Universitat Autònoma de Barcelona

# THE USE OF BACTERIOPHAGES FOR THE DETECTION OF BACTERIA IN ENVIRONMENTAL WATER SAMPLES

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Doctoral Thesis in Microbiology

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Aproval of the thesis supervisor,

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To my parents

“Nothing in life is to be feared, it´s only to be understood. Now is the time to understand more, so that we may fear less.“

Marie Curie

# Contents

**Non-standard abbreviations**

| | |
|---|---|
| ACT | Acetaminophen |
| AgNPs | Silver Nano Particles |
| ASs | Artificial Sweeteners |
| ALP | Alkaline phosphatase |
| AuNPs | Gold Nano Particles |
| BNG | 6-Bromo-2-Naphtyl-ß-d-Galactopyranoside |
| CBZ | Carbamazepine |
| CPR | Chlorophenol Red |
| CPRG | Chlorophenol Red ß-D-Galactopyranoside |
| CS | Conductometric Sensing |
| DAS1 | Diaminostilbene |
| DSBP | Distyrylbiphenyl |
| DTT | Dithiothreitol |
| EDTA | Ethylene Diaminetetra Acetic Acid |
| ELISA | Enzyme-Linked Immuno Sorbent Assay |
| ETEC | Entro Toxigenic *E. coli* |
| FDG | Fluorescein Di-ß-d-Galactopyranoside |
| FISH | Fluorescent In Situ Hybridization |
| FWA | Fluorescent Whitening Agents |
| GBPs-ALP | Gold Binding Peptides-Alkaline Posphate |
| GOX | Glucose Oxidase |
| HPC | Heterotrophic Plate Counts |
| HRP | Horseradish Peroxidase |
| IFA | Immuno Fluorescence Assay |
| IMS | Immuno Magnetic Separation |
| IPTG | Isopropil-ß-D-1-tiogalactopiranosidae |
| LB | Luria-Bertani |
| LuGal | D-Luciferin-O-ß-Galactopyranoside |
| MAS | Magnetic Analyte Separation |
| MB-PCR | Molecular Beacons-based PCR |
| MEA | Mercapto Ethyl Amine |
| MOI | Multiplicity of Infection |
| MPN | Most Probable Number |

| | |
|---|---|
| MUG | 4-Methylumbelliferyl-ß-d-Galactopyranoside |
| MU-Glu | 4-Methylumbelliferyl-ß-d-Glucuronide |
| $Na_2CO_3$ | Sodium Carbonate |
| NASBA | Nucleic Acid Sequence-Based Amplification |
| ONP | 2-Nitrophenol |
| ONPG | 2-Nitrophenol ß-d-Galactopyranoside |
| PAP | P-Aminophenol |
| PAPG | 4-Aminophenyl-ß-d-Galactopyranoside |
| PBS | Phosphate-Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PG | Phenyl-ß-d-Galactopyranoside |
| pH | Potential of Hydrogen |
| PNPG | P-Nitrophenol-ß-d-Galactopyranoside |
| PPCPs | Pharmaceuticals and Personal Care Products |
| QCM | Quartz Crystal Microbalance |
| q-PCR | quantitive Polymerase Chain Reaction |
| qRT-PCR | quantitative Reverse Transcriptase PCR |
| RFU | Relative Fluorescence Units |
| RGP | Resorufin-ß-d-Galactopyranoside |
| R2A | Reasoners´s 2A agar |
| SA | Salicylic Acid |
| SAW | Surface-Acoustic Wave |
| SDS | Sodium Dodecyl Sulfate |
| SELEX | Systematic Evolution of Ligands by Exponential Enrichment |
| SSs | Sterols and Stanols |
| TFOBS | Tapered Fiber Optical Biosensors |
| TMB-$H_2O_2$ | Tetramethyl Benzidin Hydrogen Peroxide |
| UNESCO | United Nations Educational, Scientific and Cultural Organization |
| X-GAL | 5-bromo-3-indonlyl-ß-d-Galactopyranoside |
| VBNC | Viable But Non Culturable |

**Units**

| | |
|---|---|
| $^{o}C$ | degrees Celsius |
| cfu | colony forming units |
| g | gram |
| min | minutes |
| mL | mililiters |
| M | molar |
| mM | milli Molar |
| h | hours |

## Summary

According to WHO (World Health Organization) fecal contamination of drinking water affects more than 2 billion people worldwide and results in more than 500.000 deaths every year. Current approaches for microbiological water quality monitoring rely heavily on the use of true and tested culture-based methods that provide good results but require long incubation times. The growing trend in this field has been the progressive introduction of alternative fast methods that can provide results in a fraction of the time, allowing for a more agile management of waterborne hazards. These methods include nucleic acid-based approaches (PCR, qPCR), antibody or aptamer-based reactions (ELISA) or specific enzyme assays (ATPase, ß-galactosidase, ß-glucuronidase). Although these methods provide remarkably good result, they are often plagued by a host of problems. In most cases the methods require expensive reagents, the use of sophisticated equipment, or the participation of highly skilled personnel. Therefore, the quest for a simple, fast and specific method for the detection of *E. coli* in water samples remains open. This thesis tries to contribute to this quest by developing an enzyme-based method that uses an optimized nonspecific ß-galactosidase assay to provide specific detection of *E. coli* through the use of a T4 phage.

Optimizing the ß-galactosidase assay to achieve maximum sensitivity has required the determination of optimal values for the main parameters affecting its performance. The results provide a rough estimate of how departure from optimal values reduces the output of the assay potentially decreasing its sensitivity. The optimized assay requires induction of the samples using 0.2 mM IPTG during 180 minutes. Permeabilization of the samples is mandatory as lack of it results in an almost 60% reduction in assay output. The choice of enzyme substrate is critical as different substrates yield products with different extinction coefficients or fluorescence yields. The concentration of substrate used must be high enough (around 3 to 4 times $K_m$) to ensure that the activity measured is not substrate-limited. Finally, as the color/fluorescence of the reaction products is highly dependent on pH, care must be taken to ensure that pH at the time of reading is high enough to provide maximum signal.

The assay is performed in 96 well plates, uses MUG (4-methylumbelliferyl-ß-D-galactopyranoside) as the enzyme substrate and has a total length of 90 minutes. The method is able to detect 75 cells of *E. coli*. Under the conditions of the assay this corresponds to a concentration of $1.49 \cdot 10^3$ cells·$mL^{-1}$ of sample. For the analysis of field samples, we produced an extended version of the assay that incorporates preconcentration and preincubation steps with a total running length of 7.5 hours. When tested with field samples and compared with Colilert-18, the extended method performed well with a limit of detection of 96 cells·100 $mL^{-1}$. The assay is robust and has the potential to be further modified and adapted to user friendly formats such as a paper-based assay.

## Resumen

Según la OMS (Organización Mundial de la Salud), la contaminación fecal de agua potable afecta a más de 2.000 millones de personas en todo el mundo y provoca más de 500.000 muertes cada año. La estrategia actual para el control de la calidad microbiológica del agua dependen en gran medida del uso de métodos de cultivo bien validados que proporcionan buenos resultados, pero que requieren tiempos largos de incubación. La tendencia en este campo ha sido la introducción progresiva de métodos alternativos rápidos que proporcionan resultados en una fracción del tiempo y permiten una gestión más ágil de los riesgos asociados al agua de consumo. Estos métodos incluyen aproximaciones basadas en la utilización de ácidos nucleicos (PCR, qPCR), reacciones basadas en anticuerpos o aptámeros (ELISA) o ensayos enzimáticos específicos (ATPasa, ß-galactosidasa, ß-glucuronidasa). Aunque estos métodos ofrecen un resultado bastante bueno, a menudo sufren de una serie de inconvenientes. En la mayoría de los casos, los métodos requieren reactivos caros, el uso de equipos sofisticados o la participación de personal altamente cualificado. Por lo tanto, la búsqueda de un método sencillo, rápido y específico para la detección de *E. coli* en muestras de agua continúa abierta. Esta tesis intenta contribuir a esta búsqueda desarrollando un método basado en enzimas que utiliza un ensayo inespecífico para β-galactosidasa optimizado para proporcionar una detección específica de *E. coli* mediante el uso de un bacteriófago T4.

La optimización del ensayo de ß-galactosidasa para conseguir la máxima sensibilidad ha requerido la determinación de valores óptimos para los principales parámetros que afectan su rendimiento. Los resultados proporcionan una estima aproximada de cómo la desviación de los valores óptimos reduce el rendimiento del ensayo y disminuye su sensibilidad. El ensayo optimizado requiere la inducción de las muestras mediante IPTG 0.2 mM durante 180 minutos. La permeabilización de las muestras es necesaria, ya que su ausencia provoca una reducción de casi el 60% en el rendimiento del ensayo. La elección del sustrato enzimático es fundamental ya que diferentes sustratos producen productos con diferentes coeficientes de extinción o rendimientos de fluorescencia. La concentración del sustrato utilizado debe ser lo suficientemente elevada (alrededor de 3 a 4 veces Km) para garantizar que

la actividad medida no esté limitada por el sustrato. Finalmente, dado que el color / fluorescencia de los productos de reacción depende mucho del pH, se debe procurar que el pH en el momento de la lectura sea suficientemente elevado como para proporcionar la máxima señal.

El ensayo se realiza en placas de 96 pozos, utiliza MUG (4-metilumbeliferil-ß-D-galactopiranosido) como sustrato enzimático y tiene una duración total de 90 minutos. El método es capaz de detectar 75 células de *E. coli*. En las condiciones del ensayo, esto corresponde a una concentración de $1.49 \cdot 10^3$ células$\cdot mL^{-1}$ de muestra. Para el análisis de muestras de campo se desarrolló una versión ampliada del ensayo que incorpora pasos de preconcentración y preincubación con una duración total de 7.5 horas. Cuando se probó con muestras de campo y se comparó con Colilert-18, el método ampliado tuvo un buen rendimiento con un límite de detección de 96 células$\cdot 100\ mL^{-1}$. El ensayo es robusto y se puede modificar por adaptarlo a formatos más fáciles de utilizar, como un ensayo en papel.

## Resum

Segons l'OMS (Organització Mundial de la Salut), la contaminació fecal d'aigua potable afecta més de 2.000 milions de persones a tot el món i provoca més de 500.000 morts cada any. L'estratègia actual per al control de la qualitat microbiològica de l'aigua depenen en gran mesura de l'ús de mètodes de cultiu ben validats que proporcionen bons resultats, però que requereixen temps llargs d'incubació. La tendència en aquest camp ha estat la introducció progressiva de mètodes alternatius ràpids que proporcionen resultats en una fracció del temps i permeten una gestió més àgil dels riscs associats a l'aigua de consum. Aquests mètodes inclouen aproximacions basades en la utilització d'àcids nucleics (PCR, qPCR), reaccions basades en anticossos o aptàmers (ELISA) o assajos enzimàtics específics (ATPasa, ß-galactosidasa, ß-glucuronidasa). Tot i que aquests mètodes ofereixen un resultat força bo, sovint pateixen d'una sèrie d'inconvenients. En la majoria dels casos, els mètodes requereixen reactius cars, l'ús d'equips sofisticats o la participació de personal altament qualificat. Per tant, la cerca d'un mètode senzill, ràpid i específic per a la detecció d'*E. coli* en mostres d'aigua continua oberta. Aquesta tesi intenta contribuir a aquesta cerca desenvolupant un mètode basat en enzims que utilitza un assaig inespecífic per β-galactosidasa optimitzat per proporcionar una detecció específica d'*E. coli* mitjançant l'ús d'un bacteriòfag T4.

L'optimització de l'assaig de ß-galactosidasa per aconseguir la màxima sensibilitat ha requerit la determinació de valors òptims per als principals paràmetres que afecten el seu rendiment. Els resultats proporcionen una estima aproximada de com la desviació dels valors òptims redueix el rendiment de l'assaig i disminueix la seva sensibilitat. L'assaig optimitzat requereix la inducció de les mostres mitjançant IPTG 0.2 mM durant 180 minuts. La permeabilització de les mostres és necessària, ja que la seva absència provoca una reducció de gairebé el 60% en el rendiment de l'assaig. L'elecció del substrat enzimàtic és fonamental ja que diferents substrats produeixen productes amb diferents coeficients d'extinció o rendiments de fluorescència. La concentració del substrat utilitzat ha de ser prou elevada (al voltant de 3 a 4 vegades Km) per garantir que l'activitat mesurada no estigui limitada pel substrat. Finalment, com que el color / fluorescència dels productes de reacció

depèn molt del pH, s'ha de procurar que el pH en el moment de la lectura sigui prou elevat per proporcionar la màxima senyal.

L'assaig es realitza en plaques de 96 pous, utilitza MUG (4-metilumbeliferil-ß-D-galactopiranosid) com a substrat enzimàtic i té una durada total de 90 minuts. El mètode és capaç de detectar 75 cèl·lules d'*E. coli*. En les condicions de l'assaig, això correspon a una concentració d'$1.49 \cdot 10^3$ cèl·lules·$mL^{-1}$ de mostra. Per a l'anàlisi de mostres de camp es va desenvolupar una versió ampliada de l'assaig que incorpora passos de preconcentració i preincubació amb una durada total de 7.5 hores. Quan es va provar amb mostres de camp i es va comparar amb Colilert-18, el mètode ampliat va tenir un bon rendiment amb un límit de detecció de 96 cèl·lules·100 $mL^{-1}$. L'assaig és robust i es pot modificar per adaptar-lo a formats més fàcils d'utilitzar, com ara un assaig en paper.

# CHAPTER 1

# INTRODUCTION

# 1 INTRODUCTION

## 1.1 Importance of microorganism detection in public health

One of the main challenges for water managers and environmental scientists is the contamination of water supplies and water distribution networks by pathogenic microorganisms. In 2015, 2.1 billion people did not have access to safely managed drinking water services, roughly 29% of the global human population (UNESCO World Water Assessment Programme 2019). The same year, the World Health Organization (WHO) reported that at least 2 billion people used drinking water sources contaminated with feces (WHO 2019 Drinking Water Fact Sheets), mainly due to the lack of facilities to ensure the quality of their water supply. This lack of adequate water supply results in 3.5 million people dying each year due to water-borne diseases, many of them children (Prüss-Üstün et al 2008). In the same line, in 2014, UNICEF (United Nation Children's Fund) estimated that 4000 children die each day as a consequence of consuming contaminated water (UNICEF 2019).

The agents responsible for waterborne diseases belong to a diverse constellation of bacteria, viruses and protozoa (WHO 2011). Monitoring each of the possible organisms contributing to sanitary risk is not feasible and therefore a number of approaches have been developed to assess water quality and detect the existence of sanitary risk. These approaches involved quantification of "indicators", this is biological or chemical agents that are typically released with feces of human or other warm-blooded animals and that, although not necessarily harmful by themselves, can indicate the possible presence of pathogens released alongside them. In this category belong organisms such as *Escherichia coli*, thermotolerant coliforms such as *Citrobacter*, *Klebsiella* and *Enterobacter*, total coliforms, heterotrophic bacteria, *Clostridium perfringens*, coliphages, phages of *Bacteroides fragilis*, selected enteric viruses or a number of recently described chemical compounds (Lim et al 2017).

Probably the most widely used indicator organisms for fecal water pollution is *E. coli*. The use of this organism, however, is not free of controversy. The presence of *E. coli* evidences the existence of fecal contamination in drinking water. However, this doesn't mean that in the absence of *E. coli,* water is free from possible pathogens. Enteric viruses and protozoa are more resistant to disinfection (Horan 2003, Medema et al 2003) and might survive when *E. coli* is no longer detectable. Also,

under certain circumstances bacteriophages of fecal bacteria and bacterial endospores can survive in water under conditions in which *E. coli* cannot, thus providing better estimates for the risk posed by viruses or sporulated pathogens. These microorganisms could be usable as alternative indicators for detection of infectious organisms in water system.

## 1.2 Water quality indicators

As mentioned above, water quality indicators are microbiological or chemical agents that, although not harmful by themselves, are usually released to the medium by sources susceptible to release pathogenic organisms. These indicators can therefore be used as surrogates to assess the possible presence of these pathogens. An ideal fecal contamination indicator should have the following features (Bonde, 1962., World Health Organization 1993, Grabow 1996, Godfree et al, 1997, Horan 2003):

1) Released in large amounts by the potential sources of pathogenic organisms.
2) Non-hazardous/non-pathogenic.
3) Suitable for all categories of water.
4) Present and detectable whenever pathogens are present.
5) Easy to detect using fast and, preferably, low-cost methods.
6) Persistence in the environment must be equal or better that persistence of pathogens.
7) It can only originate in the pathogen source.
8) It cannot multiply in water.

The group of microorganisms that has been most extensively used by water quality managers as suitable indicators for detecting water pollution, have been coliform bacteria, more specifically, *E. coli*. Nevertheless, alternative microbial and chemical markers have also been proposed during the years that can be used as indicators for water and wastewater treatment. Most of them present advantages and disadvantages when compared with coliforms. They are discussed in the following sections.

### 1.2.1 Bacterial indicators

Early after its initial description by Theodor Escherich in 1885, *Bacillus coli*, promptly renamed as *Escherichia coli,* was identified as a fecal pollution marker. Ever since, research in microbial water quality monitoring has been an expanding field (Griffin et

al 2001). Because *Escherichia coli* was difficult to differentiate from other enteric bacteria such as *Klebsiella*, *Enterobacter* or *Citrobacter*, the term "coliforms" was coined to include all these microorganisms. The first standard description of methods for microbial quality assessment based on microbial indicators was published in 1905 by the American Public Health Association in their "Standard Methods for the Examination of Water and Wastewater". In these methods, Enterococci and *Clostridium perfringens* were used alongside coliform bacteria as water quality indicators (Ashbolt et al 2001, Hutchinson and Ridgway 1977). Since then until now, the set of methods available for microbial water quality monitoring has been steadily expanding and, although there is no universal agreement about which is the best indicator, their combined use provides a solid basis for the assessment of microbiological risks in different types of water (Noble et al 2003).

#### *1.2.1.1* Coliform bacteria and *E. coli*

Coliform bacteria are rod-shaped, Gram-negative microorganisms able to ferment lactose with production of acid and gas at 35 $^{o}$C in a period of 48 h. and including fecal and non-fecal coliforms. Fecal coliforms bacteria are growing at 44$^{o}$C. Because many coliforms were of environmental origin and did not correlate well with the existence of water pollution, the term "fecal coliform" was introduced referring to a subset of coliforms that was able to grow and ferment lactose at a temperature of 44.5 $^{o}$C and produce indole from tryptophan (American Public Health Association, 1998). The concept has been later modified and now fecal coliforms are defined as member of the family Enterobacteriaceae that possess the gene encoding for ß-galactosidase (Horan 2003). As defined, the group includes members of the genera *Enterobacter*, *Citrobacter, Klebsiella, Escherichia, Yersinia, Serratia* and *Hafnia*.

*Escherichia coli* is an abundant component in the gut microbiota of humans and animals where it plays a commensal role. A limited number of *E. coli* strains, that can be classified according to their virulence factors, can cause diverse pathologies, diarrheal disease, urinary tract infections (UTI), and even sepsis. (Pearson et al 2016, Tandogdu and Wagenlehner 2016, Vila et al 2016). But it is it's high abundance in human feces that has made this organism the preferred choice as a reliable, inexpensive and rapid indicator for the assessment of microbiological hazards in water quality management (Edberg et al 2000, Yates 2007).

#### 1.2.1.2 Fecal Enterococci

This group of microorganisms includes some members of genera *Enterococcus,* and *Streptococcus* which are inhabitants of the intestinal tract in humans and animals. They are considered as suitable indicators as they outnumber bacterial and viral pathogens in wastewater and do not multiply in environmental waters. The fact that they are not totally specific to humans but can be also found in farm animals (cattle and pigs) reduces their utility as indicators of human fecal pollution but, on the other hand, the fact that they are more resistant to disinfection and have a higher survival in the environment than fecal coliforms constitutes a bonus, as they provide a warning on the possible malfunction of water disinfection facilities (Godfree et al, 1997, Gerba 2015).

#### *1.2.1.3 Clostridium perfringens*

*Clostridium perfringens* is a Gram-positive, anaerobic, spore-forming sulfite-reducing bacterium, present in humans and animal feces. The ability to sporulate allows this organism to survive for long periods of time, indicating the existence of fecal pollution long after coliforms or enterococci died off. The resistance of its spores to toxic compounds means that this bacterium can survive in industrial wastewater in conditions in which fecal coliforms or enterococci cannot, therefore making it a good candidate as a microbial indicator in industrial wastewater (Horan 2003).

#### 1.2.1.4 Heterotrophic bacteria

The term heterotrophic bacteria is customarily used to refer to all bacteria present in water that use organic compounds as a carbon and energy source. Heterotrophic bacteria are usually determined through viable counts in agar plates containing a suitable culture medium such as R2A (Reasoner and Geldritch 1985) and the results obtained are referred to as Heterotrophic Plate Counts or HPC. Values of HPC are extremely dependent on the type of carbon source used, the concentration of nutrients, the temperature of incubation, the length of the incubation or even the method used to inoculate the plates (pour plate, spread plate or membrane filtration) (Allen et al 2004). As a rule, no single method can recover and count all heterotrophic bacteria present in the sample an it is now an accepted fact that the information provided by HPC must be interpreted in the context of all these variables. Also, the value of HPC for the assessment of microbial hazards in different types of

water has been questioned as there seems to be no correlation between high HPC values and increased health risk (Allen et al 2004). In spite of this, heterotrophic plate counts are often included as a convenient control variable, as it provides interesting information that can be used to assess the possible interference of high levels of heterotrophs in the quantification of conventional fecal indicators. In addition to this, HPC values provide information about the efficiency of water disinfection, the existence of bacterial regrowth in drinking water distribution networks, the possible presence of biofilms shedding planktonic bacteria in water distribution systems, or just the existence of conditions suitable for active microbial growth and survival (Reasoner 1990).

#### 1.2.1.5 Bacteriophages (coliphages)

One of the main concerns when managing microbial water quality is the use of proper methods to assess the risk of waterborne viral diseases. From the point of view of survival in different types of natural waters, conventional fecal indicators do not necessarily mimic the survival of pathogenic viruses. Actually, in a study carried out in Santa Monica Bay, Noble and Fuhrman (2001) were unable to establish a good correlation between Enteroviruses detected by PCR and the levels or fecal indicators. In an attempt to find a better surrogate for the behavior of pathogenic viruses, bacterial viruses have been proposed as indicators. Interest in this field has centered mainly in coliphages and in phages from *Bacteroides*. Coliphages are phages that are highly specific for *Escherichia coli*. They are 4 to 5 order of magnitude more abundant than infectious enteric viruses (McMinn et al 2017) and have similar survival characteristics in water (Payment and Locas 2011). Moreover, coliphages can be quantified using relatively simple and inexpensive methods.

Coliphages are classified into somatic coliphages with receptors in the surface of the outer membrane, and F-specific coliphages that target the F pili. Both types of coliphages have been used to assess fecal pollution in different types of water (Leclerc et al 2000, García-Aljaro et al 2018), however, F-specific phages seem to provide an extra edge as their morphological characteristics allow them to better mimic the behavior of enteroviruses during water treatment (Havelaar and Nieuwstad 1985, Havelaar and Pot-Hogeboom 1988, Grabow 2001).

The other group of phages that has emerged as an important indicator of fecal water contaminations are *Bacteroides* phages. *Bacteroides* are anaerobic bacteria that are

found in much higher numbers than coliforms in the human and animal gut but that are readily inactivated when exposed to oxygen in the environment (Salyers 1984). *Bacteroides* phages are highly resistant to disinfectants and water treatments and due to their size and composition, mimic quite well the fate of enteroviruses, both in the environment and in water treatment facilities. At the moment, their use as tools for microbial source tracking is restricted to phages of some strains clearly associated to human fecal pollution, as phage GA17 of *Bacteroides thetaiotamicron* (Payan et al 2005) and phage GB124 of *Bacteroides fragilis* (Ebdon et al 2012).

### 1.2.2 Chemical indicators

Recent years have seen the appearance of a number of studies that consider the possibility of using chemical tracers to detect wastewater contamination in aquatic environments. These chemical tracers include pharmaceuticals and personal care products (PPCPs), artificial sweeteners (ASs), fluorescent whitening agents (FWAs), fecal sterols and stanols (SSs), nitrate and nitrogen isotopic signatures, or alternative potential tracers such as boron, 1-aminopropanone or trihalomethanes (Lim et al 2017).

***PPCPs*** are present on a large scale in the environment. Most of them originate from feces and urine excreted from humans and, therefore, provide strong evidence of the presence of fecal contamination in water. The main advantages of these markers are their high specificity and fast analysis (Harwood 2014). This category includes compounds such as caffeine, acetaminophen, ciprofloxacin, diclofenac, carbamazepine, and many others. Carbamazepine (CBZ) is a stable and resistant chemical compound that, when detected in the environment together with labile compounds acetaminophen (ACT) and salicylic acid (SA) indicate the existence of wastewater pollution of groundwater or surface waters (Tran et al 2014b). In other studies, caffeine and carbamazepine and diclofenac have been proposed as possible markers of anthropogenic wastewater discharges in natural waters (Vystvna et al 2012).

