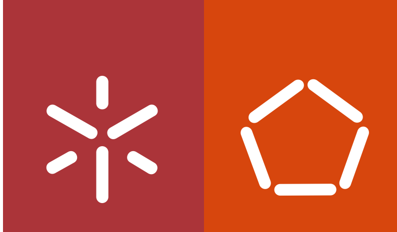
Universidade do Minho
Escola de Engenharia

Diana Cristina Mendes de Lima e Silva

Development of enzymatic and molecular methods for the detection of *Escherichia coli*, total coliforms and *Vibrio cholerae* in water samples

July 2014



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Development of enzymatic and molecular methods for the detection of *Escherichia coli*, total coliforms and *Vibrio cholerae* in water samples

PhD in Chemical and Biological Engineering

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“O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis.”

Fernando Pessoa

À minha família.

Porque são insubstituíveis!

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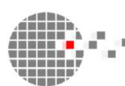

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ABSTRACT

DEVELOPMENT OF ENZYMATIC AND MOLECULAR METHODS FOR THE DETECTION OF *ESCHERICHIA COLI*, TOTAL COLIFORMS AND *VIBRIO CHOLERAE* IN WATER SAMPLES

Water is one of the major sources of transmission of pathogen microorganisms worldwide and outbreaks of waterborne diseases (e.g. cholera, typhoid fever) are a reality. Globally, these outbreaks have an enormous impact, which is particularly noticed in developing countries, becoming essential to constantly evaluate the microbiological quality of the water. For such purpose, methods based in the detection of total coliforms and, in particular, *Escherichia coli* are used as indicators of faecal contamination and of enteric pathogens in water. The methods currently available for microbiological water quality monitoring provide results with a considerable delay, lacking on specificity and sensitivity.

The aim of this thesis was to develop methods for the detection of indicator microorganisms in water samples, having as final goal the application of these methods to water quality monitoring. This study was initiated on November 2009 and finished in October 2013, and was divided in three main goals: development of an enzymatic culture medium for the detection of *E. coli* and total coliforms in environmental samples, development of a sample preparation method to concentrate and recover microbial cells from 100 mL of water samples to detect *E. coli* and total coliforms by standard polymerase chain reaction (PCR) and real-time polymerase chain reaction (rtPCR), and development of a method to detect *Vibrio cholerae* in ballast water, using rtPCR.

The enzymatic culture medium was based in identifying the activity of the enzymes β-D-glucuronidase and β-D-galactosidase, which are specific to the indicators *E. coli* and total coliforms, respectively. A medium in the form of a powder was developed, being capable of detecting as little as one target indicator in 1 mL of drinking and river waters after 14 to 18 h of incubation, and in seawater, after 24 h of incubation.

In order to evaluate the adaptability of PCR-based methods to detect total coliforms and *E. coli* in water quality monitoring and with the purpose of reducing the assay time, a sample preparation method to concentrate and recover cells from water samples was developed, without the need of culturing the microorganisms. The final protocol was able to detect 10^4 CFU/100 mL in approximately 6 h by standard PCR and in 3 h by real-time PCR in bacterial suspensions of water. Nevertheless, PCR-based methods are highly sensitive to contaminations and inhibitions, being necessary further research in order to adapt this technique to environmental samples of water. The addition of a molecular decontamination and a molecular enrichment steps will undoubtedly drive this method further by reducing contamination and increasing the limit of detection.

Ballast water is one of the major sources of transmission of waterborne microorganisms worldwide and has been correlated to outbreaks of cholera disease. In order to improve the water quality monitoring of the ballast water, we aimed at creating a methodology able to detect the three major *Vibrio* human pathogens: *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus,* by coupling an efficient sample preparation method to concentrate and recover bacterial cells with a molecular enrichment step, and detection of the target microorganisms by multiplex-rtPCR. This led to the development of a new method, named as *Vibrio* CRENAME-rtPCR, capable to detect 14 CFU of *V. cholerae,* 4 CFU of *V. vulnificus* and 12 CFU of *V. parahaemolyticus* in 100 mL of artificial seawater, in less than 5 h.

In conclusion, the methods developed in this thesis have shown to have high potential to be used for assessing the microbiological quality of water, providing the results in a reduced time.

RESUMO

DESENVOLVIMENTO DE MÉTODOS ENZIMÁTICOS E MOLECULARES PARA DETETAR *ESCHERICHIA COLI*, COLIFORMES TOTAIS E *VIBRIO CHOLERAE* EM AMOSTRAS DE ÁGUA

A água é uma das principais fontes de transmissão de microrganismos patogénicos em todo o mundo e epidemias de doenças transmitidas pela água (por exemplo: cólera, febre tifoide) são uma realidade. Estes surtos têm um enorme impacto no mundo, particularmente nos países em desenvolvimento, tornando-se essencial avaliar constantemente a qualidade microbiológica da água. Para cumprir este objetivo, são utilizados métodos baseados na deteção de coliformes totais e, em particular, da bactéria *Escherichia coli* como indicadores de contaminação fecal e da presença de patogénicos entéricos na água. Os métodos atualmente disponíveis para monitorizar a qualidade microbiológica da água fornecem os resultados com um atraso considerável, carecendo em especificidade e sensibilidade.

Esta tese teve como objetivo desenvolver métodos para a deteção de microrganismos indicadores em amostras de água, tendo como propósito final aplicar estes métodos na monitorização da qualidade da água. O trabalho foi iniciado em Novembro de 2009 e terminado em Outubro de 2013, tendo sido dividido em três objetivos principais: o desenvolvimento de um meio de cultura enzimático para a deteção de *E. coli* e coliformes totais em amostras ambientais, o desenvolvimento de um método de preparação de amostras para concentrar e recuperar as células microbianas a partir de amostras de água de 100 ml para a deteção de *E. coli* e coliformes totais através das técnicas de reação em cadeia da polimerase (PCR) e de reação em cadeia da polimerase em tempo real (rtPCR), e o desenvolvimento de um método para detetar *Vibrio cholerae* na água de lastro, usando o rtPCR.

O meio de cultura enzimático baseou-se na identificação da atividade das enzimas β-D-glucuronidase e β-D-galactosidase, que são específicas para os indicadores *E. coli* e

coliformes totais, respetivamente. Foi desenvolvido um meio sob a forma de pó, sendo capaz de detetar até uma célula do indicador alvo em 1 mL de água potável e de rio após 14 a 18 horas de incubação, e na água do mar após 24 h de incubação.

No sentido de avaliar a capacidade de adaptação dos métodos baseados em PCR para detetar coliformes totais e *E. coli* na monitorização da qualidade da água, e com a finalidade de reduzir o tempo de análise, foi desenvolvido um método de preparação das amostras com o objetivo de concentrar e recuperar as células a partir de amostras de água, sem incluir a cultura dos microrganismos. O protocolo final foi capaz de detetar 10^4 unidades formadoras de colónias (UFC)/100 mL de suspensões bacterianas de água em cerca de 6 horas pelo PCR convencional, e em cerca de 3 horas pelo rtPCR. No entanto, os métodos baseados em PCR são altamente sensíveis a contaminações e inibições, pelo que é necessário continuar o estudo da adaptação desta técnica a amostras ambientais de água. Incluir no método etapas de descontaminação molecular e de cultura molecular irá, sem dúvida, aperfeiçoar este método por reduzir a contaminação e aumentar o limite de deteção.

A água de lastro é um dos mais importantes veículos de transmissão de microrganismos através da água em todo o mundo e tem sido relacionada com surtos de cólera. No sentido de contribuir para um avanço na monitorização da qualidade da água de lastro, visamos desenvolver uma metodologia capaz de detetar os três principais patogénicos humanos *Vibrio*: *Vibrio cholerae*, *Vibrio parahaemolyticus* e *Vibrio vulnificus*, associando um método eficiente para preparar as amostras por concentração e recuperação das células bacterianas, com um passo de cultura molecular, e deteção dos microrganismos alvo por multiplex-rtPCR. Isto levou ao desenvolvimento de um novo método, denominado como *Vibrio* CRENAME-rtPCR, capaz de detetar 14 UFC de *V. cholerae*, 4 UFC de *V. vulnificus* e 12 UFC de *V. parahaemolyticus* em 100 mL água do mar artificial, em menos de 5 horas.

Em suma, os métodos desenvolvidos nesta tese provaram ter um elevado potencial de serem aplicados na avaliação da qualidade microbiológica da água, sendo os resultados visíveis num tempo reduzido.

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ABBREVIATIONS

BHI – brain heart infusion

BSA – bovine serum albumin

CCRI – Collection of Centre de Recherche en Infectiologie

cDNA – complementary DNA

CFU – colony-forming units

C_T – threshold cycle

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide

EDTA – ethylenediaminetetraacetic acid

gc – genome copies

GE – genomic equivalents

HCl – hydrochloric acid

HPC – heterotrophic plate count

IBDG – indoxyl-β-D-glucuronide

IMO – International Maritime Organization

IPTG – isopropyl β-D-1-thiogalactopyranoside

ITS – internal transcribed spacer region

KCl – potassium chloride

KH_2PO_4 – potassium phosphate monobasic

LB – Luria Broth

Met-Glu – methyl β-D-glucuronide sodium salt

$MgCl_2$ – magnesium chloride

MPN – most probable number

mRNA – messenger ribonucleic acid

MUG – 4-methylumbelliferyl-β-D-galactopyranoside

$Na_2HPO_4.7H_2O$ – sodium monohydrogen phosphate heptahydrate

NaCl – sodium chloride

NaOH – sodium hidroxide

NASBA – nucleic acid based sequence amplification

NC – negative control

NTC – no template control

OD – optical density

ONPG – *o*-nitrophenyl-β-D-galactopyranoside

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PMA – propidium monoazide

qrtPCR – quantitative real-time polymerase chain reaction

r – correlation coefficient

R^2 – coefficient of determination

RNA – ribonucleic acid

rRNA – ribossomal ribonucleic acid

RT-PCR – reverse transcriptase polymerase chain reaction

rtPCR – real-time polymerase chain reaction

Salmon-Gal – 6-bromo-3-indolyl-β-galactopyranoside

SDS – sodium dodecyl sulfate

TAE – Tris-acetate-EDTA buffer

TBE – Tris-borate-EDTA buffer

TE – Tris-EDTA buffer

Tris-HCl – Tris(hydroxymethyl)aminomethane hydrochloride

US EPA – United States Environmental Protection Agency

VBNC – viable but non-culturable cells

WGA – whole genome amplification

WHO – World Health Organization

X-Gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

X-Glu – 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

Symbols

$\varnothing$ – diameter

$\bar{x}$ – average number of colonies

CHAPTER 1

MOTIVATION AND RESEARCH AIMS

The motivation and research aims of this work are presented in this chapter, providing a general overview of the thesis.

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1.1 | THESIS MOTIVATION

Even though we live in a world where technology and science are quickly and astonishingly evolving, the transmission of pathogenic microorganisms through water is still of major concern [1-6]. It is not possible to permanently decontaminate all the water in the planet in order to avoid the outbreaks of diseases that are a constant occurrence [6, 7]. The solution lies, therefore, in prevention by constantly monitoring the microbiological safety of water. However, so far, there is not a reliable method for providing this information at the moment of sampling. Therefore, this thesis aims at contributing to the development of methodologies for fast and reliable detection of microorganisms in water.

1.2 | Research aims

This thesis was developed as part of a *PhD Studentship in Industry* (BDE), granted by the *Foundation for the Science and Technology* (FCT) and by the Portuguese company *Frilabo*. The main purpose was to develop methods for fast detection of the microorganisms *Escherichia coli* (*E. coli*), total coliforms and *Vibrio cholerae* (*V. cholerae*) in water samples:

- ***Escherichia coli* and total coliforms**:
  - Enzymatic detection: The purpose was to develop an enzymatic culture medium, similar to the medium Colilert®, a commercially available enzymatic culture medium.
  - Molecular detection: The main purpose was to study the adaptability of polymerase chain reaction (PCR) and real-time PCR (rtPCR) to the water quality monitoring. In order to reduce the time of analysis, the pre-enrichment of the samples was discarded and the developed activities were focused in developing a sample preparation method aiming at the concentration and recovery of microbial cells from 100 mL water samples, followed by the identification of the microorganisms by PCR or rtPCR.
- ***Vibrio cholerae***:
  - Molecular detection: The objective was to develop a fast method to detect *V. cholerae* in the ballast water of ships (*Vibrio* CRENAME-rtPCR), based in the method developed by Maheux and coworkers for the detection of *E. coli*/*Shigella* in potable water (*E. coli*/*Shigella* CRENAME rtPCR) [8].

In all methods, the objectives to be achieved were:

- A sensitivity of 1 coliform forming unit (CFU) of the target microorganism per 100 mL of water sample;
- Specificity to the target microorganisms;
- Simplicity of the procedure;
- Fast results.

Note: The work concerning the enzymatic detection and the molecular detection (using standard PCR) to detect *E. coli* and total coliforms was initiated during the Master course dissertation of the author and further developed during this thesis [9].

1.3 | REFERENCES

1. WHO, Water recreation and disease - Plausibility of associated infections: acute effects, sequelae and mortality 2005, International Water Association Publishing.
2. Friesema, I., Sigmundsdottir, G., van der Zwaluw, K., Heuvelink, A., Schimmer, B., de Jager, C., Rump, B., Briem, H., Hardardottir, H., Atladottir, A., Gudmundsdottir, E., and van Pelt, W., An international outbreak of Shiga toxin-producing *Escherichia coli* O157 infection due to lettuce, September-October 2007. Euro surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin, 2008. 13, 3029-3035.
3. Wade, T.J., Pai, N., Eisenberg, J.N.S., and Colford, J.M., Do US Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environmental Health Perspectives, 2003. 111, 1102-1109.
4. Gray, N.F., Chapter Thirty - Pathogen control in drinking water, in *Microbiology of Waterborne Diseases (Second Edition)*, S.L. Percival, et al., Editors. 2014, Academic Press: London, 537-569.
5. Gibson, K.E., Viral pathogens in water: occurrence, public health impact, and available control strategies. Current Opinion in Virology, 2014. 4, 50-57.
6. de Man, H., van den Berg, H.H.J.L., Leenen, E.J.T.M., Schijven, J.F., Schets, F.M., van der Vliet, J.C., van Knapen, F., and de Roda Husman, A.M., Quantitative assessment of infection risk from exposure to waterborne pathogens in urban floodwater. Water Research, 2014. 48, 90-99.
7. WHO, Water Quality and Health Strategy 2013-2020. 2013.
8. Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.L.T., Huppe, V., Picard, F.J., Berube, E., and Bergeron, M.G., Rapid concentration and molecular enrichment approach for sensitive detection of *Escherichia coli* and *Shigella* species in potable water samples. Applied and Environmental Microbiology, 2011. 77, 6199-6207.
9. Mendes, D., Desenvolvimento de um kit de detecção da bactéria *Escherichia coli* e coliformes totais em amostras de água, in Centre of Biological Engineering. 2008, University of Minho. Master degree dissertation.

CHAPTER 2

GENERAL INTRODUCTION

This chapter reviews the main topics on water quality monitoring and the technology used for its assessment, as well as the main advantages and limitations. The review is focused on the detection of *E. coli* in water since it is, still, the main recommended faecal indicator bacteria and the most frequently used worldwide. PCR-based methods are considered in detail since they are the most emergent molecular methods being developed for water quality monitoring.

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2.1 | INTRODUCTION

Water is frequently contaminated with human and/or animal feces [1-6], and the presence of faecal-derived pathogenic microorganisms (bacteria, virus, protozoa) in water is responsible for several infectious diseases [7, 8]. Some of the pathogens that can cause water-related diseases are listed in Table 2.1.

Table 2.1 – Examples of water-transmitted pathogens and their associated diseases

MICROORGANISM	ASSOCIATED DISEASES	REFERENCES
BACTERIA		
Escherichia coli (pathogenic strains), *Shigella* spp.	Gastroenteritis	[7, 9-12]
Campylobacter spp.	Campylobacteriosis	[13]
Helicobacter pylori	Gastritis, gastric cancer	[7, 14-16]
Leptospira spp.	Leptospirosis	[17, 18]
Salmonella typhi	Typhoid fever	[19, 20]
Legionella pneumophila	Pneumonia	[21, 22]
Vibrio cholerae	Cholera	[23-26]
Vibrio parahaemolyticus and Vibrio vulnificus	Septicemia, gastroenteritis	[26, 27]
VIRUS		
Adenovirus, Norovirus	Conjunctivitis, pharyngitis, pneumonia, appendicitis, bronchiolitis, gastroenteritis	[28, 29]
Hepatitis A virus	Hepatitis A	[29, 30]
Hepatitis E virus	Hepatitis E	[29, 31]
Coxsackieviruses	Meningitis, pharyngitis, myocarditis, encephalitis	[7, 32]
PROTOZOA		
Cryptosporidium spp.	Cryptosporidiosis	[33]
Giardia spp.	Giardiasis	[34]

Outbreaks of waterborne diseases remain a major challenge to public health providers worldwide, claiming millions of lives annually [13, 35-41]. The World Health Organization (WHO) estimates that 2.5 million people died with diarrheal disease in 2008 and that the number of cholera cases increased 85% in 2011, compared to 2010 [42].

Whereas developing countries are particularly susceptible to these epidemics due to their poor sanitation and water quality control, the developed countries are also affected by emerging pathogens resistant to water treatment, technological failure and/or inappropriate detection measures [43-45]. Therefore, it is imperative to provide microbiologically safe water in order to protect Public Health.

2.1.1 - Indicator Microorganisms

It is not feasible to identify each one of the pathogens that can be found in water due to the excessive costs and labour involved. Instead, one or more microorganisms should be chosen to indicate the possible presence of pathogens in water. The ideal faecal indicator microorganism should meet some conditions: (1) be consistently present in faeces of human and warm-blooded animals; (2) be unable to proliferate outside of the intestinal tract; (3) be strongly related to the presence of pathogenic microorganisms; (4) be at least as resistant to environmental conditions and to water treatment processes as the pathogens; (5) and allow simple laboratory methods for detection [46].

At the end of the 19th century, the heterotrophic plate count (HPC) was introduced to water microbiology and has been used since then as a tool to monitor the efficacy of water treatment processes and the cleanliness of the water distribution systems [47, 48]. Nevertheless, there is no correlation between HPC and health-risk conditions [48]. Moreover, these microorganisms are universally present in all types of water, soil, food, vegetation and air [48]. Therefore, this analysis is not adequate for accessing the microbiological safety of water.

Historically, many countries have been using faecal indicator bacteria, comprising total coliforms, faecal coliforms, *E. coli* and enterococci, as a monitoring tool to predict the presence of bacterial, viral and protozoan pathogens originated in faecal contamination [46]. The European Commission (EC) [49-51], the United States Environmental Protection Agency (USEPA) [52-54] and the WHO [55] recommend the use of *E. coli* as the most effective available faecal indicator bacteria for predicting the presence of pathogens in water, and *Enterococcus sp.* as the most appropriate for marine waters.

Total coliforms have been used as indicators of the efficiency of the water treatment, as well as to evaluate the cleanliness and integrity of the water distribution systems and the potential presence of biofilms [56-58]. However, they are not useful to indicate the presence of faecal pathogens in recreational water, as these microorganisms are widely spread in nature and have the ability to survive and grow in water [59]. For drinking water, total coliforms are still used as a standard test to indicate contamination by an outside source.

Faecal coliforms are a subgroup of total coliforms, more specific to faecal contamination. However, this group contains some species that are not necessarily related to faecal origin. *E. coli* is widely accepted as an indicator bacteria [60] because it is a species of faecal coliform bacteria that is specific to the intestines of humans and other warm-blooded animals but not normally pathogenic, it is easy to detect and culture, and it is found at higher concentrations than other pathogens in waters [54, 55]. Moreover, its persistence in water and the effects of water treatment in cells viability is similar to that of waterborne pathogens [1, 61, 62].

The salinity of marine waters can affect the persistence of the indicators in the water. Enterococci are recommended for these waters because they can mimic more closely the persistence of many pathogens in this environment than other indicators due to their ability to survive in salt water [54]. Ballast water discharges from ships in seawater is one of the major sources of transmission of aquatic microorganisms across the world [23]. One of these microorganisms is the pathogenic *V. cholerae*, which causes the cholera disease, and their transmission by the discharges of ballast water has already been correlated with cholera outbreaks [63]. Therefore, the International Maritime Organization (IMO) established guidelines requiring that ships must control the microbiological quality of water, not only by detecting *E. coli* and enterococci, but also the pathogenic *V. cholerae* [64]. Therefore, in order to be discharged, ballast water must contain <1 CFU of toxigenic *V. cholerae*, <250 CFU of *E. coli* and <100 CFU enterococci per 100 mL [64].

Recently, some studies reported the need for new indicators of the quality of water. These findings suggest that some genotypes of *E. coli* are adapted to grow in extra-enteric environments [53, 65]. In some cases, there was a poor correlation between bacterial indicators and virus, which can survive in water for a longer period of time than the indicators currently used [66]. Hence, the relationship between faecal indicator bacteria and the occurrence of diseases may not be accurate in all the locations of sampling [53, 67]. Enteric viruses and bacterial viruses (as adenovirus, norovirus and, in particular, coliphages) have been suggested as alternative indicators of faecal contamination due to their ability to survive for a longer period of time in water than faecal indicator bacteria [66, 68]. *Pseudomonas aeruginosa* is one of the new indicators of health significance as this microorganism is extremely adaptable, has minimal growth requirements, has the ability to survive for long periods of time in diverse habitats and has shown significant resistance to antibiotics [69-71]. Nevertheless, there is no indicator with the ability to predict illness consistently in all environments [52].

2.1.2 – Portuguese legislation concerning the microbiological quality of water

The purpose of legislation is to promote a sustainable and appropriate use of the water, to reduce the sources of pollution, to ensure the supply of clean and safe water and to protect the quality of the water and the public health [72].

The concept of the microbiological quality of the water depends on the type of water and its final use. The European Commission states that all water intended for human consumption or food production, must have less than 1 colony forming units (CFU)/100 mL of *E. coli* as a parametric value [49, 51]. Concerning the quality of bathing water, the European Commission states that water is evaluated as *Excellent*, *Good* or *Sufficient* quality when *E. coli* is present at a maximum of 500, 1000 and 900 CFU/100 mL in inland waters, and of 200, 500 and 500 CFU/100 mL in coastal and transitional waters, respectively (values based upon a 90-percentile evaluation) [50].

In Portugal, the law divides water into four groups, according to its use: water for human consumption, water for aquaculture, irrigation water and bathing water [73]. The regulations specify the physical, chemical and microbiological parameters to be assessed in order to assure the quality of the water, the frequency of sampling and the laboratory methods to be used. Following, the microbiological limits required by the Portuguese law to ensure the quality of the different types of water are described.

2.1.2.1 – Legal microbiological parameters applied to water intended for human consumption

Water for human consumption is defined as water in its original state, or after treatment, intended for drinking, cooking, prepare food, personal hygiene or other domestic purposes. It also applies to the water used in the food industry in the manufacturing, processing, preservation or commercialization of products intended for

human consumption, as well as the water used to clean surfaces, objects and materials that may be in contact with food [72-74].

The regulations DL 236/98 and DL 306/2007 defining the guidelines for water intended for human consumption include the water supplied by distribution networks, fountains, trucks or tankers, reservoirs and food industry (Table 2.2), the water sold in bottles or other containers (Table 2.3) and inland surface water used in the production of water for human consumption (Table 2.4). Although it is not specific to the enteric environment, the bacteria *P. aeruginosa* is part of the regulations listed in Table 2.3 as one of the new indicators of increased health significance due to its pathogenicity, not being exclusive to the enteric environment and resistance to difficult environments [69]. The total number of colonies grown at 22 °C and 37 °C is not an indicator of faecal contamination but of the potential enrichment by organic matter. It is useful to detect changes in the number of CFU by comparison with the historical records, revealing new pollution focus.

Table 2.2 – Microbiological parametric values applied to water used for human consumption supplied by distribution networks, fountains and reservoirs not connected to the distribution network, delivery places, trucks, tankers and water used in food industry [74]

Microorganism	Parametric Value
Escherichia coli	0 CFU/100 mL
Enterococcus	0 CFU/100 mL

Table 2.3 – Microbiological parametric values applied to water sold in bottles or other containers [74]

Microorganism	Parametric Value
Escherichia coli	0 CFU/250 mL
Enterococcus	0 CFU/250 mL
Pseudomonas aeruginosa	0 CFU/250 mL
CFU at 22 °C [(1)]	100 CFU/mL
CFU at 37 °C [(1)]	20 CFU/mL

The inland surface water can be used in the production of water for human consumption and is labelled in three groups: A1, A2 and A3 [73]. According to the legislation DL 236/98, these groups are divided by the type of water treatment used: A1 - physical treatment and disinfection; A2 - physical and chemical treatment and disinfection; A3 - physical treatment, chemical adjustment and disinfection [73]. The microbiological quality of each group is described in Table 2.4. The underground water is considered suitable for the production of water for human consumption when it is observed evidence of equal or superior quality as the group A1 of inland surface water (Table 2.4).

Table 2.4 – Microbiological parametric values applied to inland surface water used in the production of water for human consumption, according to the categories A1, A2 and A3, (corresponding to the water treatment used): A1 - physical treatment and disinfection; A2 - physical and chemical treatment and disinfection; A3 - physical treatment, chemical adjustment and disinfection [73]

		Maximum Recommendable Value		
Microorganism	**Parametric Value**	**A1[(1)]**	**A2**	**A3**
Total coliforms	CFU/100 mL	50	5000	50000
Faecal coliforms	CFU/100 mL	20	2000	20000
Streptococcus	CFU/100 mL	20	1000	10000
Salmonella		0/5000 mL	0/1000 mL	–

[(1)] This topic is also applicable to underground water used in the production of water for human consumption.

The minimum frequency of sampling for the microbiological water quality control in water for human consumption is described in Table 2.5, according to the type of control. The routine control 1 is the most frequently performed, where *E. coli* and total coliforms are tested.

The analytical methods of reference to detect the microorganisms are based in culturing the microorganisms and are the following [74]:

- *E. coli* and coliforms: ISO 9308-1
- *Enterococcus*: ISO 7899-2

- *P. aeruginosa*: EN ISO 12780
- Number of CFU at 22 °C and 37 °C: EN ISO 6222
- *C. perfringens*: membrane filtration, anaerobic incubation in m-CP agar at 44 °C ± 1 °C for 21 ± 3 hours, and enumeration of the yellow colonies that become pink or red after exposure, for twenty to thirty seconds, to vapours of ammonium hydroxide.

Table 2.5 – Minimum frequency of sampling and microbiological water quality control in water for human consumption supplied by distribution networks, fountains, trucks and water used in food industry, according to the type of control: routine control 1, routine control 2 and inspection control [74]

TYPE OF CONTROL	MICROORGANISM	VOLUME OF WATER SUPPLIED (m^3/day)	MINIMUM SAMPLING (NUMBER/YEAR)
Routine 1	*Escherichia coli* Total coliforms	< 100	6
		≥ 100	12 / 5000 hab
Routine 2	*Clostridium perfringens* (including spores) [(1)] Number of CFU at 22 °C Number of CFU at 37 °C *Pseudomonas aeruginosa* [(2)]	< 100	4
		> 100 and ≤ 1000	4
		> 1000	4 + 3 for each 1000 m^3/day + 3 by fraction of the remaining total volume
Inspection	Enterococcus	< 1000	1
		> 1000 and ≤ 10000	1 + 1 for each 3300 m^3/day + 1 by fraction of the remaining total volume
		> 10000 and ≤ 100000	3 + 1 for each 10000 m^3/day + 1 by fraction of the remaining total volume
		> 100000	10 + 1 for each 25000 m^3/day and by fraction of the remaining total volume

[(1)] When the source of water is not superficial nor is under influence of superficial water, this parameter is part of the inspection control.
[(2)] To be tested only in water sold in bottles or other containers.

2.1.2.2 – LEGAL MICROBIOLOGICAL PARAMETERS APPLIED TO WATER USED IN AQUACULTURE AND IRRIGATION WATER

The legislation states that the microbiological quality control in aquaculture is performed only in the water used for the growth of shellfish. Moreover, the control is not performed directly in the water, instead it is performed in the body of the mollusks, by identification of the presence of faecal coliforms, as detailed in Table 2.6 [73].

Table 2.6 – Microbiological maximum recommendable value required for aquaculture and the minimum frequency of sampling. The analysis is performed in the shellfish flesh and intervalvular liquid of the mollusks for human consumption [73]

MICROORGANISM	MAXIMUM RECOMMENDABLE VALUE	MINIMUM SAMPLING (NUMBER/YEAR)
Faecal coliforms	≤ 300 MPN(1)/100 mL	Quarterly

(1) Counting according to the MPN method (Most Probable Number).

The importance of the microbiological control of the quality of irrigation water is due to the need of protecting the quality of surface and underground waters, the cultures and soil used in agriculture, and the public health [73]. Therefore, the parameters described in Table 2.7 are required by law to access the quality of irrigation water.

Table 2.7 – Microbiological parameters used in the evaluation of quality of irrigation water, and the minimum frequency of sampling [73]

MICROORGANISM	MAXIMUM RECOMMENDABLE VALUE(1)	MAXIMUM ADMISSIBLE VALUE(2)
Faecal coliforms	100 CFU/100 mL	–
Eggs of enteric parasites	–	1/L

(1) This parameter should not be exceeded and ensures the health of the consumer and the supply of his needs.
(2) When it is not possible to ensure the maximum recommendable value, the maximum admissible value is considered. It is not expected a significant risk in the health of the consumer.

Usually, the irrigation water does not have significant values of faecal coliforms and eggs of enteric parasites. Therefore, screening tests are recommended and, whenever a degradation in the quality of the water is identified , the authorities should determine the frequency of sampling [73].

The analytical method of reference for the faecal coliforms is the fermentation in multiple tubes, followed by confirmation of the positive results by culturing in a medium for this purpose and enumeration by the method of the most probable number. The eggs of enteric parasites are to be identified by enumeration in the microscope [73].

2.1.2.3 – LEGAL MICROBIOLOGICAL PARAMETERS APPLIED TO BATHING WATER

Bathing water is defined by the law 58/2005 as surface water, either inland, transitional or coastal waters, where it is predicted a significant number of people bathing, and where bathing is not permanently forbidden or advised against [72]. The main purpose is to protect the public health and to preserve, protect and improve the quality of the environment [75].

The legislation DL 135/2009 describes the parameters for assessing the quality of bathing water, and the microbiological control is divided according to the type of water: inland bathing water (Table 2.8) and coastal and transitional bathing water (Table 2.9) [75].

Table 2.8 – Microbiological parametric values applied to inland bathing water [75]

		Quality		
Microorganism	**Parametric Value**	**Excellent**	**Good**	**Sufficient**
Escherichia coli	CFU/100 mL	500	1000	900
Enterococcus	CFU/100 mL	200	400	330

Table 2.9 – Microbiological parametric values applied to coastal and transitional bathing water [75]

Microorganism	Parametric Value	Quality		
		Excellent	Good	Sufficient
Escherichia coli	CFU/100 mL	250	500	500
Enterococcus	CFU/100 mL	100	200	185

Sampling should be performed to a maximum of 15 days before the beginning of each bathing season, with a minimum of 4 samples per bathing season. Moreover, the dates of sampling should be evenly distributed by the bathing season and do not exceed one month of interval. However, in places where the bathing season does not exceed eight weeks or are located in special geographic conditions, only three samples per bathing season are required [75].

The analytical methods recommended for bathing water are ISO 7899-1 or ISO 7899-2 for the identification of faecal *Enterococcus*, and ISO 9308-3 or ISO 9308-1 for the identification of *E. coli* [75].

2.2 | Methods for Microbiological Water Analysis

The purpose of an analysis to the microbiological quality of water is to determine the presence or absence of pathogens, to quantify the microorganisms and/or to assess the different species present in a sample.

Exhaustive research has been conducted to achieve ideal methods to detect indicators, but among the different methods developed thus far, an "*ideal*" one has not been achieved. The goal is to have fast and reliable results so that, in case of contamination, safety measures can be taken in order to avoid the spread of a disease.

Presently, these methods are at different stages of development, and can be divided in two major groups, as described in Figure 2.1: culture-based methods and molecular methods.

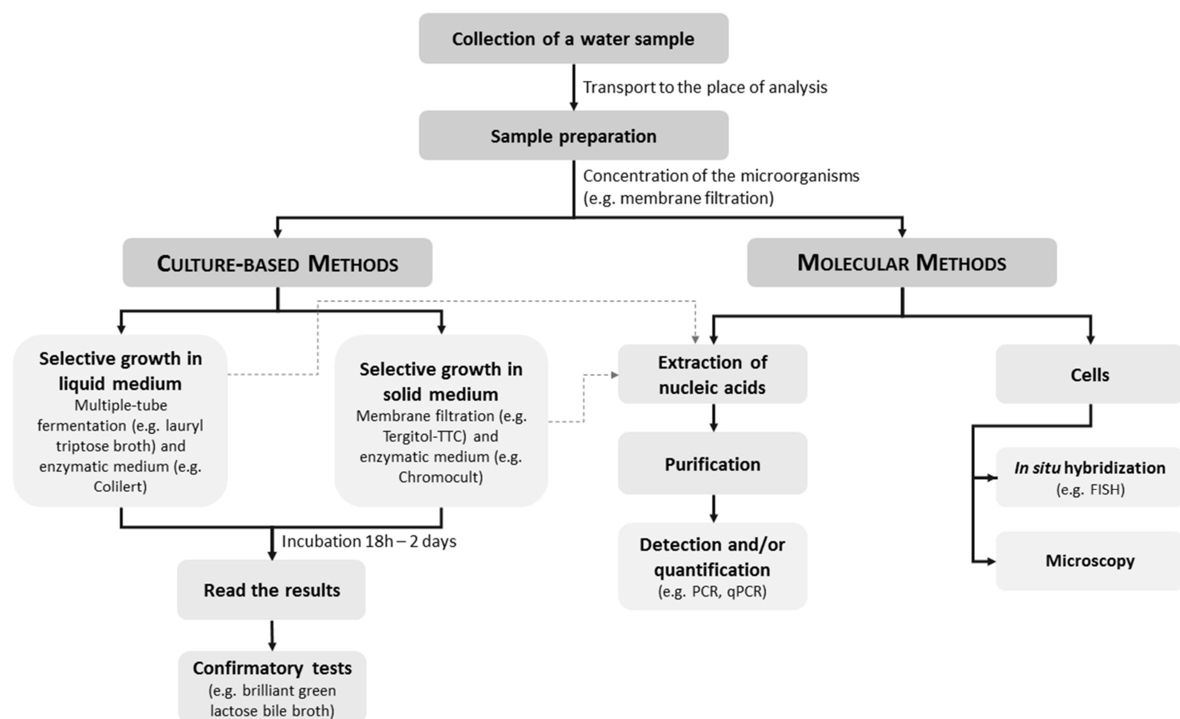


Figure 2.1 – Current methods used for the detection of bacteria in water samples (adapted) [13, 29, 76].

2.2.1 – Culture-based methods

Culturing microorganisms is a laborious process, involving microbial enrichment and the use of selective media in an effort to isolate the indicators from the background bacteria [29]. Nevertheless, since the Pasteur era, the microbiological quality of water has been monitored worldwide mainly through officially approved culture-based methods. These include the classical methods (e.g. multiple-tube fermentation, membrane filtration) and the enzymatic methods (e.g. based on the visual measurement of the activity of the enzymes β-D-galactosidase or β-D-glucuronidase) [76, 77].

Classical methods are based on the capability of the microorganisms to exhibit a biochemical reaction. Multiple-tube fermentation involves the inoculation of a series of tubes with decimal dilutions of the water to be analyzed, and the observation, after an incubation period of 48 h, of presumptive positive responses (production of gas, acid formation) [76]. The membrane filtration method consists on filtering the water with a sterile membrane to retain the microorganisms, which is subsequently placed on a plate with a solid selective medium and, after an incubation period, the colonies formed are enumerated. The results of these two methods are correspondingly expressed as most probable number (MPN) and colony-forming unit (CFU), which allow an estimation of the concentration of the microorganisms in the water [78]. Since these methods are based on biochemical reactions, they are not fully specific to the indicators, as interference from other microorganisms occurs. Hence, additional confirmatory tests are necessary. As a consequence, results are provided only after 3 to 4 days from sampling and actions to prevent the spreading of pathogens are delayed [79].

In the European Union and, thus, in Portugal, the analytical methods recommended by the legislation are based in the classical methods for assessing the microbiological quality of the water. The purpose is to standardize the methods across

the sampling sites, enabling similar sensitivity and specificity in order to be comparable. The detection of *E. coli* is described by ISO 9308-1 and is based in a membrane filtration procedure, with an incubation period of 21 ± 3 h in TTC medium. This culture medium is relatively non-selective, producing large numbers of false positive results and is only recommended for use with high quality waters [80].

To diminish the interference in the detection from other microorganisms and avoid confirmation tests, the multiple-tube fermentation and membrane filtration techniques have evolved to new and increasingly attractive methods that focus on the enzymatic properties of the target microorganisms [79]. These enzymatic methods rely on chromogenic and fluorogenic substrates that correspondingly produce color and fluorescence after cleavage by specific enzymes and are able to provide the results within a working day (18h to 24h) [76]. Chromogenic and fluorogenic substrates are widely used in microbiology and have been proven as powerful tools in the identification of microorganisms, as they allow the detection of specific enzymes produced by the target microorganism. In these methods, the enzymes cleave the chromogenic or fluorogenic substrate that points up the microorganism (grown colonies or liquid culture medium) by color or fluorescence differentiation, thus offering an enhanced accuracy and easy microbial detection and identification [76, 77]. The enzymes most frequently used in water microbiological quality analysis are β-D-glucuronidase (encoded by the *uidA* gene) and β-D-galactosidase (encoded by the *lacZ* gene). The first one is the target to identify *E. coli,* present in more than 97% of the isolates of this bacteria. The second one is assessed for the detection of total coliforms, and classical methods are based on this enzyme [79, 81, 82]. Each of these enzymes can use different chromogenic or fluorogenic substrates, which allows to simultaneously distinguish and quantify different microorganisms within the same culture-medium, thus saving time when the purpose is to analyze several microorganisms in the same sample [76, 81]. Among the chromogenic substrates that can be used to detect the presence of *E. coli* in a sample are the indoxyl-β-D-glucuronide (IBDG) and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Glu), both

producing blue color [83]. Nonetheless, the most frequently used is the fluorogenic substrate known as 4-methylumberlliferyl-β-D-glucuronide (MUG), which exhibits blue fluorescence when exposed to UV light at 366 nm [76, 81, 84]. To detect the presence of total coliforms, chromogenic substrates such as 6-bromo-3-indolyl-β-galactopyranoside (Salmon-Gal), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and, more frequently, *o*-nitrophenyl-β-D-galactopyranoside (ONPG) can be added to culture-medium, resulting in salmon, blue and yellow colonies or colored liquid medium, respectively [76, 81, 84]. The evaluation of the positive or negative results is, therefore, performed visually, which is an advantage over classical methods that are based on biochemical responses more difficult to identify (e.g. gas production) [76].

More recently, some devices based on spectroscopy were developed to detect the fluorescence produced by the MUG substrate used to detect *E. coli* at earlier stages of the culturing process. The major advantage of this technology is that it enables the measurement of the intensity of the fluorescence before it becomes visible to the human eye and, therefore, allows the earlier identification of water contamination [85].

Despite the several advantages of these new enzymatic methods, lack of coverage may still occur when it is used a culture medium based on the specific enzymatic activity of the indicators, since the enzymes are not always found in all the strains of a target indicator [86]. An example of this is the detection of *E. coli* using a culture medium based on β-glucuronidase activity, which is encoded by the *uidA* gene [87]. This gene is found in the majority of *E. coli* strains, but some of them (e.g. the pathogenic *E. coli* O157:H7) do not exhibit the corresponding enzymatic activity [43, 88], which can lead to false-negative results [89-91]. Moreover, indicators may be severely underrepresented when injured by disinfectants used on water treatment processes; these are known as viable but non-culturable cells (VBNC) since they have lost the ability to form colonies [92-94]. Low specificity is also a problem, as interference from other microorganisms can occur, resulting in false-positive outcomes

[80, 86, 95-99]. For example, the enzyme β-D-glucuronidase is found in several microorganisms, including some *Salmonella* and *Shigella* strains, and some *Yersinia, Edwardia, Citrobacter* and *Hafnia* strains. *Flavobacterium* spp., *Bacteroides* spp., *Staphylococcus* spp. and *Clostridium* are also capable of producing β-D-glucuronidase. Numerous other microorganisms carry the β-D-galactosidase enzyme, including Gram-negative bacteria (e.g. some strains of *Enterobacteriaceae*, *Vibrionaceae*, *Pseudomonadaceae*, *Neisseriaceae*), yeast, fungi, protozoa and several Gram-positive bacteria [82]. Thus, it is advised the use of antibiotics in the culture medium to reduce interference from other non-target microorganisms.

2.2.2 – Molecular methods

Currently, culture-based enzymatic methods are the first choice for water quality monitoring due to their advantages over classical methods. However, the limitations associated have induced an increasing interest in molecular methods to achieve a more efficient monitoring of microbiological water quality.

Most of the molecular techniques are based on protocols that amplify and detect nucleic acids. These methods, specifically those based on the PCR technique, amplify *in vitro* specific segments of the genome from the indicators or pathogens (DNA or RNA) [29]. The advantages of these methods over culture-based techniques are due to the possibility of achieving a higher level of specificity and sensitivity on a faster period of time, without the need for complex cultivation or confirmation steps [29]. Moreover, both culturable and non-culturable microorganisms can be detected with this technology within hours [76]. When time is not a concern, a pre-enrichment step may be included to increase the amount of the target microorganisms, which is particularly helpful when their number in the sample is insufficient for its detection.

Despite the outstanding advantages of PCR-based techniques over classical methods, in what concerns water quality monitoring time is money and, more importantly, time is life. Therefore, it is essential to remove the pre-enrichment step from the sample preparation to reduce the assay time. This comprises the major challenge in the adaptation process of molecular methods to water quality analysis, since it directly influences the sensitivity of the method. The microorganisms are often disperse in a large volume of water and it becomes necessary to include a sample preparation step to concentrate them, ideally to the volume used in the molecular method (in PCR-based methods, it is usually used a volume of 1 or 2 μL) [13, 29, 100, 101]. Given the high importance of this procedure for the successful adaptation of these methods to the detection of microorganism in water, some of the techniques used are following described.

2.2.2.1 - SAMPLE PREPARATION

In what concerns water quality monitoring, the sensitivity of detection, time of analysis, volume assayed and the presence of inhibitory substances in the water are critical factors for implementing molecular methods [102, 103]. Depending on the final purpose of the analysis, different paths can be chosen when designing a new sample preparation protocol, as including or not a pre-enrichment step, extracting the nucleic acids or performing a direct detection by PCR, among others. An overview of the different paths that can be adopted for sample preparation is exemplified in Figure 2.2. The main challenge is to avoid culturing the microorganisms and, thus, reduce the assay time in order to increase the competitive potential of molecular methods over the culture-based methods currently available.

Sample preparation procedures usually comprise three demanding steps: concentration of the target organism (e.g. filtration, centrifugation), extraction of the nucleic acids (e. g. heat-shock; chemical, mechanical and/or enzymatic lysis) and purification (e. g. chemical precipitation, solvent extraction, magnetic separation) to eliminate possible inhibitors [104]. Therefore, an ideal sample preparation protocol must efficiently release the nucleic acids from the indicator, protect them from degradation, eliminate or neutralize the inhibitors of the amplification reaction and provide the necessary analytical sensitivity [105].

Environmental samples are naturally diluted [106], which implies a small concentration of the target microorganisms in a large volume of water. PCR-based technologies use small volumes of sample (usually, 1 or 2 μL of template) and the minimum volume to be analyzed by law is typically 100 mL, which, inevitably, arises the problem of insufficient sensitivity [102]. This problem is usually minimized by including a concentration step that increases by several times the number of target microorganisms in an increasingly smaller volume of sample [102, 106].

In what concerns nucleic acid extraction procedures, an impressive variety of commercial solutions are available, but these are only efficient in the presence of a considerable amount of the target microorganisms, which challenges the requirement of identifying the indicator up to only 1 cell present in the water sample.

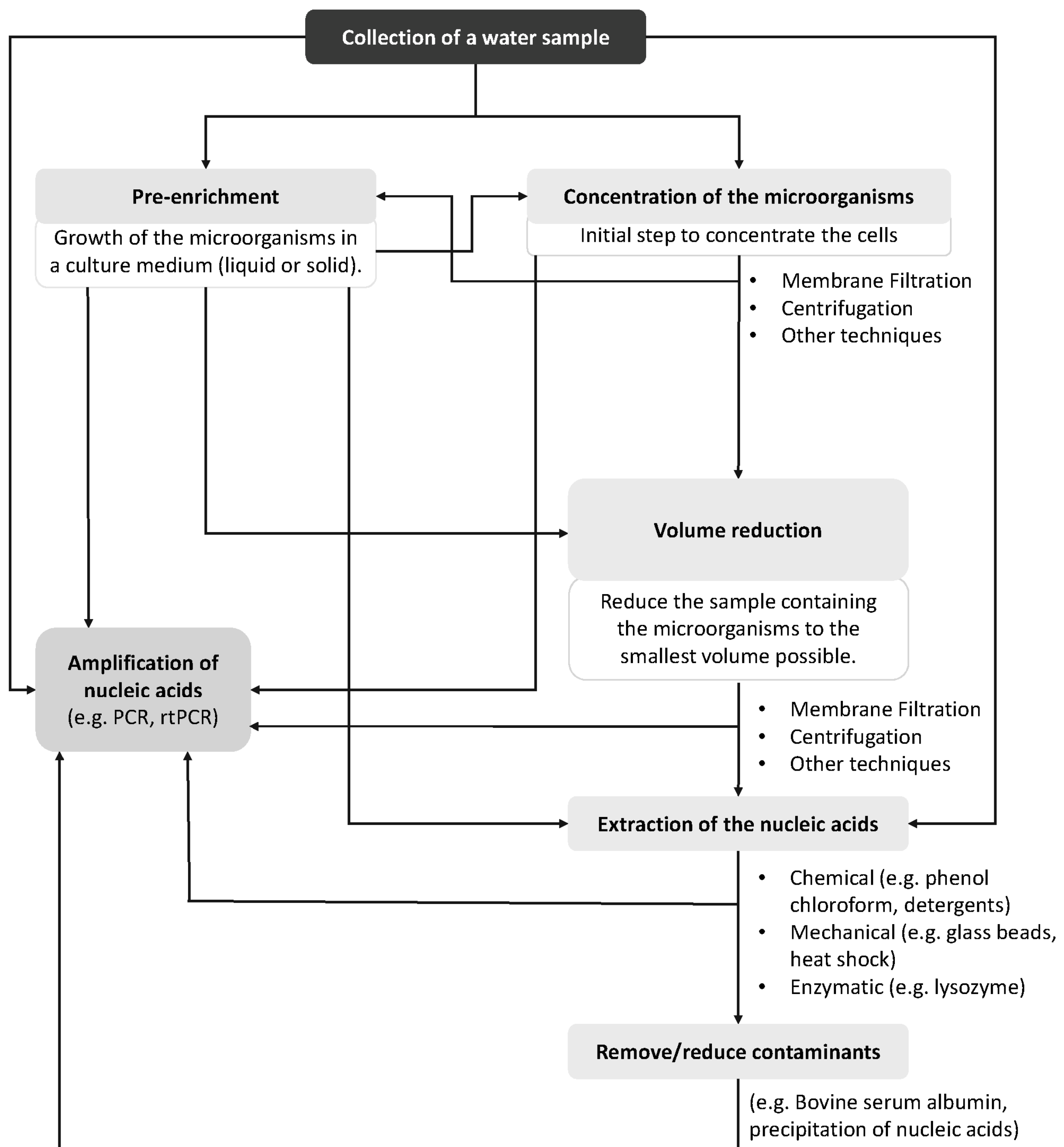


Figure 2.2 – Perspective of the different procedures that can be used in a protocol for the preparation of the water sample to be analysed by amplification of the nucleic acids from the target microorganism (adapted) [29, 44, 76].

