SENSING PLATFORM DESIGN FOR FAECAL INDICATOR BACTERIAL DETECTION IN RECREATIONAL WATERS

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Thesis submitted for the award of PhD

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD for ‘Sensing platform design for Faecal indicator bacterial detection in Marine waters’ is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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“You can never be overdressed or overeducated.”

— Oscar Wilde
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Contents

DECLARATION .............................................................................................................................................. II
ACKNOWLEDGEMENTS ............................................................................................................................... IV
CONTENTS .................................................................................................................................................. V
LIST OF TABLES .......................................................................................................................................... X
LIST OF FIGURES ....................................................................................................................................... XI
LIST OF ABBREVIATIONS AND ACRONYMS .............................................................................................. XIV
ABSTRACT ................................................................................................................................................ XIX

1 LITERATURE REVIEW ............................................................................................................................. 1
   1.1 FAECAL POLLUTION AND ILLNESS ................................................................................................. 2
   1.2 FAECAL INDICATORS ....................................................................................................................... 2
   1.3 CLASSIFICATION OF FAECAL INDICATORS ...................................................................................... 3
      1.3.1 Microbiological indicators ......................................................................................................... 3
      1.3.2 Chemical indicators .................................................................................................................... 5
      1.3.3 Physical indicators ..................................................................................................................... 6
   1.4 ISSUES WITH THE USE OF FAECAL INDICATORS ........................................................................... 7
   1.5 CURRENT LEGISLATION .................................................................................................................... 7
   1.6 FAECAL INDICATOR DETECTION ...................................................................................................... 8
      1.6.1 Culture based methods ................................................................................................................. 9
      1.6.2 Enzymatic methods ...................................................................................................................... 9
      1.6.3 Molecular methods ..................................................................................................................... 11
      1.6.4 Biosensors .................................................................................................................................. 12
      1.6.5 Flow cytometry .......................................................................................................................... 13
      1.6.6 Microbial source tracking (MST) ............................................................................................... 14
      1.6.7 Optical ....................................................................................................................................... 15
      1.6.8 Predictive Modelling ............................................................................................................... 16
   1.7 ENZYME ASSAYS FOR E. COLI DETECTION ..................................................................................... 18
      1.7.1 E. coli ........................................................................................................................................ 18
      1.7.2 Survival and significance of VBNC bacteria ............................................................................... 21
      1.7.3 Sample pre-concentration .......................................................................................................... 22
      1.7.4 Substrates and hydrolysis ........................................................................................................... 22
      1.7.5 Continuous and discontinuous detection methods .................................................................. 23
      1.7.6 Fluorescence overview .............................................................................................................. 24
   1.8 INSTRUMENTATION FOR FLUORESCENCE ENZYME ASSAYS .................................................... 26
      1.8.1 Online automated systems ......................................................................................................... 26
2 B-Glucuronidase assays for E. coli ........................................ 31

1.8.2 Laboratory based systems ........................................... 27
1.8.3 Field Portable systems ............................................. 27
1.8.4 Deployable autonomous systems .................................. 28
1.9 CONCLUSION ..................................................................... 29
1.10 RESEARCH PROPOSAL AND OBJECTIVES ...................... 30

2 B-Glucuronidase assays for E. coli ........................................ 31

2.1 INTRODUCTION .......................................................... 32
2.1.1 Substrate selection ................................................... 32
2.1.2 Matrix effects ......................................................... 33
2.1.3 Raw water Metabolism based assay .............................. 33
2.1.4 Sample variability .................................................... 34
2.1.5 Aims of Chapter 2: β-Glucuronidase assays for E. coli. ........ 34

2.2 MATERIALS ................................................................. 35

2.3 METHODS ........................................................................ 35
2.3.1 Substrate selection ................................................... 35
2.3.2 Matrix effects ......................................................... 36
2.3.3 Environmental water sampling procedure ....................... 37
2.3.4 Variability within a sample ......................................... 37
2.3.5 Continuous direct fluorescence assay ............................ 38

2.4 RESULTS AND DISCUSSION .............................................. 40
2.4.1 Substrate selection ................................................... 40
2.4.2 Matrix effects ......................................................... 50
2.4.3 Variability within a sample ......................................... 52
2.4.4 Continuous direct fluorescence assay ............................ 54

2.5 CONCLUSION ..................................................................... 62

3 COLISENSE 1 DESIGN AND TEST ......................................... 63

3.1 INTRODUCTION .......................................................... 64
3.1.1 Aims of Chapter 3: ColiSense 1 design and test .................. 67

3.2 MATERIALS AND METHODS .......................................... 68
3.2.1 Chemicals and reagents ............................................. 68
3.2.2 Engineering components ........................................... 68
3.2.3 Sample vial reaction vessel ........................................ 68
3.2.4 Fluorescence detection system development .................... 69
3.2.5 Fluorescence detection system characterisation ................. 76
3.2.6 Commercial GUS kinetics .......................................... 76
3.2.7 Environmental sample dilution series .............................................. 76
3.2.8 Field trial ..................................................................................... 77
3.2.9 Raw water and extra cellular enzyme testing .................................. 78
3.3 RESULTS AND DISCUSSION ............................................................. 79
  3.3.1 Fluorescence detection calibration .................................................. 79
  3.3.2 Commercial GUS kinetics ............................................................. 83
  3.3.3 Environmental sample testing ....................................................... 83
  3.3.4 Field trial ..................................................................................... 85
  3.3.5 Raw water and extra cellular enzyme .......................................... 88
3.4 CONCLUSION .................................................................................... 89
4 COLISENSE 2 DESIGN AND TEST ...................................................... 91
  4.1 INTRODUCTION ................................................................................ 92
    4.1.1 Sample pre-concentration method ............................................... 92
    4.1.2 Filtration and metabolic assay concept ....................................... 93
    4.1.3 Aims of Chapter 4: ColiSense 2 design and test ......................... 94
  4.2 MATERIALS AND METHODS ............................................................ 95
    4.2.1 Chemicals and reagents ............................................................. 95
    4.2.2 Engineering components .......................................................... 95
    4.2.3 Filter system concept ............................................................... 95
    4.2.4 Filter system development ......................................................... 97
    4.2.5 Detection system development ................................................. 98
    4.2.6 Filter system testing ............................................................... 99
    4.2.7 Optical characterisation ........................................................... 99
    4.2.8 Commercial GUD kinetics ..................................................... 100
    4.2.9 Environmental sample dilution series ....................................... 100
    4.2.10 Field trial .............................................................................. 101
  4.3 RESULTS AND DISCUSSION ......................................................... 102
    4.3.1 Filter system tests .................................................................. 102
    4.3.2 Fluorescence detection system characterisation .......................... 104
    4.3.3 Commercial GUS kinetics ...................................................... 106
    4.3.4 Environmental sample dilution series ...................................... 107
    4.3.5 Field trial .............................................................................. 108
  4.4 CONCLUSION ................................................................................... 110
5 FIELD EVALUATION OF COLISENSE 1 & 2 .................................... 111
  5.1 INTRODUCTION ............................................................................... 112
5.1.1 Aims of Chapter 5: Field evaluation of ColiSense 1 & 2....................113

5.2 MATERIALS AND METHODS .................................................................114
  5.2.1 Chemicals and reagents .................................................................114
  5.2.2 Field sampling ...............................................................................114
  5.2.3 Physical and chemical water quality ..............................................116
  5.2.4 Microbiological analysis .................................................................116
  5.2.5 Rainfall and river level .................................................................117

5.3 RESULTS AND DISCUSSION ...............................................................118
  5.3.1 Rainfall and river flow .................................................................118
  5.3.2 Physical and chemical water quality ..............................................119
  5.3.3 Microbiological water quality .......................................................121
  5.3.4 Comparison with standard methods .............................................125

5.4 CONCLUSION ......................................................................................130

6 CULTURE BASED ASSAYS FOR E. COLI & ENTEROCOCCI ..............131

6.1 INTRODUCTION ...................................................................................132
  6.1.1 Culture media ..............................................................................132
  6.1.2 Detection methods .......................................................................134
  6.1.3 Aims of Chapter 6: Culture based assays for E. coli and Enterococci..136

6.2 MATERIALS AND METHODS ..............................................................137
  6.2.1 Chemicals and reagents .................................................................137
  6.2.2 Test for variability within a sample ...............................................137
  6.2.3 Growth media in ColiSense ............................................................137
  6.2.4 Time to detect TTD .......................................................................138
  6.2.5 Dilute to specification assay ..........................................................139
  6.2.6 Miniature MPN assay ....................................................................141

6.3 RESULTS AND DISCUSSION ...............................................................142
  6.3.1 Sample variability .........................................................................142
  6.3.2 Growth media in ColiSense 1 .......................................................144
  6.3.3 Time to detect ...............................................................................147
  6.3.4 Dilute to specification ...................................................................149
  6.3.5 Miniature MPN ............................................................................153

6.4 CONCLUSIONS ....................................................................................155

7 CONCLUSIONS AND FUTURE WORK ..............................................157

7.1 CONCLUSIONS FROM LITERATURE REVIEW .................................158

7.2 CONCLUSIONS FROM EXPERIMENTAL WORK ...............................159
7.3 RECOMMENDED ENGINEERING ADVANCEMENTS ........................................... 160
  7.3.1 Redesign of ColiSense 1 ...................................................................... 160
  7.3.2 Redesign of ColiSense 2 ...................................................................... 160
  7.3.3 Automated system design ................................................................. 162
7.4 RECOMMENDED ASSAY DEVELOPMENTS .................................................. 164
7.5 RECOMMENDED FURTHER TESTING ...................................................... 165
7.6 COMMERCIALISATION ........................................................................... 166
A1: PUBLICATIONS ..................................................................................... A-1
A 2: COLISENSE 1 DESIGN DETAILS .......................................................... A-6
A 3: COLISENSE 1 FUNCTIONAL TESTING ................................................... A-14
List of Tables

**TABLE 1-1:** BATHING WATER STANDARDS, INLAND ......................................................... 8

**TABLE 1-2:** BATHING WATER STANDARDS, COSTAL AND TRANSITIONAL....................... 8

**TABLE 1-3:** COMPARISON OF DETECTION METHODS ..................................................... 17

**TABLE 2-1:** CHEMICAL AND PHOTO-PHYSICAL PROPERTIES OF 3 FLUOROPHORES. .......... 32

**TABLE 2-2:** SUBSTRATE ABSORBANCE AND EMISSION WAVELENGTHS, ......................... 50

**TABLE 2-3:** *E. coli* CONCENTRATIONS ACHIEVED USING CENTRIFUGATION. .................. 54

**TABLE 3-1:** COLISense 1 FIELD-TRIAL BACKGROUND DATA ........................................... 86

**TABLE 4-1:** FILTER TRAPPING EFFICIENCY ................................................................. 104

**TABLE 5-1:** DUBLIN BAY BATHING WATERS QUALITY .................................................... 112

**TABLE 5-2:** SAMPLE POINT DESCRIPTION AND COORDINATES ...................................... 115

**TABLE 5-3:** DUBLIN RIVER STUDY SAMPLING SCHEDULE .............................................. 116

**TABLE 5-4:** WEATHER CONDITIONS DURING SAMPLING PERIOD ..................................... 118

**TABLE 5-5:** PHYSICAL AND CHEMICAL WATER QUALITY ............................................. 120

**TABLE 5-6:** DUBLIN RIVERS 5 DAY AVERAGES ............................................................... 121

**TABLE 5-7:** COLISense COMPARISON WITH COMMERCIAL METHODS ............................. 128

**TABLE 5-8:** COMPARISON OF 4 GUS ACTIVITY DETECTION METHODS .............................. 129

**TABLE 6-1:** GROWTH MEDIA INGREDIENTS ................................................................. 133

**TABLE 6-2:** BWD, VOLUMES PROBABLY CONTAINING 1 CFU ........................................... 135

**TABLE 6-3:** TRUTH TABLE FOR SEMI-QUANTITATIVE 3-VIAL MPN METHOD .................... 140

**TABLE 6-4:** SAMPLE VARIABILITY AT 0.8 ML SAMPLE VOLUME ........................................ 143

**TABLE 6-5:** RELIABILITY OF DILUTE TO SPECIFICATION CLASSIFICATIONS .................... 153
List of Figures

FIGURE 1-1: TOTAL COLIFORMS. ................................................................. 3
FIGURE 1-2: SANDWICH ELISA DETECTION PRINCIPLE.......................... 11
FIGURE 1-3: PRINCIPLE OF BIOSENSORS. ............................................. 13
FIGURE 1-4: SCHEMATIC ILLUSTRATING THE PRINCIPLE OF FLOW CYTOMETRY ...............14
FIGURE 1-5: SCHEMATIC ILLUSTRATING THE BIOSENTRY MALS SYSTEM .......... 15
FIGURE 1-6: B-GLUCURONIDE METABOLIC PATHWAY OF E.COLI, .................. 18
FIGURE 1-7: FACTORS CONTRIBUTING TO GUS ACTIVITY IN ENVIRONMENTAL WATERS .20
FIGURE 1-8: JABLONSKI DIAGRAM ......................................................... 25
FIGURE 1-9: INNER FILTER EFFECT ....................................................... 26
FIGURE 2-1: 4-N ABSORBANCE CALIBRATION AT 405 NM.......................... 40
FIGURE 2-2: 4-MU FLUORESCENCE CALIBRATION CURVE ......................... 41
FIGURE 2-3: 3D FLUORESCENCE RESPONSE OF 3 FLUOROPHORES ................... 43
FIGURE 2-4: EXCITATION SCANS OF 3 FLUOROPHORES .............................. 44
FIGURE 2-5: SUBSTRATE ABSORBANCE SPECTRA. .................................... 47
FIGURE 2-6: SUBSTRATE EMISSION SPECTRA ........................................ 48
FIGURE 2-7: EFFECT OF TURBIDITY ON FLUORESCENCE OF 4-MU ..................... 50
FIGURE 2-8: FLUORESCENCE LOSS IN A 96-WELL PLATE............................ 51
FIGURE 2-9: VARIABILITY IN 1ML SAMPLES MEASURED WITH PETRIFILM ............ 53
FIGURE 2-10: FRESHWATER 6-CMUG RESPONSE ....................................... 55
FIGURE 2-11: BRACKISH WATER 6-CMUG RESPONSE ................................ 56
FIGURE 2-12: FRESHWATER 6-CMUG RESPONSE VS E.COLI CONCENTRATION ........ 57
FIGURE 2-13: BRACKISH WATER 6-CMUG RESPONSE VS E.COLI CONCENTRATION .... 58
FIGURE 2-14: FRESHWATER 6-CMUG RESPONSE ON PLATE-READER ................ 59
FIGURE 2-15: BRACKISH WATER 6-CMUG RESPONSE ON PLATE READER ............ 60
FIGURE 3-1: FLUORESCENCE BASED ENZYME ASSAY PRINCIPLE .................. 66
Sensing platform design for Faecal indicator bacterial detection in recreational waters

Figure 3-2: ColiSense optical design. ................................................................. 70
Figure 3-3: ColiSense incubation and fluorescence detection system .......... 70
Figure 3-4: ColiSense instrument component parts. .................................. 72
Figure 3-5: ColiSense 1 software components ........................................... 75
Figure 3-6: Field trial of ColiSense 1. ............................................................... 78
Figure 3-7: ColiSense 1 and LS50B dynamic range for 6-CMU .................... 79
Figure 3-8: ColiSense 1 sensitivity optimisation ......................................... 80
Figure 3-9: ColiSense 1 system characterisation ........................................ 82
Figure 3-10: ColiSense ITarget analyte testing ........................................... 84
Figure 3-11: ColiSense 1 River Tolka field trial ........................................... 86
Figure 3-12: GUS activity of raw water and extracellular enzyme ............... 88
Figure 4-1: Depth filter pre-concentration .................................................... 96
Figure 4-2: ColiSense 2 filter system components and setup ....................... 97
Figure 4-3: ColiSense 2 construction ............................................................. 98
Figure 4-4: Auto-fluorescence of Eastar™ copolyester EB062 ...................... 102
Figure 4-5 Filtration rate vs turbidity ....................................................... 103
Figure 4-6: ColiSense 2 fluorescence calibration ........................................ 105
Figure 4-7: ColiSense 2 commercial GUS activity .................................... 106
Figure 4-8: ColiSense 2 environmental sample serial dilution ....................... 107
Figure 4-9: ColiSense 2 progress curves for an environmental sample .......... 108
Figure 4-10: ColiSense 2 field trial results .................................................... 109
Figure 5-1: Map of Dublin rivers ................................................................. 114
Figure 5-2: River levels during sampling period ......................................... 119
Figure 5-3: Time study of Dublin rivers ..................................................... 123
Figure 5-4: Pollution tracing studies on 5 Dublin rivers .............................. 124
Figure 5-5: Log-log relationships GUS activity vs MPN standard methods .. 126
Figure 5-6: Coefficient of variation of GUS detection methods ................... 127
Figure 6-1: Bacterial growth curve ................................................................. 132
Figure 6-2: 3-vial method for *E. coli* ................................................................. 140
Figure 6-3: ColiSense 1 response to Colilert 18 media ..................................... 145
Figure 6-4: ColiSense 1 response using different growth media ..................... 145
Figure 6-5: Time to detect (TTD) principle ...................................................... 147
Figure 6-6: Time to detect method on ColiSense 1 ......................................... 148
Figure 6-7: Dilute to specification response ..................................................... 150
Figure 6-8: Well-plate setup for dilute to specification and Mini-MPN .......... 151
Figure 6-9: Dilute to spec, distribution of classification occurrences .......... 152
Figure 6-10: MPN detection limit for 96 well plate ....................................... 154
Figure 6-11: Mini MPN performance ............................................................... 155
Figure 7-1: Portable and disposable filtration setup ........................................ 161
Figure 7-2: Top down fluorescence measurement ........................................... 162
Figure 7-3: Biofouling of sample vials ............................................................. 163
Figure 7-4: Automated carousel system for repeat sampling ......................... 163
List of Abbreviations and Acronyms

3-CU: Carboxyumbelliferyl
3-CUG: Carboxyumbelliferyl β-D-glucuronide
4-MU: 4-Methylumbelliferone
4-MUD: 4-Methylumbelliferyl β-D-Galactopyranoside
4-MUG: 4-Methylumbelliferone-β-D-glucuronide
4-N: 4-Nitrophenol
6-CMU: 6-Chloro-4-Methylumbelliferone
6-CMUG: 6-Chloro-4-Methylumbelliferone-β-D-glucuronide
AWISS: Autonomous Wireless In Situ Sensor
BWD: Bathing Water Directive
CARD: Chemistry and Reagent Device
CBT: Compartment Bag Test
CFU: Colony Forming Unit
CRM: Certified reference material
CS 1: ColiSense 1
CS 2: ColiSense 2
CSO: Combined sewer overflow
DCC: Dublin City Council
DNA: Di-Ribonucleic Acid
E. coli: Escherichia Coli
EIS: Electrochemical Impedance Spectroscopy
ELISA: Enzyme Linked Immunosorbent Assay
EPA: Environmental Protection Agency
EU: European Union
FC: Faecal Coliforms
Sensing platform design for Faecal indicator bacterial detection in recreational waters

**FI**: Faecal Indicator

**FISH**: Fluorescent In-Situ Hybridisation

**FS**: Faecal Streptococci

**GC**: Gas Chromatography

**GI**: Gastro-Intestinal

**GUS / GUD**: Beta Glucoronidase

**H₂S**: Hydrogen Sulphide

**HPC**: Heterotrophic Plate-Count

**HPLC**: High Performance Liquid Chromatography

**IFA**: Immuno-Fluorescence Assay

**LB**: Luria Broth

**LED**: Light Emitting Diode

**LOD**: Limit of Detection

**MALS**: Multi-Angle Light Scattering

**MF**: Microfiltration

**MPN**: Most probable number

**MS**: Mass Spectrometry

**MST**: Microbial Source Tracking

**MTF**: Multiple Tube Fermentation

**NASA**: National Aeronautics and Space Administration

**nm**: nano meter

**NTU**: Nephelometric turbidity unit

**OB**: Optical Brightener

**P/A**: Presence / Absence

**PCR**: Polymerase Chain Reaction

**PE**: PolyEthylene

**PP**: Polypropylene
**PPT:** Parts per thousand

**qPCR:** Quantitative PCR

**RNA:** Ribonucleic Acid

**SOB:** Sub Optimal Broth

**SPE:** Solid Phase Extraction

**TB:** Terrific Broth

**TC:** Total Coliforms

**TSS:** Total suspended solids

**TTC:** Thermo-Tolerant Coliforms

**TTD:** Time To Detect

**USEPA:** United States Environmental Protection Agency

**UV:** Ultra Violet

**VBNC:** Viable But Not Culturable

**VC:** Viable Culturable

**WFD:** Water Framework Directive
Abstract

Faecal indicator detection in recreational waters is of growing importance in Europe and the rest of the developed world for the safeguarding of the health of users. The EU Bathing Water Directive (BWD) dictates the microbiological water quality standards for European waters using the Faecal indicators: *Escherichia coli* (*E. coli*), and *Enterococci*. Waters are classified as ‘Excellent’, ‘Good’ or ‘Sufficient’. To measure compliance, culture-based tests are widely used and accepted e.g. Colilert 18 from IDEXX or membrane filtration. These methods are reliable and proven but they are slow, typically taking 18 hours or more to produce a result. These are limited to detecting only Viable-Culturable (VC) cells but not Viable-But-Not-Culturable (VBNC) cells. More rapid results incorporating VBNC detection would allow for more timely and accurate decision-making by governing bodies.

This thesis investigates the use of rapid assays based on enzymatic detection to allow for sub 4 hour quantification of *E. coli* and *Enterococci* in recreational waters. The work involved improving upon existing enzymatic assays through the introduction of novel reagents and the development of field portable instrumentation for On-site analysis of samples.

In this work an enzyme assay for *E. coli* detection based on β-Glucoronidase activity and the fluorescent substrate 6-Chloro-4-Methylumbelliferone-β-D-glucuronide (6-CMug) was developed. 6-CMug is only recently available (2010) and offered higher fluorescence yield and lower pH sensitivity than previously available substrates such as 4-Methylumbelliferone-β-D-glucuronide (4-Mug). The assay developed offered significant improvements in speed, LOD and sensitivity over existing assays based on 4-Mug. As there is no specific enzyme for the detection of *Enterococci* culture based assays with specific media were also investigated and a number of detection methodologies were developed.

A sensitive field-portable fluorimeter with incubating capability and triplicate sample chambers was designed and built for the on-site analysis of water samples. This development moved beyond state of the art, which was based upon laboratory fluorimeters. The system named ColiSense 1 was designed to conduct a continuous direct enzyme assay for *E. coli* where the cells were filtered and lysed to release β-Glucuronidase. Data from a one day field trial demonstrated the ability of the system to deliver results on-site within a 75 minute period.
An upgraded system named ColiSense 2 was designed to incorporate sample preparation and pre-concentration in order to reduce the total time from sample to answer. The assay used in this was based on continuous direct metabolism of 6_CMUG by *E.coli* thus removing the lysing step. On the same one day field trial as before data demonstrated the ability of the system to deliver results *on-site* within a 30 minute period.

Both the ColiSense 1 and 2 systems achieved sub 1 hour detection and quantification while conducting measurements on-site. Limits of detection (LOD) achieved for both methods were 125 CFU/100 mL. When compared with the standard method: IDEXX Colilert 18 the LODs are much higher but they are below the ‘Excellent’ standards as dictated by the BWD. Thus these devices are suitable for rapid warnings of pollution events.
1 LITERATURE REVIEW
1.1 Faecal pollution and illness

Faecal pollution of recreational waters occurs due to the leakage of human or animal waste into the water body and also due to the presence of wildlife such as gulls. The largest risk to human health comes from the presence of human wastes [1][2] which allows for the recycling of pathogens e.g. Cryptosporidium, Giardia, back to bathers. Numerous epidemiological studies have linked the presence of faecal matter to gastrointestinal diseases and allowable concentrations of pollution have been established [3]-[10].

1.2 Faecal indicators

Faecal Indicators (FI) include microbiological, chemical, and physical parameters which can be used in detection as a surrogate for pathogens which are often difficult to detect. Many have been suggested and evaluated since their first use in the 19th century and with advancements in detection technology new indicators are being found and old indicators are becoming viable once again [11]-[13].

FI have two main functions:

1. to index i.e. relate to health risks, and
2. to indicate i.e. demonstrate the effectiveness of water treatment [14].

An ideal FI should:

- Be easily detected using simple laboratory tests,
- Generally not be present in unpolluted waters,
- Be non-pathogenic,
- Appear in concentrations greater than that of the pathogens,
- Be present in a direct ratio to pathogens,
- Be unable to reproduce outside of the gut, and
- Have a similar lifespan in the environment to the pathogens of interest [11].
1.3 Classification of faecal indicators

1.3.1 Microbiological indicators

There are numerous types of microbiological indicators of faecal pollution which vary in specificity and suitability for different environments [15], [16].

*Total coliforms (TC):* A group of bacteria which can originate from human faeces and other sources in the environment. They are little used as an indicator for recreational waters but are used for drinking waters [14]. Figure 1-1 shows the classes of bacteria within Total coliforms.

![Figure 1-1: Total coliforms. Venn diagram showing the classes of bacteria within Total coliforms. Adapted from [17].](image)

*Faecal coliforms (FC):* Bacteria which generally originate from faeces. However this is not exclusive, so depending on local conditions, alternative more specific indicators may be required.

*E.coli:* A Gram negative, thermo-tolerant rod shaped coliform bacterium commonly found in the lower intestine of warm blooded animals. The US EPA recommends *E. coli* as the best indicator of health risk from water contact in recreational fresh waters [13], [18].

*Faecal streptocci (FS):* These originate in the intestines of humans and warm blooded animals. They are now rarely used as indicator bacteria [19]. The *Faecal coliform/Faecal*
Streptococci (FC/FS) ratio has been used to differentiate between human and animal sourced pollution [11].

Clostridium perfringens: Anerobic spore-forming gram-positive rods. They originate exclusively in faeces. They survive longer in water than E. coli or Streptococci but are not useful indicators in recreational waters as they can be re-suspended from sediments causing false positives in detection long after a pollution event [11], [20].

Enterrococci: Gram positive cocci which are a subgroup of faecal streptococcus. They survive in salt water and are more human specific than the rest of the faecal streptococcus group. The US EPA recommends them for use as an FI in recreational salt-waters but they can also be used in freshwater [21].

Bacteriophages: Bacterial viruses which depend upon the presence of bacteria to replicate e.g. the somatic coliphage targets E. coli so its presence in water infers the presence of E. coli [22]-[25].

Bifidobacteria: Gram-positive anaerobic rods found in human and animal faeces. They are considered useful indicators in the tropics as they won’t multiply outside the gut. They are not widely used as they have a short survival time in the environment and are difficult to detect [21].

Rhodococcus: Anerobic bacteria sourced exclusively in farm animal faeces. It survives longer in water than other indicators. It has been used as an indicator of farm animal pollution but it is slow to detect taking 17-18 days to obtain a result [21].

Heterotrophic plate count (HPC) bacteria: Aerobic or facultative anaerobic gram-negative bacteria. They are non-specific as they can arise in vegetation as-well as sewage and can multiply in the environment. Therefore they are not a direct indicator of faecal pollution but can be used as an indicator of water quality [11].

Pseudomonas aeruginosa: A pathogen which multiplies in the environment. This is not a good indicator as it is not specific to faeces [21].

Bacteroides: Anaerobic bacteria, more numerous in human faeces than E. coli. They die rapidly in water and are difficult to enumerate [12], [25], [26]. These have been suggested for use with PCR detection as an indicator in marine waters as they display less cross reactivity than other microbiological indicators [27].

Candida albicans: A yeast found in faeces has been used but was found not to be reliable as it can originate from various sources [11].
Of the microbes listed here, *E. coli* and Enterococci are widely regarded as the best indicators of faecal pollution due to their prevalence in faecal matter and their relative ease of detection. The preference for these two indicators is borne out in legislation in most developed countries with standard methods for the detection of both embedded in the European Bathing Water Directive. [28][11], [29], [30]

### 1.3.2 Chemical indicators

Certain chemical markers of exclusively faecal or urine origins have been suggested as FI, these include:

*Faecal stenols & stanols:* Reliable indicators of faecal pollution. Copropstanol in particular, which derives from cholesterol, is specific to sewage. Detection however requires analysis by Gas-Chromatography, a complex technique utilising expensive instrumentation [31].

*Urobilins:* A product of intestinal micro-flora, specific to mammals. Detection requires HPLC a complex technique which limits their usefulness as an indicator [11].

*Bile acids and Aminoacetone* can also be used as markers but their detection involves complex techniques such as GC-MS and HPLC [32]-[34].

*H₂S:* Produced by faecal bacteria including: *Citrobacter, Enterobacter, Salmonella, Clostridium perfringens.* It is detectable using simple inexpensive colour-change chemistry. The H₂S paper strip test is popular in resource poor locations as its cost is far lower than that of standard coliform tests. It can be used for presence/absence (P/A) tests or semi quantitatively by measuring the time taken to change colour or by MPN. Accuracy and specificity are variable. Results are obtained in 12 to 120 hours [35], [36].

*Caffeine:* Specific to humans but not necessarily to sewage as it can be disposed of in storm drains or solid waste. It has not been proven as a reliable indicator [37], [38].

*Human Pharmaceuticals:* chlorpropamide, phensuximide and carbamazepine have been used as indicators of human waste pollution. Detection can be complex and some pharmaceuticals are common to animals and humans [39].

*Boron:* From detergents, once proposed as an indicator has fallen out of favour as many detergent manufacturers have replaced Boron with alternatives in their manufacturing processes [40].
Optical brighteners (OB)’s: From detergents are easily detectable by simple fluorescence measurements. They however are not necessarily related to sewage as they may be a result of industrial pollution [41].

Of the chemical indicators listed here none are commonly used in developed countries however various chemicals are now being included in Microbial Source Tracking (MST) techniques which use a range of analyses to identify the origin of bacterial pollution [42]. H$_2$S is used in low resource settings due to its cost effectiveness, but it is not regarded as quantitative test and is mainly used for presence/absence measurements [43].

1.3.3 Physical indicators

A number of physical and optical parameters can be used to infer the presence of pollution in water, but not necessarily identify it. These can be used to monitor conditions and trigger more specific testing to identify and or quantify the pollution.

Turbidity: Turbidity is the cloudiness of a liquid caused by minute particles suspended within. In environmental waters turbidity can originate from natural sources such as mud re-suspension or from anthropogenic pollution such as sewage inflow. Quantification can be performed using a simple and direct optical measurement. There is however no clear relationship between faecal pollution and turbidity, and each site will have different characteristics. Turbidity can be used to detect a change in the environment but not to quantify pollution [44].

Colour: Indirectly related to pollution. E.g. increased nutrients in water due to a faecal pollution incident may lead to increased growth in chlorophyll producing algae causing a green tint to the water. The relationship is non-specific and tenuous so like turbidity can only indicate a change in the environment.

UV absorbance: Can indicate the presence of dissolved organics e.g. tryptophan, and infer the presence of faecal pollution [45], [46].
1.4 Issues with the use of faecal indicators

The use of FI to imply the presence of pathogens is a debated topic, and has been for decades. Water quality and the presence of pathogens are not necessarily directly related to the presence of FI bacteria. Using FI bacteria numbers as a standard can lead to ambiguous results and false negatives due the lack of a definitive relationship [11], [47]. FI such as *Escherichia Coli* (*E.coli*), while heavily used in temperate climates are not reliable in tropical climates due to their background presence in the environment and their ability to in some circumstances multiply in that environment, particularly in sediments. Risk based approaches such as sanitary surveys are more commonly used than indicator enumeration in tropical climates to determine the quality of a water source.

Despite the above issues there is wide agreement that *E.coli* and *Enterococci*, are the best available indicators of water microbiological quality. They are widely used across the developed world and they seem set to remain so for the foreseeable future [29], [30], [48], [49]. More recent studies on the uses of FI use in the tropics have reconfirmed *E. coli* as the best indicator for both recreational and drinking waters [50]-[52].

1.5 Current legislation

In 2000 the European Union adopted the Water Framework Directive 2000/60/EC (WFD) to manage and protect European water supplies and in 2006 the Bathing Water Directive set the standards for recreational waters. It classified them into “Poor, Sufficient, Good or Excellent.” The BWD set out the microbiological parameters, threshold levels and methods of analysis for quality control. These are displayed in Table 1-1 and Table 1-2.