***ASs***: In recent years, artificial sweeteners have also been considered as alternative markers for anthropogenic wastewater pollution (Tran et al 2014b). This type of molecules, that include compounds like acesulfame, saccharin, sucralose, aspartame or cyclamate, are extensively used and most of them are poorly metabolized. As a consequence, they are excreted to the environment through feces

and urine with little or no loss (Lange et al 2012). Additionally, some of these compounds (sucralose and acesulfame) are recalcitrant enough to survive transit through a wastewater treatment plant (Brorström-Lundén et al 2008) making them ideal markers for the presence of point and diffuse wastewater-derived environmental pollution (Mead et al 2009). Several studies carried out in the USA, Canada, Germany and a number of different European countries have shown that ASs can be extensively found in all types of surface and groundwaters (Buerge et al 2009), with acesulfame and sucralose showing the highest survival when exposed to wastewater treatment and drinking water treatment processes (Gan et al 2013).

***FWA***: Fluorescent Whitening Agents are present in laundry detergents and are released into sewage systems in large amounts. Their high solubility in water and their ability to accumulate in activated sludge results in poor removal during wastewater treatment (Poiger et al 1998), with an estimated 13% of the total FWAs consumed being released into the environment (Stoll and Giger 1998). As a consequence, these compounds have been considered interesting candidates for monitoring the possible impact of wastewater inputs into natural water systems. FWAs emit blue fluorescence when exposed to UV light, a characteristics that allows their detection using a rapid and simple fluorometric procedure (Hartel et al 2007). Some of them (DAS1 and DSBP) have been successfully used as markers of sewage pollution in coastal waters (Hayashi et al 2002, Managaki et al 2006) as well as in surface continental waters (Stoll et al 1998). However, the lack of correlation between the presence of FWAs and fecal bacteria questions their validity as chemical surrogates of the presence of microbiological hazards in water (Hartel et al 2007).

***SSs***: Sterols and stanols are lipid constituents of the cells. They are present in plant cells (ß-sitosterol, stigmasterol, campesterol) as well as in animal cells (cholesterol, coprostanol). The sterols are converted into stanols in the process of digestion in warm-blooded animals thanks to the metabolic activity of commensal bacteria (Leeming et al 1996) and, therefore, the presence of stanols in the environment can be considered a sign of fecal contamination. Coprostanol is the most abundant stanol in human feces (Leeming et al 1996, Martins et al 2007) and thus, could be used as a marker of anthropogenic waste (Wang et al 2010). The application of this type of marker in real world is tainted by the complex interactions between stanols

and sediments that precludes a straightforward interpretation of the values found in the environment (Writer et al 1995).

## 1.3 Methods of bacterial detection and identification

During the years, a number of methods have been developed and used for monitoring microbiological water quality. Some of these methods attempt to carry out a direct detection of hazardous organisms. Others quantify the presence of surrogate organisms that provide an indication of the risk. The methods range from simple, tested and proved culture-based methods, to more advanced methods based on the use of nucleic acids, antibodies, aptamers, phages, or enzyme activities. The trend, in all cases has been to develop methods with improved specificity, better sensitivity. Lower limits of detection and, more importantly, shorter detection times. They are summarized in Table 1. All of them with their advantages and pitfalls are described and discussed below.

### 1.3.1 Culture-based methods

One of the most common strategies for detecting bacteria in food and environmental samples is the use of culture-based methods. Conventional water analysis methods for detecting and identifying environmental relevant microorganisms include the **standard plate count method**, the **most probable number method**, and the **membrane filtration technique**. Very often, water quality regulations require that detection of indicators microorganisms is performed using 100 ml volumes of water samples. The presence of pre-enrichment/enrichment stages allow to include observation of morphological, and microscopy features (gram stain). There is a number of instances, culture-based methods require confirmation tests carried out sub culturing colonies in selective and differential media like lactose fermentation media for coliform bacteria, or other tests depending on the biochemical features, metabolic characteristics, enzyme activities, oxidation-reduction reactions, antibiotic-resistant, motility, etc. Most of these methods require incubation at 25-37 $^{o}$C for 24-48 h. If an enrichment step is involved, the total time required to obtain a verifiable result can increase up to 4 or more days.

**Plate counts.** In plate count methods, the microorganisms present in a certain volume of sample are extended evenly across the surface of a culture plate containing solid culture medium. The composition and characteristics of the culture

media determine which organism will grow or whether the information obtained will refer to a specific organism or group or organisms (*E. coli*, coliforms) or to a broad category (heterotrophic bacteria). According to how the method is performed we distinguish between the pour plate method and the spread plate method. In the **pour plate** method, a 1 mL sample of water is mixed with 15-20 mL of melted agar at 40 ºC and allowed to solidify in a Petri dish. In the **spread plate** method, a volume of 0.1 to 0.5 mL of sample is spread on the surface of a plate containing solid medium and allowed to dry. In both cases plates are incubated at the number of colonies is counted. Plate counts provide reliable results albeit strongly conditioned by the choice of culture medium and the conditions of incubation. Due to the small volumes analyzed plate counts as described above are suitable for the detection and quantification of microorganisms present in the sample at concentrations equal or higher to 10-100 viable cells per mL (Chigbu and Parveen 2014).

**Most Probable Number (MPN).** In the most probable number method, samples are serially diluted to extinction in tubes containing culture medium adequate to the organisms being investigated. Each dilution contains several replicate tubes, and the number of microorganisms is probabilistically calculated using the mass function of the Poisson distribution from the proportion of tubes showing positive growth at the highest dilution showing growth (American Public Health Association 1998). The method is sensitive and has a high dynamic range but it is time consuming and cumbersome due to the high number of tubes involved. Variations of this method have been adapted for the development of quantitative assays such as Colilert (IDEXX), suitable for the detection and quantification of coliforms and *E. coli* in water.

**Table1.** Summary of the main methods used for microbiological quality monitoring

| Detection method | Based on | Microorganism | LOD (CFU·mL$^{-1}$) | References |
|---|---|---|---|---|
| **Culture-based** | HPC | Hetrotrophic bacteria | 10-100 | Chigbu and Parveen 2014 |
| **Nucleic acid** | PCR | *Acrobacter spp,*<br>*Campylobacter spp.* | $10^3$ (direct)<br>1 (enrichment) | Moreno et al 2003 |
| | PCR | *Escherichia coli* | 10 | Sandhya et al 2008 |
| | qPCR | *Escherichia coli* | 0.025 | Lam et al 2014 |
| **Immunology** | Lateral flow immunoassay | *Escherichia coli* O157:H7 | 133 | Hassan et al 2019 |
| **Aptamers and SELEX** | Conductimnetry | *Escherichia coli* | $2.3x10^4$ | Zhang et al 2020 |
| **Enzyme activities** | ß-glucuronidase | *Escherichia coli* | 18 | Satoh et al 2020 |
| | ß-galactosidase | Coliform bacteria | 0.1 | Sicard et al 2014 |
| | ATP | *Escherichia coli* O157:H7 | $10^4$ | Ngamson et al 2017 |
| | ß-galactosidase | *Escherichia coli* O157:H7 | $3x10^2$ | Park et al 2020 |
| **Phage-mediated** | bioluminescent | *Aeromonas hydrophila*<br>*Entrobacter cloacae*<br>*Escherichia coli*<br>*Salmonella typhimurium* | 10 | Burnham et al 2014 |
| | Magnetic separation | *Escherichia coli* | $1x10^4$<br>10 (after enrichment) | Chen et al 2015 |
| | Electrochemical | *Escherichia coli* | $10^5$ (after 4 h)<br>0.01 (after a 9 h preincubation) | Wang et al 2019 |

**Membrane filtration**. Bacterial counting by membrane filtration could be, somehow considered as a variation of the viable plate count. The technique was first described by Windle-Taylor et al. in 1953 and has since constituted one of the workhorses of drinking water microbiology monitoring. The main idea behind this method is that a large volume of water (usually between 100 and 200 mL, but can be larger) is filtered through 0.45 or 0.22 μm membrane filters in such a way that microbial contaminants are retained at the filter surface. The filter is then placed on top of a culture plate containing suitable medium. As the setup is incubated, nutrients from the solid medium diffuse through the filter allowing growth and colony formation at the filter surface. The system is well suited to handle samples with very low concentrations of microorganisms (i.e. drinking water that must contain less than 1 *E. coli* per 100 mL).

Culture-based methods are inexpensive, reproducible and simple to implement. However, they are also time-consuming, labor-intensive, and relatively slow. Also, they are limited by the fact that the fraction of microorganisms present in the sample that can grow in culture media is small (0.01-1%) (Watkins and Jian 1997). One of the concerns about culture-based media is their inability to deal with the presence of Viable But Not Culturable bacteria (VBNC) (Ashbolt 2003). VBNC cells are cells that have lost their ability to grow in routine microbiological media and develop into colonies. These cells are viable for metabolic activities (Oliver 2000) and given the appropriate circumstances could constitute a microbiological hazard.

### 1.3.2 Nucleic acid-based methods

Nucleic acid-based methods are rapid, specific and sensitive enough to detect low concentrations of microbial contaminants during analytical monitoring of water quality. These techniques provide specific detection of microorganisms in the environment based on genotype features, unique target sequences that allow accurate fingerprinting (Sayler and Layton 1990). For the detection of specific microorganisms, specific labeled probes are used to hybridize target nucleic acid sequences. Conserved 16S rRNA-encoding gene sequences have been extensively applied to detect the presence of microbial contaminants using polymerase chain reaction (PCR) (Jenkins et al 2012). Although PCR by itself has limited sensitivity, combination of this method with enrichment steps allows the detection of 1 cell of *E.*

*coli* in 100mL of water (Tsen et al 1998). The PCR techniques that have been used for quantitative detection of microorganisms in environmental water samples are quantitative PCR (qPCR) and quantitative reverse transcriptase PCR (qRT-PCR). qPCR uses fluorescent-labeled probes as reporters to identify the presence of specific sequences. qRT-PCR includes an additional reverse transcriptase reaction that enables probing for the presence of mRNA sequences in the environments, qRT-PCR provides a useful alternative for the detection of RNA viruses (Leifels et al 2016), but it is particularly interesting when targeting signature mRNA molecules. mRNAs have a very short half-life and, therefore, their detection provides better correlation with the presence of viable organisms (Yaron and Matthews 2002, McIngvale et al 2002), one of the main shortcomings of DNA-based methods, that can easily provide false positives derived from the presence of recalcitrant DNA or dead cells as early reported in Sayler and Layton (1990), and Josephson et al (1993). Discrimination of viable vs non-viable microorganisms using PCR-based methods has also been attempted using v-qPCR (v stands for viability). This method uses propidium monoazide (PMA), a DNA-disrupting reagents that prevent PCR reaction, but only in dead cells, as it can only enter the cytoplasm in cells with damaged membranes (Nocker et al 2006, Pan and Breidt 2007). qPCR has been evaluated for detection of *E. coli* in marine water. The method has proved to be sensitive with a limit of detection of 25 cells in 100mL (Lam et al 2014).

Another nucleic acid amplification-based technique is Nucleic Acid Sequence-Based Amplification (NASBA). This method can amplify RNA from DNA or RNA templates (Girones et al 2010, Tanchou 2014) and can be used to carry out as a single step direct identification of the target microorganism in environmental samples, or as an alternative step to identify specific microorganism after isolation from environmental samples (Cook 2003, Toze 1999, Jofre and Blanch 2010)

Fluorescent In Situ Hybridization (FISH) can be applied to the detection of specific microorganism in environmental water samples in the presence of a complex microbial community. In this technique, a probe with a fluorescent-label or an incorporated reporter molecule is applied to a sample immobilized for microscopic observation, labeling the microorganisms either with a fluorescent tag or with an enzyme tag that allows counting or the labeled target organism under the microscope (Girones et al 2010, Ratan et al 2017).

Some nucleic acid-based methods, such as Molecular Beacons-based PCR (MB PCR), multiplex PCR, and FISH, used to identify *E. coli* and other aquatic bacteria, are highly sensitive. These techniques can detect less than 10 $CFU \cdot mL^{-1}$ in water samples, after adding a 18-24 h pre-enrichment step (Moreno et al. 2003, Sandhya et al. 2008, Bonetta et al. 2011).

Even though nucleic acid-based methods have made significant progress in the detection and quantification of low numbers of microbial contaminants in water samples, there are still some limitations that must be taken into account, particularly the existence of false positives derived from the presence of free DNA originating from dead or partially lysed organisms (Toze 1999). This last is important in samples that have undergone treatment with disinfectants such as chlorine that might have undermined the integrity of their cell envelope. Although nucleic acid-based methods are sensitive, they cannot match the requirements of many water quality directives that demand absence of target microorganisms in 100 mL of sample. In this cases PCR must be accompanied either by preconcentration, pre-enrichment, or both. At a more technical level, the presence in the environment of natural or man-made chemicals, such as heavy metals, phenolic compounds or humic acids, can inhibit or interfere with the PCR reaction. As consequence, their presence in the samples must be taken into account, particularly when the target organism is present at low concentrations. Finally, there are some commercial products available that apply sensitive and fast real-time PCR such as iQ-check® for *E.coli* O157:H7 (Lauer et al 2009, Baranzoni et al 2014), MicroSEQ® (Patel et al 2000, Hall et al 2003), IsoQuick kit, NucliSens kit, and QIAamp kit (Holland et al 2000). They all provide results after enrichment in a nonselective medium.

### 1.3.3 Immunology-based methods

Immunological methods use monoclonal or polyclonal antibodies to bind to specific antigens present in the target organism. These methods are able to detect down to about $10^5$ bacteria per mL (Tanchou 2014) but their sensitivity can be improved by combining the method with enrichment and preconcentration steps, or with PCR or qPCR as a signal amplification system (Immuno-PCR, Adler et al 2008). These methods are able to detect a wide range of pathogens and they are efficient at low cell concentrations in complex environmental samples. Immunological methods can be applied as Enzyme-Linked Immunosorbent Assay (ELISA), Immuno Fluorescence

Assay (IFA) and, for target analytes present at very low concentrations, as Immunomagnetic Separation (IMS) (Byrne et al 2009, Ramírez-Castillo et al 2015, Deshmukh et al 2016).

**Enzyme-Linked Immunosorbent Assay (ELISA).** In this type of assay samples are added to a multi well plate where the analyte adheres to the bottom of the wells. The samples are then treated with a primary antibody that binds to the surface antigens of the target analyte. The excess antibody is rinsed and the samples are treated with a secondary antibody attached to a reporter enzyme. This enzyme catalyzes the conversion of a substrate to a product that can be detected either colorimetrically, fluorometrically or electrochemically. In the case of bacteria detection a "sandwich" ELISA is often performed in which the bottom of the well is covered with immobilized antibodies specific for the target organism. When the water sample is placed in the well, target organisms are captured by the immobilized antibodies. After the capture step and posterior neutralization and rinsing, a second antibody specific for the target organism is applied, but in this case, the antibody carries the reporter enzyme attached (usually alkaline phosphatase, horseradish peroxidase or ß-galactosidase) that generates the detection signal as described above (Yeni et al 2014).

In the **Immuno Fluorescence Assay (IFA)** the specific antibody is conjugated to a fluorescent dye directly or with an additional step. The presence of pathogens in the samples is followed by epifluorescence microscopy or using flow cytometry (Amman 1990, Karo 2008). **Immunomagnetic separation (IMS)**, on the other hand, allows the detection and enumeration of low numbers of pathogens in water and it has been used successfully for the detection of *E. coli* O157 (Ashbolt 2003). The method uses monoclonal antibodies that can bind to the LPS of the target organism. The antibodies are conjugated to magnetic Dynabeads® that allow magnetic concentration of the antibody attached target organisms in a short period of time (Bennett et al 1996, Park et al 2020):

### 1.3.4 Aptamers and SELEX

Aptamers are molecular recognition elements consisting of single strand DNA or RNA oligonucleotide with high affinity and specificity to bind a certain target analyte (Hong and Sooter 2015). Tuerk and Gold described aptamers and the systematic evolution of ligands by the exponential enrichment (SELEX) method twenty years ago (Tuerk and Gold 1990). Aptamer based methods are cost-effective, have high

selectivity as well as good chemical stability and reproductability after synthesized (Teng et al 2016). Utilizing a fluorescent reporter does not affect the affinity with which aptamers bind to their target (Citartan et al 2012). Aptamer-based methods have been used for the successful detection *E.coli, Salmonella*, spores of *Bacillus anthracis, Mycobacterium tuberculosis* and *Campylobacter jejuni* (Wang et al 2012a). Aptamers target specific structures of the bacterial surface. For instance, the specific aptamers designed for *E. coli* O111:B4 bind to LPS (Bruno et al 2008) while enterotoxigenic *E.coli* (ETEC) K88 is detected using a DNA aptamer that binds to fimbriae protein (Li et al 2011). Biosensors that make use of aptamers as biorecognition elements components are known as aptasensors. In aptasensors, the reaction between target and aptamers results in physical, chemical, electrical, or optical signal changes. Combination of aptamers with nanomaterials like graphene, carbon nanotubes, metal nanoparticles (gold nanoparticles), magnetic nanoparticles or quantum dots can increase their sensitivity and improve signal amplification (Zhang et al 2018). Adding separation methods in the SELEX aptamer selection process is important to demonstrate the affinity of aptamer sequences to the target. These separation methods include traditional methods like a filtration, centrifugation, affinity chromatography or magnetic beads, as well as new techniques like flow cytometry, atomic force microscopy or capillary electrophoresis. (Wang et al 2012a). Combination of magnetic analyte separation (MAS) and conductometric sensing (CS) has been successfully applied to the detection of viable *E.coli* in water samples. (Zhang et al. 2020).

### 1.3.5 Enzyme activity

Enzymes are specific functional proteins that possess catalytic activity essential for bacterial success in their environment. Often, some of these enzymes are specific for a species or a certain taxonomic group. Enzymes such as a ß-glucuronidase, ß-galactosidase, adenylate kinase, alpha-amylase, alpha-glucosidase, neuraminidase, esterases, lipases, phosphates, DNAases, peptidases, proteases, coagulase have been used for the specific detection of bacteria. The presence of these enzymes is detected using specific assays that depend to a large extent on external factors like pH, temperature, ionic strength, as well as on the adequate concentration of their target substrate (Bisswanger 2014). For the detection of *E. coli* and coliform bacteria in water, the assays commonly used target the enzymes ß-glucuronidase, and ß-

galactosidase. These assays are most often colorimetric (chromogenic), fluorometric (fluorogenic) or based on bioluminescence reactions.

**Chromogenic reaction assays:** In this assays enzyme activities are evaluated through color producing reactions. Some common and popular reagents for chromogenic ß-galactosidase assays are 2-nitrophenol ß-D-galactopyranoside (ONPG), chlorophenol red ß-D-galactopyranoside (CPRG), 5-bromo-4-chloro-3-indonlyl-ß-D-galactopyranoside (X-GAL), p-nitrophenyl-ß-D-galactopyranoside (PNPG), and 6-bromo-2-naphthyl-ß-D-galactopyranoside (BNG) (Manafi et al 1991). In the presence of lactose, the bacterial cells (coliforms) produce and release the ß-galactosidase enzyme that hydrolyzes the substrate cleaving it to chromophore and galactose. The ß-glucuronidase enzyme is a glycosyl hydrolase that hydrolyzes glucuronic acid from the reporter tag leading to color production. The activity of the enzyme can be measured by color intensity using substrates analogous to those used in the ß-galactosidase assay, but containing glucuronic acid as the sugar moiety.

An alternative approach for measuring specific enzyme activities relies on the use on metal nanoparticles. In one example reported by Chen et al (2016) the presence of ß-galactosidase catalyzes the hydrolysis of p-aminophenyl ß-D-galactopyranoside (PAPG) releasing PAP that reduces silver ions to metallic silver. The metallic silver is deposited on the surface of AuNPs changing the color of the suspension (Chen et al 2016). In a similar assay based on silver nanoparticles (AgNPs), urease-containing bacteria bind to specific antibodies tethered to the surface of AgNPs. Upon addition of urea the pH increases and changes the color of phenol red present in the medium (Singh et al 2019). In a radically different approach, amine functionalized metal NPs inhibit the activity of the ß-Gal enzyme due to the electrostatic binding between the enzyme and the amine groups of NPs. The ß-Gal activity is restored whenever bacteria bind to the NPs surface. ß-gal activity is measured using chlorophenol red ß-D-galactosidase (CPRG) and the increase in activity is proportional to the amount of cells present in the medium (Miranda et al 2011).

A similar competitive inhibition-based approach uses glucose oxidase (GOX) that catalyzes oxidation of glucose by oxygen producing hydrogen peroxide ($H_2O_2$). In a second step $H_2O_2$ reacts with starch-iodide paper resulting in a deep blue color. In the present of bacteria, glucose is biologically consumed and the above reaction is competitively inhibited (Sun et al 2019).

**Fluorogenic reaction assays:** This assays use reagents that release fluorescent dyes after enzymatic cleavage reaction by the target enzyme. Methods based on fluorogenic substrates have high sensitivity and are often used for the detection of bacterial activity in environmental samples. The most common fluorogenic substrates that are relevant to ß-glucuronidase and ß-galactosidase assays are 4-methylumbelliferyl-ß-D-glucuronide (MU-Glu) and 4-methylumbelliferyl-ß-D-galactopyranoside (MU-Gal), respectively. Other alternative fluorescent compounds also used in bacterial enzyme assays are resorufin-ß-D-galactopyranoside (RGP) or fluorescein di (ß-D-galactopyranoside) (FDG). Rapid enzymatic techniques based on the use of MU-Gal and MU-Glu fluorogenic substrates have allowed the detection of $10^2$-$10^3$ fecal coliforms per 100 ml in sewage-polluted coastal waters (Berg and Fiksddal 1988, Fiksdal et al 1994). Recently, 4-Methylumbelliferyl β-D-glucuronide (MU-Glu) has been used as a fluorescent reagent for the detection of *E. coli* in wastewater samples by means of its ß-D-glucuronidase activity, using an automated plate reader, and with a limit of detection of 22 cells·mL$^{-1}$ (Satoh et al 2020). In spite of not being intrinsically fluorescent, Chlorophenol Red (CPR) has been used, bound to polylysine, to develop a fluorogenic ß-D-galactosidase assay for coliform detection in water (Sicard et al 2014). Finally, from a commercial perspective, Colilert is a commercial test for detection of *E. coli* and total coliform based on the ß-D-glucuronidase catalyzed reaction, for the detection of *E. coli* in water in 18 or 24 hours. Despite its broad use, these tests are still subject to the possible existence of false-positive and false-negative results (Pisciotta et al 2002, Chao et al 2004).

**Bioluminescence assays:** Most of these methods detect bacteria through the quantification of ATP (adenosine triphosphate) present in all living cells. In the presence of ATP the enzyme luciferase promotes the conversion of the substrate luciferin into oxyluciferin with the simultaneous emission of light. The mechanism is called bioluminescence and the amount of light emitted is proportional to the amount of ATP present in the sample (Squirrell et al 2002). ATP based methods are fast and labor efficient, however, they are limited by their inability to distinguish between intracellular ATP of viable microorganisms and extracellular ATP of organic materials like dead cells, blood cells, or human secretions (Sakakibara et al 1997, Venkateswaran et al 2003, Arroyo et al 2017). In its basic configuration ATP detection is nonspecific, however, when combined with another method like immunoassay (ELISA) or immuno magnetic separation (IMS), it allows for specific

and sensitive detection of the target organisms (Eed et al 2016, Ngamson et al 2017). Finally, bacteriophages able to transfer the lux (light emission) genes from luminescent bacteria to their host-specific target bacteria, have been used successfully for the detection of enteric bacteria in the food industry with a limit of detection of 10 $CFU \cdot g^{-1}$ after a 4h enrichment of the samples (Dostalek and Branyik 2005).

### 1.3.6 Bacteriophage-based methods

Bacteriophages are the most abundant biological agents in the environment. They can only infect their specific target bacteria through specific receptors on the host bacteria's surface. Phages can be specific, at the level of strain, species, genera, or only in a serotype of bacteria and, depending on the type of phages or the physiological conditions of the host cell, can develop a lysogenic or a lytic life cycle. In the lytic cycle, phages capture and infect the cells by injecting their viral genome (DNA/RNA). Cell function is highjacked and directed to the production of new viral particles. The process leads to lysis of the host and release of the newly formed viral particles that can propagate and infect new cells.

