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ANALYTICAL DETECTION OF SOMATIC COLIPHAGES IN WATER SAMPLES AS INDICATORS OF VIRAL OR FAECAL CONTAMINATION

Final Degree Project

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BIOCHEMISTRY AND MOLECULAR BIOLOGY

CONTENTS

1. ABSTRACT

This study explores the detection of somatic coliphages in water as indicators of viral or faecal contamination, highlighting their role as reliable surrogates for microbial water quality. Coliphages share similarities with human enteric viruses, such as resistance to environmental stress and water treatment, making them superior to traditional indicators such as E. coli. A comprehensive review identified the double agar layer (DAL) technique as the most suitable due to its high sensitivity, specificity and compliance with regulatory standards.

Experimental validation at Laboratori Analític Valls demonstrated the effectiveness of the DAL method in detecting coliphages, providing accurate results in plaque forming units (PFU). Out of 32 water samples analysed, coliphages were detected in only one, confirming the reliability of the method in identifying viral or faecal contamination. The results highlight the importance of including coliphage detection in water quality standards to improve public health protection through better detection and management of waterborne pathogens.

Keywords: double agar layer method, faecal contamination, **s**omatic coliphages, viral contamination, water quality

2. INTRODUCTION

The detection of coliphages in water is crucial due to their potential as accurate indicators of viral contamination originating from faecal sources. Unlike traditional bacterial indicators, such as *Escherichia coli* (E. coli), coliphages—particularly somatic and F-specific types—share many characteristics with human enteric viruses, such as resistance to environmental stress and water treatment processes (Grabow, 2001; Gerba, 2009). As a result, they are increasingly recognised as reliable surrogates for monitoring viral contamination in water, providing a more specific and effective assessment of microbial water quality (Ashbolt et al., 2001; Jofre et al., 2016).

This study aims to explore the analytical detection of somatic coliphages in water as indicators of viral or faecal contamination. Specifically, it involves a review of existing methods and their effectiveness, along with the practical application of the double agar layer (DAL) technique, a method validated for its sensitivity and specificity in detecting somatic coliphages in water samples (Gerba & Rose, 1990; Jofre et al., 2016). This practical work was conducted at the "Laboratori Analític Valls" using water samples from the local area. By combining a comprehensive literature review with practical experiments, this work aims to contribute to the broader adoption of coliphages as reliable indicators for water quality monitoring and public health protection (Pusch et al., 2005; Samhan et al., 2016).

2.1. What are coliphages?

Bacteriophages, or simply phages, are viruses that infect and destroy bacteria. The term "bacteriophage" comes from Greek and means "bacteria eater." They are composed of at least a nucleic acid molecule, which is the genome, encased in a protein shell known as a capsid. These viruses are highly specific, as each type of bacteriophage usually infects only a particular type of bacteria. Coliphages are bacteriophages that specifically infect coliform bacteria, basically Escherichia coli (E. coli), after infecting it through the cell wall. They have been investigated as potential indicators of human-derived viral contamination since the early 1980s (Jamalludeen et al., 2009). They are commonly present in both human and animal faeces. Structurally, many coliphages are small, icosahedral, and non-enveloped, resembling numerous human enteric viruses (Bertani & Bertani, 1974). Their environmental transport and survival characteristics are similar to those of enteric viruses, although they vary by season and by group (Ashbolt et al., 2001). They do not typically replicate in natural waters at significant levels, making their presence a clear sign of recent faecal contamination (Muniesa and Jofre, 2004). Moreover, their resilience to environmental conditions makes coliphages a better indicator of faecal contamination in water compared to faecal bacteria. (Grabow 2001; Contreras-Coll et al. 2002; Yates 2007; Jurzik et al. 2010). Coliphages can replicate within bacterial hosts, which allows them to survive longer in environmental conditions compared to many human-associated enteric viruses (Oliveira et al., 2009).

2.2. Groups of coliphages

Ashbolt et al. (2001) claimed that there are two primary groups of coliphages used in water quality assessments: somatic coliphages and F-specific (male-specific) coliphages (Figure 1, Jofre et al., 2016). Somatic coliphages infect E. coli through the cell wall and are predominantly found in the Myoviridae, Siphoviridae, Podoviridae, and Microviridae families. F-specific coliphages, including F-specific RNA bacteriophages, infect bacteria via the sex pili, which is coded by the F plasmid. These RNA bacteriophages are part of the Leviviridae family and consist of a simple capsid with cubic symmetry, containing a single-stranded RNA genome.

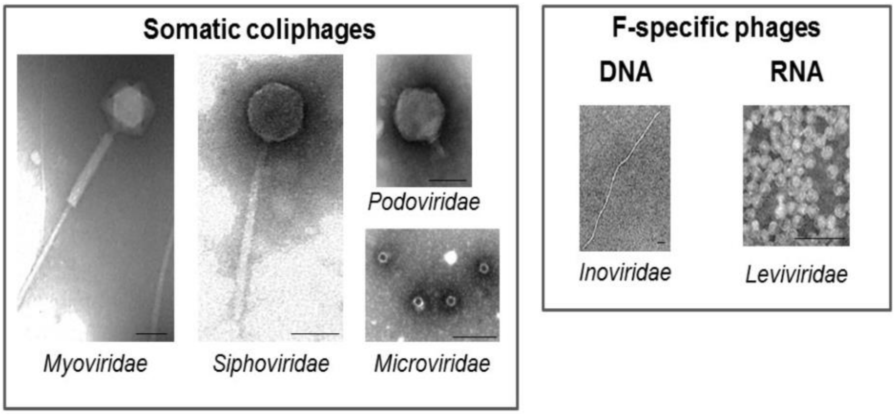


Figure 1: Major families of coliphages (Jofre et al., 2016)

As depicted in Table 1 (Singh et al., 2022), bacteriophages are classified into diverse taxonomic groups based on their replication mechanism, mode of infecting hosts, morphological characteristics, and genomic content.

Table 1: Characteristics of major groups of bacteriophages

Order	**Family**	**Phage genus**	**Representative species**	**Type**	**Nucleic acid**
Levivirales	*Fiersviridae* (earlier *Leviviridae*)	*Levivirus*	*Escherichia virus MS2*	F-specific RNA phage	ssRNA linear genome
			Escherichia virus BZ13		ssRNA linear genome
		Allolevirus	*Escherichia virus Qbeta*		ssRNA linear genome
			Enterobacteria phage SP		ssRNA linear genome
Tubulavirales	*Inoviridae*	*Inovirus*	*Enterobacteria virus M13*	F-specific DNA phage	dsDNA circular genome
Kalamavirales	*Tectiviridae*	*Alphatectivirus*	*Salmonella virus PRD1*	Somatic	dsDNA linear genome
Caudovirales	*Myoviridae*	*T4 virus*	*Enterobacteria virus T4*		dsDNA linear genome
	Siphoviridae	*Lambdavirus*	*Enterobacteria virus lambda*		dsDNA linear genome
	Podoviridae	*T7 virus*	*Enterobacteria virus T7*		dsDNA linear genome
Petitvirales	*Microviridae*	*phix174*	*Escherichia virus phix174*		ssDNA circular genome

Somatic coliphages are usually more numerous than F-specific coliphages. However, under certain conditions, such as after a single application of UV inactivation treatment in reclaimed water (Montemayor et al., 2008), in clayey sediments (Skraber et al., 2009) or in some groundwater from certain aquifers (Lucena et al., 2006), F-specific coliphages may be more prevalent. Therefore, it may be beneficial to monitor both groups of coliphages simultaneously in certain contexts. The E. coli strain C3000 (ATCC 15597) has been widely used in the USA for simultaneous detection, although it detects fewer somatic coliphages than the CN13 and WG5 strains. More recently, the custom E. coli strain CB390 has been developed to detect similar numbers of somatic coliphages as WG5 and as many F-specific phages as the host strains used in standard procedures, and this has been validated in different studies by different authors (Bailey et al., 2017; Guzmán et al., 2008).

2.3. Coliphages replication

Coliphages reproduce in the human and animal gut by infecting gut bacteria such as E. coli. Coliphages infect host cells by first adsorbing to the host cell and injecting their genetic material (DNA or RNA). Subsequently, the coliphage nucleic material circularises and enters either the lytic (virulent phage), lysogenic (temperate phage), or chronic cycle (temperate phage). During the lytic cycle, coliphages replicate within their host cells to produce new coliphage DNA or RNA and proteins, which are then assembled into phage virions. These virions are subsequently released into the environment in large numbers through the lysis-protein-mediated rupture of the host cell wall. There are similarities between the lytic and lysogenic cycles. In the lysogenic cycle, the coliphage attaches to the host cell and injects its genetic material into the bacterial host. During these stages, the coliphage DNA integrates stably into the host bacterium's chromosome, forming a prophage. This prophage is non-infectious and non-lethal, replicating along with the host DNA and increasing its concentration with each bacterial replication cycle. Occasionally, environmental triggers cause the prophage to excise from the bacterial chromosome and switch to the lytic cycle. This change from the lysogenic to the lytic state is known as induction. After induction, the host cells undergo lysis, resulting in the release of new phage virions.

