Pathogen Detection Methodologies for Wastewater and Reservoirs

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The Urban Water Security Research Alliance (UWSRA) is a $50 million partnership over five years between the Queensland Government, CSIRO's Water for a Healthy Country Flagship, Griffith University and The University of Queensland. The Alliance has been formed to address South-East Queensland's emerging urban water issues with a focus on water security and recycling. The program will bring new research capacity to South-East Queensland tailored to tackling existing and anticipated future issues to inform the implementation of the Water Strategy.

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Cover Photograph:

Description: the aeration tank of an Activated Sludge Plant
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FOREWORD

Water is fundamental to our quality of life, to economic growth and to the environment. With its booming economy and growing population, Australia's South East Queensland (SEQ) region faces increasing pressure on its water resources. These pressures are compounded by the impact of climate variability and accelerating climate change.

The Urban Water Security Research Alliance, through targeted, multidisciplinary research initiatives, has been formed to address the region's emerging urban water issues.

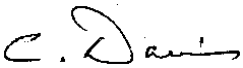
As the largest regionally focused urban water research program in Australia, the Alliance is focused on water security and recycling, but will align research where appropriate with other water research programs such as those of other SEQ water agencies, CSIRO's Water for a Healthy Country National Research Flagship, Water Quality Research Australia, eWater CRC and the Water Services Association of Australia (WSAA).

The Alliance is a partnership between the Queensland Government, CSIRO's Water for a Healthy Country National Research Flagship, The University of Queensland and Griffith University. It brings new research capacity to SEQ, tailored to tackling existing and anticipated future risks, assumptions and uncertainties facing water supply strategy. It is a $50 million partnership over five years.

Alliance research is examining fundamental issues necessary to deliver the region's water needs, including:

- ensuring the reliability and safety of recycled water systems.
- advising on infrastructure and technology for the recycling of wastewater and stormwater.
- building scientific knowledge into the management of health and safety risks in the water supply system.
- increasing community confidence in the future of water supply.

This report is part of a series summarising the output from the Urban Water Security Research Alliance. All reports and additional information about the Alliance can be found at http://www.urbanwateralliance.org.au/about.htmL.



Chris Davis
Chair, Urban Water Security Research Alliance

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EXECUTIVE SUMMARY

The Purified Recycled Water (PRW) project was tasked to undertake research on issues relating to the production of PRW in South East Queensland (SEQ). A recognised shortfall in routine monitoring of microbiological water quality is that many pathogens are not represented by the conventional faecal indicator organisms that are detected by culture-based methods only. Another problem with culture-based methods is that a final result takes two to five days to produce. In an attempt to work towards improving detection methods for waterborne pathogens, quantitative molecular genetic methods were optimised and validated against conventional culture-based techniques. This involved addressing problems with extracting clean genetic material from wastewater samples, removing inhibitors that interfere with amplifying the DNA and developing an internal standard so that false positive or negatives are unequivocally determined.

A thorough review of the literature and commercially available kits for removal of inhibiting substances was carried out. As there are no guarantees of previously published methods or kits being successful, an attempt to characterise polymerase chain reaction (PCR) inhibiting compounds was trialled using mass spectroscopic techniques. Humic acids were used as model compounds due to their abundance in fresh water and their ability to covalently bind to DNA, inhibiting the polymerase chain reaction. Examination with matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) spectroscopy or direct injection electrospray spectroscopy failed to detect humic acids when trialled as standards or in samples. Attempts were made to indirectly detect humic acids by exploiting their capacity to bind DNA. A readily detected 25 mer single-stranded DNA oligonucleotide standard was used, assuming that binding with humic acids would prevent its detection. Little change was observed between the humic acid treated DNA standard and the untreated control. Therefore, the investigation was halted and a "trial and error" approach to removing potential PCR inhibiting compounds from wastewater samples resulted in using the MoBio kit for sample clean-up.

The second problem with molecular genetic methods when analysing material from environmental samples is the inclusion of a suitable internal control. Quality control indicators are necessary at both the initial sample processing steps, to ensure that extracted DNA is representative of the sample, and also during preparation and analysis of the genetic material. To address this, the gene for enhanced green fluorescent protein (EGFP) was chosen as a novel DNA species that would not be present within the wastewater samples. The genetic method chosen to validate against culture-based methods was the quantitative polymerase chain reaction (qPCR) for targeted pathogens. To assess the efficiency of qPCR assays and the effects of inhibitors to the reaction, duplex reactions were designed, targeting the pathogen of interest and the GFP gene. Plasmid containing the GFP gene was then seeded into all reactions in known concentrations to act as an internal standard and assess the reaction efficiency.

The project examined pathogen numbers, both before and after treatment in three wastewater treatment plants, using both conventional culture-based methods and qPCR. This offered not only comparison of modern genetic techniques for pathogen detection in a water matrix that often presents difficulties using DNA-based analyses, but also assessed wastewater treatment efficacy in pathogen removal from water destined for further purification and reuse.

Interestingly, the qPCR pathogen detection methods generally detected higher concentrations of bacteria than culture-based methods for the primary effluent at microbial numbers in excess of 5×10^6 cells per 100 mL. Detection levels by qPCR ranged from 2 to 80 times higher than culture-based methods ($p \geq 0.0001$). There was no statistically significant difference at most sampling locations between qPCR and culture-based methods in the treated effluent at cell numbers around 10^4 per 100 mL. It can be speculated that the increased level of detection with qPCR methods could be due to: increased sensitivity of qPCR methods over culture-based methods; bacterial targets entering a viable-but-unculturable state, rendering then undetectable to culture-based methods; and the qPCR reaction detecting nucleic acids from lysed, non-viable bacteria. The primary cause of the disparity in detection levels between qPCR and culture-based methods at high concentrations of pathogens deserves further investigation. However, as both culture and qPCR techniques gave comparable organism numbers at lower pathogen concentrations, differences in microbial risk management would be minimal. Quantitative PCR offers the distinct advantage of greatly decreased time and higher throughput over culture-based techniques, allowing water managers to take preventative action much sooner if organisms of public health significance are detected.

1. INTRODUCTION

Current water quality monitoring requirements are based mainly on numbers of indicator organisms (total coliforms, faecal coliforms and *Escherichia coli*) (NHMCR-ARMCANZ, 1996; WHO, 1996); and for water recycling requirements, selected reference pathogens such as *Campylobacter*, *Cryptosporidium, Giardia* and enteric viruses (NRMMC, EPHC and NHMRC, 2008). However, the use of indicator organisms to assess public health risks can have serious limitations and have not always protected public health to the desirable level (Allwood *et al.,* 2003; Baggi *et al.,* 2001; Efstratiou *et al.,* 1998; Fattouh *et al.*, 2004; Horman *et al.,* 2004; Jin *et al.,* 2004; Tallon *et al.,* 2005). One notable example of where indicator organisms have failed include the Milwaukee drinking water contamination incident where over 400,000 people were infected with *Cryptosporidium* when the indicator organism tests apparently demonstrated acceptable cell numbers (MacKenzie *et al.,* 1994) and there have been many more since.

The NHMRC's (2003) review of the use of coliforms as indicator organisms, and the revised Australian Drinking Water Guidelines, have recognised the inadequacies of culture-based methods and are advocating the role of gene technology for the direct detection of pathogens. Since this recommendation, there has been a number of studies attempting to show these inadequacies (Savichtcheva and Okabe, 2006) and continuing to question the suitability of indicator organisms as indicators of the occurrence and concentration of human viruses (Bosch *et al.*, 2008) and protozoan cysts (Lipp *et al.*, 2001; Schriewer *et al.,* 2010). Indicator bacteria such as *E. coli* are much more sensitive to inactivation (through treatment processes, natural competition and the elements such as sunlight) than pathogenic bacteria, viruses and protozoa (Carey *et al.*, 2004; Sinclair *et al.*, 2009). Other limitations of the present application of indicator organisms include the facts that they are not necessarily from a non-exclusive faecal source (Simpson *et al.,* 2002), their ability to multiply in the environment and they have a low correlation with the presence of many waterborne pathogens (McFeters *et al.,* 1974; Farnleitner *et al.,* 2000; Savichtcheva and Okabe, 2006; Stedtfeld *et al.*, 2007). As the use of indicator organisms does not always meet all established criteria for water quality and protection of public health, there is a relatively urgent need to investigate alternative approaches and methods.

In recent years, advancements in molecular genetics have paved the way for the development of many techniques which exploit the high degree of organism-specific sequence structure DNA. The conservative, yet variable, nature of DNA allows accurate detection and quantification of organisms of public health significance (Schneegurt and Kulpa, 1998; Farnleitner *et al.,* 2005; Gilbride *et al.,* 2006). Foremost among these methods is the polymerase chain reaction (PCR) and, with the recent development of quantitative PCR (qPCR) (Behets *et al.,* 2007; Lee *et al.,* 2008; Rothrock *et al.,* 2009; Viau and Peccia, 2009; Klein *et al.,* 2010; Skotarczak, 2010), this technique allows the highly accurate quantification of the amount of starting material used in the reaction, and therefore the ability to enumerate organisms present in a sample.

Molecular genetic based methods have many advantages over traditional culture-based methods for pathogen detection, including: much higher organism specificity; greatly decreased time required for analysis (potentially as little as four hours processing and analysis time in a developed method); much higher through-put; and they do not require further biochemical confirmation of the species being analysed. Though PCR-based methods offer many advantages for pathogen detection, the water industry has been slow to adopt them in preference to culture-based methods. Some of the reasons for this delayed uptake of advanced methods include:

- Risk of false negatives due to inhibition of the amplification of genetic material
- High cost of base equipment
- Level of skills required to handle and process samples
- Ability to obtain quantitative results
- Internal standards for quality control and assurance of the results
- The ability to determine whether a cell or viral particle is viable and/or capable of causing infection (infectivity).

The requirement for expensive specialised equipment to conduct qPCR and the high degree of technical skill that is required to use the methods, especially in methods development and troubleshooting, can be another deterrent for commercial laboratories to adopt these methods. In addition to this, there is a shortfall in quality control for qPCR and preliminary sample preparation. DNA extraction from environmental samples requires sufficient controls to reliably measure efficiency of the extraction method and ensure DNA extracted from the sample is representative of the original microbial population. The integrity of the extracted DNA needs to be accurately measured to assess the amount of contaminants within the extract and ensure that minimal degradation or shearing has occurred in the DNA. Lastly, controls need to be in place to monitor the efficiency of the qPCR as, in many environmental and clinical samples, PCR-inhibiting substances co-purify with DNA resulting in either complete or partial inhibition of the reaction, providing a false result.

DNA extracts from environmental and clinical samples often contain substances that co-purify with DNA, which can severely affect downstream genetic analysis (Straub and Chandler, 2003; Straub *et al.,* 2005; Behets *et al.,* 2007). These inhibiting substances have a wide range of mechanisms of interference with genetic techniques. The main classes of inhibitors are: those that interact directly with DNA, effecting DNA-DNA hybridisation or enzymatic binding; those that interact with enzymes through either binding to active sites or inhibiting polymerase elongation; and those that can sequester co-factors, such as magnesium, essential for enzymatic reactions during DNA replication steps. In many cases, great effort has been made to remove inhibiting substances during the DNA extraction process and a wide array of commercial kits for DNA extraction is available, claiming effective inhibitor removal from various sample types. A summary of the literature and commercial kits available is given in Appendix II.

The main shortfall of many of the current approaches to remove PCR inhibitors is that often they involve time consuming and expensive pre-treatment steps and can cause significant reductions in the final DNA yield. Additionally, environmental DNA extracts are not routinely checked for DNA quality beyond UV spectrophotometry testing for carbohydrate (at 230 nm), proteins (at 280 nm) and occasionally the absorbance at 340 nm detecting polyphenolics such as humic acids. Often the "PCR-ability" of an environmental DNA extract is used as a measure of the presence of inhibition, involving serially diluting the template DNA until a successful PCR can be performed. The drawback of using methods such as these is they do not give true indications of the content of the extract and can result in false estimates of the starting template quantity.

In the present project, we have attempted to use a combination of mass spectrometry to ascertain the chemical composition of the DNA extracts and internal standards which co-amplify with target sequences to try and overcome these hurdles. It is hoped that this work could lead to tailored DNA extraction methods for different water matrices to maximise high quality yields of template DNA for further analyses. We have also attempted to address several of the quality control issues and have undertaken some preliminary investigations into alternative methods for detecting PCR product other than fluorescent tags or dyes routinely used in qPCR. An attempt was made to develop controls and monitoring methods to assess DNA extraction efficiency, quality of DNA extracts and internal standards to assess the reaction efficiency of the qPCR.

The viability and infectivity of pathogenic bacteria in treated water is a major issue for water safety and risk assessments to public health (Girones *et al.,* 2010). It is important that this issue be adequately addressed if molecular genetic techniques are to be adopted for determining water quality. Approaches for determining infectivity and viability of microbes that have been evaluated by others include using DNA-intercalating dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) or propridium iodide (PI) (Amjad *et al.,* 2004; Call, 2005; Rudi *et al.,* 2005; Cenciarini-Borde *et al.,* 2009; Nocker and Camper, 2009). These DNA stains only enter cells that have a damaged membrane, indicating that the cell is dead or dying. Another possible approach is to not only target DNA but also the messenger RNA (mRNA) as it is known to degrade quickly after cell death. However, there is now much debate about the integrity of this approach as there is evidence that the persistence of mRNA in the environment after cell death may not be as short as once thought. A further issue is that some mRNA molecules are not transformed in viable non-culturable cells and would result in false

negatives (Girones *et al.,* 2010). The most promising approach may be the combination of PCR-PMA assays for *Cryptosporidium* that allow genotyping and viability to be carried out (Brescia *et al.,* 2009). The issue of viability has not been specifically addressed in the current study in the method development but is included in the pressing future steps to allow for the validation of these techniques, wider reaching uptake and acceptance by water authorities.

Pathogens that might enter a sewage treatment plant will largely depend on the disease reservoir within the community. In Australia, we have eradicated and prevented some diseases through the collection and treatment of wastewater, provision of treated drinking water, strict quarantine laws at our borders and immunisation. Typical concentrations of pathogens found in raw sewage in developing and developed countries are listed in Table 1. Few studies have been carried out in Australia that fully characterise the pathogens that may be found in treated sewage. Those that have been carried out have detected 2,000 *Cryptosporidium*, 8,000 rotavirus and 7,000 *Campylobacter* per litre (as 95th percentiles) (NRMMC–EPHC 2006).

Table 1. Reported concentration of indicator and pathogenic microorganisms in raw sewage in studies from around the world.

Organism	Numbers in Raw Sewage per Litre
Bacteria	
Escherichia coli (indicator)	10^5 -10^{10}
E. coli (pathogenic)	Low
Enterococci (indicator)	10^6 -10^7
Shigella	10^1 -10^4
Salmonella	10^3 -10^5
Clostridium perfringens (indicator)	10^5 -10^6
Viruses	
Enteroviruses	10^2 -10^6
Adenoviruses	10^1 -10^4
Noroviruses	10^1 -10^4
Rotaviruses	10^2 -10^5
Somatic coliphages (indicator)	10^6 -10^9
F-RNA coliphages (indicator)	10^5 -10^7
Protozoa	
Cryptosporidium sp.	0 -10^4
Entamoeba histolytica	4.5 x 10^4
Giardia sp.	10^2 -10^5
Helminth ova	
Ascaris lumbricoides	10^2
Hookworm	10 - 10^2

Adopted from [Bitton, 1994; Feachem and others, 1983; NRMMC and EPHC, 2005].

Campylobacter, Shigella and *Salmonella* are the most frequent agents of bacterial gastroenteritis recorded in Australia (Hall, 2004) and world wide (Girones *et al.,* 2010). While these organisms are isolated from water samples in much lower numbers than indicator organisms and phages, such as faecal coliforms and enterococci (Sidhu and Toze, 2009), these low numbers of pathogenic bacteria can still cause a public health threat. Therefore, it is important to take this into account when carrying out risk assessment and management exercises. The risk of only relying on indicator organisms for assessing water quality monitoring is that the lower number of viable and infective pathogens may be missed. Therefore, there is a need to develop reliable, rapid and accurate methods to better detect pathogenic microorganisms in water. This project set out to examine methods available for rapid detection of microbial pathogens and developments to enable quantitative real-time PCR assays to be used for water quality monitoring in SEQ.

2. METHOD DEVELOPMENT

The optimisation and implementation of genetic molecular techniques requires many steps in preparation to ensure that data is reliable and reproducible. Many steps were required in this project to ensure that we could compare qPCR with culture-based methods for enumerating reference pathogens and indicator organisms in wastewater samples. These included: addressing potential PCR inhibition; development of an internal standard; and cross-checking and optimisation of the primers selected or designed for each organism. A brief description of each of these steps and the final methods used is given in this Section. As each of these tasks occupied a significant amount of time and resources through the various steps required, a full description of the method development is provided in Appendices I, II and III.

