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Microbiological Parameters under the Drinking Water Directive

Current state of art on somatic coliphages and Clostridium perfringens and spores

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Executive summary

Background

In November 1998, the European Council adopted a directive, the Drinking Water Directive (DWD), concerning the quality of water intended for human consumption. It includes a certain number of microbiological, chemical or physical criteria or parameters to monitor, to ensure that i) it is "clean", ii) the distribution network is safe and iii) to react promptly in case of contamination (Directive 98/83/EC)[1].

The Directive has been implemented by Member States, but its approach to monitoring quality at the point of consumption is defined by parameters determined over twenty years ago. After the submission of the European citizens' initiative "Right2Water" to the Commission in December 2013, the Commission invited Member States to improve the access to a minimum water supply and the management of water in a sustainable manner.

Following the WHO recommendations[2], the Commission made a recast proposal either for the microbiological or for the chemical parameters in 2018. After discussions, the European Parliament and the Council reached a provisional agreement on the recast Drinking Water Directive (DWD) on December 2019. The formal agreement was published on February 2020 and the new directive will soon enter into force after its publication in the Official Journal of the European Union (RECAST DWD)[a]. Among the microbiological parameters, somatic coliphage (virus infecting *Escherichia coli*) has been proposed as new parameter, while bacterium *Clostridium perfringens* (*C. perfringens*) and its spores are already included in the Directive.

Rationale

The present report provides an overview on the current knowledge of these two microbiological parameters, their biological characterisations, relevance and suitability as indicators for human faecal contamination in the drinking water treatment. Finally, the report illustrates the available and standardised methods for their detection in water, listing as well the new and most promising ones with advantages/disadvantages and costs.

Furthermore, the report provides a list of recommendations in order to elucidate the role of these two microbiological parameters for drinking water quality management.

Main Findings

Bacteriophages have been proposed as surrogates to study viral persistence in different water environments. They are naturally present in the environment polluted with faeces and have size and morphology similar to enteric viruses. They are used as an indicator for the presence of enteric viruses during wastewater treatment process. Their significant removal ensures an efficient reduction of viruses in wastewater before release of effluent.

Particularly, we investigated whether somatic coliphages could reliably predict the viral contamination of surface waters. Several publications showed that there is no linear correlation between the presence of somatic coliphages and enteric viruses in raw water, but in some studies a partial correlation has been observed, but not with all types of enteric

[a] https://ec.europa.eu/environment/water/water-drink/review_en.html

viruses. However, since somatic coliphages are more resistant than bacteria, if detected in raw water, they could serve as an indicator in the verification process, for removal efficiency of small particles.

Bacterium *C. perfringens* behaves as vegetative cells, that can differentiate into spores when the conditions turn unfavorable. Spores are able to germinate (turn back to vegetative cells) when the conditions turn favorable. *C. perfringens* spores, on the contrary of vegetative cells, are very persistent in the environment and during wastewater treatment. For their reliability as surrogates for the presence of *Cryptosporidium* oocystis and *Giardia* cysts during wastewater treatment, spores have been proposed as an indicator for operational monitoring in drinking water (DW) treatment studies. The number of publications on the co-occurrence of *C. perfringens* spores*, Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts during DW processes is very limited. Indeed, most of the time, inactivation of *C. perfringens* spores during drinking water process is evaluated together with *Escherichia coli* and coliphages, not with parasites. However, due to their persistence and resistance, *C. perfringens* spores could be an indicator for the removal efficiency along the drinking water system.

Recommendations

Somatic coliphage

- Somatic coliphage could be an indicator for verification of the removal efficiency for small and more resistant particles such as viruses during the treatment process of surface water as raw water. However, this would not ensure protection from all enteric viruses since only a partial correlation has been reported between somatic coliphages and some human enteric viruses.
- For groundwater as DW source, the somatic coliphage should be measured only in case of leakage from Waste Water Treatment Plant (WWTP) (sewage pipe breakage close to the groundwater wells) or flood risks due to storm water, and in case the wells are not protected.
- If detected in raw water, the somatic coliphage should be measured along the train barrier for its removal efficiency. No need of any reference value.
- The Water Safety Plan (WSP) should also take into account the resistance (decay rate) of coliphages and enteric viruses due to different environmental factors (temperature, pH, UV light).
- The standardised methods (ISO 10705-2, ISO10705-3, USEPA 1601 and USEPA 1602) should be considered for detection of somatic coliphages and a suitable method should be used based on the range of volume.

Clostridium perfringens and spores

- *Clostridium perfringens* spores are very persistent to water treatment process. Their presence in finished water could indicate the possible contamination by *Cryptosporidium* and *Giardia* (oo)cysts. As an indicator for the validation of the drinking water system process, their absence should be verified according to the risk assessment approach within the WSP in order to see whether the removal of

Cryptosporidium and *Giardia* (oo)cysts (log removal) at each barrier is in the same range.

- ISO 14189 is convenient for the detection of *C. perfringens* spores during drinking water. The parametric value "0 CFU/100 mL" should be reported in raw water.
- When using ISO 14189 for the enumeration of bacteria resulting from the germination of *C. perfringens* spores, the possibility to conclude as "presumed *C. perfringens and spores*" could be left to the laboratories as the confirmation step requires the use of a carcinogenic reagent.
- Alternatively to ISO 14189, ISO 6461 could be used for the enumeration of sulphite-reducing bacteria resulting from the germination of spores of all Clostridia species.
- *Cryptosporidium* oocysts are more resistant to free chlorine than *C. perfringens* spores; in case this disinfection is the only treatment process, *Cryptosporidium* oocysts should be measured.
- For groundwater as drinking water source, this indicator should be measured in case of contamination due to WWTP leakage or flood risks (due to storm water).

1. Drinking water, a right for all citizens

The States have to ensure water quality for their citizens, from water intended for human consumption (drinking water) to recreational water. Constant efforts are made to improve the access to water supplies by a series of treatments, for providing safe water considered as free from microbes and harmful chemicals.

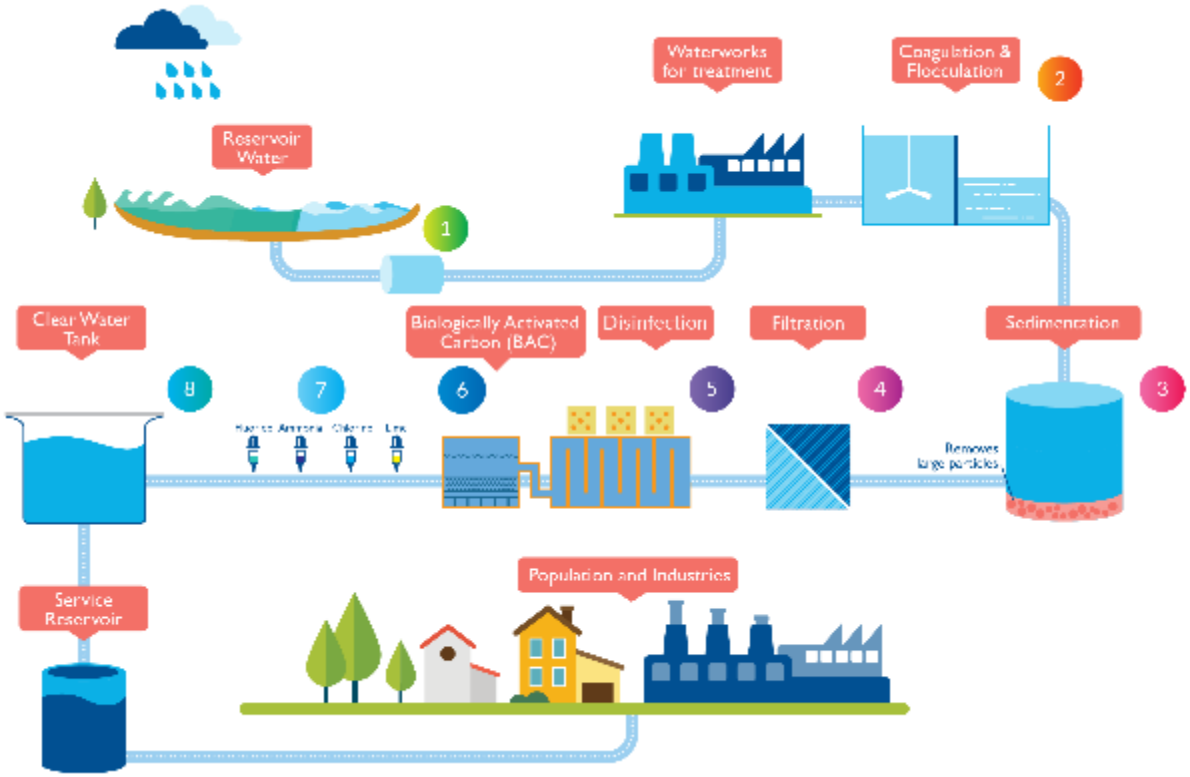

Figure 1. Example of water treatment process from source (here surface water) to tap. From the PUB (Public Utilities Board - Singapore's National Water Agency's website, https://www.pub.gov.sg/watersupply/watertreatment).

Water intended for human consumption, generally from surface water (Figure 1) or groundwater, undergoes a treatment before arriving to the customer's tap. This treatment includes:

- pumping and conveyance of raw water (surface water or groundwater) to the waterwork by pipelines, where particles greater than 1 mm are removed;

- coagulation/flocculation: chemical treatment where coagulants are added to make particles smaller than 1 mm, as sand, flocculate;

- sedimentation: particules fall at the bottom of the tank and are removed;

- filtration: water passes through either rapid sand filters or membrane to remove particle of up to 0.02 micrometer;

- disinfection: UV treatment (not obligatory), chlorination or ozonation to kill harmful bacteria and viruses;

- filtration through activated carbon filters: last step to ensure removal of organic matter;

- residual treatment (to monitor the pH, quality);

- storage of "finished water" in a clear water tank before transport to reservoirs or direct distribution to customers.

To ensure tap water is clean and safe, some additional steps can take place. Water samples are regularly collected by water suppliers (daily and periodically tested) and analysed chemically and microbiologically in water testing laboratories at various stages of treatment from the source until the distribution network.

Not all citizens have access to clean water (http://www.euro.who.int/en/health-topics/environment-and-health/water-and-sanitation/data-and-statistics). Smaller water units and also private wells (not submitted to regular tests) are potentially threats. Even for water coming from water supply, some accidents can occur due to leakage or works on the network, leading to contamination and exposure of customers to potential diseases.

1.1 Drinking water can be source of infections

Despite the efforts made to provide safe water, contamination of the treatment chain by microorganisms and chemicals can occur (pollution of water is often linked to pollution of water faecal or non-faecal pollution).

Among microorganisms, some can be potentially pathogens as listed in Table 1. They are numerous and diverse as they include bacteria, viruses, protozoa and helminths. Some of them can resist to treatment (e.g. chlorine treatment) and then persist into the water supplies over one month causing a threat to the consumer.

In drinking water, the main route of infection is expected to be the ingestion with gastrointestinal disease (gastroenteritis) as the main symptom. However, other routes of infection can occur, such as inhalation or aspiration (leading to respiratory diseases), or direct contact leading to diverse pathologies including infections of the skin, eyes, mucous membranes and wounds (e.g. for bathers) (Figure 2).

In recent years, many waterborne infections, often qualified as outbreaks, have been reported all over the world. The studies described in Annex I of the report highlight the constant need to limit the effects of emerging pathogens among viruses and parasites.

Table 1. Pathogens transmitted through drinking water. The table lists pathogens for which there is some evidence of health significance related to their occurrence in drinking water supplies. (a) Health significance relates to the incidence and severity of disease, including association with outbreaks. (b) Detection period for infective stage in water at 20°C: short, up to 1 week; moderate, 1 week to 1 month; long, over 1 month. Modified from WHO, 2017[3]

Pathogen	Health significance[a]	Persistence in water[b] supplies
Bacteria		
Burkholderia pseudomallei	High	May multiply
Campylobacter jejuni, C. coli	High	Moderate
Escherichia coli – Pathogenic	High	Moderate
E. coli – Enterohaemorrhagic	High	Moderate
Francisella tularensis	High	Long
Legionella spp.	High	May multiply
Leptospira	High	Long
Mycobacteria (non-tuberculous)	Low	May multiply
Salmonella Typhi	High	Moderate
Other salmonellae	High	May multiply
Shigella spp.	High	Short
Vibrio cholerae	High	Short to long
Viruses		
Adenoviruses	Moderate	Long
Astroviruses	Moderate	Long
Enteroviruses	High	Long
Hepatitis A virus	High	Long
Hepatitis E virus	High	Long
Noroviruses	High	Long
Rotaviruses	High	Long
Sapoviruses	High	Long
Protozoa		
Acanthamoeba spp.	High	May multiply
Cryptosporidium hominis/parvum	High	Long
Cyclospora cayetanensis	High	Long
Entamoeba histolytica	High	Moderate
Giardia intestinalis	High	Moderate
Naegleria fowleri	High	May multiply
Helminths		
Dracunculus medinensis	High	Moderate
Schistosoma spp.	High	Short

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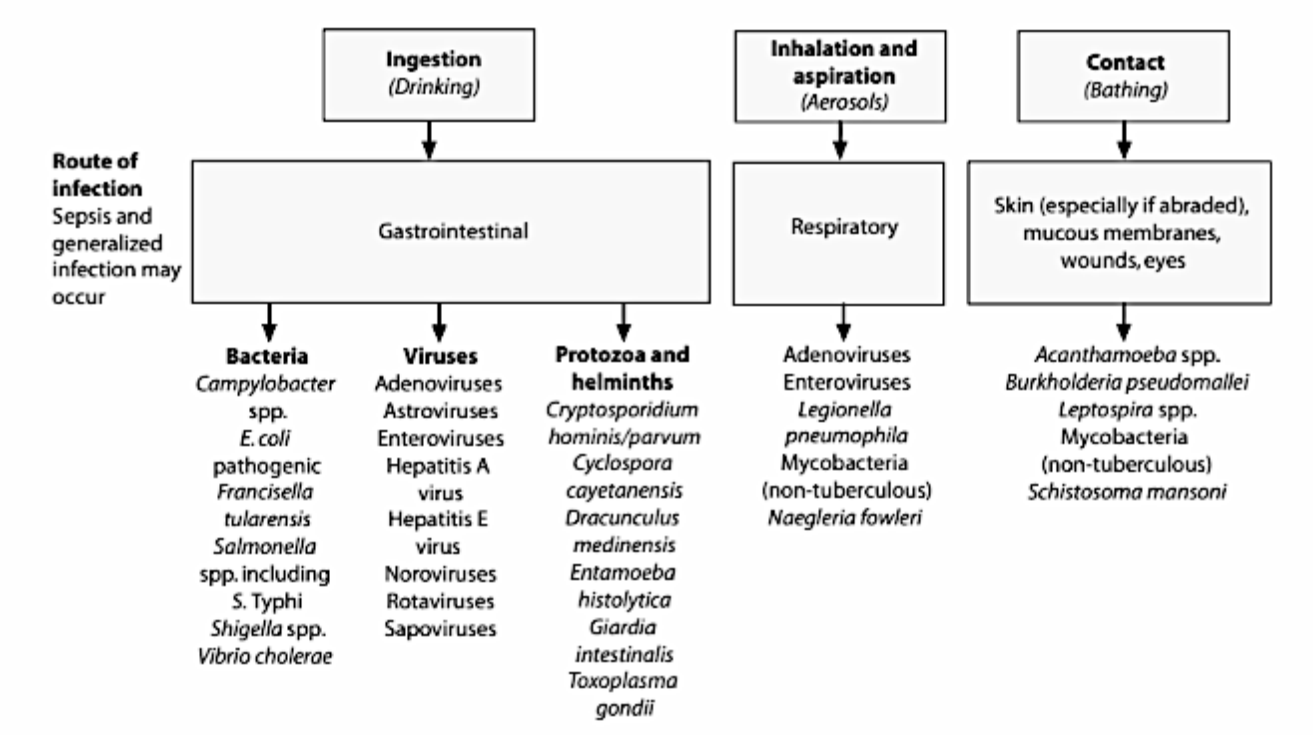

Figure 2. **Transmission pathways for water-related pathogens.** From WHO, 2017[3].

2. Human Enteric Viruses

Viruses are infectious agents classified as obligate intracellular parasites due to their inability to multiply outside a host cell, that results from very limited gene pool encoding only some biomolecules necessary for self-replication. The production of multiple copies of viral particles, called virions, occurs by hijacking the reproductive machinery and employing the metabolism of a host cell through a process of infection. Enteric viruses are capable of primarily infecting and replicating in the gastrointestinal tract of humans and warm-blooded animals. Their genetic material can be either DNA or RNA organised in a single- or double-stranded form stored within a protein structure (capsid) composed of different morphological subunits that confer peculiar characteristics to each virus. To date, there are more than 200 recognised enteric viruses among which 140 serotypes known to cause infections in humans following the feacal-oral transmission route[4].

Although enteric viruses are unable to replicate in the environment, they are shed in extremely high quantities into the feaces of infected individuals and transported through drinking and surface water, groundwater and wastewater. Their environmental persistence is further enhanced, in most cases, by the lack of lipid envelope (Table 2) which makes them resistant to adverse conditions and water treatment processes[5].

Enteric viruses associated with waterborne diseases include adenoviruses, astroviruses, noroviruses, hepatoviruses, rotaviruses, enteroviruses, coronaviruses, parvoviruses, and toroviruses[6,7] (Table 2). As summarized in Table 2, infections caused by these genera may cause symptoms ranging from mild to acute that regard different body compartments with gastroenteritis as a common feature. Notably, low infectious dose is the reason for which the risk of infections caused by enteric viruses ingested with contaminated water may be up to 10000-fold greater compared to bacteria at similar exposures[5]. To limit the incidence of such infections, efforts are being undertaken by national authorities to establish strategies aimed at reducing the presence of enteric viruses and other pathogens in drinking water. The United States Environmental Protection Agency (US EPA) has settled a risk management approach that, in the first instance, foresees the characterisation of a water source, the description of treatment barriers already in place, the identification of circumstances in which contamination may occur and the definition of measures to decrease risks. The US EPA also requires drinking water systems to achieve a 4 log removal and/or inactivation of enteric viruses, meaning elimination of 99.99% of viral particles[8]. Similar recommendations have been expressed in Guidelines for Canadian Drinking Water Quality[9]. The World Health Organisation (WHO) recommends providing control measures within a safety plan in order to reduce potential risks from enteric viruses[3].

Given a wide distribution of enteric viruses in the environment, monitoring of all species and genera would be too demanding in terms of time, cost and feasibility. Strategies based on detection of indicator organisms have been developed to restrict the number of viral pathogens to the most relevant infectious agents. Alternatively, other indicators have been considered over last decades as surrogates to enteric viruses. Some microorganisms making part of faecal microbiota were proposed referring to the common faecal-oral route through which enteric viruses and faecal bacteria may be transmitted[10], however reliance on bacterial model strains would not guarantee water to be free from enteric viruses. Indicators more closely related to enteric viruses such as bacteriophages were further suggested. These viruses target bacterial cells and may reflect pollution by faecal bacteria when considering bacteriophages that specifically infect hosts residing in the gastrointestinal tract. Phages specifically infecting *Escherichia coli* (*E. coli*), namely coliphages, have been selected as the best candidates provided the abundance and role of

their host in current methods employed for detection of faecal contamination. For the same reason, the use of coliphages to evaluate the efficacy of wastewater treatment processes in the elimination of faecal contamination and related infectious agents is under investigation. In particular, bacteriophages have been proposed as indicators for the removal efficiency of enteric viruses from water due to their similarity in size and morphology. For a better comprehension of their potential as indicators reveiling the presence of enteric viruses in water environments, the next chapater describes the current state of the art on bacteriophages with focus on coliphages.

Table 2. Symptoms and morphology of human enteric viruses that may be transmitted in waterbodies.

Enteric viruses Genus and common name(s)	Symptoms	Nucleic acid	Structure
Astrovirus Astrovirus	Mild diarrhoea (lasting 2-3 days but not significant dehydration), headache, malaise, nausea, vomiting, mild fever	Spherical ssRNA	Nonenveloped
Calcivirus Norovirus	Nausea, vomiting, diarrhoea, mild fever, abdominal pain and fever, chronic gastroenteritis for immunocompromised patients, other symptoms than gastroenteritis, fatal issue in some cases	Spherical ssRNA	Nonenveloped
Coronavirus Coronavirus	Upper-respiratory tract illnesses and sometimes lower-respiratory tract illnesses, such as pneumonia or bronchitis, which are more common in immunocompromised people, infants and older adults	Linear ssRNA	Enveloped
Enterovirus Poliovirus, Coxsackievirus A & B, Echovirus	Wild range of clinical symptoms, from mild symptoms (fever, malaise, sore throat vomiting, rash, upper respiratory tract illnesses, less commonly acute gastroenteritis) to more rare and severe symptoms (association with myalgia, Guillain-Barré syndrome, hepatitis, conjunctivitis, meningitis, encephalitis, poliomyelitis, myocarditis) and other chronic diseases	Linear ssRNA	Nonenveloped
Enterovirus Hepatitis A virus	Fever, malaise, anorexia, nausea, abdominal pain, jaundice, rare complication as liver damage that can lead to death	Spherical ssRNA	Nonenveloped
Hepevirus Hepatitis E virus	Fever, malaise, anorexia, nausea, abdominal pain, jaundice, liver damage can lead to death	Spherical ssRNA	Nonenveloped
Mastadenovirus Adenovirus	Upper and lower respiratory tract infections, gastroenteritis, pneumonia, urinary tract infections, conjunctivitis, hepatitis, myocarditis, encephalitis especially in immunocompromised patients, children and the elderly	Linear dsDNA	Nonenveloped
Parvovirus Parvovirus	Responsible for a variety of diseases including acute respiratory tract infections, persistent anemia in immunocompromised patients, transient aplastic crises, hydrops fetalis in pregnant women, and arthropathy. Many people infected by parvoviruses do not have any symptoms. Several parvoviruses are emerging viruses associated with human diseases of unclear clinical significance	Linear ssRNA	Nonenveloped
Reovirus *Reovirus*	Infections usually restricted to the upper respiratory and gastrointestinal tracts and often asymptomatic	Linear dsRNA (segmented)	Nonenveloped
Rotavirus Rotavirus	Group A rotavirus, the most widespread group worldwide: gastroenteritis (vomiting then severe diarrhoea in infants and children leading to hospitalization in most of the cases), before introduction of vaccination in some countries. Symptoms of infections with group B and C rotavirus: asymptomatic sometimes in adults except elderly and immunocompromised patients and sporadic case of diarrhoea in children in a few countries	Spherical dsRNA (segmented)	Nonenveloped
Torovirus Torovirus	Gastroenteritis leading to acute and chronic diarrhea	Linear ssRNA	Enveloped

ss: single-strain; ds: double-strain. Table modified from US EPA, 2015[11].

3. State of the art on coliphages

3.1 Bacteriophages as the starting point

Nearly by the same time, two independent scientists, the English physician Frederick Twort (1915) and the French-Canadian microbiologist Felix d'Herelle (1917), discovered the ability of some viruses to infect bacteria[12]. Twort, while attempting to propagate vaccinia virus (the primary component of the smallpox vaccine), observed transparent spots on agar plates which later revealed to be clear areas deprived of microbial cells within a confluent bacterial layer (Figure 3), interpreted by d'Herelle in the concept of viral parasitism[13,14]. Such clearance zones, today called plaques, correspond to plaque forming units (PFU) of a bacteriophage used to determine the degree of faecal contamination through culture-based methods.

Figure 3. Plaques of bacteriophage AP22 on *Acinetobacter baumannii* 1053 cell lawn. Plaques (clear zones) produced by plating bacteriophage AP22 on a lawn of the host *Acinetobacter baumannii* 1053. The plaques indicate the ability of the bacteriophage to replicate inside the susceptible host cell. From Dubrovin *et al.*, 2012[15].

Further studies showed that bacteriophages display a remarkable diversity and are ubiquitous. The number of phage species in natural environments is estimated in the range of tens of millions, while the concentration of phage particles correlates with the presence of bacteria, making them supposedly the most abundant replicating entity on Earth[16].

Bacteriophages pose an indirect threat to human health by contributing to the evolution of pathogenic bacteria from commensal microorganisms as evidenced by phage remnants integrated into bacterial genomes along with phage-encoded virulence and/or fitness factors[17]. On the other hand, they help combat bacterial colonisation and are employed to reduce bacterial infections through phage therapy that recently has attracted attention as a promising strategy against the globally recognised phenomenon of antibiotic resistance when antimicrobial treatments result inefficient[18].

