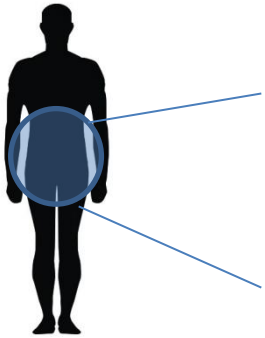
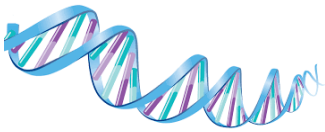
Graphical Abstract

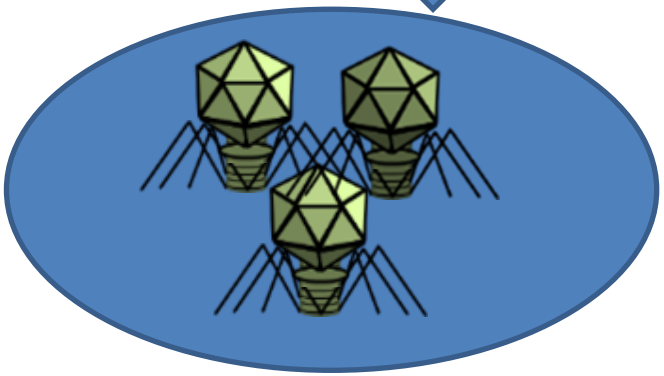


Metagenomics



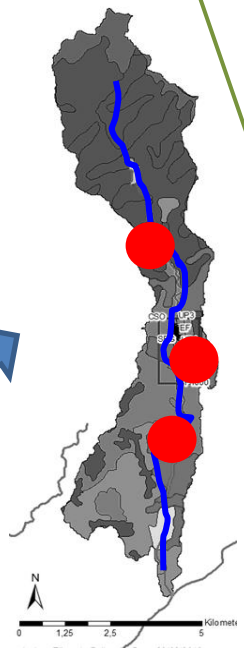
crAssphage DNA

qPCR

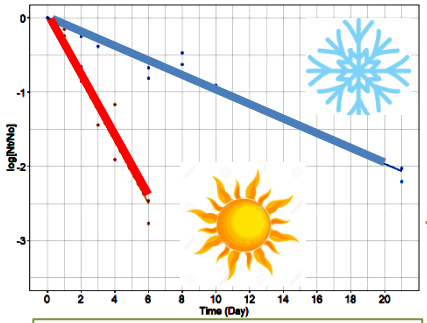


crAssphage detection
in waterbodies

River +
sediment

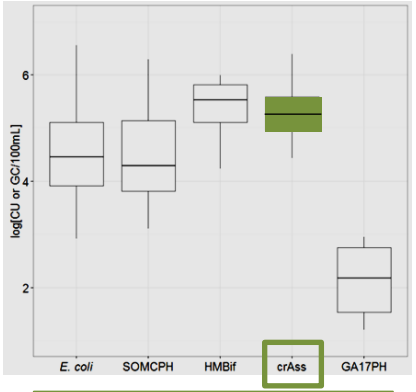


mesocosm



Persistent

Human specific



Abundant

Human MST marker

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

E. Ballesté *, M. Pascual-Benito, J. Martín-Díaz, A.R. Blanch, F. Lucena, M. Muniesa, J. Jofre, C. García-Aljaro.

Department of Genetics, Microbiology and Statistics, Microbiology section. Faculty of Biology. University of Barcelona. Diagonal 643. E-08028 Barcelona. Spain.

*To whom correspondence should be addressed. Elisenda Ballesté: Tel:+349340309040; Fax:+34934039047. eballeste@ub.edu

Running Title: crAssphage dynamics in the environment

Keywords: crAssphage; Microbial Source Tracking; Faecal pollution; Environmental dynamics; 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 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-

110 Aljaro et al., 2017a). Both markers showed similar abundance in human samples
111 and were absent in animal samples or found in lower amounts than in human
112 samples (Ahmed et al., 2018; García-Aljaro et al., 2017a; Stachler et al., 2017).

113 The evaluation of the abundance, persistence, inactivation, and resuspension of FIB
114 and MST in the environment requires an understanding of their behaviour and the
115 availability of data for modelling purposes, as shown in previous studies (Ballesté et
116 al., 2018; Bell et al., 2009; Blaustein et al., 2013; Drummond et al., 2014; Garcia-
117 Aljaro et al., 2017b; Jamieson et al., 2005). To assess the potential of crAssphage as
118 a human MST marker in the environment, the aim of the present work was to
119 measure its dynamics in potentially human-polluted water samples. Thus, the
120 abundance of crAssphage was determined in sewage effluents and rivers with
121 different pollution impacts, including water and sediments. CrAssphage removal in
122 a river with human point pollution was monitored to determine the effect of
123 environmental parameters. Additionally, inactivation was assessed *in situ* using
124 previously developed mesocosms experimental approaches (Ballesté et al., 2018).
125 The dynamics of crAssphage were compared with those of other bacterial and viral
126 faecal indicators and MST markers detected by culture-based and molecular
127 techniques: *E. coli*, somatic coliphages (SOMCPH), bacteriophages infecting
128 *Bacteroides* GA17 strain (GA17PH) (Gómez-Doñate et al., 2011; Payan et al.,
129 2005), the human-associated *Bifidobacterium* marker HMBif (Gómez-Doñate et al.,
130 2012) which has been designed and applied in the area of study (Casanovas-
131 Massana et al., 2015; Yahya et al., 2017) and the human-associated *Bacteroides*
132 marker HF183 (Haugland et al., 2010) which is being used as MST in a wide
133 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 1200 $m^3 s^{-1}$ 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 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) < 13ºC), 3 at an intermediate temperature (Tmax between 13 and 25ºC), and 2 at a high temperature (Tmax > 25ºC) (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 4ºC 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 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 25ºC) and winter (water temperature of around 14.5ºC). 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 44.5ºC for 24 h (ISO, 2000a). Results were expressed as CFU 100 ml^{-1}. Somatic coliphages were enumerated according to ISO10705-2 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 -20ºC 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., 2017a), the human-associated

249 *Bifidobacterium* marker HMBif (Gómez-Doñate et al., 2012) and the HF183 marker
250 (Haugland et al., 2010), using the primers, probes and protocols previously
251 described. Amplification was performed in a 20 µl reaction mixture using TaqMan
252 Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA) in a
253 StepOne Real-Time PCR System (Applied Biosystems). Each mixture contained the
254 corresponding concentration of each primer and probe (García-Aljaro et al., 2017a;
255 Gómez-Doñate et al., 2012; Haugland et al., 2010) and 5 µL of the DNA sample.
256 The thermal-cycler conditions were 10 min at 95°C, 40 denaturation cycles of 15 s
257 at 95°C, and 1 min of annealing and extension at 60°C.

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

267

268 **2.6. Data analysis**

269 Microbial abundances were log-transformed and analysed for descriptive statistics.
270 Data were analysed and plotted using the statistical software R version 3.5.1 (R
271 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

295 correlation (Table 3, Table S1). Lower concentrations of all the markers were
296 detected in the WWTP using nutrient removal, but otherwise no clear pattern
297 associated with the sewage treatment was observed (Fig. S1).

298 After monitoring the crAssphage in the effluent of the same WWTP for two years to
299 assess temporal variability (intra-effluent comparison), an average abundance of
300 5.34 $\log_{10}$ gc 100 ml^{-1} of crAssphage was found(with a range from 4.65 to 6.72 $\log_{10}$
301 gc 100 ml^{-1}). No seasonality was detected: the minimum and maximum
302 concentration were observed in January and February 2016, respectively, with a
303 return to average levels after 15 days (Fig. S2). An abundance similar to crAssphage
304 was observed for HMBif (5.53 $\log_{10}$ gc 100 ml^{-1}) and HF183 (5.32 $\log_{10}$ gc 100 ml^{-1}),
305 a 1 $\log_{10}$ lower concentration for *E. coli* (4.42 $\log_{10}$ CFU 100 ml^{-1}) and SOMCPH
306 (4.26 $\log_{10}$ PFU 100 ml^{-1}), and almost 4 logs lower for GA17PH (1.69 $\log_{10}$ PFU
307 100 ml^{-1}) (Fig. 2b and Table 2). CrAssphage correlated best with HF183, and
308 moderately well with HMBif and *E. coli* (Table 3, Table S1).