2.1. Construction of an Internal Standard - *Escherichia coli* Chromosomal Insertion of EGFP Gene

To accurately assess the DNA extraction efficiency of an unknown environmental sample, the approach adopted was to introduce in known quantities a detectable DNA sequence that would not be normally present within the sample but that would behave like DNA within the targeted organism. To achieve this, the gene for enhanced green fluorescent protein (EGFP) was chosen as a novel DNA species that would not be present within the wastewater samples that were being examined in the project. A chromosomal insertion of the EGFP gene into the laboratory strain of *Escherichia coli* DH5α was attempted. A site-directed method of chromosomal insertion of the EGFP gene into the single copy gene *ydiA* was chosen to give a single copy of the EGFP gene in the modified chromosome, to allow accurate quantification using qPCR targeting the EGFP gene. Known numbers of *E. coli* containing the EGFP gene chromosomal modification could then be seeded into environmental samples and compared to levels of the EGFP gene detected in the DNA extracts. The obvious limitation to this approach is that extraction efficiencies could only be estimated for gram negative bacteria with cell wall characteristics similar to *E. coli*. It is hoped that this could lead to future work utilising DNA markers in other classes of organisms, such as gram positive bacteria, viruses and encysted protozoa or helminth eggs, to assess DNA extraction efficiencies for these groups of organisms. Details of the process undertaken to construct this internal standard are given in Appendix I.

2.2. Assessing the Presence of PCR Inhibiting Compounds

Humic acids were used as a model PCR inhibiting compound for this study. Matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectroscopy and electrospray ionisation mass spectroscopy (ESI-MS) were used to detect humic acids. Both direct and indirect detection of humic acids were attempted using the methods outlined in Mugo and Bottaro (2004). Indirect detection involves exploiting the ability of humic acids to covalently bind to DNA. Detection methods were optimised for a 25 mer single-stranded DNA oligonucleotide standard. It was reasoned that the addition of humic acids would cause either a mass shift or completely suppress the detection of the oligonucleotide standard and the amount of inhibition can therefore be semi-quantified. For a full description of the methods, please refer to Appendix II.

2.3. Wastewater DNA Extraction and Concentration

Three of the six wastewater treatment plants (WWTPs) involved in the Western Corridor Recycled Water Scheme were selected as the targets for sampling to determine the removal rates of select pathogens. The three WWTPs were Luggage Point, Oxley Creek and Bundamba. Luggage Point feeds directly to the adjacent Luggage Point advanced water treatment plant (AWTP), while Oxley Creek and Bundamba WWTPs supply treated wastewater to the Bundamba AWTP. The sampling took place at each plant over 24-hour periods, so the influent was taken both morning and evening at 12-hour intervals.

Sampling methods and the culture methods are as described in Toze *et al.* (2011). Select bacterial pathogens were cultured from these samples and at the same time a sub-sample was processed for preparation for DNA analysis as described below.

2.3.1. Sample Processing

Wastewater treatment plant influent: 100 mL of plant influent was transferred to two 50 mL sterile centrifuge tubes and centrifuged for 15 minutes at 4,000 g. 45 mL of supernatant was removed from each tube and the pellet was resuspended in the remaining 5 mL. The 5 mL concentrates were then pooled in a 10 mL centrifuge tube and centrifuged a second time at 4,000 g for 15 minutes. 7 mL of the supernatant was then removed and the pellet resuspended in the remaining 3 mL.

Wastewater treatment plant effluent: 1 L of plant effluent was filtered through 49 mm 0.45 µm cellulose acetate filters (Millipore) and the filtrate discarded. Filters were transferred aseptically to 10 mL centrifuge tubes. 1 mL of sterile glass beads was added to the tube and the tubes filled to the 5 mL increment with sterile TE buffer pH 7.6 (10 mM Tris, 1 mM EDTA, pH balanced using HCl). These where then fixed to rotary plate shaker and shaken for 20 minutes at 400 rpm. Liquid was then removed and transferred to 10 mL centrifuge tubes. A second amount of TE buffer pH 7.6 was added to the tube containing the filter paper and the tube shaken for a further 20 minutes at 400 rpm. The liquid from the second wash was then pooled with that off the first wash. The filter wash aliquots were then centrifuged for 15 minutes at 4,000 g and all but 3 mL of the supernatant removed and the pellet resuspended.

2.3.1.1. DNA Extraction

The same procedure was used for the concentrated WWTP influent and effluent. The method applied was a modified version of that reported by Yu and Mohn (1999). In brief, 1 mL of sterile 1 mm glass beads were added to plant concentrates contained in 10 mL centrifuge tubes. Lysozyme (Sigma) was then added to a final concentration of 100 µg/mL. Tubes where fixed to a shaker incubator at 37^{O}C and shaken for 30 minutes at 400 rpm. Proteinase K (Invitrogen) (final concentration 500 µg/mL) and extraction buffer was added to the tube (final Concentration: 50 mM Tris, 5 mM EDTA, 3 % SDS: pH 8) and returned to shaker incubator and shaken for 30 minutes at 60^{O}C with 400 rpm shaking.

Tubes where then balanced with sterile, nuclease-free, deionised water (Invitrogen) and spun at 6,000 rpm for 10 minutes. The supernatant was then transferred to a clean 10 mL tube and the original tube discarded. Ammonium acetate (Sigma) was then added to the supernatant to final concentration of 2 M. Tubes were incubated at room temperature for 5 minutes then centrifuged at 6,000 g for 30 minutes. The supernatant was removed transferred to a new 10 mL tube and the pellet discarded. An equal volume was of ice-cold isopropanol was added to incubated on ice for 5 minutes.

Tubes where then centrifuged for 40 minutes at 6,000 rpm at 4^{O}C. The supernatant was removed and the pellet washed in 500 µl of 70 % molecular grade Ethanol (Sigma) in nuclease-free water. This was then centrifuged for a further 10 minutes at 6,000 g and the supernatant discarded. The pellet was then dried to remove residual ethanol and the pellet resuspended in 400 µl of nuclease-free water.

The DNA extract was then transferred to a 1.7 mL centrifuge tube and the DNA precipitated a second time using the procedure just described. DNA extracts were finally resuspended in 50 µL nuclease-free water and stored at -20^{O}C until analysis.

2.3.1.2. Reduction of Inhibition

Post-extraction clean-up methods were explored for WWTP DNA purification to increase removal of any PCR inhibiting compounds that co-purified during extraction and assess the "PCR-ability" of the sample. The DNA extracts were obtained during the routine sampling of the Oxley Creek WWTP and performed in parallel with the sample processing for extracts used for qPCR analysis using the methods outlined earlier. The samples used corresponded to Oxley Creek WWTP influent at 6/4 am, 6/4 pm 8/4 am and 8/4 pm and plant effluent at 7/4 am, 7/4 pm, 9/4 am and 9/4 pm. The clean-up methods trialed included: the commercially available PowerClean® DNA clean-up kit (MoBio); heating and centrifugation of the samples to remove particulate matter; and dilution of the extracts.

DNA quality and "PCR-ability" were assessed before and after clean-up. DNA quality was assessed spectrophotometrically using a Biophotometer by measuring the absorbance at 260 nm to quantify DNA concentration and at 360 nm to assess contaminants, specifically humic acids.

The "PCR-ability" of samples was assessed by standard PCR using the primers and reaction conditions for the lacZ reaction outlined in Appendix III. Standard PCR samples were spiked with genomic DNA extracted from *Escherichia coli* strain ATCC 15766 to ensure that negative results were due to inhibition rather than lack of template DNA. All standard PCRs were performed on a BioRad C1000 thermocycler with the following modifications to the method. Go Taq green PCR master mix (Promega) was used with the following reaction conditions - 10 µL Master mix; 1 µL Template; 500 nM Primers; up to 20 µL Water - with a three step reaction (95^0C for 30 sec, 60^0C for 30 sec and 72^0C for 30 sec) repeated 31 times. Reactions were visualised on a 3 % Agarose gel stained with 10 µg/mL of ethidium bromide.

2.3.1.3. Inclusion of Internal Standard to Assess Reaction Inhibition

An internal standard was used to assess inhibitor effects and provide confidence in the pathogen quantification data. This involved designing duplex reactions targeting the organism of interest, coupled with a reaction targeting the gene for green fluorescent protein (GFP), allowing for known a concentration of plasmid containing the GFP gene to be spiked into the reaction. The GFP gene was used as the likelihood of this gene being present within wastewater samples was extremely low, which was confirmed using negative controls not spiked with plasmid (data not shown). All samples for qPCR analysis were diluted 1:1, 1:5 and 1:25 and the duplex assay performed on all dilutions. Controls only containing plasmid were run on every plate so the inhibitor effects could be assessed by examining the difference in Ct values between the neat internal standard and the internal standard run with the sample. Only the dilution which showed comparable amplification values to the control internal standard was used for the final analysis to work out cell numbers. The development of the internal standard is detailed in Appendix I.

2.4. Nucleic Acid Primer and Probe Design

Nucleic acid primers for each of the selected pathogens were designed and optimised using control cultures. The DNA sequence used for each of the primers is given in Table 10, Appendix III.

A duplex taqman reaction was used, coupling the detection reaction for the pathogen of interest with a taqman reaction targeting the EGFP gene contained within the plasmid pEGFP. The taqman EGFP reaction was designed specifically for this study. An extensive literature review was conducted to review published primers and probes sequences that have been used previously for detection of the pathogens of interest examined within this study. A combination of published detection oligonucleotides and sequences designed specifically for this project was finally used. All published oligonucleotide sequences were validated for specificity using desktop bioinformatic tools before ordering using the following methods.

Primer sequences where BLAST analysed using the blastN algorithm against the NCBI sequence database to confirm specificity. DNA sequences of the target gene from the organism of interest where then obtained from the NCBI genebank database from as many strains of the target organism as possible, with a minimum of five strain sequences being used. DNA sequences were then converted into FASTA format within a single file and aligned using ClustalX software (Larkin *et al.*, 2007). A consensus sequence was obtained and primer and probe sequences where aligned against this using the BioEdit software package (Hall, 1999). Self homology (hairpin formation) and dimerisation with other oligonucleotide used in the duplex taqman reaction was analysed using web-based resources from the Integrated DNA Technologies (IDT) website.

2.4.1. Primer Optimisation

For all optimisations and standard curve generation, genomic DNA extracted from pure cultures was used as template DNA. Optimal concentrations of primer and probe where generated by running dilution series of primer and probe for single plex and duplex reactions with the EGFP reaction for all pathogen detection reactions. Annealing temperature was optimised by running single plex and EGFP duplexed pathogen detection four-point, 10-fold dilution standard curve reactions over a temperature gradient. Optimal annealing temperature was chosen according to the reaction efficiency of the pathogen detection reaction. Specificity of the reactions was confirmed by single plex and duplex reactions against genomic DNA from both distant and closely related bacterial species. All optimisations were performed using a Bio-Rad CFX96 lightcycler and Brilliant III mastermix as their reaction chemistry.

2.4.2. Reaction Conditions

All reactions were performed on a Bio-Rad CFX96 light cycler. All wastewater DNA extracts where five-fold serially diluted to final concentration of 1 in 25. qPCR analysis was performed in triplicate for all pathogens of interest using 1 µL of the 1:1, 1:5 and 1:25 dilutions of wastewater DNA extracts as template for the reaction. One pg of pEGFP was added to each reaction to act as the internal standard to access inhibitor affects. All qPCR plates examining wastewater extracts contained three normaliser wells containing replicates of a duplex reaction containing 10^6 chromosomal copies of the pathogen of interest and 1 pg of pEGFP. This was added to allow later normalisation for inter-run variability and quantification against standard curves. All reactions used Brilliant III mastermix (Agilent) as their reaction chemistry as per the manufacturer's instructions. Concentrations of primers and probe for each pathogen detection reaction are outlined in Table 11, Appendix III. In brief, all reactions had an initial 10 minute denaturation step at 95^{O}C to activate the polymerase used in the Brilliant III mastermix. This was followed by 40 two-step amplification cycles consisting of 95^{O}C for 15 seconds and one minute annealing using the temperature outlined in Table 11, Appendix III. Fluorescent signal acquisition was performed during the annealing step. The details of the reactions are given in Appendix III.

2.4.3. Normalisation of Inter-Run Variance and Data Analysis

All qPCR reactions were run on 96 well plates. As it was prohibitive in both time and consumables to add a standard curve to every plate, inter-run normaliser wells where added. Inter-run variance, such as optimisation of threshold values for determining Ct values, could then be normalised for between runs using the normaliser standards on each plate. This allowed the unknown reactions to be quantified against standard curves generated from earlier runs. The inter-run normalisers consisted to three replicate wells containing a duplex reaction targeting the organism of interest and the GFP gene seeded with 1 x 10^6 genome copies of the target organism and 1 pg of the pEGFP plasmid. To reduce manual handling errors, a single stock solution was made with both genomic DNA and plasmid which could be used for plates.

Acquisition of all quantitative PCR data and preliminary quality analysis was performed using the CFX Manager software package (BioRad). All Ct values were then exported to the Excel software package (Microsoft) for manual normalisation and generation of averaged standard curves and quantification of unknowns. Details of the process used are given in Appendix III.

3. RESULTS AND DISCUSSION

3.1. The Hunt for PCR Inhibitors

Many compounds present in environmental and clinical samples can inhibit the polymerase chain reaction, some of which will co-purify with DNA during nucleic acid extractions. This inhibition can range from a decrease in reaction efficiency to total suppression of the PCR. This can have severe implications when PCR-based methods are used for surveillance of organisms of public health concern; causing anything from an under-estimation of pathogen numbers to a false negative result. This project aimed to explore methods for screening DNA extracts for the presence of PCR inhibiting compounds, providing an additional external control to give veracity to PCR-based methods of pathogen detection.

Humic acids are a broad class of high molecular weight organic compounds containing multiple phenolic groups and were used here as model PCR inhibiting substances. They were chosen as they make up 50-80 % of the dissolved organic matter in freshwater systems (Wetzel, 2001) and readily co-purify with DNA (Robe *et al.,* 2003). The mechanism by which humic acids inhibit PCR is thought to be both through the phenolic groups directly disrupting the enzyme by binding to amides in the protein or being oxidised to quinones which then covalently bind directly to DNA (Young *et al.,* 1993).

Both matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) spectroscopy and electrospray ionisation mass spectroscopy (ESI-MS) techniques were trialled as detection methods for humic acids. Neither method was successful for directly detecting humic acids in this study. Several authors report successful detection of humic acids in the literature using MALDI-TOF (Pacheco and Havel, 2002; Mugo and Bottaro, 2004; Shinozuka *et al.,* 2004). This study directly replicated the methods outlined in Mugo (2004b) without success after several attempts. Both MALDI-TOF and electrospray mass spectroscopy rely on generating ions of the analyte which are then detected, though they use different methods for doing so. MALDI-TOF relies on a high energy laser to ablate the analyte from a surface and transfer it into a high-energy state. Conversely, electrospray ionisation nebulises a polar analyte in solution under a heated gas flow to remove the target molecule from the bulk liquid. As both techniques are heavily reliant on generating ions of the analyte, they are easily inhibited by compounds that will either electrochemically stabilise the analyte or more efficiently form ions, effectively stealing energy from the analyte. In the present case, the humic acids were not being detected because they were not being effectively ionised, either through deficiencies in the method, charge dampening contamination or the innate chemistry of the analyte inhibiting ionisation.

As direct detection of humic acids was unsuccessful, indirect detection was attempted. This involved detecting a single stranded oligonucleotide standard and then looking for either a mass shift in the oligonucleotide standard or its absence, indicating it was bound to humic acids. Indirect detection lacks the ability to characterise the inhibitor but could have valuable practical applications telling the "PCR-ability" of a DNA extract, at least in terms of DNA binding PCR inhibitors. Once again, the humic acids failed to be detected, but they also failed to inhibit the detection of the oligonucleotide standard, as it was as readily detected in the humic acid treated samples as the untreated controls. Several factors could be responsible for the ineffective indirect detection of humic acids, including:

1. MALDI-TOF is not quantifiable, even though the humic acid was binding the oligonucleotide standard, there was insufficient unbound DNA to be detected; and
2. The phenol groups of the humic acid where not sufficiently oxidised to quinones to covalently bind the DNA standard.

The project was halted at this point to save on further time and expense. As mentioned previously, humic acids are a major class of PCR inhibiting substance in water and being unable to effectively detect them made the project unfeasible. Therefore, a trial and error approach of other published methods was taken as outlined in 2.3.1.1 above, the outcomes of which is described here in 3.1.1.

3.1.1. Reduction and Accounting for PCR Inhibition Effects

As mentioned previously, the effect of PCR inhibitors is a serious concern using qPCR methods as it can cause either an underestimation of cell numbers or even total suppression of the reaction, resulting in the false negative. Inhibition was addressed using several methods in this study. Removal of inhibitor affects was assessed by examining treatment strategies of DNA extracts. Accurate quantification of inhibitor effects was achieved through use of an internal standard run in parallel with all samples.

Direct measures of spectrophotometric indicators of DNA quality and "PCR-ability" before and after post-extraction clean up were measured. The results and methods comparison are outlined in Table 2 and Table 3 for spectrophotometry measures and "PCR-ability" respectively. It should be noted that all PCR assays for all samples were run in parallel with an aliquot spiked with *E. coli* strain ATCC15766 genomic DNA to ensure that negative results are due to inhibition rather than lack of template. Overall, the commercially available MoBio kit was the most efficient at removing contaminants as measured by the absorbance at 340 nm. However, this came at the cost of drastic reductions in DNA yield. Reductions in the DNA yield would have serious implications for the accuracy of molecular quantification methods, due to loss of target DNA sequences. To avoid this shortfall in the MoBio clean-up procedure, the heating and spinning method was chosen to clean-up samples.