It is frequent to encounter environmental samples with PCR inhibitors such as humic acids, organic and inorganic compounds, cellular debris and heavy metals [76, 102, 107]. Many of these inhibitors have solubility properties similar to those of the nucleic acids [108] and, as a consequence, they are concentrated along with the nucleic acids [102], not being removed from the sample during the preparation steps [61]. In these cases, a purification step should be included in order to avoid inhibition of the polymerase activity. However, some of the reagents used in this step (e.g. ethanol) may be difficult to eliminate from the sample and cause inhibition of the amplification as well [107].

Numerous molecular procedures [103, 109-112] still include a culture step before extracting the genetic material of *E. coli* to increase the number of cells in the sample. In recent years, and in the time frame of this thesis, some authors [60, 86, 101, 111, 113-123] started pursuing the highest sensitivity without a pre-enrichment step by using different strategies. Some strategies that resulted in interesting results (concerning the sensitivity, rapidity, environmental sampling) are described as follows.

- Clark et al. (2011) tested ten different methods for recovering waterborne bacterial pathogens, combining membrane filtration and centrifugation to concentrate the cells, with mechanical, chemical and enzymatic lysis techniques for nucleic acid extraction, providing results in 12 h with 73.3% of recovery. Purification was performed using a commercial kit, but not all inhibitors were removed [86].

- An interesting and innovative procedure was developed by Maheux et al. (2011), designated as CRENAME (concentration and recovery of microbial particles, extraction of nucleic acids, and molecular enrichment) [122, 123]. This method used filtration and centrifugation to concentrate cells, and dissolved the membrane to recover cells that could be trapped between the cellulose fibers. After the extraction of nucleic acids, these were amplified using a whole genomic

amplification (WGA) kit, increasing the amount of total DNA available for analysis and, as a consequence, increasing the sensitivity. The whole procedure allowed a detection of 4.5 enterococcal and 1.8 *E. coli*/*Shigella* CFU per 100 mL of potable water in less than 5 hours.

- A simple protocol based on a repeated centrifugation of large volumes of water samples was developed by Ram et al. (2011), where DNA was extracted by boiling, and detection was carried out by rtPCR [101]. A sensitivity of 100 genomic equivalents (GE) per mL, which refers to the amount of DNA needed to assure that one copy of the full genome of the organism is present, was achieved in less than 2 h.

- Heijnen and Medema (2009) accomplished a detection limit of 1 viable CFU of *E. coli* in 100 mL of water through a complex procedure that included filtration, heat-shock and several extraction reagents, as well as magnetic extraction [114]. Despite its complexity, the method was performed in 3-4 h.

- Mull et al. (2009) and Zhang et al. (2012) stated that the membrane filtration of turbid surface waters can be limited due to filter clogging, since organic matter and colloids are a common presence in water [115, 120]. To avoid this problem, Mull et al. (2009) suggested hollow-fiber ultrafiltration as a primary step for concentrating microorganisms and approximately 50 CFU could be recovered from a 40-liter surface water sample. However, there is no indication of the length of this protocol, and, since it includes an incubation period of 20 h to 24 h, it has a disadvantage over other faster methods [115]. Zhang et al. (2012) used a chemical flocculation protocol. *E. coli* cells were captured and concentrated inside the flocs by a lanthanum-based flocculation, which were dissolved with EDTA, filtered and, after extraction, DNA was detected by rtPCR. This method detected 15 cells of *E. coli*/mL in raw and finished water [60].

Difficulties still prevail, particularly, when these techniques are applied to environmental samples, since it is not yet possible to completely eliminate the losses of target cells and/or genetic material, nor to remove the inhibitors during the sample preparation procedures. The most significant technical problems associated with sample preparation are listed in Table 2.10. Nevertheless, remarkable improvements have been accomplished in the recent years, providing attractive results concerning sensitivity and a reduced assay time.

Table 2.10 – Technical challenges associated with the preparation of the samples for the detection of microorganisms in water by molecular methods, when pre-enrichment is avoided (adapted) [29, 107, 108, 124-126]

LIMITATIONS OF WATER SAMPLES PREPARATION FOR MOLECULAR DETECTION
– Need to concentrate the microorganisms from variable volumes of water due to the dilution effect (low concentration of microorganisms per volume), or to perform a previous culture step in order to increase the number of microorganisms in the sample
– Contaminants are concentrated along with the target microorganisms
– Membrane-clogging during filtration of environmental samples
– Loss of target cells and/or nucleic acids during the several steps of the samples preparation (concentration, extraction, purification), which causes an inefficient concentration of the target microorganisms to be detected and, as a consequence, false-negatives may occur
– Lengthy and complex procedures
– Inhibitors are difficult to remove and are not totally eliminated (some have the same solubility properties as nucleic acids)

2.2.2.2 - POLYMERASE CHAIN REACTION-BASED TECHNIQUES

Polymerase chain reaction is a technique used to exponentially amplify (copy) a segment of DNA or RNA, generating thousands to millions of copies of this particular section [127, 128]. This method is based on thermal cycling, a method in which a solution containing the genetic material is repeatedly heated and cooled, causing the enzymatic replication of the specific segment of the DNA or RNA [127]. A typical PCR reaction essentially requires the presence of the DNA sequence to be amplified, a set of primers, nucleotides and a DNA synthesis enzyme (e.g. Taq DNA polymerase). Each reaction typically requires 20 to 40 cycles, divided in three steps of different temperatures for DNA denaturation, primer annealing and DNA synthesis.

The selectivity of the PCR technique is originated in the use of a primer set that is complementary to the DNA region targeted for amplification under specific thermal cycling conditions. The exponential amplification of the target sequence significantly increases the probability of detecting a rare sequence or relatively low numbers of target microorganisms in a sample [76, 129]. Most of the published PCR assays focused on the genes coding the same enzymes identified in the culture-based methods as the DNA sequences targeted for the detection of *E. coli* and total coliforms: β-D-glucuronidase (*uidA* gene) and β-D-galactosidase (*lacZ* gene), respectively [76, 87, 96, 129, 130]. The distinction here is that PCR-based methods can detect and identify *E. coli* strains that carry the *uidA* gene but do not exhibit β-D-glucuronidase activity, whereas culture-based methods cannot. This is particularly important considering that one of these strains that remains undetected by culture-based methods is the pathogenic *E. coli* O157:H7, which was recently the cause of outbreaks in Europe [131, 132]. On the other hand, *uidA* and *lacZ* genes are not exclusive to *E. coli* spp., but can also be found in other closely related bacteria (e.g. *Salmonella* sp.) [76, 133, 134]. Therefore, primers targeting other genes were studied for these indicators to increase specificity (ability to target only the desired species) and ubiquity (ability to detect all strains of targeted species) [79, 105]. Maheux et al. (2009) compared nine PCR primer

sets targeting other genes (including the *uidA* gene) that were designed to detect *E. coli* and *Shigella* in water [135]. Traditionally, *E. coli* and *Shigella* have been considered as two different genera, but more recently some authors introduced the idea that *Shigella* is, in fact, "*E. coli* in disguise" [136] and that genetically they belong to the same species [135-138]. Therefore, Maheux and colleagues [135] tested the ability of the primers to identify both *E. coli* and *Shigella*, and concluded that only the primers targeting the *tuf* gene were able to detect all the strains of both microorganisms. However, *tuf* gene was also identified in *Escherichia fergusonii*, which means that none of the primers was totally ubiquitous and should be selected according to the purpose of the analysis.

The PCR method has often been described for the detection and identification of microorganisms in foods, soils, sediments and waters [13, 15, 60, 107, 139]. Several adaptations of the basic PCR protocol have been developed for the detection of the indicator microorganisms in water samples. In spite of the reported success in the works presented in Table 2.11, common limitations were identified as challenges to be overcome. Pathogens are naturally at low number in environmental samples due to the dilution effect [106], which leads to one of the most significant limitations: the inefficient recovery of all the target genetic material (necessary to achieve the required detection limit of a single cell in the sample) due to the precondition of avoiding the pre-enrichment step as a requirement to increase the rapidity of the assay [106]. Moreover, the volumes used in PCR-based assays are of only a few microliters, being mandatory to apply the concentration step before the nucleic acids extraction. A purification procedure is also advised to reduce or, ideally, eliminate the inhibitors naturally present in environmental samples [29]. These three main steps (concentration, extraction and purification) bring, as a consequence, the loss of a considerable amount of the target cells and/or nucleic acids, due to the complexity of the procedures and the excessive manipulation of the samples. In Table 2.12, the main advantages and difficulties observed in the several research works were summarized for a more comprehensive perspective.

The different methods that have been studied to detect *E. coli* and diarrheagenic *E. coli* in water samples were Multiplex-PCR [140-142] RT-PCR [110], rtPCR and qrtPCR [60, 101, 112, 114, 120, 121, 143, 144], NASBA rtPCR [114], and Multiplex rtPCR [109, 115, 117]. Some of these adaptations of the standard PCR protocol are here described with further detail:

- **Real-time PCR (rtPCR)** is based on the cycling principle of standard PCR with the difference that successful amplification at each cycle is monitored by the release of a fluorescent signal, which brings the possibility to follow the detection in real time [100]. Moreover, measuring the intensity of the fluorescent signal at the end of each cycle allows a quantitative assessment of the initial concentration of the target in the reaction tube, which can be achieved by employing a standard curve, and is designated by quantitative real-time PCR (qrtPCR) [100]. rtPCR-based technologies have emerged in recent years as a leading technology for rapid detection of microorganisms due to their high degree of sensitivity and specificity, introducing the possibility of a much faster detection of the target microorganism in real time, with no need for additional time to detect the rtPCR products by electrophoresis [106, 145]. Furthermore, quantification of the target is also possible by qrtPCR [100]. Khan et al. (2007) used rtPCR in the detection of *E. coli* from agriculture watersheds by amplifying newly designed species-specific oligonucleotide primers derived from conserved flanking regions of the 16S rRNA gene, the internal transcribed spacer region (ITS) and the 23S rRNA gene [146]. This analysis led to the development of the first rtPCR assay in the ITS region for detection and enumeration of *E. coli*, as an attempt to solve the limitations of other primers normally used (for *lacZ, lamB, uidA, malB* genes), which are not totally specific for *E. coli*, or are of insufficient ubiquity [60, 105]. However, no specificity evaluation was performed in this research to confirm the advantage of these primers over others.

An appealing sensitivity was achieved by Maheux et al. (2011) with the *Enterococcus* CRENAME-rtPCR and *E. coli/Shigella* CRENAME-rtPCR methods [122]. These authors used molecular enrichment (WGA) as an alternative to culture enrichment. By performing an unspecific amplification of nucleic acids, improvements in the sensitivity were obtained within a considerably reduced period of time, when compared to culturing (3 h). However, this unmeasured amplification of the genetic material makes it impossible to quantify the amount of the target originally present in the sample. A more comprehensive evaluation of the adaptability of this method to other water samples, such as polluted river water and seawater, as well as its behavior in the presence of higher levels of inhibitors, should be carried out in order to assess its adaptability to water quality monitoring.

- **Multiplex-PCR/rtPCR** is rather useful as it allows the simultaneous detection of different gene sequences and/or different microorganisms through the introduction of different primer-pairs in the same reaction tube [147]. Horakova et al. (2008) developed a multiplex-PCR method for reliable detection of *E. coli* isolated from water samples, which enables the differentiation from biochemically and phylogenetically related bacteria [141]. To improve the specificity of the PCR-based method for *E. coli* identification, in this method four target genes were used: *uidA*, *lacZ*, *lacY* (coding for lactose permease) and *cyd* (coding for cytochrome bd complex) genes, whose products could be considered as biochemical hallmarks of *E. coli* spp. [141]. The four fragments were observed only on *E. coli* strains, including those that did not exhibit β-D-glucuronidase, and not on other close relatives.

- **Nested PCR** comprises two consecutive rounds of PCR amplification, being the second round to increase the PCR product to detectable levels [56]. The first primer set is used to amplify a sequence which will serve as a template for the second amplification performed with a second primer set [148]. Juck et al.

(1996) used this method targeting the *uidA* gene of *E. coli* by designing two sets of primers: the first pair produced an amplicon of 486 bp that served as template for the second primer pair, which resulted in a fragment of 186 bp [149].

PCR-based technologies are used to determine whether the target DNA is present in the sample or not, not being able to provide information about the viability of the microorganisms, a key factor for their pathogenicity. When released in water, the microorganisms often enter in a VBNC state due to starvation or inefficient water treatment procedures [29]. Even when the water treatment is successful and is able to kill all the microorganisms, DNA is very stable after cell-death, which may result in the detection of both viable and dead cells, becoming difficult to conclude about the safety of the water tested. It is also frequent to have a contamination of the PCR reagents with trace amounts of DNA from the target microorganism, as the recombinant polymerases used are frequently produced in *E. coli*. Therefore, assessing the viability of the indicators is an absolute prerequisite for the application to water quality monitoring and to fulfill the requirements of the legislation [124]. Some adaptations of the PCR technology have been introduced as a way to distinguish viable from dead cells:

- **Reverse Transcriptase PCR (RT-PCR)** is a two-stage process: a target messenger RNA (mRNA) sequence is first transcribed into a complementary DNA (cDNA) sequence, which subsequently can be directly amplified by PCR [92]. Liu et al. (2008) applied this method to river water [110]. Samples were filtered with a low-protein-binding membrane, RNA was extracted and purified directly. Target *E. coli* O157:H7 was detected using a combination of RT-PCR and electronic microarray, with a sensitivity of 50 VBNC cells in 1 liter of river water (the duration of the assay is not referred). It is known that mRNA has a very short half-life (seconds) and is able to provide a much better association with the viability of the cell. Even though mRNA has a potential advantage for

distinguishing viable from non-viable cells, the complexity of the method and practical difficulties in extracting detectable levels of intact mRNA from only a few cells can be a serious drawback [61, 92].

Ribosomal RNA (rRNA) has also been studied as a possible target of detection [61, 92, 144], since it is more abundant and easier to detect than mRNA. However, its longer half-life and instability makes it a weaker option.

- **Nucleic Acid Based Sequence Amplification (NASBA)** is an improvement of RT-PCR, as it enables selective amplification of a RNA fragment, without interference of background DNA [92]. Heijnen and Medema (2009) developed a real-time NASBA (NASBA-rtPCR) for the detection of *E. coli* in water samples using a molecular beacon probe for the amplification of a fragment of mRNA coding for the *clpB* heat shock protein [114, 150]. Different inactivation procedures (starvation, chlorine treatment, UV-irradiation and chlorine) were used to test the correlation between culturability and the ability to detect *E. coli* with NASBA [151]. A 100% specificity of the NASBA assay was demonstrated in the tested strains and the sensitivity of 1 viable/100 mL was determined using serially diluted spiked tap water samples. Moreover, a good correlation was observed between the number of colonies on the culture plates and the results obtained with NASBA. It should be taken into account that the VBNC cells do not form colonies on the culture plates but can interfere in the NASBA detection and, thus, alter that correlation. With an assay time of 3-4 h, this study has demonstrated that the NASBA method has potential as a rapid test for microbiological water quality monitoring.

- **Propidium monoazide rtPCR (PMA-rtPCR)** has been introduced recently as an alternative method to distinguish viable from dead cells. Propidium monoazide (PMA) is a DNA intercalating dye that, when photo-activated, forms a stable covalent bond with DNA, thus resulting in permanent DNA

modification [152]. This dye is cell membrane-impermeable, which means that live cells with intact membranes can exclude PMA, and free DNA or the DNA from cells with the integrity of the membrane compromised are irreversibly modified (preceding the PCR amplification) [124]. As a result, a selective PCR amplification of the DNA from only live cells occurs. This method has proven to be effective in evaluating the sterilization rate of water [152] and in significantly reducing the false positive signal from the amplification of DNA from dead cells, even in a background of a highly abundant and complex microflora [124]. However, matrices with high solid content can hamper the detection [153].

Table 2.11 assembles some of the PCR-based techniques reported in the literature to assess the microbiological quality of water, selected either due to the interesting approaches used in the development of a new protocol, or for the promising results obtained. The technology applied, microorganism and type of sample, whether a pre-enrichment step is included or not, and the length of the whole procedure is indicated.

The PCR-based technology has been investigated as a possible methodology for detection and, in some cases, quantification of target microorganisms in water quality monitoring due to its attractive advantages over culture-based technology. Many improvements and adaptations of PCR-based methods have been published, reinforcing the high potential of these techniques for the detection of microorganisms, particularly when it is possible to apply a pre-enrichment step. In addition to the increased sensitivity and specificity, PCR-based methods made possible to test multiple targets in the same analysis (not only the indicators), to detect the pathogenic *E. coli* O157:H7 and VBNC cells, which are not identified by culture-based methods, and to reduce the assay time. However, several tests were performed using samples of clean water spiked with cultured strains of bacteria, or using simple dilutions of DNA. Even though this procedure is the first step in developing a valid protocol for the detection of microorganisms, a follow-up step must be taken in environmental water samples, so

that the developed methods can be completely validated and its applicability range determined.

Table 2.11 – Selected PCR-based methods for microbiological water quality monitoring described by method used, microorganism selected, sample preparation procedures, type of sample tested, detection target, sensitivity, time of analysis and the presence of a pre-enrichment step in the procedure

METHOD	MICROORGANISM	SAMPLE PREPARATION	TYPE OF SAMPLE	TARGET	PRE-ENRICHMENT	SENSITIVITY	TIME OF ANALYSIS	REFERENCE
PCR	*E. coli* O157:H7	Filtration, membrane and filtrates re-suspended, boiled, extraction (phenol-chloroform-isoamyl alcohol), precipitation	Wastewater	*rfbE*	No	200 CFU/L in pure water Inhibition on wastewater samples	Not reported	[119]
RT-PCR and electronic microarray	*E. coli* O157:H7	Filtration, RNA extraction (ethanol, phenol-chloroform, TRIzol reagent)	Drinking water River water	*rfbE* and *fliC*	No	3-4 CFU/L in drinking water 7 CFU/L in river water 50 VBNC/1 L in river water	Not reported	[110]
NASBA rtPCR (molecular beacon probe)	*E. coli*	Filtration, lysis buffer, heat-shock, magnetic nucleic acids extraction	Drinking water Treated sewage Surface water	*clpB*-mRNA	No	1 viable/100 mL	3-4 h	[114]
qrtPCR (SYBR Green)	*E. coli*	Centrifugation, cells lysis and purification	Agriculture watersheds	Internal transcribed spacer (ITS) region between 16S-23S rRNA subunit genes	No	10 cells/mL	< 3 h	[60]
Multiplex rtPCR (SYBR Green)	*E. coli* O157:H7	1 colony suspended in water, heat-shock, centrifugation, supernatant used for amplification	Laboratorial	*stx1* and/or *stx2*	Yes (overnight)	8.4×10^{3} CFU/mL	Not reported	[154]
Multiplex rtPCR (SYBR Green)	Enterohemorrhagic *E. coli*	Boiling, sonication, centrifugation	Laboratorial	*stx1, stx2* and *eae*	Yes (overnight)	10^{3} to 10^{4} CFU/mL	Not reported	[155]

METHOD	MICROORGANISM	SAMPLE PREPARATION	TYPE OF SAMPLE	TARGET	PRE-ENRICHMENT	SENSITIVITY	TIME OF ANALYSIS	REFERENCE
qrtPCR (molecular beacon probe)	Shiga toxin-producing *E. coli* (STEC)	Repeated centrifugation, boiling, precipitation	River water Drinking water	*stx2*	No	10 GE/PCR 100 GE/100 mL	< 2 h	[101]
qrtPCR (molecular beacon probe)	Enterotoxigenic *E. coli* (ETEC)	Repeated centrifugation, boiling, precipitation	Surface water	*LT1*	No	2 CFU/mL in spiked water $1.2×10^3$ to $1.4×10^6$ CFU/100 mL in polluted river water	Not reported	[143]
rtPCR (TaqMan probe)	*E. coli* O157:H7	Ultra-filtration, incubation, immunomagnetic-separation, incubation, colony DNA extraction	Surface water	*stx1, stx2* and *rfbE*	Yes (> 24 h)	50 cells / 40 L	Not reported	[115]
rtPCR (TaqMan probe)	*Enterococcus* sp. E*nterococcus faecalis/faecium* *E. coli and Shigella spp.*	Filtration, dissolution of the filtration membrane, concentration by repeated centrifugation, lysis with glass beads, whole genome amplification	Drinking water	23S rRNA, *mtlf*, *ddl*, *atpD*	No (molecular enrichment: 3 h)	4.5 enterococcal CFU/100 mL 1.8 *E. coli/Shigella* CFU/100 mL	< 5 h	[117, 123]
Multiplex qrtPCR (Minor groove binding probes)	Stressed *E. coli* O157:H7	Nucleic acids extraction from 1 colony	Drinking water	*eae*, *stx1* and *stx2*	Yes (≈24 h)	3-4 cells/L	24 h	[112]
qrtPCR (TaqMan probe)	*E. coli* *Helicobacter pylori*	Lanthanum-based flocculation	Raw and finished water	*lacZ*	No	15 *E. coli* cells/mL	Not reported	[120]
qrtPCR (SYBR Green)	ETEC	Boiling and precipitation	Potable waters derived from civic water supply	*LT1 and ST1*	No	1 CFU/PCR	Not reported	[121]

Table 2.12 - Summary of the main advantages and limitations observed in PCR-based methods for microbiological water quality monitoring (adapted) [13, 29, 101, 107, 108, 124, 125, 156]

METHOD	ADVANTAGES	LIMITATIONS
Standard PCR	– Higher sensitivity and specificity when compared to culture-based methods – Possibility of Multiplex-PCR for multiple pathogen detection – Detects VBNC cells – Simultaneous detection of different targets is possible (multiplex-PCR)	– Post-PCR confirmation step needed (electrophoresis) – Non-quantitative – No distinction between viable and dead cells (detects both) – Inhibition of the amplification when environmental samples are analyzed due to the presence of contaminants (e.g. organic, inorganic and biomass content) – Low nucleic acid concentration causes frequent variability on the results, which lead tube-to-tube variability
RT-PCR	– Distinguishes viable from dead cells	– Complexity of the procedures – Short half-life of RNA – Technical expertise in necessary – Environmental samples can inhibit the detection
rtPCR	– Faster than conventional PCR – High level of sensitivity and specificity – Real-time detection – Quantification of the target in the sample is possible	– Inhibition of the amplification when environmental samples are analyzed due to the presence of contaminants – No distinction between viable and dead cells (detects both)
NASBA-rtPCR	– Distinguishes viable from dead cells – No interference from background DNA	– The same as in RT-PCR
PMA-rtPCR	– Distinguishes live from dead cells and from free DNA – Simple to perform	– Possible inhibition from high solid content samples (further research is required) – Use of an extremely toxic compound (e.g. propidium monoazide)

Another key issue is the observed variability in molecular detection. It was shown that the extremely low concentrations of target microorganisms in water samples can cause high unpredictable fluctuations in the PCR efficiency, which leads to tube-to-tube variability and, thus, to false negatives [104]. Another cause of variability concerns the differences in composition of the water between samples and place of

sampling [157]. Additionally, extracellular DNA that persists in the environment can affect the detection, increasing the risk of false-positives [158]. However, Maheux et al. (2011) [122] found that free DNA in drinking water could flow through the filter during the filtration step without interfering with the detection protocol. Thus, the detection of microorganisms in environmental water samples requires the adjustment and optimization of the protocol to each type of water and place of sampling, as the contaminants (inhibitors and background DNA), the concentrations and types of indicators, the weather and other variables differ from location to location [53, 158-160]. Standardization of these methods is, therefore, a challenge for a routine use, since accuracy and reproducibility are still hindered by these problems. Some studies concerning other indicators, with the purpose to achieve standardization and optimization for specific locations, are already having some promising results [68, 125, 159-162].

Molecular methods are promising tools to provide sensitive, rapid and quantitative analysis for the detection of the indicators currently used in microbiological water quality monitoring, VBNC cells, and new emerging pathogens and indicators. The currently available or under development techniques still require further improvement before being standardized and adapted to the different characteristics of environmental samples of water, each having its own particularities.

2.3| FUTURE PERSPECTIVES

Numerous approaches used for the detection of indicator microorganisms and pathogens are being deeply explored with the purpose of efficiently identifying harmful contaminations in water.

Molecular PCR-based methods have shown immense potential in the characterization and recognition of targets, identifying non-culturable microorganisms and providing information about the presence of indicators and/or pathogens in water. This is particularly accurate when the pre-enrichment step is performed. However, as faster results are pursued, the pre-enrichment step is avoided and thus, the detection limit of only 1 CFU per sample becomes a challenge. Therefore, the complexity of these methods, the partial loss of the target cells and/or genetic material during these processes, and the presence of inhibitors and contaminants in environmental samples are barriers not yet fully overcome. Achieving the required levels of reliability, precision and robustness is essential for water quality monitoring and, so far, analysis in replicate and an adaptation of the protocol to each location of sampling are still a necessity. Nevertheless, detection using rtPCR techniques is growing quickly. In particular, US EPA is moving toward the implementation of qrtPCR for the detection of *Enterococci* and *Bacteroidales* for ambient water quality monitoring.

The new methods to be developed for the detection of microorganisms should overcome the current methods on what concerns speed, specificity and sensitivity [163]. Novel molecular methodologies such as loop-mediated isothermal amplification (LAMP) of DNA techniques have emerged as an alternative to the use of PCR-based methods [13, 164]. This new technique is faster than other PCR-based techniques as the cycling equipment is unnecessary, thus being a simpler and cheaper method for

the identification of target microorganisms, capable of providing equivalent sensitivity and specificity [164].

A recent interest is growing in coupling the molecular-based techniques with electronic devices (biosensors), as they promise to combine the advantages of a higher sensitivity and specificity of the molecular methods with the portability and easy-to-use technology of sensors and microchips [163, 165]. It is expected that in the near future these technologies will bring the possibility of *in situ* real-time monitoring using low cost technology [106, 166-168].

Research must continue its efforts on detecting indicators in their natural environment and on its subsequent sample preparation steps. Miniaturization strategies, by confining a reaction within a micro or nanoscale fluidic channel, can benefit sensitivity, since the losses of sample are reduced by the possibility to comprise multiple operations together in one device [163]. Coupled with new materials and multiplexing, these efforts are envisaged to bring new methods capable of detecting several relevant pathogens at once, with the desired sensitivity, specificity and speed.

2.4 | References

1. Ahmed, W., Neller, R., and Katouli, M., Host species-specific metabolic fingerprint database for *Enterococci* and *Escherichia coli* and its application to identify sources of fecal contamination in surface waters. Applied and Environmental Microbiology, 2005. 71, 4461-4468.
2. Higgins, J.A., Belt, K.T., Karns, J.S., Russell-Anelli, J., and Shelton, D.R., *tir*- and *stx*-positive *Escherichia coli* in stream waters in a metropolitan area. Applied and Environmental Microbiology, 2005. 71, 2511-2519.
3. Scott, T.M., Rose, J.B., Jenkins, T.M., Farrah, S.R., and Lukasik, J., Microbial source tracking: current methodology and future directions. Applied and Environmental Microbiology, 2002. 68, 5796-5803.
4. Davis, M.A., Cloud-Hansen, K.A., Carpenter, J., and Hovde, C.J., *Escherichia coli* O157:H7 in environments of culture-positive cattle. Applied and Environmental Microbiology, 2005. 71, 6816-6822.
5. Walters, S.P., Gannon, V.P.J., and Field, K.G., Detection of Bacteroidales fecal indicators and the zoonotic pathogens *E. coli* O157 : H7, *Salmonella*, and *Campylobacter* in river water. Environmental Science & Technology, 2007. 41, 1856-1862.
6. Gibson, K.E., Viral pathogens in water: occurrence, public health impact, and available control strategies. Current Opinion in Virology, 2014. 4, 50-57.
7. WHO, Water recreation and disease - Plausibility of associated infections: acute effects, sequelae and mortality 2005, International Water Association Publishing.
8. Straub, T.M. and Chandler, D.P., Towards a unified system for detecting waterborne pathogens. Journal of Microbiological Methods, 2003. 53, 185-197.
9. Bettelheim, K.A., Bennett-Wood, V., Lightfoot, D., Wright, P.J., and Marshall, J.A., Simultaneous isolation of verotoxin-producing strains of *Escherichia coli* O128:H2 and viruses in gastroenteritis outbreaks. Comparative Immunology, Microbiology and Infectious Diseases, 2001. 24, 135-142.
10. Schroeder, S., Caldwell, J., Vernon, T., White, P., Granger, S., and Bennett, J., A waterborne outbreak of gastroenteritis in adults associated with *Escherichia coli*. The Lancet, 1968. 291, 737-740.
11. Geldreich, E.E., Fox, K.R., Goodrich, J.A., Rice, E.W., Clark, R.M., and Swerdlow, D.L., Searching for a water supply connection in the Cabool, Missouri disease outbreak of *Escherichia coli* 0157:H7. Water Research, 1992. 26, 1127-1137.
12. Hsu, B.-M., Wu, S.-F., Huang, S.-W., Tseng, Y.-J., Ji, D.-D., Chen, J.-S., and Shih, F.-C., Differentiation and identification of *Shigella* spp. and enteroinvasive *Escherichia coli* in environmental waters by a molecular method and biochemical test. Water Research, 2010. 44, 949-955.

13. Pitkanen, T., Review of *Campylobacter* spp. in drinking and environmental waters. Journal of Microbiological Methods, 2013. 95, 39-47.

14. McDaniels, A.E., Wymer, L., Rankin, C., and Haugland, R., Evaluation of quantitative real time PCR for the measurement of *Helicobacter pylori* at low concentrations in drinking water. Water Research, 2005. 39, 4808-4816.

15. Ghosh, P. and Bodhankar, S.L., Determination of risk factors and transmission pathways of *Helicobacter pylori* in asymptomatic subjects in Western India using polymerase chain reaction. Asian Pacific Journal of Tropical Disease, 2012. 2, 12-17.

16. Velázquez, M. and Feirtag, J.M., *Helicobacter pylori*: characteristics, pathogenicity, detection methods and mode of transmission implicating foods and water. International Journal of Food Microbiology, 1999. 53, 95-104.

17. Murgia, R., Riquelme, N., Baranton, G., and Cinco, M., Oligonucleotides specific for pathogenic and saprophytic *Leptospira* occurring in water. FEMS Microbiology Letters, 1997. 148, 27-34.

18. Parker, J. and Walker, M., Survival of a pathogenic *Leptospira* serovar in response to combined *in vitro* pH and temperature stresses. Veterinary Microbiology, 2011. 152, 146-150.

19. Kumar, S., Balakrishna, K., and Batra, H.V., Enrichment-ELISA for detection of *Salmonella typhi* from food and water samples. Biomedical and Environmental Sciences, 2008. 21, 137-143.

20. Kumar, A., Arora, V., Bashamboo, A., and Ali, S., Detection of *Salmonella typhi* by polymerase chain reaction: Implications in diagnosis of typhoid fever. Infection, Genetics and Evolution, 2002. 2, 107-110.

21. Zhou, G., Wen, S., Liu, Y., Li, R., Zhong, X., Feng, L., Wang, L., and Cao, B., Development of a DNA microarray for detection and identification of *Legionella pneumophila* and ten other pathogens in drinking water. International Journal of Food Microbiology, 2011. 145, 293-300.

22. Cassier, P., Landelle, C., Reyrolle, M., Nicolle, M.C., Slimani, S., Etienne, J., Vanhems, P., and Jarraud, S., Hospital washbasin water: risk of Legionella-contaminated aerosol inhalation. Journal of Hospital Infection, 2013. 85, 308-311.

23. Fykse, E.M., Nilsen, T., Nielsen, A.D., Tryland, I., Delacroix, S., and Blatny, J.M., Real-time PCR and NASBA for rapid and sensitive detection of *Vibrio cholerae* in ballast water. Marine Pollution Bulletin, 2012. 64, 200-206.

24. Reidl, J. and Klose, K.E., *Vibrio cholerae* and cholera: out of the water and into the host. FEMS Microbiology Reviews, 2002. 26, 125-139.

25. Thompson, C.C., Freitas, F.S., Marin, M.A., Fonseca, E.L., Okeke, I.N., and Vicente, A.C.P., *Vibrio cholerae* O1 lineages driving cholera outbreaks during seventh cholera pandemic in Ghana. Infection, Genetics and Evolution, 2011. 11, 1951-1956.

26. Neogi, S.B., Chowdhury, N., Asakura, M., Hinenoya, A., Haldar, S., Saidi, S.M., Kogure, K., Lara, R.J., and Yamasaki, S., A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Letters in Applied Microbiology, 2010. 51, 293-300.

27. Robert-Pillot, A., Guénolé, A., Lesne, J., Delesmont, R., Fournier, J.-M., and Quilici, M.-L., Occurrence of the *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. International Journal of Food Microbiology, 2004. 91, 319-325.

28. Cashdollar, J.L., Brinkman, N.E., Griffin, S.M., McMinn, B.R., Rhodes, E.R., Varughese, E.A., Grimm, A.C., Parshionikar, S.U., Wymer, L., and Fout, G.S., Development and evaluation of EPA Method 1615 for detection of enterovirus and norovirus in Water. Applied and Environmental Microbiology, 2013. 79, 215-223.

29. Girones, R., Ferrús, M.A., Alonso, J.L., Rodriguez-Manzano, J., Calgua, B., de Abreu Corrêa, A., Hundesa, A., Carratala, A., and Bofill-Mas, S., Molecular detection of pathogens in water – The pros and cons of molecular techniques. Water Research, 2010. 44, 4325-4339.

30. De Paula, V.S., Diniz-Mendes, L., Villar, L.M., Luz, S.L.B., Silva, L.A., Jesus, M.S., da Silva, N.M.V.S., and Gaspar, A.M.C., Hepatitis A virus in environmental water samples from the Amazon Basin. Water Research, 2007. 41, 1169-1176.

31. Swain, S.K., Baral, P., Hutin, Y.J., Rao, T.V., Murhekar, M., and Gupte, M.D., A hepatitis E outbreak caused by a temporary interruption in a municipal water treatment system, Baripada, Orissa, India, 2004. Transactions of the Royal Society of Tropical Medicine and Hygiene, 2010. 104, 66-69.

32. Sinclair, R.G., Jones, E.L., and Gerba, C.P., Viruses in recreational water-borne disease outbreaks: a review. Journal of Applied Microbiology, 2009. 107, 1769-1780.

33. Gómez-Couso, H., Fontán-Sainz, M., Navntoft, C., Fernández-Ibáñez, P., and Ares-Mazás, E., Comparison of different solar reactors for household disinfection of drinking water in developing countries: evaluation of their efficacy in relation to the waterborne enteropathogen *Cryptosporidium parvum*. Transactions of the Royal Society of Tropical Medicine and Hygiene, 2012. 106, 645-652.

34. Robertson, L.J., Chapter Nineteen - *Giardia duodenalis*, in *Microbiology of Waterborne Diseases (Second Edition)*, S.L. Percival, et al., Editors. 2014, Academic Press: London, 375-405.

35. Cabral, J.P.S., Water microbiology. Bacterial pathogens and water. International Journal of Environmental Research and Public Health, 2010. 7, 3657-3703.

36. Cheun, H.-I., Kim, C.-H., Cho, S.-H., Ma, D.-W., Goo, B.-L., Na, M.-S., Youn, S.-K., and Lee, W.-J., The first outbreak of giardiasis with drinking water in Korea. Osong Public Health and Research Perspectives, 2013. 4, 89-92.

37. Yang, Z., Wu, X., Li, T., Li, M., Zhong, Y., Liu, Y., Deng, Z., Di, B., Huang, C., Liang, H., and Wang, M., Epidemiological survey and analysis on an outbreak of gastroenteritis due to water contamination. Biomedical and Environmental Sciences, 2011. 24, 275-283.

38. Gelting, R.J., Baloch, M.A., Zarate-Bermudez, M.A., and Selman, C., Irrigation water issues potentially related to the 2006 multistate *E. coli* O157:H7 outbreak associated with spinach. Agricultural Water Management, 2011. 98, 1395-1402.

39. Jones, E.L., Gaither, M., Kramer, A., and Gerba, C.P., An analysis of water quality in the Colorado river, 2003–04; an investigation into recurring outbreaks of norovirus among rafters. Wilderness & Environmental Medicine, 2009. 20, 6-13.

40. Breathnach, A.S., Cubbon, M.D., Karunaharan, R.N., Pope, C.F., and Planche, T.D., Multidrug-resistant *Pseudomonas aeruginosa* outbreaks in two hospitals: association with contaminated hospital waste-water systems. Journal of Hospital Infection, 2012. 82, 19-24.

41. Walser, S.M., Gerstner, D.G., Brenner, B., Höller, C., Liebl, B., and Herr, C.E.W., Assessing the environmental health relevance of cooling towers – A systematic review of legionellosis outbreaks. International Journal of Hygiene and Environmental Health, 2012.

42. WHO, Water Quality and Health Strategy 2013-2020. 2013.

43. Vale, F.F., Silva, A.M., Granja, A.T., Vale, M.J., and Vieira, H., Simultaneous detection of micro-organisms in water samples for future chip applications: Coliform bacteria, non-mandatory bacteria, hepatitis A virus and noroviruses. Physica Status Solidi (C) Current Topics in Solid State Physics, 2009. 6, 2184-2189.

44. Brettar, I. and Höfle, M.G., Molecular assessment of bacterial pathogens - a contribution to drinking water safety. Current Opinion in Biotechnology, 2008. 19, 274-280.

45. de Man, H., van den Berg, H.H.J.L., Leenen, E.J.T.M., Schijven, J.F., Schets, F.M., van der Vliet, J.C., van Knapen, F., and de Roda Husman, A.M., Quantitative assessment of infection risk from exposure to waterborne pathogens in urban floodwater. Water Research, 2014. 48, 90-99.

46. Savichtcheva, O. and Okabe, S., Alternative indicators of fecal pollution: Relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. Water Research, 2006. 40, 2463-2476.

47. (WHO), W.H.O., Heterotrophic plate counts and drinking-water safety, J.C. J. Bartram, M. Exner, C. Fricker, A. Glasmache, Editor. 2003, International Water Association Publishing: London, UK.

48. Allen, M.J., Edberg, S.C., and Reasoner, D.J., Heterotrophic plate count bacteria - what is their significance in drinking water? International Journal of Food Microbiology, 2004. 92, 265-274.

49. Counsil Directive 2009/54/EC of 18 June 2009 on the exploitation and marketing of natural mineral waters. Official Journal of the European Union. 164 (26.6.2009) 45-58.

50. Counsil Directive 2006/7/EC of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC. Official Journal of the European Union. 64 (4.3.2006) 37-51.

51. Counsil Directive 98/83/CE of 3 November 1998 on the quality of water intended for human consumption. Official Journal of the European Communities. L330 (5.12.98) 32-53.

52. Wade, T.J., Pai, N., Eisenberg, J.N.S., and Colford, J.M., Do US Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environmental Health Perspectives, 2003. 111, 1102-1109.

53. Boehm, A.B., Ashbolt, N.J., Colford Jr, J.M., Dunbar, L.E., Fleming, L.E., Gold, M.A., Hansel, J.A., Hunter, P.R., Ichida, A.M., McGee, C.D., Soller, J.A., and Weisberg, S.B., A sea change ahead for recreational water quality criteria, in Journal of Water & Health. 2009, IWA Publishing, 9-20.

54. Agency, U.S.E.P., Implementation guidance for ambient water quality for bacteria. Draft., O.o. water, Editor. 2002.

55. WHO, Guidelines for drinking-water quality. 2011, WHO: Geneva, Switzerland.

56. Tallon, P., Magajna, B., Lofranco, C., and Kam, T.L., Microbial indicators of faecal contamination in water: A current perspective. Water, Air, and Soil Pollution, 2005. 166, 139-166.

57. Martiny, A.C., Albrechtsen, H.-J., Arvin, E., and Molin, S., Identification of bacteria in biofilm and bulk water samples from a nonchlorinated model drinking water distribution system: detection of a large nitrite-oxidizing population associated with *Nitrospira* spp. Applied and Environmental Microbiology, 2005. 71, 8611-8617.

58. Eichler, S., Christen, R., Holtje, C., Westphal, P., Botel, J., Brettar, I., Mehling, A., and Hofle, M.G., Composition and dynamics of bacterial communities of a drinking water supply system as assessed by RNA- and DNA-based 16S rRNA gene fingerprinting. Applied and Environmental Microbiology, 2006. 72, 1858-1872.

59. Tortorello, M.L., Indicator organisms for safety and quality-uses and methods for detection: Minireview. Journal of AOAC International, 2003. 86, 1208-1217.

60. Khan, I.U.H., Gannon, V., Kent, R., Koning, W., Lapen, D.R., Miller, J., Neumann, N., Phillips, R., Robertson, W., Topp, E., van Bochove, E., and Edge, T.A., Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agriculture watersheds. Journal of Microbiological Methods, 2007. 69, 480-488.

61. Lemarchand, K., Masson, L., and Brousseau, R., Molecular biology and DNA microarray technology for microbial quality monitoring of water. Critical Reviews in Microbiology, 2004. 30, 145-172.

62. McLellan, S.L., Daniels, A.D., and Salmore, A.K., Clonal populations of thermotolerant *Enterobacteriaceae* in recreational water and their potential interference with fecal *Escherichia coli* counts. Applied and Environmental Microbiology, 2001. 67, 4934-4938.

63. Dobbs, F., Goodrich, A., Thomson, F., III, and Hynes, W., Pandemic serotypes of *Vibrio cholerae* isolated from ships' ballast tanks and coastal waters: assessment of antibiotic resistance and virulence genes (*tcp*A and *ctx*A). Microbial Ecology, 2013. 65, 969-974.

64. International Maritime Organization (IMO), 2008. Guidelines for approval of ballast water management systems (G8), MEPC 58/23/, Annex 4, Res. MEPC 174 (58).

65. Luna, G.M., Vignaroli, C., Rinaldi, C., Pusceddu, A., Nicoletti, L., Gabellini, M., Danovaro, R., and Biavasco, F., Extraintestinal *Escherichia coli* carrying virulence genes in coastal marine sediments. Applied and Environmental Microbiology, 2010. 76, 5659-5668.

66. Colford, J.M.J., Wade, T.J., Schiff, K.C., Wright, C.C., Griffith, J.F., Sandhu, S.K., Burns, S., Sobsey, M., Lovelace, G., and Weisberg, S.B., Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. Epidemiology, 2007. 18, 27-35 10.1097/1001.ede.0000249425.0000232990.b0000249429.

67. Colford Jr., J.M., Schiff, K.C., Griffith, J.F., Yau, V., Arnold, B.F., Wright, C.C., Gruber, J.S., Wade, T.J., Burns, S., Hayes, J., McGee, C., Gold, M., Cao, Y., Noble, R.T., Haugland, R., and Weisberg, S.B., Using rapid indicators for *Enterococcus* to assess the risk of illness after exposure to urban runoff contaminated marine water. Water Research, 2012. 46, 2176-2186.

68. Ashbolt, N.J., Schoen, M.E., Soller, J.A., and Roser, D.J., Predicting pathogen risks to aid beach management: The real value of quantitative microbial risk assessment (QMRA). Water Research, 2010. 44, 4692-4703.

69. Loveday, H.P., Wilson, J.A., Kerr, K., Pitchers, R., Walker, J.T., and Browne, J., Association between healthcare water systems and *Pseudomonas aeruginosa* infections: a rapid systematic review. Journal of Hospital Infection, 2014. 86, 7-15.

70. Weiser, R., Donoghue, D., Weightman, A., and Mahenthiralingam, E., Evaluation of five selective media for the detection of *Pseudomonas aeruginosa* using a strain panel from clinical, environmental and industrial sources. Journal of Microbiological Methods, 2014. 99, 8-14.

71. Kerr, K.G. and Snelling, A.M., *Pseudomonas aeruginosa*: a formidable and ever-present adversary. Journal of Hospital Infection, 2009. 73, 338-344.

72. Lei nº 58/2005 de 29 de Dezembro. Diário da República - I Série-A. 249 (29.12.2005) 7280-7310.

73. Decreto-Lei nº 236/98 de 1 de Agosto. Diário da República - I Série-A. 176 (1.8.98) 3676-3722.

74. Decreto-Lei nº 306/2007 de 27 de Agosto. Diário da República - I Série. 164 (27.8.2007) 5747-5765.

75. Decreto-Lei nº 135/2009 de 3 de Junho. Diário da República - I Série. 107 (3.6.2009) 3460-3468.

76. Rompré, A., Servais, P., Baudart, J., de-Roubin, M.-R., and Laurent, P., Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. Journal of Microbiological Methods, 2002. 49, 31-54.

77. Fiksdal, L. and Tryland, I., Application of rapid enzyme assay techniques for monitoring of microbial water quality. Current Opinion in Biotechnology, 2008. 19, 289-294.

78. Cho, K.H., Han, D., Park, Y., Lee, S.W., Cha, S.M., Kang, J.-H., and Kim, J.H., Evaluation of the relationship between two different methods for enumeration fecal indicator bacteria: Colony-forming unit and most probable number. Journal of Environmental Sciences, 2010. 22, 846-850.

79. Maheux, A.F., Huppe, V., Boissinot, M., Picard, F.J., Bissonnette, L., Bernier, J.L.T., and Bergeron, M.G., Analytical limits of four beta-glucuronidase and beta-galactosidase-based commercial culture methods used to detect *Escherichia coli* and total coliforms. Journal of Microbiological Methods, 2008. 75, 506-514.

80. Fricker, C.R., Bullock, S., Murrin, K., and Niemela, S.I., Use of the ISO 9308-1 procedure for the detection of *E. coli* in water utilizing two incubation temperatures and two confirmation procedures and comparison with defined substrate technology. Journal of Water and Health, 2008. 6, 389-397.

81. Manafi, M., New developments in chromogenic and fluorogenic culture media. International Journal of Food Microbiology, 2000. 60, 205-218.