309

310 **3.2. CrAssphage evaluation in a river highly impacted by pollution**

311 CrAssphage abundance were measured in water samples collected in the Llobregat
312 River over one year (n=11). All the samples were positive for crAssphage, with an
313 average abundance of 5.42 $\log_{10}$ units gc 100 ml^{-1} (Table 2, Fig. 2c). These values
314 were similar to those already obtained for the molecular markers HMBif and *E. coli*
315 (detected by qPCR), 1 $\log_{10}$ unit higher than for culture-detected *E. coli* and
316 SOMCPH, and 3 $\log_{10}$ units higher than GA17PH (Garcia-Aljaro et al., 2017). A
317 significant correlation was observed with GA17PH and *E. coli* by qPCR (Table 3,
318 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 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 to 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 waterbody. 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 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 28ºC (mean of 25ºC) 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 8.5-11ºC and maximum global solar radiation of 273-871 W m^{-2}, the marker was detected for up to 21 days (the duration of the

394 experiment) (Fig. 5). The average obtained T_{90}s were 2.41 days in summer and
395 10.21 days in winter. These values were similar to or slightly higher than for *E. coli*
396 and other MST markers in summer (T_{90} of 1.05 days for HF183, 2.20 days for
397 HMBif, 1.52 days for *E. coli* culture and 2.62 days for *E. coli* qPCR) and much
398 higher in winter (T_{90} of 3.26 days for HF183, 6.12 days for HMBif, 2.06 days for *E.*
399 *coli* culture and 4.26 days for *E. coli* qPCR) (Table S2) (Ballesté et al., 2018). T_{90}
400 values for SOMCPH were similar to crAssphage in winter (9.90 ± 1.58 days) and
401 higher in summer (4.84 ± 0.30 days).

402

403 **4. DISCUSSION**

404 The recently described *Bacteroides* bacteriophage crAssphage is reported to be one
405 of the most abundant viruses in sewage and human faecal samples (Dutilh et al.,
406 2014; Shkoporov et al., 2018; Stachler and Bibby, 2014). Due to its prevalence, it
407 has been postulated as a potential human MST marker and two different methods
408 have already been developed for its application. The assay designed in the USA
409 (CPQ_056 and CPQ_064) showed the marker was highly abundant in raw sewage
410 (9.1-10.0 $\log_{10}$ gc L^{-1}), with a high specificity and sensitivity, although cross-
411 reactivity has been observed, mainly with poultry samples (Ahmed et al., 2018;
412 Stachler et al., 2017). The other method developed in Europe, was adapted and
413 validated to the crAssphage sequence detected in the area, that shows few
414 differences with the crAssphage sequence in USA (García-Aljaro et al., 2017a). This
415 assay revealed a similar marker abundance to that of CPQ_056 and CPQ_064 in raw
416 sewage (8.4-9.9 $\log_{10}$ gc L^{-1}) and high sensitivity, although with some occurrence in
417 animal samples. However, the concentration in non-targeted samples was found to

418 be 2-4 orders of magnitude lower than in untreated sewage samples (García-Aljaro
419 et al., 2017a; Stachler et al., 2017). Given that this marker targets a virus, and its
420 concentration is at least two orders of magnitude higher than enteric viruses
421 (Ahmed et al., 2018), it could be a more effective pathogen indicator than the
422 classical faecal indicators *E. coli* and enterococci. Another advantage is that it is a
423 molecular method and the same DNA extraction can be used to analyse
424 complementary faecal indicators to improve the source identification (Casanovas-
425 Massana et al., 2015). However, to date no studies have determined crAssphage
426 dynamics in the environment, which is fundamental for its application for water
427 management purposes.

428 In our study, crAssphage was detected in WWTP effluents at higher concentrations
429 (1 order of magnitude) than the general culture-based indicators SOMCPH and *E.*
430 *coli*, whereas its abundance in raw sewage was similar to that of other human
431 molecular MST markers like HMBif and HF183 (García-Aljaro et al., 2017a;
432 Stachler et al., 2017). In addition, crAssphage showed a concentration 3-4 logs
433 higher than infectious *Bacteroides* strain GA17 bacteriophages (GA17PH).
434 GA17PH has proved to be a good human-associated marker in the studied area and
435 has been used as a culturable indicator of human enteric viruses (Jofre et al., 2014;
436 Muniesa et al., 2012). The intra-effluent comparison showed that crAssphage
437 abundance was quite stable along the year, with variations possibly related to
438 rainfall events, which overwhelmed WWTP capabilities by increasing the flow.
439 Comparing crAssphage concentrations in the WWTP effluents in the current study
440 with those previously reported in untreated sewage (García-Aljaro et al., 2017a)
441 (mean of 8.24 $\log_{10}$ units gc 100 ml^{-1}) reveals a decrease of around 3 $\log_{10}$ after the

442 wastewater treatment. However, the crAssphage concentration in the effluents
443 discharged to the environment was similar to that of bacterial molecular markers and
444 higher than cultured bacterial and infectious viral markers. Similar values for faecal
445 indicators and human source tracking markers other than crAssphage were observed
446 in treated wastewaters from Europe and Northern Africa than in those of this work
447 (i.e. *E. coli* and SOMCPH around 4 $\log_{10}$ CFU and PFU 100 ml^{-1}, GA17PH between
448 0.1 to 3 $\log_{10}$ PFU 100 ml^{-1} and HF183 between 4 and 6 $\log_{10}$ gc 100 mL^{-1}) (Mayer
449 et al., 2018, 2016; Yahya et al., 2015; Wery et al., 2008). Meanwhile values of
450 around 2.5 $\log_{10}$ and 1-2 $\log_{10}$ gc 100 mL^{-1} were observed for human adenoviruses
451 and polyomaviruses in effluents in Europe (Bofill-Mas et al., 2006; Mayer et al.,
452 2016), 3-4 $\log_{10}$ lower concentration than crAssphage.

453 The abundance of crAssphage was measured in two different kinds of rivers: the
454 Llobregat River, which has a medium-high flow and a high human pollution impact,
455 and the temporal "Riera de Cànoves", which has a low flow and is impacted by
456 diffuse pollution (upstream of the WWTP effluent discharge). The same pattern of
457 abundances was observed in the two rivers and also in the WWTP effluents. The
458 molecular markers detected by qPCR (crAssphage and HMBif) were 2 orders of
459 magnitude more abundant than *E. coli* and SOMCPH detected by culture-based
460 methods, and almost 3 orders of magnitude higher than GA17PH. The concentration
461 of crAssphage in the Llobregat River remained stable throughout the 11 sampling
462 campaigns, with values similar to those of the WWTP effluents, thereby confirming
463 the low water quality of this river at this site (Casanovas-Massana et al., 2015). A
464 high concentration of crAssphage was also found in the sediments in the lower
465 transect of the river, similar to other qPCR markers and indicators (Garcia-Aljaro et

al., 2017b). 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 mobilization of sediments which, as already suggested may become a reservoir of faecal indicators (Calero-Cáceres et al., 2017; Garcia-Aljaro et al., 2017b). 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

514 al., 2018). Thus, DNA extractions stored at -80ºC were used to evaluate the
515 crAssphage inactivation during high and low temperature seasons and it was
516 compared with that of other human MST markers like HMBif and HF183 and faecal
517 indicators like *E. coli* and SOMCPH. A higher inactivation was observed in
518 summer, as already found for bacterial indicators, but the T_{90}s of crAssphage were
519 higher than those of the other MST markers and *E. coli* during both seasons,
520 suggesting a greater resistance to environmental parameters. This effect was
521 particularly significant in winter, when crAssphage was detected on more than 21
522 days compared to 10 days for the other MST markers, which had between 1.66 and
523 5-fold lower T_{90}. In winter the decay of crAssphage was similar to that of SOMCPH
524 (10.21 and 9.90 days, respectively), although in summer SOMCPH persisted for
525 longer. Other studies have reported similar results for infectious SOMCPH and
526 *Bacteroides* phages with T_{90}s of 3-4 days in summer and 14-16 days in winter
527 (Casanovas-Massana and Blanch, 2013) and 1.72 days in summer and 13.54 days in
528 winter (Wu et al., 2016). Thus, crAssphage has similar persistence to SOMCPH, the
529 slight variation being possibly due to the different methodological and
530 environmental parameters of each study.

