Wastewater Monitoring of *Mycobacterium Tuberculosis* Complex, *Mycobacterium Tuberculosis*, and *Mycobacterium bovis*

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Abstract

Tuberculosis (TB) remains the leading cause of death among infectious diseases, with 1.25 million fatalities reported globally in 2023. The World Health Organization estimates that 10.8 million individuals developed TB in the same year, with Southeast Asia and Africa accounting for the majority of cases. Despite low TB incidence rates overall in Canada, Indigenous populations in Canada, particularly the Inuit of Nunangat, continue to experience disproportionately high incidences of TB, outnumbering all other population groups in the country. Systemic inequalities, including overcrowded housing, food insecurity, and limited access to healthcare, present persistent challenges to equitable TB prevention and management. Current strategies for TB control in remote regions like Inuit Nunangat rely heavily on community-wide screenings and contact tracing. However, logistical barriers, including geographic isolation and insufficient health infrastructure, hinder the effectiveness of these interventions. Additionally, historical trauma associated with TB testing and treatment has led to medical hesitancy among Indigenous communities, further complicating public health monitoring. Wastewater and environmental monitoring (WEM) offers a non-invasive, anonymous alternative for TB monitoring, enabling the early detection of outbreaks without the need for individual clinical diagnoses. Although the detection of *Mycobacterium tuberculosis* species (MTBC) in wastewater has been demonstrated, current methodologies such as intercalating dye-based polymerase chain reaction (PCR) employ clinical assays that lack the sensitivity and specificity required for wastewater and environmental applications. Moreover, effective WEM implementation, requires an understanding of the partitioning behavior of TB markers in wastewaters to optimize nucleic acid extraction to improve detection and quantification methods, which has not yet been established.

This study focuses on developing and validating probe-based quantitative PCR assays for the detection and quantification of MTBC species, including *Mycobacterium tuberculosis* and *Mycobacterium bovis*, in wastewater. By addressing the sensitivity and specificity challenges in current methodologies, and incorporating partitioning insights, this research aims to provide a foundation for implementing WEM as a disease monitoring system for TB in Northern Indigenous communities, ultimately advancing equitable public health solutions for underserviced, priority populations.

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List of Abbreviations

Abbreviation	Description
ALOD	Assay limit of detection
ALOQ	Assay limit of quantification
BCG	bacille Calmette-Guérin
COVID-19	Coronavirus disease in 2019
dPCR	Digital polymerase chain reaction
ddPCR	Digital droplet polymerase chain reaction
DOTS	Directly observed therapy
DR-TB	Drug-resistant tuberculosis
HAV	Hepatitis A virus
HIV	Human immunodeficiency virus
HMPV	Human metapneumovirus
INF-γ	Interferon-γ
MB	*Mycobacterium bovis*
MDR-TB	Multidrug-resistant tuberculosis
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NTM	Non-tuberculosis mycobacteria
PIV	Parainfluenza
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PLA	Polylactic acid
qPCR	Quantitative polymerase chain reaction
RSV	Respiratory syncytial virus
RT-PCR	Real-time polymerase chain reaction
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
TB	Tuberculosis
TST	Tuberculin skin test
WEM	Wastewater and environmental monitoring
WHO	World Health Organization
XDR-TB	Extensive Drug Resistant Tuberculosis

Chapter 1.
Introduction & Background

1.1 Introduction

Tuberculosis (TB) is considered the oldest active pandemic in the world, having killed over one billion people since its discovery in 1882 (Moutinho, 2022). TB is a disease caused by a group of Mycobacteria named the *Mycobacterium tuberculosis* complex (MTBC) (Daniel, 2006). Species in the MTBC include *M. tuberculosis* (MTB), *M. bovis* (MB), *M. africanum*, *M. canetti*, *M. caprae*, *M. microti*, and *M. pinnipedii*. However, the dominant species causing TB in humans is MTB, responsible for approximately 98% of infections (Lin & Desmond, 2014; Živanović et al., 2014). It wasn't until the year 1993 that TB was declared as a global public health emergency by the World Health Organization (WHO), setting a goal to eliminate TB globally by 2050 (Gupta & Sachdeva, 2020; Sotgiu et al., 2017; Zumla et al., 1999). By 2006, the WHO launched the Stop TB Strategy (now known as the End TB Strategy), expanding high-quality treatment systems such as directly observed therapy (DOTS), emphasizing outreach to underserved and vulnerable populations, and increasing community engagement (Lienhardt et al., 2012). While great progress has been achieved since the launching of the End TB Strategy, challenges such as the spread of multidrug-resistant TB (MDR-TB), human immunodeficiency virus (HIV) co-infection, and social determinants of health have posed a threat to achieving the goal by 2050 (Sotgiu et al., 2017).

1.2 Tuberculosis Epidemiology

In 2023, the WHO reported approximately 10.8 million new TB cases and 1.25 million TB-related deaths globally (World Health Organization, 2024). Despite being a preventable and usually curable disease, TB was the leading infectious cause of death in the world in 2023 (World Health Organization, 2024). TB is a reportable disease in all countries including the United States of America, United Kingdom, China, Canada, and countries of the European Union (Ditah et al., 2008; Dye et al., 2008; Falzon & Aït-Belghiti, 2007; Huang et al., 2014; Min et al., 2020; Pillaye

& Clarke, 2003; Vachon et al., 2018; World Health Organization, 2023). Five countries accounted for 56% of the total worldwide cases in 2023: India (26%), Indonesia (10%), China (6.8%), the Philippines (6.8%), and Pakistan (6.3%) (Figure 1) (World Health Organization, 2024). The majority of people who developed TB in 2023 were men (55%), whereas 33% were women, and 12% were children and young adolescents (aged 0 to 14 years) (World Health Organization, 2023). It is estimated that a quarter (25%) of the world population is infected with TB in a form known as latent tuberculosis infection (LTBI). LTBI occurs when individuals are infected with MTB but do not exhibit symptoms and are not infectious (Houben & Dodd, 2016; Jasmer et al., 2002). Individuals with LTBI have a 5 to 10% risk of developing active TB later in life. However, small children and immunocompromised individuals are at a greater risk of developing active TB (Zellweger et al., 2020). In addition, social determinants of health inequalities and inequities such as food insecurity, malnutrition, poor housing, and accessibility of health systems give rise to disparities of TB infection between populations (Hargreaves et al., 2011; Lienhardt, 2001).

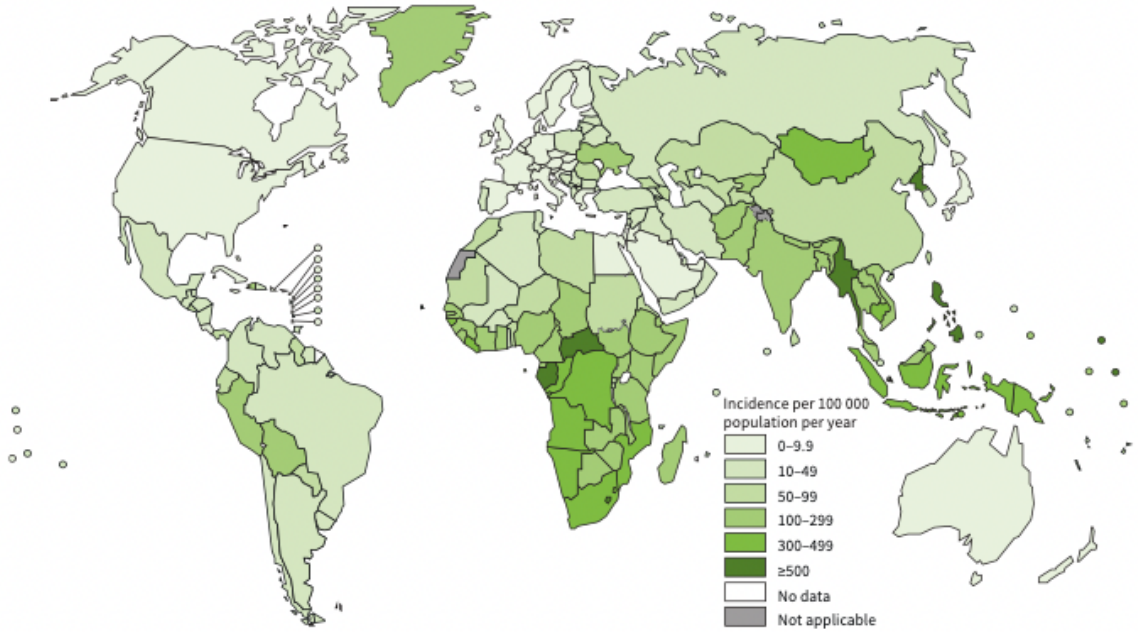

Figure 1.1. Incidence of TB globally in 2023 (World Health Organization, 2024)

1.3 Tuberculosis in Canada

The rate of newly reported cases of TB per year has remained relatively stable, from 4.6 to 5.1 per 100 000, between 2012 and 2022 (Figure 1.2) (Public Health Agency of Canada, 2024a). In 2022, there were 1,971 new cases of active TB reported in Canada, resulting in a rate of 4.8 per

100 000. For reference, the WHO estimated that the global TB incidence to be between 125 and 145 cases per 100 000 population (World Health Organization, 2023). Among new reported cases, 76.7% were in individuals born outside Canada, 16.9% were in Indigenous Peoples, and 3.6% were in non-Indigenous Canadian-born individuals. Among people born outside of Canada, over 70% originated two regions: the Western Pacific region (37.6%) and South-East Asia region (35.6%) (Public Health Agency of Canada, 2024a). The majority of active TB notifications in Canadian non-Indigenous populations are discovered through symptoms or incidental findings (81%), followed by 18% being in the "other" and "unknown" categories, and 1% being in contact investigations (Public Health Agency of Canada, 2024b).

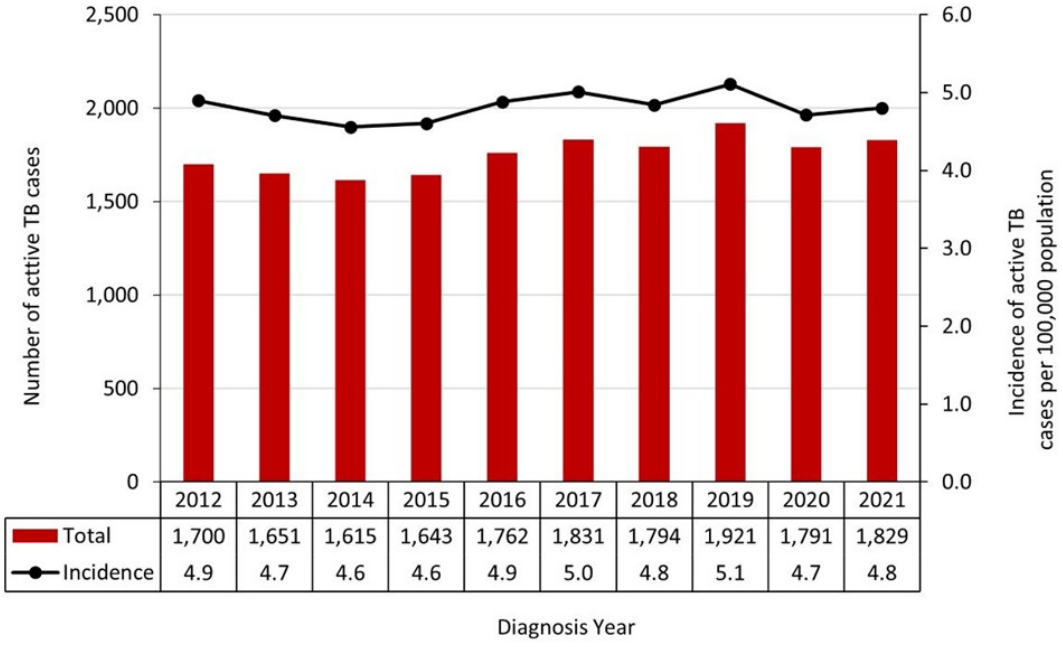

Figure 1.2. Number and Incidence of active TB in Canada from 2012 to 2021 (Public Health Agency of Canada, 2024a)

1.4 Tuberculosis in Inuit Populations

The incidence of TB in Indigenous populations is much higher than non-Indigenous Canadian-born individuals, at 16.6 per 100 000, compared to just 0.3 per 100 000, respectively. Within Indigenous populations, Inuit communities face the highest rates of active TB in Canada, with an incidence of 136.7 per 100 000, more than 450 times higher than that of non-Indigenous populations (Public Health Agency of Canada, 2024a). This rate is comparable with the global

incidence, which includes countries with the highest TB notifications (Public Health Agency of Canada, 2024a; World Health Organization, 2023).

According to the 2021 Census, 69.7% of the Inuit population resides in the Inuit Nunangat (Figure 1), which means "Inuit homeland" in Inuktitut (ᐃᓄᐃᑦ ᓄᓇᖅᑐᑦ). The Inuit Nunangat makes up approximately 35% of Canada's land mass and is comprised of 51 communities and villages across four Northern regions, from West to East: Inuvialuit (within Yukon and Northwest Territories), Nunavut, Nunavik (Northern Québec), and Nunatsiavut (Northern Labrador) (Figure 1.3) (Sheremata, 2018). Many of these areas are remote and accessible only by plane, resulting in challenges to receive support from all levels of government (Layton, 2023; Patterson et al., 2018).

Figure 1.3. Map of Inuit Nunangat (Statistics Canada, 2015).

Like other Indigenous populations in Canada, the Inuit Nunangat population faces disproportionate challenges such as overcrowded housing, inadequate access to healthcare, food insecurity, water insecurity, and higher unemployment rates compared to non-Indigenous Canadians (Indigenous Services Canada, 2023; Inuit Tapiriit Kanatami, 2018; Kilabuk et al., 2019; Layton, 2023; Richmond, 2009; Shankar et al., 2013). These challenges are considered as direct risk factors for disease transmission and development (Kim & Swaminathan, 2021; Narasimhan

et al., 2013). The Inuit Statistical Profile of 2018 reports that among Inuit in the Nunangat, 52% live in crowded homes, six times that of non-Indigenous populations. Due to the lack of available housing, this makes the delivery of infrastructures such as health systems and social services from the South more difficult to coordinate and achieve (Inuit Tapiriit Kanatami, 2018; Kilabuk et al., 2019; Riva et al., 2020; Sultan, 2023). Like many other Indigenous populations, Inuit populations have experienced and still experience a long-standing history of colonization and forced relocation by the Government of Canada, negatively impacting their health and livelihoods (Farrell et al., 2021; Kulmann & Richmond, 2011; Richmond & Ross, 2009). Due to this complex history which includes forced medical treatment and relocation, many Inuit individuals are hesitant to receiving TB testing and treatment, leading to an often under-representation of individuals with TB in Indigenous communities. This hesitancy is evident in the differences of case finding methods between Inuit populations and non-Indigenous populations, with only 36% of cases in Inuit populations being reported due to symptoms compared to 81% in non-Indigenous populations (Figure 1.4) (Public Health Agency of Canada, 2024b). Due to the disproportionately high rates of TB reported in Inuit populations, the Government of Canada has made a commitment to the elimination of TB across Inuit Nunangat by 2030 (Indigenous Services Canada, 2018).

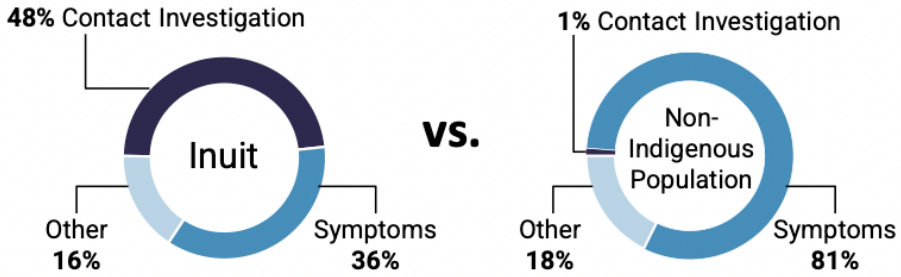

Figure 1.4. Case Finding of TB Methods in Canada (Public Health Agency of Canada, 2024b)

1.5 Tuberculosis Diagnosis and Treatment

TB diagnosis is a lengthy and expensive process, which is often not available to underserved populations (Campbell & Bah-Sow, 2006). Healthcare professionals will often begin diagnosis by administering a tuberculin skin test (TST), an intradermal injection of mycobacterial antigens to observe an immune response, which typically presents as localized swelling. Following

the administration of a TST, the suspected patient must return to the healthcare professional within 48-72 hours to analyze the injection site for a reaction to the antigens (Snider, 1982). This test is often used as a primary screening tool due to its low cost, however, the TST can produce a high degree of false-positive result in individuals who have received the bacille Calmette-Guérin (BCG) vaccine (Farhat et al., 2016). Following a positive TST result, healthcare professionals will confirm diagnosis using a variety of methods such as sputum culture or smear, chest radiographs, interferon-γ (INF-γ) assays, or molecular methods such polymerase chain reaction (PCR) (Campbell & Bah-Sow, 2006; Farhat et al., 2016; Tagmouti et al., 2014). Newer technology such as the cartridge-based GeneXpert MTB/RIF Ultra system, which uses PCR to rapidly detect MTB and drug-resistant MTB (DR-MTB) have been distributed but can be cost-prohibitive to underserved communities (Pantoja et al., 2013; Vassall et al., 2011; Zeka et al., 2011). Due to the limitations of each method, healthcare professionals will often use various combinations of multiple methods to conclude the diagnosis of TB (Dosanjh et al., 2008; Shingadia & Novelli, 2003).

Following the diagnosis of active TB can be a lengthy and often aggressive course of treatment using a combination of antibiotics such as rifampicin, isoniazid, pyrazinamide, and ethambutol for several months. In addition to the combination of the four drugs for several months, continuation of the first-line drugs rifampicin and isoniazid is required for several more months (World Health Organization, 2008). In addition, patients receiving treatment may experience adverse effects such as gastrointestinal issues and hepatitis caused by the antibiotic drugs. Due to the long treatment duration, patient adherence can be difficult to achieve (Gebremariam et al., 2010). As a result, strains of TB that are resistant to treatment known as DR-TB, MDR-TB, and extensively drug-resistant TB (XDR-TB) have become more common, threatening the WHO's goal to eliminate TB by 2050 (Seung et al., 2015; World Health Organization, 2024).

1.6 Tuberculosis in Wastewater

Since the late 19th century, MTB bacteria has been found in various types of environmental samples such as soil and water (Martinez et al., 2019; Velayati, Farnia, & Mirsaeidi, 2015). The source of these pathogens in environmental samples are often due to contamination with human

excreta, typically urine and stool (Abaye et al., 2017). In the 1950s, viable MTB bacteria was reported in wastewater samples by numerous studies (Bergsman & Vahlne, 1951; Greenberg & Kupka, 1957; Jensen, 1954; Kelly et al., 1955; Pramer & Heukelekian, 1950; Velayati, Farnia, & Mirsaeidi, 2015). These studies confirmed viable MTB in wastewater by inoculating guinea pigs with discharged wastewaters from institutions treating TB patients such as sanatoria. Following inoculation, the guinea pigs not only developed evidence of TB infection, but also evidence of DR-TB (Gao et al., 2018; Jensen, 1954; Kelly et al., 1955). In addition to the successful inoculation of animals, multiple cases were reported of children developing TB after being immersed in wastewater-contaminated waters were reported in the 1950s, demonstrating the viability and infectious nature of MTB bacteria in contaminated water sources (Miller & Anderson, 1954). More recently, molecular studies of MTB and MB infection in livestock have reported and quantified fecal shedding in infected mammals such as bovine and porcine species both *in vivo* and in environmental samples (Emma Travis et al., 2019; Santos et al., 2015). The presence of MTB has also been demonstrated in drinking water due to animal and fecal contamination in drinking water supplies of Rio de Janeiro, Brazil (Bianco et al., 2020; Mtetwa et al., 2022b).

Current methods for the detection of MTBC in the environment can be divided into two categories: culture-based and molecular-based (Cai & Zhang, 2013; Khan et al., 2017; Mtetwa et al., 2023b; Velayati, Farnia, Mozafari, et al., 2015; Verma et al., 2022). Culture-based methods require the isolation and culturing of MTBC from wastewater and environmental samples. In order to isolate MTBC, sample disinfection is performed to eliminate nontarget microorganisms, typically incorporating antimicrobials. Following isolation of MTBC in the sample, is concentration of MTBC cells, often using filtration or centrifugation (Mtetwa et al., 2022b). However, culture-based methods present several limitations such as the slow growing nature of *Mycobacterium spp.,* having a growth rate of 18-20 hours and the presence of viable but non-culturable MTBC organisms (Falkinham, 2009, 2022; Lambrecht et al., 1988; Mtetwa et al., 2022b). More novel molecular-based methods have since been employed, such as conventional PCR, quantitative (qPCR) using intercalating dyes (ie. SYBR Green), droplet digital PCR (ddPCR), and high-throughput shotgun sequencing (Cai & Zhang, 2013; Mtetwa et al., 2023b). Such methods have recently been employed in TB affected regions throughout Africa in the context of wastewater environmental monitoring (WEM) (Mtetwa et al., 2021, 2022a, 2023a, 2023b). TB

WEM has recently been demonstrated as a means to detect and monitor antibiotic resistance within communities associated with TB treatment (Mtetwa et al., 2021, 2023b). WEM of TB has the capability of providing an anonymous and non-invasive monitoring technique to collect meaningful public health data, especially in communities where the topic may be sensitive or stigmatized (D'Aoust et al., 2022; Hegazy et al., 2022; Mercier et al., 2023; Parkins et al., 2024; The Lancet Microbe, 2024).