Table 2. DNA extract spectrophotometric quality control measures.

Sample	Native		After MoBio clean up kit		After heat+spin	
	ng/uL	A340	ng/uL	A340	ng/uL	A340
Oxley creek primary 6/4 am	155	0.256	-7	-	130	0.072
Oxley creek primary 6/4 pm	431	0.2	-8	-	286	0.055
Oxley creek secondary 7/4 am	4	0.007	-11	-	-12	-
Oxley creek secondary 7/4 pm	0	0	-11	-	-14	-
Oxley creek primary 8/4 am	155	0.466	-12	-	115	0.255
Oxley creek primary 8/4 pm	188	0.882	-12	-	156	0.281
Oxley creek secondary 9/4 am	18	0.031	-12	-	-10	-
Oxley creek secondary 9/4 pm	13	0.111	-12	-	-13	-

Table 3. PCR-ability of the WWTP extracts after treatment, where a "-" is a negative result and "yes" indicates that a PCR product was detected.

Sample	Dilution	No Post-Extraction Treatment	Post-Extraction MoBio Clean Up	Post-Extraction Heat+Spin
Oxley creek primary 6/4 am	neat	-	yes	-
"	1:5	-	yes	yes
"	1:25	yes	yes	yes
Oxley creek primary 6/4 pm	neat	-	yes	-
"	1:5	-	yes	-
"	1:25	yes	yes	yes
Oxley creek secondary 7/4 am	neat	yes	yes	yes
"	1:5	yes	yes	Yes
"	1:25	yes	yes	yes
Oxley creek secondary 7/4 pm	Neat	yes	yes	yes
"	1:5	yes	yes	Yes
"	1:25	yes	yes	yes
Oxley creek primary 8/4 am	Neat	-	yes	-
"	1:5	yes	yes	-
"	1:25	yes	yes	yes
Oxley creek primary 8/4 pm	Neat	-	yes	-
"	1:5	-	yes	-
"	1:25	yes	yes	yes
Oxley creek secondary 9/4 am	Neat	-	yes	yes
"	1:5	yes	yes	yes
"	1:25	yes	yes	yes
Oxley creek secondary 9/4 pm	Neat	yes	yes	yes
"	1:5	yes	yes	yes
"	1:25	yes	yes	yes

3.2. Construction of an Internal Standard to Assess Sampling and DNA Extraction Efficiency

The accuracy of molecular methods for pathogen identification is pivotal to extracting nucleic acids for analysis that are representative of the microbial population of the sample. Exploration of the literature shows that little work has been done to produce viable measures to ensure that DNA extraction, processing and storage steps yield high quality material for analysis and have instead concentrated on standards for the analytical techniques. This is a serious impediment in the implementation of genetic methods as some studies have found as little as 0.98 % recovery rates of spiked DNA using standard extraction procedures for DNA purifications in water matrices (Weinbauer *et al.,* 2002; Stoeckel *et al.,* 2009). Without an accurate measure of nucleic acid extraction efficiency, the accuracy of molecular pathogen detection methods can be called into question. To address this, genetic modifications to a the laboratory strain *Escherichia coli* DH5α were attempted, involving insertion of the gene for green fluorescent protein (GFP) and kanimycin resistance (kan^R) into the chromosome of the organism. The final genetically modified *E. coli* could then be seeded into samples at known concentrations to assess the efficiency of the sampling and extraction processes.

The novel genes chosen for this study were contained on a DNA construct that was created, which would then be inserted into the chromosome of DH5α. A chromosomal insertion was considered the best way to stably and quantifiably import the novel genes to the bacteria. Introduction of the genes on a plasmid to *E. coli* by transformation was discounted as the plasmid would have a variable copy number for each cell, disallowing accurate quantification by qPCR, and be susceptible to horizontal gene transfer to other bacteria within the sample. The *E. coli* strain DH5α had the added advantage of not containing the *lacZ* gene, the target of *E. coli* qPCR assay, so insertion of the standard would not skew qPCR quantification of *E. coli* within the sample.

Technical problems creating the DNA construct in addition to early finalisation of the project precluded completion of the modified *E. coli* strain which could be used as an internal standard. Appendix I provides a full technical discussion of the methods used and hurdles encountered. The internal standard would add much greater confidence in pathogen quantification data and microbial risk assessment predictions. As such, generating standards, not only for *E. coli* but also other classes of microorganisms of public health concern such as gram positive bacteria, viruses and parasitic protozoa is worthy of future research.

3.3. Comparison of Culture-Based Methods and qPCR on Wastewater Samples

The results are presented as a comparison of numbers (log cells/100mL) recorded using the culture-based methods (detailed in Toze *et al.*, 2011) to quantitative qPCR. Figures 1 to 3 compare the log removal of *E. coli* cells as determined by culture and qPCR at the three WWTPs. While the log removal was generally similar on average between the two methods (Table 4), the qPCR method tended to over-estimate the whole numbers of cells. Whether this is an over estimation of the numbers or a more accurate assessment of the actual numbers present in the samples requires further confirmation with more replicates and inclusion of specific methods for the detection of viable cells as suggested below. The log removal of *E. coli* from the three activated sludge processes included in the study ranged from 2.7 to 3.27 log as determined by culture-based methods and 3.18 to 4.44 log by qPCR. The indicative log reductions for *E. coli* by secondary treatment as given by the Augmentation of Drinking Water Supplies – Australian Water Recycling Guidelines (NRMMC, EPHC and NHMRC, 2008) is a range from 1.0-3.0 log, so both methods were within this range, although the qPCR technique has over-estimated the log removal at the Luggage Point WWTP by 1.5 log.

Table 4. Comparison of the average log removal of *E. coli* cells from three wastewater treatment plants based on both culture (plate counts) and quantitative PCR.

Wastewater Treatment Plant	Log Removal: Culture (±SE)	Log Removal: qPCR (±SE)	T-test p Value
Luggage Point	3.27 ± 0.07	4.44 ± 0.12	3.4×10^{-5}
Oxley Creek	2.77 ± 0.19	3.53 ± 0.11	0.011
Bundamba	2.70 ± 0.14	3.18 ± 0.08	0.015

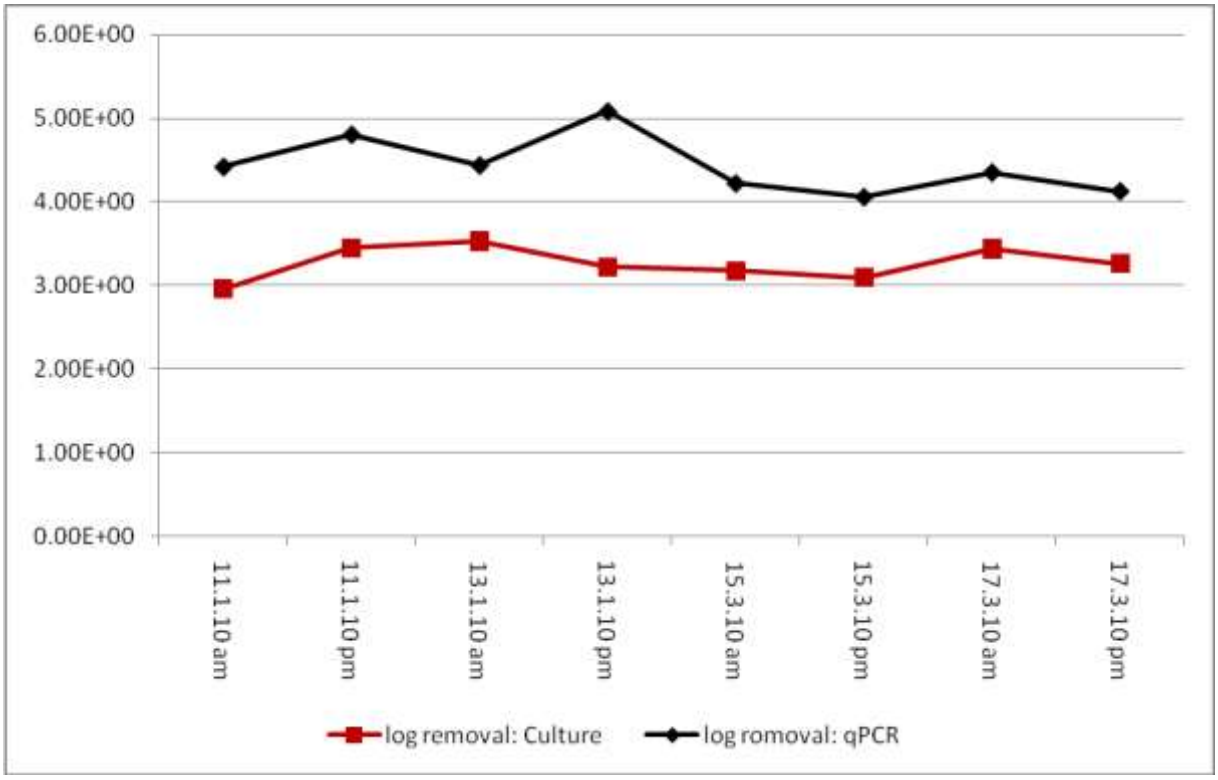


Figure 1. Comparison of log removal of *E. coli* cells as determined by culture and qPCR using the Luggage Point WWTP samples. The sample dates 1 – 10 were from 11 Jan to 17 March 2010.

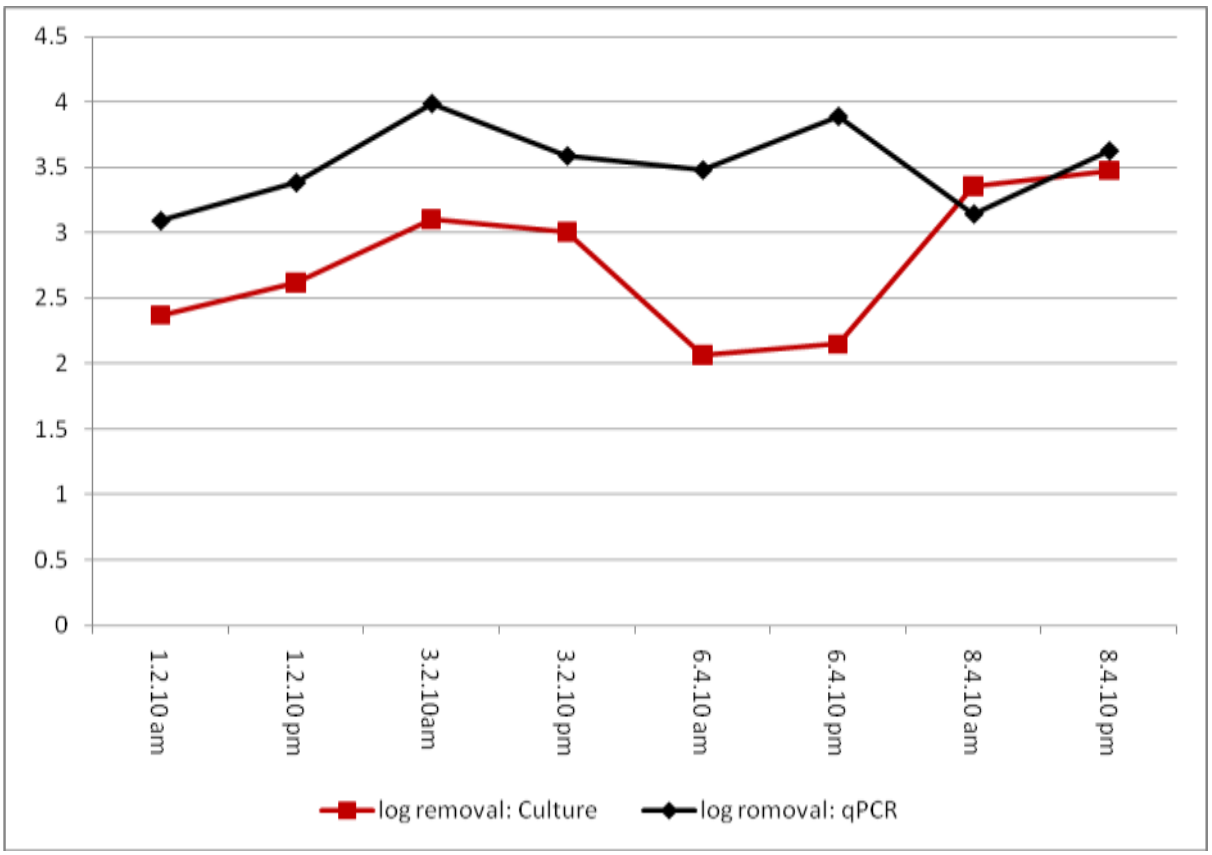


Figure 2. Comparison of log removal of *E. coli* cells as determined by culture and qPCR using the Oxley Creek WWTP samples. The sample dates 1 – 10 were from 1 Feb to 8 April 2010.

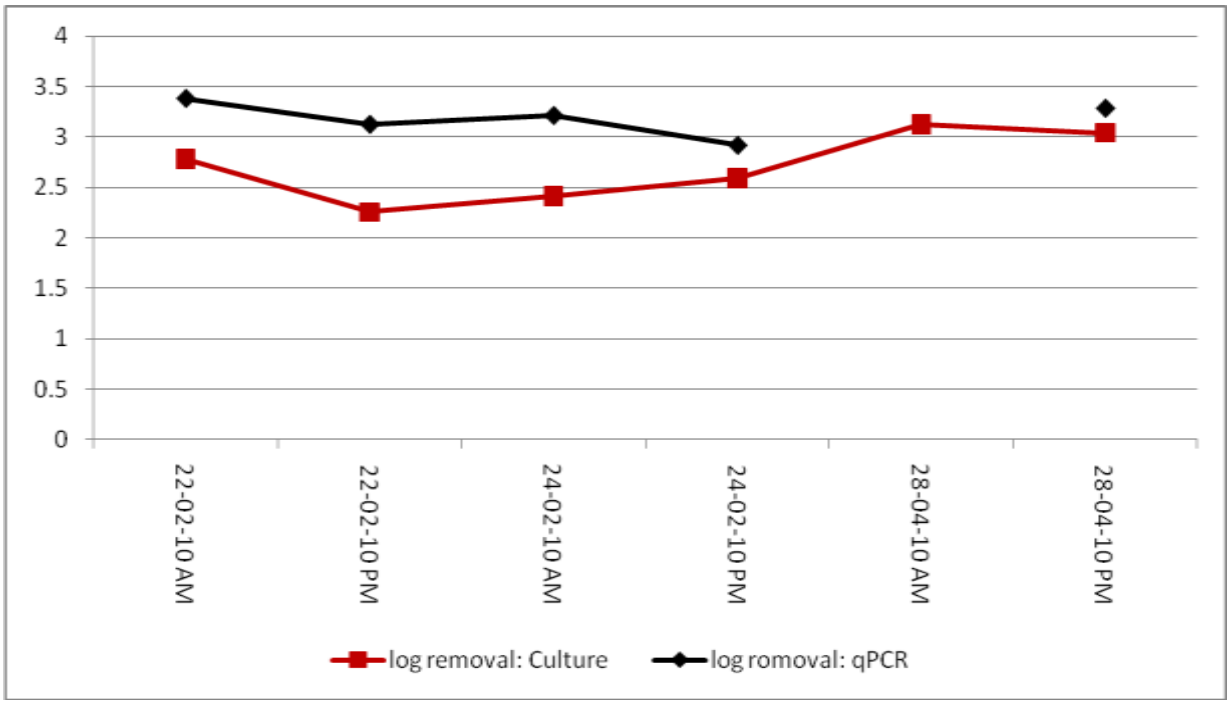


Figure 3. Comparison of log removal of *E. coli* cells as determined by culture and qPCR using the Bundamba WWTP samples. The sample dates 1 – 10 were from 22 Feb to 28 April 2010.

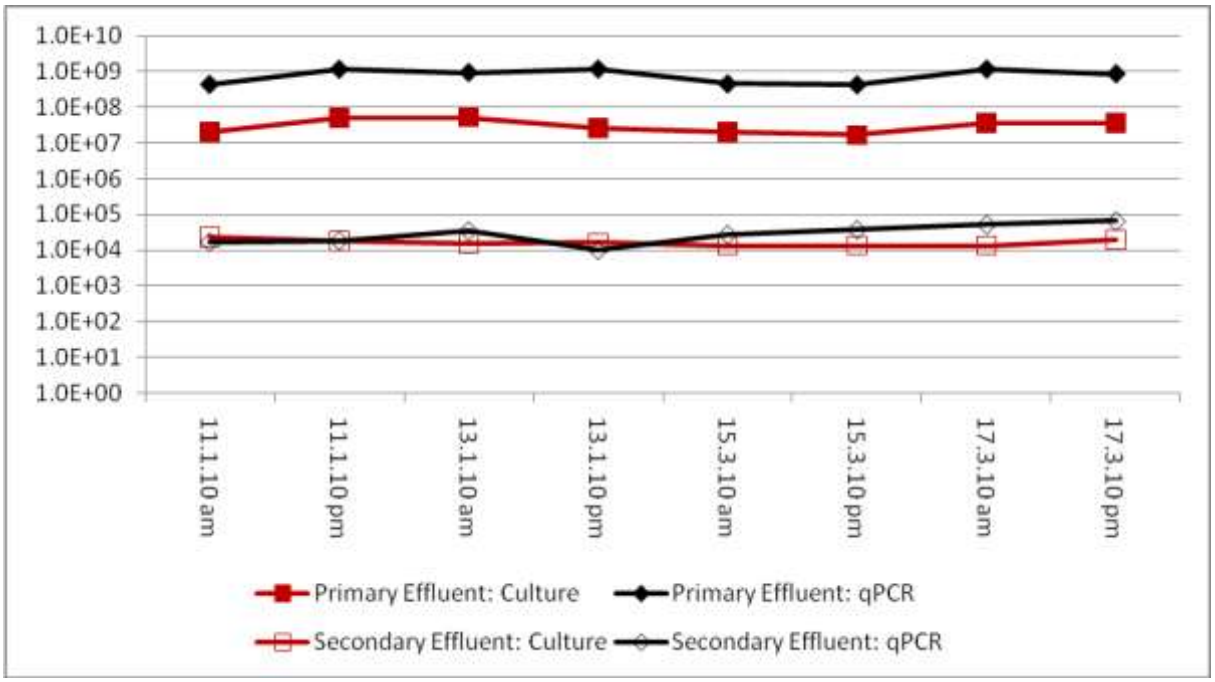


Figure 4. Comparison of whole number of *E. coli* cells (log) as determined by culture and qPCR in the Luggage Point WWTP samples. The sample dates 1 – 10 were from 11 Jan to 17 March 2010.