531 These results confirm that in summer there are several inactivation parameters,
532 including higher temperature, higher irradiation and more active predators like
533 grazing protozoa and bacteria (Barcina et al., 1997; Menon et al., 2003; Wanjugi
534 and Harwood, 2013), which exert a similar effect on the different MST markers and
535 indicators. In contrast, in winter the intrinsic characteristics of the marker become
536 more significant (Ballesté et al., 2018), in this case being the higher resistance of a
537 viral particle. It should be noted that ΦCrAss001, the only bacteriophage isolated

538 from the crAssphage family, has an unusual way of replication; it can generate free
539 virions, and although not a temperate phage it interacts with *Bacteroides intestinalis*
540 without causing lysis in a sort of carrier state life cycle (Shkoporov et al., 2018).
541 Thus, phage virions should be able to coexist with bacterial cells carrying the non-
542 integrated phage. The proportion of each state in natural samples is unknown and
543 may be related to both intrinsic and extrinsic parameters. Therefore, the persistence
544 would vary depending on the crAssphage state (bacterial or virion). Likewise, the
545 virion state of crAssphage can maintain good correlation with SOMCPH despite the
546 differences in persistence shown by the bacterial state. In this work, crAssphage was
547 analysed by molecular methods from the total DNA isolated from samples, hence it
548 was impossible to discern whether the detected phage was in the virion state or
549 inside the bacterial cell. Differences in the phage state in different environmental
550 conditions (after inactivation or seasonal) could explain the variable correlations
551 with bacterial molecular markers or infectious phages found in this study.

552 In general, crAssphage correlated highly with other human-associated markers like
553 HMBif and HF183 and with *E. coli* in the wastewater effluents and in the "Riera de
554 Cànoves". In the highly polluted Llobregat River correlation was found only with
555 GA17PH and *E. coli* measured with molecular methods. The high level of pollution
556 in this river, with some sources far upstream, may alter the correlation between
557 parameters. On the other hand, crAssphage correlated with SOMCPH in the inter-
558 effluent comparison and also in sediments, where crAssphage correlated with all the
559 markers tested. The reduction of crAssphage also correlated with all the observed
560 indicators. Ahmed et al. (2018) observed similar trends in crAssphage and HF183 in
561 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 0.22µm 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. 2017a). 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.

586 Some controversy appears when proposing molecular or culture-based methods for
587 MST. Molecular MST markers do not depend on the use of a right host (for phages
588 or viruses) or on the viable-but not culturable state of the marker, allowing the
589 detection of the markers regardless their physiological state. In contrast, molecular
590 detection does not provide information of the infectivity of the marker, although this
591 should not pose a serious drawback for MST that aims to know the faecal source of
592 the pollution. Nevertheless, it is highly advisable to use different MST markers that,
593 in combination with other strategies such as conditional probabilities or machine
594 learning approaches, can provide the maximum accuracy in determining the source
595 of faecal pollution (Ballesté et al., 2010; Kildare et al., 2007; Mayer et al., 2018;
596 Sánchez et al., 2011).
597
598
599 **5. CONCLUSIONS**

600 • CrAssphage is highly sensitive, abundant, and shows high correlation with other
601 faecal indicators (viral and bacterial, cultured and molecular). Thus, crAssphage
602 enlarges the toolbox of potential MST markers and can be used for water
603 management and monitoring.

604 • CrAssphage is highly abundant and stable in WWTP effluents, showing similar
605 abundances to and high correlation with human bacterial source tracking
606 markers and higher abundance than *E. coli* and other viral indicators.

607 • CrAssphage was detected in human-impacted rivers at similar abundance to
608 other human MST markers and at higher concentration than faecal indicators for

609 both point and diffuse pollution. In the latter case, abundance was related with
610 rainfall events.

611 • CrAssphage inactivated more rapidly at high than at low temperatures. During
612 the low temperature season it showed a T_{90} of around 10 days, which is similar
613 to SOMCPH and much higher than other bacterial human source tracking
614 markers.

615 • Sediments can become a reservoir of crAssphage together with other molecular
616 markers, which can be mobilized after rainfall events.

617 • CrAssphage persisted longer in waterbodies than other molecular MST markers
618 like HMBif and HF183 but less than culture viral markers SOMCPH and
619 GA17PH. Thus, factors other than natural inactivation, including particle
620 adsorption, sedimentation or the bacteriophage state (virion or inside the cell),
621 may affect crAssphage reduction.

622

623 **ACKNOWLEDGMENTS**

624 This work was supported by the European Union AQUAVALENS project (FP7-
625 KBBE.2012.2.5-01, grant agreement No.: 311846), the Spanish government
626 (research projects CGL2014-59977-C3-3-R and AGL2016-75536) and the Catalan
627 government (2017 SGR 170).

628 **CONFLICT OF INTEREST**

629 The authors declare no conflict of interest.

630

631 **REFERENCES**

632 Ahmed, W., Lobos, A., Senkbeil, J., Peraud, J., Gallard, J., Harwood, V.J., 2018.
633 Evaluation of the novel crAssphage marker for sewage pollution tracking in storm
634 drain outfalls in Tampa, Florida. Water Res. 131, 142–150.
635 doi:10.1016/j.watres.2017.12.011

636 Ahmed, W., Sidhu, J.P.., Smith, K., Beale, D., Gyawali, P., Toze, S., 2015.
637 Distributions of fecal markers in wastewater from varying climatic zones for
638 human fecal pollution tracking in Australian surface waters. Appl. Environ.
639 Microbiol. 82, AEM.03765-15. doi:10.1128/AEM.03765-15

640 Ballesté, E., García-Aljaro, C., Blanch, A.R., 2018. Assessment of the decay rates of
641 microbial source tracking molecular markers and faecal indicator bacteria from
642 different sources. J. Appl. Microbiol. 125, 1938–1949. doi:10.1111/jam.14058

643 Ballesté, E., Bonjoch, X., Belanche, L.A., Blanch, A.R., 2010. Molecular indicators
644 used in the development of predictive models for microbial source tracking. Appl.
645 Environ. Microbiol. 76, 1789–1795.

646 Barcina, I., Lebaron, P., Vives-Rego, J., 1997. Survival of allochthonous bacteria in
647 aquatic systems: A biological approach. FEMS Microbiol. Ecol. 23, 1–9.
648 doi:10.1016/S0168-6496(97)00016-0

649 Bell, A., Layton, A.C., McKay, L., Williams, D., Gentry, R., Sayler, G.S., 2009. Factors
650 influencing the persistence of fecal Bacteroides in stream water. J.Environ.Qual.
651 38, 1224–1232.

652 Bernhard, A.E., Field, K.G., 2000. Identification of nonpoint sources of fecal pollution
653 in coastal waters by using host-specific 16S ribosomal DNA genetic markers from
654 fecal anaerobes. Appl.Environ.Microbiol. 66, 1587–1594.

