Development of Environmental Surveillance Methods for the Detection of *Salmonella enterica* serovar Typhi in Wastewater

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A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

Spring 2020

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Program Authorized to Offer Degree:

Environmental and Occupational Health Sciences

University of Washington

Abstract

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Typhoid fever continues to be a leading cause of global morbidity and mortality, especially in developing countries with limited access to safe drinking water and adequate sanitation. Environmental surveillance – the process of examining samples contaminated with fecal matter for the presence of pathogens - is a useful tool for monitoring the circulation of typhoid fever in populations. Many common methods for the detection of *Salmonella enterica* serovar Typhi in environmental samples benefit from an enrichment step. Selenite-based media - the enrichment media commonly recommended for *S.* Typhi - poses occupational and environmental health concerns. This study proposes an alternative enrichment media that does not contain hazardous material. Additionally, this new enrichment media outperforms Selenite F media for the enrichment of both pure cultures of *S.* Typhi and wastewater cultures seeded with *S.* Typhi. This study also reports the estimated limit of detection for the combination of a common wastewater sampling method – membrane filtration – with an enrichment step utilizing this new media. Finally, this study presents a dynamic computational model, designed to predict the concentration of *S.* Typhi bacteria and the probability of detection of *S.* Typhi at various

sampling locations in a wastewater system. This model is utilized in conjunction with a quantitative microbial risk model to estimate the occupational risk of contracting Typhoid fever for environmental samplers handling contaminated wastewater in the field.

Introduction

Typhoid fever is an enteric disease caused by the etiologic agent *Salmonella enterica* serovars Typhi and Paratyphi.[1] Patients with typhoid fever commonly experience prolonged fever, headaches, and abdominal pain.[2] As of 2017, the burden of typhoid fever in low and middle-income countries (LMICs) was estimated to be 17.8 million cases per year, with incidence peaking among children aged 2-4 years old.[3] In 2018, the World Health Organization prequalified the first typhoid conjugate vaccine and has adopted the use of TCVs in parts of the world with a high burden of typhoid.[4,5] Countries considering how and where to provide TCVs need accurate and geographically representative information about typhoid incidence in urban and rural areas.[6] However, the true burden of typhoid is difficult to determine, and data on the incidence of typhoid fever in LMICs remains insufficient.[3] Typhoid fever surveillance is traditionally reliant on clinical-specimen culture surveillance, which requires the detection of *S.* Typhi in blood, bone marrow, stool, or urine of individual patients.[2] This type of surveillance is resource-intensive, requires large numbers of participants, and depends on robust laboratory and medical infrastructure.[4]

Environmental surveillance, defined as the process of monitoring the circulation of an infectious disease in a population by examining local wastewater (or sewage) and wastewater-impacted natural waters for the presence of the disease-causing pathogen, has been proposed as an alternative for routine surveillance.[4,7] Environmental surveillance offers an anonymous, non-invasive approach to monitor disease circulation, providing the potential for better geographical and temporal resolution for disease incidence estimates.[4] Additionally, a proportion of individuals infected with *S.* Typhi are known to become asymptomatic, chronic carriers, capable

of shedding large numbers of bacteria into the environment.[8,9] Therefore, environmental surveillance may be an ideal initial method for monitoring typhoid transmission in populations.

Environmental surveillance strategies for *S.* Typhi present several challenges. As noted by Hovi et al. in a review on poliovirus environmental surveillance, it is difficult to identify representative sampling sites in parts of the world that lack sewer networks.[7] The identification of an optimal sampling time that does not miss peak pathogen concentrations in sewage presents an additional challenge.[7] Finally, the sensitivity of an environmental surveillance strategy is limited by the sensitivity of laboratory procedures applied to the detection of the relevant pathogen.[10]

The goal of the studies presented here are centered around improving sampling and detection strategies for Typhoid environmental surveillance and examining potential occupational safety concerns for both field samplers and laboratory workers involved in environmental surveillance.

Specific Aims

Aim 1: Develop an enrichment broth to selectively enrich *Salmonella enterica* serovar Typhi bacteria in wastewater samples and improve the sensitivity of detection methods for *S.* Typhi.

Aim 2: Calculate the limit of detection of membrane filtration with the addition of an enrichment step in combination with a qPCR assay for the detection of *Salmonella* Typhi.

Aim 3: Build a dynamic mathematical model to predict the probability of detection of *S.* Typhi in wastewater and use model output to predict the risk of illness for environmental surveillance samplers interacting with contaminated waters.

Aim 1. Develop an enrichment broth to selectively enrich *Salmonella enterica* serovar Typhi bacteria in wastewater samples and improve the sensitivity of detection methods for *S.* Typhi.

Introduction

Current methods used for the detection of one of the causative agents of typhoid fever, *Salmonella enterica* serovar Typhi (*S.* Typhi), in environmental samples benefit from an enrichment step that promotes the growth of *S.* Typhi and inhibits other competing bacterial species.[19] In environmental samples, the target organism may be present in numbers as low as one to ten colony forming units (CFUs) among 10^4 to 10^7 CFUs of background flora.[11] Inhibition of background flora is especially relevant in raw sewage, which is estimated to have at least 4.4 x 10^7 actively respiring, culturable bacteria per milliliter, which may include between 17 and 268 different bacterial species.[12,13]

Additional motivation for enrichment arises from the fact that detection assays for pathogens, such as quantitative PCR and immunoassays, often have a relatively high detection limits; qPCR assays tend to have detection limits in the range of 10^3 to 10^4 CFUs per mL.[11] More specifically, the qPCR assay used in this study for the identification of *S.* Typhi has a reported detection limit of 10^2 CFU per mL of blood.[14] The detection limit of this assay in wastewater samples may be even higher than that reported, since reduced detection sensitivity is known to be an issue when qPCR is performed in sewage or sludge, given the suspected presence of many known PCR inhibitors, such as metal ions, polysaccharides, and humic acid.[15] Given this potentially high detection limit, enrichment offers a way to improve the sensitivity of methods for detecting *S.* Typhi in wastewater by allowing low numbers of target bacteria in a concentrated sample to multiply and reach numbers that are detectable by qPCR.

There are few enrichment options available for the selective recovery of *S.* Typhi or *S.* Paratyphi from wastewater samples. Enrichment media that have often been recommended for the recovery of *Salmonella spp.* include selenite media (such as Selenite F and Selenite Cystine), tetrathionate (TT) media, and Rappaport-Vassiliadis (RV) media. Various formulations of both TT media and RV media have been developed, but none of these formulations are ideal for the recovery of *S.* Typhi from environmental samples. RV media, which contains malachite green as a selective inhibitor, has been repeatedly shown to slow or inhibit the growth of *S.* Typhi and is ineffective for recovering low numbers of *S.* Typhi.[16–18] Studies with TT media have also demonstrated that it is also relatively ineffective for the recovery of *S.* Typhi bacteria and may inhibit its growth.[17] A version of tetrathionate broth supplemented with brilliant green was completely ineffective at recovering *S.* Typhi or *S.* Paratyphi bacteria from pure cultures.[16] Variations of selenite media, including Selenite F and Selenite Cystine, consistently demonstrate the best performance for isolating *S.* Typhi bacteria, as compared to other media developed for *Salmonella spp.*[19–21] A selenite medium was first used for the isolation of *S.* Typhi in 1916, and this original medium was further improved upon by Einar Leifson in 1936.[22] This medium's ability to recover *S.* Typhi has been corroborated by multiple other studies.[16] It was concluded in 1959 that selenite broth was the most suitable enrichment broth for *S.* Typhi and, since that time, this recommendation has remained consistent.[19]

However, various studies have shown that selenite media may not be ideal for the isolation of *Salmonella* spp. from water samples containing stressed or injured cells, due to the toxicity of the media for *Salmonella* bacteria.[17,23] A study comparing the growth of several *Salmonella* spp. in

multiple enrichment broths found that all eight strains of *Salmonella spp.* tested (including *S.* Paratyphi) showed an initial decrease in viable cells upon inoculation into Selenite F media, which was followed by recovery and exponential growth. Although *S.* Typhi was not tested in this study, if a similar decrease in viable *S.* Typhi bacteria occurs in Selenite F media, this could decrease the ability to recover these bacteria from environmental samples, which may have very low numbers of *S.* Typhi.[24] A study evaluating an enrichment step prior to detection via sandwich ELISA assay determined that better ELISA values were obtained for *S.* Typhi cultures grown in BPW, a pre-enrichment medium with no inhibitory agents, than for cultures grown in Selenite Cystine broth.[25]

Selenite media also poses risks for laboratory workers and the aquatic environment. The inhibitory component of selenite media is sodium hydrogen selenite. Safety data sheets of these media indicate that this component is toxic if swallowed or inhaled, potentially fatal if swallowed or inhaled, can cause damage to organs through prolonged exposure, and is severely toxic to aquatic life.[26,27] Selenite media should be handled and prepared with either adequate respiratory protection (including a full-face supplied air respirator) or with appropriate engineering controls, such as a fume hood or a Type B2 Biosafety Cabinet, both of which offer protection for the operator from chemical hazards.[26,27] However, many laboratories in low and middle-income countries, where environmental surveillance for Typhoid is likely to be performed, may not have these engineering controls or the necessary personal protective equipment.

A common method for the decontamination of spent media in laboratories is autoclaving. This is concerning for selenite media, because selenium dioxide can be generated when selenium is heated. This compound is a respiratory irritant and mutagen that can cause a number of acute health problems (headache, nausea, vomiting, abdominal pain, fatigue, and pulmonary edema) and chronic health problems (damage to the liver, kidneys, and nervous system).[28,29] The disposal of spent media containing selenium, is further complicated by its classification as a hazardous waste by the United States Environmental Protection Agency; it cannot be allowed to enter waterways, sewers or drains.[30] High concentrations of selenium can cause severe reproductive impairments in fish and aquatic invertebrates and can bioaccumulate in the aquatic food chain.[31]

In light of the limitations pertaining to the safe use and disposal of selenite media in laboratories, the goal of this study is to develop a new enrichment media that does not pose significant occupational and environmental health challenges and that will selectively grow *S.* Typhi while inhibiting most gram-positive bacteria and at least some gram-negative bacteria.

Bovine bile and extracted bile acids were considered as possible inhibitory agents for this new enrichment media. Bile was considered due to its inhibitory effects on many gram-positive bacteria.[32] In addition to inhibiting many gram-positive bacteria, there is some evidence that *S.* Typhi may be more resistant to the deleterious effects of bile than other common gram-negative gastrointestinal bacteria, due to *S.* Typhi's ability to colonize the gall bladder. In the human gallbladder, concentrations of bile can reach 8%, as compared to intestinal concentrations ranging from 0.2-2%.[33] Reported Minimal Bactericidal Concentrations for *S.* Typhi range from

30% to over 60% bile.[33] Therefore, although many gastrointestinal bacteria are adapted to survive the stress imposed by bile, there is some evidence that, at high concentrations of bile, *S.* Typhi may have an advantage over other gastrointestinal bacteria.

Extracted bile acids were evaluated in two forms. Sodium choleate – a crude bile extract containing the sodium salts of taurocholic, glycocholic, deoxycholic, and cholic acid - was considered.[34] This extract was considered according to an assumption that extracted and purified bile acids may pose a stronger selective pressure on organisms that are not as well-adapted to bile as *S.* Typhi.

Sodium deoxycholate – a purified extract of a single type of bile acid - was also evaluated as a potential inhibitor. This bile acid was chosen because *S.* Typhi has been shown to be particularly resistant to this bile acid (with a reported minimum inhibitory concentration (MIC) of 20%) as compared to *Salmonella typhimurium* (with a reported MIC of 5%).[35] Additionally, sodium deoxycholate has been shown to be the bile acid that most effectively inhibits the growth of both stressed and non-stressed *Escherichia coli*.[36]

In addition to inhibitory ingredients, Ferrioxamine E was also added to media formulations due to its potential to provide a selective advantage for *Salmonella spp.* bacteria over other closely related bacterial species, including *E. coli*. A previous study with *Salmonella enterica* serovar Typhimurium showed that microbial trihydroxamate siderophore ferrioxamine E was extremely effective in resuscitating *Salmonella* bacteria after long-term stress in water microcosms.[37] The selective advantage is specific for bacteria that are capable of using ferrioxamines as a source of

iron, and will not effectively resuscitate *E. coli*.[38] The gene (*foxA*), which has been shown to be required for the utilization of ferrioxamine molecules B, E, and G, is also expressed in *Salmonella typhi*.[38]

Materials and Methods

Bacterial Strains

S. Typhi Ty2 cultures were obtained courtesy of the laboratory of Dr. Stephen Libby in the Department of Laboratory Medicine at the University of Washington. The Ty2 strain was chosen for these initial studies because it is a well-studied pathogenic strain of *S.* Typhi that does not contain plasmids or drug resistance cassettes.[39] A latex agglutination test to confirm the presence of the Vi capsular antigen was performed prior to all experiments with cultures of our Ty2 strain in order to confirm that our experimental cultures had not undergone significant mutations over time. An ATCC Strain 29212 of *Enterococcus faecalis* was used to model gram-positive bacterial response to various enrichment media.

Wastewater Samples

Grab samples of raw influent wastewater were obtained courtesy of the Wastewater Treatment Division of King County, Washington. Samples were collected from the West Point Treatment Plant, located in Seattle, Washington.

Preparation of Media

Selenite F (HiMedia M052) and Universal Pre-Enrichment Broth (BD Biosciences 223510) were prepared according to manufacturer's instructions. Buffered peptone water was prepared by dissolving 10 g Soya Peptone, 5 g NaCl, 3.5 g Disodium Phosphate, and 1.5 g of Monopotassium phosphate in one liter of water, followed by autoclave sterilization. Buffered peptone water with

bile was prepared by adding bovine bile (Sigma-Aldrich B3883) at a concentration of 0.3 g/L after autoclaving and re-sterilizing the media by boiling for 10 minutes. Sodium choleate and sodium deoxycholate were prepared by dissolving sodium salts in water at two times the desired final concentration and filter sterilizing the solution with 0.22um filters. Filter-sterilized 2x solutions were then mixed with autoclaved 2x solutions of buffered peptone water to create final 1x solution of BPW containing the desired percent concentration (wt/vol) of sodium deoxycholate (Sigma-Aldrich D6750) or sodium choleate (Sigma-Aldrich S9875). A working stock of Ferrioxamine E stock (Sigma-Aldrich 38266) was prepared by dissolving Ferrioxamine E in distilled water and filter sterilizing. Stock was then added to BPW to a final concentration of 75 ng/mL.