1.7 Current Limitations and Gap of Knowledge

WEM has regained research interest during the surge of the COVID-19 pandemic and has since expanded to include other the monitoring of Influenza A and B, respiratory syncytial virus (RSV), enterovirus D68, noroviruses, and mpox (formerly known as monkeypox) (Boehm et al., 2023; Choi et al., 2018; D'Aoust et al., 2021; Malla et al., 2024; Mercier et al., 2022, 2023; Philo et al., 2024; C. H. Wong et al., 2023; Zhang et al., 2024). As WEM and more specifically, TB WEM continues to expand and provide meaningful public health data, there is presently no standardized approach to detect and quantify TB in wastewater. Studies have demonstrated the successful monitoring of MTBC in wastewater using conventional PCR and ddPCR using intercalating dyes in regions of Africa with high TB incidence (Mtetwa et al., 2022a). However, it is well-established that probe-based qPCR offers a higher degree of both sensitivity and specificity compared to intercalating dye-based qPCR due to its requirement of the successful hybridization of three unique and specific oligonucleotides compared to two oligonucleotides required in the latter for detection (Ahmed et al., 2022; Alvarez & Doné, 2014; Crockett & Wittwer, 2001; W. Wong et al., 2015). In the context of WEM, probe-based qPCR is commonly employed compared to other molecular methods due to its high sensitivity and specificity (Ahmed et al., 2020, 2022; Haramoto et al., 2020; La Rosa et al., 2020; Länsivaara et al., 2024; Medema et al., 2020; Nagelkerke et al., 2023; Shrestha et al., 2023; Thakali et al., 2024). With respect to detecting and quantifying TB in wastewater, current clinical qPCR assays, such as the IS6110 assay, lack the specificity required for wastewater applications due to its non-specific hybridization to extraneous organisms present in wastewater (Coros et al., 2008; Gillespie et al., 1997; Hellyer et al., 1996; Müller et al., 2015). In addition, there is no current available study which establishes the partitioning behaviour of TB-causing bacteria in wastewater samples, which is critical to understanding how best to concentrate

and extract TB-causing bacteria DNA prior to qPCR. As a result, an understanding of TB-causing bacteria partitioning in wastewater align with the development and validation of highly sensitive and specific probe-based qPCR assays are required to accurately detect and quantify TB-causing bacteria in wastewater. In addition, the methods need to be validated in both large municipal and small remote communities to ensure the wide applicability to all populations.

1.8 Research Objectives & Significance of Work

This thesis addresses two key challenges in the detection and quantification of TB in wastewater: (i) the lack of probe-based qPCR assays that are validated for application in wastewater samples, and (ii) establishment of the partitioning behaviour of TB markers in wastewater. The specific objectives of this research are as follows:

1. Design and validate probe-based qPCR assays for TB quantification in wastewaters.
2. Determine sensitivity to detect and quantify MTBC, MTB, and MB in wastewaters collected from both low- and high-prevalence regions.
3. Determine specificity to effectively differentiate between MTBC, MTB, and MB in wastewater, a biologically diverse sample.
4. Determine the partitioning behaviour of TB markers in both municipal influent wastewater and primary clarified sludge.

Results from this work will provide a means to overcome the current limitations of WEM of TB and advance its application, specifically in disproportionately affected and underserved communities in Northern Canada.

1.9 Thesis Structure

This thesis is subdivided into three chapters of which the first chapter is an introduction to the research topic, the second chapter is submitted in the Water & Health peer reviewed journal, and the third chapter is a conclusion of the work. A description of the contents of each chapter is outlined below.

Chapter 1. "*Introduction & background.*" This chapter provides an overview of the public health challenges associated with TB and the potential of WEM as a tool for meaningful public health monitoring in underserved communities, particularly Indigenous communities. It highlights the limitations of current WEM methods and establishes the significance of this research. The chapter also outlines the study's objectives, scope, and the structure of the thesis.

Chapter 2. "*Partitioning and probe-based quantitative PCR assays for the wastewater monitoring of Mycobacterium tuberculosis Complex, M. tuberculosis, and M. bovis.*" This chapter details the development and validation of three probe-based quantitative qPCR assays adapted from clinical

methodologies for use in WEM applications. It also explores the partitioning behavior of TB markers in wastewater, providing a framework for employing WEM as an anonymous and effective public health monitoring tool.

Chapter 3. "*Conclusions and recommendations*" This chapter iterates on how the research objectives were met. Overall recommendations for future work are also provided.

1.20 Contribution of Authors

The thesis includes a manuscript, which is based on the findings of this study. The manuscript has been submitted to the peer-reviewed Journal of Water & Health. The authors' contributions are described below:

Manuscript 1: T. B. Nguyen, É. Mercier, C. H. Wong, N. Hegazy, M. P. Kabir, E. Tomalty, F. Addo, L. Ward, E. Renouf, S. Wan, Y. Tcholakov, S. Guilherme, and R.Delatolla. *Partitioning and probe-based quantitative PCR assays for the wastewater monitoring of Mycobacterium tuberculosis Complex, M. tuberculosis, and M. bovis.* Submitted to the Journal of Water & Health.

T. B. Nguyen: Conducted literature review, designed assays, performed nucleic acid extraction, performed qPCR analysis, performed formal analysis, and wrote the manuscript.

É. Mercier: Provided expertise and guidance for experimental methodology, assisted with formal analysis, review and editing of the manuscript.

C. H. Wong: Provided expertise and guidance for experimental methodology, review, and editing of the manuscript.

N. Hegazy: Review and editing of the manuscript.

M. P. Kabir: Provided expertise and guidance for experimental methodology, review, and editing of the manuscript.

E. Tomalty: Review and editing of the manuscript.

F. Addo: Review and editing of the manuscript.

L. Ward: Provided expertise and guidance for experimental methodology, review, and editing of the manuscript.

E. Renouf: Provided supervision in analysis and validation of results, and review and editing of the manuscript.

S. Wan: Provided expertise and guidance for experimental methodology, review, and editing of the manuscript.

Y. Tcholakov: Provided supervision in analysis and validation of results, and review and editing of the manuscript.

S. Guilherme: Provided supervision in analysis and validation of results, and review and editing of the manuscript.

R. Delatolla: Conceptualized study, provided expertise and guidance for experimental methodology, supervision in analysis and validation of results, editorial guidance and contribution, and funding acquisition for the research.

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Chapter 2.

Experimental Methods

2.1 qPCR Assay Design

To detect and quantify different the *Mycobacterium tuberculosis* complex (MTBC) in wastewater samples, three quantitative polymerase chain reaction (qPCR) assays were designed and adapted from previously published methods. These assays target specific genetic markers that distinguish MTBC, *M. tuberculosis* (MTB), and *M. bovis* (MB). The MTBC *rv0577* probe-based qPCR assay which is associated with virulence and found in all MTBC species. It was adapted from an assay developed by Chae *et al.* (2017) to detect MTBC bacteria, including MTB and MB (Chae et al., 2017). The *rv0577* gene is unique to MTBC, detecting this gene confirms the presence of any MTBC species in a sample (Chae et al., 2017; Gu et al., 2016; Qasim et al., 2023). The MTB RD9 probe-based qPCR assay is specific to MTB and is based on the genomic region of difference 9 (RD9), located between two genes (*rv2072c* and *rv2073c*). RD9 is present in MTB but absent from most other MTBC species, such as MB, *M. africanum*, and *M. orygis*, allowing for specific identification of MTB (Brosch et al., 2002; Huard et al., 2003; Smith et al., 2006; Teo et al., 2013). The MB RD4-deletion probe-based qPCR assay was designed to specifically detect MB by targeting the genomic deletion of the region of difference 4 (RD4) in MB, exclusively. By detecting this deletion, the assay can distinguish MB from closely related bacteria, such as MTB (Kapalamula et al., 2021; Ru et al., 2017; Sales et al., 2014).

The reference assays were utilized to identify genomic locations to produce sensitive and specific probe-based qPCR assays for the application of wastewater and environmental monitoring using the Primer3Plus tool (https://www.primer3plus.com). To validate that each assay was specific to their intended targets, the designed probe-based qPCR assays were aligned using MUSCLE to determine *in situ* specificity with reference genome sequences from commonly

referred MTB variants (H37Rv and HN878), MTBC species (*M. canettii*, *M. africanum*, and *M. microti*), commonly referred MB variants (Mb3602, Danish 1331, bacille Calmette-Guérin (BCG)), and commonly referred NTM species (*M. avium hominissuis, M. kansasii, and M. intracellulare*) to determine *in silico* assay specificity (Figure 1) using genome sequences obtained from GenBank (National Institutes of Health) (Edgar, 2004). The sensitivity of each assay was analyzed using the IDT OligoAnalyzer Tool to determine melting temperature, hairpin structures, homo-dimerization, and hetero-dimerization. All primers and probes included a 5'-FAM reporter, a 3' minor groove binder (MGB), and a nonfluorescent quencher for increased sensitivity and specificity during qPCR.

2.2 qPCR Conditions Optimization

The optimal conditions for the qPCR assays targeting MTBC (*rv0577*), MTB (RD9), and MB (RD4-deletion) were established using the CFX96 Real-Time PCR Detection System. Reactions were carried out with the TaqMan Fast Advanced Master Mix, and quantified DNA templates were obtained from cultured MTB H37Rv and MB BCG strains, measured using digital PCR (dPCR). To determine the optimal denaturation duration for each assay, qPCR was performed with technical triplicates along with non-template controls and cycling conditions of denaturation at 95°C for 30 seconds, followed by 44 cycles consisting of 95°C for 5, 10, 15, 20, and 30 seconds, paired with an extension step at 60°C for 30 seconds (Table X). The optimal annealing temperatures were identified using qPCR cycles at 95°C for 30 seconds, followed by 44 cycles of 95°C for 15 seconds, and annealing temperatures ranging from 57°C to 63°C in 0.5°C increments for 30 seconds.

Table 1.1. Determination of optimal qPCR cycling conditions

Temperature	Duration (seconds)
95°C	30
	44 cycles
95°C	5 / 10 / 15 / 20 / 30
60°C	30

2.3 Assay Sensitivity

The amplification efficiencies of the MTBC *rv0577,* MTB RD9, and MB RD4-deletion qPCR assays were determined using technical triplicates of serially diluted DNA, previously purified from cultured MTB cells, and diluted using molecular-grade water (Appendix A). Non-template controls using molecular-grade water were included in all runs to monitor for contamination. DNA concentrations were quantified using dPCR on the AbsoluteQ system (Applied Biosystems), employing the AbsoluteQ Universal DNA Digital PCR Master Mix with each respective assay. The calculated reaction efficiencies for the MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays were 99.85%, 99.71%, and 99.65%, respectively, as determined using standard curve analysis consistent with optimal qPCR performance criteria.

2.4 Assay Specificity

Specificity of the MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays was assessed using genomic DNA extracted from a panel of reference *Mycobacterium spp.* strains. DNA was quantified using the NanoDrop™ One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). MTB DNA was used as a positive control for the MTBC *rv0577* and MTB RD9 assays, and as a negative control for the MB RD4-deletion assay. Conversely, MB BCG DNA served as a positive control for the MTBC *rv0577* and MB RD4-deletion assays and a negative control for the MTB RD9 assay. Additionally, DNA from *M. kansasii* and *M. smegmatis*, both non-tuberculous mycobacteria (NTM) was included as negative controls for all three assays, given that these species belong to the *Mycobacterium* genus but are not tuberculosis-causing bacteria. Each assay was evaluated at two template concentrations (10 ng/µL and 1 ng/µL) to assess specificity DNA. All qPCR reactions were conducted in technical triplicates under the previously optimized cycling conditions, with non-template controls (Figure X). Amplification was considered positive when at least two of the three technical replicates demonstrated quantification cycle (Cq) values within 40 cycles.

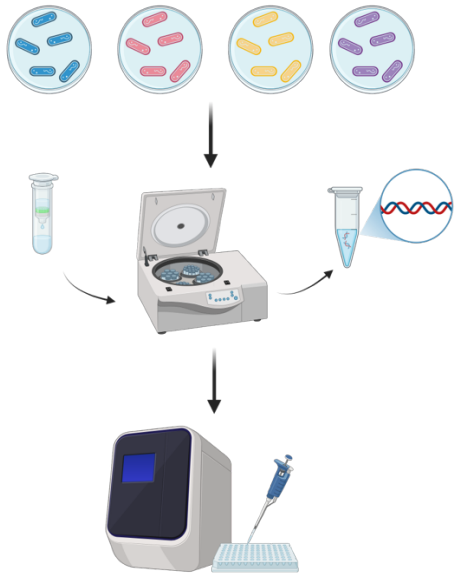

Figure 2.5. Assay specificity evaluation protocol using cultured *Mycobacterium spp.* strains

2.5 Locations of collected wastewater with endogenous targets

A panel of wastewater samples with endogenous MTBC was employed to validate the three newly developed probe-based qPCR assays targeting MTBC *rv0577*, MTB RD9, and MB RD4-deletion. This panel consisted of 88 samples collected from three distinct geographic and epidemiological contexts: 70 samples from Ottawa, Ontario, Canada, a moderate-risk, high-income metropolitan setting; two samples from Mumbai, India, a high-risk, middle-income metropolitan region; and 16 samples from a high-risk, remote Indigenous community in the Inuit Nunangat region of Northern Quebec (Figure 2.2). Each location had documented cases of active TB during the corresponding sampling periods. Ottawa, the capital region with an approximate population of one million, has an average pre-tax annual household income of $64,500 CAD (Statistics Canada, 2023). Mumbai, India's most populous city with roughly 12.5 million inhabitants, has an average pre-tax annual household income of ₹230,000 INR (~$3,778 CAD) (Rathore, 2024). The Inuit Nunangat region, comprising 14 remote communities with a collective population of approximately 13,000, has an estimated average pre-tax annual household income of $35,000 CAD (Nunavik Statistics Program, 2021).

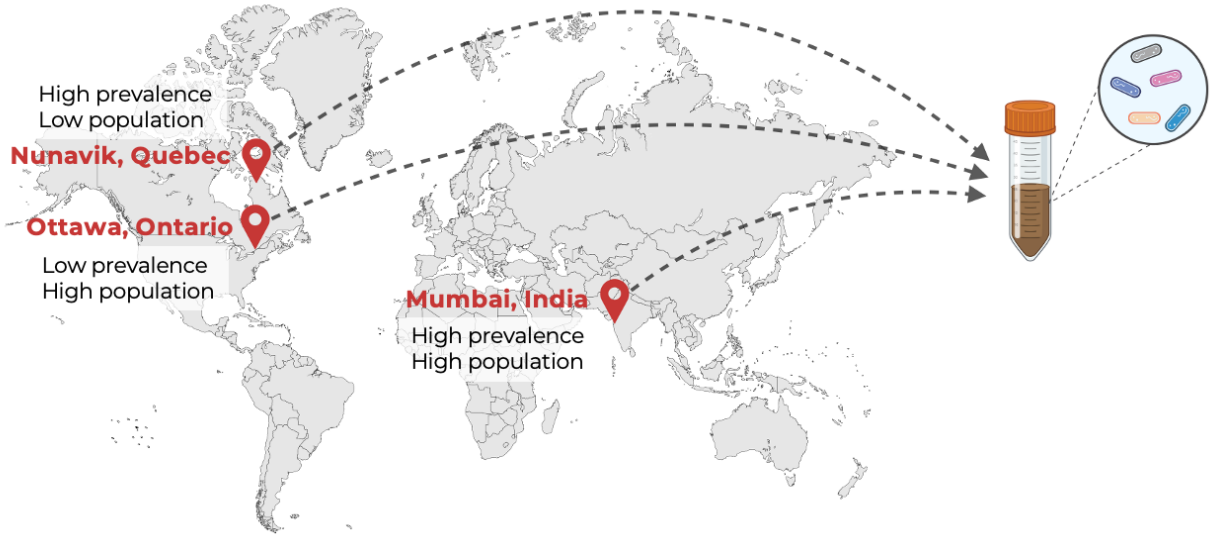

Figure 6.2. Locations of collected wastewater with endogenous targets for the validation of proposed assays.

2.6 Wastewater collection, transport, and storage prior to analysis

In Ottawa, 500 mL 24-hour composite primary sludge samples were collected from the Robert O. Pickard Environmental Centre WRRF, which serves approximately one million people and treats an average of 436 million litres of wastewater per day. Samples were stored at 4°C immediately after collection and transported on ice to the University of Ottawa for processing. In Mumbai, 2.0 L grab samples of influent wastewater and 24-hour composite primary sludge were obtained from the Powai and Ghatkopar WRRFs, which treat an estimated 5.8 and 180 million litres of wastewater per day, respectively. These samples were stored at 4°C and shipped internationally via courier on ice. In the Inuit Nunangat community, samples were collected from the village's decentralized wastewater lagoon system using 3D-printed COVID-19 Sewer Cage (COSCa) passive samplers containing sterile gauze (Figure 2.3) (Hayes et al., 2021). These were 3D-printed using PLA filament on an UltiMaker 2+ printer (Figure 2.4). After field collection, the samples were stored at 4°C and shipped via express courier to the University of Ottawa.

Figure 2.7. COSCa passive sampling deployed at the Inuit Nunangat village's wastewater lagoon.

Figure 2.8. 3D printed COSCa passive sampler with sterile gauze.

40 mL of homogenized primary sludge samples from Ottawa were centrifuged at 10,000 × g for 45 minutes at 4°C, with a secondary centrifugation step applied to the pellet. Total nucleic acids (DNA and RNA) were extracted using the AllPrep PowerViral DNA/RNA Kit (Qiagen), following protocols previously described protocols (D'Aoust et al., 2021b; Wong et al., 2023). Upon reception, 30 mL aliquots of influent wastewater settled solids samples from both Powaii and Ghatkhopar WRRFs were treated with

a PEG-8000/NaCl solution (final concentrations: 80 g/L PEG, 0.3 M NaCl), as described in Wong et al. (2023), followed by ultracentrifugation. Total nucleic acids (DNA and RNA) were extracted using the AllPrep PowerViral DNA/RNA Kit (Qiagen), following protocols previously described protocols (D'Aoust et al., 2021b; Wong et al., 2023). Upon reception of the Inuit Nunangat samples, gauze from COSCa samplers was immersed in 300 mL of deionized water, allowed to settle for 15 minutes, and 40 mL of the settled solids was extracted using the previously mentioned protocol. Total nucleic acids (DNA and RNA) were extracted using the AllPrep PowerViral DNA/RNA Kit (Qiagen), following protocols previously described protocols (D'Aoust et al., 2021b; Wong et al., 2023). All extractions included blank controls using molecular-grade water processed in parallel to rule out contamination.

2.7 Wastewater qPCR conditions

All qPCR reactions were performed in technical triplicates, with non-template controls included to confirm assay integrity and rule-out contamination. Standard curves were generated using five-point serial dilutions (100, 40, 20, 5, 2.5 copies/µL) of dPCR-quantified DNA from cultured MTB and MB BCG, diluted in molecular-grade water. MTB DNA served as the assay control for MTBC *rv0577* and MTB RD9 assays, while MB BCG DNA was used as the control for the RD4-deletion assay. To assess potential qPCR inhibition, PMMoV (pepper mild mottle virus), a commonly used fecal contamination indicator (D'Aoust et al., 2021a; D'Aoust et al., 2021b; Haramoto et al., 2013; Rosario et al., 2009; Wong et al., 2023), was quantified in 1/10 and 1/40 dilutions. RT-qPCR efficiency ranged between 90–100%, with coefficients of determination (R^2) exceeding 0.95. Singleplex probe-based RT-qPCR targeting the PMMoV RAP gene was conducted using primers, probes, and thermocycling conditions as described by Haramoto et al. (2013) and D'Aoust et al. (2021a; 2021b) (D'Aoust 2021a; 2021b; Haramoto et al., 2013).

2.8 Wastewater sample measurements

Wastewater measurements for MTBC *rv0577*, MTB RD9, and MB RD4-deletion targets are expressed in three metrics: target DNA copies per qPCR reaction (copies), per gram of wet wastewater solids (cp/g), and normalized to PMMoV fecal indicator levels (cp/cp).

2.9 Partitioning of Endogenous TB in Municipal Influent Wastewater and Primary Sludge

To determine the partitioning behavior of endogenous MTBC and MTB in wastewater samples, experiments were conducted using grab influent wastewater and 24-hour composite primary sludge samples collected from the Powai and Ghatkopar WRRFs in Mumbai, India. Samples from Mumbai were utilized for partitioning experiments due to the high burden of TB in the metropolitan region, compared to those of Ottawa and the remote Indigenous community. All samples were shipped on ice and processed within 24 hours following arrival at the University of Ottawa. DNA was extracted from each sample fraction within six hours of the partitioning procedure.

To determine the partitioning of endogenous MTBC and MTB in influent wastewater, a 2.0 L grab sample was collected from both Powai and Ghatkopar WRRFs. Both influent wastewater samples were divided into four fractions (n=21): liquid, filtered solids, centrifuged solids, and polyethylene glycol (PEG)-precipitated ultracentrifuged solids. A total of 5 biological replicates of the liquid fractions, 6 biological replicates of the filtered solids fraction, 5 biological replicates of the centrifuged solids fraction, and 5 biological replicates of the PEG-precipitated ultracentrifuged solids of the samples were produced (Mercier et al., 2022; Wong et al., 2023). Upon reception, the samples were initially settled for 2 hours at 4°C, followed by pipetting 40mL of the settled solids for centrifugation at 10 000 x g for 45 minutes at 4°C. The settled solids supernatant was separated and preserved at 4°C for further study. The centrifuged settled solids pellet was centrifuged a second time at 10 000 x g for 10 minutes at 4°C, also preserving the settled solids supernatant. Nucleic acids were extracted from the centrifuged settled solids using the AllPrep PowerViral DNA/RNA Kit (Qiagen) following previously described work (D'Aoust 2021b; Wong et al., 2023). 40mL of the settled solids supernatant of each sample was serially filtered through 30 kDa-15mL Amicon cartridges (Millipore Sigma) at 4 000 x g for eight repeats of 30 minutes at 4°C, discarding the flow-through after each repeat. Nucleic acids were extracted from the settled solids supernatant using the QIAamp Viral RNA Mini Kit (Qiagen) using a QIAcube Connect automated extraction protocol as per the manufacturer's instructions. 400 mL of the post-settling supernatant

from the samples were serially diluted through 0.45-µm GF6 mixed cellulose ester filter using vacuum filtration. 32 mL of elution buffer (0.05 M KH2PO4, 1.0 M NaCl, 0.1% (v/v) TritonX-100 pH 9.2) was passed through the filters using vacuum filtration. Nucleic acids were extracted from the filtered solids using the AllPrep PowerViral DNA/RNA Kit (Qiagen) following previously described work (D'Aoust et al., 2021b; Wong et al., 2023). Finally, 32mL of each influent wastewater sample was treated with a PEG 8 000 solution to reach a final concentration of 80g/L PEG, 0.3M NaCl, pH 7.3 in a final volume of 40mL. The sample was homogenized and incubated overnight at 4°C, centrifuged at 100 000 x g for 1 hour at 4°C. The supernatant was discarded. Nucleic acids were extracted from the PEG-precipitated ultracentrifuged solids using the AllPrep PowerViral DNA/RNA Kit (Qiagen) following previously described work (D'Aoust et al., 2021b; Wong et al., 2023).