Since the discovery of bacteriophages, their classification (taxonomy) has been determined as described in Annex II and is subjected to continuous changes. Indeed, following the isolation of several novel bacteriophages and hosts along with new capabilities in genomics and metagenomics, more than 400-600 genomes of novel phages have been annually deposited between 2008 and 2016 to GenBank and to the NCBI phage genome database (most of them carry dsDNA), including a multitude of bacteriophages non classified yet. The classification is performed based on the general structure of a bacteriophage shown in Figure 4, according to morphology and composition of genomes encapsulated in a

symmetric, usually icosahedral, capsid composed of repeat protein subunits. Similar to enteric viruses, phage genomes display a great heterogeneity consisting of both single- or double-stranded DNA and RNA, be linear or circular and, for the RNA genomes, be either positive sense (directly translated into protein) or negative sense (requiring conversion to positive sense RNA before translation) (see Table 2). The main difference among phages is the presence or absence of a "tail" structure.

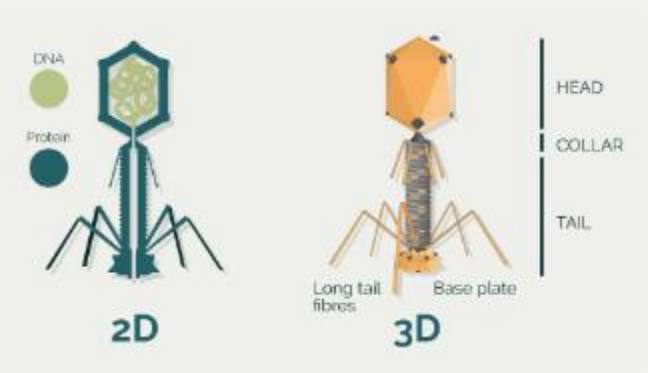

Figure 4. 2D and 3D structure of tailed bacteriophages. The structure of a phage consists of a "head" or capside (symmetric, non-enveloped) enclosing the genome (generally DNA), and a contractile or non-contractile "tail" with spiral shape, harbouring a base plate and long fibers which enable phage attachment or adsorption to the surface of a host cell and the injection of the genome into the bacterial cytoplasm. Both parts are connected by a "collar". From https://coliphages.com.

3.2 The reproduction of bacteriophages

3.2.1 Attachment of bacteriophages to the bacterial host cell

Bacteriophage tropism is conditioned by specific attachment via proteins considered as key receptors on the surface of the bacterial host. This phase, named attachment or adsorption of the virion, is followed by penetration, viral synthesis, maturation/assembly and finally release of new virions.

Receptors are located on two different sites. Some phage receptor sites, located on bacterial sex fimbriae expressed for reproduction purposes, are used by F-specific (alternate name "male-specific") phages. They are produced by bacteria in the logarithmic growth phase under optimal growth conditions. Some phage receptor sites are located on the bacterial cell wall and present/expressed all the time. These receptor sites are recognised by somatic phages which can also attach to dead bacteria. Figure 5 shows the two distinct groups of phages infecting *E. coli*: F-specific coliphages and somatic coliphages.

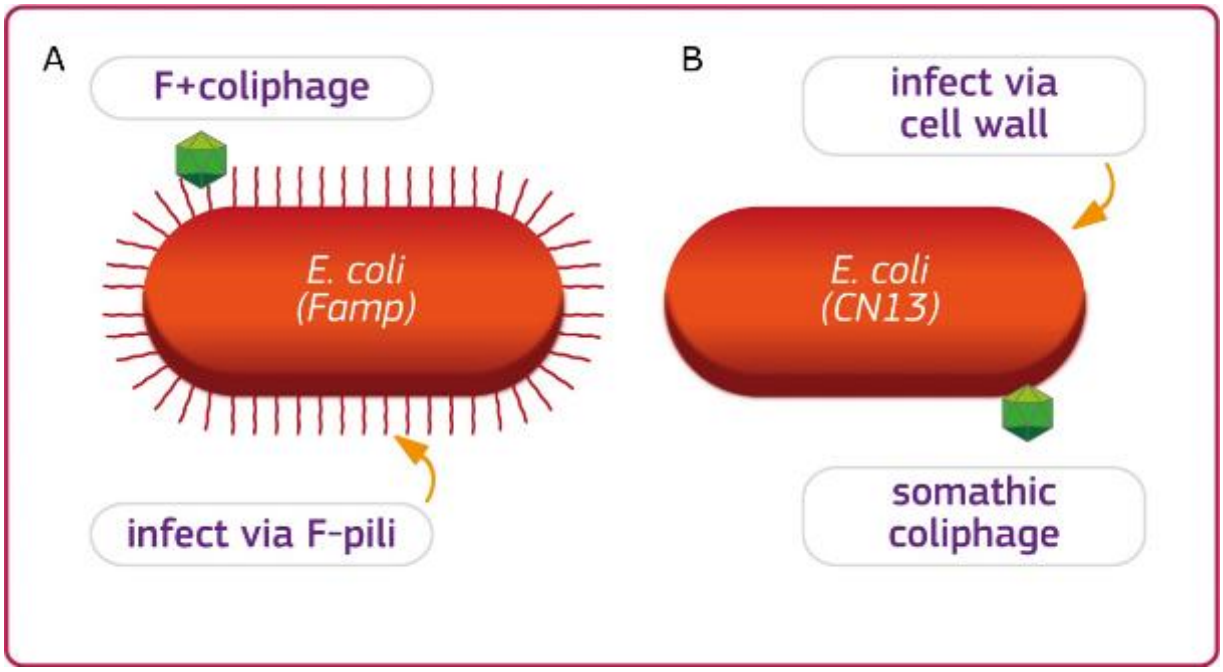

Figure 5. Schematic representation of F-specific (or male-specific) coliphages, somatic coliphages and their host cells. (A) F-specific coliphages infect host cells (e.g. *E. coli* $_{Famp}$, *Salmonella typhimurium* WG49) through the sex pili encoded by the F-plasmid. (B) Host strains of somatic coliphages include *E. coli* (e.g. *E. coli* CN13) and related species which are infected through the cell wall.

3.2.2 Replication and release of new viral particles

Phages are commonly divided into two major groups according to their mode of replication: **lytic phages** and **lysogenic phages** (Figure 6). Both kinds of phages use the host cell machinery for the replication of their genetic material and for a correct assembly of viral subunits (capsomers into capsid surrounding the genome, and eventually collar and tail, and fibres) to produce mature viral particles or virions.

Lytic phages start replication immediately after infection of the host cell (Figure 6), releasing new virions in less than 30 minutes for some phages (usually between 100 and 200 minutes, depending on the bacteriophage) and displaying a halo of lysis around bacterial colonies cultured on a solid culture medium.

Lysogenic phages are able to integrate the viral genome into the nucleic acid of the host cell or maintain it as a circularised DNA in the cytoplasm. The viral genome is replicated alongside the host genome without producing new virions (Figure 6). These phages are referred to as "prophages". The production of virion particles can occur following a switch from a lysogenic cycle to a lytic cycle.

Two additional phage lifecycles have been well studied: the pseudolysogenic and chronic infection[19,20] (Figure 6).

Phages in pseudolysogenic lifecycle are able to insert the genome into the host cell or may maintain it as free circularised DNA in the cytoplasm[21] (Figure 6). In both cases, the viral genome resides within the cell in a non-active state, so it does not multiply as in the lytic lifecycle and its replication is not synchronised with the host cell cycle as in the lysogenic phages. This phage-host cell interaction is due to host cell starvation conditions and therefore to an insufficient energy for the phage to initiate a lytic or lysogenic process.

Depending on the environmental stimuli, these phages undergo lytic or lysogenic infection. Finally, phages capable of chronic infection produce viral progeny but do not lyse the host cells (Figure 6).

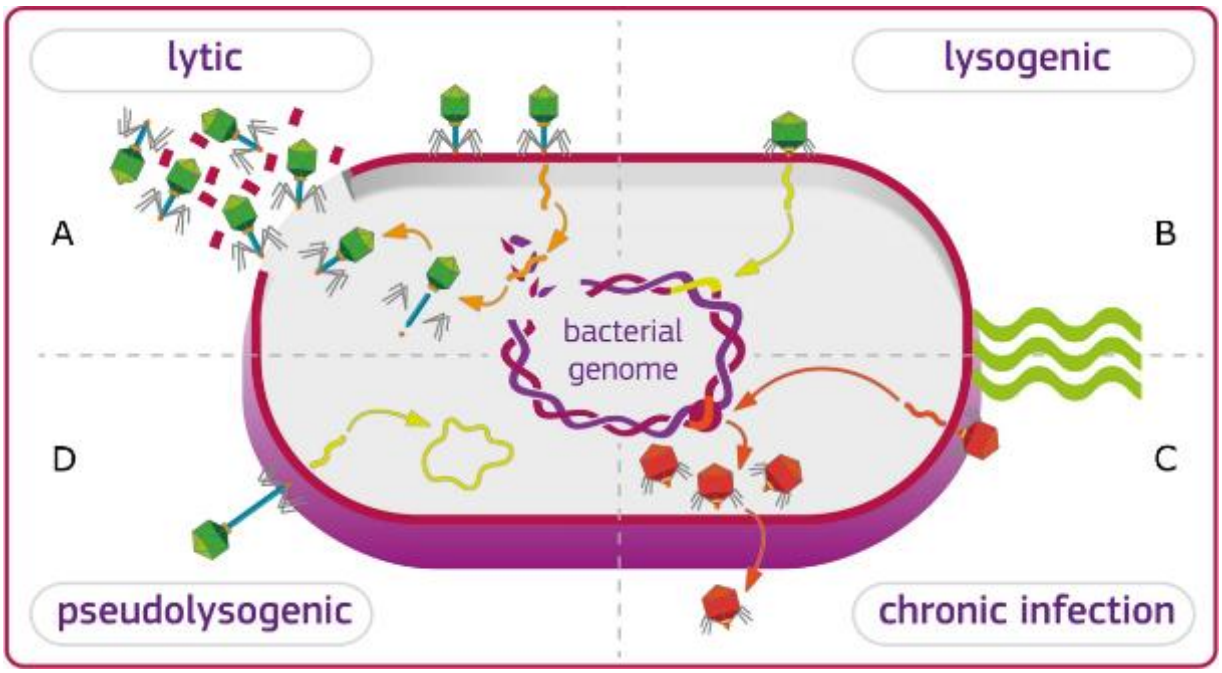

Figure 6: Illustration of the most studied bacteriophage lifecycles. (A) In the lytic cycle, bacteriophages replicate and lyse the host bacterial cells. (B) Lysogenic bacteriophages incorporate their nucleic acid in the host genome, or may maintain it in the cytoplasm, and no virion is released from the host cell. (C) Chronic phages are actively replicating in the host and produce viral progeny without lysing their host cell. (D) The pseudolysogenic infection involves the insertion of the viral DNA into the host. The viral genome can remain free in the cytoplasm or can be integrated in the host genome without producing virion particles. Depending on the environmental conditions, these phages undergo lytic or lysogenic lifecycle. Adapted from Lawrence *et al*., 2019[20].

3.3 Coliphages in the assessment of water quality

The potential of coliphages as indicators of general faecal contamination with regard on human viral pathogens has been linked to their natural presence and excretion in faeces of humans and warm-blooded animals[11,22]. Similar to waterborne human enteric viruses described in Table 2, many coliphages are non-enveloped and share similar nucleic acid structure (shown in Table 3). F-specific RNA coliphages (*Leviviridae*) are morphogically similar to enteroviruses, caliciviruses, astroviruses, and hepatitis A and E viruses, while somatic coliphages are more similar to adenoviruses[11,23]. Possible applications of bacteriophages infecting other bacteria such as *Bacteroides fragilis*, *Salmonella typhimurium* and *Enterococcus* spp. are presented in Annex III.

Somatic coliphages refer to a wide spectrum of lytic members of the families *Myoviridae*, *Siphoviridae*, *Podoviridae* and *Microviridae*, characterised by linear or circular single- or double-stranded DNA genomes[11] (Table 3). Controversies exist regarding a possible application of somatic coliphages as reliable indicators for the detection of enteric viruses. Some studies point out that they are DNA and not RNA phages, therefore the genetics of enteric viruses, which mostly have DNA genomes, is not fully mimicked by somatic coliphages. Uncertainties regard also the ability of somatic coliphages to replicate in *E. coli*

under environmental conditions or in water treatment facilities, as well as correlations with the abundance of enteric viruses[11,24]. Nonetheless, coliphages are used in some countries for water quality assessment[25,26]. Somatic coliphages have been detected at higher concentrations than F-specific coliphages in river and marine water environments (Table 4), with much lower percentage of positive samples obtained from groundwater (Table 5).

The number of studies assessing the abundance of somatic coliphages and F-specific DNA or RNA coliphages in surface water and groundwater is scarce. In a review by Jofre and colleagues (2016)[27], 10 publications are cited for surface water (river water, fresh and marine water, reservoir) at different geographic latitudes, showing a great variability in the number of positive samples (Table 4). For groundwater (wells, springs), concentrations of phages are not indicated in the review but an indicative percentage of positive samples is provided as the criterion of coliphage presence/absence in a given volume (Table 5).

Recently, most efforts are dedicated to studies on detection of faecal contamination or enteric viruses in wastewater, where somatic coliphages have been found to outnumber F-RNA phages by a factor of ~5. They have also been reported at high levels in sewage in different studies over the past decades (10^6-10^8 PFU per liter)[28-33].

Male- or F-specific coliphages mostly refer to *Inoviridae* and *Tectiviridae* (untailed circular ssDNA and linear dsDNA phages) and *Leviviridae* (linear ssRNA phages) (Table 3). F-specific DNA coliphages (*Inoviridae*) have received less attention as reliable indicators of faecal contamination due to their minor abundance compared to F-specific RNA coliphages and major morphological differences with enteric viruses. In turn, F-specific RNA coliphages (*Leviviridae*) have been proposed for water monitoring. Their host cells produce receptor sites on the fertility fimbriae which are expressed only during the logarithmic growth phase under optimal growth conditions. It is thought, therefore, that F-specific RNA coliphages unlikely replicate in environments other than the gastrointestinal tract, although contrasting studies exist[26,34]. Their great resistance against water treatment processes and adverse environmental conditions involving the presence of chemical substances, heat, sunlight, ultraviolet light, salinity and chlorination has been reported[11]. F-specific RNA coliphages have been divided into two genera based on the nucleotide sequence similarities: the *Levivirus* and *Allolevivirus*[23]. Taking into account serological properties and other experiments, *Levivirus* genus has been further subdivided into genogroup I and II, and *Allolevivirus* into genogroups III and IV[35,36] (Table 3). Subsequent studies showed their possible application to discriminate between human and non-human faecal sources as human excreta contain higher populations of genogroups II and III, whilst the animal waste is rich in populations belonging to genogroups I and IV (but not excluding the other genogroups).

Several studies evaluating faecal contamination in recreational water, recycled water (reclaimed water) and, to a lesser extent, in drinking water (water supply) and groundwater suggested F-RNA coliphages as indicators but with lower counts compared to somatic coliphages.

Table 3. Morphology of selected families of somatic and F-specific coliphages. From US EPA, 2015[11].

Type	Family (Examples)	Nucleic acid	Structure
Somatic coliphages	*Myoviridae* (T2, T4)	Linear dsDNA	Nonenveloped, contractile tail, consisting of a sheath and central tube
Somatic coliphages and *Bacteroides* bacteriophages	*Siphoviridae* (λ, T1, T5)	Linear dsDNA	Nonenveloped, long noncontractile tail
Somatic coliphages	*Podoviridae* (T3, T7, P22)	Linear dsDNA	Nonenveloped, short noncontractile tail
Somatic coliphages	*Microviridae* (ΦX174)	Circular dsDNA	Nonenveloped, isometric
F-specific DNA coliphages	*Tectiviridae* (PR772)	Linear dsDNA	Nonenveloped, cubic capsid (isosahedral), no tail
F-specific RNA coliphages (Genogroups I, II, III, IV)	*Leviviridae* (MS2, Qβ, F2)	Linear ssRNA	Nonenveloped, isometric
F-specific DNA coliphages	*Inoviridae* (M13)	Circular ssDNA	Nonenveloped, filamentous

Table 4. Literature data on the concentration of somatic and F-specific coliphages in surface water. The concentration is measured by either US EPA or ISO methods. A mean of the number of coliphages is shown per each site and expressed as PFU/100 mL. The percentage of positive samples is indicated in brackets The asterisk indicates that mean values for somatic coliphages varied from 8.8 to 430 PFU/100 ml between 10 sampling sites. From Jofre *et al.*, 2016[27].

Samples	Methods	Number of Samples	Geographical Location	Somatic Coliphages (% +)	F-Specific (% +)	RNA F-Specific (% +)
River water	ISO	392	Spain, France, Colombia, Argentina	6.2×10^3 (?)		5×10^2 (?)
Freshwater reservoir	USEPA	65	Singapore	2.2×10^2 (98)	1.1×10^2 (98)	
Sea water	ISO	806 for somatic and 427 for RNA F-specific phages	Spain	32.8 (72.6)		8 (25.5)
Fresh and sea water	ISO	139	9 European countries, 13 sampling sites	1.7×10^2 (92)		12 (50)
Fresh and sea water	USEPA	12	California	$2.0–3.3 \times 10^2$ (100)	<0.02–30 (25)	
River water	USEPA	120	California	$6.0–10^3$ (?)	$5.0–1.1 \times 10^2$ (?)	
River water	ISO	96	France	1.7×10^3 (100)	2.0×10^2 (92)	
Sea water	ISO	20	Spain	$<10–1.2 \times 10^4$ (95)		0–84 (15)
River water	ISO	75 (10 sites)	The Netherlands	$^*8.8–4.3 \times 10^2$ (100)	0.04–93.6 (?)	
Sea water	USEPA	436	California	3.1–4.9 (median)	0.3 (median)	

Table 5. Literature data on the presence of somatic, F-specific and RNA F-specific coliphages in groundwater. The authors report the percentage of positive samples for each type of coliphages. From Jofre *et al*, 2016[27].

Sampling site	Method	Number of Samples	Geographical Location	Somatic Coliphages	F-Specific Phages	RNA F-Specific Phages
Wells of varied characteristics	USEPA	160	Canada	8.7	1.8	
Variety of wells and springs	ISO	197	Argentina, Colombia, France, Spain	41.7		28.8
Variety of wells	USEPA	39	Korea	12.5	7.5	
Wells and springs	ISO	125	Spain	53.6		36.0
Variety of wells	F+ ISO, somatic coliphages C3000	444	USA	10.8	9.5	

3.4 Persistence of coliphages in the environment

The effects of environmental factors such as temperature, sunlight, salinity, predation and enzymatic degradation on decay rate of coliphages and/or human enteric viruses have been widely described in the US EPA review (2015)[11]. Some studies have found that coliphages may be equally or more resistant to environmental stressors than enteric viruses depending on viral subgroup and characteristics of water site. Morphological features are largely associated with the ability of coliphages to survive in the environment. The presence of a tail and a large and mechanically stable capsid, along with the lack of a lipid envelope which can be more easily disrupted than the other parts of a virus, are generally thought to increase resistance against temperature changes, osmotic pressure, dessication and chemical disinfectants. It has been observed that synergistic action of environmental stressors, mainly temperature, sunlight and salinity, may reduce coliphage persistence and influence viral aggregation that further affects the number of plaque forming units (PFU) detected in a sample.

Besides physico-chemical conditions, biological factors such as planktonic or biofilm-associated microbial community residing in aquatic systems may reduce the number of coliphages via direct predation or by releasing proteolytic enzymes which degrade the viral capsid. Adsorption to larger and heavier particles (organic and inorganic matter) is thought to confer protection to bacteria (from predation) and virions, thus participating to their spread in the environment[11]. On the other hand, solid particles, especially those containing photosensitizers, may produce reactive oxygen species upon exposure to sunlight resulting in reduced survival of viruses.

For all factors, persistence and decay rate of coliphages and enteric viruses differ with the intensity of exerted stress and not always the effects correlate between bacteria and human viruses.

3.5 Somatic coliphages as potential indicators for monitoring faecal contamination and viral contamination in drinking water

Bacterial indicators such as *E. coli* and Enterococci (also referred to as fecal indicator bacteria, FIB) are generally used for water quality management.

Recently, the issue of eventual presence of viruses (and especially enteric viruses) in the aquatic environment has been highlighted[5]. Unfortunately, the detection of viral particles is very complicated, and there have been several suggestions to use other measurements to assess the presence of viruses. In particular, bacteriophages which are naturally present in the environment polluted with faeces, have been proposed as surrogates to study viral persistence in different water environments. Two types of bacteriophages have been proposed: i) somatic phages, especially **somatic coliphages** infecting *E. coli* via attachment to the cell wall; ii) **F-specific coliphages** that infect *E. coli* via F-pili.

The available peer-reviewed literature reports conflicting results on whether **somatic coliphages** can reliably predict the viral contamination of surface waters[37]. We identified twenty-five studies (published between 1999 and 2019) addressing the correlation between enteric viruses and somatic and/or F-specific coliphages in ambient water and groundwater.

A brief summary and key relevant details of each article are reported below, while Table 6 summarises the identified literature. Culture methods are used for detection of coliphages while molecular methods are used for enteric viruses.

In a study of 2001, Baggi *et al*. did not show any association between somatic coliphages (by culture method) and enteric viruses (RT-PCR and nested-PCR) in the case of water receiving waters from a wastewater treatment plant (WWTP) based on four treatment stages, while there was an association for water receiving effluents from a three-treatment stages WWTP[38]. No regression study was performed.

Jiang *et al*. (2001) did not find any correlation between the presence of total coliphages (somatic and F-specific together) and the presence of adenoviruses (PCR, nested-PCR) in a study on coastal waters[39].

Hot *et al*., (2003) did not show any association between somatic coliphages (by culture method) and enteroviruses in a model of culture method that enables to count infectious enterovirus, and between somatic coliphages and different types of enteric viruses by molecular method (RT-PCR on six types)[40].

Jiang *et al*., (2004) could not observe any association between the presence of somatic coliphages and adenoviruses, enteroviruses and hepatitis A virus (determination by PCR or RT-PCR, not by viral culture)[41].

Ballester *et al*., (2005) showed that the presence of enteric viruses in marine coastal waters impacted by WWTP was significantly correlated with the presence of somatic and F-specific coliphages[42]. The presence of somatic coliphages was significantly correlated with the presence of adenoviruses, but less significantly with the presence of rotaviruses and enteroviruses and non-correlated with the presence of astroviruses (Pearson linear correlation). Therefore, it was difficult to conclude that they could act as surrogate for all enteric viruses.

Choi *et al*., (2005), did not observe any association between the presence of somatic coliphages and adenoviruses and enteroviruses (cell culture or qPCR) in Californian rivers[43].

Boehm *et al*, (2009) did not show any correlation between the presence of somatic coliphages and enteroviruses and did not detect other types of enteric viruses or adenoviruses in a study on samples from a Californian beach impacted by sewage (leakage)[44].

In a study on two rivers impacted by wastewaters in Germany, Jurzik and coworkers (2010) did not find any significant correlation between the presence of somatic coliphages and the presence of adenoviruses, noroviruses, nor rotaviruses[45].

In a study of Dutch rivers as a source of drinking water**,** Lodder *et al*. (2010) observed a correlation between somatic and F-specific coliphages and enteroviruses but no correlation was found between somatic coliphages and the other enteric viruses (reoviruses, noroviruses, rotaviruses)[46].

Payment and Locas (2011) did not find any correlation between the presence of somatic coliphages and enteroviruses in a study on surface water (Canadian river). In the same study, they did not show any association between the presence somatic and F-specific coliphages and the presence of noroviruses in a large study on groundwater[47].

Viau *et al*., (2011) did not show any association between the presence of somatic coliphages and the presence of adenoviruses, noroviruses and enteroviruses in Hawaiian streams[48,49].

In a study on two Californian recreational beaches, Love *et al*. (2014) concluded that the presence of somatic coliphages was not correlated with the presence of noroviruses and adenoviruses (nested-PCR and RT-PCR)[50].

In a study on a tropical reservoir used as source for potable water in Singapore (2014), Rezaeinejad *et al*. reported that the presence of somatic coliphages was not significantly correlated with the presence of enteric viruses, while the presence of F-specific coliphages correlated with the presence of noroviruses but not with the presence of astroviruses, rotaviruses and adenoviruses[51].

Performing a study on a tropical reservoir source for potable water in Singapore (2015), Liang and coworkersdid did not report any correlation between coliphages (somatic and F-specific coliphages) and six types of enteric viruses (qPCR, RT-qPCR method)[52].

Mackowiak *et al*. did not observe any association between the presence of somatic coliphages and adenoviruses, noroviruses, enteroviruses, rotaviruses in a study on a German lake-river (2018)[53].

Kauppinen *et al*. (2018) did not report any association between the presence of somatic coliphages (nor F-specific coliphages) and noroviruses (qPCR and RT-qPCR) in groundwater after disinfection procedures following two waterborne outbreaks in Finland in 2011[54]. Norovirus and adenovirus persisted after disinfection.

