To develop and optimise methods for the detection and isolation of *Salmonella* Typhi from the environment.

Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor in Philosophy

By Jonathan Richard James Rigby

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I. Collaborators

This thesis is the result of my own work, though collaborations with others have helped achieve aspects of the project. Where relevant, this has been explained in the related chapters, however, is reiterated here for clarity.

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Chapter 5 is a published paper, whilst written primarily by me, all co-authors listed contributed to the results, analysis or writing of the manuscript.

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III. Publications

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Related Peer-review publications

Rodwell, E. V., Wenner, N., Pulford, C. V., Cai, Y., Bowers-Barnard, A., Beckett, A., . . . Perez-Sepulveda, B. M. (2021). Isolation and Characterisation of Bacteriophages with Activity against Invasive Non-Typhoidal *Salmonella* Causing Bloodstream Infection in Malawi. *Viruses, 13*(3). doi:10.3390/v13030478

Byrne, R. L., Cocker, D., Alyayyoussi, G., Mphasa, M., Charles, M., Mandula, T., . . . Edwards, T. (2022). A novel, magnetic bead-based extraction method for the isolation of antimicrobial resistance genes with a case study in river water in Malawi. *J Appl Microbiol*, 2021.2004.2023.439981. doi:10.1111/jam.15755

Related, pre-peer review publications

Zhou, N. A., Ong, A. Q. W., Fagnant-Sperati, C. *S.*, Harrison, J. C., Kossik, A. L., Beck, N. K., . . . Meschke, J. S. (2022). Evaluation of sampling and concentration methods for *Salmonella enterica* serovar Typhi detection from wastewater. *medRxiv*, 2022.2006.2007.22275929. doi:10.1101/2022.06.07.22275929 (as part of the Typhoid ES working Group)

Uzzell, C. B., Troman, C. M., Rigby, J., Mohan, V. R., John, J., Abraham, D., . . . Grassly, N. C. (2021). Environmental surveillance for *Salmonella* Typhi as a tool to estimate the incidence of typhoid fever in low-income populations. *medRxiv*, 2021.2005.2021.21257547. doi:10.1101/2021.05.21.21257547

Unrelated peer reviewed publications

Painset, A., Day, M., Doumith, M., Rigby, J., Jenkins, C., Grant, K., . . . Swift, C. (2020). Comparison of phenotypic and WGS-derived antimicrobial resistance profiles of *Campylobacter jejuni* and *Campylobacter coli* isolated from cases of diarrhoeal disease in England and Wales, 2015-16. *J Antimicrob Chemother, 75*(4), 883-889. doi:10.1093/jac/dkz539

Ngwira, L. G., Sharma, B., Shrestha, K. B., Dahal, S., Tuladhar, R., Manthalu, G., . . . Mvundura, M. (2022). Cost of wastewater-based environmental surveillance for SARS-CoV-2: Evidence from pilot sites in Blantyre, Malawi and Kathmandu, Nepal. *PLOS Global Public Health, 2*(12), e0001377. doi:10.1371/journal.pgph.0001377

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VII. Glossary of Terms

AMR: Antimicrobial Resistance

AST: Antimicrobial Susceptibility Testing

ATCC: American Type Culture Collection

Bile$_{cy}$: Bile^{-} broth with Cycloheximide

Bile$_{s}$: Bile^{-} broth with sucralose

BLAST: Basic Local Alignment Search Tool

BMGF: Bill and Melinda Gates Foundation

BPW: Buffered Peptone Water

BSA: Bismuth Sulphite Agar

CASE: Chromogenic Agar *Salmonella* esterase

CBA: Columbia Blood Agar

cDNA: copy Deoxyribonucleic Acid

CFU: Colony Forming Units

CFU mL^{-1}: Colony Forming Units per Millilitre

CL3: Containment Level three

COM: College of Medicine (former name for KUHeS)

Ct: Cycle Time

DCA: Deoxycholate Citrate Agar

DNA: Deoxyribonucleic Acid

DRUM: Drivers of Resistance in Uganda and Malawi

EE: Enterobacteriaceae Enrichment

EM: Electron Microscopy

ES: Environment Surveillance

FWE: Food, Water and Environment

g: grams

GBRU: Gastro-intestinal Bacteria Reference Unit

GIS: Geographic Information System

GMO: Genetically Modified Organism

GPS: Global Positioning System

h: Hours

HRM: High-resolution Melt

ICL: Imperial College London

IgG: Immunoglobulin G

IgM: Immunoglobulin M

iNTS: invasive Non-typhoidal *Salmonella*

KUHeS: Kamuzu University of Health Sciences

LIMS: Laboratory Information Management System

LMIC: Low- and Middle-income county

LOD: Limit of Detection

LOD$_{50}$: Limit of Detection

LOD$_{95}$: Limit of Detection of 50%

LOQ: Limit of Quantification of 95%

LSTM: Liverpool School of Tropical Medicine

mCASE: modified Chromogenic Agar *Salmonella* esterase

MDR: Multi-drug resistant

mg: Milligrams

mH$_2$O: Nuclease free water/molecular water

mL: millilitre

MLW: Malawi-Liverpool Wellcome Trust

MRD: Maximum Recovery Diluent

NAAT: Nucleic Acid Amplification Technique

NCTC: National Collection of Type Cultures

nd: Not detected

NTC: Non-Template Control

NTS: Non-Typhoidal *Salmonella*

ODK: Open Data Kit

OMP: outer membrane proteins

OR: Odds Ratio

PCR: Polymerase Chain Reaction

PEG: Polyethylene Glycol

PFP: PowerFaecal Pro

PHE: Public Health England (former name of UKHSA)

PPCO: Polypropylene Copolymer

PPE: Personal protective equipment

PR: Positivity Ratio

PW: Pathway

RLS: Ringers' Lactate Solution

RNA: Ribonucleic Acid

SAGE: Scientific Advisory Group of Experts

***S.* Typhi**: *Salmonella enterica* serovar Typhi

SC: Selenite Cystine

SEM: Standard Error of the Mean

SF: Selenite Faecal

SPC: Sample Processing Control

T3SS: Type 3 Secretion System

TCV: Typhoid Conjugate Vaccine

TDS: Total Dissolved Solids

TyVAC: Typhoid Vaccine Acceleration Consortium

UKHSA: United Kingdom Health Security Agency

ULT: Ultra-Low Temperature

UNAIDS: Joint United Nations Programme on HIV/AIDS

UoL: University of Liverpool

UPE: Universal Pre-enrichment Broth

ViCPS: Vi Capsular Polysaccharide

WASH: Water, Sanitation and Hygiene

WDCM: World Data Centre for Microorganisms

WHO: World Health Organisation

XLD: Xylose Lysine Deoxycholate Agar

µg: Micrograms

µL: Microlitres

µm: Micrometres

VIII. Abstract

To develop and optimise methods for the detection and isolation of *Salmonella* Typhi from the environment. By Jonathan Rigby
Supervisors: Professor Nicholas Feasey; Doctor Nicola Elviss and Doctor Adam Roberts

Introduction: *Salmonella* Typhi is a globally important pathogen that causes Typhoid fever, responsible for an estimated 11.9-26.9 million cases and 129,000-216,510 typhoid-related deaths per year worldwide. Surveillance of clinical disease based on quality assured diagnostic clinical microbiology services is often not performed, making it difficult to understand the true burden of typhoid. Environmental surveillance (ES) has the potential to be a cheaper alternative to clinical surveillance of Typhoid and *S*. Typhi in endemic settings that does not rely on patients attending hospitals or consenting to research. Between April 2015 and January 2017, 546 culture-confirmed cases were reported at Queen Elizabeth Central Hospital in Malawi, however it is likely that case numbers are under-reported, and ES data can provide supplementary insight for spatially targeted interventions.

Methods: A novel culture method for *S.* Typhi was developed at the UK Health Security Agency in London and optimized in Blantyre, Malawi with environmental samples collected in 2019, including water (via trap and gran methods), sediments, food, and biofilms. The method used two enrichment broths (2% bile, then selenite F broths) before plating onto modified chromogenic agar for Salmonella esterase agar. Isolates were identified by real-time PCR and confirmed by biochemistry and serology. The method was validated and used for city-wide surveillance in Blantyre, Malawi between May 2021 and April 2022, alongside a method proposed by the Bill and Melinda Gates Foundation *S*. Typhi ES working group. After findings between these two methods, further refinements to the culture pathway were started.

Results: The six-month pilot study, from 2019 to 2020, isolated six *S*. Typhi cultures from three Moore swabs, two water samples, and one biofilm. PCR positivity was confirmed by biochemistry and serology. Non-typhoidal salmonellae (NTS) accounted for 377 isolates, 16 of which amplified *staG*. Between 2021 and 2022, 33 samples were *S*. Typhi positive and 80 positives for NTS by direct PCR detection only, with two *S*. Typhi and 255 NTS positive isolates cultured. An alternative extraction method for PCR was shown to have good performance under laboratory conditions, but the inclusion of antimicrobial-containing broths required further development.

Conclusion: This work demonstrates that ES of Typhoid by culture is achievable in low- and middle-income countries. Moore swabs yielded a higher detection rate than water samples. Direct detection by PCR appeared to be more sensitive than culture, but still had a low positivity rate. This low positivity rate coincided with the lowest rates of blood culture positivity in a decade and the SARS-CoV-2 pandemic. Work to integrate a PCR screening tool before culture is ongoing but shows potential viability.

1 Introduction

1.1 Introduction

Typhoid fever remains a disease of global public health concern in low- and middle-income countries (LMIC), where access to water, sanitation and hygiene (WASH) infrastructure is limited and often inadequate (Parry *et al*., 2002, Feasey *et al*., 2015, Schwenk, 2020). Humans are the only known reservoir of *Salmonella enterica* serovar Typhi (*S.* Typhi). Whilst cases are ultimately transmitted from human to human, transmission may be direct/person-to-person or indirect, following excretion of the pathogen into the environment. This has been referred to as long-cycle transmission (Levine *et al*., 1982, González-Guzmán, 1989, Baker *et al*., 2011, Akullian *et al*., 2015, Gauld *et al*., 2018).

With a global estimate from the World Health Organisation (WHO) of 11.9-26.9 million cases and 129,000-216,510 typhoid related deaths per annum (Darton *et al*., 2017, Meiring *et al*., 2017) cases have re-emerged and increased in areas where incidence were previously low, perhaps due to the emergence of antimicrobial resistant strains (Feasey *et al*., 2015) and a lack of effective vaccination programmes in endemic regions (Darton *et al*., 2017, Meiring *et al*., 2017). Other causes of enteric fever include *Salmonella enterica* serovariants Paratyphi A, B and C - typically called paratyphoid. These can often be found in regions endemic for typhoid caused by *S.* Typhi as mode of transmission is the same. Areas where typhoid is endemic can also be associated with separate invasive non-typhoidal *Salmonella* (iNTS) infections. The most common serovariants for iNTS are *Salmonella enterica* serovar Typhimurium or Enteritidis but may be caused by any non-typhoidal *Salmonella* (NTS) that infects a patients' bloodstreams. (Feasey *et al*., 2010, Adhikary *et al*., 2013).

Large scale phase 3 vaccine trials with a new typhoid conjugate vaccine (TCV) have recently concluded in Blantyre, Malawi and other endemic locations in Nepal and Bangladesh (Neuzil *et al*., 2019, Shakya *et al*., 2019, Patel *et al*., 2021). Following the success of these, it is hoped that role out of TCV will eventually reduce burden of disease. Key to the control of infectious disease is the capacity to survey the problem. This project aims to develop a universal tool for environmental surveillance (ES) of typhoid, which can be used in any endemic region for the identification of public health interventions such as improved WASH infrastructure and community support as well as help identify areas most heavily affected by shedding into the environment.

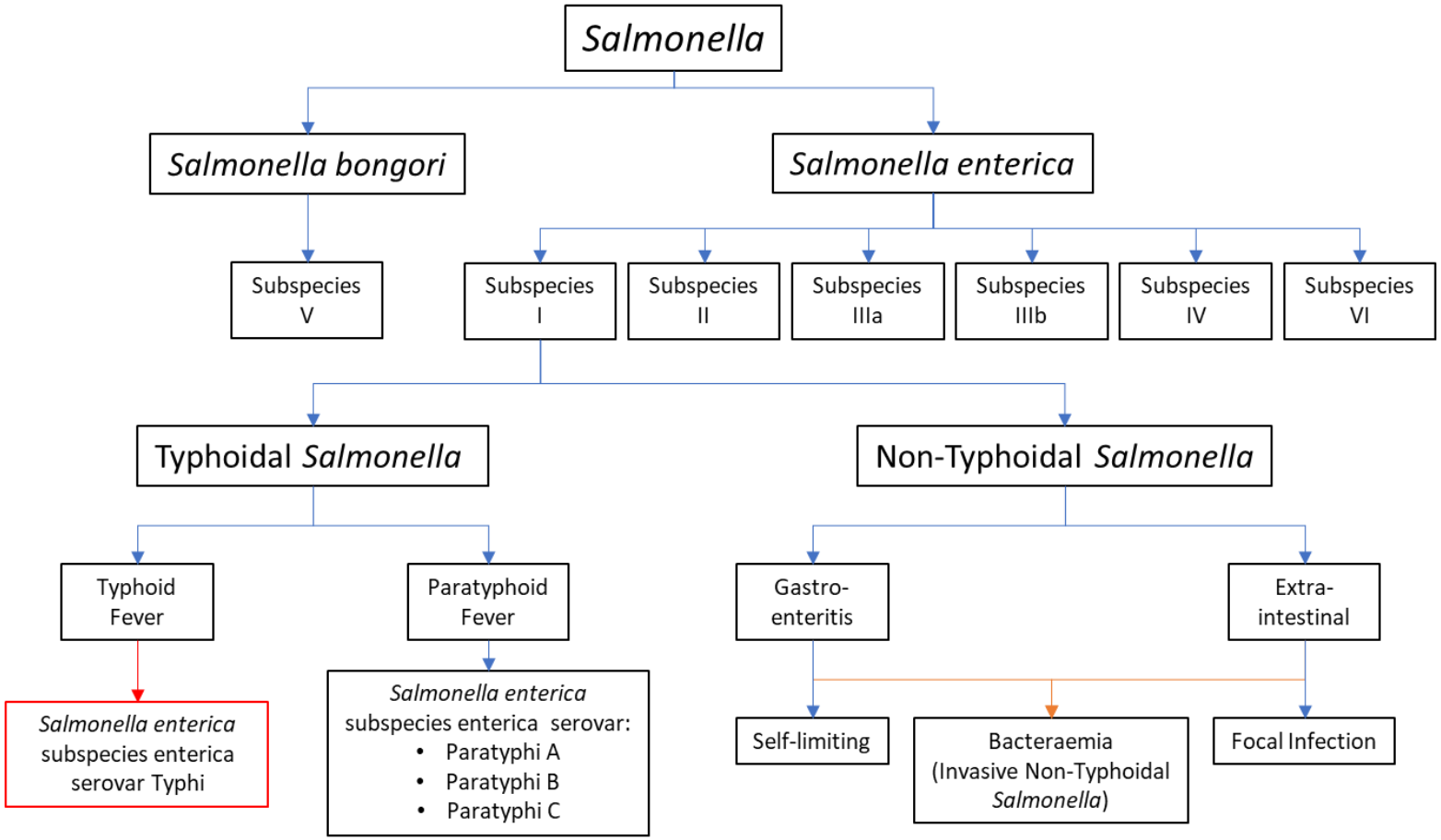


Figure 1.1: Overview of Salmonella spp. classification, showing relationship between Typhoid, non-typhoidal infections strains, and between the two species, Salmonella bongori and Salmonella enterica adapted from Achtman *et al*. (2012).

1.2 The Bacterium

Salmonella is a genus of Enterobacteriaceae, which is a flagellated, Gram-Negative rod, comprising of two species: *Salmonella enterica* and *Salmonella bongori*, as seen in Figure 1.1. The *S. enterica* group contains six subspecies with over 2,600 serovars. It is a motile, aerobic to facultatively anaerobic, non-spore-forming organism typically associated with gastrointestinal disease, when caused by a NTS, or enteric fever when caused by *S.* Typhi, *S. enterica* serotype Paratyphi A, B or C. *Salmonella* enterocolitis is one of the four leading causes of diarrhoeal disease, however NTS *Salmonella* serovars can also cross the gut wall to cause systemic invasion, with bacteraemia and/or focal disease such as meningitis or osteomyelitis.

Salmonella Bongori, like *S. enterica*, is pathogenic, causing salmonellosis also, but is typically associated with cold-blooded animals such as reptiles. It is a leading cause of gastroenteritis and diarrhoeal disease in pets of this nature but has also been documented occurring asymptomatically in some birds and mammals, in addition to causing opportunistic infections in humans, although uncommon (Giammanco Giovanni *et al*., 2002, Foti *et al*., 2011, Wang *et al*., 2019)

The remainder of this introduction will focus on *S.* Typhi, which is human restricted and highly adapted to be a human pathogen and disseminates in areas with poor sanitation or WASH infrastructure. Historically a public health problem in Europe and the USA, it is currently endemic in South Asia and Sub-Saharan Africa, particularly where there is overcrowding, such as informal settlements(Parry *et al*., 2002, Kariuki *et al*., 2019).

In the 19th century, typhoid had begun to be recognised as its own distinct clinical disease from the vague group of “continued fevers of unknown cause”. Prior to 1880, Karl Liebermeister had proposed a microorganism might be responsible for typhoid fever and spread similarly to cholera. Independently, William Budd (1838) observed that the causative agent of typhoid fever was infectious and present in human secretions. In 1838, an outbreak of fever occurred in 1,300 people from Taw Valley, in the UK, under Budd’s care. He observed cases where those who visited or assisted bedridden fever patients later developed the disease themselves and subsequently proposed the concept of “intestinal fever” (Smith, 1984, Moorhead, 2002).

In 1847, due to an outbreak among the residents of Richmond Terrace, Bristol, Budd hypothesised the association of intestinal fever with that of contaminated water due to residents in the same block not all being affected due to some houses being on a separate water supply. This was observed alongside cases of cholera being associated with water within the city. In 1859, Budd's observations were published in The Lancet and, whilst opposed by supporters of the miasma theory, were consistent with similar experiences from fellow country doctors, such as William Cook, who made comparable observations during outbreaks.

Later, in 1879, Karl Joseph Eberth successfully discovered the bacillus responsible for typhoid fever in patients from samples of their abdominal lymph nodes and spleen, naming the organism *Eberthella typhosa*. This discovery was confirmed by other bacteriologists, most notably, Georg Gaffky, who isolated the organism for the first time in 1884 using gelatin, as well as Robert Koch independently shortly after. After discovery by Gaffky, the organism was renamed *Bacillus typhosus* and would eventually be renamed *Salmonella enterica* serovar Typhi in 1934; with the genus *Salmonella* being named after American veterinarian Daniel Elmer Salmon. Other names that *S.* Typhi acquired before the nomenclature was finalised included:

- Bacillus des Abdominal Typhus of Eberth, 1880;
- Typhus Bacillus of Gaffky, 1884;
- *Bacillus typhosus*, Zopf, 1885;
- *Bacillus* Typhi, Schroter, 1886;
- *Bacillus* Typhi *abdominalis*, Fliigge, 1886;
- *Vibrio typhosus*, Trevisan, 1889;
- *Eberthella* Typhi, Buchanan, 1918;
- *Eberthus typhosus*, Castellani and Chalmers, 1919;
- *Salmonella* Typhi, Warren and Scott, 1929-30, Schuitze, 1930;
- *Salmonella typhosus*, Bruce White, 1929-30;
- Typus-Typhus of Kauffmann, 1931.
    - (Salmonella Subcommittee of the Nomenclature Committee of the International Society for, 1934)

The bacterium *S.* Typhi is a gram-negative flagellated bacilli of the *Salmonella enterica* subspecies group I (Table 1.1).

Table 1.1: Taxonomic hierarchy of *S.* Typhi (Achtman *et al*., 2012, ITIS, 2012).

Taxonomic Hierarchy	**Title**
Kingdom	Bacteria
Sub-kingdom	Negibacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacterales
Family	Enterobacteriaceae
Genus	*Salmonella*
Species	*Salmonella enterica*
Subspecies	*Salmonella enterica* subspecies I
Serovariant	*Salmonella enterica* subspecies enterica serovar Typhi

One of the defining characteristics of *S.* Typhi is the expression of the Vi antigen. This is encoded by the *via* complex, which contains the *viaB* locus. Within *viaB* are the genes *tviABCDE* and *vexABCDE*. The *via* complex is located on the *SPI-7* pathogenicity island, although some rare strains of *S.* Typhi have lost the Vi antigen due to loss or disruption of *SPI-7* (Nair *et al*., 2004, Wain *et al*., 2005). However, this gene is not unique to *S.* Typhi and can also be found in *S.* Paratyphi C and a rare variant of *S.* Dublin, accession number CP074226, contains Vi surface antigens with a *viaB* locus. A similar gene can also be found in other *S.* Dublin variants and *Citrobacter baumanii* which also gives Vi expression to the organism, but does not contain the same gene, *tviB,* within the *viaB* locus (Hashimoto and Khan, 1997, Nair *et al*., 2019); as such, *tviB* presents a PCR target potentially specific to *S.* Typhi, *S.* Paratyphi C and a variant of *S.* Dublin.

1.3 The Disease

Whilst the exact Infectious dose is unknown, it is estimated between 1,000 to 1 million bacteria from work done with human challenge models during as part of vaccine trials with volunteers (Parry *et al*., 2002, Waddington *et al*., 2014, Jin *et al*., 2017). The infectious dose is likely at the higher end of this estimate in healthy individuals due to the use of sodium

bicarbonate being administered in human challenge studies to ensure infection by neutralising stomach acid that would ordinarily kill invading oral organisms.

Nearly all cases of infection occur with strains containing the virulence capsular polysaccharide, or Vi antigen, which is believed to help prevent phagocytosis in the host and helps provide serum resistance. Typically, the loss or disruption of the *SPI-7* pathogenicity island or *viaB* locus, which encode for Vi, leads to a less virulent and less infectious strain (Felix and Pitt, 1951), there have been documented cases of Vi-negative strains causing clinical illness in Pakistan (Baker *et al*., 2005). This *SPI-7* pathogenicity island also encodes one of the type III secretion systems (T3SS) and type IVB pilus.

The T3SS is required for the invasion of non-phagocytic cells, whilst the IVB pilus and fimbriae are important adhesion factors for host invasion and cellular interactions. Other virulence factors involved in *S.* Typhi infection, survival and disease include endo-, entero- and cytotoxins, somatic O antigen, lipopolysaccharide containing outer membrane proteins (OMPs) and flagella. The endotoxin involved in fever and cytotoxin inhibits host cell protein production. The OMPs are split into two groups, the porin substances required in forming pore channels for solute uptake, and non-porin substances which are structural proteins. Most *S.* Typhi strains are monophasic and display the *fliC* gene of the antigen H:d directly, although some strains have mutated through in-frame deletions and selective pressure in the immune system due to the virulence of H:d *fliC*. As shown in Table 1.2, *S.* Typhi is motile, and monophasic, typically with the H:*d* H phase flagellar antigens, but some mutant strains have also exhibited other H antigens, primarily H:*j* and *Hz66* (Hatta *et al*., 2011, Schreiber *et al*., 2015).

As seen in Figure 1.2, *S.* Typhi utilises the faecal-oral route of transmission and must survive the low gastric pH in the stomach first, before exposure to bile, which, due to its alkalinity, neutralises the stomach acid and allows the invasion in the small intestine. Whilst in high concentration of bile, *S.* Typhi may be inhibited, it has been shown that the bacilli generate anti-oxidants in the presence of bile to protect themselves from the oxidative stress (Walawalkar *et al*., 2016). After exposure to bile in the host, the organism adheres to the mucosal cells found in the small intestine. Using M cells as the site of internalisation, *S.* Typhi is transported to the underlying lymphoid tissue before translocation to the intestinal lymphoid follicles, draining mesenteric lymph nodes and the reticuloendothelial cells of the liver and spleen. Mononuclear phagocytic cells are also vital to *S.* Typhi's survival, as they

invade these cells to evade further immune responses and can multiply intracellularly before release into the bloodstream (Parry *et al*., 2002, Feasey *et al*., 2013).

The incubation period of typhoid fever is typically 7 to 14 days, after which bacteraemia occurs, accompanied by symptoms of malaise and fever (Figure 1.3). During the bacteraemic phase, the organism disseminates through the body leading to secondary infections, which most commonly occur in the liver, spleen, bone marrow, gallbladder and Peyer's patches. Gallbladder invasion can occur from the blood or infected bile during initial invasion.

During acute infection, the median concentration of free bacteria is one cell per millilitre in blood, as 66% of cells during bacteraemia are internalised in phagocytic cells and 10 cells per millilitre in bone marrow. Typhoid induces both humoral and cellular immune responses, but this does not confer complete protection as reinfection can occur. In severe cases when left untreated, necrosis of the Peyer's patches occurs and leads to the late complication of perforation of the bowel, which is typically fatal without surgery, however mortality from treated patients in uncomplicated disease is <1%.

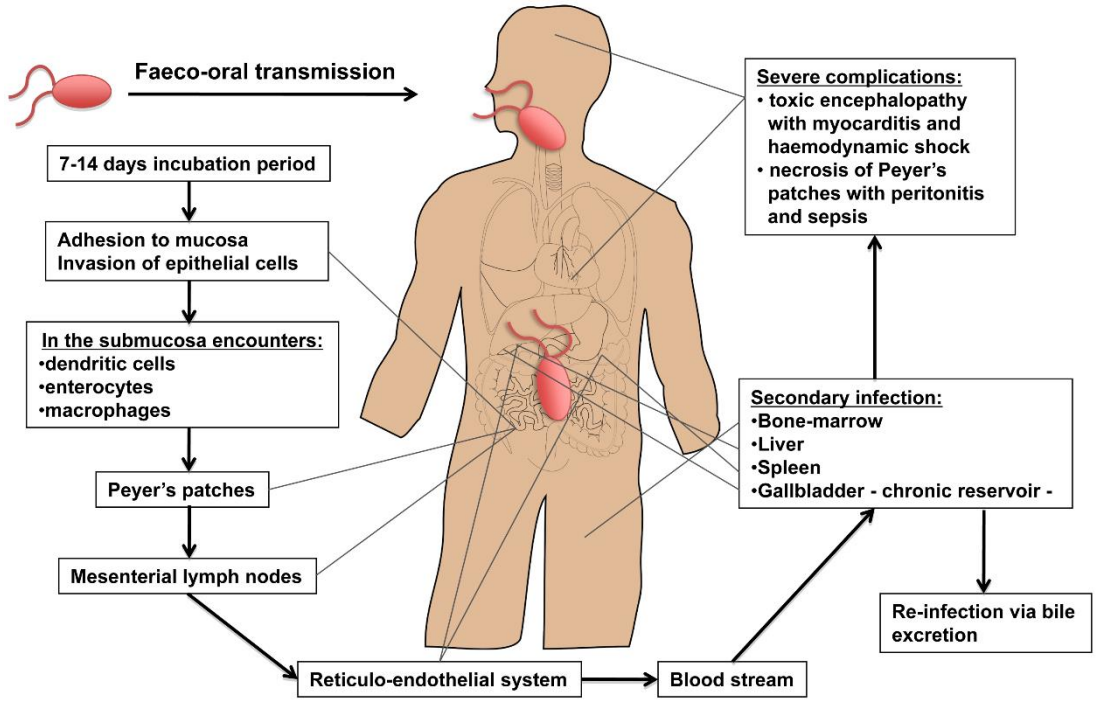


Figure 1.2: Dissemination of *S.* Typhi in the host during a systemic infection. Used with permission (de Jong *et al*., 2012) 10.1371/journal.ppat.1002933 .

S. Typhi can also produce biofilms in the gallbladder (Prouty *et al*., 2002) allowing persistence inflammation and chronic infection – this is associated with the development of gallbladder stones and an increase in gallbladder cancer but primarily allows the excretion of *S.* Typhi in bile. Organisms excreted in the bile can re-invade the intestinal wall or be excreted in faeces.

Clinical Features of typhoid fever is shown in Figure 1.3. Most cases of typhoid are in ages 5 – 25 but community studies in endemic regions have shown that many adult patients and those <5 years old, have non-specific illness not recognised clinically as typhoid. This non-specific illness may lead to misdiagnosis and inappropriate treatment, whilst also affecting local, national and global burden estimates. Between 60 to 90% of people with typhoid do not receive treatment or are treated as outpatients with oral antimicrobials. If patients attend healthcare facilities for the disease, it is usually after the onset of early symptoms, such as fever, after the first week post infection.

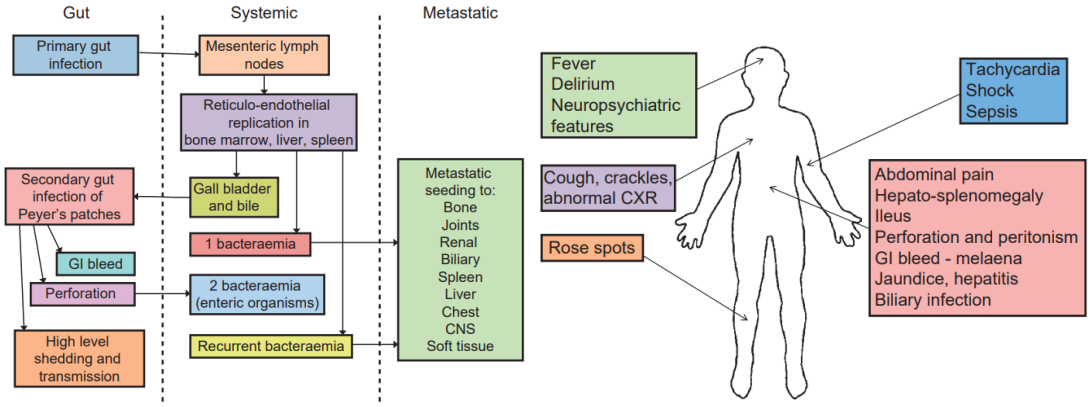


Figure 1.3: Diagram of the clinical presentations of *S.* Typhi during infection from Principles of Medicine in Africa, 2013 [Used with permission Feasey, N, Cambridge University Press 2013, 10.1017/cbo9780511751660.023].

1.3.1 Burden of Disease

Salmonella Typhi is a major cause of febrile illness and severe bacterial infection across sub-Saharan Africa, most commonly affecting children and young adults and arising in both high-population and low population density settings (Marks *et al*., 2017). Typhoid infections are typically associated with contaminated food and water, or poor hygiene, due to being transmitted through the faecal-oral route. Whilst endemic in many LMICs, high income countries have sporadic cases, typically associated with returning travellers from endemic regions. Outbreaks are associated with diverse risks, including contaminated water supplies in communities, close contact with those who have developed fever or are asymptomatic

carriers, food that is improperly prepared or typically eaten uncooked, or food that is improperly stored, such as the tinned corned beef from Argentina responsible for an outbreak in Aberdeen (Aberdeen outbreak), or food items that increase stomach pH such ice cream or drinks containing ice (Parry *et al*., 2002).

The burden of disease has been difficult to estimate with any precision as countries with endemic typhoid often have inadequate microbiological surveillance, which limits reported cases, with fever often being treated empirically if the patient is negative for Malaria. The most recent estimate of global burden concluded that in 2017 there were 14.3 million cases (ranging from 12.5 to 16.3m) of typhoid and paratyphoid fever worldwide. Whilst this showed a reduction of 44.6% of cases from 1990, 135,900 deaths were attributed to typhoid, showing a reduction of 41%. Of this estimate of typhoid fever, *S.* Typhi was responsible for 76.3% of cases (approx. 10.9m) (Stanaway *et al*., 2019), 1.5 million cases of typhoid were estimated for sub-Saharan Africa, of which, 728,100 were reported in south-eastern sub-Saharan Africa (Stanaway *et al*., 2019). The high incidence of typhoid in south-eastern sub-Saharan Africa could be due to an increase in reporting in addition to the causes seen in the rest of Africa and Asia (Kim *et al*., 2019, Kim *et al*., 2022).

In 2016, it was estimated that there were at least 16,144 cases of typhoid in Malawi, of which 227 resulted in death (Typhoid Vaccine Acceleration Consortium and Typhoid, 2018). In Queen Elizabeth Central Hospital (QECH), Blantyre, Typhoid cases between 1998 and 2010 had a mean occurrence of 14 microbiological diagnoses per year. With the introduction of the multi-drug resistant (MDR) H58 lineage in 2010, cases rose to 67 in 2011 and 782 in 2014 (Feasey *et al*., 2015). Between April 2015 and January 2017; 546 patients presented to QECH with typhoid symptoms, with infection confirmed by isolation of *S.* Typhi from blood (Gauld *et al*., 2021). In 2020, there were 178 cases reported at QECH, and within the first six months of 2021, 54 cases were reported (personal communication – Priyanka Patel/Melita Gordon). However, as cases require patients to be diagnosed for disease incidence, the burden of disease in Blantyre is likely much higher. In 2014, whilst there were 782 cases in QECH, the estimated incidence was 184.1 per 100,000 population, with a city-wide population of 800,264 in 2018 (Feasey *et al*., 2015, Brinkhoff, 2020).

Table 1.2: A brief timeline of the history of vaccines; a select number of outbreaks; successful clinical isolation; development of WASH infrastructure; treatment; and antimicrobial resistance of *S*. Typhi.

Year	1830	1840	1850	1860	1870	1880	1890	1900	1910	1920
Vaccine							First vaccine (Wright, 1896/Pfieffer and Kolle, 1896)	Carroll's challenge model 1904 (Woodward, 1980)	Isolation of Ty2 1918	
Outbreaks					Oxford Univeristy outbreak 1870					
Clinical isolation	Observation of disease by infection (Budd, 1838)	Infection cause proven (Budd, 1847)	Cause of disease published in The Lancet (Budd, 1859)		Discovery of Causative Organism (Eberth, 1879)	Isolation of causative organism (Gaffky, 1884)				
WASH		Oxford starts health reforms 1849			Introduction of Government loans for improved WASH infrastructure		Maistone introduces Water Chlorination	Licoln Introduces Water chlorinatioin - other urban areas begin to follow suite		
Treatment and Antimicrobial Resistance										

Year	1930	1940	1950	1960	1970	1980	1990	2000	2010	2020
Vaccine	First description of the Vi Antigen 1935 (Felix and Pitt,)				Creation of Ty21a 1977					
Outbreaks				Last UK outbreak (Aberdeen 1964)	Cholera outbreak in Santiago, Chile, identifies source of infection for *S*. Typhi also				Introduction of Haplotype H58 into Malawi (Feasey *et al.*, 2011	
Clinical isolation										
WASH										
Treatment and Antimicrobial Resistance		Introduction of Chloramphenicol treatment 1948	Chloramphenicol Resistance observed 1949/1950			Emergence of H58 Haplotype in Asia	Fluoroquinilone resistance documented; H58 established as dominant strain in Asia		Azithromycin reisstance in Bangledesh discovered	

1.4 Control Measures

Typhoid can be controlled through treatment of cases eradicating carriage, vaccination, improved WASH infrastructure, or some combination of these. Whilst many high- and middle-income countries have managed to eliminate endemic Typhoid solely through improvements to WASH infrastructure, for many endemic regions this is more challenging particularly with many countries, like Malawi, which have large rural regions; or areas with informal settlements that do not have adequate water treatment or access to clean, safe and affordable water. Figure 1.2 shows a brief timeline of major events or publications from the history of *S.* Typhi.

1.4.1 Vaccine

The first known record of typhoid vaccines was an inactivated whole cell vaccine isolated from clinical cases and killed through heating. Whilst there is some debate over whom created this vaccine first, or whether it was created independently, between Almorth Edward Wright (Wright, 1896) and Pfeiffer and Kolle (Gröschel and Hornick, 1981, Williamson *et al*., 2021), both vaccines were published a month apart in 1896 and used the same method based on the Haffkine's anti-cholera inoculations from 1892. The vaccine proposed by Wright was trialled in two men, with one being exposed to live typhoid after inoculation and did not develop illness (Waddington *et al*., 2014), as such, this vaccine was later used with the British and Indian armies.

In 1904, Carroll attempted to use the same method on volunteers including himself, however the inoculums were prepared incorrectly and resulted in most of the volunteers developing typhoid fever, though they did survive. Accidentally, this was the first description of a human challenge model for typhoid (Waddington *et al*., 2014) (Woodward, 1980).

In 1935, Arthur Felix and Margaret Pitt described the Vi antigen, noting some strains were more virulent than other strains (Felix *et al*., 1935). The discovery would lead to the development of the Vi capsular polysaccharide (ViCPS) vaccine in the 1970-80s, made by the purification of the ViCPS, and was first licensed in 1994. Additionally, the strain Ty2, which was used extensively by Felix and Pitt, would eventually be attenuated into Ty21a, which would develop into a live, attenuated vaccine in 1977 and licensed in 1983 (Germanier and Füer, 1975, Gilman *et al*., 1975, Waddington *et al*., 2014).

The next generation of typhoid vaccines, polysaccharide conjugate vaccines, have recently gone through phase 3 trials, most notably by the Typhoid Acceleration Consortium, (TyVAC), involving multiple groups and funders to research and implement these vaccines. To date, results have been good for safety and efficacy data (Jin *et al*., 2017, Meiring *et al*., 2017, Shakya *et al*., 2019, Yousafzai *et al*., 2021, Patel *et al*., 2021). Previously, only Pakistan had licensed the vaccine, due to an outbreak of ceftriaxone resistant *S.* Typhi (Yousafzai *et al*., 2019, Yousafzai *et al*., 2021), but more recently has been approved by Scientific Advisory Group of Expers (SAGE) and the WHO, whilst also licensed for use in Zimbabwe and Liberia (Birkhold *et al*., 2021) with a scheduled for rollout in Malawi in October 2022.

1.4.2 WASH

With the observations of many, such as William Budd, that typhoid is transmittable by water, and often associated with similar infections such as cholera, various interventions began to be implemented over the 19th and 20th century. Interventions were not linear or evenly distributed across the UK, USA or western Europe (Kirchhelle *et al*., 2019, Vanderslott *et al*., 2019). A good example is the city of Oxford in the UK. Sanitarians and civic engineers were able to slowly introduce reforms between 1849 and 1860, despite rejection by the community, university, local council, residents and adherents to the theory of miasma. However, it wasn't until an outbreak of typhoid at the University of Oxford that resulted in student's deaths in the 1870s, that sanitation issues of the city were acknowledged, with the working classes being the worst affected (Vanderslott *et al*., 2019). This story is far from popular myth of a centralised push toward improved WASH infrastructure in Victorian Britain. Instead, a series of cheap government loans with lengthy repayment schemes (up to 200 years) led to many municipalities across England investing in improved sanitation. Most notably better piped water, drainage, introduction of plumbed water closets, and sewage farms – instead of disposal into local rivers and estuaries such as the Thames. Later, slow sand filtration and improved water reservoirs were introduced (Vanderslott *et al*., 2019).

In 1897, a town in Kent called Maidstone was one of the first to implement chlorination of all domestic water and was one of the first to enforce this for the entire water supply permanently (Majesty, 1897). Similarly, a typhoid outbreak in Lincoln in 1905 led to a chlorination programme for all domestic water (Majesty, 1905) with other towns and cities eventually adopting the practice. Despite increasing improvements in sanitation in the UK occurring in the mid-19th century, outbreaks still occurred in the UK until the Aberdeen

outbreak in 1964, which related to importation of corned beef from Argentina (Forbes, 1879, Hart, 1895, Aberdeen, 1966).

Over the course of the 20th century, the USA and European countries improved sewage treatment and adopted sanitary practices to improve domestic water quality. More recently, middle-income countries also have had success in eliminating or significantly reducing typhoid, such as Santiago, Chile in the 1970s, or Vietnam in the 2000s, due to improved economies leading to better WASH infrastructure and education. In Santiago, improvement in WASH infrastructure at point of access had effectively reduced, but not eliminated, typhoid cases in the city. An outbreak of cholera within the city led to the discovery that untreated sewage was being discharged directly into rivers, which were used for irrigation of crops. Once this practice had stopped, typhoid cases stopped (Sears *et al*., 1984). Between 1998 and 2014, Vietnam experienced a large economic boom, providing a better standard of living for the average citizen, along with better healthcare provision, WASH infrastructure and vaccination programmes (Nga *et al*., 2018).

1.4.3 Antimicrobial Chemotherapy

Current treatment of *S.* Typhi is typically based on one of three classes of antibiotics, due to the emergence of MDR strains and emergence of extreme drug resistance, which include fluoroquinolones (typically ciprofloxacin), the azalide azithromycin and 3rd generation cephalosporins such as ceftriaxone. Therapy should be chosen based on local resistance patterns, availability of oral medication and the clinical setting (Torok *et al*., 2016).

Examples of antimicrobial regimens for typhoid include:

- Fluoroquinolones:
    - Ciprofloxacin at a dose of 500 mg orally twice per day for 7-10 days
        - Bactericidal, intracellular and concentrates in bile.
- Third generation cephalosporins:
    - Ceftriaxone, 2 g daily IV for 10-14 days
        - Used in severe disease or fluoroquinolone resistant cases
    - Cefixime, 20 mg per patient weight in kg, halved and taken twice per day for 7-14 days, orally
- Azithromycin (Macrolide), 1 g orally on admission/diagnosis followed by 500 mg to 1 g daily for 7 days.

In the event of a relapse, patients will be given an additional course of which ever antimicrobial the infecting strain is susceptible to (Torok *et al*., 2016). Currently, in QECH, Blantyre, Malawi, the empirical management of suspected sepsis is IV ceftriaxone, followed by oral ciprofloxacin once susceptibilities are known. In the event of severe illness, surgery may be required to repair damage from early ileal perforation. Intravenous fluids may be necessary if prolonged fever and diarrhoea are preventing patients from taking on sufficient fluids orally. For convalescent carriers, prolonged antimicrobial therapy is required, typically a course of fluoroquinolones for four weeks and often the gallbladder may be removed due to biofilm establishment and persistence in infected individuals (Torok *et al*., 2016).

1.4.3.1 Antimicrobial Resistance

In 1948, chloramphenicol was introduced as a treatment for *S.* Typhi, however, less than two years later, resistance to chloramphenicol was documented to be emerging. Chloramphenicol inhibits protein synthesis by binding to the 50S ribosomal subunit and inhibiting peptide chain elongation, whilst resistance is primarily due to the acquisition of chloramphenicol acetyltransferase, which causes acetylation, preventing binding. In 1972, resistance to chloramphenicol caused major problems for several countries with resistant outbreaks due to the acquisition of an IncH1 plasmid containing chloramphenicol hydrolysing enzymes, and other antimicrobial resistance (AMR) genes conferring resistance to tetracycline, sulphonamides and streptomycin.

By the 1980-90s resistance to all first line antibiotics for typhoid treatment, chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole had emerged due to clonal dissemination MDR isolates. These led to the adoption of fluoroquinolones in some regions of Asia. Resistance to fluoroquinolones subsequently emerged, with genetic analysis showing dominant strains in the area having mutations in the *gyrA* gene, one of the main targets of actions for fluoroquinolones. In the 1990s, an emerging subtype: Haplotype H58, established itself in Asia, containing both the MDR IncHI1 plasmid and fluoroquinolone resistance mutations in *gyrA*. No further reports of mutations in the other topoisomerase were reported in H58, allowing fluoroquinolones to still be useful, even with decreased susceptibility. Whole genome sequencing of CT18, which was an MDR strain though not a H58 haplotype, revealed that the IncHI1 plasmid contained; *dhfr1b* for trimethoprim resistance, *sul2* for sulfonamides resistance, *cat*I for chloramphenicol resistance, *blu* for TEM-1 and ampicillin resistance, *tetA/tetC* for tetracycline and *strAB* for streptomycin resistance (Parkhill *et al*., 2001).

With the emergence of MDR, including fluoroquinolone resistance in Asia, ceftriaxone and azithromycin became common drugs for the treatment of typhoid, with azithromycin being preferred in uncomplicated cases as it can be taken orally. Ceftriaxone resistance was once sporadic, however there has recently been a massive outbreak of cephalosporin resistant typhoid in Pakistan (Saha *et al*., 1999, Yousafzai *et al*., 2019). Additionally, an azithromycin resistant strain was identified in Bangladesh, with the SNP responsible for this change in susceptibility being identified as arginine (R) 717- on the *arcB*, with R being replaced by either glutamine (Q) or leucine (L) so far (Ahsan and Rahman, 2019).

In Malawi, cases of typhoid were low in the early 2010s until emergence of the H58 lineage in sub-Saharan Africa, with 99% of cases becoming MDR by late 2011, leading to a large outbreak in late 2012 to early 2013 as seen in Figure 1.5 (Feasey *et al*., 2015).

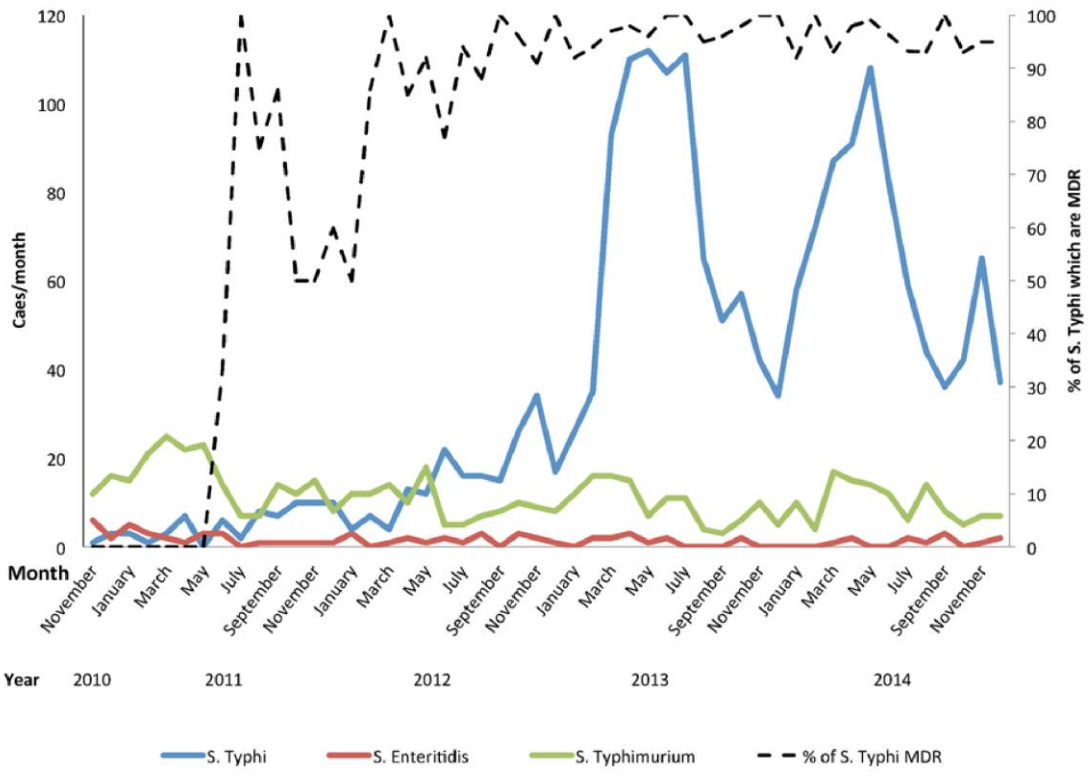


Figure 1.4: Monthly trends in bloodstream invasive Salmonella diagnosed at QECH from November 2010-October 2014. Provided by Feasey (Feasey *et al*., 2015).

1.5 Diagnosis of typhoid fever

In endemic regions, typhoid will often be diagnosed clinically and treated empirically without microbiology testing, however this is a deeply flawed approach as typhoid is clinically indistinguishable from many causes of febrile illness early in its course. Ideally, blood specimens should be taken for culture on automated systems such as the Bact/Alert (BioMerieux) or Bactec (Becton, Dickinson and Company) systems.

When blood cultures flag as positive, organisms can be further identified using biochemistry, for example with an API 20E, with the test results for *S.* Typhi shown in Table 1.2 for biochemical reactions; or with the use of MALDI-TOF platform. Using the Kauffmann-White classification (Salmonella Subcommittee of the Nomenclature Committee of the International Society for, 1934, Grimont and Weill, 2007), the strain can be identified using serology based on identifying O antigens Vi, O9 and O12 and H antigen, Hd, surface antigens. In circumstances where culture is not possible, PCR on clinical specimens can be performed, with many targets available, primarily *staG*, *stgA* or *fliC* that encode for two fimbrial proteins and a flagellin, respectively (Baker *et al*., 2005, Nga *et al*., 2010, Yin Ngan *et al*., 2010).

One of the more controversial tests used for diagnostics of typhoid fever is the Widal Test. Introduced in 1896 by Georges-Fernand Widal, the test is an indirect agglutination test, where *S.* Typhi is mixed with serum from a patient. However, this method is affected by both IgG and IgM – meaning long term immunity due to either previous infection, or vaccination, can give a false positive. Whilst studies report a sensitivity of 80-90%, some report as low as 30% (Khan *et al*., 2012), specificity is much lower at 10-20%, with multiple false positives and negatives, leading to misdiagnosis if the test is relied upon (Abraham *et al*., 1981, Olopoenia and King, 2000, Willke *et al*., 2002, Mawazo *et al*., 2019). Whilst improved antigen rapid tests have since come to market, when compared to other forms of diagnostics, these still perform poorly.

Table 1.4 shows the full range of biochemical tests that can be performed for the identification of *S.* Typhi, including sugar fermentation reactions and enzyme activity, most biochemistry would be done with a BioMerieux API20E, which contains a subset of 12 reactions that can positively identify *S.* Typhi from other NTS strains (Holmes *et al*., 1978).

1.5.1 Detection in the environment

Given the association of Typhoid with the environment, environmental isolation seemed a rational next step, and many attempts have been made. These attempts have shown that whilst not impossible, it is difficult to culture *S.* Typhi from natural waters, despite infection being associated with contaminated water sources. Figure 1.3 show timelines of the methods and uses of methods to isolate *S.* Typhi from sewage, food and other environmental samples over the past century.

Multiple media have been utilised over the last century, however, the media that remained in use for the longest time seemed to be Selenite based media, primarily Selenite F broth, Deoxycholate Citrate agar (DCA), Bismuth Sulphite Agar (BSA) and Xylose Lysine Deoxycholate (XLD) (Figure 1.6). The primary issue with these older media today is either their lack of selectivity or their toxicity. Some of these media have been replaced with products that perform better, whilst others have been replaced with less toxic, but less selective alternatives that make isolation from complex background flora more difficult.

Table 1.3: A synopsis of key publications between 1928 and 2022 that discuss the isolation of *S.* Typhi from environmental sources.

1928	● Use of MacConkey lactose bile salt agar and bismuth sulphite agar (BSA) - Wilson, 1928
1931	● Continued development of BSA for isolation from sewage - Wilson & Blair, 1931
1933	● Detection of *S.* Typhi in domestic damn water using biochemistry and BSA - Wilson, 1933
1938	● Showed *S.* Typhi could proliferate in milk and butter - Pullinger & Kemp, 1938
1945	● Introduction of double strength Selenite-F broth to increase recovery of *S.* Typhi - Hobbs & Allison, 1945
1945	● Improved recovery with pre-enrichment in liquid deoxycholate citrate media ● Demonstrated inhibitory effect of Brilliant Green on *S.* Typhi - Gell, Hobbs, & Allison, 1945
1948	● Creation of sewage swab, referred to as the Moore swab. ● Use of double strength selenite-F broth subcultured onto deoxycholate citrate agar (DCA) - Moore, 1948
1951	● Use of the Moore method of Moore swab, cultured in selenite-F broth and subcultured on both DCA and BSA to improve recovery - Lendon & Mackenzie, 1951
1951	● Compared culture of Moore swab in selenite-F and tetrathionate broths, showing selenite-F as superior for pre-enrichment, but both superior to direct plating on DCA - Jones, 1951
1952	● Use of Moore swabs to detect asymptomatic carriers in Sidmouth ● Washed swabs in nutrient broth transferring into selenite-F and subcultured onto BSA - Moore, Perry, & Chard, 1952
1955	● Deployed Moore swabs for multiple organisms and described use of modified Endo medium for *S.* Typhi isolation - Kelly, Clark, & Coleman, 1955
1955	● Use of Moore/Alison and Hobbs method with double strength selenite-F but introduced serial dilutions at 24 and 48 hours before subculture - Murdock & Lawson, 1955
1959	● Use of Moore method to track a carrier in High Sierra, California. Positive sample after 3 days, negative for following 5 days, then positive for 3 consecutive days. - Shearer, Browne, Gordon, & Hollister, 1959
1968	● Deployment of 2,300 Moore swabs during Aberdeen out break. Multiple methods used, but described first use of Ringer's solution as an osmotically stable diluent for samples. - Callaghan & Brodie, 1968
1972	● Use of sanitary towels instead of Moore swabs and addition of lactose to selenite-F broth - Conn, Heymann, Jamieson, McWilliam, & Scott, 1972
1984	● Use of Moore swabs in river and irrigation canals of Santiago, Chile. Swabs were cultured in selenite-F broth then subcultured onto BSA, DCA and Shigella-Salmonella agar, with suspect colonies confirmed by triple iron sugar agar. - Sears et al., 1984
1986	● Deployed swabs in known carrier's household's sewage pipes. ● Use of dilution series when subculturing from swabs. One diluted sample positive of the negative undiluted samples - Sears, Ferreccio, & Levine, 1986
1995	● Moore swabs used in domestic waters, cultured in Selenite F broth, subcultured onto XLD and confirmed by API20E - al-Quarawi, el Bushra, Fontaine, Bubshait, & el Tantawy, 1995
2011	● Use of membrane filtration for culture and molecular environmental surviellance ● Cultured attempted by Tryptone Soya Broth, Mueller Hinton, Xylose Lysine Deoxycholate and MacConkey - Baker et al., 2011
2021	● Discussion on the revival of Moore swabs for the isolation of *S.* Typhi from sewage and rivers. - Sikorski & Levine, 2020
2021	● Included in this thesis, method proposed for the detection and isolation of S. Typhi from River's in Blantyre, Malawi – See Chapter 3 - Rigby et al., 2022
2022	● Method using Moore Swab submitted to the Bill and Melinda Gates Foundation. - VanTassell, Raymond, Wolfe, Liu, & Moe, 2022

With these difficulties and the improvement of molecular detection techniques, many studies moved towards molecular detection alone for identifying *S.* Typhi in the environment, typically based on nucleic acid amplification technique (NAAT). NAAT has problems of its own, not least of which is these approaches do not yield a whole genome sequence, which permits discrimination between isolates, neither can organism viability be confirmed. In this thesis, I have therefore returned to culture-based methodologies.

Advancement of microbiology has occurred over the 20th century, particularly in the clinical microbiology field, where isolation of the bacterium from stool, urine, blood and especially bone marrow, improved significantly. Culture for environmental samples has been primarily limited to sewage or heavily faecally contaminated water, or foods with expected high numbers of bacteria, which are typically cultured in the same way as clinical stool samples. Table 1.3 identifies various media used for the isolation and culture of *S.* Typhi outlining the microbiological developments.

The literature review identified media which are listed in Table 1.5 for the isolation of *S.* Typhi and allowed thirty to be selected for further consideration. Of these, 16 were eliminated as being unsuitable. The reason for elimination is shown in Table 1.5 included logistics, shelf-life and a reliance on unreliable distinguishing criteria for the identification of *Salmonella* spp. (Wilson, 1948, Kunz and Ewing, 1965, Aksoysan *et al*., 1981). Further work on the selection of these media are detailed in Chapter 3.

Table 1.4: List of biochemical, sugar, enzyme and anti-sera reactions of *S.* Typhi; + indicates a positive reaction and – indicates a negative or no reaction.

Biochemistry of *S.* Typhi									
Characteristic	Capsule	Catalase	Citrate	Flagella	Gas	Gelatine Hydrolysis		Growth in KCN	
Reaction	-	+	-	+	-	-		-	
Characteristic	Gram Staining		H2S	Indole	Motility	Methyl Red	MUG Test	Nitrate Reduction	
Reaction	-		+	-	Motile	+	-	+	
Characteristic	Oxidase	Pigment	Shape	Spore	Triple Sugar Iron Agar		Urease	Voges Proskauer	
Reaction	-	-	Rod	-	Alkali/Acid		-	-	
Sugar fermentation									
Fermentation	Adonitol	Arabinose	Arabitol	Cellobiose	DNase	Dulcitol	Erythritol	Esculin Hydrolysis	Glucose
Reaction	-	-	-	-	-	-	-	-	+
Fermentation	Glycerol	Inositol	Lactose	Malonate	Maltose	Mannitol	Mannose	Melibiose	Mucate
Reaction	-	-	-	-	+	+	+	+	-
Fermentation	Myoinositol	Raffinose	Rhamnose	Salicin	Sorbitol	Sucrose	Tartrate	Trehalose	Xylose
Reaction	-	-	-	-	+	-	+	+	+
Enzymatic Reactivity									
Enzyme	Acetate Utilization	Arginine Dehydrolase	Esculin Hydrolysis	Lipase	Lysine	Ortho-Nitrophenyl-β-galactosidase	Ornithine Decarboxylase	Peroxidase	Tyrosine Hydrolysis
Reaction	-	-	-	-	+	-	-	-	-
Antisera Reactivity									
Serology	H Phase 1		H Phase 2		O Group		O antigens		
Reaction	d		No phase 2 antigen		D		O9, O12, Vi		

Table 1.5: List of media for the isolation and culture of Salmonella spp. identified from literature review; their purpose, advantages and disadvantages.

Media	Purpose	Pro	Con	Reference
Brilliant Green Agar	Used for the isolation of Salmonellae since 1925	NTS culture only	Selectivity is reliant of the addition of brilliant green, triarylmethane dye, which is inhibitory to *S.* Typhi.	(Read and Reyes, 1968)
Bromo-Thymol-Blue Lactose Agar	Isolation from faeces. Used in conjunction with Litmus Lactose Agar.	Outdated for *S.* Typhi, but does successfully culture	Reliant on Lactose fermentation to distinguish colonies	(Vogelsang and Bøe, 1948)
BSA	Bismuth Sulphite Agar is a modification of the original Wilson and Blair selective medium for the isolation and preliminary identification of *Salmonella* Typhi	Successfully cultures *S.* Typhi and still in use, but outdated	Reliant on hydrogen sulphite production	(Wilson, 1928)
CASE	A novel chromogenic agar in use for the selection of NTS	Chromogenic agar using esterase which is a more reliable enzyme in *Salmonella* spp.	Weakly grows *S.* Typhi	https://www.neogen.com/categories/microbiology/harlequin-chromogenic-agar- *Salmonella*-esterase/
Columbia Blood Agar (CBA)	A multi-purpose medium, the addition of blood increases growth of fastidious organisms.	Very luxuriant *Salmonella* spp. growth	Non-selective and use for pure growth rather than isolation.	(Ellner *et al*., 1966)
DCA	A modification of Leifson's medium for the isolation of salmonellae and shigellae.	Outdated for *S.* Typhi, but does successfully culture	Reliant on Lactose fermentation to distinguish colonies	(Hynes, 1942)

Endo's Agar	Originally used to isolate *S.* Typhi, currently used for coliform bacteria	Outdated for *S.* Typhi, but does successfully culture	Reliant on Lactose fermentation to distinguish colonies	(Genung, 1926)
Eosin Methylene Blue (EMB) Agar	Used for the identification of Gram-negative coliforms and faecal bacteria and inhibits Gram-positive and fastidious Gram-negative organisms.	Outdated for *S.* Typhi, but does successfully culture	Differentiation based on lactose fermentation but is a non-selective agar, allowing false positives and competitive organisms to grow.	(Levine, 1918)
Hektoen Enteric (HE) Agar	A selective and differential agar used for the distinction between *Shigella* and *Salmonella* spp.	Useful for identification of NTS and *Shigella* spp.	Unable to distinguish *S.* Typhi from other *Salmonellae* with lower recovery rates for *S.* Typhi than other media and known failures to produce H_2S reaction.	(King and Metzger, 1968)
LB Miller Agar	A rich multi-purpose medium	Very luxuriant *Salmonella* spp. growth	Non-selective and use for pure growth rather than isolation	(Kingsley *et al*., 2018)
Litmus Lactose Agar	Isolation from faeces when used in conjunction with Bromo-Thymol-Blue Agar.	Outdated for *S.* Typhi, but does successfully culture	Reliant on Lactose fermentation to distinguish colonies	(Vogelsang and Bøe, 1948)
MacConkey	A differential medium for intestinal pathogens, reliant on bile salts for selection	*Salmonella* spp. are able to grow	Reliant on Lactose fermentation to distinguish colonies	(Elazhary *et al*., 1973)

Mueller Hinton Agar	A rich media, typically used for antimicrobial susceptibility testing	Very luxuriant *Salmonella* spp. growth	Non-selective and use for pure growth rather than isolation	(Mueller and Hinton, 1941)
Other *Salmonella* Chromogenic Agar	Use of Chromogens is more sensitive and specific than the use of lactose fermentation and production of H_2S.	Easily identifiable *Salmonella* spp. colonies	Comparison of ABC to CASE media showed that the chromogens selected for CASE was more specific than those used in ABC agar and the other chromogenic agar.	(Rambach, 1990)
***Salmonella*-Shigella Agar**	A modification of Deoxycholate Citrate Agar, this is a selective and differential plate used for the distinction between *Shigella* and *Salmonella* spp.	Useful for identification of NTS and *Shigella* spp.	Does not readily distinguish between *Salmonella* spp.; reliant on hydrogen sulphite production and contains brilliant green ,which is inhibitory to *S.* Typhi	(Leifson, 1935)
XLD	Medium for the selection of *Shigella* and *Salmonella* spp. utilising sodium deoxycholate as the selective agent	*Salmonella* spp. are able to grow	Reliant on Lactose fermentation to distinguish colonies	(Taylor and Schelhart, 1968)
Brain Heart Infusion broth	A highly nutritious broth used for fastidious organisms	Very luxuriant *Salmonella* spp. growth	Non-selective and use for pure growth rather than isolation.	(Wain and Hosoglu, 2008)
Enterobacteriaceae - Enrichment (EE) Broth	A rich media for recovery of sub-lethally damaged Enterobacterales	NTS culture only	The inclusion of the triarylmethane dye, brilliant green, is inhibitory to typhoidal *Salmonellae*. This media was adapted as the 2%	(Mossel *et al*., 1963)

			bile broth used in the study was a modification of EE broth with the dye removed.	
Luria broth	A highly nutritious general-purpose broth for bacteria	Very luxuriant *Salmonella* spp. growth	Non-selective and use for pure growth rather than isolation.	(Kingsley *et al*., 2018)
Rappaport Vassiliadis Broth	Preferred selection broth for *Salmonella* spp.	NTS culture only	Inappropriate for *S.* Typhi due to its sensitivity to the triarylmethane dye, malachite green.	(Konforti *et al*., 1956)
Selenite Cystine broth	Selenite F broth with the addition of cystine to reduce toxicity.	Highly selective for *Salmonella* spp.	Biselenite, used to produce the selective component of the media, is toxic, especially when heated. Discontinued in large quantities.	(Leifson, 1936)
Selenite F broth	A historic selective enrichment broth for the cultivation of *Salmonella* spp. and S*higella* spp., still in use today	Highly selective for *Salmonella* spp.	Biselenite, used to produce the selective component of the media, is toxic, especially when heated	(Leifson, 1936)
Selenite Mannitol Broth	Assessed historically with Selenite F and Selenite Cystine broths. Replaces the lactose found in Selenite F with mannitol, making it a comparable medium to	Highly selective for *Salmonella* spp.	More recent studies imply there is no significant difference in performance to Selenite Cystine and Selenite F broth. Less readily available than Selenite F broth.	(Leifson, 1936)

	Tetrathionate broth for the isolation of *S.* Paratyphi.		Biselenite, used to produce the selective component of the media, is toxic, especially when heated	
Tetrathionate Broth	A selective growth media for *Salmonella* spp. including *S.* Typhi. Typically used in clinical settings, but also for sewage and food microbiology. Often used in parallel with selenite-based media for *S.* Typhi and Paratyphi isolation.	Selective broth media effective for selection of *Salmonella* spp. including *S.* Typhi	Limited shelf-life due to self-generating tetrathionate selective element makes it impractical as reaction begins once iodide and iodine added to the broth and so this media could only be used on day of production.	(Pollock and Knox, 1943)
Tryptone Soya broth	A highly nutritious general-purpose broth for bacteria	Very luxuriant *Salmonella* spp. growth	Non-selective and use for pure growth rather than isolation.	(Baker *et al*., 2011)
Universal Pre-Enrichment broth	Used for sub-lethally damaged cells from environmental samples.	Very luxuriant *Salmonella* spp. growth	Non-selective and less readily available in the UK due to production and supply being based in the USA.	(Juven *et al*., 1984)

1.5.2 Molecular Methods

Due to the difficulty of laboratory set up required for culture, molecular methods have replaced isolation of the organism from environmental sampling for much of the late 20th and early 21st century, with only a few studies still attempting culture, and largely with limited success. A variety of targets have been assessed for PCR detection and quantification of *S.* Typhi from clinical samples, these are listed in Table 1.4.

Table 1.6: Various PCR primer targets used for *S.* Typhi

Gene target	Function	Present/Absent	Organism	Reference
ttr	Tetrathionate respiration	+	Pan-*Salmonella*	(Hopkins *et al.*, 2009)
staG (STY0201)	Fimbrial protein	+	Typhi	(Nga *et al.*, 2010)
stgA	Fimbrial protein	+	Typhi	(Forest *et al.*, 2007)
sseJ	Decreases cytotoxicity	-	Typhi, Paratyphi A. B	(Nair *et al.*, 2019)
tviB	Part of ViaB operon-Vi polysaccharide synthesis	+	Typhi, Paratyphi C	(Nair *et al.*, 2019, Nair *et al.*, 2004)
*inv*A	Invasion flagellin	+	Pan-*Salmonella*	(El-Sayed *et al.*, 2015)
*fli*C	flagellin	+	Typhi	(El-Sayed *et al.*, 2015)
*cly*A (*hly*E)	Cytolysin	+	Typhi Paratyphi	(Tennant *et al.*, 2015)
*fli*C-d	Flagella	+	Typhi	(Zhou and Pollard, 2010)
STY0307	Hypothetical protein	+	Typhi	(Goay *et al.*, 2016)
STY0322	Hypothetical protein	+	Typhi	(Goay *et al.*, 2016)
STY0326	Conserved hypothetical protein	+	Typhi	(Goay *et al.*, 2016)
STY2020	Putative bacteriophage protein	+	Typhi	(Goay *et al.*, 2016)
STY2021	Putative bacteriophage protein	+	Typhi	(Goay *et al.*, 2016)
16S rRNA	mRNA gene	+	Typhi	(Karkey *et al.*, 2016)

Several gene targets were identified from the literature as being used for *S.* Typhi PCR detection, including *staG*, *stgA*, *tviB*, *fliC-d*, *prt* (Baker *et al.*, 2005, Wain and Hosoglu, 2008,

Yin Ngan *et al*., 2010, Goay *et al*., 2016, Nair *et al*., 2019). Most of these targets are not unique to *S.* Typhi and have cross-reactivity with one or more non-typhoidal organisms, including: *S.* Paratyphi C; *S.* Dublin; *S.* Cholerae-suis and *Citrobacter freundii* (Townsend *et al*., 2001, Goay *et al*., 2016, Nair *et al*., 2019). The list of other potential targets is increasing over time as whole genome sequence data collections improve, including genes that are less well characterised such as hypothetical proteins and putative bacteriophage proteins (Goay *et al*., 2016). The Nair *et al.* (2019) assay includes a series of single target real time PCR reactions to identify the typhoidal salmonellae *S.* Typhi and *S.* Paratyphi A, B and C from NTS based on seven primer pairs and probes and was chosen to be modified due the work performed on those target assays utilising an *in-silico* PCR that had been applied to the collection of *Salmonella* spp. genomes (n=1,882).

1.5.3 Environmental Sampling

At present, methods for the detection of *S.* Typhi from environmental sources are unreliable. Whilst sewage has intermittently been successful for the culture of *S.* Typhi, in Malawi, sanitation systems are infrequently present and even where they exist, are often inoperable. Instead, we must look to river water, however this tends to be more challenging due to rivers being a constantly changing environment with large volumes of water diluting the sample, UV radiation from sunlight, changing chemical composition and fluctuating level of contamination among other considerations.

Approaches to sampling river water can be divided into trap and grab samples. Trap samples are defined as anything that remains in the sampling environment for a longer period allowing concentration of the sampling matrix to occur onsite, giving the potential of increasing the load of target organism at the expense of being able to quantify the samples, or loss of sampling apparatus. Grab samples are individual samples taken from the environment – most notably a water sample, which would give a "snapshot" of how the biological make-up is at the time of sampling, when collecting. These can be quantified and are always available for collection but are often less sensitive than trap samples. One of the more successful approaches to "trapping" *S.* Typhi from the environment prior to detection using culture has been the deployment of Moore swabs. These were invented in the 1950's by Moore and are made from a sterile piece of surgical gauze 15 cm wide by 120 cm long and folded until 8-ply and tied in the centre with fishing line, as shown in Figure 1.7 (Sikorski and Levine, 2020).

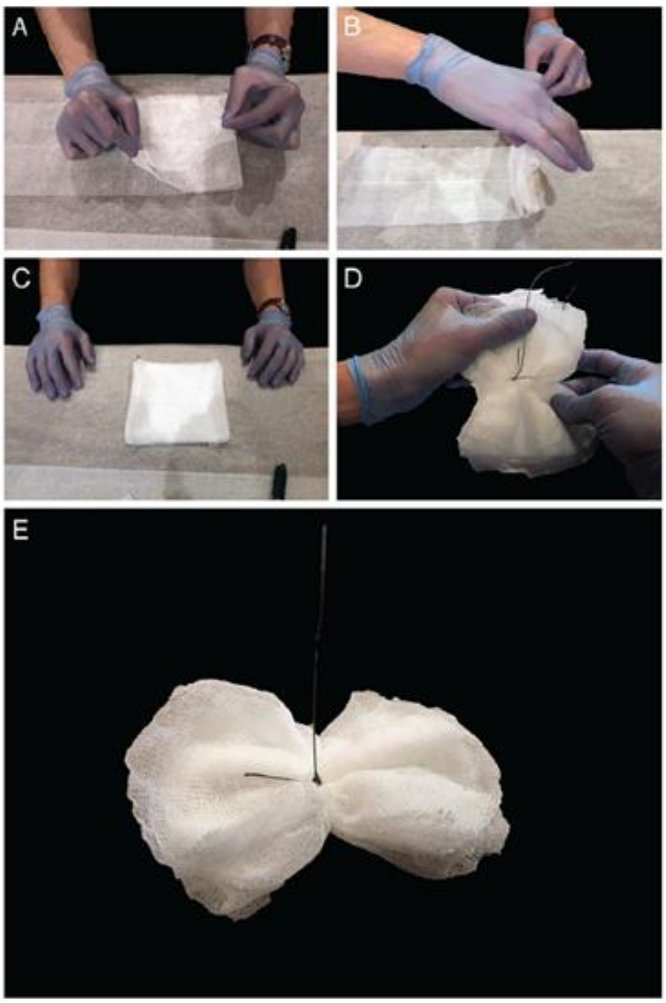


Figure 1.5: "Constructing a Moore swab. (A and B) A length of gauze 6 inches by 48 inches [152.4 x 1219.2 mm], is folded onto itself in a pleated pattern to form a pad. (C and D) The gauze is tied at the center with high-test fishing line. (E) The Moore swab may be suspended in flowing sewers of surface waters." Instructions for the creation of a Moore swab, from Sikorski and Levine (2020).

These swabs are then deployed in areas with flowing water for 24 – 96 hours depending on publication. A recent example of their successful deployment was in Santiago, Chile (Sears *et al*., 1984). This area had been associated with typhoid cases, but direct plating of the water yielded no *S.* Typhi positive isolates, as such, following the traditional Moore method, swabs were placed in the river for 48-72 hours and then retrieved and cultured in Selenite-F broth. Whilst recovery of organisms was low, four swabs were culture positive out of 45 recovered. Swabs were also deployed in irrigation channels, with eight out of 76 being culture positive. Subsequently, the efficacy of Moore swabs were assessed in the community (Sears *et al*., 1986). Deploying swabs in the drains of households of ten asymptomatic carriers, only 25%

returned a culture positive (six out of 24 swabs) whilst 50% of the households were confirmed a culture positive (five out of ten households).

This study shows the importance of the sample collection method used for culture, which is the first series of questions: What, when and how? As seen in Figure 1.6, starting from an assumed burden of disease based on clinical data, or using population density data within the region of interest, locations across an area, in this case, Blantyre, Malawi, need to be selected and a frequency of sample to be decided. Time of day is also important, as some studies have shown that not just seasonality, but time of day affects likelihood of target detection.

Figure 1.6: Diagram of ES challenges and outcomes as they are considered, encountered and bridged to create an ES programme.

The next major question is whether the culture methods of today are sufficient. Environmental isolates of pathogenic organisms are often sub-lethally damaged by unfavourable conditions and when isolated by laboratories, are often put in a media such as Buffered Peptone Water (BPW) as an osmotically stable diluent. However, as a human restricted organism, *S.* Typhi is easily outcompeted by environmental organisms and other

faecal contaminants such as NTS, *E*. *coli* and coliform bacteria. As such, media selected for the culture, isolation and successful identification of *S.* Typhi would both need to help the organism recover from the sub-lethal damage, but also help put selective pressure on the sample to prevent being outcompeted.

The extensive use of molecular methods for environmental detection is a viable alternative until a robust culture method is developed, however these still harbour some distinct flaws. With the use of PCR, DNA can be detected, however DNA is known to persist and can be detected after long periods of time (Zhou *et al*., 2018). Reverse transcriptase PCR is an alternative for detecting live pathogens, but RNA has been shown to be stable and detectable for up to 21 days free in the environment (Tsai *et al*., 1995). As such, molecular methods cannot reliably inform the origin or even exact location of a viable organism, which adversely affects its use as a surveillance tool to inform the best locations for WASH interventions and vaccination programmes. One of the other major drawbacks to a molecular only approach is the search for specific genes that can be targeted by primers whilst also being sensitive enough to detect low titres within the environment. The current primer of choice is STY0201 (also known as *staG*), which encodes a putative fimbrial protein, for the detection of *S.* Typhi in clinical blood samples via PCR (Nga *et al*., 2010). However, whilst there is appeal in a single target assay, other studies have argued that this is not specific enough, a paper from United Kingdom Health Security Agency (UKHSA) shows that the *staG* targ*et al*one is not sufficient due to the *in silico* analysis showing it gave 41 serovars a false positive out of 952 NTS strains (Nair *et al*., 2019).

Once identification of the organism is confirmed, there is the potential for a more accurate disease burden within the city to be determined, with hotspots of infection identified, whether that be due to where people are living with their infection, or whether they are shedding faeces into those areas – interventions can be considered to prevent further spread of the pathogen within the environment and thereby reduce community transmission. Once the primary technical issue of detection of the organism has been resolved, other questions can start to be addressed, for example:

- What is the environmental load of the pathogen?
- How frequently is it present in the environment and therefore how frequently do we need to sample?
- What volumes of sample need to be taken?

When these questions have been answered, we might start to answer questions about the utility of ES of *S.* Typhi in different contexts. For example:

- Will this always be a research tool?
- Can it be delivered in a cost-effective manner?
- Can it inform targeted deployment of vaccine and WASH strategies?

1.6 Objectives and Aims

The aim of this study was to demonstrate that *S.* Typhi can utilise the environment, including river water, sewage, sediment and food, as a mode of long cycle transmission within endemic communities. Whilst this has been explored by other studies, this study aimed to create a toolset that could be utilised for ES in other endemic regions for the detection, isolation and confirmation of *S.* Typhi.

The objectives of this study included:

- To develop and optimise a novel culture pathway for the isolation of *S.* Typhi from complex matrices, such as river water;
- To identify *S.* Typhi from the environment by PCR;
- To confirm *S.* Typhi isolates by PCR;
- To establish an ES programme in Blantyre, Malawi, where *S* .Typhi is endemic.

2 Materials and Methods

2.1 Summary

This chapter covers the basic methodologies used throughout this project and thesis. Whilst there are variations on some methods for optimisation work, particularly discussed in Chapters 3, 4 and 7, the methods here are listed in their final format.

Blantyre, Malawi, is an endemic region for *S.* Typhi with access to a centralised hospital, QECH, and research institutes which have close ties with this hospital, in particular Malawi-Liverpool Wellcome Trust (MLW) and Kamuzu University of Health Sciences (KUHeS). In order to deliver this project, a new laboratory space had to be established, which was done in collaboration with KUHeS, due to being located within the Blantyre College of Medicine, which later merged with the Nursing college to form KUHeS. Whilst preliminary work was completed in London with the UKHSA FWE service laboratories; details of sampling strategy, method, processing culture, molecular and phenotypic confirmation tools were finalised in country, with the methods used described here.

2.2 Introduction

2.2.1 The setting

Malawi is a small, low-income country located in southeast of sub-Saharan Africa with a population of 18.628 million in 2019 (World-Bank, 2022). The Gross National Income was $636.80 in 2020, with a Gross Domestic Product of $11.025 billion, in United States Dollars (World-Bank, 2022). As of 2019, the World Bank also states a life expectancy of 64.263 with the human development index being low, ranking 174 out of 189 countries assessed with low ratings in all sectors, notably health care, education and quality of life (UNDP, 2020). The United Nations Development Programme also states 70.3% of the population lives below the poverty line, stated as living on less than $1.90 per day, with 18.5% being in severe multidimensional poverty. Malawi is subtropical, with a large proportion of the economy and population relying on rural agriculture – with 82.6% of people living in rural communities as of 2020 (World-Bank, 2022).

Figure 2.1: Border of Malawi with the three largest cities, Lilongwe, Blantyre and Mzuzu, plotted. Taken from Open Street Map (https://www.openstreetmap.org/)

Malawi is endemic to Malaria, human immunodeficiency virus (HIV) and Tuberculosis (TB), as well as typhoid. According to the WHO report on Malaria, 2019, Malawi was amongst the top 20 countries for high prevalence of the disease, with 208 cases per 1,000 population; accounting for 2% of the global burden (WHO, 2019). The UNAIDS project reports that Malawi has 990,000 cases of HIV, 930,000 of which are adults aged 15 and above, and 62,000 children, aged 0 to 14 (UNAIDS, 2020). The World Bank also lists 141 cases of TB per 100,000 population in Malawi (World-Bank, 2022). Figure 2.1 shows the border of Malawi, with the three major cities identified. A large proportion of the country is taken up by Lake Malawi and other river systems, however, only 70% of the population has access to basic drinking water – this breaks down into 86.5% in urban areas and 66.6% in rural areas (World-Bank, 2022).

