5: Mosaic plot where all sample groups and the 20 most abundant ASV where used. Standardized residuals are represented as a method to detect sample patterns under the null model (independence) between samples and ASV. Seasonality is indicated in each sample with W or S, corresponding to winter or summer, respectively. Sampling campaign is indicated with a number from 1 to 4 depending on the season, before the sampling site name.

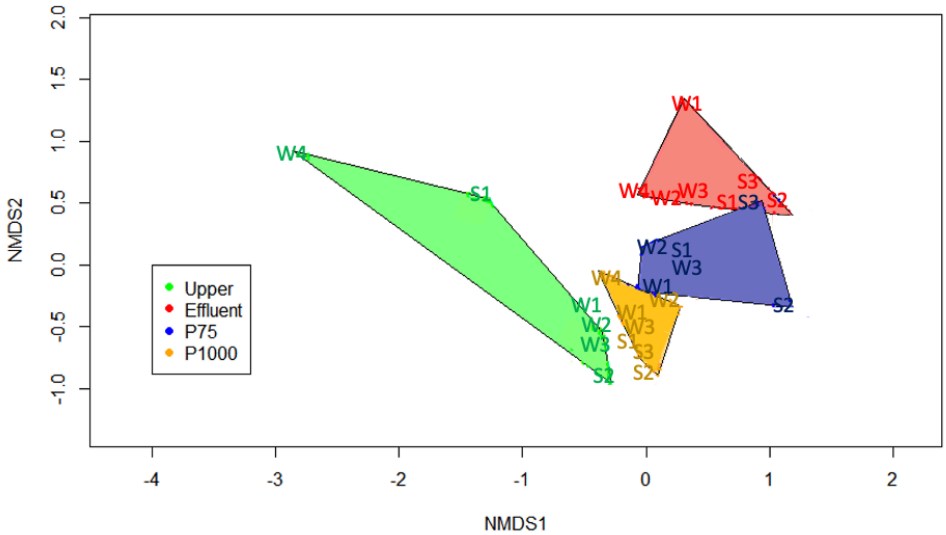


ASV1, *Flavobacterium*; ASV4, *Polynucleobacter*; ASV5, Family_Burkholderiaceae; ASV6, Order_Absconditabacteriales_(SR1); ASV7, *Rhodoferax*; ASV8, *Sediminibacterium*; ASV9, Family_Neisseriaceae; ASV10, Rhodocyclaceae C39; ASV11, *Arcobacter*; ASV12, *Simplicispira*; ASV14, *Flavobacterium*; ASV15, Class_Gracilibacteria; ASV16, Babeliales Family_UBA12409; ASV17, *Aquabacterium*; ASV18, *Arcobacter*; ASV19, Family_Hydrogenophilaceae; ASV20, Order_Candidatus_Magasanikbacteria; ASV24, Order_Candidatus_Moranbacteria; ASV25, Order_Candidatus_Campbellbacteria; ASV26, Aurantimicrobium.

Supplementary Fig. 6: Pearson's correlation of the temperature (T, in °C) and alpha diversity (Shannon index).



Supplementary Fig. 7: Multidimensional scaling plot of the dissimilarity between the sampling points. Seasonality is indicated in each sample with W or S, corresponding to winter or summer, respectively. Sampling campaign is indicated with a number from 1 to 4 depending on the season.



4.4 ARTÍCULO 4

Assessment of dead-end ultrafiltration for the detection and quantification of microbial indicators and pathogens in the drinking water treatment processes

Miriam Pascual-Benito, Pere Emiliano, Raquel Casas-Mangas, Cristina Dacal-Rodríguez, Mercedes Gracenea, Rosa Araujo, Fernando Valero, Cristina García-Aljaro y Francisco Lucena

Artículo sometido en International Journal of Hygiene and Environmental Health

Desde el año 2004 la Organización Mundial de la Salud ha apostado por la implantación de los Planes Sanitarios del Agua (PSA), que consisten en el análisis de riesgo en todas las etapas del tratamiento de potabilización de agua, ya que éste es un sistema multibarrera diseñado para garantizar la obtención de agua segura para la salud. Para llevar a cabo la evaluación de riesgo microbiológico es importante el análisis tanto de indicadores microbiológicos como de patógenos de referencia. Éstos últimos, al encontrarse normalmente en concentraciones bajas, requieren la concentración de grandes volúmenes de muestra. De los diferentes métodos desarrollados la ultrafiltración se ha mostrado como el método más eficaz para la concentración simultánea de bacterias, virus y protozoos, siendo el método de ultrafiltración sin salida (en inglés dead-end ultrafiltration, DEUF) el más empleado para aguas potables.

Los objetivos de este estudio fueron: evaluar la eficacia del método DEUF en la concentración de los diferentes indicadores y patógenos de referencia en muestras ambientales y su idoneidad para evaluar la eliminación de indicadores y patógenos en las diferentes etapas de tratamiento del agua.

En este estudio se analizó la eficacia del método DEUF en las diferentes etapas de tratamiento de dos ETAP que tratan agua con diferentes características fisicoquímicas. La ETAP A trata agua de un río con elevados niveles de turbidez, conductividad y contaminación fecal en cuatro etapas: clarificación, filtración por arena, filtración por carbón activo y cloración. Por otro lado, la ETAP B trata agua de un río después de su paso por un sistema de embalses por lo que sus aguas se caracterizan por bajos niveles de turbidez, conductividad y contaminación fecal. La ETAP B consta de tres etapas de tratamiento: clarificación, filtración por carbón activo y cloración.

Las muestras se analizaron directamente (sin concentración previa) y mediante concentración con el método DEUF. Se analizaron 4 indicadores bacterianos (*E. coli*, enterococos fecales, coliformes totales y esporas de clostridios reductores de sulfito), 3 indicadores víricos (colifagos somáticos, colifagos totales y colifagos F-específicos) y 4 patógenos (*Campylobacter* spp., enterovirus, *Cryptosporidium* spp. y *Giardia* spp.).

A pesar de ser muestras con características muy diferentes se pudieron concentrar grandes volúmenes (100 - 500 l). Además, los resultados mostraron que el agua en la captación de la ETAP A presenta elevados niveles de contaminación fecal, detectando todos los indicadores y patógenos en prácticamente todas las muestras y alrededor de 1-2 logaritmos más que en la captación de la ETAP B. Las concentraciones de los indicadores en las muestras concentradas mediante DEUF fueron inferiores de forma estadísticamente significativa para gran parte de los microorganismos indicadores en las dos ETAP. No obstante, el método de concentración permitió el análisis de mayor volumen de muestra, reduciendo el límite de detección de los microorganismos. Así, al analizar la eliminación de los indicadores de contaminación fecal se observó una eliminación mayor estadísticamente significativa en el caso de las muestras concentradas mediante DEUF.

El método de concentración DEUF presentó una recuperación de los indicadores muy variable en función del microorganismo y la muestra. A pesar de la variabilidad y teniendo en cuenta todos los indicadores, la recuperación media fue del 43,8 ± 17,5 %, ligeramente superior en los indicadores bacterianos (45.5 ± 24,0 %) respecto a la de los indicadores víricos (22,4 ± 9,0 %) y no se observaron diferencias significativas entre las recuperaciones obtenidas en las dos plantas. Sin embargo, aunque no se pudieron encontrar correlaciones estadísticamente significativas entre la recuperación y los parámetros ambientales, sí que se observaron correlaciones negativas estadísticamente significativas entre el volumen filtrado y el carbono orgánico total (TOC), la turbidez y la conductividad. Estos resultados confirmaron el porqué del menor volumen filtrado en las muestras de la captación de la ETAP A, ya que estas muestras presentaron elevados TOC, turbidez y conductividad.

La detección tanto de los microorganismos indicadores como de los patógenos se produjo en las primeras etapas del tratamiento de agua en ambas ETAP. Sin embargo, sí que se detectaron algunos indicadores después de la filtración por carbón activo, aunque en ningún caso después de la etapa de cloración. Sólo *Cryptosporidium* spp. fue detectado en una

muestra después del proceso final de cloración en la ETAP A, coincidiendo con un episodio de crecida del caudal y de la turbidez del río que mantuvo la planta parada.

El hecho de que se detectaran sobretodo coliformes totales a partir de la filtración por carbono activo podría estar indicando un posible recrecimiento en los filtros de carbón por la presencia de materia y orgánica y de partículas que favorecen la persistencia y el crecimiento bacteriano. Sin embargo, el agua tratada de las dos ETAP cumplió con la normativa establecida para el agua de bebida y el método DEUF permitió demostrar una mayor reducción de los microorganismos indicadores y patógenos a lo largo de todo el proceso de tratamiento del agua.

De este estudio se pueden extraer las siguientes conclusiones:

- La concentración mediante DEUF permite la concentración de grandes volúmenes (100-500 l) de agua de río con diferentes características fisicoquímicas (turbidez y conductividad) y diferentes niveles de contaminación fecal. No obstante, el volumen concentrado se correlaciona negativamente con la turbidez, la conductividad y el TOC.
- El método DEUF se muestra eficaz en la concentración de los microorganismos indicadores y patógenos ensayados.
- El porcentaje de recuperación del método DEUF en ambas plantas no presenta diferencias, lo que sugiere que es independiente de las características fisicoquímicas de las muestras a analizar. El porcentaje medio de recuperación de los microorganismos indicadores es del 43%.
- En las dos ETAP los microorganismos indicadores y patógenos son detectados principalmente en las primeras etapas del proceso de tratamiento. La clarificación y filtración por arena en la ETAP A y la clarificación en la ETAP B son las etapas que eliminan la mayor cantidad de microrganismos indicadores y patógenos.
- El DEUF permite reducir el límite de detección de los microorganismos indicadores y patógenos y como consecuencia, detectar una reducción de 1-1,5 logaritmos superior en ambas plantas de tratamiento respecto a las muestras no concentradas.
- La filtración por carbón en ambas plantas puede ser un punto crítico del tratamiento de potabilización y que requiere el seguimiento por el posible recrecimiento de los microorganismos y la formación de biofilms.

- Los eventos de crecimiento de caudal y aumento de turbidez pueden afectar a los diferentes tratamientos de la ETAP A, disminuyendo la calidad del agua tratada y suponiendo un riesgo para la salud.
- La mejora en la detección de microorganismos indicadores y patógenos de referencia puede ser útil para el conocimiento de sus abundancias relativas.
- El método DEUF se muestra como un método muy eficaz para la evaluación de indicadores y patógenos en las diferentes etapas del tratamiento de las ETAP, ayudando también en la identificación de los puntos críticos de tratamiento. Esto hace que pueda ser implementado para el análisis de riesgo del sistema multibarrera que son las ETAP.

Assessment of dead-end ultrafiltration for the detection and quantification of microbial indicators and pathogens in the drinking water treatment processes

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ABSTRACT

A safe water supply requires different treatments and monitoring to guarantee the absence of pathogens and substances potentially hazardous for human health. In this study we assessed the efficiency of the dead-end ultrafiltration (DEUF) method to concentrate faecal indicator organisms (FIO) and pathogens in water samples with different physicochemical characteristics. Water samples were collected at the different treatment stages of two drinking water treatment plants to analyse the concentration of a variety of 7 FIO and 4 reference pathogens: *Campylobacter* spp., enteroviruses, *Cryptosporidium* spp. and *Giardia* spp. The samples were analysed before and after concentration by DEUF. Percent recoveries were highly variable with a mean of 43.8 ± 17.5 %, depending on the FIO and inherent sample characteristics. DEUF enabled FIO concentration in high volumes of water (100 - 500 l), allowing a reduction in the detection limit compared to the non-concentrated samples due to the high volume processing capabilities of the method. As a consequence, the detection of FIO removal was 1.0 to 1.5 logarithms greater in DEUF-treated water compared to unfiltered samples.

The DEUF method improved the detection of target indicators and allowed for the detection of pathogens in low concentrations in water after the different treatment stages, confirming the suitability of DEUF to concentrate high volumes of different types of water. This method could be useful for microbial analysis in water treatment monitoring and risk assessment, allowing the identification of potential hazards in water destined for different uses. And critical points during the water treatment process.

Keywords: water quality, drinking water, *Campylobacter*, enteroviruses, *Cryptosporidium*, *Giardia*

INTRODUCTION

The accessibility of safe water is a major global concern: in 2016, 1,870,998 deaths are estimated to have been caused by inadequate water, sanitation and hygiene (WHO, 2019). To guarantee its safety, water is submitted to different treatments that ensure the absence of pathogens and harmful substances hazardous for consumer health. The expected quality of water depends on its final use, the highest being required for drinking water.

Pathogen detection and quantification by culture methods is costly and time-consuming, and it is unfeasible to analyse all waterborne pathogens. The development of molecular techniques has improved monitoring speed but does not provide information about pathogen viability and/or infective capacity. Consequently, the assessment of faecal indicator organisms (FIO) remains the main strategy for water quality monitoring.

Faecal indicator bacteria, such as *Escherichia coli* (EC) or intestinal enterococci (IE), have been used for many years in water supply management (Anderson et al., 2005; Hijnen et al., 2000; Tallon et al., 2005; Van Donsel et al., 1967). However, the efficiency of bacteria as indicators of viruses and protozoa has been questioned (Gerba et al., 1979; Keswick et al., 1984), because of differences in their structure, life cycle, persistence and survival in water. Bacteriophages and spores of sulphite-reducing clostridia (SSRC) have been recommended as more effective indicators of viruses and protozoa, respectively (Agulló-Barceló et al., 2013; IAWPRC Study Group on Health Related Water Microbiology, 1991; Payment and Franco, 1993), and some drinking water regulations now require their monitoring to guarantee water quality (Health Canada, 2019; NHMRC, 2011), besides pathogen analysis and removal.

Since 2004, the World Health Organization has promoted the implementation of water safety plans, which consist of risk assessment and management at all steps of the multibarrier treatment of drinking water, from the catchment to the end-user (WHO, 2011). In Spain, where the current study was performed, the implementation of water safety plans is obligatory in zones supplying water to 50,000 inhabitants or more. To perform the microbial risk assessment, in addition to the analysis of FIO, it is necessary to monitor different reference pathogens.

The infectious dose for some pathogens is as little as 1-10 cfu, as in the case of *E. coli* O157:H7 or *Shigella* spp.(Kothary and Babu, 2001) and pathogen analysis and removal requires the analysis of high volumes of water, as pathogens are frequently present in lower numbers compared to FIO. Although standardized protocols are available for the detection

and quantification of each pathogen, new approaches are needed that allow the simultaneous concentration of multiple kinds of pathogens. With this aim, different concentration methods have been extensively tested, including glass wool filtration, monolithic affinity techniques, and ultrafiltration, the latter allowing the concentration of the highest volumes of water and different microorganisms (Bridle, 2014; Polaczyk et al., 2008).

The ultrafiltration procedure is based on size exclusion and can be carried out by tangential flow or dead-end concentration. Compared to other techniques, dead-end ultrafiltration (DEUF) has the advantage of being able to handle higher volumes of water. Previous studies have tested DEUF in drinking water samples, reclaimed water or spiked water samples, with the physicochemical parameters and microorganism concentration set under controlled conditions (Liu et al., 2012; Mull and Hill, 2012; Smith and Hill, 2009). However, little is known about its performance in environmental samples with different characteristics or samples under natural conditions.

The aim of this study was to assess the ability of DEUF to concentrate high volumes of water samples from the different stages of two drinking water treatment plants (DWTPs) for the analysis of FIO and bacterial, viral and protozoan pathogens. In addition, we studied the ability of the method to better assess the FIO and pathogen removal efficiency of the different treatment steps in both DWTPs compared to traditional sample processing. To perform this research, we analysed four different faecal indicator bacteria: total coliforms (TC), EC and IE as non-conservative parameters, and SSRC as a conservative parameter indicator of resistance forms. Three bacteriophages were analysed as viral indicators: somatic coliphages (SOMCPH), F-specific RNA coliphages (FRNAPH) and total coliphages (CB390PH). In this study, we also analysed four different reference pathogens that follow the faecal-oral transmission route and are crucial in assessing the microbial risk of drinking water. We chose *Campylobacter* spp. as a bacterial pathogen, as it is the major source of bacterial gastroenteritis globally (European Centre for Disease Prevention and Control, 2019; Kaakoush et al., 2015; WHO, 2012) and has a low infective dose of 360 MPN (Hara-Kudo and Takatori, 2011). Enteroviruses (EV) were chosen as viral pathogens because they have a low minimum infective dose and can cause serious diseases, not only gastroenteritis, but also meningitis and myocarditis; for this reason, they are included in some regulations as a reference pathogen (Health Canada, 2019; NHMRC, 2011; USEPA, 1998). Finally, we analysed *Cryptosporidium* spp. and *Giardia* spp., two protozoa that are another major cause of gastroenteritis worldwide (Fletcher et al., 2012). Moreover,

the importance of *Cryptosporidium* and *Giardia* lies in their ability to form oocysts and cysts, respectively, resistance forms that can persist after different water treatments. In order to perform this study, we analysed: i) the concentration of different FIO in the catchment of the DWTPs; ii) the recoveries of the DEUF method through FIO concentration; iii) the removal of FIO in both DWTPs, comparing the direct and concentration methods; iv) the pathogen concentrations and their removal in the DWTPs.

MATERIAL AND METHODS

Samples and sampling site

This research was performed in two DWTPs located in Catalonia (Northeast of Spain) that supply drinking water to more than 4.5 million inhabitants in the Barcelona Metropolitan Area. DWTP A treats the surface water of a river in its lower course, and DWTP B treats the water of a river in its middle course after a reservoir system. The two types of raw water therefore have very different characteristics: pollution and values of turbidity and conductivity are much higher in the water of the DWTP A catchment area compared to DWTP B, and consequently the treatment required is also different. DWTP A has a maximum capacity of 3.2 m3/s with 5 different stages: catchment, clarification, sand filtration, active carbon filtration and chlorination with NaClO. Before the chlorination, part of the water is treated by reversed electrodialysis to reduce its high conductivity. DWTP B has a maximum capacity of 8 m3/s and consists of 4 stages: catchment, clarification, active carbon filtration and chlorination with NaClO.

A total of 12 sampling campaigns were performed in DWTP A and 9 in DWTP B from January 2018 to February 2019. Samples were taken at catchment intake and after each stage of the drinking water treatment. Sample volumes ranged between 150 and 506 l for the DEUF method and between 0,01 and 100 ml for the direct analysis of the different FIO using conventional methods as stated below.

Physicochemical parameters

Different physicochemical parameters were measured *in situ* to characterise each water sample: turbidity, temperature, total organic carbon (TOC) and conductivity.

Turbidity was measured with a 2100-N and 2100-P Turbidimeter (Hach, USA) in DWTP A and DWTP B, respectively. Temperature was measured with a Thermometer 0560 1113 (Testo, Australia) and TOC was registered with a TOC-V CSN and TOC-L CSH (Shimazdu, Japan).

Finally, the conductivity was measured with a multiparametric analyser Crison Multimeter MM41 (Danaher, United States) in DWTP A and with a Crison GLP-32 analyser (Danaher, United States) in DWTP B.

Assessment of the DEUF method

For the assessment of the DEUF method samples were concentrated using Rexeed-25A™ hollow fiber filters (Asahi Kasei Medical America Inc, Japan) following a previously described method (Hill et al., 2007). The Rexeed-25A™ filters were pre-treated by recirculating 400 ml of 6.25% of sterile foetal bovine serum (FBS) blocking solution for 5 minutes to avoid bacterial adsorption to the filters. Filters were stored at 4°C for 72 hours until use. Samples were concentrated at 2 l/min by connecting the filter directly to a faucet that was available for sampling after each water treatment step, except for the raw and clarified water from DWTP A, which was concentrated using a peristaltic pump at 2.9 l/min. Filters were eluted using 500 ml of phosphate-buffered saline supplemented with 0.5 ml of 1% Antifoam A (Sigma-Aldrich, USA)/10% Tween 80 (Scharlab, Spain) and 0.5 ml of 10% NaPP (Sigma-Aldrich, USA). A back-flush elution was performed using a peristaltic pump at 0.65 l/min and the eluate was recovered obtaining a final volume between 480 ml and 640 ml.

In addition, we also collected 1 l of each sample to be analysed without DEUF concentration. Direct and concentrated samples were analysed to detect and quantify the FIO and pathogens as stated below.

Enumeration of faecal indicator organisms

Escherichia coli (EC) and total coliform bacteria (TC) were analysed following ISO 9308-1:2014 (ISO, 2014). Samples were filtered through 0.45 µm diameter pore size nitrocellulose membranes and the filters were then incubated on Chromocult® agar (Merck, Germany) at 37°C for 20 hours. Dark blue and purple colonies were enumerated as *E. coli.* The sum of pink colonies plus dark blue and purple colonies were enumerated as TC.

Intestinal enterococci (IE) were enumerated following ISO 7899-2:2000 (ISO, 2000a). Samples were filtered through 0.45 µm diameter pore size nitrocellulose membranes and the filters were then incubated on BD Difco Enterococcus agar (Thermo Scientific®, USA) at 37°C for 48 hours. In order to confirm the positive colonies, filters were transferred to Bile Esculin Azide Agar (Scharlab, Spain) and incubated at 44°C for 4 hours.

Spores of sulphite-reducing clostridia (SSRC) were enumerated as described previously (Ruiz-Hernando et al., 2014). Samples were subjected to a thermal shock at 80°C for 10

minutes and anaerobically cultured in *Clostridium perfringens* selective agar (Scharlab, Spain) at 44 °C for 24 hours.

For bacterial indicator analyses, the maximum volume analysed per sample was 100 ml in direct samples and 2 ml in samples concentrated by DEUF. For volume samples less than 10 ml sample volume was increased up to 10 ml by adding sterile PBS. Therefore, the theoretical detection limit of the used method to analyse direct samples was 1 cfu/100 ml, and in the DEUF samples it was about 0.05 cfu/100 ml, according to the volume concentrated and the volume obtained in the elution process. The DEUF estimated detection limit was calculated by taking into consideration the theoretical detection limit and the efficiency in the recovery of FIO by the DEUF method compared to the non-concentrated samples, which was about 0.02 cfu/100 ml.

Somatic coliphages (SOMCPH), F-specific RNA coliphages (FRNAPH) and total coliphages (CB390PH) were enumerated by the double agar layer technique following the protocols described in ISO 10705-2:2000, ISO 10705-1:1995 and Agulló-Barceló et al (2016), respectively (Agulló-Barceló et al., 2016; ISO, 2000b, 1995). A maximum of 10 ml of each sample was analysed in direct samples and 2 ml of the eluate in concentrated samples. Therefore, the theoretical detection limit was 10 pfu/100 ml and about 0.05 pfu/100 ml in direct and concentrated samples, respectively. The estimated detection limit was calculated as for the FIO, resulting in 0.01 pfu/100 ml.

Enumeration of pathogens

The pathogen concentrations were only analysed in DEUF samples due to their low values that made necessary to concentrate high volumes of water. To quantify *Campylobacter* spp., 150 ml of eluate (equivalent to 50-125 l of the original sample) was further concentrated by centrifugation at 7,700 g for 20 min at 20 °C and the pellet was resuspended in 5 ml of the discarded eluate. The enumeration of *Campylobacter* spp. was performed following ISO 17995:2005 with some modifications in order to adapt the protocol to a Most Probable Number (MPN) method, as previously described (Rodríguez and Araujo, 2012). Briefly, this method consisted in 3 tenfold serial dilutions of the samples and selective enrichment in Preston *Campylobacter* Selective Enrichment Broth (Oxoid, United Kingdom) at 42 °C for 48 h in microaerobic conditions. Samples were inoculated in *Campylobacter* Agar Base Blood Free (Oxoid, United Kingdom) and incubated at 42 °C for 48 h in microaerobic conditions. Grey colonies were considered presumptive colonies of *Campylobacter* spp. and were confirmed

by Gram stain. The theoretical detection limit of *Campylobacter* spp. was about 0.0001 MPN/100 ml.

The detection and quantification of infective enteroviruses also required eluate concentration. Different volumes of eluate, ranging from 70 to 192 ml (equivalent to 50-160 l), were concentrated using Centricon® Plus-70 Centrifugal Filter Units (Merck Millipore, Germany) to obtain a final volume of 1 ml. 780 µl of the concentrate was resuspended in 20 ml of Eagle's Minimum Essential Medium (Sigma-Aldrich, USA) and filtered through 0.22 µm pore size hydrophilic polyethersulfone membrane to remove non-viral microorganisms. Infective enteroviruses were quantified by a double agar layer plaque assay in Buffalo green monkey kidney cells as described (Mocé-Llivina et al., 2004). The theoretical detection limit of EV was about 0.001 pfu/100 ml.

In order to detect and quantify *Cryptosporidium* spp. and *Giardia* spp. a volume of 50 ml of eluate (equivalent to 15-50 l) was further concentrated by centrifugation at 3,000 g for 10 min and the pellet was recovered and resuspended in 5 ml of phosphate buffered saline to detect and quantify *Cryptosporidium* spp. and *Giardia* spp., as previously described (USEPA, 2012). Samples were subjected to Ziehl-Neelsen staining (Henriksen and Pohlenz, 1981) and merthiolate-iodine-formaldehyde staining (Sapero et al., 1951) to detect and quantify *Cryptosporidium* spp. and *Giardia* spp., respectively, after their observation by optical microscope. The volumes analysed resulted in a detection limit of about 0.2 oocysts/100ml of *Cryptosporidium* spp. and 0.1 cyst/100 ml of *Giardia* spp.

Statistical analysis

In order to analyse the results, FIO and pathogen concentrations were $\log_{10}$-transformed. We used the value corresponding to the detection limit as a result for negative results.

The recovery was calculated as the result of the fraction of the values obtained by the DEUF method and the values obtained in the direct samples.

The normality distribution of the data was checked by Shapiro-Wilk's test and data analysis and plots were performed using R Studio software v. 1.2.5001. Finally, we analysed the correlations among different parameters using Spearman's correlation test. Spearman's coefficient, r, with *P* values lower than 0.05 were considered statistically significant.

RESULTS

Concentration of samples by DEUF

Different volumes of each sample were concentrated by DEUF which varied according to their physicochemical parameters (Table 1). For most samples, the filtered volume was approximately 500 l, which was eluted in 560 ml and consequently, 1 ml of the eluate was equivalent to 0.9 l of the direct sample. The exception was the raw and clarified water samples of DWTP A, for which the equivalents were 0.3 l and 0.4 l, respectively.