The reference methods listed are for Enterococci: ISO 7899-1 (miniaturized Most Probable Number (MPN)) and ISO 7899-2 (Membrane Filtration (MF)) and for E. coli: ISO 9308-3 (miniaturized Most Probable Number (MPN)) and ISO 9308-1 (Membrane Filtration (MF)).
Table 1-1: Bathing water standards, inland [28].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Excellent quality</th>
<th>Good quality</th>
<th>Sufficient quality</th>
<th>Reference methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal Enterococci</td>
<td>200(*)</td>
<td>400(*)</td>
<td>330(*)</td>
<td>ISO 7899-1 or ISO 7899-2</td>
</tr>
<tr>
<td>(CFU/100mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>500(*)</td>
<td>1,000(*)</td>
<td>900(**)</td>
<td>ISO 9308-3 or ISO 9308-1</td>
</tr>
<tr>
<td>(CFU/100mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(*) Based upon a 95-percentile evaluation, (**) Based on a 90-percentile evaluation.

Table 1-2: Bathing water standards, coastal and transitional [28].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Excellent quality</th>
<th>Good quality</th>
<th>Sufficient quality</th>
<th>Reference methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal Enterococci</td>
<td>100(*)</td>
<td>200(*)</td>
<td>185(*)</td>
<td>ISO 7899-1 or ISO 7899-2</td>
</tr>
<tr>
<td>(CFU/100mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>250(*)</td>
<td>500(*)</td>
<td>500(**)</td>
<td>ISO 9308-3 or ISO 9308-1</td>
</tr>
<tr>
<td>(CFU/100mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.6 Faecal indicator detection

A multitude of determination methods with varying specificity, repeatability, and response time have been developed over the years to identify various faecal indicators. Traditional methods such as micro-filtration are inexpensive and reliable but their response time is in the order of 24 to 48 hours which is too great for many modern applications. There is a drive towards real-time monitoring of faecal indicators, for environmental applications, water-supply and food processing. Thus there have been many recent developments in this area with a focus on reduced analysis time [53]-[58].

‘Rapid’ enzyme assays which give results on the same working day show promise. Nobel and Weisberg recommend that to qualify as rapid a detection method must take 4 hours or less to respond. This is regarded as the maximum time allowable to enable municipalities to respond to a positive result within the same day [58].
Chapter 1: Literature review

Most microbiological measurements display a level of uncertainty much higher than that for chemical or physical measurements. The uncertainty is typically 10% or higher [59]-[61].

1.6.1 Culture based methods

*Multiple tube fermentation (MTF)* involves placing dilutions of the water sample in tubes and incubating for 48 hr at 35°C. The production of gas, acid or growth within the tubes indicates a positive sample. This is also known as Most Probable Number (MPN) method. Colilert 18 is an advancement on this technique and is now the industry standard for recreational water monitoring.

*Membrane filtration (MF)* or culture and colony-count involves filtering a water sample through a sterile 0.45 micron filter (*E. coli* are typically 1μm in diameter and 2μm in length) [62], trapping the bacteria on the membrane, incubating the filter on selective medium and counting the number of colonies formed using visual methods. MF has been accepted as the standard for drinking water monitoring in many countries [13]. *Potatest* from Wagtech is portable water test kit using micro-filtration, culture and colony counting methods. It is part of a range of detection equipment intended for use in field locations in particular in humanitarian crises. The system replicates standard laboratory methods for field use. Similar field test kits include, ELE Paqualab, Hach MEL portable laboratory series, and the Oxfam / DelAgua kit [63][64].

1.6.2 Enzymatic methods

Monitoring of enzyme activity allows more specificity in indicator detection than classical techniques. β-D-Glucuronidase is one enzyme largely specific to *E.coli*. Its action on fluorogenic or chromogenic substrates is used to indicate the presence of and/or enumerate *E.coli* [13], [65]-[67]. Other enzymes such as Galactosidase have been used successfully [68]. Amperometric detection using substrates such as p-aminophenyl-β-D-glucuronide has also been used [69][70].

Presence/absence detection and enumeration is an improvement upon MTF which uses a specific substrate to promote growth of the target bacteria (*E.coli* or Total coliforms) and a fluorogenic or chromogenic substrate for detection. Samples are incubated in multiple
tubes and the optical change due to enzymatic action on the substrates indicates the presence or absence of the target species. Quantification of the target species can be incorporated using the MPN technique. The IDEXX Colilert 18 is based on this principle and has become the standard test in most European countries [13].

MF with enzymatic detection involves standard MF techniques coupled with fluorogenic or chromogenic substrate added to agar media. Enzymatic activity due to the presence of the target species causes optical change allowing faster enumeration of CFUs than standard MF [13], [66], [71], [72].

Direct fluorescence detection of enzyme activity allows rapid estimation of coliform numbers using a fluorescence threshold detection method. The time taken for an incubated sample in the presence of a substrate to produce a particular level of fluorescence is used to indicate the initial number of coliforms in the sample. The sensitivity of these methods is low, thus they are not suitable for drinking water monitoring. A second issue with this approach is that the direct enzyme assay detects extracellular enzyme in the water, however extracellular GUD can persist in the environment long after cell lysis and death, thus causing false positives [34]. The method has been used in early warning systems e.g. CoilFast automated analyser [13].

*E. coli* and *Enterococci*, the two most commonly used FIs can be detected using enzymatic methods. *E. coli* synthesise the enzyme β-D-Glucuronidase (GUD) to metabolise glucuronides. 97% of *E. coli* strains are GUD positive. GUD activity thus can be used to indicate the presence of the *E. coli* [71]. *Enterococci* synthesise β-D-Glucosidase and use this to metabolise glucosides. A synthetic fluorescent substrate e.g. 4-Methyleumbelliferyl-β-D-Glucoside (MUD) can be used to detect β-D-Glucosidase activity and indicate the presence of *Enterococci*. β-D-Glucosidase is however not specific to *Enterococci* so a selective step is normally included in these assays [72].

Coliplage from Veolia, is a detection method which involves filtration and GUD activity detection using 4-MUG and claims results in 1 hour with an LOD of 100 *E.coli*/100ml. Equipment required includes a lab based spectrophotometer [73].

Enzymatic detection with solid phase cytometry, involves using MF to trap cells which are then labelled with a fluorescent marker such as fluorescein-di-β-D-glycoside and then enumerated using a laser scanning device e.g. a ChemScan RDI. The technique can be used to enumerate coliforms rapidly without the need for culturing [74], [75].
1.6.3 Molecular methods

Molecular methods based on DNA allow rapid analysis with high specificity and sensitivity without the need for cultivation. Response times can be just a few hours [13]. These techniques are not easily applicable for the routine monitoring of bathing water quality, due to their complex implementation, the need for an equipped laboratory with an experienced technician and their cost. However molecular techniques are the best available method for detecting many pathogens and new indicators as efficient culture systems do not exist [76]-[79].

Immunological methods are based on the specific recognition between antibodies and antigens. Immuno-capture of cells can be carried out by Enzyme-Linked Immuno-Sorbent Assay (ELISA) and/or target cells can be detected by Immuno-Fluorescence Assay (IFA) or Immuno-Enzyme Assay (IEA), Figure 1-2 shows a schematic of the ELISA method [13]. Recently aptamers specific to *E. coli* have been developed and appear to be a better option than antibodies for field tests due to their higher stability [80].

Figure 1-2: Sandwich ELISA detection principle showing the capture of a target antigen and the subsequent release of a chromophore from the substrate mediated by the enzyme attached to the detection antibody [81]

Polymerase chain reaction (PCR) involves the amplification of target fragments of DNA through cycling replication. The technique gives high specificity by amplifying a target
gene to detectable levels and low response times, in the order of a few hours. Two methods MPN and competitive PCR are used to quantify DNA. PCR is susceptible to inhibition by, and giving false positive results due to environmental contaminants [13], [34], [55], [82]. As PCR is highly sensitive, it can’t be used to quantify microbes, rather only to indicate their presence [53]. The PCR principle has been used by Rheonix for their field portable Chemistry and Reagent Device (CARD) [83]

qPCR is an advancement upon standard PCR whereby a standard curve is built by analyzing known concentrations. Unknown concentrations in samples are then fitted to the curve [84]. Dorevitch compared qPCR against membrane filtration as a method for indicating the presence of pathogens giardia and cryptosporidium in recreational waters. He concluded that the method provided a similar level of accuracy to the widely used MF technique, and that given its faster response time, he predicted that qPCR would be adopted as the standard method [56]. qRT-PCR denotes quantitative Real-time PCR or quantitative Reverse Transcription PCR. The terms are used interchangeably to refer to the same process as qPCR [26], [85], [86].

FISH uses oligonucleotide probes to detect complementary nucleic acid sequences. It can target DNA or RNA molecules with high specificity. Specific probes are commercially available for different applications e.g. the “Colinsitu” probe for E.coli detection in urine, water and food [13]. However FISH without a viable count step can’t distinguish between VC and VBNC cells. Direct Viable Count (DCV)-FISH was developed in 2010 by Baudart [87]. The method is based on a combination of membrane filtration, fluorescence conjugation and laser scanning cytometry (Scan RDI). This is a promising technique but suitable only to laboratory use.

1.6.4 Biosensors

A biosensor can be defined as an integrated receptor transducer device, which is capable of providing selective quantitative or semi-quantitative analytical information using a biological recognition element or Bioreceptors. In environmental analysis these can be whole cells, enzymes, antibodies or DNA [53], [88]. Figure 1-3 shows a schematic of the general principles of a biosensor.
Transduction elements of biosensors convert a biological signal e.g. binding of an antibody to a target, into a usable signal. There are four main detection methods based on: electro-chemical, optical (including absorbance and fluorescence), mass-sensitive and thermal effects [53], [88], [90]-[92].

The Biohazard water analyser from Early-Warning is an online pathogen detection system intended for water supply and food production industries. This system samples 10 litres of water and uses ultra-filtration to concentrate the sample. It uses a nano-tube-based biosensor licensed from NASA that can detect 10 or more specific pathogens per test. Total pathogens and viable pathogens can be quantified. The sensing element was developed as part of NASA’s Mars programme and then spun out in 2009. High specificity and repeatability are claimed [93].

**1.6.5 Flow cytometry**

Flow cytometry is a rapid non culture method of enumerating particular cells in a sample by passing them in single file through an optical detector and counting them. This is achieved by hydraulically focusing the sample. Often cells are bound to beads to aid flow through the device. A fluorescent labelled oligonucleotide probe or antibody probe is typically used so a target cell fluoresces as it passes through the detector [94]. Flow Cytometry was developed from the Coulter counter which used impedance effects of a particle passing through a channel, to establish the size of the particle [95], [96]. It has been suggested that flow cytometry is not suitable for recreational water analysis (for
Enterococci) due to a lot of non-specific antibody binding [97]. Figure 1-4 depicts the principle of flow cytometry.

Figure 1-4: Schematic illustrating the principle of flow cytometry [98]

1.6.6 Microbial source tracking (MST)

Much has been published recently on MST which is a collection of techniques (molecular, biochemical and chemical) for identifying the source of faecal pollution (i.e. human, ruminant, gull etc.) which allows for better estimation of the risk of illness to bathers. MST can be based on any of the aforementioned detection methods which target host specific genes or chemicals. One early method was the faecal coliform/faecal streptococci ratio used to differentiate human and domestic animal pollution. This has been superseded
by methods such as Ribotyping and PCR, however these are all still in the development phase and have not yet become widespread or achieved regulatory approval. [12], [99]-[101].

1.6.7 Optical

*BioSentry* is a sensing technology which quantifies and characterizes microbiological contamination in drinking water using Multi-Angle Light Scattering (MALS). Known Pathogens are identified by a “bio-optical signature” comparable to a fingerprint. The system is intended for online measurements in water utilities for detecting the presence of pathogens. The system is claimed to be cost effective as it uses no reagents, operates remotely and can monitor continuously [102], [103]. Figure 1-5 shows the MALS principle.

![Figure 1-5: Schematic illustrating the BioSentry MALS system based on the multi-angle light scattering detection method [104]](image-url)
1.6.8 Predictive Modelling

In the absence of a reliable real-time faecal indicator monitoring system, municipalities have resorted to models to predict high levels of faecal indicators. Typically regression analysis of large volumes of historical data (e.g. rainfall, land-usage) vs recorded pollution levels is carried out to develop the model [105], [106]. The SmartCoasts project between University College Dublin (UCD) and University of Aberystwyth investigated real-time prediction of coastal water quality using wireless sensor networks on a catchment feeding a coastal bathing area. The project involved monitoring of *E.coli*, *Enterococci* and microbial source markers and modelling against rainfall and land use [107]-[109]. This study concluded that the use of ‘collaborative’ or networked sensors with distributed intelligence provide more reliable predictions of water quality than traditional sampling programmes.

The Ribble study based in the UK used remote sensing, digital land use data, hydrographic data and water sampling to model FI flows in the watershed. It concluded that FI flows are closely related to hydrographic events and so sampling plans should be designed to be responsive to these events [110].

Table 1-3 compares the detection methods listed above. Of the detection methods available culture-based methods offer a combination of high specificity with ease of use and low equipment requirements. They are however slow with results delivered in a minimum of 18 hours. Flow cytometry on the other hand offers high accuracy and specificity combined with fast results. The drawback however is the complexity and cost of the instrument required. Enzymatic assays offer a middle ground. These can be rapid (sub 4 hours) and can have enough specificity and accuracy to serve as monitoring tools for bathing water compliance. The size and cost of incubators and spectrophotometers can be reduced to allow for field portability of enzymatic assays, thus these are the most promising of the methods investigated here.
Table 1-3: Comparison of detection methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Example</th>
<th>Commercially available</th>
<th>Time to result</th>
<th>Equipment</th>
<th>Sample handling and pretreatment</th>
<th>Training required</th>
<th>LOD</th>
<th>Accuracy</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Based</td>
<td>Colilert 18 from Idexx</td>
<td>Yes</td>
<td>18 to 48 hours</td>
<td>Incubator UV light</td>
<td>Medium</td>
<td>Low</td>
<td>Single cell</td>
<td>± 30%</td>
<td>High</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>Coliplage</td>
<td>No</td>
<td>1 to 4 hours</td>
<td>Incubator Spectrophotometer</td>
<td>Medium</td>
<td>Medium</td>
<td>Multiple cells</td>
<td>± 15%</td>
<td>Medium</td>
</tr>
<tr>
<td>Molecular</td>
<td>Rhenonix PCR card</td>
<td>Yes</td>
<td>Minutes</td>
<td>Thermal cycler</td>
<td>High</td>
<td>Medium</td>
<td>Single cell</td>
<td>Often not quantitative</td>
<td>High</td>
</tr>
<tr>
<td>Biosensors</td>
<td>Microprint Bio-Card</td>
<td>Yes</td>
<td>Minutes</td>
<td>Depends on transduction method</td>
<td>Low</td>
<td>Low</td>
<td>Single cell</td>
<td>Medium</td>
<td>High</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>LSR II Flow Cytometer</td>
<td>Yes</td>
<td>Minutes</td>
<td>Flow cytometer</td>
<td>Medium</td>
<td>Medium</td>
<td>Single cell</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Direct optical</td>
<td>V-Lux fluorimeter from Chelsea instruments</td>
<td>Yes</td>
<td>Instantaneous</td>
<td>Optical probe</td>
<td>None</td>
<td>Low</td>
<td>Multiple cells</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>MST</td>
<td>Service available from 'Source Molecular' certified laboratory</td>
<td>Yes</td>
<td>Depends on methods used</td>
<td>Depends on methods used</td>
<td>High</td>
<td>High</td>
<td>Depends on methods used</td>
<td>Depends on methods used</td>
<td>High</td>
</tr>
<tr>
<td>Predictive modeling</td>
<td>SmartCoasts project Dublin</td>
<td>No</td>
<td>Results before event</td>
<td>Computer historical data-sets and weather forecasts</td>
<td>None</td>
<td>Automated</td>
<td>Not applicable</td>
<td>Warning levels</td>
<td>Low</td>
</tr>
</tbody>
</table>
1.7 Enzyme assays for E. coli detection

1.7.1 E. coli

*E. coli* is a facultatively aerobic, motile, Gram-negative, thermo-tolerant rod shaped coliform bacterium commonly found in the lower intestine of warm blooded animals. There are many strains of *E.coli* present in human faeces only a few of which are pathogenic.

1.7.1.1 Metabolic pathways

*E.coli* can utilise various food sources through different pathways including the Krebs cycle. In the absence of oxygen e.g. when in the human gut, an important energy source are glucuronides. A glucuronide is a glucoronic acid bound to an aglycon which in the case of synthetic glucuronides e.g. 4-MethylUmbelliferyl-β-D-Glucuronide (4-MUG), can be a fluorescent molecule. Figure 1-6 outlines the metabolism process for glucuronides. The glucuronide is transported through the cell wall via a permease. Within the cytoplasm the glucuronide is hydrolysed by the enzyme GUD to separate the glucoronic acid from the Aglycon. The aglycon is then removed from the cell while the glucuronic acid is retained [111].

![B-Glucuronide metabolic pathway of E.coli](image)

**Figure 1-6: B-Glucuronide metabolic pathway of E.coli, Adapted from [111]**
1.7.1.2 β-Glucuronidase

β-GUD is a homo-tetramer meaning that it consists of 4 identical monomers. Substrate recognition and binding takes place through a series of polar interactions at the active site. Its activity is dependent upon it remaining in this configuration [112]. Contact with glass is one mechanism through which it can be deformed and deactivated. Not all *E.coli* utilize GUD e.g. the entero-pathogenic strain O157:H7 is GUD negative. However for the purpose of detection of faecal indicators in environmental waters, GUD is 97% specific to *E.coli*.

1.7.1.3 GUS activity

Garcia-Armisen calculated an activity per cell based on experiments using lysing to extract enzyme from induced cells and hydrolysis of 4-MUG to indicate activity. The reported activity was between 1 and 100 femto-Mol of substrate per minute per cell [113]. This however is approximate as the activity is dependent upon a number of factors as illustrated by Figure 1-7. Culturable, VBNC and dead *E. coli* plus GUS positive non-*E.coli* faecal bacteria all contribute to the GUS activity of a sample the remaining inputs all contribute positively but are deemed as interferences which can lead to false positives. Baudart reports from Michaelis Mentin modeling that maximum GUS activity is achieved at a substrate concentration of 600 µM [114].
1.7.1.4 GUD induction

The presence of glucuronides such as 4-MUG induces the production of Glucuronidase within the *E.coli*. Gratuitous inducers such as I-8350 from BioSynth trigger the production but are not consumed. The use of such inducers can increase the rate of GUD production considerably and thus potentially increase the speed of the assay. It has however been shown that *E.coli* of faecal origin are already GUD-induced due to glucuronides in the intestinal tract [115].

1.7.1.5 Enzyme Inhibitors

An inhibitor can be a molecule with similar characteristics to the enzymes target molecule which can bind to the active site of the enzyme thereby preventing the binding of the target molecule and reducing the enzyme activity. Glucuronidase activity can also be inhibited by a range of substances these include metal ions such as Cu$^{2+}$, Ca$^{2+}$, Hg$^{2+}$, and sugars such as D-Glucuronic acid [116]. Measures to combat inhibition include chelation / sequestration of inhibitors using EthyleneDiamineTetraAcetic acid EDTA [117], [118].

Figure 1-7: Factors contributing to GUS activity in environmental waters. Grey boxes are faecal sources of GUS, Orange boxes are non-faecal interferences. Adapted from [66]
1.7.1.6 Enzyme Disassociation & deactivation

β-Glucoronidase can lose its form and consequently it’s activity through dilution, thermal denaturation and in a process called proteolysis break down to its constituent polypeptides and amino acids. This breakdown can be prevented through the addition of activators and or stabilizers (e.g. various types of DNA, BSA). Some commercial enzymes from Sigma Aldrich is supplied activated with Dithiothreitol (DTT) and stabilized with Polyethylene glycol (PEG). In the case of assays where the *E.coli* retains the enzyme intra-cellularly, deactivation may not occur. However in an assay where cells are lysed, activators and/or stabilisers may need to be added to maintain activity of the extra-cellular enzyme.

1.7.1.7 Interferences

Baudart reports that the main cause of interference in GUD enzyme assays is GUD positive *Vibrio* bacteria while other non-*E.coli* bacteria may contribute an insignificant amount of GUD activity [114]. Fiksdal and Tryland conclude that rapid GUD based tests should not be rejected on the basis of interference from non-target bacteria [119]. Davies found that plant and algal species in water can contribute GUD activity and potentially interfere with rapid assays but that this should be only significant in waters with high biomass content [120], [121].

1.7.2 Survival and significance of VBNC bacteria

On entering environmental waters faecal bacteria are subject to stresses including osmotic stress, UV irradiation and lack of food sources. Therefore they begin to shut down certain functions as a survival strategy and eventually die [122]-[124]. Bacteria which when introduced to a growth medium will recover and begin to multiply are classed Viable Culturable (VC) while bacteria which are sub-lethally stressed and can metabolise foods but have lost the ability to replicate are called Viable But Not culturable (VBNC). With time the ratio of VBNC to VC increases [125]-[130]. Thus assays such as Colilert 18 which only report VC do not show a full picture of the faecal bacteria numbers in environmental waters. Huq reports that VBNC bacteria are of epidemiological significance [131] and assays and methods have been developed to detect them along with VC bacteria [66], [132].
1.7.3 Sample pre-concentration

The coastal and transitional water ‘Excellent’ standard for *E. coli* is 250 CFU.100 mL$^{-1}$ or 2.5 CFU.mL$^{-1}$. Also FI bacteria are often non-uniformly distributed throughout a sample due to their attachment to each other as clumps or through attachment to particulate matter [133][134]. Thus with sample volumes of 1 mL or so there can be large variation [135]. In order to achieve accurate measurement at such low concentrations, the larger the volume of water sampled the better. However analysing large volumes consumes power and reagents, thus many systems incorporate a sample pre-concentration step, e.g. ColiGuard filters up to 3 litres to obtain a sample [136]. Measurement technologies often accept small volumes e.g. a standard 1 cm optical cuvette for fluorescence detection accepts 3 mL thus at 2.5 CFU.mL$^{-1}$ the number of cells present is 7.5. Pre-concentration is necessary to increase this number achieve acceptable precision [58].

1.7.4 Substrates and hydrolysis

A substrate is a glycoside which consists of a glycone i.e. a sugar (e.g. glucose, glucopyranose) bound to an aglycone i.e. a non-sugar, normally a chromophore, fluorophore or a chemiluminescent molecule [74]. Upon hydrolysis by an appropriate enzyme, the aglycone is released from the sugar allowing optical detection. Numerous substrates are available for the detection of enzymatic activity. Some are suitable for use in solid (gel) assays while others are suitable for fluid assays [137]-[139]. Fluorescence detection is regarded as being in the order of 1000 times more sensitive than absorbance detection due to the absence of background light. Thus fluorogenic substrates for fluid assays are only considered here. Some examples are:

1. 4-methylumbelliferone β-D-glucuronide (4-MUG)/(MUGLcU)
2. Fluorescein β-D-glucuronide
3. Resorufin β-D-glucuronide
4. Carboxyumbelliferyl β-D-glucuronide
5. 6-Chloro-4-Methylumbelliferone-β-D-glucuronide (CMUG)

4-methylumbelliferone (4-MU) and its associated substrates including *Enterococci* substrate 4-MUD have been used extensively but, it has been suggested that it is unsuitable for use in an automated sensor, due to its insolubility in water at natural
temperatures [65]. Also the fluorescence of 4-MU is pH dependant with an optimum pH of 9.0. This is due to its high pKa of 7.8.

3-carboxyumbelliferyl-β-D glucuronide (CUGlcU) is a substrate with a pKa close to that of MUG but with higher fluorescence at neutral pH’s and better solubility. It is still however pH dependant with an optimum fluorescence observed at pH 9 [65].

6-chloro-4-methylumbelliferone-β-D glucuronide (6-CMUG) is produced by Glycosynth, UK. It behaves similarly to MUG with stronger (x2) fluorescence at pH7.4 (seawater pH varies about 8.05), and better solubility at natural water temperatures [138], [139].

1.7.5 Continuous and discontinuous detection methods

Petit and Servais describe a discontinuous direct enzyme fluorescence assay where the activity of enzyme lysed from cells is monitored over time by repeatedly analysing aliquots from a reaction vessel. This method involves adding NaOH thus raising the pH of the solution to pH10 in order to maximise fluorescence and also to stop the reaction [115]. The method proved reliable but has the disadvantage of requiring a large sample volume i.e. the number of aliquots analysed times the volume of each aliquot. Also, a lot of sample handling is required. A continuous assay using 4-MUG was used by Fior for analysis of GUS activity in plants. The advantage of this assay was greatly reduced sample handling [140].

Geary describes a continuous assay where the enzyme activity of non-lysed cells is monitored over time by directly interrogating the sample for fluorescence resultant from the hydrolysis of a substrate, without stopping the reaction or adding any further reagents. This approach offers the advantage of less sample handling than the discontinuous method and smaller sample volume however the LOD achieved (1.00 × 10³ CFU. 100 mL⁻¹) was too high to be useful for recreational monitoring [65].

Lysing E.coli cells to release intra-cellular GUD is a rapid method of quantifying E.coli which also allows for the detection of VBNC cells. Fiksdal developed a method including a lysing reagent and based on 4-MUG fluorescence for marine E.coli quantification [141]. Garcia used a similar method which involved a selective pre-culture step [113]. George adapted these methods to freshwater analysis [115]. These 3 methods are discontinuous due to the requirement for addition of a base to raise pH to achieve optimal fluorescence
from 4-MU. A continuous method based on 6-CMUG developed by Briciu Burghina offers the advantage of reduced sample handling [142].

1.7.6 Fluorescence overview

Fluorescence is the emission of light from a molecule due the absorbance of light of a shorter wavelength. Absorbance of light is governed by the Beer-Lambert law. The absorbed light causes an excited state in the molecule which then releases a photon returning the molecule to ground state. The Jablonski diagram in Figure 1-8 shows photon absorption and the excited state reached then the subsequent release of light through fluorescence and phosphorescence thus returning the molecule to ground state. Fluorescence emitted is usually at a lower energy than the absorbed (excitation) light. Thus the wavelength of the emitted light is longer than that of the excitation. The difference in nanometers between the two is called the Stokes shift. The fluorescence lifetime of many common fluorophores is in the order of a few nano-seconds. Fluorophores with short lifetimes are preferable to those with long lifetimes as they are less susceptible to excited state quenching. Phosphorescence is usually emitted with a time delay (micro-seconds up to hours for some compounds) and at a longer wavelength thus lower energy than fluorescence.
The efficiency of a substance fluorescing is denoted by the quantum yield which is the ratio of photons absorbed to photons emitted [143]. Molar absorptivities ($\varepsilon$) and quantum yields ($\Phi$) of most fluorophores can be found in literature e.g. 4-MU exhibits $\lambda_{\text{max}}$ at 360 nm, $\lambda_{\text{em}}$ at 450nm, $\varepsilon=1.7\times10^{-3}/\text{M/cm}$ and $\Phi=0.63$ in pH 10 phosphate buffer [144]. Fluorescence response can be limited by a number of factors including quenching, the inner filter effect and photo bleaching these are discussed below.

### 1.7.6.1 Quenching and photo-bleaching

Quenching is the reduction of fluorescence intensity which can occur through a number of mechanisms. Collisional quenching occurs when an excited molecule loses its energy in an interaction with another molecule and so returns to the ground state but neither molecule is chemically altered [143]. Static quenching is the formation of non-fluorescent complexes of the fluorophore in its ground state with other molecules such as halides in seawater [145], [146].

Photo-bleaching is loss of signal due to prolonged irradiation of the fluorophore. It’s prevalent in solid samples but negligible in liquid samples where molecules can move around. Bleaching can be minimised by reducing excitation intensity and exposure time [147].

### 1.7.6.2 Inner filter effect

The inner filter effect is another form of quenching of signal reduction where the absorptivity of the fluorophore itself causes a reduction in signal. The effect becomes pronounced when the total absorbance at the excitation and emission wavelengths exceeds 0.08 [148]. The fluorescence response continues to degrade until the limit of solubility is reached. The effect is shown below in Figure 1-9 where the apparent fluorescence of 4-MU increases with concentration up to a maximum at 80 $\mu\text{M}$ before reducing with further increases in concentration. The linear range of the fluorescence response of 4-MU can be seen to extend from zero to about 20 $\mu\text{M}$.

In fluorescence based enzyme assays further reductions in fluorescence may be observed due to the absorption of excitation or emitted light by the substrate or other absorbing
species within the solution. This effect limits the maximum detectable concentration of fluorophore in enzyme assays to the low µM range. Thus detection instruments used must be capable of sub µM detection.

![Inner Filter Effect](image)

Figure 1-9: Inner filter effect. The apparent fluorescence of increasing concentrations of 4-MU at pH10 excited at 355nm and emission recorded at 460nm. Adapted from [149].

1.8 Instrumentation for fluorescence enzyme assays
A range of instrumentation is currently available for the performance of fluorescence assays for FI bacteria detection. These can be categorised as fixed systems which are mainly used in water treatment plants, laboratory based systems, field portable systems and deployable autonomous systems which can function remotely in the field.

1.8.1 Online automated systems
ColiGuard from MBonline detects *E.coli* through activity of β-Glucuronidase and detects coliforms through activity of β-Galactosidase. It uses fluorescence measurement without incubation. Sample volumes can vary between 20 and 3000 mL with 1000 mL being typical. The system is intended for online pathogen detection in water-supply, the food and the pharmaceutical industries. It is also proposed as an early warning system for monitoring drinking and bathing waters using a green, amber, red traffic-light system. An auto sampling system to work with the ColiGuard is commercially available [136].
ColiMinder and BACTcontrol are two devices which evolved from the ColiGuard system. They have recently been trialled in sediment laden streams. Results were inconclusive but demonstrated the potential for autonomous on-site monitoring of GUD activity [150].

Kolisoon is a bench-top on-line auto-sampling system using fluorimetry to detect GUD activity. It is intended for waste-water treatment plant outflows. Its components are an auto-sampler, filtration unit, reagent doser, reaction carousel and a fluorimeter. The system was developed as part of a plan to address the need for monitoring of treated wastewater outflows to satisfy the EU Water Framework Directive. The principle it is based upon is the automation of classical laboratory techniques for microbiological detection. Response times of 4-6 hours are claimed [104].

Colifast alarm is an automated at-line faecal coliform detection system which uses enzymatic methods to produce a presence/absence result. This is a similar technology but lower specification version of the Coliguard system [151], [152]

The ColiLine Portable Microbe Enrichment Unit (PMEU) uses IDEXX Colilert culture medium in an automated system to detect low concentrations (LOD = 1 CFU/100 mL) of *E. coli* using a time to detect TTD method. (i.e. the higher the concentration the faster the detection) typically 5 hours [153], [154].

### 1.8.2 Laboratory based systems

The BioSense BDS1000 was purpose designed for conducting 4-MUG assays. It incorporates a sample incubation chamber and separate fluorescence detection chamber. This system achieved detection times below 120 minutes [155].

The Tecta bench-top microbial detector from Veolia Edentech uses enzymatic methods with incubation to detect *E.coli* and total coliforms. Single cell sensitivity for both with a response-time of 2-18 hours, depending on the level of contamination are claimed. It boasts “All-in-one” pathogen detection cartridges utilizing a proprietary substrate [156], [157].

### 1.8.3 Field Portable systems

There are numerous examples of field portable fluorimeters usually consisting of an LED source, optical filter and Photo-Multiplier Tube (PMT) or silicone photodiode detector [158]-[160]. These can be built with fixed wavelengths to suit most target fluorophores.
including 4-MUG or similar. However there is none currently available which combines sample incubation, necessary for a continuous assay.

1.8.4 Deployable autonomous systems

Systems for faecal indicator detection capable of being deployed in a marine/costal environment for prolonged periods do not exist. The main reason for this is biofouling and contamination of sampling apparatus (which must be repeatedly used) leading to measurement error [161], [162].

In 2009 the University of Notre Dame in cooperation with the US army developed the Autonomous Wireless In-Situ Sensor (AWISS) a deployable *E.coli* sensor using the GUD method with fluorescence detection. It incorporated automatic sampling, reagent mixing, incubation and fluorescence detection. Quantification of faecal indicators was estimated indirectly using a Time to Detect (TTD) method by measuring the time taken for the fluorescence due to enzyme activity to cross a threshold value. AWISS was deployable for up to 2 days with power being the limiting factor (The author notes that this can be improved). The current cost of manufacture is 8000 USD due largely to the use of a high grade Ocean Optics spectrometer [161]. Another critical issue demonstrated in this system was biofouling of the detection chamber leading to memory effects and false positives [163].
1.9 Conclusion

FI and pathogen detection are a hot topic in research at the moment. Manuscripts describing new methods and systems are published regularly. There is a trend towards miniaturization with reduced response time, increased specificity and sensitivity and greater cost effectiveness. A cross section of the available technologies and research in the field has been examined in this report. From these, the following conclusions were drawn.

Of the faecal markers examined, the microbiological indicators *E.coli* and *Enterococci* are the most useful. Culture based methods, either MPN or colony counting are still the most reliable and widely accepted. They have however the disadvantage of being slow.