To determine the partitioning of endogenous MTBC and MTB in primary sludge, a 160 mL grab sample was collected from both Powai and Ghatkopar WRRFs. Both influent wastewater samples were divided into three fractions (n=15): liquid, centrifuged solids, and PEG-precipitated ultracentrifuged solids. Upon reception, the samples were initially settled for 2 hours at 4°C, followed by pipetting 40mL of the settled solids for centrifugation at 10 000 x g for 45 minutes at 4°C. The centrifuged settled solids pellet was centrifuged a second time at 10 000 x g for 10 minutes at 4°C, also preserving the settled solids supernatant. Nucleic acids were extracted from the centrifuged settled solids using the AllPrep PowerViral DNA/RNA Kit (Qiagen) following previously described work (D'Aoust et al., 2021b; Wong et al., 2023). 40mL of the settled solids supernatant of each sample was serially filtered through 30 kDa-15mL Amicon cartridges (Millipore Sigma) at 4 000 x g for eight sessions of 30 minutes at 4°C, discarding the flow-through each session. Nucleic acids were extracted from the centrifuged settled solids using the QIAamp Viral RNA Mini Kit (Qiagen) using a QIAcube Connect automated extraction protocol as per the manufacturer's instructions. Finally, 32mL of each primary sludge sample was treated with a PEG 8 000 solution to reach a final concentration of 80g/L PEG, 0.3M NaCl, pH 7.3 in a final volume of 40mL. The sample was homogenized and incubated overnight at 4°C, centrifuged at 100 000 x g for 1 hour at 4°C. The supernatant was discarded. Nucleic acids were extracted from the PEG-

precipitated ultracentrifuged solids using the AllPrep PowerViral DNA/RNA Kit (Qiagen) following previously described work (D'Aoust et al., 2021b; Wong et al., 2023).

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Chapter 3.
Partitioning and probe-based quantitative PCR assays for the wastewater monitoring of *Mycobacterium tuberculosis* Complex, *M. tuberculosis*, and *M. bovis*

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Abstract

Three new probe-based quantitative PCR assays were designed based on Chae *et al.* (2017), Pérez-*Osorio et al.* (2012), and Sales *et al.* (2012) to quantitate *Mycobacterium tuberculosis* complex (MTBC) species, *M. tuberculosis* (MTB), and *M. bovis* (MB) in wastewater targeting genomic regions *rv0577,* RD9, and the deletion of RD4, respectively. The assays were validated for specificity using four Mycobacterial species, including two MTBC species and two non-tuberculosis Mycobacteria species, and endogenous wastewater samples from Ottawa, Ontario, Canada; Mumbai, India; and a remote Northern Indigenous community in Inuit Nunangat with known ongoing tuberculosis cases or outbreaks. The three assays demonstrate high sensitivity and are suitable for use in wastewater. Partitioning experiments performed on endogenous MTBC and MTB in collected wastewaters from Mumbai, India with known tuberculosis outbreaks show that the targeted genomic regions of *rv0577* (MTBC) and RD9 (MTB) used to quantitate human tuberculosis disease predominately partition to solids fraction of wastewaters. The partitioning results of this study, in combination with the presented probe-based PCR assays, provide guidance on how to best enrich wastewaters and rapidly and economically quantify tuberculosis with high specificity and sensitivity in wastewaters.

2.1 Introduction

Tuberculosis (TB) infections were ranked the most lethal infectious disease globally in 2023, resulting in over 1.25 million fatalities. Approximately 10.8 million people became ill with TB worldwide in 2023, with most cases occurring in Southeast Asia (45%) and Africa (24%). The 30 top TB-burdened countries account for 87% of all incident cases globally, including India (26%), Indonesia (10%), and China (6.8%) (World Health Organization, 2024). TB is caused by *Mycobacterium tuberculosis* complex (MTBC) species, a group of closely related mycobacterial strains capable of causing infections in various animal species, including humans. MTBC species share highly conserved genomes that result in potential zoonotic TB (zTB) infection potential across animal species (Bayraktar et al., 2011; Correa-Macedo et al., 2019; Jia et al., 2017; Kanabalan et al., 2021; Kock et al., 2021; Langer & LoBue, 2014; Lawn & Zumla, 2011; Romha et al., 2018). Individuals who are frequently in close contact with infected animals, such as livestock, are hence at risk of zTB infection of other MTBC species, such as *M. bovis* (MB), *M. orygis,* and *M. caprae* (Duffy et al., 2024; Neil M. Ampel, 2024; Olea-Popelka et al., 2017). Although zTB infection is possible in humans, *M. tuberculosis* (MTB) is the primary MTBC species responsible for human infection (Gagneux, 2012). Human TB infection most often begins when an individual inhales droplets or bodily fluids containing viable MTBC bacteria from an infected human host (Sakamoto, 2012), with 90-95% of exposure events resulting in latent TB infection (LTBI) (Greenaway et al., 2011). LTBI is a state of persistent immune response to MTB antigens, with no evidence of clinically manifest active TB (World Health Organization, 2019). It is estimated that up to one-third of the world population is latently infected with MTB (World Health Organization, 2019).

In 2022, Canada documented 1,971 incident active TB cases, with a rate of active TB disease being 5.1 cases per 100 000 individuals. Within this group, 74.5% of documented cases had occurred in foreign-born individuals, at 12.3 cases per 100 000 individuals. The lowest rates of active TB occurrence in Canada are in non-Indigenous, Canadian-born individuals, at a rate of 0.3 cases per 100 000 individuals (Public Health Agency of Canada, 2024). Indigenous populations in Canada have the highest rate of incident active TB cases, at a rate of 16.6 cases per 100 000 individuals. (Statistics Canada, 2019). The Inuit population of Inuit Nunangat (or ᐃᓄᐃᑦ ᓄᓇᖅᑐᑦ

in Inuktitut) is located in Canada's Arctic region and is home to 69.1% of the Inuit population in Canada (Statistics Canada, 2022). The Inuit Nunangat population is disproportionately affected by TB, with the average incidence rate being more than 290 times higher than non-Indigenous, Canadian-born individuals (Patterson et al., 2018). The Inuit Nunangat population experiences systemic inequality and inequity issues such as crowded housing, food insecurity, lack of access to adequate healthcare, water insecurity, and unemployment as compared to non-Indigenous Canadians. These social determinants of health are known to exasperate TB disease prevalence (Cassivi et al., 2023; Clark et al., 2002; Patterson et al., 2018; Sinha et al., 2019; Tuite et al., 2017). Despite an overall low prevalence of TB in Canada, these specific communities and populations are still disproportionately impacted by TB.

Currently, a common approach to responding to community outbreaks in Inuit Nunangat are community-wide screenings, which can be difficult and expensive to conduct due to the lack of health infrastructure available. Since the communities of this region are often remote, issues such as limited available space and housing pose significant barriers to obtaining the necessary health infrastructures to conduct effective screening (Patterson et al., 2018). Furthermore, TB disease identification in Inuit Nunangat traditionally first requires an infected person to present with symptoms and then volunteer to complete clinical diagnosis, followed by reporting to public health officials. Indigenous communities, including Inuit populations, in Inuit Nunangat and across Canada have experienced a well-documented, complex, and traumatic medical history related to TB testing. This history has resulted in medical testing hesitancy and, hence, often an under-representation of individuals with TB in Indigenous communities by public health agencies across the country (Basta & de Sousa Viana, 2019; Hick, 2019; Jetty, 2020; Vachon et al., 2018).

Wastewater and environmental monitoring (WEM) provides an anonymous and non-invasive means to potentially provide Inuit Nunangat communities with an early warning system of TB illness and prevalence (Williams et al., 2024). MTB has been detected in stool from infected patients using both culture- and molecular-based methods (Greenberg & Kupka, 1957; Jensen, 1954; Kesarwani et al., 2022; Kokuto et al., 2015; Mesman et al., 2019; Mtetwa et al., 2022a; Oramasionwu et al., 2013). Since the early 20th century, MTB has been detected in wastewater, from sanatorium wastewater to, more recently, wastewater monitoring in water recovery and reuse

facilities (WRRF) in high-prevalence regions (Greenberg & Kupka, 1957; Mtetwa et al., 2022a). Wastewater from facilities housing TB patients, such as sanatoria, has been used to detect viable bacteria and successfully used as a vector to infect animals such as Guinea pigs with TB (Brown et al., 1916; Greenberg & Kupka, 1957; Jensen, 1954; Kroger & Trettin G, 1951). After the onset of the COVID-19 pandemic, there has been a pronounced resurgence in WEM to track disease incidence and provide early identification of outbreaks and disease transmission in communities around the world (Robins et al., 2022; Schmidt, 2020). In particular, WEM has been expanded to monitor not only SARS-CoV-2, but also influenza A & B, respiratory syncytial virus (RSV), mpox (formerly known as monkeypox), human metapneumovirus (HMPV), parainfluenza (PIV), norovirus GII, rotavirus, adenovirus group F, enterovirus D68, *Candida auris*, poliovirus, hepatitis A virus (HAV) and others (Boehm et al., 2023a; Boehm et al., 2023b; Kitakawa et al., 2023; Mercier et al., 2022; Sherchan et al., 2023; Tedcastle et al., 2022).

Similar to clinical diagnoses, many current protocols used to detect MTB in wastewater have utilized culture-based methods. Newer methodologies, such as polymerase chain reaction (PCR) and shotgun sequencing, have since been used (Mtetwa et al., 2022b). In clinical settings, the IS6110 PCR target is utilized for MTB detection via PCR. However, this PCR target lacks specificity for wastewater applications due to non-specific hybridization to extraneous microbial species found in wastewater (Coros et al., 2008; Müller et al., 2015). More recently, successful surveillance of MTB in wastewater has been demonstrated using conventional non-probe-based PCR and intercalating dye-based digital droplet PCR (ddPCR) in regions of Africa with high TB incidence (Mtetwa et al., 2022a). Intercalating dye-based PCR assays are known to demonstrate lower specificity and sensitivity and are more susceptible to false positive results and non-specific binding to other organisms compared to probe-based quantitative (qPCR) assays (Navarro et al., 2015). In order to meet its potential as an early warning system for TB disease in Northern Indigenous communities, WEM TB methods require highly specific assays due to the biologically diverse nature of wastewater and high genomic homology across mycobacterial species, highly sensitive assays to ensure detection of TB disease cases in the community (McEvoy et al., 2012). Currently, there is a lack of available probe-based qPCR assays that demonstrate the sensitivities and specificities required for wastewater applications. The objective of this research is to develop and validate wastewater probe-based qPCR assays to detect and quantify not only MTB but also

MTBC and MB in both low- and high-incidence municipal wastewater from Ottawa, Ontario, Canada, Mumbai, India, and an Inuit Nunangat, Northern Indigenous community with known ongoing TB cases or outbreaks. The developed assays are specific to their respective targets and were validated to rule out non-specific hybridization to extraneous microbial species. The three probe-based qPCR assays were developed based on existing PCR assays (Araújo et al., 2014; Chae et al., 2017; Pérez-Osorio et al., 2012; Sales et al., 2014). An assay targeting the *rv0577* region is used to detect and quantitate MTBC species, the region of difference 9 (RD9) is targeted to detect and quantitate MTB, and the RD4 deletion is targeted to detect and quantitate MB. The MTBC *rv0577* and MTB RD9 assays in this study were also used to determine the partitioning behaviour of endogenous MTBC and MTB related to human TB disease in municipal influent wastewater and primary clarified sludge to guide best practices related to the enrichment and concentration of these genetic targets and the application for WEM for TB monitoring. This research provides the necessary information to implement sensitive and specific TB WEM, and hence also provides a pathway towards non-invasive and economical TB monitoring in Northern Indigenous communities in Canada.

3.2 Materials and Methods

3.2.1 qPCR Assay Design

Assay design

The MTBC *rv0577* qPCR assay is designed to measure all MTBC species, including MTB and MB, and is a modified version of the Chae *et al.* (2017) *rv0577* assay (Chae et al., 2017). The MTB RD9 assay is designed to specifically quantitate MTB and is a modified version of the Pérez-Osorio *et al.* (2012) RD9 assay (Pérez-Osorio et al., 2012). The MB RD4-deletion assay is designed to quantitate MB and is a modified version of the Sales *et al.* (2017) Mb.400 assay (Table 3.1) (Ru et al., 2017; Sales et al., 2014). The virulence-associated gene, *rv0577,* is found explicitly within the MTBC and used to confirm MTB and MB detections (Chae et al., 2017; Gu et al., 2016; Qasim et al., 2023). The RD9 intergenic region is targeted within the MTB RD9 assay and is found between the *rv2072c* and *rv2073c* genes that is specific to MTB and not found in other MTBC species (except *M. canetti*) (Chae et al., 2017). Other TB-causing mycobacteria and non-TB mycobacteria (NTM) lack the RD9 intergenic region, allowing for molecular differentiation of MTB from other species of MTBC, such as *M. africanum*, *M. orygis, and* MB (Brosch et al., 2002;

Huard et al., 2003; Smith et al., 2006; Teo et al., 2013). MB lacks the RD4 intergenic region compared to other closely related MBTC species, such as MTB, allowing for molecular differentiation from other species of MTBC (Kapalamula et al., 2021; Ru et al., 2017). As a result, the MB RD4-deletion assay is used to measure the deletion of the RD4 region in MB. The existing referred PCR products were modified using Primer3Plus (https://www.primer3plus.com) to produce probe-based qPCR assays and subsequently aligned using MUSCLE with sequences of commonly referred MTB variants (H37Rv and HN878), MTBC species (*M. canettii*, *M. africanum*, and *M. microti*), commonly referred MB variants (Mb3602, Danish 1331, bacille Calmette-Guérin (BCG)), and commonly referred NTM species (*M. avium hominissuis, M. kansasii, and M. intracellulare*) to determine *in silico* assay specificity (Figure 3.1) using genome sequences obtained from GenBank (National Institutes of Health) (Edgar, 2004). The assays employed DNA primers and probes with a 5'-FAM reporter molecule, a 3' minor groove binder, and a nonfluorescent quencher (Applied Biosystems Integrated).

Table 3.2. qPCR MTBC, MTB, and MB probe-based assays for TB WEM.

Target Locus	Oligo Name	Sequence	Genomic Location	Size	Reference
MTBC *rv0577*	rv0577_F	5'- GCC CAA GAG AAG CGA ATA CAG G	671,168-671,189	124 bp	*Chae et al.* (2017)
	rv0577_R	5'- GAC CGG GTT GTC GTC GTA A	671,273-671,291		
	rv0577_P	5'- CCC AGC CGA ACA ACG ATG TGT AGA ACT T	671,244-671,271		
MTB RD9	RD9_F	5'- TCG GCG GTG ACG GTA TC	2,330,010-2,330,026	88 bp	*Pérez-Osorio et al.* (2012)
	RD9_R	5'- AGC ATT CTC GCT CCG AAT TG	2,330,078-2,330,097		
	RD9_P	5'- CAA GTT GCC GTT TCG AGC CGT AAA	2,330,038-2,330, 061		
MB RD4-deletion	RD4_deletion_F	5'- GTC GCC GCT CCC AAA AAT TAC C	1,697,369-1,697,390	118 bp	*Sales et al.* (2017)
	RD4_deletion_R	5'- CCG TTG TAG GCC ACT CCA AGA G	1,697,645-1,697,486		
	RD4_deletion_P	5'- AAG CCG TAG TCG TGC AGA AGC GC	1,697,438-1,697,460		

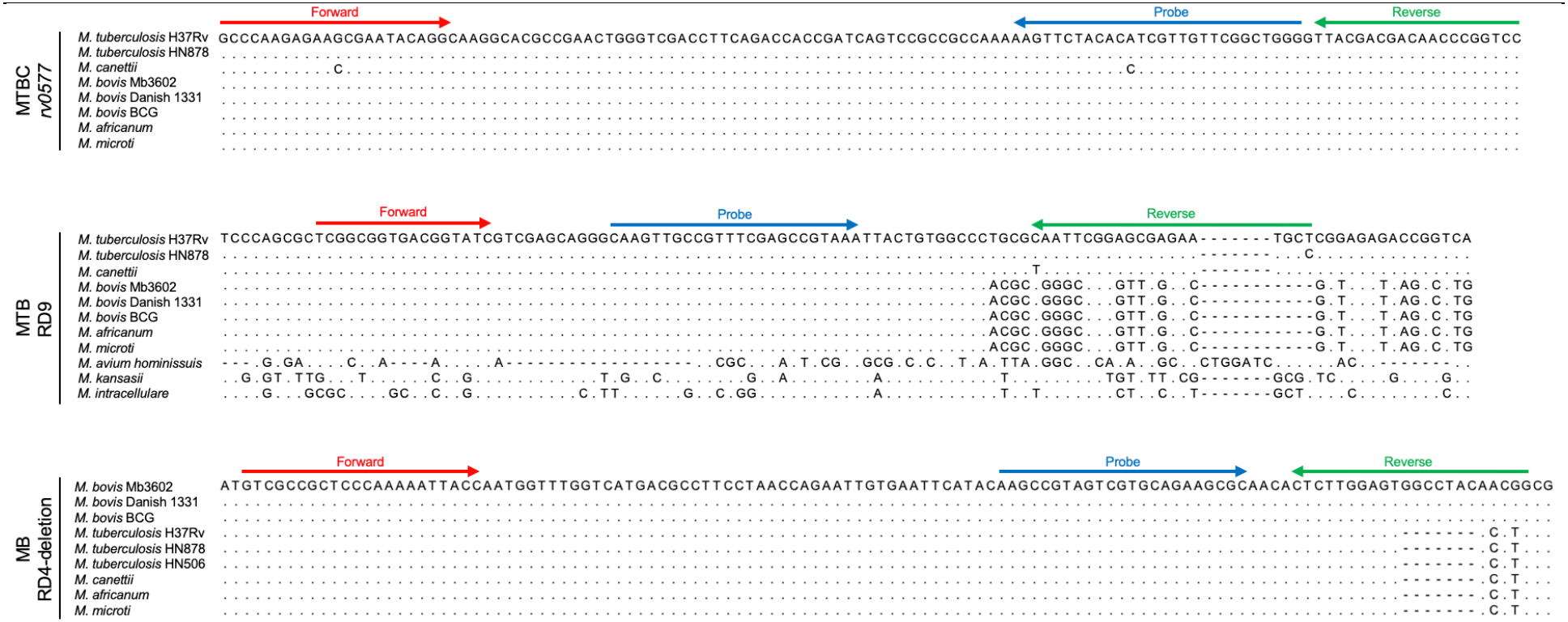

Figure 3.1. Alignment of primers and probes with mycobacterial DNA genome sequences. The primers and probe for the MTBC rv0577, MTB RD9, and MB RD4-deletion real-time qPCR assays are aligned with the targeted DNA sequence within several mycobacterial species. Mycobacterial strains: *M. tuberculosis* H37Rv (NC_000962), *M. tuberculosis* HN878 (AP018036), *M. canettii* (HE572590), *M. bovis* (NZ_CP096843), *M. bovis* (CP039850), *M. bovis* BCG (AM408590), *M. africanum* (FR878060), MTB *microti* (NZ_LR882496), *M. avium hominissuis* (CP018019), *M. kansasii* strain (CP006835), *M. intracellulare* (CP085945). Dots represent conserved nucleotides, and dashes represent nucleotide gaps

3.2.2 qPCR Assay Validation and Optimization - Laboratory Propagated Cell Lines

DNA Controls

DNA controls were obtained from a panel of laboratory-propagated MTBC cell lines: MTB H37Rv, MB BCG, and a panel of NTM cell lines: *M. kansasii,* and *M. smegmatis.* All cell lines were cultured as previously described in *Madden et al.* (2023) (Madden et al., 2023). DNA was extracted following a previously described protocol by *Wong et al.* (2023) (Wong et al., 2023).

qPCR conditions optimization

The optimal qPCR conditions of the MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays were measured on the CFX96 Real-Time PCR Detection system (BioRad, Hercules, USA) using TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA) and digital PCR (dPCR) quantified DNA from cultured MTB cells and MB BCG respectively (Table 3.2). Optimal denaturation durations were determined by running technical triplicates and non-template controls with qPCR reactions conducted at 95°C at 30s, 44 cycles of 95°C for 5, 10, 15, 20, and 30s, and 60°C for 30s. Optimal melting temperatures were determined by running technical triplicates with non-template controls conducted at 95°C at 30s, 44 cycles of 95°C 15s, and annealing temperatures of 0.5°C increments from 57°C to 63°C for 30s. The determined optimal qPCR conditions are comparable to other employed PCR assays for the detection of MTBC, MTB, and MB in clinical and wastewater applications (Lyu et al., 2020; Mtetwa et al., 2023; Wang et al., 2019).

Table 3.2. Optimal qPCR conditions.

MTBC *rv0577*	MTB RD9	MB RD4-deletion
95°C for 30s	95°C for 30s	95°C for 30s
44 cycles:	44 cycles:	44 cycles:
95°C for 15s	95°C for 15s	95°C for 15s
59°C for 30s	60°C for 30s	60°C for 30s

Assay sensitivity

The efficiencies of the MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays were measured using technical triplicates of serially diluted, previously purified, DNA from cultured MTB cells with non-template controls. The DNA was quantified using AbsoluteQ (ABI) dPCR using the AbsoluteQ Universal DNA Digital PCR Master Mix (ABI), MTBC *rv0577*, MTB RD9,

and MB RD4-deletion assays, respectively. The reaction efficiencies for MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays are 99.85%, 99.71%, and 99.65%, respectively (data not shown) (Ruijter et al., 2013).

Assay specificity

The MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays were evaluated for specificity using DNA from the above-mentioned cultured *Mycobacterium spp.* strains. The DNA was quantified using the NanoDrop™ One Microvolume UV Spectrophotometer (ThermoFischer Scientific). MTB was used as a positive control to evaluate the MTBC *rv0577*, MTB RD9 assays, and as a negative control for the RD4-deletion assay. MB BCG was used as a positive control for the MTBC *rv0577* and RD4-deletion assays and a negative control for the MTB RD9 assay. Both *M. kansasii* and *M. smegmatis* were used as negative controls for all three assays, as they belong to the same genus (*Mycobacterium*) but are classified as NTM, because these species do not belong to the MTBC. For each assay, both positive and negative controls were tested at two concentrations, 10 ng/µL (high) and 1 ng/µL (low), using the qPCR conditions as mentioned above. Each test was run in technical triplicates alongside non-template controls to rule out contamination. Positivity was considered when at least two of the three technical replicates showed amplification within 40 qPCR cycles.