The physicochemical parameters observed confirmed the differences between the catchment of DWTP A and DWTP B, referred to henceforth as catchment A and catchment B, respectively. As well as high turbidity, water from catchment A presented high conductivity values. A statistically significant correlation between the streamflow and the turbidity was observed ($r=0.46$; $P<0.01$) (Fig. A.1) using the values registered in the DWTP during the studied period.

The conductivity values were statistically negatively correlated with the streamflow ($r= -0.75$; $P<0.01$), meaning that high streamflow produced a dilution of the electrolyte concentration and reduced the conductivity of the raw water.

The water samples of catchment B showed low values of turbidity, conductivity and TOC, making it possible to concentrate 500 l at each stage.

FIO concentration in the DWTP catchments by conventional analysis

DWTPs are designed to remove particles, microorganisms and substances that could affect consumer health, incorporating different stages to optimise the treatment. In order to assess the DWTP performance, it was necessary to characterise the raw water at the point of intake. Considerable differences were found in FIO concentrations between the two catchments (Fig. A.2). In direct samples of catchment A, concentrations ranged from 2 to 4 $\log_{10}$(cfu/100ml) or $\log_{10}$ (pfu/100ml); the exceptions were for TC, 3 to 5 $\log_{10}$cfu/100ml), and FRNAPH, 1 to 3 $\log_{10}$ (pfu/100ml). In direct samples of catchment B, FIO concentrations were roughly at the detection limit, ranging from 0 to 1.5 $\log_{10}$ (cfu/100ml) or log10(pfu/100ml); the exception was for TC, 1.5 to 2.5 $\log_{10}$ (cfu/100ml).

The concentration of faecal bacterial indicators in samples of catchment A was about 2-2.5 $\log_{10}$ higher than in catchment B. There was a similar difference in concentration of bacteriophage viral indicators (2 $\log_{10}$), with the exception of FRNAPH, which was only 1 $\log_{10}$higher in catchment A. Viral indicators were present at lower concentrations than

bacterial indicators; in catchment B, bacteriophages were detected at roughly the detection limit, or in some samples not at all.

FIO concentration in the DWTP catchments by DEUF-method

We observed lower FIO values in DEUF-concentrated samples compared to the direct samples, with differences of about 1 log_{10} in both catchments, suggesting a loss of microorganisms during the concentration process and a recovery lower than 100%. These differences were statistically significant for TC, SSRC, SOMCPH and CB390PH in catchment A ($P<0.05$) and for IE, SOMCPH, CB390PH and FRNAPH in catchment B ($P<0.05$).

FIO recoveries after concentration by DEUF

The percentage of microorganism recovery is key in the assessment of a concentration method. In order to analyse the effectivity of concentration by DEUF, we enumerated the concentrations of 7 FIO in direct and DEUF concentrated samples and we calculated the recovery of each FIO at every sampling campaign (Fig. 1). We obtained 1 to 3 outlier values for each FIO, which were removed for a better understanding of the results. Taking into account all the FIO and both DWTPs, the mean recovery was 43.8 ± 17.5 %, while the recovery of bacterial indicators was 45.5 ± 24.0 and the bacteriophages recovery was 22.4 ± 9.3.

The recoveries of each FIO differed considerably between samples and DWTPs. This could be explained by the variable physicochemical characteristics of environmental water, which can affect the efficiency of the concentration method. In our case, more variable recovery was obtained in bacterial than viral indicators in both DWTPs, but especially in DWTP A, where bacterial concentration was higher. Although recovery percentages were slightly higher in DWTP B for faecal bacterial indicators and in DWTP A for viral indicators, the differences were not statistically significant ($P > 0.05$).

To better understand how the environmental conditions of the water influence microorganism recovery in the DEUF method, we correlated FIO recoveries with the main physicochemical parameters (Fig. 2), finding no statistical significance in the correlations overall. However, the results showed that some parameters were highly relevant for the concentration method, including conductivity ($r=-0.8$; $P<0.01$), TOC ($r=-0.61$; $P<0.01$) and turbidity ($r=-0.59$; $P<0.01$), which presented strongly and moderately significant negative correlations with the filtered volume.

FIO removal in the DWTPs

In this research, we analysed the presence of FIO after each treatment stage (Table 2). In catchment A, FIO were detected in all direct and concentrated samples. In catchment B, faecal bacterial indicators were also detected in almost all the samples; however, bacteriophages were found in only a few direct samples and FRNAPH not at all, whereas in concentrated samples, SOMCPH and CB390PH were detected in 88.9% and 100%, respectively, and FRNAPH only in 44% of the samples.

The clarification stage removed part of the FIO in both DWTPs; in DWTP B removal was almost total, with only TC still detected in clarified water. In the clarified water of DTWP A, a high percentage of the direct samples still showed faecal bacterial indicators (83.3% for TC, EC and IE and 91.7% for SSRC), whereas fewer were positive for bacteriophages (66.7% for SOMCPH and CB390PH, and 33% for FRNAPH); a high percentage of concentrated samples were also positive for faecal bacterial indicators (66.7% - 83.3%) and viral indicators (33.3% to 75.0%).

After the sand filtration in DWTP A, the target microorganisms were almost all absent, except TC, which was detected in 1 direct and 3 concentrated samples (8.3% and 25% of positive samples, respectively). Additionally, 3 concentrated samples (25%) were also positive for *E. coli*.

After the active carbon filtration in DWTP A, TC was detected in 41.7% of the concentrated samples and SSRC in one sample (8.3%). In DWTP B, TC was also found in direct (11.1%) and concentrated (2.2%) samples.

The final samples from both DWTPs did not show the presence of the tested FIO, achieving the quality expected for drinking water. In general, a lower number of positive results were obtained in direct than in concentrated samples when the concentrations were roughly the detection limit. This was noticeable in the advanced stages of the treatment process of both DWTPs and in catchment B.

To assess the operation of both DWTPs, FIO removal was compared in direct samples and samples concentrated by DEUF. FIO removal (Fig. 3) was calculated as the difference between the FIO $\log_{10}$ concentrations in the catchment and treated water. In samples without detectable microorganisms, the detection limit was used. FIO removal was approximately 2 $\log_{10}$ higher in DWTP A than in DWTP B, a reflection of the difference in FIO concentrations in raw water, which was about 2 $\log_{10}$ higher in catchment A. The lower detection limit of the

DEUF method meant that most FIO values were higher in the filtered than direct samples (by 0.4 - 1.8 log_{10}), which facilitated the monitoring of FIO removal throughout the water treatment process. Removal was statistically significantly higher in DEUF samples for all the FIO in both DWTPs ($P<0.05$), except for SSRC in DWTP A and FRNAPH in DWTP B ($P>0.05$).

Pathogen concentrations in the catchments and their removal in DWTPs

In order to evaluate DEUF as a method for pathogen monitoring in environmental samples, we assessed the concentration and the presence of four different kinds of pathogens in all the stages of both DWTPs: *Campylobacter* spp. as a bacterial pathogen, enteroviruses as viral pathogens and *Cryptosporidium* spp. and *Giardia* spp. as parasites (Table 3). We detected EV in 83% of the samples and the mean concentration of EV in catchment A was 0.01 pfu/100 ml. while the concentrations of *Campylobacter* spp. in catchment A, ranging from 0.3 to 5.2 MPN/100 ml.

Concentrations of *Cryptosporidium* spp. in catchment A ranged from 3.3 oocysts/l to 52 oocysts/l and a mean concentration of 18.4 oocysts/l while we detected *Giardia* spp. in 11 out of 12 (91%) catchment A samples, with a mean concentration of 4.6 cysts/l.

We studied the correlations between the FIO and pathogen concentrations in the catchments of the both DWTPs. In the DWTP A we found a statistically significant correlation between IE and *Campylobacter* spp. ($r=0.69$; $P<0.05$), the SSRC and *Cryptosporidium* spp. ($r=0.89$; $P<0.01$) and between the SSRC and *Giardia* spp. ($r=0.94$; $P<0.01$) while in DWTP B we did not find statistically significant correlations.

In DWTP A, the clarification stage removed the highest percentage of pathogens, after which 41.7% of samples were positive for *Campylobacter* spp., 16.7% for EV, 33.3% for *Cryptosporidium* spp. and 8.3% for *Giardia* spp. However, after the sand filtration, all pathogens had been removed with the exception of *Cryptosporidium* spp. detected in one sample of treated water in DWTP A at a concentration of 1.02 oocysts/l.

In DWTP B, pathogens were observed in concentrations of roughly the detection limit, with the exception of EV, which was not detected. The raw water samples for catchment B were 85.7% positive for *Campylobacter* spp. with concentrations ranging from 0.003 MPN/100 ml to 0.05 MPN/100 ml; 62.5% for *Giardia* spp. with a mean concentration of 0.78 cysts/l and *Cryptosporidium* spp. were detected in 100% with concentrations ranging from 2.3 oocyst/l to 6.9 oocysts/l. The clarification stage of DWTP B removed almost all the pathogens, resulting

in only 1 out of 8 samples (12.5%) positive for *Cryptosporidium* spp. After the active carbon filtration, *Cryptosporidium* was also detected in 1 out of 8 samples (12.5%).

DISCUSSION

In order to ensure the absence of pathogens and harmful substances in tap water, DWTPs provide a multibarrier system where each stage is optimised to achieve the best water quality. The detection and quantification of faecal indicators and reference pathogens in each stage of the drinking water treatment process is crucial for the water quality management.

The DEUF method performed in this study allowed the concentration of high volumes from the different stages of the both DWTPs. The lower volumes of filtered raw and clarified water from DWTP A can be explained by the procedure, as they were concentrated by a peristaltic pump. Moreover, another relevant factor in the catchment water was high turbidity, which resulted in an earlier saturation of the filters by particles. The higher conductivity of water at catchment A can be explained by its passing through an area of salt mines. The low values of turbidity, conductivity and TOC at catchment B allowed for the concentration of 500 l. In this case, the reservoir system works as a huge sedimentation tank, clarifying the water.

In both DWTPs water presented low turbidity and TOC values after the clarification stage. However, the conductivity in DWTP A was not reduced until the end of the treatment process, where part of the water was subjected to reversed electrodialysis.

The levels of faecal pollution were also different in both catchments. The concentrations of faecal indicator bacteria in catchment A were similar to those reported in previous studies, whereas SOMCPH concentrations were slightly higher (by 0.5 $\log_{10}$) (Montemayor et al., 2005; Muniesa et al., 2012). The lower FIO concentrations in catchment B were due to the reservoir system, located above the intake point, which removed part of the faecal pollution. These results agree with previous studies performed at the same waterbody (Araujo et al., 1997). The bacteriophage concentrations detected in this study agree with (Lucena et al., 2003), who reported that concentrations of SOMCPH were also about 1 $\log_{10}$ higher than FRNAPH in rivers in South America, France and Spain.

In this study we analysed the recovery of FIO using DEUF method, considering the values obtained by the direct analysis of 100 ml as reference for the calculations. The recovery of the DEUF method presented high variability in bacterial and viral indicators that can be caused by their attachment to particles (LeChevallier et al., 1988; Templeton et al., 2008). This trend was

also described by Liu and collaborators, who reported high variability in the recoveries of microorganisms, primarily bacteria, after DEUF of reclaimed water (Liu et al., 2012). The outlier values, which ranged from 124% to 900%, could be attributed to different factors, such as the variation in the filtration process. The concentration of catchment samples took 3.7 ± 1.3 hours in DWTP A and 4.6 ± 0.7 hours in DWTP B, and FIO concentrations can change during this time. Another factor could be the disaggregation of flocs containing microorganisms during the elution process, which would increase the FIO concentration in the eluate compared to the direct sample (Hill et al., 2007).

The concentration method tested in this study was developed by CDC and USEPA in order to concentrate and detect biothreat agents in drinking water with recoveries higher than 50% (USEPA and CDC, 2011). Several filtration methods such as glass wool, nanoCeram, continuous flow centrifugation or electropositive cartridge have been developed to concentrate pathogens, but they are optimised for detecting one type of microorganism (Francy et al., 2013; Karim et al., 2009). The DEUF method has been used to concentrate bacterial, viral and protozoan pathogens, leading to recoveries of 60 - 80% in drinking water (Gunnarsdottir et al., 2020). The recoveries obtained here, ranging from 9% to 121% and with a mean recovery of 43.8 %, agree with the results of (Bosch et al., 2016), who reported recoveries of 9% - 102%, depending on the microorganism and the sample characteristics. However, it is necessary to take into account that the assays in previous studies were performed in a laboratory, using spiked samples under controlled conditions, which could improve microorganism recoveries compared to the environmental samples and natural conditions analysed here.

The physicochemical parameters of the water can affect the microorganism recovery. TOC, turbidity and conductivity are parameters that quantify the presence of organic matter, particles and electrolytes, respectively, which can saturate the filter and reduce the filtered volumes. In spite of the physicochemical parameters interfere with the DEUF method, different studies have promoted its use because it allows for the concentration of different types of microorganisms and pathogens with high microbial recoveries (Francy et al., 2013; Smith and Hill, 2009).

In both DWTPs the highest percentages of FIO and pathogen removal were achieved in the clarification stage but in general, each stage contributed to the removal of FIO and pathogen concentrations in both DWTPs, working as an effective multibarrier system. Nevertheless, However, the increased detection of TC after the active carbon filtration could suggest that

the organic matter retained by the filters provides the nutrients required for bacterial growth. Furthermore, the carbon particles could protect the bacteria against the treatments by allowing the formation of biofilms (Gibert et al., 2013), which is a critical issue in drinking water treatment.

FIO concentrations were higher in the direct samples of both catchments compared to the values obtained by the DEUF method. However, the lower detection limit in the treated water samples concentrated by DEUF resulted in a logarithmic increase in FIO removal detected at all stages of the system since for some samples the FIO values by the direct method were below the limit of detection. In addition, the concentration allowed to improve the percentages of detection, especially in that stages where the FIO concentrations were very low. This issue is crucial for the microbial risk assessment in water for the reason that the DEUF method could increase the safety of the drinking water.

The suitability of the DEUF method to perform the concentration of reference pathogens was not compared with direct sample analysis since it is well known that a concentration method is usually needed to quantify pathogens due to the low numbers present in the environment. However, if we compare the obtained results with values obtained in previous studies performed by our research group in the same catchment area, the EV mean concentration detected in this study (0.01 pfu/100 ml) was very similar to that reported by Costán-Longares and co-workers in a previous study, which reported a mean concentration of 0.04 pfu/100 ml in different rivers in Catalonia using electropositive filter cartridges to concentrate virus by adsorption (Costán-Longares et al., 2008). However, *Campylobacter* spp. concentrations were slightly lower than those of a previous study at the same waterbody using the centrifugation of 3 l as the concentration method (Rodríguez and Araujo, 2010). This difference could have two possible explanations: the increased ecological flow in catchment A over the last decade producing a dilution effect, and fewer sources of *Campylobacter* spp. pollution. Nevertheless, we detected *Campylobacter* spp. in 100% of catchment A samples, which was higher than the 81% of positive samples in the previous study. Our results for *Campylobacter* spp. confirm the high pollution pressure in catchment A and are similar to those of other studies reporting a high percentage of *Campylobacter* spp. in polluted surface waters (Eyles et al., 1998; Stelzer and Jacob, 1991).

The *Cryptosporidium* spp. concentrations detected in this study were higher than the 0.43 - 1.36 oocysts/l previously reported at the same waterbody using Envirocheck® filters

(Montemayor et al., 2005). While both studies report 100% of positive samples in catchment A, the DEUF method could explain why we obtained higher concentrations despite an increased ecological flow in catchment A in the last years. The positive result obtained in treated water from DWTP A was obtained after a heavy rainfall event, during which DWTP A stopped operating for 2 days. The sampling campaign was performed 2 days after the DWTP A resumed functioning, when the treated flow, 0.6 m3/s, was still lower than the normal flow of 1.2 m3/s, which was subsequently achieved on the same day as the sampling. The turbidity in catchment A was still high (130 NTU) and the concentration of *Cryptosporidium* spp. in the catchment sample was 52 oocysts/l, the highest concentration obtained during the studied period. It should be taken into account that the detection and quantification method performed in this study do not provide information about the viability and infective capacity of the parasite. Previously reported viability levels range from 16% to 28% (Montemayor et al., 2005). Thus, this incident further suggests the important role of heavy rainfall in the mobilisation of waterborne pathogens (Curriero et al., 2001; García-Aljaro et al., 2017; Kistemann et al., 2002; Tryland et al., 2011), which affects the quality of the surface water utilized by the DWTPs and entails a risk if the pathogens can overcome the different treatment stages.

The treated water of both DWTPs achieved the drinking water quality standards (CEU, 1998; WHO, 2012), as the current law assessing drinking water treatment processes only requires the absence of *E. coli* and Enterococci in 100 ml and the analysis of Clostridium perfringens. The monitoring not only of FIO but also pathogens, especially protozoa and resistance forms, in the last stages of drinking water treatment is crucial because they can become attached to particles and infrastructures and form biofilms (Wingender and Flemming, 2011). Several waterborne pathogens can be released from biofilms to water and constitute a hazard for consumers (Helmi et al., 2008; Searcy et al., 2006). Moreover, the ability of *Cryptosporidium* to excyst and grow in an environment without host cells such as biofilms has been recently described (Clode et al., 2015; Thompson et al., 2016). This capacity opens a new scenario in water quality monitoring, focusing attention on the treatment stages where multiplication is possible, and the possible addition of new stages to the multibarrier system.

The concentrations of protozoa in this research were in agreement with the results of previous studies, where *Cryptosporidium* spp. were detected more frequently and in higher

concentrations than *Giardia* spp. in surface waters (Prystajecky et al., 2014). However, the inverse trend, where the concentrations of *Giardia* spp are higher than the concentrations of *Cryptosporidium* spp. has also been reported by some authors (Burnet et al., 2014; Mons et al., 2009). Several factors such as human and animal parasitization (Fletcher et al., 2012), the physicochemical characteristics of the water, water discharges, and the persistence of oocysts and cysts can contribute to the different densities and predominance of one or another protozoan in surface waters (Wilkes et al., 2009; Xiao et al., 2013).

Although we only found some statistically correlations between SSRC and the analysed protozoan and IE and *Campylobacter* spp. in the DWTP A, this method could be useful to select the most suitable surrogate microbial indicators and pathogens for testing.

Finally, the main advantage of this method is that allows the easy concentration of different microorganism with high recoveries for all of them. The use of the DEUF method to concentrate environmental samples before performing microbial analysis can provide valuable information for water management and for the quantitative microbial risk assessment included in water safety plans. As well as drinking water, DEUF can be applied to concentrate water for usage requiring less quality, such as bathing or irrigation.

CONCLUSIONS

The DEUF method was effective for FIO and pathogen concentration in high volumes of water with different physicochemical characteristics. The physicochemical factors determining the volume concentrated by DEUF were turbidity, conductivity and TOC. The concentration method reduced the FIO detection limit, increasing the logarithms of FIO removal in both DWTPs.

FIO and pathogen removal occurred at all the stages of the DWTP multibarrier systems but in both DWTPs the highest removal was achieved in the clarification stage. The increased detection of indicators after active carbon filtration showed this stage to be a critical point for water quality monitoring.

Concentration by DEUF represents an effective method for monitoring the quality of water for different uses and performing the quantitative microbial risk assessment required by water safety plans. Moreover, it allows for the identification of critical points during the water treatment processes and conditions that can compromise the microbial water quality.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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TABLES AND FIGURES

Table 1: Number of samples, mean and standard deviation of concentrated sample volumes; turbidity, conductivity and TOC at the different stages of DWTP A and DWTP B.

	Stage	n	Filtered volume (l)	Turbidity (NTU)	Conductivity (µS/cm)	TOC (mg/l)
DWTP A	Catchment	12	175.6 ± 27.2	36.6 ± 37.6	1246 ± 368	3.8 ± 0.8
	Clarification	12	244.0 ± 98.1	1.3 ± 0.5	1256 ± 365	3.0 ± 0.6
	Sand Filtration	12	504.0 ± 8.3	0.4 ± 0.2	1251 ± 367	2.9 ± 0.6
	Active carbon filtration	12	504.4 ± 8.0	0.3 ± 0.1	1250 ± 368	1.8 ± 0.4
	Chlorination	12	505.1 ± 6.8	0.4 ± 0.3	862 ± 394	1.4 ± 0.3
DWTP B	Catchment	9	503.3 ± 5.7	1.5 ± 0.6	416 ± 31	2.9 ± 0.5
	Clarification	9	506.1 ± 14.5	0.6 ± 0.2	448 ± 34	2.5 ± 0.5
	Active carbon filtration	9	464.6 ± 43.0	0.3 ± 0.2	450 ± 36	2.0 ± 0.4
	Chlorination	9	501.2 ± 14.3	0.3 ± 0.2	451 ± 25	1.9 ± 0.4

Table 2: Percentage of positive FIO results in direct and concentrated samples at the different stages of the DWTPs (n=12 in DWTP A; n=9 in DWTP B).

			TC	EC	IE	SSRC	SOMCPH	CB390PH	FRNAPH
DWTP A	Catchment	Direct	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		Concentrated	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	Clarification	Direct	83.3	83.3	83.3	91.7	66.7	66.7	33.3
		Concentrated	83.3	83.3	66.7	83.3	75.0	58.3	33.3
	Sand filtration	Direct	8.3	0.0	0.0	0.0	0.0	0.0	0.0
		Concentrated	25.0	25.0	0.0	0.0	0.0	0.0	0.0
	Active carbon filtration	Direct	0.0	0.0	8.3	0.0	0.0	0.0	0.0
		Concentrated	41.7	0.0	0.0	8.3	0.0	0.0	0.0
	Chlorination	Direct	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Concentrated	0.0	0.0	0.0	0.0	0.0	0.0	0.0
DWTP B	Catchment	Direct	100.0	100.0	88.9	100.0	22.2	22.2	0.0
		Concentrated	100.0	100.0	100.0	100.0	88.9	100.0	44.4
	Clarification	Direct	11.1	0.0	0.0	0.0	0.0	0.0	0.0
		Concentrated	11.1	0.0	0.0	0.0	0.0	0.0	0.0
	Active carbon filtration	Direct	11.1	0.0	0.0	0.0	0.0	0.0	0.0
		Concentrated	22.2	0.0	0.0	0.0	0.0	0.0	0.0
	Chlorination	Direct	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Concentrated	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 3: Percentage of positive pathogen results after the different stages of both DWTPs (n=12 in DWTP A; in DWTP B n=9 for EV, n=8 for *Cryptosporidium* and *Giardia*, n=7 for *Campylobacter*).

		Campylobacter	EV	*Cryptosporidium*	*Giardia*
DWTP A	Catchment	100.0	83.3	100.0	91.7
	Clarification	41.7	16.7	33.3	8.3
	Sand filtration	0.0	0.0	0.0	0.0
	Active carbon filtration	0.0	0.0	0.0	0.0
	Chlorination	0.0	0.0	8.3	0.0
DWTP B	Catchment	85.7	0.0	100.0	62.5
	Clarification	0.0	0.0	12.5	0.0
	Active carbon filtration	0.0	0.0	12.5	0.0
	Chlorination	0.0	0.0	0.0	0.0

Fig. 1: Percentage of FIO recoveries in a) DWTP A catchment and b) DWTP B catchment. (n=12 in DWTP A and n=9 in DWTP B).

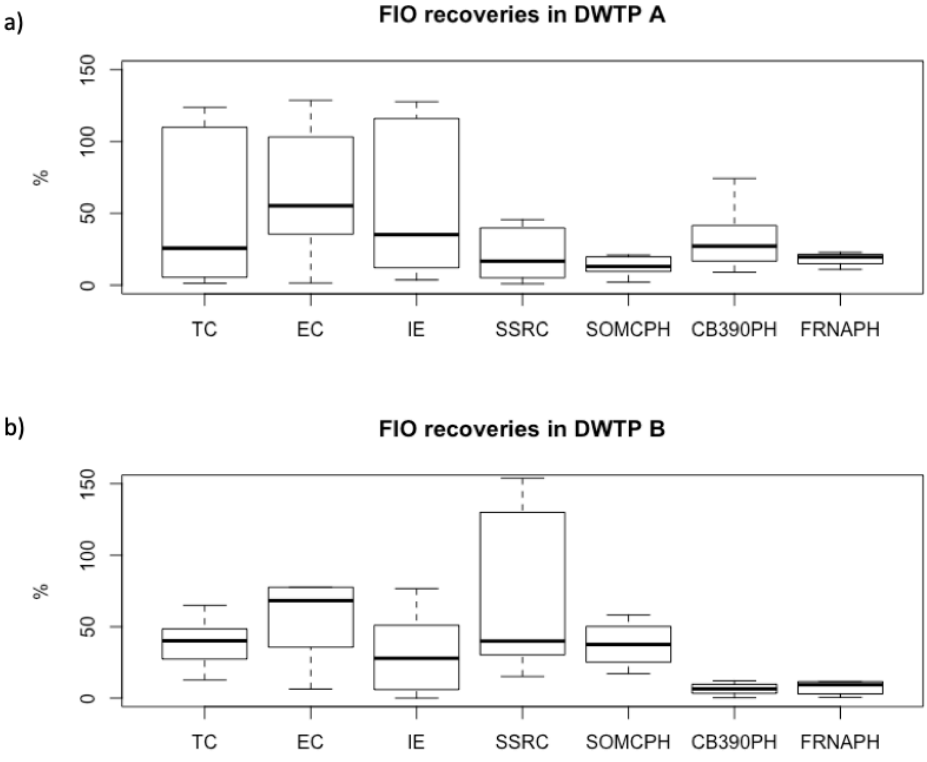


Fig. 2: Statistically significant Spearman's correlations (*P* <0.01) between FIO recoveries and the main physicochemical parameters.