Adapted Miller-Mallinson (MM) media was formulated according to the formula outlined in Mallinson et al., with X-gal, Tergitol (Niaproof) 4, and agar removed.[40] For these experiments, MM media consisted of following amounts of each ingredient, per liter of deionized water: sodium thiosulfate (6.8 g), ferric ammonium citrate (0.8 g), Trizma base (0.7 g), Trizma hydrochloride (2.3 g), alpha-lactose (10.0 g), D(+) cellobiose (5.0 g), D-Mannitol (1.2 g), D(+) trehalose dihydrate (1.33 g), Sodium chloride (3.0 g), Yeast extract (3.0 g), Beef extract (2.0 g), and Polypeptone peptone (3.5 g).[40]im These ingredients were dissolved in 1 liter of deionized water and the solution was heated to boiling for 10 minutes.

100x “aromix” was prepared according to instructions provided courtesy of Dr. Stephen Libby. This 100x stock consists of L-Phenylalanine (4 mg/mL), L-Tryptophan (4 mg/mL), 2,3-Dihydroxybenzoic Acid (1 mg/mL), and Para-Aminobenzoic Acid (1 mg/mL). The ingredients

were dissolved in deionized water and filter sterilized. This “aromix” stock was then added to various media to achieve final 1x concentrations.

Preparation of Inocula

For pure enrichment cultures, an overnight culture of *S.* Typhi Ty2 was diluted in PBS to the desired concentration. 1 mL of this seed stock was added to 99 mL of enrichment media.

For wastewater enrichment cultures, an overnight culture of *S.* Typhi Ty2 was diluted in wastewater at the desired concentration. 1 mL of this wastewater seed stock was added to 99 mL of enrichment media.

For enrichment experiments with higher volumes of wastewater, an overnight culture of *S.* Typhi Ty2 was diluted in PBS. The diluted bacteria were then seeded at the desired concentration into 100 mL of wastewater. This seeded sample was divided into two 50 mL volumes, which were each combined with 50 mL of 2x enrichment media.

Growth Curves

Pure and wastewater enrichment cultures were both incubated in 250-mL vented Erlenmeyer flasks (Thermo Scientific 4115-0250) at 37$_{o}$C at 225 RPM. For wastewater cultures, after 24 hours, cultures were removed from the incubator and divided into 50 mL conical tubes. The conical tubes were then centrifuged at 3500 x g for 30 minutes. The supernatant was removed, and pellets were stored at -20$_{o}$C for extraction. For pure cultures, 1 mL aliquots of the enrichment culture were removed at regular time points over 24 hours. The goal was to sample every 2-3 hours, although time points were occasionally missed, or bacteria overgrew and were

not countable. These 1 mL aliquots were serially diluted and plated on LB agar with 1x "aromix." Plated bacteria were allowed to grow for 24 hours at 37$_{o}$C and then counted.

qPCR

DNA extractions were performed using the QIAamp PowerFecal Pro DNA Kit (Qiagen 12830) according to the kit's instructions. Final extracted DNA was eluted in 60 uL of elution buffer. Real-time qPCR was performed according to the assay described in Nga et al., 2010.[14]

The primer sequences were as follows:

Forward: 5' - CGC GAA GTC AGA GTC GAC ATA G – 3'

Reverse: 5' – AAG ACC TCA ACG CCG ATC AC – 3'

Probe: 5' - [6FAM] CAT TTG TTC TGG AGC AG GCT GAC GG [TAMRA] – 3'

These primers and probers were designed to produce a 131 bp amplicon within the gene STY0201 (known as the *staG* gene).[41] PCR reactions were cycled according to the following steps: 15 minutes at 95$_{o}$C followed by 45 cycles of 30 sec at 95$_{o}$C, 30 seconds at 60$_{o}$C, and 30 sec at 72$_{o}$C.

qPCR Quantification

Log$_{10}$CFU estimates were calculated from CQ values in qPCR assays according to a standard curve methodology. In order to create standard curves for each qPCR assay, 1 mL Ty2 cultures, with known CFU quantities, were extracted. The pure extracts were then used to make 10-fold serial dilutions and these dilutions were assayed using the qPCR assay described above. For each qPCR assay performed, Log$_{10}$CFU estimates were then calculated by using the CQ value for each unknown sample and the linear equation created by the standard curve to estimate the log$_{10}$CFU in that sample. Similar to reports from Nga et al., 2010, the lower limit of quantification with this standard curve appeared to correspond to 10$_{2}$ CFU.

Statistical Analyses

The statistical significance of results were evaluated using Welch's two-sample t-test, which does not assume equal variances between samples.[42] These t-tests were performed using the base t-test package in R 3.6.1, with a significance level of 0.05.[43]

Results

Growth Cultures of Pure *S.* Typhi Ty2

In order to analyze the proposed enrichment media, growth curves of pure cultures of *S.* Typhi Ty2 (hereafter referred to as Ty2) inoculated into 100 mL of various media were performed.

Initially, comparison growth curves were performed with three media bases, without any inhibitory chemicals added, in order to determine if one of these media was preferable for growing Ty2. (Figure 1). Because Ty2 grew equally well in all three media, the same three media were tested with 3% bile added as an inhibitory agent

Figure 1. Growth Curves of Ty2 in 3 Media Bases

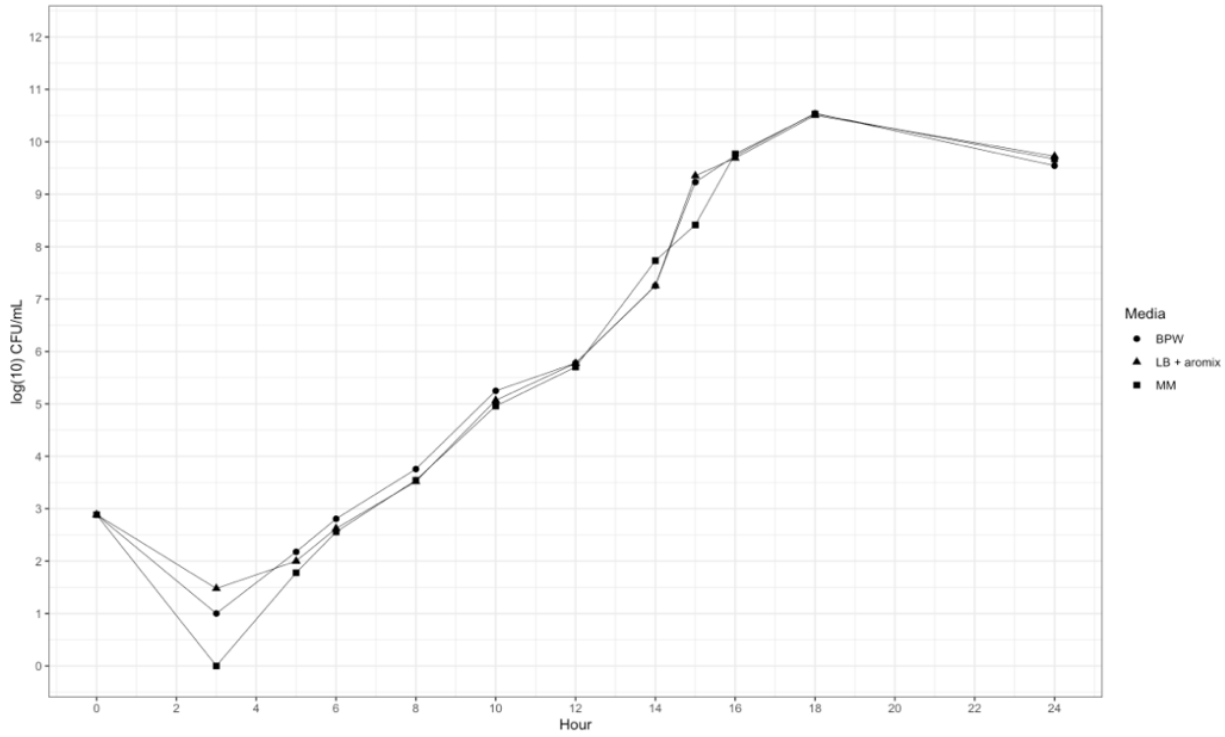

Figure 1. Growth curves of Ty2 bacteria in BPW, MM, or LB.

Figure 2. Growth Curves of Ty2 in 3 Media Bases with 3% Bovine Bile

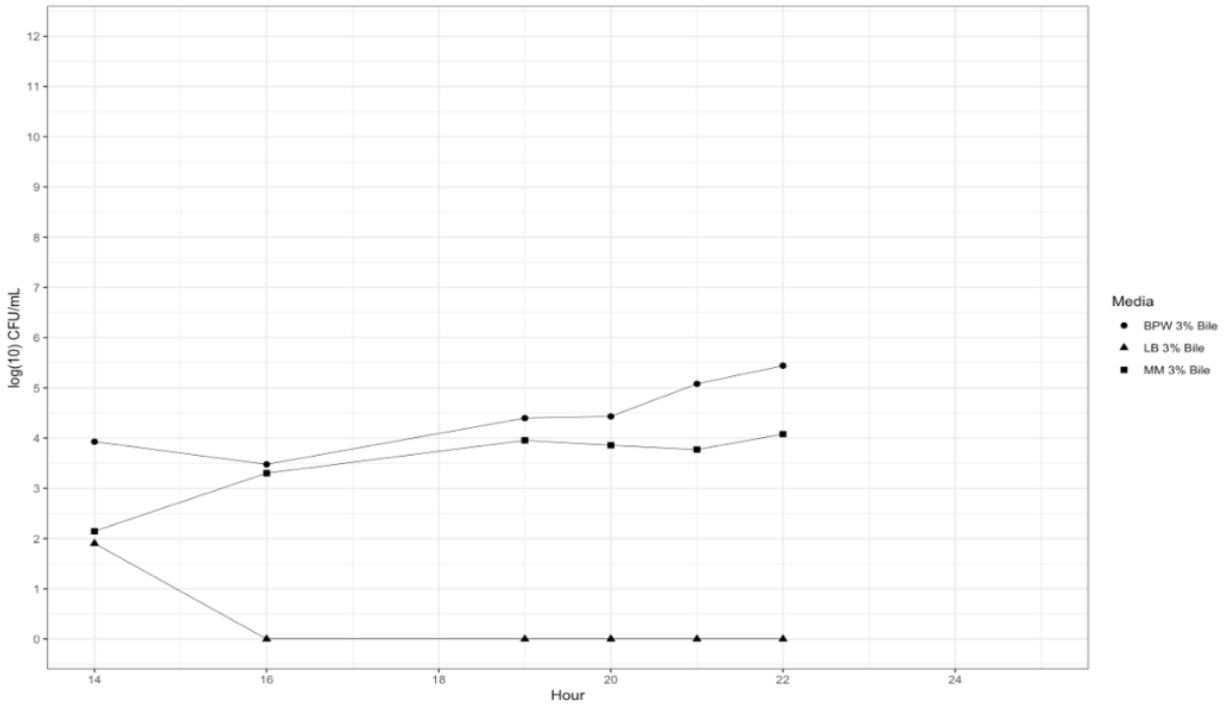

Figure 2. Growth curves of Ty2 bacteria in BPW, MM, or LB with 3% wt/vol bovine bile.

For growth curves of Ty2 with 3% bovine bile, preliminary experiments had not shown any growth before 14 hours, so time points were only collected between 14 and 24 hours. 3% bile appeared to substantially inhibit the growth of Ty2 (Figure 2). Of the three media, BPW allowed for the most robust growth of Ty2 in the presence of bile, although the final concentration still demonstrated a 4-log reduction, as compared to BPW without bile. LB media with 3% bile did not result in any growth of Ty2. Adapted MM media resulted in growth comparable, if slightly lower, then BPW. Thus, BPW was chosen as the optimal base media moving forward. Additionally, BPW is simpler to make than adapted MM, requires fewer ingredients, and is less expensive, further supporting the decision to move forward with buffered peptone water (See Materials and Methods for preparation details).

For the analysis of extracted bile acids, growth curves were first performed with *E. faecalis* to determine whether these acids, at various concentrations, could inhibit a common gastrointestinal gram-positive bacterium. Brain Heart Infusion Broth was used as a control media.

Figure 3. Growth of *E. faecalis* in Media with Varying Concentrations of Bovine Bile Acids

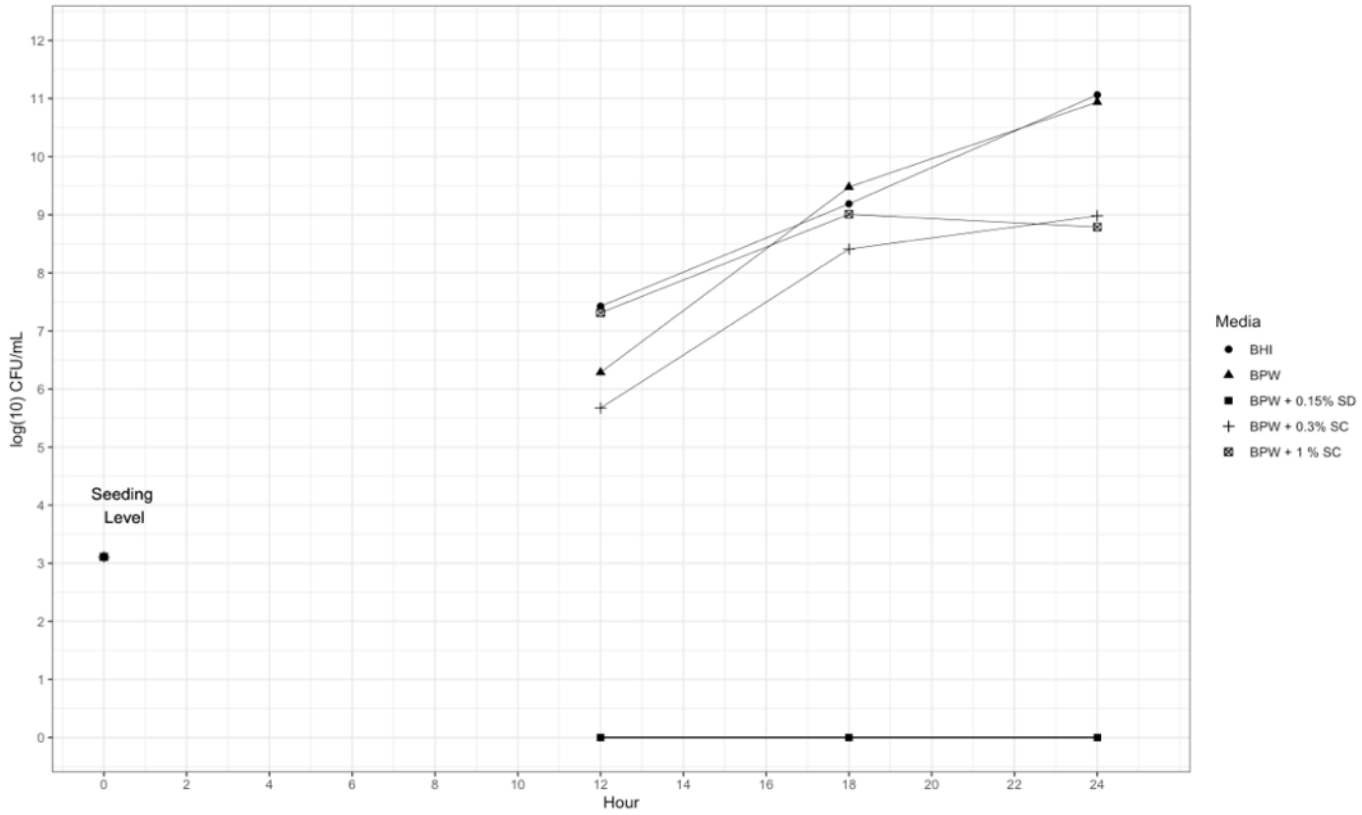

Figure 3. Measurements of E. faecalis growth were taken in control media (Brain Heart Infusion broth (BHI)) and compared to growth in BPW with varying concentrations of extracted bovine bile acids.