655 Blaustein, R. a., Pachepsky, Y., Hill, R.L., Shelton, D.R., Whelan, G., 2013.
656 *Escherichia coli* survival in waters: Temperature dependence. Water Res. 47, 569–
657 578. doi:10.1016/j.watres.2012.10.027

658 Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-
659 Manzano, J., Allard, A., Calvo, M., Girones, R., 2006. Quantification and Stability
660 of Human Adenoviruses and Polyomavirus JCPyV in Wastewater Matrices. Appl.
661 Environ. Microbiol. 72, 7894–7896. https://doi.org/10.1128/AEM.00965-06

662 Cahn, M.L., Conner, M.M., Schmitz, O.J., Stephenson, T.R., Wehausen, J.D., Johnson,
663 H.E., 2011. Disease, population viability, and recovery of endangered Sierra
664 Nevada bighorn sheep. J. Wildl. Manage. 75, 1753–1766. doi:10.1002/jwmg.232

665 Calero-Cáceres, W., Méndez, J., Martín-Díaz, J., Muniesa, M., 2017. The occurrence of
666 antibiotic resistance genes in a Mediterranean river and their persistence in the
667 riverbed sediment. Environ. Pollut. 223. doi:10.1016/j.envpol.2017.01.035

668 Cao, Y., Sivaganesan, M., Kelty, C.A., Wang, D., Boehm, A.B., Griffith, J.F.,
669 Weisberg, S.B., Shanks, O.C., 2018. A human fecal contamination score for
670 ranking recreational sites using the HF183/BacR287 quantitative real-time PCR
671 method. Water Res. 128, 148–156. doi:10.1016/j.watres.2017.10.071

672 Casanovas-Massana, A., Gomez-Donate, M., Sanchez, D., Belanche-Munoz, L.A.,
673 Muniesa, M., Blanch, A.R., 2015. Predicting fecal sources in waters with diverse
674 pollution loads using general and molecular host-specific indicators and applying

675 machine learning methods. J Env. Manag. 151, 317–325.
676 doi:10.1016/j.jenvman.2015.01.002

677 Casanovas-Massana, A., Blanch, A.R., 2013. Determination of fecal contamination
678 origin in reclaimed water open-air ponds using biochemical fingerprinting of
679 enterococci and fecal coliforms. Environ. Sci. Pollut. Res. 20, 3003–3010.
680 doi:10.1007/s11356-012-1197-1

681 Cho, K.H., Pachepsky, Y.A., Oliver, D.M., Muirhead, R.W., Park, Y., Quilliam, R.S.,
682 Shelton, D.R., 2016. Modeling fate and transport of fecally-derived
683 microorganisms at the watershed scale: State of the science and future
684 opportunities. Water Res. 100, 38–56. doi:10.1016/j.watres.2016.04.064

685 Cinek, O., Mazankova, K., Kramna, L., Odeh, R., Alassaf, A., Ibekwe, M.A.U.,
686 Ahmadov, G., Mekki, H., Abdullah, M.A., Elmahi, B.M.E., Hyöty, H., Rainetova,
687 P., 2018. Quantitative CrAssphage real-time PCR assay derived from data of
688 multiple geographically distant populations. J. Med. Virol. 90, 767–771.
689 doi:10.1002/jmv.25012

690 Drummond, J.D., Davies-Colley, R.J., Stott, R., Sukias, J.P., Nagels, J.W., Sharp, A.,
691 Packman, A.I., 2014. Retention and remobilization dynamics of fine particles and
692 microorganisms in pastoral streams. Water Res. 66, 459–472.
693 doi:10.1016/j.watres.2014.08.025

694 Dutilh, B.E., Cassman, N., McNair, K., Sanchez, S.E., Silva, G.G.Z., Boling, L., Barr,
695 J.J., Speth, D.R., Seguritan, V., Aziz, R.K., Felts, B., Dinsdale, E.A., Mokili, J.L.,
696 Edwards, R.A., 2014. A highly abundant bacteriophage discovered in the unknown
697 sequences of human faecal metagenomes. Nat. Commun. 5, 4498.

698 doi:10.1038/ncomms5498

699 Ebdon, J., Muniesa, M., Taylor, H., 2007. The application of a recently isolated strain of
700 *Bacteroides* (GB-124) to identify human sources of faecal pollution in a temperate
701 river catchment. Water Res. 41, 3683–3690. doi:10.1016/j.watres.2006.12.020

702 Edberg, S.C., Rice, E.W., Karlin, R.J., Allen, M.J., 2000. *Escherichia coli*: the best
703 biological drinking water indicator for public health protection. J. Appl. Microbiol.
704 88, 106S–116S. doi:10.1111/j.1365-2672.2000.tb05338.x

705 Fauvel, B., Gantzer, C., Cauchie, H.M., Ogorzaly, L., 2017. In Situ Dynamics of F-
706 Specific RNA Bacteriophages in a Small River: New Way to Assess Viral
707 Propagation in Water Quality Studies. Food Environ. Virol. 9, 89–102.
708 https://doi.org/10.1007/s12560

709 Fauvel, B., Cauchie, H. M., Gantzer, C., & Ogorzaly, L., 2016. Contribution of
710 hydrological data to the understanding of the spatio-temporal dynamics of F-
711 specific RNA bacteriophages in river water during rainfall-runoff events. Water
712 Research, 94, 328–340. doi:10.1016/j.watres.2016.02.057.

713 García-Aljaro, C., Ballesté, E., Muniesa, M., Jofre, J., 2017a. Determination of
714 crAssphage in water samples and applicability for tracking human faecal pollution.
715 Microb. Biotechnol. 10, 1775–1780. doi:10.1111/1751-7915.12841

716 Garcia-Aljaro, C., Martin-Diaz, J., Viñas-Balada, E., Calero-Caceres, W., Lucena, F.,
717 Blanch, A.R., 2017b. Mobilisation of microbial indicators, microbial source
718 tracking markers and pathogens after rainfall events. Water Res. 112, 248–253.
719 doi:10.1016/j.watres.2017.02.003

720 Gerba, C.P., Betancourt, W.Q., Kitajima, M., 2017. How much reduction of virus is
721 needed for recycled water: A continuous changing need for assessment? Water
722 Res. 108, 25–31. doi:https://doi.org/10.1016/j.watres.2016.11.020

723 Gómez-Doñate, M., Ballesté, E., Muniesa, M., Blanch, A.R., 2012. New molecular
724 quantitative PCR assay for detection of host-specific Bifidobacteriaceae suitable
725 for microbial source tracking. Appl. Environ. Microbiol. 78, 5788–95.
726 doi:10.1128/AEM.00895-12

727 Gómez-Doñate, M., Payán, A., Cortés, I., Blanch, A.R., Lucena, F., Jofre, J., Muniesa,
728 M., 2011. Isolation of bacteriophage host strains of *Bacteroides* species suitable for
729 tracking sources of animal faecal pollution in water. Environ. Microbiol. 13, 1622–
730 1631. doi:10.1111/j.1462-2920.2011.02474.x

731 Gourmelon, M., Caprais, M.P., Segura, R., Le, M.C., Lozach, S., Piriou, J.Y., Rince, A.,
732 2007. Evaluation of two library-independent microbial source tracking methods to
733 identify sources of fecal contamination in French estuaries.
734 Appl.Environ.Microbiol. 73, 4857–4866.

735 Green, H.C., Haugland, R.A., Varma, M., Millen, H.T., Borchardt, M.A., Field, K.G.,
736 Walters, W.A., Knight, R., Sivaganesan, M., Kelty, C.A., Shanks, O.C., 2014.
737 Improved HF183 Quantitative Real-Time PCR Assay for Characterization of
738 Human Fecal Pollution in Ambient Surface Water Samples. Appl. Environ.
739 Microbiol. 80, 3086–3094. doi:10.1128/AEM.04137-13

740 Harwood, V.J., Boehm, A.B., Sassoubre, L.M., Vijayavel, K., Stewart, J.R., Fong, T.-
741 T., Caprais, M.-P., Converse, R.R., Diston, D., Ebdon, J., Fuhrman, J.A.,
742 Gourmelon, M., Gentry-Shields, J., Griffith, J.F., Kashian, D.R., Noble, R.T.,

743 Taylor, H., Wicki, M., 2013. Performance of viruses and bacteriophages for fecal
744 source determination in a multi-laboratory, comparative study. Water Res. 47,
745 6929–6943. doi:https://doi.org/10.1016/j.watres.2013.04.064

746 Haugland, R.A., Varma, M., Sivaganesan, M., Kelty, C., Peed, L., Shanks, O.C., 2010.
747 Evaluation of genetic markers from the 16S rRNA gene V2 region for use in
748 quantitative detection of selected Bacteroidales species and human fecal waste by
749 qPCR. Syst Appl Microbiol 33, 348–357. doi:10.1016/j.syapm.2010.06.001

750 Hsu, F.C., Shieh, Y.S., van Duin, J., Beekwilder, M.J., Sobsey, M.D., 1995. Genotyping
751 male-specific RNA coliphages by hybridization with oligonucleotide probes. Appl.
752 Environ. Microbiol. 61, 3960 LP-3966.