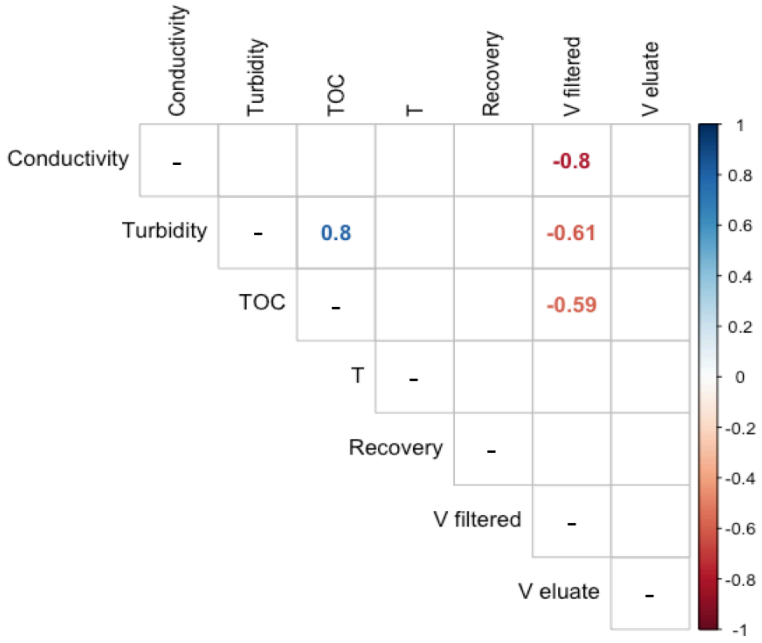
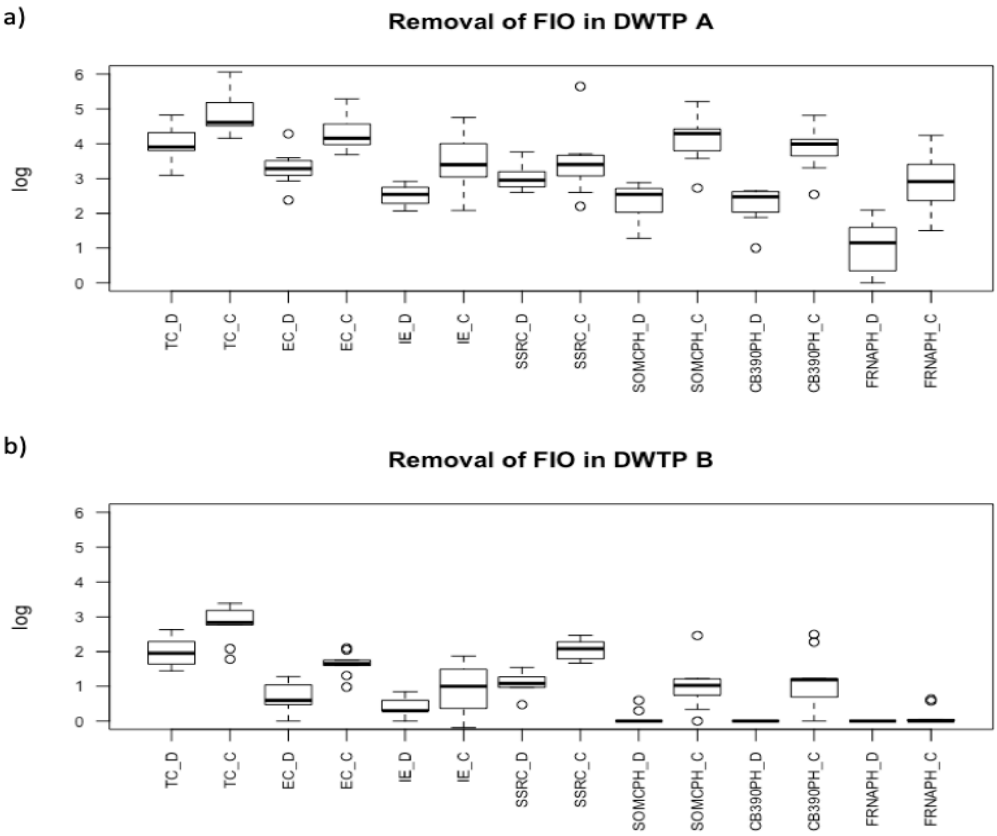


Fig. 3: Removal of faecal indicator organisms in direct samples (D) and concentrated samples (C) from the catchment to treated water in a) DWTP A and b) DWTP B.



APPENDIX A: Supplementary Data

Fig. A.1: Spearman's correlation of streamflow ((log (Q), in log(m^3/s)) and Turbidity (log (Turbidity), in log (NTU)) in the catchment of DWTP A.

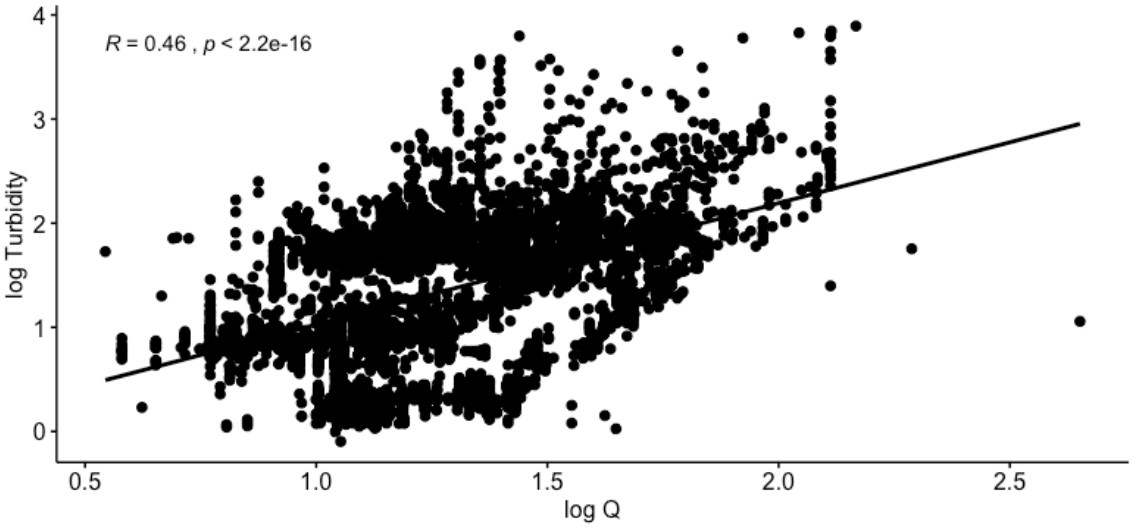
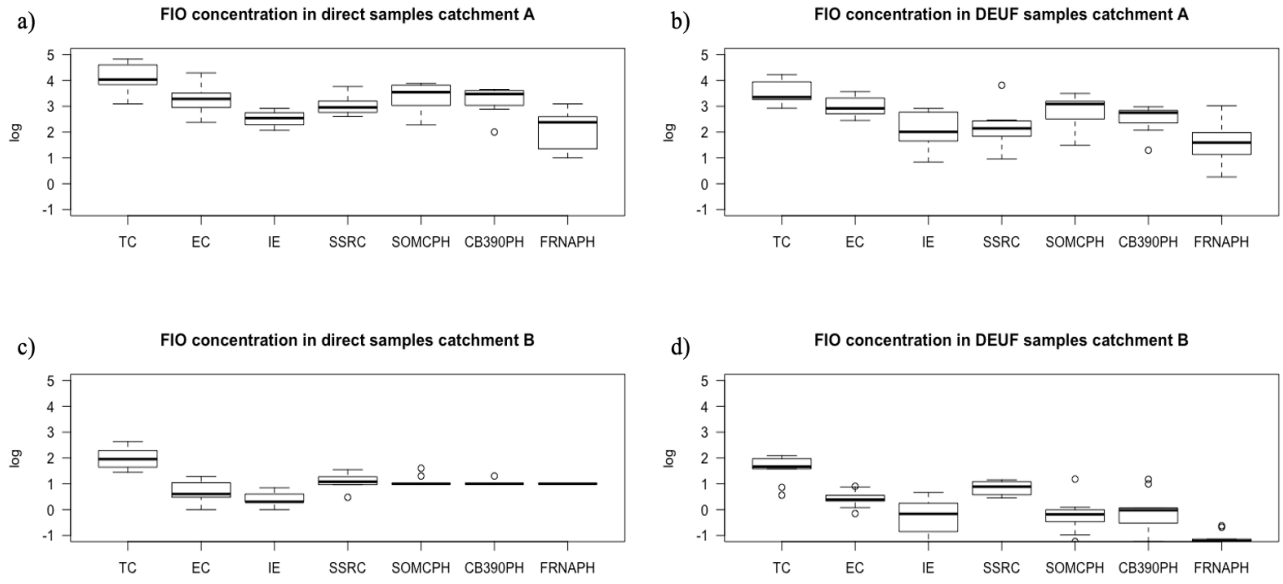


Fig. A.2: Concentration of faecal indicator organisms in a) direct samples from catchment A, b) concentrated samples from catchment A, c) direct samples from catchment B and d) concentrated samples from catchment B.



5. DISCUSIÓN GENERAL

Los recursos hídricos son indispensables para el desarrollo de la vida y de las actividades socioeconómicas del ser humano. Sin embargo, la disponibilidad y el acceso al agua y saneamiento es muy desigual en todo el planeta, ya sea por las características climáticas de la zona o por las capacidades económicas, que imposibilitan la construcción de infraestructuras que permiten el tratamiento del agua. La baja disponibilidad de recursos hídricos en la zona mediterránea y el descenso pronosticado en las próximas décadas, fruto del cambio climático, suponen un gran desafío para su gestión. La gestión actual de los recursos hídricos se fundamenta en dos pilares básicos: el aumento de los recursos existentes y el control de la calidad de los recursos disponibles. Esta investigación se ha centrado en el control de la calidad microbiológica del agua desde una visión integral de los recursos bajo las perspectivas de *One Health* y *Global Health*.

Los diferentes estudios realizados en esta tesis doctoral muestran la transversalidad con la que puede llevarse a cabo el control de la calidad microbiológica del agua. Para ello se han analizado aguas de orígenes muy diferentes (residual, residual tratada, distintos ríos, diferentes etapas de tratamientos de potabilización y aguas potables), además de sedimentos de río. En ellos se han estudiado microorganismos indicadores de contaminación fecal, patógenos, sus comunidades bacterianas, así como los procesos naturales de inactivación y tratamientos para la eliminación de los microorganismos. Varios de los estudios realizados estuvieron localizados en la riera de Cànoves. El tramo de estudio, donde se produce la entrada de contaminación fecal directa por el efluente secundario de una EDAR, está afectado por una marcada irregularidad hidrológica, típica de los ríos mediterráneos. La peculiaridad de la zona permitió estudiar las dinámicas de inactivación de diferentes microorganismos indicadores y patógenos, así como los cambios en la estructura y diversidad de las comunidades bacterianas a lo largo de un transecto longitudinal aguas abajo del vertido de la EDAR. La relativa simplicidad del ecosistema, sin otras grandes entradas de contaminación fecal ni extracciones de agua, se planteó como un buen punto de partida para el análisis del impacto de la contaminación fecal del efluente secundario sobre la calidad microbiológica del agua.

En los diferentes estudios se han empleado un amplio abanico de técnicas: métodos de cultivo para la detección y cuantificación de microorganismos, microscopía, técnicas moleculares como la qPCR y la secuenciación masiva. También se han utilizado, evaluado y desarrollado metodologías como MST, la modelización, la secuenciación masiva y la

concentración mediante ultrafiltración con el objetivo de implementarse como herramientas útiles en la gestión de la calidad microbiológica del agua.

La detección de la contaminación fecal es muy importante para la gestión de la calidad del agua. Además, la legislación actual está basada en el principio de quien contamina paga, razón por la que la determinación del origen de la contaminación es fundamental. Los marcadores de MST son muy diversos, pero como ya se ha visto en la introducción los requisitos de estos marcadores son numerosos y uno de los principales inconvenientes que presentan es que su eficacia varía en función de la zona geográfica de la que procedan las muestras. Por sus características, crAssphage podría ser a priori un marcador humano de MST útil. Asimismo, numerosos estudios y normativas avalan la utilización de los bacteriófagos como indicadores víricos, pero la heterogeneidad dentro de los diferentes grupos dificulta su análisis mediante técnicas moleculares (Muniesa et al., 1999; Ogorzaly and Gantzer, 2006), técnicas que sí son empleadas para la detección de crAssphage.

En esta tesis se ha evaluado la idoneidad de crAssphage como marcador de MST a través de la comparación con otros microorganismos indicadores de contaminación fecal generales (SOMCPH y *E. coli*) y otros marcadores de MST (HMBif, HF183 y GA17PH). Los resultados obtenidos del estudio realizado de crAssphage con diferentes matrices demostraron una elevada prevalencia de crAssphage en aguas de efluentes secundarios de diferentes EDAR. A pesar de haber estado sometidas al proceso de depuración, las aguas de efluentes secundarios presentaron concentraciones de alrededor de 5 log (CG/100ml), en torno a 3 unidades logarítmicas menos que las reportadas en estudios en aguas residuales de entrada de EDAR (García-Aljaro et al., 2017a; Stachler et al., 2017). En los efluentes secundarios también se observó que las concentraciones de crAssphage fueron similares a las de otros marcadores moleculares y superiores, en torno a 1 logaritmo, a las de otros indicadores analizados por método de cultivo como los colifagos somáticos y *E. coli*. Además, las concentraciones de crAssphage fueron bastante estables tanto entre los diferentes efluentes secundarios analizados como entre las muestras pertenecientes al mismo a lo largo del tiempo. Esta baja variabilidad en las concentraciones también es observada en agua residual de entrada en los estudios anteriores y en otros estudios de crAssphage llevados a cabo posteriormente. (Crank et al., 2019; Tandukar et al., 2020).

La estabilidad en las concentraciones de crAssphage se confirmó también en las muestras de los ríos. A pesar de ser dos ríos con diferentes niveles de contaminación se observó el

mismo patrón de abundancia relativa, siendo crAssphage el más abundante seguido de cerca por el otro marcador molecular analizado (HMBif) y con concentraciones en torno a 2 logaritmos más que los SOMCPH y *E. coli* y 3 logaritmos más que GA17PH. El hecho de que crAssphage se encuentre en concentraciones más elevadas que otros microorganismos indicadores permite un mejor seguimiento de la contaminación fecal en el medio ambiente en el tiempo y el espacio.

Numerosos estudios apuntan a los sedimentos de río como un reservorio de microorganismos que pueden ser movilizados. También es el caso de crAssphage, que presentó concentraciones más elevadas en los sedimentos del río Llobregat que en el agua del río. El sustrato del río podría conferir una protección contra los factores que provocan la inactivación de los microorganismos fecales en el medio ambiente (Brookes et al., 2004; Martín-Díaz et al., 2017). La movilización de los sedimentos, como consecuencia de un aumento repentino de caudal, puede aumentar la concentración de microorganismos indicadores de contaminación fecal y patógenos en el agua superficial (García-Aljaro et al., 2017b), poniendo así en peligro el funcionamiento de los tratamientos del agua.

La utilización de marcadores e indicadores de contaminación fecal requiere el conocimiento de su comportamiento en el medio ambiente. En el caso de crAssphage se observó una inactivación natural aguas abajo de la EDAR en la riera de Cànoves que puede ser atribuida a diferentes factores bióticos y abióticos, pero en el que la temperatura podría jugar un papel importante. Esto es lo que se deduce también de los resultados observados de la persistencia de crAssphage en mesocosmos. CrAssphage mostró una persistencia superior a la de los marcadores moleculares bacterianos (HMBif y HF183). Sin embargo, su persistencia resultó muy similar a la de los SOMCPH a baja temperatura mientras que en verano fue inferior a la de los SOMCPH, pero superior a la de los indicadores bacterianos analizados (*E. coli*, HMBif y HF183). Este hecho hace de crAssphage un marcador humano de MST semiconservativo, porque su persistencia, elevada en invierno, se ve alterada por los factores que se acentúan en verano. Factores abióticos como la temperatura y la radiación solar aceleran la inactivación de los microorganismos alóctonos del agua, pero también se ven incrementados los procesos bióticos como la depredación por parte de otros organismos (Blaustein et al., 2013; Wanjugi and Harwood, 2013).

El estudio de la dinámica de inactivación de cinco microorganismos indicadores (*E. coli*, SSRC, SOMCPH, GA17PH y HMBif) en la riera de Cànoves fue el punto de partida para poder

realizar la modelización del impacto de la EDAR en la calidad microbiológica del agua del río. Los resultados mostraron una inactivación diferencial en los microorganismos tanto en el espacio (aguas abajo de la EDAR) como en el tiempo (a lo largo de las diferentes estaciones). Se confirmó el carácter no conservativo de *E. coli*, el semiconservativo de los bacteriófagos (SOMCPH y GA17PH) y el indicador molecular de MST (HMBif) y el carácter no conservativo de SSRC (Agulló-Barceló et al., 2013; Davies et al., 1995; Sinton et al., 1999). Es decir, los indicadores presentaron una tendencia a la inactivación, pero a un ritmo diferente en función de las características del microorganismo. La inactivación de los microorganismos en la riera de Cànoves permitió la cuantificación de la distancia de autodepuración (SDD), una medida de la capacidad del río para recuperar las concentraciones previas al vertido de la EDAR. La distancia ya ha sido utilizada en otros estudios de modelización y su principal ventaja es que permite la integración de diferentes parámetros (Fauvel et al., 2017; Jonsson and Agerberg, 2015). En este estudio, las SDD obtenidas se explicaron principalmente por dos factores: el caudal y la temperatura. En la temporada seca, principalmente el verano, el caudal bajo o nulo antes de la entrada del efluente de la EDAR y la elevada temperatura aumentaron la inactivación de los microorganismos indicadores de contaminación fecal. En cambio, en la temporada húmeda, con un mayor caudal y una menor temperatura, la temperatura adquirió más relevancia para explicar el comportamiento de los microorganismos, que presentaron una mayor SDD. Las SDD máximas modelizadas teniendo en cuenta todos los microorganismos fueron de 3 km en la temporada seca y 15 km en la temporada húmeda, proporcionando información para el uso del agua aguas abajo.

En las muestras de la riera de Cànoves se aplicaron diversas técnicas como la siembra y recuento en placa, la microscopía o las técnicas de secuenciación masiva con el fin de valorar el impacto de la EDAR en la estructura y diversidad de las comunidades bacterianas. El estudio demostró que el efluente de la EDAR tiene un impacto sobre las comunidades bacterianas autóctonas del río, no sólo modificando las abundancias de los diferentes taxones sino también reduciendo la diversidad alfa del río (Drury et al., 2013; Mansfeldt et al., 2019). Sin embargo, en 1 km aguas abajo, las comunidades bacterianas ya no mostraron diferencias significativas con las comunidades autóctonas del río, confirmando su recuperación del impacto producido por la entrada de la contaminación fecal del efluente. La recuperación del impacto también se observó en las concentraciones de microorganismos indicadores de contaminación fecal y el patógeno estudiados, volviendo en 1 km a concentraciones muy

similares a las propias de la riera antes de la entrada del efluente de la EDAR. Asimismo, la diversidad alfa, fuertemente disminuida por la entrada del efluente, también se vio recuperada aguas abajo.

El efecto de la estacionalidad sobre las comunidades bacterianas se hace patente mediante la obtención de una correlación negativa estadísticamente significativa entre la riqueza de especies (diversidad alfa) y la temperatura del río, observando los niveles más bajos de diversidad alfa en las muestras de verano. Estos resultados, en consonancia con lo reportado por otros autores (Kent et al., 2004; Rubin and Leff, 2007), contrastan con el estudio de modelización donde se apunta a un incremento de la capacidad de autodepuración del río en verano. Estos resultados adquieren relevancia en un contexto de cambio global con unas predicciones de aumento de temperatura y disminución de caudal en los ríos mediterráneos, ya que por un lado se podría aumentar la capacidad de autodepuración de los ríos, pero por otro lado se podría ver reducida la riqueza de especies y, por tanto, alterar las funciones que llevan a cabo en los ecosistemas acuáticos (Comte and Del Giorgio, 2010). Este hecho supone que la sensibilidad, resistencia y resiliencia de las comunidades bacterianas frente a los impactos son claves para el funcionamiento de los ecosistemas acuáticos (Lindh and Pinhassi, 2018).

La gestión de la calidad microbiológica del agua y el control del impacto de la contaminación fecal sobre los recursos hídricos debería llevarse a cabo no sólo mediante el análisis de microorganismos indicadores de contaminación fecal y patógenos, sino también el de las comunidades propias del agua. Por otro lado, el uso de herramientas como la modelización permite anticipar las afectaciones en la calidad microbiológica de los recursos hídricos en función de los parámetros modelizados. En esta tesis doctoral las variables empleadas para la modelización (caudal y temperatura) están muy relacionadas con el cambio climático. Así pues, en un contexto de cambio global, el disponer de modelos que permitan integrar la información y teorizar sobre los efectos en los recursos puede ser de gran ayuda en la toma de decisiones para la gestión de éstos.

El conocimiento generado podría ayudar a potenciar la autodepuración natural de los ríos, lo que supondría una ayuda en la gestión que podría conllevar incluso la reducción de los costes del tratamiento del agua. Este hecho adquiere especial relevancia en zonas como la mediterránea donde un porcentaje muy elevado de su población se abastece con agua superficial, principalmente de ríos. El control de la calidad microbiológica del agua

tradicionalmente se ha llevado a cabo mediante el análisis de microorganismos indicadores de contaminación fecal y patógenos. A menudo el análisis de patógenos de referencia requiere la concentración de grandes volúmenes de agua, razón por la cual en los últimos años se han intentado desarrollar protocolos unitarios que permitan concentrar los diferentes patógenos bacterianos, víricos y protozoarios de forma simultánea. La ultrafiltración, y más concretamente el método DEUF se ha mostrado como el método más eficaz para esta tarea, pero su eficacia sólo se ha testado en muestras de agua potable, aguas regeneradas o muestras de agua dopadas en el laboratorio con concentraciones conocidas de microorganismos indicadores y patógenos. En este contexto, el estudio del método DEUF llevado a cabo en esta tesis supone un avance en el conocimiento de esta metodología que puede presentar grandes ventajas para el control de la calidad microbiológica del agua desde la captación hasta la salida del agua tratada, pasando por todas las etapas del tratamiento.

En el estudio se analizó la eficacia del método DEUF en la concentración de grandes volúmenes de muestras procedentes de la captación de dos ETAP que tratan aguas de características fisicoquímicas y microbiológicas muy diferentes. Los porcentajes de recuperación de los microorganismos indicadores analizados fueron muy variables. Sin embargo, la recuperación media del 43.8 % resultó muy próxima a los datos reportados en los estudios previos, mostrando también una elevada variabilidad (Bosch et al., 2016; Liu et al., 2012; USEPA and CDC, 2011). No obstante, el hecho de trabajar con muestras ambientales reales donde los microorganismos pueden estar agregados o adheridos a partículas y que el agua puede cambiar sus características a lo largo del proceso de concentración, pueden acentuar esta variabilidad en la recuperación de los microorganismos indicadores y patógenos. Los estudios donde se comparan los diferentes métodos de concentración muestran también variabilidad en la recuperación de los diferentes tipos de microorganismos. Algunos métodos pueden presentar mayores porcentajes de recuperación para un tipo de microorganismo en concreto pero es la ultrafiltración la que muestra los mejores porcentajes de recuperación para bacterias, virus y protozoos en su conjunto (Bridle, 2014; Francy et al., 2013). Esto es debido a que la ultrafiltración, a diferencia de métodos como la adsorción-elución, retiene en función de la medida de partícula y no de propiedades fisicoquímicas como la carga, por lo que quedan retenidos los diferentes tipos de microorganismos. Además, la detección de algunos microorganismos requiere una concentración secundaria, por lo que su recuperación también se puede ver afectada por la eficiencia de ésta. Otra de las principales

ventajas que presenta el método DEUF respecto a los otros métodos de concentración es que puede llevarse a cabo in situ, lo que facilita a nivel logístico la concentración de mayores volúmenes.

El método DEUF también demostró su eficacia en el seguimiento de los procesos de tratamiento del agua. Al permitir la concentración de grandes volúmenes de agua, se pudo garantizar una mayor reducción de los microorganismos indicadores desde la entrada a la salida de las plantas potabilizadoras, donde no pudieron ser detectados. El método DEUF posibilita un mejor seguimiento de los indicadores y patógenos en todos los puntos de las etapas de tratamiento. Este hecho presenta dos principales ventajas: por un lado, aumenta el conocimiento de las relaciones entre las concentraciones de los microorganismos indicadores y los patógenos de referencia y, por otro lado, permite una mejor identificación de los puntos críticos del proceso de tratamiento del agua. Los resultados observados en ambas ETAP muestran los filtros de carbón activo como un punto crítico donde la formación de biofilms y el recrecimiento de bacterias pueden suponer un problema y a los que hay que prestar debida atención (Clode et al., 2015; Gibert et al., 2013). En todo el estudio sólo hubo detección de 1 ooquiste de *Cryptosporidium* spp en las muestras tratadas y fue en la ETAP A, aunque no se dispone de información sobre su viabilidad que es lo que determinaría el riesgo de consumo de agua. El hecho de que el único positivo en el agua tratada fuera tras un evento de fuertes lluvias, constata también el especial seguimiento que hay que establecer durante estos episodios y los días posteriores, ya que puede verse alterada la eficacia de las etapas del tratamiento. Las predicciones de cambio climático en la zona mediterránea prevén un aumento de este tipo de episodios (IPCC, 2013), por lo que el seguimiento del funcionamiento de los tratamientos del agua en estas condiciones adquiere una especial relevancia a la hora de reducir los riesgos que pueden suponer para la salud humana.

En la actualidad se está realizando el QMRA de los resultados obtenidos del estudio, poniendo de manifiesto la idoneidad del método DEUF para la evaluación de riesgo microbiológico en la potabilización del agua que requieren los planes sanitarios del agua. Además, por sus características el método también puede ser empleado para el análisis del riesgo microbiológico en otros usos del agua como el regadío o las aguas de baño, ayudando en la gestión de estos recursos.

