# Integration of culture- and molecular-based water quality monitoring tools to protect human health

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A thesis submitted in partial fulfilment of the requirements of the University of Brighton for the degree of Doctor of Philosophy

January 2017

University of Brighton

## Abstract

Monitoring and improving the microbiological quality and safety of surface waters used for various purposes, including drinking water abstraction and recreation is paramount as degradation may pose a serious risk to human health and cause significant economic losses as a result of the closure of beaches and shellfish harvesting areas. With the aim of providing new knowledge and tools with which to manage more effectively faecal contamination of water resources, this study focused on three goals: 1) determining the fate and suitability of new bio-indicators for virus removal during wastewater treatment; 2) elucidating the levels and sources of faecal pollution in the River Tagus (Rio Tejo) using a blend of newly-developed and existing microbial source tracking (MST) markers; and 3) critically evaluating various pre-treatments to distinguish between infectious and non-infectious viruses. To this end, raw and treated wastewater were collected and tested for the presence of traditional faecal indicator bacteria (FIB), and four viral bio-indicators (namely, somatic coliphages (SC), GB124 phages, human adenovirus (HAdV) and JC Polyomavirus (JCPyV)).

In order to demonstrate whether the novel bio-indicators might be suitable indicators of risk to human health, Norovirus genogroup II (NoVGII) were also analysed, in parallel. FIB, SC and GB124 phages were analysed using standardised culture methods (membrane filtration and plaque assays) and HAdV, JCPyV and NoVGII were analysed using widely used molecular (qPCR) methods. Samples of river water were collected over a thirteen-month period and analysed for both non source-specific indicators of faecal contamination (*Escherichia coli* (EC), intestinal enterococci (IE), and SC) and source-specific contamination markers ((GB124 phages, HAdV) and four mitochondrial DNA markers (HMMit, CWMit, PigMit and PLMit)). EC, IE, SC and GB124 phages were detected by culture methods and HAdV and mitochondrial markers were detected by molecular (qPCR) methods. Furthermore, domestic animal markers (based on the detection of mitochondrial DNA) were also developed for dog and cat and tested during the catchment study. Finally, in order to determine accurately the level of risk to human health, heat-, chlorine-, and UV-inactivated Enterovirus and Mengovirus were subjected to PCR pre-treatments using enzymatic digestion and viability dyes, in order to determine infectivity. Detection of inactivated Mengovirus ($MC_0$) was performed by RT-qPCR and detection of inactivated Enterovirus (EntV) was performed by both RT-qPCR and cell culture.

The results demonstrated that the traditional bacterial indicators (FIB) were more effectively removed during wastewater treatment than GB124 phages, SC, HAdV and JCPyV, the removal levels of which were more similar to those of NoVGII. Spearman's correlation showed that SC and GB124 phages correlated positively with NoVGII at a relatively high level and that HAdV and EC correlated positively at a moderate level. Discriminant analysis revealed that whilst no organism could predict the presence or absence of NoVGII in treated wastewater, GB124 phages in combination with other parameters did result in higher percentages of correct classification. GB124 phages plus HAdV appeared to be good candidates as alternative indicators of enteric virus removal during wastewater treatment.

Results from the catchment study demonstrated that certain sites on the River Tagus are relatively highly impacted by faecal contamination (as indicated by EC, IE and SC concentrations). Moreover, the MST markers revealed that this contamination appears to be not only of human origin, but also originates from a range of other animal sources. The HMMit marker was the most prevalent and was found at the highest mean concentrations, followed by the CWMit marker. Two-way ANOVA revealed a correlation between concentrations of non source-specific indicators (and the CWMit marker) and season. Physico-chemical parameters,

such as temperature and UV radiation, were found to be related to to levels of the CWMit, EC, IE, and SC. Interestingly, rainfall levels were found to be related to concentrations within the river of the PLMit marker and of the newly-developed dog and cat markers. Weak to no correlations were found between non source-specific indicators and the various MST markers, providing further evidence that these faecal indicators were unsuitable for determining the source(s) of contamination in this study. In contrast, the relatively high sensitivity and specificity of the mitochondrial DNA markers supported their use as appropriate markers of the origin of faecal contamination in this scenario.

The results from the viral infectivity study demonstrated that results of 'viability PCR' (involving viability dyes) of chlorine- and UV-treated viruses did not correlate with those from cell culture assay. However, data from RNase-RT-qPCR from chlorine- and UV-inactivated viruses were consistent with the cell culture assay, achieving full PCR signal reduction in several instances. Heat treatment appeared to play an important role, since a significant reduction in the RT-qPCR signal was achieved. Different pre-treatments were able to achieve full removal of RT-qPCR signal for non-infectious heat-treated EntV and $MC_0$. Therefore, enzymatic treatment may represent a rapid and inexpensive tool for discriminating between infectious and non-infectious viruses and as such should improve understanding of risks to human health.

This research has demonstrated that the currently-used methodologies and approaches to assess the potential human health impact of wastewater discharges to environmental waters are limited in their ability to predict the prevalence of important agents of human waterborne disease. Furthermore, these findings provide evidence to support the development and application of alternative and potentially more effective approaches, which could better protect human health in the future.

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## Abbreviations

| | |
|---|---|
| A | Adenine |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pair |
| C | Cytosine |
| cRNA | complementary Ribonucleic Acid |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| dUTP | Deoxyuridine thriphosphate |
| ECDC | European Centre for Disease Prevention and Control |
| EEA | European Environment Agency |
| EEC | European Economic Union |
| EFSA | European Food Safety Authority |
| FAO | Food and Agriculture Organization of the United Nations |
| G | Guanine |
| Glu | Glutamic acid |
| HBV | Hepatitis B virus |
| HCV | Hepatitis C virus |
| HIV | Human Immunodeficiency virus |
| IAWPRC | International Association on Water Pollution Research and Control |
| ICTV | International Committee on Taxonomy of Viruses |
| ISO | International Standards Organisation |
| NADH | Nicotinamide adenine dinucleotide |
| NCBI | National Centers for Biotechnology Information |
| NRDC | Natural Resources Defense Council |
| OIE | World Organisation for Animal Health |
| ORF | Open reading frame |
| qMST | Quantitative microbial source tracking markers |
| RNA | Ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| RNase | Ribonuclease |
| rpm | revolutions per minute |
| RT-qPCR | Quantitative reverse transcription-polymerase chain reaction |
| SD | Standard deviation |
| Ser | Serine |
| T | Thymine |
| UNICEF | United Nations Children's Fund |
| US EPA | United States Environmental Protection Agency |

| | |
|---|---|
| UV | Ultraviolet radiation |
| Val | Valine |
| WHO | World Health Organisation |
| w/v | weight per volume percent |
| v/v | volume per volume percent |

## Dedication

Dedicated to my dad Cipriano Monteiro, my mum Maria Monteiro, my brother João Monteiro, my grandmothers Maria Amélia and Maria Luzia, and my late grandfathers Manuel Duílio and Manuel Monteiro.

**Acknowledgements**

I would like to acknowledge my supervisors Professor Huw Taylor, Dr James Ebdon of the University of Brighton for all the support (academic and personal) and knowledge transfer. My huge gratitude goes to Dr. Ricardo Santos at Laboratório de Análises Instituto Superior Técnico for his support through this long and bumpy road, for always keeping me positive and helping me during my craziest moments.

I would also like to acknowledge Dra. Maria Cândida Vaz, Dra. Manuela Cadete and Professor Margarida Santos Romão at Laboratório de Análises of Instituto Superior Técnico for providing me with the opportunity to pursue the doctoral programme and for all the incentive and support. I would like to think to all the laboratory personnel and especially to Mário Freire and Greice Brondani.

I wish to thank my colleagues in the Environment and Public Health Research Group (EPHReG), University of Brighton – Dr Dave Diston, Dr Sarah Purnell, Dr Diogo Trajano, Dr Lakshmi Yaliwal, Dr Austen Buck, Dr Emanuele Sozzi, Dr Edgard Diaz, and Mario Rodrigues Peres.

Finally, I would like to thank to my dad Cipriano Monteiro, my mum Maria Monteiro, my brother João Monteiro, my grandmothers Maria Amélia and Maria Luzia for their unconditional love and support through this long and hard journey.

## Author's declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed: 

Dated: 12 December 2016

# CHAPTER 1 : INTRODUCTION

## 1. INTRODUCTION

### 1.1. Background: a brief history of water

Water is essential to all forms of life and consists of a molecule made up of two hydrogen atoms and an oxygen atom. Hydrogen is a primordial element and is thought to be the first to be formed following the beginning of the Universe. Oxygen is the third most abundant element found in the sun and constitutes up to 21 percent of the Earth's atmosphere (by volume). It consititutes up to 75 percent of the human body and 90 percent of the mass of water and roughly half of the Earth's crust mass is made up of oxygen (Whitten *et al.*, 2007).

In spite of its importance to all living organisms, water is also an important vehicle for microbial pathogens and has been associated with many major illnesses and worldwide, several major outbreaks have been reported since the 19$^{th}$ century. The first well-documented waterborne outbreaks were reported in the UK and Sweden between the 1830s and 1880s (Snow, 1849; Andersson and Bohan 2001).

### 1.2. Burden of disease

The ability to describe accurately the burden of illnesses and their associated risk factors amongst a human population is extremely important in order to support effective health decision-making and planning processes. However, much existing data on mortality and healthwere, until relatively recently, unpredictable and incongruent in most regions of the World. Therefore, there has been an increasingly urgent need to create a structure capable of integrating, validating, analysing and disseminating such information. Premature death, loss of health and disability can then be evaluated in terms of the relative importance of risk factors, injuries and illnesses. To this end, the World Bank commissioned the first Global Burden Disease (GBD) study in 1990. This document provided information on the quantification of health effects of more than a hundred diseases and injuries for eight regions of the world during the year 1990 (Murray and Lopez, 1996). Not only did their study produce consistent information of mortality and morbidity by region, gender and age but also proposed a new concept for measuring the quantity of burden, disease and risk, the 'disability-adjusted life-year' (DALY). This metric combines the years of life lost from premature death and years of life lived under less than optimal health (generally termed 'disability'). One DALY can be understood as one lost year of life lived with perfect

health. The disease burden is then the difference between a normative reference population and the health status of the population in the study.

Environmental factors are shown to be the primary influence on disease burden, especially in less economically-developed countries (LEDC). One of the most important risks is linked to unsafe water, poor sanitation and hygiene. Diarrhoeal disease accounted for an estimated 4.3% of the total DALY global burden of disease, as of 2011. An estimate of 58% of the total global burden has been attributed to unsafe water supply, sanitation and hygiene (WHO, 2014a).

Significant improvements have been made, in terms of access to 'safe' drinking water, largely as a result of the UN Millennium Development Goals (MDGs). In 2012, 90% of the world population had improved sources of drinking water compared with 76%, as reported in 1990. However, the improvements observed were inconsistent amongst different regions, between rural and urban areas and between different social strata. Furthermore, basic sanitation was not readily available to all. In 2012, 2.5 billion people had no access to improved sanitation facilities, with almost half of these people still practicing open defecation.Moreover, because of rapid growth in the size of urban populations, the number of people living in urban areas, without access to proper sanitation is increasing (WHO, 2015). Currently, diarrhoeal disease is still the second leading cause of death in children under the age of five years, with around 760,000 deaths reported annually (approximately 9% of all deaths among children under the age of fiveglobally) (WHO, 2013; UNICEF, 2016). Figure 1.1 shows the percentage of deaths in 2015 among children under the age of five as a result of diarrhoea (UNICEF, 2016). Nevertheless, during the past fifteen years, a decrease of more than 50% in deaths within this age group has been achieved. Additionally, almost 1.7 billion cases of diarrhoeal disease occur worldwide (WHO, 2013).

Africa is still the region most affected by diarrhoeal disease, with a high level of mortality and with a DALY of more than 5000 per 100,000 population (WHO, 2014b,c). Conversely, Europe is still the least affected WHO region, and here diarrhoea does not feature amongst the twenty leading causes of death, with a DALY of 181 per 100,000 population (Table 1.1).

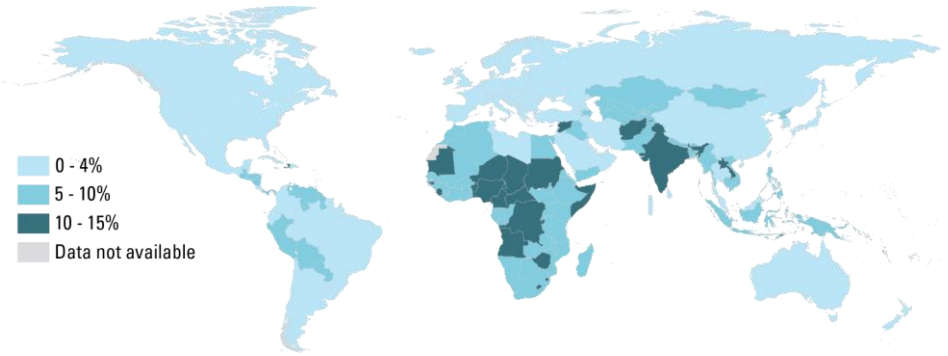


**Figure 1.1 -** Percentage of mortality among children under the age of five as a result of diarrhoeal diseases in 2015 (UNICEF, 2016).

**Table 1.1 -** Disease burden from diarrhoeal disease, total deaths, and DALYs per region, 2012 (WHO, 2014b,c)

| Region/mortality in children and adults | Deaths per 100,000 population | DALYs per 100,000 population[a] |
|---|---|---|
| African Region | 67.5 | 5168 |
| Region of the Americas | 4.37 | 281 |
| South-East Asia Region | 35.5 | 1852 |
| **European Region** | 2.76 | 181 |
| Eastern Mediterranean Region | 22.9 | 1855 |
| Western Pacific Region | 1.97 | 213 |
| Global | 21.2 | 1409 |

Nonetheless, a substantial decrease was observed between the years 2000 and 2012, in parallel with improvements to sanitation, drinking water sources and better sanitary practices (WHO, 2014b,c). However, the global health burden associated with poor water quality, sanitation and hygiene still represents major.

### 1.3. Pathogens in water

Pathogenic microorganisms can be transmitted via several routes, including air, person-to-person transmission, zoonosis, and through contaminated food and water (Eames *et al.*, 2009; Santos and Monteiro, 2013; Kirk *et al.*, 2015). A wide range of pathogenic microorganisms (including bacteria, viruses and protozoa) may be present in untreated or poorly treated wastewaters, or in waters used for recreational purposes, shellfish

harvesting, or drinking water abstraction. For instance, it has been estimated that more than 100 viruses can be found, generally, in environmental waters (Havelaar *et al.,* 1993; Leclerc *et al.,* 2000). Table 1.2 introduces the widely considered to be most important pathogens and toxins found in water. Several pathogens transmitted through contact with contaminated drinking or recreational waters are transmitted via the faeco-oral route.

Wastewater is a complex mixture of human excreta, suspended solids, detritus and several different chemical compounds resulting from residential areas, and/or commercial and industrial activities. Raw (untreated) wastewater is a major source of environmental contamination and human excreta-borne diseases, especially those arising from enteric pathogens (Bosch, 1998). The treatment of raw wastewater prior to release into the receiving waters is therefore of primary importance, though the use of combined sewer overflow (CSO) in some countries (such as the UK) ensures that this is not always the case.

**Table 1.2 -** Pathogens and toxins associated with waterborne diseases (Hunter, 2003; Bosch *et al.*, 2008; Beer *et al.*, 2015; Monteiro and Santos, in press)

| Risk Type / aetiological agent | | Host/ environmental reservoir | Route of transmission | Symptoms |
|---|---|---|---|---|
| **Viral** | | | | |
| Human Adenoviruses (HAdV) | | Human | Faecal-Oral, respiratory | Respiratory disease, gastroenteritis, conjuntivitis |
| Aichi viruses | | Human | Faecal-Oral | Gastroenteritis |
| Astroviruses | | Human | Faecal-Oral | Gastroenteritis |
| Enteroviruses (EntV) | Coxsackievirus A, B virus<br>Echovirus<br>Poliovirus | Human | Faecal-Oral | Herpangina, meningitis, fever, respiratory disease, hand-foot-and-mouth disease, myocarditis, heart anomalies, rush, pleurodynia, diabetes |
| | | Human | | Meningitis, fever, respiratory disease, rush, gastroenteritis |
| | | Human, mammals | | Paralysis, meningitis, fever, poliomyelitis |
| Hepatitis A viruses (HAV) | | Human | Faecal-Oral | Hepatitis |
| Hepatitis E viruses (HEV) | | Human (zoonotic) | Faecal-Oral, zoonoses | Hepatitis |
| Noroviruses (NoV) | | Human | Faecal-Oral, person-to-person contact | Gastroenteritis |
| Rotaviruses (RoV) | Rotavirus A, B, C | Human | Faecal-Oral | Gastroenteritis |

**Table 1.2 (continued) -** Pathogens and toxins associated with waterborne diseases (Hunter, 2003; Bosch *et al.*, 2008; Beer *et al.*, 2015; Monteiro and Santos, in press)

| | | | | |
|---|---|---|---|---|
| Sapovirus (SoV) | | Human | Faecal-Oral | Gastroenteritis |
| **Bacterial** | | | | |
| *Campylobacter* | | Humans, rodents, mammals and birds | Faecal-Oral. Faecally contaminated waters and food | Campylobacteriosis – diarrhoea, cramping, abdominal pain, and fever. May be asymptomatic. Occasional spreading to bloodstream causing serious life-threatening infection |
| *Escherichia coli* | VTEC | | Faecal-Oral | Stomach cramps, diarrhoea (often bloody), vomiting, haemolytic uremic syndrome |
| | ETEC | | | Profuse watery diarrhoea, abdominal cramping, fever, nausea, vomiting |
| *Legionella* | | Environmental waters, wastewaters, manmade environment | Water – usually airborne (hot tubs, cooling towers, hot water tanks, large plumbing systems, air-conditioning) | Pontiac fever and Legionnaires' disease |
| *Mycobacterium avium* complex (MAC) | *M. avium* | Survive and grow in infected animal macrophages. Ubiquitous in the environment – soils, natural waters, drinking water | Oral and aerosol, environmentally acquired. | Disseminated infection usually associated with HIV infection. Less commonly, pulmonary colonisation in non-immunocompromised persons. Cervical lymphadenitis in children. |
| | *M. intracellulare* | | | |

**Table 1.2 (continued) -** Pathogens and toxins associated with waterborne diseases (Hunter, 2003; Bosch *et al.*, 2008; Beer *et al.*, 2015; Monteiro and Santos, in press)

| | | | | |
|---|---|---|---|---|
| *Salmonella* | *S.* serotype Typhimurium and Enteritidis | | Faecally contaminated waters, eggs | Salmonellosis – diarrhoea, fever and abdominal cramps |
| *Shigella* | | | Faecal-Oral, person-to-person, contaminated food | Dysentery, diarrhoea, fever, stomach cramps |
| *Vibrio* | *V. cholera* | | Faecal-Oral – contaminated water and food | Cholera – watery diahrroea, vomiting, circulatory collapse and shock. Asymptomatic to deadly |
| *Yersinia* | | | Faecal-Oral, contaminated food | Yersiniosis – fever, abdominal pain, and diarrhoea often bloody |
| **Parasite** | | | | |
| *Acanthamoeba* | | Human, free-living cells | Commonly found in lakes, swimming pools, tap water, and heating and air conditioning units | Granulomatous Amoebic Encephalitis (GAE), eye infection |
| *Cryptosporidium* | *C. parvum* | Domestic livestock, predominantly cattle, Human | Faecal-Oral, zoonotic, faecally contaminated waters | Cryptosporidiosis – stomach cramps or pain, dehydration, nausea, vomiting, fever, weight loss |
| | *C. hominis* | Human | Faecal-Oral, faecally contaminated waters | |
| | *C. meleagridis* | Zoonoses, Human | Faecal-Oral, zoonotic | |

**Table 1.2 (continued) -** Pathogens and toxins associated with waterborne diseases (Hunter, 2003; Bosch *et al.*, 2008; Beer *et al.*, 2015; Monteiro and Santos, in press)

| | | | | |
|---|---|---|---|---|
| *Cyclospora* | *C. cayetanensis* | Human | Faecal-Oral, faecally contaminated waters and food | Cyclosporiasis – watery diarrhoea, cramping, bloating,nausea, fatigue, occasional vomiting and low-grade fever |
| *Entamoeba* | *E. hystolitica* | Human | Faecal-Oral, faecally contaminated waters and food | Amoebic dysentery, fever, diarrhoea. May be asymptomatic |
| *Giardia* | *G. intestinalis* | Human, zoonoses – found in dogs, cats, primates, beavers | Faecal-Oral, faecally contaminated waters, zoonotic | Giardiasis – diarrhoea, stomach or abdominal cramps, nausea/vomiting. May be asymptomatic |
| *Naegleria* | *N. fowleri* | Free-living cells | Present in contaminated waters – warm fresh water (e.g. lakes, rivers, hot springs) | Primary Amoebic Meningoencephalitis (PAM) – fever, nausea, vomiting, coma, hallucinations, seizure, altered mental status |
| *Toxoplasma* | *T. gondii* | Cats – definitive hosts; Warm blood animals including humans and birds – intermediate hosts | Foodborne, zoonotic, mother-to-child, more rarely organ transplant and infected blood via transfusion | Toxoplasmosis. Immunocompetent individuals may present fever, lymphadenopathy, muscle aches, and headache. In pregnant infected women, the risk of miscarriage or stillborn child is present. Congenitally infected children may suffer mental retardation. Immunosuppressed patients may suffer encephalitis |
| Phytoplankton | | | Found in the aquatic environment | Rash, hives, skin blisters, sore throat, asthma-like symptoms, or allergic reactions. |

**Table 1.2 (continued) -** Pathogens and toxins associated with waterborne diseases (Hunter, 2003; Bosch *et al.*, 2008; Beer *et al.*, 2015; Monteiro and Santos, in press)

| | | | | |
|---|---|---|---|---|
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| Phytoplankton | | | Found in the aquatic environment | Rash, hives, skin blisters, sore throat, asthma-like symptoms, or allergic reactions. |

**Table 1.2 (continued) -** Pathogens and toxins associated with waterborne diseases (Hunter, 2003; Bosch *et al.*, 2008; Beer *et al.*, 2015; Monteiro and Santos, in press)

| | | | | |
|---|---|---|---|---|
| Chemical – Toxins | Endotoxins | Gram-negative bacteria and cyanobacteria | Found in treated drinking water, water for dyalisis | Septicemias, meningites, respiratory problems (asthma), autoimmune diseases, and pneumonia, among others |
| Cyanobacteria | Anatoxin-a | Cyanobacteria and some strains of *Anabaena* | Found in environmental waters, drinking waters | Neurotoxicity. Symptoms begin within 5 min of ingestion and include cyanosis, convulsions, cardiac arrhythmia, and respiratory paralysis leading ultimately to death by suffocation. Non-lethal human poising manifests as gastrointestinal disorders (e.g., nausea, vomiting, diarrhoea). |
| | β-*N*-methylamino-L-alanine (BMAA) | Cyanobacteria | Found in fresh, marine and drinking waters, food, and soil | Neurotoxicity – amyotrophic lateral sclerosis/parkinsonism dementia complex |
| | Cylindrospermopsin | Cyanobacteria | Found in environmental waters. Ingestion of contaminated waters. | Initial stage of toxicity includes anorexia, constipation, vomiting, fever, headache, abdominal pain. In the later stage acidotic shock, bloody diarrhoea and hyperemic or bleeding mucous membrane indicating adverse effect to the liver and kidneys. |
| | Microcystins | Cyanobacteria | Found in contaminated fresh waters, drinking water | Liver toxicity, liver tumour promotion |

### 1.4. Waterborne and water-related outbreaks

The removal of viruses and protozoa during municipal wastewater treatment is generally not as great as that for bacteria and these organisms may subsequently be released into the environment in treated effluents, often in such low numbers as to make their detection difficult, though in numbers high enough potentially to cause infection in humans. A high number of outbreaks of waterborne disease in Europe and in the US have been associated with drinking water contaminated with enteric enteric pathogens potentially originating in municipal wastewaters (EFSA and ECDC 2010, 2011, 2012, 2013, 2014; Beer *et al.*, 2015).

Data collected by the Centers for Disease Control and Prevention (CDC) in Atlanta (US) have shown that in spite of the major advances accomplished during the last few decades with regard to sanitation and water management, several water-related outbreaks still have occurred in the US (Beer *et al.*, 2015). In Europe, several outbreaks were reported during the period 2008 and 2012, as can be seen in Table 1.3 (EFSA and ECDC 2010, 2011, 2012, 2013, 2014). It is interesting to note that a large number of outbreaks have been linked to drinking water distribution systems. From 2009 to 2012, a total of 62 outbreaks were recorded, with 42,744 cases and 122 corresponding hospitalisations. *Cryptosporidium* was the aetiological agent responsible for the highest number of cases and hospitalisations, 32,711 (76%) and 49 (40%), respectively.

**Table 1.3 -** List of worldwide reported and verified waterborne outbreaks from 2008 to 2012 (EFSA and ECDC 2010, 2011, 2012, 2013, 2014)

| Agents | Country | Setting | Strong-evidence outbreaks | | | | Additional information |
|---|---|---|---|---|---|---|---|
| | | | N | Cases | Hospitalised | Deaths | |
| **2012** | | | | | | | |
| Calicivirus –Norovirus (NoV) | Denmark | | 1 | 183 | 0 | 0 | Contaminated drinking water after repairment work on a water pipe |
| | Greece | School, kindergarten | 1 | 79 | 0 | 0 | Tap water in a primary school |
| Calicivirus - Sapovirus (SoV) | Finland | Household/domestic kitchen | 1 | 225 | 0 | 0 | Water distribution system |
| *Cryptosporidium – C. parvum* | Ireland | Disseminated cases | 1 | 11 | 3 | 0 | Treated public surface water surface |
| *Escherichia coli* (EC), pathogenic – Verotoxigenic EC (VTEC) – VTEC 0157 | Ireland | Household/domestic kitchen | 2 | 2 | 1 | 0 | |
| | | Household/domestic kitchen | 1 | 2 | - | - | Private water supply |
| | | Disseminated cases | 1 | 27 | - | - | Treated well water |
| | | Household/domestic kitchen | 3 | 3 | 2 | 0 | Well water |
| | | Other setting | 1 | 6 | 0 | 0 | Well, untreated groundwater |
| EC, pathogenic – VTEC – VTEC 026 | Ireland | Household/domestic kitchen | 1 | 1 | 0 | 0 | |
| | | Household/domestic kitchen | 1 | 2 | - | 0 | Well, groundwater |

**Table 1.3 (continued)-** List of worldwide reported and verified waterborne outbreaks from 2008 to 2012 (EFSA and ECDC 2010, 2011, 2012, 2013, 2014)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Rotavirus | Greece | Household/domestic kitchen | 1 | 552 | 2 | 0 | Treated tap water from a rural area's water supply system |
| Unknown | Finland | Household/domestic kitchen | 1 | 20 | 0 | 0 | |
| **EU Total** | | | **16** | **1,113** | **8** | **0** | |
| **2011** | | | | | | | |
| Calicivirus – including NoV | Finland | Household/ domestic kitchen | 3 | 54 | 0 | 0 | In one outbreak, water treatment failure was reported as well as the isolation of *Campylobacter* |
| | | Other setting | 1 | 8 | 0 | 0 | |
| *Campylobacter – C. jejuni* | Belgium | Temporary mass catering (fairs, festivals) | 1 | 64 | 0 | 0 | Unprocessed contaminated ingredient |
| | Finland | Household/ domestic kitchen | 1 | 10 | 0 | 0 | |
| *Cryptosporidium hominis* | Sweden | Disseminated cases | 1 | 20,000 | 46 | 0 | |
| EC, pathogenic – VTEC – VTEC O157 | Ireland | Household/ domestic kitchen | 1 | 3 | - | - | |
| | | Disseminated cases | 1 | 20 | 7 | 0 | Ground water source suspected to have been contaminated with animal faeces. |
| | | Household/ domestic kitchen | 1 | 2 | 0 | - | |

**Table 1.3 (continued)-** List of worldwide reported and verified waterborne outbreaks from 2008 to 2012 (EFSA and ECDC 2010, 2011, 2012, 2013, 2014)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Unknown | Finland | Household/ domestic kitchen | 1 | 6 | 0 | 0 | Water distribution system |
| **EU Total** | | | **11** | **20,167** | **53** | **0** | |
| **2010** | | | | | | | |
| Calicivirus – including NoV | Belgium | Disseminated cases | 1 | 3,000 | - | - | |
| | Finland | Well water | 1 | 17 | 0 | 0 | |
| | Ireland | Restaurant, café, pub, bar, hotel | 1 | 50 | 0 | 0 | |
| | Sweden | Disseminated cases | 3 | 1,015 | 0 | 0 | |
| | | Restaurant, café, pub, bar, hotel | 1 | 40 | 0 | 0 | |
| *Campylobacter* spp., unspecific | Denmark | Tap water including well water | 1 | 400 | 0 | 0 | |
| | Denmark [1] | Seawater | 1 | 400 | 0 | 0 | Seawater swallowed during swimming at a triathlon event |
| | United Kingdom | Private drinking water supply | 1 | 44 | 0 | 0 | |
| *Salmonella* Enteritidis | Poland | Household/ domestic kitchen | 1 | 11 | 4 | 0 | |
| Other bacterial agents | Poland | Restaurant, café, pub, bar, hotel | 2 | 56 | 0 | 0 | |
| *Cryptosporidium hominis* | Sweden | Disseminated cases | 1 | 12,700 | 0 | 0 | |

**Table 1.3 (continued)-** List of worldwide reported and verified waterborne outbreaks from 2008 to 2012 (EFSA and ECDC 2010, 2011, 2012, 2013, 2014)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **EU Total**<br>[1]Other agents detected: ETEC, *Giardia* | | | **14** | **17,733** | **4** | **0** | |
| **2009** | | | | | | | |
| Calicivirus – including NoV | Finland | Untreated well water | 1 | 74 | 0 | 0 | |
| | | Waste water leakage | 1 | 117 | 0 | 0 | |
| | Sweden | Unknown | 1 | 173 | 0 | 0 | |
| *Campylobacter – C. jejuni* | Denmark | Other setting | 1 | 500 | 3 | 0 | |
| | Greece | Treated tap water | 1 | 60 | 14 | 0 | Treated tap water of a rural area's water supply system on a Greek island was the implicated foodstuff of this outbreak |
| *Campylobacter* spp. | France | Unkown | 1 | 11 | 1 | 0 | |
| EC, pathogenic – VTEC – VTEC O157 | Ireland | Household | 4 | 8 | 3 | 0 | |
| EC, pathogenic | Sweden | Unknown | 1 | 4 | 0 | 0 | |
| *Shigella flexneri* | France | Unknown | 1 | 4 | 4 | 0 | |
| *Shigella* spp., unspecified | France | Unknown | 1 | 15 | 10 | 0 | |
| Unknown | France | Unknown | 1 | 15 | 10 | 0 | |
| | Poland | Household | 1 | 6 | 0 | 0 | |
| **EU Total** | | | **15** | **987** | **45** | **0** | |
| **2008** | | | | | | | |

**Table 1.3 (continued)-** List of worldwide reported and verified waterborne outbreaks from 2008 to 2012 (EFSA and ECDC 2010, 2011, 2012, 2013, 2014)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Campylobacter – C. jejuni* | Switzerland | Distribution system | 1 | 185 | - | 0 | Technical problem in the tap water distribution system |
| *Campylobacter* spp. | France | Camp, picnic | 1 | 15 | 0 | 0 | |
| EC | France | Other setting | 1 | 8 | 6 | 0 | |
| EC, pathogenic – VTEC – VTEC 0157 | Ireland | Private well | 3 | 14 | 5 | 0 | Contamination of family private well |
| Calicivirus – including NoV | Sweden | | 1 | 2,000 | - | - | |
| | Hungary | Distribution system | 1 | 597 | 4 | 0 | The plumbing of the township was very old with no sewage system. Consequently, the tap water was contaminated through leakage in the water pipes. |
| *Shigella flexneri* | France | Household | 1 | 3 | 3 | 0 | |
| *Francisella tularensis* | Norway | Private water | 1 | 15 | 3 | 0 | The outbreak was caused by contamination pf the private water sources with dead rodents or infected rodent faeces. The agent was only isolated from human cases. |
| *Salmonella* Enteritidis | Spain | Household | 1 | 8 | 0 | 0 | Private water supply supplying more than one household. |
| *Salmonella* typhimurium | Spain | Household | 1 | 2 | 1 | 0 | Individual household supply (private well). |
| **EU Total** | | | **12** | **2,847** | **22** | **0** | |

*Escherichia coli* is shown to be responsible for the highest percentage of outbreaks (35%), with a low number of cases (0.24%), but high levels of hospitalisations (20%). Enteric viruses caused twenty outbreaks (32%), with 8,184 reported cases (19%) but with low levels of hospitalisations (5%). No deaths were reported during this period.

The high number of waterborne outbreaks emphasises the need for better and improved treatments and disinfection procedures, as well as the need for appropriate monitoring tools.

### 1.5. Behaviour of viruses in wastewater treatment and disinfection

If an indicator is to be of use, either for identifying sources of faecal contamination or for indicating the potential presence of pathogens, then it is important that it survives wastewater treatment at least as well as the pathogen or pathogens that it is representing. Treatment at wastewater treatment plants (WWTP) is crucial for the removal of pollutants (especially biological pathogens) and in Europe and North America commonly consists of a range of physical, biological and chemical processes, including sedimentation ('primary treatment'), and either activated sludge or trickling filters ('secondary biological treatment'). The determination of microbiological removal efficiency within a WWTP has traditionally been based on the detection of faecal indicator bacteria that tend to be less resistant to treatment than are enteric viruses and certain protozoan pathogens. Therefore, in order to protect human health it is also important to understand why viruses are capable of withstanding treatment and disinfection and to elucidate the mechanisms behind their survival.

Enteric viruses are mostly negatively charged at neutral pH, and are found in two different living forms: free-floating or adsorbed to suspended solids. The use of physical processes in drinking water treatment plants, such as coagulation, flocculation, sedimentation and filtration helps to remove viruses in water by removing the particles to which they are attached (Templeton *et al.,* 2008). However, these processes do not ensure the removal of particles with diameters less than 10 μm, which will act as a shield for viruses against further aggressions from disinfection treatments. Studies using bacteriophages (as surrogates for enteric viruses) have demonstrated that the removal of these microorganisms by physical processes is no more than about 90 to 99% (1 log removal only) (Payment *et al.,* 1986; Nieuwstad *et al.,* 1988; Havelaar *et al.,* 1993; Lucena *et al.,* 2004; Harwood *et al.,* 2005; Lodder and Husman 2005; Mandilara *et al.,* 2006; Costán-Longares *et al.,* 2008; Ebdon *et al.,* 2012). According to Schijven and Hassanizadeh 2000, the efficiency of

removal using physical procedures is greatly affected and dependent on viral adsorption affinity to adsorbents. Several possible adsorbents exist naturally in waters, including sand, bacterial cells, suspended colloids, clay, and sediments (Templeton *et al.,* 2008).

The removal rates depend not only on the adsorption of viruses, but also on physicochemical parameters, such as substrate saturation, dissolved oxygen and redox potential. They also depend to a considerable degree on pH due to the properties of viral capsids (outer shell of the virus). The capsids of most viruses are composed of proteins that give the virus a net charge and which are related to the presence of amino acids containing carboxylic and amino groups, namely histidine, tyrosine, glutamic acid and aspartic acid. At pH levels above 5.0, and as a result of the presence of these particular amino acids, the capsid is negatively charged. However, the electronic cloud changes to positive at pH levels below 5.0 (Olson *et al.*, 2005). Therefore, the attachment of viruses to adsorbents occurs through the existence of electrostatic forces, which are influenced by the isoelectric point of the virus, of the absorbent particle and of its hydrophilic level. The flow rate and ionic strength also been suggested (Charles *et al.,* 2008) to play an important role in the adsorption of viral particles to suspended solids. At higher flow rates, the capacity of viral adsorption is limited as they have less time in contact with the adsorbents. Rainfall has low ionic strength and so has the capacity to detach viruses, whereas high ionic strength waters, such as septic tank effluents, increase the potential for viral adsorption (Charles *et al.*, 2008).

Physical treatment processes can be coupled with disinfection processes to improve the removal of pathogens in wastewaters, but these disinfection treatments again tend to remove bacteria more effectively than they do enteric viruses (Tyrrell *et al.*, 1995; Tree *et al.,* 1997; Gehr *et al.,* 2003; Jacangelo *et al.,* 2003; Costán-Longares *et al.,* 2008; Gomila *et al.,* 2008). Chlorine is the disinfection procedure used most regularly, but others, such as ozone, peracetic acid and UV light are also widely available (Mezzanotte *et al.,* 2007). The efficacy of disinfection is highly influenced/reduced by the presence of even low levels of suspended solids, as these methods require direct contact between the chemical compounds, or low-wavelength photons (UV light) and the organism (viruses). A comprehensive knowledge and understanding of the inactivation processes of viruses is rather difficult to achieve because of the different disinfection kinetics often observed in strikingly similar viruses when using the same biocide (Dennis *et al.,* 1979; Brien and Newman 1979; Floyd and Sharp 1979; Sharp and Leong 1980; Nuanualsuwan and Cliver 2003; Li *et al.,* 2004; Baxter *et al.,* 2007; Hotze *et al.,* 2009; Cromeans *et al.,* 2010; Sano *et al.,* 2010; Wigginton *et al.,* 2010).

The differences in performance of chemical and physical disinfection treatments highlighted above poses a serious question as to their efficiency and ability to remove the broad range of viral pathogens that may be present in wastewaters and receiving waters.

### 1.6. Enteric viruses health risks

To complicate matters further, risk to human health varies from person to person (as a result of the individual immune response and general level of health of each individual), so that the infectious dose required to cause illness also differs from person to person. Infections with waterborne pathogens range from asymptomatic to fatal (Table 1.4). The most common illness is gastroenteritis, followed by skin infections.

**Table 1.4 -** Probability of human infection from exposure to one virus and the dose needed for 1% chance of infection and mortality rates for human enteric viruses in industrialised countries (adapted from Bosch, 1998; WHO, 2014d)

| Virus | Probability of infection from exposure to one virus | Dose required for 1% chance of infection |
|---|---|---|
| Poliovirus 1 | 0.0149 | 0.67 |
| Poliovirus 2 | 0.0310 | 0.32 |
| Echovirus 12 | 0.0170 | 0.59 |
| Rotavirus | 0.3100 | 0.03 |
| Virus | Mortality rate (%) | |
| Poliovirus 1 | 0.90 | |
| Coxsackievirus A | 0.12 – 0.50 | |
| Coxsackievirus B | 0.59 – 0.94 | |
| Echovirus | 0.27 – 0.29 | |
| Hepatitis A | 0.20 | |
| Hepatitis E | 0.5 – 4.0 | |
| Rotavirus | 0.01 – 0.12 | |
| Norovirus | 0.0001 | |
| Adenovirus | 0.01 | |

In industrialised nations, outbreaks of human waterborne infectious disease are for the most part non-fatal. However, some can be serious and cause fatalities, with Hepatitis E virus having the highest mortality rate (Bosch (1998); WHO, 2014e). In addition, the extent of waterborne outbreaks is under reported makes it difficult to ascertain the

relationship between disease and water exposure. In low-income countries, waterborne disease continues to cause high levels of infantile mortality (WHO, 2014a).

### 1.7. Sources of faecal contamination and routes of transmission

Water-related illnesses are associated with exposure to a wide range of environmental matrices. Whilst waterborne pathogens are mainly transmitted via the faecal-oral route, the processes of transmission from the first shedding until the new host can be extremely complex and involve a number of different matrices, including food and water transmission or even through fomites. Consequently, waterborne pathogens can be spread via numerous pathways, not only through waters used for drinking or recreational purposes but also through direct contact from person-to-person, or through contaminated fomites (surfaces) or aerosols. Water used for agricultural purposes is another area of concern, as crops may be irrigated with faecally contaminated waters, increasingly within managed wastewater reuse systems. Moreover, shellfish grown and harvested in contaminated waters are though to be one of the main routes for pathogen transmission, as well as an important source of gastroenteritis and hepatitis outbreaks (Sánchez *et al.,* 2002; Le Guyader *et al.,* 2006). Whilst drinking water may no longer pose a major threat to human health in many more economically developed countries (MEDCs), the contamination of foodstuffs with faecal material can have a significant impact both in less economically developed countries (LEDCs) and MEDCs as a consequence of global commerce (Ashbolt, 2004; Le Guyader *et al*., 2006; Widdowson and Vinjé, 2008; Verhoef *et al*., 2010).

Faecal contamination of water bodies is commonly a blend of contamination from different sources: both point- and non-point (diffuse) sources. Figure 1.2 exemplifies the various sources of faecal contamination commonly found in surface and ground waters. Contamination inputs originating from point sources, most commonly treated wastewater effluents, tend to be more easily detected and addressed by legislation, such as EU Directives that are then transposed to legislation within each EU Member State, e.g., the Urban Wastewater Treatment Directive (91/271/EEC, CEC, 1991) and the Water Framework Directive (2000/60/EC, CEC, 2000), whereas non-point sources, such as urban run-off, illegal uncontrolled spillage, and inputs from domestic or wild animals (e.g., avian sources) can be also be more difficult and costly to manage.

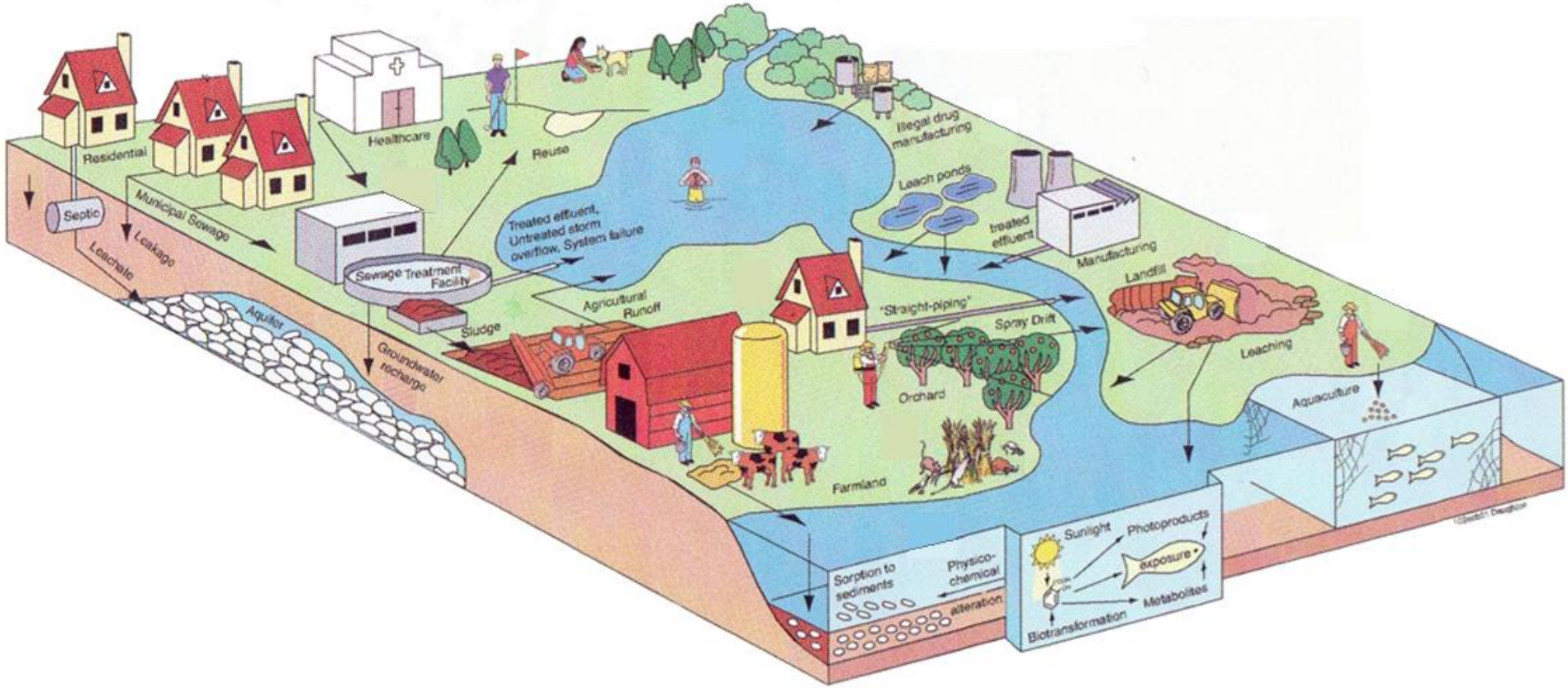


**Figure 1.2 -** Sources through which surface and groundwaters can become contaminated (US EPA, 2006a)

### 1.7.1. Point source faecal contamination (PS)

The U.S. Environmental Protection Agency (EPA) defines point source contamination as "any single identifiable source of pollution from which pollutants are discharged, such as a pipe, ditch, or ship" (Hill, 1997). Common point sources of faecal contamination include industrial and andmunicipal wastewater discharges. In industrialised countries, some factories (e.g., paper mills) often have their own treatment facilities, whereas others send their waste to WWTP to be processed, prior to release into receiving waters. In many places (e.g., the UK), combined sewer systems (CSS) are widely used. CSS may simultaneously contain both wastewater and water resulting from urban run-off.

Farm drainage systems are sometimes intentionally, or unintentionally connected (or misconnected) to domestic sewers that were designed principally to receive municipal wastewater. Therefore, domestic sewage can contain not only anthropogenic pollution but also a component (albeit smaller) of contamination from non-human sources, including agricultural inputs (e.g., livestock) and surface run-off (e.g., wildlife). The incorporation of both types of faecal contamination into one sewer makes the correct distinction of human and non-human sources even more challenging. This means that even though municipal wastewaters are often considered as point sources of faecal contamination, they may in fact also include contamination from non-point sources.

### 1.7.2. Non-Point source faecal contamination (NPS)

Because of their unpredictability, non-point sources are much harder to identify and to control than point sources (Figure 1.3). Non-point sources (NPS), or diffuse sources of contamination are mostly associated with agricultural activities, wildlife, and run-off and are generally non-anthropogenic in origin (though leaking septic tanks may also be regarded as an additional diffuse source in rural catchments). Regardless of the type of source, contamination of water bodies by human and/or non-human faecal material can increase the probability of the onward transmission of viral, protozoan and bacterial pathogens presenting different levels of risk to human health.

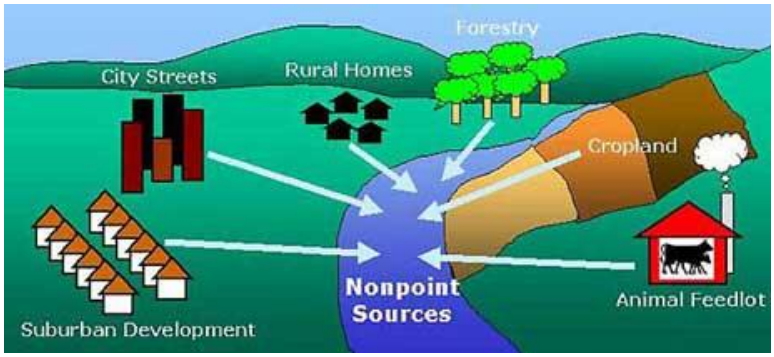


**Figure 1.3 -** Non-point sources of faecal contamination (adapted from NOAA, 2008)

Besides the presence of pathogens associated with wastewaters, zoonotic pathogens may also be transmitted from diffuse animal sources to humans, leading to zoonotic infections, such as hepatitis E, toxoplasmosis or cryptosporidiosis and giardiasis (WHO/FAO/OIE, 2004).

The proportion of non-point and point sources of faecal pollution at any given time is highly variable, both spatially and temporally and the levels and type of pathogens associated with both sources is highly dependent on the health state of the population, which varies seasonally, annually, and geographically.

In Portugal, few measures have been adopted to mitigate the issues arising from diffuse pollution apart from the existence of combined sewer systems. As mentioned, these systems are designed to collect domestic sewage, industrial wastewater and rainwater run-off in the same pipe. They are generally transported to a WWTP, prior to release into the receiving waters following treatment. Nevertheless, combined sewers are often of insufficient size to transport the volume of run-off associated with heavy rainfall events. Therefore, the wastewater volume in the combined sewer exceeds the capacity of the sewer system and/or the treatment plant. For this reason, combined sewers are designed to overflow and discharge the excess volume directly into the water bodies. These overflows, named combined sewer overflows (CSO's, considered a point source of faecal contamination), can contain high loads of contaminants not only from urban run-off but also from untreated human and industrial waste, toxic materials, and debris, posing a potentially serious threat to human and animal health.

### 1.7.3. The Urban Wastewater Treatment Directive (UWWTD)

The UWWTD (CEC, 1991) requires secondary treatment, or equivalent treatment at WWTP for agglomerations with a population equivalent (p.e.) greater than 10,000 and is

more stringent when the discharges are made into freshwater and estuaries (with a lower cut off at 2,000 p.e.). The UWWTD also establishes a stricter treatment for wastewaters discharged into sensitive areas. Table 1.5 illustrate the most recent situation in 27 EU Member States with regard to the implementation levels of the UWWTD (adapted from the EEA, 2015). With regards to provisions in Article 3 (collecting systems), Article 4 (biological treatments) and Article 5 (more stringent treatment, applied to sensitive areas) of the UWWTD, the EU as a whole has to reached 94%, 82% and 77%, respectively (CEC, 2012). Countries such as Austria, Germany and the Netherlands have reached levels of 100% of compliance with the three articles of the UWWTD. On the other hand, other countries have attained consistently rates below 20% for all articles (namely, Bulgaria, Cyprus, and Latvia). Although the situation in Portugal has improved over the past five years, with regard to compliance with Article 3 (reaching 97%), much remains to be done to address Articles 4 and 5, where compliance is only 47 % and 20%, respectively. In the EU as a whole, improvements still need to be achieved with regard to all three articles but attention should perhaps be focussed on addressing Articles 4 and 5, where compliance rates are still very low for several countries (including Portugal) (CEC, 2012).

Although, the use of tertiary processes (such as UV, or ozonation) during wastewater treatment has been increasing in Europe and in the US in recent years, further changes are still necessary to guarantee discharge of wastewater into the river catchments that is less likely to affect the health of downstream users.

**Table 1.5 -** National compliance rates with Articles 3, 4 and 5 of the EU UWTD (2009-2010) (colours show extent of compliance: red, 0%-20%; orange, >20%-40%; yellow, >40%-60%; green, >60%-80%; blue, >80%-100%; white, no data or transition period still pending) (data adapted from CEC (2012))

| Member State | Article 3 compliance rate (%) | Article 4 compliance rate (%) | Article 5 compliance rate (%) |
|---|---|---|---|
| Austria | 100 | 100 | 100 |
| Belgium | 78 | 73 | 52 |
| Bulgaria | 15 | 6 | 2 |
| Cyprus | 0 | 0 | 0 |
| Czech Republic | 100 | 81 | 20 |
| Denmark | 100 | 99 | 94 |
| Estonia | 30 | 31 | 21 |
| Finland | 100 | 97 | 97 |
| France | 96 | 84 | 87 |
| Germany | 100 | 100 | 100 |
| Greece | 100 | 99 | 100 |
| Hungary | 100 | 100 | 48 |
| Ireland | 100 | 40 | 2 |
| Italy | 87 | 64 | 86 |
| Latvia | 0 | 0 | 0 |
| Lithuania | 100 | 98 | 85 |
| Luxembourg | 100 | 57 | 38 |
| Malta | 100 | 5 | 0 |
| Netherlands | 100 | 100 | 100 |
| Poland | 71 | 24 | 10 |
| Portugal | 97 | 47 | 20 |
| Romania | Transition period pending | Transition period pending | Transition period pending |
| Slovakia | 100 | 90 | Transition period pending |
| Slovenia | 32 | 23 | 23 |
| Spain | 98 | 86 | 54 |
| Sweden | 100 | 99 | 87 |
| United Kingdom | 100 | 98 | 63 |
| EU 27 | 94 | 82 | 77 |

### 1.8. Microbial source tracking, tracing sources of faecal pollution

Microbial Source Tracking (MST) is still a relatively new and continually developing branch of environmental microbiology that grants scientists the ability to discriminate between different sources of faecal pollution in environmental waters. Full implementation of such tools and the information provided by them will potentially allow the implementation of more effective and targeted remediation measures, which will not only result in economic benefits, but should lead to better protection of public health and environmental resources. For example, the partial or complete closure of shellfishing harvesting areas in the US has been estimated to lead to losses of hundreds of millions of dollars annually (Meschke and Boyle, 2007). The closure and advisory of beaches and shellfishing is performed regardless of the source of contamination. However, acknowledgement of the dominant source responsible for the faecal pollution would allow for the implementation of more targeted remedial actions and would improve understanding of potential risks to human health.

The detection of all potential pathogens is time-consuming, labour-intensive, and infeasible in most parts of the world because of the need for specialised laboratories and personnel. To overcome these issues, faecal indicator organisms (FIO) have been employed over the last 150 years, with the main objective of indicating the presence of enteric bacteria, viruses and protozoa. However, it is well recognized that these microorganisms are not host-specific, occurring not only in human intestinal flora but also in many other warm-blooded animals. Moreover, *Escherichia coli* (EC) have been found in tropical and subtropical soils (Byappanahalli and Fujioka, 1998; Byappanahalli and Fujioka, 2004) and have further been isolated from coastal temperate forest soils in Indiana (Byappanahalli *et al.,* 2006). It has also been demonstrated proved that EC is able to grow in non-amended, nonsterile soils (Byappanahalli *et al.,* 2006; Ishii *et al.,* 2006). The increasingly recognised ubiquity of these organisms impairs the suitability of FIO to anticipate the presence of human or animal faecal contamination hazards and thereby to predict potential risks to human health.