82. Tryland, I. and Fiksdal, L., Enzyme characteristics of β-D-galactosidase- and β-D-glucuronidase-positive bacteria and their interference in rapid methods for detection of waterborne coliforms and *Escherichia coli*. Applied and Environmental Microbiology, 1998. 64, 1018-1023.

83. Sharpe, A.N., Parrington, L.J., Diotte, M.P., and Peterkin, P.I., Evaluation of indoxyl-β-d-glucuronide and hydrophobic grid membrane filters for electronic enumeration of *Escherichia coli*. Food Microbiology, 1989. 6, 267-280.

84. Carmen Balebona, M., Moriñigo, M.A., Cornax, R., Borrego, J.J., Torregrossa, V.M., and Gauthier, M.J., Modified most-probable-number technique for the specific determination of *Escherichia coli* from environmental samples using a fluorogenic method. Journal of Microbiological Methods, 1990. 12, 235-245.

85. Wildeboer, D., Amirat, L., Price, R.G., and Abuknesha, R.A., Rapid detection of *Escherichia coli* in water using a hand-held fluorescence detector. Water Research, 2010. 44, 2621-2628.

86. Clark, S.T., Gilbride, K.A., Mehrvar, M., Laursen, A.E., Bostan, V., Pushchak, R., and McCarthy, L.H., Evaluation of low-copy genetic targets for waterborne bacterial pathogen detection via qPCR. Water Research, 2011. 45, 3378-3388.

87. Bej, A.K., DiCesare, J.L., Haff, L., and Atlas, R.M., Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for uid. Applied and Environmental Microbiology, 1991. 57, 1013-1017.

88. Fricker, C.R., DeSarno, M., Warden, P.S., and Eldred, B.J., False-negative beta-d-glucuronidase reactions in membrane lactose glucuronide agar medium used for the simultaneous detection of coliforms and *Escherichia coli* from water. Letters in Applied Microbiology, 2008. 47, 539-542.

89. Maheux, A.F., HuppÃ©, V., Boissinot, M., Picard, F.J., Bissonnette, L., Bernier, J.L.T., and Bergeron, M.G., Analytical limits of four b-glucuronidase and b-galactosidase-based commercial culture methods used to detect *Escherichia coli* and total coliforms. Journal of Microbiological Methods, 2009. 75, 506-514.

90. Martins, M.T., Rivera, I.G., Clark, D.L., Stewart, M.H., Wolfe, R.L., and Olson, B.H., Distribution of uidA gene sequences in *Escherichia coli* isolates in water sources and comparison with the expression of beta-glucuronidase activity in 4-methylumbelliferyl-beta-D-glucuronide media. Applied and Environmental Microbiology, 1993. 59, 2271-2276.

91. Chao, K.-K., Chao, C.-C., and Chao, W.-L., Evaluation of Colilert-18 for detection of coliforms and *Eschericha coli* in subtropical freshwater. Applied and Environmental Microbiology, 2004. 70, 1242-1244.

92. Keer, J.T. and Birch, L., Molecular methods for the assessment of bacterial viability. Journal of Microbiological Methods, 2003. 53, 175-183.

93. Juhna, T., Birzniece, D., Larsson, S., Zulenkovs, D., Sharipo, A., Azevedo, N.F., Menard-Szczebara, F., Castagnet, S., Feliers, C., and Keevil, C.W., Detection of *Escherichia coli* in

biofilms from pipe samples and coupons in drinking water distribution networks. Applied and Environmental Microbiology, 2007. 73, 7456-7464.

94. Rockabrand, D., Austin, T., Kaiser, R., and Blum, P., Bacterial growth state distinguished by single-cell protein profiling: does chlorination kill coliforms in municipal effluent? Applied and Environmental Microbiology, 1999. 65, 4181-4188.

95. Sousa, O.V., Evangelista-Barreto, N.S., Catter, K.M., Fonteles-Filho, A.A., Macrae, A., and Vieira, R.H.S.F., Specificity of a defined substrate method used to monitor balneability of tropical coastal waters impacted by polluted stormwater, in Journal of Water & Health. 2010, IWA Publishing, 543-549.

96. Bej, A.K., McCarty, S.C., and Atlas, R.M., Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: comparison with defined substrate and plating methods for water quality monitoring. Applied and Environmental Microbiology, 1991. 57, 2429-2432.

97. Pisciotta, J.M., Rath, D.F., Stanek, P.A., Flanery, D.M., and Harwood, V.J., Marine bacteria cause false-positive results in the Colilert-18 rapid identification test for *Escherichia coli* in Florida waters. Applied and Environmental Microbiology, 2002. 68, 539-544.

98. Tryland, I. and Fiksdal, L., Enzyme characteristics of beta-D-galactosidase- and beta-D-glucuronidase-positive bacteria and their interference in rapid methods for detection of waterborne coliforms and *Escherichia coli*. Applied and Environmental Microbiology, 1998. 64, 1018-1023.

99. Omar, K.B., Potgieter, N., and Barnard, T.G., Development of a rapid screening method for the detection of pathogenic *Escherichia coli* using a combination of Colilert® Quanti-Trays/2000 and PCR, in Water Science and Technology: Water Supply. 2010, 7-13.

100. Hahn, S. and Lapaire, O., Polymerase Chain Reaction, Real-Time Quantitative, in *Brenner's Encyclopedia of Genetics (Second Edition)*, S. Maloy and K. Hughes, Editors. 2013, Academic Press: San Diego, 396-397.

101. Ram, S., Vajpayee, P., Dwivedi, P.D., and Shanker, R., Culture-free detection and enumeration of STEC in water. Ecotoxicology and Environmental Safety, 2011. 74, 551-557.

102. Noble, R.T. and Weisberg, S.B., A review of technologies for rapid detection of bacteria in recreational waters. Journal of Water and Health, 2005. 3, 381-392.

103. Higgins, J.A., Jenkins, M.C., Shelton, D.R., Fayer, R., and Karns, J.S., Rapid extraction of DNA from *Escherichia coli* and *Cryptosporidium parvum* for use in PCR. Applied and Environmental Microbiology, 2001. 67, 5321-5324.

104. Jofre, J. and Blanch, A.R., Feasibility of methods based on nucleic acid amplification techniques to fulfil the requirements for microbiological analysis of water quality. Journal of Applied Microbiology, 2010. 109, 1853-1867.

105. Boissinot, M. and Bergeron, M.G., Toward rapid real-time molecular diagnostic to guide smart use of antimicrobials. Current Opinion in Microbiology, 2002. 5, 478-482.

106. Quilliam, R.S., Williams, A.P., Avery, L.M., Malham, S.K., and Jones, D.L., Unearthing human pathogens at the agricultural-environment interface: A review of current methods for the detection of *Escherichia coli* O157 in freshwater ecosystems. Agriculture, Ecosystems & Environment, 2011. 140, 354-360.

107. Green, H.C. and Field, K.G., Sensitive detection of sample interference in environmental qPCR. Water Research, 2012. 46, 3251-3260.

108. Schriewer, A., Wehlmann, A., and Wuertz, S., Improving qPCR efficiency in environmental samples by selective removal of humic acids with DAX-8. Journal of Microbiological Methods, 2011. 85, 16-21.

109. Ibekwe, A.M., Watt, P.M., Grieve, C.M., Sharma, V.K., and Lyons, S.R., Multiplex fluorogenic real-time PCR for detection and quantification of *Escherichia coli* O157:H7 in dairy wastewater wetlands. Applied and Environmental Microbiology, 2002. 68, 4853-4862.

110. Liu, Y., Gilchrist, A., Zhang, J., and Li, X.-F., Detection of viable but nonculturable *Escherichia coli* O157:H7 bacteria in drinking water and river water. Applied and Environmental Microbiology, 2008. 74, 1502-1507.

111. Takahashi, H., Kimura, B., Tanaka, Y., Shinozaki, J., Suda, T., and Fujii, T., Real-time PCR and enrichment culture for sensitive detection and enumeration of *Escherichia coli*. Journal of Microbiological Methods, 2009. 79, 124-127.

112. Sen, K., Sinclair, J.L., Boczek, L., and Rice, E.W., Development of a sensitive detection method for stressed *E. coli* O157:H7 in source and finished drinking water by culture-qPCR. Environmental Science & Technology, 2011. 45, 2250-2256.

113. Wolffs, P.F.G., Glencross, K., Thibaudeau, R., and Griffiths, M.W., Direct quantitation and detection of *Salmonellae* in biological samples without enrichment, using two-step filtration and real-time PCR. Applied and Environmental Microbiology, 2006. 72, 3896-3900.

114. Heijnen, L. and Medema, G., Method for rapid detection of viable *Escherichia coli* in water using real-time NASBA. Water Research, 2009. 43, 3124-3132.

115. Mull, B. and Hill, V.R., Recovery and detection of *Escherichia coli* O157:H7 in surface water, using ultrafiltration and real-time PCR. Applied and Environmental Microbiology, 2009. 75, 3593-3597.

116. Omiccioli, E., Amagliani, G., Brandi, G., Bruce, I.J., and Magnani, M., Simultaneous direct detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk samples by magnetic extraction and multiplex PCR. Journal of Rapid Methods and Automation in Microbiology, 2009. 17, 195-213.

117. Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.-L.T., Huppé, V., Bérubé, È., Boudreau, D.K., Picard, F.J., Huletsky, A., and Bergeron, M.G., Method for rapid and sensitive detection of *Enterococcus* sp. and *Enterococcus faecalis/faecium* cells in potable water samples. Water Research, 2011. 45, 2342-2354.

118. Horáková, K., Mlejnková, H., and Mlejnek, P., Evaluation of methods for isolation of DNA for polymerase chain reaction (PCR)-based identification of pathogenic bacteria from pure cultures and water samples. Water Science & Technology, 2008, 995-999.

119. Bertrand, R. and Roig, B., Evaluation of enrichment-free PCR-based detection on the rfbE gene of *Escherichia coli* O157 - Application to municipal wastewater. Water Research, 2007. 41, 1280-1286.

120. Zhang, Y., Riley, L.K., Lin, M., and Hu, Z., Determination of low-density *Escherichia coli* and *Helicobacter pylori* suspensions in water. Water Research, 2012. 46, 2140-2148.

121. Patel, C.B., Vajpayee, P., Singh, G., Upadhyay, R.S., and Shanker, R., Contamination of potable water by enterotoxigenic *Escherichia coli*: qPCR based culture-free detection and quantification. Ecotoxicology and Environmental Safety, 2011. 74, 2292-2298.

122. Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.L.T., Huppe, V., Berube, E., Boudreau, D.K., Picard, F.J., Huletsky, A., and Bergeron, M.G., Method for rapid and sensitive detection of *Enterococcus* sp and *Enterococcus faecalis/faecium* cells in potable water samples. Water Research, 2011. 45, 2342-2354.

123. Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.L.T., Huppe, V., Picard, F.J., Berube, E., and Bergeron, M.G., Rapid concentration and molecular enrichment approach for sensitive detection of *Escherichia coli* and *Shigella* species in potable water samples. Applied and Environmental Microbiology, 2011. 77, 6199-6207.

124. Gensberger, E.T., Sessitsch, A., and Kostic, T., Propidium monoazide-quantitative polymerase chain reaction for viable *Escherichia coli* and *Pseudomonas aeruginosa* detection from abundant background microflora. Analytical Biochemistry, 2013. 441, 69-72.

125. Mulugeta, S., Hindman, R., Olszewski, A.M., Hoover, K., Greene, K., Lieberman, M., and Mauro, S.A., Contamination level and location of recreational freshwater influence the ability to predict *Escherichia coli* concentration by qPCR targeting *Bacteroides*. Journal of Environmental Management, 2012. 103, 95-101.

126. Rodríguez, R.A., Thie, L., Gibbons, C.D., and Sobsey, M.D., Reducing the effects of environmental inhibition in quantitative real-time PCR detection of adenovirus and norovirus in recreational seawaters. Journal of Virological Methods, 2012. 181, 43-50.

127. Caetano-Anollés, D., Polymerase Chain Reaction, in *Brenner's Encyclopedia of Genetics (Second Edition)*, S. Maloy and K. Hughes, Editors. 2013, Academic Press: San Diego, 392-395.

128. Hoy, M.A., Chapter 8 - DNA amplification by the Polymerase Chain Reaction: molecular biology made accessible, in *Insect Molecular Genetics (Third Edition)*, M.A. Hoy, Editor. 2013, Academic Press: San Diego, 307-372.

129. Bej, A.K., Steffan, R.J., DiCesare, J., Haff, L., and Atlas, R.M., Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Applied and Environmental Microbiology, 1990. 56, 307-314.

130. Farnleitner, A.H., Hocke, L., Beiwl, C., Kavka, G.C., Zechmeister, T., Kirschner, A.K.T., and Mach, R.L., Rapid enzymatic detection of *Escherichia coli* contamination in polluted river water. Letters in Applied Microbiology, 2001. 33, 246-250.

131. Friesema, I., Sigmundsdottir, G., van der Zwaluw, K., Heuvelink, A., Schimmer, B., de Jager, C., Rump, B., Briem, H., Hardardottir, H., Atladottir, A., Gudmundsdottir, E., and van Pelt, W., An international outbreak of Shiga toxin-producing *Escherichia coli* O157

infection due to lettuce, September-October 2007. Euro surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin, 2008. 13, 3029-3035.

132. Frank, C., Werber, D., Cramer, J.P., Askar, M., Faber, M., an der Heiden, M., Bernard, H., Fruth, A., Prager, R., Spode, A., Wadl, M., Zoufaly, A., Jordan, S., Kemper, M.J., Follin, P., Müller, L., King, L.A., Rosner, B., Buchholz, U., Stark, K., and Krause, G., Epidemic profile of Shiga-toxin–producing *Escherichia coli* O104:H4 outbreak in Germany. New England Journal of Medicine, 2011. 365, 1771-1780.

133. Feng, P., Lum, R., and Chang, G.W., Identification of *uidA* gene sequences in b-D-glucuronidase-negative *Escherichia coli*. Applied and Environmental Microbiology, 1991. 57, 320-323.

134. Rice, E.W., Allen, M.J., and Edberg, S.C., Efficacy of b-glucuronidase assay for identification of *Escherichia coli* by the defined-substrate technology. Applied and Environmental Microbiology, 1990. 56, 1203-1205.

135. Maheux, A.F., Picard, F.J., Boissinot, M., Bissonnette, L., Paradis, S., and Bergeron, M.G., Analytical comparison of nine PCR primer sets designed to detect the presence of Escherichia coli/Shigella in water samples. Water Research, 2009. 43, 3019-3028.

136. Lan, R. and Reeves, P.R., *Escherichia coli* in disguise: molecular origins of *Shigella*. Microbes and Infection, 2002. 4, 1125-1132.

137. Zuo, G., Xu, Z., and Hao, B., *Shigella* strains are not clones of *Escherichia coli* but sister species in the genus *Escherichia*. Genomics, Proteomics & Bioinformatics, 2013. 11, 61-65.

138. Paradis, S., Boissinot, M., Paquette, N., Bélanger, S.D., Martel, E.A., Boudreau, D.K., Picard, F.J., Ouellette, M., Roy, P.H., and Bergeron, M.G., Phylogeny of the *Enterobacteriaceae* based on genes encoding elongation factor Tu and F-ATPase *β*-subunit. International Journal of Systematic and Evolutionary Microbiology, 2005. 55, 2013-2025.

139. Staley, C., Chase, E., and Harwood, V.J., Detection and differentiation of *Vibrio vulnificus* and *V. sinaloensis* in water and oysters of a Gulf of Mexico estuary. Environmental Microbiology, 2013. 15, 623-633.

140. Lang, A.L., Tsai, Y.L., Mayer, C.L., Patton, K.C., and Palmer, C.J., Multiplex PCR for detection of the heat-labile toxin gene and shiga-like toxin I and II genes in *Escherichia coli* isolated from natural waters. Applied and Environmental Microbiology, 1994. 60, 3145-3149.

141. Horakova, K., Mlejnkova, H., and Mlejnek, P., Specific detection of *Escherichia coli* isolated from water samples using polymerase chain reaction targeting four genes: cytochrome bd complex, lactose permease, beta-D-glucuronidase, and beta-D-galactosidase. Journal of Applied Microbiology, 2008. 105, 970-976.

142. Gómez-Duarte, O.G., Bai, J., and Newell, E., Detection of *Escherichia coli, Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. Diagnostic Microbiology and Infectious Disease, 2009. 63, 1-9.

143. Ram, S., Vajpayee, P., and Shanker, R., Rapid culture-independent quantitative detection of enterotoxigenic *Escherichia coli* in surface waters by real-time PCR with molecular beacon. Environmental Science & Technology, 2008. 42, 4577-4582.

144. Chern, E.C., Siefring, S., Paar, J., Doolittle, M., and Haugland, R.A., Comparison of quantitative PCR assays for *Escherichia coli* targeting ribosomal RNA and single copy genes. Letters in Applied Microbiology, 2011. 52, 298-306.

145. Mackay, I.M., ed. Real-time PCR in microbiology: from diagnosis to characterization. 2007, Caister Academic Press: Norfolk, UK.

146. Pérez-Luz, S., Adela Yaì nÌƒez, M., and Catalaì n, V., Identification of waterborne bacteria by the analysis of 16S-23S rRNA intergenic spacer region. Journal of Applied Microbiology, 2004. 97, 191-204.

147. Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., and Adley, C., An overview of foodborne pathogen detection: In the perspective of biosensors. Biotechnology Advances, 2010. 28, 232-254.

148. Shi, X.-M., Long, F., and Suo, B., Molecular methods for the detection and characterization of foodborne pathogens. Pure and Applied Chemistry, 2010. 82, 69-79.

149. Juck, D., Ingram, J., Prévost, M., Coallier, J., and Greer, C., Nested PCR protocol for the rapid detection of *Escherichia coli* in potable water. Canadian Journal of Microbiology, 1996. 42, 862-866.

150. Min, J. and Baeumner, A.J., Highly sensitive and specific detection of viable *Escherichia coli* in drinking water. Analytical Biochemistry, 2002. 303, 186-193.

151. Whitman, R.L., Nevers, M.B., Korinek, G.C., and Byappanahalli, M.N., Solar and temporal effects on *Escherichia coli* concentration at a Lake Michigan swimming beach. Applied and Environmental Microbiology, 2004. 70, 4276-4285.

152. Xing-Long, X., Cong-Cong, L., Yang, Q., Yi-Gang, Y., and Hui, W., Molecular monitoring of *Escherichia coli* O157: H7 sterilization rate using qPCR and propidium monoazide treatment. Letters in Applied Microbiology, 2013. 56, 333-339.

153. Bae, S. and Wuertz, S., Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide. Applied and Environmental Microbiology, 2009. 75, 2940-2944.

154. Jothikumar, N. and Griffiths, M.W., Rapid detection of *Escherichia coli* O157:H7 with multiplex real-time PCR assays. Applied and Environmental Microbiology, 2002. 68, 3169-3171.

155. Chassagne, L., Pradel, N., Robin, F., Livrelli, V., Bonnet, R., and Delmas, J., Detection of *stx1*, *stx2*, and *eae* genes of enterohemorrhagic *Escherichia coli* using SYBR Green in a real-time polymerase chain reaction. Diagnostic Microbiology and Infectious Disease, 2009. 64, 98-101.

156. Maheux, A., Bissonnette, L., and Bergeron, M., Rapid detection of the *Escherichia coli* genospecies in water by conventional and real-time PCR, in *PCR Detection of Microbial Pathogens*, M. Wilks, Editor. 2013, Humana Press, 289-305.

157. Kozuskanich, J., Novakowski, K.S., and Anderson, B.C., Fecal indicator bacteria vriability in samples pumped from monitoring wells. Ground Water, 2011. 49, 43-52.

158. Lavender, J.S. and Kinzelman, J.L., A cross comparison of QPCR to agar-based or defined substrate test methods for the determination of *Escherichia coli* and *enterococci* in municipal water quality monitoring programs. Water Research, 2009. 43, 4967-4979.

159. Shanks, O.C., Sivaganesan, M., Peed, L., Kelty, C.A., Blackwood, A.D., Greene, M.R., Noble, R.T., Bushon, R.N., Stelzer, E.A., Kinzelman, J., Anan'eva, T., Sinigalliano, C., Wanless, D., Griffith, J., Cao, Y.P., Weisberg, S., Harwood, V.J., Staley, C., Oshima, K.H., Varma, M., and Haugland, R.A., Interlaboratory comparison of real-time PCR protocols for quantification of general fecal indicator bacteria. Environmental Science & Technology, 2011. 46, 945-953.

160. Whitman, R.L., Ge, Z.F., Nevers, M.B., Boehm, A.B., Chern, E.C., Haugland, R.A., Lukasik, A.M., Molina, M., Przybyla-Kelly, K., Shively, D.A., White, E.M., Zepp, R.G., and Byappanahalli, M.N., Relationship and variation of qPCR and culturable *Enterococci* estimates in ambient surface waters are predictable. Environmental Science & Technology, 2010. 44, 5049-5054.

161. Benedict, R.T. and Neumann, C.M., Assessing Oregon's twenty-six coastal beach areas for recreational water quality standards. Marine Pollution Bulletin, 2004. 49, 624-629.

162. Haugland, R.A., Siefring, S.C., Wymer, L.J., Brenner, K.P., and Dufour, A.P., Comparison of Enterococcus measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Research, 2005. 39, 559-568.

163. Connelly, J.T. and Baeumner, A.J., Biosensors for the detection of waterborne pathogens. Analytical and Bioanalytical Chemistry, 2012. 402, 117-127.

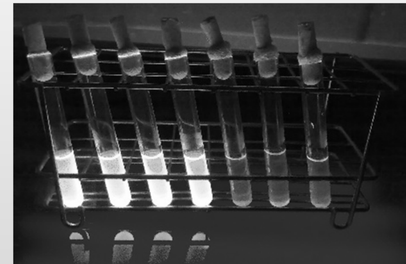
164. Niessen, L., Luo, J., Denschlag, C., and Vogel, R.F., The application of loop-mediated isothermal amplification (LAMP) in food testing for bacterial pathogens and fungal contaminants. Food Microbiology, 2013. 36, 191-206.

165. Sanvicens, N., Pastells, C., Pascual, N., and Marco, M.P., Nanoparticle-based biosensors for detection of pathogenic bacteria. Trends in Analytical Chemistry, 2009. 28, 1243-1252.

166. Tosar, J.P., Brañas, G., and Laíz, J., Electrochemical DNA hybridization sensors applied to real and complex biological samples. Biosensors and Bioelectronics, 2010. 26, 1205-1217.

167. Skottrup, P.D., Nicolaisen, M., and Justesen, A.F., Towards on-site pathogen detection using antibody-based sensors. Biosensors and Bioelectronics, 2008. 24, 339-348.

168. Lopez-Roldan, R., Tusell, P., Courtois, S., and Cortina, J.L., On-line bacteriological detection in water. Trends in Analytical Chemistry, 2013. 44, 46-57.



CHAPTER 3

DEVELOPMENT OF AN ENZYMATIC CULTURE MEDIUM FOR THE DETECTION OF *ESCHERICHIA COLI* AND TOTAL COLIFORMS IN WATER

This chapter presents the development of an enzymatic culture medium for a qualitative detection of *E. coli* and total coliforms in water samples.

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3.1 | INTRODUCTION

To prevent the dissemination of diseases, the microbiological safety of water is monitored using one or more indicators of pollution. Traditionally, *E. coli* and total coliforms have been used as the main indicators of fecal contamination in water [1-5].

Classical methods for assessing the hygienic quality of water are based in the biochemical reaction of the target microorganisms (e.g. gas production) [6] and usually require 24 to 72 h to be completed, using either the membrane filtration or multiple-tube fermentation [7]. Since these methods lack specificity, it is necessary to perform a confirmation step, which delays the results by 2 to 3 days. Moreover, the presence of background bacteria can decrease the recovery of the indicators [8]. The classical methods are the analytical reference methods recommended by the legislation to assess the microbiological quality of water. However, it has been shown that the methods recommended by ISO 9308-1, stated by the European drinking water Directive 98/83/CE, failed to detect a significant number of the indicator microorganisms *E. coli* and coliforms, and allowed the growth of other non-target microorganisms, particularly *Klebsiella oxytoca*, causing false-positive results [9-13]. The inherent limitations of these methods make them unable to provide timely results and useful information concerning the quality of water and the safety of public health.

In order to circumvent these limitations, methods based on the enzymatic properties of the indicator microorganisms have been developed. Defined substrates, either chromogenic or fluorogenic, are added to the culture medium in order to be metabolized by the specific enzymes of the indicator microorganisms [8, 14, 15]. Chromogenic substrates are hydrolyzed by the specific enzyme and the product

released provides color to the grown colony or to the liquid medium, and the fluorogenic substrates, when hydrolyzed by the specific enzyme, release a compound that exhibit fluorescence by the colony or liquid medium, when exposed to a UV light [14]. Therefore, when the indicator microorganisms are present in the water sample, their specific enzymes convert the chromogenic and/or fluorogenic substrates into products that exhibit a specific color and/or fluorescence, and a positive result is achieved when these modifications in the color and/or fluorescence become visible to the human eye. Due to this enzymatic specificity, coupled with other components in the culture medium that support the growth of the indicator microorganisms, no confirmatory steps are necessary and, thus, enzymatic methods can provide the results faster and relatively inexpensively, when compared to classical methods [16, 17].

The enzymes β-D-glucuronidase and β-D-galactosidase are the most commonly used specific enzymes for the identification of *E. coli* and total coliforms, respectively [8, 18-20]. These choices are based in the fact that, for *E. coli*, the gene (*uidA*) encoding the enzyme β-D-glucuronidase is specific and present in 97.7 % of the isolates [21] and, for total coliforms, the classical coliform monitoring methods are based in detecting the presence of the enzyme β-D-galactosidase, encoded by the *lacZ* gene [8]. To identify these enzymes, the most commonly used substrates are the fluorogenic 4-methylumbelliferyl-β-D-galactopyranoside (MUG) to identify *E. coli*, and the chromogenic 2-nitrophenyl-β-D-galactopyranoside (ONPG) to identify total coliforms (Figure 3.1) [8, 22, 23].

Several enzymatic culture media, either liquid or solid, have been developed to identify microorganisms is water. Readycult® Coliforms 100 (Readycult), Chromocult® Coliform agar (Chromocult) and Colilert® (Colilert) are examples of available commercial enzymatic culture media based in the detection of the activity of the β-D-galactosidase and β-D-glucuronidase enzymes to assess the microbiological quality of water within 24 h [8]. Colilert®, in particular, has become

one of the most frequently used enzymatic culture media used worldwide. Previous studies have shown the advantages of this medium over the classical methods, as it is more sensitive and specific to the indicator microorganisms, it is user friendly as it does not require complex cultivation procedures or an expert professional to perform the analysis, and provides faster results (within 24 h) [9-11, 24].

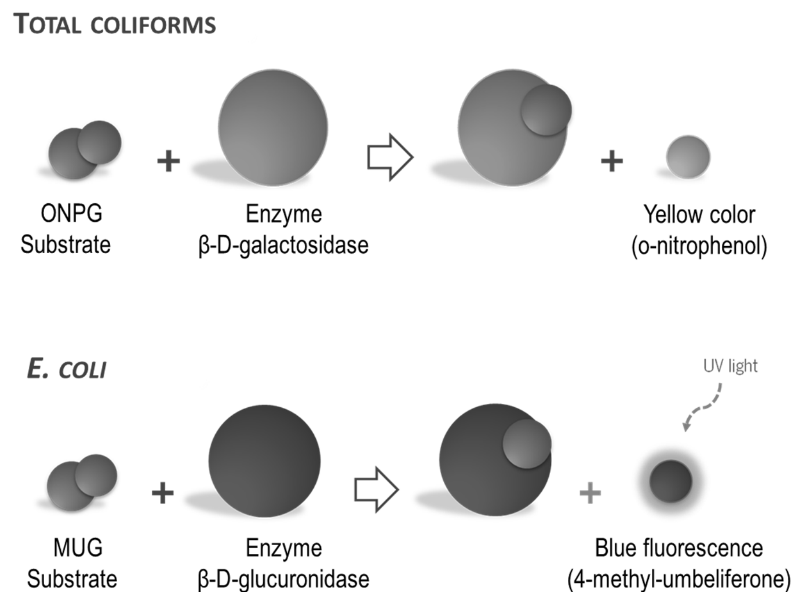


Figure 3.1 – Illustration of the color/fluorescence identification process of an enzymatic culture medium. The chromogenic ONPG (2-nitrophenyl-β-D-galactopyranoside) and fluorogenic MUG (4-methylumbelliferyl-β-D-galactopyranoside) substrates are used to detect the specific enzymes β-D-galactosidase and β-D-glucuronidase as indicators of the presence of total coliforms and *E. coli*, correspondingly (adapted) [8, 11, 14, 19, 20, 25].

However, it is known that the enzymes β-D-glucuronidase and β-D-galactosidase can be found in several other microorganisms (e.g. some strains of *Salmonella*, *Yersinia* and *Edwardia,* yeasts, fungi, protozoa). There is not a culture medium currently available with the ability of detecting flawlessly the indicator microorganisms in water samples and, thus, false-positive and false-negative results are a reality [23, 26-28].

The main purpose of this research work was to develop a liquid enzymatic culture medium, similar to the commercially available product named as Colilert® (Colilert, IDEXX Laboratories, Westbrook, ME, USA), to detect *E. coli* and total coliforms in (i) drinking water, (ii) river water and (iii) seawater, for the company Frilabo.

3.2 | MATERIALS AND METHODS

3.2.1 – Microorganism

To test the ability of the enzymatic culture medium under development to detect the indicator microorganisms, a frozen stock culture of *E. coli* K12, chosen for being biologically safe to handle, was obtained from the culture collection at the Centre of Biological Engineering, University of Minho (Braga, Portugal) and was used to assess the identification of both enzymes β-D-glucuronidase and β-D-galactosidase [29]. *E. coli* was grown overnight in Luria-Bertani (LB) agar (tryptone, 10g/L; yeast extract, 5 g/L; sodium chloride, 10 g/L; agar, 20 g/L; NaOH 10M, 175 µL/L) at 37 °C (aerobic incubator) [30]. This pure culture was used to artificially inoculate distilled water and environmental samples that were not naturally contaminated with the indicator microorganisms.

3.2.2 – Collection of environmental water samples

With the purpose of evaluating the capability of the enzymatic culture medium under development to detect the indicator microorganisms in different water samples, it was tested using different types of environmental water samples: 20 samples of drinking water were collected from the fountain Fonte das Águas Férreas (Braga), 5 samples of water from the distribution network (tap water, collected at the laboratory *Plataforma de Biologia Molecular e Sintética* at Centre of Biological Engineering (University of Minho, Portugal), 96 samples of river water from the Este river at the location of the *Parque da Rodovia* (Braga), and 15 samples of seawater from the beaches of Castelo do Queijo (Porto), Ofir, Marinhas, Suave Mar,

Esposende and Apúlia (Esposende), all in Portugal and between 2008 and 2013. The sampling sites of Rio Este and Castelo do Queijo were specifically chosen for their known high level of pollution.

In order to preserve the water samples in the best conditions during the transport from the sampling site to the laboratory and until the moment of the assay, the water samples were collected taking into consideration the standardized guidelines EN 25667-2 (ISO 5667-2:1991) on sampling techniques. Namely, the person collecting the samples had the hands and the clothes clean, the recipients for collecting the samples were made of polypropylene and sterilized at 121 °C for 20 minutes and remained closed before and after the moment of sampling. After collecting the samples, they were kept at ~4 °C and protected from light until the moment of analysis.

3.2.3 – Enumeration of the indicator microorganisms

In the assays using water samples previously sterilized and artificially inoculated with the pure culture of *E. coli*, the concentration of *E. coli* in each sample was confirmed by plating 100 µL of each suspension, in triplicate, in LB agar plates. The plates were incubated at 37 °C overnight (aerobic incubator) prior to the determination of colony counts. The number of CFU per mL was found using the Equation 3.1, in which $\bar{x}$ is the average number of colonies counted (plates in triplicate) in a given dilution (*dil*) and V_{inoc} is the volume of sample spread in the plates.

$$CFU/mL = \bar{x}.\frac{1}{dil}.\frac{1}{V_{inoc}}$$ [Equation 3.1]

In the assays using environmental water samples naturally contaminated with the indicator microorganisms, the bacterial count was verified by filtering 100 mL of the water samples (in triplicate), using mixed cellulose membrane filters (47 mm diameter, 0.22 µm pore size; Advactec, Japan) placed in a standard filtration system. The filters were incubated ChromoCult® coliform agar plates (Merck Millipore, Germany) at 37 °C (aerobic incubator) for 24 h. After incubation, colony counts by color were recorded, according to the manufacturer instructions: dark-blue to violet colonies were counted as *E. coli*; salmon to red and dark-blue to violet colonies were counted as total coliforms.

3.2.4 – Enzymatic culture medium

3.2.4.1 – COMPOSITION OF THE ENZYMATIC CULTURE MEDIUM

As requested by the company Frilabo, the enzymatic culture medium was developed to resemble the commercially available enzymatic culture medium Colilert®. Therefore, the enzymatic medium was developed based in the Colilert® patent EP 1403378 for general guidance, with significant changes [31]. The final composition of the enzymatic culture medium developed is described in Table 3.1.

In order to provide the same visual results as Colilert®, the fluorogenic substrate MUG (4-methylumbelliferyl-β-D-glucuronide) was selected to identify *E. coli*, and the chromogenic substrate ONPG (2-nitrophenyl-β-D-galactopyranoside) was selected for the identification of total coliforms [14, 31].

Table 3.1 – Final composition of the enzymatic culture medium developed (adapted) [15, 23, 31-36]

NUTRIENT		CONCENTRATION USED	
Amino acids	Alanine	0.8	g/L
	Arginine	0.8	g/L
	Aspartic acid	0.85	g/L
	Cysteine	0.05	g/L
	Glutamic acid	2.07	g/L
	Glycine	1.5	g/L
	Histidine	0.20	g/L
	Isoleucine	0.46	g/L
	Leucine	0.8	g/L
	Lysine	0.68	g/L
	Methionine	0.23	g/L
	Phenylalanine	0.37	g/L
	Proline	0.93	g/L
	Serine	0.44	g/L
	Threonine	0.32	g/L
	Tryphtophan	0.05	g/L
	Tyrosine	0.18	g/L
	Valine	0.56	g/L
Elements	Cobalt	0.00015	mg/L
	Copper	0.00015	mg/L
	Iron	0.0165	mg/L
	Plumb	0.0002	mg/L
Vitamins	Biotin	0.0016	mg/L
	Choline	1.0	mg/L
	Cyanocobalamin	0.0002	mg/L

	Folic acid	0.014	mg/L
	Inositol	0.014	mg/L
	Niacin	0.070	mg/L
	Nicotinic acid	0.3	mg/L
	PABA	0.38	mg/L
	Pantothenic acid	0.13	mg/L
	Pyridoxine	0.21	mg/L
	Riboflavin	0.058	mg/L
	Thiamine	0.026	mg/L
	Thymidine	0.2	mg/L
Enzyme inducers	IPTG	0.015	g/L
	Met-Glu	0.200	g/L
Other compounds	Ammonium Sulfate	5	g/L
	HEPES (free acid)	6.864	g/L
	HEPES (Na+ salt)	5.292	g/L
	D-Gluconic acid	0.145	g/L
	Sodium sulfite	0.040	g/L
	Amphotericin B (solubilized)	0.0010	g/L
	Magnesium sulfate	0.100	g/L
	ONPG	0.500	g/L
	MUG	0.075	g/L
	Zinc sulfate (heptahydrate)	0.0005	g/L
	Manganese sulfate	0.0005	g/L
	Piruvic acid (Na^+ salt)	0.005	g/L
	Sodium chloride	0.100	g/L
	Vancomycin or Bacitracin	0.005	g/L
	Phosphate sources(1)	0.1	g/L

(1) The phosphate source used was potassium diphosphate [32].

3.2.4.2 – Preparation of stock solutions of the nutrients

Due to the extremely low concentrations of the majority of the nutrients, it was not possible to weight them all. Therefore, the nutrients stock solutions were prepared at higher concentrations: all the nutrients were provided by Frilabo and dissolved in distilled water, ethanol, HCl or NaOH solutions, accordingly to the solubility information provided by the manufacturer, sterilized using syringe filters (cellulose acetate filters, Ø 25 mm, 0.20 µm of pore size, sterile; Frilabo, Portugal) [37], and stored away from the light, each at the temperature recommended by the manufacturer and used at the moment of the preparation of the enzymatic culture medium

3.2.4.3 – Preparation of the enzymatic culture medium from the nutrients stock solutions

To prepare the enzymatic culture medium, the volumes of each of the nutrients were measured from the stock solutions and added to a sterile recipient, taking into consideration the final concentration intended and the sample volume. The specific nutrients ONPG, MUG, Met-Glu and IPTG were weighted and added directly to the sterile recipient, at the concentrations described in Table 3.1. The medium was immediately used by adding the volume of water sample to be tested to the sterile recipient containing the nutrients, and adjusting the pH to 7.0 with a 10 M NaOH stock solution.

3.2.4.4 – Preparation of the enzymatic culture medium with lyophilization

In order to be *easy-to-use*, it was aimed to achieve an enzymatic culture medium in the final form of a powder to be added in the water sample. Therefore, to evaluate the performance of the culture medium as a powder, the medium was prepared as described in 3.2.4.3 (sterile distilled water was added to the recipient containing the nutrients instead of water sample in order to achieve an homogenous solution), the final solution was sterilized using syringe filters (cellulose acetate filters, Ø 25 mm, 0.20 μm of pore size, sterile; Frilabo, Portugal) [37], lyophilized using a Christ Alpha 1-4 LSC Freeze Dryer equipment, and stored. Stability of the medium in the powder form was tested by storing it at room temperature, 4 °C and -20 °C, for up to 6 months.

3.2.4.5 – Antibiotics tested in order to inhibit interference from non-target microorganisms

In order to select the antibiotics to be included in the enzymatic culture medium under development, the following were individually added with the other nutrients to the enzymatic culture medium in the steps 3.2.4.3 and 3.2.4.4, and were tested at the following concentrations:

- Antibiotics against Gram-positive bacteria tested: vancomycin (0.005 g/L), bacitracin (0.005 g/L) [35, 36, 38];
- Antibiotics against Gram-negative bacteria tested: colistin (0.005 g/L, 0.001 g/L), cefsulodin (0.010 g/L, 0.005 g/L) [34, 39].

3.2.5 – Detection of *E. coli* and total coliforms with the enzymatic culture medium developed

Tests were performed comparing the capability of the enzymatic culture medium under development to detect the indicator microorganisms, before and after the lyophilization process.

Some of the nutrients were particularly expensive and, thus, tests were performed using reduced volumes of the enzymatic culture medium (1.35 mL per test-tube). Therefore, the detection was evaluated by the number of CFU per mL and by the corresponding number of CFU per test-tube.

In all assays, and after being added to enzymatic culture medium, the water samples were incubated at 37 °C (aerobic incubator). Every hour, a visual assessment of the water samples developing a yellow color (total coliforms) in the test-tubes and/or blue fluorescence (*E. coli*), by placing the test-tubes under a UV lamp with a wave length of 366 nm, was performed. The visual assessments were recorded as a positive or negative result until a total of 24 h of incubation was completed.

3.2.5.1 – Detection using the non-lyophilized enzymatic culture medium

The enzymatic culture medium was prepared as described previously (see section 3.2.4.3). The following steps were performed according to the type of water sample to be tested (see sections 3.2.5.1.1 and 3.2.5.1.2).

3.2.5.1.1 - Water not naturally contaminated and artificially inoculated

The water samples were added to the recipient containing the nutrients of the enzymatic culture medium, previously prepared in accordance to the section

3.2.4.3 and taking into consideration the final concentration described in Table 3.1, and mixed thoroughly to dissolve all the nutrients. The pH was immediately adjusted to 7.0 using the solution of NaOH 10 M. The resulting solution was then sterilized using syringe filters (cellulose acetate filters, Ø 25 mm, 0.20 µm of pore size, sterile; Frilabo, Portugal) [37]. In order to simulate a decreasing concentration of the indicator microorganisms, a ten-fold serial dilution [40] was prepared by adding a volume of 1.5 mL of the sterile solution to the first test-tube, and volumes of 1.35 mL to as many test-tubes as the estimated to be necessary to achieve a concentration of less than 1 CFU of *E. coli* in the last test-tube, plus one test-tube to be used as the negative control, as described in Figure 3.2 (the number of dilutions may vary according to the estimated initial concentration of CFU in the first test-tube). The first test-tube was artificially inoculated with one small colony from the overnight pure culture of *E. coli* (prepared according to the section 3.2.1), which was resuspended in the 1.5 mL of the culture medium by mixing (~10^7 CFU/mL), and ten-fold serially diluted by pipetting 150 µL of each dilution to the following test-tube (see Figure 3.2). The last test-tube was kept sterile as a negative control. The test-tubes were then incubated at 37 °C for up to 24 h.

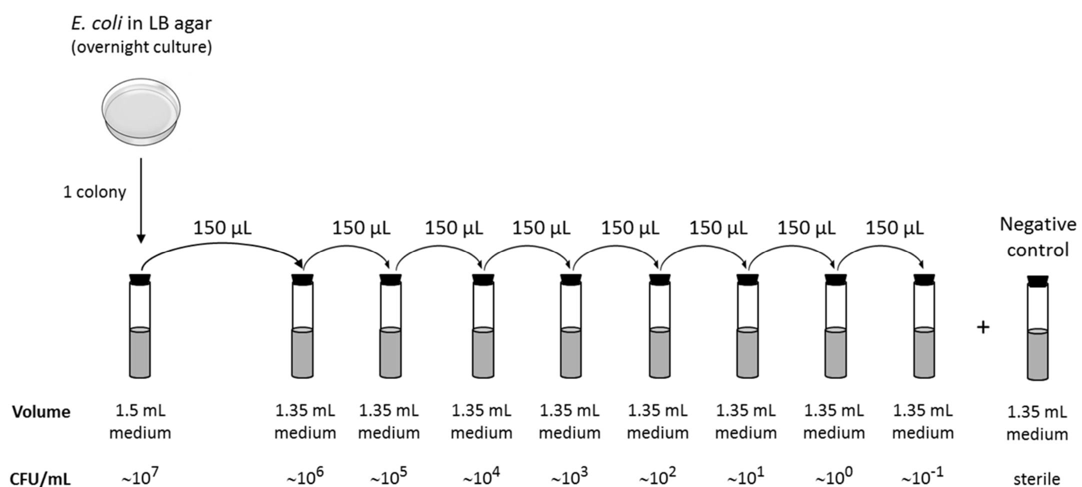


Figure 3.2 – Representation of the bacterial suspensions ten-fold serially diluted in the enzymatic culture medium resuspended in water not naturally contaminated and artificially inoculated with one small colony of a pure culture of *E. coli* to mimic an estimated

decreasing concentration of the indicator microorganism in the water sample up to less than 1 CFU in the last test-tube.

3.2.5.1.2 - WATER NATURALLY CONTAMINATED WITH THE INDICATOR MICROORGANISMS

When the microorganisms to be detected were naturally present in the water sample, this was added directly to the recipient containing the nutrients of the enzymatic culture medium, previously prepared in accordance to the section 3.2.4.3 and taking into consideration the final concentration described in Table 3.1, and it was mixed thoroughly to dissolve all the nutrients. The pH was immediately adjusted to 7.0 using the solution of NaOH 10 M. Then, 1.5 mL of the resulting solution (contaminated water sample with the enzymatic culture medium) was added to the first test-tube of a ten-fold serial dilution prepared to simulate a decreasing concentration of the indicator microorganisms, as described in Figure 3.3 (the number of dilutions may vary according to the estimated initial concentration of CFU in the first test-tube). The remaining solution was sterilized using syringe filters (cellulose acetate filters, Ø 25 mm, 0.20 µm of pore size, sterile; Frilabo, Portugal) and volumes of 1.35 mL were added to as many test-tubes as the estimated to be necessary to achieve a concentration of less than 1 CFU of *E. coli* in the last test-tube, plus one test-tube to be used as the negative control. The first test-tube, containing the indicator microorganisms naturally present in the water sample, was ten-fold serially diluted by pipetting 150 µL of each dilution to the following test-tube (see Figure 3.3). The last test-tube was kept sterile as a negative control. The test-tubes were then incubated at 37 °C for up to 24 h.

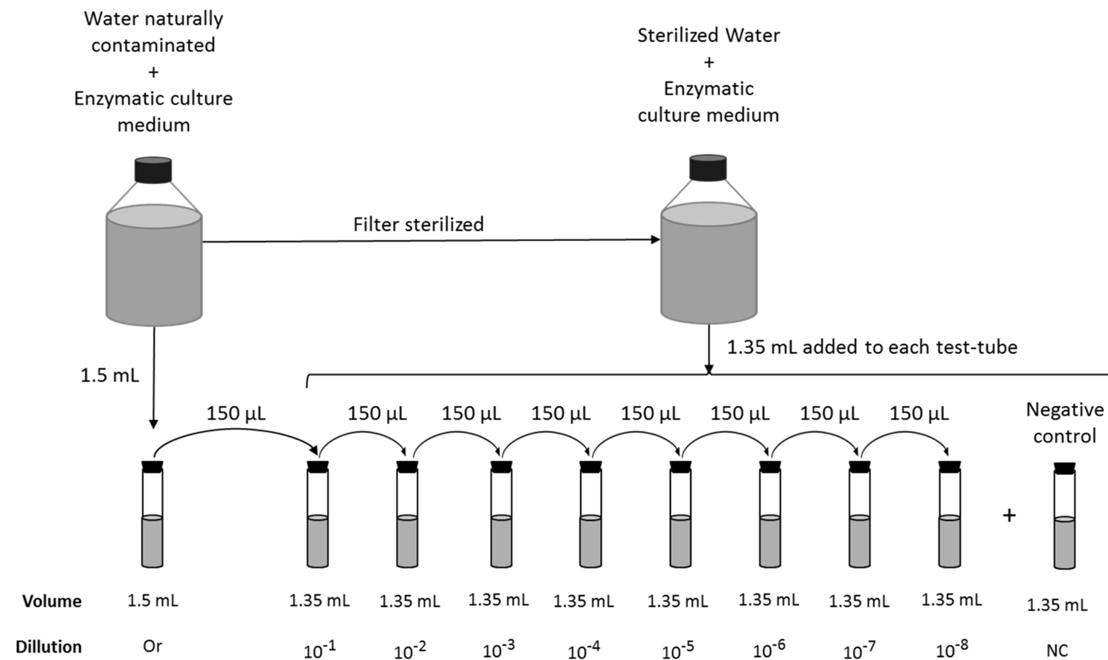


Figure 3.3 – Representation of the ten-fold serial dilution using the enzymatic culture medium resuspended in water naturally contaminated, to mimic an estimated decreasing concentration of the indicator microorganism in the water sample up to less than 1 CFU in the last test-tube.

3.2.5.2 – DETECTION USING THE LYOPHILIZED ENZYMATIC CULTURE MEDIUM

The enzymatic culture medium was prepared as previously described in section 3.2.4.4. The following steps were performed according to the type of water sample to be tested (see sections 3.2.5.2.1 and 3.2.5.2.2).