En resumen, a lo largo de esta tesis doctoral se ha generado información sobre diferentes parámetros relacionados directa o indirectamente con la contaminación fecal a lo largo del

ciclo del agua, incluyendo microorganismos indicadores de contaminación fecal, patógenos, parámetros para determinar el origen de la contaminación fecal y su comportamiento, así como también metodologías para ayudar a la monitorización de la calidad microbiológica del agua y a la gestión de los recursos hídricos. La información generada adquiere una especial relevancia en el contexto actual, de disminución de recursos y aumento de demanda hídrica, y ante el desafío de una gestión integral de los recursos basada en los principios de *One Health* y *Global Health*, porque de esta manera será más sencillo controlar todos los factores que puedan poner en riesgo la salud humana. La información obtenida puede ser útil para los gestores del agua implicados principalmente en los procesos de depuración y potabilización del agua. Asimismo, los resultados obtenidos pueden ser de ayuda para los organismos responsables de las guías y normativas sobre la calidad microbiológica del agua.

6. CONCLUSIONES

CONCLUSIONES

Las conclusiones extraídas de los diferentes estudios realizados en esta tesis doctoral son:

- CrAssphage está presente en grandes concentraciones en el agua residual tratada que es vertida al medio ambiente y en aguas contaminadas fecalmente.
- El comportamiento de crAssphage en el medio ambiente es similar al de otros microorganismos indicadores de contaminación fecal. Su persistencia como indicador semiconservativo y sus concentraciones superiores a las de los bacteriófagos analizados por métodos de cultivo hacen de crAssphage un marcador humano de MST útil para ser empleado junto con otros marcadores.
- Los sedimentos son un reservorio de microorganismos indicadores, incluido crAssphage y de patógenos que pueden entrar en el ciclo del agua poniendo en riesgo la salud humana.
- El efluente de la EDAR es una fuente de contaminación fecal que incrementa significativamente las concentraciones de todos los microorganismos indicadores de contaminación en la riera de Cànoves. También altera significativamente la estructura de las comunidades bacterianas y la diversidad microbiana del río.
- Los microorganismos indicadores se inactivan aguas abajo de la EDAR en función de sus características intrínsecas, aunque la autodepuración natural del río está condicionada por factores ambientales como son el caudal y la temperatura. La distancia de autodepuración disminuye con el descenso de caudal y el aumento de temperatura, condiciones que mejoran la inactivación de los microorganismos alóctonos.
- A la vez que el aumento de temperatura da lugar a una mayor inactivación de los microorganismos indicadores de contaminación fecal, también se observa una disminución de la diversidad alfa o riqueza de especies bacterianas en el ecosistema acuático.
- Las comunidades bacterianas muestran una elevada resiliencia frente al impacto de la EDAR, recuperando su estructura y la riqueza de especies en tan sólo 1 km aguas abajo de la EDAR. No obstante, los factores ambientales también pueden modificar la estructura de las comunidades bacterianas.
- El método DEUF es un método efectivo para la concentración de bacterias, virus y protozoos en grandes volúmenes de agua superficial con diferentes características fisicoquímicas.

- El método DEUF permite hacer un mejor seguimiento del tratamiento de potabilización del agua, asegurando una mayor reducción de microorganismos indicadores y patógenos desde su captación hasta la salida del agua tratada.
- El método DEUF puede implementarse para realizar el análisis de riesgo en todas las etapas del tratamiento de potabilización del agua como requieren los planes sanitarios del agua.
- Las predicciones de cambio climático en la zona mediterránea como el aumento de temperatura y de los eventos de lluvias intensas pueden alterar las concentraciones y dinámicas naturales de los microorganismos indicadores de contaminación fecal, patógenos y comunidades bacterianas autóctonas de los ecosistemas acuáticos. Asimismo, también pueden poner en riesgo la eficacia de los tratamientos del agua.
- La gestión de la calidad microbiológica del agua se puede llevar a cabo mediante el análisis de microorganismos indicadores de contaminación fecal y patógenos. Sin embargo, herramientas como la modelización de éstos y el conocimiento de la afectación sobre las comunidades bacterianas pueden ayudar a escoger estrategias que faciliten y mejoren la gestión integral de los ecosistemas acuáticos que tengan como fundamento *One Health* y *Global Health.*

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8. ANEXOS

8.1 ANEXO 1: Otras publicaciones

M. Pascual Benito, C. García-Aljaro, S. Casanovas-Massana, A.R. Blanch, F. Lucena. Effect of hygienization treatment on the recovery and/or regrowth of microbial indicators in sewage sludge. Journal of Applied Microbiology (2015), Vol. 118 (2); 412-418; (DOI: 10.1111/jam.12708).

Julia Martín-Díaz, Cristina García-Aljaro, **Míriam Pascual-Benito**, Belén Galofré, Anicet R. Blanch, Francisco Lucena. Microcosms for evaluating microbial indicator persistence and mobilization in fluvial sediments during rainfall events (2017), Vol. 123; 623-631; (DOI: 10.1016/j.watres.2017.07.017).

M. Muniesa, E. Ballesté, L. Imamovic, **M. Pascual-Benito**, D. Toribio-Avedillo, F. Lucena, A.R. Blanch, J. Jofre. Bluephage: A rapid method for the detection of somatic coliphages used as indicators of fecal pollution in water (2018), Vol. 128; 10-19; (DOI: 10.1016/j.watres.2017.10.030).

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ORIGINAL ARTICLE

Effect of hygienization treatment on the recovery and/or regrowth of microbial indicators in sewage sludge

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Keywords
anaerobic digestion, *Escherichia coli*, indicators, pasteurization, regrowth, *Salmonella* spp., sewage sludge, thermophilic, viable-but-not-culturable.

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Abstract

Aims: *Escherichia coli* (EC) is the primary indicator micro-organism in regulations for sewage sludge reuse. The aim of this work was to assess the ability of EC to enter and recover from a viable-but-not-culturable state (VBNC) after sludge hygienization treatments.

Methods and Results: The persistence of EC, somatic coliphages (SOMCPH), spores of sulphite-reducing clostridia (SRC) and *Salmonella* spp. was assessed in digested sludge after different pasteurization treatments and storage conditions. Pasteurization at 55°C produced EC-injured cells that were resuscitated during the first 24 h. Different sludge treatments altered the inactivation kinetics of EC, while SOMCPH and SRC did not resuscitate and showed lower die-off than EC. No regrowth was observed in stored sludge for up to 60 days.

Conclusions: EC monitoring by culturable methods is not by itself a suitable method for assessing the hygienization achieved in sludge as EC can enter into VBNC from which it can recover during the first hours of storage.

Significance and Impact of the Study: The regulations should indicate the time when monitoring of EC should be performed to avoid the period when EC can resuscitate from VBNC or add alternative microbial indicators, such as SOMCPH, which do not have a VBNC state.

Introduction

In the search for sustainable resources, the reuse of sewage sludge after anaerobic digestion, which is a by-product of wastewater treatment, has been applied in different areas. Amongst others, sewage sludge can be applied to agricultural lands as fertilizers or used to restore abandoned or degraded soils because of its high nutrient content (Wallace *et al.* 2009). Conventional wastewater treatment processes are designed to reduce the number of pathogenic micro-organisms in water, but, at the same time, they tend to concentrate the micro-organisms that are already present in the raw wastewater of the resulting biosolids. Amongst others, the presence of enteric viruses, protozoa or pathogenic bacteria, such as *Campylobacter*, *Salmonella* or *Shigella*, and other emerging pathogens, such as *E. coli* O157:H7 or *Helicobacter pylori*, are of concern in the reuse of biosolids (Sidhu and Toze 2009). Therefore, the search for microbial indicators in finished sewage sludge is of great importance to minimize the exposure of humans or animals to these pathogenic micro-organisms and protect the environment. However, the behaviour of different pathogenic and traditional microbial indicators for biosolids have not been studied in detail due to methodology limitations or because different studies cannot be compared due to lack of standardized techniques.

In spite of these limitations, different countries have adopted different regulations for the disposal of biosolids for agricultural applications. Traditional microbial indicators, such as *Escherichia coli* (EC), have been included as indicator micro-organisms in regulations that have been adopted by many countries. In the United States, biosolids are divided into two classes, Class A and Class B, depending on the levels of pathogenic and indicator micro-organism of the sludge. The land application of certain

types of biosolids requires a Class B designation, and this designation can be met by three different criteria, one of which includes monitoring of faecal coliforms, whose density cannot surpass 2×10^6 MPN per gram (CFR 2002). In Europe, the use of sludge for agriculture is subject to the Directive 86/278/EEC regulations, which has no limits related to pathogens, although European countries are allowed to include their own parameters in their national regulations. For instance, in Spain, the regulation regarding fertilizing products to be used in agriculture cannot exceed 1000 MPN g^{-1} for EC, and *Salmonella* must be absent in 25 g of the product (Anonymous 2005). In spite of the differences in these regulations, it is important to highlight that, although sludge can meet these criteria after digestion processes when being measured in a wastewater treatment plant, these values should remain below this limit during the storage and transportation of these biosolids to their final destination. In this respect, the suitability of certain indicator micro-organisms of faecal contamination, such as EC, is controversial because they have been shown to grow after the anaerobic digestion process and during sludge storage under certain conditions (Monteleone *et al.* 2004; Qi *et al.* 2007; Higgins *et al.* 2007). Some investigators have explained that this regrowth occurs during storage for long periods of time in samples from thermophilic and mesophilic-digestion processes that use centrifuge dewatering (Chen *et al.* 2011). Others have added that coliform levels increase at 25 and 37°C, continue for up to 5 days, and then gradually decline (Qi *et al.* 2007). However, some reports point to an improvement in detection by the reactivation of non-culturable organisms because, during storage, conditions allow for their recovery and them again becoming culturable (Higgins *et al.* 2007). On the other hand, pathogenic bacteria, such as *Salmonella* spp., which are closely related to EC, have also been reported to grow in Class B biosolids (Higgins *et al.* 2008). The inactivation of human pathogens and their respective indicator micro-organisms have recently been reviewed (Sidhu and Toze 2009).

The aim of this study was to monitor EC regrowth and/or recovery of EC-injured cells from a viable-but-not-culturable state after sludge hygienization treatments. In addition, a range of microbial indicators, including EC, somatic coliphages (SOMCPH) and spores of sulphite-reducing *Clostridium* (SRC), and their relation with the presence of pathogenic *Salmonella* spp. were monitored in fresh, thermophilic-digested sludge and dewatered, mesophilic- and thermophilic-digested sludges that were collected from four different wastewater treatment plants, after different inactivation treatments and under different storage conditions.

Materials and methods

Sample collection

Sludge samples after thermophilic and mesophilic anaerobic digestion treatment followed by dewatering and fresh, thermophilic-digested samples were collected from four different waste water treatment plants and transported to the laboratory for further analysis. The thermophilic treatment included aerobic digestion of sludge at 55°C for 20–25 h, while mesophilic treatment consisted in anaerobic digestion at 35°C for 20–21 days. The samples were then subjected to different pasteurization treatments and storage conditions as indicated below, and the traditional faecal indicators enumerated.

Enumeration of bacterial indicators and *Salmonella* spp. in sewage sludge

Sludge samples were mixed at a 1 : 10 ratio in phosphate-buffered saline (pH 7·0) and homogenated by shaking for 30 min at room temperature before analysis. Culturable *Escherichia coli* (cEC) were enumerated using the pour plate method in Chromocult agar (Astals *et al.* 2012) and incubation at 37°C for 24 h. SOMCPH were extracted from sludge as described previously (Guzmán *et al.* 2007) and counted using the double layer agar method (Anonymous 2000). Spores of SRC were enumerated using mass inoculation in SPS agar and incubated for 24 h at 44°C, as previously described (Astals *et al.* 2012). *Salmonella* was detected using presence/absence techniques on 50, 5 and 0·5 g of sludge following the ISO 6579:2002/A1:2007 with slight modifications, as described previously (Guzmán *et al.* 2007). Briefly, the samples were mixed in a 1 : 10 (w/v) ratio in Buffered Peptone Water (BPW) pH 7 and incubated at 37°C for 24 h. The pre-enrichment BPW (0·1 ml) was inoculated in 10 ml of Rappaport-Vassiliadis *Salmonella* enrichment broth and incubated at 42°C for 24 h. Then, it was inoculated in SMS® agar and incubated at 42°C for 24 h. At this point, it was possible to determine the absence of *Salmonella* spp. The presence of this species was confirmed by seeding in Hektoen Agar (incubated at 37°C for 24 h) and then in TSA (incubated at 37°C for 24 h). Finally, the presence of *Salmonella* was confirmed using the MUCAP, oxidase and API-20E test kits.

Enumeration of the *Escherichia coli* 16S rRNA gene using quantitative PCR

The EC 16S rRNA gene was enumerated by qPCR as an indirect measure of total EC genomes using a TaqMan® assay with the probe and primers previously described

(Huijsdens *et al.* 2002) and the TaqMan® Environmental Master Mix 2·0 (Applied Biosystems, Barcelona, Spain) to minimize the negative effect of potential inhibitors in the sludge samples. DNA was extracted from the sludge samples (0·25 g) using the Power soil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. A fragment of the EC 16S rRNA gene was cloned into the pGEM-T Easy vector, purified and serially diluted in PCR-grade water. Different dilutions of the plasmid were subjected to qPCR to construct standard curves. The $\log_{10}$GC (gene copies) per gram for each sample was calculated using the threshold cycle (*C*t) values (mean of 3 replicates) using the following equation derived from the standard curves:

$$\log_{10}\text{GC} = -0{\cdot}2973(Ct) + 12{\cdot}168$$

with a limit of detection of 2·35 GC per PCR tube (equivalent to 1·34E2 GC g^{-1}).

The absence of PCR-inhibitors in the samples was assessed by diluting the samples at a 1 : 10 ratio in PCR-grade water.

Inactivation kinetics of bacterial indicators in fresh sludge after sewage sludge pasteurization

To study the inactivation kinetics and/or regrowth of EC in fresh sewage sludge, samples from fresh, thermophilic-digested sludge were pasteurized at 55°C and 80°C for 2, 3 and 4 h and incubated at 37°C for 8 days. The microbial indicators were enumerated as described above at different intervals.

Persistence of bacterial indicators in finished sludge at different storage temperatures

To study the possibility of regrowth of the finished sludge during storage, samples from thermophilic and mesophilic digested-dewatered sludge were stored at different temperatures, 22 and 37°C, for 60 and 20 days, respectively, and the microbial indicators and *Salmonella* were enumerated and detected, respectively, at different time intervals.

Results

Inactivation kinetics of bacterial indicators in fresh sludge after sewage sludge pasteurization

A series of experiments were conducted to study the inactivation kinetics and/or regrowth of EC in fresh sewage sludge after thermophilic digestion treatment. The effect of pasteurization on EC recovery was studied by monitoring the EC 16S rRNA gene and cEC at different intervals at two temperatures, 55 and 80°C. As shown in Fig. 1, the cEC were inactivated significantly slower at 55°C than at 80°C, with a T90 of approx. 100 min and 1 min at 55 and 80°C, respectively. The T90 of the EC 16S rRNA gene was higher than 240 min and could not be calculated. In view of these results, the subsequent experiments were performed at 55°C.

The effect of sewage sludge pasteurization at 55°C on EC regrowth and recovery from an injured state was assessed in fresh sludge samples from thermophilic digestion treatment by counting the cEC by standard methods and the EC 16S rRNA gene by qPCR. The duration of the pasteurization treatment had a significant effect on the recovery of cEC (Fig. 2). A maximum increase in cEC of approximately threefold was observed in the sample that was subjected to pasteurization for 3 h after around 20 h of treatment (Fig. 2c). This increase could be attributed to regrowth of EC in the sample after the treatment. However, this increase was not reflected in the enumeration of the EC 16S rRNA gene using qPCR, suggesting that, in addition to a slight regrowth, a resuscitation or recovery of injured EC had occurred after incubation of the samples at 37°C for 20 h. In fact, only

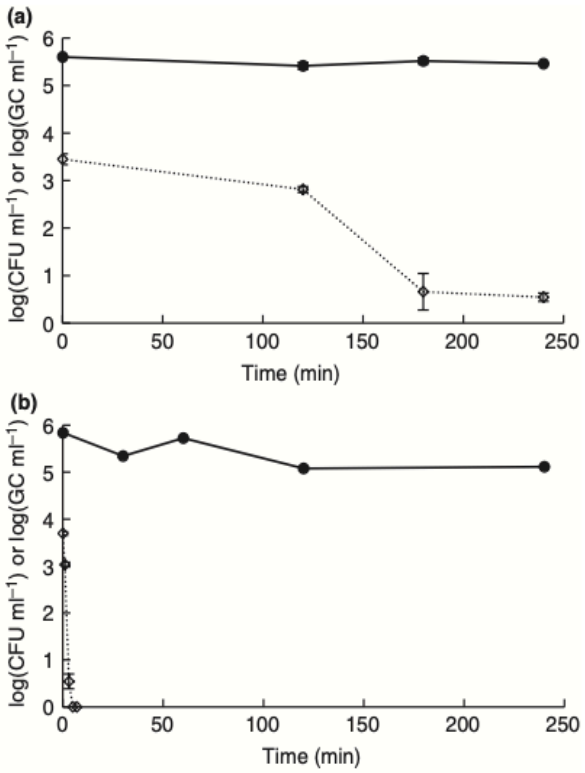

Figure 1 Inactivation of EC (EC 16S rRNA gene and cEC) after pasteurization at different temperatures: (a) 55°C; (b) 80°C; ● EC 16S rRNA gene; ◇ cEC.

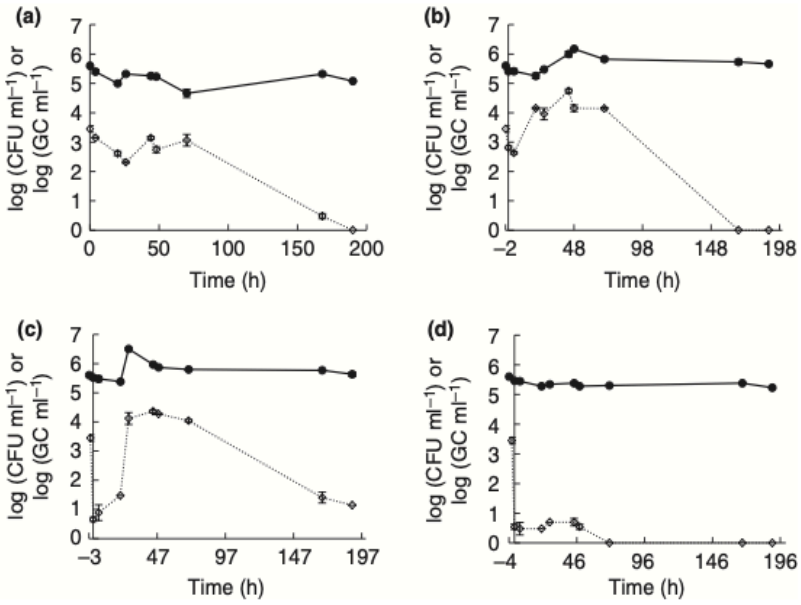

Figure 2 EC regrowth (EC 16S rRNA gene and cEC) in fresh sewage sludge from thermophilic digestion treatment after pasteurization at 55°C for different time periods: (a) no treatment; (b) 2 h; (c) 3 h; (d) 4 h; ● EC 16S rRNA gene; ◇ cEC. The duration of the treatment is reflected in the *x* axis of figures starting in 0, −2, −3, and −4, respectively.

an increase in EC of approximately onefold with respect to the initial sample was observed. In view of these results, the following experiments were performed under these experimental conditions. It must be noted that the ratio between cEC and the EC 16S rRNA gene was highly variable throughout the experiment and ranged from 1:10^2 to 1:10^6.

The inactivation kinetics of the other bacterial indicators are shown in Fig. 3. Of the different bacterial indicators, SRC were the most resistant to the treatment and showed no significant variations throughout the experiment. A decrease in SOMCPH of approximately onefold was observed after pasteurization, and they were subsequently slowly inactivated at a T90 of >197 h. Consequently, the ratios of SRC, SOMCPH and cEC were highly variable, depending on the incubation time after pasteurization, which has several implications that will be discussed below. Moreover, the recovery of cEC during the first 20 h after treatment showed significant variations in the three independent samples, as shown in Fig. 3, although the initial number of cEC was approx. 10^4 UFC g^{-1} in all the samples. This finding indicated high variability in the injured state of cEC after the digestion treatment.

Persistence of bacterial indicators in finished sludge at different storage temperatures

The regrowth of bacterial indicators and *Salmonella* spp. during sludge storage was monitored at 22 and 37°C for up to 60 and 20 days, respectively, in digested-dewatered sludge samples from thermophilic- and mesophilic-digestion treatments. The storage temperatures were chosen to simulate the average and maximum storage temperatures of sludge that can be achieved in a Mediterranean country. As shown in Fig. 4, the initial concentrations of all analysed bacterial indicators were higher in the samples originated from a mesophilic-digestion treatment, which was expected due to the harsh conditions that were used in the thermophilic treatment. No significant regrowth was observed for any of the analysed indicators throughout the analysed period in any of the sludge samples stored at 22 or 37°C. This trend was observed in all of the analysed sludge samples from the different sewage treatment plants (data not shown). On the other hand, *Salmonella* spp. were detected in all of the samples and presented cEC counts that were higher than 10^2 CFU ml^{-1} and decreased during the studied period.

The persistence of SRC was the highest amongst the analysed bacterial indicators in the solid sludge samples and did not significantly decrease after storage at 22°C (Fig. 4a,c) or 37°C (Fig. 4b,d). Of the analysed bacterial indicators, cEC showed the highest inactivation rate of T90 > 20 days and T90 ~2·5 days when the sludge was stored at 22 and 37°C. The inactivation kinetics of SOMCPH was higher than that of SRC, but was lower than that of cEC, with a T90 of >60 days and T90 of >10 days when stored at 22 and 37°C, respectively. No significant differences in the inactivation rates were observed for the any of the analysed bacterial indicators in the sludge samples from the thermophilic- or mesophilic-digestion processes.

Discussion

Several investigators have recently reported higher densities of indicator bacteria after dewatering anaerobically

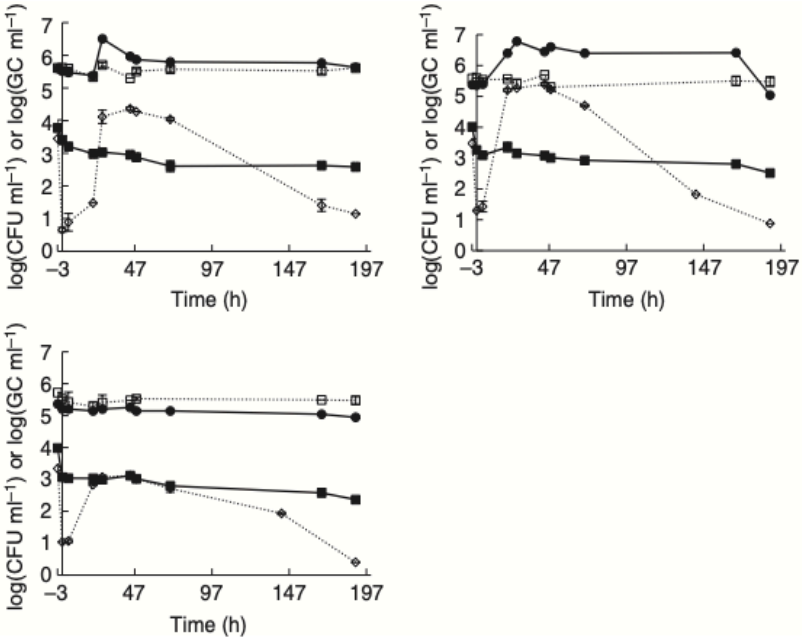

Figure 3 Inactivation of microbial indicators in sewage sludge from thermophilic digestion in three independent samples after pasteurization at 55°C for 3 h. The results show high variability in the recovery of cEC during the first 20 h; ● EC 16S rRNA gene; ◇ cEC; ■ SOMCPH; □ SRC.

□

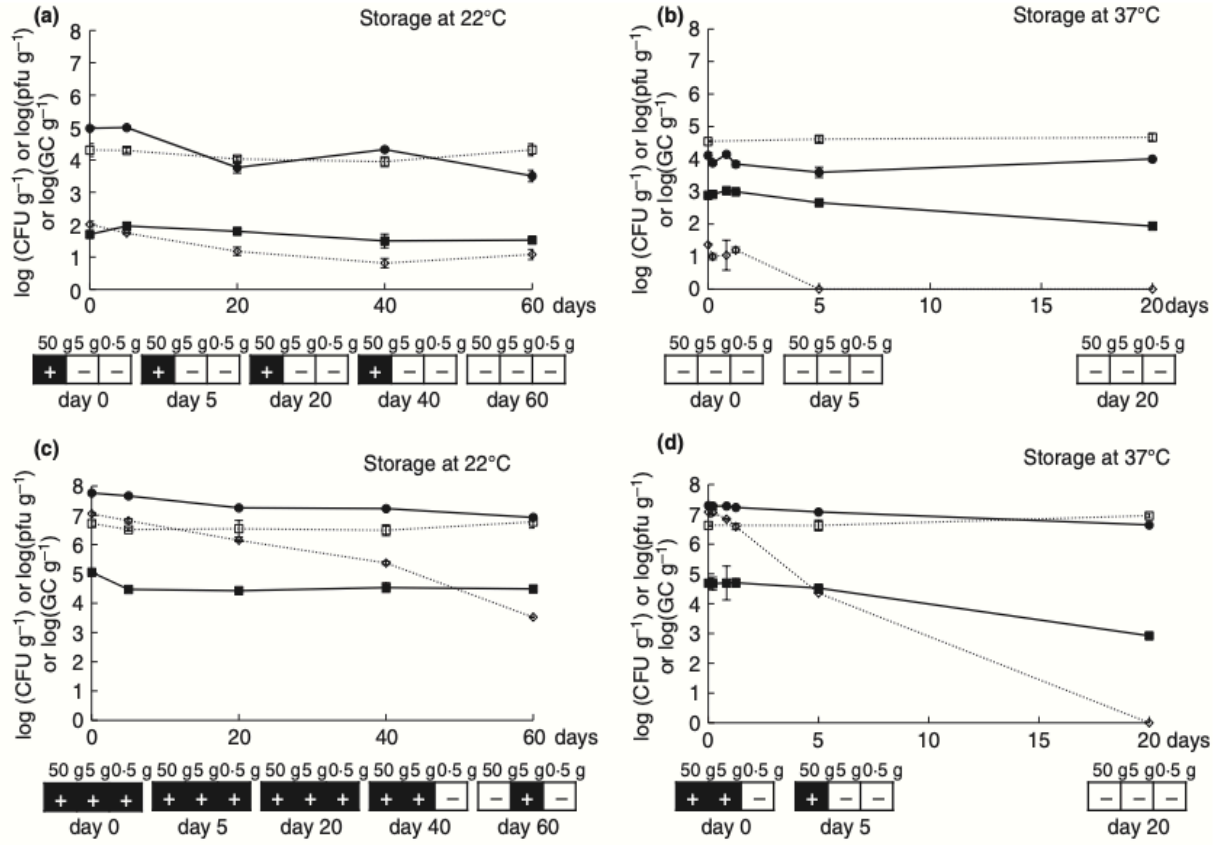

Figure 4 Example of the inactivation kinetics of the microbial indicators and *Salmonella* spp in finished sludge from thermophilic (a and b) and mesophilic (c and d) processes after storage at 22 and 37°C. Black and white boxes indicate the presence and absence, respectively, of *Salmonella* spp in 50, 5 and 0·5 ***g*** of sample; ● EC 16S rRNA gene; ♦ cEC; ◆ SOMCPH; □ SRC.

digested biosolids; however, the cause of this increase is controversial. The increases appear to occur at two points in the biosolids process: the first, which is referred to as a 'sudden increase', occurs immediately after dewatering; and the second, or 'regrowth', occurs during storage over longer periods (Higgins *et al.* 2007). Other authors reported an increase in the faecal coliform load after centrifugal dewatering of digested biosolids that was attributed to regrowth that continued for up to 5 days. In thermophilic-digested samples, the increase was more likely from reactivation of injured cells (Qi *et al.* 2007). In this work, an experimental approach was developed that can explain the differences obtained by different authors when analysing cEC in digested biosolids.

