Detection and Quantification of Bacteriophages in Wastewater Samples by culture and molecular methods

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Abstract

Bacteriophages are promising tools for the detection of fecal pollution in water bodies and particularly for viral pathogen risk assessment. Having similar morphological and biological characteristics, bacteriophages are perfect surrogates for the study of the fate and transport of enteric viruses, generally better than any other group of indicators.

Different groups of bacteriophages, such as somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting selected strains of *Bacteroides*, have been comprehensively tested as indicators of fecal pollution. Somatic coliphages and F-specific RNA bacteriophages can be used as indicators of general fecal contamination, whereas *Bacteroides* phages can be used to detect a particular fecal source, for instance human, bovine, porcine, or poultry fecal contamination.

Feasible and cost-effective protocols standardized by the International Standardization Organization and the United States Environmental Protection Agency for the detection of infectious bacteriophages belonging to these three groups are available. Molecular methods for the detection of some particular phages have also been developed. Here we introduce those methods for the detection, enumeration, and isolation of bacteriophages in wastewater samples.

1. Introduction

Bacteriophages, or viruses that infect bacteria, are probably the most abundant entities on Earth. They can only replicate inside susceptible and metabolically active host bacteria. By infecting and lysing their hosts, phages contribute to the mortality of the bacterial populations and have an important impact on the cycling of organic matter in the biosphere [1]. Many phages can also mobilize genetic material among different host bacteria in a process known as transduction. A given phage can only infect certain bacteria to the point that different strains of the same species differ in their susceptibility to phage infection [2, 3]. This host-specificity of phages is determined by receptor molecules on the surface of the bacteria (capsule, cell wall, flagella, or pili). Their extracellular form, named virion, consists of a genome of either RNA or DNA covered by a protein coat or capsid. Many phages can also present additional capsid structures such as tails, fibers and spikes. Due to their simple structure and composition, virions persist quite successfully in the environment and are moderately resistant to natural and anthropogenic stressors [4]. This resistance and persistence are the reason why bacteriophages infecting enteric bacteria have been proposed as alternative indicators of fecal and viral pollution.

In water bodies, among the sources of fecal pollution, the discharge of human and animal fecal waste is one of the most important. If poorly managed, enteric pathogens, including viruses, can spread into aquatic environments. However, monitoring the presence of every specific pathogen is impracticable for routine control purposes. Besides the technical difficulties, it would be extremely time-consuming and prohibitively expensive, especially for those countries in most urgent need of efficient water quality control.

Fecal indicator microorganisms represent then a useful tool to monitor the microbiological quality of water, since their presence reveals fecal contamination and potentially the existence of fecal pathogens. The most used are fecal indicator bacteria (FIB), including total coliforms,

fecal coliforms, *Escherichia coli*, streptococci and enterococci [5]. However, they do not provide information on the source of fecal contamination, being frequently found in the microbiota of many animals. Another drawback is that they correlate poorly with human viruses or parasites in natural aquatic environments and wastewater treatment plants, displaying different behavior and lower survival rates [5].

In contrast, bacteriophages from fecal origin provide a more accurate information about viral fecal pathogens. As indicators of fecal contamination, phages are abundant in feces, do not replicate in natural environments [6], spread into the environment in a similar way to enteric viral pathogens, and have similar fates and survival patterns [7]. Therefore, easy-to-detect bacteriophages have been proposed as indicators of fecal and viral pollution and are now included in multiple water quality regulations and guidelines worldwide [8].

Bacteriophages used as fecal pollution indicators are those capable of infecting enteric bacteria and are generally classified into three taxonomically diverse groups: somatic coliphages, F-specific coliphages, and phages infecting *Bacteroides* spp.

Phages that bind to the receptors located in the cell wall are typically known as somatic phages while those infecting through the sex pilus, which is encoded by the F-plasmid, are known as F-specific phages. Somatic coliphages infect specific species of *Escherichia coli* through the cell wall [9]. They are the most abundant group of indicator bacteriophages in almost all environmental samples [10].

F-specific coliphages are the second most widespread indicator phages in the environment [10]. They were first detected in *E. coli* K-12 and since F-plasmid is transferable to a wide range of gram-negative bacteria via conjugation [11], different host strains that produce sexual pili can be infected with F-specific bacteriophages. F-total coliphages include two groups of F-specific coliphages, F-DNA and F-RNA coliphages, and the use of RNase in culture methods permits

the differentiation between them. Standardized methods use specific *E. coli* or *Salmonella enteritidis* sv. Typhimurium strains that express the F-pili for their detection.