The simplest and most robust of the rapid methods is enzymatic detection of *E. coli*. This method offers the following advantages over culture methods:

- Able to detect Viable But Non-Culturable cells (VBNC);
- Cost-effective;
- Enzyme mediated reactions are rapid and time saving;
- Circumvents the time consuming culturing period;
- Enables the exploitation of a range of enzyme synthetic substrates;
- Has the potential to be implemented in continuous-remote monitoring.

Methods developed to date have used discontinuous assays and laboratory based fluorescence detectors, so there is significant room for development in this area. The rapid enzyme assay is not an option for *Enterococci* detection as it lacks a specific enzyme marker as *E.coli* has. Thus a selective culture step is required for *Enterococci* assays.

Of the systems reviewed the majority are aimed at the detection of pathogens and or faecal indicators in municipal water supplies, in industry or at the outflow of sewerage treatment plants. AWISS is the closest existing technology to the aims of this project as it performs in-situ analysis of fresh and marine water using enzymatic detection and does so in a rapid (sub 4 hour) timeframe. There is however great scope for improvement upon this system in terms of reducing its cost and power consumption, improving upon its accuracy and sensitivity and in reducing the number of false positives.
1.10 Research proposal and objectives

The aim of this work is to develop a field portable system capable of conducting ‘rapid’ (sub 4 hours) microbial analysis of recreational waters. The system should have an LOD lower than the ‘excellent’ standard as specified by the BWD. To achieve this aim, these specific objectives are addressed:

1. Identify a suitable substrate for β-D-Glucuronidase detection which gives higher specificity and faster response time than currently used substrates.
2. Develop & test enzyme-based fluorescent assays for *E.coli* which have comparable performance to standard methods but offer more rapid results.
3. Develop a portable detection system to implement the assays *in-situ* or *on-site* with comparable precision to laboratory based systems.
4. Test and validate the system and assay against standard methods to establish LOD, sensitivity and reliability.
5. Test the system in the field to determine the robustness and time required to process samples.
2 \textbf{B-GLUCURONIDASE ASSAYS FOR} \textit{E. coli}

2.1 Introduction

2.1.1 Substrate selection

A wide range chromogenic and fluorogenic of β-Glucuronidase substrates are available for rapid assays. A subset of these were detailed previously in section 1.7.4. From this list three fluorogenic substrates including 4-methylumbelliferone-β-D-glucuronide, 3-carboxyumbelliferyl-β-D-glucuronide and 6-chloro-4-methylumbelliferone-β-D-glucuronide were selected for further investigation. The investigation sought to identify the most suitable substrate for FI indicator detection in recreational waters by comparing on a number of parameters. These included Limit Of Detection (LOD), pH dependence of fluorescence and the absorbance spectrum of the substrates. Table 2-1 details the photo-physical properties of each of the fluorophores from the selected substrates.

Table 2-1: Chemical and photo-physical properties of 3 fluorophores. [65], [138], [142], [144], [164]-[167].

<table>
<thead>
<tr>
<th></th>
<th>4-methylumbelliferone</th>
<th>3-carboxyumbelliferyl</th>
<th>6-chloro-4-methylumbelliferone</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS number</td>
<td>90-33-5</td>
<td>779-27-1</td>
<td>19492-02-5</td>
</tr>
<tr>
<td>Formula</td>
<td>C₅H₇O₆</td>
<td>C₅H₇O₆</td>
<td>C₅H₇C₅O₄</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>176.17</td>
<td>206.15</td>
<td>210.63</td>
</tr>
<tr>
<td>pKa</td>
<td>7.8±0.6</td>
<td>7.38±0.6</td>
<td>6.12±0.3</td>
</tr>
<tr>
<td>Excitation wavelength</td>
<td>365 nm (pH 10.2)</td>
<td>386 nm (pH 9)</td>
<td>365 nm (pH 7)</td>
</tr>
<tr>
<td>Emission wavelength</td>
<td>445 nm (pH 10.2)</td>
<td>448 nm (pH 9)</td>
<td>445 nm (pH 7)</td>
</tr>
<tr>
<td>Extinction coefficient</td>
<td>17,000 /M/cm at pH 10</td>
<td>36,700 /M/cm at pH 10</td>
<td>18,500 /M/cm at pH 7</td>
</tr>
<tr>
<td>Fluorescence lifetime</td>
<td>not available</td>
<td>not available</td>
<td>not available</td>
</tr>
<tr>
<td>Bleaching</td>
<td>22% (33 mins illumination at 360 nm)</td>
<td>not available</td>
<td>not available</td>
</tr>
<tr>
<td>Quantum yield</td>
<td>0.63</td>
<td>0.7</td>
<td>not available</td>
</tr>
<tr>
<td>Photostability</td>
<td>&gt; 1 hour at pH 10</td>
<td>not available</td>
<td>not available</td>
</tr>
<tr>
<td>Solubility</td>
<td>methanol (50 mg/ml) with heating, Insoluble in cold water</td>
<td>Water, DMF, DMSO, Alcohols</td>
<td>DMSO, Slightly in cold water</td>
</tr>
<tr>
<td>Coliform growth inhibition</td>
<td>Significant above 8 μmol / L</td>
<td>none</td>
<td>medium</td>
</tr>
</tbody>
</table>

Structure

![Structure](image-url)
2.1.2 Matrix effects

The literature suggests that absorbance of samples increases due to turbidity and thus reduces fluorescence response. In some cases however an apparent fluorescence response can increase due to turbidity depending on the properties of the particulate matter causing the turbidity (e.g. due to high reflectivity, or scatter effects such as Rayleigh, Raman or Tyndall) [143].

Both Rayleigh and Raman scatter are due to the interaction of light with molecules in solution. Tyndall scatter on the other hand is due to the effect of colloidal particles in suspension (turbidity). Rayleigh scattering is elastic meaning that the wavelength of the scattered light is the same as that of the incident light. Raman scattering is inelastic meaning that the wavelength of the scattered light is changed (shortened) from that of the incident light. The Tyndall effect causes shorter wavelengths to be scattered while longer wavelengths are passed through the sample.

The Raman effect is weak so only affects sensitive assays. Rayleigh and Tyndall scatter however can cause significant errors in fluorescence measurements if not guarded against. Measure to reduce scatter effects include selecting a fluorophore with a large stokes shift and filtering (high-pass or band-pass) the emitted light to allow only the fluorescence response to reach the detector.

In environmental waters it can be assumed that most particulate matter will not be highly reflective and therefore absorb light. Thus fluorescence will be negatively affected by an increase in turbidity. Turbidity levels in Irish coastal waters are generally in the region of 1 to 50 Nephelometric Turbidity Units (NTU) [168] with peaks due to unusual events reaching into the hundreds of NTU. Fluorescence response is also affected by further matrix factors including the inner filter effect, quenchers, temperature and pH.

2.1.3 Raw water Metabolism based assay

As described in 1.7.1 *E. coli* can metabolise a fluorescent substrate such as 4-MUG, directly to produce a fluorescent response. This detection method was implemented in an autonomous system by Geary using 3-CUG [161]. However, at typical marine water pH (approx. 8.2) the fluorophore 3-CU does not fluoresce optimally. By using 6-CMUG instead it was proposed that a higher fluorescent response can be achieved and thus more accurate quantification of *E. coli* with lower detection limits. This was tested here by
introducing substrate directly to environmental samples and monitoring fluorescence response. The test was conducted both manually using an LS50B fluorimeter and then semi-automated using a Tecan Saffire 2 96-well plate-reader.

2.1.4 Sample variability

In bacterial analysis small sample volumes mean low reagent use and low waste. However the lower the sample volume the higher the variability between measurements due to non-homogeneous dispersion of target bacteria in the sample. In this chapter the repeatability of 1 mL samples from a range of \( E.\ coli \) concentrations (the range corresponding to BWD limits) was examined.

2.1.5 Aims of Chapter 2: \( \beta \)-Glucuronidase assays for \( E.\ coli \).

The aim of this chapter is to investigate the performance of and the factors affecting \( E.\ coli \) detection assays based upon the enzyme \( \beta \)-Glucuronidase.

Objectives include

- Selection of a suitable substrate for \( \beta \)-Glucuronidase assays in environmental waters
- Identification and investigation of matrix effects which may influence the performance of a \( \beta \)-Glucuronidase assay
- Quantification of variability in \( E.\ coli \) concentrations within environmental samples
- Demonstration of a \( \beta \)-Glucuronidase assay in environmental waters
2.2 Materials
A chromophore: 4-Nitrophenyl (4-N) and fluorophores: 4-Methyl umbelliferyl (4-MU), 7-hydrocoumarin-3-carboxylic acid (3-CU) were obtained from Sigma Aldrich. A fluorophore 6-chloro-4-methylumbelliferone- (6-CMU) was obtained from CarboSynth, UK. Fluorogenic substrate 4-Methyl Umbelliferyl β-D glucorinide (4-MUG) was obtained from (Sigma Aldrich). Fluorogenic substrate 3-carboxyUmbelliferyl β-D glucorinide (3-CUG) was obtained from (CarboSynth, UK). Fluorogenic substrate 6-chloro-4-methylumbelliferone- β-D glucuronide (6-CMUG) was obtained from (GlycoSynth, UK). Hach, formazin turbidity standard was obtained from Sigma Aldrich. 3M Petri-Film for E.coli was ordered from TechnoPath Ireland. E.coli ATCC 11775 from certified reference materials was obtained from Sigma Aldrich. PVDF membrane filters, (0.45 µm pore size, 47 mm diameter), black Greiner PP well plates type 655209, clear Greiner PS well plates type 655161 and Nunc well-plate sealers type 232702 were obtained from Sigma Aldrich. Water was passed through a Milli-Q water purification system to achieve a conductivity of 0.55 μS.

2.3 Methods

2.3.1 Substrate selection

2.3.1.1 Absorbance and fluorescence range
To test the dynamic range of absorbance measurements, a range of 4-N concentrations (0.001µM to 100 µM) were prepared in 1mM, pH7 phosphate buffer (Sodium Phosphate Monobasic/ Sodium Phosphate Dibasic). These were placed in 1 cm path-length polystyrene (PS) cuvettes and their absorbance at 405 nm was recorded on a Lambda 900 spectrophotometer.

To test the dynamic range of fluorescence measurements, a range of 4-MU concentrations (0.001µM to 100 µM) were prepared in pH7 phosphate buffer. These were placed in a quartz cuvette and their fluorescence recorded at excitation 362 nm, emission 444 nm using an LS50B spectro-fluorimeter.
2.3.1.2 Fluorescence analysis

Three fluorophores 4-MU, 3-CU and 6-CMU were analysed to establish their optimal excitation and emission wavelengths at a range of pHs. Solutions of 1 µM of each fluorophore were prepared in triplicate in a range of buffers with pHs 3 to 11 on a black Polypropylene (PP) 96 well-plate. 3D fluorescence scans of each fluorophore were carried out on a Tecan Saffire 2 96-well plate-reader. Excitation wavelengths from 300nm to 400 nm at 5 nm intervals were used, while fluorescence emission was measured from 400 nm to 500 nm at 5 nm intervals.

2.3.1.3 Substrate absorbance

A total of 3 substrates 4-MUG, 3-CUG and 6-CMUG were analysed to find their absorbance spectrums at a range of pHs. Solutions of 500 µM of each substrate were prepared in triplicate in a range of buffers with pHs 3 to 11 on a polystyrene 96 well-plate. Absorbance scans of each were carried out on a Tecan Saffire 2 96-well plate-reader.

2.3.1.4 Substrate fluorescence

To examine the fluorescence response of parent substrates, standards of 4-MUG, 3-CUG and 6-CMUG (all 500 uM) were prepared in a range of pHs from 7 to 11. These standards were placed in triplicate in a black PP 96 well-plate. Using a Tecan Saffire 2 96-well plate-reader their emission spectra were recorded when excited at their respective absorption maxima (320 nm for 4-MUG, 330 nm for 3-CUG, 325 nm for 6-CMUG) and at the optimal excitation of their respective fluorophores (365 nm for 4-MU, 385 nm for 3-CU, 365 nm for 6-CMU). Plates were sealed with Nunc well-plate sealers to avoid evaporation.

2.3.2 Matrix effects

To investigate the effect of turbidity on fluorescence, standards of 4-MU (1 µM) in Phosphate buffer pH7 were prepared in a black PP 96 well-plate. These were spiked in triplicate with formazin turbidity standard from 5 NTU to 320 NTU (The range typically
encountered in environmental waters). Fluorescence response of the 4-MU was recorded at (ex 365 nm, em 445 nm) using a Tecan Saffire 2 96-well plate-reader.

To investigate the effect of quenchers present in environmental waters on fluorescence, a marine water sample was filtered using a 0.45 micron membrane to remove solids and micro-organisms. This was spiked with concentrations of 6-CMU from 0.5 µM to 20 µM on a black PP 96 well-plate. The plate was incubated at 42°C and fluorescence was measured each minute for a period of 12 hours using a Tecan Saffire 2 96-well plate-reader.

### 2.3.3 Environmental water sampling procedure

Samples were taken from the freshwater Tolka River at Griffith Park, Dublin and the estuarine Liffey River at Poolbeg Marina, Dublin. The sampling for microbiological and chemical and physical water quality parameters was carried out according to international best practice detailed in ISO 5667-6 (Guidance on sampling of rivers and streams) and ISO 19458:2006 (Sampling for microbiological analysis). In brief, the sampling procedure was as follows:

Samples were taken at each location in sterilised 1 litre Nalgene (high density polypropylene (HDPP)) bottles. The bottles were rinsed three times with sample water before filling in order to condition them. Samples were then placed on ice and returned to the laboratory within 2 hours. The samples were analysed within 4 hours of collection. Prior to any analysis the 1 L samples were allowed to settle for 30 minutes to remove heavy sediment.

### 2.3.4 Variability within a sample

*E.coli* Certified Reference Material (CRM) was prepared in DI water to a range of concentrations from 50 CFU.100 mL⁻¹ to 1000 CFU.100 mL⁻¹. These were each plated onto 3M PetriFilm in triplicate at 1 mL volumes. Plates were incubated at 44°C for 21 hours and counted.
2.3.5 Continuous direct fluorescence assay

2.3.5.1 Environmental sample analysis using 20 mL sample vials

To achieve a range of *E. coli* concentrations, samples taken from the freshwater Tolka River at Griffith Park, Dublin and the estuarine Liffey River at Poolbeg Marina, Dublin were pre-concentrated using micro-filtration and re-suspension in a smaller volume of filtrate from the original sample. The re-suspension volume was selected to achieve concentrations of the original concentration, the original concentration X 10 and the original concentration X 20 plus a blank sample.

The full details of the pre-concentration procedure were: For the x10 pre-concentration step, 100 mL of the real environmental sample was vacuum filtered through 0.45 µm membrane filter. For the x20 pre-concentration step 200 mL was filtered. This procedure was carried out in triplicate. The filters were then each individually placed in a conical flask containing 10 mL of filtrate from the sample. The flasks were then vortexed for 1 min to re-suspend the bacteria from the filter and into the 10 mL sample. To prepare a blank or control sample (i.e. containing 0 CFU), the filtrate from a sample was used.

To measure achieved concentrations, 1mL aliquots of sample from each flask were plated on 3M Petrifilm in triplicate. These plates were incubated at 44°C for 21 hours and then counted. A range of concentrations from 2,900 CFU /100 mL to 27,000 CFU / 100 mL were recorded.

To conduct the fluorescent assay, 20 mL samples of each concentration were prepared in borosilicate glass vials. The substrate 6-CMUG at 100 µM was introduced to the sample and the vials were incubated at 44°C. An aliquot from each vial was removed each hour for 12 hours and placed in a quartz cuvette. Fluorescence was recorded with excitation at 365 nm and emission at 445 nm using an LS50B fluorescence spectrometer. The aliquot was then returned to the sample vial to maintain sample volume.

2.3.5.2 Environmental sample analysis using 96 well Plate reader

A Tecan Saffire 2 96-well plate-reader was employed to conduct a continuous fluorescent assay with reduced sample handling on water samples obtained from the freshwater Tolka River at Griffith Park, Dublin and the estuarine Liffey River at Poolbeg Marina, Dublin.

A range of 5 concentrations including the original concentration X1, X2, X5, X10 and a blank were derived from each sample using the following method: Samples were placed
into 50 ml centrifuge tubes. Tubes were spun on an Eppendorf Centrifuge 5810R at 4,000 RPM for 30 min at 4°C. Supernatant from each tube was poured off to achieve the required pre-concentration. (i.e., for X2 pour off 25 mL, for X 5 pour off 40 mL, for X 10 pour off 45 mL). Each sample was agitated by pipetting to re-suspend the pellet in the remaining supernatant to achieve the desired concentration. The blank was prepared by microfiltration with a 0.45 micron filter to remove all *E.coli*. Aliquots (1 mL) of each of the derived concentrations were plated in triplicate on to 3M PetriFilm plates for *E.coli*. The plates were incubated at 44°C for 21 hours and the CFUs were counted.

To conduct the fluorescent assay, 300 µL aliquots of each sample were placed in triplicate in a black PP 96 well-plate. 100 µM of 6-CMUG added to each well. The 96 well plate was incubated in the Tecan Saffire 2 96-well plate-reader at 42°C. Fluorescence at ex 365/ em 445 nm was recorded for each well at 10 min intervals for 18 hours.
2.4 Results and discussion

2.4.1 Substrate selection

2.4.1.1 Absorbance vs. fluorescence

Figure 2-1 shows a calibration curve for 4-N over its detectable range using a Lambda 900 spectrophotometer. The red data points represent the Coefficient of Variation (CV) expressed as a percentage for triplicate samples (n = 3). The CV rises sharply at concentrations below 10 µM. Eisenthal recommends that for absorbance based enzyme assays in micro-titer plates, absorbance values below 0.2 should not be used in order to maintain CVs within 10% [149]. With the Lambda 900 the CVs of triplicate measurements were less than 10% at absorbance values greater than 0.1. The acceptable range of absorbance measurement was concluded to be 2 µM to 100 µM. The 100 µM upper limit represents the highest concentration of 4-N tested in this experiment.

![Absorbance vs. fluorescence](image)

Figure 2-1: 4-N absorbance calibration at 405 nm recorded @ 25 deg C (0.001 mM to 0.1 mM) 0.1 mM pH7 phosphate buffer on a (Lambda 900 spectrophotometer) (n = 3) Black data points represent the average of triplicate absorbance measurements. Red data points represent the Coefficient of Variance (CV) for each of the triplicate measurements expressed as a percentage.
A calibration for 4-MU over its linear range using an LS50B fluorescence spectrometer is shown in Figure 2-2. The red data points represent the Coefficient of Variation (CV) expressed as a percentage for triplicate samples. The CV increased at concentrations below 2 µM but remained below 10% for concentration as low as 0.01 µM. The useful range of fluorescence measurement was concluded to be 0.01 µM to 10 µM. Above 10 µM the calibration becomes nonlinear demonstrating the inner filter effect. This feature is not shown here as this was shown previously in Figure 1-9.

Figure 2-2: 4-MU fluorescence calibration curve recorded @ 25 °C (0.01 uM to 10 uM) (@362/445 nm) in 0.1 mM pH7 phosphate buffer on an (LS50B Spectro-fluorimeter) (n = 3) Black data points represent the average of triplicate fluorescence measurements. Red data points represent the Coefficient of Variance (CV) for each of the triplicate measurements expressed as a percentage.

While the absorbance linear detection range (2 µM to 100 µM) was larger than the fluorescence based detectable range (0.01 µM to 10 µM), it was found that fluorescence measurements had significantly lower variability at concentrations lower than 10 µM. E.g. for 4-MU at 2.5 µM the CV of triplicate fluorescence measurements was 2% for the same concentration of 4-N the CV of triplicate absorbance measurements was 10%, while for 4-MU and 4-N both at 1 µM the CV of triplicate fluorescence measurements was 5%.
and the CV of triplicate absorbance measurements was 86%. This is attributable to the higher sensitivity of fluorescence measurements than those of absorbance due to the absence of background light in fluorescence measurements. From this it was concluded that fluorescence measurements would allow lower LODs than absorbance measurements and thus only fluorescent substrates were investigated further in this work.

### 2.4.1.2 Solubility

Table 2-1 provides information on the solubility of the 3 fluorophores 4-MU, 3-CU and 6-CMU. Of these 3-CU is the most soluble in water while both 4-MU, 6-CMU suffer from poor solubility in cold water [65]. However due to the fact that in enzyme assays they are released from the parent substrate in low concentrations typically less than 10µM, poor solubility is not a big problem. In selecting a substrate, pH dependence and fluorescence response is of greater importance.

### 2.4.1.3 Fluorescence analysis

3D fluorescence scans of the fluorophores 4-MU, 3-CU and 6-CMU at pH 7 and pH 10.8 are shown in Figure 2-3. It was found that for each fluorophore the optimal excitation wavelength was dependent on pH. However the peak emission wavelength for each fluorophore occurred at 445 nm across the range of pHs. From panel A and B, it can be seen that the optimal excitation wavelength of 4-MU changes from 325 nm to 360 nm as pH is increased from 7 to 10.8. The 4-MU at pH7 displayed a maximum fluorescence of 500 Relative fluorescence units (RFU). From panel C and D, it can be seen that 3-CU has a broad excitation peak centred on 345 nm at pH 7 while at pH 10.8 it has a narrow excitation peak centred on 380 nm. The 3-CU at pH10.8 displayed a maximum fluorescence of 1500 Relative fluorescence units (RFU). From panel E and F, it can be seen that 6-CMU has a narrow excitation peak centred on 365 nm at pH 7. This did not change with increased pH, though the peak did become broader. The 6-CMU displayed a maximum fluorescence of 1500 Relative fluorescence units (RFU).

From this data it was concluded that 6-CMU was the highest yielding fluorophore at neutral pH. It also had the narrowest excitation peak which makes it the least susceptible to optical interferences.
Figure 2-3: 3D fluorescence response of 3 fluorophores. A. 4-MU (1µM) at pH 7, B. 4-MU (1µM) at pH 10.8, C. 3-CU (1µM) at pH 7, D. 3-CU (1µM) at pH 10.8, E. 6-CMU (1µM) at pH 7, F. 6-CMU (1µM) at pH 10.8, Temperature = 25°C.
Figure 2-4: Excitation scans of 3 fluorophores. A. Excitation scan of 4-MU (1 µM) vs pH, B.C. Excitation scan of 3-CU (1 µM) vs pH, E. Excitation scan of 6-CMU (1 µM) vs pH. For all scans, emission wavelength = 445 nm, n = 3, error bars show 1 standard deviation.
As the peak emission wavelength of each fluorophore did not vary with pH, data was extracted from the 3D scans to create 2D excitation scans with emission fixed at 445 nm. Figure 2-4, panels (A, C and E) show excitation spectra for the fluorophores 4-MU, 3-CU and 6-CMU at a range of pHs (pH3 to pH 11). Panels (B, D and F) show the peak fluorescence of each fluorophore at the 2 excitation maxima across the range of pHs.

The shifting of the excitation optimum for each fluorophore with increasing pH is caused by the deprotonation of the fluorescent molecule. They each fluoresce most strongly in their deprotonated state which occurs at pHs above their respective pKa’s. The pKa of each of fluorophore was inferred from the intersection points in Panels (B, D and F) of Figure 2-4. These were the 4-MU pKa = 7.8, 3-CU pKa = 7.04 and 6-CMU pKa = 6.1.

From Figure 2-4, panel A, the optimal excitation for 4-MU was found to be at 325 nm for pH values below 8. At pH’s 9 and above the optimal fluorescence was achieved at 365 nm. Maximum fluorescence was 600 RFU at pH 10.8.

From Figure 2-4, panel B, the optimal excitation wavelength for 3-CU was found to be at 335 nm for pHs between 5 and 6.5. At pH’s 3 and 4 different excitation peaks were observed but not investigated further as no assay will be performed in this low pH region. The pH values 7 to 8 had a wide excitation peak. At pH’s 9 and above, the optimal fluorescence was found at 386 nm. Maximum fluorescence was 1200 RFU at pH 10.8.

From Figure 2-4, panel C, the optimal excitation for 6-CMU was found to be at 325 nm for pHs below 5. Between pH 5.5 and 6.5 there was a wide excitation peak. At pH’s 7 and above the optimal fluorescence excitation occurred at 365 nm. The emission maximum was found at 445 nm. Maximum fluorescence was 1200 RFU at pH 10, however at pH 8. 6-CMUG had reached 95% of its full scale value maximum fluorescence.

Comparing the 3 fluorophores, it was found that 6-CMU was the best performer as it exhibited the highest fluorescence and approached maximum fluorescence at the neutral pH. The optimum pH for *E. coli* β-Glucuronidase enzyme activity is between 6 and 7 while the natural pH of freshwater and seawater are typically 7 to 8 and 8.2 respectively. Therefore 6-CMU offers an advantage over both 4-MU and 3-CU as it does not require pH adjustment before being measured. This makes it the most suitable fluorophore for continuous assays.
2.4.1.4 Substrate absorbance

The substrate is present in an assay at much higher concentration (typically 100µM or higher) than the fluorophore being detected (typically sub 1 µM). To maximise the excitation light available to the fluorophore (e.g. 4-MU) in an assay it is necessary to select an excitation wavelength which is outside the absorption range of the parent substrate (e.g. 4-MUG).

Figure 2-5 show absorption spectra of 4-MUG, 3-CUG, 6-CMUG. The spectra are normalized to 1. For 4-MUG and 6-CMUG the absorbance of the substrate does not vary with pH so only the pH 7 spectra are shown. For 3-CUG absorbance does vary with pH so the spectra at pH 7 and the extremes measured pH3 and pH9 are included.

In Figure 2-5 a, the optimal fluorescence for 4-MU which occurs at pH 9 and above is significantly clear of the substrate absorbance peak. However at neutral pH the optimum excitation is 320 nm which is within the substrate absorbance range. Thus it can be concluded that for 4-MU at neutral pH, significant fluorescence yield would be lost due to substrate absorbance of the excitation light.

In Figure 2-5 b, the optimal fluorescence for 3-CU which occurs at pH 8 and above is significantly clear of the substrate absorbance peak. However at neutral pH the optimum excitation is 340 nm which is within the substrate absorbance range. Thus it can be concluded that significant fluorescence yield would also be lost to substrate absorbance of the excitation light, for 3-CU at neutral pH.

In panel C the optimal fluorescence for 6-CMU which occurs at pH 6.5 and above is significantly clear of the substrate absorbance peak. Thus it can be concluded that for 6-CMU there will be minimal loss of fluorescence yield due to substrate absorbance of excitation light.
Figure 2-5: Substrate absorbance spectra. (a) 4-MUG (500 µM)(n = 3) pH7; (b) 3-CUG (500 µM) (n = 3) pH3,7,9; and (c) 6-CMUG (500 µM) (n = 3) pH7, for all error bars not displayed as CV <= 6%

2.4.1.5 Substrate fluorescence

From Figure 2-5 it can be seen that some excitation radiation is absorbed by the parent substrate, i.e. for each substrate the absorption at the optimal excitation wavelength for the fluorophore is 1 to 5% of the absorption maximum for the substrate. Figure 2-6 shows the fluorescence emission spectra for each of the 3 parent substrates 4-MUG, 3-CUG and 6-CMUG (all 100 µM) excited at the absorbance maxima of the substrates and the optimal excitation wavelengths of the fluorophores. It can be seen that each substrate fluoresces strongly when excited at its absorbance maximum. This effect is independent of pH. A range of pHs from pH3 to pH11 were tested but for clarity only pH7 and pH11 are shown here. The peak emission of each substrate occurred at 385 nm for 4-MUG, 400 nm for 3-CUG and 390 nm for 6-CMUG. These emission maxima are significantly lower wavelength than the emission maximum for each of the fluorophores i.e. 445 nm for each.

When excited at the optimal excitation wavelength for each fluorophore (365 nm for 4-MU, 385 nm for 3-CU and 365 nm for 6-CMU), the fluorescence emitted from each substrate is at least an order of magnitude lower than when excited at the substrate absorbance maximum. For each substrate particularly 6-CMUG, an emission peak can be seen at 445 nm.
Sensing platform design for Faecal indicator bacterial detection in recreational waters

Brendan Heery – September 2018

Figure 2-6: Substrate emission spectra. A. Emission spectra of 100 µM of 4-MUG in pH 7 & 11 buffers excited at 320 nm and 365 nm. B. Emission spectra of 100 µM of 3-CUG in pH 7 & 11 buffers excited at 330 nm and 385 nm. C. Emission spectra of 100 µM of 6-CMUG in pH 7 & 11 buffers excited at 325 nm and 365 nm. For all n = 3, CV <=5%, error bars not shown.
To understand the impact of substrate fluorescence it is necessary to look at the emission response of the respective fluorophores at typical assay concentrations and under the same conditions. Figure 2-4 showed the fluorescence response of 1 µM solutions of each of the 3 fluorophores at a range of pHs. At pH 11 and excited at 365 nm, 4-MU had an emission response of 600 RFU, this compares here to a substrate (100 µM 4-MUG) emission response of 50 RFU. For 3-CU At pH 11 and excited at 385 nm an emission response of 1,200 RFU was recorded, this compares here to a substrate (100 µM 3-CUG) emission response of 250 RFU. At pH 11 and excited at 365 nm, 6-CMU had an emission response of 1,250 RFU, this compares here to a substrate (100 µM 6-CMUG) emission response of 250 RFU.

The emission peaks observed at 445 nm for each substrate can be caused by substrate fluorescence but also by the presence of fluorophore released into solution due to auto-hydrolysis of the substrate [142]. The effect can be a significant source of error.

Substrate fluorescence can cause interference in sensitive measurements but this and other background effects can be minimised by careful optical setup. Also in enzyme assays where the rate of fluorophore production is proportional to enzyme concentration, static background interferences such as substrate fluorescence can be corrected for. They should however be minimised to optimise instrument sensitivity.

In assays conducted at neutral pH, 6-CMU has an advantage that it can be excited at 365 nm (significantly above the absorbance maximum of 6-CMUG). Thus the effect of substrate fluorescence is lower for 6-CMU than the other 2 fluorophores.
Table 2-2: Substrate absorbance and emission wavelengths, and fluorophore excitation and emission wavelengths. All substrates measured at a concentration of 500 µM, all fluorophores measured at a concentration of 1 µM.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;</th>
<th>Absorbance peak</th>
<th>Substrate emission max</th>
<th>Excitation max</th>
<th>Emission max</th>
<th>Combined fluorescence max at pH7</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-MUG</td>
<td>7.8</td>
<td>320</td>
<td>380</td>
<td>365 nm</td>
<td>445 nm</td>
<td>450 RFU</td>
</tr>
<tr>
<td>3-CUG</td>
<td>7.4</td>
<td>330</td>
<td>400</td>
<td>380 nm</td>
<td>445 nm</td>
<td>500 RFU</td>
</tr>
<tr>
<td>6-CMUG</td>
<td>6.1</td>
<td>325</td>
<td>390</td>
<td>365 nm</td>
<td>445 nm</td>
<td>1000 RFU</td>
</tr>
</tbody>
</table>

**2.4.2 Matrix effects**

Figure 2-7 shows the effect of turbidity on fluorescence response. The response is shown to decrease with increased turbidity at a rate of 0.125 Relative Fluorescence Units per NTU.

![Figure 2-7: Effect of turbidity on fluorescence of 4-MU (1 µM) in pH 7 phosphate buffer](image)

\[ y = -0.125x + 149.05 \]

\[ R^2 = 0.8562 \]

Figure 2-7: Effect of turbidity on fluorescence of 4-MU (1 µM) in pH 7 phosphate buffer (excitation 362, emission 445 nm) \( (Y = -0.13X + 149) \ (n = 1) \)
Panel A of Figure 2-8, shows a reduction in fluorescence response over a period of 2 hours for filtered seawater samples spiked with various concentrations of 6-CMU and placed on a black PolyPropelyne (PP) well plate and sealed to prevent evaporation. Each spiked seawater sample exhibited a decrease in fluorescence over approximately 2 hours until a steady state was reached. The effect was more significant for lower concentrations of fluorophore. Panel B shows that concentrations 2 µM or greater, fluorescence was reduced to approximately 80% of its original value whereas for lower concentrations the effect is a reduction to below 30% of the initial value.

This effect was first attributed to quenching of fluorescence by substances present in the seawater sample. However, repeat experiments using the same experimental setup but using firstly phosphate buffer and secondly DI water instead of seawater showed similar results. To eliminate the PP well-plate the experiment was repeated in glass vials using the ColiSense 1 system detailed in Chapter 3. In this setup the effect was not visible, i.e. solutions of 6-CMU identical to those shown in Figure 2-8 maintained their fluorescence for a prolonged period (10hrs). This indicated that the effect was caused by the use of the PP well-plates on the Tecan Saffire 2 plate-reader. The effect could either be caused by adsorption or photo-bleaching effects. Adsorption can take place in well plates when molecules are attracted to the well walls through hydrophobic adsorption or Van Der
Waals forces [169], while photo bleaching can occur when the fluorophore of interest is exposed to excessive amounts of excitation light [147]. From these results it was not possible to conclude which effect caused the loss in fluorescence. It was however decided at this point to discontinue the use of the PP well plates and the Tecan plate reader and move to a purpose built fluorescence detection system as detailed in Chapter 3.