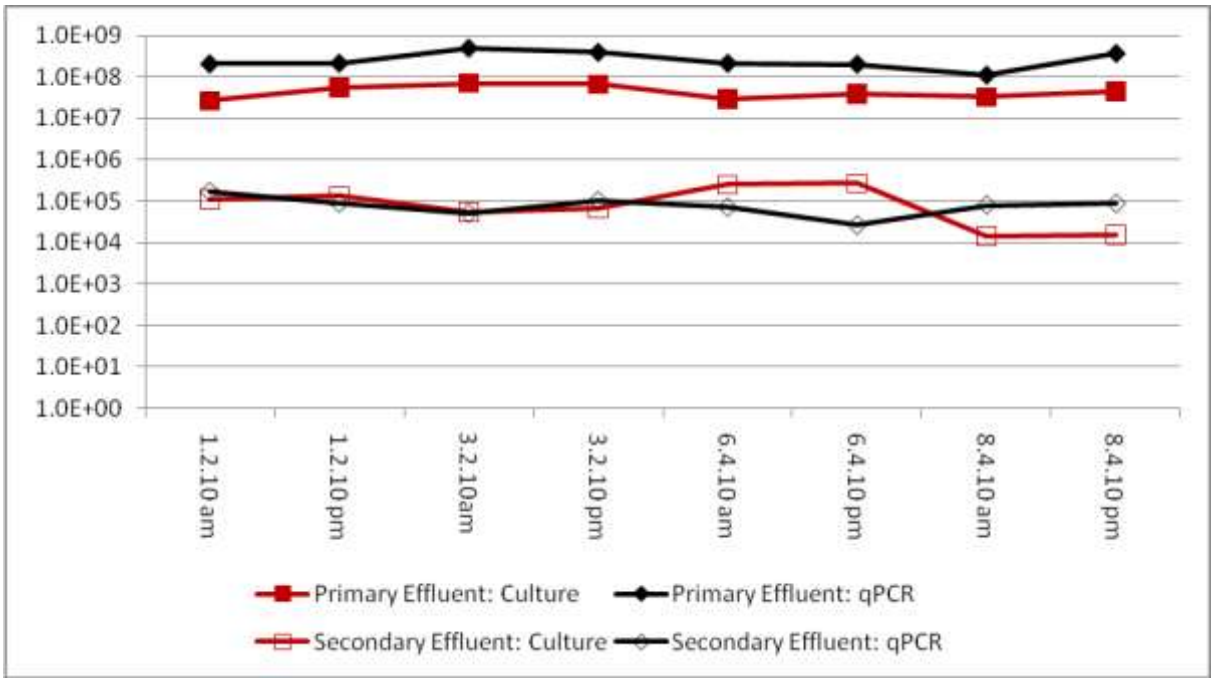


Figure 5. Comparison of whole number of *E. coli* cells (log) as determined by culture and qPCR in the Oxley Creek WWTP samples. The sample dates 1 – 10 were from 1 Feb to 8 April 2010.

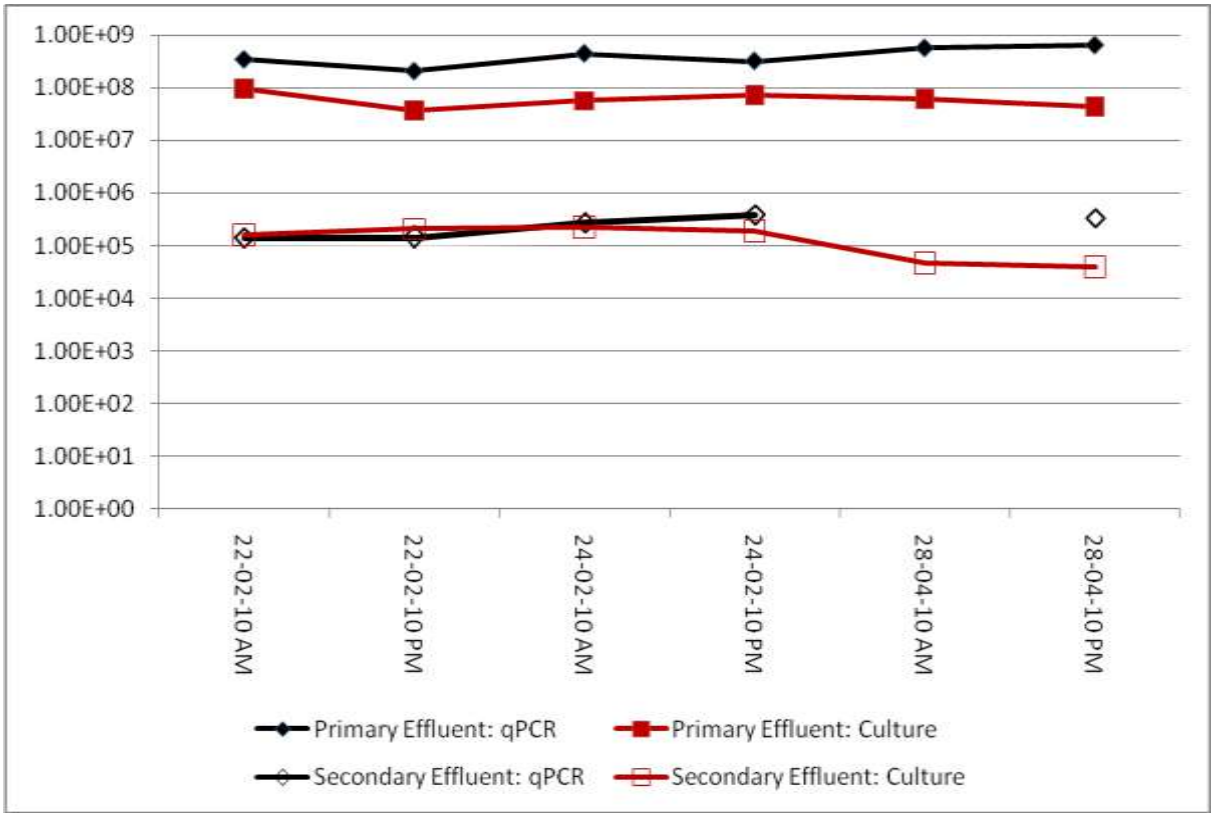


Figure 6. Comparison of whole number of *E. coli* cells (log) as determined by culture and qPCR in the Bundamba WWTP samples. The sample dates 1 – 10 were from 22 Feb to 28 April 2010.

Influent and effluent of the three WWTPs were screened for *E. coli* cell numbers using culture-based and qPCR quantification methods. Figures 4 to 6 compare the whole number of *E. coli* cells (log) as determined by culture and qPCR at the three WWTPs. Table 5 summarises the average *E. coli* counts over the sampling period using culture-based and qPCR techniques with statistical analysis of the variance between the data obtained by the two methods. Highly comparable results were observed between the two methods when assessing the *E. coli* numbers in the effluent of all three plants. This is reflected in the statistical analysis as no significant variation occurred between the effluent cell numbers in any plant using qPCR or culture-based methods. In contrast to this, qPCR analysis consistently provided between 5 and 10-fold higher cell numbers than culture-based techniques, with a high degree of statistically significant variation between the two methods.

Table 5. Average *E. coli* numbers over the sampling period with statistical analysis of the difference between the quantification techniques.

Sample	Mean E. coli numbers - Culture	Standard Error	Mean E. coli numbers - qPCR	Standard Error	T-Test P value
Oxley Creek Influent	4.6×10^7	6.05×10^6	2.78×10^8	4.51×10^7	0.0007
Oxley Creek Effluent	1.2×10^5	3.55×10^4	8.45×10^4	1.46×10^4	0.4888
Luggage Point Influent	3.3×10^7	4.87×10^6	8.39×10^8	1.22×10^8	0.0002
Luggage Point Effluent	1.74×10^4	3.47×10^3	3.29×10^4	6.69×10^3	0.0508
Bundamba Influent	6.2×10^7	8.74×10^6	4.16×10^8	6.56×10^7	0.0033
Bundamba Effluent	1.4×10^5	3.27×10^4	2.55×10^5	4.81×10^4	0.2282

Several studies have assessed the differences in pathogen detection and enumeration between qPCR and culture-based methods. Increases of up to one log unit in pathogen numbers using qPCR over culture-based methods has been reported in *Enterococcus* species in river and lake water (Haugland *et al.,* 2005), *E. coli* O157:H7 in soils (Ibekwe *et al.,* 2004) and river water (Ram *et al.,* 2008). The reasons for this may lie in the modes of detection between the two methods. Culture-based techniques are dependent on harvesting actively growing viable cells, whereas qPCR methods simply require the DNA template for the assay to be present. Due to this, qPCR has the potential to include dead cells or free DNA in the analysis, giving the propensity to overestimate cell numbers. Conversely, as culture-based methods only detect actively growing organisms, viable bacteria in arrested growth states can be overlooked, causing an underestimation in growth states.

The viable-but-non-culturable (VBNC) growth state has been the subject of much research and debate (Oliver, 2010). Organisms in this growth state can retain their pathogenicity though are undetectable using culture-based methods. This phenomenon can be triggered by a variety of environmental stimuli including temperature shifts, nutrient limitation, osmotic stress, heavy metals, chemical disinfectants and even visible light, and is well reported in *E. coli,* including pathogenic strains (Oliver, 2010). In an earlier study, it was found that *E. coli* cells exposed to mild ultra violet (UV) and heat stress and nutrient depletion resulted in a large portion of the population becoming unculturable, while still retaining metabolic activity (Fiksdal and Tryland, 1999). Investigations of *E. coli* O157:H7 in chlorinated water and river water found that VBNC organisms could cause underestimations of up to 90 % and 14 % of cell numbers using culture-based methods in the two water matrices respectively (Liu *et al.,* 2010).

A study that enumerated *Enterococci* sp. in biosolids using 23S rRNA qPCR and membrane filtration with mEI-agar culture analyses demonstrated that the values were not significantly different for a given treatment ($P > 0.05$, paired t-test). Both assays showed differences in biosolid treatment effectiveness - anaerobic digestion treatments averaged 5-5.5 log genomic units (GU) and colony forming units (CFU)/dry g (Viau and Peccia, 2009). This is encouraging, particularly given that biosolids are probably one of the most difficult matrices to recover cells and DNA from.

As qPCR increasingly becomes the method of choice for microbial community analysis and enumeration, greater research has been undertaken to overcome its shortfalls, specifically targeting dead organisms or free DNA. Studies examining the persistence of extracellular DNA in soil and sediment have found quantities of DNA detectable by PCR after 20 days, though extremely high levels of fragmentation had occurred (Ceecherini *et al.,* 2007). In contrast, another study examining the contribution of extracellular DNA using PCR methods for microbial enumeration in seawater reported that seeded extracellular DNA persisted for around 24 hours at levels close to the limits of detection (Bae and Wuertz, 2009). In an attempt to only target viable organisms using PCR methods, a number of strategies has been attempted, including the effectiveness of targeting mRNA. This has had variable success as often mRNA expression levels have little bearing on cell numbers, though several detection assays for viable *E. coli* targeting mRNA from strain specific and stress response genes have been attempted (Sharma, 2006; Zhao *et al.,* 2006).

Another method to minimise the influence of dead cells or extracellular DNA on PCR-based microbial quantification methods has been to treat samples with propidium monoazide (PMA). The PMA molecule is a PCR inhibitor which covalently binds DNA but is impermeable to intact cell membranes, effectively stopping the amplification of DNA targets from dead cells and extracellular DNA (Nocker *et al.,* 2007; Nocker *et al.,* 2010). This has been used effectively to discriminate live targets for quantification by qPCR (Bae and Wuertz, 2009; Agusti *et al.*, 2010; Kobayashi *et al.,* 2010) as well as microbial community analysis using microarray (Yergeau *et al.,* 2010) and 454 pyrosequencing (Nocker *et al.,* 2007). Future studies for enumeration of pathogens in wastewater should explore the use of PMA to limit the detection to viable cells.

The limitations of inhibition and detecting only viable cells when using genetic techniques is a feasible explanation for the discrepancy between qPCR and culture-based methods in the WWTP influent and effluent. It can be assumed that bacteria in both influent and effluent will be in a variety of growth states and viability. Interestingly, in a similar study He and Jiang (2005) examined *Enterococci* cell numbers in effluent from two WWTPs and found comparable results from qPCR and culture methods. These authors also found that chlorination of the effluent resulted in no detection using culture-based methods but only a slight reduction in qPCR results, strongly inferring the detection of nucleic acids from non-viable organisms and/or the adoption of VBNC cell state.

Both culture-based and qPCR methods for determining *E. coli* cell numbers were highly comparable in this present study. PCR-based methods do have many advantages over culture-based methods for microbial pathogen monitoring and risk assessment, most significantly providing accurate pathogen numbers in a matter of hours rather than days. Though PCR methods offer unique confounds when compared to culture-based methods, such as reaction inhibition, this study illustrates the utility of qPCR methods, with the use of proper controls, for accurately monitoring pathogen numbers.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Key Findings

The application of qPCR for the quantification of *E. coli* in wastewater samples was comparable to culture-based methods, indicating that, with some further work, full validation of these techniques is possible and could replace culture-based methods in the future.

This study aimed to provide further weight to using qPCR methods for surveillance of pathogens by addressing some of the key deficiencies of the method. This included controlling for efficiency in nucleic acid purifications from the sample and both detection and controlling for the effects of PCR-inhibiting compounds that often co-purify with nucleic acids. Generation of a genetically modified *E. coli* strain containing a novel chromosomal DNA insertion for use as a seeding control and mass spectrometric detection of inhibitors was attempted to address DNA extraction and detection of inhibition respectively.

Though these proved too ambitious due to early finalisation of the project, qPCR reaction efficiency was adequately addressed through the inclusion of an internal standard in all qPCR assays, by seeding reactions with a known amount of plasmid containing the gene for green fluorescent protein (GFP). All pathogen detection assays were designed as duplex taqman reactions targeting both the pathogen of interest and the GFP gene. This allowed PCR inhibition in the samples to be assessed, giving confidence that the pathogen quantification data did not contain either false negatives or depressed pathogen numbers due to reduced efficiency of the reaction. This work has shown the efficacy of the qPCR for pathogen monitoring in water matrices but has also highlighted the need for further controls to ensure the accuracy of the method.

4.2. Future Research

To finalise this work, as detailed in Appendix III, a set of detection methods for all of the reference pathogens listed in the current water recycling guidelines would need to be completed. This could include primer sets that have been optimised for *Campylobacter jejuni* and *C. coli*, *Salmonella* sp., Enterococci sp. and *E. coli* 0157:H7 and are ready for validation in the same way that *E. coli* was as described in Section 3.2. Immediate future work would:

- Continue to improve DNA extraction methods to reduce reaction inhibition;
- Refine internal standards, including a chromosomal insertion of the control gene;
- Continue to improve primer and probe design, specifically to include infectivity/viability; and
- Extend the list of pathogens to include viruses, protozoa and helminths that are validated against conventional and culture-based methods, ultimately to develop multiplex assays to test multiple organisms in one reaction.

APPENDIX I:
Methods and Outcomes: *Escherichia coli* Chromosomal Insertion of EGFP Gene

A shortfall in the present molecular techniques to access to access pathogen numbers from environmental samples is adequate quality controls to ensure that detection levels are representative of the original samples. One area which requires such controls is initial DNA extraction. The accuracy of downstream pathogen quantification using molecular techniques hangs on successful DNA extraction from the organism of interest. At present, adequate controls are not in place to routinely access extraction efficiency for each sample to be analysed. The work in this project attempted to address this shortfall by generating a control which could be seeded into samples prior to sample concentration and DNA extraction which could give an accurate measure of the efficiency of initial sample processing methods.