The third group of bacteriophages proposed as indicators are those infecting certain strains of *Bacteroides* spp., one of the most abundant species in the human gut. The concentrations of virulent phages infecting *Bacteroides* detected in feces or in fecally contaminated samples are usually lower compared to coliphages [12]. However, several recent studies place a new group of phages infecting *Bacteroides*, named crAssphages, as the most abundant inhabitants in the human gut. So far, this group has been detected by molecular methods, not by enumeration of infectious particles [13].

Indeed, phages can be detected by culture-dependent methods, which provide qualitative or quantitative information about infectious phages in samples [14]. Some of these methods have already been registered as standardized protocols, mainly by two regulatory bodies: the International Standardization Organization (ISO) and the United States Environment Protection Agency (USEPA). Standardized culture methods are simple, robust, cost-effective, and easily prepared, especially for coliphages, which do not require anaerobic growth conditions. The methods can be scaled to different sample volumes, maintaining the same proportions between medium, host strain and sample. Material, media, reagents, and labor have a similar cost to the methods currently used in routine analysis laboratories to detect fecal coliforms / *E. coli*. Some culture-dependent methods for coliphage detection have been adapted to a user-friendly format, providing a qualitative colorimetric detection protocol that allows reducing the detection time of infectious coliphages to one working day. One of these methods, the Bluephage® method, has recently been optimized for somatic and F-specific coliphages [15, 16] and allows quantitative detection of coliphages by applying the Most Probable Number to the qualitative results.

On the other hand, phages can also be detected by molecular methods. The major drawback of molecular detection is that it cannot provide information about phage infectivity, and, therefore, additional steps are required. Without infectivity data, the viral concentration and the real risks for human health are often overestimated [17] Molecular methods are based on plaque hybridization, employing specific probes or, more frequently, on qPCR / RTqPCR assays. They have been used to detect F-specific phages [18] or for the detection of CrAssphage of *Bacteroides* [19, 20] but have limited application for the large and heterogeneous group of somatic coliphages, though PCR and qPCR techniques have been developed for specific families of coliphages [21]. Moreover, phages harboring particular genes can also be detected by molecular methods. This is the case of phages carrying toxin genes [22] or antibiotic resistance genes [23, 24], whose detection is being usefully applied for the study of horizontal gene transfer.

In this chapter we provide information about the most common culture-based methods as well as some molecular methods used to isolate and quantify bacteriophages in wastewater samples.

2. Materials

2.1. Isolation and quantification of reference bacteriophages in wastewater

2.1.1 Host strain stock culture preparation

1. Bacteriophage host strain.
2. Specific broth media.
3. Specific 90 mm agar plates (1.5% agar)
4. Orbital incubator or shaker (180 rpm) at 37 ºC.
5. Glycerol: Sterilize at 121 ºC for 15 min and store at room temperature (25 ºC).

138 2.1.2 Host strain inoculum culture preparation for bacteriophage quantification

139 1. Host strain stock culture (section 3.1.1).
140 2. Specific broth media.
141 3. Orbital incubator or shaker (180 rpm) at 37 ºC.
142 4. Spectrophotometer equipped with 500–650 nm (±10 nm) filter.

143 2.1.3 Enumeration of reference bacteriophages by a double agar layer plaque assay

144 1. PES (polyethersulfone) or PVDF (polyvinylidene fluoride) low protein
145 binding membrane filters (0.22 μm pore size).
146 2. Filter holders or sterile syringes.
147 3. Host strain inoculum culture (section 3.1.2).
148 4. Reference bacteriophage stock (section 3.1.6).
149 5. Saline peptone: 1 g of peptone, 8.5 g of NaCl, 1 L of distilled water, adjust
150 pH to 7.2. Sterilize at 121 ºC for 15 min (*see* **Note 1**).
151 6. Specific soft agar (0.7% agar).
152 7. Specific 90 mm agar plates (1.5% agar).
153 8. 10 mL sterile tubes.
154 9. Water bath at 44 ºC.
155 10. Incubator at 37 ºC.

156 2.1.4 Bacteriophage isolation from plaques

157 1. Saline peptone: 1 g of peptone, 8.5 g of NaCl, 1 L of distilled water, adjust pH
158 to 7.2. Sterilize at 121 ºC for 15 min.

159 2. Sterile needle.

160 3. Chloroform.