Cooksey *et al*. did not find any correlation between the presence of somatic coliphages and adenoviruses (qPCR) in a study published in 2019 on a subtropical brackish estuarine lake in Louisiana[55].

In a study of nineteen samples collected from different residential canals in Florida potentially impacted by a septic tank, Griffin *et al.* (1999) did not report any association between the presence of total coliphages (somatic and F-specific) or F-specific coliphages and enteric viruses (coliphages were detected in only 2 out of 19 samples)[56].

Skraber *et al.* found a correlation between the presence of somatic coliphages and enteroviruses and noroviruses (culture method combined to molecular methods for enteroviruses) in a tudy of a French fresh river in 2004[57].

Mocé-Llivina *et al.* (2005) showed a correlation between the presence of somatic coliphages and enteroviruses (culture method, RT-PCR) in samples from Spanish beaches and rivers impacted by WWTP and their effluents[58].

In the study of River Meuse, Westrell *et al*. (2006) observed that the seasonal peak corresponding to norovirus did not coincide with the peak of F-specific *Salmonella* spp. Phages. The association between F-specific coliphages and enteric viruses was not measured[59].

In 2007, Jiang and collaborators did not observe any correlation between F-specific phages and enteroviruses nor with adenoviruses in different sites of a Californian estuary zone serving as recreational water and ecological reserve[60].

In a study of a tropical aquatic system in Mexico, Espinosa *et al*., (2009) reported a positive correlation between the presence of F-specific coliphages and enteroviruses, but they did not observe any correlation between the presence of F-specific coliphages and the presence of adenoviruses and astroviruses (RT-PCR)[61].

In a small study on nine samples from groundwater wells and one from a polluted river in Nepal in 2011, Haramoto and collaborators could not conclude to any association between coliphages and enteric viruses due to few samples. However, of the six samples that did not contain F-specific coliphages, two samples contained enteric viruses (adenovirus or norovirus); of three samples positive for F-specific coliphages, two were positive for enteric viruses (qPCR, RT-qPCR)[62].

Updyke *et al*. did not observe any association between coliphages and enteric viruses in freshwater in Hawaii in 2015[63].

It is important to note that these studies have been performed with very different methods and sampling protocols. For example, water sources are different (from freshwater in European rivers to saline or brackish water in tropical canals, high altitude tropical reservoir, coastal waters, creeks, beaches, influenced by sewage or non-impacted), as well as the numbers and volumes of samples, sampling methods (season, frequency, number of sample, temperature) and detection methods for coliphages and enteric viruses.

Taken together, seventeen of the twenty-five studies show no correlation between the presence of somatic coliphages and the presence of enteric viruses. Therefore, at the moment, there is no robust experimental evidence supporting the use of somatic coliphages as reliable indicators of water quality.

Table 6. Studies on coliphages as possible indicators of faecal contamination in water. (Source: US EPA, 2015[11] and Dorevitch, 2016[64]). Abbreviations: FIB: faecal indicator bacteria; AstroV: astrovirus; HAV: hepatitis A virus; HAdV: human adenovirus; EV: enterovirus; HpyV: human polyomavirus; ReoV: reovirus; RoV: rotavirus; NoV GI/GII: norovirus (former Norwalk virus) group I or II; N: sumber of samples; ICC: integrated cell culture, nPCR: nested Polymerase Chain Reaction; qPCR: quantitative PCR, RT: retro-transcription; VIRADEN method: "virus adsorption enumeration" based on the direct enumeration of viruses adsorbed into nitrate-acetate cellulose membranes.

Water type	Sampling	detected coliphages	coliphage detection method	detected virus	virus detection method	Comments or conclusion to the correlation study or the comparison test	Reference
Lake Pontchartrain, subtropical brackish estuarine lake, Louisiana, USA	March 2017-August 2017 Weekly sampling 9 recreational sites water samples (N=222, volume: 1L for coliphages and 100 L per FIB) water samples (N=54; volume of 20 L for AdV)	somatic coliphages and F-specific coliphages	USEPA Method 1602	HAdV	qPCR	No correlation between somatic coliphages, F-specific coliphages (and other FIB) and HAdV	Cooksey *et al*., 2019[55]

Water type	Sampling	detected coliphages	coliphage detection method	detected virus	virus detection method	Comments or conclusion to the correlation study or the comparison test	Reference
Groundwater after two waterborne outbreaks linked to contamination of groundwater supplies, Finland	August 2011 Water samples (N=5; septic tank wastewater, collection tank, ground water well and tap water)	somatic coliphages and F-specific coliphages	US EPA Method 1601: for somatic coliphages and F-specific coliphages US EPA Method 1602: for F-specific coliphages	NoV GI, NoV GII, HAdV	RT-qPCR (NoV GI, NoV GII) qPCR (HAdV)	After the disinfection process, no correlation could be assigned as F-specific coliphages could not be detected in septic tank neither in collection tank or groundwater well. Somatic coliphages were detected only from the collection tank, whereas NoV GI, NoV GII, AdV could still be measured (removal efficiency lower than FIB).	Kauppinen *et al*., 2018[54]
Lake Baldeney and Ruhr river (urban river), Germany	July- September 2015 3 sampling sites (upstream the lake, at the lake and downstream) water samples (N= 24), biofilms (N= 24), and sediments (N=24), weekly collected	somatic coliphages	ISO 10705-2	HAdV, NoV GII, EV, RoV	RT-qPCR (NoV GII, EV, RoV), qPCR (HAdV)	Not determined	Mackowiak *et al*., 2018[53]

Water type	Sampling	detected coliphages	coliphage detection method	detected virus	virus detection method	Comments or conclusion to the correlation study or the comparison test	Reference
Eighteen fresh and offshore recreational waters, Hawaii some sampling points could be impacted by sewage treatment plant	water samples (N=108)	F-specific coliphages	PCR	EV, NoV GI, NoV GII	PCR, qPCR	No correlation between F-specific coliphages and EV. Some samples are positive for enteric viruses.	Updyke, 2015[63]
Surface water as a reservoir of a tropical urban area serving as catchment area for potable-water use and recreation, Singapore	December 2011-March 2012 and July 2012- April 2013 N=148 water samples (volume of 10L for enteric viruses)	somatic coliphages and F-specific coliphages	US EPA 1601	RoV, AstroV, NoV GI, NoV GII, HAdV, HpyV	qPCR	No correlation between somatic coliphages and F-specific coliphages, and NoV and AdV.	Liang, 2015[52]
Surface water as a reservoir of a tropical urban area serving as catchment area for potable-water use and recreation, Singapore	1-year period water samples (N= 65) monthly sampling (different sampling points)	somatic coliphages and F-specific coliphages	US EPA Method 1602	HAdV, NoV GI, NoV GII, AstroV, RoV	qPCR, RT-qPCR	Correlation between F-specific coliphages and NoV.	Rezaeinejad *et al*., 2014[51]

Water type	Sampling	detected coliphages	coliphage detection method	detected virus	virus detection method	Comments or conclusion to the correlation study or the comparison test	Reference
Two recreational beaches with a history of beach closures, Southern California USA	4-month study Avalon beach (N=324 water samples) and Doheny Beach (N=112 water samples) For coliphages detection: 2L water samples; for virus detection: 40 L samples	somatic coliphages and F-specific coliphages	modified version of US EPA method 1601	HAdV, NoV	nested RT-PCR HAdV and NoV	- At Avalon beach: correlation between F-specific coliphages and AdV (but marginally significant) - At Doheny beach: beween F-specific coliphages and AdV: Inverse correlation between somatic coliphages, F-specific coliphages and NoV: no correlation between somatic coliphages and HAdV: no correlation	Love *et al*., 2014[50]
Twenty-two streams that discharge to coastal waters adjacent to beaches, Hawaii	December 2009 (5 consecutive days) and March 2010 (5 consecutive days) 3L-samples (early morning and high noon)	F-specific coliphages (96% of the samples positive)	membrane filtration and US EPA Method 1601	HAdV, EV, Nov GI, NoV GII Samples: 15% positive to AdV, 22% to NoV GI, NoV GII (12.5%), EV (6%)	qPCR, RT-qPCR	No correlation between F-specific coliphages and viruses (AdV, NoV, EV).	Viau *et al*., 2011[48,49]
Saint Lawrence River, and groundwater, Province of Québec, Canada	3 datasets (sewage, surface water and groundwater) River samples (N=379) and groundwater (N=242)	somatic coliphages and F-specific coliphages	US EPA Methods 1601 and 1602	NoV	cell culture, RT-PCR	In surface water: correlation not reported as no information on coliphages concentration. In groundwater: no correlation between somatic coliphages, F-specific coliphages and enteric viruses	Payment and Locas, 2011[47]

Water type	Sampling	detected coliphages	coliphage detection method	detected virus	virus detection method	Comments or conclusion to the correlation study or the comparison test	Reference
Groundwater wells and polluted river, Kathmandu Valley, Nepal	groundwater (N=9) and river water samples (N= 1)	F-specific coliphages	qPCR (Ct values are mentioned, but quantification is not reported)	NoV, HAdV	qPCR, RT-qPCR	Correlation not reported between F-specific coliphages and enteric viruses (NoV, HAdV).	Haramoto, 2011[62]
Fresh rivers for the production of drinking water (10 locations), The Netherlands	4-year study (1999-2002) with regular sampling N= 75	somatic coliphages and F-specific coliphages	ISO 10705-2 and ISO 10705-1	EV, ReoV, NoV, RoV	RT-PCR	Correlation between F-specific coliphages, somatic coliphages and EV.	Lodder *et al*., 2010[46]
Ruhr and Rhine Rivers impacted by wastewater, Germany	20-months study N= 190	somatic coliphages	double agar layer assay (probably ISO10705-2)	HAdV, HpyV, EV, group A RoV, NoV	qPCR	No correlation (not statistically significant) between somatic coliphages and HAdV, NoV, RoV.	Jurzik *et al*., 2010[45]
Tropical high – altitude aquatic system that receives rainwater, treated and non-treated wastewater; used for irrigation, and groundwater for drinking water, South of Mexico City	a two-year study N= 80	F-specific coliphages	Double Agar layer method (probably US EPA method 1601)	EV, RoV, AstroV	RT-PCR	Correlation between F-specific coliphages and EV No correlation between F-specific coliphages and AdV No F-specific coliphages and AstroV	Espinosa *et al*., 2009[61]

Water type	Sampling	detected coliphages	coliphage detection method	detected virus	virus detection method	Comments or conclusion to the correlation study or the comparison test	Reference
Avalon Beach (impacted by sewage, leakage), California, USA	samples collected every hour during 3 days in August 2008	somatic coliphages, F-specific coliphages (DNA and RNA)	concentration (no other indication)	EV, HAdV	RT-PCR for EV and nested-PCR for AdV (no AdV were detected, but EV were detected)	No correlation between somatic coliphages, F-specific coliphages and EV	Boehm *et al*., 2009[44]
Fifteen locations around the Newport Bay watershed (estuary), place used for water recreation and ecological reserve no information if impacted by sewage	a one-year study N=206	F-specific coliphages	US EPA method 1601	EV, HAdV	PCR (only 5% of the samples are positive for EV, AdV)	No correlation between F-specific coliphages and EV, AdV	Jiang *et al*., 2007[60]
River Meuse, at the intake of reservoirs that serve as the raw water supply for several waterworks in the Netherlands	1-year study 2001:200-500L samples (monthly) End 2002-beginning 2003: 1 month of weekly sampling (10 L samples)	F-specific coliphages (host *Salmonella typhimurium* WG49, not *E. coli*)	ISO 10705-1	NoV, EV, RoV	culture or RT-PCR	No association between F-specific coliphages and NoV (NoV peaks during the intensified sampling did not coincided with the peak in F-specific coliphages)	Westrell *et al*., 2006[59]

Water type	Sampling	detected coliphages	coliphage detection method	detected virus	virus detection method	Comments or conclusion to the correlation study or the comparison test	Reference
Two beaches impacted by WWTP and rivers that carry the effluents, Barcelona, Spain	2000, 2001 and 2002 (June-October) N= 20	somatic and F-specific coliphages	ISO 10705-2, ISO 10705-1	culturable EV	3 methods: concentration from a 10-L sample and plaque assay with the eluted viruses; double-layer plaque assay; VIRADEN and RT-PCR	Correlation between somatic coliphages and EV	Mocé-Llivina *et al.*, 2005[58]
Two Urban rivers, California (one river received tertiary effluents from WWTPs)	114 river samples from 5 different locations 1-year period	coliphages and F-specific coliphages	US EPA method 1601 and US EPA method 1602	HAdV, EV	Cell culture or qPCR (to discriminate between infectious and non-infectious particles)	No correlation between somatic coliphages, F-specific coliphages and HAdV, EV	Choi *et al*., 2005[43]
Marine coastal water impacted by WWTP, Massachusetts, USA	5-year study No indication on the number of samples	somatic coliphages, F-specific coliphages	US EPA Method 1602	AstroV, EV, RoV, HAdV (type 40 and 41)	ICC-nPCR; RT-PCR-nPCR	Correlation between somatic coliphages and HAdV No correlation between somatic coliphages and EV, RoV Correlation between F-specific coliphages and RoV, HAdV No correlation between F-specific coliphages and AstroV	Ballester *et al*., 2005[42]

Water type	Sampling	detected coliphages	coliphage detection method	detected virus	virus detection method	Comments or conclusion to the correlation study or the comparison test	Reference
River Moselle, eastern France	February 2000-May 2002 5 sampling sites N= 170	somatic coliphages	ISO 1075-2	EV, NoV GII	Infectious EV: cell culture, ICC-RT-PCR, RT-PCR NoV GII: RT-PCR	Association between somatic coliphages and EV, NoV GII (the number of positive samples for pathogenic viral genome increased with increasing densities of coliphages)	Skraber *et al*., 2004[57]
Eleven urban rivers and creeks, potentially submitted to run-off or impacted by WWTP effluents, Southern California, USA	July-August 2000 N= 21	somatic coliphages, F-specific coliphages	Double agar layer (probably US EPA Method 1601)	HAdV, EV, HAV	nPCR, RT-PCR	No clear relationship between the concentrations of human viruses (HAdV, EV, HAV) and the concentration of coliphages (somatic coliphages or F-specific coliphages)	Jiang, 2004[41]
Four fresh rivers, North of France	Monthly or semimonthly, February 1999-January 2000 Water samples of 20L N= 68	somatic coliphages	Single Agar Layer	Infectious EV, EV, HAV, NoV GI, NoV GII, AstroV, RoV	Infectious EV: cell culture EV, HAV, NoV GI, NoV GII, AstroV, RoV: RT-PCR	No correlation between somatic coliphages and culturable EV No correlation between somatic coliphages and viruses by RT-PCR	Hot *et al*., 2003[40]

Water type	Sampling	detected coliphages	coliphage detection method	detected virus	virus detection method	Comments or conclusion to the correlation study or the comparison test	Reference
Marine Coastal waters from twelve beaches impacted by run-off, between Los Angeles and Mexico, California, USA	February- March 1999 20- and 40-L water samples Number of samples not specified	total coliphages, and F-specific coliphages	US EPA Method 1601	HAdV	PCR and nPCR	No correlation between somatic coliphages and HAdV Correlation between F-specific coliphages and AdV	Jiang *et al*., 2001[39]
Rivers receiving treated waters from WWTP using three, or four treatment stages, Switzerland	Upstream and downstream WWTPs (N=35) Raw sewage (N=32) Treated water before release (N=32)	somatic coliphages, F-specific coliphages (*E. coli* and *Salmonella typhimurium* phages)	ISO 10705-1	EV, RoV, HAV	RT-PCR and nPCR	Correlation between all 3 classes of phages and EV, RoV, HAV in the case of rivers impacted by three-treatment stages-WWTPs No correlation between all 3 classes of phages and EV, RoV, HAV in the case of rivers impacted by a four-treatment stages-WWTP	Baggi *et al*., 2001[38]
Residential canals of the Florida Keys (September 1997-October 1997 and August 1998), potentially impacted by 30000 septic tanks in the Keys	Each site sampled once (small study) 19 sites=19 samples	coliphages, F -specific coliphages	Non-specific coliphage assay (DNA and RNA coliphages), genotyping of , F -specific coliphages using nucleic probes directed against GI, GIIa, GIIb, GIII, GIV groups	Poliovirus, coxsackie A and B viruses, echoviruses, HAV, NoV, small round-structured viruses (SRSVs)	RT-PCR (110-L water samples concentrated)	Association between Coliphages and several viral pathogens No conclusion on somatic coliphages as only 2 of 19 detected somatic coliphages	Griffin, 1999[56]

3.6 Standardised methods for detection of bacteriophages in water

Methods to detect bacteriophages in water are being developed and further standardised. For culture-based methods, detection of coliphages consists mostly in direct observation of circular clearance zones corresponding to host cell lysis (plaque assay). The results are expressed as plaque-forming units (PFUs) or plaque-forming particles (PFPs) for a given sample. A plaque-forming unit (PFU) is an entity, usually a single virion, but it may also be a clump of virions that gives rise to a single plaque of lysis in a host strain monolayer. PFU are used in US EPA methods, while PFP are used in the ISO standards. These methods are considered easy, reliable and cheap but they employ up to 2 days to results (if pre-culture of host cells or enrichment step and monolayer culture of the host cell is taken into account).

Table 7 shows an overview of standardised methods for the detection of some bacteriophages in drinking water (somatic, F-specific coliphages and other phages). These ISO and US EPA methods do not cover all subgroups of bacteriophages. They use the same or close host strains and differ in minor details relating to the media and assay conditions (volumes, time of contact, quality assurance description). Some other methods are further cited but these are not suitable for drinking water. Figures 7 and 8 represent the methods used for detection (presence/absence) and quantification of coliphages.

ISO 10705, describes the most commonly employed methods in Europe for the detection and the enumeration of bacteriophages in water. ISO 10705 is composed of four parts. Three parts of the ISO show the detailed procedure for the detection or the quantification of a specific type of bacteriophage (ISO 10705-1, 10705-2, 10705-4). One part, ISO 10705-3, gives indications on the minimal performance of methods for the concentration of bacteriophages. These methods are applicable to all kinds of water, sediments, sludge extracts and shellfish. Dilution or preconcentration may be necessary in some specific cases.

ISO 10705-1[65] (published in 1995) includes two procedures, one for the detection and the second one for the enumeration of F-specific RNA bacteriophages, a subgroup of F-specific bacteriophages. The use of the RNase enzyme, an enzyme that interferes with the infection of F-specific RNA phages, enables the specific detection of this subgroup. This ISO describes a protocol for the detection of MS2 coliphage in water samples using *E. coli* K12 Hfr (or *Salmonella enterica* serovar *typhimurium* WG49) as the host strain, and its selection on Tryptone–yeast extract-glucose agar (TYGA) plates.

ISO 10705-2[66] (published in 2000) includes two procedures for the detection and the enumeration of somatic bacteriophages. The sample (1 mL) is mixed with a small volume of semi-solid nutrient medium. Host cells are then plated and the culture is incubated for a determined period.This method recommends the use of ΦX174 coliphage as the control bacteriophage. *E. coli* strain C is also used in case of samples with expected low bacterial counts (e.g. drinking water or unpolluted natural waters) whereas *E. coli* strain CN (also named WG5), in case of polluted natural waters or wastewater (with high bacterial background flora). Nalidixic acid is added to the medium (Modified Scholten's Agar – MSA) for selection of the CN strain in order to reduce interference by the background flora.

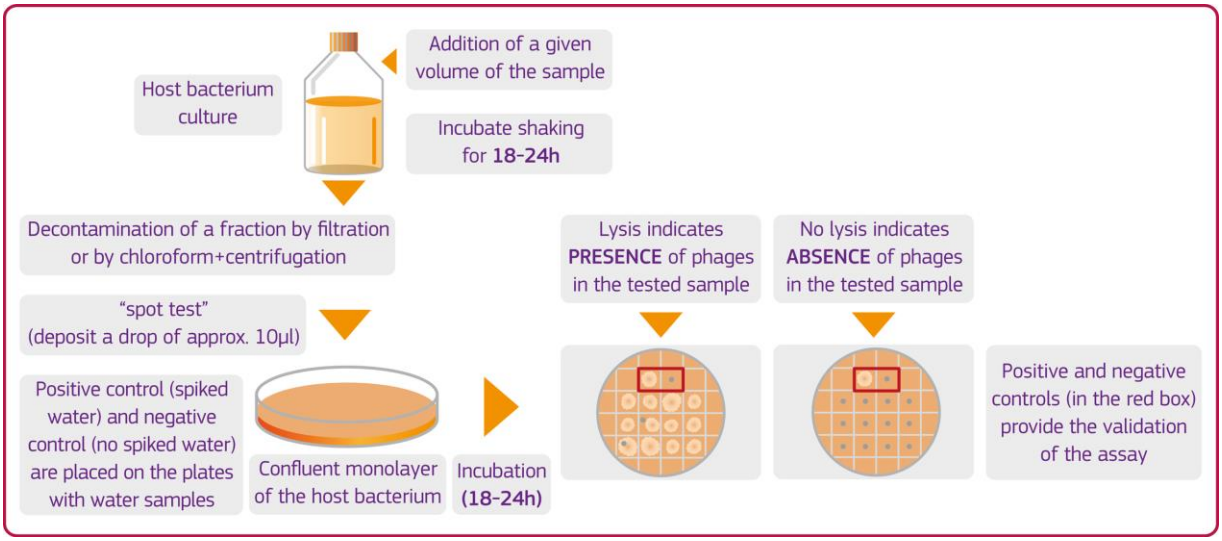

Figure 7. Phage detection by the qualitative presence/absence enrichment test. This procedure is included in ISO 10705-1, ISO 10705-2 and US EPA Method 1601 and 1602. A culture of host bacteria is mixed with an aliquot of the sample (1 mL for ISO 10705-1 and 10705-2, 100 mL or 1L for US EPA Method 1601, 100 mL for US EPA Method 1602). The culture is then filtrated to collect bacteriophages and drops of liquid phage sample are transferred onto a plate covered by a confluent monolayer of the host bacterium. The Petri plate is incubated overnight upside down and the presence of bacteriophages is indicated by the loss of turbidity in correspondence of the drops. Modified from https://coliphages.com

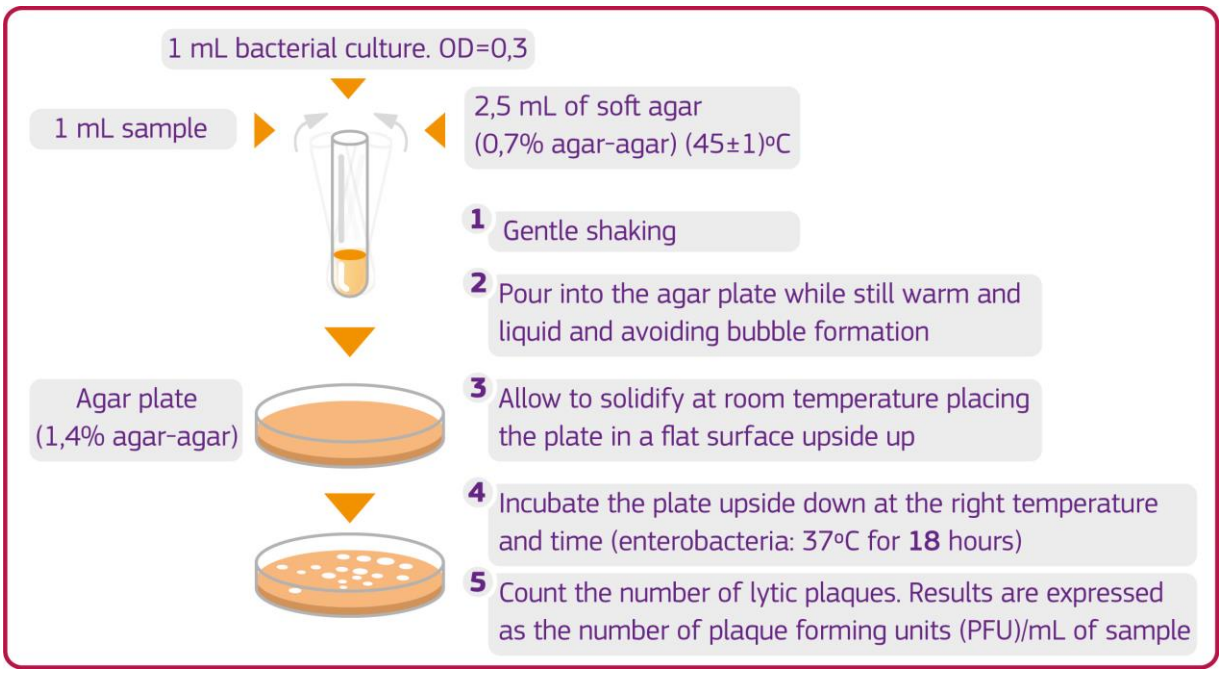

Figure 8. Phage enumeration by the quantitative plaque assay. This procedure is included in ISO 10705-1, ISO 10705-2 and USEPA Method 1602. An agar monolayer is prepared in a Petri dish by mixing a bacterial culture (1 mL) with the sample (1 mL in the case of ISO ISO 10705-1 and 10705-2, 100 mL for US EPA Method 1602) potentially containing bacteriophages. The Petri dish (MSA medium for ISO 10705, TSA for US EPA Method 1602) is then incubated upside down at 37°C for 18 hours and the lytic plaques (clear zones on the bacterial lawn) are counted. Each clear area is caused by a bacteriophage or by a clump of bacteriophages infecting only one bacterium and the results are expressed as plaque forming units (PFU)/mL of sample. OD: optical density. Modified from https://coliphages.com

A high number of plates should be used in parallel to allow a reliable detection of 1 PFU in 100 mL water, the volume mentioned in the DWD, and in case of water with a low phage number. Due to the high consumption of culture media, it may be advisable to use concentration methods. ISO 10705-3 includes a procedure for this approach.