However, the suitability of FIO can be significantly improved if they can be used alongside MST methodologies that are able to determine the origin of these microorganisms. The approach of using a toolbox of different organisms and techniques to predict the origin of faecal contamination is potentially powerful and may include methods, which are either library-dependent, or library-independent. The latter can be further subdivided into culture-dependent and culture-independent, as shown in Figure 1.4.

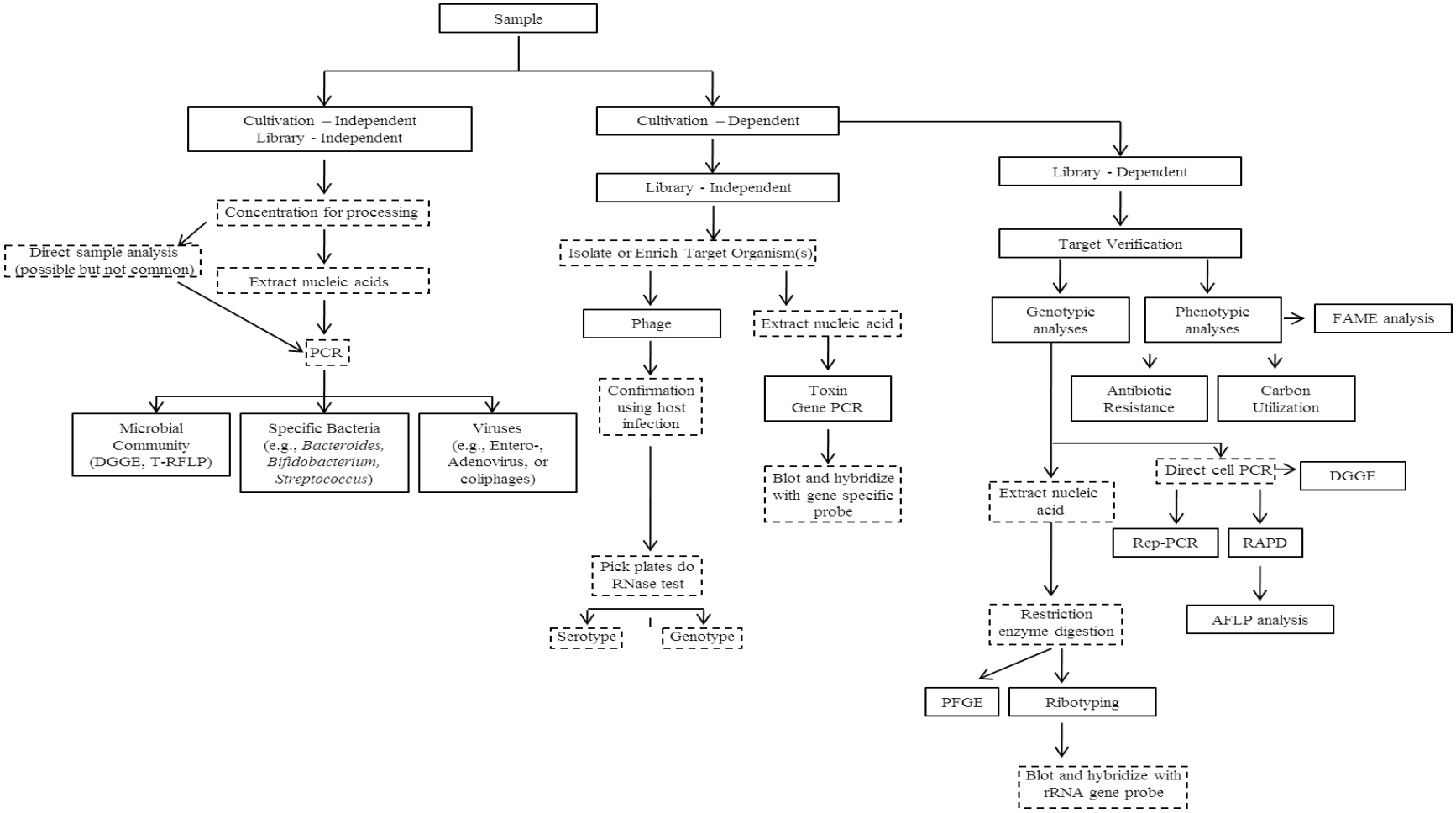


**Figure 1.4 -** Schematic portrayal of currently available MST methods (AFLP – Amplified Fragment Length Polymorphism; DGGE – Denaturing Gradient Gel Electrophoresis; FAME – Fatty Acid Methyl Ester; PFGE – Pulsed-Field Gel Electrophoresis; RAPD – Random Amplification of Polymorphic DNA; Rep-PCR – Repetitive Element Palindromic-PCR; T-RFLP – Terminal Restriction Fragment Length Polymorphism)

### 1.8.1. Library-independent approaches (LI)

The term 'library-independent' derives from the fact that the methods within this category rely solely on the presence or absence of a certain target or gene and consequently avoid the need for a 'library' of organisms or genetic markers, against which to compare the outcomes. Library-independent molecular techniques are not based on isolate-to-isolate typing of bacteria cultured from different samples, but rather rely on the direct detection of a specific, host-associated genetic marker in a DNA extracted for amplification with the Polymerase Chain Reaction (PCR) (Bernhard and Field, 2000; Carson *et al.,* 2001; Field *et al.,* 2003; Scott *et al.,* 2005). Nevertheless, some targets are present in such low levels that it is first necessary to enrich the sample or to obtain isolates. Rapid development in the genomics and biotechnological fields have permitted the application of culture-independent techniques and by avoiding the culturing steps, these methodologies tend to be expeditious and sometimes, less expensive, as they have the ability to target for a wide range of microorganisms that because of difficulties in their cultivation could not otherwise be quantifiable or detected at all.

### 1.8.2. Library-dependent approaches (LD)

The origin of 'library-dependent' methods dates back to the beginning of the 1970's and has its roots in research performed with *E. coli*, centred around population biology and antibiotic resistance patterns (Milkman, 1973; Cooke, 1976; Bell, 1978; Bell *et al.,* 1983; Krumperman, 1983; Ochman *et al.,* 1983; Caugant *et al.,* 1984; Kaspar *et al.,* 1990). Most culture-dependent methods demand the creation of a reference 'library', which is conceived by using isolates from specific and known hosts (or known environmental sources). A library is in this particular subject, a cluster of microorganisms from different faecal sources and/or environments. The majority of the isolates are obtained from the faeces themselves, whether by collecting the faeces directly from the animal (e.g., via direct rectal retrieval) or by collecting as soon as possible after voiding to avoid cross-contamination. The collection of isolates from environmental samples may be, nonetheless, more representative in terms of the survival of the microorganisms which would provide a higher degree of representative isolates more likely to be detected in the environment. Initial library-dependent studies relied on the commonly-used indicator bacteria (faecal coliforms, *E. coli*, faecal enterococci, or coliphages) for the construction of an isolate or host origin database (Dombek *et al.,* 2000; US EPA, 2005a). After isolation, further characterisation was needed. This could be achieved either using genotypic or phenotypic

approaches (Wiggins, 1996; Parveen *et al.*, 1999; Hagedorn *et al.*, 1999; Dombek *et al.*, 2000; Harwood *et al.*, 2000; Carson *et al.*, 2001).

The isolates are compared to their category source, either by subtype matching, or by using statistical methods (Wiggins, 1996; Hagedorn *et al.*, 1999; Parveen *et al.*, 1999; Dombek *et al.*, 2000; Harwood *et al.*, 2000, 2003; Ritter *et al.*, 2003; Meays *et al.*, 2006). Most libraries are collected in a certain geographical area and temporal setting, and issues have been raised mainly regarding library sizes, different sampling and analysis methods. A guide to available MST library-dependent and library–independent methods is presented in Tables 1.6, and 1.7. The MST methods with immediate pertinence to the programme of research presented herein are discussed in greater detail in Chapter Two.

**Table 1.6 -** Library-based phenotypic and genotypic methods (US EPA 2005a; Hagedorn et al. 2011)

| **Method** | **Advantages** | **Disadvantages** | **References** |
|---|---|---|---|
| Amplified Fragment Length Polymorphic DNA (AFLP) analysis | Highly reproducible; Robustness | Labour-intensive; Requires specialized personnel; Costly; Library dependent; Libraries geographically and temporally specific | Vos *et al.*, 1995; Blears *et al.*, 1998; US EPA, 2005a; Yan and Sadowsky, 2007 |
| Antibiotic Resistance Analysis (ARA) | Expeditious; easy to perform; Low cost | Library dependent; Libraries geographically and temporally specific | Scott *et al.*, 2002; Simpson *et al.*, 2002; Meays *et al.*, 2004; US EPA, 2005a; Ebdon and Taylor, 2006; Mott and Smith, 2011 |
| Carbon Utilization Profiles (CUP) | Expeditious; easy to perform<br>Standardized | Library dependent; Libraries geographically and temporally specific; Stability; results often inconsistent | Konopka *et al.*, 1998; Hagedorn *et al.*, 2003 |
| Denaturating Gradient Gel Electrophoresis (DGGE) | Fingerprinting of total community | Library independent; Suitability of selected gene target; Technically challenging | Farnleitner *et al.*, 2000; Meays *et al.*, 2004; D'Elia *et al.*, 2007; Neave *et al.*, 2014 |
| Fatty Acid Methyl Ester (FAME) analysis | Potentially may require a small library | Library independent; Still at the proof-of-concept and biological likelihood level of testing | Seurinck *et al.*, 2006; Duran *et al.*, 2006, 2009; Field and Samadpour, 2007 |
| Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectroscopy (MALDI-TOF-MS) | Expeditious | Limited information on the method | Siegrist *et al.*, 2007; Giebel *et al.*, 2008 |

**Table 1.6 (continued)-** Library-based phenotypic and genotypic methods (US EPA 2005a; Hagedorn et al. 2011)

| | | | |
|---|---|---|---|
| Pulse-Field Gel Electrophoresis (PFGE) | Extremely reproducible; High sensitivity; Highly discriminatory | Library dependent; Labor-intensive and time-consuming<br>Libraries may be geographically and temporally specific; Requires an extensive library due to sensitivity | Olive and Bean, 1999; Scott *et al.*, 2002; Lu *et al.*, 2004; Furukawa *et al.*, 2011 a,b; Furukawa and Suzuki, 2013) |
| Random Amplified Polymorphic DNA (RAPD) analysis | Expeditious; easy to perform; Effective | Low levels of reproducibility<br>Libraries may be geographical and temporally specific; Lack of standardization | Hopkins and Hilton 2000 a,b; US EPA, 2005a |
| Rep-PCR | Highly reproducible; Expeditious; easy to perform; Cost effective; Less technically challenging than other genotypic methods | Libraries may be geographical and temporally specific; Moderate reproducibility | Albert *et al.*, 2003; Seurinck *et al.*, 2005a; Kon *et al.*, 2009; Lyautey *et al.*, 2010 |
| Ribotyping | Extremely reproducible; Highly sensitive<br>Can be automated | Libraries may be geographical and temporally specific<br>Expensive | Carson *et al.*, 2001, 2003; Hartel *et al.*, 2002; Moore *et al.*, 2005 |

**Table 1.7 -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| Animal Host | Target Organism | Target | Advantages | Disadvantages | References |
|---|---|---|---|---|---|
| Human | Sorbitol-fermenting *Bifidobacteria* | Cultivable anaerobic cells | Inability to reproduce; Easy to perform; Not library dependent | Survival highly variable; Rapid die-off | Mara and Oragui, 1983; Rhodes and Kator, 1999; Blanch *et al.*, 2004; Plummer and Long, 2007; Venegas *et al.*, 2015 |
| | *Bifidobacterium adolescentis*<br><br>*Bifidobacterium dentium* | 16S rRNA | Inability to reproduce; Not library dependent | Survival highly variable; Rapid die-off; False-positive/negative signal | Bonjoch *et al.*, 2004; Blanch *et al.*, 2006; Bonjoch *et al.*, 2009; Gourmelon *et al.*, 2010 |
| | *Enterococcus faecium* | *esp* gene | High specificity; Not library dependent | Detected in animal faeces; Low predominance in enterococci population | Scott *et al.*, 2005; Ahmed *et al.*, 2008a; Layton *et al.*, 2009 |
| | *Faecalibacterium* | 16S rRNA | No false-positive signal; Indication of global distribution; Not library dependent | Lack of information in field studies | Zheng *et al.*, 2009; Sun *et al.*, 2016 |
| | *Methanobrevibacter smithii* | *nifH* | Highly specific; Not library dependent | Limited application in field studies; Low sensitivity | McQuaig *et al.*, 2009, 2012; Johnston *et al.*, 2010; Ahmed *et al.*, 2012 |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | | STIb gene<br><br>STh gene | Detection of human sewage; Not library dependent | Lack of information<br>Lack of specificity<br>Lack of positive environmental samples | Oshiro and Olson, 1997; Field *et al.*, 2003; Khatib *et al.*, 2003; Jiang, *et al.*, 2007 |
| | *Lachnospiraceae* | 16S rRNA | Strong correlation to human sewage and human markers; Not library dependent | Lack of information<br>(sensitivity and specificity) | Newton *et al.*, 2011; McLellan *et al.*, 2013 |
| | *Bacteroidales* | 16S rRNA<br>*HF183* | High sensitivity and specificity; Low global diversity; Not library dependent | High level of false-positives using SYBR Green qPCR | Bower *et al.*, 2005; Seurinck *et al.*, 2005b; Shanks *et al.*, 2006a; Walters and Field, 2006; Gourmelon *et al.*, 2007; Santoro and Boehm, 2007; Ahmed *et al.*, 2008b, 2009a, b; Mieszkin *et al.*, 2009; McLain *et al.*, 2009; McQuaig *et al.*, 2009; Rosario *et al.*, 2009; Saunders *et al.*, 2009; Jenkins *et al.*, 2009; Ballesté *et al.*, 2010; Layton *et al.*, 2013; Lin and Ganesh, 2013; Green *et al.*, 2014 |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | | *BacHum* | 100 % sensitivity raw sewage samples; Not library dependent | 67 % sensitivity human faecal samples<br>Low sensitivity | Ahmed *et al.*, 2009a, b; Bae and Wuertz, 2009a, b; Jenkins *et al.*, 2009; McLain *et al.*, 2009; Silkie and Nelson, 2009; Walters *et al.*, 2009; Dick *et al.*, 2010; Schriewer *et al.*, 2010; Wang *et al.*, 2010a; Layton *et al.*, 2013; Reischer *et al.*, 2013 |
| | | *BacH* | High sensitivity (~100 %) and specificity (> 99 %); Applied in field testing; Not library dependent | Positivity in fish faeces | Reischer *et al.*, 2007, 2008, 2011, 2013; Ahmed *et al.*, 2009a, b; McLain *et al.*, 2009; Layton *et al.*, 2013 |
| | | *HuBac* | High sensitivity; Not library dependent | Low specificity | Layton *et al.*, 2006; Ahmed *et al.*, 2009a, b; McLain *et al.*, 2009 |
| | | *HumanBac I* | High sensitivity (limited number of human faecal samples); Not library dependent | Extremely low specificity; Geographically unstable | Okabe and Shimazu, 2007; Okabe *et al.*, 2007 |
| | | *BFD* | Not library dependent | Lack of information | Converse *et al.*, 2009 |
| | | *YHF* | Not library dependent | Lack of information | Jeong *et al.*, 2010 |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | | *B. thetaiotomicron* α-mannanase, *B. theta α* | Highly specific and sensitive; Detection of *B. thetaiotaomicron*, species only found in human faeces; Not library dependent | Lack of information and testing | Yampara-Iquise *et al.*, 2008 |
| | | *B. fragilis gyr*B | Highly sensitive for human fecal contamination; Not library dependent | Detection of contamination from pig origin; Lack of information and further testing | Lee and Lee, 2010 |
| | | Hypothetical protein *HumM2*<br>Putative RNA polymerase sigma factor *HumM3* | High levels of sensitivity<br>Specific; Not library dependent | Signal detected in non-human samples<br>Lack of information | Shanks *et al.*, 2009 |
| | F+ RNA Coliphages<br>Group II and III F-RNA phages | Replicase and coat protein<br>Viral Genome | Low levels of false-positive rates with viral detection approach; Specificity; Not library dependent | Low sensitivity; Identified in animal-source samples; Differential survival rates | Griffin *et al.*, 2000; Brion *et al.*, 2002; Schaper *et al.*, 2002 a,b; Cole *et al.*, 2003; Blanch *et al.*, 2006; Stewart-Pullaro *et al.*, 2006; Kirs and Smith, 2007; Wolf *et al.*, 2010; Harwood *et al.*, 2013 |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | Viruses | Pepper Mild Mottle Virus (PMMV)<br>Viral genome | Highly sensitive and specific; Major component of viral metagenome in human faeces; Not library dependent | Cross-reactivity with chicken and gull faeces and intestinal homogenates | Zhang *et al.,* 2006; Rosario *et al.,* 2009; Wong *et al.,* 2012 |
| | | Polyomaviruses JC and BK (HPyVs)<br>T antigen | Highly specific for human contamination; Highly sensitive; Not library dependent | Better concentration methods required for environmental samples | Albinana-Gimenez *et al.,* 2009a; Harwood *et al.,* 2009; McQuaig *et al.,* 2009; Hundesa *et al.,* 2010 |
| | | Noroviruses | Tests against faecal samples from humans and animals, only host-specific viruses were amplified – high level of sensitivity; Not library dependent | Variable rates of viruses shedding; Demarked seasonality | Wolf *et al.,* 2010; Harwood *et al.,* 2013 |
| Different animal sources | *Methanobrevibacter ruminantium* - ruminant | *nifh* | Specific and sensitive for domesticated bovine; Abundant in rumen fluid; Not library dependent | Lack of information and further testing | Ufnar *et al.,* 2007 |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | *Bacteroides* - ruminant | 16S rRNA - BacR | Specific and sensitive; Not library dependent | Cross-reaction with a range of non-target faecal samples | Reischer *et al.,* 2006, 2008, 2011, 2013; Boehm *et al.*, 2013 |
| | | 16S rRNA - Rum-2-BacqPCR | Specific and sensitive; Capable of discriminating between cattle and horse contamination; Not library dependent | Lack of studies and information | Mieszkin *et al.,* 2010; Mauffret *et al.,* 2012; Oladeinde *et al.,* 2014 |
| | *Bacteroides* - Bovine | 16S rRNA - BoBac | Sensitivity (82%); Not library dependent | Low specificity locally (47%) and in a large worldwide study (59%) | Layton *et al.,* 2006; Shanks *et al.,* 2010; Reischer *et al.,* 2013 |
| | *Bacteroides* - Cow | 16S rRNA – CF128F/Bac708R; CF193F/Bac708R | High rates of true-positive detection to DNA samples of cow feces; Tested worldwide; Not library dependent | Detection in non-target samples (low specificity) - false-positive detection in pig samples (20–90%), chicken (20–50%), dog (up to 30%); High rates of positive detection in sewage, dog, and horse samples | Gawler *et al.,* 2007; Gourmelon *et al.,* 2007; Kildare *et al.,* 2007; Lamendella *et al.,* 2007; Ufnar *et al.,* 2007; Ahmed *et al.,* 2008a,b,c; Fremaux *et al.,* 2009; Silkie and Nelson, 2009; Ballesté *et al.,* 2010; Shanks *et al.,* 2010 |
| | | 16S rRNA - BacCow | High sensitivity; Not library dependent | Detection in non-target samples (low specificity) | Kildare *et al.,* 2007; Reischer *et al.,* 2013; Odagiri *et al.,* 2015 |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | | 16S rRNA – Cow-Bac 1, 2, 3 | High specificity; Not library dependent | Field testing only with Cow-Bac 2; Lack of information | Okabe and Shimazu, 2007, Okabe *et al.*, 2007 |
| | | 16S rRNA – CI125f, Bac708r, 1408r | Not library dependent | Lack of information; No field testing | Stricker *et al.*, 2008 |
| | | 16S rRNA - YCF | Specific and sensitive (no cross-reaction with non-target samples); Not library dependent | Lack of knowledge | Jeong *et al.*, 2010 |
| | | Membrane-associated and secreted protein genes – Bac2, Bac3, CowM2, CowM3 | Highly specific; Not library dependent | Lower abundance in bovine population; False-negative results obtained | Shanks *et al.*, 2006a, b, 2008, 2010; Boehm *et al.*, 2013 |
| | *Bifidobacterium* – Bovine | CWBif – 16S rRNA | Sensitive and high specificity; Not library dependent | Low numbers in environmental waters (below limit of detection); More information required | Gómez-Doñate *et al.*, 2012; Casanovas-Massana *et al.*, 2015 |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | Viruses - Bovine | Bovine Enterovirus | High degree of host-specificity; Enteric viruses of different host species are easily identified and differentiated based on sequence differences in genus-common genes; Not library dependent | Dependent on the levels of infection of the population<br>Usually in low levels in environmental waters | Ley *et al.*, 2002; Fong *et al.*, 2005; Jiménez-Clavero *et al.*, 2005 |
| | | Bovine Adenovirus | | | Maluquer de Motes *et al.*, 2004; Wong and Xagoraraki, 2010, 2011; Ahmed *et al.*, 2013) |
| | | Bovine Polyomavirus | | | Hundesa *et al.*, 2006, 2010; Wong and Xagoraraki, 2011 |
| | *Bacteroides* - Porcine | 16S rRNA – PF163/Bac708R | Sensitive (animal faecal samples); Not library dependent | Found in a low number of environmental waters; False-positive results in different nontarget samples: cattle (40%), human (30%), chicken (50%), raccoon (4%) and horse (67%); Qualitative information | Dick *et al.*, 2005a, b; Lamendella *et al.*, 2009; Boehm *et al.*, 2013 |
| | | 16S rRNA – PigBac1, 2 | PigBac1 and PigBac2 are sensitive; Not library dependent | PigBac1 and PigBac2 found in nontarget samples; PigBac1 found in relatively lower numbers than PigBac2 | Okabe *et al.*, 2007; Fremaux *et al.*, 2009; Lamendella *et al.*, 2009 |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | | 16S rRNA – Pig-1-Bac, Pig-2-Bac | Sensitive and highly specific; Not library dependent | Found in substantial lower numbers that total *Bacteroides* in pig faeces; Little information on the persistence in the environment; Different studies reported non-identical results | Mieszkin *et al.,* 2009, 2010; Heaney *et al.,* 2015 |
| | *Neoscardovia* – Porcine | | Highly specific; Not library dependent Sensitive | Low numbers in environmental samples (below LoD) | Gómez-Doñate *et al.*, 2012; Casanovas-Massana *et al.*, 2015 |
| | Viruses - Porcine | Porcine Adenovirus – PadV | High degree of host-specificity; Enteric viruses of different host species are easily identified and differentiated based on sequence differences in genus-common genes; Not library dependent | Dependent on the levels of infection of the population; Usually in low levels in environmental waters | Jiménez-Clavero *et al.*, 2003; Maluquer de Motes *et al.*, 2004; Hundesa *et al.*, 2006, 2009; Wong and Xagoraraki, 2010; Rusiñol *et al.*, 2014 |
| | | Porcine Teschovirus – PTV | | | |
| | *Streptococcus* spp. - Birds | Gull 3 – 16S rRNA | Suited to detect faecal contamination from specific gull species; Not library dependent | Low prevalence in gull faecal samples; Cross-amplification with nonavian faecal samples | |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | *Catellicoccus marimammalium* - *Birds* | Gull 2 and 4 | Highly abundant in gull faeces; Found in environmental samples known to be impacted by gulls; Low number to no detection in environmental samples known to be impacted by nongull faecal contamination; Not library dependent | Gull maker found with great fluctuations in tested gull faeces; Cross-amplification with nonavian faecal samples although in low percentages; Moderate levels of cross-amplification with nongull avian faecal samples | Lu *et al.*, 2008; Ryu *et al.*, 2012a; Boehm *et al.*, 2013; Sinigalliano *et al.*, 2013 |
| | | Cat marker - gull | Specific and sensitive; Not found in wastewaters; Not library dependent | Found in high numbers in chicken faeces; The sequences obtained from 5 chicken faecal samples identical to that of *C. marimammalium* | Boehm *et al.*, 2013; Lee *et al.*, 2013; Sinigalliano *et al.*, 2013 |
| | *Bacteroides* - goose | CGOF1-*Bac* and CGOF2-*Bac* – 16S rRNA | High specificity; Relative temporal stability; Tested in environmental samples; Not library dependent | Low sensitivity | Fremaux *et al.*, 2010 |
| | *Bifidobacterium* - poultry | PLBif – 16S rRNA | Highly specific and sensitive; Not library dependent | Only one study published to date | Gómez-Doñate *et al.*, 2012 |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | *Brevibacterium avium* – poultry | LA35 – 16S rRNA | Specific (93% true-positive); Not library dependent | Low sensitivity levels; Lack of information with few only one study conducted | Weidhaas *et al.*, 2010 |
| | *Lactobacillales* – sandhill crane | Crane 1 – 16S rRNA | Highly specific; Not library dependent | Low sensitivity; Lack of information and field application | Ryu *et al.*, 2012b |
| | Parvovirus – chicken and turkey | ChPV/TyPV – VP1/VP2 region | Highly specific; Sensitive; Not library dependent | Lack of information and field testing | Carratalà *et al.*, 2012 |
| | Mitochondrial DNA - ovine | Ovmito 1, 11 – Nadlt gene from ovine mitochondrial DNA | Allows the direct detection of the contaminating agent; Extremely specific and sensitive; Not library dependent | Lack of field testing and information<br>Qualitative detection | Martellini *et al.*, 2005; Boehm *et al.*, 2013 |
| | Mitochondrial DNA - sheep | *cytb* sheep – cytochrome *b* | Allows the direct detection of the contaminating agent; High specificity and sensitivity; No dietary carryover ; Not library dependent | More field testing is needed | Schill and Mathes, 2008 |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | Mitochondrial DNA - Ovine | Ovine 2, N2 - cytochrome *c* oxidase subunit 3-ND3 | Species specific and sensitive; Not library dependent | Lack of field testing and information | Kortbaoui *et al.*, 2009 |
| | Mitochondrial DNA - dog | NADH and cytochrome *b* | Species specific and sensitive; Not library dependent | Lack of field testing and information; Lower sensitivity – tested two faecal samples with one giving a positive result the other negative | Schill and Mathes, 2008; Caldwell and Levine, 2009 |
| | *Bacteroides* spp. – dog | DF475F/Bac708R | Specific methodology; Not library dependent | Lack of information; Difficult and time consuming procedure; Sensitivity level low | Dick *et al.*, 2005b |
| | | BacCan | Fast and easy to perform; Not library dependent | Low levels of specificity and sensitivity | Kildare *et al.*, 2007; Ahmed, 2008b; Silkie and Nelson, 2009; Schriewer *et al.*, 2010; Wang *et al.*, 2010a |
| | Mitochondrial DNA - deer | Cytochrome *b* | High specificity and sensitivity; Not library dependent | Lack of field testing and information | Schill and Mathes, 2008 |
| | *Bacteroides* spp. - deer | EF447F/990R | Specific methodology; Not library dependent | Lack of information; Difficult and time consuming procedure | Dick *et al.*, 2005b |

**Table 1.7 (continued) -** Widely used library-independent MST approaches and their associated advantages and disadvantages (US EPA 2005a; Hagedorn et al. 2011)

| | | | | | |
|---|---|---|---|---|---|
| | Mitochondrial DNA - horse | Cytochrome *b* | High specificity and sensitivity; Not library dependent | Lack of field testing and information | Schill and Mathes, 2008 |
| | *Bacteroides* spp. - horse | HoF597 and HorseBact | High specificity and sensitivity; Not library dependent | Lack of field testing and information | Silkie and Nelson, 2009 |

### 1.9. Aim of the study

To provide new knowledge and tools with which to manage more effectively faecal contamination of water sources and resources. This aim was tackled by focussing on three research objectives.

### 1.10. Objectives of the study

- To determine the utility and behaviour of a new bio-indicator for viral pathogen (Norovirus) removal during wastewater treatment and comparing this to existing methods (Chapter 5);
- To investigate levels of faecal contamination in the River Tagus catchment and to identify the dominant sources of contamination using a toolbox of novel and established MST methods. (Chapter 6);
- To evaluate citically various pretreatments (enzymatic and intercalating dyes) in order to distinguish accurately between infectious and non-infectious viral particles and to determine the effects of different inactivation treatments (e.g., heat, chlorine, and UV-light). This involved seeding samples with Coxsackievirus B3 (CV-B3) from the *Enterovirus* genus, and *Mengovirus*, a murine virus from the *Picornaviridae* family and a process control and surrogate for Hepatitis A virus and Norovirus.

# CHAPTER 2 : THE HYGIENIC QUALITY OF WATER

## 2. INDICATORS AND INDEX ORGANISMS TO MONITOR WATER QUALITY

### 2.1. Traditional indicators

In Europe, measurement of the hygienic quality of water has long relied on the detection and quantification of faecal indicator bacteria (FIB), such as total coliforms (TC), faecal coliforms, *E. coli* (EC), intestinal enterococci (IE), *Clostridium perfringens* (CP) and total heterothrophic colony counts. Whilst FIB have been used for considerable time now (Berg, 1978), their presence is not necessarily associated with the presence of pathogens and *vice versa*. It has been suggested (Grabow, 1996) that there is insufficient evidence of a direct correlation between the concentrations of any faecal indicator and enteric pathogens. A more useful approach would be to develop appropriate index organisms through epidemiological studies. However, most of the epidemiological studies conducted thus far fail to correlate levels of FIB with pathogens, mostly as a result of poor design and/or due to wide variations in the ratio of pathogens to faecal indicators and the fluctuation on infection patterns of the pathogens (Fleisher, 1990, 1991).

Moreover, the usefulness of any indicator system is also directly and constantly affected by the rates of removal and inactivation/destruction of the indicator compared to the pathogen. Their validity is influenced by different survival rates during treatment processes in drinking water treatment plants (DWTP) and wastewater treatment plants (WWTP), persistence in the environment and even by the possibility of replication of certain FIB in the environment. Therefore, bacteria, viruses, helminths and protozoa are not expected to all behave in the same way and in all circumstances. Additionally, some pathogens are not continually present as part of the regular faecal microbiota of humans, but instead are only excreted by infected individuals. The presence of pathogens is also variable according to their specific seasonal patterns (Berg and Metcalf, 1978).

As a result of these considerable variations in the presence, behaviour and survival of these organisms, there is no universal indicator of the risks associated with faecal contamination of waters. However, several groups of organisms have to be considered and widely used, with varying degrees of success.

#### 2.1.1. Coliforms

Reference to bacteria as indicators of the water quality likely began in 1880 when *Klebsiella pneumoniae* and *K. rhinoscleromatis* were described as microorganisms found

in human faeces by Von Fritsch (Geldreich, 1978). In 1885, the first routine analysis of water quality was performed in London by Percy and Grace Frankland (Hutchinson and Ridgway, 1977). By 1891, the Franklands described the concept that microorganisms found in sewage must be identified in order to provide insights into potentially dangerous pollution (Hutchinson and Ridgway, 1977). Even though the coliform bacteria concept has been extensively used for many year in the UK, this group is extremely broad and includes bacteria not only of faecal origin but also of non-faecal origin.

Consequently, *E. coli* (EC) was proposed as an indicator of sewage, since it was largely of faecal origin (Winslow and Walker, 1907). EC are the major component of the facultative anaerobic portion of the natural intestinal flora of warm-blooded mammals (Krieg and Holt, 1984). Several studies have conclusively shown that EC is the only bacterium within the coliform group that is a natural inhabitant of the gastrointestinal tract (Geldreich, 1966; Dufour, 1977).

By the end of the 1970s, it was concluded that EC was specific to and extremely abundant in human and animal faeces, with an average estimated concentration of $10^9$ per gram. EC is found in many environmental and anthropogenic reservoirs, such as in sewage, treated effluent, and all natural waters and soils subjected to mammalian faecal contamination. EC has good features of a faecal indicator, such as not usually being pathogenic to humans, and it is present at concentrations much higher than the pathogens it predicts. However, different studies have shown that EC may not be a dependable indicator in tropical and subtropical environments because of its capacity to replicate in contaminated soils (Desmarais *et al*, 2002; Solo-Gabrielle *et al*, 2000).

### 2.1.2. Faecal streptococci and enterococci

Faecal streptococci, a group of Gram-positive bacteria, have also been used as an important bacterial indicator (Houston, 1900; Winslow and Hunnewell, 1902). The use of faecal streptococci (FS) as an indicator was stalled by the difficulties in differentiating faecal from non-faecal streptococci (Kenner, 1978). However, three important characteristics as an indicator are attributed to faecal streptococci: (1) they are found in relatively high numbers in the natural gut microbiota of humans and other warm-blooded mammals; (2) they are present in wastewaters and polluted waters; (3) they are usually absent from clean waters, soils and environments with no contact with human and animal life. Finally, in 1957, Slanetz and Bartley (1957) made available a selective medium for the detection and enumeration of faecal streptococci. The FS group is composed by different

*Enterococcus* spp., *Streptococcus bovis* (*S. bovis*) and *S. equinus* (WHO, 1997). Among the FS group, the most relevant and accepted indicators are the intestinal enterococci. Enterococci were formerly classified as streptococci but through DNA analysis they were reclassified into their own species (Kalina, 1970; Collins *et al.,* 1984; Schleifer and Kilpper-Balz, 1984). *E. faecalis* and *E. faecium* are the species most commonly found in the human gut. IE have been used widely and with success as indicators of faecal contamination and are especially reliable as indicators of potential risk to health in recreational waters and marine environments (Cabelli *et al*, 1982; Cabelli, 1983). However, it has been established that IE have environmental reservoirs and that once introduced into the environment there is the possibility of replication (Desmarais *et al*, 2002).

## 2.2. Alternative indicators

### 2.2.1. Bacteriophages

Bacteriophages, commonly known as phages, were discovered at the beginning of the 20$^{th}$ century by two scientists working independantly, Frederick William Twort and Félix Hubert d'Hérelle. Phages are parasitic particles with the ability to infect prokaryotic bacteria (Ackermann and DuBow, 1987), playing a crucial role in the regulation of the bacterial ecosystem and even the in the higher ecosystem domains (Kutter and Sulakvelidze, 2005).

Phages are capable of infecting the plurality of bacterial families (Ackermann and DuBow, 1987; Ackermann, 1999) with over 140 bacterial genus hosts. Since 1959, over 6000 prokaryote infectious agents have been described morphologically, including 6196 bacterial and 88 archaeal infectious agents. The first phage review, published in 1967, listed 111 negatively stained phages, mostly tailed, but also phage $\phi$X174 (*Microviridae*), *Leviviridae* or ssRNA phages, and nine filamentous phages of the *Inoviridae* family (Eisenstark, 1967). A more recent report published in 2007 included 5500 phages (Ackermann, 1996, 2001, 2007).

In all the reports published, the vast majority (over 96%) were tailed phages, infecting bacteria (98.5%), and belonging to the same three families: *Myoviridae*, *Siphoviridae*, and *Podoviridae*. Phages have been described in 12 prokaryote phyla, which include 16 archaeal and 163 bacteria genera (Table 2.1).

**Table 2.1 -** Overview of bacterial phages

| Shape | Virus Group | Nucleic Acid | Features | Example |
|---|---|---|---|---|
| Tailed | *Myoviridae* | DNA, DS, L | Tail contractile | T4 |
| | *Siphoviridae* | | Tail long, noncontractile | λ |
| | *Podoviridae* | | Tail short | T7 |
| Polyhedral | *Microviridae* | DNA, SS, C | Conspicuous capsomers | $\phi$X174 |
| | *Corticoviridae* | DS, C, S | Complex capsid, lipids | PM2 |
| | *Tectiviridae* | DS, L | Inner lipid vesicle, pseudotail | PRD1 |
| | *Leviviridae* | RNA, SS, L | Poliovirus-like | MS2 |
| | *Cystoviridae* | DS, L, seg | Envelope, lipids | $\phi$6 |
| Filamentous | *Inoviridae* | DNA, SS, C | a. long filaments | Fd |
| | | | b. short rods | MVL1 |
| Pleomorphic | *Plasmaviridae* | DNA, DS, C, S | Envelope, lipids, no capsid | L2 |

C - circular; L - linear; S - superhelical; seg - segmented; SS – single-stranded; DS – double-stranded (Adapted from Ackermann and Prangishvili, 2012)

Bacteriophages (Table 2.1) can be tailed, polyhedral, filamentous or pleomorphic and can be composed of either single- stranded, or double-stranded DNA or RNA. There is only one family assigned, the *Caudovirales* (Figure 2.1), constituted by tailed infectious particles with icosahedral heads or prolate capsids and comprising three families: *Myoviridae*, *Siphoviridae*, and *Podoviridae*. The *Caudovirale* capsid is generally icosahedral composed of monomers (quasi-equivalent protein subunits). This structure allows for an economic genome. The three families are further grouped by tail structure.

Bacteriophages are “obligate parasites” since they can only replicate inside the bacterial host cell, manipulating all the cellular machinery (ribosomes, amino acids, energy generating systems and protein-synthesising factors) to multiply inside a living bacterial cell (Bruessow and Kutter, 2005). The life cycle stages of phages differ in length thus yielding taxonomically relevant information. The critical stages of the phage life cycle include the adsorption of the phage to the membrane surface of the bacterial host, the

transfer of genetic material from the phage to the bacterial host cell, the synthesis and assembly of the phage inside the hosts cell.

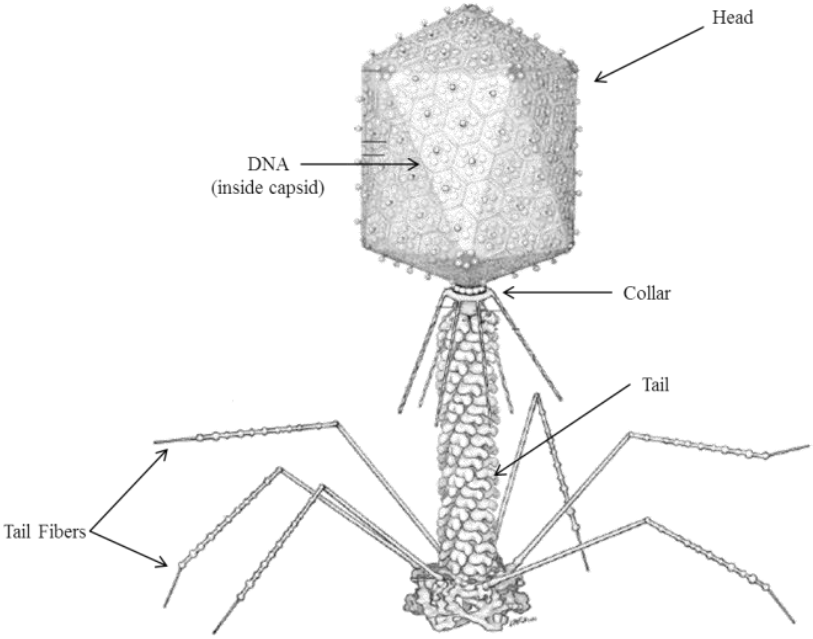


**Figure 2.1 -** Structure of phages from the *Caudovirales* order (adapted from Mosig and Eiserling, 2006)

Naturally lytic phages may enter a lysogenic state when faced with environmental stresses. Lysogenic phages may provide new properties to the bacterial host including the possibility to express genes coding for toxins (Waldor and Mekalanos, 1996). Lytic phages create clear distinct plaques in a lawn of bacterial host whereas the lysogenic phages produce faint plaques, since the release is at a lower frequency.

**Phages as indicators of faecal contamination**

To overcome the issues associated with the use of FIB, different groups of phages have been proposed as indicators of faecal contamination, including those from human sources (Jofre *et al.*, 1986; Payan *et al.*, 2005; Ebdon *et al.*, 2012). Phages are persistent in the environment and resistant to disinfection treatment, are quantified by easy and economical assays and are non-pathogenic (Ackermann and DuBow, 1987; Grabow *et al.*, 1995; Van Cuyk and Siegrist, 2007). It has also been proposed to use phages as surrogate organisms for enteric viruses since they have similar structure, morphology, size and composition (Jofre *et al.*, 1986; IAWPRC, 1991; Grabow *et al.*, 1995; Leclerc *et al.*, 2000 Contreras-

Coll *et al.*, 2002; Mandilara *et al.*, 2006) and as model organisms to evaluate the efficacy of wastewater treatment processes (Durán *et al.*, 2003; Mocé-Llivina *et al.*, 2003).

The main groups of phages that have been suggested as faecal indicators are: coliphages (phages infecting coliform bacteria) and phages infecting *Bacteroides* spp. Interest in such approaches is increasing. For example, the US EPA is currently considering the use of F-specific and somatic coliphages, as possible viral indicators of faecal contamination in ambient water (US EPA, 2015). Coliphages (detected by EPA Method 1601 (2001a), 1602 (2001b), or approved equivalent methods) are also one of the faecal indicator organisms that can be selected for microbial monitoring of groundwater systems in the US (US EPA, 2006b). Furthermore, they are also contained in the drinking water quality regulations of the Canadian Province of Quebec and Australia (E.O.Q., 2016a).

The detection and enumeration of phages in different matrices (all kinds of waters, sediments and sludge) is based on ISO standard methods. These include ISO 10705-1:1995 for enumeration of F-specific RNA bacteriophages, ISO 10705-2:2000 for enumeration of somatic coliphages, and ISO 10705-4:2001 for the enumeration of bacteriophages infecting *Bacteroides fragilis*.

#### 2.2.1.1. Somatic coliphages

Somatic coliphages (SC) are the most studied group of phages (Toranzos *et al.*, 2002) and are capable of infecting members of the *Enterobacteriaceae* family (Grabow, 2001; Gerba, 2006). Somatic coliphages are a diverse group of phages belonging to the *Mycoviridae*, *Siphoviridae*, *Podoviridae*, and *Microviridae* families. They are lytic phages with an infection cycle of approximately 30 minutes, creating plaques with extremely different sizes and morphology (ISO 10705-2; Gerba 2006). Coliphages can be easily isolated from the human, cattle, pigs, chicken faeces, among others (Dhillon *et al.*, 1976; Havelaar *et al.*, 1986, 1990). They are usually detected in levels varying from $10^4$ to $10^7$ plaque-forming units (PFU) per gram (Geldreich, 1978) and at concentrations ranging from $10^4$ to $10^5$ PFU/mL in sewage (Dhillon *et al.*, 1970; Furuse, 1987; Havelaar *et al.*, 1984).

Since somatic coliphages were first proposed as indicators of faecal contamination by Kott (1966), several studies have recommended their use as faecal indicators or indicators of enteric viruses (Kott *et al.*, 1974; Grabow *et al.*, 1984; El-Abagy *et al.*, 1988; Borrego *et al.*, 1987; Mocé-Llivina *et al.*, 2005).

There are countless advantages of using SC as indicators beyond the simple and economical aspects. SC are a heterogeneous group, presenting different responses to environmental factors such as pH and temperature (Gerba, 2006) and tend to persist for longer periods than traditional FIB and pathogenic viruses (Scarpino, 1978; Borrego *et al.*, 1990; US EPA, 2007). Some studies have shown positive correlation between enteric viruses and SC (Borrego *et al.*, 1987, 1990; Dutka *et al.*, 1987). Generally, SC are present in higher numbers than human enteric viruses (Grabow *et al.,* 1993). SC tend to be more frequently detected in polluted waters (wastewater and wastewater impacted waters) than are F-specific RNA phages and *B. fragilis* phage (Grabow *et al.,* 1993). SC are also able to be detected in small sample volumes, being therefore a useful tool as a general indicator of faecal contamination (Gerba, 2006). The disadvantages include the heterogeneity of the group, with differences in inactivation kinetics, the possibility of infecting the *Enterobacteriaceae* family, which are capable of replicating in the environment promoting the environmental replication of SC. Further, little to no information is available on SC ecology, mainly host range and pattern distribution in different geographical areas. SC are also linked to non-point contamination and are detected in areas not impacted by faecal pollution (Paul *et al.,* 1997; Seeley and Primrose, 1980).

Although good positive correlations between SC and enteric viruses have been described, other papers have discouraged the use of SC as surrogate organisms of human pathogenic viruses (Gantzer *et al.,* 1998; Hot *et al.,* 2003; Monteiro, 2007). This results essentially from different excretion patterns (concentrations and timing – Jiang *et al.,* 2001). As a result, SC are good indicators of the presence of faecal contamination and of water quality since they can be found in high concentrations in faecal material and in faecally contaminated environments but cannot be used to discriminate between human and animal sources.

#### 2.2.1.2. F-specific RNA bacteriophages

F-specific phages are sub-divided into two categories according to the genetic material: F-RNA and F-DNA phages. F-specific RNA (FRNA) phages are constituted by a cubic capsid (usually between 21 and 30 nm in diameter – ISO 10705-1) carrying ssRNA as the genome and they belong to the *Leviviridae* family and constitute more than 90% of all the F-specific bacteriophages, infecting members of the coliform bacteria (Jofre, 2002). Based on serological and physicochemical characteristics, these phages can be further classified into four genogroups (I, II, III, and IV). Genogroups I and IV are primarily associated with

human faecal contamination, whereas genogroups II and III are associated with animal contamination (Furuse, 1987). These features are useful when considering F-specific phages as MST tools (see Table 1.9). Further exploration of mechanisms of infectivity, environmental persistence and behaviour is beyond the remit of this study (Kott *et al.*, 1974; Dhillon *et al.*, 1976; Havelaar *et al.*, 1986, 1990, 1993; Lucena *et al.*, 1994; Griffin *et al*., 2000; Grabow, 2001; Fujioka, 2002; Jofre, 2002; Schaper *et al.*, 2002 a; Durán *et al.*, 2003; Arraj *et al.*, 2005; Nappier *et al.*, 2006; Muniesa *et al.*, 2009; Haramoto *et al.*, 2012; Hata *et al.*, 2013).

#### 2.2.1.3. Phages infecting *Bacteroides* spp.

The genus *Bacteroides* is constituted of anaerobic bacteria that have the human intestinal tract as their main ecological niche and the same has been argued for its phages (Jofre *et al.*, 1986; Gerba, 2006). It is one of the major constituents of the human gut (Finegold *et al.*, 1983), found in numbers ranging from $10^9$ to $10^{10}$ per gram of faeces (Geldreich, 1978; Salyers, 1984), being more prevalent than faecal coliforms with most environmental monitoring studies being conducted using the species *Bacteroides fragilis* (Jofre *et al.*, 1986; Tartera and Jofre, 1987; IAWPRC, 1991; Lucena *et al.*, 1994, 1995; Gantzer *et al.*, 2002; Formiga-Cruz *et al.*, 2003; Moce-Llivina *et al.*, 2005; Payan *et al.*, 2005).

Phages infecting *B. fragilis* are lytic and belong to the *Siphoviridae* family (Puig and Gironés, 1999; Gómez-Doñate *et al.*, 2011; Diston *et al.*, 2012). They present a binary morphology, with an icosahedral capsid varying from 50 to 70 nm and a non-contractile, straight or slightly curved tail measuring approximately 130 to 190 nm.

Despite belonging to the same group and being possibly serologically similar, *Bacteroides* phages do not have an identical host range, many being specific to a single *Bacteroides* strain (Kory and Booth, 1986). *Bacteroides* phages only have the capacity to infect other strains within the original species (Booth *et al.*, 1979). The specificity of host range has been further documented by Keller and Traub (1974), Tartera and Jofre (1987) and Bradley *et al*. (1999). Several reports have been made into the prevalence of phages infecting *Bacteroides* spp. in human population (Kai *et al.*, 1985; Grabow *et al.*, 1995; Gantzer *et al.*, 2002). Research has shown that the prevalence of these phages ranged from 5 to 13%, with concentrations varying between 70 to $3x10^5$ PFU per gram of faecal material. Generally, the concentration of *B. fragilis* phages is between $10^2$ and $10^3$ PFU/100 mL in treated wastewater (Tartera and Jofre, 1987; Tartera *et al.*, 1989; Puig *et al.*, 1999).

The ratios between the three phage groups presented in this chapter (namely SC, FRNA phages and *Bacteroides* spp. phages) are normally stable in untreated sewage and wastewaters ranging approximately between 10 to 100 for the ratio of FRNA phages and the strains RYC2056 and HSP40 (Puig *et al*, 1999; Contreras-Coll *et al.,* 2002) and from 100 to 200 for the ratio of SC to strain RYC2056 (ISO 10705-4; Contreras-Coll *et al.*, 2002). These ratios reduce significantly in seawater samples, varying from 0.7 to 1.2 for ratio of somatic coliphages to *B. fragilis* phages and 0.5 logs for *B. fragilis* RYC2056 phages to FRNA phages. These results suggest that *B. fragilis* phages, even though in lower numbers in faeces, are more persistence in the environment than the other phages discussed previously. Thereafter, *B. fragilis* phages have been suggested as index organisms of remote faecal contamination (Lucena *et al.,* 1996).

*Bacteroides* spp. phages also appear also to be more resistant to drinking water treatments (Jofre *et al.,* 1995). Different inactivation experiments performed with specific phages have shown that even though they appear to be less prevalent in the environment they were also more resistant to natural and physicochemical inactivation (Araujo *et al.,* 1997; Durán *et al.*, 2002, 2003; Gomila *et al.*, 2008).

The predominance of *Bacteroides* phages belonging to *Siphoviridae* family is not related to geographical differences (Kory and Booth, 1986). The main advantage of these bacteriophages resides in the fact that their hosts are obligate anaerobes. Therefore, the possibility of replication in the environment, outside the gut, is negligible, and accordingly so is the probability of phages infecting them. Furthermore, the high levels of host specificity shown by these phages mean that they are potentially powerful tools for MST studies.

As a result of geographical limitations in the efficacy of certain *Bacteroides* hosts to recover phages in certain parts of the world, a method for isolating *Bacteroides* spp. from a given source and useful in distinct geographical areas was developed (Payan *et al.,* 2005). The most prominent strains arising from this study were *B. thetaiotaomicron* HB-13, isolated in Colombia, GA-17, isolated in Spain, and *B. fragilis* GB-124, isolated in the UK. Since then, several other works have described the isolation of host-specific *Bacteroides* species (Gómez-Doñate *et al.,* 2011; Wicki *et al.,* 2011).

Research has been performed on the correlation between *B. fragilis* phages and the presence of enteric viruses (enterovirus, adenovirus and Hepatitis A virus) in different matrices (Jofre *et al*., 1989; Pina *et al.,* 1998; Gantzer *et al.,* 1998).

Even though *Bacteroides* spp. phages present good characteristics for an ideal surrogate, they also show a crucial shortcoming, lack of sensitivity that arises from the fact that they are shed in relatively low numbers, mainly compared to the other phage groups discussed. Finally, another fact that may potentially hinder the successful use of *Bacteroides* spp. phages relates to a lack of geographic stability of certain hosts.

#### 2.2.1.3.1. *B. fragilis* strain GB-124 phages

The strain and respective phages were first isolated in a collaborative study involving different European laboratories in 2004 interested in isolating a human-specific *Bacteroides* spp. host strains capable of being used in different geographical regions (Payan *et al.*, 2005). Ebdon *et al.* (2007) substantiated the specificity of this host strain during a study involving more than 300 samples of river water, municipal wastewater and faeces from animals. This *Bacteroides* host was classified by molecular biology techniques as belonging to *B. fragilis* (Ogilvie *et al.*, 2012) and their respective phages as belong to the *Siphoviridae* family.

A study by Diston *et al.* (2012) showed that GB-124 phages were likely to be inactivated by UV-C light used in wastewater UV disinfection processes, with similar inactivation rates to EntV, HAV and RoV, evidence of the possible utility of these phages as surrogates for these particular viruses. GB-124 phages were used to correctly discriminate between human and non-human faecal contamination as part of an integrated analysis of water quality parameters from the River Ouse (Nnane *et al.*, 2012) in the UK. Additionally, phages infecting GB-124 strain were identified in different geographical areas, including Cuba, North America, Brazil, and Cyprus, even though this bacterial strain was initially isolated in Southeast England (Ebdon *et al.*, 2007, 2012; McMinn *et al.*, 2014).

### 2.2.2. Enteric viruses

Enteric viruses are an important cause of waterborne and water-related diseases, with over 100 virus species reported to be present in sewage contaminated waters (Bosch *et al.*, 2008). These include gastroenteritis, meningitis, hepatitis, fever, and conjunctivitis (Table 2.2).

**Table 2.2 -** Possible waterborne transmissible human viruses (adapted from Bosch, 1998, Bosch *et al.,* 2008, ICTV, 2012)

| Family | Genus | Name | Illness |
|---|---|---|---|
| *Picornaviridae* | *Enterovirus* | Poliovirus | Paralysis, meningitis, fever |
| | | Coxsackievirus, A, B | Herpangina, meningitis, fever, respiratory disease, hand-foot-and-mouth disease, myocarditis, rush, pleurodynia |
| | | Echovirus | Meningitis, rush, respiratory disease, gastroenteritis, fever |
| | *Hepatovirus* | Hepatitis A virus | Hepatitis |
| *Hepeviridae* | *Hepevirus* | Hepatitis E virus | Hepatitis |
| *Reoviridae* | *Rotavirus* | Human Rotavirus | Gastroenteritis |
| *Adenoviridae* | *Mastadenovirus* | Human Adenovirus | Gastroenteritis, conjunctivitis, respiratory illness |
| *Caliciviridae* | *Norovirus* | Human norovirus | Gastroenteritis |
| | *Sapovirus* | Human sapovirus | Gastroenteritis |
| *Astroviridae* | *Mamastrovirus* | Human astrovirus | Gastroenteritis |
| *Coronaviridae* | *Coronavirus* | Human coronavirus | Respiratory disease, gastroenteritis |
| | *Torovirus* | Human torovirus | Gastroenteritis |
| *Parvoviridae* | *Parvovirus* | Human parvovirus | Gastroenteritis |
| *Polyomaviridae* | *Polyomavirus* | JC Polyomavirus | Progressive multifocal leukoencephalopathy |

Enteric viruses are shed in extremely high numbers, with patients suffering from diarrhoea or hepatitis excreting up to $10^{13}$ and $10^{10}$ virus particles per gramme of stool, respectively (Costafreda *et al.*, 2006; Ozawa *et al.*, 2007) and as described previously, common water treatments do not guarantee the absolute removal of viruses, which then are released in environmental waters in high enough numbers to pose a potential risk to human health, but in low enough concentrations to make their detection difficult (Bosch *et al.,* 2008).