Sodium deoxycholate, event at a concentration as low as 0.15%, appeared to completely inhibit the growth of *E. faecalis* in BPW (Figure 3). Sodium choleate, in concentrations ranging from 0.3% to 1% wt/vol, did not appear to effectively inhibit the growth of *E. faecalis* (Figure 3). Thus, sodium deoxycholate was identified as the more suitable inhibitory agent.

The growth of *S.* Typhi Ty2 was then analyzed in two different concentrations of sodium deoxycholate. Although 1% SD appears to slow the growth of Ty2 slightly, it does not substantially affect the final concentration (Figure 4). 0.15% SD does not appear to inhibit the growth of Ty2 at all, resulting in a growth curve very similar to BPW without any bile acids (Figures 1 and 4). Because 0.15% SD was sufficient to completely inhibit *E. faecalis* while allowing Ty2 bacteria to grow at a normal rate, this concentration of sodium deoxycholate was identified as the concentration that may exhibit the best performance for enriching *S.* Typhi bacteria in wastewater.

Figure 4. Growth of Ty2 in the Presence of Sodium Deoxycholate

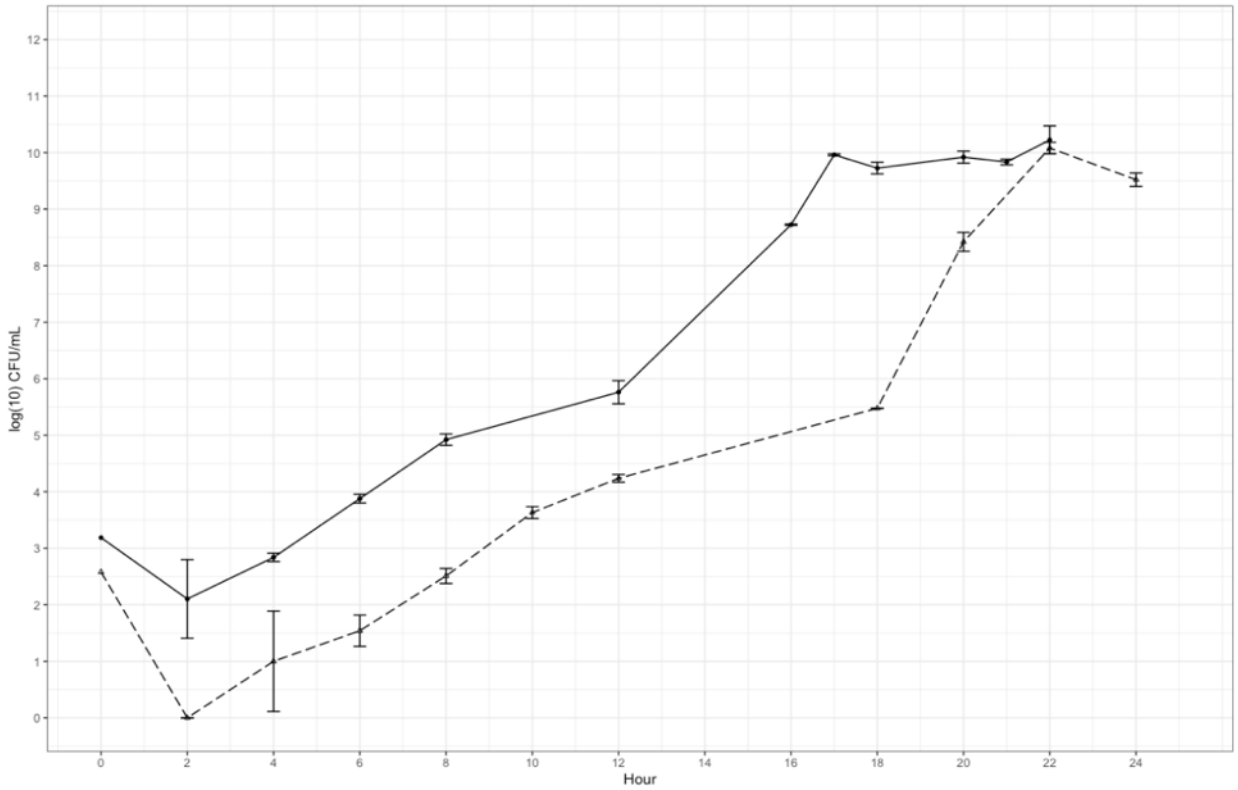

Figure 4. Growth curves of Ty2 bacteria in BPW with 0.15% wt/vol sodium deoxycholate (solid line) vs. 1% wt/vol sodium deoxycholate (dashed line).

In order to compare the new enrichment media (BPW with 0.15% SD) to traditional media, this media was tested against Selenite F broth for the growth of pure Ty2 cultures. Figure 5 clearly demonstrates that Selenite F substantially inhibits Ty2, resulting in a 24-hour concentration that is nearly 5-log lower than the final concentration of Ty2 in BPW with 0.15% SD. This appears to be promising evidence that a new enrichment media may be as effective or more effective than Selenite media for wastewater enrichment.

Figure 5. Growth of Ty2 in Selenite F vs. BPW with 0.15% wt/vol Sodium Deoxycholate

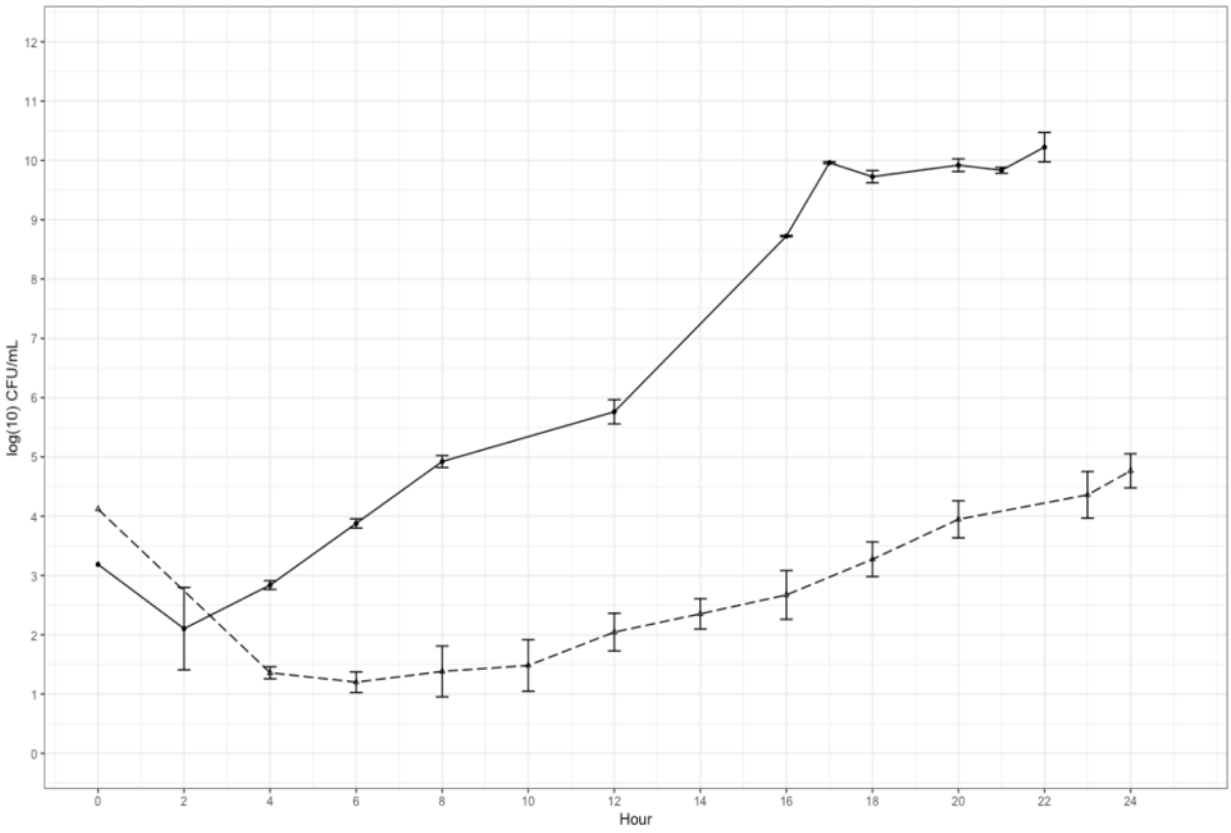

Figure 5. Growth curves of Ty2 bacteria in BPW with 0.15% wt/vol sodium deoxycholate (solid line) vs. Selenite F media (dashed line).

Wastewater Enrichment:

First, wastewater enrichments were performed by adding 1 mL of raw influent wastewater, seeded with pure Ty2 culture, to 99 mL of various media and were allowed to grow for 24-hours before centrifugation and extraction of the resulting pellet. For the experiments outlined in Table 1, the initial seeding of Ty2 ranged from 50 – 500 CFU / 100 mL.

Table 1: 24-Hour Enrichment of 1 mL of Ty2-Seeded Wastewater

	Media					
	BPW	BPW 0.15% SD	BPW 3% Bile	BPW 1% SD	LB 3% Bile	Selenite F
n	3	3	9	3	6	3
Mean Log Change in CFU	3.294	3.091	2.698	1.988	1.030	-0.428
SD	0.188	0.038	1.074	1.127	0.524	1.264

Table 1 shows the estimated mean log change in Ty2 bacteria, given the various initial seeding amounts and the final estimated concentration (as calculated by a standard curve). Compared to all of the possible enrichment media tested, Selenite F performed the worst. Upon analyzing these results, one concern was that these experiments were performed on different days, with different seeding concentrations and different wastewater samples, possibly introducing excess variability and producing incomparable results.

To address this variability, a side-by-side enrichment experiment was performed with four media, seeded on the same day, with the same wastewater sample, and the same seed stock. All samples were seeded with approximately 50 CFU / 100 mL. The results are shown in Table 2.

Table 2: Side-by-Side Enrichment of 1 mL of Ty2-Seeded Wastewater

	Media			
	BPW	BPW + 0.15% SD	BPW + 1% SD	Selenite F
n	3	3	3	3
Log_{10} CFU Seeded	1.7	1.7	1.7	1.7
Mean Final Log_{10} CFU	4.993	4.798	3.687	1.271
Mean Log_{10} Change	3.3	3.1	2.0	-0.4
SD	0.188	0.038	1.127	1.264

The final mean log_{10}CFU estimates of cultures enriched in BPW + 0.15% SD were 3.55 higher than the final mean log_{10}CFU estimates of cultures enriched in Selenite F ($p = 0.01$) (Table 2). The final mean log_{10}CFU estimates of cultures enriched in BPW + 1% SD were 2.18 higher than the final mean log_{10}CFU estimates in Selenite F, although this difference in means did not achieve statistical significance ($p = 0.098$). There was no statistically significant difference between the final mean log_{10}CFU estimates in BPW with 0.15% SD and those in BPW with no SD.

These results seem to indicate that BPW without any inhibitory agent performs just as well as BPW with SD added. This was unexpected. It may be that, when enriching or pre-enriching

samples with low levels of competing bacteria for analysis by qPCR, an inhibitory agent is unnecessary. However, because these experiments were performed with only 1 mL of wastewater, it may be that larger volumes of wastewater, with larger numbers of competing organisms, would require some kind of inhibitory agent to prevent the growth of some gram-positive bacteria and potentially slow down the growth of some gram-negative bacteria. In light of this assumption, experiments assaying larger volumes of wastewater compared the three highest performing media with inhibitory agents – BPW with 1% Sodium Deoxycholate, 0.15% Sodium Deoxycholate or 3% Bile.

Enrichment experiments with 100 mL of primary influent wastewater and 100 mL of 2x Enrichment media demonstrate that all three of the media tested performed similarly with larger volumes of wastewater, although 0.15% Sodium Deoxycholate performed slightly better than the other two. (Table 3). The differences between the different enrichment media were not statistically significant. BPW + 0.15% SD exhibited the lowest variability between replicates.

Table 3: Enrichment of 100 mL Ty2-Seeded Wastewater with 100 mL 2x Enrichment Media

	Media		
	BPW + 0.15% SD	BPW + 1% SD	BPW + 3% Bile
n	3	3	3
Mean Log_{10}Change	1.80	1.49	1.38
SD	0.060	0.097	0.578

Given the results in Table 3 and all previous experiments, it appears as if the highest performing media in this study is BPW + 0.15% SD. This media consists of Soya Buffered Peptone Water with 75 ng/mL of Ferrioxamine E, 1x "aromix", and 0.15% wt/vol Sodium Deoxycholate. This media allows for robust growth of Ty2 (Fig. 4), completely inhibits the model gram-positive organism used in this study (Fig. 3) and performs well with seeded primary wastewater (Tables 2 and 3).

Discussion

This study proposes an alternative to selenite-based media for the isolation and detection of *S.* Typhi from water and wastewater samples. Although many media options exist for the enrichment of other *Salmonella* spp. from food and environmental samples, no alternative media has been proposed that is capable of effectively isolating *S.* Typhi bacteria in addition to other *Salmonella* spp. In light of the safety and environmental concerns related to the use of selenite and its poor performance in the context of stressed *Salmonella* bacteria, the development of an alternative media was warranted.