3.2.5.2.1 - WATER NOT NATURALLY CONTAMINATED AND ARTIFICIALLY INOCULATED, USING THE LYOPHILIZED ENZYMATIC CULTURE MEDIUM

Approximately 26.9 g/L of the previously lyophilized enzymatic culture medium (prepared as described in section 3.2.4.4) was added to the water samples

and mixed until completely dissolved. It was not necessary to adjust the pH to 7.0. The resulting solution was then sterilized using syringe filters (cellulose acetate filters, Ø 25 mm, 0.20 µm of pore size, sterile; Frilabo, Portugal). In order to simulate a decreasing concentration of the indicator microorganisms, a ten-fold serial dilution was prepared by adding a volume of 1.5 mL of the sterile solution to the first test-tube, and volumes of 1.35 mL to as many test-tubes as the estimated to be necessary to achieve a concentration of less than 1 CFU of *E. coli* in the last test-tube, plus one test-tube to be used as the negative control, as described in Figure 3.2 (the number of dilutions may vary according to the estimated initial concentration of CFU in the first test-tube). The first test-tube was artificially inoculated with one small colony from the overnight pure culture of *E. coli* (prepared according to the section 3.2.1), which was resuspended by mixing (~10^7 CFU/mL), and ten-fold serially diluted by pipetting 150 µL of each dilution to the following test-tube (see Figure 3.2). The last test-tube was kept sterile as a negative control. The test-tubes were then incubated at 37 °C for up to 24 h.

3.2.5.2.2 - Water naturally contaminated with the indicator microorganisms, using the lyophilized enzymatic culture medium

When the microorganisms to be detected were naturally present in the water sample, approximately 26.9 g/L of the previously lyophilized enzymatic culture medium (prepared as described in section 3.2.4.4) was added to the water samples and mixed until completely dissolved. It was not necessary to adjust the pH to 7.0. Then, 1.5 mL of the resulting solution (contaminated water sample with the enzymatic culture medium) was added to the first test-tube of a ten-fold serial dilution prepared to simulate a decreasing concentration of the indicator microorganisms, as described in Figure 3.3 (the number of dilutions may vary according to the estimated initial concentration of CFU in the first test-tube). The remaining solution was sterilized using syringe filters (cellulose acetate filters, Ø 25 mm, 0.20 µm of pore size, sterile; Frilabo, Portugal) and volumes of 1.35 mL were

added to as many test-tubes as the estimated to be necessary to achieve a concentration of less than 1 CFU of *E. coli* in the last test-tube, plus one test-tube to be used as the negative control. The first test-tube, containing the indicator microorganisms naturally present in the water sample, was ten-fold serially diluted by pipetting 150 µL of each dilution to the following test-tube (see Figure 3.3). The last test-tube was kept sterile as a negative control. The test-tubes were then incubated at 37 °C for up to 24 h.

3.2.5.3 – DETECTION USING SEAWATER SAMPLES

In the particular case of assays using seawater, it was known that its high concentration of sodium chloride (NaCl) can negatively affect the growth of the indicator microorganisms [41]. According to Palmer and his coworkers [42], it should be used a reduced concentration of NaCl in the culture medium in order to avoid harming the indicator microorganisms. Therefore, in assays using seawater samples, the NaCl specified in Table 3.1 was not added to the enzymatic culture medium.

To evaluate the effect of the salinity of seawater in the survival of the indicator microorganisms and the capability of enzymatic culture medium to detect these microorganisms, assays were performed using different dilutions of seawater in distilled water: 100, 75, 50, 25 and 0 % of seawater artificially inoculated. To perform this assay, the nutrients of the enzymatic culture medium were added to two different sterile recipients, taking into consideration the final concentration described in Table 3.1 and previously prepared accordingly to the section 3.2.4.3. To the first recipient, it was added the seawater sample, and to the second one it was added distilled water. The two solutions were mixed thoroughly to dissolve all the nutrients and the pH was immediately adjusted to 7.0 using a solution of NaOH 10 M. The two solutions were then sterilized using syringe filters (cellulose acetate filters, Ø 25 mm, 0.20 µm of pore size, sterile; Frilabo, Portugal). In order to simulate

a decreasing concentration of the indicator microorganisms, the recipient containing the seawater sample was artificially inoculated with two colonies from the overnight pure culture of *E. coli* (prepared according to the section 3.2.1), which were resuspended by vigorously mixing. The ten-fold serial dilutions of each concentration of seawater were prepared by adding a volume of 1.5 mL of the artificially inoculated seawater solution to the first test-tube of each concentration of seawater, named as "Or", as described in Figure 3.4. To each remaining test-tubes of each concentration of seawater, it was added the following volumes of the solutions of the enzymatic culture medium dissolved in seawater or distilled water, plus one test-tube to be used as the negative control:

- 100 % seawater: 1.350 mL of seawater and 0 mL of distilled water;
- 75 % seawater: 1.013 mL of seawater and 0.338 mL of distilled water;
- 50 % seawater: 0.675 mL of seawater and 0.675 mL of distilled water;
- 25 % seawater: 0.338 mL of seawater and 1.013 mL of distilled water;
- 0 % seawater: 0 mL of seawater and 1.350 mL of distilled water;

The ten-fold serial dilutions were performed by pipetting 150 µL of the "Or" test-tube to the following test-tube. The last test-tube was kept sterile as a negative control. The test-tubes were incubated at 37 °C for up to 24 h.

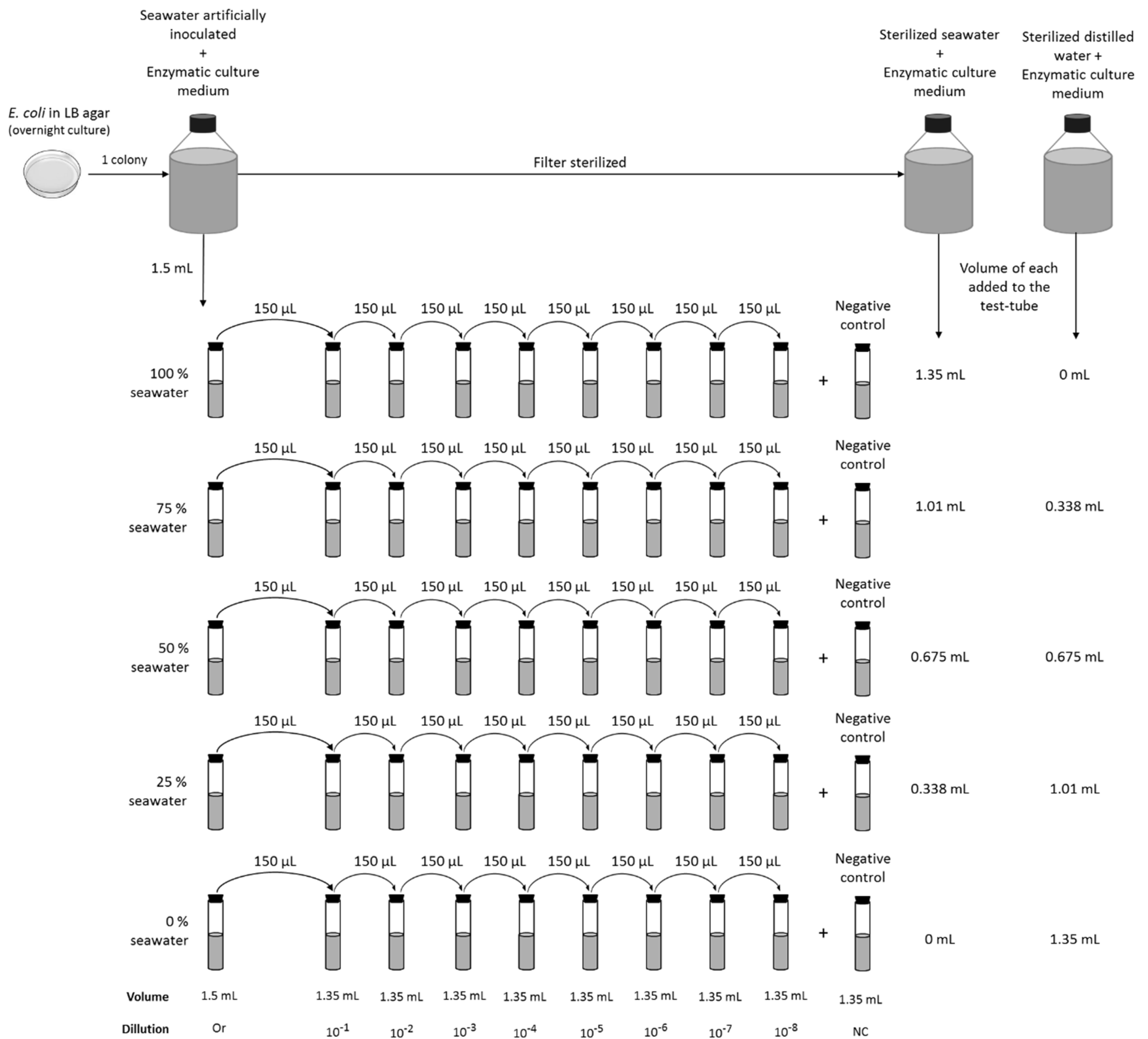


Figure 3.4 – Representation of the ten-fold serial dilutions using the enzymatic culture medium resuspended in seawater artificially inoculated with a pure culture of *E. coli*, to mimic an estimated decreasing concentration of the indicator microorganism in the water sample up to less than 1 CFU in the last test-tube. Each ten-fold serial dilution was performed with the following concentrations of seawater diluted with sterile distilled water: 100 %, 75 %, 50 %, 25 % and 0 % of seawater.

3.3 | Results and Discussion

The main purpose of this work was to develop an enzymatic culture medium, similar to the commercially available Colilert®, capable of detecting *E. coli* and total coliforms in water samples. To accomplish this purpose, the enzymatic culture medium developed was based in the patent published for the Colilert® medium (EP 1403378) [31]. However, the patent does not contain the exact formulation of the enzymatic culture medium. Instead, some general examples of several possible nutrients that may or may not be used in the culture medium are provided for general guidance. Moreover, a range of concentration of several nutrients is presented as an example of a possible medium to culture microorganisms, which are not necessarily the nutrients and concentrations used in Colilert®. It is also referred that these concentrations may be 3, 10 or even 20 times above the suggested, the nutrients may be not all the described, may vary their relative concentrations, be from different sources, and can change with several factors as sample size and concentration of the target microorganisms. Therefore, the patent was used in this work merely for general guidance.

3.3.1 – Chromogenic and fluorogenic detection in bacterial cells suspensions

The purpose of the tests at a laboratorial scale using distilled water artificially inoculated with *E. coli* was to evaluate, under controlled conditions, the enzymatic culture medium while it was being developed. *E. coli* suspensions in sterile distilled water samples were used to mimic a decreasing concentration of the target

microorganisms (ten-fold serial dilution), up to less than 1 CFU/100 mL in order to evaluate the limit of detection of the enzymatic culture medium under development.

The incubation temperature of 37 °C and the pH of 7.0 were selected for this work based in the findings of George *et al.* (2000), which compared the impact of pH and temperature on the specific enzymes of the indicator microorganisms. This study showed maximum activity at pH 7.2 and 37 °C with β-D-galactosidase, and pH 6.9 and 44 °C with β-D-glucuronidase. Other studies showed that, when the incubation temperature was shifted from 37 °C to any temperature in the range 40 to 45 °C, the growth rate of *E. coli* immediately assumed a lower value, and 37 °C was recommended as the incubation temperature for *E. coli* [43-45].

3.3.1.1 – Nutrients specific to *E. coli* and total coliforms

The patent suggests several possible chromogenic/fluorogenic nutrients, but they are not limited to these, that could be used to identify total coliforms and *E. coli*, including 2-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG) [31]. These nutrients were selected as the chromogenic and fluorogenic specific nutrients to identify total coliforms and *E. coli* in this enzymatic culture medium, correspondingly, for being able to provide the same visual results as the Colilert® medium (yellow color and blue fluorescence, respectively), as requested by Frilabo [15, 31]. Moreover, these were the most frequently used substrates to identify the β-D-galactosidase and β-D-glucuronidase enzymes, respectively, and were adapted to a multitude of tests to detect total coliforms and/or *E. coli* in food, drinking water, seawater, freshwater, clinical and faecal samples [14, 16, 22].

Several other nutrients, including amino acids, vitamins and other elements, were suggested by the patent as possible nutrients that could be used to grow *E. coli* and total coliforms, being these not necessarily part of the Colilert® culture medium.

Therefore, the amino acids, vitamins and elements suggested in the patent were used in the initial enzymatic culture medium, at the maximum value of the range of concentrations indicated (ten times less the concentrations described in Table 3.1), along with the chromogenic and fluorogenic nutrients [31]. Preliminary tests using these substrates in samples inoculated with *E. coli*, confirmed the easily distinguishable yellow color at naked eye, and blue fluorescence under an UV lamp (366 nm), respectively [32]. It was also noticed that the test-tubes with the highest concentration of *E. coli* produced the yellow color and blue fluorescence significantly faster that the lower concentrations, suggesting a correlation between the concentration of microorganisms and the time to obtain the visual results. This is due to the fact that a higher concentration of the indicator bacteria outcomes in a higher concentration of the specific enzymes and, therefore, the specific substrates are hydrolyzed faster; as a consequence, color and fluorescence are visible faster [22]. Although the enzymatic culture medium was able to achieve the aimed sensitivity of 1 CFU in the water sample, the time of incubation was of almost 30 h [32]. This incubation time was considered not competitive with the other commercially available enzymatic culture media, including Colilert®, which claim to provide the results in 18 h to 24 h [46].

At this point, the composition of the enzymatic culture medium did not include any enzyme inducers. Therefore, as the purpose was to reduce the incubation time, the concentration of the reagents was increased ten-fold (Table 3.1), tested and compared to the previous results: the detection of 1 CFU in the test-tubes was achieved after 24 h of incubation, which shows a clear improvement in the enzymatic culture medium by reducing the incubation time [32].

Although the time for detection had been successfully reduced, we aimed to develop an enzymatic culture medium that could be commercially competitive for providing fast results. Therefore, to increase the metabolic activity of the target enzymes, inducers were tested. The patent recommended the use of enzyme

inducers to reduce the incubation time, suggesting isopropyl β-D-1-thiogalactopyranoside (IPTG) as a possible inducer for the enzyme β-D-galactosidase but no inducer was suggested or described for the enzyme β-D-glucuronidase. Therefore, for β-D-glucuronidase, it was selected as inducer the methyl β-D-glucuronide sodium salt (Met-Glu) since this is commonly used in commercial culture media and its effectiveness was confirmed by other studies [22, 23] and for β-D-galactosidase it was selected, by comparison with the use of lactose as an inducer, the isopropyl β-D-1-thiogalactopyranoside (IPTG) [31, 47-50]. Both Met-Glu and IPTG were known to be noncompetitive inducers, i.e. non hydrolysable substrates by the specific enzymes [22].

The evaluation of the inducers was carried out through the comparison of the time of detection of total coliforms/*E. coli* and the intensity of color/fluorescence. For the enzyme β-D-glucuronidase, Met-Glu was added to the enzymatic culture medium at the concentration of 0.2 g/L, since it was observed by Tryland and Fiksdal that the enzyme was induced in *E. coli* but other β-D-glucuronidase-positive bacteria were not induced or were only slightly induced at this concentration, thus contributing to the selectivity of the enzymatic culture medium [23]. This clearly resulted in a faster and more intense blue fluorescence, which confirmed the potential of the inducer in increasing the enzymatic activity, as described by Tryland and Fiksdal [23]. Lactose was firstly tested as a possible inducer for β-D-galactosidase, since it has been traditionally used in classical methods [51-53], and its effectiveness was compared to IPTG. Including IPTG in the medium resulted in faster observation of the yellow color, when compared to lactose [32]. Although the intensity of the yellow color was relatively less intense with IPTG, it was clearly visible and it was selected as the inducer for this enzymatic medium due to its faster results.

Therefore, the efficacy of Met-Glu and IPTG as inducers was demonstrated, in accordance to the literature, and the detection time was reduced to approximately 18 h of incubation [23]. Moreover, Tryland and Fyksdal showed that the other

environmental non-target microorganisms were less inducible with these reagents than the enzymes of coliforms and *E. coli* [23]. Thus, Met-Glu and IPTG were the inducers selected for the enzymatic culture medium.

3.3.1.2 – ANTIBIOTICS AGAINST NON-TARGET MICROORGANISMS

Although both ONPG and MUG are specific nutrients commonly used to identify the specific enzymes of the indicator microorganisms, it is known that other non-target microbes can use these nutrients and, thus, produce false-positives and interfere in the analysis of the results [8, 23, 54]. The β-D-galactosidase and β-D-glucuronidase enzymes were found in Gram-positive (e.g. *Pseudomonas* spp.) and Gram-negative bacteria (e.g. *Salmonella* spp.), as well as in some yeasts, protozoa and fungi [23]. Therefore, it was considered important to include antibiotics and antifungal in the enzymatic culture medium in order to minimize false-positive results. Thus, the antibiotics should be capable of inhibiting the non-target microorganisms and prevent their regrowth. Since *E. coli* and total coliforms are Gram-negative, antibiotics should inhibit Gram-positive and non-coliform Gram-negative bacteria.

For Gram-positive bacteria, both vancomycin and bacitracin were selected to be tested due to their known efficacy over these microorganisms [38, 55]. Since *E. coli* and total coliforms are Gram-negative bacteria, it was intended to choose an antibiotic against Gram-negative microorganisms capable of inhibiting the growth of all the non-target microorganisms, without affecting negatively the growth the indicator microorganisms. Thus, colistin was chosen to be tested as one of the antibiotics suggested in the patent EP 1403378, and cefsulodin was chosen as an antibiotic specified in the literature for the Chromocult® agar medium, cefsulodin, since some studies in the literature describe these antibiotics as having activity against other non-target Gram-negative microorganisms known for being the cause

of false-positive results (e.g. *Pseudomonas* and *Klebsiella*), and to which *E. coli* has shown resistance [32-34, 39, 56]. In all the assays, it was considered the effect of the antibiotics on the growth of *E. coli* and on its detection, since it is aimed at no inhibition of the indicator microorganisms.

As expected, both vancomycin and bacitracin showed no interference in the growth of *E. coli*, understandably due to the fact that these are antibiotics that hinder the growth of Gram-positive bacteria [38, 55], and that our indicators are Gram-negative [18]. Assays in naturally contaminated river water, using either vancomycin or bacitracin, showed that the positive results observed in the test-tubes with less than 1 CFU of *E. coli* in the tube were eliminated, suggesting a reduction in false-positive results by Gram-positive microorganisms. Either of these antibiotics could be used in this enzymatic culture medium. Nevertheless, the choice for vancomycin or bacitracin to be used as the antibiotic against Gram-positive microorganisms in the enzymatic culture medium should take in consideration their cost and stability for longer periods of time (during the storage and incubation conditions).

Even though colistin was suggested by the patent, this antibiotic severely inhibited the growth of *E. coli*, even when lower concentrations of colistin were used [31]. Studies have shown that this antibiotic is rapidly bactericidal against Gram-negative bacteria, causing a disruption of the bacterial cell membrane [57], and an excellent activity against *E. coli* [58]. As it is aimed to detect up to 1 CFU of *E. coli* and total coliforms in a water sample, colistin was not included in the final composition of the enzymatic culture medium since it would inevitably cause the death of the indicator microorganisms and provide false-negative results.

Cefsulodin was tested as a possible antibiotic against the non-coliform Gram-negative microorganisms. This antibiotic is part of the Chromocult® Coliform Agar as being active against *Aeromonas* spp. and *Pseudomonas* spp., which have the enzyme β-D-galactosidase [39]. These microorganisms are ubiquitous in the environment and

can be a source of false-positives in the detection of total coliforms [23, 39]. The concentration of 10 mg/L was tested, as described by Alonso and coworkers for chromogenic coliform media [39]. However, when chromogenic and fluorogenic identification of pure cultures of *E. coli* was attempted, with and without cefsulodin, it was observed a partial inhibition of the detection, even though not as severe as the inhibition observed with colistin. Thus, cefsulodin was not included as well in the final composition of the enzymatic culture medium since it was considered preferable to have a false-positive result, than a false-negative that could endanger the public health (e. g. *Pseudomonas aeroginosa* is a known pathogen capable of producing false-positives) [59, 60].

Amphotericin B was suggested by the patent EP 1403378 as the antifungal to be used [31]. This antifungal exhibits an extensive spectrum of activity against fungal and protozoan pathogens with relatively rare resistance [61]. Tests comparing the detection with and without amphotericin B added (0.0010 g/L) showed no visual difference in the color of medium, which was a previous concern since this is in the form of an orange powder, resulting in an orange stock solution. Moreover, no interference in the detection of the indicator microorganisms was observed [32]. Therefore, amphotericin B was included in the final composition of the enzymatic culture medium. These results were equally confirmed during the tests using environmental samples.

In order to reduce the interference of non-target microorganisms in the detection and avoid false-positive results, more research studies using other antibiotics should be performed.

3.3.1.3 – Effect of the lyophilization of the enzymatic culture medium in the detection of the indicator microorganisms

Lyophilization, also known as freeze-drying, was understood as an essential step to reduce the exhaustive and highly hard-working preparation of the enzymatic culture medium before each detection (e.g. weighting and measuring the volumes of each one of the many nutrients) and develop instead a pre-dosed powder that could be added directly to the water sample to be analyzed. This is a relatively simple procedure in which dehydration is typically used to preserve a perishable material [62]. In this process, the enzymatic culture medium resulted in a white powder (Figure 3.5) that was easily dissolved in water. Tests were performed in order to assess the sensitivity and rapidity of the detection with this medium before and after lyophilization (Table 3.2).

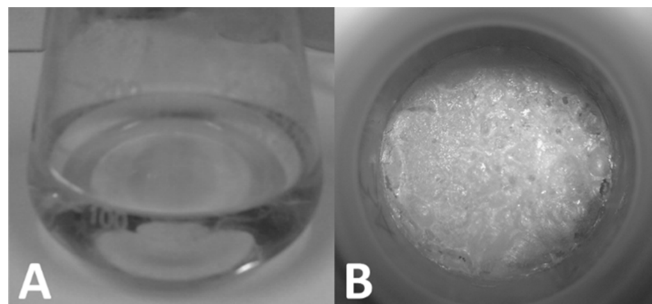


Figure 3.5 – Appearance of the culture medium developed (A) before and (B) after the process of lyophilization.

Table 3.2 – Comparison of the detection of *E. coli* using the enzymatic culture medium developed, serially diluted in distilled water: not lyophilized and lyophilized. The corresponding number of CFU/mL estimated for each dilution is indicated. "NC" refers to the negative control of the assay, where no microorganisms were added

	(3.4 ± 0.40) CFU/mL	$x10^7$	$x10^6$	$x10^5$	$x10^4$	$x10^3$	$x10^2$	$x10^1$	$x10^0$	$x10^{-1}$	NC
Not lyophilized	Total coliforms	+	+	+	+	+	+	+	+	+	-
	E. coli	+	+	+	+	+	+	+	+	+	-
Lyophilized	Total coliforms	+	+	+	+	+	+	+	+	+	-
	E. coli	+	+	+	+	+	+	+	+	+	-

Note: For the detection, are considered: detected, **+** ; not detected, **-** .

Results have shown that the process of lyophilization preserved the capability of the enzymatic culture medium to accurately detect total coliforms (yellow color) and *E. coli* (blue fluorescence), which is confirmed by the fact that lyophilization is a well-established method for the preservation of pharmaceutical products, and has been commonly applied to overcome their physical and/chemicals instabilities [62, 63]. The limit of detection achieved up to approximately 1 CFU of *E. coli* in the test-tube, after an incubation period of 16 h.

Tests to the stability of the lyophilized enzymatic culture medium were performed by comparing the limit of detection after a storage period at three different temperatures: room temperature (≈ 22 °C), 4 °C and -20 °C. It was observed that, at room temperature, the capacity of the enzymatic culture medium to detect the indicator microorganisms was significantly and almost totally reduced in 2 to 3 days. When stored at 4 °C, the properties of the enzymatic culture medium remained stable for a longer period of time (1 to 2 months) but this was not considered sufficient when commercialization is in perspective. Therefore, storage at -20 °C was

attempted for a period of time of 6 months and, although the intensity of color and fluorescence was visibly reduced, the limit of detection of 1 CFU per sample was achieved. Nevertheless, further tests aiming to increase the stability and shelf-life of the enzymatic culture medium should be performed.

Since a detection of total coliforms was achieved between 14 to 16 h and of *E. coli* between 16 h to 18 h, using artificially inoculated distilled water samples, was achieved in all the assays, the enzymatic culture medium was considered ready to be tested in environmental water samples.

3.3.2 – Chromogenic and fluorogenic detection in environmental water samples

The main purpose of these assays was to test the applicability of the enzymatic culture medium developed to environmental water samples and evaluate its capability to assess the microbiological safety of water. Results of the assays performed using samples of drinking, river and seawater are here presented and discussed.

3.3.2.1 – Detection using the enzymatic culture medium in drinking and river water samples

Water from the distribution network (tap water) and water from a public fountain were chosen as the most common examples of drinking water and where the water quality monitoring is more significant to the human health. According to the legislation, water from the distribution network must be disinfected (usually,

using chlorine) and it is advised to add at the end of the water treatment a concentration of residual chlorine between 0.2 and 0.6 mg/L to ensure the microbiological quality of the water in all points of distribution network [4]. For the period of time at which the water samples were collected, the distribution network declared a concentration of residual chlorine between 0.11 and 0.81 mg/L [64]. Therefore, water samples from the distribution network were tested to evaluate the survival of the indicator microorganisms in the potential presence of residual chlorine (as well as other elements, such as aluminum, iron and nitrates) and its effect in the detection with the enzymatic culture medium. Confirming that water from the distribution network and fountain were safe for human consumption, no microorganisms were found in the water samples and, therefore, water samples were artificially inoculated with *E. coli*.

Samples of river water, however, were collected in the Este river due to its high level of contamination. The main purpose was to challenge the enzymatic culture medium with a complex background of other non-target microorganisms and contaminants. Thus, these samples were not artificially inoculated and the detection was performed with the microorganisms naturally present in the samples and the number of CFU of *E. coli* and total coliforms was determined by filtering the river water samples and plating the membrane filters in Chromocult® agar (as described in section 3.2.3).

In Figure 3.6 it is visible the chromogenic and fluorogenic detection in three types of samples: tap, fountain and river water. The corresponding estimation of the number of CFU and the limit of detection is found in Table 3.3. In the three types of water samples, the limit of detection was of, approximately, 1 CFU in the sample, with periods of incubation between 14 to 16 h in the tap and fountain water samples, and between 12 to 14 h in river water samples. When compared to river water samples, the relatively longer incubation period necessary to detect the indicator microorganisms in tap and drinking water samples can be explained, in one hand, by

the severe lack of nutrients and, in the other hand, due to the potential presence of residual chlorine in tap water, creating a stressful environment for the indicator microorganisms [65-68]. Moreover, the river water contains several contaminants and possibly organic and inorganic nutrients at unknown concentrations that have been shown to support the survival and growth of the indicator microorganisms, resulting in a faster growth and detection with the enzymatic culture medium [69]. Although it was noticed a slightly less intense fluorescence in the tap water test-tubes containing the lower concentration of microorganisms, the yellow color was not affected and sensitivity of both chromogenic and fluorogenic detection was equivalent to the other water samples.

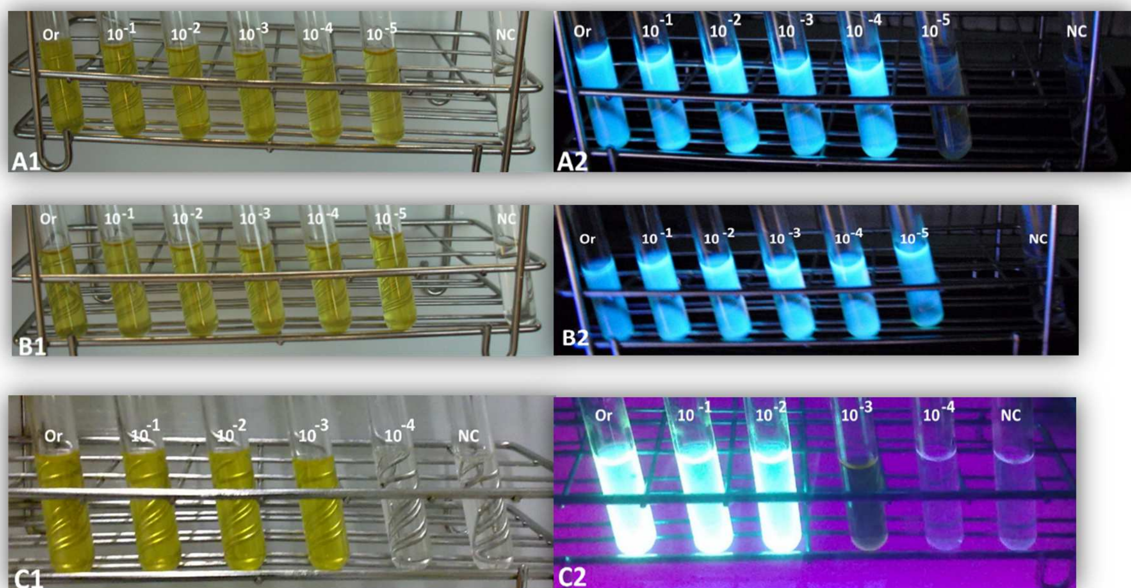


Figure 3.6 – Detection in tap water from the distribution network (A) and fountain water (B) inoculated with *E. coli*, and in river water (C) with the naturally present microorganisms, after 16 h of incubation. Yellow color at the left (A1, B1, C1) indicates the chromogenic detection of total coliforms, and the blue fluorescence at the right (A2, B2, C2) indicates the fluorogenic detection of *E. coli* (exposed at UV light, 366 nm). Each tube is named after its dilution, from left to right: “Or” is the first tube of the serial ten-fold dilutions up to 10^{-5}, and “NC” is the negative control (without microorganisms). Estimation of the number of CFU and limits of detection are shown in Table 3.3.

Table 3.3 – Comparison of the detection of *E. coli* and total coliforms in the samples of tap, fountain and river waters presented in Figure 3.6, using the enzymatic culture medium developed, after 14 to 16 h of incubation for tap and fountain waters, and 12 to 14 h of incubation for river water. The respective number of CFU/mL estimated for each dilution is indicated. "NC" refers to the negative control of the assay, with no microorganisms added.

Water source	Dilution	Or	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	NC
Tap	(7.3 ± 1.3) CFU/mL	$x10^4$	$x10^3$	$x10^2$	$x10^1$	$x10^0$	$x10^{-1}$	0
	Total coliforms	+	+	+	+	+	+	-
	E. coli	+	+	+	+	+	+	-
Fountain	(7.3 ± 1.3) CFU/mL	$x10^4$	$x10^3$	$x10^2$	$x10^1$	$x10^0$	$x10^{-1}$	0
	Total coliforms	+	+	+	+	+	+	-
	E. coli	+	+	+	+	+	+	-
River	(8.4 ± 0.62) CFU/mL	$x10^2$	$x10^1$	$x10^0$	$x10^{-1}$	(1)	(1)	0
	Total coliforms	+	+	+	+	-	-	-
	(1.8 ± 0.15) CFU/mL	$x10^2$	$x10^1$	$x10^0$	$x10^{-1}$	(1)	(1)	0
	E. coli	+	+	+	-	-	-	-

(1) For this dilution, it is considered: 0 CFU/mL.
Note: For the indicators detection, are considered: detected, **+** ; not detected, **-** .

Another possible cause for the different incubation times observed, is the possible interference of a non-target microorganism present in the river water samples, resulting in false-positive results [23]. Although the enzymatic culture medium includes antibiotic against Gram-positive bacteria and amphotericin B against fungi and protozoa, other non-coliform Gram-negative microorganisms capable of metabolize ONPG and MUG specific nutrients can grow and contribute to a faster visual result [15].

3.3.2.2 – SEAWATER SAMPLES

The main purpose was to test the adaptability of this enzymatic culture medium to seawater, which has a high content of sea salts in its composition (approximately, 3.7 %), in its majority sodium chloride (NaCl), and is known to be a challenge for the *E. coli* growth and survival in seawater [41, 70-72]. Since it is not possible to remove the sea salts from the seawater, assays comparing dilutions of seawater in distilled water (100, 75, 50, 25 and 0 % of seawater) were performed to perceive the extent of salinity impact in the detection (Figure 3.7). The corresponding estimation of the number of CFU and the limit of detection is presented in Table 3.4.

As expected, both the chromogenic and fluorogenic visual results show that it was achieved a higher sensitivity in the seawater samples more diluted, a clear evidence that an inhibition of the detection of the indicator microorganisms has occurred. This inhibition was observed not only in the limit of detection but also in the time to obtain the results. The first obvious difference between this water sample and the other types of water previously tested is the high salinity of seawater, which is known to significantly contribute to the inactivation and death of *E. coli* cells [70, 72]. When released to seawater, the enteric bacteria is immediately subjected to an osmotic shock which can explain the difficulty observed in culturing *E. coli*, even when longer periods of incubation were attempted [41]. Therefore, some *E. coli* cells may have remained active, others entered a viable but non-culturable state, and others may have lost their viability [41].

Figure 3.7 – Detection in (A) 100 %, (B) 75 %, (C) 50 %, (D) 25% and (E) 0 % of seawater diluted in distilled water and artificially inoculated with *E. coli*. Yellow color at the left (A1 to E1) identifies the chromogenic detection of total coliforms, and the blue fluorescence at the right (A2 to E2) identifies the fluorogenic detection of *E. coli* (exposed at UV light, 366 nm). Each tube is named after its dilution, from left to right: “Or” is the first tube of the serial ten-fold dilutions up to 10^{-8}, and “NC” is the negative control (without microorganisms). Estimation of the number of CFU and limits of detection are shown in Table 3.4.

Table 3.4 – Comparison of the detection of *E. coli* and total coliforms in the 100 %, 75 %, 50 %, 25% and 0 % of seawater samples diluted in distilled water, artificially inoculated with *E. coli* (Figure 3.7), using the enzymatic culture medium developed. The respective number of CFU/mL estimated for each dilution is indicated. "NC" refers to the negative control of the assay, with no microorganisms added. t_{inc} is the time of incubation at which the limit of detection was achieved to each sample and detection has stopped

Seawater	Dilution	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	NC	t_{inc} (h)
	(1.5 ± 0.43) CFU/mL	$x10^4$	$x10^3$	$x10^2$	$x10^1$	$x10^0$	$x10^{-1}$	(1)	0	
100 %	Total coliforms	+	+	-	-	-	-	-	-	12
	E. coli	+	-	-	-	-	-	-	-	12
75 %	Total coliforms	+	+	+	-	-	-	-	-	14
	E. coli	+	+	-	-	-	-	-	-	12
50 %	Total coliforms	+	+	+	+	+	-	-	-	18
	E. coli	+	+	+	-	-	-	-	-	24
25 %	Total coliforms	+	+	+	+	+	-	-	-	16
	E. coli	+	+	+	+	-	-	-	-	20
0 %	Total coliforms	+	+	+	+	+	+	-	-	16
	E. coli	+	+	+	+	+	+	-	-	18

(1) For this dilution, it is considered: 0 CFU/mL.

Note: For the indicators detection, are considered: detected, **+** ; not detected, **-** .

Results have shown that a dilution of 50 or 25 % allowed the recovery and detection of a low number of *E. coli* cells, even though with a longer period of incubation (20 to 24 h). These results are in accordance with previous survival experiments of *E. coli* in seawater that perceived an inverse correlation of the survival of *E. coli* with the salinity of seawater, and it was the found an optimum survival rate at 25 % of seawater (74 % of survival) [41]. Even though the dilution of seawater has allowed successful identification of *E. coli*, it should be taken in consideration that, when a contamination of seawater occurs, the *E. coli* cells are rapidly harmed and are less resistant in this environment than other pathogenic bacteria (e.g. *V. cholerae*)

[41]. Therefore, a negative result in the detection of *E. coli* in seawater does not allow to conclude with confidence that other pathogen microorganisms are not present in the seawater sample. Choosing a more appropriate indicator microorganism, with survival characteristics more similar to that of pathogens in seawater, is advised.

3.3.3 – Adaptability of the enzymatic culture medium to the microbiological water quality monitoring

According to the legislation (see Chapter 2, section 2.1.2), drinking water is to have 0 CFU of *E. coli* per 100 mL, river water is to have 20 CFU of faecal coliforms and 50 CFU per 100 mL (as inland water used in the production of water for human consumption) and 500 CFU of *E. coli* per 100 mL (as bathing water), and seawater is to have 500 CFU of *E. coli* per 100 mL [2-5]. Comparing the results obtained in the several water samples tested with the microbiological parameters described in the Portuguese legislation, the enzymatic culture medium developed was capable to fulfill and surpass the requirements of sensitivity as it was capable of detecting 1 CFU of the indicator microorganisms in all types of environmental water samples.

Moreover, the time to obtain the results was significantly reduced when compared to the analytical recommended methods by legislation (e.g. ISO 9308-1 and ISO 9308-3), which require 24 h to 2 or 3 days to provide the results due to the need of confirmatory steps and are based in a culture medium that produces large numbers of false positive results and is only recommended for use with high quality waters [11].

However, more research is necessary in order to study other antibiotics against non-coliform Gram-negative microorganisms, to perform specificity tests and

storage conditions before the enzymatic culture medium can be used for microbiological water quality monitoring.

3.3.4 – Procedure of the enzymatic culture medium as a commercial kit to detect *E. coli* and total coliforms in water

The lyophilized enzymatic culture medium developed can be used as a detection kit as described in Figure 3.8.

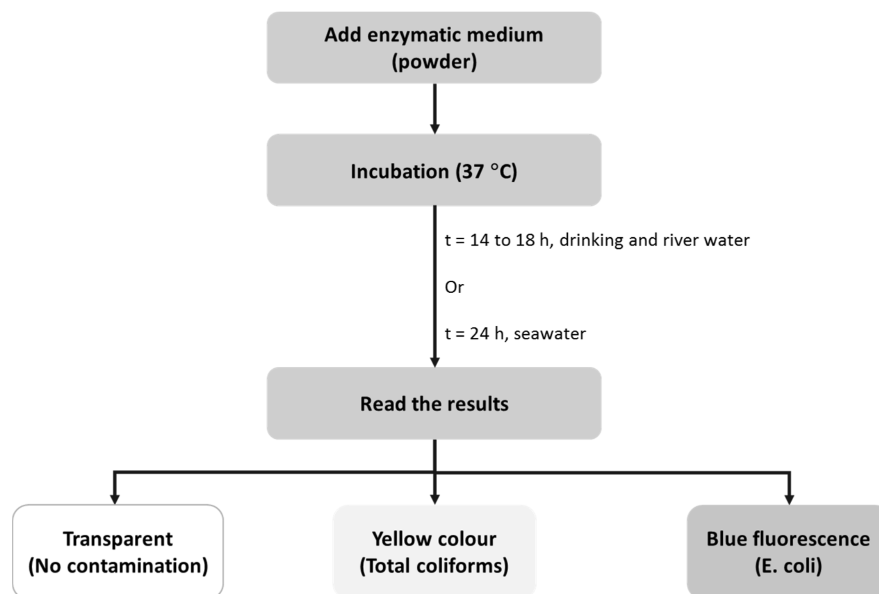


Figure 3.8 – Illustration of the procedure of the enzymatic culture medium developed as a commercial kit.

3.4 | Conclusions

A simple to use enzymatic culture medium was successfully developed, with the ability to detect the indicator microorganisms total coliforms and *E. coli* in water samples, up to the desired sensitivity of 1 CFU in the sample, within a period of time of 14 to 18 h.

Considering the microbiological limits established by the legislation, the enzymatic culture medium developed has shown to be capable to detect E. coli and total coliforms at the sensitivity required, in a reduced time when compared to the analytical methods recommended (e.g. ISO 9308-1 and ISO 9308-3) [2-5].

Moreover, this enzymatic culture medium is easy to adapt to a commercial kit as a lyophilized powder. To assess the microbiological water quality, the client would need to:

1. Add the powder of the enzymatic culture medium to the water sample (for seawater, dilute to 25 % using sterile distilled water)

2. Mix to dissolve until a clear solution is obtained;

3. Incubate accordingly to the water sample: up to 14 - 18 h for drinking and river water, and up to 24 h for seawater samples (37 °C);

4. Read the results: yellow color indicates the presence of total coliforms and the risk of pathogens in the water; blue fluorescence indicates the presence of *E. coli* and confirms the risk of pathogens in the water sample.

This enzymatic medium includes an antibiotic to inhibit interference from Gram-positive bacteria (either vancomycin or bacitracin). However, no antibiotic against non-coliform Gram-negative microbes was included in the final composition since the antibiotics tested showed to have activity against the indicator microorganisms. It was considered to be preferable to risk a false-positive result and take preventive measures than to risk false-negatives and compromise the safety of public health.

Although it was possible to use the enzymatic culture medium to detect total coliforms and *E. coli* in diluted seawater samples, it should be taken in consideration that other pathogens may survive longer than *E. coli* in this environment [41].

In order to reduce further the time to obtain the results, this enzymatic culture medium could be coupled to devices based in spectroscopy or optical sensors to detect the color and fluorescence produced by the specific substrates used to identify the indicator microorganisms at earlier stages of the culturing, when the results are not yet visible to the human eye [73, 74].

3.5 | REFERENCES

1. Rompré, A., Servais, P., Baudart, J., de-Roubin, M.-R., and Laurent, P., Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. Journal of Microbiological Methods, 2002. 49, 31-54.
2. Lei nº 58/2005 de 29 de Dezembro. Diário da República - I Série-A. 249 (29.12.2005) 7280-7310.
3. Decreto-Lei nº 236/98 de 1 de Agosto. Diário da República - I Série-A. 176 (1.8.98) 3676-3722.
4. Decreto-Lei nº 306/2007 de 27 de Agosto. Diário da República - I Série. 164 (27.8.2007) 5747-5765.
5. Decreto-Lei nº 135/2009 de 3 de Junho. Diário da República - I Série. 107 (3.6.2009) 3460-3468.
6. Njoku-Obi, A. and Skinner, C.E., Boric acid lactose broth as a medium for the detection of fecal coliform bacteria. Applied Microbiology, 1957. 5, 80-82.
7. Fiksdal, L., Pommepuy, M., Caprais, M.P., and Midttun, I., Monitoring of fecal pollution in coastal waters by use of rapid enzymatic techniques. Applied and Environmental Microbiology, 1994. 60, 1581-1584.
8. Maheux, A.F., Huppe, V., Boissinot, M., Picard, F.J., Bissonnette, L., Bernier, J.L.T., and Bergeron, M.G., Analytical limits of four beta-glucuronidase and beta-galactosidase-based commercial culture methods used to detect *Escherichia coli* and total coliforms. Journal of Microbiological Methods, 2008. 75, 506-514.
9. Bonadonna, L., Cataldo, C., and Semproni, M., Comparison of methods and confirmation tests for the recovery *Escherichia coli* in water. Desalination, 2007. 213, 18-23.
10. Pitkänen, T., Paakkari, P., Miettinen, I.T., Heinonen-Tanski, H., Paulin, L., and Hänninen, M.-L., Comparison of media for enumeration of coliform bacteria and *Escherichia coli* in non-disinfected water. Journal of Microbiological Methods, 2007. 68, 522-529.
11. Fricker, C.R., Bullock, S., Murrin, K., and Niemela, S.I., Use of the ISO 9308-1 procedure for the detection of *E. coli* in water utilizing two incubation temperatures and two confirmation procedures and comparison with defined substrate technology. Journal of Water and Health, 2008. 6, 389-397.
12. Niemi, R.M., Mentu, J., Siitonen, A., and Niemela, S.I., Confirmation of *Escherichia coli* and its distinction from *Klebsiella* species by gas and indole formation at 44 and 44.5 degrees C. Journal of Applied Microbiology, 2003. 95, 1242-1249.
13. Counsil Directive 98/83/CE of 3 November 1998 on the quality of water intended for human consumption. Official Journal of the European Communities. L330 (5.12.98) 32-53.

14. Orenga, S., James, A.L., Manafi, M., Perry, J.D., and Pincus, D.H., Enzymatic substrates in microbiology. Journal of Microbiological Methods, 2009. 79, 139-155.

15. Manafi, M., Fluorogenic and chromogenic enzyme substrates in culture media and identification tests. International Journal of Food Microbiology, 1996. 31, 45-58.

16. Nikaeen, M., Pejhan, A., and Jalali, M., Rapid monitoring of indicator coliforms in drinking water by an enzymatic assay. Iranian Journal of Environmental Health Science and Engineering, 2009. 6, 7-10.

17. Brenner, K.P., Rankin, C.C., Roybal, Y.R., Stelma, G.N., Scarpino, P.V., and Dufour, A.P., New medium for the simultaneous detection of total coliforms and *Escherichia coli* in water. Applied and Environmental Microbiology, 1993. 59, 3534-3544.

18. Trepeta, R.W. and Edberg, S.C., Methylumbelliferyl-beta-D-glucuronide-based medium for rapid isolation and identification of *Escherichia coli*. Journal of Clinical Microbiology, 1984. 19, 172-174.

19. Rice, E.W., Allen, M.J., and Edberg, S.C., Efficacy of b-glucuronidase assay for identification of *Escherichia coli* by the defined-substrate technology. Applied and Environmental Microbiology, 1990. 56, 1203-1205.

20. Rice, E.W., Allen, M.J., Brenner, D.J., and Edberg, S.C., Assay for beta-glucuronidase in species of the genus *Escherichia* and its applications for drinking-water analysis. Applied and Environmental Microbiology, 1991. 57, 592-593.

21. Martins, M.T., Rivera, I.G., Clark, D.L., Stewart, M.H., Wolfe, R.L., and Olson, B.H., Distribution of uidA gene sequences in Escherichia coli isolates in water sources and comparison with the expression of beta-glucuronidase activity in 4-methylumbelliferyl-beta-D-glucuronide media. Appl. Environ. Microbiol., 1993. 59, 2271-2276.

22. George, I., Petit, M., and Servais, P., Use of enzymatic methods for rapid enumeration of coliforms in freshwaters. Journal of Applied Microbiology, 2000. 88, 404-413.

23. Tryland, I. and Fiksdal, L., Enzyme characteristics of β-D-galactosidase- and β-D-glucuronidase-positive bacteria and their interference in rapid methods for detection of waterborne coliforms and *Escherichia coli*. Applied and Environmental Microbiology, 1998. 64, 1018-1023.

24. Fricker, E.J. and Fricker, C.R., Use of two presence/absence systems for the detection of *E. coli* and coliforms from water. Water Research, 1997. 30, 2226-2228.

25. Chao, K.-K., Chao, C.-C., and Chao, W.-L., Evaluation of Colilert-18 for detection of coliforms and *Eschericha coli* in subtropical freshwater. Applied and Environmental Microbiology, 2004. 70, 1242-1244.