Two criteria used in design of the sample processing efficiency control. Firstly, it would contain novel genetic material that would not be present within the samples being tested. Secondly, it would have physical and chemical characteristic similar to the target organism so that it would behave in a comparable manner to target organisms during sample processing. For the present project, assessing pathogens within wastewater, a genetically modified strain of *Escherichia coli* containing a detectable DNA sequences was chosen to meet these criteria.

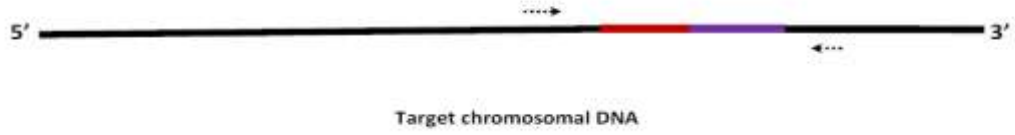
A DNA insert containing the genes for Enhanced Green Fluorescent Protein (EGFP) and kanymicin resistance (kan^R) was chosen to be inserted into the *E. coli* strain. These were chosen as successful expression of EGFP and kan^R would provide further markers that could be used for quality control indicators using other detection methods such as direct culture. Finally, it was decided that a chromosomal insertion of a genetic cassette containing the marker genes would be attempted as this result in a defined number of copies of the marker genes in modified organism. Simple transformation of the *E. coli* strain with a plasmid containing the marker genes would result in a highly variable copy number of the plasmid in each cell, disallowing accurate quantification of cell numbers seeded into the sample using a qPCR assay detecting the marker genes.

The laboratory *E. coli* strain DH5α was chosen for the host organism as it has been routinely used for genetic experimentation, is non-pathogenic, and has been genetic altered to remove the *lacZ* gene. The *lacZ* gene was the gene target chosen for the *E. coli* qPCR detection assay so addition of the genetically modified DH5α strain as a processing control would not skew downstream qPCR detection of *E. coli* from wastewater samples.

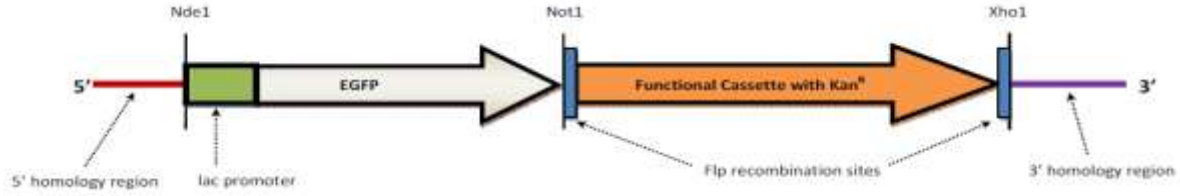
A site-directed method of chromosomal insertion of the marker genes was chosen so the number of copies of chromosomal insert could be controlled. The Quick and Easy *E. coli* Gene Deletion Kit (Gene Bridges) was chosen to perform the insertion. The kit relies on a site directed recombination of the chromosomal DNA of the organism to be modified to insert DNA containing the kan^R gene into the gene to be knocked out, causing a frameshift in gene and loss of function in the expressed protein. This requires a 50 bp arm to be added to both the 5' and 3' ends of the insertion cassette homologous to the chromosomal insertion site. This allows for the target insertion site in the chromosome of the organism to be modified to be strictly regulated. For the purposes of this study, a DNA construct would be created to include the EGFP gene into the insertion cassette. Refer to Figure 7 for a flow chart of methods.

Stage 1 of the project involved selection of a site within the chromosome of the DH5α chromosome. A literature review was undertaken to find an appropriate site within the *E. coli* genome for the insertion which would not adversely affect the physiology of the host organism. The *ydiA* gene, encoding for a putative phosphatase transporter, was chosen as a suitable site for insertion of the marker genes as previous work (for example, Baba and others, 2006) has shown little effect on cellular growth in a variety of media when this gene is non-functional.

Stage 1: Selection of chromosomal insertion site into *E. coli* strain DH5α.



Stage 2: Generation of the DNA construct containing marker genes for Chromosomal Insertion.



Stage 3: Insertion of DNA construct into target *E. coli* chromosomal site.

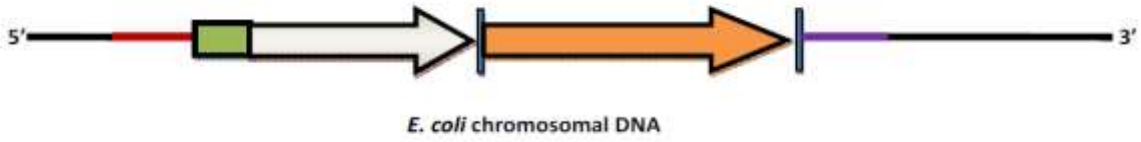
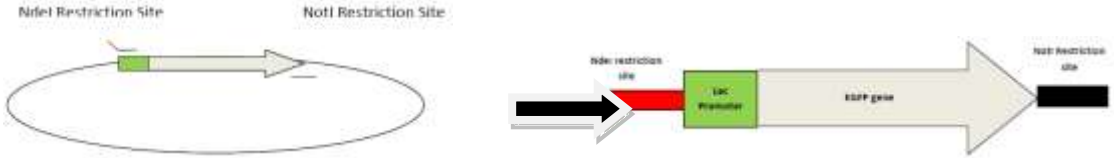


Figure 7. Flow Chart of methods for insertion of marker genes into the *E. coli* DH5α chromosome.

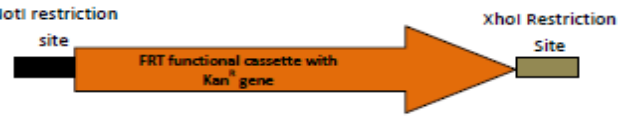
The DNA sequence of *ydiA* from several strains of *E. coli* was obtained for the NCBI genbank database. These were aligned using the ClustalX software and a consensus sequence was obtained using the BioEdit software package. The protein sequence of the YdiA protein from the *E. coli* strain K12 was obtained from the NCBI protein database. Theoretical functional regions based on amino acid sequences were determined to ascertain which regions on the gene would be relatively conserved due to function. Theoretical regions which may be unconserved and specific to the YdiA protein were highlighted and correlated back to consensus gene sequence of the *ydiA* gene. The 3' tail region of the gene was shown to have no direct physiological function according to the amino acid sequence. BLAST analysis using the blastN algorithm using the NCBI genbank data base of a 320 bp sequence in the 3' tail region of 834 bp gene proved have high specificity for the *ydiA* gene. PCR primers were designed to amplify the 307 bp region of the gene from bases 508 to 815. The 307 bp fragment containing the putative insertion site were PCR amplified and sequenced in DH5α. Table 6 of Appendix 1 contains the primer sequences used for PCR amplification and sequencing this region of the DH5α genome. A 100 bp region from bases 710 to 810 was chosen as the gene insertion site as it had little sequence homology to the rest of the *E. coli* chromosome and conformed to the design criteria for the chromosomal insertion site specified by the Gene Bridges kit.

Stage 2 of the project involved generation of the DNA insertion construct. Figure 8 below shows a diagrammatic flow chart of methods for generation of the construct. The pEGFP (Clonetech) expression plasmid containing the EFP gene preceded by the lac promoter was kindly donated by Dr Chris Brown, Griffith University. The plasmid DNA sequence, including restriction endonuclease sites, was obtained from Clonetech. In brief, restriction mapping was performed using web based restriction mapping tools on the DNA fragment containing the insertion vector and the kan^R gene and its promoter included with the Quick and Easy *E. coli* Gene deletion kit. It was found that a NotI restriction site was located immediately after the stop codon of the 3' end of EGFP gene in the pEGFP plasmid. No restriction sites were available in the 5' region preceding the lac promoter on the pEGFP plasmid. Restriction mapping of the insertion cassette fragment revealed that the 5' region preceding the insertion cassette contained a NotI site and the 3' region of the fragment contained a XhoI site after the cassette. As no other sites were present for NotI or XhoI in either the insertion cassette or the EGFP gene and lac promoter these were chosen as ligation sites.

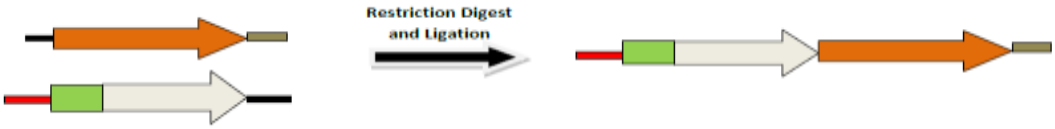
Step 1: PCR amplification of EGFP gene and lac promoter from pEGFP plasmid.



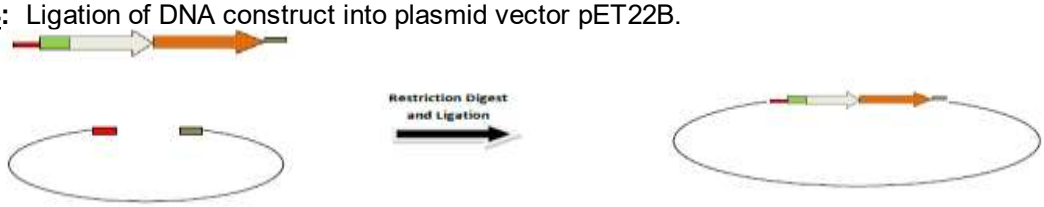
Step 2: PCR amplification of FRT insertion cassette containing Kan^R gene.



Step 3: Restriction endonuclease digestion and ligation.



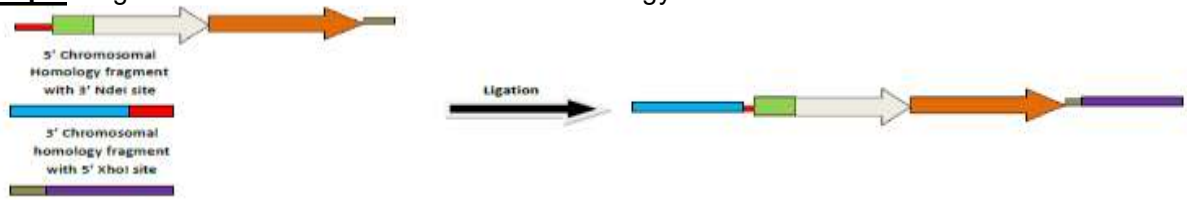
Step 4: Ligation of DNA construct into plasmid vector pET22B.



Step 5: Transformation of pET22B with insert into *E. coli* DH5α and confirmation of expression of marker genes.

Step 6: Plasmid extraction, restriction digest and purification of insert.

Step 7: Ligation of chromosomal insertion site homology arms.



Step 8: PCR amplification of DNA construct.

Figure 8. Diagrammatic flow chart of methods for generation of the DNA construct.

PCR primers were designed to amplify the region containing the lac promoter and EGFP gene from the pEGFP plasmid. The reverse primer included the NotI restriction site. As no suitable restriction sites where available at the 5' end region of interest in the pEGFP plasmid, a non-priming NdeI site was included at the 5' end of the forward amplification primer for the lac promoter and EGFP gene. An additional 6 non-priming bases where included at the 5' end of the primer before the NdeI site. These six additional bases are required for successful binding and activity of the NdeI restriction endonuclease (New England Biolabs; product information). NdeI was chosen as there were no sites for this enzyme anywhere throughout the EGFP gene and lac promoter or the insertion cassette. Amplification primers were designed for the 5' and 3' ends of the insertion cassette to include the NotI and XhoI sites respectively. Table 6, Appendix 1 shows the primer sequences and reaction conditions used for PCR amplification of components of the DNA construct.

Table 6. Primer sequences used. For primers EGFP-NdeI-F and EGFP-NotI-R, restriction enzyme sites are highlighted in red.

Primer Name	Target	Sequence	Conc.	Annealing Temp.
ydiA Seq-F	ydiA	TCT GGC AAT GCA ATT TGG TA	500 nM	58
ydiA Seq-R	ydiA	AGG CCC ATG ATA TCG AGG AT	500 nM	58
ydiA Homo-F	ydiA	CGC GGA AGT AGA AGC GTT GTA	500 nM	59
ydiA Homo-R	ydiA	CAT GAT ATC GAG GAT CTT GGT	500 nM	59
FRT-F	Insertion cassette	AAT TAA CCC TCA CTA AAG GGC GG	500 nM	58
FRT-R	Insertion cassette	TAA TAC GAC TCA CTA TAG GGC TCG	500 nM	58
EGFP-NdeI-F	pEGFP	TAA TTA CAT ATG GCG GGC AGT GAG CGC AAC GC	500 nM	58
EGFP-NotI-R	pEGFP	AGA GTC GCG GCC GCT TTA CTT GTA	500 nM	58
FRT-F end- Seq	Insertion cassette	CTA CTT CCA TTT GTC ACG TCC TGC A	500 nM	55
FRT-R end -Seq	Insertion cassette	TAT TGC TGA AGA GCT TGG CGG CGA	500 nM	55
EGFP-F end-Seq	EGFP	GTC AGC TTG CCG TAG GTG GCA T	500 nM	55
EGFP-R end-Seq	EGFP	AAG AAC GGC ATC AAG GTG AAC TT	500 nM	55

Oligonucleotides were also designed targeting the chromosomal insertion site in the *ydiA* gene of DH5α. These consisted of 50 bp arms to be ligated to the 5' and 3' ends of the construct homologous to chromosomal insertion site. To achieve this, the 5' arm would require an NdeI site on the 3' end and conversely the 3' arm would require a XhoI site on the 5' end. For each homology arm, the single stranded forward and reverse complement oligonucleotides where synthesised so that when annealed they would provide the necessary ligation site (see Table 7, Appendix I). Oligonucleotides of the homology arms were diluted to 10 µM in TE buffer pH 8.0 for both complementary stands and annealed through heating to 95 C for 15 minutes. These were then allowed to slowly cool to room temperature. Successful annealing of the complementary strands was confirmed by electrophoresis on a 3 % Agarose gel and visualised through ethidium bromide staining. Amplification primers were designed simultaneously for 5' and 3' ends of the homology arms for final amplification of the completed construct.

Table 7. Oligonucleotide design for chromosomal homology arms at the 5' and 3' end of the insertion construct. Green colour for the annealed sequence indicates chromosomal homology region. Red colour for the annealed sequence indicates ligation site.

Oligonucleotide Name	Sequence	Restriction Site
5' Homology Arm	**Forward:** CGCGGAAGTAGAAGCGTTGTACCGTAAAAATCAGATCCCGTGGATTAACACA **Reverse Complement:** TATGTGTTAATCCACGGGATCTGATTTTTACGGTACAACGCTTCTACTTCCGCG **Annealed:** CGCGGAAGTAGAAGCGTTGTACCGTAAAAATCAGATCCCGTGGATTAACACA GCGCCTTCACTCTTCGCAACATGGCATTTTTAGTCTAGGGCACCTAATTGTGTAT	**NdeI site:** ...CA TATG... ...GTAT AC...
3' Homology Arm	**Forward:** TCGAGGTACCAATTATTCGGTAGAAGAGATTGCCACCAAGATCCTCGATATCATG **Reverse Complement:** CATGATATCGAGGATCTTGGTGGCAATCTCTTCTACCGAATAATTGGTACC **Annealed:** TCGAGGTACCAATTATTCGGTAGAAGAGATTGCCACCAAGATCCTCGATATCATG CCATGGTTAATAAGCCATCTTCTCTAACGGTGGTTCTAGGAGCTATAGTAC	**XhoI site:** ...C TCGAG... ...GAGCT C...

The next step in generation of the DNA construct was to be ligation of PCR amplification of the lac promoter and EGFP gene fragment and the insertion cassette. These would then be cloned into an expression vector and transformed into *E. coli* DH5α. This step was required to ensure that both the EGFP gene and Kan^R gene where being expressed within the construct. These genes were to fulfil duel roles of being markers in the final modified organism and also acting as selection markers for DH5α mutants that had successfully been modified. Thus, it was essential for the process that these genes successfully express functional protein.

To perform the cloning the expression, plasmid pET22B (Novagen) was kindly donated by Dr Chris Love, Griffith University. Calcium chloride competent DH5α cells were made specifically for this project.

The EGFP gene and insertion cassette were PCR amplified. Multiple banding patterns were evident after electrophoresis of PCR product of both reactions, indicating a lack of specificity (Figure 9). Bands corresponding to the correct size for the lac promoter/EGFP fragment and insertion cassette were excised from the gel and purified using a DNA gel extraction kit (Qiagen). The purified fragments were then sequenced and subsequent analysis confirmed that the fragments were correct.

Two methods of restriction digestion and subsequent ligation were performed. The first method involved a double digestion using NdeI and NotI for the EGFP fragment, NotI and XhoI for the insertion cassette, and finally NdeI and XhoI for the pET22B expression vector. The products of the restriction double digest were then electrophoesed and gel purified. These were then used for a 3-way ligation. The ligation reaction products were then directly used in a heat-shock transformation of DH5α competent cells. Culturing of the transformed cells on nutrient agar plates containing 100 μg mL^{-1} ampicilin (the selection marker the pET22B plasmid) resulted in no viable colonies. Other researchers, on separate projects, used the DH5α competent cells generated for this project for successful transformations. This was considered sufficient proof that the competent cells were viable. This method was repeated again to ensure that experimental error was not responsible for the initial failure.