ISO 10705-3[67] (published in 2003) describes a procedure for the validation of methods for bacteriophage concentration from sample with relatively large volumes (water volumes of 100 mL to several litres are concentrated to 20 mL). This method can be applied to all kinds of waters expected to contain < 3 PFP/mL and in which the amount and nature of suspended solids and/or dissolved matter do not interfere with the concentration procedure. Specific methodological details are not provided. Samples are treated according to a method of choice for which protocols of selected concentrations, detection methods, target bacteriophages, types of water and volumes analysed must be provided.

ISO 10705-4[68] (published in 2001) regards the enumeration of bacteriophages infecting *Bacteroides fragilis*. This method recommends the use of the bacteriophage B56-3 and its host *B. fragilis* RYC2056, an obligate anaerobe bacterium, as reference material for controls. This method is not currently used for the enumeration of coliphages in drinking water, but in sewage and sludge.

As a conclusion, among ISO methods, only ISO 10705-2 and ISO10705-3 can be taken into account for the detection and the quantification of somatic coliphages in drinking water. The cost of analysis performed for replicates according to ISO 10705-2 and ISO 10705-3 is estimated to be roughly 4.65 €.

US EPA Method 1601[69] and **Method 1602**[70] (April 2001) are commonly applied for detection of somatic coliphages as indicators of faecal contamination, in groundwater and surface water in other countries. These methods can also be used to detect faecal contamination in drinking water. Despite methodological differences with possible impact on results, performances of these multi-step methods are often compared.

The cost of analysis performed for one sample is estimated to be roughly 9.68 €.

US EPA Method 1601[69] specifies steps for detection (qualitative method) of male-specific (F+) and somatic coliphages by a two-step enrichment procedure in water using model coliphages (MS2 for F-specific and ΦX174 for somatic coliphages) and host strains. Method 1601 describes two procedures: a double agar layer procedure (DAL, first procedure) for the preparation and enumeration of a coliphage stock (to be spiked in water samples and used as positive control in the second procedure), and a two-step enrichment procedure for the analysis of 100 mL water samples (second procedure). The two-step enrichment procedure consists in amplifying bacteriophage concentration, if present in the original sample. An aliquot of 5 mL of CN-13 log-phase host bacteria and 50 mL of concentrated Tryptic soy broth (10X TSB) are added to 100 mL water samples. The mix is incubated at 36°C for 16-24h. Ten µL of this culture are then spotted on a layer of Tryptic Soy Agar (TSA) in which host bacteria have been added and the plate incubated at 36°C for 16-24h. Method blank and positive controls are spotted on the same plate. . This method also describes the protocol for the detection of somatic coliphages in 1L water samples. Method 1601 also includes a dechlorination procedure for chlorinated waters (sodium thiosulfate is recommended).

US EPA Method 1602[70] (April 2001) specifies two procedures, as US EPA Method 1601, for detection and enumeration of male-specific (F+) and somatic coliphages in water samples (100 mL only). This method can be qualitative (detection) and quantitative (enumeration). Detection and enumeration of coliphages in water samples is directly

performed by Single Agar Layer (SAL), not after two-enrichment step as described in US EPA Method 1601.

Other US EPA methods, including 1642[71] and 1643[72] as well as Standard Method 9224[73] are not applicable to the drinking water process. US EPA Method 1642 is employed for the detection of coliphages in recreational waters and wastewater by ultrafiltration (UF) and single agar layer (SAL) procedure, while Method 1643 is used for the detection of coliphages in secondary wastewater (no disinfection) by SAL procedure only. Details on these methods are provided in Annex IV.

The cost of analysis performed for one sample is estimated to be roughly 9.68 €.

Table 7. Summary of the normalised and validated methods for the detection of somatic and F-specific coliphages in water sources for drinking water. The table reports culture-based methods taking up to 48 h to result due to pre-culture of the host cell before agar plaque assay, incubation and reading.

Method	Purpose	Recommended control coliphage	Host cell	Water type	Required sample volume	Output	Time to results	Sensitivity	Cost
ISO10705-1 (1995)	Detection and enumeration of bacteriophages- Enumeration of F-specific RNA bacteriophages	F-specific RNA bacteriophage MS2	*Salmonella enterica* serovar *typhimurium* WG49 or E.coli K12 Hfr	all kinds of water drinking water, bathing water, sediments, sludge, shellfish	1 mL (or 5mL when expected low counts)	Plaque-Forming Units (PFU)/volume	24-48h	1 PFU per sample	5.10 €/sample
ISO10705-2 (2000)	Detection and enumeration of bacteriophages- Enumeration of somatic coliphages	somatic coliphage ΦX174	*E. coli* strain C (drinking water, unpolluted natural waters) *E. coli* strain CN, also known as WG5 (polluted natural waters or wastewaters)	all kinds of water drinking water, bathing water, sediments, sludge, shellfish	1 mL (or 5mL when expected low counts)	Plaque-Forming Units (PFU)/volume	24-48h	1 PFU per sample	4.65 €/sample
ISO10705-3 (2003)	Validation of methods for concentration of bacteriophage from water	F-specific RNA and somatic coliphages used with other parts of ISO10705	to define according to the detection method	water samples expected to contain < 3 PFU/mL	from 100 mL, up to 10 L, the sample is concentrated in 20 mL	Plaque-Forming Units (PFU)/volume	A few hours, depending on the detection method	depends on the method for concentration	Not reported
ISO10705-4 (2001)	Enumeration of bacteriophages infecting *Bacteroides fragilis*	phage B56-3	*B. fragilis* RYC2056	all kinds of water, sediments and sludge extracts, shellfish extracts	Dilution or pre-concentration of samples is allowed	Plaque-Forming Units (PFU)/volume	24-48 h	1 PFU per sample	Not reported
US EPA 1601 (2001)	Detection and quantification of coliphages by a two-step enrichment procedure	male-specific coliphage (MS2) and somatic coliphage (ΦX174)	*E. coli* F_{amp} (for male-specific coliphage and *E. coli* CN-13 for somatic coliphage	groundwater (only validated for groundwater) and other waters	100 mL, 1 L	Plaque-Forming Units (PFU)/volume	24-48 h	1 PFU per sample	9.68 €/sample
US EPA 1602 (2001)	Detection and quantification of coliphages by Single Agar Layer (SAL) procedure	male-specific coliphage (MS2) and somatic coliphage (ΦX174)	*E. coli* F_{amp} (for male-specific coliphage and *E. coli* CN-13 for somatic coliphage	groundwater (only validated for groundwater)	100 mL	Plaque-Forming Units (PFU)/volume	24 h-48h	1 PFU per sample	9.68 €/sample

3.7 Promising culture-based and non-culture based methods

In the last decade, novel methods (culture-based and non-culture based methods) have been developed to generate reliable, easier to settle, time-saving and cost-effective protocols, with focus on quantitative instead of qualitative analysis. These methods are described below and summarised in Table 8 and Table 9.

3.7.1 Culture-based methods

Fast Phage Modified Method 1601[74] is a qualitative US EPA-accepted alternative method for the detection of somatic or F-specific coliphages indicative of faecal contamination in compliance with the United States Ground Water Rule[75] (2006). By employing commercial kits, this method provides positive prediction within 8 h, enabling early warning, and confirmation (plaque test) in 16-24 h, with detection of one coliphage per 100 mL of water sample. The technology is based on detection of a fluorescent substrate (methylumbelliferyl) cleaved from the culture medium containing methylumbelliferyl-galactoside by extracellular β-galactosidase which is released from host cells (*E. coli*) upon coliphage-induced lysis. The test is adapted for quantification as the most probable number (MPN) in two available formats (TEMPO card and Quanti-Tray/2000 enabling detection of <0.25 PFU per 1-4 mL sample and <1 PFU/100 mL, respectively) with results comparable to plaque enumeration methods such as US EPA Method 1602 and double-layer agar techniques.

The cost of analysis performed for three replicates is estimated to be roughly 12.52 €.

Bluephage method employs commercial kits and is able to detect somatic or F-specific coliphages in raw and treated wastewater, surface water, drinking water, recreational water, shellfish extracts, sediments and sludge extracts. Bluephage technology is based on the detection of a chromogenic substrate, analogous to glucuronic acid synthesised by a modified *E. coli* host strain (CB 10 strain). The *uidB* and *uidC* genes for transport of glucuronic acid inside the cells have been mutated, but the β-glucuronidase enzyme encoded by *uidA* gene is overexpressed and accumulates in the cytoplasm while the strain is unable to internalise the substrate. After phage infection, cell lysis occurs and the enzyme is released to the medium where it metabolises the chromogenic substrate leading to a change of colour from yellow to dark blue[76]. To adapt to simultaneous detection of somatic and F-specific coliphages, the method has been recently modified by Toribio-Avedillo *et al*. (2019)[77].

The cost of analysis performed for three replicates is estimated to be roughly 78-195 €.

Quanti Phage Assay is a recently published method[78] employing cellulose absorbent pad materials to support coliphage growth and colorimetric detection in place of agar that is used in the conventional plaque assay. It enables enumeration of somatic coliphages in 1.5-2 h and F-specific coliphages in 2.5-3 h. The limit of detection is 1 PFU per volume of sample analysed (1 mL, 10 mL or 100 mL) and depends on the type of water.

A new development is a gelatin-immobilisation method enabling preparation of the host cells in 40-60 min instead of 20 h, depending on the assay format. It has been applied for the quantification of somatic coliphages in wastewater and surface water samples instead of conventional plaque assay.

3.7.2 Non culture-based methods

These methods, mostly molecular and immunology-based, are considered faster than culture-based methods since results are provided in few hours.

Reverse Transcription PCR or quantitative PCR and multiplex. Conventional polymerase chain reaction (PCR) methods enable the amplification of a target DNA or RNA fragment in 2-3 hours and a qualitative (presence/absence) evaluation respect to a reference control. The quantitative PCR (qPCR), considered as more sensitive than conventional PCR, is a method based on quantification of a fluorescent signal emitted from the reactional medium in 1-1.5 h. It can be directly performed on nucleic acids extracted from water samples (RNA or DNA) even with low content of biological material. The closed-tube format of these techniques reduces the risk of carry-over contamination, ensures wide dynamic range of quantification and possibilities of automation[79]. The reverse transcription PCR (RT-PCR) employs a supplementary step consisting on retro-transcription of extracted RNA into a complementary DNA (cDNA) strand, that is then amplified following the classical PCR or qPCR protocol.

Molecular techniques are now being used routinely for virus detection, and qPCR has become the method of choice. The international ISO/CEN committee CEN/TC275/WG6/TAG 4 recommended this method as the basis for the forthcoming international standards for the detection of noroviruses and hepatitis A virus[80].

Finally, multiplex qPCR and RT-qPCR assays enabling quantification of multiple targets in one samples have been adapted to detect F-specific coliphages by targeting replicase gene in several types of samples, such as seawater, and further in shellfish - an important source of gastroenteritis[81]. Other primers and probes specifically designed for each coliphage family are required[36,81-83].

Although qPCR-based technologies can be used to rapidly detect viral genomes, they do not distinguish infectious versus non-infectious viral particles.

Digital PCR. PCR methods have been recently improved by digitalisation on microfluidic chips available now as platforms. The main application for the moment is the detection of MS2 (F-specific) coliphage in wastewater, not somatic coliphages.

In-gel loop-mediated isothermal amplification (gLAMP) system. This method based on a simple and easy-to-use membrane system displays a similar sensitivity compared to RT-qPCR (1 PFU/reaction) and has been used to detect F-specific coliphages in a rapid (30 min) and low-cost manner (~0.10 $)[84]. Its advantage is that users do not need to enter the cleanroom for complex chip fabrication and, contrarily to other digital systems, no specialised equipment is required. However, small droplet size formed on the membrane are the cause of high detection limit for nucleic acids at current stage of development in this method (10 copies/µL) (Figure 9)[85].

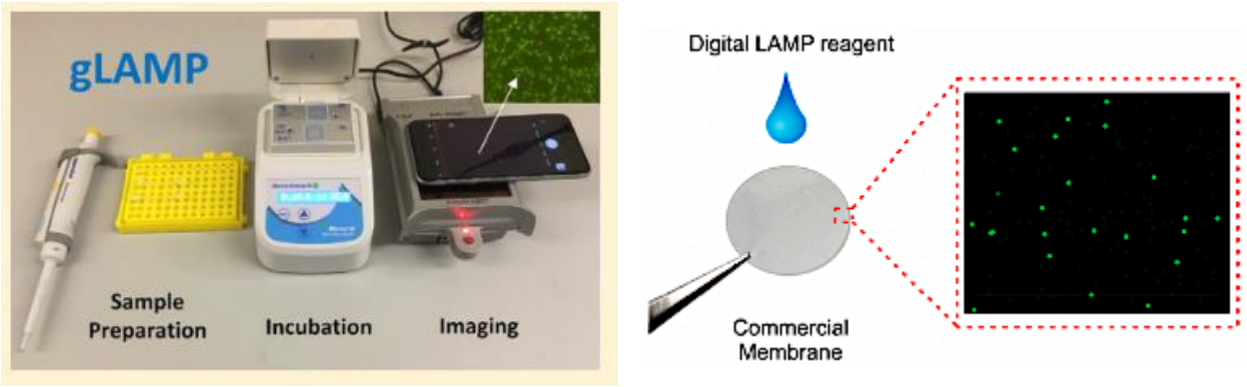

Figure 9. Device and principle of the in-gel loop-mediated isothermal amplification (gLAMP) detection. The gLAMP uses filtered samples containing F-somatic coliphages (MS2) resuspended in a buffer prior to RNA extraction. The material is subjected to reverse-transcription in the presence of fluorophore-labelled primer in an incubation chamber (9X9 mm) (left) and incubation for polymerization of the gel (5-15 min) prior to reaction (25 min). The gel is then stained with a LAMP dye (15 min) and results are visible as a picture of the amplicon dots which can be sent to a smartphone or observed on a fluorescence microscope (left). From Huang *et al*., 2018 and Li *et al.*, 2019[84,85].

Culture Latex Agglutination and Typing (CLAT). This immunology-based method is qualitative and consists on a two-step enrichment process which has been validated for the detection of F-specific coliphages associated with faecal contamination on beaches[86-88] and applied to some groups of somatic coliphages[89]. Results are visible on the agglutination card as clumps formed in 30-60 seconds when the antigen (coliphage-derived target molecule) is sequestered by a specific antibody (Figure 10). Although the entire procedure takes 5-24 h due to the pre-enrichment step and improvement of sensitivity/specificity are still needed, this very low-cost method can be used on site and help in differentiation of coliphages.

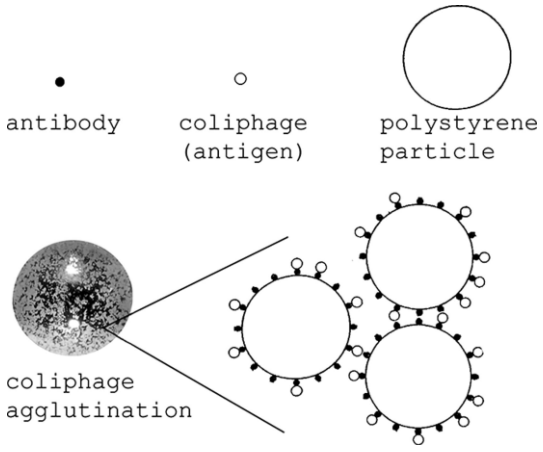

Figure 10. Culture Latex Agglutination and Typing (CLAT). Coliphage agglutination is visible to the naked eye after mixing equal volumes of coliphage enrichments with antibody-labelled polystyrene particles for 30 seconds. From Bercks and Querfurth, 1971[90].

Table 8. Promising culture-based methods for the detection of somatic and F-specific coliphages

Method	Purpose/ Type	Target coliphage	Host cell	Sample type tested	Required sample volume	Sensitivity	Time to results	Output	Advantages/ Disadvantages	Cost
Fast Phage	**qualitative method** (presence/ absence)	somatic or F-specific coliphages	*E. coli* CN-13 for somatic F_{amp} for male-specific coliphage	groundwater (validated by US EPA), drinking water	100 mL	1.5 PFU/100 mL	prediction (visual fluorescence test: 8 h) followed by confirmation (plaque test: 16 h) Total:24 h	PFU/100mL	Advantages: - no need overnight preculture for host cell (ready-to use tablet) - fluorescence-based prediction enables early warning Disadvantage: - not quantitative - time to result comparable to classic culture-based methods	for somatic coliphages: 346 $ = 313 €/ Kit (25 tests) 12.52 €/sample
	quantitative method for somatic or F-specific coliphages in all kinds of samples	somatic coliphages	*E. coli* no other information on the strain	drinking water	TEMPO format: 4 mL or MPN format: 100 mL	<0.25 PFU/ 4 mL (TEMPO format) or <1 PFU/100 mL (MPN format)	5.5-6 h	PFU/4mL (TEMPO format) or PFU/100mL (MPN format)	Advantages: - quantitative Disadvantages: - initial cost of the devices (expensive)	price kit not publicly available cost of the devices

Method	Purpose/ Type	Target coliphage	Host cell	Sample type tested	Required sample volume	Sensitivity	Time to results	Output	Advantages/ Disadvantages	Cost
Bluephage	**quantitative method**	somatic or F-specific coliphages	*E. coli* WG5 strain for somatic coliphages or *E. coli* $_{Famp}$ for F-specific coliphages	drinking and bottled water	100 mL	1 PFU/ 100mL	18-24 h	PFU/100mL	Advantages: - no need overnight culture but 2 h of pre-growth of the host strain Disadvantage: - results not available in the same working day (incubation time for plate 18$\pm$2 h)	for somatic coliphages: 830- 934 € (kit 10 tests, without or with Petri dishes) 83-93.4 €/sample
	quantitative method	somatic or F-specific RNA coliphages	*E. coli* WG5 strain for somatic coliphages	raw or treated wastewater, surface water, recreational water, shellfish extracts, sediments, sludge extracts	1 mL (dilution if necessary)	1 PFU/ sample 1mL	18-24 h	PFU/mL	Advantages: - no need overnight culture but 2 h of pre-growth of the host strain Disadvantage: - results not available in the same working day	for somatic coliphages: 741-1171 € (kit 70 tests, without or with Petri dishes) 78-195 €/sample depending on the level of contamination and number of dilutions and replicates
Quantiphage	**quantitative method**	somatic or F-specific RNA coliphages	*E. coli* CN-13 for somatic F_{amp} for male-specific coliphage	surface water, drinking water, recreational water, wastewater	1, 10 mL, 100 mL	1 PFU/mL (when 1 mL analysed), or 1 PFU/10 mL (when 10 mL surface water)	1.5-2 h for somatic coliphages, 2.5-3 h for F-specific coliphages	PFU/volume	Advantages: - visual Disadvantage: - need preparation of host cells overnight culture	not publicly available

Table 9. Non-culture based methods for the detection of somatic and F-specific coliphages.

Method	Purpose	Target coliphage	Sample type tested	Required sample volume	Sensitivity	Time to results	Output	Advantages/ Disadvantages
Reverse Transcription quantitative PCR (RT-qPCR) **Quantitative PCR (qPCR)** **Multiplex quantitative PCR**	detection and quantification of bacteriophages in a variety of samples	developed for F-specific coliphages, can be used for somatic coliphage and other bacteriophage	Various kinds of samples	1, 100 mL (after concentration)	increased respect to the culture	total time: 4-5 h for F-specific RNA coliphages (RNA extraction, retro-transcription and real-time PCR)	DNA copy number or genome unit (gu) or genome copy number (gc)	Advantages: - faster than traditional USEPA or ISO culture based- method (4 h vs 24-48 h), but as fast as the new culture based-methods - multiple assays could be used to target more than one family of bacteriophages Disadvantages: - does not provide the infectivity status of the detected genome - it should be coupled to other methods
Digital PCR	detection and quantification of bacteriophages in a variety of samples	developed for F-specific RNA coliphage (MS2), can be used for somatic coliphage and other bacteriophage	mostly wastewater	not reported	Increased respect to the culture	4 h	DNA copy number or genome unit (gu) or genome copy number (gc)	Advantages: - rapid - ~10 $/ sample Disadvantage: - initial investment (instrument)
In-gel loop-mediated isothermal amplification (gLAMP) system	detection and quantification of bacteriophages in a variety of samples	developed for F-specific RNA coliphage (MS2), can be used for somatic coliphage and other bacteriophage	environmental waters, wastewater	20 mL	0.7 PFU per reaction or 10 DNA copies/µl	RNA extraction: approx. 2 h gLAMP:30 min Total time: 2 h	amplicon	Advantages: - rapid, visual (dye, fluorescence) - ~0.1$/ sample Disadvantages: - initial investment (instrument) - fluorescence microscope

Method	Purpose	Target coliphage	Sample type tested	Required sample volume	Sensitivity	Time to results	Output	Advantages/ Disadvantages
Culture Latex Agglutination and Typing (CLAT)	Qualitative immunoassay combines a two-step enrichment process and latex agglutination serotyping to monitor the presence of coliphages.	F-specific coliphages, under development for somatic coliphages	Environmental waters, animal-derived samples, validated in beach waters	not reported	5×10^3 to 1×10^5 PFU and 1×10^6 to 5×10^6 for F+ RNA and DNA coliphages, respectively	culture followed by agglutination 1 min	PFU	Advantages: - detects F-specific coliphages in water samples in 5 to 24 hours - inexpensive (need agglutination card and antibody, reagents can be stored at ambient temperature for months) - portable on site Disadvantages: - qualitative - needs enrichment step

3.8 Discussion on somatic coliphages as indicator of viral contamination in drinking water

In the last years, concern for viruses and their impact on human health has increased. Water-transmitted viral pathogens have been classified by the World Health Organization (WHO)[3] as having a moderate to high health significance and include adenoviruses, astroviruses, hepatitis A and E viruses, rotaviruses, noroviruses and other caliciviruses, and enteroviruses, including in turn coxsackieviruses and polioviruses[91].

Their monitoring and the removal is very difficult. For this reason, somatic (and F-specific) coliphages, being viruses infecting *E. coli* and sharing characteristics with human enteric viruses (morphology, replication, resistance to degradation), have been investigated for their possible use as indicators of viral removal following water treatment process.

Although many studies reported correlation between the concentrations of coliphages and those of enteric viruses, as well as their respective removal performances in wastewater[11,27,92], the co-occurrence of coliphages and subtypes of enteric viruses in surface water and groundwater (as potential sources for drinking water) is not clear.

Over the period 1999-2019, 25 studies have been selected (21 on surface water, 3 on groundwater and 1 on both waterbodies) showing both concentrations of coliphages, particularly somatic coliphages, and enteric viruses. Several types of water were studied (e.g. lakes, rivers, canals, beaches, reservoirs, groundwater, brackish and saline water).

Seventeen of the 25 publications showed that there is no linear correlation between the presence of somatic coliphages and enteric viruses in raw water, suggesting that somatic coliphages could not be considered as surrogate indicator for the removal for all enteric viruses. However, a partial correlation could be observed in some studies[40,46]. Doubts about the use of somatic coliphages as indicator for the presence of enteric viruses in all situations, and also in distribution systems, were expressed by Figueras and Borrego (2010)[93]. Nonetheless, somatic coliphages, being more resistant to water treatment than bacteria, could be an indicator in the verification process, if detected in raw water, for removal efficacy of small particles but without ensuring a complete protection from all human enteric viruses in finished water.

For groundwater, since few data are available that would suggest a strict correlation with the presence of enteric viruses[47], somatic coliphages should be measured only in case of leakage from wastewater treatment plant or contamination due to the floods.

Any indicator for the removal efficacy should be monitored along the train barrier to ensure an optimal removal/inactivation performance. In case of somatic coliphages, to avoid contamination by pathogens, it is essential for suppliers to monitor the efficacy of the barriers in place. They have to determine the removal/inactivation performance.