26. Fricker, C.R., DeSarno, M., Warden, P.S., and Eldred, B.J., False-negative beta-d-glucuronidase reactions in membrane lactose glucuronide agar medium used for the simultaneous detection of coliforms and *Escherichia coli* from water. Letters in Applied Microbiology, 2008. 47, 539-542.

27. Sousa, O.V., Evangelista-Barreto, N.S., Catter, K.M., Fonteles-Filho, A.A., Macrae, A., and Vieira, R.H.S.F., Specificity of a defined substrate method used to monitor balneability of tropical coastal waters impacted by polluted stormwater, in Journal of Water & Health. 2010, IWA Publishing, 543-549.

28. Pisciotta, J.M., Rath, D.F., Stanek, P.A., Flanery, D.M., and Harwood, V.J., Marine bacteria cause false-positive results in the Colilert-18 rapid identification test for *Escherichia coli* in Florida waters. Applied and Environmental Microbiology, 2002. 68, 539-544.

29. Kuhnert, P., Nicolet, J., and Frey, J., Rapid and accurate identification of *Escherichia coli* K-12 strains. Applied and Environmental Microbiology, 1995. 61, 4135-4139.

30. Lin, S., Liang, R., Meng, X., OuYang, H., Yan, H., Wang, Y., and Jones, G.S., Construction and expression of mutagenesis strain of aroG gene from *Escherichia coli* K-12. International Journal of Biological Macromolecules, 2014. 68, 173-177.

31. Ehrenfeld, E.F., Colin; Townsend, David, Medium for detecting target microbes in a sample. Patent EP 1403378. 1996. Idexx Laboratories, inc.

32. Mendes, D., Desenvolvimento de um kit de detecção da bactéria *Escherichia coli* e coliformes totais em amostras de água, in Centre of Biological Engineering. 2008, University of Minho. Master degree dissertation.

33. Li, J., Nation, R.L., Turnidge, J.D., Milne, R.W., Coulthard, K., Rayner, C.R., and Paterson, D.L., Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. The Lancet Infectious Diseases, 2006. 6, 589-601.

34. Li, J., Nation, R.L., Milne, R.W., Turnidge, J.D., and Coulthard, K., Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. International Journal of Antimicrobial Agents, 2005. 25, 11-25.

35. Ciesiołka, J., Jeżowska-Bojczuk, M., Wrzesiński, J., Stokowa-Sołtys, K., Nagaj, J., Kasprowicz, A., Błaszczyk, L., and Szczepanik, W., Antibiotic bacitracin induces hydrolytic degradation of nucleic acids. Biochimica et Biophysica Acta (BBA) - General Subjects, 2014. 1840, 1782-1789.

36. Nailor, M.D. and Sobel, J.D., Antibiotics for Gram-positive bacterial infection: vancomycin, teicoplanin, quinupristin/dalfopristin, oxazolidinones, daptomycin, telavancin, and ceftaroline. Medical Clinics of North America, 2011. 95, 723-742.

37. Jeong, K.C., Hiki, O., Kang, M.Y., Park, D., and Kaspar, C.W., Prevalent and persistent *Escherichia coli* O157:H7 strains on farms are selected by bovine passage. Veterinary Microbiology, 2013. 162, 912-920.

38. Nagarajan, R., Antibacterial activities and modes of action of vancomycin and related glycopeptides. Antimicrobial Agents and Chemotherapy, 1991. 35, 605-609.

39. Alonso, J.L., Amoros, I., and Alonso, M.A., Differential susceptibility of aeromonads and coliforms to cefsulodin. Applied and Environmental Microbiology, 1996. 62, 1885-1888.

40. Yu, G., Niu, J., Shen, M., Shao, H., and Chen, L., Detection of *Escherichia coli* O157 using equal-length double-stranded fluorescence probe in a real-time polymerase chain reaction assay. Clinica Chimica Acta, 2006. 366, 281-286.

41. Rozen, Y. and Belkin, S., Survival of enteric bacteria in seawater. FEMS Microbiology Reviews, 2001. 25, 513-529.

42. Palmer, C.J., Tsai, Y.L., Lang, A.L., and Sangermano, L.R., Evaluation of Colilert-marine water for detection of total coliforms and *Escherichia coli* in the marine environment. Applied and Environmental Microbiology, 1993. 59, 786-790.

43. Ron, E.Z. and Davis, B.D., Growth rate of *Escherichia coli* at elevated temperatures: limitation by methionine. Journal of Bacteriology, 1971. 107, 391-396.

44. Ron, E.Z. and Shani, M., Growth rate of *Escherichia coli* at elevated temperatures: reversible inhibition of homoserine trans-succinylase. Journal of Bacteriology, 1971. 107, 397-400.

45. Nataro, J.P. and Kaper, J.B., Diarrheagenic *Escherichia coli*. Clinical Microbiology Reviews, 1998. 11, 142-201.

46. Hörman, A. and Hänninen, M.-L., Evaluation of the lactose Tergitol-7, m-Endo LES, Colilert 18, Readycult Coliforms 100, Water-Check-100, 3M Petrifilm EC and DryCult Coliform test methods for detection of total coliforms and *Escherichia coli* in water samples. Water Research, 2006. 40, 3249-3256.

47. Nakada, D. and Magasanik, B., The roles of inducer and catabolite repressor in the synthesis of β-galactosidase by *Escherichia coli*. Journal of Molecular Biology, 1964. 8, 105-127.

48. Wyborski, D.L. and Short, J.M., Analysis of inducers of the *E.coli lac* repressor system mammalian cells and whole animals. Nucleic Acids Research, 1991. 19, 4647-4653.

49. Mok, M.T.S. and Henderson, B.R., A two-step fluorescence-activated cell sorting approach to isolate genetically modified mammalian cells with isopropyl-beta-D-thiogalactoside (IPTG)-inducible gene expression. Cytometry Part A, 2012. 81A, 101-104.

50. Nwoguh, C.E., Harwood, C.R., and Barer, M.R., Detection of induced β-galactosidase activity in individual non-culturable cells of pathogenic bacteria by quantitative cytological assay. Molecular Microbiology, 1995. 17, 545-554.

51. Nagamachi, E. and Kanemasa, Y., Development of a new device for the detection of gas production in coliform group bacteria determination. Water Research, 1991. 25, 1131-1135.

52. Yáñez, M.A., Valor, C., and Catalán, V., A simple and cost-effective method for the quantification of total coliforms and *Escherichia coli* in potable water. Journal of Microbiological Methods, 2006. 65, 608-611.

53. Anglès d'Auriac, M.B., Roberts, H., Shaw, T., Sirevåg, R., Hermansen, L.F., and Berg, J.D., Field evaluation of a semiautomated method for rapid and simple analysis of recreational water microbiological quality. Applied and Environmental Microbiology, 2000. 66, 4401-4407.

54. Fiksdal, L., Tryland, I., and Nelis, H., Rapid detection of coliform bacteria and influence of non-target bacteria. Water Science and Technology, 1997. 35, 415-418.

55. Scogin, D.A., Mosberg, H.I., Storm, D.R., and Gennis, R.B., Binding of nickel and zinc ions to bacitracin A. Biochemistry, 1980. 19, 3348-3352.

56. Byamukama, D., Mach, R.L., Kansiime, F., Manafi, M., and Farnleitner, A.H., Discrimination efficacy of fecal pollution detection in different aquatic habitats of a

high-altitude tropical country, using presumptive coliforms, *Escherichia coli*, and *Clostridium perfringens* spores. Applied and Environmental Microbiology, 2005. 71, 65-71.

57. Catchpole, C.R., Andrews, J.M., Brenwald, N., and Wise, R., A reassessment of the in-vitro activity of colistin sulphomethate sodium. Journal of Antimicrobial Chemotherapy, 1997. 39, 255-260.

58. Galani, I., Kontopidou, F., Souli, M., Rekatsina, P.-D., Koratzanis, E., Deliolanis, J., and Giamarellou, H., Colistin susceptibility testing by Etest and disk diffusion methods. International Journal of Antimicrobial Agents, 2008. 31, 434-439.

59. Breathnach, A.S., Cubbon, M.D., Karunaharan, R.N., Pope, C.F., and Planche, T.D., Multidrug-resistant *Pseudomonas aeruginosa* outbreaks in two hospitals: association with contaminated hospital waste-water systems. Journal of Hospital Infection, 2012. 82, 19-24.

60. Loveday, H.P., Wilson, J.A., Kerr, K., Pitchers, R., Walker, J.T., and Browne, J., Association between healthcare water systems and *Pseudomonas aeruginosa* infections: a rapid systematic review. Journal of Hospital Infection, 2014. 86, 7-15.

61. Chudzik, B., Tracz, I.B., Czernel, G., Fiołka, M.J., Borsuk, G., and Gagoś, M., Amphotericin B–copper(II) complex as a potential agent with higher antifungal activity against *Candida albicans*. European Journal of Pharmaceutical Sciences, 2013. 49, 850-857.

62. Kodama, T., Takeuchi, M., Wakiyama, N., and Terada, K., Optimization of secondary drying condition for desired residual water content in a lyophilized product using a novel simulation program for pharmaceutical lyophilization. International Journal of Pharmaceutics, 2014. 469, 59-66.

63. Kasper, J.C., Winter, G., and Friess, W., Recent advances and further challenges in lyophilization. European Journal of Pharmaceutics and Biopharmaceutics, 2013. 85, 162-169.

64. Agere, Relatório trimestral de análises: divulgação de resultados obtidos na água destinada a consumo humano. Acedido em Junho de 2014: http://www.agere.pt/web1/uploads/documentos/image/PDFs/analises/1trim2011.pdf. 2012.

65. Özkanca, R., Saribiyik, F., Isik, K., Sahin, N., Kariptas, E., and Flint, K.P., Resuscitation and quantification of stressed *Escherichia coli* K12 NCTC8797 in water samples. Microbiological Research, 2009. 164, 212-220.

66. Tallon, P., Magajna, B., Lofranco, C., and Kam, T.L., Microbial indicators of faecal contamination in water: A current perspective. Water, Air, and Soil Pollution, 2005. 166, 139-166.

67. Rockabrand, D., Austin, T., Kaiser, R., and Blum, P., Bacterial growth state distinguished by single-cell protein profiling: does chlorination kill coliforms in municipal effluent? Applied and Environmental Microbiology, 1999. 65, 4181-4188.

68. Lemarchand, K., Masson, L., and Brousseau, R., Molecular biology and DNA microarray technology for microbial quality monitoring of water. Critical Reviews in Microbiology, 2004. 30, 145-172.

69. Hong, H., Qiu, J., and Liang, Y., Environmental factors influencing the distribution of total and fecal coliform bacteria in six water storage reservoirs in the Pearl River Delta Region, China. Journal of Environmental Sciences, 2010. 22, 663-668.

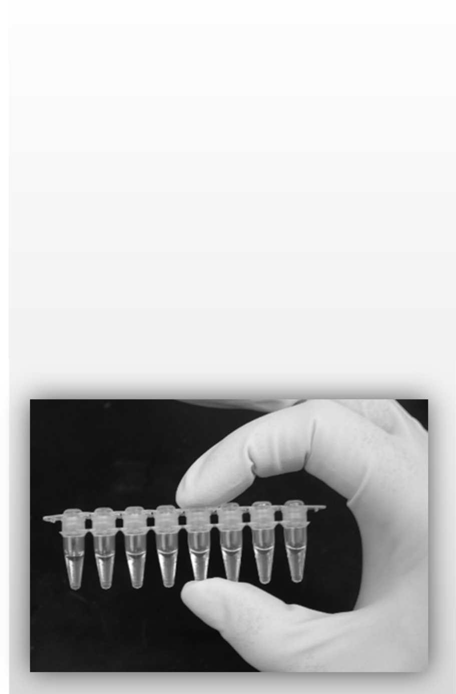
70. Carlucci, A.F. and Pramer, D., An evaluation of factors affecting the survival of *Escherichia coli* in sea water. II. Salinity, pH, and nutrients. Applied Microbiology, 1960. 8, 247-250.

71. Chandran, A. and Mohamed Hatha, A.A., Relative survival of *Escherichia coli* and *Salmonella typhimurium* in a tropical estuary. Water Research, 2005. 39, 1397-1403.

72. Sørensen, S.J., Survival of *Escherichia coli* K12 in seawater. FEMS Microbiology Letters, 1991. 85, 161-167.

73. Geary, J.R., Nijak Jr, G.M., Larson, S.L., and Talley, J.W., Hydrolysis of the soluble fluorescent molecule carboxyumbelliferyl-beta-d-glucuronide by *E. coli* beta-glucuronidase as applied in a rugged, *in situ* optical sensor. Enzyme and Microbial Technology, 2011. 49, 6-10.

74. Wildeboer, D., Amirat, L., Price, R.G., and Abuknesha, R.A., Rapid detection of *Escherichia coli* in water using a hand-held fluorescence detector. Water Research, 2010. 44, 2621-2628.



CHAPTER 4

TOWARDS METHODS OF DETECTION OF *ESCHERICHIA COLI* AND TOTAL COLIFORMS IN WATER BY POLYMERASE CHAIN REACTION

In this chapter, it was developed a sample preparation method to concentrate and recover microorganisms from samples of water, to be used in the detection of *E. coli* and total coliforms using PCR and rtPCR as molecular methods.

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4.1 | Introduction

Standard methods used for the detection of total coliforms and *E. coli* in water samples deeply rely in cultivation on a selective growth medium and on conventional biochemical tests [7-10]. The analytical techniques recommended by the legislation are based in these methods and require between 18 to 72 h to perform and achieve the results [11-14]. This delay in providing information about the water quality, the inability to detect VBNC bacteria [15-18] and the failure in detecting some *E. coli* strains, particularly the pathogenic *E. coli* O157:H7 [4, 17, 19, 20], highlighted the need for other detection methods.

PCR allows amplification of one or more DNA target sequences, by cycling replication, and has distinct advantages over culture-based methods in the detection of microbiological pathogens: higher level of sensitivity and specificity without the need for a complex cultivation step or additional confirmation, reduced time to obtain the results, accuracy and capacity to detect small amounts of target nucleic acids in a sample [1, 18, 21, 22]. Another important advantage of PCR assays is the ability to detect the microorganisms known as viable but non-culturable (VBNC); when released in water, faecal bacteria can lose their ability to grow on culture media while preserving some metabolic activities and their virulence as VBNC bacteria [15, 23].

In recent years, rtPCR technology has been preferred over standard PCR due to the additional advantages of providing the results faster, in real time, with the possibility of quantification and without the need to run the amplified products on an agarose gel to obtain the results (electrophoresis) [24]. This has become possible by including a fluorescent molecule in the reaction tube that signals an increase in the amount of DNA with a proportional increase in the fluorescent signal. Two types

of fluorescent dyes can be used: DNA-binding dyes (e.g. SYBR Green I, Eva Green) and fluorescently labelled sequence-specific primers or probes (e.g. TaqMan probe, molecular beacon) [25]. DNA-binding dyes, which bind non-specifically to double-stranded DNA, are the most commonly used as they are less expensive and easier to use. Sequence-specific primers and probes are designed to bind to the target and, thus, the fluorescent signal is only detected in the presence of the targeted amplicon [25].

In order to detect the indicator bacteria, most primers have been based on genes coding the specific enzymes widely expressed in the several strains of the indicator microorganisms. For total coliforms, primers based on the *lacZ* gene have been used as a target for PCR amplification since the classical methods of coliform monitoring are based on the detection of the expression product of this gene: the enzyme β-D-galactosidase [2, 4]. For the specific detection of *E. coli*, it was firstly suggested a region of the *lamB* gene, which encodes the outer membrane protein entitled LamB [2, 26]. However, *Shigella* and *Salmonella* spp. could also be detected using this primer. It was then proposed the *uidA* gene to identify *E. coli* [4, 6]. The *uidA* gene encodes the β-D-glucuronidase enzyme, the specific enzyme that most enzymatic culture media use for the identification of *E. coli* and whose set of primers is specific for *E. coli* and *Shigella* spp. [27]. The importance of using molecular methods in the detection of this gene relies on the fact that it becomes possible to identify *E. coli* strains that do not exhibit activity of the enzyme β-D-glucuronidase (culture with the substrate MUG does not detect these strains), although they carry the *uidA* gene (e.g. the pathogenic *E. coli* O157) [4, 19, 21, 28].

Despite the *uidA* gene has been considered as the ideal candidate for DNA-based assays to identify *E. coli*, the molecular analysis of genes from *E. coli* and *Shigella* strains have shown a very close evolutionary relationship between those species and sustain that *Shigella* should be considered as, in fact, *E. coli* [5, 19, 29]. Having this in consideration, other genes were evaluated as possible alternatives to

identify all the strains of *E. coli/Shigella* spp. [27]. Since primers based in the *uidA* gene were not able to identify all *E. coli/Shigella* strains, it was suggested a detection method based in the housekeeping gene *tuf* (elongation factor Tu) [1, 5, 27]. However, this gene also identified *Escherichia fergusonii* (*E. fergusonii*) as a non-specific target and, thus, if it becomes necessary to differentiate between *E. fergusonii* and *E. coli*, the *uidA* gene should be the choice for the molecular detection [27].

PCR-based methods are well established as a technique capable of efficiently identify microorganisms in samples from various sources, achieving a sensitivity of 1 CFU per tube when pre-enrichment of the microorganisms in the sample is part of the procedure [22, 30-33]. However, the pre-enrichment itself requires an incubation period of several hours and, in some cases, of days to achieve a concentration of microorganisms sufficient for the PCR-based detection [18, 21]. In water quality monitoring, it is imperative to reduce the time required to obtain the results since a timely response to the contamination of the water with pathogenic microorganisms is essential to reduce the risk of an outbreak and efficiently protect the public health. For that reason, it is important to remove the pre-enrichment of the microorganisms from the sample preparation procedure in order to reduce the assay time and, thus, provide results faster than the analytical reference methods (e.g. ISO 9308-1, based in culturing the microorganisms) recommended in the legislation [12-14]. Removing the pre-enrichment from the procedure, however, results in an unsatisfactory sensitivity due to the requirement to detect 1 CFU of the indicator microorganism in a water sample of 100 mL by using only 1 µL of the sample in the PCR-based detection.

Therefore, the aim of this research was to evaluate the effectiveness of PCR and rtPCR as molecular methods for the detection of total coliforms and *E. coli*, as well as their possible adaptation to microbiological water quality monitoring. For this purpose, the pre-enrichment step was avoided in order to provide faster results than

those achieved with the culture-based methods. This protocol attempted at simple handling, high efficiency and the sensitivity of 1 CFU per water sample (100 mL).

4.2 | MATERIALS AND METHODS

4.2.1 – Water samples preparation without pre-enrichment

Several strategies were tested in an effort to obtain an effective but simple protocol to collect and concentrate the cells of the indicator microorganisms present in the water samples, and extract the nucleic acids to be used for amplification by PCR or rtPCR. The more significant results were discussed and included in the final protocol.

4.2.1.1 – MICROORGANISM

E. coli K12 was the microorganism selected to perform the assays due to its non-pathogenicity [34]. It was cultured from a frozen stock culture of *E. coli* K12, from the culture collection at the Centre of Biological Engineering, University of Minho (Braga, Portugal). A pure culture of *E. coli* K12 was cultured overnight at 37 ˚C in Luria-Bertani (LB) agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 20 g/L agar, 175 µL/L NaOH 10 N) (Merck Millipore, Germany) [35]. This pure culture was used to artificially inoculate distilled water and the environmental samples that were not naturally contaminated with the indicator microorganisms.

4.2.1.2 – MEMBRANE FILTRATION OF THE WATER SAMPLES TO CONCENTRATE THE MICROORGANISMS

In order to increase the sensitivity of the method, it is necessary to pre-concentrate the water samples since most of them have a small number of the target bacteria dispersed in a large volume of water. To accomplish this purpose, it was chosen to include a filtration step in the procedure.

In a closed sterile glass flask, 100 mL of sterile distilled water were artificially inoculated with two colonies from the overnight pure culture of *E. coli* (prepared according to the Section 4.2.1.1), which were resuspended by vigorously mixing. In order to simulate a decreasing concentration of the indicator microorganisms, a ten-fold serial dilution was prepared by adding a volume of 90 mL of the sterile distilled water to as many sterile glass flasks as the estimated to be necessary to achieve a concentration of less than 1 CFU of *E. coli* per 100 mL in the last glass flask, plus one flask to be used as the negative control, as described in Figure 4.1 (the number of dilutions may vary according to the estimated initial concentration of CFU in the first flask). The inoculated flask was then ten-fold serially diluted by pipetting 1 mL of each dilution to the following flask (see Figure 4.1). The last flask was kept sterile as a negative control. The enumeration of the concentration of *E. coli* in the water samples was performed by plating 100 µL of each dilution, in triplicate, in LB agar plates (as described in Chapter 3, Section 3.2.3).

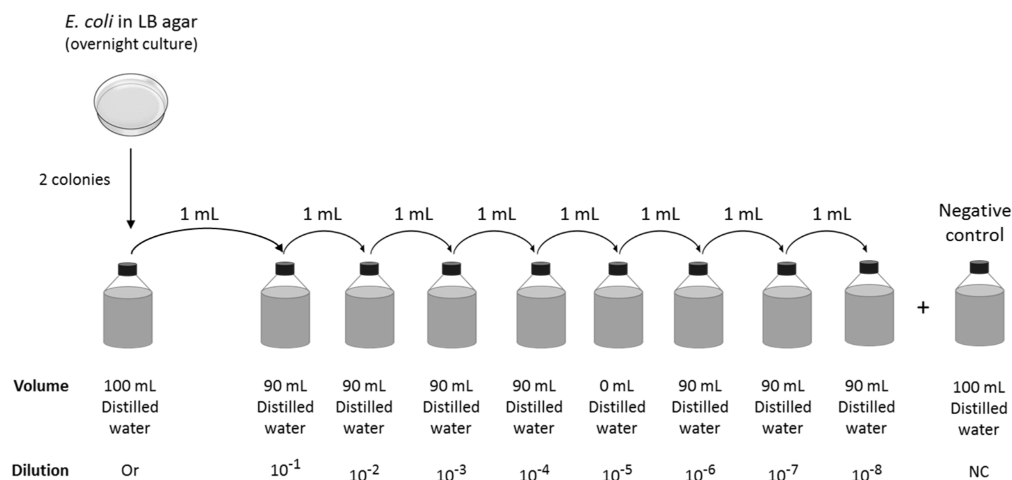


Figure 4.1 – Illustration of the ten-fold serial dilution of the water samples artificially inoculated with *E. coli*, to mimic an estimated decreasing concentration of the indicator microorganism in the water sample up to less than 1 CFU in the last test-tube.

The *E. coli* filtration and recovery from the filters was based in a method developed by Wolffs and coworkers (2006) to detect *Salmonella* sp. in biological

samples [36, 37]. Therefore, the microorganisms were captured using vacuum-filtration on cellulose acetate membrane (∅ 47 mm) filters with 0.2 µm pore-size (Advantec, Japan), as described in Figure 4.2A. To recover the cells of *E. coli*, the filters were folded and transferred to a 15 mL tube, containing 1 mL of phosphate buffered saline (PBS: 8 g/L NaCl, 0.2 g/L KCl, 2.68 g/L $Na_2HPO_4.7H_2O$, 0.24 g/L KH_2PO_4) (Merck Millipore, Germany), followed by vortexing for 15 s at maximum speed. The liquid was then transferred to a 1.5 mL tube (Frilabo, Portugal).

To test the possibility of further reducing the final volume of the sample, the previous filtration system was replaced by a cellulose acetate membrane of reduced diameter (∅ 13 mm, 0.2 µm; Advantec, Japan) placed in a syringe filter holder (Frilabo, Portugal) (Figure 4.2B). The water samples were then forced through the filter holder using a sterile syringe (BD, Canada), the membranes were removed from the holder, folded and transferred to a 2 mL tube (Frilabo, Portugal) with ~ 0.0080 g of glass beads (acid-washed) (710-1.180 µm; Sigma-Aldrich, Germany) [38]. 150 µL of PBS with SDS (0.05 g/L; Sigma-Aldrich, Germany) was added to the 2 mL tube, followed by vortexing for 15 s at maximum speed.

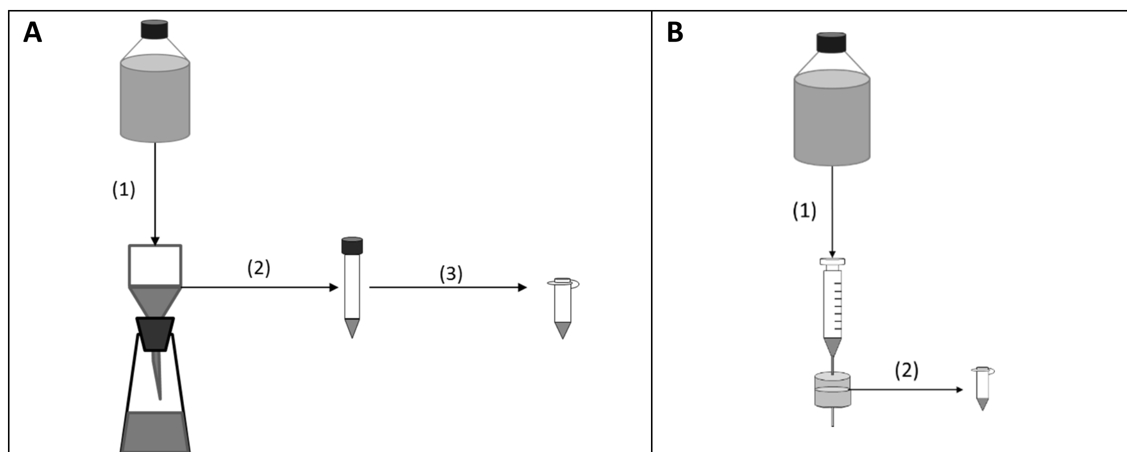


Figure 4.2 – Illustration of the membrane filtration during sample preparation. A – (1) The water sample is filtered using a standard filtration system to concentrate the microorganisms on a 47 mm diameter membrane, (2) the membrane is transferred to a 15

mL tube to be washed with PBS and by vortexing for 15 s, (3) and the PBS with the microorganisms is transferred to the 1.5 mL tube. B - (1) The water sample is filtered to concentrate the microorganisms on a 13 mm diameter membrane placed in a syringe filter holder, (2) and the membrane is transferred to a 2 mL tube, with glass beads, to be washed with a solution of PBS and SDS (0.05 g/L) and by vortexing for 15 s.

4.2.1.3 – Extraction of the nucleic acids using cell lysis agents

After the filtration step, each of the following cell lysis agents were added to the 2 mL tubes (Frilabo, Portugal) to the final concentration described: lysozyme (0.5 mg/mL), lysozyme (10 mg/mL) with proteinase K (0.2 mg/mL), according to Clark et al. [39]), Triton X-100 (1%) and SDS (0.05 mg/mL) (Sigma-Aldrich, Germany). The tubes were then placed in a water bath at 95 °C to perform a heat shock, as described in Section 4.2.1.4, in order to evaluate their ability to efficiently release the nucleic acids [40-42].

4.2.1.4 – Extraction of the nucleic acids using heat shock

Cellular lysis can also be achieved through rapid changes of temperature. The 1.5 mL and 2 mL tubes containing the concentrated cells were placed in a water bath at 95 °C [43] during approximately 15 minutes, after which, they were immediately immersed in ice for 10 minutes. In another approach, a cycling method was used to attempt enhancing the lysis effect by submitting the sample to six cycles of variable temperatures: tubes were placed in a water bath at 95 °C, after 3 minutes the tubes were put in ice for another 3 minutes; this process of placing the tubes in the water bath and in ice was repeated every 3 minutes, until a total of six cycles was completed and then tubes were kept on ice for 10 minutes.

4.2.1.5 – DNA RECOVERY BY PRECIPITATION OF THE NUCLEIC ACIDS

This step was tested in order to increase the recovery of DNA from the samples and as a purification process to achieve a higher sensitivity and reduce contaminants. After heat shock, half-volume of cold isopropanol (Frilabo, Portugal) was added to the samples, which were then placed at -20 ˚C for 30 min, centrifuged for 20 min at 13 200 rpm (4 ˚C) (Microcentrifuge 5415R, Eppendorf, Germany), the supernatant was rejected, the pellet was washed with 1 mL of cold ethanol (70%) (Frilabo, Portugal), followed by centrifugation for 20 min at 13 200 rpm at 4 ˚C (Microcentrifuge 5415R, Eppendorf, Germany). The ethanol was rejected and the pellet was left to dry at room temperature. Finally, it was resuspended in 100 mL of TE buffer (Tris-HCl at 100 mM, EDTA at 1 mM, pH 8.0) (VWR International, Germany) [37, 44].

4.2.1.6 – ADAPTABILITY OF THE PHUSION BLOOD DIRECT PCR KIT (THERMO SCIENTIFIC) TO THE DIRECT PCR DETECTION OF *E. COLI*

An assay was performed to test the Phusion Blood Direct PCR kit (Thermo Scientific, USA) by filtering 10 mL distilled water artificially spiked with one colony of a pure culture of *E. coli* (prepared according to the Section 4.2.1.1), the cellulose acetate membrane (∅ 13 mm, 0.2 µm; Advantec, Japan) was cut square-shapped (3 mm) and placed in a 200 mL tube (Frilabo, Portugal) before being amplified by standard PCR as described in Section 4.2.4.

The amplification of DNA from a pure culture of *E. coli* (prepared according to the Section 4.2.1.1) extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA), was tested by adding 1 µL of *E. coli* DNA directly to the reaction tube, and by adding 1 µL of DNA to the filter, which was then cut and placed in the 200 mL tube (Frilabo, Portugal). The standard PCR amplification was performed as described in Section 4.2.4.

4.2.1.7 – OTHER TESTS PERFORMED TO CONCENTRATE AND RECOVER *E. COLI*

During membrane filtration, after filtering the water samples using an acetate cellulose membrane (∅ 13 mm, 0.2 µm; Advantec, Japan) placed in a filter holder (Frilabo, Portugal) (see Section 4.2.1.2), the membranes were washed by reversing the flow in the filter holder using a sterile syringe (BD, Canada) with 300 mL of the PBS solution (PBS: 8 g/L NaCl, 0.2 g/L KCl, 2.68 g/L $Na_2HPO_4.7H_2O$, 0.24 g/L KH_2PO_4) (Merck Millipore, Germany) into a 2 mL tube (Frilabo, Portugal) containing ~0.0080 g of glass beads (acid-washed) (710-1.180 µm; Sigma-Aldrich), followed by vortexing for 15 s at maximum speed.

In another approach, during the heat shock (Section 4.2.1.4), the 1.5 mL and 2 mL tubes were left open in the water bath at 95 °C until most of the volume was evaporated to a final volume of ~50 µL. Samples were immersed in ice for 10 minutes and analysed by standard PCR as described in Section 4.2.4.

4.2.2 – Method CRENAME (concentration and recovery of microbial particles, extraction of nucleic acids, and molecular enrichment)

This method, named as *E. coli* CRENAME-rtPCR [1, 5, 45], was tested during an internship at the Centre de Recherche en Infectiologie de l'Université Laval (Québec City, Canada). The purpose was to evaluate the reproducibility of the method developed for the detection of *E. coli* and *Shigella* spp. in water samples by rtPCR.

4.2.2.1 – Water samples preparation

All cultures were obtained from the Collection of Centre de Recherche en Infectiologie (CCRI). *E. coli* ATCC 11775 (CCRI-467) was grown at 37 °C to logarithmic phase (OD 600, 0.5 - 0.6) in BHI liquid medium (BD, Canada) and the culture adjusted to the McFarland 0.5 standard (used in microbiology to standardize the approximate number of bacteria in a liquid suspension by turbidity comparison with a standard) [5]. The culture was serially diluted ten-fold in PBS and the dilution 10^{-5} was used to inoculate sterile ultra-pure water to produce bacterial suspensions containing approximately 100, 50 and 0 (negative control) CFU/100mL [5]. A process control was performed, accordingly to Picard et al. [46], by using approximately 50 spores/100 mL of *Bacillus atrophaeus* subsp. *globigii* (CCRI-9827) that were added to all water samples. Detecting the spores of *B. atrophaeus* subsp. *globigii* along with *E. coli* has the purpose of evaluating the integrity of the sample preparation, nucleic acid extraction and molecular enrichment techniques and identify a possible inhibition of the whole-genome amplification (WGA) and/or rtPCR.

4.2.2.2 – Membrane filtration of the water samples to concentrate the microorganisms

Bacterial counts were confirmed by filtering 100 mL of each spiked water sample prepared as described in 4.2.2.1, in triplicate, through a membrane filter (GN-6, ∅ 47 mm, 0.45 µm pore size; PALL Corporation, USA) on a 3-place standard manifold (Millipore Corporation, Billerica, MA) (Figure 4.3) and placed in sheep blood agar plates (BD, Canada) and incubated for approximately 24 h at 35 °C (aerobic incubator). The number of colonies was counted (*E. coli*: beige colonies; *B. atrophaeus* subsp. *globigii*: orange colonies) and the number of CFU/mL was calculated using the Equation 3.1 (Chapter 3, Section 3.2.3).

Figure 4.3 – Filtration system (Millipore Corporation, Billerica, MA): At the right, the 3-place standard manifold used for filtering the samples and, at the left, the UV box to sterilize the filter holders and funnels between filtrations [5].

The spiked water samples, prepared as described in 4.2.2.1, were filtered using a membrane filter (GN-6, ∅ 47 mm, 0.45 µm pore size; PALL Corporation, USA) on the 3-place standard manifold (Millipore Corporation, Billerica, MA). Each sample was filtered in triplicate and the membrane was placed in a 15 mL tube (BD, Canada). Between each filtration, the 3-place standard manifold was sterilized by putting the filter holders and funnels in a UV box (Millipore Corporation, Billerica, MA) for 2 min [5]. Membranes were then in the CRENAME procedure (Section 4.2.2.3).

4.2.2.3 – CRENAME PROCEDURE TO CONCENTRATE AND RECOVER THE MICROORGANISMS

Membranes with the captured microorganisms (prepared in Section 4.2.2.2) were dissolved into small pieces by adding 8.5 mL of methanol (BD, Canada) by gently mixing. The tubes were then centrifuged for 5 min at 4100 rpm (Jouan, Thermo Scientific, USA), and the supernatant was removed and discarded. 1 mL of acetone (BD, Canada) was added to tubes and the pellet was dissolved by vigorous agitation. The resulting clear solution was transferred to a 2 mL screw-cap tube (BD, Canada) containing acid-washed glass beads (710-1.180 µm and 150-212 µm; Sigma-Aldrich), centrifuged for 3 min at 15 800 x g, room temperature (Eppendorf, Germany); the supernatant was removed and discarded. The 15 mL tube was rinsed with 1 mL of acetone and the resulting solution was transferred to the 2 mL screw-cap tube previously used. The tubes were centrifuged at 15 800 x g for 3 min (Eppendorf, Germany) and supernatant was removed and discarded. 1 mL of TE (Tris-HCl at 100 mM, EDTA at 1 mM, pH 8.0) (BD, Canada) was added to the screw-cap tubes and these were then centrifuged 3 min at 15 800 x g (Eppendorf, Germany). Supernatant was removed and discarded. Samples were used either for molecular enrichment (Section 4.2.2.4) or directly for amplification by rtPCR (Section 4.2.5.1).

4.2.2.4 – WHOLE GENOME AMPLIFICATION (WGA) FOR THE MOLECULAR ENRICHMENT OF NUCLEIC ACIDS

20 µL of sample buffer (part of the Illustra GenomiPhi DNA amplification kit; GE Healthcare, Québec, Canada) was added to the screw-cap tubes containing the samples prepared in Section 4.2.2.3, and were placed on a vortex mixer at maximum speed for 5 min, as described by Maheux and coworkers [5]. After, tubes were placed for 3 min in a water bath of 95 °C and kept on ice for 3 min. A mixture containing 45 µL of the GenomiPhi reaction buffer with 5 µL of the Genomiphi ϕ29 DNA polymerase, both part of the Illustra GenomiPhi DNA amplification kit (GE Healthcare, Québec, Canada), was added to each tube and gently mixed by finger tapping. Then

the tubes were incubated for 3 h at 30 °C, and the enzymatic reaction was stopped by an incubation of 10 min at 65 °C. 1 µL of each sample was used as the template for *E. coli* and *B. atrophaeus* subsp. *globigii* rtPCR amplifications (Section 4.2.5.1).

4.2.3 – Primers and rtPCR dyes

A pair of 24 bp (base pair) primers located within the coding region of the *lacZ* gene [3] was chosen for total coliforms detection (Table 4.1) and a pair of 20 and 21 bp primers located within the *uidA* structural gene [3] were selected for identification of *E. coli*. A set of 24 bp primers was also used to amplify a segment of the coding region of the *lamB* gene [2]. These primers were tested individually, as well as simultaneously on a Multiplex-PCR reaction. A primer pair targeting the *uidA* gene developed by Takahashi and colleagues [6] to identify *E. coli* in food samples was also tested, as well as the primers targeting the *tuf* gene developed by Maheux *et al.* [1, 5] for the identification of both *E. coli/Shigella*. The primers are detailed in Table 4.1, were ordered to Thermo Scientific (USA) and used in both standard PCR and rtPCR (Sections 4.2.4 and 4.2.5, respectively).

Two types of rtPCR amplification were performed by using two types of dyes: Sso Fast™ EvaGreen® (Bio-Rad, USA) and TaqMan (Biosearch Technologies, Inc., USA) probe. EvaGreen® dye has similar properties to SYBR® Green I, which means that it binds non-specifically to all double-stranded DNA, but is less sensitive to PCR inhibitions and can produce greater fluorescence signals [25]. The TaqMan probe (Table 4.1) was developed by Maheux *et al*. [1, 5] and was used for the *E. coli* CRENAME-rtPCR tests, as well as the primers and probe for *B. atrophaeus* subsp. *globigii*, which was the process control of the method.

Table 4.1 – Sequence of the primers and the targeted genes used in the detection of total coliforms (*lacZ*) and *E. coli* (*uidA*, *lamb*), and the TaqMan probes used for the detection of *E. coli/Shigella* in water samples (adapted) [1-6]

TARGET GENE	PRIMERS/PROBE	PRIMERS SEQUENCE (5´→ 3´)	TANNEALING	AMPLICON SIZE	REFERENCE
lacZ	ZL-1675 ZR-2548	5´ – ATG AAA GCT GGC TAC AGG AAG GCC – 3’ 5´ – GGT TTA TGC AGC AAC GAG ACG TCA – 3’	55 °C	264 bp	[2, 3]
uidA	uidAF uidAR	5´ – AAA ACG GCA AGA AAA AGC AG – 3’ 5´ – ACG GCT GGT TAC AGT CTT GCG – 3’	50 °C	147 bp	[4]
lamB	BL-4910 BR-5219	5´ – CTG ATC GAA TGG CTG CCA GGC TCC – 3’ 5´ – CAA CCA GAC GAT AGT TAT CAC GCA – 3’	53 °C	309 bp	[2]
uidA	ECN1254F ECN1328r	5´ – GCA AGG TGC ACG GGA ATA TT – 3’ 5´ – CAG GTG ATC GGA CGC GT – 3’	50 °C	75 bp	[6]

E. coli (*tuf*) (primers)	TEcol553 TEcol754	5´ – TGG GAA GCG AAA ATC CTG – 3’ 5´ – CAG TAC AGG TAG ACT TCT G – 3’	58 °C	212 bp
E. coli (TaqMan probe)	TEco573T1-B1	5´ – TET – AAC TGG CTG GCT TCC TGG – BHQ –1 – 3’	62 °C	–
B. atrophaeus subsp. *globigii* (primers)	ABgl158 ABgl345a	5´ – CAC TTC ATT TAG GCG ACG ATA CT – 3’ 5´ – TTG TCT GTG AAT CGG ATC TTT CTC – 3’	60 °C	211 bp
B. atrophaeus subsp. *globigii* (TaqMan probe)	ABgl-T1-A1	5´ – FAM-GTC CCA ATG TTA CAT TAC CAA CCG GCA CT (BHQ-1) – GAA ATA GG – 3’	71 °C	

4.2.4 – Standard PCR amplification to detect *E. coli* in water samples

The standard PCR amplification was performed in a thermal cycler (Piko, Fynnzymes, Finland), using a final volume of 50 µL containing 1 µL of template DNA, 1 x reaction buffer, 200 µM of dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer and 1 µL of *Taq* DNA polymerase (DyNAzyme II^{TM}, Finnzymes, Finland).

DNA was initially denatured at 94 ˚C for 2 min. Then a total of 45 PCR cycles were run under the following conditions: denaturation at 94 ˚C for 45 s, primer annealing at 50, 53 and 55 ˚C (primers uidAF/uidAR, BL-4910/BR-5219 and ZL-1675/ZR-2548*,* correspondingly), DNA extension at 72 ˚C for 45 s. After the last cycle, samples were kept at 72 ˚C for 10 s to complete elongation. Each PCR included a positive control, which included 1 µL of DNA extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) from a pure culture of *E. coli* (prepared as described in Section 4.2.1.1). Negative controls were also included: a negative control of the whole protocol developed (1 µL), and the negative control of the amplification (NTC) where the 1 µL of template DNA was replaced by 1 µL of ultra-pure deionised water. After amplification, amplicons were detected by electrophoresis (Section 4.2.6).

4.2.5 – rtPCR amplification to detect *E. coli* in water samples

The amplification by rtPCR was performed in a CFX-96 thermal cycler (Bio-Rad, USA), using a final volume of 20 µL containing 1 µL of template DNA, 10 µL of SsoFast™ EvaGreen® Supermix (Bio-Rad, USA), 0.5 µM of each primer and ultra-pure deionized water to complete the final volume of 20 µL.

DNA was initially denatured at 98 °C for 2 min. Then a total of 40 cycles were run under the following conditions: denaturation at 98 °C for 5 s, primer annealing at the annealing temperature for 5 s, DNA extension at 72 °C for 5 s. After the last cycle, a high resolution melting was performed between 72 °C and 95 °C, with an increment of 1 °C (adapted from the manufacturer recommendations: Sso Fast™ EvaGreen®; Bio-Rad, USA). The signal was acquired using the SYBR/FAM channel. Each amplification included a positive control with 1 µL of DNA extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) from a pure culture of *E. coli* (prepared as described in Section 4.2.1.1). A negative control of the whole protocol developed (1 µL) was also included, as well as the negative control of the amplification (NTC) where the 1 µL of template DNA was replaced by 1 µL of ultra-pure deionised water.

4.2.5.1 – TaqMan CRENAME-rtPCR to detect *E. coli* and *B. atrophaeus* subsp. *globigii* in water samples

4.2.5.1.1 – *E. coli* and *B. atrophaeus* subsp. *globigii* pure DNA dilutions

In order to acquire the rtPCR standards, amplification was performed using pure DNA dilutions for both *E. coli* and *B. atrophaeus* subsp. *globigii*, from stock DNA of the Collection of Centre de Recherche en Infectiologie, as described by Maheux and coworkers [1]. Stock DNA of *B. atrophaeus* subsp. *globigii* was diluted from a concentration of 7 ng/µL to 10 000, 5 000, 1 000, 500, 100, 50, 10 and 5 genome copies (gc)/µL in 1 x TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). *E. coli* DNA was diluted from a stock concentration of 17 ng/µL to 24 300, 12 150, 2 430, 1 215, 243, 122, 24 and 12 gc/µL in TE buffer (. 1 µL of each dilution was added to the reaction tube and samples were amplified by rtPCR, as described in Sections 4.2.5.1.2 and 4.2.5.1.3.

4.2.5.1.2 – Detection of *B. atrophaeus* subsp. *globigii* by rtPCR

1 µL of the DNA dilutions (prepared as described in Section 4.2.5.1.1) or of the templates (prepared as described in Section 4.2.2) were added to a 200 µL reaction tube (BD, Canada) containing a 24 µL mixture of 7.5 µL PCR premix buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100), 13.8 µL of PCR water, 1 µL of each primer (0.4 µM), 0.5 µL of the TaqMan probe (0.2 µM; Biosearch Technologies, Inc., Canada) and 0.19 µL of *Taq* DNA polymerase (Promega, USA). The positive amplification control was prepared by pipetting 1 µL of the 100 gc/µL *B. atrophaeus* subsp. *globigii* DNA dilution to the reaction tube. Three rtPCR negative controls (NTC) were prepared by adding to the reaction tube 1 µL of PCR water: NTC 1 was prepared after making the master mix and before leaving the PCR reagent preparation room; in NTC 2 the PCR water was added to the tube when entering the sample preparation room and for NTC 3 the PCR water was added after preparing all the reaction tubes and before leaving the sample preparation room. Thermal cycling was performed in a molecular amplification room using a Rotor-Gene 3000 (QIAGEN, Canada) with the following conditions: 3 min at 95 °C, 45 cycles of 15 s at 95 °C and 60 s at 60 °C, acquiring the fluorescent signal in the green channel [1, 5].

4.2.5.1.3 – Detection of *E. coli* by rtPCR

Following the instructions of Maheux and coworkers [1, 5], 1 µL of the DNA dilutions (prepared as described in Section 4.2.5.1.1) or of the templates (prepared as described in Section 4.2.2) were added to a tube containing a 24 µL mixture of 1.25 µL of bovine serum albumin (BSA, 3.30 mg/mL), 2.5 µL of 25 mM $MgCl_2$, 2.5 µL of 10x Taq PCR buffer (Promega), 1.25 µL of dNTPs (0.2 mM), 0.6 µL of 8-methoxypsoralen (0.06 µg/µL), 0.19 µL of *Taq* DNA polymerase (Promega), 13.2 µL of PCR water, 1 µL of each primer (0.4 µM) and 0.5 µL of the TaqMan probe (0.2 µM). Molecular decontamination of the rtPCR master mix was achieved by placing the tube with the master mix in a Spectrolinker^{TM} model XL-1000 UV crosslinker (Spectronics

Corporation) [47] with the purpose of reducing the occurrence of false-positives since *E. coli* is the host of choice for the production of several enzymes and the PCR reagents are frequently contaminated. The positive amplification control was prepared by pipetting 1 µL of the 100 gc/µL DNA dilution to the reaction tube. Three rtPCR negative controls (NTC) were prepared by adding to the reaction tube 1 µL of PCR water: NTC 1 was prepared after making the master mix and before leaving the PCR reagent preparation room; in NTC 2 the PCR water was added to the tube when entering the sample preparation room and for NTC 3 the PCR water was added after preparing all the reaction tubes and before leaving the sample preparation room. Thermal cycling was performed in a molecular amplification room using a Rotor-Gene 6000 (QIAGEN, Canada) with the following conditions: 3 min at 95 °C, 45 cycles of 2 s at 95 °C, 10 s at 58 °C and 20 s at 72 °C, acquiring the fluorescent signal in the yellow channel.

4.2.6 – Electrophoresis

An aliquot (10 µL) of the amplification product was loaded in a 1% agarose gel containing ethidium bromide (Frilabo, Portugal) and run by electrophoresis in 1 x TAE buffer for 1 h at 100 V. The bands corresponding to the amplification were visualized and photographed on a UV transilluminator (ChemiDoc, Bio-Rad). A 100 bp DNA ladder (Fermentas, Canada) was loaded on each gel as a DNA size standard.