2.2.3 qPCR Assay Validation - Endogenous Targets in Wastewater

Locations of collected wastewater with endogenous targets

A panel of MTBC-positive wastewater samples was utilized to validate the herein proposed three new probe-based qPCR assays. The samples include 88 wastewater samples collected from Ottawa, Ontario, Canada, a moderate-risk high-income country metropolitan area (n=70), Mumbai, India, a high-risk middle-income country metropolitan area (n = 2) and a high-risk remote Northern Indigenous community in the Inuit Nunangat region (n=16). All locations have reported active TB cases during the respective sampling periods. Ottawa is a moderate-risk, high-income country metropolitan area located in the National Capital Region, where approximately 1 million people reside. The average annual household income in Ottawa is approximately $64,500 Canadian Dollars (CAD) before taxes (Statistics Canada, 2023). Mumbai is a high-risk, middle-

income country metropolitan area where approximately 12.5 million people reside and India's largest city. The average annual household income in Mumbai is approximately ₹230,000 Indian Rupees ($3,778 CAD before taxes)(Rathore, 2024). The Inuit Nunangat, Northern Indigenous community is a high-risk remote village located in the Nunavik region of the province of Quebec. Approximately 13 thousand people reside in the Nunavik region across the 14 isolated communities. The average annual household income in the Nunavik region is approximately $35,000 CAD before taxes (Nunavik Statistics Program, 2021).

Figure 3.2. Sampling locations. Red location markers locate Ottawa, Mumbai, and the Inuit Nunangat, Northern Indigenous Community, where wastewater samples containing endogenous TB targets were collected.

Wastewater collection, transport, and storage prior to analysis

500 mL 24-hour composite primary sludge samples from the city of Ottawa were collected at the Robert O. Pickard Environmental Centre WRRF. This WRRF processes, on average, 436 million litres of wastewater per day, servicing an estimated 1 million residents. Upon collection, the primary sludge samples were immediately refrigerated at 4°C at the WRRF and transported on ice to the University of Ottawa and processed upon receipt. 2.0 L grab samples of influent wastewater and 24-hour composite primary sludge samples from the city of Mumbai were

collected at both the Powai and Ghatkhopar WRRFs. These WRRFs process on average, 5.8 and 180 million litres of wastewater per day, servicing an estimated 29 thousand and 900 thousand people, respectively. Upon collection, both the influent wastewater and primary sludge samples were immediately refrigerated at 4°C. The samples were then shipped on ice to the University of Ottawa by express international courier and processed upon receipt. Samples from the Inuit Nunangat, Northern Indigenous community were collected from the village's decentralized wastewater treatment lagoon, where sewage trucks deposit wastewater samples from homes and buildings in the village and transport the wastewater to the treatment lagoon daily. Wastewater was collected from the transport trucks using 3D-printed COVID-19 Sewer Cage (COSCa) passive samplers filled with sterile gauze (Hayes et al., 2021). COSCa balls were printed using UltiMaker 2+ (Ultimaker) and polylactic acid filament (PLA). Upon collection, the samples were stored at 4°C. The samples were then shipped on ice to the University of Ottawa by express courier and analyzed upon receipt.

Wastewater sample nucleic acid extraction

40 mL of homogenized primary sludge samples from Ottawa were centrifuged at 10,000 x g for 45 minutes at 4°C. With the supernatant removed, the pellets were centrifuged again at 10,000 x g for 10 minutes at 4°C. The pellet was stored at -20°C for long-term storage. For retrospective analysis, pellets were thawed overnight at 4ºC. Both DNA and RNA were extracted with the AllPrep PowerViral DNA/RNA Kit (Qiagen) using the previously described protocol in Wong *et al.* (2023), D'Aoust *et al.* (2021a), and D'Aoust *et al.* (2021b) and analyzed within 12 hours of DNA extraction (D'Aoust et al., 2021a; D'Aoust et al., 2021b; Wong et al., 2023). 30 mL of influent wastewater settled solids from both Powaii and Ghatkhopar WRRFs were aliquoted. The aliquots were treated with a polyethylene glycol (PEG) 8,000 (Millipore Sigma) solution to reach a final working concentration of 80 g/L PEG, 0.3M NaCl using the previously described protocol in Wong *et al.* (2023)(Wong et al., 2023). After ultracentrifugation, the resulting pellet was used for both DNA and RNA extraction with the AllPrep PowerViral DNA/RNA Kit (Qiagen) using the previously described protocol in *Wong et al.* (2023), D'Aoust *et al.* (2021a), and D'Aoust *et al.* (2021b) and analyzed within 12 hours of DNA extraction (D'Aoust et al., 2021a; D'Aoust et al., 2021b; Wong et al., 2023). Upon reception of the COSCa samplers used in the Inuit Nunangat,

Northern Indigenous community, the gauze was removed from the COSCa sampler and mixed with 300 mL of deionized water to elute the collected solids in a clean beaker. The beaker was left to allow the solids to settle for 15 minutes, and 40 mL of the settled solids was pipetted and followed the above primary sludge nucleic acid extraction protocol. All samples were co-extracted with extraction blanks simultaneously using the protocol to rule out contamination in the extraction kits.

Wastewater qPCR conditions

All qPCR reactions were run in technical triplicates with non-template controls to ensure accuracy and reliability. A five-point standard curve was prepared using serial dilutions of dPCR-quantified DNA from cultured MTB and MB BCG at concentrations of 100, 40, 20, 5, and 2.5 copies/µL, diluted in molecular-grade water (ThermoFisher Scientific). MTB DNA was used as the control for the MTBC *rv0577* and MTB RD9 assays, while MB BCG DNA was used as the control for the RD4-deletion assay. Inhibition was tested by running pepper mild mottle virus (PMMoV) dilutions at 1/10 and 1/40 with molecular grade RNase-free water (ThermoFisher Scientific). RT-qPCR efficiency ranged from 90-100%, and the coefficient of determination (R^2) values were greater than 0.95. PMMoV is a common fecal matter indicator used in environmental samples (Haramoto et al., 2013; Rosario et al., 2009). Singleplex, probe-based, single-step RT-qPCR analysis of the PMMoV's RAP (replication-associated protein) encoding gene was performed. Primers and probes, PCR cycling conditions, and reagent concentrations used for the PMMoV analysis as stated in *Haramoto et al.* (2013), *D'Aoust et al.* (2021a) and *D'Aoust et al.* (2021b) (D'Aoust et al., 2021a; D'Aoust et al., 2021b; Haramoto et al., 2013).

Wastewater sample measurements

Measurements of the MTBC *rv0577*, MTB RD9, and MB RD4-deletion targets in wastewater using the herein presented assays are expressed as copies of target DNA per qPCR reaction volume (copies), copies of target DNA per copy per gram of wet wastewater solids (cp/g) and also copies of target DNA per copy per copies of the PMMoV fecal matter indicator (cp/cp).

3.2.4 Partitioning of Endogenous TB in Municipal Influent Wastewater and Primary Sludge

The partitioning experiments of this study were performed on grab influent wastewater samples and 24-hour composite primary sludge samples from two WRRFs in Mumbai, India. Samples from Mumbai were utilized for partitioning experiments due to the high burden of TB in the metropolitan region, compared to those of Ottawa and the remote Indigenous community. The samples were collected and then shipped on ice via international courier to the University of Ottawa. All samples were subject to the same storage, transport, and holding times prior to the partitioning experiments. The DNA of each fractioned portion of the samples was extracted within 6 hours of performing the partitioning procedure.

To determine the partitioning of endogenous MTBC and MTB in influent wastewater, a 2.0 L grab sample was collected from both Powai and Ghatkopar WRRFs, and each was split into four fractions (n=21): liquid, filtered solids, centrifuged solids, and PEG-precipitated ultracentrifuged solids. MB partitioning experiments were not performed in this study, as the intent of this research is to develop sensitive assays for the measurement of human TB targets in wastewaters. To study influent wastewater partitioning, the samples were processed using the protocol as previously described by *Wong et al.* (2023) and *Mercier et al.* (2022) to produce a total of 5 biological replicates of the liquid fractions, 6 biological replicates of the 0.45-µm vacuum filtered solids fraction, 5 biological replicates of the centrifuged solids, 5 biological replicates of the PEG-precipitated ultracentrifuged solids of the samples (Mercier et al., 2022; Wong et al., 2023). Nucleic acids from the liquid fractions were extracted using the QIAmp Viral RNA Mini Kit (Qiagen) on a QIAcube Connect (Qiagen) automated extraction platform as per the manufacturer's instructions. Nucleic acids were extracted from the 0.45-µm filtered solids, centrifuged solids, and the PEG-precipitated ultracentrifuged solids fractions using the protocol previously described by *Wong et al. (2023)* and *D'Aoust et al.* (2021) and the AllPrep PowerViral DNA/RNA Kit (Qiagen) (D'Aoust et al., 2021b; Wong et al., 2023). All sample fractions in this portion of the partitioning study were analyzed using probe-based qPCR using the above-mentioned respective reagents and qPCR cycling conditions within 24 hours of nucleic acid extraction (Figure 3.3).

To determine the partitioning of endogenous MTBC and MTB in primary sludge, a 160mL primary sludge sample was collected from both the Powai and Ghatkopar WRRFs, and each was split into three fractions (n=15): liquid, centrifuged solids, and PEG-precipitated ultracentrifuged solids. To study primary sludge partitioning, the samples were processed using the protocol as previously described by *Wong et al.* (2023) and *Mercier et al.* (2022) to produce a total of 5 biological replicates of the settled solids, 5 biological replicates of the PEG-precipitated ultracentrifuged solids, and 5 biological replicates of the supernatant fractions of the samples (Mercier et al., 2022; Wong et al., 2023). Nucleic acids from the liquid fractions were extracted using the QIAmp Viral RNA Mini Kit (Qiagen) on a QIAcube Connect (Qiagen) automated extraction platform as per the manufacturer's instructions. Nucleic acids were extracted from the centrifuged solids and the PEG-precipitated ultracentrifuged solids fractions using the protocol previously described by *Wong et al.* (2023) and *D'Aoust et al.* (2021) and the AllPrep PowerViral DNA/RNA Kit (Qiagen) (D'Aoust et al., 2021b; Wong et al., 2023). All sample fractions in this portion of the partitioning study were analyzed using probe-based qPCR using the above-mentioned respective reagents and qPCR cycling conditions within 24 hours of nucleic acid extraction (Figure 3.3).

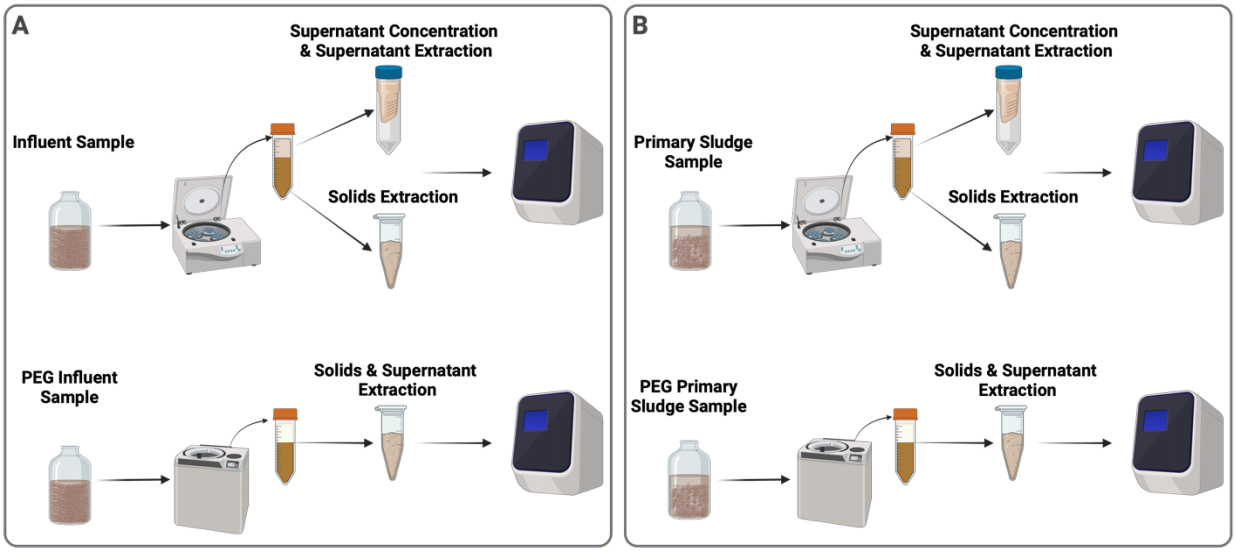

Figure 3.3. Processing flowcharts of (A) influent samples and (B) primary sludge to identify the partitioning of MTBC and MTB signals within the supernatant and solids with and without PEG addition to optimize extraction of TB-markers in endogenous wastewaters.

3.3 Results

3.3.1 Laboratory Propagated Cell Line Validation of qPCR Assays

Assay sensitivity

The assay limits of detection (ALOD) and assay limits of quantification (ALOQ) are measures of analytical sensitivity and concentrations below the ALOD and ALOQ are not false positives, rather having a lower probability of detection (Stokdyk et al., 2016). for the MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays were determined using dPCR quantified MTB and MB BCG DNA, respectively (Forootan et al., 2017). The purified DNA was serially diluted to concentrations ranging from 1 to 100 copy/µL. The ALOD was determined for MTBC *rv0577* assay, MTB RD9, and MB RD4-deletion at 2.5, 2.5, and 3.2 copies per reaction, respectively, with 95% confidence using conventional methods. The ALOQ was determined for MTBC *rv0577*, MTB RD9, and MB RD4-deletion to be 6.5, 4.5, and 5.4 copies per reaction, respectively. The sensitivity of the probe-based qPCR assays of the herein study using laboratory propagated cell lines are shown to be very similar to SARS-CoV-2, influenza A, influenza B and respiratory syncytial virus sensitivity and hence sufficient for wastewater monitoring (Mercier et al., 2022, 2023; Vogels et al., 2020).

Assay specificity

The MTBC *rv0577* assay detected MTB and MB BCG DNA extracts, while the NTM (*M. kansasii* and *M. smegmatis*) species tested were negative in all the performed tests. The MTB RD9 assay detected DNA extracted from the MTB DNA extract, and all other Mycobacteria DNA extracts tested (MB BCG, *M. kansasii*, and *M. smegmatis*) were negative. The MB RD4-deletion assay only detected the MB BCG DNA extract, and all other Mycobacteria DNA extracts tested (MTB, *M. kansasii* and *M. smegmatis*) were negative (Table 3.3). This demonstrates the proposed assays can effectively differentiate between their respective TB-causing bacteria strains and NTM. As such, the specificity of the probe-based assays of the herein study were validated using laboratory propagated cell lines.

Table 3.3. Validation of assays using laboratory propagated cell lines.

Organism	qPCR Assay		
	MTBC *rv0577*	MTB RD9	MB RD4-deletion
M. tuberculosis	+	+	-
M. bovis BCG	+	-	+
M. kansasii	-	-	-
M. smegmatis	-	-	-

3.3.2 Endogenous Targets in Wastewaters Validation of qPCR Assays

A panel of 88 wastewater samples was collected from locations with known TB cases: Ottawa, which is a moderate-risk, high-income country metropolitan area (n=70); Mumbai, which is a high-risk middle-income country metropolitan area (n = 2) and a high-risk Inuit Nunangat, Northern Indigenous community (n=16) during periods of known ongoing TB cases or outbreaks was used to validate the MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays using endogenous TB target samples. The detection of MTBC, MTB, and MB was considered when the at least 2 out of 3 technical replicates had a non-zero qPCR quantification. Of the 70 samples from Ottawa, Ontario, Canada, 8 tested positive for both MTBC and MTB (11.4%), and none for MB (0.0%) using the herein proposed MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays (Table 3.3). However, all positive samples from Ottawa yielded concentrations below the reported ALOD and ALOQ. All the samples (100%) from Mumbai detected MTBC, MTB, and MB using the proposed MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays. 2 of the 16 samples from the high-risk Inuit Nunangat, Northern Indigenous community samples tested positive for MTBC (12.5%), the same 2 were positive for MTB (12.5%), and no samples tested positive for MB using the proposed MTBC *rv0577*, MTB RD9, and MB RD4-deletion assays (Table 3.4). All positive samples from the Inuit Nunangat, Northern Indigenous community yielded concentrations below the reported ALOD and ALOQ.

Cases of active TB in Ottawa fluctuate between 40 to 70 cases reported per year in the city of approximately 1M people, with more than half being cases of pulmonary TB (Ottawa Public Health, n.d.; Public Health Ontario, 2024). The city's public health unit, Ottawa Public Health reported 61 cases of active TB in 2021 and 59 cases in 2022, resulting in incidences of 5.8 and 5.5

per 100,000 people, respectively. This is observed in the detection of MTBC and MTB using the proposed assays in primary sludge samples collected from Ottawa (Table 3.4).

In 2022, an estimated 354 million people in India were presumed to have active TB disease, the highest burden globally (Chauhan et al., 2023). In addition, Mumbai experienced approximately 67,000 cases of active TB, resulting in an incidence of 313 cases per 100,000 people in 2022, which is reflected in the detection and high concentrations of MTBC and MTB using the proposed assays (Shah et al., 2024). The high detection rate of MB (100%) in Mumbai likely reflects the endemic nature of MB in the region, where close human-animal interactions and agricultural practices may occur (Table 3.4) (Ramanujam & Palaniyandi, 2023).

In 2023, the Inuit Nunangat, Northern Indigenous community's public health unit, the Nunavik Regional Board of Health and Social Services reported 78 cases of active TB in Nunavik. This equates to an estimated incidence of 643 reported cases per 100,000 people in the Nunavik region. These reported cases highlight the disproportionate burden of TB in Indigenous communities compared to that of non-Indigenous, Canadian-born individuals (0.3 per 100 000) (Public Health Agency of Canada, 2024). Despite this higher incidence, the MTBC and MTB measurement concentrations in wastewater samples from the Inuit Nunangat, Northern Indigenous community were similar to those observed in Ottawa. This similarity may reflect that the 78 reported cases in Nunavik were distributed across 14 villages, potentially with significant lower case numbers in the specific location where we collected samples. Additionally, the case count at the time of sampling, when there was not a major outbreak TB identified, may have been low at this specific sampling location, leading to lower concentrations detected in the samples. Nonetheless, the detection of the MTBC and MTB signals using the proposed assays confirms the utility of WEM for TB in this Inuit Nunangat, Northern Indigenous community, as well as other remote and underserved communities (Table 3.4).

Table 3.4. Validation of assays on wastewater samples.

Sample Location	Sample Date *DD/MM/YY*	Sample Type	qPCR assays [a]									Notes on active cases in sampling locations
			MTBC *rv0577*			MTB RD9			MB RD4-deletion			
			copies	cp/g	cp/cp [b]	copies	cp/g	cp/cp	copies	cp/g	cp/cp	
Ottawa	10/11/21	WRRF	0.54	70.90	$4.75x10^{-8}$	0.17	21.87	$1.93x10^{-6}$	nd	nd	nd	61 confirmed cases in Ottawa in 2021
Ottawa	27/11/21	WRRF	0.93	120.17	$8.12x10^{-8}$	1.07	138.34	$1.21x10^{-5}$	nd	nd	nd	
Ottawa	04/12/21	WRRF	0.49	63.89	$4.16x10^{-8}$	0.07	8.65	$3.75x10^{-7}$	nd	nd	nd	
Ottawa	19/04/22	WRRF	0.86	110.25	$1.39x10^{-7}$	0.13	16.45	$9.38x10^{-7}$	nd	nd	nd	59 confirmed cases in Ottawa in 2022
Ottawa	13/06/22	WRRF	0.12	15.86	$1.53x10^{-7}$	0.11	14.28	$6.39x10^{-6}$	nd	nd	nd	
Ottawa	17/06/22	WRRF	0.69	89.60	$1.19x10^{-7}$	0.19	24.91	$1.14x10^{-6}$	nd	nd	nd	
Ottawa	04/07/22	WRRF	0.38	49.16	$9.45x10^{-8}$	0.18	23.23	$2.04x10^{-6}$	nd	nd	nd	
Ottawa	15/11/22	WRRF	0.36	47.14	$1.58x10^{-8}$	0.15	19.59	$8.05x10^{-7}$	nd	nd	nd	
Mumbai - P	12/07/23	WRRF	12622.98	$1.68x10^{6}$	$4.34x10^{0}$	5012.99	$6.68x10^{5}$	$1.73x10^{0}$	$2.46x10^{2}$	$3.27x10^{4}$	$8.47x10^{-2}$	An estimate of 67 000 cases of active TB in Mumbai in 2023
Mumbai - G	13/07/23	WRRF	8313.63	$1.42x10^{6}$	$1.22x10^{0}$	2843.98	$3.79x10^{5}$	$0.39x10^{0}$	$6.92x10^{1}$	$9.20x10^{3}$	$2.42x10^{-2}$	
Inuit Nunangat	14/02/23	Sewage Truck	0.73	93.98	$1.98x10^{-4}$	0.16	20.87	$5.64x10^{-3}$	nd	nd	nd	78 confirmed cases in the Inuit Nunangat, Northern Indigenous community in 2023
Inuit Nunangat	28/02/23	Sewage Truck	1.60	206.53	$2.40x10^{-3}$	0.34	43.39	$6.5x10^{-2}$	nd	nd	nd	

[a] The cycle when the fluorescence signal crosses the threshold (C_T) for each positive sample is shown. nd, not detected. Each reaction mixture contained 1X TaqMan™ Fast Advanced Master Mix (with UNG) (Biosystems, Foster City, CA), 500 nmol of each primer, 250 nmol of each TaqMan probe, and 3 µL of template DNA. Thermal cycling conditions for the BioRad CFX96: one cycle of 95°C for 30 s; 44 cycles of 95°C for 15 s, and 59°C for 30 s for MTBC (*rv0577*) assay, one cycle of 95°C for 30 s; 44 cycles of 95°C for 15 s, and 60°C for 30 s for MTB (RD9) and MB (RD4-deletion) assays.
[b] The number of copies normalized for PMMoV
Mumbai - P: Powaii WRRF
Mumbai - G: Ghatkhopar WRRF

3.3.3 Partitioning of Endogenous TB in Municipal Influent Wastewater and Primary Sludge

Endogenous partitioning experiments for MTBC and MTB were conducted on influent wastewater and primary sludge samples collected from two WRRFs in Mumbai, India where TB measurement magnitudes were sufficient to fractionate the samples. These experiments employed the proposed probe-based MTBC *rv0577* and MTB RD9 assays. MB RD4-deletion was not tested in the partitioning portion of this study as the herein study is focussed on the development of wastewater monitoring assays for human TB. In influent wastewater, 86.4 ± 15.7% of the total MTBC and 97.3 ± 2.4% of the total MTB signal was detected in the PEG-precipitated ultracentrifuged solids fraction (Bonferroni adjusted $p = 0.49$) (Jafari & Ansari-Pour, 2018). The centrifuged solids fraction accounted for 31.0 ± 1.6% of the MTBC signal and 14.8 ± 1.4% of the MTB signal (adj. $p = 0.00006$). In contrast, the liquid fraction contained <0.1% of the total signal for MTBC, and no signal was detected for MTB (adj. $p = 0.045$) (Figure 3.5). No measurement of both MTBC and MTB was observed in the 0.45-µm filtered solids of the influent wastewater. Note that the above partitioned percentages of MTBC compared to MTB in influent wastewater are not statistically distinct from one another, except for the centrifuged solids fraction. This distinction in centrifuged solids fraction partitioning may be attributed to the heterogeneity in sample composition, as the dispersed nature of influent wastewater solids could influence their partitioning behaviour. Overall, these findings indicate that the vast majority of MTBC and MTB in influent wastewater partitions to the wastewater solids and is further enhanced with the addition of PEG-precipitation compared to the liquid extraction method.