The use of bacteriophages as biorecognition elements for detecting bacteria has been exploited in recent years in combination with different transduction methods. Production of phages is easy and unexpensive allowing for the development of low-cost detection methods. Phage-based assays can achieve good limits of detection when combined with concentration methods such as the use of magnetic beads. Magnetic beads conjugated to the bacteriophages are used to capture bacteria from water samples and concentrate them. In the next step cells are lysed by the phage and their contents are released. At this point, the ß-galactosidase released from the cells is measured with a colorimetric assay. Using this procedure, the assay achieves a limit of detection of $10^4$ $CFU \cdot mL^{-1}$ in 2.5 hours. Adding a 6 hours pre-enrichment decreases the limit of detection down to 10 $CFU \cdot mL^{-1}$ (Chen et al 2015). In a similarly designed assay, the use of a bioluminescent substrate (6-O-ß-galactopyranosyl-luciferin, Beta-Glo), allowed for the reliable detection of 10 $CFU \cdot mL^{-1}$ in 5.5 h (Burnham et al 2014). The sensitivity of this approach has been further improved by using engineered phages to express gold-binding peptides fused to alkaline phosphatase (GBPs-ALP). After cell lysis the released alkaline phosphatase attaches to the surface of gold electrodes through the gold-binding

peptides and the alkaline phosphatase activity is measured electrochemically using linear sweep voltammetry (Wang et al 2019).

## 1.4 New trends in bacterial detection

In addition to the methods described above, the emergence of technological developments applied to microbiological detection has allowed the appearance of a number of sensing technologies that improve detection and allow for fast and unexpensive solutions for microbiological water monitoring. These developments are briefly shown in Table 2 and are discussed below.

### 1.4.1 Biosensors

Biosensors are devices that detect the target analyte by converting the response of a biological transducer into an electrical signal. In 1962 Clark and Lyons described the first biosensor. The device combined glucose oxidase with a typical Clark type oxygen electrode. It was dedicated to glucose detection and quantification. Ever since, the technology has expanded and nowadays there are several types of biosensors involved in the specific detection of microbial contaminants.

In general, biosensors can be classified into two groups, **Direct Biosensors** and **Indirect Biosensors**. Direct Biosensors, are devices that rely on the biological reaction changes taking place at the transducer surface to directly (label-free) observe and measure the signal in real-time. On the other hand, Indirect Biosensors have an initial binding of the target analyte to an immobilized biorecognition element, followed by the binding of secondary biorecognition element (nucleic acid, antibody, aptamer, enzyme, cell, etc.) that is responsible for generating the analytical response. Immunosensors are the example of this sort of biosensors. Beyond this general classification, biosensors are often categorized into four main groups according to their transducing elements as: optical, electro-chemical, mass-based, and thermal (Ivnitski et al 1999, Leonard et al 2003, Sentürk et al 2018).

#### 1.4.1.1 Optical biosensors

Optical biosensors can detect changes that occur due to the emission or reflection of light from immobilized cells at the surface of the sensor. Biosensors based on this type of transduction have high efficiency due to the sensitivity, selectivity, and rapid response for the detection of toxins, drugs, and pathogenic bacteria (Willardson et al 1998, Tschmelak et al 2004). Several measuring principles used in optical

biosensors include Surface Plasmon Resonance (SPR), Tapered Fiber Optical Biosensors (TFOBS) and colorimetry.

Surface plasmon resonance (SPR) measures the changes from reflected light from varied refractive indices on a thin metal conductor layer at which cells bind to their receptors (Cooper 2003, Sentürk et al 2018). The method is applied for environmental and food safety analysis and allows the detection of $1.25x10^5$ $CFU \cdot mL^{-1}$ (Mazumdar et al 2007, Wang et al 2013). SPR can determine the affinity and adsorption phenomena involved in the determination of antigen-antibody reaction kinetics (Lazcka et al 2007). There are some commercial SPR biosensor equipment available in the market that provide label-free analysis in real time such as Bioffin's BiacoreTM (Moller-Jason et al 2006).

Tapered Fiber Optical Biosensors (TFOBS) are fabricated in silica or polystyrene and have a tapered end. TFOBS can transmit and receive light signals through the tapered end. They can be used to measure the cells, protein, and DNA but the technique is usually applied combined with immunological antibody-antigen reactions for the specific detection of pathogens in clinical samples. They are a good alternative to classical immunoassays due to their low cost, efficiency, and effectiveness. TFOBS has been used to successfully detect 70 $cells \cdot mL^{-1}$ of *Escherichia coli* O157:H7 via an immobilized antibody on the surface of the biosensor (Rijal et al 2005, Leung et al 2007). More recently, several authors have described a device that can automatically detect *E. coli* based on colorimetric and fluorometric fiber-optics measurements (Tok et al 2019).

Finally, colorimetric based biosensors are optical biosensors that can identify target microorganisms by changes in color without using any analytical devices. They rely on metallic nanoparticles, especially gold nanoparticles. The methods use conjugation of DNA/RNA to the nanoparticle and can recognize a wide range of analytes (Liu and Lu 2004, Yuan et al 2014).

**Table 2.** Summary of advanced technologies applied microbiological quality monitoring

| Detection sensor | Based on | Sample type | Microorganism | LOD (CFU·mL$^{-1}$) | References |
|---|---|---|---|---|---|
| **Biosensor** | SPR | Milk | *Salmonella typhimurium* | 1.25x10$^{5}$ | Mazumdar et al 2007 |
| | Optical fibre | - | *Escherichia coli* O157:H7 | 70 | Rijal et al 2005 |
| | QCM | - | *Escherichia coli* O157:H7 | 10$^{2}$-10$^{5}$ | Ngo et al 2014 |
| | Impedimetric | Waste water | *Escherichia coli* | 1.9x10$^{3}$ | Rengaraj et al 2018 |
| | Amperometric | Milk and chicken extract sample | *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni* | 10 | Chemburu et al 2005 |
| **Nanoparticles** | | - | *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Xanthamonas campestris* | 10$^{2}$ | Peng et al 2019 |
| | | Water | *Escherichia coli* O157:H7 | 4 | Amin et al 2020 |
| **Paper-based** | | Chinese cabbage | *Escherichia coli* O157:H7 | 10$^{4}$ | Pang et al 2018 |
| | | Sewage sludge | - | 10$^{3}$-10$^{6}$ | Rengaraj et al 2018 |
| | | - | *Escherichia coli* | 10$^{5}$ | Jahanshahi-Anbuhi et al 2017 |

#### 1.4.1.2 Electrochemical biosensors

In electrochemical biosensors the transduction system converts the amount of analyte into a measurable electrical signal. These sensors have high sensitivity and are not affected by sample turbidity. Moreover, determinations can be carried out with relatively low-cost equipment and using small sample volumes (Lazcka et al 2007, Teng et al 2016, Sentürk et al 2018). Different types of electrochemical biosensors are categorized by the variable they measure as: amperometric (current), potentiometric (voltage), impedimetric (impedance), and conductometric (conductance) biosensors.

***Amperometric biosensors*** are probably the most widely used type of sensor for microbial detection. These sensors measure the electrical current generated when applying a constant potential from the electrochemical oxidation or reduction reaction of an electroactive metabolic, or from a redox reaction catalyzed by the target organism or by a reporting element (usually an enzyme tag) (Shah and Wilkins 2003, Su et al 2011). The advantages of amperometric biosensors are their high sensitivity, short detection times and low cost (Ghindilis et al 1998, Soni et al 2018) with a good sensitivity that allows the detection of down 10 cells·mL$^{-1}$ (Chemburu et al 2005). The downside of these techniques, however, is the potential interference of active chemical redox species present in the medium generating false positive (Soni et al 2018).

***Potentiometric biosensors*** measure the variation of voltage between two electrodes as a consequence of changes in the concentration of redox active species. These sensors are used in clinical, food, and environmental samples (Pisoschi et al 2016). Potentiometric biosensors have some drawbacks that can be addressed: first, the signals are affected by temperature; second, they only detect free ions; third, they can provide erroneous readings at low concentration due to the presence of interfering ions and, fourth, the membrane potential is affected by the absorption of solution components to the electrode surface (Cosio et al 2012, Bratov et al. 2010, Vlasov et al. 2010).

***Impedimetric biosensors*** are of great importance in the identification of bacteria in the food industry as well as in the monitoring of drinking water pollution. Impedimetric biosensors come in two formats. In the first one, impedance changes due to the binding of target microorganisms to receptors immobilized at the electrode surface (antibodies or aptamers). The second type of impedance biosensor detects

the metabolites produced by the target organism. Recent incarnations of impedimetric biosensors incorporate elements such as nanomaterials, microfluidics and/or biorecognition elements like bacteriophages and lectins that improve their sensitivity (Wang et al 2012). However some authors question whether impedimetric biosensors have adequate efficiency and stability to detect and measure unknown amounts of target bacteria in separate replicated measurements (Kivirand et al 2019). In an attempt to simplify the technology and make it more affordable, carbon electrodes screen printed on hydrophobic paper and functionalized with Concanavalin A, have been used to detect bacteria in water samples with a limit of detection of $1.9x10^3$ $CFU \cdot mL^{-1}$ and a linear range between $10^3$ and $10^6$ $CFU \cdot mL^{-1}$ (Rengaraj et al 2018).

***Conductometric biosensors***: This type of sensor rely on the immobilization of the biological material on a thin-film of a conductive transducer surface. Conversion of complex substances to charged species that modify the electrical conductive properties of the medium. Conductometric biosensors are widely used in the analysis of food and water samples. Some of the advantages of these sensors are that they are insensitive to environmental light, they are sensitive to a very wide range of analytes, do not require a reference electrode, have low power consumption and their technology is well suited for miniaturization in the fabrication of mass produced low-cost devices (Jaffrezic-Renault et al 2008; Dzyadevych et al 2008). However, there are some limitations to the method as its applicability is limited in ion rich media and cannot distinguish between reactions unless linked to enzymes or cells with specific catalytic activities (Dzyadevych et al 2008).

#### 1.4.1.3 Mass-based biosensors

Mass-based biosensors can detect small changes in mass. They are based in the use of quartz crystal microbalance (QCM) or surface-acoustic wave (SAW) devices to measure the mass of the samples in a different way. In QCM biosensor the quartz crystal oscillates with a frequency proportional to its mass. Whenever this mass changes because microorganisms specifically attach to the crystal surface by means of aptamers or antibodies, the oscillation frequency changes in a measurable way. In SAW biosensor the attachment of the target organisms is detected through changes in the oscillation of acoustic wave that generates by the piezoelectric crystal. Mass-based biosensors have been applied to the detection of pathogens and toxins in

recent years (Fu et al 2019). Thus, in a study using sensors based on quartz crystal microbalances functionalized with antibodies specific for *E. coli* O157:H7, the authors were able to achieve reliable detection of the target organism in water in the range $10^2$ to $10^7$ CFU·mL$^{-1}$ (Ngo et al 2014).

#### 1.4.1.4 Thermal sensors

Thermometric biosensors measure the evolution and absorption of heat during enzyme or cell-catalyzed reactions. These devices have been used for the fast and stable detection of enzymes activities such as ß-galactosidase as well as for monitoring of clinical metabolites, antibiotics, environmental analytes, or lactate and urea in milk fermentation process (Ramanthan and Danielsson 2001, Ramanathan et al 2001, Chen et al 2011, Zhou et al 2013).

### 1.4.2 Paper based methods

One of the recent trends in the development of fast analytical methods is the simplification of the analytical devices in the direction of lowering production costs and reducing the need for sophisticated equipment or skilled technicians. In this sense, the transition towards paper-based method has become common in many studies published in recent years. Different types of paper with different features are combined with technologies such as wax printing and screen printing to produce a number of paper based devices for multiple applications (Peixoto et al 2019). Paper based methods are fast, inexpensive, portable and labor efficient and, despite the fact of having accuracy and sensitivity problems early on, most of these issues have been progressively solved. Fusion of a ß-gal based assay with a paper platform has been used successfully for the detection of *E. coli* in water sample in a litmus paper-like format (Gunda et al 2017). This relatively simple technology can detect between $2x10^5$ and $4x10^4$ CFU·mL$^{-1}$ of *E. coli* in 60-65 min. In this method the chemotactic properties of *E. coli* have been exploited to increase the concentration of target bacteria in the part of the paper where detection occurs. In a more complex design Hossain et al (2012) described a multi-layer paper sensor fabricated via ink-jet printing and applied to detection of bacterial contaminants. In this design some of the reagents are entrapped within sol-gel silica layers. The method is sensitive enough to detect a single bacterium in 2 mL of water after a 5 h enrichment (Hossain et al

2012). In a more complex iteration of a similar design, a multi-layer paper-based assay for detecting *E. coli* allows rapid detection of $1x10^5$ CFU·mL$^{-1}$ by using a pullulan film containing lysing reagent that releases ß-galactosidase. A second adjacent layer of paper coated with poly arginine contains the reagents needed for ß-galactosidase quantification (Jahanshahi-Anbuhi et al 2017). A combination of ELISA and paper-based assay has significant sensitivity of $10^4$ CFU·mL$^{-1}$ for bacterial isolation. The colorimetric reaction occurred after adding TMB-$H_2O_2$ on the sensing area of Whatman paper when the initial antibody attached to the bacteria and HRP labeled by the second antibody. The format platforms have been further adapted for the implementation of more complex detection principles such as paper based ELISA (Pang et al 2018) or impedimetric paper based biosensors fabricated by screen-printing carbon electrodes on hydrophobic paper. The sensitivity of the device is enough to provide direct detection of $10^3$-$10^6$ CFU·mL$^{-1}$ (Rengaraj et al 2018).

### 1.4.3 Nanoparticle mediated assays

During the past decade a lot of attention has been paid to the applied aspects of some unique properties of nanoparticles. These properties include small size, large surface to volume ratio, high stability, excellent biocompatibility, low toxicity, and assembly-disassembly properties. Also, gold nanoparticles have localized surface plasmon resonance that causes light absorption due to the oscillation of the conduction band electrons of gold nanoparticles by the electromagnetic field (Yeh et al 2012, Wang, et al 2014). Nanoparticles have recently been used as a colorimetric detection element to identify bacterial species present in water. Many studies have been done on the measurement of bacterial enzymes and nanoparticles using gold to detect bacteria as has also been described above when discussing the enzyme activity assays. In one study, bacteria were detected through the simultaneous use of gold nanoparticles and chimeric phages. The engineered M13 phages bind to AuNPs through thiol bonds while at the same time recognizing the bacteria through phage-host specific interactions. The interaction between phage and bacteria results in aggregation of the nanoparticles that can be observed through color changes in the suspension (Peng and Chen 2019). A similar approach was also attempted using mercaptoethylamine (MEA), an organic compound that binds to bacteria through an electrostatic link while at the same time forming thiol bonds with the AuNPs. The

aggregation of gold nanoparticles in the presence of bacteria leads to changes in color that can be detected visually (Su et al 2012). More recently, a point-of-care system for the detection of *E. coli* O157:H7 has been developed using gold nanoclusters as signal reporters quenched by gold nanoparticles functionalized with antibodies specific for the target organism. In the presence of bacteria the quencher attaches to the target and the gold nanoclusters are able to emit fluorescence. The response of the system can be followed with the naked eye, providing a limit of detection of 4 $CFU \cdot mL^{-1}$ in a single step measurement (Amin et al 2020).

## OBJECTIVES

The main objective of this thesis is the **development of a fast, affordable and highly specific assay for the detection of bacteria in water**. We propose a method that combines the power and ease of use of enzyme-based assays with the specificity provided by the bacteriophage-host interaction. As a model organism we use *Escherichia coli* and the detection is carried out using a general ß-galactosidase assay on cells specifically permeabilized using bacteriophage T4.

To achieve this main goal the work has been organized around three secondary objectives:

1.- **Optimization of the ß-galactosidase assay to maximize sensitivity.**
The ß-galactosidase assay was initially conceived as a tool to monitor the expression of the *lacZ* gene in molecular biology studies. Since these studies are carried out at high cell concentrations, the assay was never optimized to maximize sensitivity. As a first step in this work, an analysis has been carried out to determine the optimal values of the main assay variable (concentration of inductant, length of the induction period, type of enzyme substrate, concentration of substrate used, lysis/permeabilization of the cells, and addition of carbonate at the end of the assay. The results obtained are presented in chapter 2 of this thesis.

2.- **Development of the basic phage-enzyme assay and assessment of its performance.** The information gained in the optimization study has been used to develop a fast basic assay for the direct analysis of liquid samples. The assay uses bacteriophage T4 to lyse the previously induced target organism present in the sample, which is then analyzed using a ß-galactosidase assay. A description of the assay and a characterization of its performance are presented in chapter 3 of this thesis.

3.- **Adaptation and validation of the phage-enzyme assay for the analysis of real-world samples.** We have developed an extended version of the assay that includes preconcentration and preincubation steps in order to match the sensitivity requirements of current environmental and water supply regulations. The

performance of this extended assay has been validated using real world samples, using Colilert-18 as a reference method. The results are also presented in chapter 3 of this thesis.

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# CHAPTER 2

## The ß-galactosidase assay in perspective: critical thoughts for biosensor development

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# 2 The ß-galactosidase assay in perspective: critical thoughts for biosensor development

**Abstract**

Recently, the ß-galactosidase assay has become a key component in the development of assays and biosensors for the detection of enterobacteria and *E. coli* in water quality monitoring. The assay has often performed below its maximum potential, mainly due to a poor choice of conditions. In this study we establish a set of optimal conditions and provide a rough estimate of how departure from optimal values reduces the output of the assay potentially decreasing its sensitivity. We have established that maximum response for detecting low cell concentrations requires an induction of the samples using IPTG at a concentration of 0.2 mM during 180 minutes. Permeabilization of the samples is mandatory as lack of it results in an almost 60% reduction in assay output. The choice of enzyme substrate is critical as different substrates yield products with different extinction coefficients or fluorescence yields. The concentration of substrate used must be high enough (around 3 to 4 times $K_m$) to ensure that the activity measured is not substrate limited. Finally, as the color/fluorescence of the reaction products is highly dependent on pH, care must be taken to ensure that pH at the time of reading is high enough to provide maximum signal.

## 2.1 Introduction

The first description of a standardized assay for the detection of ß-galactosidase activity in microbial samples was made by Miller in 1972. The assay contemplated permeabilizing the cells with SDS-chloroform and quantifying ß-galactosidase through the increase of yellow color resulting from the ß-galactosidase-mediated hydrolysis of ONPG (o-nitrophenyl β-D-galactopyranoside) to ONP (o-nitrophenol). The assay was extremely successful and has been broadly used, ever since, to monitor the expression of *lacZ* reporter gene fusions in gene expression studies

(Casadaban and Cohen 1979, Silhavy and Beckwith 1985, Hautefort and Hinton 2000, Silhavy 2000). Through the years the assay has been the object of several modifications including the use of alternative reagents such as CPRG (chlorophenol red β-D-galactopyranoside) (Henderson et al 1986), MUG (4-methylumbelliferyl β-D-galactopyranoside) (Berg et al 1988), FDG (fluorescein di(β-D-galactopyranoside)) (Huang 1991) or a host of other galactoside derivatives (Browne et al 2009); the improvement of the permeabilization method using commercial reagents such as PopCulture® (Thibodeau et al 2004, Schaefer et al 2016); or even adapting the assay for high throughput applications using microtiter plates (Arvidson et al 1992, Griffith and Wolf 2002, Thibodeau et al 2004, Vidal-Aroca et al 2006, Schaefer et al 2016). Despite all these changes, the assay has evolved mainly in the direction of improving its ease of use and facilitating the analysis of large sample numbers. Sensitivity of the assay has seldom been under scrutiny, mainly because gene expression assays are carried out using samples with high cell densities that ensure ample levels of enzyme. During the 1980's and 90's several analytical tools appeared that used ß-galactosidase levels as surrogate measurements to assess the existence of fecal contamination in water or food samples using ONPG (Edberg et al 1988, Apte et al 1995), MUG (Apte and Batley 1994, Fiksdal et al 1994, Davies and Apte 1999) and other compounds such as D-luciferin-o-ß-galactopyranoside (LuGal) (Masuda-Nishimura et al 2000). These measurements showed excellent correlation with the actual number of coliforms determined through plate counts across several orders of magnitude (George et al 2000). In an attempt to develop compact and affordable systems for water quality monitoring, the colorimetric, fluorometric or luminometric assays mentioned above were later brought to an electrochemical format through the use of substrates such as PG (phenyl ß-D-galactopyranoside) (Serra et al 2005) or PAPG (4-aminophenyl ß-D galactopyranoside) (Neufeld et al 2003, Laczka et al 2010, Ettenauer et al 2015, Adkins et al 2017). These substrates, upon hydrolysis, yield electroactive products that can be measured amperometrically using relatively simple and inexpensive instrumentation.

In recent years this technology has evolved into several new and promising approaches including the use of paper-based formats for ease of use (Hossain et al 2012, Kim HJ 2019), using bacteriophage-based amplification to provide higher

signal and more specificity (Derda et al 2013), encapsulation of the enzyme substrate in alginate microbeads (Kikuchi et al 2020) or the use of the ß-galactosidase assay with an electrochemically active substrate to modify the surface plasmon resonance of a suspension of gold nanoparticles (Chen et al 2016).

In all cases, and no matter what technology or platform is used for the detection, the response produced by the assay should be proportional to the number of target microorganisms present in the sample. However, this proportionality is strongly affected by factors such as the level of induction of the genes encoding the ß-galactosidase enzyme, the efficiency of the permeabilization step, the concentration of enzyme substrate, the molar extinction coefficient of the reaction products (in the case of optical measurements), and the pH of the reaction mix, to name the most important. Use of the wrong set of conditions can lead both to a decrease in sensitivity and/or to extended detection times. As a rule, most of the work carried out until now has devoted little or no attention to these factors, and thus, it is unlikely that they reached their full analytical potential. In this work we attempt to analyze the effect of these factors on the performance of the ß-galactosidase assay, and to provide a set of conditions that help maximize the output of ß-galactosidase-based detection assays and biosensors for environmental, industrial or diagnostic applications.

## 2.2 Materials and methods

### 2.2.1 Microorganisms and growth conditions

*Escherichia coli* DSMZ-613 (DSMZ, Germany) was grown overnight at 37 ºC in Luria-Bertani (LB) liquid medium using an Infors HT Ecotron orbital incubator operating at 100 rpm. Growth of the cultures was monitored by following $OD_{600}$ using a SmartSpec Plus spectrophotometer (Bio-Rad, California, USA). For the determination of cell concentration we carried out plate counts in LB medium at 37C, of samples serially diluted in 0.1M phosphate buffer.

### 2.2.2 ß-galactosidase induction

For induction of ß-galactosidase, 0.2 mL of an overnight culture were inoculated into 5 mL of fresh LB medium an allowed to grow for 1 hour at 37 ºC in a Ecotron orbital

shaker (Infors HT, Switzerland) at 100 rpm. When induction of ß-galactosidase was required the cultures were supplemented at mid exponential phase ($OD_{600}$ of 0.4-0.5) with isopropyl ß-D-1-thiogalactoside (IPTG) at a final concentration of 1 mM an incubated for a further 3 hours to allow complete induction of ß-galactosidase. After induction the culture was centrifuged, resuspended in 0.1 M phosphate buffer and diluted down to a standard $OD_{600}$ of 0.3. From there culture the culture was serially diluted in phosphate buffer (0.1 M) to obtain the required cell concentration. In specific instances, when we wanted to explore the dependency of induction levels on IPTG concentration or induction time, different concentrations of IPTG (0, 0.1, 0.2, 0.5, 1.0, 1.5 and 2.0 mM) and different induction times (0, 45, 90, 120, 180 and 225 minutes) were tested.

### 2.2.3 Cell permeabilization

In the ß-galactosidase enzyme assay described by Miller in 1972 and many other publications thereafter, cells were permeabilized with a combination of toluene or chloroform and SDS. Because the chloroform and toluene are not compatible with the 96 well polystyrene plates used for high throughput measurements, we tried several compatible commercial reagents. Samples (50 µL) containing *E. coli* were permeabilized using PopCulture® (20 µL), rLysozyme™ (10 µL), a combination of PopCulture® and rLysozyme™ (20+10 µL) or a combination of B-PER and rLysozyme™ (20+10 µL). The rLysozyme™ reagent was prepared by mixing a 300 KU rLysozyme solution (Novagen®) with 1 mL of rLysozyme™ dilution buffer containing (100 mM NaCl, 50 mM Tris-HCl, 1 mM DTT, 0.1 mM EDTA, 50 % Glycerol, 0.1 % Triton X-100, pH 7.5). PopCulture and B-PER were used directly as provided by the supplier. Controls without permeabilization reagents were also prepared adding equivalent volumes of 0.1 M PBS. The reagents were incorporated into the reaction mix as described in the following section.