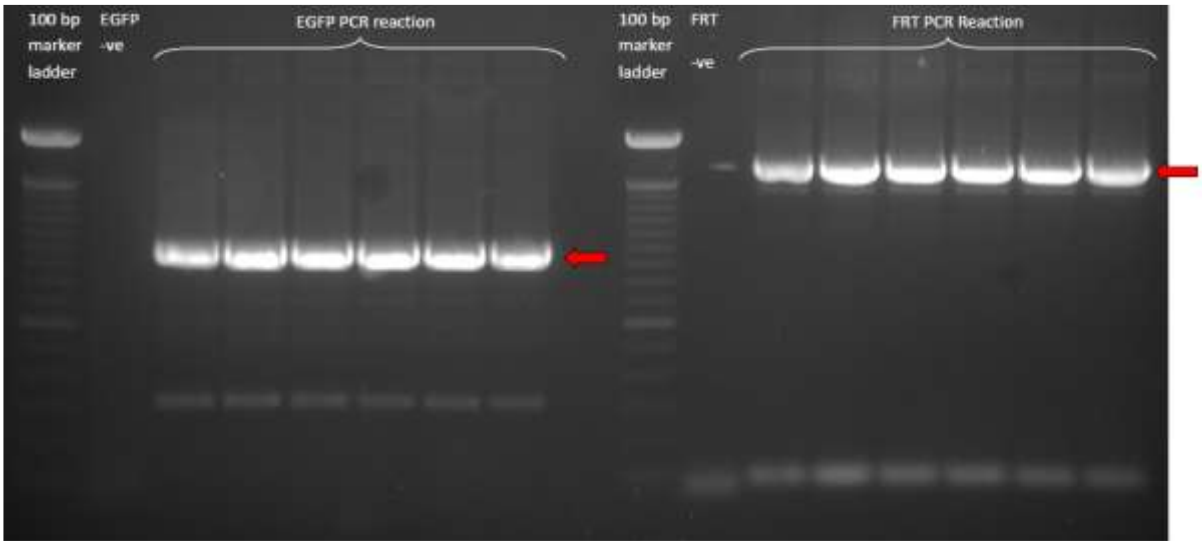


Figure 9. Ethidium bromide stained 1% Agarose gel of the PCR reaction for EGFP and FRT insertion cassette. Both reactions were repeated six times to provide enough DNA for later digestion and ligation. Multiple banding patterns indicate that non-specific amplification was occurring. Red arrows indicate the band corresponding to the amplification product corresponding in size to the lac promoter/EGFP gene and FRT insertion cassette respectively.

The second method involved a single digest of the EGFP and insertion cassette fragments using NotI. These were then gel purified and used for a single ligation reaction. Direct electrophoresis and visualisation of the ligation reaction showed multiple banding occurring in the ligation products (Figure 10). The assumption was made that multiple banding patterns at sizes in excess of that of the largest fragment would be due to blunt end ligation. Gel extraction was attempted for the middle of the top three fragments, as this corresponded to rough size of the EGFP-insertion cassette ligation product. The forward primer targeting the EGFP fragment and the reverse primer targeting the insertion fragment were used to amplify the ligated fragment. This resulted in several bands being observed in the PCR product.

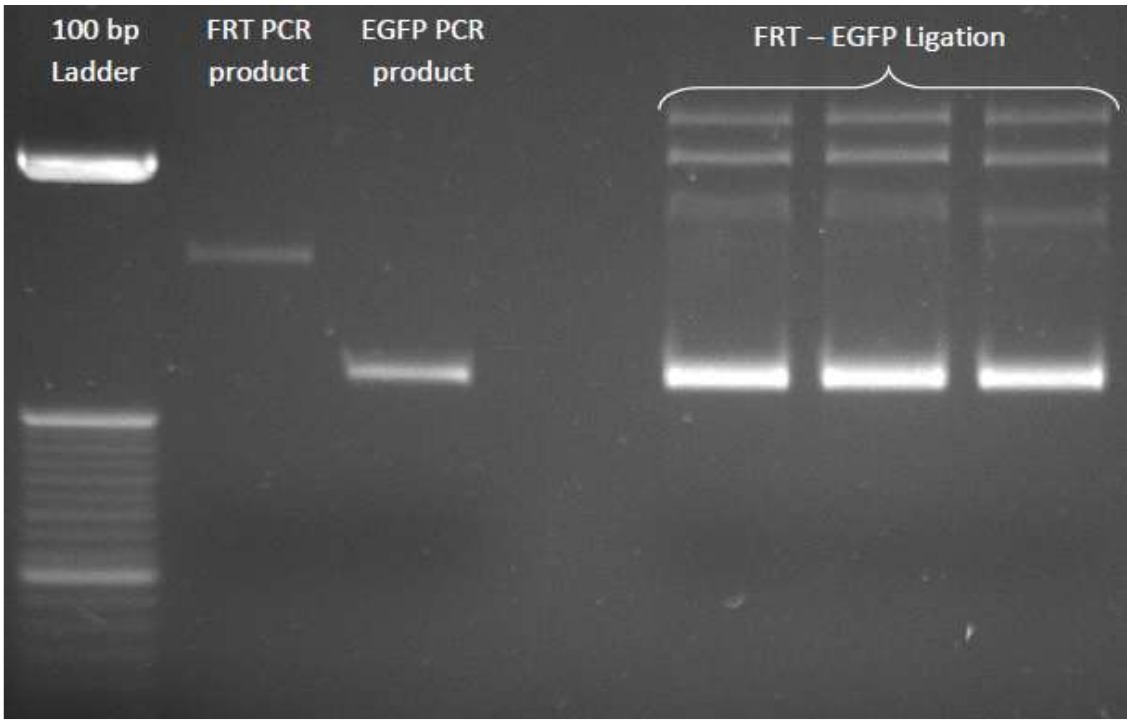


Figure 10. Products of the ligation reaction for the NotI digested FRT and EGFP fragments.

This would stand to reason as, if blunt end ligations were occurring, the extracted band from the ligation would contain product in several conformations, with EGFP either at the front or rear of the fragment. The ligation was repeated a second time with a 15 minute incubation time rather than the two hours used in the initial reaction. As sticky ended ligations, such as those created by NotI restriction digests, are more energetically favourable then blunt end ligations, it was hoped that decreasing the ligation time would remove any blunt end interactions. This resulted in no ligation of the products. Control runs had shown that NotI successfully linearised the pEGFP plasmid so it was assumed that NotI reaction was sufficient to digest the fragments. To confirm this, internal primers were designed to sequence the end regions of both the EGFP and insert cassette fragments. Sequence analysis confirmed that the NotI site in both fragments was being digested.

Further development of the mutant *E. coli* containing a chromosomal gene insertion was abandoned at this point due to time and budgetary constraints. Though it was beyond the scope of the present project it is hoped that work on the mutant strain can be re-established in the near future. The completed mutant would offer an invaluable tool in assessing quality control of DNA extractions and in other aligned research projects.

APPENDIX II:
Detection and Characterisation of PCR Inhibition Compounds that Co-Purify with DNA

Many substances will co-purify with DNA from environmental and clinical samples, many of these can cause deleterious effects on analysis of the sample using techniques based on the polymerase chain reaction. The effects on the PCR can range from a decrease in the reaction efficiency to total inhibition of the reaction. The presence of PCR inhibiting compounds can have severe repercussions using quantitative PCR methods for pathogen detection from an under estimate of pathogen numbers to a false negative result. Many studies have been published in designing and optimising procedures to remove PCR inhibiting compounds from samples and these are summarised in Table 9. The present study attempted to develop a screening tool for the presence of PCR inhibitors within DNA extracts this project attempted to develop methods to detect and identify PCR inhibitors using mass spectroscopy techniques.

Humic acids were chosen as model PCR inhibitors for the purposes of method development. Humic acids were chosen as they are ubiquitous within fresh water environments and will readily co-purify with DNA using standard extraction methods. Humic acids are a group of large organic molecules characterised by their multiple phenolic groups. Inhibition of the PCR can occur either through disruption of the polymerase via the phenolic groups interacting to the amide groups of the protein, or through oxidation of the phenol to a quinone which will covalently bind DNA.

Humic acid standards, isolated from river water, were obtained from the International Humic Substance Society (IHSS) along with a fulvic acid from the same source. The minimum inhibitory concentration of the humic acid standard was determined by seeding the solubilised humic acid standard into PCRs at known concentrations. The PCR reaction targeting the *lacZ* gene in *Escherichia coli,* as described previously, was used and 100 ng of *E. coli* genomic DNA was seeded into all reactions, baring the negative control and visualised on a 3 % Agarose gel stained with ethidium bromide (see Figure 11, Appendix II). From these results, it was observed that the humic acid standard fully inhibited the PCR targeting *lacZ* at concentrations greater than 1 µg/mL. All matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) experiments used concentrations between 100 µg/mL and 1 µg/mL humic acid for experiments.

Detection and characterisation of the humic acid standard was attempted using MALDI-TOF mass spectroscopy. After a review of the literature of previously attempted methods, the procedure reported in Mugo *et al.* (2004) was used. In brief; a number of solvents where used to solubilise the humic acid standards for sample preparation, including water, methanol, 1:1 acetonitrile (ACN)/water and ACN with 1 % trifluoroacetic acid (TFA). Several matrices were also trialled including α-cyano-4-hydroxycinammic acid (CHCA), 2-(4-hydroxyphenylazo) benzoic acid (HABA) and 2,5-dihydroxybenzoic acid (DHBA) and a laser desorption ionisation (LDI) method using no matrix. All matrices were prepared as saturated solutions as reported in Mugo *et al.* (2004) immediately prior to preparation for each run. Samples were prepared using an off-plate spotting method involving mixing the solubilised sample with the matrix in a ratio of 1:100 and mixed using a vortex mixer. One µl of the humic acid/matrix mix was spotted onto a MALDI-TOF sampling plate and allowed to co-crystallise. Plates where then analysed using Kratos PC Axima using the parameters outlined in Mugo *et al.* (2004).

It has been noted by several authors that the laser source can have effects on the ionisation of humic acids with 10.6 nm and 1.06 nm laser predominately producing negative ions of humic acids (Brown and Rice, 2000), while a 337 nm laser produced predominately positive ions (Gajdosova and others, 2003; Mugo and Bottaro, 2004). This is due to the different functional groups of humic acids being more effectively ionised at different wavelength lasers, resulting in differing mass to charge spectra for the same molecule. The Axima MALDI-TOF mass spectrometer used for this study utilised a 337 nm nitrogen laser as did the instrument in the used in the Mugo *et al.* 2004 study.

The humic acid standard was not detected in any of the solvents using any of the matrices or LDI methods. The protein size standards where readily detectable.

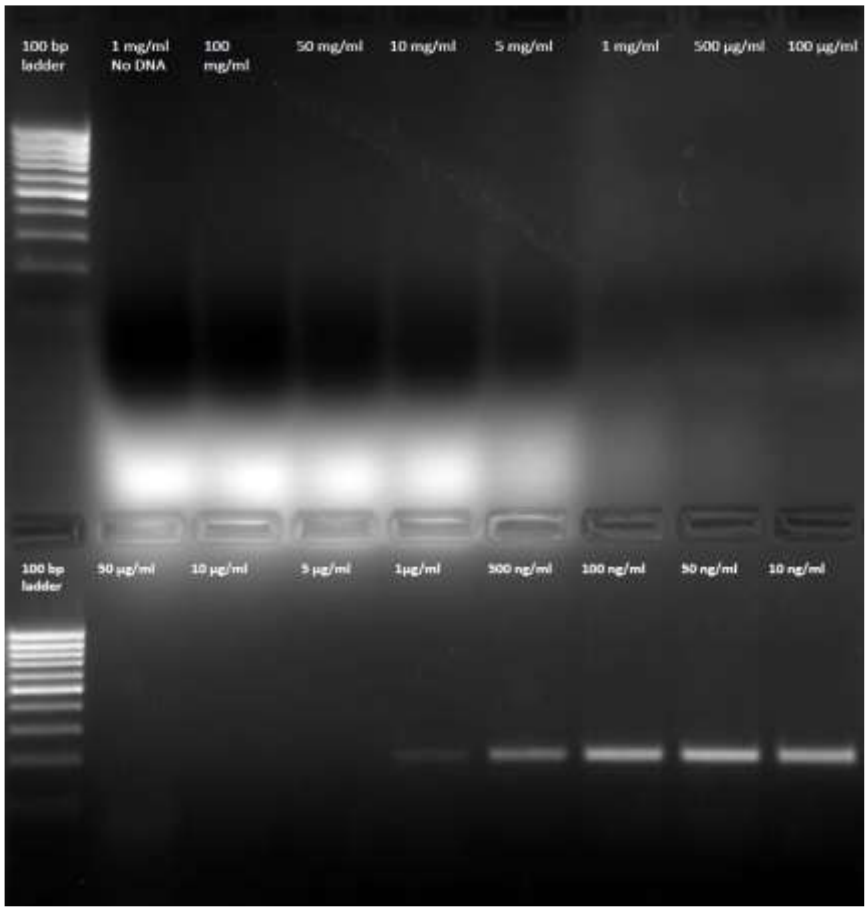


Figure 11. PCR inhibitory concentrations of the IHSS Humic Acid standard. PCRs targeting the *lacZ* gene of *Escherichia coli* were seeded with humic acid concentration ranging from 100 mg/mL to 10 ng/mL and 100 ng of *E. coli* genomic DNA. Humic acid concentrations are represented on the gel image. The PCR was completely inhibited at concentrations greater the 1 µg/mL. Estimation of band intensity implies that partial inhibition was evident at 1 µg/mL and 500 ng/mL humic acid.

The methods were repeated a second time to ensure that operator error was not responsible for the inability to detect the humic acid standard. The second run also included fulvic acid standards, obtained from IHSS, as they are similarly functionalised to humic acids only smaller organic molecules. This was performed in case the semi-polymeric nature of the humic acids resulted in molecule sizes which were too large to be detected using MALDI-TOF. The fulvic acids were prepared using the same solubilisation methods and matrices as previously outlined for the humic acid standards. Neither the humic or fulvic acid samples were detected.

As direct detection of humic acids was not achieved, indirect detection was attempted through the DNA binding capacity of the molecule. This involved detecting a DNA standard which would then either increase in mass due to interactions with the DNA binding compound or fail to be detected. It was hoped that this assay could form the basis of a detection assay for DNA binding PCR inhibitors, which could possibly be expanded to include protein binding compounds with the inclusion of polymerase standard.

MALDI-TOF has been used extensively for analysis of nucleic acids. The methods used in this study nucleic acids analysis were obtained from the Shimatzu user's manual for the Axima MALDI-TOF mass spectroscope. A 20 bp single-stranded DNA oligonucleotide obtained from Invitrogen (5'-ACACACACACACACACACAC-3') was used as a standard. Three different matrices where used in conjunction with two sample preparation methods. Table 8 outlines the methods used for optimisation of detection of the oligonucleotide standard with each of the three matrices trialled. The results of the optimisation are shown in Figure 12. Off-spot mixing using THAP as a matrix was used for the remainder of the study as this was the most time-efficient method which provided clear detection of the oligonucleotide standard.

Table 8. Matrices and sample preparation methods used for DNA nucleotide methods development.

Matrix	Matrix Preparation and Reagents	Spotting Technique
2,4,6 trihydroxyacetophenone (THAP)	-50 mg/mL THAP in ethanol -50 mg/mL dihydrogen ammonium citrate (DHAC) in water	**Off spot:** -1:1:1 THAP/DHAC/100 µM Oligo mixed in 1.5 mL tube -1 µl of mix spotted onto plate **Sandwich:** -0.3 µl THAP spotted onto plate and allowed to dry. -1 µl of 1:1 DHAC/40 µM Oligo spotted onto plate and allowed to dry. -0.3 µl THAP spotted and allowed to dry.
3-hydroxypicolinic acid (HPA)	-50 mg/mL HPA in 1:1 acetonitrile/water -50 mg/mL DHAC in water	**Off spot:** -1:1:1 HPA/DHAC/100 µM Oligo mixed in 1.5 mL tube -1 µl of mix spotted onto plate **Sandwich:** -1 µl HPA spotted onto plate and allowed to dry. -1 µl of 1:1 DHAC/40 µM Oligo spotted onto plate and allowed to dry. -1 µl HPA spotted and and allowed to dry.
6-aza-2-thiothymine (ATT)	-50 mg/mL ATT in 1:1 ethanol/acetonitrile. -50 mg/mL DHAC in water	**Off spot:** -1:1:1 ATT/DHAC/100 µM Oligo mixed in 1.5 mL tube -1 µl of mix spotted onto plate **Sandwich:** -0.5 µl ATT spotted onto plate and allowed to dry. -1 µl of 1:1 DHAC/40 µM Oligo spotted onto plate and allowed to dry. -0.5 µl ATT spotted and and allowed to dry.

The humic acid standard was then added to the oligonucleotide standard and the detection was attempted using the THAP off spot mixing method. Humic acids were added in concentrations of 100 µg/mL, 10 µg/mL and 1 µg/mL to the oligonucleotide standard. Treatment with humic acid at any concentration failed to cause a mass shift or reduce detection of the oligonucleotide standard as compared to controls.