In the development of this study, a qPCR assay was chosen that is currently in use for environmental surveillance of Typhoid.[4,14,44] One limitation of this study pertains to the potential non-specificity of this qPCR assay used for the detection of *S.* Typhi in wastewater cultures. When unseeded enriched wastewater or unseeded nonenriched wastewater samples are extracted and examined by qPCR, these samples sporadically demonstrate amplification of the target amplicon (a 131-base pair-long portion of the *staG* gene). The CQ values of the non-specific amplification are generally higher than 34 (with a range of 34-40) and are rarely as low as 29 or

30. This non-specific amplification does not occur with every unseeded control experiment, but it occurs often enough to be of concern. It brings into question the validity of positive results related to the enrichment of wastewater cultures, since it is difficult to differentiate between true positives with high CQ values (> 34) and false positives in the same CQ range. Further work is currently being done to evaluate whether the non-specific amplification is a result of laboratory contamination.

The previously reported specificity of the qPCR assay used in this study is reported to be 100% in blood cultures.[14] However, the specificity of this assay in environmental samples is not as well understood. A more recent study evaluated 114 nontyphoidal *Salmonella* isolates via qPCR, and, of those tested, 12 demonstrated amplification of the same 131 base pair-long amplicon found in the *staG* gene.[41] Of these 12, at least five of the *Salmonella* species have been associated with food-borne outbreaks in the United States, and some, including *Salmonella* Choleraesuis, *Salmonella* Cerro, and *Salmonella* Napoli, have been isolated from various livestock, including bovine isolates, swine isolates, and poultry isolates.[45–48] Although none of the nontyphoidal isolates identified in this study were common human isolates, they may be present in Seattle wastewater. Another possible explanation is that this qPCR assay is occasionally detecting very low levels of *S.* Typhi bacteria in Seattle wastewater, potentially associated with travel-related Typhoid fever or individuals in the area receiving live attenuated Typhoid vaccines.

Despite the concerns surrounding non-specificity in wastewater enrichment cultures, this study includes experiments evaluating the performance of various media for pure cultures of *S.* Typhi.

Even in pure cultures, with no competing organisms or contaminants, the new proposed medium substantially outperforms the selenite-based medium tested (Figure 5).

Another limitation of this study pertains to the quantification estimates performed based on the qPCR standard curve. The standard curves were performed with pure Ty2 cultures and ten-fold serial dilutions in molecular grade water. Because of this, these linear standards may not be appropriate for estimating the true Log_{10}CFU that may be present in a wastewater sample, given known concerns about inhibition in sewage.[15] Therefore, the quantification estimates may be incorrectly estimating the true number of organisms represented in each sample. A standard curve with ten-fold serial dilutions in wastewater extracts was attempted to address this concern, but the linear curves for these wastewater standard curves reflected very poor assay efficiency, with slopes greater than 4.5 and R_2 values less than 0.90. Further analysis is warranted to determine how to gain more accurate quantitative CFU estimates for wastewater extracts. Despite this concern about quantification, the CQ values of different media are still comparable in a within-assay setting. When comparing different media performances, quantitative PCR assays were performed with all DNA extracts included on one 96-well plate, in one single assay. Therefore, although the quantification estimates may not be accurate, the estimated log_{10}CFU values for different media can be compared within each assay to determine which media exhibited the best performance (see Tables 1 and 2)

This study demonstrates that BPW, with or without 0.15% SD, performed significantly better than Selenite F in side-by-side enrichments of Ty2 seeded into 1 mL of wastewater. Follow-up

experiments with 100 mL of wastewater were performed with BPW containing 0.15% SD, 1% SD, or 3% Bile. Therefore, the question remains whether the addition of 0.15% SD to BPW, as it was formulated in this study, improves the detection of Ty2, as compared to BPW with no SD. Further experiments with 100 mL of wastewater may help clarify this, as larger volumes of wastewater are expected to contain larger numbers of competing organisms. A small percentage of sodium deoxycholate, which may inhibit many gram-positive bacteria and potentially slow the growth of some gram-negative bacteria, may confer a greater advantage in this more challenging environment, as compared to only 1 mL of wastewater, but that assumption is not yet confirmed.

By evaluating both the growth dynamics of pure *S.* Typhi cultures in various enrichment media and the growth estimates of *S.* Typhi-seeded wastewater samples in those same enrichment media, this study makes a strong case that selenite-based media is not the ideal enrichment media for *Salmonella* Typhi. Among pure and wastewater cultures, it was the lowest performing media tested. Given these results, and the occupational safety concerns cited above, it would be preferable to phase out the use of this media in favor of other enrichment media. Another strength of this study is that it proposes a potential candidate for a replacement enrichment media, although further studies are warranted to replicate these results and further determine an optimal concentrations of sodium deoxycholate.

Future Directions

Further studies would be useful to optimize enrichment conditions. For example, different enrichment times (either longer or shorter than 24 hours) may improve recovery. Different enrichment temperatures may also affect recovery. Previous studies have suggested that elevated

enrichment temperatures between 40-43^{o}C may increase the chances of isolating various *Salmonella spp.* strains in complicated food and water matrices by inhibiting more heat-sensitive bacteria.[20,30,49] None of the cited studies were performed specifically with *S.* Typhi, so it is not yet certain whether an elevated temperature will improve the chances of detections of this particular pathogen.

Further work may also be warranted to improve the specificity of the qPCR assay used in this study. This future work is especially relevant in light of the fact that other research groups conducting environmental surveillance for Typhoid have used this assay; those studies likely face the same concerns about specificity. The target amplicon could be lengthened, or the current assay could be multiplexed with another target that is specific to *S.* Typhi, as suggested by Nair et al., 2019.[41]

Finally, various concentration methods exist to allow for assaying of large volumes of water or wastewater for the presence of various bacterial, viral, and protozoan organisms. Such methods include membrane filtration, Moore swabs, differential centrifugation, the Bag-Mediated Filtration System, and ultrafiltration.[50–54] It is likely that an optimized enrichment step, wherein concentrates or eluates from these concentration methods are enriched prior to DNA extraction or culture-based detection, will help improve the sensitivity of these sampling methods for the detection of *S.* Typhi. Further studies may be warranted to determine whether the incorporation of an enrichment step using the media proposed in this study (or some variation of the formulation presented here) will help improve the performance of these existing sampling methods.

Aim 2: Estimate the limit of detection of membrane filtration with the addition of an enrichment step in combination with a qPCR assay for the detection of *Salmonella* Typhi.

Introduction

Membrane filtration, followed by incubation on M-FC agar, has been utilized by wastewater treatment plants in the United States as a method to detect the presence of fecal coliforms in chlorinated sewage effluents.[55] A study with fecal coliforms has suggested that a pre-enrichment step improved the isolation of fecal coliforms in secondary chlorinated sewage by culture methods.[55] Similarly, a study on the recovery of *Pseudomonas aeruginosa* from raw sewage, secondary sewage effluent, and swimming pool water samples determined that various selective enrichment media improved recovery of *P. aeruginosa* by culture methods.[56] An additional study evaluating membrane filtration followed by subculture in enrichment media reported success for the detection of *Listeria monocytogenes* by culture methods.[57]

To our knowledge, no such studies combining membrane filtration with enrichment for *Salmonella spp.* have been published. Similarly, we have not found published studies examining the combination of membrane filtration, enrichment, and detection via qPCR as a possible multi-step sampling method for the detection of *S.* Typhi in water or wastewater.

This study seeks to fill this gap by evaluating this multi-step method for the detection of *S.* Typhi in raw, influent wastewater. This method may be useful for environmental surveillance studies

for Typhoid fever. For this study, the parameter of interest for the proposed method is the limit of detection (LOD), defined as "the lowest concentration where a true positive is still identifiable."[58] The limit of detection of a method is a useful performance metric because it allows a sampler to properly interpret a negative result.[11,58] To determine the LOD, five replicate experiments are performed with samples, seeded with the target organism, at three sequential serial dilutions. The highest seeding concentration should be at a level at which 100% detection (or 5/5 positive) is expected. By evaluating the number of positive and negative replicates at each dilution, an LOD, or a range in which the true LOD exists, can be estimated.[11]

Methods

Media Preparation

All media used in this aim (BPW with 75 ng/mL Ferrioxamine E and 1x "aromix", with 0.15% sodium deoxycholate, 1% sodium deoxycholate, or 3% bile) were prepared as described in Aim 1.

Inocula Preparation

Cultures of *S.* Typhi Ty2 bacteria were grown and prepared as described in Aim 1.

Membrane Filtration and Enrichment

For the membrane filtration tests, diluted overnight cultures were seeded at the desired concentrations into 100 mL of wastewater. The wastewater was then filtered by membrane filtration through a 0.45 um cellulose filter (Millipore HAWG047S6). The filter was then placed in a small Whirl-pack© bag (WPB01018WA) with 10 mL of the enrichment media being test. The filter was massaged gently until it appeared "clean." The 10 mL of media was then added to 90 mL of the same enrichment media, along with the membrane filter.

100 mL enrichment cultures, with the membrane filters included, were incubated in 250-mL vented Erlenmeyer flasks (Thermo Scientific 4115-0250) at 37$_0$C at 225 RPM for 24 hours. After 24 hours, cultures were removed from the incubator and divided into 50 mL conical tubes. The conical tubes were then centrifuged at 3500 x g for 30 minutes. The supernatant was removed, and pellets were stored at -20$_0$C prior to extraction.

Extraction, qPCR, and Quantification

A DNA extraction and a qPCR detection assay were performed as described in Aim 1 in order to detect the presence and estimate the quantity of *S.* Typhi bacteria in enrichment cultures. Quantification estimates were obtained according to the methods outlined in Aim 1.

Statistical Analyses

The statistical comparisons between different experiments were evaluated using Welch's two-sample t-test, which does not assume equal variances between samples.[42] A one-tailed t-test was used to compare experimental results to a lower limit. These t-tests were performed using the base t-test package in R 3.6.1, with a significance level of 0.05.[43]

Results

An initial membrane filtration experiment tested the proposed multi-step sampling method with three different enrichment media. At a seeding level of approximately 50 CFU/mL (1.7 Log_{10}CFU/mL), this experiment resulted in 100% detection in all three media used, suggesting that this method is a viable choice for assaying 100 mL of wastewater (Table 1). Because 0.15% sodium deoxycholate was identified as a consistently high-performing enrichment media (see Aim 1), this media was chosen for the LOD analysis. The media used for the LOD experiment

consists of Soy Buffered Peptone Water, 1x “aromix”, 75 ng/mL Ferrioxamine E, and 0.15% wt/vol sodium deoxycholate.

Table 1: Initial Membrane Filtration at a seeding level of 1.7 Log_{10}CFU/mL (50 CFU/mL)

	Inhibitory Agent					
	3% Bile		1% Sodium Deoxycholate		0.15% Sodium Deoxycholate	
Percent of Culture Assayed	100%		100%		1%	
Replicates	CT	Log_{10}CFU	CT	Log_{10}CFU	CT	Log_{10}CFU
a	28.36	3.75	27.51	4.00	30.9	3.03
b	26.80	4.20	29.43	3.45	31.1	2.97
c	28.38	3.75	28.16	3.81	34.5	2.00
Mean	27.85	3.90	28.37	3.75	32.17	2.67
SD	0.976	0.259	0.907	0.279	2.02	0.58

For the LOD experiment, a maximum seeding concentration of 1 CFU/mL was chosen. This choice was based on previous membrane filtration experiments performed with *S.* Typhi with no enrichment step, which indicated 100% detection was expected at this seeding level (published data not yet available). The lowest seeding level tested was 0.01 CFU/mL. All qPCR was performed on the same 96-well plate, under the same conditions and quantified using the same standard curve. CQ values for this assay demonstrate the varying ability of this assay to detect *S.* Typhi at the three seeding levels tested (Table 2).

Table 2: CQ Values in Replicate Membrane Filtration Enrichment Samples at 3 Seeding Levels

BPW + 0.15% SD	**Approximate Seeding Level**		
Replicate	1 CFU/mL	0.1 CFU/mL	0.01 CFU/mL
a	31.5	31.4	NaN
b	28.8	33.1	35.8
c	29.9	33.7	NaN
d	28.8	33.5	36.3
e	30.0	32.1	NaN
Mean	29.8	32.8	36.1
SD	1.11	0.979	0.354

Table 2: Grey highlighted CQ values under the 0.01 CFU/mL seeding level indicate that these CQ values are above the estimated limit of quantification for this qPCR assay. Additionally, CQ values above 34 may not reflect true positive amplification of Ty2 DNA, as control wastewater samples occasionally produce CQ values in this range.

Table 3: Estimated Log_{10}CFUs in Replicate Membrane Filtration Enrichment Samples at 3 Seeding Levels

BPW + 0.15% SD	**Total CFU Seeded in 100 mL Wastewater**		
Replicate	1 CFU/mL (0 Log_{10}CFU/mL)	0.1 CFU/mL (-1 Log_{10}CFU/mL)	0.01 CFU/mL (-2 Log_{10}CFU/mL)
	Estimated Log_{10}CFU in Sample Post-Enrichment		
a	2.86	2.88	NaN
b	3.63	2.41	1.62
c	3.33	2.23	NaN
d	3.63	2.29	1.48
e	3.28	2.67	NaN
Mean	3.35	2.50	1.55
SD	0.317	0.273	0.10

Table 3: Grey highlighted CQ values under the 0.01 CFU/mL seeding level indicate that these CQ values are above the estimated limit of quantification for this qPCR assay. Although log CFU's were estimated for these replicates using the standard curve, it should be noted that these estimates are outside the limit of quantification and may not be accurate estimates and may not reflect true positives.

Estimated final Log_{10}CFUs in enrichment cultures demonstrate that, among all positive results at seeding levels of 1 CFU/mL and 0.1 CFU/mL, the average change in log_{10}CFU is 1.421 (95% Confidence Interval: 1.21 – 1.63) (Table 3). At a seeding level of 0.1 CFU/mL or higher, the mean log change in CFU is greater than 1 ($p = 0.0007$). The mean log change in CFU was not significantly different between these two seeding levels, as analyzed by Welch's two sample t-test.

It appears that the multi-step membrane filtration method yields a limit of detection below 0.1 CFU/mL (or 10 CFU / 100 mL) and above 0.01 CFU/mL (or 1 CFU / 100 mL). Although amplification of the gene target occurred in 2 of the 5 replicates at the 0.01 CFU/mL (1 CFU/100mL) seeding level, these CQ values are as high as the occasional background CQ values that periodically occur when unseeded wastewater is extracted and amplified (see Aim 1 Discussion). This uncertainty about the specificity of amplification curves with CQ values over 34 precludes us from confirming whether the two positive results at the 0.01 CFU/mL seeding level are true positives or whether they reflect non-specific amplification of the gene target in the wastewater.