753 Huijsdens, X.W., Linskens, R.K., Mak, M., Meuwissen, S.G.M., Vandenbroucke-
754 Grauls, C.M.J.E., Savelkoul, P.H.M., 2002. Quantification of Bacteria Adherent to
755 Gastrointestinal Mucosa by Real-Time PCR. J. Clin. Microbiol. 40, 4423–4427.
756 https://doi.org/10.1128/JCM.40.12.4423-4427.2002

757 Hurwitz, B.L., U'Ren, J.M., Youens-Clark, K., 2016. Computational prospecting the
758 great viral unknown. FEMS Microbiol. Lett. 363, 1–12.
759 doi:10.1093/femsle/fnw077

760 ISO, 2000a. Water Quality – Detection and Enumeration of Escherichia coli and
761 Coliform Bacteria – Part 1: Membrane Filtration Method (ISO 9308-1: 2000).

762 ISO, 2000b. ISO 10705-2:2000 - Water quality -- Detection and enumeration of
763 bacteriophages -- Part 2: Enumeration of somatic coliphages. Int. Organ. Stand.

764 Jamieson, R., Joy, D.M., Lee, H., Kostaschuk, R., Gordon, R., 2005. Transport and

765 deposition of sediment-associated Escherichia coli in natural streams. Water Res.
766 39, 2665–2675. doi:https://doi.org/10.1016/j.watres.2005.04.040

767 Jofre, J., Blanch, A.R., Lucena, F., Muniesa, M., 2014. Bacteriophages infecting
768 Bacteroides as a marker for microbial source tracking. Water Res. 55, 1–11.
769 doi:10.1016/j.watres.2014.02.006

770 Jofre, J., Lucena, F., Blanch, A.R., Muniesa, M., 2016. Coliphages as model organisms
771 in the characterization and management of water resources. Water (Switzerland).
772 doi:10.3390/w8050199

773 Jofre, J., Bosch, A., Lucena, F., Gironés, R., Tartera, C., 1986. Evaluation of
774 Bacteroides fragilis Bacteriophages as Indicators of the Virological Quality of
775 Water. Water Sci. Technol. 18, 167. doi:10.2166/wst.1986.0126

776 Kildare, B.J., Leutenegger, C.M., McSwain, B.S., Bambic, D.G., Rajal, V.B., Wuertz,
777 S., 2007. 16S rRNA-based assays for quantitative detection of universal, human-,
778 cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. Water Res. 41,
779 3701–3715.

780 Lavender, J.S., Kinzelman, J.L., 2009. A cross comparison of qPCR to agar-based or
781 defined substrate test methods for the determination of *Escherichia coli* and
782 enterococci in municipal water quality monitoring programs. Water Res. 43, 4967–
783 4979. doi:https://doi.org/10.1016/j.watres.2009.08.010

784 Liang, L., Goh, S.G., Vergara, G.G.R.V., Fang, H.M., Rezaeinejad, S., Chang, S.Y.,
785 Bayen, S., Lee, W.A., Sobsey, M.D., Rose, J.B., Gin, K.Y.H., 2015. Alternative
786 fecal indicators and their empirical relationships with enteric viruses, *Salmonella*
787 *enterica*, and *Pseudomonas aeruginosa* in surface waters of a tropical urban

788 catchment. Appl. Environ. Microbiol. 81, 850–860. doi:10.1128/AEM.02670-14

789 Martín-Díaz, J., García-Aljaro, C., Pascual-Benito, M., Galofré, B., Blanch, A.R.,
790 Lucena, F., 2017. Microcosms for evaluating microbial indicator persistence and
791 mobilization in fluvial sediments during rainfall events. Water Res. 123, 623–631.
792 https://doi.org/10.1016/j.watres.2017.07.017

793 Mayer, R.E., Reischer, G.H., Ixenmaier, S.K., Derx, J., Blaschke, A.P., Ebdon, J.E.,
794 Linke, R., Egle, L., Ahmed, W., Blanch, A.R., Byamukama, D., Savill, M., Mushi,
795 D., Cristóbal, H.A., Edge, T.A., Schade, M.A., Aslan, A., Brooks, Y.M., Sommer,
796 R., Masago, Y., Sato, M.I., Taylor, H.D., Rose, J.B., Wuertz, S., Shanks, O.C.,
797 Piringer, H., Mach, R.L., Savio, D., Zessner, M., Farnleitner, A.H., 2018. Global
798 Distribution of Human-Associated Fecal Genetic Markers in Reference Samples
799 from Six Continents. Environ. Sci. Technol. 52, 5076–5084.
800 doi:10.1021/acs.est.7b04438

801 Mayer, R.E., Bofill-Mas, S., Egle, L., Reischer, G.H., Schade, M., Fernandez-Cassi, X.,
802 Fuchs, W., Mach, R.L., Lindner, G., Kirschner, A., Gaisbauer, M., Piringer, H.,
803 Blaschke, A.P., Girones, R., Zessner, M., Sommer, R., Farnleitner, A.H., 2016.
804 Occurrence of human-associated Bacteroidetes genetic source tracking markers in
805 raw and treated wastewater of municipal and domestic origin and comparison to
806 standard and alternative indicators of faecal pollution. Water Res. 90, 265–276.
807 https://doi.org/10.1016/j.watres.2015.12.031

808 McMinn, B.R., Ashbolt, N.J., Korajkic, A., 2017. Bacteriophages as indicators of faecal
809 pollution and enteric virus removal. Lett. Appl. Microbiol. 65, 11–26.
810 doi:10.1111/lam.12736

811 Méndez, J., Audicana, A., Isern, A., Llaneza, J., Moreno, B., Tarancón, M.L., Jofre, J.,
812 Lucena, F., 2004. Standardised evaluation of the performance of a simple
813 membrane filtration-elution method to concentrate bacteriophages from drinking
814 water. J. Virol. Methods. doi:10.1016/j.jviromet.2003.11.013

815 Menon, P., Billen, G., Servais, P., 2003. Mortality rates of autochthonous and fecal
816 bacteria in natural aquatic ecosystems. Water Res. 37, 4151–4158.

817 Mieszkin, S., Furet, J.P., Corthier, G., Gourmelon, M., 2009. Estimation of pig fecal
818 contamination in a river catchment by real-time PCR using two pig-specific
819 *Bacteroidales* 16S rRNA genetic markers. Appl.Environ.Microbiol. 75, 3045–
820 3054.

821 Muniesa, M., Lucena, F., Blanch, A.R., Payán, A., Jofre, J., 2012. Use of abundance
822 ratios of somatic coliphages and bacteriophages of *Bacteroides thetaiotaomicron*
823 GA17 for microbial source identification. Water Res. 46, 6410–8.
824 doi:10.1016/j.watres.2012.09.015

825 NHMRC, 2011. Australian Drinking Water Guidelines 6 2011, National Health and
826 Medical Research Council, National Resource Management Ministerial Council,
827 Commonwealth of Australia,. (National Health and Medical Research Council).
828 doi:1864965118

829 Ogorzaly, L., Tissier, A., Bertrand, I., Maul, A., Gantzer, C., 2009. Relationship
830 between F-specific RNA phage genogroups, faecal pollution indicators and human
831 adenoviruses in river water. Water Res. 43, 1257–1264.
832 doi:10.1016/j.watres.2008.12.011

833 Payan, A., Ebdon, J., Taylor, H., Gantzer, C., Ottoson, J., Papageorgiou, G.T., Blanch,

834 A.R., Lucena, F., Jofre, J., Muniesa, M., 2005. Method for isolation of Bacteroides
835 bacteriophage host strains suitable for tracking sources of fecal pollution in water.
836 Appl.Environ.Microbiol. 71, 5659–5662.