At the moment, no data are available for both the concentrations or occurrence of somatic coliphages and enteric viruses at each step (raw, settled, filtered, finished water) in drinking water system plants. We presume that, as for wastewater treatment plants (WWTP), the performance of the treatment train is site-specific, depending on the design of the process. There are different types of filtration with different recovery rates. When applied, concentration and time of contact with chlorine or chloramine, ozone, UV wavelength and water pressure are all individual parameters than can influence the general performance of a water treatment plant.

Some studies have found that coliphages are more resistant to environmental stressors (e.g. temperature, sunlight, salinity) than human viruses, but resistance depends on the

characteristics of water, on the subgroup of coliphages (somatic or F-specific) and the type of enteric virus[11].

These effects should be taken into account in the risk-based assessment approach of the water safety plan. Each water supply should be characterised as a pilot case study and be tested for the log removal efficiency (decay rate) of somatic coliphages and the main enteric viruses along the train barriers.

Available detection methods for somatic coliphages have been extensively described in this report, listing the existing methods (culture-based or culture-independet, standardised/non standardised) as well as the promising ones including relevant information (e.g. advantages/drawbacks and cost).

For the standardised methods, ISO 10705-2, ISO 10705-3, US EPA Method 1601 and Method 1602 have been developed, the latter providing a method for larger volumes, while the ISO is applicable only after adapting the procedure as described in ISO 10705-3 in a quite complex protocol. Therefore, US EPA Method 1602 adapts better to a possible implementation.

In the last years, methods which aim at shortening virus detection time have been developed and listed in Tables 8 and 9, such as i) ready-to-use kits with calibrated strains, control bacteriophages, medium and plates; ii) molecular and immunology-based metods. They could be time-saving or even more sensitive (after improvments), however do not allow determination of viral infectivity.

In conclusion, somatic coliphages may be implemented as verification parameters for the removal efficiency of small particles (e.g. viruses) keeping in mind that the removal of somatic coliphages occurs with the removal of one or more subtype of enteric viruses but not all subtypes. A water safety plan should be put in place and developed case by case. Although culture-based and standardised methods are available, faster methods should be considered, especially in case of leakage/contamination of the distribution system or to ensure a better management of the water quality.

3.9 Recommendation

As the presence of coliphages does not correlate significantly with enteric viruses in studies on raw water (surface and groundwater), at this stage the recommendations are:

1. To include somatic coliphages for verification of the removal efficiency for small particles and more resistant subgroups for the efficiency of the treatment process in surface water as raw water, however this would not ensure protection from all enteric viruses.

2. For groundwater as DWD source, somatic coliphages should be measured only in case of WWTP leakage (sewage pipe breakage close to the growndwater wells) or flood risks due to the storm water and in case the wells are not protected.

3. The Water Safety Plan (WSP) should also take into account the resistance (decay rate) of coliphages and enteric viruses due to different environmental factors

(temperature, pH, UV light). For the risk-based assessment approach of the water supply, a pilot case of the water system should be performed to verify the log removal of somatic coliphages and enteric viruses.

4. The standardised methods (US EPA 1601, 1602 and ISO 10705-2) should be included for culture methods and the detection of coliphages in a range of volumes (1 mL to 100 mL). ISO 10705-3 should also be considered (procedure for the validation of methods for concentration of high volumes further applied to ISO 10705-2). The US EPA methods would allow to select a larger volume.

5. In case of larger volumes, (up to 1 L as suggested by US EPA), US EPA Method 1601 would be recommended for the study of surface water and groundwater.

6. No reference value should be reported in raw water as studies reported differences in concentrations of somatic coliphages. Being an indicator for the verification of water treatment process, if detected in raw water, somatic coliphages should be measured along the train barrier for their removal efficacy.

7. New methods enabling rapid (within the same day) detection of somatic coliphages at acceptable costs are available or under development and can be applicable. The obtained results instead of PFU reported by agar-based methods, should be expressed as "number of indicator particles (virions)".

8. We recommend that every six years, based on scientific evidence, this parameter should be evaluated and eventually replaced with a better indicator, e.g. specific enteric viruses.

3.10 New perspectives and outlook for monitoring human viruses

Detection and inactivation of human enteric viruses in water to ensure safer quality is still a challenge. To date, their detection employs a great diversity of methods and provides results further affected by variable factors influencing conditions within the same and among different water types or sample collection sites. Additionally, exhaustive descriptions of study conditions are often missing. Ultimately, there is no any water treatment able to inactivate all virus types independently of water quality. For example, human adenovirus is nearly five times more resistant to the monochromatic UV inactivation compared to the other enteric viruses.

Thus far, collected data suggest coliphages as a better indicator of human viruses associated with faecal contamination than a representative selection of enteric viruses relevant for human health safety. The morphological similarity between coliphages and enteric viruses has been proved to correlate with more similar behavior under different environmental conditions in natural habitats and during water treatment processes compared to faecal indicator bacteria. However, no bacteriophage studied to date

accurately represents enteric virus behavior for all disinfectants. Regardless, from a regulatory standpoint, a major barrier is that not one disinfection system is effective against all viruses and applicable to all water quality conditions[91].

Therefore, detection and inactivation of human enteric viruses deserve more research to overcome the traditional cell culture-based viral growth assay (unavailable for several genera, e.g. norovirus) and which is quite time consuming and expensive. Advanced methods like immunology-based or qPCR should be improved since at the moment they cannot distinguish the infectious *vs* non-infectious particles. Gall and coauthors listed the new approaches, which could be implemented in the future[91].

Advanced technologies such as functional viral metagenomics could give more hints than existing approaches regarding, in the first instance, detection of unknown viruses, investigation of the molecular mechanism underlying their resistance over the treatment processes, as well as determination of viral infectivity by detecting related genes and their products[94].

4. State of the art on *Clostridium perfringens* and spores

4.1 Description of *Clostridium perfringens* bacterium and spores

Clostridium perfringens is an anaerobic, Gram-positive, spore-forming, rod-shaped bacterium (Figure 11).

Figure 11. *Clostridium perfringens* visualised by electron microscopy.

It was first isolated and identified by William H. Welch in 1891 from the autopsy of a man where gas bubbles were observed within infected blood vessels. The bacterium was then called *Clostridium wellchii*. The lactose-fermenting spore-forming anaerobic aspect was described the following year by Welch and Nutall (1892)[95] and other microbiologists, leading to the new denomination as *Bacterium enteritidis sporogenes*. During the following decade, based on the description of new characteristics, microbiologists improved the classification of this bacterium renamed *Clostridium perfringens* (*C. perfringens*) (Figure 12 and 13).

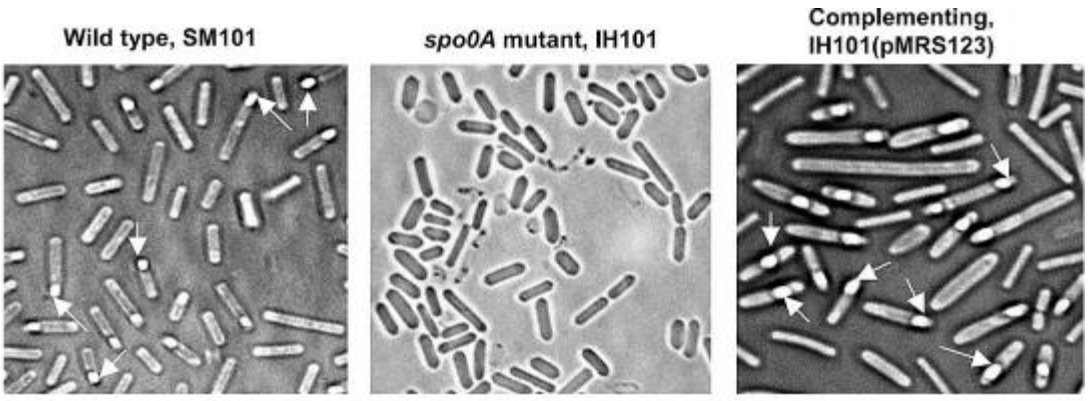

Figure 12. Phase-contrast microscopic analysis of sporulating *C. perfringens* cultures. *C. perfringens* wild-type SM101, *spo0A* mutant IH101 and complemented IH101 (pMRS123) strains were grown in DS medium at 37°C for 8–24 h. Endospores were visualised using a phase contrast microscope (Zeiss) with 1000 magnification. Endospores are indicated by arrows and were observed in 8 h-grown cultures of both SM101 and IH101 (pMRS123). No detectable spores were found for *spo0A* mutant IH101 even after 24 h of growth. From Huang *et al*., 2004[96].

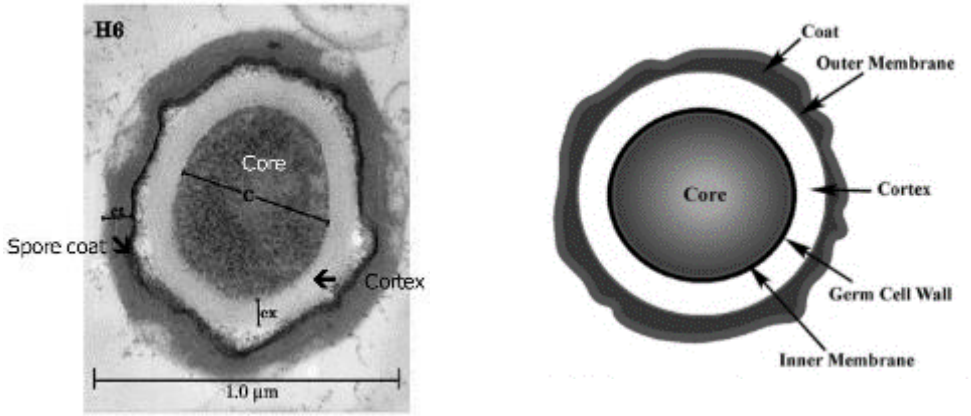

Figure 13. Ultrastructure of *C. perfringens* spores. Transmission electron micrograph of a spore from *C. perfringens* strain H-6, a food poisoning strain (left). Three areas can be observed: proteinaceous spore coat layers, cortex region, and the core with ribosomes giving a granular appearance. A scheme explaining the different layers is shown in the right part of the figure. From Novak *et al.*, 2003[97].

4.2 Infections associated with *Clostridium perfringens*

Low levels of *Clostridium* spores, in themselves, are unlikely to present a significant risk to healthy individuals directly from consuming contaminated drinking water. However, spores can enter in contact with food (e.g. vegetables, meet, fish), where they find suitable conditions for germination and then multiplication. Consumption in large quantities of incorrectly cooked food increases the risk of infection, in particular the risk of serious gastrointestinal diseases. *C. perfringens* is not only responsible for gas gangrene and food poisoning, but also for non-foodborne diarrhea, enterocolitis and necrotising enterocolitis (NEC) in preterm infants (symptoms range from mild abdominal pain until peritonitis)[98,99].

4.3 History of *Clostridium perfringens* as indicator to assess water quality

C. perfringens is found in the environment (soil, water) and in the gut and faeces of warm-blooded animals and humans (only 13-35% of human faeces would harbor *C. perfringens*)[100]. It is employed in different countries worldwide as a criterion for detection of faecal contamination in water supply by measuring both before and after disinfection or train process, until finished water.

A few reviews and two studies in particular, have been the starting point towards the choice of such a bacterium as an indicator for the European Drinking Water Directive (1998)[1,101-103].

In 1925, Wilson and Blair showed a relationship between the presence of anaerobic sulphite-reducing spore-forming bacteria and the presence of *E. coli* in water[104]. The same scientists suggested that since *Clostridium* was essentially a faecal microorganism (bacteria and spores could be excreted by both humans and warm-blooded animals), it could be found in soils, food and sewage. *Clostridium* spores may persist longer than other indicators of contamination such as coliform bacteria and, for this reason, *C. perfringens* was considered a possible indicator of intermittent pollution.

Since the 1960s, *C. perfringens* has been used in Europe, in conjunction with other sulphite-reducing clostridia, to detect faecal contamination in water. However, Bonde (1963) suggested that *C. perfringens* but not all sulphite-reducing clostridia could serve as an indicator of faecal pollution in receiving waters[105].

C. perfringens is much less prevalent than other bacterial indicators (i.e. bifidobacteria), but its ability to form spores allows it to survive outside the gut, in aquatic and estuarine receiving waters[106]. Soon after this statement, Bisson and Cabelli (1979)[107] developed a two-step membrane filtration method for concentration and enumeration of bacteria from wastewater and natural waters and suggested *C. perfringens* as an indicator of sewage pollution.

Based on works done on tropical streams (Hawaiian streams) that contained high concentrations of faecal coliforms and faecal streptococci, Fujioka and Shizumura (1985)[101] suggested the use of *C. perfringens* as an alternative indicator. They concluded that its concentrations correlated with the presence of wastewater in streams, making of it a reliable indicator of stream water quality (tropical waters). They also recommended a quality parameter of 50 CFU/100 mL for freshwaters.

4.4 *Clostridium perfringens* and spores as an indicator for the presence of parasites in drinking water

Parasites include free and enteric parasites. Most of them are free-living organisms that can reside in freshwater and pose no risk to human health. Contrarily, enteric protozoa are pathogenic and have been associated with drinking water outbreaks. The main water-related parasites are *Cryptosporidium* and *Giardia* (Annex I and Annex V).

4.4.1 *Cryptosporidium* spp.

Cryptosporidium is a protozoan parasite (order Coccidia). It was first recognised as a potential human pathogen in a previously healthy three-year-old child[108]. A second case of cryptosporidiosis (name of the associated disease) occurred two months later in an individual who was immunosuppressed as a result of drug therapy[109]. The disease became best known in immunosuppressed individuals exhibiting symptoms now referred to as acquired immunodeficiency syndrome, or AIDS[110].

The symptoms of cryptosporidiosis occur between 2 and 12 days after ingestion of oocysts. They include water diarrhoea, abdominal pain, nausea, vomiting and low fever that can last up to 3 weeks and be recurrent. Immunocompromised people and young children are at particular risk.

To date, twenty-nine species of *Cryptosporidium* have been recognised. The main species of *Cryptosporidium* associated with illness in humans are *C. hominis* and *C. parvum* (Table 10). They account for more than 90% of human cryptosporidiosis cases[111]. *C. hominis* appears to be more prevalent in North and South America, Australia and Africa, whereas *C. parvum* is responsible for more infections in Europe[112-115].

Humans and other animals, especially cattle, are important reservoirs for *Cryptosporidium*. Reported prevalence rates of human cryptosporidiosis range from 1 to 20%, with higher rates reported in developing countries[116,117]. Livestock, especially cattle, are a significant source of *C. parvum*[118].

Oocysts are easily disseminated in the environment (sewage and surface waters and occasionally in groundwater sources) and are transmissible via the faecal–oral route. Major pathways of transmission for *Cryptosporidium* include person-to-person, contaminated drinking water, recreational water, food and contact with animals, especially livestock.

Cryptosporidium oocysts have been shown to survive in cold waters (4°C) under laboratory conditions for up to 18 months. In warmer waters (15°C), *Cryptosporidium parvum* has been shown to remain viable and infectious for up to seven months[119]. In general, oocyst survival time in the environment decreases as temperature increases[120-122].

Smith *et al*. (1993) found that oocyst viability in surface waters is often very low[123]. A study by LeChevallier *et al*. (2003) reported that 37% of oocysts detected in natural waters were infectious[123,124]. Additionally, a study by Swaffer *et al*. (2014) reported that only 3% of the *Cryptosporidium* detected was infectious[125].

Table 10. Cryptosporidium species. From "Guidelines for Canadian Drinking Water Quality: Guideline Technical Document – Enteric Protozoa: *Giardia* and *Cryptosporidium*", 2019[126]

Species (genotype)	Major host	Human health concern[a]
C. andersoni	Cattle	+
C. baileyi	Poultry	-
C. bovis	Cattle	+
C. canis	Dogs	++
C. cuniculus	Rabbits	++
C. erinacei	Hedgehogs and horses	+
C. fayeri	Marsupials	+
C. felis	Cats	++
C. fragile	Toads	-
C. galli	Finches, chickens	-
C. hominis (genotype H, I or 1)	Humans, monkeys	+++
C. huwi	Fish	-
C. macropodum	Marsupials	-
C. meleagridis	Turkeys, humans	++
C. molnari	Fish	-
C. muris	Rodents	+
C. parvum (genotype C, II or 2)	Cattle, other ruminants, humans	+++
C. rubeyi	Squirrel	-
C. ryanae	Cattle	-
C. scophthalmi	Turbot	-
C. scrofarum	Pigs	+
C. serpentis	Reptiles	-
C. suis	Pigs	+
C. tyzzeri	Rodents	+
C. ubiquitum	Ruminants, rodents, primates	++
C. varanii	Lizards	-
C. viatorum	Humans	++
C. wrairi	Guinea pigs	-
C. xiaoi	Sheep, goats	+

[a] Human health concern is based solely on the frequency of detection of the species from human cryptosporidiosis cases, designation may change as new cases of cryptosporidiosis are identified
+++ Most frequently associated with human illness
++ Has caused human illness, but infrequently
+ Has caused human illness, but only a few very rare cases (very low risk)
- Has never been isolated from humans

Upon ingestion by humans, the parasite completes its life cycle in the digestive tract (Figure 14). It evolves in six major stages. The formation of an oocyst starts with the excystation and release of sporozoites that are capable of asexual reproduction (merogony), followed by the formation of gametes (gametogony), and then the formation of a zygote protected by a resistant cell wall. The formation of a "wall" in the middle of the oocyst leads to the formation of four new sporozoites (sporogony). The four sporozoites become mature oocysts which are shed in the faeces.

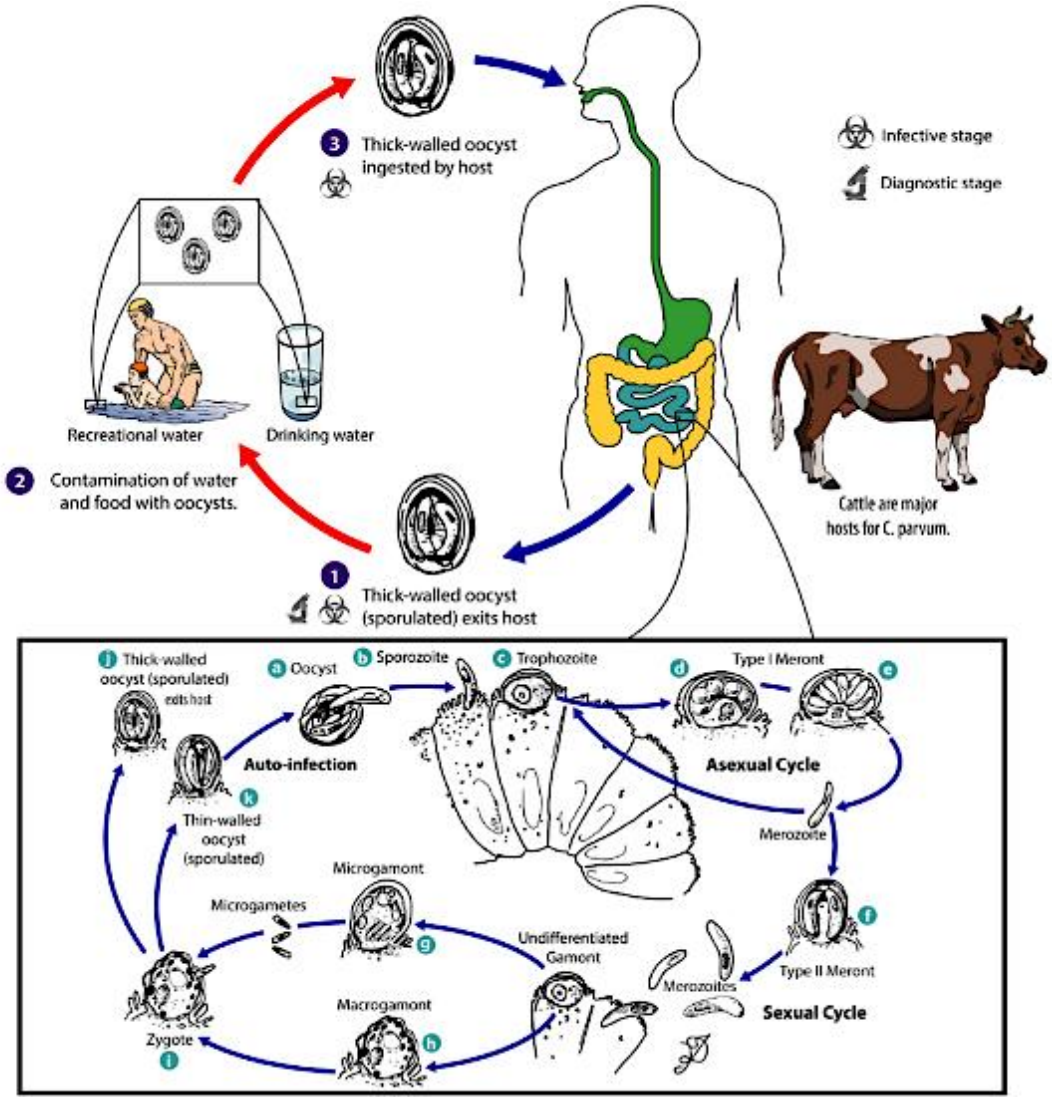

Figure 14. Lifecycle of *Cryptosporidium* spp. From CDC website https://www.cdc.gov/dpdx/cryptosporidiosis/index.html[127].

Sporulated oocysts, containing 4 sporozoites, are excreted by the infected host through feces ❶ (and possibly other routes such as respiratory secretions). Transmission of *Cryptosporidium* spp. occurs mainly through ingestion of fecally contaminated water (e.g., drinking or recreational water) or food (e.g., raw milk) or following direct contact with infected animals or people ❷ . Following ingestion (and possibly inhalation) by a suitable host ❸ , excystation ❹ occurs. The sporozoites are released and parasitize the epithelial cells (❺ , ❻) of the gastrointestinal tract (and possibly the respiratory tract). In these cells, usually within the brush border, the parasites undergo asexual multiplication (schizogony or merogony) (❼ , ❽ , ❾) and then sexual multiplication (gametogony) producing microgamonts (male) ❿ and macrogamonts (female) ⓫ . Upon fertilization of the macrogamonts by the microgametes (⓬) that rupture from the microgamont, oocysts develop and sporulate in the infected host. Zygotes give rise to two different types of oocysts (thick-walled and thin-walled). Thick-walled oocysts are excreted from the host into the environment ⓭ , whereas thin-walled oocysts are involved in the internal autoinfective cycle and are not recovered from stools ⓮ . Oocysts are infectious upon excretion, thus enabling direct and immediate fecal-oral transmission. Extracellular stages have been reported, but their relevance in the overall life cycle is unclear.

Giardia is a flagellate protozoan (order Diplomonadida) recognised as a human pathogen in the 1960s[128].

The main species infecting humans is *Giardia lamblia*. The alternative name, *Giardia duodenalis* or *intestinalis*, is also used.

The taxonomy of *Giardia* is in constant revision as new species or "assemblages" are described. It relies mainly on the shape of the median body, the organelle composed of microtubules that is most easily observed in the trophozoite. Six species have been described; *G. lamblia* (*G. intestinalis* or *G. duodenalis*) assemblages A and B are associated with human giardiasis (and can infect animals), while assemblages C, D, E, F, G seem to infect only animals (Table 11).

It is associated to giardiasis, one of the most frequently diagnosed intestinal parasitic disease in the United States and the most commonly reported food- and waterborne parasitic disease in the European Union (EU)/European Economic Area (EEA) (with 19 437 confirmed cases in 2017, in constant increase compared to 2010-2013 period). Cases of giardiasis were reported by 22 European Member States, Iceland and Norway, the majority of which (60.1%) were domestically acquired except for three Nordic countries (Iceland, Norway, Sweden) where 71-83% of cases were travel-associated[129].

Signs and symptoms may vary and can last for 1 to 2 weeks or longer. In some cases, people infected with *Giardia lamblia* have no symptoms. Acute symptoms of giardiasis include: diarrhea, stomach or abdominal cramps, nausea, vomiting and dehydration leading to a weight loss. Less common symptoms include itchy skin, hives, and swelling of the eye and joints. In children, severe giardiasis might delay physical and mental growth and slow development.

G. lamblia is found in the small intestine of humans and other animals with prevalence rates of 1% to 5% in humans, 10% to 100% in cattle, and 1% to 20% in pigs. The life cycle displays two states: trophozoite and cyst. The trophozoite is a mobile form than cannot persist outside the host. Pear-shaped and flagellated binucleated trophozoites are normally attached to the surface of the intestinal villi. After detachment, they start multiplying and dividing (by longitudinal binary fission) leading to the ovoid form called cyst, an immobile state that is very resistant to environmental stressors and contributes to dissemination of *Giardia* in the faeces (Figure 15).