**Enteric viruses as indicators of faecal contamination**

Generally, enteric viruses such as Norovirus (NoV), Enterovirus (EntV), Rotavirus (RoV), Hepatitis A virus (HAV) and Hepatitis E virus (HEV) have been shown to vary both in their geographical and seasonal distribution. This major diversity in epidemiological patterns is indicative of the disadvantage of choosing a single viral organism to satisfy all the criteria of a good indicator (Girones and Bofill-Mas, 2013). Because no indicator is able to fulfil all the criteria, it has been established that a set of different indicators are required to reflect the variety and behaviour of pathogens. Appropriate viral indicators could obviate the need to detect specific viral pathogens.

Viruses transmitted through contaminated food or water are usually more stable to environmental stressors than other viruses because of the absence of a lipid envelope. In general terms, viruses are more resilient to environmental stressors than the current FIB, explaining to some extent the frequent lack of correlation between these organisms. Because of the high diversity and low concentrations of viruses in natural water, the detection of human enteric viruses is still challenging. The use of better concentration, elution and detection methods would help to understand better these organisms and to develop new risk-based guidelines for improving water microbiological water quality. The main proposed viral groups include human Enterovirus, human Adenovirus (HAdV), and human Polyomavirus (HPyV). From the proposed viral groups, HAdV and HPyV are readily detected in most geographical areas and have not presented marked seasonality.

### 2.2.2.1. Human Adenovirus (HAdV)

The *Adenoviridae* family consists of a non-enveloped icosahedral capsid with fibre-like projections from each vertex measuring between 70 and 90 nm in diameter, protecting a single, linear molecule of dsDNA ranging from 26,163 to 48,395 bp (ICTV, 2012, Girones and Bofill-Mas, 2013) as seen in Figure 2.2.

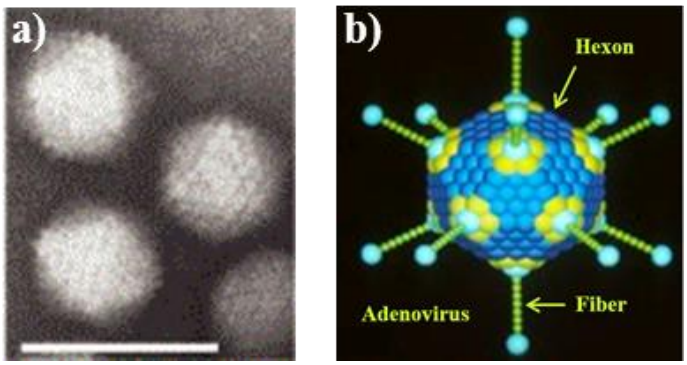


**Figure 2.2 -** Transmission electron microscopy of human adenovirus type 41. Scale bar, 100 nm (Stone *et al.*, 2007)

HAdV are a vast group, consisting of seven species (A-G) with 52 types (HAdV1-HAdV52; Jones *et al.*, 2007, Mena and Gerba, 2009). Several types of HAdV (HAdV 1, 2, 5, 7, 12, 31, 40 and 41) have been detected in contaminated waters and shellfish (Formiga-Cruz *et al.*, 2002; Bofill-Mas *et al.*, 2006; Wyn-Jones *et al.*, 2011; Haramoto *et al.*, 2012; Sales-Ortells *et al.*, 2015). HAdV are widely found in sewage throughout the year, with no known seasonality in their temporal distribution (Bofill-Mas *et al.*, 2000; Fong *et al.*, 2010; Simmons and Xagoraraki, 2011) in concentrations up to $10^3$ PFU/L (Mena and Gerba, 2009) and ranging between 2 and 4 logs per mL in raw sewage (Bofill-Mas *et al.*, 2006; Rodriguez-Manzano *et al.*, 2012). HAdV have also been detected in different geographical areas (Gironés *et al.*, 2010) and environmental waters, including seawater, groundwater, river and surface water (Calgua *et al.*, 2008; Albinana-Gimenez *et al.*, 2009 a,b; Bofill-Mas *et al.*, 2010; Haramoto *et al.*, 2010; Ogorzaly *et al.*, 2010; Guerrero-Latorre *et al.*, 2011; Wyn-Jones *et al.*, 2011; Souza *et al.*, 2012) being highly stable in such complex matrices (Bofill-Mas *et al.*, 2006; Girones *et al.*, 2010; Girones and Bofill-Mas, 2013). Furthermore, they have also been reported in swimming pools and drinking water (D'Angelo *et al.*, 1979; Turner *et al.*, 1987; Genthe *et al.*, 1995; Papapetropoulou and Vantarakis, 1998; Grabow *et al.*, 2001; Lee and Jeong, 2004; Lee *et al.*, 2005; van Heerden *et al.*, 2005; Albinana-Gimenez *et al.*, 2006, 2009a, b; Jiang *et al.*, 2006).

Adenoviruses were suggested as viral indicators of faecal pollution in water and as indicators of wastewater treatment efficiency in the 1990's (Puig *et al.*, 1994; Pina *et al.*, 1998) and they possess several characteristics of an ideal indicator, including: stability, persistence and distribution in a large variety of water environments, including wastewaters, rivers and drinking waters (Pina *et al.*, 1998; Bofill-Mas *et al.*, 2006; Katayama *et al.*, 2008; Dong *et al.*, 2010); and lack of seasonality in untreated wastewater,

though there have been some reports on the existence of environmental waters seasonal variation (Pina *et al.*, 1998; Haramoto *et al.,* 2007; Hewitt *et al.,* 2011; Poma *et al.,* 2012; Carducci and Verani, 2013). Other important features include detection during the several stages at a water treatment plant, prominent resistance to disinfection by UV radiation, particularly HAdV40 and 41 (Gerba *et al.,* 2002; Thurston-Enriquez *et al.,* 2003a) and lack of environmental replication (Girones and Bofill-Mas, 2013).

The information currently known about HadV substantiates the usefulness of these viruses as indicators of microbiological quality.

#### 2.2.2.2. Polyomavirus

Polyomaviruses are non-enveloped viruses of approximately 40 to 45 nm in diameter, belonging to the *Polyomaviridae* family (ICTV, 2012). The capsid is icosahedral and composed of 72 capsomers in a skewed lattice arrangement (Figure 2.3).

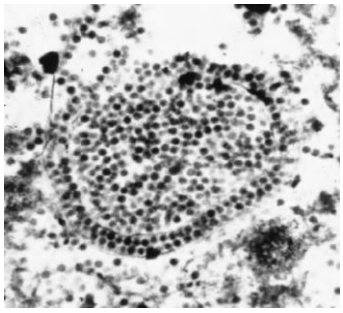

**Figure 2.3 -** Electron micrograph displaying the assembly of JC Polyomavirus (JCPyV) particles (ICTV)

Polyomaviruses are capable of infecting different vertebrates including humans. HPyV can be divided into classical and new HPyV (Gardner *et al.,* 1971; Padgett *et al.,* 1971; Gironés and Bofill-Mas, 2013). JCPyV, considered a classical HPyV, is distributed globally and the respective antibodies has been detected in over 80% of humans (Weber *et al.,* 1997; Gironés e Bofill-Mas, 2013). JCPyV are excreted in urine and are therefore present in wastewater (Bofill-Mas *et al.,* 2000). Since, JCPyV are globally distributed, are found in wastewater and in relatively high numbers, they have been suggested as indicators of human faecal pollution (Bofill-Mas *et al.,* 2000).

JCPyV have been detected in distinct types of environmental matrices and have been found in concentrations up to $10^7$ genome copies (GC)/100 mL in wastewater (Bofill-Mas *et al.,* 2006; Fumian *et al.,* 2010) and in extremely high numbers in river water ($10^3$ GC/100 mL;

Albinana-Gimenez *et al.,* 2009a; Hamza *et al.,* 2009; Haramoto *et al.,* 2010). HPyV have also been detected in fresh and marine waters (Abdelzaher *et al.,* 2010; Hellerin *et al.,* 2011), in coastal waters (Korajkic *et al.,* 2011; Moresco *et al.,* 2012), surface waters (Jurzik *et al.,* 2010; Gibson *et al.,* 2011) and drinking water (Gibson *et al.,* 2011). Several characteristics found in JCPyV seem to be indicative of a good indicator of water quality including: the fact that they infect only humans, do not replicate within the environment, do not display seasonal variances and exhibit excretion levels that appear to be independent of population infectivity (Gironés and Bofill-Mas, 2013). Additionally, PyV present high resistance to chlorine and persistence in sewage samples (Bofill-Mas *et al.,* 2006; Corrêa *et al.,* 2012; Gironés and Bofill-Mas, 2013).

### 2.3. Mitochondrial DNA (mtDNA)

The concept of using host cell nucleic acids instead of targeting microorganisms from the gut lumen began in 2000 (Caldwell *et al.,* 2011). Mitochondrial DNA is found in the mitochondria (organelles found in most eukaryotic cells). Mitochondria are found in multiple numbers and each one has its individual genome in multiple copies. The number of copies in which mitochondria and their genome are found in each eukaryotic cell is highly variable depending not only on the cell type but also on the physiological conditions (Alberts *et al.,* 2002; Cooper and Hausman 2006).

mtDNA has evolved differently in different species, for instance, human and chimpanzee mtDNA have 9% of non-identities. This characteristic is important when designing primers and probes that are completely species-specific for use in real-time quantitative PCR (qPCR). mtDNA have different field applications including as a way to identify species, known as "DNA barcoding" (Caldwell *et al.,* 2011; Barcode of Life 2015a) in forensic analysis (Hopwood *et al.,* 1996; Andreasson *et al.,* 2002a; Budowle *et al.,* 2003) and as a monitoring tool to prevent fraudulent description of food content (Zhang *et al.,* 2007; Fujimura *et al.,* 2008; Caldwell *et al.,* 2011).

### 2.4. Microbial Source Tracking (ST)

Organisms such as *B. fragilis* phages, including GB-124 phage, members of the enteric viruses family mainly HAdV and the detection of eukaryotic genomic sequences, namely mitochondrial DNA, have been proposed as indicators of the origin of faecal contamination (Maluquer de Motes *et al.,* 2004; Martellini *et al.,* 2005; Caldwell *et al.,*

2007, 2009; Schill and Mathes, 2008; Hundesa *et al.*, 2009, 2010; Ballesté *et al.*, 2010; Ebdon *et al.*, 2012; Harwood *et al.*, 2013; McMinn *et al.*, 2014; Carratalà *et al.*, 2012; Rusiñol *et al.*, 2014). MST markers have to be used in a 'toolbox' approach, in which multiple methods are used, allowing for a more accurate interpretation of the data (Stewart *et al.*, 2003; US EPA, 2005a; McDonald *et al.*, 2006; Ballesté *et al.*, 2010).

**Advantages and Disadvantages of selected MST markers**

The selected markers for this study (GB-124 phages, HAdV and mtDNA) have several advantages and disadvantages. GB-124 phages and HAdV have some common advantages, such as increased persistence in the environment and resistance to disinfection treatment and the fact that replication is not possible outside of the host. GB-124 phages have also the advantage of being detected and quantified according to a standard ISO method, a methodology that is simple, easy to perform, non-expensive and suitable for use in low resource settings. Detection is performed through the visualisation of plaques of lysis, guaranteeing that only infectious (viable) intact particles are detected. The main disadvantage of using a phage that infects a given bacterium (or group of bacteria) is that the presence of the host (and consequently the phage) can vary both geographically and temporally (most likely as a result of diet and climate). Early studies reported that the absence of *B. fragilis* phages in highly polluted waters, including sewage, and the low levels recorded in environmental waters are certainly disadvantages associated with the use of these organisms (Scott *et al.*, 2002). The main advantages in using enteric viruses, besides the ones discussed previously, are that these organisms also exhibit a high degree of host specificity. Moreover, HAdV do not show a marked seasonality, indicating that they are not dependent solely on the levels of infection among the population and that the most commonly used technique for their detection (qPCR), is relatively easy to perform using readily available commercial kits.

However, viruses are usually found in low numbers in environmental samples and larger volumes of water have to be concentrated through often-expensive filters and/or membranes, often involving complicated, time-consuming, concentration and elution steps. Interestingly, the potential use of mtDNA has an advantage over the other approaches mentioned (HAdV and GB-124 phages), in that identification of the contamination source is carried out directly rather than indirectly as measured by the detection of microorganisms belonging to, or infecting a certain species. Therefore, the detection of mtDNA obviates ambiguity regarding the sources of contamination. The sensitivity of qPCR for the detection of mtDNA is extremely high as a result of the large numbers in

which it is excreted (Caldwell *et al.*, 2011). Furthermore, mtDNA genomes are highly conserved (intraspecies), but evolved differently between species, promoting the design of primers species-specific for use in qPCR.

The major draw-back associated with this parameter is the possibility of 'carry-over'. That is, the presence of non-degraded animal mtDNA in human faeces that would then be detected by qPCR (Caldwell *et al.*, 2007), although some reports indicated that no such event occurs (Martellin *et al.*, 2005; Schill and Mathes, 2008). In addition, mtDNA from non-faecal sources (sputum, skin, industrial manufacturing wastewaters or abbatoirs) can theoretically be detected by qPCR. Despite some issues regarding the three parameters, they all present several positive characteristics that makes them potentially useful to include as part of the MST 'toolbox' investigated in this study.

# CHAPTER 3 : DISCRIMINATION BETWEEN INFECTIOUS AND NON-INFECTIOUS VIRUSES USING PCR

There has been significant progress in the development of water and wastewater treatment technologies in recent years but human waterborne diseases are still a major concern with large public health and socio-economic impact, not only in LEDCs but also in MEDCs.

The detection of enteric viruses, found in low levels in the environment, is a rather intricate process that can be divided into two different steps: namely concentration of large volumes of sample and virus detection. Viruses can be detected through a multitude of distinct techniques, including cell culture, enzyme-linked immunosorbent assay (ELISA), PCR or reverse transcription (RT)-PCR, quantitative PCR and RT-quantitative PCR, loop-mediated isothermal amplification (LAMP or rt-LAMP) and nucleic acid sequence-based amplification (NASBA) (Mattison and Bidawid, 2009; Hamza *et al.*, 2011).

The 'gold standard' method for detection and isolation of enteric viruses is cell culture. Although some viruses are easy to cultivate (enteroviruses and adenoviruses), most enteric viruses of human health interest are difficult to cultivate (e.g., rotaviruses and hepatitis A viruses), only cell culture system was made available recently (human noroviruses) or no cell line is available. Since cell culture assays are expensive, require specialised training and equipment, are difficult to perform and time-consuming, it is complicated to use on a routine basis. Molecular biology techniques, such as qPCR, are easy to perform, relatively inexpensive and provide fast and sensitive results. However, qPCR detects not only nucleic acids from infectious viral particles but also naked nucleic acids and virions with disrupted capsids. As a result, qPCR does not discriminate between infectious and non-infectious viral particles, which is of extreme importance in determining risks to human health (Choi and Jiang, 2005; Hamza *et al.*, 2011). This chapter outlines two different approaches currently used to determine infectivity in order to counter the limitations of cell culture (Figure 3.1).

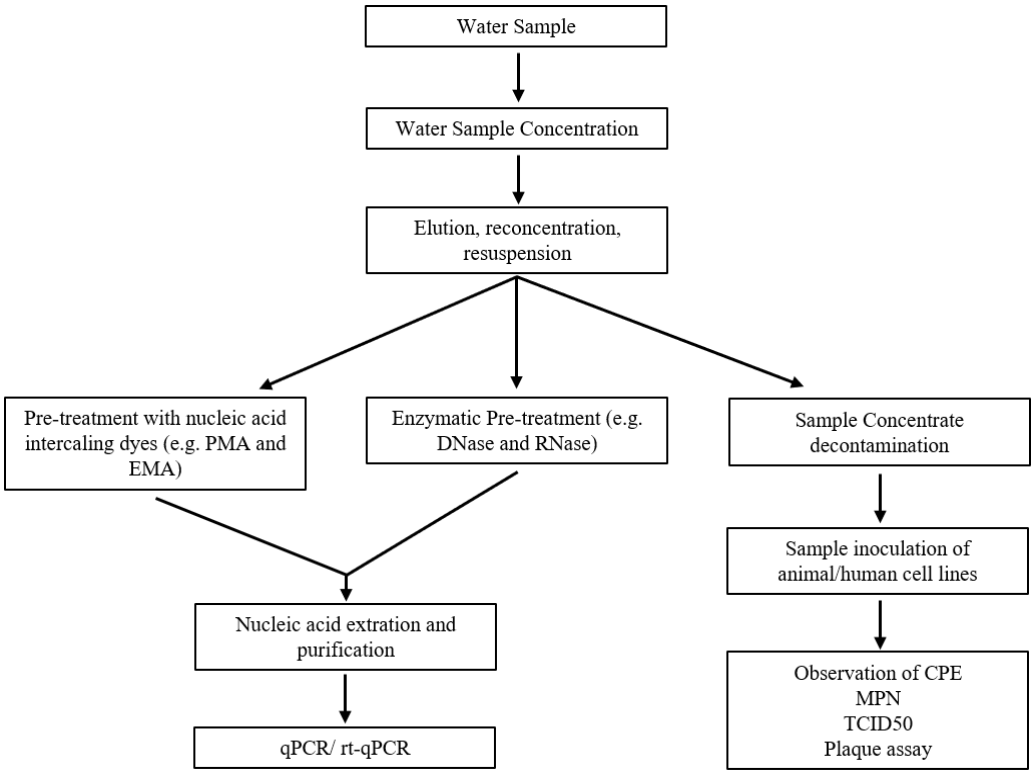


**Figure 3.1** - Scheme of commonly used approaches to determine infectivity in environmental water samples

### 3.1. Cell Culture

Viruses are considered infectious if they are able to penetrate a susceptible cell with the expression at least one viral gene and/or replication of their genome without the need for the ultimate concentration in a real living host.

No single cell culture system is able to correctly detect all viruses even within the same viral group. A typical example are EntV that although very close structurally and antigenically and easy to detect by cell culture lines are not all able to propagate within a single cell culture line (Leland and Ginocchio, 2007).

Cell infection by viruses occurs by adsorption of the viruses to a cell culture monolayer or double layer, applying the inoculum directly to the cell layers. Two different infectivity assays exist: the (i) quantal and the (ii) quantitative:

(i) A quantal infectivity assay does not quantify the number of infectious viral particles in the sample but indicates their presence in a certain volume of sample. The assays are based on liquid culture media, are dependent on the development of cytopathogenic alterations promoted in monolayers of susceptible host cells and are observed by microscopy. The most frequently used quantal methods are the most probable number of cytopathogenic units (MPNCU) and the 50% tissue culture infectious dose (Clesceri *et al.,* 1998; Mocé-Llivina *et al.,* 2004).

(ii) In quantitative assays, also known as plaque assay, cell destruction by infectious viral particles occurs in a small area, the plaque, by incorporation of agar in the medium, which can be done using a cell monolayer, using cell suspensions or even a mixture of both techniques (the double layer plaque assay – Figure 3.2). In this case, plaque formation occurs after incubation under strict conditions and plaques are counted following incubation or as soon as they become visible. For enteroviruses, the development of plaques usually appears after three or four days of incubation. Since suspended cells provide a larger number of adsorption sites for the viruses it is three to four times more sensitive than the monolayer assay.

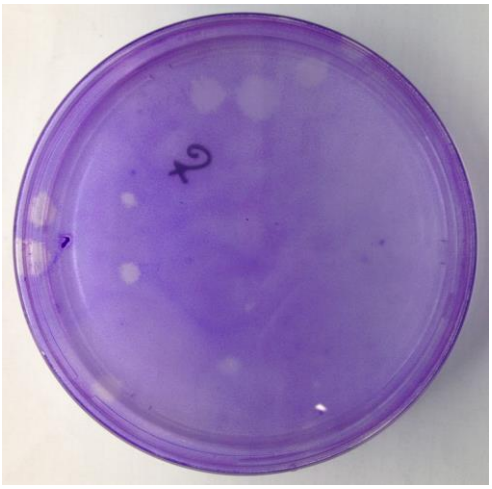

**Figure 3.2 -** Example of the double-layer plaque assay for detection of enteroviruses. BGM cell line (dyed using a solution of crystal-violet). Transparent spots correspond to cell lysis by enteroviruses (Image: Author's own)

A study by Mocé-Llivina *et al.* (2004) tested the efficiency of recovery for different cell culture assays: monolayer plaque assay, most probable number of cytopathogenic units (MPNCPU), suspended-cell plaque assay, and a novel developed technique termed the double-layer plaque assay. The latter combines the monolayer plaque assay and plaque assay on suspended cells. The authors determined that the efficiency of the different methods for quantification of enteroviruses ranked as follows: double-layer plaque assay ≥ suspended-cell plaque assay ≥ MPNCPU ≥ monolayer plaque assay. Although there may be more virions included as a result of virus association mainly with organic and inorganic matter, one plaque is considered as the progeny of one infectious particle.

The infectivity of enteric viruses may also be determined in a liquid culture system, where a viral inoculum is added to the cell culture and are afterwards observed for a specific cytopathic effect (CPE). Through computational analysis of positives it is possible to determine a titre in terms of MPN units or Tissue Culture $Dose_{50}$ ($TCD_{50}$) units.

Although cell culture is widely regarded as the ultimate tool to access viral infectivity, it also presents some drawbacks including: 1) costs and time to produce results; 2) viral counts should be interpreted carefully and several serotypes do not form plaques, which are important facts especially when used in quantitative microbial risk assessments (QMRA) for the estimation of public health impact; 3) they are sometimes not specific for a particular virus with the need for further identification; 4) viral clump formation is extremely frequent, and so it is possible that a plaque may not actually arise from only one virion; 5) cytotoxicity of co-concentrated compounds from environmental samples which may lead to false-positive results or to a decrease in the sensitivity, as a result of hindered

cell culture systems; 6) some viruses grow slowly or are unable to grow in cell culture systems (Hamza *et al.*, 2011).

### 3.2. Enzymatic Pretreatment

Damage to viral capsid or genome, inducing loss of infectivity may still produce a positive signal by PCR. In an attempt to achieve infectivity through molecular tools, Nuanualsuwan and Cliver (2002) suggested the use of a combined proteinase K and RNase pretreatment. The objective behind this hypothesis was that proteinase K would digest damaged viral capsids (not affecting intact viral capsids) preventing therefore RNA protection and the detection of residual signal by PCR. The authors determined that inactivated viruses (HAV, poliovirus-1 and feline calicivirus) treated with proteinase K and RNase previous to RT-PCR produced negative signals. Pretreatment with proteinase K and RNase has been applied in further studies with different levels of success (Table 3.1; Baert *et al.*, 2008; Lamhoujeb *et al.*, 2009; Pecson *et al.*, 2009; Tang *et al.*, 2010; Diez-Valcarce *et al.*, 2011).

Combined use of proteinase K and RNase has declined as a result of difficulties in controlling and reproducing results for several reasons (Knight *et al.*, 2013). Further information is provided by Wieger and Hilz (1971) and Mormann *et al.* (2010). As a result, several studies have used RNase treatment alone, which produces partial or complete elimination of false-positive PCR results (Table 3.1; Topping *et al.*, 2009; Diez-Valcarce *et al.*, 2011; Schielke *et al.*, 2011; Li *et al.*, 2012).

Topping *et al.* (2009) used RNase ONE enzyme to determine the effect of increasing temperature in the capsid of NoV GII.4 (a non-cultivable virus) and feline calicivirus (FCV-9), a cultivable norovirus surrogate. The authors observed that RT-qPCR signals of FCV-9 and NoV GII.4 decreased 2- to 3-log units after RNase ONE pretreatment, not achieving complete removal. A study performed with NoV GII.3 in different foodstuffs subjected to different treatments have also shown similar results with reductions ranging from 0-log units to more than 7-log units (Mormann *et al.*, 2010).

### 3.3. Viability PCR

A different approach involving the use of PCR or qPCR coupled with nucleic acid intercalating dyes such as propidium monoazide (PMA) or ethidium monoazide (EMA), known as viability PCR has been suggested (Novga *et al.*, 2003; Nocker and Camper, 2006; Nocker *et al.*, 2006; Table 3.1). It is based on the assumption that viability dyes penetrate only into compromised membrane cells and once inside the cell, the dye intercalates covalently into the nucleic acid following exposure to strong visible-UV light,

preventing nucleic acid amplification. The methodology has been successfully implemented in bacteria, viruses, fungi and protozoa.

Several aspects should be considered when applying PMA and EMA and these include (Fittipaldi *et al.,* 2012): 1) dye concentration, incubation time and temperature; 2) target organism; 3) length of the target gene and target sequence; 4) sample physicochemical parameter (salt concentration, turbidity, and pH); 5) fraction of dead/non-infectious to live/infectious cells; 6) light source and exposure time; 7) inactivation process. Moreover, both dyes were compared and though their behaviour as intercalating agents is equivalent, they show different permeation rates through the membrane. EMA, given its smaller chemical composition, is relatively more effective than PMA in reducing the false-positive signal but PMA is more efficient in discriminating between live and dead cells, infectious vs. non-infectious viral particles (Fittipaldi *et al.*, 2012).

PMA/EMA pretreatment has been used successfully to discriminate between infectious and heat inactivated Enterovirus, HAV and MNV (Parshionikar *et al.,* 2010; Kim *et al.*, 2011; Sanchez *et al.,* 2012; Karim *et al.,* 2015). Other publications have described that EMA or PMA did not manage to reduce totally the false-positive signal in PCR (Karim, *et al.*, 2015; Leifels *et al.*, 2015). The fluctuations found between the works are possibly due to different inactivation temperatures tested, qPCR gene targeted, and the difference in the viruses themselves (Coudray-Meunier *et al.*, 2013; Karim *et al.*, 2015; Leifels *et al.,* 2015). Further details on viability PCR differences are reported by Fittipaldi *et al.,* (2010), Moreno *et al.* (2015) and Prevost *et al.* (2016). However, the effectiveness and usefulness of enzymatic and dye pretreatments to discriminate between infectious and inactivated viruses in complex water matrices has yet to be explored.

**Table 3.1 -** Usefulness of PCR pretreatments for the discrimination of infectious viruses

| Target organism | Inactivation process | Pretreatment used | Successful pretreatment | References |
|---|---|---|---|---|
| HAV<br>PV1<br>FCV | UV radiation<br>Heat treatment<br>Chlorination | Proteinase K + RNase | Yes | Nuanualsuwan and Cliver, 2002 |
| MNV | Heat treatment | Proteinase K + RNase | No | Baert *et al.*, 2008 |
| FCV<br>NoV | Heat treatment | Proteinase K + RNase | Yes | Lamhoujeb *et al.*, 2009 |
| MS2 | Heat treatment<br>Singlet oxygen ($^1O_2$)<br>UV radiation | Proteinase K + RNase | No<br>No<br>Partially | Pecson *et al.*, 2009 |
| MNV | High-pressure processing (HPP) | Proteinase K + RNase | Partially | Tang *et al.*, 2010 |
| MNV | HPP<br>Heat treatment<br>UV radiation | Proteinase K + RNase<br>RNase | No | Diez-Valcarce *et al.*, 2011 |
| FCV<br>NoV | Heat treatment | RNase | No | Topping *et al.*, 2009 |
| HEV | Heat treatment | RNase | Yes (except for treatment at 56 ºC for 15 min) | Schielke *et al.*, 2011 |

| **Table 3.1** - Usefulness of PCR pretreatments for the discrimination of infectious viruses | | | | |
|---|---|---|---|---|
| MNV<br>NoV | Heat treatment | RNase | No | Li *et al.*, 2012 |
| HAV | Heat treatment | RNase | No | Sanchez *et al.*, 2012 |
| Coxsackievirus (CV)<br>Echovirus (EV)<br>PV<br>NoV | Heat treatment<br>Hypochlorite | PMA | Yes<br>Yes<br>Yes<br>Yes by rt-PCR. No by rt-qPCR | Parshionikar *et al.*, 2010 |
| T4 | Heat treatment<br>Proteolytic treatment | PMA | No (except for treatment at 110 ºC for 15 min) | Fittipaldi *et al.*, 2010 |
| MNV<br>PV | Heat treatment | EMA | Yes | Kim *et al.*, 2011 |
| MS2<br>MNV | Heat treatment | PMA | Yes<br>No | Kim and Ko, 2012 |
| HAV | Heat treatment<br>Hypochlorite treatment<br>HPP | PMA | Yes<br>Yes<br>Partially | Sanchez *et al.*, 2012 |
| HAV<br>RoV (Wa)<br>RoV (SA11) | Heat treatment | PMA<br>EMA | Partially | Coudray-Meunier *et al.*, 2013 |

| **Table 3.1** - Usefulness of PCR pretreatments for the discrimination of infectious viruses | | | | |
|---|---|---|---|---|
| PV<br>MNV<br>NoV | Heat treatment, chlorine, UV radiation | PMA | Yes, Yes, No<br>No, Yes, No<br>No, No, No | Karim *et al.*, 2015 |
| HAdV<br>PV<br>RoV<br>MNV<br>φX174 | Heat treatment, hypochlorite treatment, UV radiation, | PMA<br>EMA | No, Partially, No<br>No, Yes, No<br>No, Yes, No<br>No, Yes, No<br>No, Partially, No | Leifels *et al.*, 2015 |
| HAV | Heat treatment | PMA<br>EMA | Significant reduction | Moreno *et al.*, 2015 |
| HAdV<br>CV | Heat treatment, hypochlorite treatment, UV radiation | PMA<br>EMA | Partially, Close to cell culture, No<br>Partially, Close to cell culture, No | Prevost *et al.*, 2016 |

# CHAPTER 4 : MATERIALS AND METHODS

## 4.1. Evaluation of traditional and alternative surrogate organisms for removal of enteric viruses during municipal wastewater treatment processes

### 4.1.1. Sampling

Samples of raw and treated municipal wastewater were collected from 26 wastewater treatment plants (WWTP) in Portugal for the detection and quantification of SC, GB-124 phage and the three human-specific viruses: HAdV, NoV, and JCPyV. WWTP were chosen with reference to population equivalent (p.e.) and type of treatment, and included secondary and tertiary (UV disinfection) treatment (Table 4.1). According to the EU Urban Wastewater Treatment Directive (EU UWWT), small-scale WWTP (<2000 p.e.) often employ only primary treatment to remove a significant proportion of the suspended solids. In municipalities with a higher population (i.e. > 10,000 p.e.), and/or with high seasonal influx of tourists, the treatment requirements are greater and typically consist of three main phases (Figure 4.1).

**Table 4.1.** Number of samples analysed and type of treatment for each WWTP chosen

| WWTP N. | N. samples/Type of WWTP | Type of treatment |
|---|---|---|
| WWTP01 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP02 | 2 – D. | Primary + Secondary (activated sludge) |
| WWTP03 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP04 | 1 – D. + I. | Primary + Secondary (activated sludge) + Disinfection (UV) |
| WWTP05 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP06 | 1 – D- | Primary + Secondary (activated sludge) |
| WWTP07 | 5 – D. + I. | Primary + Secondary (activated sludge) + Disinfection (UV) |
| WWTP08 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP09 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP10 | 2 – D. | Primary + Secondary (activated sludge) |
| WWTP11 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP12 | 1 – D. + I. | Primary + Secondary (activated sludge) + Disinfection (UV) |
| WWTP13 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP14 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP15 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP16 | 1 – D. + I. | Primary + Secondary (activated sludge) + Disinfection (UV) |
| WWTP17 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP18 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP19 | 1 – D. + I. | Primary + Secondary (activated sludge) + Disinfection (UV) |
| WWTP20 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP21 | 2 – D. | Primary + Secondary (activated sludge) |
| WWTP22 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP23 | 1 – D. | Primary + Secondary (activated sludge) |
| WWTP24 | 3 – D. | Primary + Secondary (activated sludge) |
| WWTP25 | 2 – D. | Primary + Secondary (activated sludge) |
| WWTP26 | 1 – D. + I. | Primary + Secondary (activated sludge) + Disinfection (UV) |

D. – domestic influent; I. – industrial influent

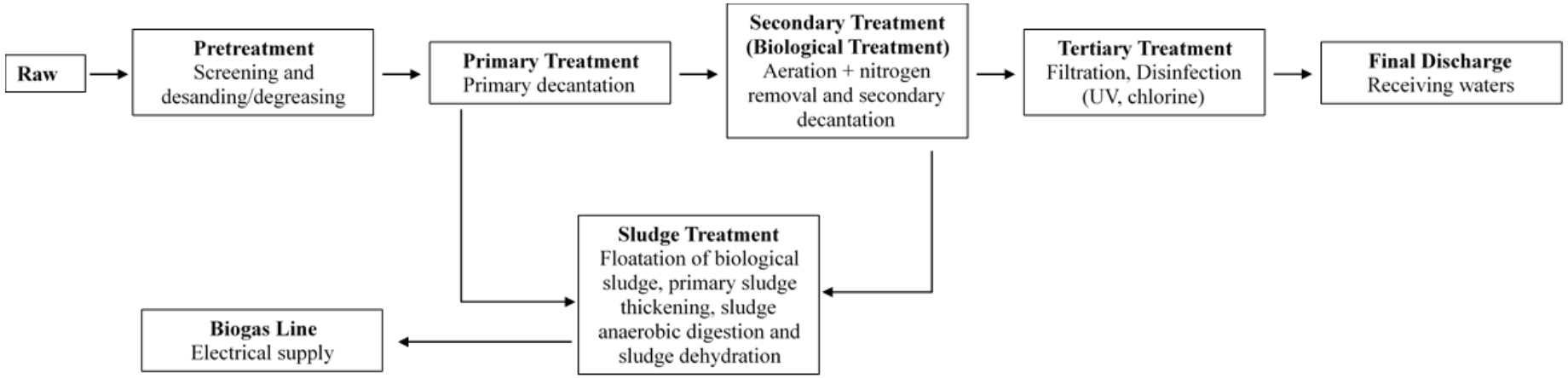


**Figure 4.1 -** Description of the various treatment stages of a typical Portuguese WWTP (Source: SimTejo, 2013)

Raw and treated wastewater samples were collected in 500 mL sterile polyethylene bottle (Vidrolab 2, Portugal), transported to the laboratory facilities at 5 (± 3) ºC and analysed for the presence of EC, IE, SC, and GB-124 phage, upon arrival at the laboratory (within six hrs of sampling). A selected sample was analysed in duplicate at every sampling date.

### 4.1.2. Enumeration of faecal indicator bacteria

EC and IE were enumerated in raw and treated wastewater by the Most Probable Number (MPN) method using the Colilert and Enterolert systems (IDEXX Laboratories, USA), according to standard methods (ASTM, 1999; UK Environment Agency, 2009). For both raw and treated effluents, dilutions were performed as necessary to achieve bacterial concentrations within a suitable range for the method. Enumeration of EC and IE was carried out accordingly to UK Environment Agency (2009) and ASTM (1999). Each sample, or dilution thereof was poured into a 100 mL-sterile plastic bottle and mixed with sterile $H_2O$ up to a final volume of 100 mL. The appropriate substrate (Colilert-18 or Enterolert) was added to the 100mL-bottle and the mixture was allowed to settle (for up to 10 mins) until dissolved. The contents were then poured into a Quanty-tray® and the tray was placed, carefully, in the pre-warmed heat sealer (IDEXX Laboratories, USA). The sealed Quanty-tray® was then incubated at 37 ºC for 18-22 h and at 41 ºC for 18-22 h, for EC and enterococci, respectively. Positive and negative controls were added both for EC and for IE. Samples were analysed in duplicate. Following the incubation period, the trays were observed under UV light and wells showing fluorescence were counted (Figure 4.2).

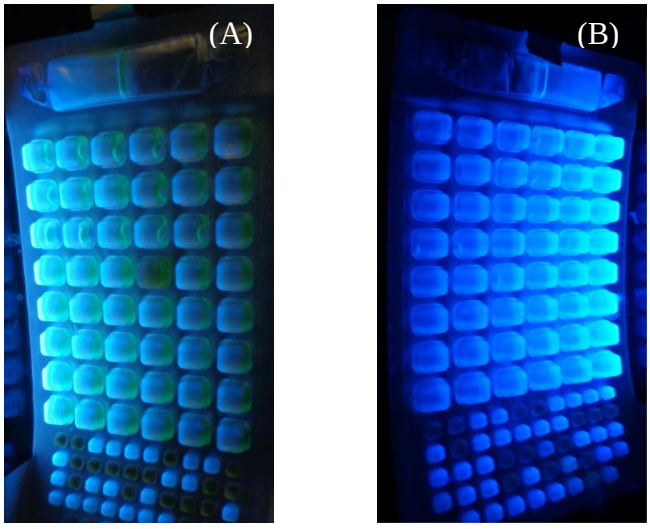


**Figure 4.2 -** MPN tray results, (A) Colilert; (B) Enterolert. Fluorescence under UV light demonstrates the activity of the enzymes (Images: Author's own)

The number of positive wells was then cross-referenced to the relevant MPN table (IDEXX, 2015b) in order to obtain the most probable number of organisms per 100 mL in the original sample (subject to correction for dilution). Both Colilert and Enterolert are based on defined substrate technology (DST). The defined substrate in Colilert is 4-methylumbelliferyl-β-D-glucuronide, the major available carbon source, which is metabolized by *E. coli* using the enzyme β-glucuronidase. For enterococci, the defined substrate is 4-methylumbelliferyl-β-D-glucoside, which is degraded by enterococci using β-glucosidase. Each enzyme hydrolyses the respective substrate, producing a compound that fluoresces under UV light (4-methylumbelliferone). The level of false-positives and false-negatives associated with this technology is relatively low as most of microorganisms either do not express these specific enzymes, or the few capable of doing so are supressed by the specific matrix of the substrate.

### 4.1.3. Detection and enumeration of SC and GB-124 phage

The detection and enumeration of bacteriophages was performed in duplicate using the double-layer method specific for the detection of SC and *B. fragilis* (GB-124) phages (ISO 10705-2; ISO 10705-4). Prior to analysis, water samples (raw and treated wastewater) were passed through a 0.22 µm polyvinylidene fluoride (PVDF) filter (PALL, UK) with low protein binding, which retains bacteria and particulate organic matter whilst permitting the passage of bacteriophages (Tartera *et al.*, 1992; Mocé-Llivina *et al.*, 2003).

For determining the level of SC, 1 mL of *E. coli* inoculum culture was added to 2.5 mL of semi-solid agar medium and to 1 mL of sample (or dilution or concentration), vortexed, poured over a layer of solid agar and left to set. The plates were then inverted and incubated at 36 (± 2) ºC for 18 (± 2) h.

For enumeration of *Bacteroides* GB-124 phages the procedure was similar. For each sample tested, 1 mL of the *Bacteroides* GB-124 inoculum culture was added to 2.5 mL of semi-solid agar medium (*Bacteroides* phage recovery medium agar; 1% agar; ISO 10705-4). One mL of sample (or dilution, or concentration (see procedure below)) was added subsequently to the mixture. The latter was slowly vortexed in order to avoid the formation of air bubbles, poured over the medium with 2% agar, and left to set. The plates were then inverted and placed in anaerobic jars containing an anaerobic sachet (Oxoid, UK), an anaerobic indicator and incubated at 36 (± 2) ºC for 18 (± 2) h. Each sample was analysed in duplicate and positive and negative controls were added. Following incubation, infection of the specific hosts (*E. coli* or GB-124 culture) by their specific bacteriophages (somatic coliphages or *Bacteroides* GB-124 bacteriophage) generated bacterial lysis, which

produced clear plaques, with each plaque representing one plaque-forming unit (PFU) (Figure 4.3). Complete media constitution available in Appendix 1.

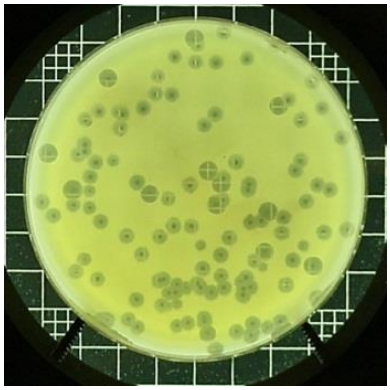

**Figure 4.3 -** Zone of lysis (plaques)

Samples demonstrating no plaques were further concentrated according to the following protocol: 1.5 % of 4.14 M $MgCl_2.6H_2O$ were added to the sample and the mixture was allowed to rest for a couple of minutes. Samples were filtered through a 0.22 µm mixed cellulose esters membrane filter (Whatman, GE Healthcare, US). The membrane filter was then cut into eight pieces and these were placed in 5 mL of eluent in a 50 mL glass flask. The bacteriophages were then recovered by ultrasonication for 5 min at 45 Hz. The concentrated samples were then assayed as described previously.

Numbers of both bacteriophages (PFU/mL) were calculated as follows:

$$n_{pfp} = \frac{N}{(n_1 V_1 F_1) + (n_2 V_2 F_2)}$$

Where:

- $n_{pfp}$ - number of plaque forming particles/units of somatic coliphage/ GB-124 bacteriophage per millilitre (PFU/mL)
- N - total number of plaques counted on plates
- $n_1$, $n_2$ – number of replicates counted for dilutions $F_1$ and $F_2$, respectively
- $V_1$, $V_2$ – test volumes used with dilution $F_1$ and $F_2$, respectively
- $F_1$, $F_2$ – dilution or concentration factors used for the test portions $V_1$ and $V_2$, respectively (F = 1 for undiluted, F = 0.1 for ten-fold dilution, F = 10 for a ten-fold concentration, etc.).

### 4.1.4. Concentration of viruses from water samples

The recovery of viral particles from raw wastewater samples was achieved using an ultracentrifugation method (Puig *et al.,* 1994; Albinana-Gimenez *et al.,* 2006). In brief, 42 mL of sample were ultracentrifuged for 1 h at 110 000 x g at 4 ºC. The ultracentrifugation allows the sedimentation of viruses together with the suspended solids. The pellet was then eluted with 3.5 mL of glycine buffer (0.25 M, pH 9.5). The samples were placed on ice for 30 min and 3.5 mL of 2 x Phosphate Buffered Saline (PBS) added and the contents centrifuged for 20 min at 12 000 x g. This centrifugation achieved separation of the suspended solids from the supernatant. A final step of ultracentrifugation, for 1h at 110 000 x g and at 4 ºC, was again executed to pellet down the viruses. The supernatant was carefully removed and the pellet was resuspended in 8 mL of PBS. The viral suspension was stored at -80 ºC prior to use (which was within six months).

To concentrate enteric viruses from 1-L treated effluent, a method based on direct organic flocculation was used, with the adhesion of viral particles to pre-flocculated skimmed milk (Calgua *et al.,* 2008; Wyn-Jones *et al.,* 2011). In brief, the pH of the samples was pre-regulated to pH 3.5 by adding 1 N HCl. 5 mL of pre-flocculated 1 % (w/v) skimmed milk was then added to the sample, so that the final concentration of skimmed milk was 0.01 % (w/v). The samples were agitated at room temperature for 8 h using a magnetic stirrer and fleas (Fisher Scientific). This step allows the binding of the viruses to the organic matter in suspension. The material in suspension was allowed to settle, by gravity for a further 8 h and the supernatant was carefully removed. The flocculated sample was then centrifuged at 12 ºC for 30 min at 7 000 x g. Following centrifugation, the supernatant was carefully removed and the sediment was resuspended in 8 mL of 0.2 M phosphate buffer (pH 7.5). The phosphate buffer was prepared by using a solution of 0.2 M of sodium monohydrogen phosphate ($Na_2HPO_4$) and a solution of sodium phosphate ($NaH_2PO_4$) at a proportion of 1:1. The volume of the resuspended viral particles was finally adjusted to 10 mL. The solution was kept at -80 ºC until further use.

The efficiency of viral concentration procedure was evaluated with spiking of Mengovirus MC0 ($MC_0$) with a known concentration of approximately $10^4$ genome copies/µL.

### 4.1.5. Extraction of nucleic acids from samples

The nucleic acids were extracted from 140 µL of concentrated samples using commercially available kits, QIAamp® Viral RNA Mini Kit (Qiagen GmbH, Germany), following the manufacturer's instructions (Figure 4.4). The kit is based on a well-known procedure used for the extraction of viral nucleic acid. The method relies on the Boom method (Boom,

1990) which conjugates the application of guanidinium thiocyanate, a chaotropic agent with lysing and nuclease-inactivating properties, with the nucleic acid binding properties of the silica-gel based membrane (QIAamp Mini column). Prior to extraction, the samples and AVE buffer were equilibrated to room temperature. Lysis of the cells was performed by adding 140 µL of each sample to 560 µL of pre-prepared AVL-carrier RNA buffer. The AVL buffer contains RNase inactivating properties and the AVL-carrier RNA buffer was prepared as follows:

$$n \times 0.56\, mL = y\, mL$$

$$y\, mL \times 10\ {}^{\mu L}/_{mL} = z\, \mu L$$

Where:

n – number of samples to be processed simultaneously

y – calculated volume of AVL buffer

z – volume of carrier RNA to be added to buffer AVL.

Samples were mixed vigorously for 15 s to ensure complete homogenisation of the solution so that an efficient lysis was carried out. The samples were incubated for 10 min at room temperature. The solution was then briefly centrifuged at 8 000 rpm for 30 s to remove the drops from the lid. 560 µL of ethanol (96 %) were added to the samples and these solutions were vortexed vigorously to allow complete homogenisation, followed by brief centrifugation at 8000 rpm for 30 s to remove the drops from the lid. The addition of ethanol provides the necessary interactions to efficiently bind the sample to the silica gel-based membrane. 630 µL of the solution were applied to the QIAamp mini spin columns and centrifuged at 8 000 rpm for 1 min. The filtrate was discarded and the mini column was placed in a new collection tube. The last two steps were then repeated. To wash any residual contaminants, two short centrifugations were performed using wash buffers (AW1 and AW2). The first washing step was carried out by applying 500 µL of AW1 buffer and centrifuging at 8 000 rpm for 1 min. The filtrate was then discarded and the mini column placed into a new 2 mL eppendorf® tube. 500 µL of AW2 buffer was added to the spin column and the sample was centrifuged for 3 min at 14 500 rpm. The filtrate was again discarded and a new centrifugation step performed for 1 min at 14 500 rpm. This additional

centrifugation step removes residual AW2 buffer that was not removed during the previous centrifugation, as AW2 buffer may cause problems in the subsequent assays. The mini spin column was then placed in a new microcentrifuge tubes (1.5 mL) and the collection tubes with filtrate were ditched. A double elution of the spin column was performed using 2 times 40 µL of buffer AVE, followed by incubation at room temperature for 1 min and centrifugation for 1 min at 8 000 rpm. The viral nucleic acid was kept at -80 ºC prior to further processing.

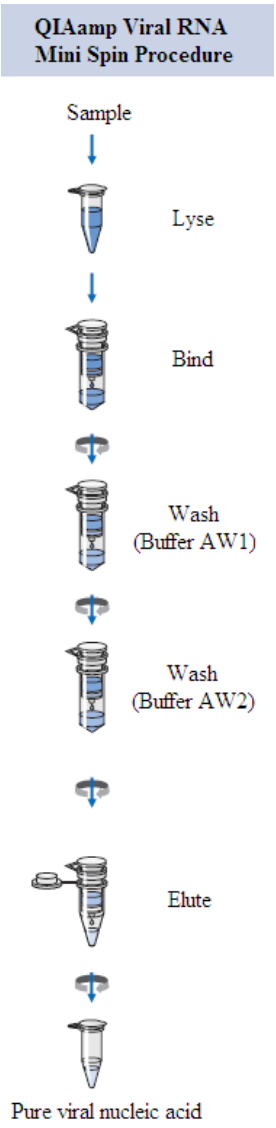


**Figure 4.4 -** QIAamp viral RNA procedure (QIAGEN, 2012)

### 4.1.6. cDNA synthesis

NoV is a single-stranded RNA virus and in order to quantify it, a reverse transcription (RT) to single-stranded complementary DNA (cDNA) step is required. cDNA synthesis was

carried out using the High Capacity cDNA reverse transcription kit (Applied Biosystems, US) following the manufacturer's instructions (Figure 4.5).

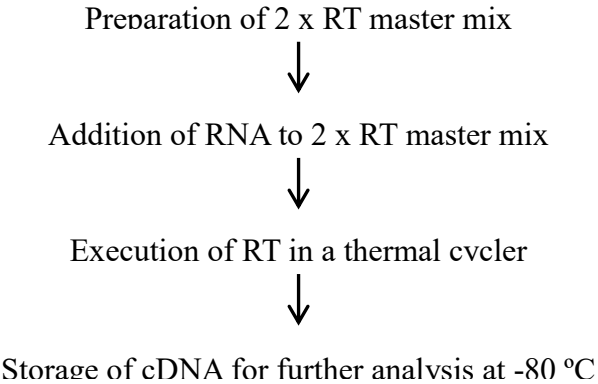


**Figure 4.5 -** Scheme of cDNA synthesis using High Capacity cDNA RT kit

The volumes of each component used per reaction are presented in the table below (Table 4.2):

**Table 4.2 -** RT master mix reagents and respective volumes

| **Reagent** | **Volume/Reaction (µL)** |
|---|---|
| 10 x RT buffer | 2.0 |
| 25 x dNTP mix (100 mM) | 0.8 |
| 10 x NoV Reverse primer (1319-r) | 2.0 |
| MultiScribe™ Reverse Transcriptase | 1.0 |
| Nuclease-free $H_2O$ | 4.2 |
| Total per reaction | 10.0 |

Reverse transcription of NoV was performed using reverse primer 1319-r (Jothikumar *et al.*, 2005; La Rosa *et al.*, 2009). Ten µL of extracted RNA sample were added to 10 µL of 2 x RT master mix in a PCR tube (0.2 mL, MβP Molecular BioProducts, Thermo Fisher, US). The tubes were then briefly centrifuged and loaded in the thermocycler (Verity® Thermal Cycler, Applied Biosystems, US) using the appropriate program (Appendix 1.B). cDNA samples were stored at -80 ºC prior to further processing by real-time PCR.

### 4.1.7. Real-time PCR

To compare the levels of SC and GB-124phages with those of enteric viruses, a qPCR based on TaqMan probe system was performed for the latter. qPCR is a variation of

conventional PCR for the quantification of DNA samples in which fluorescence is measured and the increase of the fluorecense signal is proportional to the amount of amplicon (product of amplification). The result of a real-time PCR reaction is displayed as an amplification curve, or plot (Figure 4.6). The amplification curve displays all the information required of a real-time PCR. The line of 'Threshold' represents the point where the intensity of the reporter signal surpasses the intensity level of fluorescence in the background. For a more accurate reading, the threshold line is located at the exponential phase of the amplification. The Cycle Threshold (Ct) is the cycle at which the sample reaches this level. The set of primers and probes for the detection of AdV, JCPyV and NoV were chosen for their capacity to amplify only the human strains of these viruses (Hernroth *et al.,* 2002; Jothikumar *et al.,* 2005; Pal *et al.,* 2006; La Rosa *et al.,* 2009). The primers and probes used for the detection and quantification of HAdV were selected on a conserved region of the AdV hexon gene (Hernroth *et al.,* 2002). The combination of the two probes allows the detection of all the AdV genome variations (from serotype A to F). For the detection of NoV, the construction of the primers and probes was performed on the highly conserved ORF1/ORF2 junction region of this virus genome (Jothikumar *et al.,* 2005; La Rosa *et al.,* 2009). The sequences of primers and probes used, the product length, and their position in the genome are presented below (**Erro! A origem da referência não f oi encontrada.**).