837 Peduzzi, P., Luef, B., 2008. Viruses, bacteria and suspended particles in a backwater
838 and main channel site of the Danube (Austria). Aquat. Sci. 70, 186.
839 doi.org/10.1007/s00027-008-8068-3

840 Peed, L.A., Nietch, C.T., Kelty, C.A., Meckes, M., Mooney, T., Sivaganesan, M.,
841 Shanks, O.C., 2011. Combining land use information and small stream sampling
842 with PCR-based methods for better characterization of diffuse sources of human
843 fecal pollution. Env. Sci Technol 45, 5652–5659. doi:10.1021/es2003167

844 R Core Team, 2016. R: A Language and Environment for Statistical Computing, R
845 Foundation for Statistical Computing. Doi:10.1007/978-3-540-74686-7

846 Roslev, P., Bukh, A.S., 2011. State of the art molecular markers for fecal pollution
847 source tracking in water. Appl.Microbiol.Biotechnol. 89, 1341–1355.

848 Sánchez, D., Belanche, L., Blanch, A.R., 2011. A Software System for the Microbial
849 Source Tracking Problem. J. Mach. Learn. Res. 17, 7.

850 Schaper, M., Jofre, J., Uys, M., Grabow, W.O.K., 2002. Distribution of genotypes of F-
851 specific RNA bacteriophages in human and non-human sources of faecal pollution
852 in South Africa and Spain. J. Appl. Microbiol. doi:10.1046/j.1365-
853 2672.2002.01600.x

854 Shanks, O.C., Atikovic, E., Blackwood, A.D., Lu, J., Noble, R.T., Domingo, J.S.,
855 Seifring, S., Sivaganesan, M., Haugland, R.A., 2008. Quantitative PCR for

856 detection and enumeration of genetic markers of bovine fecal pollution.
857 Appl.Environ.Microbiol. 74, 745–752.

858 Shkoporov, A., Khokhlova, E. V, Fitzgerald, C.B., Stockdale, S.R., Draper, L.A., Ross,
859 R.P., Hill, C., 2018. ΦCrAss001, a member of the most abundant bacteriophage
860 family in the human gut, infects *Bacteroides*. bioRxiv. doi:10.1101/354837

861 Stachler, E., Kelty, C., Sivaganesan, M., Li, X., Bibby, K., Shanks, O.C., 2017.
862 Quantitative CrAssphage PCR Assays for Human Fecal Pollution Measurement.
863 Environ. Sci. Technol. 51, 9146–9154. doi:10.1021/acs.est.7b02703

864 Stachler, E., Bibby, K., 2014. Metagenomic Evaluation of the Highly Abundant Human
865 Gut Bacteriophage CrAssphage for Source Tracking of Human Fecal Pollution.
866 Environ. Sci. Technol. Lett. 1, 405–409. doi:10.1021/ez500266s

867 Tartera, C., Lucena, F., Jofre, J., 1989. Human origin of *Bacteroides fragilis*
868 bacteriophages present in the environment. Appl.Environ.Microbiol. 55, 2696–
869 2701.

870 USEPA, 2016. 2016 Coliphage Experts Workshop: Discussion Topics and Findings.
871 EPA 823-F-16-001 1–2.

872 USEPA, 2012. 2012 Recreational Water Quality Criteria Documents.

873 USEPA, 2006. National Primary Drinking Water Regulations: Ground Water Rule. Fed.
874 Regist. 71.

875 Wade, T.J., Calderon, R.L., Sams, E., Beach, M., Brenner, K.P., Williams, A.H.,
876 Dufour, A.P., 2006. Rapidly Measured Indicators of Recreational Water Quality
877 Are Predictive of Swimming-Associated Gastrointestinal Illness. Environ. Health

878 Perspect. 114, 24–28. doi:10.1289/ehp.8273

879 Wanjugi, P., Harwood, V.J., 2013. The influence of predation and competition on the
880 survival of commensal and pathogenic fecal bacteria in aquatic habitats. Environ.
881 Microbiol. 15, 517–526. doi:10.1111/j.1462-2920.2012.02877.x

882 Wery, N., Lhoutellier, C., Ducray, F., Delgenes, J.P., Godon, J.J., 2008. Behaviour of
883 pathogenic and indicator bacteria during urban wastewater treatment and sludge
884 composting, as revealed by quantitative PCR. Water Res. 42, 53–62.

885 WHO, 2001. Guidelines, Standards and Health: Assessment of risk and risk
886 management for water-related infectious disease.

887 Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New
888 York.

889 Wickham, H., 2007. Reshaping Data with the {reshape} Package. J. Stat. Softw. 21, 1–
890 20.

891 Wu, J., Cao, Y., Young, B., Yuen, Y., Jiang, S., Melendez, D., Griffith, J.F., Stewart,
892 J.R., 2016. Decay of Coliphages in Sewage-Contaminated Freshwater: Uncertainty
893 and Seasonal Effects. Environ. Sci. Technol. acs.est.6b03916.
894 doi:10.1021/acs.est.6b03916

895 Wu, J., Rees, P., Storrer, S., Alderisio, K., Dorner, S., 2009. Fate and transport
896 modeling of potential pathogens: the contribution from sediments. J. Am. Water
897 Resour. Assoc. 45, 35e44. http://dx.doi.org/10.1111/j.1752-1688.2008.00287.x.

898 Yahya, M., Blanch, A.R., Meijer, W.G., Antoniou, K., Hmaied, F., Ballesté, E., 2017.
899 Comparison of the Performance of Different Microbial Source Tracking Markers

among European and North African Regions. J. Environ. Qual. 46, 760. doi:10.2134/jeq2016.11.0432

Yahya, M., F. Hmaied, S. Jebri, J. Jofre, and M. Hamdi. 2015. Bacteriophages as indicators of human and animal fecal contamination in raw and treated wastewaters from Tunisia. J. Appl. Microbiol. 118:1217–1225. doi:10.1111/jam.12774

905 **Table 1.** Meteorological data obtained from an automatic station close to "Riera de Cànoves"
906 stream during the sampling campaigns. Season: winter (W), summer (S), medium (M). Tmax:
907 Maximum temperature (ºC). Tmin: Minimum temperature (ºC). R: Rainfall of the sampling
908 day. RR: Rainfall of the sampling day and 1 day before (mm). RRR: Rainfall of the sampling
909 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

910 na: Not available data

911

912 **Table 2.** Descriptive statistics of faecal indicators: *E. coli*, SOMCPH and human
913 microbial source tracking markers: GA17PH, HMBif, HF183 and crAssphage from:
914 inter WWTPs effluents comparison, intra WWTP effluent comparison for 2 years, in
915 Llobregat River (water and sediments), and "Riera de Cànoves" stream. Data is
916 given in log_{10} gc (*E. coli*, HMBif, HF183 and crAssphage), PFU (SOMCPH and
917 GA17PH), CFU (*E. coli*) per 100 ml for water samples or per gr of dry weight for
918 sediment samples. The data for the Llobregat River of *E. coli* (culture and qPCR),
919 SOMCPH, GA17PH and HMBif is already published in García-Aljaro et al (2017b).
920 na: Not available.
921

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

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

	E. coli	***E. coli* qPCR**	**SOMCPH**	**GA17PH**	**HMBif**	**HF183**
Inter effluent comparison	0.729[1]	na	0.665[1]	nsc	0.757[1]	na
Intra effluent comparison	0.585[2]	na	nsc	nsc	0.603[2]	0.642[1]
Llobregat River Water	0.589[2]	nsc	nsc	0.708[1]	nsc	na
Llobregat River Sediments	0.804[1]	0.878[1]	0.843[1]	0.755[1]	na	na
Riera de Cànoves Stream	0.589[2]	na	nsc	nsc	0.794[1]	0.877[1]
Reduction along the stream	nsc	na	0.875[1]	nsc	0.802[1]	0.759[1]

924 [1]Significant at $p \leq 0.05$
925 [2]Significant at $p \leq 0.1$ and ≥ 0.05
926 nsc: Not significant correlation
927 na: Not available

928 **Table 4.** Distance (km) with a 90% reduction of the initial bacterial or phage
929 concentration (D_{90}) obtained in "Riera de Cànoves" catchment for *E. coli* culture-
930 based, SOMCPH, GA17PH, HMBif, HF183 and crAssphage. The distance sampled
931 to calculate the D_{90} was 925 m.
932

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[1]	**0.36**	**0.72**	**0.82**	**0.40**	**0.25**	**0.28**

933 [1] Mad: Median absolute deviation
934
935

FIGURE LEGENDS

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

Figure 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).