Our results are in agreement with the results obtained by Higgins *et al.* (Higgins *et al.* 2007). In our experimental approach, biosolids that were pasteurized at 55°C showed decreased cEC followed by an increase in these levels with respect to the original sample a few hours after pasteurization. According to our results, this increase could be mostly related to the recovery of injured cells after pasteurization, although a slight increase due to regrowth was also observed in some samples, as reflected by the EC 16S rRNA gene counts. In any case, the increase was highly variable and depended on the duration and temperature of pasteurization. Several other factors, including nutrient deficiency, oxidative and osmotic stresses and the presence of toxic substances, have been reported to be linked to the entry into the viable-but-not-culturable state (Erkan and Sanin 2013). Those factors were not evaluated in this study, and only temperature and time of treatment were considered.

The other analysed bacterial indicators, that is SOMCPH and SRC, did not recover or 'resuscitate'. Moreover, as shown in Fig. 3, the outcome of pasteurization in terms of inactivation and recovery of EC-injured cells showed a certain degree of variability, and, although an approximately twofold reduction was achieved for the different samples, the cEC increased between twofold and fourfold after the injured cells recovered 20 h after pasteurization. Interestingly, no regrowth was observed after this sudden increase. In view of these results, it is important to include the time after digestion treatment in the regulations to ensure that complete inactivation, and not just entry into the viable-but-not-culturable state, of EC has occurred because the recovery of injured cells in the first 20 h can be misleading to an analyst, who could underestimate the number of cEC. Additional bacterial indicators could also be included in the regulations to circumvent this problem. Amongst the bacterial indicators that were analysed, the use of SOMCPH is recommended because it was more informative with respect to the treatment and showed inactivation kinetics with a T90 that was higher than that of cEC. Moreover, bacteriophages do not have an injured state from which they can recover because they can only be infective or noninfective. SRC were shown to be highly conserved, but were not significantly inactivated after pasteurization. Therefore, SRC cannot be used to evaluate and validate thermal treatment processes, as was previously suggested (Moce-Llivina *et al.* 2003). On the other hand, the EC 16S rRNA gene (representing EC genomes) showed more than a twofold difference between samples from mesophilic and thermophilic processes, and the results obtained after inactivation at 80°C showed the high resistance of bacterial DNA. The suitability of a bacterial genome, such as EC, to be used as an indicator in treatment processes is therefore of limited use.

In our study, the regrowth of different bacterial indicators and *Salmonella* spp. in finished sludge from mesophilic and thermophilic digestion treatments at 22 and 37°C to mimic biosolid storage conditions has been assessed. No regrowth of the analysed bacterial indicators or pathogenic *Salmonella* spp. was shown during storage at either temperature.

In conclusion, our experimental approach confirmed that cEC monitoring is complicated due to the possibility of this micro-organism entering a viable-but-not-culturable state depending on the hygienization treatment applied. It has been shown that different sludge treatments can alter the inactivation kinetics of this micro-organism. Consequently, it would be advisable to incorporate alternative microbial indicators that do not enter into a viable-but-not-culturable state in sludge regulations. Otherwise, the regulations should restrict the time by which monitoring of EC should be performed to avoid the conflictive period when EC can resuscitate from a viable-but-not-culturable state. Our results showed that the density of cEC was lower than in the initial sample in all of the analysed biosolids approx. 5 days after treatment. It would be interesting to determine whether our results are the same in sludge samples subjected to other treatments to ascertain whether the obtained results can be extrapolated to different biosolid treatments.

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Conflict of Interest

Authors declare that do not have any conflict of interest.

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Microcosms for evaluating microbial indicator persistence and mobilization in fluvial sediments during rainfall events

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ABSTRACT

Mediterranean rivers, which are subject to long, dry periods and heavy rainfall events, could be particularly useful for understanding future climate scenarios. This study generated microcosms that mimicked riverbank sediment resuspension into the water of a typical Mediterranean river as a consequence of heavy rainfall. The mobilization and inactivation of six fecal pollution indicators and microbial source tracking markers were evaluated. The T_{90} values in the sediments were: 4 days for sorbitol-fermenting *Bifidobacterium*, 11 days for culturable *E. coli*, 36 days for bacteriophages infecting *Bacteroides thetaiotaomicron* strain GA17 and more than 42 days for qPCR-detected *E. coli*, somatic coliphages and sulfite-reducing clostridia spores. Bacteriophages and bacteria showed different resuspension and sedimentation patterns. The data obtained could be used in predictive models to assess the effects of climate change on surface water quality. Pathogen mobilization into the water column poses a risk for humans, animals and the natural environment, and breaches the One Health approach.

1. Introduction

Climate change and demographic evolution are expected to increase the intensity and frequency of heat waves and extreme precipitation events in several places on Earth (IPCC, 2014). The IPCC scientists have also forecasted, with *"medium evidence"* and *"high agreement"* that the interaction of increased temperature; increased sediment, nutrient and pollutant loadings from heavy rainfall; increased concentrations of pollutants during droughts; and disruption of treatment facilities during floods will reduce raw water quality and pose risks to drinking water quality (IPCC, 2014). Rivers in the Mediterranean region are typically characterized by medium-to-low water flow that increases during rainfall events until flooding occurs, thereby causing the resuspension of nearby sediments into the water column. Due to these characteristics, rivers in this area may be useful for the study of climate change effects. The majority of microorganisms present in water bodies are usually attached to sediment particles that contain nutrients and provide them with protection from predators and UV light (De Brauwere et al., 2014; Jamieson et al., 2005). This fact increases survival rates in sediments compared to water (Craig et al., 2004; Walters et al., 2014) and allows microorganisms to remain active even after weeks of being released into the environment. Resuspension of these sediments due to heavy rainfall events involves pathogen mobilization that causes a deterioration in water quality and subsequent problems in the purification process, and thus a potential increase in outbreaks of waterborne diseases (Craig et al., 2004; Jamieson et al., 2005; Nichols et al., 2009; Walters et al., 2014). In addition, deterioration of water quality poses a risk to animal health and degrades the natural environment, thereby breaching the One Health concept. This approach is based on an interdisciplinary analysis of all aspects of human, animal and environmental health (AVMA, 2008). Despite the attention traditionally paid to surface water quality monitoring, several questions concerning bacterial and viral survival and transport remain unanswered (Walters et al., 2014). The characterization of microbial loads, survival rates and transport patterns for pathogens present in surface waters would therefore lead to the proper management of these situations and a reduction in the associated impact on health. Numerous systems to simplify the study of environmental

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0043-1354/

conditions affecting waterborne pathogens have been developed (Ahmed et al., 2014; Anderson et al., 2005; Craig et al., 2004; Jamieson et al., 2005; Mocé-Llivina et al., 2005; Stocker et al., 2015; Walters et al., 2014). However, each situation comes with specific variables, dynamics and scientific questions, and therefore requires its own specific system (De Brauwere et al., 2014). The development of microcosms that simulate Mediterranean rivers would be very helpful for enhancing predictive models for understanding waterborne pathogen behavior in relation to climate change. Examples of predictive models are the Soil and Water Assessment Tool (SWAT) and the Hydrological Simulation Program - FORTRAN (HSPF) (De Brauwere et al., 2014; Kim et al., 2010). Microcosms may simulate conditions as varied as a calm river, rain falling on soil or water flowing inside a flume (Anderson et al., 2005; Durán et al., 2002; Stocker et al., 2015; Walters et al., 2014). These systems allow physical, chemical or microbiological data, among others, to be obtained easily under controlled conditions and examined in order to gain a better understanding of real situations. A wide range of pathogens could be present in environmental samples and the lack of effective extraction, concentration and detection methods makes their monitoring difficult and cumbersome. In this context, the use of fecal indicators is an appropriate tool for the study of waterborne pathogen behavior. A universal indicator does not exist, however, since no individual microorganism can significantly predict the presence of all pathogens in water (Wu et al., 2011). The most adequate approach is the control of a group of microorganisms that represent a broad pathogen spectrum such as bacteria, viruses and parasites. It is also important to include non-conservative, semi-conservative and conservative microorganisms that indicate pathogens with low, medium or high resistance to natural inactivating factors. In addition, the inclusion of microbial source tracking (MST) markers is helpful for discriminating between different fecal pollution origins. Regarding detection methods, molecular techniques such as qPCR are fast, sensitive and useful since it is only necessary to detect part of a microorganism to indicate pollution. Nevertheless, the detection of culturable microorganisms continues to be essential for indicating the infectious capacity of pathogens and, therefore, the real health risk associated with the pollution.

Microcosms were created as part of this study in order to evaluate indicator persistence and mobilization in fluvial sediments from a typical Mediterranean river. Flow, turbidity and *E. coli* data for the Llobregat River (NE Spain) were evaluated over a 14-year period. Microcosms were generated to mimic the natural conditions and to complement the data obtained by a previous study (García-Aljaro et al., 2017) in the same location. To this end, six fecal pollution indicators were monitored under simulated rainfall events and calm periods. Two indicators that signify bacterial contamination were analyzed in the microcosms: *E. coli*, both culturable (cEC) and total *E. coli* detected by qPCR (tEC), and sorbitol-fermenting *Bifidobacterium* (SFBIF). cEC is a traditional, non-conservative indicator that features in the great majority of water regulations worldwide. By contrast, *E. coli* detected by qPCR is a conservative indicator, since DNA can persist for much longer periods. SFBIF is a non-conservative indicator that acts as a MST marker of human fecal pollution (Bonjoch et al., 2005). As representatives of enteric viruses, two semi-conservative indicators were analyzed: somatic coliphages (SOMCPH) and bacteriophages infecting *Bacteroides thetaiotaomicron* strain GA17 (GA17PH). While SOMCPH are related to general fecal pollution, GA17PH is a human-specific MST marker (Jofre et al., 2014). Finally, the spores of sulfite-reducing clostridia (SSRC), as a representative of the most conservative pathogens (sporulating bacteria, protozoa oocysts and helminth ova), were analyzed (Agulló-Barceló et al., 2013). To obtain a better understanding of indicator behavior in the natural environment, all microorganisms tested were indigenous to the samples collected. This approach is more realistic than spiking samples with laboratory microorganisms, since it eliminates the need for cumbersome mixing and better reflects the natural inactivation kinetics and microorganism-sediment associations.

2. Materials and methods

2.1. Experimental design

This study was divided into three parts. In the initial phase, the river was characterized by analyzing the flow, turbidity and *E. coli* data collected over a period of 14 years (Appendix A). In the second phase, microcosms were generated and their performance was evaluated through the analysis of the turbidity and *E. coli* results obtained. In the final phase, the entire 42-day microcosm experiments were conducted in duplicate in order to study persistence and mobilization in water and sediments for the six fecal indicators analyzed.

2.2. Sample origin

Sediment and water samples were collected from the final stretch of the Llobregat River (NE Spain), downstream from a drinking water treatment plant. According to the regional water authority (Catalan Water Agency), this river runs through a heavily urbanized area and is mainly subjected to the influence of several effluents from sewage treatment plants. This watercourse is a typical Mediterranean river and undergoes both long, dry periods and heavy rainfall events, combined with occasional severe droughts. As a first step, the natural conditions of the river at the sampling point were studied and analyzed. Daily information about the mean flow and mean turbidity over a period of 14 years (2000–2013) was collected. A total of 4836 flow and turbidity data records were used in this study. Weekly information about the cEC levels of the river water over a period of 7 years, from January 2005 to February 2012, was also collected, for a final count of 352 data records. Arithmetic mean for flow and turbidity, geometric mean for cEC, and standard deviation, minimum and maximum values were calculated. These three parameters were analyzed to search for a possible relationship between them. Statistical analyses were performed using Statgraphics Centurion XVII.

River water and fluvial sediments were collected from the sampling point in sterile plastic containers the day after a rainfall event to ensure high indicator concentrations in the sediments used to create the microcosms. Riverbank sediments in contact with water were sampled and only the layer in the upper 2 cm was collected, since this is where the highest indicator concentrations are expected (Hassard et al., 2016). Water was manually collected at the depth of 50 cm, close to the riverbank. Sediment and water samples were maintained at 4 °C until the experiments started within a 48-hour period. Sediment particle size was determined by granulometry using a Beckman Coulter LS 230, VSM Plus Instrument (Beckman Coulter Inc., CA, USA). Particle size results were used to calculate sorting using the following equation:

$$So = \frac{P75}{P25} \quad (1)$$

where *P75* is the 75th percentile and *P25* is the 25th percentile. Sediment samples were classified as: very well sorted ($So < 2.5$), well sorted ($2.5 < So < 3.5$), moderately sorted ($3.5 < So < 4.5$) and poorly sorted ($So > 4.5$).

2.3. Microcosms

Microcosms were created in accordance with the scheme shown in Fig. 1. Since flooding leads to the resuspension of sediment in the riverbank, sediment and water samples were used to simulate the Llobregat water column covering the riverbank sediments. This system was evaluated under two different conditions: heavy rainfall events and calm periods without rainfall. At the same time, the sediment sample was also used to simulate the riverbank sediment not covered by the water column, and this system was studied during a calm period. Erlenmeyer flasks with a capacity of 500 ml were used to create the microcosms. One hundred grams of fluvial sediments were placed in the bottom of the flasks to form a 2.5 cm-thick layer. To guarantee that all indicators studied (including molecular detection) were indigenous to the sediment sample, river water was subjected to five regular autoclave cycles. A 400 ml sample of this sterilized river water was used to cover the sediments, which constituted a 5 cm-thick upper layer. In order to simulate heavy rainfall events, the Erlenmeyer flasks filled with water and sediments were agitated by orbital shaking for 2 h at 250 rpm. The aim was to achieve turbidity results similar to those found in natural conditions. For the non-covered sample, 100 g of fluvial sediments were placed in plastic containers.

Before starting the microcosm experiments, their performance was evaluated. To achieve this, three sampling campaigns were carried out and three microcosm replicas were created from each sampling campaign. Turbidity results and cEC concentrations in the water phase before and immediately after each agitation event were recorded. Turbidity and cEC data were analyzed in order to search for a possible relationship between them. A total of 18 turbidity and cEC measurements were recorded.

The microcosm experiments were conducted as follows: firstly, all flasks were agitated under the conditions described, thereby simulating an initial heavy rainfall event for all samples in order to homogenize the starting conditions. After this, the flask and container caps were loosened to guarantee oxygen exchange and samples were maintained at 20 ± 2 °C in the dark. The day after this simulation represented time 0 and the first sampling. Samples were taken regularly from both the water and sediment phases of the microcosms over a six-week period. The day before each sampling, two Erlenmeyer flasks and one plastic container were taken. One of the flasks was subjected to agitation to simulate a heavy rainfall event and the other was not agitated to simulate calm conditions. The three samples were maintained at 4 °C until analysis the next day, which represented a 24 h decantation period for the agitated flask. The outcomes obtained for the agitated microcosms therefore corresponded to those expected for a fictitious sampling campaign in the river the day after a rainfall event. The entire six-week microcosm experiment was performed twice, to constitute two replicas. Turbidity and pH were recorded for the microcosm water phase in each sampling. Fig. 1 shows the different phases obtained in the microcosms: agitated (AW) and non-agitated (NW) water, agitated (AS) and non-agitated (NS) covered sediments, and non-covered sediments (NCS).

2.4. Bacterial detection and enumeration

Sediment samples were mixed at a ratio of 1:10 in phosphate buffered saline (PBS), pH 7.2, and homogenized for 30 min at room temperature and 900 osc/min, using a wrist action shaker. Samples were then subjected to centrifugation at 300 × g for 3 min at 4 °C and the supernatant was used for the quantification of bacteria. cEC was enumerated using the pour plate method in ChromoCult agar supplemented with *E. coli*/coliform-selective supplement (Merck, Darmstadt, Germany). Plates were incubated at 44 °C overnight and dark-blue/purple cEC colonies were counted (Astals et al., 2012). For SSRC, the supernatant was subjected to thermal shock at 80 °C for 10 min. Samples were then anaerobically cultured by mass inoculation in *Clostridium perfringens* selective agar (Scharlab, Barcelona, Spain) and incubated at 44 °C overnight (Ruiz-Hernando et al., 2014). The typical black spherical colonies with black halos were counted as SSRC. SFBIF were incubated in human bifidobacteria sorbitol-fermenting agar (HBSA) (Mara and Oragui, 1983) for 48 h at 37 °C under anaerobic conditions (GasPak Jar, BBL, Hampshire, UK) with CO_2 atmosphere generators (Anaerocult A, Merck, Darmstadt, Germany). DNA for the enumeration of tEC was directly extracted from sediment samples (0.25 g) using the Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) in

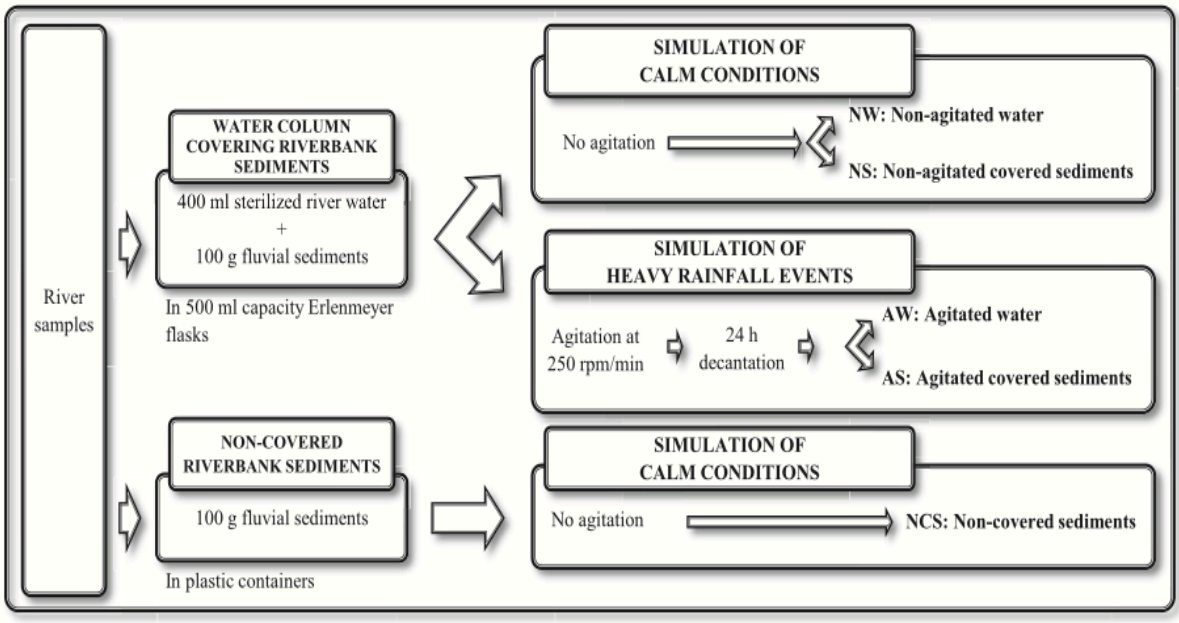

Fig. 1. Microcosm diagram.

accordance with the manufacturer's instructions. A qPCR protocol was then applied and all the quality and inhibition controls described were followed (Pascual-Benito et al., 2015). Water samples were directly analyzed with the culture and qPCR methods described.

2.5. Bacteriophage detection and enumeration

SOMCPH and GA17PH were extracted from sediments as previously described (Guzmán et al., 2007), a method in the process of standardization by the European Committee for Standardization (CEN, 2007). Water samples were filtered using polyethersulfone non-protein-binding membrane filters with a 0.22 μm pore size (Millipore, MA, USA). Bacteriophages from sediments and water were then counted using the double agar layer method indicated in ISO standards 10705-2 and 10705-4 (ISO, 2000, 2001). For the detection of human-specific bacteriophages, the latter method was modified by using *Bacteroides thetaiotaomicron* strain GA17 (Muniesa et al., 2012).

2.6. Calculation of resuspension parameters

The quantity of each indicator that was resuspended in the water phase of the microcosms after each agitation event was calculated using the following equation:

$$Log_{10}(Q_{AW} - Q_{NW}) \quad (2)$$

where Q_{AW} is the total quantity of microorganisms (CFU or PFU) present in the AW (in 400 ml) and Q_{NW} is the total quantity present in the NW (in 400 ml). The contribution (in percentage) of each agitation event to the microbial load in the water column was calculated as follows:

$$\frac{C_{AW} - C_{NW}}{C_{AW}} \cdot 100 \quad (3)$$

where C_{AW} is the concentration in the AW (CFU/100 ml or PFU/100 ml) and C_{NW} is the concentration in the NW (CFU/100 ml or PFU/100 ml).

2.7. Calculation of inactivation parameters

Inactivation rates in sediments were calculated for each indicator assuming the first order exponential model, defined by the equation:

$$k = \frac{Log_{10}(N_t/N_0) \cdot 2.303}{t} \quad (4)$$

where N_t and N_0 are microorganism concentrations (in CFU or PFU/g dw) at the time t and the initial time (in days), respectively, and k is the decay rate coefficient (in day^{-1}). In order to calculate the inactivation parameters, $Log_{10}(N_t/N_0)$ was plotted versus t for each sampling point and the regression line was generated. The slope of this line was used to calculate k, as follows:

$$slope = \frac{k}{2.3} \quad (5)$$

Finally, T_{90} or the time required to reduce the microorganism concentration by 1 log_{10} unit, was isolated as follows:

$$T_{90} = -2.3/k \quad (6)$$

3. Results and discussion

3.1. Microcosm evaluation

In order to evaluate the performance of the microcosms, turbidity and cEC concentrations were analyzed in the water phase before and immediately after each agitation. Turbidity results obtained before the agitation were between 6 and 47 NTU, with a mean value of 28 NTU and a standard deviation of 15 NTU. These results, always below the mean value of 181 NTU obtained in the river (Appendix A), satisfactorily reflected calm conditions. After the agitation, turbidity was between 253 and 2100 NTU, with a mean value of 802 NTU and a standard deviation of 703 NTU. These results were always above the mean in the river (Appendix A) and showed how the microcosms satisfactorily reproduced the turbidity values reached in the river after rainfall events and the consequent sediment resuspension. Again, a positive and statistically significant relationship was found between turbidity and cEC in microcosms, as occurred with the natural river conditions. A linear regression, represented by the following equation, was generated, with a p-value of 0.0446.

$$Log_{10}\ cEC = 2.80954 + 0.367735 \cdot Log_{10}\ turbidity \quad (7)$$

where *cEC* was expressed in CFU/ml and *turbidity* in NTU.

3.2. Microcosm experiments

3.2.1. Physical-chemical parameters

Turbidity results for the water phase of the microcosms in each sampling (for both the AW and the NW) are shown in Table 1. Each agitation produced an abrupt rise in turbidity due to the sediment resuspension and the turbidity decreased steadily over the following hours until the sampling event. It is important to remark that the samplings took place after the 24 h decantation period in order to simulate the river conditions the day after a heavy rainfall event. In that regard, the turbidity results obtained for the AW ranged between 36 and 334 NTU, with a mean of 152 NTU, and therefore satisfactorily reflected the natural conditions after rainfall event (Appendix A). In fact, results higher than 181 NTU (the mean obtained in the river) were still detected in 40% of the AW samples. By contrast, the turbidity results obtained for the NW ranged between 9 and 82 NTU, with a mean of 39 NTU. These results, which were always under 181 NTU, satisfactorily reflected the effects of calm conditions in the water column after the simulation of the first rainfall event (Appendix A). Although the conditions applied for each agitation were exactly the same (250 rpm, 24 h decantation) and the sediment sample was the same for each microcosm, the turbidity results obtained were heterogeneous. The factors affecting this are complex and diverse. Theoretically, particle sedimentation speed could be calculated using Stokes' law. However, the real behavior of sediment particles floating in the AW is difficult to predict, since the particles may be frequently present in the form of mineral-organic matter aggregates, with different densities and diameters than separated particles. In addition, sorting values for the sediments used in both microcosm experiments were higher than 4.5, i.e., poorly sorted, thus indicating that very different particle sizes were present in the same sample. The microcosms conducted in the present study implied the destruction of the natural sediment-water structure, followed by the subsequent restoration under laboratory conditions, which could have led to a certain bias between laboratory and natural sediment conditions. To overcome this limitation, Frey et al. (2015) developed *in situ* rainfall simulators capable of reducing the bias between natural and laboratory conditions. For the pH, the results were

Table 1
Turbidity values reached in the non-agitated water (NW) and the agitated water (AW) during microcosm experiments. Results in NTU. * = NW data for day 0 do not exist since all flasks were agitated at the beginning of the microcosm experiments.