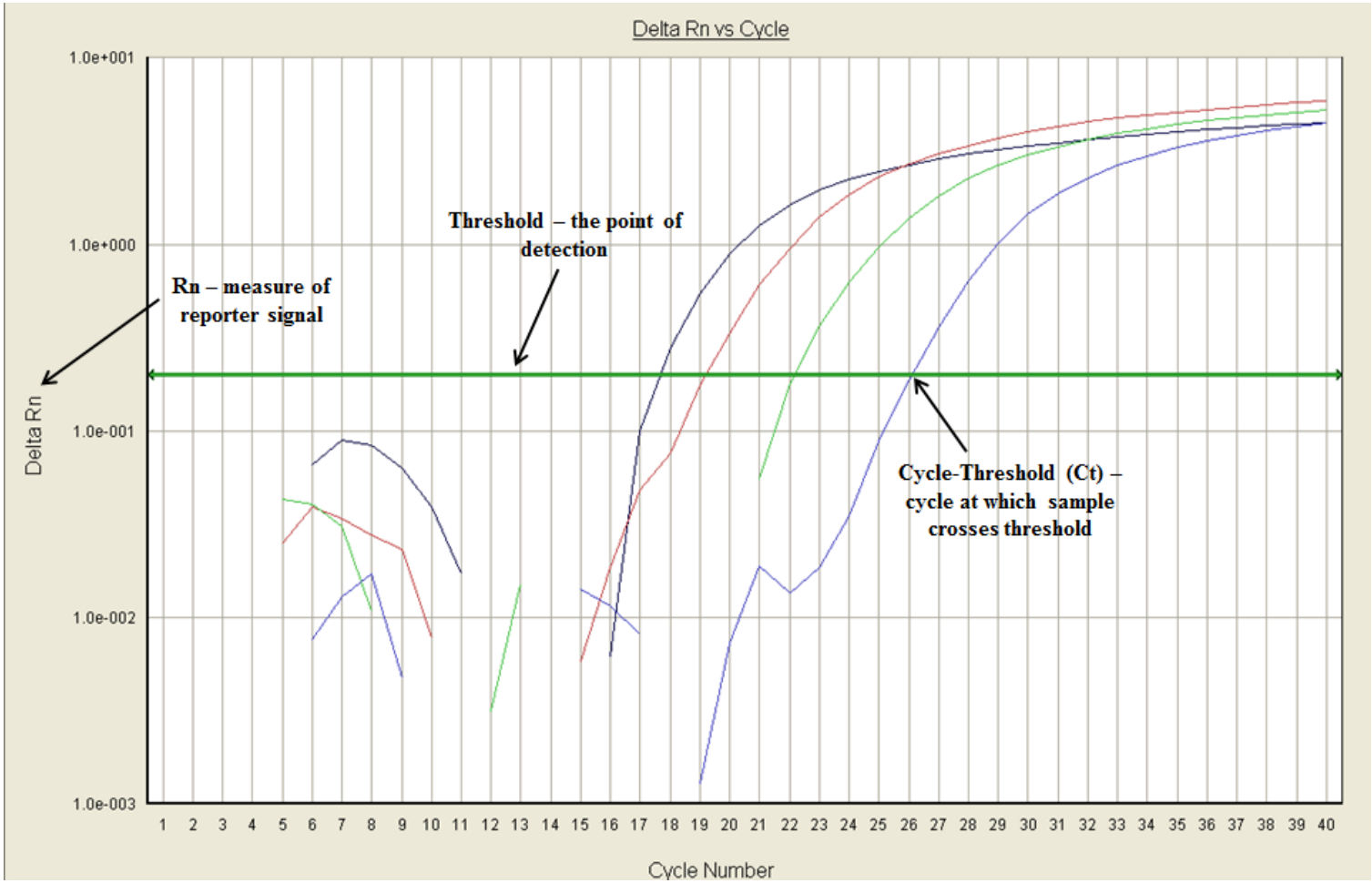


**Figure 4.6 -** Amplification curve obtained in a real-time PCR (Images: Author's own)

**Table 4.3 -** Selected primers and probes for the detection of HAdV, JCPyV and NoV

| Virus | Oligonucleotide | Sequence[a] | Product Length (bp) | Location/ Annealing Temperature | Reference |
|---|---|---|---|---|---|
| HAdV | HAdV_F | CWT ACA TGC ACA TCK CSG G | 68 | 18373-18441[b] / 60 ºC | Hernroth *et al*., 2002 |
| | HAdV_R | CRC GGG CRA AYT GCA CCA G | | | |
| | Ad:ACDEF | (6FAM)-CCG GGC TCA GGT ACT CCG AGG CGT CCT-(BHQ1) | | | |
| | Ad:B | (6FAM)-CCG GAC TCA GGT ACT CCG AAG CAT CCT-(BHQ1) | | | |
| JCPyV | JE3_F | ATG TTT GCC AGT GAT GAT GAA AA | 88 | 4251-4339[c] / 60 ºC | Jothikumar *et al*., 2005; La Rosa *et al*., 2009 |
| | JE3_R | AAA GGT AGA AGA CCC TAA AGA CTT TCC | | | |
| | JE3_P | (6TAMRA)-AGG ATC CCA ACA CTC TAC CCC ACC TAA AAA GA-(BHQ2) | | | |
| NoV | 1380-F | CAAGAGYCAATGTTYAGRTGGATGAG | 98 | 5003-5100[d] / 60 ºC | Pal *et al*., 2006 |
| | 1319-R | TCGACGCCATCTTCATTCACA | | | |
| | 1379-P | (6JOE)-TGGGAGGGCGATCGCAATCT-(BHQ1) | | | |

BHQ = Black Hole Quencher; bp = base pair; 6FAM – 6-Fluorescein Amidite; JOE – 4-5-Dichloro Carboxy Fluorescein; TAMRA - Carboxytetramethylrhodamine [a] W = A or T; K = G or T; S = C or G; R = A or G; Y = C or T; [b] Sequence position refer to the Human adenovirus 16 strain E26, complete genome sequence; [c] Sequence position refer to JCPyV Mad-1, complete genome sequence; [d] Sequence position refer to Lordsdale virus, complete genome

The assay for each virus was designed and inserted in the real-time PCR software program (7300 System SDS Software, Applied Biosystems, US). The assay contained the identification of the samples, a standard curve, negative control and a positive control (Appendix 1.B).

The amplifications were performed in 25 µL of reaction mixture using the TaqMan® Universal Master Mix II (Applied Biosystems, US). The master mix is provided in a 2 x concentration and is constituted by AmpliTaq Gold® DNA Polymerase, uracil-N glycosylase (UNG), dNTP mixture (with dUTP), ROX as the passive reference dye and an optimized buffer solution. 12.5 µL of the master mix was mixed with 5 µL of Internal Control (IC), 0.28 µM of IC primers, 0.07 µM of IC probe, and the corresponding primers and TaqMan probes at their corresponding concentration (Appendix 1.C). The volume was adjusted to 20 µL with sterile DNA and RNA-free water.

A volume of 20 µL of master mix reaction were distributed in a 96-well PCR microplate (Thermo Scientific, US). The standard curve, the samples (or dilutions), and the controls were added in their respective well. The plate was covered with a plastic band and inserted into the real time PCR instrument (7300 Real-Time PCR System, Applied Biosystems, US) and the program was set to run. The first step occurs at 50 ºC for 2 min, followed by activation of the AmpliTaq Gold DNA Polymerase at 95 ºC for 10 min, only activated when subjected to high temperatures (95 ºC for 5-10 min).

The two steps described herein are performed during 40 cycles. The first of the two steps corresponds to the dissociation, at 95 ºC for 15s, of the double-stranded DNA into single-stranded DNA. This allows for the annealing of the primers to each of the opposite strands and also the annealing of the probe. The annealing reaction is performed at 60 ºC for 1 min. The annealing temperature is extremely important as it prevents primers self-annealing, probe self-annealing, primer-probe annealing and non-specific annealing of primers and probes annealing to other regions of the DNA. In this step, the polymerisation of the DNA also occurs, which results in cleavage of the probe and the detection of the reporter signal. The amount of DNA was then calculated with regard to the standard curve and calculated as the number of genome copies (GC) per mL (GC/mL).

### 4.1.8. Quality control of real-time PCR

To minimise any cross-contamination between samples, positive controls and PCR reagents, the master mix and the respective distribution onto the PCR microplates were undertaken in a separate laboratory facility from the one in which the inoculation of the

samples, standard curve and controls had been performed. The standard curve for quantification of HAdV was constructed using the Amplirun® Adenovirus DNA control (Vircell, Spain). For the quantification of JCPyV, NoV, human, cattle, porcine and poultry mitochondrial DNA (HMMit, CWMit, PGMit, and PLMit), the standard curves were obtained by amplification of each target using conventional PCR. After amplification, the amplicons were run in a 4% electrophoresis agarose gel and followed by staining with ethidium bromide. After visualisation of each band and confirmation of the length of the target amplicon, the bands were excised from the gel and DNA was purified using the illustra GFX PCR DNA and Gel band purification kit (GE Healthcare Life Sciences, US) following manufacturer's instructions (GE Healthcare Life Sciences, 2008). The protocol was as follows:

1 – a 1.5 mL DNA-free microcentrifuge tube was weighted. The excised DNA band was transferred into the microcentrifuge tube and weighted to know the band weight;

2 – 10 µL Capture buffer type 3 was added for each 10 mg of gel slice;

3 – the gel slice was mixed by inversion and incubated at 60 ºC for 15-30 min until the band was liquefied. In the meantime, the mixture was mixed by inversion every 3 min;

4 - the Capture buffer type 3 – sample mixture was vortexed to collect the liquid at the bottom of the tube;

5 – 800 µL of the Capture buffer type 3 – sample mixture was transferred onto a GFX MicroSpin column placed previously into one collection tube;

6 – the mixture was incubated at r.t. for 1 min, followed by centrifugation at 16000 x g for 1 min;

7 – the flow was discarded;

8 – steps 5 to 7 were repeated until all sample was loaded;

9 – 500 µL of Wash buffer type 1 was added to the GFX MicroSpin column;

10 – the assembled spin column and collection tube were centrifuged at 16000 x g for 30 s;

11 – the collection tube was discarded and the column was placed into a new 1.5 mL DNA-free microcentrifuge tube;

12 – 50 µL of Elution buffer type 4 was added to centre of the spin column membrane and incubated at r.t. for 1 min;

13 – following incubation, the spin column was centrifuged at 16000 x g for 1 min to recover the purified DNA;

14 – the DNA was stored at -35 ºC until further analysis.

Following purification of a DNA stock for each quantitative parameter, the concentration of each stock was quantified using the NanoDrop ND-1000 spectrophotometer

(ThermoScientifics, DE) and expressed in ng. To calculate the genome copies (GC) of each standard stock, the following formula was applied:

$$GC\ \# = \frac{amount\ of\ amplicon\ (ng)\ x\ 6.0221x10^{23}}{length\ of\ amplicon\ (bp)\ x\ 660\ x\ 1x10^{9}}$$

where:

- amount of amplicon expressed in ng
- the Avogadro's number expressed in molecules/mole
- length of amplicon expressed in bp
- 660 g/mole being the average mass of 1 bp dsDNA.

Serial dilutions of each stock were prepared in DNA/RNA-free water in order to prepare the standard curve for qPCR. The standard dilutions were further aliquoted and stored at -35 ºC until use. An effective internal control (IC) was included in each assay. The concentration of the IC, and the respective primers and probe concentration were optimised and validated previously in the laboratory. The IC used was quantified DNA commercially available (Appendix 1.E). The IC was used to detect PCR amplification inhibitions. In order to consider a real time PCR assay to be valid, the following conditions have to be fulfilled. The standard curve must be composed of at least five of the aforementioned points and the coefficient of determination ($R^2$) has to be at least 0.980. No amplification should be detected for the negative control. If such a situation occurs in this study, the assay was rejected and a new one was performed. If the situation persisted, an evaluation of the problem was performed by determining the causes (contamination of the primers, probe, master mix, water, etc.). For the positive control, an amplification curve had to be obtained. If any of the previous conditions were not fulfilled, the assay was considered invalid and was executed again.

To ensure that no cross-contamination existed during the complete protocol several quality control steps were added, e.g., negative process control, negative extraction control and PCR control. The process control accompanied all the procedure from centrifugation until PCR included. The negative extraction control followed the process from the extraction until the PCR and the blank control was added to each PCR reaction. The negative control consisted of DNA/RNA-free water. Additionally, a positive control was added to each conventional PCR and qPCR run. The results of the water quality monitoring are presented in Chapters 5, 6 and 7.

### 4.2. Microbial Source Tracking (MST) – River Tagus (Rio Tejo) study catchment

The River Tagus catchment is highly developed for most of its course. The catchment was chosen as a study area as it is subjected to a distinct range of potential contaminant sources, catchment features and land management practices. The latter comprises areas classified as:

- rural agricultural areas -including crop cultivation and livestock production;
- Mediterranean vegetation;
- High density urban areas, large numbers of properties connected to a mains sewerage system.

Several dams and diversions have been created to supply drinking water to parts of central Spain and Portugal. The river is highly impacted by wastewater effluent along its course and particularly in the vicinity of Lisbon where it receives effluent from seven WWTP (Figure 4.7; Table 4.4). Although the WWTPs are based in urban areas, they also receive inputs from agriculture and livestock and from industrial sources. The effluent is considered acceptable for a discharge in environmental waters if concentrations of EC are below $10^4$ CFU/100mL. This implies that the River Tagus is subject to high levels of microbiological degradation, particularly in the vicinity of areas with high number of inhabitants. In recent decades there has been great investment and a concerted effort to clean up the River Tagus. The reappearance of dolphins is perhaps evidence of the improving situation (Diário de Notícias, 2016). Nonetheless, high concentrations of FIB are still routinely detected in the river, with concentrations of EC ranging between $10^3$ and $10^4$ CFU/100 mL and IE between $10^2$ and $10^4$ CFU/100 mL (Pers. com.; Pereira *et al.*, 2013) which is still impacted by uncontrolled spillage that may transport contamination from different sources. Moreover, during rainfall events urban run-off present in combined sewers may also be released in to the river system. The objective of focusing on this particular catchment has arisen largely from the lack of detailed information on the actual levels of contamination from both human and non-human sources. As a result four sampling points were selected, site (1) Vila Franca de Xira, site (2) Marina Parque das Nações, site (3) Alcântara, and site (4) Belém (Figure 4.7). Vila Franca de Xira sampling point was selected because of its location at the upper most extent of the tidal river. This sampling point in particular is not impacted from waters from the Atlantic Ocean during high tide, a situation quite distinct from the other sampling points. Moreover, Vila Franca de Xira is a well-known location for bull fighting and for the existence of livestock (mainly cattle, but also pigs). The sampling point, Marina Parque das Nações was chosen because it

is influenced by two important WWTP (WWTP 3 and 4, Figure 4.7). WWTP 4 receives wastewater from livestock farms and discharges into one of the River Tagus tributaries (Rio Trancão). It is important to understand whether the Rio Trancão increases the contamination in River Tagus and if so, which sources are the most important to control. This area is also highly urbanized and used for recreational purposes from cycling to pet walking. The Alcântara sampling site was chosen because of its proximity to the discharge point of the largest WWTP in Lisbon (WWTP 6). This WWTP receives and treats effluents from the majority of Lisbon area and is therefore a main focus of human faecal contamination in the catchment. This sampling point is also highly influenced by the entrance of oceanic waters during high tide. Finally, the Belém sampling point was selected because of its popularity as a recreational site and the fact that it is heavily influenced by the Atlantic Ocean. Sampling was conducted over a period of twelve months (February 2015 to March 2016), in order to understand the influence of seasonality on the concentration of MST markers.

The samples collected in the River Tagus catchment were principally used to test the performance and suitability of the selected MST assays, rather than to serve as an in-depth assessment of the entire catchment. However, the parameters provide potentially useful information regarding the sources of faecal contamination that impact the catchment and as such, help elucidate the associated risks to human health. To determine the levels of faecal contamination of the River Tagus, general indicators of faecal contamination (EC, IE and SC) were measured. To discriminate between sources of faecal pollution, different markers were selected: i) human – human specific phage GB-124, HAdV, and human mitochondrial marker; ii) cattle – cattle mitochondrial marker; iii) pig – pig mitochondrial marker; iv) poultry – poultry mitochondrial marker. Additionally, two novel mitochondrial markers were designed during this study to detect contamination from domestic pet animals (cats and dogs).

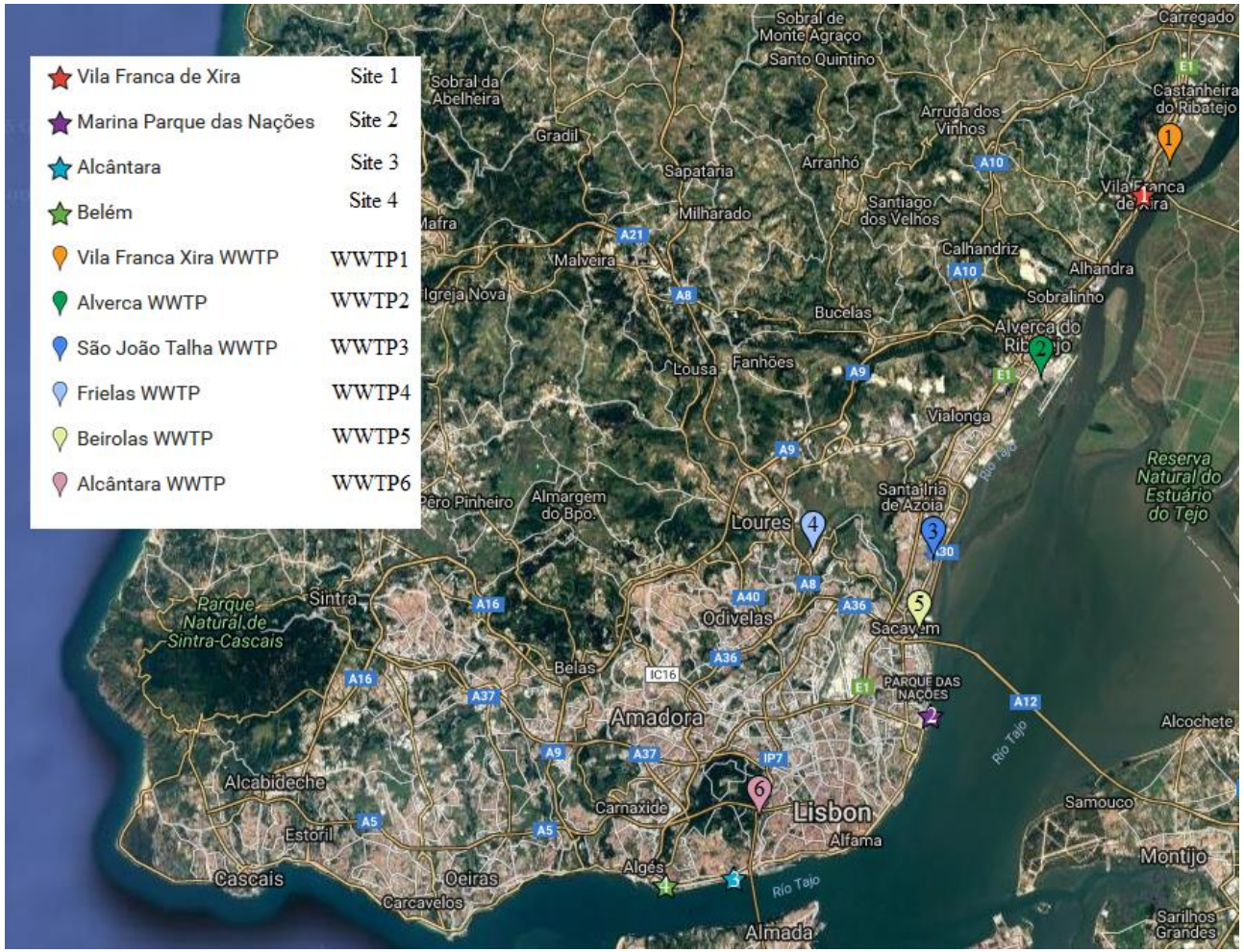


**Figure 4.7 -** The River Tagus **(**Rio Tejo) catchment, sampling sites and WWTPs location (Google, 2016)

**Table 4.4 -** Characteristics of relevant WWTP in Lisbon area (SimTejo, 2011, 2015)

| **WWTP #** | **Description / population served** | **Average daily flow ($m^3$/day)** | **Type of treatment** |
|---|---|---|---|
| 1 – Vila Franca Xira | Urbanised centre; Receives ww from rural areas (mainly bovine) / 136,886 | 9445 | Secondary (activated sludge) |
| 2 – Alverca | Urban centre; Industry / 31,070 | 16157 | Secondary (activated sludge) |
| 3 – São João Talha | Urban centre; High burden of animal waste / 17,252 | 61786 | Secondary (activated sludge) + disinfection (UV treatment) |
| 4 – Frielas | Urban centre; Industry / 28,052 | 12058 | Secondary (activated sludge) |
| 5 – Beirolas | Urban centre / 21,025 | 47667 | Tertiary (UV treatment) |
| 6 – Alcântara | Urban centre; Receives wastewater from outskirt of Lisbon – animal waste / 800,000 | 165390 | Secondary (activated sludge) + disinfection (UV treatment) |

### 4.2.1. FIB, SC, GB-124 phage and HAdV

River water samples were concentrated for these parameters following the methods described previously (see sections 4.1.2 to 4.1.5 and 4.1.7). FIB were quantified using Colilert and Enterolert system (ASTM, 1999; UK Environment Agency, 2009), SC and GB-124 phage were enumerated by ISO standard methods (ISO 10705-2; ISO 10705-4) and HAdV using a skimmed milk organic flocculation (Calgua *et al.*, 2008; Wyn-Jones *et al.*, 2011).

### 4.2.2. mtDNA

Water samples for the analysis of mtDNA were concentrated by centrifugation (Martellini *et al.*, 2005; Caldwell *et al.*, 2007). Samples were centrifuged in 50 mL sterile centrifuge tubes at 9000 x g for 15 min and the supernatant was carefully discharged until all the volume was centrifuged. Pellets were then resuspended in 10 mL of sample. After concentration, mtDNA were extracted using a commercially available kit, QIAamp Viral RNA Mini Kit (Qiagen GmbH, Germany) following manufacturer's instructions as described in section 4.1.5. mtDNA from human origin and from livestock (bovine, pig, and poultry) were quantified using previously described primers and probes (Table 4.4) (Schill and Mathes, 2008). The primers and probes were chosen based on previous studies conducted where these combinations performed better in terms of specificity and sensitivity using faecal matter from the different sources (unpublished data). mtDNA from domestic animals was also detected using a set of original primers designed specifically for this study.

**Table 4.5 -** mtDNA primers and probes used in this study (Schill and Mathes, 2008)

| Target | Sequence (5'-3') | Length (bp) |
|---|---|---|
| Human | | |
| Forward | AGTCCCACCCTCACACGATTCTTT | 184 |
| Reverse | AGTAAGCCGAGGGCGTCTTTGATT | |
| Probe | ACCCTTCATTATTGCAGCCCTAGCAGCACT | |
| Cow | | |
| Forward | AATGCATTCATCGACCTTCCAGCC | 172 |
| Reverse | ACGTCTCGGCAGATATGGGTAACA | |
| Probe | TCGGTTCCCTCCTGGGAATCTGCCT | |
| Pig | | |
| Forward | CGACAAAGCAACCCTCACACGATT | 115 |
| Reverse | TAGGGTTGTTGGATCCGGTTTCGT | |
| Probe | ATCCTGCCATTCATCATTACCGCCCT | |
| Chicken | | |
| Forward | TAGCCATGCACTACACAGCAGACA | 104 |
| Reverse | TTTGCGTGGAGATTCCGGATGAGT | |
| Probe | ACTTGCCGGAACGTACAATACGGCT | |

bp = base pairs

For the specific detection and quantification of targeted mitochondrial DNA genomes, 5 µL of the 10-fold and 100-fold dilutions of every DNA extraction was also assayed to avoid possible inhibition due to the high sensitivity to inhibitors of qPCR assays. Real-time qPCR was carried out in a 25 µL reaction volume using the TaqMan® Environmental MarterMix 2.0 (Applied BioSystems, ThermoFisher Scientific, US). To 12.5 µL MasterMix were added 0.28 µM of each primer and 0.07 µM of the target probe and the reaction mixture was brought to a final volume of 20 µL with DNA and RNA-free water. The cycling conditions were equal to the cycling conditions used for HAdV amplification (see section 4.1.7.) and the analysis of the results calculated with regards to the standard curve and expressed as the number of genome copies (GC) per mL (GC/mL).

#### 4.2.2.1. Design of primers for mtDNA from domestic pets – *Canis lupus familiaris* (dog) and *Felis catus* (cat)

Genome from mitochondria is conserved intra-species but varies between species. This aspect is important to the design of primers that are species-specific for use in PCR. To increase the specificity of the results for dog and cat mtDNA, a nested PCR was developed for this study. The design of the primers started with the selection of the region of interest. For this, mtDNA sequences from dog and cat were obtained from NCBI (NCBI, 2012).

Mitochondrion sequences from cat and dog were aligned with mitochondrion sequences from the remaining targets using ClustalW software (European Bioinformatics Institute, UK). After the alignment of the different sequences, locations in the cat and dog mtDNA with the greatest divergence were chosen and inserted in the Primer Express® 3 software (Applied Biosystems, US).

Two sets of primers were chosen for each target based on primer length, melting temperature, the contents of guanine-cytosine (GC) content and clamp, and on the possibility of forming hairpins (primer capacity of forming a secondary structure), self-dimers (formed by intermolecular interactions between two homologous primers) and cross-dimers (formed by intermolecular interaction between sense and antisense primers where homology is present). Candidate primers were further analysed using BLAST to detect and eliminate those with interspecies reactivity (Altschul *et al.*, 1990). Each set of primer pairs were analysed in conjugation and the first PCR and second PCR primers were analysed separately to contemplate their use. Primers were purchased from Thermo Biopolymers (Thermo Biopolymers, Germany). For each species, a specific-primer pair was selected (Table 4.5) and used for nested PCR.

**Table 4.6 -** List of primers designed during this study and used in this study (PCR and nested PCR)

| **Target** | **Sequence (5'-3')** | **Length (bp)** |
|---|---|---|
| Cat[a] | | |
| Single PCR primers | | |
| CatMito1-F | CCTGTCCACACTACTTGTACTCATCGC | 539 |
| CatMito1-R | AGATGGTTGTTTAGGATGGCTACG | |
| Nested PCR primers | | |
| CatMito2-F | ATTTGATCCTATAGGGTCCGCC | 350 |
| CatMito2-R | CCTATGAGCGACATGATGAAAGC | |
| Dog[b] | | |
| Single PCR primers | | |
| DogMito1-F | ATGGCTCTAGCCGTTCGATTAAC | 638 |
| DogMito1-R | GGCTAGGAGGACTGAGGTGTTGAG | |
| Nested PCR primers | | |
| DogMito2-F | CATTAGGATTCACAACCAACCTGTTA | 236 |
| DogMito2-R | AATAATGCCGGTAGGAGGTCAG | |

[a]*Felis catus*. [b]*Canis lupus familiaris*.

The cycling conditions are vital in PCR because they affect the performance of the reaction. Accordingly, optimisation of the annealing temperature (Ta) is a critical issue for the PCR specificity (Wang and Seed, 2006). Choosing a low Ta may cause non-target amplification of PCR products. On the other hand, setting a very high Ta can result in a reduction in the yield of the target PCR product. The optimal annealing temperature for the dog and cat PCR assays was determined using the gradient PCR step available in the thermocycler (Veriti 96-well Thermal cycler, Applied Biosystems, US). A gradient of 55-60 ºC, with an increase of 1 ºC between each individual block, was tested with the remaining PCR cycling conditions kept equal. Table 4.6 displays the best cycling conditions for both assays.

**Table 4.7 -** PCR cycling conditions for the designed assays

| | | Temperature (ºC) | Cycle # | Time (s) |
|---|---|---|---|---|
| Step | | Dog and cat | | |
| 1st Denaturation | | 94 | 1 | 300 |
| 1st Annealing | | 59 | 1 | 300 |
| Amplification | Extension | 72 | 35 | 120 |
| | Denaturation | 94 | | 40 |
| | Annealing | 59 | | 60 |
| Final Extension | | 72 | 1 | 600 |

The amplicons were visualised after electrophoresis on 2.0% agarose gels and ethidium bromide staining. The specificity of each primer set was substantiated by confirmation of the expected length of the relative amplicon in the appropriate target DNA.The validation of the assays consisted of two steps. First, targeted single-component faecal suspensions were analysed by the two designed assays in a nested PCR to test and determine the specificity and sensitivity. Secondly, each assay was scrutinised for possible cross-reactivity with other species such as human, cow, pig, poultry, pigeon, gulls and rats. The cross-reactivity assay consisted of the analysis of mixed faecal suspensions composed of the non-target species mentioned above by the assays developed.

### 4.2.4. Sampling

Four water quality monitoring sites were selected at specific points along the course of the River Tagus and samples were collected monthly over a 13-month period (February 2015 to March 2016) and each sampling site was sampled at least once a month. 10-L water samples were collected at each site (Vila Franca de Xira (*n* = 25), Marina Parque das Nações (*n* = 27), Alcântara (*n* = 25) and Belém (*n* = 28) in sterile polyethylene terephthalate containers (VWR, US) for analysis of HAdV. Furthermore, a total of 6-L water sample were collected in sterile high-density polyethylene containers (VWR, US) and 2-L was saved to be analysed for:

- FIB;
- SC and GB-124;
- mtDNA (human, bovine, pig, poultry, and domestic pets).

All water samples were collected using gloves, especially sample to be analysed for mtDNA to avoid problems with potential cross-contamination. Samples were transported to the laboratory at 4 ºC in the dark and processed within four hrs for the parameters previously described.

## 4.3. Discrimination between infectious and non-infectious viral particles

### 4.3.1. Viral stock preparation (cell culture)

Buffalo Green Monkey kidney (BGM) cells were grown in Eagle Minimum Essential Medium (EMEM, Gibco – Thermo Fisher Scientific, US) supplemented with 5% foetal bovine serum (FBS), non-essential amino acids (Lonza, Switzerland) and antibiotics (Lonza, Switzerland). The cells were grown for four days before inoculation. Coxsackievirus B3 (CV-B3) strain was cultivated on previously grown monolayer cultures of BGM cells at 37 ºC with 5% $CO_2$ during ten days. Following the incubation period, viruses were harvested by lysing the cells through three consecutive freeze (80ºC) -thaw cycles (room temperature (r.t.)). The supernatant containing the released viral particles were clarified by centrifugation at 1500 x g for 10 min. Viruses were aliquoted and stored at -80 ºC until further analysis. The stock quantity for CV-B3 from the EntV family contained approximately $10^8$ PFU/mL. *Mengovirus* strain VMC0 ($MC_0$), a commonly used NoV surrogate, was purchased from CEERAM (CEERAM, France) with a known quantity of approximately $10^8$ PFU/mL. CV-B3 will be denominated as EntV henceforward.

### 4.3.2. Binding of dyes to purified viral RNA

The influence of different ethidium monoazide (EMA) and propidium monoazide (PMA) treatment processes on EntV and $MC_0$ RNA in 100 µL of 1x phosphate-buffered saline (PBS), pH 7.0 was assessed by testing the chosen concentrations for each dye (20 µM for EMA and 50 µM for PMA (Coudray-Meunier *et al.,* 2013; Moreno *et al.,* 2015)) with an incubation period of 5 min at r.t. in the dark followed by sample exposure to light for 15 min using the PhotoActivation System for Tubes – PhAST – equipment (GenIUL, Spain). To verify if the dyes inhibited the RT-qPCR capacity to detect viruses, controls consisting of viral RNA treated with the dyes without the photoactivation step were included. An additional procedure was added to reduce the possible inhibitory effect of free dye in rt-qPCR with every sample being purified using the QIAamp Viral RNA mini kit (Qiagen GmbH, Germany) following manufacturer's instruction (see Section 4.1.5.). A negative and a positive control were added to the experiment. The negative control consisted of non-treated 1x PBS and the positive control was a non-treated EntV and $MC_0$ RNA sample in 1x PBS. To determine the effect of the equipment on viral RNA, a purified non-treated RNA control sample was subjected to photoactivation. The assays were performed in triplicate.

### 4.3.3. Thermal inactivation of viruses

Viruses were inoculated in concentrated river water. 35 µL aliquots were incubated for 10 min in dry bath set at 95 ºC to achieve full viral inactivation. To assess the matrix factor, the concentrated river sample was 10-fold diluted and processed in parallel with the original sample. One aliquot of virus suspension was stored on ice during thermal treatment and used as a control. After heat treatment, samples were maintained on ice and further subjected to EMA, PMA, reagent D and RNase treatment, followed by RT-qPCR and infectivity assay.

### 4.3.4. Chlorine inactivation of viruses

CV-B3 and $MC_0$ were inoculated into a concentrated river sample. Buffer demand free water (BDF) was prepared by dissolving 0.54g of $Na_2HPO_4$ (anhydrous) and 0.88g of $KH_2PO4$ (anhydrous) per litre of ultrapure water (Milli-Q® Integral Water Purification System, Merck Millipore, US), as described by Thurston-Enriquez *et al.* (2003b). A stock solution of 150 mg/L of free chlorine (FChl) was obtained by dilution of 7% of sodium hypochlorite (PanReac AppliChem, Germany) in BDF buffer. The chosen final concentration of free chlorine was 2 mg/L. Viral stocks were diluted in a concentrated river

sample with a concentration of 2mg/L of free chlorine and incubated at room temperature for 2 min. To neutralise any remaining free chlorine and stop the inactivation, a solution of 0.5% of thiosulphate was added. To address the matrix effect, a ten-fold dilution of the concentrated water sample was processed equally to the original sample. A process control corresponding to untreated virus suspension was prepared and used as a positive control in the experiments. Chlorine inactivated viruses aliquots were treated with the dyes and RNase followed by RT-qPCR or cell culture to measure infectivity. All experiments were conducted in triplicate.

### 4.3.5. UV inactivation of viruses

UV inactivation was chosen because it is extensively used as a disinfectant in drinking water and wastewater systems and plays an important role on the inactivation of microorganisms in the environment. CV-B3 and $MC_0$ were diluted in concentrated river water and the viral suspensions were transferred into 24-wells tissue culture plates removing the lid for direct UVC light exposure. The average irradiance was an output of the UV absorbance of the test suspensions at 254 nm, the sample depth and the incident average irradiance. The lamp was pre-warmed for at least 30 min before each UV treatment. The plates were placed 20 cm below a low pressure germicidal UV lamp, in a UV flow cabinet. The UV lamp emitted a monochromatic germicidal light at a wavelength of 254 nm, with a UV intensity of $4.58x10^2$ mW/cm$^2$ as measured by UVX radiometer (UVP, US). Immediately before exposing viruses to UVC light the fluence rate was measured and this was used to calculate the overall fluence. The fluence received by the sample is given by (Metcalf and Eddy, 2003):

$$D = It$$

Where: $D$ = fluence (mJ/cm$^2$)

$I$ = fluence rate (mW/cm$^2$)

$t$ = exposure time

Different irradiation times were assayed: 30 s, 60 s, 200 s and 600 s. These were equivalent to fluence of $1.374x10^4$, $2.748x10^4$, $5.496x10^4$, and $2.748x10^5$ mJ/cm$^2$, respectively. The matrix effect was once again analysed as described previously. A suspension of viruses without exposure to UV light was prepared and used as a positive control in the experiments. For each virus and each UV dose, experiments were conducted in triplicate. Following the UV treatment, inactivated EntV and $MC_0$ were stored at -70 ºC. EntV

infectivity was measured by cell culture and both viruses were subjected to dye and RNase pre-treatments prior to RT-qPCR.

### 4.3.6. Plaque Assay

For the infectivity of EntV following the different inactivation treatments, the double-layer plaque assay was used (Mocé-Llivina *et al.*, 2004). The virus suspension was transferred to sterile tube and 260 µL of BGM cell suspension was added to the virus suspension. Two millilitres of 2x MEM containing extra antibiotics, gentamicin (50 µg/mL), nystatin (50 µg/mL) and ceftazidime (20 µg/mL) was then added to the sample. Finally, 2 mL of 2% agar previously melted and kept at 55 ºC was added and after mixing, the mixture was poured immediately onto a pre-grown confluent monolayer on a 90-mm-diameter Petri plate. The cell growth medium of the Petri plates was discarded prior to infection of the monolayer. The plates were then incubated, non-inverted, at 37 ºC, in the presence of 5% $CO_2$ under relative humidity of more than 80% for 4 days. Following the incubation period, the agar was removed and the monolayer stained with 0.1% crystal violet and the number of PFU were enumerated.

### 4.3.7. Enzymatic Digestion

The enzymatic pre-treatment of thermal-, chlorine-, and UV-inactivated viruses was performed as described previously with minor modifications (Topping *et al.*, 2009). To each inactivated viruses, a final concentration of 100 µg/mL of RNase A (ThermoScientific, US) was added. The mixtures were incubated at 37 ºC for 30 min after which 1 U/µL final concentration of RiboLock RNase Inhibitor (ThermoScientific, US) was added to inactivate the RNase. The inactivated virus suspensions were incubated at 37 ºC for 4h. After digestion, viral suspensions were subjected to RNA extraction using QIAamp Viral RNA mini kit (Qiagen GmbH, Germany) and tested by rt-qPCR.

### 4.3.8. Dye pretreatment prior to amplification

PMA (50 µM), EMA (20 µM) and reagent D were added to 35 µL of inactivated viruses. All the tubes were vortexed and incubated for 5 min at r.t. in the dark, followed by photoactivation for 15 min using the PhAST equipment (GenIUL, Spain). The samples were extracted using QIAamp Viral RNA mini kit (Qiagen GmbH, Germany) and the extracts were kept at -80 ºC until analysis by rt-qPCR.

### 4.3.9. Quantitative real-time PCR

The reaction was performed using AgPath-ID™ One-Step RT-PCR (Ambion, Thermo Fisher Scientific, US). The kit is constituted by a 25X RT-PCR Enzyme Mix that includes

a highly efficient ArrayScript™ Reverse Transcriptase and AmpliTaq Gold® polymerase, a 2X RT-PCR buffer, a ROX™ dye and a detection enhancer. All the reactions were conducted in a 25 µL reaction volume containing 5 µL of nucleic acid template. EntV primers and probe (Table 4.7) were used at a final concentration of 0.28 µM and 0.07 µM, respectively. For $MC_0$ the final concentration of forward and reverse primers and probe (Table 4.7) were 0.5 µM, 0.9 µM and 0.25 µM, respectively. 12.5 µL of the 2X RT-PCR buffer was mixed with 1 µL of ArrayScript™ Reverse Transcriptase, 1.67 µL of detection enhancer and the corresponding primers and probes concentrations. The volume was adjusted to 20 µL with DNA and RNA-free water. The cycling conditions were conducted following the manufacturer's instruction (Appendix 1.D).

**Table 4.8 -** Selected primers and probes for the detection of EntV and $MC_0$

| Virus | Oligonucleotide | Sequence (5'-3') | Product Length (bp) | Location / Annealing Temperature | Reference |
|---|---|---|---|---|---|
| EntV (CV-B3) | EntV_F | CCC CTG AAT GCG GCT AAT C | 147 | 455-602[a] / 60 ºC | Monpoeho *et al.*, 2000 |
| | EntV_R | GAT TGT CAC CAT AAG CAG C | | | |
| | EntV_P | FAM-CGG AAC CGA CTA CTT TGG GTG TCC GT-BHQ1 | | | |
| $MC_0$ | Mengo110 | GCG GGT CCT GCC GAA AGT | 99 | 110–209[b] / 60 ºC | Pintó *et al.*, 2009 |
| | Mengo209 | GAA GTA ACA TAT AGA CAG ACG CAC AC | | | |
| | Mengo147 | VIC-ATC ACA TTA CTG GCC GAA GC-MGB | | | |

BHQ – Black Hole Quencher; FAM – Fluorescein Amidite; MGB – Minor Groove Binder

[a] Sequence position refer to the Human Poliovirus 1, complete genome sequence

[b] This primer set targets a product of 99 bp corresponding to nucleotides 110-209 of the deletant $MC_0$ strain. This corresponds to nucleotides 110-270 of the non-deletant mengo virus isolate M (GenBank accession number L22089)

# CHAPTER 5 : THE APPLICATION OF NOVEL BIO-INDICATORS FOR VIRUSES IN WASTEWATER TREATMENT PLANTS

## 5.1. Microbial Concentration in WWTP

A total of 94 raw (influent) and treated wastewater (effluent) samples were collected from municipal WWTPs situated in Portugal and analysed for traditional FIB (EC and IE), for potential bio-indicators (SC, GB-124 phage, HAdV, JCPyV) and for specific pathogenic enteric viruses (NoVGII), as outlined in Chapter 4, Section 4.1. Although HAdV are also pathogenic viruses, they have also been described as possible surrogates for other enteric viruses in environmental waters and for wastewater treatment processes, as described in Chapter 2. NoVGII were chosen as the specific pathogen because they are the leading cause of food and waterborne gastroenteritis (Bosch *et al.*, 2008) and were tested for as part of this research in order to understand whether there is a correlation with traditional FIB, or with any of the proposed bio-indicators tested. The WWTPs were chosen as a result of the distinct treatments used, e.g. secondary versus secondary plus disinfection. This work intended to elucidate the fate and use of the potential novel bio-indicators as surrogates for human-specific norovirus (NoVGII) during wastewater treatment and sought to elucidate the influence of seasonality on final effluent quality. In addition, the influence of different levels of treatment was also explored. The $Log_{10}$ concentrations of all parameters in raw and treated wastewater are shown in Table 5.1 and Figure 5.1.

FIB and SC were found at high concentrations in all raw wastewater samples analysed (mean of 6.85 log MPN/mL, 5.83 log MPN/mL and 4.27 $log_{10}$ PFU/mL for EC, IE, and SC, respectively). HAdV were found in 45 out of 53 (85%) raw wastewater samples analysed, with a mean concentration of 4.26 log GC/mL. Conversely, GB-124 phages were the bio-indicator found at the lowest concentration and in the lowest number of raw wastewater samples, with a percentage of positive samples of only 66% and a mean concentration of 1.17 log PFU/mL. NoVGII, the primary pathogen of interest, was found in more than half of the raw wastewater samples tested (58%) and was detected at high levels (up to 5.92 log GC/mL). Although JCPyV were found in a high percentage of raw wastewaters (72%), the concentrations were slightly lower compared to HAdV and NoVGII.

**Table 5.1 -** $Log_{10}$ concentrations of FIB, phages, and viruses

| WWTP sample | | | | |
|---|---|---|---|---|
| | Raw wastewater | | Treated wastewater | |
| **Parameter ($Log_{10}$ units)** | **Mean** (min – max) | **% Positive samples** | **Mean** (min – max) | **% Positive samples** |
| EC (MPN/mL) | 6.85 (4.61 – 7.68) | 100 (53/53) | 3.12 0.30 – 6.56 | 95 (39/41) |
| IE (MPN/mL) | 5.83 (3.33 – 7.38) | 100 (53/53) | 2.40 0.30 – 5.08 | 80 (33/41) |
| SC (PFU/mL) | 4.27 (2.40 – 6.34) | 100 (53/53) | 1.49 0.00 – 4.33 | 73 (30/41) |
| GB-124 (PFU/mL) | 1.17 (0.00 – 2.82) | 66 (35/53) | 0.18 0.00 – 2.03 | 12 (5/41) |
| HAdV (GC/mL) | 4.26 (2.64 – 7.06) | 85 (45/53) | 2.53 1.66 – 6.35 | 56 (23/41) |
| NoVGII (GC/mL) | 3.95 (1.51 – 5.92) | 58 (31/53) | 1.65 0.53 – 6.14 | 46 (19/41) |
| JCPyV (GC/mL) | 3.12 (1.08 – 5.32) | 72 (26/36) | 1.67 0.93 – 4.44 | 38 (9/24) |

MPN = Most Probable Number; PFU = Plaque-Forming Unit; GC = Genome Copies

As expected, the mean levels of all parameters was lower in treated wastewater, though EC and IE were still present at high levels (mean of 3.12 log and 2.40 log MPN/mL, respectively) and in a high percentage of samples (95 and 80%, respectively). The bio-indicator showing the highest levels in treated wastewater (i.e., lowest level of removal) was HAdV (mean = 1.73 log GC/mL), although being found only in just over half of the tested samples (56%). SC were found in a high number of samples (30 out of 41 samples) but the levels decreased to 1.34 log PFU/mL. GB-124 phages were once again the organisms found at the lowest levels and were found in only 5 of the 41 samples analysed (12% detection). NoVGII levels decreased almost 1 log GC/mL between treated and raw wastewater. qPCR for all viruses tested was not highly affected with respect to the different matrices and concentration methodologies adopted. FIB exhibited a larger variation of concentrations in treated wastewater compared to the data obtained for untreated influent, most likely as a result of different removal levels at the WWTP (Figure 5.1).

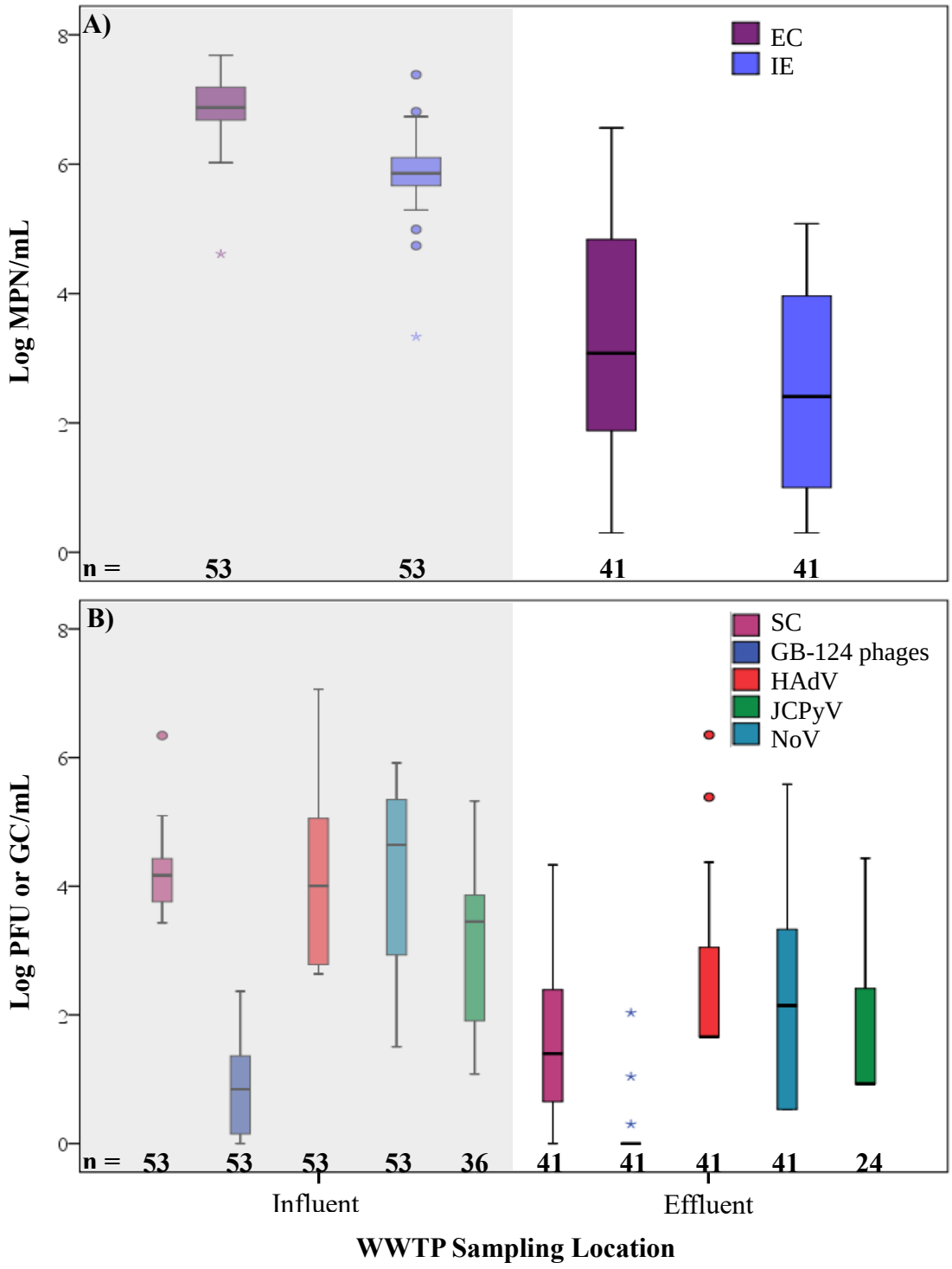


**Figure 5.1 -** Concentrations of markers in raw and treated wastewaters for A) FIB and B) new bio-indicators. Results were transformed into logarithmic format after addition of 1 to each value to account for the non-detected values. Outliers (observations >1.5 times the interquartile range) are represented by a *. Boxes, 25$^{th}$ and 75$^{th}$ percentile; lines inside the boxes, median; whiskers, 10$^{th}$ and 90$^{th}$ percentile, respectively; n, number of samples in each assay. Grey boxes correspond to influent samples and white boxes correspond to effluent samples.

The same trend was observed for SC (Figure 5.1). However, this trend was not observed for the other bio-indicators (GB124 phages, HAdV, and JCPyV) and NoVGII that exhibited a large spread of values both for both untreated influent and treated effluent. The spread of GB124 phage concentrations in the influent was broader than that observed for the other phages group tested, but smaller compared to that of HAdV, JCPyV and NoV. In effluent, the levels found for GB124 phages were consistently low (mean = 0.18 log PFU/mL), or below the limit of detection (<1 PFU per 1 mL).

The influence of seasonality (an important factor to consider when determining a suitable indicator/index organism), that is, the variation in the concentration and prevalence of a given organism throughout the different seasons (Spring-Summer and Autumn-Winter) was estimated for the observed levels of each parameter in the inffluent, analysis of variance following one-way factor was conducted in order to obtain the significance level of the *F* test statistics. The *p*-values for the one-way factor analysis of variance of microorganism concentration in the influent are summarised in Table 5.2.

**Table 5.2 -** *p*-values from the statistical tests (ANOVA) between the two seasons

| Source of Variation | Microorganism | | | | | | |
|---|---|---|---|---|---|---|---|
| | EC | IE | SC | GB124 phages | HAdV | NoVGII | JCPyV |
| Season | 0.076 | <0.05 | 0.654 | 0.700 | <0.05 | 0.095 | <0.05 |

From Table 5.2, it is noticeable that the variance of the concentration of SC and GB124 phages cannot be attributed to the seasonal factor ($p > 0.05$), with both phages presenting very little correlation with respect to seasonality. The concentration of EC and NoVGII in the influent were also not significantly affected by season during which the samples were collected. Interestingly, the concentration of IE was highly associated with seasonality ($p < 0.05$), the same being observed for HAdV and JCPyV ($p < 0.05$).

The seasonal patterns of FIB, new bio-indicators and NoVGII in raw and treated wastewater are shown in Figure 5.2. The concentration of EC, IE and SC in raw wastewater was predominantly steady throughout the study period, whereas the concentration of GB124 phages, HAdV, NoVGII and JCPyV showed irregular patterns of concentration within the same period. The levels of all tested organisms varied significantly in treated wastewater, with no definite pattern for FIB and phages.

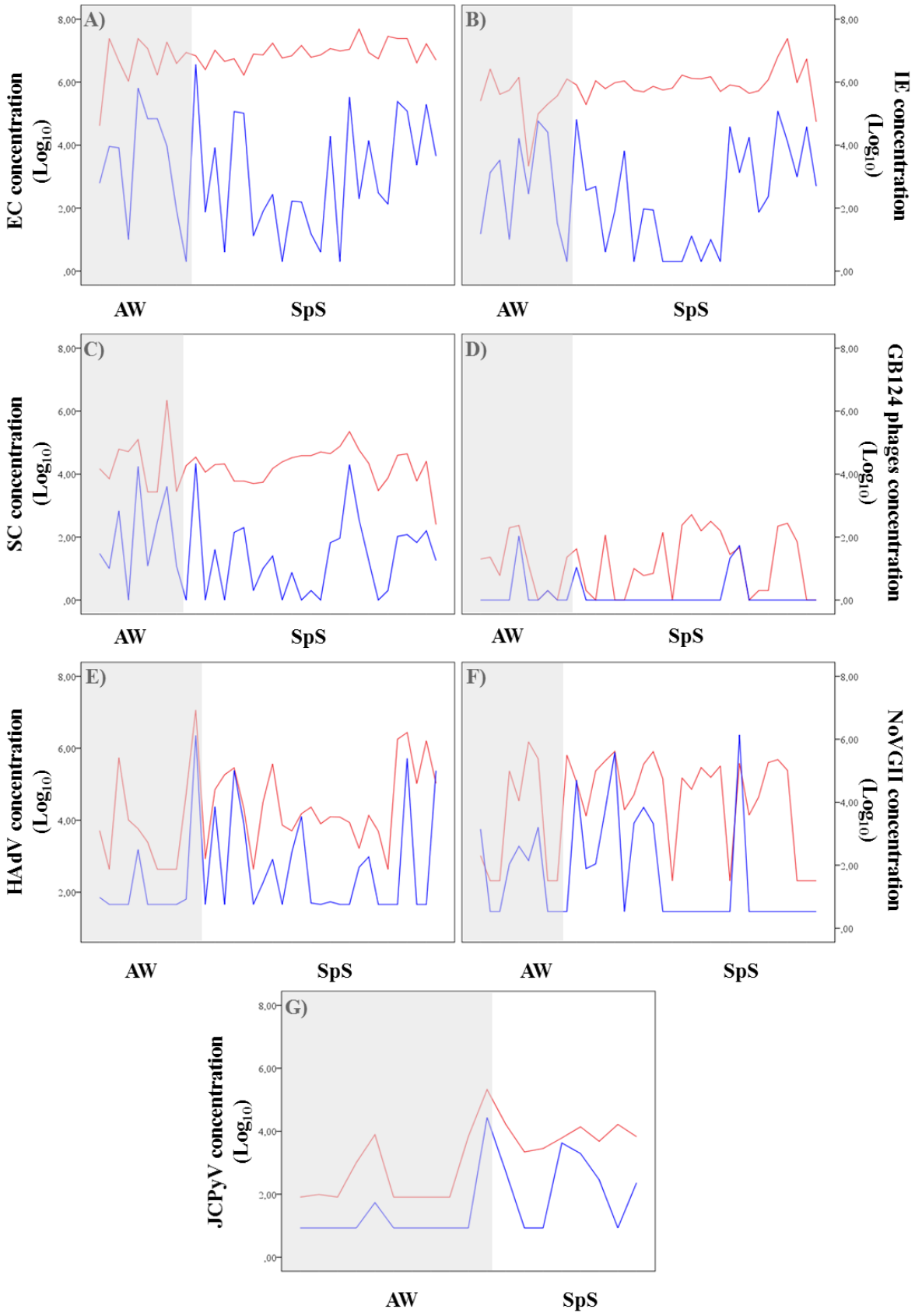


**Figure 5.2** - Seasonal profile of (A) EC, (B) IE, (C) SC, (D) GB124 phages, (E) HAdV, (F) NoVGII, and (G) JCPyV in influent (-) and effluent (-) of WWTPs in Portugal. Grey boxes represent SpS samples and white boxes represent AW samples.