The study focuses on Blantyre city (Figure 2.2), located in the southern Shire highlands, and has an elevation of 1000m. The population within the city was 995,000 as of 2022 according to calculations from the United Nations - World Population Prospects by Macrotrends https://www.macrotrends.net/cities/21798/blantyre-limbe/population.

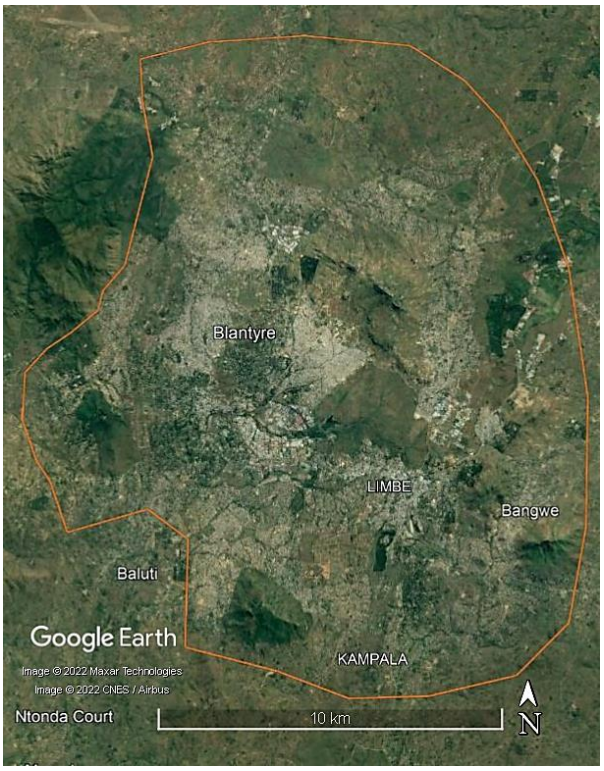


Figure 2.2: Border of Blantyre City from Google Earth

Also aggregated by the World Bank from their climate change portal is averages of monthly temperature rates and rainfall from 1901 to 2009, Figure 2.3. The seasonality of Malawi is split into three distinct periods, the warm wet season from November to April, the cold season from June to July, and then the warm dry season between August and October. Diseases like typhoid may have a seasonal component, so these trends are of importance to this study.

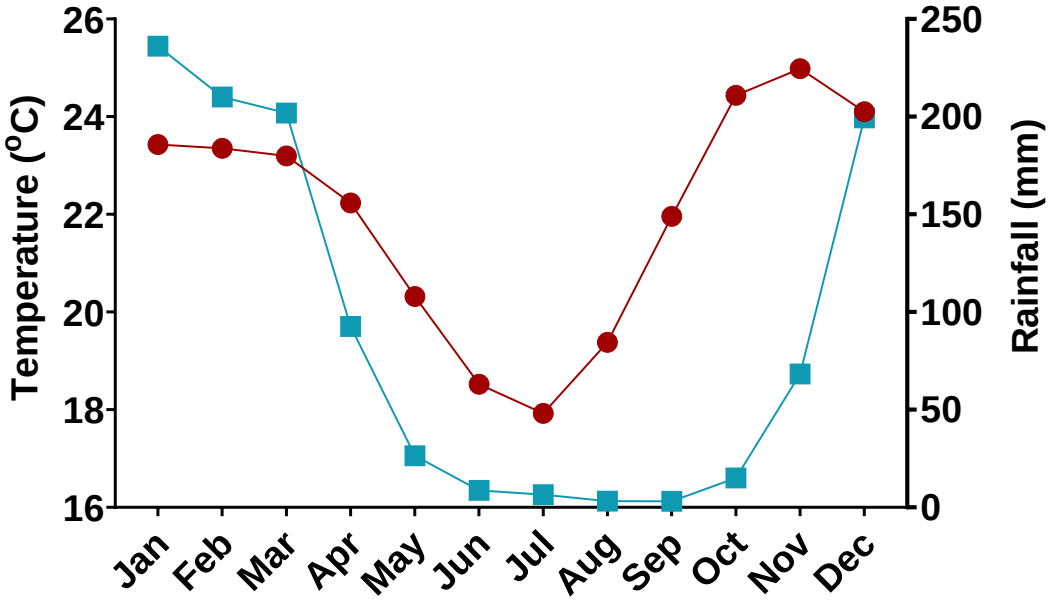


Figure 2.3: Average Temperature (red) and Rainfall (blue) in Malawi between 1901 and 2009.

2.2.2 Malawi-Liverpool-Wellcome Trust

The Malawi-Liverpool-Wellcome Trust was founded in 1995 and is affiliated locally with the KUHeS and Queen Elizabeth Central Hospital (QECH), Blantyre, Malawi. Internationally, MLW is mainly affiliated with the Liverpool School of Tropical Medicine (LSTM) and University of Liverpool (UoL). It is located on the grounds of QECH and aims to perform high-quality research, improve health and facilitate training of the next generation of Malawian researchers.

2.2.3 Kamuzu University of Health Sciences

Originally two separate institutions, KUHeS was established in 2019 through the merger of the Kamuzu College of Nursing and the College of Medicine. The university aims to produce innovative and high-quality education in health and research.

2.2.4 UK Health Security Agency Food, Water and Environmental Laboratories.

The UKHSA Food, Water and Environmental Laboratories (FWE) has three main sites across England, with this study mainly collaborating with the Colindale site in London, UK. The UKHSA FWE laboratories provide accredited specialist microbiology testing, expert advice

and public health outbreak investigations. The research and development aim to introduce and develop new techniques to detect emerging and evolving pathogens.

Due to neither MLW nor KUHeS having an established FWE style laboratory previously, training by members of the UKHSA laboratories was of great importance in helping establish a novel workspace for the use with environmental samples and One Health research.

2.3 Establishing an Environmental Microbiology Laboratory in Blantyre, Malawi

For me to deliver my PhD, I first had to establish an environmental microbiology laboratory. I identified a suitable space at the University of Malawi College of Medicine Department of Microbiology (KUHeS COM). The design considered:

- Specimen flow
- Human movement
- Data-flow
- Health and safety
- Other concurrent studies requiring ES capacity
- And ensuring the space was viable for long term usage with subsequent environmental microbiology projects.

Due to the nature of samples being handled, a one-way system was chosen, where sample concentration, culture, archive, and waste management would flow in one direction towards the exit of the room. Figure 2.4 shows the planned layouts of the laboratory before and after SARS-CoV-2 restrictions were implemented, which included social distancing.

In addition to the equipment required for the project, a new handwash basin was installed by the entrance. Due to regular water shortages, a 20-litre cistern was installed above the sink to ensure adequate water supply for staff and students would always be available for hand washing. Due to concerns from the faculty at the College of Medicine (KUHeS) during the pandemic, once patient testing and the ES for SARS-CoV-2 started, sample handling and filtration work was moved into a separate laboratory, which also contained the necessary equipment for initial processing by the SARS-CoV-2 ES study.

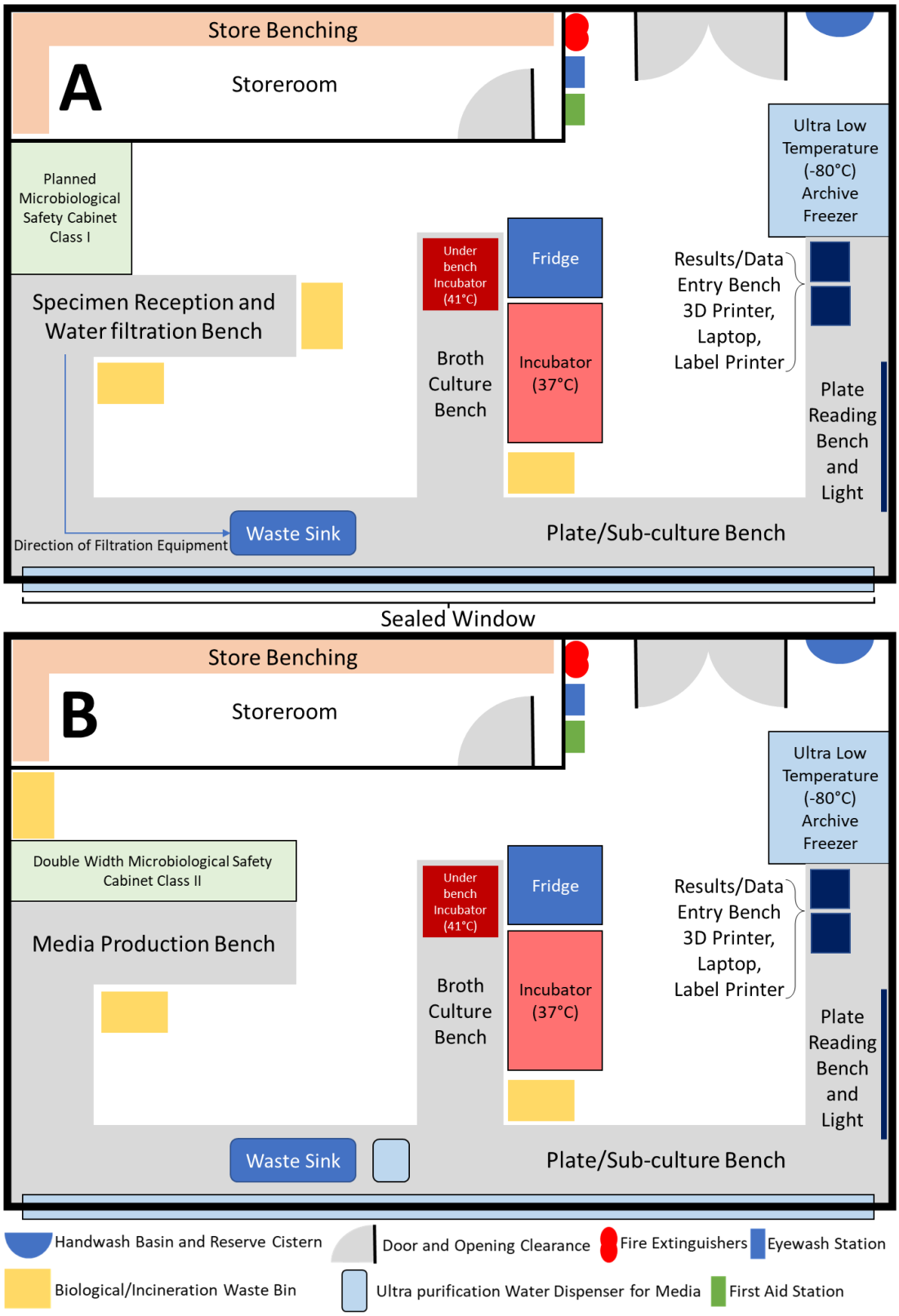


Figure 2.4: Schematic for the Environmental Laboratory at KUHeS, not to scale. A) displays the initial design for the laboratory layout, B) shows the changes to layout as used for the study. The major changes seen were due to being provided a larger Microbiological Safety Cabinet (MSC) than planned for, and the request by KUHeS to move specimen reception into a separate laboratory due to SARS-CoV-2 restrictions.

Many challenges were faced during the establishment of this workspace, for example, in addition to water shortages, the building would often experience power cuts. An uninterruptible power supply unit was purchased; however, the voltage was too low for the larger equipment and reserved for the laptops; as such, surge protectors designed to protect the larger pieces of equipment were purchased for the Ultra-Low Temperature (ULT) freezer and incubators.

The other major challenge faced by the laboratory was reliable requisitions of equipment. Consumables and smaller items were purchased through the supply channel established by the LSTM and UoL with the Wellcome Trust, but each shipment took two months to arrive. Larger pieces of equipment had to be purchased locally, with the exception of the ULT freezer. Figure 2.5 shows the progress of the laboratory from its handover by the previous occupants to the current layout with equipment in place.

In addition to equipping the laboratory with items necessary for the work being performed, a low-cost 3D-printer (Comgrow Creality Ender 5) was also purchased in order to produce reusable items used day to day. Due to the limitations of the technology, it was primarily used for medium scale objects that would not be in direct contact with cultures, and therefore could be cleaned with surface disinfectants rather than autoclaving; this included items such as tube, eppendorf and petri dish racks, cryo-boxes, coat pegs and glove dispensers. Later, it proved vital for the production of face visors during the pandemic whilst the local hospital, Queen Elizabeth Central Hospital, faced personal protective equipment (PPE) shortages. Over 200 visors were produced for the local hospital using a 3D design provided by LSTM and UoL for similar initiatives in the UK. Examples of each of these products can be seen in Figure 2.6.

Field forms (Appendix 10.1) were completed using the KoBoToolbox (https://kf.kobotoolbox.org/) fork of the open data kit (ODK). In place of a digital laboratory information management system (LIMS) for sample management, a paper laboratory form was designed, as seen in Appendix C. These were filled out per sample and tracked through all downstream processes. Upon completion of the sample, whether it discarded as negative, or sent for PCR identification, the form was photocopied, with data entered into a second KoBoToolbox entry form, Appendix D.

Figure 2.5: Transition from laboratory layout by previous occupants (Molecular space for MORDOR Study), to DRUM Environmental Laboratory pre-COVID.
A, B and C display the laboratory prior to the establishment of the ES Laboratory, which included wooden benching.
D, E and F display the changes made to the laboratory – including the installation of an ULT freezer, Class II Microbiological Safety Cabinet (MSC), and a new handwashing sink at the exit of the laboratory with a 20 L cistern for water storage due to the College of Medicine regularly losing piped water.

Figure 2.6: Examples of the items produced using the 3D printer for the DRUM Laboratory to improve workflow.

A. Stack of face shields printed from a Standard Tessellation Language file (STL) provided by LSTM for the 3D design
B. Examples of the face shields with visors and headbands attached
C. Face shield stack as printed on the Creality Ender 5 3D printer
D. Multi-stack petri dish: https://www.thingiverse.com/thing:3031170
E. Example of a 3D file when in the software used to prepare it for 3D printing: https://ultimaker.com/software/ultimaker-cura
F. A printed, and in use, modification of a microcentrifuge tube rack: https://www.thingiverse.com/thing:1881429
G. A Rack for 15 mL and 50 mL centrifuge tubes: https://www.thingiverse.com/thing:79529
H. Glove holders, to keep boxes of sterile gloves away from dirty benchtops: https://www.thingiverse.com/thing:1265367
I. Rack for holding cultures grown in bags, such as Moore swabs, designed by me
J. Two alternative designs for petri dish racks: https://www.prusaprinters.org/prints/100382-petri-dish-rack-low-material-9-x-90-mm and https://www.prusaprinters.org/prints/100383-petri-dish-rack-square-9-x-90-mm respectively
K. Safety glasses rack: https://www.thingiverse.com/thing:2126436

Once the data had been collected into KoBoToolbox and an updated data-log was created and uploaded to a local database developed in Microsoft Access. Datasets downloaded from KoBoToolbox were first cleaned through Excel Pivot Tables, which were automatically updated whenever a new run was added to the folder. PCR run files were exported from the PCR software into an excel document before being uploaded to the MLW data management system.

A full list of all samples and their PCR results combined and organised could then be downloaded, which would then be uploaded to the database. This allows secure storage of the data as the original data set is left unmodified, and output data is stored in a stable programme. Minor changes had to be made to the database due to the differences between the pilot and full-scale ES field forms.

2.4 Methods for field work and sample collection

In a previous study, cases of Typhoid that presented to QECH were recorded, with the patients or patient's guardians being recruited for interview to determine sources of infection within the city. Several risk factors were identified, with the primary environmental factors including the use of river water for cooking and cleaning, sourcing water from an open dug well, having more than one source of drinking water and the cultivation of crops within households (Gauld *et al*., 2020, Gauld, 2020). Of 658 cases during the period of the study, 297 applicable patients, or their guardians, consented to their household locations being mapped. Isolates from the patients were typed by whole genome sequencing providing six distinct clades of *S.* Typhi. When isolates were mapped with river water data included, clades split along the river and sewage catchment areas (Gauld *et al*., 2021). Using the areas where cases accumulated along these rivers, and the concentration of cases near rivers, hot spots across the city could be identified, with these areas being selected as sampling locations by this project.

I and my team received training on field sampling and safety from my supervisor Dr Nicola Elviss and Rob Johnston, a UKHSA outposted field epidemiologist. Prior to each collection visit to every location, a dynamic risk assessment was undertaken as changes in weather year-round, some sites were not always appropriate or safe to access. Once at the riverside, each of the sample collection containers were prepared with the unique barcode for each and a marker pen to ensure it could be identified when returned to the laboratory in case the label was lost or damaged in transit. PPE was worn, nitrile gloves and 3-ply surgical face

masks (Figure 2.7), and each sample collected as per the description above. If the sample was not obtainable, then the reason would be noted down.

Figure 2.7: Example of PPE worn whilst collecting samples: including gloves, facemasks and safety boots. Field workers present Pamela Kamanga and Enock Winess – pictures used with consent.

Each sample would be disinfected by ethanol wipes (Ref. 115-0128, VWR) and their barcodes scanned into the field form using a tablet (Samsung Galaxy Tab A 10.1-Inch 32 GB LTE), before being placed into the sample collection cooler box. The cooler box would also be wiped down and PPE discarded into an appropriate waste bag, which would be disposed of in the laboratory after samples are delivered. Hand sanitiser (Ref. 129-0272, Micronova NovaCleanz, VWR) was also provided.

Where water depth allowed without damaging the probe or endangering the field worker, these metrics were taken directly from the water streams. Otherwise, the probe would be placed inside of the sampling bottle in order to take the readings. This probe records the Global Positioning System (GPS) of each unique sample, so data can be linked to sample collection forms based on date, save file number and GPS. Other collection data tools are outlined in Table 2.1.

A form was devised to track all samples, as well as record water quality metrics. To develop this, KoBoToolbox (https://kf.KoBoToolbox.org/) was selected for its versatility, user friendliness as a tool for building forms and collecting data and compatibility with MLW's preferred data capture system ODK (https://opendatakit.org/). The most important aspect of the form was to ensure a physical sample could be matched to its metadata, such as GPS location and sample type, via a unique barcode.

Forms were created using the wizard. The form used during the pilot study (Appendix 10.1.1) only allowed one sample and meta data per entry. The subsequent ES form (Appendix 10.1.2) was made more efficient, with site metadata entry completed per site visited, and to four samples with distinct barcodes entered per site. For the pilot, water, sediment, food, algae, biofilms, and swabs could be selected as sampling options from a drop-down menu

Table 2.1: List of Probes used to measure water metrics during field collection

Product Name	Data Metrics Collected	Manufacturer	Product Code
AP-2000	• Temperature • Barometric pressure • pH • Oxidation Reduction Potential • Dissolved Oxygen (DO) • Electrical Current (EC) • Electrical Resistivity • Total Dissolved Solids (TDS) • Salinity • Sea Water Specific Gravity • Turbidity	AquaRead, Kent, UK	AP-2000
DO sensor InLab OptiOx	• DO • Temperature • Altitude	Mettler Toledo, Leicester, UK	51344621
pH Probe	• pH	Ketotek, Xiamen, China	KT-B0181c
TDS and EC Probe	• TDS • EC		KT-B0181c
pH indicators and test papers dispenser	• pH	Whatman, Massachusetts, USA	PAP1100

2.4.1 Grab Samples

One litre water samples were collected from representative water sources – primarily from rivers, but also from boreholes, water kiosks and from washing produce at the market. Each of these samples was collected in reusable, autoclavable polypropylene copolymer (PPCO), wide mouth sample bottles (Ref. 215-7276; Thermofisher Scientific Nalgene, Basingstoke, UK). Food products from the markets were purchased and placed into Whirl-Pak® sampling

bags (Ref. B00994, Scientific Laboratory Supplies, Nottingham, UK). Food products chosen were ready-to-eat fruit and vegetables commonly associated with salmonellae in other regions, or dried spices.

2.4.2 Trap Samples

2.4.2.1 Moore Swabs

Moore Swabs were assembled in Malawi by Tiyamake Sewing, a local Non-Government Organisation (https://www.tiyamikesewing.com), as described by Sikorski and Levine (2020). Modification had to be made to the materials used due to different availability of resources.

Two gauze products were trialled. The first was a 10 cm x 10 cm 16-ply double wrapped bandage (Ref. EMI283, Unisurge international LTD, Newport, UK), which was unfolded until 1-ply to ensure appropriate width, then folded back in half and layered four times to give an 8-ply bundle. The second was a 10 cm x 4.5 m stretchable conforming bandage (Ref. B07BDGP2LQ, General Medi, China). These bandages were rolled out and cut into 1 m strips before being folded 4 times to give an 8-ply bundle.

The thread used was a camouflage-coloured monofilament fishing line with a 10 lb/4.5 kg breaking strain (Ref. B01C6CZFDG, Next Generation Tackle, UK). This would be secured by threading it through the centre of the gauze and tied by wrapping it around the swab across the shortest length, to ensure a tight and secure fixture before being knotted.

These swabs were deployed for 48 to 96 h within the water course. The swab was lowered into a stream and then secured to any nearby features such as large rock formations or trees. Swabs were collected in Whirl-Pak® sampling bags after draining excess water without compressing the swab and returned to the laboratory.

2.4.2.2 Biofilms

A biofilm constituted a surface coating on a hard surface from within the water course and they were examined, as they potentially represented naturally formed capture vessels for bacteria. This was done through the collection of small stones (no more than 5 cm in diameter), taken from below the water surface with visually confirmed, established biofilm. These would be placed in small sample bags (Ref. MINIMGRL2P0304, VWR, Leicestershire, UK,) and returned to the laboratory. Additionally, algae and other surface matter, and sediment, were collected from rivers into the small sampling bags before returning them to the laboratory.

For each of the samples collected, a unique barcode was generated and attached to each container, with a sample collection form being completed electronically at the point of collection. Two versions of this form were used (Appendix 10.1). The purpose of these forms were to describe the sample type collected, date and time of collection and attach GPS coordinates for identification of exact location during analysis.

2.5 Culture methods for the isolation of *S.* Typhi

2.5.1 Media Production

Several media were identified for evaluation to determine the best media, or combination of media, for the culture of *S.* Typhi. The bile$^-$ and bile$^+$ broths are a modification of the Enterobacteriaceae Enrichment (EE) Broth, removing the triarylmethane dye brilliant green. The recipe for this broth is detailed in Table 2.2; this media required autoclaving after production, before use. Bile$^+$ indicated the addition of iron by including 0.2 g of iron (III) pyrophosphate (Ref. P6526, Merck, Darmstadt, Germany).

Table 2.2: Recipe for bile$^-$ broth.

Component	Volume/Weight
Distilled Water (in-house)	1,000 mL
Ox Bile (Ref. NCM0240A; Neogen, Manchester, UK)	20 g
Dextrose (Ref. NCM0241A; Neogen)	5 g
Peptone from gelatin, pancreatic digest (Ref. 70176, Merck, Hertfordshire, UK)	10 g
Sodium phosphate dibasic dihydrate (Ref. 71643, Merck)	8 g
Potassium dihydrogen phosphate (Ref. NIST200B, Merck)	2 g

For selenite F broth, the manufacturer's instructions were followed (Table 2.3), but often used in double strength per volume, as the sample is used in 1:1 with selenite F, diluting it to regular strength with a larger volume of sample (Hobbs and Allison, 1945):

Table 2.3: Recipe for selenite F broth

Component	Volume/Weight	
	Normal Strength	Double Strength
Distilled Water	1,000 mL	1,000 mL
Selenite Broth Base (Ref. CM0395, Oxoid, Basingstoke, UK)	19 g	38 g
Sodium Biselenite (Ref. LP0121, Oxoid)	4 g	8 g

As high temperature inactivates the media, and vapours of sodium biselenite are toxic, autoclaving was not performed with selenite-based media, instead they were sterilised in a boiling water bath, for 10 minutes and allowed to cool sufficiently before handling. The modified chromogenic agar for salmonellae esterase (mCASE; Ref. NCM1016S, Neogen) was produced per manufacturer's instruction, by adding 49.9 g of the powder to 1000 mL of distilled water and sterilised in a boiling water bath, or in free-flowing steam, and not in an autoclave, as overheating of the media causes the reagents to separate and the chromogen agents to inactivate. Other media, found in Table 2.4, were evaluated during the project and produced following the manufacturer's instruction without modification, and included:

Table 2.4: List of media looked at in this project

Media	Product code	Manufacturer
MacConkey	CM0109	Oxoid, Basingstoke, UK
Chromogenic Agar for Salmonellae Esterase (CASE)	NCM1006S	Neogen
ABC		Neogen
Deoxycholate Citrate Agar (DCA)	CM0035	Oxoid
Xylose-Lysine-Deoxycholate Agar (XLD)	CM0469	Oxoid
Bismuth Sulphite Agar (BSA)	CM0201	Oxoid
Buffered Peptone Water (BPW)	CM1049	Oxoid
Selenite Cystine (SC)	CM0699	Oxoid
Universal Pre-enrichment Broth (UPE)	91366	Merck, Hertfordshire, UK
Bromothymol Lactose Blue Agar (BTLB)	M1822	HiMedia, Mumbai, India

All cultures on solid media were incubated for 18 ± 1 h at 37 ± 1 °C. Broth based culture incubation details are provided within Chapter 3, and this process was refined throughout my PhD.

2.5.2 Water Sample Processing

Water samples were filtered using a 47 mm, 0.45 µM sterile cellulose nitrate membrane (Ref. 515-0228, Sartorius, Epsom, UK), placed into a sterile filter cup (Ref. 516-7594, Pall, UK), attached to a multi-cup Vacuum filtration manifold (Ref. 513-3451, Thermofisher Nalgene) and a liquid transfer pump (Ref. EZSTREAM1, Merck). For each sample of water, up to five membranes were used within a defined two-hour processing time slot, with each membrane then being placed inside of a culture tube and primary broth added. The remainder of the sample was discarded.

Membranes from water filtration were immersed in 10 mL of the bile$^-$ broth in the 30 mL glass tubes and incubated at 37 ± 1 °C for 18 ± 1 h.

2.5.3 Moore Swabs Processing

Moore swabs required no sample manipulation upon receipt in the laboratory, as sample concentration is performed by the nature of the collection method. As such, 50 mL of bile$^-$ broth was added to the Whirl-Pak® sampling bag of each swab and gently massaged to ensure full penetration of the culture media.

If a DNA extraction method was performed on the Moore swab directly, UPE broth was used instead, and the overnight culture filtered using the same method as the water samples.

2.5.4 Biofilm and Algal Sample Processing

Like the Moore Swabs, these samples needed no manipulation upon arrival to the laboratory. To culture, 20 mL of bile$^-$ was added to each sample. The sample bags were then rubbed to ensure the biofilm was sufficiently removed from the stones for culture.

2.5.5 Sediment and Food Sample Processing

Sediment and food samples were measured out into a 1:9 ratio of sample to bile$^-$ broth. Sediment samples were weighed, with two grams being transferred to a culture tube and 18 mL of bile$^-$ added. Similarly, food was weighed out to five grams, with the remainder discarded, and 45 mL of bile$^-$ broth being added to the Whirl-Pak® sampling bag.

All samples in bile$^-$ were incubated for 18 ± 1 h at 37 ± 1 °C. After incubation, 5 mL of the surface of the bile$^-$ broth was transferred into 5 mL of double strength selenite F broth in 15 mL glass tubes. Enrichment broths were incubated at 41 ± 1 °C for 18 ± 1 h. Longer than 12 hours has a detrimental effect on *Salmonella* and a reduction in potency against non-salmonellae organisms (Chattopadhyay and Pilfold, 1976).

Each sample was plated onto mCASE using the streak plate method and diluted 1:100 and 1:1000 in RLS (Ref. BR0052, Oxoid) solution (RS). These two dilutions were then spread plated by adding 100 µL to the plate and then using an L-shaped spreader to create a lawn. These mCASE plates were incubated for 18 ± 1 h at 37 ± 1 °C. Any blue/green colonies were then subcultured for purity and confirmed by PCR.

2.6 Identification and Molecular methods for the detection of *S.* Typhi

2.6.1 Extraction and Purification of DNA

For real time PCR with pure culture, thermal lysis, henceforth referred to as "boilates", were used. UltraPure™ DNase/RNase-Free Distilled Water (Ref. 10977035, Thermofisher Scientific Invitrogen, Basingstoke, UK) was the standard diluent used for extraction however, other liquid mediums were also utilised: maximum recovery diluent (MRD; Ref. CM0733, Oxoid), Ringer's lactate solution (RLS), BPW, UPE and saline.

To generate a boilate from the bacterial growth on agar, one mL of the nuclease free water (mH_2O), or other diluent, was added to a 1.5 mL screw-cap microcentrifuge tube. Two colonies from the agar plate were picked using a 10 µL sterile plastic loop and suspended in the diluent by agitation of the loop or by vortexing the sample at full speed for five seconds. Using a dry heat block, preheated to 96 ± 2^{o}C, each sample was heated for ten minutes to inactivate and lyse the cells. After heating, tubes were placed into a microcentrifuge and pulse centrifuged (max speed/16,000 g for 5 seconds). Samples could then be refrigerated (4-8^{o}C) for short term storage, frozen (-20^{o}C) or at ultra-low temperatures (ULT; -80^{o}C) for long term storage before use.

Alternatively, if the sample was already in a liquid medium, such as overnight culture, 500 µL of the sample was transferred to a microcentrifuge tube. The remainder of the extraction was performed in the same way.

For samples that required higher purification, such as samples for whole genome sequencing, or to extract from raw samples, three types of Qiagen kits were utilised at various points in the project:

1. DNeasy Blood and Tissue 96 well extraction kits (Ref. 69582, Qiagen, Manchester, UK) with the QIAvac 96 well plate vacuum extraction kit (Ref. 19504, Qiagen) used to replace a centrifuge.
2. Qiagen DNeasy power kits, utilising individual spin columns for purification, four versions of this kit were used, but instructions and reagents within the kits were similar:
    a. PowerFecal Kit (Ref. 12830-50, Qiagen),
    b. PowerWater Kit (Ref. 14900-100-NF, Qiagen),
    c. PowerFecal Pro (PFP) Kit (Ref. 51804, Qiagen),
    d. PowerSoil Pro Kit (Ref. 47016, Qiagen).
3. QiaSymphony DSP Viral/Bacteria Mini kit (Ref. 937055, Qiagen)

For each of these methods, the manufacturer's instructions were followed, using a suspect culture positive, or directly from the filter membranes before culture.

The last extraction method utilised was MagNA extract and was developed by my fellow PhD student Rachel Byrne, who shared the method she developed, which forms a chapter of her own thesis. This method utilised magnetic beads bound to DNA released after thermal inactivation. Briefly, for this method, an overnight culture in a liquid medium such as BPW, UPE or Bile$^-$ was required. Of this cultured broth, 200 µL was aliquoted into a fresh microcentrifuge tube. Each tube was then lysed by the boilate method.

After extraction, each sample required purification. Samples were centrifuged at 6,150 x *g* for five minutes, with 100 µL of the supernatant then being transferred to a fresh microcentrifuge tube. The MagNA extract beads were pre-prepared in-house by Rachel Byrne, and 100 µL is added to each 100 µL sample aliquot. Using the 24 microtube adaptor for the vortex genie, these were vortexed for 5 seconds on high speed. The vortex speed was then reduced to setting 3 (highest shaker setting) and left to incubate at room temperature for 5 minutes.

Each tube was then placed into a magnetic rack to pellet the magnetic particles; the supernatant was then removed. Using pre-prepared 70% ethanol (made by adding 7 parts

≥99.8% molecular grade ethanol (Ref. 51976 , Merck) to 3 parts mH_2O), the pellet was resuspended. Samples were vortexed on high speed and then pulse centrifuged, to remove liquid from the caps. The tubes were then placed into the magnetic rack to pellet the magnetic particles again. This was then repeated with 200 µL of the 70% ethanol for the second wash step. After the supernatant was removed after the second wash, the sample was then air dried for 30 seconds.

Once dry, the sample was removed from the magnetic rack and resuspended in 30 µL of mH_2O. These were incubated for two minutes at room temperature before the samples were returned to the magnetic rack. Once pelleted, the supernatant was transferred to a fresh microcentrifuge tube, ready for use or storage.

2.6.2 Real time PCR Assay

The real time PCR utilised was adapted from the Public Health England Hazard Group 3 PCR protocol (Nair *et al*., 2019). The dyes on each probe were changed to allow for multiplexing, with a duplex and triplex being utilised for routine identification. The master mix for the assay was made with the Takyon Low ROX Probe 2X master mix dTTP blue (Ref. UF-LPMT-B0710, Eurogentec, Seraing, Belgium) and the primer pairs and probes listed in Tables 2.6 and 2.7. Table 2.5 shows the concentrations and volumes of each reagent used to create the master mix for both assays. Nuclease free distilled water was used to dilute the master mix to the required concentration.

Table 2.5: Concentration for PCR assay master mix.

Screening Duplex			
Reagent	**Volume (µL)**	**Concentration (µM)**	**Final Concentration (nM)**
Takyon Low ROX Probe 2X master mix dTTP blue	12.5	-	-
mH_2O	8	-	-
ttr Forward (*ttr*_F)	0.25	20	200
ttr Reverse (*ttr*_R)	0.25	20	200
ttr Probe (*ttr*_P)	0.5	5	100
sseJ Forward (*sseJ*_F)	0.5	20	400
sseJ Reverse (*sseJ* _R)	0.5	20	400
sseJ Probe (*sseJ* _P)	1	5	200
Total	23.5	-	-
DNA	2.5	-	-
Confirmation Triplex			
Takyon master mix	12.5	-	-
mH_2O	5	-	-
ttr Forward (*ttr*_F)	0.25	20	200
ttr Reverse (*ttr*_R)	0.25	20	200
ttr Probe (*ttr*_P)	0.5	5	100
tviB Forward (*tviB*_F)	0.5	20	400
tviB Reverse (*tviB* _R)	0.5	20	400
tviB Probe (*tviB* _P)	1	5	200
staG Forward (*staG*_F)	0.5	20	400
staG Reverse (*staG* _R)	0.5	20	400
staG Probe (*staG* _P)	1	5	200
Total	22.5	-	-
DNA	2.5	-	-

Table 2.6: List of all PCR primer sequences, their purpose, manufacturer and reference of each forward (F) and reverse (R) pair.

Name	Sequence 5'-3'	Purpose/Target/ Accession Number	Reference	Manufacturer
*ttr*_F	CTCACCAGGAGATTACAACATGG	Tetrathionate respiratory, pan-Salmonellae, AF282268	(Hopkins *et al*., 2009)	Eurogentec; Sigma Aldrich (Hertfordshire, UK)
*ttr*_R	AGCTCAGACCAAAAGTGACCATC			
*tviB*_F	TGTGGTAAAGGAACTCGGTAAA	Vi polysaccharide biosynthesis protein, *S.* Typhi and *S.* Paratyphi C, NC_003198	(Nair *et al*., 2019)	
*tviB*_R	GACTTCCGATACCGGGATAATG			
*staG*_F	CGCGAAGTCAGAGTCGACATAG	Fimbrial protein, *S.* Typhi and some NTS. AL513382	(Nga *et al*., 2010)	
*staG*_R	AAGACCTCAACGCCGATCAC			
*sseJ*_F	CGAGACTGCCGATGCATTTA	Secreted effector protein, NTS and *S*. Paratyphi C, AF294582	(Nair *et al*., 2019)	
*sseJ*_R	GTACATAGCCGTGGTGAGTATAAG			

Table 2.7: List of all real time PCR probe (P) sequences, fluorophore dyes used and manufacturer and reference of each.

Name	Sequence 5'-3'	Final 5' Modification	3' Modification	Alternate 5' Modifications	Reference	Manufacturer
*ttr*_P	CACCGACGGCGAGACCGACTTT	FAM	BHQ-1	Cy3	(Hopkins *et al*., 2009)	Eurogentec Sigma Aldrich
*tviB*_P	TGGATGCCGAAGAGGTAAGACGAGA	TET	BHQ-2	TAMRA, Cy3, FAM	(Nair *et al*., 2019)	
*staG*_P	CATTTGTTCTGGAGCAGGCTGACGG	Yakima Yellow	BHQ-2	Cy3, Texas Red	(Nga *et al*., 2010)	
*sseJ*_P	TGGAGGCGGCCAGTAATATTGGTT	Cy5	BHQ-2	N/A	(Nair *et al*., 2019)	

Both the duplex and triplex assay formats were performed with 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 10 seconds using both the Applied Biosystems ViiA 7 and QuantStudio 7 platforms (ThermoFisher Scientific), with 0.2 mL 96 clear well plates. Detection channels used were: Blue (FAM); Green (TET); Yellow (Yakima Yellow); Red (Cy5) with a passive reference set to ROX. Thresholds for the assay were set automatically as these gave reproducibly suitable values (between 0.08 ΔRn and 0.2 ΔRn) in the linear phase of exponential amplification. A positive amplification was defined as amplification crossing the threshold between cycles 10 and 30 if extracts were from a pure isolate, or 10 to 35 if from a direct sample extraction, with any amplifications up to 40 being repeated or further investigated.

2.6.3 High-Resolution Melt PCR Assay

A high-resolution melt (HRM) PCR was designed utilising the same primers as the real time PCR but replaced the probes with EVAGreen™ x20 (25 µM; Ref. 31000, Biotium, VWR, UK) intercalating dye in water and performed in a quadruplex. The Master Mix concentrations and volumes are shown in Table 2.8.

Table 2.8: Master mix recipe for the HRM assay.

Reagent	Volume (µL)	Concentration (µM)	Final Concentration (nM)
Takyon Master mix	12.5	-	-
mH2O	5	-	-
ttr Forward	0.25	20	200
ttr Reverse	0.25	20	200
tviB Forward	0.5	20	400
tviB Reverse	0.5	20	400
staG Forward	0.5	20	400
staG Reverse	0.5	20	400
sseJ Forward	0.5	20	400
sseJ Reverse	0.5	20	400
EVAGreen	1	25	1,000
Total	22.5	-	-
DNA	2.5	-	-

The setup of the PCR was the same as the real time assay, but instead of selecting targets for each probe, only the SYBR channel was used, and no passive reference selected. The finalised addition of the melt curve after the 40 cycles of amplification comprised of 95 °C for 15

seconds, 72 °C for 1 minute and 95 °C for 15 seconds, with readings being taken continuously every 0.05 °C increase in temperature.

2.6.4 Interpretation of PCR Assay

For a sample to pass the screening assay, *ttr* should amplify to confirm the isolate was a *Salmonella*. spp. If the sample additionally amplifies with *sseJ*, then the sample was listed as an NTS or potential *S*. Paratyphi C. Any isolate that was negative for both targets was discarded, whilst any that were positive for *sseJ* but negative for *ttr* were repeated and investigated before discarding. For the confirmation triplex, the *ttr* was retained to ensure the sample was still amplifying as positive. For a definitive *S.* Typhi result, both *tviB* and *staG* needed to be positive in addition to *ttr*. If *staG* alone, with or without the *sseJ* from the screening duplex, amplified, then the sample was regarded as an NTS. If *tviB* amplified, with or without *sseJ*, it was regarded as a potential *S*. Paratyphi C, which could only be confirmed by WGS, although if *sseJ* had also amplified, then the likelihood of it being *S*. Paratyphi C was considered to be higher. Any isolates that tested positive for *staG* and/or *tviB* were then confirmed by conventional culture-based phenotypic methods.

2.6.5 Phenotypic identification

Phenotypic identification of colonies that were *ttr, tviB* and *staG* positive were undertaken using API® 20E (Ref. 20100, BioMerieux, Basingstoke, UK) and an anti-sera agglutination test. For this, I used the sera for O9 surface antigen (Ref. PL6015, Pro-Lab Diagnostics, Birkenhead, UK), Vi antigen (Ref. PL6040, Pro-Lab Diagnostics) and Hd flagella antigen (Ref. PL6113, Pro-Lab Diagnostics) on all isolates screened, following manufacturer's instructions, to confirm the results of the real time PCR against traditional *Salmonella* typing methods. Anti-microbial susceptibility testing (AST) was also performed as H58-*S.* Typhi are typically MDR, by disc diffusion method following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST, 2021) on Mueller Hinton (Ref. CM0337, Oxoid) agar. The definition of MDR for *S.* Typhi is resistance to three first line antimicrobials: co-trimoxazole (25 µg; Ref. CT0052B, ThermoFisher), chloramphenicol (30 µg; Ref. CT0013B, ThermoFisher), and ampicillin (10 µg; Ref. CT0003B, ThermoFisher). All *Salmonella*, whether NTS or *S.* Typhi, were stored on cryo-preservative beads (Ref. PL.170C, Pro-Lab Diagnostics) and archived at -80 °C in a ULT freezer.

3 Optimising culture-based detection for the isolation of *Salmonella* Typhi from the environment.

3.1 Summary

This chapter discusses the identification of appropriate media and controls for developing culture methods to isolate *S.* Typhi from environmental samples, primarily natural waters. Assessment of each media was primarily done through enumeration of diluted control strains. Once the best media had been identified, broths and agar were combined into culture "pathways" so that cells could recover from sub-lethal damage and increase selective pressure of the methods assessed. These were narrowed down to eight candidate pathways utilising modifications of the Enterobacteriaceae - Enrichment Broth, selenite-based broths and a novel modification of a chromogenic agar called chromogenic agar for *Salmonella* esterase (mCASE). The candidate pathways were trialled with an enumeration challenge, mixed culture challenge and used when a spiked sample of pond water with *S.* Typhi could no longer be detected by direct agar culture. Finally, these methods were compared *in situ* in Malawi, Blantyre, with real environmental samples and a final candidate was selected based on its ability to successfully culture *Salmonella spp*.. Some modifications were made to the selected pathways when used in Malawi to combat specific issues encountered, such as the introduction of an anti-fungal – however, further work is required to assess the efficacy of this addition.

3.2 Introduction and summary of previous work this chapter was based on

Culture of *S.* Typhi from environmental samples has proven to be difficult historically, but possible. From the early 20th century until the 1970s, many culture methods were used for ES of *S.* Typhi, however, these investigations primarily focussed on sewage with only a small number looking at the wider environment and use of the methods declined as Typhoid fever declined in high income settings. Regions with poor WASH infrastructure, areas prone to flooding, and places where there is environmental contamination with untreated human waste have seen ongoing transmission of *S.* Typhi. Regions that might benefit most from ES often have least access to it. Here, I have re-evaluated methods for the isolation of *S.* Typhi from environmental samples and developed new pipelines for this purpose.

Some work had previously been done on this project by Rory Miles, my predecessor in this role, who spent time identifying current methods used for the isolation of *Salmonella* spp. for food and water microbiology, with a focus on culture of *S.* Typhi. This work was, however, far from complete, the results generated by him were fragmented and much of it was repeated. A summary of the work he undertook is presented within this Chapter introduction section (3.2), with work that I exclusively performed beginning in section 3.4 and the last paragraph of 3.3.

Media screened during this period was done by direct culture of *S.* Typhi strains (Table 3.1) on the candidate agars, with elimination based on quality of growth and selectivity. The first agars eliminated included MacConkey agar and CASE and ABC from Neogen. MacConkey agar was eliminated as it had broad selectivity for enteric, Gram-negative bacteria making it difficult to distinguish *Salmonella* spp. from other *Enterobacterales*. CASE gave poorer growth for *S.* Typhi due to the presence of a selective agent within the media. The other Neogen chromogenic agar, ABC, also proved to be unhelpful as the α-galactosidase chromogen gave false negative results for some *S.* Typhi strains.

As such, a discussion with the manufacturer who had been investigating improved methods for the isolation of *Salmonella* spp. from complex matrices (Elmerhebi, 2018) led to modifying the chromogenic agar for *Salmonella* esterase (CASE) early in the selection process. Originally, this media performed worse when compared to typical agar used for the isolation of *S.* Typhi, such as BSA, also known as Wilson and Blair medium. In collaboration with the manufacturer, the agar was modified into three additional variants. This chromogenic agar was based on the esterase activity of *Salmonella* spp. and the lack of esterase activity or presence of ß-glucosidase activity of non-target organisms. It also included two selective compounds within the recipe, which were the suspected causes of *S.* Typhi inhibition, but their identity could not be shared by the company. The first modification removed both selective agents included in the recipe and is henceforth referred to as called CASE-; the second removed one selective agent and was called CASE+1; whilst the third removed the other selective agent and named CASE+2.

Work performed with the strains of *S.* Typhi (Table 3.1) showed that both CASE- and CASE+2 had superior performance for *S.* Typhi growth, compared not only CASE and CASE+1, but also compared to DCA, BSA and XLD, which were the other candidate agars under review. Due to

the performance of CASE- and CASE+2 being the same, with CASE+2 containing that additional selective agent, CASE+2 was selected and named modified CASE (mCASE).

Table 3.1: *S.* Typhi strains used in culture method evaluation experiments, which were provided from the UKHSA Gastro-intestinal Bacterial Reference Unit collection (Rigby *et al*., 2022). [A]Accessible from https://www.ncbi.nlm.nih.gov/sra/ (last accessed 9th November 2020). UKHSA, United Kingdom Health Security Agency; LSTM, Liverpool School of Tropical Medicine; MLW, Malawi-Liverpool-Wellcome Trust. A, Ampicillin; C, Chloramphenicol; Su, Sulfamethoxazole Tm, Trimethoprim; Nx, Nalidixic Acid; Cp, Ciprofloxacin

No.	Provided	Isolated from	Year	Country of travel recorded	Antimicrobial susceptibility status	Haplotype (where available)	Sequence Type	eBurst Group	Accession ID [A]
1	UKHSA	Human Faeces	2009	Nepal	A,C,Su,Tm,Nx,Cp	H58	1	13	SRR7165748
2	UKHSA	Human Blood	2012	Malawi	A,C,Su,Tm	H58	1	13	SRR5949979
3	UKHSA	Human Blood	2012	Vietnam	Nx,Cp		1	13	SRR1645294
4	UKHSA	Human Blood	2012	Dem. Rep. Congo	A,Su,T,Tm		2	13	SRR1645361
5	UKHSA	Human Blood	2013	Sudan			2	13	SRR5886991
6	UKHSA	Human Faeces	2013	Niger	Nx,Cp		2	13	SRR5974884
7	UKHSA	Human Faeces	2013	Nigeria			2	13	SRR7165353
8	UKHSA	Human Blood	2014	Cameroon			1	13	SRR7165415
9	UKHSA	Human Faeces	2014	India			2	13	SRR1967790
10	UKHSA	Human Blood	2014	India	Nx,Cp		1	13	SRR1966683
11	UKHSA	Human Blood	2014	Ethiopia			2	13	SRR3048982
12	UKHSA	Human Blood	2014	Ghana	Tm,Nx,Cp		2	13	SRR7165399
13	UKHSA	-	2014	Zimbabwe	A,C,S,Tm,Nx,Cp		1	13	SRR1967049
14	UKHSA	Human Blood	2015	Angola			1	13	SRR1963294
15	UKHSA	Human Blood	2015	United Rep. Tanzania	A,C,Su,Tm		1	13	SRR1960208
16	UKHSA	Human Blood	2015	Pakistan			1	13	SRR3048958
17	UKHSA	Human Faeces	2015	India	A,C,Su,Tm,Nx,Cp		1	13	SRR1967675
18	UKHSA	Human Blood	2015	Uganda	A,C,Su,Tm,Nx,Cp		1	13	SRR1967963
19	LSTM	Ty21a							
20	UKHSA	CT18							
21	MLW	Human Blood	Undisclosed	Malawi	Undisclosed	N/A	N/A		N/A

Subsequently, some initial spiked water experiments were also performed using the 18 strains of *S.* Typhi listed in Table 3.1. The complex matrices of these spiked samples showed it was not possible to sufficiently distinguish *S.* Typhi from the natural background flora using three of the agars, DCA, BSA, XLD so these were eliminated, leaving mCASE as the preferred agar. Furthermore, several broth media had been identified for use in pre-enrichment prior to plating onto agar, these included selenite cystine, 2% bile broth and BPW.

The formulation for the 2% bile broth was based on the Enterobacteriaceae-enrichment broth, by removing the selective component, brilliant green triarylmethane dye, which is inhibitory towards *S.* Typhi. Work performed between my predecessor and the manufacturer showed it was effective for the enrichment of *S.* Typhi. To further improve the efficacy of this broth, multiple sources of ox bile were tested, as well as concentration and the inclusion of iron pyrophosphate. The ox bile acquired by Neogen at a 2% concentration seemed most effective for the enrichment of pure *S.* Typhi and NTS, whilst not favouring competitive organisms such as *E. coli*. The addition of iron to the media also showed an increase in growth from *S.* Typhi strains. To differentiate between the broth that contained the iron, compared to that without, the broths were named bile^{+} and bile^{-} respectively. Other media that had been reviewed initially and rejected can be seen in Table 3.2

Table 3.2: List of media reviewed and excluded.

Media	Reason for Consideration	Reason for Exclusion	Reference
Enterobacteriaceae - Enrichment Broth	A rich media for recovery of sub-lethally damaged Enterobacterales	The inclusion of the triarylmethane dye, brilliant green, is inhibitory to typhoidal Salmonellae. This media was adapted as the 2% bile broth used in the study was a modification of this broth with the dye removed.	(Mossel *et al*., 1963)
Rappaport Vassiliadis Broth	Preferred selection broth for *Salmonella* spp.	Inappropriate for *S.* Typhi due to its sensitivity to the triarylmethane dye, malachite green.	(Konforti *et al*., 1956)
Tetrathionate Broth	A selective growth media for *Salmonella* spp. including *S.* Typhi. Typically used in clinical, sewage and food microbiology.	Limited shelf-life due to self-generating tetrathionate selective element. Impractical as reaction begins once iodine added to the broth and so this media could only be used on day of production.	(Pollock and Knox, 1943)
Brain Heart Infusion broth	A highly nutritious broth used for fastidious organisms	Non-selective and use for pure growth rather than isolation or sub-lethal recovery.	(Wain *et al*., 2008)
Tryptone Soya broth	A highly nutritious general-purpose broth for bacteria	Non-selective and use for pure growth rather than isolation or sub-lethal recovery.	(Baker *et al*., 2011)
Luria broth	A highly nutritious general-purpose broth for bacteria	Non-selective and use for pure growth rather than isolation or sub-lethal recovery.	(Kingsley *et al*., 2018)
***Salmonella*-Shigella Agar**	Modification of DCA, a selective and differential plate distinguishing between *Shigella* and *Salmonella* spp.	Does not differentiate between Salmonellae; with known failures to produce hydrogen sulphide (H_2S) reaction. Also contains Brilliant Green, triarylmethane dye, which is known to be inhibitory to *S.* Typhi.	(Leifson, 1935)
Hektoen Enteric Agar	A selective and differential agar used for the distinction between *Shigella* and *Salmonella* spp.	Unable to distinguish *S.* Typhi from other Salmonellae with lower recovery rates for *S.* Typhi than other media and known failures to produce H_2S reaction.	(King and Metzger, 1968)
Eosin Methylene Blue Agar	Identification of Gram-negative coliforms and faecal bacteria, inhibits Gram-positive and fastidious Gram-negative organisms.	Differentiation based on lactose fermentation but is a non-selective agar, allowing false positives and competitive organisms to grow.	(Levine, 1918)
Brilliant Green Agar	Used for the isolation of Salmonellae since 1925	Selectivity is reliant of the addition of brilliant green, triarylmethane dye, which is inhibitory to *S.* Typhi.	(Read and Reyes, 1968)
Other *Salmonella* Chromogenic Agar	Chromogens more sensitive and specific than the lactose fermentation and H_2S production.	Comparison of ABC to CASE media showed that chromogens selected for CASE were more specific than those used in ABC agar and the other chromogenic agar.	(Rambach, 1990)

3.3 Selection of experimental *S.* Typhi control strains

Prior to selecting a method for use within the field, laboratory tests needed to be performed on appropriate control strains, representing the target organism *in situ*. Several strains were considered, each with advantages and disadvantages. The first organism considered was the vaccine strain Ty21a, which has been attenuated so it can be handled under containment level two conditions (Germanier and Füer, 1975). This was used initially but was clear early on that it was not representative due to the degree of attenuation. A genetically modified organism (GMO) modification of Ty21a was also considered, with either green fluorescent protein or mCherry ght red monomeric fluorescent protein. This would have allowed more scope in spiking experiments, allowing the identification of the GMO Ty21a more easily in a broth culture, or when spiking complex background matrices, such as food or contaminated water. However, this was not continued due to the same limitations as the ordinary Ty21a was under in addition to modifying a wild-type strain being difficult due to GMO regulations.

The UKHSA Gastro-intestinal Bacteria Reference Unit (GBRU) *Salmonella* section provided 18 *S.* Typhi clinical isolates and CT18 for this study. Whilst LSTM provided the vaccine strain, Ty21a. The CT18 strain is a well characterised whole genome sequencing strain, used primarily in validating the real time PCR, whilst the 18 strains were representative of typhoid endemic countries globally (Figure 3.1). Isolation dates ranged from 2009 to 2015 and included H58 haplotype isolates, an MDR lineage that is predominant in many endemic regions. These clinical isolates were then used in the initial media selection comparisons to ensure diverse *S.* Typhi strains could be cultured. Later, when more in-depth analysis work was being performed, moving from testing single media to testing culture pathways, the 01 Nepal and 02 Malawi strains were selected. The Malawian strain was selected as it was an H58 strain from Malawi, where subsequent fieldwork would be performed. The Nepalese strain was chosen as an H58 representative of what strains circulating in Southeast Asia.

Finally, work done on validating and improving the methods selected whilst in Malawi was done with a local clinical strain. This was chosen as importation of control and reference strains proved difficult the Hazard Group and laboratory containment level of *S.* Typhi. It was also decided that as this was a recent strain circulating clinically in Malawi, it may be more relevant than the one from the culture collection, isolated in 2012; however, as part of its adoption, the strain was anonymised, without genomic or antimicrobial susceptibility data provided to comply with MLW's guidelines.

Figure 3.1: Global distribution of strains used in optimisation & evaluation of environmental isolation provided by Satheesh Nair, UKHSA

3.4 Validation of previous work: media selection

To verify the results of my predecessor, the modified media mCASE and bile$^{-/+}$ were revaluated. They were first discussed with the manufacturer (specifically, Dr Ezzeddine Elmerhebi). Next, multiple control strains which were used commonly at the UKHSA FWE microbiology laboratories, Colindale, were cultured in these media to confirm what was described previously. This was done by subculturing the controls strains listed in Table 3.3, except for *S.* Typhi, onto mCASE in a Containment Level two laboratory. The colours seen on the chromogenic agar were described and were compared with the information provided by the manufacturer.

When culturing non-target organism, that contain no esterase or ß-glucosidase activity, such as an *E. coli*, the colonies will appear colourless on the agar, whilst an organism with ß-glucosidase activity, such as *Raoultella planticola*, whether it has esterase activity, will grow with a black pigmentation (Figure 3.2). Some non-target organisms will have esterase activity, such as *Bacillus subtilis*, however, these can be distinguished by colony morphology typically, showing as a much brighter blue colour, extracellular halos or being larger and either dryer or wetter than typical *Salmonella* spp. on this agar. *Salmonella* spp. typically have a more teal or blue/green colouration (Figure 3.3).

Table 3.3: Reference strains used in the study and their growth characteristics on mCASE [A] National Collection of Type Cultures; [B] World Data Centre for Microorganisms; [C] Based on at least triplicate data; [D] American Type Culture Collection; N/A, Not Applicable

Strain	**NCTC [A]**	**WDCM [B]**	**Colour**
***S.* Typhi**	Multiple Wild Types		Blue/Green
Bacillus cereus	7464	ATCC 10876	Blue
Bacillus subtilis	10400	00003	Blue
Enterococcus faecalis	775	00009	Blue/Black
Escherichia coli	9001	00090/00155	Colourless
Escherichia coli	13216	00202	White
***Escherichia coli* O157**	12900	00014	Colourless
Listeria innocua	11288	00017	Black
Listeria monocytogenes	11994	00019	Blue
Mycobacterium fortuitum	10394	ATCC 6841	Blue
Mycobacterium chelonae	946	ATCC[D] 35752	Blue
Pseudomonas aeruginosa	10662	00114	Blue
Raoultella planticola	9528	N/A	Black
Saccharomyces cerevisiae	10716	00058	Blue
***Salmonella* Nottingham**	7832	N/A	Blue/Green
***Salmonella* Typhimurium**	Wild-Type	N/A	Blue/Green
Staphylococcus aureus	6571	00035	Blue
Staphylococcus epidermidis	11047	00132	Blue
Vibrio furnissii	11218	00186	No Growth
Vibrio parahaemolyticus	10885	00185	Blue

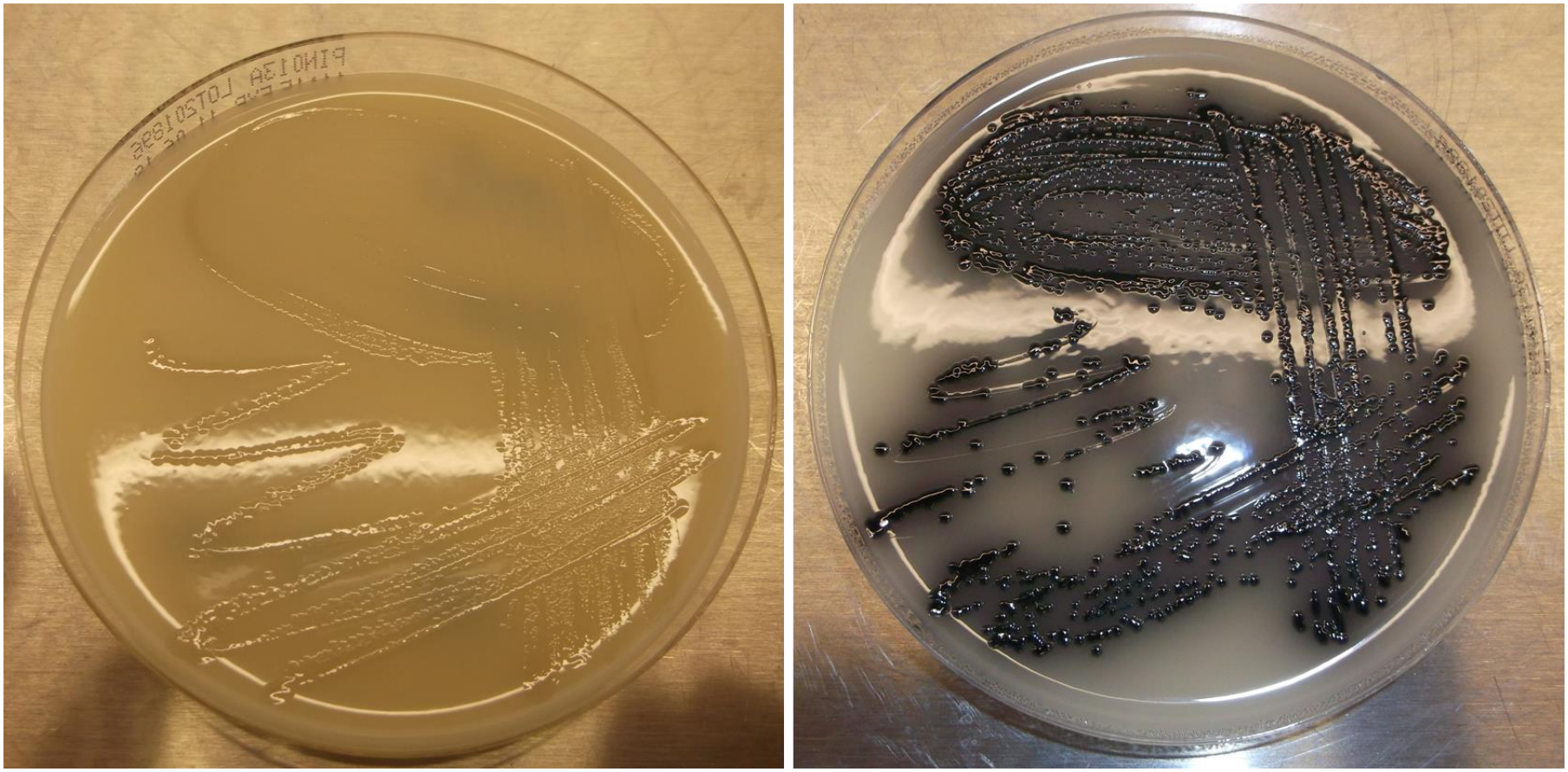


Figure 3.2: Examples of E. coli (left) and Raoultella planticola (right) on mCASE. Due to neither esterase nor ß-glucosidase activity, the E. coli colonies are colourless, whilst the Raoultella planticola is black due to ß-glucosidase activity. *Salmonella* spp. turn a blue/green colour due to their esterase activity, but lack of ß-glucosidase activity.

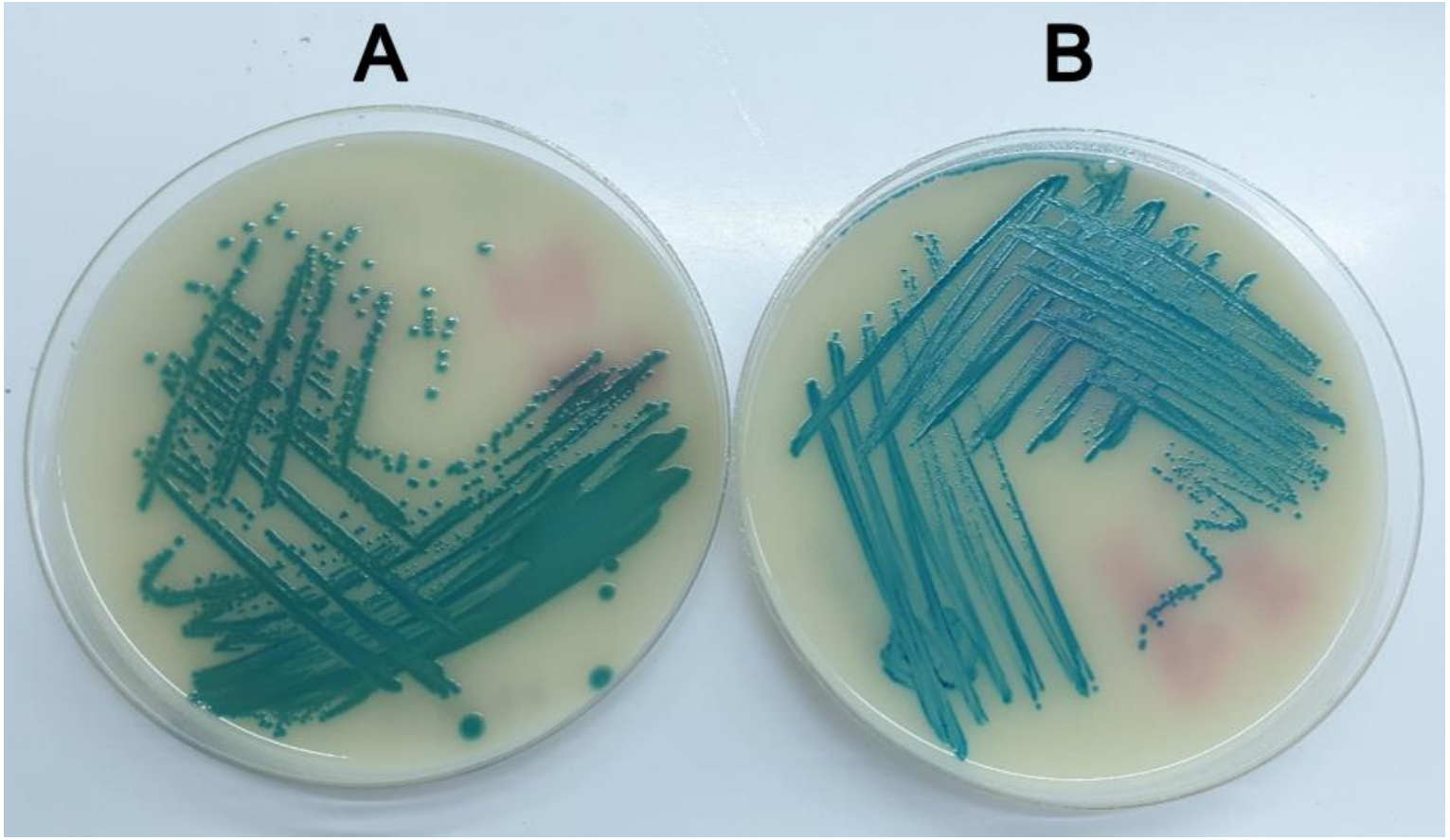


Figure 3.3: Colony morphology of *S.* Typhimurium (A) and *S.* Typhi (B) on mCASE. There is a slight difference in the shade of blue between the two colonies, which may be due to differing levels of esterase activity, however, the main distinction between colony morphology is in their size; *S.* Typhimurium colonies are much larger after 18 ± 1 h of growth than that of *S.* Typhi.

Whilst completing Containment Level three (CL3) laboratory training, I also familiarised myself with the Miles, Misra and Irwin (1938) spot titre method for enumeration, using a *Salmonella* Nottingham control strain. This was done by creating a 0.5 MacFarland standard density of culture in MRD, then serially diluting it ten-fold to 10^{-8}, and then pipetting 20 µL 3 times per dilution onto an mCASE agar plate. Additionally, 1 mL of this suspension was added to bile$^-$ and selenite cystine broth. These were incubated for 18 ± 1 h at 37 ± 1 °C. After incubation, the two broths were also enumerated using the same method, whilst the colony forming units per millilitre (CFU mL^{-1}) of the inoculum was calculated. After the agar plates for the subculture broths were incubated, the CFU mL^{-1} were calculated for these too (Table 3.4).

Table 3.4: CFU mL^{-1} of S. Nottingham when made to a 0.5 MacFarland standard density, and after culture in bile$^-$ and selenite cystine, showing the viability of the media and the enumeration method.

Medium	**CFU mL^{-1} average**
MRD	1.44 x 10^8
Bile$^-$	3.71 x 10^8
Selenite cystine	2.53 x 10^8

Upon completion of CL3 training, the first task performed was to ensure that all media selected by my predecessor was suitable for *S.* Typhi culture, as per his results. This was done by direct culture using the collection series of *S.* Typhi strains provided by the UKHSA GBRU from their *Salmonella* culture collection, which included each strain's metadata and, in most cases, whole genome sequence data (Table 3.1). Figure 3.4 shows the morphology and performance of an *S.* Typhi strain cultured on each candidate agar. Columbia Blood Agar (CBA) was used as a non-selective control to ensure the strains were still viable, whilst XLD, DCA, BSA, CASE (Figure 3.4) and mCASE (Figure 3.5) were all reviewed for their ability to grow pure *S.* Typhi.

Amongst agars tested, mCASE showed the best performance for the culture of *S.* Typhi from a pure, laboratory grown source (Appendix 10.3). Further, previous work was performed, challenging each agar with *S.* Typhi spiked into complex background matrices. From this work, mCASE was selected due to the increased performance over the other agars for

culture, and its consistency for identifying *Salmonella* spp. (Appendix 10.3). Unfortunately, *S.* Typhi cannot be differentiated from NTS unlike BSA media, however, the sulphite production of *S.* Typhi can be unreliable, especially when culturing from contaminated sources. When cultures were pure, there was some indication that colony morphology can be used to differentiate between *S.* Typhi and *S.* Typhimurium (Figure 3.5) or *S*. Nottingham, however, as the comparison was performed with two NTS only, this is not a sufficient sample size for it to be a reliable indicator of *S.* Typhi alone.

After the initial media were evaluated, two more agars were identified from historical documentation (Vogelsang and Bøe, 1948) for use in the isolation of *S.* Typhi from stool, these were Bromo-Thymol-Blue Lactose Agar (HiMedia) and Litmus Lactose Agar (HiMedia). These media were used in tandem to isolate *S.* Typhi, with colonies being identified based on their lactose and mannitol fermentation properties (negative and positive, respectively, for *S.* Typhi). Plating the control strains (Table 3.1) onto these agars, however, proved that for the use with *S.* Typhi, these media were inferior to more specialised agar that had already been screened during this project. All control strains formed colonies on the agar (Figure 3.6), but when compared to media specifically formulated for the growth of *S.* Typhi, or to chromogenic media for the identification of *Salmonella* spp. it was determined these would not provide a more reliable result than those already tested.

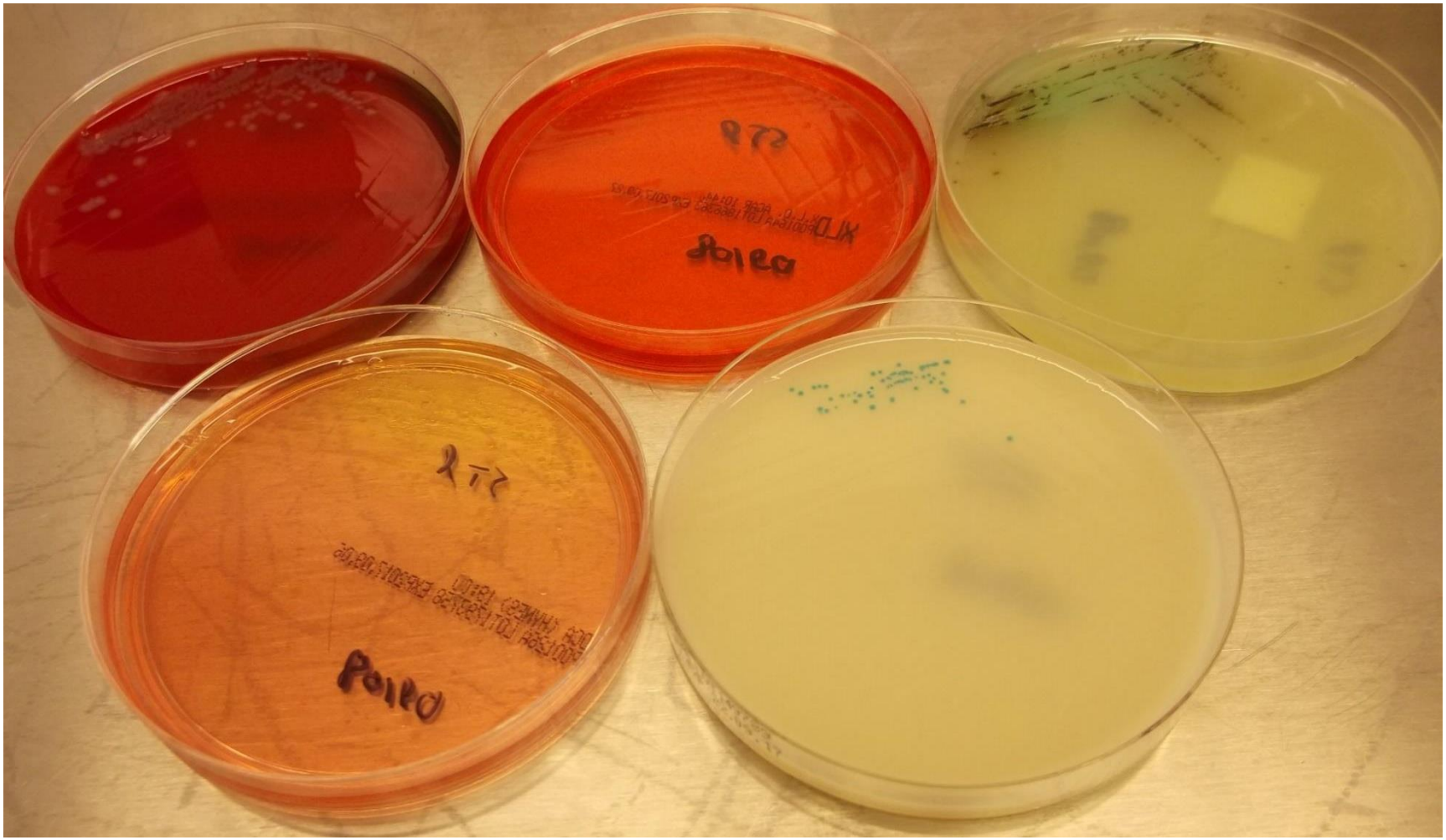


Figure 3.4: Example of an *S.* Typhi control growth cultured on: CBA; XLD; BSA; DCA and CASE, from left to right

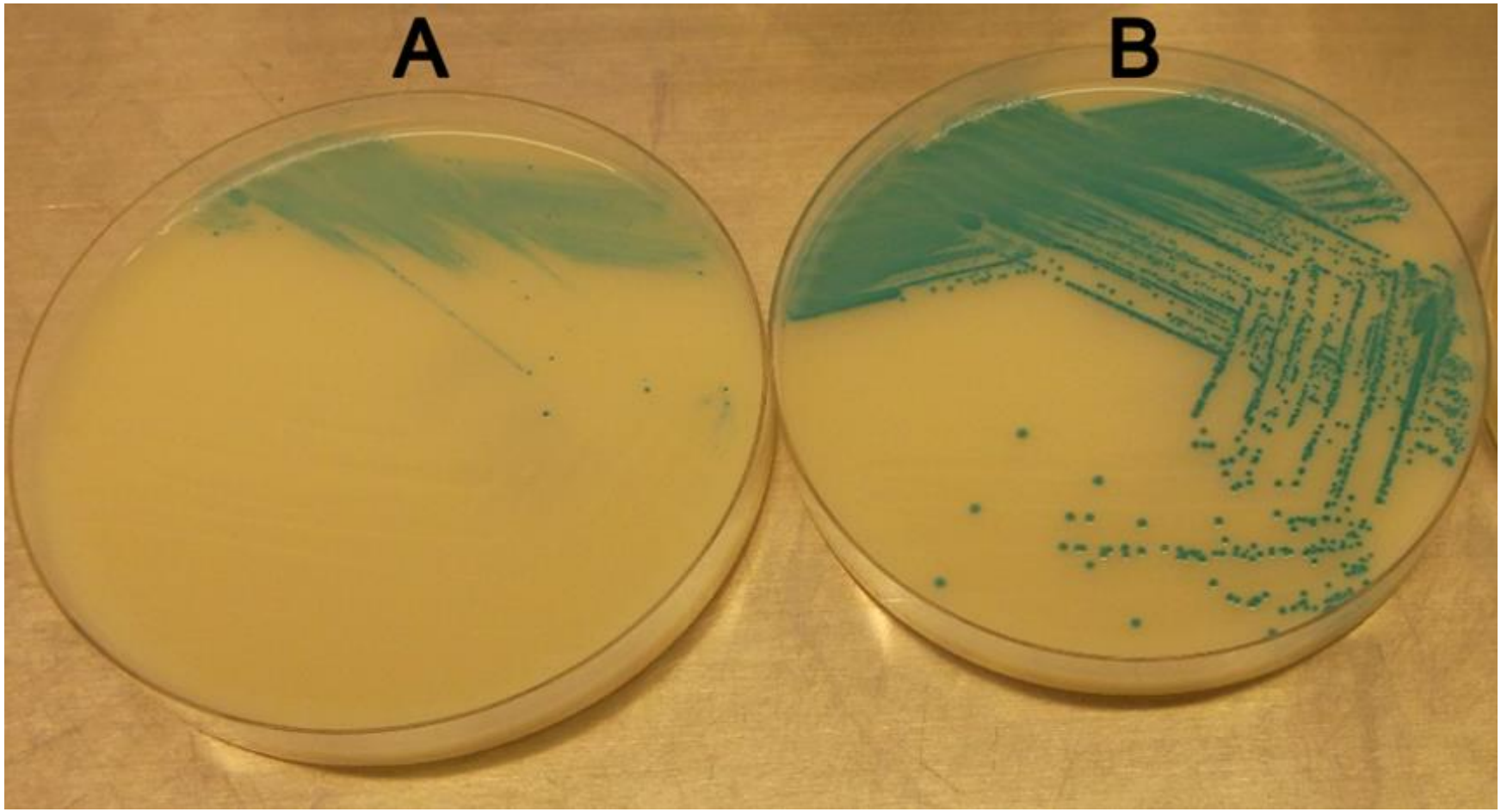


Figure 3.5: Comparison between CASE agar and mCASE when culturing *S.* Typhi, with much weaker performance of most *S.* Typhi strains on CASE compared to mCASE.

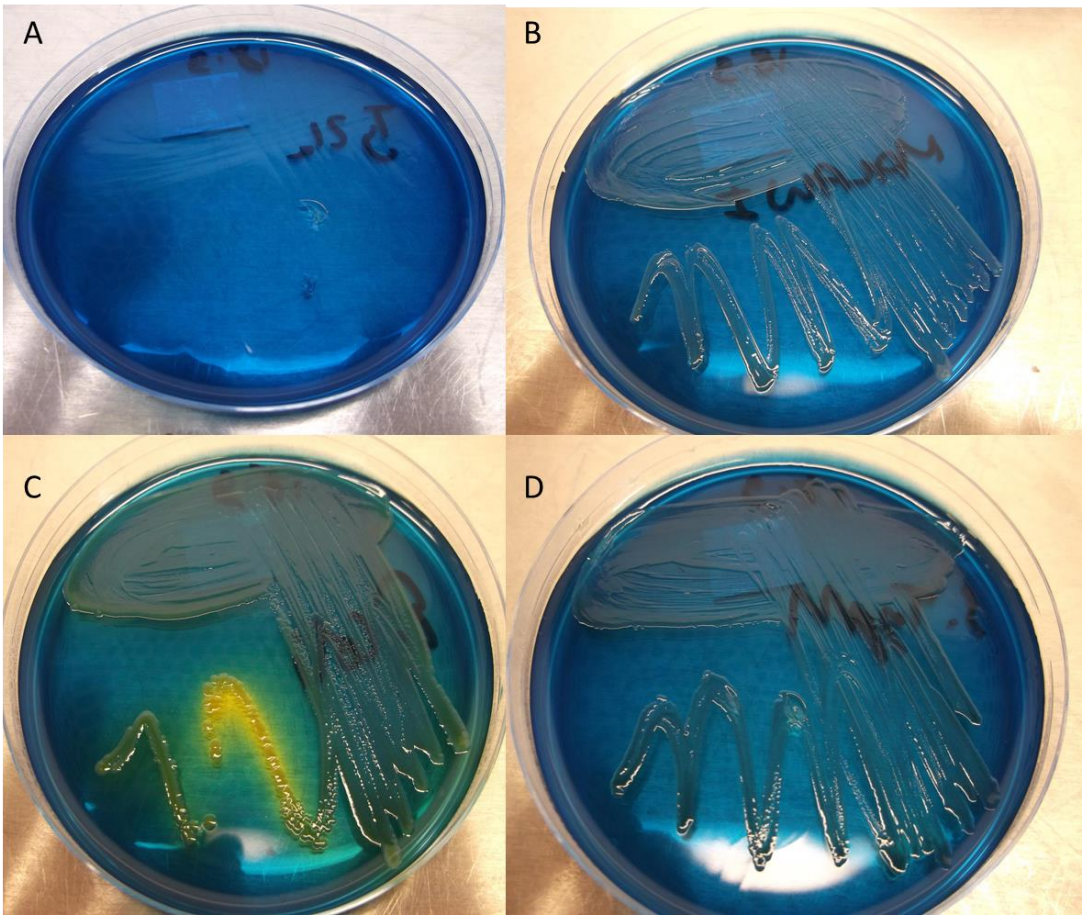


Figure 3.6: Bromo-Thymol-Blue agar, with colonies of Ty21a (A), *S.* Typhi control strain 2 (B), E. coli (C) and *S.* Typhimurium (D). Whilst Ty21a did not grow on this media, the control strains of *Salmonella* spp. including all strains of *S.* Typhi, *S.* Typhimurium and S. Nottingham all had the same appearance on the agar. E. coli is lactose fermenting, and producing the yellow colouration, however, the lack of any colouration when mCASE is used is easier to distinguish than that of the lactose positivity seen here.

