Fate and Transport of Synthetic DNA in Surface Water

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Unlike conventional salt or dye tracers, artificial/synthetic DNA hydrologic tracers are essentially non-toxic and several can be used simultaneously. These features have implications for DNA to be used to better understand environmental processes and map hydrological pathways in complex environments, such as watersheds. Synthetic DNA tracers also have the potential to help with a better understanding of the behaviour of environmental DNA (eDNA) and its application in biomonitoring. Some components of eDNA exist as free/naked DNA not bound to other substances or protected by cellular material. The goal of the current research was to assess the fate and transport of naked DNA (short single stranded DNA sequences) in a small stream as a potential environmental tracer. As a proof-of-concept, two unique DNA tracers were released into an upstream location in Washington Creek (southern Ontario). After releasing the tracers, water samples were collected 100 m and 350 m downstream and breakthrough curves of tracer concentration were plotted over time. Both tracers behaved similarly with a mass recovery of 71% (T11) and 80% (T22) at the 100 m downstream sampling location and about 70% for both tracers at 350 m downstream. The downstream tracer peak arrival times were 15 – 16 min and 30 – 31 min at the 100 m and 350 m sampling sites, respectively, demonstrating that naked DNA injected into the stream can quickly travel downstream. This suggests that eDNA, in the naked form, may survive considerable distances downstream from the source and has implications for biomonitoring strategies. Additional unique DNA tracers were designed and optimized for future experiments. DNA tracers create many opportunities for applications in environmental sciences, especially if they can be combined with other substances to alter their environmental properties and fate (i.e., nanoparticles). DNA tracers can be attached to nanoparticles to protect DNA degradation in harsh environments or influence their zeta potential.

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1. Introduction

Many different human activities are threatening freshwater environments (Thomsen & Willerslev, 2015a). With climate change, there is an ever increasing need to improve biodiversity monitoring and track the state of valuable water resources. Emerging contaminants, specifically those that represent an environmental risk in low concentrations, are a growing concern for drinking water sources and ecosystems. Synthetic DNA tracers could be a vital tool to better understand environmental processes and support tracking and modelling the fate and transport of a subset of these potential contaminants. In addition, DNA tracers may help to inform the rapidly developing field of environmental DNA biomonitoring (Thomsen & Willerslev, 2015b).

Stream injection experiments provide realistic information about solute transport, retention, and reactivity in flowing surface water systems (Stream Solute Workshop, 1990). Common tracers include salts, fluorescent dyes, isotopes, silica, and bromide due to their simple and inexpensive use. In most cases, these traditional tracers are conservative. However, some conventional tracers have limitations such as potential toxicity, background noise, and interferences (Foppen et al., 2011; Liao et al., 2018). DNA tracers have the advantage of being environmentally inert (e.g., friendly) and allow for the application of numerous tracers simultaneously. Simultaneous application of multiple DNA tracers is possible due to the essentially unlimited number of unique DNA sequences that can be produced and applied concurrently. Conventionally, using multiple tracers including salts, fluorescent tracers, and environmental isotopes requires multiple analysis methods such as ion chromatography, or fluorescent, absorption, or mass spectrometry. DNA tracing allows for multiple tracers to be used and only requires one type of laboratory technology (Dahlke et al., 2015). In addition, the physical and chemical properties of a DNA tracer could be deliberately modified by encapsulation or association with other participles (e.g., nanoparticles) which could alter their transport and reactive behaviour. Encapsulation can protect the DNA from degradation in harsh environments and reduce mass losses (Liao et al., 2018). Functionalized coatings ensure that if multiple tracers are used, they will all exhibit identical transport behaviour but each has a unique DNA signature (Mikutis et al., 2018). Other benefits of DNA tracers include safely tracking surface water, groundwater, and their interactions (Foppen et al., 2011; Sabir et al., 1999, 2000). Synthetic DNA tracers may be used to track sources of pollution as they can be easily applied and individually identified in the natural environment due to their unique DNA sequence. DNA tracers are biologically inactive and much lower concentrations are required compared to conventional tracers, reducing the concern for toxicity

as DNA tracers are completely non-toxic (Liao et al., 2018). They also do not cause any changes to water density or viscosity that may alter the fate of the tracer (Aquilanti et al., 2016). The sensitive analysis techniques for DNA (e.g., quantitative polymerase chain reaction (qPCR)) allows for a single molecule to be detected. Therefore, even though DNA can undergo considerable dilution, the DNA signal can be detected at very low concentrations, allowing it to be a very sensitive indicator (Liao et al., 2018).

Tracers studies may help to understand the fate and transport of stream solutes, such as nutrients, organic compounds, and environmental DNA (eDNA) (Shogren et al., 2017). Environmental DNA (eDNA) is an emerging approach to assess the biodiversity of a system using samples from the environment rather than the organism itself. eDNA is any DNA extracted from environmental samples such as air, water, or sediment and originates from the decomposition of organisms, shedding of epidermal cells, or bodily secretions (blood, gamete, urine, mucous) (Cristescu & Hebert, 2018; Shogren et al., 2017). Although eDNA can be in various physical forms (e.g., particles, colloids, and free fragments) it is generally considered to be easily transported through water (Cristescu & Hebert, 2018). Analyzing the transport of naked DNA (not attached to anything) may be a good surrogate with many advantages over traditional approaches and help to better understand the pathways and cycling of eDNA (or at least specific components) and other solutes in streams (Abbott et al., 2016). eDNA is a substance in streams that can be used by biologists and ecologists to derive critical understanding of the natural system. DNA tracers represent a specific tracer that is related to some components of eDNA and may help to better understand its fate and distribution in the environment. Although the fate of DNA has not been fully explored in streams it represents considerable promise as an environmental tracer. In this project, a case study assesses the fate of two naked DNA tracers and their potential as hydrological tracers.

The headwater portion of Washington Creek (Grand River watershed in southern Ontario) is a cold-water stream that has been a site of multiple eDNA studies focused on brook trout (*Salvelinus fontinalis*). Understanding how DNA travels at this site may inform eDNA study design, sampling, and interpretation. The sensitivity of the brook trout does not allow for the use of conventional tracers such as salts. A proof-of-concept for the use of DNA tracers in these types of critical stream habitats would greatly enhance our understanding and enable the application of future tracer studies to be applied to investigate a variety of environmental processes.

1.1. Research objectives

The objectives of this research were to:

1. Assess the potential for synthetic free/naked DNA to be used as an environmental tracer (proof-of-concept) in a small headwater stream;
2. Design several unique DNA sequences and test their detection and uniqueness in water samples from a small headwater stream for potential use in future environmental stream tracer studies.

2. Background

2.1. DNA in the natural environment

The basic function of any living organism is defined by the genetic information stored within their cells' deoxyribonucleic acid (DNA). DNA are molecules made up of a phosphate-deoxyribose backbone and four nitrogenous bases: guanine (G), cytosine (C), adenine (A), and thymine (T). Naturally occurring DNA is typically found as a double helix made up of two complementary strands bound together by hydrogen bonding. The strands are connected by the complementary base pairs: G and C, and A and T. The sequence of bases in DNA provide specific information to the cell to define its function (Davis et al., 1986). Environmental DNA (eDNA) is any genetic material shed from organisms into the environment and is used as a proxy for assessing ecological diversity. Non-invasive environmental samples collected in natural surface water environments can contain eDNA, which can provide useful site information about the biological communities. For instance, eDNA could be applied to indicate which fish species are most abundant at specific times of the year (Cristescu & Hebert, 2018).

2.2. Stream tracer tests

Stream tracer tests have several purposes in terms of gathering hydrological information to characterize surface water systems. Discharge, stream velocity, and advective and dispersive transport can be characterized and calculated from downstream sampling data (Foppen et al., 2013; A. N. Sharma et al., 2012). Storage zones and exchange rates can be determined through stream tracer data modelling (Foppen et al., 2013). Flow paths can also be studied and mapped (A. N. Sharma et al., 2012). Stream tracer tests can help predict the downstream transport of contaminants such as urban run-off or wastewater effluent. In addition, small scale surface water tracer tests in creeks can be a preliminary step before planning larger scale tracer tests or groundwater tracer tests.

The application of eDNA sampling can be informed by a better understanding of the fate of DNA tracers. Understanding the fate and transport of eDNA will improve the accuracy of their interpretation. In terms of eDNA samples collected from a river, knowing how far downstream eDNA could travel from its source can allow for a better understanding of the site and distribution of the biological signal. There can be a relationship between eDNA quantity and fish abundance (Marjan et al., 2021). However, as the DNA may travel rapidly downstream understanding the fate can help to select representative sites and give a better representation of fish presence and abundance. DNA

tracers can be used as a surrogate for the naked component of eDNA due to the similarity in composition. Multiple DNA tracer tests could be conducted at locations where fish eDNA is collected to compare downstream DNA tracer concentrations and assess which conditions allow for the greatest level of detection. Varying environmental conditions such as increased flow, turbidity in a stream (e.g., after a heavy rainfall or intense snowmelt), UV exposure, or temperature changes throughout the seasons could impact eDNA interpretation. Using DNA tracers to understand the influence of environmental factors could improve eDNA sampling and interpretation.

2.3. DNA as a hydrological tracer in surface water

Tracers are substances that can be used to track the transport and behaviour of components of the natural environment (Liao et al., 2018). Several, studies have shown that naked DNA molecules can be used as a tracer in small stream systems (Foppen et al., 2011, 2013; McCluskey et al., 2021). DNA has also been applied to understand water flow pathways through large surface water systems, such as bedrock and proglacial rivers (Bovolin et al., 2014; Dahlke et al., 2015). Naked DNA is the free form of DNA which is not associated with cellular material or attached to other materials that may create a protective coating or alter its behaviour. In many applications, the DNA tracer is effective, but the properties and fate of the DNA can be controlled by association with other materials. Encapsulating the DNA in nanoparticles is a way of changing and controlling the behaviour of the DNA and allows for additional applications. Encapsulated DNA, where a nanomaterial coats the DNA to prevent degradation and/or adsorption to the surrounding media, has been shown to be a potential and affordable surface water tracer (Pang et al., 2020; A. N. Sharma et al., 2012).

Hydrological tracing is conducted with DNA by releasing it into a water body at an upstream point, then collecting water samples to determine downstream concentrations (Liao et al., 2018). Reported surface water DNA tracer tests (Table 1) have been conducted in the following countries: Netherlands and Belgium (Foppen et al., 2011, 2013), Sweden (Dahlke et al., 2015), Italy (Bovolin et al., 2014), New Zealand (Pang et al., 2020), and the United States of America (McCluskey et al., 2021; A. N. Sharma et al., 2012). There are limited studies on naked DNA tracer tests, and none recorded in Canadian watersheds. Past DNA tracer experiments have generally focused on the temporal distribution and transport downstream (Bovolin et al., 2014; Cristescu & Hebert, 2018; Dahlke et al., 2015; Foppen et al., 2011, 2013). There has been little mention of the ecological influence of DNA tracers to improve eDNA sampling (Barnes & Turner, 2016), and understanding hydrological processes (Foppen et al., 2011), although a recent study utilized eDNA as a tracer to gather

hydrological and biodiversity characteristics of an Alpine catchment (Mächler et al., 2021). Additional research using DNA tracers and eDNA may greatly enhance our understanding of the ecology of aquatic environments.

2.3.1. DNA tracer design

Unique DNA sequences can be designed with an arrangement of bases that do not match any existing DNA in the environment. These sequences can be ordered from suppliers such as Integrated DNA Technologies and Thermo Fisher Scientific. DNA tracers can be single stranded or double stranded and are typically less than 500 base pairs (bp's) long (Liao et al., 2018). Previous stream tracer tests with naked DNA have most frequently involved ssDNA of around 100 bp's (Table 1). Although double stranded DNA (dsDNA) is more robust than single stranded DNA (ssDNA), the adsorption characteristics of the complementary strands may differ (Foppen et al., 2011). The degradation of DNA may depend on the length of the sequence as shorter sequences of less than 100 bp's have been shown to degrade less rapidly than longer sequences of approximately 500 bp's (Bylemans et al., 2018; Shogren et al., 2018). In terms of shearing, which is when the strand of DNA is fragmented by the separation of base pairs due to structural strain, the size of the DNA is irrelevant (Thorstenson et al., 1998). Shearing is important to consider as breakage of DNA could be a source of mass loss during a tracer experiment. Quantitative polymerase chain reaction (qPCR) detection techniques must have enough intact sequence for appropriate analysis (Demeke & Jenkins, 2010).

Table 1. Summary of synthetic DNA tracers from the literature and their applications. qPCR efficiency should range from 90 – 110% for reliable DNA quantification. Note that qPCR amplification efficiency values were not reported for all tracers.