2.4.3 Variability within a sample

Figure 2-9 shows results obtained from triplicate PetriFilm measurements of *E. coli* concentration for standards ranging from 50 CFU.100 mL⁻¹ to 1000 CFU.100 mL⁻¹. Black data points represent an average of 3 measurements with error-bars showing 1 SD. Red data points show CV expressed as a percentage. Variability decreased with increasing concentration up to the upper LOD of the method which is at 10K CFU/ 100 mL [135]. At concentrations above 500 CFU/ 100 mL, CVs are 20% or lower. Below 500 CFU/ 100 mL CVs reach as high as 90%. This variability is attributed to the non-homogeneous distribution of target bacteria in the sample and to sampling errors such as variation in the sampled volume. These results were achieved using CRM standards which were homogenised by vortexing. It is expected that the variability within real environmental samples would be higher than for these results.
Figure 2-9: Variability in 1 mL samples measured with Petrifilm. Data points represent the average of 3 measurements, error bars represent 1 standard deviation (n= 3) (Y = 0.89X + 51) (R2 = 0.81). CV is the ratio of SD to measured value expressed as a percentage.
2.4.4 Continuous direct fluorescence assay

2.4.4.1 Sample vial (20 mL) based experiment

A range of concentrations of *E. coli* were achieved through centrifugation of freshwater and marine samples. The resultant concentrations measured using PetriFilm are detailed in Table 2-3.

Table 2-3: *E.coli* concentrations achieved using centrifugation.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Nominal</th>
<th>Freshwater measured (CFU)</th>
<th>Marine water measured (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>Zero</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>X1</td>
<td>Original concentration</td>
<td>19233</td>
<td>1033</td>
</tr>
<tr>
<td>X2</td>
<td>10 times the original concentration</td>
<td>29067</td>
<td>1967</td>
</tr>
<tr>
<td>X5</td>
<td>10 times the original concentration</td>
<td>49067</td>
<td>3800</td>
</tr>
<tr>
<td>X10</td>
<td>10 times the original concentration</td>
<td>83333</td>
<td>7267</td>
</tr>
</tbody>
</table>

The fluorescence response 3 freshwater samples containing a range of *E. coli* concentrations and spiked with 100 µM 6-CMUG over a 5 hour incubation period are shown in Figure 2-10. The measured fluorescence increased over time for each sample with the *E. coli* containing samples increasing at a higher rate than the blank (filtered) sample. This result indicates that the 6-CMUG based assay can respond to different concentrations of *E. coli* but the sample size is too small to draw any conclusions about its performance.
Figure 2-10: Freshwater 6-CMUG response. Response of 100 µM 6-CMUG in freshwater from Tolka river Dublin with various concentrations of *E. coli*. (n = 3) error bars represent 1 standard deviation, Temperature = 42°C. Fluorescence response in RFU recorded on a LS50B fluorescence spectrometer (Ex: 365 nm, Em: 445 nm)
The fluorescence response of 3 brackish water samples containing a range of *E. coli* concentrations and spiked with 100 µM 6-CMUG are shown in Figure 2-11. Over the 5 hour incubation period the two intermediate samples (7,800 CFU and 10,300 CFU, 100 mL⁻¹) had similar responses, both higher than the blank, while the high concentration (20,200 CFU, 100 mL⁻¹) sample had a large response.

The variability in triplicate measurements was notably high as indicated by error-bars representing 1 standard deviation, shown on the graph. These results again indicated that the 6-CMUG can differentiate between sample concentrations, this time in brackish water, but no conclusions could be drawn due to the small sample number.

Figure 2-11: Brackish water 6-CMUG response. Response of 100 µM, 6-CMUG in brackish water from Poolbeg marina Dublin with various concentrations of *E. coli*. (n = 3) error bars represent 1 standard deviation, Temperature = 42°C. Fluorescence response in RFU recorded on a LS50B fluorescence spectrometer (Ex: 365 nm, Em: 445 nm)
Linear regressions of both the end-point fluorescence value (at 5 h) and the slope of the line vs measured sample concentration for the freshwater samples are shown in Figure 2-12. Those for the brackish water samples are shown in Figure 2-13. Each of the regressions showed increases in GUS activity with increasing sample *E. coli* concentration. However these results were based on a sample number of 4 and therefore should not be considered conclusive.

Figure 2-12: Freshwater 6-CMUG response Vs *E. coli* concentration. Black data points represent fluorescence magnitude after 5 hours and red data points represent rate of increase of fluorescence Vs *E. coli* concentration. Fluorescence response in RFU recorded on a LS50B fluorescence spectrometer (Ex: 365 nm, Em: 445 nm).
Figure 2-13: Brackish water 6-CMUG response Vs E. coli concentration. Black data points represent fluorescence magnitude after 5 hours and red data points represent rate of increase of fluorescence Vs E. coli concentration. Fluorescence response in RFU recorded on a LS50B fluorescence spectrometer (Ex: 365 nm, Em: 445 nm)

In the freshwater and brackish water assays there was a significant level of fluorescence for the measurement at time zero. Thus in the regressions of the absolute fluorescence at 5 hours against concentration there were significant constant terms for both water types, 5.5 for the freshwater and 1.9 for the brackish water. An ideal regression model goes through the origin and thus has no constant term [170]. This is better approximated by the regression of the slopes of the line against the E. coli concentration which gave constant terms of 0.3 for the freshwater and 1.5 for the brackish water. Thus it is preferable to use the slope (rate of fluorophore production) as an indicator of E. coli concentration.

This assay, conducted in 20 mL glass vials was not truly continuous as the sample was not analysed in the glass vial. Rather at each 1 h interval an aliquot was removed, analysed on a Perkin Elmer LS50B fluorimeter and returned to the vial. This measurement approach was simpler than the discontinuous ColiPlage method [73] as it did not involve a pH adjustment. However it still involved a lot of sample handling and the number of
measurements which could be taken during an assay was limited by practical considerations.

### 2.4.4.2 Plate reader based experiment

To automate the assay previously conducted in 20 mL sample vials, the procedure was repeated in PP 96 well-plates. Figure 2-14, shows the fluorescence response over 5 hours of 4 freshwater samples (salinity 5 ppt) containing a range of *E. coli* concentrations and spiked with 100 µM 6-CMUG while Figure 2-15, shows the same for 4 brackish water samples (salinity 21 ppt).

![Figure 2-14: Freshwater 6-CMUG response on plate-reader. Response of 100 µM 6-CMUG in freshwater from Tolka river Dublin with various concentrations of *E. coli*. (n = 3) error bars not shown (CV typically < 10%) Temperature = 42°C](image-url)
Figure 2-15: Brackish water 6-CMUG response on plate reader. Response of 100 µM 6-CMUG in brackish water from Poolbeg marina Dublin with various concentrations of *E. coli*. (n = 3) error bars not shown (CV typically < 10%), Temperature = 42°C.

The freshwater samples having *E. coli* concentrations of 19,230 CFU. 100 mL\(^{-1}\) and greater showed clear differentiation in activity levels for samples of different concentrations. The brackish water samples having *E. coli* concentrations 7,270 CFU. 100 mL\(^{-1}\) and lower showed no differentiation in activity between samples of different concentration. In the brackish water sample of concentration 1,970 CFU. 100 mL\(^{-1}\) a decrease in fluorescence was observed between time zero and 2 h. It is proposed that this is due either photo-bleaching or to the fluorophore being adsorbed to the PP well-plate as previously shown in Figure 2-8.

These results demonstrate that 6-CMUG can be used for rapid automated assays for *E. coli* β-Glucuronidase. Standard culture-based *E. coli* detection methods take 18 to 24 h to achieve a result, whereas results here were achieved in 5 h.
The 96 well-plate assay allowed for a high sample rate (1 fluorescence measurement / 10 min) which allowed the progress curves to be observed in detail. However for measuring *E. coli* concentrations lower than 10,000 CFU. 100 mL^-1 the PP well-plate was found not to be suitable due to the effect of fluorophore adsorption to the PP material or to photo-bleaching by the Tecan plate-reader.

While relationships between fluorescence response and *E. coli* concentration can be seen 3 of the 4 experiments above, there is an inherent problem with this relationship. β-Glucuronidase activity which is measured by the fluorescence response is contributed to by VC, VBNC, and dead bacteria and also extra cellular enzyme, whereas PetriFilm which was used to quantify the *E. coli* concentration, measures only VC bacteria. Garcia addressed this issue by correlating GUS activity with Direct Viable Count Fluorescent In-situ Hybridisation (DVC-FISH) which counts all live and dead *E. coli* in a sample. The correlations achieved with this method were higher than correlations with culture based methods [113].
2.5 Conclusion
The aim of this chapter was to investigate β-Glucuronidase assays for *E. coli* detection and to identify the factors affecting the performance of these assays.

Results have indicated that 6-Chloro-4-Methylumbeliferyl-β-D-Glucuronide is the best available substrate for *E.coli* β-D-Glucuronidase detection due to its fluorophore’s low pKa (6.1) which allows it to fluoresce strongly at near neutral pH where enzyme assays perform optimally (see Figure 2-3, Figure 2-4). It also exhibits separate absorbance maximums for the substrate and the Fluorophore at neutral pH which minimizes optical interference by the parent substrate with the fluorescence of the fluorophore (see Figure 2-5).

Semi continuous and continuous assays with environmental water samples have been demonstrated using 6-CMUG as a substrate (see Figure 2-10 and Figure 2-11 ). These assays were rapid as they were performed within 5 hours. The continuous assay conducted using a 96 well-plate reader minimised sample handling but it was found that PP well plates were unsuitable for sensitive assays due to fluorophore adsorption to the plate (see Figure 2-14 and Figure 2-15).

The LOD of the method appeared to be approximately 10,000 CFU / 100 mL for both the semi-continuous and the continuous methods. (i.e. Below this concentration there was little distinction between samples). This LOD is too high to satisfy the requirements for detection to BWD specified limits for E.coli (i.e. below 250 CFU / 100 mL ).

It was concluded that a method using 6-CMUG which includes a pre-concentration step with a factor of 40 or higher and simultaneously removes matrix effects and extra cellular enzyme, would have greatly increased sensitivity and lower LOD and therefore would meet the BWD requirements.
3 ColiSense 1 Design and Test

3.1 Introduction

*E. coli* and Enterococci are widely used as bacterial Faecal Indicators (FI) for recreational waters [30], [171]. Table 1-1: Bathing water standards, inland and Table 1-2: Bathing water standards, costal and transitional showed the specified maximum limits in Colony Forming Units (CFU) for marine and transitional waters as per the EU Bathing Water Directive 2006/7/EC. Standard culture based detection methods are slow to produce a result e.g. Colilert 18, a Most Probable Number (MPN) method, requires 18 hours incubation and Petri-Film, a colony counting method, requires 22 hours incubation. The incubation period, plus the time to take the sample and transport it to the laboratory, means that a result is not obtained until the following day. There is a demand for “Rapid” or same day test methods preferably on-site and autonomous [58], [172]. Enzyme assays have been suggested as the best solution for this [73].

Enzyme assays have long been suggested as a rapid alternative to culture based FI assays. β-D-Galactosidase (GAL) and β-D-Glucuronidase (GUS) have been used as marker enzymes in assays for *E. coli* [65], [66], [113], [114], [136], [141], [173] while Glucosidase has been used in assays for Enterococci. Of these target enzymes GUS is the most specific, being present in 94-97% of *E. coli* strains tested [66], [174].

There are a number of key differences between enzyme activity assays and culture based methods. Enzyme assays measure the activity of (i) Viable Culturable (VC), (ii) Viable But Not Culturable Bacteria (VBNC) plus dead bacteria, and (iii) free enzyme depending on the particular method used; whereas culture based methods count only the VC portion of bacteria present in a sample [132], [173]. If clusters of aggregated or particle bound *E. coli* are present in a sample, culture based methods count clusters as single units thus underestimating the number of cells present whereas enzyme assays account for the activity of each cell thus giving a better representation of the total number of cells present. For these reasons it is difficult to correlate the two approaches although this is commonly done due to a lack of a practical alternative standard reference method [66], [113], [115].

There is a growing body of evidence pointing to the virulence of VBNC bacteria and suggesting the importance of measuring their numbers in environmental waters [126], [131], [175]. GUS activity assays are suitable for this purpose and thus are investigated in this thesis.

GUS activity assays for *E. coli* do not have a selective growth step (as culture-based methods do). Thus they are susceptible to interference from other GUS sources [66]. Sources include plant and algal biomass [120], free (extracellular) enzyme [176], dead
target bacteria and GUS positive non-target bacteria. GUS positive non-target bacteria generally have GUS activities which are several orders of magnitude less than those of GUS induced E. coli, thus present little interference unless at very high concentrations. However certain species e.g, A. viridans, Bacillus spp [119] and Vibrio harveyi (particularly in the marine environment) [114] are highly GUS positive and may interfere with an assay if present at similar numbers to the target bacteria. Further interferences such as enzyme inhibition can occur due chemicals in the water matrix [117].

Chromogenic and fluorescent synthetic substrates have been used for enzyme assays and of the two; fluorescence offers much greater sensitivity by up to 1000X. As a consequence 4-Methyl-Umbelliferyl-β-D-Glucuronide (4-MUG) has been used extensively in discontinuous assays. Its fluorophore 4-Methyl-Umbellierone (4-MU) has a pKa of 7.8 and is highly fluorescent at values over pH9, [66], [114], [173]. Recent work in our research group [142] demonstrates the use of 6-Chloro-4-Methyl-Umbelliferyl-β-D-Glucuronide (6-CMUG) for continuous GUS assays with greatly reduced sample handling. Its fluorophore 6-Chloro-4-Methyl-Umbelliferone (6-MUG) has a lower pKa value (6.12) than 4-MU and at pH 6.8 is almost fully dissociated into its highly fluorescent anionic form. Furthermore GUS catalysis rates for the two substrates are quite similar, $K_{cat} = 222 \pm 13.4$ S$^{-1}$ for 4-MUG and $K_{cat} = 207 \pm 8.5$ S$^{-1}$ for 6-CMUG at 37°C and pH 6.8 [142].

Enzyme assays typically involve sample filtration, lysing, incubation, and detection steps. Figure 3-1 outlines the principle of the continuous 6-CMUG assay. E. coli cells are trapped and lysed, releasing GUS which catalyses the hydrolysis of 6-CMUG to a glucuronic acid and the fluorescent molecule 6-CMU. As shown in Figure 2-5 the substrate 6-CMUG has low molar absorptivity at 361nm (optimal excitation wavelength for 6-CMU) thus little substrate florescence occurs. When the fluorophore is released it has high molar extinction coefficient at 361nm thus fluoresces strongly. The amount of fluorophore (6-CMU) released in a certain period of time is directly proportional to the number of E. coli cells trapped. The assay performs optimally at 44° C and at pH 6.8 [142].
Figure 3-1: Fluorescence based enzyme assay principle. Cell lysis and release of β-D-Glucuronidase (GUS), substrate: 6-Chloro-4-Methyl-umbelliferyl-β-D-Glucuronide (6-CMUG) hydrolysis to 6-Chloro-4-Methyl-umbelliferyl (6-CMU) catalysed by GUS, fluorescence increase over time [177].

In the literature, GUS activities per E. coli are reported within the range of 0.1 to 100 fmol min\(^{-1}\) per culturable E. coli depending on method used. Garcia-Armisen [113] using a method from George [115] based on 4-MUG, reports GUS activities of approximately 100 fmol (4-MUG) min\(^{-1}\) per culturable E. coli for lightly contaminated freshwater samples (i.e. 100 to 1,000 E. coli. 100 mL\(^{-1}\) as established by MPN method). Lebaron [178] using the same method reports GUS activities per culturable E. coli of approximately 20 fmol (4-MUG) min\(^{-1}\) per culturable E. coli for seawater samples.

Instrumental detection of hydrolysis products of assays has commonly been conducted using standard laboratory bench fluorimeters [114], [115]. There have been a few attempts to conduct analysis on-site with portable fluorimeters [163], [179], but there remains a need for a rapid, sensitive on-site test for FI bacteria.

The ColiSense system was designed to perform E. coli enzyme assays on-site and was optimised for conducting a continuous 6-CMUG based assay. The system was designed with triplicate sample chambers to facilitate statistical analysis of results. Simultaneous assays were the only option for achieving triplicates as sequential assays were not possible in microbiological analysis of environmental waters due to sample aging.
To remain relevant to bathing water standards [28] the system was required to detect GUS activities in water with corresponding *E. coli* concentrations below 250 culturable *E. coli* per 100 mL. Assuming GUS activity of 100 fmol (6-CMU) min$^{-1}$ calculated per culturable *E. coli* [113], this meant detection of sample activities below (250 culturable *E. coli* per 100 mL) X (100 fmol (6-CMU) min$^{-1}$ per culturable *E. coli*) = 25,000 fmol (6-CMU) min$^{-1}$ 100 mL$^{-1}$. To achieve this, the system was designed with a nano-molar 6-CMU fluorescence detection range and sensitivity less than 1 nM 6-CMU.

Some authors suggest that the enzyme GUD be used directly as a faecal indicator, rather than as an indicator of the presence of *E.coli* [34], [65], [161]. As yet there has been no epidemiological study to relate GUD levels to disease occurrences.

### 3.1.1 Aims of Chapter 3: ColiSense 1 design and test

The aim of this chapter is to detail the design construction and test of a prototype system for conducting β-Glucuronidase assays in the field.

Objectives include

- Design and construction of a field portable incubating fluorescence detector
- Characterisation of the system and calibration using fluorescence standards
- Evaluation of system performance using commercial β-Glucuronidase
- Evaluation of the system using dilution series of environmental samples
- Trial of the system in the field
- Analysis of different sample fractions using the ColiSense 1 system
3.2 Materials and methods

3.2.1 Chemicals and reagents

The fluorophore, 6-chloro-4-methylumbelliferone (6-CMU) (97%) was obtained from CarboSynth, UK. The fluorogenic substrate, 6-chloro-4-methylumbelliferyl-β-D-glucuronide (97%) (6-CMUG) was obtained from GlycoSynth, UK. The enzyme: β-D-glucuronidase type VII-A (27%) from E. coli and 1,4-dithiothreitol (DTT) were obtained from Sigma Aldrich Ireland. The Colilert-18®/Quanti-Tray 2000® system from IDEXX Laboratories used for the enumeration of coliforms and E. coli was obtained from TechnoPath Ireland. Corning syringe filters with cellulose acetate surfactant-free membranes diameter 28 mm, pore size 0.45 μm were obtained from Sigma Aldrich, Ireland. Bacterial PELB buffer and PELB lysozyme were obtained from VWR Ireland. Water was passed through a Milli-Q water purification system to achieve a conductivity of 0.55 μS. Stock solutions of fluorophore and substrate (100 mM) were prepared in 1 mL DMSO (99.5%) and stored at 4°C. Water was passed through a Milli-Q water purification system to achieve a conductivity of 0.55 μS.

3.2.2 Engineering components

Ultraviolet LEDs (FG360-R5-WC015) with peak emission wavelength at 361 nm were obtained from ATP, USA. Photodiodes (BPW21R), operational amplifiers (MCP601), voltage regulators (LM317), Darlington transistor array (ULN2803), digital temperature sensor (DS18b20) and silicone matt heater (1.25 W, 50 mm x 25 mm) were obtained from Radionics Ireland. Optical filters (GG-420, Long Pass, diameter 12.5mm) were obtained from Edmund Optics, UK. A Wixel micro-controller board was obtained from Cool-Components, UK. The instrument enclosure (Diatec S White) was obtained from OKW, UK. Glass sample vials (TVL-050-040) were obtained from SciChem Ireland. The heating block was machined in-house from aluminium.

3.2.3 Sample vial reaction vessel

The optical properties of sample vials to be used as incubation, reaction and detection chambers were investigated using UV-Vis spectroscopy. This is detailed in appendix A 3.1: Glass vial optical characterization.
3.2.4 Fluorescence detection system development

A portable incubating fluorimeter (ColiSense) was designed and built to conduct the detection step of the continuous, 6-CMUG based assay. The system shown in Figure 3-4 featured three sample chambers for performing assays in triplicate as recommended by Lebaron due to the high Coefficient of Variance (CV) (15%) of enzymatic methods [178]. Each chamber was set to incubate at 44°C and control to within 0.5°C. A fluorescence detection system with excitation at 361 nm and emission at 445 nm was integrated into each chamber.

3.2.4.1 Optical and mechanical design

Figure 3-2 shows the normalised absorbance and emission spectra for the chemical components of the enzyme assay at near neutral pH (pH 6.8) and shows the characteristic spectra of the optical components of the system. An LED (Type FG360-R5-WC015) with peak emission at 361 nm and spectral width of 20 nm was selected to excite the 6-CMU close to its maximum while exciting the 6-CMUG as little as possible. A photodiode with peak sensitivity at 570 nm and enhanced sensitivity in the blue region (65 % of max at 445 nm) was selected as the detector. A high pass optical filter with 420 nm cut-off was selected to reduce any interference from substrate fluorescence and block the excitation light from the detector. Glass sample vials (2 mL) were used as cuvettes due to their disposable nature and low cost. The optical characteristics of the vials were tested and results are shown in A 3.1: Glass vial optical characterization.
Figure 3-2: ColiSense optical design. Normalised spectra of chemical components of the GUS assay and optical components of the fluorescence detection system [177].

Figure 3-3: ColiSense incubation and fluorescence detection system [177].

An incubation block, shown in Figure 3-4, with 3 detection chambers was machined from aluminium. A self-adhesive silicone foil heater was attached to this and cork insulation
(6 mm) was applied to exposed surfaces of the heating block to increase thermal efficiency. A digital temperature sensor was inserted in the block and fixed in place with thermally conductive epoxy. The components of the fluorescence detection system were incorporated into the heating block in an off axis (90°) arrangement as shown in the schematic in Figure 3-3 and the image in Figure 3-4. The glass sample vials were inserted into the incubation block where the LED excited the fluorescence from below and fluorescence was emitted at right angles, filtered and captured by the photodiode. The heating block and the fluorescence detection system were incorporated into the instrument enclosure shown in Figure 3-4.
3.2.4.2 Electronic circuit design

The electronic control system was designed around a Texas instruments CC2511F32 based micro-controller board called Wixel (https://www.pololu.com/product/1336). This was programmed in a variant of C via USB comms. The board offers features including a 3.3 V regulator, USB, low power radio, 12 bit differential Analog to Digital Converter (ADC). It has a small form factor and is highly versatile. The ADC with 2047 quantization levels was set to use an internal reference of 1.25 V. Thus the resolution was 1.25 V /2047 = 0.61mV.

An LED light source was chosen for the device as it offers low power consumption, small size, low weight, high robustness and high monochromaticity. The emission spectrum is shown in Figure 3-2. To maximise measurement sensitivity, the LED was powered at its maximum continuous rating (20 mA) though a constant current supply based on a LM317 regulator delivering a radiant power of 750 µW. To reduce any possible bleaching of the fluorophore by over exposure to the excitation source, the sample rate was set to 0.1 Hz with a sample illumination duty cycle of 0.5 %, i.e. the sample was illuminated for 50 ms each 10 s.

A photodiode was chosen as the optical detector for the device as it offers low power consumption, small size, low weight and high robustness. The acceptance spectrum of the chosen photodiode is shown in Figure 3-2. The photodiode was used in photovoltaic mode with a trans-impedance amplifier based on an MCP601 operational amplifier to convert its output to a voltage. The voltage was recorded by the 12 bit ADC on the controller board. Details of trans-impedance amplifier gain resistor selection are shown in section 3.2.5 and in Figure 3-8.

A silicone foil heater was selected as the heat source. This delivered 5 W of heat while powered with 24 V and drawing 200 mA. Temperature control was performed using a Dallas 1-wire digital temperature sensor (DS18B20) allowing control to within 0.5 °C. Details of temperature and power testing are presented in Appendix A3. A Darlington transistor array (ULN2803) controlled by the Wixel was used to switch the LEDs and heater. This component can switch loads up to 500 mA per channel at up to 36 V. The system was powered by a 24V switch mode plug top supply for laboratory use and by a 24V battery for field use. Communications to the PC was via USB using serial protocol at 9,600 baud rate.
3.2.4.3 Software design

The Wixel development platform around which the electronics of the system was based was programmed in C. Function prototypes were created to control each of the system components. Fluorescence levels in each sample chamber plus the temperature of the incubating block were transmitted to the PC and displayed on ExtraPutty terminal or graphed using a Graphical User Interface (GUI) designed in Java, while simultaneously being recorded in a log file in Comma Separated Value (CSV) format. The timestamp feature on ExtraPutty V0.26 was used to append a date and time to each reading stored in the log-file. The log-files were subsequently imported into Microsoft Excel for analysis. Figure 3-5 shows a flow diagram of the firmware on the ColiSense instrument and software on the attached PC including the programmed Java GUI.
Figure 3-5: ColiSense 1 software components. (A) Instrument firmware and PC software flow diagrams and (B) detailed view of Graphical User Interface (GUI) [177].
3.2.5 Fluorescence detection system characterisation

To select optimum gain settings for the photodiode trans-impedance amplifier and establish the range and sensitivity of the ColiSense, the system response was tested against a range of 6-CMU concentrations (0.1 nM to 130 µM in sodium phosphate buffer at pH 6.8). The same procedure was carried out on an LS50-B spectro-fluorimeter to benchmark the response of ColiSense.

An optimum sensitivity setting with a trans-impedance amplifier feedback resistance of 100 MΩ (with a parallel 12 pF ceramic capacitor for stability) was selected and the following calibration procedure was carried out. 6-CMU concentrations from 0.1 nM to 10 µM were prepared in 2 mL glass vials also containing 0.5 mM 6-CMUG, 5% PELB (v/v), 0.05 mg mL⁻¹ PELB lysozyme and 20 mM DTT in sodium phosphate buffer at pH 6.8. Analysis was carried out in triplicate. The three vials were placed in channel A, B, C of the ColiSense instrument, incubated at 44°C and fluorescence response was recorded.

3.2.6 Commercial GUS kinetics

Commercial GUS at a range of concentrations (0.02 to 0.42 ng.mL⁻¹) was inoculated into 2 mL glass vials containing 2 mL of 500 µM 6-CMUG in phosphate buffer at pH 6.8. Analysis was carried out in triplicate. The three vials were placed in channel A, B, C of the ColiSense instrument where they were incubated at 44°C and fluorescence increase was recorded during 30 minutes at intervals of 10 seconds.

3.2.7 Environmental sample dilution series

Fresh water and salt water samples were collected from the river Tolka and the river Liffey estuary respectively, both in Dublin, Ireland using the sampling procedure outlined in section 2.3.3.

For the determination of E. coli (MPN), the Colilert-18 enumeration protocol was followed in accordance with manufacturer’s instructions. Aliquots of 10 mL from the original water samples were diluted 1:10 with sterile deionised water and placed into 100 mL bottles. After the addition of Colilert-18 media, samples were inoculated into Quanti-Trays and sealed. For E. coli and coliform enumeration, samples were incubated at 37.0
°C for 18 to 20 hours. Following incubation the Quanti-Tray wells were read visually for yellow colour indicating the presence of coliforms and for blue fluorescence indicating the presence of *E. coli*.

GUS activity was measured using the following protocol developed by Briciu Burghina [142][180]. The sample was filtered through 0.45 μm syringe filters for bacteria capture and pre-concentration using 50 mL syringes, followed by PELB lysing agent addition (100 μL). In the next step, the filters were sealed using screw caps and incubated at 37° C for 30 min. In the third step, 1.9 mL of buffer (pH 6.8) was flushed through the filter using 2.5 mL syringes and the samples were recovered in 2 mL glass vials. The vials were placed into the ColiSense to allow pre-warming to 44 °C after which a 10 μL aliquot of 100 mM 6-MUG in DMSO was added and the vials were vigorously mixed. The vials were placed back into the ColiSense, allowed to equilibrate and GUS activity was monitored/recorded for 30 min. Triplicate blanks were also prepared by adding 1.9 mL of buffer, 100 μL lysing agent and a 10 μL aliquot of 100 mM 6-MUG in DMSO to 2 mL vials. These were placed in ColiSense and their activity was monitored for 30 min to detect auto-hydrolysis of 6-CMUG.

To mimic a dilution series, a range of *E. coli* concentrations (trapped in filters) were prepared by filtering various volumes of water sample (100 mL to 2 mL) through syringe filters. These samples were analysed for GUS activity as described above.

### 3.2.8 Field trial

On 27th February 2015 a field trial was conducted to demonstrate the portability of the ColiSense system. The ColiSense system and miniature incubator for use in the lysing procedure were placed in a van for transport and powered from the van’s battery source. Seven points along the Tolka River, Dublin, Ireland (shown in Figure 3-6) were sampled and analysed *on-site* in 1 day. Sampling points 1 to 5 were within the urban area at approximately 2 km spacing’s while points 6 and 7 were in the rural catchment 1 km apart with point 6 being 6 km west of point 5. The sample capture and testing began in the early morning downstream just above the tidal range and concluded in the evening in the upstream catchment.
GUS activity was measured in triplicate using the protocol detailed in section 3.2.7, with filtered volume fixed at 50 mL. Additional water quality parameters were measured including *E. coli* MPN and total coliforms MPN, pH, temperature, turbidity, conductivity measured by Hydrolabs DS5x multi-parameter sonde, while phosphate, nitrate and nitrite were measured using a Hach DR900 Nutrient Analyser.

### 3.2.9 Raw water and extracellular enzyme testing

A range of freshwater samples from waterbodies in the Dublin area using the sampling procedure detailed in section 3.2.7. The *E. coli* (MPN) of each sample was enumerated using the protocol detailed in the same section.

Raw Water GUS activity was recorded as follows: Raw water was placed into 2 mL glass vials. 6-CMUG was added to achieve a concentration of 500 µM. Analysis was carried out in triplicate. The three vials were placed in channel A, B, C of the ColiSense instrument where they were incubated at 44°C and fluorescence response was recorded for 30 minutes at intervals of 10 seconds. To record extracellular GUS activity, raw water samples were filtered through 0.45 µM membrane filters. The filtrate was placed into 2 mL glass vials. 6-CMUG was added at 500 µM and fluorescence response recorded as before.
3.3 Results and discussion

3.3.1 Fluorescence detection calibration

3.3.1.1 Linear range and inner filter effect

Figure 3-7 shows the response of an LS50B spectro-fluorimeter to a range of concentrations of 6-CMU (0.1 to 100 µM) in phosphate buffer at pH 6.8, and shows the response of the ColiSense 1 system to the same concentrations. The response for both was approximately linear until 20 µM. At concentrations higher than 40 µM the fluorescence response decreased due to the inner filter effect [143].