Most *Giardia* cysts are not viable (only 3.5-18% are viable, most of them are empty cysts or "ghosts" as verified by 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy. However, they can persist for a long time in the environment: up to 15-30 days in human faeces and animal faeces (cattle), 28-56 days in surface water and several weeks in wastewater. Bingham *et al*. (1979) observed that *Giardia* cysts can survive up to 77 days in tap water at 8°C compared with 4 days at 37°C[130]. Exposure to ultraviolet (UV) light can also shorten the survival time of *Giardia*[131,132] or predation[133].

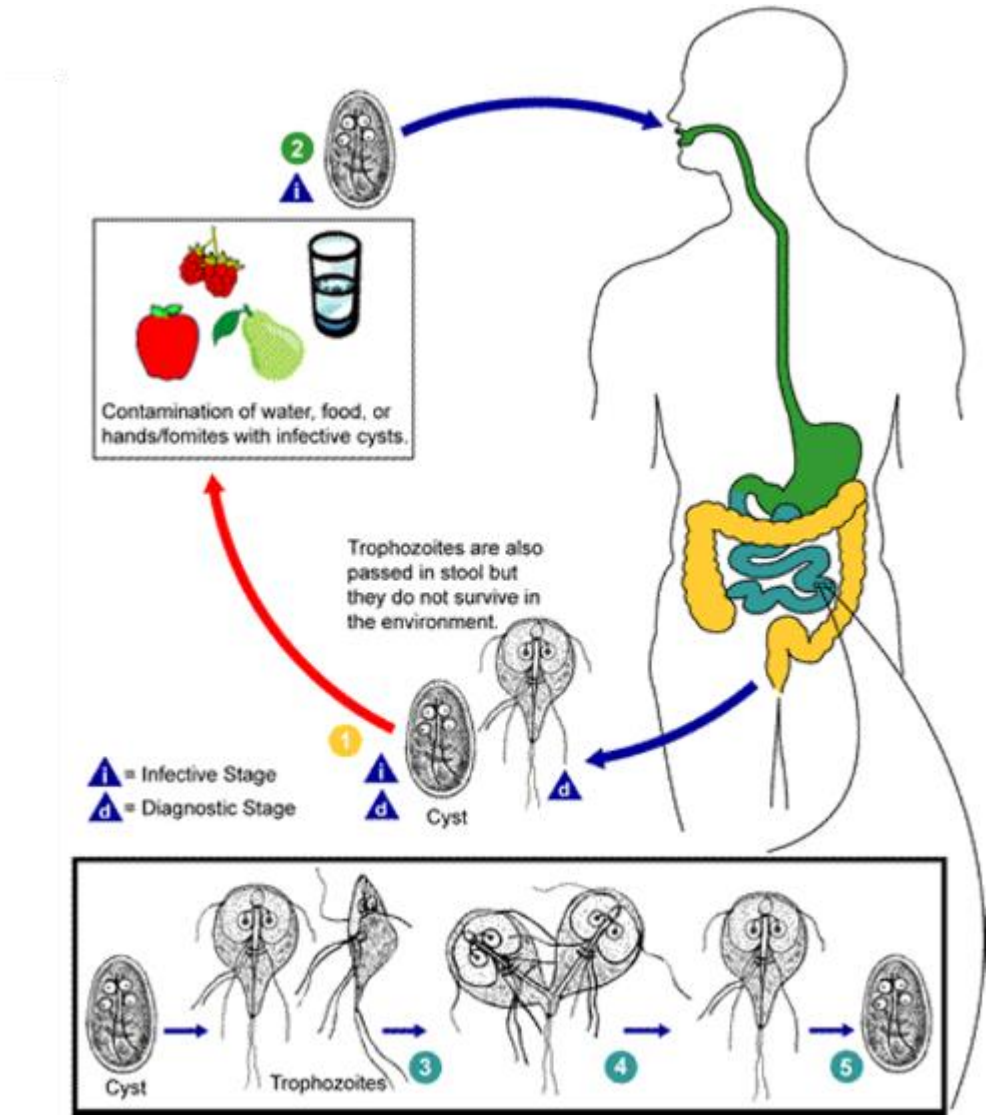

Figure 15. *Giardia* life cycle. Cysts are resistant forms and are responsible for transmission of giardiasis. Both cysts and trophozoites can be found in faeces (diagnostic stages) (**1**). The cysts are hardy and can survive several months in cold water. Infection occurs by the ingestion of cysts in contaminated water, food, or by the faecal-oral route (hands or fomites) (**2**). In the small intestine, excystation releases trophozoites (each cyst produces two trophozoites) (**3**). Trophozoites multiply by longitudinal binary fission, remaining in the lumen of the proximal small bowel where they can be free or attached to the mucosa by a ventral sucking disk (**4**). Encystation occurs as the parasites transit toward the colon. The cyst is the stage found most commonly in nondiarrheal faeces (**5**). Because the cysts are infectious when passed in the stool or shortly afterward, person-to-person transmission is possible. While animals are infected with *Giardia*, their importance as a reservoir is unclear. From CDC website https://www.cdc.gov/dpdx/giardiasis/index.html[134].

Table 11. Giardia species. From "Guidelines for Canadian Drinking Water Quality: Guideline Technical Document – Enteric Protozoa: *Giardia* and *Cryptosporidium*", 2019[126].

Species (assemblage)	Major host(s)
G. agilis	Amphibians
G. ardeae	Birds
G. lamblia, syn. *G. intestinalis*, syn. *G. duodenalis*	
(A)	Humans, livestock, other mammals
(B)	Humans
(C)	Dogs
(D)	Dogs
(E)	Cattle, other hoofed livestock
(F)	Cats
(G)	Rats
G. microti	Muskrats, voles
G. muris	Rodents
G. psittaci	Birds

4.4.3 Persistence of *Clostridium perfringens*, *Cryptosporidium parvum* and *Giardia lamblia* in the environment

At present, there is not any review available showing the influence of environmental factors (such as temperature, sunlight, salinity, predation or enzymatic degradation) on the survival of *C. perfringens* spores and oocysts of protozoan parasites in aquatic environments.

C. perfringens spores have been shown to be highly resistant to temperature, even more resistant than vegetative cells. Wang and collaborators showed that more than 90% of *C. perfringens* spores were inactivated when incubated in water at 90-100°C for 10-20 minutes[135]. More generally, an increase of temperature leads to a significant reduction of spores. *Cryptosporidium* oocysts remain viable for 7 to 18 months and infectious for over 12 weeks at low temperatures (4-15°C)[119,121]. A 4 log reduction of viability has been observed after 8-12 weeks at medium temperatures (20-25°C) in diverse water types (King *et al*., 2005). *Giardia* oocysts have been shown to persist 77 days at 8°C, 26 days at 21°C and 6 days at 37°C[130]. *C. parvum* oocysts can withstand a variety of environmental stresses, including freezing (but the viability is greatly reduced) and exposure to seawater. However, *C. parvum* oocysts are susceptible to desiccation. Only 3% of oocysts were still viable within two hours in a desiccation assay[136]. A small fraction of *Cryptosporidium* oocysts and *Giardia* cysts withstand a freeze cycle (less than 1% of *Giardia* cysts survived freezing at -13°C for 14 days).

Several studies reported that variability in *Giardia* cyst concentrations in river and lake water may depend on temperature[137-140]. Other factors such as exposure to ultraviolet (UV) light[131,132] or predation[133] can also shorten the survival time of *Giardia* cysts. No relationship was found between *Giardia* cyst survival and other factors such as water pH, dissolved oxygen, turbidity, color, hardness, ammonia, nitrate or phosphorous.

The rates reported for infectious oocysts in water are very different from one study to another[124,125,141], and depend on the method of detection. Most of the oocysts would be "empty", non viable, thus non infectious ("ghost" oocysts).

4.4.4 Occurence of *Clostridium perfringens*, *Cryptosporidium parvum* and *Giardia lamblia* in water

Cryptosporidium and/or *Giardia* (oo)cysts are often reported in wastewater and surface water, less often in groundwater or drinking water. They have been demonstrated as the etiologic agents of waterborne diseases, especially in the USA and Canada.

European Union Summary Reports on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks (from EFSA and ECDC) reported the association of *Cryptosporidium* and/or *Giardia* (oo)cysts with some waterborne outbreaks. The association between *Cryptosporidium hominis* and a waterborne outbreak in Sweden in 2010 has been demonstrated (12700 cases), and between *Cryptosporidium parvum* and a waterborne outbreak in UK in 2014 (24 cases). In these outbreaks, treatment deficiencies have been pointed out but, most of the time, the studies showed a weak evidence for the association of an agent and a waterborne outbreak. Moreover, in Europe, not all the countries use the latest case definitions for cryptosporidiosis and giardiasis and not all have settled surveillance systems and report to ECDC (in 2017, 24 of 31 countries EU/EEA countries reported confirmed giardiasis data, 25 reported confirmed cryptosporidiosis). Predicting the real number of cryptosporidiosis and giardiasis and the number of cases linked to water seems very difficult. A reason could be that routine monitoring *of C. parvum* and G. *lamblia* in water is expensive. Thus, a clear map of *Cryptosporidium* and *Giardia* as source of waterborne outbreaks in Europe is not available.

Several surrogates for the removal of *Cryptosporidium* oocysts and *Giardia* cysts have been evaluated, among which spores from aerobic (*Bacillus subtilis*) and anaerobic bacteria (Clostridia). Spores of sulphite-reducing clostridia and of *C. perfringens* in particular have been used extensively. *C. perfringens* spores have been proposed as indicators for the presence of *C. parvum* oocysts in river water due to their slower die-off rates versus those of *E. coli* and enterococci[142] and as surrogates for *Cryptosporidium* oocysts in water treatment studies[143]. Then, *C. perfringens* spores have been proposed as indicators for the presence of *Giardia* cysts in river water.

Korajkic and collaborators[144] recently published a report on the use of *C. perfringens* as an alternative indicator (alone or together with coliphages) for the presence of *Cryptosporidium*, *Giardia* oocysts or other pathogens in water (Table 12). Another study conducted at 25 freshwater recreational and water supply sites showed that *C. perfringens* was not always detected in samples where other indicators *(*e.g. *E. coli)* were present and no relationship was found between *C. perfringens* and *Cryptosporidium* and/or *Giardia* (oo)cysts (Table 12)[145]. Overall, in freshwater and marine/brackish waters, 8 of 11 studies did not report a relationship between *C. perfringens* spores and *Cryptosporidium* and/or *Giardia* (oo)cysts (Table 12), suggesting that *C. perfringens* seems not to be the best indicator for the presence of *Cryptosporidium* and/or *Giardia* oocysts in aquatic ecosystems[145-155].

Table 12. Relationship between *C. perfringens* as indicator of faecal pollution and pathogens in freshwater and marine/brackish waters (modified from Korajkic *et al*., 2018[144] and Till *et al*., 2008[145]).

Indicator	Pathogen(s)	Location	Relationship between indicators and pathogens	Reference
Freshwater				
C. perfringens	*Campylobacter* spp., *Salmonella* spp., *P. aeruginosa*, *Giardia* and *Cryptosporidium* (oo)cysts, *Aeromonas* spp.	River Ruhr (recreational water and raw water source for drinking water) and barrier lakes, Germany	not reported	Strathmann *et al*., 2016[146]
C. perfringens	Human adenovirus, *Giardia* and *Cryptosporidium (*oo)cysts	Rivers in France	not reported	Jacob *et al*., 2015[147]
C. perfringens (and F-specific RNA coliphages)	Campylobacter spp, *Giardia* and *Cryptosporidium* (oo)cysts	Avon River (impacted by sewage discharge), Christchurch, New Zealand	F-specific RNA coliphages more strongly correlated with Campylobacter spp, *Giardia* and *Cryptosporidium* oocysts than *C. perfringens*	Devane *et al*.,2014[148]
C. perfringens (and FIB)	*L. monocytogenes*, *Salmonella* spp. , *E. coli* O157:H7 , *Campylobacter* spp., *Cryptosporidium* and *Giardia* (oo)cysts	South Nation River basin, Canada	positive, but weak relationships between *C. perfringens* and pathogens other than *Cryptosporidium* and *Giardia* oocysts, no relationship with *Cryptosporidium* and *Giardia* oocysts weak correlation between *C. perfringens* and FIB	Wilkes *et al*., 2009[149]
C. perfringens and F-RNA coliphages	*Cryptosporidium* and *Giardia* (oo)cysts, Salmonella, Campylobacter	recreational and water supply sites, New Zealand	not reported	Till *et al*., 2008[145]
C. perfringens	*Cryptosporidium* spp, *Salmonella* spp., *Campylobacter* spp.	Lake Parramata (recreational water), Australia	not reported	Roser *et al*., 2006[150]

Indicator	Pathogen(s)	Location	Relationship between indicators and pathogens	Reference
Marine and brackish waters				
C. perfringens (and F-specific RNA coliphages)	*Salmonella* spp., *Campylobacter* spp., *Cryptosporidium* and *Giardia* oocysts, adenoviruses, enteroviruses	Docklands, South Yarra and Abbotsford estuaries, Melbourne Australia	not reported	Henry *et al*., 2016[151]
C. perfringens	*Cryptosporidium* and *Giardia* oocysts	Coastal beaches, contaminated with domestic sewage, Venezuela	no significant correlation	Betancourt *et al*., 2014[152]
C. perfringens (and F-specific RNA coliphages)	*V. vulnificus*, *S. aureus*, enterovirus, norovirus, hepatitis A virus,*Cryptosporidium* and *Giardia* oocysts	Coastal Beaches, Miami Dade County, Florida, USA	not reported	Abdelzaher *et al*., 2011[153]
C. perfringens	*V. vulnificus*, *S. aureus*, enterovirus , norovirus , hepatitis A virus, *Cryptosporidium* and *Giardia* (oo)cysts	Virginia Key Beach, Florida, USA	not reported	Abdelzaher *et al*., 2010[154]
C. perfringens (and coliphages)	*Cryptosporidium* and *Giardia* (oo)cysts, enteroviruses	Sarasota Bay, coastal waters, Florida, USA	not reported	Lipp *et al*., 2001[155]

4.5 Removal or inactivation during drinking water process

Due to the persistence of *C. perfringens* spores in the environment and their reliability as surrogates for the presence of *Cryptosporidium* and *Giardia* oocysts during wastewater treatment, they have been proposed as a surrogate indicator in water treatment studies[47,143].

For public water systems in the United States, the US EPA requires producing filtered water with a minimum of 2 log removal or inactivation of *Cryptosporidium* oocysts (99%) and a 3 log removal or inactivation (99.9 %) of *G. lamblia* cysts (Surface Water Treatment Rules since 1998)[156] and a minimum performance for the different barriers in place.

Table 13 lists the principal studies published on the physical inactivation and dinsinfection of *C. perfringens* spores and both protozoan parasite oocysts. The number of publications on the co-occurrence of *C. perfringens* spores, *C. parvum oocysts* and *G. lamblia* cysts during drinking water production processes is very limited compared to the literature published on the occurrence of these organisms during wastewater treatments. Most of the time, inactivation of *C. perfringens* spores during drinking water treatment process is mentioned together with *E. coli* and coliphages (as an alternative indicator), not with parasites.

Coagulation is an important barrier for *Cryptosporidium* and *Giardia* oocysts during water treatment with a minimum of 3 log removal[9]. This value is very different from another study where coagulation and dissolved air flotation (DAF) provided a 1.08-1.79 log removal of parasites (oo)cysts[157]. The assessed processes seem more efficient than coagulation-flotation combined action used on *C. perfringens* spores[158].

The ideal indicator should have the same concentration in raw water as (oo)cysts and the same inactivation (removal) rate. Hijnen and colleagues initially evaluated the removal of spores of sulphite-redicung clostridia (SRC) as a 2 log removal (99%)[159]. They used larger water volumes to determine the concentration of spores after different treatment stages. All the barriers applied to reduce the load of pathogens in water did not have the same performance; this difference was compensated by combination with other barriers to reach a number of pathogens detected under the acceptable limit (determined after epidemiological studies). Hijnen and collaborators observed that inactivation kinetics of *C. perfringens* and *C. parvum* at low temperature and during ozonation was in the same order of magnitude[160]. *C. parvum* and *G. lamblia* oocysts were more susceptible than *C. bifermentans* spores (other species of SRC) to GAC filtration[161]. Also, they were highly resistant to chemical disinfection and UV radiation; *C. parvum* oocysts were more resistant to free chlorine than *C. perfringens* spores but had similar inactivation rate when mixed oxidants were used (they are considered not producing by-products that could be harmful to consumers)[143]. *C. parvum* oocysts and *G. lamblia* cysts are more susceptible than *C. perfringens* spores to UV light[162].

According to some studies, the barriers of the train process do not display the same inactivation rate for indicators and pathogens. For *Clostridium* spp. spores, Hokajärvi *et al.* (2018) found a 5.2 to 7.5 log removal in pilot scale waterworks and 0.8 to 3.1 log mean removal in full-scale waterworks[158]. These results show the importance for water suppliers to determine the efficiency of each stage of the train process and to define a Water Safety Plan (WSP) so that the efficacy of the system may be constantly controlled and actions immediately taken in case of failure at one barrier.

Comparability of inactivation rates between *C. perfringens* spores and parasite (oo)cysts is often difficult due to difference in materials, doses and contact times evaluated. Further studies should provide new information to conclude whether *C. perfringens* spores are reliable surrogates as indicators for the presence of *Cryptosporidium* and *Giardia* (oo)cysts during water treatment processes.

Table 13. Average of log removal of *C. perfringens* spores (or *C. bifermentans or sulphite-reducing clostridia –SRC-*), *C. parvum* and *G. lamblia* (oo)cysts during water treatment processes.

Unit process	*C. perfringens* spores (except * *C. bifermentans,* ***SRC*)	*C. parvum* oocysts	*G. lamblia* cysts	Comments	Reference
Coagulation		>2.90	>3.2		Guidelines for Canadian Drinking Water Quality, 2019[9]
Coagulation and flotation	1.9-2.4				Hokajärvi *et al*., 2018[158]
Coagulation, dissolved air flotation (DAF)		1.08-1.42	1.31-1.79		Andreoli and Sabogal-Paz, 2019[157]
Sand filtration: - Slow sand filtration	3.6	4.7		- *C.parvum* is more susceptible to slow sand filtration than *C. perfringens* - Because of a high persistence due to attachment to the sand more efficient for spores of *C. perfringens*, spores of SRC are unsuited for use as a surrogate for oocyst removal by slow sand filter (too conservative)	Hijnen *et al*., 2007[163]
- Rapid sand filtration	1.0-1.2				Hokajärvi *et al*., 2018[158]
Ozonation	0.8** -0.2	0.8		same magnitude	Hijnen *et al*., 2002[160] Hokajärvi *et al*., 2018[158]
Granulated Activated Carbon (GAC) Filtration	0.9-1.1*	1.1-2.7	2.0-2.2	*C. parvum* and *G. lamblia* oocysts more susceptible than *C. bifermentans* spores to (fresh or loaded) GAC filtration	Hijnen *et al*., 2010[161]
	-0.03-0.9				Hokajärvi *et al*., 2018[158]
UV disinfection (Range)	3 (UV:48-64 mJ/cm^2)	3 (UV: 13 mJ/cm^2)	2.5 (UV: 1.5 mJ/cm^2)	*C. parvum* and *G. lamblia* oocysts more susceptible than *C. perfringens* spores to UV (need less energy for inactivation)	Hijnen *et al*., 2006[162]

Unit process	***C. perfringens*** **spores (except * *C. bifermentans,* *****SRC*****)**	***C. parvum*** **oocysts**	***G. lamblia*** **cysts**	**Comments**	**Reference**
Chemical disinfection: - chlorine disinfection	0.05 (global)				Hokajärvi *et al*., 2018[158]
- free chlorine (in 4 h)	1.4	0		*C. parvum* is more resistant than *C. perfringens* to free chlorine	Venczel *et al*., 1997[143]
- mixed oxidants	3	3		similar inactivation by mixed oxidants is observed	Venczel *et al*., 1997[143]
UV + chlorine disinfection	0.3-3.1				Hokajärvi *et al*., 2018[158]

In the Drinking Water Directive 98/83/EC[1] dating from 1998, Annex I-Part C (indicator parameters) defines the content of "*Clostridium perfringens* (and spores): 0 number/100 mL water" meaning no bacteria should be present in 100 mL of water intended for human consumption, while Annex II (Monitoring)-Table A mentions that for *C. perfringens* (including spores), this parameter has to be monitored only if drinking water originates from or is influenced by surface water. Annex III also specifies the analytical method (membrane filtration followed by anaerobic incubation of the filter on mCP agar).

4.6 Methods for the detection of *Clostridium perfringens* and/or its spores in water

Different methods have been developed for the isolation, identification and characterisation of *C. perfringens* in water; they include culture-based methods and non-culture based methods.

4.6.1 Culture-based methods

In culture-based methods, two solid agar media are used for the detection of *C. perfringens* vegetative cells and/or spores in water: the modified *Clostridium perfringens* (mCP) agar and the Tryptose-Sulfite-Cycloserine (TSC) medium.

The **mCP agar** was first described by Bisson and Cabelli in 1979[107] for the specific quantification of *C. perfringens* in water and is now included in the Directive 98/83/EC[1] for testing the quality of water intended for human consumption.

In this method, water sample is filtered and the filter is then placed onto mCP solid medium and incubated under anaerobic conditions at 44°C for 24 ± 2 h. It allows only the growth of *C. perfringens* at 44°C, whereas the growth of other clostridia is inhibited. Filtration membrane containing straw yellow-coloured colonies are then transferred to pads saturated with ammonium hydroxide. After 20 to 30 seconds of exposure, opaque yellow colonies that turn pink or red to magenta are considered as *C. perfringens* (Figure 16 and Table 14).

However, this simple and low-cost method has limitations due the use of mCP medium. Many colonies obtained on mCP agar plates can fail to grow after isolation. Also, problems to stain presumptive colonies on mCP after exposure to ammonia fumes are sometimes encountered resulting in the presence of yellow colonies that can remain colorless (considered as mCP-negative).

The cost of analysis performed for one sample is estimated to be roughly 2 €.

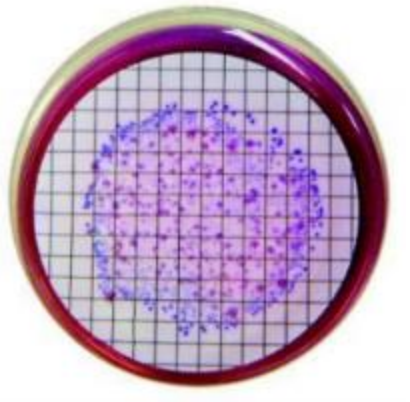

Figure 16. Enumeration of *C. perfringens* and spores on mCP agar medium after exposure to ammonium hydroxide. The pink or red to magenta colonies are confirmed as *C. perfringens.* From Manafi *et al*., 2015[164].

ISO 14189 (2013)[165] (Figure 17 and Table 14) is a TSC-based method that enables the detection and enumeration of *C. perfringens* and/or its spores in different types of water. It was proposed to replace the mCP method in the last proposal for DWD recast (2019)[166]. TSC is a selective medium that incorporates D-cyloserine. Like mCP medium, it allows the enumeration of vegetative bacterial cells and/or spores, depending if pasteurisation is used. Sample pasteurisation inactivates vegetative cells and enables the selective detection and enumeration of spores (turning to vegetative cells after germination during plate incubation). After water filtration, membranes are put onto TSC agar, incubated under anaerobic conditions and as sulphite-reducing bacteria reduce sulphite to sulfide in the presence of the appropriate iron salt, black ferrous sulphide precipitates around individual colonies[167]. Compared to mCP, the TSC medium is more selective and normally allows higher recoveries, it produces fewer false-positive results. However, more false-negatives are detected as TSC selects for all sulphite-reducing clostridia[168], therefore a confirmation step is necessary. For this purpose, a subculture of black-grey presumptive colonies is performed onto blood agar plates under anaerobic conditions. The colonies are then put onto a filter paper and 2-3 drops of phosphate acid are added. All colonies that turn purple within 3-4 minutes are confirmed positive. Subculture and confirmation steps take in total 48 hours[169].

The cost of analysis according to ISO 14189 is estimated to be 3.15 € for one sample dilution.

As the confirmation step requires the use of a carcinogenic reagent, some laboratories interpret positive results as "presumed *C. perfringens* spores". For that reason, **ISO 6461** could be proposed as an alternative method, since made for the enumeration of all clostridial spores (sulphite-reducing bacteria) in water.

ISO 6461 (1986) consists of two parts: a method by enrichment in a liquid medium (ISO6461-1), a method by membrane filtration (ISO6462-2). **ISO 6461-1** procedure is applicable to all types of water, including turbid water. **ISO 6461-2** procedure is applicable to all types of water, except when a large amount of particulate material is liable to be retained by the membrane. The principle covers several steps from selection by applying heat to destroy vegetative bacteria to the indication by inoculating volumes of the sample into media followed by incubation at 27°C in anaerobic conditions. The method includes filtration of the water sample through a membrane filter having a suitable pore size (0.2

µm) to retain the spores. The filter is then placed on a selective culture medium, followed by incubation and counting of black colonies.