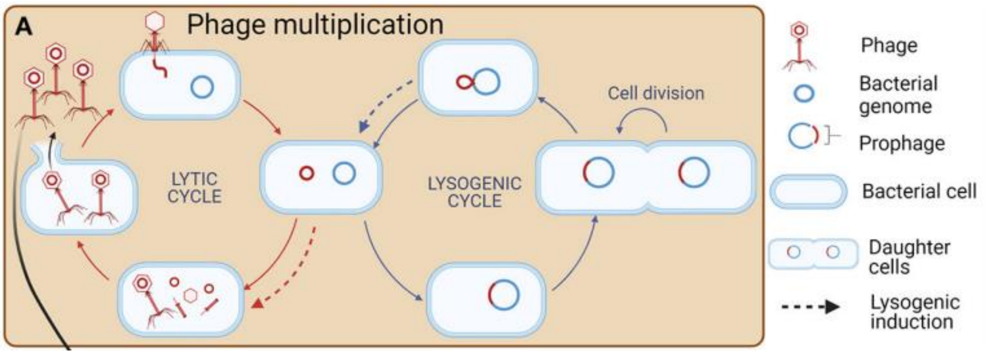


Figure 2: Bacteriophage multiplication (extracted from Singh et al., 2022)

2.4. Why are coliphages analysed in water?

The assessment of microbiological water quality is essential to protect public health from waterborne diseases, which affect millions of people annually (Zamxaka et al., 2004; Wilkes et al., 2009). Traditional bacterial indicators such as E. coli and faecal coliforms are commonly used, but they do not always correlate with the presence of viral pathogens (Baggi et al., 2001; Morens et al., 2004). Coliphages, due to their structural and behavioural similarities to enteric viruses, offer a more reliable indication of viral contamination in water (Gerba, 2009).

Coliphages have been proposed as indicators for several reasons. They are present in high numbers in sewage and polluted water environments and are easily detectable using straightforward laboratory methods (Grabow, 2001). Their presence correlates well with human-derived viral contaminants, and they exhibit similar resistance to environmental stresses and disinfection processes as enteric viruses (Ashbolt et al., 2001). This makes coliphages valuable for assessing the efficacy of water treatment processes and the potential presence of pathogenic viruses (Beaudoin et al., 2007). These pathogens can lead to gastrointestinal, respiratory, and other infections (Simonet et al., 2006). Moreover, coliphages, particularly somatic coliphages, are known to be more resistant to disinfection treatments than coliform bacteria. Therefore, their presence after treatment indicates the potential ineffectiveness of the disinfection process in eliminating viral pathogens. This increases the risk of viral infections, such as those caused by norovirus and rotavirus, which are common agents of acute gastroenteritis outbreaks (Payment & Locas, 2011).

Studies have shown that coliphages and enteric viruses are removed from water at comparable rates during treatment processes, indicating their utility as surrogate indicators (Gerba & Rose, 1990; Cho et al., 2000). However, the presence of coliphages does not always guarantee the presence of human viruses, and vice versa (Hauri et al., 2005). This limitation notwithstanding, coliphages are still considered valuable indicators because they fulfil many criteria for an ideal microbial indicator, including persistence in the environment and ease of detection (Samhan, 2005).

Although coliphages are not directly pathogenic to humans, their presence indicates shared transmission routes with human enteric viruses, like adenoviruses and enteroviruses. These viruses can cause severe illnesses, especially in vulnerable populations such as children, the elderly, and immunocompromised individuals (Sassi et al., 2018).

In summary, coliphages are analysed in water because they provide a reliable, cost-effective method for assessing faecal contamination and the potential presence of enteric viruses. Their detection helps in monitoring and managing water quality to prevent waterborne diseases, making them an essential component of public health protection in both developed and developing regions (Baker et al., 2003).

2.5. Sources of coliphages in contaminated water

Coliphages come from a variety of sources in contaminated waters. These sources are often related to the presence of faecal matter, as they are commonly found in the intestine of animals and humans. Some of the most common sources are included in this list:

- Wastewater Discharges: Coliphages are often introduced into water bodies through the discharge of untreated or inadequately treated wastewater from treatment plants. During overflows or malfunctions, these facilities can release significant quantities of coliphages into the environment (Nappier et al., 2019)

- Sewer Overflows: Heavy rainfall or infrastructure failures can lead to sewer overflows, mixing sewage with stormwater and subsequently contaminating surface waters with coliphages (Muniesa and Jofre, 2004; Franke et al., 2009)
- Domestic Wastewater: Leaks or failures in septic systems can lead to the infiltration of coliphages into groundwater and surface waters. These systems, if not properly maintained, contribute to the spread of these viruses (Hassard et al., 2016)
- Animal Excrement: Livestock, wildlife, and pets can introduce coliphages into water bodies directly through defecation. Agricultural runoff, particularly from fields treated with manure, can also carry coliphages into nearby streams and rivers (Jebri et al., 2017)
- Agricultural Activities: Manure application in agricultural fields can result in coliphages, viruses that infect E. coli, being washed into nearby water bodies during rain or irrigation. This runoff is a significant contributor to water contamination by coliphages. (Jofre et al., 2016)

2.6. Regulatory and practical implications

Regulatory bodies, including the European Commission, recognize the importance of monitoring coliphages to assess water quality and treatment efficacy. Guidelines and standards are being developed to include coliphages as indicators of water safety, reflecting their utility in both environmental monitoring and public health protection (ISO 10705-1, 2001; ISO 10705-2, 2001; WHO, 2017).

According to Ley 14/1986, of April 25, "General de Sanidad", public health administrations are required to prioritise their actions towards health promotion and disease prevention. This law stipulates that activities and products that may have negative health consequences, either directly or indirectly, must be subject to control by public administrations.

Following the transposition of Directive (EU) 2015/1787, which amended Annexes II and III of Directive 98/83/EC on the quality of water intended for human consumption, the Spanish government introduced a legal obligation to test for the presence of somatic coliphages in water for certain specific uses. This transposition was carried out by Real Decreto 902/2018, of 20 July, which amends Real Decreto 140/2003, of 7 February, establishing the health criteria for the quality of water for human consumption.

Therefore, as of the publication of Real Decreto 902/2018 on 21 July 2018, it has been a legal requirement to analyse the presence of somatic coliphages in water in order to comply with the quality standards for water intended for human consumption in Spain.

The analysis of coliphages in water is guided by specific regulations, primarily focusing on ensuring water quality and safety. Coliphages, especially somatic coliphages, serve as indicators for viral contamination and faecal pollution in various water sources, including drinking water, recreational waters, and wastewater. The key legislative documents include the Real Decreto 3/2023 and the Directive (EU) 2020/2184. These regulations mandate the monitoring of coliphages as indicators of water quality, aligning with broader European standards for water safety. The official analytical methods for somatic coliphages are included in UNE-EN ISO 10705-2. (Water quality. Detection and enumeration of bacteriophages. Part 2: Enumeration of somatic coliphages) and UNEISO 10705-3 (Water quality. Detection and enumeration of bacteriophages. Part 3: Validation of methods for the concentration of bacteriophages in water).

The Real Decreto 3/2023 establishes the parametric values for coliphages in drinking water. Specifically, the presence of somatic coliphages must not exceed 0 UFP/100 mL. Somatic coliphages are a microbiological parameter that must always be analysed in comprehensive water tests. If coliphages are detected in treated water, immediate corrective actions must be taken. Additionally, operators are encouraged to conduct a quantitative microbial risk assessment according to World Health Organization guidelines if feasible.

The Directive (EU) 2020/2184, which focuses on the quality of water intended for human consumption, includes somatic coliphages as a critical parameter for assessing water safety. The directive emphasises the importance of using coliphages as viral indicators due to their structural and persistence similarities with enteric viruses, making them reliable markers for potential pathogenic viral contamination.

3. HYPOTHESIS AND OBJECTIVES

As outlined in the introduction, the detection of coliphages in analytical laboratories specialising in Quality Control is crucial from both a legal and a health perspective. Currently, there are various methods for testing for the presence of these viruses in water. However, due to the recent (2018) obligation to detect this parameter, it has not yet been implemented in many laboratories.