3.4.1 Evaluation of remaining media for use in a multi-step culture pathway

Having selected mCASE, several pre-enrichment culture broths were then examined for use in a multi-step culture pathway. Initially, the broth media that were examined included bile$^{-/+}$, selenite cystine and BPW. Selenite cystine was also examined as it is a modified form of selenite F broth, a media that has historically been valuable for the isolation of *S.* Typhi, and that is still in use for clinical isolation of *S.* Typhi in the UK. BPW was also of interest as it is routinely used for NTS and other Enterobacteriaceae pre-enrichment work in Environmental microbiology laboratories, however, preliminary results from my predecessor, included in appendix 10.3 indicated that it was not as effective as bile^{-}. Selenite F and selenite mannitol broths were also considered, with the former being initially disregarded until availability of

selenite cystine became an issue, and selenite mannitol not being considered due to many previous studies showing it was no more effective at isolating for *S.* Typhi from faeces than selenite F broth (Wain *et al*., 2008). The previous work enabled me to create pathways from the remaining candidate media (Table 3.5), through combining primary and secondary stages of the candidate broths before culture on mCASE.

Table 3.5: The remaining eight pathways evaluated during this study.
SC, Selenite Cystine; mCASE, Modified Chromogenic agar *Salmonella* Esterase with the second selective agent removed; SF, Selenite F; Bile$^-$, modified Enterobacteriaceae Enrichment broth; Bile$^+$, Bile broth with 0.2 g/L iron pyrophosphate.

Pathway	**Primary Broth**	**Secondary Broth**	**Agar**
PW1	Bile$^-$	SC	mCASE
PW2	Bile$^+$	SC	
PW3	SC	Bile$^-$	
PW4	SC	Bile$^+$	
PW5	Bile$^-$	SF	
PW6	Bile$^+$	SF	
PW7	SF	Bile$^-$	
PW8	SF	Bile$^+$	

Initially, only pathways PW1 to PW4 were assessed, using the Nepalese, Malawian and Ethiopian strains. This was done by inoculating 1 mL of a 0.5 McFarland density suspension into each of the primary broths, which were incubated at 37 ± 1 °C for 18 ± 1 h. From the primary broth, 1 mL was inoculated into the secondary broth and incubated at 37 ± 1 °C for 18 ± 1 h. Each stage (inoculum, primary and secondary culture) was enumerated using the Miles, Misra and Irwin method. From these initial results, PW1 and PW2, which used bile$^-$ and bile$^+$ broths respectively, appear to have the highest counts from the primary and secondary incubations, with bile$^+$ (PW2) performing fractionally better (Figure 3.7). The Ethiopian strain used first did not have the inoculum enumerated, so the experiment was repeated with the Malawian and Nepalese strains with inoculum calculated for comparison across pathways to determine level of growth from inoculum to primary.

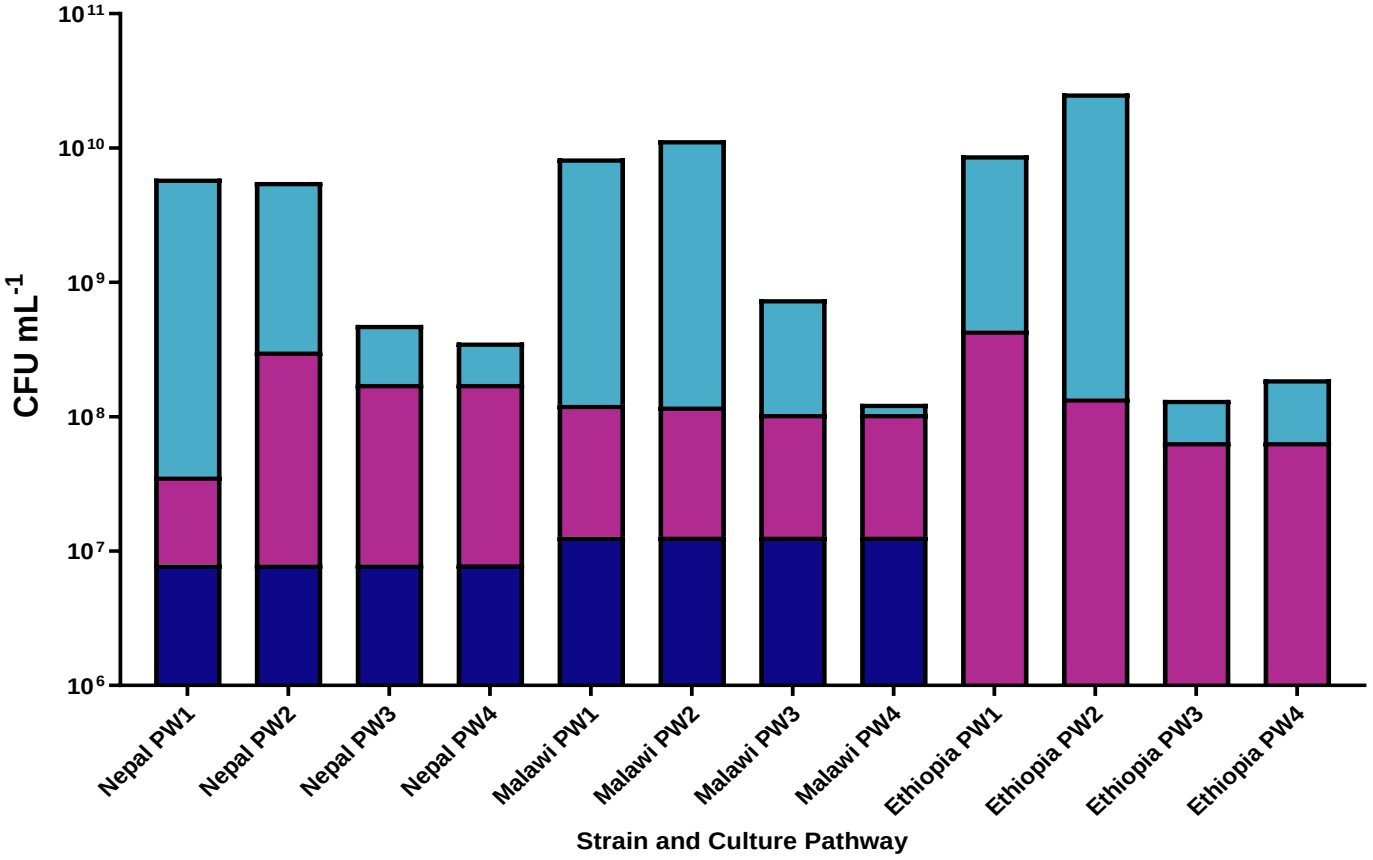


Figure 3.7: Shows the cumulative growth of each control strain through pathways one to four. Blue represents the CFU mL^{-1} of the Inoculum, Purple represents the CFU mL^{-1} of the primary broth after incubation and the Teal represents the CFU mL-1 for the Secondary Broth. The Ethiopian strains inoculums were not enumerated due to an issue with the starting culture colony counts. Each in this initial experiment was done as a single replicate.

At this point, however, selenite F broth was discussed as an alternative media due to some manufacturers ending production of selenite cystine, making it harder to procure, as well as selenite F being used more commonly internationally. As such, the enumeration experiments were repeated with the newly added PW4 to PW8 pathways, in addition to repeating PW1 to PW4 using the Malawian clinical strain (Strain 21, Table 3.1).

Growth was quantified by the spot titre method (Miles *et al*., 1938), from the primary broth and the secondary broth after incubation. It was identified that culture pathways had to be seeded with a minimum of 100 CFU mL^{-1}, the 10^{-6} dilution, for consistent growth to be observed (Table 3.6). Therefore, I determined the limit of detection (LOD) of my culture pathway to be 10^{2} CFU mL^{-1} as inocula of 10^{1} CFU mL^{-1} did not allow for sufficient growth within the primary or secondary broth incubations to allow robust detection.

All eight pathways were tested in triplicate, to determine their reproducibility and selective advantage. PW1, 2, 4, 5, 7 and 8 appeared to perform best by pure culture enumeration when the averages of results are taken (Figure 3.8), however, pathways PW1, 5 and 8 demonstrated consistent growth from inoculum through both primary and secondary broth culture (Figure 3.9). Pathways PW2, 3, 4, 6 and 7 all demonstrated a reduction in the level of growth after the transition from primary to secondary broth. However, as the difference observed when using the enumeration of pure cultures was small, all eight pathways were retained and evaluated through challenging with a mixed culture broth.

Table 3.6: Attempt at pathway culture comparisons with a low starting inoculum. A 0.5 MacFarland Standard of the control was made in RLS and diluted 10-fold eight times. The 10^{-7} dilution have a starting inoculum of 10^{1} CFU mL^{-1} but did not grow sufficiently in the primary broth for subculture in secondary broth. The 10^{-6} gave a starting inoculum of 10^{2} CFU mL^{-1} and was successfully plated after subculture in primary and secondary broth.

Pathway	Inoculum CFU mL^{-1}	Primary CFU mL^{-1}	Secondary CFU mL^{-1}	Change in CFU mL^{-1} between Inoculum and Primary broth	Change in CFU mL^{-1} between Primary and Secondary broths
PW1 10^{-7}	1.00×10^{1}	5.00×10^{1}	0	4.00×10^{2}	-5.00×10^{1}
PW1 10^{-7}	2.67×10^{1}	5.00×10^{2}	0	4.73×10^{2}	-5.00×10^{2}
PW1 10^{-7}	2.17×10^{1}	4.75×10^{2}	0	4.53×10^{2}	-4.75×10^{2}
PW1 10^{-6}	2.90×10^{2}	2.77×10^{5}	2.58×10^{7}	2.76×10^{5}	2.55×10^{7}
PW1 10^{-6}	1.62×10^{2}	7.46×10^{5}	3.58×10^{6}	7.46×10^{5}	2.84×10^{6}
PW1 10^{-6}	2.68×10^{2}	1.39×10^{6}	3.67×10^{6}	1.38×10^{6}	2.28×10^{6}

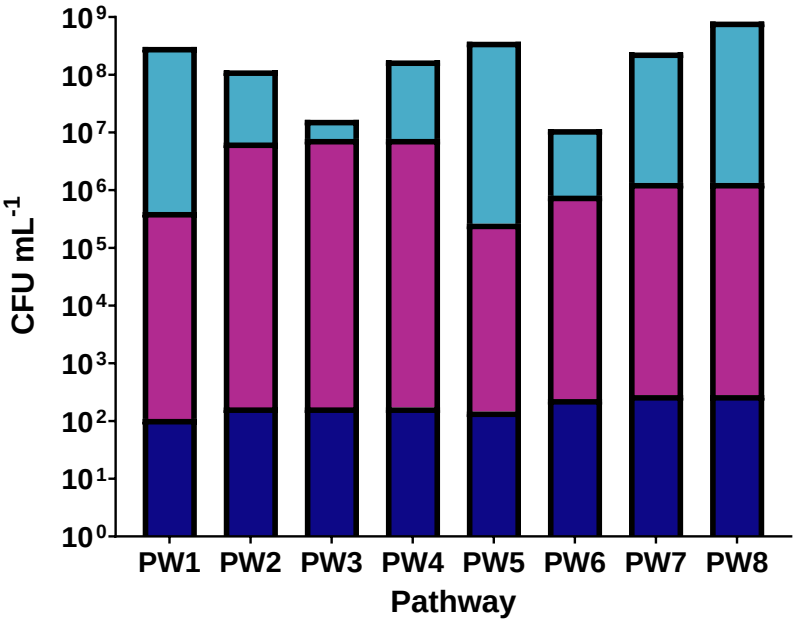


Figure 3.8: Shows the cumulative growth for the Malawian clinical strain through pathways one to eight. Blue represents the CFU mL^{-1} of the Inoculum, Purple represents the CFU mL^{-1} of the primary broth after incubation and the Teal represents the CFU mL^{-1} for the Secondary Broth. The inoculum selected for this experiment was using samples diluted ten-fold, six times, as per Table 3.6 demonstrated diluted seven times prevented detectable growth in subsequent broths. Each of these were performed in triplicate and the mean plotted above.

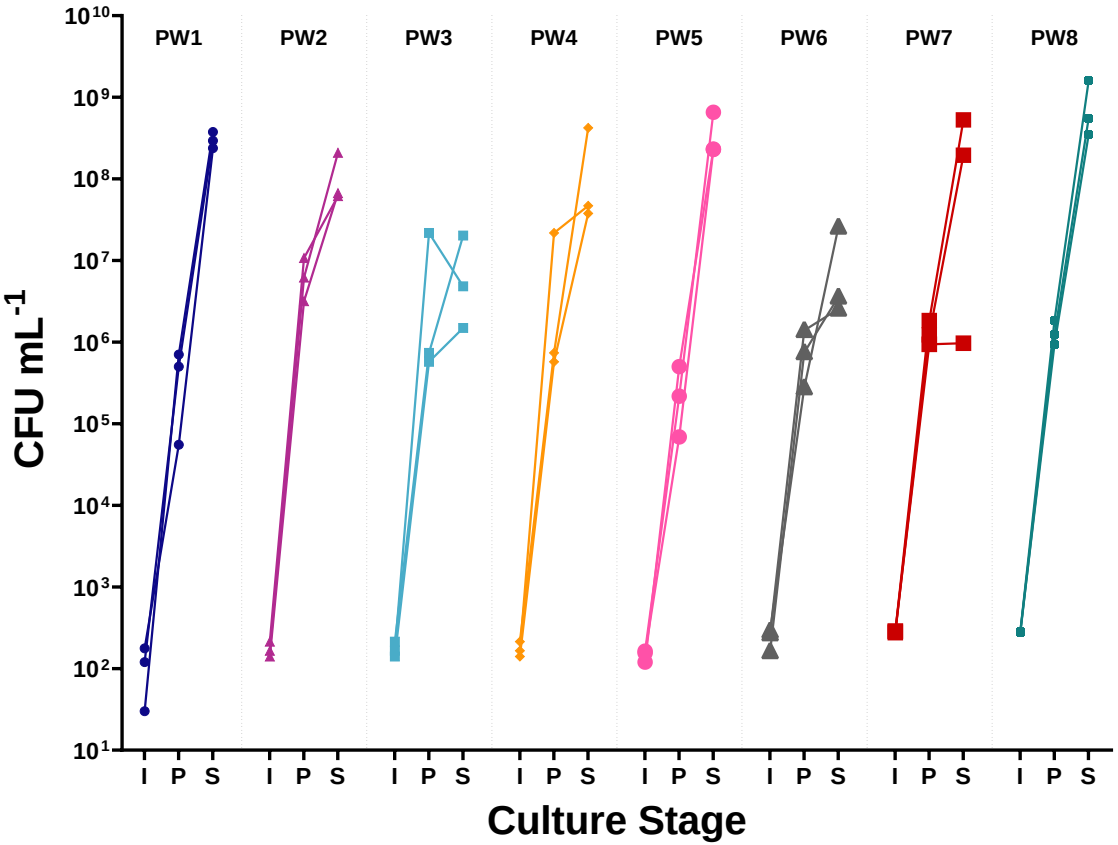


Figure 3.9: Growth data for each culture pathway (Table 3.5) using the Malawian clinical control strain. I Is the CFU mL^{-1} of the inoculum, P is the CFU mL^{-1} of the primary broth after incubation and S is the CFU mL^{-1} of the secondary broth after incubation. Each pathway was performed in triplicate, the graph maps out each of these replicates per pathway as advised for publication (Chapter 5) by the MLW head statistician, Dr Marc Henrion, due to the replicates being too few for statistical analysis.

3.5 Long-term survival of *S.* Typhi in water

In parallel to work isolating *S.* Typhi from complex matrices, I evaluated its long-term viability in water. Samples of water were collected from a domestic pond and the local stream, called the Silk Stream in Colindale, before aliquoting 199 mL into 250 mL conical flasks (Figure 3.10), with three different variations. The first was untreated, the second was filtered through a 0.45 µm filter membrane and the third was autoclaved (121 °C with a holding time of 15 minutes). To each of these, an inoculum of the Malawian strain 02 (Table 3.1) was prepared to a 0.5 MacFarland standard density in 10 mL of MRD media and 1 mL added to each flask. Each flask based on water and treatment type were performed in triplicate.

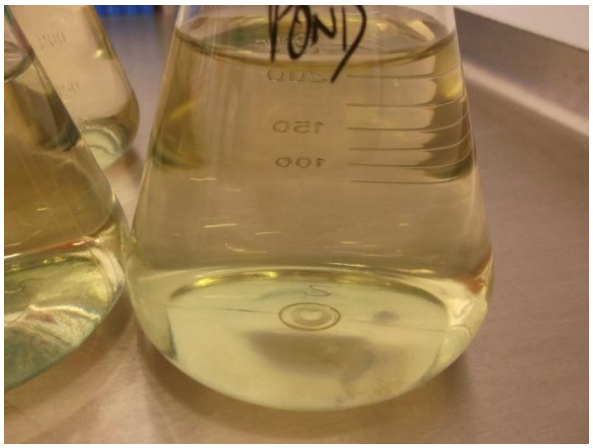


Figure 3.10: Example of the conical flasks used, this one specifically being the pond water, as left in the CL3 laboratory during this experiment.

Each day, six samples were taken from each bottle and enumerated onto mCASE until *S.* Typhi could no longer be detected through direct plating (Figure 3.11 and Figure 3.12). Following this, each water was then cultured through the pathways PW1 to PW4 for evaluation of the capacity for the pathway to recover sub lethally damaged cells.

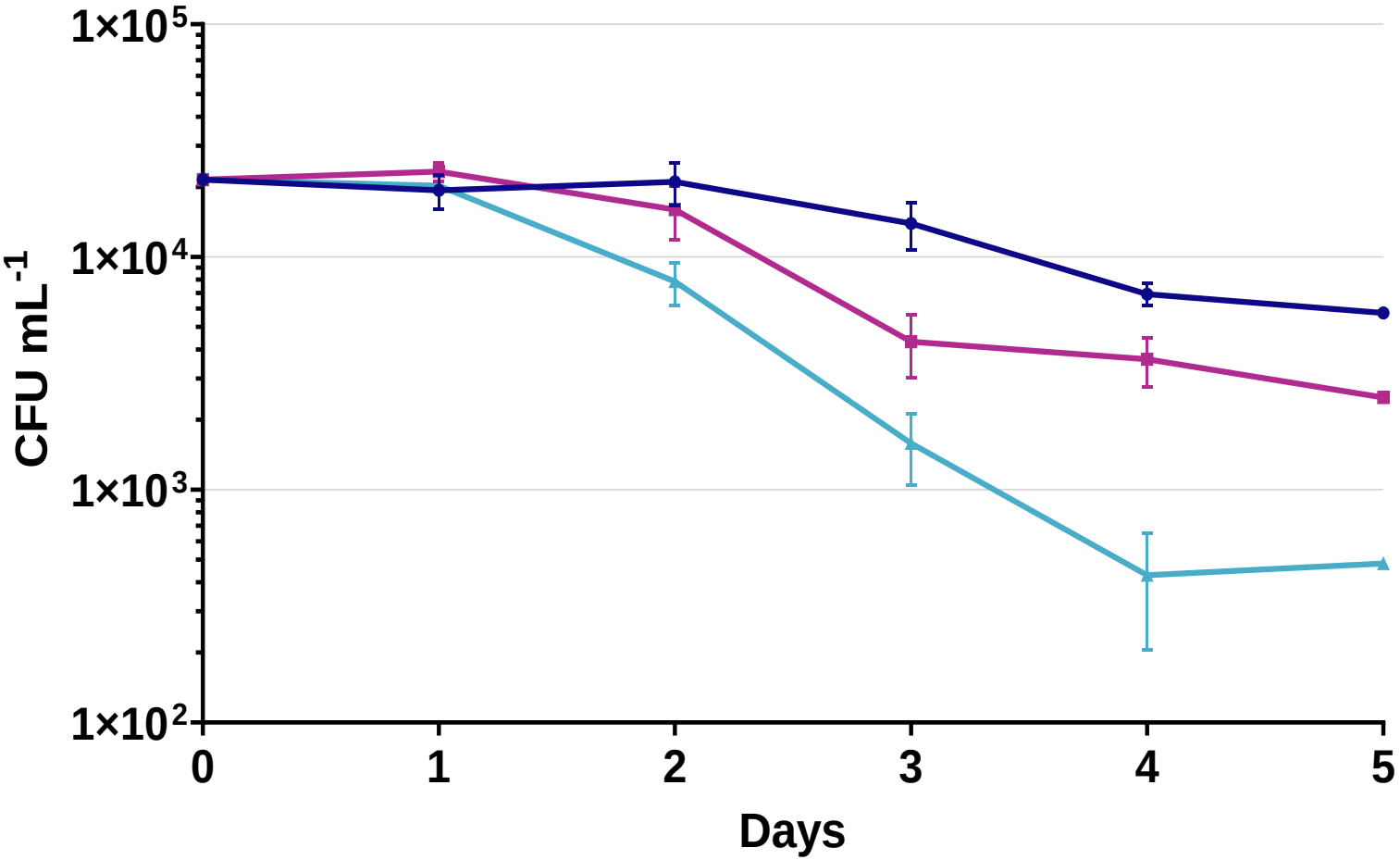


Figure 3.11: Mean colony counts in CFU mL^{-1} of *S.* Typhi strain 02 when inoculated into stream water and cultured onto mCASE daily. Dark Blue: water filtered through a 0.45 µm filter before inoculation, Purple: water autoclaved before inoculation, Teal: stream, unmodified. Error bars represent standard error of the mean (SEM) in colony count calculations from the spot titre method used (Miles *et al.*, 1938).

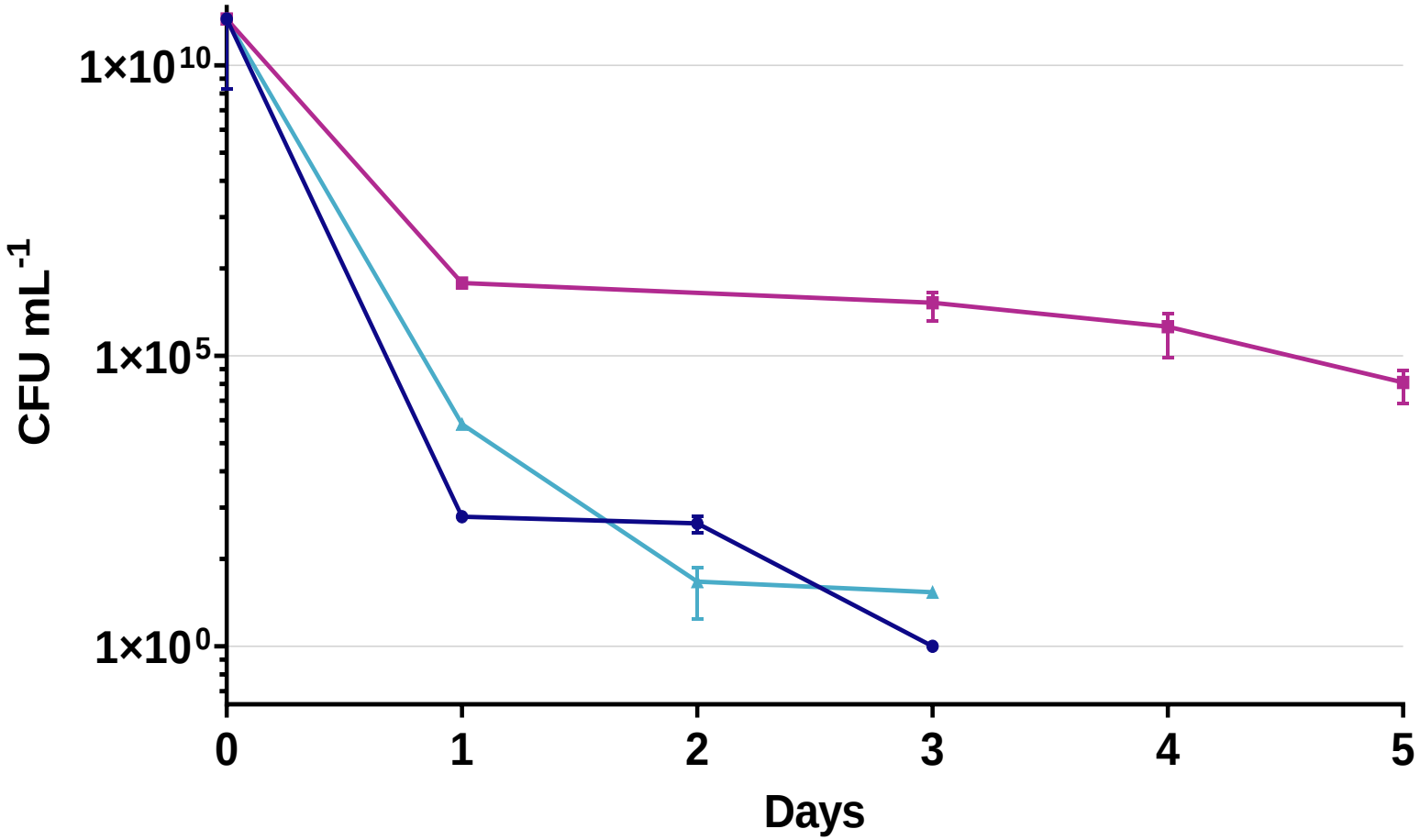
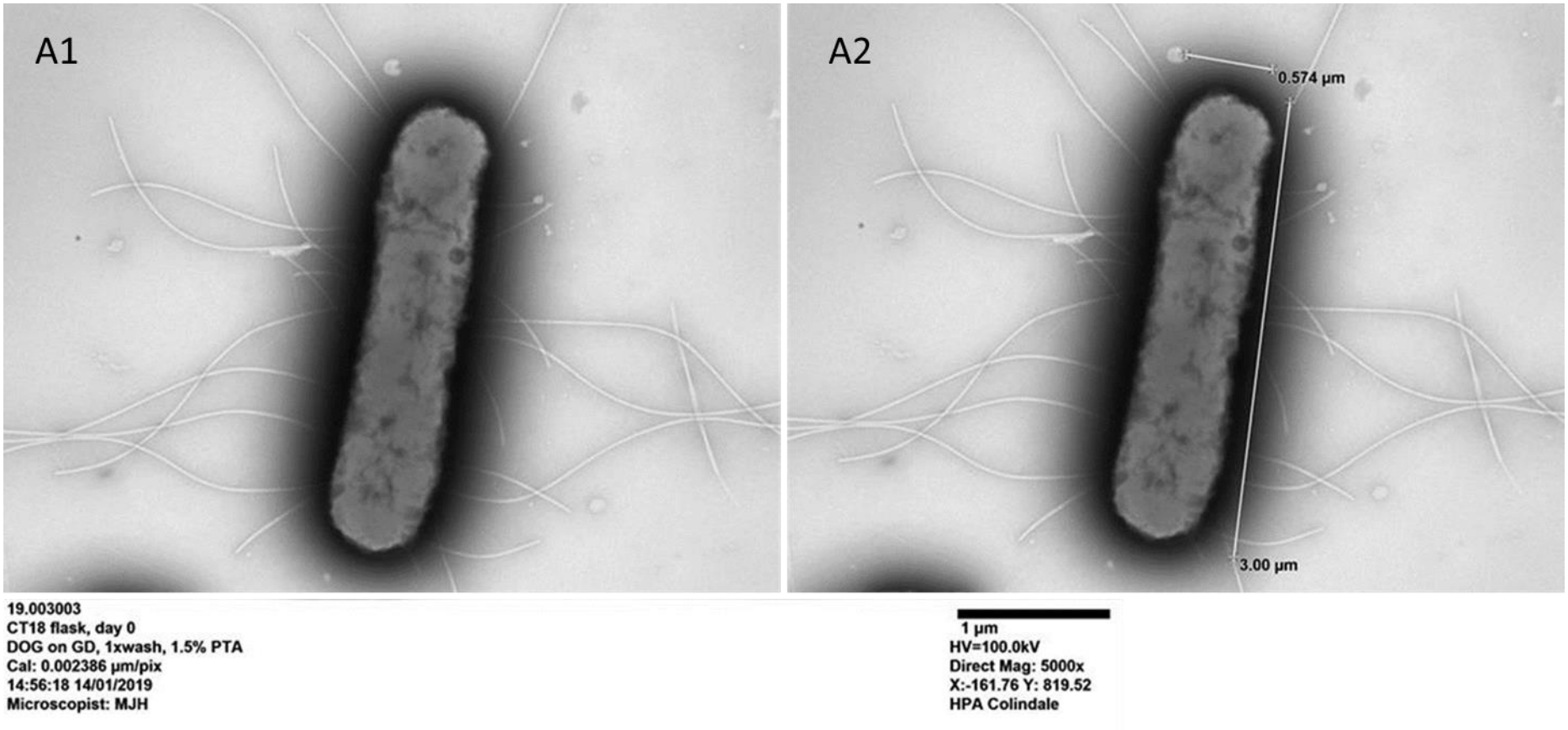
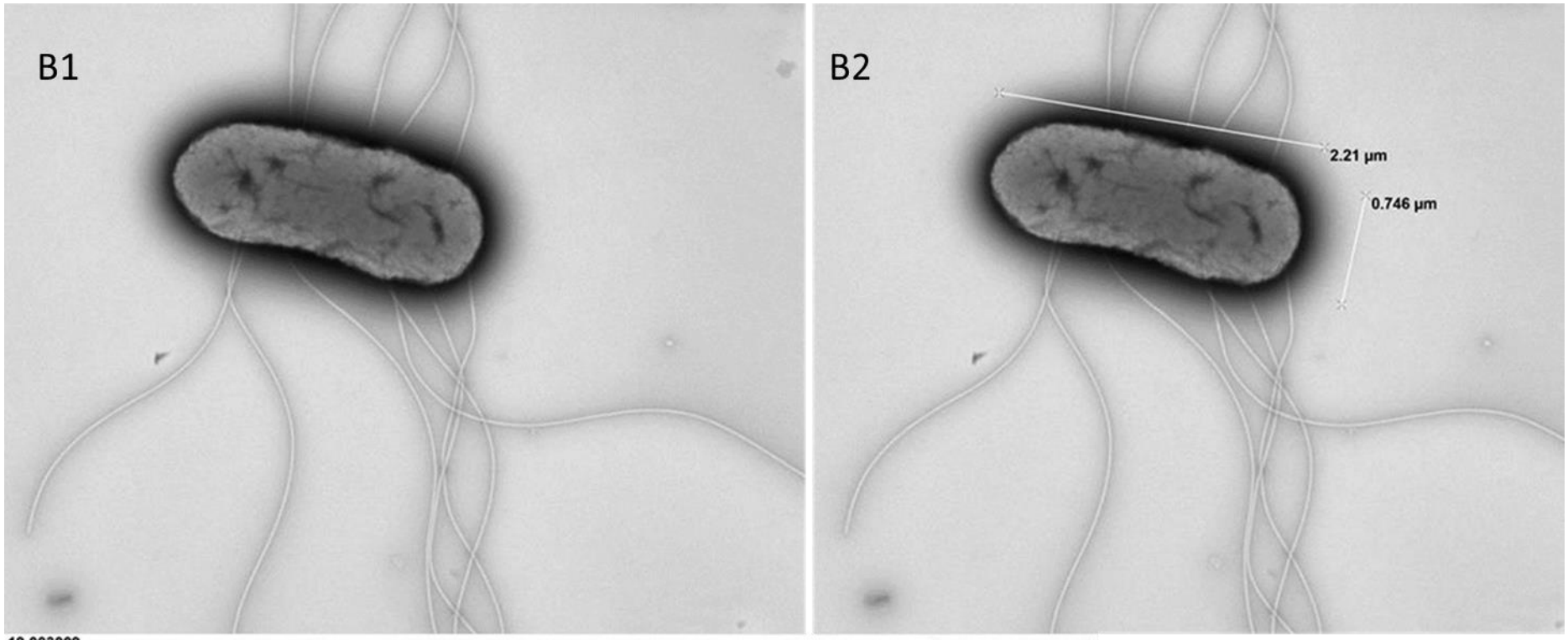


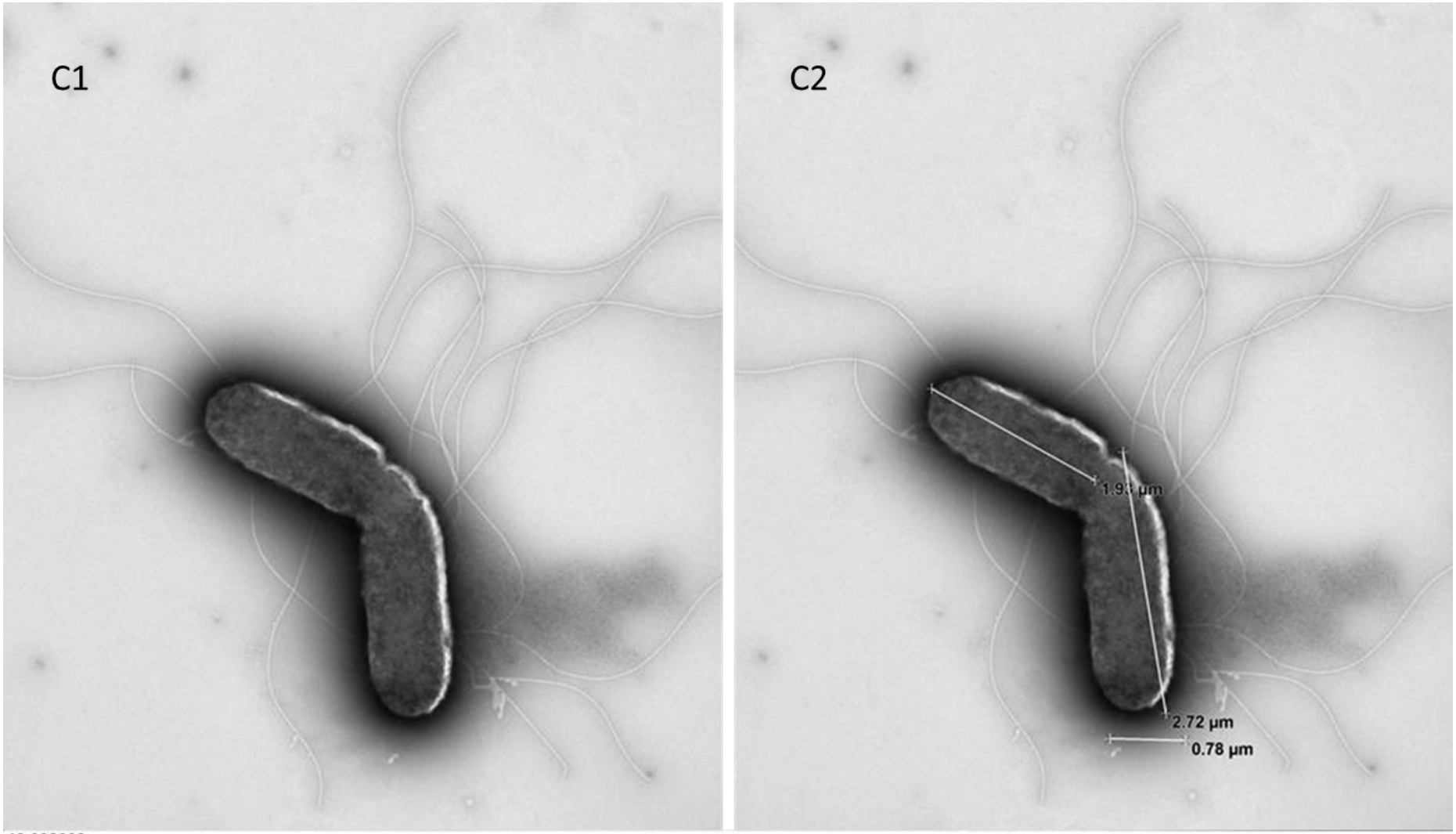
Figure 3.12: Mean colony counts in CFU mL^{-1} of *S.* Typhi strain 02 when inoculated into pond water and cultured onto mCASE daily. Dark Blue: water filtered through a 0.45 µm filter before inoculation, Purple: water autoclaved before inoculation, Teal: pond stream, unmodified. Error bars represent SEM in colony count calculations from the spot titre method used (Miles *et al.*, 1938).

After six days, growth could no longer be observed by direct plating of the water samples, for the filtered and unmodified water samples. These samples were cultured using PW1, PW2, PW3 and PW4. *S.* Typhi was successfully isolated using Pathways PW1 and PW2 from the filtered and unmodified samples of the stream and the unmodified pond water sample, but not through pathways PW3 and PW4. The filtered pond water did not give a positive through any of the pathways, whilst the autoclaved was still detected through direct plating.

In addition to attempted culture and detection by PCR, each sample had an aliquot taken for electron microscopy (EM) at the point of inoculation and after two weeks to compare the cells within the samples under EM (Figure 3.13) Each of the samples were inactivated and submitted to the Colindale electron microscopist, Dr Matthew Hannah.


A1
A2
0.574 µm
3.00 µm
19.003003
CT18 flask, day 0
DOG on GD, 1xwash, 1.5% PTA
Cal: 0.002386 µm/pix
14:56:18 14/01/2019
Microscopist: MJH
1 µm
HV=100.0kV
Direct Mag: 5000x
X:-161.76 Y: 819.52
HPA Colindale


B1
B2
2.21 µm
0.746 µm
19.003009
CT18 flask, day 0
DOG on GD, 1xwash, 1.5% PTA
Cal: 0.002386 µm/pix
15:08:31 14/01/2019
Microscopist: MJH
1 µm
HV=100.0kV
Direct Mag: 5000x
X:-493.88 Y: 753.28
HPA Colindale



19.002002
JR, #9 CT18, tube, W2
DOG on GD, no wash, 1.5% PTA
Cal: 0.003977 µm/pix
11:56:53 04/01/2019
Microscopist: MJH

1 µm
HV=100.0kV
Direct Mag: 3000x
X:132.58 Y: 765.01
HPA Colindale

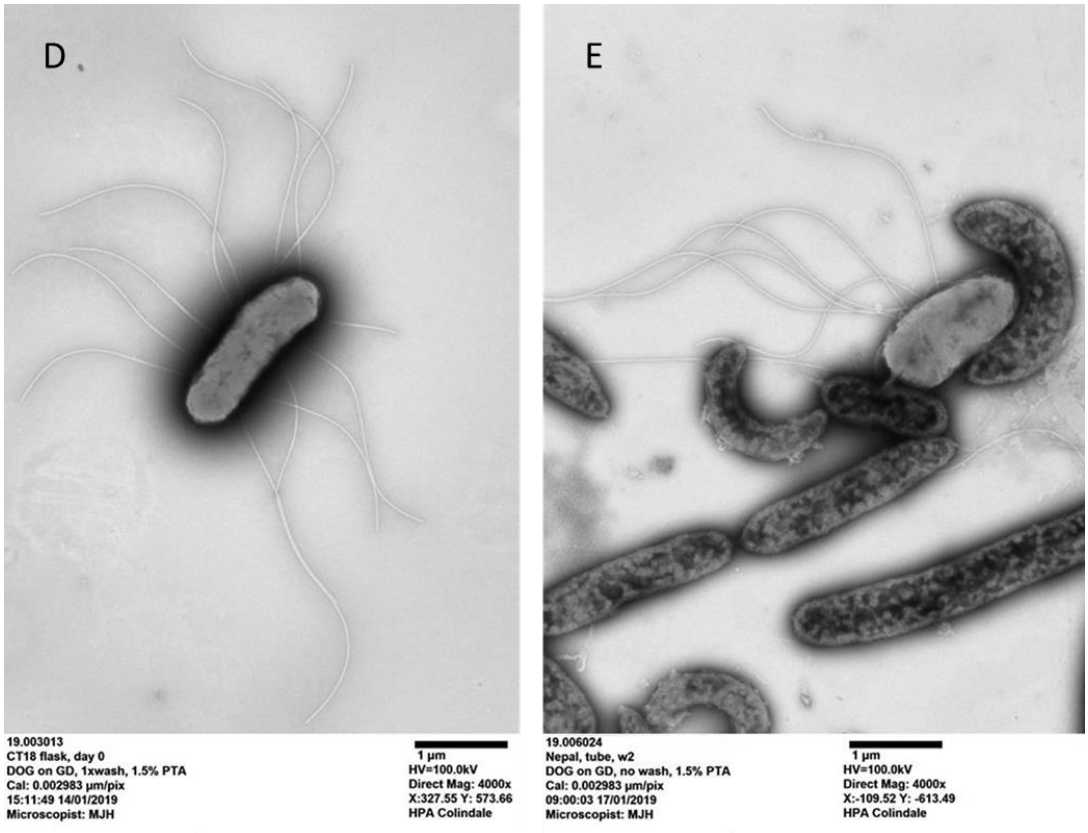


Figure 3.13: Electron Micrographs of *S.* Typhi from long term survival samples in Water, as taken by Dr Matthew Hannah:

A. CT18 immediately after exposure to water (day 0) in conical flask from Figure 3.10, with A2 showing measurements of the organism

B. CT18 at day 0, with clearly visualised flagella

C. CT18 after two weeks in water undergoing binary fission

D. CT18 at day 0, with clearly visualised flagella

E. Nepalese strain, strain 1, mixed with non-salmonellae from the unsterilised water, two weeks after exposure

3.6 Mixed culture challenges

For the eight pathways assessed by me, labelled PW1 to PW8, a blinded study was performed. Dr Nicola Elviss created five culture broths containing a mixture of control strains which included a variety of non-typhoidal organisms (Table 3.7). This was done by inoculating five separate 50 mL falcon tubes, containing 10 mL of RLS, by taking growth with a 10 µL loop of subcultures from CBA of each NCTC control strain and then vortexing until homogenous.

Table 3.7: List of organisms in each of the blinded mixtures used to trial the remaining eight pathways

Mixture 1	*Bacillus subtilis*
	Enterococcus faecalis
	Escherichia coli
	Pseudomonas aeruginosa
	Staphylococcus aureus
Mixture 2	*Bacillus cereus*
	Enterococcus faecalis
	Escherichia coli
	Raoultella planticola
	Salmonella Nottingham
Mixture 3	*Escherichia coli*
	Salmonella Nottingham
	Staphylococcus epidermidis
	Salmonella Typhi
Mixture 4	*Bacillus cereus*
	Listeria innocua
	Pseudomonas aeruginosa
	Raoultella planticola
	Saccharomyces cerevisiae
	Salmonella Typhi

Mixture 5	*Salmonella* Nottingham
	Vibrio furnissii
	Salmonella Typhi

All five broths were then cultured through the eight pathways and colonies typical of *Salmonella* spp. were tested with *Salmonella* anti-sera and identity was confirmed by real time PCR. Three out of five mixed cultures contained *S.* Typhi. For the three *S.* Typhi mixes, all eight pathways had isolates with typical morphology on mCASE (Figure 3.14). In pathway PW2 and 6, the *S.* Typhi could not be isolated as pure culture from mixtures three and five, and was only identifiable as a *Salmonella* species through phenotypic identification due to the excessive growth on the agar of NTS.

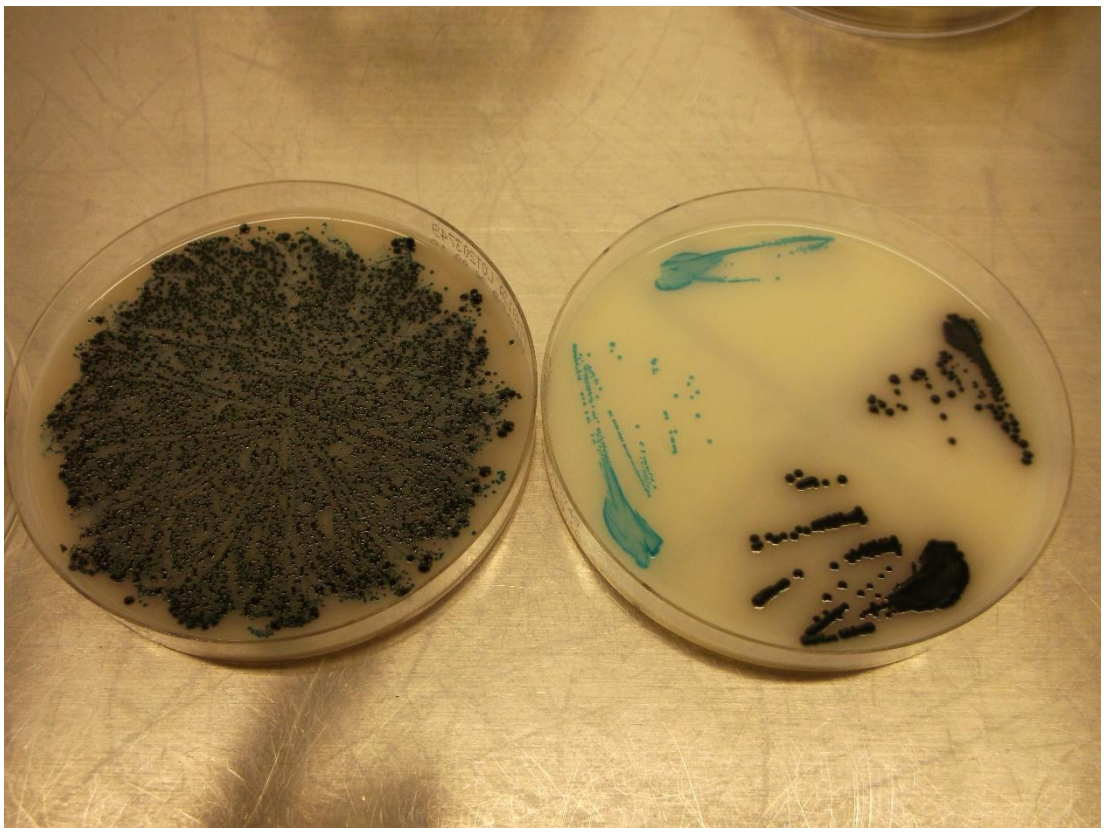


Figure 3.14: When the blinded cultures were plated onto mCASE, some of the colonies were more difficult to isolate than others, as seen here with growth of “contaminants” from the

environmental microbiology control samples, with non-target organisms being colourless, or, in this case, black with production of β-galactosidase.

3.6.1 Final pathway selection and validation in Malawi

Over a three-month period of sample collection (March to May 2019), the pathways PW1 and PW8 were used in parallel in the field. Whilst no *S.* Typhi were identified as being isolated from the 27 water samples collected, a retrospective PCR of all isolates in archive found two strains had been isolated, but they were unrecoverable due to inappropriate storage that was available during the validation period, these had been isolated through PW5 ($bile^-$ and selenite F broth). Observations from growth on the mCASE identified that pathway PW5 demonstrated better recovery of NTS than pathway PW8. Pathway PW8 also allowed greater growth of contaminating organisms including, *E. coli*, swarming bacteria, and fungi, which appeared to impact NTS recovery and therefore was likely to reduce the success of isolating *S.* Typhi. This is work is detailed in Chapter 5.

3.6.2 Culture Pathway modifications

With the results of the pre-pilot, some additional modifications to the pathway were initially explored to increase selectivity without impacting on its performance for the culture of *S.* Typhi. Cycloheximide powder was added at a concentration of 20 mg/L to the bile broth ($bile_{Cy}$) to reduce fungal contamination. When performing an enumeration experiment on the $bile_{Cy}$, there is a growth curve significantly lower than that of $bile^-$ (Figure 3.15). This was also added to some samples in parallel to their usual processing. When plates were compared between $bile_{Cy}$ and $bile^-$, there seemed to be little to no reduction of colony types suspected to be fungal contaminants and the dilution of samples before plating seemed more effective at reducing contamination of non-target organisms.

Sucralose was also added to $bile^-$ ($bile_S$), replacing the dextrose, to help inhibit sucrose fermenting bacteria such as *E. coli*. This was done by modifying the recipe with a direct swap of the dextrose with an equal mass of sucralose. Being a synthetic modification of sucrose, by substituting three specific hydroxyl groups with chlorine atoms, sucralose can still be recognised as sucrose. Due to the lowered energy gain from metabolising sucralose, it may have a bacteriostatic effect (Wang *et al.*, 2018) whilst *S.* Typhi may metabolise the peptones instead of the sugars immediately. When compared to $bile^-$, $bile_S$ shows a very similar quantity of growth over 24 and 48 hours when incubated at 37 ± 1 °C (Figure 3.15).

Immuno-magnetic beads, tagged with anti-sera for antigens O9, Vi and a Pan- *Salmonella* antigen were also tested. When compared with pure suspensions of *S.* Typhi, each of these seemed to perform equal to, or worse than direct subculture – likely due to the much lower volume being transferred. When used with mixed culture, each allowed *E*. *coli* to be carried over. This was likely due to washing stages not being sufficient, however the vigour of washing was determined as there was concern over damaging the *Salmonella* spp. cells.

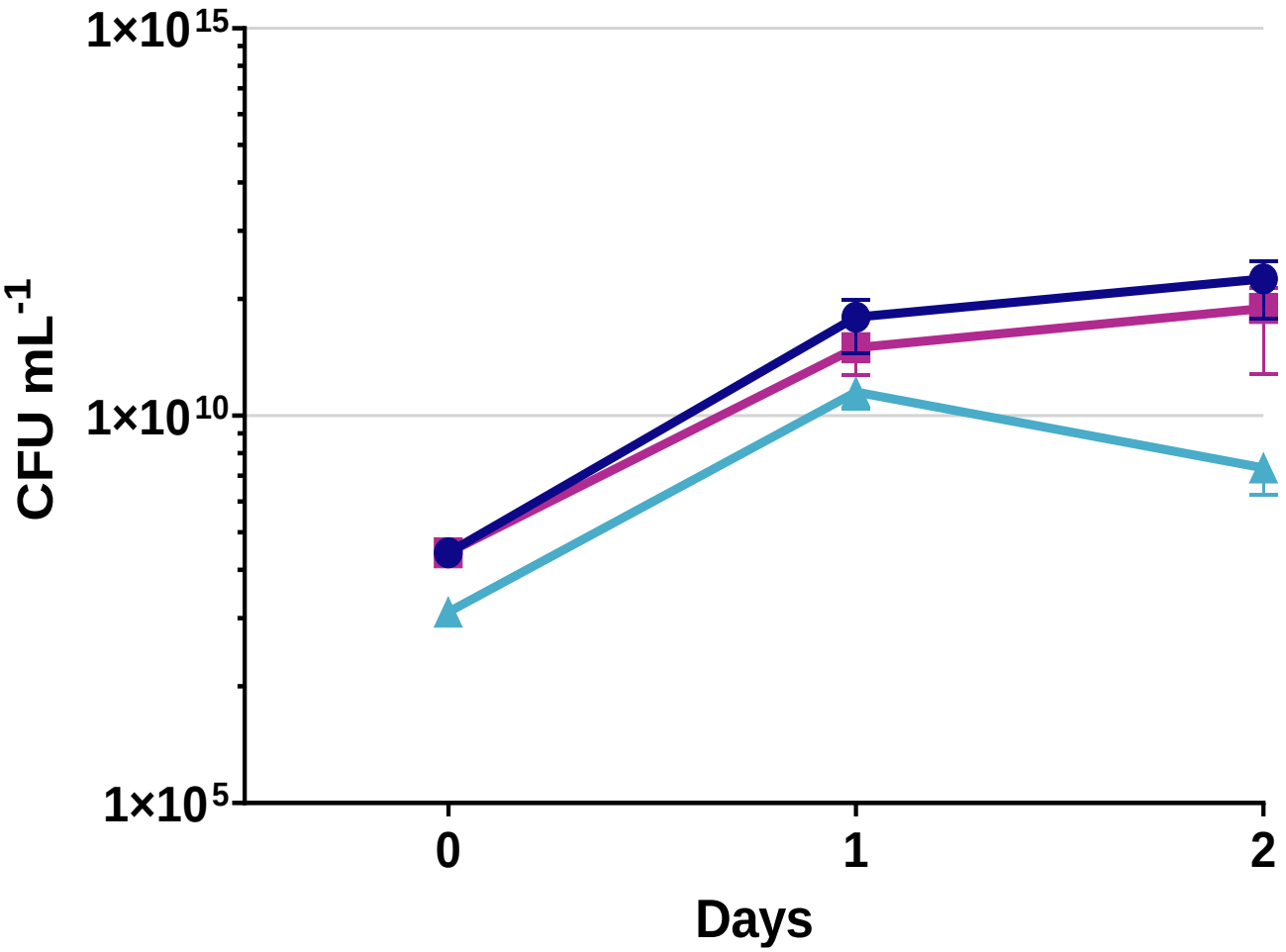


Figure 3.15: Growth of *S.* Typhi control strain 21 in two different bile$^-$ broth modifications from the inoculum (day 0), 24-hour and 48-hour incubation. Dark blue: unmodified bile$^-$, purple: bile$^-$ with sucralose, teal: bile$^-$ with Cycloheximide 20 mg/L. Error bars show SEM.

3.7 Discussion

The aim of this study was to develop a method that would allow the isolation of *S.* Typhi from environmental samples. I began by comparing the media that was likely to be appropriate for the isolation of *S.* Typhi from complex background matrices and refined my understanding of their performances based on direct culture, enumeration through both individual and combined culture pathways, and the selectivity of the media by mixed culture challenge.

With the assistance of the manufacturer, a novel chromogenic agar was produced which has been demonstrated here to be effective for the culture of *S.* Typhi, and whilst it doesn't specifically select for *S.* Typhi, it allows for successful isolation of serovariants of the genus *Salmonella* from complex background matrices. Whilst the production of H_2S in BSA media is theoretically a*ttr*active for its ability to indicate that a colony is *S.* Typhi, this proved to be inconsistent and therefore was deemed unreliable. Other agar used for the isolation of *S.* Typhi are not specific or also rely on distinction by a negative lactose fermentation reaction or H_2S production, therefore being no more specific for our purposes than the mCASE. Further, mCASE was modified specifically for this project to achieve a more optimal performance for *S.* Typhi; with commercially produced media focussing on the isolation of NTS from food, water and environmental samples, additional work could be pursued in the future to modify each of the media used to increase their performance for *S.* Typhi recovery.

Multiple broths were then considered to allow sub-lethally damaged cells to recover from the environment. Media were rejected if they were being inappropriate for the isolation of *S.* Typhi specifically or if thought to be difficult to acquire in a low-income setting, or to have limited shelf life in contexts where procurement can be challenging and expensive. The final candidates tested were thus selenite cystine, selenite F, bile$^{+/-}$ broths and BPW. Work performed by my predecessor determined that BPW was not sufficient in its ability to recover *S.* Typhi once introduced to competitive organisms, such as *E. coli* and NTS. Selenite cystine was initially favoured for its selectivity against the competitive organisms, but due to supply, selenite F was assessed also and found to have better performance when used in conjunction with a bile$^{+/-}$ pre-enrichment. Whilst the toxicity of selenite-based media is a disadvantage, both in its powder form (inhalation), as well as the danger it poses to aquatic life and environments, this can be controlled through comprehensive risk assessment and limitation of the volume to 10 mL per sample with appropriate disposal systems in place (Neogen, 2019).

Due to the harshness of selenite media and the low concentrations of *S.* Typhi in contaminated water samples, a pre-enrichment broth that provided selectivity for *Salmonella* spp. was considered a requirement. As part of my evaluation of culture pathways, 2% bile broth was selected. The bile broth, a modified version of EE broth with the Typhi-inhibitory malachite green removed, was selected for the recovery of sub-lethally injured *S.* Typhi. This pre-enrichment broth also mimicked the known exposure to bile in the human host during *S.* Typhi infection of the duodenum (Parry *et al*., 2002). Bile^{+} showed the best performance in enumeration but caused higher levels of contaminant organisms when assessed in mixed culture. With the addition of iron, it appeared to make competition more robust when sub-cultured into selenite F or cystine broth.

The long-term survival study of the Malawian strain 02 in pond and river water showed different results between the two water types, where in the stream, the filtered water sample performed better compared to autoclaved and unmodified, but all followed a similar decline over the five days cultured; the pond water showed a much more rapid decline for the filtered and unmodified, with the autoclaved sample following a decline similar to that of the stream. The longer persistence of, and higher CFU mL^{-1} of the autoclaved pond water could be due to the much higher inoculum compared to that of the stream, but the rapid decline of the filtered and unmodified could be due to the presence of a biological component such as a bacteriophage that would be destroyed or denatured by the autoclaving. The high inoculum and rapid decline in one day for the pond water highlights the importance of ensuring samples are processed as quickly as possible once collected. Furthermore, that the isolation of *S.* Typhi from these water samples after direct culture was no longer possible showed that the pathways that used a bile broth for pre-enrichment were more effective, possibly due to the stress the organisms are under when in non-optimal environments.

Laboratory results in London indicated that pathways PW1 (bile^{-} to selenite cystine), 5 (bile^{-} to selenite F) and 8 (selenite F to bile^{+}) all had potential for use *in situ*, and as such, were all validated in Malawi with a locally acquired clinical isolate and river water samples collected from hotspots identified within the city for high levels of typhoid cases. When utilised in Malawi, PW5 appeared to perform best when isolating NTS, as no *S.* Typhi had been recoverable during this period, as the two archived samples that tested positive by PCR could not be confirmed due to the Cryobeads being unviable due to long-term storage at -20 °C instead of the recommended -80 °C.

Lastly, I attempted some modifications to the pathways to improve their selectivity, primarily the introduction of cycloheximide to the bile broth. The introduction antifungal powder caused a decrease in growth for the *S.* Typhi control strain when compared to unmodified bile$^-$ broth. Further work would need to be performed with complex background matrices spiked with the control strain to determine if the reduction in enrichment is balanced by the increase in selectivity, and thus reduction in competition for nutrients.

I also tested alternative sugars in the bile through the replacement of dextrose with sucralose and immuno-magnetic bead separation. The use of bile$_S$ compared to bile$^-$ showed some promise, with the growth levels of the *S.* Typhi control being unaffected significantly, however, whilst some studies on this artificial sweetener have shown its bacteriostatic capabilities (Wang *et al*., 2018), others have shown that it is only short-term inhibition (Shahriar *et al*., 2020) or ineffective with increased growth of *E*. *coli* and coliforms (Shil and Chichger, 2021), suggesting other artificial sweeteners may have higher levels of inhibition. The immune-magnetic beads used for isolation of *S.* Typhi were prepared with the 09, Vi and a pan- *Salmonella* anti-sera, and whilst of the three of these, the pan- *Salmonella* bead appeared most effective, under laboratory conditions, none of these targets gave a sufficient recovery compared to subculture in selective media to justify the cost of using these.

3.7.1 Limitations

The major limitation with the work at this point is the use of laboratory culture. Whilst many of the control strains have been isolated from clinical sources, they are healthy strains maintained to keep them viable for future studies. As a human restricted pathogen, *S.* Typhi will either be sub-lethally damaged, or have some unknown environmental survival mechanism or niche, such as adhering to the naturally formed biofilms from other organisms, or an intracellular niche within predatory amoeba.

Additionally, whilst some validation work was performed in Malawi with local water sources, most of the complex matrices were sourced in London and may not be representative of the challenges faced by *S.* Typhi circulating in Blantyre, Malawi. Furthermore, many of the media reviewed for this project have been validated in the field of clinical diagnostic microbiology, where the background matrices are vastly different to what may be seen in the environment. Whilst faecal contamination needs to be high for *S.* Typhi to be present in infectious doses for healthy individuals, there are other aquatic, sediment and plant-based organisms that may be present in environmental samples that are not considered in the clinical setting.

Determining the full environmental microbiome to account for these organisms was, however, beyond the scope of this project.

3.7.2 Next Steps

Due to the proposed culture pathway being unable to verify *S.* Typhi alone, and with biochemistry and serological testing being expensive and time consuming, an effective confirmation method had to be chosen. Due to its flexibility, a real time PCR assay was selected, as this could be used for direct detection from any environmental sample, broths stage or on pure isolates from agar, and is detailed in the next chapter.

4 Optimising PCR-based detection for *Salmonella* Typhi Culture

4.1 Summary

An effective, accurate and high-throughput method of *S.* Typhi confirmation from isolates was an integral part of creating a routine ES lab. In reflection of the requirements for the type of work being performed, a PCR assay was optimised from a clinical diagnostic assay. Using this tool, six isolates were positively identified as *S.* Typhi and confirmed by gold-standard methods including an API 20E (BioMerieux) biochemical panel, anti-sera agglutination testing (Pro-Lab diagnostics) and antimicrobial susceptibility testing (EUCAST). Further modifications to the assay and the extraction methods were explored, such as an HRM multi-target assay for isolates and the use of commercial and in-house extraction methods such as Magna Extract (Byrne *et al*., 2022) or Qiagen 96-well DNeasy Blood and Tissue kit, or the QIAamp PFP kits.

4.2 Introduction

To confirm the identity of presumptive *S.* Typhi isolates from cultured samples, molecular methods were chosen over traditional phenotypic identification due to their lower total cost, high throughput capability, and flexibility in application. A real time PCR was selected based on a literature review and ultimately, a modification of the assay described by Nair *et al* (2019) was selected due to compelling evidence that a multi-target assay for *S.* Typhi from environmental samples was superior to the mono-target assay typically used.

Any isolates that showed potential to be *S.* Typhi positive were confirmed using gold standard culture-based methods used by clinical diagnostic laboratories. Putative *S.* Typhi had their identify confirmed by a combination of API (BioMerieux) and anti-sera agglutination testing (Pro-Lab diagnostics). Lastly, ASTs were performed due to the circulation of MDR H58 strains being seen in the clinical setting.

Additionally, an HRM assay was assessed due to its potential cost savings, as a singular intercalating dye, such as SYBR and EVAGreen is significantly cheaper than the use of a TaqMan™ or other branded fluorophore dye and specific probe region. Whilst not as versatile as a screening tool for a direct detection-based assay, like a real time PCR assay, it has the potential benefit to be cost saving when confirming the identity of isolates generated

by culture. Further, there is potential scope to expand an HRM assay to target typhoidal serovars *S*. Paratyphi A, B and C, and to key nontyphoidal serovars associated with iNTS disease in Africa such as *S*. Enteritidis and *S.* Typhimurium; giving an all-purpose tool for ES of salmonellae associated with invasive disease.

Whilst the initial aim was to identify isolates from the culture pipeline, it would be desirable to be able to screen the pathway developed in Chapter 3 by PCR and thus reduce downstream workload. The challenge is to extract high quality DNA from complex samples like natural water sources, which is significant with known PCR inhibitors, denaturing of the organisms in transport and processing, as well as nucleases present in samples - making the process difficult and potentially causing assay inhibition of the gene targets, destruction of the DNA extractions, or inhibition of the dyes or master mix during testing. I therefore also tested different methods for extraction of DNA from both complex matrices and directly cultured samples before a final method was selected.

4.3 Results

4.3.1 Real Time PCR Assay

For the identification of *S.* Typhi, a real time PCR was developed through the modification of the assay described by Nair *et al.* (2019). The original assay was a series of duplex primers to identify *S.* Typhi and *S*. Paratyphi A, B and C so they could be separated from NTS. The modified assay described here utilised the targets for *S.* Typhi (*tviB* and *staG*), the pan-Salmonellae target (*ttr*) and a gene which is not present in *S.* Typhi to distinguish a *tviB* positive strain as either *S.* Typhi or *S*. Paratyphi C (*sseJ*). This modification of the assay replaced the fluorophores used as dyes for each probe target to allow multiplexing, with each variant at each stage outlined in Table 4.1.

Table 4.1: Shows which fluorescent dye was used in the probe of each gene target at various points within development and validation

Target Gene	Probes used at each stage of optimisation			
	Stage 1	**Stage 2**	**Stage 3**	**Stage 4**
ttr	FAM	FAM	FAM	FAM
tviB	Texas Red	Cy3	TAMRA	TET
staG	Cy5	Cy5	Cy5	Cy5
sseJ	Cy3	Yakima Yellow	Yakima Yellow	Yakima Yellow

These targets were selected as they allowed the use of a Sample Processing Control (SPC, Eurogentec) which acted as an extraction and internal positive control using the Yakima Yellow fluorophore in its probe. The real time PCR assay was performed on the Applied Biosystems ViiA 7 for these first screening tests, using boilates of the *S.* Typhi strains 1 to 20 from Table 3.1 and an *S.* Typhimurium NCTC 12116. A non-template control and PCR negative was run with every assay, and the controls used here to challenge the assay for optimisation were cultured strains originally from clinical cases.

In monoplex, each of these targets amplified with Cts in an acceptable range, however, once used in a quadruplex (Figure 4.1), the Texas Red and ROX fluorophore that was used as a passive reference dye could not be reliably distinguished, which adversely affected all amplification curves. *Escherichia coli* strain NCTC 9001 did not cross-react with any primer target. However, *tviB* did not amplify with Ty21a.

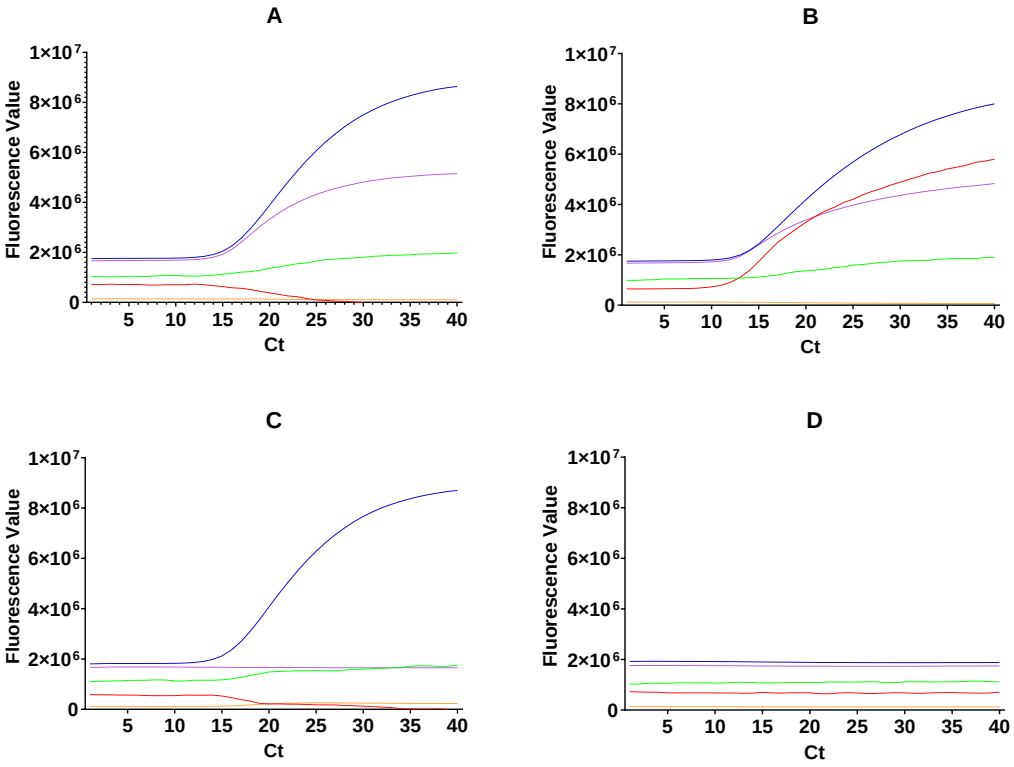


Figure 4.1: These graphs show the amplification curves of fluorescent values for the first quadruplex assay in its multicomponent format, using FAM for *ttr* (Blue), Cy5 for *staG* (Purple), Texas Red for *tviB* (Red) and Cy3 for *sseJ* (Orange. A) Shows the assay for Ty21a, where *sseJ* and *tviB* are both undetected. B) shows the assay for CT18, where all *S.* Typhi genes amplified. C) shows the assay for *S.* Typhimurium, the amplification for *sseJ* using Cy3 is indistinguishable, and when compared to the baseline ROX, would be regarded as not amplifying due to interference from the Texas Red being present. D) shows the E. coli assay, with all primer targets being negative. Passive reference dye, ROX, is in green.

As such, the assay was repeated with *tviB* utilising the Cy3 fluorophore (Figure 4.2) and *sseJ* using Yakima Yellow (Table 4.1, Stage 1); the reasoning for this change was that *sseJ* is not a reliable target when used in a complex sample directly as it is found in all NTS strains as well as *S*. Paratyphi C, and the SPC would only be used for direct DNA extraction from environmental samples – therefore, being able to distinguish both targets in the same assay was not necessary.

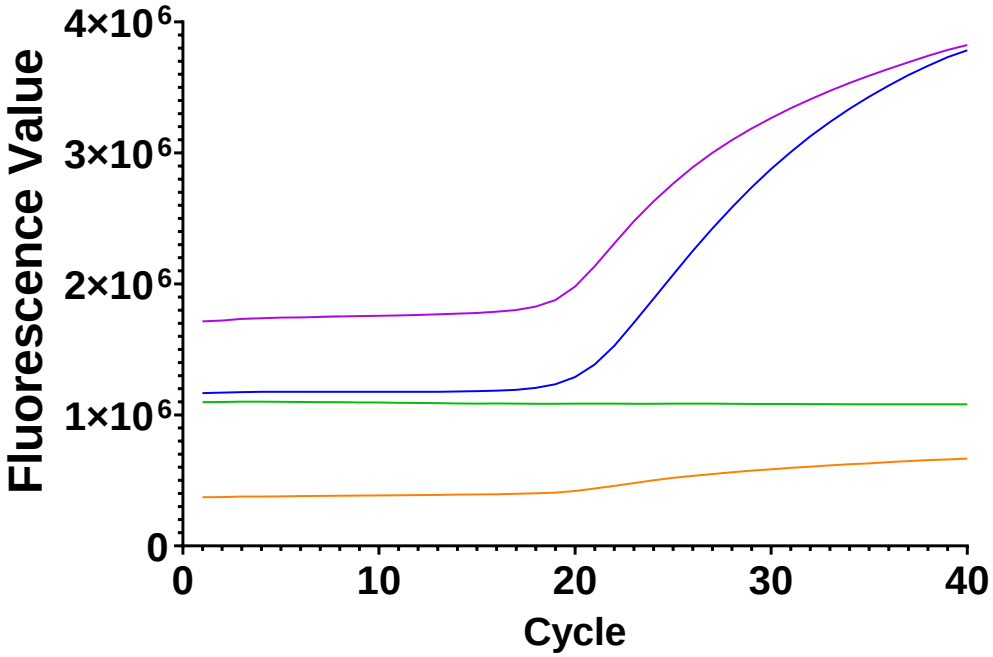


Figure 4.2: Graph shows the multicomponent fluorescence values of an *S.* Typhi control strain when *sseJ* is removed, and the *tviB* probe uses Cy3 fluorophore. Blue = FAM (*ttr*); orange = Cy3 (*tviB*); purple = Cy5 (*staG*); green=ROX (passive reference).

When comparing the amplification of *tviB* using Cy3 to the previous assay utilising Texas Red, the signal strength in the multiplex assay was unacceptable, with true amplification being almost indistinguishable. When revisiting previous assays (Figure 4.1), *sseJ* using Cy3 showed poor amplification for NTS also, demonstrating Cy3 was not a favourable dye. As such, TAMRA was used for *tviB* (Table 4.1, Stage 3; Figure 4.3) in another attempt to create a viable quadruplex. Initially, Quasar 705 was also considered, but calibration of the dye within the time available proved to be difficult.

The amplification for TAMRA did not appear as effective as other widely used TAQMAN style fluorophores. Due to time restrictions, it was decided that *tviB* would use the TET fluorophore and the assay should be performed as two assays – the screening duplex containing *ttr* with FAM and *sseJ* with Yakima Yellow, and the confirmation triplex, utilising *ttr* with FAM, *staG* with Cy5 and *tviB* with TET (Figure 4.4).

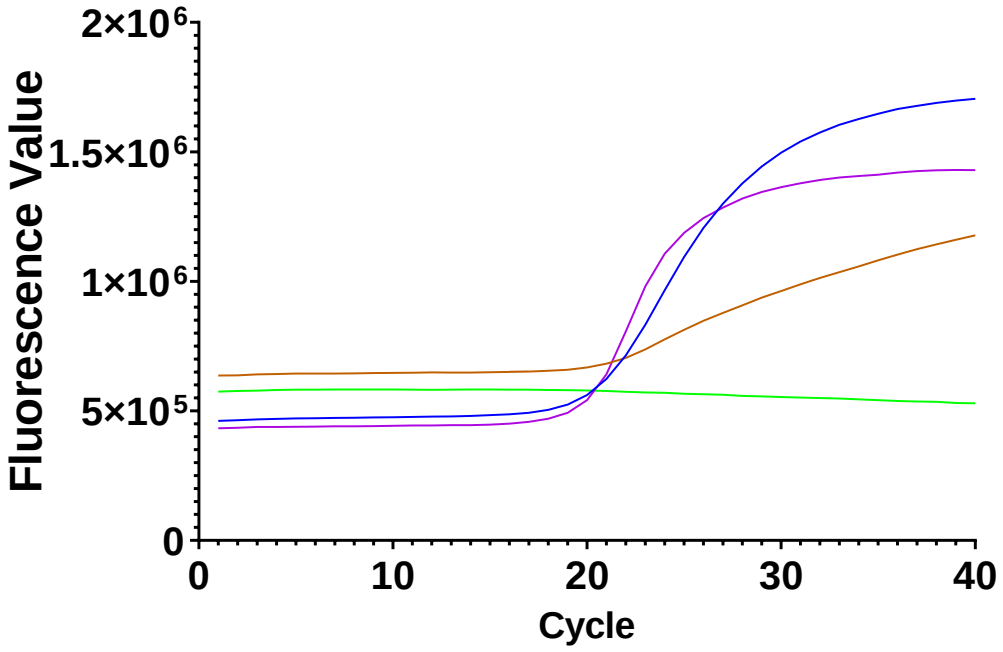


Figure 4.3: Graph shows the multicomponent fluorescence values of an *S.* Typhi control strain when *sseJ* is removed, and the *tviB* probe uses TAMRA fluorophore. Blue = FAM (*ttr*); brown=TAMRA (*tviB*); purple = Cy5 (*staG*); green=ROX (passive reference).

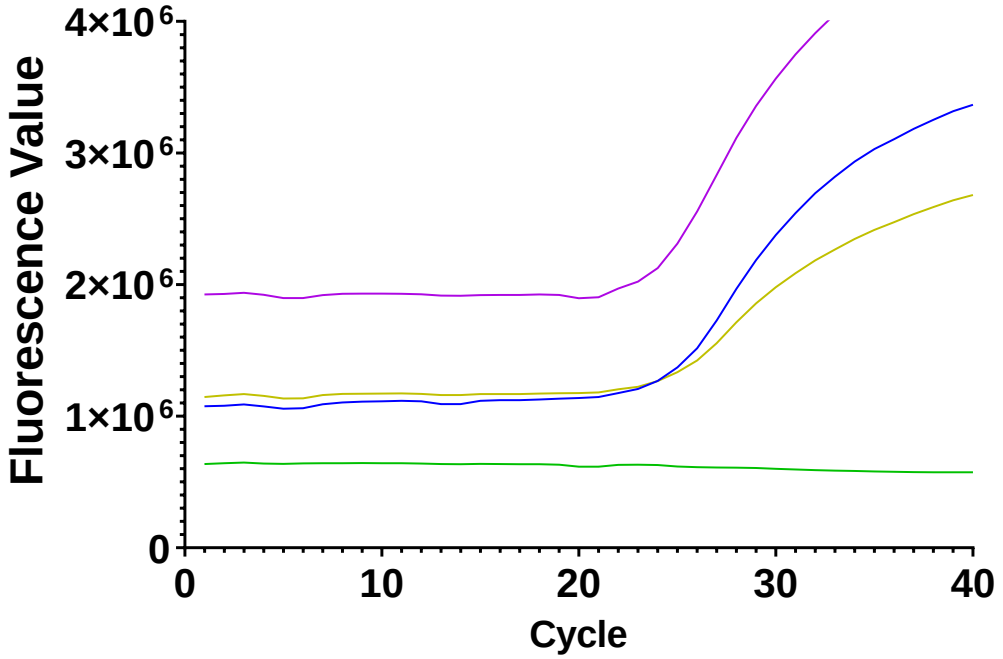


Figure 4.4: Graph shows the multicomponent fluorescence values of an *S.* Typhi control strain when *sseJ* is removed, and the *tviB* probe uses TET fluorophore. Blue = FAM (*ttr*); yellow = TET (*tviB*); purple = Cy5 (*staG*); green=ROX (passive reference).

The TET tagged *tviB* showed strong and consistent amplification with all the *S.* Typhi control strains, including Ty21a. As such the design of the two-stage PCR with two multiplex assays was adopted. To finalise the assay, the concentrations of each primer and probe was revised due to *ttr* amplifying more significantly than other targets. This was done by performing the triplex assay with the *ttr* at four different concentrations: the original concentration from Nair *et al* (2019) – 400 nM for each primer, and 200 nM for the probe; 200 nM for the primers, 100 nM for the probe; 100 nM primers, 50 nM for the probe and 50 nM for primers, 25 nM for the probe (Figure 4.5).