Figure 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.

Figure 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.

Figure 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).

FIG1

Click here to download high resolution image

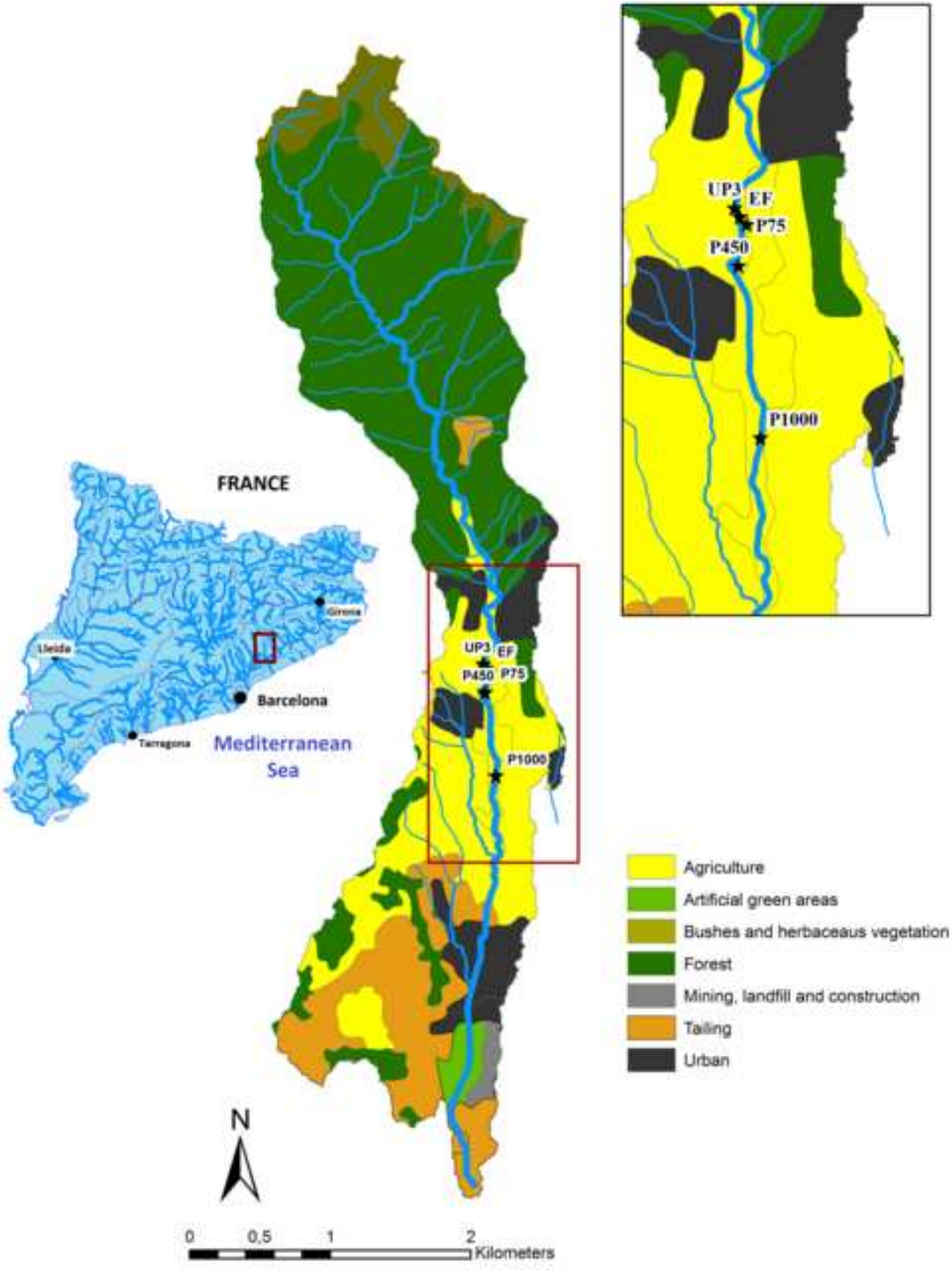


FIG2