In primary sludge, most of the MTBC and MTB signal was found in the PEG-precipitated ultracentrifuged solids fraction. Specifically, 77.0 ± 31.0% of the total MTBC and 77.3 ± 28.0% of the total MTB signals were detected in this fraction (adj. $p = 0.99$). The centrifuged solids accounted for 7.2 ± 2.1% of the MTBC signal and 9.0 ± 1.1% of the MTB signal (adj. $p = 0.07$). In contrast, the liquid fraction contained <0.1% of the total signals for both MTBC and MTB (adj. $p = 0.43$) (Figure 3.5). Again, it is noted that all above partitioned percentages of MTBC compared to MTB in primary sludge are not statistically distinct from one another. These findings again indicate that the majority of MTBC and MTB in primary sludge partitions to the solids and is

further enhanced with the addition of PEG-precipitation compared to the liquid extraction method. These results highlight the importance of targeting the solids fraction of wastewaters during sample collection and again during sample processing to enrich and optimize the recovery of MTBC and MTB markers. While the solids-based extraction method is effective, the addition of PEG-precipitation using ultracentrifugation is particularly effective for concentrating MTBC and MTB signals in both influent wastewater and primary sludge, which can be used to guide sample enrichment and augment detection sensitivity in WEM applications, especially during periods of low signal.

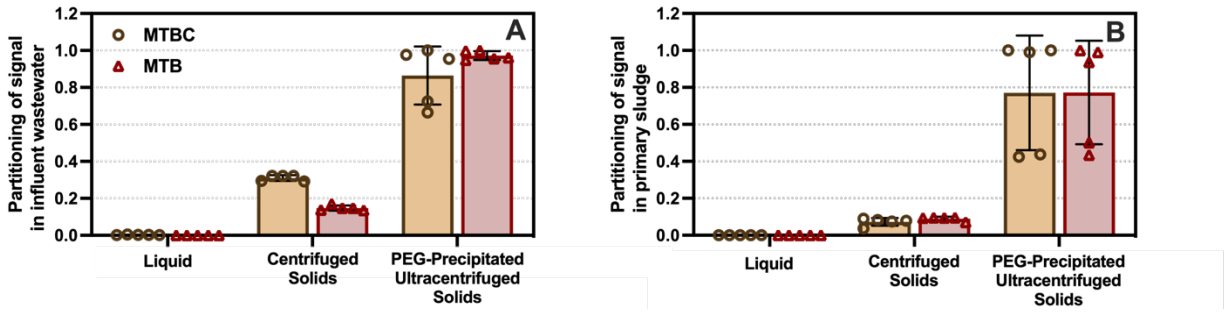

Figure 3.4. Partitioning of measured endogenous MTBC and MTB signal present in (A) influent wastewater (n=2) and (B) primary sludge (n=2). Mean percentages and standard deviations are displayed. Where the standard deviation is too small, the error bars are not displayed.

3.4 Discussion

3.4.1 Contextualization of TB WEM in Inuit Nunangat, Northern Indigenous Community Wastewaters as an Early Warning System and to Monitor Transmission and Recovery

The Inuit Nunangat, Northern Indigenous community described in this study experiences a disproportionate burden of TB compared to the non-Indigenous, Canadian-born population. Indigenous communities in Canada, and more specifically, Inuit populations, consistently face TB incidences that are several hundred times than the national average, driven by a combination of socio-economic, institutional, and historical factors. Overcrowded housing, food insecurity, limited access to healthcare, geographic isolation, and systemic inequalities and inequities in social determinants of health contribute significantly to this disparity (Kilabuk et al., 2019). This burden is exasperated by a complex medical history, which includes forced treatment and relocation by the Government of Canada originating from forms of colonialism (Allan and Smylie, 2015). As a result, many Indigenous and Inuit individuals are hesitant to obtain medical diagnosis or treatment for TB (Jetty, 2020). In this context, TB WEM offers an alternative for TB monitoring in these communities where there are historical and ongoing barriers toward medical diagnoses. Detecting and quantifying TB in wastewater allows for non-invasive and anonymous monitoring, avoiding the need for direct individual participation (Doorn, 2022). This approach respects privacy and minimizes potential stigmatization, which is particularly important in communities where TB is not only a public health issue but also a stigmatized issue. Additionally, WEM provides a population-level snapshot of TB prevalence, offering insights into outbreaks without clinical data (Wong et al., 2023). In order to effectively identify increases in transmission and infection of TB, longitudinal analysis may be conducted to establish a baseline of TB disease in the community. In Indigenous communities, especially remote communities, access to healthcare services is often limited. The herein proposed assays and the partitioning results provide an approach that can hence serve as an early warning system, identifying trends in transmission and trends in recovery. It is necessary, however, to clearly state that any adoption of TB WEM in Northern Indigenous communities must occur in collaboration with the Indigenous communities themselves. This adoption requires culturally sensitive and respectful approaches. Monitoring strategies and techniques must be co-developed with community members and stakeholders to ensure that the methodology and goals align with the community's values and priorities. By incorporating

Indigenous knowledge and perspectives into the development process, WEM can become a valuable tool to reduce TB transmission in priority populations rather than a perpetuation of historical trauma.

3.5 Conclusion

This study presents the development and validation of three probe-based qPCR assays for the detection of MTBC, MTB, and MB in wastewater, offering a sensitive and specific tool for TB monitoring. The assays were validated using propagated cell lines and wastewater samples from three geographically and socioeconomically diverse locations with confirmed TB disease: Ottawa, Canada; Mumbai, India; and a remote Northern Indigenous community in Inuit Nunangat, Canada. These settings represent a spectrum of TB incidence rates and infrastructure contexts, demonstrating the versatility and applicability of the assays in both high- and low-burden areas. The partitioning of MTBC and MTB in influent wastewaters and primary sludge both demonstrated strong partitioning to the solids fraction of wastewaters and recovery is further enhanced with the addition of PEG-precipitation and ultracentrifugation. By offering a non-invasive, anonymous, and cost-effective approach to monitoring TB at a population level, these assays and the partitioning information provides a new monitoring tool that addresses key challenges in traditional monitoring methods. This is particularly valuable for remote, underserved, priority communities, where healthcare infrastructures may be limited and difficult to obtain. Overall, this study highlights the potential of a WEM approach to address global health challenges, emphasizing the critical role of developing innovative solutions for TB control and informed public health interventions.

3.6 Ethical considerations

Before commencing this research, the authors received advice from the research ethics board of the University of Ottawa and the Canadian Research Ethics Board. The clinical data used in this study was gathered and consolidated by local public health units, adhering to applicable regulations and guidelines, and was entirely anonymous.

3.7 Acknowledgements

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3.8 Author contributions statement

R.D. conceived the experiments, T.B.N. and S.W. conducted the experiments, T.B.N., E.M, and R.D. analyzed the results, T.B.N., E.M., N.H., P.K., S.W., S.G., and R.D. edited the manuscript. All authors reviewed the manuscript.

3.9 Competing interest statement

The authors declare that no known competing financial interests or personal relationships could appear to influence the work reported in this manuscript.

3.10 Data availability statement

Data will be available upon reasonable request by contacting the corresponding author.

3.11 Funding

This research was supported by the CIHR Applied Public Health Research Chair in Environment, Climate Change and One Health, awarded to Dr. Robert Delatolla. The funding had no involvement in the study design, data collection, data analysis, data interpretation, nor the writing or decision to submit the paper for publication.

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Chapter 4.

Conclusions & Recommendations

4.1 Summary

The objective of this thesis was to address two key challenges of the wastewater environmental monitoring (WEM) of tuberculosis (TB); the lack of a sensitive and specific probe-based quantitative polymerase chain reaction (qPCR) for WEM applications, and the lack of partitioning behaviour information of TB markers in wastewater. Results from this work are targeted to optimize current enrichment and quantification methods of *Mycobacterium tuberculosis* complex (MTBC), *Mycobacterium tuberculosis* (MTB), and *Mycobacterium bovis* (MB) in wastewater.

4.2 Conclusions

This thesis examined the development and validation of three probe-based qPCR assays for the detection of MTBC, MTB, and MB in wastewater. The three assays were designed using genomic regions of commonly employed clinical PCR assays, MTBC *rv0577*, MTB RD9, and MB RD4-deletion to detect MTBC, MTB, and MB, respectively in wastewater. To validate the sensitivity of the assays, assay limits of detection (ALOD), assay limits of quantification (ALOQ), and standard curve efficiency tests were used to demonstrate excellent sensitivity for application in both low- and high-prevalence populations. Specificity was demonstrated using extracted DNA from a panel of laboratory-propagated mycobacteria cell cultures, and wastewater from three locations with confirmed TB cases: Ottawa, Canada; Mumbai, India; and an Inuit Nunangat, Northern-Indigenous community. Effective application of the three assays were demonstrated in a low-prevalence large municipality, high-prevalence large municipality, and high-prevalence small community context. The partitioning behaviour of MTBC and MTB was found to partition to the solids fraction of wastewater, being optimally concentrated and extracted with the addition of

PEG-precipitation. As a result, the three newly designed assays demonstrate a means to apply probe-based qPCR technology to the WEM of TB to address public health challenges and provide a method to rapidly and economically track TB in both municipalities and remote communities.

4.3 Recommendations for Further Research

The conclusions drawn from this research has led to the following recommendations for further research to assist in optimizing WEM for the monitoring of TB and other pathogenic diseases:

Optimization of wastewater sampling methods in Northern Indigenous communities. Future work should focus on optimizing wastewater sampling methods tailored to the unique environmental, infrastructural, and socio-cultural contexts of Northern Indigenous communities, including those in Inuit Nunangat. Given the diverse and often remote settings of these communities, sampling strategies must be adaptable to varying wastewater treatment systems, seasonal fluctuations, and logistical constraints. Additionally, it is essential that these methods be co-developed in collaboration with Indigenous stakeholders and community members to ensure cultural appropriateness, ethical practices, and community engagement. By addressing key geographic and infrastructural challenges such as arctic weather conditions, decentralized wastewater treatment systems, and limited accessibility, WEM can serve as a valuable tool for TB monitoring and treatment. This approach has the potential to generate real-time data that can inform targeted public health responses, contributing to the reduction of TB disease prevalence and the advancement of health equality in these historically underserviced populations.

Expansion of assay panel to quantify Drug Resistant-TB in wastewater. The emergence and persistence of drug-resistant TB (DR-TB), including multidrug-resistant (MDR-TB) and extensively drug-resistant TB (XDR-TB), continue to challenge global TB elimination efforts. Wastewater and environmental monitoring (WEM) offers a promising approach for the early and rapid detection of DR-TB at the community level, providing valuable epidemiological data without the need for individual clinical diagnoses. By integrating WEM into public health monitoring systems, officials can obtain real-time insights into the prevalence and distribution of DR-TB, enabling more efficient resource allocation and targeted intervention strategies. This approach is

particularly critical for remote and underserviced communities, where timely access to diagnostics and treatment is often limited. The ability to assess community-level DR-TB burden through WEM can allow for tailored public health responses, ultimately supporting more effective TB control and elimination efforts worldwide.

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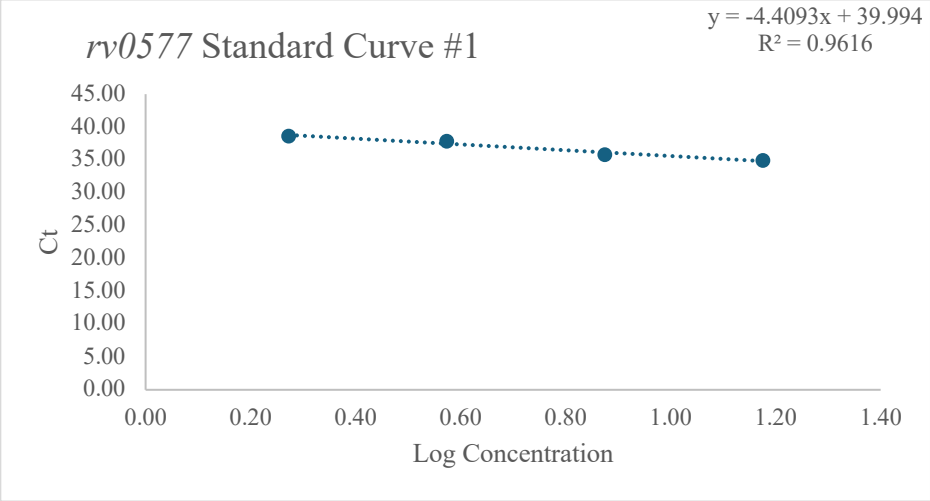
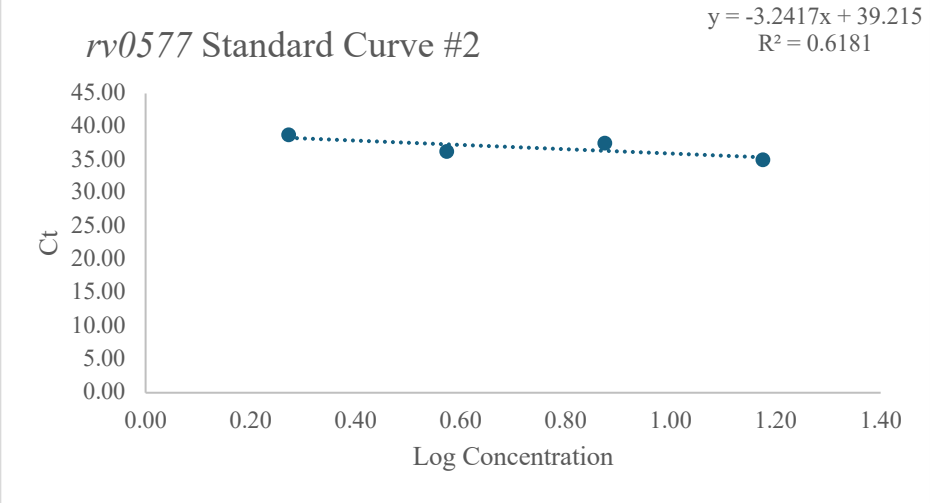
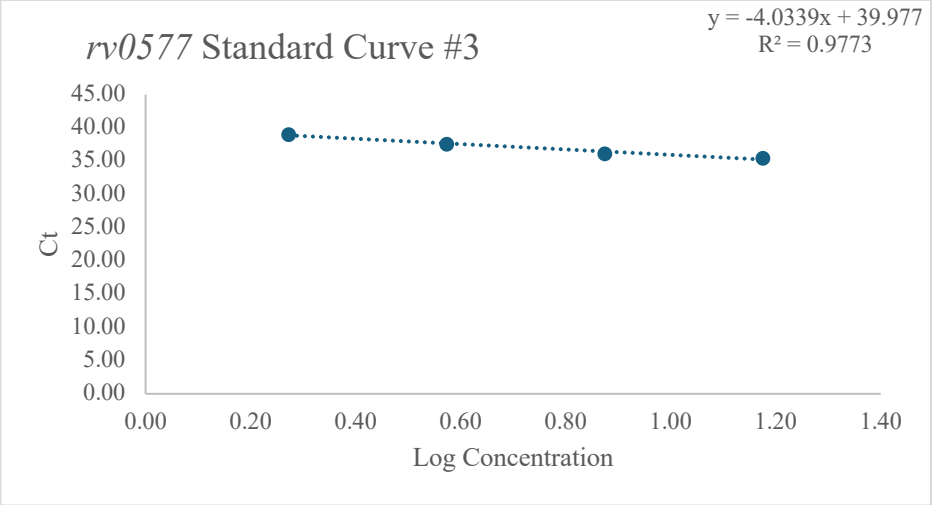
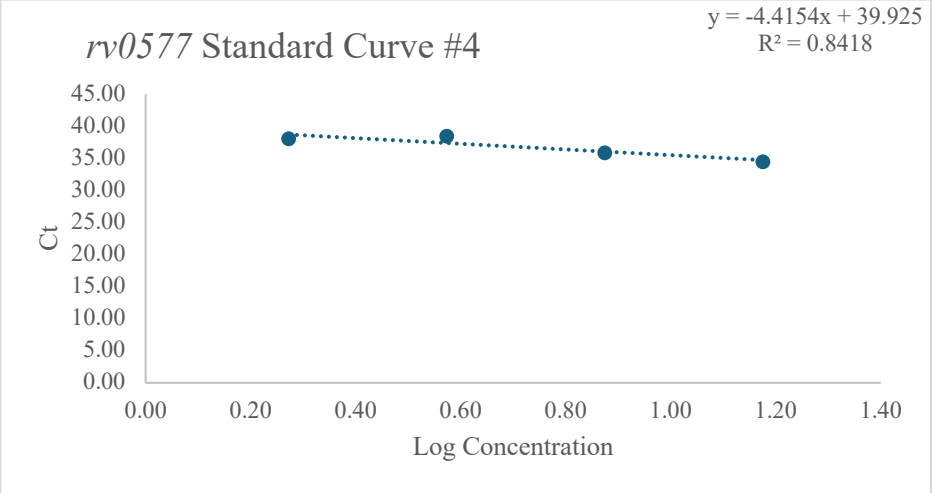
Appendix A

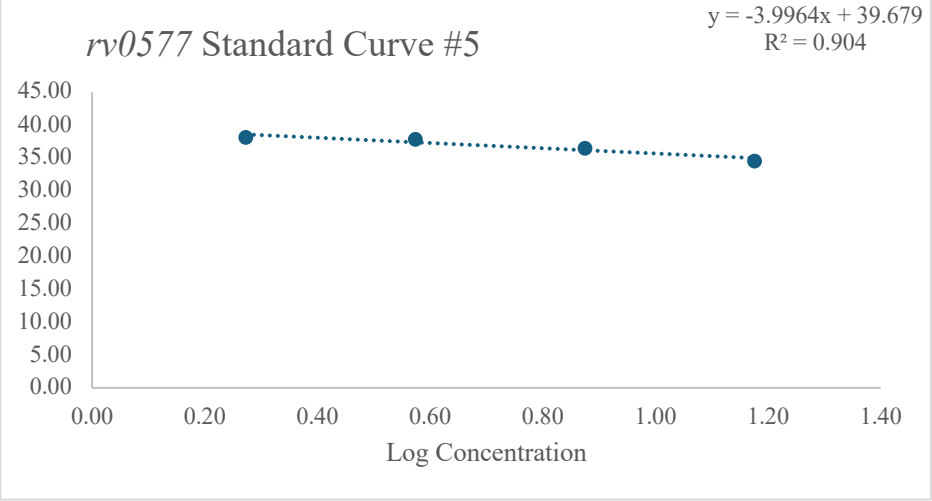
Standard calibration curves for the calculation of assay limits of detection (ALOD) and assay limits of quantification (ALOQ).