4.2.7 – Detection of *E. coli* using environmental water samples

To evaluate the adaptation of the protocol developed to the water quality monitoring, environmental water samples were collected from the Este river (Braga,

Portugal), taking into consideration the standardized guidelines EN 25667-2 (ISO 5667-2:1991) on sampling techniques, as described in Chapter 3, Section 3.2.2. Water samples from the Este river were chosen for being naturally contaminated with total coliforms, *E. coli* and several other unknown microorganisms. The water samples were then filtered to concentrate the microorganisms (according to Section 4.2.1.1), submitted to the heat shock (according to Section 4.2.1.4) to extract the nucleic acids and amplified by standard PCR (as described in Section 4.2.4). The products of the amplification were loaded in an agarose gel (electrophoresis, Section 4.2.6).

Nevertheless, since the concentration of the microorganisms in the Este river fluctuates according to the weather conditions, a second group of Este river water samples (50 mL) were artificially spiked with 5 colonies from a pure culture of *E. coli* (prepared according to the Section 4.2.1.1) in order to perform a direct detection of *E. coli* in highly contaminated environmental samples. 1 µL of the artificially inoculated samples was added directly to the 200 µL reaction tube (Frilabo, Portugal) and were amplified by standard PCR, as described in Section 4.2.4. The products of the amplification were loaded in an agarose gel (electrophoresis, Section 4.2.6).

The number of CFU of *E. coli* and total coliforms was determined by filtering 100 mL of dilutions of the water samples and placing the membranes filters (47 mm diameter, 0.22 µm pore size; Advactec, Japan) in ChromoCult® coliform agar plates (Merck Millipore, Germany), according to the procedure described for environmental samples in Chapter 3, Section 3.2.3.

4.3 | RESULTS AND DISCUSSION

PCR-based methods are well established as a technique capable of efficiently identify microorganisms in samples from various sources, achieving a sensitivity of 1 CFU per tube when pre-enrichment of the microorganisms in the sample is part of the procedure [22, 30-33]. In this work, however, the pre-enrichment step was removed from the water samples preparation as the purpose was to detect *E. coli* in a reduced time, preferably, faster than the culture-based methods (i.e. in less than 18 h). As a result, developing a sample preparation protocol without a pre-enrichment step became the main challenge of the molecular detection of *E. coli*, since it was necessary to concentrate all the cells of the microorganisms found in a sample of water (usually, with a volume of 100 mL) to a volume of only few microliters to be used in the molecular detection and, thus, to pursue the 1 CFU/100 mL sensitivity.

Therefore, the research of this work was focused on sample preparation, as we aimed for the absence of pre-enrichment prior amplification, in order to obtain a simple and highly sensitive (1 CFU/100 mL) procedure, presenting fast results.

4.3.1 – Development of a sample preparation procedure to concentrate and recover *E. coli* from water samples

Numerous attempts were performed in the pursuit for a sample preparation method capable of providing the maximum concentration of the indicator microorganism and extraction of the nucleic acids, while avoiding cells losses during the handling of the samples, and inhibition by the reagents used. The main

techniques that resulted in the more positive and negative impact in the detection of *E. coli* are here discussed.

4.3.1.1 – Membrane filtration of the water samples to concentrate the microorganisms

Membrane filtration was a necessary step to concentrate the microorganisms since the standard volume legislated for water sampling is of 100 mL and 250 mL [12-14], and the PCR-based technologies use template volumes of only 1 or 2 µL [25].

The first membrane filtration assays were performed using a standard filtration system and the regular size of membrane (∅ 47 mm) normally used in culture-based methods and in the analytical reference methods recommended by the legislation to assess the microbiological quality of water [12-14, 28, 48, 49]. The recovery of the target cells from the filter was based in the method of Wolffs *et al*. (2006), which obtained 103 ± 7 % of recovery of *Salmonella* cells by vortexing the filter in a 15 mL tube with 1 mL of PBS for 15 s. However, a final volume of 1 mL is still 1000-fold the volume used in a PCR assay, but the necessary volume to wash all the filter during the vortexing step. Therefore, in order to reduce the final volume of the cells suspension, the filtration system was altered to a syringe-based system, which allowed to use a membrane filter of considerable reduced diameter (∅ 13 mm) that was placed inside a filter holder, and the water sample was forced through the filter holder using a syringe. These modifications allowed that, after filtration, the membrane could be transferred to a 2 mL tube, and a reduced volume of PBS (150 µL) was used to wash and recover the *E. coli* cells from the filter, resulting in an increased final concentration of cells. In another experiment, it was observed that the membrane filter should be removed from the 2 mL tube after the vortexing step, which resulted in more intense bands in the agarose gel. A possible explanation for this difference observed is that keeping the membrane in the tube during the other steps of the sample preparation could contribute to the release of the inhibitory

chemicals from the membrane to the suspension of cells, which are known to be a source of inhibition [36]. This method allowed to concentrate the target microorganisms from water samples of 100 mL to a final volume of 150 µL.

4.3.1.2 – Extraction of the nucleic acids using heat shock

The heat shock step was tested for its known negative effect in the cellular membrane of bacteria and subsequent cell lysis [42, 44]. In order to induce a higher release of the genetic material to the solution, the heat shock effect was enhanced by using a 6-cycles system, which involved placing the tubes at 95 °C for 3 min, and then on ice for another 3 min. Through comparison of the obtained agarose gels (Figure 4.4), it was evident that the bands from the serial dilution treated with thermal shock (B) were more visible and intense than those observed in the dilutions A without the heat shock effect. Thus, the heat shock considerably improved the release of genetic material to the solution. At this point, the limit of detection was approximately (6.76 ± 1.8) x 10^5 CFU/100 mL and, thus, further improvement of the method was necessary. Nevertheless, heat shock was a fast and simple step to improve the release of genetic material and, for that reason, was included in the final protocol.

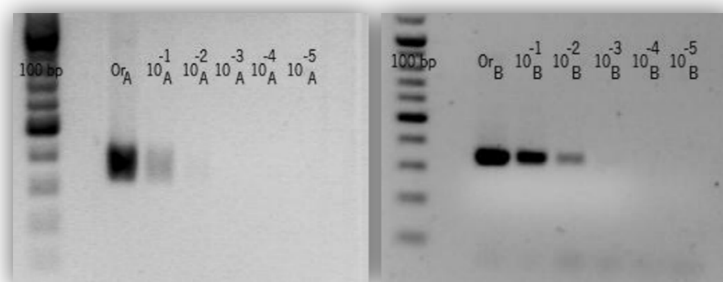


Figure 4.4 – Agarose gel with the products of amplification by PCR, using the primers targeting the *lamB* gene (309 bp), of the samples without the heat shock effect (left) and with

the heat shock effect (right). The samples are ten-fold serially diluted, starting from the *Or* tubes, which had approximately (6.76 ± 1.8) x 10^7 CFU of *E. coli* in 100 mL.

4.3.1.3 – Extraction of the nucleic acids using cell lysis agents

Additional attempts to increase the extraction of nucleic acids into the extracellular environment were carried out by applying cell lysis agents of chemical and biochemical nature (lysozyme, Triton X-100 and SDS) [37, 39]. Results showed that lysozyme has not enabled a positive effect in this test (Figure 4.5), possibly causing inhibition of the amplification during the PCR [50, 51]. Clark and coworkers [39] reported a method for cell recovery where lysozyme and proteinase K were used with 73.3 % ± 5.2 % of recovery. Therefore, it was attempted to include this procedure in our protocol. However, similarly to our previous results, this assay also resulted in inhibition of the PCR amplification, which was also observed in other works [41, 51]. Therefore, it was not included in the final protocol.

The detergents SDS and Triton X-100 were known to be used for cell disruption and DNA extraction [39, 52]. Results in Figure 4.5 show a positive influence in the detection, indicating an increase in the amount of DNA released, without inhibition of the amplification process. Comparing the results for both SDS and Triton X-100, it was possible to observe that the bands for the SDS (0.05 g/L) amplification were visibly more intense, which was related to a more efficient release of nucleic acids and is in accordance to previous studies [39]. Moreover, this is a procedure easy to perform with a major improvement in the genetic material release and microorganism detection. Therefore, the detergent SDS was included in the protocol, at a concentration of 0.05 g/L, being part of the PBS solution used to recover the cells from the membrane.

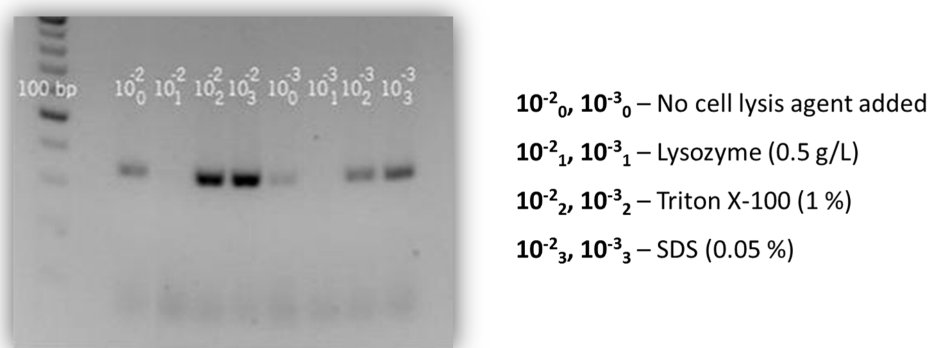


Figure 4.5 – Agarose gel with the products of amplification by PCR, using the primers targeting the *lamB* gene (309 bp), comparing the effect of three cell lysis agents: lysozyme (0.5 g/L), 1 % Triton X-100, SDS (0.05 g/L). CFU count: (6.76 ± 1.8) x 10^5 CFU of *E. coli* in the dilution 10^{-2} and (6.76 ± 1.8) x 10^4 CFU of *E. coli* in the dilution 10^{-3}, per 100 mL.

4.3.1.4 – DNA recovery by precipitation of the nucleic acids

At this point, a standard DNA precipitation assay was performed with the purpose to increase the concentration of the nucleic acids in a reduced final volume of sample, since PCR-based methods usually require as template for amplification a volume of 1 µL [18]. This assay comprised two serial dilutions in parallel comparing the procedure with and without precipitation of the nucleic acids. In this assay, it was possible to observe that the DNA precipitation enhanced slightly the intensity of the bands, however, no difference in the limit of detection was observed. Since this is a long and hard-working procedure that did not produce a significant advantage, it was not included in the final protocol to detect *E. coli*. However, this assay was performed using distilled water artificially inoculated with cells from a pure culture of *E. coli* and, thus, an insignificant amount of contaminants are present in this sort of water samples. Isolation of bacterial DNA from environmental samples, though, is more

complex since the water samples may contain large amounts of substances capable of interfering with PCR amplification [53, 54]. Therefore, further research in developing a method of DNA precipitation and purification is advised to reduce the compounds that inhibit the PCR detection.

4.3.1.5 – Mechanical lysis of the cells using glass beads

Glass beads are frequently used as a mechanical disruption method [5, 45]. When glass beads are added to the tube containing the microorganisms and the tube is shaken (e.g. vortex), collisions occur between the beads and the cells, resulting in the cell lysis [55, 56]. In an attempt to increase the lysis of the *E. coli* cells, glass beads were added to each tube before vortexing the samples. As expected, this step clearly increased the release of nucleic acids to the solution and, as a consequence, the limit of detection, which is in accordance to the literature as this is an established technique of lysis [38, 44]. Moreover, some studies have shown that glass beads can be used as a support for the immobilization of DNA, which can contribute to an increased stability of the nucleic acids in the tube [57, 58]. Since it was a fast and straightforward procedure to perform, it was included in the final protocol.

4.3.1.6 – Adaptability of the Phusion Blood Direct PCR Kit (Thermo Scientific) to the direct PCR detection of *E. coli*

The Phusion Blood Direct PCR kit (Thermo Scientific, USA) captured our interest since this kit was designed for the amplification of blood DNA directly from the membrane filter: blood is added to the filter, a portion of the filter is put in the reaction tube, the PCR reagents are added to the tube and the amplification is performed, without any prior DNA extraction or purification [59, 60]. Moreover, the manufacturer claimed that the modified Phusion Hot Start II High-Fidelity DNA polymerase (part of the Phusion blood direct PCR kit, Thermo Scientific, USA) is resistant to the PCR inhibitors present in blood [60]. Therefore, the easiness of the method and the alleged high resistance to inhibitors were important advantages that

served as motivation for developing an assay to test its capability of detecting *E. coli* in water samples. Results showed that the DNA polymerase efficiently amplified DNA from *E. coli*, but it was strongly inhibited whenever the membrane filter was included in the reaction tube, not allowing to detect (1.7 ± 0.9) x 10^8 CFU of *E. coli*/100 mL that were concentrated in the membrane filter. Previous studies showed that the chemicals found in membrane filters can strongly inhibit the PCR amplification [36, 61]. Considering that this kit was developed for samples of high DNA concentration, the strong inhibition observed, and the limit of detection required for water quality monitoring, it was decided to reject this procedure from our protocol.

4.3.1.7 – Other assays performed to concentrate and recover *E. coli*

Further changes in the protocol were attempted but not included in the final procedure due to the unsatisfying results. Washing the membrane filters by reversing the flow in the filter holder increased the loss of the target cells in the dead volume inside the filter holder. Furthermore, the excessive handling of the membrane increases the possibility of contamination of the samples.

Evaporating part of the PBS volume in the tube to reduce the final volume of the sample (by keeping the tubes in the bath at 95 °C) had the purpose to increase the concentration of *E. coli* DNA in the sample. This test resulted in a reduced intensity of the bands, which could be caused by an increased inhibition of the amplification by the salts found in the PBS-SDS solution, along with other contaminants (e.g. chemicals from the membrane filter) that were also concentrated [53, 54]. Moreover, the evaporation rates between tubes was not equal (i.e. in some samples evaporated a larger volume than in other samples) which does not allow to easily standardize the method or the quantification of the microorganisms. Finally, the need for having the tubes opened during the procedure in order to allow the vapour to be released was perceived as an increment in the possibility of contamination of the samples. Hence, this step was not included in the final protocol.

4.3.1.8 – Final sample preparation protocol to detect *E. coli* in water samples

Considering the results obtained, it was achieved the final protocol presented in Figure 4.6.

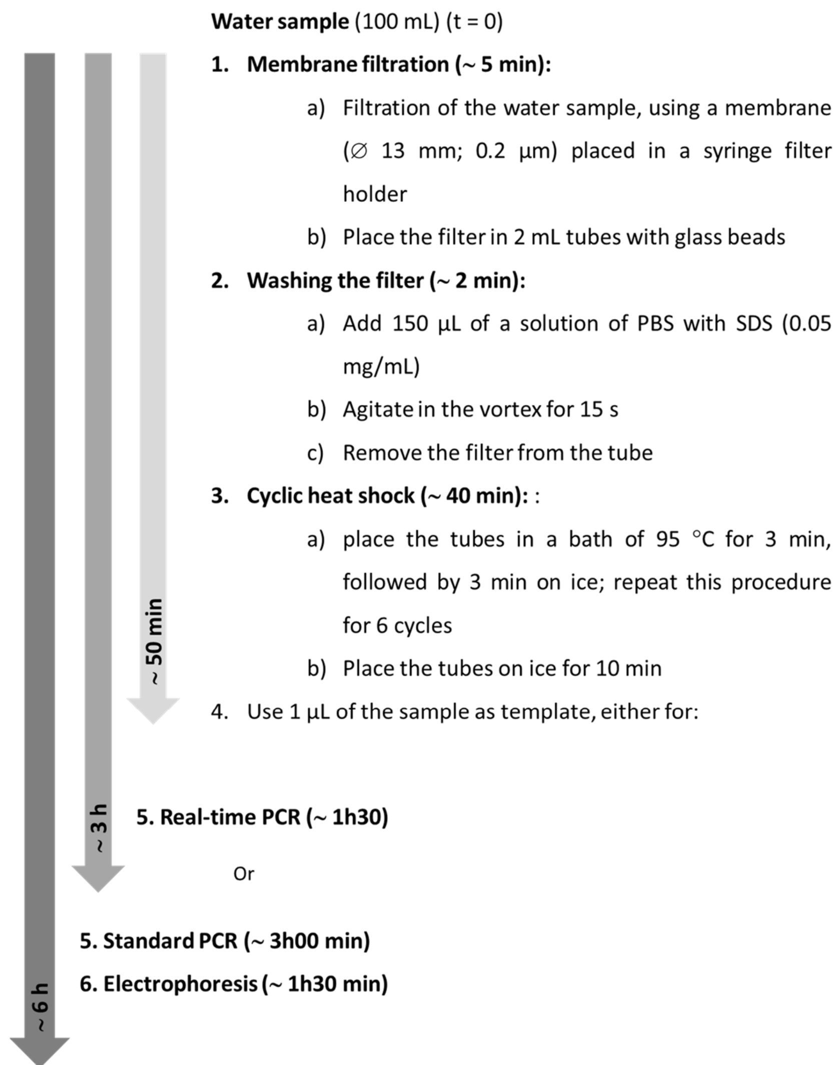


Figure 4.6 – Comparative performance of the detection of *E. coli* using the sample preparation protocol developed and amplification by standard PCR or rtPCR.

4.3.2 – Thermal cycling amplification to detect the indicator microorganisms

With the sample preparation method developed, it was possible to detect approximately 10^4 CFU of *E. coli*/100 mL by standard PCR (using the primers uidAF/uidAR, BL-4910/BR-5219 and ZL-1675/ZR-2548). This was a promising result taking into consideration the simplicity of the protocol, which is an advantage over more complex methods when commercialization is in perspective, and the ~ 6 h to provide the results which is a significant over culture-based methods [2-4]. A multiplex-PCR including these three pairs of primers was also possible by using an annealing temperature of 53 °C, which is an important improvement as it allows to detect both total coliforms and *E. coli* in the same reaction tube, and to confirm the presence of *E. coli* by having two primer pairs targeting this microorganism. However, sensitivity tests should be performed to the multiplex-PCR in order to evaluate its efficiency with lower concentrations of the target bacteria.

The sample preparation method developed for the detection of total coliforms and *E. coli* was also tested by using rtPCR amplification, and results were compared with the standard PCR detection. The adaptability to the rtPCR technology and the efficiency of each primer pair were tested by using pure DNA ten-fold diluted and comparing the amplification, melting and standard curves for each pair (Figures 4.7, 4.8, 4.9 and 4.10). By comparing the results of the rtPCR curves for the four primer pairs, it was possible to observe that all the primers successfully amplified their target, even though that the first three (ZL-1675/ZR-2548, uidAF/uidAR, BL-4910/BR-5219) were designed for standard PCR. Since it was used EvaGreen®, a non-specific dye that binds to all double-stranded DNA, a melt-curve analysis was used at the end of the amplification. Melt curves are used to identify different reaction products, including non-specific products, by their corresponding melting temperature peak [25].

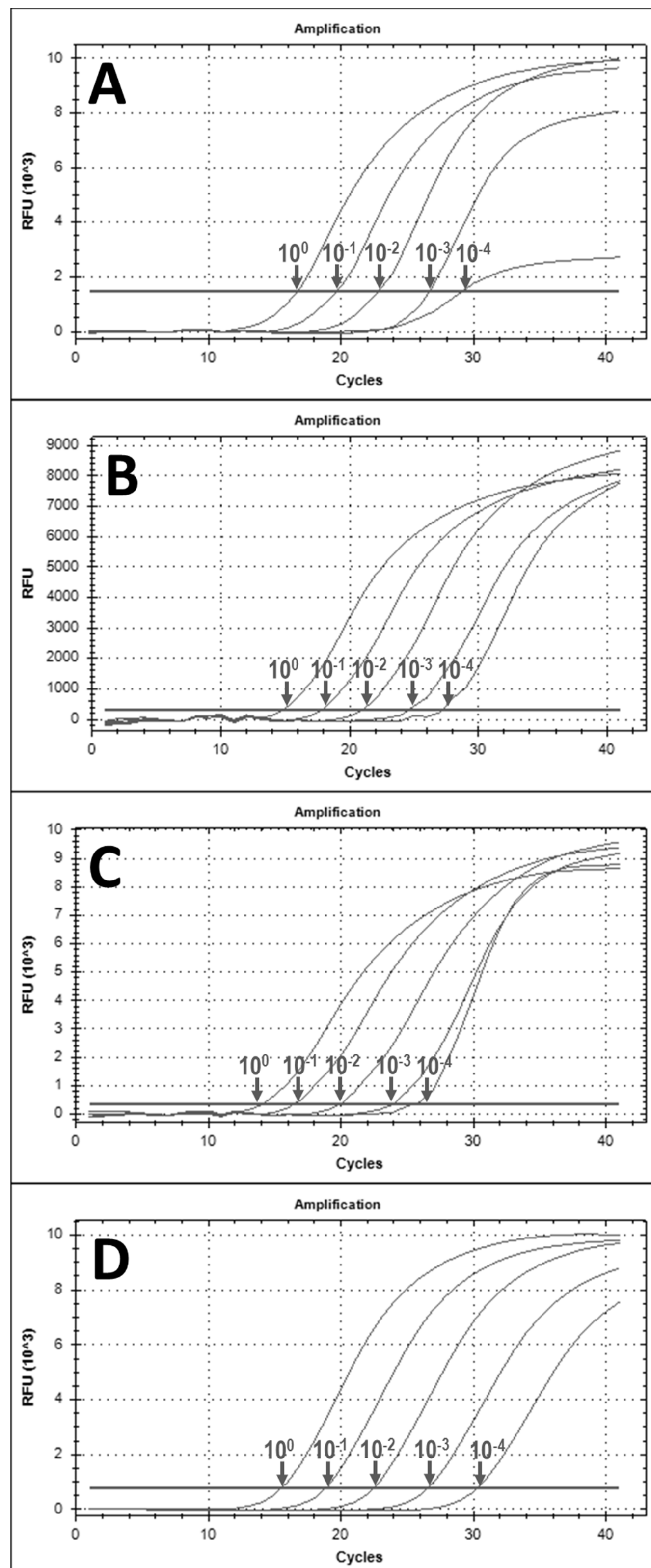


Figure 4.7 – rtPCR amplification curves of ten-fold serially diluted pure *E. coli* DNA using the primer pairs (A) ZL-1675/ZR-2548, (B) uidAF/uidAR, (C) BL-4910/BR-5219 and (C) ECN1254F/ECN1328R.

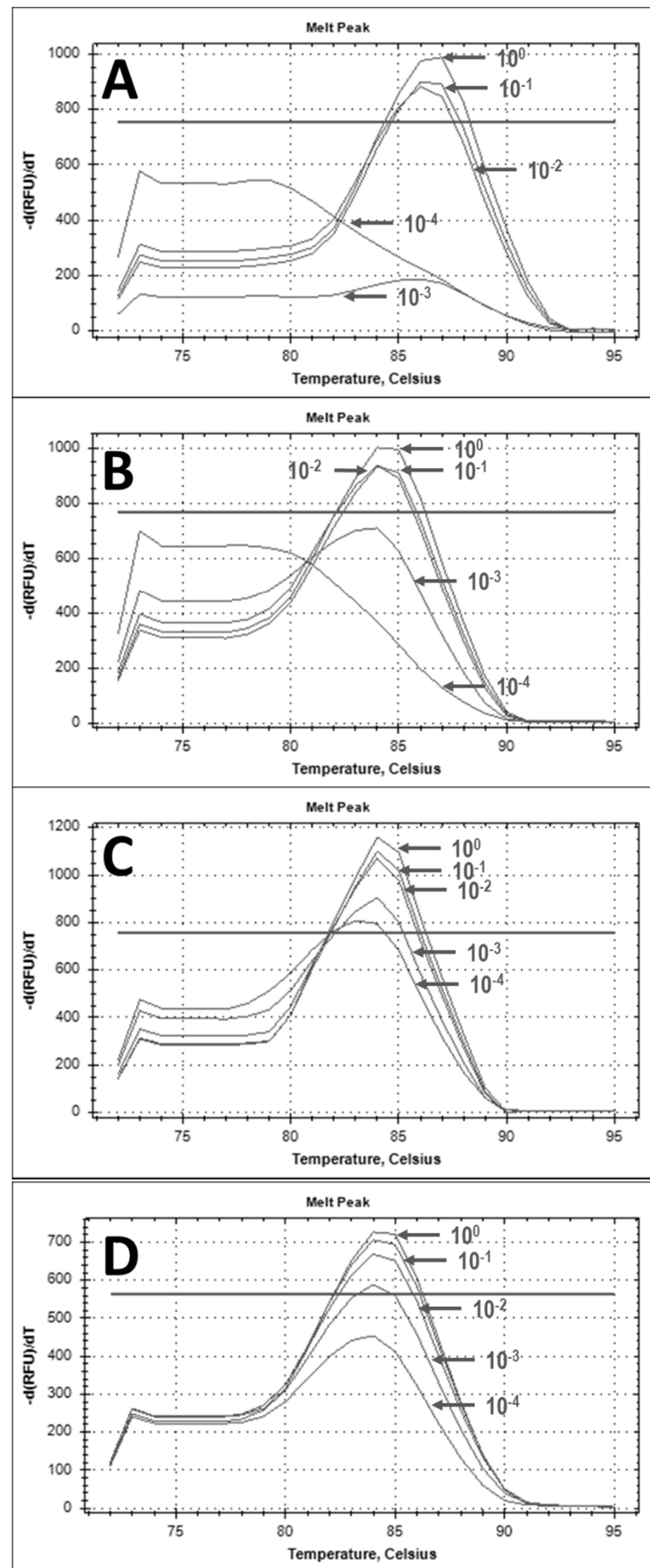


Figure 4.8 – rtPCR melt curves of ten-fold serially diluted pure *E. coli* DNA using the primer pairs (A) ZL-1675/ZR-2548, (B) uidAF/uidAR, (C) BL-4910/BR-5219 and (C) ECN1254F/ECN1328R.

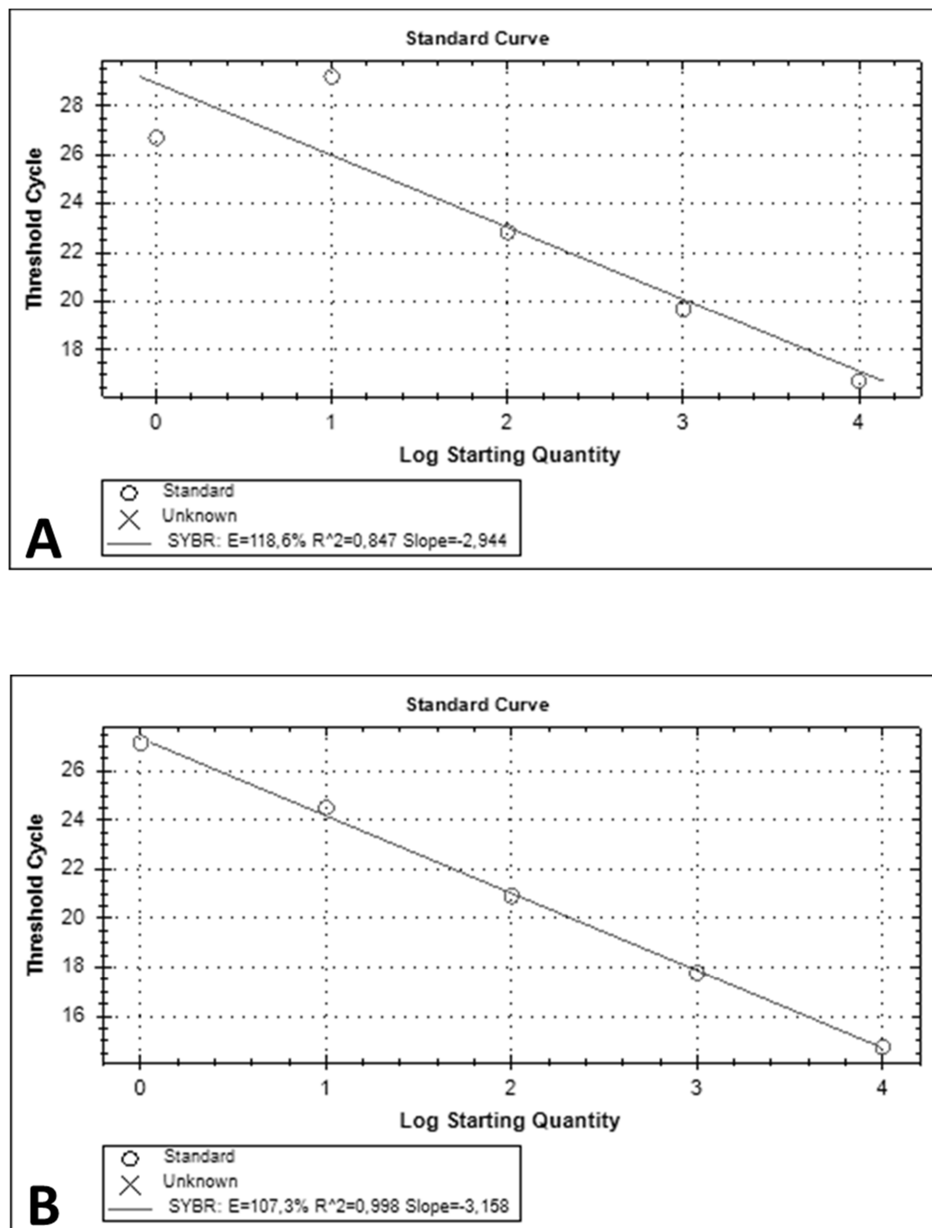


Figure 4.9 – rtPCR standard curves of ten-fold serially diluted pure *E. coli* DNA using the primer pairs (A) ZL-1675/ZR-2548 and (B) uidAF/uidAR.

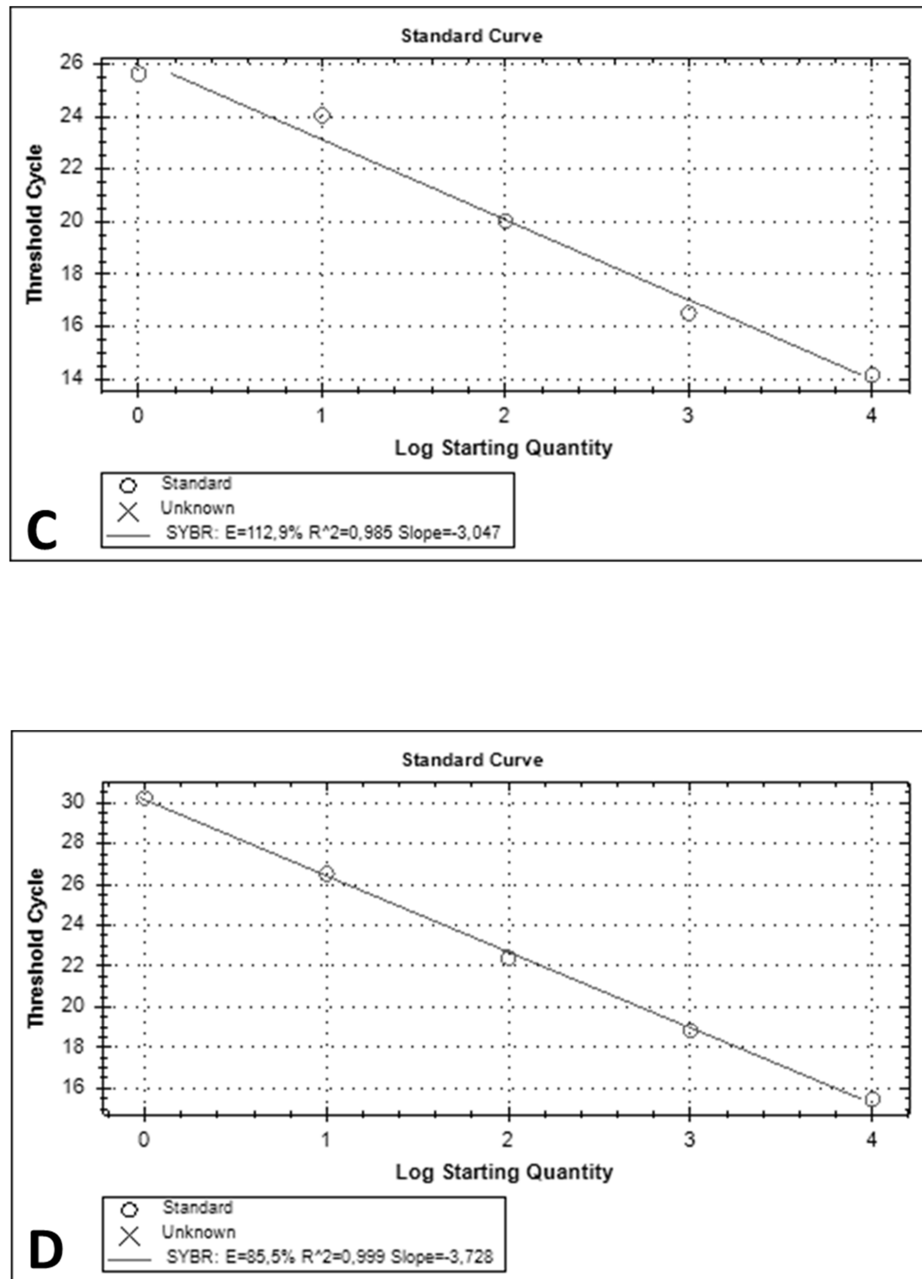


Figure 4.10 – rtPCR standard curves of ten-fold serially diluted pure *E. coli* DNA using the primer pairs (C) BL-4910/BR-5219 and (C) ECN1254F/ECN1328R.

Each amplification product has a characteristic melting temperature that distinguishes it from other amplification products [25]. Although all the samples had the same target DNA, both the amplification curves and the melt curves presented a noticeable difference between the four primer pairs: the three pairs that were not designed for rtPCR revealed significant variation in the curves of the less concentrated dilutions, only the primers ECN1254F/ECN1328r had the same melting temperature in all samples (84 °C). The variability observed in the melt curves with the first three primers can be caused by interference of primers-dimer, i. e. primers that have complementary sequences and bind to each other. As a consequence, primer-dimers form a double stranded sequence that can be amplified by the DNA polymerase, the EvaGreen® dye binds to this by-product and an interference in the fluorescent signal occurs [25].

Even though none of the standard curves had a high amplification efficiency (90% - 105%), the primers ECN1254F/ECN1328r were selected since all the samples showed the same melting temperature of 84 °C, suggesting that no interference from primer-dimers has occurred. This is particularly important since the final purpose of the method developed during this research is to detect *E. coli* in environmental samples that are known to contain other background microorganisms and contaminants [62, 63]. Thus, the melt curve analysis is an essential tool to provide the confirmation that the target bacteria was detected. However, it was not possible to compare these results with those obtained by Takahashi and colleagues (2009) since it was not provided information about the efficiency of the primers [6].

Having the primers selected, it was performed a detection by rtPCR to water samples artificially spiked with *E. coli*. The results in Figure 4.11 show that amplification has occurred in all the samples, including the negative control of the sample preparation procedure and of the amplification (NTC: no template control). Observing the melt curves and the corresponding melting temperatures, there is a difference of 1 °C between the samples. Therefore, only the samples exhibiting a

peak at 84 °C (the same temperature obtained with pure DNA of *E. coli*) were considered as positives, and the samples exhibiting a peak at 83 °C were perceived as a non-specific amplification.

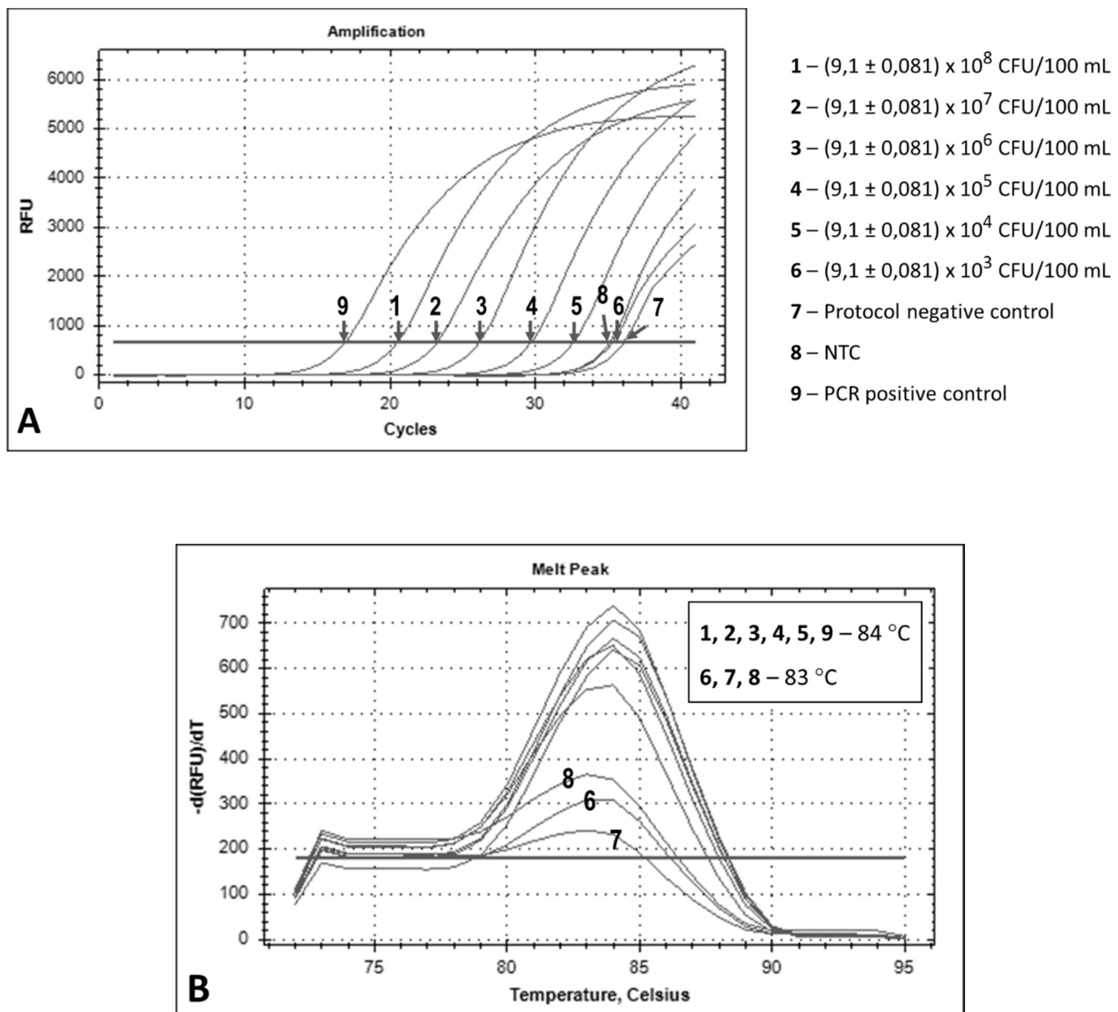


Figure 4.11 – rtPCR (A) amplification and (B) melt curves of ten-fold serially diluted *E. coli* suspensions, using the primers ECN1254F/ECN1328R.

Although pure cultures of *E. coli* were used to artificially inoculate the water samples, a non-specific amplification has occurred, even in the negative controls. Considering that the NTC consists in the reagents used in the master mix and 1 µL of ultra-pure water, the non-specific amplification could have been caused by primer-dimer. Therefore, in order to evaluate the impact of primer-dimer in the detection, tests to the limit of detection using pure DNA at lower concentrations should be performed.

The sensitivity observed using PCR and rtPCR was, in both methods, of 10^4 CFU/100 mL. However, further research is necessary (using duplicates and triplicates) in order to confirm these results. Preferably, optimizing the sample preparation procedure using the rtPCR detection in order to conclude more accurately about the impact of the modifications in the protocol by using the tools that the rtPCR allow (e.g. melt curve analysis). Non-specific binding dyes are more frequently used since no probe-design is necessary and are less expensive than the other options. However, considering that the purpose is to adapt molecular methods to water samples that can contain several contaminants and a complex background of microorganisms [63, 64], the results may become difficult to analyse. Therefore, it is advised to use a probe specific to the target microorganism and, thus, avoid non-specific amplification.

More than 300 PCR amplifications were performed during this thesis with the purpose to amplify and detect *E. coli* DNA concentrated from the bacterial suspensions prepared. However, several results were discarded due to the inhibition and/or contamination observed. This has revealed that this technique is highly variable and sensitive to the modifications in the protocols. Frequent contaminations of the amplification occurred and it became difficult to conclude if an adjustment in the protocol caused an improvement in the detection. It is known that many reagents and enzymes (including DNA polymerase) are produced by *E. coli* and are frequently contaminated with nucleic acids of this microorganism, which was most probably the main cause of the contaminations and false-positives [47]. A highly sensitive and

specific detection method as this, that aims to identify a single cell in a sample of water of 100 mL, was extremely affected by undesired contamination. Therefore, it becomes imperative that laboratories performing this type of research/detection have the necessary equipment available to perform a molecular decontamination of the master mix (before adding the sample) in order to reduce the incidence of contamination and false-positive results [5, 27, 47].

4.3.3 – Assays using the *E. coli* CRENAME-rtPCR method

The CRENAME method has proved to be an efficient method to concentrate and recover the cells of microorganisms from water samples, coupled to a molecular enrichment step and detection by rtPCR [5, 45]. This method claims to detect as few as 1 microbial target per volume of potable water in less than 5 hours [5, 45]. Therefore, this method provided remarkable results that were in accordance to the purpose of this thesis. The main objective of these experiments was to evaluate the adaptability of this method to a new user and compare the implementation of the method in a different laboratory.

4.3.2.1 – Limit of detection of thermal amplification of *B. atrophaeus* subsp. *globigii* and *E. coli*

Highly sensitive detection of nucleic acids should incorporate a non-target nucleic acid capable of being used as an internal control of the amplification [65]. The spores of *B. atrophaeus* subsp. *globigii* are used in this method as the internal control of both the sample preparation and the rtPCR amplification. Therefore, it was necessary to define the limits of detection of both *B. atrophaeus* and *E. coli* using dilutions of purified genomic DNA. Results in Figures 4.12 and 4.13 confirm that it was possible to detect all the dilutions of DNA, either by standard or rtPCR, even at the lowest concentrations of target DNA, 5 gc/µL and 12 gc/µL, correspondingly. Even though some variability in the curves was observed in the more diluted water samples for both standard curves, it was an expected result since DNA concentrations as low as these are more difficult to amplify. It is impossible, based in the Poisson distribution, to guarantee that a single genome copy is placed into a specific reaction tube [66]. Thus, an excellent detection was possible using these primers and probes.

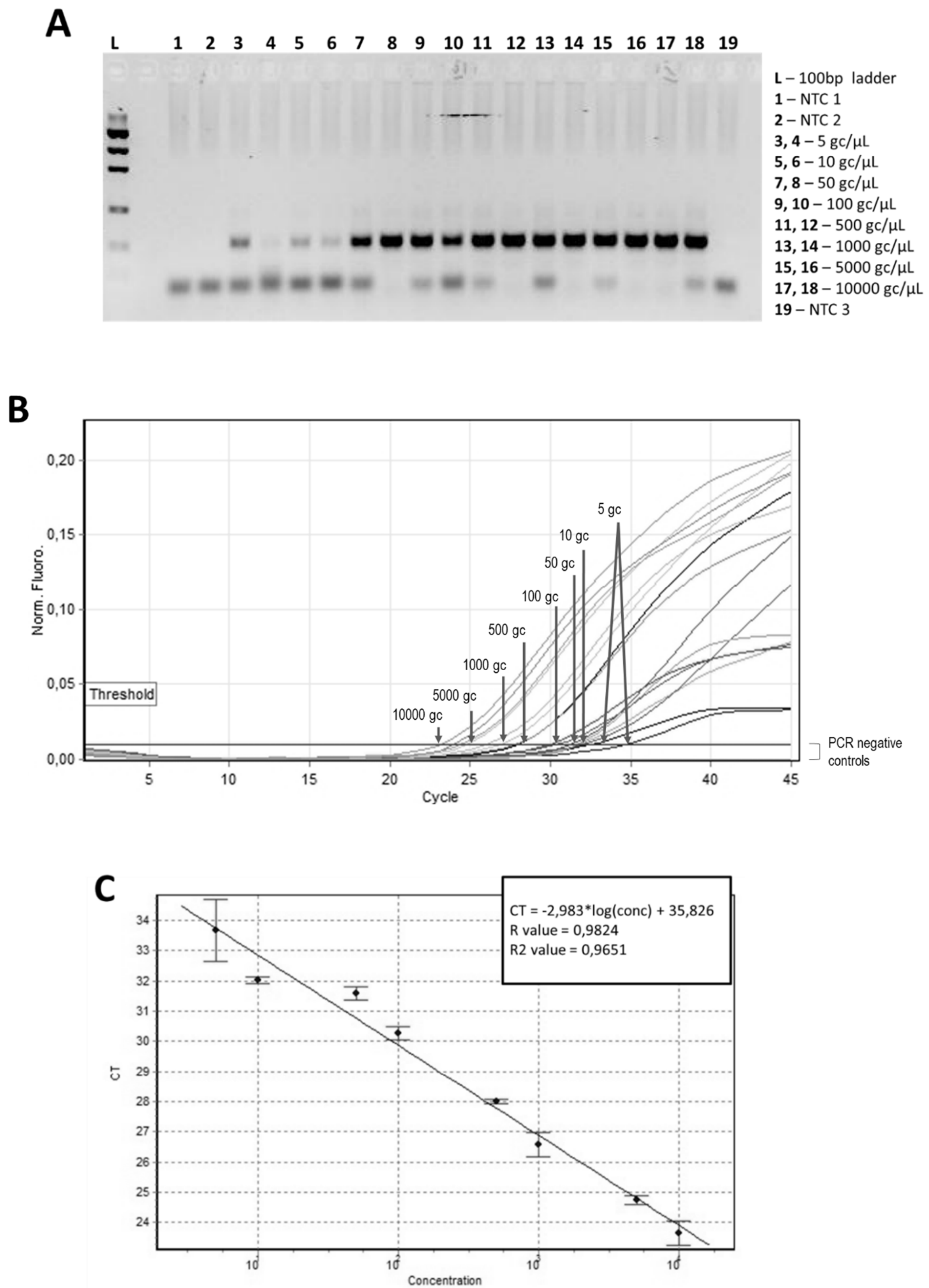


Figure 4.12 – PCR and rtPCR limits of detection of *B. atrophaeus* subsp. *globigii* using the primers Abgl158/Abgl345a and the TaqMan probe Abgl-T1-A1. (A) agarose gel (1.5%), (B) amplification curves and (C) standard curve derived from the amplification in B.