		Day 0	Day 7	Day 21	Day 29	Day 42
Microcosm 1	NW	*	49	58	18	10
	AW	165	334	172	201	182
Microcosm 2	NW	*	35	49	82	9
	AW	223	109	50	49	36

similar for the AW and the NW and were between 7.4 and 8.0.

3.2.2. Indicator resuspension and mobilization

Initial indicator concentrations in the sediments used to create the microcosms were the following, expressed as $\log_{10}$ [(CFU, FPU or GC/g dw)]: 4.94 cEC, 6.11 tEC, 4.59 SSRC, 4.34 SFBIF, 5.27 SOMCPH and 2.30 GA17PH for microcosm 1 and 4.54 cEC, 6.04 tEC, 4.08 SSRC, 3.71 SFBIF, 4.61 SOMCPH and 1.21 GA17PH for microcosm 2. For all indicators, higher levels were found in the first microcosm than in the second. In order to study the microbial resuspension produced in the microcosm experiments, cEC, SOMCPH and SSRC were chosen as models for non-conservative, semi-conservative and conservative indicators, respectively. Fig. 2 shows the resuspension patterns for the three indicators. These results represent the quantity of each microorganism that was resuspended from sediments into the water column after each agitation event, calculated using equation (2); the trend lines generated are also shown. As time passed, the quantity of cEC and SOMCPH resuspended in the water phase decreased due to the inactivation affecting these indicators. By contrast, the quantity of SSRC that was resuspended was similar for all agitation events, which indicates high resistance throughout the entire six-week experiment. These observations were in accordance with the results from the previous study conducted in the same transect of the river where the sediments were gathered, in which the concentration of SSRC remained stable in the sediments throughout the studied period independently on the weather conditions (García-Aljaro et al., 2017). In any case, the differences associated with the resistance to inactivation were reflected in the trend lines that were generated. In fact, despite differences in the initial concentrations in sediments for microcosms 1 and 2, the slopes were very similar for each indicator analyzed. Fig. 2 also represents the contribution of each agitation event to the microbial load present in the water column, calculated using equation (3) and expressed as percentages. Obviously, 100% of the microbial load in the water column after the first agitation event came from sediments, as the water was initially sterilized. After this, each indicator was affected by sedimentation to a different degree. As a consequence, the indicator concentrations detected in the AW after a new agitation event were the sum of the microorganisms that had not settled since the previous agitation and those that were mobilized from sediments into the water column due to this new agitation. In general, more than 80%–90% of the cEC and SSRC detected in the AW came from resuspension. However, the contribution of agitation to SOMCPH concentrations in the AW was lower and more heterogeneous, and ranged from 28% to 90%. These behavioral differences between bacteria cells or spores and bacteriophages could be explained by differences in microbial size and composition that would influence sedimentation. In addition, microbes attached to sediment particles and floating in the water phase were subjected to different sedimentation patterns than those freely floating. Attached microorganisms undergo the same physical processes as the material they are attached to (De Brauwere et al., 2014). However, it is often assumed that free microorganisms do not settle at all, but some authors believe that this is not the case (De Brauwere et al., 2014). In any case, since bacteria-sediment and bacteriophage-sediment associations are expected to be different, this should also apply to sedimentation patterns.

3.2.3. Indicator levels in the different microcosm phases

Table 2 summarizes the indicator levels detected in microcosms for the three simulations: the water column covering riverbank sediments subjected to both calm conditions and heavy rainfall events, and the non-covered riverbank sediments subjected to calm conditions. Results in the water column and the sediments at the initial time and after 42 days are shown for all indicators analyzed. Different inactivation patterns were obtained for each microorganism and sample type. In the NW, the factors affecting the quantity of microorganisms detected were: (i) death or inactivation mainly due to nutrient exhaustion and predators coming from sediments (water was sterilized in order to create the microcosms), (ii) sedimentation, related to microbial size and density, the microorganism-sediment particle association and the microorganism-microorganism association. Grazing by protozoa has been reported as the most important cause of death among bacteria in natural water (De Brauwere et al., 2014). Some authors (Anderson et al., 2005) have not taken the effects of sedimentation into account when analyzing the water phase in microcosms, while others have (Craig et al., 2004), thus revealing the importance of this factor. Several other factors may be implied in microbial inactivation, such as redox potential, pH, sediment structure, rainfall intensity, etc. Solar radiation and high temperatures have been also reported to inactivate microorganisms in nature (De Brauwere et al., 2014); however, in order to simplify the number of factors that influence inactivation, the present study was carried out in the dark at a constant temperature of 20 ± 2 °C. Craig et al. (2004) found higher inactivation rates for cEC in microcosms containing only marine water than in the water phase of microcosms that reproduce the water column covering the underlying sediments. For this reason, the microcosms in the present study were designed to reproduce this natural scheme. Forty-two days after the beginning of the experiments, the only two indicators detected in the NW were SSRC and SOMCPH. Therefore, it seems that a significant number of these bacteriophages and spores were not affected by sedimentation and that these indicators resisted inactivation more satisfactorily than the others. On the other hand, SFBIF were not detected in any of the microcosm phases, even after 21 days, and thus represent the most sensitive indicator. Since *E. coli* was the most abundant microorganism in the NW at the initial time, a positive detection using qPCR could be expected at time 42. However, the tEC detection limit in the water phase was 4.67 $\log_{10}$ (GC/100 ml), which clearly influenced this result. By contrast, the low GA17PH levels found at the initial time, compared with the other indicators, would explain the negative result obtained for this indicator at time 42 in the NW. Indeed, GA17PH was found to resist inactivation even better than SOMCPH in marine-water microcosms (Mocé-Llivina et al., 2005). In the AW, as explained earlier in section 3.2.2, the indicator levels detected were strongly related to resuspension from sediments and, therefore, to indicator survival in those sediments. In fact, the majority of the indicators were still detected in the AW after 42 days, which serves

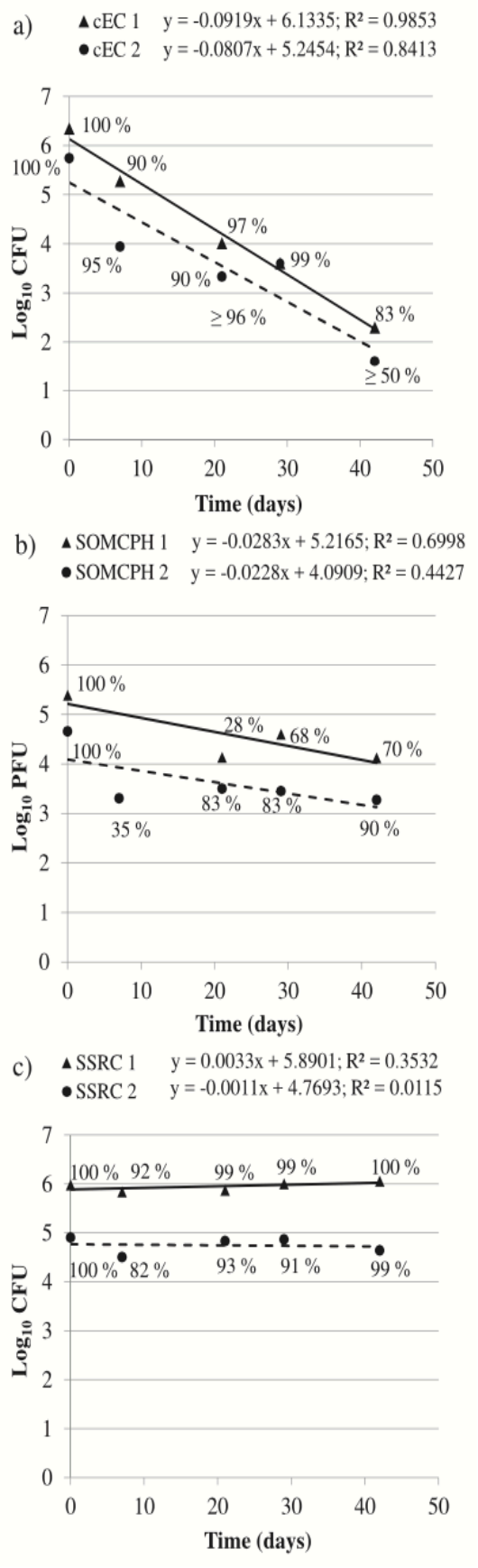

Fig. 2. Resuspension patterns and indicator mobilization in microcosms 1 and 2 for: a) culturable *E. coli*, cEC, b) somatic coliphages, SOMCPH and c) spores of sulfite-reducing clostridia, SSRC.

as a warning about the capacity of fluvial sediments to act as pathogen reservoirs. Thus, under the conditions assayed in this study, resuspension from sediments led to deterioration of the water quality, even six weeks after microbial depositions in the riverbank. Indicator inactivation patterns in the NCS, NS, AW and AS phases are discussed in greater detail in the following sections. These data agreed with the results from García-Aljaro et al. (2017), in which indicators such as cEC, SOMCPH and SSRC, among others, survived in the sediments even after 15 days without a rainfall event.

3.2.4. Microbial inactivation

Regarding the sediment phases of the microcosms, the particle sizes were classified as follows: particles with a diameter smaller than 4 μm were defined as clay, particle diameters between 4 and 63 μm were defined as silt, and particle diameters greater than 63 μm were defined as sand. For microcosm 1, the particle size distribution for the sediment was 20.7% clay, 26.6% silt and 52.7% sand; for microcosm 2, it was 21.0% clay, 49.9% silt and 29.1% sand. Indicator resistance to inactivation was compared in the NCS with respect to the NS to evaluate the protection that the water column conferred to the microbes present in the riverbank sediments. Those pathogens with the capacity to resist the environmental conditions that affect riverbank sediments over time could potentially mobilize from sediments into the water during the subsequent rainfall event. Inactivation results for NCS and NS are shown in Fig. 3 and Table 3. These results may be used to include in predictive models such as the SWAT, the HSPF and other in-house developed programs that act at the catchment scale (De Brauwere et al., 2014; Kim et al., 2010). In general, the indicators studied in this research behaved differently in each sediment phase. Inactivation was slightly higher for non-covered sediments than for those covered by the water column. Although dryness is a key inactivation factor in the microbial world, the moisture percentage of the NCS appeared to be high enough to confer good protection for the indicators analyzed. The moisture percentage was between 26.6% and 36.2%. For the NS this percentage was between 49.6% and 69.1%. The most resistant indicators were SSRC and tEC. SSRC remained unaltered throughout the whole experiment in both sediment phases, which was expected for these spore-forming bacteria. For tEC, a slight decay was observed, although the T_{90} values were higher than 42 days in the two phases. On the other hand, the most sensitive indicators in this study were culturable bacteria, i.e. cEC and SFBIF. The cEC concentrations decreased by 1 $\log_{10}$ after 10 and 11 days for the NCS and the NS, respectively. Similar results for cEC were found in sediments subjected to simulated rainfalls and maintained at 18 °C (Stocker et al., 2015). On the contrary, the microcosms developed by Anderson et al. (2005) showed lower resistance to inactivation for fecal coliforms. This was probably due to the fact that their microcosms were incubated outdoors and were therefore subjected to weather variations. Similarly, T_{90} values of between 2.04 and 4.55 days have been obtained for cEC in water-covered sediments incubated at 20 °C (Craig et al., 2004). However, these authors used laboratory-grown *E. coli* strains, which are known to present less resistance than environmental microorganisms. In addition, several studies (Craig et al., 2004; Jamieson et al., 2005; Walters et al., 2014) have found that microorganisms resist for longer in sediments with small particle sizes such as silt and clay than in sandy sediments. In that regard, the sediments used by Craig et al. (2004) had high percentages of sand. With respect to SFBIF, their levels reduced to 10% in barely 3 days in the NCS and just 4 days in the NS. This behavior was consistent with the nature of obligate anaerobes that rapidly decline outside the gut (Bonjoch et al., 2009). This indicator is therefore useful for detecting recent pollution events. Finally,

Table 2
Indicator levels detected in microcosms 1 and 2 at the initial time (t_0) and after 42 days (t_{42}). Results in $\log_{10}$ [CFU, PFU or GC/100 ml of water or 1 g of sediments (dry weight)]. NW = non-agitated water, AW = agitated water, NS = non-agitated covered sediments, AS = agitated covered sediments, NCS = non-covered sediments. cEC = culturable *E. coli*, tEC = total *E. coli*, SSRC = spores of sulfite-reducing clostridia, SFBIF = sorbitol-fermenting *Bifidobacterium*, SOMCPH = somatic coliphages, GA17PH = bacteriophages infecting *Bacteroides thetaiotaomicron* strain GA17.

	cEC		tEC		SSRC		SFBIF		SOMCPH		GA17PH	
	t_0	t_{42}	t_0	t_{42}	t_0	t_{42}	t_0	t_{42}	t_0	t_{42}	t_0	t_{42}
Microcosm 1												
NW	5.75	<1	5.27	<4.67	5.38	2.93	5.04	<2	4.79	3.15	2.79	<0.60
AW	5.75	1.78	5.27	4.71	5.38	5.46	5.04	<2	4.79	3.67	2.79	0.20
NS	4.94	1.20	5.16	4.73	4.59	4.81	4.34	<1.39	5.27	4.16	2.30	1.60
AS	4.89	1.47	5.49	4.94	4.83	4.96	4.55	<1.48	4.84	4.13	1.31	0.59
NCS	4.94	1.14	5.16	4.60	4.59	5.03	4.34	<1.17	5.27	2.58	2.30	−0.26
Microcosm 2												
NW	5.15	<1	4.77	<4.67	4.30	2.18	3.78	<2	4.06	1.70	1.20	<0.60
AW	5.15	1.30	4.77	<4.67	4.30	4.04	3.78	<2	4.06	2.72	1.20	<0.60
NS	4.54	0.33	5.11	3.86	4.08	3.90	3.71	<1.39	4.61	3.79	1.21	−0.07
AS	4.60	1.48	4.93	3.93	4.40	4.06	2.66	<1.48	4.33	3.74	1.64	0.00
NCS	4.54	0.87	5.11	3.39	4.08	3.60	3.71	<1.17	4.61	2.96	1.21	−0.25

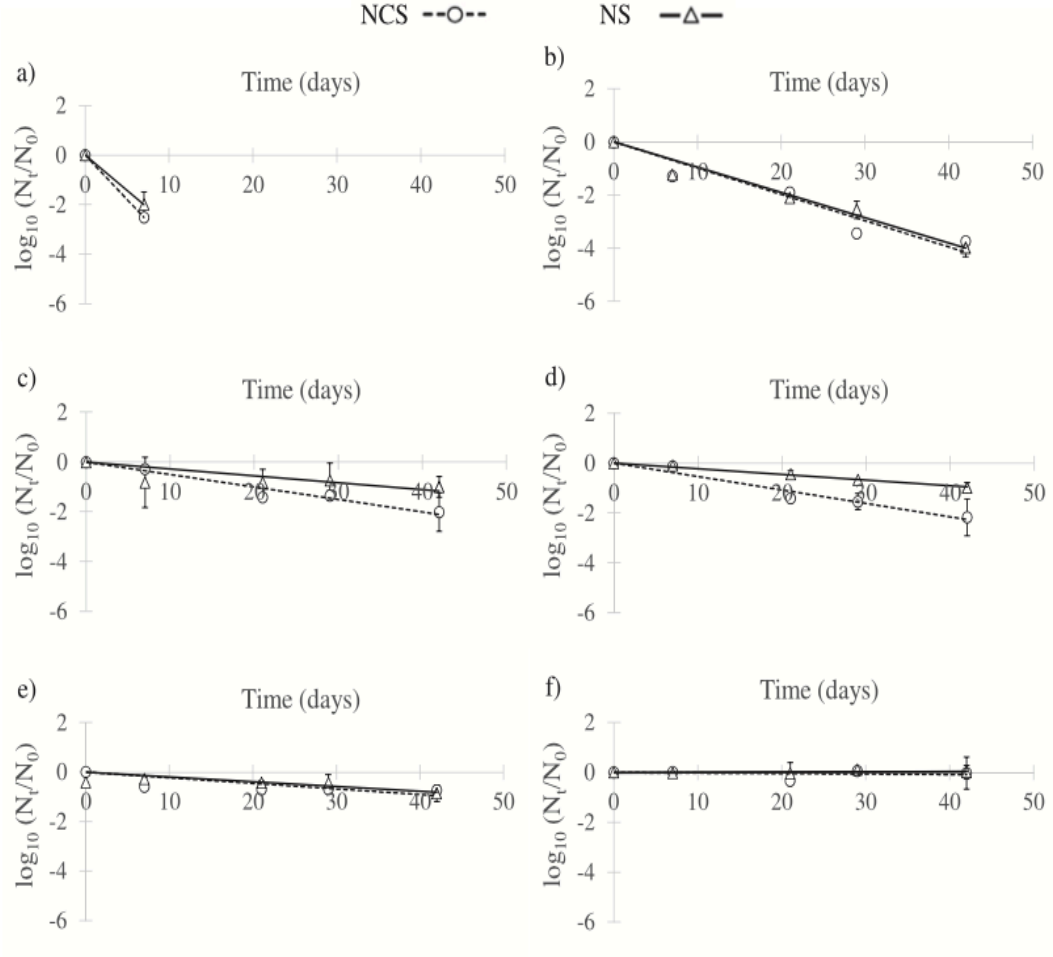

Fig. 3. Inactivation patterns in the non-covered sediments (NCS) and the non-agitated covered sediments (NS) for a) SFBIF, sorbitol-fermenting *Bifidobacterium*, b) cEC, culturable *E. coli*, c) GA17PH, bacteriophages infecting *Bacteroides thetaiotaomicron* strain GA17, d) SOMCPH, somatic coliphages, e) tEC, total *E. coli*, f) SSRC, spores of sulfite-reducing clostridia. Mean values for the two microcosms tested. Error bars represent standard deviation.

bacteriophages showed intermediate resistance to inactivation in the microcosms and remained detectable throughout the entire experiment. In this case, T_{90} values clearly differed between covered and uncovered sediments. Twenty days were needed to reduce the GA17PH concentrations in the NCS by 1 $\log_{10}$, and this value was 36 days for the NS. Regarding SOMCPH, T_{90} values were 19 and more than 42 days for the NCS and the NS, respectively. These inactivation results were similar for both groups of bacteriophages studied. As with the water phase of the microcosms, nutrient exhaustion and grazing are expected to be the most important causes of microbial death or inactivation in the sediment phase. On the other hand, the positive effect of sedimentation could

Table 3
Inactivation parameters obtained in the non-covered sediments (NCS) and the non-agitated covered sediments (NS). R^2 and slopes corresponded to the regression lines showed in Fig. 3 and defined by the equation "$\log_{10}(N_t/N_0)$ = slope · time". SFBIF = sorbitol-fermenting *Bifidobacterium*, cEC = culturable *E. coli*, GA17PH = bacteriophages infecting *Bacteroides thetaiotaomicron* strain GA17, SOMCPH = somatic coliphages, tEC = total *E. coli*, SSRC = spores of sulfite-reducing clostridia.

		SFBIF	cEC	GA17PH	SOMCPH	tEC	SSRC
NCS	Slope	−0.3595	−0.0986	−0.0501	−0.0537	−0.0225	−0.002
	R^2	1.0000	0.9076	0.943	0.9573	0.2321	−0.036
	k (days^{-1})	−0.83	−0.23	−0.12	−0.12	−0.15	0.00
	T_{90} (days)	3	10	20	19	>42	>42
NS	Slope	−0.2846	−0.0948	−0.0278	−0.0225	−0.019	0.001
	R^2	1.0000	0.9581	0.1981	0.9887	−0.153	0.1522
	k (days^{-1})	−0.66	−0.22	−0.06	−0.05	−0.14	0.00
	T_{90} (days)	4	11	36	>42	>42	>42

be neglected in this case, since the quantity of microorganisms resuspended into the water column during the first agitation event was insignificant compared to the quantity that remained in the sediments (Table 2). Indeed, several studies conducted under both environmental and laboratory conditions have shown that greater numbers of microorganisms are attached to sediments than float free in the water column (Craig et al., 2004). The nature and proportion of particle attachment is still under study and appears to depend on the properties of both the solid particles and the microorganisms studied (De Brauwere et al., 2014), in addition to temperature, salinity and other environmental factors. Studies have reported percentages of fecal indicator bacteria attached to particles of 38%, 30%, 50%, 80%, between 30 and 55%, and percentages varying according to the season (De Brauwere et al., 2014).

Indicator behavior in the agitated phases of microcosms (AW and AS) are shown in Fig. 4. Detection in the AS was strongly related to indicator resistance to inactivation. Thus, the conservative indicators (SSRC and tEC) showed lower levels of decay over time

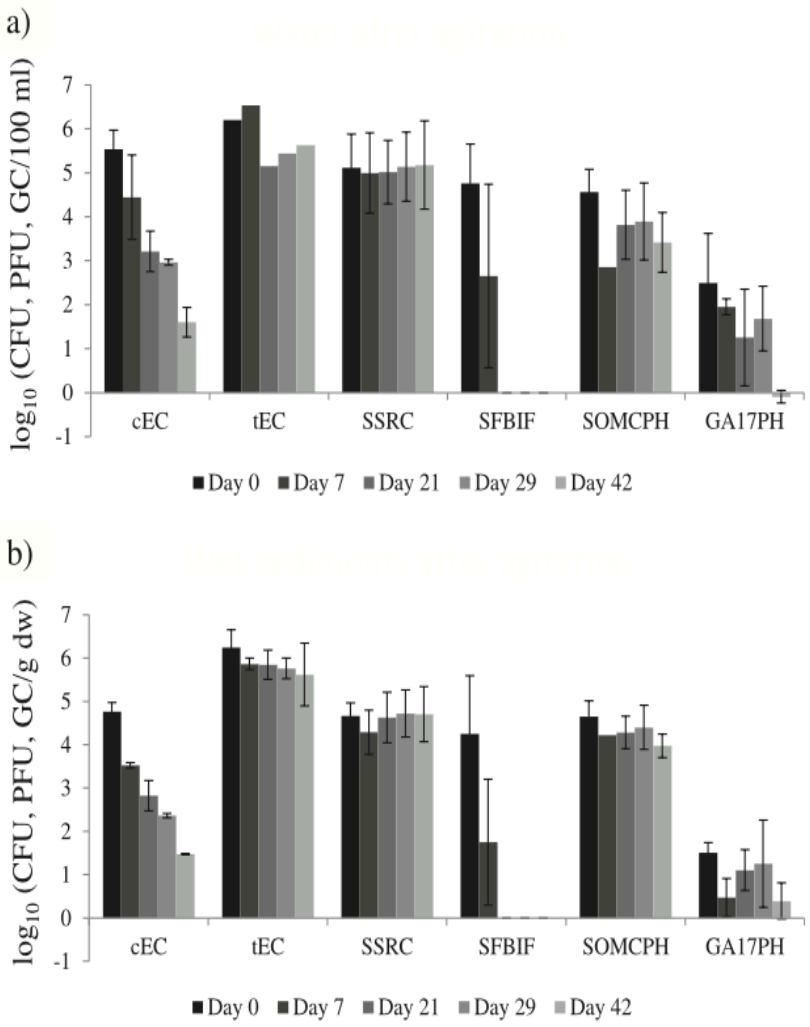

Fig. 4. Indicator concentrations found during the 42-day-microcosms in a) the agitated water, AW and b) the agitated covered sediments, AS. Mean values for the two microcosms tested. Error bars represent standard deviation. No standard deviation is shown for tEC in the AW and for SOMCPH at time 7 in the AW and AS. Data shown for these parameters correspond to microcosm 1.

than the semi-conservative bacteriophages (SOMCPH and GA17PH) or the non-conservative bacteria (cEC and SFBIF). Since the microorganisms present in the water phase mobilized due to the sediment resuspension produced in each agitation event, the AS and the AW were clearly interrelated (Fig. 4). It is important to highlight the presence of almost all indicators in the sediments after 42 days and how the sediment resuspension with the consequent microbial mobilization led to deterioration of the water quality. These results support previous observations (García-Aljaro et al., 2017) in which all the indicators analyzed with the exception of SFBIF and GA17PH were present in both sediment types (covered and non-covered by the water column) and in the presence or absence of rainfall events, being at lower concentrations in the latter.

4. Conclusions

The experimental microcosms from this study mimicked the resuspension of fluvial sediments into the water column produced as a consequence of heavy rainfall events. This methodology made it possible to calculate the T_{90} for six fecal pollution indicators and MST markers in the sediments and study their mobilization due to sediment resuspension.

- The inactivation patterns obtained in water and sediments during the 42-day simulation were determined, and were as follows: cEC and SFBIF were the most sensitive, SOMCPH and GA17PH showed intermediate resistance and SSRC and tEC were the most resistant.
- Bacteriophages and bacteria showed different resuspension and sedimentation patterns, probably related to microbial size and composition and to different microorganism-sediment particle and microorganism-microorganism associations.
- Five out of the six indicators were still detected in the sediments after 42 days, which serves as a warning about the risk of pathogen mobilization due to rainfalls.
- The present study has generated valuable data about waterborne pathogen behavior for inclusion in climate change predictive models that evaluate the effects of water scarcity and heavy rainfall events.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2017.07.017.

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Appendix A. Supplementary data - Microcosms for evaluating microbial indicator persistence and mobilization in fluvial sediments during rainfall events

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In the initial phase of the study, the natural conditions of the river at the sampling point were determined. Figure S1 shows the flow and turbidity data in chronological order, represented as logarithms for better visualization. The mean river flow during the 2000-2013 period was 11 m^3/s (Figure S1a). The alternation between drought periods and heavy rainfall events gave rise to a minimum of 0 m^3/s, a maximum of 314 m^3/s and a standard deviation of 17 m^3/s, the typical variability of a Mediterranean river. Since the sampling point was located at the final stretch of the river, the factors that may determine flow include rainfall events throughout the river basin and snowmelt phenomena in spring. In addition, the presence of four reservoirs in the Llobregat basin affects and regulates the flow. However, the effect of these latter two factors should be a gradual increase in flow. The most important factor in sudden flow increases was therefore expected to be heavy rainfall events produced somewhere upstream of the sampling point and downstream of the reservoirs. The largest concentration of rainfall in the area occurs between October and March (García-Herrera, Paredes, et al., 2007). It is therefore not surprising that flow data higher than 28 m^3/s (mean + standard deviation) were mostly distributed throughout this period and spring, when snowmelt takes place. River flow data lower than 1 m^3/s corresponded to occasional events (lasting 1 to 6 days) in 2000, 2007 and 2008, and to a long period during the severe drought that affected the Iberian Peninsula in 2005. Indeed, the 2004-2005 hydrological year represented one of the worst droughts ever recorded on the Iberian Peninsula (García-Herrera, Paredes, et al., 2007).