y = -4.0339x + 39.977
R² = 0.9773
rv0577 Standard Curve #3
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -4.4154x + 39.925
R² = 0.8418
rv0577 Standard Curve #4
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -3.9964x + 39.679
R² = 0.904
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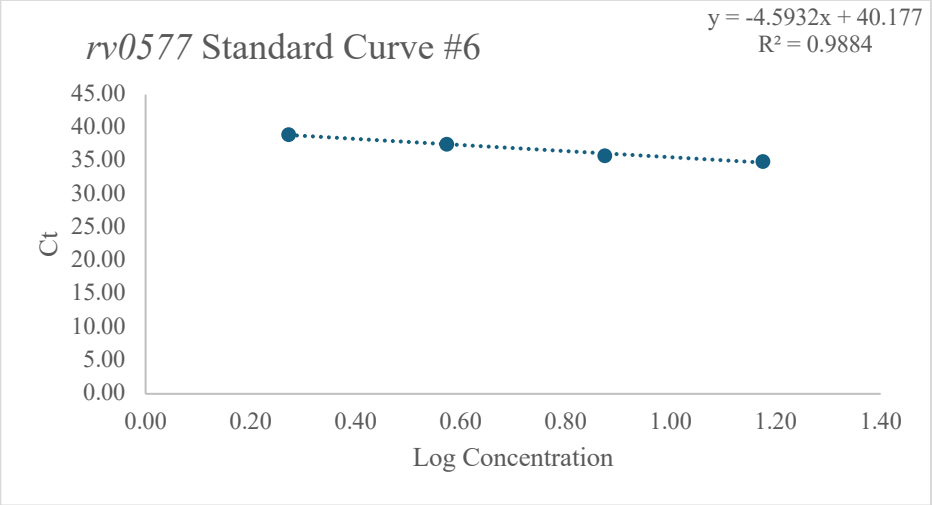
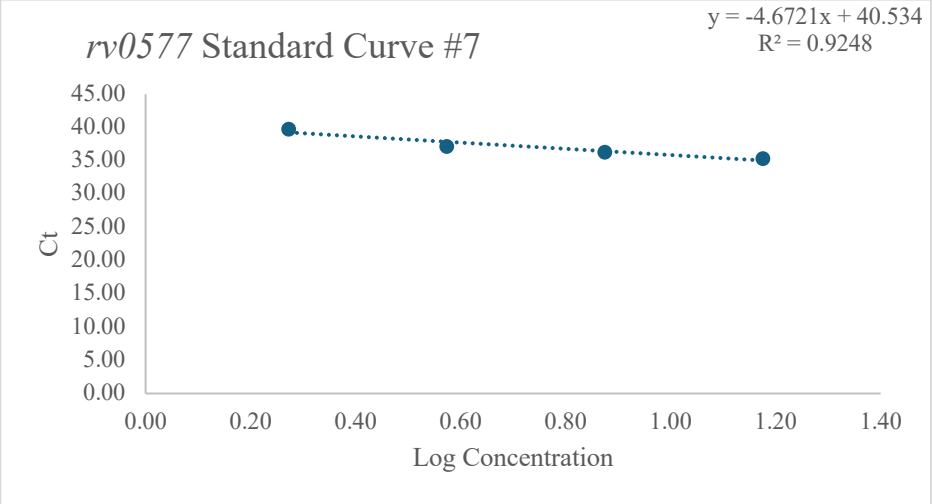
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -4.5932x + 40.177
R² = 0.9884
rv0577 Standard Curve #6
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -4.6721x + 40.534
R² = 0.9248
rv0577 Standard Curve #7
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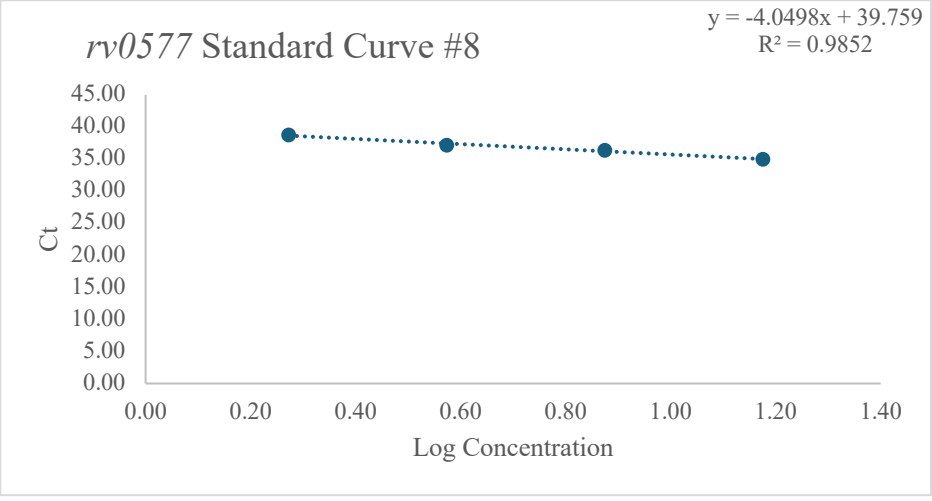
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -4.0498x + 39.759
R² = 0.9852
rv0577 Standard Curve #8
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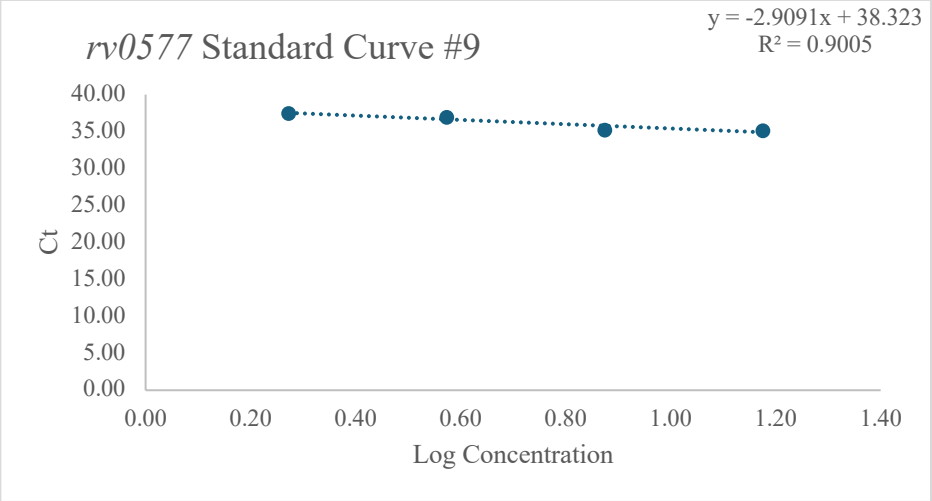
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -2.9091x + 38.323
R² = 0.9005
rv0577 Standard Curve #9
Ct
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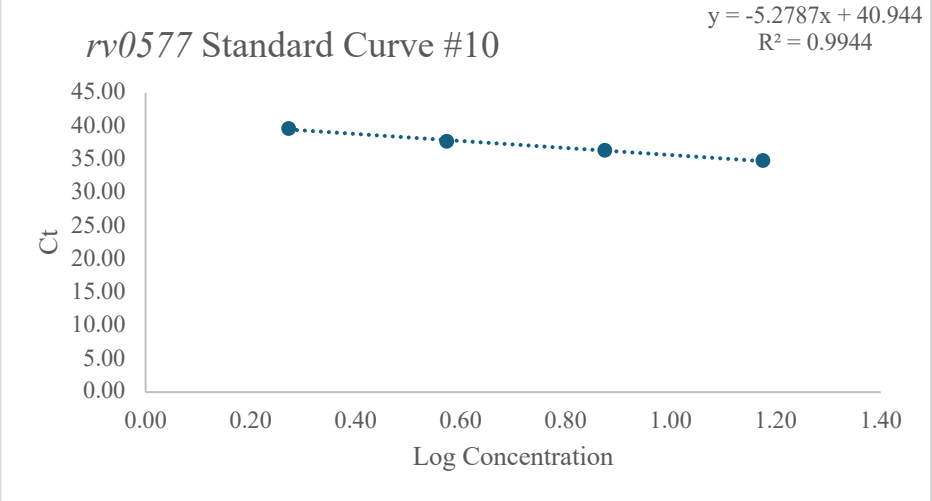
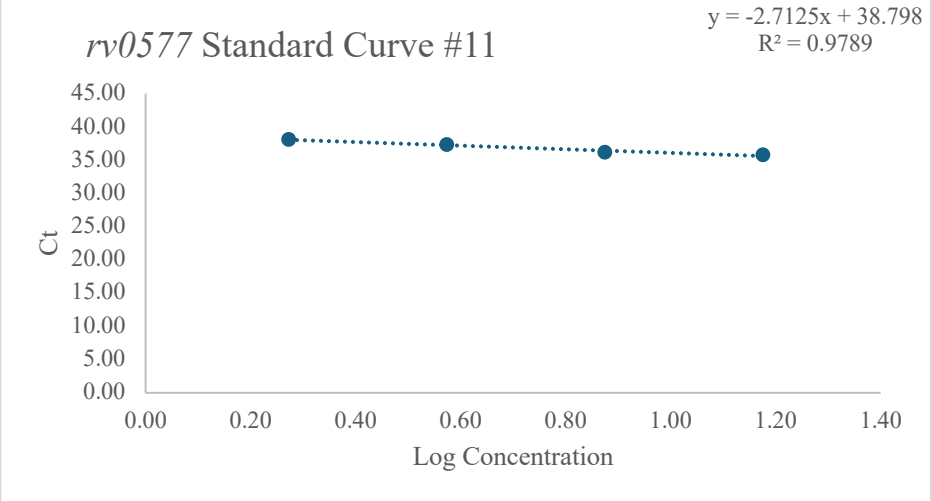
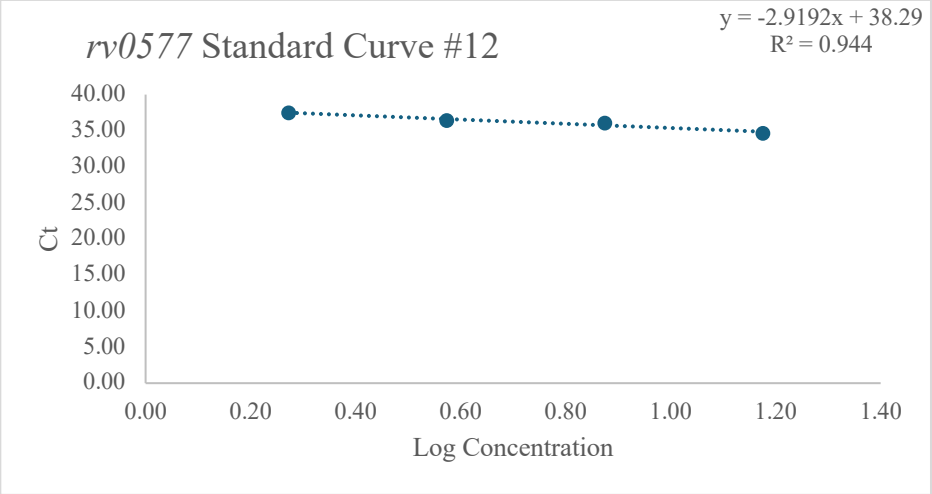
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -5.2787x + 40.944
R² = 0.9944
rv0577 Standard Curve #10
Ct
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -2.7125x + 38.798
R² = 0.9789
rv0577 Standard Curve #11
Ct
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -2.9192x + 38.29
R² = 0.944
rv0577 Standard Curve #12
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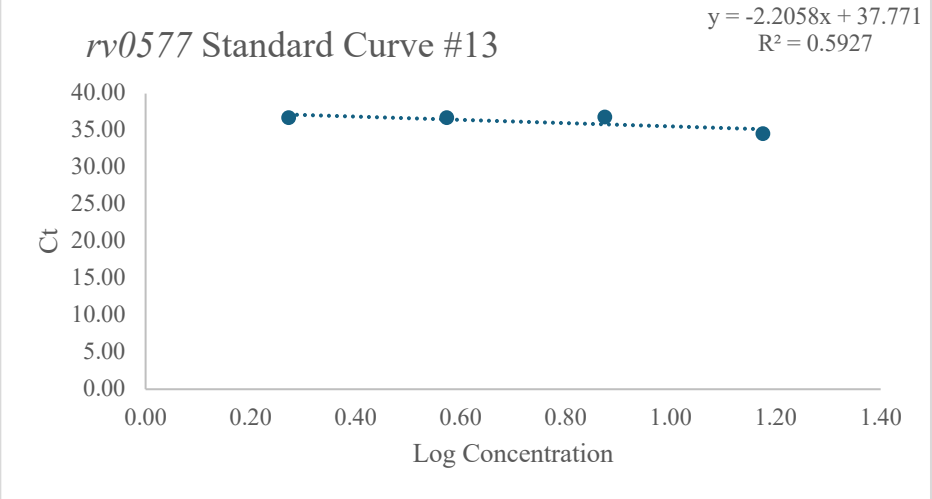
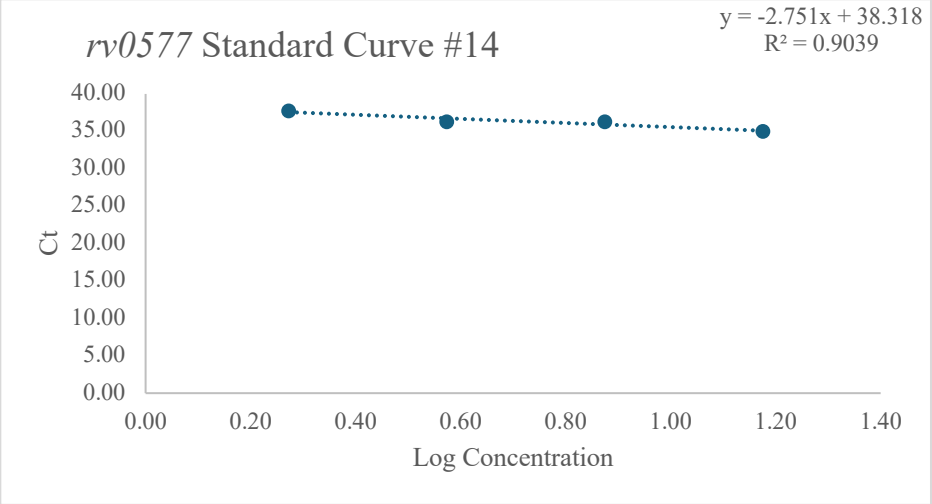
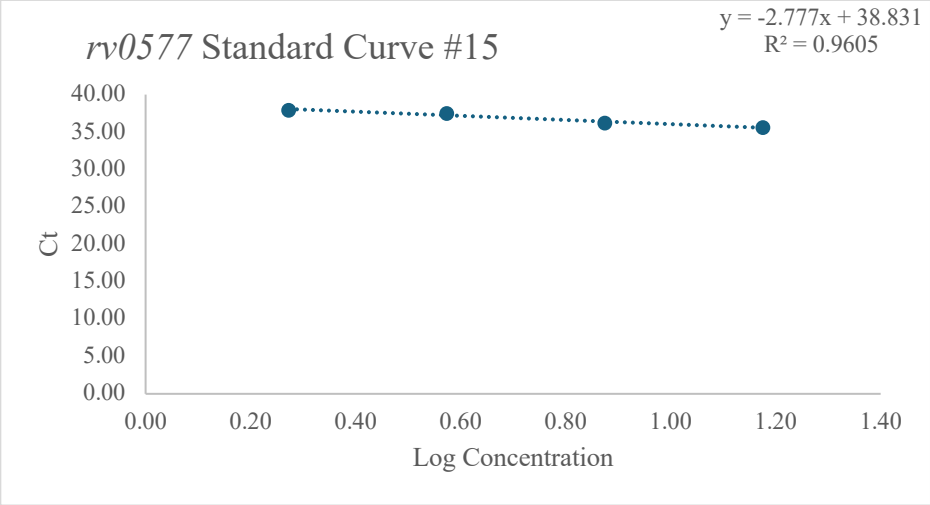
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -2.2058x + 37.771
R² = 0.5927
rv0577 Standard Curve #13
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -2.751x + 38.318
R² = 0.9039
rv0577 Standard Curve #14
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -2.777x + 38.831
R² = 0.9605
rv0577 Standard Curve #15
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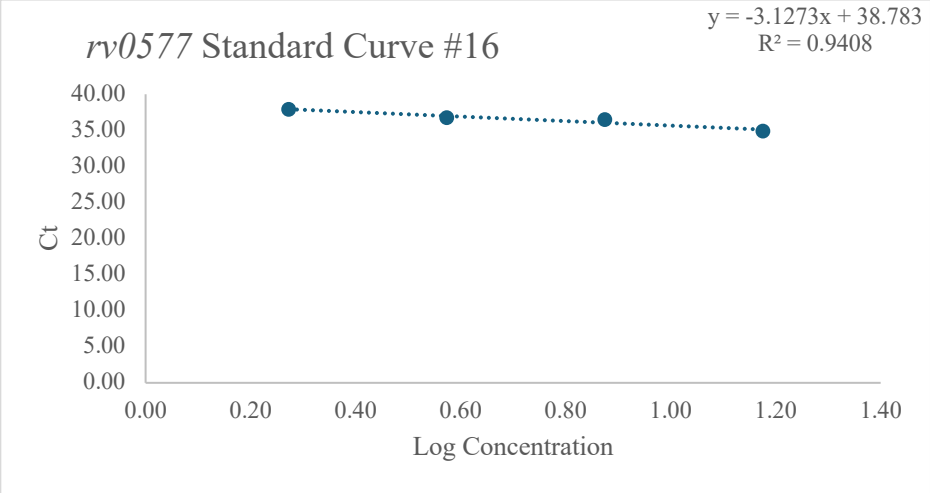
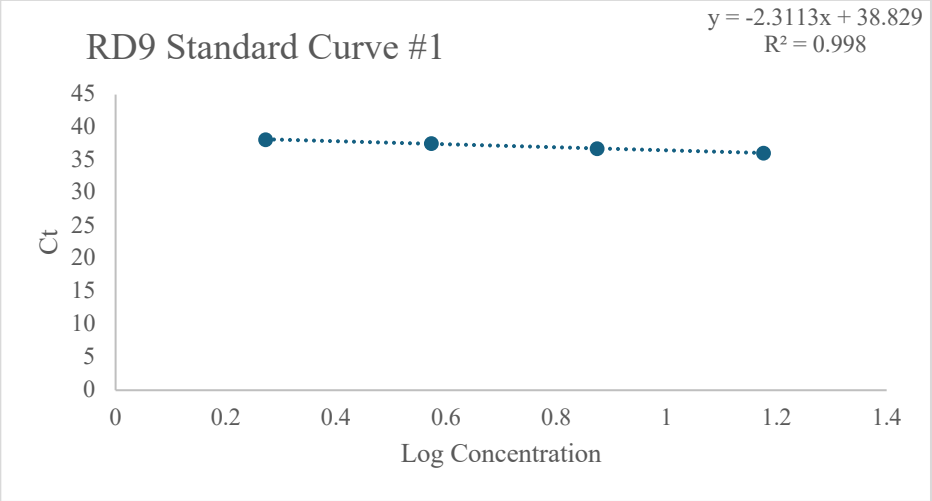
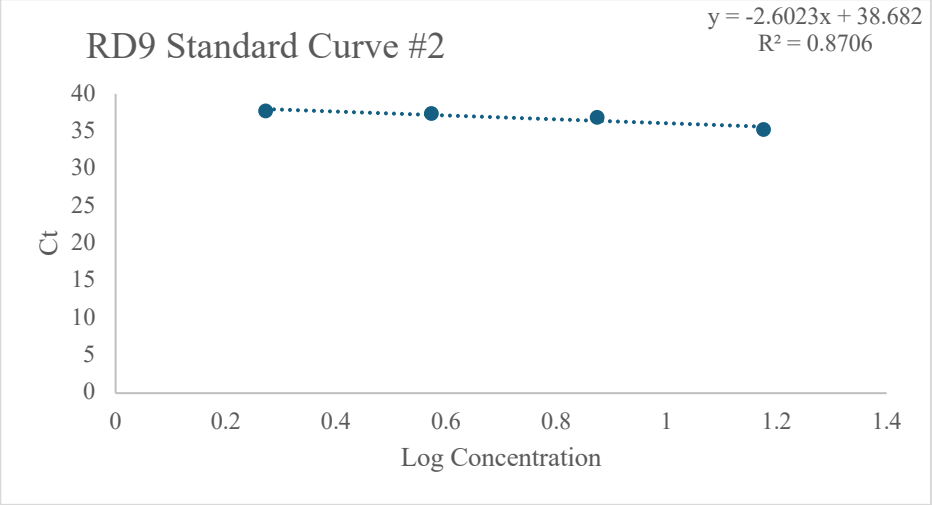
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -3.1273x + 38.783
R² = 0.9408
rv0577 Standard Curve #16
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ct
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
Log Concentration


y = -2.3113x + 38.829
R² = 0.998
RD9 Standard Curve #1
Ct
Log Concentration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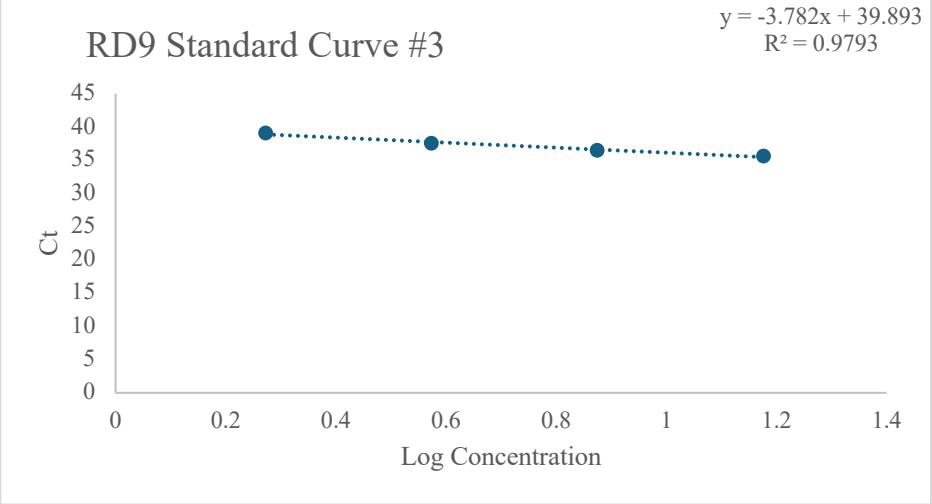
R² = 0.8706
RD9 Standard Curve #2
Ct
Log Concentration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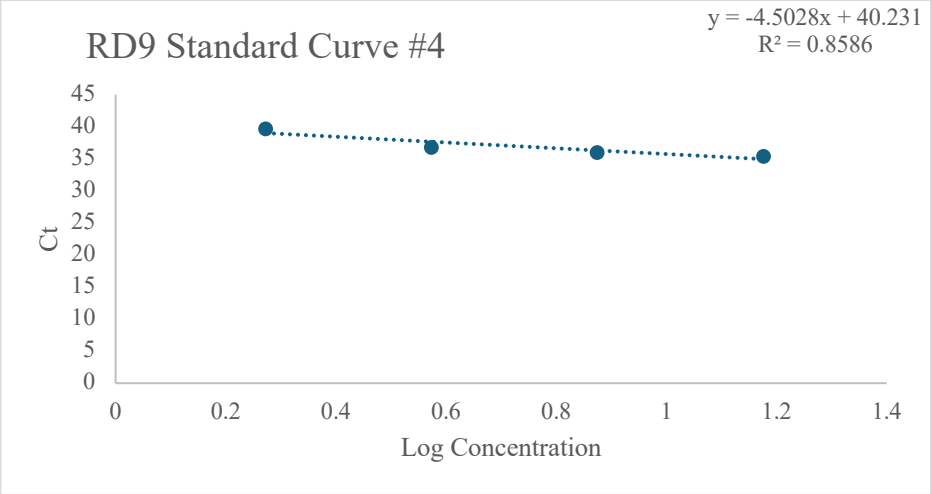
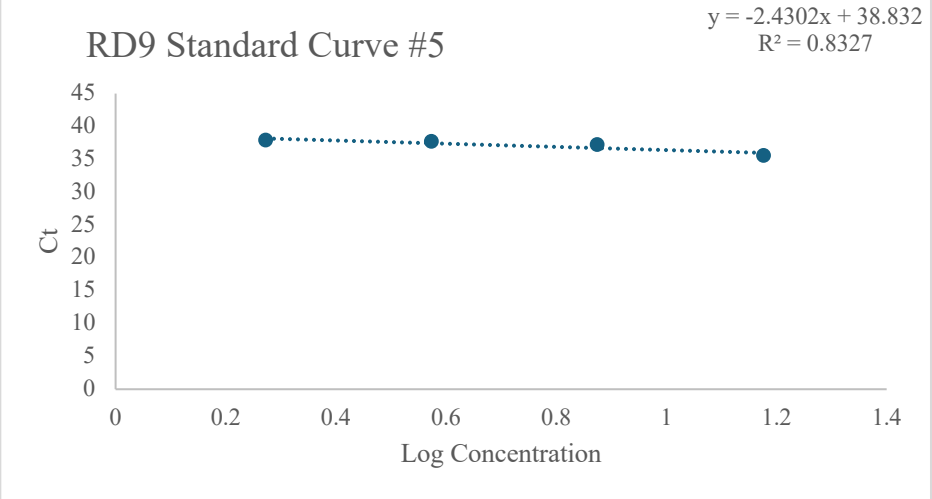
R² = 0.9793
RD9 Standard Curve #3
Ct
Log Concentration