### 2.2.4 ß-galactosidase assay

For the ß-galactosidase assay, 50 µl of a previously induced sample containing a known amount *E. coli* were added to 120ul of Z-buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM ß-mercaptoethanol, pH=7) and the volumes of lysis reagents stated above. The reaction started after adding 10 µl of the ß-galactosidase substrate. In order to compare their efficiency, we tested 2-

nitrophenyl-ß-D-galactopyranoside (ONPG) 2 mM, chlorophenol red-ß-D-galacto-pyranoside (CPRG) 2 mM and 4-methylumbelliferyl-ß-D-galactopyranoside (MUG) 1 mM. The assay was carried out in 96-well plates. The plates were incubated at 37$^{o}$C and measured in a Varioskan Flash Plate reader (Thermo Scientific, Massachusetts, USA). Measurements were taken every 3 minutes during 1 hour. Samples were shaken before every measurement. For the CPRG- and ONPG-based assay, optical density was measured at 420 and 574 nm. In the MUG-based assay fluorescence was measured using an excitation wavelength of 360 nm and an emission wavelength of 438 nm. For the $K_m$ determination experiments, the concentrations of the different reagents were modified accordingly to explore the adequate range.

### 2.2.5 Determination of the % reduction of the assay output under non-optimal conditions

For each of the variables analyzed we have determined the Optimal value that maximizes the assay response providing an Optimal Output ($Out_{opt}$). Also, for each variable we have made an educated and realistic choice of the variable that would result in a Suboptimal Output ($Out_{subopt}$). The % of reduction in the assay output has been calculated as:

*Equation 1:*

$$\%\, Reduction\ = \frac{Out_{opt} - Out_{sub}}{Out_{opt}} \cdot 100$$

for each of the variables analyzed.

### 2.2.6 Data treatment, curve fitting and parameter estimation

Each set of conditions was assayed as triplicates and the data points plotted in the graphs represent either the average of these replicates, with the error bars indicating the standard error of the measurements, or the ß-galactosidase activities estimated by linear regression, with the error bars indicating the 95% confidence intervals of the estimates. ß-galactosidase activities were calculated as the slopes of the absorbance (Abs) or fluorescence (RFU) vs time graphs included as Supplement Figures 1A, 1B, 3A, 3B and 3C for each of the variables tested. The estimates of $K_m$ for each substrate for each substrate were obtained after nonlinear fitting of the Michaelis-Menten equation to each set of data. In the case of IPTG and due the existence of inhibition at high concentration we fitted the data to a Haldane substrate

inhibition equation that provide estimates of both, $K_m$ and $K_i$. Both linear regression and nonlinear fitting were carried out with GraphPad - Prism using pre-defined functions included in the package.

## 2.3 Results and discussion

### 2.3.1 ß-galactosidase induction

Because ß-galactosidase expression in *E. coli* is not constitutive, its levels in the cell are highly dependent on the level of induction of the lac operon. In order to guarantee full expression and maximum levels during detection, enzyme production must be induced through a preincubation of the samples in the presence of either lactose or IPTG. The length of this induction period and the concentration of inducer used have not been standardized and are seldom used in a consistent way. In order to assess the **effect of IPTG concentration** on ß-galactosidase induction, we exposed cultures of *E. coli* to different concentrations of IPTG ranging between 0 and 2 mM as described in Materials and Methods. The kinetics of $OD_{420}$ increase were registered (Supplement 1A), and the level of ß-galactosidase activity was determined as the slope of the OD vs time curve for each IPTG concentration. The results, represented in Figure 1A, indicate that maximum activity was achieved at relatively low IPTG concentrations, around 0.2 mM. Increasing the concentration of inducer above this value resulted in a decrease in the level of enzyme indicating the existence of inhibition at high IPTG concentrations. Non-linear fitting of the data to a Haldane substrate inhibition equation provided an excellent fit with an $R^2$ of 0.995 and allowed us to estimate a $K_m$ for IPTG of 12 µM and a Ki of 5.65 mM. Lack of data in the low concentration range made for some uncertainty in the estimate of $K_m$ with a 95% confidence interval of 0 to 31 µM, in any case much below the values commonly used. The existence of inhibition at high IPTG concentrations in assays performed in nonpermeabilized cells can be explained by the existence of competition between IPTG and the ß-galactosidase substrate at the transport step, because IPTG is transported into the cell using a lactose permease (Fernández-Catané et al 2012) and transport seems to be the main bottleneck in the metabolism of lactose and ONPG (Cohen and Monod 1957, Kepes 1971). In the experiment described above, however, the assays were carried out in permeabilized cells in

which transport was not rate-limiting. This means that inhibition had to occur at the enzyme level. Inhibition of enzymatic ONPG hydrolysis by high concentrations of IPTG was hinted at in some of the early works on the ß-galactosidase of *E. coli* (Herzenberg 1959) and was confirmed later on in *Pseudomonas* (Hidalgo et al 1977), however, this effect has often been overlooked as a host of papers on ß-galactosidase-dependent detection of *E. coli* often induce expression of the enzyme using concentrations of IPTG close to $K_i$, well in the inhibitory range (4.2, 4.19, 5.0 mM, Apte et al 1994, Sotah et al 2020, Burnham et al 2014) while others, completely overlook the induction step (Ryzinska-Paier et al 2014, Adkins et al 2017). According to the results plotted in Figure 1A, we establish the *optimal concentration* of IPTG as 0.2 mM, the concentration providing the highest activity. For the sake of comparison and considering the concentrations used in the literature, we have arbitrarily taken a *suboptimal concentration* of 5.7 mM equal to $K_i$, and we have calculated the percentual reduction in assay output using Equation 1 and the activities measured under optimal and suboptimal conditions. The result of the calculation, shown in Table 1, indicates a reduction of 45.2% in assay output under suboptimal conditions.

To investigate how the **length of the induction period** affects the levels of ß-galactosidase activity, we incubated a culture of *E. coli* in the presence of 1 mM IPTG during 0, 45, 90, 120, 180 and 225 minutes. At the end of the induction period, cell concentration was normalized to a constant value of $10^8$ cells·mL$^{-1}$ (see Materials and Methods) and a ß-galactosidase assay was performed. The results of the experiment have been represented in Figure 1B as the initial slope of the $OD_{420}$ vs time curves (Supplement 1B) for each of the induction times tested. As can be observed, ß-galactosidase activity is strongly influenced by the length of the induction period up to approximately 3 hours. Increasing the induction period beyond that point does not seem to result in any significant improvement of the activity observed. We have thus established 3 h as the *optimal induction time* providing maximum enzyme activity. Again, in order to provide a counterpoint to the optimal value, we have picked 45 min as a *suboptimal induction time* and we have determined the resulting % reduction in assay output using the activities from Figure 1B. The results, collected in Table 3, indicate that shortening the induction time from 3h to 45 minutes results in a 58% reduction in measured activity. In general, methods published in the literature that include an inductions step use very different induction times ranging from 1-2 h (Boyaci et al 2005, Lackzka et al 2010) to as

much as 10 or 16 h (Sotah et al 2020, Sicard et al 2014). Most of them, however, are in the range 4 to 6 hours (Browne et al 2009, Ettenauer et al 2015, Masuda Nishimura et al 2000, Sicard et al 2014), adequate to provide enough induction for the assay in a reasonably short time frame.

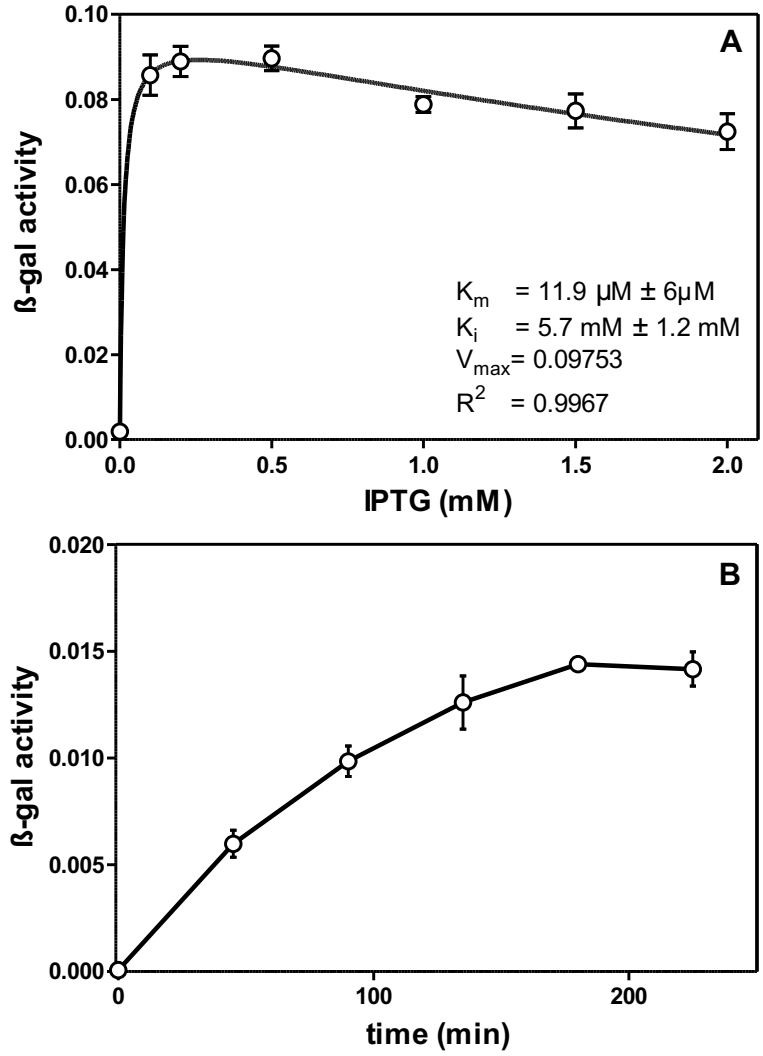


**Figure 1.** Effect of induction conditions on the levels of ß-galactosidase activity in cultures of *E. coli*. Enzyme activity was determined using ONPG as a substrate and is expressed as $A_{420}$·$min^{-1}$. Estimates of $K_m$ and $K_i$ for IPTG, plus minus their standard errors were obtained after nonlinear fitting of a Michaelis-Menten equation in Figure 1A or a Haldane substrate inhibition equation in Figure 1B. (**A**) ß-galactosidase activity as a function of IPTG concentration in samples containing $10^8$ cells·$mL^{-1}$ preincubated 180 min in the presence of the inductant. (**B**) ß-galactosidase activity as a function of induction time in samples containing $10^7$ cells·$mL^{-1}$ and 1 mM IPTG. Error bars indicate the 95% CI of the ß-galactosidase activity calculated as the slope of the $Abs_{420}$ vs time line at each IPTG concentration (Supplement Figure 1A) and each induction time (Supplement Figure 1B).

### 2.3.2 Cell permeabilization

In order to check whether and to what extent permeabilization and lysis of the cells increased the response of the assay, a sample of a fully induced *E. coli* culture containing $10^7$ cells·$mL^{-1}$ was assayed for ß-galactosidase using different lysis

reagents added at the beginning of the assay as described in materials and methods. The results have been represented in Figure 2 for an untreated control, and for samples treated with PopCulture®, a combination of PopCulture® and rLysozyme™, a combination of B-PER and rLysozyme™ and a control without lysis reagents. Figure 2 shows the increase in optical density at 420 nm resulting from the ß-galactosidase-dependent hydrolysis of ONPG. The slope of the Abs vs time line is proportional to the amount of ß-galactosidase available. Untreated samples not subject to permeabilization or lysis displayed a certain level of activity, as ONPG can be slowly transported by the lactose permease system present in induced cells (Kepes 1971). However, the slope of the curve is considerably higher in samples treated with PopCulture®, a detergent-based cell permeabilization reagent. Addition to the reaction mix of rLysozyme™, a commercial high-yield recombinant lysozyme, did not result in any significant improvement. Similar results were obtained with a combination of B-PER™ (also a detergent based commercial reagent) and rLysozyme™ (Fig 2).

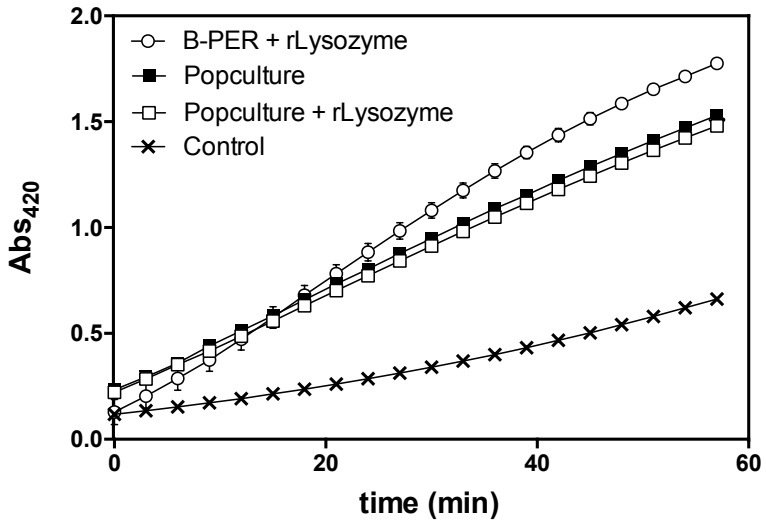


**Figure 2.** Effect of adding different permeabilization reagents to the reaction mix, on the development of the ß-galactosidase assay. (■) PopCulture (□) PopCulture + rLysozyme (○) B-PER + rLysozyme (✕) control without permeabilization reagents. Error bars represent the standard deviation of the data.

The results indicate that for biosensing and detection applications based on ß-galactosidase measurements, permeabilization or lysis of the sample is paramount

to ensure maximum sensitivity, as in fully induced cells, transport of ONPG by lactose permease seems to be the main rate-limiting step for the activity of the enzyme (Cohen and Monod 1957). The fact that this step can be optimized using only a detergent-based reagent greatly simplifies the procedure as it avoids the use of less robust enzyme preparations. Using the results shown in Figure 2 we have estimated a 56.7 % reduction in assay output between samples permeabilized with PopCulture and a non-permeabilized control (Table 3) that provides an indication of the loss of sensitivity that can result from overlooking this factor. While some studies published during the years have chosen not to use any permeabilization step (Wutor et al 2007, Hossain et al 2012, Hesari et al 2016, Chiu and Watson 2017) most publications guarantee access of the substrate to the enzyme, by including treatments with surfactants (SDS, Triton X-100) (George et al 2000, Griffith et al 2002, Ryzinska-Paier et al 2014), surfactant-based commercial reagents (PopCulture, B-PER) (Thibodeau et al 2004, Zakir-Hossain et al 2012, Sicard et al 2014, Schaefer et al 2016, Gunda et al 2016, Gunda et al 2017, Jahanshahi-Anbuhi et al 2017), organic solvents (chloroform, toluene) (Vidal-Aroca et al 2006,Browne et al 2009), or a more strict lysis by sonication (Adkins et al 2017) or induced by phages (Derda et al 2013, Ettenauer et al 2015, Chen et al 2016).

**Table 3.** Effect of using optimal or suboptimal values of several variables, on the output of the ß-galactosidase assay.

| Variable | Optimal | Suboptimal | % Reduction |
|---|---|---|---|
| IPTG Concentration[1] | 0.2 mM | 5.7 mM | 45.2 |
| Induction time | 3 h | 45 min | 58.4 |
| Lysis/permeabilization[2] | YES | NO | 56.7 |
| Enzyme substrate[3] | | | |
| ONPG | 4.5 mM | 1.12 mM | 37.5 |
| CPRG | 51.4 mM | 12.84 mM | 37.5 |
| MUG | 0.25 mM | 0.063 mM | 37.5 |
| Carbonate addition | | | |
| ONPG | YES | NO | 47.6 |
| CPRG | YES | NO | 2.3 |
| MUG | YES | NO | 73.9 |

% Reduction has been calculated as the difference between the assay output under optimal and suboptimal conditions referred to the output of the assay under optimal conditions.[1]Optimal value obtained from Figure 1A as the concentration of IPTG that results in maximum ß-galactosidase activity. As an example of suboptimal activity we have taken the value of $K_i$ for IPTG, which is close to values often used in the literature. [2]Optimal and suboptimal conditions correspond to the results shown in Figure 2 for samples permeabilized with Pop Culture and untreated controls. [3]Optimal conditions correspond to concentrations of substrate equaling 4 time $K_m$ and providing an activity of 80% $V_{max}$. As an example of suboptimal conditions we have taken the value of $K_m$ that provides activity levels equaling 50% of $V_{max}$.

### 2.3.3 Optimal reagent concentration

The rate at which enzyme-catalyzed reactions proceed is strongly dependent on the concentration of substrate as described in the Michaelis-Menten equation. Optimization of the ß-galactosidase assay for maximum sensitivity in bacterial detection requires that the activity measured is proportional to the amount of enzyme found in the sample, and that this activity is not hampered by low concentrations of substrate. The concentrations of substrate described in the literature for detection of *E. coli* through ß-galactosidase vary considerably in a way that suggests that, in some cases, the authors were little aware of the effect of this variable. We have analyzed the effect of substrate concentration on the rates of galactoside hydrolysis for three commonly used substrates: ONPG, CPRG and MUG. To this end we determined ß-galactosidase activity in fully induced cultures of *E. coli* containing $10^7$ cells·mL$^{-1}$, using different concentrations of each substrate. We monitored the reaction at 3 minute intervals for a total of 60 minutes (Supplement 3A, 3B, 3C). We

calculated reaction rates as the slope of the optical density or fluorescence vs time curves. The results have been represented in Figure 3 as a function of substrate concentration for each of the substrates tested. In all cases the results indicate the existence of a saturation behavior as befits an enzyme-catalyzed reaction. The data obtained were used to perform a non-linear fit to a Michaelis-Menten function that allowed to estimate the $K_m$ for each substrate. In the case of ONPG (Figure 3A), the estimated $K_m$ was 1.12 ± 0.08 mM. In the case of CPRG (Figure 3B) $K_m$ was estimated at 12.84 ± 3.14 mM. The large error observed in this case is a consequence of the impossibility to test high concentrations as CPRG possesses a relatively low solubility in water (6 $mg \cdot mL^{-1}$, Burnham et al 2014) equivalent to 9.88 mM. Finally, $K_m$ for MUG derived from data shown in figure 3C is 0.309 ± 0.088 mM, the lowest of them all. The values of $K_m$ obtained are interesting because they provide some insight in the loss of sensitivity that can be expected when using suboptimal concentrations. In general, achieving 75% of $V_{max}$ for an enzyme assay requires using a concentration of substrate of 3 times $K_m$. Increasing this percentage to 80% or 90% of $V_{max}$ would require concentrations of substrate of 4 times $K_m$ and 9 times $K_m$ respectively. Obviously, in most cases a compromise must be reached between maximizing sensitivity by approaching $V_{max}$, and keeping the concentration of reagent at reasonable levels based on solubility and cost constrains. Based on such grounds we define an optimal concentration as the value of 4 times $K_m$ that allows reaching an 80% of $V_{max}$, and we will consider 80% of $V_{max}$ as the maximum attainable reaction rate in practice. In the case of ONPG, the optimal reagent concentration is thus 4.5 mM. Compared to this value, the concentrations of ONPG that have been used in the ß-gal assays for bacteria detection vary quite a lot and, while some authors use suitable concentrations (e.g. 10 mM, Apte et al 1995), many others use concentrations (2.21 mM, Griffith 2002; 2.5 mM, Sicard 2014; 3.65 mM, Schaefer 2016) quite below the optimum, indicating the existence of room for improving the sensitivity of their assay. CPRG is a rapid and sensitive substrate that has been used for the ß-galactosidase-based detection of *E. coli* (Sicard et al 2014). Although CPRG is used at concentration higher than ONPG of (10.24 mM, Burnham et al 2014; 8 mM, Chen et al 2015; 9.27 mM, Gunda et al 2016) the concentrations used are lower than the optimum value of 51 mM required to achieve 80% of $V_{max}$, but are the maximum concentrations that can be used considering that the solubility of CPRG in water is 9.88 mM. In the case of fluorometric ß-galactosidase coliform

and *E. coli* detection assays, MUG (MU-Galactoside) has been the substrate of choice. The concentration of MUG used in these assays ranges between 0.5 and 2.95 mM (Dudak et al 2008, Apte et al 1994, Davies et al 1998, Sicard et al 2014, Sotah et al 2020) all of them higher than the optimum 0.3 mM defined as 4 times $K_m$ and 80% of $V_{max}$.

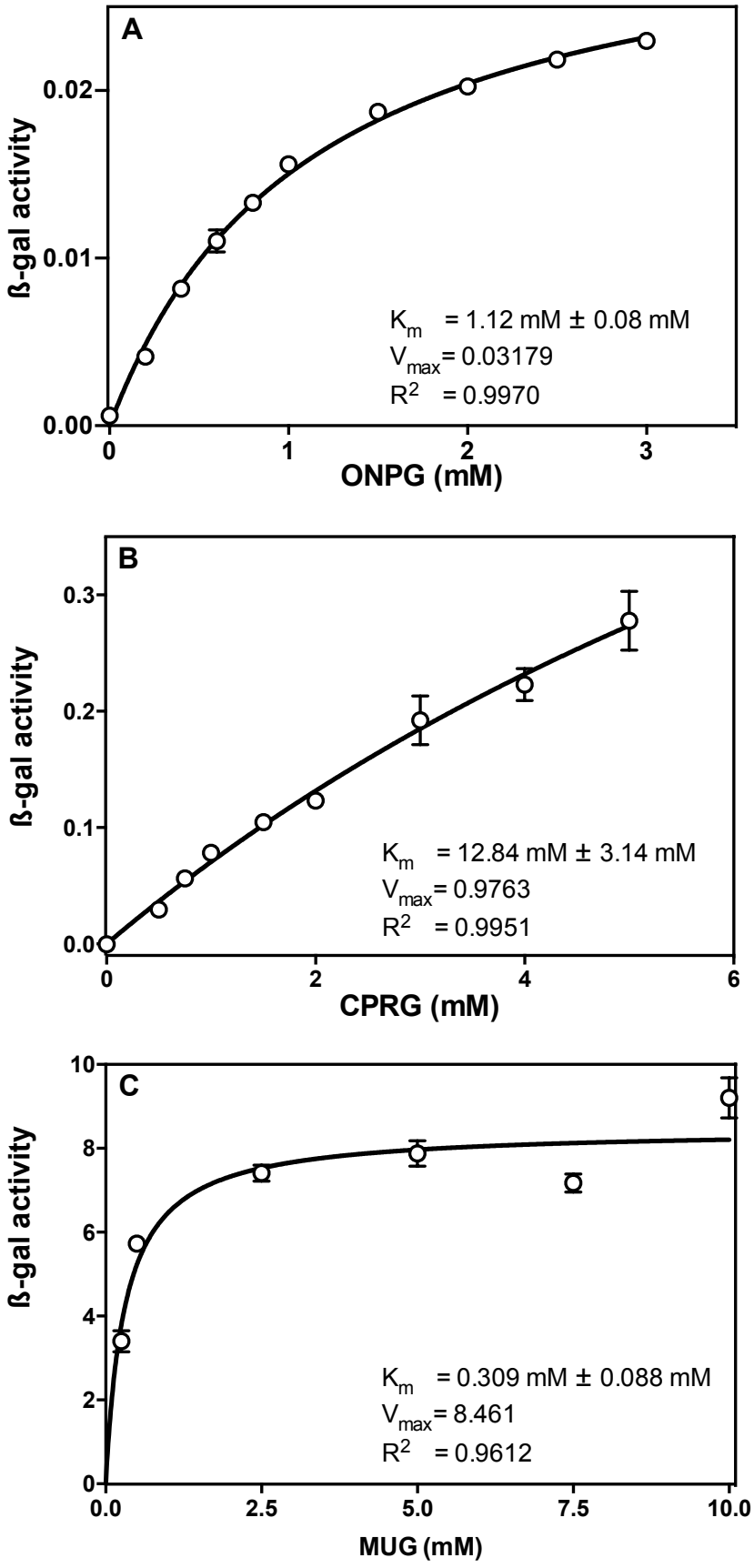


**Figure 3.** Effect of substrate concentration on reaction rates for each of the three substrates tested. The assays were carried out with IPTG induced samples (1 mM IPTG, 180 min) containing $10^7$

cells·mL$^{-1}$. Estimates of $K_m$ for each substrate plus minus their standard errors were obtained after nonlinear fitting of the Michaelis-Menten equation to each set of data. (**A**) ß-galactosidase activity (expressed as $A_{420}$·min$^{-1}$) as a function of ONPG concentration. (**B**) ß-galactosidase activity (expressed as $A_{574}$·min$^{-1}$) as a function of CPRG concentration. (**C**) ß-galactosidase activity (expressed as RFU·min$^{-1}$) as a function of MUG concentration. Error bars indicate the 95% CI of the ß-galactosidase activity calculated as the slope of Absorbance or Fluorescence vs time line at each substrate concentration (Supplement Figure 3A. 3B and 3C).