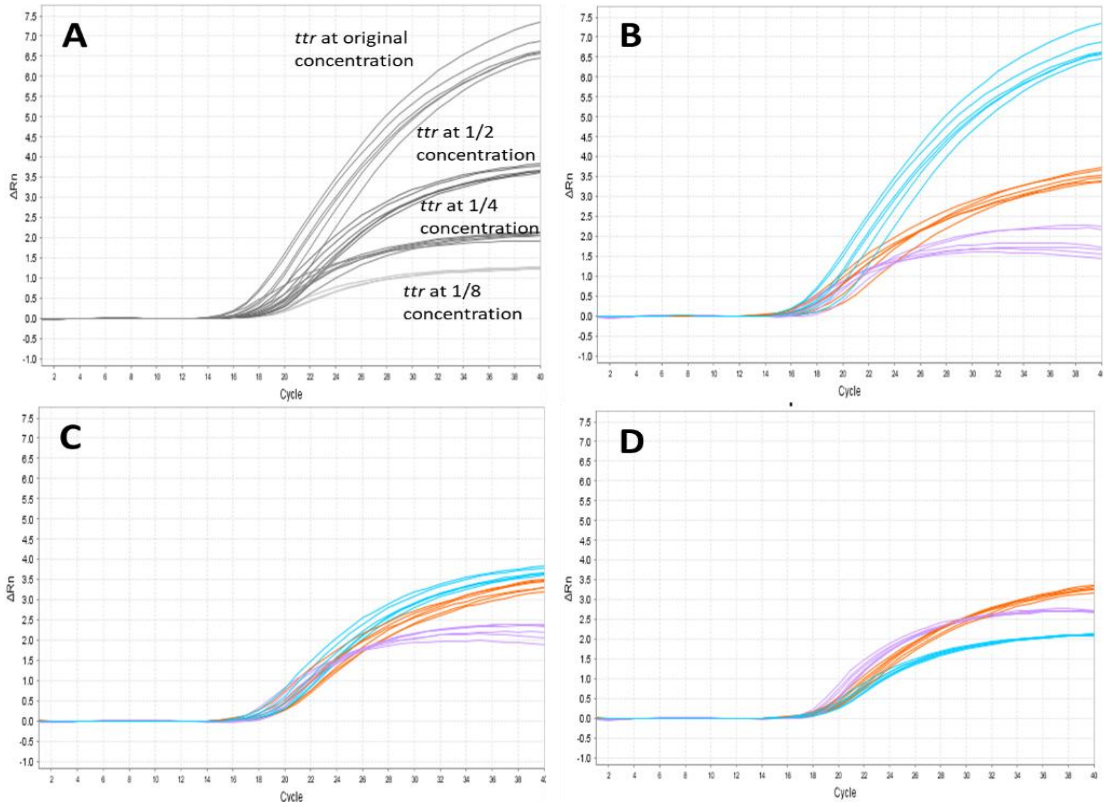


Figure 4.5: Amplification plots for the triplex real time assays using difference concentrations of *ttr* primers and probes. Blue = FAM (*ttr*); yellow = TET (*tviB*); purple = Cy5 (*staG*). A) demonstrates the amplification curve of *ttr* only at all four concentrations tested. B) demonstrates the amplification curve of *ttr* 400 nM for each primer, and 200 nM for the probe multiplexed with *tviB* and *staG*. C) demonstrates the amplification curves for *ttr* at 200 nM for the primers, 100 nM for the probe in triplex with *tviB* and *staG*. D) demonstrates the amplification curves for *ttr* at 100 nM primers, 50 nM for the probe in triplex with *tviB* and *staG*. ΔRn=change of fluorescent signal at each cycle. The negative controls are not displayed in these figures as they would be the same colours as the targeted positive strain.

A local, anonymised, clinical strain of *S.* Typhi (Table 3.1, strain 21), which had been isolated from blood by the MLW CORE diagnostics laboratory, was used as a control strain to re-validate the assay. This was due to potential differences in the background matrix that would be seen in Malawi, in addition to work being performed on a different model of PCR machine, the Applied Biosystems QuantStudio 7 Flex.

To ensure the validity of the assay as an identification tool, the efficiency (Eff%), coefficient of determination (R^2), LOD and limit of quantification (LOQ) needed to be calculated. This was done by creating 12 replicates of a 0.5 MacFarland concentration of *S.* Typhi control in 5 mL RLS. Each of these suspensions were enumerated using the Miles, Misra and Irwin (1938) spot titre method on mCASE and DNA extracted through the boilate method. After extraction, a serial dilution of each sample was produced through eight, 10-fold dilutions and the real time PCR assay was run in its triplex format. The triplex was assessed only due to *sseJ* not being present in *S.* Typhi (Figure 4.6).

Using the Malawian strain of *S.* Typhi, the primer Eff%, R^2, LOD and LOQ were calculated. The Eff% and R^2 values fell within 100% and 110%, and 0.99 to 1, respectively: *ttr* 106.6%, 0.993; *tviB* 101.2%, 0.997; *staG* 108.7%, 0.995. The LOD was determined using the probit model analysis method and determined that the LOD_{50} was 4.84 x 10^1 CFU mL^{-1}, 6.85E x 10^2 CFU mL^{-1} and 1.18 x 10^2 CFU mL^{-1} for primer pairs for *ttr*, *tviB* and *staG,* respectively. The LOD_{95} was 3.60 x 10^2 CFU mL^{-1}, 3.61 x 10^3 CFU mL^{-1} and 8.97 x 10^2 CFU mL^{-1}, respectively. The LOQ for all assays was 1.74 x 10^3 CFU mL^{-1} (Figure 4.7).

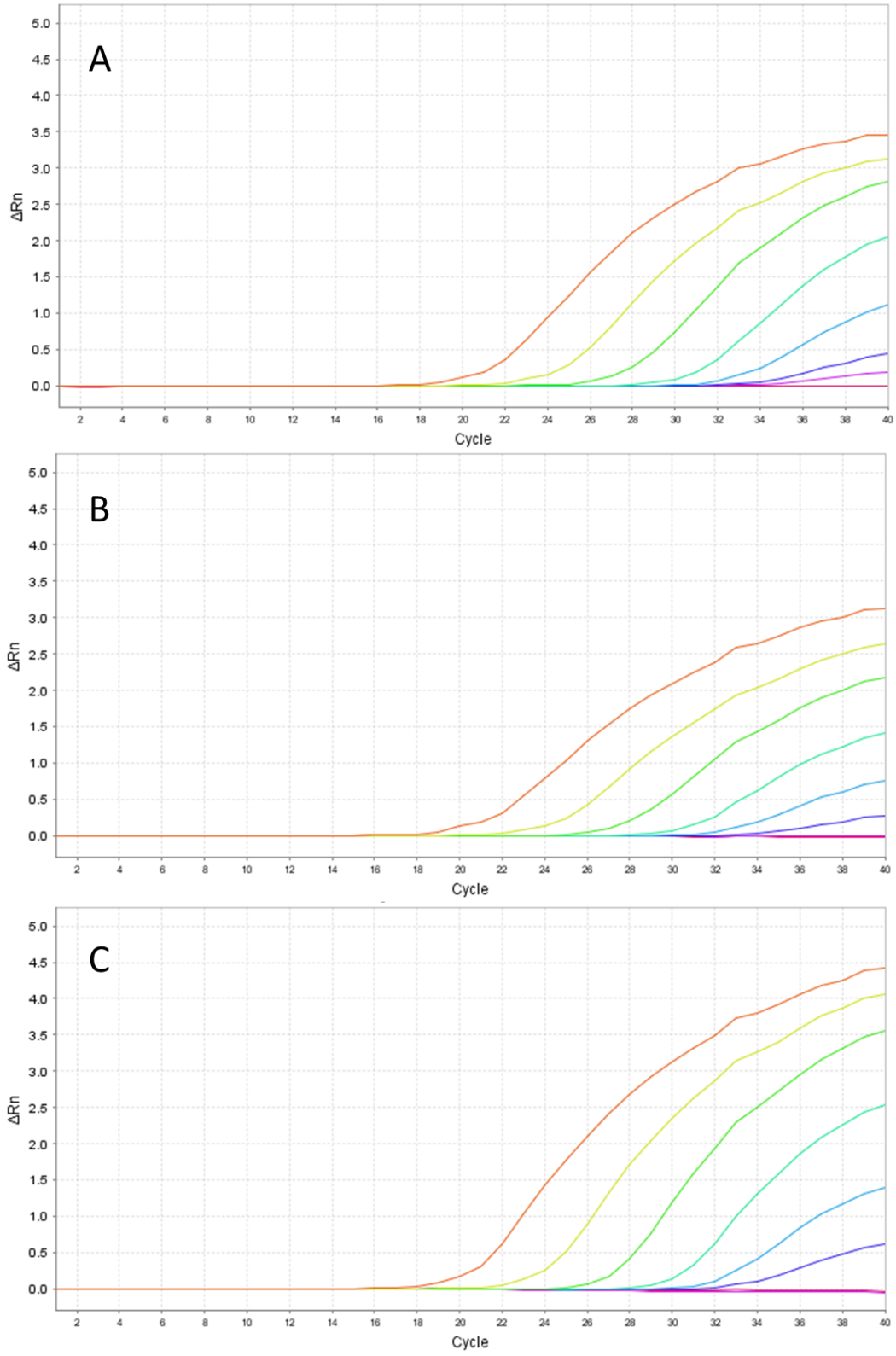
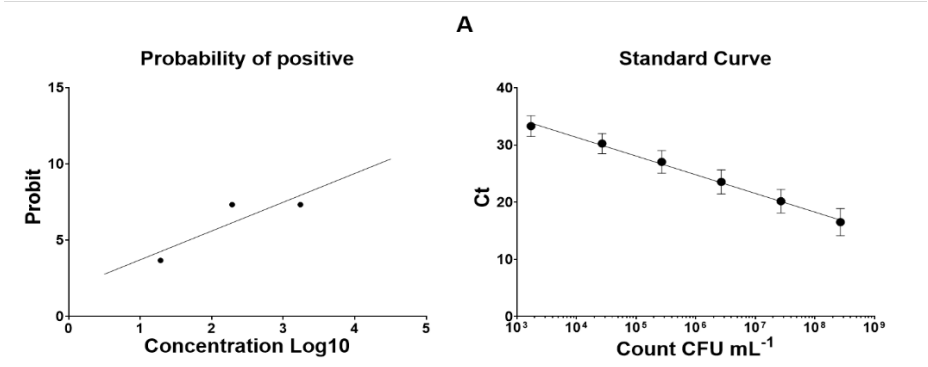
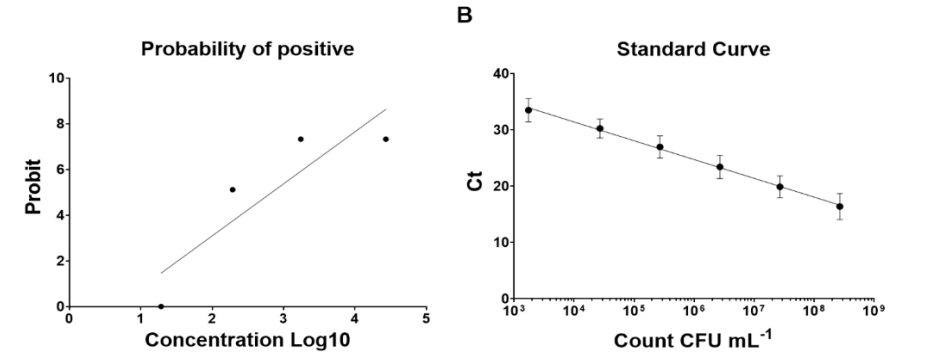


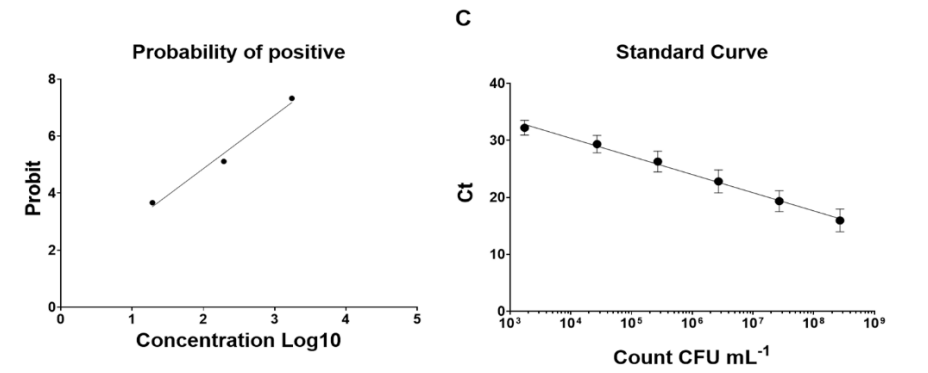
Figure 4.6: Serial dilutions of an *S.* Typhi control strain for the calculation of the LOD and LOQ. The assay was performed as a triplex, but A) shows the amplification for *ttr* only, B) shows the amplification for *tviB* only and C) shows the amplification of *staG* only. ΔRn=change of fluorescent signal at each cycle. A negative control is not displayed in these figures due to its visibility being unclear against the lowest dilution.



Primer	Eff%	R2	LOD 50%	LOD 95%	LOQ
ttr	106.6189	0.9934	4.84 x 10^1 CFU mL^{-1}	3.60 x 10^2 CFU mL^{-1}	1.74 x 10^3 CFU mL^{-1}



Primer	Eff%	R2	LOD 50%	LOD 95%	LOQ
tviB	101.1826	0.9969	6.85 x 10^2 CFU mL^{-1}	3.61 x 10^3 CFU mL^{-1}	1.74 x 10^3 CFU mL^{-1}



Primer	Eff%	R2	LOD 50%	LOD 95%	LOQ
staG	108.6584	0.9952	1.18 x 10^2 CFU mL^{-1}	8.97 x 10^2 CFU mL^{-1}	1.74 x 10^3 CFU mL^{-1}

Figure 4.7: The Ct values and CFU mL^{-1} of each serial dilution were taken as an average and mapped out as a standard curve for each primer pair, shown on the right, with the probability of finding a positive result based on cell concentration within a sample was mapped on the graphs on the left. A) shows the graphs and results for *ttr*, B) for *tviB* and C) for *staG*. With this data, the efficiency (Eff%), coefficient of determination (R^2), LOD and limit of quantification (LOQ) could be calculated and attached to each graph.

4.3.2 Identification and confirmation of suspect *S.* Typhi colonies

During the assay validation period and pilot study for ES, all suspect *Salmonella* spp. colonies from the culture pathway were screened by the two real time PCR assays, screening duplex and confirmation triplex (Table 2.5) . A total of 1,053 presumptive *Salmonella* spp. isolates from 424 unique samples were screened by the real time PCR assays, 33 of which demonstrated positive amplification for either *staG* or *tviB*; or both. Of those, 16 samples were further investigated by biochemistry, serology and ASTs.

Of these six of the 16 isolates that had *ttr*, *tviB* and *staG* genes detected, but not *sseJ*, were confirmed as *S.* Typhi by both API 20E and antisera agglutination. For all six, the API 20E returned one of two profiles, 4005540 and 4405540, both of which indicate a 99.9% match to the profile for *S.* Typhi. All six isolates were associated with an agglutination reaction against all three of the O9, Vi and Hd antigen target sera. In addition, their antimicrobial susceptibility testing (AST) profiles were determined, and resistance identified to ampicillin, chloramphenicol, and cotrimoxazole. Nine of the remaining isolates tested were confirmed as NTS strains but could not be identified further with the anti-sera and biochemical tests available. Upon repeating the PCR assay, these nine strains had amplification for both *staG* and *sseJ* and were negative for *tviB*; the latter two results implying the PCR assay and biochemical results accurately matched. The remaining isolate was identified as a *Plesiomonas shigelloides* by the API20E™ (Table 4.2) despite a positive result for some targets. Further purification work was carried out on ERST_100919_02, but salmonellae genes were not detected on subsequent extracts. Furthermore, the extraction for ERST_290920_1B was from the archive bead and not a plated isolate, subsequent attempts to purify this sample have only NTS results by PCR, implying a mixed archived sample.

Additionally, a further 331 unique isolates of NTS were identified with the real time PCR assay by testing positive for *ttr* and *sseJ*, but negative for *staG* and *tviB*. Of these, 55 were from the 1L grab samples, 31 from Moore swabs, 5 from biofilms and a further 30 from other sources (algae, sediment and other water surface plants and debris).

Table 4.2: Summary of each sample that was evaluated by biochemistry, serology and AST after a PCR result to show the importance of a multi-target assay. *nd*: not detected

Sample ID	Collection Date	Sample Type	PCR Result			API20E Result	Serology Result			AST Result		
			ttr Ct	*tviB* Ct	*staG* Ct		O9	Vi	Hd	Ampicillin	Chloramphenicol	Trimethoprim / Sulfamethoxazole
ERST_230919_04	23/09/19	Water	16.96	16.67	16.14	4005540 *S.* Typhi	+	+	+	Resistant	Resistant	Resistant
ERST_041119_16	04/11/19	Swab	20.16	19.73	19.01	4005540 *S.* Typhi	+	+	+	Resistant	Resistant	Resistant
ERST_041119_19	04/11/19	Biofilm	22.84	22.55	21.79	4004540 *S.* Typhi	+	+	+	Resistant	Resistant	Resistant
ERST_041119_23	04/11/19	Biofilm	17.59	*nd*	21.06	4005540 *S.* Typhi	+	+	+	Resistant	Resistant	Resistant
ERST_041119_26	04/11/19	Swab	21.84	22.62	20.92	4005540 *S.* Typhi	+	+	+	Resistant	Resistant	Resistant
ERST_041119_27	04/11/19	Swab	22.26	23.04	21.35	4004540 *S.* Typhi	+	+	+	Resistant	Resistant	Resistant
ERST_100919_02	10/09/19	Water	16.24	*nd*	18.32	*P. shigelloides*	-	-	-	Intermediate	Sensitive	Sensitive
ERST_111119_10	11/11/19	Water	14.73	*nd*	*nd*	*Salmonella spp.*	-	-	-	Sensitive	Sensitive	Sensitive
ERST_041119_15	04/11/19	Swab	18.23	*nd*	22.09	*Salmonella spp.*	-	-	-	Sensitive	Sensitive	Sensitive
ERST_181119_06	18/11/19	Water	14.34	*nd*	17.18	*Salmonella spp.*	-	-	-	Sensitive	Sensitive	Sensitive
ERST_181119_07	18/11/19	Water	21.03	*nd*	19.76	*Salmonella spp.*	-	-	-	Sensitive	Sensitive	Sensitive
ERST_191020_20	19/10/20	Sediment	21.33	*nd*	20.22	*Salmonella spp.*	-	+	-	Sensitive	Sensitive	Sensitive
ERST_021219_12	02/12/19	Swab	21.73	*nd*	*nd*	*Salmonella spp.*	-	-	-	Sensitive	Sensitive	Sensitive
ERST_121020_06	12/10/20	Water	21.94	*nd*	20.76	*Salmonella spp.*	-	-	-	Sensitive	Sensitive	Sensitive
ERST_251119_06	25/11/19	Water	24.70	*nd*	23.79	*Salmonella spp.*	-	-	-	Sensitive	Sensitive	Sensitive
ERST_290920_1B	29/09/20	Water	17.67	27.62	17.52	*Salmonella spp.*	+/-	+	-	Sensitive	Sensitive	Sensitive

4.3.3 High Resolution Melt PCR Assay

The primer pairs used for the real time PCR assay were also investigated using a HRM assay format. After initial screening with *S.* Typhi and *S.* Typhimurium strains, *ttr* and *staG*, and *tviB* and *sseJ* appeared to have melt temperatures too close to be distinguished by HRM, therefore, only a duplex was functional (Figure 4.8). As such, new primers were generated with the Basic Local Alignment Search Tool (BLAST), Molecular Evolutionary Genetics Analysis (MEGA) sequence aligner and Primer3 web for each gene target in attempt to adjust the melt temperatures.

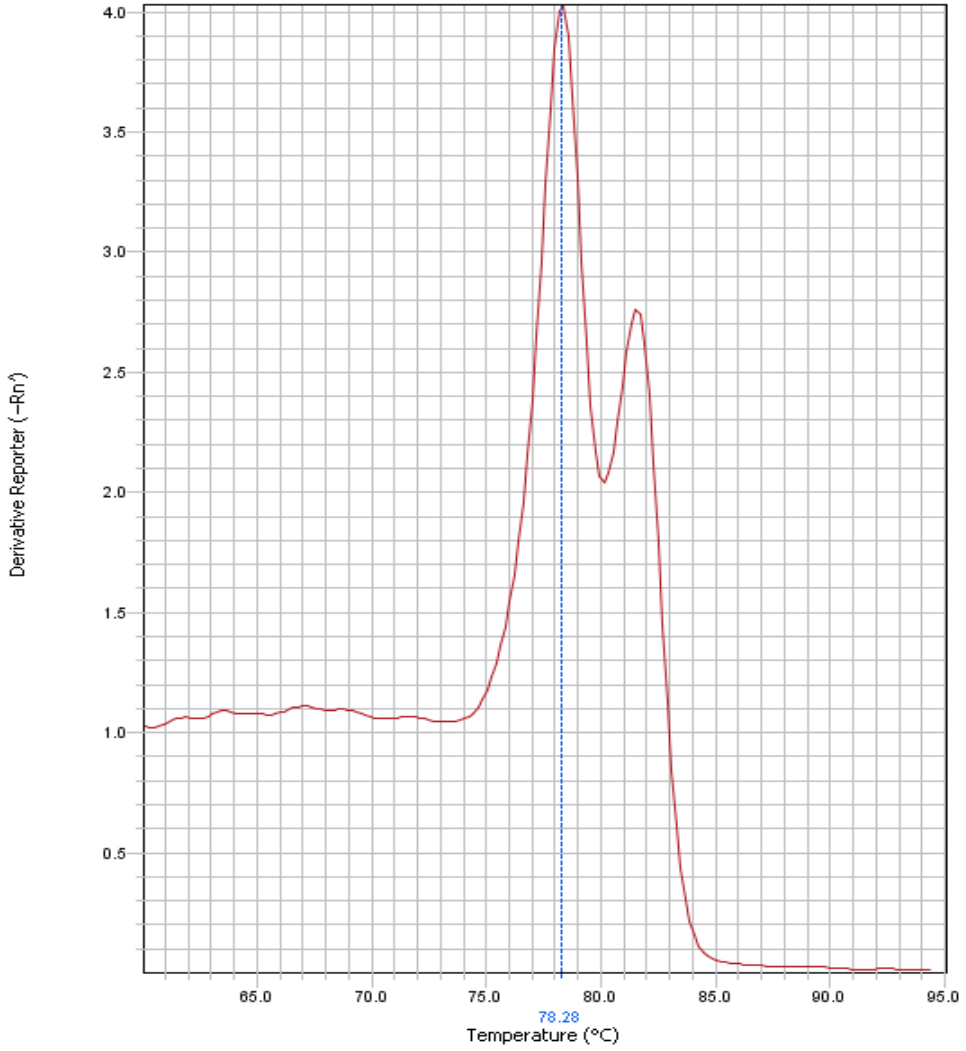


Figure 4.8: Melt curve of an *S.* Typhi control, utilising all four primer pairs and EVAGreen intercalating dye. The melt temperature for *ttr* and *staG* is shown at 78.28 °C, whilst *tviB* is at ~81.5 °C. As an *S.* Typhi was used, *sseJ* is not demonstrated here, but has the melting temperature of ~81.5 °C like *tviB*, making the result indistinguishable from an *S.* Typhi and NTS isolate.

To produce these primer pairs, multiple *S.* Typhi genomes were downloaded from GenBank using BLAST nucleotide suite (https://blast.ncbi.nlm.nih.gov/) to identify multiple genomes that did not have 100% match sequences (Accession numbers). These FASTA files were compared for conserved regions using the MEGA align sequences tool, Multiple Sequence Comparison by Log- Expectation. Once these conserved regions were identified, the original sequences for the genes of interested were identified using the primer pairs found in Table 2.6.

No viable alternatives for *tviB* were identified, as such, the focus was made to move the melt temperature of *sseJ*. A melt curve PCR was performed with all 17 alternate primers listed in Table 4.3, as well as the original primer sequences (as listed in Table 2.6). The results of each primer pair is displayed in Table 4.4.

Table 4.3: Additional sequences generated for use with the HRM PCR assay.

Name	Forward Primer	Reverse Primer
ttr 1	ACCAGGAGATTACAACAT	TTAAATTAGCCATGTTGTA
ttr 2	CGCTGAACGGACTCACCA	TAATCTCCTGGTGAGTCC
ttr 3	AGAGCTGGGGCTTTACGG	CCAGGGCGCCGTAAAGCC
staG 1	CAAGCGTTAGCCTTTTCTGG	CCAACAATCAATCCAGGTGA
staG 2	CAAGCGTTAGCCTTTTCTGG	CACCGCTCAATTTTCAATCA
staG 3	ATAACGTACTGGATGCCGGA	CCAGTGAACATGATGGCCCA
staG 4	TGGGTCAGTTGAAGGTAGGT	ACAATTTTGGGCAGACCATC
staG 5	GGAGTCGCCGTTTTTAGACA	AGCCTGCTCCAGAACAAATG
staG 6	CACCTGGATTGATTGTTGGA	CCACCAATAACACCGGAGAC
sseJ 1	GATGAAAGCATCGCTCACAA	CCGCCTCCATTATCACCTTA
sseJ 2	ACATCGGCAAGCTATTCCTG	CCTGGTGAGAAGGGGTGTAA
sseJ 3	AATTTTGCTGAAGGGGGAAG	CCTGGTGAGAAGGGGTGTAA
sseJ 4	ATTTTGCTGAAGGGGGAAGT	GGGGTGTAAGATGCGACTTG
sseJ 5	TCAATACTTTGGCGGAAGGT	CAGGAATAGCTTGCCGATGT
sseJ 6	TTGCTGAAGGGGGAAGTACA	CCTGGTGAGAAGGGGTGTAA
sseJ 7	GATGAAAGCATCGCTCACAA	ATATTACTGGCCGCCTCCAT
sseJ 8	ATTTTGCTGAAGGGGGAAGT	TGCGACTTGTCTGTCCGTAT

Table 4.4: Amplification and melt curve results for all primer pairs screened for use as potential HRM targets.

Primer Name	Amplification (Ct)	Melt Temperature (°C)
Original *ttr*	17.44	83
***ttr* 1**	No Amplification	N/A
***ttr* 2**	No Amplification	N/A
***ttr* 3**	3.41	76.5
Original *staG*	16.09	82.5
***staG* 1**	No Amplification	N/A
***staG* 2**	16.76	79.5
***staG* 3**	16.26	84
***staG* 4**	16.29	84
***staG* 5**	16.82	83
***staG* 6**	15.96	83
Original *sseJ*	18.07	79
***sseJ* 1**	20.34	79
***sseJ* 2**	21.64	80
***sseJ* 3**	No Amplification	N/A
***sseJ* 4**	24.26	80
***sseJ* 5**	21.42	79
***sseJ* 6**	No Amplification	N/A
***sseJ* 7**	26.48	79
***sseJ* 8**	22.55	79
Original *tviB*	16.55	79

The resolution of a HRM assay can be as little as 1 °C (Williams *et al*., 2019), therefore some of the targets showed promise, such as *staG* 3 and 4 and *sseJ* 2 and 4, however the amplification curves showed reduced fluorescence compared to the original primer pairs, suggesting reduced efficiency. For primer *ttr* 3, the cycle time (Ct) for the amplification was much earlier than would be acceptable by most real time PCR assays and would require further testing to determine if it is genuine and specific amplification of the target gene. Due to most primer pairs not being a significant and reliable improvement over the original ones, and with limited time available, the HRM assay was dropped, with the real time multiplex assays being utilised for isolate identification.

4.3.4 DNA Extraction Direct from Complex Matrices

For extraction from samples directly, the use of the QIAvac system was first trialled with the DNeasy Blood and Tissue 96 well plates kit. To ensure this extraction method's efficacy, a control strain was grown on mCASE at 37 °C for 18 ± 1 h. A 10 µL loop was used to harvest five colonies of growth, which was homogenised in 1 mL RLS, this was repeated eight times. Each of these eight control samples were serially diluted to 10^{-8} and extracted alongside a non-template control (NTC) and a sample of the water used for the serial dilutions. Using the QIAvac as a replacement for the centrifuge, manufacturer's instructions were followed for the DNeasy blood and tissue kit, starting with 200 µL of homogenised sample being transferred into the appropriate well on the spin column plate. After extraction, these samples were tested using the established triplex (*ttr*, *tviB* and *staG*) real time PCR method.

Whilst the amplification of the extracts was of good quality, the NTC demonstrated contamination within the PCR. After repeating the assay to determine if it was the extraction or the loading of the 96-well PCR plate, it was determined the extraction had been contaminated, due to the NTC amplifying again, whilst the PCR negatives and extraction water were both negative. Upon observation of the extraction process, bubbles were identified at the tips of each filter funnel, which were in close proximity to one another (Figure 4.9).

Figure 4.9: Image of the Qiagen 96 well filter extraction plate.

Subsequently, another assay was performed to determine if this was caused by contamination during extraction. Four controls in RLS were created, with two of these having 1 µL of an anti-foaming agent (QIAGEN Ref ID: 19088) added. An NTC with the anti-foaming agent and one without was also made using 3 mL of RLS each, which would be divided between 13 wells for both, surrounding all four *S.* Typhi control extractions - Figure 4.10 demonstrates the layout used for the extraction prior to screening with the Real Time PCR.

Figure 4.10: Layout of the samples used to assess contamination during extraction with the QiaVAC and DNeasy 96 well kits, for both extraction and the real time PCR assay. NTC AF: Non-template control with anti-foaming agent, ST AF: *S.* Typhi control with anti-foaming agent. NTC F and ST F: Non-template control and *S.* Typhi control without anti-foaming agent, respectively.

These samples were extracted using manufacturer's instructions and 200 µL of sample per appropriate well. The real time PCR assay used was the triplex assay, alongside PCR negative samples below the run. The PCR negative samples were master mix and primers without any sample added.

The PCR from the QIAvac extracted samples showed significant contamination in each of the NTC wells, both for the samples extracted with and without the anti-foaming agent. Figure 4.11 shows the amplification of the antifoaming agent (A and C), where in graph A, the green lines represent the control samples, whilst the red shows the NTC; similarly, in Graph B, which shows the amplification of the samples without anti-foaming agent, the grey displays the amplification of the controls, whilst the orange is the NTC. Graphs C and D display the gene targets for *ttr* in blue and *staG* in purple. Whilst the controls have a very low Ct of 12; the NTC amplifies later at a Ct of 34 to 38. These contamination events, even with an anti-foaming agent included, demonstrate sufficient crossover that could have resulted in false positives had the method been utilised for direct environmental extractions that it was not explored further.

As an alternative to the QIAvac system, a novel extraction method, called Magna Extract (Byrne *et al*., 2022), was also investigated. The development of this methods is described in full in Byrne *et al*. (2022) and what is presented here relates to identification of *S.* Typhi from complex matrices. This was seen as a potential alternative to commercial extraction kits for environmental samples due to lower costs and comparable results when utilised for environmental AMR genes.

The first stage of this extraction method includes a liquid culture step, and as such, several selective and non-selective broth media were trialled. This was done by creating a 0.5 MacFarland standard suspension in RLS, which was serially diluted to 10^{-8} and enumerated onto mCASE using the spot titre method (Miles and Misra, 1938). Subsequently, 1 mL of the 10^{-7} and 10^{-8} solutions were transferred into 9 mL of: bile^{-}; UPE; BPW; Luria Broth and Tryptone Soya Broth. After incubation at 37 ± 1 °C for 18 ± 2 h, these broths were extracted as detailed in section 2.3.1. These extracts were assessed through the use of the triplex real time PCR assay (Figure 4.12).

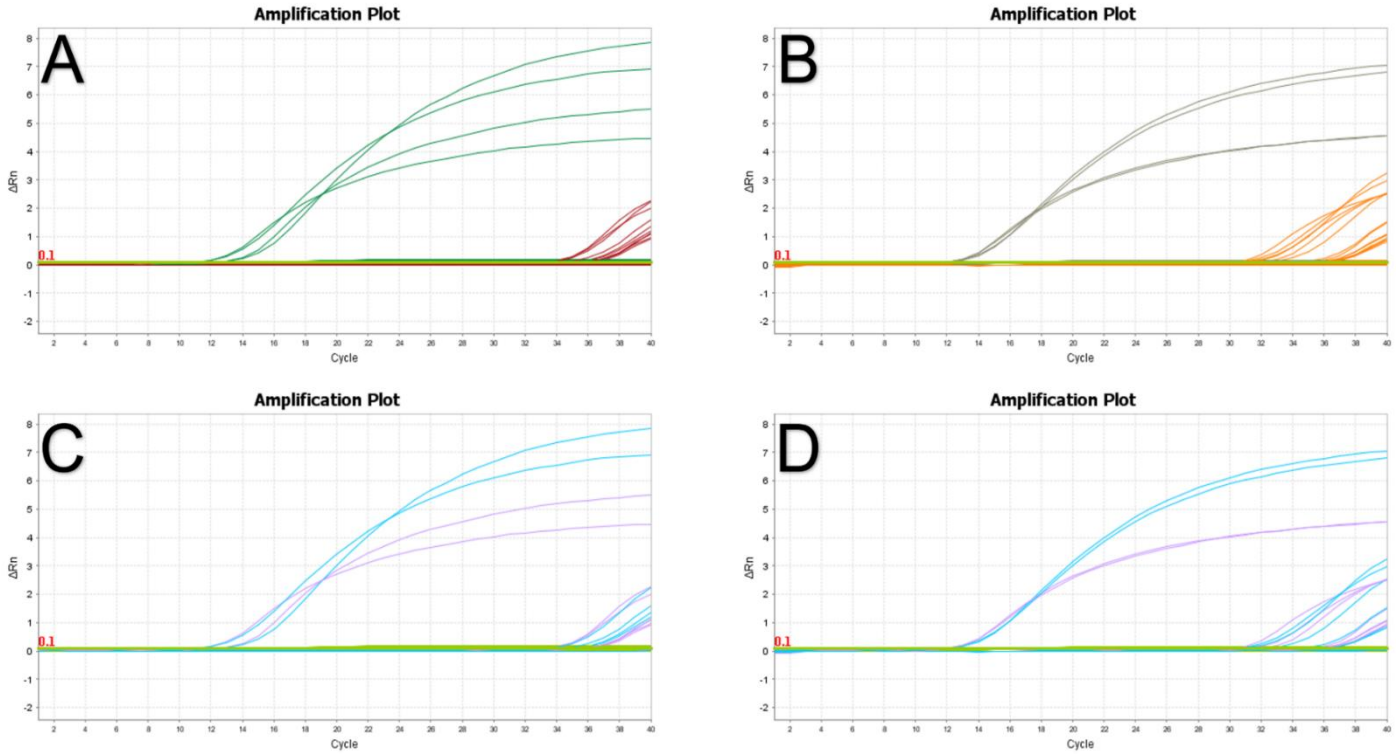


Figure 4.11: Amplification results of the PCR assay from the QIAvac contamination assessment: A) shows the amplification of the *S.* Typhi targets containing the anti-foaming agent, with red indicting the NTCs surrounding it during extraction. B) demonstrates *S.* Typhi in grey and the NTCs in orange for the extractions not containing anti-foaming agents. C) demonstrates the same extraction as A, but with the light blue highlighting *ttr* and the purple highlighting *staG* gene targets, demonstrating clear contamination in the NTC's with *S.* Typhi. D) shows the same as C, but for the samples not using the anti-foaming agent from graph B. ΔRn=change of fluorescent signal at each cycle.

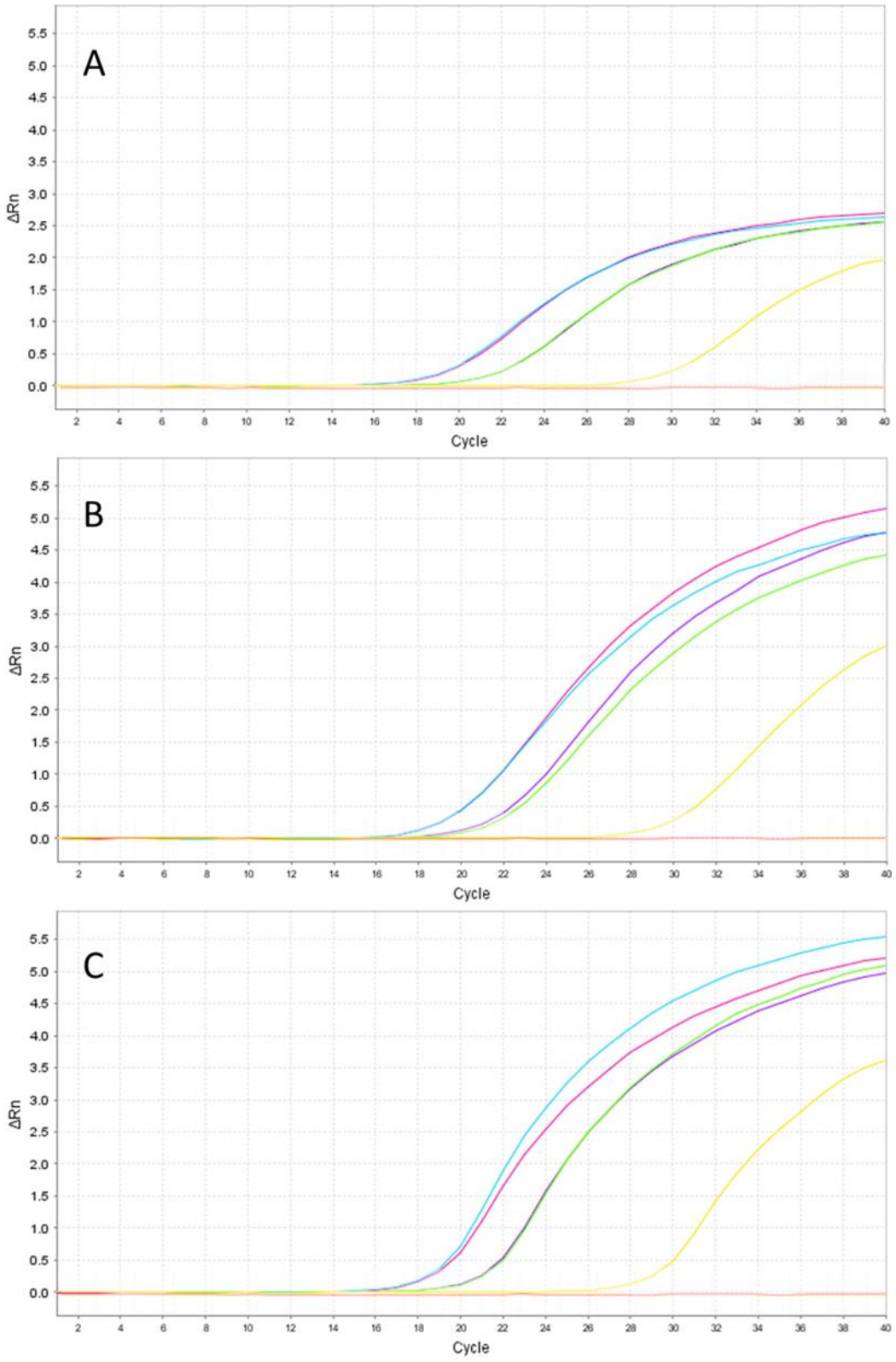


Figure 4.12: This shows the amplifications of an *S.* Typhi control in various broth culture after extraction with Magna Extract. The amplification curve in purple was bile$^-$ broth, green was BPW, pink was UPE, blue was LB and yellow was TSB. A) Shows the amplification curves for *ttr*, B) the amplification curves for *tviB* and C) the amplification curves for *staG*. The orange curve was the non-template control

The PCR results from the various broth cultures demonstrated that the Magna extract protocol purified the samples sufficiently that the inhibitors present in broths such as bile$^-$ did not affect the assay. When compared to a boilate protocol, the cultures from bile$^-$ were not detected. When Magna was compared to a boilate of pure culture from a non-inhibitory broth, such as BPW, the Ct values were comparable. Additionally, the concentration of cells in the initial inoculum of RLS was too low to be effectively extracted by either boilate or Magna.

To confirm viability of the extraction method from bile$^-$, another run was performed, with 20 biological replicates of *S.* Typhi control, cultured in bile$^-$, from a 10^{-7} and 10^{-8} dilution of a 0.5 MacFarland standard suspension in RLS. Of the 20 replicates, 16 amplified the target genes, although some Cts were very late, additionally, four of the replicates did not amplify. When enumeration of the inocula were complete, the four that did not amplify showed no growth at the dilution used to culture the broths, whilst those that amplified late, showed a lower CFU mL^{-1} than expected, accounting for the lack of detection or late Ct's due to low genome copies.

4.4 Discussion

The purpose of the molecular assay within this project was initially as a confirmation tool to screen isolates from the culture processes consequent upon its high-throughput and cost-effective nature when compared to culture-based methods. It was adapted from Nair *et al* (2019) to a multiplex format. As such, the probes had to be adjusted so that they were compatible with the PCR machines in use. Additionally, the concentration of each probe and primer had to be adjusted for the targets to be compatible, as the *ttr* amplified more readily than *staG* or *tviB*, which had an adverse effect on the reaction in multiplex.

Whilst the original aim was to create an effective assay using a quadruplex containing all four targets, I was not able to achieve this due to time constraints, therefore an appropriate triplex was developed as a compromise, with *sseJ* being removed from the confirmation tool and used in duplex with *ttr* to create a screening assay. As such, all isolates were screened with this duplex first to determine whether an isolate was a *Salmonella* spp. with the subsequent confirmation triplex being used on only those that were *ttr* positive, irrelevant of their *sseJ* status. This became a more optimal solution than the originally intended assay due to a high number of environmental NTS being isolated.

It was deemed essential that a multi-target approach be taken to comprehensively determine the identification of an isolate as *S.* Typhi, as it has been hard to establish a single primer pair with requisite sensitivity and specificity (Nair *et al* 2019). The primers targets selected included *ttr* gene primers as a pan- *Salmonella* assay that confirms genus of the isolate. The *staG* primers have been used exclusively in some studies for direct detection of *S.* Typhi from environmental samples, the *sta* operon, in which *staG* is located, is known to be detected in other salmonellae such as *S*. Sendai, *S*. Gallinarum (Pu3 and Pu4), *S*. Dublin, *S*. Enteritidis and *S*. Derby (Townsend *et al*., 2001). Whilst *tviB* is more specific, only being found in *S.* Typhi and *S*. Paratyphi C; there have been reports of *S.* Typhi pathogenic strains without the *SPI-7* pathogenicity island, within which the gene for the Vi antigen is encoded (Baker *et al*., 2005). Lastly, as I performed PCR on single picks, *sseJ*, which is not found in *S.* Typhi was used to detect nontyphoidal serovariants. All isolates that were *staG* positive, but *tviB* negative were *sseJ* positive and phenotypically confirmed to be NTS. In contrast, all isolates that were *ttr*, *staG* and *tviB* positive and *sseJ* negative were phenotypically confirmed to be *S.* Typhi.

The real time PCR efficiency and reproducibility fell within the acceptable range, and the LOD and LOQ were in the range of a well performing real time PCR assay (Taylor *et al*., 2019). The adaptability of the assay allows it to be used as a screening tool from direct environmental samples, once an appropriate DNA extraction method is implemented. Whilst the use of the QiaVAC to allow high-throughput extraction of samples without the expense of an automated extraction robot like the QiaSymphony was a*ttr*active, the reagents used and design of the container for the 96 well spin column kits proved to be far too prone to cross-contamination due to the foaming nature of the regents and proximity of the extraction columns. Spin column kits, such as the PFP kit from Qiagen are effective but prohibitively expensive for large scale rollout, therefore an in-house extraction method, the MagNA Extract method has been developed and it shows potential with salmonellae, though requires further work. To ensure this method is robust for *S.* Typhi, several experiments were performed to ensure its efficacy and practicality as a screening tool through adjustments to the volumes and preparation methods prior to purifying the DNA through the Magna Extract method – these are detailed in Chapter 7.

Direct detection from environmental samples was explored by replacing *sseJ*, which would not provide valuable information in a direct environmental sample, with the Eurogentec SPC, which is detailed in the later Chapter 6: Environmental Surveillance.

The development of the HRM assay did not give a viable tool due to the issue with the melting temperatures overlapping on the primer targets that worked well, and replacement primers for those target genes with separate melting temperatures performed poorly or did not amplify at all. However, with further work on these targets, and the addition of other primers of interest such as STM4200 for *S.* Typhimurium and SEN1392 for *S*. Enteritidis (Heymans *et al*., 2018) to allow ES of potential iNTS which are also highly prevalent in areas endemic with Typhoid, it could become a useful tool for ES of invasive salmonellae.

The purpose of utilising real time PCR in this study was to provide a low-cost, high-throughput confirmation tool for isolates of *Salmonella* spp.. Whilst work remains for direct detection from environmental samples to screen prior to culture, the assay has proven to be effective and sensitive when working with pure isolates. The use of the DNeasy Blood and Tissue 96 well kit was, in hindsight, not as appropriate for extractions from a complex matrix heavily soiled in the way environmental water and sediment samples would be, with the Qiagen PowerFaecal pro or PowerSoil pro kits being designed for this purpose. Additionally, preliminary results with other extraction methods that give higher purification and better-quality DNA show promise for direct detection with this assay.

5 Optimised methods for detecting *Salmonella* Typhi in the environment using validated field sampling, culture and confirmatory molecular approaches.

5.1 Summary of my role in this manuscript

Following my relocation from London to Malawi, it was necessary for three strands of work to happen in parallel, the selection of the final culture pathway (Chapter 3), optimisation of the molecular confirmation method (Chapter 4) and optimisation of field sampling and sample concentration. The culture and molecular work needed to be described in granular detail for my thesis as individual chapters, but all of this work was brought together in a single peer reviewed publication, which is appended below, and is published online here (https://sfamjournals.onlinelibrary.wiley.com/doi/10.1111/jam.15237). Supplementary materials that were published in this paper are available in Appendix A.5, although some tables and figures have been used in earlier chapters where relevant.

The published manuscript is submitted as Chapter 5 of my thesis in line with guidance from the LSTM Postgraduate Research department as it describes in full detail:

1. My approach to field sampling including:
    a. Site selection;
    b. Health and safety at the site;
    c. Specific methods for both grab and trap sampling;
2. My approach to sample concentration;
3. Culture and molecular results of this pilot work;
4. Statistical methods I used to compare grab vs trap methods.

I led all aspects of the work described in this manuscript, with expert support from my supervisors and the disciplinary support from the individuals named in this manuscript. I trained and supervised the field and laboratory team, and collated and analysed all the results, then led the writing of this manuscript.

5.2 The Manuscript: Optimised methods for detecting *Salmonella* Typhi in the environment using validated field sampling, culture and confirmatory molecular approaches.

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ORIGINAL ARTICLE

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Optimized methods for detecting *Salmonella* Typhi in the environment using validated field sampling, culture and confirmatory molecular approaches

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Abstract

Aims: This study evaluated detection methods for *Salmonella* Typhi (*S.* Typhi) in the environment, to establish a novel pathway from field sampling to isolation of viable organisms and molecular confirmation from complex environmental samples, thus enabling environmental surveillance of typhoid.

Methods and Results: Multiple media were assessed using clinical isolates from the Public Health England's (PHE) Culture collection. The culture pathway selected consisted of a primary 2% bile broth and secondary Selenite F broth, followed by modified Chromogenic Agar for Salmonella Esterase (mCASE). A qPCR assay was adapted from a validated *S.* Typhi PCR panel for confirmation of isolates, with comparison to biochemical and serological tests showing good specificity. Sampling locations in Blantyre, Malawi were used to compare sampling methods. Viable *S.* Typhi were isolated from a mixture of trap and grab river water samples on six occasions.

Conclusions: Culture of viable *S.* Typhi from environmental samples was possible using effective capture and culture techniques.

Significance and impact of study: Whilst several studies have attempted to detect *S.* Typhi from the environment, this is the first successful attempt to isolate the organism from river water since the 1980s. Supplementing clinical data with environmental screening offers the potential for enhanced surveillance, which might inform interventions and assess vaccination programmes.

KEYWORDS

bile broth, biofilm, biofilms, Identification, Malawi, mCASE, Moore swabs, PCR (polymerase chain reaction), river water, salmonella, selenite broth, typhoid, water

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INTRODUCTION

Typhoid fever remains a public health problem of global concern, particularly in Low and Middle-Income Countries (LMICs) where water, sanitation and hygiene infrastructure are frequently inadequate (Feasey et al., 2015; Parry et al., 2002; Schwenk, 2020). Humans are the only known reservoir of *Salmonella enterica* serovar Typhi (*S.* Typhi). Whilst cases are ultimately transmitted from human to human, transmission may occur through direct or indirect exposure following excretion of the pathogen into the environment. This has been referred to as long-cycle transmission (Akullian et al., 2015; Baker et al., 2011; Gauld et al., 2018; González-Guzmán, 1989; Levine et al., 1982).

Whilst *S.* Typhi can often be readily detected in symptomatic patients by blood culture, environmental detection has proved more challenging. Gram Negative bacteria, including nontyphoidal *Salmonella* (NTS), coliform bacteria, *Escherichia coli* and other *Enterobacterales*, have been shown to suffer sublethal stress and injury when recovered from the environment which adds challenges to their isolation from samples (Rhodes & Kator, 1988); however, little is documented for *S.* Typhi due to its literature description as a human restrictive pathogen (Parry et al., 2002). It would, however, be advantageous to reliably detect *S.* Typhi from such samples, as environmental surveillance would considerably advance understanding of the epidemiology of typhoid fever and assist policy makers in establishing public health interventions (Sikorski & Levine, 2020). Previous attempts to identify how the environment is involved in this abiotic transmission have associated typhoid with water sources but they have not successfully cultured the organism (Baker et al., 2011). Without culture-based methods, the viability of *S.* Typhi and, therefore, its capability to cause human infection cannot be ascertained.

Whilst it has been possible to culture *S.* Typhi from environmental sources in the past, as has been described in high income settings in the mid-20th century (Moore, 1951; Moore et al., 1952), this has become restricted by microbiological laboratory capacity in endemic settings of the 21st Century (Sears et al., 1986) and a shift towards media production focusing on NTS due to its higher incidence in high-income countries (Kirchhelle et al., 2019; Majowicz et al., 2010; Oxford-Vaccine-Group, 2019; Stanaway et al., 2019). In recent years, molecular techniques have become the preferred method to detect environmental *S.* Typhi despite the culture-based identification approach remaining the gold standard (Mather et al., 2019). Molecular methods such as quantitative PCR (qPCR) have their own limitations and challenges, particularly in the endemic LMICs. There are concerns about sensitivity and specificity, and DNA from dead bacteria may persist and thus not be a true representation of viable bacteria that can cause infection (Zhou et al., 2018).

Despite the challenges of culture-based detection methods, there are clear advantages for their use. Where it has been successful (Sears et al., 1984), culture not only proves the presence of viable *S.* Typhi, but it also offers the opportunity to undertake further characterization of the organism, for example by whole genome sequencing (WGS). These techniques allow environmental isolates to be compared against the strains that cause human disease and thereby facilitate the investigation of transmission pathways and the associated epidemiology. In this study, we have optimized sample collection techniques, culture-based pathways and qPCR to establish methods that can reliably be used in endemic areas.

MATERIALS & METHODS

Methodologies were assessed and evaluated in the laboratory setting at the Public Health England (PHE) Food, Water and Environmental Microbiology Laboratory, London. They were then applied in the field in Blantyre, Malawi, where an outbreak of Typhoid fever, associated with use of river water for cooking and cleaning, began in 2011 (Gauld et al., 2020). Figure 1 and Table 1 summarize the workflow.

Strain information

Eighteen *S.* Typhi cultures from PHE's Gastrointestinal Bacteria Reference Unit (GBRU) were selected to represent strains recently identified from human cases of infection, isolated between 2009 and 2015 (Table 2). Isolates were from individuals who had recent travel history to Africa, the Indian subcontinent and South East Asia. Isolates included representatives of the H58 haplotype (genotype 4.3.1), which remains widespread in Malawi (Feasey et al., 2015) and included an isolate from a patient with recent travel history to Malawi.

The isolates were selected to challenge a range of media described in the scientific literature for the isolation of *Salmonella* species or *S.* Typhi (Tables 1 and 2). Isolates of different microbial species from PHE's culture collection were also utilized to determine media selectivity (Table 3).

Media Selection

Media were identified through a literature review and assessed on the availability, stability and safety status of

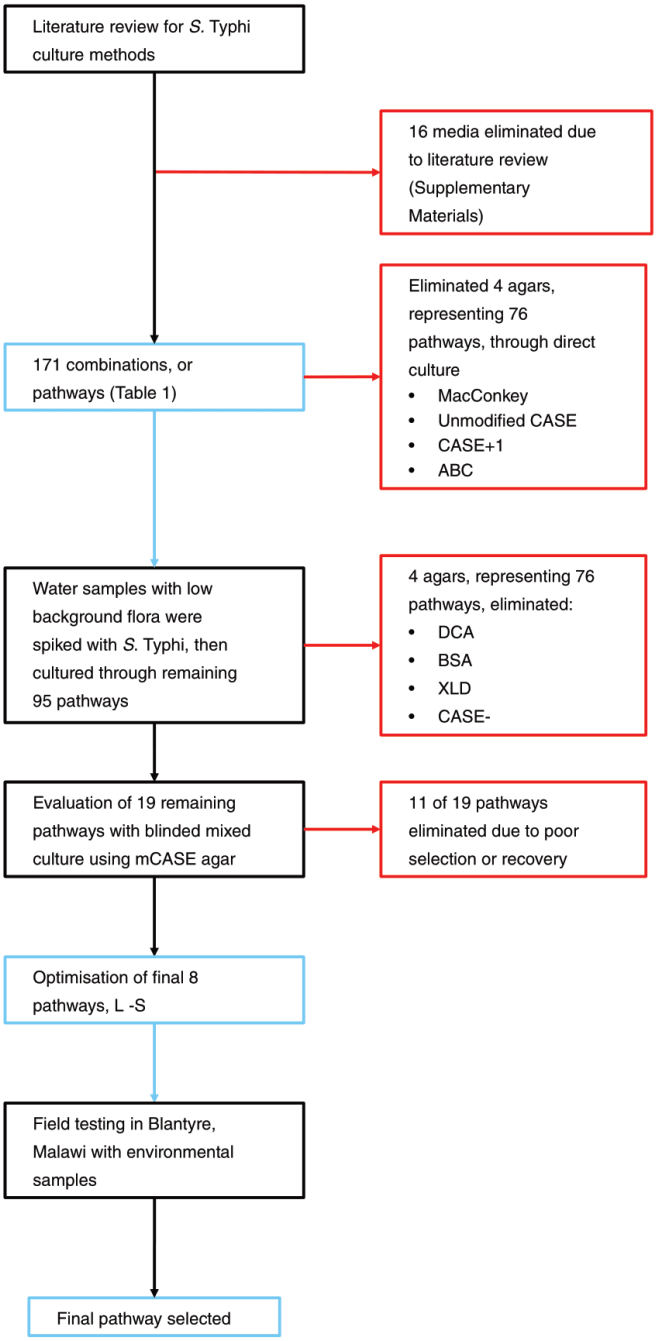

FIGURE 1 Workflow diagram showing the experiments and decisions taken to select the final pathway for *S.* Typhi culture. Black boxes, action taken; Red boxes, action outcome; Blue boxes, pathway information

their ingredients. A full list of the media eliminated without laboratory evaluation is provided in Table S1a,b.

Media performance was evaluated based on the growth of *S.* Typhi isolates (Table 2) on the following: xylose lysine deoxycholate agar (XLD; Oxoid); deoxycholate citrate agar (Hyne's media; Oxoid); bismuth sulphite agar (Wilson and Blair media; Oxoid); Harlequin ABC agar (Neogen); Chromogenic Agar for Salmonella

TABLE 1 The culture pathways evaluated in this study utilizing five broths and nine agars in 19 different broth combinations

Pathway	Primary broth	Secondary broth	Tertiary broth	Agar
A	BPW	SC	—	MacConkey
B	BPW	SC	Bile$^-$	CASE
C	SC +Bile$^-$	—	—	mCASE
D	SC	—	—	CASE+1
E	BPW	Bile$^-$	SC	CASE−
F	BPW	Bile$^-$	—	ABC
G	BPW	SC +Bile$^-$	—	DCA
H	Bile$^-$	BPW	SC	XLD
I	Bile$^-$	—	—	BSA
J	BPW +Bile$^-$	—	—	
K	BPW +Bile$^-$	Bile$^-$	—	
L	Bile$^-$	SC	—	
M	Bile$^+$	SC	—	
N	SC	Bile$^-$	—	
O	SC	Bile$^+$	—	
P	Bile$^-$	SF	—	
Q	Bile$^+$	SF	—	
R	SF	Bile$^-$	—	
S	SF	Bile$^+$	—	

Abbreviations: Bile$^-$, modified Enterobacteriaceae Enrichment broth; Bile$^+$, Bile broth with 0.2 g/L iron pyrophosphate; BSA, Bismuth Sulphite Agar; CASE−, CASE media with selective agents removed; CASE, Chromogenic agar Salmonella Esterase; CASE+1, CASE with one selective agent removed; DCA, Deoxycholate citrate agar; mCASE, CASE with the second selective agent removed; PW, Buffered Peptone Water; SC, Selenite Cystine; SF, Selenite F; XLD, Xylose lysine deoxycholate.

Esterase (CASE; Neogen); selenite cystine broth (SC; Sigma Aldrich and Oxoid); selenite F broth (SF; Neogen and Oxoid); buffered peptone water (Oxoid); bile$^-$ broth (modified Enterobacteriaceae Enrichment or EE broth; Neogen); bile$^+$ broth (bile$^-$ broth; Neogen, with 0.2 g/L iron pyrophosphate; Oxoid).

The base formulation of CASE was developed for the selective isolation and identification of *Salmonella* species. Identification is achieved using a dual chromogenic system utilizing esterase and β-glucosidase detection. Isolation is achieved by the incorporation of all the necessary target growth requirements (i.e., amino acids, vitamins and trace elements), as well as selective agents such as bile acids and metal and phosphate salts to inhibit common nontarget micro-organisms (Neogen, 2019). Additionally, two antibiotics are included in the base medium; the first was included to enhance selectivity against Gram positive and nontarget Gram negative *Enterobacterales*, whilst the second was to inhibit the growth of *Pseudomonas* spp. as these can exhibit strong esterase activity, giving false positives. The CASE media (Neogen) was modified for this project to generate three further agars to improve *S*. Typhi growth. The CASE− agar was the base agar with both antibiotics removed. The CASE+1 was the base agar with just the first antibiotic. The CASE+2 (later described as modified CASE; mCASE) was the base agar with just the second antibiotic.

Development of test pathways

Following a literature review, 171 test pathways were devised to represent possible combinations of the five broths and nine agars (Figure 1 and Table 1).

Qualitative studies

Candidate agars were initially screened with pure cultures of different strains of *Salmonella* serovariants, including *S*. Typhi and *E. coli* NCTC 9001; the *E. coli* was used as a negative control for those agars that selected or allowed identification of *Salmonella* spp. by inhibition or biochemical reactions. Each was prepared to a 0.5 McFarland standard (the inoculum) in Ringer's solution (Oxoid), which was then diluted to a 10^{-6} dilution. The dilutions were inoculated in triplicate onto each of the agars by spreading 100 μL onto each plate. After incubation at 37 ± 1°C for 18 ± 1 h, the growth was recorded as a qualitative score; +++ luxuriant growth, ++ good growth, + weak growth,

TABLE 2 *Salmonella* Typhi strains used in culture method evaluation experiments, which were provided from the Public Health England Gastro-intestinal Bacterial Reference Unit collection

Number	Isolated from	Year	Country of travel recorded	Antimicrobial susceptibility status	Haplotype (where available)	Sequence Type	eBurst Group	Accession ID[a]
1	Human faeces	2009	Nepal	A,C, Su, Tm, Nx, Cp	H58	1	13	SRR7165748
2	Human blood	2012	Malawi	A,C, Su, Tm	H58	1	13	SRR5949979
3	Human blood	2012	Vietnam	Nx, Cp		1	13	SRR1645294
4	Human blood	2012	The Democratic Republic of the Congo	A, Su,T, Tm		2	13	SRR1645361
5	Human blood	2013	Sudan			2	13	SRR5886991
6	Human faeces	2013	Niger	Nx, Cp		2	13	SRR5974884
7	Human faeces	2013	Nigeria			2	13	SRR7165353
8	Human blood	2014	Cameroon			1	13	SRR7165415
9	Human faeces	2014	India			2	13	SRR1967790
10	Human blood	2014	India	Nx, Cp		1	13	SRR1966683
11	Human blood	2014	Ethiopia			2	13	SRR3048982
12	Human blood	2014	Ghana	Tm, Nx, Cp		2	13	SRR7165399
13	—	2014	Zimbabwe	A,C,S, Tm, Nx, Cp		1	13	SRR1967049
14	Human blood	2015	Angola			1	13	SRR1963294
15	Human blood	2015	United Republic of Tanzania	A,C, Su, Tm		1	13	SRR1960208
16	Human blood	2015	Pakistan			1	13	SRR3048958
17	Human faeces	2015	India	A,C, Su, Tm, Nx, Cp		1	13	SRR1967675
18	Human blood	2015	Uganda	A,C, Su, Tm, Nx, Cp		1	13	SRR1967963

Abbreviations: A, Ampicillin; C, Chloramphenicol; Cp, Ciprofloxacin; Nx, Nalidixic Acid; Su, Sulphamethoxazole Tm, Trimethoprim.

[a]Accessible from https://www.ncbi.nlm.nih.gov/sra/ (last accessed 9th November 2020).

− absence of growth. The media that showed lower performance, no or weak growth, across the control strains were removed from further testing.

After removing the agar which allowed no or weak growth of *S.* Typhi, all culture pathways (Figure 1) of our proposed *S.* Typhi isolation protocol were used in conjunction with the remaining agar. Using spiked water samples that had low background flora from a local silt stream and tap water, 9 mL aliquots were inoculated with a 0.5 McFarland standard density of *S.* Typhi. Primary broths were inoculated with 1 mL of the water sample and incubated at 37 ± 1°C for 18 ± 1 h. A 1 mL volume was transferred into 9 mL of the secondary broth (Table 1), which was again incubated at 37 ± 1°C for 18 ± 1 h. After the incubation of the primary and secondary broths, a 50 μL volume was spread onto each agar (DCA, BSA, XLD, CASE− and mCASE) and incubated at 37 ± 1°C for 18 ± 1 h and growth was scored using the qualitative approach.

Incubation times were chosen based on manufacturer's recommendations and were not changed as a variable. This was due to the methods being developed for a surveillance programme that would not be able to operate at high sample numbers with incubation times requiring further processing same day.

Mixtures of known but undisclosed micro-organisms or mixed culture challenges

Mixtures of known but undisclosed culture collection micro-organisms were used to challenge the broth media pathways and mCASE (Tables 2 and 3; Table S3). Experiments were undertaken using a range of 18 NCTC strains and a wild-type *S.* Typhimurium, likely to challenge identification of suspected *S.* Typhi, these strains are listed in Table 3. These organisms were either other Salmonellae bacteria with similar morphology to *S.* Typhi (same colour on mCASE) or organisms likely to be found in the environment that might cause overgrowth on media. Combinations of these strains alongside a Malawian *S.* Typhi strain 2 (Table 3) were prepared as 10 blinded solutions by an independent laboratory worker, and these were used as inocula for each of the broth-based isolation pathways and agar to challenge selectivity.

TABLE 3 Reference strains used in the study and their growth characteristics on mCASE

Strain	NCTC[a]	WDCM[b]	Colour	Growth[c]
Bacillus cereus	7464	ATCC[d] 10876	Blue	+
Bacillus subtilis	10400	00003	Blue	+
Enterococcus faecalis	775	00009	Blue/Black	+
Escherichia coli	9001	00090/00155	Colourless	+++
Escherichia coli	13216	00202	White	+
Escherichia coli O157	12900	00014	Colourless	+++
Listeria innocua	11288	00017	Black	+
Listeria monocytogenes	11994	00019	Blue	+
Mycobacterium fortuitum	10394	ATCC 6841	Blue	+
Mycobacterium chelonae	946	ATCC[d] 35752	Blue	+
Pseudomonas aeruginosa	10662	00114	Blue	++
Raoultella planticola	9528	N/A	Black	+++
Saccharomyces cerevisiae	10716	00058	Blue	+
Salmonella Nottingham	7832	N/A	Blue/Green	+++
Salmonella Typhimurium	Wild-type	N/A	Blue/Green	+++
Staphylococcus aureus	6571	00035	Blue	+
Staphylococcus epidermidis	11047	00132	Blue	+
Vibrio furnissii	11218	00186	No Growth	−
Vibrio parahaemolyticus	10885	00185	Blue	+

Note: Key: +++ luxuriant growth, ++ good growth, + weak growth, − absence of growth.

Abbreviation: N/A, not applicable.

[a]National Collection of Type Cultures.

[b]World Data Centre for Micro-organisms.

[c]Based on at least triplicate data.

[d]American Type Culture Collection.

Enumeration studies

To assess the pathways that had previously performed best, enrichment was tested with the use of a known amount of inoculum to challenge the limit of detection (LOD). Using a Malawian strain, a suspension was made to a 0.5 McFarland standard density in Ringer's Lactate solution. A serial dilution was performed and the inoculum's CFU mL^{-1} was assessed through a spot-titre method (Miles et al., 1938) on Columbia blood agar (5% *v*/*v* horse blood; Oxoid) and incubating at 37 ± 1°C for 18 ± 1 h and quantified the following day. Using 1 mL of each of the 10^{-4}, 10^{-5} and 10^{-6} dilutions of the inoculum were transferred into independent 9 mL preparations of each primary broth (Table 1). After completing each of the culture steps, these samples were again enumerated with the spot-titre method to confirm growth of the test strain.

After this initial assessment, the culture media's LOD was determined and used for further enrichment comparisons on the selected agar from previous assessments, mCASE. Subsequently, three biological replicates of the Malawian *S*. Typhi strain were prepared as independent inoculums per pathways L to S (Table 1), as described above. Each inoculum was enumerated as per the Miles, Misra and Irwin method, and 1 mL of the dilution identified as the LOD (10^{-6}) was transferred to a 9 mL volume of the primary broth for each pathway. Primary broths were incubated at 37 ± 1°C for 18 ± 1 h. A 1 mL volume of primary broth postincubation was transferred into the secondary broth (9 mL), which was again incubated at 37 ± 1°C for 18 ± 1 h. An enumeration (Miles et al., 1938) was performed after preparation of the inoculum and after each broth (primary and secondary) incubation to allow quantification at each step.

Molecular confirmation of *S*. Typhi

Nucleic acid extraction was performed by boiling two colonies of pure growth in 500 μL of molecular grade water (Sigma) in a 1.5-mL centrifuge tube. After vortexing, the tube was heated at 95°C for 10 minutes in a dry block heater, after which, the tube was pulse centrifuged (five

TABLE 4 Primer and probe sequences used in multiplex quantitative PCR assays for the identification of *S*. Typhi

Gene	Gene purpose	Primer and probe sequences (5' – 3')		Accession number	Reference
ttr	Tetrathionate respiratory	Forward:	CTCACCAGGAGATTACAACATGG	AF282268	Hopkins et al., (2009)
		Reverse:	AGCTCAGACCAAAAGTGACCATC		
		Probe:	FAM-CACCGACGGCGAGACCGACTTT-BHQ1		
tviB	Vi polysaccharide biosynthesis protein	Forward:	TGTGGTAAAGGAACTCGGTAAA	NC_003198	Nair et al., (2019)
		Reverse:	GACTTCCGATACCGGGATAATG		
		Probe:	TET-TGGATGCCGAAGAGGTAAGACGAGA-BHQ2		
staG	Fimbrial protein	Forward:	CGCGAAGTCAGAGTCGACATAG	AL513382	Nga et al., (2010)
		Reverse:	AAGACCTCAACGCCGATCAC		
		Probe:	CY5-CATTTGTTCTGGAGCAGGCTGACGG-BHQ2		
sseJ	Secreted effector protein	Forward:	CGAGACTGCCGATGCATTTA	AF294582	Nair et al., (2019)
		Reverse:	GTACATAGCCGTGGTGAGTATAAG		
		Probe:	YY-TGGAGGCGGCCAGTAATATTGGTT-BHQ2		

seconds at 13,300 RPM/17,000g) and stored at 6 ± 2°C until real-time qPCR testing.

Molecular identification used a multiplexed adaptation of the Nair et al., (2019) qPCR focusing on the target genes *ttr*, *tviB*, *staG* and *sseJ* (Table 4). To utilize these assays in a multiplex format, new fluorophores were attached to the established probes enabling amplicon differentiation, and all 18 reference strains (Table 2) were screened against the assays (Supplementary Materials, Table S5).

The assays were performed in two stages: a duplex and a triplex format (Supplementary Materials, Table S4). The duplex screened isolates using *ttr*, the pan-*Salmonella* target and *sseJ*, found only in *S*. Paratyphi C and other nontyphoidal Salmonellae. All isolates that were *ttr* positive and *sseJ* negative would then be further screened by the triplex of *ttr*, *staG* (an established *S*. Typhi-specific target) and *tviB* (a target specific for *S*. Typhi and *S*. Paratyphi C) that was modified for use from gel electrophoresis assays to real-time PCR in Nair et al., (2019). However, the Vi antigen can also be found in some *Citrobacter freundii* (Snellings et al., 1981) with a 78% per identity match when a blast search is performed against *Citrobacter* spp., as such necessitating the use of *ttr* to confirm all isolates as a *Salmonella* spp.

A two-stage PCR was decided upon so that the sensitivity was not too adversely affected for the *S*. Typhi-specific primers and to minimize use of reagents as NTS are more common than *S*. Typhi, allowing samples that are *sseJ* positive, or *ttr* negative to be disregarded. Therefore, the *ttr*, *tviB* and *staG* would all need to generate an amplicon to determine the presence of *S*. Typhi DNA.

The assay used Takyon Low ROX Probe 2X dTTP blue MasterMix (Eurogentec); the primer and probe concentrations of each multiplex, DNA and total reaction volume are listed in Table S5. The fluorophores and quenchers are listed in Table 4.

Both the duplex and triplex assay formats were performed with 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 10 s using either the Applied Biosystems ViiA 7 and QuantStudio 7 platforms (Thermo Fisher Scientific), with 0.2-mL volume 96 well plates. Detection channels used were Blue (FAM), Green (TET), Yellow (Yakima Yellow) and Red (Cy5). Thresholds for the assay were set automatically as these gave reproducibly suitable values (between 0.08 ΔRn and 0.2 ΔRn) in the linear phase of exponential amplification. A positive was defined as amplification crossing the threshold between cycles 10 and 30.

Using 11 biological replicates of overnight culture of the Malawian strain of *S*. Typhi, suspensions were made to a 0.5 McFarland standard in Ringer's Lactate solution. DNA was extracted and serially diluted from 10^{-1} to 10^{-8}, and a standard curve was produced for *ttr*, *tviB* and *staG* as a triplex. These replicates were enumerated by culture prior to extraction to give a CFU mL^{-1} for each extract and serial dilution. With these results, the PCR efficiency (Eff%), Coefficient of Determination (R^2), the 50% and 95% limit of detections (LOD^{50} and LOD^{95}) and Limit of Quantification (LOQ) were calculated.

The Eff% was calculated by determining the slope of the average CFU mL^{-1} at each dilution as a Logarithmic 10 value and calculating the slope when plotted against the average CT value at each dilution and then using the formula (Svec et al., 2015):

$$\text{Eff}\% = 10^{\left(-\frac{1}{\text{slope}}\right)} - 1.$$

The LOD was determined using the probit model (CLSI, 2012). The LOQ was determined using the coefficient of

variance (Cv) between the replicates at each dilution, with the lowest dilution below a Cv of 25% selected (Kralik & Ricchi, 2017).

Phenotypic identification

Phenotypic identification was undertaken using API 20E (BioMerieux) and an anti-sera agglutination test, using the sera for O9 surface antigen, Vi antigen and Hd flagella antigen (Pro-Lab Diagnostics) on all isolates screened to confirm the results of the qPCR against traditional *Salmonella* typing methods. Anti-microbial susceptibility testing (AST) was also performed by disc diffusion method following EUCAST guidelines (EUCAST, 2021) on Mueller Hinton (Oxoid) agar to further differentiate isolates. The definition of multi-drug resistant (MDR) for *S.* Typhi is resistance to all three first line antimicrobials: co-trimoxazole (25 μg), chloramphenicol (30 μg) and ampicillin (10 μg) (Oxoid).

Field comparison of pathways P to S

The final candidate pathways L to S (Figure 1 and Table 1) were then deployed on field samples in Blantyre, Malawi to determine the most consistent culture method for isolation of *S.* Typhi from the environment.

Samples of 1 L water and soil were collected from and alongside four different water courses (rivers). The four sampling sites were selected using data provided from a case control study in which typhoid patient households were geospatially located (Gauld et al., 2019; Gauld et al., 2020). Sites were prioritised downstream of river junction points that had a large number of cases living upstream.

As per the Standing Committee of Analysts recommended method for *Salmonella* spp. (SCA, 2016), water samples were filtered through a 0.45 μM membrane under vacuum and the membrane was then placed into 10 mL of the primary broth. Soil was immersed in 18 mL of the primary broth in a 1:9 ratio, using two grams of soil. Primary broths were incubated at 37 ± 1°C for 18 ± 1 h. A 1 mL volume was transferred into the secondary broth (9 mL), which was again incubated at 37 ± 1°C for 18 ± 1 h. Using a 10-μL loop, the surface of an mCASE plate was inoculated to enable isolation of individual colonies. Two ten-fold dilutions were also made from the secondary broth after incubation, and 0.5 mL of the 10^{-1} and 10^{-2} preparations were spread over the surface of an mCASE plate using a sterile L-shaped spreader.

Confirmation was performed by qPCR and phenotypic methods, as described above.

Field studies in Malawi using final selected pathway

Field studies on the final selected pathway were established in **eight locations, and within each of these, there were 10 defined environmental sample collection sites. Four sampling sites were as described above, with additional sites including a sewage plant, which eluted into the Mudi river and three communities: Zingwangwe, Ndirande and Mbayani (map in Supplementary Materials, Figure S4). At each of the 10 sample collection sites per location, up to five different sample types were collected based on availability. Targeted sample types included 1L water collections, food (vegetables, fruits and spices), soil, riverbed rock samples coated in biofilms (2–5 cm in diameter) and Moore swabs (Moore, 1951; Sikorski & Levine, 2020).

Pathway P was selected for the field studies. Water samples were filtered through a 0.45 μM membrane and then placed into 10 mL of the primary broth. All solid samples were cultured at a ratio of 1:9 of solid sample to media, as such two grams of soil was immersed in 18 mL and rock biofilms were immersed in 20 mL of the primary broth (biofilm was scraped off in culture media, but stone not removed); similarly, five grams food and Moore swabs were immersed in 50 mL. Primary broths were incubated at 37 ± 1°C for 18 ± 1 h. A 1 mL volume was transferred into the secondary broth (9 mL), which was again incubated at 37 ± 1°C for 18 ± 1 h. Using a 10-μL loop, the surface of an mCASE plate was inoculated to enable isolation of individual colonies. Three ten-fold dilutions were also made from the secondary broth after incubation, and 0.5 mL of the 10^{-2} and 10^{-3} preparations were spread over the surface of an mCASE plate using a sterile L-shaped spreader.

Confirmation of presumptive *S.* Typhi was by qPCR. If qPCR positive, phenotypic analysis was undertaken using an API 20E biochemical panel, by serology and AST. Sampling methods were split into grab (1L water samples) and trap samples (Moore swabs and biofilms) and then compared using a Fisher's exact test to compare efficacy between sampling approaches.

RESULTS

Thirty media were selected from the literature review and of these, 16 were eliminated as being unsuitable (Supplementary Materials, Table S1a,b). The reasons for elimination included logistics, shelf-life and a reliance in traditional media on the absence of lactose fermentation or the production of hydrogen sulphide (H_2S) to distinguish colonies despite these being unreliable for the identification of *Salmonella* spp. (Aksoysan et al., 1981; Kunz & Ewing, 1965; Wilson, 1948).

Preliminary screening of agars to reduce candidate pathways

Direct culture of *S*. Typhi strains on the nine candidate agars led to four agars being eliminated based on quality of growth and selectivity: MacConkey; Unmodified CASE; CASE+1; ABC (Figure 1). MacConkey agar was eliminated as it had broad selectivity for enteric, Gram-negative bacteria making it difficult to distinguish *Salmonella* spp. from other *Enterobacterales*. Unmodified CASE and CASE+1 gave poorer growth than the other two formulations: mCASE and CASE− (Figure 2). The other Neogen chromogenic agar, ABC, also proved to be less favourable as the α-galactosidase chromogen gave false negative results for some *S*. Typhi strains.

The 95 remaining pathways were challenged through spiked water experiments (Figure 1). It was not possible to sufficiently distinguish *S*. Typhi from the natural background flora using four of the agars, DCA, BSA, XLD and CASE−, so these were eliminated, removing 76 pathways; descriptions of colony morphology for each of the agar can be found in Supplementary materials (Table S2). The mCASE agar, in contrast, gave consistent recovery of *S*. Typhi through the remaining 19 pathways.

Mixed culture challenges

Using five blinded mixes of culture collection strains (Table 3) and *S*. Typhi, all remaining 19 pathways (Table 1) were evaluated using only the mCASE agar. This led to the removal of a further 11 pathways, A to K, due to their use of nonselective broth media and subsequent overgrowth of *S*. Typhi by the other genera represented in the culture collection strains. It was also observed that colony morphology was impacted as a result of the order in which broth media was used.

For the remaining eight pathways, L to S, a further blinded study was performed in which two out of five mixed cultures contained *S*. Typhi. For the two *S*. Typhi mixes, all eight pathways had isolates with typical morphology on mCASE (Figure 3). In pathway M and Q, the *S*. Typhi could not be isolated as pure culture and only identified as a *Salmonella* species through phenotypic identification.

Quantification of broth recovery, pathways L to S

Primary broths used for pathways L to S were seeded using a Malawian clinical strain of *S*. Typhi. Growth was quantified from the primary broth and the secondary broth after incubation. It was identified that culture pathways had to be seeded with a minimum of 100 CFU mL^{-1}, the 10^{-6} dilution, for consistent growth to be observed. Therefore, we determined the limit of detection of our culture pathway to be 10^2 CFU mL^{-1} as inocula of 10^1 CFU mL^{-1} did not allow for sufficient growth within the primary or secondary broth incubations to allow robust detection.

Twenty-four biological replicates were used to perform all eight pathways in triplicate, to determine their reproducibility and selective advantage. As presented in Figure 4 (additional information in Supplementary Materials Figure S1 and Table S4), pathways L, P and S demonstrated consistent growth from inoculum through both primary and secondary broth culture, where all replicates showed similar performance and the secondary broth showed good growth after the primary broth. Pathways M, Q, N, R and O all demonstrated a reduction in the level of growth after the transition from primary to secondary broth. Analysis of the difference of log10 for pathway L, P and S (Figure 5) showed similar growth between the inoculum to primary and primary to secondary stages of the experiment. After a review of the growth experiment

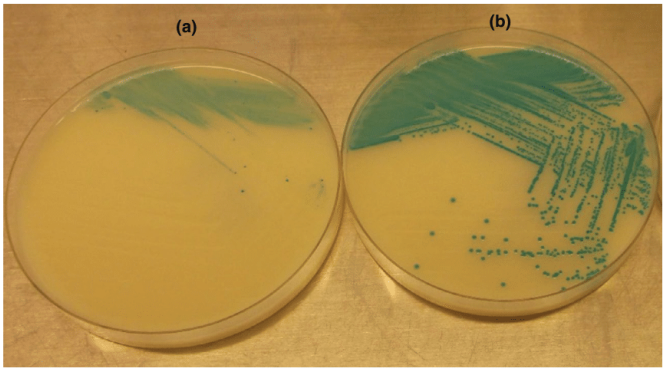

FIGURE 2 Comparison of *S*. Typhi growth luxuriance on (a) unmodified CASE and (b) modified CASE (mCASE)

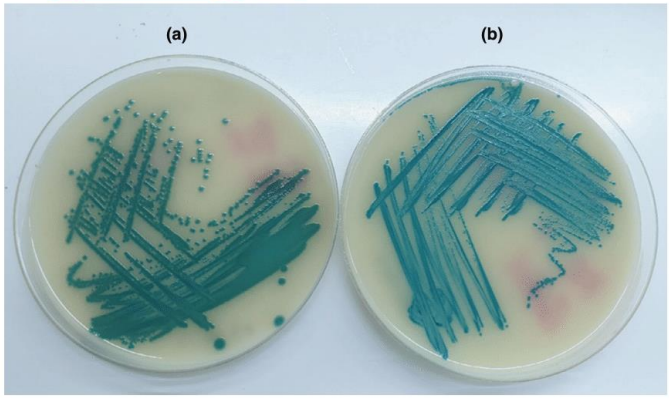

FIGURE 3 Growth of (a) *Salmonella* Typhimurium and (b) *S*. Typhi on mCASE to demonstrate the difference in colony colour and morphology. The *S*. Typhi colonies metabolise the chromogen, generating a bluer colony colour and a smaller colony size than other tested serovariants, with translucent halo. This difference is subtle but appreciable with experience

results alongside consideration of the availability of the broth used, pathway L was eliminated as selenite cystine broth was not consistently available from suppliers, with some discontinuing the product. The pathways P and S were taken through into field trials in Malawi to confirm that performance was equivalent in naturally contaminated samples.