4. Host strain inoculum culture (methods section 3.1.2).

5. Specific broth media.

6. PES or PVDF low protein binding membrane filters (0.22 μm pore size).

7. Filter holders or sterile syringes.

8. Bench centrifuge.

9. Vortex.

2.1.5 High titre bacteriophage suspension preparation

1. Reference bacteriophage or phage suspension (isolated previously from plaques, section 3.1.4).
2. Host inoculum culture.
3. Specific soft agar (0.7% agar).
4. Specific 90 mm agar plates (1.5% agar).
5. Saline peptone: 1 g of peptone and 8.5 g of NaCl and add 1 L of distilled water, adjust pH to 7.2. Sterilize at 121 ºC for 15 min.
6. Chloroform.
7. PES or PVDF low protein binding membrane filters (0.22 μm pore size).
8. Filter holders or sterile syringes.
9. Bench centrifuge.
10. Vortex.

2.1.6 Reference bacteriophage stock preparation

1. High titre bacteriophage suspension (section 3.1.5).
2. Saline peptone: 1 g of peptone, 8.5 g of NaCl, add 1 L of distilled water, adjust pH to 7.2. Sterilize at 121 ºC for 15 min.

3. Sterile glycerol: Sterilize at 121 ºC for 15 min and store at room temperature (25 ºC).
4. -80 ºC freezer.

2.2 Isolation and quantification of somatic coliphages

For the isolation and quantification of somatic coliphages, use the materials described in section 2.1. However, note the different strains, media, and reagents specific for somatic coliphages described below.

1. Host strains: the host strains included in the standardized methods are *E. coli* CN, more frequently referred as WG5 (ATCC 700078) in the ISO 10705-2 method or *E. coli* CN13 (ATCC 700609) in the USEPA 1601, 1602, 1642 and 1643 methods [25–29].
2. Bacteriophage reference material: bacteriophage ϕX174 (ATCC 13706-B1) can be used as a reference bacteriophage for both host strains [25–29].
3. 1 M calcium chloride solution (additive): 14 g $CaCl_2.2H_2O$ in 100 mL distilled water, filter-sterilize through 0.22 µm mixed cellulose esters (MCE) membrane filter, store in the dark at 4 °C.
4. Modified Scholtens' Broth (MSB) for *E. coli* CN (ATCC 700078): 10 g peptone, 3 g yeast extract, 12 g meat extract, 3 g NaCl, 5 mL Na_2CO_3 solution (150 g/L), 0.3 mL 4.14M $MgCl_2$ solution, adjust to 1 L with distilled water. Adjust the pH to 7.2. Sterilize at 121°C for 15 min.
5. Modified Scholtens' Agar (MSA): MSB with 1.5% agar.
6. Soft Modified Scholtens' Agar (ssMSA): MSB with 0.7% agar. After sterilization add 1.2 mL of 1M $CaCl_2$ solution to a 200 mL of ssMSA (*see* **Note 2**).

7. Tryptone Soy Broth (TSB) for *E. coli* CN13 (ATCC 700609): 17 g tryptone, 3 g soytone, 2.5 g dextrose, 2.5 g dipotassium phosphate, 5 g NaCl adjust to 1 L with distilled water. Sterilize at 121°C for 15 min. Before use, aseptically add nalidixic acid (final concentration of 100 µg/mL).
8. Tryptone Soy Agar (TSA): TSB with 1.5% agar. Before use, aseptically add nalidixic acid (final concentration of 100 µg/mL).
9. Soft Tryptone Soy Agar (ssTSA): TSB with 0.7% agar. Before use, aseptically add nalidixic acid (final concentration of 100 µg/mL).

2.3 Isolation and quantification of F-specific bacteriophages

For the isolation and quantification of F-specific bacteriophages, use the materials described in section 2.1. However, note the different strains, media, and reagents specific for F-specific bacteriophages described below.

1. Host strain: The host strains included in the standardized methods are *Salmonella enterica* serovar Typhimurium (NCTC 12484) in the ISO 10705-1 method and *E. coli* HS (*E. coli* Famp, ATCC 700891) in the USEPA 1601, 1602, 1642 and 1643 methods [26–30].
2. Bacteriophage reference material: Bacteriophage MS2 (ATCC 15597-B1) can be used as reference bacteriophage for both host strains [26, 28–30].
3. Calcium glucose solution (additive): 3 g $CaCl_2.2H_2O$, 10 g glucose in 100 mL distilled water, filter-sterilize through 0.22 µm MCE filter, store in the dark at 4 °C.
4. Medium for *Salmonella enterica* serovar Typhimurium (NCTC 12484): Tryptone-yeast extract-glucose broth (TYGB): 10 g trypticase peptone, 1 g yeast extract, 8 g NaCl, adjust to 1 L with distilled water. Adjust the pH to 7.2. Sterilize at 121°C for 15 min. Add 2 mL of calcium glucose solution into 200 mL of the sterilized medium.