### 2.3.4 Choice of enzyme substrate and effect of $Na_2CO_3$ addition

In the Miller assay (Miller 1972) sodium carbonate was added as a final step to increase the pH of the mix, stop the reaction and ensure that ortho nitrophenol (ONP) reached its maximum extinction coefficient and displayed an intense yellow color. However, addition of $Na_2CO_3$ presents a dilemma when enzyme activity requires neutral pH and maximum color is obtained at alkaline pH. As a consequence this step is often omitted, particularly when attempting to develop one-step, compact, non-sequential systems for biosensing applications.

In order to assess to what extent the presence or absence of carbonate affected the output of the assay, we carried out an experiment in which we determined ß-galactosidase activity of fully induced cultures of *E. coli* diluted to a concentration of $10^5$ and $10^6$ when using CPRG and MUG as substrate and $10^6$ and $10^7$ when the substrate was ONPG. After 60 minutes the absorbance or fluorescence of the samples was measured before and after the addition of $Na_2CO_3$. The output of the experiment is shown in Figure 4. In the case of ONPG the results indicate a reduction of 48% when carbonate is omitted and measurements are performed at pH close to 7. This is consistent with the fact that the 2-nitrophenol solutions are colorless below pH 5 and reach their maximum hue at pH 8.5 and above (Zhang et al 2015). When the substrate was CPRG, carbonate addition had little effect on the readings with only a 2% reduction in the measured absorbance. This can be explained by the fact that chlorophenol red is a well-known pH indicator that changes color from yellow to red in the pH range 5.4 - 6.8 (Haynes 2016). In experiments carried out at neutral pH CPR already has maximum intensity as pH is beyond the highest point of its turning interval. Finally, when using MUG as a substrate we observed a 74% drop in the absence of $Na_2CO_3$. This is a striking difference which results from 4-methylumbelliferone having a fluorescence strongly dependent on pH, increasing in the range pH 6-pH 9 (Chen 1968, Graber et al 1986), and remaining stable at pH 9 and above. Leaving aside the effect of pH, data shown in Figure 4

indicate that CPRG is a much better choice than ONPG. While ONPG yields an absorbance reading of 0.11 after a 60 min incubation with samples containing $10^6$ cells of *E. coli* per mL, CPRG incubated with the same samples under exactly the same conditions displays an absorbance of 3.2, more than an order of magnitude higher. This agrees with the fact that chlorophenol red has an extinction coefficient 21-fold higher than ortho-nitrophenol as reported by Eustice et al (1991).

Summarizing, despite having been known for many years and being used extensively for bacteria detection and biosensing purposes, the ß-galactosidase assay is sometimes used below its maximum potential, mainly due to a poor choice of conditions. Table 3 collects the main variables analyzed in this study and pinpoints what we have found to be the optimal conditions as well as an assessment of the penalty derived from the choice of a particular set of suboptimal conditions. In most cases, making one wrong choice results in around 50% loss of sensitivity. Choosing the wrong value for 2 or 3 parameters decreases the assay response by 75-90%, seriously hampering its ability to carry out fast or sensitive detection of the target analyte.

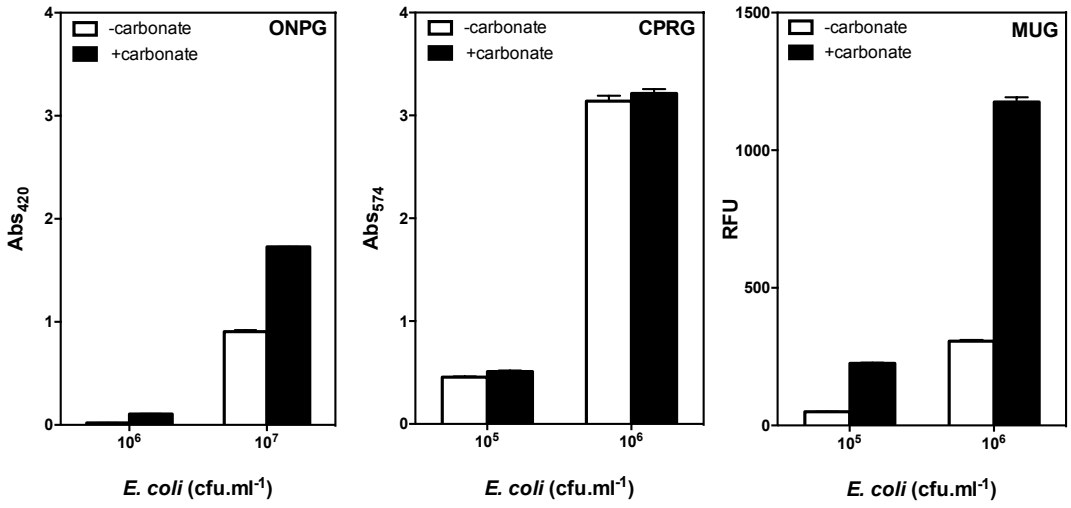


**Figure 4.** Effect of $Na_2CO_3$ addition on the response of the ß-galactosidase assay as a function of the substrate used. The assays were carried out using samples with different concentrations of fully induced cultures of *E. coli* ($10^6$ and $10^7$ cfu·mL$^{-1}$ for ONPG, and $10^5$ and $10^6$ cfu·mL$^{-1}$ for CPRG and MUG). Error bars represent the standard deviation of the data.

## 2.4 Conclusions

In this paper, we assess the effect of different variables (presence and concentration of enzyme inducer, induction time, cell permeabilization, type and concentration of enzyme substrate and pH) all critical for the outcome of the ß-galactosidase assay, particularly when used for biosensing applications such as detection of coliform bacteria or *E. coli*. Our results define a set of optimal conditions that maximize the response of the ß-galactosidase assay, and shed some light on the effects a poor choice of parameters when developing sensors or sensing procedures that rely on this enzyme. Maximizing the response of the assay requires an induction of the samples with 0.2 mM IPTG for 180 minutes, as well as the permeabilization or lysis of the cells using conditions that preserve the structure and activity of the enzyme. The choice of enzyme substrate is critical as different substrates yield products with vastly different extinction coefficients or fluorescence yields. The concentration of substrate used must be high enough (around 3 to 4 times $K_m$) to ensure that the activity measured is not substrate-limited. Finally, as the color/fluorescence of the reaction products is highly dependent on pH, care must be taken to ensure that pH at the time of reading is high enough to maximize color or fluorescence.

## 2.5 Acknowledgements

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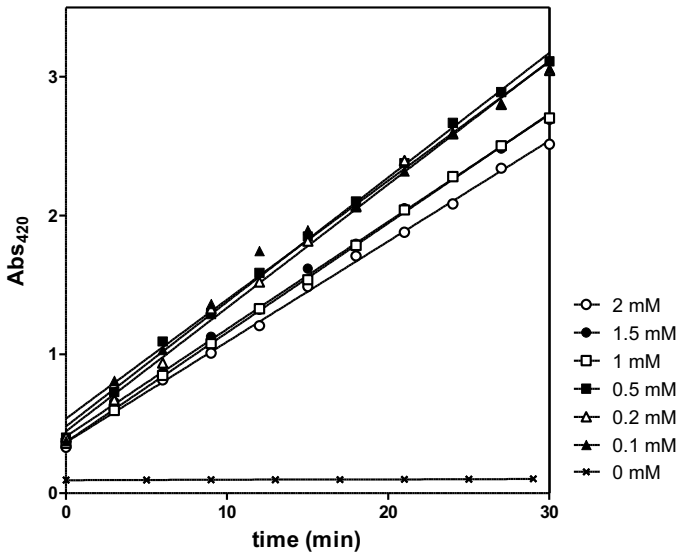
## Supplementary data

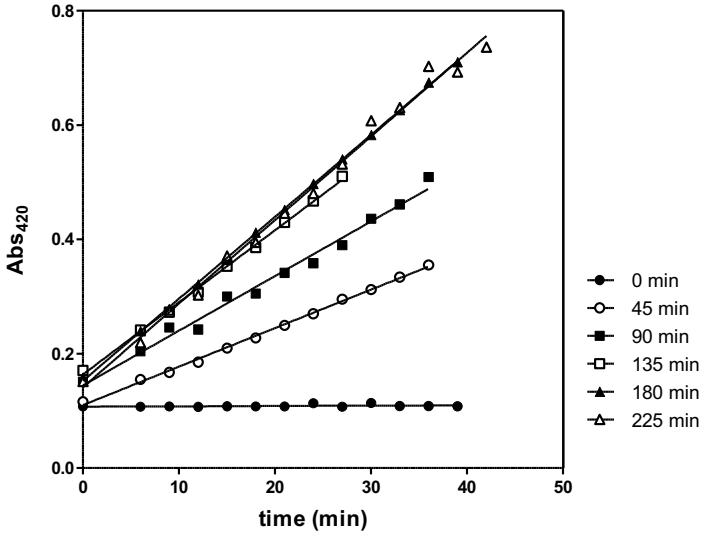
# Supplement 1A




| IPTG (mM) | Slope | 95% Confidence Interval | Y-intercept | $R^2$ |
|---|---|---|---|---|
| 2.0 | 0.07247 ± 0.0020 | 0.06823 to 0.07670 | 0.3664± 0.03685 | 0.9752 |
| 1.5 | 0.0773 ± 0.0019 | 0.07335 to 0.08132 | 0.4099 ± 0.03468 | 0.9806 |
| 1.0 | 0.0788 ± 0.0008 | 0.07701 to 0.08066 | 0.3691 ± 0.01588 | 0.9960 |
| 0.5 | 0.0896 ± 0.0013 | 0.08680 to 0.09258 | 0.4825 ± 0.02460 | 0.9952 |
| 0.2 | 0.0889 ± 0.0001 | 0.08539 to 0.09250 | 0.4468 ± 0.03090 | 0.9883 |
| 0.1 | 0.0857 ± 0.0023 | 0.08096 to 0.09048 | 0.5382 ± 0.04141 | 0.9776 |
| 0.0 | 0.0012 ± 0.0033 | 0.00137 to 0.00247 | 0.1722 ± 0.00465 | 0.7054 |

**Supplement 1A.** Effect of IPTG concentration on the kinetics of the ß-galactosidase reaction. The assays have been carried out with samples of *E. coli* containing $10^8$ cells·$mL^{-1}$ induced during 180 min with different concentrations of IPTG. The graph represents $A_{420}$ as a function of time for each of the concentrations tested. ß-galactosidase activities have been established as the slope of each line estimated by linear regression and are summarized at the bottom of the page together with the corresponding $R^2$ values.

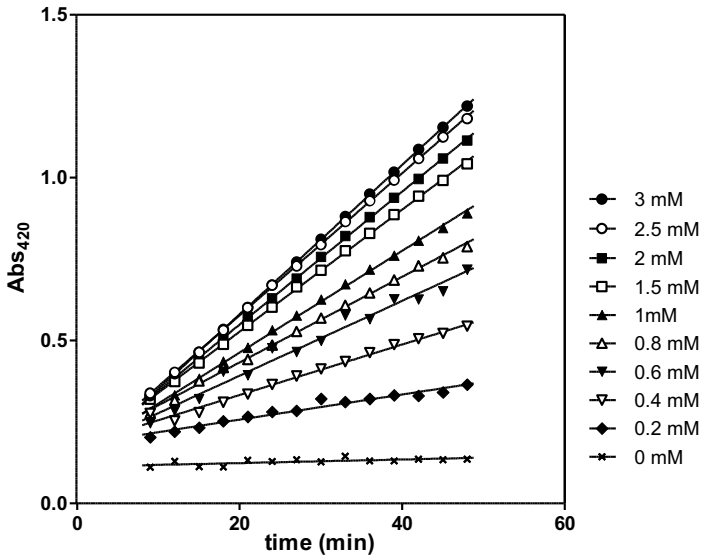
# Supplement 1B




| time (min) | Slope | 95% Confidence Interval | Y-intercept | $R^2$ |
|---|---|---|---|---|
| 225 | 0.0146 ± 0.0002 | 0.01404 to 0.01523 | 0.1411 ± 0.00748 | 0.9842 |
| 180 | 0.0143 ± 6.65e-005 | 0.01421 to 0.01448 | 0.1525 ± 0.00158 | 0.9992 |
| 135 | 0.0126 ± 0.0006 | 0.01123 to 0.01400 | 0.1635 ± 0.01131 | 0.9340 |
| 90 | 0.0095 ± 0.0003 | 0.00881 to 0.01032 | 0.1444 ± 0.00818 | 0.9514 |
| 45 | 0.0067 ± 0.0001 | 0.00065 to 0.00695 | 0.1100 ± 0.00235 | 0.9915 |
| 0 | 6.034e-005 ± 5.38e-005 | -4.87e-005 to 0.0001 | 0.1073 ± 0.00128 | 0.0328 |

**Supplement 1B.** Effect of induction time on the kinetics of the ß-galactosidase reaction. The assays have been carried out with samples of *E. coli* containing $10^8$ cells·$mL^{-1}$ induced with 1 mM IPTG for different periods of time. The graph represents $A_{420}$ as a function of time for each of the concentrations tested. ß-galactosidase activities have been established as the slope of each line estimated by linear regression and are summarized at the bottom of the page together with the corresponding $R^2$ values

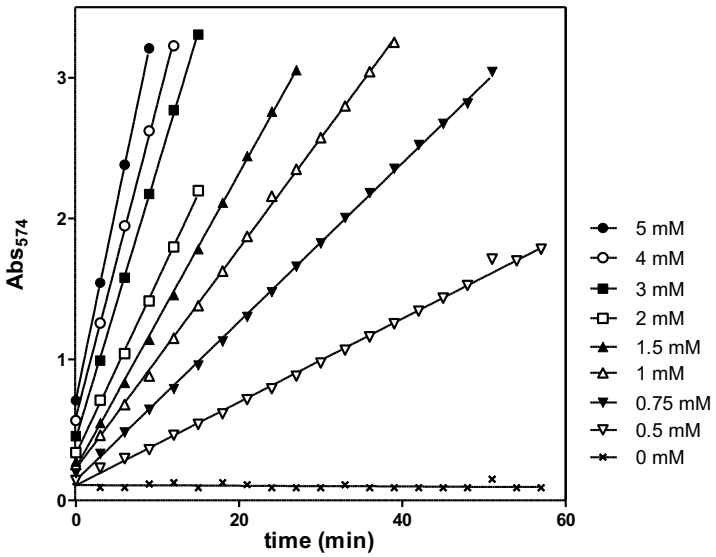
# Supplement 3A




| ONPG (mM) | Slope | 95% Confidence Interval | Y-intercept | $R^2$ |
|---|---|---|---|---|
| 3.0 | 0.02294 ± 8.093e-005 | 0.02278 to 0.02310 | 0.1216 ± 0.002506 | 0.9995 |
| 2.5 | 0.02182 ± 0.0001244 | 0.02156 to 0.02208 | 0.1409 ± 0.003853 | 0.9992 |
| 2.0 | 0.02040 ± 0.0002113 | 0.01998 to 0.02083 | 0.1409 ± 0.006541 | 0.9957 |
| 1.5 | 0.01872 ± 0.0001762 | 0.01837 to 0.01908 | 0.1527 ± 0.005456 | 0.9965 |
| 1.0 | 0.01562 ± 9.837e-005 | 0.01543 to 0.01582 | 0.1504 ± 0.003045 | 0.9984 |
| 0.8 | 0.01315 ± 0.0001812 | 0.01278 to 0.01351 | 0.1697 ± 0.005608 | 0.9925 |
| 0.6 | 0.01148 ± 0.0005215 | 0.01043 to 0.01254 | 0.1624 ± 0.01614 | 0.9238 |
| 0.4 | 0.007741 ± 0.000299 | 0.00713 to 0.0083 | 0.1780 ± 0.009268 | 0.9436 |
| 0.2 | 0.003860 ± 0.000213 | 0.003427 to 0.0042 | 0.1800 ± 0.006623 | 0.8906 |
| 0.0 | 0.0005515 ± 0.00020 | 0.000134 to 0.0009 | 0.1127 ± 0.006395 | 0.1513 |

**Supplement 3A.** Effect of ONPG concentration on the kinetics of the ß-galactosidase reaction. The assays have been carried out with samples of *E. coli* containing $10^7$ cells·mL$^{-1}$ induced with 1 mM IPTG for different periods of time. The graph represents $A_{420}$ as a function of time for each of the ONPG concentrations tested. ß-galactosidase activities have been established as the slope of each line estimated by linear regression and are summarized at the bottom of the page together with the corresponding $R^2$ values.

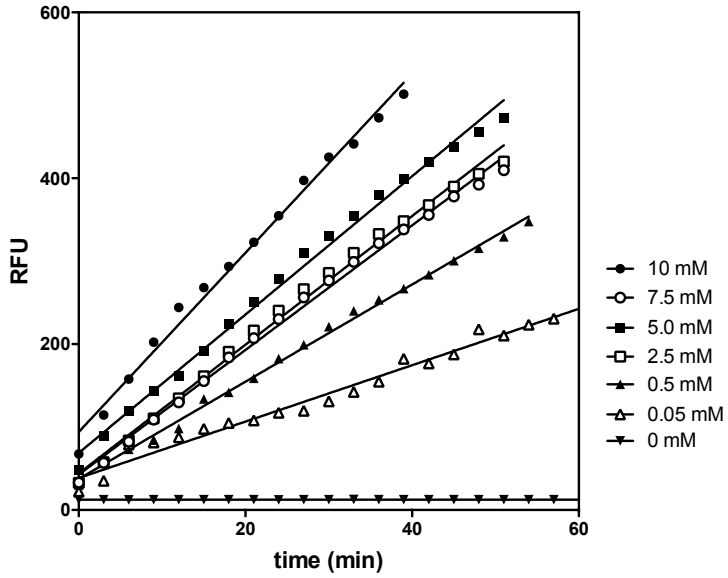
# Supplement 3B




| CPRG (mM) | Slope | 95% Confidence Interval | Y-intercept | $R^2$ |
|---|---|---|---|---|
| 5.0 | 0.2778 ± 0.01133 | 0.2526 to 0.3030 | 0.7111 ± 0.06357 | 0.9836 |
| 4.0 | 0.2229 ± 0.006357 | 0.2091 to 0.2366 | 0.5877 ± 0.04671 | 0.9895 |
| 3.0 | 0.1922 ± 0.009372 | 0.1713 to 0.2131 | 0.4382 ± 0.08512 | 0.9768 |
| 2.0 | 0.1232 ± 0.002305 | 0.1180 to 0.1283 | 0.3270 ± 0.02094 | 0.9965 |
| 1.5 | 0.1046 ± 0.002302 | 0.0998 to 0.109 | 0.2302 ± 0.03687 | 0.9866 |
| 1.0 | 0.07828 ± 0.00210 | 0.0740 to 0.0825 | 0.2229 ± 0.04820 | 0.9720 |
| 0.75 | 0.05631 ± 0.00041 | 0.05547 to 0.0571 | 0.1416 ± 0.01254 | 0.9971 |
| 0.5 | 0.02961 ± 0.00067 | 0.02825 to 0.0309 | 0.1057 ± 0.02255 | 0.9706 |
| 0.0 | -0.00025± 0.00022 | -0.00070 to 0.0002 | 0.1089 ± 0.00756 | 0.0205 |

**Supplement 3B.** Effect of CPRG concentration on the kinetics of the ß-galactosidase reaction. The assays have been carried out with samples of *E. coli* containing $10^7$ cells·mL$^{-1}$ induced with 1 mM IPTG for different periods of time. The graph represents $A_{574}$ as a function of time for each of the CPRG concentrations tested. ß-galactosidase activities have been established as the slope of each line estimated by linear regression and are summarized at the bottom of the page together with the corresponding $R^2$ values.

# Supplement 3C




| MUG (mM) | Slope | 95% Confidence Interval | Y-intercept | $R^2$ |
|---|---|---|---|---|
| 10.0 | 10.80 ± 0.4046 | 9.985 to 11.62 | 93.99 ± 9.284 | 0.9469 |
| 7.5 | 7.515 ± 0.1117 | 7.291 to 7.739 | 42.63 ± 3.336 | 0.9886 |
| 5.0 | 8.343 ± 0.1651 | 8.011 to 8.674 | 68.75 ± 4.931 | 0.9801 |
| 2.5 | 7.761 ± 0.09411 | 7.572 to 7.950 | 44.01 ± 2.812 | 0.9924 |
| 0.5 | 5.861 ± 0.08004 | 5.700 to 6.021 | 37.45 ± 2.530 | 0.9898 |
| 0.05 | 3.401 ± 0.1258 | 3.149 to 3.652 | 38.55 ± 4.414 | 0.9229 |
| 0.0 | 0.0012 ± 0.0033 | -0.0055 to 0.0080 | 12.39 ± 0.1187 | 0.0022 |

**Supplement 3C.** Effect of MUG concentration on the kinetics of the ß-galactosidase reaction. The assays have been carried out with samples of *E. coli* containing $10^7$ cells·$mL^{-1}$ induced with 1 mM IPTG for different periods of time. The graph represents fluorescence (RFU) as a function of time for each of the MUG concentrations tested. ß-galactosidase activities have been established as the slope of each line estimated by linear regression and are summarized at the bottom of the page together with the corresponding $R^2$ values.

# CHAPTER 3

## Specific detection of *Escherichia coli* using a phage-assisted ß-galactosidase assay

# 3 Specific detection of *Escherichia coli* using a phage-assisted ß-galactosidase assay

**Abstract**

Fast and reliable detection of microbial contaminants in food, water and environmental samples is critical for an efficient management of public health. This detection has relied traditionally in culture-based methods that require long incubations and are not well suited for situations that require a rapid response. Faster methods of bacterial detection have evolved during the last decades, targeting specific cell elements like nucleic acid sequences (PCR, qPCR, FISH), surface antigens (ELISA) or specific enzyme activities (ATPase, ß-galactosidase, ß-glucuronidase). Most of these methods provide reliable results although many of them have a number of drawbacks ranging from low sensitivity to the need of sophisticated equipment, the use of expensive reagents or the participation of highly skilled personnel.

In this paper we develop a non-specific ß-galactosidase assay that uses a T4 phage to provide selective detection and quantification of *E. coli*. The assay is performed in 96 well plates, uses MUG (4-methylumberlliferyl-ß-D-galactopyranoside) as the enzyme substrate and has a total length of 90 minutes. The method is able to detect 75 cells of *E. coli*. Under the conditions of the assay this corresponds to a concentration of $1.49 \cdot 10^3$ cells·mL$^{-1}$ of sample. For the analysis of field samples we produced an extended version of the assay that incorporates preconcentration and preincubation steps with a total running length of 7.5 hours. When tested with field samples and compared with Colilert-18 performed well, with a limit of detection of 96 cells·100 mL$^{-1}$.

## 3.1 Introduction

One of the most important challenges in the water sector is providing access to safe and high-quality drinking water for a healthy life. In 2018, the United Nations

Children's Fund (UNICEF) reported that about 570 million children worldwide lacked basic services for drinking water at schools (Drinking water, sanitation and hygiene in schools 2019). According to a report from the World Health Organization (WHO) in 2019, at least 2 billion people use drinking water sources contaminated with feces (WHO 2019 Drinking Water Fact Sheets), mainly due to the lack of facilities to ensure the quality of their water supply (WHO 2019 Drinking Water Fact Sheets). *E. coli* is the most widely used indicator for the existence of microbiological hazards as it warns of the presence of fecal contamination in water, mainly originating from feces of warm-blooded animals.

Common methods used for the detection of bacteria for environmental, industrial, and health applications include culture-based methods (Allen et al. 2004), immunological methods such as enzyme-linked immunosorbent assays (ELISA) (Ma et al. 2014, Wang et al. 2020), polymerase chain reaction (PCR) (Moreno et al. 2003, Sandhya et al. 2008), and enzyme-based (ß-D-galactosidase or ß-glucuronidase assays) methods (Satoh et al. 2020). Although some of the methods like ELISA or PCR have good sensitivity and a short response time, these methods are limited by some drawbacks such as false positives and negatives as well as by their inability to distinguish between live and death microorganisms (Ramirez-Castillo et al. 2015, Deshmukh et al. 2016). As a result, traditional culture-based methods, despite being time-consuming and labor-intensive (Chapman et al. 2016), are still commonly used as the primary diagnostic tools for detecting fecal indicators in water quality monitoring.