The mean river turbidity value recorded in the 2000-2013 period was 181 NTU (Figure S1b). As occurred with flow, extreme variability was detected, with a maximum of 16,083 NTU, a minimum of 3 NTU and a standard deviation of 677 NTU. However, in contrast to river flow, turbidity showed a marked periodicity, with the lowest values (less than 10 NTU) corresponding to winter every year, and more specifically to

December and January. These low turbidity results were always accompanied by flows below the mean, i.e., less than 11 m^3/s, except for January in 2000, which recorded higher flow values. One may expect that the concentration of suspended solids in the water column would produce a rise in turbidity when the river flow is low. However, this phenomenon was not observed. On the other hand, turbidity values higher than the mean, i.e., 181 NTU, corresponded to short events (lasting 1 to 5 days) and were mostly accompanied by marked increases in short-duration flow. In addition, extreme turbidity values of more than 858 NTU (mean + standard deviation) always occurred within those events. Therefore, although it was not possible to rule out snowmelt and water release in the reservoirs as the causal agents, the short-duration flow-turbidity increases were most likely due to rainfall events throughout the river basin. Thus, a heavy rainfall event would produce a sudden increase in river flow. This would lead to sediment resuspension accompanied by an abrupt rise in turbidity. In fact, the majority of flow results higher than 50 m^3/s were accompanied by turbidity values higher than 858 NTU.

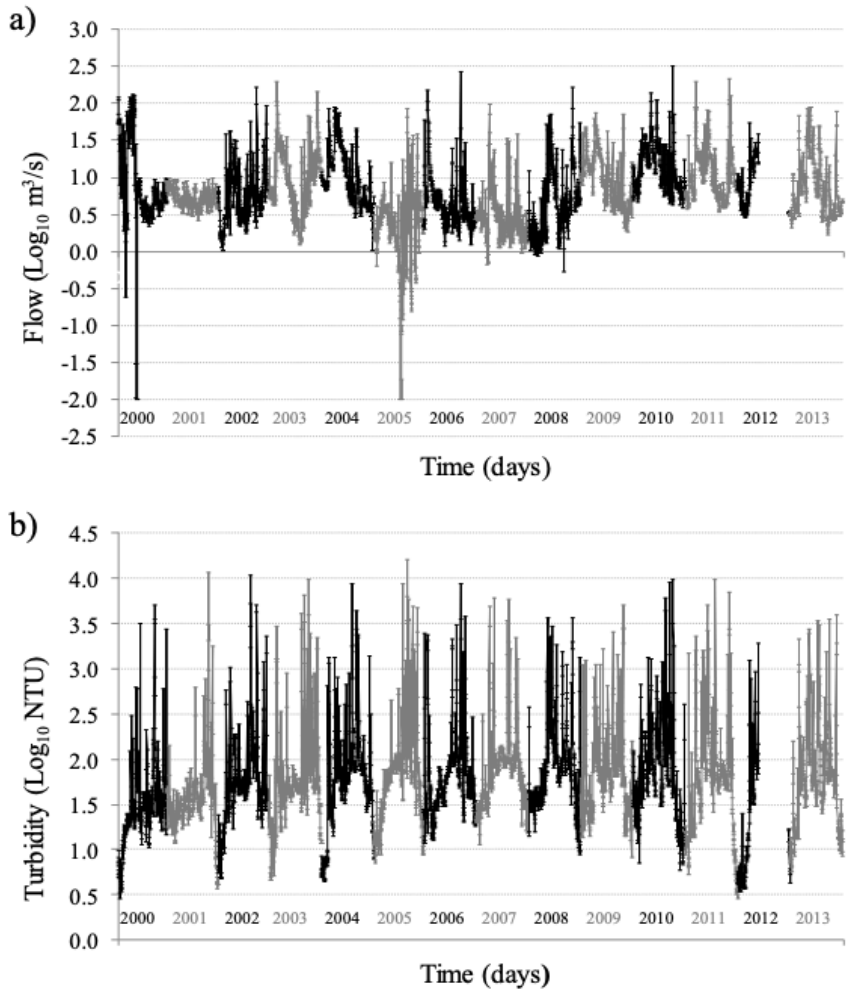

Figure S1. Chronological parameters observed during a 14- year period (2000 - 2013) in the Llobregat River: a) Flow b) Turbidity.

Indeed, a positive and statistically significant relationship between river flow and turbidity was found. In that regard, a linear regression was generated, defined by the following equation, with a p-value of 0.0000.

$$Log_{10}\, turbidity = 1.41565 + 0.427544 \cdot Log_{10}\, flow \qquad \text{(S1)}$$

where *turbidity* was expressed in NTU and *flow* in m^3/s.

The weekly results for cEC concentrations in the Llobregat River over a seven year period are shown in Figure S2. The mean was 3.42 log_{10} (CFU/100 ml) and the standard deviation was 0.71 log_{10} (CFU/100 ml), with minimum and maximum values of 1.20 log_{10} (CFU/100 ml) and 5.97 log_{10} (CFU/100 ml), respectively. Sediment resuspension produced as a consequence of a heavy rainfall event was expected to lead to water quality deterioration and therefore increase pathogen and indicator concentrations. In fact, some authors have recognized bacteria resuspension as a potentially significant but extremely variable and poorly understood source of fecal indicator bacteria (De Brauwere, Ouattara, et al., 2014; Jamieson, Joy, et al., 2005). In that regard, a positive and statistically significant correlation was found between cEC and turbidity. A linear regression, represented by the following equation, was generated, with a p-value of 0.000.

$$Log_{10}\, cEC = 2.71379 + 0.399123 \cdot Log_{10}\, turbidity \qquad \text{(S2)}$$

where *cEC* was expressed in CFU/100 ml and *turbidity* in NTU.

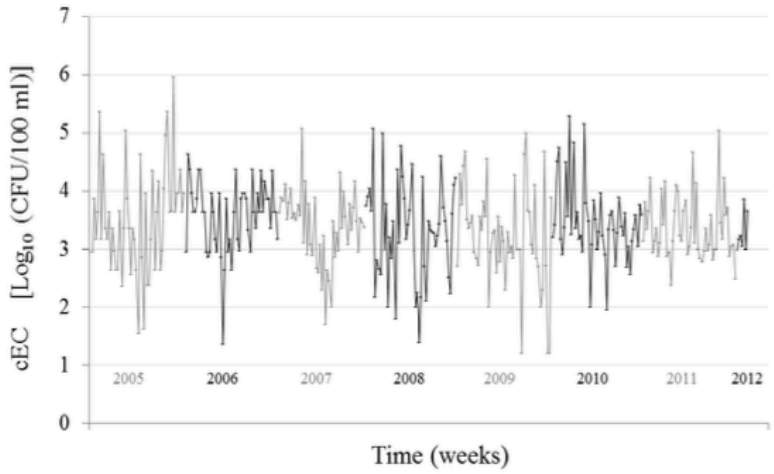

Figure S2. Weekly distribution of culturable *E. coli* (cEC) levels over a seven-year period in the Llobregat River (NTU). Results in log_{10} (CFU/100 ml)

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Bluephage: A rapid method for the detection of somatic coliphages used as indicators of fecal pollution in water

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ABSTRACT

The use of somatic coliphages as indicators of fecal and viral pollution in water and food has great potential due to the reliability, reproducibility, speed and cost effectiveness of methods for their detection. Indeed, several countries already use this approach in their water management policies. Although standardized protocols for somatic coliphage detection are available, user-friendly commercial kits would facilitate their routine implementation in laboratories. The new method presented here allows detection of up to 1 somatic coliphage in under 3.5 h, well within one working day. The method is based on a modified *Escherichia coli* strain with knocked-out *uidB* and *uidC* genes, which encode the transport of glucuronic acid inside cells, and overexpressing *uidA*, which encodes the enzyme β-glucuronidase. The enzyme accumulated in the bacterial cells only has contact with its substrate after cell lysis, such as that caused by phages, since the strain cannot internalize the substrate. When the enzyme is released into the medium, which contains a chromogen analogous to glucuronic acid, it produces a change of color from yellow to dark blue. This microbiological method for the determination of fecal pollution via the detection of culturable microorganisms can be applied to diverse sample types and volumes for qualitative (presence/absence) and quantitative analysis and is the fastest reported to date.

1. Introduction

Fecal water pollution is an important cause of pathogen transmission and is associated with several thousand human mortalities per day around the world (LeChevallier and Kwok-Keung, 2004). The indicators most commonly used to determine fecal pollution levels in different water sources are bacterial, but fecal-contaminated water also contains viral and protozoan pathogens. This has raised doubts within the water microbiology community concerning the capacity of bacterial indicators to measure water quality and predict waterborne viral disease hazards (Borchardt et al., 2004; Gerba et al., 1979; Grabow, 2001; Keswick et al., 1984; Payment et al., 1997). Therefore, additional indicators are advisable to predict the presence and behavior of viruses in water.

Several groups of bacteriophages have been proposed as indicator microorganisms to assess fecal and viral contamination of water. The potential value of different groups of phages that infect enteric bacteria as quality indicators in water, biosolids and food has been studied in depth and reviewed (Armon and Kott, 1996; Bosch et al., 2001; Goyal, 2006; Grabow, 2001; Hsu et al., 2002; IAWPRC Study group on health related water microbiology, 1991; Jofre, 2007). One of these bacteriophage groups are somatic coliphages, which replicate in *Escherichia coli* after infection through the cell wall (IAWPRC Study group on health related water microbiology, 1991). Somatic coliphages are abundant in fecally contaminated samples, and there are standardized methods for their detection and quantification (Anonymous, 2000; U.S. EPA, 2001a; 2001b). The standardized ISO and U.S. EPA methods are faster than the tests to identify bacterial indicators, easy to perform, cost effective and reasonably robust (Mooijman et al., 2005; U.S. EPA, 2003a; 2003b). However, current methods are still multi-step processes that require several media and operations and more than 8 h (that is, more than one ordinary working day) to yield results.

Although the use of somatic coliphages as fecal indicators was proposed years ago (IAWPRC Study group on health related water

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microbiology, 1991), they have only recently been included in water and sludge quality guidelines: for example, those affecting water reclamation (North Carolina Administration, 2011; Queensland Government, 2005), groundwater (NHMRC and NRMMC, 2011; U.S. EPA, 2006), biosolids applied in agriculture (Republica de Colombia, 2014; Western Australian Government, 2012) and aquaculture practices (Food and Drug Administration and Interestate Shellfish Sanitation Comission, 2015). Additionally, the U.S. regulatory authorities (U.S. EPA, 2016; U.S. EPA, 2015) are currently studying the application of coliphages in the quality control of bathing and recreational surface water. As the use of somatic coliphages expands, simpler and faster detection methods will be required. The availability of straightforward ready-to-use tests would facilitate routine implementation of the methods in laboratories and encourage their adoption in guidelines for water, biosolids and food quality management. Unfortunately, the use of molecular techniques such as PCR, which would provide a rapid detection, is not possible, because somatic coliphages are a heterogeneous group belonging to different phage families (Muniesa et al., 1999) that do not share common DNA sequences.

Among various approaches to devise such a method, some are based on the measurement of the phage-mediated release of intracellular molecules, such as the intracellular enzyme β-galactosidase (Ijzerman et al., 1994, 1993; Salter and Durbin, 2012) or adenylate kinase (Guzmán-Luna et al., 2009). However, this approach has certain drawbacks that still need to be resolved, including long incubation times when the phage density is low or frequent false positive results. The present study reports further progress in this field with a method based on an engineered host bacterium.

Herein we describe a somatic coliphage detection method based on a modified *E. coli* host strain. The modification is located in a genetic operon specific for *E. coli*: the β-glucuronidase system. An assay based on β-glucuronidase has already been applied to detect *E. coli* in agar plates (e.g., Chromocult® Merck Laboratories. Darmstadt, Germany) or liquid media (Colilert®, IDEXX Laboratories, Inc. Maine, US), and has been approved by some food management agencies for drinking water and processed food samples (Anonymous, 2001). The media contain the substrate X-glucuronide, which is cleaved by ß-D-glucuronidase, a characteristic enzyme of *E. coli*. *E. coli* grown in this media develop a dark blue coloration or fluorescence (Frampton et al., 1988; Kilian and Bülow, 1976).

The β-glucuronidase system in *E. coli* is composed of several genes (Liang et al., 2005). For our purpose, we focused on the *uidA*, *uidB* and *uidC* genes that encode the UidA, B and C proteins. UidA is the active subunit: an intracellular acid glucuronoside glucuronosohydrolase with broad substrate specificity (Novel and Novel, 1976). UidB, which is located in the inner membrane, is a proton-dependent transporter specifically for importing α- and β-glucuronides into the cell, making it essential for glucuronide transport. UidC, which is associated with the outer membrane, does not confer transport activity, but it improves the efficiency of transport by UidB through an unknown mechanism (Liang et al., 2005). β-glucuronide molecules are imported by the activity of UidC and UidB and once inside the cell are cleaved by UidA (Novel and Novel, 1976).

The objective of this work was to develop a method that allows the rapid detection of somatic coliphages at low densities while minimizing false negative results. The method, named *Bluephage*, incorporates a tailored *E. coli* strain with two features that improve its sensitivity for phage detection. On the one hand, it overexpresses the *uidA* gene, causing an accumulation of the β-glucuronidase enzyme in the cytoplasm. On the other, it lacks the genes (*uidB* and *uidC*) involved in introducing the substrate into the cell. The culture medium used in this test incorporates the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, which turns blue after its cleavage by β-glucuronidase. Consequently, only after the host strain cells are lysed, as occurs after phage infection, will the enzyme degrade the substrate, which in turn causes the medium to change color, thereby providing fast phage detection in a given sample.

2. Materials and methods

2.1. Medium and growth conditions

The media and conditions used here are those included in the ISO protocol for the culture of *E. coli* WG5 strain (Anonymous, 2000). The method employs a modified version of Modified Scholten's Broth (MSB) (Anonymous, 2000). The modified MSB medium is supplemented with 0.05% (*w/v*) arabinose, 0.5% (*v/v*) glycerol, 15 µl $CaCl_2$ 1 M and 0.1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid. When necessary for the selection of colonies transformed with the pBAD vector, ampicillin (ap) (100 µg/ml) was added to the liquid medium.

The modified bacterial strain and bacteriophages were inoculated into MSB media and incubated at 37 °C for variable periods (from 1 to 18 h), unless specified otherwise.

2.2. Plasmids

Plasmid pKD46 (GenBank AY048746), expressing the Red recombinase system, was used to insert DNA fragments into the *E. coli* chromosome (Datsenko and Wanner, 2000). The tetracycline resistance gene (*tet*) was amplified from plasmid pACYC184 (Genbank X06403) (Rose, 1988). The pBAD-TOPO vector (Invitrogene) was used to clone and overexpress the *uidA* gene. All the vectors were purified using Qiagen Plasmid Midi purification kits (Qiagen Inc., Valencia, USA).

2.3. Bacteriophages

The bacteriophages in this study were used from laboratory stocks of known concentration (10^9 PFU/ml) containing a pure culture of one bacteriophage, or naturally occurring somatic coliphages. Coliphages were purified from 1 ml of raw urban sewage from the influent of a wastewater treatment plant that serves approximately 500,000 inhabitants (containing *ca* 10^{4-5} PFU/ml), or 50 ml of Llobregat river water (*ca* 10^3 PFU/ml). Both sampling points are located in the Barcelona area, Spain. Phage ΦX174 (ATCC 13706-B1) was used as a reference phage for somatic coliphages, in accordance with the ISO protocol (Anonymous, 2000). To prepare a phage mixture, two phages (SOM3 and SOM23) (Muniesa et al., 2003), which belong to *Myoviridae* and *Siphoviridae* morphological types, respectively, were used together with phage ΦX174 (Anonymous, 2000).

The environmental samples used were filtered through 0.22 µm pore size, low-protein-binding (PES) membranes (Millipore, USA) to remove bacteria and other particulate material.

2.4. PCR techniques

PCRs were performed with a GeneAmp 2400 PCR system (Perkin-Elmer, PE Applied Biosystems, Barcelona, Spain.). The oligonucleotides used in this study are shown in Table 1 and were designed from the sequence available for *E. coli* K-12 (GenBank Accession number NC_000913).

Table 1
- Oligonucleotides used in this study.

Name	Oligonucleotides	Target gene	ref
uidA-For2	CTTAATGAGGAGTCCCTT	*uidA*	This study
uidA-Rev2	CCAGGAGAGTTGTTGATT	*uidA*	This study
uidB-up	CTGGACTGGCATGAACTTC	*uidB*	This study
uidC-lp	ACTTCAGCATAAAGTCATACT	*uidC*	This study
uidB-Tc5	CTTATATCGTATGGGGCTGACACTGTCCACCACTCGTCCG	*uidB* overlapping *tet*	This study
uidC-Tc3	CTAACGGATTCACCACTCCAACGCTGACATTTGCACCGAT	*uidC* overlapping *tet*	This study
Tc5	TCAGCCCCATACGATATAAG	*tet*	(Serra-Moreno et al., 2006)
Tc3	TGGAGTGGTGAATCCGTTAG	*tet*	(Serra-Moreno et al., 2006)
pBADf	ATGCCATAGCATTTTTATCC	pBAD Plasmid construct	Invitrogene
pBADr	GATTTAATCTGTATCAGG	pBAD Plasmid construct	Invitrogene
RR46-lp	GAGCTCTAAGGAGGTTAT	pKD46	(Serra-Moreno et al., 2006)
RR46-up	GTGCAGTACTCATTCGTT	pKD46	(Serra-Moreno et al., 2006)

2.5. Electroporation

Electrocompetent cells were prepared from 50 ml of culture in super optimal broth (SOB) medium (Hanahan, 1983) with 0.05% L-arabinose as previously described (Sambrook and Russell, 2001), mixed with the corresponding amount of DNA (plasmid or PCR-amplified, 0.5 μg) and transferred to a 0.2 cm electroporation cuvette (Bio-Rad, Inc.). The cells were electroporated at 2.5 kV, 25 F and 200 Ω. Cells containing the construct were recovered on MSB agar with the corresponding antibiotic.

2.6. Generation of a tailored CB10 strain

The *E. coli* strain WG5 (the wild-type strain: wt) recommended in the ISO standard method for the detection of somatic coliphages (Anonymous, 2000) was genetically modified in several steps.

2.6.1. *uidA* overexpression

A fragment containing the gene encoding the β-glucuronidase enzyme (*uidA*, 1847 bp) was amplified by PCR with primers UidA-For2/UidA-Rev2 (Table 1) from wt *E. coli* and cloned using a pBAD-TOPO® TA Expression Kit. The latter contains a pBAD-TOPO vector that allows gene insertion under the control of an *ara*BAD promoter (Invitrogene Corporation, Barcelona, Spain). The *uidA* gene was inserted into the plasmid following the manufacturer's instructions. The construct was separately transformed in electro-competent cells prepared with wt *E. coli*. In the resulting construct, pBAD:*uidA*, the gene was positioned in the correct orientation immediately downstream of the P_{ara} inducible promoter, as confirmed by PCR using primers pBADf/pBADr (Table 1) and sequencing.

The expression of *uidA* in the pBAD vector was optimized by adding arabinose to the medium at different final concentrations (ranging from 0 to 0.02%) as described by the manufacturer (Invitrogen Corporation, Barcelona, Spain). Finally, arabinose was added to a final concentration of 0.05% in the media. Both vectors contain an ampicillin marker, therefore the strains containing the plasmid construct were grown in the presence of ampicillin in an initial stage.

2.6.2. Construction of the uidB-tet-uidC fragment

The amplimer containing the *tet* gene was constructed, replacing the whole fragment comprising *uidB* and *uidC* genes. The primer pair Tc5-Tc3 (Table 1) was used for the amplification of the *tet* gene (1343 bp). The primer pair uidB-up/uidB-Tc5 amplified a 5′ region of the *uidB* initial codon including a 20 bp tail homologous with the 5′ region of *tet* (279 bp). Primers uidC-Tc3/uidC-lp amplified the 3′ region of the *uidC* subunit (261 bp) (Fig. 1) including a 20 bp fragment homologue of the 3' region of *tet*. The

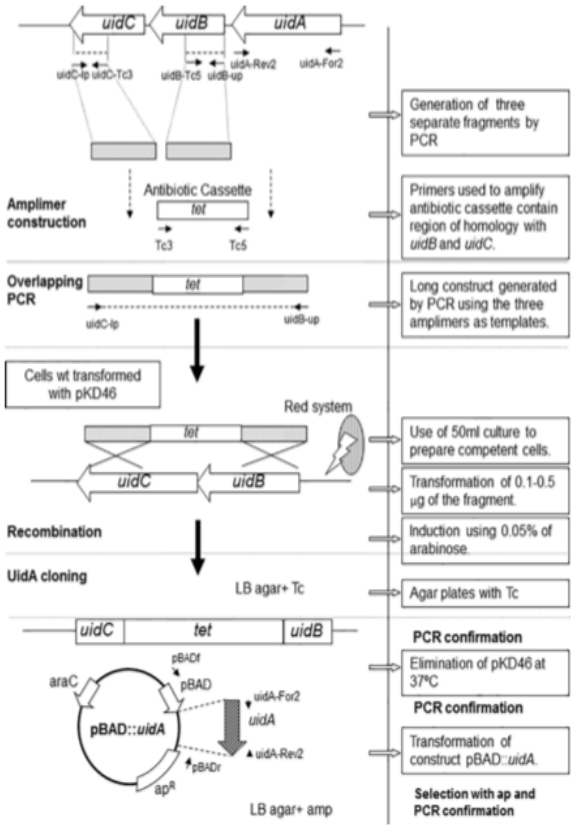

Fig. 1. Scheme showing the construction of strain CB10.

conditions used for all primer combinations were an annealing temperature of 50 °C and an elongation time of 60 s.

Fragments *uidB* and *tet* were annealed at their overlapping region (underlined letters in Table 1). They were then amplified by PCR as a single fragment with the external primers uidB-up and Tc3 (generating a fragment of 1602 bp) with an annealing temperature of 55 °C and an elongation time of 2 min. Fragments UidC and *tet* were annealed at their overlapping region with the external primers uidC-lp/Tc5 (underlined letters in Table 1) generating a fragment of 1584 bp. Finally, the two fragments, of 1602 and 1584 bp, were annealed at their overlapping region (the *tet* gene) and were amplified by PCR as a single fragment with the external primers uidB-up/uidC-lp, generating the final fragment (1843 bp), in which the sequence between genes *uidB* and *uidC* was replaced by the *tet* gene. The uidB-tet-uidC fragment was excised from the gel and purified using the Qiaquick Gel Extraction Kit (Qiagen Inc.,

Valencia, USA). The final product was used for the electro-transformation in the wt strain.

2.6.3. Transformation of pKD46

Plasmid pKD46 (GenBank AY048746) was transformed by electroporation in electrocompetent wt *E. coli* cells prepared as described above. Transformed colonies were selected by resistance to ampicillin and the presence of pKD46 was confirmed by PCR, using the primer pair RR46-up/RR46-lp and sequencing.

2.6.4. Recombination of uidB-tet-uidC

The transformation of 30 µl of the fragment 6 (UidB-tet-UidC) PCR amplimer (corresponding to 0.1–0.5 µg of amplified DNA) was performed in electrocompetent wt cells containing vector pKD46. These cells were prepared from 50 ml cultures (approximately 5×10^{10} CFU/ml) grown at 30 °C in SOB medium with ampicillin and 0.05% L-arabinose to an OD_{600} of 0.6. The transformed cells were recovered in the presence of Tc (10 µg/ml). Colonies in which *uidB* and *uidC* were replaced by fragment 6 were confirmed by PCR, using the *uidB-UidC* primers, and by sequencing.

The strain was forced to lose the plasmid pKD46 by several growing steps at 37 °C (pKD46 cannot replicate over 30 °C) without ampicillin. The loss of the pKD46 plasmid was confirmed by PCR.

2.6.5. Construction of CB10

The mutant lacking the *uidB* and *uidC* genes was transformed with plasmid pBAD:*uidA* generating strain CB10, Δ*uidBuidC*:*tet* (pBAD::*uidA*). The presence of plasmid pBAD was confirmed.

The performance of strain CB10 in detecting somatic coliphages was compared with that of the wt strain using a suspension of phages SOM3, SOM23 and ΦX174 propagated in the laboratory and with phages occurring in environmental samples.

2.7. Assays of the Bluephage method

Strain CB10 was inoculated in standard MSB with ampicillin, and incubated overnight at 37 °C. One ml of the overnight culture was 1/100 diluted in fresh MSB with ampicillin and grown to the exponential growth phase, monitored by an optical density (OD) at 600 nm of 0.3. At this stage, 0.5 ml of this culture was added to 2.5 ml of the MSB medium containing 0.05% arabinose, 0.5% glycerol, 15 µl CaCl 1 M and 0.1 mg/ml of X-glucuronide (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt; $C_{20}H_{26}BrClN_2O_7$) as the substrate. As indicated in each experiment, aliquots of dilutions of the corresponding phage suspension were added to each tube and one tube without phages was kept as a control. The tubes were statically incubated at 37 °C and periodically evaluated for color change from light yellow to blue. The experiment lasted until the phage-free control tube turned blue (a minimum of 7 h). The method was also adapted for quantification using the most probable number (MPN) method.

2.8. Spot test confirmation of phages detected by Bluephage

From each tube inoculated using the *Bluephage* method, after the reported incubation period, 15 µl aliquots were taken and spiked onto an agar monolayer containing strain CB10 according with the ISO method (Anonymous, 2000). The plates were side-up incubated at 37 °C. Plaques appearing in the spot area were considered positive for the presence of phages in a given sample. Positive results for this spot test were compared with positive results in the *Bluephage* tube to evaluate the sensitivity of the new method.

For a quantitative comparison, the MPN method was applied to the positive spot tests and the positive tubes obtained with the *Bluephage* method, and compared with the number of phages detected by the conventional ISO method (Anonymous, 2000).