Hypothesis: The detection of somatic coliphages in water samples using the double agar layer method is a reliable indicator of the presence of faecal and viral contamination.

All this considered, the objectives of this study are:

- To select the most appropriate method, conditions and tools to analyse the presence of coliphages in drinking water samples at the "Laboratori Valls".
- To verify the effectiveness of the double agar layer method in the laboratory and to design a protocol to carry it out.
- To study the amount of water samples contaminated by coliphages analysed at "Laboratori Valls"

4. METHODOLOGY

4.1. Selection of the most appropriate method

4.1.1. Search strategy

The literature search for this review was carried out using the PubMed database to identify relevant peer-reviewed articles and empirical studies focused on the detection of somatic coliphages as indicators of water contamination. The search was conducted using the following specific queries: "somatic coliphages detection AND bacteriophages indicators," "somatic coliphages detection AND faecal contamination water," "somatic coliphages detection AND double agar layer," and "somatic coliphages detection AND viral contamination water." These search terms were carefully chosen to encompass a broad spectrum of research on the role of somatic coliphages in detecting both faecal and viral contamination in water, and to evaluate various detection methodologies.

The search initially resulted in a total of 215 articles. After removing duplicates, i.e. items that appeared in more than one search, 71 unique items remained. From these 71 unique articles, the 14 most relevant and pertinent studies were selected, which closely matched the objectives of this review.

4.1.2. Inclusion and exclusion criteria

To ensure the inclusion of the most recent and relevant studies, articles published within the last 10-15 years were prioritized. The review focused on peer-reviewed and empirical studies that specifically addressed the detection of somatic coliphages as indicators of water contamination, particularly in relation to faecal and viral pollutants. Studies exploring different detection methods for coliphages, with a particular emphasis on their practical application and effectiveness in various water environments, were included in the review.

Articles not available in English were excluded to maintain consistency and ease of analysis. Studies published before 2008 were generally excluded unless they were considered foundational or seminal works that significantly contributed to the understanding of coliphages as indicators of water quality. Additionally, non-peer-reviewed sources, such as opinion pieces or articles with unverified data, were excluded to ensure the reliability and scientific rigor of the review.

4.1.3. Data extraction and analysis

Once the relevant articles were identified, data were extracted focusing on several critical aspects. This included a thorough comparison of the methodologies used for detecting somatic coliphages, particularly in terms of their sensitivity, specificity, and suitability across different types of water environments. The review also assessed the role of somatic coliphages as reliable indicators of faecal and viral contamination in water, comparing their effectiveness with traditional bacterial indicators like E. coli. Furthermore, the analysis considered the practical implementation of these detection methods in laboratory settings, with particular attention to compliance with regulatory standards, such as ISO 10705-2.

The following table shows the 14 articles selected to extract information on the methods used to detect coliphages in water. This table shows the title and authors of each study, as well as the year of publication and the main data of each study. In table 2, the 14 articles are divided into 3 categories according to their main subject:

Table 2: Main findings of the 14 selected articles, together with title, authors and year of publication, classified into 3 groups according to the main topic of each study.

Authors	Title	Year	Topic	Main Results
Bailey, E. S., et al.	E. coli CB390: An alternative E. coli host for simultaneous detection of somatic and F+ coliphage viruses in reclaimed and other waters	2017	Method optimisation and improvement	E. coli CB390 was validated as a reliable host for simultaneous detection of somatic and F+ coliphages, showing no significant differences compared to standard hosts.
Ballesté, E., et al.	Bacteriophages in Sewage: Abundance, Roles, and Applications	2022	Water quality indicators	Bacteriophages are abundant in sewage and have multiple applications, including serving as indicators for faecal contamination and for monitoring sewage treatment efficiency.
Blanch, A. R., et al.	Fast and Easy Methods for the Detection of Coliphages	2020	Methods and detection of coliphages	Developed simplified methods for coliphage detection, emphasizing ease of use and rapid processing, while maintaining sensitivity and accuracy.
Dias, E., et al.	The Application of Bacteriophages as Novel Indicators of Viral Pathogens in Wastewater Treatment Systems	2018	Water quality indicators	Phages were more reliable indicators of viral pathogen removal in wastewater treatment than traditional faecal indicators. SOMPH was the most effective phage indicator.
García-Aljaro, C., et al.	Surface Plasmon Resonance Assay for Real-Time Monitoring of Somatic Coliphages in Wastewaters	2008	Methods and detection of coliphages	SPR technique was effective for real-time detection of somatic coliphages in water, with a detection limit of about 102 PFU/ml.
Harwood, et al.	Validation of Human Viral Markers in Coastal Waters for Fecal Source Tracking	2013	Water quality indicators	Human viral markers were validated as effective tools for tracking fecal contamination in coastal waters, with specificities and sensitivities varying by region.
Helmi, et al.	Comparison of Two Filtration-Elution Procedures to Improve ISO Methods for Bacteriophage Detection in Water Samples	2011	Method optimisation and improvement	The study found that modified filtration-elution procedures enhanced the recovery of bacteriophages compared to standard ISO methods, particularly in surface waters.
Huang, et al.	Smartphone-Based in-Gel Loop-Mediated Isothermal Amplification (gLAMP) System Enables Rapid Coliphage MS2 Quantification in Environmental Waters	2018	Methods and detection of coliphages	The study introduced a smartphone-based gLAMP system for rapid coliphage MS2 quantification in water, delivering results in 30 minutes. The method showed a strong correlation with plaque assays and comparable sensitivity to RT-qPCR. It also demonstrated high tolerance to wastewater inhibitors.
McMinn, B. R., et al.	Comparison of Somatic and F+ Coliphage Enumeration Methods with Large Volume Surface Water Samples	2018	Methods and detection of coliphages	The D-HFUF-SAL method outperformed other methods for coliphage detection in large water volumes, offering higher sensitivity and lower non-detection rates.

Muniesa, M., et al.	Bluephage: A Rapid Method for Detecting Infectious Bacteriophages in Water Samples	2017	Methods and detection of coliphages	Bluephage was demonstrated as a rapid and effective method for detecting infectious bacteriophages in water, reducing detection time significantly.
Pascual-Benito M., et al.	Comparison of Methods for the Enumeration of Coliphages in 100 mL Water Samples	2022	Methods and detection of coliphages	Comparison of different coliphage enumeration methods in various water matrices, highlighting the strengths and weaknesses of each method.
Rames, E., et al.	Viral Indicators of Water Quality: A Review and Case Studies	2017	Water quality indicators	The review highlights the effectiveness of various viral indicators for assessing water quality and discusses case studies demonstrating their application.
Salter, R., et al.	Proposed Modifications of Environmental Protection Agency Method 1601 for Detection of Coliphages in Water	2010	Method optimisation and improvement	Proposed modifications to EPA Method 1601 improved the detection of coliphages, making it more effective for water quality monitoring.
Toribio-Avedillo, D., et al.	Simultaneous Detection of Somatic Coliphages and F-Specific RNA Coliphages as Indicators of Fecal Pollution	2020	Methods and detection of coliphages	Developed a method for simultaneous detection of somatic and F-specific RNA coliphages, improving efficiency in assessing faecal contamination in water.

The selected articles were grouped into 3 main topics:

- **Methods and detection of coliphages**: To explore the methods developed for the detection of coliphages in water samples.

- **Water quality indicators**: To evaluate the use of coliphages and other viruses as indicators of water quality and their relationship with faecal and viral pathogens.

- **Method optimisation and improvement**: Investigate innovations and seek further improvements in coliphage detection methods.

4.2. Double agar layer method

After carrying out the literature review, a suitable method for the detection of coliphages at the 'Laboratori Analític Valls' was chosen. The reasons for this choice are explained in the results and discussion section below.

The chosen method for the detection of somatic coliphages in water was the ISO-10705-2 standard method (International Standardisation Organisation, 2000), which uses the double agar layer (DAL) plaque test to quantify PFU. This method has a theoretical minimum detection limit of 5 plaque forming units (PFU) per 100 ml (El-Abagy et al., 1988; Samhan et al,.2016). The similarity between coliphages and many human enteric viruses supports their use as water quality indicators (Beaudoin et al., 2007; Samhan et al., 2016). Additionally, coliphages have been shown to indicate faecal pollution effectively, particularly in urban watershed areas (Dini & De Urraza, 2010; Samhan, et al., 2016).