Some of the most the reliable methods extensively used in the microbiological analysis of water are based on the detection of *ß-D-galactosidase* specific from coliforms, or *ß-glucuronidase* specific for *E. coli*. The assays rely on the use of chromogenic or fluorogenic galactoside or glucuronide precursors that develop color or fluorescence after cleavage by the target enzymes (Goodridge and Griffiths 2002). One of the main challenges in these assays is allowing contact between the extracellular substrate and the intracellular enzyme. Long time methods (18-24 h) rely on the transport systems of the microorganism to transport of the substrates into the cell. Because the assay has long duration and cleavage of the substrate occurs concomitant to growth, there is ample time to generate a signal strong enough for detection. On the other hand, whenever the enzyme assays are implemented in a fast format, contact between the enzyme and the substrate must be facilitated using

membrane permeabilization or cell lysis. Several techniques can be used to disrupt the bacterial cells, including sonication, addition of toluene, chloroform, sodium dodecyl sulfate (SDS), Polymyxin B, commercial reagents (PopCulture®, B-PER®), and bacteriophages (Feliu et al. 1997, Griffith and Wolf 2002, Schaefer et al. 2016, Miller 1972). The lysis methods allow access of the enzyme substrate to the target intracellular enzymes but, except for the case of bacteriophages, they lyse the cells in a highly unspecific way. The use of bacteriophages as lysis reagents provides an extra layer of specificity that can be used to approach detection using relatively unspecific reagents.

In this study, we focus on the development of a phage-assisted enzyme-based assay for the specific detection of *E. coli*. The assay uses lytic phages to release the content of the target organisms, coupled to a colorimetric/fluorometric ß-galactosidase enzyme kinetic assay. Phages are ideal for specific cell lysing as they are inexpensive, easy to prepare, and provide robust and specific lysis of the target cells (Tawil et al. 2014, Richter et al. 2018), enabling detection of specific bacteria in samples containing a mix of microorganisms. In this study we compare the efficiency of a phage treatment with the efficiency of a commercial non-specific lysis reagent (PopCulture®) on the release of the intracellular target enzyme and its availability for the enzyme assay. Using this principle, we have developed a full-fledged detection assay that has been tested in the lab in terms of sensitivity and limit of detection and has been later on validated for qualitative application using real world samples.

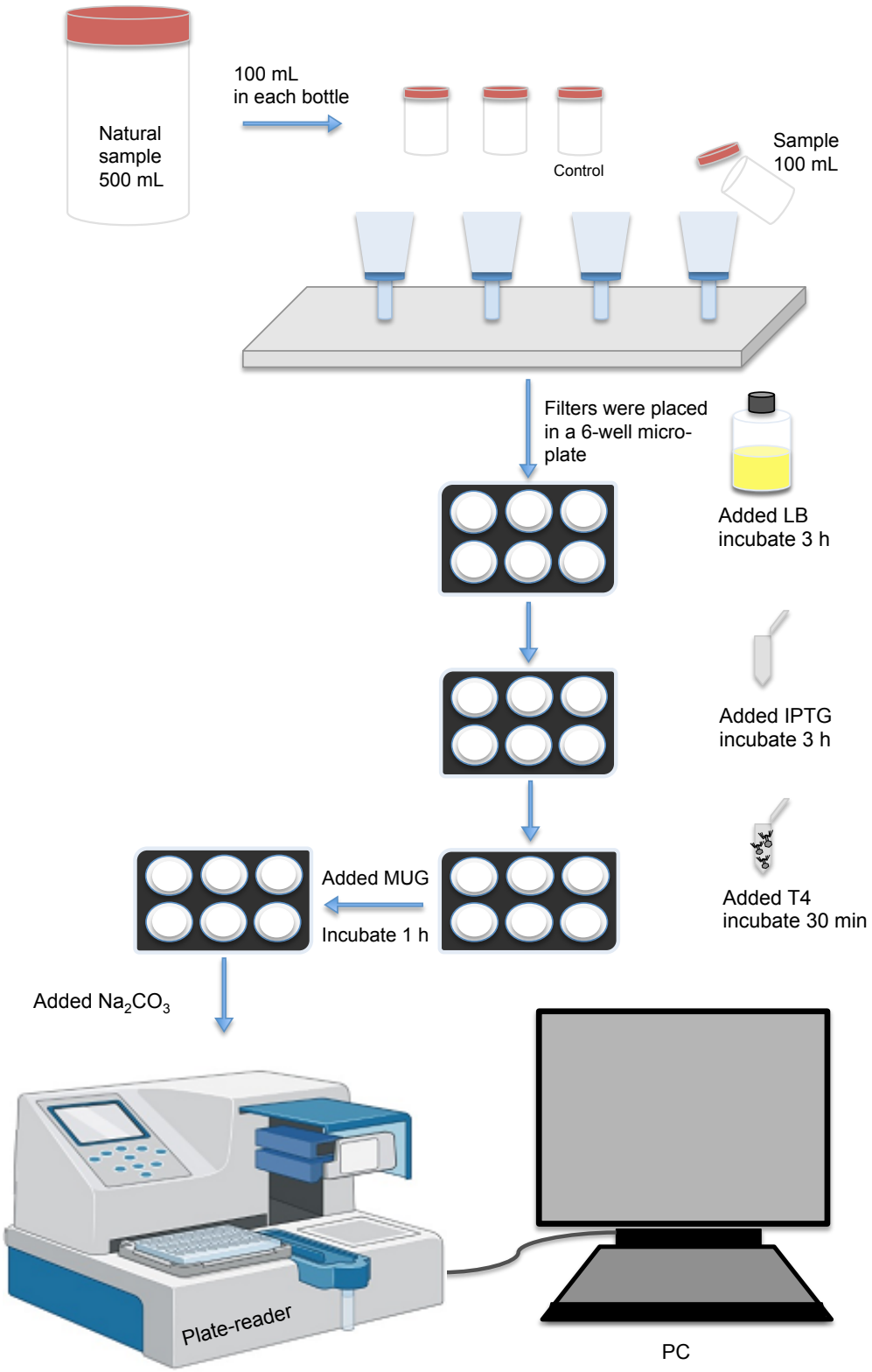



**Figure 1.** Diagram of the experimental procedure applied to analyze environmental samples using a phage-based ß-galactosidase assay.

## 3.2 Material and method

### 3.2.1 Microorganisms and growth conditions

*Escherichia coli* strains DSM613 and MC1061 were used throughout the work. DSM613 is a wild type strain, while MC1061 is a mutant that does not express ß-galactosidase under normal conditions (Casadaban and Cohen 1980). Bacterial cultures were grown overnight in Luria-Bertani (LB) liquid medium using an Infors HT Ecotron orbital incubator operating at 100 rpm at 37$^{o}$C. Bacteriophage T4 was kindly provided by Dr. M. Llagostera from the Department of Genetics and Microbiology of the Autonomous University of Barcelona.

### 3.2.2 Bacteriophage propagation

Phage lysates were prepared following the protocol of Bonilla et al (2016) using *E. coli* wild type strain DSM613 or MC1061 (mutant strain unable to express *lacZ*) as hosts. 10 mL of an *E. coli* overnight culture in LB broth were added to 100 mL of LB broth supplemented with $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM). The culture was incubated in a shaking incubator at 37$^{o}$C for 1 hour. After that, the culture was infected with 0.1 mL of a high titer T4 bacteriophage suspension containing > $10^9$ pfu/mL. Once lysis was completed, the culture was centrifuged at 4000 g for 20 min. The supernatant was filtered through a 0.22 µm cellulose acetate membrane filter (Whatman) and subsequently treated with chloroform to remove lipids. The resulting suspension was concentrated by ultrafiltration using Amicon Ultra-15 centrifuge tubes with a nominal molecular weight limit of 100 kDa. The concentrate was stored in SM buffer at 4°C (Bonilla et al 2016). The concentration of bacteriophage was determined as plaque-forming units (pfu) using the double layer agar method described by Adams (1959) after serially diluting the lysate in PBS buffer. Prior to their use, virus suspensions were diluted in LB to achieve the desired final concentration.

### 3.2.3 Preparation of *E. coli* standards

Laboratory standards of *E. coli* for the calibration of the assay were prepared inoculating 0.2 mL of an overnight culture in 5 mL of fresh LB medium and incubating under aerobic conditions at 37 $^{o}$C for approximately 1 hour until reaching the middle of the stationary phase ($OD_{600}$ of 0.4-0.5). At that point the culture was supplemented with 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) and incubated

for a further 3 hours to fully induce the expression of ß-galactosidase and maximize the enzyme content. After induction the culture was centrifuged at 4000 g for 10 minutes and then resuspended in PBS buffer. The fully induced culture was diluted to an $OD_{600}$ of 0.3 ($10^8$ cfu·mL$^{-1}$) and was serially diluted in phosphate buffer (0.1 M) to obtain the required cell concentration.

### 3.2.4 Microscopy assessment of lysis and permeabilization treatments

In order to assess the efficiency of the lysis procedure using PopCulture and T4, samples treated with either of the two reagents were filtered through 0.2 µm polycarbonate filters and the filters were stained with the Invitrogen LIVE/DEAD™ BacLight™ Bacterial Viability Kit, for microscopy. The stained filters were observed in a Zeiss AXIO Imager A1 fluorescence microscope. In the preparations, live bacteria stained with SYTO9 display green fluorescence (630 nm) while dead bacteria, retaining their cell wall but with the membranes permeabilized are stained with propidium iodide and present red emission (530 nm). Cells which have been completely lysed do not show up in the preparations as their cell walls have been destroyed and their contents have been dispersed in the medium.

### 3.2.5 Microplate-based ß-galactosidase assay

The method used for the ß-galactosidase assay is an adaptation of the 96 well plate method described by Schaeffer et al (2016) as modified by Hosseini and Mas (2021). The modification accounts for the testing different substrates as well as different lysis and permeabilization reagents. To carry out the assay, 50 µL of the appropriate dilution of a fully induced *E. coli* culture were added to a microplate well containing 120 µL Z-buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM ß-mercaptoethanol, pH=7), 20 µL of of lysis reagents and 10 µL PBS. The reaction was started by adding 10 µL of ß-galactosidase substrate. To provide specific disruption of the envelope and prevent organisms different from *E. coli* contributing false positives, we have used bacteriophage T4 as the lysis reagent. As a control for the efficiency of phage lysis we have used a non-specific, commercial, detergent-based reagent (PopCulture). As substrates for the reaction we used MUG (4-methylumbelliferyl-ß-D-galactopyranoside), ONPG (O-nitrophenyl-ß-D-galacto-pyranoside) and CPRG (Chlorophenol red-ß-D-galactopyranoside) depending on the experiments.

Each of the samples was assayed as 3 independent replicates. In addition, in all cases, a control without *E. coli*, was simultaneously carried out. The reaction was carried out using 96-well plates incubated at 37 $^{o}$C in a Varioskan Flash Plate reader (Thermo Scientific, Massachusetts, USA) for 1 hour. OD (optical density) at 420 and 574 nm for ONPG and CPRG and fluorescence at 360/438 nm (excitation/emission) for MUG were recorded at 3 min intervals with shaking steps set between recordings. Final readings were taken at 60 minute after addition of carbonate, if required to increase pH and maximize the signal of the reaction product.
The Limit of detection (LOD) was calculated as:

$$LOD = 3.3 \cdot \sigma_{BLANK}$$

where $\sigma_{BLANK}$ is the standard deviation (SD) of the response at zero or extremely low concentrations.

### 3.2.6 Analysis of field samples

The type and limits of acceptable standards for the microbiological quality of water varies depending on the type of use as well as on the country. In most cases, however, standards for drinking water require absence of *E. coli* in a 100 mL sample (Wen et al. 2020). Certifying absence of *E. coli* in 100 mL samples using a short time assay requires both concentrating the samples through filtration, and amplifying the concentrate by preincubation previous to the assay. The method of analysis used for field samples uses both approaches
Water samples from natural sources were collected using sterile 2 L disposable plastic bottles, carried to the lab and analyzed immediately thereafter. Sample treatment has been represented in Figure 1. For the analyzes, 100 mL subsamples were filtered through 0.22 µm sterile nitrocellulose filters and the filters were placed in a 6-well plate. After that, 1.715 mL of LB broth was added to each well completely covering the filter. The plates containing filters and LB broth were incubated in an orbital incubator (100 rpm) at 37 $^{o}$C during 3 hours to allow for growth of the bacteria collected at the filter surface. After this preincubation, IPTG was added to each well at a final concentration of 1 mM and a second 3 hour incubation was performed to allow for further bacterial growth, and maximize ß-galactosidase induction. Once the induction period reached its end 200 µL of a T4 lysate (>$10^{9}$ pfu.mL$^{-1}$) were added to each well. Lysis was allowed to proceed for 30 minutes after which 0.1 mL of MUG

(5 mM) were added to the wells. The reaction was allowed to proceed in an orbital incubator (100 rpm) at 37 $^{o}$C for 60 minutes after which 500 µL of 1M $Na_2CO_3$ were added to increase the pH, stop the reaction and maximize fluorescence of the reaction product. At this point the samples were inspected under UV light for a qualitative reading. After this, 210 µL of each filter overlay were transferred to a 96 microwell plate for quantitative determination of the fluorescence at 360/438 nm. Overall, the procedure lasted 7.5 h. As a reference method all samples were also analyzed using Colilert-18 (IDEXX Laboratories 2013).

## 3.3 Results

### 3.3.1 Interference of lysis and permeabilization reagents with the ß-galactosidase assay.

Detection of bacteria using enzyme-based assays requires careful monitoring of the degradation of specific substrates by the action of the target enzyme. In the case of ß-galactosidase the enzyme catalyzes the hydrolysis of the galactoside moiety of the substrate releasing colored (o-nitrophenol or chlorophenol red), or fluorescent (4-methylumbelliferone) products that are used for detection.

One of the main concerns when carrying out this type of enzyme-based detection is that hydrolysis of the substrate occurs spontaneously or as result of the presence of some component present in the medium. While most of the components of the Z-buffer used for the assay (Miller 1972) have been tested time and again and do not interfere with the results, the lysis and permeabilization agents that we used for this work (PopCulture and bacteriophage T4) could potentially contribute to hydrolyze the galactoside bond. Therefore, we decided to carry out a control experiment without bacteria to assess the extent of the effect of the lysis reagents on substrate (2 mM ONPG) hydrolysis. The results of this experiment can be found in Figure 2.

As expected, increase of optical density in the control without lysis reagents is virtually zero, indicating that spontaneous hydrolysis of the reagent is inexistent during a 80 minutes incubation. Utilization of the detergent-based reagent PopCulture shows a slight increase in optical density that suggests a minor effect of this reagent on ONPG hydrolysis. But the most striking result was found when adding a T4 lysate. In this case, a steady increase in $OD_{420}$ was observed indicating

a considerable hydrolysis of ONPG. Since the there is no known mechanisms through which the T4 phage can catalyze the hydrolysis of ONPG, we concluded that the hydrolytic activity came from ß-galactosidase carried over from the wild type (DSM613) *E. coli* culture used for propagation. The purification method used concentrates the phage by ultrafiltration using high molecular weight (100 kDa) cutoff membranes, but it turns out that ß-galactosidase from *E. coli* is found as a 465 kDa tetramer (Jacobson and Matthews 1992) that was retained and concentrated along with the bacteriophage during the purification procedure. To overcome this problem we prepared a new lysate in which the phage was propagated using *E. coli* (MC1061), a mutant unable to express ß-galactosidase due to a lack of promotor. The effect of using this phage in the assay is also shown in Figure 2. The results indicate no effect in ONPG hydrolysis and thus, absence of phage-related ß-galactosidase activity. The rest of the experiments were, therefore, performed with this ß-galactosidase-free phage.

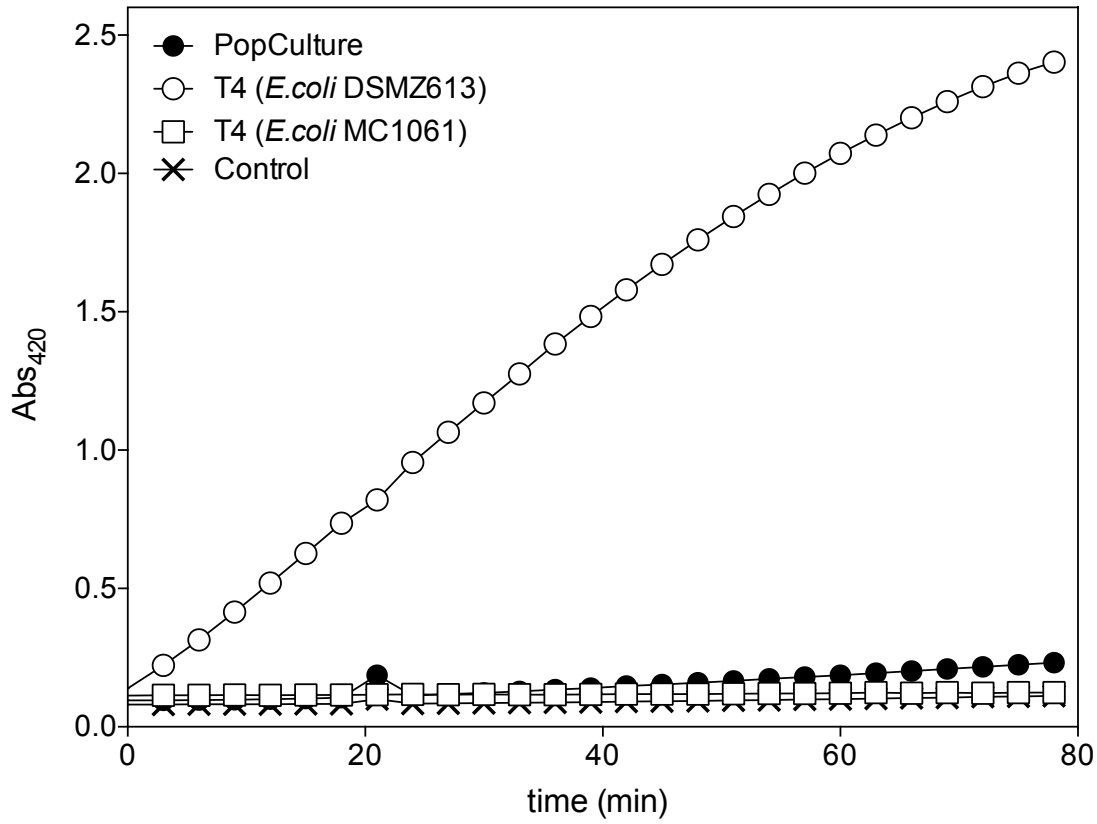



**Figure 2.** Effect of lysis agents on the hydrolysis of the ß-galactosidase substrate: (●) PopCulture, (○) T4 (propagated in *E. coli* DSMZ613, (□) T4 (propagated in *E. coli* MC1061), (×) Control (reaction mix without lysis reagent). Determinations were carried out in cell-free reaction mixes using ONPG as a substrate. Error bars represent the standard error of the data.

### 3.3.2 Comparison of phage-based and detergent-based permeabilization.

In order to assess the efficiency of T4 phage treatment as an organism-specific permeabilization step, we compared it with a standard detergent-based commercial reagent (PopCulture®) looking at both, procedural and mechanistic aspects. From a procedural point of view we wanted to determine: **a)** if phage addition had an instantaneous effect on cell permeabilization and, if that was not the case, **b)** what was as time required between phage addition and intracellular enzyme availability and what preincubation should be used between phage addition and the performance of the assay. Also, from a mechanistic point of view we wanted to establish **c)** if substrate-enzyme contact was promoted through membrane permeabilization and substrate entry inside the cell (enzyme activity was associated to particulate phase) or, on the contrary, through cell lysis and enzyme release into the extracellular medium (enzyme activity was found in the liquid phase).

#### 3.3.2.1 Procedural aspects. Time required for permeabilization.

In the assay protocol used in this work, both, permeabilization reagents and enzyme substrate are present in the reaction mix together with the sample is added and the reaction starts. This works relatively well when using detergent or solvent-based reagents with a quick action mode. However, we wanted to test if that was also the case when using T4 as a lysis agent. Cell lysis as a result of phage infection usually requires that the phage infects the cell, multiplies and finally lyses the cell to release the phage progeny. However, it has been long known that T even phages can induce *"lysis from without"*. The term was coined by Max Delbruck in 1940, and refers to a phenomenon of adsorption-induced lysis dependent on tail protein gp5 which displays lysozyme activity (Abedon 2011), that occurs when cells are infected at high multiplicities (MOI > 100) and phage titers higher than $10^9$ pfu/mL. Thus, we carried out an assay in which we added the lysis agent (PopCulture or T4) at time zero to a reaction mix that contained CPRG as enzyme substrate. The reaction was monitored every three minutes during the following 60 minutes. The results have been represented in Figure 3A. When PopCulture was used, the reaction proceeded at a constant rate from the beginning, indicating efficient and immediate permeabilization

of the cells and unrestricted access of the substrate to the enzyme. When T4 was used, the results were different. There was no detectable ß-galactosidase activity during the first 30 minutes. After that, the slope of the curve increased indicating a progressive permeabilization of the cell, with maximum activity achieved 10 minute later, at time 40 minutes. The results indicate quite clearly that 30 minutes is enough to reach maximum signal when using an instant detergent-based permeabilizing reagent like PopCulture, but it is insufficient to provide minimum lysis when using T4. This means that specific permeabilization using phages will require a preincubation period prior to the assay to allow full release of the intracellular enzyme.

Figure 3B shows the results obtained when adding a 30 minute preincubation before the assay for both T4 and PopCulture. Although in both cases ß-galactosidase activity is present at the beginning of the assay, the results indicate higher activity (as evidenced by a steeper slope) in the culture lysed with T4. This was not completely unexpected as it was also observed in Figure 3A. The higher activity is not a result of a more efficient lysis, but probably is a result of the fact thar the sample treated with T4 has lysed 30 minutes later, time enough to allow for a duplication of an *E. coli* culture growing under the conditions of the assay.

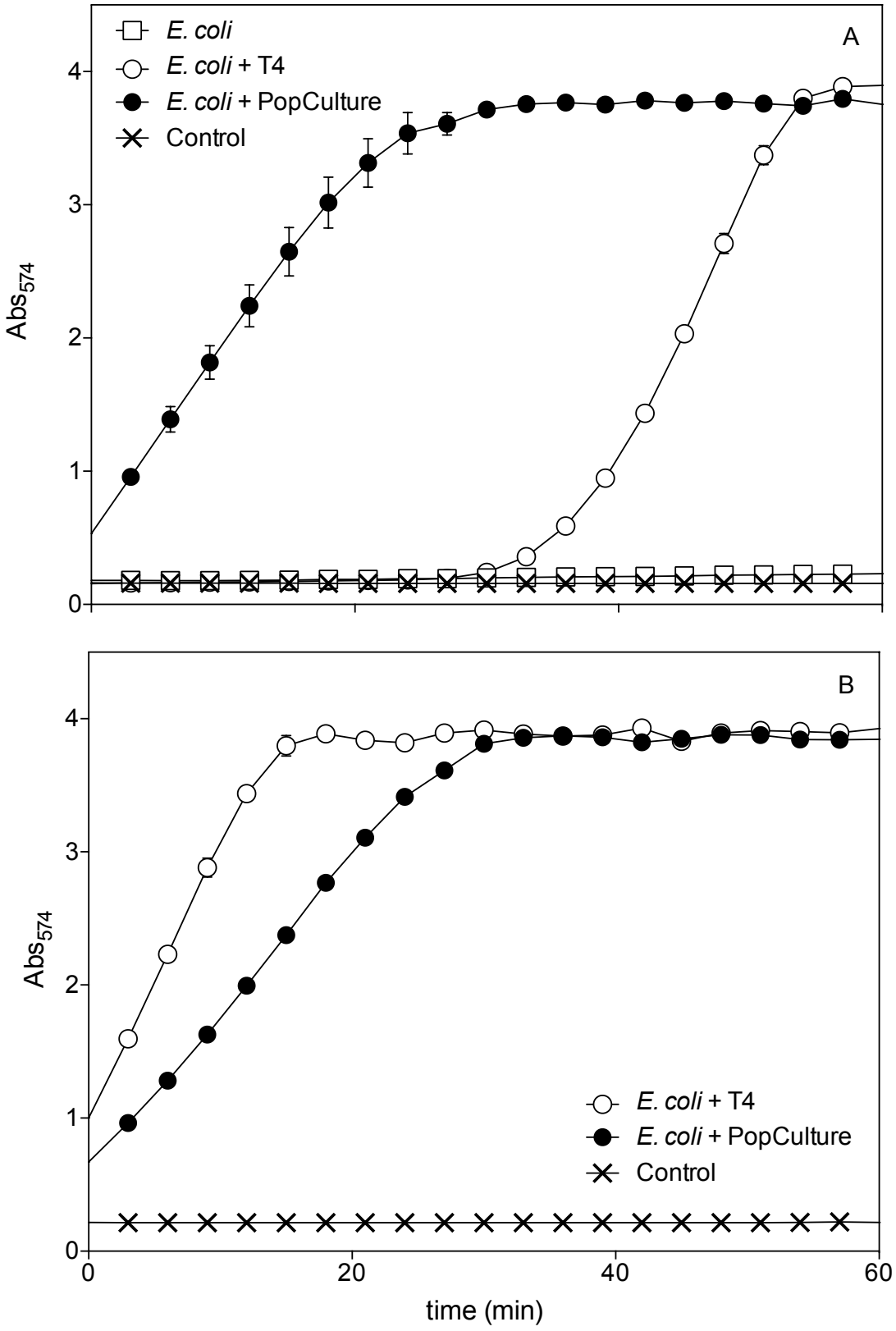



**Figure 3.** Effect of lysis preincubation on the kinetics of the ß-galactosidase reaction in cultures of *E. coli* treated with PopCulture and with T4. **A.** Lysis reagents and ß-galactosidase substrate were added simultaneously and recording of the reaction started immediately thereafter: (●) *E. coli* ($10^7$

cells·mL$^{-1}$ + PopCulture), (○) *E. coli* ($10^7$ cells·mL$^{-1}$ + T4), (□) *E. coli* ($10^7$ cells·mL$^{-1}$ without lysis reagent), (×) Control (reaction mix without cells). **B.** Lysis reagents were added and the reaction mix was incubated for 30 minutes. After that period the ß-galactosidase substrate was added and recording of the reaction started: (●) *E. coli* ($10^7$ cells·mL$^{-1}$ + PopCulture), (○) *E. coli* ($10^7$ cells·mL$^{-1}$ + T4), (×) Control (reaction mix without cells) Error bars represent the standard error of the data.