The concentration of FIB was mostly constant in the influent during both seasons, with upper levels of 6.56 and 5.39 log MPN/mL for both spring-summer (SpS, corresponding to the period between the end of March and end of September) period and at 6.94 and 5.97 log MPN/mL for the autumn-winter (AW, corresponding to the period beginning at the end of September and ending by the end of March) for EC and IE, respectively. The same trend was observed in the effluent with levels of 3.67 and 2.91 log MPN/mL during SpS and at 2.93 and 2.23 log MPN/mL during AW for EC and IE, respectively. The levels of both phages in influent and effluent were also highly constant throughout the seasons, 4.36 and 1.06 log PFU/mL (SpS) and 4.24 and 1.20 log PFU/mL (FW) for SC and GB124 phages in the influent and 1.98 and 0.26 log PFU/mL (SpS) and 1.33 and 0.15 log PFU/mL (FW) for SC and GB124 phages. The profiles of HAdV, NoVGII and JCPyV in influent were highly similar, with higher amounts detected in AW season at 4.52, 4.21 and 3.98 log GC/mL, respectively, and less abundant during SpS at 3.46, 3.18 and 2.17 log GC/mL, respectively. The data from the effluent followed a similar pattern at lower levels for HAdV and JCPyV with greater concentrations during AW, 2.75 and 2.26 log GC/mL, respectively, against 1.85 and 1.02 log GC/mL for SpS. The mean concentration of NoVGII during SpS and AW in the effluent was almost constant at 1.70 and 1.64 log GC/mL, respectively. Regression analysis between the concentrations obtained for the influent and the effluent throughout the sampling campaign showed that correlation between influent and effluent were statistically significant for SC ($r$ = 0.410, $p$ = 0.05), HAdV ($r$ = 0.520, $p$ = 0.01), JCPyV (($r$ = 0.703, $p$ = 0.01), and NoVGII (($r$ = 0.418, $p$ = 0.05).

The profile obtained for viral bio-indicators (HAdV and JCPyV) and NoVGII in treated wastewater exhibited the most stable ratio of concentration between raw and treated wastewater (Figure 5.2, Table 5.3).

**Table 5.3.** Removal ratio and standard deviation between concentrations in the influent and effluent

| Microorganism | Mean ratio ± SD |
|---|---|
| EC | 3.73 ± 1.80 |
| IE | 3.43 ± 1.66 |
| SC | 2.79 ± 1.18 |
| GB-124 phages | 0.99 ± 0.97 |
| HAdV | 1.73 ± 1.27 |
| JCPyV | 0.76 ± 0.97 |
| NoVGII | 1.92 ± 1.57 |

This is especially true for JCPyV, where raw and treated wastewater data followed a similar trend within the majority of the samples analysed.

### 5.2. Indicator role of microbial bio-indicators (survival to treatments)

One of the characteristics of an 'ideal indicator' is an ability to survive wastewater treatment, at least as well as the most resistant pathogens of concern. This quality was assessed for each of the various microorganisms and expressed in Table 5.4 and Figure 5.3. Mean $log_{10}$ reductions observed during secondary treatments (consisting of biological treatment by activated sludge) for bacterial indicators were highly similar to one another (3.42 and 3.40log MPN/mL for EC and IE, respectively), with removal levels ranging from 0.30 to 6.70log MPN/mL for EC and 0.88 to 5.92log MPN/mL for IE. The mean reduction obtained for NoVGII was 2.54log GC/mL and the lowest inactivation rates were obtained for GB124 phages, HAdV and JCPyV, with mean reductions of 1.42log PFU/mL, 1.73 and 1.33log GC/mL, respectively. The percentage of removal of the various organisms varied between 81% and 99%. EC, IE and SC showed similar percentages of removal between raw and treated wastewater (97%, 99% and 96%, respectively), whereas interestingly, GB124 phages, HAdV, JCPyV displayed percentages of removal highly similar to the targeted pathogen NoVGII (81%, 82%, 82% and 83%, respectively).

**Table 5.4 -** Percentage of removal and mean $Log_{10}$ removal levels in WWTP using secondary and tertiary treatment for FIB, bacteriophages and enteric viruses

| **Parameter** | **% Removal (min-max)** | **Average $Log_{10}$ Removal Levels (min-max)** |
|---|---|---|
| Secondary Treatment (ST) | | |
| EC | 97 (47-100) | 3.42 (0.30-6.70) |
| IE | 99 (87-100) | 3.40 (0.88-5.92) |
| SC | 96 (38-100) | 2.69 (0.21-4.72) |
| GB-124 phages | 81 (18-100) | 1.42 (0.00-2.71) |
| HAdV | 82 (0-100) | 1.73 (0.00-4.59) |
| NoVGII | 83 (0-100) | 2.54 (0.00-4.96) |
| JCPyV | 82 (30-100) | 1.33 (0.15-2.91) |
| Tertiary Treatment | | |
| EC | 100 (100) | 6.03 (4.53-7.08) |
| IE | 100 (100) | 5.15 (2.72-7.08) |
| SC | 100 (100) | 3.77 (2.37-4.64) |
| GB-124 phages | 63 (0-100) | 1.20 (0.00-2.44) |
| HAdV | 98 (89-100) | 2.81 (0.98-3.89) |
| NoVGII | 95 (89-100) | 2.10 (0.97-4.48) |
| JCPyV | 96 (90-100) | 1.62 (0.98-2.52) |

For WWTP featuring secondary treatment followed by disinfection (UV-treatment), the mean log removal was usually higher for the organisms tested. FIB removal levels increased by more than 2 log and significant increases (around 1 log) were also obtained for SC, HAdV, and NoVGII. Log reductions of GB124 phages, JCPyV and NoVGII ($\rho$ > 0.05) in secondary treated and UV-disinfected wastewater were not significantly different, with less than 1-log reduction between WWTP with secondary treatment and those with additional disinfection.

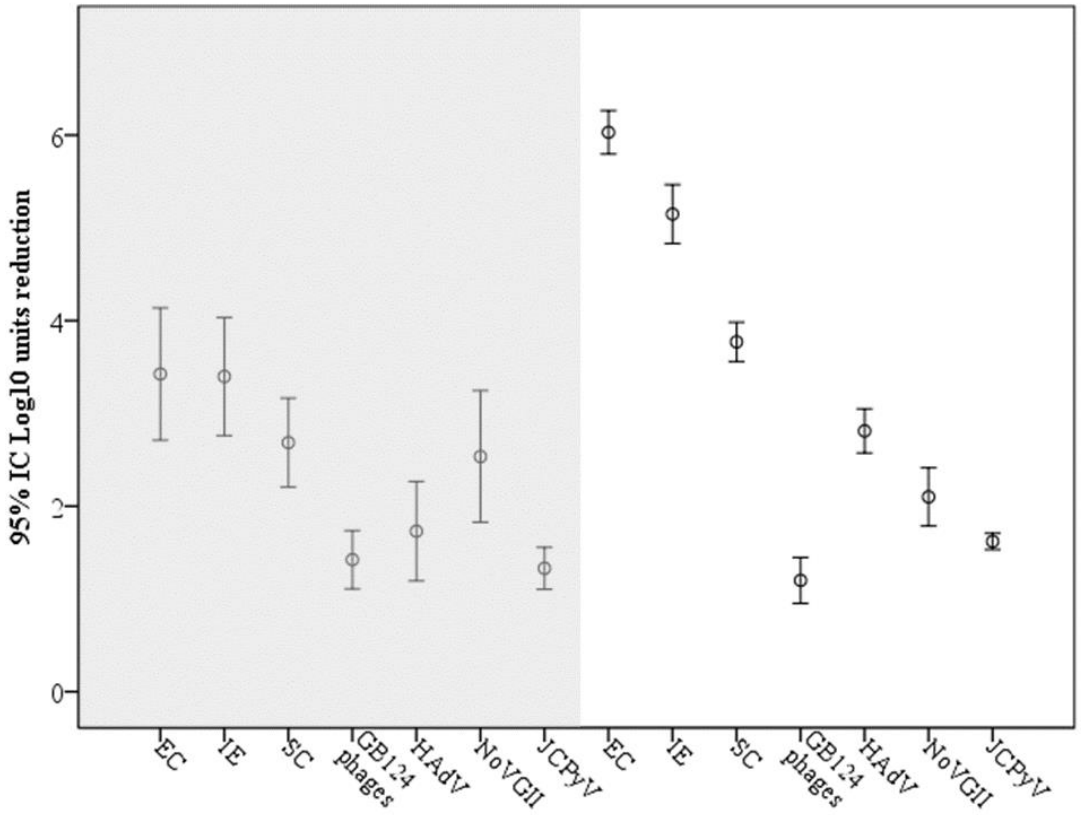


**Figure 5.3 -** Microbial inactivation (log10 unit reduction) obtained during the secondary (grey box) and tertiary (white box) treatments studied.

Figure 5.4 depicts the probability plots illustrating the log10 removal obtained for NoVGII and the various microbial bio-indicators in all the WWTP studied.

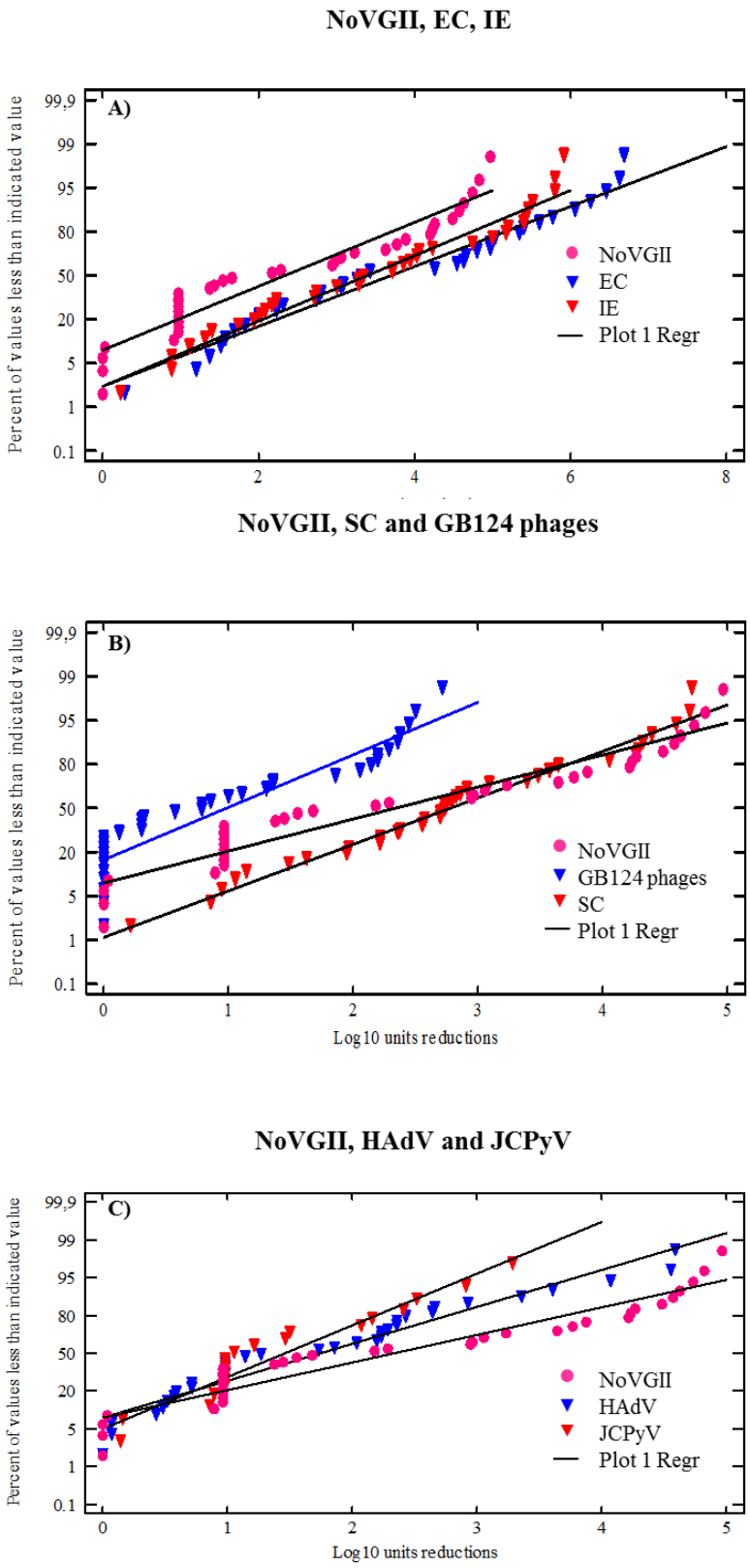


**Figure 5.4 -** Probability plots illustrating levels of inactivation obtained for indicator organisms and NoVGII in all WWTP studied. A) NoVGII, EC, and IE, B) NoVGII, SC, and GB124 phages, and C) NoVGII, HAdV, and JCPyV.

As shown in Figure 5.4A, reductions corresponding to EC and IE were higher than those achieved for NoVGII. Conversely, log10 reduction levels for GB124 phages were consistently lower than for NoVGII, behaviour not followed by SC (Figure 5.5B). When comparing NoVGII removals with those of HAdV and JCPyV, HAdV had generally lower levels of removal compared to NoVGII, the same being observed for JCPyV although the difference is not as marked for the former comparison.

## 5.3. Index role of bio-indicators

### 5.3.1. Quantitative correlation between concentrations of bio-indicators and NoVGII

In order to determine whether any of the various indicators could be considered as index bio-indicators, that is, if their presence could predict the presence of a certain pathogen, Spearman's correlation was performed on the log removal data. The results revealed that, for some of the tested bio-indicators, a significant correlation was evident (Table 5.5). In particular, the correlations between EC and IE and between FIB and SC were very high (r = 0.723, 0.800, and 0.737, for EC vs. IE, EC vs. SC and IE vs. SC, respectively).

**Table 5.5** - Spearman's correlation between the log10 removal of traditional (EC, IE) and proposed bio-indicators (SC, GB124, HAdV, JCPyV) and NoVGII in WWTPs

| | EC | IE | SC | GB124 phages | HAdV | JCPyV | NoVGII |
|---|---|---|---|---|---|---|---|
| EC | - | **0.723**[a)] | **0.800**[a)] | 0.236 | 0.270 | **0.690**[a)] | 0.357 |
| IE | | - | **0.737**[a)] | -0.103 | 0.099 | **0.517**[b)] | 0.301 |
| SC | | | - | **0.425**[b)] | 0.278 | **0.593**[b)] | 0.459[b)] |
| GB124 phages | | | | - | 0.318 | 0.160 | **0.527**[a)] |
| HAdV | | | | | - | 0.427 | 0.365 |
| JCPyV | | | | | | - | 0.224 |
| NoVGII | | | | | | | - |

[a)] **Two tailed significance, p < 0.01**.

[b)] **Two tailed significance, p < 0.05**.

JCPyV correlated with FIB and SC at a high level showing r levels between 0.517 ($p \leq 0.05$) and 0.690 ($p \leq 0.01$). No correlation was also found between FIB and GB124 phages or HAdV ($0.099 \leq r \leq 0.270$) but a good correlation was obtained between both phages (r =

0.425, $p \leq 0.05$). Interestingly, the highest correlation of an indicator with NoVGII was observed for GB124 phages (r = 0.527, $p \leq 0.01$). NoVGII correlated also with SC showing r value of 0.459 ($p \leq 0.05$). Significant but weak correlations were observed between NoVGII and HAdV, NoVGII and SC, and IE and NoVGII with r values ranging from 0.301 and 0.365 and no correlation was established between JCPyV and NoVII (r = 0.224).

Simple regression analysis (Figure 5.5) performed with the data confirmed that the most closely correlated parameters were GB124 phages and NoVGII log removal (r = 0.582), followed by SC log removal (r = 0.492). The remaining parameters were not considered to be appropriate models for predicting NoVGII log reductions since r values were low ($0.293 \leq r \leq 0.338$).

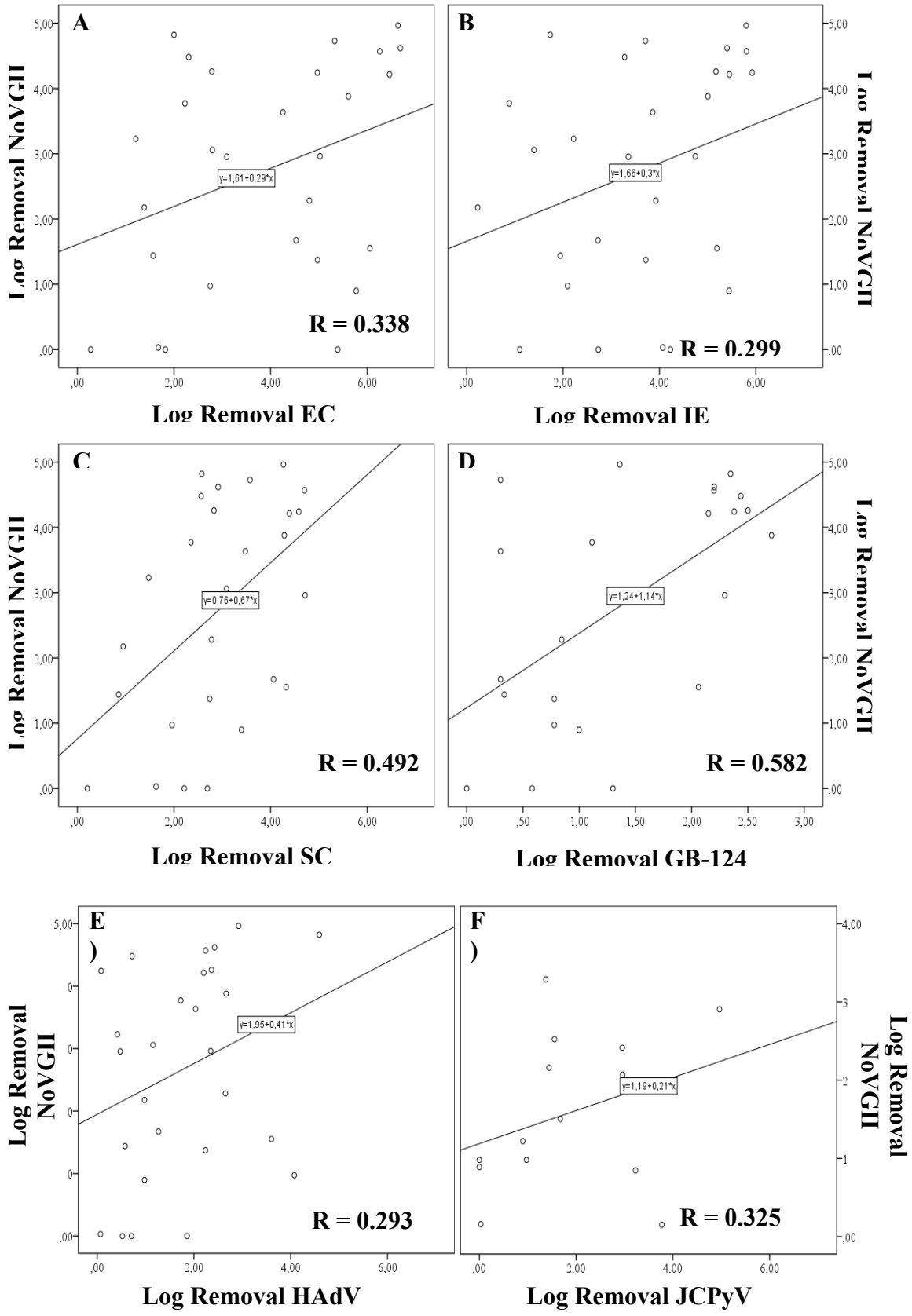


**Figure 5.5 -** Linear regression analysis between log10 reduction of microorganisms (EC, IE, SC, GB124 phages, HAdV, JCPyV) and log10 reduction of NoVGII

Based on the initial results for the correlation between log removal of NoVGII and microbial bio-indicators and additionally the levels of removal during wastewater treatment, JCPyV were excluded from further laboratory and statistical analyses. A discriminant statistical analysis was performed in order to establish which parameter, or group of parameters was most capable of predicting the presence/absence of NoVGII (Table 5.6). Discriminant analysis demonstrated that there were no statistically significant discriminant functions ($p > 0.05$) for predicting the presence of NoVGII in wastewater effluents when using either a single, or a group of bio-indicators.

The concentration of one bacterial indicator alone, or of combinations of two or more showed low percentages of well classified samples (61.1%). The same results were obtained for SC, and HAdV with levels of correct classification of 61.1%. GB124 phages displayed the highest percentage of correctly classified samples (66.7%). The lowest correct classification of the presence of NoVGII was achieved for the combination of FIB and HAdV at 58.3%. The combination of GB124 phages with two other parameters resulted in higher percentages of correct classification, being the same for the combination of GB124 phages with SC, or HAdV with levels of correct classification of 66.7%. However, the additional incorporation of different microbial indicators did not increase the percentage of well classified samples above the result obtained solely with GB124 phages and the percentages ranged between 61.1 and 66.7%.

**Table 5.6 -** Discriminant analyses for prediction of the presence NoVGII in effluents through microbial concentrations

| | Correctly Classified (%) | Sensitivity (%) | Specificity (%) |
|---|---|---|---|
| EC | 61.1 | 100 | 0 |
| EC+IE | 61.1 | 100 | 0 |
| EC+IE+SC | 61.1 | 95.5 | 7.1 |
| EC+IE+SC+GB124 phages | 63.9 | 90.9 | 21.4 |
| EC+IE+SC+GB124 phages+HAdV | 63.9 | 90.9 | 21.4 |
| EC+IE+GB124 phages | **66.7** | 95.5 | 21.4 |
| EC+IE+GB124 phages+HAdV | 63.9 | 90.9 | 21.4 |
| EC+IE+HAdV | 58.3 | 86.4 | 14.3 |
| EC+SC | 61.1 | 100 | 0 |
| EC+SC+GB124 phages | 66.7 | 95.5 | 21.4 |
| EC+SC+GB124 phages+HAdV | 63.9 | 90.9 | 21.4 |
| EC+SC+HAdV | 63.9 | 90.9 | 21.4 |
| EC+GB124 phages | **66.7** | 95.5 | 21.4 |
| EC+GB124phages+HAdV | **66.7** | 95.5 | 21.4 |
| EC+HAdV | 61.1 | 90.9 | 14.3 |
| IE | 61.1 | 100 | 0 |
| IE+SC | 61.1 | 95.5 | 7.1 |
| IE+SC+GB124 phages | **66.7** | 95.5 | 21.4 |
| IE+SC+GB124 phages+HAdV | 63.9 | 90.9 | 21.4 |
| IE+GB124 phages | **66.7** | 95.5 | 21.4 |
| IE+GB124 phages+HAdV | **66.7** | 95.5 | 21.4 |
| SC | 61.1 | 100 | 0 |
| SC+GB124 phages | **66.7** | 95.5 | 21.4 |
| SC+GB124 phages+HAdV | 63.9 | 90.9 | 21.4 |
| SC+HAdV | 61.1 | 90.9 | 14.3 |
| GB124 phages | **66.7** | 95.5 | 21.4 |
| GB124 phages+HAdV | **66.7** | 95.5 | 21.4 |
| HAdV | 61.1 | 90.9 | 14.3 |

**Bold denotes the highest rate of correct classification**

### 5.3.2. Qualitative relationship (presence/absence) of bio-indicators and NoVGII

Figure 5.6 displays the breakdown of the percentage of treated wastewater samples where the presence (or absence) of individual parameters corresponded to the presence or absence of NoVGII. Positive results for NoVGII that were also positive for the microbial bio-indicator was considered a 'true positive', the absence of both NoVGII and the bio-indicator was considered a 'true negative', samples where the pathogen was present but the bio-indicator was absent were considered 'false negatives' and samples with negative results for NoVGII but positive for the bio-indicator were considered 'false positives'.

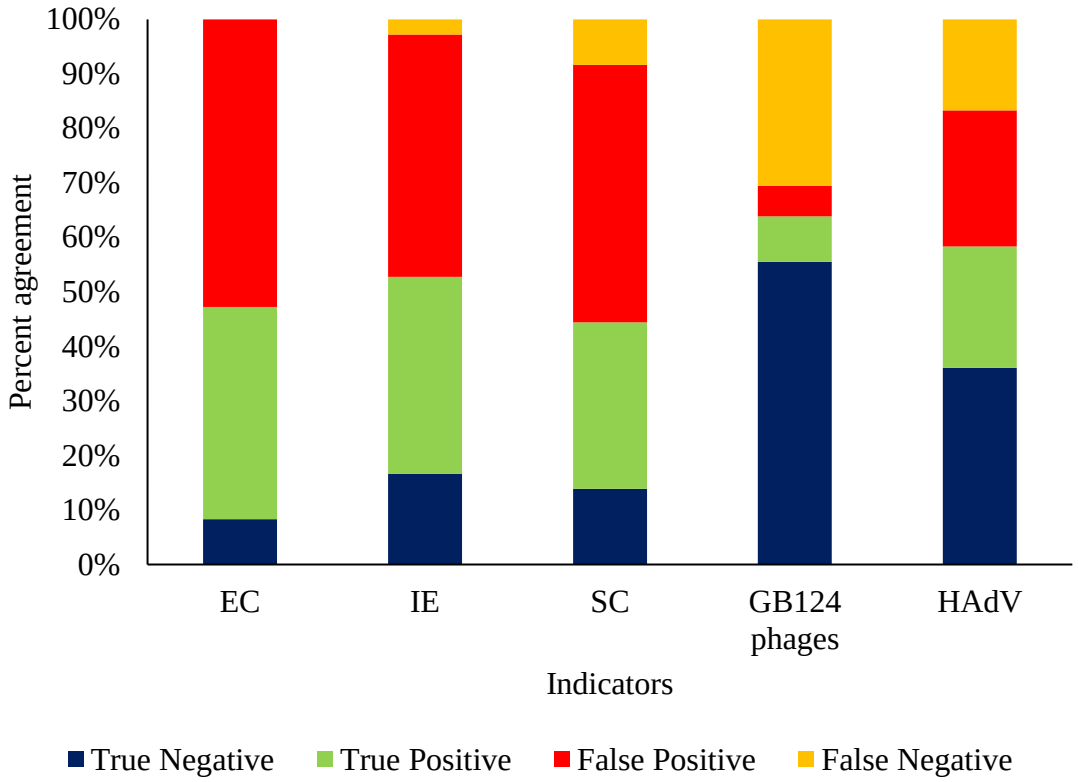


**Figure 5.6 -** Breakdown of detection of individual microbial indicators and NoVGII in final WWTP effluents

The percentage of 'true positive' results for NoVGII and bio-indicators was 39% for EC, 36% for IE, 31% for SC, 8% for GB124 phages, 22% for HAdV, and 32% for JCPyV. The percentage of 'true negative' results, where both pathogen and indicator were absent in the final effluent were 8% for EC, 17% for IE, 14% for SC, 55% for GB124 phages, 36% for HAdV, and 26% for JCPyV. The percentage of samples with NoVGII, but no indicator organism, 'false negative' results, was 0% for EC, 3% for IE, 8% for SC, 31% for GB124 phages, 17% for HAdV, and 37% for JCPyV. Finally, in the case of 'false positive' results,

where the indicator was present but the pathogen was absent, the percentages were 53% for EC, 44% for IE, 47% for SC, 6% for GB124 phages, 25% for HAdV and 5% for JCPyV. Figure 5.7. shows the percentage accuracy between the detection of microbial bio-indicators and NoVGII. To understand the potential need for using more than one indicator parameter to assess the presence of NoVGII and for determining the quality of effluent in receiving waters, a simulation of current and possible scenarios for indicator levels prescribed in water reuse legislation were examined and compared to the absence of NoVGII. The current and potential water reuse standards evaluated are presented on Table 5.7.

**Table 5.7 -** Current or potential water reuse standards

| **Current or potential water reuse standards** |
|---|
| 0 EC/100mL (USEPA, 2004) |
| < 100 EC/100mL |
| < 1000 EC/100mL (WHO, 1989) |
| < 100 EC/100mL and < 100 SC/mL |
| < 100 EC/100mL and < 100 GB124 phages/mL |
| < 100 EC/100mL, < 100 SC/mL and < 100 GB124 phages/mL |
| < 100 EC/100mL and < 100 HAdV/mL |
| < 100 EC/100mL, < 100 GB124 phages/mL and < 100 HAdV phages/mL |
| < 100 EC/100mL, < 100 SC/mL and < 100 HAdV phages/mL |
| < 100 GB124 phages/mL and < 100 HAdV/mL |

By using these standards, it was possible to construct a graph showing the percentage of samples where the presence (or absence) of bio-indicators corresponded to the presence or absence of NoVGII (i.e. 'true positive' and 'true negative' results), respectively, and samples where 'false positive', or 'false negatives' were obtained. That is, where the indicator was present but the pathogen was absent, or where the indicator was absent but the pathogen present. Furthermore, the percentage of treated wastewater samples that would comply with the current and proposed legislation was also calculated. Analysing the different legislations chosen, the specification of 0 EC/100mL would provide the highest percentage of samples, complying with the guidelines and additionally this quality standard provided the highest percentage of 'true negative' results (pathogen and indicator absence), but with a reduced percentage of 'false negative' results. The combinations of bio-indicators giving the highest percentage of 'false negatives', (samples where the indicator

was present but the pathogen was absent) were < 100 EC/100mL, < 100 SC/mL and < 100 GB124 phages/mL and < 100 EC/100mL, < 100 SC/mL and < 100 HAdV/mL.

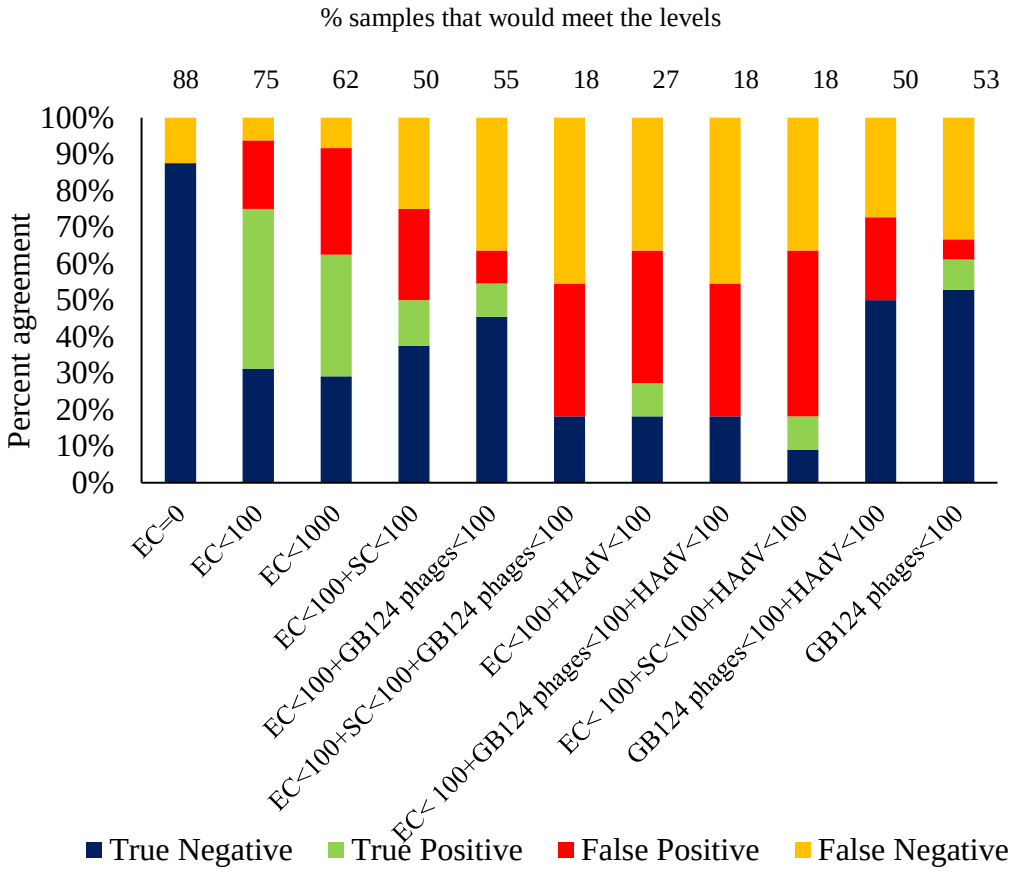


**Figure 5.7 -** Precision between the detection of NoVGII and microbial indicators in WWTP effluents with respect to current, or potential water reuse standards.

From Figure 5.7 it is interesting to observe that the use of EC values of 0 CFU/100 mL would lead to few false positive results and a high degree of certainty on the true negative results. However, it would not provide information on the presence of NoVGII in treated wastewater, which is of course extremely important. The adoption of legislation aimed at achieving less than 100 EC/ 100mL, although increasing the number of false positive results (suggesting presence of NoVGII, when in reality it is not present), would also enable one to predict with high degree of certainty that there is the potential for NoVGII to exist in the final treated wastewater, thereby providing further information on the potential risk to public health. Potential water reuse standards relying on the detection of fewer than 100 EC/100mL and fewer than 100 SC/mL, standard levels of fewer than 100 GB124 phages/mL and fewer than 100 HAdV/mL, or fewer than 100 GB124 phages/mL yielded good agreements with the regulations although presenting higher levels of 'false positive' and 'false negative' results.

# CHAPTER 6 : RESULTS OF MOLECULAR AND CULTURE-BASED ASSAYS TO TARGET HUMAN AND NON-HUMAN FAECAL CONTAMINATION IN THE RIVER TAGUS

## 6.1. Qualitative and quantitative distribution of general indicators of faecal contamination and MST markers in the River Tagus

As mentioned, the discrimination of sources of faecal contamination is paramount for the future application of better and target-based remediation measures. Results from Chapter 5 have suggested that EC levels with a mean concentration of 3.12 log MPN/100 mL are common in discharges from the WWTW monitored in this study. Additionally, uncontrolled releases of faecal contaminants are another important input, which can exert a high negative impact on water quality. The study also sought to assess the effectiveness of the various indicators and markers during catchment-level application. A total of 105 samples were collected at four different locations along the River Tagus, as shown in Chapter 4, Figure 4.7. The mean concentration of each parameter and percentage of positive samples are presented in Table 6.1.

**Table 6.1 -** Quantitative results and % of positive samples for FIB, bacteriophages, and human, bovine, pig and poultry faecal markers in River Tagus

| Marker | Average Concentration (min-max) | % Positive samples |
|---|---|---|
| EC (MPN/100mL) | 2.73 (1.52 – 5.24) | 99 (104/105) |
| IE (MPN/100mL) | 2.07 (0.78 – 4.29) | 98 (103/105) |
| SC (PFU/100mL) | 2.57 (0.00 – 4.31) | 99 (104/105) |
| GB-124 phages (PFU/100mL) | 0.00 (0.00 – 0.30) | 1 (1/105) |
| HAdV (GC/100mL) | 2.53 (2.23 – 6.30) | 32 (34/105) |
| HMMit (GC/100mL) | 4.25 (2.54 – 11.17) | 83 (87/105) |
| CWMit (GC/100mL) | 3.77 (2.33 – 9.02) | 73 (77/105) |
| PigMit (GC/100mL) | 3.29 (2.54 – 7.69) | 56 (59/105) |
| PLMit (GC/100mL) | 2.88 (2.33 – 8.10) | 39 (41/105) |

FIB and SC (general indicators of faecal contamination), were present in the majority of river water samples with similar mean concentrations (2.73 log EC MPN/100mL, 2.07 log IE MPN/100mL and 2.57 log SC PFU/100mL). GB-124 phages, HAdV, and HMMit (human-specific markers of faecal contamination) displayed a different distribution. HMMit was the most frequently detected marker (present in 83% of samples) and was

present at the highest concentrations (4.25 log GC/100mL). HAdV were found in 32% of samples (at mean concentration close to those of FIB and SC). GB-124 phages were detected in a single river water sample and at a low concentration. CWMit marker was found at a high concentration (3.77 log GC/100mL) and in a large number of samples (73%). The PigMit was detected in over 50% of the samples at a mean concentration of 3.29 Log GC/100mL. Finally, PLMit was the mitochondrial DNA marker present in the lowest amount of samples (39%) and at the lowest concentrations (2.88 Log GC/100mL). Figure 6.1 and 6.2 present the data, plotted separately for each parameter, sampling location and season, displaying the percentage of positive samples and mean values.

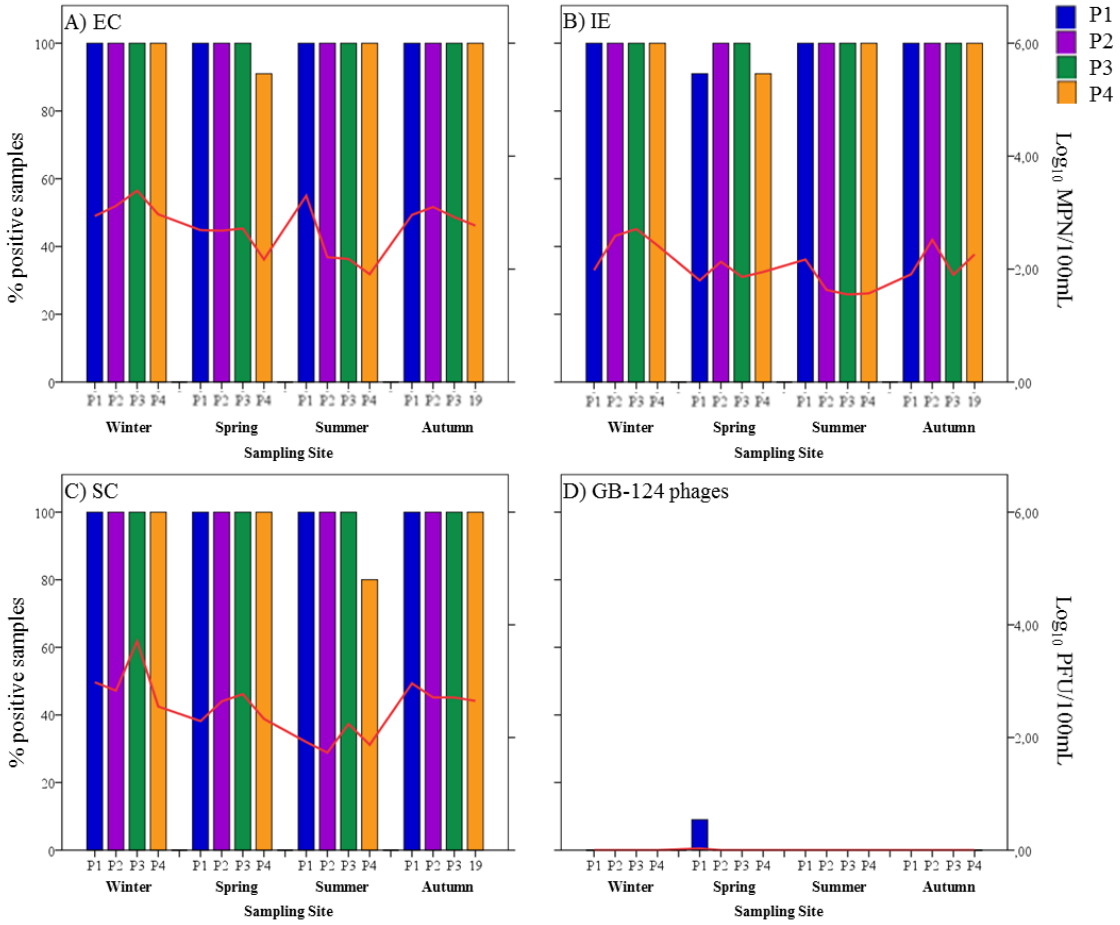


**Figure 6.1** - Quantitative results for culture-based markers in different sampling points along the River Tagus. P1 - VFX; P2 - MPdN; P3 - Alc; P4 - Bel. Columns represent the prevalence, the % of positive samples, in each sampling site and the curve represents the mean concentration values.

Figure 6.1 represents the data obtained for non-specific markers (EC, IE, SC) and for human-specific GB-124 phages. The results for all these markers were performed using culture-based methods. General markers of faecal contamination were detected throughout the year. EC, and IE were detected in all of the samples tested during winter and autumn,

whereas during spring and summer, the prevalence in some of the sampling points was approximately 90%. SC were detected in all samples tested except two during the summer (P4 site). GB-124 phages were not detected in the River Tagus catchment, with the exception of one single collected sample (at P1 site). The markers showed some variations also in their concentrations in the different sampling points and seasons. The mean concentration and standard deviation of each parameter are summarised in Table 6.2. The results for the molecular markers are also summarised in Figure 6.2 (A - E).

Human faecal contamination (presence of HAdV and HMMit) was detected all year around, but generally with lower prevalence during the summer. HAdV were not detected during the summer and also during winter in P1 site and no HAdV were detected at the P4 site during autumn. HAdV generally exhibited low prevalence at P4 site with values ranging from non-detected to 40%. The highest percentage of positive samples for HAdV was registered for the P3 site during winter and spring and the P1 site in autumn. The HMMit marker was detected at least once at all sampling points, with higher prevalence levels than HAdV, although with a lower percentage of positive samples obtained during warmer months. The prevalence of HMMit was mostly above 75% with the exception of P2 site site during winter.

CWMit marker was also detected frequently (irrespectively of time of year) with higher prevalence observed during the warmer seasons (spring and summer). From the tested non-human markers, the bovine marker (CWMit) was found to be present with a high prevalence throughout the year (Table 6.2). The pig marker (PigMit) was also consistently detected with similar prevalence between the different seasons. The poultry marker (PLMit) showed higher prevalence during summer with positive results found at all sampling locations (percentage positive between 40% and 75% positive). The mean concentration patterns for each parameter throughout the year differed significantly, with contamination of human and bovine origin obtained using HMMit and CWMit marker showing less stability in their values compared to the remaining markers (Figure 6.2 B and C). The summary for each location and by season can be found in Appendix 2.A and 2.B.

The results showed that the concentration of non-specific markers decreased during the summer and spring seasons (with the exception of site 1, for EC and IE that demonstrated a slight increase during the summer). Data analysis revealed that no strong variation was observed in the levels of the non-specific markers with respect to sampling site.

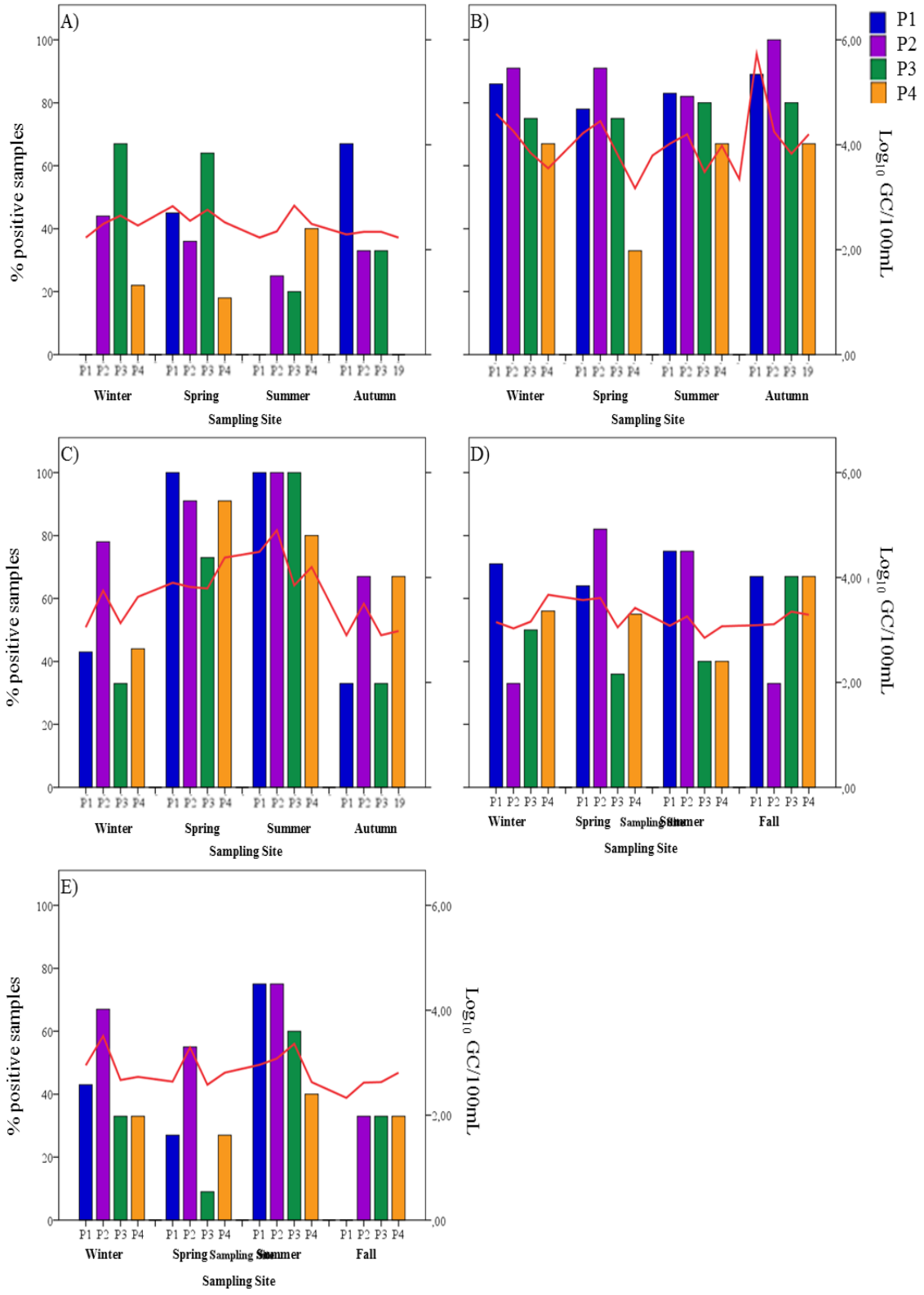


**Figure 6.2** - Quantitative qPCR results for qPCR-based methods. A) HAdV, B) HMMit, C) CWMit, D) PigMit, and E) PLMit. P1 - VFX, P2 - MPdN, P3 - Alc, and P4 - Bel. In columns the prevalences (% of positive samples) and curve represents mean concentration values.

Mean HAdV and PigMit concentrations were also fairly similar throughout the year and with respect to the different sampling points. HAdV was not detected in any samples collected during winter and spring for site 1 and autumn for site 4. Examining Appendix 2.A and 2.B it is clear that human mitochondrial DNA marker exhibits a pattern similar to that of the non-specific faecal markers with greater mean concentrations observed during the colder months (winter and autumn) and lower levels during the warmer seasons. The variation of the mean concentration does not appear to be sampling location specific, as similar trends were detected at all multiple sites. These results and the seasonal dependency is further explored below (Table 6.2). In contrast with the results obtained for the HMMit marker, the CWMit marker showed a higher increase in the mean concentration during spring and summer with lower concentrations observed during the colder months. Once again, the concentration does not appear to be sampling site dependent. The mean concentration of poultry mitochondrial marker showed no distinct variation throughout the year and from sampling site to sampling site. Similarly to HAdV, no positive sample was detected during autumn at site 1 displaying also a lower concentration for this season at site 2.

In order to better estimate whether the concentrations of each parameter were influenced by sampling site and season, analysis of variance following a two-way ANOVA test were performed to attain the *p*-value of the *F*-test. The *p*-values are outlined in Table 6.2. To account with the heteroscadicity of the values, data was transformed and normalised using the following equation:

$$X_2 = \frac{(X - \bar{X})}{SD}$$

Where:

- X2 is the normalised data;
- X is the initial log transformed data;
- $\bar{X}$ is the mean concentration;
- SD is the standard deviation

**Table 6.2 -** *p*-values of *F*-test by the analysis of variances of two-way factor (four seasons and four locations along River Tagus) for each parameter

| **Parameter** | **Location** | **Season** |
|---|---|---|
| EC | **< 0.05** | **< 0.001** |
| IE | 0.271 | **< 0.001** |
| SC | 0.059 | **< 0.001** |
| GB-124 | 0.366 | 0.702 |
| HAdV | 0.518 | 0.252 |
| HMMit | 0.352 | 0.213 |
| CWMit | 0.552 | **0.010** |
| PigMit | 0.545 | 0.584 |
| PLMit | 0.104 | 0.558 |

The concentration of the non-specific markers is highly affected by seasonality ($p < 0.05$ and $< 0.001$) and for EC the sampling site also plays an important role ($p < 0.05$). The variance of the concentration of most MST markers is not clearly attributed to either sampling site or seasonal factor ($p > 0.05$) with the exception of the CWMit that shows a statistically significant difference with respect to season ($p < 0.05$). These results corroborate the results and analysis performed previously when analysing Appendix 2.A and 2.B. Based on the comparison of the *p*-values it is evident that the variance derived from seasonality was found to be more relevant in the occurrence of EC, IE, SC, and CWMit. Table 6.3 discriminates the determinant season for the variance of the concentration that were statistically significant.

**Table 6.3 -** *p*-values of *F* test calculated by analysis of variances of two-way factor for EC, IE, SC, and CWMit to determine the most dominant combination of seasons

| | **Parameter** | | | |
|---|---|---|---|---|
| Season | EC | IE | SC | CWMit |
| Autumn – Summer | 0.020 | 0.090 | **0.010** | **0.008** |
| Autumn – Spring | **0.015** | 0.240 | 0.052 | **0.015** |
| Autumn - Winter | 0.305 | 0.085 | 0.255 | 0.450 |
| Spring - Summer | 0.285 | 0.212 | 0.002 | 0.252 |
| Summer - Winter | **< 0.001** | **< 0.001** | **< 0.05** | **0.020** |
| Spring - Winter | **< 0.05**[a] | **< 0.05**[a] | **< 0.001** | 0.057 |

Table 6.3 highlights the combination of seasons that are most important and that accounted for the greatest variation in the concentrations of the specific parameters. These are summer - winter ($p < 0.001$ and $< 0.05$) and spring - winter ($p < 0.001$ and $< 0.05$), except for the case of CWMit concentrations that were mostly affected by the combination of autumn – summer, although also impacted by the autumn –spring and summer - winter ($p < 0.05$). The data contained in Table 6.3 demonstrate that seasonal variations have a significant impact on FIB, SC and also on one of the bovine MST marker. EC concentrations were also shown to be affected by the sampling site with site 4 influencing primarily the observed differences in Table 6.3 and Appendix 2.C.

## 6.2. Correlation between physico-chemical parameters and general markers of faecal contamination/MST markers in the River Tagus

Different physico-chemical parameters may influence the behaviour of microorganisms in the environment, which include temperature, UV radiation, water temperature and salinity, and rainfall (IPMA, 2016; MARETEC, 2016) . The first two factors are also known to influence the inactivation levels of microorganisms. To understand whether temperature and/or UV influenced the changes in the concentrations of each parameter as observed previously, a one-way ANOVA was carried out to obtain the *p*-value. Table 6.4 displays the most relevant results, which as expected follow the results from Table 6.4, were found for EC, IE, SC, and CWMit. Furthermore, HMMit marker also showed high correlation with UV radiation but no relationship was found with temperature.

**Table 6.4 -** Influence of physicochemical environmental parameters on the concentration of EC, IE, SC, and CWMit

| | **Physicochemical Influence** |
|---|---|
| **Parameter** | **Radiation** |
| EC | **< 0.001** |
| IE | **< 0.001** |
| SC | **< 0.001** |
| HMMit | **< 0.05** |
| CWMit | **< 0.05** |
| | **Temperature** |
| EC | **< 0.05** |
| IE | **< 0.001** |
| SC | **< 0.001** |
| HMMit | **0.105** |
| CWMit | **<0.001** |

Results from Table 6.4 demonstrates that both factors (UV radiation and temperature) play a very important role on the distinct behaviour followed by non-specific markers of faecal contamination and CWMit. The remaining MST markers did not show any impact on their yearly behaviour as a result of the two physicochemical parameters ($p > 0.05$) and these results are in accordance with those from Table 6.3.

Another important factor in the quality of environmental waters is rainfall events. One-way ANOVA analysis was performed to determine whether rainfall influenced the concentration of the parameters. Data showed that for the majority of the parameters concentrations were not affected by the occurrence of rainfall (Appendix 2.D; $p > 0.05$). However, the one-way analysis of variance identified significant impact between IE, SC, and PLMit marker and rainfall ($p < 0.05$) and through the analysis of behaviour of tested parameters during the year in the different locations, a small influence of rainfall was noticeable for SC and PLMit at sites 3 and 4, respectively (Appendix 2.D, 2.E and 2.F). The remaining parameter concentrations did not show any correlation with rainfall (Appendix 2.D).

Although some parameters were influenced by the occurrence of rain events, the correlation between the levels of rainfall and the concentration of parameters showed a different pattern (Appendix 2.D). Data from rainfall levels have shown to be correlated with HAdV concentration ($r = 0.417$, $p$-value = 0.043) but showed no correlation with the remaining parameters.

The influence of water temperature and salinity on the concentration of the different parameters was also evaluated. Spearman's correlation was performed to determine the correlation between water temperature and salininty at the study sites (Table 6.5).

**Table 6.5 -** Correlation between tested parameters and water temperature/salinity. Significant correlations are in bold ($p < 0.01$)

| | **Physical parameter** | |
|---|---|---|
| **Parameter** | Water Temperature | Salinity |
| EC | **-0.379** | **-0.268** |
| IE | **-0.404** | -0.112 |
| SC | **-0.389** | -0.109 |
| GB124 phages | 0.152 | -0.131 |
| HAdV | 0.014 | 0.046 |
| HMMit | -0.190 | -.026 |
| CWMit | **0.293** | 0.062 |
| PigMit | 0.102 | -0.050 |
| PLMit | -0.006 | -0.140 |

The results revealed that water temperature negatively affected concentrations of EC, IE, and SC ($r$ = -0.379, $p < 0.01$; $r$ = -0.404, $p < 0.01$; $r$ = -0.389, $p < 0.01$). Weak but positive correlation was found between water temperature and CWMit ($r$ = 0.293, $p < 0.01$). The remaining parameters did not correlate with water temperature. Water salinity only weakly correlated with the concentration of EC ($r$ = -0.268, $p < 0.01$). Although no correlation was found between other tested parameters and salinity, this parameter influenced most of them negatively ($r$ below zero).