Phages are quantified using direct plaque assays (Adams, 1959), which give results in plaque forming units (PFU). ISO standards also refer to these as plaque-forming particles (PFP). A PFU represents an entity, typically a single virion, but occasionally a cluster of virions that form a plaque.

The method, conditions and strain of E. coli were selected in accordance with the references consulted, as detailed in the results section. “Laboratori Analític Valls” did not carry out this analysis, so all the necessary reagents and materials had to be purchased.

This DAL is used to detect and count somatic coliphages in water intended for human consumption. In this case, 32 water samples from Valls and the surrounding area were analysed, so a total of 33 Petri dishes were required to conduct the test, comprising 32 for the samples and one for the positive control. It is based on mixing the sample with a semi-solid nutrient medium, to which a culture of the host soil is added and seeded on a solid nutrient medium. The plates are incubated and then examined.

The results are expressed as the number of plaque forming particles (pfu) per unit of sample volume.

4.2.1. Reagents and culture media

Different culture media were prepared to carry out this method:

- <u>Modified Scholten’s Broth (MSB):</u>

  The dehydrated medium was mixed with distilled water in accordance with the ratio of 28 g of MSB dehydrated to 1,000 ml of distilled water, as indicated in Table 3. Once combined, the liquid medium was subjected to autoclaving at 121°C for 15 minutes. Once prepared, the medium should be stored refrigerated at 5 ± 3 ºC for a maximum of one month.

- Modified Scholten's Agar (MSA):

  This medium is prepared by mixing dehydrated MSB with Bacto Agar, a solidifying agent in which extraneous matter, pigments and salts have been reduced to a minimum. The recommended ratio for preparing liquid MSA is 14 grams of dehydrated MSB and 6.8 g of Bacto Agar for 500 ml of distilled water, as outlined in Table 3. The components of MSA were mixed and then the medium was boiled. Once prepared, it is kept refrigerated at (5 ±3) ºC for a maximum of 8 weeks. The amounts for the preparation of the medium have been calculated for a volume of 500 ml, as this is the medium that is placed on the plates and each plate contains between 12-15ml.

- Calcium chloride solution (1M):

  The calcium chloride solution is prepared in a glass bottle following the proportions shown in table 3, mixing 14.6 ml of solid $CaCl_2$-$2H_2O$ with 100 ml of distilled water. Once it has been prepared, the whole solution is filtered and stored in a glass jar covered with silver paper in a dark place.

Once both the MSA and the calcium chloride solution have been prepared, the MSA is melted and left to cool at 45-50ºC. Then, 3,0 ml of calcium chloride solution are added per 500 ml of MSA.

- Semi-solid modified Scholten's agar (ssMSA):

  To prepare this medium, dehydrated MSB and Bacto Agar are used as for the liquid MSA, but in different proportions. For each 500 ml of distilled water, 14 g of dehydrated MSB and 3.4 g of Bacto Agar are added.

  A larger volume than necessary was prepared in order to be able to measure the quantities correctly with the granular scale. From this medium, the required amount was separated for mixing with the calcium chloride solution. For each 100 ml of ssMSA, 600 µl of calcium chloride solution were added.

Table 3: Summary of ratios for the preparation of each reagent or medium

MSB		**MSA (liquid)**			**$CaCl_2$-$2H_2O$**		**ssMSA**		
28,0 g MSB dehydrated	1,000 ml H_2O d	14,0 g MSB dehydrated	6.8 g Bacto Agar	500 ml H_2O d	14.6 ml solid $CaCl_2$-$2H_2O$	100 ml H_2O d	14,0 g MSB dehydrated	3.4 g Bacto Agar	500 ml H_2O d

4.2.2. Petri dishes preparation

The solid culture medium in the sterile 55 mm diameter Petri dishes is MSA medium with calcium chloride. The medium and calcium chloride solution are prepared according to Table x and x respectively. When the MSA medium has cooled to 45-50ºC, the calcium chloride solution is added according to the proportions indicated.

This mixture is then poured into Petri dishes and allowed to solidify. A total of 35 plates were prepared (2 left over).

4.2.3. Sample information

In the course of their activities, different companies, town councils or individuals need to analyse drinking water or waste water to detect the presence of coliphages. This is why "Laboratori Analític Valls" carries out this type of analysis. For this experiment, a total of 32 water samples were taken from Valls and the surrounding area.

Samples must be protected from heat and sunlight, therefore an isothermal cooler with frozen gel packs was used for transport. Samples may be stored at 5 ± 3 ºC for a maximum of 18 hours prior to analysis.

To check the reliability of the method and to see if contaminated samples could be detected, a plate was also incubated with a positive control.

4.2.4. Verification of method effectiveness

Before performing the procedure with the real samples, to check that the method was working, this assay was performed in the same way on different days using only the bacteriophage positive control vial. This sample contains bacteriophages, therefore, once the procedure is performed, plaque-forming units should grow in the petri dish with MSA medium.

These Positive Controls have a mean value of 61 PFU/ml with a lower and upper control limit of 40 PFU/ml and 80 PFU/ml respectively. They should be stored at -20°C in the freezer.

4.2.5. E. coli incubation

The E. coli sample has to be prepared before starting the analysis. The strain used was CECT 4177.

Two days before the start of the procedure, 2 inoculum pellets of E. coli strain were sown in the culture medium RAPID E. COLI. The plate was incubated at 36°C for 18 hours.

Figure 3: E. Coli plate incubated at 36ºC for 18h

The next day, multiple colonies were observed growing on the plate as shown in figure 3. Using a Kolle loop, 3 colonies were picked up and suspended in a plastic tube containing 50 ml of MSB medium. This tube containing E. coli was placed in an oven at 36°C for 18 hours.

4.2.6. Sample filtration

The following day, a membrane filtration equipment was prepared by sterilising and thoroughly flaming the various parts of the ramp. This filtration equipment consists of a vacuum pump connected to a pipe which is connected to a filtration chute with 3 stainless steel funnels.

A 0.45 µm porous cellulose membrane filter was placed on the porous plate of the apparatus, previously flamed and cold, and held with sterilised tweezers. 500 ml of each sample was then filtered.

32 plastic tubes, labelled with the number of the sample to be analysed, were prepared with 5 ml of MSB. The filters of each filtered water sample were cut into small pieces using sterile scissors and placed in the corresponding MSB tube. These tubes were placed in an ultrasonic bath for 10 minutes to mix the contents thoroughly.

4.2.7. MSAss test tubes

A 100 ml volume of MSAss was separated and mixed with 600 µl of calcium chloride solution in a water bath at 45°C to prevent the MSAss from solidifying. 2.5 ml of this mixture was placed in each of 33 glass tubes, also in a gradette in a bath. Then 1 ml of E. coli, which had been incubated in an oven at 36ºC since the day before, was added to each tube. Finally, 1 ml of the MSB tube containing the filter of each sample is added to each test tube containing MSAss and E. coli, except for the control tube to which 1 ml of a control+ vial is added.

The contents of the tubes were poured into the MSA plates (previously labelled with the sample number) so that each Petri dish had two layers: a lower layer of MSA medium and an upper layer of MSAss. These plates were incubated in an oven at 36ºC for 24 hours.

5. RESULTS AND DISCUSSION

5.1. Review and comparison of existing methodologies

Detection of coliphages, viruses that specifically infect E. coli, is essential for water quality monitoring as they are indicators of faecal contamination and potential viral pathogens. Several methods have been developed for the detection of coliphages, each with its own advantages and disadvantages. This review compares the most commonly used methods, such as the double agar layer (DAL) assay, single agar layer (SAL) assay, modified SAL/ISO method, enzymatic and non-agar-based methods, and USEPA methods 1642 and 1643, as well as other emerging methods.

Like all bacteriophages, coliphages can only reproduce within metabolising host cells. Infectious bacteriophages are typically identified by the effects they have on the host bacteria, especially lysis. Various strains of a bacterial species like E. coli, along with different assay media, result in differing counts and types of coliphages (Bailey, E. S., et al., 2017; Pascual-Benito M., et al.., 2022).

5.1.1. Standard methods

Standard methods for the detection of somatic coliphages are available from ISO (International Standardization Organization), USEPA (United States Environmental Protection Agency) and Standard Methods. However, there are also many alternative methods for detecting both somatic and F-specific coliphages.

The ISO-10705-2 standard method (International Standardization Organization, 2000) employs a technique known as the DAL plaque assay to quantify plaque-forming units (PFU). This standardization ensures consistency and reliability across laboratories, allowing for comparable results and compliance with water quality regulations. The standardized nature of the DAL method facilitates its use in various regulatory and research settings, making it a versatile and reliable choice for coliphage detection (Pascual-Benito et al., 2022). This method also incorporates a presence/absence test, that can be modified to a most probable number (MPN) format.