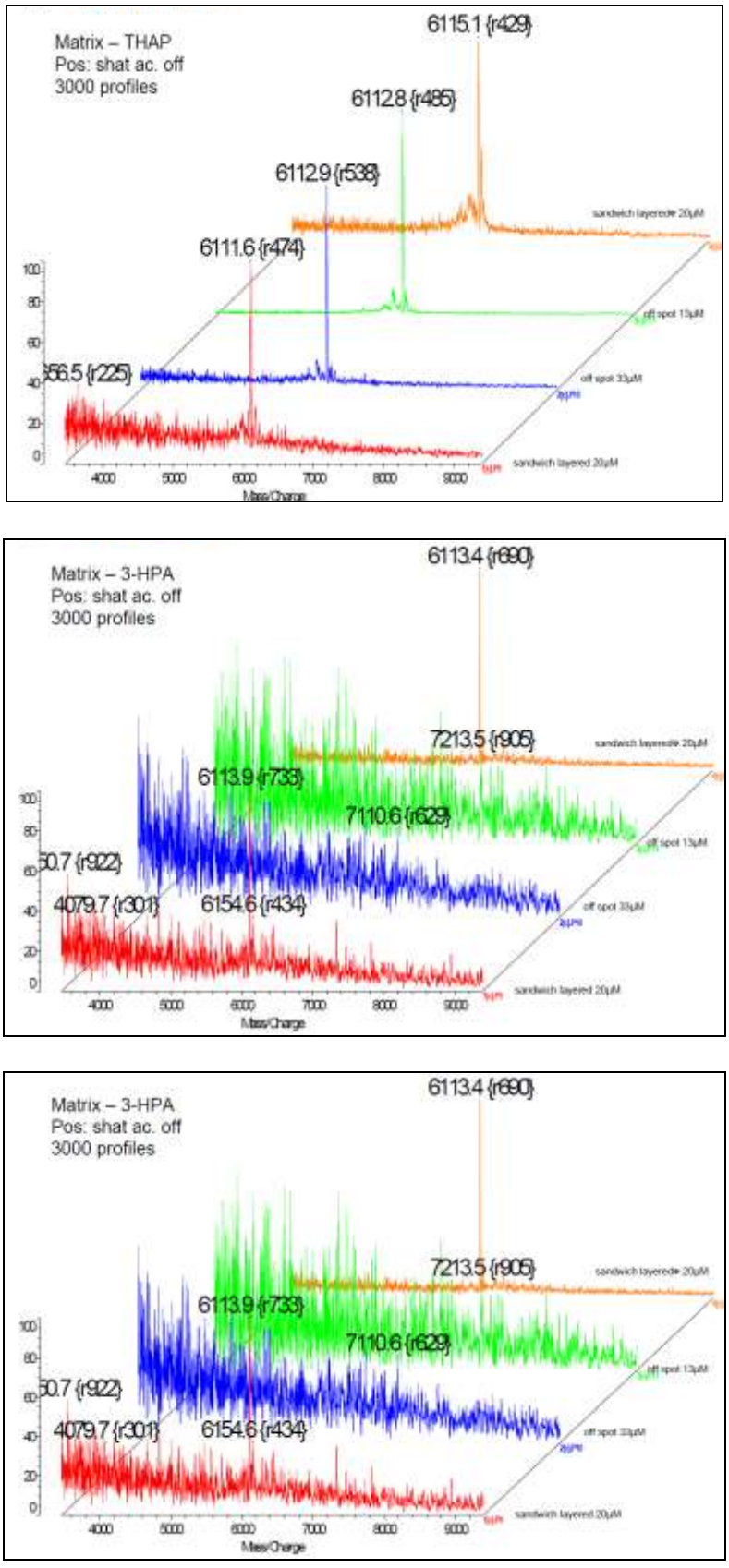


Figure 12. Results using the three different matrices. THAP gave the best result using both spotting methods.

Direct injection electrospray ionisation (ESI) mass spectroscopy was used to detect both the oligonucleotide standard and the humic acid standard. This involved directly injecting 20 µM of oligonucleotide or 100, 10 or 1 µg/mL of humic acid solubilised in water for mass spectroscopic analysis. The fragmentation pattern of the oligonucleotide was readily and detected and deconvoluted to provide approximately the correct mass to charge ratio (see Figure 13). Accurate mass estimation was not possible as only a preliminary ESI-MS analysis was attempted and the instrument was not calibrated. As with MALDI-TOF, ESI-MS failed to detect the humic acid standard and the addition of the humic acid standard failed to cause a mass shift or diminish the signal of the oligonucleotide standard.

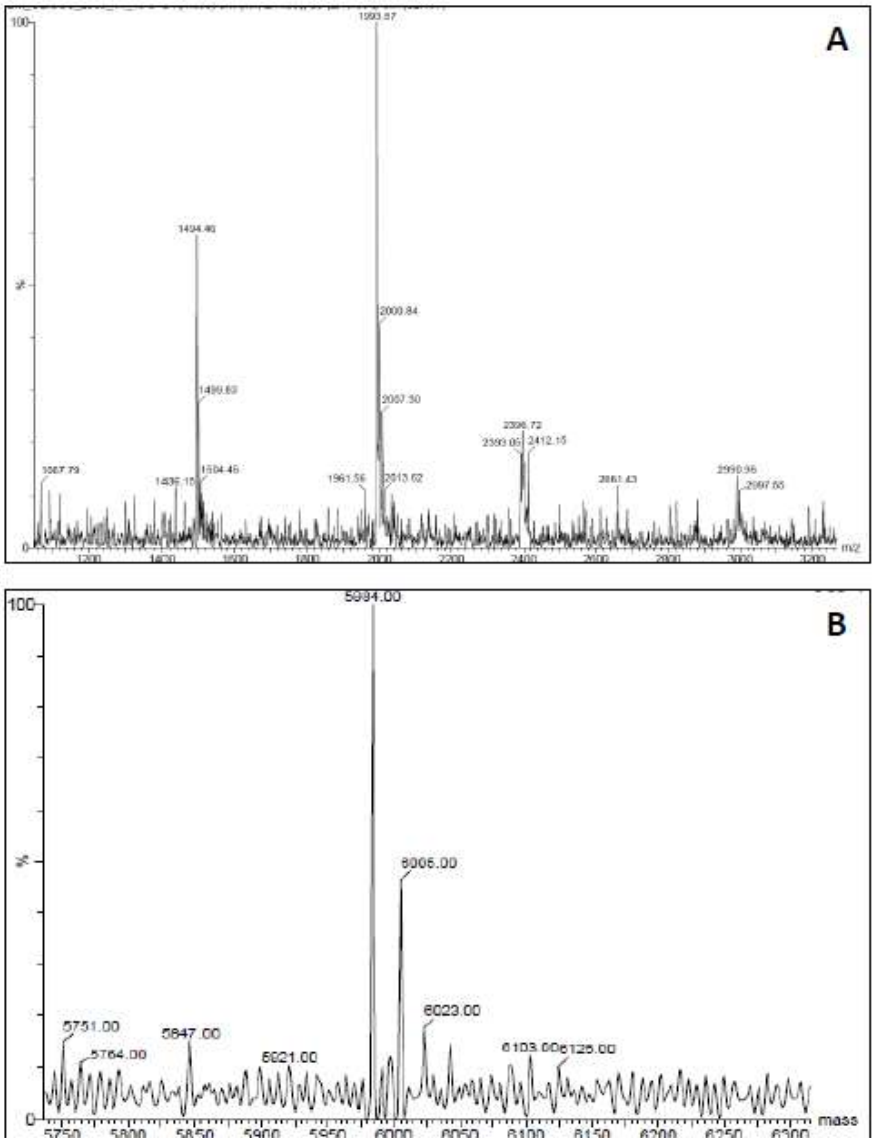


Figure 13. ESI-MS profile of 20 bp oligonucleotide standard. Panel A shows the direct fragmentation profile of the oligonucleotide standard. Panel B shows the deconvoluted fragmentation pattern with a primary mass peak of 5994 m/z and two secondary isotopic peaks.

As humic acids are a major PCR inhibiting substance present in fresh water samples, the inability to detect them made continuation of the this portion of the project unviable. The detection of PCR inhibitors was abandoned at this point to minimise further loss of time or funds.

Table 9. Nucleic Acid extraction and PCR inhibitor removal from wastewater.

Material	Pretreatment	Extraction Technique	Comments	Ref
Soil	Chemical flocculation with multivalent cations	Ultraclean Soil DNA extraction kit	Aluminium ammonium sulphate ($AlNH_4(SO_4)_2$) provided the greatest removal of inhibitors with little effect on DNA recovery.	[Braid et al 2003]
Sediment	Wash with Triton-X100 and EDTA or Powered activated carbon (PAC) then ion exchange chromatography with amberlite	CTAB then phen/chlor or Ultraclean Soil DNA	PAC and amberlite chromatography using CTAB and phenol/chloroform extraction resulted in greatest yield and little PCR inhibition	[Desai and Madamwar 2006]
Soil	Treated DNA extracts with $Al_2(SO_4)_3$ to chemically flocculate humics	SDS lysis then Chloroform or Ultraclean Soil DNA	$Al_2(SO_4)_3$ treatment effectively removed humics with little loss of DNA at pH 6. SDS/chloroform method resulted in greater DNA yields	[Dong et al 2006]
Compost		SDS and bead beating lysis with polyethylene glycol (PEG) precipitation sephadex chromatography purification	Yielded DNA which could be used for PCR and restriction digests	[Howeler et al 2003]
Ancient Skeletal remains	Either *N*- phenacylthiazolium bromide (PTB) or Repeat silica extraction	Modified phenol/chloroform	PTB ineffective at removing inhibitors. Repeat Silica treatments highly effective at removing inhibitors with little loss of DNA.	[Kemp et al 2006]
Soil	Wash in PBS containing EDTA	Extraction: Bead-beating or microwave or liquid nitrogen Purification: microspin columns, PVPP wash or PVPP and sepharose 2B column elution	Bead beating extraction with PVPP and sepharose resulted in greatest yields. All DNA extractions using this method could be used for molecular methods	[Lakay et al 2007]
Compost	Wash in 1.5 M NaCl, 100 mM EDTA yields maximal results	Extraction: bead beating with SDS at 60^{O}C had greatest results. Followed by chloroform treatment Purification: Sepharose 4B chromatography or agrose gel electrophoresis	Greatest yield using high salt buffers during extraction. Purification with Sepharose 4B columns resulted in all DNA samples being amplifiable.	[LaMontagne et al 2002]
Water Various sources	N/A	Addition of GeneRelease^{TM} solution and 5 min microwave	Samples from most water sources had amplifiable DNA without requiring additional GeneRelease clean ups	[Menking et al 1999]
Soil and Sediment		Compared 9 different extraction methods Lysis: Bead beating with SDS and chloroform most effective Purification: Evaluated 4 different methods Sephadex G-200 most effiecient	Gel electrophoresis efficient at removing inhibitors though large DNA loss. Sephadex purification greatest retention of DNA while removing inhibitors. Note: All samples required dilution	[Miller et al 1999]
Faeces	Catrimox-14^{TM} (cationic surfactant)	MicroSpin Columns	Hmmmm…	[Uwatoko et al 1996]
Biogas Plant waste		Analysed range of extraction techniques. Modified Phenol/Chloroform provided higher yields then commercial kits. Purification: Three methods tested; electrophoresis and electroelution, spermine precipitation, and dialysis.	Dialysis provided the best removal of inhibitors. Samples still had to be diluted 1 in 100. Note: Dialysis resulted in loss of 66% of DNA	[Weiss et al 2007]

Material	Pretreatment	Extraction Technique	Comments	Ref
Sludge	N/A	Bead beating with SDS and EDTA for lysis Precipitating the sample with ammonium acetate		[Yu and Mohn 1999a]
Respiratory biopsy		AMPLICOR respiratory specimen preparation kit	Internal control for real-time PCR using homologous probes of known concentration. Probes for *mycobacterium tubercolosis,* hepatitis B virus, herpes simplex virus and varicella zoster virus.	[Burggraf and Olgemoller 2004]
Clinical samples spiked with inhibitors			Assessed a taqman internal positive control to detect PCR inhibition. Used site directed mutagenesis to generate probes	[Hartman et al 2005]
Wastewater and Soil		Used alkaline lysis and lysates used as template for subsequent amplification.	Used multiple displacement amplification for a whole genome amplification prior to specific PCR amplification. Increased sensitivity of the reaction with the addition of humic and fulvic acid.	[Gonzalez et al 2005]
Bone			Addition of 1.25 U of Taq polymerase overcomes inhibition by 100 ng humic acid in real-time PCR	[Sutlovic et al 2005]
Cultures spiked with humic acid		Bio 101 FastDNA SPIN kit for soil	Used fluorescent intensity of alternately binding probe of known concentration to assess sample concentration. Used either PCR or LAMP amplification. Gave comparable results to RT-PCR. Efficient in up to 8 µg/mL humic acid.	[Tani et al 2007a;2007b]
Wastewater settlement pond		Freeze/thaw lysis Phenol/chloroform extraction	Magnetic bead hybridised to target specific DNA sequence where used to extract either low copy number samples or samples in the presence of inhibitors	[Tsai et al 2003]

APPENDIX III:
Materials and Methods: qPCR

Wastewater DNA Extraction

Concentration

WWTP influent: 100 mL of WWTP influent was transferred to two 50 mL sterile centrifuge tubes and centrifuged for 15 min at 4,000 G. 45 mL of supernatant was removed from both tubes and the pellet was resuspended in the remaining 5 mL. The 5 mL concentrates were then pooled in a 10 mL centrifuge tubes and centrifuged a second time at 4,000 G for 15 min. 7 mL of the supernatant was then removed and the pellet resuspended in the remaining 3 mL.

WWTP effluent: 1 L of WWTP effluent was filtered through 49mm 0.45µm cellulose acetate filters (Millipore) and the filtrate discarded. Filters were transferred aseptically to 10 mL centrifuge tubes. 1 mL of sterile glass beads were added to the tube and the tubes filled to the 5 mL increment with sterile TE buffer pH 7.6 (10 mM Tris, 1 mM EDTA, pH balanced using HCl). These were then fixed to rotary plate shaker and shaken for 20 min at 400 RPM. Liquid was then removed and transferred to 10 mL centrifuge tubes. A second amount of TE buffer pH 7.6 was added to the tube containing the filter paper and the tube shaken for a further 20 min at 400 RPM. The liquid from the second wash was then pooled with that off the first wash. The filter washed were then centrifuged for 15 min at 4,000 G and all but 3 mL of the supernatant removed and pellet resuspended.

DNA Extraction

The same procedure was used for the concentrated WWTP influent and effluent. The method used was a modified version of that reported by Yu and Mohn (1999). In brief, 1 mL of sterile 1 mm glass beads were added to plant concentrates contained in 10 mL centrifuge tubes. Lysozyme (Sigma) was then added to a final concentration of 100 µg/mL. Tubes were fixed to a shaker incubator at 37^{O}C and shaken for 30 min at 400 RPM. Proteinase K (Invitrogen) (final concentration 500 µg/mL) and extraction buffer was added to the tube (final Concentration: 50 mM Tris, 5 mM EDTA, 3 % SDS: pH 8) and returned to the shaker incubator and shaken for 30 min at 60^{O}C at 400 RPM shaking.

Tubes where then balanced with sterile, nuclease-free, deionised water (Invitrogen) and spun at 6,000 RPM for 10 minutes. The supernatant was then transferred to a clean 10 mL tube and the original tube discarded. Ammonium acetate (Sigma) was then added to the supernatant for a final concentration of 2M. Tubes were incubated at room temperature for 5 minutes then centrifuged at 6,000 G for 30 min. The supernatant was removed and transferred to new 10 mL tube and the pellet discarded. An equal volume was of ice-cold isopropanol was added to incubate on ice for 5 minutes. Tubes where then centrifuged for 40 minutes at 6,000 RPM at 4^{O}C. The supernatant was removed and the pellet washed in 500 µl of 70 % molecular grade ethanol (Sigma) in nuclease-free water. This was then centrifuged for a further 10 minutes at 6,000 G and the supernatant discarded. The pellet was then dried to remove residual ethanol and the pellet resuspended in 400 µl of nuclease-free water. The DNA extract was then transferred to a 1.7 mL centrifuge tube and the DNA precipitated a second time using the procedure just described. DNA extracts were finally resuspended in 50 µl nuclease-free water and stored at -20^{O}C until analysis.

Primer and Probe Design

This project utilised a duplex taqman reaction coupling the detection reaction for the pathogen of interest with a taqman reaction targeting the EGFP gene contained within the plasmid pEGF. The taqman EGFP reaction was designed specifically for this study (details of the process carried out to do this are given in Appendix I). An extensive literature review was conducted to review published primers and probes sequences that have been used previously for detection of the pathogens of interest examined within this study. A combination of published detection oligonucleotides and sequences designed specifically for this project was finally used. All published oligonucleotide sequences were validated for specificity using desktop bioinformatic tools before ordering using the following

methods. Primer sequences where BLAST analysed using the blastN algorithm against the NCBI sequence database (Altschul, 1990) to confirm specificity. DNA sequences of the target gene from the organism of interest were then obtained from the NCBI genbank database from as many strains of the target organism as possible, with a minimum of five strain sequences being used. DNA sequences were then converted into FASTA format within a single file and aligned using ClustalX software (Larkin, 2007). A consensus sequence was obtained and primer and probe sequences were aligned against this using the BioEdit software package (Hall, 1999). Self homology (hairpin formation) and dimerisation with other oligonucleotide used in the duplex taqman reaction were analysed using web-based resources supplied from Integrated DNA Technologies (IDT).