5. Tryptone-yeast extract-glucose agar (TYGA): TYGB with 1.5% agar.
6. Soft tryptone-yeast extract-glucose agar (ssTYGA): TYGB with 0.7% agar (*see* **Note 3**).
7. Tryptone Soy Broth (TSB) for *E. coli* HS (ATCC 700891): 17 g tryptone, 3 g soytone, 2.5 g dextrose, 2.5g dipotassium phosphate, 5 g NaCl adjust to 1 L with distilled water. Sterilize at 121°C for 15 min. Before use aseptically add streptomycin sulfate and ampicillin sodium salt (final concentration of 15 µg/mL).
8. Tryptone Soy Agar (TSA): TSB with 1.5% agar. Before use aseptically add streptomycin sulfate and ampicillin sodium salt (final concentration of 15 µg/mL).
9. Soft Tryptone Soy Agar (ssTSA): TSB with 0.7% agar. Before use aseptically add streptomycin sulfate and ampicillin sodium salt (final concentration of 15 µg/mL).
10. RNAse (additive): Dissolve 100 mg RNAse in 100 mL of distilled water. Filter-sterilize through a 0.22 µm MCE membrane filter and store in small aliquots in the dark at -20 °C.

2.4 Isolation and quantification of *Bacteroides* (anaerobic bacteria) bacteriophages

For the isolation and quantification of *Bacteroides* bacteriophages, use the materials described in section 2.1. However, note the different strains, media, and reagents specific for *Bacteroides* bacteriophages described below.

1. Host strain: *B. fragilis* RYC2056 (ATCC 700786) is recommended in the ISO 10705-4:2001 [31, 32]. This strain allows detection of phages from fecal origin but does not

259 distinguish between phages from different fecal sources. For the latest purpose, see
260 references [33, 34].
261 2. Bacteriophage reference material: Bacteriophage B56-3 (ATCC 700786-B1) that
262 infects *Bacteroides fragilis* RYC2056 is recommended for the preparation of
263 reference materials [31, 32].
264 3. $CaCl_2$ stock solution (additive; 0.05 g/mL): 5 g of $CaCl_2 \cdot 2H_2O$ in 100 mL of distilled
265 water while heating gently. Cool to room temperature (25 ºC) and filter-sterilize
266 through a 0.22 µm MCE membrane filter. Store in the dark at 4°C for no longer than 6
267 months.
268 4. Bacteroides phage recovery medium broth (BPRMB): 10 g meat peptone, 10 g casein
269 peptone, 2 g yeast extract, 5 g NaCl, 1.8 g glucose, 0.5 g monohydrated L-cystein,
270 0.12 g $MgSO_4$.$7H_2O$, adjust to 1 L with distilled water. Add 1 mL of $CaCl_2$ solution
271 (0.05 g/mL). Sterilize at 121°C for 15 min. Store in the dark at 4°C. The complete
272 broth should be prepared immediately before use. Aseptically add 10 mL of hemin
273 solution, 25 mL of 1 M Na_2CO_3, kanamycin monosulfate solution (final concentration
274 of 100 µg/mL) and nalidixic acid (final concentration of 100 µg/mL) to 1 L of basal
275 broth. The pH should be adjusted to 6.8 by adding HCl.
276 5. Bacteroides phage recovery medium agar (BPRMA): BPRM with 1.5% agar.
277 6. Soft *Bacteroides* phage recovery medium agar (ssBPRMA): BPRM with 0.7% agar
278 (*see* **Note 4**).
279 7. Hemin solution (additive): 0.1 g of hemin, 0.5 mL of 1M NaOH and 99.5 ml of
280 distilled water. Dissolve by magnetic stirring (*see* **Note 5**). Filter-sterilized through a
281 0.22 µm MCE filter. Store at room temperature (25 ºC) for no longer than 6 months.

8. 1 M disodium carbonate solution (Na_2CO_3) (additive): 10.6 g of Na_2CO_3 in 100 mL of distilled water and filter-sterilize through a 0.22 µm MCE filter. Store at room temperature for no longer than 6 months.
9. Cryoprotectant for *Bacteroides*: 10% bovine serum albumin and 20 % sucrose in water w/v and filter-sterilize through a 0.22 µm MCE filter.
10. Other materials: Anaerobic jar or chamber, Hungate glass tube with rubber stopper and screw cap.

2.5 Extraction of DNA from bacteriophages

1. PES or PVDF low protein binding membrane filters (0.22 µm pore size).
2. Filter holders or sterile syringes.
3. 100 mg/mL DNase I.
4. 20 mg/mL Proteinase K.
5. Buffer for proteinase K: 1 mL 1 M Tris-HCl (pH 8.0), 2 mL 0.5M EDTA (pH 8.2), 10 mL 10% SDS, up to 100 mL double distilled H_2O. Sterilize at 121°C for 15 min.
6. Chloroform.
7. Phenol-chloroform (1:1) (v:v).
8. Absolute ethanol.
9. 70% ethanol.
10. 3M sodium acetate.
11. Double-distilled sterile water.
12. Bench centrifuge.
13. Incubator at 37 ºC.
14. Thermal block at 80 ºC.