2.9. Sensitivity of the Bluephage method

To evaluate the sensitivity of the method and the minimum incubation time needed for the analysis, somatic coliphages were enumerated in natural water samples using the ISO method (Anonymous, 2000). The samples were filtered through 0.22 µm low-binding membranes (PES, Millipore) and diluted to reach estimated lower densities of phages. Each dilution was evaluated in triplicate using the *Bluephage* method. The minimum time to obtain results was reported.

The performance of the test was assayed at 20 °C, 25 °C, 30 °C, 37 °C and 44 °C.

Wastewater and river water samples filter-sterilized through high-protein binding membranes of 0.22 µm (Millex-MF Millipore) to remove phages were used as additional controls.

2.10. Assay protocol in solid samples: sludge and mussels

To evaluate the performance of the method in matrices other than water, sludge samples from the digestor of a wastewater treatment plant in the Barcelona metropolitan area, which serves approximately 500,000 people, and mussels obtained from local supermarkets were processed. The method for phage extraction from sludge corresponds to the methods used in established policies, as described previously by Lasobras et al. (1999). with minor modifications. Briefly, the samples were mixed in a 1:10 (w/v) ratio with PBS (pH = 7.4) and homogenized by magnetic stirring for 30 min at room temperature. The suspension was then centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was subsequently filtered through low-protein-binding 0.22 µm pore size membrane filters (Millex-GP, Millipore, Bedford, MA). The suspension was diluted and used to enumerate phages using the standard ISO method and analyzed in triplicate using *Bluephage*.

For the study of phages in mussels, samples were spiked with somatic coliphages from raw sewage to reach a final estimated concentration of 10^2-10^3 PFU/g of mussel. Phages were incubated with the samples for 2 h at 23 °C. There is no established guideline for phage extraction from mussels, so previously reported methods were used (Lucena et al., 1994). Briefly, mussel meat from a minimum of 10 mussels per sample was homogenized in a blender for 5 min. Elution of phage particles was performed on 100 ml of the homogenate by magnetic stirring for 15 min in 400 ml of borate buffer (0.01 M) containing 3% beef extract (pH 9.5). After neutralization to pH 7.2, the treated homogenate was clarified by centrifugation at 2500 × *g* for 15 min at 4 °C. Phages present in the supernatant were enumerated by the ISO standard procedure and detected by the *Bluephage* method.

2.11. Scaling up and down of the Bluephage method

To evaluate the performance of the method in different sample volumes, 100 ml bottles were used with 50 ml of river water samples diluted to contain phage densities of 10 PFU/ml. Similarly, small volumes of 1.5 ml containing densities from 10^4 to 0.1 phages were used. Both assays were performed keeping the same proportion of nutrients, substrate and strain CB10 as in the tubes.

2.12. Statistical analysis

Computation of data and statistical tests were performed using the Statistical Package for Social Science software (SPSS). The ANOVA test was used to evaluate the differences between MPN,

spot test and *Bluephage* results; evaluations were based on 5% of significance levels.

3. - Results

3.1. - Method rationale and strain performance

The genetically modified *E. coli* strain (referred to henceforth as strain CB10) described in this study was grown in the presence of the substrate without its consumption, since the substrate cannot be incorporated inside the cell in the absence of transporter genes. Only when phage infection caused cell lysis was β-glucuronidase, which is overexpressed in the presence of arabinose and accumulated in the cell cytoplasm, released into the medium where it encountered the substrate. Even as few as 1 to 5 somatic coliphages were able to lyse a number of cells exceeding 10^8 in less than 3 h, thus liberating a large amount of enzyme into the medium. The enzyme in the medium cleaves the D-glucuronide in the 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid molecules, releasing the blue substrate that turns the broth (originally light yellow) dark blue. The time necessary for this color change varied depending on the number of bacteria lysed and hence on the concentration of phages added to the cell culture and the time elapsed after infection.

The performance of CB10 in the presence of the substrate was evaluated before using it for phage detection. To do this, the *E. coli* WG5 (wt) strain containing pBAD:*uidA* and CB10 (with and without phages) were compared when inoculated into the broth for the *Bluephage* method. We observed a slight but visible color change with the wt *E. coli* (Fig. 2). Meanwhile, strain CB10 had no effect on the color in the absence of phages, because of its incapacity to incorporate the substrate caused by replacement of the *uidBuidC* complex. The highest expression of the enzyme was observed in the wt strain containing pBAD:*uidA* in the presence of 0.02% arabinose, which produced a darker blue than the wt strain. Finally, on adding phages from wastewater (we used a high quantity of 10^3 PFU to test the method under optimal conditions), CB10 caused a change of color after only 1.5 h of incubation (Fig. 2).

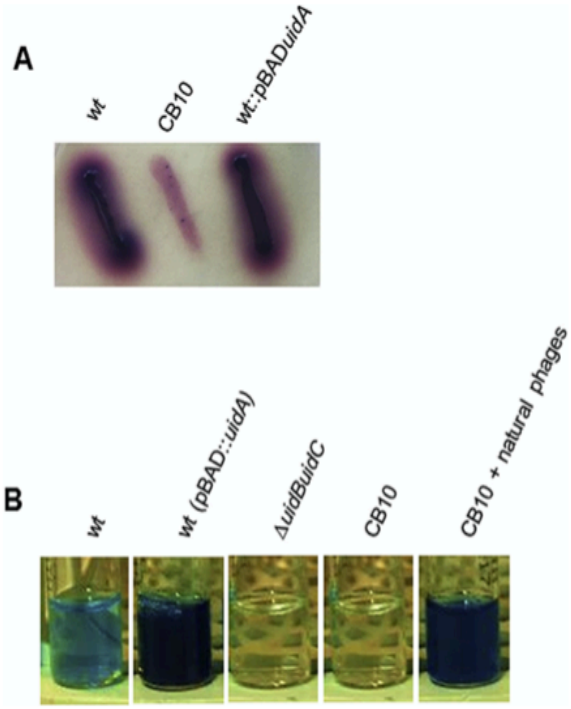

Fig. 2. - Confirmation of the incapacity of the strain CB10 to incorporate the substrate in the absence of phages and of the overexpression of the *uidA* gene under the control of the arabinose promoter. A: Growth on a Chromocult® agar plate containing X-glucuronide. B: Growth in *Bluephage* liquid medium. The tube containing natural occurring phages was inoculated with 10^3 phages.

3.2. - The CB10 strain and the standard method detected the same number of phages

To confirm that the modification of the wt strain did not cause a decrease in the number of phages detected, its performance was compared with the wt strain, according to the ISO method (Anonymous, 2000). The phage counts obtained with both strains showed no significant differences ($P > 0.05$) (Table 2).

3.3. Performance of the Bluephage method with environmental samples

Filtered wastewater and river water samples were 10-fold diluted and added to the *Bluephage* tubes containing strain CB10, at descending phage densities. In Fig. 3, the tubes correspond to estimated 500, 50, 5 and ≤ 1 PFU/tube (calculation based on the dilution of the sample), and one control tube that contained only CB10 and no phages. The cultures were incubated at 37 °C and the color change was monitored over time (Fig. 3). The first positive results were observed at 1:30 h for concentrations of 50 and 500 phages. Cultures containing 5 phages turned blue after 2:15 h. Nevertheless, the tubes were incubated until the control culture turned blue, which required a minimal incubation time of 7 h. Samples were considered negative for the presence of phages if blue coloration did not occur before the time needed for the control sample to change color (Fig. 3).

Potentially polluted wastewater and river samples were 10-fold diluted and the number of somatic coliphages was evaluated using the ISO method (Table 3). The color change was monitored after 2:30 h, 3:30 h and 4:30 h, times that were selected to generate results within one working day. The resulting number of positive tubes out of the total number of tubes assayed at the different times for each phage density are presented in Table 3. Notably, at higher phage densities, detection occurred after 2:30 h. Some samples with high phage densities turned blue even earlier, at 1:30 h (data not shown). In general, tubes with high phage densities changed color earlier than those with low densities (Table 3), although a clear correspondence between the number of phages and the time required for the color change was not always observed, as discussed below.

The minimal time to observe color change in tubes with <5 PFU was 2:30 h, as for example in tubes with densities of 0.7 PFU/tube in wastewater or 3.4 PFU/tube in river water (Table 3). Nevertheless, at 4:30 h, all the tubes containing >10 PFU/tube were positive and 46% of the tubes containing 1–10 PFU/tube had also turned blue. This time is well within the time frame in which the phage-free negative control remained without changing color. In parallel, no changes were observed in the filter-sterilized diluted wastewater and river water control samples from which the phages had been removed.

3.4. Results using the Bluephage method at different temperatures

The above mentioned results were obtained when incubating at 37 °C, with the same results obtained at 44 °C. In contrast, at 20 °C and 25 °C, longer incubation times (5 h) were required before the color change was detected in the tubes containing 500 PFU. Accordingly, the phage-free control tube also remained unaltered for longer, and no change of color was observed before 12 h of

Table 2
- Enumeration of phages by the ISO method comparing the wt and the mutant CB10 as host strains in wastewater (WW), river water (RW) and using laboratory phages as controls.

Sample Ref.	Source	Wt (PFU/ml)	CB10 (PFU/ml)	Log_{10} difference
WW1	Wastewater	4.95×10^4	3.80×10^4	0.11
WW2	Wastewater	7.27×10^3	8.56×10^3	−0.07
WW3	Wastewater	1.95×10^4	2.06×10^4	−0.02
WW4	Wastewater	7.91×10^3	3.34×10^3	0.37
WW5	Wastewater	7.26×10^3	5.12×10^3	0.15
RW1	River Water	4.77×10^a	3.36×10^a	0.15
ΦX174	Laboratory	5.70×10^{10}	3.20×10^{10}	0.25
Mixture of phages[a]	Laboratory	$2.54 \cdot 10^6$	$2.14 \cdot 10^6$	0.07

[a] Mixture is composed by phage ΦX174 (Anonymous, 2000), and environmental *Myoviridae* (SOM3) and *Siphoviridae* (SOM23) phages.

incubation.

3.5. Sensitivity and specificity of the Bluephage method

To confirm that the color change in inoculated tubes was due to the presence of phages, the supernatant of each tube (including the negative tubes) underwent a spot test on strain CB10. Positive results of both *Bluephage* and the spot test were used to enumerate the phages using the MPN method. The results recorded after 3.5 h of incubation by the *Bluephage* method and by the spot test were used to determine the phage densities using MPN calculations (Fig. 4). In all cases, the values obtained with *Bluephage* were the same as or slightly lower than the spot test values. However, the results showed no statistically significant ($P > 0.05$) differences between them.

As indicated above, only results obtained within an acceptable time frame for the method, during which the control did not change color, were considered and used for the calculations.

3.6. The Bluephage method is applicable to phages extracted from solid samples

To evaluate any limitation of the method when using somatic coliphages extracted from matrices other than water, two types of solid samples related with water pollution, sludge and mussels, were evaluated. The presence of phages in wastewater sludge and mussels was detected by the *Bluephage* method after 2:30 h of incubation, when no change was observed in the control (Table 4) or before 7 h of incubation. As in the water samples, most samples with densities of up to 10 PFU/tube, and some with even lower densities (Table 4), were already positive at 1:30 h (data not shown). All the samples with fewer than 3 phages/tube were negative, since no change of color was observed before the control turned blue.

3.7. The Bluephage method is applicable at different scales

When using larger volumes, phages were detected by the *Bluephage* method whenever the proportions of nutrients, substrate and the host strain CB10 were maintained. Volumes of 50 ml of samples containing low phage densities (10 PFU/l of sample, which corresponds to 500 PFU in each bottle) produced positive results visible after 1:30 h of incubation (Fig. 5A).

Similarly, the method was successfully down-scaled, which is useful for quantification based on the MPN technique. As in the tubes, the *Bluephage* method allowed detection of somatic coliphages in sample dilutions containing 100 PFU/well or over at 1:30 h and 10 PFU/well or over at 3:30 h (Fig. 5B). Even a well containing just 1 PFU started to turn blue after 3:30 h (Fig. 5B, 3:30 h, 2nd row).

4. Discussion

The standardized ISO and U.S. EPA protocols for the detection and enumeration of somatic coliphages consist of a multiple-step procedure involving coliphage replication in exponential-growth-phase host cells. ISO-10705-2 (Anonymous, 2000) includes both a double-agar-layer (DAL) plaque assay for the quantification of PFU and the presence/absence test that can also be adapted to the MPN format. U.S. EPA Method 1601 (U.S. EPA, 2001a) is a presence/absence method and U.S. EPA Method 1602 (U.S. EPA, 2003b) a single-agar-layer (SAL) plaque assay. Plaque assays provide results more rapidly, and in both methods, plaques can be obtained after 6 h. However, up to two additional hours are needed for pre-growth of the host strain, and the overall recommended assay length is 18 h.

Alternative protocols based on the detection of a molecule released by host cell lysis have also been reported (Guzmán-Luna et al., 2009; Ijzerman et al., 1993). These methods provide insight into the number of infectious bacteriophages in a given sample. They are also relatively fast because of the short time needed for the initial and subsequent replication cycles, which allows a high number of lysed cells to be obtained in 2–3 h. Thus, Ijzerman et al. (1993). report a method based on rapid extracellular release and detection of a β-galactosidase enzyme during the coliphage-induced lysis; the drawbacks are false positives and long incubation times required when the phage number is low. Later, Guzmán Luna et al (Guzmán-Luna et al., 2009). described a method based on the detection of somatic coliphages via a bioluminescence assay measuring the phage-mediated release of adenylate kinase and subsequent adenosine 5′-triphosphate detection. These methods have been improved and adapted to multiple tube serial dilutions based on the U.S. EPA and ISO presence/absence standards (Salter and Durbin, 2012; Salter et al., 2010), with results available within one working day. Additionally, the test is facilitated by the incorporation of lyophilized *E. coli* tablets.

The *Bluephage* method presented here takes advantage of the overexpressed β-glucuronidase enzyme, which is not secreted into the medium until the cells are lysed. Therefore, in the absence of bacteriolysis, contact between the intracellular enzyme and its specific substrate is precluded. As the substrate remains uncleaved, false positive results are avoided during the time needed to complete the screening. In the presence of bacteriophages, the bacterial cells are lysed, releasing the enzyme into the medium where it finds and cleaves the substrate, inducing a change of color when enough cells are lysed. The general trend observed was that higher phage densities caused a faster change of color than lower densities, although with some exceptions. The variability observed at low phage densities has two explanations. First, the possibility of inoculating a single phage can vary from tube to tube when the densities are low. Second, the somatic coliphages are heterogeneous, as they belong to different families with a variety of burst

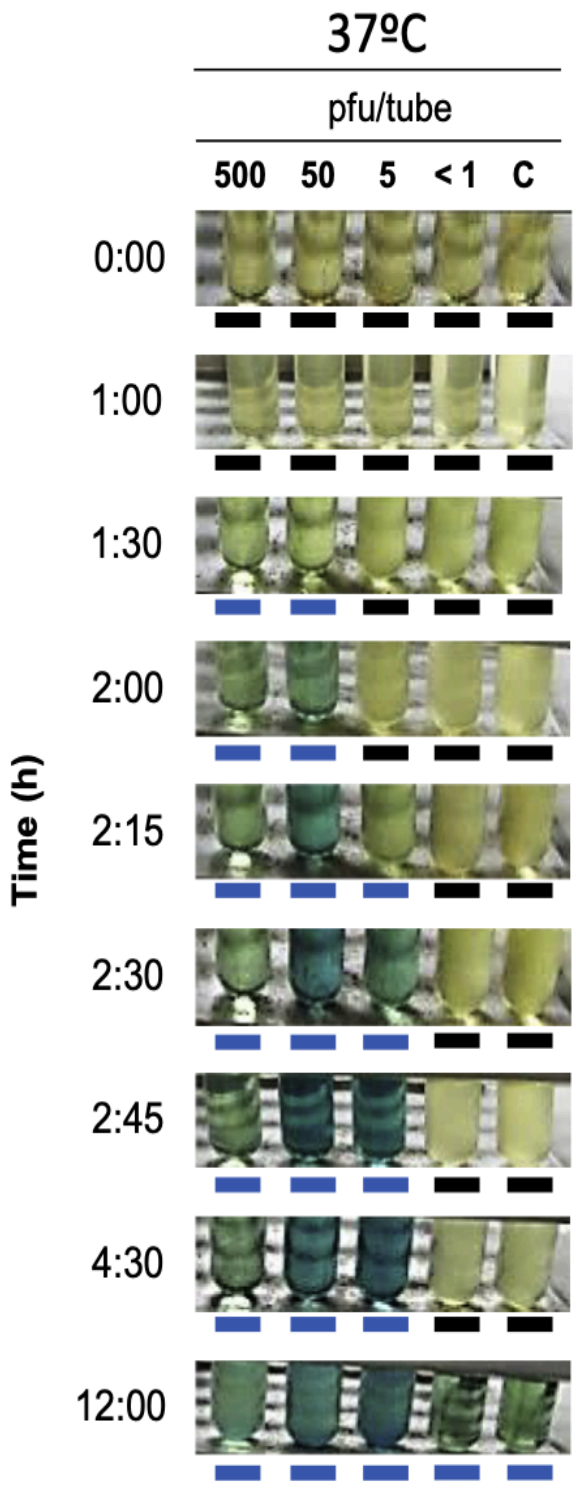

Fig. 3. - Example of color change of the *Bluephage* tubes in the presence of decreasing phage densities at different times. Blue indicates a positive result while yellow indicates negative results.

sizes and times required for replication (Muniesa et al., 1999).

Considering that 1.5–2 h are required to prepare strain CB10 at an OD_{600} of 0.3 for inoculation into the tubes, and that the method allows detection of one phage per tube in 3.5 h, the maximum test duration is of 5.5 h: well within one habitual working day. It should also be considered that the host strain can be grown to an OD_{600} of

Table 3
Comparison between the number of phages enumerated by the ISO method or by using *Bluephage*in dilutions of river water, urban raw wastewater and a mixture of phages.

Sample	ISO PFU/tube[a]	*Bluephage*		
		Positive tubes/total tubes		
		2:30 h	3:30 h	4:30 h
River 1	33.6	3/3	3/3	3/3
	16.8	2/3	2/3	3/3
	3.4	1/3	1/3	2/3
	1.7	0/3	0/3	2/3
	0.3	0/3	0/3	0/3
River 2	43	1/3	2/3	3/3
	21.5	1/3	2/3	3/3
	4.3	0/3	1/3	1/3
	2.1	0/3	0/3	1/3
	0.4	0/3	0/3	0/3
Wastewater 1	85.6	3/3	3/3	3/3
	17.1	1/3	3/3	3/3
	8.6	1/3	3/3	3/3
	1.7	0/3	2/3	2/3
	0.9	0/3	0/3	1/3
	0.2	0/3	0/3	0/3
Wastewater 2	33.4	3/3	3/3	3/3
	6.7	3/3	3/3	3/3
	3.3	2/3	3/3	3/3
	0.7	1/3	2/3	2/3
	0.1	0/3	0/3	0/3
Phages mixture[b]	49.9	1/1	1/1	1/1
	16.6	1/2	1/2	2/2
	8.3	1/2	1/2	2/2
	5.0	0/2	0/2	0/2
	1.7	0/2	0/2	1/2
	0.5	0/2	0/2	0/2

[a] Measured in the water samples by ISO method, numbers correspond to the estimated calculation based on the dilution of the sample.
[b] Mixture composed by phage ΦX174, one *Siphoviridae* phage and one *Myoviridae* phage.

0.3 before the sample arrives at the laboratory (Anonymous, 2000). Compared to the aforementioned methods based on the detection of a molecule or molecules released by host cell lysis, the method reported herein is faster, and gives a much lower percentage of false positive results; furthermore, it needs minimal laboratory equipment.

The change of color observed in the phage-free controls after 7 h was attributed to spontaneous cell lysis after a certain incubation period, but this is sufficient time to ensure phage detection in the sample. It cannot be excluded that the presence of bacteriolytic agents other than phages can lead to false positive results, which could also be caused by the presence of high concentrations of the glucuronidase enzyme or glucuronidase-positive bacterial strains in the samples. This can occur with highly polluted undiluted wastewater, and the high density of phages occurring found in such samples produces a color change before glucuronidase can have the same effect. In any case, the *Bluephage* method requires the previous filtration of the sample to reduce the possibility of other bacteriolytic agents and particularly for the removal of other bacterial strains possessing glucuronidase activity. Filtration of natural samples through 0.22 μm low-protein binding membranes is already used in the standard methods for somatic coliphage detection (Anonymous, 2000), although it is not mandatory.

Importantly, the *Bluephage* method shows the same sensitivity as the standard ISO method. The CB10 strain does not lose its capacity to detect somatic coliphages after its modification, and it was confirmed that all positive enzymatic activity results were due to the presence of phages in the sample. Moreover, *Bluephage* is useful for the analysis of water and solid matrices such as biosolids and

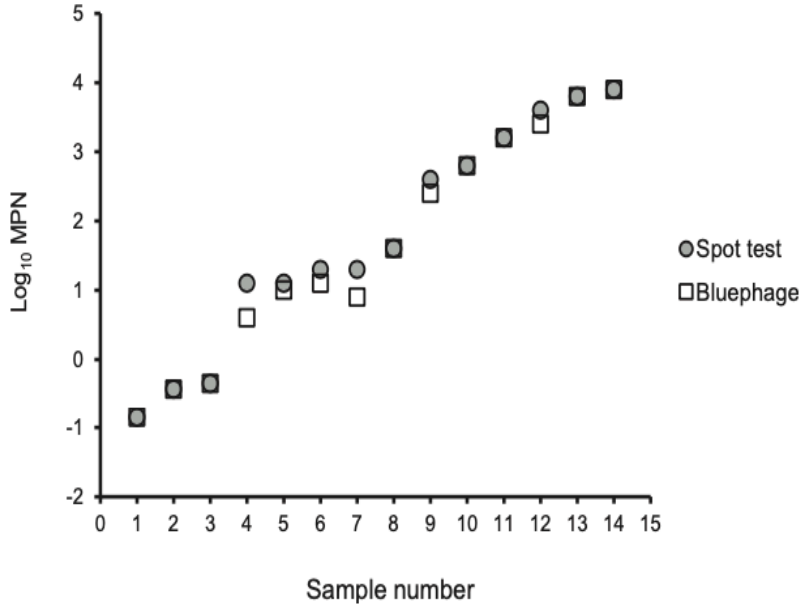

Fig. 4. Mean MPN for the *Bluephage* method (n = 14) and the spot test (n = 14) compared across samples containing different concentrations of somatic coliphages. Mean MPN were not significantly different (p > 0.05) according to ANOVA test.

Table 4
Performance of the method in 2:30 h for phages in solid matrices.

Sample	wt strain (PFU/ml or g)	Dilution	Theoretical PFU/ml	Positive tubes at 2:30 h	Control performance (2:30 h)
Sludge 1	$1.89.10^3$	D	945	3/3	No change
		−1	94.5	3/3	
		−2	9.45	3/3	
Sludge 2	$1.03.10^3$	D	515	3/3	No change
		−1	51.5	3/3	
		−2	5.15	2/3	
Sludge 3	$8.91.10^2$	D	445	3/3	No change
		−1	44.5	3/3	
		−2	4.45	1/3	
		−3	0.45	0/3	
Mussels 1	$1.55 .10^2$	D	155	3/3	No change
		−1	15	3/3	
		−2	1.5	0/3	
Mussels 2	$4.09.10^3$	D	1600	3/3	No change
		−1	33.5	3/3	
		−2	3	1/3	
		−3	<0.25	0/3	

shellfish. The method can also be scaled up for large volumes and adapted to small volumes for MPN quantification, mimicking kits available for the detection of bacterial indicators. In the current work, our main intention was to establish the suitability of the method for use in different conditions and matrices, but a more exhaustive analysis should be done in the future to evaluate the significance of low PFU in these formats.

The rapid performance of the method, providing results within the same day of sampling, would be an advantage when a fast, immediate or continuous response to the quality of a water body is required: for instance, to provide safe drinking water, trace the origin of a water-borne outbreak, evaluate the quality of recreational surface waters in the morning before the users arrive, monitor a treatment plant or a disinfection device, or evaluate the water used in food production.

The method could be improved still further without involving substantial changes by using fluorogenic instead of colorimetric detection and lyophilized strains that are easier to store, although in this case, the time frame necessary to generate visible results should be validated. An adaptation of *Bluephage* to detect β-glucuronidase activity using already available online platforms (Hesari et al., 2015; Togo et al., 2007) seems feasible and would provide an automated method.

5. Conclusions

- The widespread incorporation of somatic coliphage detection in water management policies requires user-friendly methods. *Bluephage* can facilitate routine implementation of somatic coliphage determination in laboratories with minimal requirements.
- *Bluephage* allows the detection of up to 1 somatic coliphage in under 3.5 h, well within a working day. This improves the sensitivity reported for other available methods
- The method has been successfully applied to diverse sample types and volumes, using presence/absence and quantitative analysis, which confirms its suitability for use with a variety of food and water samples

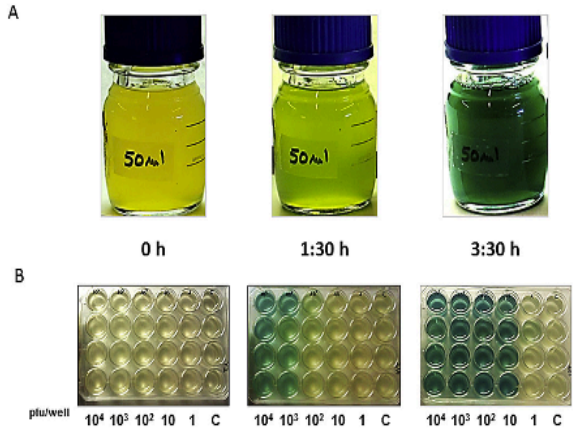

Fig. 5. - A: *Bluephage* method for volumes of sample of 50 ml containing phages at a concentration of 10 PFU/ml after different times. B: Scale-down of *Bluephage* method using replicates inoculated with phages from wastewater samples at different concentrations and times.

- This microbiological method for the determination of fecal pollution via detection of culturable microorganisms is the fastest reported to date, and can provide results where rapid information of fecal pollution is essential.

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