### 6.3. Relationship between source-specific and non-specific markers

To determine whether the source-specific markers were quantitatively correlated to general microbiological markers of faecal contamination, Spearman's correlation was performed for the dataset. Among the general non-specific markers of contamination, a significant correlation was found (Table 6.6). Unsurprisingly, the strongest correlation was between the FIB (EC and IE) ($r$ = 0.673, $p < 0.01$) and the weakest for IE and SC ($r$ = 0.469, $p < 0.01$). Significant but weak correlations were observed between EC and HAdV ($p < 0.05$), EC and PigMit ($p < 0.05$), IE and PigMit ($p < 0.05$), GB124 phages and HAdV ($p < 0.05$), HAdV and CWMit ($p < 0.05$) and CWMit and PigMit ($p < 0.01$) (Table 6.6). Correlations between the remaining pairs were not significant.

**Table 6.6 -** Spearman's correlation between the $Log_{10}$ concentration of non-specific faecal markers and the different source-specific markers.

| | EC | IE | SC | GB124 phages | HAdV | HMMit | CWMit | PigMit | PLMit |
|---|---|---|---|---|---|---|---|---|---|
| EC | - | **0.673**[a)] | **0.511**[a)] | 0.055 | 0.204[b)] | -0.091 | -0.107 | 0.221[b)] | 0.093 |
| IE | | - | **0.469**[a)] | -0.042 | 0.083 | -0.059 | -0.069 | 0.247[b)] | 0.193[b)] |
| SC | | | - | -0.097 | 0.134 | -0.079 | -0.188 | 0.080 | 0.058 |
| GB124 phages | | | | - | 0.202[b)] | 0.019 | 0.046 | 0.132 | -0.075 |
| HAdV | | | | | - | -0.012 | 0.223[b)] | 0.014 | 0.144 |
| HMMit | | | | | | - | 0.061 | 0.022 | -0.056 |
| CWMit | | | | | | | - | 0.255[a)] | -0.010 |
| PigMit | | | | | | | | - | -0.043 |
| PLMit | | | | | | | | | - |

[a)] **Two tailed significance, p < 0.01**.

[b)] **Two tailed significance, p < 0.05**.

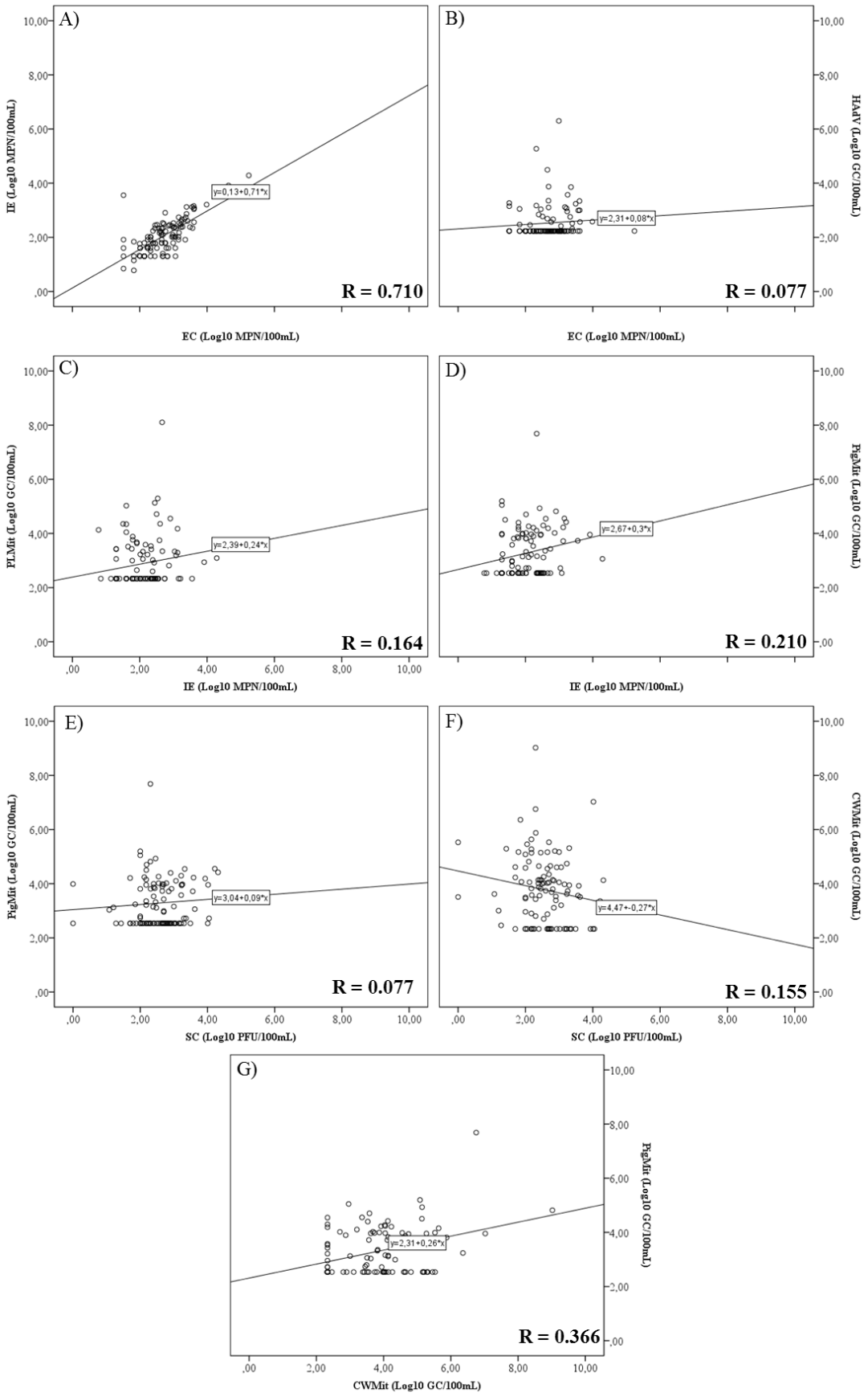


**Figure 6.3 -** Quantitative correlation between different parameters. A) EC vs IE; B) EC vs HAdV; C) IE vs PLMit; D) IE vs PigMit; E) SC vs CWMit; F) SC vs PigMit; G) CWMit vs PigMit. Equations and coefficients of determination for regressions are given in the figures.

Interestingly, the concentration of human- and bovine-associated markers tended to decrease with the concentration of non-specific markers of faecal contamination (Figure 6.3). To understand whether the source-specific MST markers are correlated quantitatively to the non-specific faecal markers, correlation analysis using Spearman rank correlation coefficients was performed for rainfall (Table 6.7). PigMit was moderately positively correlated with IE during rain events ($r$ = 0.444, $p$-value = 0.05). Although not statistically correlated at a $p$-value level of 0.05, PigMit always presented the highest correlation coefficients with EC and SC. In stark contrast, the remaining markers showed low, or no significant correlations with the non-specific markers.

**Table 6.7 -** Spearman's correlation between log10 concentration of non-specific faecal markers and the different source-specific markers in samples associated with rainfall events

| | EC | IE | SC |
|---|---|---|---|
| GB 124 phages | n.a. | n.a. | n.a. |
| HAdV | -0.189 | -0.191 | 0.027 |
| HMMit | -0.160 | -0.272 | -0.240 |
| CWMit | -0.094 | 0.031 | -0.104 |
| PigMit | 0.366 | **0.444** | 0.227 |
| PLMit | 0.012 | 0.252 | 0.091 |

### 6.4. Method development (domestic animal markers)

Cat and dog mitochondrial markers (CatMito and DogMito, respectively) were designed as described in Section 4.2.2 of Chapter 4. These domestic animal markers were developed and trialled in order to understand whether any of the sampling sites were influenced by urban run-off following rainfall. Faecal matter of cat and dog origin were used to extract DNA using single- and nested-PCR, which were performed using gradients of annealing temperatures (Ta) to determine the most suitable PCR amplification cycle (Figure 6.4) as explained previously in Chapter 4, Section 4.2.2.

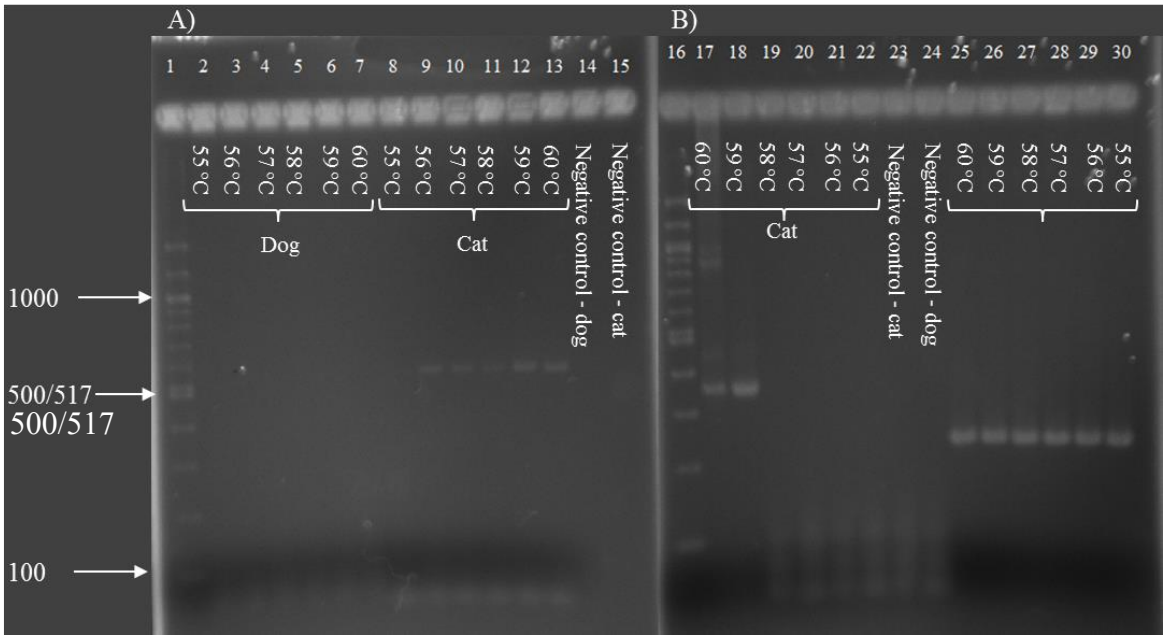


**Figure 6.4 -** Detection and optimisation of annealing temperature of mitochondrial DNA by PCR. (A) DNA extracted from faecal matter of each species was used to run single-PCR with species-specific primers. 100 bp molecular marker (lane 1; New England Biolabs, US), dog Ta (lanes 2-7), cat Ta (lanes 8-13), negative control (lanes 14-15). (B) nested-PCR performed on amplicons from single-PCR. 100 bp molecular marker (lane 1), cat Ta (lanes 17-22), negative control (lanes 23-24), dog Ta (lanes 25-30).

Figure 6.4 shows that no amplification was obtained for the single-PCR using the DogMito marker at the different Ta, the same result obtained for cat single-PCR at annealing temperature 55 ºC. At Ta of 56, 57, 58, 59, and 60 ºC specific amplification occurred for cat, with a slightly better definition for Ta = 59 ºC. The same temperatures were used to perform nested-PCR on the amplification products of single-PCR. No amplification product was obtained for cat at low Ta (55 to 58 ºC). Specific cat amplicons were achieved at 59 and 60 ºC. As a result of the formation of non-specific amplifications at 60 ºC, the chosen optimal Ta to use for CatMito was 59 ºC. For dog nested-PCR, amplicons existed for all Ta, although no amplification was observed in the agarose gel from single-PCR. A test was run to determine whether the first PCR would be unnecessary for dog faecal contamination detection. The results showed that a first PCR was indeed necessary, as no consistent amplification would be obtained just by using the nested primers (Appendix 2.G). The amplification protocol used in subsequent analysis was as described previously in Table 4.7, Section 4.2.2 in Chapter 4.

Following optimisation of PCR conditions, the set of primers were tested against faecal matter from different species, including humans, cow, pig, poultry, pigeons and gulls. These animals were chosen specifically since they are the main sources of faecal

contamination in urban and river environments found within the River Tagus catchment. The results are shown in Figure 6.5.

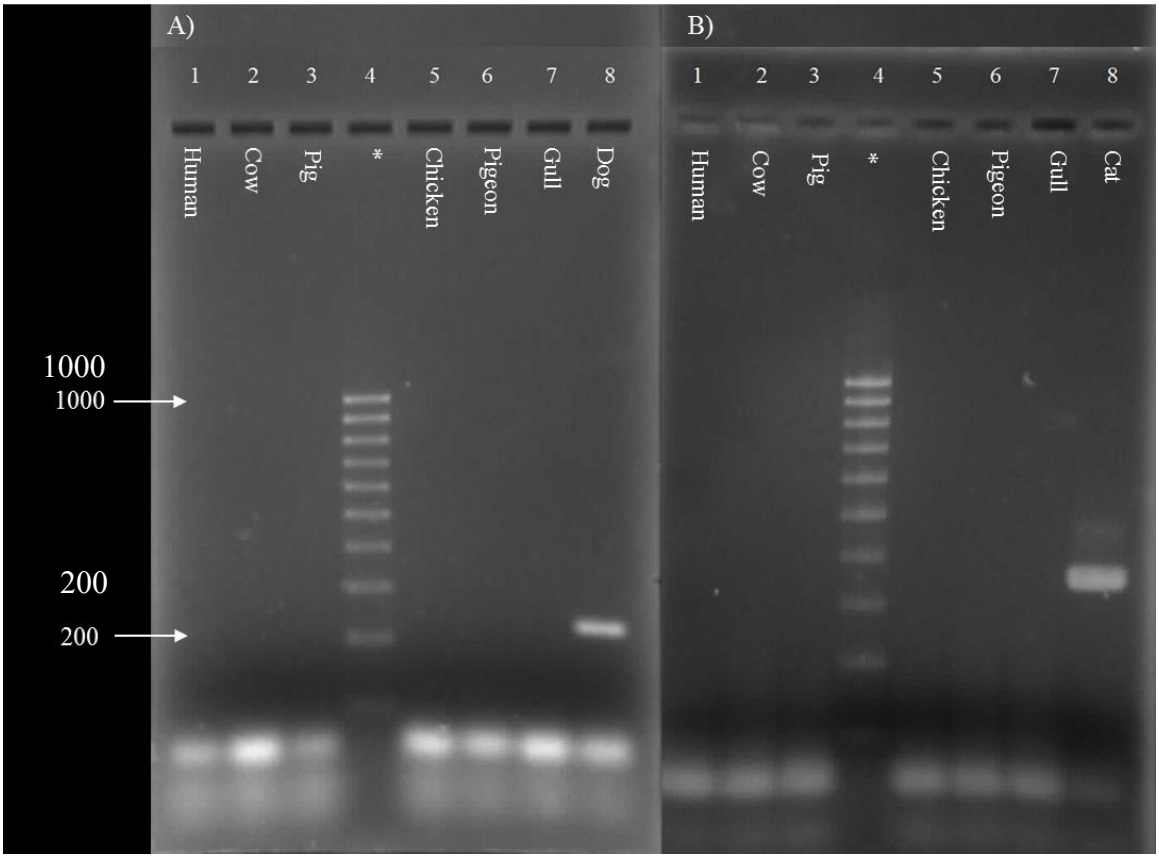


**Figure 6.5 -** Nested PCR of faecal matter from different source origin. (A) DNA extracted from faeces of each species with dog specific primers. *100 bp molecular marker (Fermentas, ThermoScientific, US. (B) DNA extracted from faeces of each species with cat specific primers. *100 bp molecular marker (Fermentas, ThermoScientific, US). 2.0% agarose gel with ethidium bromide staining.

No cross-reactivity was observed for each species-specific primer with the different origins of contamination of interest. The assays developed for the specific detection of contamination from domestic animals produced positive results only when the corresponding species was analysed with no extra bands appearing in the remaining tested organisms representing the absence of nonspecific amplifications in the faecal samples. Sensitivity tests were made for each set of nested-PCR by decreasing the quantity of the corresponding targeted animal DNA in the PCR reaction (Figure 6.6).

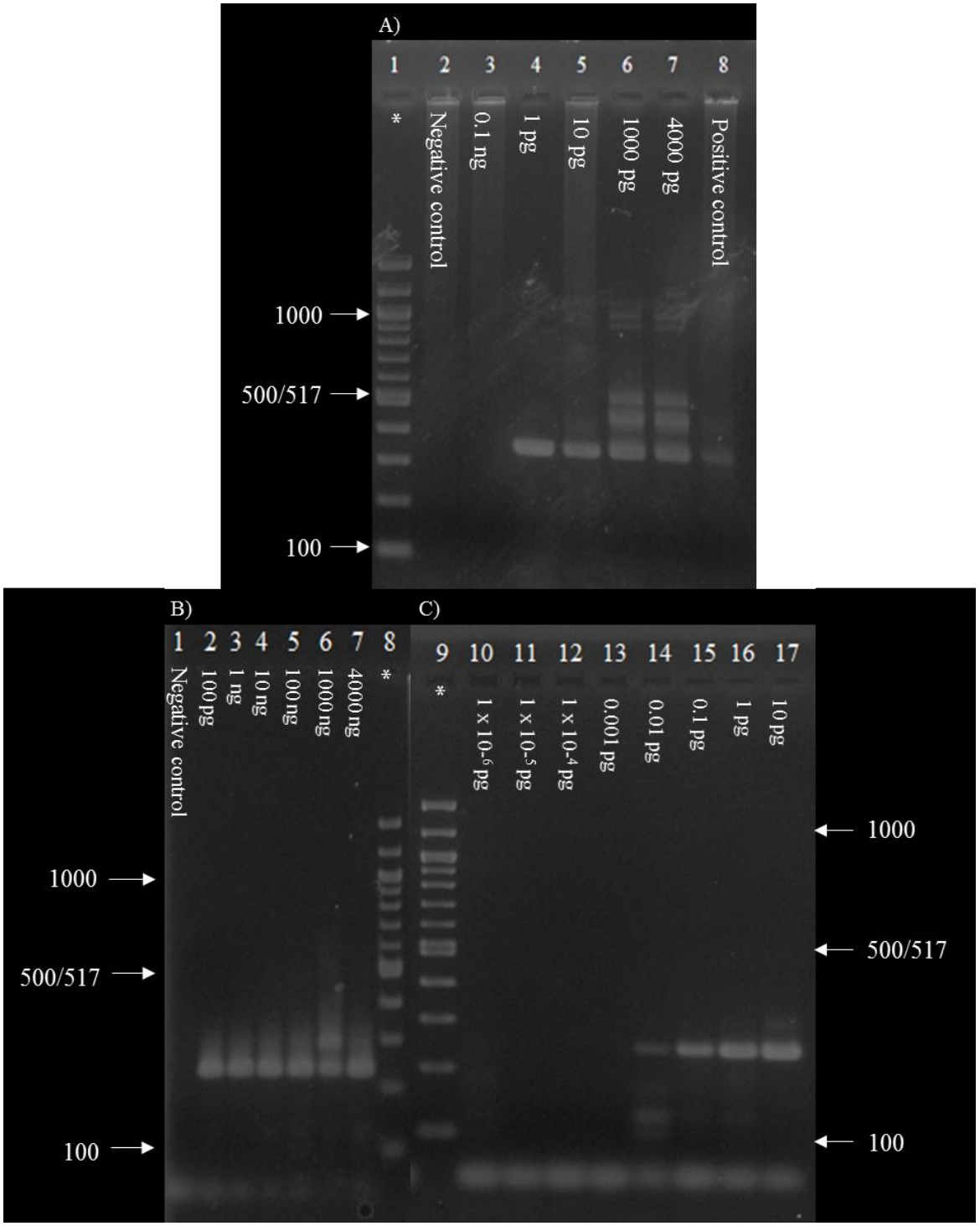


**Figure 6.6 -** Sensitivity tests for nested-PCR. (A) DNA extracted from cat faeces. * 100 bp molecular marker (New England Biolabs, US). (B) and (C) DNA extracted from dog faeces. * 100 bp molecular marker (New England Biolabs, US).

The sensitivity limit was around 1 pg for cat mitochondrial marker and 0.01 pg for dog mitochondrial marker following nested PCR. Water samples from the River Tagus were tested for contamination from dog and cat origin. The overall percentage of positive samples for cat and dog faecal contaminationof the river, the percentages of positive results during dry and rainy days and by sampling location are summarised in Figure 6.7.

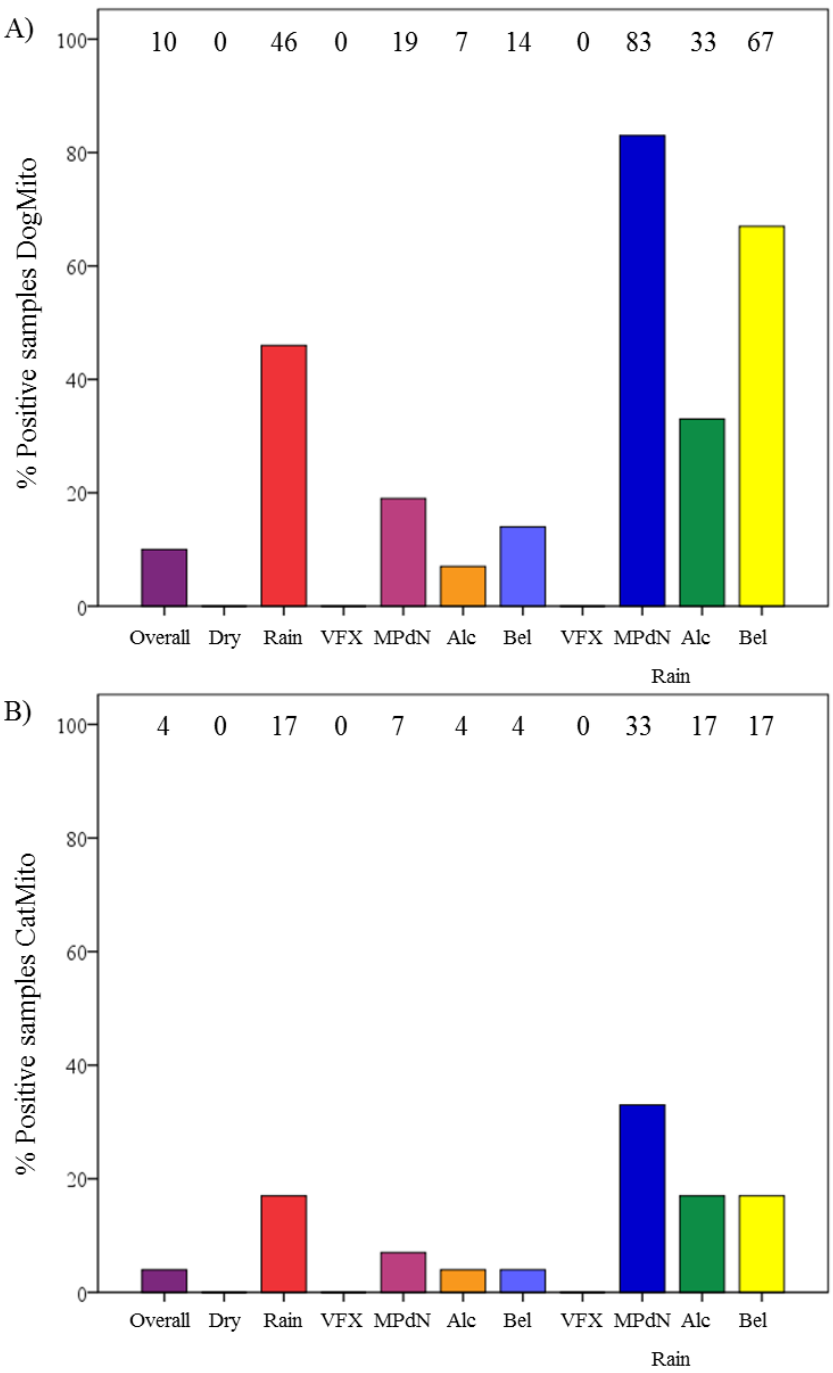


**Figure 6.7 -** Percentage of positive samples for cat and dog faecal contamination in the River Tagus. A) DogMito results. B) CatMito results.

The results showed that amplification of DNA from such faecal contamination was only obtained in samples following higher levels of rainfall, which would suggest input from urban runoff. Faecal contamination from pet animals was detected at low prevalence, with higher percentage of positive samples for dog (10 %). PCR signals for each marker were only detected following or during periods of rainfall within the catchment, with a relatively

high percentage of samples positive for dog faecal contamination under such conditions. No positives were obtained for samples collected on dry days. Site 2 (MPdN) was the sampling site with the highest prevalence of both pet animal markers with 19 % for dog and 7 % for cat. As a likely result of rainfall events, the prevalence of CatMito and DogMit for this particular sampling site showed a steep increase with percentages over 30% and 80%, respectively. Sites 3 (Alc) and 4 (Bel) were also positive for both markers with percentages ranging from 17% to over 67%. Site 1 (VFX) was always negative for both markers, regardless of rainfall events.

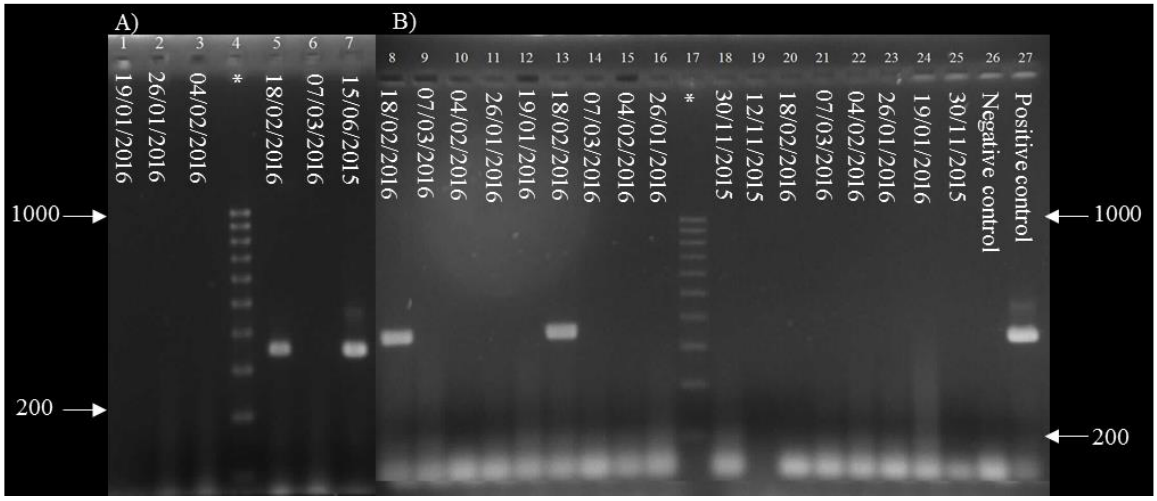


**Figure 6.8 -** Cat faecal contamination in the R. Tagus water samples. (A) Sites 3 (Lanes 1-3 and 5-6) and 2 (lane 8) sampling sites, *100 bp molecular marker (Fermentas, ThermoScientific, US). (B) Sites 2 (Lanes 8 to 12), 4 (lanes 13-16 and 18-19) and 1 (lanes 20-25) sampling sites, *100 bp molecular marker (Fermentas, ThermoScientific, US).

Site 2, a highly urbanized centre used also for recreation like pet walking was positive in two different sampling dates for CatMito: 15 June 2015 and 18 February 2016 (Figure 6.8). In previous days and on the day of sampling itself, rainfall occurred. 18 February 2016 sampling date was also positive for CatMito at sites 3 and 4. The remaining dates were negative for this marker in the different sampling points. Dog faecal contamination was detected on several days where rainfall occurred (Figure 6.9). Samples collected at 18 February and 7 March 2016 were positive in sites 2, 3, and 4 sampling points.

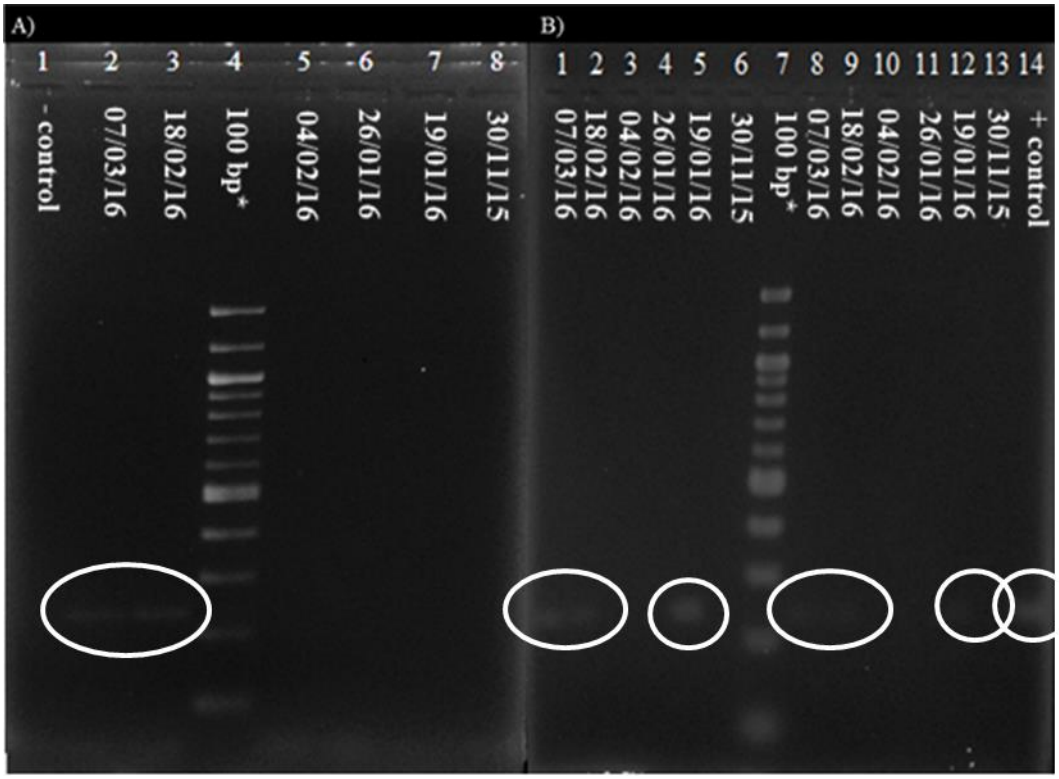


**Figure 6.9 -** Dog faecal contamination in R. Tagus water samples. (A) Sites 3, (B) 2 (Lanes 1-6) and 4 (lanes 8-13). *100 bp molecular marker (New England Biolabs, US). The circles represent then positive samples for dog faecal contamination.

Sampling site 4 was positive for DogMito on four sampling occasions: 15 June 2015, 19 January 2016, 18 February 2016, and 7 March 2016. Site 2 was the sampling point with the highest percentage of positive samples with positive results on five sampling dates: 4 May 2015, 15 June 2015, 19 January 2016, 18 February 2016, and 7 March 2016. Subsequent to the analyses of the results obtained by nested-PCR for CatMito and DogMito, the influence of rainfall events on the observed data for each marker was assessed by analysing variance following two-way factors (two-way ANOVA; Appendix 2.F). A significant correlation was found between rainfall events and detection of domestic (cat and dog) faecal contamination (Appendix 2.H). Additionally, both markers also showed strong correlation with the sampling location, though stronger correlations were obtained using DogMito ($p$-value < 0.001). However, no correlation was observed for DogMito/CatMito, rainfall events at site X (VFX) ($p$-value > 0.05).

# CHAPTER 7 : NOVEL PRETREATMENT METHODS TO IDENTIFY INFECTIOUS VIRUSES BY RT-QPCR

## 7.1. Evaluation of enzymatic and intercalating dye pretreatment to determine infectivity by RT-qPCR

In Chapter 5, it was shown that RT-qPCR could detect enteric viruses in samples in which a culture-based method failed to detect FIB or phages. Therefore, it is important to elucidate whether the culture-based approach is failing to detect infective viruses or whether qPCR is detecting non-infectious viral particles. Two contrasting approaches have previously been tested and employed to estimate infectivity more effectively using RT-qPCR, namely, enzymatic treatment with RNase in combination with proteinase K or not and treatment of samples with nucleic acid intercalating dyes, as described previously in Chapter 3.

The objective of this particular component of the research study was to develop a methodology, based on pre-treatment, that could be combined with RT-qPCR to distinguish between infectious and thermal-, chlorine-, and UV- inactivated viruses in spiked river water. Enzymatic and viability dye pretreatments (ethidium monoazide (EMA), propidium monoazide (PMA) and Reagent D (RD)) were chosen. EntV was chosen for their cultivability, whereas $MC_0$, a murine virus of the *Picornaviridae* family, were chosen because their structural and physicochemical properties are close to those of hepatitis A virus (HAV). $MC_0$ have also been used as surrogates for NoV and HAV, which are major causative agents of gastroenteritis and hepatitis worldwide.

To understand whether a complex matrix such as river water following skimmed milk flocculation for the concentration of viruses would interfere with the different pretreatments, a 10-fold dilution of the river sample was also spiked with the viruses and subjected to the same processes.

## 7.2. Chlorine Treatment

Figure 7.1 shows the $\log_{10}$ removal determined for EntV and $MC_0$ following free chlorine (FChl) treatment and PCR pre-treatment in undiluted and diluted river water samples.

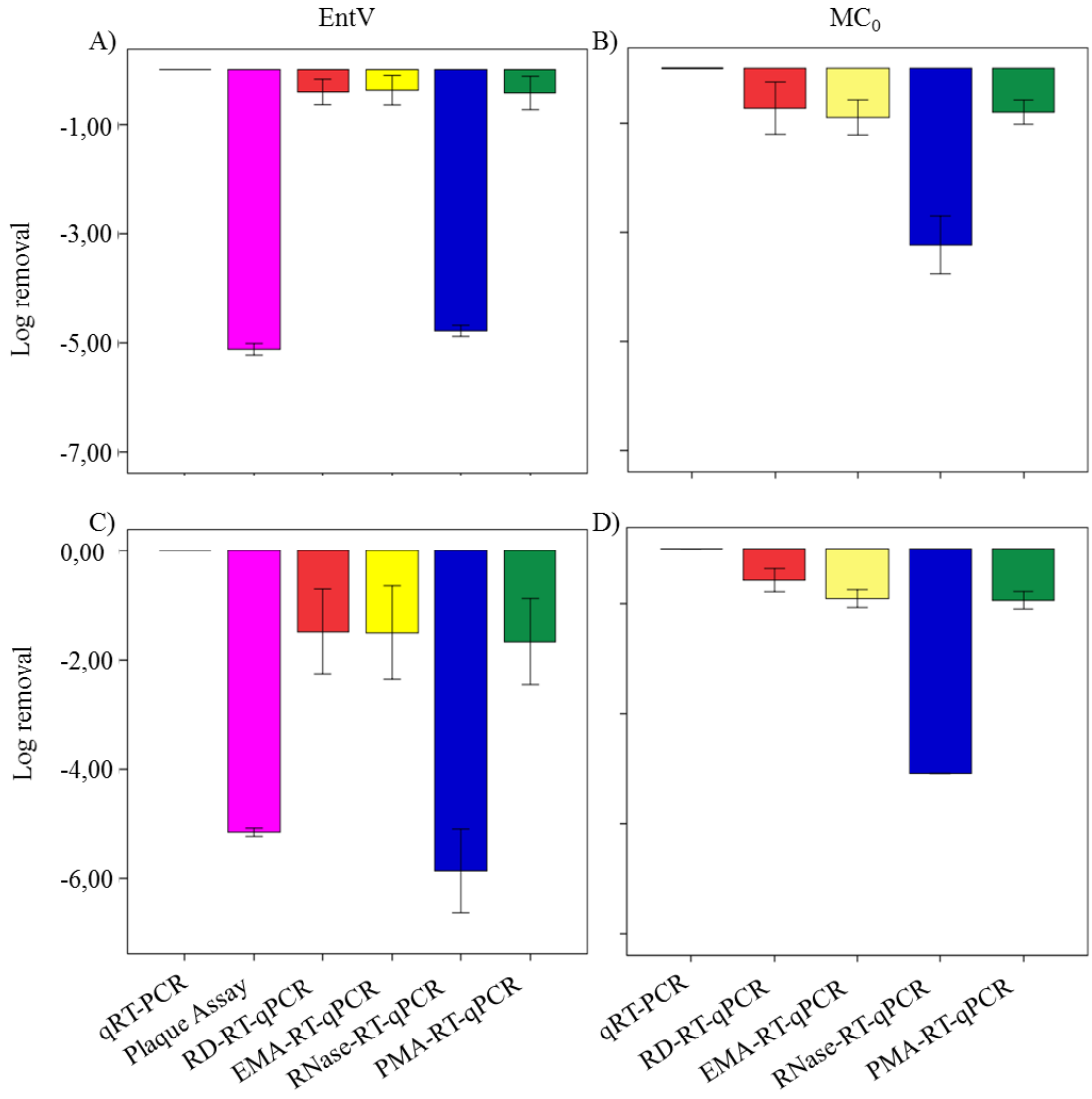


**Figure 7.1** - Discrimination between infectious and non-infectious viral particles using Reagent D-, EMA-, RNase-, and PMA-RT-qPCR. A) Direct river water sample for EntV; B) Direct river water sample for $MC_0$; C) 10-fold diluted river water sample for EntV; D) 10-fold diluted river water sample for $MC_0$. Each bar represents mean ± SD.

Viral inactivation measured by plaque assay (PA) resulted in > 5 log inactivation for EntV in both diluted and un-diluted samples. An example of infectivity measured by plaque assay is shown in Appendix 3.A. Although FChl affected EntV and $MC_0$ in a slightly different way, RT-qPCR by itself caused false-positive results for both viruses.

Interestingly, results for RNase-RT-qPCR of chlorine-inactivated viruses were equivalent to those obtained by plaque assay. In addition, for chlorine-treated EntV, pre-treatment with RNase was able to distinguish between infectious and inactivated EntV (Appendix 3.A).

Table 7.1 displays the results obtained for the reduction of non-infectious viruses using the different pre-treatments. A statistically significant five-fold reduction in non-infectious EntV was demonstrated when exposed to FChl ($p$-value < 0.001), for undiluted river water samples. For $MC_0$, the reduction levels were lower (three-fold), but were still statistically significant ($p$-value < 0.001). EMA dye yielded similar results for chlorine-treated EntV but demonstrated a higher degree of free RNA removal for $MC_0$, although not entirely removing the signal.

**Table 7.1 -** Inactivation of EntV and $MC_0$ by FChl as determined by cell culture and qPCR using pre-treatments

| **Virus** | **Treatment** | **Fold-reduction** |
|---|---|---|
| EntV | | |
| Plaque assay | | 5.12 |
| Non-infectious | RT-qPCR | 0.00 |
| | RD-RT-qPCR | 0.41 |
| | EMA-RT-qPCR | 0.37 |
| | RNase-RT-qPCR | 4.78[a] |
| | PMA-RT-qPCR | 0.42 |
| $MC_0$ | | |
| Non-infectious | RT-qPCR | 0.00 |
| | RD-RT-qPCR | 0.72 |
| | EMA-RT-qPCR | 0.89 |
| | RNase-RT-qPCR | 3.23[a] |
| | PMA-RT-qPCR | 0.80 |

[a] There was a statistically significant difference between these values and the remaining reduction values ($p$ < 0.001).

For EntV, RD-qPCR showed statistically significant levels of removal ($p$-value < 0.05). Distinguishing between infectious and non-infectious FChl-treated viruses using the three different dyes was not statistically significant ($p$-value > 0.05) whereas the use of RNase demonstrated statistically significant compared to the other treatments ($p$-value < 0.001 for EntV and $p$-value < 0.05 for $MC_0$). The inhibitory effect of the matrix was also evaluated by testing a ten-fold dilution of the initial concentrated river water sample (Figure 7.1). Chlorine-treated $MC_0$ and EntV detection using the different RT-qPCR was not significantly influenced by a matrix effect ($p$-value > 0.05). Additionally, in diluted river

water samples, RNase was able to discriminate completely between infectious and non-infectious EntV. Again, the use of dyes did not fully remove the qPCR signal.

### 7.3. UV Treatment

To determine whether viral infectivity following UV irradiation could be detected by RT-qPCR, skimmed-milk concentrated river water samples, spiked with EntV and $MC_0$, were submitted to UVC irradiation for different time periods (30, 60, 200 and 600 s) followed by the use of RNase or viability dyes and detected by RT-qPCR. EntV were also detected by cell culture plaque assay. The results are presented in Figure 7.2 and Appendix 3.B.

The results demonstrated that a 200 s exposure to UV radiation was sufficient to achieve full inactivation of EntV, as measured by plaque assay. A 30 s exposure to UV radiation led to more than a four-log decrease in infectivity levels. There is an evident difference between the EntV infectivity assay and treatment using dyes. RT-qPCR detection following UV exposure did not effectively reduce the concentration of inactivated viruses observed, therefore presenting 'false positive' results. The results for UV inactivated viruses were in agreement with those for FChl, with RNase providing a more effective reduction in the qPCR signal. Treatment with RNase led to more than a four-log decrease in the reduction levels for EntV. Nevertheless, after 600 s of exposure to UV radiation, the inactivation of the 'false-positive' signal for EntV was less effective (Figure 7.2 A). Pre-treatment with RD, PMA, and EMA only resulted in a 1.2 log reduction in EntV infectivity.

The results obtained for $MC_0$ were similar to those obtained for EntV. Again, only a partial removal of the qPCR signal was achieved using the pre-treatments investigated. RD/EMA/PMA-RT-qPCR provided a similar decrease in the values, with up to a 1.2 log reduction in infectivity. RNase-RT-qPCR of inactivated $MC_0$, although still presenting positive PCR signal, showed more than a 3.0-log decrease in the signal. The mean log reductions in the qPCR signal for $MC_0$ were comparable to those of EntV, following a similar pattern, with the exception of the 600 s UV exposure time.

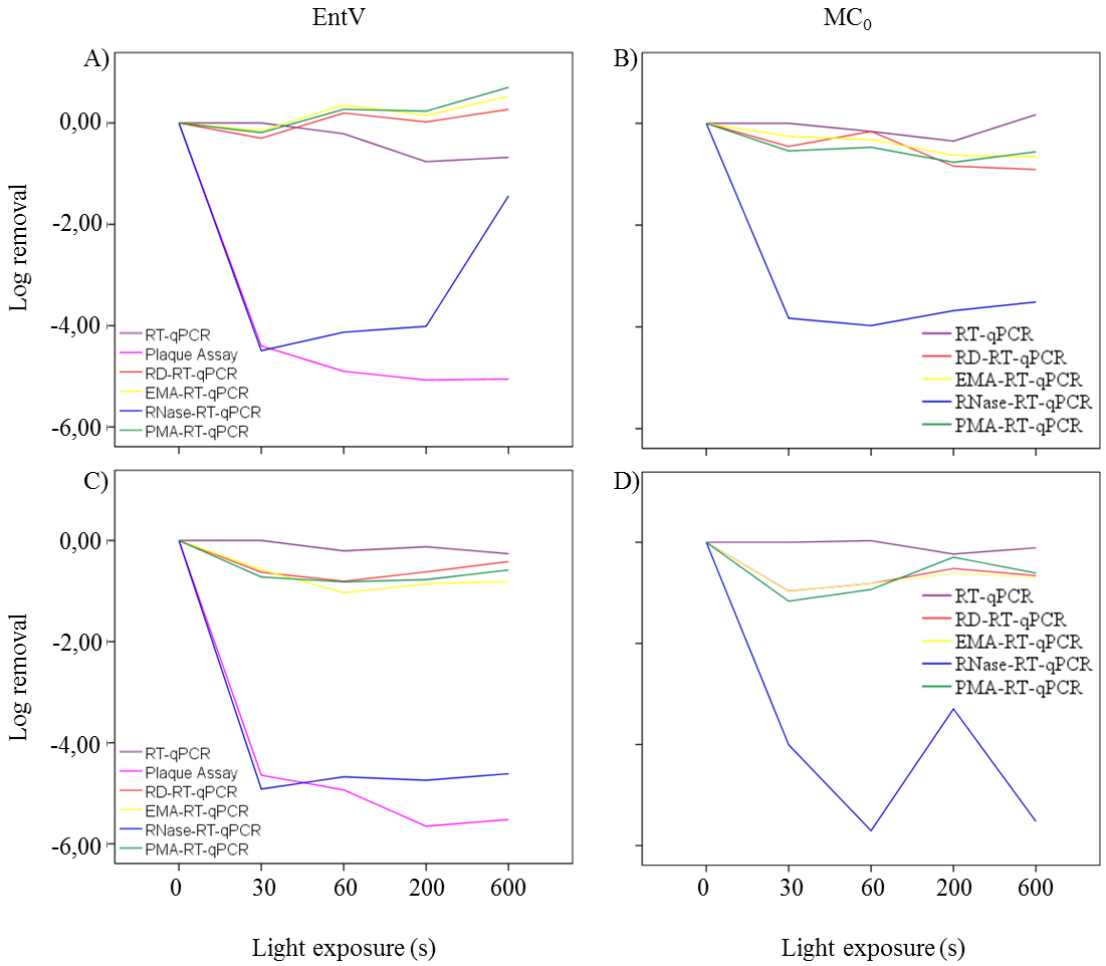


**Figure 7.2 -** The ability of different pre-treatment-qPCR assays to discern viable viruses following exposure to UV. A) Undiluted river water for EntV; B) Undiluted river water for $MC_0$; C) Diluted river water for EntV; D) Diluted river water sample for $MC_0$.

EntV RD/PMA-RT-qPCR did not demonstrate any significant difference in the reduction of infectivity across the UV exposure times investigated ($p$-value > 0.05). The reduction in infectivity by plaque assay, showed that EMA/RNase-RT-qPCR was statistically different from the first exposure time of 30 s to the final exposure time of 600 s ($p < 0.05$). Results for $MC_0$ demonstrated that no difference could be detected between 30 s of exposure time and 600 s, for all treatments ($p$-value > 0.05).

Table 7.2 shows the level of significance between different pre-treatments during the UV various UV exposure times for EntV and $MC_0$. It is noticeable that for all tested exposure times, the difference in the removal values obtained for EntV plaque assay (PA) were always statistically different to PCR (qPCR) with no pre-treatment. The same trend was also found between PA and all pre-treatments, with the exception of RNase for the first tested exposure time ($p > 0.05$). RNase treatment also produced removal levels that were significantly different to those achieved using the membrane impermeant dyes for both viruses. The distinct dyes did not show substantial differences in their effect on removal level of PCR signal.

The effect of a complex matrix was also evaluated by spiking a ten-fold dilution of the original concentrated river water sample with EntV and $MC_0$ and subjecting it to the same procedures that were adopted for the original sample. Analysing Figures 7.2 C and D it is interesting that a higher degree of reduction in infectivity was achieved for both viruses using the RNase treatment. Complete removal of the RNase-RT-qPCR signal was obtained for EntV in the diluted river water sample with reduction levels of more than 4.6 logs. For EntV, results from 30 s of exposure to UV did not show significant differences between diluted and undiluted samples ($p > 0.05$). However, using other UV exposure times did produce statistically significant differences between levels of reduction in infectivity, particularly for RNase/PMA-RT-qPCR. For $MC_0$, identical results were obtained, with a higher reduction in infectivity achieved when using RNase in the diluted sample compared to the undiluted one. Reduction levels varied between 3.7 and 5.7 logs for RNase-RT-qPCR and results for 30 s of UV exposure resulted in a strong difference between undiluted and diluted sample ($p < 0.05$) for all pre-treatments with the exception of RNase-RT-qPCR. Following 200 and 600 s of UV exposure, no significant variation was found between undiluted and diluted sample ($p > 0.05$).

**Table 7.2 -** *p*-values of One-way ANOVA calculated for EntV and $MC_0$ for no pre-treatment PCR, PA, RD, EMA, RNase, and PMA

| | EntV | | | | $MC_0$ | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | 30 | 60 | 200 | 600 | 30 | 60 | 200 | 600 |
| qPCR-PA | < 0.001 | < 0.001 | < 0.001 | < 0.001 | - | - | - | - |
| qPCR-RD | < 0.05 | 0.198 | 0.267 | < 0.05 | 0.159 | 0.226 | < 0.05 | < 0.05 |
| qPCR-EMA | 0.092 | 0.195 | < 0.05 | < 0.05 | 0.078 | < 0.05 | < 0.05 | 0.059 |
| qPCR-RNase | < 0.001 | < 0.001 | < 0.001 | 0.160 | < 0.05 | < 0.001 | < 0.05 | < 0.05 |
| qPCR-PMA | 0.468 | 0.106 | < 0.05 | < 0.05 | < 0.05 | < 0.05 | < 0.05 | < 0.05 |
| PA-RD | < 0.001 | < 0.001 | < 0.05 | < 0.001 | - | - | - | - |
| PA-EMA | < 0.001 | < 0.001 | < 0.001 | < 0.001 | - | - | - | - |
| PA-RNase | 0.119 | < 0.05 | < 0.05 | < 0.05 | - | - | - | - |
| PA-PMA | < 0.001 | < 0.001 | < 0.001 | < 0.001 | - | - | - | - |
| RD-EMA | 0.203 | 0.739 | 0.858 | 0.458 | 0.910 | 0.253 | 0.376 | 0.616 |
| RD-RNase | < 0.001 | < 0.001 | < 0.05 | < 0.05 | < 0.05 | < 0.001 | < 0.05 | < 0.05 |
| RD-PMA | 0.678 | 0.844 | 0.770 | 0.300 | 0.219 | 0.172 | 0.253 | 0.307 |
| EMA-RNase | < 0.001 | < 0.001 | < 0.001 | < 0.05 | < 0.05 | < 0.001 | < 0.05 | < 0.05 |
| EMA-PMA | 0.888 | 0.849 | 0.854 | 0.645 | 0.215 | 0.484 | 0.573 | 0.863 |
| RNase-PMA | < 0.001 | < 0.001 | < 0.001 | < 0.05 | < 0.05 | < 0.001 | < 0.05 | < 0.05 |

### 7.4. Thermal Treatment

To determine whether there was a difference in the application of RT-qPCR pre-treatments between physical treatments (UV, heat) and chemical oxidants (FChl), skimmed milk concentrated river water was again spiked with EntV and $MC_0$ and the viruses were inactivated at 95 ºC for 10 mins in a dry bath. Heat-inactivated viruses were treated with viability dyes (RD, EMA, and PMA) and RNase and detected by RT-qPCR. EntV infectivity was also determined by plaque assay. Figure 7.3 shows that quantification using RT-qPCR resulted in a mean decrease of 1.6- and 3.0 logs in the reduction of qPCR signal for EntV and $MC_0$, respectively.

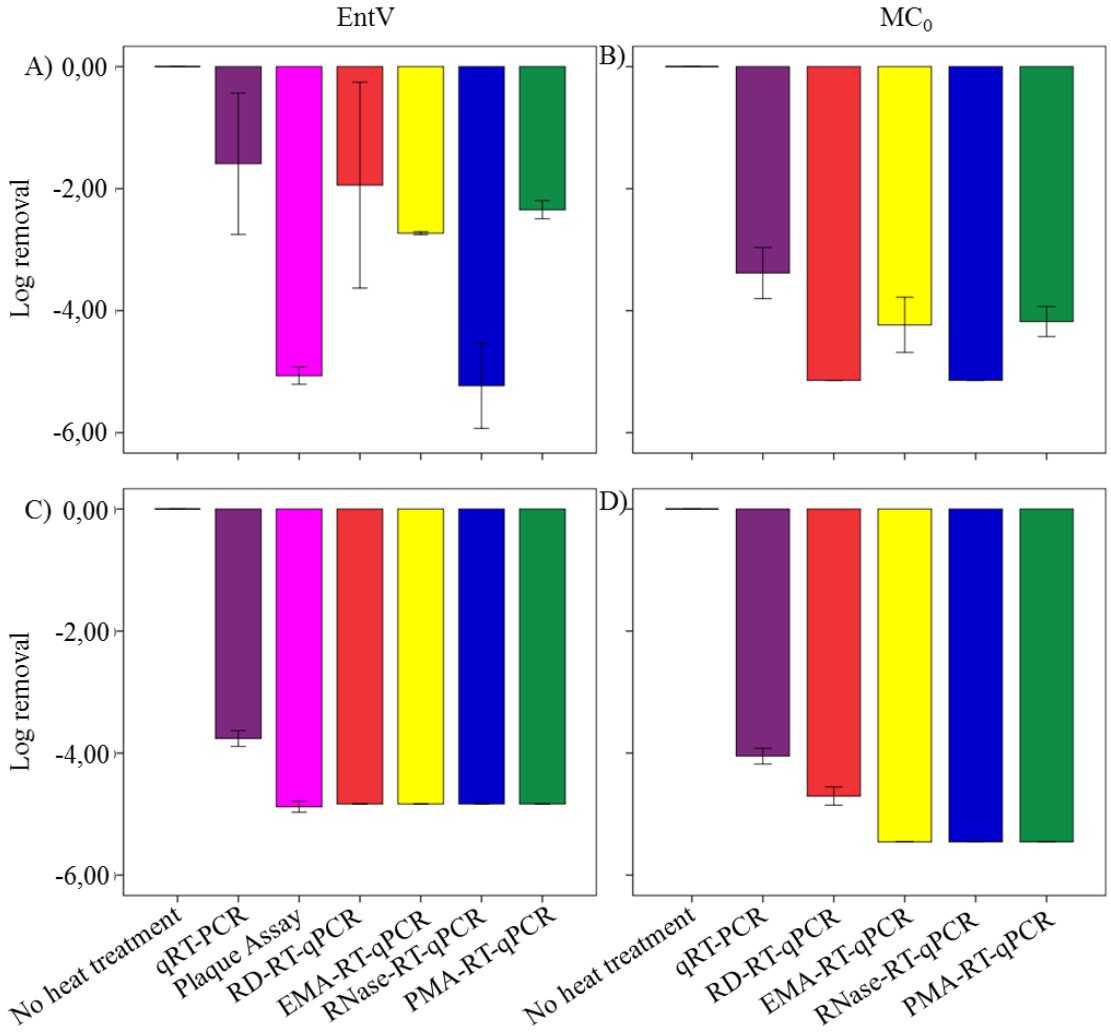


**Figure 7.3 -** Capacity of dye and RNase treatment to discriminate between infectious and non-infectious viruses following thermal inactivation at 95 ºC for 10 min. A) Undiluted river water and EntV; B) Undiluted river water and $MC_0$; C) Diluted river water and EntV; D) Diluted river water and $MC_0$. Each bar represents mean ± SD.