2.6 Absolute quantification of bacteriophages by molecular methods

1. Customer manufactured double-strand DNA fragments or plasmid construct vector.
2. Set of primers and TaqMan probe.
3. Master mix designed for use in probe-based assay.
4. Real-time PCR thermal cycler.
5. Molecular grade water.

3 Methods

3.1 Isolation and quantification of reference bacteriophages in wastewater

The general procedure for isolation and quantification of the desired bacteriophage group in wastewater, including preparing the host strain inoculum and reference bacteriophage stock, is described. Nevertheless, the host, media and reference bacteriophage should be adapted according to the target bacteriophage and its corresponding host strain. Table 1 shows the media, host and reference bacteriophage for the bacteriophage groups commonly used to assess water quality, such as somatic coliphages, F-specific bacteriophages and bacteriophages infecting *Bacteroides*. Note that different host strains infecting the same bacteriophage result in a different number of plaque counts.

3.1.1 Host strain stock culture preparation

1. Rehydrate the reference culture of the host strain and streak on a 90 mm agar plate containing the specific medium (1.5% agar). Incubate for 18 h at 37 °C (*see* **Note 6 and 7**).

2. Select 2-3 colonies from the plate, inoculate with 50 ml of the specific broth media and incubate at 37 °C for 18 h with agitation (*see* **Note 7 and 8**).
3. Add glycerol in a ratio of 1:4, mix gently and distribute in small aliquots. Store the aliquots at -80 °C (*see* **Note 8**).

3.1.2 Host strain inoculum culture preparation for bacteriophage quantification

1. Thaw an aliquot of the host strain stock culture at room temperature (25 °C). Inoculate in a ratio 1:100 (v/v) of broth media (*see* **Note 9**) and incubate with agitation at 37 ºC for 18 h. Alternatively, a liquid culture of the strain grown at 37 ºC for 18 h can be used to prepare the inoculum culture (1:100 (v/v)) (*see* **Note 10**).
2. After 1 h, measure absorbance at 600 nm every 30 min until the culture reaches OD_{600} 0.3-0.5, which will correspond to approximately 10^8 colony forming units (CFU)/mL (*see* **Note 11 and 12**). Once the culture reaches the optical density, quickly cool the culture until used. Use the culture within the same working day (*see* **Note 13**).

3.1.3 Enumeration of target bacteriophages by a double agar layer plaque assay (Fig. 1)

1. Filter the wastewater sample through 0.22 µm PES or PVDF low protein binding membrane filter (Fig. 1, top left).
2. Dilute the sample by decimal serial dilutions using saline peptone (Fig. 1, top left) (*see* **Note 14 and 15**).
3. Distribute 2.5 mL of melted soft agar (0.7% agar) into tubes and place them in a water bath at 44 ºC (*see* **Note 16 and 17**).
4. Add 1 mL of the host strain inoculum grown at OD_{620} 0.3-0.5 and 1 mL of the sample to the tube containing 2.5 mL of melted soft agar (Fig. 1 top right

image and bottom image) (0.7% agar). Enumerate phages from each sample at least in duplicate.

5. Mix the tube avoiding the formation of bubbles but without inverting the tube to avoid contamination.
6. Pour the liquid into a 90 mm agar plate containing the specific medium (1.5% agar).
7. Additional controls: Media control (pour 2.5 mL of melted soft agar), host strain inoculum control (add 1 mL of the host strain inoculum grown to 2.5 mL of melted soft agar and pour) and reference bacteriophage control [add 1 mL of the reference bacteriophage stock (section 3.1.6), 1 mL of the host strain inoculum grown to 2.5 mL of melted soft agar, mix gently and pour the mixture onto the agar plate].
8. Allow to solidify and incubate the plates upside down at 37 ºC for 18 h (*see* **Note 18**).
9. Count the number of lytic plaques (count the petri dish with 30-300 plaques) and calculate the bacteriophage concentration in plaque forming units (PFU)/mL).

3.1.4 Bacteriophage isolation from plaques

1. Gently excise a selected isolated plaque from the over layer of soft agar using a sterile needle.
2. Resuspend the plaque in 200 µL of saline peptone.
3. Add chloroform in a ratio of 1:10 (v:v) and vortex vigorously for 5 min. Centrifuge at 16000 *g* for 5 min at 4 ºC.
4. Recover the supernatant.