PCR validation

A modification of the real-time qPCR assays as described by Nair et al., (2019) was developed in this study to allow isolate identification and quantification of *S*. Typhi from environmental samples. All 18 of the *S*. Typhi strains (Table 2) used to challenge this assay amplified with *ttr*, *staG* and *tviB*, whilst the *S*. Nottingham (Table 3) and a *S*. Typhimurium strain amplified *ttr* and *sseJ*. *Escherichia coli* strain NCTC 9001 did not cross-react with any primer target.

Using the Malawian strain of *S*. Typhi (Table 2), the primer efficiency (Eff%), coefficient of determination (R^2), limit of detection (LOD) and limit of quantification (LOQ) were calculated (Supplementary Materials, Figure S2). The Eff% and R^2 values fell within 100% and 110%, and 0.99 to 1, respectively: *ttr* 106.6%, 0.993; *tviB* 101.2%, 0.997; *staG* 108.7%, 0.995. The LOD was determined using the probit model analysis method and determined that the LOD^{50} was 4.84×10^1 CFU mL^{-1}, 6.85E $\times 10^2$ CFU mL^{-1} and 1.18×10^2 CFU mL^{-1} for primer pairs for *ttr*, *tviB* and *staG*, respectively. The LOD^{95} was 3.60×10^2 CFU mL^{-1}, 3.61×10^3 CFU mL^{-1} and 8.97×10^2 CFU mL^{-1}, respectively (Supplementary Materials, Figure S2). The LOQ for all assays was 1.74×10^3 CFU mL^{-1}.

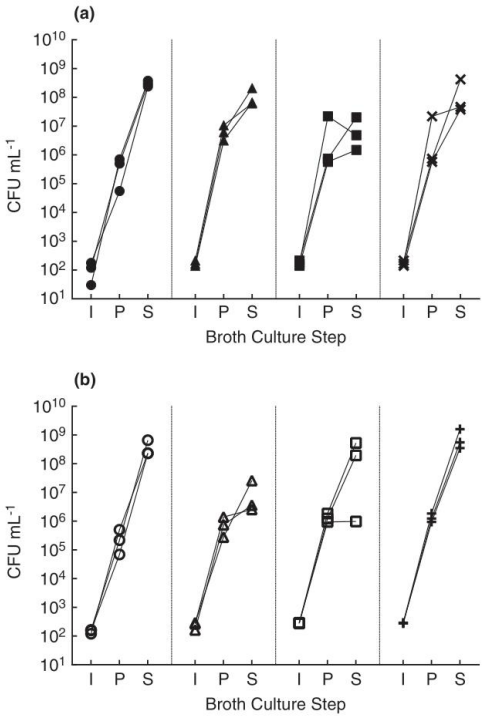

FIGURE 4 *Salmonella* Typhi growth across pathways L to S, representing colony counts at inoculation (I), postincubation of the primary (P) and secondary (S) enrichment broths. Data is divided between the selenite cystine (a) and selenite F (b). A. shows pathways L, M, N and O (Left to right): $bile^-$ to selenite cystine (●), $bile^+$ to selenite cystine (▲), selenite cystine to $bile^-$ (■) and selenite cystine to $bile^+$ (x). B. shows pathways P, Q, R and S (left to right): $bile^-$ to selenite F (○), $bile^+$ to selenite F (Δ), selenite F to $bile^-$ (□) and selenite F to $bile^+$ (+)

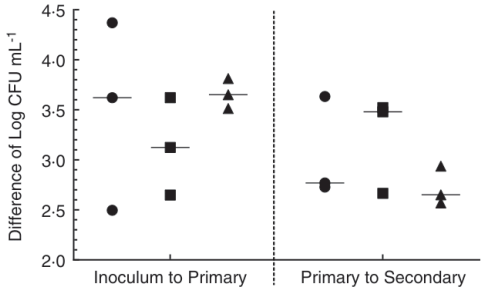

FIGURE 5 Graph showing the difference of log for inoculum to postincubation primary (I to P) broth colony counts and postincubation primary to secondary (P to S) broth colony for pathways L (●), P (■) and S (▲)

In situ use of culture pathways in Malawi

Over a three-month period of sample collection (March to May 2019), the pathways P and S (Table 3) were used in parallel in the field. No *S*. Typhi was isolated from the 27 water samples collected. Observations from growth on the mCASE identified that pathway P demonstrated better recovery of NTS than pathway S. Pathway S also allowed greater growth of contaminating organisms including, *E. coli*, swarming bacteria and fungi, which appeared to impact NTS recovery and therefore was likely to reduce the success of isolating *S*. Typhi.

Field application of Pathway P

Between June 2019 and January 2020, 592 samples were collected across the eight sampling locations in Blantyre, Malawi. These sample types could be separated into two categories: grab and trap samples. We define grab samples as comprising of 1 L water samples (532 collected of a total 592 samples), which provide a snapshot of the *S*. Typhi status of the river collected in the bottle at the particular time and location the sample is taken; and trap samples (60/592), as objects that remain in the river for a longer period (48–72 h), concentrating material *in situ* and increasing the likelihood for the target organism to be captured. Trap samples included Moore swabs (19 swabs out of 60 trap samples), which capture particulates and organisms within the gauze over the period of deployment due to river flow; and rocks covered in biofilms (41/60) (Table 5), which also capture organism due to the nature of biofilms. The number of *S*. Typhi positives from grab samples (1/532) were then compared to the number of positives from the trap samples (5/60) using Fisher's Exact test, and a p value of 5.07×10^{-5} was calculated (Table 5). This demonstrates statistically that trap samples are more likely to be positive for *S*. Typhi (risk ratio from Table 5 = 44.33, i.e., trap samples were 44.33 times as likely to be positive for S. Typhi in this study's field experiment). No *Salmonella* spp. was isolated from food or soil.

TABLE 5 Number of each sample type collected between June 2019 and January 2020, and the number that were positive by culture for *S*. Typhi. It provides the number of grab samples (1L waters sampling) and trap samples (Moore swabs and biofilms) collected

Sample type	Number negative	Number positive
Water	531	1
Total grab samples	*531*	*1*
Moore swab	16	3
Biofilm	39	2
Total trap samples	*55*	*5*

Note: Comparing the positivity of grab to trap samples using Fisher's Exact test a p value of 5.07×10^{-5} was calculated with a risk ratio of 44.33, meaning trap samples are 44.33 times more likely to be positive.

In this study, geospatial data were combined with current local knowledge of river usage and access points to identify field sampling sites. Of the six positive samples, *S*. Typhi was isolated from: one sample collected from a hotspot located by the geospatial data; four samples from areas with geolocated cases but not identified as a high priority area; and one collected from a site selected independently from the model. Of these samples, five were collected from a river with a busy market located upstream, which demonstrates the importance of combining modelled and observation data (Baker et al., 2011; Gauld et al., 2019; Mirembe et al., 2019; Pitzer et al., 2019).

Additionally, a further 121 unique isolates of NTS were identified with the qPCR assay by testing positive for *ttr* and *sseJ*, but negative for *staG* and *tviB*. Of these, 55 were from the 1L grab samples, 31 from Moore swabs, 5 from biofilms and a further 30 from other sources (algae, soil and other water surface plants and debris).

Confirmation of environmental isolates

The qPCR was performed on all presumptive *Salmonella* spp. isolates, of which six had *ttr*, *tviB* and *staG* genes detected but not *sseJ*, identifying them as *S*. Typhi. The six *S*. Typhi isolates were then screened by API 20E and antisera agglutination for additional confirmation. For all six, the API 20E returned one of two profiles, 4005540 and 4405540, both of which indicate a 99.9% identification for *S*. Typhi. All six isolates were associated with an agglutination reaction against all three of the O9, Vi and Hd antigen target sera. In addition, their AST profiles were

determined, and resistance identified to ampicillin, chloramphenicol and sulfamethoxazole.

DISCUSSION

This study describes a comprehensive approach to environmental detection of *S*. Typhi. Our work has addressed the whole process from the suitability of sample type through to isolate confirmation, considering field sampling, sample processing, bacterial enrichment and isolation. This study is important because it provides a method to evidence long cycle typhoid transmission, which is not as well quantified as short-cycle transmission, but which plays a key role in the epidemiology of typhoid fever (Gauld et al., 2018).

We reviewed 30 culture media for the isolation of *S*. Typhi (Supplementary Materials, Table S1a), 12 were disregarded due to their shelf-life and reliability of supply chain, as well as their reliance on unreliable distinction methods (absence of lactose fermentation and/or H_2S production), compared to more robust modern media, such as chromogenic agars. Of the remaining 19, selenite-based media provided best selectivity. Selenite F broth was our preferred option, due to a lack of availability of selenite cystine, with little difference in performance between the two being evident. The toxicity of selenite-based media is a disadvantage, both in its powder form for inhalation and the danger it poses to aquatic life and environments. This can be controlled through comprehensive risk assessment and limitation of the volume to 10 mL per sample with appropriate disposal systems in place (Neogen, 2019). Further, mCASE was modified specifically for this project to achieve a more optimal performance for *S*. Typhi; with commercially produced media focussing on the isolation of NTS from food, water and environmental samples, additional work could be pursued in the future to modify each of the media used to increase their performance for *S*. Typhi recovery. Additionally, further variations could be assessed, such as incubation times with the use of items like incubator shakers.

Due to the harshness of selenite media and the low concentrations of *S*. Typhi in contaminated water samples, a pre-enrichment broth that provided selectivity for *Salmonella* spp. was considered a requirement. As part of our evaluation of culture pathways, 2% bile broth was selected. The bile broth, a modified version of EE broth with the Typhi-inhibitory malachite green removed, was selected for the recovery of sublethally injured *S*. Typhi. This pre-enrichment broth also mimicked the known exposure to bile in the human host during *S*. Typhi infection of the duodenum (Parry et al., 2002). When compared with more traditional enrichment media for sublethal injury, such as buffered peptone water, 2% bile broth gave much better recovery whilst also providing some selective pressure due to the bile salts within the medium, showing its utility for *S*. Typhi isolation. Further, the addition of iron to this broth to increase *S*. Typhi cell recovery had the unintended consequence of increasing the growth rate of competitive organisms, and assisting in their survival when subcultured into selenite media, making isolation on agar more challenging.

The purpose of utilizing real-time qPCR in this study was to provide a low-cost, high-throughput confirmation tool for isolates of *Salmonella* spp., including *S*. Typhi. The assay used four primer pairs previously described (Nair et al., 2019). It was deemed essential that a multi-target approach be taken to comprehensively determine the identification of an isolate as *S*. Typhi as it has been hard to establish a single primer pair with requisite sensitivity and specificity (Nair et al 2019). We decided upon *ttr* gene primers as a pan-salmonellae assay that confirms genus of the isolate. The *staG* primers have been used exclusively in some studies for direct detection of *S*. Typhi from environmental samples; however, the *sta* operon, in which *staG* is located, is known to be detected in other salmonellae such as *S*. Sendai, Gallinarum (Pu3 and Pu4), Dublin, Enteritidis and Derby (Townsend et al., 2001). Whilst *tviB* is more specific, only being found in *S*. Typhi and Paratyphi C, there have been reports of S. Typhi pathogenic strains without the *SPI-7* pathogenicity island, within which the gene for the Vi antigen is encoded (Baker et al., 2005). Lastly, as we were performing PCR on single picks, *sseJ*, which is not found in *S*. Typhi was used to detect non-typhoidal serovariants. All isolates that were *staG* positive, but *tviB* negative were *sseJ* positive and phenotypically confirmed to be NTS. In contrast, all isolates that were *ttr*, *staG* and *tviB* positive and *sseJ* negative were phenotypically confirmed to be *S*. Typhi. Our data therefore support this primer combination. The qPCR's efficiency and reproducibility fell within the acceptable range and are therefore suitable to be used as a confirmation tool for *S*. Typhi identification. Further, the LOD and LOQ were in the range of a well-performing qPCR assay (Taylor et al., 2019).

Whilst other studies have isolated *S*. Typhi from sewage and heavily contaminated domestic use water (Roy et al., 2016), here we present the first description for the isolation of *S*. Typhi from river water and river-borne environmental samples since the 1980s (Sears et al., 1986; Sears et al., 1984). We previously developed a methodology that places the genomes of clinically isolated organisms in the spatial context of human cases of infection to predict environmental hotspots of typhoid transmission (Gauld, 2020; Gauld et al., 2019). Geolocating the homes of typhoid fever patients allowed for the development of an optimized method for field sampling, targeting the

environmental sampling in areas of known transmission and large numbers of cases. These hotspots provided locations where new and traditional sampling methodologies could be applied to determine whether a capture (grab) method or capture and concentration (trap) method was most effective for *Salmonella*. Whilst we detected *S*. Typhi using both trap and grab samples, trap samples were significantly more effective. Due to intermittent shedding of the organism, Moore swabs were created to "trap" the organism when sampling and used to great effect in previous outbreaks, including for organisms other than *S*. Typhi (Sikorski & Levine, 2020). This is despite variation in the volumes in culture media used, reducing volumes of selenite F media from typical volumes of 200–500 mL down to 10 mL (Sears et al., 1986; Sikorski & Levine, 2020).

Potential loss of Moore swabs presents a problem with only three-quarters of the swabs deployed in this study being recovered; further, two visits to the field are required per Moore swab (deployment and collection 48–72 h later). To reduce sample loss, we attempted and successfully demonstrated the use of environmental biofilms as a sampling tool for *S*. Typhi. Naturally occurring and available in any river water source, this could prove to be a suitable, low-cost, widely available environmental sample. Rocks covered in biofilms, or biofilm scrapings cannot be lost in the same way as a Moore swab, or other deployed tool for long-term collection. The only disadvantage is we do not know the duration that the *S*. Typhi has resided in the biofilm; however, as we are not aware that typhoid has a true reservoir outside of humans, we consider this a minor limitation when compared to cost savings.

In application of the model for this study, there was an assumption used that the 2015–2016 clinical case data would be reflective of current disease presentation across the city, which was potentially not reliable. As no comparator testing process was included in the study, the sensitivity and specificity of this approach cannot be determined due to the lack of a reference standard. However, the analytical validation of each stage of the processing indicates this combination is likely a sensitive methodology for the detection of *S*. Typhi in environmental samples. Additionally, linking in more recent spatially referenced case data would assist in further testing of these methods, in the case that spatial patterns of incidence have changed over time.

This study describes a holistic approach to *S*. Typhi capture, concentration, culture and confirmation in the environment. We combine historical experience of environmental surveillance of *S*. Typhi with molecular approaches, to improve our chances of isolating it from complex environmental matrices. With the increase in antimicrobial resistant strains of *S*. Typhi, the identification of long-cycle reservoirs for typhoid are important to allow for targeted intervention programmes to reduce incidence, and thereby, burden of the disease. We believe this approach will support impact assessment following typhoid conjugate vaccine introduction. The culture-based approach also allows for the identification of NTS, which may be of interest due to the high prevalence of invasive nontyphoidal salmonella disease in regions with endemic *S*. Typhi. The use of an environmental surveillance programme would not only allow identification of areas where interventions could be implemented but could also be used as an effective tool for the monitoring of vaccination programmes world-wide, and their impact on the local transmission and exposure of typhoid.

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CONFLICT OF INTEREST

No conflict of interest declared by any author.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article, further data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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6 A year of Environmental Surveillance for *Salmonella* Typhi

6.1 Summary

In this chapter I present the results of the one-year of ES for *S.* Typhi in Blantyre, Malawi, from May 2021 to April 2022. This ES programme used two methods for the detection of *S.* Typhi. The first was the culture method detailed in Chapters 3 to 5 of this thesis. The second, which I subsequently refer to as the "BMGF method", utilises a real time PCR assay, either directly or on DNA extracted from broth culture, to detect *S.* Typhi from ES samples.

Substantially more NTS were detected than *S.* Typhi, with each method having an apparent difference in seasonality. For the BMGF method, peak detection of *S.* Typhi and NTS was seen in the warm dry months immediately after the rains. *S.* Typhi was only isolated by culture twice (0.0005%), whereas isolation of NTS by culture was common peaking in the cool season (July-August) and the hot season prior to the rains (November and December).

Moore swabs were positive more frequently overall but were often lost/swept away in the wet season. The *S.* Typhi positivity from Moore swabs tested by the BMGF method peaked in the warm dry season after the rains (May-July), whereas grab/water samples gave a much lower positivity, implying water samples are less sensitive. Moore swabs performed best for the detection of NTS by both methods.

The Manase Sewage Plant, where I was able to sample wastewater was a hotspot for detection. When sampling river water, hotspots of *S.* Typhi detection were areas previously reported to have high incidence of typhoid fever such as Mbayani and Zingwangwe. Overall, detection of *S.* Typhi by ES was low by both methods, however this coincided with a decline in typhoid fever. Whether this was due to a change in healthcare seeking behaviour resulting in fewer presentations to QECH during the COVID-19 pandemic, or a genuine fall in typhoid cases due to improved hand-hygiene in response to the pandemic is uncertain and prolonged ES will be required to determine this.

6.2 Introduction

Having optimised field and laboratory methods for *S.* Typhi detection (Chapter 5) I aimed to undertake a year-long environmental surveillance programme. This was set back due to the COVID-19 pandemic, finally beginning in May 2021, shortly after the SARS-CoV-2 Beta-variant

of concern peak, the second wave of COVID-19 in Malawi. Seasonal rains commenced two months later than usual, in January (Figure 6.1).

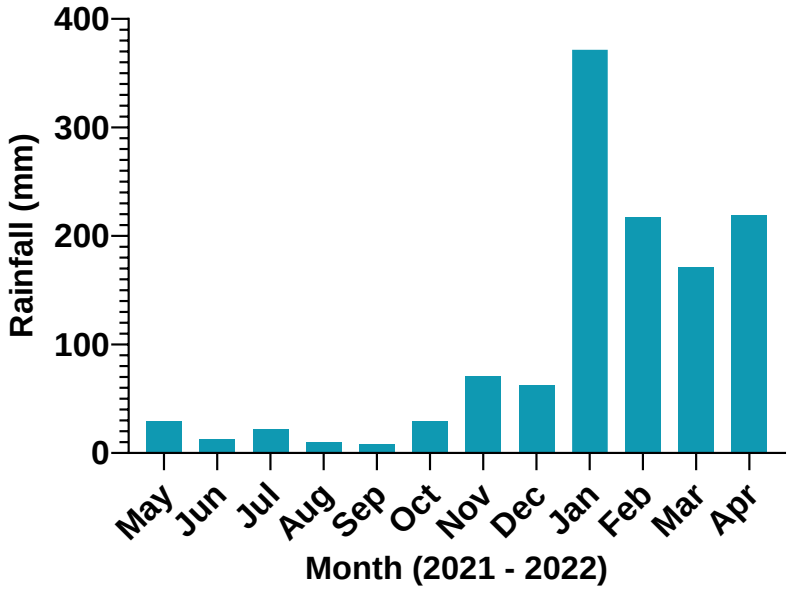

Figure 6.1: Rainfall data from May 2021 to April 2022. Adapted from data available from Climate Hazards Group InfraRed Precipitation with Station data (Funk *et al*., 2014)

This work was part of a wider programme of ES research for *S.* Typhi funded by BMGF which had a primary aim of deploying environmental surveillance methods for the detection of *S*. Typhi in endemic regions at scale, to determine whether they would be viable and valuable. Multiple approaches to Typhoid ES were tested and the results considered by an expert advisory group, which made recommendations about which methods to take forwards. Following their recommendations, during the ES period, I deployed both my methods (Chapter 3-5) hereafter referred to as the culture method, and the methods selected by the Bill and Melinda Gates Foundation (BMGF) *S.* Typhi ES steering group, hereafter referred to as the BMGF method (Zhou *et al*., 2022). The key distinction is that my method aimed to culture individual colonies of *S.* Typhi and formally confirm them, whereas the BMGF method was based on direct PCR or culture-PCR of complex matrices.

The next stage in BMGF ES programme was to develop epidemiological methods to support ES, led by Prof Nick Grassly at Imperial College London (ICL). This programme aimed to develop these surveillance methods in Blantyre, Malawi, Vellore (led by Christian Medical College (CMC) Vellore) and in Agogo, Ghana (led by Kwame Nkrumah University of Science and Technology (KNUST)), and to compare environmental incidence across endemic areas to

inform a more robust method and selection criteria for future studies. Additionally, the BMGF programme study aims to compare clinical incidence of typhoid fever with ES sample positivity from each of these sites, and the potential viability of their use with vaccination programmes to monitor trends and incidence reduction after administration (PI Nick Grassly, ICL). Whilst the Vellore site was entirely focussed on sewage collection, and Agogo, on a mixture of sewage and natural water, Blantyre principally sampled river water due to the lack of formal sewerage across much of the city, or its reliance on septic tanks for wealthier communities (Uzzell et al., 2021). This chapter describes my work in Malawi.

6.3 Methods

6.3.1 Site Selection and sampling

Site selection was undertaken in partnership with Dr Chris Uzzell, a spatial statistician at ICL. My role was to "ground truth" sites proposed by his models, shown in Figure 6.2 and assess their suitability (Uzzell *et al*., 2021). In brief, sites were selected to ensure that the study would obtain 80% power to confirm that the incidence of typhoid seen in Blantyre, Malawi was half that of Vellore, India, and to allow a reasonable calculation of typhoid incidence within both sites. The justification of why the environmental incidence in Blantyre was expected to be half the rate seen in Vellore was based on previous epidemiological studies of Typhoid including STRATAA in Malawi, where the cases in Vellore were estimated to be double those seen in QECH. To achieve this by the end of the study when a mixed effects logistic regression model is used, once per month sampling of 47 sites across the 214m^2 municipality of Blantyre, Malawi, was needed due to the estimated incidence of 1% per year of observation for children <15 years old.

The geospatial method employed by Dr Chris Uzzell is described in brief here and performed by him to generate the GPS coordinates which I validated through site visits. Using a Geographic Information System (GIS) based framework, all river confluence points within the city were selected. Geographical catchment areas were then generated using a topological dataset which was created using publicly available elevation data and the standardised AGREE watershed delineation approach (Liu *et al*., 2016, Fenta *et al*., 2017, Uzzell *et al*., 2021). Population density was assigned to the identified catchment areas using WorldPop and high-resolution settlement layer datasets (Stevens *et al*., 2015, Uzzell *et al*., 2021). To make the data generated comparable to the Vellore, India, and the Agogo and Kumasi, Ghana, sampling sites, medium- and high- population density areas were selected, defined as population groupings of small <9,000; medium 9,000 – 35,000; and large >35,000; and

other regions within the city that had industrial or agricultural land use, were removed (Uzzell et al., 2021).

The city was then divided into 500m^2 polygons, with catchment areas mapped. If more than one GPS location was in the same polygon, on the same river or water system, the location furthest upstream within the polygon was selected as a candidate sampling location. These candidate sites were stratified by population density and assigned to small, medium or large categories. Priority sites were then decided based on which location had the greatest population served estimate per category.

These sites were packaged into a dataset including their GPS coordinates and topological information, and site identifier number and sent to me for on-site field assessment to ensure viability of the location before ES could begin. I considered the site's proximity to the nearest vehicle access; the accessibility of the water by foot with sampling equipment; potential hazards; and the likely availability of sufficient water year-round for a sample. At each location, a site information form was completed (see Appendix 10.1.3), which included estimations for water depth, width, flow rate, direction and colour.

When a list of viable sampling locations were compiled, they were checked by Dr Uzzell and I to ensure all areas of the city of interest were sufficiently covered, and where necessary, alternatives were provided where the original site was not viable for any of the reasons outlined above. This list was then combined with the locations collected during the pre-pilot and initial SARS-CoV-2 ES work, with any of the old sites being removed if they were <100m from a new site and not previously isolated an *S.* Typhi. The only exception to this was the defunct Manase sewage plant which was still sampled at the inlet, lagoon and outlet to the Mudi river.

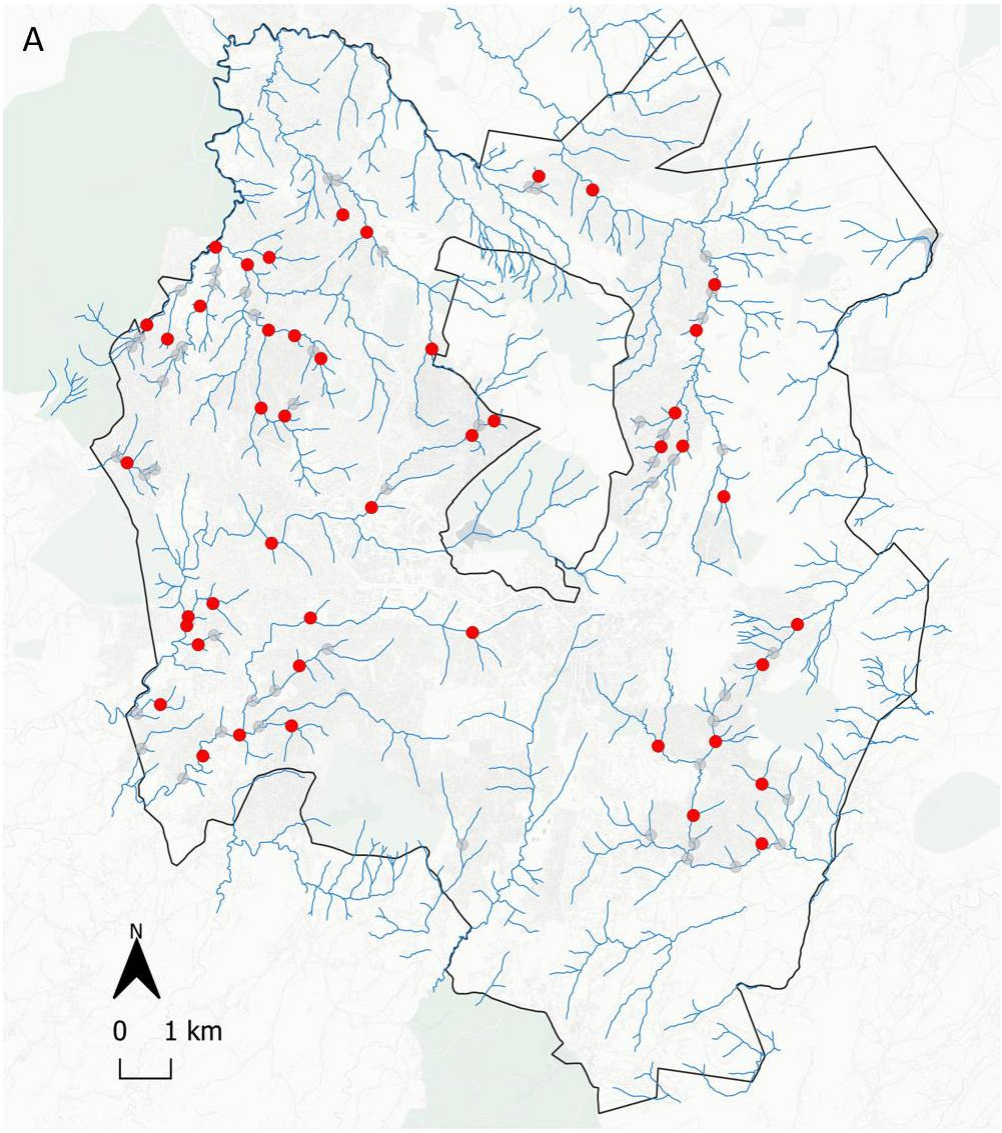

Figure 6.2: Geographic distribution of ES sites for Blantyre, Malawi. Candidate and ultimately confirmed ES sites are shown as grey and red dots, respectively. Blue lines represent rivers. Maps were plotted using QGIS 3.10 using base map tiles sourced from CartoDB (using data by OpenStreetMap made available under the Creative Commons Attribution (CC BY) 4.0 license). (Uzzell *et al*., 2021)

Following site identification, I considered the logistics of sampling. Sites were divided up into 16 groups of three to six sites, depending on proximity and distance from the laboratory. All new sites were assigned to morning collections, from 08.00 to 11.00 on a Monday and Tuesday as requested by the collaborators at ICL. The old sites were assigned to afternoon slots, 13.00 to 16.30. This was due to defecation frequency being most common in the morning, and therefore pathogen recovery from sewage could be greater in the morning through capturing the morning toilet flush (Heaton *et al*., 1992), however in the Malawi context we were not sampling sewage, but river systems. These time slots were split between

two teams of a minimum of two field workers, whilst collections were performed on a fortnightly rota – whilst collections were performed weekly, each site was only visited twice per month. Wednesdays were reserved for overspill, so that sites that were not collected from for unforeseeable issues, such as traffic, lack of vehicular access or staff shortages, could be visited still; with Thursdays and Fridays being reserved for Moore swab deployments for the sites the following Monday and Tuesday. These routes were created using the website Routes XL (https://www.routexl.com/), a free application aimed at delivery companies, with the weekly schedules programmed into the calendars of the field sampling tablets.

During collection, four sample types were collected in duplicate, except for the biofilms:

1. 1 Litre water sample for Typhoid ES;
2. 50 mL water samples for SARS-CoV-2 ES;
3. Moore Swabs for Typhoid ES;
4. Biofilms on rocks, or scrapings from established biofilms for Typhoid (not collected in duplicate).

River water metadata was collected using the AquaRead AP-2000, with results recorded on the updated field collection form with the sample barcodes, created using KoBoToolbox.

6.3.2 Sample Processing

Samples were collected in duplicate in order to be processed via two different methodologies, the first was the culture pathway as discussed previously, whilst the second was the method recommended by the BMGF. The workflow for both methods is shown in Figure 6.3.

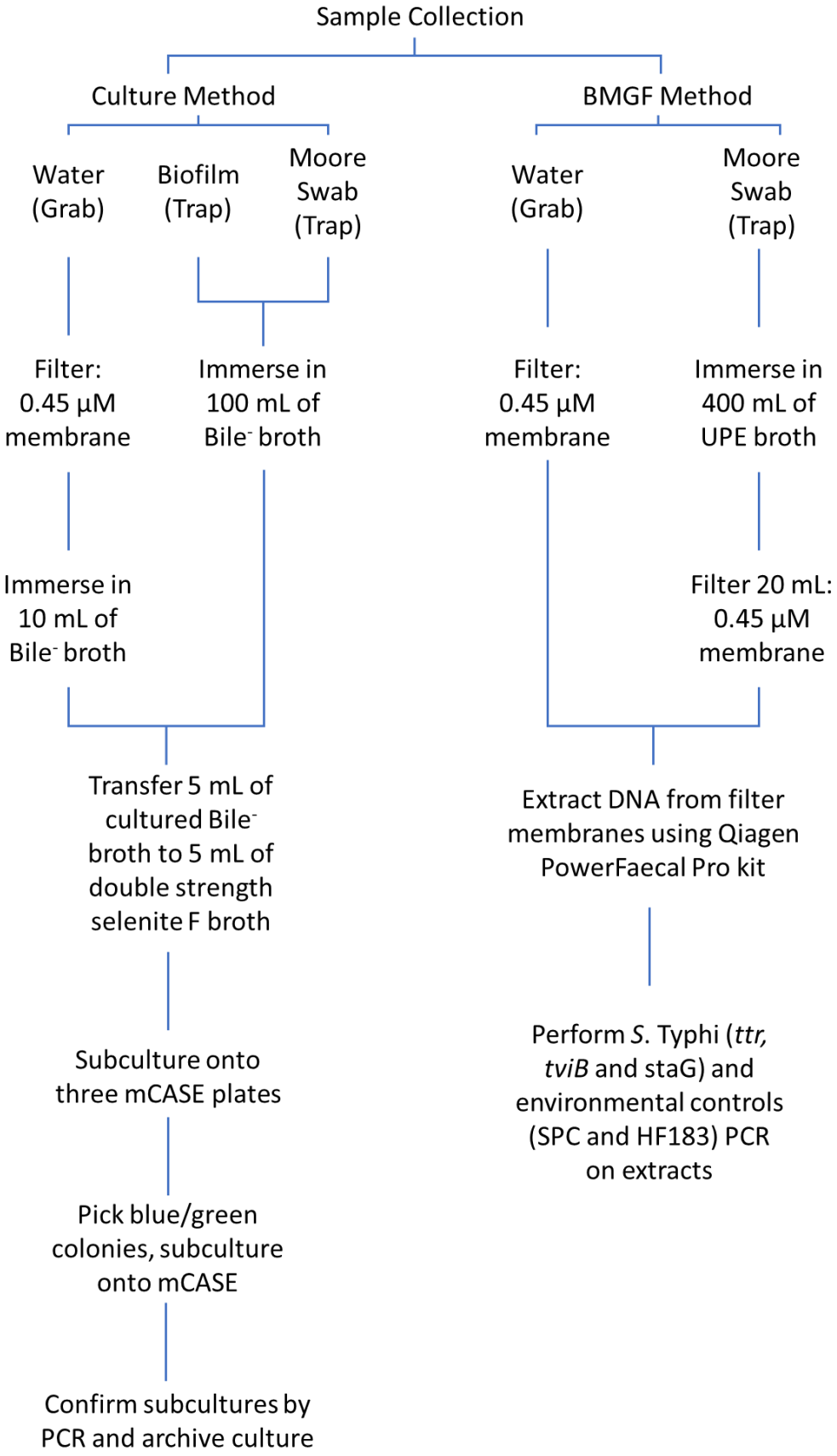

Figure 6.3: Workflow diagram of the culture method and the BMGF method in parallel

6.3.2.1 Culture method

The culture method was used for the Moore swabs, water and biofilms. Moore Swabs and biofilms were immersed in 50 mL of bile$^-$ broth in a 500 mL whirl pack bag, whilst water samples were filtered through a 0.45 µm filter membrane for 1 hour, until five membranes were saturated with filtrate, or the complete sample was filtered; with all of the membranes per sample being added to 10 mL of bile$^-$ broth in 30 mL culture tubes. After 18 to 24 hours at 37 ± 1 °C incubation, 5 mL of the bile$^-$ broth was transferred to 5 mL of double strength selenite F broth in 15 mL narrow culture tubes. These were incubated for 12 to 18 hours at 41 ± 1 °C; due to the toxic nature of selenite F, the shorter incubation is preferred, so samples were inoculated at the end of the workday and subcultured onto mCASE the following morning.

Selenite F broth samples were plated onto mCASE using a standard plate streaking method with a 10 µL loop, and two spread plates by inoculating the centre of the plate with 50 µL of 1:10 and 1:100 dilutions before being spread evenly across the surface of the agar until dry using a wedge- or L-shaped cell spreader. Plates were incubated for 18 to 24 hours at 37 ± 1 °C. Any blue/green colonies observed were subcultured onto a second mCASE plate and incubated the same, to check for typical *Salmonella* spp. morphology. Any isolates that match the typical *Salmonella* spp. morphology were extracted through heat lysis boilates and confirmed by the real time PCR described in Chapter 2 and 4 using targets *ttr*, *sseJ*, *staG* and *tviB*.

6.3.2.2 BMGF Molecular Only Method

For the BMGF method, a direct real time PCR was performed on parallel samples of the "grabbed" water samples, whilst Moore swabs were cultured in a different broth before extraction from the culture. Water samples were filtered with a 0.45 µm filter membrane for 1 hour, until five membranes were used, or until the sample was completely filtered. These membranes were then folded using sterile forceps, with the sedimented sides facing outwards, and placed in either 1.5 mL or 2 mL micro-centrifuge tubes. The Moore swabs were placed in 1 litre PPCO bottles, like those used to collect the water samples, and 400 mL UPE broth was added to each sample. These were incubated for 18 to 24 hours at 37 ± 1 °C. After incubation, 20 mL of the UPE broth was filtered through a 0.45 µm filter membrane, which was also placed in microcentrifuge tubes like the water samples.

To these tubes, glass lysis beads from the Qiagen PFP kits were added. DNA extraction then followed the instruction manual, opting for the 50 µL elution volume. Extracts were tested using a modified real time PCR assay containing *ttr*, *tviB* and *staG* targets as a triplex in one reaction, whilst a SPC from Eurogentec and a primer and probe combination for HF183 was used in a control panel duplex. HF183 is a human restricted *Bacteroides* bacteria used as a human faecal contamination indicator. HF183 was only screened for in the BMGF samples, and not culture, as PCR was not performed on incoming samples, and *Bacteroides dorei* would not be isolatable by the culture method implemented.

6.4 Results

In total, 4,117 unique samples were collected in duplicate between May 2021 to April 2022. One set of the duplicates were processed through the culture pathway comprising a mixture of paired grab (water) samples (n=1,965) and trap samples; Moore Swabs (n=1,472) and biofilms (n=680) sample. A second set of samples, (n=1,042, 554 Moore swabs and 488 water samples), were processed via the BMGF method. Excess sample collections were stored in archive prior to extraction for the BMGF samples as secondary samples where required. In total, 33/1,042 (3.2%) were positive for *S.* Typhi and 80/1,042 (7.7%) were positive for NTS by the BMGF methods (Table 6.1) and for the culture method, 2/4,117 (0.05%) were positive for *S.* Typhi and 255/4,117 (6.2%) were positive for NTS (Table 6.2).

Of the 80 samples identified as NTS by the BMGF protocol, 21 samples were also positive for *staG*, but were negative for *tviB*, even when a PCR was repeated. From the 255 NTS samples cultured, six isolates were also positive for *staG*, and of those, four were also positive for *sseJ*, whilst none were *tviB* positive, even after further subculture, re-extraction and repeat PCR. Biofilm collections ceased in January 2022 due to the poor weather conditions making it difficult to retrieve items from the riverbed, such as the stones used for biofilm samples.

Table 6.1: Number of NTS and *S.* Typhi positive samples detected during year of ES using the BMGF method, comparing grabbed water samples to trap (Moore swabs) samples.

Month	NTS		*S.* Typhi	
	Grab	Trap	Grab	Trap
May-21	10/45 (22.2%)	8/49 (16.3%)	2/45 (4.4%)	5/49 (10.2%)
Jun-21	0/37 (0%)	4/63 (6.3%)	1/37 (2.7%)	2/63 (3.2%)
Jul-21	1/35 (2.9%)	18/63 (28.6%)	1/35 (2.9%)	6/63 (9.5%)
Aug-21	8/45 (17.8%)	2/47 (4.3%)	1/45 (2.2%)	5/47 (10.6%)
Sep-21	0/38 (0%)	6/40 (15%)	1/38 (2.6%)	2/40 (5%)
Oct-21	0/38 (0%)	1/73 (1.4%)	1/38 (2.6%)	1/73 (1.4%)
Nov-21	5/35 (14.3%)	1/54 (1.9%)	0/35 (0%)	0/54 (0%)
Dec-21	2/39 (5.1%)	0/42 (0%)	0/39 (0%)	0/42 (0%)
Jan-22	3/34 (8.8%)	0/2 (0%)	1/34 (2.9%)	0/2 (0%)
Feb-22	3/54 (5.6%)	0/38 (0%)	1/54 (1.9%)	1/38 (2.6%)
Mar-22	3/43 (7%)	1/41 (2.4%)	1/43 (2.3%)	0/41 (0%)
Apr-22	0/45 (0%)	4/42 (9.5%)	0/45 (0%)	1/42 (2.4%)
Total	**35/488 (7.2%)**	**45/554 (8.1%)**	**10/488 (2%)**	**23/554 (4.2%)**

Table 6.2: Number of NTS and *S.* Typhi positive samples detected during one year of ES using the culture method, comparing grabbed water samples to trap (Moore swabs and biofilm) samples.

Month	NTS			*S.* Typhi		
	Grab	Swabs (Trap)	Biofilms (Trap)	Grab	Swabs (Trap)	Biofilms (Trap)
May-21	1/206 (0.5%)	6/155 (3.9%)	0/12 (0%)	0/202 (0%)	0/155 (0%)	0/12 (0%)
Jun-21	6/191 (3.1%)	22/181 (12.2%)	4/88 (4.5%)	0/191 (0%)	0/181 (0%)	0/88 (0%)
Jul-21	4/150 (2.7%)	27/139 (19.4%)	2/97 (2.1%)	0/150 (0%)	0/139 (0%)	0/97 (0%)
Aug-21	6/134 (4.5%)	25/125 (20%)	2/70 (2.9%)	0/134 (0%)	1/125 (0.8%)	0/70 (0%)
Sep-21	6/167 (3.6%)	12/157 (7.6%)	3/118 (2.5%)	0/167 (0%)	0/157 (0%)	0/118 (0%)
Oct-21	0/152 (0%)	3/141 (2.1%)	0/109 (0%)	0/152 (0%)	0/141 (0%)	0/109 (0%)
Nov-21	5/199 (2.5%)	39/178 (21.9%)	3/136 (2.2%)	0/199 (0%)	0/178 (0%)	0/136 (0%)
Dec-21	2/184 (1.1%)	26/88 (29.5%)	1/50 (2%)	0/184 (0%)	0/88 (0%)	0/50 (0%)
Jan-22	0/131 (0%)	1/4 (25%)	-	0/131 (0%)	0/4 (0%)	-
Feb-22	4/138 (2.9%)	6/70 (8.6%)	-	0/138 (0%)	0/70 (0%)	-
Mar-22	2/174 (1.1%)	23/123 (18.7%)	-	0/174 (0%)	0/123 (0%)	-
Apr-22	2/139 (1.4%)	12/111 (10.8%)	-	0/139 (0%)	1/111 (0.9%)	-
Total	**38/1,965 (1.9%)**	**202/1,472 (13.7%)**	**15/680 (2.2%)**	**0/1,965 (0%)**	**2/1,472 (0.1%)**	**0/680 (0%)**

Enough *S.* Typhi samples were detected by the BMGF method to permit statistical comparison of paired samples. Of the 23 *S.* Typhi positive Moore swabs by the BMGF method, 22 were culture negative, with the 23rd isolating only NTS. All 10 *S.* Typhi positive water samples through the BMGF method were culture negative. Of the two *S.* Typhi cultured, only one was tested by BMGF and gave a PCR negative result.

Initially, site IDs of some samples collected did not match their GPS coordinates; when this was reviewed, gaps in the samples processed to date were identified. Whilst the samples missing were rectified as much as possible, some gaps remained. Sites inadequately sampled included 1067/961; 1122/1072; 1132/1116; 1150/1099; 1151/1108; 1158/1076; 1161/1067 and 1167/1026. Sites 1122/1072; 1132/1116; 1150/1099; 1151/1108; 1158/1076; 1161/1067 and 1167/1026 were located in or around Bangwe, where a serious safety incident occurred, preventing teams from entering that community for the majority of this study, as such, samples were not collected from this area from March 2021 to January 2022.

Some locations were over-sampled, particularly between May and July 2021, with the primary sites oversampled being 2002/931 and 1119/932. Site 2002/931 is the sewage plant, where three sample sites are located – the inlet, lagoon and outlet; additionally, the COVID project requested samples be collected from this site on a twice-per-week basis. Further, its proximity to site 1119/932, which was located on the river 80m (from the lagoon) to 180m (from the outlet) north of the sewage plant, has caused issues during collection where these sites have been merged, so frequency of sampling increased for both. The number of samples, per site, per month, is shown in Table 6.3.

The peak in rainfall in January 2022 coincides with the peak Moore swabs loss. Swabs were only recovered from five site in this January and all other samples were water samples.

Table 6.3: Number of samples tested by the BMGF method from each site for each month. Every site should have two samples: a water (grab) and a Moore Swab, highlighted in green. Blue cells indicate sites which have not been collected from during this study, whilst yellow cells indicate more than two were processed from that site that month.

Site ID	Date (Year/Month)											
	21/05	21/06	21/07	21/08	21/09	21/10	21/11	21/12	22/01	22/02	22/03	22/04
1011/961	1	2	2	2	3	0	0	0	1	2	2	2
1019/893	2	2	3	2	2	2	2	1	1	2	2	2
1024/904	2	1	3	2	2	3	3	1	1	2	2	2
1030/877	2	1	3	2	1	1	0	1	1	2	2	3
1033/864	2	1	3	2	2	3	2	2	1	2	1	1
1038/1028	3	3	2	2	2	3	2	2	1	2	2	2
1053/929	2	2	1	2	3	3	2	2	1	2	2	2
1054/912	2	2	2	2	2	3	2	2	0	3	2	2
1056/1039	3	3	0	0	0	2	3	2	1	2	2	2
1060/929	2	2	3	2	2	2	2	2	1	2	2	2
1064/1015	3	3	2	2	2	3	3	1	0	2	2	2
1067/961	4	4	4	2	3	3	3	2	0	3	2	2
1076/944	2	1	3	2	2	3	2	2	1	2	2	2
1077/963	4	4	3	2	2	4	4	3	0	2	2	2
1078/1109	3	3	2	2	2	3	3	2	0	2	2	3
1083/1095	2	3	1	2	2	3	2	2	0	3	2	2
1085/1092	5	3	2	2	2	3	2	2	1	2	1	2
1086/1098	4	3	2	3	2	3	2	2	2	2	2	2
1093/945	2	1	3	2	2	3	2	2	1	0	0	0
1102/1146	0	0	0	2	1	3	2	2	1	3	2	1
1103/1039	2	2	1	0	2	2	3	1	0	1	2	2
1116/971	2	3	2	2	2	3	2	2	1	2	2	2
1119/932	4	9	10	9	7	9	6	9	1	1	2	2
1120/1033	3	4	4	4	2	4	1	2	1	2	2	2
1122/1072	0	0	0	0	0	0	0	0	1	2	1	2
1123/1090	2	3	2	1	2	4	2	2	1	2	2	2
1127/951	3	5	2	3	2	3	2	3	1	3	2	3
1132/1116	0	0	0	0	0	0	0	0	2	2	1	2
1133/1006	2	6	5	5	2	2	3	2	1	1	3	2
1138/916	3	2	1	3	2	3	2	3	1	2	2	2
1144/1017	2	3	4	3	3	3	2	2	1	2	2	2
1149/979	2	2	4	4	2	2	2	2	1	3	2	2
1150/1099	0	0	0	0	0	0	0	0	0	2	2	2
1151/1108	0	0	0	0	0	0	0	0	1	3	2	3
1154/960	3	2	4	3	2	2	2	2	1	2	2	2
1158/1076	0	0	0	0	0	0	0	0	1	2	2	2
1161/1067	0	0	0	0	0	0	0	0	1	3	2	2
1167/1026	0	0	0	0	0	0	0	0	1	2	1	2
1178/1073	5	2	1	2	4	2	3	2	1	3	2	2
2001/1009	4	2	3	2	1	4	2	2	0	2	2	2
2002/931	2	7	6	7	4	7	8	8	2	1	4	2
2003/849	2	1	3	3	1	5	3	2	1	5	3	3
2004/969	3	3	2	2	1	3	3	2	0	2	3	2

6.4.1 Seasonality

6.4.1.1 BMGF method

Salmonella Typhi was detected throughout the year, but NTS were typically detected much more frequently (Table 6.1). There is a trend towards higher rate of positivity in the dry season (May- August) for all serovars (Figure 6.4). January was most heavily affected by the inability to collect samples and a higher number of Moore swab losses due to heavy rainfall.

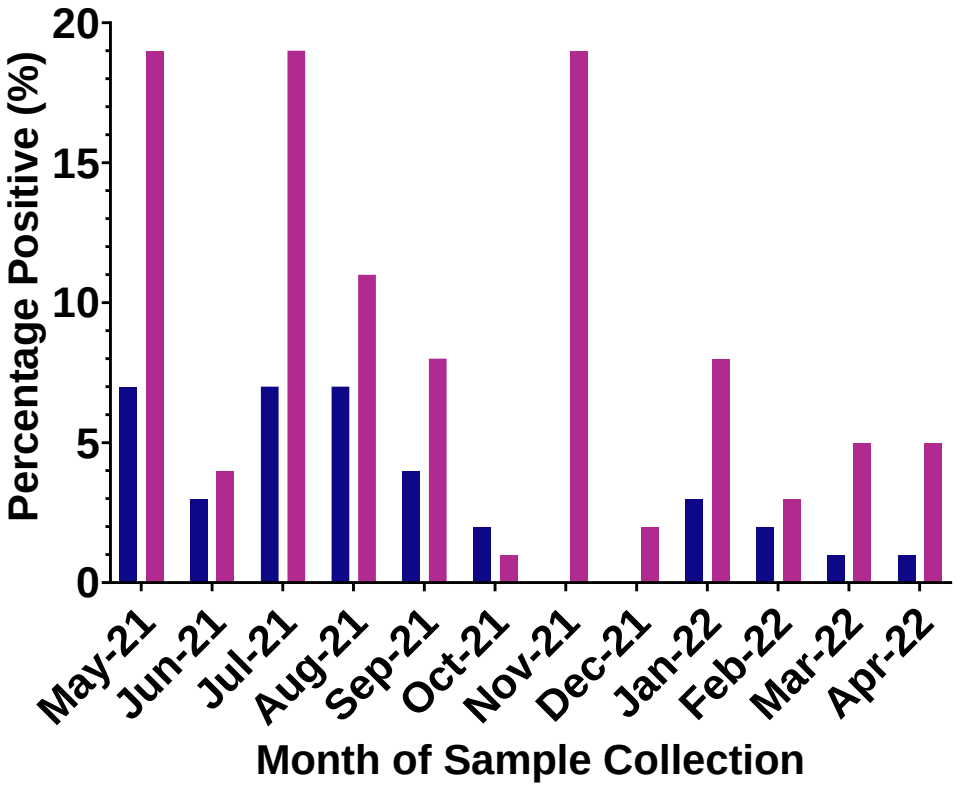

Figure 6.4: Denominated positivity of *S.* Typhi (dark blue) and NTS (pink) positive samples compared to all BMGF samples collected per month.

6.4.1.2 Culture method

More NTS samples were isolated by culture than detected by the BMGF PCR method, however, significantly fewer *S.* Typhi isolates were obtained by culture than PCR; only two *S.* Typhi isolates were cultured, one in August 2021 and one in April 2022 (Table 1.2 and Figure 6.5). There was no clear seasonal trend; at least one sample per month were culture positive for NTS with an average positivity rate of 7.5% accounting for more than 10 NTS per month, except for May and October 2021 and January 2022, which had less than 10 NTS isolates

cultured, no *S.* Typhi and only accounted for 1-2% of the total samples collected during those months.

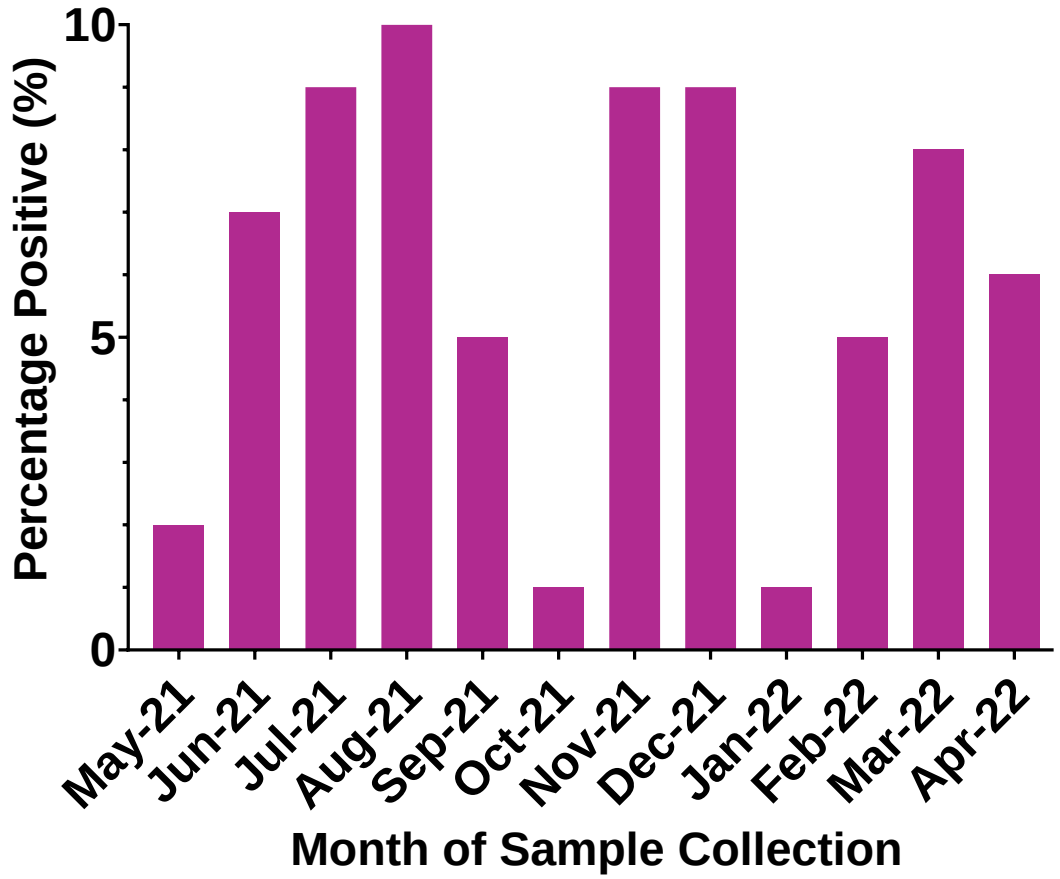

Figure 6.5: NTS positivity by culture method. *S.* Typhi is not plotted due to only two isolates being cultured.

6.4.2 Yield from different environmental sample types

6.4.2.1 BMGF Method

Moore swabs were positive more frequently than the water filtration samples, with 23/554 (4.2%) *S.* Typhi positive, whilst 10/488 (2.2%) water samples were PCR *S.* Typhi positive (Figure 6.6). For Moore swabs, positivity appeared to follow a seasonal pattern, whereas, water filtration samples were consistently positive at a very low level all year round. This pattern was repeated with NTS with 45/554 (8.1%) Moore swab samples, whilst water samples accounted for 35/488 (7.2%) positives (Figure 6.7).

As the samples were paired, McNemar's test was performed (Table 6.4). For *S.* Typhi an odds ratio (OR) of 1.833 (CI 95%: 0.621, 6.037) and chi-squared (χ^2) with 1 degree of freedom (n=315) equal to 0.941 and p = 0.332. This shows no statistically significant association between sample type and *S.* Typhi positivity. Similarly, no statistically significant association between sample type and NTS positivity was observed either, χ^2 (1, n=315) = 0.026, p = 0.871 (OR = 1.111 [CI 95%: 0.558 to 2.228]).

Table 6.4: BMGF sample results based on paired samples between Moore swabs and water samples.

S. Typhi BMGF		Moore Swab	
		Positive	Negative (+/-)
Water	Positive	2	6
	Negative (-/+)	11	296
NTS BMGF		Moore Swab	
		Positive	Negative
Water	Positive	5	18
	Negative	20	272

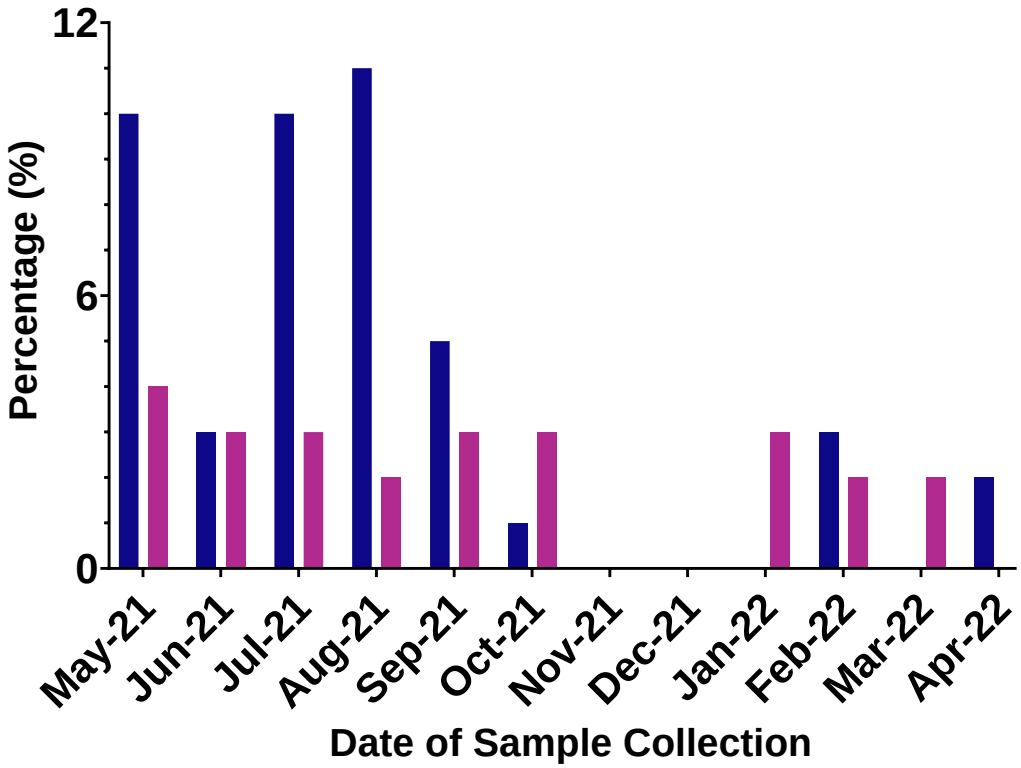

Figure 6.6: Percentage positive for *S.* Typhi of samples tested for the BMGF protocol. Dark blue shows the rate for Moore swabs tested, pink for water (grab) samples.

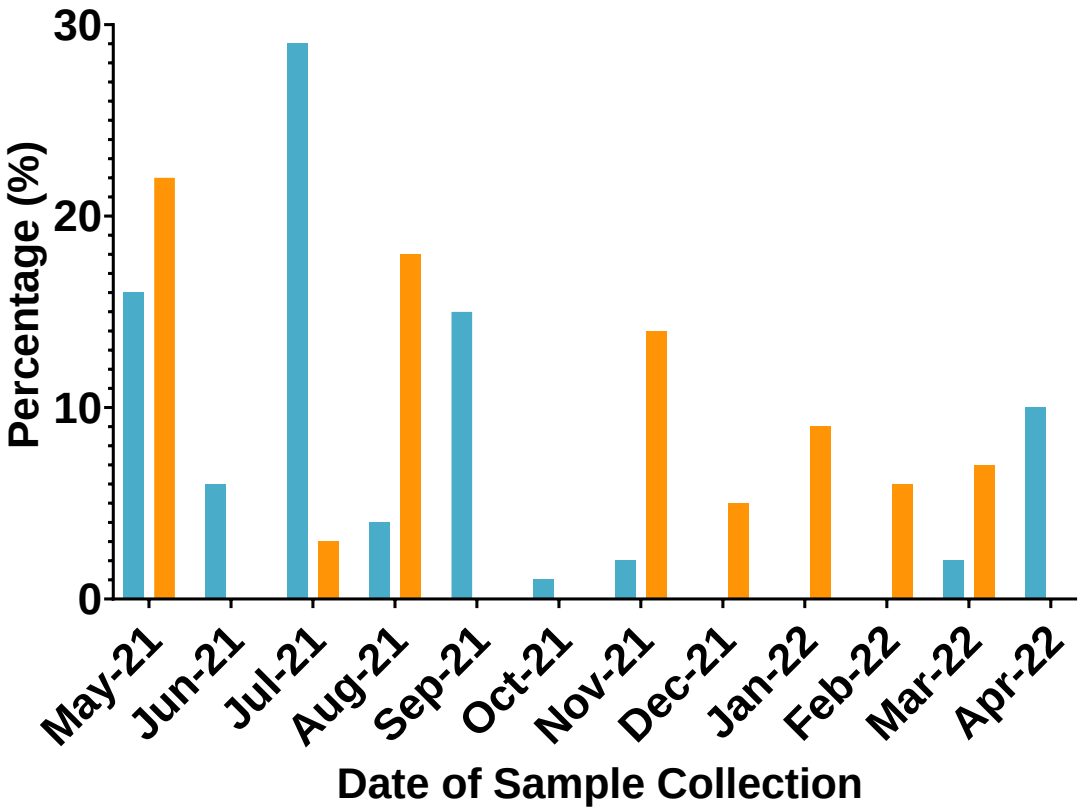

Figure 6.7: Percentage positive for NTS of samples tested for the BMGF protocol. Light blue shows the rate for Moore swabs tested and orange for water (grab) samples.

6.4.2.2 Culture Method

As *S.* Typhi was isolated only twice, further analysis was not possible, however both samples were found from Moore Swabs. Once again, for NTS, the Moore swab provided the highest frequency of positive samples. The proportional positivity of samples for NTS by Moore swab were four times, or more, that of water or biofilms in all months. Moore swabs accounted for a total of 202 NTS positive isolates, whilst water only accounted for 38 and biofilms accounted for 24 positive isolates throughout the year, despite similar numbers of collections (Figure 6.8).

Following on from Chapter 5, the one year of ES used multiple sample types – most prominently Moore swabs and water samples. As biofilms were not collected during January to April 2022, their statistical comparison has been omitted rather than combining them with Moore swabs, like was done previously, as both are examples of Trap sampling. Samples were paired (Table 6.5) and a McNemar's test was performed on NTS positivity; χ^2 (1, n=1,507) =

135.087, p = < 0.0001 (OR = 0.117 [CI 95%: 0.073 to 0.181]), showing that there was a highly statistically significant association between sampling method and positivity for NTS.

Table 6.5: Paired culture method samples based on NTS isolation

NTS Culture Method		Moore Swab	
		Positive	Negative
Water	Positive	12	23
	Negative	196	1276

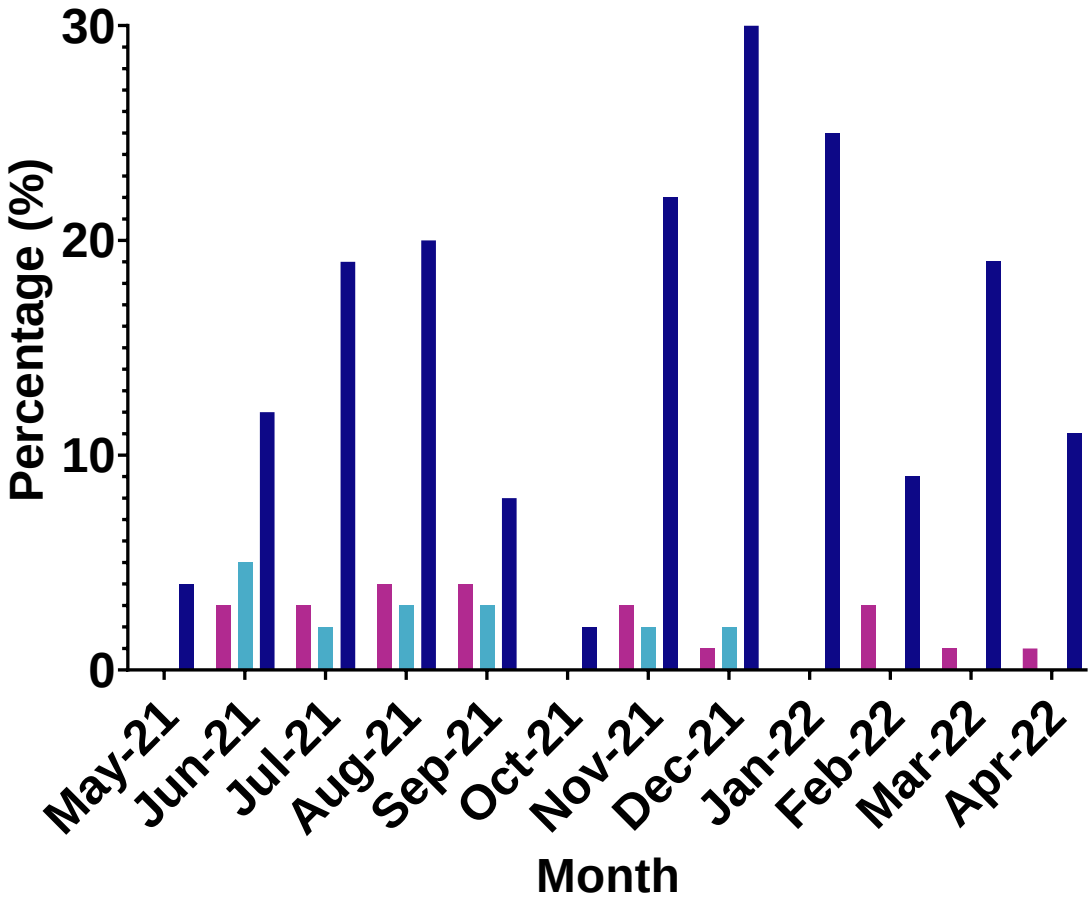

Figure 6.8: Percentage positive for NTS by different environmental sample type. Dark blue shows the PCR positivity rate for Moore swabs, pink for water, and light blue for biofilms, when compared with the total number of samples collected (n=4,117), including culture negative.

6.4.3 Moore Swab Loss

Figure 6.9 shows the rate of Moore swab loss. Throughout the year, the total loss rate was only 10%, with a clear seasonal peak. An additional 315 sample collections took place over the one-year surveillance period where no swabs were deployed – primarily due to sites not

having suitable fixture points at certain times of the year with heavy rainfall removing stones that would be typically used as anchor points, or due to sites not being safe to access during deployment but accessible for collection the following week.

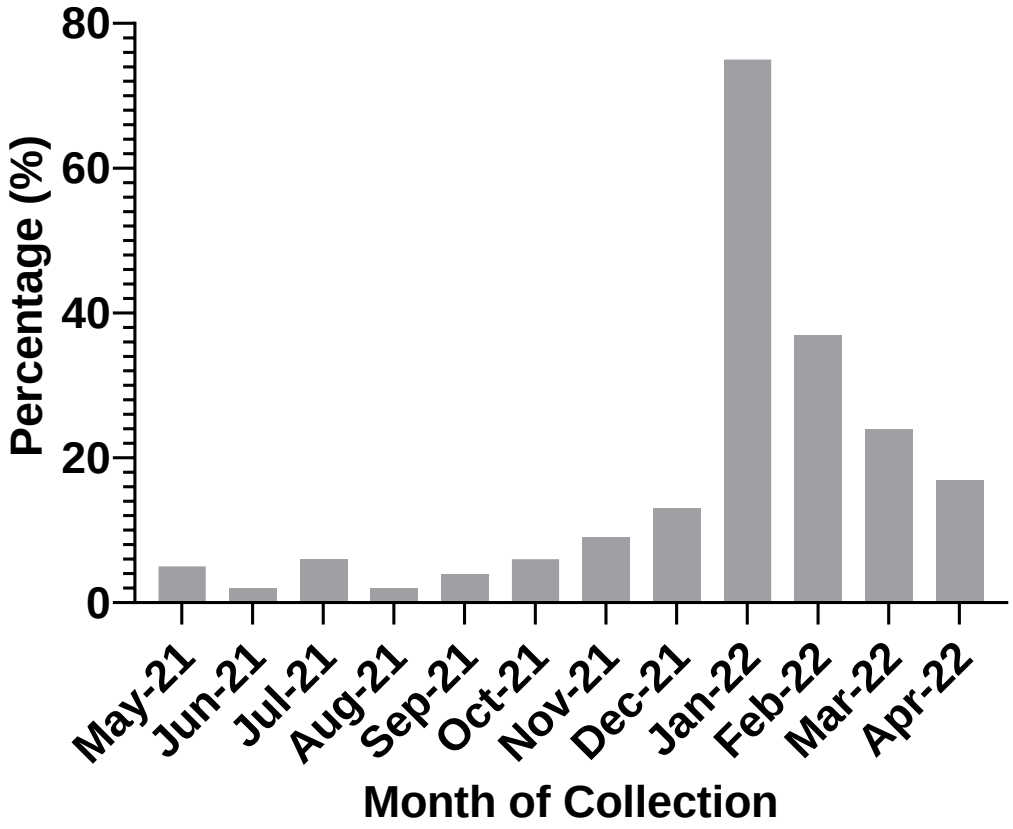

Figure 6.9: Percentage loss rate of Moore swabs over the one-year surveillance programme

6.4.4 Sample site Distribution

6.4.4.1 BMGF

The maps shown in Figure 6.10 plot the frequency of PCR positive samples of the BMGF method, from the GPS coordinates logged by the field team during sampling in a frequency heat map. The warmer the colour, up to red, the more frequently that location was positive for *S.* Typhi (A) or NTS (B). These maps show a higher number of positive samples for both *S.* Typhi and NTS in east Blantyre, especially along the Mudi river and Manase sewage plant (14 *S.* Typhi positive and 15 NTS). Hotspots are also in Ndirande, Mbayani and Bangwe. Negative results are more distributed across the city with no obvious patterns for negative samples that aren't also positive for *S.* Typhi or NTS except for the centre north sites.

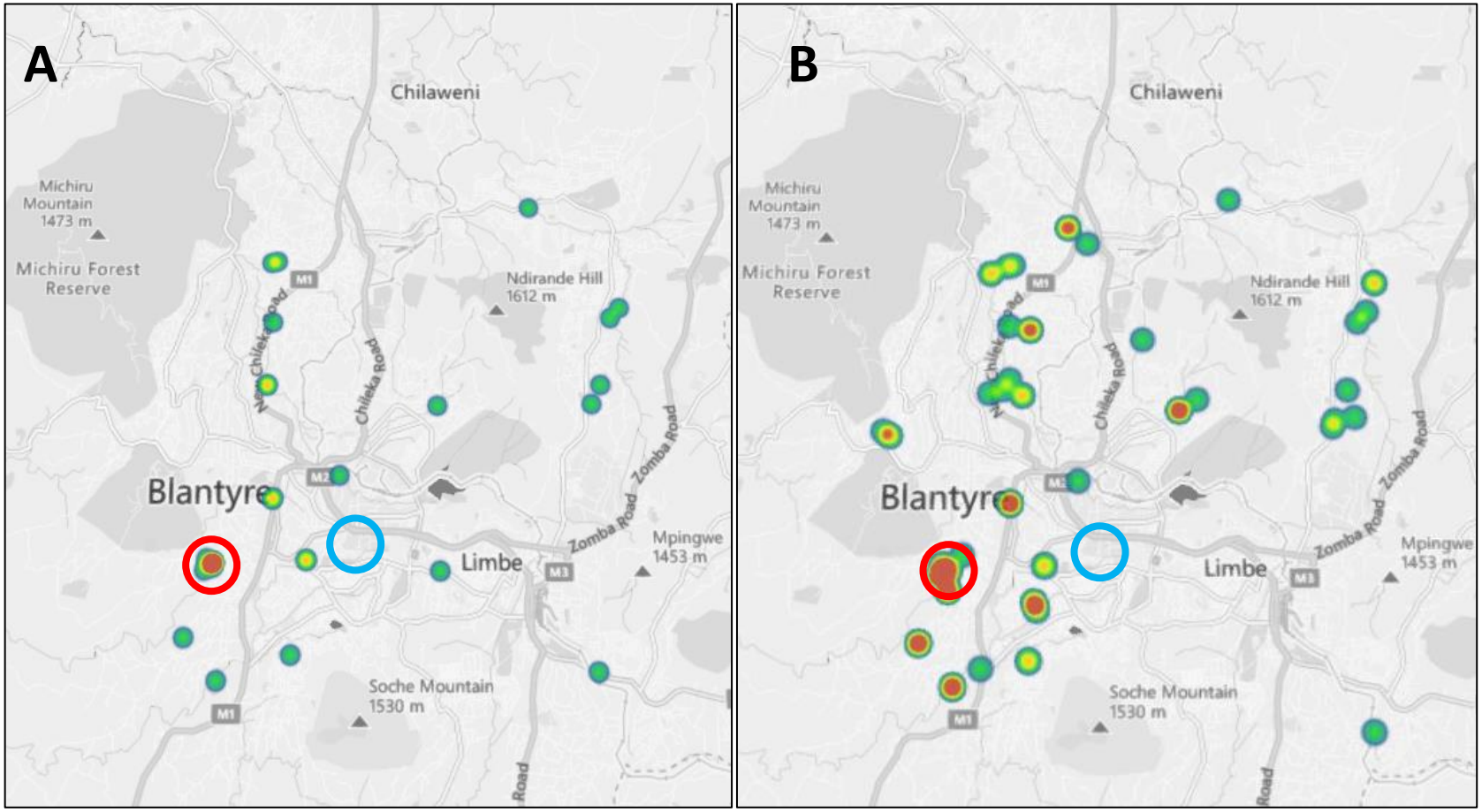

Figure 6.10: Map distribution of BMGF PCR results. Map A shows the locations of *S.* Typhi positive samples and Map B is for NTS positive samples. The red circle indicates the location of the Manase Sewage plant, whilst the blue circle indicates the location of QECH and MLW. Maps generated with Excel 3D Maps, Bing.

6.4.4.2 Culture

Figure 6.11 shows the location of the two water samples positive for *S.* Typhi, one from a similar location to the positives found in 2019, described in Chapter 5. This isolate has been cultured from a Moore swab sample taken directly from the sewage plant lagoon at the Manase sewage plant. The second sample is from the Lunzu river, in the southern part of Nkolitkuti where a busy main road, market and residential area are located.

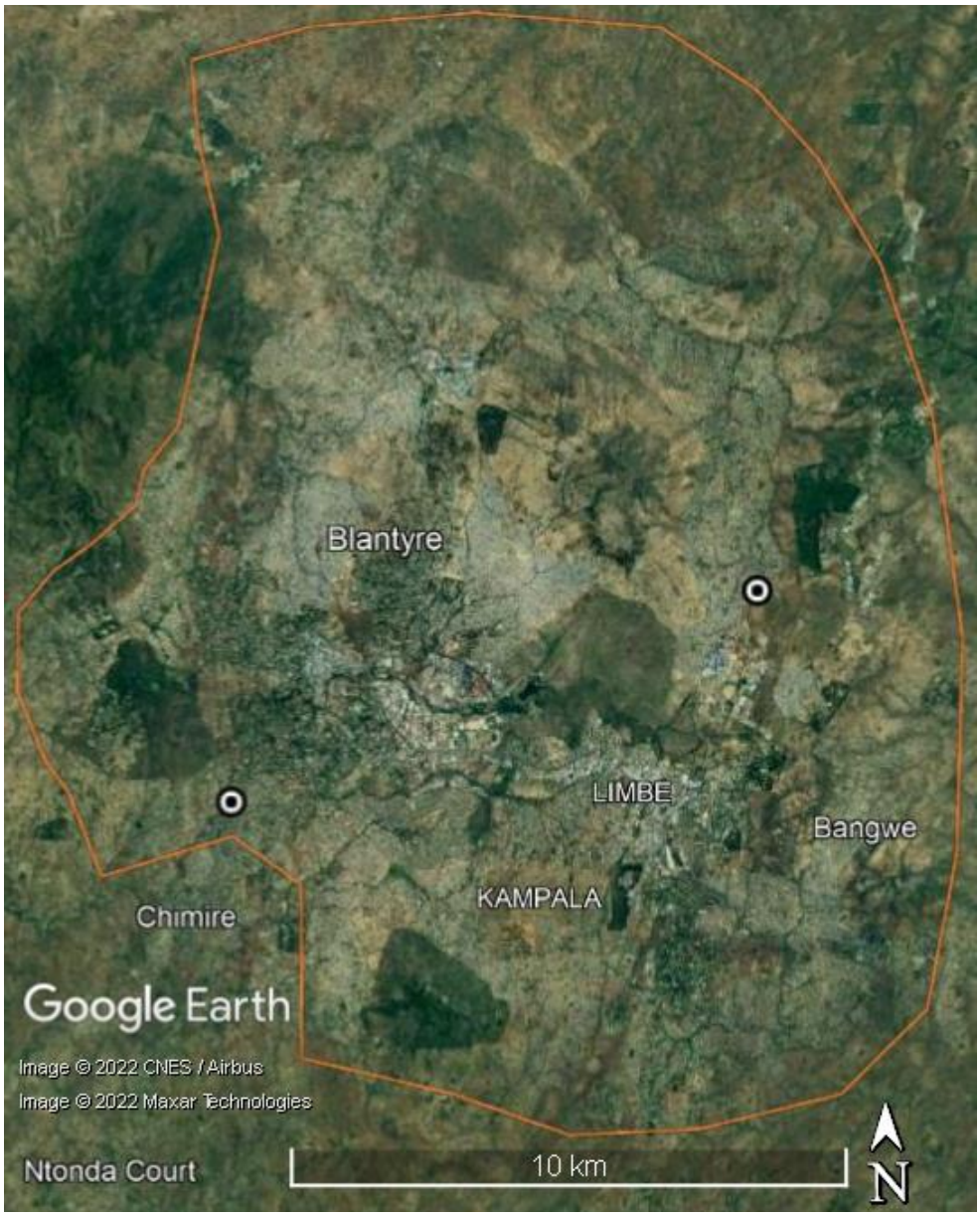

Figure 6.11: Location of the two positive *S.* Typhi isolates from the culture pathway during the one-year surveillance. One isolate came from the defunct Manase Sewage Plant on the Mudi river, whilst the second was from a township in eastern Blantyre on the Lunzu river.

Figure 6.12 is a frequency heatmap of NTS isolates of the culture method, from across the city. They appear to have a wider distribution than those from the BMGF PCR detection samples, showing large concentrations of isolates from Ndirande, Mbayani, Bangwe, and south-west around the Mudi river. However, positive NTS isolates have been sampled at least once from across the entire city's sampling locations.