The DAL assay is a traditional and well-established method that involves layering a sample containing potential coliphages with a host bacterial strain in agar. The interaction between coliphages and host bacteria results in visible plaques that can be counted to quantify coliphage concentrations.

In this method, a sample is mixed with a host bacterial culture and molten top agar, which is then poured onto a solidified base agar layer. After incubation, the coliphages form clear zones or plaques where they have infected and lysed the host bacteria, allowing them to be enumerated (Grabow, 2001).

One of the primary advantages of the DAL method is its high sensitivity and specificity. The method's sensitivity stems from the clear visualization of plaques resulting from coliphage-induced lysis of host bacteria, making it a gold standard for accurately detecting coliphages in various water matrices (Garcia-Aljaro et al., 2008). Additionally, the DAL assay provides quantitative results in plaque-forming units (PFU), which are essential for regulatory compliance and research purposes (Bailey et al., 2017). Unlike some rapid detection methods that provide only qualitative data or relative estimations, the DAL method offers precise quantification, which is crucial in studies that require accurate measurements of coliphage concentrations.

Despite its advantages, the DAL method has some disadvantages. One of the main disadvantages is that it is time consuming, requiring 18 to 24 hours for plaques to develop, which can be a significant limitation when rapid results are required (Bailey et al., 2017). This long incubation period can delay decision making in scenarios where immediate assessment of water quality is critical. In addition, the DAL method is labour intensive. Preparation of double agar layers, and precise pipetting and plating steps require skilled personnel and careful attention to detail. This complexity may limit its practicality in laboratories that lack experienced staff or need to process a high volume of samples quickly (Garcia-Aljaro et al., 2008).

In the ISO-10705-2 presence/absence test, the sample is added to a nutrient broth containing a host bacterium. If coliphages are present, they will infect the bacteria, resulting in visible changes such as turbidity or colour change due to bacterial lysis. This method can also be adapted to a Most Probable Number (MPN) format, using multiple tubes and different sample volumes to estimate the concentration of coliphages based on statistical probability (Jofre et al., 2016).

The SAL assay is a simplified version of the DAL method. It uses a single layer of agar in which the host bacteria and sample are directly combined, allowing for faster and simpler plaque development. The main advantage of the SAL method is its relative simplicity and speed compared to the DAL method. This simplification reduces preparation time and the potential for error during the stratification process. It is particularly advantageous for laboratories requiring faster turnaround times for coliphage detection (Pascual-Benito et al, 2022).

The SAL method is particularly suitable for samples with low coliphage concentrations, such as clean water samples where coliphage levels are typically low. This method eliminates the need for a concentration step, saving time and reducing procedural complexity (Blanch et al., 2022).

However, the SAL assay has limitations. In samples with higher coliphage concentrations, it tends to underperform compared to DAL due to potential coliphage adsorption on the dish surface, which can result in lower plaque counts (Pascual-Benito et al, 2022). In addition, plaques formed by SAL can be more difficult to visualise and count, particularly in complex or turbid samples. This reduced sensitivity may limit its use in samples with high coliphage loads or in matrices that require highly accurate quantification.

The single agar layer (SAL) plaque assay and the double agar layer (DAL) plaque assay both aim to detect and quantify coliphages, but they differ in their methodologies (International Standardization Organization, 2000; USEPA, 2001b; Pascual-Benito M., et al., 2022; Helmi, et al.,2011):

- Layer Structure: The SAL (Single Agar Layer) involves a single layer of agar that contains both the host bacteria and the sample. The mixture is poured onto a solidified agar plate and incubated. Plaques form directly on this single layer. In contrast the technique DAL (Double Agar Layer) uses two layers of agar. The sample and host bacteria are mixed with molten top agar and poured over a solidified base agar layer. After incubation, plaques form on the top layer.
- Complexity: The SAL is generally simpler and quicker because it involves only one layer of agar, making it easier to prepare and handle and the double is more complex due to the requirement of two agar layers, which can provide a more stable environment for plaque formation but is more time-consuming to set up.
- Applications: The simple agar is suitable for scenarios where simplicity and speed are essential, and where resources for preparing multiple layers may be limited. On the other hand, DAL is used when more precise plaque formation is required, as the double layer can improve plaque visibility and separation.
- Plaque Visualisation: Plaques form directly on the single layer, which can sometimes lead to less distinct plaques due to the absence of a supportive base layer. In the DAL, the bottom agar layer supports the top agar layer, potentially resulting in more distinct and well-formed plaques.

The modified SAL/ISO method combines the SAL plaque assay with ISO bacterial host strains, aiming to combine the simplicity of SAL with the robustness of ISO standard methods. This approach offers several advantages, including the elimination of the concentration step required in some methods, thereby reducing procedural steps, time and the potential for sample contamination. The SAL/ISO method provides results comparable to the DAL assay in samples with less than 100 PFU/100 mL (Pascual-Benito et al., 2022). This makes it a viable option for coliphage analysis in clean water samples where coliphage concentrations are low. However, its performance can vary depending on coliphage concentration and sample type, which may affect its reliability in different matrices. Therefore, while the SAL/ISO method offers a simplified and efficient alternative for certain applications, it may not be universally applicable to all water types.

In contrast, the USEPA Method 1601 (USEPA, 2001a) is centred around a presence/absence test, which is a simpler and more straightforward approach to detecting specific contaminants. On the other hand, USEPA Method 1602 (USEPA, 2001b) uses the single agar layer (SAL) plaque assay. This method allows the testing of 10 mL of sample per plate using 150 mm diameter plates, making it suitable for detailed analysis of microbial presence in larger sample volumes.

The USEPA Method 1601 presence/absence test is designed to detect coliphages in water samples by determining whether they are present or absent. The method involves adding a water sample to a nutrient-rich medium containing a host bacterium (Fout et al., 2003). The results are observed in the same way as in the test described above in ISO-10705-2. If coliphages are present, changes in turbidity or colour of the medium are observed when the bacteria are infected. The USEPA Method 1602 single agar layer (SAL) plaque assay is used to detect and quantify coliphages. Water samples are mixed with a host bacteria and molten agar, and then poured onto a solidified agar plate. After incubation, coliphages infect the bacteria, forming clear zones or plaques where bacterial cells have been lysed. The number of plaques is counted to determine the concentration of coliphages in the sample, with each plaque representing one plaque-forming unit (PFU) (Salter, R., et al., 2010).

USEPA Methods 1642 and 1643 are advanced versions of EPA Methods 1601 and 1602 specifically designed for recreational and wastewater samples. These methods use ultrafiltration to concentrate large volumes of water prior to performing the SAL plaque assay. Their main advantage is the increased sensitivity they offer for detecting low coliphage concentrations in large volumes of water, making them particularly suitable for applications such as secondary wastewater and recreational waters (Pascual-Benito et al., 2022). However, these methods are more complex and time consuming due to the additional concentration steps required. The need for ultrafiltration equipment adds further complexity and may limit the practicality of these methods for routine use in many laboratories.

5.1.2. Other methods

Enzymatic and non-agar-based methods are emerging alternatives that rely on enzyme activity or other non-agar supports to detect coliphages. These methods represent a new approach to traditional agar-based techniques. One of their main advantages is the speed with which they provide results, often within a few hours. This rapid turnaround makes them ideal for real-time monitoring and rapid decision making (Pascual-Benito et al., 2022). In addition, these methods minimise the need for technical expertise, reducing the risk of cross-contamination and handling errors. However, they lack standardisation and require further validation in different water types and coliphage concentrations before they can be widely used in regulatory contexts. The accuracy and precision of these methods can vary with water type and sample matrix, limiting their current utility in standardised laboratory settings.

- **Enzymatic methods:**

Enzymatic methods for the detection of coliphages are generally based on the detection of enzymes released during phage-induced cell lysis. An example is the β-galactosidase method, first reported by Ijzerman et al. in 1993, which involves the induction of β-galactosidase in E. coli using isopropyl-β-D-1-thiogalactopyranoside, a compound that inactivates the lac repressor. When lysis occurs, β-galactosidase is released into the medium where it hydrolyses a yellow chromogenic substrate, which turns red if coliphages are present. This method is noted for its simplicity, cost-effectiveness and sensitivity, although it can produce false positives when phage numbers are low (Blanch et al., 2020).