5. Inoculate 100 µL of supernatant with 1 mL of host strain log-phase inoculum (section 3.1.2) and 9 mL of broth media and incubate at 37 °C for 18 h with agitation.
6. Add chloroform in a ratio of 1:10 (v:v) and vortex vigorously for 5 min. Centrifuge at 16000 *g* for 5 min at 4 ºC.
7. Carefully transfer the aqueous supernatant to a sterile empty tube avoiding solvent phase. Filter the supernatant through 0.22 µm PES or PVDF membrane filter (*see* **Note 19**).
8. Enumerate the bacteriophages in the suspension using the double agar layer plaque assay (section 3.1.3). If higher bacteriophage titer is desired, repeat steps 5-8 using 1 mL of the suspension obtained.

3.1.5 High titre bacteriophage suspension preparation

1. Use 1 mL of phage suspension (obtained in section 3.1.4) and perform the double layer agar plaque assay (section 3.1.3). If the reference bacteriophage is purchased, rehydrate the vial, make decimal serial dilutions, and perform the double layer agar plaque assay.
2. Select a petri dish with confluent lysis and place 5 mL of saline peptone onto the surface of the plate. Incubate at 4 ºC for 15 min.
3. Recover the liquid from the surface with a sterile pipette and add chloroform in a ratio of 1:10 (v:v) and vortex vigorously for 5 min. Centrifuge at 16000 *g* for 5 min at 4 ºC.
4. Take the supernatant and filter it through 0.22 µm PES or PVDF membrane filter.
5. Determine the concentration of phages by performing the double layer agar plaque assay.

403 3.1.6 Reference bacteriophage stock preparation

404 A positive control for the enumeration of bacteriophages is required in each
405 analysis. A stock of known concentration should be used to validate the stability
406 of the host strain. Bacteriophage stocks can be prepared or purchased, thus
407 simplifying procedures and reducing time.

408 1. Dilute the high titre phage suspension in saline peptone solution until it reaches
409 a dilution containing between 40 and 100 PFUs/mL.
410 2. Add sterile glycerol (1:10 v/v) to the diluted phage suspension selected.
411 3. Distribute into aliquots of 1 mL and store at -80 ºC.
412 4. Check the intra- and inter-vial homogeneity (*see* **Note 20**).

413

414 **3.2 Extraction of DNA from bacteriophages**

415 1. Filter 5 mL of the wastewater sample through 0.22 µm PES or PVDF
416 membrane filter to obtain 1 mL of the filtrated wastewater to proceed with the
417 DNA extraction (*see* **Note 21**).
418 2. Chloroform treatment: add chloroform (1:10 v/v) to 1 mL of the filtrated or
419 concentrated sample. Vortex and centrifuge at 16000 *g* for 5 min at room
420 temperature (25 ºC) and collect the supernatant (*see* **Note 22**).
421 3. DNase treatment: Place the supernatant into a new tub and add 20 µL of DNase
422 (100 mg/mL) and incubate at 37 ºC for 1 h (*see* **Note 23**). Afterwards, inactivate
423 DNase enzyme at 80 ºC for 10 min. Take a 5 µL aliquot of the sample as a
424 control of non-encapsidated DNA removal.
425 4. Proteinase K treatment: Add 6 µL of Proteinase K (20 mg/mL) and 250 µL of
426 Proteinase K buffer to 500 µL of the sample and incubate at 55 ºC for 1 h.

5. Mix the sample with phenol-chloroform (1:1 v/v) and centrifuge at 16000 *g* for 5 min at room temperature (25 ºC).
6. Place the supernatant into a new tube and add chloroform (1:1 v/v) and centrifuge again at 16000 *g* for 5 min at room temperature (25 ºC).
7. Place the supernatant into a new tub and mix with 0.1 volume of 3 M sodium acetate and 2 volumes of absolute ethanol and keep at 4 ºC for 24 h to enhance DNA precipitation. Centrifuge at 16000 *g* for 30 min at 4 ºC.
8. Remove the supernatant and wash with 300 µL ethanol (70%) and centrifuge at 16000 *g* for 30 min at 4 ºC.
9. Carefully remove the supernatant and air dry the pellet for 2 hours. After the pellet is air dried elute the pellet in 100 µL with sterile double-distilled water.

3.3 Absolute quantification of bacteriophages by molecular methods

The genetic diversity and plasticity of the bacteriophages within the same group hinders the design of a single molecular assay. In addition, there is not a universal marker for phages such as the 16S rRNA used for bacteria. Nevertheless, bacteriophages that produce barely visible plaques or have conservative genes can be enumerated using a molecular approach, such as real-time PCR. For example, there are assays targeting conservative encoding Shiga toxin genes harboured in temperate bacteriophages infecting *Escherichia coli* [35]. CrAssphage, an abundant human gut-specific bacteriophage suggested as a universal human marker for faecal pollution, has only been quantified so far using molecular assays [19, 20]. Real-time PCR allows absolute quantification if the results are compared to a standard curve with known gene copy concentrations.