Because of this uncertainty, our conclusion here is that the limit of detection for this detection method is lower than 0.1 CFU/mL, with 100% detection at this level, and higher than 0.01 CFU/mL, with 40% potential, but unconfirmed, detection at this level. Further precision in the detection limit cannot be determined from this experiment alone.

Discussion

This study proposes a combination of a well-known wastewater sampling method with a novel enrichment step for detecting very low levels of *S.* Typhi bacteria in wastewater. In combination with a qPCR assay (which enables specific detection of *S.* Typhi), the method demonstrated here may be used for sampling wastewater or environmental waters with high concentrations of competing organisms. An advantage of membrane filtration in conjunction with an enrichment step is that the volume of wastewater can be increased. This study evaluated 100 mL volumes of

wastewater. However, it is conceivable that similar results could be achieved with 300 mL or 500 mL volumes of wastewater containing the same concentrations of *S.* Typhi bacteria.

The same weakness described in Aim 1 relating to the potential non-specificity of the qPCR assay used for these experiments applies to Aim 2. As in Aim 1, this non-specificity makes it difficult to discern true positives from false positives and thus prevents a confident estimate of where the true limit of detection lies. As stated above, further analysis of frozen extracts from this experiment using an improved qPCR assay may clarify the results of this LOD experiment and allow for a more precise estimation of the LOD range. Although the true LOD may be lower than the limit presented here, this study demonstrates strong evidence that the true limit of detection is at least as low as 0.1 CFU/mL and potentially lower.

An immediate proposed future direction of this study is to repeat this experiment to ensure that the estimated LOD remains consistent between experiments. Another future study could apply this sampling method with a different detection assay. For example, another detection assay, called droplet digital PCR, has recently become more widely utilized in food microbiology. This assay has the potential to lower the detection limit of this assay even further. This technology has been shown to provide higher sensitivity for the detection of foodborne pathogens in food microbiology and has been shown to provide better performance in inhibition-prone samples than qPCR.[59,60] Further analysis of DNA extracts from this study using droplet digital PCR may reveal a lower limit of detection than that proposed in this study.

Aim 3:

Build a dynamic computational model to predict the probability of detection of *S.* Typhi in wastewater and use model output to predict the risk of illness for environmental surveillance samplers interacting with contaminated waters.

Introduction

In order to inform environmental sampling for the purpose of environmental surveillance for *S.* Typhi in wastewater, a dynamic mathematical model is presented which attempts to predict the probability of detecting *S.* Typhi at a given sampling site in a system. Ranta et al., 2001 designed a similar model in order to quantify the environmental sensitivity of surveillance for poliovirus, given various transmission scenarios, sampling schemes, and laboratory techniques.[10] Ranta et al. focused on sampling within a single sewage network and at a single sampling location in Helsinki, Finland, relying on a probability gamma distribution to estimate the delay between fecal output of poliovirus into the sewage system and its arrival at the sampling site.[10] This current study takes a different approach, by attempting to model the fate and transport of *S.* Typhi bacteria travelling through a flexible wastewater system that can accommodate most open or closed-channel sewage networks. The model builds a network from user-inputted parameters describing the system and predicts the concentration of viable *S.* Typhi bacteria at any given point along the network.

Similar to Ranta et al., 2001, this model then estimates the probability of the detection of *S.* Typhi, given the estimated concentration of *S.* Typhi bacteria in a sample. This estimation is obtained given user-provided information about the sampling and laboratory techniques in use.

In order to incorporate uncertainty, the dynamic model incorporates certain variables as distributed parameters (Table 2). The model then runs for a specified number of simulations, resulting in final output with means and standard deviations for concentration estimates of bacteria and probability of detection estimates.

Finally, the model provides an estimation of the risk of illness for environmental surveillance samplers that are responsible for collecting contaminated wastewater. Since splashing, spilling, and contamination of hands and skin are all possible during field sampling, it is likely that there is some risk of Typhoid fever for samplers who are working with wastewater with high concentrations of *S. typhi* bacteria. This estimation is performed by utilizing an exposure model and a dose-response model for *S.* Typhi, according to the Quantitative Microbial Risk Assessment (QMRA) Framework.[58]

Methods

Data Collection

Data for the model was gathered from literature searches in order to obtain information about bacterial survival dynamics in water and wastewater, estimated wastewater flows in developing countries, estimated daily fecal output, *S.* Typhi shedding rates in feces, infiltration rates, diurnal variations in wastewater flow, and diurnal variations in fecal output (Publication Date Range: 1992 – 2020).

Model Code

The code for this model was written in the programming language Python v. 3.7.0.

Model Description

This model requires two data tables as input in order to create the desired sewer line network and run the specified scenario. The first data table, described in Table 1, includes fixed parameters that do not change over the course of multiple model simulations. The second data table, described in Table 2, describes parameters with defined distributions. Values for these distributed parameters are randomly selected from the specified distributions every time a model simulation is performed.

Every time the model is run, the user can select the number of days and number of simulations. The model will then collect output for the given number of simulations. Each simulation runs for the specified number of days before storing the data from that final day for that simulation. The result is an estimate of the mean and standard deviation of each of the output parameters, calculated from the array of outputs from all simulations. The advantage of running the model for multiple days within each simulation is that it allows the model to reach a "steady state" within that simulation before calculating bacterial concentrations in the system. This is important because the model starts from zero at the beginning of each simulation, and it takes two or more days for the system to reach a steady state that accommodates data from the previous day and ongoing inputs from the current day. The user is encouraged to determine the number of days required to reach a steady state by first running the model with all parameters fixed (i.e., plug in point estimates for all fixed *and* distributed parameters) and determine the number of days required, in one simulation, for the output to remain constant between days.

Initialization

The wastewater system of interest – a stream, river, open channel, or sewer – is divided into branches. The user will need to create a crude map of the system that defines how different branches connect. For example, if a branch has two parent branches (i.e. two branches that feed into the branch), then this will need to be defined as an input parameter. A branch may have no more than two parent branches. Each branch in the system is given a number ID, and the branch's parent branches are defined. If a branch has no parent branches, then this branch's parents are labelled as '-1'.

Table 1: Fixed Parameter Inputs for Each Branch

Input Parameter	Units
Branch ID	Numerical ID
Parent 1	Numerical ID or -1 (for no parent)
Parent 2	Numerical ID or -1(for no parent)
Velocity	meters/hour
Length (of branch)	meters
Local Population along branch	# persons
Prevalence along branch	# shedding typhoid cases / total population

The model inputs listed for Table 1 are defined for every branch of the system. The user can change fixed parameters for each branch, depending on knowledge about the system and how the population and prevalence of typhoid fever are distributed, in a .csv file. The model then creates

a “map” using the branch inputs that define how each stream or sewer line branch feeds into other branches.

The model inputs listed in Table 2 are the distributed parameters. Each of these parameters is included as defined in the table, with defined distributions. Each time the model runs, it randomly selects estimates for each of these parameters from the specified distributions.

Table 2: Distributed Parameter Inputs for System

Input Parameter	Units	Distribution	Values Required
Fecal Output Rate	grams / day	Normal	Mean, Standard Deviation
Shedding Rate	pathogen / gram of feces	Normal	Mean, Standard Deviation
Wastewater Flow	Liters / person / day	Normal	Mean, Standard Deviation
Environmental Flow (including background flow in a stream and infiltration)	Liters / hour	Normal	Mean, Standard Deviation
Bacterial Decay Rate	Proportion remaining after 1 hour	Discrete Uniform	3 point-estimates

Estimates for each of these distributed inputs have been gathered from estimates in relevant papers and studies. Those parameter inputs, including references, are defined in the a supplementary table at the end of this document. The model is intended to be flexible, allowing

the user to input their own estimate for each parameter, if they have a preferred estimate. However, the user can choose to use these provided estimates.

Model Run

Once the system has been initialized, the model will run for a specified number of simulations, and, within each simulation, for a specified number of days, and store the output data for the final day. Each daily update includes data from the previous day in the calculations for the current day. (i.e. if a branch of the river has bacteria in it at Hour 24, those bacteria are included in the estimated concentration of bacteria at Hour 0 on the following day.)

Wastewater flows and defecation rates are variable throughout a 24-hour daily cycle. Therefore, the model is designed to update every hour according to the estimated wastewater flows and defecation rates at that hour of the day. Data was pulled from a 1996 study that analyzed multiple aspects of wastewater characteristics and flow in developing countries, including variations in wastewater flow over the course of the day.[3] This study analyzed communities in Belo Horizonte, Brazil between the years 1986-1987. The study divided the city into categories based on total family income and housing standards. The estimates in Table 2 reflect the study's estimates of flow cycles in middle-income communities (the study only reports flow cycles for "high class" and "middle class" communities, without defining the exact estimated incomes for these categories). This study was the only published study found providing wastewater flow estimates outside of Europe or the United States, but these estimates do not necessarily accurately reflect flow variation for countries where Typhoid is endemic. Table 2 contains the breakdown of the approximate daily variation in flow, according to this study.

Similarly, data for the hourly variations in defecation rates over a 24-hour cycle were pulled from a 1992 study analyzing defecation frequency, timing, and stool formation in 1,897 participants from East Bristol, Wisconsin. Again, this population does not necessarily reflect populations where endemic Typhoid exists, and better studies may be found that more accurately capture the characteristics of relevant communities. This study reports the percent of total defecations that occur at each hour of a 24-hour day.[4] Table 3 contains the breakdown of the daily variations in defecation rates, according to this study.

Table 3: Diurnal Variations in Wastewater Flow (Hour 0 corresponds to midnight)[61]

Hour	% of total daily WW flow	Hour	% of total daily WW flow
0	1.79	12	8.96
1	0.896	13	8.36
2	0.896	14	4.78
3	0.299	15	7.16
4	0.896	16	4.18
5	0.299	17	4.18
6	1.19	18	4.48
7	5.37	19	4.18
8	6.57	20	3.88
9	7.16	21	4.18
10	7.76	22	3.28
11	5.97	23	3.28

Table 4: Diurnal Variations in Defecation Rates (Hour 0 corresponds to midnight)[62]

Hour	% of Total Daily Defecation	Hour	% of Total Daily Defecation
0	2	12	2.5
1	0.3	13	3.5
2	0.3	14	3.25
3	0.05	15	2.5
4	0.25	16	3.25
5	1.25	17	2.25
6	6	18	4.25
7	19.5	19	4
8	17.5	20	2.5
9	10.25	21	1.75
10	7.75	22	2
11	5.5	23	1.75

Hourly Updates

Pathogen Loading

At every hour, the model starts at the bottom of the system and calculates pathogen concentration for each section along each branch at that hour. The following equations are used to estimate the final concentration of bacteria in the system at any location or time.

Eq 1: Number of pathogens entering system at any location along the section of a branch of the system (*N*):

$$N = percent * [(bp * p * d * sh) / l]$$

bp = branch population (persons)

p = local prevalence of Typhoid along branch (% of population)

d = estimated defecation rate (g/day*person)

sh = shedding rate (CFU / gram)

l = length of branch (m)

percent = % of total daily defecation occurring at the specified hour

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Eq 2: Distance Travelled (D)

$$D = t * v$$

t = time since last update (1 hour)

v = velocity of flow in the relevant branch (m/h)

Eq 3: Estimated number of pathogens in a section of a branch along the system at a given time (P).

$$P = (path * dec) + N$$

path = # of pathogens feeding into current section from previous sections (CFU)

dec = decay rate (hour^{-1})

N = Number of pathogens entering the system at that hour from the local population (CFU)

If the pathogens feeding into the current section of a branch have travelled farther than the length of that branch, the model recursively calculates the total pathogen load from the parent branches,

given the estimated velocity of each branch and the total distance travelled since the previous hour.

Flow Loading

To calculate the dilution factor, the model calculates the input of wastewater flow along each branch at each hour and the background flow along each branch.

Eq 4: Estimated volume of wastewater and/or environmental water in the system at a given section of a given branch (W)

$$W = inf + (percent * fl) + env$$

inf = influx of wastewater from previous branches flowing into the current branch, given the values of the previous hour. (L)

fl = total wastewater flow into a section of a branch, given the population along that branch (L / hour)

percent = % of total daily wastewater flow occurring at the specified hour

env = environmental flow per section of system (in a sewage system, this value will reflect infiltration estimates. In a river or a stream system, this value will reflect the estimated volume of the river or stream plus infiltration estimates) (L/hour)

Similar to the pathogen loading calculations, if the combined environmental flow and wastewater feeding into the current section of a branch have travelled farther than the length of that branch since the previous hour, the model recursively calculates the total flow load from parent branches.

Pathogen Concentration

Given the pathogen load estimate (P) and the dilution factor (W) (or, estimated volume of combined wastewater and environmental water) at each section of each branch, the model then calculates and stores the estimated concentration of *S.* Typhi at each section at the given hour.

Eq 5: Estimated pathogen concentration (C)

$$C = \frac{P}{W}$$

Probability of Detection

The probability of detection is calculated according to the formula described in Ranta et al., 2001. First, the user must pre-define the sensitivity of the method with a Beta parameter, which can be calculated according to the following formula.

Eq 6: Probability of Detection (β)

$$\beta = \frac{-ln(1-P)}{k}$$

Where:

P = the probability of a positive result, given k

k = the number of *S.* Typhi bacteria in 1 Liter of wastewater.

Then, for each branch and each section, the estimated concentration of bacteria per liter from the dynamic model is input into the following Poisson distribution in order to estimate the number of bacteria (k) that are captured in a single sample.

Eq 7: Estimated Concentration in Sample

$$p(k) = \text{Poisson}[k;\ S * C]$$

Where:

p(k) = the estimated number of bacteria in a sample, randomly chosen from a Poisson distribution

S = the volume sampled (Liters)

C = the estimated concentration of bacteria in the wastewater (CFU / Liter)

This estimated number of bacteria in a collected sample is then used to calculate the probability of a positive laboratory result for *S.* Typhi, given the Beta parameter of the detection method being used.

Eq 8: Probability of a Positive Laboratory Result

$$p(+) = 1 - exp(-\beta\ x\ k)$$

Where:

p(+) = the probability of a positive result

β = laboratory method sensitivity parameter

k = estimated number of bacteria in the sample, according to Poisson distribution.

This k estimation (according to a Poisson distribution) and probability of detection calculation is repeated 100 times in a simple Monte Carlo procedure, and the mean and standard deviation of the calculated probabilities are reported in the model output.