![Figure 3-7: ColiSense 1 and LS50B dynamic range for 6-CMU. Response of ColiSense 1 and an LS50B spectro-fluorimeter to 6-CMU concentrations in pH 6.8 phosphate buffer. For both systems (Excitation = 365 nm, Emission = 445 nm) LS50B set to 1% attenuation [177].](image-url)
3.3.1.2 Sensitivity optimization and comparison with standard instrument

Figure 3-8 shows calibrations for Channel A of ColiSense 1 with 3 different transimpedance amplifier gain resistances (1 MΩ, 10 MΩ and 20 MΩ) for 6-CMU concentrations up to 4000 nM in pH 6.8 phosphate buffer. System sensitivity increased with gain resistance but background level also increased as is shown by the Y axis intersects. Background level was subsequently reduced by blackening the internal surfaces of the incubation block to reduce spurious reflections. The data was also plotted against the response of the LS50B spectro-fluorimeter to the same concentrations. This graph is not shown here. For each of the 3 different transimpedance amplifier gain resistances the correlation was linear with R² above 0.98. This indicates that the ColiSense can be used as an accurate wavelength specific fluorimeter.

\[
\begin{align*}
Y &= 0.0107x + 17.507 \\
R^2 &= 0.9851 \\
Y &= 0.1139x + 204.42 \\
R^2 &= 0.987 \\
Y &= 0.2193x + 420.43 \\
R^2 &= 0.9859
\end{align*}
\]

Figure 3-8: ColiSense 1 sensitivity optimisation. Responses to 6-CMU concentrations of 0 µM to 4 µM in pH 6.8 phosphate buffer are shown for 3 sensitivity settings on the ColiSense 1 (set by the trans-impedance amplifier gain resistor). For 1 MΩ, (Y = 0.011X + 17), for 10 MΩ, (Y = 0.11X + 204), for 20 MΩ, (Y = 0.22X + 420) [177].
3.3.1.3 Calibration

Figure 3-9.a shows calibration curves for channel A, B, C (each with a 100 MΩ trans-impedance amplifier feedback resistor) of the ColiSense instrument for concentrations of 6-CMU up to 900 nM in pH 6.8 phosphate buffer. Points represent the average of triplicate samples and vertical error bars represent the standard deviation of those samples. Channel A and C showed a sensitivity of 1.54 and 1.57 quantisation levels (ADC units) per nM 6-CMU. Channel B was slightly more sensitive at 1.76 quantisation levels per nM 6-CMU. Resolution was less than 1 nM for each channel. The LOD for each channel was 5 nM (6-CMU). Each curve crossed the Y axis at 100 fluorescence units or more. This is due to a combination of leakage of the excitation source light to the detector, the fluorescence of the substrate (6-CMUG) itself and the presence of small concentrations of fluorophore (6-CMU) from auto-hydrolysis of the substrate. The substrate, was present in the assay at much higher concentration than the fluorophore (500 µM vs >1 µM respectively), thus any substrate fluorescence emitted was a significant interference. As demonstrated in section 2.4.1 the substrate (6-CMUG) absorbed maximally at 325 nm and emitted maximally at 400 nm while its hydrolysed fluorophore (6-CMU) absorbed maximally at 365 nm and emitted maximally at 445 nm. The high pass optical filter with 420 nm cut-off reduced interference from substrate fluorescence and leakage of the excitation light to the detector while allowing the 6-CMU fluorescence to pass with minimal attenuation.
Figure 3-9: ColiSense 1 system characterisation. (A) Calibration of fluorescence response of ColiSense channels A, B, and C with concentrations of 6-CMU up to 1,000 nM in pH 6.8 phosphate buffer with 500 µM 6-CMUG and PELB, n = 3, (for channel A, Y = 1.54X + 107; for channel B, Y = 1.76X + 141; for channel C, Y = 1.57X + 110). Error bars show SD of triplicate measurements. (B) Progress curves for 0.43 ng.L\(^{-1}\) GUS added to 500 µM 6-CMUG in pH 6.8 phosphate buffer with PELB in ColiSense Channels A,B,C. (C) Progress curves from panel B expressed as 6-CMU concentration. (D) Enzyme activity per 100 mL recorded by ColiSense for concentrations of GUS up to 1 ng.L\(^{-1}\) (n = 3) (Y = 63X - 0.13) (R\(^2\) = 0.98). Error bars show SD of triplicate measurements. Circled point is the average enzyme activity calculated from the 3 progress curves shown in panel C [177].
3.3.2 Commercial GUS kinetics

Figure 3-9.b shows progress curves for 0.42 ng mL\(^{-1}\) GUS added to 500 µM 6-CMUG in Channels A, B, C of the ColiSense instrument. The curves were linear to 30 minutes. Channel B showed a larger value response than A or C due to its higher sensitivity, (see Figure 3-9.a). Figure 3-9.c shows the same progress curves converted to 6-CMU concentration by dividing each sample point by the channel sensitivity found from Figure 3-9.a. Points represent the average of triplicate samples and vertical error bars represent the standard deviation of those samples. The circled point in Figure 3-9.d shows the mean and standard deviation of the same progress curves converted to activity levels in picomoles 6-CMU per minute per 2 mL (cuvette volume = 2 mL) and plotted against enzyme concentration. Figure 3-9.d shows further data obtained in the same manner for GUS concentrations from 0.02 to 0.42 ng mL\(^{-1}\). The activity of 0.1 pmol (6-CMU) min\(^{-1}\) ml\(^{-1}\) recorded for the blank (i.e. without the addition of enzyme) is due to substrate autohydrolysis where 6-CMUG spontaneously separates into 6-CMU and glucuronic acid in the presence of water.

The system was shown to detect GUS activities as low as 0.1 pmol (6-CMU) min\(^{-1}\) ml\(^{-1}\) for the blank and up to 14.5 pmol (6-CMU) min\(^{-1}\) ml\(^{-1}\) for the highest enzyme concentration tested. The LOD was lower than the design requirement for the system i.e. detection lower than 25 pmol (6-CMU) min\(^{-1}\) 100 mL\(^{-1}\) or 0.25 pmol (6-CMU) min\(^{-1}\) ml\(^{-1}\) as detailed in section 3.1. Thus it was concluded that the ColiSense system could be used to analyse samples containing 250 E. coli per 100 mL without any pre-concentration. The coefficient of variance (CV) for the method depended on enzyme concentration, with a CV of 14 % calculated for the lowest GUS concentration (0.02 ng mL\(^{-1}\)) and a CV of 1.8 % calculated for the highest concentration (0.42 ng mL\(^{-1}\)).

3.3.3 Environmental sample testing

An MPN of 3,873 E. coli per 100 mL was recorded for the freshwater sample (salinity 4 ppt) while an MPN of 8,164 E. coli per 100 mL was recorded for the seawater sample (salinity 32 ppt). By varying the volume of sample filtered, a range of MPNs from 193 to 3,873 E. coli for freshwater and from 163 to 8,164 E. coli for seawater were achieved. Figure 3-10 shows graphs of recorded GUS activity (pmol (6-CMU) min\(^{-1}\) 100 mL\(^{-1}\) of sample) vs E. coli MPN for freshwater and seawater. Points represent the average of
triplicate samples and vertical error bars represent the standard deviation of those samples.

Figure 3-10: ColiSense Target analyte testing. (A) Enzyme activity per 100 mL sample vs E. coli concentration in River Water (Salinity = 4 ppt) (n = 3) (Y =0.24X + 2.19) (R^2 = 0.99), Error bars show SD of triplicate measurements. (B) Enzyme activity per 100 mL sample vs E. coli concentration in Sea Water (Salinity = 32 ppt) (n = 3) (Y =0.91X + 66) (R^2 = 0.99), Error bars show SD of triplicate measurements [177].

From the data plotted it can be seen that both samples show linear relationships between GUS activity and E. coli MPN down to E. coli concentrations lower than the excellent standard (MPN 250 E. coli) as stipulated by the BWD [28]. The slopes of the curves however differ significantly with the seawater sample having 4 times more GUS activity per E. coli than the freshwater. This may be attributed to a higher proportion of VBNC to VC E. coli in the marine environment than in freshwater. This is not proven here experimentally but it has been addressed adequately by other researchers [123], [125], [132]. Another potential influence is interference from GUS positive marine biomass including plant and algal matter as detailed by Davies [120]. Plant based interference occurs through the release of GUS into the water body. In this work a sample filtration
step is included so this interference is removed. However, GUS positive algae which Davies states are more common in the Marine than in Freshwater [120] remain a potential interference as they are retained during filtration.

The coefficient of variance for the method varies depending on the E. coli concentration being measured with higher variability at lower concentrations. For the freshwater sample CV decreases from 6% at the lowest E. coli MPN (193) to 1.5% for the highest E. coli MPN (3,873) while similarly for the seawater sample CV decreases from 23% at the lowest E. coli MPN (163) to 0.3% for the highest E. coli MPN (8,164).

### 3.3.4 Field trial

The field trial began at 7 am and concluded at 7 pm on the same day. With 7 sites sampled in 12 hours, this gave an average time per sample of 103 min including total analysis time (approximately 75 min) and transit between sites. This demonstrated the field portable nature and rapidity of the device and assay.

Figure 3-11 shows the collected E.coli MPN and GUS activity data recorded at each location along the Tolka River. Error bars on MPN measurements represent the upper and lower 95% confidence limits of the method, while error bars on the activity measurements represent 95% confidence intervals for triplicate samples. The horizontal red line indicates the acceptable upper limit for recreational waters i.e. MPN 1,000 E. coli per 100 mL. Table 3-1 shows additional water quality parameters measured at each sample location during the field trial.
Figure 3-11: ColiSense 1 River Tolka Field Trial. *E. coli* MPN and GUS activity by location. Vertical error bars on GUS activity show the 95% confidence intervals of triplicate samples in each channel of ColiSense, Vertical error bars on *E. coli* MPN represent the upper and lower 95% confidence intervals for the Colilert 18 MPN method. Red line indicates the BWD (Good quality) level for *E. Coli* in inland waters. [177].

Table 3-1: ColiSense 1 field-trial background data. Water quality parameters measured during field trial [177].

<table>
<thead>
<tr>
<th>Sample point</th>
<th>Water Temp (°C)</th>
<th>Air Temp (°C)</th>
<th>pH</th>
<th>ORP</th>
<th>Sp Cond (µS/cm)</th>
<th>Sal (ppt)</th>
<th>TDS (g/l)</th>
<th>Turb (NTU)</th>
<th>Chlorophyll A (µg/l)</th>
<th>Phosphate (mg/L PO₄³⁻)</th>
<th>Nitrate (mg/L NO₃⁻)</th>
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<td>0.41</td>
<td>0.5</td>
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<td>0.5</td>
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<td>0.5</td>
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<td>0.5</td>
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<td>0.5</td>
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<td>2.36</td>
<td>0.25</td>
<td>1.8</td>
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</tbody>
</table>

In Figure 3-11, point 1 shows elevated *E. coli* MPN (15 times the acceptable upper limit for recreational waters) and GUS activity. There had been heavy rain in the hours prior
to the sample being taken. Therefore the high *E. coli* levels may have been due to a combined sewer overflow (CSO) as is known to occur on the Tolka [181]. *E. coli* levels of this order have been recorded previously at the same site under similar conditions [182]. In the case of a CSO *E. coli* MPN would be expected to be high and GUS activity correspondingly high [132]. Points 2 to 5 show lower *E. coli* MPNs (3 to 6 times the acceptable upper limit for recreational waters) and correspondingly lower GUS activities. This decrease from point 1 agrees with previous research where it was shown that bacterial counts reduce with progress upstream in the river Tolka [182] and it is supposed that this is due to decreased urbanisation.

Points 6 and 7, both in the agricultural catchment of the Tolka River, showed high levels of GUS activity relative to measured *E. coli* MPN. It is suggested that this was due to the recent application of farm slurry to land in the catchment. The slurry spreading seasons had opened on 15th February in the catchment area. Furthermore phosphate levels at were elevated at points 6 and 7, these were 0.26 and 0.25 mG.L\(^{-1}\) (PO\(_4^{3-}\)) respectively. The highest of the other 5 points was point 3 at 0.01 mg L\(^{-1}\) (PO\(_4^{3-}\)). Slurry on farmland would contribute a large proportion of VBNC compared to VC *E. coli* due to it being aged either on land or in the farmyard before reaching the river [125]. Thus a high level of GUS activity can be expected while the MPN of culturable *E. coli* is low.

The coefficient of variance for the method depended on the *E. coli* concentration being measured, with higher variability at lower concentrations. CV decreased from 15.6 % at the lowest *E. coli* MPN (624) to 2.6 % for the highest *E. coli* MPN (14,136). These figures agree with the work of Lebaron who in a much more extensive study using the discontinuous 4-MUG method for measuring GUS activity reported CVs less than 15% [178]. By comparison, culture based MPN methods typically report CVs of 15 to 30 % [183].
3.3.5 Raw water and extra cellular enzyme

Raw water can contain varying proportions of VC, VBNC *E. coli* and extra-cellular GUS. Figure 3-12 shows the activities of a range of raw freshwater samples and a range of filtrate from freshwater samples. GUS activity in the filtrate is due to extracellular enzyme as all cells were removed by filtration. From this it can be seen that extra-cellular enzyme present in a raw sample can have a significant contribution to overall GUS activity. In this case extracellular GUS activity contributes 22% of total activity (slope of filtrate as a percentage of the slope of raw water). Results from the raw water analysis were obtained within 15 to 25 minutes of sampling.

![Graph showing GUS activity of raw water and extracellular enzyme](image)

Figure 3-12: GUS activity of raw water and extracellular enzyme. A number of filtrate samples were omitted for operational reasons. Data points represent the average of 3 measurements. Error bars represent 1 standard deviation. For raw water \(Y = 0.0059X + 26\), for filtrate \(Y = 0.0013X + 24\) [177].
3.4 Conclusion

The ColiSense detection system detailed here utilised the β-D-Glucuronidase (GUS) enzyme assay. The system and the method combined now offer a rapid (75 min) on-site solution for FI detection and quantification to below 250 CFU E. coli 100 mL⁻¹.

The ColiSense system is a sensitive purpose built fluorescence detection and incubation system with three sample chambers for triplicate analysis. When combined with an efficient GUS extraction protocol and a continuous fluorometric assay based on 6-Chloro-4-Methyl-Umbelliferyl-β-D-Glucuronide (6-CMUG) enzyme substrate, it provided a sensitive and rapid method of on-site analysis of surface waters for E. coli.

The system has demonstrated an LOD of 5 nM (6-CMU) with a resolution of less than 1 nM (6-CMU) and detection of GUS activities below 1 pmol (6-CMUG) min⁻¹ mL⁻¹. This allowed detection of GUS activities for E. coli concentrations lower than 250 MPN per100 mL (the upper limit for excellent marine recreational water as per the Bathing Water Directive). The CV of the method has been shown to be dependent on E. coli concentration (23 % at the lowest concentrations measured to <1 % at the highest concentrations). The triplicate analysis facility allows for greater confidence in results.

The system and assay detect VBNC along with VC E. coli and GUS from sources other than E. coli which leads to some disagreement with culture based methods but as an alternative to standard culture based methods of E. coli detection it offers greater rapidity and portability and is capable of meeting the need for rapid faecal indicator detection to ensure recreational water quality standards.

ColiSense also demonstrated the ability to conduct raw water and extracellular enzyme assays. Extra cellular enzyme has been shown to make up a significant portion of raw water activity. The raw water assay took as low as 15 minutes to perform (from sample to result). It is limited in accuracy but its speed may make it interesting for tracing studies which use relative levels of contamination to locate pollution sources.
Sensing platform design for Faecal indicator bacterial detection in recreational waters

Brendan Heery – September 2018
4 ColiSense 2 Design and Test
4.1 Introduction

The measurement range of interest for an *E. coli* assay for recreational waters is between 100 CFU.100 mL\(^{-1}\) and 1,000 CFU.100 mL\(^{-1}\) which equates to 1 CFU.mL\(^{-1}\) and 10 CFU.mL\(^{-1}\). Due to non-uniform distribution of *E. coli* in a sample [135], it is necessary to sample a significant volume to obtain a representative assay result. The BWD standard volume is 100 mL [28]. For measurement instruments with sample capacities of the order of a few mL, pre-concentration of sample is used widely [58], [136].

In section 2.5 it was concluded that a pre-concentration step by a factor of 40 or more was required to lower the LOD of a 6-CMUG based GUS assay to 250 CFU.100 mL\(^{-1}\) and below to satisfy the requirements of monitoring for BWD standards.

The ColiSense 1 instrument has a sample chamber of 2 mL [177] and used a syringe filtering procedure to concentrate a 100 mL sample and then lyse the cells to release GUS. This procedure involved significant sample handling and took 75 minutes to prepare and analyse a single sample. This chapter investigates alternative procedures and assays to simplify sample preparation and reduce time to result. Pre-concentration and filtration to achieve these goals were therefore examined and are presented in this chapter.

4.1.1 Sample pre-concentration method

Various pre-concentration methods exist including magnetic bead separation using antibodies specific to *E. coli* or *Enterococci* and microfiltration. Of the methods available filtration is the simplest, most robust and most suitable to on-site assays. Filtration allows the concentration of target bacteria while also removing matrix interferences such as heavy metals, halides and extracellular free enzymes. Extra-cellular GUS is a significant contributor to raw water activity as shown in section 0.

Filtration also allows for the replacement of the sample liquid fraction with a solution designed to optimise *E. coli* metabolism and assay conditions. The solution can be designed to buffer pH to optimise transportation across cell inner membrane and fluorescence response, and to be isotonic for the target bacteria. The disadvantage of filtration is that it is non-specific as it also concentrates non-target bacteria.
4.1.2 Filtration and metabolic assay concept

The metabolic pathways of *E.coli* were detailed in section 1.7.1 and an assay based on the metabolism of 6-CMUG by *E.coli* was detailed in section 2.3.5. This assay required significantly less sample handling than the lysing based assay detailed in section 3.2.7 and utilised with ColiSense 1 [177].

The lysing based assay had a pre-concentration step and a cell lysing step performed in a syringe filter before transfer of lysate to a reaction vessel for detection of fluorescence response. In this chapter the aim is to reduce sample handling by combining the pre-concentration step and the reaction/detection step in the same vessel. The cell lysing step is eliminated through the use of a metabolism based assay where *E.Coli* remains intact and transports 6-CMUG across the cell membrane. The resultant fluorophore is then released back into solution. This principle was investigated for a membrane filtration and a depth filtration technique.

Enclosed membrane filter units with Leur fittings for pressurised flow such as Sterivex GP have the features required to perform pre-concentration and detection in a simple chamber. A drawback of this design is the availability of the filter units only in plastic (Eastar™ Copolyester EB062) which is can be unsuitable for fluorescence measurement due to the auto-fluorescence of the plastic itself under UV. The system may however be suitable for colorimetric detection or fluorescence with excitation in the visible range.

Depth filters, such as glass fibre paper, trap particles within the material of the filter rather than on the filter surface as is the case for membrane filtration. Depth filtration is more suitable for particle laden samples than membrane filtration as it allows for higher flow-rates and thus larger filtered volumes for turbid samples.
4.1.3 Aims of Chapter 4: ColiSense 2 design and test

The aim of this chapter is to detail the design, construction and test of a system based on ColiSense 1 but including features which enable sample pre-concentration and which reduces sample handling and total sample time for conducting for field-based β-Glucuronidase assays.

Specific objectives include:

- Design and construction of a field portable sample pre-concentration system
- Incorporation of the pre-concentration system into an incubating fluorescence detector based on ColiSense 1
- System characterisation including pre-concentration system performance and fluorescence detection performance.
- Evaluation of system performance using commercial β-Glucuronidase
- Evaluation of the system using dilution series of environmental samples
- Trial of the system in the field
4.2 Materials and methods

4.2.1 Chemicals and reagents

The fluorophore, 6-chloro-4-methylumbelliferone (97%) (6-CMU) was obtained from CarboSynth, UK. The fluorogenic substrate, 6-chloro-4-methylumbelliferyl-β-D-glucuronide (97%) (6-CMUG) was obtained from GlycoSynth, UK. The enzyme: β-D-glucuronidase type VII-A (27%) from *E. coli* was obtained from Sigma Aldrich Ireland. The Colilert-18®/Quanti-Tray 2000® system from IDEXX Laboratories used for the enumeration of coliforms and *E. coli* was obtained from TechnoPath Ireland. Water was passed through a Milli-Q water purification system to achieve a conductivity of 0.55 µS.

Stock solutions of fluorophore and substrate (100 mM) were prepared in 1 mL DMSO (99.5%) and stored at 4°C.

4.2.2 Engineering components

The ColiSense 2 system was designed using the same components as ColiSense 1 (See section 3.2.2) plus the following: Sterivex GP 0.45 µm membrane filter capsules were obtained from Sigma Aldrich Ireland. Borosilicate glass SPE cartridges with 20µm porosity PTFE frits, Stainless Steel (SS) frits were produced from SS mesh 0.5 mm porosity, Corning Leur caps and Whatman glass fibre 0.7 µm depth filtration paper were obtained from Sigma Aldrich Ireland. Corning Leur caps were obtained from Sigma Aldrich Ireland. Nitrile rubber O-Rings (Diameter 13mm), Pelicase and electronic components were obtained from Radionics Ireland.

4.2.3 Filter system concept

A concept design was developed for an integrated pre-concentration and detection system based on depth filtration in modified glass Solid Phase Extraction (SPE) cartridges with a glass fibre filter material disk supported by a PTFE frit. This is shown in Figure 4-1.

Step 1: 100 mL of sample is passed through the chamber containing the filter by applying a vacuum (0.68 bar). Bacteria are trapped on and inside the depth filter while filtrate containing extra-cellular enzyme and other interferences are removed.
Step 2: 1.5 mL of reagent (substrate solution) is added and the chamber is sealed. This gives a 67 times concentration of the sample. The Chamber is then incubated and the assay is carried out. As the substrate is broken down by the *E. coli* trapped in the filter the resultant fluorophore diffuses throughout the volume of reagent. Fluorescence detection is carried out through the glass vial.

Figure 4-1: Depth filter pre-concentration, Proposed filtration based pre-concentration system with integrated fluorescence detection.
4.2.4 Filter system development

An integrated filtration and detection chamber was developed using borosilicate glass SPE cartridges with a glass fibre depth filter supported by a porous PTFE frit and held in place by a nitrile rubber O-ring. Figure 4-2 shows the features of this system including the vacuum filtration apparatus and the fluorescence response visible within the detection chamber.

Figure 4-2: ColiSense 2 filter system components and setup. A. Top cap, B. Borosilicate glass SPE cartridge, C. Nitrile O-Ring filter retainer, D. Filter paper, E. Porous PTFE frit, F. Bottom cap, G. UV light source, H. Fluorescence response of 10µM 6-CMUG to 365 nm excitation in the filter system, I. Rubber bung, J. Retort stand, K. Vacuum flask.

A cleaning protocol for the filter system was developed. The steps followed were.

1. Dismantle the filter system.
2. Remove the used filter and dispose.
3. Place all rubber and plastic parts in a glass bottle with a lid containing tap water.
4. Shake vigorously for 1 minute and pour off the water. Refill and repeat 3 times.
5. Refill the bottle with ethanol. Shake, pour off and repeat 3 times.
6. Refill the bottle with DI water. Shake, pour off and repeat 3 times.
7. Rinse the glass SPE cartridge with tap-water for 1 minute.
8. Rinse with Ethanol and brush internally with a test tube brush.
9. Rinse the glass SPE cartridge with DI-water for 1 minute.
10. Place all parts in a drying oven overnight 8 hrs at 100˚ C to dry and sterilise.

4.2.5 Detection system development

The ColiSense 2 system was designed using the same technology as ColiSense 1 (detailed in section 3.2.4) with a number of added features (shown in Figure 4-3)

Additional features were:

- Redesigned incubation & detection blocks to accommodate the glass SPE cartridge and horizontal optical interrogation of the sample.
- Independently temperature controlled incubation blocks
- IP67 field portable housing

Figure 4-3: ColiSense 2 construction. Incubation and detection block and operational setup. A. Cork insulation, B. UV led, C. Heater connection, D. Photodiode, E. Data-logging, F. Power supply, G. IP67 enclosure, H. USB connection, I. Indication led, J. Triplicate sample chambers.
4.2.6 Filter system testing

4.2.6.1 Auto-fluorescence

To test for auto fluorescence of the Sterivex Filter cartridges, empty cartridges were irradiated with UV light at 361 nm. The fluorescence response was observed visually and photographed with a Canon PowerShot SD1100IS 8MP Digital Camera.

4.2.6.2 Filter flow rate

To compare the performance of membrane filtration against depth filtration for turbid samples, environmental samples with a range of recorded turbidity were passed through 0.45 µm membrane filters and 0.7 µm depth filters. The flow rate for each was recorded.

4.2.6.3 Filter trapping efficiency

Two sizes (12.5 mm and 16 mm diameter) of filter paper circles (0.7 µm porosity) were produced by cutting from a sheet using a paper punch. These were inserted into the SPE cartridges using a push-rod and fixed in place using a nitrile rubber O-ring.

The trapping efficiency of both setups was tested by passing an environmental sample through the filter in the SPE cartridge using vacuum filtration as shown in Figure 4-2. Vacuum pressure applied was 0.68 bar. The MPN of *E. coli* and Total Coliforms were measured in the raw sample and the filtrate. The ratio of MPN in the filtrate to MPN in the raw sample was expressed as a percentage to represent filter trapping efficiency.

4.2.7 Optical characterisation

Solutions of 0.5 mM 6-CMUG in 1mM Phosphate buffer at pH 6.9 were prepared in 3 glass SPE cartridges with bottom caps. The three vials were placed in channel A, B, C of the ColiSense 2 instrument, incubated at 44°C and fluorescence response was recorded. The cartridges were swapped to alternative channels so that each channel recorded the fluorescence response of 3 different cartridges. Each cartridge was spiked with known concentrations of 6-CMU to achieve a range of concentrations from 0.01 nM to 1400 nM. The response of each instrument channel was recorded for each concentration in triplicate.
4.2.8 Commercial GUD kinetics

Solutions of 0.5 mM 6-CMUG in 1mM Phosphate buffer at pH 6.9 were prepared in triplicate in glass SPE cartridges with bottom caps. Commercial GUS at a range of concentrations (0.02 to 0.42 ng.mL\(^{-1}\)) was inoculated the SPE cartridges. Three cartridges were placed in channel A, B, C of the ColiSense 2 instrument where they were incubated at 42\(^\circ\)C and fluorescence increase was recorded for 30 minutes at intervals of 10 seconds. This process was repeated for each GUS concentration in the range.

4.2.9 Environmental sample dilution series

Fresh water and salt water samples were collected from the river Tolka and the river Liffey estuary respectively, both in Dublin, Ireland using the sampling procedure outlined in section 2.3.3. *E. coli* (MPN) for each sample was determined using Colilert-18.

A range of trapped *E. coli* concentrations were achieved by filtering different volumes (100 mL to 2 mL) of sample through the ColiSense 2 glass SPE cartridges containing 0.7 \(\mu\)m glass fibre filter paper. This was carried out in triplicate. Each cartridge was the rinsed with de-ionised water to remove interferences such as salts from the filter paper, then the cartridges were capped.

2.5 mL of phosphate buffer at pH 6.9 containing 0.5 mM 6-CMUG was added to each SPE cartridge. The cartridges were then placed in the ColiSense 2 detection chambers where they were incubated at 44\(^\circ\)C and their fluorescence response was recorded for 30 minutes to determine GUS activity.
4.2.10 Field trial

The ColiSense 2 system was tested on the same field trial as the ColiSense 1 as detailed in section 3.2.8. This consisted of seven points along the Tolka River, Dublin, Ireland which were sampled and analysed for GUS activity on-site in 1 day. The ColiSense 2 system was powered from the 12V battery of the van used for transport, while a laptop was used to record data from the instrument. Further water quality parameters including *E. coli* MPN were as detailed in section 3.3.4.

Samples to be analysed by ColiSense 2 were prepared using a field sample filtration setup, consisting of a 100 mL filtration flask, manual vacuum pump, funnel and a graduated cylinder. The sample was taken from the source with the sampling jug and poured into the graduated cylinder to achieve a fixed volume of 100 mL. The sample was then poured into the glass SPE cartridge containing 0.7 µm glass fibre filter paper through the funnel and filtered while a vacuum of 0.68 bar was applied using a hand pump. 2.5 mL of phosphate buffer at pH 6.9 containing 0.5 mM 6-CMUG was added to each SPE cartridge. The cartridges were then placed in the ColiSense 2 detection chambers where they were incubated at 44°C and their fluorescence response was recorded for up to 30 minutes to determine GUS activity.
4.3 Results and discussion

4.3.1 Filter system tests

4.3.1.1 Auto fluorescence of membrane filter cartridges

Figure 4-4 shows the fluorescence response of Sterivex filter (Eastar™ Copolyester EB062) cartridges to excitation at 365 nm. Panel A shows an empty cartridge whereas Panel B shows a cartridge containing 10 µM of 6-CMU. Visibly auto-fluorescence has a significant effect so it was concluded that these filters were unsuitable for fluorescence detection.

Figure 4-4: Auto-fluorescence of Eastar™ Copolyester EB062, A, auto-fluorescence response of the Sterivex Microfilter to UV excitation at 365 nm. B, Fluorescence response of 6-CMU (10 µM) in a Sterivex Microfilter to UV excitation at 365 nm. C, White light image of the Sterivex Microfilter showing the 361 nm LED light source.
4.3.1.2 Filter flow rate

Figure 4-5 shows a comparison of flow rates between membrane filtration and depth filtration. Flow rates achieved for membrane filtration were lower than but comparable with those achieved by depth filtration for turbidity below 3 NTU. However above 3 NTU, flow stopped in the membrane filters while the depth filters continued to function at turbidity levels above 20 NTU. From this data it was concluded that Depth filtration is the preferable option for the pre-concentration of typical environmental samples.

Figure 4-5 Filtration rate Vs turbidity For a micro-pore filter and a glass fibre depth filter both 12.5 mm diameter. Conditions: Vacuum pressure = 0.68 Bar.
4.3.1.3 Filter trapping efficiency

The trapping efficiency of two different cut-out sizes of filter paper circles when placed in the SPE cartridge are shown in Table 4-1. The 12.5 mm cut-out had an efficiency of approximately 95% for TC and E. coli (i.e. 5% of the bacteria passed the filter). The 16 mm cut-out performed better than the 12.5, with 100% trapping of E. coli and above 99% trapping of TC.

Table 4-1: Filter trapping efficiency. Trapping efficiency is the ratio of the number of bacteria in the sample before filtration to the number in the filtrate expressed as a percentage.

<table>
<thead>
<tr>
<th>Size</th>
<th>Sample source</th>
<th>Sample MPN</th>
<th>Trapping efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TC</td>
<td>E.coli</td>
</tr>
<tr>
<td>12.5 mm</td>
<td>Tolka</td>
<td>&gt; 24916</td>
<td>93.30</td>
</tr>
<tr>
<td></td>
<td>Camac</td>
<td>&gt; 24916</td>
<td>95.17</td>
</tr>
<tr>
<td></td>
<td>Poolbeg</td>
<td>&gt; 24916</td>
<td>93.43</td>
</tr>
<tr>
<td>16 mm</td>
<td>Tolka</td>
<td>17329</td>
<td>99.99</td>
</tr>
<tr>
<td></td>
<td>Royal canal</td>
<td>1299.7</td>
<td>99.95</td>
</tr>
<tr>
<td></td>
<td>Grand canal</td>
<td>920.8</td>
<td>99.74</td>
</tr>
<tr>
<td>filter disk</td>
<td>Poolbeg</td>
<td>19863</td>
<td>99.98</td>
</tr>
</tbody>
</table>

4.3.2 Fluorescence detection system characterisation

It was discovered during early testing that the PTFE frit used to support the filter paper in the glass SPE cartridge caused contamination or a memory effect between measurements, i.e. the fritt would adsorb fluorophore from one assay and release it back into solution in the following assay. (Results not shown) To eliminate this problem, the PTFE frit was replaced with a disk of fine (0.5 mm) stainless steel mesh (grade 308). Results presented here were produced with the SS mesh setup. No memory effect was observed when using the SS mesh frits and using the cleaning protocol detailed in section 4.2.4.

Figure 4-6 shows the response of the 3 channels of ColiSense 2 for a range of concentrations of 6-CMU in 0.5 mM of 6-CMUG. The responses are linear to below 50
nM of 6-CMU with CV of triplicate measurements less than 5%. The slopes of the 3 channels vary from each other by up to 10%. This can be explained by engineering tolerances in the detection chamber and in electronic components. The system demonstrated a resolution of approximately 1 nM and an LOD below 50 nM.

Figure 4-6: ColiSense 2 fluorescence calibration. The response of ColiSense 2 channels A, B, and C with concentrations of 6-CMU up to 1,400 nM in pH 6.9 phosphate buffer with 500 µM 6-CMUG (n = 3). Error bars show SD of triplicate measurements. Channel A : (Y = 1.12X + 92) (R² = 1), Channel B : (Y = 1.02X + 84) (R² = 1), Channel C : (Y = 1.23X + 101) (R² = 1)
4.3.3 Commercial GUS kinetics

Figure 4-7 shows the enzyme activity recorded by ColiSense 2 for a range of commercial GUS concentrations inoculated into 0.5 mM 6-CMUG in pH 6.9 phosphate buffer. Triplicate measurements show CVs of less than 5%. The response is approximately linear across the range. Deviations from linearity at lower concentrations can be partly attributed to variations in GUS activity due to aging and inactivation of enzyme in samples. This occurred as a range of sample concentrations were prepared simultaneously but analysed sequentially. The system demonstrates the ability to detect activities of less than 100 pmol (6-CMU). min\(^{-1}\) 100 ml\(^{-1}\). This was a suitable level of precision for BWD levels of \(E.\) \(coli\) as discussed in section 3.1.

![Graph showing ColiSense 2 commercial GUS activity. Enzyme activity per 100 mL recorded by ColiSense 2 for concentrations of GUS up to 1 ng L\(^{-1}\) (n = 3). Error bars show SD of triplicate measurements. \((Y = 2195.7X + 92.88) (R^2 = 0.9844)\)
4.3.4 Environmental sample dilution series

A freshwater sample from the river Tolka had an *E. coli* MPN of 3,100 / 100 mL. By filtering different volumes as detailed in Figure 4-1, a range of concentrations between 200 / 100 mL and 3100 / 100 mL were achieved. Figure 4-8, shows the activities recorded for each concentration. A salt-water sample from the river Liffey estuary had an *E. coli* MPN of 7,200 / 100 mL. This was filtered to achieve a range of concentrations between 200 / 150 mL and 7,200 / 100 mL. Figure 4-8, shows the activities recorded for each concentration.