1. Standard curve: For the preparation of the standard curve, serial decimal dilutions of a plasmid construct vector containing cloned target gene or customer manufactured double-strand DNA fragments can be used. The standard curve should contain dilutions between 10^8 and 1 gene copies. The limit of detection and quantification can be determined following previous reports [36].
2. Calculate the number of reactions, considering triplicate reactions for the enumeration of each sample (*see* **Note 24**), and a minimum of 3 dilutions of the standard curve and negative controls (qPCR negative control and bacteriophage DNA extraction controls).
3. Bacteriophage DNA extraction controls: To ensure amplification of the genetic material inside the bacteriophage capsid, the following controls should be incorporated:

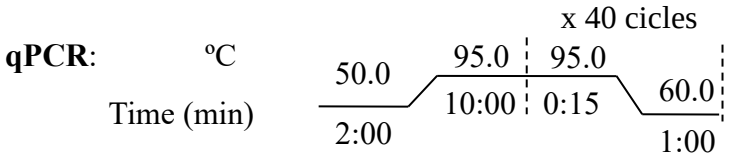
Removal of non-package DNA. Use an aliquot of the sample taken after DNAse treatment (section 3.2.3). Amplification should be negative to discard the presence of DNA outside the phage capsids.

Good performance of DNAse treatment: Serial decimal dilutions of the standard are treated with DNAse followed by the heat inactivation of the enzyme. Amplification should be negative to prove a correct enzymatic activity of the DNAse to remove DNA outside the phage capsids, even if this is present at high concentrations.

Effective inactivation of the DNAse enzyme: Serial decimal dilutions of the standard are added to the heat inactivated standards treated with DNAse. Amplification should be equal as the original serial decimal standard dilutions if the heat treatment effectively inactivated the enzyme.

4. Prepare qPCR mixture as shown in table 2.

5. Amplify DNA using standard thermal cycle conditions for qPCR below.



6. Determine the efficiency of each reaction using the $E = 10^{(1/slope)}-1$ equation [37].

4. Notes

1. Alternative buffer: 10 mL 1 M Tris (pH 7.5), 10 mL 1 M $MgSO_4$, 4 g NaCl, and add 980 mL double distilled water, adjust pH to 7.2. Sterilize at 121 ºC for 15 min.
2. For samples with high bacteria background add nalidixic acid (final concentration 250 µg/mL). Background bacteria interfere with the visualization of plaques.
3. For samples with high bacteria background that interfere with the visualization of plaques, add nalidixic acid (final concentration 100 µg/mL).
4. For samples with high bacteria background that interfere with the visualization of plaques, add kanamycin monosulfate solution (final concentration of 300 µg/mL).
5. Complete dissolution of hemin is required and may take 30-60 min with magnetic stirring.
6. *Bacteroides fragilis* is an anaerobic bacterium, however, it can be handled shortly in aerobic conditions; agar plates must be incubated until 48 h at 37 °C under anaerobic conditions (anaerobic jars or anaerobic cabinet).
7. WG49 host strain:
    - 7.1 Rehydrate the host strain WG49 culture and streak on a McConkey agar plate. Incubate at 37 °C for 18 h.
    - 7.2 Select 2-3 lactose-positive colonies from the plate, inoculate in 50 mL of broth media and incubate at 37 °C for 18 h with agitation.

8. *Bacteroides* host strain*:*

8.1. Inoculate at least ¼ of the cells with 10 mL of BPRM and incubate at 37 °C for 18 h without agitation.

8.2. Add BSA+sucrose as cryoprotectant in a 1:1 ratio (v/v). Distribute to aliquots and store the aliquots at -80 °C.

9. Prewarm the culture media.

10. *Bacteroides* host strain*:* Thaw one vial of stock culture at room temperature (25 ºC) and inoculate in a ratio of 1:10 (v/v) to a prewarmed BPRM broth. Fill the screw-capped tube completely with the broth media. Incubate at 37 °C without agitation.

11. *Bacteroides* host strain: After 2 h, measure absorbance every 30 min until OD_{620} 0.3-0.5. If the cell density does not reach 10^8 CFUs/mL in 3 h, increase the inoculum ratio.