Type	**Length (# bases)**	**qPCR Efficiency (%)**	**Detection method**	**Application**	**Reference**
Naked ssDNA	80	Not reported	SYBR Green dye	Stream tracer	(Foppen et al., 2011)
Naked ssDNA	80	T22: 108 – 119 T23: 89 – 104	Probe	Stream tracer	(Foppen et al., 2013)
Naked ssDNA	80	104	Probe	Bedrock river tracer	(Bovolin et al., 2014)
Naked dsDNA	250	N/A	SYBR Green dye	Stream tracer	(McCluskey et al., 2021)
Encapsulated DNA	100	Not reported	SYBR Green dye	Column test, and stream tracer	(A. N. Sharma et al., 2012)
Naked & encapsulated ssDNA	82 – 102	T4M: 96 T11: 81	SYBR Green dye	Proglacial river tracer	(Dahlke et al., 2015)
Naked ssDNA	72	100	SYBR Green dye	Column test	(Aquilanti et al., 2013)
Encapsulated dsDNA	65 – 120	65 – 106	Not reported	Transport through a fractured rock	(Kittilä et al., 2019)
Encapsulated dsDNA	105	Aerobic effluent: 100 Anoxic effluent: 79	SYBR Green dye	Wastewater tracking	(Grass et al., 2014)
Encapsulated ssDNA	100	Not reported	SYBR Green dye	Septic system tracer	(Georgakakos et al., 2019)
Naked & encapsulated dsDNA	352	96 – 110	SYBR Green dye	Stream and groundwater tracer	(Pang et al., 2020)

2.4. DNA analysis

Synthetic DNA analysis only requires very small volumes of water, on the scale of mL's, to be collected. These water samples must be preserved by freezing (or immediately extracted) to prevent degradation (Dahlke et al., 2015). When DNA is encapsulated, a washing/extraction step is incorporated to release the DNA from its protective coating (Grass et al., 2014). If water samples contain high levels of humic acids (degraded plant matter), qPCR readings could be inhibited, and dilution steps may be required. Once all washing and diluting steps have been completed, water samples are assayed using qPCR. qPCR will amplify (replicate) specific sequences of DNA through multiple thermal cycles (Cq) with the target DNA doubling through each cycle. The resulting curves are compared to a standard curve under the same conditions to determine the starting quantity in the sample. The quantity is determined through a fluorescent signal where the amount of fluorescence is proportional to the amount of target DNA detected. In most tracer studies, the DNA is added at relatively high concentrations hence a concentration step may not be required. In contrast, eDNA requires collection of higher volumes, as well as additional filtration and extraction steps to concentrate and prepare the DNA for qPCR analysis (Baldigo et al., 2017; Larson et al., 2017; Smart et al., 2015).

2.5. Challenges and limitations

The use of DNA tracers has increased over the past 20 years due to advancements in molecular biology. Obtaining synthetic DNA and analyzing it has become less time consuming in recent years. However, the novelty of the technology means there still remain gaps in knowledge such as the sources of mass loss and predicting its behaviour in various environments (Liao et al., 2018). Other major limitations are the challenge of handling, sampling, caring for samples, and analyzing the DNA tracer. Analysis for DNA tracers is also relatively expensive, time consuming, and requires specialized training and instrumentation. Although, qPCR is very sensitive and allows for multiple tracers (e.g., sequences) it can not currently be applied in real time.

2.5.1. DNA mass losses and water quality

The sources of apparent mass loss (e.g., degradation or adsorption) in the stream are important to consider when using naked DNA as a tracer (Foppen et al., 2011). eDNA degradation appears to decrease in cold water, therefore mass loss of naked DNA is likely decreased in cool groundwater fed streams (Lance et al., 2017; Nevers et al., 2018). The warmer conditions may increase the activity of

microbes and extracellular nucleases. Field and lab studies led to the conclusion that naked DNA is unlikely to be available for more than 18 to 32 hours after being released into surface water (McCluskey et al., 2021). The persistence of a DNA tracer in a stream system is likely shorter compared to eDNA, where the DNA is often protected by inclusion in cells and tissue, or association with particles. eDNA may persist for a few days to a couple weeks in a stream system (Cristescu & Hebert, 2018; Dejean et al., 2011; Piaggio et al., 2013; Thomsen et al., 2011). In a batch adsorption experiment with DNA, where the DNA was expected to degrade by microbial breakdown and/or photodegradation, no degradation was detected (Foppen et al., 2013). Water quality may therefore be an important consideration for both tracer studies and eDNA biomonitoring.

The detection of DNA may be adversely affected by the presence of qPCR inhibitors in the sample. Natural organic matter is an example of a substance that may inhibit DNA detection (Foppen et al., 2013; Shogren et al., 2016). Conducting eDNA collections after a physical disruption or hydrologic event may result in high loads of organics in the stream that have the potential to introduce inhibitors into the DNA analysis. For eDNA analysis, where large volumes of water must be filtered and extracted, the presence of inhibitors means the sample may need to be diluted or cleaned prior to qPCR. Inhibitors could be held on filters and their presence becomes more concentrated. However, by using small sample volumes (µLs) in DNA tracer studies, inhibition is likely to be minimal and less of a factor (although this must be tested) due to a decreased concentration of inhibitors.

2.5.2. DNA and surrounding sediments

Batch tests have shown how sediment characteristics will affect apparent mass loss of DNA (Aquilanti et al., 2013; Foppen et al., 2013; Gardner & Gunsch, 2017; Grass et al., 2014; Schmidt & Martínez, 2017). Sediment may be suspended in the water column of surface water during times of high flow. Adsorption of DNA onto suspended particles could alter their fate and their apparent concentration depending on the method used to isolate the DNA. In addition, sorption to organic matter/particles may result in the need to extract the samples prior to qPCR analysis. DNA tracing in a proglacial stream with high sediment loads showed significant mass losses, however DNA was still detectable (Dahlke et al., 2015).

3. Methods

3.1. Designing and selecting synthetic DNA

Single stranded DNA (ssDNA) was selected over double stranded DNA because ssDNA is less expensive, more widely used in the literature as a tracer, and avoids potential differences in behaviour of complementary DNA strands. The initial step of DNA design was carried out with assistance from Dr. Michael Lynch of the Biology Department at the University of Waterloo. DNA sequence design began with the generation of a random DNA sequence using an online software (https://faculty.ucr.edu/~mmaduro/random.htm), with the guanine-cytosine (GC) content set to 50%. The concentration of these bases dictates the stability and potential for primer dimer (secondary structure) formation. The probability of secondary structures was then verified for the DNA sequences through an online application (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Primer sequences for each target sequence were designed using Integrated DNA Technology's (IDT) online tool (https://www.idtdna.com/Primerquest/Home/Index). Throughout the design process, the sequences were occasionally modified by adding or removing base pairs to ensure a low probability of secondary structures and to design primers with melting temperatures ranging between 50 and 60°C. Optimally designed forward and reverse primer sets include similar melting temperatures, G's and C's at each end to promote binding, and GC content ranging from 35 - 60%. Finally, the alignment of each of the DNA's was verified to ensure each target DNA sequence would not interfere with naturally occurring DNA in the environment or with the other target DNA sequences. The target sequences were run through the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information's Nucleotide Primer-BLAST Tool database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) for any known matches with organisms and no matches were found. Table 2 lists the selected synthetic DNA sequences with forward and reverse primer regions bolded. Ten sequences were initially designed (Table A - 1) however only four were selected (Table 2) due to reasons which are further explained in the Appendix.

In addition to designing unique sequences at the University of Waterloo, two sequences were also ordered based on surface water studies that were conducted and reported in the literature (e.g., Table 1). Table 3 lists sequences from the literature that were ordered. These sequences (target, forward primer, reverse primer) were also run through BLAST to verify the sequences do not match with anything naturally present in the environment (e.g., Washington Creek). All DNA sequences and

primers listed in Table 2 and Table 3 were ordered from Thermo Fisher Scientific (www.thermofisher.com).

Table 2. Synthetic DNA sequences designed at the University of Waterloo showing forward and reverse primer regions (bolded), GC content (%), and sequence length in base pairs (bp).

Tracer	DNA Oligo sequence (5' to 3')	Length	GC (%)
S2	AT**CTCCTGGAGATCAAGGAAATGT**TTCTTGTCCAA GCGGACAGCGGTTCTACGGAATGGAGGCCATAT**TCT ACGTTACTGCCTGCATAAG**GTC	93	48
S21	ATCATGGCCTG**CACGGCAAATGACGCTTAT**AATGGAC TTCGACATGGCAATAACGCGTACTC**GTTTCTACGTCAG GAGGAGAATAG**TATAAA	92	45
S22	ATC**GGCGGCACGAATAACATACT**CATCTCTATACAT TCTCGACAATCTATCGAGCGAGTCGATTATCAACGG **GTTGTCATGCAGTTTAATCGGG**TTAA	98	43
S28	G**CTGGGTCCTACTGCAGCGGGACTTTCTAA**AGGAG GCGTTGAGAGGAGCAGTCGTCAGACCACATAGC**TTTCA TGTCCTGATCGGAAGG**ATC	92	54

Table 3. DNA sequences and primer sets (bold) selected from the literature for the Washington Creek proof-of-concept DNA tracer test.

Tracer	DNA Oligo sequence (5' to 3')	Source
T11	T**CCCTAAGTGTAAGACCTGAGATCGA**CCTGCAG CACACTGACCTCGGAATTACTGCGAAGAGCA**CGT AGAAAGGGGATGTAAGTTAGCC**T	(Dahlke et al., 2015)
T22	**TAGCGAGGAATGAAGGTCGA**TGATACTTTTAGG CCATCACATTCGAACTCTCCTACCTGT**TTCATAGT ACACAAGAGCGC**	(Foppen et al., 2013)

3.2. Optimizing DNA and primer sets through qPCR

Prior to releasing a DNA tracer into a stream, analysis for quantifying the DNA in water must be validated in the laboratory. All analysis was conducted under a PCR hood and all surfaces were sanitized with ethanol and UV light prior to starting laboratory work. Fisherbrand™ Premium Microcentrifuge Tubes (catalogue #05-408-125 and #05-408-137) and Fisherbrand™ SureOne™

Filter Tip Pipette Tips (catalogue #02-707-474, #02-707-475, and #02-707-480) were utilized throughout analysis. Preliminary studies were conducted to assess the upper and lower concentration limits of the qPCR to the target DNA in a molecular water solution using T11 and T22. Serial dilutions of the DNA sample were also conducted to determine the lowest detection limit for qPCR amplification for these sequences. All primers were optimized by testing the qPCR over a range of temperatures and concentrations to obtain an ideal amplification efficiency for the standard curve. The standard curve was created by a series of standards (target DNA and molecular water) diluted 10-fold $1*10^{-4}$ ng/µL to $1*10^{-10}$ ng/µL. The DNA sequences chosen for the tracer study must contain primer sets that result in qPCR amplification efficiencies ranging from 90 to 110% and must not amplify naturally present DNA at the study sites. Each target DNA's amplification was initially prepared using BioRad's SsoFast™ EvaGreen Supermix (catalogue #1725200). Table 4 outlines the contents of each PCR well for dye-based qPCR reactions.

Table 4. Contents of a 20 µl well for qPCR using SsoFast™ EvaGreen Supermix.

Component	**Volume (µl)**
SsoFast™ EvaGreen Supermix	10
Forward Primer Mix	2.5
Reverse Primer Mix	2.5
Sample (DNA or Molecular Grade Water for blanks)	5

The qPCR methods were determined through considerable lab testing and modification of conditions. In total, 10 DNA sequences were designed and assessed for their ability to be optimized, but only 4 were selected as suitable tracers. The criteria for selection were based on acceptable amplification efficiencies, melt-curve analysis showing a single product, and no amplification of non-template controls (NTCs) or environmental blanks. The selected tracers and their optimal qPCR cycling conditions are listed in Table 5. qPCR cycling steps are outlined in Table 6. Further details with regards to qPCR optimization are outlined in the Appendix.

Table 5. Optimal forward and reverse primer concentrations for selected synthetic DNA tracers (T11, T22, S2, S21, S22, and S28) in the BioRad C1000 Touch Thermocycler machine using SsoFast™ EvaGreen Supermix.

DNA Tracer	Forward primer concentration (nM)	Reverse primer concentration (nM)	Annealing & extension temperature (°C)	Standard curve efficiency (%)
T11	600	600	60	92
T22	300	300	62	91
S2	700	900	55	92
S21	500	500	67	98
S22	500	500	62	90
S28	700	700	57	92

Table 6. qPCR cycling protocols optimized for T11 with the SsoFast™ EvaGreen Supermix dye.

Cycling step	Temperature (°C)	Time	# Cycles
Enzyme activation	98	30 sec	1
Denaturation	95	5 sec	40
Annealing / extension	Variable, see Table 5	10 sec	
Melt curve	65 – 95	5 sec/step	1

Amplification efficiencies for the DNA assays were determined by creating standard curves with DNA spiked into molecular grade water using the Bio-Rad CFX Maestro 1.1 software. Reaction efficiency is determined by:

$$E = \left(10^{-\frac{1}{s}} - 1\right) \cdot 100\% \quad (1)$$

where E is efficiency in %, and s is the slope of the standard curve. The standard curve is the best fit line between the log transformed starting quantity of the first standard and the qPCR quantitation cycle (Cq) (Foppen et al., 2013).

3.3. Case study: Washington Creek DNA tracer test

A field tracer test was conducted in the fall on October 17th, 2019, at Washington Creek (43°20'21"N 80°36'8"W). The field site has an elevation of 319 m and is in the Grand River watershed of southern Ontario (Figure 1). The tracer study was conducted in the headwater portion of Washington Creek.