This project used primers and probes designed specifically for this project (see Table 10, Appendix III) and Taqman probes designed for existing monoplex SYBR green based reactions. Both processes used the same processes for screening potential primer and probe sequences. In brief, sequence information for target gene or amplicon was obtained from the NCBI genebank database. Multiple sequence alignments using ClustalX were performed and a consensus sequence generated using BioEdit. Primer sequences were then aligned against the consensus sequence to give the amplicon sequence for existing primers. Putative probe and primer sequences where then designed using the web based primer design tool Primer 3 Plus (Untergasser, 2007) and the amplicon sequence of putative primers ascertained. Putative Taqman probes where screened using the probe design criteria using online resources supplied by Applied Biosystems. Predicted secondary structure at the primer Tm for the probe hybridisation strand of the amplicon was generated using Mfold (SantaLucia, 1998). Putative probes were then screened against the predicted secondary structure of the amplicons to ensure that the probe binding region did not containing excessive secondary structure to inhibit hybridisation. Finally, putative probes were then screened for self homology and dimerisation with other oligonucleotides in the duplex reaction using tools in the IDT website.

Primer Optimisation

For all optimisations and standard curve generation, genomic DNA extracted from pure cultures was used as template DNA. Optimal concentrations of primer and probe were generated by running dilution series of primer and probe for single plex and duplex reactions with the EGFP reaction for all pathogen detection reactions. Annealing temperature was optimised by running single plex and EGFP duplexed pathogen detection four-point, 10-fold dilution standard curve reactions over a temperature gradient. Optimal annealing temperature was chosen according to the reaction efficiency of the pathogen detection reaction. Specificity of the reactions was confirmed by single plex and duplex reactions against genomic DNA from both distant and closely related bacterial species. All optimisations were performed using a Bio-Rad CFX96 light cycler and Brilliant III mastermix as their reaction chemistry.

Reaction Conditions

All reactions were performed on a Bio-Rad CFX96 light cycler. All wastewater DNA extracts where five-fold serially diluted to final concentration of 1 in 25. qPCR analysis was performed in triplicate for all pathogens of interest using 1 µl of the 1:1, 1:5 and 1:25 dilutions of wastewater DNA extracts as template for the reaction. 1 pg of pEGFP was added to each reaction to act as an internal standard to assess inhibitor effects. All qPCR plates examining waste water extracts contained three normaliser wells containing replicates of a duplex reaction containing 10^6 chromosomal copies of the pathogen of interest and 1 pg of pEGFP. This was added to allow later normalisation for inter-run variability and quantification against standard curves. All reactions used Brilliant III mastermix (Agilent) as their reaction chemistry as per the manufacturer's instructions. Concentrations of primers and probe for each pathogen detection reaction are outlined in Table 11, Appendix III. In brief, all reactions had an initial 10 min denaturation step at 95^{O}C to activate the polymerase used in the Brilliant III mastermix. This was followed by 40 two-step amplification cycles consisting of 95^{O}C for 15 seconds and one minute annealing using the temperature outlined in Table 11, Appendix III. Fluorescent signal acquisition was performed during the annealing step.

Table 10. Primer and probe DNA sequences used for qPCR pathogen quantification.

Target Organism	Name	Sequence	Temp (°C)	5' Modification	3' Modification	Amplicon Size	Gene Target	Source
Escherichia coli	lacZF	CTT AAT CGC CTT GCA GCA CA	58	None	None	180	*lacZ*	Foulds, 2002; Wery, 2008
	lacZR	CAG TAT CGG CCT CAG GAA GA	60.5	None	None			Foulds, 2002; Wery, 2008
	lacZPr	ATT CGC CAT TCA GGC TGC GCA A	64.5	VIC	BHQ			This Study
pEGFP Internal Standard	GFP-F	TGC TCA GGT AGT GGT TGT CG	60.5	None	None	135	EGFP gene	This Study
	GFP-R	AGA ACG GCA TCA AGG TGA AC	58.4	None	None			This Study
	GFP-Pr	ACG CTG CCG TCC TCG ATG TT	62.5	6FAM	BHQ			This Study
***E. coli* Serotype O157:H7**	O157 F	CGG ACA TCC ATG TGA TAT GG	58.4	None	None	259	*rgbD*	Ahmed, 2007
	O157R	TTG CCT ATG TAC AGC TAA TCC	57.5	None	None			Ahmed, 2007
	O157Pr	TCG TGA CAA CCA TTC CAC CTT CAC C	67.4	VIC	BHQ			This Study
Campylobacter jejuni	mapA-F	GGT TTT GAA GCA AAG ATT AAA GG	57.6	None	None	94	*mapA*	Ahmed, 2009
	mapA-R	AAG CAA TAC CAG TGT CTA AAG TGC	62	None	None			Ahmed, 2009
	mapA-Pr	TGG CAC AAC ATT GAA TTC CAA CAT CGC TA	67.4	Cy5	BHQ2			This Study
***Salmonella* spp.**	Sal-F	CGT TTC CTG CGG TAC TGT TAA TT	60.9	None	None	66	*invA*	Shannon, 2007
	Sal-R	AGA CGG CTG GTA CTG ATC GAT AA	62.9	None	None			Shannon, 2007
	Sal-Pr	ACC ACG CTC TTT CGT CTG GCA	63.2	Cy5	BHQ2			This Study
Enterococcus faecalis	Ent-R	AGG AAT TGT TCT TGC ATC CGT T	58.4	None	None	209	*groES*	This Study
	Ent-F	TGG CAA TAA TGT CTT TGG CTG A	58.4	None	None			This Study
	Ent-Pr	ATC GCA GTA GGT GAA GGT CGT GTG C	69.1	Cy5	BHQ2			This Study
Clostridium perfringens	Clost-F	TGA CAC AGG GGA ATC ACA AA	56.4	None	None	180	*plc*	This Study
	Clost-R	AAC ATG TCC TGC GCT ATC AA	56.4	None	None			This Study
	Clost-Pr	TCC ATA TCA TCC TGC TAA TGT TAC TGC CG	60.3	Cy5	BHQ2			This Study

Table 11. Annealing temperature and concentrations of primers and probe for each pathogen detection reaction.

Target Organism	Annealing Temp. (°C)	Primer Conc.	Probe Conc.	Control Strains
EGFP gene	As per the duplex reaction	500 nM	300 nM	**Postive:** pEGFP (Clonetech) **Negative:** As per the duplexed reaction
E. coli	60	500 nM	300 nM	**Positive:** *E. coli* ATCC15766 *E. coli* O157:H7 ATCC **Negative:** *Salmonella Typhimurim* ATCC pEGFP *Enterobacter aerogenes* *Enterococcus faecalis*
***E. coli* O157:H7**	60	500 nM	300 nM	**Positive:** *E. coli* O157:H7 ATCC **Negative:** *E. coli* ATCC15766 pEGFP *Salmonella typhimurim* ATCC *Enterobacter aerogenes*
Campylobacter jejuni	62	800 nM	500 nM	**Positive:** *C. jejuni* ATCC **Negative:** pEGFP *E. coli* ATCC 15766 *Clostridium perfringens* ATCC *Enterococcus faecalis* ATCC *Enterobacter aerogenes* ATCC
***Salmonella* spp.**	60*	500 nM*	300 nM*	**Positive:** *Salmonella typhimurim* ATCC **Negative:** pEFGP *E. coli* ATCC15766[#] *E. coli* O157:H7 ATCC *Enterococcus faecalis* ATCC
Entercoccus faecalis	60*	500 nM*	300 nM*	**Positive:** *Enterococcus faecalis* ATCC **Negative:** pEGFP *Enterococcus faecium* ATCC[#] *Enterobacter aerogenes* ATCC *E. coli* ATCC15766
Clostridium perfringens	60*	500 nM*	300 nM*	**Postive:** *Clostridium perfringens* ATCC **Negative:** *Clostridium* pEGFP

Normalisation if Inter-run Variance and Data Analysis

Chromosomal DNA was obtained from extractions of pure cultures of the pathogen of interest using the method described previously for nucleic acid extraction from wastewater samples. RNA was removed by the addition of 5 µl of RNase A (Invitrogen) to the 50 µl aliquots of pure culture DNA extracts. This was then incubated for 30 min at room temperature followed by ethanol precipitation, as has been described previously, and resuspended in 50 µl of nuclease-free water. DNA concentrations were then quantified by spectrophotometry by measuring the absorbance at 260 nm using a Biophotometer (Eppendorf) and the concentration calculated in ng μl^{-1}. The theoretical weight of the chromosome of the organism of interest was calculated based on the number of base pairs in the chromosome using the method reported by Applied Biosystems (http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf).

Chromosomal DNA extracts where diluted to a concentration of 10^7 chromosomal copies. Eight point standard curves were generated using chromosomal DNA diluted 10-fold from 10^7 to 10^0 chromosomal copies and 10-fold diluted pEGFP from 100 pg to 1 ag as template. Each point in the standard curve was repeated in triplicate. Standard curves for each pathogen of interest were repeated on three separate runs to ensure that the reaction efficiency was repeatable and for later analysis to inter-run variability.

The software package CFX Manager (Bio-Rad) was used to analyse the raw data and determine Ct values for individual runs. The default settings within the CFX Manager software were used to determine the threshold value, which was then used to calculate CT values. Data analysis used to normalise for inter-run variance and quantify unknown samples against the averaged standard curves was performed using the Excel software package (Microsoft).

Standard curves for every pathogen of interest were repeated three times and average Ct value plotted against the log starting concentration for each reaction. The reaction efficiency of each individual standard curve was ascertained using the slope of the line of best fit using the following website (http://www.finnzymes.com/java_applets/qpcr_efficiency.htmL). The reaction efficiency of each individual standard reaction was shown to be comparable.

Normalisation for inter-run variability was performed as follows: Normaliser wells containing 10^6 chromosomal copies of the pathogen of interest and 1 pg of pEGFP where included in every plate analysing unknown samples. These concentrations corresponded to concentrations included in the standard curve. The average Ct value for each concentration on the three standard curves and the normaliser wells for a plate were calculated and the mean Ct value for a concentration determined. The difference between the mean Ct for a concentration and the average of the three replicates for each run was calculated. This value was termed the Δ norm for a concentration. Interestingly, a large degree of variability was observed in the Δ norm at different concentrations within the standard curve for all standard curves being tested.

A second normalisation was performed to account for the concentration variation in the normalisation protocol. This involved averaging the Δ norm for each concentration in a standard curve run. The difference between the run average Δ norm and the Δ norm at the inter-run normalisation concentration was determined for each standard curve replicate. The absolute value for this difference from all three standard curve runs was averaged and this value was termed the concentration normalisation factor. The concentration normalisation factor was then either added or subtracted from the Δ norm at the inter-run normalisation concentration to bring it back towards the mean, to give the final value used for inter-run normalisation. This value was termed the $\Delta\Delta$norm. The $\Delta\Delta$norm was then subtracted from all Ct values from a run to normalise for inter-run variability.

Averages of the three normalised standard curves were determined and a normalised average standard curve generated by plotting the normalised average Ct value against the log of the starting concentration. A line of best fit was calculated for the normalised average standard curve. The theoretical efficiency of the normalised standard curve was determined using the slope of the line of best fit using the online efficiency calculator software supplied by Finzyme http://www.finnzymes.fi/. Concentrations of unknown samples were calculated using their normalised Ct values against the equation of the line of best fit of the normalised average standard curve. Standard Error was calculated using the variance in the ΔΔ normalised Ct values for the inter-run calibrators on the unknown plates and standard curve replicates.

As proof of concept for the inter-run normalisation method used, unknown concentrations of chromosomal DNA for the pathogen being tested and pEGFP were added in triplicate to each standard curve plate. These were quantified using both the standard curve they were run with and the inter-run normalised standard curve. The quantification value obtained from the standard curve on the plate the unknown was run with was considered the actual value, whereas the value obtained from the inter-run normalised standard curve was considered the predicted value. All actual values fell within the standard error range of the predicted values obtained from the normalised standard curve (refer to Table 12, Appendix III).

This project utilised primers and probes designed specifically for this project and Taqman probes designed for existing monoplex SYBR green based reactions. Both processes used the same processes for screening potential primer and probe sequences. In brief, sequence information for target gene or amplicons was obtained from NCBI genbank database. Multiple sequence alignments using ClustalX (Larkin, 2007) were performed and a consensus sequence generated using BioEdit (Hall, 1999). Primer sequences were then aligned against the consensus sequence to give the amplicon sequence for existing primers. Putative probe and primer sequences were then designed using the web based primer design tool Primer 3 and the amplicon sequence of putative primers ascertained (Untergasser, 2007). Putative Taqman probes were screened using the probe design criteria from Applied Biosystems. Predicted secondary structure at the primer Tm for the probe hybridisation strand of the amplicon was generated using Mfold. Putative probes were then screened against the predicted secondary structure of the amplicons to ensure that the probe binding region did not contain excessive secondary structure to inhibit hybridisation. Finally, putative probes where then screened for self homology and dimerisation with other oligonucleotides in the duplex reaction using tools in the IDT website.

Table 12. Efficiencies and of each replicate used for generating the normalised standard curve used for quantification of unknown samples for each organism. As proof of concept an unknown sample (triplicate) was included on each standard curve plate. The actual value (A) of the unknown as determined when plotted against standard curve on the plate it was run on and compared to the normalised predicted (P) value when plotted against the normalised averaged standard curve. In all cases the predicted value fell with the standard error of the normalisation process except one of the *C. jejuni* replicates highlighted in red.

Duplex Reaction	Standard Curve 1	Unknown Standard 1	Standard Curve 2	Unknown Standard 2	Standard Curve 3	Unknown Standard 3	Normalised Standard Curve
***E. coli*/GFP**	***E. coli*** Eff: 93.45% R^2 : 0.9968 **GFP** Eff: 109.94% R^2 : 0.9967	***E. coli*** A: 1.27 x 10^6 P: 1.17 x 10^6 **Standard Error** 1.07-1.27 x 10^6 **GFP** A: 1.59 ng P:1.35 ng **Standard Error** 1.03-1.67 ng	***E. coli*** Eff: 90.68% R^2: 0.9891 **GFP** Eff: 107.67% R^2: 0.9892	***E. coli*** A: 1.27 x 10^6 P: 1.17 x 10^6 **Standard Error** 1.08-1.28 x 10^6 **GFP** A: 1.53 ng P: 1.53 ng **Standard Error** 1.16-1.90 ng	***E. coli*** Eff: 90.5% R^2: 0.9922 **GFP** Eff: 116.65% R^2: 0.9953	***E. coli*** A: 1.28 x 10^6 P: 1.4 x 10^6 **Standard Error** 1.28-1.52 x10^6 **GFP** A: 1.25 ng P: 1.49 ng **Standard Error** 1.13-1.85 ng	***E. coli*** Eff: 91.52% R^2: 0.9954 **GFP** Eff: 111.3% R^2: 0.9972
***E. coli* O157:H7/GFP**	**O157** Eff: 95.71% R^2: 0.9969 **GFP** Eff: 94.22% R^2: 0.9972	**O157** A:1.04 x 10^7 P: 8.75 x 10^6 **Standard Error** 8.38 x 10^6- 1.25 x 10^7 **GFP** A: 0.996 ng P: 1 ng **Standard Error** 0.92-1.07 ng	**O157** Eff: 92.99% R^2: 0.9947 **GFP** Eff:103.29% R^2: 0.9983	**O157** A: 6.03 x 10^6 P: 6.99 x 10^6 **Standard Error** 4.86-7.19 x10^6 **GFP** A: 1.09 ng P: 1.04 ng **Standard Error** 1-1.18 ng	**O157** Eff: 93.70% R^2: 0.9996 **GFP** Eff:92.86% R^2: 0.9982	**O157** A: 1.36 x 10^7 P: 1.30 x 10^7 **Standard Error** 1.1-1.62 x 10^7 **GFP** A: 1.01 ng P: 1.02 ng **Standard Error** 0.935-1.09 ng	**O157** Eff: 94.67% R^2: 0.9979 **GFP** Eff: 94.99% R^2: 0.9988
***C. jejuni*/GFP**	***C. jejuni*** Eff: 103.82% R^2: 0.9989 **GFP** Eff: 78.39% R^2: 0.9984	***C. jejuni*** A:N/A P:N/A **Standard Error** **GFP** A:N/A P:N/A **Standard Error**	***C. jejuni*** Eff: 93.89% R^2: 0.9975 **GFP** Eff: 83.37% R^2: 0.9979	***C. jejuni*** A: 8.2 x 10^4 P: 5.68 x 10^4 **Standard Error** 6.92-9.49 x 10^4 **GFP** A: 1.02 ng P: 0.79 ng **Standard Error** 1.01-1.04 ng	***C. jejuni*** Eff: 98.33% R^2: 0.9968 **GFP** Eff: 93.26% R^2: 0.9997	***C. jejuni*** A: 1.27 x 10^5 P: 1.11 x 10^5 **Standard Error** 1.06-1.47 x 10^5 **GFP** A: 1.15 ng P:1.13 ng **Standard Error** 1.13-1.17 ng	***C. jejuni*** Eff: 100.57% R^2: 0.9986 **GFP** Eff: 82.7% R^2: 1

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