Another enzymatic method involves the detection of adenylate kinase (AK) and adenosine 5′-triphosphate (ATP). Following phage-induced cell lysis, AK converts exogenous ADP to ATP, which is then detected by a bioluminescent assay using firefly luciferase. While this method can detect low numbers of phage in a relatively short time, it does not significantly improve the detection limits or speed of the β-galactosidase-based approach (Blanch et al., 2020).

These methods are useful in situations where sensitive, rapid and cost-effective detection of coliphages is required, such as water quality monitoring and assessment of contamination levels in various environmental samples. They can be used for presence/absence testing and can be adapted to the MPN format for quantification.

- **Bluephage method:**

One example of an enzymatic method is Bluephage. The Bluephage method was developed by a team led by Maite Muniesa and Juan Jofre at the University of Barcelona. The technique has been commercialised by Bluephage S.L., a company based in Barcelona, with the aim of providing a faster and more reliable method for detecting coliphages in water (Toribio-Avedillo D. et al., 2020).

The Bluephage method involves a genetically engineered strain of *Escherichia coli,* designated as CB10, which has been modified by knocking out two genes, *uidB* and *uidC,* that are responsible for the transport of glucuronic acid inside the cell, while overexpressing the *uidA* gene encoding β-glucuronidase. This modification ensures that the enzyme accumulates within the cells and is only released when cell lysis occurs due to the presence of coliphages. The assay uses a chromogenic substrate, 5-bromo-4-chloro-3-indolyl β-D-glucuronide, which turns from yellow to dark blue upon enzymatic action following cell lysis, allowing for a colorimetric detection of coliphages (Muniesa, M., et al., 2017; Toribio-Avedillo D. et al., 2020).

This method allows for both the presence/absence detection and quantification of coliphages through the Most Probable Number (MPN) format. It has a detection limit of one plaque-forming unit (PFU) per 100 mL, with results available within 3.5 hours, plus an additional 2.5 hours needed to prepare a lyophilized host strain (Muniesa, M., et al., 2017; Toribio-Avedillo D. et al., 2020).

Recent modifications of the Bluephage method, such as the introduction of the *E. coli* CB12 strain, enable the simultaneous detection of somatic and F-specific RNA coliphages. This strain includes an F plasmid encoding the pili necessary for F-specific coliphage infection, maintaining the sensitivity and effectiveness of the CB10 strain while expanding its detection capabilities (Toribio-Avedillo D. et al., 2020).

The method is suitable for use in various scenarios where rapid detection of faecal contamination is essential, such as recreational water quality monitoring, wastewater analysis, river water testing, and biosolids and shellfish testing. The method can be scaled up for large sample volumes or adapted for smaller sample sizes using microplates, making it a versatile tool for both field and laboratory applications (Muniesa, M., et al., 2017).

- **Molecular methods (PCR and RT-qPCR):**

The application of molecular techniques such as PCR to the detection of coliphages began in the 1990s. Since then, techniques have been refined, particularly for F-specific RNA coliphages, and methods such as RT-qPCR have been developed for microbial source tracking. Researchers such as Jofre and colleagues have contributed to the development and refinement of these molecular detection methods (Blanch et al., 2020).

These include nucleic acid amplification techniques, such as polymerase chain reaction (PCR), and serological detection methods that are applied directly to viral particles in a sample. However, these methods do not distinguish between infectious and non-infectious phage particles when applied directly. In addition, molecular techniques developed for specific phages may not be effective for detecting somatic or F-specific coliphages, as these groups encompass a broad and diverse range of bacteriophages (Blanch et al., 2020).

PCR-based methods, particularly reverse transcription quantitative PCR (RT-qPCR), have been developed to detect F-specific RNA coliphages and their different genogroups. These methods can be applied directly to determine the number of genome copies in a sample or to enriched cultures to improve sensitivity. The direct RT-qPCR test takes about 2 hours and has a detection limit of about 100 PFU per mL, while the enriched culture approach takes about 5 hours and can detect as little as 1 PFU per mL (Sala-Comorera, L., et al. 2024).

Molecular methods are particularly useful for microbial source tracing. For example, RT-qPCR techniques targeting F-specific RNA phages can help distinguish human from animal sources of faecal contamination by identifying specific serogroups. This is because different sero- or genogroups of these phages are typically associated with different sources: subgroups II and III are generally found in human faeces, while subgroups I and IV are associated with animal faeces (Blanch et al., 2020).

- **QuantiPhage (QP) Assay:**

The QuantiPhage Assay is a rapid colorimetric method for the detection of coliphages, developed by Emily Rames and Joanne Macdonald in 2018. This innovative assay uses cellulose pad materials instead of traditional agar, significantly reducing the detection time of coliphages in water samples, making it highly relevant for water quality monitoring.

This method replaces agar with cellulose pads to promote growth and detection of coliphages by colorimetric changes. This method detects somatic coliphages within 1.5 to 2 hours and F-specific coliphages within 2.5 to 3 hours. The assay uses a colour change (yellow to red/purple) caused by the enzymatic reaction of bacterial β-galactosidase released during coliphage-induced lysis to indicate the presence of coliphages.

It is ideal for assessing water quality in contexts where rapid results are required, such as after environmental contamination, in recreational water testing or in recycled water monitoring. It is particularly useful where timely decision making is critical, allowing faster responses compared to traditional plaque tests (Rames et al., 2018).

- **Surface Plasmon Resonance (SPR):**

The SPR technology has evolved significantly since its initial use for measuring protein-protein interactions. The adaptation of SPR for detecting bacteriophages and other pathogens is relatively recent, with substantial advances in the last few decades. Its application for detecting somatic coliphages represents a novel use of the technology, expanding beyond traditional biosensing applications such as hormone and pesticide detection.

The SPR Assay is a biosensor technology that can detect molecular interactions in real-time by measuring changes in the refractive index near a sensor surface. SPR works by coupling visible light into an electron field (plasmon wave) in a thin metal film, typically gold or silver. The resonance angle, where light maximally couples with the plasmon wave, changes when molecules bind to the sensor surface, allowing real-time monitoring of these interactions (García Aljaro et al., 2008).

SPR has been adapted to detect somatic coliphages, which are bacteriophages that infect *Escherichia coli*. García Aljaro et al. (2008) claimed that, in this approach, the host bacterium *E. coli* WG5 is immobilized on a gold sensor chip using an avidin-biotin interaction. The bacteriophages are then added, and their binding is monitored. The assay can detect bacteriophages at concentrations as low as 102 PFU/ml after an incubation period of about 120 minutes. This sensitivity is due to the observed structural changes in the bacteria upon interaction with the bacteriophages, such as cell collapse, which affects the mass density on the sensor chip. It is particularly useful for detecting somatic coliphages in wastewater.

5.1.3. Advantages and disadvantages

Table 4: Advantages and disadvantages of each mentioned methodology

METHOD	ADVANTAGES	DISADVANTAGES
DAL	- **High Sensitivity and Specificity**: The DAL method is highly sensitive and specific due to the clear visualization of plaques that result from coliphage infection and lysis of host bacteria. - **Quantitative and Reproducible**: It provides accurate quantitative results in the form of plaque-forming units (PFU), which is critical for regulatory compliance and research purposes. - **Standardization**: The method is widely standardized (e.g., ISO 10705-2, EPA Methods 1601/1602), making it the gold standard in coliphage detection for both research and regulatory contexts.	- **Time-Consuming**: Requires a long incubation period (18–24 hours) to allow for plaque development, which can be a drawback in situations requiring rapid results. - **Labor-Intensive**: The method involves multiple preparation steps, including the preparation of agar plates and careful layering, making it more labor-intensive than newer methods.
SAL	- **Faster and Easier to Perform**: The method is faster and simpler to set up compared to DAL, reducing the preparation time and complexity. - **Suitable for Low-Coliphage Concentration Samples**: It is particularly effective in clean water samples with low coliphage concentrations, avoiding the need for a concentration step	- **Lower Sensitivity in High-Concentration Samples**: In samples with higher coliphage concentrations, the SAL method tends to underperform compared to DAL due to potential coliphage adsorption on the dish surface. - **Less Accurate Plaque Counting**: Plaques formed in SAL can be more challenging to visualize and count, especially in complex or turbid samples