This section of the creek is mostly groundwater fed and has been a site for multiple brook trout population studies conducted by Trout Unlimited and the University of Waterloo's Biology Department. Approximately 80 m downstream of the tracer release point is Bridge St. where the creek must pass through a culvert and under the road (Figure 2). The creek bed is hard packed with highly permeable sediments ranging from sand to cobble (Figure 3). Watercress (*Nasturtium officinale*) is abundant along the stream channel (Figure 4). The upper portion of the tracer site, upstream of Bridge St., is open and dominated by grasses and sedges with only a few trees providing shade above the creek. The creek is generally narrower along this upstream stretch and most of the shade above the creek is near the bank of the creek and provided by overgrown grasses and shrubs. The downstream portion of the creek, south of Bridge St., is in a wooded area with evergreen and deciduous trees. The tree canopies provide significant shade above the creek (Figure 5).

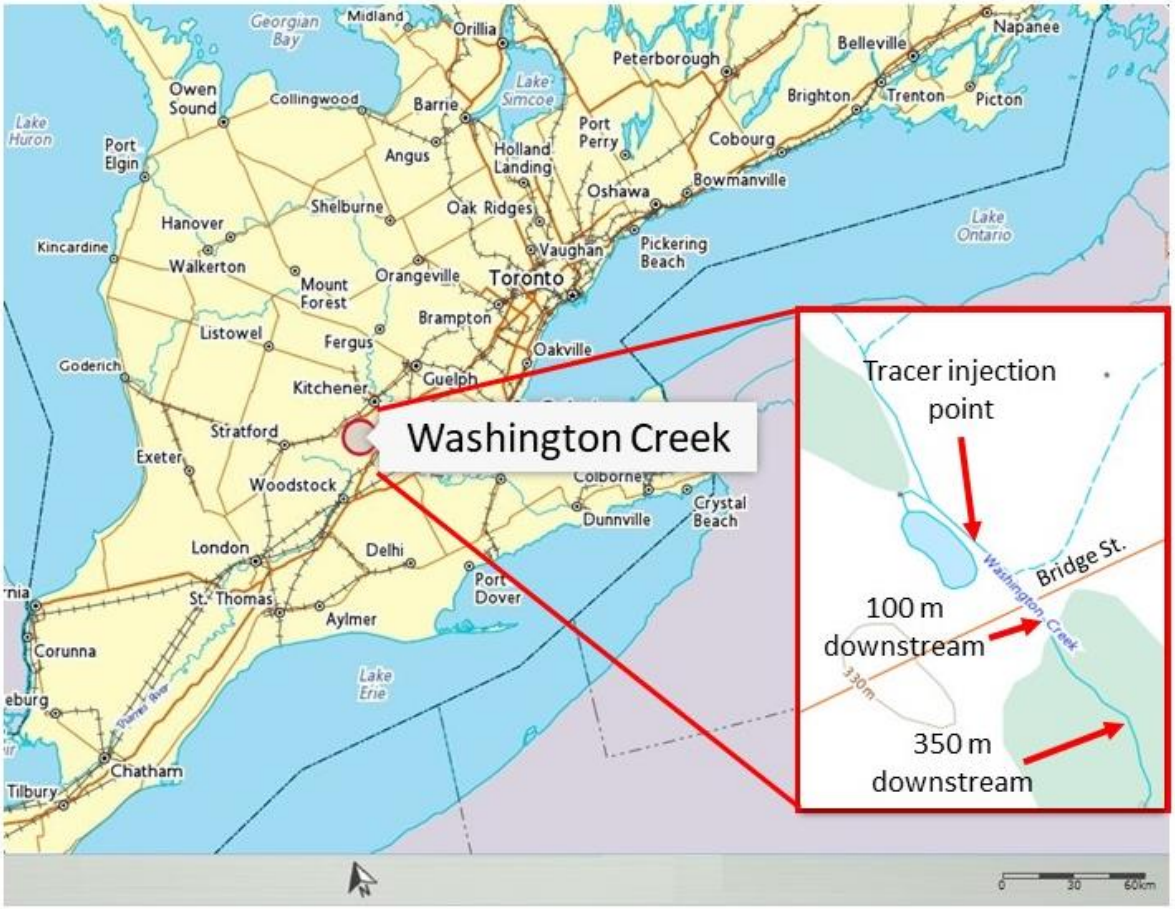

Figure 1. Washington Creek in the Grand River watershed of southern Ontario is where the DNA tracer test was conducted. The tracer release point as well as the downstream sampling sites are indicated on the map. The map data was obtained from Toparama (https://atlas.gc.ca/toporama/en/index.html).

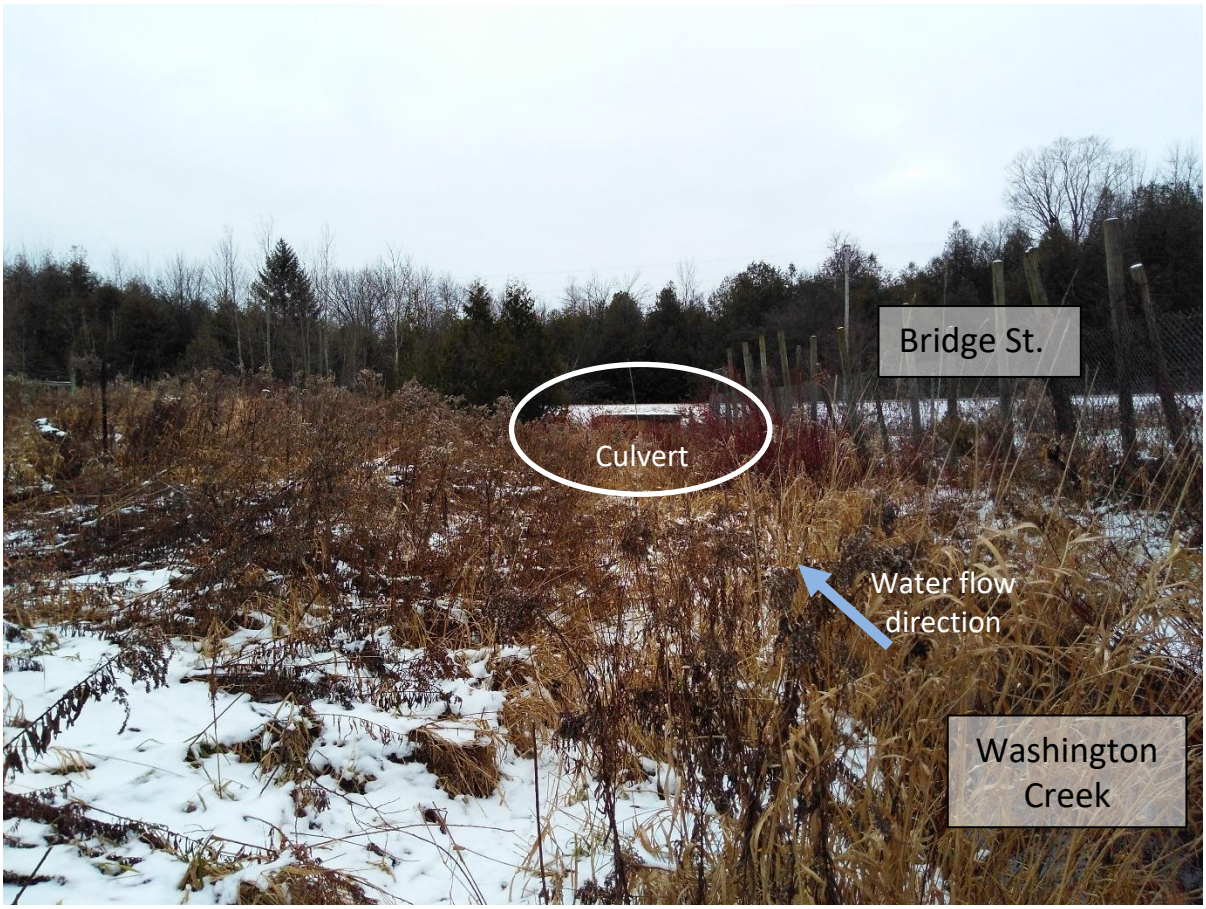

Figure 2. Photo from December 2018 of the Washington field site. The photo was taken from approximately 50 m downstream of the tracer release point and faces downstream toward the wooded areas where water sampling occurred. Approximately 80 m downstream of the release point is a culvert (circled) which passes under Bridge St.

Figure 3. Hardpacked streambed with mostly cobble sized material surrounded by some sand. This photo was taken near the upper reach of the stream close to the tracer release point with fewer trees and shade cover. Most of the sediments in the streambed are dark grey, black, and reddish-brown in colour.

Figure 4. Photo of Washington Creek from November 2018 approximately 90 m downstream of the tracer release point showing the presence of watercress (*Nasturtium officinale*) vegetation (circled) along the stream channel. Watercress is a strong indicator of groundwater seepage (Nichols & Shaw, 2002).

Figure 5. Washington Creek has been restored in some areas including meanders where engineered riparian zones (circled) have been built. These areas are composed of sticks, logs, and stakes to restore fish habitat and prevent erosion.

Two naked DNA tracers (T11, T22) were injected into the headwater of Washington Creek and followed at two downstream sites. Both tracers were obtained from the literature and used in naked DNA surface water tracer studies conducted in Europe (Dahlke et al., 2015; Foppen et al., 2013). The tracers were already named by their creators and their original names are used in this study. A concentrated solution (100 µM) of naked DNA was suspended in 100 mL of MilliQ water and triplicate samples were taken to validate the initial injected mass. The suspended tracer solution was released on the surface of the water in the middle of the stream over approximately 2 s at the "Injection Point" (Figure 1). The first downstream site was approximately 100 m downstream of the injection point and the second site was approximately 350 m downstream. The approximate dimensions for each downstream sampling site and the upstream injection point were measured in the field and are illustrated in Figure 6. Wooden boards were set up across the creek at two locations to

allow for sampling along the width of the stream (Figure 7). The boards allowed sampling without people stepping into the upstream stream sites, thus preventing sediment disruption and mobilization of sediment into the water column that could potentially affect DNA detection at downstream sites.

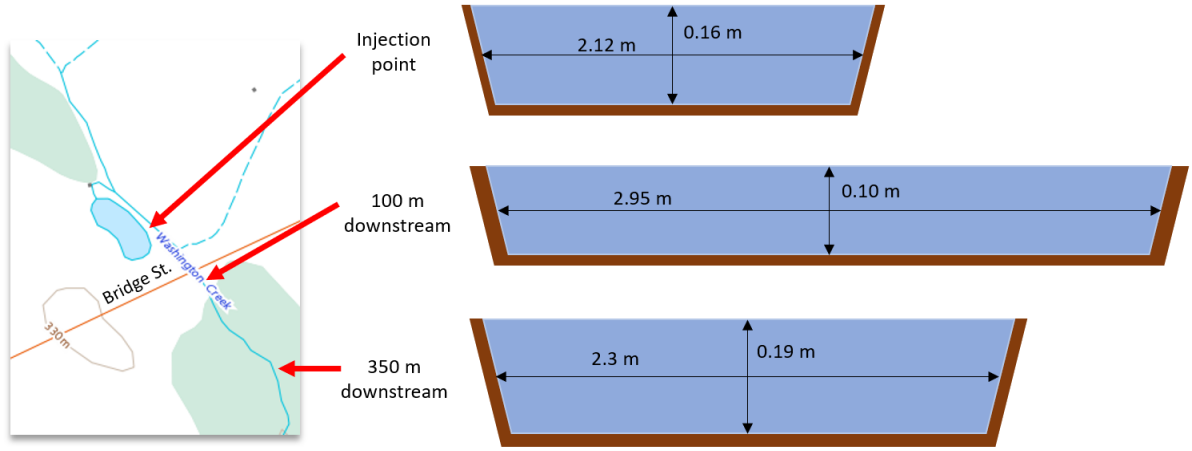

Figure 6. Average channel dimensions for each of the downstream sampling sites and the injection point. Diagrams are not to scale. The map was modified from Toparama (https://atlas.gc.ca/toporama/en/index.html).

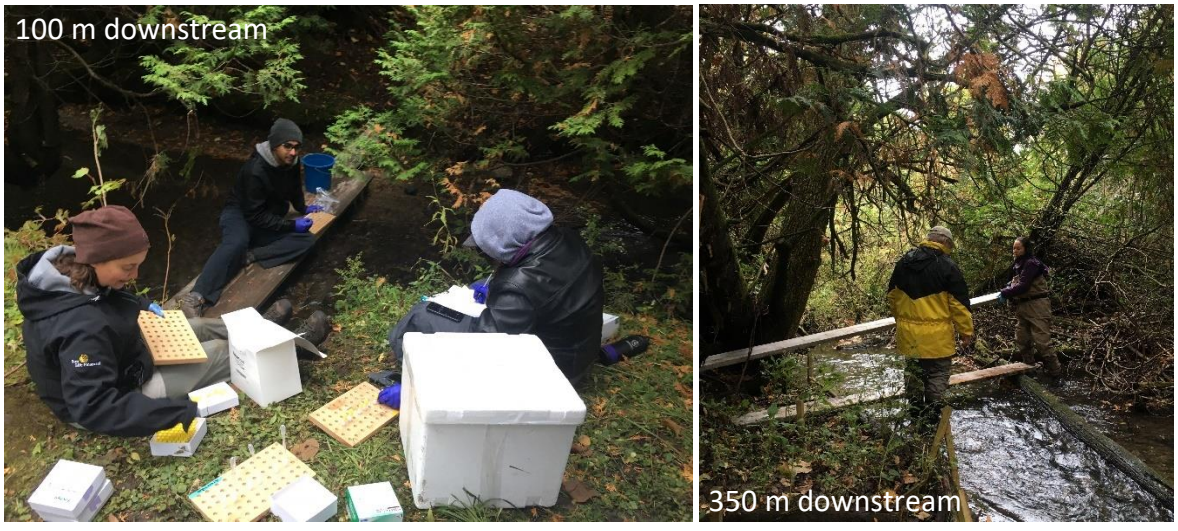

Figure 7. Wooden boards were set up at each sampling site to prevent disruption of the streambed during the tracer test. Depicted are the Downstream 1 (100 m downstream, left) sampling site and Downstream 2 (350 m downstream, right).