Model Output

After a run of the specified number of days, the code produces a .csv file with the hard data from the run. The file is organized with rows specifying which branch and which section of the branch the estimate corresponds to and columns specifying the hour of the day, as shown below (where hour 0 corresponds to midnight):

Branch	Section	H0	H1	H2	H3	H4	H5	H6	…	H23
0	**1**	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	…	CFU/L
0	**2**	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	…	CFU/L
1	**1**	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	…	CFU/L
1	**2**	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	CFU/L	…	CFU/L

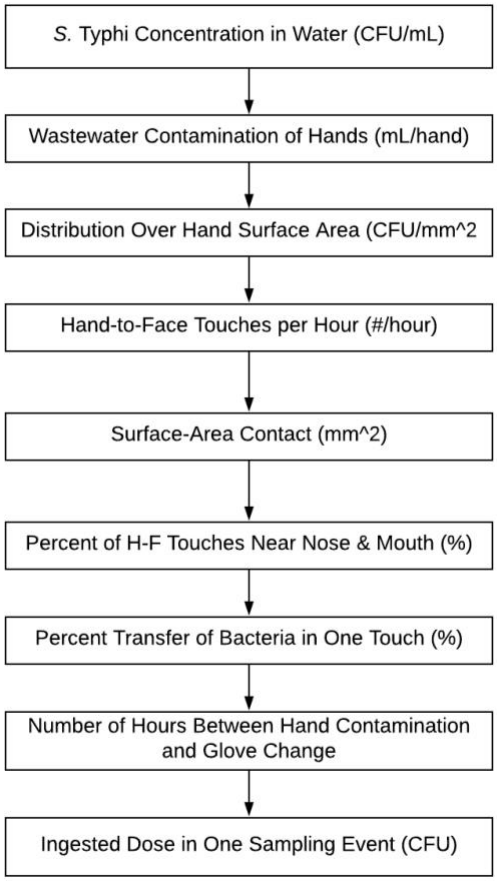
Four .csv files are created, all in the same format, with output data for each branch and section estimating the mean concentration of bacteria per liter, the standard deviation of these concentration, the mean probability of detection, given the bacterial concentration, and the standard deviation of these probabilities.

The model also produces various figures, which are described in the sample run below. For ease of visualization, some of these figures use heat maps to reflect the estimated concentrations of pathogens or estimated probabilities of detection, with the actual numerical estimates reported along the heat map key. The model keeps track of the minimum and maximum calculated pathogen concentration (anywhere in the system) and uses these values to build the heat map.

Exposure Model

The mathematical framework of the risk assessment model is simple. A fairly direct chain of events is described, from the source of the contamination (contamination of wastewater to be sampled) to the point of ingestion. Equation 9 defines the mathematical equation corresponding to the exposure model in Figure 1. The input parameters for the exposure model are defined in Table 5.

Figure 1: Exposure Model for Environmental Samplers



Eq 9: Mathematical Exposure Model Equation:

$$D = (WW * C * L)/h * k * m * p * h$$

Where D is the estimated Dose (CFU) that a sampler may ingest during a single sampling instance and the other variables are defined in Table 5.

Table 5: Input Parameters for Exposure Model (All distributions are truncated at zero)

Variable	Definition	Units	Distribution	Estimate	Source
WW	Concentration of S. Typhi.	CFU/mL	Normal	Estimated from dynamic model	Estimated from dynamic model
C	Contamination of fingers during sampling	mL/finger	Normal	$\mu = 1$ $\sigma = 0.8$	Assumption
L	Loading of bacteria onto hands	CFU/hand	-	WW * C	
h	Hand surface-area	mm^2	Normal	$\mu = 448$ (males) $\mu = 392$ (females)	[63]
k	Hand-to-face touches among laboratory personnel	#/hour	Lognormal	$\mu = 2.79$ $\sigma = 2.14$	[64]
m	Hand-to-face touches near the nose or mouth	%	Point Estimate	48.9	[64]
p	Percent transfer of bacteria from finger to face in one touch	%	Normal	$\mu = 34$ $\sigma = 3$	[65]
h	Estimated number of hours before a sampler may either sanitize hands or change gloves	#	Normal	$\mu = .5$ $\sigma = 0.25$	Gathered from Experience

Dose Response Assessment

The dose response assessment was performed with data points from two dose response feeding studies in humans.[66,67] In order to determine the best-fit model, the data from each study were evaluated using a maximum likelihood estimation. Briefly, a formula denoting the -2-log likelihood ratio (termed the deviance) was used to determine how much the actual response data from each study deviates from the predicted response data, given a dose-response model. The optimum maximum likelihood parameters were obtained for each model by finding the parameters that minimize the deviance. This maximum likelihood routine was performed for the exponential dose-response model and the beta-Poisson dose response model. For both studies, the beta-Poisson model provided a better fit to the data (determined by comparison to a Chi-squared distribution, with an alpha of 0.05) (Figure 1).

In order to conduct the best-fit dose response model and generate the optimal distribution of model parameters, dose response data were pooled from these two studies, and the maximum likelihood estimation was repeated with the pooled data. Pooling data, when valid, results in a larger number of data points in the parameter estimation, which, in turn leads to a smaller range of the confidence region. These two studies together provide data points from 351 total patients at eight different doses. The pooling of these two experiments is justified for a few reasons. Both of these studies were performed in humans, using the Quailes strain of *Salmonella typhi*, so it is expected that the dose response did not differ significantly between these two studies.[66,67] Additionally, the best-fit model for each of these studies separately is the beta-Poisson model.

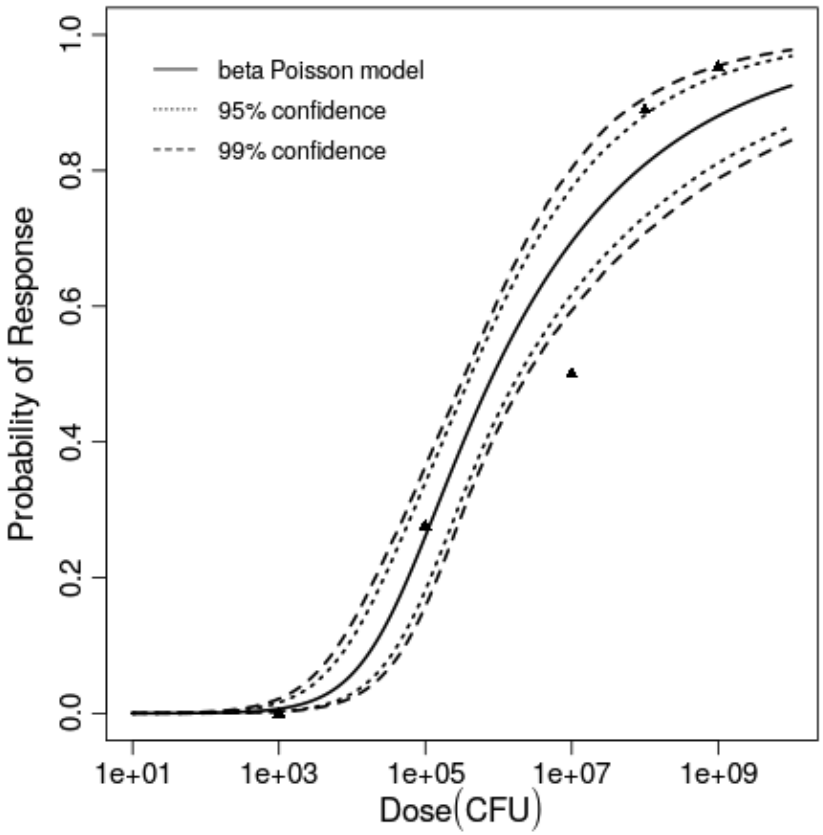
Finally, a bootstrap routine was performed with the beta-Poisson model and our pooled dose response data to produce 10,000 estimates of model parameters, which were paired and sampled together from a uniform distribution within the model.

In order to calculate the single event risk of becoming ill with typhoid fever, given the dose ingested during a single sampling event, the beta-Poisson parameters α and N_{50}, obtained from the bootstrap routine described above, were plugged into the following equation, along with the dose estimate from the exposure model.

Eq. 10: Risk of Illness, given dose

$$Single\ Event\ Risk\ (P(dose)\ =\ 1\ -\ \left[1\ -\ \frac{dose}{N_{50}}\ x\ \left(2^{\frac{1}{\alpha}}\ -\ 1\right)\right]^{-\alpha}$$

Figure 1. Probability of Illness, Given Dose, as Estimated by Beta-Poisson Model



The cumulative risk in one day, over the course of two sampling events in a day, is then calculated with the following equation, assuming that the risk of becoming ill from either sampling events is independent.

Eq. 11: Cumulative Risk, Given a Number of Sampling Events

$$Cumulative\ Risk\text{: } P(\#\ sampling\ events) = Risk_{Event1} + Risk_{Event2}$$

Example Runs:

To demonstrate the model, two example runs were performed based off of a wastewater system in Vellore City, India.

In order to set up this model, information about the sewer lines in a densely populated area of Vellore City, India was collected from research partners who are currently performing environmental surveillance for *S.* Typhi in the city {data not currently available for citation} and from the Environmental Surveillance Digital Elevation Map Catalogue.[7] The Environmental Surveillance Digital Elevation Map Catalogue uses a 30m Digital Elevation Model (DEM) to derive the topology and synthetic streams and waterways for a given area. The model uses the topology and slopes of the land to estimate the direction of flow for all waterways. The mapping function of this catalogue also allows for measurements (in meters) of stream segments. These flow directions and length measurements were used to set up the branches of the system for this example run.

To allow for ease of visualization, this model was limited to areas with denser populations within the watershed. A more comprehensive model, however, would take into account the entire watershed.

Figure 2: Model Map in Vellore, India[7]

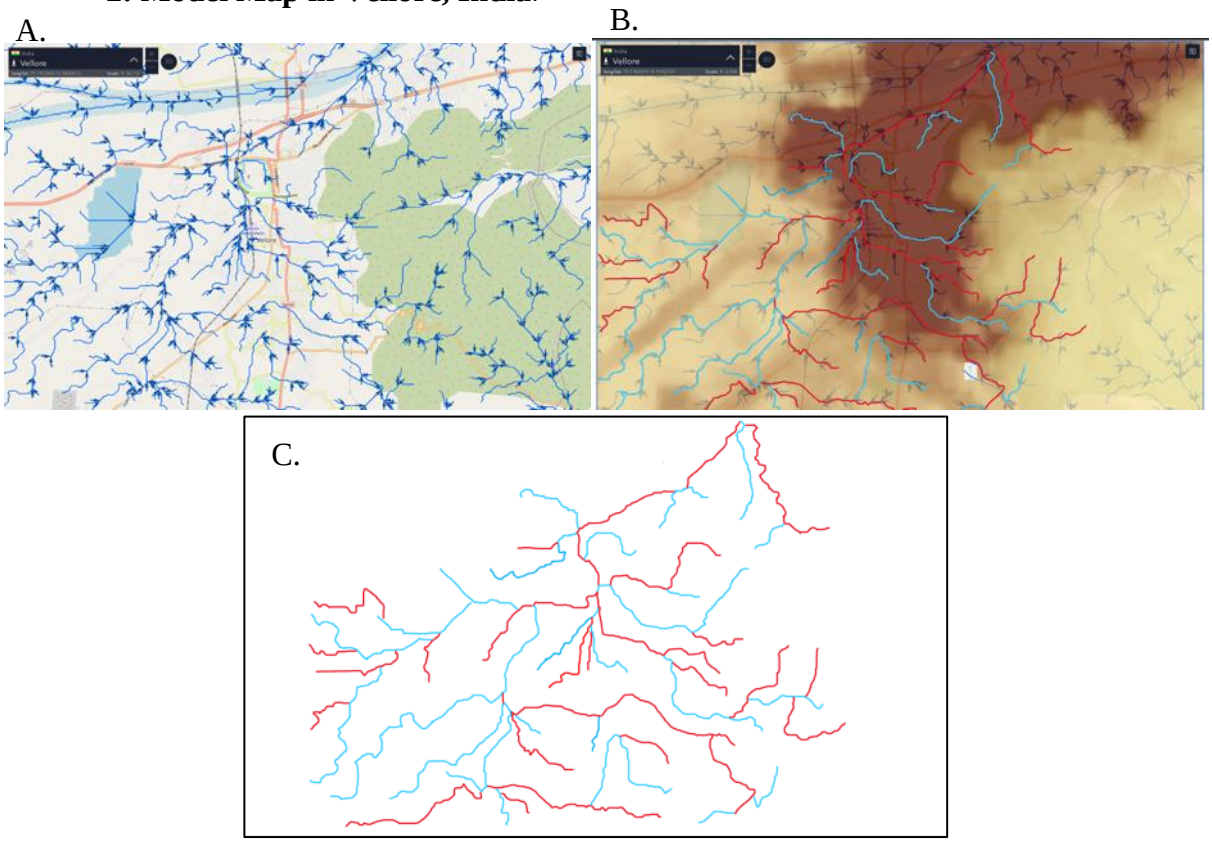


Figure 2. Digital Elevation Model Map of Vellore City, India[2] (2A) Representation of population density in model region (2B). Illustration of a model based off this city (2C).

Figure 2 represents a map of the wastewater system used in this example run, built using the Environmental Surveillance Digital Elevation Map. Figure 2B visualizes the relative population density along this system, as estimated by the Digital Elevation Map Catalogue.[5] When assigning populations along branches for the fixed parameter setup, the total population of this area was distributed along the system according to relative population density.

The distributed parameters table for the example run was populated with estimates from literature sources, which are defined in the appendix. The fixed parameter table, with branches, branch lengths, and distributed populations, was populated according to the maps defined in Figures 2.

Output from Example Run 1

In the first example run, an evenly distributed prevalence of 50 cases per 100,000 persons was analyzed. The sampling method for this run included the collection of 1 liter of wastewater, which is then analyzed by a membrane filtration laboratory method. According to experimental data, this method has a probability of a positive result of 0.67 for a 1-liter wastewater sample that contains 100 bacteria.[6] This experimental data was used to estimate the Beta parameter for the probability of detection.

The model can output a number of possible figures. Figure 3 provides an overview of concentration estimates in the entire system over the course of the day. The branches are ordered along the x-axis by their given ID number, so the order along the x-axis does not necessarily

reflect the flow progression or the order of the system. Rather, it reflects the way the user chose to order the branches during set-up.

Because this system has over 50 branches, Figure 3 does not allow for a precise evaluation of which branches may be ideal sampling locations. However, it does provide insight into how the estimated concentrations are changing over the course of a 24-hour cycle in this system.