USEPA 1601 and 1602	- **High Sensitivity:** These methods allow the detection of low concentrations of coliphages - **Detailed analysis of microbial presence in larger sample volumes:** allows the testing of 10 mL of sample per plate using 150 mm diameter plates. - **Suitable for regulatory compliance**: accepted and standardised methods. - **Qualitative results**: qualitative data on coliphage presence.	- **Not quantitative results:** presence/absence test. If coliphages are present, changes in turbidity or colour of the medium are observed. - **Time-Consuming**: It requires long incubation periods (18 hours for enrichment and additional time for the spot plate test). This can delay obtaining results.
USEPA 1642 and 1643	**- Enhanced Sensitivity for Low-Coliphage Samples**: Suitable for detecting low coliphage concentrations in large water volumes, such as recreational and secondary wastewater.	- **Complex and Time-Consuming**: The requirement for ultrafiltration equipment and additional concentration steps makes these methods less practical for routine use in many labs.
Modified SAL/ISO	- **No Concentration Step Required:** Reduces procedural steps, time, and the potential for sample contamination. This is particularly advantageous for clean water samples where coliphage concentrations are low. - **Comparable Results to ISO Methods**: In samples with less than 100 PFU/100 mL, the results are comparable to those obtained by the DAL assay with ISO strain	- **Variable Results in Polluted Samples:** The performance can vary depending on the coliphage concentration and sample type, which might affect reliability in different matrices.
Enzymatic methods	- **Simplicity and Cost-Effectiveness**: These methods are easy to perform, do not require specialized equipment, and are relatively inexpensive. - **Rapid Results**: Results can typically be obtained within one working day.	- **Need for Confirmation Steps**: The results may be ambiguous and require additional steps to confirm the presence of phages. - **Potential for False Positives**: If the host culture undergoes spontaneous colour changes over extended incubation periods.
Bluephage	- **Speed**: It is the fastest available for detecting infectious somatic coliphages,	- **Potential False Positives in Highly Polluted Samples**: While rare, false

	with a total processing time of about 5.5 hours. - **Flexibility**: The method can handle different types of samples (e.g., water, seafood, sludge) and varying volumes, and it can be adapted for both qualitative and quantitative assays. - **Reduced False Positives**: Unlike other enzymatic detection methods, this method minimizes false positives by ensuring that the β-glucuronidase enzyme is not released until cell lysis occurs, thereby preventing premature substrate interaction. - **Ease of Use**: Requires only basic laboratory equipment.	positives may occur due to the presence of other bacteriolytic agents or bacterial strains that naturally produce glucuronidase in samples with high contamination levels. This can be mitigated through pre-filtration. - **Requires Preparation Time**: There is a need for 1.5–2 hours of preparation time to achieve the appropriate cell density before performing the test.
Molecular methods	- **High Sensitivity:** These methods allow the detection of low concentrations of coliphages - **High Specificity**: They target particular nucleic acid sequences or serogroups. - **Rapid Results**: Much faster than traditional culture-based methods. Direct RT-qPCR can deliver results in about 2h.	- **No Distinction Between Infectious and Non-infectious Phages**: A major drawback is that they do not differentiate between viable and non-viable phages. - **Complexity and Cost**: Specialized equipment, such as thermocyclers for PCR, and trained personnel to handle the processes, make them more complex and expensive than traditional methods.
QP Assay	- **Speed**: Detects coliphages in a shorter time compared to traditional methods. - **Simplicity**: Uses readily available materials like cellulose pads, making it easier and cheaper to implement. - **Versatility**: Can be used for both somatic and F-specific coliphages.	- **Specificity**: May not distinguish between different phage types as effectively as more sophisticated molecular methods. - **Adoption**: Being relatively new, it may not be widely recognized or accepted in all regulatory frameworks.
SPR Assay	- **Real-Time Detection**: SPR provides kinetic data on molecular interactions, which allows the monitoring of binding	- **Complex Setup**: The SPR assay requires sophisticated equipment and careful calibration, making it more complex than conventional methods.

	events as they occur, a feature that traditional methods cannot match. - **High Sensitivity**: The assay can detect low concentrations of bacteriophages. - **Specificity**: It relies on the selective interaction between the bacteriophage and its host bacteria, ensuring that the assay targets the intended pathogens. - **Versatility**: SPR can be adapted to detect other bacteriophages and pathogens by modifying the host used on the sensor.	- **Cost and Accessibility**: The need for specialized instrumentation and materials, such as gold-coated sensor chips, can make the technique expensive and less accessible. - **Sample Preparation**: Proper immobilization of the host bacteria can be time-consuming and technically demanding.

5.1.4. Selected method: DAL

Overall, the choice of method depends on the specific application, available resources and the required sensitivity and specificity for detecting coliphages in water samples. Each method offers a balance of speed, accuracy, cost and ease of use that must be weighed when selecting the appropriate detection strategy.

When compared to these methods, the DAL assay remains a strong candidate for use in analytical laboratories due to its high sensitivity, specificity and standardisation. Despite the time and labour requirements, the method's ability to provide accurate, quantitative results that are widely accepted and standardised makes it invaluable for regulatory compliance and research. The DAL method is particularly suitable for applications where accurate and reproducible results are critical, such as regulatory testing of drinking and recreational water, or in research settings where detailed analysis of coliphage presence is required. The quantitative capabilities of the DAL method, combined with its proven reliability, make it the most appropriate choice for a comprehensive coliphage detection strategy in an analytical laboratory.

In summary, the reasons why DAL was chosen to reproduce at the 'Laboratori Analític Valls' were as follows:

- <u>The availability of reagents and equipment:</u> At the 'Laboratori Analític Valls', due to the fact that similar assays are usually carried out, many of the materials and equipment required for this method were already available, allowing a quick start to the implementation.
- <u>Cost:</u> The DAL does not involve very high costs in terms of the purchase of reagents and culture media and, above all, very complex materials and equipment.

- High Accuracy and Quantitative Results: The DAL method provides precise quantification of coliphages by forming distinct plaques, making it ideal for compliance with regulatory standards and conducting research studies that require accurate data.
- Widely Standardized and Accepted: The DAL assay is recognized and standardized by major health and environmental organizations such as the ISO and USEPA. This standardization ensures consistency in results across different laboratories and studies.
- Applicability to a Wide Range of Sample Types: The DAL method is versatile and can be applied to various water matrices, from drinking water to wastewater, making it a flexible tool in an analytical laboratory's arsenal.
- Low Risk of False Positives: Due to its clear plaque formation and counting method, the DAL assay minimizes the risk of false positives, providing reliable data that can be confidently used for decision-making.

In conclusion, while several methods offer alternatives that may provide speed or simplified procedures, the DAL method remains the gold standard for coliphage detection in analytical laboratories. Its combination of sensitivity, specificity, quantitative capability, and standardization makes it the preferred method for reliable and accurate coliphage analysis in various water matrices. The conditions under which the DAL method is conducted are crucial for maximizing its effectiveness and ensuring high-quality data, making it the method of choice.

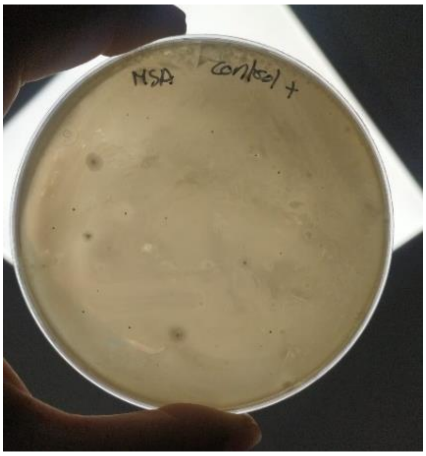
5.1.5. Optimal conditions for conducting DAL

To obtain optimal results with the DAL method, certain conditions must be met. The host bacterial strain must be highly susceptible to the coliphages to be detected, such as E. coli WG5 or Salmonella Typhimurium WG49, to ensure effective adsorption and plaque formation (Garcia-Aljaro et al., 2008). The growth medium should support robust bacterial growth and phage adsorption, such as modified Scholten's agar (MSA) for somatic coliphages and tryptone-yeast extract-glucose agar (TYGA) for F-specific coliphages. In addition, the incubation temperature should be maintained at 37°C, which is optimal for bacterial growth and phage activity, with an incubation time of at least 18-24 hours to allow sufficient plaque development for accurate enumeration (Pascual-Benito et al, 2022). Ensuring a controlled environment with sterile techniques and proper handling is also essential to minimise the risk of contamination and ensure reliable and reproducible results.

5.2. DAL effectiveness evaluation

After selecting the most appropriate method for the conditions of the “Laboratori Analític Valls”, an analytical determination of positive controls containing coliphages was carried out to ensure the reliability of the results.

The controls were first checked for correct growth. 3 positive controls were seeded before the drinking water samples were analysed. Two of them grew successfully, as observed in Figure 5, and one did not grow as expected, as shown in Figure 4.