Water samples were collected just below the surface, within approximately 5 cm from the top of the water's surface at the centre and halfway to each bank (Figure 8). Duplicate 1.5 mL water samples were obtained using clean disposable pipettes (Fisherbrand™ Blood Bank Disposable Transfer Pipettes, Catalogue #13-711-5AM) where a single squeeze draws up to 2.4 mL. Samples were taken every minute for a total of 10 min before the injection, every minute for 30 min after the injection, then every 5 min for another 20 min. This timeframe was calculated by going to the field site the day before and getting a rough estimate of the stream's volumetric flow. A rough estimate of stream flow (approximately 120 L/s) was obtained using an orange as a float instead of measuring velocity directly in the creek with an apparatus. Using a current velocity meter would involve stepping in the streambed and this was to be avoided within the few days before the experiment as undisturbed conditions were hoped to be maintained for the upcoming tracer test. Stream flow was measured with a float. A similar surface water tracer test conducted by Foppen et al. (2013) in a brook with a discharge of 89 L/s reported a tracer peak arrival time of about 17 min at the 300 m downstream sampling site. Based on the crude discharge calculated and comparison to surface water tracer tests from the literature, a conservative estimate was made for how far downstream and where to sample. Water samples were placed into microcentrifuge tubes (Sigma Aldrich, catalogue # DWKW985864) then immediately placed onto dry ice. Samples were also obtained from upstream of the injection point to verify nothing naturally present in the system would be amplified by the primer set. In total, 485 water samples were obtained from the preliminary tracer experiment and all samples were stored at -80°C.

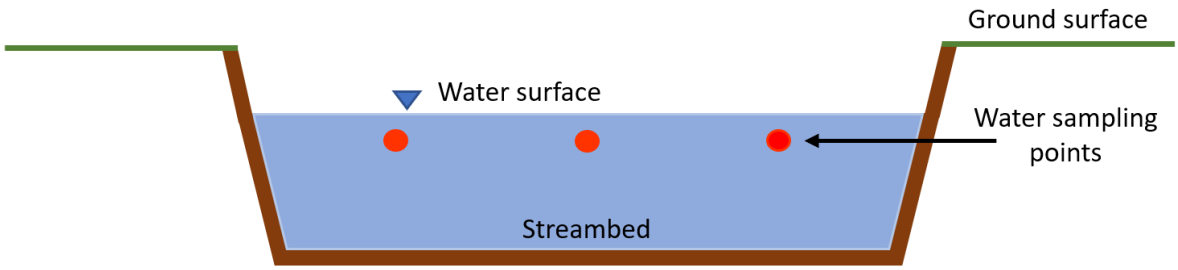

Figure 8. Cross section of three water sampling locations in the stream equally distributed across each downstream location of the Washington Creek field site.

After the tracer experiment, dissolved oxygen (DO), temperature, specific conductance, conductivity, and total dissolved solids were measured with a YSI Professional Plus handheld multiparameter meter. Turbidity data was obtained with a LaMotte 2020i portable turbidity meter.

Water quality was sampled at each downstream site and the injection point. Stream discharge was obtained by measuring depth and flow at three locations across the stream width. Flow measurements were obtained at the injection point, Downstream 1, and Downstream 2.

Two negative controls were used: a “transfer blank” and a “trip blank”. Three transfer blanks (done in the field) were taken at each downstream sampling site at the start of the tracer test as well as at 1 min, 30 min, and 50 min after the tracer injection. The transfer blank involved pipetting MilliQ water from one vial to another to test for potential contamination of target DNA during sampling. The trip blank was transported between the field and lab to verify contamination of vials did not occur. Handling of equipment was conducted with clean gloves and gloves were changed frequently to avoid contamination.

A salt tracer test was not conducted at Washington Creek as it is a cold-water creek with sensitive species, such as rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). If a salt tracer test were to have been conducted, the amount of salt required to be above the detection limit of the conductivity meter would be close to the LC50 of chloride for fish. Although the salt plume would be passing quite quickly, this was not a risk that was acceptable at this site. Permission to conduct the tracer test was granted by the Ontario Ministry of the Environment, Conservation and Parks (MECP), reference number 7575-BDGPMB (MECP). Email communication was carried out with Jacqueline Lamport (jacqueline.lamport@ontario.ca, 519-240-4327) and Dana Mohammed (dana.mohammed@ontario.ca, 519-820-3083) from the MECP. Written permission was granted by Mili New, the director of the West Central Region of the MECP.

3.4. Water sample analysis by qPCR

3.4.1. Analyzing tracer test samples

DNA quantification of field tracer samples was conducted through the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) according to conditions outlined in Table 4, Table 5, and Table 6 in section 3.2. Each qPCR plate included at least 6 no template controls (NTCs) and a 7-point standard curve. The purpose of the NTCs was to show if any contamination may have occurred during plate preparation and to demonstrate the effects of random amplification (noise). The standard curve was created with target DNA diluted 10-fold with field water samples to account for any environmental variability introduced by creek water. Before choosing to use creek water, a comparison of standard curves for molecular water versus creek water was conducted to determine any differences (Figure 9).

Although the standard curves shown are below 90%, the standard curve utilized for quantifying field samples was within an acceptable range (Figure A - 6 and Figure A - 7). Cq is the quantification cycle (also called the Ct, threshold cycle) that represents the thermal cycle in which fluorescence was detected at a set threshold by the qPCR instrument. Cq is proportional to the amount of DNA in the sample and is quantified by comparison to the standard curve. The Cq values were similar however a slight shift toward a higher Cq and earlier amplification was noted for creek water (Table 7) (Thermo Fisher Scientific, 2021).

Table 7. Comparison of the Cq of a standard curve of molecular water and creek water for T11.

	Starting concentration (ng/uL)	**Molecular water**	**Creek water (Downstream 1)**	**Creek water (Downstream 2)**
Standard 1	$1*10^{-4}$	16.24	16.59	16.36
Standard 2	$1*10^{-5}$	21.25	20.51	20.42
Standard 3	$1*10^{-6}$	25.25	24.05	24.24
Standard 4	$1*10^{-7}$	28.98	28.26	28.37
Standard 5	$1*10^{-8}$	32.96	32.17	32.15
Standard 6	$1*10^{-9}$	37.14	36.42	36.51

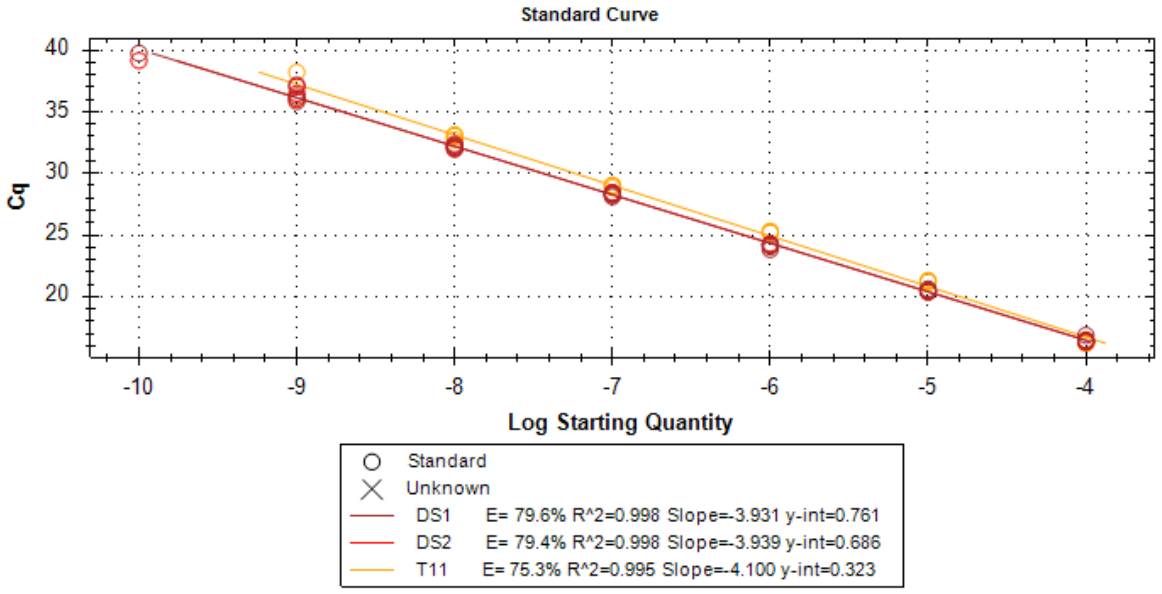

Figure 9. Standard curves for T11 spiked into molecular water, Downstream 1 creek water, and Downstream 2 creek water.

3.4.2. Testing new DNA tracers with creek water

In November 2020, Washington Creek water samples were obtained and synthetic DNA (S2, S21, S22, S28) was spiked into them to assess if the primers detected any inhibition of the qPCR (Figure 10 to Figure 13). The four selected sets of primers had acceptable standard curves with efficiencies between 90 – 100% and a single product (melt curve). Negative controls were included when analyzing each target DNA to determine if the primers would amplify any naturally present DNA in Washington Creek. The negative control samples did not appear to detect any naturally present DNA in Washington Creek water samples.

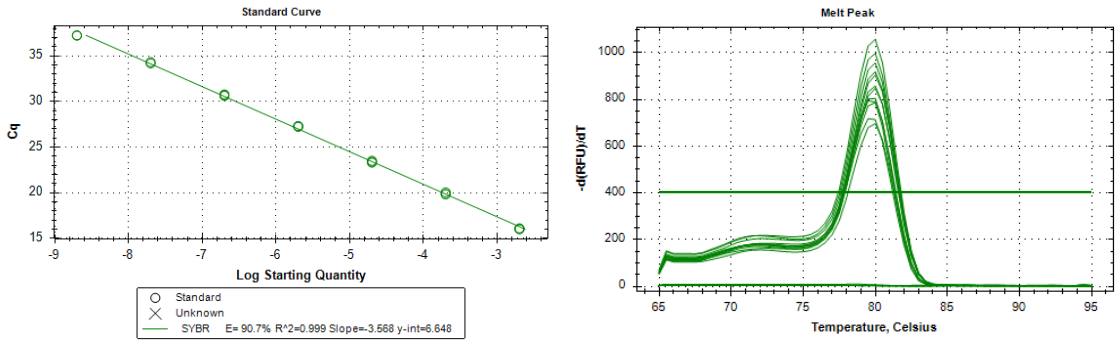

Figure 10. Left: Ten-fold dilution standard curve for S2 which had an efficiency (E) of 90.7%; Right: Melt-curve output for S2 showing a melt-temperature range of 79.5 – 80.0°C

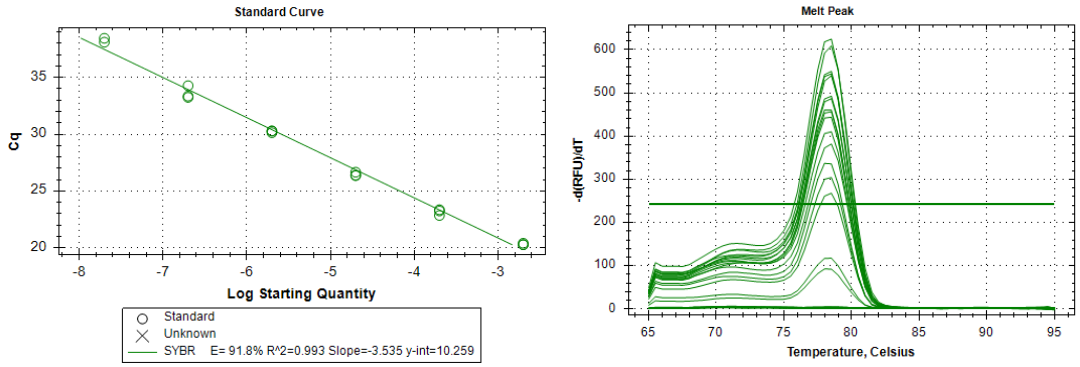

Figure 11. Left: Ten-fold dilution standard curve for S21 which had an efficiency (E) of 91.8%; Right: Melt-curve output for S21 showing a melt-temperature of 78.5°C.