Click here to download high resolution image

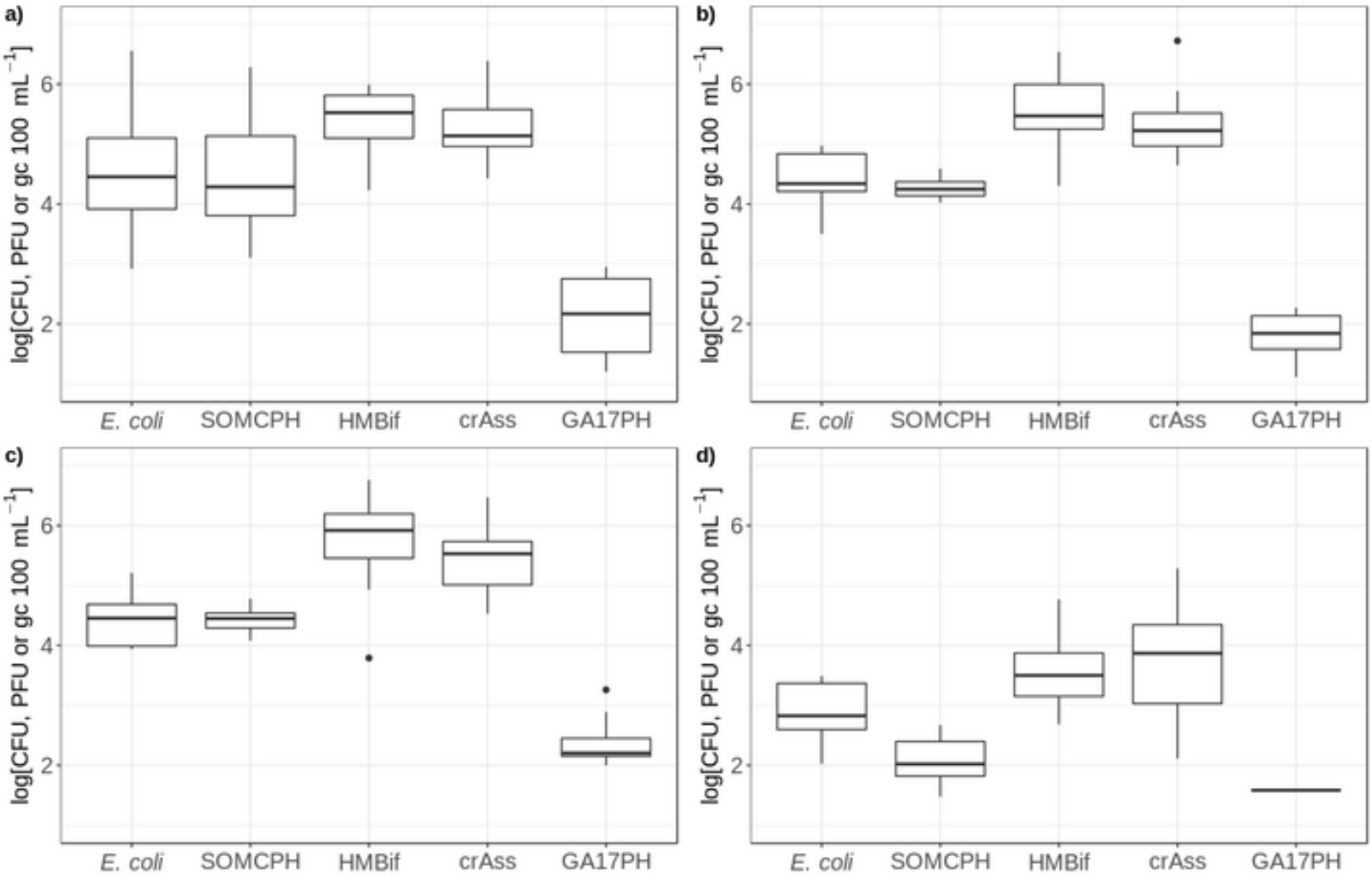


FIG3

Click here to download high resolution image

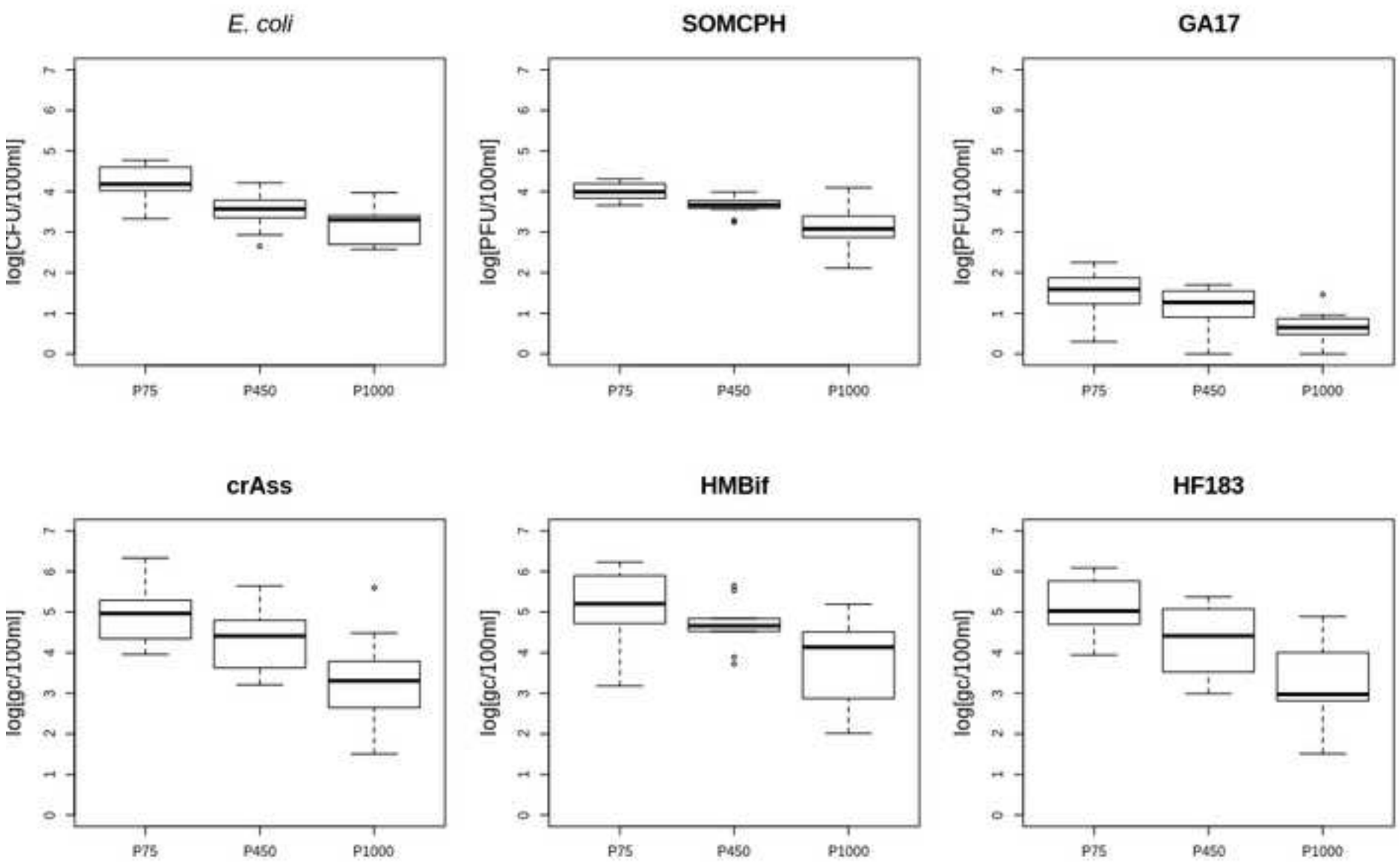


FIG4

Click here to download high resolution image

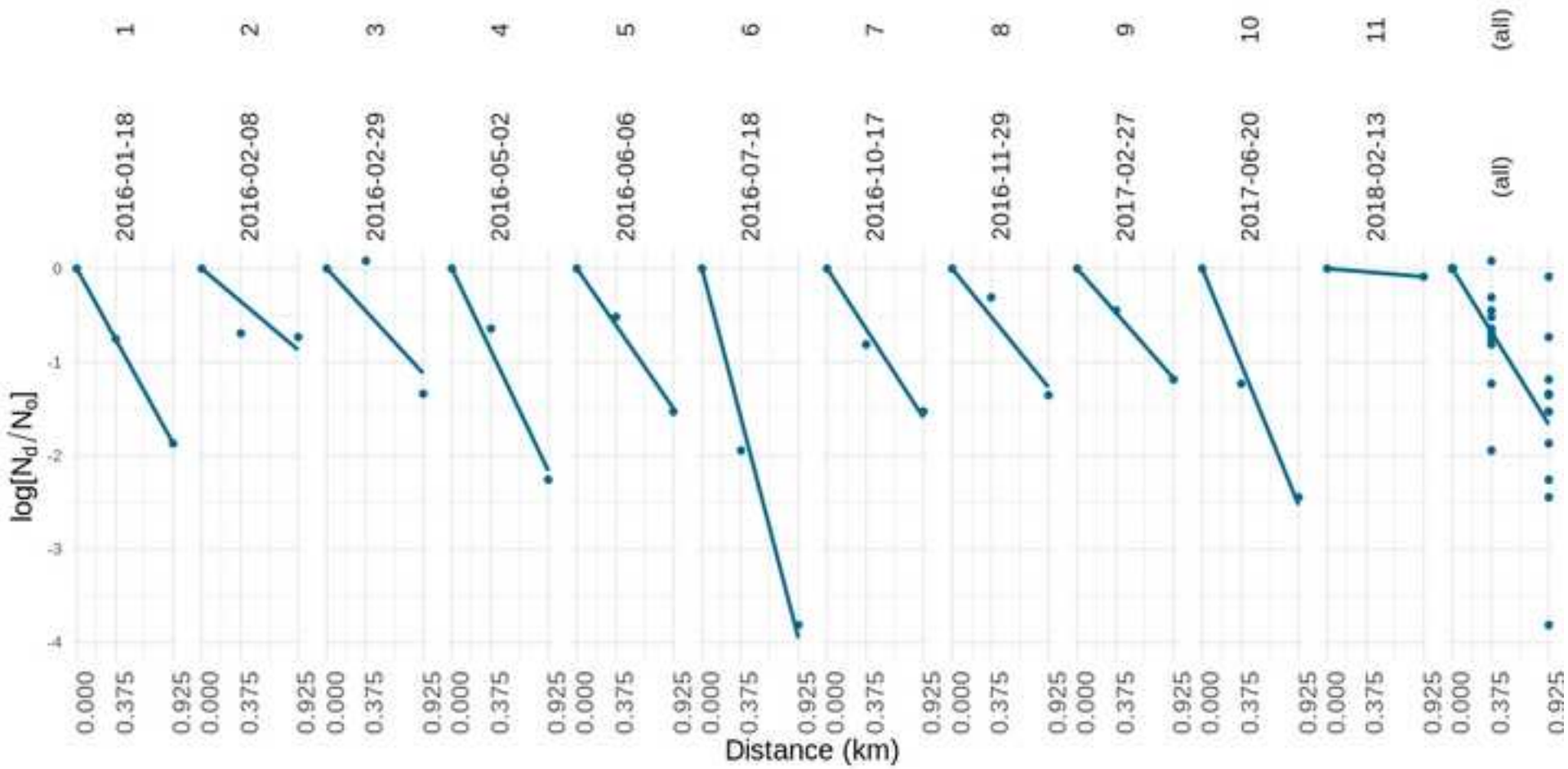


FIG5

Click here to download high resolution image