Figure 3: Hourly Plots of Estimated Concentrations Along Branches

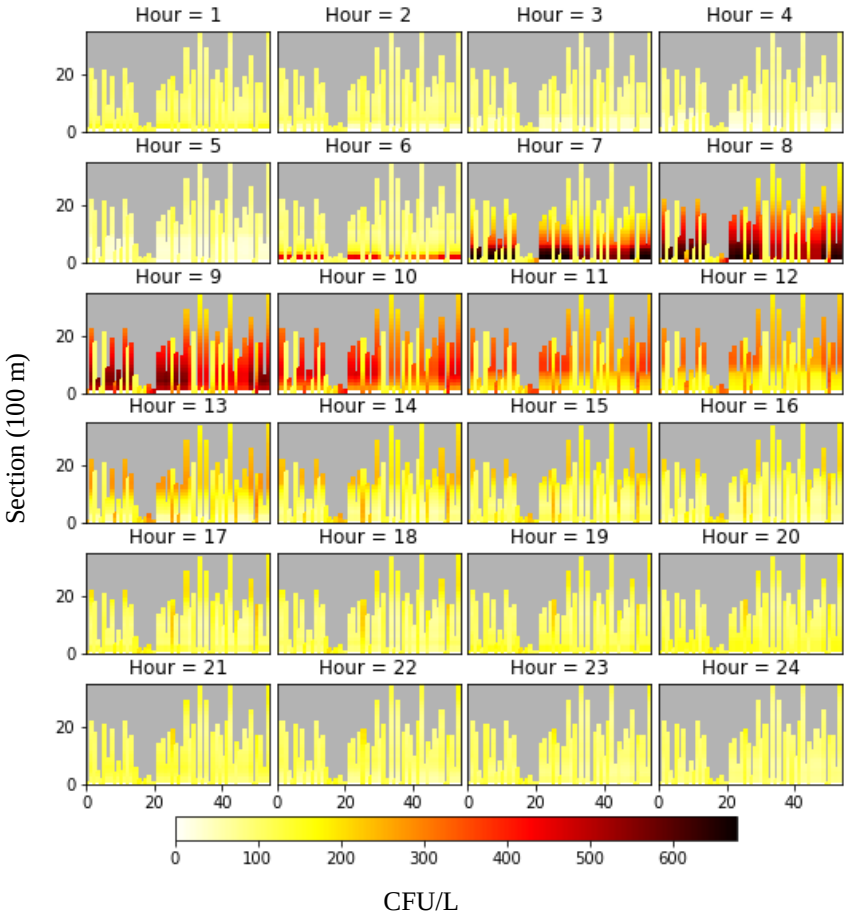


Figure 3 One plot is displayed for each hour. At each hour, each branch is plotted along the x-axis, with the section indicated on the y-axis. The estimated concentration (CFU/L) is indicated by a heatmap

Figures 4-7 demonstrate the plots that can be generated for any branch in the system. Figures 4 and 6 reflect estimated concentrations and probability of detection estimates over entire branches. Figures 5 and 7 reflect those same estimates for a particular section along a particular branch, over the course of the day. The user can use these plots to determine when and where to sample to achieve the highest probability of detection.

Figure 4: Branch 13 Heat Maps of Bacterial Concentration and Probability of Detection

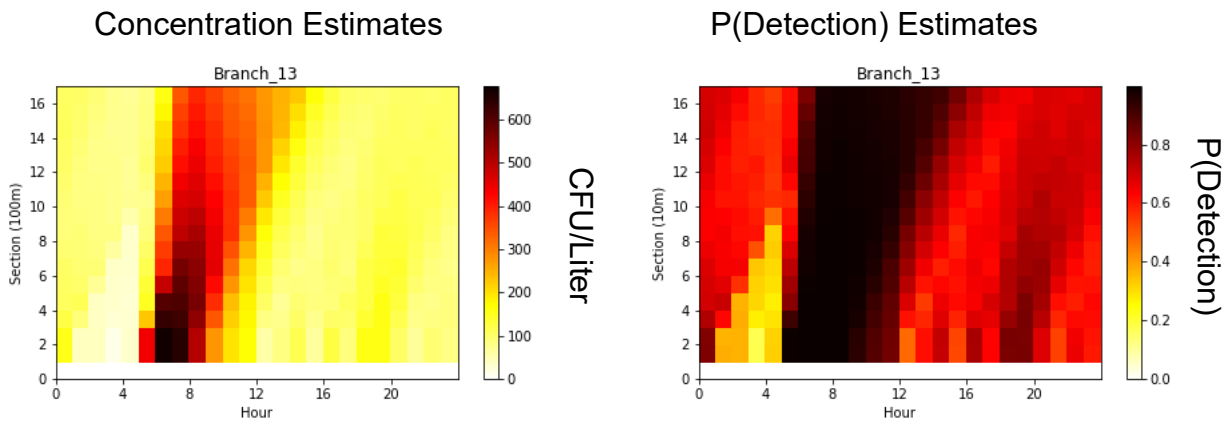


Figure 5: Branch 13, Section 4

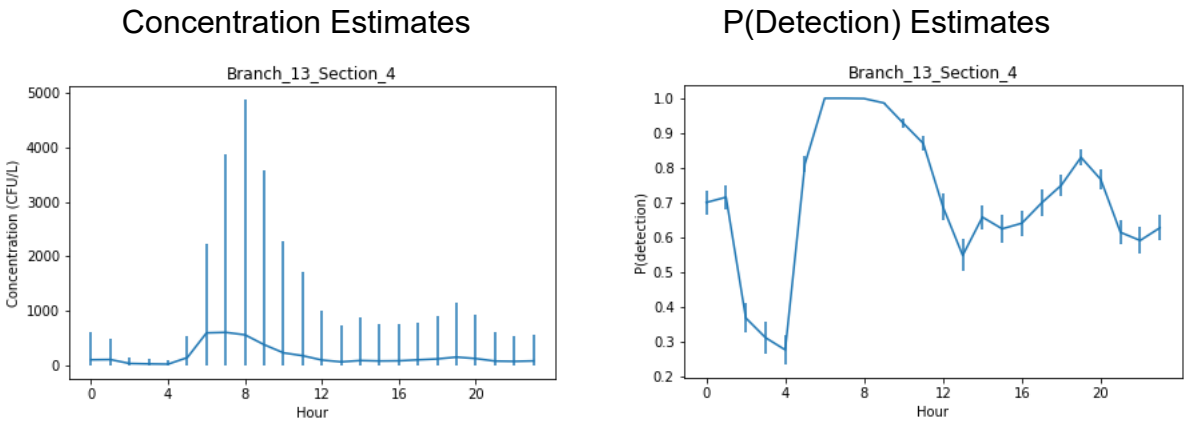


Figure 6: Branch 5 Heat Maps of Bacterial Concentration and Probability of Detection

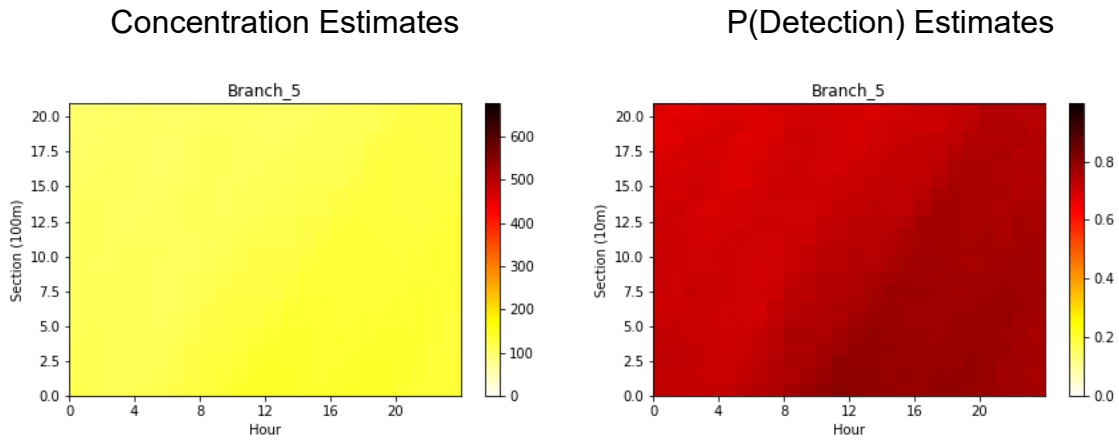
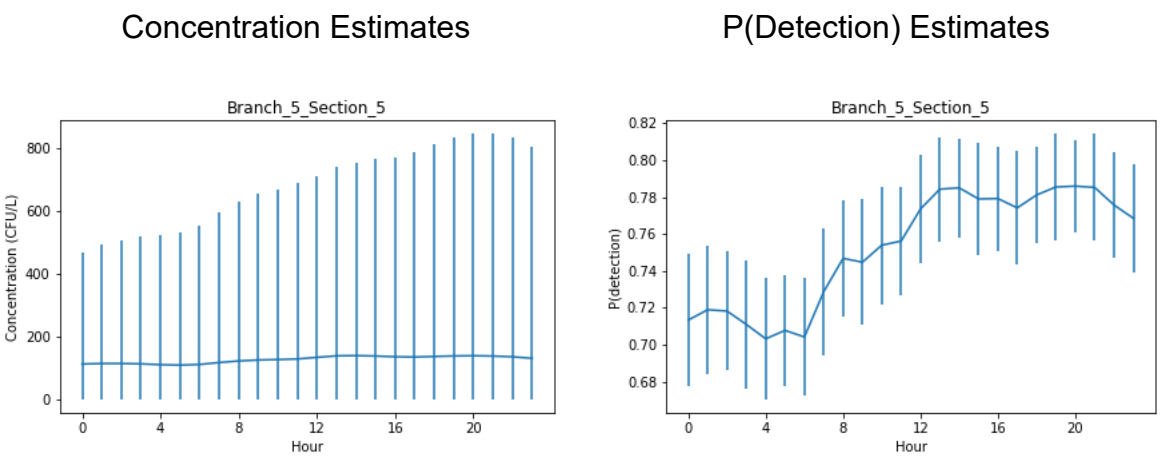


Figure 7: Branch 5, Section 5



Assuming these two sampling locations are accessible, it appears that sampling at Branch 13, Section 14 will have the highest probability of detection between 7:00 am and 10:00 am and sampling at Branch 5, Section 5 will have the highest probability of detection at approximately 2:00 pm. Risk calculations were performed using the estimated concentrations of *S.* Typhi at these two locations and time points. (Table 5)

Table 5: Estimations of Risk of Disease for Sampler

Single Sampling Event Risk (Branch 13, Section 4, 9 am) Mean (SD)	Single Sampling Event Risk (Branch 5, Section 5, 2 pm) Mean (SD)	Cumulative Risk from Both Samples Mean (SD)
0.00099 / 100 samplers (0.00097)	0.00099 / 100 samplers (0.00098)	0.0020 / 100 samplers (0.0019)

Example Run 2

In the second example run, a small, localized cluster of Typhoid cases along Branches 17-21, 23, and 25 was analyzed. In this run, these four branches were given a higher Typhoid fever prevalence of 250 cases / 100,000 persons. The rest of the system was given a low background prevalence of 1 case / 100,000 persons. The same sampling and laboratory method as Run 1 were used (1 L sample and membrane filtration). For sampling locations, this run analyzed Branch 5, Section 5 (same as Run 1) and Branch 10, Section 2.

Figure 8 demonstrates another plot that the model can produce, which provides an overview of P(Detection) estimates over the course of the day. Similar to Figure 3, this plot is unable to provide visual precision as to which branch may be the ideal place to sample. It does demonstrate that some of the higher-numbered branches are clearly not ideal, and the user should explore the individual branch plots more to determine where to sample.

Figure 8. Hourly Plots of Estimated P(Detection) Along Branches

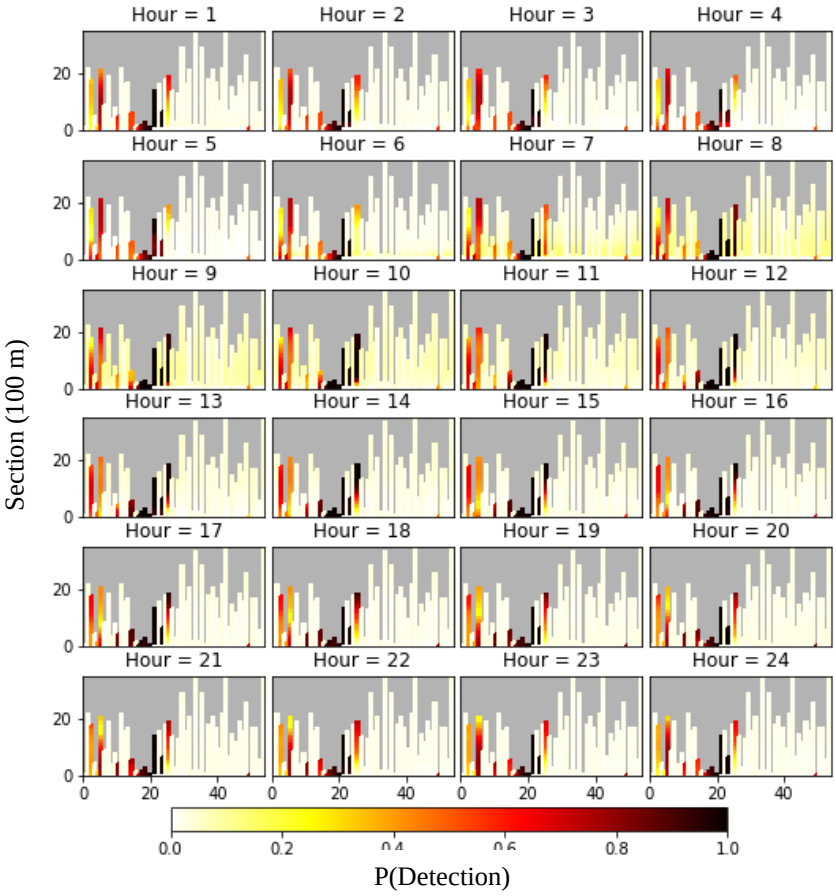


Figure 10. One plot is displayed for each hour. At each hour, each branch is plotted along the x-axis, with the section indicated on the y-axis. The P(Detection) is indicated by a heatmap.

Similar to Run 1, Figures 9-12 illustrate information about two specific branches and two specific sections.