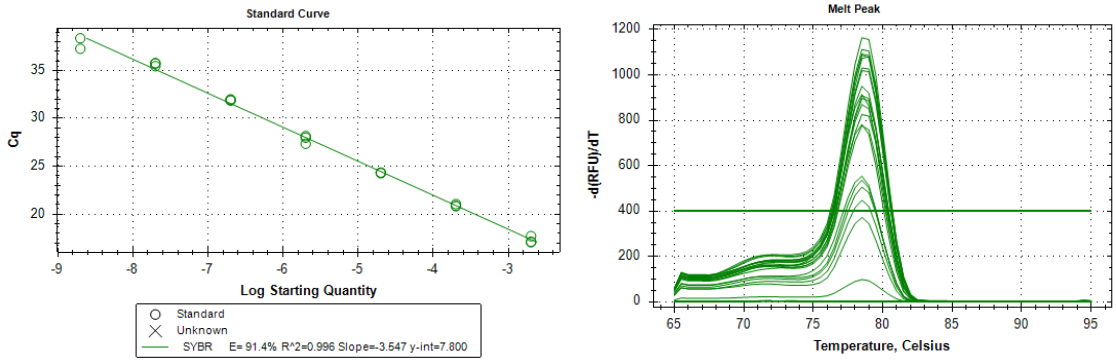

Figure 12. Left: Ten-fold dilution standard curve for S22 which had an efficiency (E) of 91.4%; Right: Melt-curve output for S22 showing a melt-temperature range of 78.5 – 79.0°C.

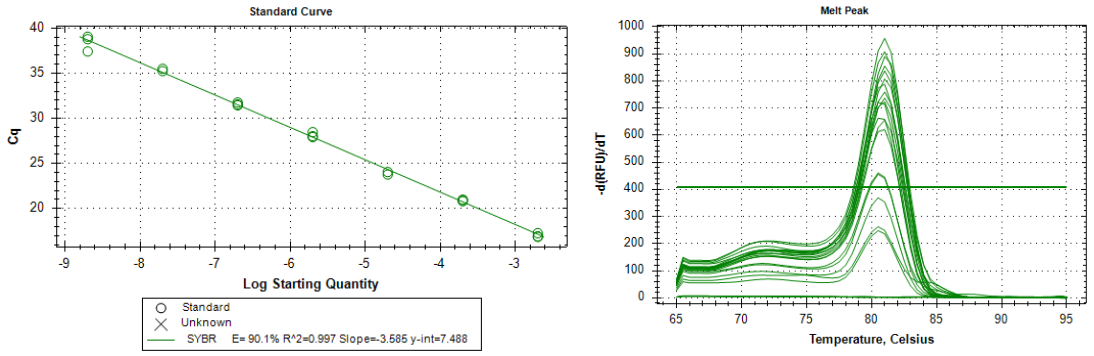

Figure 13. Left: Ten-fold dilution standard curve for S28 which had an efficiency (E) of 90.1%; Right: Melt-curve output for S28 showing a melt-temperature 81.0°C.

3.5. Creating breakthrough curves with water sample data

Breakthrough curves (BTCs) of relative concentration (C/C_0) versus time were plotted for each target DNA at each sampling site (Figure 15, Figure 16). The concentration (C) of each sample was determined by comparing the sample's qPCR Cq to the average of all standard curves. C_0 was the theoretical concentration of the tracer at the injection point in the creek, assuming 2 s of dilution and immediate dispersion. Each qPCR plate included a standard curve, however, to account for inter-plate variability, an average standard curve was created and applied for quantifying T11 (Figure A - 6) and T22 (Figure A - 7) field samples. The injected mass, as measured from triplicate samples taken from the concentrated tracer solution was 9,980 ng for T11 (approximately 10^{13} copies) and 13,470 ng for T22 (approximately 10^{13} copies). The triplicate samples were diluted 1000-fold, analyzed through

qPCR, then quantified using a standard curve. The equation below outlines how the approximate number of copies is calculated (Prediger, 2017):

$$number\ of\ copies\ (molecules) = \frac{X\ ng * 6.0221 * 10^{23} \frac{molecules}{mole}}{\left(N * 330 \frac{g}{mole}\right) * 1 * 10^{9} \frac{ng}{g}} \quad (2)$$

The number of copies is in terms of molecules, *X* is the amount of DNA in ng, and *N* is the length of the ssDNA amplicon. 330 g/mole is the average mass of 1 base pair of ssDNA. C_0 was calculated using the dilution formula:

$$C_1 V_1 = C_2 V_2 \quad (3)$$

where C_1 and V_1 are the concentration and volume, respectively, of the 100 mL concentrated tracer solution that was injected into the creek. C_2 and V_2 are the concentration and volume, respectively, in the creek. The calculated C_2 from the equation is the C_0 value utilized in the BTCs. The measured flow at the upstream site where the tracer was released was 106 L/s. It took two seconds to pour the tracer into the creek and this resulted in a dilution volume of 212 L. This volume was obtained by multiplying 106 L/s by 2s to give 212 L. Immediate and complete mixing across the creek was assumed. Using the starting concentration values in the 100 mL starting solution for T11 ($9.98*10^{-2}$ ng/µL) and T22 ($1.35*10^{-1}$ ng/µL) tracers, their C_0 values were determined: T11 ($4.71*10^{-5}$ ng/µL) and T22 ($6.36*10^{-5}$ ng/µL). The y-axis values for the BTC are the concentration of DNA in the sample over C_0. The y-axis values are relative concentration and are unitless. BTC relative concentration values (C/C_0) were averaged for each pair of duplicates to create one BTC for each sampling site. Reporting averages of duplicate concentration values was used in previous tracer tests in the literature (Georgakakos et al., 2019). The differences between duplicates are further illustrated in Table A - 3, Table A - 4, Table A - 5 of the Appendix which shows absolute concentration. A 30% difference in relative concentration was noted between some duplicates therefore averaging provided a clearer pattern of the tracers' downstream transport. The differences between duplicates were concluded to be because of variability between qPCR runs. Due to the number of samples and limitation of number of reactions for PCR plate (maximum of 96 reactions), field sample duplicates were included on separate plates. Some duplicates were excluded because of degradation caused by an extra freeze-thaw cycle, or if the samples showed signs of contamination through NTCs that amplified. Samples that did not meet QA/QC criteria were not included. Additional details with regards to which samples were excluded are further elaborated upon in the Appendix.

3.6. Breakthrough curve analysis

3.6.1. Calculating mass flux at each sampling site

The mass flux at each sampling site is determined by analyzing the downstream breakthrough curves (BTCs). At each downstream sampling site, sampling was conducted at three time points therefore three separate breakthrough curves were created. Each downstream sampling site was subdivided into three segments according to sampling. The area for each segment is the sum of the depth and width in m^2 (Table 8 and Table 9). Flow velocity was obtained from a 60% depth (Genç et al., 2015), and measured with a flow meter in m/s (Swoffer model 3000). It was assumed that discharge (volumetric flow) was constant and uniform throughout each stream width segment during the tracer test. Mass recovery (M_R) for each tracer was determined for each sampling site to assess the reliability of the tracer. M_R is a product of the area under the BTC and is given by:

$$M_R = \int_{t=0}^{\infty} (Q * C)\, dt \quad (4)$$

where Q is volumetric flow in L/s, C is concentration in ng/µL, and (Q*C) is flux (Goldscheider et al., 2008). Discharge (m^3/s) was calculated for each of the three sections at each downstream sampling site (Table 8). Discharge is calculated by multiplying flow velocity (m/s) by the depth (m) and width (m) of the sampling segment's cross section. The residence time of the tracer and the boundary time points for Equation 4 were chosen based on when sample concentrations exceeded background levels. Residence time is the time range in which the tracer was detected above background levels and is essentially the width of the BTC along the x-axis (time) (Figure 14). The boundary time points define the upper- and lower-time limits of the residence time. The left boundary time point is when the tracer was first detected above background amplification noise at each downstream sampling site. The right boundary time point of the BTC is when the tracer plume has passed the sampling site and amplification has returned to background levels. The method for determining the area under the BTC to determine mass recovery (M_R) at each sampling segment is trapezoidal integration. Trapezoidal integration approximates area by subdividing the region under the BTC into trapezoids connected by two adjacent sampling points, then calculating the sum area of all trapezoids. About 15 trapezoids were used on average because the average residence time for the tracer mass at each sampling point was about 15 minutes. The number of trapezoids represents the precision of the approximated area and depends on the sampling frequency. Each field sample represents a time point and for every minute (sampling frequency), there is an associated mass (area

of one trapezoid). Each trapezoid represents the mass at that time point. When the masses from all time points are determined, the individual masses from each trapezoid are summed and the resulting sum is the mass flux of that segment. The mass flux for each of the three segments were added together to obtain the total mass flux for each downstream sampling site. The percent of mass recovery for downstream sites was determined through a mass balance by dividing the starting mass of the tracer released upstream by the mass estimate at each downstream sampling site. This mass balance method assumes conservation of mass and is not based on tracer volume.

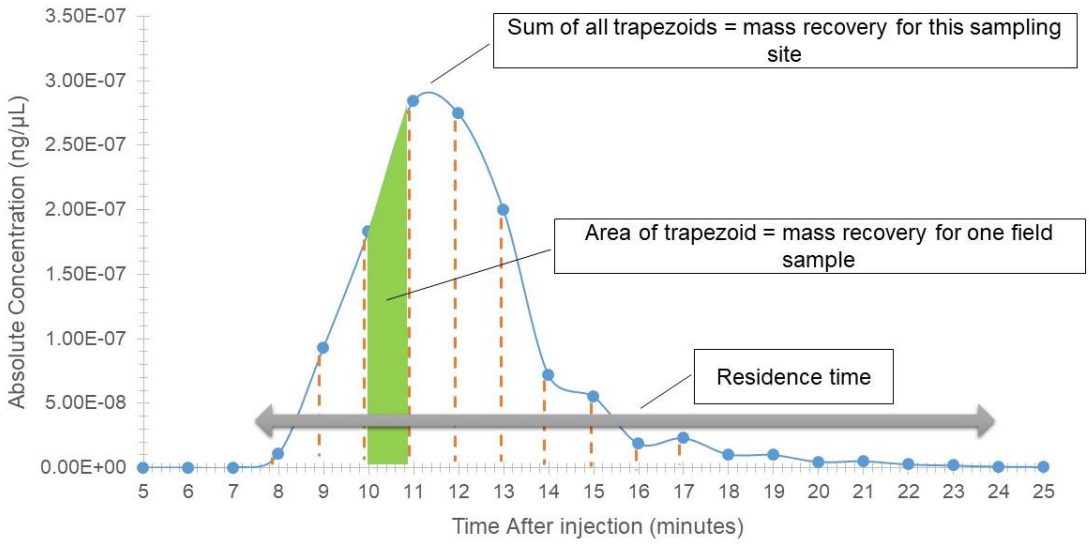

Figure 14. Schematic representing how trapezoidal integration was used to determine mass recovery for each downstream sampling site. Illustrated is a sample breakthrough curve where each data point represents a water sample that was obtained.

3.6.2. Calculating downstream arrival time

Because a conservative tracer (e.g., salt) was not released into Washington Creek, a theoretical peak arrival time of the centroid (centre of mass) of the tracer was determined assuming travel purely by advection (Augustine et al., 2020). The centre of mass for each curve was calculated as the average residence time of the tracer mass. Average residence time was calculated between when the tracer was first detected at the site and when it was no longer above the detection limit. The theoretical arrival time of a tracer (e.g., salt) was determined by:

$$t = d/v \tag{5}$$

where d is the distance from the injection point to the downstream sampling site in metres, v is the measured velocity in the creek in m/s, and t is time in minutes. Rough estimates for velocity were estimated for each of the left, centre, and right segments of each downstream sampling site as a worst-case estimate. This simple model assumes the creek is essentially a straight pipe with minimal mixing, which of course is not the case.

4. Results

4.1. Pilot scale DNA tracer test

4.1.1. Site conditions

The air temperature at Washington Creek was 9°C upon arriving at the site and the sky was overcast. Water quality was consistent along the reach of the creek where water quality was measured three times in total at the left, centre, and right sections of Downstream 1 (DS1), Downstream 2 (DS2), and the Injection Point (IP). Parameters measured were temperature, specific conductance, conductivity, total dissolved solids, pH, and turbidity (Table 8). A one-way ANOVA was conducted to compare water quality between each site and results are reported as P-values. There were statistically significant ($p \leq 0.05$) differences between sites (IP, DS1, and DS2) for the following parameters: specific conductance, conductivity, and pH. There did not appear to be statistically significant ($p > 0.05$) differences between sites for temperature, total dissolved solids, and turbidity. The turbidity values for DS2 were excluded as they may have been influenced by field crew stepping in the stream. Stream flow and dimensions varied across and between sites (Table 9). Turbidity was measured after the field test so field crew stepping in the stream did not interfere with the field tracer test.

Table 8. Average water quality values from triplicate sampling at Washington Creek on October 17th, 2019.

Parameter	**Injection Point**	**Downstream 1**	**Downstream 2**	**P-value**
Temperature (°C)	9.1	9.0	8.5 ± 0.9	0.4
Specific conductance (µS/cm)	576 ± 0.1	575 ± 0.1	575 ± 0.06	$3*10^{-6}$
Conductivity (µS/cm)	401 ± 0.6	400	398 ± 1	0.03
Total dissolved solids (mg/L)	408	374	408 ± 57	0.4
pH	7.7 ± 0.006	7.8 ± 0.01	7.9 ± 0.01	$4*10^{-6}$
Turbidity (NTU)	2.4 ± 0.6	2.1 ± 0.3		0.6

Table 9. Measured stream dimensions and flow used for volumetric discharge calculations.