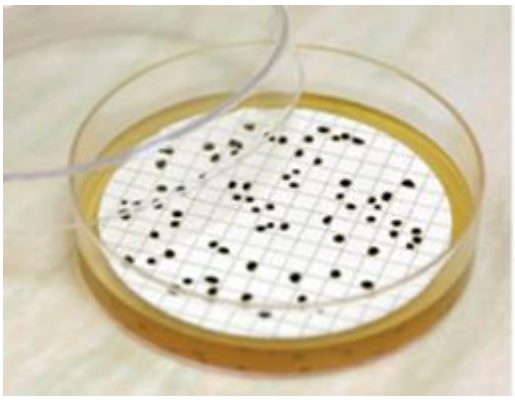

Figure 17. Appearance of *C. perfringens* colonies after enumeration in drinking water samples according to ISO 14189 (2013). Image from https://www.itwreagents.com/united-states/en/ip-046-news-en

Watkins and Sartory developed a new medium, the **New Tryptose Cycloserine agar** (TCA)[169], which contains sodium pyruvate instead of sodium metabisulfate to improve recovery. This method also includes a procedure of a membrane filter transfer onto reagent-soaked filters for the immediate testing for acid phosphatase production. This method is considered as equivalent to ISO 14189 TSC medium method. It enables the isolation and confirmation of *C. perfringens* within 18–24 h, half the time required for ISO 14189.

Another culture medium, the **CP ChromoSelect agar**, has been recently described by Stelma in his review (2018)[170]. It allows for better recoveries and greater specificity than mCP (Figure 18). Used after membrane filtration, this medium would be more reliable and easier to handle than mCP and TSC media. CP ChromoSelect Agar avoids the disadvantages of mCP agar such as problems of evanescence of the red color and of colonies damaged by the presence of ammonium hydroxide[164]. The green colour of colonies is specific for *C. perfringens* and does not diffuse to the agar, therefore confirmation is not required (in contrat to TSC agar). In addition, the homogeneity of colour observed on CP ChromoSelect Agar enables the detection of false negative colonies more easily. This method takes 24 hours of incubation before results are available.

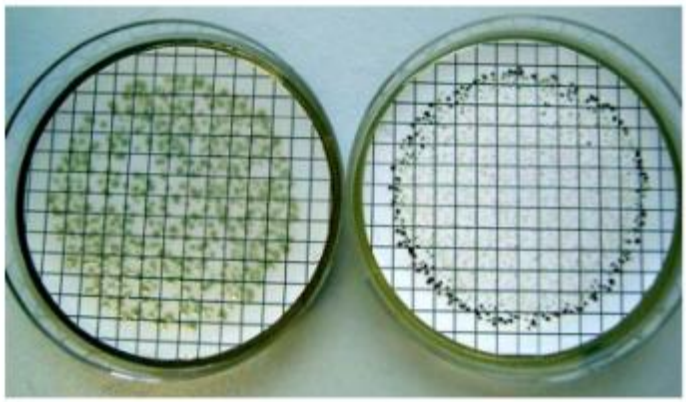

Figure 18. Drinking water sample with *C. perfringens* ATCC 10873 strain cultured on CP ChromoSelect agar (left) and TSC Agar (right). From Manafi *et al*., 2013[164].

Table 14. Standardised culture-based methods for the detection and enumeration of *C. perfringens* and/or spores in drinking water.

Method	Purpose	Water type	Required sample volume	Sensitivity	Time to results	Output	Costs
mCP	Enumeration of *C. perfringens* and spores by membrane filtration	Drinking water	100 mL	< 1 CFU/mL	24-25 h	CFU/mL	2 €
ISO 14189 (2013)	Enumeration of *C. perfringens* and spores by membrane filtration	All water samples without particulate or colloidal matter	100 mL	< 1 CFU/mL	24-25 h	CFU/mL	3.15 €

Additional method, such as the **Fung double tube method** is mentioned by Stelma[170] . This method is based on culture in glass tubes with Shahidi Ferguson Perfringens medium as shown in Figure 19. It is the first rapid method that creates anaerobic conditions allowing germination and specific enumeration of *C. perfringens* directly in tubes. This test has been originally developed for the detection of clostridia spores in food stuffs but it is also used for the detection of faecal contamination in Hawaiian recreational waters[171]. Vijayavel (2009)[172] and other laboratories have provided some modifications such as the use of CP AnaSelect Oxyplate medium, the heat pre-treatment of water samples (to enumerate spores only), inclusion of the phosphatase reaction, an increase of the volume of the tube (5 to 10 mL). Addition of 4-methylumbelliferyl phosphate (MUP) prior to incubation should generate the fluorescence of black colonies which would be confirmed as *C. perfringens* within 5-6 hours[170]. This method would enable early warning as detection of *C. perfringens* in water samples would take 5-6 hours instead of 13 days required by the classical methods or even 24-25 to 48 hours necessary for new culture-based methods. Improvements would be needed before its use in drinking water routine detection.

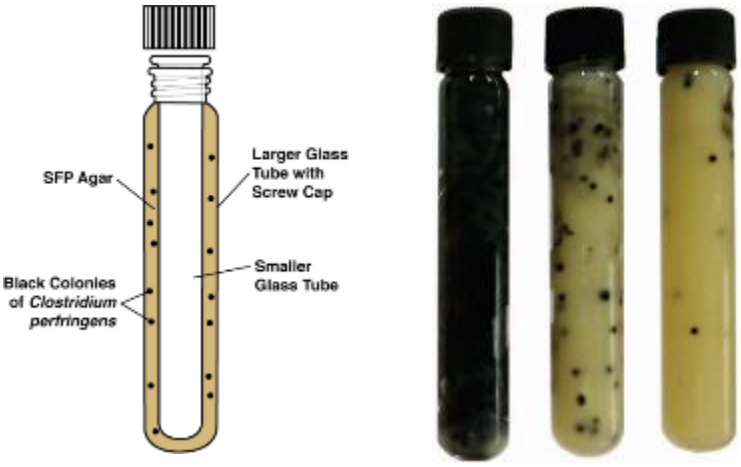

Figure 19. Fung double tube method. Description of the system (left) and examples of a test using chicken intestines extracts (right). From Barrios *et al*., 2013[173].

4.6.2 Non-culture based methods

Molecular methods for the detection of *C. perfringens* have been developed starting from **conventional polymerase chain reaction (PCR)** based on toxins-encoding genes as targets. Due to the great panel of toxins produced by the bacterium and its spores, the **multiplex-PCR**, initially involving the simultaneous detection of 4 toxin-encoding genes, has been then developed for helping the classification of clinical isolates[174].

Grant *et al*. (2008) developed a **quantitative real-time PCR (qRT-PCR)** assay targeting the *cpe* gene encoding for the enterotoxin expressed by spores, with the aim of investigating potential waterborne or foodborne outbreaks (*cpe* strains are responsible for most food poisoning cases) and having a better understanding of the disease transmission routes[175].

In 2013, Maheux *et al*. developed a method, called "Concentration Recovery Extraction of Nucleic Acids and Molecular Enrichment" (CRENAME), for the detection of *C. perfringens*

spores in drinking water (water samples were spiked with spores and then filtered)[176]. This approach is composed of a method for the concentration and recovery of microbial particles, a nucleic extraction procedure and a molecular enrichment combined with the amplification of the *cpa* gene by qPCR. The *cpa* alpha-toxin-encoding gene is specific for *C. perfringens*.

Comparing results obtained from a culture-based method (on mCP agar) and a non-culture based method (CRENAME), it has been shown that the CRENAME method can detect non culturable bacteria originating from spores and invalidate colonies that grew on mCP agar (considered after as false positive). The detection of *C.perfringens* (as low as 1 CFU/100 mL) in drinking water took 5 hours using the CRENAME method and 25 hours with the mCP method[176]. The CRENAME method provides therefore promising results in terms of detection and time necessary to obtain results respect to a culture-based method employing mCP agar.

A list of alternative and promising methods for the detection of *C. perfringens* and/or spores in water along with their advantages/disadvantages are reported in Table 15 and Table 16.

Table 15. Promising methods for detection of *Clostridium perfringens* in water matrices

Method	Purpose	Sample type tested	Required sample volume	Sensitivity	Time to results	Output
Culture-based methods						
CP Chromo Select Agar	Detection of *C. perfringens* by membrane filtration and incubation	Different water samples	Not reported	< 1 CFU/mL	24 h	CFU/mL
Fung Double Tube	Detection of *C. perfringens* by membrane filtration and incubation	Sewage-contaminated and environmental water	5-10 mL	< 1 CFU/mL	5-6 h	CFU/mL
Molecular methods						
Conventional PCR (*cpe* gene)	Detection of *C. perfringens* through genetic screening	All kinds of water samples	Not reported	high	< 2.5h	Agarose gel band
Real-time PCR (*cpe* gene) (qualititative)	Detection of *C. perfringens* through genetic screening	Environmental waters, drinking water, sludge, WWTP	100 mL	3.57 spores/100 mL	4 h	DNA copy number
Multiplex PCR (*cpa*, *cpb*, *ia*, *etx*, *cpb2*, *cpe* genes)	Detection and quantification of C*. perfringens* through genetic screening	Spiked water, drinking water, animal faeces	Not reported	100 pg/µL	4 h	DNA pg or copy number
CRENAME	Detection and quantification of C*. perfringens* through genetic screening	Drinking water	100 mL	1- 4 CFU/100 mL	5 h	CFU/mL

Table 16. Advantages and disadvantages of the promising methods for the detection of *C. perfringens* and/or spores in water.

Method	**Advantages**	**Disadvantages**
CP Chromo Select Agar	Better recovery and specificity than m-CP Agar method Specific (no confirmation of results required) Reduced number of false negative results compared to m-CP and TSC Colonies can be used for further biochemical testing	Qualitative Culture-requiring Requires confirmation of results through visual enzymatic tests (additional 4 h)
Fung Double Tube	Rapid (5-6 hours) Low cost External anaerobic generating systems non required	Qualitative Culture-requiring
Conventional PCR	Avoiding culture Rapid	Qualitative PCR instrument and specific reagents required Gel electrophoresis required to visualize results
Real-time PCR	Quantitative Avoiding culture Rapid Efficient for small sample volumes or low biological material	PCR instrument and specific reagents required Major costs compared to conventional PCR
Multiplex PCR	Quantitative Avoiding culture Rapid Simultaneous detection of different strains/genes	PCR instrument and specific reagents required Major costs compared to conventional PCR
CRENAME	Quantitative Avoiding culture Detection of non cultivable bacteria from spores Reduced frequency of false positive and false negative results Possibility to couple with multiplex PCR	Real-time PCR system and specific reagents required

4.7 Discussion on *Clostridium perfringens* and spores as surrogates for detection of *Giardia* and *Cryptosporidium* in drinking water

C. perfringens is a Gram-negative anaerobic spore-forming bacterium. Interestingly, it can persist in the environment for several weeks as a spore, which is more resistant to heat than the vegetative form and traditional faecal indicator bacteria (FIB) used to detect faecal contamination or sewage pollution.

Cryptosporidium and *Giardia* are protozoan parasites often excreted by human and warm-blooded animals. They have been associated with waterborne diseases (cryptosporidiosis and giardiasis) in different publications. (Oo)cysts are the form responsible for persistence and infectivity of these parsites.

Due to a similarity in size, morphology and, in some extent, the life cycle, *C. perfringens* and in particular its spores are considered as surrogates to detect the presence of *Cryptosporidium and Giardia* (oo)cysts in sewage and during wastewater treatment. They are also surrogates for the presence of *Cryptosporidium and Giardia* oocysts in drinking water since the adoption of the Directive 98/83/EC in 1998.

There are numerous publications on the co-occurence of *C. perfringens* and its spores with *Cryptosporidium and Giardia* (oo)cysts in sewage and during wastewater treatment, however few studies on other types of water exist. Recently, a meta-analysis performed by Korajkic *et al* (2018) and results presented in a previous publication by Till *et al* (2008) provided data to conclude that *C. perfringens* and/or spores are not a good indicator of water quality in ambient waters (fresh, marine and brackish waters) [144,145]. Considering the presence of *C. perfringens* and *Cryptosporidium and Giardia* (oo)cysts, 10 of the 11 studies did not show any association or correlation between the indicator and both parasites. Only one study reported such an association (and with other pathogens) but to a weaker extent compared to F-specific coliphages (Wilkes *et al*, 2009) (Table 12).

The global inactivation rate of *C. perfringens* and of parasite (oo)cysts during drinking water treatment is difficult to predict. For the drinking water supplies in the United States, US EPA requires a minimum removal or inactivation of 3 log for *Giardia* and 2 log for *Cryptosporidium*. Only a few studies evaluating their removal during drinking water treatment are available (Table 13). Most of these studies showed only the removal of *C. perfringens* with other indicators (FIB) or the removal of *Cryptosporidium* and *Giardia* (oo)cysts. For instance, there is no common study showing the removal of *C. perfringens* spores and parasite (oo)cysts during coagulation[9]. In a study of the drinking water processes, removal/inactivation of *C. perfringens* and *Cryptosporidium* oocysts (as the model for all parasites) were compared during the five steps of the treatment train (before and after slow sand filtration, ozonation, GAC filtration, UV and chlorine disinfection)[163]. Slow sand filtration seemed very efficient in the removal of *C. perfringens* and *Cryptosporidium* oocysts (>3 log), however the authors found *C. perfringens* spores unsuitable for the use as a surrogate indicator for oocysts removal in this treatment step as *C. perfringens* spores attach more efficiently to sand[163]. During ozonation, the removal was of the same magnitude between *C. perfringens* spores and parasites, while *Cryptosporidium and Giardia* (oo)cysts resulted more susceptible than Clostridia spores during GAC filtration[160,161]. Also, *Cryptosporidium and Giardia* (oo)cysts were found much more susceptible to UV disinfection than *C. perfringens* spores[158,162]. For chlorination, two situations should be considered depending on the disinfectant used (free chlorine and mixed-oxidants). *Cryptosporidium* oocysts are more resistant to free chlorine than *C. perfringens* spores, while mixed-oxidants are very efficient against both agents (3 log

removal)[143,158]. These data suggest that *C. perfringens* spores could be a surrogate indicator for *Cryptosporidium* and *Giardia* (oo)cysts removal during ozonation and mixed-oxidant disinfection only (Table 13).

In conclusion, *C. perfringens* spores could be used as a microbiological indicator parameter in addition to *Escherichia coli* and Enterococci to ensure tap water safety.

For the detection and enumeration of *C. perfringens,* culture-based methods based on mCP and on TSC media (ISO 14189) are prevalently used. Pasteurisation is used most of the time in the studies on water quality (but not always) in order to enumerate exclusively spores - the most resistant form to water treatment. These methods provide results in 48 hours, however they are incompatible with early warning in case of contamination.

Alternatively to this parameter, when the confirmation step is not performed, the choice could be left to conclude analysis by "presumptive colonies". Otherwise, sulphite-reducing bacterial spores (all Clostridia) could be also considered. ISO 6461 (1 and 2)[177,178] method is available for their detection in different types of water.

Furthermore, other culture media have been developed for the detection and enumeration of *C. perfringens* and spores (Chromoselect Agar, TCA media). They are more robust in terms of results (less false-positive and false-negative results) but with a similar time to results. Another culture medium has been developed for study, in tube, instead of plates. It enables detection of *C. perfringens* spores within 5-6 hours and has been successfully used for the detection of faecal contamination in Hawaiian recreational waters.

Non-culture based methods have also been developed for the detection of *C. perfringens* in water, including molecular techniques (e.g. polymerase chain reaction - PCR), that could be interesting in terms of time to results and sensitivity compared to the reference methods.

4.8 Recommendations

C. perfringens vegetative cells are present in raw water but they can not be detected after water treatment process. Only spores (to be precise, bacteria resulting from spore germination) can be measured as more resistant.

C. perfringens spores are the surrogate for the presence of *Cryptosporidium* and *Giardia* (oo)cysts during wastewater treatment. *Cryptosporidium* and *Giardia* are protozoan parasites. Waterborne outbreaks associated with parasites have been described in Europe (Annex I and V), USA and Canada (Annex V). The removal efficiency of *C. perfringens* spores is generally considered similar to the one of *Cryptosporidium* and *Giardia* (oo)cysts which enables the release of water with acceptable quality into rivers. *C. perfringens* spores are also an indicator parameter for faecal contamination in the DWD 98/83/EC, currently under revision.

In Europe, not all the countries use the latest case definitions for cryptosporidiosis and giardiasis and not all have settled surveillance systems and report to ECDC (in 2017, 24 EU/EEA reported giardiasis data, 25 reported cryptosporidiosis data). The number of cases of cryptosporidiosis and giardiasis linked to water is probably underestimated.

At this stage, our recommendations are:

1. *C. perfringens* spores could be used as a microbiological indicator parameter and should be measured in drinking water. The reference value should be 0 CFU/100 mL in drinking water. No reference value should be mentioned in raw water as, based on studies in fresh and marine waters or brackish water, the presence of *C. perfringens* spores is not correlated with the presence of *Cryptosporidium* and *Giardia* (oo)cysts.

2. If reported in drinking water, investigations should be performed as it indicates a potential risk of a former or recent contamination by protozoans.

3. For groundwater as drinking water source, this indicator should be measured in case of contamination due to WWTP leakage or flood risks (due to storm water).

4. Since *Cryptosporidium* oocysts are more resistant to free chlorine than *C. perfringens* spores, *Cryptosporidium* oocysts should be measured in case this type of disinfection is the only treatment process.

5. Large volumes should be analysed as peak concentrations of spores and oocysts may persist for a long time.

6. Using ISO 14189 as the method of detection in drinking water allows the detection of *C. perfringens* spores in a wide range of water types after concentration by membrane filtration. ISO 14189 allows selection by applying heat to destroy vegetative bacteria. The filter is placed on a selective culture medium, followed by incubation and counting of black colonies resulting from spore germination.

7. The possibility to use another method, such as ISO 14189, provides a confirmation step for presumptive colonies requiring the use of a carcinogenic agent, potentially harmful for

technicians. ISO 6461 could be used, as it allows the detection all other sulphite-reducing bacteria (all Clostridia) spores in a wide range of water types.

8. Every six years, this indicator should be evaluated based on scientific evidences, considering also the development of easy and low-cost methods for measuring *Cryptosporidium* and *Giardia (*oo)cysts directly in raw water and along the train barrier of the drinking water process.

Annex I

Recent waterborne outbreaks associated with viruses, bacteria and parasites. Investigation on the water source or the type of water supply

This section proposes a non-exhaustive list of the most important waterborne outbreaks either in terms of number of outbreaks or number of cases for countries that reported to national or international Health Authorities.

1.1 Outbreaks in Europe

In Europe, waterborne outbreaks caused by enteric viruses have been reported in Iceland (2004), Finland (2007), Montenegro (2008) and Italy (2011)[9]. Noroviruses were identified as one of the main causative agents and sewage contamination was among the attributable causes of the outbreaks (Table 1)[180-184]. Some other enteroviruses were also strongly associated with waterborne outbreaks (Table 2).

Waterborne outbreaks due to protozoan parasites (*Giardia* and *Cryptosporidium*) are probably underestimated as not all EU/EEA Member States (and also countries from the pan-European region) report data to ECDC/EFSA (different case definition, no legal obligation to report outbreaks apart from those that are considered food-borne) (Table 2) [179].

Table 1. Selected viral outbreaks in Europe in the period 2007-2011. From Guidelines for Canadian Drinking Water Quality**,** April, 2019[9]. This report summarises the well-documented viral outbreaks related to drinking water in North America (46 in USA and Canada) and in other countries (5) for the period 1971-2012. Of 5 outbreaks outside the USA and Canada, 4 occurred in EU/EEA countries (Iceland, Finland, Montenegro and Italy between 2000 and 2011). Several hundreds of cases were reported. Investigations showed that these outbreaks could be attributed to virus – mainly norovirus, after detection in untreated groundwater or water contaminated by sewage.

Date	Location	Causative agent	Estimated cases	Water system	Attributable causes	References
2004	Iceland (Lake Myvatn)	norovirus	> 100	small rural supply	untreated groundwater	Gunnarsdóttir *et al*., 2013[180]
2007	Finland (Nokia)	at least 7 pathogens, including norovirus	6500	municipal system (water source: groundwater and artificial groundwater); including filtration and chlorine disinfection	sewage contamination	Maunula *et al*., 2009[181] Laine *et al*., 2010[182] Rimhanen-Finne *et al*., 2010[183]
2008	Montenegro (Podgorica)	viral	1700	municipal system (water sources: karstic spring water and groundwater); chlorinated but no residual	sewage contamination	Werber *et al*., 2009[184]
2011	Italy (Sicily)	norovirus	156	public (municipal) system	contamination of the well and springs supplying the public water network	Giammanco *et al*., 2014[185]

In a report collecting epidemiological data on cases of infectious diseases (including infections by enteric viruses) in the pan-European region during the period 2000-2013, approximately 18% of the investigated outbreaks were linked to water (Table 2)[179]. According to the Global Infectious Disease and Epidemiology Network (GIDEON) database, a total of 1039 outbreaks were reported in in the pan-European region and the majority of these outbreaks were caused by contaminated drinking water supplies. Other identified sources included lakes, swimming pools, spas, water parks, heating and cooling towers, or public fountains. Leptospirosis, cryptosporidiosis, giardiasis and legionellosis associated with water showed the highest percentages of outbreaks. Of the 53 reporting countries of the pan-European region, 45 countries represented Southern, Northern, Western and Eastern Europe (the remaining 8 countries represented Central Asia and Caucasus). Over the period 2000-2013, these countries recorded 1004 out of 1039 documented outbreaks. A total of 174 outbreaks could be potentially linked to water (mean of 17%)[179].

In contrast to the GIDEON database, data included in the Centralized Information System for Infectious Diseases (CISID) and the European Surveillance System (TESSy) databases did not provide information on the number of infectious diseases related to water. However, both of them showed that campylobacteriosis, hepatitis A and giardiasis were the most commonly reported gastrointestinal infectious diseases in the Pan-European Region for the 2000-2010 (CISID) and 2006-2013 (TESSy) time period[179].

The same report gives also information on the number of cases and outbreaks for five specific diseases (cholera, shigellosis, enterohemorrhagic *E. coli*, hepatitis and typhoid fever) for the 2010-2012 time period[179]. A total of 279 outbreaks were reported for 9 out of the 23 participating countries but no information on the number of outbreaks was directly linked to water. Aside from systematic reporting on these five diseases, a few countries provided national reports on some specific diseases. In particular, water-related disease outbreaks, mainly caused by noroviruses or *Campylobacter* and primarily associated with private wells and small groundwater supplies serving fewer than 500 people, were reported in Finland. In 2011, an outbreak of Pontiac fever, associated with spa pool water contaminated by *Legionella anisa* bacterium, affected 11 people.

A report on waterborne outbreaks in European Nordic countries provided data on a total of 175 waterborne outbreaks notified in Denmark, Finland, Norway (1998-2012) and Sweden (1998-2011). The outbreaks affected 86 000 people and a total of 124 out of 163 cases were linked to contaminated groundwater or to single-household water supplies, affecting a small number of people (often less than 100 people per outbreak)[186].

In Hungary, 485 out of 778 cases of gastroenteritis registered in 2011 were associated with the following etiological agents: noroviruses, rotaviruses, *Salmonella, Shigella, Campylobacter* and *Clostridium difficile*. Drinking water was confirmed as the transmission route for only one outbreak. One case was probably due to adenovirus infection, and 20 cases of a probable or confirmed nosocomial legionellosis were reported (domestic hot water system being the most likely the source for 12 cases).

Table 2. Outbreaks attributed to water according to publications in GIDEON (2000–2013). The GIDEON database contains information about documented infectious diseases reported by 53 countries (through national health ministry reports) of the pan-European Region. This term refers to the WHO European Region and Liechtenstein. The WHO European Region comprises the following 53 countries: Albania, Andorra, Armenia, Austria, Azerbaijan, Belarus, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, Czechia, Denmark, Estonia, Finland, France, Georgia, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Luxembourg, Malta, Monaco, Montenegro, Netherlands, Norway, Poland, Portugal, Republic of Moldova, Romania, Russian Federation, San Marino, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Tajikistan, the former Yugoslav Republic of Macedonia, Turkey, Turkmenistan, Ukraine, United Kingdom and Uzbekistan. Of 1039 outbreaks recorded in GIDEON over the period 2000-2013, 185 (18%) were specifically linked to water and represented 18 diseases (Table 4). The majority of these outbreaks were caused by contaminated drinking-water supplies. Other sources were also identified. The pathogens showing the highest percentages of outbreaks linked to water are leptospirosis, cryptosporidiosis, giardiasis and legionellosis. From Kulinkina *et al*., 2016[179].