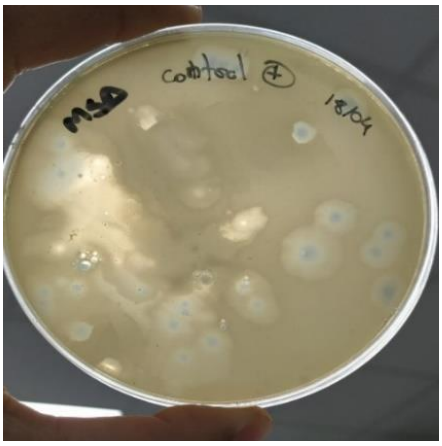


Figure 4: positive control not showing the expected growth

Figure 5: positive control with expected growth

This growth is the result of incubating the coliphages together with E. coli, where plaque-forming units can be observed. These plates can be read with the naked eye as the growth of halos around the E. coli bacteria is clearly visible.

The positive control plate tested on day 14/03 (PC1) shows a growth of 34 PFU as shown in figure x and the positive control on day 18/04 (PC2) counts 41 PFU. As each plate contains 1 ml of the control vial, the final concentration is 34 PFU/ml and 41 PFU/ml respectively.

On the other hand, a positive control was carried out on 29/04 (PC3) where the expected growth was not observed, as shown in Figure 6. This could be due to the fact that the incubation of E. coli was not successful. This was then verified by seeding 1 ml of MSB medium (where the 3 E. coli colonies were incubated at 36ºC) in a Petri dish with Rapid E. coli medium. After incubation of this plate, no significant bacterial growth was observed. This means that there were not enough E. coli bacteria and therefore no plaque-forming units were growing.

As the controls were successful on two of the three occasions and the reason for the failure on the third was known, the water samples were analysed and a further control (PC4) was carried out at the same time to ensure the validity of the results. The growth of the positive control in this case was 47 PFU/ml, therefore the other samples were considered valid.

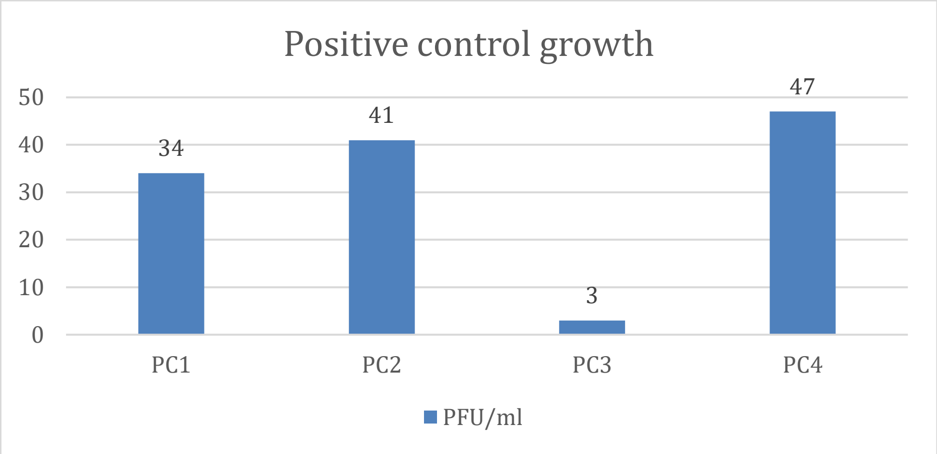


Figure 6: growth of the positive controls carried out before the analysis of the samples.

5.3. Application of the method to water samples

After verifying the efficacy of the method by analysing the positive controls, the samples could be analysed. The DAL method was carried out, and after the appropriate incubation period, it was observed that no coliphage PFU growth was observed in 31 of the 32 Petri dishes, as shown in Figure 7.

This result was to be expected as most of the water samples were intended for human consumption and if the presence of these bacteriophages had been detected, the treatment of these samples would have to be reviewed and the customer or company would have to be alerted.

Figure 7: Examples of plates where coliphages are not present

In the one sample where the presence of coliphages was detected, the growth of 23 plate forming units (PFU), as shown in Figure 8. This water sample was waste water and the colour and odour of the water indicated that it might be contaminated.

Therefore, we can consider the presence of coliphages as an indication of viral or faecal contamination of the water.

Figure 8: contaminated sample

6. CONCLUSION

Based on the results of this study, which included both a comprehensive literature review and the experimental validation of a detection method at the "Laboratori Analític Valls", several important conclusions can be drawn. The work highlights the importance of using coliphages as reliable indicators of water contamination, especially in cases where traditional bacterial indicators such as E. coli do not consistently correlate with the presence of viral pathogens. This study provides a dual contribution: a theoretical review of existing methods and their effectiveness, and a practical application of the double agar layer (DAL) method, highlighting its potential for widespread application in water quality monitoring.

The primary findings from both the literature review and the experimental component underscore that somatic coliphages, due to their structural and behavioural similarities to enteric viruses, provide a more reliable indication of viral contamination in water than traditional bacterial indicators. The literature supports that coliphages are more resistant to environmental stresses and water treatment processes, making them particularly useful for water quality monitoring (Gerba & Rose, 1990; Grabow, 2001). The DAL method, as implemented in the study carried out at Laboratori Analític Valls, was validated as a sensitive and specific technique for the detection of somatic coliphages in water samples. The ability of this method to detect low concentrations of coliphages is consistent with previous studies suggesting that coliphages serve as effective surrogates for human enteric viruses. The experimental results indicated that most water samples did not show the presence of coliphages, suggesting effective treatment processes or the absence of significant faecal contamination. However, the detection of coliphages in one sample highlighted the need for continuous monitoring using robust methods such as DAL.

The results of the literature review are consistent with studies that support the use of coliphages, particularly somatic coliphages, as indicators of faecal contamination. For example, studies by Jofre et al. (2016) and Ballesté et al. (2022) highlight the reliability of coliphages in assessing water quality, particularly in settings where faecal pollution is suspected. The ability of somatic coliphages to replicate within host bacteria and survive longer in environmental conditions than many human-associated enteric viruses makes them ideal indicators. In addition, the review highlighted that alternative methods such as Bluephage assay and filtration-elution techniques can also provide accurate results, although they may not offer the same sensitivity or practicality as DAL in all scenarios (Salter et al., 2010; Helmi et al., 2011). The integration of these methods, combined with the results of experimental validation, strengthens the argument for wider adoption of coliphages as viral indicators.

The practical implications of these findings for coliphage research are significant. The study reinforces the idea that incorporating coliphage detection, particularly using optimised methods such as DAL, into regular water quality monitoring protocols can significantly improve the detection of potential viral pathogens. This approach is particularly valuable in settings where human health is at risk from waterborne diseases. The use of coliphages as viral indicators is consistent with broader regulatory trends observed in Europe and elsewhere, where the limitations of traditional bacterial indicators are increasingly recognised (WHO, 2017; ISO, 2001). The study also suggests that further refinement and standardisation of coliphage detection methods, including DAL and other emerging techniques, could provide even more reliable tools for water quality management and public health protection.

However, the study is not without its limitations. One of the main challenges identified was the occasional inconsistency in the growth of the E. coli used in the DAL method, which could potentially affect the reliability of the results. For example, one of the positive controls failed due to insufficient E. coli growth, highlighting the need for strict quality control in the preparation and handling of bacterial cultures. Future research could focus on optimising bacterial culture conditions and reagent preparation to minimise such problems. In addition, while this study focused primarily on somatic coliphages, further research is already exploring the simultaneous detection of F-specific coliphages and other viral indicators to provide a more comprehensive assessment of water quality. Research by Toribio-Avedillo et al. (2020) has demonstrated the potential for methods that simultaneously detect multiple types of coliphages, which could provide enhanced detection capabilities in different water environments.

Another limitation is the geographical and sampling scope of the study. The samples analysed were limited to the Valls and surrounding area, which may not represent wider environmental conditions or the diversity of potential contaminants found in other regions. Future studies could expand the geographical scope of sampling to include different environments, such as coastal, urban and agricultural areas, to better understand the effectiveness and limitations of coliphage detection methods in different contexts. Furthermore, the study's reliance on a single detection method, while effective, suggests that combining multiple detection approaches may provide a more robust framework for water quality monitoring.

In conclusion, this study, through its comprehensive literature review and experimental validation, provides valuable insights into the use of somatic coliphages as indicators of viral and faecal contamination in water. It validates the effectiveness of the DAL method for this purpose and supports the wider use of coliphages in water quality monitoring. The results are consistent with recent regulatory trends and highlight the need for ongoing research to refine detection methods. By addressing the identified limitations and exploring new lines of research, the scientific community can develop even more effective tools to ensure water safety and public health protection.

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