Figure 9: Branch 5 Heat Maps of Bacterial Concentration and Probability of Detection

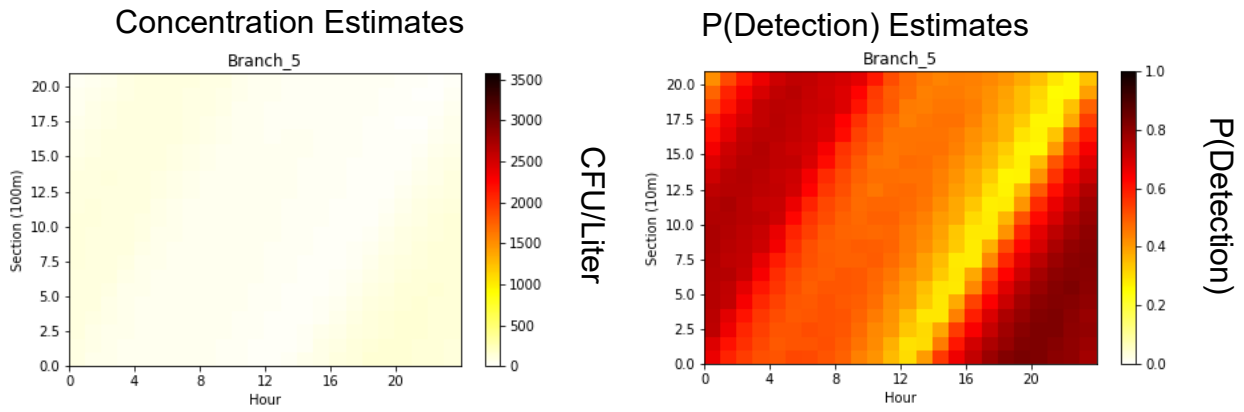


Figure 10: Branch 5, Section 5

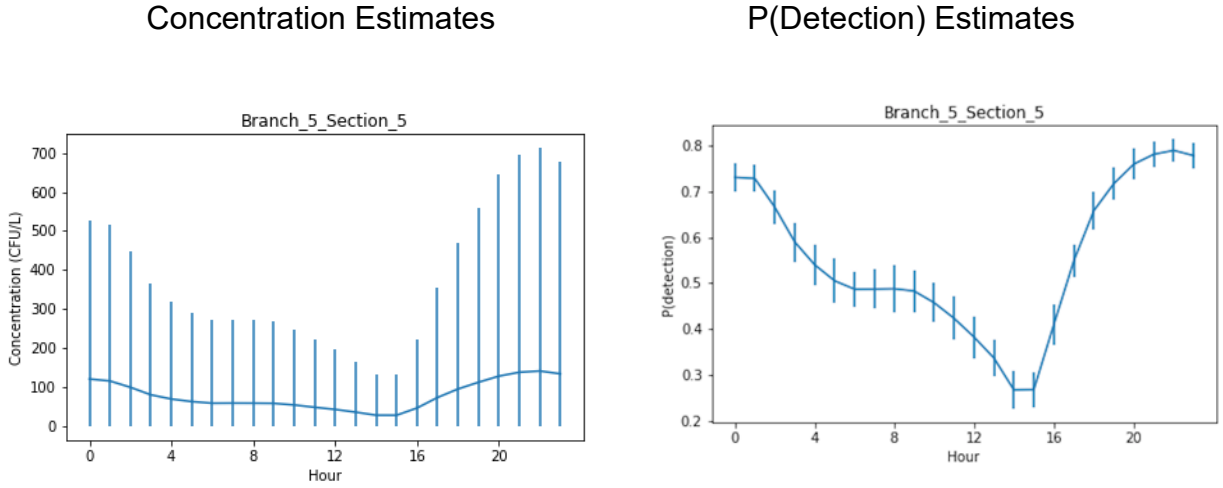


Figure 11: Branch 10 Heat Maps of Bacterial Concentration and Probability of Detection

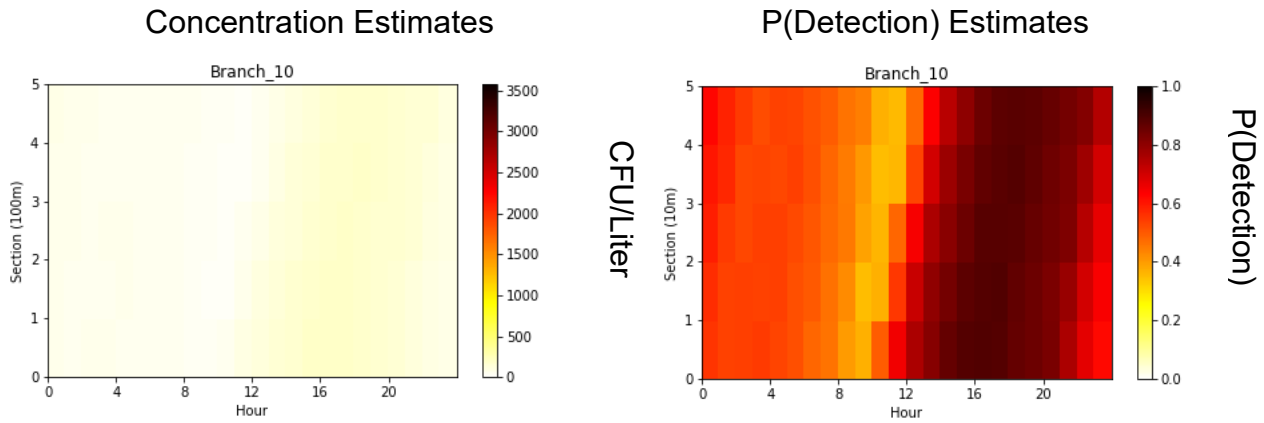


Figure 12: Branch 10, Section 2

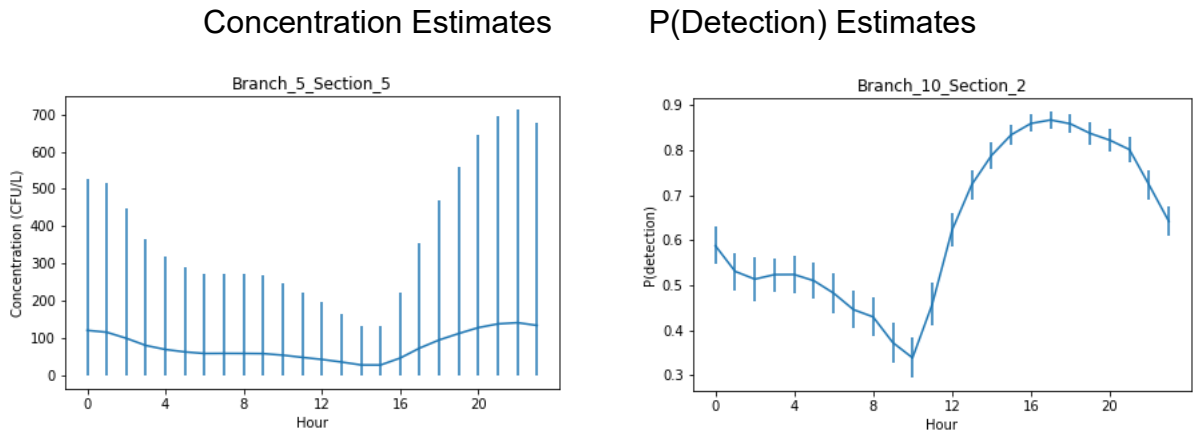


Table 6: Estimations of Risk of Disease for Sampler

Single Sampling Event Risk (Branch 5 Sect 5 Hour 12) Mean (SD)	Single Sampling Event Risk (Branch 10 Sect 2 Hour 16) Mean (SD)	Cumulative Risk from Both Samples Mean (SD)
0.0010 / 100 samplers (0.00098)	0.0010 / 100 samplers (0.00098)	0.0020 / 100 samplers (0.0019)

Given the estimates in Figures 9-12, the sampling locations and times listed in Table 6 were used in the risk model to estimate sampler risk. These risk estimates are very similar to those provided in Table 5. The estimated CFU/L for all of these sampling sites is between 100-1000 CFU/L. Although these concentration estimates produce variable probabilities of detection, it appears as if they are all so low that they produce very similar risk estimates. As Figure 1 illustrates, variability in the probability of illness is only introduced above a dose of 10_3 CFU. At doses below this, the risk of illness appears relatively uniform and extremely low.

Despite these low risk estimates, it was of interest to evaluate a potential intervention to reduce risk to samplers even further. In the original risk models, it is estimated that approximately 30 minutes (+/- 15 minutes) pass between the moment that a sampler's gloves get contaminated with wastewater and the moment that the sampler changes gloves. This is a large range, and different samplers may vary significantly in how often they change gloves, depending on training (no cited data, based on laboratory experience). Therefore, glove-changing was identified as an area for potential intervention to reduce risks. To test this intervention, the same risk model was run, while requiring that every sampler change gloves exactly 5 minutes after handling wastewater. Table 7 summarizes the results of this new risk model, using the output from Example Run 2, with the intervention included.

Table 7: Estimations of Risk of Disease for Sampler with Glove Intervention

Single Sampling Event Risk (Branch 5 Sect. 5 Hour 12) Mean (SD)	Single Sampling Event Risk (Branch 10 Sect 2 Hour 16) Mean (SD)	Cumulative Risk from Both Samples Mean (SD)
0.00032 / 100 samplers (0.0003)	0.00032 / 100 samplers (0.0003)	0.0006 / 100 samplers (0.0006)

Although the risk was already low, this intervention does reduce it even further. (Table 7). This is of interest because it demonstrates that more regular glove changes appear to limit health risks for environmental samplers interacting with wastewater. This risk reduction is likely to apply in other scenarios, where a sampler is interacting with more highly contaminated wastewater than that evaluated here. Therefore, based on this model, it may be an appropriate recommendation that samplers be trained to change gloves or decontaminate gloves at least every 5 minutes while actively handling raw sewage.

Discussion

The central function of this model is to track sections and branches along a wastewater system and record how the concentrations of a target organisms may change over time and location. Because of the built-in flexibility of this model, the user has almost complete control over all parameters of the system and can adjust them based on more accurate regional knowledge and knowledge about local disease prevalence. Because of this flexibility, the accuracy of predictions presented here directly reflects the accuracy of the input data.

From this risk model, it appears that the risk of becoming ill with Typhoid is low for samplers, assuming that samplers wear appropriate PPE. This risk can be further reduced by implementing more regular glove changes or glove decontamination procedures.

Weaknesses

Figures 5, 7, 10, and 12 illustrate the high degree of variability in the estimated CFU concentrations produced by these model runs. The standard deviations for many of these estimates are much larger than the estimated means. The shedding rate estimates (which range from 0 CFU/gram of feces to 10^7 CFU/gram of feces) appear to be introducing a large proportion of this variability. This may reflect natural variability in human populations, but further laboratory analyses of the feces of Typhoid cases. This may help clarify the distribution of shedding rates in a human population and determine whether the high degree of variability in reported shedding rates reflects true natural variability or small sample sizes.

A major weakness of this model is that it does not currently incorporate accurate estimates of streamflow and velocity. The estimates used in the example runs were based off best guesses from photos and maps. They do not reflect actual measured streamflow estimates. This weakness is extremely important, because the estimated concentrations of the target pathogen in a sample of wastewater directly relate to the flow of the stream or channel being sampled. Without accurate estimates of the expected dilution factor, this model cannot accurately estimate pathogen concentrations.

Another weakness of the model is a direct result of the intentionally built-in flexibility. Because the model is designed to be adapted to any wastewater system, the set-up requires a large amount of work on the user's end. The user must map out the system of interest, determine the lengths of each branch, and determine the flow directions and parent branches for each branch. This extra work does not require programming knowledge or technical skills and could be performed by hand, with the help of maps and photos. However, it does make model implementation very time- and labor-intensive.

Strengths

The flexibility of the model, although it results in a time- and labor-intensive set-up process, is also a strength of this model. By manipulating input parameters, the user could apply this model to any open channel system and, theoretically, to any closed-channel sewer network (although a sewer network has not yet been tested). The model was designed to be applied in any region or community. By changing the shedding rate and survival parameters, the same model could also be applied to other target pathogens.

Another strength of this model is that it provides intuitive output to help users create sampling plans. Even if the concentration and probability estimates are incorrect (given the weaknesses cited above), the model reflects patterns that occur over a system and over the course of the day. The user can utilize those patterns to identify branches and times that have the highest probability of detection, even if the actual probability and concentration estimates reflect under- or over-estimates of the true probability and concentrations.

Finally, the risk model provides estimates of risks for workers handling raw sewage. Health risks for wastewater workers has been considered before, as workers interacting with sewage are exposed to high numbers of viral, bacterial, and protozoan pathogens.[68,67] This study acknowledges that samplers collecting wastewater are exposed to similar risks, evaluates one potential exposure pathway, and proposes a low-cost and simple risk-reduction intervention.

Future Directions

A clear opportunity exists to improve this model by incorporating better stream flow and velocity estimates. Additionally, the model could benefit from incorporating variation due to weather and seasonality. For example, Vellore City is situated in the Paler River Basin, which experiences a monsoon season from June to December, during which 80% of the average annual rainfall occurs.[68]

The accuracy of this model could be greatly improved by incorporating accurate hydrological and meteorological data. This has been done before in microbial risk assessment models assessing complex environmental systems.[69] Models and tools already exists in the field of hydrology for estimating stream dynamics.[70] If such tools could be integrated with this model, the accuracy could be substantially improved.

Once these improvements are implemented, this model could also be applied to other wastewater networks and other infectious diseases.

Another important next step is to test the performance of this model against actual sampling data. Currently, such sampling data is unpublished and unavailable. As sampling data from various regions, such as Vellore City, are collected, they should be compared against this model's estimates. Such comparisons will help determine the accuracy of the model estimates and may help identify other areas for improvement.

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Supplemental Table: Distributed Parameter Estimates from Literature

Parameter	Units	Distribution	Estimates	Source	Notes
Fecal Output	Grams / day / person	Normal	Low Fiber: Mean 94.1 (11.1) g/day/person Medium Fiber: Mean 137.9 (13.2) g/day/person High Fiber: Mean 182.7 (22.3) g/day/person	[71]	Study results from 5 young men, ages 23.8 +/- 1.6 years, in Wisconsin, US
Shedding rate	Pathogens / gram of feces	Normal	Mean: 9.49 x 10^4 (1.05 x 10^5) cells / gram feces	Gathered from unpublished laboratory data.	
Wastewater Flow	Liters / person / day	Normal	Mean: 118.33 (SD: 42.39) Range (71-208)	[61]	Belo Horizonte, Brazil. City with 2 million inhabitants.
Infiltration	Liters / meter / hour	Normal	Mean: 0.535 SD: 0.606	[61]	9 Sampling Areas - Populations ranging from 286 - 3012.
Bacterial Decay Parameters in wastewater	Proportion of bacteria remaining after 1 hour	Point Estimate	Mean: 0.986 (SD: 0.0026)	[72]	*Salmonella enterica* serovar Typhi
		Point Estimate	Mean: 0.940 (SD: 0.0081)	[73]	*Salmonella enterica* serovar Typhimurium
		Point Estimate	Mean: 0.958 (SD: 0.0340)	[74]	*Salmonella enterica* serovar Enteritidis