12. It is highly recommended to perform a growth curve of the strain to correlate optical density with colony counts.

13. If more inoculum is needed, several inoculum growth cultures can be prepared and mixed before use.

14. Test the different dilutions the same working day. Dilutions cannot be stored at 4 °C and used the following day.

15. Prewarm the sample to room temperature (25 ºC) to avoid a sharp decrease in the temperature of the soft agar when mixing.

16. Immediately before use, add the additives to the soft agar, described in steps 2.2.3, 2.3.3, 2.3.10, 2.4.3, 2.4.7, and 2.4.8.

17. F-specific bacteriophages: For the enumeration of F-specific RNA bacteriophages, two enumerations must be performed: F-specific total bacteriophages and F-specific DNA bacteriophages, the latest being evaluated as the former but by adding 40 µg/mL of RNase solution (step 2.3.10) to the soft agar to inhibit the F-specific RNA bacteriophages. The

522 subtraction of the F-specific total bacteriophages minus F-specific DNA bacteriophages
523 provides the value of F-RNA bacteriophages.
524 18. For preliminary results, plaques of somatic coliphages can be visualised and counted within
525 6 h, but incubation for 18 h can provide more accurate counts.
526 19. Bacteriophages can be purified using a $CsCl_2$ density gradients [38] to remove cell DNA.
527 This purification is optional and may reduce the number of phages obtained; however, it is
528 mandatory for some analysis such as proteomics or phage genomic studies.
529 20. Reference bacteriophage stock must be assessed for intra-vial homogeneity (homogeneous
530 distribution of bacteriophages in a tube) and for inter-vial homogeneity (homogeneous
531 distribution of bacteriophages withing all tubes) [39].
532 21. For low concentrated targets: Centrifuge 50 mL of the sample at 3000 *xg* and 4 ºC for 10
533 min. Filter the supernatant through 0.22 µm PES or PVDF membrane filter. Concentrate
534 the samples using protein concentrator devices (100 kDa cut-off) that allow protein
535 concentration by centrifuging at 3000 *xg* and 4 ºC for 10 min. Repeat the centrifugation
536 cycle until the volume is 0.5 mL. Recover the phage suspension with a sterile pipette. Some
537 samples may require longer centrifugation times. If necessary, additional double distilled
538 water can be used to rinse the upper part of the concentrator tube and increase recovery.
539 22. Chloroform treatment enhances the disruption of extracellular membrane vesicles.
540 23. Adjust the DNAse concentration according to the sample. Free DNA is hydrolysed in this
541 treatment.
542 24. For lower concentrated target, a previous propagation step that increase the number of
543 phages (hence the target gene) can be done. Nevertheless, in this situation, the abundance
544 of phages in the original samples could not be estimated since the numbers would have
545 been biased during the propagation step. In this case, only a qualitative result
546 (presence/absence) will be obtained. For propagation, filter the sample through 0.22 µm

PES or PVDF membrane filter. Dilute 1:100 a 18 h culture of the host strain in fresh broth media and incubate until OD_{600} 0.3 - 0.4. Inoculate the filtrated samples in a ratio of 1:10 (v/v) in the host strain log-phase culture and incubate at 37 °C for 18 h. Centrifuge the tube at 3000 *g* for 10 min at 4 ºC. Filter the supernatant through 0.22 µm PES or PVDF low protein binding membrane filter and proceed with the DNA extraction.

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674 **Table 1. Host strains, media and reference bacteriophage to be selected according to the reference bacteriophage group.**

Reference bacteriophage group	Host strain	Broth media	Soft agar	Agar plate	Reference bacteriophage	Standard method
Somatic coliphages	*E. coli* CN (ATCC 700078)	MSB	ssMSA	MSA	ϕX174 (ATCC 13706-B1)	ISO 10705-2
	E. coli CN13 (ATCC 700609)	TSB	ssTSA	TSA		USEPA 1601, 1602, 1642 and 1643
F-specific phages	*Salmonella enterica* serovar Typhimurium, WG49 (NCTC 12484)	TYGB	ssTYGA	TYGA	MS2 (ATCC 15597-B1)	ISO 10705-1
	E. coli HS (ATCC 700891)	TSB	ssTSA	TSA		USEPA 1601, 1602, 1642 and 1643
Bacteroides phages	*Bacteroides fragilis* (ATCC 700786)	BPRMB	ssBPRMA	BPRMA	B56-3 (ATCC 700786-B1)	ISO 10705-4

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676 **Table 2. qPCR reaction mixture.**

Reagent	Reaction
Taqman Environmental Master Mix 2X	10 µL
Forward primer	1 µM (final concentration)
Reverse primer	1 µM (final concentration)
TaqMan probe	0.250 µM (final concentration)
Template DNA (Sample or standards)	1 µL
Final volume	20 µL

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