Both the Fresh-water and the Salt-Water responses show good linearity to concentrations below 1,000 MPN / 100 mL which is the range relevant to the BWD. The CV of triplicate measurements for each concentration was within 10% which is acceptable for an environmental microbiological measurement. The salt-water response shows a much higher (0.47 vs 0.003 pmol. min⁻¹. MPN⁻¹) GUS activity per MPN than the freshwater response. This may be due to the higher proportion of VBNC *E. coli* present in sea-water.

![Figure 4-8: ColiSense 2 environmental sample serial dilution. GUS activity recorded for various *E. coli* concentrations achieved by varying filtered volume. (n = 3) Error bars represent 1 standard deviation. Freshwater sample from Tolka river Dublin, ( Y = 0.047X + 2) (R² = 0.89), Brackish water sample from Poolbeg marina Dublin. (Y = 0.52X + 84) (R² = 0.99).](image-url)
4.3.5 Field trial

Total sample preparation time including the field filtration of triplicate samples, capping and addition of substrate buffer solution and placement into the ColiSense 2 detection chambers was found to take less than 10 minutes.

Figure 4-9 shows the progress curves for 3 replicates of a sample containing *E. coli* MPN of 14,136 / 100 mL. The progress curves were seen to fluctuate more than the progress curves recorded for the lysing method with ColiSense 1 and shown in Figure 3-9. A possible explanation for the increased fluctuation may be the diffusion of fluorophore from the filter paper containing trapped bacteria into the bulk solution. The slope of the progress curves is evident from 20 minutes or less. Thus with 10 minutes sample preparation and 20 minutes detection time a total time from sample to result of 30 minutes was achieved.

![Page Image](image.png)

Figure 4-9: ColiSense 2 progress curves for an environmental sample. The *E. coli* MPN of the sample was 14,136 / 100 mL. Channel A ( $Y = 10.435X + 193.68$) ($R^2 = 0.9925$), Channel B ( $Y = 11.746X + 86.822$) ($R^2 = 0.9976$), Channel C ( $Y = 10.766X + 78.973$) ($R^2 = 0.9981$).
Figure 4-10 shows GUS activity and *E. coli* MPN for each sample point in the field trial. No attempt is made here to correlate the 2 variables as this trial was intended solely to demonstrate the on-site use of the ColiSense 2 system. The results are visibly similar to those achieved with the ColiSense 1 lysing method (see Figure 3-11). Further field sampling programs were conducted with a larger number of samples to demonstrate the analytical performance of the instrument. These are detailed in Chapter 5.

Figure 4-10: ColiSense 2 field trial results. GUS data points are the average of three measurements recorded with ColiSense 2. Error bars represent 1 standard deviation (n = 3). *E. coli* MPN was recorded with Colilert 18. Error bars represent the upper and lower 95% confidence limits. Red line indicates the BWD (Good quality) level for *E. coli* in inland waters. [176].
4.4 Conclusion

ColiSense 2 is an upgraded version of ColiSense 1 which is modified to incorporate sample pre-concentration via depth filtration and fluorescence detection into one vessel thus reducing sample handling and by using a metabolism based assay, eliminated the lysing procedure used with ColiSense 1. This simplified sample handling and simplified assay protocol led to a reduction in sample to result time of 30 minutes compared with 75 minutes for ColiSense 1 using the lysing based method while maintaining similar analytical performance to the ColiSense 1. Both methods are trialled further in Chapter 5.
5 Field Evaluation of ColiSense 1 & 2
5.1 Introduction

The previous chapters detailed the design construction and characterisation of the ColiSense 1 and 2 systems for *E. coli* detection. This chapter details a study conducted on 5 rivers in the Dublin area to provide a large range of samples to validate the 2 systems against standard methods.

There are 5 main rivers draining Dublin city. The Dodder, Poddle and Camac all join the Liffey before entering Dublin Bay through the port, while the Tolka enters the bay north of the port. These rivers contribute in varying amounts to pollution (including faecal) entering Dublin bay and the subsequent contamination of designated bathing areas: Dollymount, Sandymount, Merrion and Seapoint. Table 5-1 shows the annual water quality classifications of each bathing area from 2012 to 2015.

Table 5-1: Dublin Bay bathing waters quality Classification 2014-2015, [168], [184].

<table>
<thead>
<tr>
<th>Bathing area</th>
<th>2012</th>
<th>2013</th>
<th>2014</th>
<th>2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dollymount</td>
<td>Sufficient</td>
<td>Good</td>
<td>Good</td>
<td>Sufficient</td>
</tr>
<tr>
<td>Sandymount</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
<td>Sufficient</td>
</tr>
<tr>
<td>Merrion</td>
<td>Sufficient</td>
<td>Sufficient</td>
<td>Sufficient</td>
<td>Poor</td>
</tr>
<tr>
<td>Seapoint</td>
<td>Good</td>
<td>Good</td>
<td>Excellent</td>
<td>Excellent</td>
</tr>
</tbody>
</table>

Dublin City has a network of combined sewers which carry wastewater and rainfall run-off from industrial and residential areas to treatment plants along the coast. The largest of these plants is at Poolbeg at the entrance to Dublin Port. During periods of heavy rainfall the combined sewer network is subject to overflows (CSO) which enter the rivers in the Dublin area and then proceed to contaminate coastal bathing areas [181].

The location of most of these overflows are known as they were designed into the drainage network, however some are unknown due to evolution of the network over more than 100 years and the loss of historical records [185]. The sewerage treatment plants also overflow during high rainfall but the effects of this are not investigated here as the outflows go directly to coastal waters. This study is concerned only with freshwater within the city.
5.1.1 Aims of Chapter 5: Field evaluation of ColiSense 1 & 2

The aim of this chapter is to evaluate the performance of the ColiSense 1 and 2 systems in real-world conditions by conducting a study on the waterbodies in the Dublin region and to compare their performance with methods published in the literature.

Objectives include

- Sampling water from 5 different rivers in the Dublin region over the course of 1 month
- Monitoring weather patterns and river flows during the sampling period
- Recording a range of physical and chemical water quality parameters which could interfere with or provide background information to GUS activity measurements.
- Microbial analysis of the water samples including standard methods and the rapid enzymatic methods based on ColiSense 1 and 2.
- Comparison of enzymatic assay results with those from published literature to evaluate the performance of ColiSense 1 and 2
5.2 Materials and methods

5.2.1 Chemicals and reagents

As detailed in sections 3.2.1 and 4.2.1.

5.2.2 Field sampling

In August 2015 each of 5 rivers in the Dublin urban area were sampled on separate days. In addition on each sampling day the most downstream point on each river was sampled. Sampling locations are shown on the map in Figure 5-1. The exact location of these points and a site description are provided in Table 5-2 and the sampling schedule is shown in Table 5-3. Samples were taken at each location using the procedure detailed in section 2.3.3. These were placed on ice, returned to the laboratory and analysed for GUS activity and microbiology, TSS and nutrients within 12 hours. Supplementary data including pH, temperature, turbidity, conductivity were measured on-site using a Hydrolabs DS5x multi-parameter sonde.

Figure 5-1: Map of Dublin rivers showing sampling points plus designated bathing areas in Dublin Bay.
### Table 5-2: Sample point description and coordinates.

<table>
<thead>
<tr>
<th>River</th>
<th>Point</th>
<th>Site name</th>
<th>Latitude, Longitude</th>
<th>Site description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolka</td>
<td>T1</td>
<td>Griffith Pk</td>
<td>53.36994, -6.261958</td>
<td>Fast moving through an urban park with a weir just above the tidal range.</td>
</tr>
<tr>
<td>Tolka</td>
<td>T2</td>
<td>Addison Pk</td>
<td>53.375535, -6.277723</td>
<td>Flows along the boundary of Glasnevin cemetery.</td>
</tr>
<tr>
<td>Tolka</td>
<td>T3</td>
<td>Tolka valley Pk</td>
<td>53.376288, -6.301376</td>
<td>Urban park below a large residential area. Red pond treating the Finglas Stream overflows into the Tolka downstream from the sampling point.</td>
</tr>
<tr>
<td>Tolka</td>
<td>T4</td>
<td>Scribblestown</td>
<td>53.377822, -6.333357</td>
<td>Semi rural site, close to the disused Dunsink landfill.</td>
</tr>
<tr>
<td>Tolka</td>
<td>T5</td>
<td>MSO bridge</td>
<td>53.384682, -6.291392</td>
<td>Slow moving section of river flowing from an industrial and residential area.</td>
</tr>
<tr>
<td>Camac</td>
<td>C1</td>
<td>Bow bridge</td>
<td>53.345254, -6.296498</td>
<td>Fast moving channelised section of river below St James’s hospital.</td>
</tr>
<tr>
<td>Camac</td>
<td>C2</td>
<td>Turvey ave</td>
<td>53.341583, -6.311097</td>
<td>Fast flowing section, Urban park in the center of Inchicore, an old and densely populated area of Dublin.</td>
</tr>
<tr>
<td>Camac</td>
<td>C3</td>
<td>Lansdown Valley Pk</td>
<td>53.383901, -6.37249</td>
<td>Slow moving section through an urban park, leading from an industrial area.</td>
</tr>
<tr>
<td>Camac</td>
<td>C4</td>
<td>Sheldon Pk</td>
<td>53.329875, -6.344725</td>
<td>Fast flowing channelised section through an industrial estate.</td>
</tr>
<tr>
<td>Camac</td>
<td>C5</td>
<td>Corkagh Pk</td>
<td>53.317540, -6.449056</td>
<td>Large urban park with the river flowing from a large green area where the river rises in a pond.</td>
</tr>
<tr>
<td>Dodder</td>
<td>D1</td>
<td>Herbert PK</td>
<td>53.326927, -6.231309</td>
<td>Fast moving section, just above the tidal range through a residential and commercial area.</td>
</tr>
<tr>
<td>Dodder</td>
<td>D2</td>
<td>Bankside cottages</td>
<td>53.306641, -6.48405</td>
<td>Fast moving section, through an urban park downstream from the Milltown Golf club.</td>
</tr>
<tr>
<td>Dodder</td>
<td>D3</td>
<td>Orwell bridge</td>
<td>53.305775, -6.266949</td>
<td>Slow moving tree-lined section through the residential area of Rathfarnam.</td>
</tr>
<tr>
<td>Dodder</td>
<td>D4</td>
<td>Bushy Pk</td>
<td>53.300729, -6.287725</td>
<td>Fast moving section, through a wooded urban park.</td>
</tr>
<tr>
<td>Dodder</td>
<td>D5</td>
<td>Mount Carmel Pk</td>
<td>53.288566, -6.332494</td>
<td>Slow moving section in a sub-urban park just above Bulrothery weir.</td>
</tr>
<tr>
<td>Dodder</td>
<td>D6</td>
<td>Old Bawn Bridge</td>
<td>53.276015, -6.355558</td>
<td>Fast moving section of river flowing from the Dublin mountains and the Bohernabreena reservoirs.</td>
</tr>
<tr>
<td>Liffey</td>
<td>L1</td>
<td>Eastlink bridge</td>
<td>53.346416, -6.272732</td>
<td>Tidal, upstream from Dublin port. Opposite bank from the confluence of the Dodder river with the Liffey and the entrance to the Grand Canal basin.</td>
</tr>
<tr>
<td>Liffey</td>
<td>L2</td>
<td>Milenium Bridge</td>
<td>53.346038, -6.280649</td>
<td>Tidal section in the urbanised center of Dublin. Downstream from the confluence of the Poddle and the Liffey.</td>
</tr>
<tr>
<td>Liffey</td>
<td>L3</td>
<td>Heuston bridge</td>
<td>53.347241, -6.290690</td>
<td>Tidal section in the urbanised center of Dublin. Downstream from the confluence of the Camac and the Liffey.</td>
</tr>
<tr>
<td>Liffey</td>
<td>L4</td>
<td>Rowing club</td>
<td>53.346106, -6.19743</td>
<td>Slow moving freshwater section, just above the Islandbridge weir which marks the tidal limit of the river. Opposite the Phoenix park.</td>
</tr>
<tr>
<td>Liffey</td>
<td>L5</td>
<td>Strawberry hall</td>
<td>53.361959, -6.385509</td>
<td>Slow moving section through tree-lined valley between the sub-urban residential areas of Blanchardstown and Lucan.</td>
</tr>
<tr>
<td>Poddle</td>
<td>P1</td>
<td>Gandon Close</td>
<td>53.332543, -6.280536</td>
<td>Fast flowing channelised section, bordering Mt Jerome Cemetery. From here the river goes underground until it reaches the Liffey.</td>
</tr>
<tr>
<td>Poddle</td>
<td>P2</td>
<td>Mount argus</td>
<td>53.332089, -6.28763</td>
<td>Slow moving section through an urban park with a retention pond for flood control.</td>
</tr>
<tr>
<td>Poddle</td>
<td>P3</td>
<td>Poddle Pk</td>
<td>53.314166, -6.28000</td>
<td>Fast flowing channelised section through a small park in a densely populated area.</td>
</tr>
<tr>
<td>Poddle</td>
<td>P4</td>
<td>Thymon Pk</td>
<td>53.304352, -6.321929</td>
<td>Slow section flowing out from the large Thymon Park where the river rises in Thymon Lake [a pond].</td>
</tr>
</tbody>
</table>
Table 5-3: Dublin river study sampling schedule.

<table>
<thead>
<tr>
<th>Date</th>
<th>Sampling points</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-Aug-15</td>
<td>L1 D1 P1 C1 T1 T2 T3 T4 T5</td>
</tr>
<tr>
<td>19-Aug-15</td>
<td>L1 D1 P1 C1 T1 C2 C3 C4 C5</td>
</tr>
<tr>
<td>21-Aug-15</td>
<td>L1 D1 P1 C1 T1 D2 D3 D4 D5 D6</td>
</tr>
<tr>
<td>24-Aug-15</td>
<td>L1 D1 P1 C1 T1 P2 P3 P4</td>
</tr>
<tr>
<td>29-Aug-15</td>
<td>L1 D1 P1 C1 T1 L2 L3 L4 L5</td>
</tr>
</tbody>
</table>

5.2.3 Physical and chemical water quality

Nutrients: Phosphate, nitrate and nitrite were measured using a Hach DR900 Nutrient Analyser. Nitrate was measured using the chromotropic acid test, N Tube method. Nitrite was measured using the diazotization test, N tube method and phosphate was measured using the molybdovanadate test, N tube method. TSS was recorded by filtration drying and weighing of samples according to Standard Method 2540 D [186].

5.2.4 Microbiological analysis

Gus activity was measured in the laboratory using 4 different methods:

1. Lysing method as detailed in section 3.2.7 with filtered volume fixed at 50 mL, measured on ColiSense 1.
2. Raw water Gus activity as detailed in section 3.2.9 measured on ColiSense 1.
3. Extra cellular Gus activity as detailed in section 3.2.9, measured on ColiSense 1.
4. Metabolic Gus activity, detailed in section 4.2.9 with filtered volume fixed at 50 mL, measured on ColiSense 2.

Extra-cellular GUS activity was not measured on Day 5 for operational reasons. For all GUS results (n=3). E. coli MPN and total coliforms MPN were recorded using Colilert 18. Enterococci MPN was recorded using Enterolert.

Microbiological data from point 1 on each river was averaged over the 5 sampling days and used to rank the 5 rivers in order of faecal pollution load. The same data was graphed against time, to show temporal variation in the pollution load of each river. Microbiological data from each sampling point on each river was graphed longitudinally to determine the location of faecal pollution sources along each river.
5.2.5 Rainfall and river level

Hourly average rainfall measurements were collected from the Irish Meteorological Service (Met Éireann) from three meteorological stations in Dublin area: Dublin Airport (53°25'40" N, 6°14'27" W, 71 m above sea level), Casement Aerodrome (53°18'20" N, 6°26'20" W, 94 m above sea level) and Phoenix Park (53°21''50" N, 6°20'00" W, 48 m above mean sea level). Hourly average sunshine data was available only from the Dublin Airport station.

Water level data for the Camac, Dodder, Poddle and Tolka rivers was obtained from Dublin City Council (DCC) who maintain a network of hydrometric stations in the Dublin region. No level data was obtained for the Liffey river. In order to compare rivers to each other water level in meters was normalised to percentage change in water level, relative to the maximum recorded for each river for the month of August.
5.3 Results and discussion

5.3.1 Rainfall and river flow

The first half of August 2015 in Dublin was dry and river levels were low. On the 19th there was a heavy but short rainfall event, and then on the 25th there was a heavy and sustained rainfall event followed by a period of generally wet weather. Daily totals for rainfall and sunshine hours and average temperature are shown in Table 5-4.

Table 5-4: Weather conditions during sampling period. (Recorded at Dublin Airport)

<table>
<thead>
<tr>
<th>Date</th>
<th>Rainfall (mm)</th>
<th>Temperature (°C)</th>
<th>Sunshine (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>13.9</td>
<td>8.7</td>
</tr>
<tr>
<td>2</td>
<td>5.8</td>
<td>14.2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>16.1</td>
<td>8.6</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>15.1</td>
<td>3.7</td>
</tr>
<tr>
<td>5</td>
<td>5.1</td>
<td>14.4</td>
<td>0.1</td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
<td>13.7</td>
<td>4.5</td>
</tr>
<tr>
<td>7</td>
<td>0.4</td>
<td>13.3</td>
<td>2.6</td>
</tr>
<tr>
<td>8</td>
<td>0.4</td>
<td>14.1</td>
<td>6.5</td>
</tr>
<tr>
<td>9</td>
<td>0.5</td>
<td>16.4</td>
<td>2.4</td>
</tr>
<tr>
<td>10</td>
<td>0.1</td>
<td>15.6</td>
<td>5.3</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>13.3</td>
<td>8</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>13.0</td>
<td>12.7</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>13.4</td>
<td>11.8</td>
</tr>
<tr>
<td>14</td>
<td>0.3</td>
<td>13.0</td>
<td>1.3</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>11.7</td>
<td>3.1</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>14.0</td>
<td>4.3</td>
</tr>
<tr>
<td>17</td>
<td>0.1</td>
<td>13.7</td>
<td>12</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>16.0</td>
<td>7.6</td>
</tr>
<tr>
<td>19</td>
<td>11.4</td>
<td>15.5</td>
<td>3.4</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>16.5</td>
<td>2.8</td>
</tr>
<tr>
<td>21</td>
<td>0.1</td>
<td>17.8</td>
<td>6.3</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>14.1</td>
<td>1.1</td>
</tr>
<tr>
<td>23</td>
<td>35.8</td>
<td>13.2</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0.1</td>
<td>13.5</td>
<td>5.2</td>
</tr>
<tr>
<td>25</td>
<td>10</td>
<td>11.5</td>
<td>0.7</td>
</tr>
<tr>
<td>26</td>
<td>7.5</td>
<td>13.7</td>
<td>6.9</td>
</tr>
<tr>
<td>27</td>
<td>1.9</td>
<td>13.0</td>
<td>8.6</td>
</tr>
<tr>
<td>28</td>
<td>2.4</td>
<td>14.1</td>
<td>8.6</td>
</tr>
<tr>
<td>29</td>
<td>6.3</td>
<td>14.0</td>
<td>4.4</td>
</tr>
<tr>
<td>30</td>
<td>0.1</td>
<td>12.7</td>
<td>3.7</td>
</tr>
<tr>
<td>31</td>
<td>11</td>
<td>11.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>
River levels in Dublin were low during the dry period at the beginning of the month but rose sharply in response to the rainfall events from the 19th onwards. These events are shown as percentage change in river level in Figure 5-2.

![River Levels Graph](image)

Figure 5-2: River levels during sampling period. Relative change in water height on 4 Dublin rivers involved in the study. No data for the river Liffey was recorded.

### 5.3.2 Physical and chemical water quality

A range of physical and chemical water parameters were measured to provide background to the microbiological data. The results of these measurements are tabulated in Table 5-5.
### Sensing platform design for Faecal indicator bacterial detection in recreational waters

**Endan Heery – September 2018**

Table 5-5: Physical and chemical water quality.

<table>
<thead>
<tr>
<th>Date</th>
<th>Sample Point</th>
<th>E. coli</th>
<th>Enterococci TSS (mg L⁻¹)</th>
<th>Turbidity (NTU)</th>
<th>Temp (°C)</th>
<th>SpCond (μS cm⁻¹)</th>
<th>Salinity (ppt)</th>
<th>pH</th>
<th>DO (mg mL⁻¹)</th>
<th>Nitrate (mg L⁻¹ N-NO₃⁻)</th>
<th>Nitrite (mg L⁻¹ N-N₂O₅⁻)</th>
<th>Phosphate (mg L⁻¹ P-P₂O₅⁻)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27/5/2016</td>
<td>11</td>
<td>222</td>
<td>37.20</td>
<td>14.08</td>
<td>15.14</td>
<td>235</td>
<td>0.11</td>
<td>48.95</td>
<td>0.15</td>
<td>1.40</td>
<td>0.00</td>
<td>0.15</td>
</tr>
<tr>
<td>28/5/2016</td>
<td>11</td>
<td>222</td>
<td>37.20</td>
<td>14.08</td>
<td>15.14</td>
<td>235</td>
<td>0.11</td>
<td>48.95</td>
<td>0.15</td>
<td>1.40</td>
<td>0.00</td>
<td>0.15</td>
</tr>
<tr>
<td>29/5/2016</td>
<td>11</td>
<td>222</td>
<td>37.20</td>
<td>14.08</td>
<td>15.14</td>
<td>235</td>
<td>0.11</td>
<td>48.95</td>
<td>0.15</td>
<td>1.40</td>
<td>0.00</td>
<td>0.15</td>
</tr>
<tr>
<td>30/5/2016</td>
<td>11</td>
<td>222</td>
<td>37.20</td>
<td>14.08</td>
<td>15.14</td>
<td>235</td>
<td>0.11</td>
<td>48.95</td>
<td>0.15</td>
<td>1.40</td>
<td>0.00</td>
<td>0.15</td>
</tr>
<tr>
<td>31/5/2016</td>
<td>11</td>
<td>222</td>
<td>37.20</td>
<td>14.08</td>
<td>15.14</td>
<td>235</td>
<td>0.11</td>
<td>48.95</td>
<td>0.15</td>
<td>1.40</td>
<td>0.00</td>
<td>0.15</td>
</tr>
</tbody>
</table>
5.3.3 Microbiological water quality

5.3.3.1 Average faecal pollution load

*E. coli* and Enterococci levels for each of the waterbodies in the Dublin region were well above the levels permitted by the bathing water standards. Table 5-6 shows the 5 day averages of the most downstream point of each river. The Camac river had the highest levels of faecal pollution for both *E. coli* and Enterococci MPN with averages of 7,433 and 3,506 MPN /100 mL respectively. None of the rivers are designated bathing areas so from a regulatory point of view, this is not of concern. However it can be expected that they contribute to the contamination of bathing areas on the coast.

Table 5-6: Dublin rivers 5 day averages of *E. coli*, Enterococci and GUS levels

<table>
<thead>
<tr>
<th>Sample source</th>
<th><em>E. coli</em> CFU 100 mL⁻¹</th>
<th>Enterococci CFU 100 mL⁻¹</th>
<th>Raw water GUS pMol min⁻¹ 100 mL⁻¹</th>
<th>Extracellular GUS pMol min⁻¹ 100 mL⁻¹</th>
<th>Lysed cell GUS pMol min⁻¹ 100 mL⁻¹</th>
<th>Metabolic GUS pMol min⁻¹ 100 mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camac</td>
<td>7433</td>
<td>3506</td>
<td>3506</td>
<td>851</td>
<td>51.76</td>
<td>8.29</td>
</tr>
<tr>
<td>Poddle</td>
<td>4091</td>
<td>2226</td>
<td>2526</td>
<td>587</td>
<td>54.14</td>
<td>5.71</td>
</tr>
<tr>
<td>Tolka</td>
<td>3960</td>
<td>1482</td>
<td>1535</td>
<td>194</td>
<td>43.13</td>
<td>3.43</td>
</tr>
<tr>
<td>Dodder</td>
<td>3180</td>
<td>2526</td>
<td>2226</td>
<td>727</td>
<td>32.60</td>
<td>23.13</td>
</tr>
<tr>
<td>Liffey</td>
<td>2085</td>
<td>1535</td>
<td>1482</td>
<td>227</td>
<td>24.72</td>
<td>7.85</td>
</tr>
</tbody>
</table>

5.3.3.2 Time studies on waterbodies

The FI load of each river varied widely over the sampling period. Figure 5-3 shows the temporal variation in *E. coli* and Enterococci levels plus GUS activities measured by 4 different methods for the lowest sampling point (e.g. T1, L1…) each day on each of the 5 Dublin rivers during the sampling period. During the dry weather at the beginning of the sampling period the FI levels were relatively low. In the case of the Dodder and the Liffey the levels were below the BWD standards. The rainfall event on the 19th August and the subsequent larger rainfall event beginning on the 25th coincided with a large increase in FI load in each of the rivers. The GUS based methods also showed increased activity during and after the rainfall events. It can be concluded from this, that rainfall is a significant factor in determining the FI loads of the rivers in Dublin, probably through the mechanism of combined sewer overflows.
5.3.3.3 Spatial study on 5 Dublin waterbodies

FI loading also varied widely along the course of each of the Dublin rivers. Figure 5-4 shows the spatial variation in *E. coli* and Enterococci levels plus GUS activities measured by 4 different methods for the each sampling point (e.g. T1, T2…) on each of the 5 rivers on alternate days during the sampling period. This study allowed the tracing of point sources of faecal pollution as was achieved in a similar study on the Seine drainage network in Paris [187] and as modelled for the Scheldt drainage network in Belgium [188]. The highest recorded *E. coli*, TC and Enterococci levels were at P2 on the Poddle and C4 on the Camac. In both these cases the next upstream points P3 and C5 respectively showed much lower levels. The pollution source for the Camac could thus be located somewhere between C4 and C5 (a distance of 3.8 kilometers) and for the Poddle between P2 and P3 (a distance of 1.1 km). Further sampling within these points would allow the precise location of the sources within a rapid timeframe. The high FI load at C4 on the Camac was reduced at points C3, C2 and C1 this demonstrates the natural remediation of rivers due to dilution and sedimentation of faecal pollution. The high faecal loading on the Camac River appears to be due to a sewerage over flow just upstream from the Sheldon Park sampling point. It was not possible to determine the exact source but it was possible to say that this was a persistent problem due to the high levels of contamination recorded in the Camac over the whole sampling period.
Figure 5-3: Time study of Dublin rivers. *E. coli* and Enterococci MPN are single Colilert 18 and Enterolert measurements. (Associated confidence intervals are omitted here for clarity). GUS activity measurements are in triplicate. (Error bars omitted for clarity. CVs for each method are Metabolic CV 5.7%, Lysing CV 8.1%, Extracellular CV 6.5%, Raw water CV 5.4%)
Figure 5-4: Pollution tracing studies on 5 Dublin rivers. *E. coli* and Enterococci MPN are single Colilert 18 and Enterolert measurements. (Associated confidence intervals are omitted here for clarity). GUS activity measurements are in triplicate. (Error bars omitted for clarity. CVs for each method are Metabolic CV 5.7%, Lysing CV 8.1%, Extracellular CV 6.5%, Raw water CV 5.4%)
5.3.4 Comparison with standard methods

5.3.4.1 Log-log relationships

To validate the GUS based methods it was necessary to show a relationship between their results and those from the standard methods. The data from the 5 rivers were aggregated and regression analysis performed against the *E.coli* and Enterococci MPN methods. Only freshwater samples were included (Liffey points 1 to 3 excluded), in the regressions as GUS activities have been shown previously to be significantly higher in saline waters.

Figure 5-5 shows log-log regressions for each of the 4 GUS methods against *E.coli* and Enterococci MPN. The raw water method (n = 38) and the extracellular method (n = 20) showed weak relationships with the standard methods. In Figure 5-3 it is clear that extracellular GUS makes up a large portion of the activity of raw water. E.g. for the Camac river (panel A) on the 24th Aug the Raw water GUS activity was 60, Extracellular GUS activity was 40, while the metabolic GUS method, which removes the extra-cellular enzyme, had an activity of 20 ppm. min\(^{-1}\). 100 mL\(^{-1}\). In this example extra-cellular GUS made up two-thirds of the total sample activity. The standard MPN methods neglect extra-cellular enzyme thus the relationships are weak.

The lysing based method (n = 38) showed a correlation with *E. coli* MPN of \((R^2 = 0.56)\) and strong correlation with Enterococci MPN \((R^2 = 0.75)\). The metabolic GUS method (n = 36) showed a poor correlation with *E. coli* MPN \((R2 = 0.35)\) (Pearson) and good correlation with Enterococci MPN \((R2 = 0.64)\). In each case the slope of the line is less than 1. This was addressed by Garcia and Servais [189] who concluded that at lower *E. coli* concentrations in natural waters there are a higher proportion of VBNC cells than at higher concentrations. This means that at higher concentrations (typically shortly after a pollution event) the GUS activity is mainly due to VC cells, thus enzymatic methods can accurately represent the number of cells present. However at lower concentrations the activity contribution of VBNC cells is higher. This is detected by the enzymatic methods but not by the MPN methods, thus the relationship deteriorates at lower concentrations.

Enterococci are more durable than *E. coli* in environmental waters and enter a VBNC state later [190]. This explains the stronger correlation between the GUS methods and the Enterococci MPN than for the *E. coli* MPN.
Figure 5-5: Log-log relationships GUS activity vs MPN standard methods. GUS activity measured using 4 different methods and E. coli and Enterococci MPN. Time study of Dublin rivers. E. coli and Enterococci MPN are single Colilert18 and Enterolert measurements. (Associated confidence intervals are omitted here for clarity). GUS activity measurements are in triplicate. (Error bars omitted for clarity. Average CVs for each method are Metabolic CV 5.7%, Lysing CV 8.1%, Extracellular CV 6.5%, Raw water CV 5.4%)
### 5.3.4.2 Intra sample variability

Each data point shown in Figure 5-5 is the average of triplicate measurements of a single sample. To visualise the variability of measurements within a single sample the CV of each of the data points was plotted Vs average activity for each of 4 GUS methods. The results are shown in Figure 5-6. The lowest average CV was for the raw water method at 5.4% followed by the metabolic method with 5.7%, the extra cellular method with 6.5% and the lysing method average with 8.1%. The raw water method is probably the lowest due to the simplicity of the assay (i.e. a single sample handling step). The lysing method has numerous sample handling steps which can each contribute to variability. The average values reported here compare well with those from a previous study which reported approximately 15% variability for enzymatic methods [178].

![Figure 5-6](image)

**Figure 5-6**: Coefficient of variation of GUS detection methods. (CV %) determined by triplicate samples for each data point for each of 4 GUS detection methods. Metabolic method average CV 5.7% (n = 45), Lysing method average CV 8.1% (n = 45), Extra cellular average CV 6.5% (n = 24), Raw water average CV 5.4% (n = 45). The red line in each panel highlights the 15% CV deemed acceptable for enzyme assays by Lebaraon [178].
5.3.4.1 Performance and cost

The performance of an assay can be judged on a number of measures including it’s time to result, number of sample handling steps, equipment requirement and cost per measurement. Table 5-7 summarises the performance of a number of commercially available methods and some methods being developed including ColiSense (developed in this work) and ColiPlage (developed by Veolia.)

The cost per sample of an enzyme assay is significantly lower than the cost of a culture based MPN method while the time to result is reduced less than 1 hour. The ColiSense 1 method with lysing offers good analytical performance (as shown in Table 5-8) with a total on-site sampling and analysis time of 75 minutes. The ColiSense 2 method offers similar analytical performance to the ColiPlage method for a similar cost and with the same analysis time. It has the advantage however of performing the analysis on site using low cost equipment (ColiSense 2, detailed in Chapter 4) whereas the ColiPlage method uses standard laboratory based instrumentation. Additionally the ColiSense 2 method has only 2 sampling handling steps (filtration and substrate addition) due to the use of a continuous assay whereas the ColiPlage method which uses a discontinuous assay requires aliquot removal at regular intervals during the analysis.

Table 5-7: ColiSense comparison with commercial methods. *includes sample retrieval, assay conducted on-site.