y = -4.5028x + 40.231
R² = 0.8586
RD9 Standard Curve #4
Ct
45
40
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -2.4302x + 38.832
R² = 0.8327
RD9 Standard Curve #5
Ct
45
40
35
30
25
20
15
10
5
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1
1.2
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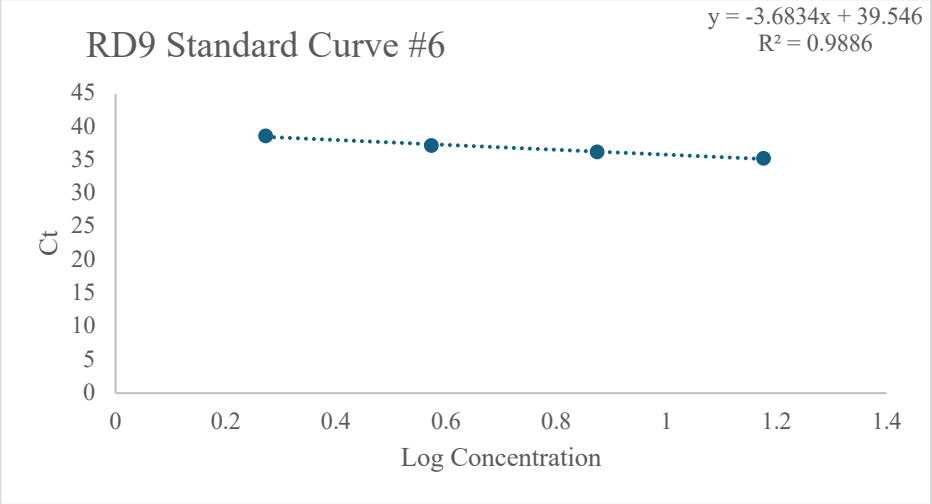
Log Concentration


y = -3.6834x + 39.546
R² = 0.9886
RD9 Standard Curve #6
Ct
45
40
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30
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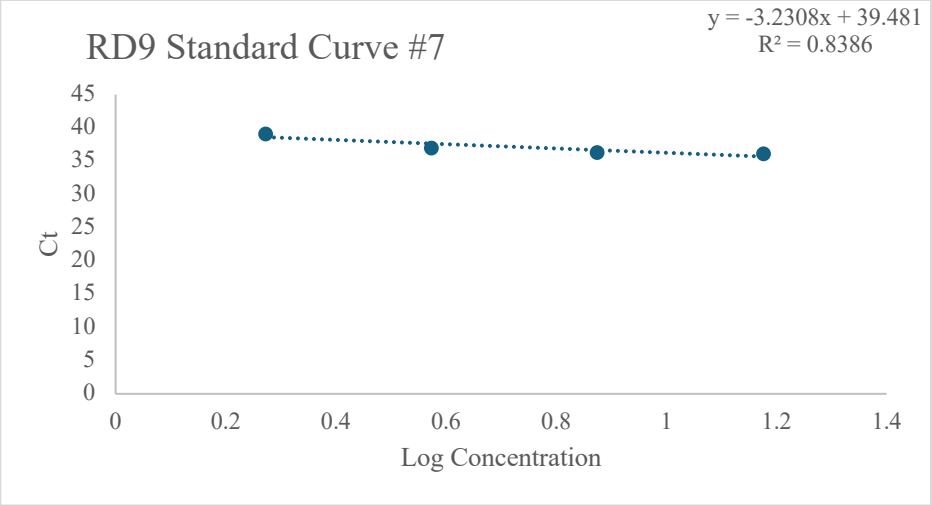
Log Concentration


y = -3.2308x + 39.481
R² = 0.8386
RD9 Standard Curve #7
Ct
45
40
35
30
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20
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0.8
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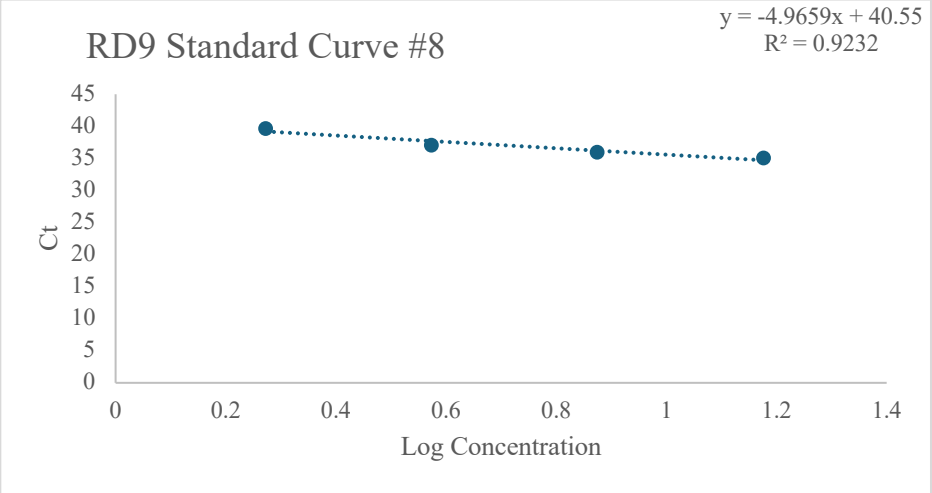
1.2
1.4
Log Concentration


y = -4.9659x + 40.55
R² = 0.9232
RD9 Standard Curve #8
Ct
45
40
35
30
25
20
15
10
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0
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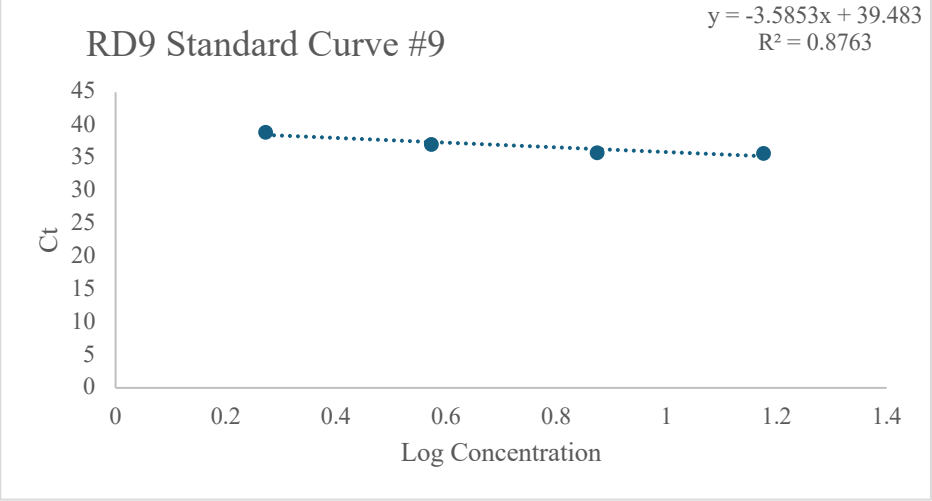
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -3.5853x + 39.483
R² = 0.8763
RD9 Standard Curve #9
Ct
45
40
35
30
25
20
15
10
5
0
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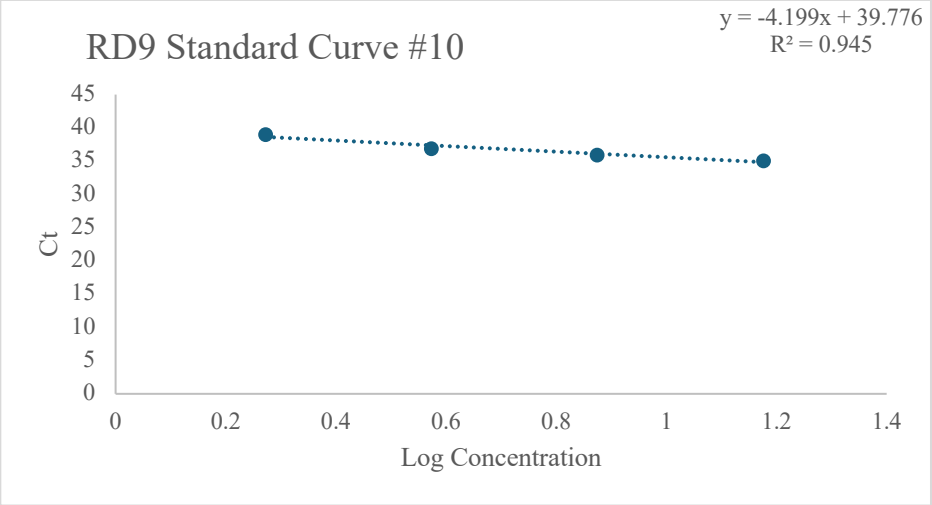
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -4.199x + 39.776
R² = 0.945
RD9 Standard Curve #10
Ct
Log Concentration
45
40
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30
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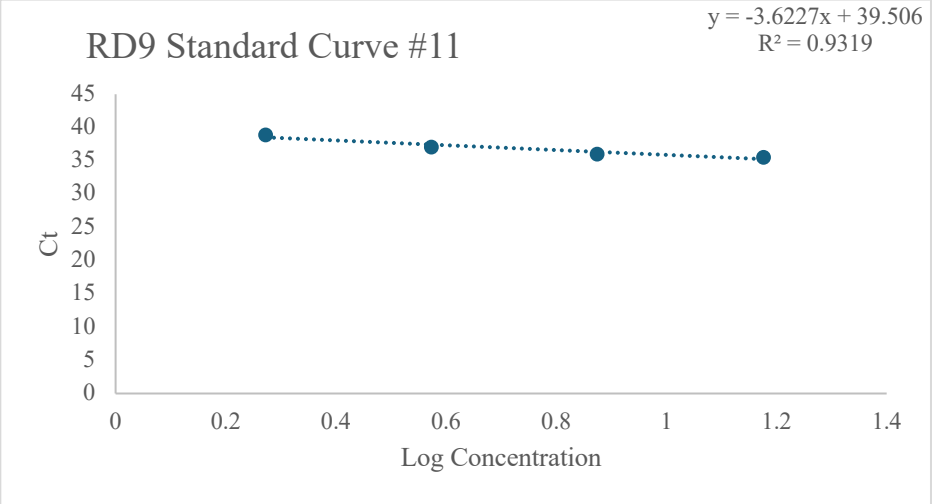
y = -3.6227x + 39.506
R² = 0.9319
RD9 Standard Curve #11
Ct
Log Concentration
45
40
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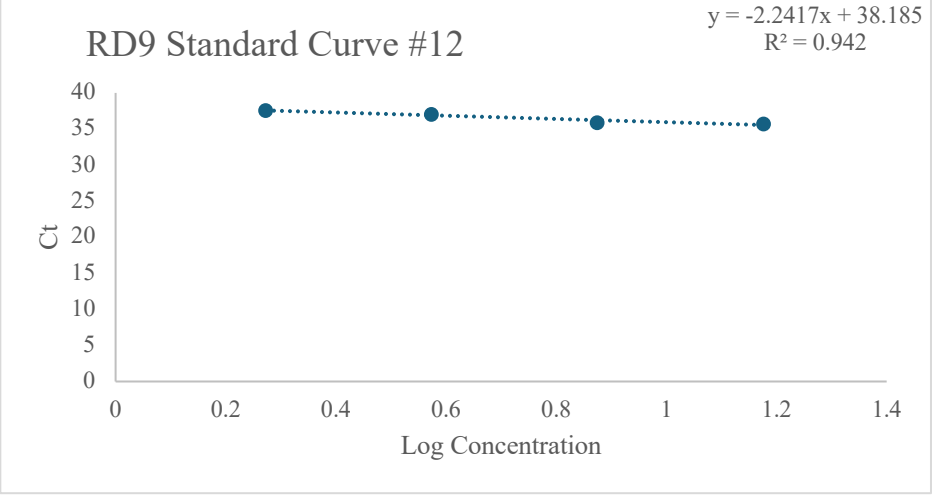
y = -2.2417x + 38.185
R² = 0.942
RD9 Standard Curve #12
Ct
Log Concentration
40
35
30
25
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15
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0.8
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1.2
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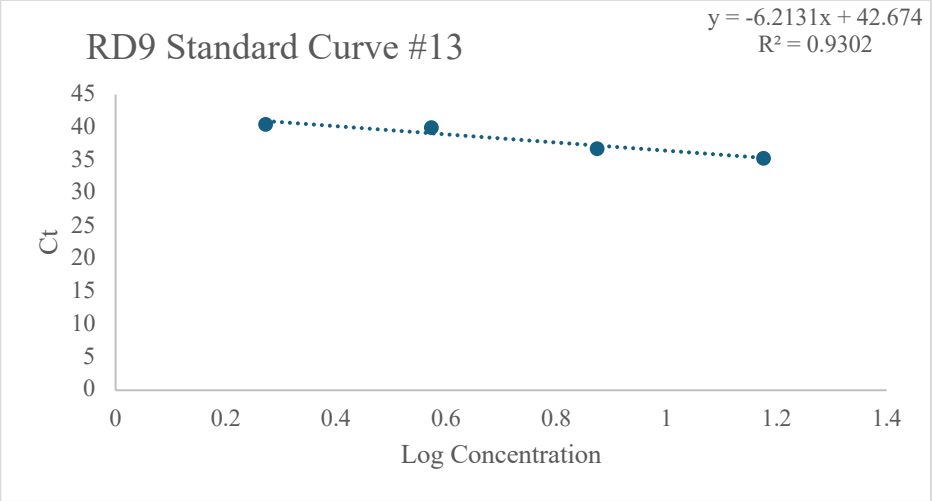
y = -6.2131x + 42.674
R² = 0.9302
RD9 Standard Curve #13
Ct
Log Concentration
45
40
35
30
25
20
15
10
5
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0.2
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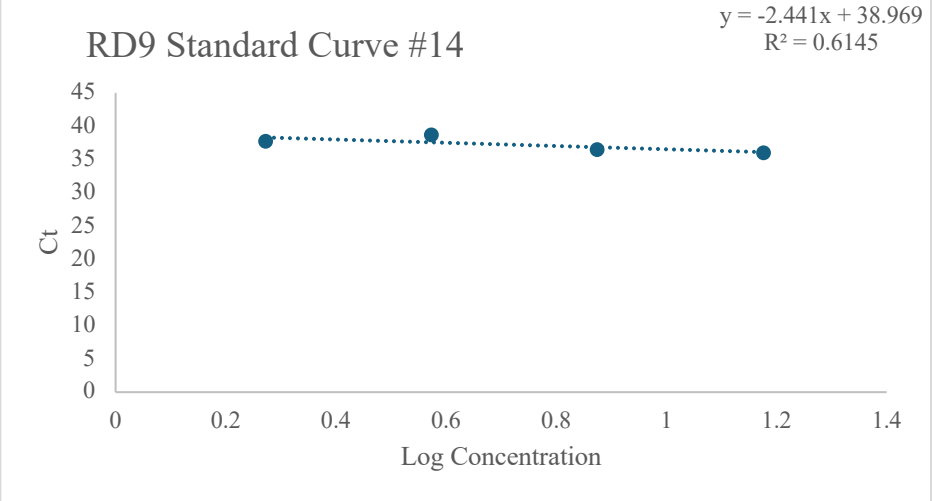
0.6
0.8
1
1.2
1.4


y = -2.441x + 38.969
R² = 0.6145
RD9 Standard Curve #14
Ct
Log Concentration
45
40
35
30
25
20
15
10
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0.2
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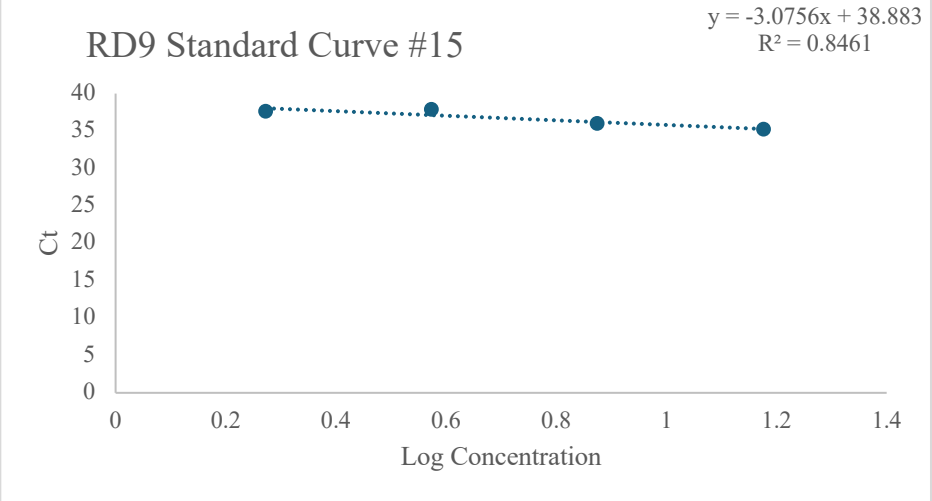
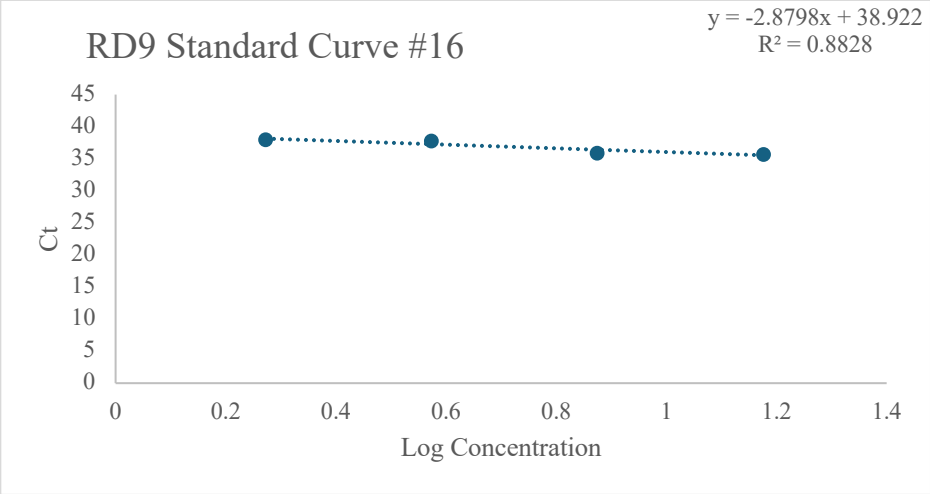
0.6
0.8
1
1.2
1.4


y = -3.0756x + 38.883
R² = 0.8461
RD9 Standard Curve #15
Ct
Log Concentration
40
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4