Overall the results obtained with T4 were somewhat unexpected, as *"lysis from without"* did not occur event though we infected the culture with a MOI of (400 pfu/bacterial cell) using T4 which is one of the phages in which this phenomenon is regularly observed. The cause for this probably can be found in the fact that both, bacteria and phage were added as part of the reaction mix when preparing the ß-galactosidase assay. Under these conditions, the concentration of phage in the reaction mix ended up being somewhat lower than $10^9$ resulting in slower adsorption kinetics and the possibility that bacteria develop resistance to lysis from without, a phenomenon observed when massive phage attachment occurs a few minutes after primary infection (Visconti 1953, Abedon 2011).

3.3.2.2 Mechanistic aspects: Permeabilization vs Lysis.

The type of mechanism involved in allowing the access of the substrate to the enzyme is highly relevant if we want to use phages as an element providing specificity to an enzyme assay. If cells are completely lysed, intracellular enzymes are released from the cells and, thus, can be find in the liquid phase. If, on the contrary, cells have been only permeabilized to allow entry of the substrate, enzyme activity will remain in the particulate fraction. This is particularly relevant when analyzing natural samples, as discrimination between enzyme activity coming from a small number of specifically phage-lysed target organisms or from a large number of intact non-target organisms also present in the sample requires excluding non-lysed cells by filtration. To check whether enzymes were found in the liquid or in the particulate fraction we carried out an experiment in which we included a filtration step (Whatman Puradisc FP 30 mm Cellulose Acetate Syringe Filter, 0.2 µm), between the phage/detergent treatment and a CPRG-based ß-galactosidase assay. The results, expressed as the increase in optical density at 574 nm resulting from CPRG hydrolysis, have been represented in Figure 4 as a function of time. As can be observed, the filtrate of the phage-treated sample displays a strong activity, comparable to the activity found in a non-filtered T4 treated culture of *E. coli* (see

Figure 3B). On the contrary, the filtrate of a PopCulture treated sample (Figure 4) does not display any ß-galactosidase activity, as the values of absorbance at 574 nm are virtually indistinguishable from the control. The same sample in the absence of filtration (Figure 3B) shows a marked ß-galactosidase activity that brings $OD_{574}$ up to 4 in a 30 min. period. The results indicate clearly that phage treatment completely releases the intracellular enzyme into the medium while, on the contrary, the use of a detergent-based reagent like PopCulture permeabilizes the membrane and allows the entry of substrate into the cell, but while retaining 100% of enzyme activity in the particulate fraction.

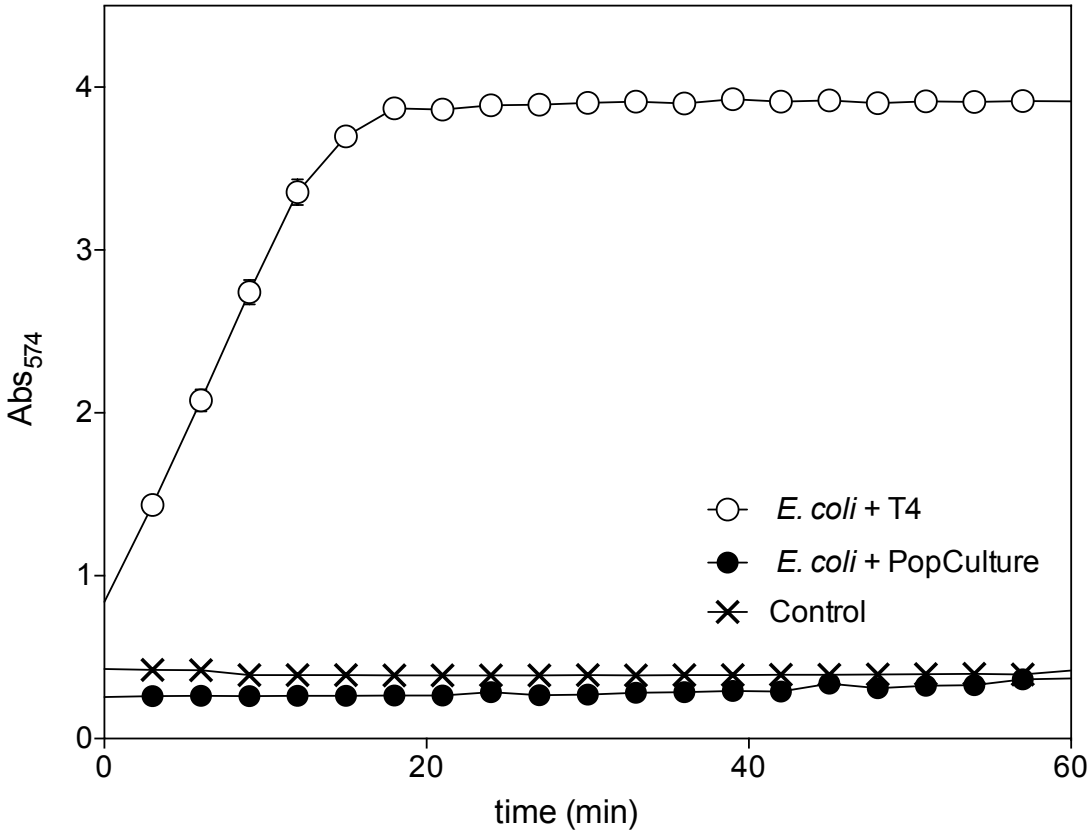



**Figure 4.** Effect of sample filtration on the ß-galactosidase kinetics of *E. coli* samples ($10^7$ cells·$mL^{-1}$) treated with bacteriophage T4 (○) and with PopCulture (●), (×) Control (reaction mix without cells). Error bars represent the standard error of the data.

To support these observations we carried out fluorescence microscopy observations of Live/Dead stained samples of *E. coli* treated with T4 (Fig. 5 upper panel) and PopCulture (Fig. 5 lower panel). The T4 treated sample is devoid of cell remains that could be stained using the Live/Dead kit. The PopCulture treated sample shows orange stained cells corresponding to cells with compromised membrane integrity

that were penetrated and stained by the propidium iodide component of the Live/Dead stain.

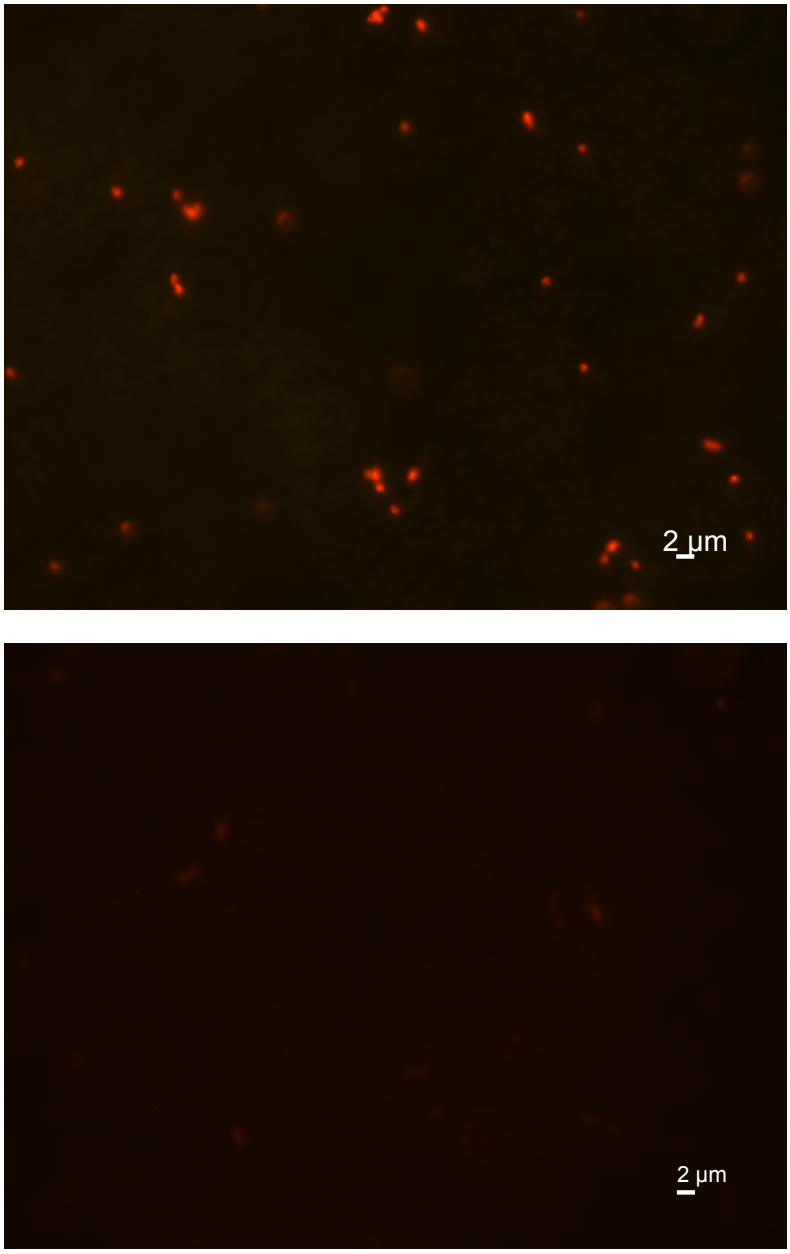



**Figure 5.** Fluorescence microscopy images of Live/Dead stained *E. coli* cultures treated with permeabilizing agents. **Upper panel.** Culture treated with PopCulture. Orange color indicates loss of membrane integrity. Cells, nonetheless, maintain their cell wall and structural integrity. **Lower panel.** Culture treated with T4. Cells have been completely lysed and no structure can be observed.

### 3.3.3 Calibration using standardized cultures.

The phage-based assay as described in the methods section was tested with samples of *E. coli* of known concentrations in order to calibrate and assess the dynamic range of the assay as well as determine its limit of detection. Testing was carried out colorimetrically and fluorometrically using CPRG and MUG respectively as enzyme substrates. The assay response was monitored at 3 minute intervals, either as $OD_{574}$ for CPRG, or as relative fluorescence units (RFU) for MUG. Final values, recorded after 60 minutes of reaction, were subject to a logarithmic transformation. The results have been plotted in Figures 6 and 7 as a function of the logarithm of cell concentration.

Figure 6 shows the calibration curve of the assay when using CPRG as the assay substrate for samples containing *E. coli* at concentrations ranging between $10^3$ and $10^8$ cells·mL$^{-1}$. Data have been used to fit a linear function. The function was fitted in the $10^5$ to $10^6$ cells·mL$^{-1}$ interval as higher concentrations yielded values of $OD_{574}$ that saturated the plate reader and yielded values well outside the linear range. The limit of detection, calculated as described above, is $5.35 \cdot 10^4$ cells·mL$^{-1}$, corresponding to an $OD_{574}$ of 0.273.

In a similar way the results obtained when using MUG as enzyme substrate have been represented in Figure 7 as a function of cell concentration. Since the product of MUG hydrolysis, methylumbelliferone emits fluorescence at 438 nm, the response of the assay has been recorded as Relative Fluorescence Units (RFU). The concentrations tested range between $10^1$ and $10^8$ cells·mL$^{-1}$ and both, the logarithm of cell concentration and the logarithm of the assay response have been used to fit a regression line in the interval $1.5 \cdot 10^3$ - $3 \cdot 10^6$ ($R^2$=0.993) that provided a 3 log detection range. The limit of detection, calculated from the mean and the standard deviation of the blank was $1.49 \cdot 10^3$ cells·mL$^{-1}$, corresponding to a fluorescence of 18 RFU. As the limit of detection when using MUG was considerably lower than when using CPRG, we decided to use MUG for the validation of the method using field samples.

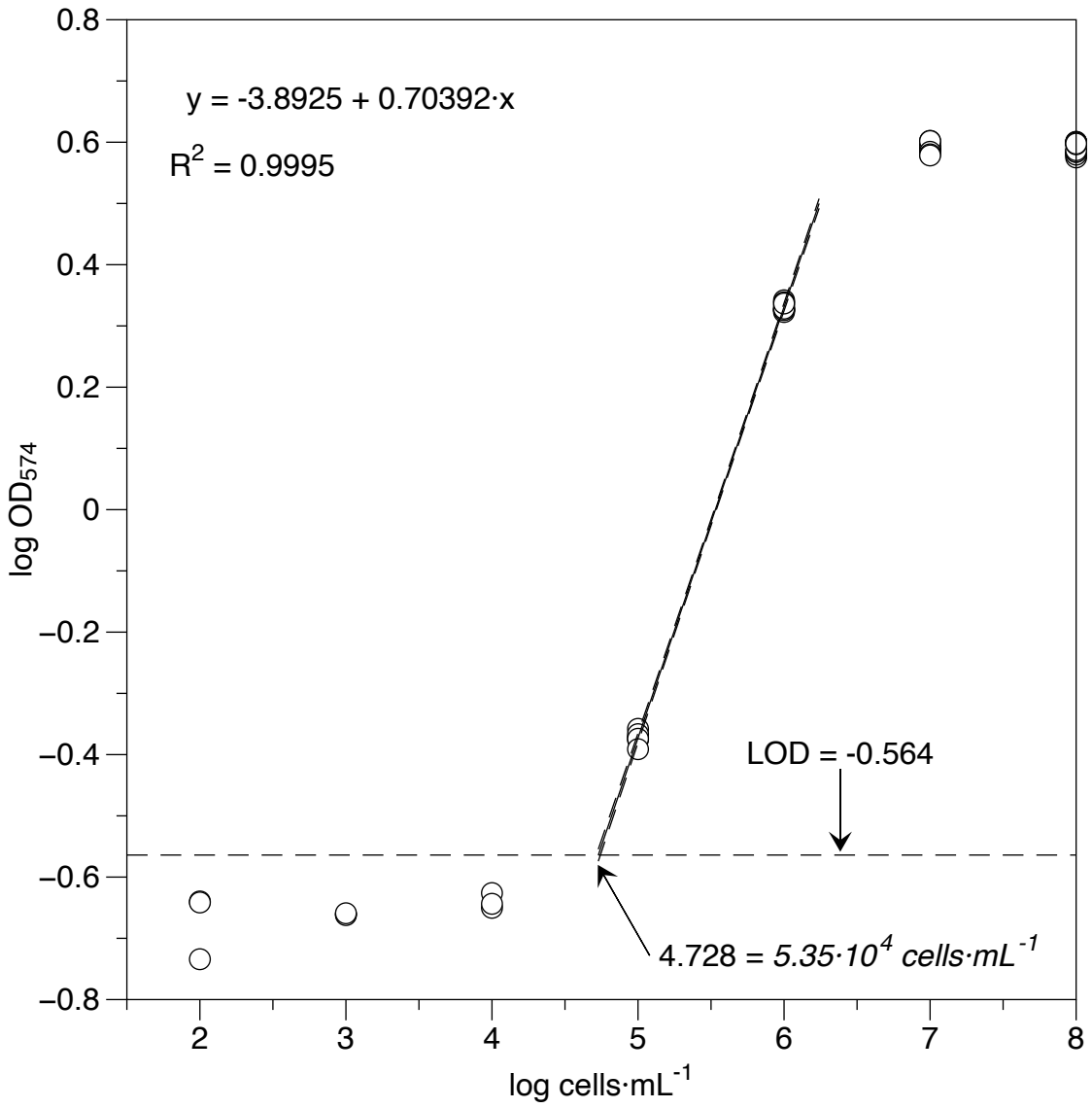



**Figure 6.** ß-galactosidase-based detection of *E. coli* using **CPRG** as enzyme substrate. The graph shows the logarithm of $OD_{574}$ after 60 minutes of assay plotted as a function of the logarithm of cell concentration. The Limit of Detection, corresponding to $5.35 \cdot 10^4$ cells·mL$^{-1}$, is represented as a horizontal dashed line. The assay has a short two-log dynamic range.

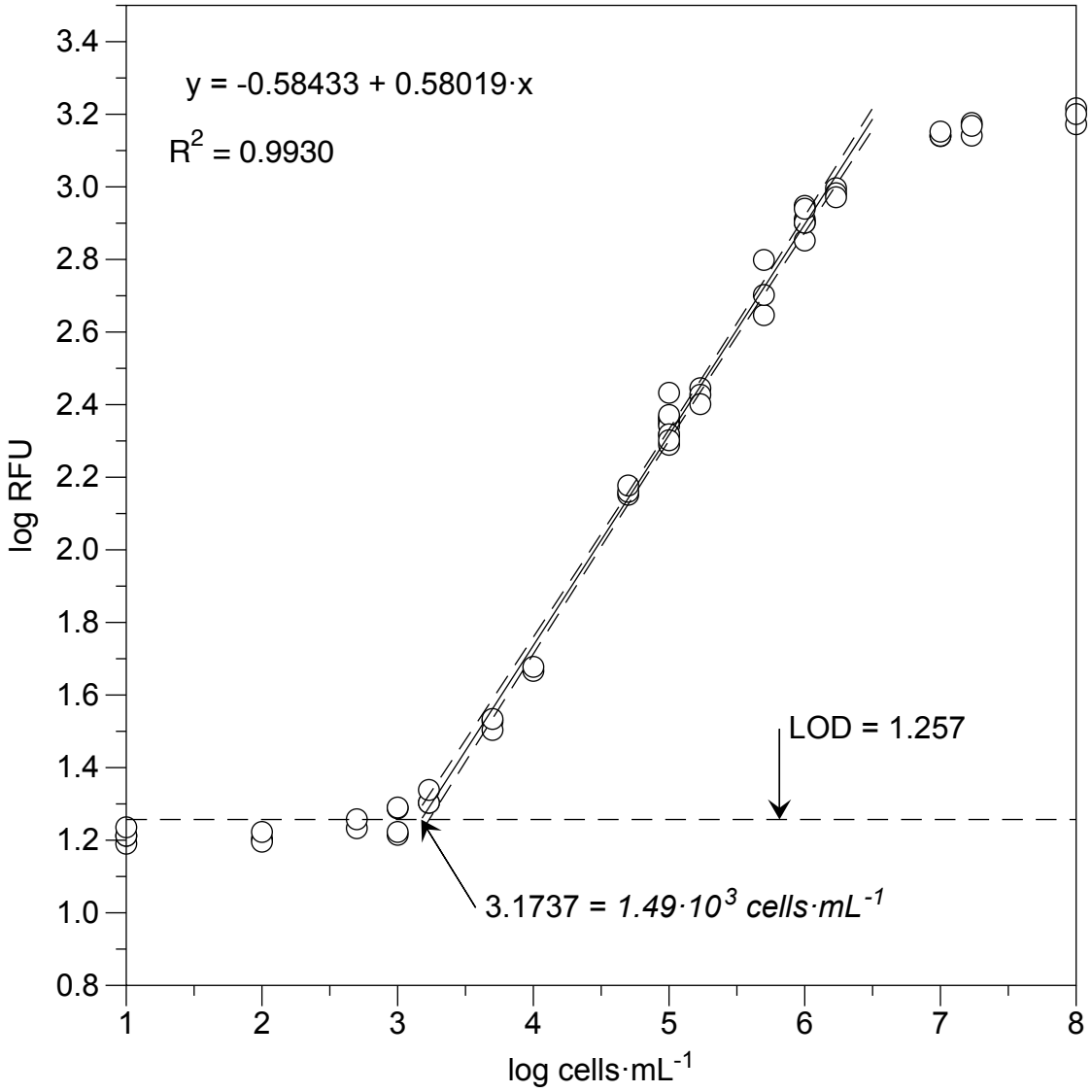



**Figure 7.** ß-galactosidase-based detection of *E. coli* using **MUG** as enzyme substrate. The graph shows the values of fluorescence (expressed as the logarithm of relative fluorescence units RFU) measured after 60 minutes of assay, plotted as a function of the logarithm of cell concentration. The assay has a three log dynamic range, between $2 \cdot 10^3$ - $2 \cdot 10^6$. The limit of detection, represented as a horizontal dashed line, corresponds to $1.49 \cdot 10^3$ cells·mL$^{-1}$. Data in the $2 \cdot 10^3$ - $2 \cdot 10^6$ cells·mL$^{-1}$ interval have been used to fit a linear regression. The 95% CI of the regression line is represented as a set of dashed lines plotted at each side of the regression line.

### 3.3.4 Validation of the protocol for field samples.

To fulfill the levels of sensitivity often required for the analysis of water samples we design a extended analytical protocol (see Materials and Methods, Figure 1) that includes a preconcentration of the samples by filtration followed by a 6 hour preincubation stage to allow for growth amplification and ß-galactosidase induction, and finally a ß-galactosidase assay with phage lysis that uses MUG as enzyme substrate. Using this protocol we analyzed a number of samples obtained from different wastewater treatment plants as well as from different creeks surrounding the university facilities. *E. coli* concentrations in the water samples were determined using Colilert 18 as a standard reference method. The results of the extended phage-based ß-galactosidase assay, recorded as relative fluorescence units and transformed as logarithms, have been plotted as a function of the logarithm of cell concentration in Figure 8. The results indicate the existence of two data domains. Below 50 cells·100 $mL^{-1}$ the assay output does not correlate with changes in cell concentration indicating lack of sensitivity in this range. Above this value, fluorescence signal correlated rather well with cell concentration. Fitting a linear regression to this data domain allowed us to calculate a regression coefficient of $R^2$=0.9571. To determine the limit of detection of the extended assay we calculated the mean and the standard deviation of the set of data below 50 cells·100 $mL^{-1}$ and calculated LOD as described In the methods section. The value obtained is 96 cells·100 $mL^{-1}$ and corresponded to and assay output of 43 RFU (horizontal dashed line in Figure 8).

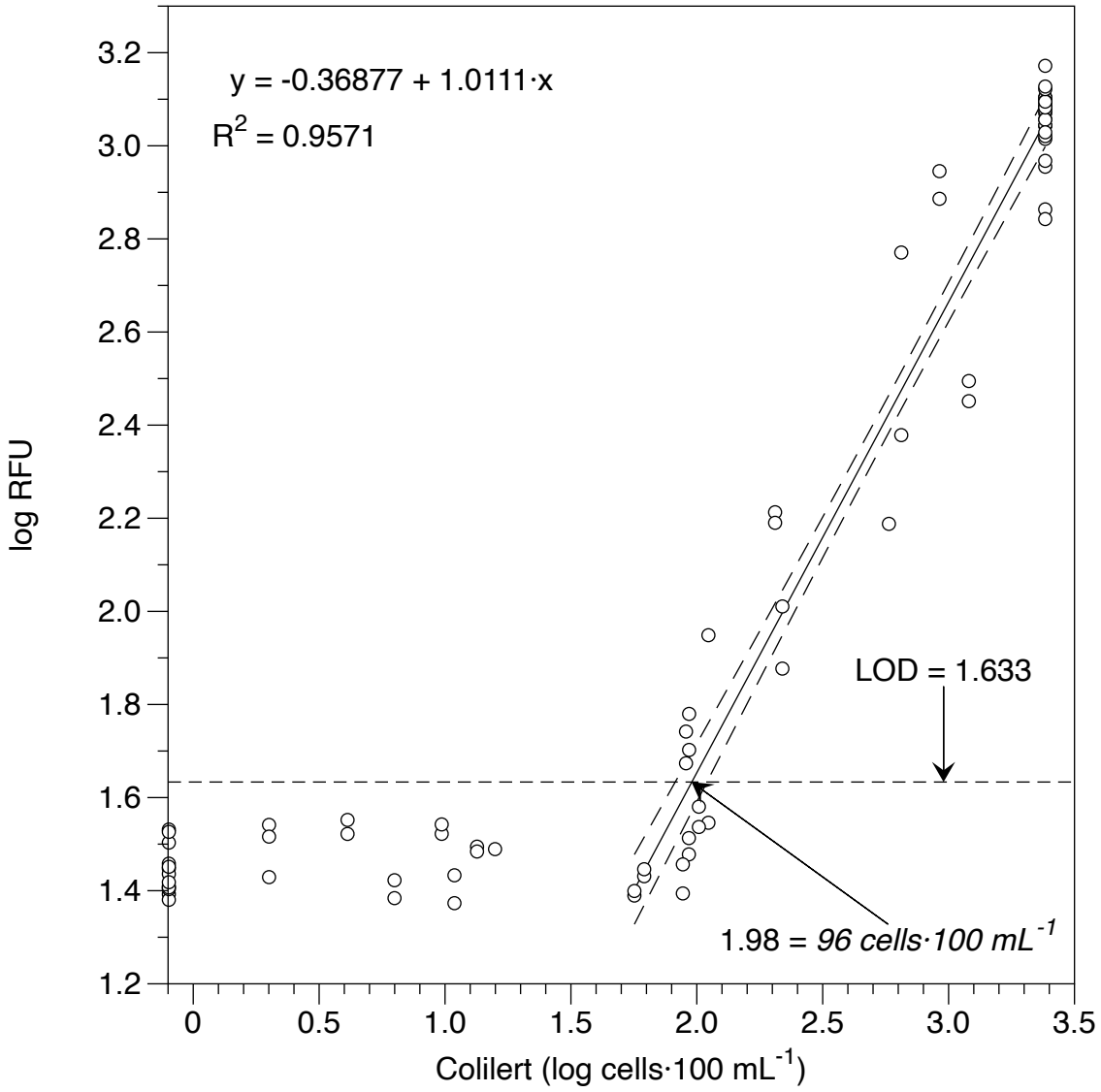



**Figure 8.** ß-galactosidase-based detection of *E. coli* using **MUG** as the enzyme substrate. The graph shows the values of fluorescence measured after 60 minutes of assay and expressed as logarithms, represented as a function of the logarithm of cell concentration determined with our reference method. The assay has a 1.5 log dynamic range, between $10^2$ - $3 \cdot 10^3$ cells·mL$^{-1}$. The limit of detection, represented as a horizontal dashed line, corresponds to 96 cells·100 mL$^{-1}$. Data have been used to fit a linear regression. The 95% CI of the regression line is represented as a set of dashed lines plotted at each side of the regression line.