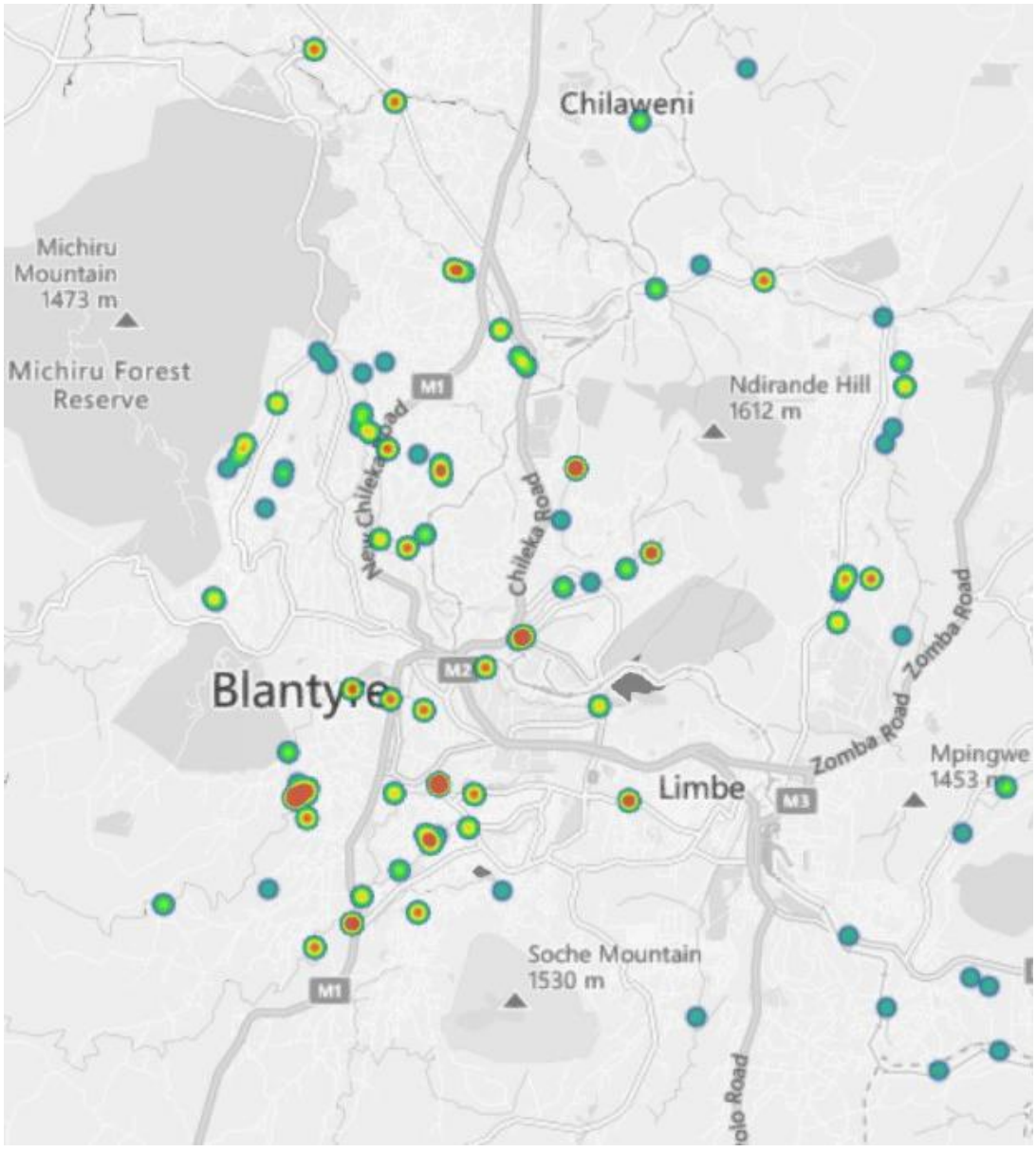

Figure 6.12: Heatmap of NTS isolates cultured during the one-year ES.

6.4.5 Positivity by time of day

6.4.5.1 BMGF

Upon request by the ICL team, samples for the collaborative study were collected in the morning as soon as possible. NTS and *S.* Typhi results are broken down by their positivity to time of collection (Figure 6.13). Rate of *S.* Typhi detection peaked at 8:00 to 9:00 for the BMGF method, but remained less than 7% positive at any other time of day, whereas NTS peaked in the early afternoon.

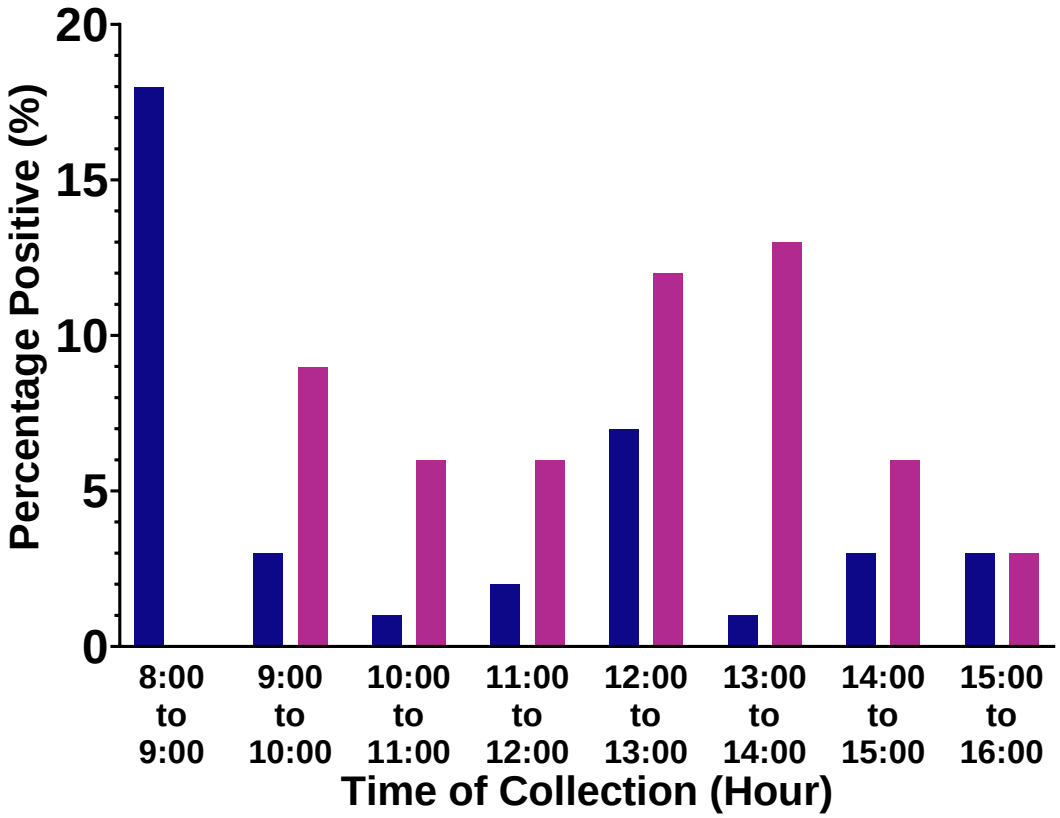

Figure 6.13: PCR positive samples from BMGF protocol for *S.* Typhi (dark blue) and NTS (pink) mapped against hour of collection.

6.4.5.2 Culture

In contrast, time of collection did not impact on culture positivity rate (Figure 6.14).

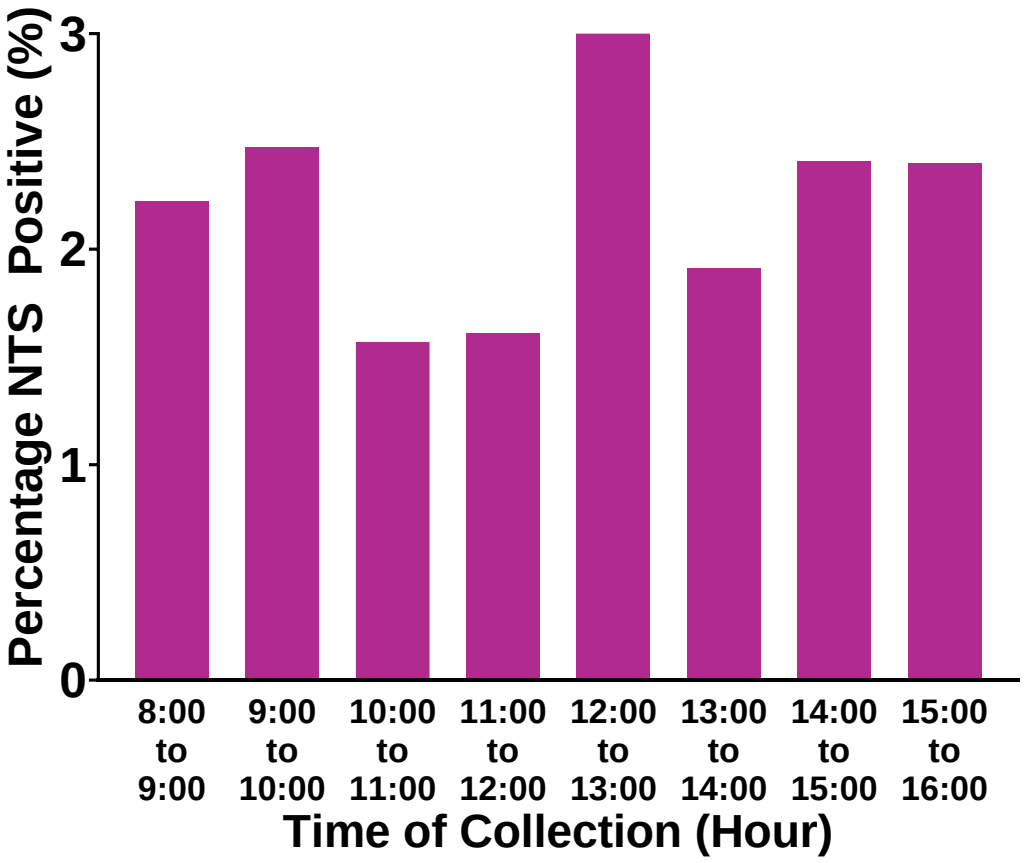

Figure 6.14: PCR positive samples from culture for NTS mapped against hour of collection.

6.4.6 Human faecal contamination indicator

The human faecal HF183 marker gene, sourced from *Bacteroides dorei* originally, is used as an alternative to determine contamination levels of water with human waste (Green *et al*., 2014, Ahmed *et al*., 2019), however this was only tested for on BMGF method samples. Figure 6.15 shows a high percentage of faecal contamination all year round, with a total percentage positivity of 55.6%. When broken down by sample type throughout the year, only 47.5% of Moore swabs were 47.5% positive, compared to 64.8% water samples perhaps because *Bacteroides dorei* is an obligate anaerobe, and the first stage of Moore swab processing is aerobic incubation in UPE.

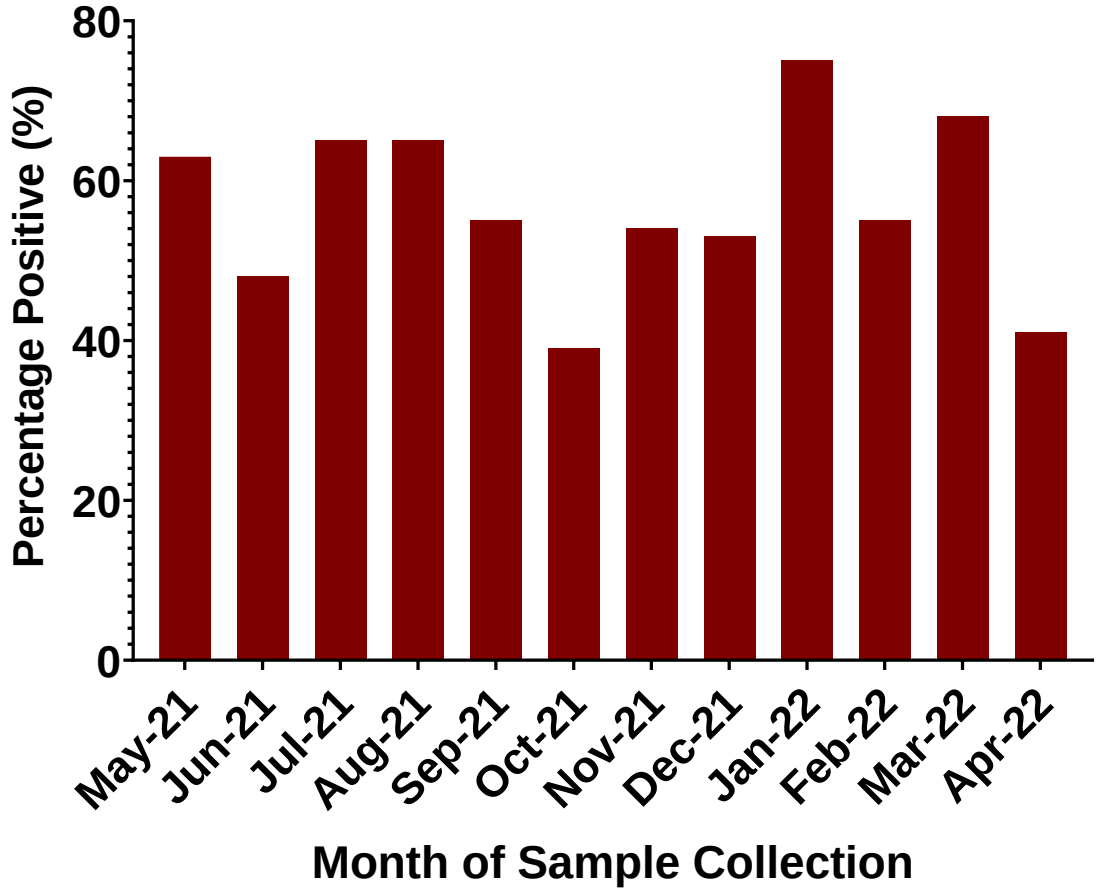

Figure 6.15: Number of samples that were tested by the BMGF molecular only method that were positive for HF183 human faecal contamination indicator.

Figure 6.15 shows the spatial distribution of PCR positive samples for HF183. Like with the cultured NTS, HF183 has been detected in all BMGF sampling locations at least once throughout the year, showing heavy human faecal contamination across the natural waters within the city of Blantyre.

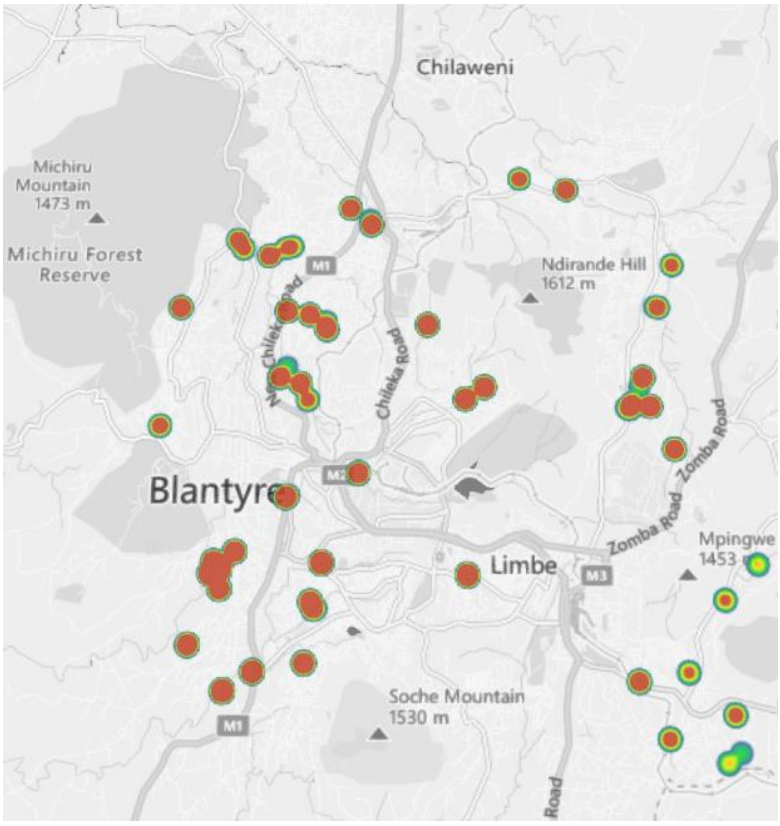

Figure 6.16: Heatmap of HF183 positive locations during the one-year *S.* Typhi surveillance.

Table 6.6 compares the rates of HF183 positivity when a sample was positive for either NTS or *S.* Typhi. Samples that were NTS positive were also HF183 positive 3.8 times more often than HF183 negative, accounting for 11.1% of all samples tested, whilst *S.* Typhi was 7.25 times more often than HF183 negative, accounting for 5% of all samples. For the *S.* Typhi positive samples by the BMGF method, there was a significant association with the positivity of HF183, with samples being six times more likely to be positive if HF183 was present (p = 0.0001, PR = 5.797 [CI 95%: 2.149, 15.730]). Similarly, the samples positive for NTS by the BMGF were three times more likely to be positive if HF183 was present ($p = 3.27 \times 10^{-6}$, PR = 3.199 [CI 95%: 1.892, 5.435]).

Table 6.6: The number of PCR positive NTS and *S.* Typhi samples that were either HF183 positive or negative

	NTS Positive	***S.* Typhi Positive**
HF183 Positive	64 (11.1%)	29 (5%)
HF183 Negative	16 (3.5%)	4 (0.9%)

6.5 Discussion

In this chapter, I demonstrate the implementation of an ES programme for *S.* Typhi and demonstrate the real-world effectiveness of two pathways for identifying *S.* Typhi, the "BMGF" culture-PCR method and my own method, designed to isolate single colonies of viable *S.* Typhi. Both approaches yielded positives and have strengths and weakness. In total, 4,117 unique samples were collected in duplicate between May 2021 to April 2022. Of the BMGF samples, 33/1,042 (3.2%) were positive for *S.* Typhi and 80/1,042 (7.7%) were positive for NTS, whilst for the culture method, 2/4,117 (0.05%) were positive for *S.* Typhi and 255/4,117 (6.2%) were positive for NTS.

6.5.1 *S.* Typhi vs NTS

NTS were detected more frequently than *S.* Typhi and this is unsurprising given that *S.* Typhi is only one of 2,600 serovars of *Salmonella enterica*. Especially as many NTS are environmentally adapted, whereas the only known reservoir of *S.* Typhi is humans, despite it having a poorly understood environmental phase of its transmission cycle. For the BMGF method, NTS was detected almost twice as often as *S.* Typhi except for October 2021. *S.* Typhi was only isolated twice; in contrast NTS were commonly isolated and samples identified as NTS were archived, as some may be of clinical interest such as pathovars associated with iNTS disease, like ST313 *S.* Typhimurium.

Another aspect of interest was the number of NTS samples positive for *staG,* as this gene has been widely used as a single target, specific primer pair for *S.* Typhi. Whilst only six isolates in this category were detected by culture, four of which were *sseJ* positive and therefore could not be *S.* Typhi, and the remaining two of which were subsequently confirmed as not *S.* Typhi by serology and biochemistry. Twenty-one BMGF samples were also PCR positive for *staG* in the BMGF samples and here *sseJ*, serology and biochemistry could not be performed. Broth samples that were *ttr* and *staG* positive, but *tviB* negative have been prioritised for submission for shotgun metagenomic genome sequencing. Results of these WGS submission are not yet complete, so will cannot be discussed in this thesis.

6.5.2 A comparison of culture vs BMGF Methods

As currently operated, the BMGF method is more sensitive than culture alone, with 33 *S.* Typhi samples detected by this method compared to the two isolates confirmed from culture, despite a larger number of samples processed by culture (1,127 vs 4,117). This may be because the BMGF method permits detection of DNA from sub-lethally degraded or dead *S.*

Typhi that are unable to form colonies. Alternatively, this may be because of the use of a much higher volume of sample for DNA extraction in the BMGF methods than is inoculated onto plates by the culture pathway, likely increasing chances of *S.* Typhi detection. In contrast, when divided by number of samples collected, the difference for NTS between the two sample types is much closer (7.7% vs 6.1%).

6.5.3 A comparison of "Grab" vs "Trap" Sample Types

Based on frequency of positivity alone, it appears that Moore swabs or trap samples are the more effective sampling method, however, when denominated by number of samples collected and tested for statistically significant association, the superiority of trap samples is less clear. Using the BMGF method, there was similar performance between trap collected and grab samples χ^2 (1, n=315) = 0.941, p = 0.332 (OR = 1.833 [CI 95%: 0.621 to 6.037]). Of the samples positive for *S.* Typhi, only one sample gave a positive result by both water/grab and Moore swab with the BMGF method, whilst the remaining *S.* Typhi positive samples were only positive from either a water/grab sample (n=9) or Moore swab (n=22) only.

Like for *S.* Typhi, there is no statistically significant association for the BMGF method between Moore swab and water/grab samples for the probability of a sample testing positive for NTS χ^2 (1, n=315) = 0.026, p = 0.871 (OR = 1.111 [CI 95%: 0.558 to 2.228]). For NTS, there is a cross over between four samples positive by Moore swab having their corresponding four water samples also positive.

In contrast, evaluation of the culture method, which largely isolated NTS, favoured Moore swabs, χ^2 (1, n=1,507) = 135.087, p = < 0.0001 (OR = 0.117 [CI 95%: 0.073 to 0.181]). Whilst the only two *S.* Typhi isolates were both Moore swabs; NTS positives were seven times more likely by Moore swab, with 186 NTS positive swabs being culture negative in the water samples.

This loss rate of Moore swabs, caused by the late wet season and high volumes of rain, gives the impression of seasonal positivity – however, this is likely due to Moore swabs being washed away during the heavy rains, as the proportion of water samples that are positive for NTS remains consistent by both methods throughout the year. Biofilms could not be compared during the seasonal changes, as the increased volume of water made collection impossible during 2022 to the end of this data set.

For future culture work, Moore swabs alone would be preferable, even though they are prone to loss during heavy rains and involve field workers visiting locations twice per sample, they are much easier to process in the laboratory. The most challenging bottleneck faced in this study was filtration of one litre water samples, which led to delays that could have contributed to samples being compromised and lowering positivity rate. Alternative methods were not explored as a part of this study, such as ultra-filtration, as the necessary equipment was not available, and too expensive to purchase at this point in the project. However, the time and money saved by dropping processing of the water samples would allow more frequent and more rigorous testing of Moore swabs in future (see Chapter 7).

Additionally, a new method for the collection of biofilms will be implemented, so that a larger number of sites can be sampled. Previously, only small stones were taken from riverbeds where available, but now, natural sponges have been purchased, sterilised, cut into 3 cm by 3 cm swatches, and with the use of a scraping tool, biofilms will be collected from larger surfaces at sites. This was due to the lack of available samples for biofilm utilisation with the previous method, due to natural availability of appropriately sized stones with established biofilms, and a safety risk, due to the depths of some rivers.

6.5.4 Geography

Figure 6.10 A and B, Figure 6.11 and Figure 6.12 show the distribution of *S.* Typhi and NTS positive samples across the city. In all four of these maps, the biggest hotspot for positivity is the Manase Sewage Plant. This is not surprising as sewage plants primarily contain human waste, even at the inlet of the plant. STPs thus present a sample much more likely to be positive for a human-restricted faecal-oral pathogen. Other areas where NTS and *S.* Typhi were consistently detected were the townships of Mbayani, Ndirande, Zingwangwe and Nkholikuti. Each of these are informal settlements with varying quality of accommodation, busy markets and no piped sewage facilities, and all known typhoid fever hotspots (Gauld *et al*., 2020, Gauld, 2020, Gauld *et al*., 2021). These are therefore areas we expected to find *S.* Typhi present. A further predicted hotspot was Bangwe, however, due to a safety incident, sampling in Bangwe was not possible for most of the study period.

Not accounted for is whether the areas where Moore swabs are lost are those where the highest rates of positivity are detected. When this is mapped out, as seen in Figure 6.17, Moore swab loss (B) is seen at all sites across the city but most frequent in areas where the NTS positive samples are commonly detected. They would help explain why there is such a

drop in sensitivity during the wet season. Swab loss was minimal at the Manase sewage plant due to the design of the plant making it difficult to deploy swabs throughout the plant.

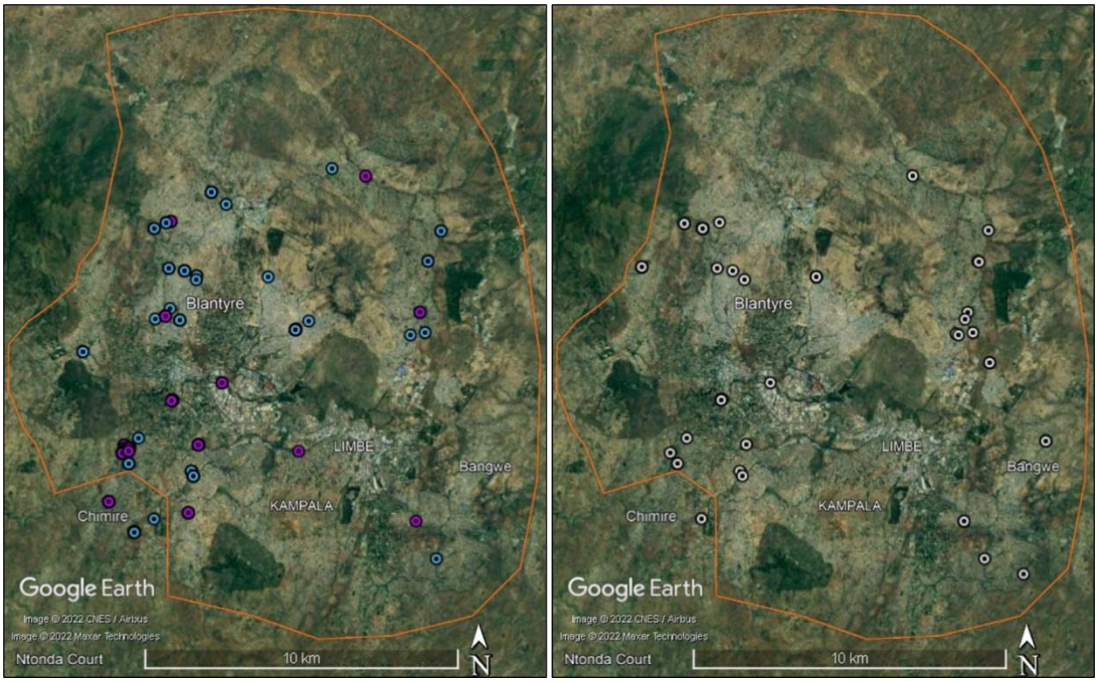

Figure 6.17: Map comparison between locations of *S.* Typhi (purple) and NTS (blue) positive BMGF locations and Lost Moore swab locations (White) for the BMGF samples only.

6.5.5 PCR Screening

Comparing the positive detection rate of the BMGF samples to that of culture, a much higher percentage positivity is seen: 2.9% positive compared to 0.05% positive, which implies many *S.* Typhi are not being successfully cultured (although we must also explore the possibility that the PCR positives from broth are false positives, perhaps due to the three genes being detected across three different bacteria). Combining these methods, however, may increase the chances of positive culture, as samples could be screened either upon arrival from the field, or after a pre-enrichment step to allow sub-lethally damaged cells to recover. After screening these samples, only the ones that are positive would be processed, allowing more laboratory time to interrogate these samples much further. However, the commercial kits available are expensive, so an alternative method has been identified, Magna Extract, which was assessed briefly in Chapter 4, and further in Chapter 7.

6.5.6 Limitations

The study coincided with the lowest rates of clinical typhoid seen in Blantyre, Malawi, as seen in Figure 6.18; with cases presenting to QECH being at their lowest since 2013; although cases have since begun to increase to pre-pandemic levels in 2022. This two-year period, 2020 to 2021, coincides with the worst of the SARS-CoV-2 outbreaks seen in Malawi, which would have had a direct impact on *S.* Typhi cases presenting to QECH. What is unknown, however, is whether the reduction in typhoid fever was due to a change in health seeking behaviour leading to reduced hospital attendance, or to reduced transmission of *S.* Typhi due to increased hand washing, adoption of hand sanitiser, and a reduction in socialisation, with markets and bars also being under a curfew during the COVID-19 pandemic, or a combination of the two. This highlights the limitation of only one year of collection, and the pandemic making the year unusual.

6.5.7 Conclusion

I have demonstrated the viability of ES of *S.* Typhi, and that detection of *S.* Typhi by PCR is much higher than by culture using the current SOPs. Further I have shown that Moore swabs have equal or better performance than grab-water samples, and although Moore swabs may be lost in heavy rains, the grab samples are challenging to process in the laboratory. Some sites are more likely to yield a positive result than others, and these tend to be in areas where we expect high numbers of typhoid cases. Samples that were positive for *S.* Typhi by Moore swab were different sample collections to water samples, which could mean that positive samples are lost by dropping one, but statistically, the association between the two and *S.* Typhi positivity is non-significant, showing that the probability of a positive is equally likely through either method.

The next stage of ES for *S.* Typhi by culture is to reduce sampling, to ensure that the samples get back to the laboratory for processing sooner; that sites are selected based on their probability of positivity, or in areas where information is of interest, such as communities where the conjugate vaccine is being trialled. However, before those are implemented, improvements to the culture method are required – a screening PCR should be assessed and implemented, so that negative samples are discarded, and even if a sample is culture negative, we would still be able to detect the transient presence of *S.* Typhi based on a PCR positive. In turn, with the number of samples PCR positive ranging from 2.9% for *S.* Typhi and 7.3% for NTS, smaller numbers of samples will be handled by the laboratory, and more work can be done to ensure the highest chances possible of a culture positive, utilising parallel

broths or alternative methods – including pelleting of the sample by centrifugation, for culture. A larger volume of bile$^-$ broth may also be of value, but keeping processing volumes of selenite-based media would be preferable due to its toxicity. With a lower number of samples, more agar plates per sample could also be considered, using a differing dilution volume when plated, and still reduce workload and cost based on the low percentage positives observed in this ES programme.

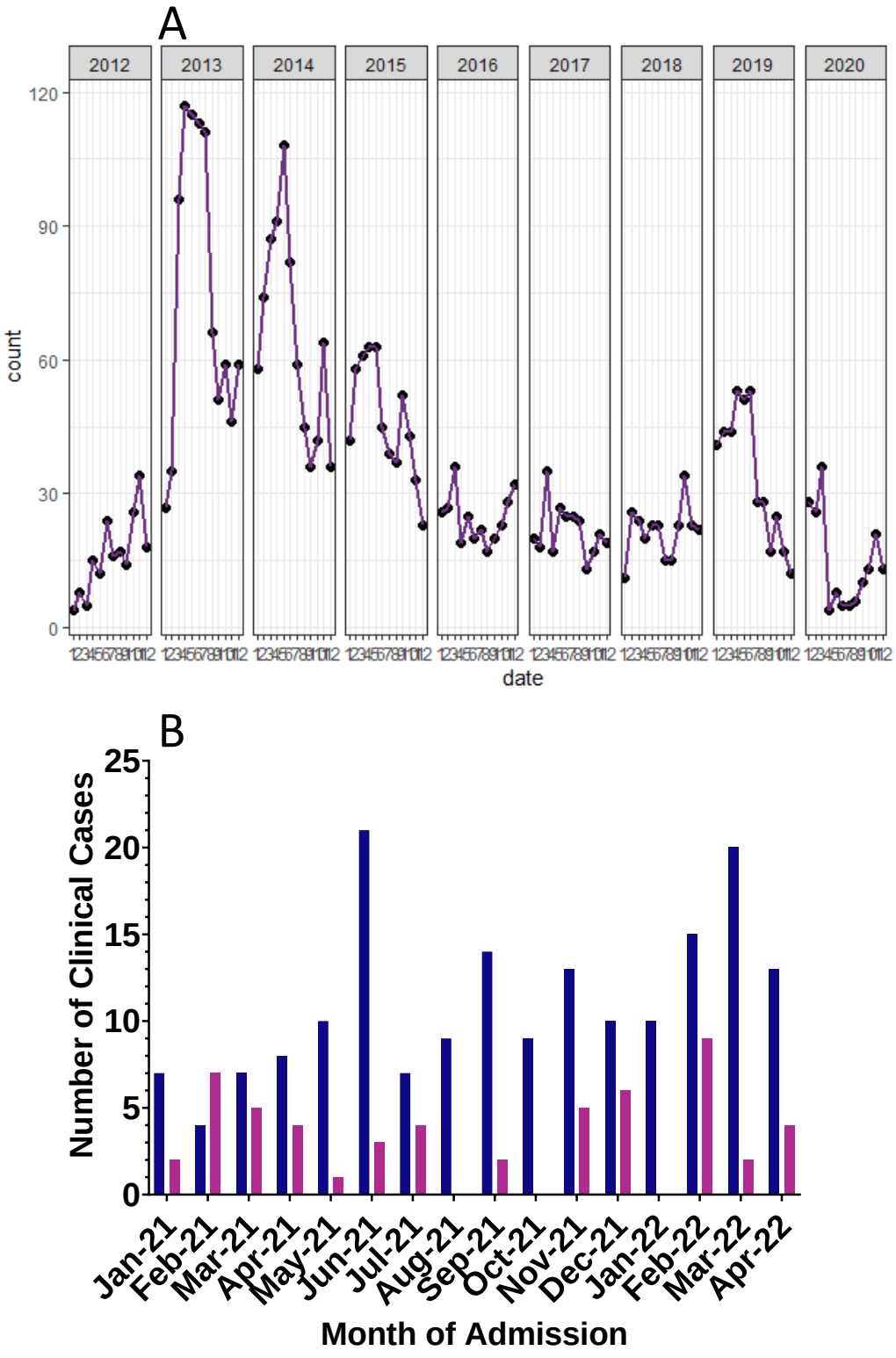

Figure 6.18: A: Number of confirmed cases of *S.* Typhi cases from Queen Elizabeth Central Hospital, Blantyre, Malawi, between 2012 to 2021. B: Number of confirmed cases of invasive *S.* Typhi (blue) and NTS (pink) cases from Queen Elizabeth Central Hospital, Blantyre, Malawi, between January 2021 and April 2022

7 Modifications to culture pathway for future improvements

7.1 Summary

This chapter discusses potential improvements to the culture pathway described in chapter 3, as a higher level of *S.* Typhi detections were made by the BMGF PCR assay when compared to culture alone. All experiments emulated *in situ* ES samples, through the culture of the control strain in bile$^-$ broth, with a ten-fold serial dilution used to determine the lowest detection concentration, as well as a second series using bile$^-$ broth spiked with cultured river water as a diluent.

A PCR screening method was assessed for incoming samples using a low-cost method of DNA purification from thermal lysis method extraction, lowering the per sample cost ten-fold. The BMGF method used during ES extracted samples from 20 mL of broth, whilst the original Magna Extract method uses 200 µL. As such, extraction volume modifications were made. The original Magna Extract had a lower level of detection at 391.33 CFU mL^{-1} than the Qiagen PFP kit (32.65 CFU mL^{-1}); whilst performance was equitable with the control strain when the extraction volume was increased to 20 mL (Range: 20.13 to 39.13 CFU mL^{-1}). When challenged with incubated river water (mixed culture) with control, the 20 mL Magna Extract methods reduced in sensitivity.

Comparison of the current secondary broth, selenite F, was undertaken with five broth formulations: tetrathionate broth; selenite F with chloramphenicol (8 ug mL^{-1}); selenite F with ampicillin (8 ug mL^{-1}); selenite F with pimaricin (2 ug mL^{-1}); selenite F with cycloheximide (8 ug mL^{-1}). All five broths had inferior performance to unmodified selenite F, however tetrathionate broth performed best of the five. Selenite F with either chloramphenicol or ampicillin enabled higher counts of *S.* Typhi from the spiked river water, potentially suppressing competitor bacteria, whilst pimaricin and cycloheximide inhibited all bacterial growth.

7.2 Introduction

The major objective of this study was the development of a protocol for the detection and isolation of *S.* Typhi from natural river water. In the 2019 pilot, *S.* Typhi was successfully isolated from six ES samples in Blantyre, Malawi. However, in the one-year surveillance programme that followed (2021 – 2022), the isolation rate of *S.* Typhi was reduced both compared to the pilot phase (n= 2) and when compared to the direct detection rate by

molecular methods (n=30). Noting the apparently superior rate of detection by nucleic acid amplification, here I revisited the entire pipeline to:

- Streamline the process
- Reduce the cost
- Improve the culture methodology

A faster, better and cheaper method is desirable in LMICs due to resource constraints. In Blantyre and other endemic settings, such as Vellore, India, and Agogo, Ghana, collaborators have expressed an interest to utilise the culture methods developed in this thesis, as they currently rely on solely molecular approaches.

The primary aim of this chapter is to assess the potential for a cheap and easy to implement DNA extraction method which could be used as a PCR screening stage between the first stage culture, or day 1, potentially even being utilised on day 0 when samples arrive, so that PCR negative samples are removed from further downstream culture processes. This seeks to reduce the number of samples that are followed through for culture allowing more focus and the use of more sensitive culture methods, as well as reducing costs due to fewer samples being tested.

7.3 Methods

7.3.1 Novel extraction method for screening incoming samples

The extraction method for the screening real time PCR reviewed for this study was originally developed by another PhD student, Rachel Byrne, for the extraction of AMR genes from ESBL-producing *E. coli* and *Klebsiella pneumoniae* from river water in Blantyre (Byrne *et al*., 2022). The extraction method is referred to as Magna Extract, and is described in Chapter 2. Initial results discussed in Chapter 4 showed promise for the method, which is explored more fully here, where I have reviewed the method and adapted it to increase sensitivity. This adaptation was required due to the lower levels of *S.* Typhi in natural samples than that of *E. coli*, *K. pneumoniae* and NTS.

Firstly, I reviewed the input volumes into the extraction methods to ensure they are comparable. The Qiagen extraction method (Chapter 2 section 2.6.1) used during the surveillance programme utilised 20 mL of cultured broth for the Moore Swabs, and up to one litre of water; both filtered through a 0.45 µm filter membrane followed by the addition of glass lysis beads and vortexed before further extraction. The 200 µL of cultured broth used

for the original Magna Extract method was therefore a 100-fold lower test volume and not directly comparable. Table 7.1 shows the volume modifications to both the sample test and AmpPure bead volumes. I also assessed the use of an automated magnetic bead robot: the Bead Retriever (Thermofisher, formally Dynal), which uses magnets to transfer beads between wash buffers from sample to an elution buffer. Lastly, I trialled the addition of 0.1 mm glass lysis beads (Qiagen, 13118-50) before the thermal lysis/boilate step of Magna Extract, to mechanically disrupt the material deposited on the membranes used on the filtered versions of the method to ensure maximum possible filtrate was extracted, similar to the pre-processing step used for the BMGF method.

Table 7.1: List of six different methods of Magna Extraction reviewed. These methods had various modifications to volume modifications or pre-extraction preparation to increase sensitivity.

Method	**Sample Volume**		**Bead Volume**
	Lysis Volume	**Purification Volume**	
Direct from culture broth			
i	200 µL	100 µL	100 µL
ii	800 µL	500 µL	500 µL
Filtered 20 mL culture broth; membrane washed in RLS			
iii	200 µL	100 µL	100 µL
iv	800 µL	500 µL	500 µL
Filtered 20 mL culture broth: membrane bead-beaten in 1 gram of glass lysis beads and RLS			
v	300 µL	100 µL	100 µL
vi	800 µL	500 µL	500 µL

For methods iii and iv, filtrate was washed off the membranes with RLS (volume in Table 7.1) and transferred to a sterile 1.5 mL centrifuge tube. Additionally, three sets of control samples were tested with a pre-lysis step where the membranes were added to one gram of glass lysis beads (Qiagen), and vortexed on full speed for 10 minutes in RLS, methods v and vi. The alternates of Magna Extract that performed best in triplicate were repeated 12 times alongside the original method, i, so a LOD could be determined.

Due to the Bead Retriever being an older piece of equipment designed for immunomagnetic bead separation of culture samples, concerns were raised that the machine could cause cross-contamination, as identified by the FWE laboratory that donated the equipment. Due to the sensitivity of molecular assays to cross-contamination, the Bead Retriever was assessed for this phenomenon. This was done in two rounds of the purification step, where each well had a positive or negative control in an alternating pattern, as seen in Figure 7.1, which allowed for each well to be assessed for splashing.

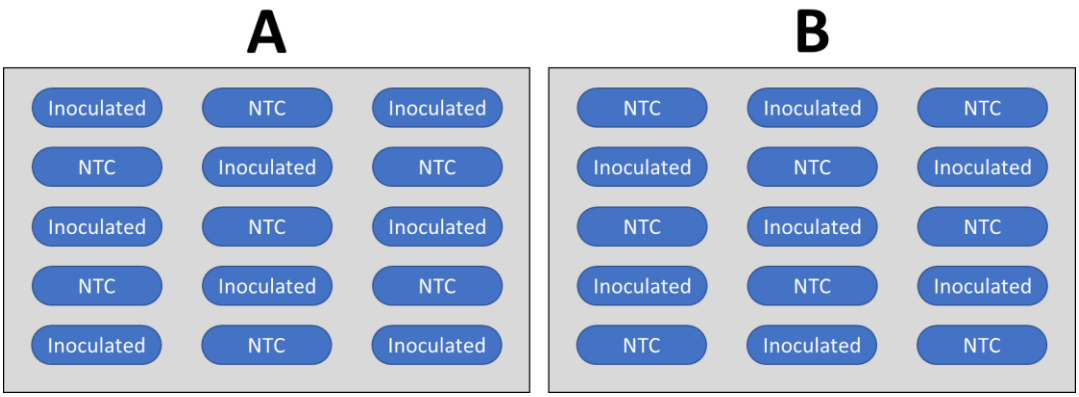

Figure 7.1: To confirm whether the Bead Retriever could be used as a DNA extraction tool, the cross-contamination aspect of the machine was tested by extracting a high concentrated sample (10^8 CFU mL^{-1}) and NTCs using sterile mH_2O in alternative patterns as shown above.

Following experiments with pure samples, and due to concerns that contaminant DNA or inhibitors present in the samples would negatively impact the assay, I conducted a second round of experiments using river water. In this second set of experiments, 100 mL of river water was added to four litres of bile$^-$ broth and incubated overnight at 37 ± 1 °C. This cultured broth was then used as a diluent for a further 12 replicates of the three selected final alternates to give a more representative mixture of what environmental samples would contain and how that might affect PCR screening.

To prepare the samples for extraction, a fresh culture of *S.* Typhi control strain 21 was plated out from archived Cryobeads, before a 0.5 MacFarland standard density was produced in bile$^-$ broth and incubated overnight. After incubation, a dilution series was produced in sterile bile$^-$ broth to 10^{-9} and enumerated using the Miles, Misra and Irwin method (Miles *et al.*, 1938). To simulate more complex samples, bile$^-$ broth was incubated with river water and each dilution used for extraction was made from the sterile 10-fold dilution series into their respective spiked broth, as see in Figure 7.2.

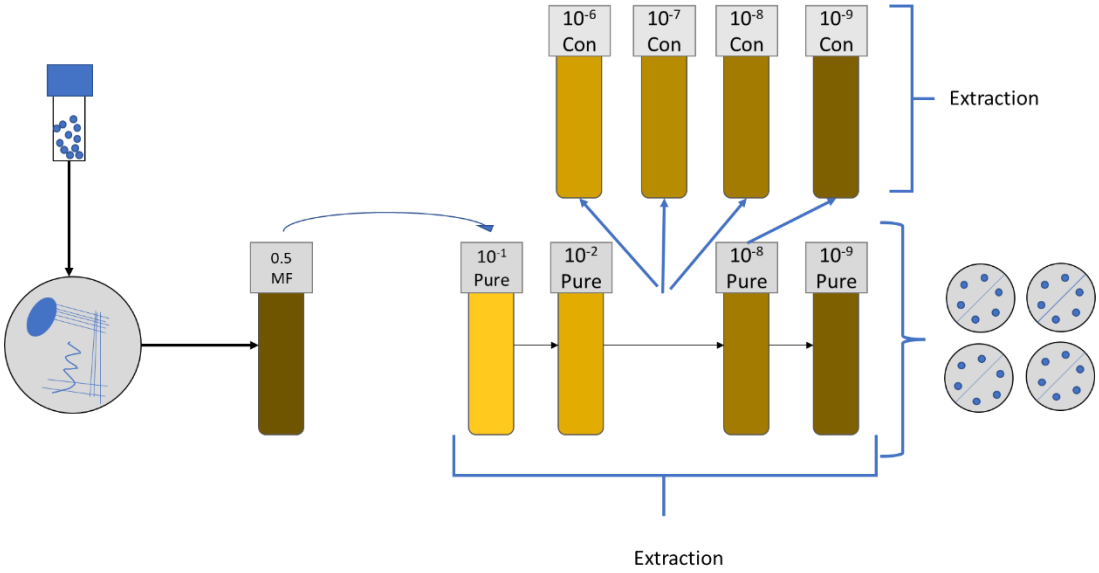

Figure 7.2: Workflow of how broths were set up for Magna Pure extraction comparison. Cryobeads were taken from the archive and plated out onto mCASE agar. Colonies were then taken to create a 0.5 MacFarland standard density in bile$^-$, before a serial dilution was made in pure bile$^-$ to 10^{-9}. This dilution was enumerated with the Miles, Misra and Irwin method before also diluted into a spiked bile$^-$. Both dilution series were then extracted by Magna Extract.

7.3.2 Broth modifications to improve culture

By implementing the above sample screening, I aimed to release resource (staff time and money) through screening out negative samples by PCR and allowing more focus to be given to PCR positive samples through culture methods. As such, one media type that was initially rejected due to the logistical challenge, tetrathionate broth was reassessed. One of the major reasons for its preliminary rejection was that it needed to be used the same day it was produced, and with up to 160 samples being processed in a day, this was deemed unfeasible,

but with a PCR positivity rate of 3%, this would be less of a concern than before. Additionally, the use of antimicrobials to make the broths more selective was also reassessed.

To test these media modifications, a control strain was cultured in bile$^-$ broth overnight at 37 ± 1 °C for 18-24 hours. The following day a ten-fold dilution series was produced, diluted 10-fold to 10^{-9}, and enumerated (Miles *et al*., 1938). The serial dilution was performed in RLS for the pure control strain, whilst a 1 mL aliquot of each dilution was transferred to 9 mL of cultured river water in bile$^-$ broth, as per the method described in Figure7.2, except the extraction step was replaced with sub-culture. From the 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilutions, 1 mL was transferred to an unmodified selenite F broth and a challenge broth in pairs for direct comparison between the current method and a potential modification. Each comparison was made in triplicate, and included the following alternates:

- Tetrathionate broth
- Selenite F with Chloramphenicol (8 µg mL^{-1})
- Selenite F with Ampicillin (8 µg mL^{-1})
- Selenite F with Pimaricin/Natamycin (2 µg mL^{-1})
- Selenite F with Cycloheximide (5 µg mL^{-1})

The inoculated broths were then incubated at their appropriate temperatures (37 ± 1 °C for tetrathionate and 41 ± 1 °C for selenite F alternates, each for 12-18 hours). After incubation, each broth was diluted 10 – fold to 10^{-9} and an enumeration (Miles *et al*., 1938) was performed on mCASE to determine CFU mL^{-1} of each alternate for comparison.

The antimicrobials were prepared in solution using molecular grade ethanol, except for ampicillin, which was prepared in mH_2O (water solubility 50 mg mL^{-1}). Each antimicrobial was prepared to a concentration two $\log_{10}$ higher than required in the broth, so that 100 µL could be added from a stock stored at -80 °C, to 10 mL of culture broth. The concentrations for chloramphenicol and ampicillin were selected based on the clinical break points (EUCAST, 2021) for *Salmonella* spp. as circulating strains in QECH Blantyre, Malawi, have been MDR since the introduction of the H58 haplotype in the early 2010s (Feasey *et al*., 2015).

7.4 Results

7.4.1 Magna Extract for complex sample extraction

7.4.1.1 Magna Extract compared to Qiagen PowerFaecal Pro

The initial experiment (Figure 7.3), which compares the original Magna Extract method, described as method i, (i in Table 7.1) to that of the Qiagen PFP kit, shows the DNA extraction performance for each dilution. Each PCR target at each dilution has a higher Ct value in the Magna Extract sample than that of the Qiagen kit, indicating a lower DNA concentration, or genome copies. Furthermore, the lowest detection in this triplicate is the 10^{-6} dilution for the Magna Extract method, based on a positive amplification of all three targets, denoting *S.* Typhi, whilst the Qiagen kit was the 10^{-8} dilution, 200 times higher than Magna Extract. However, as the extraction volume of the BMGF method is 100-fold higher than that of method i, this disparity was expected, and necessitated the need for reviewing the other methods listed in Table 7.1.

Whilst the lowest detection for an *S.* Typhi positive by Magna Extract was at 10^{-6}, which, when converted into colony counts based on the enumeration of the samples prior to extraction, gave an average 391.33 CFU mL^{-1} (Range: 216 to 570 CFU mL^{-1}), both *ttr* and *tviB* were detectable at a 10-fold lower concentration, 10^{-7}, or 39.13 CFU mL^{-1}. Similarly, the Qiagen's lowest *S.* Typhi detection was one log higher due to the *ttr* target's lowest detection being the 10^{-8} dilution (32.65 CFU mL^{-1} [range: 2.16 to 57 CFU mL^{-1}]), both *tviB* and *staG* were detected at the 10^{-9} dilution, which would have made the lowest detection concentration 3.37 CFU mL^{-1}. Figure 7.4 shows the range of CFU mL^{-1} of the extracted broths at their lowest detected concentration, based on a three-target positive for *S.* Typhi. This demonstrates that whilst the Qiagen detection was 10-fold lower in average CFU mL^{-1}, the range was much wider at that lower concentration due to the incredibly low numbers of colonies counted.

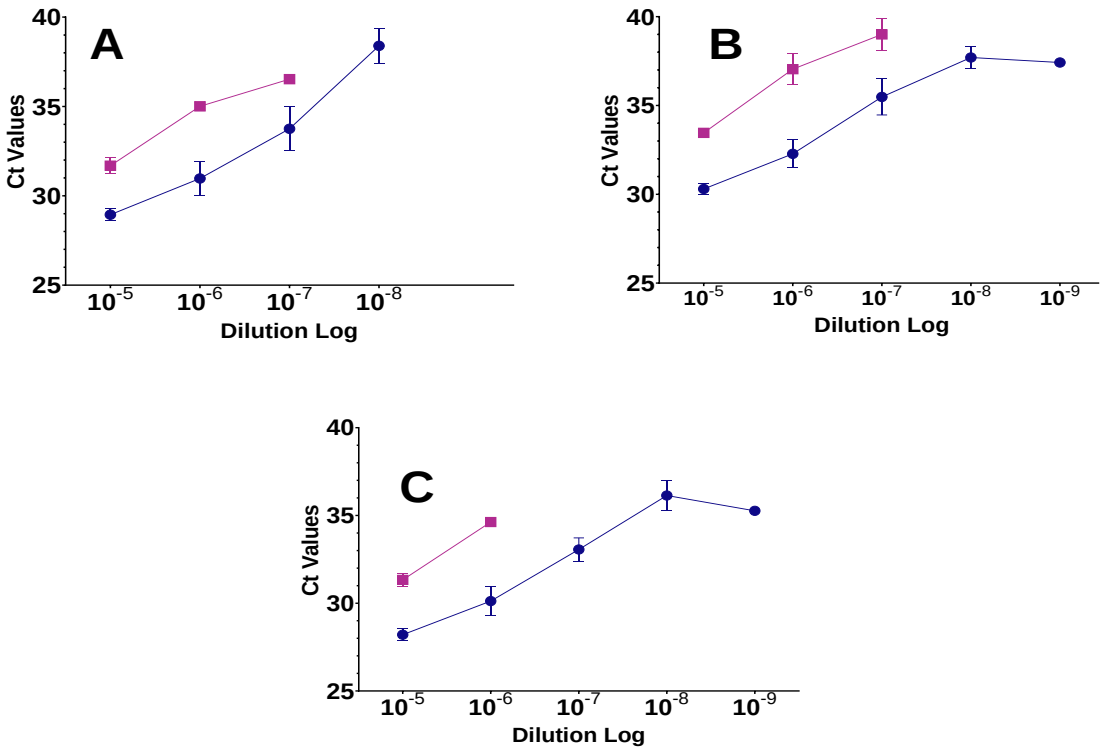

Figure 7.3: Ct values of *S.* Typhi control extracted by Qiagen PFP (dark blue circles) versus Magna Extract (purple squares, Table 7.1 – i) plotted against the serial dilution factor of the 0.5 MacFarland standard density control broth. A shows the values for *ttr*, B for *tviB* and C for *staG*. The LOD from A and B is 10^{-7} and 10^{-6} for *staG* by Magna Extract, whilst the LOD for the Qiagen extraction was 10^{-8} for *ttr* and 10^{-9} for *tviB* and *staG*. However, the extraction volume of Qiagen is 100-fold higher than that of Magna Extract. Error bars represent SEM.

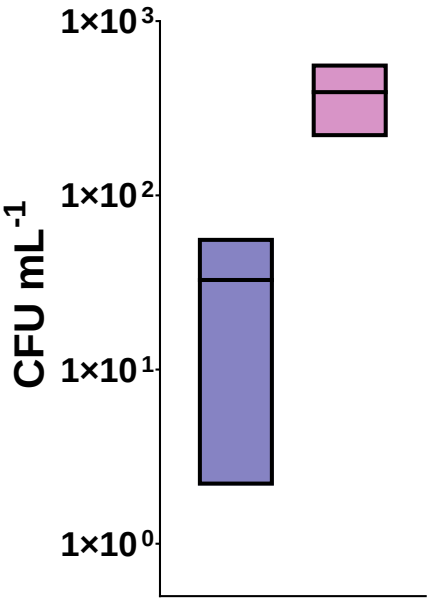

Figure 7.4: Shows the lowest broth dilution at which *S.* Typhi was detected, based on all three PCR targets being positive from Figure 7.3 when CFU mL^{-1} is calculated. Dark purple shows the extraction from the Qiagen PFP kit, and pink from Magna Extract (Table 7.1 – i). The Qiagen is showing the calculated CFU mL^{-1} of the 10^{-8} dilution, 32.65 CFU mL^{-1} (range: 2.16 to 57 CFU mL-1), whilst the Magna Extract is at 10^{-6} dilution, 391.33 CFU mL^{-1} (range: 216 to 570 CFU mL^{-1}).

7.4.1.2 Extraction Volumes and Filtration

The next series of extractions assessed the methods i, ii, iii and iv (Table 7.1). As seen in Figure 7.5, the original method (method i) had the lowest detection at a dilution of 10^{-6}. Similarly, method ii had the lowest overall detection of all the new alternates (ii – iv) at the 10^{-7} dilution extract (*ttr* and *staG*), a $\log_{10}$ higher than the original, but *tviB* was still detected down to the 10^{-9} dilution, equal to the lowest dilution Qiagen detected. Method iii, which used the 20 mL of broth filtered through a 0.45 µm filter, followed by washing the filtrate off, into a 1.5 mL tube, and processed like the original method, showed one $\log_{10}$ higher in the LOD, with *tviB* and *staG* being positive until the 10^{-8} dilution.

The final method, iv, had the lowest detection, with all three replicates giving a positive *S.* Typhi result at 10^{-8}, and one of the replicates being *S.* Typhi positive until the 10^{-9} dilution, despite the calculated CFU mL^{-1} for that broth being 0.6. The LOD of each alternate calculated to CFU mL^{-1} is showing in Figure 7.6, with the average for method i being the same as in Figure 7.3, whilst method ii LOD was 275 CFU mL^{-1} (Range: 39 to 570 CFU mL^{-1}); method iii at 39.13

CFU mL^{-1} (Range: 21.6 to 57 CFU mL^{-1}) and modification D at 20.3 CFU mL^{-1} (Range: 0.6 to 38.8 CFU mL^{-1}).

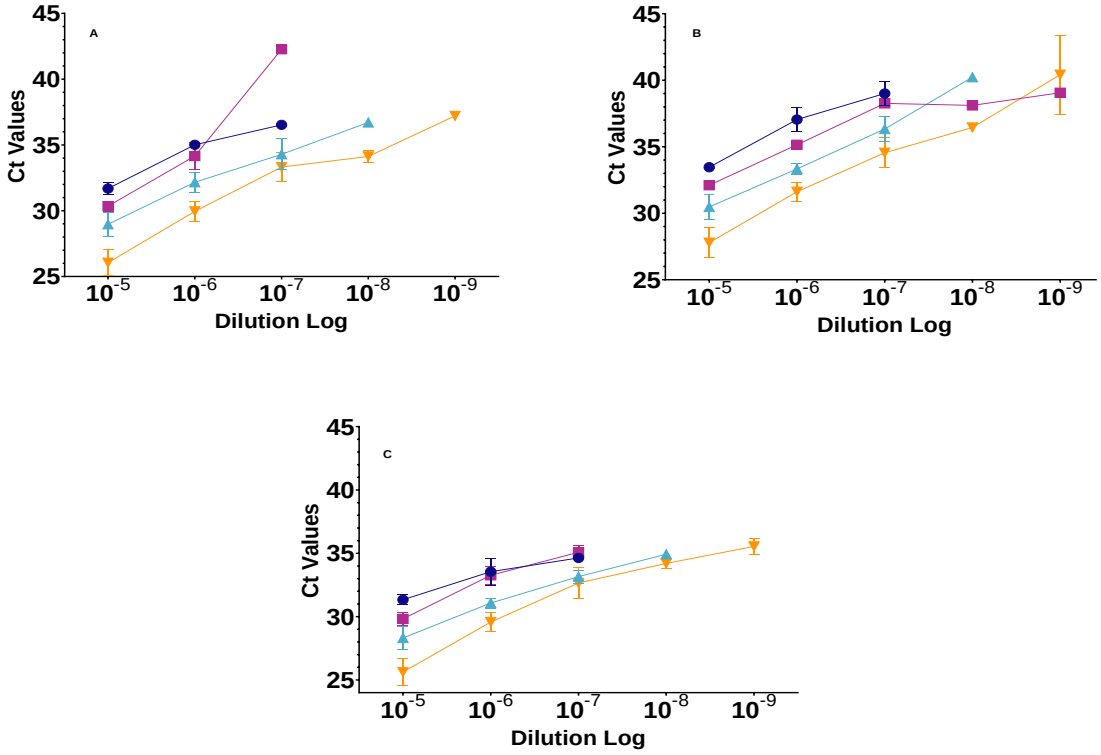

Figure 7.5: Ct values of *S.* Typhi control extracted by modified Magna Extract plotted against the serial dilution factor of the 0.5 MacFarland standard density control broth. The dark blue circles are the standard method (Table 7.1 – i), the purple squares are an increased volume direct method (Table 7.1 – ii), the light blue triangles are 20 mL of sample broth filtered through a 0.45 µm membrane and using RLS as a wash buffer in 200 µL, similar to the original method (Table 7.1 – iii) and the orange inverted triangles are an increased volume version using the membrane filtration method (Table 7.1 – iv). A shows the values for *ttr*, B for *tviB* and C for *staG*. Error bars represent SEM.

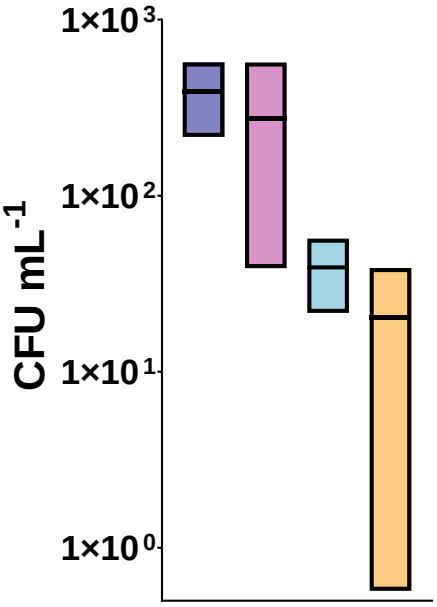

Figure 7.6: Shows the LOD from calculated CFU mL^{-1} in each extracted control at their lowest dilution where *S.* Typhi was correctly identified (positive for all three targets). Dark purple is method i, pink is method ii, light blue is method iii and orange is method iv. Whilst having the largest range between the CFU mL^{-1} of its replicates, D has the lowest detection concentration, and so the highest sensitivity, with light blue being closest based on the mean represented by the bars in the centre of the box.

Using the calculated CFU mL^{-1} from Figure 7.4 and Figure 7.6, the average CFU per extraction was calculated (Table 7.2). This identified that the Magna Extract methods most comparable to the Qiagen kit were iii and iv, which used the sample membrane filtration step, with *S.* Typhi being detected consistently at the 10^{-8} dilution, with a CFU mL^{-1} in the range of 20 to 40 cells. Methods i and ii, which used 1% and 4%, respectively, of the extraction volume compared to the Qiagen kit, or methods iii and iv, gave a minimum detection at the 100-fold and 10-fold dilution broths, with their lowest CFU mL^{-1} detection being only 1 log higher than the extracts using almost 100 × the extraction volume.

Table 7.2: Calculated CFU per extraction for the Qiagen PFP kit and the four Magna Extract Alternates assessed, i to iv

Method	Volume of Sample extracted (µL)	CFU mL^{-1} of lowest dilution *S.* Typhi positive	Lowest dilution with three replicates	Estimated CFU per extraction
Qiagen	20,000	32.65	10^{-8}	653
Method i	200	391.33	10^{-6}	78.3
Method ii	800	275	10^{-7}	220
Method iii	20,000	39.13	10^{-8}	782.6
Method iv	20,000	20.13	10^{-9}	402.6

7.4.1.3 Bead Retriever versus Manual Extraction

Figure 7.7 shows a comparison of the original method (i) of Magna Extract against method iv (500 µL of supernatant after 20 mL culture broth membrane filtered and washed with 800 µL of RLS centrifuged) and vi (500 µL of supernatant after 20 mL culture broth membrane filtered and immersed in 800 µL of RLS with 1 g of lysis beads, vortexed) through both a manual extraction and a semi-automated platform using the Bead Retriever.

The enumeration plates used for the controls extracted for this experiment failed to grow clear, countable colonies, so the exact CFU mL^{-1} could not be calculated. As such, all results below are discussed to their dilution factor from a starting broth that was inoculated to a 0.5 MacFarland standard density control, which is estimated to be the equivalent to approx. 1.5 x 10^8 CFU mL^{-1}.

Here, the original method, i, performs to the dilution factor 10^{-7}, therefore estimating the LOD to be approx. 1.5 x 10^1 CFU mL^{-1}. The original method also gave the highest Ct values of the extractions assessed in this experiment. Method iv, when performed manually, also only performed to the 10^{-7} dilution (approx. 1.5 x 10^1 CFU mL^{-1}) for the identification of *S.* Typhi, due to *tviB*, whilst *ttr* and *staG* was detectable in the 10^{-8} (approx. 1.5 x 10^0 CFU mL^{-1}) dilution. This is a $\log_{10}$ lower than in the previous assessment. The use of the bead retriever gave a consistent detection to the 10^{-9} dilution (approx. 1.5 x 10^{-1} CFU mL^{-1}) for all targets across all three replicates. Figure 7.7 D, E and F show the same sample, except when extracted using method vi, for both manual extraction and the use of the bead retriever. *S.* Typhi is detectable

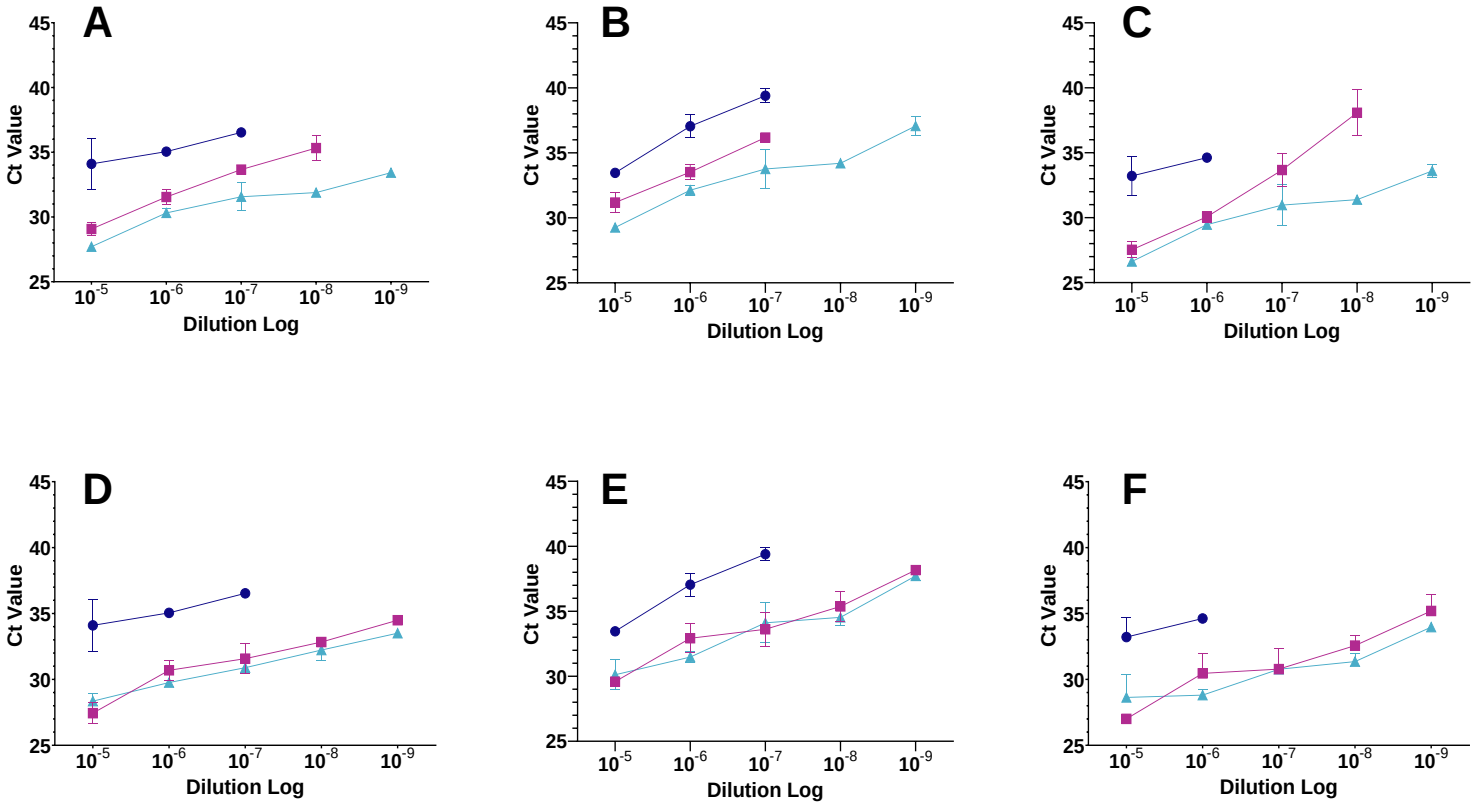

Figure 7.7: Comparison between manual extraction using the Magna Extract method and a semi-automated method with the bead retriever. Additionally, this figure compares the use of a washed membrane to one that was bead beaten using 0.1mm glass lysis beads like the first step of a Qiagen PFP kit. In all six figures, the dark blue circles is the extraction using the original method (Table 7.1 – i), the purple squares in A, B and C depict the manual method and light blue triangles represent the bead retriever method using 500 µL of washed membrane eluate (Table 7.1 – iv); whilst the purple squares in D, E and F are the manual method and the light blue triangles are the bead retriever method using 500 µL from a membrane vortexed in ceramic beads with 500 µL of wash solution (Table 7.1 – vi). A and D are the results for the *ttr* target, B and E for the *tviB* target and C and F for the *staG* target. Error bars represent SEM.

to the 10^{-9} dilution (approx. 1.5 x 10^{-1} CFU mL^{-1}), with all Ct values being lower than 35 for *ttr* and *staG*, though only <38 for *tviB*. The amplification at a calculated approx. 1.5 x 10^{-1} CFU mL^{-1}, however, implies the concentration of the starting culture to likely be at least 1 log_{10} higher than expected. Figure 7.8 shows the results of the bead retriever cross-contamination assessment. Whilst all positive controls gave successful amplification, demonstrating the utility of the platform, 12 of the 15 negative controls also amplified targets for *S.* Typhi, showing widespread contamination during the operation of the robot.

In summary, whilst the samples that were extracted using the bead retriever allowed for *S.* Typhi detection at the lowest concentration, it does suffer from cross-contamination. The use of bead lysis on the membranes, rather than washing them, also gives a more sensitive LOD.

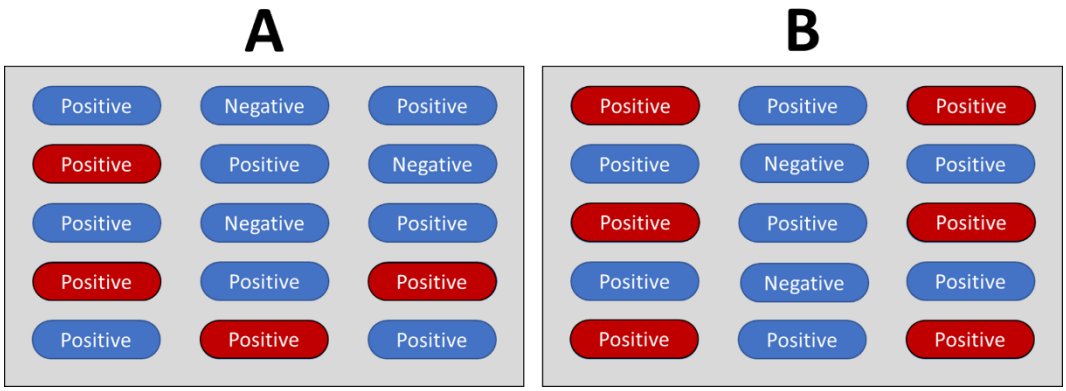

Figure 7.8: Shows the results of the cross-contamination assessment of the bead retriever. Wells highlighted in red are NTCs that amplified with positive *S.* Typhi DNA, indicating a contamination during the purification process.

7.4.1.4 Limit of Detection and spiked broth culture

To determine the LOD of this novel extraction and purification method, 12 replicates of the control were extracted. Figure 7.9 shows the plots of Ct values to dilution factor for methods i (dark blue circles), v (purple squares) and vi (light blue triangles). The average Ct is plotted with the bars representing the range of results. When the pure control strain in $bile^-$ was used for extraction (Figure 7.9 A); Ct values were consistent across replicates, with positive amplification to the 10^{-8} dilution. However, not all replicates were positive, even at the 10^{-5} dilution, and all 10^{-9} dilutions were negative; the percentage of broths positive at each dilution is shown in Appendix x.

When combining a triple positive required for an *S.* Typhi positive result, only half of the replicates were positive at the 10^{-5}, 10^{-6} and 10^{-7} dilutions, whilst all three methods showed less than half were *S.* Typhi positive at 10^{-8} (i = 42%, v = 33% and vi = 42% of the 12 replicates were positive for *S.* Typhi at this dilution factor).

Figure 7.9 B show the average Ct values of the samples when diluted in the spiked bile^{-} broth. Whilst the lowest dilution *S.* Typhi was detected at remained the same for methods i and vi, the lowest dilution detected for v was 10^{-7}; however, the percentage of the 12 replicate broths that were positive at those dilutions was much lower, with only two broths positive using method i and v each. At dilution 10^{-7}, four broths were positive with i, three were positive using vi, and only one was positive with v. Unlike, when challenged with the pure strain of *S.* Typhi, however, which had a 100% positivity at the 10^{-4} dilution, before detection dropped dramatically (i = 58%, v = 75% and vi = 50%), method i had positive *S.* Typhi genes across all 12 broths at the 10^{-5} dilution, compared to a dramatic drop in performance by methods v and vi at 17%, with two broths positive.

Whilst detection at the lower concentrations using method i was not as good in the spiked sample compared to the pure sample (17% versus 42% respectively), method i performed better at each dilution compared to the other two methods in these two experiments. As such, the spiked extracts were diluted 1:10 and the PCR was repeated to see if inhibitors or too much DNA was causing the drop in detection.

Figure 7.9 C are the results from the diluted samples, a standard practice for extracts with potential inhibitors. In doing so, the Ct values of the broths that were positive were more consistent, as seen in Figure 7.9, and caused an increase in the percentage of replicates positive for methods v and vi, compared to the undiluted extracts. Here, at the lowest detected dilution, 10^{-8}, method i increased from 17% to 67%, method v increased from 0% to 67% and method vi increased from 17% to 50%. The performance of method v matched that of method i, with both performing better than method vi at the lower dilutions.

In summary, the original Magna Extract method, which had inferior performance to that of the Qiagen kit originally, performed best with the undiluted samples here, potentially due to lower levels of inhibitors in the final product. Method v performed similarly to method i in both the pure and diluted samples but performed worst of all three methods when challenged with spiked river water and the extracts used undiluted. Method vi seemed to perform the least well of all three methods reviewed under all three circumstances.

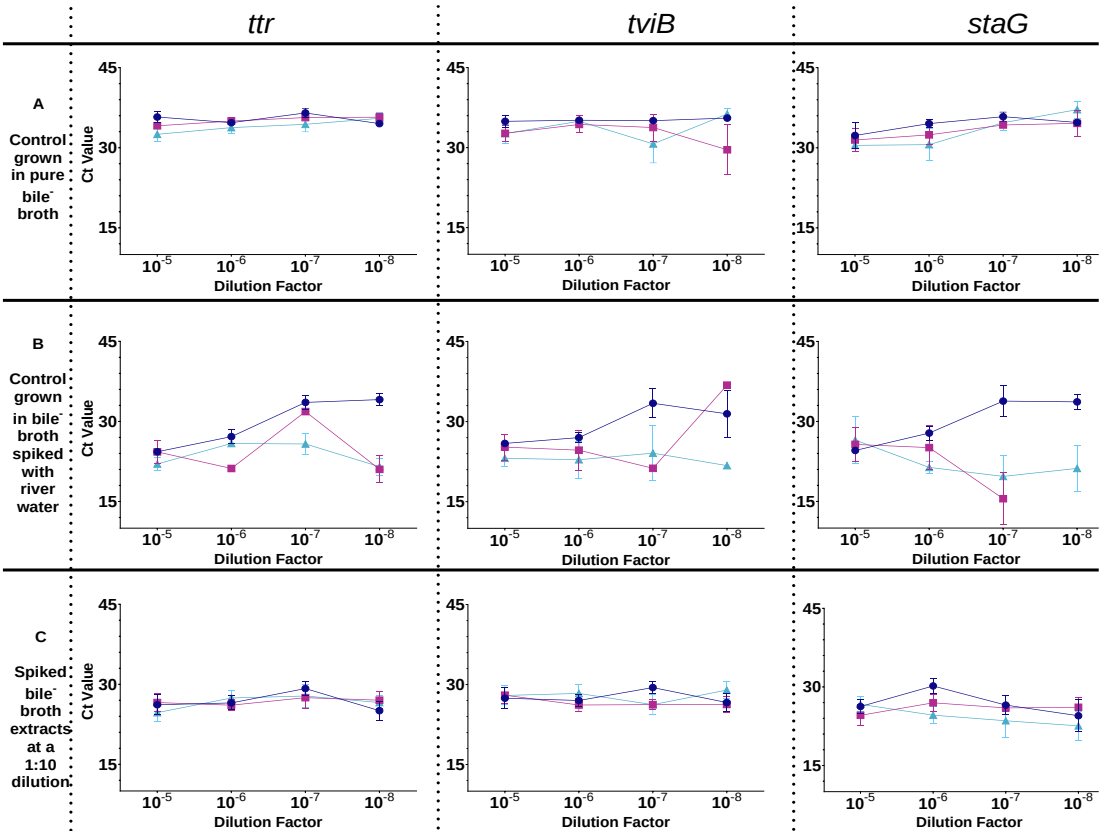

Figure 7.9: Shows the average Ct values, with error bars representing the range, of Magna Extract using the three alternates selected to compare for true LOD analysis. Each sample was performed in 12 replicates. Dark blue circles represent the original method (Table 7.1 – i), purple squares were method v (Table 7.1), and the light blue triangles was method vi (Table 7.1). A, B and C in rows show the type of control challenge, described on the graph, whilst columns divided by primer target. Ct values show detection across the different methods to a 10^{-8} dilution factor, however, not all 12 replicates were positive – the percentage positivity is shown in Appendix 10.6.1. Error bars represent the SEM.

7.4.2 Tetrathionate broth

When comparing tetrathionate broth, as a secondary culture step, against the current method of selenite F, Figure 7.10 demonstrates that selenite F performs best overall. The dilution of the inoculum to 10^{-5} (average: 3.81 x 10^{3} CFU mL^{-1} in, 2.94 x 10^{8} and 1.67 x 10^{5} CFU mL^{-1} out) and 10^{-6} (average: 3.81 x 10^{2} CFU mL^{-1} in, 7.66E x 10^{7} and 1.38E x 10^{6} CFU mL^{-1} out) for both pure and spiked control strains, as well as 10^{-7} (average: 3.81 x 10^{1} CFU mL^{-1} in, 7.5 x 10^{5} CFU mL^{-1} out) and 10^{-8} (average: 3.81 x 10^{0} CFU mL^{-1} in, 1.65 x 10^{5} CFU mL^{-1} out) for the pure strains only; allowed for a much larger increase in growth. Whilst selenite F supported the isolation of *S.* Typhi at all four plotted concentrations of the spiked bile^{-}, tetrathionate appeared to have a much larger increase at 10^{-7} (2.5 x 10^{5} versus 2.04 x 10^{7} CFU mL^{-1}) and 10^{-8} (2.75 x 10^{3} versus 1.93 x 10^{9} CFU mL^{-1}) respectively.

The higher concentration of tetrathionate broth positive isolates was from the lower dilution plates from the Miles, Misra and Irwin method (Miles *et al.*, 1938), where one colony has a large effect on the final calculation. The negative growth seen in tetrathionate from the spiked broth at 10^{-5} and 10^{-6} inoculums was due to overgrowth of competitive organisms, as such, no *S.* Typhi were isolated with this method, despite the control strain concentrations being higher.

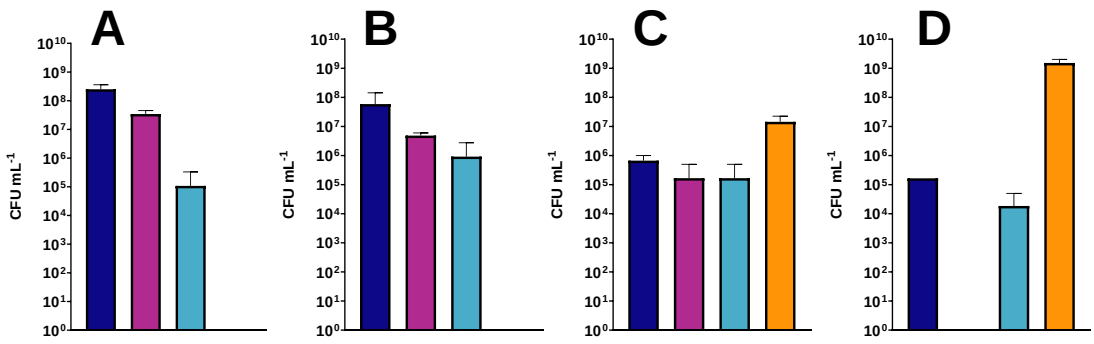

Figure 7.10: Shows the mean, with upper range due to triplicate data in Log_{10}, change in CFU mL^{-1} of each broth between the control in bile^{-} used for inoculation and after incubation in tetrathionate broth. Dark blue is the pure control strain in selenite F broth, purple is the pure control strain in tetrathionate broth, light blue is the control strain diluted in bile^{-} containing cultured river water with selenite F broth, and orange is the control strain diluted in bile^{-} containing cultured river water with tetrathionate broth. A is using a starting culture diluted to 10^{-5}, B using the dilution 10^{-6}, C is the dilution 10^{-7} and D is the dilution 10^{-8}. Error bar shows the range of CFU mL^{-1} across replicates.