	Injection Point			Downstream 1			Downstream 2		
	Left	Centre	Right	Left	Centre	Right	Left	Centre	Right
Depth (cm)	18	15	14	8	9	14	20	20	16
Velocity (m/s)	0.285	0.395	0.281	0.36	0.179	0.348	0.226	0.461	0.261
Total Width (m)		2.12			2.95			2.3	
Mean volumetric flow (L/s)		106			92			137	

4.1.2. Background DNA in Washington Creek

Background water samples of Washington Creek that were collected before the start of the experiment did not contain any naturally occurring DNA that amplified with the target primers. The trip blanks (molecular water transferred in the lab but transported in the coolers) did not show any signs of contamination. The transfer blanks for T11 were free of any contamination. For T22, one out of the three transfer blanks showed some contamination. This transfer blank was conducted at the end of the tracer test at 50 minutes after tracer injection and was at DS2. The contamination of the T22 DS2 transfer blank was equivalent to a concentration of 8.54×10^{-10} ng/µL which was below background amplification noise (Cq > 37). T22 transfer blanks for the IP and DS1 were free of contamination.

4.1.3. Breakthrough curves

The breakthrough curves for T11 and T22 as relative concentration (C/C_0) (y-axis) versus time since injection in minutes (x-axis) are shown in Figure 15 and Figure 16, respectively. The concentrations of each tracer at the same time were similar across the stream (left, right, centre) suggesting the stream was well mixed by the time the tracers reached the first downstream site. The tracer arrived at DS1 at about 7 minutes and the peak was at 11-12 min with a residence time of about 16 min. The tracer arrived at DS2 at 22 – 23 min with a peak at approximately 30 min and a residence time of around 20 min. The peak of the centre of mass was 15 – 16 min for DS1 and 30 – 31 min for DS2 which is much slower than the theoretical predicted values of 4.6 – 9.3 min and 13 – 26 min, respectively (Table 10). The relative concentration declined from 0.006 – 0.008 C/C_o at DS1 to less than 0.002 C/C_o at DS2 for both T11 and T22. The downstream curves were lower and wider and

showed a longer tail. The mass recovery of T11/T22 was estimated at DS1 as 71/80%, and at DS2 as 68/71%, respectively (Figure 17). The sample concentrations show the pattern of a relatively smooth curve and exhibit a shape that is typical for BTCs.

Table 10. Predicted and actual arrival of centre of mass for T11 and T22 tracers at downstream sampling sites. The predicted values assume only straight advective flow with no mixing.

	Downstream 1			**Downstream 2**		
Site	Predicted	Actual (T11)	Actual (T22)	Predicted	Actual (T11)	Actual (T22)
Left	4.6	16	16	26	30	31
Centre	9.3	16	15	13	30	31
Right	4.8	16	15	22	30	31

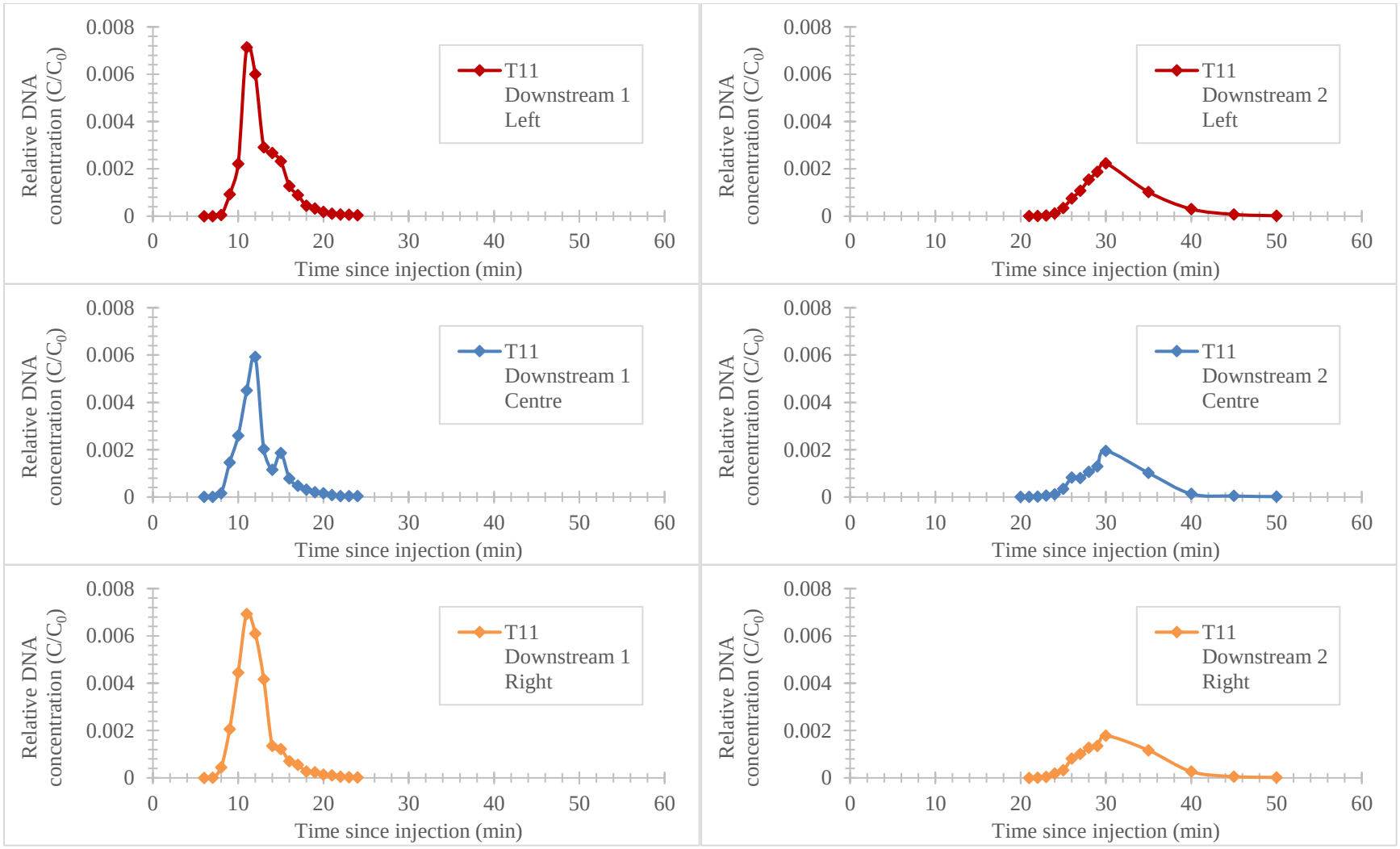

Figure 15. Breakthrough curves of relative concentration (C/C_0) for T11. Downstream 1 sampling site was 100 m downstream of the injection point and Downstream 2 was 350 m downstream.

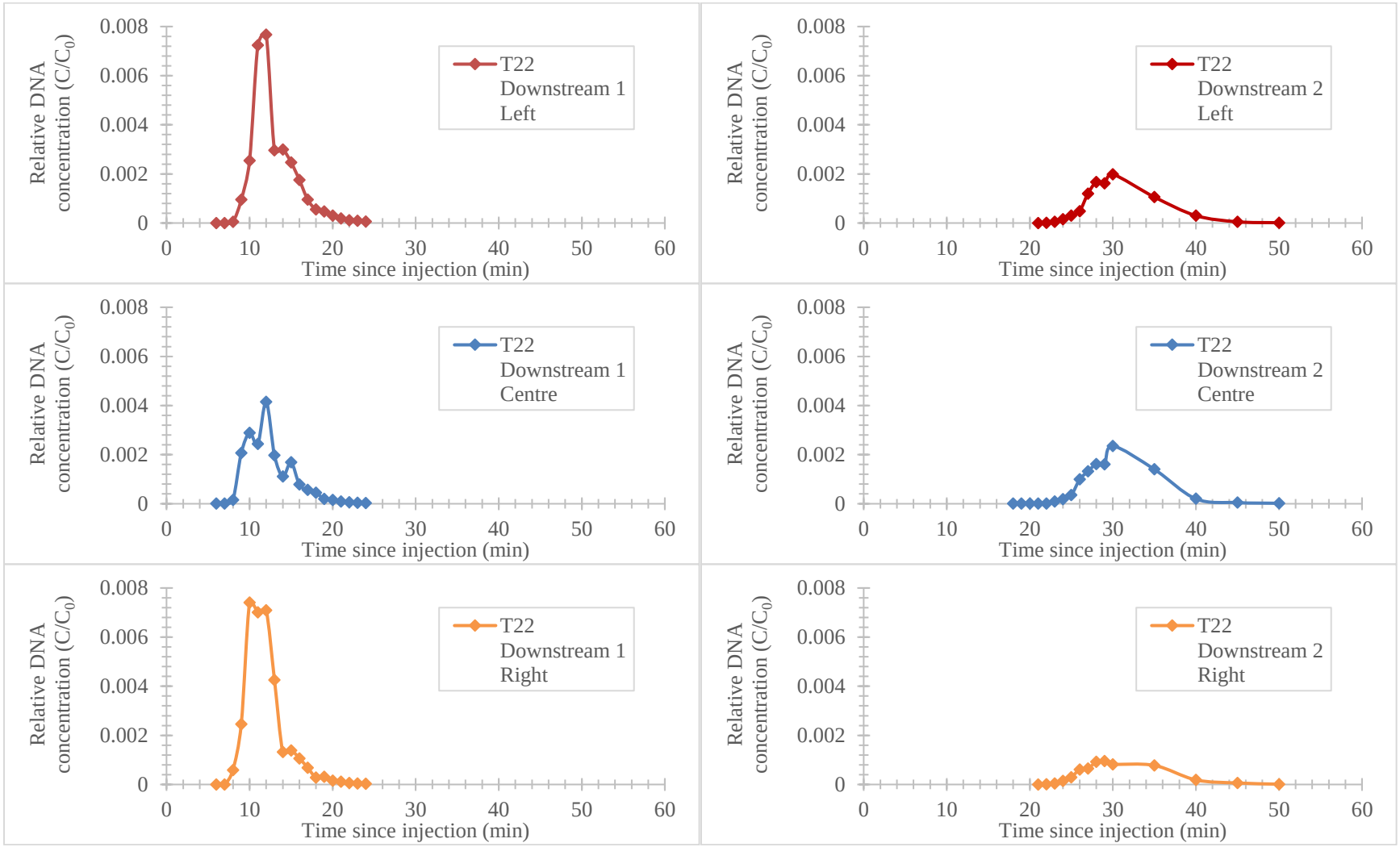

Figure 16. Breakthrough curves of relative concentration (C/C_0) for T22. Downstream 1 sampling sites appear on the left and were 100 m downstream of the injection point and Downstream 2, 350 m downstream, are shown on the right.

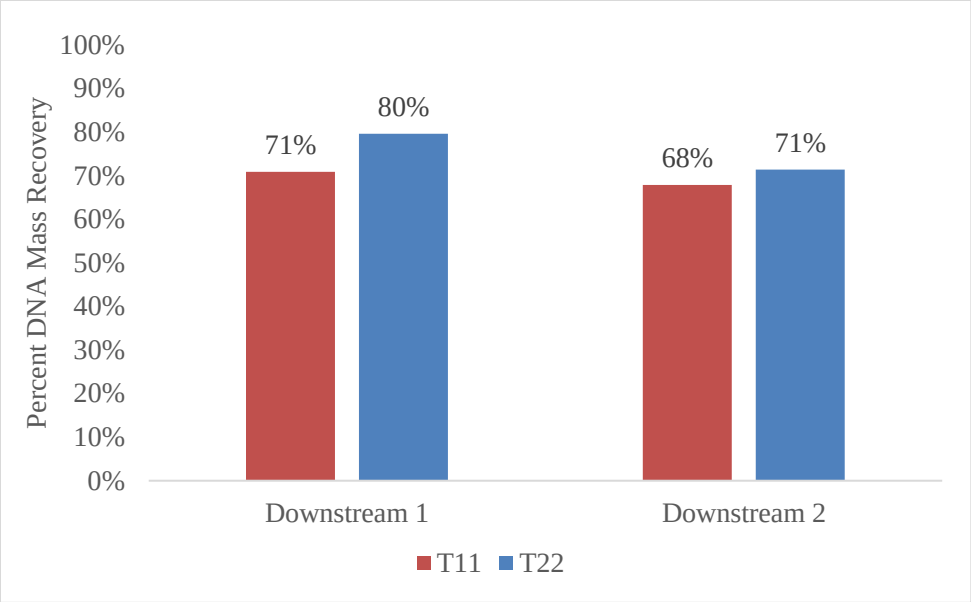

Figure 17. Percent mass recovery for each DNA tracer at Downstream 1 (100 m downstream), and Downstream 2 (350 m downstream).

4.2. Optimized synthetic DNA

The second objective of this research project was to design unique DNA sequences to be used for future experiments in an environment such as Washington Creek. Ten unique sequences were designed, however only four of the sequences met the criteria based on the formation of primer dimers and the efficiency of the qPCR (i.e., S2, S21, S22, and S28). The six sequences that were not selected demonstrated the formation of primer dimers or multiple products other than the target based on the melting curve or the qPCR efficiencies were unable to be optimized to an acceptable range (see Appendix).