Disease	Outbreaks linked to water	Number of outbreaks	Proportion linked to water (%)	Countries	Most common sources
Legionellosis	37	100	37	15	Drinking-water, water heater, cooling tower, spa
Gastroenteritis – viral	24	206	12	12	Drinking-water, swimming area, spa
Cryptosporidiosis	20	50	40	6	Drinking-water, swimming pool
Hepatitis A	18	155	12	8	Drinking-water, sauna
Campylobacteriosis	14	45	31	11	Drinking-water
Leptospirosis	13	21	62	8	Drinking-water, outdoor recreational area
Rotavirus	10	37	27	7	Drinking-water
Shigellosis	9	64	14	8	Drinking-water, fountain
Typhoid and other enteric fever	9	38	24	4	Drinking-water
Tularaemia	8	42	19	4	Drinking-water
E. coli diarrhoea	5	109	5	4	Drinking-water, swimming pool
Giardiasis	5	14	36	5	Drinking-water
Cercarial dermatitis	4	4	100	4	Outdoor swimming and bathing areas
Adenovirus	3	26	12	3	Drinking-water, swimming pool
Mycobacteriosis – nontuberculous	2	12	17	1	Swimming pool
Yersiniosis	2	23	9	2	Drinking-water
Aeromonas & marine *Vibrio* infection	1	1	100	1	Swimming area
Blastocystis hominis infection	1	1	100	1	Drinking-water

France registered an increase of legionellosis cases since the late 1990s, with more than 1200 outbreaks in 2012 probably linked to water[187]. Over the 1998-2008 period, the French Institute for Publich Health Surveillance (INVS) reported 10 water-related outbreaks linked to drinking water supply networks. Cases of acute gastroenteritis were most of the time caused by noroviruses and *Cryptosporidium* spp.*,* but also by *Campylobacter* and rotaviruses, which indicated faecal contamination of the water.

Many countries are concentrating their efforts to reduce the number and the impact of outbreaks of water-related diseases in connection with the Protocol on Water and Health[179]. The Epidemic Intelligence Information System for Food- and Waterborne Diseases and Zoonoses (EPIS-FWD), coordinated by the European Centre for Disease Prevention and Control (ECDC), is a surveillance system for the detection of multicountry food- and waterborne diseases outbreaks and for the assessment of the related risk. During the 2008-2013 time period, 215 outbreak alerts, also known as "urgent inquiries" (UI), were launched in Europe[188]. Epidemiological and microbiological investigations revealed that for 110 UI (51%) a food vehicle of infection was either suspected or confirmed, for 93 UI, the vehicle of infection remained unknown, for 7 UI the infection was due to contact with animals, for 4 UI, it was water and for 1 UI, it was a laboratory-acquired infection. Three waterborne outbreaks were related to cholera in countries outside the EU, the remaining outbreak was a local outbreak of cryptosporidiosis after contamination of drinking water.

In Belgium, 64 children at a youth camp became ill after using water from a local source contaminated by *Campylobacter jejuni*. Denmark reported a waterborne outbreak with over 400 cases recorded due to *Campylobacter jejuni*[189,190].

Waterborne outbreaks of giardiasis due to inadequate treatment of drinking water are frequently reported in Europe. Infants and children are at a particularly increased risk for infection but no numbers of water-linked outbreaks were reported in the ECDC report published in 2014[190].

In 2012, 10 European countries reported to the European Food Safety Authority (EFSA) a total of 61 outbreaks caused by verocytotoxin-producing *Escherichia coli* (VTEC) detected in food and water[191]. Ten outbreaks were caused by water and all the infection cases were reported by Ireland. Of these 10 outbreaks, 7 were reported to be linked to private water supplies or wells.

Denmark faced an outbreak of Shiga toxin-producing *E. coli* (STEC)/VTEC *E. coli* O157:H7 infections in 2012. A high proportion of hemolytic uremic syndrome (HUS) (62% of cases) was reported and epidemiological investigations suggested ground beef as the vehicle of the outbreak[190].

The waterborne transmission of congenital toxoplasmosis (due to the change in the European Union case definition for toxoplasmosis in 2008, and change in reporting since 2009) is also described in an ECDC Surveillance Report as an emerging public health risk worldwide[191]. Water contaminated with faeces of infected cats is one of the transmission routes for humans exposed to *Toxoplasma gondii*[192] and standard disinfection processes, including UV radiation, are not always able to eliminate the protozoan parasite from drinking water[193,194].

Although outbreaks of great size (more than 1000 ill people) are rare, authors often highlight the need for increased awareness, correct water treatment follow up, constant management and maintenance of the water supply and distribution systems.

1.2 Outbreaks in Canada

In a recent report, Canadian Health Autorities reported cases of endemic Acute Gastrointestinal Illness (AGI) from all sources (food, water, animal, person-to-person). Approximatively 20.5 million cases were reported per year for 35 millions of Canadians over the period 2000-2010[9].

Almost 1.7% of these cases (335000 cases) were estimated to be associated with the consumption of tap water from municipal systems that serve >1000 people in Canada[195] and on which relied 29 millions of Canadians (84% of the population) in 2012. Twenty-five millions relied on surface water sources, the remaining 4 millions, on groundwater sources. Murphy *et al*. (2016) estimated that among these systems, those who did not include treatment, or applied a minimal treatment, or chlorine or chlorine dioxide treatment, accounted for the majority of the estimated cases (50121), whereas systems using multiple treatment barriers were associated with 15991 cases[195]. The authors also estimated that over 35% of the 335000 cases were attributed to the distribution system.
Approximatively 103230 cases were associated with *Giardia*, *Cryptoporidium*, *Campylobacter*, *E. coli* O157:H7 and norovirus and were also associated with private wells or small community water systems (using ground or surface water) in Canada[195]. Most of the 103230 cases were attributable to contaminated private wells (75% cases while 25% attributed to contaminated small groundwater or surface water systems). Regards the 5 pathogens, 73% cases were associated with the presence of norovirus (27%, to the presence of at least one of the 4 other pathogens cited above). Taken together, 53% of the total case number were associated with norovirus in private wells, and 19.25% with norovirus in small system(groundwater or surface water). Canadians served by private wells or small water supplies are thus at greater risk of exposure to pathogens (especially to noroviruses) and to develop waterborne AGI. Other studies showed the presence of enteric viruses in groundwater sources[7,196]. They estimated the AGI incidence in 14 communities, serving 1300 to 8300 people and supplied by untreated groundwater, and analysed tap water for the presence of noro-, adeno- and enteroviruses. They observed strong association only with noroviruses and established that from 6 to 22% of the AGI was attributable to enteric viruses[7]. Lambertini and coworkers performed a study on the same area before and after the introduction of a UV disinfection step in the treatment process. They enumerated enteric viruses post UV disinfection and already at that time observed an increase in virus concentration between the location of UV disinfection and household taps which was attributed to viruses entering into the distribution system[196].

1.3 Outbreaks in the USA

The American Public Health Agencies report on waterborne disease outbreaks recorded 15 outbreaks of water-related viral illnesses between 1991 and 2002 (3487 cases; while 77 outbreaks of unknown etiology with 16036 cases). Twelve outbreaks were attributed to noroviruses, one to a "small round-structured virus" and two outbreaks to the hepatitis A virus (HAV)[197].

Between 2003 and 2012, the Center for Disease Control and Prevention (CDC) reported 138 outbreaks associated with drinking water. Enteric viruses were identified as the single causative agent in 13 outbreaks (noroviruses in 10 and HAV in 3) and the majority of viral outbreaks were attributed to the consumption of untreated or inadequately treated groundwater[9].

During the 2013-2014 period, a total of 42 drinking water-associated outbreaks were reported, accounting for at least 1006 cases of illness. *Legionella* was the most common causative agent, responsible for over half of outbreaks (57%). Eight outbreaks were caused by *Cryptosporidium* or *Giardia.* The origin of these outbreaks was investigated and associated with water system deficiencies. As shown in Figure 1, each outbreak was assigned to one or more deficiency classification. For example, for outbreaks caused by *Legionella*, the bacteria were identified inside premise plumbing systems[198].

Recently, an online platform has been settled by CDC to inform the public on i) the latest waterborne outbreaks (https://waterandhealth.org/safe-drinking-water/outbreaks/); ii) the current water treatment (https://waterandhealth.org/safe-drinking-water/treatment/) and iii) the presence of antimicrobial resistance in drinking water and wastewater (https://waterandhealth.org/safe-drinking-water/drinking-water/, https://waterandhealth.org/safe-drinking-water/wastewater/).

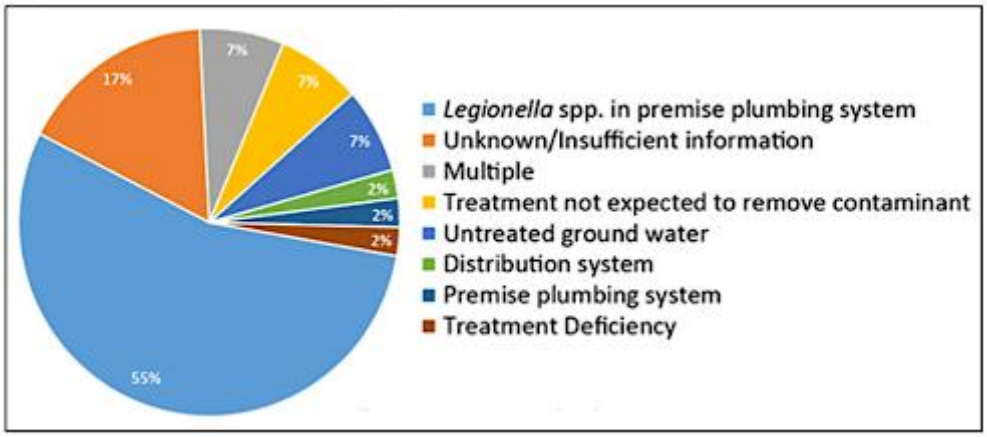

Figure 1. Deficencies related to drinking water-associated outbreaks (2013-2014) in the USA (Center for Disease Control and Prevention (CDC), 2017). The figure summarises the information on water system deficiencies related to outbreaks in 2013–2014. From https://waterandhealth.org/safe-drinking-water/recent-trends-in-legionella-and-waterborne-disease-outbreaks-and-their-causes/

1.4 Outbreaks in Australia

Reports on waterborne outbreaks of gastroenteritis are rare in Australia and there have been no reviews of water-associated outbreaks. OzFoodNet, an Australian national network for the surveillance of foodborne diseases, reports information on outbreaks of gastroenteritis for all transmission routes since 2001. Outbreak reports recorded as 'waterborne' or 'suspected waterborne' from 2001 to 2007 were extracted and fifty-four outbreaks were classified as either 'waterborne' (44) or 'suspected waterborne' (10). Drinking water was the suspected source for 19% (10/54) of the outbreaks and 78% (42/54) were attributed to recreational water. Dale and collaborators showed that waterborne outbreaks of gastrointestinal diseases in Australia are predominantly associated with recreational exposure[199].

Three outbreaks of suspected waterborne diseases were attributed to rainwater collected from facility roofs. To prevent disease outbreaks, the authorities have to ensure that rainwater tanks have a scheduled maintenance and disinfection program[200].

1.5 Outbreaks in New Zealand

Waterborne outbreaks caused by enteric viruses have been reported in New Zealand in 2006, due to the presence of noroviruses in the water supply of a ski resort contaminated by human sewage[201].

In August 2016, 5000 out of 14000 residents in a North Island town of New Zealand became ill. Drinking water came from untreated groundwater, and was found contaminated with *Campylobacter* bacteria. The explanation was that after heavy rains, runoff water contaminated a pond with sheep faeces. The pond water seeped into the ground, contaminating the aquifer serving a nearby shallo-bored well that was used as a surface source[202].

Annex II
Bacteriophage taxonomy

In 1937, Burnet showed for the first time that phages differed in size and resistance to physical agents. In 1943, Ruska proposed the first classification of phages based on their morphological differences observed using electron microscopy[203]. A few years later, in 1948, Holmes proposed a classification based on host range and symptoms of diseases. Subsequently, in 1962, Lwoff, Horne and Tournier settled the basis of the future International Committee on Taxonomy of Viruses (ICTV) and proposed a new classification based on the properties of the virions and the nucleic acid molecules.

The current ICTV classification relies on the size, shape and complexity of the virion, and in particular on i) the nucleic acid molecule (either double-stranded –ds or single-stranded –ss, DNA or RNA); ii) the protein coat or capsid (made of assembled capsomers), and iii) the lipid membrane envelope present in some of them[203].

In 2007, Ackermann mentioned that more than 5500 phages of eubacteria and archaea had been examined by electron microscopy since the introduction of negative staining in 1959 and 96% of them showed to be dsDNA and tailed phages while the remaining 4% was represented by polyhedral, filamentous, or pleomorphic phages[203].

In 2017, the ICTV proposed a new classification of the major order Caudovirales (tailed phages) which encompasses 88 genera and 249 species[204].

Annex III
Other bacteriophages studied in water

Host species	type of coliphages	Uses
Salmonella enterica serovar *Typhimurium* (e.g. WG 49)	F-specific coliphages	*S. typhimurium* is a Gram-negative bacterium in which F_{amp} plasmid has been transferred. It has resistance markers (resistance to ampicilline and capacity to use lactose) in contrast to *E. coli* HS (F_{amp}) strain. Due to theses markers, re-selection is easy. This strain can be used according to ISO 10705-1 for the enumeration of F-specific coliphages or the enumeration of F-specific RNA bacteriophages. The number of F-specific RNA bacteriophages is the difference between the number of phages counted in the absence and in the presence of RNAse in the assay medium, since this enzyme interferes with the infection of F-specific RNA bacteriophages. It can be used according to ISO 10705-1, or US EPA Method 1601 or 1602.
Bacteroides fragilis (e.g. *HSP40, RYC 2056)*	Bacteroides spp. Phage B56-3	*Bacteroides fragilis* is a Gram-negative bacterium of the intestinal tract of humans and warm-blooded animals, that can harbour phages of the *Siphoviridae* family. This phage seems to be specific for the *Bacteroides fragilis* HSP40 and RYC 2056 strains and its presence has been detected in human but not in animal faeces. In turn, it can not be used to trace animal faecal pollution. Being the bacterial host susceptible to environmental conditions, it is unlikely that phages of *Bacteroides fragilis* replicate. Its distribution appears to be geographically contained overlappig with the prevalence of their host cells (lower concentrations have been recorded in sewage and environmental waters). It can be used according to ISO 10705-4.
Enterococcus faecalis	Enterophages	*Enterococcus faecalis* is a Gram-positive bacterium frequently identified in human intestines but absent in animal faeces. Enterophages have been shown to have comparable persistence rate to human enteric viruses in both fresh and marine waters except tropical and subtropical zones[205]. Further studies on other host species and from other regions of the world could help implementing the data on this possible indicator.

Annex IV

Standardised methods for the enumeraton of coliphages in other types of water

~~S~~tandard Method 9224[73] Membrane filtration (2017)

This method is used for the detection of enteric viruses in water and wastewaters, after membrane filtration of 100 mL sample or larger volumes and is very similar to Method 1601 and Method 1602, which are a single-agar layer (SAL) methods.

The method is based on the detection of F-RNA coliphages using *E. coli* F_{amp} or *Salmonella typhimurium* WG49 as hosts, and of somatic coliphages using *E. coli* strain C or WG4. The *E. coli* strain C is a mutant in which genes encoding nuclease enzymes have been deleted. This strain is susceptible to a broad range of coliphages and it is the host most frequently used for detecting the presence of somatic coliphages in water environments[26]. It is based on a single layer plaque assay.

The advantage of this method is the use of high sample volumes, therefore a better sensitivity is expected. However, a low recovery rate of bacteriophages is likely to occur during filtration and elution. It still requires an overnight culture before reading.

US EPA Method 1642[71] (April 2018) Male-specific (F+) and somatic coliphages in Recreational Waters and Wastewater by Ultrafiltration (UF) and Single Agar Layer (SAL) Procedure

Method 1642 describes a dead-end ultrafiltration (UF) concentration procedure with enumeration by the single agar layer (SAL) procedure.

This method is used to concentrate large sample volumes (2 L) (fresh and marine water) as required for recreational water monitoring. Wastewater from advanced treatments can also be used.

Samples of fresh and marine waters are collected by hand or with a sampling device if the sampling site has difficult access such as a dock, bridge or bank adjacent to the surface water. The sampling depth for surface water samples should be of 15-30 centimetres below the surface water.

For wastewater, 2 L of wastewater effluent samples are used. When samples such as chlorinated wastewaters are collected, a dechlorinating agent (2 mL of a 10% sodium thiosulfate solution per 2 L sample) must be added into the sample container.

After UF using a hollow-fiber ultrafilter, the final sample volume is 200 mL. It is then splitted into two 100 mL aliquots, which can then be assayed for both somatic and male-specific coliphages using the SAL procedure.

In addition to recreational water, this method has also been validated in an interlaboratory study on advanced treatment wastewater effluents[71].

Smaller volumes can also be used in advanced treatment wastewater effluents.

The SAL procedure takes between 24 and 48 h depending on whether the host strain has been prepared.

US EPA Method 1643[72] (April 2018) Male-specific (F+) and somatic coliphages in Secondary (No Disinfection) Wastewater by the Single Agar Layer (SAL) Procedure

It is a modification of US EPA Method 1602 based on a sampe volume of 100 mL of secondary wastewater samples (undiltuted or diluted 1:10).

Interferences can be caused by high background levels of microorganisms that may prevent the host bacteria from producing a confluent lawn of growth.

This method has been validated in an interlaboratory study on secondary wastewater samples and unspiked and spiked phosphate buffered saline (PBS) samples as control blank and positive control.

Annex V
Giarda and *Cryptosporidium* outbreaks

Giardia and *Cryptosporidium* have the ability to produce cysts or oocysts that are extremely resistant to environmental stresses. These microorganisms may be found in water following direct (contaminated drinking water or recreational water) or indirect contamination caused by infected faeces of humans or animals. Uncooked food or food contaminated after cooking can also cause infections. Person-to-person transmission is the major route of exposure to *Giardia* and *Cryptosporidium*.

Two major outbreaks associated with *Cryptosporidium* were identified in the late 80s and beginning of the 90s and were directly linked to treated water. The first one was reported in Swindon and Oxfordshire (UK) in 1989, and affected 5000 people; the second one occurred in 1993, in Milwaukee, Wisconsin (United States) and affected more the 400 000 people.

For the 1971-2006 period, Craun and collaborators reported more than 243 outbreaks in the United States linked to groundwater (the aetiologic agent was identified for 38% of the outbreaks), and 123 linked to surface water (the aetiologic agent was identified for 62% of the outbreaks)[206]. Acute Gastrointestinal Illness (AGI) was the most common disease. Drinking water-related outbreaks have been reported for both *Giardia* and *Cryptosporidium*[206]. *Giardia* was the most frequently identified aetiological agent associated with waterborne outbreaks in the United States between 1971 and 2006, accounting for 16% of outbreaks (126/780), while *Cryptosporidium* accounted for 2% (15/780). These outbreaks were associated with 28127 cases of giardiasis and 421 301 cases of cryptosporidiosis[206]. Most of the cryptosporidiosis cases (95.65%) were associated with the Milwaukee outbreak in 1993[206].

Giardia and *Cryptosporidium* are common causes of waterborne infectious disease outbreaks in Canada. Between 1974 and 2001, *Giardia* and *Cryptosporidium* were the first and the third most commonly reported causative agents, respectively, associated with infectious disease outbreaks related to drinking water in Canada[207]. *Giardia* was linked to 51 of the 138 outbreaks for which causative agents were identified and *Cryptosporidium* was linked to 12 of the 138 outbreaks. The majority of *Giardia* and *Cryptosporidium* outbreaks (75 and 92%, respectively) were associated with public drinking water systems. From 2002 to 2016, only one outbreak of giardiasis linked to a drinking water source has been reported in Canada[208,209]. No outbreaks of cryptosporidiosis related to drinking water have been reported in the same time period.

In a worldwide review on waterborne protozoan outbreaks, *Giardia lamblia* and *Cryptosporidium* accounted for 40.6% and 50.6%, respectively, of the 325 outbreaks reported between 1954 and 2003 from all water sources, including recreational water[210]. The largest reported drinking water-related *Giardia* outbreak occurred in 2004, in Norway, with an estimation of 2500 cases[211,212]. Between 2004 and 2010 and between 2011 and 2016, 199 and 381 respective protozoan outbreaks were also reported[208, 212]. *Giardia* accounted for 35.2% and 37% of outbreaks, and *Cryptosporidium* for 60.3% and 63%, respectively.

Several authors have investigated whether there are commonalities in the causes of the drinking water outbreaks related to enteric protozoa. For the outbreaks identified in Canada, contamination of water sources from human sewage and inadequate treatment (e.g. poor or no filtration, relying solely on chlorination) appears to have been major contributing factors[207]. An analysis by Risebro *et al*. (2007) showed that in the European Union (1990–2005), the majority of outbreaks have more than one contributing factor[213]. Indeed, similar to the findings of Schuster *et al*. (2005), contamination of the water source with sewage or livestock

faecal waste (usually following rainfall events) and treatment failures (filtration problems) were frequently detected in enteric protozoa outbreaks. Risebro *et al*. (2007) also noted that long-term treatment deficiencies resulted in drinking water outbreaks. Although less common, distribution system issues were reported to have been responsible for outbreaks, mainly related to cross-connection control problems[209,213].

A recent review, focusing on outbreaks occurring between 2000 and 2014 in North America and Europe, reported very similar problems[209]. Some of the water sources were described as untreated groundwater supplies. Wallender et al. (2014) reported that 248 outbreaks registered in the US between 1971 and 2008 involved untreated groundwater. Briefly, 14 outbreaks (5.6%) were due to *Giardia intestinalis*, two (0.8%) due to *Cryptosporidium parvum* and *Giardia intestinalis* and five (2%) to multiple causative agents. The same study also reported that 70% of these 248 outbreaks were related to semi-public and private drinking water supplies using untreated well water[214].

List of abbreviations and definitions

AGI	Acute Gastrointestinal Illness
AIDS	Acquired Immune Deficiency Syndrome
AstroV	Astrovirus
CDC	Center for Disease Control and Prevention
cDNA	complementary cDNA
CEN	Comité Européen de Normalisation
CFU	Colony forming unit
CISID	Centralised Information System for Infectious Diseases
CLAT	Culture Latex Agglutination and Typing
CRENAME	Concentration Recovery Extraction of Nucleic Acids and Molecular Enrichment
DAF	Dissolved air flotation
DAL	Double Agar Layer
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
ds	double-stranded
DWD	Drinking Water Directive
ECDC	European Center for Disease Control
EEA	European Economic Area
EFSA	European Food Safety Authority
EPIS-FWD	Epidemic Intelligence Information System for Food- and Waterborne Diseases
EV	Enterovirus
EU	European Union
FIB	Faecal Indicator bacteria
GI, GII	Genotype I, Genotype II
GAC	Granulated Activated Carbons
GIDEON	Global Infectious Disease and Epidemiology Network
gLAMP	In-gel Loop-Mediated Isothermal Amplification
GUDI	Groundwater Under Direct Influence
h	hour
HAdV	Human Adenovirus
HAV	Hepatitis A virus
HPyV	Human Polyomavirus
HUS	Hemolytic uremic syndrome
ICTV	International Committee on Taxonomy of Viruses
INVS	Institut national de veille sanitaire
ISO	International Organization for Standardization
mCP	modified Clostridium Perfringens
MPN	Most Probable Number
MSA	Modified Scholten's Agar
NEC	Necrotising enterocolitis
NoV	Norovirus
PCR	Polymerase Chain Reaction
PFP	Plaque-forming Particle
PUB	Public Utilities Board
PFU	Plaque-forming unit
qPCR	quantitative PCR
RNA	Ribonucleic acid
ReoV	Reovirus
RoV	Rotavirus
RT-PCR	Retro-Transcription-Polymerase Chain Reaction
RT-qPCR	Retro-Transcription-quantitative Polymerase Chain

SAL	Single Agar Layer
SCA	Standing Committee of Analysts
spp	subspecies
SRC	Sulphite Reducing Clostridia
ss	single-stranded
STEC	Shiga-toxin-Producing *Escherichia coli*
TESSy	The European Surveillance System
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSC	Tryptose-Sulfite-Cycloserine
TYGA	Tryptone–yeast extract-glucose agar
UF	Ultrafiltration
UI	Urgent inquiries
US EPA	United States Environmental Protection Agency
UV	Ultra violet
VTEC	Verotoxigenic *Escherichia coli*
WHO	World Health Organization
WSP	Water Safety Plan
WWTP	Waste Water Treatment Plant

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