7.4.3 Antimicrobials in Selenite F broth

7.4.3.1 Chloramphenicol

Figure 7.11 compares the growth of *S.* Typhi control strain 21 in the unmodified selenite F broth to that with the addition of 8 µg mL^{-1} of chloramphenicol from the inoculum in pure $bile^-$ (dark blue and purple columns) and $bile^-$ spiked with river water (light blue and orange columns).

The Selenite F with chloramphenicol was significantly outperformed by unmodified selenite F when challenged with pure culture from $bile^-$ broth at the 10^{-8} (average 0.75 CFU mL^{-1}) dilution, with enumeration done selenite F giving a calculated CFU mL^{-1} of 3.3 x 10^5, whilst there were no countable colonies on the pure control plates for the chloramphenicol broths. The unmodified and chloramphenicol selenite F broth had similar performances at dilutions 10^{-5} (7.5 x 10^2 CFU mL^{-1}) and 10^{-6} (7.5 x 10^3 CFU mL^{-1}), with the unmodified growing to 1.00 x 10^8 CFU mL^{-1}; the chloramphenicol to 1.64 x 10^8 CFU mL^{-1}, and the unmodified to 1.52 x 10^6 CFU mL^{-1} versus the chloramphenicol to 6.75 x 10^5 CFU mL^{-1} respectively. At the 10^{-7} (7.5 CFU mL^{-1}) dilution there was an almost 10-fold difference between the calculated CFU mL^{-1} for the unmodified broth (1.83 x 10^6 CFU mL^{-1}) and the one with the chloramphenicol additive (3.3 x 10^5 CFU mL^{-1}).

However, when challenged with the control in $bile^-$ with spiked river water, the chloramphenicol broth appears to perform better overall than the unmodified selenite F. At the 10^{-5} dilution step, no *Salmonella* spp. colonies could be counted due to overwhelming non-target organisms growing on the enumeration mCASE plates used, whilst, across the enumeration dilutions, almost 50 isolates with *Salmonella* spp. morphology could be identified for the chloramphenicol broth, giving a concentration of 1.73 x 10^9 CFU mL^{-1}. At the lowest dilution, 10^{-8}, which had an average inoculum of 0.75 CFU mL^{-1} across the three replicates, no bacteria grew on the enumeration plates for the unmodified selenite F whilst the broth with chloramphenicol allowed for one colony to grow, which gave a calculated 1.65 x 10^7 CFU mL^{-1}; however, due to being performed only in triplicate, the result is unreliable. With six colonies across the enumeration plates for the chloramphenicol broth, and only one for the unmodified selenite F broth at the inoculum dilution 10^{-7}, it appears that chloramphenicol outperforms unmodified selenite F (3.50 x 10^8 CFU mL^{-1} versus 1.65E x 10^7 CFU mL^{-1}); however, the enumeration plates used for the unmodified selenite F broth up to 10^{-5} were too contaminated to count *Salmonella* spp. colonies, whilst the amount of

contaminants seen on the chloramphenicol plate disappeared at the 10^{-4} dilution plate showing an increase in inhibition of all organisms.

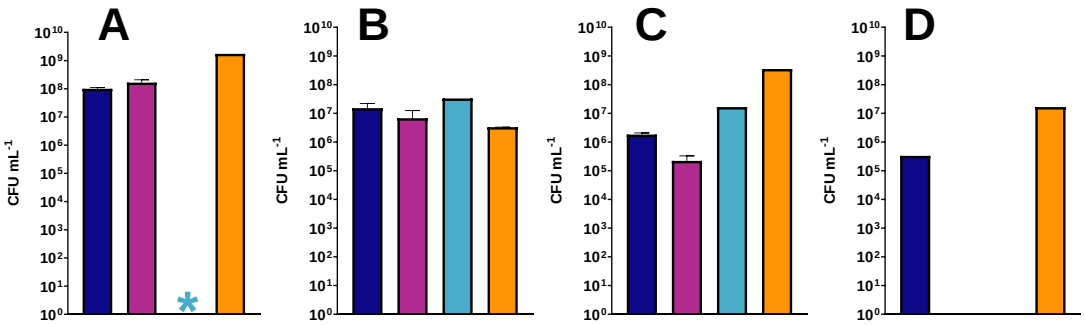

Figure 7.11: Shows the mean, with upper range due to triplicate data in Log_{10}, change in CFU mL^{-1} of each broth between the control in bile$^-$ used for inoculation and after incubation in chloramphenicol in selenite F broth. Dark blue is the pure control strain in selenite F broth, purple is the pure control strain in chloramphenicol in selenite F broth, light blue is the control strain diluted in bile$^-$ containing cultured river water with selenite F broth, and orange is the control strain diluted in bile$^-$ containing cultured river water with chloramphenicol in selenite F broth. A is using a starting culture diluted to 10^{-5}, B using the dilution 10^{-6}, C is the dilution 10^{-7} and D is the dilution 10^{-8}. * Overgrowth of contaminants prevented colony counting for this experiment. Error bar shows the range of CFU mL^{-1} across replicates.

7.4.3.2 Ampicillin

Figure 7.12 compares the growth of *S.* Typhi control strain 21 in the unmodified selenite F broth to that with the addition of 8 µg mL^{-1} of ampicillin from the inoculum in pure bile$^-$ (dark blue and purple columns) and bile$^-$ spiked with river water (light blue and orange columns).

The results of selenite F with ampicillin when challenged with the pure strain of *S.* Typhi, reflect that of chloramphenicol above, where the unmodified selenite F broth performed best, particularly at dilution 10^{-8}, where no *S.* Typhi were cultured from the selenite F with ampicillin after inoculation with pure *S.* Typhi. Inoculum dilutions 10^{-5} (7.5×10^2 CFU mL^{-1}) gave a calculated CFU mL^{-1} of 1×10^8 for the unmodified selenite F compared to 1.6×10^7 for the ampicillin broth. For the inoculum dilution, 10^{-6} (7.5×10^3 CFU mL^{-1}), , and 1.52×10^7 for unmodified and 1.74×10^6 for the ampicillin respectively. The last pure *S.* Typhi control challenge, 10^{-7} (3.62 CFU mL^{-1}), allowed growth to 1.83×10^6 CFU mL^{-1} for the unmodified, and 1.65×10^5 CFU mL^{-1} for the ampicillin.

When challenged with an *S.* Typhi inoculum diluted in bile$^-$ broth spiked with river water, ampicillin allowed for colonies with *Salmonella* spp. morphology to be isolated and counted, whilst the unmodified selenite F it was compared to is the same as the one above for chloramphenicol, and as such, was too contaminated to allow *S.* Typhi colonies to be counted. Whilst the calculated CFU mL^{-1} for the ampicillin broth appear high in Figure 7.12, these are due to very low colony counts at a high dilution, due to the enumeration plate dilutions being too contaminated to count to a much higher dilution than that of unmodified selenite F or chloramphenicol, with the inoculum dilutions 10^{-5} and 10^{-6} having one colony at enumeration dilution 10^{-7} and 10^{-6} respectively. Whilst the inoculum dilution 10^{-7} and 10^{-8} allowed five and two colonies, respectively, after culture in ampicillin. This shows, that whilst Figure 7.12 implies ampicillin selenite F performed better than the unmodified selenite F when challenged with cultured river water, it is performing less well than that of the chloramphenicol broth.

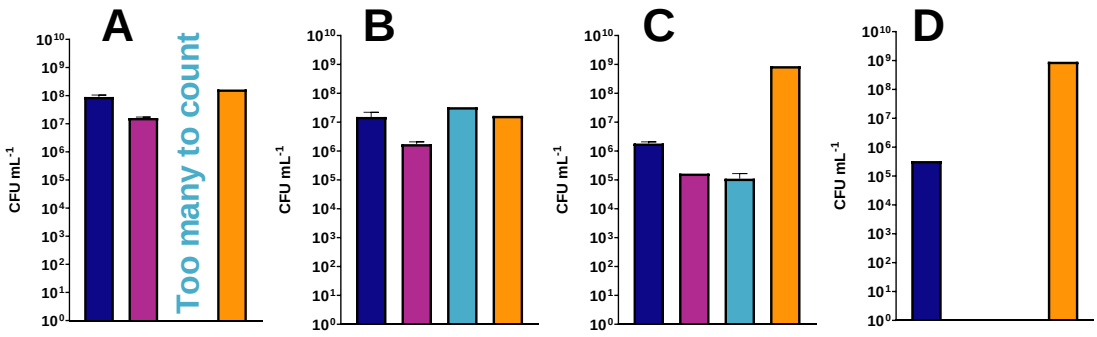

Figure 7.12: Shows the mean, with upper range due to triplicate data in Log_{10}, change in CFU mL^{-1} of each broth between the control in bile$^-$ used for inoculation and after incubation in ampicillin in selenite F broth. Dark blue is the pure control strain in selenite F broth, purple is the pure control strain in ampicillin in selenite F broth, light blue is the control strain diluted in bile$^-$ containing cultured river water with selenite F broth, and orange is the control strain diluted in bile$^-$ containing cultured river water with ampicillin in selenite F broth. A is using a starting culture diluted to 10^{-5}, B using the dilution 10^{-6}, C is the dilution 10^{-7} and D is the dilution 10^{-8}. Error bar shows the range of CFU mL^{-1} across replicates.

Across tetrathionate, and selenite F with ampicillin and chloramphenicol, the control inoculum at 10^{-4} dilution gave growth that was too densely cultured to count, whilst 10^{-9} was negative from the original bile$^-$ inoculum, leaving no *S.* Typhi to be cultured in the secondary broth, as such, none of these comparisons plot either of these concentrations, despite being tested.

7.4.3.3 Pimaricin/Natamycin

Whilst this broth combination was attempted, no growth was seen in the selenite F containing pimaricin 2 µg mL^{-1} for the pure control, nor on the plates from the enumeration. When plated, the only organisms that grew were spiked non-target organisms, with no evidence of *Salmonella* spp.

7.4.3.4 Cycloheximide

As seen in Figure 7.13, when 5 µg mL^{-1} cycloheximide is added to the selenite F broth, *S.* Typhi appears to be inhibited at all concentrations using the pure strain, and in all but 10^{-5} dilution of the spiked bile$^-$ broth. However, this CFU mL^{-1} was derived from a very low number of colonies at a low dilution value (eight colonies counted at the enumeration dilution of 10^{-4}, and two colonies at 10^{-5}) of one replicate.

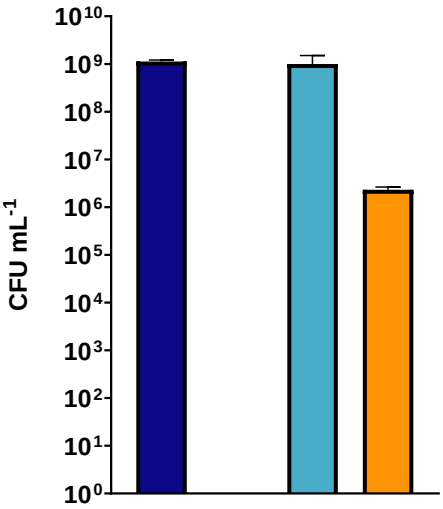

Figure 7.13: Shows the mean, with upper range due to triplicate data in Log_{10}, change in CFU mL^{-1} of each broth between the control in bile$^-$ used for inoculation and after incubation in cycloheximide in selenite F broth. Dark blue is the pure control strain in selenite F broth, purple is the pure control strain in cycloheximide in selenite F broth, light blue is the control strain diluted in bile$^-$ containing cultured river water with selenite F broth, and orange is the control strain diluted in bile$^-$ containing cultured river water with cycloheximide in selenite F broth. This graph is the results of the control dilution at 10^{-5} only. Error bar shows the range of CFU mL^{-1} across replicates.

7.5 Discussion

7.5.1 Comparing Magna Extract to commercial extraction for sample screening

The initial assessment of Magna Extract from Chapter 4 showed promise in the method with the broths used during routine ES culture methods (bile$^-$ broth), in addition to other culture broths commonly used for *S.* Typhi. These included BPW, Luria broth and tryptone-soya broth, however selenite-based media were not trialled as Magna Extract relies on thermal lysis of the cells before purification, and bi-selenite vapours are toxic. This early work did show that the culture step was vital for successful extraction through this method, as the diluted inoculums did not amplify when screened.

As the screening step utilising this extraction method was planned to be integrated into the current ES protocol, the method discussed in this chapter exclusively used the bile$^-$ broth, so inhibition caused by the media in-use would be accounted for.

Initially, the original method appeared to be less sensitive to the current in-use Qiagen kit, so volume modifications to the Magna extract method to make the input volume of the two methods comparable were explored. When used to extract from pure culture, each of the methods appeared to be more sensitive than the original method (i), with methods ii, iv, v and vi (which used 20 mL broth via a filtration membrane) performing best of the replicates. When the samples were filtered and membranes bead beaten with glass lysis beads (v and vi), in the same way that samples are processed prior to extraction by the Qiagen kit, it gave non-inferior results.

However, when these methods were challenged using a spiked broth, to simulate a natural sample; the reliability of the methods using higher input volumes of samples did not perform as well as the original method. This will be due to inhibitors present in the mixed culture which may not be fully purified from the samples due to the high quantity present in the sample binding to the tubes used for the extraction, or due to the non-specific binding nature of the AmpPure beads. The lower starter volume of the original extraction method may be lower in sensitivity, with reduced chances of containing the target organism due to the 100-fold lower volume, but does prevent the final extract from being overwhelmed by inhibitor (Jansson and Hedman, 2019). This being the potential cause of reduced sensitivity with complex samples is supported by the increased sensitivity when those extracts were diluted, as would be done routinely for extracted samples with potential inhibitors such as wastewater or faecal samples. The use of glass lysis beads on the membranes for the broth

culture increased sensitivity with the pure control strain, but was affected by the same phenomenon described above, overloading the assay with non-target DNA.

The bead retriever would have allowed for a larger number of samples to be extracted at once, consistently, as the run time for to perform the bead purification on 15 samples was similar to me doing five samples manually using a magnetic rack for bead transfer. However, due to the cross-contamination, the bead retriever is not a viable option, as the reduction in samples recommended to prevent the contamination would leave semi-automated extraction as the slower option than full manual.

Despite this, the Bead Retriever gave better, more consistent results than manual extraction. This result must be questioned due to the cross-contamination identified in this method that could have potentially skewed the apparent efficacy. Despite this concern, Figure 7.7 does show a gradual increase in Ct values at each lowering dilution, suggesting that the control strains were not as adversely affected as those in the cross-contamination assessment. Whilst this was what was available to trial at the time, MLW has recently purchased a Thermofisher Kingfisher Apex DNA purification platform that utilises magnetic beads and programmable. This platform, designed specifically to handle DNA and RNA, should allow for the Magna Extract method to be semi-automated in a consistent and reliable way, without the concern for cross-contamination.

The LOD could not be determined due to the number of replicates positive at each dilution was not sufficient for a probit calculation, which was previously used in Chapter 4 to determine an LOD_{50} and LOD_{95}. When compared with pure strain *S.* Typhi, the most sensitive methods were vi and iv (10^{-9} dilution, or 10^{0} CFU mL^{-1}), whilst the least sensitive was method i and ii (10^{-7} dilution, or 10^{2} CFU mL^{-1}). Yet, when challenged with spiked broths, method i and v performed best.

As such, whilst I think there is more work that should be done here, such as the inclusion of an internal positive control and repeating the assay sufficiently to calculate the true LOD, I would have recommended the use of Magna Extract utilising two methods in parallel, i and v, in addition to diluting extracts from method v 1:10, which is similar to some commonly used protocols for PCR from complex matrices such as wastewater and stool samples. However, this would be time consuming as each sample would be extracted twice, with the PCR performed in triplicate. Whilst the costs of the extraction and reagents would still be significantly lower than that of the BMGF method using the Qiagen PFP Kit, method i works

best with higher background flora and v works best with low background therefore, it would be more appropriate to determine a site-specific method based on TDS or some other metric, to select which extraction method. For example, I would recommend the use of method i for any sample received from the Manase sewage plant.

7.5.2 Improvements to the culture pathway

The broth alternatives assessed here did not give the same performance as the current method, utilising selenite F broth as the secondary media, but may have utility as a parallel broth when samples are reduced by pre-screening. Tetrathionate broth, selenite F with chloramphenicol and with ampicillin performed poorly when compared to the unmodified selenite F broths in almost all dilutions except the lower concentrated, spiked broths (10^{-7} and 10^{-8}). However, due to the calculation being made from such a low concentration, the effect of even a single colony would have a huge impact on the apparent CFU mL^{-1} using this method. To make the assessment more accurate, more rigorous testing with multiple replicates (≥10) would be needed as used for the pathways when tested for the original method discussed in Chapter 3.

Despite both being antifungals, pimaricin (also known as natamycin), and cycloheximide appeared to have a detrimental effect on the growth of *S.* Typhi when added to selenite F broth, which is a "harsh" media to begin with. This could be due to the antifungal reacting with, or having a synergistic effect with selenite-based media, or due to the inclusion of ethanol as the solvent. However, if it was due to the latter, the chloramphenicol would have been similarly inhibitory across the board – additionally, some older medias recommended the addition of ethanol (Wilson and Blair, 1931, Gell *et al*., 1945) for the selection of *S.* Typhi.

As with the antimicrobials and tetrathionate, more replicates would be necessary to fully assess these broths, however, at the concentration chosen, these two agents do not facilitate growth for the target organism and would initially be disregarded in favour of other antimicrobials. Repeating the experiment with different concentrations of each antimicrobial and antifungal would allow for the correct concentration to inhibit background flora but not negatively affect the target organism.

7.5.3 Limitations

One of the major limitations of this work was the lack of suitable replicates for the Magna Extract, and lack of enough replicates for the culture alternatives, therefore these samples

have not had sufficient testing to make a fair assessment, only an indication of their utility. Similarly, the antimicrobials added were done at their breakpoints for *Salmonella* spp., on the assumption clinically relevant strains of *S.* Typhi circulating in Malawi would be MDR, as these are what is seen in patients. Had time allowed, I would have preferred to do multiple broths at a variety of concentrations for the additives to determine the best balance for selectivity and enrichment, particularly due to the natural harshness of selenite-based media.

Finally, the last limitation I considered was that I used CFU mL^{-1} for the LOD and sensitivity values for the PCR, whilst the use of genome copies would have been more appropriate to allow fair comparison between the spiked and pure culture broths.

7.5.4 Future work

As an extension of the methods reviewed for this study, much of the work in this chapter is discussed further as part of the conclusion in Chapter 8. The areas of future work for this chapter would be repeating the Magna Extract replicates to determine the LOD appropriately; trial the Thermofisher Kingfisher Apex bead purification platform and run the two extraction methods (Qiagen and Magna Extract) in parallel with real samples from the field in Blantyre, Malawi. For the broth culture alternatives, reviewing the concentrations of added antimicrobial solutions and running sufficient replicates to fairly compare these to my currently implemented method.

7.5.5 Conclusion

Here I demonstrate that the Magna Extract DNA extraction method shows potential to be effective in supporting DNA extraction for PCR reactions the are required to detect target at very low concentrations, despite issues with DNA overwhelming the assay. This issue with too much DNA can be accounted for by performing the PCR in duplicate with dilutions. Therefore, this method shows promise for use in ES, due to its low cost and being a quick and easy protocol to perform.

Dependant on sample size and positive predictive value, I would use both methods in parallel for a period, screening all incoming samples with the remaining Qiagen kits, and the Magna Extract to ensure all positives detected by the Qiagen kit would also be detected by Magna Extract, to ensure no loss in sensitivity. In lieu of time or funding to perform Magna Extract and Qiagen side by side, I would recommend the use of method i and v, ensuring method v is diluted 1:10 before use. This latter step would be especially important on any samples that

are visibly more sedimented, as these tend to have the highest concentration of non-*Salmonella* cultures, and would be a prime example of when an effective screening method could help inform when more aggressive isolation methods may be beneficial.

The pilot work on secondary broths require more work to determine the most effective concentration of antimicrobials to be added to a secondary broth for use, post screening. If the screening tool is effective in reducing the number of samples being cultured, then all broths could be assessed in parallel to determine their value with natural samples.

8 Future work and Conclusions

8.1 Summary

Environmental Surveillance has the potential to be a useful tool in understanding circulating infectious diseases within a community. This project aimed to create a novel toolset for the detection and isolation of *S.* Typhi from the environment, and subsequently, determine whether that toolset had viability in an endemic setting by performing a year-long surveillance programmed in Blantyre, Malawi.

Starting with historical and contemporary culture methods used for clinical *S*. Typhi and environmental *Salmonella* spp.; selection of the most appropriate media for use *in situ* was made and combined into culture pathways. The pathway that showed the most promise was the use of a primary 2% bile broth and secondary Selenite F broth, followed by isolation on mCASE agar. To confirm the isolates as *S*. Typhi, a real time PCR assay was adopted.

With the culture and PCR methods selected, a pilot study was performed for six months in Blantyre, Malawi, taking samples of water, sediment, food, biofilms and Moore swabs from areas in the city with known high burden of typhoid, to determine the most appropriate sample collection. During this period six isolates of *S*. Typhi were cultured: one from water; two from biofilms; three from Moore swabs. A further 377 NTS were isolated, 16 of which were PCR positive for *staG*, necessitating the use of biochemistry and serology to confirm the isolates were not *S*. Typhi.

Subsequently, a one-year, Blantyre-wide, ES programme was undertaken in collaboration with a team at Imperial. In this project, I used a laboratory method proposed by the University of Washington in parallel to my culture method. This method involved the extraction of filter membranes for PCR detection only, either direct from water samples, or after Moore swabs were cultured in UPE broth. New sites were identified by geo-spatial modelling based on river catchment areas and populations served (Uzzell *et al*., 2021); with collections spanning from April 2021 to May 2022. During this period 4,117 samples were collected; 33 samples were identified as *S.* Typhi positive by PCR, whilst two isolates were cultured from samples.

The results from the one-year surveillance showed disparity in positivity between the PCR only method and culture. As such, the pathway was revisited in an attempt to improve it, primarily by comparing a low-cost alternative to the Qiagen PFP extraction, Magna Extract

(Byrne *et al*., 2022). This appeared to be a comparable method after some modifications but requires further work for validation. Similarly, alternative media were screened, including the addition of antimicrobials to the culture broths, however, these appeared to not be as effective as the method already in place, and likely needs to be further evaluated with varying concentrations of antimicrobial.

8.2 Future work

8.2.1 Further Environmental Surveillance

Firstly, we need to repeat the environmental surveillance during a normal typhoid season; there is evidence of an increase in *S.* Typhi cases reported in QECH starting 2022 following the apparent return to normal health-seeking behaviour and hand hygiene after the COVID-19 pandemic. Indeed, two or three years of sustained ES will be ideal in order to explore seasonality and whether other metrics, such as time of day, temperature, pH or phosphate levels would have a verifiable effect on detection or recovery.

8.2.2 Field sampling improvements

Field sampling improvements should also be considered, such as an evaluation of the efficacy of a Moore swab when made from different materials and sizes. One experience during my time on this study was when I went to Kumasi, Ghana, to assist in training the group setting up an ES study in Agogo, a collaborative group from the University of Washington had been using Moore swabs made of large horse bandages that were over a metre in size once folded, significantly larger than the ones used in this study. Whilst there appeared to be no difference in performance during the training session, a more rigorous comparison of parameters for Moore swab construction could be beneficial to optimise their performance. Additionally, a question often raised is what period the swab should be deployed, and whether there is a method to determine saturation and the concentrative performance of the swabs. As such, I would propose an experiment that used a sealed, circulating system, for example in Figure 8.1, where swabs can be accessed easily so that several swabs can be added and removed over time both before and after inoculation of the circulating system. This would allow swabs to be saturated prior to inoculation to determine any adverse effects on detection, in addition to leaving swabs in the system for longer periods from 12 up to 168 hours to determine whether longer exposure would positively or negatively impact recovery.

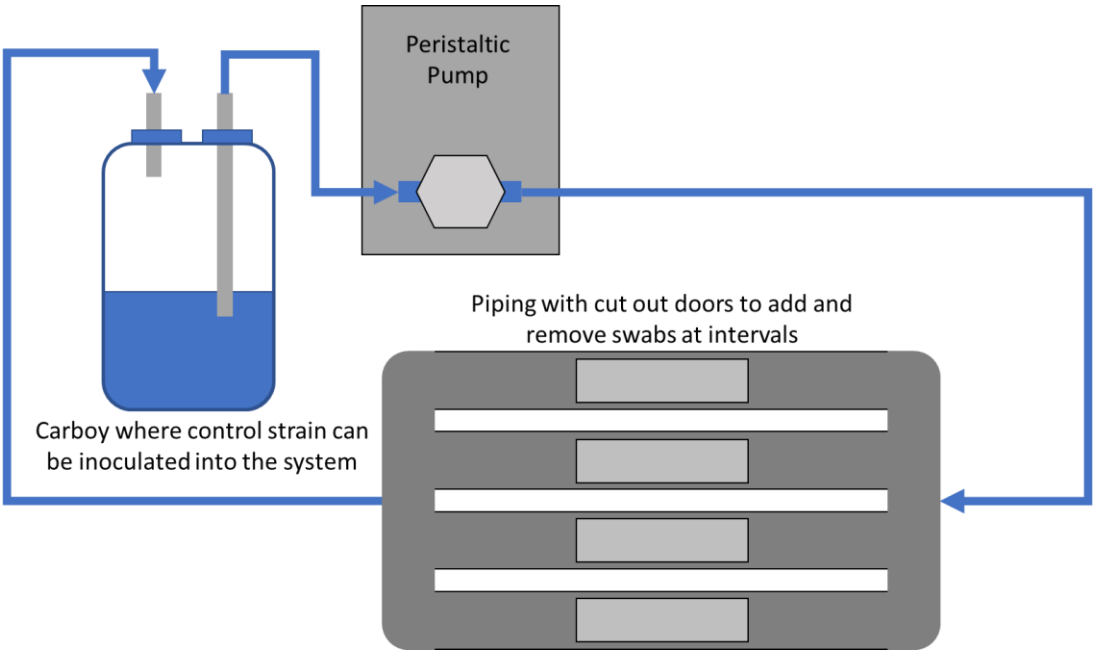

Figure 8.1: Example of a system to validate swabs for deployment periods by allowing the ease of access to the swabs in a parallel piping system under constant flow with a liquid transfer pump.

Alternative sampling methods would also be of interest. One area that was explored early on in this study but abandoned was the idea of a microfluidics sampling method, or a tool with bound antigens to stick to *Salmonella* cells as they pass through a device to ensure capture of the target organism. We have also now purchased an automated sewage sampler, the Liquiport 2010 csp44, which is a large peristaltic pump that will take samples of a predetermined volume at predetermined intervals. This will be deployed in a manhole between MLW and QECH and may offer an insight into what organisms and antimicrobials are exiting the hospital currently and may function as a "gold standard" to compare trap and grab samples too.

The defunct sewage plant sampled at present gives the highest number of positives, unsurprisingly, as these are highly contaminated/concentrated samples of human waste, giving an indication of how much more sensitive raw sewage is to that of river water. Even though there is a paucity of sewerage infrastructure, future work of interest would be to identify where human waste concentrates in Blantyre i.e., institutes with large septic tanks that receive inputs from large numbers of people.

8.2.3 Ongoing enhancements to the laboratory pathway

More work for both the novel DNA extraction method and real time PCR screening are required, as well as the alternative, parallel culture broths. Magna Extract has the potential to be effective at very low DNA concentrations, despite issues with inhibitors overwhelming the assay when large volumes were extracted. Work to determine the LOD_{50} and LOD_{95} of the Magna Extract method would help assessments for adoption both within our group and to promote the method outside of it, as it would provide quantitative data on its use for environmental *S*. Typhi that would be comparable to the performance of previously validated commercial extraction kits. Additionally, the method must be tested using an internal or sample processing control, such as the Eurogentec one used in the one-year surveillance for direct PCR extracts, as this would help guide solutions when the extracts are too heavily inhibited and ensure successful extraction and PCR reactions have been performed.

Once optimised, this extraction method could then be implemented in ES as a low-cost alternative to the commercial kits. Ideally, both methods would be used and compared in parallel for a period and on a scale determined by a sample size calculation. Furthermore, with the Bead Retriever not being a viable automated platform, there are more specific options for bead-based DNA purification methods, such as the Thermofisher Scientific Kingfisher Apex instrument recently purchased by MLW. Whilst the current workflow for the subsequent ES programme in Blantyre will not include sufficient samples to justify the use of an automated extraction platform; if this study was repeated, or a subsequent one with similar sample numbers as collected here, I would recommend the use of automation to ensure samples are extracted consistently, and within the turnaround time proposed by the new workflow.

Once DNA extraction issues have been resolved, HRM PCR could be revisited, including the full panel of *S.* Typhi and *S*. Paratyphi targets, and introducing other NTS of interest, such as *S*. Enteritidis and *S.* Typhimurium, due to their prevalence in iNTS cases within QECH. If all the above changes were to be validated and implemented, a potential *S*. Typhi ES workflow could be deployed as per Figure 8.2.

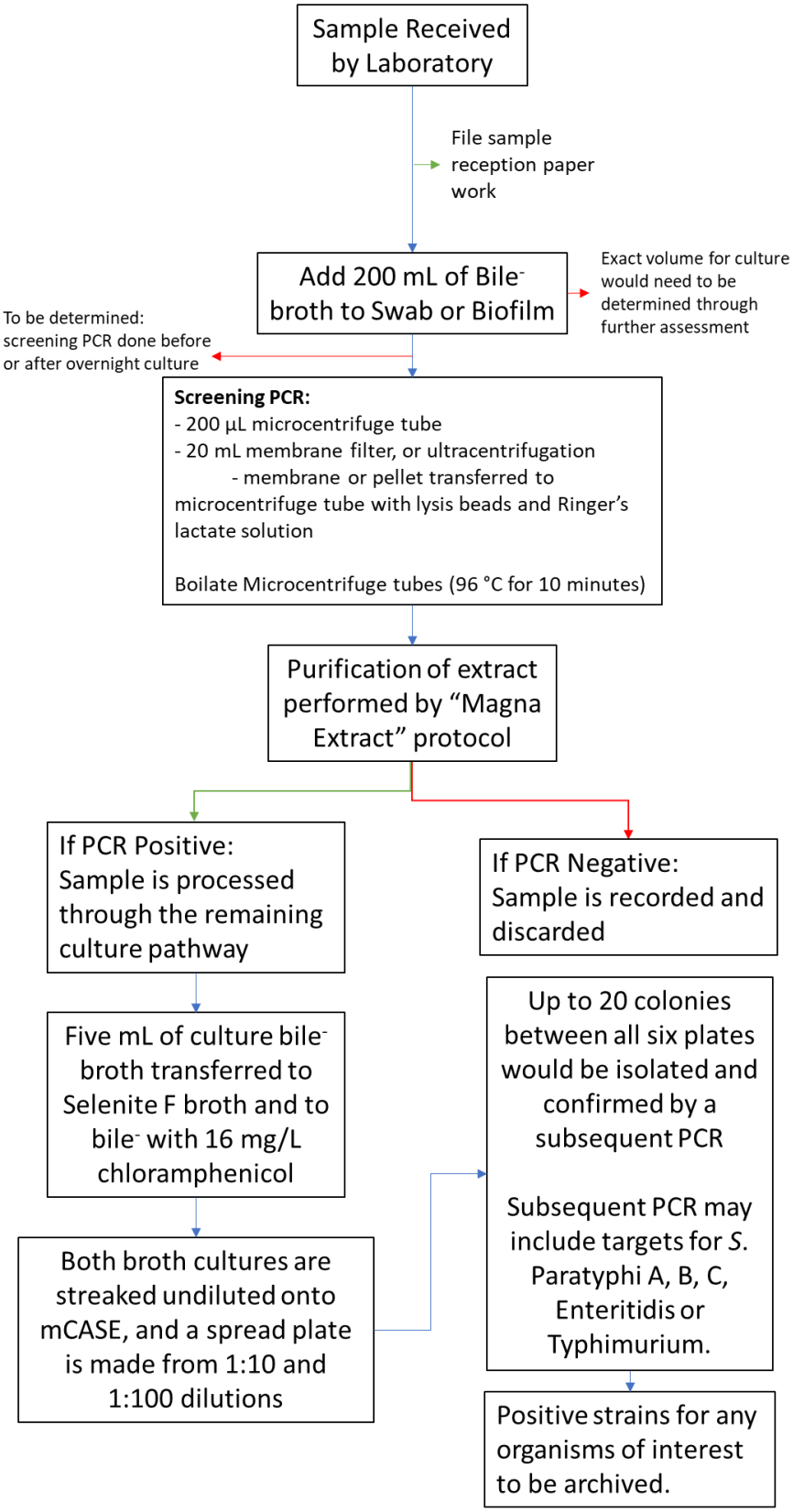

Figure 8.2: Hypothetical future *S*. Typhi culture pathway if some or all future work was to be validated and implemented.

Finally, the topic I would be most interested in expanding this work towards is a multi-pathogen ES tool.

8.3 Final remarks

My objectives were to:

- Develop and optimise a novel culture pathway for the isolation of *S.* Typhi from complex matrices, such as river water;
- Identify *S.* Typhi from the environment by PCR;
- Confirm *S.* Typhi isolates by PCR;
- Establish an ES programme in Blantyre, Malawi, where *S.* Typhi is endemic.

All of these objectives were achieved, and I have shown it is possible to perform ES on true environmental samples, such as river water, and not just wastewater, and also that culture is a viable option. Whilst positive sample numbers were low, many lessons were learnt from the study that could inform a subsequent project in Malawi, or elsewhere. Work is ongoing in Malawi to implement a screening PCR on all incoming samples, where PCR negative samples would be discarded allowing more time and resources to be dedicated to culturing the PCR positive samples.

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10 Appendix

10.1 Chapter 2: Sample Collection Forms

10.1.1 Field Collection Form Version 1 2019-2020

This form was used during initial collection, and whilst iterations were made from its first use until its last, the questions remained the same. There is a redundancy question in place "Sample ID – scan barcode" and "Sample ID – 01 to 100", the question prior, "Is the Sample Barcoded?", dictates which of these two subsequent questions will be displayed. A single form per sample was completed, however, lacked questions to ensure the correct sample was logged with the correct details, which led to some sample forms being incomplete, or assigned the wrong sample type. Location was based primarily off GPS coordinates, which were collected by the tablet using LTE (Long-Term Evolution), fourth-generation (4G) wireless communications through a SIM card purchased for each tablet, although river location was included to assist with data analysis.

ERST Field Sampling

Initials of Field Worker taking sample

- ◯ PK
- ◯ JR
- ◯ SC

Is the Sample Barcoded?

- ◯ Yes
- ◯ No

Sample ID

Scan Barcode

_____

Sample ID

01 to 100

_____

Date and Time of Sample Collection

yyyy-mm-dd _____ hh:mm _____

Site/River Name

- ◯ Mudi
- ◯ Naperi + Nearby Rivers
- ◯ Lunzu
- ◯ Mbyani
- ◯ Ndirande
- ◯ Bangwe/Thuchilla
- ◯ Michiru/Likhubula
- ◯ Kufa Stream
- ◯ Other

Site Name

_____

Location of Sample

latitude (x.y °)

longitude (x.y °)

altitude (m)

accuracy (m)

Image of Sample Source

Click here to upload file. (< 5MB)

Sample Type

- ◯ Liquid
- ◯ Solid

Water Source

- ◯ Flowing: River
- ◯ Stagnant: Pool/Pond
- ◯ Run-off: Drain pipe/Drainage Channel
- ◯ Agriculture: Run-off/Supply
- ◯ Drinking supply: Collected Water/Home-made beverage
- ◯ WASH
- ◯ Other

Solid Type

- ◯ Food
- ◯ Soil
- ◯ Moore's Swab
- ◯ Environmental Swab
- ◯ Clothing
- ◯ Other

Food Source

- ◯ Household
- ◯ Market
- ◯ Farm/Agriculture
- ◯ Other

Soil Source

- ○ River bed
- ○ Agriculture/Farm
- ○ Household/Garden
- ○ Other

Clothing Sample

- ○ Cutting
- ○ Pre-Wash
- ○ Post-Wash

Describe Sample

_____

Water Temperature (Degrees Celsius)

_____

Dissolved Oxygen

_____

pH

_____

Weather

- ☐ Sunny
- ☐ Cloudy
- ☐ Raining
- ☐ Humid
- ☐ Dry

Any other information? Environment description.

Presence and type of human activity

_____

Figure 10.1: Field collection form, version one

10.1.2 Field Collection Form Version 2 2021-2022

This form was updated to ensure that all samples would be correctly completed on site, as only one form would need to be completed for the collection, allowing water samples for both the typhoid and SARS-CoV-2 ES, Moore swabs and biofilms to have their barcodes scanned into the form. There are additional repetitions in the form shown here that would be hidden during use, as certain questions would appear based on previous answers; for example, the first question "Field Worker Completing Form" has a multiple-choice entry with initials for all field workers, myself included, whilst the second question with the same title is a free text entry field. The free text entry question will only appear if "Other (Please Specify)" is selected. With request's made from the team at ICL for the BMGF samples, and collaborators from Path for the SARS-CoV-2 ES, forms have field entries for "Site ID" based on GIS work done by Dr Uzzell (2021), and a site name so that a more "user friendly" site location could be added when the data is uploaded to Wastewater SPHERE: https://sphere.waterpathogens.org/.

There is also an addition of water metrics so the data can be compared to *S.* Typhi positivity to determine whether any correlations exist between water quality and pathogen presence.

Field Form MK.II

Field Worker Completing Form

- ◯ PK
- ◯ RM
- ◯ SC
- ◯ WM
- ◯ JR
- ◯ EW
- ◯ Other (Please Specify)

Field Worker Completing Form

Collection Team

- ◯ A
- ◯ B

Date and Time of Collection

yyyy-mm-dd hh:mm

Site ID Number

eg. 1000/900

Site Name

Blantyre 1

GPS Coordinates of Sampling

latitude (x.y °)

longitude (x.y °)

altitude (m)

accuracy (m)

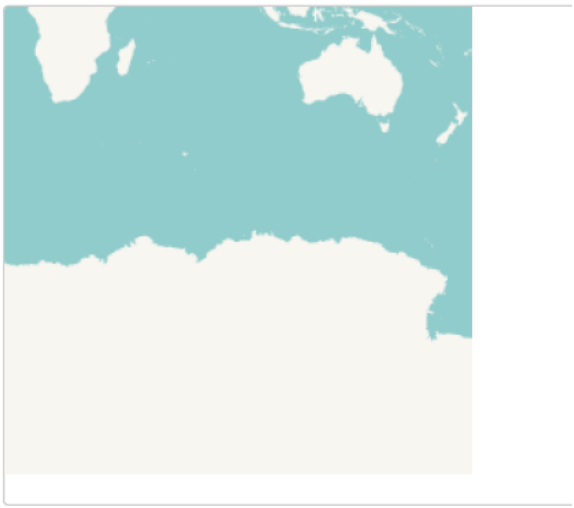


Photo of Sample Source

River

Click here to upload file. (< 5MB)

Sample Types Collected

- [ ] Water, 1 Litre
- [ ] Water, Viral x2
- [ ] Biofilm or Rock/Stone
- [ ] Moore Swab

Was a Moore Swab Deployed at this Location?

- ( ) Yes
- ( ) No

Were all Samples Barcoded?

- ( ) OK

Sample ID: Water, 1 Litre

Field Worker Collecting the Sample

- PK
- RM
- SC
- WM
- JR
- EW
- Other (Please Specify)

Field Worker Collecting the Sample

Sample ID: Water, Viral x2

Field Worker Collecting the Sample

- PK
- RM
- SC
- WM
- JR
- EW
- Other (Please Specify)

Field Worker Collecting the Sample

Sample ID: Biofilm/Rock

Field Worker Collecting the Sample

- PK
- RM
- SC
- WM
- JR
- EW
- Other (Please Specify)

Field Worker Collecting the Sample

Sample ID: Moore Swab

Field Worker Collecting the Sample

- ○ PK
- ○ RM
- ○ SC
- ○ WM
- ○ JR
- ○ EW
- ○ Other (Please Specify)

Field Worker Collecting the Sample

Sample ID: Water, 1 Litre

ERSTDATENUMBER

ERST_DATE_NUMBER

Field Worker Collecting the Sample

- ○ PK
- ○ RM
- ○ SC
- ○ WM
- ○ JR
- ○ EW
- ○ Other (Please Specify)

Field Worker Collecting the Sample

Sample ID: Water, Viral x2

ERSTDATENUMBER

ERST_DATE_NUMBER

Field Worker Collecting the Sample

- ○ PK
- ○ RM
- ○ SC
- ○ WM
- ○ JR
- ○ EW
- ○ Other (Please Specify)

Field Worker Collecting the Sample

Sample ID: Biofilm/Rock

ERSTDATENUMBER

ERST_DATE_NUMBER

Field Worker Collecting the Sample

- ○ PK
- ○ RM
- ○ SC
- ○ WM
- ○ JR
- ○ EW
- ○ Other (Please Specify)

Field Worker Collecting the Sample

Sample ID: Moore Swab

ERSTDATENUMBER

ERST_DATE_NUMBER

Field Worker Collecting the Sample

- ( ) PK
- ( ) RM
- ( ) SC
- ( ) WM
- ( ) JR
- ( ) EW
- ( ) Other (Please Specify)

Field Worker Collecting the Sample

_____

Were water metrics taken on-site?

- ( ) Yes, AquaRead
- ( ) Yes, DO metre, pH probe and TDS probe
- ( ) No

Why was a metric not taken?

_____

AquaRead Save File Number?

_____

pH

_____

Temperature, Celsius

_____

Dissolved Oxygen (DO), PPM

_____

Total dissolved solids (TDS), PPM

_____

Electic Conductivity (EC)

_____

Flow Rate of River

- ◯ Fast
- ◯ Slow
- ◯ Stagnant (not moving)
- ◯ Dry

Depth of Water

- ◯ Less than 5 cm
- ◯ 5 to 50 cm
- ◯ More than 50 cm

Width of River

- ◯ Less than 1 Metre
- ◯ 1 to 2 Metres
- ◯ More than 2 Metres

Colour of Water

- ◯ Dark Black/Grey
- ◯ Green
- ◯ Brown/Silty
- ◯ Clear
- ◯ Other

Please Describe Water Colour

_____

Any other information or comments?

_____

Figure 10.2: Field collection form, version two

10.1.3 ICL Site Questionnaire for BMGF Samples

With new sites proposed by the team at ICL from the GIS work performed by Dr Uzzell (2021), a site suitability questionnaire was created, to gather information, such as flow direction, of each site. Much of the information, such as water depth, is included in the field form (Appendix 10.1.2), as the volume, and therefore, depth and width, of the river changes between dry and wet seasons. As such, this form was only used once during site assessment, instead of being adopted as the main field form like it had been at other sites (Vellore and Agogo).

Nov 2021
Catherine Troman

Questionnaire for Environmental Site Sample Collection v1.1

1. Date & time

Date: dd / mm / yy Time: hh:mm

2. Name of person filling questionnaire

3. Site ID

4. Site name

5. GPS coordinates (Lat, Long)

(,)

6. *take a photo of ES sample site*

7. Flow speed

- ☐ Fast
- ☐ Slow
- ☐ Stagnant
- ☐ Dry

8. Depth of wastewater/sewage

- ☐ <5cm (shallow)
- ☐ 5-50cm (medium)
- ☐ >50cm (deep)
- ☐ Not sure

9. Width of wastewater/sewage

- ☐ <1m
- ☐ 1-2m
- ☐ >2m

10. Sample deployment

Moore swab

Sample ID

deployment duration (hours)

deployed but not recovered ☐

Grab sample

Sample ID

11. Water quality

Were water quality measurements taken? (Y/N)

If yes:

Save file number from probe

Probe ID

Was the probe calibrated? (Y/N)

Nov 2021
Catherine Troman

Questionnaire for Environmental Site Characteristics (do once only) v1.1

1. Date & time

Date: dd / mm / yy Time: hh:mm

2. Name of person filling questionnaire

3. Site ID

4. Site name

5. GPS coordinates (Lat, Long)

(,)

6. Qualitative assessment of flow & volume (give space for comments as well as Y/N options)

a. Does the flow or volume change throughout the day?

b. Does flow or volume vary seasonally?

7. Flow direction

Travelling to the N/NE/E/SE/S/SW/W/NW/none:

8. Assessment of catchment population

i. What is the estimated catchment population? (NA if unknown)

ii. What is the source of this estimate?

9. Which (if any) public services are located within the site's catchment?

- ☐ Primary school
- ☐ Secondary school
- ☐ Transit hub
- ☐ Food stalls/restaurants
- ☐ Hospital/health facility
- ☐ Factory
- ☐ Latrines

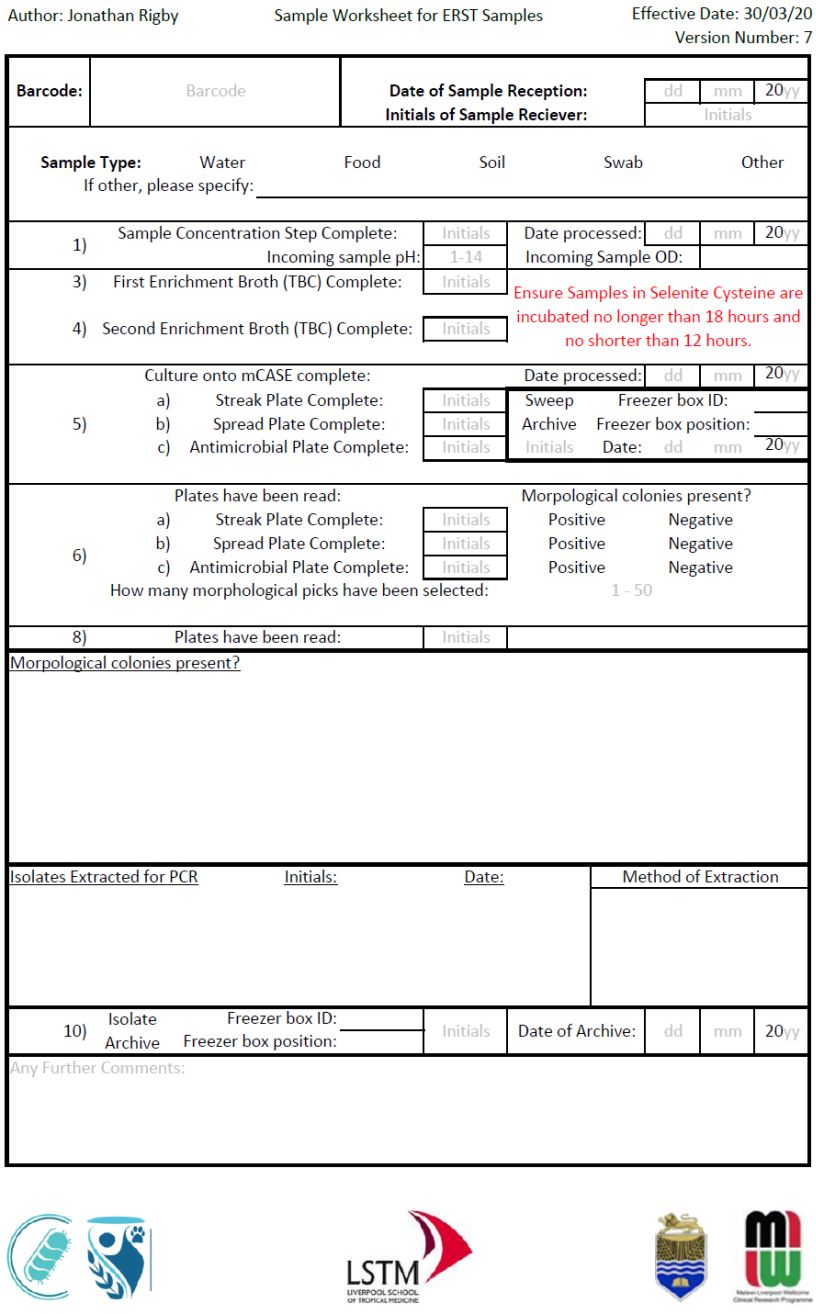
10. Any other comments that you would like to note down.

Figure 10.3: Field site assessment form

10.2 Laboratory Sample Forms

10.2.1 Laboratory Sample Paper form

This form was used in the laboratory to keep track of samples before they had finished their processing. This was due to KoBoToolbox not allowing sample forms to be updated easily, as it was not designed as a LIMS, only a data collecting tool. The laboratory team would scan the barcode from the collected sample at sample reception and stick the duplicated label to the form to minimise transcription errors.



Author: Jonathan Rigby | Sample Worksheet for ERST Samples | Effective Date: 30/03/20 Version Number: 7

Barcode:	Barcode	**Date of Sample Reception:**	dd	mm	20yy	
		Initials of Sample Reciever:	Initials			

Sample Type: Water Food Soil Swab Other
If other, please specify: _____

1)	Sample Concentration Step Complete:	Initials	Date processed:	dd	mm	20yy
	Incoming sample pH:	1-14	Incoming Sample OD:			
3)	First Enrichment Broth (TBC) Complete:	Initials	Ensure Samples in Selenite Cysteine are incubated no longer than 18 hours and no shorter than 12 hours.			
4)	Second Enrichment Broth (TBC) Complete:	Initials				
	Culture onto mCASE complete:		Date processed:	dd	mm	20yy
5)	a) Streak Plate Complete:	Initials	Sweep Freezer box ID:			
	b) Spread Plate Complete:	Initials	Archive Freezer box position:			
	c) Antimicrobial Plate Complete:	Initials	Initials Date: dd mm 20yy			

	Plates have been read:		Morpological colonies present?	
6)	a) Streak Plate Complete:	Initials	Positive	Negative
	b) Spread Plate Complete:	Initials	Positive	Negative
	c) Antimicrobial Plate Complete:	Initials	Positive	Negative
	How many morphological picks have been selected:		1 - 50	

8)	Plates have been read:	Initials	

Morpological colonies present?

Isolates Extracted for PCR	Initials:	Date:	Method of Extraction

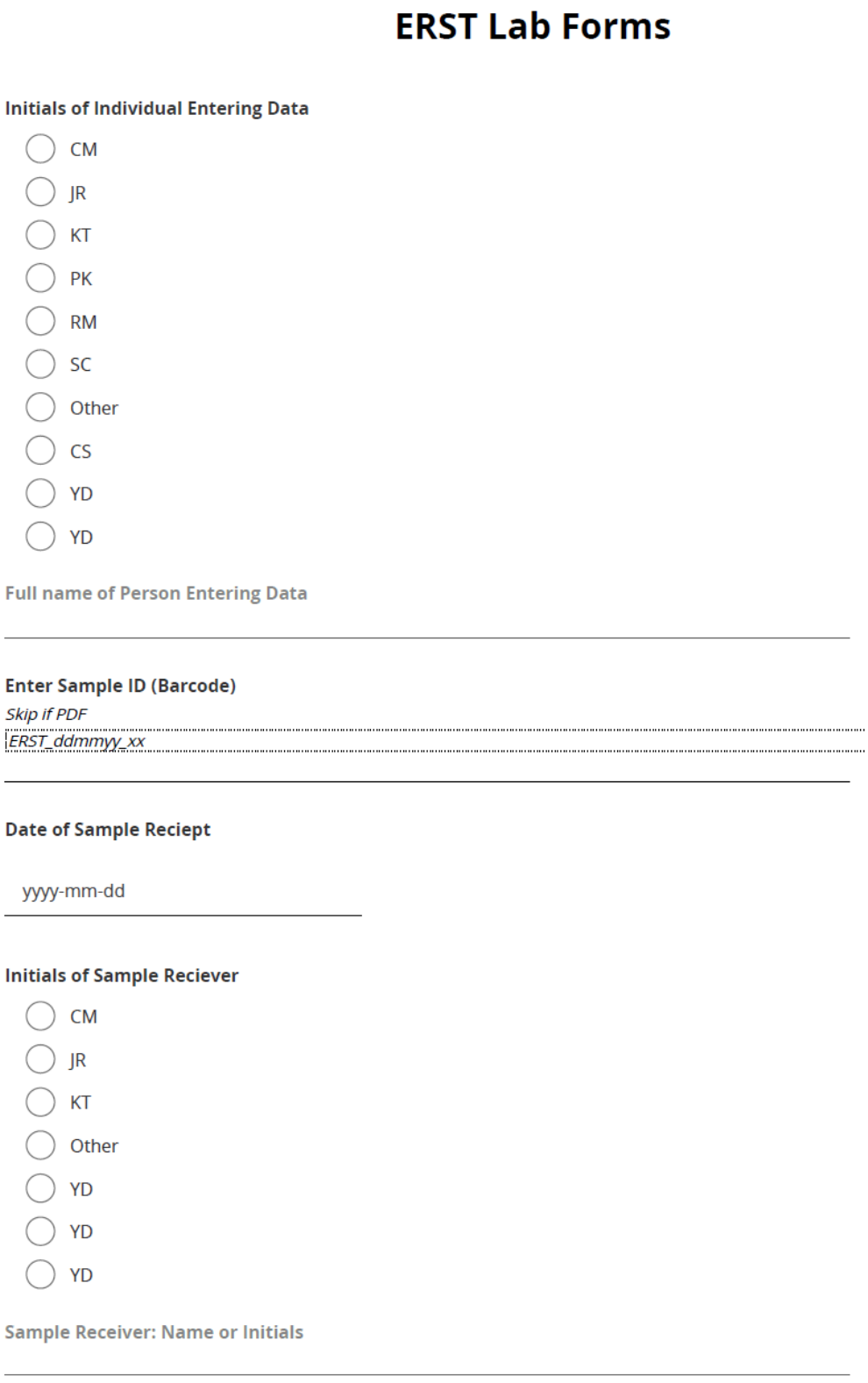
10)	Isolate Archive	Freezer box ID: _____ Freezer box position:	Initials	Date of Archive:	dd	mm 20yy

Any Further Comments:

Figure 10.4: Laboratory sample processing form

10.2.2 Laboratory Sample Digital Form

The sample data collected from the paper form (Appendix 10.2.1) required the information to be uploaded to a digital database. As such, KoBoToolbox was used once again. Data was copied from a completed laboratory form and uploaded after sample archive or discard.



ERST Lab Forms

Initials of Individual Entering Data

- ○ CM
- ○ JR
- ○ KT
- ○ PK
- ○ RM
- ○ SC
- ○ Other
- ○ CS
- ○ YD
- ○ YD

Full name of Person Entering Data

_____

Enter Sample ID (Barcode)

Skip if PDF

ERST_ddmmyy_xx

_____

Date of Sample Reciept

yyyy-mm-dd

_____

Initials of Sample Reciever

- ○ CM
- ○ JR
- ○ KT
- ○ Other
- ○ YD
- ○ YD
- ○ YD

Sample Receiver: Name or Initials

_____

Sample Type

- ◯ Water
- ◯ Food
- ◯ Soil
- ◯ Swab
- ◯ Rock/Biofilm
- ◯ Algae
- ◯ Other

Further Information on Sample Type

_____

Initial Culture Plates Culture Positive?

- ☐ Streak Plate
- ☐ Spread Plate
- ☐ Antimicrobial Plate

Number of Subculture Plates Positive

_____

Extracted for PCR?

- ◯ OK

Figure 10.5: Laboratory sample processing data upload form

10.2.3 Archive Box Plans

After samples had been stored onto Pro-Lab Microbank Cryobeads, their location within the archive had to be recorded, as such box plans were completed and uploaded to KoBoToolbox using the following form.

ERST Box Plans

Initials of staff completing document

- ◯ CM
- ◯ JR
- ◯ KT
- ◯ YD

Box Number

_____

Box Size

- ◯ 81
- ◯ 100

Position 1 Sample ID

Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

_____

Position 2 Sample ID

Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

_____

Position 3 Sample ID

Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

_____

Position 4 Sample ID

Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

_____

Position 5 Sample ID

Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

_____

Position 6 Sample ID

Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

_____

Position 7 Sample ID

Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

_____

Position 8 Sample ID

Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

_____

Position 9 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 10 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 11 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 12 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 13 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 14 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 15 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 16 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 17 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 18 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 19 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 20 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 21 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 22 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 23 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 24 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

Position 25 Sample ID
Please Ensure you follow the correct order as printed on the Cryobox, position 1 - 81

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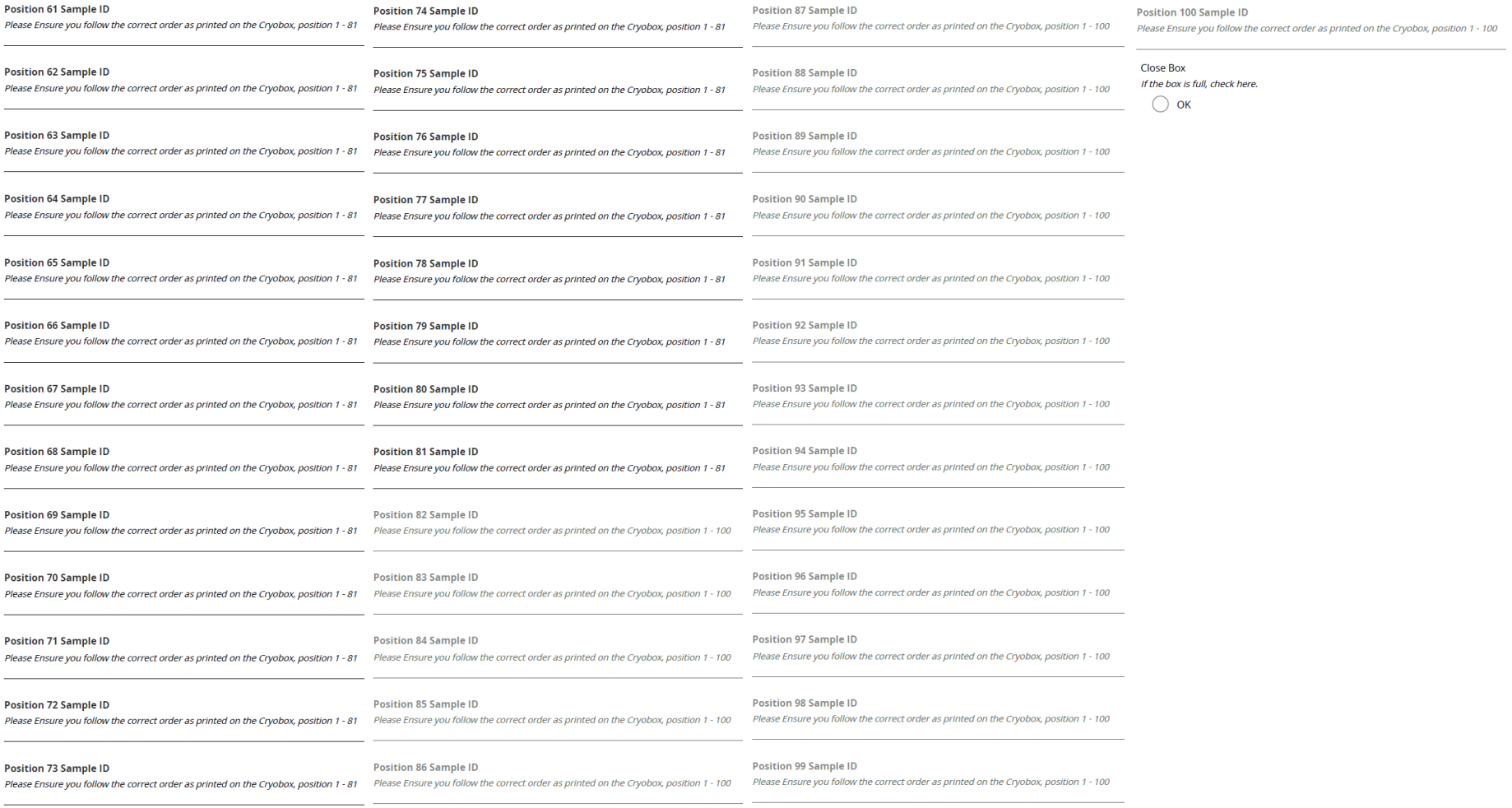

Figure 10.6: Sample archive box plan form

10.3 Results from project predecessor that informed future work

Table 10.1: Colony counts of *S.* Typhi control strains on various agar media assessed, through pathways A to J (Chapter 5 Table 1).

Pathway	*S.* Typhi Strain	Colony Counts				
		CBA	DCA	XLD	CASE	BSI
A	Strain 2	TMTC	25	0	0	80
	Strain 2	TMTC	TMTC	0	7	0
	Strain 2	TMTC	2	2	0	6
	Strain 5	TMTC	452	0	2	TMTC
	Strain 5	TMTC	52	2	0	6
	Strain 5	TMTC	145	10	0	TMTC
	Strain 8	TMTC	13	0	1	0
	Strain 8	TMTC	0	0	0	1
	Strain 8	TMTC	5	0	57	5
	Strain 10	TMTC	18	0	0	5
	Strain 10	TMTC	41	0	6	58
	Strain 10	TMTC	72	30	0	207
	E. coli	TMTC	0	30	0	0
	E. coli	TMTC	1	77	0	0
	E. coli	TMTC	3	8	0	12
B	Strain 2	TMTC	0	45	0	0
	Strain 2	TMTC	67	0	0	1
	Strain 2	TMTC	TMTC	0	0	0
	Strain 5	TMTC	77	0	0	0
	Strain 5	TMTC	12	0	0	0
	Strain 5	TMTC	0	TMTC	0	0
	Strain 8	TMTC	28	0	43	20
	Strain 8	TMTC	5	0	1	TMTC
	Strain 8	TMTC	28	10	50	36
	Strain 10	TMTC	11	46	TMTC	48
	Strain 10	TMTC	18	6	0	15
	Strain 10	460	0	0	84	26
	E. coli	TMTC	0	0	38	13
	E. coli	TMTC	0	0	0	0
	E. coli	TMTC	0	0	0	0
C	Strain 2	0	0	0	0	0
	Strain 2	TMTC	3	0	0	70
	Strain 2	0	0	0	1	1
	Strain 5	TMTC	8	0	3	2
	Strain 5	TMTC	3	0	0	70
	Strain 5	TMTC	1	1	34	60
	Strain 8	TMTC	0	0	4	1
	Strain 8	TMTC	5	1	0	4

	Strain 8	TMTC	0	0	2	3
	Strain 10	TMTC	0	0	9	0
	Strain 10	TMTC	0	25	0	0
	Strain 10	TMTC	0	0	2	10
	E. coli	TMTC	0	0	2	0
	E. coli	TMTC	31	12	0	0
	E. coli	TMTC	0	0	0	0
D	Strain 2	TMTC	6	2	0	4
	Strain 2	TMTC	0	0	0	3
	Strain 2	TMTC	7	0	0	1
	Strain 5	TMTC	15	3	0	0
	Strain 5	TMTC	15	3	0	0
	Strain 5	TMTC	0	0	9	9
	Strain 8	208	2	1	11	11
	Strain 8	TMTC	0	0	49	49
	Strain 8	TMTC	9	1	4	4
	Strain 10	TMTC	1	0	0	0
	Strain 10	TMTC	3	0	6	6
	Strain 10	TMTC	0	0	0	0
	E. coli	TMTC	0	0	0	0
	E. coli	TMTC	0	0	0	0
	E. coli	TMTC	0	0	0	0
E	Strain 2	0	0	128	0	0
	Strain 2	0	0	0	0	0
	Strain 2	0	16	0	0	0
	Strain 5	0	0	61	0	0
	Strain 5	0	77	23	0	0
	Strain 5	0	0	60	0	0
	Strain 8	0	0	0	93	0
	Strain 8	0	6	77	0	0
	Strain 8	0	56	0	0	5
	Strain 10	0	16	69	0	0
	Strain 10	0	32	55	0	88
	Strain 10	0	0	8	0	0
	E. coli	0	0	0	0	12
	E. coli	0	0	0	0	0
	E. coli	0	0	62	0	0
F	Strain 2	0	0	0	3	0
	Strain 2	0	0	0	0	0
	Strain 2	0	0	0	27	0
	Strain 5	0	0	0	0	0
	Strain 5	0	14	0	0	0
	Strain 5	0	0	0	0	0

	Strain 8	0	0	0	4	0
	Strain 8	0	4	18	0	0
	Strain 8	0	1	14	5	0
	Strain 10	113	10	7	0	0
	Strain 10	0	0	0	0	0
	Strain 10	0	0	0	0	0
	E. coli	0	10	0	0	0
	E. coli	0	0	6	5	0
	E. coli	0	0	75	0	0
G	Strain 2	0	94	38	118	0
	Strain 2	0	0	101	6	0
	Strain 2	284	0	5	8	0
	Strain 5	276	0	0	0	0
	Strain 5	0	1	6	11	10
	Strain 5	0	0	34	0	0
	Strain 8	0	28	26	0	0
	Strain 8	0	16	28	56	91
	Strain 8	0	0	48	0	0
	Strain 10	0	56	64	0	0
	Strain 10	0	0	0	0	0
	Strain 10	0	0	0	0	0
	E. coli	0	0	0	0	268
	E. coli	0	264	0	7	230
	E. coli	0	0	24	8	0
H	Strain 2	28	0	0	0	0
	Strain 2	TMTC	0	0	0	0
	Strain 2	TMTC	0	0	0	0
	Strain 5	TMTC	0	0	0	0
	Strain 5	TMTC	0	0	0	0
	Strain 5	TMTC	0	0	0	0
	Strain 8	TMTC	0	0	0	0
	Strain 8	TMTC	0	0	0	0
	Strain 8	TMTC	0	0	0	0
	Strain 10	TMTC	0	0	0	0
	Strain 10	TMTC	0	0	0	0
	Strain 10	TMTC	0	0	0	0
	E. coli	TMTC	0	0	0	0
	E. coli	TMTC	0	0	0	0
	E. coli	TMTC	0	0	0	0
I	Strain 2	TMTC	43	0	250	35
	Strain 2	TMTC	44	0	15	0
	Strain 2	TMTC	50	0	U/C	0
	Strain 5	TMTC	14	0	27	0

	Strain 5	TMTC	TMTC	0	9	0
	Strain 5	TMTC	58	0	46	0
	Strain 8	TMTC	7	0	24	30
	Strain 8	TMTC	7	0	1	6
	Strain 8	TMTC	29	0	316	13
	Strain 10	TMTC	2	0	TMTC	0
	Strain 10	TMTC	33	0	110	0
	Strain 10	TMTC	28	0	TMTC	0
	E. coli	TMTC	TMTC	0	30	0
	E. coli	TMTC	TMTC	0	3	0
	E. coli	TMTC	TMTC	0	0	0
J	Strain 2	TMTC	0	0	0	0
	Strain 2	TMTC	0	0	37	0
	Strain 2	TMTC	0	0	42	0
	Strain 5	TMTC	0	0	36	0
	Strain 5	TMTC	0	0	47	0
	Strain 5	TMTC	0	0	40	0
	Strain 8	TMTC	0	0	22	0
	Strain 8	TMTC	0	0	31	0
	Strain 8	TMTC	0	0	30	0
	Strain 10	TMTC	0	0	40	0
	Strain 10	TMTC	0	0	28	0
	Strain 10	TMTC	0	0	TMTC	0
	E. coli	TMTC	0	0	42	0
	E. coli	TMTC	0	0	38	0
	E. coli	TMTC	0	0	56	0

Table 10.2: Positivity of isolates from mixed culture challenges across culture pathway and agar type.

Spike	Experiment path	CBA	DCA	XLD	BSA	CASE
1	A	+	+	-	-	+
	B	-	+?	+?	-	+
	C	+	+	+	-	+
	D	-	+?	+?	-	+
	E	-	-	-	-	-
	F	-	+	+	-	+
	G	-	-	-	-	+
	H	-	-	-	-	+
	I	-	-	+	-	+
	J	-	-	-	-	+ (green)
	K	-	+	-	-	+
	L	-	-	-	-	-
2	A	+	+	-	-	+
	B	TMTC	-	-	-	+
	C	+	+	+	+	+
	D	+	-	-	-	+
	E	-	-	-	-	+
	F	-	-	+	-	+
	G	-	-	+	+	+
	H	-	-	-	-	+
	I	-	-	-	+	+
	J	-	-	-	-	-
	K	-	+?	-	-	+
	L	-	-	-	-	+
3	A	-	+?	-	-	+
	B	-	-	-	-	+
	C	+	-	+	-	+
	D	-	-	+	-	+
	E	-	-	+?	+	+
	F	-	-	+?	-	+
	G	-	-	+	+	+
	H	-	-	-	-	+
	I	-	-	-	+	+
	J	-	-	-	-	+
	K	-	-	-	-	+
	L	-	+	+	+	+
4	A	-	+	-	-	+
	B	-	+?	+?	-	+
	C	+	-	+	-	+
	D	-	+	-	+	+
	E	-	+	-	+	+
	F	-	+?	-	-	+
	G	-	+	-	-	+

	H	-	-	+	-	+
	I	-	-	+	-	+
	J	-	-	-	-	+
	K	-	+	+	-	+
	L	-	+	-	-	+
5	A	-	-	-	-	-
	B	-	-	-	-	-
	C	-	+?	-	+?	-
	D	-	-	-	-	-
	E	-	-	-	+	-
	F	-	-	-	-	+
	G	-	-	-	+	-
	H	-	-	-	-	-
	I	-	-	-	-	-
	J	-	-	-	-	+
	K	-	-	-	-	+
	L	-	-	-	-	-

Table 10.3: Growth quality of *S.* Typhi control strains when iron is added to bile$^-$ broth (bile$^+$). +: weak growth, ++: good growth, +++: luxuriant growth

Strain	Iron	No iron
ST12	++	+
ST10	+++	+++
ST8	++	+
ST5	++	+

Table 10.4: Growth quality of *S.* Typhi control strains when comparing the different CASE media modifications, when the selective agents are removed (CASE-), or only one is retained (+1, +2). +: weak growth, ++: good growth, +++: luxuriant growth

Strain	CASE -	CASE	CASE 1+	CASE 2+
ST2	+++	++	+	++
ST5	+++	++	+	+++
ST8	+++	+	+++	++
ST10	++	++	+	+++
ST1	+++	+++	+	++
ST9	+++	++	+	+++
ST4	+++	++	++	++
ST6	++	++	+	+++
ST11	+++	+++	+	++
ST12	+++	++	+	+++

10.4 Chapter 5: Supplementary materials from Manuscript publication

Some figures and tables have already been used earlier in this thesis that were part of the supplementary materials, covering:

- Table 2.5
- Table 3.2
- Table 3.7
- Figure 4.7

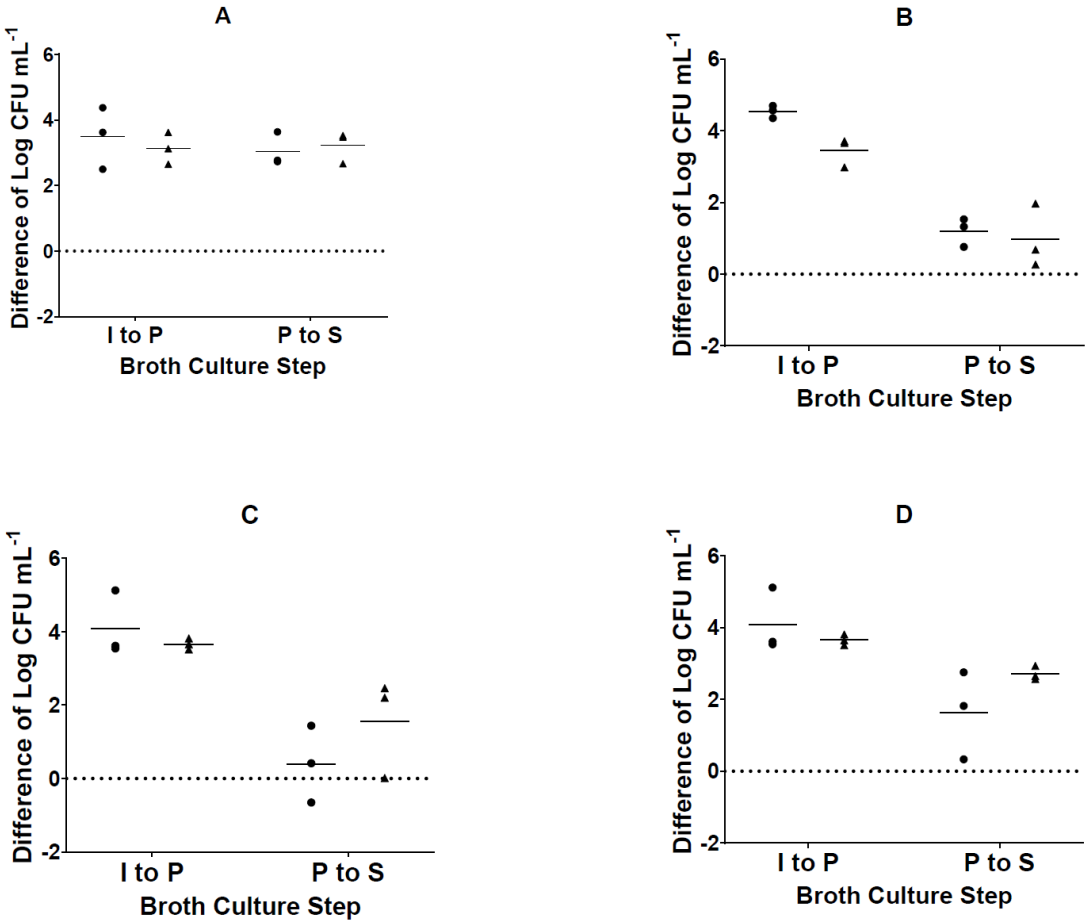

Figure 10.7: Growth data plotted using difference of log from Table S4, showing the comparison between pathways L to S. Each of these tables compare selenite cystine (l) to selenite F (p) broth across the eight pathways, L to S. A. shows the pathways L and P, representing bile$^-$ into the selenite broths; B. shows the pathways M and Q, representing bile$^+$ into the selenite broths; C. shows the pathways N and R, representing selenite broths into bile$^-$; D. shows the pathways O and S, representing selenite broths into bile$^+$.

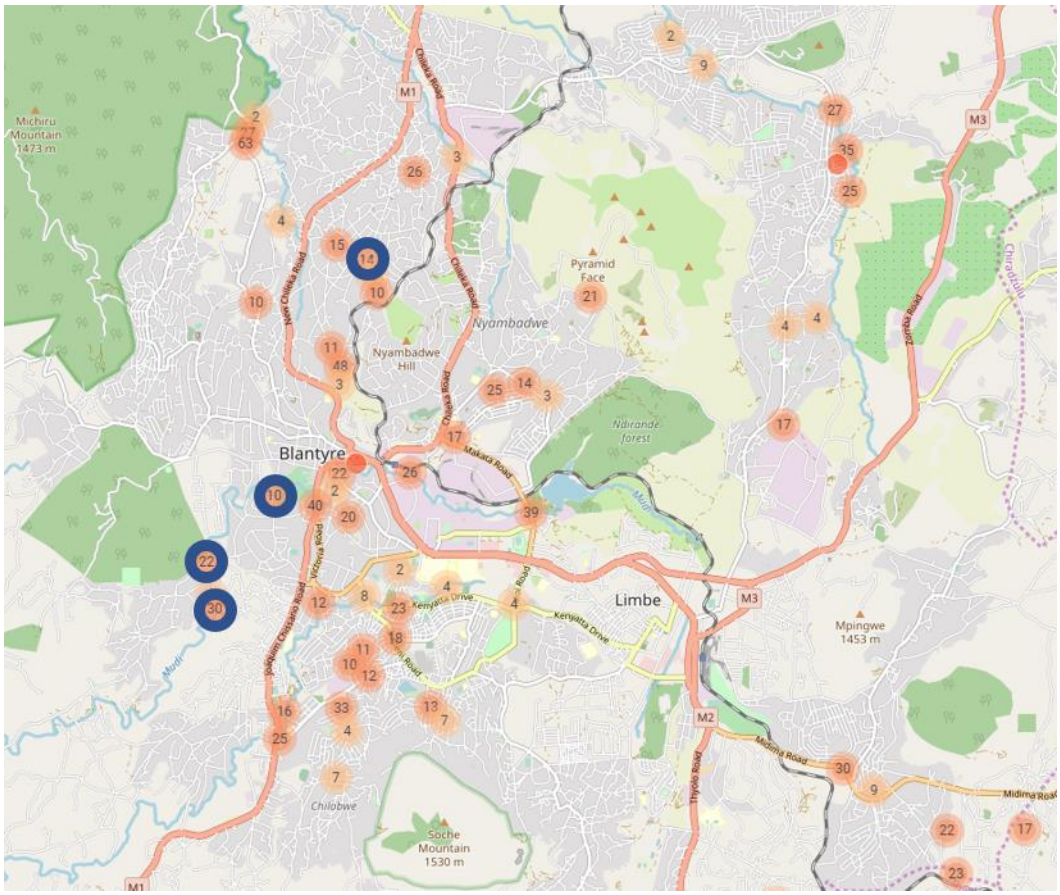

Figure 10.8: Map of Blantyre with all sampling points up to January 2020 plotted. Locations highlighted by a blue circle are those where the samples from which *S.* Typhi was isolated

Table 10.5: Colony morphology and appearance of *S.* Typhi on the agar used in this study.