## 3.4 Discussion

The assay developed in this paper is able to detect 1490 cells·mL$^{-1}$ in as short as 1.5 hour when applied without any additional treatment. In its extended version, when coupled to a pretreatment that includes filtration and preincubation, the assay is able to detect 96 cells·100 mL$^{-1}$ with a total assay length of 7.5 hours. From the point of view of sensitivity the method compares well with several recently published methods (Table 1). For instance, immunoassays for *E. coli* detection in water or milk samples are able to detect $1.3 \cdot 10^4$ and $3.0 \cdot 10^4$ cells·100 mL$^{-1}$ in 5 and 3 hours respectively (Hassan et al. 2019, Park et al 2020). Methods that concentrate the target organisms using immunocapture and carry out the detection using an ATP assay provide results in a shorter time (0.3 h), but at the expense of lower sensitivity ($10^6$ cells·100 mL$^{-1}$) (Ngamson et al 2017). On the other hand, methods based on nucleic acid detection such as PCR and qPCR tend to be fast and can provide results in a few hours, but are not sensitive enough to detect low numbers of target organism in 100 mL required for environmental ealth monitoring. To approach this level of sensitivity they usually rely on the use of preconcentration and/or preincubation methods that amplify the amount of microorganisms initially present in the sample, increasing sensitivity at the expense of assay length. Thus, Bonetta et al (2011) reported detecting 3 cells per mL using with an assay that combined PCR, filtration and preincubation with a total length of approximately 20 hours. Similarly, Sandhya et al (2008) reported the detection of 1 cell per mL, but also with a combined length of 19.5 hours. Finally, a more complex approach Wang et al. (2019) use engineered phages conjugated with magnetic beads to allow specific targeting of *E. coli*, magnetic concentration, and after phage infection and lysis, the release of a genetically engineered alkaline phosphatase fused to a gold binding peptide that immobilizes the enzyme at the surface of a gold electrode. Detection is carried out electrochemically and the sensitivity of the procedure allows detection of $10^5$ cells·mL$^{-1}$ in 4 h (equivalent to $10^7$ cells·100 mL$^{-1}$). Adding a preconcentration and preincubation step, however increases the sensitivity at the expense of assay length allowing for the detection of 1 cell·100 mL$^{-1}$ in a total of 12 hours. In comparison, Colilert-18, one of the golden standards in water quality monitoring, requires 18 hours to provide reliable results, but has a limit of detection of 1.7 and 2.3 cells·100 mL-1 when used for the analysis of drinking or bathing water respectively. In general

there is always a tradeoff between assay sensitivity and assay length with short assays having limited ability for detection and longer methods that include filtration or growth-mediated amplification steps, being more sensitive.

**Table 1.** LOD and length of the procedure for several methods for *E. coli* detection.

| Method | Sample type | LOD (cells·$10^2$ $mL^{-1}$) | Assay length | Reference |
|---|---|---|---|---|
| Colilert-18 | Drinking water | 1.7 | 18 h | AFNOR 2016 |
| | Bathing water | 2.3 | 18 h | AFNOR 2020 |
| Immunoassay | River water | $1.3 \cdot 10^4$ | 5 h | Hassan et al. 2019 |
| | Milk | $3.0 \cdot 10^4$ | 3 h | Park et al. 2020 |
| Phage-based electrochemical | Drinking water | $1.0 \cdot 10^7$ | 4 h | Wang et al. 2019 |
| | | 1 | 12 h | Wang et al. 2019 |
| ATP assay with immunocapture | Wastewater | $1.0 \cdot 10^6$ | 0.3 h | Ngamson et al. 2017 |
| PCR | Water | $3.0 \cdot 10^2$ | 20 h | Bonetta et al. 2011 |
| RT-PCR | Water | $1.0 \cdot 10^3$ | 19.5h | Sandhya et al 2008 |
| Phage-based beta galactosidase | Water | $4.9 \cdot 10^4$ | 1.5 h | this work |
| | Water | $3.7 \cdot 10^1$ | 7.5 h | this work |

The method developed in this work provides good specificity thanks to the use of a specific phage as a lysis reagent that allows fast and complete access of the assay substrate to the target enzyme. As in most examples described in Table 1, the short (1.5 h) direct version of the assay has limited sensitivity with a detection limit of $1.49 \cdot 10^3$ cells per mL (equivalent to $1.49 \cdot 10^5$ cells·100 $mL^{-1}$). While this might be good as a quick test to confirm the identity of clinical and industrial enrichments and isolates, it falls short of the analytical requirements posed by current water quality regulations. As an example, Table 2 summarizes the acceptable limits for the presence of *E. coli* in different types of water according to current EU legislation. Water for human consumption requires absence of *E. coli* in 100 mL. The limits for different qualities of bathing water are more relaxed ranging from 250 to 1000 colony forming units (cfu) per 100 mL. In the case of reclaimed water the limits range from 10 cfu·100 $mL^{-1}$ for water quality A (irrigation water for food crops where the edible part is in direct contact with reclaimed water and for root crops consumed raw) to

10.000 cfu·mL for water quality D (industrial, energy and seeded crops). Increasing the sensitivity of the assay to fulfill some or all of the above standards requires including a pretreatment of the samples that amplifies cell numbers either through concentration (e.g. membrane filtration), or growth. By including a combination of the two, the method we propose can achieve a detection limit of 96 cells in 100 mL, a 1500x increase in assay sensitivity.

**Table 2.** Acceptable limits for *E. coli* in different types and qualities of water according to current European legislation

| Type of water | Quality | Acceptable limit (cfu·100 mL$^{-1}$) | Reference |
|---|---|---|---|
| Bathing water (inland water) | Excellent | 500[a] | Directive 2006/7/EC |
| | Good | 1000[a] | |
| | Sufficient | 900[b] | |
| Bathing water (coastal and transitional waters) | Excellent | 250[a] | Directive 2006/7/EC |
| | Good | 500[a] | |
| | Sufficient | 500[b] | |
| Reclaimed water | A | 10 | Regulation (EU) 2020/741 |
| | B | 100 | |
| | C | 1000 | |
| | D | 10000 | |
| Human consumption | general | 0 | Council Directive 98/83/EC |
| | bottled | 0[c] | |

[a]Based upon a 95-percentile evaluation. [b]Based upon a 90-percentile evaluation. [c]In the case of bottled water for sale in bottles or containers the limit is expressed as cfu per 250 mL.

## 3.5 Conclusions

The use of bacteriophages as permeabilization/lysis agents allows the conversion of non-specific enzyme assays into highly specific methods for the detection of microbial contaminants. The combination of T4 with a ß-galactosidase assay using MUG as the reagent has allowed us to develop a method for *E. coli* detection that equals or outperforms many of the methods published in recent years that use approaches often more complex or expensive. The method described in this paper has room for improvement through the preconcentration of larger volumes of sample

or the use of extended growth amplification periods, and can be easily tailored to the standards required for different types of samples.

## 3.6 Acknowledgements

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# CHAPTER 4

# GENERAL DISCUSSION

# 4 GENERAL DISCUSSION

Over the years, a large number of methods have been developed for bacterial cell detection in water or beverage samples, many of them relying on the detection of intracellular compounds that are release into the medium after cell lysis. Often these methods are based on enzyme reactions. In some cases, intra-cellular enzymes are quantified after being released from the cell. In other cases, enzymes are used as a tool to detect specific intracellular compounds also released upon lysis.

One of the enzymes that can be used for the detection of ATP from bacteria is ATPase. ATP is present in all living cells and ATPase hydrolyzes ATP to ADP releasing free phosphate ions that can react with a malachite green reagent. The resulting changes in optical density can be measured at 650 nm. Another possibility for the detection of ATP in the cells is the use of luciferin-luciferase mediated bioluminescence. The luciferin-luciferase reaction emits light in the presence of the ATP released from the cells. The major drawback of this method is that it cannot distinguish between bacterial and non-bacterial cells. This drawback has been solved through a combination with other methods such as immunoassay or molecular-based methods. Moreover, the use of adenylate kinase (AK) in the ATP bioluminescence assay can increase the sensitivity of the assay (Corbitt AJ. 2001). Some species of lactic acid bacteria like *Lactobacillus gasseri* have been detected thanks to the elevated levels of phospho-ß-D-galactosidase and phospho-ß-D-glucuronidase which are released upon their lysis. These activities can be detected using o-nitrophenyl ß-D-galactosidase 6-phosphate (Honda et al 2007). Alkaline phosphatase (ALP) is another bacterial enzyme found in bacterial cells in large amounts. It was first used for the detection of pathogenic bacteria through the luminescence-based assay developed by Charm Science Inc., but the assay did not have high sensitivity. The sensitivity of the method has been improved by combining it with biosensors or modified chemoluminescence techniques (Albillos et al 2011) and, more recently, using a fluorescent probe specifically developed for the detection of alkaline phosphatase activity (Zhang et al 2020).

The ß-glucuronidase enzyme assay allows specific detection of *E. coli* in water samples. Some studies demonstrated that some non-target bacteria (*Aerococcus viridans*, *Bacillus* spp., *Corynebacterium* spp, enterococci, *Bacteroides* spp) that can be found in the environment at concentration similar to those of the target cells (*E.*

*coli*) have the same ß-glucuronidase activity. Therefore, these bacteria can interfere in the ß-glucuronidase assay (Dalhen G. 1973, Hawksworth G. 1977, Tryland I. 1998).

The presence of ß-galactosidase activity has been used for many years as a hallmark of the presence of fecal contaminants in water. There are some non-coliform bacteria that give positive results in ß-galactosidase assays when present in the samples in large amounts. However, the enzyme assay for detecting coliforms is not specific for the detection of *E. coli* which is the preferred indicator of fecal contamination by virtually all regulations (Tryland et al 1998).

Over the years, the ß-galactosidase assay has been combined with new techniques such as biosensors and paper-based assays to develop new methods of water quality assessment. In general, though, the assay has never been extensively used due to its lack of specificity and poor sensitivity.

In this thesis we have revisited the ß-galactosidase assay as a tool for the detection of *E. coli* and we have done this by first, providing specificity through the uses of a lytic phage as a permeabilizing reagent, and second, optimizing the conditions of the assay to provide the level of sensitivity required by current regulatory standards.

Achieving maximum sensitivity requires an optimal set of conditions related to IPTG induction, permeabilization procedure, concentration of enzyme reagent and pH. This study shows that induction of ß-galactosidase by preincubating the samples 180 minutes in the presence of 0.2 µM IPTG improves the sensitivity and efficiency of the assay. During the years, many studies have been using IPTG to induce *lacZ* gene expression but have often used suboptimal conditions with induction times that were to short and/or concentrations of IPTG that were two high, well in the inhibitory range. The use of these conditions can reduce the efficiency of the assay by approximately 50%.

One of the critical factors in the ß-galactosidase assay is the enzyme substrate used. The enzyme activity measured should be proportional to the amount of target organism present. However, enzyme activity is also affected by the concentration of reporting substrate used, and achieving maximum sensitivity requires using concentrations of substrate which are non-limiting, well above the $K_m$ of the enzyme for that substrate. To determine optimal substrate concentrations we determined the value of $K_m$ for three common high sensitivity colorimetric/fluorometric substrates (ONPG, CPRG and MUG). According to the Michaelis-Menten equation,

concentrations of substrate of 3 or 4 times $K_m$ provide 75-80% of $V_{max}$. Using sub-optimal concentrations of substrate can reduce assay output by approximately 40% decreasing sensitivity of the assay in a similar way.

In order to permeabilize the cell, enzyme assays usually incorporate the use of lysis reagents that are able to disrupt the bacterial envelope and facilitate the contact between the intracellular substrate and the externally supplied substrate. We have assessed that failure to add lysis reagent results in a decrease in assay output of approximately 60%. Historically, organic reagents such as toluene or chloroform, or detergents such as chloroform, SDS, and tween 20 had been used to carry out this permeabilization (Boyaci 2005, Griffith 2002). Despite their high efficiency for lysing the cells, there is a limitation to their use for bacterial detection in natural samples. Some of the reagents used (toluene and chloroform) are inappropriate for use in polystyrene materials like 96-well plates as they produce etching of the surfaces. In addition, these reagents can also produce irreversible denaturation of some enzymes (Wu et al 1993, Rothfield L 1971) and, in the particular case of ß-galactosidase, SDS causes partial denaturation of the enzyme and a significant reduction in enzyme activity (Tian et al 2015). In recent years, some commercial lysis reagents such as PopCulture™, B-PER™ or BugBuster™ have been produced for cell permeabilization and protein extraction. These reagents, also detergent-based, disrupt gram-negative bacterial cell walls and have none of the drawbacks mentioned above. However, their use does not allow selective targeting of specific microorganisms for analytical purposes.

The use of phages as biorecognition elements can provide the selectivity required in these assays through the specific release of nonspecific components from the cytoplasm of the target organism. In this study, we develop a short and simple ß-galactosidase assay through the use of bacteriophage T4 to detect specific *E. coli* in environmental water samples. Because the assay is targeted to the detection of low bacterial concentrations, a high phage titer must be used to maximize the likelihood of phage-bacteria interaction. Utilization of high phage titers requires purification of the phage lysate to remove cell debris, followed by intensive concentration of the resulting fraction. The results of this thesis clearly indicate that special care must be taken to avoid carry over of the target enzyme from the original cell lysate. Purification procedures contemplate steps such as ultrafiltration, dialysis or ultracentrifugation, however, this might be insufficient when targeting large molecular

weight enzyme multimers such as ß-galactosidase. In our case we were forced to solve this problem by propagating our phage in a *E. coli* mutant that did not express the *lacZ* gene.

The method described in this thesis can perform direct detection of $1.5x10^3$ cells·$mL^{-1}$ in only 1.5 h. An extended version of the assay including a 6h pretreatment can lower the detection limit to 96 cells per 100 mL, in a total running time of 7.5 h. This performance compares remarkably well with other methods described in the literature. For instance, Colilert-18, one of the accepted standards for the detection and enumeration of coliform and *E. coli* in water samples can detect 1 cell in 100 mL of samples, but requires 18 hours which are mainly employed in amplifying cell numbers through growth in a specific medium. The method uses the colorimetric ONPG (galactoside) substrate for the colorimetric detection of coliforms, and MUG (glucuronide) for the fluorometric detection of *E. coli* in water samples. Besides requiring a long time, some studies indicate the existence of false positives in Colilert-18 results caused by the interference of environmental microorganims, as mentioned above. On the other hand, a study carried out by Maheux et al in 2008 showed that Colilert-18 was only able to detect ß-glucuronidase activity in 51.4% of the 74 strains of *E. coli* tested (Maheux et al 2008).

Nucleic acid-based methods are accurate methods that are often considered as a fast alternative to conventional culture-based methods for the detection of bacteria in the environment. However, published work shows that, while fast and accurate when detecting high concentrations of target microorganisms, they are not well suited for the level of sensitivity required for applications like water quality monitoring. In general, the sensitivity of direct molecular methods is rather poor and, as in so many other instances of methods for bacteria detection, increasing sensitivity requires increasing the length of the assay either through the addition of preconcentration steps or of lengthy preincubations. As an example, Moreno et al (2004) developed a PCR test for the detection of bacteria from water and wastewater with a limit of detection of 1 cell per 1 mL. To reach this level of sensitivity, the method required a 48 h pretreatment consisting of concentration by filtration and preincubation in culture medium. The total length of the method was 50 hours, much longer and complex than any of the culture methods currently used. Quantitative real time or qPCR constitutes a more precise method for the quantification of bacteria in water

samples. In general, qPCR methods are fast (3 to 4 h), but achieving the sensitivities required for microbiological water quality assessment with the small sample volumes used (20-25 µL) makes mandatory the inclusion of extensive preconcentration steps. Thus, the method described by Khan et al (2007) has a detection limit of 10 cells·mL$^{-1}$, but requires concentrating 1000 mL in 25 µL in successive centrifugations, a concentration factor of 40000:1. In a similar way, the method described by Lam et al (2014) requires the filtration of 2000 mL in order to reach a detection limit of 25 cells in 100 mL.

Antibody-based and aptamer-based methods are often regarded as fast methods, but, as was the case with nucleic acid-based methods, their sensitivity is low, and they must trade sensitivity for time when low detection limits are required. For instance, Hassan et al (2019) developed an antibody-based lateral flow method for the detection of *E. coli*, with a limit of detection of 133 cells·mL$^{-1}$. The assay has a total running length of 5 hours of which 3 hours correspond to a preincubation in growth medium to allow multiplication of the target microorganism. More recently, Zhang et al (2020) have developed an aptamer-based assay to carry out conductimetric detection of *E. coli* in water samples. Despite the extended length (10 h) the method has a limit of detection of 2.3x10$^{4}$ cell·mL$^{-1}$ comparing poorly with currently established detection methods.

Enzyme assays have also been developed in an attempt to provide fast detection of *E. coli* in water and environmental samples. In this line, Sicard et al (2014) developed a method for the detection of *E. coli* through the analysis of ß-galactosidase activity using CPRG and polyR as substrates to detect 100 cfu·mL$^{-1}$ within 13 hours, 10 hours of preincubation to allow for microbial growth plus 3 hours of assay. Park et al (2020) followed a different approach combining microbial growth and immunomagnetic separation in a 2 hour pretreatment that takes 25 mL samples, allows for microbial growth in a total volume of 100 mL and collects the enriched cells by immunomagnetic capture in a final volume of 2 mL. The combined length of the assay is 3 hours and has a detection limit of 300 cells·mL$^{-1}$. Last in this category, Satoh et al (2020) have published a method with the unlikely claim of being able to detect 18 cells per mL using 180 µL samples in only 3 hours. This corresponds to roughly 3 cells per sample. The method uses a three hour preincubation and follows online development of fluorescence in the presence of a fluorogenic substrate.

Finally, fast assays that use phages as biorecognition elements have also been developed although with variable results. Burham et al (2014) developed a colorimetric and luminescent ß-galactosidase assay performed in 10 mL water samples. The samples, concentrated by filtration through 0.45 µm membrane filter, were preincubated under growth conditions for 4 hours, treated with a genetically engineered *lacZ* containing phage to enhance ß-gal expression, and tested for enzyme activity using chromogenic (CPRG) or a bioluminescent (Beta-Glo) substrates. Sensitivity was 2-20 cells·mL$^{-1}$ with an assay length of 5.5-8 h using colorimetric or bioluminescent detection. Using a radically different approach, Chen et al (2015) developed a ß-galactosidase assay that used magnetic separation using bacteriophage-conjugated magnetic beads. In this case the phage acted as a biorecognition element for the capture and, once cells were infected, as a means to release the intracellular contents an increase the efficiency of the assay. The method was able to detect $10^4$ cells·mL$^{-1}$ in 2.5 hours, or 10 cells·mL$^{-1}$ after including a 6 hours enrichment step, bringing the total assay length to 8.5 h. Last of all, Wang et al (2019) developed an electrochemical method that combines phage recognition with the expression of gold binding peptides fused alkaline phosphatase providing a limit of detection of $10^5$ cells·mL$^{-1}$ in 4 hours. Including a 9 hour preincubation enrichment lowered the detection limit to 1 cell per 100 mL, but with a total running time of 12 hours.

This thesis focuses on the detection of *E. coli* in water samples using the reporting capacity of an optimized ß-galactosidase assay and the targeting ability of a T4 bacteriophage. The method is cheap, easy to produce and implement, highly specific, and has a running time in the range of similar or even better than similar assays published in recent times. The method has the potential to be further refined by improving the preconcentration or preamplification steps, but these particular enhancements must be linked to the development of specific applications in the fields of environmental, food safety or clinical diagnostic.

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# CHAPTER 5

# CONCLUSIONS

# 5 CONCLUSIONS

1. Utilization of the ß-galactosidase assay for bacteria detection purposes requires careful optimization of the different factors that affect the assay output. A non-optimal choice of parameters can result in a decrease of sensitivity of virtually one order of magnitude.

2. The IPTG-induced levels of ß-galactosidase activity in cultures of *E. coli* are highly dependent on the concentration of inductant used. Maximum enzyme levels are detected when using a concentration of IPTG of 0.2 mM. Higher concentrations cause inhibition of the assay with an estimated inhibition constant $K_i$ of 5.7 mM.

3. IPTG-dependent induction of ß-galactosidase increases as a function of time. Maximum induction is achieved 3 hours after IPTG addition.

4. Incorporation of detergent-based reagents in the ß-galactosidase assay results in cell permeabilization and facilitates the interaction between enzyme and reagent. Lack of permeabilization treatment causes a 56.7% reduction in assay output.

5. Utilization of low concentrations of substrate in the ß-galactosidase assay reduces considerably the assay output as the enzyme is substrate-limited. Using a concentration of substrate equal to 4 times $K_m$ allows to achieve 80% of the maximum enzyme activity and it constitutes a reasonable tradeoff between good sensitivity and reagent expenditure.

6. The values of $K_m$ for ß-galactosidase activity have been determined for the three substrates used in this work, providing values of 1.12 mM for ONPG, 12.84 mM for CPRG and 61.9 µM for MUG.

7. Sodium carbonate is often used at the end of the ß-galactosidase assay to increase pH above the $pK_a$ of the reaction products and favor the transition to their colored or fluorescent forms, enhancing the sensitivity of the assay. Failure to add carbonate results in a 48% reduction in assay output when

using ONPG, in a 2% reduction when using CPRG, and in a 74% reduction when using MUG.

8. T4 phages propagated using wild type *E. coli* (DSMZ613) and purified by ultrafiltration contain high amounts of ß-galactosidase carried over from the mother strain. To avoid this problem, phages must be propagated using mutants that do not express the ß-galactosidase gene.

9. Use of lytic phages like T4 constitutes an excellent alternative to detergent-based permeabilization reagents in assays for the quantification of intracellular enzymes. Lytic phages provide complete lysis of the cell and a better release of intracellular enzymes at the expense of a delay between infection and lysis that must be factored in when designing the assay.

10. A ß-galactosidase-based phage-dependent assay for the detection of *E. coli* has been designed using phage T4 and a suitable enzyme substrate. The method has been tested using both the colorimetric substrate CPRG and the fluorometric substrate MUG. In the case of CPRG, the limit of detection was $5.4 \cdot 10^4$ cells·mL$^{-1}$. In the case of MUG, the limit of detection was $1.5 \cdot 10^3$ cells·mL$^{-1}$.

11. An extended version of the MUG-based assay was developed including preconcentration and preincubation steps to increase the sensitivity. The method was validated in natural water and wastewater samples using Colilert-18 as the reference method. The length of the assay was 7.5 hours and the limit of sensitivity was 96 cells·100·mL$^{-1}$.

12. Phage-based enzyme assays constitute a valid and viable alternative for the specific detection of microbial contaminants in environmental and industrial samples.