Heat treatment resulted in a high level of inactivation, as measured by plaque assay for EntV (Figure 7.3 and Appendix 3.C). Viability dyes did not however discriminate between infectious and non-infectious EntV and for $MC_0$, of the different dyes, only RD-RT-qPCR achieved complete inactivation (Figure 7.3 and Appendix 3.C). Treatment with membrane impermeant dyes produced higher rates of reduction for $MC_0$ than for EntV. These dyes yielded log reduction levels of between 1.9 and 2.7 for RD and EMA, for EntV and of between 3.8 and more than 5.2 for EMA and RD, for $MC_0$, respectively. Once again, RNase-RT-qPCR performed more favourably compared with the other treatments, totally removing the positive PCR signals of non-infectious viruses. RNase-RT-qPCR resulted in more than a 5.0 log reduction of EntV and $MC_0$. To evaluate the impact of heat treatment and each pre-treatment on the observed results for each virus in spiked river water, analysis of one-way ANOVA was performed to obtain the *p*-value. The results are summarised in Table 7.3.

**Table 7.3** - *p*-value of one-way ANOVA calculated for heat inactivated EntV and $MC_0$

| Treatment | No treatment | Heat treatment |
|---|---|---|
| EntV | | |
| RT-qPCR | 0.076 | - |
| PA | < 0.001 | < 0.05 |
| RD-RT-qPCR | 0.117 | 0.782 |
| EMA-RT-qPCR | < 0.001 | 0.164 |
| RNase-RT-qPCR | < 0.001 | < 0.05 |
| PMA-RT-qPCR | < 0.001 | 0.326 |
| $MC_0$ | | |
| RT-qPCR | < 0.001 | - |
| RD-RT-qPCR | n.a.[a] | < 0.05 |
| EMA-RT-qPCR | < 0.001 | < 0.05 |
| RNase-RT-qPCR | n.a.[a] | < 0.05 |
| PMA-RT-qPCR | < 0.001 | < 0.05 |

[a] n.a. - not applicable

The results presented in Table 7.3 demonstrate that thermal treatment resulted in a high degree of inactivation, as measured by RT-qPCR for $MC_0$ (*p*-value < 0.001). The reduction

of infectivity measured by RT-qPCR was shown to be less relevant for EntV ($p > 0.05$). Furthermore, all pre-treatments investigated demonstrated a high impact on the reduction of $MC_0$ infectivity signal as measured by qPCR ($p$-value < 0.05), with a similar result obtained for EntV, apart from RD-RT-qPCR ($p$-value > 0.05). Using viability dyes following the initial degradation of viral genome by inactivation at 95 ºC was not shown to have a significant impact on EntV ($p > 0.05$). A different result was obtained for $MC_0$, where all tested pre-treatments had a significant impact on the reduction of infectivity following the initial reduction by heat treatment.

Similarly, to chlorination and UV inactivation assays, the matrix effect was also evaluated with respect to thermal treatment (Figure 7.3 C and D). Figure 7.3 C and D reveals that generally higher rates of inactivation were once again achieved for the diluted river water. Complete removal of qPCR signal was observed for EntV using all pre-treatments but for $MC_0$ only partial reduction was observed using RD-RT-qPCR. Significant levels of reduction in inactivation were observed in diluted river water compared to undiluted river water heat-inactivated viruses treated with viability dyes ($p$-value < 0.001 and < 0.05). The opposite was found for RNase-RT-qPCR and plaque assay. Here, no significant difference in the reduction in infectivity reduction was observed between undiluted and diluted samples ($p$-value > 0.05).

# CHAPTER 8 : DISCUSSION AND CONCLUSIONS

## 8.1. Discussion

The research presented herein was designed to explore and elucidate the old and new paradigms of monitoring the hygienic quality of water in order to improve our understanding of what constitutes 'microbiologically safe water'. This is the first study to date, that simultaneously addresses the themes of 'index/indicator' organisms in order to (1) understand the fate and removal of four enteric viruses and indicator (index) organisms through full-scale WWTP; (2) apply a novel 'toolbox' approach to Microbial Source Tracking (MST) at a catchment-level in order to study the impact of WWTP and non-human inputs on receiving waters; and (3) attempt to overcome the drawbacks of qPCR in order to more effectively determine risk to public health from specific viral pathogens.

In this final chapter, the strength and weaknesses associated with each of the various organisms (index and indicators) are discussed and their potential suitability for the applications listed above are critically evaluated. The question as to whether GB124 phages, human adenovirus or mitochondrial markers may be used as successful and practical MST markers in river catchments is also debated. Finally, the effectiveness of different qPCR pre-treatments is discussed and improvements to the assessment of human health risks associated with waterborne infectious disease are proposed.

## 8.2. Novel bio-indicators for viruses in wastewater treatment processes

The discharge of partially, or insufficiently treated wastewater is one of the major sources of impairment to environmental waters in Portugal and much of Europe. As a result of their highly infectious nature (low infectious dose), enteric viruses are a problematic issue for public health protection. They are considered to be a major cause of human disease including gastroenteritis, hepatitis, and respiratory illness among others.

In this study, FIB and SC presented concentrations similar in both raw and treated wastewater to those published elsewhere in the scientific literature (Lucena *et al.*, 2004; Harwood *et al.*, 2005; Mandilara *et al.*, 2006; Rusiñol *et al.*, 2015). Enteric virus concentrations in wastewater influent and effluent also reflected the values previously published in other geographical areas (Bofill-Mas *et al.*, 2000; Lodder and Husman 2005; Da Silva *et al.*, 2007; Katayama *et al.*, 2008; McQuaig *et al.*, 2009; Pérez-Sautu *et al.*, 2012; Hewitt *et al.*, 2013; Rusiñol *et al.*, 2015). EC, IE, and SC were detected in 100% of

influent samples and were also present in the highest percentage of samples post treatment (95, 80 and 73%, respectively). This is also in accordance with previously published results, where these organisms were encountered in a higher percentage in raw and treated wastewater (Lucena *et al.*, 2004; Mandilara *et al.*, 2006; Costán-Longares, Mocé-Llivina, *et al.*, 2008).

Although GB124 phage concentrations in raw wastewater analysed in this study were similar to the concentrations at which *B. fragilis* phages are usually found in this type of water, the percentage of positive samples was significantly lower than other previously published studies (66% against 100%) (Tartera and Jofre 1987; Lucena *et al.*, 2004; Mandilara *et al.*, 2006; Purnell *et al.*, 2015). The existence of phages in faeces and wastewaters is highly dependent on the presence in the mammalian gut of *Bacteroides* host strains, which are homogenous in terms of receptors and modification-restriction enzymes resulting in capability of phage replication (Puig *et al.*, 1999; Ebdon *et al.*, 2007, 2012; Wicki *et al.*, 2011; McMinn *et al.*, 2014; Purnell *et al.*, 2015, 2016). Therefore, the diversity of *Bacteroides* strains is the main factor responsible for the variations in the levels of infectivity. *Bacteroides* strains have evolved over time in very well compartimentalised environments and have been segregated according to regional dietary regimens and other factors such as host age and climate (Benno *et al.*, 1989; Moore and Moore 1995; Puig *et al.*, 1999). *B. fragilis* strain GB124 was isolated in the UK and the phages infecting this strain have been shown to be almost exclusively of human origin. The diet in Southern Europe is somewhat different from that of the UK and the climate is generally cooler and wetter, two factors that may influence the different evolutionary paths in *Bacteroides* strains. These facts may explain the lower percentages at which GB124 phages were detected in raw wastewaters in Portugal. Lower levels of GB124 phages have also been reported in Spain (Payan *et al.*, 2005) and Cyprus, whereas levels in the US (McMinn *et al.*, 2014) were more similar to those in the UK.

Seasonal comparison of the quantitative presence of all microbial indicators and human-Norovirus NoVGII revealed that a significant difference between concentration detected in spring and summer (SpS) and autumn and winter (AW) was only observed for IE, HAdV, and JCPyV and that the variance in concentration for the remaining parameters was independent of the season. The results obtained for HAdV are in accordance with the results published by Katayama *et al.* (2008). IE concentration was affected by seasonality, with higher concentrations found during the colder seasons (autumn and winter) and

several factors contributed to this result. Since 1877, at least, sunlight has been a suspected stressor of bacteria (Downes and Blunt, 1877) affecting DNA by direct absorption of UV light or indirectly through the formation of endogenous and exogenous reactive oxygen species. Several studies have shown the bactericidal effect of sunlight on enterococci (Fujioka *et* al., 1981; Davies-Colley *et al*., 1994; Sinton *et al*., 1994, 1999, 2002; Noble *et al*., 2004; Kay *et al*., 2005; Schultz-Fademrecht *et al*., 2008; Byappanahalli *et al*., 2012). Predation is another important factor impacting the concentration of enterococci during the various seasons. Grazing by bacterivorous protozoa, bacteriophage infection and corresponding lysis and predation by other bacteria are among the most relevant biotic effects in controlling the abundance of bacteria in the environment. Protozoan grazing is a crucial control of bacteria populations in aquatic environments, including enterococci. Temperature is a factor that influences significantly the predation rates, with predation increasing exponentially at temperatures ranging from 12 to 22 ºC (Sherr *et al*., 1988). A direct correlation between grazing rates and temperature was found in different environments with more active grazing and an increase in the concentration of protozoa at higher temperatures (McCambridge and McMeekin *et al*., 1980; Anderson *et al*., 1983; Sherr *et al*., 1988; Barcina *et al*., 1991; An *et al*., 2002; Byappanahalli *et al*., 2012) The concentrations found in the current study for NoVGII during SpS and AW months did not differ significantly, although the percentage of negative samples was greater during the SpS months (44% versus 60%). However, the results from this study suggest that the impact of NoV outside of the winter season should not be underestimated. In temperate climates, NoV are usually detected in higher numbers and percentages during colder months (Ottoson *et al.*, 2006; Katayama *et al.*, 2008; Pérez-Sautu *et al.*, 2012; Rusiñol *et al.*, 2015) due to an increase in the number of outbreaks and a preferential circulation of NoV amongst the population during colder times of year. Nonetheless, outbreaks of NoV can occur during the summertime, known as 'off-season outbreaks', with a persistence of NoV gastroenteritis, though at a reduced rate and with the absence of epidemic spread to remote geographical areas often associated with seasonal outbreaks (Lopman *et al.*, 2004; Rohayem, 2009). A possible explanation for sporadic cases of NoV outbreaks in the off-season period may be due to the restricted circulation of the virus in this period, having a long time survival in the population and occasional seasonal reactivation. NoV illness occurs over a period of 24-48h, but shedding in the infected person's faeces can occur up to four weeks after infection and at extremely high numbers (Rohayem, 2009). In addition, asymptomatic shedding of NoV can also occur, potentially at high levels, allowing the

transmission of NoV from asymptomatic to non-infectious individuals (Rohayem, 2009). These facts could potentially account for the results obtained in this study, in which NoV were detected in a lower percentage of samples during the SpS months, but at similar concentrations to the AW months.

Levels of bacterial and phage-based indicators presented a different pattern between raw and treated wastewater with levels in influent much less variable than those detected in treated final effluents. The ratio between the levels of viruses in raw and treated wastewater was mostly stable during the sampling campaign, as shown in Figure 5.2, and presenting no distinct seasonal effect on the reduction of these viruses during wastewater treatment. This suggests that certain enteric viral pathogens (e.g. HAdV, JCPyV and NoVGII) are released in environmental waters with similar seasonal pattern as that from the influent, even after secondary and tertiary wastewater treatment processes, result that differed from those observed observed for FIB and phages. Similar results were obtained by the Katayama and coworkers (2008) that showed that NoVGI, GII and HAdV profiles in effluent did not exhibit pronounced seasonality and were also independent of the WWTP analysed.

The distributions between indicators (FIB and SC) and pathogenic NoVGII present in secondary effluent was found to be significantly different from those observed in the tertiary effluents, indicating distinct removal patterns for the aforementioned indicators and pathogens. The reductions in secondary and tertiary treated effluents for EC, IE, SC, HAdV, and NoVGII were statistically significant ($\rho < 0.05$), whereas the reductions in GB124 phages and JCPyV was not statistically significant ($\rho > 0.05$) with respect to the level of treatment. The reduction in FIB and SC were significantly higher than those observed for GB124 phages and viruses tested. This trend has been observed in previous studies (Costán-Longares *et al.,* 2008; Ebdon *et al.,* 2012; Harwood *et al.,* 2005; Mandilara *et al.,* 2006). These results are important as they suggest that these approaches provide a more conservative indicator of the persistence of viral pathogens than existing FIB and SC.

Importantly, the detection of GB124 phages was made by culture-based methods whereas the detection of HAdV, NoVGII, and JCPyV was conducted using RT-qPCR. Culture-based methods are usually less expensive than molecular techniques and provide real information on potential virus infectivity. The removal levels for GB124 phages in secondary and tertiary treatments were similar, or lower than the ones obtained for viruses. Similar results were published by Purnell *et al.* (2016). It is noteworthy that HAdV were

the organism which presented the highest percentage of positives in tertiary treated effluent samples (50% of tested samples). The log removal levels obtained for NoVGII in secondary and tertiary effluents were similar to those published by Katayama *et al.* (2008) and Pérez-Sautu *et al.* (2012).

The probability plots for log reduction of NoVGII showed no statistical similarity to FIB, which were removed at higher levels. GB124 phages showed consistently lower levels of removal than the NoVGII. The viral indicators (HAdV and JCPyV) displayed closer patterns to NoVGII, as displayed in Figure 5.4 in which it is shown that GB124 phages had a high resistance to removal by WWTP treatments. These results suggest that the proposed indicators (GB124 phages and HAdV) could be useful to go to further studies of other viral pathogens including Norovirus GI, Hepatitis A or Hepatitis E viruses. Costán-Longares *et al.* (2008) found similar results between the probability plots for enteroviruses (EntV) and log reductions of phages. It is important to note that FIB, regularly proposed in the International and national directives and regulations as 'indicators of choice' were removed more readily than phages or pathogens, with no correlation with pathogens throughout the wastewater treatment processes. This occurrence was also observed by other studies (Havelaar *et al.,* 1993; Lucena *et al.,* 2004; Harwood *et al.,* 2005; Costán-Longares, Mocé-Llivina, *et al.,* 2008). The issue of different removal efficiencies gives rise to the need for new indicators which better represent the presence and behaviour of pathogenic enteric viruses. The mean log removals and the probability plots presented here highlight the fact that phages, or certain human viruses appear to be better indicators for predicting the removal of NoVGII in secondary and tertiary effluents, than existing bacterial indicators.

The approaches used to determine the possible index function of indicators have shown that phages, especially GB124 phages, could be better at predicting the $\log_{10}$ removal levels of NoVGII in the final effluent (Spearman's correlation $r = 0.527$; $\rho < 0.01$ and single regression analysis $r = 0.582$; $\rho < 0.05$, for GB124 phages). GB-124 phages are specific to human faecal contamination and so it might be expected to occur simultaneously alongside human-specific NoVGII. However, probably unlike GB-124 phages, the presence of NoV varies amongst the population and depends mainly on the level of infectious within the human population. Therefore, although the discriminant analysis for this function provided the highest prediction levels, with a reasonable ability to

correctly classify the presence and absence of NoVGII in the effluent (66.7%), the result obtained was not statistically significant ($\rho > 0.05$).

All tested microorganisms showed different percentages of 'false positive' and 'negative' results. Nonetheless, testing for the presence/absence of GB124 phages or of HAdV would provide the best approach to determine the presence/absence of NoVGII. However, having a high percentage of 'false negative' results (31%), GB124 phages still offer the best true prediction response at 64%. On the other hand, although having a percentage of true prediction lower than that of GB124 phages (58%), HAdV also provided a relatively lower percentage of 'false negative' results (17%). FIB and SC, albeit having less 'false negative' results, also achieved lower percentages of true prediction.

The presence of 'false positive' results represents "false alarms" in terms of the estimated public health risk, suggesting that removal in WWTP is less effective than it is in reality (and potentially leading to costly unnecessary treatment). Of even greater concern is the presence of 'false negative' result which suggests that waters are safer than they are in reality. An agreement is needed for establishing better regulations regarding wastewater discharge and reclaimed water quality, where a forced choice has to be made between the two situations, or for the minimisation of both errors. In the present study, GB124 phages presented the best $\log_{10}$ removal correlation with NoVGII (albeit having a lower removal efficiency when present).

Based on the data obtained in this study, the use of GB124 phages and HAdV, simultaneously, could be used in this particular geographical region as surrogates for the presence of pathogenic NoVGII and to determine their fate and reduction levels during municipal wastewater treament. GB124 phages correlated at a high level with NoVGII whereas HAdV followed a similar removal pattern to that of NoVGII.

An important issue raised during this part of the study related to the qPCR method used for detecting viral pathogens. Many enteric viruses, most notably noroviruses, are extremely difficult to grow in a cell culture system, making the determination of infectivity very difficult using conventional qPCR (that detects infectious and non-infectious viral particles). Alternative methods to cell culture have to be performed in order to obtain a more accurate estimate of viral infectivity (and hence risk to human health). Alternative methods for improved estimation of viral infectivity using qPCR are discussed below.

### 8.3. Results of molecular and culture-based assays to target human and non-human faecal contamination in the River Tagus, Portugal

The study presented in this thesis was supported by a 13-month sampling campaign to determine the behaviour of the MST markers during the different seasons. This information is lacking in the literature for mitochondrial markers, representing very important gap in current knowledge that is crucial to elucidate in order to determine their viability as MST markers. Sampling points were chosen because of their location relative to the range of potential faecal inputs and at varying distances from the Atlantic Ocean. The sampling campaign not only encompassed seasonal fluctuations, but also investigated the influence of rainfall events, since it has been demonstrated that more than 90% of the flux of faecal contamination from diffuse sources (as determined by faecal indicator counts) can be related to hydrological events (Reischer *et al.*,, 2008). The River Tagus catchment study involved the detection of human, bovine, porcine and poultry faecal contamination using three human-specific markers (GB124 phages, HAdV and HMMit), and a single molecular marker for each remaining source (CWMit, PigMit, PLMit). Santo Domingo *et al.* (2007) set out a series of objectives for the future of MST methodologies. These objectives included high level of specificity and sensitivity for the respective faecal source that should be evaluated before applying a source tracking method in a new area. Therefore, the markers used in this study, GB124 phages, HAdV, and mitochondrial markers were previously tested, by the author, using faecal samples obtained from the same study area (unpublished results). All river water samples were positive for at least one molecular marker, which validates the geographical and temporal stability of the tested markers and demonstrates the strength and sensitivity of the method.

The results of the catchment investigation have indicated that human faecal sources are the dominant source of contamination at the sites studied here. Human markers together were detected in higher frequency (89% of the samples), with HMMit being the MST marker most prevalent and detected at the highest concentration. Faecal contamination of bovine origin also heavily influenced water quality, as supported by the data for non source-specific faecal markers and CWMit. In fact, the mean concentration of HMMit marker and of CWMit were of a similar magnitude. Nonetheless, the other sources were regularly detected, with more than half of the tested samples positive for porcine contamination and about 40% positive for poultry. Mitochondrial markers were in higher concentration than the non source-specific faecal markers. The fact that non-human markers were detected all year around in higher concentrations demonstrates the high potential impact that livestock

farming and poultry production have on water quality within certain parts of the River Tagus.

The non source-specific indicators had a generally marked seasonal pattern, with higher concentrations during the colder months and lower concentrations during the warmer months. This suggests that the decrease in non source-specific indicator concentration is correlated with the increase in summertime daylight, solar radiation and predation, as explained previously in Section 8.2. The results presented in this study are largely in agreement with previously published reports, which have demonstrated higher concentrations of FIB found during colder months (Fattal *et al.*, 1983). However, this pattern was not observed at Site 1 (VFX) where a higher concentration of FIB was detected during the summer months. The difference found in bacterial behaviour between cold and warm months at site 1 (VFX) may result from higher water temperature found in this particular sampling point. Sampling site 1 is not affected by marine waters and therefore shows greater thermal range (e.g. 10 to 24 ºC, compared with 13 to 20 ºC observed at the other sites). Therefore, the higher levels of FIB observed during summer months at this sampling site with higher water temperatures (a pattern not shared by SC, or the other sites), raises the possibility of potential bacterial indicator growth or regrowth at this location. In addition, FIB may be able to persist for longer periods of time in warmer waters. The results obtained for sampling site 1 may suggest that this particular site has a more similar pattern to tropical freshwaters during warmer months where it has been shown the potential for growth and regrowth of FIB and possibility for higher environmental persistence (Carrillo *et al.*, 1985; Jiménez *et al.*, 1989; Winfield and Groisman, 2003).

FIB and SC were also found to be highly affected by UV irradiation and atmospheric temperature ($p$-value < 0.05) and were negatively correlated with water temperature. These parameters have shown to affect the mortality rate of these particular microorganisms (Verstraete and Voets, 1976; Fattal *et al.*, 1983). Salinity influenced the concentration of EC but showed no relevant influence on the concentration of IE. The ability of enterococci to grow in the presence of salt is one of the distinguishing characteristics of the genus, displaying higher salt tolerance than fecal coliforms and EC (Byappanahalli *et al*., 2012). The Tagus river estuary is highly influenced by the entrance of waters from the Atlantic Ocean. Sampling was performed during high tide where the influence of the Atlantic Ocean was at its highest and the salinity levels also peaked (Anon., 2016d) resulting in a

negative correlation between this parameter and the concentration of EC and in no correlation with IE.

Warmer temperatures reduce levels of dissolved oxygen (DO) present in water. The decrease in the levels of oxygen solubility combined with warmer temperatures and lower levels of DO during summer months may stimulate the production of algal blooms, which have been shown to support the growth of FIB, leading also to an increase of death and decomposition rate of organic matter in the river, further promoting bacterial growth (Novotny and Olem, 1994; Byappanahalli *et al.*, 2003; Byappanahalli *et al.,* 2007). This is further supported by the results obtained for SC in this study, which exhibited a similar pattern at all sampling points, with higher concentrations found during the colder months, suggesting little or no capacity to replicate in the environment. A review by Jofre (2009) of the available literature has demonstrated that the contribution of replication in environmental waters of coliphage would have little, or no effect on the overall concentration of SC, a result further corroborated in this study. Furthermore, aquatic sediments are able to harbour substantial populations of faecal coliforms and EC (Pachepsky and Shelton, 2011). A multitude of studies have demonstrated that the concentration of FIB in sediments is many times higher than in the water column (Van Donsel and Geldreich, 1971; Goyal *et al.*, 1977; Erkenbrecher, 1981; Doyle *et al.*, 1992; Crabill *et al.*, 1999; Davies-Colley, 2007). Site 1 contained higher levels of suspended sediments throughout the sampling period, with the possibility of reintroducing FIB. Furthermore, this particular sampling site contained higher levels of suspended sediment throughout the sampling period.

Overall, the molecular MST markers exhibited no particular effect on their concentrations as a result of seasonality, with the exception of the cattle-marker (CWMit), which was detected in higher concentrations during the warmer months. The study catchment in the vicinity of Lisbon and contains a large number of livestock farms (diary and meat). The increase in levels of cattle marker observed during the warmer months is likely to be the result of a number of potential factors. First, cattle numbers are far higher in the catchment during the warmer months (as animals housed indoors over winter are moved outside); secondly, the chance of rainfall (storm events) at this time of year mobilises deposited diffuse faecal material into surface waters; and thirdly low-flow condition mean that inputs have a greater impact on water quality. These findings are in accordance with the findings of other studies, e.g., by Rusiñol *et al.* (2014), who detected contamination of animal origin

at higher concentrations during the summer in a study performed in five different countries (Brazil, Greece, Hungary, Spain and Sweden).

CWMit was influenced by atmospheric temperature and UV-radiation with a positive correlation between CWMit and both these parameters. Interestingly, rainfall appeared to have little or no influence on the concentration of non source-specific markers or the source-specific MST markers. A study by Santiago-Rodriguez *et al.* (2012) has shown that the occurrence of rainfall had no influence on the concentration of thermotolerant bacteria or SC, similar results to those find in this study. However, IE, the poultry marker (PLMit) and human adenovirus (HAdV) were shown to be affected either by the occurrence or levels of rainfall. Similar results were demonstrated in previous studies (Haramoto *et al.*, 2006; Hata *et al.,* 2014; Rodrigues *et al.,* 2015). Studies have concluded that urban runoff as a result of rainfall impacted positively the concentration of IE. A relationship between the IE and rainfall was obtained by the US EPA (2005b) (Ferguson *et al.,* 2005; Jeng *et al.*, 2005). However, this relationship was sometimes difficult to determine. Similarly, a study by Hose *et al.* (2005) concluded that rainfall by itself was unable to predict concentrations of FIB. PLMit showed a statistically significant difference between samples collected on dry or rainy days. Poultry abattoirs are usually crowded with animals, more than swine or cattle abattoirs and therefore wastewater flows from poultry abattoirs are significantly higher compared to swine or cattle abattoirs (Barros *et al*., 2007). Usually, WWTP at abattoirs are relatively small and as a result, during rainfall they may not be able to handle the additional incoming flow and reaching their maximum capacity so that untreated wastewaters may flow directly or through combined sewers into the catchment. Similar results have been found by Barros and co-workers (2007) where samples collected 100 m downstream from the effluent point of poultry abattoirs showed a steep increase in total coliforms, EC, and IE during rainfall. No such result was found when analysing waters collected 100 m downstream from swine abattoirseffluent points. In addition to the weak negative correlations found for IE and SC, a positive correlation was found between the concentration of HAdV and precipitation levels.

Enteric viruses, especially HAdV, are known to be highly resilient in the environment and to environmental stressors, surviving longer in water than enteroviruses and hepatitis A viruses (Enriquez *et al.,* 1995) and this may be one reason why they were found to correlate with rainfall, unlike the remaining parameters. For example, previous studies have shown that HAdV, particularly HAdV40 and 41, are able to survive for longer

periods than FIB in sewage and environmental waters (Enriquez *et al.,* 1995) and are extremely resistant to UV inactivation (Thurston-Enriquez *et al.,* 2003). This enhanced resistance to UV may be associated with the genome of this virus, double-stranded DNA, which when damaged can be repaired by the host cell DNA-repair mechanisms (Bernstein and Bernstein, 1991).

The results from Chapter 5 indicated that the removal of enteric viruses from WWTP in the study catchment is not entirely effective and that they are consequently discharged in significant numbers into receiving environmental waters. Their subsequent survival in the environment has shown to be prolonged by adsorption to sediments (Smith *et al.,* 1978), demonstrated by the fact that they are commonly found in higher concentrations in sediments than in the respective water column (Singer and Fraenkel-Conrat, 1961; Goyal *et al.,* 1979; Liew and Gerba, 1980; Gerba, 2007). The protective effect of sediments may result from various features, including adsorption of enzymes or other substances that would otherwise inactivate viruses, limitation of the formation of virus aggregates, enhanced stability of viral capsid, and even the possibility of diminishing the contact of viruses with virucidal substances (Gerba, 2007).

HMMit, although displaying no clear seasonality, was also influenced by the levels of UV radiation. Although the concentrations of PLMit were shown to be affected by the occurrence of rainfall events, no correlation was found with the amount of precipitation. Surprisingly little information exists on the influence of different environmental physicochemical parameters on the degradation of mitochondrial cells, or on the mitochondrial DNA itself. However, Martellini *et al.* (2005) studied the detection of mitochondrial DNA from human, bovine, and porcine sources in raw wastewater, treated wastewater (following physicochemical treatment) and disinfected effluent (UV radiation). Their findings indicated that after each treatment only human mitochondrial DNA was amplified. This may indicate that human and animal mitochondrial cells behave differently in the presence of particulate matter and sediments not only during wastewater treatment, with animal cells being more prone and attracted to sediments during physicochemical processes, but also in environmental waters where these cells may attach to river sediments that would therefore help to protect animal mitochondrial cells and mitochondrial DNA from inactivation and degradation. However, further research is necessary to corroborate this hypothesis.

In order to close the gap between general indicators of faecal pollution and source-specific (MST) markers, this study correlated quantitative MST data with standard water quality parameters during both the entire sampling campaign and also during rainfall events. Only HAdV, PigMit and PLMit demonstrated statistically significant (albeit weak) correlations with EC and IE. PigMit was weakly correlated to EC and IE during the entire sampling campaign but showed a moderate correlation with IE during rainfall events. Although not statistically significant, this marker also showed the highest correlation with EC and IE during rainfall events.

In urban areas, domestic pets may also represent a significant source of input of faecal contamination to environmental waters (Ram *et al.,* 2007; Ahmed *et al.,* 2008b; Schriewer *et al.,* 2010; Sinigalliano *et al.,* 2010). Dog faecal matter, especially, can be a considerable source of contamination as a result of a high canine population density in urban areas and their high defecation rate (Murray *et al.,* 1997; Perrin 2009; Wright *et al.,* 2009). When domestic pet faeces are not discarded properly, they can be transported directly by run-off into nearby water bodies or to storm sewers that may be linked to streams, rivers or lakes impairing water quality. Another important factor relates to the fact that dogs are often present close to recreational water, including beaches with a high risk of decreasing recreational water quality (Shibata *et al.,* 2010; Wang *et al.,* 2010; Wright *et al.,* 2009).

The dog and cat mitochondrial DNA markers developed and applied in the study catchment exhibited 100% of specificity and sensitivity. The limit of detection for nested-PCR varied between 0.01 pg for DogMito and 1 pg for CatMito. Martellini *et al.* (2005) reported similar results when analysing nested-PCR for human, cow, pig, and ovine markers. The differences found in sensitivity may be due to differences in the number of mitochondria excreted interspecies and also from the tissue analysed. DogMito and CatMito were detected occasionally and only during or immediately after rainfall events, suggesting urban run-off as an important source of contamination.

Urban run-off is an increasingly important issue for those responsible for managing and protecting surface waters used for recreation, aquaculture and drinking water abstraction, etc. With the intensity and frequency of rainfall events expected to increase in coming years due to climate change and increased usage of impervious surfaces, it is an issue that will require further research. Although single sources of non-point contamination are generally small, the cumulative impact may be significant mainly because most sources do not undergo any form of treatment before entering surface waters. Therefore, the results

from this study demonstrate that these markers are capable of identifying areas within the River Tagus catchment most at risk from contamination from domestic animals.

This study has also demonstrated the moderate capability of MST markers, in this particular case PigMit marker, to link source-specific markers concentrations of non-source specific markers. This should help facilitate the estimation of the contribution from particular sources to the total load of faecal contamination assessed by conventional faecal indicators. Interestingly, the best quantitative correlation was found between PigMit and IE. IE are often found in higher numbers in contamination from non-human animal sources, while EC tend to be found in higher numbers in human sources of contamiantion.

Interestingly, the correlation coefficients between PigMit and standard bacterial indicators of faecal contamination, especially IE; were generally higher during rainfall events. This result indicates that although PigMit and IE have different persistence times in environmental waters this does not appear to adversely influence the relationship between these two aprameters during rainfall events (i.e., periods of low hydrological retention). In periods with greater retention times, the difference in the resiliance to environmental stressors plays a much more important role,which could help explain the lower correlations in such samples.

Reischer *et al.* (2008) reported a similar result while sampling during rainfall events in Austria where the ruminant marker BacR levels correlated well with EC, especially during event sampling. Nonetheless, it is important to emphasise that most source-specific markers were not correlated to the non source-specific markers. The correlation obtained between PigMit and IE is even more interesting if looking into with regard to the revised recreational water quality criteria published by US EPA (2012a) for IE, that aims to keep the risk of gastrointestinal illness (GI) in swimmers below 30 illnesses per 1000 swimmers. IE and EC, quantified by culture methods, have been associated to GI illness after exposure to recreational water (Cabelli *et al.,* 1982; Cabelli 1983; Calderon *et al.,* 1991; Dufour, 1984; Wade *et al.,* 2003, 2006, 2008, 2010; Wiedenmann *et al.,* 2006; Marion *et al.,* 2010). Additionally, a qPCR method for IE designed by US EPA 2012b showed a statistically significant correlation with GI illness in marine and fresh recreational waters exposed to faecal contamination (Wade *et al.,* 2006, 2008, 2010).

Nonetheless, most MST markers tested in this study showed no correlation to non source-specific markers. The employment of non-source specific markers of faecal contamination as indicators of human health risk is based on multiple assumptions, including that the

presence and distribution of FIB vary in the same manner as pathogens; however, numerous studies have reported that FIB do not correlate adequately with pathogens (Lund, 1996; Bonadonna, 2002; Lemarchand and Lebaron 2003; Anderson *et al.*, 2005; Harwood *et al.,* 2005). FIB differ physiologically and phylogenically from pathogens, that include not only bacteria but also viruses and protozoa, justifying largely the lack of correlation. Moreover, epidemiological studies have also failed to correlate risks to human health and FIB, mainly when the contamination is from a non-point source (Dwight *et al.*, 2004; Colford *et al.,* 2007). This may, in part, be attributed to the fact that whilst EC and IE are able to infect different warm-blooded animals, many pathogens are limited to human hosts, especially enteric viruses (Harwood *et al.,* 1999; Souza *et al.,* 1999; Leclerc *et al.*, 2001). A high concentration of FIB, arising as a result of the presence of multiple sources of faecal contamination and where sewage from human origin has a residual impact, results generally in a decreased risk to human health (Ferguson *et al.,* 1996; Wade *et al.,* 2008). Additionally, several strains of FIB have been shown to be able to become saprophytes, persisting in many habitats, including soils, aquatic sediments, and vegetation (Solo-Gabriele *et al.,* 2000; Byappanahalli *et al.,* 2003; Byappanahalli *et al.,* 2007; Topp *et al.,* 2003; Jeng *et al.,* 2005; Ishii *et al.,* 2006; Ksoll *et al.,* 2007; Badgley *et al.,* 2011). The presence of FIB in most warm-blooded animals and in the environment hinders their use as surrogates for pathogens and also impairs the identification of sources of pollution.

This study demonstrated that the 13-month sampling campaign has contributed to an improved understanding of the effect of seasonality on MST dynamics within the study catchment and the in-depth investigation of the catchment and rainfall event sampling is evidently extremely important for an effective quantitative MST study. Moreover, the parametric analysis of rainfall events allowed for the quantitative assignment of the faecal contamination component of porcine origin. It must be emphasised here that the potential dominant sources of faecal contamination in study sites on the River Tagus were either of human or cattle origin.

Whilst it is clear that cattle, pig and poultry rearing are common at certain points within the study catchment, the prevalence and concentration of non-human markers during the sampling campaign highlight the potential impact that these non-point sources can have on water quality within the River Tagus catchment. Although human sources are a major contributor to contamination within the R. Tagus (and as such represent a high risk to public health), faecal contamination from a range of non-human sources may also present

considerable potential risks to public health. For example, several zoonotic pathogens are able to infect a range of different warm-blooded mammals (including humans), such as *Cryptosporidium* and *Giardia*, and Hepatitis E viruses, the latter of which are found in pigs. Therefore, the application of a toolbox of quantitative MST markers in a similar format as the approach used in this study should allow the identification and assignment of contaminant sources in complex catchments with multiple inputs of pollution.

### 8.4. Novel pretreatment methods for identifying infectious viruses by RT-qPCR

The maintenance of a viruses infectious capacity is dependent on the integrity of the capsid and of the genome (Nuanualsuwan and Cliver 2003; Wigginton and Kohn 2012). Regardless of advances in recent years, the 'gold standard' method to determine virus infectivity is still cell culture. However, cell culture systems remain expensive, as a result of the need for highly specialised personnel and equipment. The vast majority of cell cultures systems are fastidious and the viruses are difficult to visualise. Furthermore, several viruses still have no cell culture system readily available (e.g., Norovirus). Therefore, the method of choice for the detection of enteric viruses is qPCR. Although qPCR still requires specialised personnel, the costs are considerably lower and the technique can provide results on the same day of the sampling campaign. It is highly sensitive and specific, as several qPCR protocols have been developed for the detection of different parts of the genome of enteric viruses. In spite of the recent developments in this area, a major drawback associated with qPCR, is that it simultaneously detects both infectious and non-infectious viral particles. Therefore, this study set out to assess the suitability and efficacy of enzymatic and viability dyes treatment to determine virus infectivity through RT-qPCR. To the author's knowledge, this is the very first such study to compare fully both types of pre-treatment against one another. Heat-, UV- and chlorine-inactivated EntV were rendered non-infectious, yielding a > 5 log inactivation as measured by plaque assay. Chlorine is extensively used in the disinfection of drinking water processes and in the water distribution network. RT-qPCR by itself produced 'false positive' results for both viruses. Little to no reduction in the signal was obtained after treatment with chlorine and UV as measured by RT-qPCR solely. Similar results have been previously published (Karim *et al.,* 2015; Leifels *et al.,* 2015; Fongaro *et al.,* 2016). Partial degradation of viral RNA was obtained using by RT-qPCR following heat treatment. River

water used to spike EntV and $MC_0$ contained a high degree of microflora, particulate matter and humic substances.

A 95 ºC heat treatment for 10 min, as applied in this study, will result in the total disruption of the virus capsid and the release of viral RNA into the sample. In this study, the detection by RT-qPCR from thermal-inactivated viruses has conducted already to a reduction in the PCR signal (1.6 to 3.0 logs for EntV and $MC_0$, respectively). Extracellular nucleic acids may be chemically modified, degraded by nucleases, sheared, or stabilised by linkage to mineral surfaces and humic acids ( Maeda and Taga, 1973, 1974; Bazelyan and Ayzatullin, 1979; Lorenz *et al.*, 1981; Lorenz and Wackernagel, 1987; Ahrenholtz *et al.*, 1994; Ruiz *et al.*, 2000; Nielsen *et al.*, 2007). A study conducted by (Tenhunen, 1989) concluded that cRNA was partially degraded after 6 h and completely degraded at 60 ºC following overnight incubation. Moreover, at lower temperatures the cRNA remained intact although being incubated overnight, but at higher temperatures, such as 80 ºC, the degradation occurred in less than 2 h of incubation and therefore exposure to high temperatures may lead to the inactivation of RNA.

Comparisons between the thermal inactivation of RNA observed during this study with previously published literature are difficult because most studies were performed at lower temperatures and usually in buffered solutions that will provide further protection to extracellular RNA. Therefore, the difference observed for heat-treated EntV and MC0 RT-qPCR may result from the different levels of resistance that the viruses may present to heat inactivation. It has previously been shown that even within the same genus, certain viruses can be more resistant to inactivation than others. For instance, CV-B5 was more resistant than echovirus 1 (E1) (Kahler *et al.*, 2010). Additionally, CV-B5 was also one of the most resistant out of the 20 enterovirus, adenoviruses and reoviruses studied in Potomac estuarine water followed chlorine disinfection (Kahler *et al.*, 2010). Since differences are found between virus resistance, the level of degradation of viral capsid and, in this particular case, viral genome may also vary. This may result in smaller RNA fragments that are then not detected by RT-qPCR solely, accompanied therefore by a greater loss of the signal.

The results of dye treatment of UV- and chlorine- inactivated viruses showed that the effect of intercalating dyes is only partial, leading to the overestimation of viral persistence. A noticeable difference was found between the infectivity assay and RD/EMA/PMA-RT-qPCR for both inactivation treatments. The difference was even more

significant for the UV-treated EntV. Dye-RT-qPCR was positive for EntV whereas no infectivity was detected. Additionally, a very strong positive signal was also detected for $MC_0$.

On the other hand, RNase-RT-qPCR of chlorine- and UV-inactivated EntV were mostly in agreement with the results obtained by cell culture. The RNase-RT-qPCR signal was completely removed for EntV after chlorine-inactivation and also following 30 and 60-sec of UVC contact. The specificity of the matrix had probably an impact on the difference observed between the different UV contact times. The penetration of the UV light was probably less effective for the longer contact times and viruses were therefore more protected from inactivation. The removal of qPCR signal by RNase was less effective for chlorine- and UV-inactivated $MC_0$ although still producing a higher degree of qPCR signal reduction. The results for chlorine-inactivated viruses have shown that an alteration in the capsid conformation occurrs that facilitates the contact with RNase and also partially with the dyes of the inactivated viruses. Using RNase ONE enzyme, Nowak *et al.* (2011) measured the degradation of the viral capsid following chlorine treatment and demonstrated a good agreement between reduction in RNase-RT-qPCR signals and loss of infectivity for feline calicivirus (FCV-F9). The results of dye-treated-UV-inactivated viruses are in agreement with results found previously (Karim *et al.*, 2015; Leifels *et al.*, 2015).

However, slight differences have been found for chlorine-inactivated viruses. Karim *et al.* (2015) reported that PMA-RT-qPCR was able to differentiate between infectious and chlorine inactivated viruses when using a high dose of chlorine, a result similar to that obtained by Leifels *et al.* (2015). However, when low doses of chlorine were used, PMA-RT-qPCR was unable to differentiate between infectious and non-infectious viral particles. When a similar dosage of chlorine was applied to the one used in this study, PMA-RT-qPCR was only able to partially differentiate between infectious and non-infectious murine norovirus (MNV) (Karim *et al.*, 2015). Karim *et al.* (2015) also determined that no loss of infectivity was apparent on chlorine-inactivated NoV. For EntV the results improved when considering the diluted river sample, although the removal of the signal was weak. In accordance, the results obtained following thermal inactivation demonstrated a better performance by pre-treatment-RT-qPCR.

Similar studies performed previously on various NoVGII.4 strains in diluted (1% v/v) stool suspensions have shown an apparent higher resistance to both heat and chlorine treatment

when compared to FCV-F9 (Topping *et al.,* 2009; Nowak *et al.,* 2011). However, conducting the same experiments on diluted stools spiked with FCV-F9 exhibited a similar pattern. This is indicative that protection by the natural faecal matter on the NoV capsid increased resistance (Topping *et al.,* 2009; Nowak *et al.,* 2011). The complex matrix used in this study could also represent a challenge to the use of pre-treatment dyes. These dyes rely on photo-activation to cross-link covalently to the nucleic acid. If the photo-activation is not perfect then the dyes will not link to the nucleic acid and the RT-qPCR signal is not blocked as a result. With such a difficult matrix, the photo-activation was less than ideal and this is shown by the diluted sample that displayed higher degrees of RT-qPCR signal loss for chlorine- and heat-treated viruses, following dye pre-treatment. For UV-treated samples, the difference between the sample and its dilution were similar, which indicates that the main effect of UV is on the genome and that a small effect is on the capsid which favoured the entrance of RNase, detrimental to the entrance of the dyes.

Other factors can also affect the performance of the pre-treatments and may help further explain the difference found between pre-treatment with RNase and pre-treatment with dyes after inactivation by UV and chlorine. The low level of agreement between the results of infectivity assays and intercalating dyes RT-qPCR, in particular, may result also from different levels of susceptibility of viral genome to chlorine and UV radiation. Previous studies have analysed the influence of chlorine on rotavirus infectivity and corresponding genome integrity and have described that the genome has higher resistance to chlorine than infectivity assay (Li *et al.,* 2011; Xue *et al.,* 2013). Additionally, studies on the inactivation of common picornavirus by chlorine showed that the 5' noncoding region may be highly vulnerable to chlorine treatment (Li *et al.,* 2002). Previous studies have also highlighted the resistance of small genome fragments, with a 196-base fragment in the 5' non coding region of poliovirus 1 (PV1) and poliovirus 2 (PV2) and a 149-base fragment of PV1 in the same region being constantly detected by qualitative PCR even after high fluences (Moore and Margolin 1994; Sobsey *et al.,* 1998; Lewis *et al.,* 2000). Several studies on encapsidated PV1 have described that longer RNA fragments with over > 800 bases were at least as sensitive or even more sensitive to UV or oxidation treatment than shorter fragments with less than 145 bases (Sobsey *et al.,* 1998; Shin *et al.,* 2003; Simonet and Gantzer 2006a, b).

Therefore, in addition to differences in the sensitivity to UV and chlorine degradation, the size and location of the targeted amplicon is also highly relevant. In this study, both viruses

were detected using primers that amplified a region on the 5' noncoding region (Monpoeho *et al.,* 2000; Costafreda *et al.,* 2006). The region chosen to detect EntV is actually situated between the 196-base fragment described previously. Therefore, and although damage was made to the genome of both viruses, the destruction of small RNA fragments is not efficient, which implies the detection of both viruses by RT-qPCR. Moreover, the linkage of dyes to the genome is dependent also on the region (higher affinity of dyes for certain regions) and also the size (Coudray-Meunier *et al.,* 2013). Usually qPCR assays target short nucleic acid sequences in order to guarantee the highest efficiency and also to allow the use of probe-based qPCR methods (i.e., TaqMan).

A study using different amplicon sizes for the discrimination of heat-killed *Salmonella* and *Campilobacter* determined that targeting a sequence of less than 200 bp and applying PMA-qPCR resulted in a signal reduction of 1-3 log (Jahromi, 2013). However, an increase of the target sequence to 899 bp was able to greatly reduce the PMA-qPCR signal from dead *Campilobacter jejuni* cells (4.2 log) although not removing fully the dead cell signal. However, PMA-PCR targeting a sequence with more than 1500 bp in *Salmonella* and *Campylobacter* completely removed the dead cell signal yielding a 7 log reduction in the PCR signal. However, the comparison of results from varied studies is extremely difficult due to different dye incubation conditions and type of RNase used, including incubation time and temperature. Moreover, a vast range of lights are used to photoactivate the dyes and the differences in the exposure time and procedure may have a high influence on the photoactivation yield, especially where different lamps result in different efficiencies of light activation, possibly as a result of distinct wavelength emission.

Overall, the data obtained in this study showed the potential of the different disinfection treatments to fully inactivate EntV and $MC_0$ under a very complex and turbid matrix as measured by infectivity assays. The RT-qPCR assay by itself produced 'false positive' results resulting in an overestimation of virus infectivity. RT-qPCR following heat treatment resulted in a higher decrease in the PCR signal especially for $MC_0$. Other promising results involved the potential of RNase to be used with molecular techniques for the successful discrimination of infectious and non-infectious viruses following UV-, chlorine-, and heat-inactivation for the tested viruses. Conversely, the findings of this study suggest that the use of photoactivated dyes is not recommended when using turbid matrices with little to no RT-qPCR signal loss. Moreover, viruses present different susceptibilities to inactivation treatments, so every assay should ideally be optimised for each virus in turn.

## 8.5. Conclusions

The main findings of this research are:

1. Existing faecal indicator bacteria (EC and IE) fail to adequately predict the fate of enteric viruses such as human-specific NoVGII during wastewater treatment. The removal levels of these indicators were greater than of NoVGII after secondary and tertiary treatment, and they could not help predict the presence/absence of NoVGII. They therefore fail in their role as conservative indicators for NoVGII. Interestingly, whilst IE, HAdV, and JCPyV all exhibited seasonal fluctuations in their levels in untreated wastewater, NoVGII did not.
2. Human-specific GB-124 phages were found to the most resistant to wastewater treatment and positively correlated with NoVGII (in terms of their removal efficiency). However, several influent samples were negative for this particular phage. HAdV also demonstrated a moderate correlation with NoVGII and presented similar patterns of removal. Moreover, they were always present in higher levels in influent and the combination of GB-124 phages with HAdV allowed for a high level of prediction for the presence/absence of NoVGII. Consequently, combining GB-124 phages and HAdV would provide the best estimate for the fate of NoVGII during wastewater treatment.
3. Results from the 12-month catchment study indicated that high levels of faecal contamination still exist in several parts of the R. Tagus despite improvements to wastewater treatment. The MST markers revealed that the study sites were highly impacted by human and also bovine contamination. Furthermore, porcine and poultry sources were also commonly detected. However, GB-124 phages were not shown to be a good marker for MST in this particular catchment.
4. Precipitation was also shown to influence the concentration of IE, SC, and PLMit and also revealed that urban run-off is an important contributor of inputs from domestic animal markers. A significant input of faecal contamination during rainfall events could also be attributed to the presence of porcine contamination, as observed by the correlation between this marker, EC and IE. This observation demonstrates the applicability of quantitative MST based on the detection of mitochondrial DNA and confirms the importance of including hydrological and environmental data in future source tracking investigations.

5. Environmental conditions (UV radiation, atmospheric and water temperature and salinity) affected mostly non source-specific indicators and source-specific markers showed little or no correlation with environmental conditions.

6. Little or no correlation was found between non-specific markers of faecal contamination and chosen MST markers, highlighting that the former cannot be used to assess the sources of faecal contamination.

7. The results for virus infectivity through qPCR following different disinfection treatments showed that EntV were completely inactivated when measured by plaque assay. However, using RT-qPCR revealed little or no accompanying reduction in the strength of signal. The use of water with high levels of particulate matter also influenced the ability of the tested pre-treatments to effectively remove false-qPCR signals. Therefore, solely detecting genome fragments following disinfection procedures is unable to indicate the presence of infectious viral particles, and many factors appear to hinder the application of pre-treatments prior to RT-qPCR. However, although pre-treatment with RNase did not allow for the total discrimination between infectious and non-infectious viruses, the levels of qPCR signal removal obtained using this technique provided good indications for further studies. Moreover, this study shows that different viruses have different susceptibilities to inactivation treatments and are affected in different ways.

### 8.6. Recommendations for further research

Further research is needed to:

1. Integrate *B. fragilis* bacteriophages and human adenovirus to further verify whether these new bio-indicators could be useful tools to determine the fate and persistence of other enteric viruses of interest such as Rotavirus, Hepatitis A virus, Hepatitis E virus, NoVGI, and Enterovirus within Portuguese surface waters and elewhere.

2. Isolate other human-specific *B. fragilis* hosts that could be used more widely to determine efficacy of viral removal in wastewater processes and also in future MST studies.

3. Ensure that important information gleaned from this study is fed back to the Portuguese Water and Environmental Regulators, in order to protect public health and the environmental resources in the region more effectively.

4. Expand the sampling approach adopted in this study in order to further elucidate the major stresses on water quality in the River Tagus and particularly around the densely populated greater Lisbon area. This information could then be used to inform mitigation and management strategies within the catchment. This might provide evidence of resulting improvements in water quality through the creation of a multitask network with the Portuguese water regulator, environmental and health agencies. As a result, measures could be taken to engage the population in the different regions of Portugal to apply better remediation measures and to sensitise the populations and stakeholder to promote safe and clean water policies.

5. Further explore the persistence of mitochondrial MST markers in the environment, as this will be crucial to further determine their applicability as novel tools for water quality monitoring.

6. Further explore the suitability of other pre-treatments and approaches for establishing the infectivity of enteric viral pathogens, including long fragment qPCR coupled (both with or without pre-treatments), or integrated cell culture-PCR (ICC-PCR), where cell lines are available. Such research should also focus on testing waters from different sources and origins with distinct levels of suspended solids would allow for a better and more effective screening of the pre-treatment approaches.

7. Test the infectivity of a range of bacteria, protozoa and other viruses, such as NoV, HAdV, HAV, HEV, RoV, Aichi viruses, since each presents a different genome and capsid organization that influences not only the disinfection treatments but also the usage of the different pre-treatments. The knowledge provided by this and the previous point would help optimise the performance of qPCR and ensure that it could not only be applied to a range of different organisms and matrices, but that the issue host cell viability faced by molecular techniques could be overcome.

8. Explore the application of newly emerging chip-based techniques for molecular detection, involving the direct quantification of nucleic acid through partitioning of the sample mixture into individual wells. Such an approach has the potential to lower the costs associated with quantification of a given organisms substantially as it removes the need to rely on standard curves.

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## Appendices

Supporting data.

**Appendix 1: Materials and Methods**

**Appendix 1.A** – Bacteriophages culture media

A.1 Somatic coliphages media

A.1.1 Modified Scholtens' Broth

| Reagent | |
|---|---|
| Peptone | 10 g |
| Yeast extract | 3 g |
| Meat extract | 12 g |
| NaCl | 3 g |
| $Na_2CO_3$ solution (150 g/L) | 5 mL |
| $MgCl_2$ solution (100 g of $MgCl_2.6H_2O$ in 50 mL water | 0.3 mL |
| Distilled water | 1 000 mL |

Preparation of broth: the ingredients were dissolved in hot water and the pH was adjusted to 7.2 ± 0.2 at 45 (± 3) ºC so that after sterilization it was 7.2 ± 0.2. The medium was distributed in bottles in volumes of 200 mL and sterilized in the autoclave at 121 (± 3) ºC for 15 min.

A.1.2 Modified Scholtens' Agar (MSA)

Basal medium

| Reagent | |
|---|---|
| Peptone | 10 g |
| Yeast extract | 3 g |
| Meat extract | 12 g |
| NaCl | 3 g |
| Agar | 20 g |
| $Na_2CO_3$ solution (150 g/L) | 5 mL |
| $MgCl_2$ solution (100 g of $MgCl_2.6H_2O$ in 50 mL water | 0.3 mL |
| Distilled water | 1 000 mL |

The ingredients were dissolved in boiling water and the pH adjusted to 7.2 ± 0.2 at 55 (± 3) ºC so that after sterilization it was 7.2 ± 0.2. The medium was distributed in bottles in volumes of 200 mL and sterilized in the autoclave at 121 (± 3) ºC for 15 min.