Agar	Colony Morphology	References
Xylose Lysine Deoxycholate Agar	Red colonies with black centres due to hydrogen sulphide (H_2S) production	(Nye *et al*., 2002)
Bismuth Sulphite Agar (Wilson and Blair media)	Black colonies with a black zone and metallic sheen surrounding the colony	(Wilson and Blair, 1931, Wilson, 1938, Hobbs and Allison, 1945)
Deoxycholate Citrate Agar (Hyne's Media)	Colourless colonies with black centres due to H_2S production	(Hobbs and Allison, 1945)
MacConkey	Pale coloured, non-lactose fermenting, smooth surfaced colonies	(Pullinger and Kemp, 1938)
Harlequin ABC Agar	Green colonies	(Nye *et al*., 2002)
Chromogenic Agar for *Salmonella* Esterase (CASE)	Blue/green colonies	(Alles *et al*., 2021)
mCASE	Blue/green colonies	This manuscript
CASE -1	Blue/green colonies	This manuscript
CASE-	Blue/green colonies	This manuscript

Table 10.6: Colony forming units per millilitre (CFU/mL) of the Malawian *S.* Typhi strain used and grown within different media comparison experiments for the final candidate pathways L to S

Pathway	Biological Replicate number	CFU/mL per broth stage		
		Inoculum	Primary	Secondary
L Bile to Selenite Cystine Broth	1	1.77E+02	5.53E+04	2.38E+08
	2	1.20E+02	5.01E+05	2.94E+08
	3	3.00E+01	7.03E+05	3.76E+08
M Iron-Bile to Selenite Cystine Broth	1	1.40E+02	3.17E+06	6.67E+07
	2	2.13E+02	1.07E+07	6.14E+07
	3	1.65E+02	6.17E+06	2.09E+08
N Selenite Cystine to Bile Broth	1	1.40E+02	5.73E+05	1.49E+06
	2	2.13E+02	7.39E+05	2.02E+07
	3	1.65E+02	2.18E+07	4.83E+06
O Selenite Cystine to Iron Bile	1	1.40E+02	5.73E+05	3.78E+07
	2	2.13E+02	7.39E+05	4.22E+08
	3	1.65E+02	2.18E+07	4.67E+07
P Bile to Selenite F Broth	1	1.20E+02	5.01E+05	2.32E+08
	2	1.55E+02	6.90E+04	2.29E+08
	3	1.63E+02	2.17E+05	6.55E+08
Q Iron-Bile to Selenite F Broth	1	2.90E+02	2.77E+05	2.58E+07
	2	1.62E+02	7.46E+05	3.58E+06
	3	2.68E+02	1.39E+06	2.55E+06
R Selenite F to Bile Broth	1	2.88E+02	9.39E+05	9.68E+05
	2	2.75E+02	1.23E+06	1.95E+08
	3	2.83E+02	1.84E+06	5.29E+08
S Selenite F to Iron Bile	1	2.88E+02	9.39E+05	3.48E+08
	2	2.75E+02	1.23E+06	5.51E+08
	3	2.83E+02	1.84E+06	1.59E+09

10.5 Chapter 6: One Year Environmental Surveillance

10.5.1 Collection site information

Table 10.7: GPS coordinates (Longitude/Latitude) for each sampling site visiting during this project.

Site ID	Latitude	Longitude	Project Samples collected for
1	-15.7761000000000	35.0349000000000	ERST, COVID
2	-15.7674000000000	35.0305000000000	ERST, COVID
5	-15.7315000000000	34.9982000000000	ERST, COVID
6	-15.7320000000000	35.0463000000000	ERST, COVID
8	-15.7957000000000	35.0093000000000	ERST, COVID
9	-15.7922000000000	34.9976000000000	ERST, COVID
18	-15.8020000000000	34.9876000000000	ERST, COVID
23	-15.8079000000000	35.0045000000000	ERST, COVID
27	-15.8184000000000	35.0122000000000	ERST, COVID
28	-15.8084000000000	35.0161000000000	ERST, COVID
31	-15.8057722000000	35.0236194000000	ERST, COVID
32	-15.7949000000000	35.0365000000000	ERST, COVID
1014	-15.7724810000000	35.0419730000000	ERST, COVID
1024	-15.7840000000000	35.0251000000000	ERST, COVID
1027	-15.7808780000000	34.9826560000000	ERST, COVID
1028	-15.7818440000000	34.9807560000000	ERST, COVID
1030	-15.7786410000000	34.9757190000000	ERST, COVID
1033	-15.7652000000000	34.9840000000000	ERST, COVID
1034	-15.7605000000000	34.9865000000000	ERST, COVID
1038	-15.7590800000000	34.9874180000000	ERST, COVID
1041	-15.7590000000000	34.9782000000000	ERST, COVID
1042	-15.7575000000000	34.9796000000000	ERST, COVID
1046	-15.7490000000000	34.9871000000000	ERST, COVID
1048	-15.7455210000000	34.9934560000000	ERST, COVID
1053	-15.7478500000000	34.9932530000000	ERST, COVID
1061	-15.7532490000000	35.0006250000000	ERST, COVID
1063	-15.7596000000000	35.0115000000000	ERST, COVID
1067	-15.7690000000000	35.0080000000000	ERST, COVID
1075	-15.7418730000000	35.0240460000000	ERST, COVID
1077	-15.7289000000000	35.0140000000000	ERST, COVID
1078	-15.7303000000000	35.0521000000000	ERST, COVID
1080	-15.7292290000000	35.0155520000000	ERST, COVID
1082	-15.7300570000000	35.0508320000000	ERST, COVID
1083	-15.7421000000000	35.0833000000000	ERST, COVID
1086	-15.7483520000000	35.0843910000000	ERST, COVID
1090	-15.7530170000000	35.0827350000000	ERST, COVID
1092	-15.7715610000000	35.0714710000000	ERST, COVID
1093	-15.7737740000000	35.0758370000000	ERST, COVID
1096	-15.7786790000000	35.0740970000000	ERST, COVID

1098	-15.7824000000000	35.0739000000000	ERST, COVID
1100	-15.7782640000000	35.0777460000000	ERST, COVID
1103	-15.7763720000000	35.0866270000000	ERST, COVID
1120	-15.8124000000000	35.0963000000000	ERST, COVID
1123	-15.8199306000000	35.0876694000000	ERST, COVID
1127	-15.8245000000000	35.0855000000000	ERST, COVID
1132	-15.8323000000000	35.0831000000000	ERST, COVID
1134	-15.8384000000000	35.0993000000000	ERST, COVID
1135	-15.8463000000000	35.0979000000000	ERST, COVID
1138	-15.8464000000000	35.0821000000000	ERST, COVID
1139	-15.8505000000000	35.0898000000000	ERST, COVID
1142	-15.8491000000000	35.0810000000000	ERST, COVID
1144	-15.8449000000000	35.0742000000000	ERST, COVID
1145	-15.8469390000000	35.0396540000000	ERST, COVID
1149	-15.8263620000000	35.0021650000000	ERST, COVID
1151	-15.8273110000000	34.9952770000000	ERST, COVID
1154	-15.8357090000000	34.9883920000000	ERST, COVID
1155	-15.8304760000000	34.9806480000000	ERST, COVID
1158	-15.8242620000000	34.9798610000000	ERST, COVID
1164	-15.8102060000000	34.9937940000000	ERST, COVID
1166	-15.8197194000000	35.0053138000000	ERST, COVID
1168	-15.8218000000000	35.0009000000000	ERST, COVID
1173	-15.8125000000000	35.0146000000000	ERST, COVID
1177	-15.7493000000000	34.9990000000000	ERST, COVID
1011/961	-15.7281486092441	35.0524784991326	ERST, BMGF, COVID
1019/893	-15.7353273379426	35.0166601621778	ERST, BMGF, COVID
1024/904	-15.7383903382666	35.0210664872178	ERST, BMGF, COVID
1030/877	-15.7430186011902	35.0032399313212	ERST, BMGF, COVID
1033/864	-15.7443472120036	34.9992673488727	ERST, BMGF, COVID
1038/1028	-15.7470688173243	35.0848836961440	ERST, BMGF, COVID
1046/878	-15.7517721742979	34.9906652001669	ERST, BMGF, COVID
1053/929	-15.7552222562904	34.9809350981324	ERST, BMGF, COVID
1054/912	-15.7559308550441	35.0032417223860	ERST, BMGF, COVID
1056/1039	-15.7552412178784	35.0815978104896	ERST, BMGF, COVID
1060/929	-15.7568808487493	35.0080142179618	ERST, BMGF, COVID
1061/929	-15.7576950082023	34.9847496225384	ERST, BMGF, COVID
1064/1015	-15.7590055542845	35.0331934773353	ERST, BMGF, COVID
1067/961	-15.7608883693875	35.0128938603167	ERST, BMGF, COVID
1076/944	-15.7697895287937	35.0020241260682	ERST, BMGF, COVID
1077/963	-15.7711681688191	35.0063661062023	ERST, BMGF, COVID
1078/1109	-15.7716830021483	35.0447297330452	ERST, BMGF, COVID
1083/1095	-15.7743005703424	35.0406946349204	ERST, BMGF, COVID
1085/1092	-15.7758096448455	35.0793261364940	ERST, BMGF, COVID
1086/1098	-15.7759784794158	35.0753657091367	ERST, BMGF, COVID
1093/945	-15.7797448664535	34.9775711242977	ERST, BMGF, COVID
1102/1146	-15.7847269378742	35.0869474308946	ERST, BMGF, COVID
1103/1039	-15.7892828811710	35.0188050032647	ERST, BMGF, COVID

1116/971	-15.8045789988301	34.9935196765417	ERST, BMGF, COVID
1119/932	-15.8069559753682	34.9890264687245	ERST, BMGF, COVID
1120/1033	-15.8069800455719	35.0114287826325	ERST, BMGF, COVID
1122/1072	-15.8072871553636	35.1006672274466	ERST, BMGF, COVID
1123/1090	-15.8092824931956	35.0411400810615	ERST, BMGF, COVID
1127/951	-15.8119220576957	34.9909048556901	ERST, BMGF, COVID
1132/1116	-15.8144859013974	35.0943743721539	ERST, BMGF, COVID
1133/1006	-15.8154963194962	35.0094736802301	ERST, BMGF, COVID
1138/916	-15.8226152701048	34.9840828726030	ERST, BMGF, COVID
1144/1017	-15.8261708723240	35.0081441162891	ERST, BMGF, COVID
1149/979	-15.8278806183409	34.9986359784086	ERST, BMGF, COVID
1150/1099	-15.8282292034324	35.0858861424517	ERST, BMGF, COVID
1151/1108	-15.8291297770011	35.0753644378342	ERST, BMGF, COVID
1154/960	-15.8316739493529	34.9919487872649	ERST, BMGF, COVID
1158/1076	-15.8357001595995	35.0944593328859	ERST, BMGF, COVID
1161/1067	-15.8413910177388	35.0819476021174	ERST, BMGF, COVID
1167/1026	-15.8462886772235	35.0945562811350	ERST, BMGF, COVID
1178/1073	-15.7699551023261	35.0778592901150	ERST, BMGF, COVID
2001/1009	-15.7937730000000	35.0041660000003	ERST, BMGF, COVID
2002/931	-15.8085583330000	34.9887722220003	ERST, BMGF, COVID
2003/849	-15.7412930000000	34.9934260000000	ERST, BMGF, COVID
2004/969	-15.7304910000000	35.0623590000000	ERST, BMGF, COVID
No ID 1	-15.7525000000000	34.9984000000000	ERST, COVID
No ID 10	-15.7036820000000	35.0047320000000	ERST, COVID
No ID 11	-15.6954600000000	34.9916370000000	ERST, COVID
No ID 12	-15.7061740000000	35.0427740000000	ERST, COVID
No ID 14	-15.6992830000000	35.0373340000000	ERST, COVID
No ID 15	-15.8018640000000	35.0176930000000	ERST, COVID
No ID 16	-15.8463360000000	35.0544380000000	ERST, COVID
No ID 17	-15.8422320000000	35.0520240000000	ERST, COVID
No ID 19	-15.8137000000000	34.9868000000000	ERST, COVID
No ID 20	-15.8234140000000	34.9761670000000	ERST, COVID
No ID 21	-15.8251000000000	34.9680010000000	ERST, COVID
No ID 22	-15.8238000000000	34.9994000000000	ERST, COVID
No ID 24	-15.8134000000000	35.0162000000000	ERST, COVID
No ID 25	-15.8230010000000	35.0215000000000	ERST, COVID
No ID 26	-15.8233000000000	35.0087000000000	ERST, COVID
No ID 29	-15.8146000000000	35.0112000000000	ERST, COVID
No ID 3	-15.7770000000000	35.0310000000000	ERST, COVID
No ID 4	-15.7435000000000	35.0254000000000	ERST, COVID
No ID 7	-15.7357000000000	35.0816000000000	ERST, COVID

10.5.2 Loss rate of Moore Swabs

Table 10.8: Frequency and proportional number of sampling events during the one-year surveillance that lost all Moore swabs

Date	Swab Loss		Swab Recovered		Total
	Frequency	Percentage	Frequency	Percentage	
21/05	2	2%	92	98%	**94**
21/06	1	1%	99	99%	**100**
21/07	0	0%	98	100%	**98**
21/08	1	1%	91	99%	**92**
21/09	1	1%	77	99%	**78**
21/10	2	2%	109	98%	**111**
21/11	3	3%	86	97%	**89**
21/12	5	6%	76	94%	**81**
22/01	33	92%	3	8%	**36**
22/02	19	21%	73	79%	**92**
22/03	9	11%	75	89%	**84**
22/04	8	9%	79	91%	**87**
22/05	3	4%	82	96%	**85**
Total	**87**	**8%**	**1040**	**92%**	**1127**

10.6 Chapter 7: Pathway Modifications

10.6.1 Percentage of positive PCR extractions for Magna Extract methods.

Table 10.9: Percentage of the 12 broths that were positive for all three *S.* Typhi targets (*ttr*, *tviB* and *staG*), at each dilution factor extracted by Magna Extract for the LOD. Cells highlighted in blue represent the method that gave the best result at each dilution, whilst the red represents the lesser performing alternative.

	Pure		
	Original (Table 1.1 – i)	**Membrane, 100 µL (Table 1.1 – v)**	**Membrane, 500 µL (Table 1.1 – vi)**
10^{-5}	58% (n=7)	75% (n=9)	50% (n=6)
10^{-6}	67% (n=8)	67% (n=8)	42% (n=5)
10^{-7}	75% (n=9)	58% (n=7)	33% (n=4)
10^{-8}	42% (n=5)	33% (n=4)	42% (n=5)
	Spiked		
10^{-5}	100% (n=12)	17% (n=2)	17% (n=2)
10^{-6}	75% (n=9)	42% (n=5)	58% (n=7)
10^{-7}	33% (n=4)	8% (n=1)	25% (n=3)
10^{-8}	17% (n=2)	0% (n=0)	17% (n=2)
	Diluted		
10^{-5}	42% (n=5)	50% (n=6)	50% (n=6)
10^{-6}	50% (n=6)	50% (n=6)	67% (n=8)
10^{-7}	58% (n=7)	58% (n=7)	50% (n=6)
10^{-8}	67% (n=8)	67% (n=8)	50% (n=6)

The percentage of broths that were positive for *S.* Typhi, based on amplification of three target genes: *ttr*, *tviB* and *staG*, at each dilution of each alternate of extraction that was put forward as candidates for future implementation for sample screening: method i, v and vi. Each extract was highlighted depending on whether they performed best (in blue), or worst (in red) at each stage. Of the three methods across four dilutions, method i performed the best nine replicates out of 12, whilst giving the worst consistency twice. Comparatively, despite performing well in previous experiments, modification vi performed the worst six out of 12 times, with modification v also being the worst for six out of 12. From this data, it can be inferred that method i works best with complex, highly concentrated samples as the PCR assay is not overwhelmed post-extraction in the same way the higher concentrated extractions of methods v and vi are.

10.7 Sample Results

10.7.1 All BMGF positive Samples May 2021 to April 2022

Table 10.10: All *S.* Typhi and NTS positive samples from the BMGF collections during the one-year ES programme

Sample ID	Sample Type	BMGF Result	Culture Result	Site ID	Date, Time, Time zone	GPS Latitude	GPS Longitude	Moore Swab Loss?	*ttr* Ct	*staG* Ct	*tviB* Ct	SPC Ct	HF183 Ct
ERST_100521_37	Moore Swab	*S.* Typhi	Culture Negative	1154/960	2021-05-10 09:16:00.000+02:00	-15.8315313	34.9918552		31.32	33.28	37.07	25.73	21.03
ERST_100521_16	Moore Swab	*S.* Typhi	Culture Negative	1119/932	2021-05-10 10:52:00.000+02:00	-15.8066309	34.9892692		33.12	34.04	36.33	25.76	23.43
ERST_190521_32	Moore Swab	*S.* Typhi	Culture Negative	1056/1039	2021-05-19 12:13:00.000+02:00	-15.7552267	35.0806966		33.29	35.45	33.05	34.23	
ERST_240521_40	Moore Swab	*S.* Typhi	Culture Negative	2001/1009	2021-05-24 12:07:00.000+02:00	-15.7939468	35.0039249		34.3	38.1	37.9	29.7	23.2
ERST_310521_70	Moore Swab	*S.* Typhi	Culture Negative	1103/1039	2021-05-31 14:30:00.000+02:00	-15.7892118	35.018772		34.9	34.2	35.2	20.4	22.2
ERST_020621_09	Moore Swab	*S.* Typhi	Culture Negative	1119/932	2021-06-02 09:22:00.000+02:00	-15.8075567	34.990719		35.5	35.4	36.4	35.6	26.5
ERST_150621_54	Moore Swab	*S.* Typhi	Culture Negative	1119/932	2021-06-15 14:59:00.000+02:00	-15.8076367	34.9893902		36.94	37.41	31.46	23.78	
ERST_130721_32	Moore Swab	*S.* Typhi	Culture Negative	2002/931	2021-07-13 08:48:00.000+02:00	-15.8083945	34.9906354		32.96	33.15	38.49	30.68	22.65
ERST_130721_33	Moore Swab	*S.* Typhi	Culture Negative	1119/932	2021-07-13 09:15:00.000+02:00	-15.8075088	34.9907433		31.77	31.92	31.47	28.79	19.65
ERST_190721_19	Moore Swab	*S.* Typhi	Culture Negative	1120/1033	2021-07-19 11:06:00.000+02:00	-15.8068606	35.0114597		33.99	33.66	35.63	30.58	26.64
ERST_200721_43	Moore Swab	*S.* Typhi	Culture Negative	1030/877	2021-07-20 13:58:00.000+02:00	-15.7429707	35.0029001		33.71	33.64	37.23	33.99	32.08

ERST_210721_15	Moore Swab	*S.* Typhi	Culture Negative	1119/932	2021-07-21 08:56:00.000+02:00	-15.8075537	34.9907307		31.08	30.94	31.14	27.53	20.98
ERST_280721_04	Moore Swab	*S.* Typhi	Culture Negative	1119/932	2021-07-28 09:41:00.000+02:00	-15.8075087	34.9907531		31.94	31.25	31.49	30.15	25.12
ERST_030821_20	Moore Swab	*S.* Typhi	Culture Negative	1076/944	2021-08-03 12:01:00.000+02:00	-15.7698818	35.0022513		36.61	33.76	34.66	35.28	37
ERST_040821_04	Moore Swab	*S.* Typhi	Culture Negative	1119/932	2021-08-04 08:59:00.000+02:00	-15.80753	34.9907286		30.75	30.66	30.78	26.64	20.44
ERST_110821_07	Moore Swab	*S.* Typhi	Culture Negative	1119/932	2021-08-11 08:55:00.000+02:00	-15.8075403	34.9907432		31.84	31.24	31.93	28.28	20.58
ERST_110821_09	Moore Swab	*S.* Typhi	Culture Negative	2002/931	2021-08-11 09:32:00.000+02:00	-15.8089587	34.9887804		33.82	32.69	32.58	28.06	20.67
ERST_140921_38	Moore Swab	*S.* Typhi	Culture Negative	1054/912	2021-09-14 11:06:00.000+02:00	-15.7561476	35.003387		32.68	31.07	35.91	30.42	38.11
ERST_150921_04	Moore Swab	*S.* Typhi	Culture Negative	1119/932	2021-09-15 09:13:00.000+02:00	-15.8075064	34.9907861		33.99	36.97	34.92	23.09	21.14
ERST_111021_57	Moore Swab	*S.* Typhi	Culture Negative	1144/1017	2021-10-11 10:22:00.000+02:00	-15.8263425	35.0081085		31.54	31.26	33.61	23.42	
ERST_010222_16	Moore Swab	*S.* Typhi	Culture Negative	1120/1033	2022-02-01 12:05:00.000+02:00	-15.7698333	35.0780464		31.93	33.6	31.84	23.5	
ERST_110422_45	Moore Swab	*S.* Typhi	Culture Negative	1151/1108	2022-04-11 10:15:00.000+02:00	-15.8297755	35.0758749		30.73	19.59	31.03	24.35	19.73
ERST_100521_06	Water	*S.* Typhi	Culture Negative	2001/1009	2021-05-10 11:41:00.000+02:00	-15.7941941	35.0036079		31.79	32.88	33.01	21.83	20.76
ERST_310521_04	Water	*S.* Typhi	Culture Negative	1083/1095	2021-05-31 11:05:00.000+02:00	-15.7743214	35.0408808		33.6	32.9	34.1	31.6	27.8
ERST_090621_17	Water	*S.* Typhi	Culture Negative	1119/932	2021-06-09 09:28:00.000+02:00	-15.8075083	34.9907822		28.5	27.44	30.43	27.8	19.98
ERST_280721_01	Water	*S.* Typhi	Culture Negative	1119/932	2021-07-28 09:41:00.000+02:00	-15.8075087	34.9907531		30.3	28.8	30.9	28.9	18.1

ERST_110821_01	Water	*S.* Typhi	Culture Negative	1119/932	2021-08-11 08:55:00.000+02:00	-15.8075403	34.9907432		35.39	34.39	34.7	26.47	17.14
ERST_200921_15	Water	*S.* Typhi	Culture Negative	1123/1090	2021-09-20 11:37:00.000+02:00	-15.8091909	35.0412555		33.64	32.1	32.89	19.7	22.98
ERST_121021_13	Water	*S.* Typhi	Culture Negative	1076/944	2021-10-12 09:01:00.000+02:00	-15.7697582	35.0021413		33.51	32.18	32.8		33.8
ERST_221121_37	Water	*S.* Typhi	Culture Negative	1120/1033	2021-11-22 08:43:00.000+02:00	-15.8070932	35.0113811		31.88	30.09	32.2	22.95	32.65
ERST_100122_01	Water	*S.* Typhi	Culture Negative	1138/916	2022-01-10 08:46:00.000+02:00	-15.8228066	34.9844755	Yes	36.92	32.99	33.54	24.18	38.37
ERST_150222_02	Water	*S.* Typhi	Culture Negative	2004/969	2022-02-15 09:08:00.000+02:00	-15.7305338	35.0625244		29.95	29.16	30.39	22.12	31.91
ERST_080322_01	Water	*S.* Typhi	Culture Negative	1030/877	2022-03-08 10:10:00.000+02:00	-15.7426328	35.004298		33.66	33.25	32.76	21.55	24.13
ERST_100521_14	Moore Swab	NTS with staG	Culture Negative	1127/951	2021-05-10 09:46:00.000+02:00	-15.8120071	34.9906005		34.9	32.58		25.19	26.52
ERST_240521_17	Moore Swab	NTS with staG	Culture Negative	1120/1033	2021-05-24 11:21:00.000+02:00	-15.8068717	35.0114283		35.1	34.2		30.2	25.7
ERST_190721_15	Moore Swab	NTS with staG	Culture Negative	1154/960	2021-07-19 09:36:00.000+02:00	-15.8317117	34.9919421		31.3	31.95		33	31.29
ERST_190721_16	Moore Swab	NTS with staG	Culture Negative	1149/979	2021-07-19 10:02:00.000+02:00	-15.8279599	34.9978462		31.84	31.42		32.8	31.8
ERST_200721_45	Moore Swab	NTS with staG	Culture Negative	1076/944	2021-07-20 10:53:00.000+02:00	-15.7705871	34.9991598		35.6	35		36.5	
ERST_200721_48	Moore Swab	NTS with staG	Culture Negative	1054/912	2021-07-20 11:26:00.000+02:00	-15.7560856	35.003437		32.75	33.58		37.41	36.29
ERST_200721_46	Moore Swab	NTS with staG	Culture Negative	1019/893	2021-07-20 12:27:00.000+02:00	-15.7344288	35.016461		34.99	33.61		28.95	36.6
ERST_210721_19	Moore Swab	NTS with staG	Culture Negative	2001/1009	2021-07-21 10:18:00.000+02:00	-15.7939057	35.0038438		30.7	30.9		23.7	23.8

ERST_210721_25	Moore Swab	NTS with staG	Culture Negative	1076/944	2021-07-21 13:15:00.000+02:00	-15.7697488	35.0021544		32.58	31.51		34.97	32.31
ERST_280721_05	Moore Swab	NTS with staG	Culture Negative	1119/932	2021-07-28 09:58:00.000+02:00	-15.8076259	34.9893648		35.52	33.65		39.29	33.51
ERST_280721_06	Moore Swab	NTS with staG	Culture Negative	2002/931	2021-07-28 10:07:00.000+02:00	-15.8089577	34.9887594		35.32	32.95		32.98	30.42
ERST_240521_43	Water	NTS with staG	Culture Negative	1067/961	2021-05-24 13:47:00.000+02:00	-15.7583819	35.0116844		17.94	23.41		25.75	37.01
ERST_250521_29	Water	NTS with staG	Culture Negative	1030/877	2021-05-25 09:53:00.000+02:00	-15.7430302	35.0028697		33.44	32.95		22.03	39.62
ERST_250521_04	Water	NTS with staG	Culture Negative	1060/929	2021-05-25 10:39:00.000+02:00	-15.7569141	35.0081119		31.1	30.41		35.25	
ERST_020821_05	Water	NTS with staG	Culture Negative	2002/931	2021-08-02 11:30:00.000+02:00	-15.8088803	34.9885923		35.6	12.8		36.4	25.3
ERST_020821_08	Water	NTS with staG	Culture Negative	1154/960	2021-08-02 13:22:00.000+02:00	-15.8317305	34.9919336		36.12	34.75		34.97	27.48
ERST_100821_06	Water	NTS with staG	Culture Negative	1133/1006	2021-08-10 13:49:00.000+02:00	-15.8158603	35.0097583		35.95	18.55		33.57	27.7
ERST_081121_42	Water	NTS with staG	Culture Negative	2001/1009	2021-11-08 11:31:00.000+02:00	-15.7939376	35.0038838		33.09	32.66		36.19	29.24
ERST_100122_02	Water	NTS with staG	Culture Negative	1127/951	2022-01-10 09:12:00.000+02:00	-15.8119742	34.9906789	Yes	33.64	31.26		33.3	31.22
ERST_010222_03	Water	NTS with staG	Culture Negative	1038/1028	2022-02-01 10:35:00.000+02:00	-15.746648	35.0847346		34.9	33.64		28.15	
ERST_100521_13	Moore Swab	NTS	Culture Negative	1138/916	2021-05-10 09:07:00.000+02:00	-15.822808	34.9845407		30.97			26.68	22.14
ERST_240521_34	Moore Swab	NTS	Culture Negative	1116/971	2021-05-24 10:33:00.000+02:00	-15.8047174	34.9936124		35.93			32.32	36.8
ERST_240521_45	Moore Swab	NTS	Culture Negative	1067/961	2021-05-24 13:47:00.000+02:00	-15.7583819	35.0116844		36.6			34.9	

ERST_250521_16	Moore Swab	NTS	Culture Negative	1019/893	2021-05-25 10:00:00.000+02:00	-15.7344918	35.016474		35.41			32.04	
ERST_250521_19	Moore Swab	NTS	Culture Negative	1077/963	2021-05-25 11:35:00.000+02:00	-15.7710433	35.0064785		37.5			33.2	36.61
ERST_310521_16	Moore Swab	NTS	Culture Negative	1083/1095	2021-05-31 11:05:00.000+02:00	-15.7743214	35.0408808		36.8			24.6	34.5
ERST_140621_60	Moore Swab	NTS	Culture Negative	1056/1039	2021-06-14 10:56:00.000+02:00	-15.7552284	35.08067		36.1			24.67	
ERST_140621_48	Moore Swab	NTS	Culture Negative	1038/1028	2021-06-14 11:25:00.000+02:00	-15.7466594	35.0847257		36.41			24.18	
ERST_220621_54	Moore Swab	NTS	Culture Negative	1019/893	2021-06-22 14:31:00.000+02:00	-15.7344202	35.0165114		37.36			24.96	
ERST_190721_17	Moore Swab	NTS	Culture Negative	1144/1017	2021-07-19 10:16:00.000+02:00	-15.8263389	35.0081028		35.2			35.9	
ERST_190721_18	Moore Swab	NTS	Culture Negative	1133/1006	2021-07-19 10:36:00.000+02:00	-15.8144824	35.009194		37				
ERST_200721_41	Moore Swab	NTS	Culture Negative	1076/944	2021-07-20 09:46:00.000+02:00	-15.7676652	35.0036276		36.8			33.7	34.1
ERST_200721_42	Moore Swab	NTS	Culture Negative	1024/904	2021-07-20 12:00:00.000+02:00	-15.7380123	35.0208096		35.9			34.5	32.9
ERST_200721_47	Moore Swab	NTS	Culture Negative	1033/864	2021-07-20 14:46:00.000+02:00	-15.7445863	34.9992817		37.3			37.3	35.1
ERST_210721_17	Moore Swab	NTS	Culture Negative	2002/931	2021-07-21 09:30:00.000+02:00	-15.80901	34.9887424		33.77			31.16	27.09
ERST_210721_18	Moore Swab	NTS	Culture Negative	2002/931	2021-07-21 09:37:00.000+02:00	-15.8089664	34.9887878		36.2			31.8	28.5
ERST_210721_20	Moore Swab	NTS	Culture Negative	1093/945	2021-07-21 10:48:00.000+02:00	-15.7796672	34.9774169		34.13				36.98
ERST_270721_15	Moore Swab	NTS	Culture Negative	1133/1006	2021-07-27 12:50:00.000+02:00	-15.8159271	35.0097653		35.56			32.75	32.29

ERST_100821_14	Moore Swab	NTS	Culture Negative	1133/1006	2021-08-10 13:49:00.000+02:00	-15.8158603	35.0097583		34.5			31.6	28.2
ERST_060921_17	Moore Swab	NTS	Culture Negative	1083/1095	2021-09-06 10:25:00.000+02:00	-15.774304	35.0409121		35.34		36.37	27.98	
ERST_130921_51	Moore Swab	NTS	Culture Negative	1154/960	2021-09-13 09:45:00.000+02:00	-15.8314839	34.9918893		16.8	8.75	34.07	23.9	35.43
ERST_130921_54	Moore Swab	NTS	Culture Negative	1133/1006	2021-09-13 10:49:00.000+02:00	-15.8144791	35.0093342		20.29			26.9	34.75
ERST_140921_39	Moore Swab	NTS	Culture Negative	1067/961	2021-09-14 11:23:00.000+02:00	-15.7594992	35.0115653		23.64			28.44	37.24
ERST_150921_05	Moore Swab	NTS	Culture Negative	1119/932	2021-09-15 09:31:00.000+02:00	-15.8076751	34.9893528		31.74			25.64	32.8
ERST_181021_18	Moore Swab	NTS	Culture Negative	1083/1095	2021-10-18 10:11:00.000+02:00	-15.7743075	35.0408917		35.8			22.85	26.411
ERST_150322_38	Moore Swab	NTS	Culture Negative	1138/916	2022-03-15 13:23:00.000+02:00	-15.8227571	34.98447		33.81			23.41	29.34
ERST_040422_18	Moore Swab	NTS	Culture Negative	1119/932	2022-04-04 09:59:00.000+02:00	-15.8066921	34.9892734		35.45			22.78	
ERST_050422_20	Moore Swab	NTS	Culture Negative	1078/1109	2022-04-05 13:34:00.000+02:00	-15.7718973	35.0448755		35.47			22.85	
ERST_110422_43	Moore Swab	NTS	Culture Negative	1161/1067	2022-04-11 09:38:00.000+02:00	-15.8406679	35.081758		36.49			21.86	
ERST_180422_43	Moore Swab	NTS	Culture Negative	1127/951	2022-04-18 12:38:00.000+02:00	-15.8120399	34.9906324		36.38			24.89	
ERST_100521_29	Water	NTS	Culture Negative	1120/1033	2021-05-10 11:17:00.000+02:00	-15.8068905	35.0114369		30.73			29.97	23.17
ERST_110521_38	Water	NTS	Culture Negative	1093/945	2021-05-11 15:29:00.000+02:00	-15.7796783	34.9774665		33.86			17.48	32.29
ERST_240521_05	Water	NTS	Culture Negative	1120/1033	2021-05-24 11:21:00.000+02:00	-15.8068717	35.0114283		34.92			29.12	26.56

ERST_240521_41	Water	NTS	Culture Negative	2001/1009	2021-05-24 12:07:00.000+02:00	-15.7939468	35.0039249		35.12			29.19	23.65
ERST_250521_06	Water	NTS	Culture Negative	1077/963	2021-05-25 11:35:00.000+02:00	-15.7710433	35.0064785		10.19			35.51	33.65
ERST_310521_01	Water	NTS	Culture Negative	1011/961	2021-05-31 09:33:00.000+02:00	-15.7281957	35.0525462		33.4			30.9	35.7
ERST_310521_49	Water	NTS	Culture Negative	1178/1073	2021-05-31 10:30:00.000+02:00	-15.7698891	35.0780349		37.3			31.7	27.7
ERST_190721_02	Water	NTS	Culture Negative	1154/960	2021-07-19 09:36:00.000+02:00	-15.8317117	34.9919421		16.77			36.22	36.54
ERST_020821_01	Water	NTS	Culture Negative	1138/916	2021-08-02 09:46:00.000+02:00	-15.8228083	34.9844407		35.66			34.19	37.22
ERST_020821_02	Water	NTS	Culture Negative	1127/951	2021-08-02 10:24:00.000+02:00	-15.8119677	34.9906367		36.6		36.7	31.8	29.9
ERST_020821_06	Water	NTS	Culture Negative	2001/1009	2021-08-02 11:50:00.000+02:00	-15.7939552	35.0040309	Yes	36.9			27.2	23.7
ERST_110821_03	Water	NTS	Culture Negative	2002/931	2021-08-11 09:32:00.000+02:00	-15.8089587	34.9887804		34.65			39.51	28
ERST_110821_04	Water	NTS	Culture Negative	2002/931	2021-08-11 09:55:00.000+02:00	-15.8084606	34.9905921		34.12			38.52	36.02
ERST_011121_45	Water	NTS	Culture Negative	1103/1039	2021-11-01 13:22:00.000+02:00	-15.7892011	35.0187139		37.03			23.41	26.01
ERST_091121_41	Water	NTS	Culture Negative	1033/864	2021-11-09 09:07:00.000+02:00	-15.7446061	34.9992966	Yes	38.24			22.94	30.43
ERST_091121_06	Water	NTS	Culture Negative	1060/929	2021-11-09 10:42:00.000+02:00	-15.756922	35.0081293		32.71			24.39	20.97
ERST_221121_38	Water	NTS	Culture Negative	1144/1017	2021-11-22 09:10:00.000+02:00	-15.8264298	35.0080608		36.44			23.64	29.61
ERST_131221_04	Water	NTS	Culture Negative	1064/1015	2021-12-13 09:36:00.000+02:00	-15.7590259	35.0329805		34.45			21.13	30.39

ERST_131221_09	Water	NTS	Culture Negative	1083/1095	2021-12-13 10:39:00.000+02:00	-15.7740907	35.0410588		34.07			22.93	27.78
ERST_100122_05	Water	NTS	Culture Negative	1119/932	2022-01-10 09:58:00.000+02:00	-15.8066157	34.9892941	Yes	36.89			23.13	30.49
ERST_110122_07	Water	NTS	Culture Negative	1030/877	2022-01-11 12:08:00.000+02:00	-15.7427069	35.004278	Yes	34.21			22.27	32.16
ERST_010222_05	Water	NTS	Culture Negative	1086/1098	2022-02-01 11:39:00.000+02:00	-15.7763414	35.0751159	Yes	32.44			27.84	36.34
ERST_070222_11	Water	NTS	Culture Negative	1060/929	2022-02-07 12:52:00.000+02:00	-15.756889	35.008082	Yes	34.39			23.5	29.96
ERST_010322_30	Water	NTS	Culture Negative	1133/1006	2022-03-01 09:38:00.000+02:00	-15.8144407	35.0091641		34.97			21.72	28.78
ERST_070322_01	Water	NTS	Culture Negative	1077/963	2022-03-07 09:00:00.000+02:00	-15.7710558	35.0064451		35.65			24.23	34.22
ERST_070322_28	Water	NTS	Culture Negative	2002/931	2022-03-07 09:42:00.000+02:00	-15.8083609	34.9892798		34.74		29.93	24.65	24.63

10.7.2 All Culture positive Samples May 2021 to April 2022

Table 10.11: All samples with *S.* Typhi or NTS positive isolates during the one-year ES programme.

Sample ID	Sample Type	Culture Result	BMGF Result	Site ID Number	Date, Time, Time zone	GPS Latitude	GPS Longitude	Moore Swab Loss	*ttr* Ct	*sseJ* Ct	*staG* Ct	*tviB* Ct
ERST_230821_22	Moore Swab	*S.* Typhi	N/A	1083	2021-08-23 13:10:00.000+02:00	-15.7757041	35.0794664		23.5		31	32.4
ERST_110422_47	Moore Swab	*S.* Typhi	N/A	2002/931	2022-04-11 11:20:00.000+02:00	-15.8084077	34.990605		20.88		19.81	20.75
ERST_070621_53	Biofilm	NTS	N/A	1149/979	2021-06-07 11:04:00.000+02:00	-15.8279793	34.9979045		N/A	N/A	N/A	N/A
ERST_080621_29	Biofilm	NTS	N/A	1067/961	2021-06-08 15:09:00.000+02:00	-15.7601405	35.011892		N/A	N/A	N/A	N/A
ERST_090621_27	Biofilm	NTS	N/A	1120/1033	2021-06-09 10:58:00.000+02:00	-15.8068748	35.0114337		N/A	N/A	N/A	N/A
ERST_090621_33	Biofilm	NTS	N/A	1076/944	2021-06-09 13:00:00.000+02:00	-15.7697787	35.0021532		N/A	N/A	N/A	N/A
ERST_050721_69	Biofilm	NTS	N/A	1038	2021-07-05 10:02:00.000+02:00	-15.7591323	34.987093		N/A	N/A	N/A	N/A
ERST_050721_30	Biofilm	NTS	N/A	1063	2021-07-05 13:32:00.000+02:00	-15.7584267	35.0117027		N/A	N/A	N/A	N/A
ERST_230821_30	Biofilm	NTS	N/A	1064/1015	2021-08-23 10:01:00.000+02:00	-15.7590154	35.0329655		25.2			
ERST_230821_36	Biofilm	NTS	N/A	1086/1098	2021-08-23 13:50:00.000+02:00	-15.7752506	35.0756272		23.3			
ERST_060921_30	Biofilm	NTS	N/A	1064/1015	2021-09-06 10:18:00.000+02:00	-15.7590376	35.0329941		26.4			
ERST_060921_33	Biofilm	NTS	N/A	1085/1092	2021-09-06 14:07:00.000+02:00	-15.7757456	35.0794542		24.6			
ERST_070921_37	Biofilm	NTS	N/A	2002/931	2021-09-07 14:15:00.000+02:00	-15.8239047	34.9993211		22.5			
ERST_081121_25	Biofilm	NTS	N/A	1103/1039	2021-11-08 09:34:00.000+02:00	-15.7849142	35.0239484		25	23.63		
ERST_161121_13	Biofilm	NTS	N/A	1149/979	2021-11-16 09:44:00.000+02:00	-15.8158193	35.0099502		21.24	20.03		
ERST_301121_30	Biofilm	NTS	N/A	1077/963	2021-11-30 12:22:00.000+02:00	-15.8230213	35.0213699		20.6	19.18		
ERST_141221_07	Biofilm	NTS	N/A	1133/1006	2021-12-14 10:00:00.000+02:00	-15.8146958	35.0111795		21.78	20.38		
ERST_310521_60	Moore Swab	NTS	N/A	1067	2021-05-31 13:14:00.000+02:00	-15.7690208	35.0093044		N/A	N/A	N/A	N/A
ERST_310521_18	Moore Swab	NTS	N/A	1098	2021-05-31 13:30:00.000+02:00	-15.7824367	35.0740705		N/A	N/A	N/A	N/A

ERST_310521_74	Moore Swab	NTS	N/A	1077/963	2021-05-31 14:55:00.000+02:00	-15.79245	34.9976462		N/A	N/A	N/A	N/A
ERST_310521_77	Moore Swab	NTS	N/A	1077/963	2021-05-31 15:24:00.000+02:00	-15.7955189	35.0090096		N/A	N/A	N/A	N/A
ERST_010621_03	Moore Swab	NTS	N/A	1077/963	2021-06-01 09:50:00.000+02:00	-15.7034862	35.0044873		N/A	N/A	N/A	N/A
ERST_020621_25	Moore Swab	NTS	N/A	1120/1033	2021-06-02 12:37:00.000+02:00	-15.8083509	35.0168512		N/A	N/A	N/A	N/A
ERST_070621_19	Moore Swab	NTS	N/A	1046	2021-06-07 11:35:00.000+02:00	-15.7491499	34.9859916		N/A	N/A	N/A	N/A
ERST_070621_59	Moore Swab	NTS	N/A	1033	2021-06-07 14:03:00.000+02:00	-15.7650813	34.9840763		N/A	N/A	N/A	N/A
ERST_080621_07	Moore Swab	NTS	N/A	1033/864	2021-06-08 10:06:00.000+02:00	-15.7446008	34.9993888		N/A	N/A	N/A	N/A
ERST_090621_12	Moore Swab	NTS	N/A	32	2021-06-09 10:48:00.000+02:00	-15.7949476	35.0365435		N/A	N/A	N/A	N/A
ERST_150621_10	Moore Swab	NTS	N/A	1166	2021-06-15 09:58:00.000+02:00	-15.8199143	35.0052204		N/A	N/A	N/A	N/A
ERST_220621_56	Moore Swab	NTS	N/A	1080	2021-06-22 15:11:00.000+02:00	-15.7292923	35.0152604		N/A	N/A	N/A	N/A
ERST_220621_25	Moore Swab	NTS	N/A	1024	2021-06-22 15:12:00.000+02:00	-15.7844936	35.0246286		N/A	N/A	N/A	N/A
ERST_280621_55	Moore Swab	NTS	N/A	1098	2021-06-28 13:24:00.000+02:00	-15.7823492	35.0741144		N/A	N/A	N/A	N/A
ERST_280621_19	Moore Swab	NTS	N/A	1077/963	2021-06-28 13:49:00.000+02:00	-15.7924705	34.9976648		N/A	N/A	N/A	N/A
ERST_280621_58	Moore Swab	NTS	N/A	1083	2021-06-28 15:08:00.000+02:00	-15.7429504	35.0841955		N/A	N/A	N/A	N/A
ERST_290621_39	Moore Swab	NTS	N/A	1133/1006	2021-06-29 09:22:00.000+02:00	-15.703462	35.004492		N/A	N/A	N/A	N/A
ERST_290621_45	Moore Swab	NTS	N/A	1168	2021-06-29 14:17:00.000+02:00	-15.8239164	34.9993072		N/A	N/A	N/A	N/A
ERST_050721_59	Moore Swab	NTS	N/A	1030	2021-07-05 11:31:00.000+02:00	-15.7791515	34.9763808		N/A	N/A	N/A	N/A
ERST_050721_21	Moore Swab	NTS	N/A	1177	2021-07-05 13:54:00.000+02:00	-15.7507412	34.9994041		N/A	N/A	N/A	N/A
ERST_310521_14	Moore Swab	NTS	Negative	2004/969	2021-05-31 10:00:00.000+02:00	-15.7305554	35.0625826		N/A	N/A	N/A	N/A
ERST_310521_47	Moore Swab	NTS	Negative	1038/1028	2021-05-31 10:12:00.000+02:00	-15.7466247	35.0847593		N/A	N/A	N/A	N/A
ERST_070721_29	Moore Swab	NTS	N/A	1077	2021-07-07 10:15:00.000+02:00	-15.7289697	35.0138286		N/A	N/A	N/A	N/A
ERST_070721_59	Moore Swab	NTS	N/A	2	2021-07-07 12:58:00.000+02:00	-15.7668738	35.0305943		N/A	N/A	N/A	N/A

ERST_070721_34	Moore Swab	NTS	N/A	1075	2021-07-07 12:59:00.000+02:00	-15.7436032	35.0251588		N/A	N/A	N/A	N/A
ERST_070721_60	Moore Swab	NTS	N/A	No ID 3	2021-07-07 13:31:00.000+02:00	-15.7770448	35.0310362		N/A	N/A	N/A	N/A
ERST_070721_61	Moore Swab	NTS	N/A	1024	2021-07-07 14:25:00.000+02:00	-15.78454	35.024631		N/A	N/A	N/A	N/A
ERST_120721_28	Moore Swab	NTS	N/A	1096	2021-07-12 11:38:00.000+02:00	-15.7777191	35.0745258		25.8			
ERST_120721_12	Moore Swab	NTS	N/A	9	2021-07-12 14:04:00.000+02:00	-15.7923955	34.9978508		23.9			
ERST_130721_09	Moore Swab	NTS	N/A	1077/963	2021-07-13 09:00:00.000+02:00	-15.695557	34.9918427		N/A	N/A	N/A	N/A
ERST_130721_12	Moore Swab	NTS	N/A	1011/961	2021-07-13 10:42:00.000+02:00	-15.7064189	35.0430437		N/A	N/A	N/A	N/A
ERST_130721_13	Moore Swab	NTS	N/A	1133/1006	2021-07-13 11:43:00.000+02:00	-15.8082474	35.0044127		N/A	N/A	N/A	N/A
ERST_130721_14	Moore Swab	NTS	N/A	1077/963	2021-07-13 12:03:00.000+02:00	-15.8020589	34.9877393		N/A	N/A	N/A	N/A
ERST_210721_21	Moore Swab	NTS	N/A	1030	2021-07-21 11:12:00.000+02:00	-15.7786329	34.9759121		22.1			
ERST_210721_26	Moore Swab	NTS	N/A	1077	2021-07-21 14:09:00.000+02:00	-15.7289123	35.0138291		22.1			
ERST_210721_28	Moore Swab	NTS	N/A	1075	2021-07-21 14:53:00.000+02:00	-15.742176	35.0239629		22.9			
ERST_220721_13	Moore Swab	NTS	N/A	1177	2021-07-22 09:56:00.000+02:00	-15.7770352	35.0310313		23.1			
ERST_220721_19	Moore Swab	NTS	N/A	1177	2021-07-22 13:19:00.000+02:00	-15.7506764	34.9993656		22.7			
ERST_260721_15	Moore Swab	NTS	N/A	1078	2021-07-26 10:12:00.000+02:00	-15.7717851	35.0448021		28.4			
ERST_260721_18	Moore Swab	NTS	N/A	1038	2021-07-26 13:02:00.000+02:00	-15.7466706	35.084758		26.2			
ERST_070621_54	Moore Swab	NTS	Negative	1149/979	2021-06-07 11:04:00.000+02:00	-15.8279793	34.9979045		N/A	N/A	N/A	N/A
ERST_270721_10	Moore Swab	NTS	N/A	1077/963	2021-07-27 09:00:00.000+02:00	-15.6955766	34.9918041		24.4			
ERST_270721_12	Moore Swab	NTS	N/A	1011/961	2021-07-27 09:47:00.000+02:00	-15.6984496	35.0598398		24.1			
ERST_270721_14	Moore Swab	NTS	N/A	1077/963	2021-07-27 11:06:00.000+02:00	-15.8199255	35.0052179		22.8			
ERST_090621_28	Moore Swab	NTS	Negative	1120/1033	2021-06-09 10:58:00.000+02:00	-15.8068748	35.0114337		N/A	N/A	N/A	N/A
ERST_020821_19	Moore Swab	NTS	N/A	2	2021-08-02 13:22:00.000+02:00	-15.8317305	34.9919336		29.7			

ERST_090621_34	Moore Swab	NTS	Negative	1076/944	2021-06-09 13:00:00.000+02:00	-15.7697787	35.0021532		N/A	N/A	N/A	N/A
ERST_140621_13	Moore Swab	NTS	Negative	2004/969	2021-06-14 09:52:00.000+02:00	-15.7305606	35.0625394		N/A	N/A	N/A	N/A
ERST_140621_17	Moore Swab	NTS	Negative	1123/1090	2021-06-14 11:51:00.000+02:00	-15.8094339	35.0412679		N/A	N/A	N/A	N/A
ERST_150621_11	Moore Swab	NTS	Negative	1133/1006	2021-06-15 10:19:00.000+02:00	-15.8157302	35.0100521		N/A	N/A	N/A	N/A
ERST_160821_21	Moore Swab	NTS	N/A	1033/864	2021-08-16 11:16:00.000+02:00	-15.7535554	35.0004944		27.8			
ERST_170821_16	Moore Swab	NTS	N/A	1127/951	2021-08-17 09:27:00.000+02:00	-15.8120005	34.990723		25.5			
ERST_210621_15	Moore Swab	NTS	Negative	1127/951	2021-06-21 09:24:00.000+02:00	-15.8120826	34.9906615		N/A	N/A	N/A	N/A
ERST_050721_60	Moore Swab	NTS	Negative	1154/960	2021-07-05 13:25:00.000+02:00	-15.8315353	34.9917965		N/A	N/A	N/A	N/A
ERST_050721_62	Moore Swab	NTS	Negative	1144/1017	2021-07-05 14:16:00.000+02:00	-15.8263388	35.0081669		N/A	N/A	N/A	N/A
ERST_050721_64	Moore Swab	NTS	Negative	1120/1033	2021-07-05 15:07:00.000+02:00	-15.8069175	35.011449		N/A	N/A	N/A	N/A
ERST_260721_20	Moore Swab	NTS	Negative	1085/1092	2021-07-26 14:06:00.000+02:00	-15.7757486	35.0794878		22			
ERST_230821_26	Moore Swab	NTS	N/A	1083	2021-08-23 14:24:00.000+02:00	-15.7429432	35.0841308		23.9			
ERST_230821_27	Moore Swab	NTS	N/A	1083	2021-08-23 14:35:00.000+02:00	-15.7361348	35.081299		29.4			
ERST_240821_17	Moore Swab	NTS	N/A	1077/963	2021-08-24 09:16:00.000+02:00	-15.7035056	35.0045975		29.8			
ERST_240821_19	Moore Swab	NTS	N/A	1011/961	2021-08-24 09:57:00.000+02:00	-15.7063952	35.0430685		26.5			
ERST_240821_21	Moore Swab	NTS	N/A	1077/963	2021-08-24 11:09:00.000+02:00	-15.8159501	35.0099097		28.3			
ERST_300821_19	Moore Swab	NTS	N/A	1076/944	2021-08-30 10:54:00.000+02:00	-15.7697977	35.0021868		22.4			
ERST_300821_21	Moore Swab	NTS	N/A	1024/904	2021-08-30 12:49:00.000+02:00	-15.7380986	35.0211		29.9		32.1	
ERST_300821_23	Moore Swab	NTS	N/A	1054/912	2021-08-30 13:55:00.000+02:00	-15.7561194	35.0033926		26.2			
ERST_310821_19	Moore Swab	NTS	N/A	2002/931	2021-08-31 10:33:00.000+02:00	-15.8088801	34.9886058		21.6			
ERST_310821_22	Moore Swab	NTS	N/A	1024	2021-08-31 11:48:00.000+02:00	-15.7845228	35.0246409		27.3			
ERST_020821_17	Moore Swab	NTS	Negative	2002/931	2021-08-02 11:30:00.000+02:00	-15.8088803	34.9885923		29.8			

ERST_020821_20	Moore Swab	NTS	Negative	1149/979	2021-08-02 13:38:00.000+02:00	-15.828	34.9976522		24.2			
ERST_310821_24	Moore Swab	NTS	N/A	1144/1017	2021-08-31 13:40:00.000+02:00	-15.8262944	35.0082585		23.4			
ERST_310821_26	Moore Swab	NTS	N/A	1120/1033	2021-08-31 14:27:00.000+02:00	-15.8068957	35.01142		25.1			
ERST_020821_22	Moore Swab	NTS	Negative	1133/1006	2021-08-02 15:07:00.000+02:00	-15.8144938	35.00928		25.5			
ERST_070921_15	Moore Swab	NTS	N/A	No ID 11	2021-09-07 09:01:00.000+02:00	-15.6955479	34.9918082		21.2		23.2	
ERST_070921_22	Moore Swab	NTS	N/A	2002/931	2021-09-07 12:33:00.000+02:00	-15.8251074	34.9682023		22.7			
ERST_160821_15	Moore Swab	NTS	Negative	1177	2021-08-16 09:29:00.000+02:00	-15.755403	34.9809705		24.9			
ERST_170821_22	Moore Swab	NTS	Negative	1154/960	2021-08-17 12:03:00.000+02:00	-15.8315566	34.9918671		23.9			
ERST_070921_27	Moore Swab	NTS	N/A	2002/931	2021-09-07 13:55:00.000+02:00	-15.8082654	35.0043513		20.7			
ERST_130921_50	Moore Swab	NTS	N/A	1103/1039	2021-09-13 09:10:00.000+02:00	-15.7850089	35.0238151		21.9			
ERST_170821_24	Moore Swab	NTS	Negative	1133/1006	2021-08-17 14:16:00.000+02:00	-15.8145034	35.0093628		24.7			
ERST_170821_25	Moore Swab	NTS	Negative	1133/1006	2021-08-17 14:41:00.000+02:00	-15.8068797	35.0114228		23.8			
ERST_230821_21	Moore Swab	NTS	Negative	1083	2021-08-23 12:53:00.000+02:00	-15.7843763	35.084285		24.9			
ERST_130921_56	Moore Swab	NTS	N/A	1041	2021-09-13 13:25:00.000+02:00	-15.7590372	34.9781443		21.5			
ERST_130921_58	Moore Swab	NTS	N/A	1034	2021-09-13 14:25:00.000+02:00	-15.7603743	34.9867913		22.9			
ERST_130921_61	Moore Swab	NTS	N/A	1033	2021-09-13 15:24:00.000+02:00	-15.7786141	34.975924		22.9			
ERST_251021_55	Moore Swab	NTS	N/A	2002/931	2021-10-25 10:29:00.000+02:00	-15.8090271	34.9887438		23.12	21.63		
ERST_261021_53	Moore Swab	NTS	N/A	1046	2021-10-26 09:51:00.000+02:00	-15.7491331	34.9859521		29.99	21.63	29.975	
ERST_310821_23	Moore Swab	NTS	Negative	1149/979	2021-08-31 12:53:00.000+02:00	-15.8280037	34.9978506		29.9			
ERST_070921_26	Moore Swab	NTS	Negative	2002/931	2021-09-07 13:40:00.000+02:00	-15.8084215	34.9906351		25.3			
ERST_130921_14	Moore Swab	NTS	Negative	2002/931	2021-09-13 09:56:00.000+02:00	-15.8088489	34.9885658		22.5			
ERST_130921_52	Moore Swab	NTS	Negative	1149/979	2021-09-13 10:05:00.000+02:00	-15.8279878	34.9977035		21.5			

ERST_130921_55	Moore Swab	NTS	Negative	1120/1033	2021-09-13 11:09:00.000+02:00	-15.8068755	35.0114218		21.7			
ERST_261021_54	Moore Swab	NTS	Negative	1053/929	2021-10-26 10:19:00.000+02:00	-15.7553607	34.9808876		23.31	21.91		
ERST_011121_56	Moore Swab	NTS	N/A	1086/1098	2021-11-01 12:44:00.000+02:00	-15.7956651	35.008993		23.9	22.3		
ERST_011121_13	Moore Swab	NTS	Negative	2004/969	2021-11-01 09:26:00.000+02:00	-15.7305418	35.0625824		24.8	25.4		
ERST_081121_13	Moore Swab	NTS	N/A	1103/1039	2021-11-08 09:34:00.000+02:00	-15.7849142	35.0239484		27.1	25.96		
ERST_011121_51	Moore Swab	NTS	Negative	1085/1092	2021-11-01 09:40:00.000+02:00	-15.7757569	35.0793445		23.97	22.37		
ERST_011121_14	Moore Swab	NTS	Negative	1064/1015	2021-11-01 09:53:00.000+02:00	-15.758972	35.0329373		24.4	22.2		
ERST_011121_15	Moore Swab	NTS	Negative	1083/1095	2021-11-01 11:01:00.000+02:00	-15.7742956	35.0408806		24.8	23.9		
ERST_011121_16	Moore Swab	NTS	Negative	1078/1109	2021-11-01 11:13:00.000+02:00	-15.7719	35.044901		26.2	24.8		
ERST_011121_58	Moore Swab	NTS	Negative	1103/1039	2021-11-01 13:22:00.000+02:00	-15.7892011	35.0187139		24.8	26.3		
ERST_081121_20	Moore Swab	NTS	N/A	1042	2021-11-08 13:16:00.000+02:00	-15.7572595	34.9799147		24.85	23.61		
ERST_081121_14	Moore Swab	NTS	Negative	1154/960	2021-11-08 10:05:00.000+02:00	-15.8315419	34.9918442		24.43	23.58		
ERST_091121_59	Moore Swab	NTS	N/A	1024	2021-11-09 13:08:00.000+02:00	-15.7845501	35.0250001		26.02	24.75		
ERST_091121_25	Moore Swab	NTS	N/A	1077	2021-11-09 14:11:00.000+02:00	-15.7288687	35.013764		23.79	22.72		
ERST_081121_50	Moore Swab	NTS	Negative	1127/951	2021-11-08 10:09:00.000+02:00	-15.8119511	34.9906489		27.42	25.94		
ERST_081121_15	Moore Swab	NTS	Negative	1149/979	2021-11-08 10:24:00.000+02:00	-15.8279411	34.9977016		25.42	24.19		
ERST_161121_08	Moore Swab	NTS	N/A	1133/1006	2021-11-16 10:22:00.000+02:00	-15.8134921	35.0160773		33.47	33.72		
ERST_161121_09	Moore Swab	NTS	N/A	1133/1006	2021-11-16 10:33:00.000+02:00	-15.8083395	35.0168897		29.03	27.7		
ERST_221121_49	Moore Swab	NTS	N/A	1120/1033	2021-11-22 08:43:00.000+02:00	-15.8070932	35.0113811		30.05	30.25		
ERST_081121_51	Moore Swab	NTS	Negative	2002/931	2021-11-08 10:27:00.000+02:00	-15.8092338	34.9892233		24.12	22.88		
ERST_081121_54	Moore Swab	NTS	Negative	2001/1009	2021-11-08 11:31:00.000+02:00	-15.7939376	35.0038838		28.34	27.04		
ERST_221121_51	Moore Swab	NTS	N/A	1144/1017	2021-11-22 09:30:00.000+02:00	-15.8279514	34.9977993		27.7	28		

ERST_221121_15	Moore Swab	NTS	N/A	2002/931	2021-11-22 09:33:00.000+02:00	-15.8090338	34.9887777		26.06	24.9		
ERST_091121_16	Moore Swab	NTS	Negative	1024/904	2021-11-09 09:37:00.000+02:00	-15.7379788	35.0211232		25.5	24.32		
ERST_231121_37	Moore Swab	NTS	N/A	2003/849	2021-11-23 09:57:00.000+02:00	-15.741305	34.9924614		32.83	33.36		
ERST_291121_13	Moore Swab	NTS	N/A	2004/969	2021-11-29 09:21:00.000+02:00	-15.7305638	35.0624859		24.02	28.4		
ERST_291121_14	Moore Swab	NTS	N/A	1064/1015	2021-11-29 09:53:00.000+02:00	-15.7590274	35.0329634		17.4	16.4		
ERST_291121_16	Moore Swab	NTS	N/A	1078/1109	2021-11-29 10:40:00.000+02:00	-15.7718516	35.0448815		30.8	30.1		
ERST_291121_17	Moore Swab	NTS	N/A	1123/1090	2021-11-29 11:24:00.000+02:00	-15.8094149	35.0413049		31.8	30.2		
ERST_291121_53	Moore Swab	NTS	N/A	1086/1098	2021-11-29 11:40:00.000+02:00	-15.7761268	35.0751899		26.04	24.64		
ERST_291121_18	Moore Swab	NTS	N/A	1098	2021-11-29 12:58:00.000+02:00	-15.7823545	35.0742306		31.4			
ERST_291121_54	Moore Swab	NTS	N/A	1083	2021-11-29 13:37:00.000+02:00	-15.7957049	35.0091546		29.9	27.6		
ERST_151121_18	Moore Swab	NTS	Negative	1078/1109	2021-11-15 10:54:00.000+02:00	-15.7718667	35.0448118		21.8	20.93		
ERST_221121_50	Moore Swab	NTS	Negative	1149/979	2021-11-22 09:10:00.000+02:00	-15.8264298	35.0080608		22.8	21.9		
ERST_301121_46	Moore Swab	NTS	N/A	1077/963	2021-11-30 08:50:00.000+02:00	-15.6955336	34.9918239		18.6	22.6		
ERST_301121_47	Moore Swab	NTS	N/A	1077/963	2021-11-30 09:04:00.000+02:00	-15.7034746	35.0044257		20.4	24.8		
ERST_291121_56	Moore Swab	NTS	Negative	1077/963	2021-11-29 14:13:00.000+02:00	-15.7710596	35.0064439		29.9	30.1		
ERST_301121_50	Moore Swab	NTS	N/A	1077/963	2021-11-30 11:04:00.000+02:00	-15.8082578	35.0044036		20.7	19.07		
ERST_301121_18	Moore Swab	NTS	N/A	1077/963	2021-11-30 12:22:00.000+02:00	-15.8230213	35.0213699		21	19.48		
ERST_291121_58	Moore Swab	NTS	Negative	1103/1039	2021-11-29 14:58:00.000+02:00	-15.7890523	35.0189528		24.7	24.4		
ERST_061221_62	Moore Swab	NTS	N/A	1077	2021-12-06 13:29:00.000+02:00	-15.7535784	35.0005737		20.24	21.01		
ERST_061221_65	Moore Swab	NTS	N/A	1061	2021-12-06 13:49:00.000+02:00	-15.7526558	34.9988274		22.4	21.43		
ERST_061221_68	Moore Swab	NTS	N/A	1063	2021-12-06 14:14:00.000+02:00	-15.7583759	35.011776		22.27	20.69		
ERST_061221_17	Moore Swab	NTS	N/A	1103/1039	2021-12-06 14:46:00.000+02:00	-15.7849386	35.0239157		22.93	23		

ERST_061221_45	Moore Swab	NTS	Negative	1127/951	2021-12-06 10:04:00.000+02:00	-15.8120215	34.9907068		22.23	26.5		
ERST_071221_16	Moore Swab	NTS	Negative	1054/912	2021-12-07 10:00:00.000+02:00	-15.7559634	35.0033729		22.8			
ERST_071221_51	Moore Swab	NTS	Negative	1053/929	2021-12-07 10:11:00.000+02:00	-15.7553944	34.9809205		23.07	21.85		
ERST_071221_17	Moore Swab	NTS	Negative	1063	2021-12-07 10:13:00.000+02:00	-15.7594296	35.0115668		19.8	20.18		
ERST_071221_57	Moore Swab	NTS	N/A	32	2021-12-07 12:29:00.000+02:00	-15.79511	35.0365839		20.4	19.18		
ERST_071221_21	Moore Swab	NTS	N/A	2003/849	2021-12-07 13:20:00.000+02:00	-15.7435313	35.0252425		23.82	24.09		
ERST_071221_24	Moore Swab	NTS	N/A	1077	2021-12-07 14:44:00.000+02:00	-15.7289403	35.0137948		21.1	21.75		
ERST_071221_19	Moore Swab	NTS	Negative	1076/944	2021-12-07 11:01:00.000+02:00	-15.7710673	35.006455		23.3	22.1		
ERST_081221_04	Moore Swab	NTS	Negative	2002/931	2021-12-08 09:39:00.000+02:00	-15.8076537	34.9893163		23.3	21.8		
ERST_131221_06	Moore Swab	NTS	N/A	1064/1015	2021-12-13 09:36:00.000+02:00	-15.7590259	35.0329805		20.63	19.44		
ERST_081221_25	Moore Swab	NTS	Negative	1144/1017	2021-12-08 10:21:00.000+02:00	-15.8261844	35.0080428		20.9	19.6		
ERST_131221_41	Moore Swab	NTS	Negative	1085/1092	2021-12-13 09:46:00.000+02:00	-15.7757547	35.0794327		22.33	21.17		
ERST_131221_08	Moore Swab	NTS	Negative	1078/1109	2021-12-13 10:18:00.000+02:00	-15.7718705	35.044911		20.03	18.87		
ERST_131221_20	Moore Swab	NTS	N/A	1092	2021-12-13 14:25:00.000+02:00	-15.7529119	35.0828192		20	20.45		
ERST_141221_02	Moore Swab	NTS	Negative	2002/931	2021-12-14 09:05:00.000+02:00	-15.8084238	34.9906243		22.54	21.25		
ERST_141221_08	Moore Swab	NTS	N/A	1133/1006	2021-12-14 10:00:00.000+02:00	-15.8146958	35.0111795		29.91			
ERST_141221_14	Moore Swab	NTS	N/A	1133/1006	2021-12-14 10:56:00.000+02:00	-15.8421207	35.0519288		22.82	21.32		
ERST_141221_43	Moore Swab	NTS	N/A	1133/1006	2021-12-14 14:00:00.000+02:00	-15.8020331	34.9876952		24.23			
ERST_151221_02	Moore Swab	NTS	N/A	1119/932	2021-12-15 08:32:00.000+02:00	-15.8134742	35.0161019		25.19	25.36		
ERST_151221_04	Moore Swab	NTS	Negative	1119/932	2021-12-15 08:54:00.000+02:00	-15.8074758	34.9908682		18.74	17.87		
ERST_151221_06	Moore Swab	NTS	Negative	1119/932	2021-12-15 09:08:00.000+02:00	-15.8082796	34.9892956		22.09	20.88		
ERST_151221_09	Moore Swab	NTS	Negative	1119/932	2021-12-15 09:23:00.000+02:00	-15.8089605	34.9888236		23.27	23.73		

ERST_170122_02	Moore Swab	NTS	Negative	2002/931	2022-01-17 09:12:00.000+02:00	-15.808431	34.9906057		27.41	25.94		
ERST_140222_25	Moore Swab	NTS	Negative	1123/1090	2022-02-14 15:31:00.000+02:00	-15.8094361	35.041412		19.14	18.26		
ERST_210222_15	Moore Swab	NTS	Negative	1024/904	2022-02-21 10:00:00.000+02:00	-15.7381056	35.0210233		24.33	23.56		
ERST_210222_19	Moore Swab	NTS	Negative	1077/963	2022-02-21 11:53:00.000+02:00	-15.7710664	35.0064534		23.43	22.57		
ERST_010322_41	Moore Swab	NTS	Negative	1120/1033	2022-03-01 09:17:00.000+02:00	-15.8068733	35.0113811		23.9	22.74		
ERST_070322_13	Moore Swab	NTS	Negative	1077/963	2022-03-07 09:00:00.000+02:00	-15.7710558	35.0064451		21.17	20.2		
ERST_220222_40	Moore Swab	NTS	N/A	2002/931	2022-02-22 11:36:00.000+02:00	-15.8089978	34.9887644		23.97	23.03		
ERST_220222_43	Moore Swab	NTS	N/A	1173	2022-02-22 12:51:00.000+02:00	-15.8085276	35.0172161		22.92	23.33		
ERST_280222_52	Moore Swab	NTS	N/A	1067	2022-02-28 13:49:00.000+02:00	-15.7691115	35.0091928		26.64	25.3		
ERST_070322_42	Moore Swab	NTS	Negative	2002/931	2022-03-07 09:42:00.000+02:00	-15.8083609	34.9892798		20.88	20.04		
ERST_010322_17	Moore Swab	NTS	N/A	1024	2022-03-01 13:46:00.000+02:00	-15.7845078	35.0250249		19.83	18.86		
ERST_080322_42	Moore Swab	NTS	Negative	1083/1095	2022-03-08 10:27:00.000+02:00	-15.7741965	35.0409349		20.89	21.45		
ERST_150322_35	Moore Swab	NTS	Negative	1133/1006	2022-03-15 12:13:00.000+02:00	-15.8144864	35.0092931		20.62	25.02		
ERST_220322_13	Moore Swab	NTS	Negative	1064/1015	2022-03-22 08:45:00.000+02:00	-15.7590945	35.0329874		21.86	20.9		
ERST_150322_34	Moore Swab	NTS	N/A	1120/1033	2022-03-15 11:55:00.000+02:00	-15.8069184	35.011474		20.86	21.44		
ERST_280322_12	Moore Swab	NTS	Negative	1151/1108	2022-03-28 11:48:00.000+02:00	-15.8298408	35.0758589		24.09	22.96		
ERST_210322_14	Moore Swab	NTS	N/A	1060/929	2022-03-21 09:16:00.000+02:00	-15.7569043	35.0081707		18.81	17.96		
ERST_210322_16	Moore Swab	NTS	N/A	1054/912	2022-03-21 09:55:00.000+02:00	-15.7561277	35.0033925		23.15	22.16		
ERST_210322_44	Moore Swab	NTS	N/A	1119/932	2022-03-21 10:15:00.000+02:00	-15.8067062	34.9892431		24.1	28.57		
ERST_210322_48	Moore Swab	NTS	N/A	1042	2022-03-21 13:06:00.000+02:00	-15.7569728	34.9800755		24.81	23.73	26.01	
ERST_210322_49	Moore Swab	NTS	N/A	1046	2022-03-21 14:34:00.000+02:00	-15.7492392	34.9859874		20.2	19.38		
ERST_210322_22	Moore Swab	NTS	N/A	1139	2022-03-21 14:47:00.000+02:00	-15.8502533	35.0900579		26.32	26.8		

ERST_290322_17	Moore Swab	NTS	Negative	1086/1098	2022-03-29 11:00:00.000+02:00	-15.7762575	35.0751212		18.28	18.7		
ERST_220322_15	Moore Swab	NTS	N/A	1103/1039	2022-03-22 10:24:00.000+02:00	-15.7892984	35.0186865		23.59	22.55		
ERST_220322_17	Moore Swab	NTS	N/A	1168	2022-03-22 11:30:00.000+02:00	-15.8238454	34.9992837		19.5	20.27		
ERST_220322_39	Moore Swab	NTS	N/A	8	2022-03-22 11:55:00.000+02:00	-15.7957354	35.0090421		22.96	22.1		
ERST_220322_40	Moore Swab	NTS	N/A	9	2022-03-22 12:09:00.000+02:00	-15.7924801	34.997781		23.24	22.29		
ERST_180422_17	Moore Swab	NTS	Negative	1054/912	2022-04-18 10:02:00.000+02:00	-15.7561362	35.0033969		20.98	19.57		
ERST_180422_42	Moore Swab	NTS	Negative	2002/931	2022-04-18 12:14:00.000+02:00	-15.8090185	34.9887939		22.16	20.92		
ERST_250422_15	Moore Swab	NTS	Negative	1123/1090	2022-04-25 10:09:00.000+02:00	-15.8094239	35.0413783		20.6	19.17		
ERST_290322_40	Moore Swab	NTS	N/A	1134	2022-03-29 12:53:00.000+02:00	-15.837489	35.0979832		19.73	18.43		
ERST_290322_19	Moore Swab	NTS	N/A	32	2022-03-29 13:35:00.000+02:00	-15.7950671	35.0367752		28.92	29.38		
ERST_290322_21	Moore Swab	NTS	N/A	1	2022-03-29 14:02:00.000+02:00	-15.7762764	35.0352752		18.1	16.83		
ERST_050422_16	Moore Swab	NTS	N/A	1103/1039	2022-04-05 11:35:00.000+02:00	-15.7892705	35.0186839		21.57	20.34		
ERST_250422_41	Moore Swab	NTS	Negative	1122/1072	2022-04-25 11:54:00.000+02:00	-15.8074283	35.1006828		18.57	17.16		
ERST_120422_46	Moore Swab	NTS	N/A	1120/1033	2022-04-12 09:00:00.000+02:00	-15.8069354	35.0114106		23.94	22.54		
ERST_120422_16	Moore Swab	NTS	N/A	6	2022-04-12 09:18:00.000+02:00	-15.731732	35.0456047		19.8	18.49		
ERST_120422_25	Moore Swab	NTS	N/A	1024	2022-04-12 13:53:00.000+02:00	-15.7843141	35.0247341		19.79	18.48		
ERST_130422_08	Moore Swab	NTS	N/A	1077	2022-04-13 11:14:00.000+02:00	-15.7292228	35.0154291		24.43	22.42		
ERST_250422_42	Moore Swab	NTS	Negative	1132/1116	2022-04-25 12:23:00.000+02:00	-15.8142735	35.0937782		19.96			
ERST_250422_44	Moore Swab	NTS	Negative	1158/1076	2022-04-25 13:28:00.000+02:00	-15.8361555	35.0950685		18.3	16.95		
ERST_180422_22	Moore Swab	NTS	N/A	1135	2022-04-18 13:02:00.000+02:00	-15.8472556	35.0996139		23.32	21.93		
ERST_090621_19	Moore Swab	NTS	NTS	2002/931	2021-06-09 09:28:00.000+02:00	-15.8075083	34.9907822		N/A	N/A	N/A	N/A
ERST_301121_14	Moore Swab	NTS	NTS	1149/979	2021-11-30 09:53:00.000+02:00	-15.8158879	35.0105192		20.8	18.17		

ERST_070921_23	Moore Swab	NTS	NTS with staG	2002/931	2021-09-07 13:04:00.000+02:00	-15.8075462	34.990826		21.2			
ERST_020821_23	Moore Swab	NTS	*S.* Typhi	1120/1033	2021-08-02 15:21:00.000+02:00	-15.8069416	35.011376		26.9			
ERST_310521_03	Water	NTS	Negative	1064/1015	2021-05-31 10:27:00.000+02:00	-15.7590145	35.0329712		N/A	N/A	N/A	N/A
ERST_230821_11	Water	NTS	Negative	1038/1028	2021-08-23 14:10:00.000+02:00	-15.7465953	35.0847097		20.7			
ERST_310821_10	Water	NTS	Negative	1149/979	2021-08-31 12:53:00.000+02:00	-15.8280037	34.9978506		30.5			
ERST_010621_01	Water	NTS	N/A	1077/963	2021-06-01 09:50:00.000+02:00	-15.7034862	35.0044873		N/A	N/A	N/A	N/A
ERST_010621_14	Water	NTS	N/A	1133/1006	2021-06-01 14:37:00.000+02:00	-15.8250547	34.9679976		N/A	N/A	N/A	N/A
ERST_020621_23	Water	NTS	N/A	1173	2021-06-02 12:13:00.000+02:00	-15.8134804	35.0161092		N/A	N/A	N/A	N/A
ERST_060921_01	Water	NTS	Negative	1011/961	2021-09-06 09:53:00.000+02:00	-15.7282252	35.0525603		23.7			
ERST_070621_38	Water	NTS	N/A	1149/979	2021-06-07 11:04:00.000+02:00	-15.8279793	34.9979045		N/A	N/A	N/A	N/A
ERST_070621_05	Water	NTS	N/A	1046	2021-06-07 11:35:00.000+02:00	-15.7491499	34.9859916		N/A	N/A	N/A	N/A
ERST_151121_46	Water	NTS	Negative	1056/1039	2021-11-15 11:04:00.000+02:00	-15.7553235	35.0815867		33			
ERST_221121_01	Water	NTS	Negative	1138/916	2021-11-22 08:54:00.000+02:00	-15.8227609	34.984589		28.8	27.8		
ERST_231121_13	Water	NTS	Negative	1046/878	2021-11-23 09:44:00.000+02:00	-15.7431354	34.9939027		20.54	18.91	20.45	
ERST_131221_44	Water	NTS	Negative	1086/1098	2021-12-13 10:37:00.000+02:00	-15.7752504	35.0756013		25.47	25.99		
ERST_210222_01	Water	NTS	Negative	1024/904	2022-02-21 10:00:00.000+02:00	-15.7381056	35.0210233		23.7	22.75	23.95	
ERST_210222_05	Water	NTS	Negative	1067/961	2022-02-21 11:25:00.000+02:00	-15.759436	35.0115306		24.05			
ERST_280322_10	Water	NTS	Negative	1161/1067	2022-03-28 11:12:00.000+02:00	-15.840705	35.0818317		19.6	18.67		
ERST_280621_42	Water	NTS	N/A	1123/1090	2021-06-28 11:52:00.000+02:00	-15.8091813	35.0412027		N/A	N/A	N/A	N/A
ERST_110422_03	Water	NTS	Negative	1122/1072	2022-04-11 11:02:00.000+02:00	-15.8072253	35.1006403		25.36	24.12		
ERST_070721_06	Water	NTS	N/A	2003/849	2021-07-07 11:46:00.000+02:00	-15.7319303	35.0455644		N/A	N/A	N/A	N/A
ERST_070721_07	Water	NTS	N/A	1075	2021-07-07 12:44:00.000+02:00	-15.7421632	35.0239619		N/A	N/A	N/A	N/A

ERST_070721_46	Water	NTS	N/A	No ID 3	2021-07-07 13:31:00.000+02:00	-15.7770448	35.0310362		N/A	N/A	N/A	N/A
ERST_070721_09	Water	NTS	N/A	1067/961	2021-07-07 14:05:00.000+02:00	-15.760163	35.011895		N/A	N/A	N/A	N/A
ERST_170821_11	Water	NTS	N/A	1133/1006	2021-08-17 14:16:00.000+02:00	-15.8145034	35.0093628		22.9			
ERST_300821_06	Water	NTS	N/A	1030/877	2021-08-30 11:22:00.000+02:00	-15.7429547	35.0029089		35.6			
ERST_310821_06	Water	NTS	N/A	2001/1009	2021-08-31 11:01:00.000+02:00	-15.7939433	35.0038103		22.7			
ERST_310821_11	Water	NTS	N/A	1144/1017	2021-08-31 13:40:00.000+02:00	-15.8262944	35.0082585		25.4			
ERST_070921_09	Water	NTS	N/A	2002/931	2021-09-07 13:04:00.000+02:00	-15.8075462	34.990826		23.8			
ERST_070921_15	Water	NTS	N/A	2002/931	2021-09-07 14:41:00.000+02:00	-15.8068908	35.0114583		21.2		23.2	
ERST_130921_01	Water	NTS	N/A	2001/1009	2021-09-13 09:40:00.000+02:00	-15.7939448	35.0038439		23.9			
ERST_130921_42	Water	NTS	N/A	1120/1033	2021-09-13 11:09:00.000+02:00	-15.8068755	35.0114218		26.2			
ERST_130921_09	Water	NTS	N/A	1063	2021-09-13 14:21:00.000+02:00	-15.7535413	35.0005125		21.1			
ERST_081121_02	Water	NTS	N/A	1154/960	2021-11-08 10:05:00.000+02:00	-15.8315419	34.9918442		26.09	26.03		
ERST_231121_14	Water	NTS	N/A	2003/849	2021-11-23 09:57:00.000+02:00	-15.741305	34.9924614		23.3	24.18		
ERST_061221_46	Water	NTS	N/A	2002/931	2021-12-06 10:15:00.000+02:00	-15.8090047	34.9887419		20.8	25.07		
ERST_070222_05	Water	NTS	N/A	2001/1009	2022-02-07 10:20:00.000+02:00	-15.7942253	35.0036782		23.81	22.64		
ERST_070222_06	Water	NTS	N/A	1076/944	2022-02-07 11:08:00.000+02:00	-15.7698079	35.0021514		24.48	23.23		
ERST_220322_09	Water	NTS	N/A	1173	2022-03-22 12:39:00.000+02:00	-15.8083724	35.0169239		18.86	18.23		
ERST_260422_22	Water	NTS	N/A	1120/1033	2022-04-26 08:52:00.000+02:00	-15.8068672	35.0114092		20.47	20.35		