<table>
<thead>
<tr>
<th>Method name</th>
<th>Supplier</th>
<th>Target</th>
<th>Principle</th>
<th>Approximate Cost/sample</th>
<th>Equipment</th>
<th>Sample handling steps</th>
<th>Time to result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petrifilm</td>
<td>3M</td>
<td>E. coli</td>
<td>Colony counting</td>
<td>€1</td>
<td>Incubator</td>
<td>1</td>
<td>21 h</td>
</tr>
<tr>
<td>MUG/EC Microplates</td>
<td>BioRad</td>
<td>E. coli</td>
<td>Miniaturised MPN</td>
<td>€12</td>
<td>Incubator, UV lamp</td>
<td>1</td>
<td>24 h</td>
</tr>
<tr>
<td>ColiPlate</td>
<td>Blue Water</td>
<td>E. coli</td>
<td>Miniaturised MPN</td>
<td>€10</td>
<td>Incubator, UV lamp</td>
<td>1</td>
<td>24 h</td>
</tr>
<tr>
<td>ColiLert 1B</td>
<td>IDEXX</td>
<td>E. coli</td>
<td>MPN</td>
<td>€7</td>
<td>Incubator, UV lamp</td>
<td>1</td>
<td>18 h</td>
</tr>
<tr>
<td>Enterolert</td>
<td>IDEXX</td>
<td>Enterococci</td>
<td>MPN</td>
<td>€7</td>
<td>Incubator, UV lamp</td>
<td>1</td>
<td>24 h</td>
</tr>
<tr>
<td>-</td>
<td>DelAqua</td>
<td>E. coli</td>
<td>Microfiltration</td>
<td>&lt;€0.5</td>
<td>Field filtration unit, field incubator</td>
<td>2</td>
<td>24 h</td>
</tr>
<tr>
<td>ColiPlage</td>
<td>Not available</td>
<td>E. coli</td>
<td>Enzyme assay</td>
<td>&lt;€2</td>
<td>Lab filtration unit, Incubator, lab fluorimeter</td>
<td>8</td>
<td>30 min</td>
</tr>
<tr>
<td>ColiSense 1</td>
<td>Not available</td>
<td>E. coli</td>
<td>Enzyme assay with lysing</td>
<td>&lt;€2</td>
<td>ColiSense 1 instrument</td>
<td>4</td>
<td>75 min*</td>
</tr>
<tr>
<td>ColiSense 2</td>
<td>Not available</td>
<td>E. coli</td>
<td>Metabolism based enzyme assy</td>
<td>&lt;€1</td>
<td>ColiSense 2 instrument</td>
<td>2</td>
<td>30 min*</td>
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</table>
Table 5-8: Comparison of 4 GUS activity detection methods with literature sources.

<table>
<thead>
<tr>
<th>Method name</th>
<th>Analyte</th>
<th>Water type</th>
<th>Sample fraction</th>
<th>Equipment used</th>
<th>Reagents used</th>
<th>Assay time</th>
<th>Assay type</th>
<th>Analysis location</th>
<th>Reference method</th>
<th>Slope</th>
<th>$r^2$</th>
<th>Pearson</th>
<th>n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS1 Raw water</td>
<td>GUS</td>
<td>River</td>
<td>Raw water</td>
<td>Collisense 1</td>
<td>6-CMUG</td>
<td>30 min*</td>
<td>Continuous</td>
<td>Laboratory</td>
<td>Collett 18</td>
<td>0.26</td>
<td>0.45</td>
<td>0.67</td>
<td>38</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Entrolert</td>
<td></td>
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<td>0.27</td>
<td>0.46</td>
<td>0.66</td>
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<td>CS1 Extra-cellular</td>
<td>GUS</td>
<td>River</td>
<td>Filtrate</td>
<td>Collisense 1</td>
<td>6-CMUG</td>
<td>30 min*</td>
<td>Continuous</td>
<td>Laboratory</td>
<td>Collett 18</td>
<td>0.31</td>
<td>0.36</td>
<td>0.61</td>
<td>20</td>
<td>This study</td>
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<td>0.3</td>
<td>0.48</td>
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<td>River</td>
<td>Trapped lysed cells</td>
<td>Collisense 1</td>
<td>6-CMUG</td>
<td>75 min*</td>
<td>Continuous</td>
<td>Laboratory</td>
<td>Collett 18</td>
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<td>0.56</td>
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<td>38</td>
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<td>River</td>
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<td>Collisense 2</td>
<td>6-CMUG</td>
<td>30 min*</td>
<td>Continuous</td>
<td>Laboratory</td>
<td>Collett 18</td>
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<td>0.35</td>
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<td>0.64</td>
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<td>Lysed cells</td>
<td>Collisense 1</td>
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<td>1.2</td>
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<td>0.73</td>
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<td>Brackish</td>
<td>Trapped intact cells</td>
<td>Collisense 2</td>
<td>6-CMUG</td>
<td>30 min*</td>
<td>Continuous</td>
<td>On-site</td>
<td>Collett 18</td>
<td>0.37</td>
<td>0.13</td>
<td>0.36</td>
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<td>This study</td>
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<td>Laboratory</td>
<td>Chromocult agar</td>
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<td>GUS</td>
<td>Sea</td>
<td>Trapped lysed cells</td>
<td>Laboratory fluorometer</td>
<td>4-MUG, NaOH</td>
<td>30 min</td>
<td>Discontinuous</td>
<td>Laboratory</td>
<td>MPN microplates</td>
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<td>0.81</td>
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<td>256</td>
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<tr>
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<td>GUS</td>
<td>River &amp; Waste</td>
<td>Trapped lysed cells</td>
<td>Laboratory fluorometer</td>
<td>4-MUG, NaOH</td>
<td>30 min</td>
<td>Discontinuous</td>
<td>Laboratory</td>
<td>MPN microplates</td>
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<td>0.76</td>
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<td>166</td>
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<td>ColiPlage</td>
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<td>River &amp; Waste</td>
<td>Trapped lysed cells</td>
<td>Laboratory fluorometer</td>
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<td>Discontinuous</td>
<td>Laboratory</td>
<td>MPN microplates</td>
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<td>River &amp; Waste</td>
<td>Trapped lysed cells</td>
<td>Laboratory fluorometer</td>
<td>4-MUG, NaOH</td>
<td>30 min</td>
<td>Discontinuous</td>
<td>Laboratory</td>
<td>DVC-FISH</td>
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<td>0.99</td>
<td>-</td>
<td>41</td>
<td>Garcia (2005)</td>
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</tr>
<tr>
<td>GUS</td>
<td>Sea</td>
<td>Trapped lysed cells</td>
<td>Laboratory fluorometer</td>
<td>4-MUG, NaOH</td>
<td>4h</td>
<td>Discontinuous</td>
<td>Laboratory</td>
<td>IFA</td>
<td>0.26</td>
<td>0.44</td>
<td>-</td>
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<td>Caruso (2002)</td>
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</tr>
<tr>
<td>GUS</td>
<td>River</td>
<td>Trapped lysed cells</td>
<td>Laboratory fluorometer</td>
<td>4-MUG, NaOH</td>
<td>30 min</td>
<td>Discontinuous</td>
<td>Laboratory</td>
<td>Chromocult agar</td>
<td>0.83</td>
<td>0.9</td>
<td>-</td>
<td>98</td>
<td>Farnleitner (2001)</td>
<td></td>
</tr>
</tbody>
</table>

* Includes total sample handling time.
5.4 Conclusion
This field study on 5 of Dublin’s rivers was conducted for dual purposes:

1. to provide a wide range of samples to test the operation of ColiSense 1 & 2, and
2. to investigate the origins of faecal pollution in Dublin Waterbodies.

The rivers included were The Liffey, The Dodder, The Camac, The Poddle and The Tolka. Different sample fractions were analysed to measure 4 aspects of GUS activity: extracellular enzyme, raw water, lysed cells and metabolism. A range of supplementary water quality parameters including E. coli, TC and Enterococci MPN were also recorded at each location.

The River Camac was identified as the most highly polluted waterbody. This was most likely due to a major pollution source which was traced to within a 3.8 km section of the river. A second significant pollution source was located on the Poddle river to within a 1.1 km stretch of river.

Of the 4 GUS methods evaluated it was concluded that the raw water method was simple, highly portable and suitable for rapid pollution tracing studies. CS2 using the metabolic assay was suitable for quantitative field studies due to its portability and accuracy and reduced sample handling while CS1 using the lysing method was most suitable for rapid laboratory analysis.
6 CULTURE BASED ASSAYS FOR E. coli & ENTEROCOCCI
6.1 Introduction
Assays for faecal indicators typically require a specific step to differentiate the target bacteria from similar species and matrix interference [12], [13]. The previous chapters 3, 4, 5 focused on rapid enzyme assays for *E. coli* which are possible due to the specificity of β-Glucuronidase to *E. coli* and the suitability of GUS for rapid assays. No such specific enzyme exists for Enterococci which is regarded as a better faecal indicator than *E. coli* [191], thus a selective culture step is required in Enterococci assays.

6.1.1 Culture media
Solid or liquid media containing food sources and nutrients allowing the multiplication of bacteria are used to increase bacterial numbers to detectable levels. In the case of microfiltration, filters are placed on a solid medium and colonies form around trapped VC bacteria, whereas in MPN methods VC cells multiply in liquid media to indicate presence within a particular aliquot. In both cases fluorogenic or chromogenic substrate can be added to the media to increase signal and to improve selectivity [192]. A typical growth curve for bacteria in a medium is shown in Figure 6-1.

![Bacterial growth curve](image)

Figure 6-1: Bacterial growth curve [193]
The Lag phase is where cells inoculated into a media become acclimatised and prepare for growth. Within this there are two distinct lag phases, Lag1 where nutrient accumulation takes place but no biomass growth occurs, Lag 2 where biomass grows but no cell division occurs. These combined can range from 2 hours to 7 hours depending on growth media composition and initial state of inoculated cells [194], [195].

The Log phase follows the lag phase and consists of an exponential increase in cell population through duplication. Growth rate is dependent on temperature, pH and nutrient availability. *E. coli* has a generation time of approximately 20 minutes in ideal conditions (e.g. LB Broth) [62], [196], [197] whereas Enterococci has a generation time of approximately 30 minutes [198].

The stationary phase and death phase of the growth curve are where nutrient scarcity first slows the exponential growth of cells until it matches the rate of cells dying off, and then further so that cell die-off exceeds cell growth and the population declines. Typical ingredients of a growth media for coliform bacteria are shown in Table 6-1. Ingredients can be varied to improve different facets of the growth response.

### Table 6-1: Growth media ingredients [30], [74], [199], [200].

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Content</th>
<th>Purpose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>&lt;= 15%</td>
<td>nutrient and vitamin source</td>
<td>Berg (2000)</td>
</tr>
<tr>
<td>Enzyme inducer (IPTG / Met-Glu)</td>
<td>&lt;= 2%</td>
<td>Induce the metabolism of substrate</td>
<td>Berg (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(e.g. Galactoside or Glucuronide)</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>&lt;= 40%</td>
<td>Maintain isotonicity</td>
<td>Berg (2000)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>&lt;= 25%</td>
<td>Provide energy for cell growth</td>
<td>Berg (2000)</td>
</tr>
<tr>
<td>Bile salts</td>
<td>&lt;= 10%</td>
<td>Selective agent</td>
<td>Berg (2000)</td>
</tr>
<tr>
<td>Detergent (Triton X) (Polymixin B)</td>
<td>&lt;= 4%</td>
<td>Permeabilise cells to increase</td>
<td>Van Poucke (2000)</td>
</tr>
<tr>
<td>Substrate (4-MUG / 4-MUGal)</td>
<td>&lt;= 2%</td>
<td>Produce a detectable response when</td>
<td>Berg (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>metabolised</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>trace</td>
<td>Chelate metal ions which may inhibit</td>
<td>Hughes (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fluorescence / metabolism</td>
<td></td>
</tr>
<tr>
<td>Antibiotics</td>
<td>trace</td>
<td>Selective agent</td>
<td>Edberg (1986)</td>
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<td>Phenazine methosulphate &amp; ascorbate</td>
<td>trace</td>
<td>Aids cellular transport</td>
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</table>
6.1.2 Detection methods

Culture based assays are interrogated in various ways including optically and electrically. Visible colour change or fluorescence are by far the most common methods and are used in standard methods such as Petri-Film and Colilert. Assay performance and reliability can be improved by the use of instruments to measure the response rather than the human eye [68], [201]. When using an instrument to interrogate a culture based assay, a number of detection methodologies can be used, these include Time To Detect (TTD), dilute to specification and miniaturised MPN.

6.1.2.1 Time to detect principle

Time to detect is based on the principle that a sample inoculation with a high starting concentration of cells will reach a threshold concentration in less time than a sample with a lower initial concentration. This principle was successfully used in the ColiFast system for the quantification of total coliforms using Galactosidase activity [151].

6.1.2.2 Dilute to specification assay principle

Dilute to specification is a detection principle which utilises presence/absence tests to indicate if a concentration is above or below a certain threshold. E.g. if the threshold required is 100 VC *E.coli* per 100 mL, that equals 1 VC *E.coli* per 1 mL. Thus 1 mL of the sample is introduced to a growth medium containing substrate. If the test is positive the sample fails, if it is negative the sample passes. Foti validated this method for detection and semi-quantitative determination of *E.coli* in foods [202]. The technology has been commercialised by Biolumix under the trade-name Soleris by Neogen. The *E.coli* Compartment Bag Test (CBT) from Aquagenx operates on a similar principle [63], [203].

The dilute to specification method is based on 2 assumptions: target bacteria are distributed randomly throughout the sample, and that 1 VC target cell in a vial will multiply and give a positive result. These 2 assumptions apply to any MPN based method, so are widely accepted. However the first is a major issue here because of the small sample volume and the non-ideal nature of environmental samples. The main advantage of this method is that it allows for small sample volumes and minimal sample handling. I.e. 0.8 mL for *E. coli* and 2 mL for Enterococci inoculated with growth media in a single
step. Sample handling involves the accurate addition of sample volumes to vials. A pre-concentration step is not required.

It is proposed here that in monitoring for compliance with BWD directives, quantitative accuracy is not critical; rather that identification of exceedances of the threshold levels is key. Table 6-2 shows the BWD standards for *E. coli* and Enterococci and also the associated sample volumes theoretically containing 1 CFU. It is also proposed that a dilute to specification assay using 6-CMUG as a substrate can be designed to work on the ColiSense 1 system taking advantage of the triplicate measurement capability.

Table 6-2: BWD, volumes probably containing 1 CFU.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Excellent Coastal</th>
<th>Good Coastal</th>
<th>Excellent Inland</th>
<th>Good Inland</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>250 CFU/100 mL</td>
<td>500 CFU/100 mL</td>
<td>500 CFU/100 mL</td>
<td>1000 CFU/100 mL</td>
</tr>
<tr>
<td></td>
<td>1 CFU/0.4 mL</td>
<td>1 CFU/0.2 mL</td>
<td>1 CFU/0.2 mL</td>
<td>1 CFU/0.1 mL</td>
</tr>
<tr>
<td><em>Enterococci</em></td>
<td>100 CFU/100 mL</td>
<td>200 CFU/100 mL</td>
<td>200 CFU/100 mL</td>
<td>400 CFU/100 mL</td>
</tr>
<tr>
<td></td>
<td>1 CFU/1 mL</td>
<td>1 CFU/0.5 mL</td>
<td>1 CFU/0.5 mL</td>
<td>1 CFU/0.25 mL</td>
</tr>
</tbody>
</table>

**6.1.2.3 Miniature MPN principle**

MPN techniques involve dividing a sample into multiple aliquots and in some cases serial dilutions. Each aliquot is tested for the presence of 1 or more target bacteria and a result generated through statistical analysis [204]. Colilert 18 and Enterolert from IDEXX are recognized MPN methods [205], [206]. They both sample 100 mL of water and give results with high precision. Miniaturized MPN method based on 96 well-plates have been developed for the detection of Enterococci using β-Glucosidase activity [207]. While well plates are not suitable for sensitive enzyme assays due to fluorophore adsorption as shown in section 0, they are fine for MPN assays as these are based on presence / absence (P/A) measurements which rely on an overwhelming fluorescence signal to signify a positive response. In this case enough fluorophore is present to overcome the adsorption effect.

It is proposed that to monitor for compliance with BWD directives, the level of precision offered by the above methods is not required. This allows the sample volume to be reduced thus simplifying incorporation into a field portable device. Also with the use of 6-CMUG as a substrate, detection time can be reduced from 24 hours to < 12 hours.
6.1.3 Aims of Chapter 6: Culture based assays for *E. coli* and Enterococci

The aim of this chapter is to investigate the feasibility of implementing culture based assays for *E. coli* and Enterococci on the ColiSense 1 or a similar system involving incubation and fluorescence detection.

Objectives include:

- Determine variability within samples at the volumes relevant to ColiSense 1
- Demonstrate the use of fluorescent culture based assays on ColiSense 1
- Evaluate the Time To Detect (TTD) principle for *E. coli* assays
- Evaluate the Dilute To Specification principle for *E. coli* assays
- Evaluate a miniaturised MPN method for *E. coli* assays
6.2 Materials and methods

6.2.1 Chemicals and reagents

Fluorogenic substrate 6-chloro-4-methylumbelliferone- β-D glucuronide (6-CMUG) was obtained from (GlycoSynth, UK). Colilert 18 and 3M Petri-Film for E.coli were obtained from TechnoPath Ireland. E.coli ATCC 11775 from certified reference materials was obtained from Sigma Aldrich. Luria broth (LB), Terrific Broth (TB) and Super Optimal Broth (SOB) growth media were obtained from Sigma Aldrich. Greiner 655209 black polypropylene 96 well plates and Nunc 232702 polyolefin well plate sealing foils were obtained from Sigma Aldrich.

6.2.2 Test for variability within a sample

An environmental water sample was obtained from the Transitional Liffey River at Poolbeg marina, Dublin using the sampling method detailed in section 2.3.3. 0.8 mL aliquots of sample were plated 10 times onto 3M PetriFilm. A 10 fold dilution of the sample was prepared by dilution with DI water. 0.8 mL aliquots of the diluted sample were also plated 10 times onto 3M PetriFilm. E.coli CRMs at 250 CFU/100 mL, and 500 CFU/100 mL were prepared in DI water. These were also plated 10 times onto 3M PetriFilm at 0.8 mL aliquots of sample. The plates were incubated at 44 °C for 21 h and counted.

6.2.3 Growth media in ColiSense

The ColiSense system was tested to establish its ability to record the fluorescence response of culture based assays using Colilert 18 and then using various media incorporating 6-CMUG as a substrate.
6.2.3.1 Colilert media

Colilert 18 growth medium with substrate was added to 3 vials. These were inoculated with known concentrations of \textit{E.coli} positive fresh water sample. Fluorescence responses were recorded over a 15 h period.

6.2.3.2 Alternative growth media

Three growth media (LB, TB and SOB) were tested investigate the speed of response of the assay. 6-CMUG at 100 µM was added. Each vial was inoculated with a known concentration of \textit{E.coli} positive fresh water sample Fluorescence responses were recorded over a 15 h period.

6.2.3.3 Growth media with \textit{E. coli} CRM

Three sample vials were filled with 1.5 mL of LB containing 6-CMUG at 200 µM. Vials A and B were inoculated with concentrations of \textit{E.coli} ATCC 11775 50 and 150 CFU per 2 mL sample vial respectively. Vial C was inoculated with a blank containing no bacteria. Fluorescence responses were recorded over a 24 h period.

6.2.3.4 Growth media concentration

Three different dilutions of growth medium were tested. 0.25, 0.5, 1.0 mL / 2 mL. 6-CMUG at 100 µM was added. Each vial was inoculated with a known concentration of \textit{E.coli} positive fresh water sample. Fluorescence responses were recorded over a 15 h period.

6.2.4 Time to detect TTD

6.2.4.1 TTD theoretical model

A mathematical model of \textit{E. coli} multiplication in a 2 mL sample vial in ideal growth conditions was implemented using a duplication time of 20 minutes and neglected a lag phase. The following equation predicts the number of cells generated at a particular time since inoculation
\[
\text{Number of cells} = \text{inoculation at time zero} \times 2^{\left( \frac{\text{time in minutes}}{20} \right)}
\]

The predicted growth curves for initial inoculations of 0, 1, 10, 100 and 1,000 cells were plotted and the time for each of these to cross an arbitrary threshold level of 10 million cells were calculated.

### 6.2.4.2 TTD of environmental samples

Sample vials of 2 mL volume were filled with 1.5 mL of sterile LB broth containing 6-CMUG at 200 µM. The Vials were inoculated with environmental fresh-water samples containing known concentrations of \textit{E. coli} (0 to 1,500 CFU 100 mL\textsuperscript{-1}). The vials were placed in ColiSense 1 which incubated them and recorded their fluorescence responses over a 15 h period. A fluorescence level of 500 ADC units was chosen as a detection threshold. The time for each vial to reach this level was recorded.

### 6.2.5 Dilute to specification assay

Figure 6-2 shows a 3-vial method for the semi-quantification of \textit{E. coli} designed to meet the BWD thresholds. This method consisted of one vial containing 0.4 mL of sample and two vials containing 0.2 mL of sample. A total volume of 0.8 mL was sampled. The 3 vials also contained growth medium and fluorescent substrate. This allowed the thresholds 250 CFU/100 mL and 500 CFU/100 mL both to be tested in duplicate. The vials were incubated and fluorescence monitored. After the incubation period the vials were either positive or negative. A positive vial indicated that 1 or more VC \textit{E. coli} was present in the initial sample volume. From the number of positive wells an MPN was calculated. Table 6-3 shows a truth table for determining the initial sample concentration using the results of the 3-vial test.
Sensing platform design for Faecal indicator bacterial detection in recreational waters

Figure 6-2: 3-vial method for *E. coli*

Table 6-3: Truth table for semi-quantitative 3-vial MPN method. MPN = Number of positive tubes / sqrt(volume in negative tubes)[207].

| A (0.4 mL) | B (0.2 mL) | C (0.2 mL) | Positive tubes | Negative tubes | Volume in positive tubes (mL) | Volume in negative tubes (mL) | MPN /mL | MPN /100 mL | Classification | Status
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0.8</td>
<td>0.2</td>
<td>6.71</td>
<td>671</td>
<td>&gt;500 CFU/100 mL</td>
<td>Fail</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0.6</td>
<td>0.2</td>
<td>4.47</td>
<td>447</td>
<td>250&gt; &lt;= 500 CFU/100 mL</td>
<td>Good</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0.6</td>
<td>0.2</td>
<td>4.47</td>
<td>447</td>
<td>&lt;= 500 CFU/100 mL</td>
<td>Excellent</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0.4</td>
<td>0.4</td>
<td>3.16</td>
<td>316</td>
<td>&lt;= 250 CFU/100 mL</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0.4</td>
<td>0.4</td>
<td>1.58</td>
<td>158</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0.2</td>
<td>0.6</td>
<td>1.29</td>
<td>129</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0.2</td>
<td>0.6</td>
<td>1.29</td>
<td>129</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0.8</td>
<td>0.00</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*E. coli* CRMs were prepared in DI water to concentrations of 100, 200, 250, 300, 400, 450, 500, 550, 600, 700, 800 and 1000 CFU/ 100 ML. These were each plated onto 3M Petrifilm in triplicate at 1 mL volumes to verify concentrations. Plates were incubated at 44°C for 21 h and counted.

To increase sample throughput, 96 well-plates were used instead of the 3 vials as shown above. This allowed for multiple samples to be analysed simultaneously. The plates were
prepared with LB growth media and 6-CMUG in each well. The plates were divided to perform the 3-Vial test 8 times for each *E.coli* concentration. A single 3-Vial test was represented by 4 wells each inoculated with 0.2 mL of the prepared concentration. 2 wells represented vial A (combined volume = 0.4 mL), 1 well represented Vial B and 1 well represented Vial C. This was repeated 8 times giving a total of 32 wells inoculated with 0.2 mL for each concentration of *E.coli*.

Plates were sealed to prevent evaporation and incubated at 44°C for 24 h. They were then photographed under a long-wave (365 nm) UV lamp and the positive wells were enumerated. A total of 96 tests were conducted over 12 concentrations ranging from 100 CFU /100 mL to 1000 CFU/ 100 mL.

### 6.2.6 Miniature MPN assay

As detailed in section 6.2.5, 12 concentrations of *E.coli* CRMs were prepared and inoculated in 32 wells of 96-well-plates at a volume of 0.2 mL /well. A most probable number for each *E.coli* concentration was calculated using the following equation and each MPN was plotted against the concentration measured by the Petrifilm method as detailed previously. A mathematical model based on the formula below was generated to select the optimum number of wells of the 96-wellplate to use for the MPN calculation.

$$E.\, coli\, MPN = \left(\frac{-2.303}{V}\right) \times \log \left(\frac{S}{n}\right)$$

Where: ‘v’ is sample volume, ‘n’ is number of samples, ‘s’ is number of sterile samples. [208]
6.3 Results and discussion

6.3.1 Sample variability

Intra-sample variability is a known problem in environmental microbiology and is particularly an issue for FI assays at low sample volumes such as those used for Petrifilm (1 mL) [135] and that proposed for the dilute to specification assay (0.8 mL) as detailed in section 6.2.5.

Results of 10 replicate samples (0.8 mL) of 2 separate \textit{E. coli} CRM concentrations and 2 dilutions of a marine water sample are shown in Table 6-4. The PetriFilm showed large variability for each sample concentration: CV = 61\% for \textit{E.coli} CRM 250 CFU/100 mL, CV = 77\% for \textit{E.coli} CRM 500 CFU/100 mL and CV = 48\% for Marine water with 812 CFU/100 mL. The results of the 1/10 dilution of the marine sample were disregarded as the concentration recorded 50 CFU/100 mL was below the LOD of the Petrifilm method (100 CFU/100 mL).
Table 6-4: Sample variability at 0.8 ml sample volume on PetriFilm *E. coli* plates.

<table>
<thead>
<tr>
<th>Nominal</th>
<th>E. coli CRM Standards (0.8 mL sample volume)</th>
<th>Marine water (0.8 mL sample volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>250 CFU / 100 mL</td>
<td>500 CFU / 100 mL</td>
</tr>
<tr>
<td>Plate 1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Plate 2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Plate 3</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Plate 4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Plate 5</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Plate 6</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Plate 7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Plate 8</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Plate 9</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Plate 10</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Average / 0.8 mL</td>
<td>1.5</td>
<td>4.0</td>
</tr>
<tr>
<td>STD / 0.8 mL</td>
<td>0.9</td>
<td>3.1</td>
</tr>
<tr>
<td>CFU / 100 mL</td>
<td>187.5</td>
<td>500.0</td>
</tr>
<tr>
<td>STD /100 mL</td>
<td>115.2</td>
<td>387.3</td>
</tr>
<tr>
<td>CV %</td>
<td>61.5</td>
<td>77.5</td>
</tr>
<tr>
<td>Lower 95%</td>
<td>-38.4</td>
<td>-259.1</td>
</tr>
<tr>
<td>Upper 95%</td>
<td>413.4</td>
<td>1259.1</td>
</tr>
<tr>
<td>Lower 90%</td>
<td>-1.5</td>
<td>-135.2</td>
</tr>
<tr>
<td>Upper 90%</td>
<td>376.5</td>
<td>1135.2</td>
</tr>
</tbody>
</table>

These results indicate that any microbiological assay which uses a small sample volume such as 1 or 2 mL will suffer from poor repeatability due to the non-monodisperse nature of the *E. coli* in real samples.
6.3.2 Growth media in ColiSense 1

ColiSense 1 was shown to detect the fluorescence response of Colilert 18 medium. Figure 6-3.A shows the response to 3 dilutions (1/2, full and double strength) of Colilert 18 media inoculated with freshwater sample. Each dilution of the media produced a weak fluorescence response (25 ADC units, full scale range of the ColiSense is 2047 ADC units) beginning at 7 hours. The ½ strength dilution created the highest fluorescence response while the double strength produced the lowest response. However, the samples were visibly yellow and visibly fluorescent when illuminated with UV light.

Colilert 18 is a dual function growth media with a colorimetric and fluorescent response. The colorimetric response is produced by 4-nitrophenol which absorbs maximally at 405 nm. The fluorescence response is produced by 4-MU which is excited at 365 nm and emits at 445 nm. It is likely that the 4-N in the assay absorbs a large proportion of the 365 nm excitation and 445 emission light from the fluorophore thus reducing the fluorescence response of the media. This effect is contributed to by the current design of the ColiSense instrument which requires the excitation light to pass through a 1 cm path-length. In a highly absorbent media such as Colilert 18 this would cause significant reduction in signal. The Colilert 18 Quanti-trays are designed to be visually inspected using epi-illumination whereby the sample is illuminated and inspected through the same face. This minimises the path-length for the excitation and emitted light thus maximising the fluorescent response.

To adapt the ColiSense system to media such as Colilert 18 the system could be re-designed to use fluorescence inspection from the side of the vial rather than through the bottom as was implemented. This would reduce the absorbance effects of the Colilert media, thus improving the fluorescence response.
Figure 6-3: ColiSense 1 response to Colilert 18 media. Panel A, Colilert 18 standard media in 3 concentrations inoculated with 0.5 mL of freshwater sample containing 5400 CFU (E. coli)/100 mL. Panel B, a Colilert 18 quanti-tray showing positive wells with yellow colour.

Figure 6-4: ColiSense 1 response using different growth media. Panel A, 3 different broths each containing 200 µM 6-CMug inoculated with 0.5 mL of freshwater sample containing 2000 CFU (E. coli)/100 mL. Panel B, Different dilutions of Luria broth per 2 mL vial, the remaining volume was made up of DI water and 0.5 mL of freshwater sample containing 2000 CFU (E. coli)/100 mL.

Optically clear growth media containing fluorescent substrates were shown to give greater responses than the coloured Colilert 18 media. Figure 6-4(a) shows the response of the ColiSense to 3 different growth media (Luria Broth, Terrific Broth and Sub Optimal...
Broth) each containing 6-CMUG at 200 µM and inoculated with freshwater sample. The 3 responses are similar with *E. coli* multiplication visible between 8 and 10 hours of incubation. Luria broth is slightly faster than the others.

Figure 6-4(b) shows the response to 3 dilutions of LB broth each containing 6-CMUG at 200 µM and inoculated with freshwater sample. Each positive vial was identified at approximately 8 hours. The dilution of the LB had a large influence on the fluorescence response with the weakest dilution giving the strongest fluorescent response. This indicates that the optical density of the growth media is the critical factor in obtaining a strong fluorescence response.
6.3.3 Time to detect

6.3.3.1 Mathematical model of *E. coli* growth

The mathematical model of *E. coli* population growth from different starting concentrations shown in Figure 6-5 demonstrates the principle of TTD. The model is based on a generation time of 20 minutes and neglects a lag phase. The results show approximately 1 hour time difference in crossing a threshold level for each order of magnitude of concentration.

When applied in a 2 mL sample vial as used in ColiSense 1, assuming the volume of an *E. coli* cell to be 0.7 µM³ [209], there can be a theoretical maximum of $3.10^{12}$ *E. coli* cells in the test-cell. The maximum achievable *E. coli* cell density in LB broth is an OD$_{600}$ of 7 (i.e. $5.10^9$ cells mL$^{-1}$) but steady-state growth (Log phase) finishes at an OD$_{600}$ of 0.3 (i.e. $2.4.10^8$ cells mL$^{-1}$) [210]. Thus the cell population at the end of the log phase will be $4.8.10^8$ *E. coli* cells. An arbitrary threshold level of $1.10^8$ *E. coli* cells was used to calculate detection times. Figure 6-5(b) shows the relationship between initial concentration and the time for each concentration to cross the threshold level. This is a linear relationship with a TTD (hours) = -1.14(Log$_{10}$ (initial cell concentration)) +8.9.

Figure 6-5: Time to detect (TTD) principle. Panel A, Theoretical growth of *E. coli* from different starting concentrations. Panel B, Regression of time to cross a threshold level (10 million cells) Vs log$_{10}$ of initial number of cells. (Y = -1.149X + 8.91) (R$^2$ = 0.99)
6.3.3.2 TTD with environmental samples

In the real-life ColiSense system the threshold level was set at a measured fluorescence intensity of 500 ADC units. The fluorescence threshold represents an arbitrary *E. coli* concentration as in the mathematical model. Here the fluorescence is generated via the metabolism of 6-CMUG substrate by the multiplying *E. coli* cells. Figure 6-6 (a) shows results obtained on ColiSense for 3 different inoculation concentrations entered into each sample chamber each containing Luria broth and 200 µM 6-CMUG. The results show clear differentiation in the time domain between different starting concentrations. Baseline fluorescence was due to pre hydrolysed substrate introduced to the sample.

![Graph](image)

Figure 6-6: Time to detect method on ColiSense 1, Panel A, Example response of ColiSense 1 with different *E. coli* concentrations inoculated into each sample chamber each containing Luria broth and 200 µM 6-CMug. Panel B, Regression of detection time Vs Log10 of *E. coli* concentration. (Y = -1.7X +13.7) ( R² = 0.75)

Figure 6-6 (b) shows further results from 10 individual samples with a range of starting concentrations from 50 CFU/100 mL to 1500 CFU/100 mL. The relationship between TTD and the log of the initial concentration is linear as for the mathematical model. This is represented by the equation TTD (hours) = -1.72(Log (initial concentration)) +13.7. The constant term in this relationship is greater (by 4.8 hours) than that in the mathematical model. This is due to the lag phase of *E.coli* growth which was neglected in the model but is visible in the real samples plus the arbitrary nature of the thresholds used in the mathematical model and the environmental sample analysis.
These results indicate that TTD may be a useful detection methodology. With improvements in the ColiSense detection sensitivity and using a high fluorescent yield substrate the threshold level for detection could be reduced, thus making ‘Rapid’ detection possible. Growth media optimisation is key to this. E.g. in this experiment LB broth was used which gave the fastest response. This may not be the optimum however as it contains dextrose which is good for cell growth but prevents cells from metabolising 6-CMUG until the dextrose has been exhausted. An ideal broth would allow rapid cell growth while promoting the metabolism of the substrate. New substrates for Enterococci Glucosidase detection such as 6-Chloro-4-methylumbelliferyl β-D-glucopyranoside are now becoming available so this may also soon be feasible [165].