Calcium chloride solution ($c$ = 1 mol/L)

| Reagent | |
|---|---|
| $CaCl_2.2H_2O$ | 14.6 g |
| Distilled water | 100 mL |

Calcium chloride was dissolved in the distilled water while heating gently. After cooling to room temperature, the colution was filter-sterilized through a membrane filter of 0.2 µm.

Complete medium

| | |
|---|---|
| Basal medium | 200 mL |
| Calcium chloride solution | 1.2 mL |

The basal medium was dissolved and cooled between 45 and 50 ºC. The solution of calcium chloride was added aseptically, mixed well and poured into Petri dishes.

A.1.3 Semi-solid Modified Scholtens' Agar (ssMSA)

Basal medium

Basal medium was prepared according to A.1.2 using half the mass of agar (10 g).

A.2 *Bacteroides* GB-124 phages media

A.2.1 *Bacteroides* phage recovery medium broth (BPRMB)

Basal broth

| Reagent | |
|---|---|
| Meat peptone | 10 g |
| Casein peptone | 10 g |
| Yeast extract | 2 g |
| NaCl | 5 g |
| Monohydrated L-cysteine | 0.5 g |
| Glucose | 1.8 g |
| $MgSO_4.7H_2O$ | 0.12 g |
| $CaCl_2$ solution (0.05 g/mL) | 1 mL |
| Distilled water | 1 000 mL |

The ingredients were dissolved in hot water. The solution of $CaCl_2$ was added and the medium was mixed well and distributed in bottles of 200 mL followed by sterilization in the autoclave at 121 (± 3) ºC for 15 min.

Haemin solution

| Reagent | |
|---|---|
| Haemin | 0.1 g |
| NaOH solution (1 mol/L) | 0.5 mL |
| Distilled water | 1 000 mL |

The ingredients were dissolved in the water and the solution was filter-sterilized through a 0.2 µm pore size membrane filter.

Disodium carbonate solution (< 1 mol/L)

| Reagent | |
|---|---|
| $Na_2CO_3$ | 10.6 g |
| Distilled water | 100 mL |

Disodium carbonate was dissolved in water and filter-sterilized through a 0.2 µm pore size membrane filter.

Complete medium

| Basal broth | 1 000 mL |
|---|---|
| Haemin solution | 10 mL |
| Disodium carbonate solution | 25 mL |

The solutions were added aseptically to the basal broth and mixed gently (to avoid oxygenation of the medium). The pH was adjusted to 6.8 ± 0.5.

A.2.2 *Bacteroides* phage recovery medium agar (BPRMA)

Basal agar

| Basal medium | 1 000 mL |
|---|---|
| Agar | 20 g |

The basal broth was mixed with agar while heating and distributed into bottles, in volumes of 200 mL, and sterilized in the autoclave at 121 (± 3) ºC for 15 min. the medium was cooled to between 45 and 50 ºC for the addition of the additives.

Complete agar

| | |
|---|---|
| Basal agar | 1 000 mL |
| Haemin solution | 10 mL |
| Disodium carbonate solution | 25 mL |

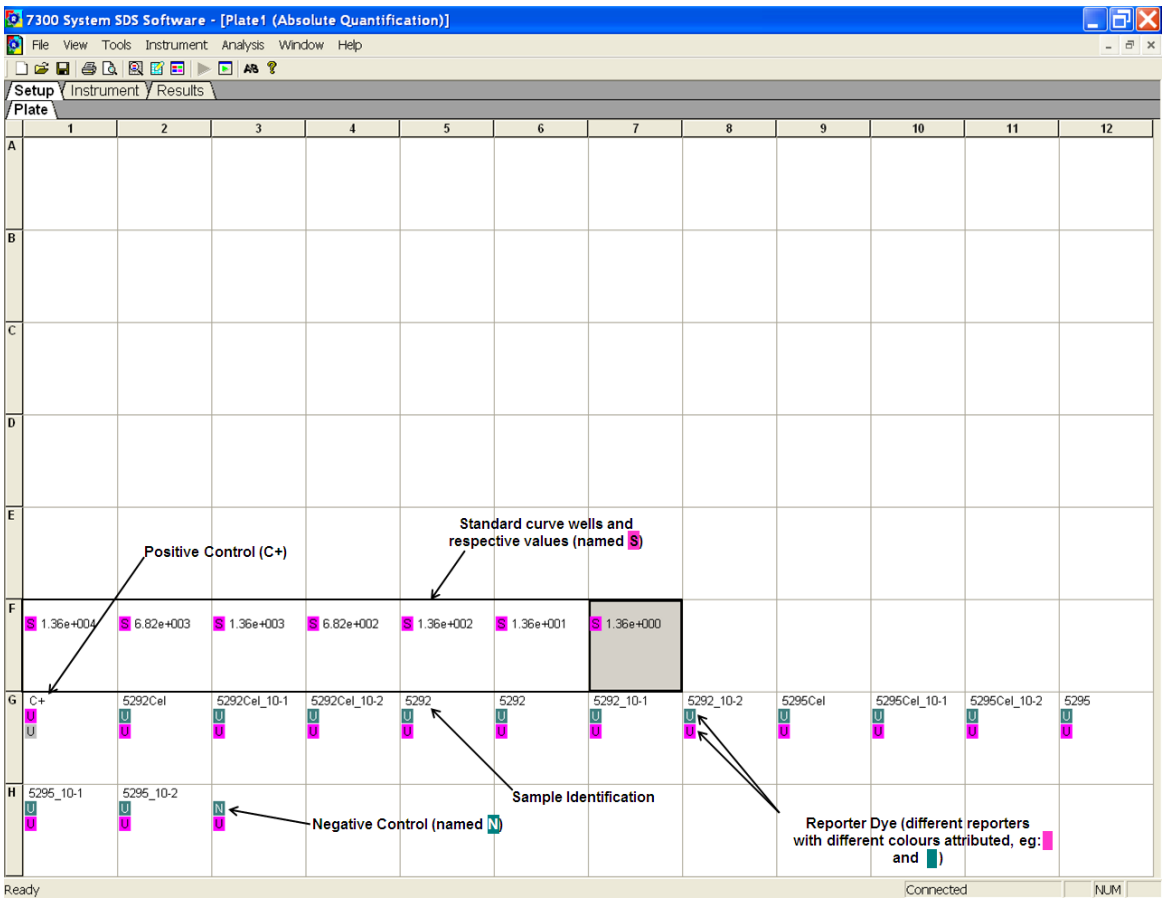
The solutions were added aseptically to the basal broth and mixed gently (to avoid oxygenation of the medium). The pH was adjusted to 6.8 ± 0.5.

A.2.3 Semi-solid *Bacteroides* phage recovery medium agar (ssBPRMA)

The basal medium was prepared according to A.2.2 but using half of the mass of the agar (10 g). Before usage, the ssBPRMA was melted and then cooled to a temperature between 45 and 50 ºC, and aseptically the solutions of haemin and Na2CO3 were added and the pH adjusted to 6.8 ± 0.5.

**Appendix 1.B -** Thermal cycler program conditions for NoV RNA reverse transcription

| | **Step 1** | **Step 2** | **Step 3** | **Step 4** |
|---|---|---|---|---|
| Temperature (ºC) | 25 | 37 | 85 | 4 |
| Time (min) | 10 | 120 | 5 | ∞ |



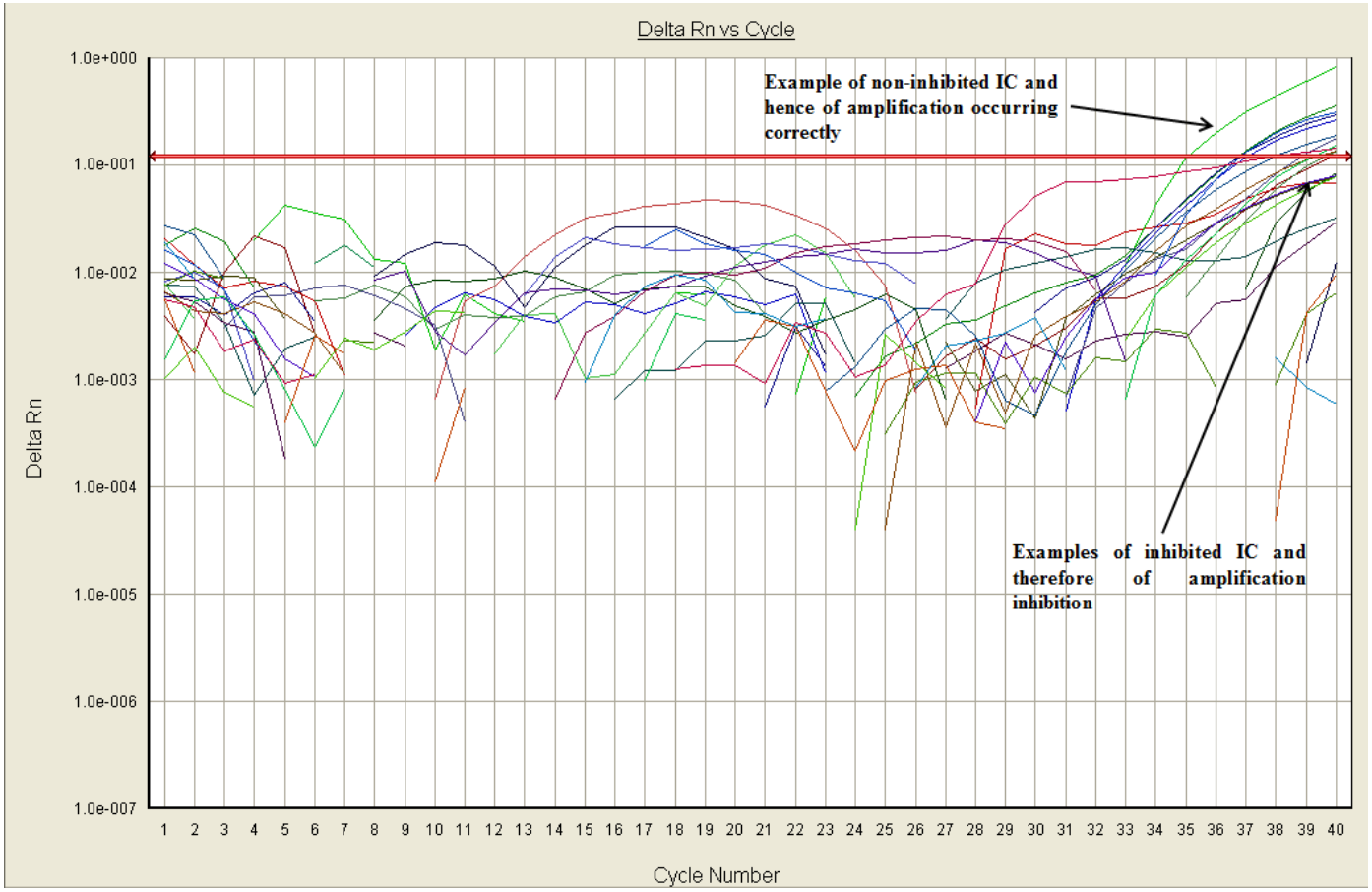

**Appendix 1.C** - Example of a design assay plate of real time PCR (Images: Author's own)

**Appendix 1.D** - Concentration of the primers and probes used for the detection of HAdV, JCPyV and NoV

| Oligonucleotide | Concentration (µM) |
|---|---|
| HAdV_F | 0.9 |
| HAdV_R | 0.9 |
| Ad:ACDEF | 0.225 |
| Ad:B | 0.225 |
| | |
| JE3_F | 0.5 |
| JE3_R | 0.5 |
| JE3_P | 0.15 |
| | |
| 1380-F | 0.8 |
| 1319-R | 0.8 |
| 1379-P | 0.2 |

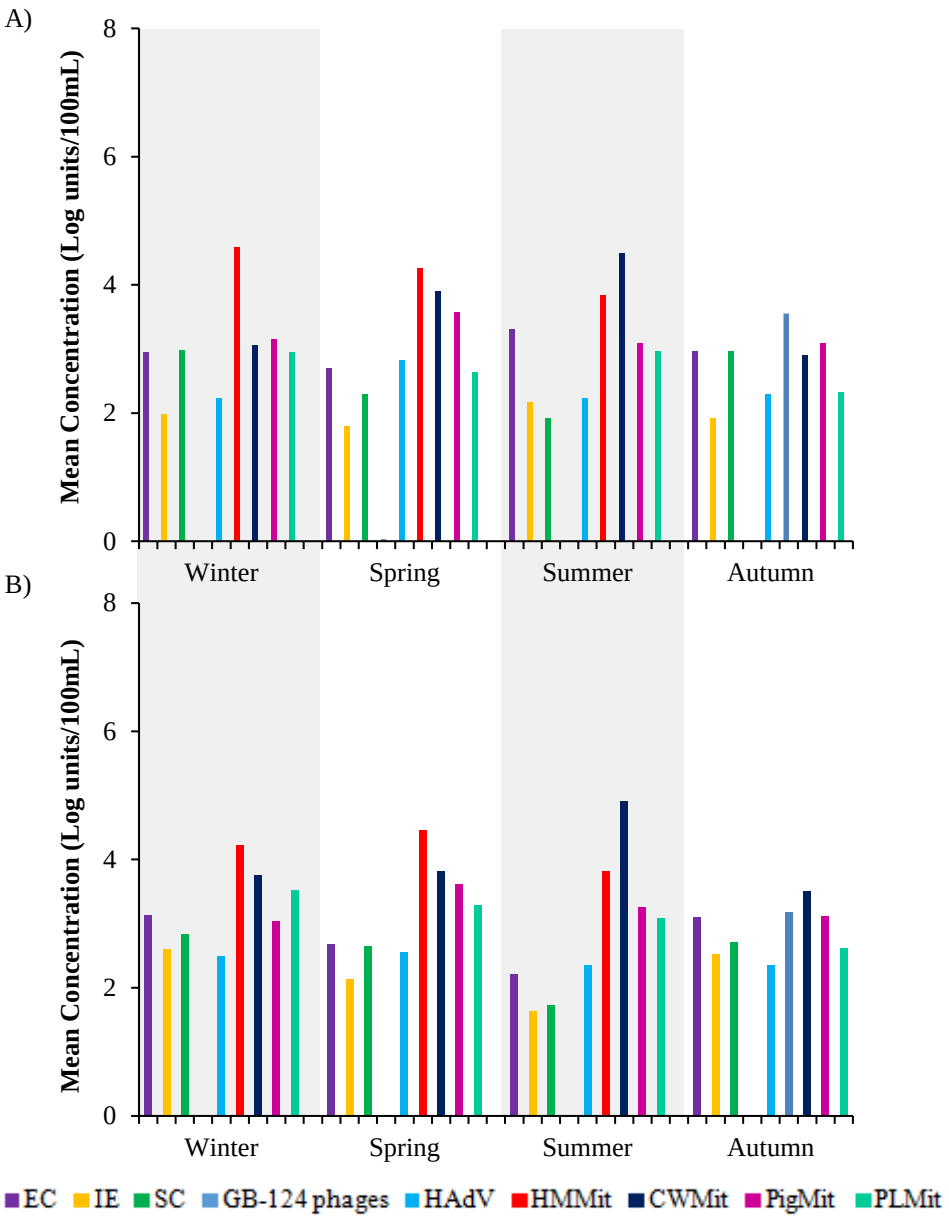
**Appendix 1.E -** Cycling rt-qPCR conditions

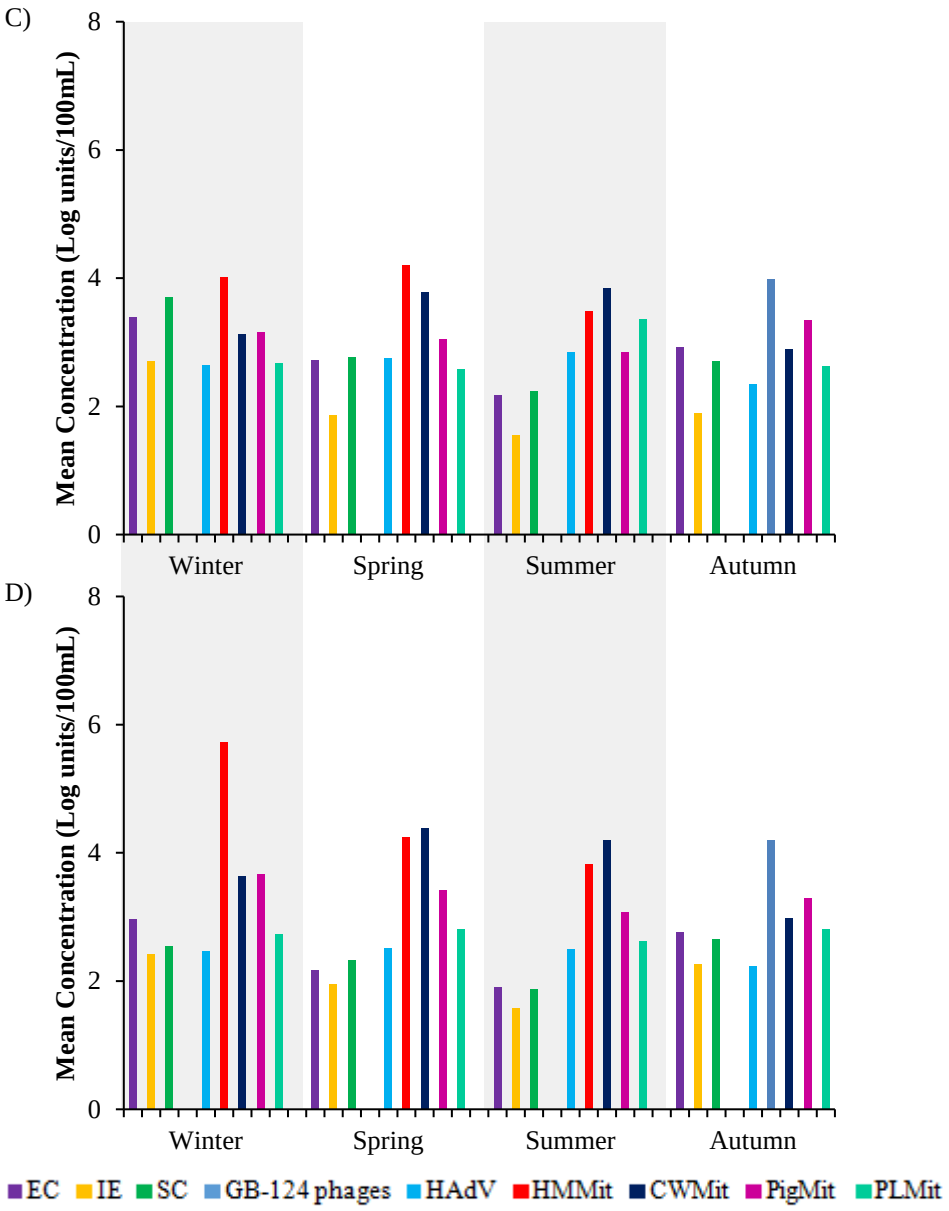
| Step | Stage | Reps | Temp (ºC) | Time |
|---|---|---|---|---|
| Reverse transcription | 1 | 1 | 45 | 10 min |
| RT inactivation./initial denaturation | 2 | 1 | 95 | 10 min |
| Amplification | 3 | 40 | 95 | 15 s |
| Annealing/Extension | | | 60 | 45 s |




**Appendix 1.F -** Example of inhibited IC and non-inhibited IC (Images: Author's own)

**Appendix 2: Results of Molecular and culture-based assays for targeting Human and non-human faecal contamination in River Tagus (Rio Tejo)**






**Appendix 2.A** Mean concentration for each parameter by season at A) site 1, B) site 2, C) site 3 and D) site 4.

**Appendix 2.B -** Mean, max and min concentrations and standard deviation for the indicator parameters per sampling site with respect to time of year (season)

**Log units/100mL**

| | **Site 1** | | | |
|---|---|---|---|---|
| **Parameter** | **Winter**<br>Mean ± SD (min-max) | **Spring**<br>Mean ± SD (min-max) | **Summer**<br>Mean ± SD (min-max) | **Autumn**<br>Mean ± SD (min-max) |
| EC | 2.94 ± 0.45 (2.01-3.42) | 2.69 ± 0.36 (2.14-3.24) | **3.30 ± 1.32 (2.45-5.24)** | 2.96 ± 0.18 (2.75-3.08) |
| IE | 1.98 ± 0.34 (1.60-2.60) | 1.80 ± 0.39 (1.30-2.59) | **2.17 ± 1.43 (1.30-4.29)** | 1.91 ± 0.38 (1.60-2.34) |
| SC | **2.98 ± 0.50 (2.00-3.36)** | 2.29 ± 0.33 (1.88-2.78) | 1.91 ± 1.48 (0.00-3.62) | 2.96 ± 0.28 (2.65-3.22) |
| GB-124 phages | 0.00 ± 0.00 (0.00-0.00) | **0.03 ± 0.09 (0.00-0.30)** | 0.00 ± 0.00 (0.00-0.00) | 0.00 ± 0.00 (0.00-0.00) |
| HAdV | 2.23 ± 0.00 (2.23-2.23) | **2.83 ± 1.21 (2.23-6.30)** | 2.23 ± 0.00 (2.23-2.23) | 2.29 ± 0.11 (2.23-2.42) |
| HMMit | **4.59 ± 1.12 (2.54-5.70)** | 4.26 ± 1.02 (2.54-5.40) | 3.84 ± 1.14 (2.54-5.29) | 3.55 ± 1.37 (2.54-5.11) |
| CWMit | 3.05 ± 0.91 (2.33-4.34) | 3.90 ± 0.78 (2.33-5.08) | **4.49 ± 1.16 (3.45-5.53)** | 2.90 ± 0.99 (2.33-4.05) |
| PigMit | 3.15 ± 0.79 (2.54-4.54) | **3.57 ± 0.95 (2.54-5.20)** | 3.08 ± 0.64 (2.54-3.99) | 3.09 ± 0.52 (2.54-3.58) |
| PLMit | 2.95 ± 0.83 (2.33-4.36) | 2.64 ± 0.54 (2.33-3.75) | **2.96 ± 0.46 (2.33-3.43)** | 2.33 ± 0.00 (2.33-2.33) |
| **Site 2** | | | | |
| EC | **3.12 ± 0.34 (2.51-3.56)** | 2.68 ± 0.48 (1.83-3.39) | 2.21 ± 0.56 (1.52-2.68) | 3.10 ± 0.49 (2.64-3.62) |
| IE | **2.59 ± 0.32 (2.33-3.12)** | 2.13 ± 0.54 (0.78-2.87) | 1.63 ± 0.38 (1.30-2.09) | 2.52 ± 0.51 (2.09-3.08) |
| SC | **2.83 ± 0.80 (1.43-3.59)** | 2.64 ± 0.30 (2.18-3.23) | 1.73 ± 0.40 (1.20-2.18) | 2.71 ± 0.15 (2.54-2.81) |

**Appendix 2.B (continued)-** Mean, max and min concentrations and standard deviation for the indicator parameters per sampling site with respect to time of year (season)

| | | | | |
|---|---|---|---|---|
| GB-124 phages | 0.00 ± 0.00 (0.00-0.00) | 0.00 ± 0.00 (0.00-0.00) | 0.00 ± 0.00 (0.00-0.00) | 0.00 ± 0.00 (0.00-0.00) |
| HAdV | 2.49 ± 0.50 (2.23-3.57) | **2.55 ± 0.54 (2.23-3.86)** | 2.35 ± 0.23 (2.23-2.69) | 2.34 ± 0.19 (2.23-2.57) |
| HMMit | 4.22 ± 1.15 (2.54-5.83) | **4.45 ± 1.18 (2.54-6.38)** | 3.81 ± 1.15 (2.54-5.23) | 3.17 ± 1.10 (2.54-4.45) |
| CWMit | 3.75 ± 1.21 (2.33-5.29) | 3.82 ± 0.85 (2.33-5.15) | **4.90 ± 1.45 (3.00-6.36)** | 3.50 ± 1.01 (2.33-4.14) |
| PigMit | 3.03 ± 0.75 (2.54-4.22) | **3.61 ± 0.75 (2.54-4.93)** | 3.26 ± 0.67 (2.54-4.15) | 3.11 ± 1.00 (2.54-4.26) |
| PLMit | **3.51 ± 1.83 (2.33-8.10)** | 3.29 ± 1.14 (2.33-5.29) | 3.08 ± 0.63 (2.33-3.88) | 2.62 ± 0.49 (2.33-3.19) |
| **Site 3** | | | | |
| EC | **3.39 ± 0.49 (2.56-3.99)** | 2.72 ± 0.64 (1.52-3.61) | 2.18 ± 0.16 (2.00-2.32) | 2.92 ± 0.22 (2.77-3.17) |
| IE | **2.71 ± 0.34 (2.34-3.21)** | 1.86 ± 0.63 (0.85-3.16) | 1.55 ± 0.17 (1.30-1.76) | 1.90 ± 0.52 (1.30-2.23) |
| SC | **3.71 ± 0.62 (2.65-4.31)** | 2.77 ± 0.56 (2.18-4.22) | 2.24 ± 0.63 (1.28-2.85) | 2.71 ± 0.31 (2.40-3.02) |
| GB-124 phages | 0.00 ± 0.00 (0.00-0.00) | 0.00 ± 0.00 (0.00-0.00) | 0.00 ± 0.00 (0.00-0.00) | 0.00 ± 0.00 (0.00-0.00) |
| HAdV | 2.65 ± 0.36 (2.23-3.07) | 2.76 ± 0.45 (2.23-3.34) | **2.84 ± 1.36 (2.23-5.27)** | 2.34 ± 0.19 (2.23-2.56) |
| HMMit | 4.02 ± 0.83 (2.54-4.96) | **4.20 ± 1.27 (2.54-6.02)** | 3.48 ± 0.81 (2.54-4.60) | 3.98 ± 1.25 (2.54-4.71) |
| CWMit | 3.13 ± 1.29 (2.33-5.31) | 3.79 ± 1.09 (2.33-5.21) | **3.85 ± 1.23 (2.46-5.53)** | 2.90 ± 0.99 (2.33-4.05) |
| PigMit | 3.16 ± 0.90 (2.54-4.42) | 3.05 ± 0.83 (2.54-4.55) | 2.85 ± 0.58 (2.54-3.87) | **3.35 ± 0.74 (2.54-3.97)** |
| PLMit | 2.67 ± 0.52 (2.33-3.34) | 2.58 ± 0.81 (2.33-5.03) | **3.36 ± 1.01 (2.33-4.35)** | 2.63 ± 0.51 (2.33-3.22) |

**Appendix 2.B (continued)** - Mean, max and min concentrations and standard deviation for the indicator parameters per sampling site with respect to time of year (season)

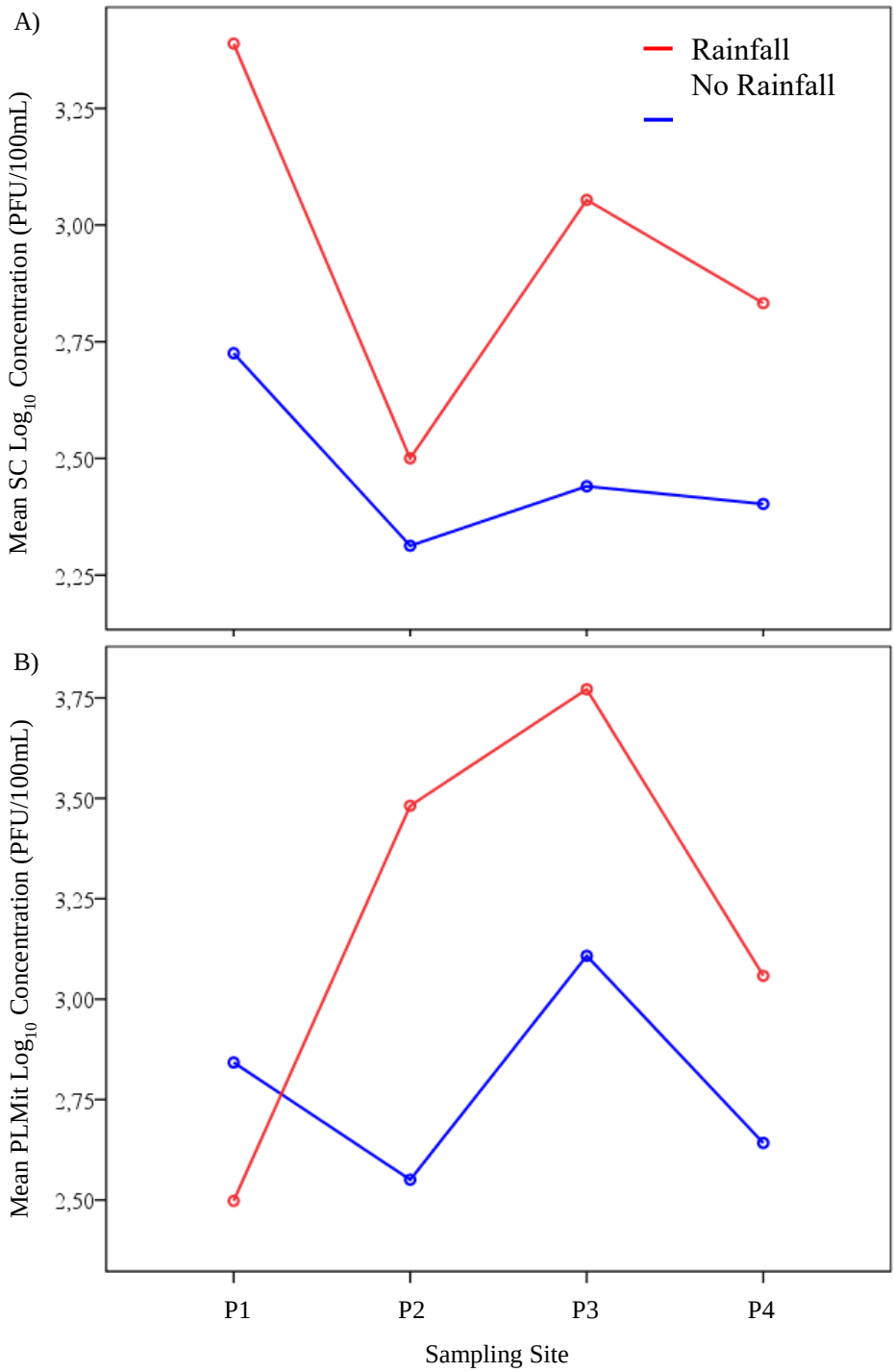
| | **Site 4** | | | |
|---|---|---|---|---|
| EC | **2.97 ± 0.71 (2.23-4.64)** | 2.17 ± 0.43 (1.52-2.76) | 1.91 ± 0.29 (1.52-2.24) | 2.77 ± 0.38 (2.39-3.16) |
| IE | **2.42 ± 0.73 (1.30-3.91)** | 1.95 ± 0.71 (1.30-3.55) | 1.57 ± 0.34 (1.15-1.91) | 2.26 ± 0.48 (1.79-2.74) |
| SC | 2.55 ± 0.92 (1.08-4.02) | 2.33 ± 0.39 (1.70-3.16) | 1.87 ± 1.06 (0.00-2.65) | **2.65 ± 0.05 (2.60-2.70)** |
| GB-124 phages | 0.00 ± 0.00 (0.00-0.00) | 0.00 ± 0.00 (0.00-0.00) | 0.00 ± 0.00 (0.00-0.00) | 0.00 ± 0.00 (0.00-0.00) |
| HAdV | 2.46 ± 0.55 (2.23-3.87) | **2.52 ± 0.71 (2.23-4.49)** | 2.49 ± 0.45 (2.23-3.27) | 2.23 ± 0.00 (2.23-2.23) |
| HMMit | **5.73 ± 3.11 (2.54-11.17)** | 4.25 ± 0.65 (3.26-5.31) | 3.83 ± 1.13 (2.54-5.29) | 4.20 ± 1.55 (2.54-5.60) |
| CWMit | 3.63 ± 1.93 (2.33-7.03) | **4.38 ± 1.73 (2.33-9.02)** | 4.20 ± 1.41 (2.33-5.88) | 2.98 ± 0.71 (2.33-3.76) |
| PigMit | **3.67 ± 1.67 (2.54-7.69)** | 3.42 ± 0.98 (2.54-5.05) | 3.07 ± 0.74 (2.54-3.96) | 3.29 ± 0.69 (2.54-3.90) |
| PLMit | 2.73 ± 0.79 (2.33-4.71) | **2.81 ± 0.84 (2.33-4.55)** | 2.63 ± 0.51 (2.33-3.51) | **2.81 ± 0.83 (2.33-3.76)** |

**Appendix 2.C -** *p*-values of *F* test calculated by the analysis of variances of two-way factor for EC to determine the most dominant combination of sampling sites

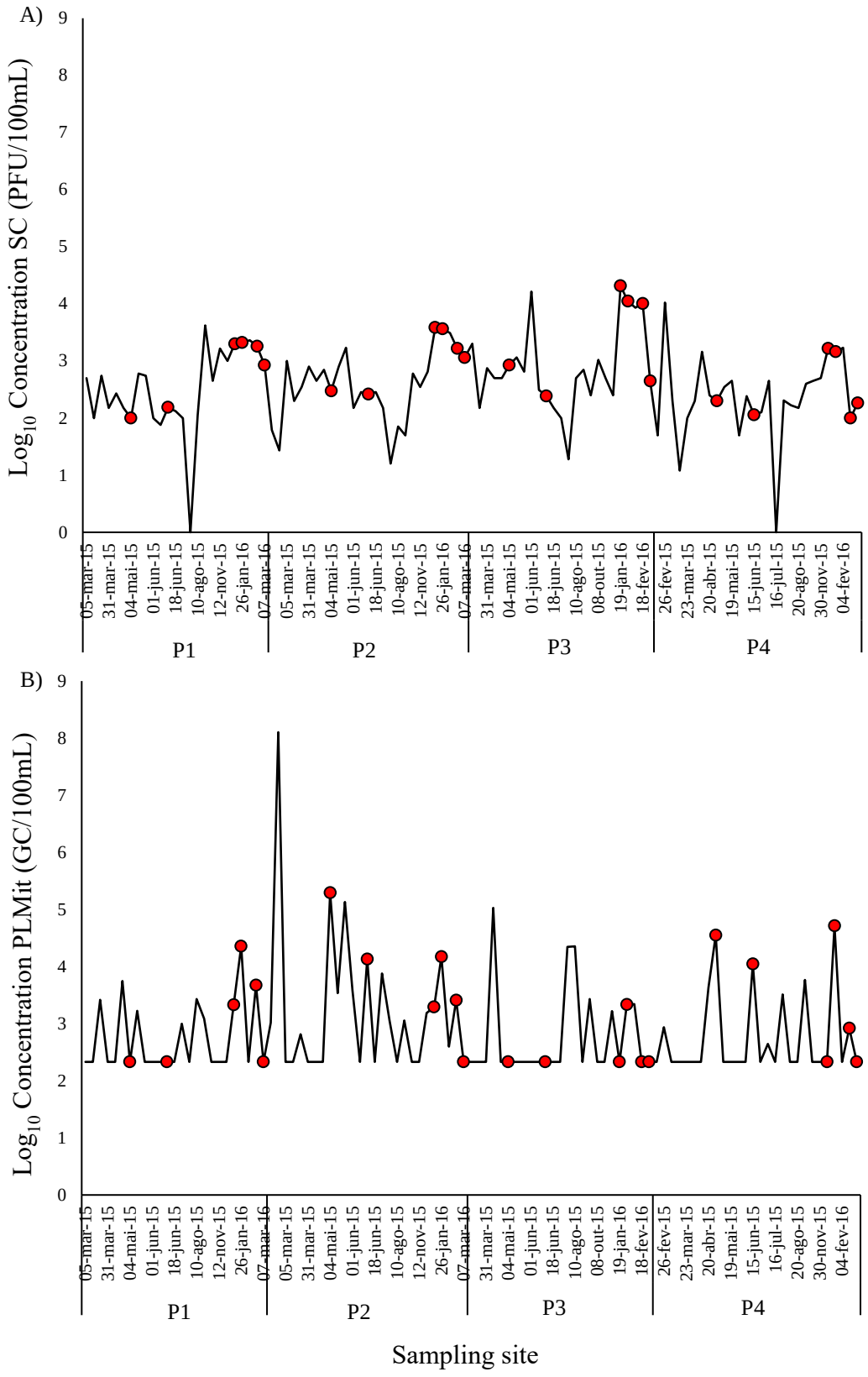
| | **Parameter** |
|---|---|
| Sampling site (name) | EC |
| 3 – 4 (Alc – Bel) | **0.030** |
| 3 - 2 (Alc – MPdN) | 0.870 |
| 3 – 1 (Alc – VFX) | 0.335 |
| 4 – 2 (Bel – MPdN) | **0.032** |
| 4 – 1 (Bel – VFX) | 0.005 |
| 2 – 1 (MPdN – VFX) | 0.230 |

**Appendix 2.D** - *p*-value calculated by one-way ANOVA for general correlation of mean concentration of parameters and rainfall events and be the analysis of variances of two-factor (rainfall and sampling location)

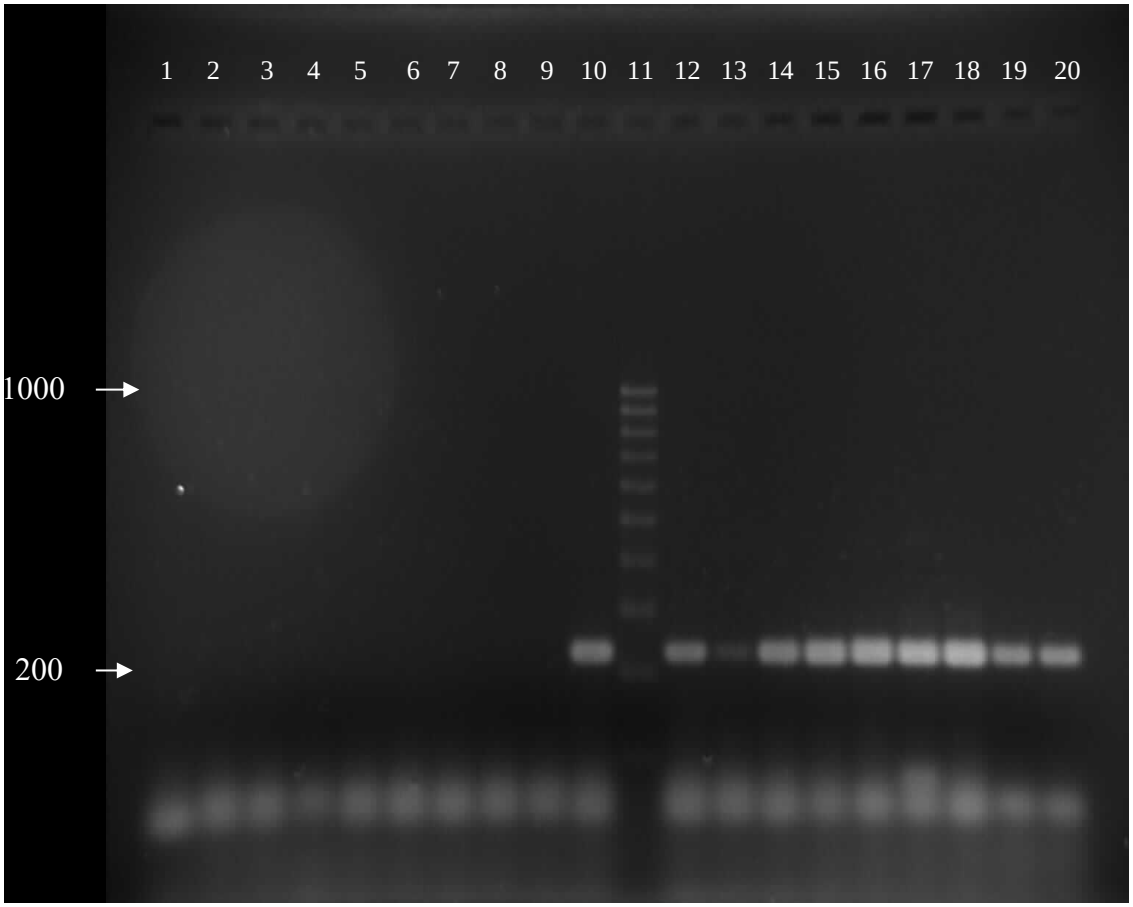
| **Parameter**<br>**Sampling Location** | **Rainfall/ Dry Sampling Dates** | **Rainfall levels (mm)** |
|---|---|---|
| EC | 0.190 | - 0.102 |
| IE | < **0.05** | - **0.286** |
| Alc | 0.071 | - |
| Bel | 0.384 | - |
| MPdN | 0.597 | - |
| VFX | 0.398 | - |
| SC | < **0.05** | - **0.256** |
| Alc | < **0.05** | - |
| Bel | 0.558 | - |
| MPdN | 0.058 | - |
| VFX | 0.187 | - |
| GB124 phages | 0.589 | n.a. |
| HAdV | 0.370 | **0.471** |
| HMMit | 0.372 | 0.141 |
| CWMit | 0.120 | - 0.167 |
| PigMit | 0.703 | - 0.254 |
| PLMit | < **0.05** | 0.385 |
| Alc | 0.403 | - |
| Bel | < **0.05** | - |
| MPdN | 0.105 | - |
| VFX | 0.313 | - |




**Appendix 2.E -** Graphic representation of the relationship between rainfall and the concentration of A) SC and B) PLMit




**Appendix 2.F -** Behaviour pattern of (A) SC and (B) PLMit during rainfall events. Small red circles mark the sampling dates where rain events occurred during the monitoring programme.

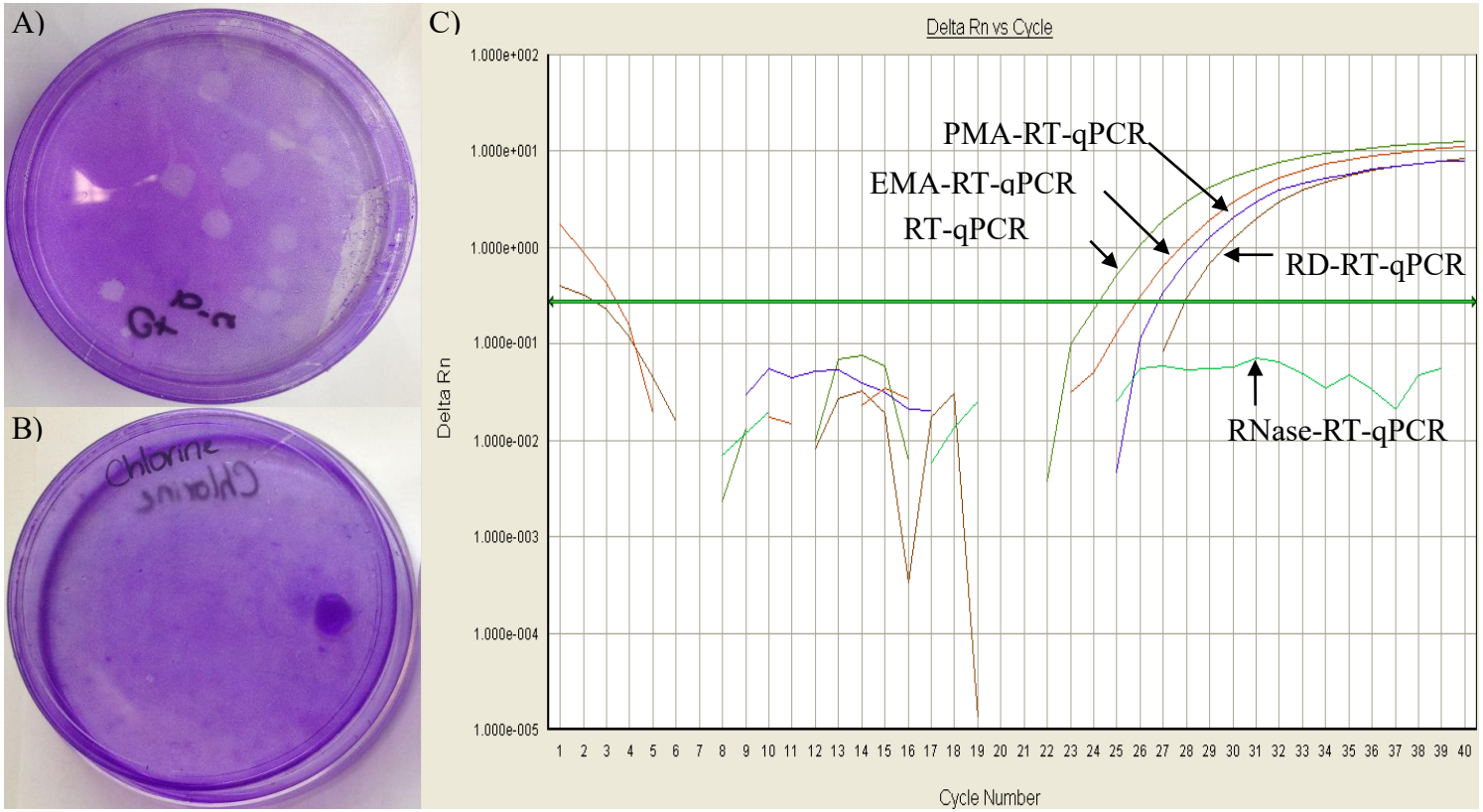



**Appendix 2.G -** Ethidium bromide stained 2.0% agarose gel for dog faecal matter single PCR using solely nested primers.

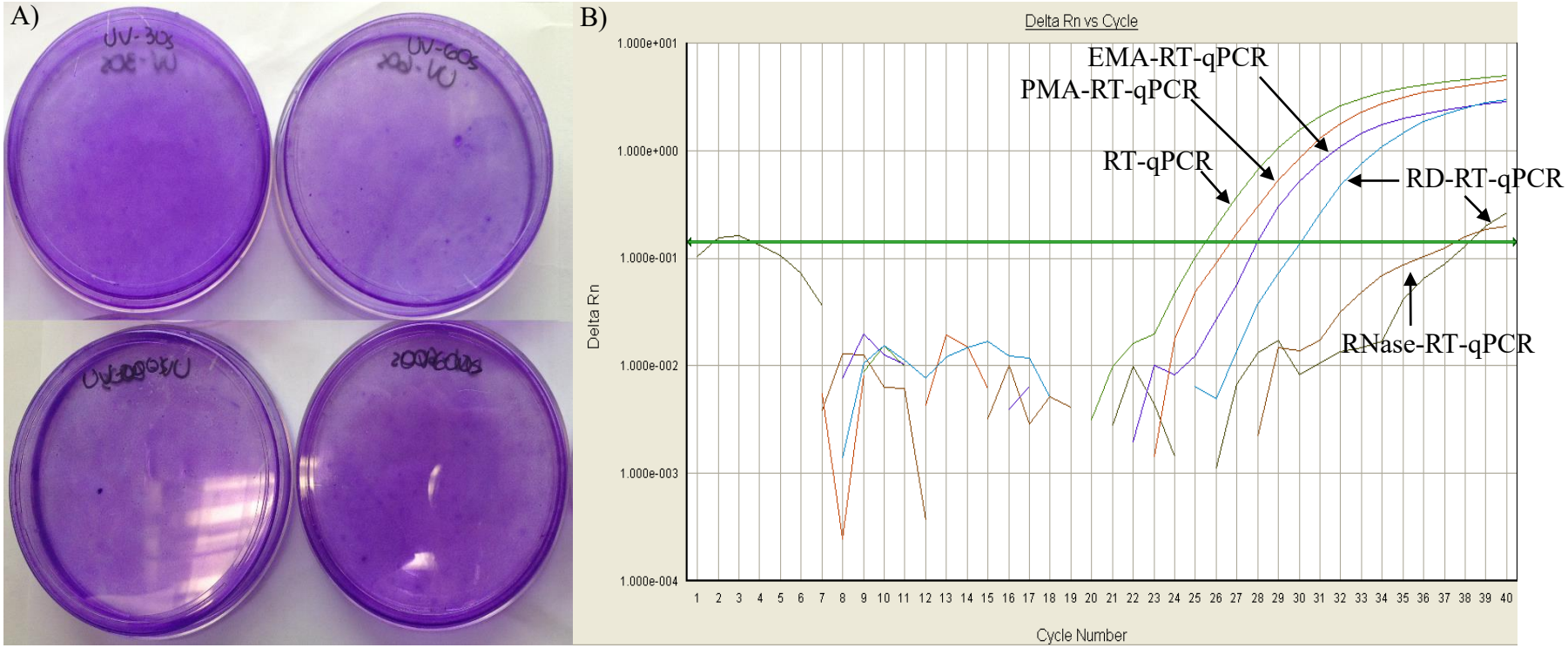
**Appendix 2.H -** *p*-values of ANOVA test calculated by the analysis of variances of two-way factor (rain events and location) for dog and cat mitochondrial marker

| Target | Rain event | Location |
|---|---|---|
| Dog | < 0.001 | < 0.001 |
| VFX | > 0.05 | |
| MPdN | < 0.001 | |
| Alc | < 0.001 | |
| Bel | < 0.001 | |
| Cat | < 0.001 | < 0.05 |
| VFX | > 0.05 | |
| MPdN | < 0.05 | |
| Alc | < 0.05 | |
| Bel | < 0.001 | |

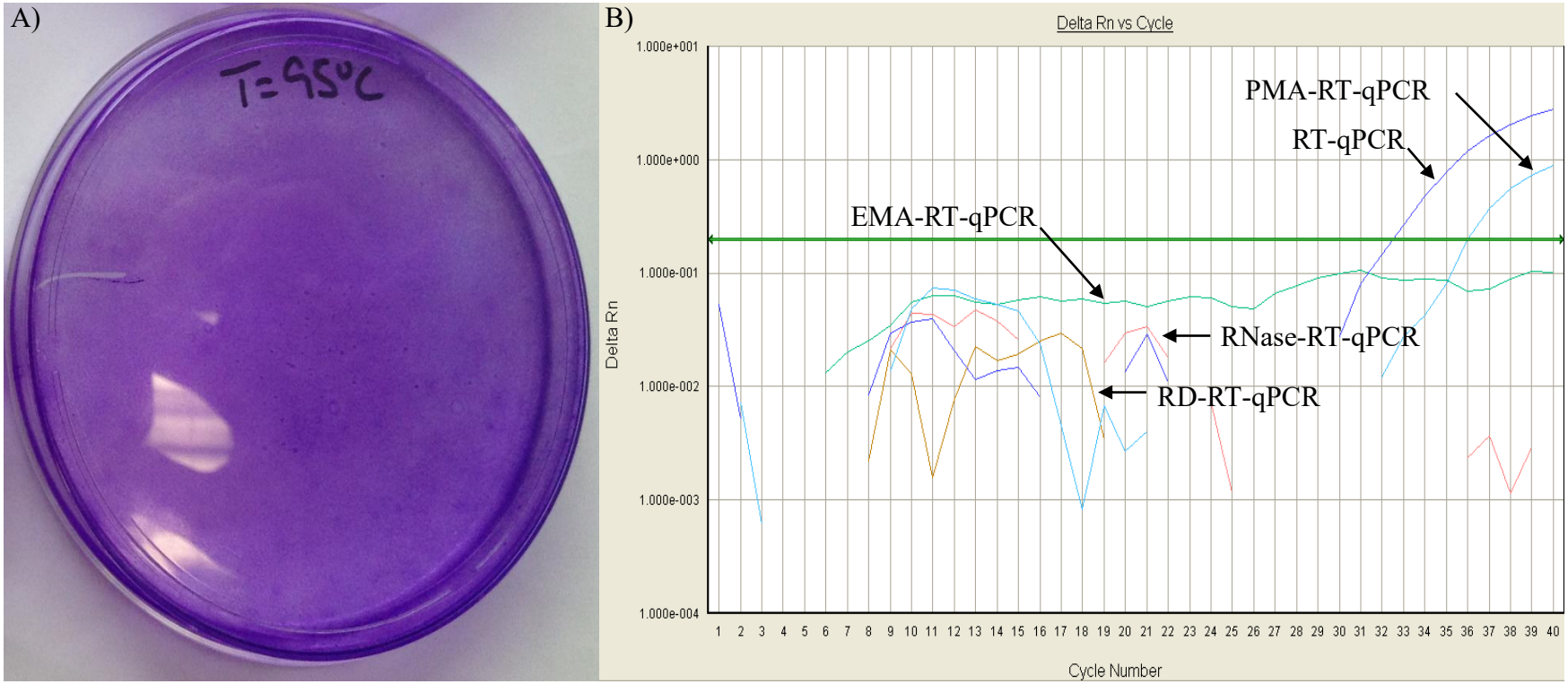
**Appendix 3: Discrimination of infectious viruses by combining pretreatments with RT-qPCR**




**Appendix 3.A** - PA and RT-qPCR of infectious and chlorine-inactivated EntV. A) PA from a river water sample spiked with EntV, without chlorine treatment. (B) PA from a river water sample spiked with EntV following chlorine inactivation. (C) RT-qPCR run from a river water sample spiked with EntV following chlorine inactivation and with different pre-treatments.




**Appendix 3.B -** PA and RT-qPCR of UV-inactivated EntV. A) PA from river water sample spiked with EntV following UV inactivation at 30, 60, 200 and 600 s. (B) RT-qPCR run from river water sample spiked with EntV following UV inactivation and with different pre-treatments. The bright blue line and olive green line represent qPCR ten-fold dilution and undiluted sample treated with RD. The lines clearly show that samples treated with RD contained qPCR inhibitors.




**Appendix 3.C** - PA and RT-qPCR of heat-inactivated EntV. A) PA from river water sample spiked with EntV following heat inactivation at 95 ºC for 10 min. (B) RT-qPCR run from river water sample spiked with EntV following heat inactivation and with different pre-treatments.