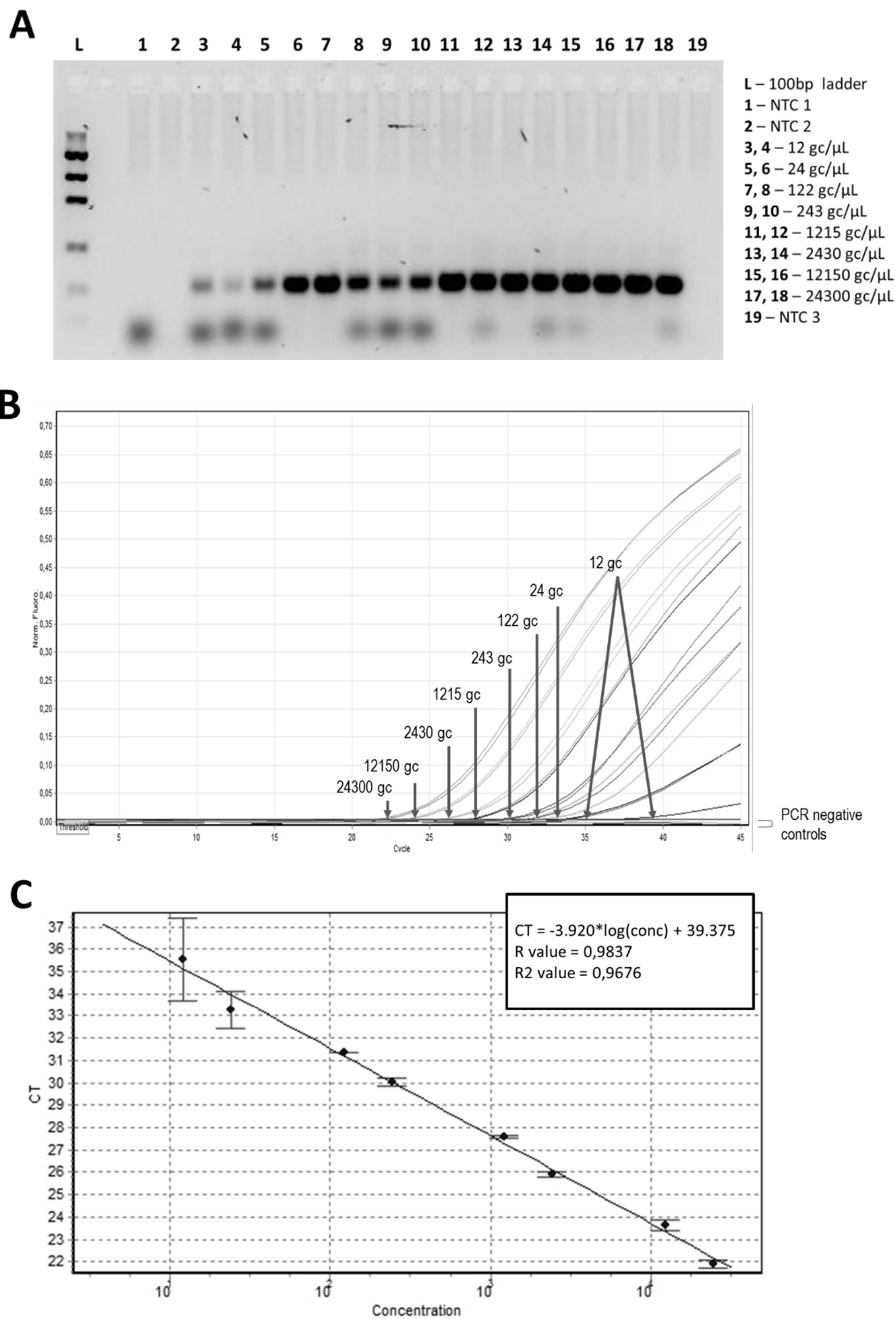


Figure 4.13 – PCR and rtPCR limits of detection of *E. coli* using the primers TEcol553/TEcol754 and the TaqMan probe TEco573-T1-B1. (A) agarose gel (1.5%), (B) amplification curves and (C) standard curve derived from the amplification in B.

4.3.2.2 – *E. coli* CRENAME-rtPCR in water samples artificially inoculated

The *E. coli* CRENAME-rtPCR was tested without the molecular enrichment step since the kit used to perform the whole genome amplification (WGA) is expensive and, at this point, the purpose was to evaluate the adaptability of the method to a new user. Artificially spiked water samples with 100, 50 and 0 CFU/100 mL of *E. coli* and with 50 spores/100 mL of *B. atrophaeus* subsp. *globigii* were tested in triplicate. Results in Figure 4.14 show that, even without the molecular enrichment step, it was possible to detect as low as 50 CFU of *E. coli* in samples of 100 mL, which is a considerable improvement in the limit of detection obtained with the sample preparation method previously developed in this thesis. The concomitant detection of *B. atrophaeus* spores in all samples increases the confidence in this technique as a method to efficiently concentrate and recover cells from a large volume of potable water.

It was also attempted to perform the full protocol of *E. coli* CRENAME-rtPCR, including the WGA step, in two different laboratories. However, no detection has occurred in both assays, except for the PCR positive control (*E. coli* DNA, 100 gc/µL). Considering that the detection of the PCR positive control has occurred, the inhibition of these assays has probably occurred during the WGA step, which is equally sensitive to contaminants. This inhibition was probably caused by the glass beads that, although they had been acid-washed previously, required to be washed for a second time in order to avoid inhibition of the WGA. Further research is necessary to conclude about the reproducibility of the CRENAME-rtPCR method at an interlaboratory scale.

It was shown that the *E. coli* CRENAME-rtPCR method was able to detect as little as 50 CFU/100 mL of potable water samples, without molecular enrichment. In addition, previous studies showed that this method was able to detect 1.8 *E. coli/Shigella* CFU/100 mL, with the WGA step [5].Therefore, this method is a

promising tool for assessing the microbiological quality of water using molecular methods in less than 5 h. However, as in other molecular methods, the success of the detection is fully dependent of an informed and trained user, so that loss of cells during the handling of the samples, inhibitions and contaminations are avoided.

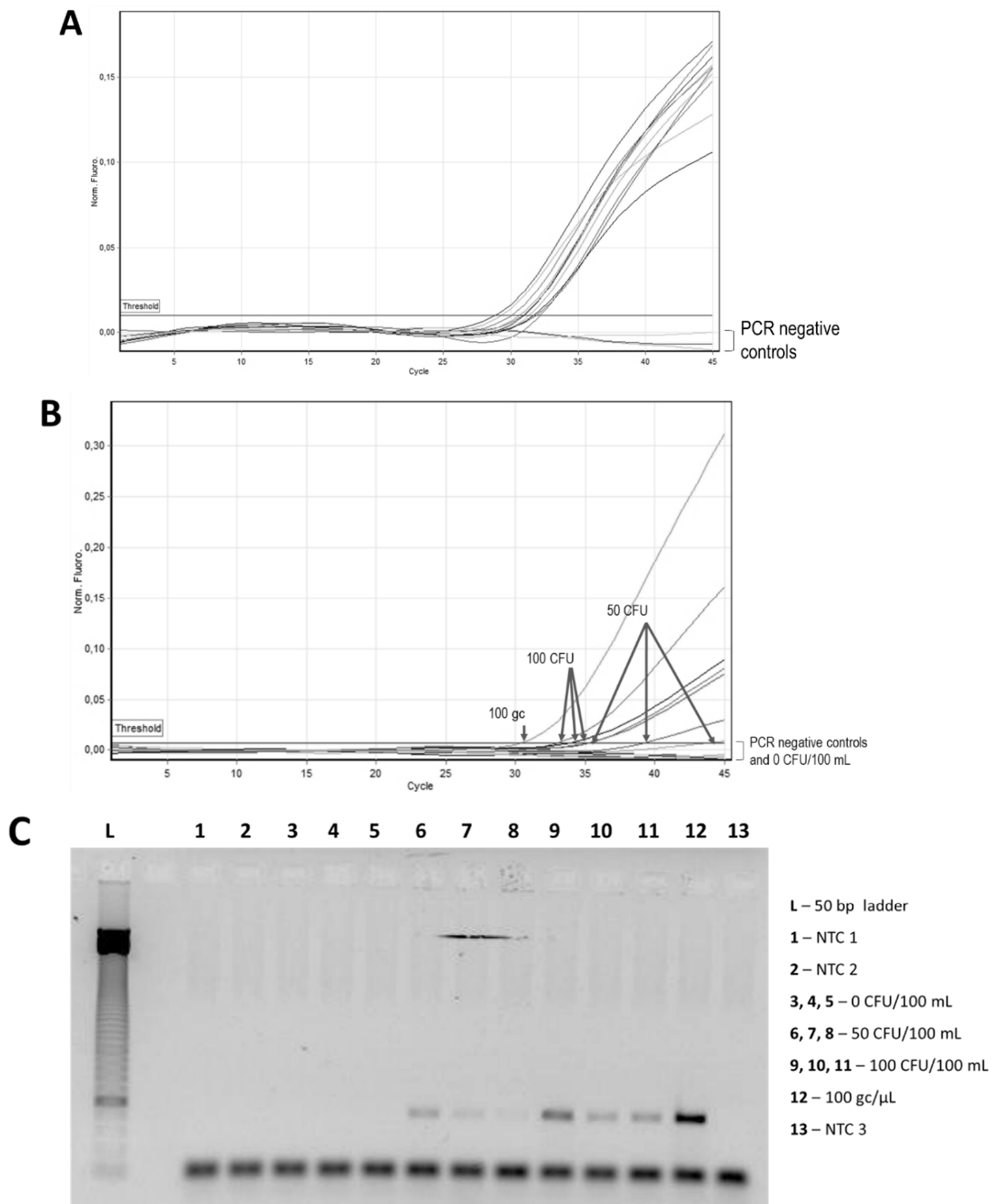


Figure 4.14 – Detection of *E. coli* by CRENAME-rtPCR, without molecular enrichment. (A) amplification curves of the internal process control (*B. atrophaeus* subsp. *globigii*), 56 ±

4.4 spores/ 100 µL and 100 gc/µL; (B) amplification curves of the *E. coli* CRENAME-rtPCR (112 ± 11.9, 57 ± 11 and 0 CFU/100 mL) and the (C) agarose gel with the products of amplification in B (*E. coli*).

4.3.4 – Detection of *E. coli* using the sample preparation protocol in river water samples

Tests were performed using highly polluted river water samples in order to obtain a first insight of the adaptability of the developed protocol to environmental samples. Even though culturable *E. coli* was present in the spiked river water at high concentration (10^5 CFU/100 mL), no detection has occurred by standard PCR. A clear inhibition of the amplification has occurred as no bands were visible in the agarose gels. Environmental samples have been reported in the literature to be extremely inhibitory of the PCR-based methods due to presence of contaminants as humic substances and colloid matter which have high affinity with DNA, which confirms the inhibition observed in this assay [18, 62-64].

4.4 | Conclusions

It is well known that PCR-based technologies have opened a new door to efficiently identify microorganisms because of its high sensitivity, specificity and rapidity, as well as the capability to detect VBNC cells. The particularity of applying these methods to the monitoring of the microbiological safety of water is the need to detect a single cell in a volume of water that is 100 000-fold the standard volume of template used in a PCR assay. Moreover, a pre-enrichment step is avoided since more rapid methods are necessary to detect contamination of a water sample before the disease becomes disseminated.

In this research, a promising protocol for sample preparation without pre-enrichment of the microorganism, using simple techniques, and detecting total coliforms and *E. coli* by both PCR and rtPCR was developed, with a limit of detection of approximately 10^4 CFU/100 mL of water. In addition, the whole procedure could be performed in about 6 h by standard PCR (sample preparation, PCR amplification, and electrophoresis) and in less than 3 h by rtPCR (sample preparation and rtPCR amplification). Even though the sensitivity needs to be improved in a future work, the time to provide the results was significantly reduced when compared to the 18 h to 72 h required by culture-based methods and the analytical reference methods recommended by the legislation to assess the microbiological quality of water. Nevertheless, further research to improve this method should be carried out since the new and fast evolving technology of rtPCR has several advantages over the standard PCR, including the possibility of quantifying the initial concentration of the target and providing faster results in real-time.

Some of the limitations associated to PCR-based techniques were observed in this research: the confidence of this approach is highly dependent on the preparation of adequate positive and negative controls, the presence of thermal cycling inhibitors

in the water samples and reagents may lead to false negative results, false-positives are frequent if no molecular decontamination of reagents used for amplification is performed, estimation of the viability of targeted bacteria is difficult (dead cells are also detected) and the membrane filtration can retain other substances that contaminate the concentrated sample.

4.5 | References

1. Maheux, A., Bissonnette, L., and Bergeron, M., Rapid detection of the *Escherichia coli* genospecies in water by conventional and real-time PCR, in *PCR Detection of Microbial Pathogens*, M. Wilks, Editor. 2013, Humana Press, 289-305.
2. Bej, A.K., Steffan, R.J., DiCesare, J., Haff, L., and Atlas, R.M., Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Applied and Environmental Microbiology, 1990. 56, 307-314.
3. Bej, A.K., McCarty, S.C., and Atlas, R.M., Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: comparison with defined substrate and plating methods for water quality monitoring. Applied and Environmental Microbiology, 1991. 57, 2429-2432.
4. Bej, A.K., DiCesare, J.L., Haff, L., and Atlas, R.M., Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for uid. Applied and Environmental Microbiology, 1991. 57, 1013-1017.
5. Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.L.T., Huppe, V., Picard, F.J., Berube, E., and Bergeron, M.G., Rapid concentration and molecular enrichment approach for sensitive detection of *Escherichia coli* and *Shigella* species in potable water samples. Applied and Environmental Microbiology, 2011. 77, 6199-6207.
6. Takahashi, H., Kimura, B., Tanaka, Y., Shinozaki, J., Suda, T., and Fujii, T., Real-time PCR and enrichment culture for sensitive detection and enumeration of *Escherichia coli*. Journal of Microbiological Methods, 2009. 79, 124-127.
7. Eckner, K.F., Comparison of Membrane Filtration and Multiple-Tube Fermentation by the Colilert and Enterolert Methods for Detection of Waterborne Coliform Bacteria, Escherichia coli, and Enterococci Used in Drinking and Bathing Water Quality Monitoring in Southern Sweden. Appl. Environ. Microbiol., 1998. 64, 3079-3083.
8. Grant, M., A new membrane filtration medium for simultaneous detection and enumeration of Escherichia coli and total coliforms. Appl. Environ. Microbiol., 1997. 63, 3526-3530.
9. Ciebin, B., Brodsky, M., Eddington, R., Horsnell, G., Choney, A., Palmateer, G., Ley, A., Joshi, R., and Shears, G., Comparative evaluation of modified m-FC and m-TEC media for membrane filter enumeration of Escherichia coli in water. Appl. Environ. Microbiol., 1995. 61, 3940-3942.
10. Fiksdal, L., Pommepuy, M., Caprais, M.P., and Midttun, I., Monitoring of fecal pollution in coastal waters by use of rapid enzymatic techniques. Appl. Environ. Microbiol., 1994. 60, 1581-1584.
11. Lei nº 58/2005 de 29 de Dezembro. Diário da República - I Série-A. 249 (29.12.2005) 7280-7310.

12. Decreto-Lei nº 236/98 de 1 de Agosto. Diário da República - I Série-A. 176 (1.8.98) 3676-3722.

13. Decreto-Lei nº 306/2007 de 27 de Agosto. Diário da República - I Série. 164 (27.8.2007) 5747-5765.

14. Decreto-Lei nº 135/2009 de 3 de Junho. Diário da República - I Série. 107 (3.6.2009) 3460-3468.

15. Wildeboer, D., Amirat, L., Price, R.G., and Abuknesha, R.A., Rapid detection of *Escherichia coli* in water using a hand-held fluorescence detector. Water Research, 2010. 44, 2621-2628.

16. Bae, S. and Wuertz, S., Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide. Applied and Environmental Microbiology, 2009. 75, 2940-2944.

17. Liu, Y., Gilchrist, A., Zhang, J., and Li, X.-F., Detection of viable but nonculturable *Escherichia coli* O157:H7 bacteria in drinking water and river water. Applied and Environmental Microbiology, 2008. 74, 1502-1507.

18. Girones, R., Ferrús, M.A., Alonso, J.L., Rodriguez-Manzano, J., Calgua, B., de Abreu Corrêa, A., Hundesa, A., Carratala, A., and Bofill-Mas, S., Molecular detection of pathogens in water – The pros and cons of molecular techniques. Water Research, 2010. 44, 4325-4339.

19. Horakova, K., Mlejnkova, H., and Mlejnek, P., Specific detection of *Escherichia coli* isolated from water samples using polymerase chain reaction targeting four genes: cytochrome bd complex, lactose permease, beta-D-glucuronidase, and beta-D-galactosidase. Journal of Applied Microbiology, 2008. 105, 970-976.

20. Friesema, I., Sigmundsdottir, G., van der Zwaluw, K., Heuvelink, A., Schimmer, B., de Jager, C., Rump, B., Briem, H., Hardardottir, H., Atladottir, A., Gudmundsdottir, E., and van Pelt, W., An international outbreak of Shiga toxin-producing *Escherichia coli* O157 infection due to lettuce, September-October 2007. Euro surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin, 2008. 13, 3029-3035.

21. Rompré, A., Servais, P., Baudart, J., de-Roubin, M.-R., and Laurent, P., Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. Journal of Microbiological Methods, 2002. 49, 31-54.

22. Hoy, M.A., Chapter 8 - DNA amplification by the Polymerase Chain Reaction: molecular biology made accessible, in *Insect Molecular Genetics (Third Edition)*, M.A. Hoy, Editor. 2013, Academic Press: San Diego, 307-372.

23. Fykse, E.M., Nilsen, T., Nielsen, A.D., Tryland, I., Delacroix, S., and Blatny, J.M., Real-time PCR and NASBA for rapid and sensitive detection of *Vibrio cholerae* in ballast water. Marine Pollution Bulletin, 2012. 64, 200-206.

24. Hahn, S. and Lapaire, O., Polymerase Chain Reaction, Real-Time Quantitative, in *Brenner's Encyclopedia of Genetics (Second Edition)*, S. Maloy and K. Hughes, Editors. 2013, Academic Press: San Diego, 396-397.

25. Bio-Rad Laboratories, I. Real-Time PCR Applications Guide. 2006 [cited 2014; Available from: http://www.gene-quantification.de/real-time-pcr-guide-bio-rad.pdf.

26. Baldwin, V., Bhatia, M., and Luckey, M., Folding studies of purified LamB protein, the maltoporin from the *Escherichia coli* outer membrane: Trimer dissociation can be separated from unfolding. Biochimica et Biophysica Acta (BBA) - Biomembranes, 2011. 1808, 2206-2213.

27. Maheux, A.F., Picard, F.J., Boissinot, M., Bissonnette, L., Paradis, S., and Bergeron, M.G., Analytical comparison of nine PCR primer sets designed to detect the presence of *Escherichia coli/Shigella* in water samples. Water Research, 2009. 43, 3019-3028.

28. Maheux, A.F., Bérubé, È., Boudreau, D.K., Cantin, P., Boissinot, M., Bissonnette, L., Rodrigue, L., and Bergeron, M.G., Ability of three DNA-based assays to identify presumptive *Escherichia coli* colonies isolated from water by the culture-based mFC agar method. Water Research, 2011. 45, 2638-2646.

29. Zuo, G., Xu, Z., and Hao, B., *Shigella* strains are not clones of *Escherichia coli* but sister species in the genus *Escherichia*. Genomics, Proteomics & Bioinformatics, 2013. 11, 61-65.

30. Son, I., Binet, R., Maounounen-Laasri, A., Lin, A., Hammack, T.S., and Kase, J.A., Detection of five Shiga toxin-producing *Escherichia coli* genes with multiplex PCR. Food Microbiology, 2014. 40, 31-40.

31. Binet, R., Deer, D.M., and Uhlfelder, S.J., Rapid detection of *Shigella* and enteroinvasive *Escherichia coli* in produce enrichments by a conventional multiplex PCR assay. Food Microbiology, 2014. 40, 48-54.

32. Liu, Y. and Mustapha, A., Detection of viable *Escherichia coli* O157:H7 in ground beef by propidium monoazide real-time PCR. International Journal of Food Microbiology, 2014. 170, 48-54.

33. Pasquali, F., De Cesare, A., Bovo, F., Serraino, A., and Manfreda, G., Relative accuracy, specificity and sensitivity of a 5′ nuclease Real-Time PCR assay for *Salmonella* detection in naturally contaminated pork cuts. Molecular and Cellular Probes, 2014. 28, 133-137.

34. Kuhnert, P., Nicolet, J., and Frey, J., Rapid and accurate identification of *Escherichia coli* K-12 strains. Applied and Environmental Microbiology, 1995. 61, 4135-4139.

35. Lin, S., Liang, R., Meng, X., OuYang, H., Yan, H., Wang, Y., and Jones, G.S., Construction and expression of mutagenesis strain of aroG gene from *Escherichia coli* K-12. International Journal of Biological Macromolecules, 2014. 68, 173-177.

36. Wolffs, P.F.G., Glencross, K., Thibaudeau, R., and Griffiths, M.W., Direct quantitation and detection of *Salmonellae* in biological samples without enrichment, using two-step filtration and real-time PCR. Applied and Environmental Microbiology, 2006. 72, 3896-3900.

37. Mendes, D., Desenvolvimento de um kit de detecção da bactéria *Escherichia coli* e coliformes totais em amostras de água, in Centre of Biological Engineering. 2008, University of Minho. Master degree dissertation.

38. Verollet, R., A major step towards efficient sample preparation with bead-beating. Biotechniques, 2008. 44, 832-833.

39. Clark, S.T., Gilbride, K.A., Mehrvar, M., Laursen, A.E., Bostan, V., Pushchak, R., and McCarthy, L.H., Evaluation of low-copy genetic targets for waterborne bacterial pathogen detection via qPCR. Water Research, 2011. 45, 3378-3388.

40. Zoetendal, E.G., Ben-Amor, K., Akkermans, A.D.L., Abee, T., and de Vos, W.M., DNA isolation protocols affect the detection limit of PCR approaches of bacteria in samples from the human gastrointestinal tract. Systematic and Applied Microbiology, 2001. 24, 405-410.

41. Miller, D.N., Bryant, J.E., Madsen, E.L., and Ghiorse, W.C., Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Applied and Environmental Microbiology, 1999. 65, 4715-4724.

42. Rees, P., Watson, J., Cumming, R.H., Liddell, J., and Turner, P., Effects of heat shock on gram negative bacteria: use of lysis by sodium dodecyl sulphate as a probe for the integrity of DNA. Bioseparation, 1996. 6, 125-132.

43. Tamanai-Shacoori, Z., Jolivet-Gougeon, A., and Cormier, M., Comparison of direct PCR and PCR amplification after DNA extraction for the detection of viable enterotoxigenic Escherichia coli in laboratory microcosms. Journal of Microbiological Methods, 1996. 26, 21-26.

44. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., and Struhl, K., Current protocols in molecular biology. 2003: John Wiley & Sons.

45. Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.-L.T., Huppé, V., Bérubé, È., Boudreau, D.K., Picard, F.J., Huletsky, A., and Bergeron, M.G., Method for rapid and sensitive detection of *Enterococcus* sp. and *Enterococcus faecalis/faecium* cells in potable water samples. Water Research, 2011. 45, 2342-2354.

46. Picard, F.J., Ke, D., Boudreau, D.K., Boissinot, M., Huletsky, A., Richard, D., Ouellette, M., Roy, P.H., and Bergeron, M.G., Use of *tuf* sequences for genus-specific PCR detection and phylogenetic analysis of 28 Streptococcal species. Journal of Clinical Microbiology, 2004. 42, 3686-3695.

47. Picard, F.J., Ménard C, B.M., and Boissinot, M., Method for the preparation of reagents for amplification and/or detection of nucleic acids that exhibit no significant contamination by nucleic acids. 2005.

48. Haugland, R.A., Siefring, S.C., Wymer, L.J., Brenner, K.P., and Dufour, A.P., Comparison of Enterococcus measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Research, 2005. 39, 559-568.

49. Zhou, G., Wen, S., Liu, Y., Li, R., Zhong, X., Feng, L., Wang, L., and Cao, B., Development of a DNA microarray for detection and identification of *Legionella pneumophila* and ten other pathogens in drinking water. International Journal of Food Microbiology, 2011. 145, 293-300.

50. Vandeventer, P.E., Weigel, K.M., Salazar, J., Erwin, B., Irvine, B., Doebler, R., Nadim, A., Cangelosi, G.A., and Niemz, A., Mechanical disruption of lysis-resistant bacterial cells by use of a miniature, low-power, disposable device. Journal of Clinical Microbiology, 2011. 49, 2533-2539.

51. Rossen, L., Norskov, P., Holmstrom, K., and Rasmussen, O.F., Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. International Journal of Food Microbiology, 1992. 17, 37-45.

52. George, S., Sokolenko, S., and Aucoin, M.G., Rapid and cost-effective baculovirus sample preparation method as a viable alternative to conventional preparation for quantitative real-time PCR. Journal of Virological Methods, 2012. 182, 27-36.

53. Horáková, K., Mlejnková, H., and Mlejnek, P., Evaluation of methods for isolation of DNA for polymerase chain reaction (PCR)-based identification of pathogenic bacteria from pure cultures and water samples. Water Science & Technology, 2008, 995-999.

54. Jofre, J. and Blanch, A.R., Feasibility of methods based on nucleic acid amplification techniques to fulfil the requirements for microbiological analysis of water quality. Journal of Applied Microbiology, 2010. 109, 1853-1867.

55. Haugland, R.A., Brinkman, N., and Vesper, S.J., Evaluation of rapid DNA extraction methods for the quantitative detection of fungi using real-time PCR analysis. Journal of Microbiological Methods, 2002. 50, 319-323.

56. Lim, C.S.Y., Tung, C.H., Rosli, R., and Chong, P.P., An alternative *Candida* spp. cell wall disruption method using a basic sorbitol lysis buffer and glass beads. Journal of Microbiological Methods, 2008. 75, 576-578.

57. Yamada, M., Kanamori, Y., and Yamada, T., Immobilization of double-stranded DNA onto glass beads by psolaren. International Journal of Biological Macromolecules, 2013. 60, 39-44.

58. Walsh, M.K., Wang, X., and Weimer, B.C., Optimizing the immobilization of single-stranded DNA onto glass beads. Journal of Biochemical and Biophysical Methods, 2001. 47, 221-231.

59. Fuehrer, H.P., Fally, M.A., Habler, V.E., Starzengruber, P., Swoboda, P., and Noedl, H., Novel nested direct PCR technique for malaria diagnosis using filter paper samples. Journal of Clinical Microbiology, 2011. 49, 1628-1630.

60. Phusion Blood Direct PCR Kit. [cited 2014; Available from: http://www.thermoscientificbio.com/uploadedFiles/Resources/tech-manual-f-547-phusion-blood-direct-pcr-kit.pdf.

61. Oyofo, B.A. and Rollins, D.M., Efficacy of filter types for detecting *Campylobacter jejuni* and *Campylobacter coli* in environmental water samples by polymerase chain reaction. Applied and Environmental Microbiology, 1993. 59, 4090-4095.

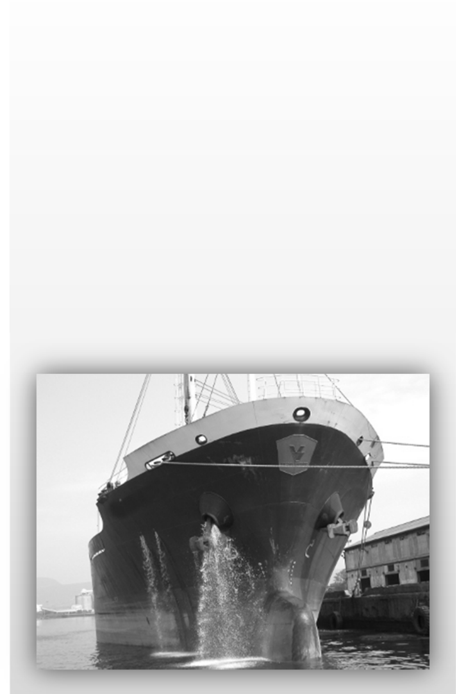
62. Rodríguez, R.A., Thie, L., Gibbons, C.D., and Sobsey, M.D., Reducing the effects of environmental inhibition in quantitative real-time PCR detection of adenovirus and norovirus in recreational seawaters. Journal of Virological Methods, 2012. 181, 43-50.

63. Schriewer, A., Wehlmann, A., and Wuertz, S., Improving qPCR efficiency in environmental samples by selective removal of humic acids with DAX-8. Journal of Microbiological Methods, 2011. 85, 16-21.

64. Green, H.C. and Field, K.G., Sensitive detection of sample interference in environmental qPCR. Water Research, 2012. 46, 3251-3260.

65. Picard, F.J., Gagnon, M., Bernier, M.R., Parham, N.J., Bastien, M., Boissinot, M., Peytavi, R., and Bergeron, M.G., Internal control for nucleic acid testing based on the use of purified *Bacillus atrophaeus* subsp. *globigii* spores. Journal of Clinical Microbiology, 2009. 47, 751-757.

66. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T., The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry, 2009. 55, 611-622.



CHAPTER 5

DEVELOPMENT OF A *VIBRIO* CRENAME-RTPCR METHOD FOR THE DETECTION OF *VIBRIO CHOLERAE* IN BALLAST WATER

This chapter presents the development of a method to detect the three major *Vibrio* pathogens by coupling a sample preparation method to concentrate microbial cells to a multiplex-rtPCR method.

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5.1 | INTRODUCTION

In nearly every aquatic system on earth, a complex microorganism assemblage can be found. Transport of ballast water in ships between harbours is one major source of transmission of aquatic organisms worldwide, including pathogenic bacteria [2-4] as represented in Figure 5.1.

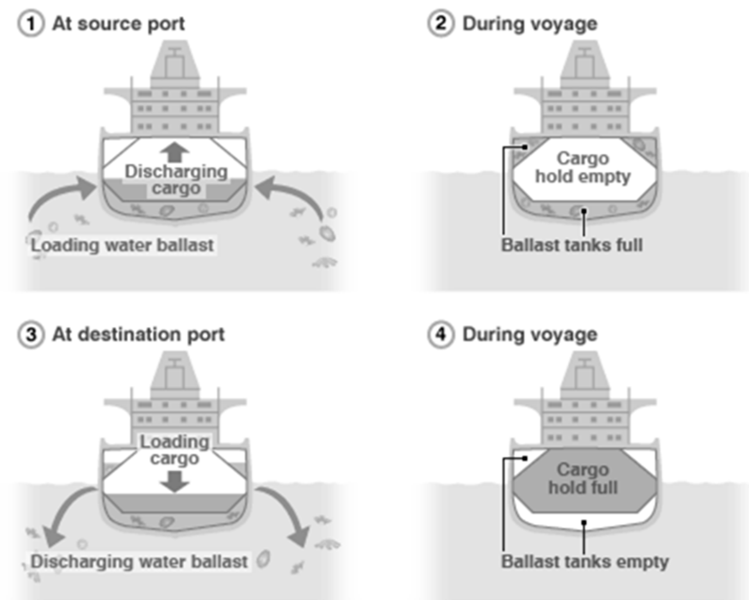


Figure 5.1 – Ballast water cycle: at the source port, while discharging the cargo, water with the local organisms is loaded into the ship to level its weight; at the destination port, while loading the cargo into the ship, water and the organisms are discharged and invade the local aquatic environment (adapted) [5].

Vibrio is a group of Gram-negative bacteria of more than 70 species that are ubiquitous in aquatic environments [6]. *Vibrio cholera*, *Vibrio parahaemolyticus* and

Vibrio vulnificus are the three species that cause the majority of human *Vibrio* infections [8]. *V. cholerae*, in particular the serogroups O1 (El Tor and classical biotypes) and O139, are responsible for cholera, which is an acute intestinal infection caused by the ingestion of contaminated food or water [9, 10]. Every year, millions of people suffer with cholera disease. Cholera outbreaks, such as the recent epidemic on Haiti, are still a common occurrence [11-13]. *V. parahaemolyticus* inhabits marine waters and is one of the leading causes of gastroenteritis by consumption of raw contaminated seafood [6, 7, 14]. *V. vulnificus* is the leading cause of death associated with consumption of raw seafood (e.g. oysters), causes gastroenteritis, wound infections and septicaemia [15, 16].

Therefore, the International Maritime Organization (IMO) established requirements for monitoring the ballast water of ships. Guidelines state that ships shall discharge less than 1 CFU of pathogenic *V. cholerae* (O1 and O139), less than 250 CFU of *E. coli* and less than 100 CFU of *Enterococci* per 100 mL of ballast water [17].

Transport in ballast water is a stressful environment for the organisms. The lack of nutrients, reduced oxygen and temperature changes kills many of the microorganisms [3]. Nevertheless, several still survive and can invade the local aquatic ecosystem. Therefore, *V. cholerae* are likely to be found in ballast water as dead, viable and VBNC cells. Culture-based methods to identify *V. cholerae* are time-consuming and VBNC cells are not detected. Therefore, there is the urgent need for a faster method capable of detecting *V. cholerae* in both viable and VBNC states.

CRENAME is a compact water sample preparation method that enables the recovery and detection of as low as 1 CFU in volumes from 100 to 1000 mL (Figure 5.2) [18, 19].

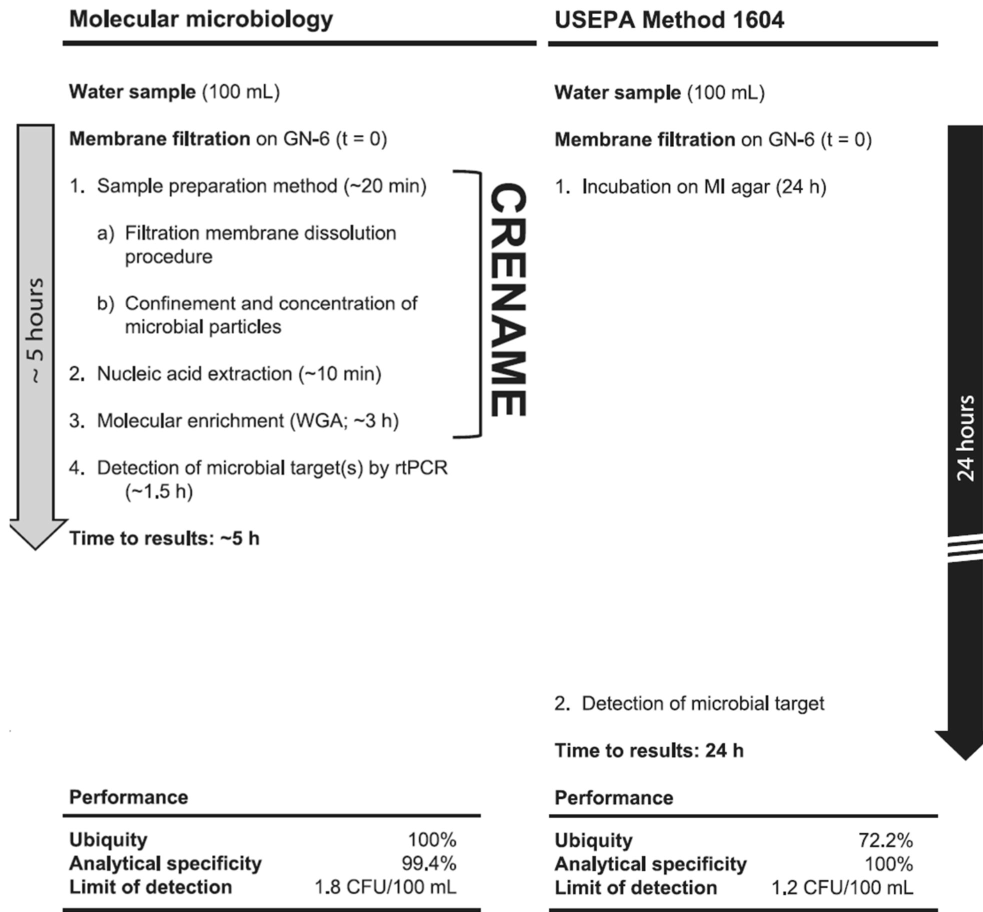


Figure 5.2 – Comparative performance of the USEPA Method 1604 and the *E. coli* CRENAME-rtPCR technology in potable water (adapted) [18].

The main objectives of this work were (i) to develop a multiplex rtPCR assay for the detection of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, which are the three major *Vibrio* human pathogens, and (ii) to develop a *Vibrio* CRENAME-rtPCR method. Such a test could be used to test the ballast water of ships, thereby preventing coastal and harbour marine invasions or pollution. *V. cholerae* was our main target since it is the *Vibrio* sp. that is regulated by the IMO. Nevertheless, the multiplex of the three major human *Vibrio* pathogens was aimed due to their increased importance for public health safety.

5.2 | MATERIALS AND METHODS

5.2.1 – Sample preparation without pre-enrichment: *Vibrio* CRENAME

5.2.1.1 - MICROORGANISMS

Bacterial cultures were obtained from the Collection of Centre de Recherche en Infectiologie (CCRI): *V. cholerae* O1 (CCRI-11019), *V. parahaemolyticus* ATCC 17802 (CCRI-8964) and *V. vulnificus* ATCC 27562 (CCRI-8942).

These *Vibrio* spp. were grown, individually, to logarithmic phase (OD 600, 0.5 - 0.6), in Brain Heart Infusion medium (BHI) (BD, Canada), at a concentration of 0.5 % and 2 % of sodium chloride (NaCl) (Sigma-Aldrich, USA) [7, 20], and the culture was adjusted to a McFarland 0.5 standard [18, 19]. Then, as described in Figure 5.3, the culture was ten-fold serially diluted by pipetting 100 µL of each dilution to a 1.5 mL tube with 900 µL of phosphate saline buffer (PBS: 8 g/L NaCl, 0.2 g/L KCl, 2.68 g/L $Na_2HPO_4.7H_2O$, 0.24 g/L KH_2PO_4) (Sigma-Aldrich, USA). As these bacteria are extremely pathogenic, they were not added directly to the water samples. Instead, 1 µL of the dilutions of the cultures of each bacteria (Figure 5.3) was added to the screw-cap tubes after the filtration and dissolution of the membrane steps, and before the cell lysis and WGA steps (see Section 5.2.1.6). CFU count was obtained by pipetting 100 µL of the dilutions in BHI 2 % NaCl agar, the plates were placed at 35 °C (aerobic incubator) for 24 h. The number of CFU/mL was calculated using Equation 3.1 (as described in Chapter 3, Section 3.2.3).

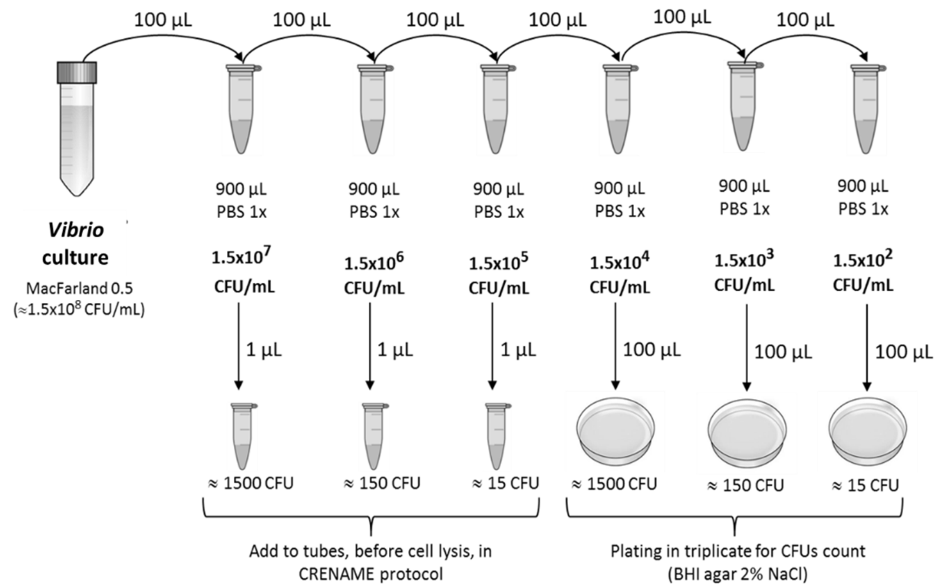


Figure 5.3 – Illustration of the dilutions performed to each culture of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* used in the *Vibrio* CRENAME-rtPCR.

5.2.1.2 – PREPARATION OF ARTIFICIAL SEAWATER

To prepare artificial seawater to be used as the water samples, a mixture of sea salts were ordered from Sigma-Aldrich (USA), containing the following: chloride 19290 mg/L, sodium 10780 mg/L, potassium 420 mg/L, calcium 400 mg/L, carbonate 200 mg/L, strontium 8.8 mg/L, boron 5.6 mg/L, bromide 56 mg/L, iodide 0.24 mg/L, lithium 0.3 mg/L, fluoride 1.0 mg/L, magnesium 1320 mg/L and <0.5 mg/L of other trace elements. According to the manufacturer instructions, a concentration of 40 g of sea salts/L was considered as the corresponding concentration of sea salts in the natural marine environment. Therefore, the following concentrations of artificial seawater were tested: 100 % artificial seawater (40 g sea salts/L), 50 % seawater (20 g sea salts/L) and 0 % seawater (0 g sea salts/L). Ultra-pure water was used to dissolve the sea salts and solutions were left mixing overnight. Artificial seawater was

sterilized at 121 °C for 20 min, and used as water samples as described in Section 5.2.1.4.

5.2.1.3 – ACID-WASH OF THE GLASS BEADS

To avoid inhibition of the whole genome amplification (WGA), glass beads were acid-washed. Enough HCl 10 N was added to two different sizes of glass beads (710-1.180 µm and 150-212 µm; Sigma-Aldrich, USA) until they were completely covered. They were then washed by stirring for 1 min. The acid was discarded and the glass beads were repeatedly washed with ultra-pure water, until pH was approximately 7.0. Glass beads were left to dry overnight at 70 °C. In the laminar flow hood, it was added to each 2 mL screw-cap tube (BD, Canada) 40 ± 8 mg of the 150-212 µm glass beads, and 15 ± 5.25 mg of the 710-1.180 µm glass beads. The tubes were closed and used in the CRENAME procedure (Section 5.2.1.5).

5.2.1.4 – MEMBRANE FILTRATION OF THE ARTIFICIAL SEAWATER SAMPLES

A volume of 100 mL of artificial seawater samples (100, 50 and 0 % of sea salts) previously prepared (as described in Section 5.2.1.2) were filtered, in triplicate, through a membrane filter (GN-6, ∅ 47 mm, 0.45 µm pore size; PALL Corporation) on a 3-place standard manifold (Millipore Corporation, Billerica, MA) and placed in 15 mL tubes (BD, Canada). As a sterility control of the filtration, 100 mL of each dilution of the artificial seawater was filtered and the membrane was placed on BHI 2% NaCl agar plates and incubated for approximately 24 h at 35 °C. Between each filtration, the filtration equipment was sterilized by putting the filter holders and funnels in a UV box (Millipore Corporation, Billerica, MA) for 2 min. The membranes in the 15 mL tubes were used in the CRENAME procedure (Section 5.2.1.5).

5.2.1.5 – CRENAME PROCEDURE

Membranes were dissolved into small pieces through the addition of 8.5 mL of methanol to the 15 mL tube (Sigma-Aldrich, USA), and were gently mixed by finger tapping. Tubes were then centrifuged for 5 min at 4100 rpm (Jouan, Thermo Scientific, USA), and the supernatant was removed and discarded. 1 mL of acetone (Sigma-Aldrich, USA) was added to tubes and the pellet was dissolved through vigorous agitation. The resulting clear solution was transferred to the 2 mL screw-cap tube with acid-washed glass beads (previously prepared as described in Section 5.2.1.3), centrifuged for 3 min at 15 800 x g (Eppendorf, Germany) and supernatant was removed and discarded. The 15 mL tube was rinsed with 1 mL of acetone and the resulting solution was transferred to the 2 mL screw-cap tube previously used. The tubes were centrifuged at 15 800 x g for 3 min (Eppendorf, Germany) and supernatant was removed and discarded. 1 mL of TE (Tris-HCl at 100 mM, EDTA at 1 mM, pH 8.0) (BD, Canada) was added to the screw-cap tubes and these were then centrifuged 3 min at 15 800 x g (Eppendorf, Germany), supernatant was removed and discarded. Samples were used in the procedure described in Section 5.2.1.6.

5.2.1.6 – MOLECULAR ENRICHMENT BY WHOLE GENOME AMPLIFICATION (WGA)

To each sample prepared in Section 5.2.1.5, 1 µL of each *Vibrio* sp. was added (see Figure 5.3), in triplicate, so that each tube had the corresponding concentration of each of the three *Vibrio* bacteria. 20 µL of sample buffer (part of the Illustra GenomiPhi DNA amplification kit; GE Healthcare, Montréal, Québec, Canada) was added to the samples, and were placed on a vortex mixer at maximum speed for 5 min. Tubes were placed for 3 min in a water bath of 95 °C and kept on ice for 3 min. A mixture containing 45 µL of the GenomiPhi reaction buffer with 5 µL of the Genomiphi ϕ29 DNA polymerase (part of the Illustra GenomiPhi DNA amplification kit; GE Healthcare, Montréal, Québec, Canada) was added to each tube and gently mixed by finger tapping. The tubes were incubated for 3 h at 30 °C and the enzymatic

reaction was stopped by incubation of 10 min at 65 °C. 1 µL of each sample was used as the template for *Vibrio* rtPCR amplifications (Section 5.2.4.3).

5.2.2 – Primers

Primers (Biosearch Technologies, Inc., Canada) were selected from two previous studies (Tables 5.1 and 5.2) since these were able to detect *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* by multiplex-PCR [1, 7]. Both sets of primers were tested with pure DNA dilutions to compare their potential as a multiplex and sensitivity. Pure stock DNA from *V. cholerae* (CCRI-10684), *V. parahaemolyticus* (CCRI-8964) and *V. vulnificus* (CCRI-8942), from stock DNA of the Collection of Centre de Recherche en Infectiologie, were diluted from a concentration of 34, 12 and 10 ng/µL, correspondingly, to 10 000, 5 000, 1 000, 500, 100, 50, 10 and 5 gc/µL in 1 x TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). 1 µL of each dilution was added to the reaction tube. Therefore, as an example, if 1 µL of the dilution 50 gc/µL was added to the reaction tube, it was the equivalent to 50 gc per reaction. Assays were performed in triplicate.

5.2.3 – TaqMan Probes

Sequence-specific TaqMan probes (Biosearch Technologies, Inc., Canada) for each *Vibrio* sp. were designed for the multiplex of Neogi and coworkers by building a multiple alignment of *toxR* and *vvhA* sequences retrieved from public databases with Mega Software (version 5.0) [7]. Specific and conserved regions for each target species were identified on the alignement and TaqMan probes compatible with the multiplex primers were design using Oligo primer analysis software (version 6.7). Selection of fluorophores was performed accordingly to the different possibilities of multiplex. The final TaqMan probes developed are listed in Table 5.3.