RD9 Standard Curve #16
y = -2.8798x + 38.922
R² = 0.8828
Ct
45
40
35
30
25
20
15
10
5
0
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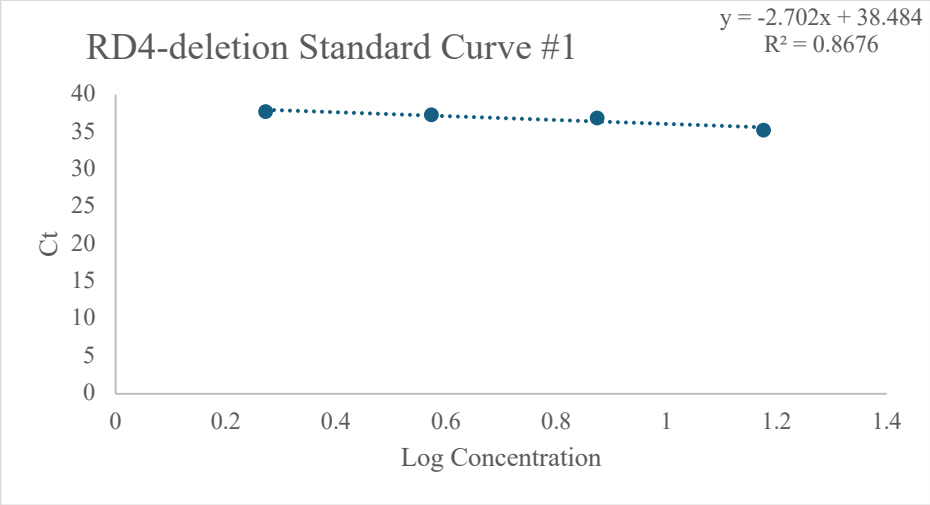
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -2.702x + 38.484
R² = 0.8676
RD4-deletion Standard Curve #1
Ct
40
35
30
25
20
15
10
5
0
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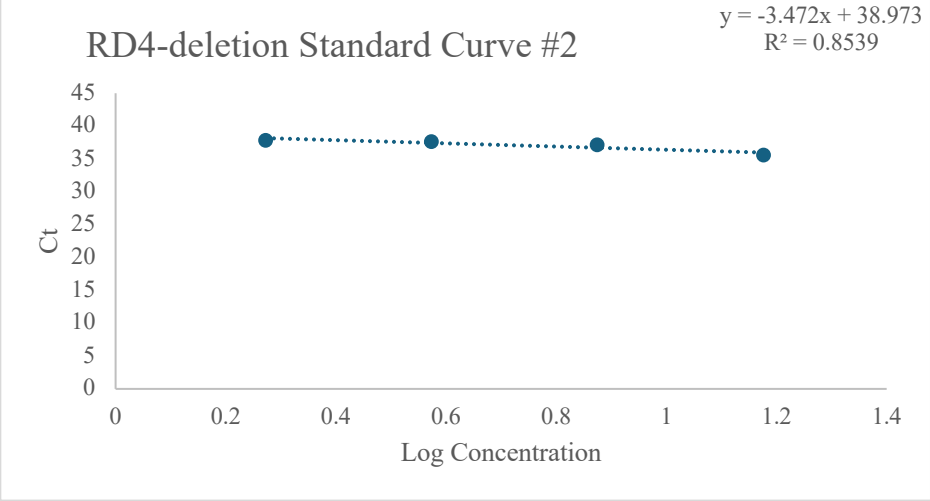
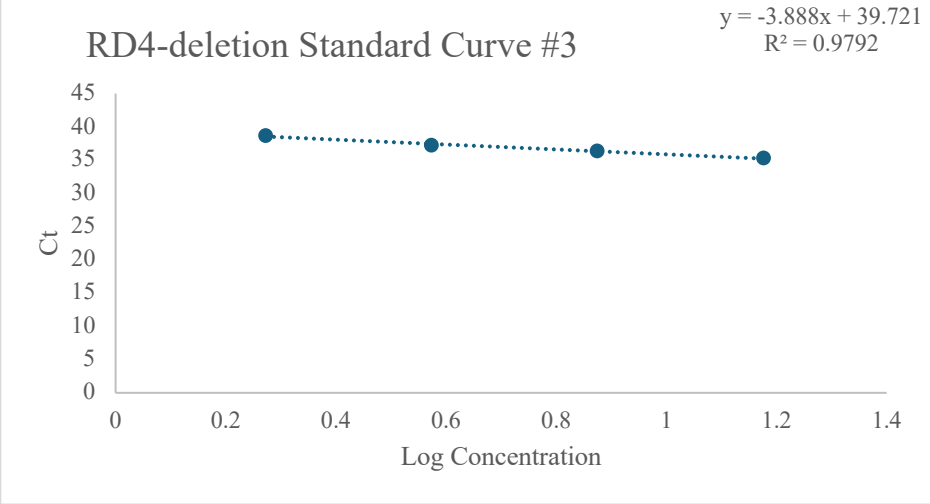
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -3.472x + 38.973
R² = 0.8539
RD4-deletion Standard Curve #2
Ct
45
40
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -3.888x + 39.721
R² = 0.9792
RD4-deletion Standard Curve #3
Ct
45
40
35
30
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15
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0
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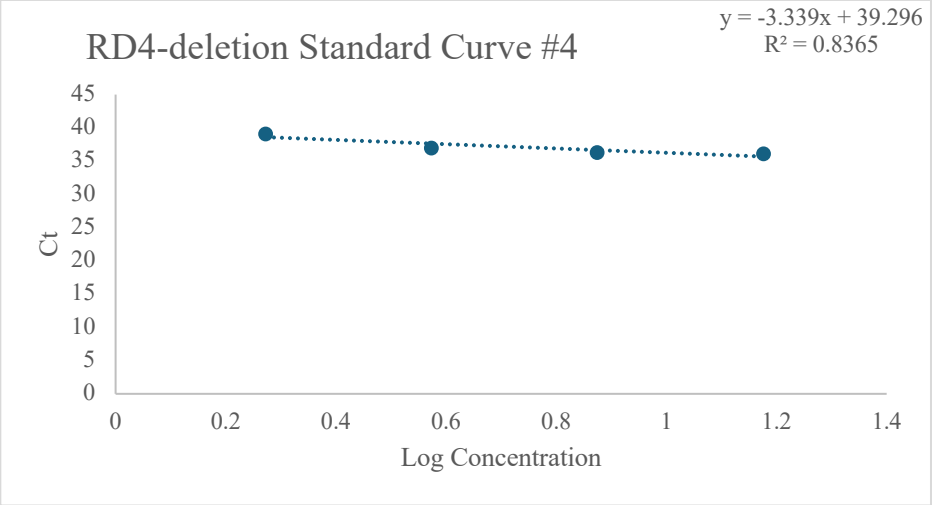
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -3.339x + 39.296
R² = 0.8365
RD4-deletion Standard Curve #4
Ct
45
40
35
30
25
20
15
10
5
0
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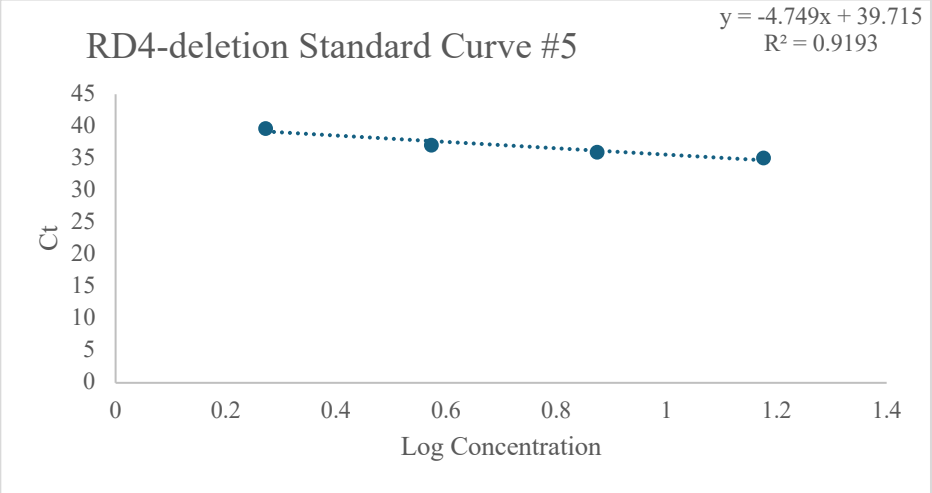
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -4.749x + 39.715
R² = 0.9193
RD4-deletion Standard Curve #5
Ct
45
40
35
30
25
20
15
10
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0
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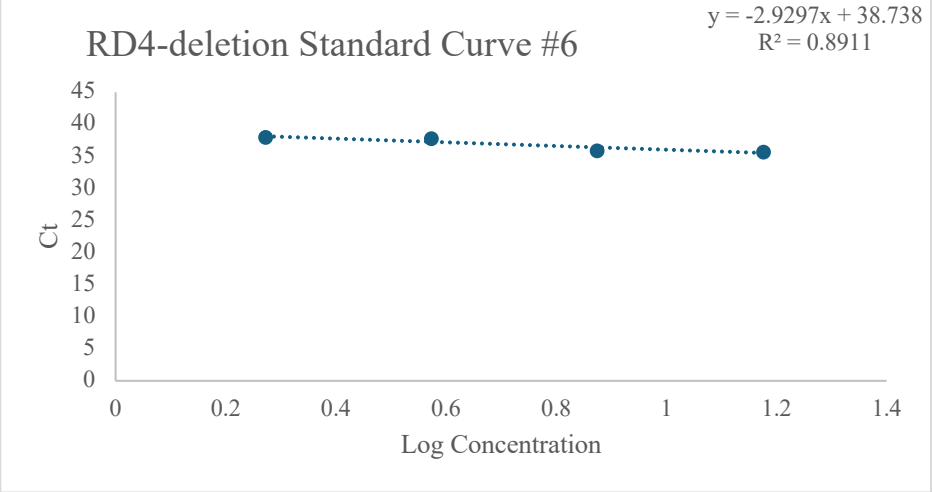
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -2.9297x + 38.738
R² = 0.8911
RD4-deletion Standard Curve #6
Ct
45
40
35
30
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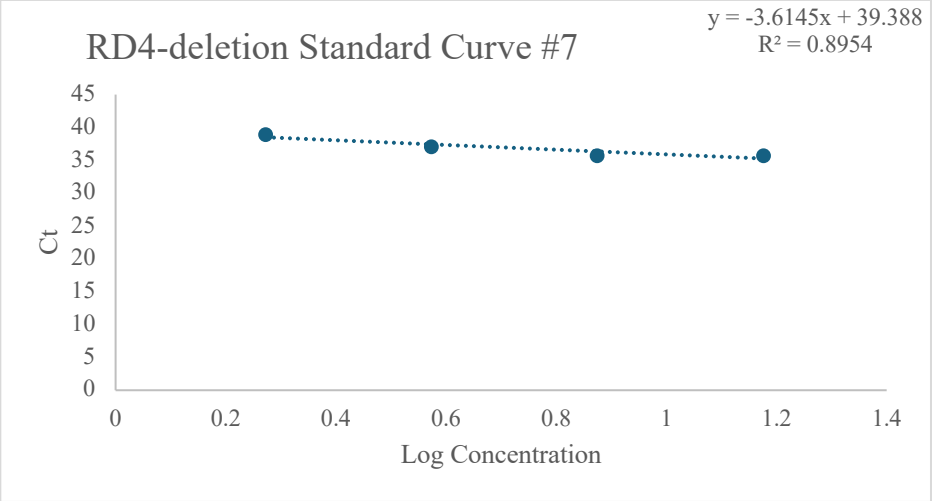
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -3.6145x + 39.388
R² = 0.8954
RD4-deletion Standard Curve #7
Ct
45
40
35
30
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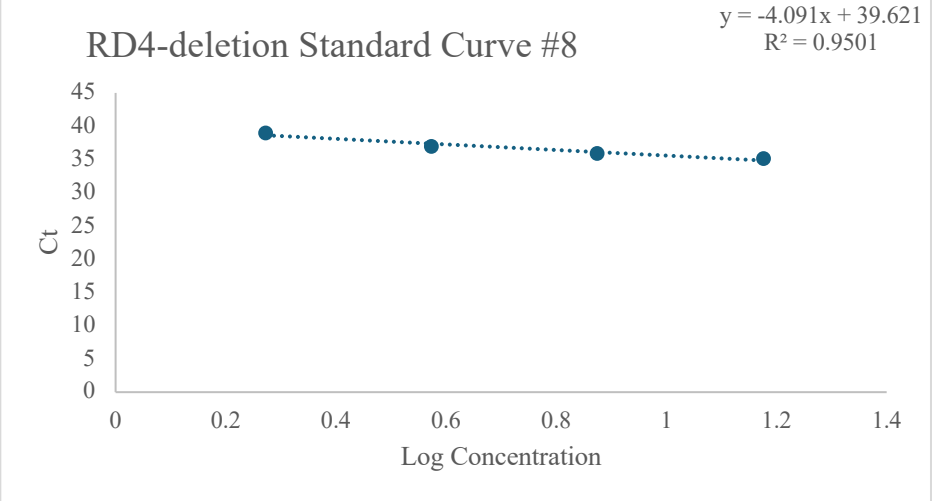
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


y = -4.091x + 39.621
R² = 0.9501
RD4-deletion Standard Curve #8
Ct
45
40
35
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0.2
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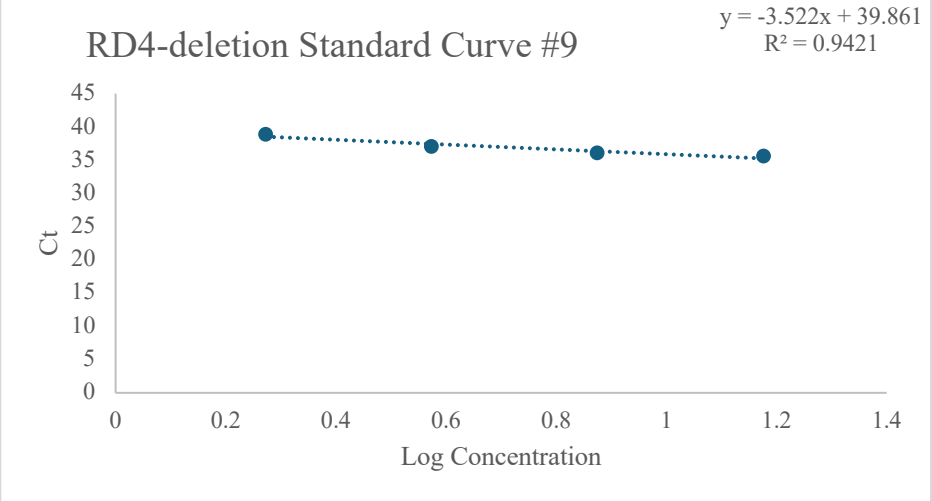
0.6
0.8
1
1.2
1.4
Log Concentration


y = -3.522x + 39.861
R² = 0.9421
RD4-deletion Standard Curve #9
Ct
45
40
35
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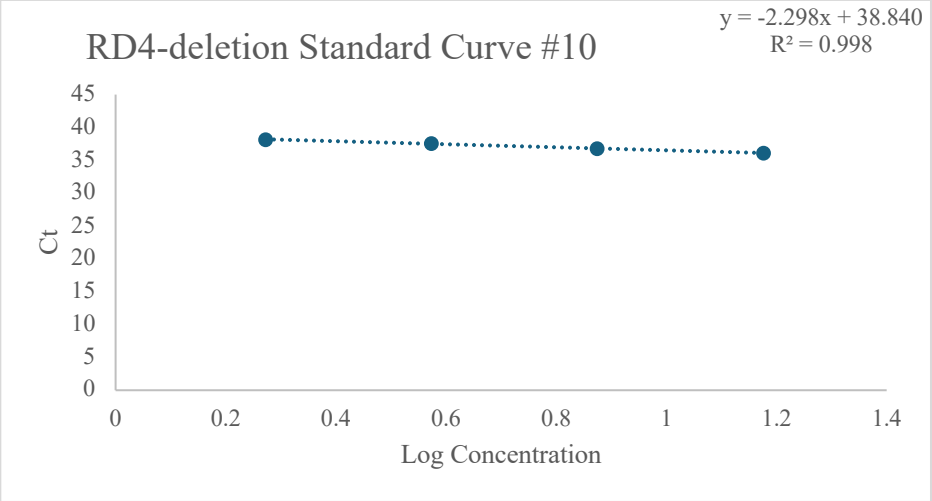
1.4
Log Concentration


y = -2.298x + 38.840
R² = 0.998
RD4-deletion Standard Curve #10
Ct
45
40
35
30
25
20
15
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5
0
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0.2
0.4
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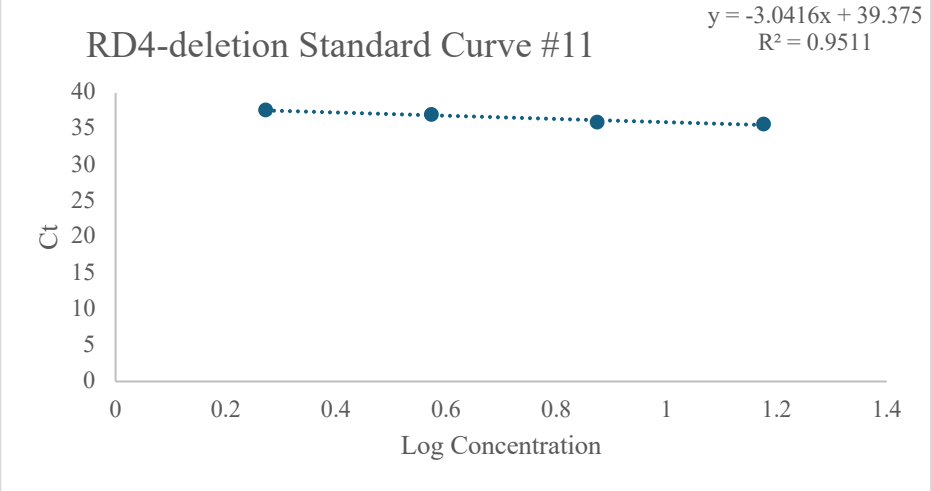
0.8
1
1.2
1.4
Log Concentration


y = -3.0416x + 39.375
R² = 0.9511
RD4-deletion Standard Curve #11
Ct
40
35
30
25
20
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0.2
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0.8
1
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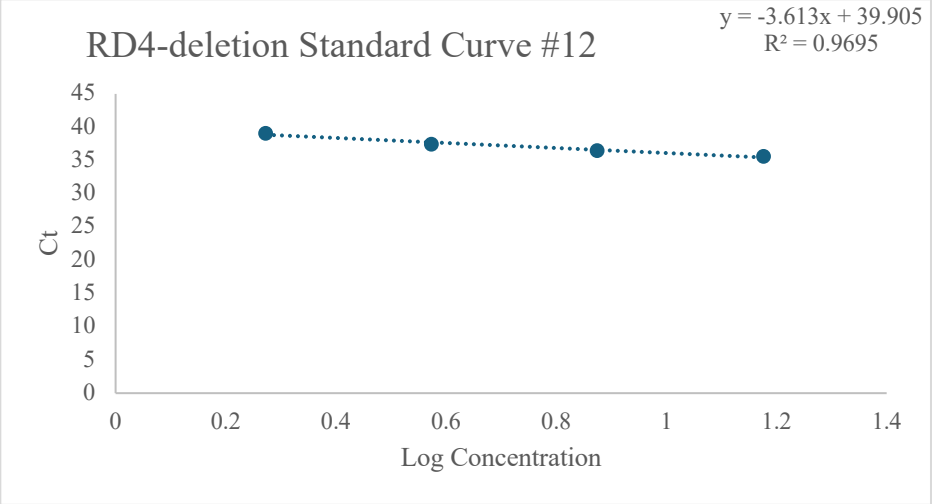
Log Concentration


y = -3.613x + 39.905
R² = 0.9695
RD4-deletion Standard Curve #12
Ct
45
40
35
30
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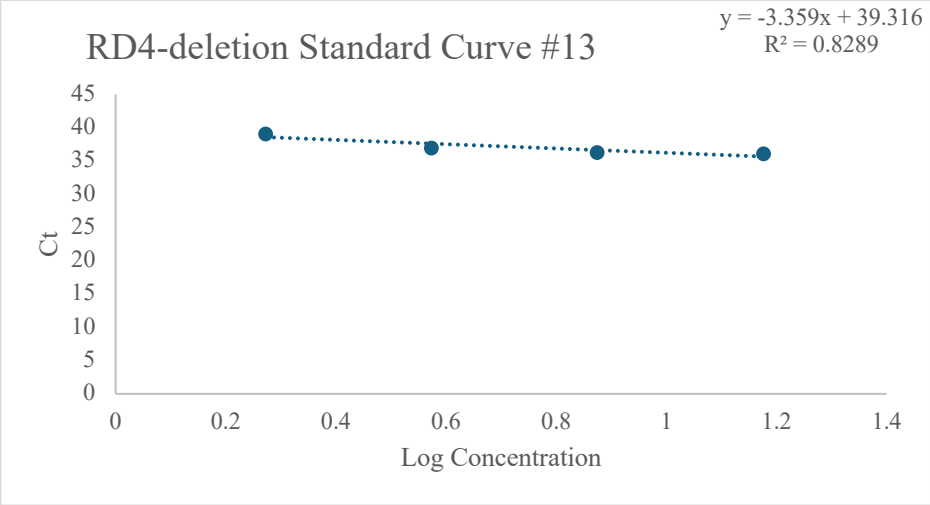
1.4
Log Concentration


y = -3.359x + 39.316
R² = 0.8289
RD4-deletion Standard Curve #13
Ct
45
40
35
30
25
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5
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0.8
1
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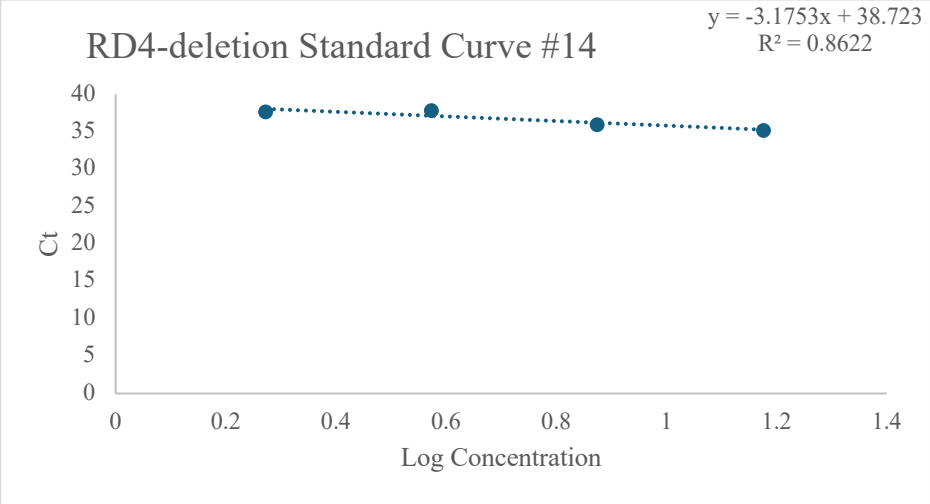
1.4
Log Concentration


y = -3.1753x + 38.723
R² = 0.8622
RD4-deletion Standard Curve #14
Ct
40
35
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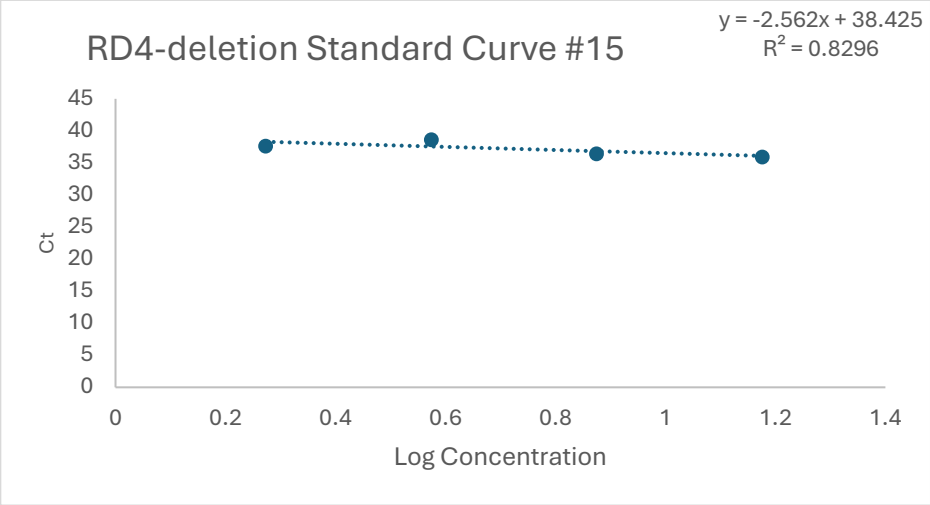
1.4
Log Concentration


RD4-deletion Standard Curve #15
y = -2.562x + 38.425
R² = 0.8296
Ct
45
40
35
30
25
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15
10
5
0
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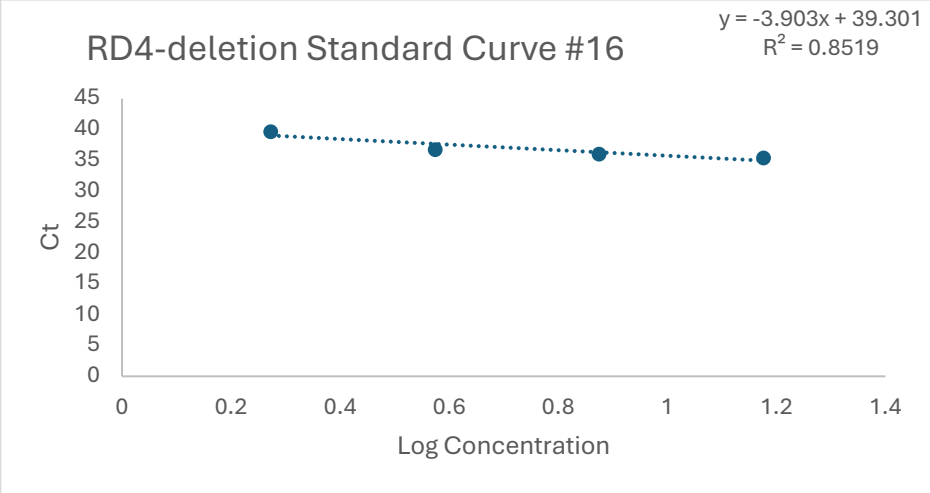
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration


RD4-deletion Standard Curve #16
y = -3.903x + 39.301
R² = 0.8519
Ct
45
40
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Log Concentration