5. Discussion

This research supports a proof-of-concept for the application of naked DNA as an environmental tracer in small headwater streams. Two single stranded DNA sequences (T11, T22) showed a very similar fate in Washington Creek with similar breakthrough curves and plume broadening downstream. The recovery of both tracers declined as they were transported downstream with only about 70% recovered 350 m downstream in the main plume. The experiments support that DNA can be applied in small streams and recovered downstream with the sensitive qPCR analysis technique. The study also supported that naked DNA can rapidly be transported downstream and may have implications for eDNA biomonitoring. Four additional sequences were validated, and their uniqueness was assessed using Washington Creek water and may be potential tracers that can be utilized in future experiments.

5.1. Analysis of field results and breakthrough curves

The tracers demonstrated a typical shape for BTCs and would permit assessment of the dispersion process. Although assessing dispersion was not in the scope of this research, the shape of the curves is a positive attribute for the use of DNA tracers. The breakthrough curves (BTCs) for DS1 showed a lag in the transport of part of the tracer mass as it travelled downstream. The shape of both BTCs for all sites generally show a normal distribution shape with some irregular “shoulders” which demonstrates the mixing pattern of the tracer. Uneven mixing patterns can be caused by decreased flow velocity along the stream channel, and eddies which introduce flow reversals (Hauns et al., 2001). The shape of the BTCs can be explained by variation in flow velocity that occurs in natural streams. The random mixing of the tracer that occurs from the injection point to the downstream sites is most likely dominated by variations in advective flow (Luhmann et al., 2012). Advection in the creek may have contributed to the lower peak, longer residence time, and greater spread of the downstream (DS2) BTCs compared to upstream ones. The downstream sampling locations for this study included several obstacles, turns, riffles, and pools.

Although the thalweg is dependent on the stream morphology, the centre segment is generally the fastest flowing and the edges are much slower. In the current study, similar concentrations across each segment of the stream indicate even mixing across the channel profile. The downstream sampling sites were a sufficient distance downstream from the injection point, and there was sufficient stream structure to ensure it was well mixed. It is commonly assumed in stream tracer

testing that uniform mixing of the tracer occurs in the streamflow (Capesius et al., 2005). Previous studies related to eDNA speculated the small free/naked forms of eDNA behave as a solute (Abbott et al., 2016; Shogren et al., 2016). This is supported by the patterns observed with naked DNA in the current study. These results indicate that at least the naked DNA component of the eDNA may travel rapidly downstream in Washington Creek. This has implications for the interpretation of eDNA studies, such as those of Marjan et al. (2021), conducted on fish (i.e. brook trout) in Washington Creek.

The velocity at which the tracer travelled downstream appears to be faster in the upper portion of the creek. The tracer took about 15 min to travel 100 m downstream (from the injection point to DS1), then 15 min to travel an additional 250 m downstream (from DS1 to DS2). The distance travelled from DS1 to DS2 was more than double the distance from the injection point to DS1, yet the time travelled were nearly identical. However, Marjan et al. (2021) showed most of the creek in the first reach is deeper and has a greater volume than the lower reaches, which would explain the slower travel time in this first reach (DS1).

The mass recovery calculations for T11 and T22 showed a slight decrease in mass as the tracer travelled downstream with approximately 71/80% mass recovery at 100 m and 68/71% at 350 m. In proglacial DNA tracer tests with 10 different DNA sequences by Dahlke et al. (2015), where the T11 tracer was also utilized, sampling was conducted at Centerjokk and Sydjokk. For Centerjokk, sampling occurred 226 m downstream and showed a 32% tracer mass recovery. For Sydjokk, sampling was conducted 313 m downstream and showed a 26% recovery. Mass recoveries in the proglacial river were highly variable overall and ranged from 1 – 66% for the 9 other DNA tracers used at Centerjokk and Sydjokk. The mass losses were likely due to the high turbidity in the water as DNA may have sorbed to suspended particles (Dahlke et al., 2015). The DNA tracer tests in small streams in Luxembourg, Belgium, and the Netherlands, also sampled around 350 m downstream and showed mass recoveries ranging from about 6 to 46%. In this same study, downstream sampling was also conducted around 600 m downstream for two different naked DNA tracer tests and recoveries of 3% and 53% were reported (Foppen et al., 2013). In a similar and earlier study that also sampled around 600 m downstream, recoveries of 52% and 61% were reported. In this same study, sampling was also conducted 1192 m downstream and 19% mass recovery was reported (Foppen et al., 2011). In comparing the Washington Creek tracer tests results to other DNA tracer tests, it is likely that the T11 and T22 tracers would continue to be detectable well beyond the DS2 sampling site.

Extrapolation of the two mass recovery values to the limit of detection showed that the DNA tracers are likely to no longer detectable beyond 37 km downstream. This was based on extrapolation of only two mass recovery values and therefore highly uncertain (Figure 18). The implication of this prediction is useful for improving eDNA sampling to estimate how far genetic material (i.e., naked DNA) may travel downstream of its source. Although eDNA can exist in several forms such as in cells or tissues, eDNA in the form of naked DNA may exhibit comparable behaviour to naked DNA tracers. eDNA shed in the first reach of Washington Creek could therefore potentially travel downstream for considerable distances very quickly and confound the interpretation of results. eDNA in other forms (associated with particles, tissues, etc.) would have different fate, but the naked DNA would likely travel downstream and be detectable for potentially dozens of kilometers. A surface water naked DNA tracer test in New Zealand sampled 1 km downstream and were able to detect the tracers (Pang et al., 2020). Another naked DNA surface water tracer test in the Netherlands noted a 19% mass recovery when sampling 1192 m downstream (Foppen et al., 2011). The naked DNA tracer study in the Netherlands included modelling of the tracer test data and they estimated detection as far as 275 km downstream may be possible (Foppen et al., 2013).

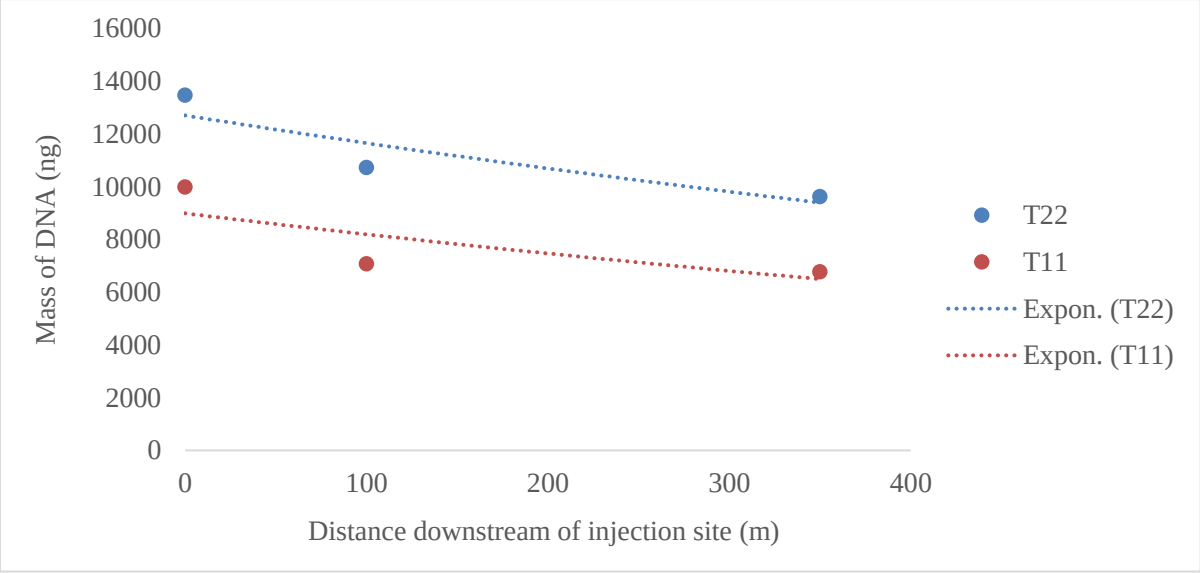

Figure 18. Mass recovery of DNA from the injection point to the downstream sampling sites for each tracer based on the starting mass. The line of best fit for T11 is $y = 8989.4e^{-0.0009x}$ and for T22 is $y = 12702e^{-0.0009x}$.

Mass loss could be due to reactivity (e.g., hydrolysis, photolysis, and biodegradation) of the tracer within the environment. If the DNA strands were degraded, broken, or adsorbed, they may not have been detected in the qPCR assay. If the DNA was adsorbed to organic material, it may have been retained in the stream for much longer and might not have been detectable in the direct qPCR analysis. The BTCs appear smooth and return to near zero (nondetectable) at the end of the tracer test. Mass loss due to not sampling long enough is likely minimal. A surface water naked DNA tracer test in small streams in Belgium and the Netherlands reported losses of the tracer at the start of the injection test. The presence of charged ions in the brook may have caused the DNA to change shape due to cation-induced condensation. The cause of the instantaneous losses were unresolved and further research is required to determine the cause (Foppen et al., 2013). The evidence of large instantaneous losses at the beginning of the experiment provide insight into how mass loss may have occurred for the Washington Creek tracer test.

When working with very low concentrations of DNA, adsorption of the DNA to the pipette tips and storage vials is a possibility. The addition of a DNA carrier in future studies may avoid potential excessive sorption to these surfaces. Although in the current study samples were placed on dry ice immediately and then stored at -80ºC, degradation during storage or freeze-thaw may also have resulted in an underestimation of the concentrations in the stream water. The calculated mass recoveries for both tracers were similar and declined moving downstream. This suggests that although there may have been some losses due to method bias there was also a reduction in mass of the DNA as it travelled downstream. Future studies should consider ways to reduce and measure losses at each step to reduce any bias. In addition, increasing the sampling resolution and physical/chemical measurements in the stream (e.g., flow, depth, and discharge) could provide greater understanding of the fate of the tracers.

The discharge may have a major impact on how far and quickly a tracer travels downstream as well as its recovery. The Washington Creek DNA tracer test site had relatively fast flowing waters at about 100 L/s across the study reach. This was comparable to the conditions of most other DNA tracer tests where fast flowing water was generally noted. The naked DNA tracer test in Helotes Creek, Texas demonstrated a discharge of 40 L/s (McCluskey et al., 2021). The groundwater fed creek in New Zealand where an encapsulated DNA tracer test was conducted also had high flow conditions above at least about 30 L/s (Pang et al., 2020). Another encapsulated DNA tracer test, which was conducted in New York, reported a discharge of 23 L/s (A. N. Sharma et al., 2012). A surface water naked DNA

tracer test conducted in a bedrock river in Italy showed a discharge of approximately 175 L/s (Bovolin et al., 2014). The surface water DNA tracer test with the highest flow conditions were the proglacial river in northern Sweden with flow conditions ranging from 460 – 1260 L/s (Dahlke et al., 2015). On the other end of the discharge spectrum were naked surface water DNA tracer tests conducted in Luxembourg and the Netherlands where the lowest discharge reported was 3 L/s (Foppen et al., 2011, 2013). Of all the surface water DNA tracer tests reported, the Washington Creek's flow conditions were in about the middle range compared to other studies.

Surface water DNA tracer tests have been conducted in various environments, but the Washington Creek tracer test is the first of its kind conducted in a Canadian watershed. Washington Creek is also a headwater creek and is a habitat for sensitive trout species. DNA tracer tests have been reported in a groundwater sourced stream in New Zealand, but was one which was heavily influenced by nutrients and algae due to nearby agricultural land use (Pang et al., 2020). A couple headwater brooks were also utilized for surface water naked DNA tracer tests, but differed from Washington Creek as they were located in the Netherlands and were dominated by sand and clay deposits (Foppen et al., 2013). Another set of naked DNA tracer tests were conducted in brooks in the Netherlands, but were characterized by reedbeds, algae, particulate matter, and surrounding land use of grasslands and maize (Foppen et al., 2011). A couple of tracer tests were reported in watersheds in the United States, including Texas (McCluskey et al., 2021), and New York (A. N. Sharma et al., 2012). Surface water DNA tracer tests have also been conducted in waters faster flowing than Washington Creek, such as in a proglacial stream with a high sediment loading (Dahlke et al., 2015), and a bedrock stream with very little losses to groundwater (Bovolin et al., 2014).

Table 11 elaborates on surface water DNA tracer tests and compares the results of this research to the site conditions, methods, and results of other studies. Naked and encapsulated ssDNA and dsDNA were concluded to be good tracers in a wide variety of stream environments around the globe (Bovolin et al., 2014; Dahlke et al., 2015; Foppen et al., 2011, 2013; McCluskey et al., 2021; Pang et al., 2020; A. N. Sharma et al., 2012). The current study showed DNA tracers were also successfully detected downstream, and the tracer test was successful at showing that two synthetic ssDNA tracers can be used as environmental tracers in a small headwater stream. The results of this proof-of-concept study align with other recent studies (Table 11) that also concluded that naked DNA is a suitable environmental tracer with many potential applications.

Table 11. The reported environmental site conditions and tracer test results of surface water DNA tracer tests from the literature. The listed studies used naked DNA, except for the study from 2015 which used both the encapsulated and naked forms (Dahlke et al., 2015), and the study from 2012 which only used encapsulated DNA (A. N. Sharma et al., 2012).