6.3.4 Dilute to specification

The ColiSense system has shown the ability to differentiate between positive and negative samples using growth based fluorescence assays. Figure 6-7 (a) shows an example response from ColiSense to an environmental sample with *E.coli* concentration of 360 CFU/100mL inoculated in 3 vials as shown in Figure 6-2 (0.4ml in A, 0.2 mL in B, 0.2 mL in C). After a period of 12 hours, vial A and vial B recorded positive responses while vial C recorded a negative response. Using the truth table for the 3-vial method in Table 6-3 an MPN of 447 E.coli /100 mL was calculated. This sample was thus classified as ‘Good’.

Figure 6-7 (b) shows a 96 well plate containing 6-CMUG and growth media and inoculated with various concentrations of *E.coli* to perform replicates of the 3-vial method. The plate displays positive and negative wells which represent positive and negative vials.
Figure 6-7: Dilute to specification response. Panel A, ColiSense 1 response to 2 positive and 1 negative inoculations (from a 360 CFU $100\text{ mL}^{-1}$ environmental sample) into Luria broth each containing 200 µM 6-CMug. Panel B, 96-well-plate displaying fluorescence positive and negative wells. Each well contained Luria broth with 200 µM 6-CMug and was inoculated with 0.2 mL of \textit{E.coli} ATCC 17755 Vitroid diluted in phosphate buffer pH 6.9. Photograph taken under UV (365 nm). The solid red rectangle highlights a single test consisting of 4 wells which all returned negative results. The dashed red rectangles highlight a further 7 tests at the same concentration.

The results of 4 separate 94 well plates each containing 3 different concentrations \textit{E.coli} inoculated into 32 wells each are shown in Figure 6-8. Each concentration is represented by a different colour with coloured wells representing positive responses and white wells representing negative responses. A single test is represented by 4 wells e.g. in plate 1 the wells A1, A2, A3, and A4 represent a single test where all the wells were negative.
Figure 6-8: Well-plate setup for dilute to specification and mini-MPN. Each well contained 0.2 mL of LB media with 200 µM 6-CMUG. Each well was inoculated with 0.2 mL of sample containing a known concentration of E. coli as indicated by the colour code (e.g. Plate 1, wells A1-A4 were inoculated with 0.2 mL of sample containing 100 CFU / 100 mL E. coli) Results are included in this figure (i.e white wells signify no fluorescence response). Red outline shows a single test consisting of 4 wells at a concentration 100 CFU / 100 mL.
The results of each single test were compared with the truth table in Table 6-3 and a classification of ‘Excellent’ (below 250 CFU / 100 mL), ‘Good’ (250 CFU / 100 mL and 500 CFU / 100 mL) or ‘Fail’ (above 500 CFU / 100 mL) was assigned to each result. These classifications of each test result are shown in Figure 6-9 graphed against the nominal E.coli concentrations at which they occurred. The ‘Excellent’ results (26 occurrences) were grouped at the lower concentrations, the ‘Good’ results (33 occurrences) appeared across the whole range but more towards the mid-range concentrations while the ‘Fail’ results (37 occurrences) appeared across the whole spectrum with a bias towards the higher concentrations.

- Correct classification is defined as where the nominal sample concentration falls within the limits of the classification. (e.g. 6 samples of 700 CFU /100 mL gave results in the fail classification).
- False positive is defined as when a ‘Fail’ is recorded for a sample with a nominal concentration within the ‘Good’ or ‘Excellent’ limits, or a ‘Good’ is recorded for a sample with a nominal concentration within the ‘Excellent’ limits.
- False negative is defined as when an ‘Excellent’ is recorded for a sample with a nominal concentration within the ‘Good’ or ‘Fail’ limits, or a ‘Excellent’ is recorded for a sample with a nominal concentration within the ‘Good’ limits.

Figure 6-9: Dilute to spec, distribution of classification occurrences for a range of E. coli concentrations showing correct, false positive and false negative classifications.

The percentages of correct, false positive and false negative classifications by the TTD method are shown in Table 6-5. The performance of this method is poor as can be seen
by the low percentages of correct classifications. This is most likely due to variability due to the small sample volume as discussed in section 6.3.1. It can be seen however from Figure 6-9 panel C that the 1000 CFU/100 mL sample was classified correctly 8 out of 8 times while the 700 and 800 CFU/100 mL samples were classified correctly 12 out of 16 times. Thus it can be concluded that confidence in results grow with increased sample concentration. When combined with a rapid culture based assay the method may be useful as an early warning of high levels of contamination but it is not useful for quantitative analysis.

Table 6-5: Reliability of dilute to specification classifications.

<table>
<thead>
<tr>
<th>Status</th>
<th>Classification</th>
<th>Correct classifications</th>
<th>False negative</th>
<th>False positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fail</td>
<td>&gt;5000cfu/100 mL</td>
<td>24</td>
<td>0</td>
<td>13</td>
<td>37</td>
</tr>
<tr>
<td>Good</td>
<td>250&gt; = 500cfu/100 mL</td>
<td>11</td>
<td>15</td>
<td>7</td>
<td>33</td>
</tr>
<tr>
<td>Excellent</td>
<td>&lt;= 250 cfu/100 mL</td>
<td>14</td>
<td>12</td>
<td>0</td>
<td>26</td>
</tr>
</tbody>
</table>

6.3.5 Miniature MPN

As shown in Figure 6-7, well plates are suitable for carrying out repeated presence absence tests so thus are suitable for use in MPN measurements. Figure 6-10, shows the maximum and minimum measurable MPN values per number of wells used on a 96 well plate with a sample volume of 200 µL. Well volume is typically 400 µL but half of this is reserved for reagents. These MPN values were calculated using the equation detailed in section 6.2.6.
Figure 6-10: MPN detection limit for 96 well plate. Maximum MPNs and resolution measurable dependant on number of wells used of a 96 well-plate, calculated using MPN = -2.303/v*log(s/n), Where: ‘v’ is sample volume (0.2 mL), ‘n’ is number of samples, ‘s’ is number of sterile samples [208].

Using fewer wells to perform an MPN means less reagent use, less waste and less financial cost. However this comes at a cost of lower resolution and range. Rapid methods for FI indicator warning however do not require the precision and the range offered by standard methods such as Colilert 18. For recreational waters, the measurement range of interest is between 200 MPN and 1000 MPN and a resolution of 100 cells or less is required to differentiate between threshold levels.

The mathematical model of maximum measureable MPN and resolution per number of wells used of a 96-well plate shown in Figure 6-10 is based on a sample volume of 0.2 mL per well. For 4 wells, the measurable limit of approximately 700 is too low and the resolution is above 100 cells. For 8 wells the upper limit is 1,000 and the resolution is below 50 cells. With increasing numbers of cells used the range and resolution continue to improve but with diminishing returns. An optimum number would be between 8 and 32 wells, [211], [212].

ColiPlate™ 96 well plates for E. coli quantification from Bluewater Biosciences, Canada cost approximately €10 per test using a single 96-wellplate. If the MPN measurement can
be carried out using only 16 wells, then 6 measurements can be conducted on each plate, reducing the cost per test to below €2.

Figure 6-11: Mini MPN performance. Correlation between MPN results and nominal \( E.\text{coli} \) concentration. Panel A, 4 wells MPN (n = 8) data points are the average of 8 readings, Error bars represent 1 standard deviation, \( (Y = 0.56\times + 549.04) \) (\( R^2 = 0.87 \)). Panel B, 8 wells MPN (n = 4) data points are the average of 4 readings, Error bars represent 1 standard deviation, \( (Y = 1\times + 98) \) (\( R^2 = 0.74 \)). Panel C, 16 wells MPN (n = 2) data points are the average of 2 readings, Error bars represent 1 standard deviation, \( (Y = 1.3\times + 4) \) (\( R^2 = 0.89 \)). Panel D, 32 wells MPN (n=1), \( (Y = 1.55\times -87) \) (\( R^2 = 0.86 \)).

6.4 Conclusions
This chapter examined methods for rapid field portable culture based assays for \( E.\text{coli} \) and Enterococci. The fluorescent substrate 6-CMUG which offers higher fluorescence
yields at neutral pH than 4-MUG [138] and other commonly used substrates was incorporated into growth media and tested in 3 different detection strategies. These included Time To Detect (TTD), Dilute to Specification both using the ColiSense 1 and miniaturised MPN using 96 well plates. These assays were each specific to E. Coli but were intended to demonstrate the feasibility of their application to Enterococci detection by using a selective medium and a 6-Chloro-Methylumbelliferyl based glucosidase substrate, a selection of which are now available [165].

In each strategy results were obtained in 14 hours or less. This was somewhat faster than the standard 18h for Colilert or 21hr for Petrifilm but ‘Rapid’ (i.e. sub 4 hr) detection was not achieved.

The dilute to specification method worked poorly for E. coli due to high sample variability. It has not been tested for Enterococci but is expected to perform better due to the larger sample volume required. The miniaturized MPN method gave promising results but it was tested with only a small number of replicates, so results are inconclusive. The TTD principle was demonstrated successfully but the sample number was too low to draw definitive conclusions on its reliability.

The typical cost per test of common culture based methods were outlined in section 5.3.4.1 with a miniaturised MPN costing approximately €10. These tests however are designed for drinking water analysis and have a level of precision which is not required for bathing water analysis. Through sacrificing some precision as detailed in Figure 6-10 it is possible to reduce the cost to below €2 per sample.
7 CONCLUSIONS AND FUTURE WORK
7.1 Conclusions from literature review

The review of available literature indicated that *E. coli* and Enterococci were the most accurate and reliable faecal indicators available at this time and the most suitable methods for rapid detection were rapid enzymatic assays.

The state of the art for *E. coli* enzyme assays was based on the fluorescent substrate 4-MUG allowing enumeration within 30 minutes [178]. While effective this substrate had the drawback of a pH dependent fluorescence response which necessitates the use of a discontinuous assay. The discontinuous assay required additional reactants and manual intervention at regular intervals. Examples were found in the literature where a continuous assay was used with the result of greatly reduced sample handling [71],[140].

This approach could be best applied using a substrate whose fluorescence was not inhibited by solutions around pH 7 which are the optimal range for enzymatic activity. The review uncovered a number of substrates only recently available [164] which have the required properties (i.e. pKa below 7) for continuous enzyme assays at neutral pH. The most promising of these was 6-CMUG.

A further limitation of the state of the art was the reliance on laboratory instrumentation for conducting analysis. While this is reliable and accurate there is a need for lower cost alternatives which can conduct assays on-site.

Thus the aim of this work was to develop a portable analyser for both *E. coli* and Enterococci based on the principle of continuous enzymatic assays.
7.2 Conclusions from experimental work

In Chapter 2, three fluorescent substrates 4-methylumbelliferone-β-D-glucuronide, 3-carboxyumbelliferyl-β-D-glucuronide and 6-chloro-4-methylumbelliferone-β-D-glucuronide along with their associated fluorophores were investigated for their suitability for rapid enzyme assays. 6-CMU was found to be most suitable, principally due to its low pKa (6.12) which allows it to fluoresce strongly at neutral pH thus enabling the use of continuous assays.

In Chapter 3, a low-cost sample incubation and fluorescence detection instrument (ColiSense 1) was developed for on-site analysis of recreational waters using a 6-CMUG based Glucuronidase assay. In laboratory testing the device was found to have comparable precision to standard laboratory instrumentation such as the LS50B fluorimeter. A lysing based assay for *E. coli* was developed and the system and assay were demonstrated successfully in a field trial on a Dublin river delivering results within 75 minutes per sample.

In Chapter 4, the ColiSense 2 instrument was developed to simplify sample handling procedures and to add a pre-concentration to the 6-CMUG based Glucuronidase assay. The system and assay were also demonstrated successfully in a field trial. The total time for retrieving a sample and analysing it on-site was reduced to 30 minutes.

In chapter 5 a field study was conducted to test both the ColiSense 1 and ColiSense 2 on a wide range of environmental water samples. Data was aggregated and compared with results of standard methods for *E. coli* and Enterococci analysis. The results from the 6-CMUG based Glucuronidase assays showed similar correlations with standard methods to those achieved in other studies using the discontinuous 4-MUG based Glucuronidase assay. This was achieved while also reducing the sample handling involved and conducting the assays on-site.

In chapter 6, a range of culture based assays using 6-CMU fluorescence were investigated with the goal of developing a detection method for Enterococci which requires a selective culture step as it has no specific enzyme. The Time to detect, Dilute to specification and miniaturised MPN methods were demonstrated using 6-CMUG and *E. coli*. It was found that by adapting the precision of some methods to suit recreational water monitoring requirements, the cost per sample can be considerably reduced.
7.3 Recommended engineering advancements

7.3.1 Redesign of ColiSense 1

The following modifications are suggested for the ColiSense 1 in order to improve detection sensitivity.

- Change fluorescence detection setup from bottom illumination to side illumination to reduce the effect of sedimentation in turbid samples.
- Improve optics to reduce background due to scattered light. I.e. use pinholes to accept only fluorescence and not scattering. Use an optical filter with a sharper cut-off to reduce background signal. Clean up the LED emission using a bandpass filter.
- Improve system electronics to reduce electronic noise thus maximising the signal to noise ratio.
- Increase the analog to digital convertor resolution from 12 bit to 16 bit to improve the sensitivity of the system.

7.3.2 Redesign of ColiSense 2

The following modifications are suggested for the ColiSense 2 in order to improve performance.

- Integrate battery power and data storage into the ColiSense 2 for stand-alone operation.
- Integrate a vacuum filtration apparatus into the system using disposable plastic filter cups. See Figure 7-1 for concept.
- Change fluorescence detection setup from side illumination and side detection to top-down illumination and top-down detection. This can reduce the volume of buffer solution added after filtration (currently 2.5 mL in order to cover the optical detection window). Reduction in this volume would give an increase in sensitivity in the assay due to lower dilution. See Figure 7-2 for design. This interrogation method would be compatible with the disposable filter cups shown in Figure 7-1.
- Improve optics to reduce background due to scattered light. I.e. use pinholes to accept only fluorescence and not scattering. Use an optical filter with a sharper
cut-off to reduce background signal. Clean up the LED emission using a bandpass filter.

- Improve system electronics to reduce electronic noise thus maximising the signal to noise ratio.
- Increase the analog to digital convertor resolution from 12 bit to 16 bit to improve the sensitivity of the system.

![Portable and disposable filtration setup](image)

Figure 7-1: Portable and disposable filtration setup
Automated systems for enzyme analysis have been developed and marketed at drinking water treatment installations, usually for early warning of source water contamination. Examples of these are the BactControl from MicroLan BV [136] or the ColiFast system [151],[152] which both use 4-MU fluorescence to monitor activity. None of these have been specifically designed for recreational water monitoring. The AWISS system [163] developed by Notre Dame University was intended for recreational water monitoring but suffered from a memory effect between measurements probably due to bio-fouling of internal surfaces in contact with the sample. Figure 7-3 shows an example of such fouling on a single-use vial used in the ColiSense system (The bio-film is stained with Methylene Blue). Disposing of vials after each sample allows the ColiSense system to avoid memory effects.
A repeat sampling mechanism based on a carousel as shown in Figure 7-4 or cartridge containing a number of sterile pre-prepared sample vials is proposed for an automated deployable monitoring system. The vials would contain the substrate 6-CMUG in desiccated or tablet form which would dissolve on the introduction of a sample by the injection mechanism. The sample would then be rotated to the incubation and detection system for analysis. To avoid memory effects the pre-concentration and sample injection system would need to be self-cleaning or to use single-use disposables to contact the sample. The 6-CMUG assay and the ColiSense incubation and detection system which have been demonstrated in this work can be utilised for this design.
7.4 Recommended Assay developments

The 6-CMUG assay used in the ColiSense 1 and 2 systems was optimised for pH (in phosphate buffer), substrate concentration and for temperature [142]. Other parameters such as osmolarity were fixed but not optimised.

The performance of both the lysing based assay used on ColiSense 1 and the metabolism based assay used on ColiSense 2 can probably be enhanced by optimising the concentration of various compounds used in the buffer solution. A number of possible optimisations are listed below.

- Add a chelating agent such as EDTA to remove inhibitors such as heavy metals from solution [116], [117], [118].
- Try out other substrates instead of 6-CMUG, such as Ethyl 7-hydroxycoumarin-3-carboxylate-b-G-galactoside which is reported to have higher fluorescence response and to be less inhibitory to bacteria growth than 6-CMUG [164].
- Trial different buffers with different salt concentrations to avoid osmotic shock of *E.coli* cells in the metabolic assay.
- Trial the addition of cell permeabilisers such as Polymoxin B which is reported to increase transport across the cell membrane [74].
- Trial the addition of phenazine methosulphate and ascorbate which is reported to increase the proton motive force and thus aid transport of substrate across the cell membrane [213].

The above optimisations could lead to lower detection limits and faster results for the GUS enzyme assays.
7.5 Recommended further testing

In Chapter 5 each of the 6-CMUG enzyme assays were tested against a range of freshwater samples and correlated with standard methods. The maximum number of samples taken for 1 method was 38 samples in triplicate. While this was enough to demonstrate the performance of the assays, further more extensive studies including marine and estuarine samples will be required to provide a weight of evidence. Lebaron for example tested 256 samples in the ColiPlage study [178].

Correlations between GUS activity measured with substrates such as 6-CMUG and culture based standard methods such as Colilert 18 or Enterolert vary between water types. This is due to the presence of varying proportions of VBNC cells which are measured by the enzyme assay but ignored by the culture based assay. This makes it difficult to establish the true performance of the enzyme assay. DVC-Fish has been used successfully as a reference for the ColiPlage method with correlations or \( R^2 > 0.9 \) achieved [113]. Testing against such a reference method which records VBNC cells could provide greater insight into the performance of 6-CMUG based enzyme assays.

An alternative to the inherently flawed approach of testing enzyme assays against culture based reference methods, is to attempt to establish a relationship between GUS activity and gastro intestinal illness. This approach would use GUS activity directly as a faecal indicator rather than indirectly as an indicator of the concentration of FI bacteria such as \( E. \ coli \). Such epidemiological studies have been carried out extensively to relate \( E. \ coli \) and Enterococci levels to the prevalence of waterborne disease [1][2] but they have yet to be carried out for GUS activity.
7.6 Commercialisation

GUS enzymatic assays for *E. coli* provide a rapid low cost-per-sample alternative to culture based methods. These assays have many potential applications from testing recreational waters, drinking waters, beach sand analysis [215], plant extract analysis [140], shell fish quality monitoring, to clinical sample analysis [216]. GUS enzyme assays however have not been widely adopted due to the equipment requirement, intensive sample handling and the lack of sufficient scientific data for assay validation [214].

In this work, the equipment requirement and the sample handling have been addressed. The ColiSense system is a low cost on-site instrument for GUS enzyme assays with comparable performance to laboratory instruments while the use of 6-CMUG based assays greatly reduces sample handling.

Currently the ColiSense systems both have an LOD of approximately 100 CFU/100 mL. This is sufficient for recreational water analysis for which they are intended but not sufficiently low for drinking water analysis in most developed countries which require an LOD of 1 CFU/100 mL. There is however a demand from the humanitarian community for systems to detect *E. coli* with an LOD of 10 CFU/100 mL. An example of this demand is the UNICEF ‘Target Product Profile for Rapid *E. coli* Detection’ published in August 2017. With some of the engineering and assay refinements recommended above, the ColiSense systems could be adapted to meet the required specifications.
REFERENCES


Sensing platform design for Faecal indicator bacterial detection in recreational waters


Appendices

A1: PUBLICATIONS

A1.1: Peer review articles


8. *Data Analysis from a Low Cost Optical Sensor for Continuous Marine Monitoring,* Kevin Murphy, Timothy Sullivan, Brendan Heery, Fiona Regan, Sensors and actuators B: Chemical 214, 211-217 (Europtrode), 2014

Sensing platform design for Faecal indicator bacterial detection in recreational waters


A1.2: Conference proceedings


3. A centrifugal lab-on-a-disc device for the in situ determination of dissolved reactive phosphate in water. Gillian Duffy, Brendan Heery, Ivan Maguire, Charles Nwankire, Jens Ducree, Fiona Regan, In: EuroAnalysis, Bordeaux, France, 6-10 September, 2015


8. Improving Data Driven Decision Making Through Integration of Environmental Sensing Technologies. Sullivan, T, Zhang, D, O’Connor, E, Armstrong, A, Briciu-


A1.3: Poster presentations


3. A Smart City needs a Smart Bay, B. Heery, J. Moreno, A. Barrett, F. Regan, Environ 2015, Sligo IT, 07-08 April 2015

4. Metabolism based fluorescence assay and field portable device for E.coli quantification in recreational waters, Brendan Heery, Ciprian Briciu-Burghina, Dermot Brabazon, and Fiona Regan. In: WWEM 2014, Telford, UK ,6-7 Nov, 2014 SWIG 2014 (3\textsuperscript{rd} place in SWIG, Early career researcher poster competition)


7. *An Optical Colour Sensor to Monitor the Marine Environment.* Kevin Murphy, Brendan Heery, Kim Lau, Lorna Fitzsimons, Dermot Diamond and Fiona Regan, In: Smart Ocean Forum, 5-6 Nov 2013, Belfast, UK


9. *Comparison of fluorogenic substrates for the detection of faecal indicator bacteria in water samples using a continuous fluorometric assay.* Briciu Burghina Ciprian Constantin and Heery, Brendan and Regan, Fiona. In: Smart Ocean Forum, 5-6 Nov 2013, Belfast, UK.


A 1.4: Invention disclosures


A 1.5: Awards

1. CIWEM, AECOM, Student Environmental award, Sligo IT, April, 2015, 1st place.
2. SWIG poster competition at WWEM 2014, 3rd place.
A 2: COLISENSE 1 DESIGN DETAILS

A 2.1: Incubation detection block mechanical design
A 2.2: ColiSense 1 Circuit design
Sensing platform design for Faecal indicator bacterial detection in recreational waters
A 2.3: ColiSense 1 firmware

// ColiSense.c
// Developed by Brendan Heery
// Date: 10/01/2014

// Some code adapted from the following
// 1. example_usb_com.c     Wixel SDK, Open source
// 2. test_adc.c            Wixel SDK, Open source
// 3. example_onewire.c     Written by Russell Nelson <nelson@crynwr.com>.

// Board setup
Pin 00 Chan A: Analog input
Pin 01 Chan B: Analog input
Pin 02 Chan C: Analog input
Pin 03 Not used
Pin 04 Not used
Pin 05 Dallas 1-Wire
Pin 10 LED A: Active HIGH
Pin 11 LED B: Active HIGH
Pin 12 LED C: Active HIGH
Pin 13 Not used
Pin 14 Red indicator: Active HIGH
Pin 15 Green indicator: Active HIGH
Pin 16 Heater: Active HIGH
Pin 17 Buzzer: Active HIGH
Pin 21 Not used
Pin 22 Not used

// Dependencies
#include <cc2511_map.h>
#include <wixel.h>
#include <time.h>
#include <math.h>
#include <stdio.h>
#include <gpio.h>
#include <string.h>
#include <stdlib.h>
#include "onewire.h"

// Global variables
const char *respondstr = NULL;
uint8 XDATA DS1820_addr[8];
uint8 is_DS18B20;
uint32 ds1820_time;
uint16 x = 0x3FFF;
BIT serialProtocolError = 0;
uint8 commandByte;
uint8 dataBytes[32];
uint8 dataBytesLeft = 0;
uint8 dataBytesReceived;
uint8 XDATA response[128];
uint8 responseLength;
BIT uartRxDisabled = 0;
BIT errorOccurredRecently = 0;
uint8 lastErrorTime;
uint8 byteCommand[32] = {0};
uint8 byteCommandCounter = 0;

/////////////////////////////////////////////////////////////////
// function prototypes
//
/////////////////////////////////////////////////////////////////
uint16 measure_a(void);
uint16 measure_b(void);
uint16 measure_c(void);
uint16 take_an_average(uint8);
void turn_everything_off(void);
void delay_seconds(int secs);
void analogInputsInit(void);
void start_DS1820(void);
void setup_DS1820(void);
int read_DS1820(void);

/////////////////////////////////////////////////////////////////
// functions
//
/////////////////////////////////////////////////////////////////
// Measure fluorescence in cell A ////////////////////////////////
uint16 measure_a()
{
    uint16 result;  // Variable to store result
    setDigitalOutput(10, HIGH);  // LED A on
    delayMs(100);  // Delay 100 ms
    result = take_an_average(0);  // Read fluorescence in cell A
    setDigitalOutput(10, LOW);  // LED A off
    return(result);  // Return result
}

// Measure fluorescence in cell B ///////////////////////////
uint16 measure_b()
{
    uint16 result;  // Variable to store result
    setDigitalOutput(11, HIGH);  // LED B on
    delayMs(100);  // Delay 100 ms
    result = take_an_average(1);  // Read fluorescence in cell B
    setDigitalOutput(11, LOW);  // LED B off
    return(result);  // Return result
}

// Measure fluorescence in cell C //////////////////////////////
uint16 measure_c()
{
    uint16 result;  // Variable to store result
    setDigitalOutput(12, HIGH);  // LED C on
    delayMs(100);  // Delay 100 ms
    result = take_an_average(2);  // Read fluorescence in cell C
    setDigitalOutput(12, LOW);  // LED C off
return(result);    // Return result
}

// Average over 20 readings at 1 ms to remove 50hz noise
uint16 take_an_average(uint8 pin) {
    uint8 i;    // Variable to count iterations
    float average = 0;   // Variable to store result
    for(i=0;i<20;i++) {
        average += adcRead(pin | ADCREFERENCEVDD | ADCBITS12);
        delayMs(1);       // 1 sample per ms for 20 ms
    }
    average = (average / 20.0f); // Divide by 20
    return(average); // Return result
}

// Delay for a specified number of seconds
void delay_seconds(int secs){
    int i; // Variable to count iterations
    for(i=0;i<secs*10;i++) {
        delayMs(100);
        boardService();   // Check for bootload signal
        usbComService();  // Check communications status
    }
}

// Initialise analog ports
void analogInputsInit(){
    P0INP = 0x3F;
}

// Start DS1820 temperature sensor
void start_DS1820(){
    onewire_reset();
    onewire_select(DS1820_addr);
    onewire_write(0x44,0);
}

// Setup DS1820 temperature sensor
void setup_DS1820(void){
    onewire_start();
    if ( onewire_search(DS1820_addr) ) {
        onewire_reset_search();
        delayMs(250);
        onewire_search(DS1820_addr);
    }
    if ( onewire_crc8(DS1820_addr, 7) != DS1820_addr[7]) {
        respondstr = "No OneWire devices found";
        return;
    }
    if ( DS1820_addr[0] == 0x10 ) {
        is_DS18B20 = FALSE;
    }
}
else if ( DS1820_addr[0] == 0x28) {
    is_DS18B20 = TRUE;
} else {
    respondstr = "No DS1820 found";
    return;
}
start_DS1820();
ds1820_time = getMs();

// Read DS1820 temperature sensor (from example_onewire.c)/////////
int read_DS1820(){
    uint8 i;
    uint8 present = 0;
    uint8 dataread[12];
    int temp_read;
    present = onewire_reset();
onewire_select(DS1820_addr);
onewire_write(0xBE,0);
    for ( i = 0; i < 9; i++)   {
        dataread[i] = onewire_read();
    }
    temp_read = ((dataread[1] << 8) | dataread[0]);
    if (!is_DS18B20)   {
        temp_read *= 8.0;
        temp_read += ( 8 * (dataread[7] - dataread[6]) )/dataread[7];
    }
    return temp_read;
}

// Switch off all peripheral devices //////////////////////////////////////////////////
void turn_everything_off() {
    setDigitalOutput(10, LOW);
    setDigitalOutput(11, LOW);
    setDigitalOutput(12, LOW);
    setDigitalOutput(13, LOW);
    setDigitalOutput(14, LOW);
    setDigitalOutput(15, LOW);
    setDigitalOutput(16, LOW);
    setDigitalOutput(17, LOW);
}

// Main function //////////////////////////////////////////////////////
void main(){
    int target_temp = 44*16;       // Target temperature times 16 is 708
    int actual_temp;               // Variable to store temperature reading
    uint16 a,b,c;                  // Variables to store fluorescence readings
    systemInit();                  // Initialise the microcontroller
   .usblInit();                    // Initialise the usb port

    // Read temperature
    temp_read = read_DS1820();
    if (temp_read < target_temp) {
        // Set motor
        motor_direction(1, 0); // Set motor direction
        motor_speed(1, 0.5);  // Set motor speed
    }
}
setup_DS1820();  // Set up DS1820 temperature sensor
analogInputsInit();  // Initialise analog ports
turn_everything_off();  // Switch off peripheral devices
while(1) {
  boardService();  // Main loop
  usbComService();  // Check for bootloader signal
  a = measure_a();  // Measure fluorescence in cell A
  b = measure_b();  // Measure fluorescence in cell B
  c = measure_c();  // Measure fluorescence in cell C
  start_DS1820();  // Start DS1820 temperature sensor
  actual_temp = read_DS1820();  // Read current temperature
  if (actual_temp >= target_temp) {
    setDigitalOutput(16, LOW);  // Heater off
    setDigitalOutput(15, LOW);  // RED led off
    setDigitalOutput(14, HIGH);  // Green led on
    delayMs(100);
  }
  else {
    setDigitalOutput(16, HIGH);  // Heater on
    setDigitalOutput(15, LOW);  // RED led off
    delayMs(100);
    setDigitalOutput(15, HIGH);  // RED led on
    setDigitalOutput(14, LOW);  // Green led off
  }
  responseLength = sprintf(response, ",%4d,%4d,%4d,%d
\n\r",a,b,c,actual_temp/16);
  usbComTxSend(response, responseLength);  // Transmit data via USB
  delay_seconds(9);  // loop delay
  delayMs(408);  // adjust total delay to 10 seconds
}
}
A 3: ColiSense 1 FUNCTIONAL TESTING

A 3.1: Glass vial optical characterization

Tests were conducted on clear borosilicate glass vials (Type TVL-050-040) to establish their optical transmission. Figure A-1 shows a Uv-Vis spectrum for the vial and highlights the cut-off point for the particular glass (i.e. just below 300 nm). Concentrations of the fluorophore 6-Chloro-4-Methyl-Umbelliferyl (6-CMU) were also scanned in the vial to highlight the region of interest for the 6-Chloro-4-Methyl-Umbelliferyl-β-D-Glucuronide (6-CMUG) based assay. The 50 µM solution of 6-CMU absorbed maximally at 365 nm. This was 65 nm above the glass cut-off wavelength. The fluorophore’s emission wavelength of 445 nm is further clear of the cut-off. Thus this particular type of vial was suitable to conduct the 6-CMUG fluorescent assay.

Figure A-1: Absorbance spectrum of Borosilicate glass sample vials, (Type TVL-050-040)
Appendices

A 3.2: Incubation temperature testing

The target temperature was set in the ColiSense device firmware to 44°C and the device was allowed to control the temperature to this value. Actual temperature values were recorded by the ColiSense and displayed on a PC. Temperature control was verified using an i-button temperature logger from Maxim attached to the heated block and cross-verified using a mercury thermometer. A mathematical thermal model of the incubating block created in Microsoft Excel predicted that at 24V power it would take 8 minutes for the block to rise from room temperature (21°C) up to 44°C.

Figure A-2 (black line) shows the temperature of the incubation block as recorded over a 1 h period by the ColiSense itself. Figure A-3 shows the temperature recorded by the attached i-button. It also displays the cool-down period of the device. The device reached the required temperature from room temperature (21 °C) in 13 minutes. The temperature was controlled to within half a degree of the target temperature of 44°C. This is an adequate level of accuracy for the purpose of incubating environmental samples. When cooling it took the incubating block approximately 2 h to reach room temperature from 44°C. An error of approximately 2°C was observed between the i-button temperature and the ColiSense recorded temperature and confirmed with the Mercury thermometer. This was subsequently corrected for in the firmware on the ColiSense system.

A 3.3: ColiSense power testing

Power was measured by placing an ammeter in line with a 24V plug-top power supply and recording the current drawn during each mode. Figure A-1 (red line) shows the power consumption measured during each mode of operation (Heating and measurement). Power usage during heating was 5.8 Watts while average power usage during the measurement phase was 2.5 Watts.
Figure A-2: Temperature of ColiSense incubating block as recorded by ColiSense during a 1 h test.

Figure A-3: Temperature of incubation block component of ColiSense during a test recorded using an i-button temperature logger.