Table 5.1 – Primers from Bauer and Rørvik (2007) for the multiplex-PCR to detect *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, targeting the *toxR* gene, which encodes trans-membrane protein involved in the regulation of virulence-associated genes. The forward primer, UtoxF, was universal for all three species, while the reverse primers were species specific [1]

TARGET	GENE	PRIMERS	PRIMERS SEQUENCE (5´→ 3´)	AMPLICON SIZE
Vibrio sp.	*toxR*	UtoxF	5´ – GASTTTCTTTGGCGYGARCAAGGTT – 3’	–
V. cholerae	*toxR*	vctoxR	5´ – GGTTAGCAACGATGCGTAAG – 3’	640 bp
V. parahaelolyticus	*toxR*	vptoxR	5´ – GGTTCAACGATTGCGTCAGAAG – 3’	297 bp
V. vulnificus	*toxR*	vvtoxR	5´ – AACGGAACTTAGACTCCGAC – 3’	435 bp

Table 5.2 – Primers from Neogi *et al.* (2010) for the multiplex-PCR, targeting the *toxR* gene for *V. cholerae* and *V. parahaemolyticus*, and the *vvhA* gene (responsible for haemolytic and cytolytic activities) for *V. vulnificus* [7]

TARGET	GENE	PRIMERS	PRIMERS SEQUENCE (5´→ 3´)	AMPLICON SIZE
V. cholerae	*toxR*	VC toxR 403F VC toxR 678R	5´ – GAAGCTGCTCATGACATC – 3’ 5´ – AAGATCAGGGTGGTTATTC – 3’	275 bp
V. parahaelolyticus	*toxR*	VP toxR 325F VP toxR 828R	5´ – TGTACTGTTGAACGCCTAA – 3’ 5´ – CACGTTCTCATACGAGTG – 3’	503 bp
V. vulnificus	*vvhA*	vvhA 870F vvhA 1236R	5´ – ACTCAACTATCGTGCACG– 3’ 5´ – ACACTGTTCGACTGTGAG – 3’	366 bp

Table 5.3 – TaqMan probes designed in this work for the primers of Neogi *et al.* (2010) to develop a multiplex-rtPCR to detect *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. The probe target gene, sequence, reporter and quencher chosen, length and the melting temperatures (T_M) are listed in the table

TARGET	GENE	PROBE	SEQUENCE (5´→ 3´)	REPORTER/QUENCHER	LENGTH	T_M (°C)
V. cholerae	*toxR*	toxRVcho-T1-F1	5´ – TGAACACACCGCAGCCAGC– 3´	Cal Red 610 / BHQ-2 (Orange channel)	19 bp	64.5
V. parahaelolyticus	*toxR*	toxRVpar-T1-G1	5´ – TGGCACTATTACTACCGATTTGCG– 3´	Quasar 670 / BHQ-2 (Red channel)	24 bp	62.9
V. vulnificus	*vvhA*	vvhAVvul-T1-A1	5´ – ACGGTCAAACAACGATCGGATG– 3´	FAM / BHQ-1 (Green channel)	22 bp	62.7

5.2.4 – rtPCR amplification

5.2.4.1 – OPTIMIZATION OF THE RTPCR AMPLIFICATION

The analytical sensitivity of the primers, probes and the thermal cycling conditions were evaluated and optimized using pure DNA dilutions of *V. cholerae* O139 (CCRI-10684), *V. parahaemolyticus* (CCRI-8964) and *V. vulnificus* (CCRI-8942) from a concentration of 34, 12 and 10 ng/µL, respectively, to 10 000, 5 000, 1 000, 500, 100, 50, 10 and 5 genome copies of each *Vibrio* sp. per µL, in 1 x TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). 1 µL of each dilution was transferred to the reaction tube for amplification by rtPCR.

5.2.4.2 – *VIBRIO* MULTIPLEX-RTPCR: TEST TO THE PRIMERS OF BAUER AND RØRVIK (2007)

1 µL of DNA dilutions of each *Vibrio* sp. was added to a tube containing a 22 µL mixture of 1.25 µL of bovine serum albumin (BSA, 3.30 mg/mL per reaction; BD, Canada), 1.5 µL of $MgCl_2$ (1.5 mM/reaction; Sigma-Aldrich, USA), 2.5 µL of 10x Taq PCR buffer (Promega, USA), 1.5 µL of dNTPs (0.2 mM, 0.19 µL of *Taq* DNA polymerase (0.025 U/reaction; Promega, USA), 12.56 µL of ultra-pure water, 1.5 µL of UtoxF primer (0.6 µM/reaction) and 1.0 µL of each vctoxR, vptoxR and vvtoxR primers (0.4 µM/reaction).

The positive amplification controls were prepared by pipetting 1 µL of the 100 gc/µL DNA dilutions of each *Vibrio* sp. to the reaction tube. Three rtPCR negative controls (NTC) were prepared by adding to the reaction tube 1 µL of ultra-pure water, in different rooms: NTC 1 was prepared after preparing the master mix and before leaving the PCR reagent preparation room to assure that the master mix was not contaminated; the ultra-pure water was added to the NTC 2 tube when entering the sample preparation room to assure that the laminar flow hood was not contaminated, and in NTC 3, the ultra-pure water was added after preparing all the

reaction tubes and before leaving the sample preparation room to determine if no cross-contamination with samples or control DNA has happened during the assembly of PCR reactions.

Thermal cycling was performed in a molecular amplification room using a Rotor-Gene 6000 (QIAGEN, Canada) with the following conditions: 4 min at 95 °C, 45 cycles of 5 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C and final extension of 7 min at 72 °C (adapted) [1].

5.2.4.3 – VIBRIO MULTIPLEX-RTPCR: TEST TO THE PRIMERS OF NEOGI *ET AL.* (2010)

1 µL of the DNA dilutions of each target *Vibrio* sp., or of the CRENAME templates was added to a reaction tube containing a mixture of 1.25 µL of bovine serum albumin (BSA, 3.30 mg/mL per reaction), 1.5 µL of $MgCl_2$ (1.5 mM/reaction), 2.5 µL of 10x Taq PCR buffer (Promega), 1.5 µL of dNTPs (0.2 mM, 0.19 µL of *Taq* DNA polymerase (0.025 U/reaction; Promega), 1.25 µL of each VCtoxR403F and VCtoxR678R primers (0.4 µM/reaction), 1.5 µL of each VPtoxR325F and VPtoxR828R primers (0.6 µM/reaction), 0.75 µL of each vvhA870F and vvhA1236R primers(0.3 µM/reaction), 0.5 µL of each of the *Vibrio* TaqMan probes (0.2 µM/reaction) and ultra-pure water to a final volume of 25 µL per reaction tube. The positive and the NTC controls were prepared as described previously.

Thermal cycling was performed in a molecular amplification room using a Rotor-Gene 6000 (QIAGEN, Canada) with the following conditions: 3 min at 95 °C, 45 cycles of 5 s at 95 °C, 15 s at 55 °C and 30 s at 72 °C and final extension of 3 min at 72 °C (adapted) [7]. When the probes were used in the reaction, the thermal cycler was set to acquire the fluorescent signal in the orange, red and green channels (Table 5.3).

5.2.5 – Electrophoresis

13 µL of the amplification product was loaded into a 1.5 % agarose gel with ethidium bromide (Sigma-Aldrich, USA) and run by electrophoresis in 1 x TBE buffer (diluted from a 10 x TBE buffer: 1 M Tris, boric acid 0.9 M, EDTA 0.01 M, pH 8.4) for 35 min at 170 V. The bands corresponding to the amplification were visualized and photographed on a UV transilluminator (ChemiDoc, Bio-Rad). A 100 bp DNA ladder was loaded on each gel as a DNA size standard.

5.3 | RESULTS AND DISCUSSION

5.3.1 – Analytical detection limit of the multiplex-rtPCR for *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*: evaluation of the primers

Two sets of primers were selected from the literature due to their ability to efficiently detect the three target *Vibrio* spp. (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) through a multiplex standard PCR [1, 7]. Nevertheless, in order to evaluate which primers best fit a rtPCR amplification, serial dilutions of pure DNA of each of the three targets were amplified and the performance of each multiplex-rtPCR was analyzed. These tests were performed in the rtPCR thermal cycler without the addition of a TaqMan probe, since it was necessary to select the most suitable primers for the multiplex-rtPCR, before developing the new TaqMan probes for the sequences amplified by the primers selected. Thus, the evaluation of the primers was based in the comparison of the amplification results by running the amplicon in an agarose gel (electrophoresis).

Bauer and Rørvik (2007) designed their multiplex PCR to be used in the detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in seafood [1]. Results in Figure 5.4-A show that these primers could be adapted to a multiplex-rtPCR, even though the amplicon sizes of all the targets are above the optimum length for rtPCR (80 - 200 bp) [21]. The limit of detection was estimated to be between 10 and 20 gc/µL, since all the triplicates at 50 gc/µL were detected but only some of the triplicate samples were detected at an amount of 10 gc/µL. Therefore, a new amplification focusing on the concentrations of DNA of 10 and 20 gc/µL was

prepared, comparing simplex versus multiplex. Results in Figure 5.4-B show that the limit of detection would be higher than 20 gc/µL as the three bands corresponding to the three targets were not completely visible, either in simplex or multiplex. It was also noticed that the bands corresponding to the detection of *V. cholerae* were the less intense, even at high concentrations of DNA. This may have been caused due to the length of 640 bp for this amplicon, which definitely exceeds the optimum amplicon length of 80 to 200 bp [21]. Since the thermal cycling in rtPCR assays are shorter in time than standard PCR, smaller amplicons will particularly lead to more efficient detection [22].

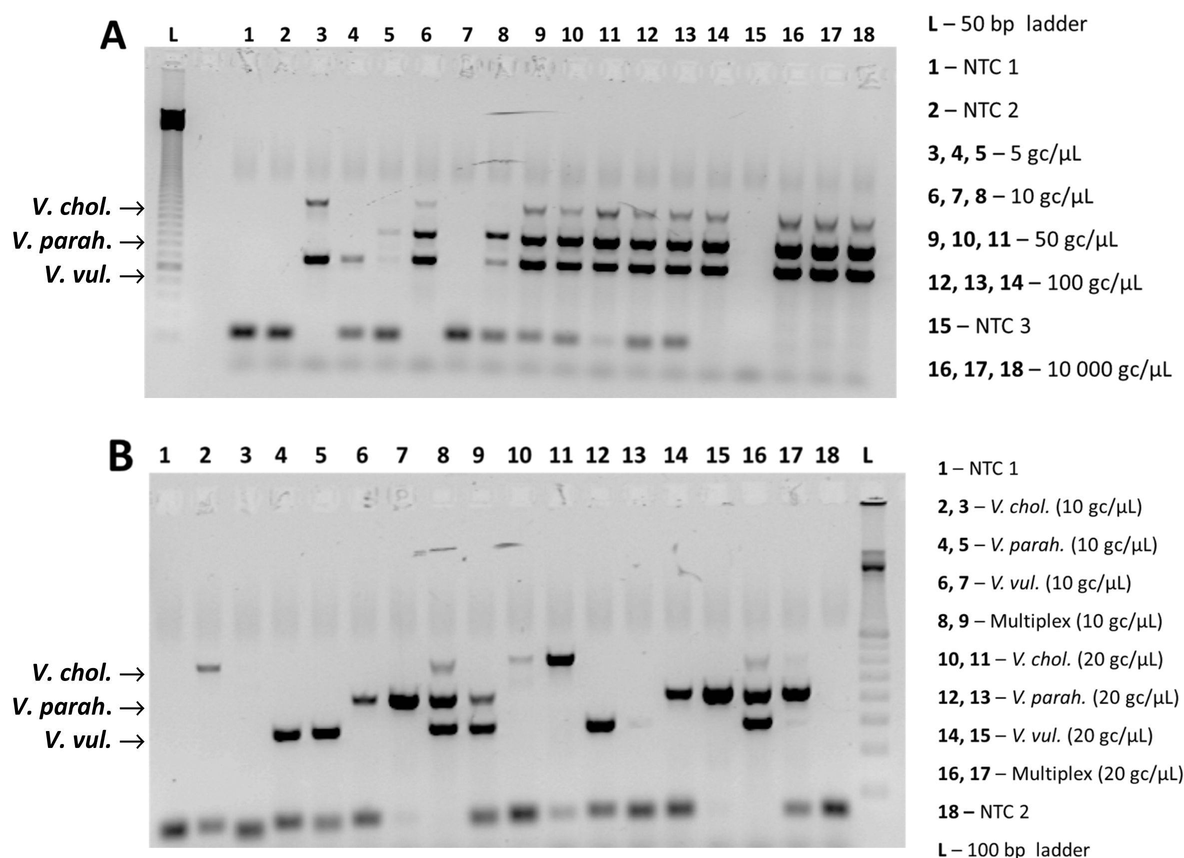


Figure 5.4 – Agarose gels of the assay testing the analytical limits of detection of the *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* Multiplex-rtPCR, using the primers from Bauer and Rørvik (2007): (A) multiplex ranging from 5 to 10 000 gc/µL, from which it was

estimated that the limit would be between 10 and 50 gc/µL; (B) simplex and multiplex amplification for 10 and 20 gc/µL.

Neogi and coworkers developed a multiplex for standard PCR to detect *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in clinical and environmental samples and claimed a limit of detection of the multiplex PCR of 10 CFU in the reaction tube, using bacterial suspensions [7]. Results in Figure 5.5-A show that the bands corresponding to *V. parahaemolyticus* were absent, despite being visible in the work of Neogi *et al.* (2010). At this stage, the concentrations of the primers used were those recommended by the authors [7], being the concentration of the primers for *V. parahaemolyticus* of 0.15 µM/reaction, a considerably low concentration when compared to the concentration of the other two primers. Therefore, an optimization of the primers concentration for this target was carried out, and it was estimated that the limit of detection would be between 20 and 50 gc/µL, to be confirmed through comparison of simplex versus multiplex (Figure 5.5-B). In this assay, the concentration of the primers VPtoxR325F/VPtoxR828R was increased to 0.40 µM/reaction, which resulted in a visible amplification for *V. parahaemolyticus* and confirmed that the above mentioned limitations on detection were due to the low concentration of the primers.

Comparing both sets of primers, it was possible to observe that the bands corresponding to *V. cholerae* were significantly more intense using the multiplex-rtPCR based in the Neogi *et al.* (2010) multiplex, even at lower concentrations of DNA, which is possibly due to the reduced length of the amplicon of 275 bp, when compared to the 640 bp of the amplicon obtained using the primers from the work of Bauer and Rørvik (2007). Moreover, the specificity tests with other closely related microorganisms performed by the authors of the multiplex PCR amplifications concluded that the primers described by Bauer and Rørvik (2007) also detected the non-target *Vibrio alginolyticus*, which is closely related to *V. parahaemolyticus* and, thus, caused false-positive results [1]. The specificity tests performed by Neogi *et al.*

(2010) concluded that their primers could detect the three target bacteria without ambiguity of false-positives, even among the closely related species of *V. parahaemolyticus* (*V. alginolyticus*) and *V. cholerae* (*Vibrio mimicus*) [7]. Therefore, the primers selected for the *Vibrio* CRENAME-rtPCR were those used in the multiplex-PCR by Neogi *et al.* (2010).

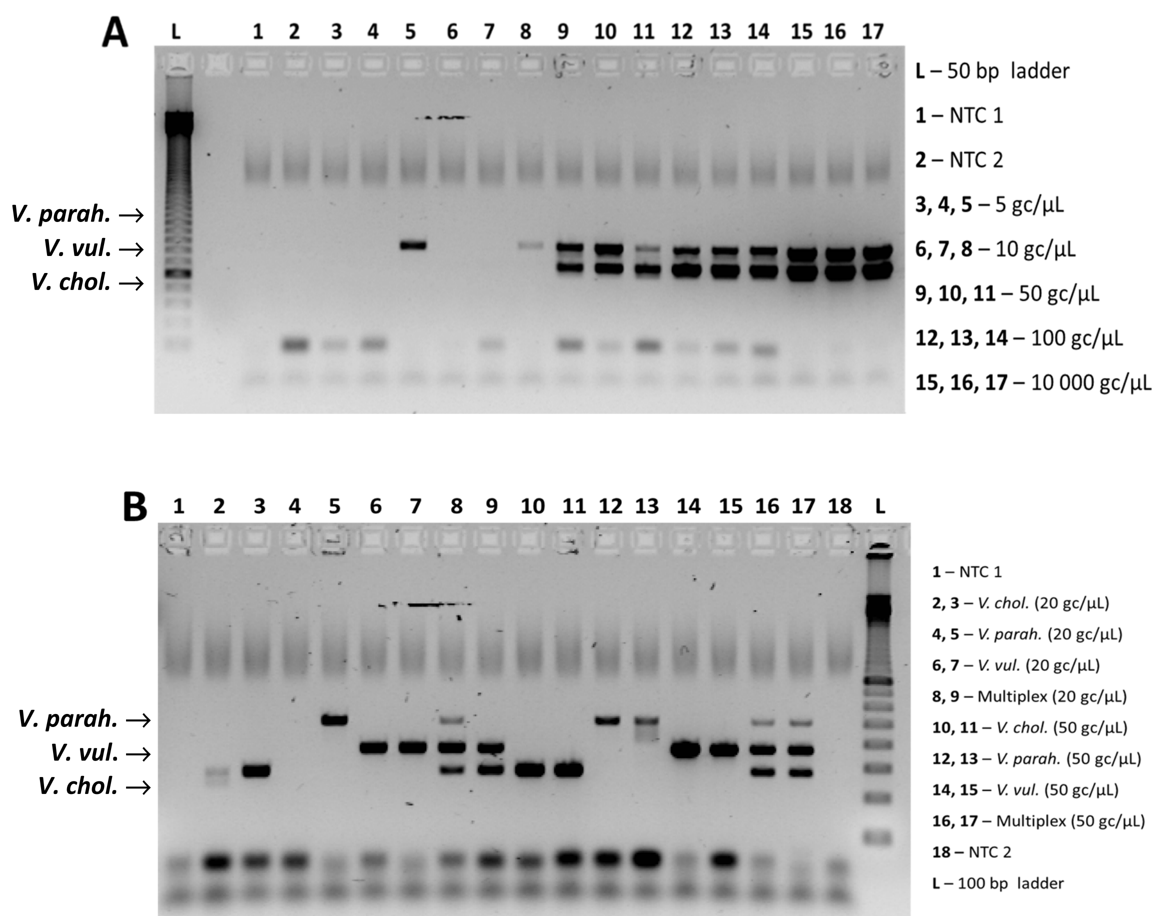


Figure 5.5 – Agarose gels of the assay testing the analytical limits of detection of the *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* Multiplex-rtPCR, using the primers from Neogi *et al.* (2010): (A) multiplex ranging from 5 to 10 000 gc/μL, from which it was estimated that the limit would be between 10 and 50 gc/μL (the bands of *V. parahaemolyticus* were not visible in this gel because the concentrations of primers were not optimized at that moment); (B) simplex and multiplex amplification for 20 and 50 gc/μL (the bands of *V. parahaemolyticus*

became visible in this gel after increasing the concentrations of primers VPtoxR325F/VPtoxR828R from 0.15 µM to 0.4 µM per reaction).

The next step was to optimize the *Vibrio* multiplex-rtPCR and develop the TaqMan probes for the target *Vibrio* spp. The optimization results of the multiplex-rtPCR to the final protocol are visible in the agarose gel in Figure 5.6. All the three bands corresponding to the three targets were intense and distinct, even at the low DNA concentrations of 20 and 50 gc/µL. Furthermore, the bands corresponding to our main target, *V. cholerae*, were the most intense, which provided more confidence in the stability of the detection of this target in particular. The analytical limit of detection achieved (20 gc/µL) was considered adequate to this work since the CRENAME protocol includes a molecular enrichment step and, consequently, the DNA template used in the detection would be at a higher concentration [23, 24].

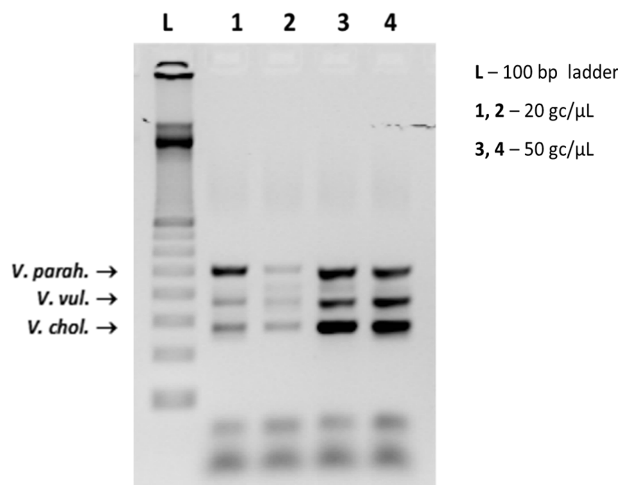


Figure 5.6 – Agarose gel showing the multiplex-rtPCR optimized, where all the bands were remarkably visible and defined, even at low concentrations of DNA (20 and 50 gc per µL, i. e. per reaction).

5.3.2 – Analytical detection limit of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* by multiplex-rtPCR: evaluation of the TaqMan probes

After selecting the primers for the *Vibrio* multiplex-rtPCR, the TaqMan probes were designed, accordingly to the literature, to bind the specific product of amplification of each of the target bacteria [21, 22]. By including these probes in the amplification, it was aimed to benefit from the main advantages of the TaqMan probes: a high specificity, low interference from background signal and the possibility to perform multiplex reactions [22].

The TaqMan probes were firstly tested using pure DNA dilutions from 5 to 10000 gc/µL in order to evaluate their limit of detection. This test was firstly performed by studying their behavior when only one target was present in the reaction tube (with all the probes) (Table 5.4) and in multiplex (Table 5.5) (all the 3 targets in the same reaction tube). A completely optimized TaqMan rtPCR should have an efficiency of the reaction between 90 and 105 %, and the coefficients for the linear standard curve should be $r > |-0.990|$ or $R^2 > 0.980$ [22]. Analysis of the results obtained in the two assays showed that the detection of *V. cholerae* achieved the best results of the three, with an efficiency of 100 % when the three *Vibrio* spp. were present in the sample. Moreover, all the samples were detected, in particular, the triplicates of the lowest concentration of DNA (10 gc/µL), with a threshold cycle (C_T) of 40 ± 0.51 cycles. The probe designed to identify *V. parahaemolyticus*, though, did not detect accurately its target. This was visible in the amplification curves, which were irregular, and by the values of efficiency and non-linearity of the standard curves. Moreover, this probe did not detect most of the samples. However, the agarose gels exhibited detection in all the 10 gc/µL of *V. parahaemolyticus* samples. Therefore, the hypothesis of a faulty amplification was excluded, being the most

probable cause a problem in the probe design. During the *in silico* tests for the multiplex with Plexiglad software, were observed strong interactions with this probe in three of the four possible regions to design the probe and, thus, the probe was designed in the region with the weaker interactions predicted by the software. As the results suggest that the probe has not bound completely to the target nucleic acids and could not be hydrolyzed by the polymerase, this probe was considered not capable to detect its target.

Table 5.4 – Threshold cycles (C_T) resulting from the tests to the TaqMan probes (toxRVcho-T1-F1, toxRVpar-T1-G1 and vvhAVvul-T1-A1) with one target per reaction tube. Amplification efficiencies were, respectively: 75 %, 137 % and 138 %. r and R^2 values were, correspondingly: r=0.9867, R^2=0.9736; r=0.6238, R^2=0.3892 and r=0.8457, R^2=0.7152.

	C_T		
DNA dilution (gc/reaction)	*V. cholerae* (toxRVcho-T1-F1)	*V. parahaemolyticus* (toxRVpar-T1-G1)	*V. vulnificus* (vvhAVvul-T1-A1)
NTC 1	–	–	–
NTC 2	–	–	–
10	39.63	–	–
10	40.40	–	–
10	40.59	–	42.09
50	37.74	34.24	36.21
50	37.92	38.25	39.26
50	36.97	39.17	36.68
100	35.12	42.72	35.63
100	35.16	–	35.91
100	36.20	36.69	36.15
10 000	28.32	31.74	32.83
NTC 3	–	–	–

The detection of the DNA dilutions of *V. vulnificus* was not as sensitive as that of *V. cholerae* because not all of the 10 gc/µL samples were detected, either when

one target or the three targets were in the sample. However, when the samples of 10 gc/µL were removed from the standard curve with the three targets, efficiency increased to 101 %, which shows an improved correlation between the threshold cycles and amount of DNA for samples with concentrations higher than the 10 gc/µL. This was in accordance with the results obtained for the analytical limit of detection of the primers, which was more variable at this low DNA concentration.

However, this multiplex rtPCR was developed to be used with CRENAME procedure as a sample preparation method, which includes a molecular enrichment step (WGA). WGA is an isothermal amplification during which all the DNA is non-specifically amplified thousands of times [23, 24] and, therefore, increases the amount of DNA to a level detectable by rtPCR. For this reason, the importance of variability observed at the extremely low amounts of DNA (10 gc/µL) was disregarded.

Table 5.5 – Threshold cycles (C_T) resulting from the tests to the TaqMan probes (toxRVcho-T1-F1, toxRVpar-T1-G1 and vvhAVvul-T1-A1) with three targets per reaction tube. Amplification efficiencies were, respectively: 100 %, 915 % and 115 %. r and R^2 values were, correspondingly: r=0.9181, R^2=0.8430; r=0.3008, R^2=0.0905 and r=0.8641, R^2=0.7467

	C_T		
DNA DILUTION (GC/REACTION)	*V. CHOLERAE*	*V. PARAHAEMOLYTICUS*	*V. VULNIFICUS*
NTC 1	–	2.18	–
NTC 2	–	3.69	–
10	33.74	2.16	42.55
10	34.55	3.76	38.01
10	36.38	5.78	–
50	33.51	8.08	38.53
50	31.00	3.47	41.68
50	31.69	10.15	38.77
100	31.17	5.25	36.86
100	31.75	5.58	38.09
100	34.06	6.80	39.31
10 000	24.63	1.31	31.16
NTC 3	–	3.60	–

Finally, the probes designed for the detection of *V. cholerae* and *V. vulnificus* efficiently detected the target DNA and were included in the *Vibrio* CRENAME-rtPCR. The probe for *V. parahaemolyticus* (toxRVpar-T1-G1) was excluded from the method since it was not able to detect its target.

5.3.3 – *Vibrio* CRENAME-rtPCR detection in artificial seawater

This method was developed having in perspective the detection of *V. cholerae* in ballast water and/or coastal waters. There was the concern that the sea salts would reduce or inhibit the efficacy of the WGA and/or the rtPCR amplifications, the two steps of the procedure that were more sensitive to contaminants. The CRENAME method required a considerable amount of each of the reagents used in the molecular enrichment step and, therefore, it was not sufficient for testing the samples in triplicate. In alternative, selected dilutions of estimated concentrations of fresh cells (accordingly to the McFarland standard 0.5 [25]) were subjected to the WGA procedure. The *Vibrio* CRENAME-rtPCR method was tested in artificial seawater at 100, 50 and 0 % of the concentration of sea salts in seawater and, due to the high pathogenicity of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* microorganisms, these were not added directly to the seawater samples in order to reduce the risk of exposure to the pathogens. Thus, since it has already been proven that CRENAME efficiently concentrates microorganisms from water samples [18, 19], fresh cells of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* were added to the tubes after the CRENAME procedure, and before the cells lysis. This was considered as equivalent to an assay using spiked seawater samples.

Threshold cycles in Table 5.6-A show that WGA was essential for the detection of the bacteria, since it resulted in a high yield of the target DNA that could be verified by the early C_T signals, which was in accordance with results observed with other microorganisms [18, 19]. In addition, some samples at higher *Vibrio* spp. concentrations (150 and 1500 CFU/100 mL) that were not submitted to the molecular enrichment, were also detected, which was confirmed by the agarose gels (Figure 5.7).

Table 5.6 – (A) Threshold cycles, C_T, obtained in the detection of *V. cholerae* and *V. vulnificus* by *Vibrio* CRENAME-rtPCR, using the TaqMan probes toxRVcho-T1-F1 and vvhAVvul-T1-A1, accordingly to the estimated concentration of CFU/100 mL and (B) the corresponding number of CFU/100 mL for each bacteria obtained by colony counts

A

Concentration of seawater	Dilution (CFU/100 mL)	WGA[1]	*V. cholerae* C_T	*V. vulnificus* C_T
	NTC 1		–	–
	NTC 2		–	–
	DNA (100 gc/µL)		39.47	40.71
0 %	0	+	–	–
	15	+	17.58	28.73
	15	-	–	–
	150	+	21.58	30.44
	150	+	15.94	24.29
	150	-	–	–
	1500	+	18.12	25.90
	1500	-	–	–
50 %	15	+	41.32	–
	15	-	–	–
	150	-	39.17	43.61
	150	+	27.46	–
	150	-	–	–
	1500	+	15.15	23.04
	1500	-	–	–
100%	15	+	22.46	29.45
	15	-	–	–
	150	-	36.98	–
	150	+	16.51	25.69
	150	-	37.58	29.90
	1500	+	13.72	19.84
	1500	-	36.43	–
	NTC 3		–	–

[1] For the WGA, are considered: with WGA, **+** ; wihout WGA, **-** .

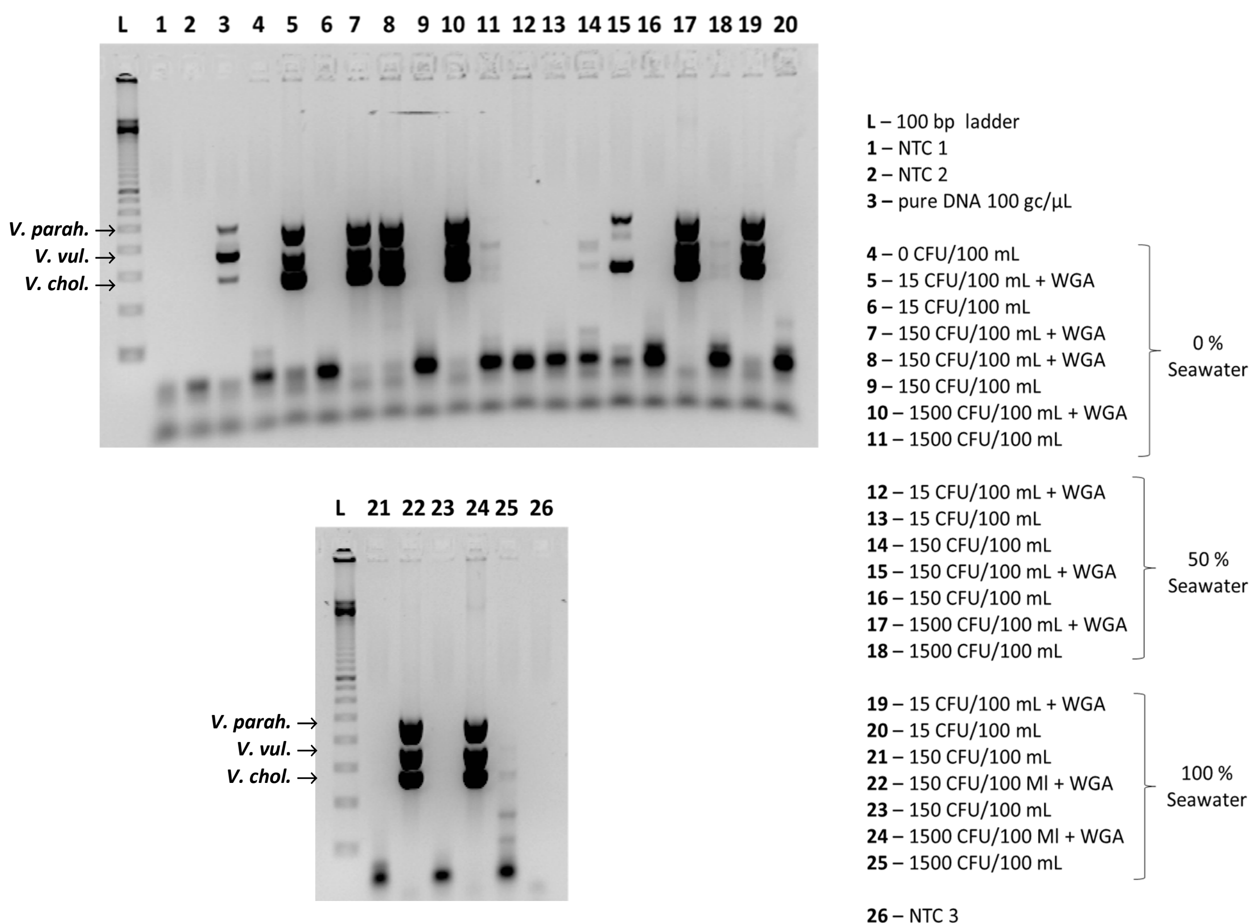


Figure 5.7 – Agarose gels for the Vibrio CRENAME-rtPCR assay in 100, 50 and 0 % of artificial seawater.

Although this experiment was not performed in triplicate, the consistency of the results between samples for the two bacteria showed that the sea salts in the artificial seawater samples, which can be a source of inhibition [26], did not reduce or inhibit the detection of the target microorganisms, suggesting that the sea salts were in the majority removed during the water sample preparation procedures.

Moreover, with this method, it was possible to detect as little as 13.6 ± 0.651 CFU/100 mL of *V. cholerae* and 4.17 ± 0.61 CFU of *V. vulnificus*. Although the probe developed for *V. parahaemolyticus* was excluded from this test, this microorganism could be detected at 12.3 ± 0.400 CFU/100 mL by running the amplification products in an agarose gel.

During the molecular enrichment step, several copies of the whole genome of the microorganisms in the sample were produced; however, this increase is not linear and, therefore, it was not possible to establish a correlation between the initial and final DNA amounts [23, 24]. Therefore, the CRENAME-rtPCR method has been considered as a qualitative technique that provides results for a presence/absence evaluation.

To our knowledge, there was no multiplex-rtPCR capable of detecting as little as 1 CFU/100 mL targeting these microorganisms in ballast water, within 5 hours or less. Fykse *et al.* (2012) reported a method to detect 1 CFU/mL in ballast water. However, this method included a pre-enrichment step of, at least, 4 h to achieve a detection of 1 CFU/100 mL, with a final assay time of 7 h [3].

This research did successfully develop a *Vibrio* CRENAME-rtPCR method, with high-potential to be used in ballast water monitoring, or other aquatic environments. In addition to have been capable of providing significantly faster results in only 5 h, when compared to the 18 h to 72 h required by the standard methods used to assess the microbiological quality of water, this method has advantage over culture-based methods used since the viable but non-culturable cells are also detected.

Nevertheless, these were preliminary tests that should be repeated in order to establish an accurate detection limit for *Vibrio* sp. and, accordingly to the previous studies with the CRENAME-rtPCR method [18, 19], this technique has the potential to detect as little as 1 cell in 100 mL.

5.4 | CONCLUSIONS

It was demonstrated that, by coupling a highly efficient sample preparation method for the concentration and recovery of microbial particles to a molecular enrichment of the extracted nucleic acids, and by using a multiplex-rtPCR assay targeting *V. cholerae, V. parahaemolyticus* and *V. vulnificus*, it was developed a molecular method for the detection of these targets in less than 5 h without a pre-enrichment step. Preliminary tests showed that the method was able to detect as little as 13.6 ± 0.651 CFU/100 mL of *V. cholerae* and 4.17 ± 0.61 CFU of *V. vulnificus* with the TaqMan probes successfully developed, and 12.3 ± 0.400 CFU/100 mL of *V. parahaemolyticus* by running the amplification products in an agarose gel.

Although a new probe for *V. parahaemolyticus* should be developed and further validation studies are still necessary to confirm these results and establish its usefulness in the water quality monitoring field, this highly effective method provides a rapid and easy approach to detect very low numbers of *Vibrio* cells, without inhibition of the sea salts in the artificial seawater. Replication studies with a large number of natural samples are, however, necessary to confirm these results.

Portugal has one of the largest Exclusive Economic Zones in Europe, and in the World, covering more than 1,700,000 km^2 [27]. Therefore, a method to assess the microbiological quality of ballast water in ships and coastal areas, such as the Vibrio CRENAME-rtPCR here developed, would be of great usefulness and commercially appealing.

5.5 | REFERENCES

1. Bauer, A. and Rorvik, L.M., A novel multiplex PCR for the identification of *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*. Letters in Applied Microbiology, 2007. 45, 371-375.
2. Dobbs, F.C. and Rogerson, A., Ridding ships' ballast water of microorganisms. Environmental Science & Technology, 2005. 39, 259a-264a.
3. Fykse, E.M., Nilsen, T., Nielsen, A.D., Tryland, I., Delacroix, S., and Blatny, J.M., Real-time PCR and NASBA for rapid and sensitive detection of *Vibrio cholerae* in ballast water. Marine Pollution Bulletin, 2012. 64, 200-206.
4. Wennberg, A.C., Tryland, I., Østensvik, Ø., Secic, I., Monshaugen, M., and Liltved, H., Effect of water treatment on the growth potential of *Vibrio cholerae* and *Vibrio parahaemolyticus* in seawater. Marine Environmental Research, 2013. 83, 10-15.
5. Partnerships, G. Cross section of ships showing ballast tanks and ballast water cycle. [cited 2014; Available from: http://globallast.imo.org/index.asp?page=problem.htm&menu=true.
6. Izumiya, H., Matsumoto, K., Yahiro, S., Lee, J., Morita, M., Yamamoto, S., Arakawa, E., and Ohnishi, M., Multiplex PCR assay for identification of three major pathogenic *Vibrio* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. Molecular and Cellular Probes, 2011. 25, 174-176.
7. Neogi, S.B., Chowdhury, N., Asakura, M., Hinenoya, A., Haldar, S., Saidi, S.M., Kogure, K., Lara, R.J., and Yamasaki, S., A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Letters in Applied Microbiology, 2010. 51, 293-300.
8. Thompson, F.L., Iida, T., and Swings, J., Biodiversity of Vibrios. Microbiology and Molecular Biology Reviews, 2004. 68, 403-431.
9. Cabral, J.P.S., Water microbiology. Bacterial pathogens and water. International Journal of Environmental Research and Public Health, 2010. 7, 3657-3703.
10. Reidl, J. and Klose, K.E., *Vibrio cholerae* and cholera: out of the water and into the host. FEMS Microbiology Reviews, 2002. 26, 125-139.
11. Notes from the field: Identification of *Vibrio cholerae* serogroup O1, serotype Inaba, biotype El Tor strain - Haiti, March 2012. Morbidity and Mortality Weekly Report, 2012. 61, 309.
12. Update on cholera - Haiti, Dominican Republic, and Florida, 2010. MMWR Morb Mortal Wkly Rep, 2010. 59, 1637-1641.
13. Pal, B.B., Khuntia, H.K., Samal, S.K., Kerketta, A.S., Kar, S.K., Karmakar, M., and Pattnaik, B., Large outbreak of cholera caused by El Tor variant *Vibrio cholerae* O1 in the eastern coast of Odisha, India during 2009. Epidemiology & Infection, 2013. 141, 2560-2567.

14. Robert-Pillot, A., Guénolé, A., Lesne, J., Delesmont, R., Fournier, J.-M., and Quilici, M.-L., Occurrence of the *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. International Journal of Food Microbiology, 2004. 91, 319-325.

15. Staley, C., Chase, E., and Harwood, V.J., Detection and differentiation of *Vibrio vulnificus* and *V. sinaloensis* in water and oysters of a Gulf of Mexico estuary. Environmental Microbiology, 2013. 15, 623-633.

16. Panicker, G. and Bej, A.K., Real-Time PCR detection of *Vibrio vulnificus* in oysters: comparison of oligonucleotide primers and probes targeting *vvhA*. Applied and Environmental Microbiology, 2005. 71, 5702-5709.

17. International Maritime Organization (IMO), 2008. Guidelines for approval of ballast water management systems (G8), MEPC 58/23/, Annex 4, Res. MEPC 174 (58).

18. Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.L.T., Huppe, V., Picard, F.J., Berube, E., and Bergeron, M.G., Rapid concentration and molecular enrichment approach for sensitive detection of *Escherichia coli* and *Shigella* species in potable water samples. Applied and Environmental Microbiology, 2011. 77, 6199-6207.

19. Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.-L.T., Huppé, V., Bérubé, È., Boudreau, D.K., Picard, F.J., Huletsky, A., and Bergeron, M.G., Method for rapid and sensitive detection of *Enterococcus* sp. and *Enterococcus faecalis/faecium* cells in potable water samples. Water Research, 2011. 45, 2342-2354.

20. Farmer, J.J. and Janda, J.M., Family I. Vibrionaceae Véron 1965, 5245,[AL] p. 491-555. 2005, *In* D. J. Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity (ed.), Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. Springer, New York, NY.

21. Mackay, I.M., ed. Real-time PCR in microbiology: from diagnosis to characterization. 2007, Caister Academic Press: Norfolk, UK.

22. Bio-Rad Laboratories, I. Real-Time PCR Applications Guide. 2006 [cited 2014; Available from: http://www.gene-quantification.de/real-time-pcr-guide-bio-rad.pdf.

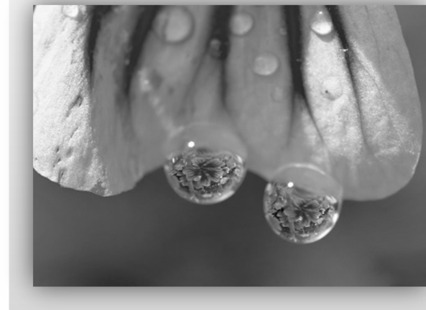
23. Balogh, M.K., Børsting, C., Sánchez Diz, P., Thacker, C., Syndercombe-Court, D., Carracedo, A., Morling, N., and Schneider, P.M., Application of whole genome amplification for forensic analysis. International Congress Series, 2006. 1288, 725-727.

24. Sujayanont, P., Chininmanu, K., Tassaneetrithep, B., Tangthawornchaikul, N., Malasit, P., and Suriyaphol, P., Comparison of phi29-based whole genome amplification and whole transcriptome amplification in dengue virus. Journal of Virological Methods, 2014. 195, 141-147.

25. Preparation of routine media and reagents used in antimicrobial susceptibility testing, in *Clinical Microbiology Procedures Handbook, 3rd Edition*. 2010.

26. Rodríguez, R.A., Thie, L., Gibbons, C.D., and Sobsey, M.D., Reducing the effects of environmental inhibition in quantitative real-time PCR detection of adenovirus and norovirus in recreational seawaters. Journal of Virological Methods, 2012. 181, 43-50.

27. Policy, E.M. EU maritime policy: facts and figures – Portugal. [cited 2014; Available from: http://www.eurocean.org/np4/file/321/portugal_en.pdf.



CHAPTER 6

GENERAL CONCLUSIONS

This chapter comprises the overall conclusions of the thesis, as well as recommendations and suggestions for future work.

6.1 | Conclusions 197

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6.1 | CONCLUSIONS

The main objective of this thesis was the development of fast, reliable and high accuracy methods to detect total coliforms, *E. coli* and *V. cholerae* in drinking and environmental waters, having in sight the commercialization of the developed methods by the sponsor company Frilabo. In particular, this work involved the development of an enzymatic culture medium, and a PCR/rtPCR-based approach to detect total coliforms and *E. coli* in drinking and environmental waters. Additionally, it was sought the development of a rtPCR-based method to detect *V. cholerae* in ballast water. The main contributions of this thesis were the following:

- An enzymatic culture medium was successfully developed, capable of detecting total coliforms and *E. coli* in water, with the presence of total coliforms indicated by the occurrence of a yellow color and of *E. coli* by a blue fluorescence.

- A culture medium in the form a powder was successfully obtained by lyophilization of the enzymatic medium. This allowed to have a straightforward protocol ready for its application by the final consumer: the client adds the powder to the water sample and, after incubation at 37 °C, reads the visual results (formation of color and/or fluorescence);

- The desired sensitivity of 1 CFU of total coliforms and *E. coli* in the water samples (1.35 mL) was achieved in a reduced period of time: 14 to 18 h for drinking and river water and 24 h for diluted seawater (25 %, 1.35 mL). Considering the regulations established by legislation, the enzymatic culture medium fully fulfills and surpasses the requirements of sensitivity, and is capable of providing faster results when compared to the time

required by the analytical methods recommended by the legislation (24 h to 72 h) (e. g. ISO 9308-1), without the need for confirmation steps;

- A protocol capable of detecting total coliforms and *E. coli* by both PCR and rtPCR techniques, with a limit of detection of approximately 10^4 CFU/100 mL of water was developed. This is an easy to perform protocol, taking ~ 6 h by standard PCR (sample preparation, PCR amplification, and electrophoresis) and less than 3 h by rtPCR (sample preparation and rtPCR amplification);

- Coupling the CRENAME sample preparation method with a multiplex-rtPCR assay targeting *V. cholerae, V. parahaemolyticus* and *V. vulnificus*, resulted in the efficient detection of these targets in artificial seawater in a significantly reduced time of less than 5 h, without a pre-enrichment step. Preliminary tests showed that this method was able to detect as little as 13.60 ± 0.65 CFU/100 mL of *V. cholerae,* and 4.17 ± 0.61 CFU of *V. vulnificus* using the TaqMan probes successfully developed, and 12.3 ± 0.40 CFU/100 mL of *V. parahaemolyticus* by running the amplification products in an agarose gel (the TaqMan probe developed for this target was rejected).

Overall, with the developed work, an enzymatic method was developed that can be easily adopted by the entities responsible for accessing the water quality due to its simplicity and sensitivity. Molecular methods were also successfully developed/improved. However, more resistance is expected for its implementation both for the associated costs and need for technically skilled personnel. Nevertheless, the increasing need for timely results without compromising sensitivity and the potential of the molecular methods to fulfil this requirement

drives its research. The results obtained in this thesis together with the ones reported recently in literature bring confidence to its broad implementation in the microbiological water quality monitoring field. The improvement in time of detection achieved by these methods over the standard methods is of major importance as it represents a decrease in the incidence of worldwide outbreaks saving human lives and health care expenses. Moreover, costs for molecular methods are constantly decreasing and thus, the incorporation of these promising methods by the corresponding entities is foreseen in the near future.

6.2 | RECOMMENDATIONS

Even though the main objectives have been accomplished, work still needs to be done in order to continue pursuing for methods to assess the microbiological quality of water capable of providing robust, reproducible and faster results. Thus, some recommendations for improvements of the present work and guidelines for future work in this field can be advanced:

- The time to obtain the results with the enzymatic culture medium could be reduced by coupling with devices based in spectroscopy or optical sensors to detect the color and fluorescence produced at earlier stages of the incubation, when the results are not yet visible to the human eye;

- The salinity of seawater causes the rapid death of *E. coli*, which reduces the capability of this microorganism to indicate the presence of pathogens in this type of water, since other pathogens can survive significantly longer in seawater. Therefore, the enzymatic culture medium could be adapted to detect an alternative indicator microorganism more suitable for seawater samples, with a prevalence similar to the pathogens, in this type of water;

- It would be important to include a molecular decontamination step in the PCR and rtPCR-based detection of *E. coli* in order to avoid the frequent contamination of the master mix reagents (either by reagents contaminated with residual nucleic acids, or by cross contamination during the preparation of the amplification);

- The sample preparation method developed for the detection of *E. coli* with standard PCR and rtPCR could be coupled with a molecular enrichment step to increase the sensitivity of detection;

- A new TaqMan probe for *V. parahaemolyticus* could be developed to be included in the multiplex Vibrio CRENAME-rtPCR assay;

- Further validation studies are still necessary to confirm these results and establish its usefulness in the microbiological water quality monitoring field.