Site conditions	**Field site water turbidity**	**Discharge / flow**	**Sampling sites (downstream of injection point)**	**Sample volume**	**Sampling interval (after tracer injection)**	**Sample storage**	**Sample and qPCR preparation**	**Mass recovery**	**qPCR lowest detection**	**Source**
Washington Creek: cold-water headwater creek in a Canadian watershed. Tracer test conducted in the fall.	2.1 – 2.4 NTU	Approx. 100 L/s	100 m and 350 m	1.5 mL	Duplicate samples every 1 min for 30 min, then every 5 min for 20 min	Field: dry ice Lab: -80ºC for 9 – 11 months	Field sample water pipetted directly into PCR well	T11: 71% (100 m) 68% (350 m) T22: 80% (100 m) 71% (350 m)	20 – 50 copies per 5 uL sample	Current research
Helotes Creek in Bexar County, Texas	Not reported	40 L/s	28 m and 42 m	1 L	Not reported	Field: on ice (no more than 6 h) Lab: -20ºC	Filtered, then extracted	Not reported	1 copy per reaction	(McCluskey et al., 2021)
Harris Drain section of Coldstream, Ashburton, New Zealand. A groundwater sourced stream where land use is dominated by agriculture. Conducted in the early spring.	Exp. 1: 6 FNU Exp. 2: 13 FNU	Exp. 1: 17 – 24 m/min Exp. 2: 14 – 18 m/min Exp. 3: 23 – 27 m/min	5 sampling sites within each range: Exp. 1: 20 – 100 m Exp. 2: 100 – 500 m Exp. 3: 600 – 1000 m	2 L	Not reported	Field: cooler with ice packs (no more than 3 h) Lab: stored at 4°C (no more than 2 weeks)	Filtered, then extracted	Not reported	5 – 250 copies per 4 µL reaction (10^{-10} – 10^{-8} mg/L)	(Pang et al., 2020)
Three proglacial streams in northern Sweden: Nordjokk, Centerjokk and Sydjokk. Conducted in August.	High turbidity	Sydjokk: approx. 990 L/s Centerjokk: approx. 1260 L/s Nordjokk: approx. 460 L/s	Sydjokk: 226 m Centerjokk: 313 m Nordjokk: 571 m	15 mL	Every 5 min for 3 h	Field: an opaque cooler Lab: -20°C	Field sample directly collected. In the lab, DNA released from encapsulation and aqueous supernatant plated for PCR.	1 – 66%	100 and 1000 copies per 4.4-µl sample volume	(Dahlke et al., 2015)
Fast flowing bedrock river in Italy	Not reported	Approx. 175 L/s	300 m	0.5 mL	Every 1 min	Field: 4°C	Undiluted water samples used in PCR plate	87%	850 particles/µl of sample	(Bovolin et al., 2014)

Two brooks in Luxembourg: Maisbich (schist lithology, conducted tracer test in February) and Heuwelerbach (sandstone and marls); and two headwater brooks, characterized by sand and clay deposits, on the border of the Netherlands and Belgium: Gelsloopken and Biezenloop. Nearby land use includes pasture, forest, and grassland. Tracer tests conducted in January and February.	Not reported	Maisbich-down: 34 L/s Maisbich-up: 11 L/s Heuwelerbach: 15 L/s Ditch: 3 L/s Gelsloopken: 89 L/s Biezenloop: 20 L/s	Maisbich-down: 100 and 400 m Maisbich-up: 150 m Heuwelerbach: 300 and 650 m Ditch: 150 m Gelsloopken: 300 m Biezenloop: 300 and 550 m	Approx. 1mL	Approx. every 30 s	Field: dry ice	If samples contained humic acids, they were diluted, otherwise field water directly pipetted into PCR wells	Maisbich-down: 32.7% ± 13.7% (100 m) 16.1% ± 6.7% (400 m) Maibich-up: 6.8% ± 2.9% (150 m) Heuwelerbach: 6.0% ± 2.5% (300 m) 2.9% ± 1.2% (650 m) Ditch: 13.6% ± 5.7% (150 m) Gelsloopken: 24.5% ± 10.3% (300 m) Biezenloop: 46.4% ± 19.5% (300 m) 52.6% ± 22.1% (550 m)	$5 \cdot 10^7$ particles/L in a 4 µl sample	(Foppen et al., 2013)
Small stream reach with high flow in New York	Not reported	23 L/s	6.1, 12.2, 36.6, and 61.0 m	100 mL	Every 20 – 30 s for 300 s, then every 40 – 60 s for 300 s, then every 120 s for 600 s	-20°C	In the lab, DNA released from encapsulation and aqueous supernatant plated for PCR	Not reported	100−1000 copies per 11 µL sample volume	(A. N. Sharma et al., 2012)
Two brooks in the Netherlands: Strijbeekse Beek (reedbeds, algae, particular matter) and Merkske. Land use dominated by grassland and maize.	Not reported	Strijbeekse Beek: approx. 38 L/s Merkske: Approx. 35 L/s	Strijbeekse Beek: 112, 621, and 1192 m Merkske: 100 and 600 m	Approx. 1mL	Every 2–5 min for 7 h	Field: cooler Lab: -20°C	Filtered water samples in the field	Strijbeekse Beek: 55% (112 m) 52% (621 m) 19% (1192 m) Merkske: 52% (100 m) 61% (600 m)	50 – 100 copies (0.01 – 0.1 fM) per qPCR well (20 uL reaction)	(Foppen et al., 2011)

5.2. Research challenges

PCR is a critical component of the application of DNA tracers, but it was also an area that presented numerous challenges. Lessons learned from the proof-of-concept study, solutions that were developed and recommendations for future DNA tracer related studies using qPCR are outlined below.

Efforts were initially put into optimizing DNA quantitation using a probe-based approach because it could be more sensitive and selective. However, various issues arose such as unacceptable non-template control (NTC) amplification and low amplification efficiencies for the sequences of interest. A probe in the context of qPCR is an oligo sequence of approximately 20 bp's that contains a reporter dye and a quencher. When the qPCR begins, the probe anneals to the target DNA. The enzyme polymerase replicates the DNA and cleaves the probe, resulting in a fluorescence signal used for quantification (Thermo Fisher Scientific, 2021). EvaGreen dye-based qPCR is an alternative analysis method that also relies on a fluorescence signal to quantify DNA. After optimization with a probe presented issues, an EvaGreen dye-based qPCR was applied and demonstrated acceptable results. When troubleshooting analysis for molecular biology techniques, there can be many factors to consider. Background water samples showed the primers used for the tracer experiment did not appear to amplify anything in the environment and the BLAST database search did not show any potential matches. It is important to verify primers do not bind to naturally present DNA as the EvaGreen dye will bind to any dsDNA. If the primers bind to and amplify naturally occurring DNA, then the fluorescence quantified through the qPCR will overestimate the quantity of the target DNA in the sample. Using a EvaGreen dye for analysis is a common practice for DNA tracer testing (Table 1). Although use of a probe should result in additional specificity, additional work would be needed to develop a probe that works with these specific sequences and environments. Through this research, EvaGreen dye analysis was an acceptable approach as it allowed for an appropriate detection limit and did not appear to be affected by other naturally occurring sequences. EvaGreen dye is more affordable than using a primer-probe set and allows for melt-curve analysis. Melt curve analysis is a way to verify if something other than the target DNA was amplified. A single melt-curve peak is required to show a single product was being produced through real-time qPCR. Due to the presence of melt-curves, it could be confirmed that no cross-linking (unintentional binding of the DNA or primers) was noted in any samples.

Hundreds of water samples were collected from a single field tracer test, and as a qPCR plate only has 96 wells, many separate plates had to be run to analyze all samples. Every time a new plate was

prepared, further variability is introduced. Randomizing which samples are run on each plate can help to account for inter-plate variability. A surface water naked DNA tracer test in Helotes Creek, Texas, used digital droplet PCR (ddPCR) for analyzing water samples to eliminate inter-plate variability between samples (McCluskey et al., 2021). In addition, primer and target DNA stocks should all be derived from the same batch for all sampling to eliminate any potential differences in concentration. Another quality control method to assess differences between qPCR plates is to include an identical reference sample with a known concentration on each plate to compare how the quantification of the reference sample differs from plate to plate. qPCR analysis may have introduced some variability with regards to quantification of results, but quality of the reaction components and the presence of a reference sample may help reduce or correct for this variability. In the current study, a full standard curve was run on every plate, allowing for some of the inter-plate variability to be controlled. Unfortunately, factors outside our control (COVID-19 pandemic) forced us to not conduct the analysis exactly as desired.

Some of the apparent initial DNA mass loss could have been due to biases associated with the methods. Such as, estimation of the initial concentrations and biases associated with sample collection, e.g., binding to collection tubes, degradation during storage, or destruction during freeze-thaw. Adding a buffer solution such as tris-EDTA to the sample vial may lead to better sample preservation when working with low concentrations of DNA. Quantifying potential mass loss during storage may also be useful and could be carried out by storing a reference sample of a known starting concentration in the freezer alongside the samples. Most samples that underwent multiple freeze-thaw cycles demonstrated degradation and while a buffer solution may help with preservation, multiple freeze-thaws should be avoided. None of the recent surface water DNA tracer tests appeared to have used a buffer solution for their field samples (Bovolin et al., 2014; Dahlke et al., 2015; Foppen et al., 2011, 2013; Pang et al., 2020; A. N. Sharma et al., 2012).

Mass loss of DNA may have potentially occurred during storage. The field samples from Washington Creek were stored but with the absence of a reference sample, therefore it is unknown if or how much sample degradation occurred during storage. Some delays were imposed on this research project due to the pandemic and the samples were kept frozen for longer than anticipated in a -80°C freezer due to limited access to the laboratory. Samples were collected in the fall of 2019, but analysis did not begin until the summer of 2020. In the literature, it was not well reported for how long others' stored field samples. Some studies stored their samples at 4°C until analysis (Pang et al.,

2020; A. N. Sharma et al., 2012), while others stored their samples in a -20°C freezer (Dahlke et al., 2015; Foppen et al., 2011; McCluskey et al., 2021). Addition of a known sample spiked into a water matrix at a similar concentration to that expected from the downstream DNA tracer samples would have helped to understand and possibly control for this issue.

Adding a carrier to the sample vial may also help reduce mass loss during qPCR preparation (Xu et al., 2009). Carrier DNA are useful when working with low concentrations or fragmented pieces of DNA. DNA is negatively charged (Liang & Keeley, 2013), and low recovery is common at dilute concentrations due to their adhesion to microcentrifuge tubes. Low-retention tubes and pipettes are vital to be used as this can be a significant source of mass loss (Ellison et al., 2006). Previously conducted DNA tracer tests have not mentioned the use of a carrier to improve mass recovery during analysis. Incorporation of a carrier could potentially reduce the instantaneous losses of DNA that were noted (Foppen et al., 2013), but this would need to be further tested.

When analysis is limited by facility operating hours or availability of space and equipment, only samples that can realistically be run that day should be thawed. An example of a step that could be taken to save time is to transfer samples from a -80°C to a -20°C freezer the day before plate preparation to decrease thawing time. Timing and organization are essential when planning for DNA sampling and analysis due to the nature of the samples being highly sensitive to degradation. Even one freeze-thaw cycle can adversely influence the integrity of the sample.

For one transfer blank sample (taken at 50 minutes after the start of the tracer release at DS2), gloves were not changed before conducting the transfer and this may have resulted in T22 DNA being detected in the blank. This contaminated control sample amplified at cycle 34. Most BTCs involved sample concentrations that amplified in the late 20’s to mid 30’s therefore the contaminated transfer blank exceeded normal background levels. The transfer blanks were intended to confirm the sampling technique was carefully conducted to prevent cross contamination between samples. The process of completing a transfer blank was to carefully transfer a pre-filled vial of molecular water that was transported from the laboratory and pipette this water into a clean vial. Perhaps the transfer blank was too stringent of a measure as it can be very easy to contaminate pure PCR-grade molecular water. qPCR is a highly sensitive technique and therefore at every step of the sample preparation, collection, and handling process, the aseptic techniques must be used to prevent trace contamination between samples.

Another challenging component to this research was working through the literature of differing fields: molecular biology and hydrology. qPCR analysis, a common molecular biology technique, is a vital step to analyzing DNA tracers. However, it is not common practice to present significant details of the molecular biology techniques in hydrology focused journal articles. When conducting interdisciplinary work, incorporating the necessary information from each field to make a complete report with adequate detail and clarity is vital for advancing the science. As DNA tracing becomes more common, the gap between the biology and earth science fields will need to be bridged through interdisciplinary collaboration. Due to the relative novelty of DNA tracing, there is still a lack of consistency in the literature in terms of reporting molecular biology practices. Inconsistencies between how target DNA and primers sets are presented, how qPCR efficiency is reported, or how standard curves are displayed has varied between articles (Table 1). Differing uses of terminology also creates barriers for researchers to communicate effectively. For example, encapsulated synthetic DNA tracers (Pang et al., 2020), DNA-labeled nanotracers (Kong et al., 2018), and silica particles with encapsulated DNA (SPED) (Mora et al., 2016) are all similar terms using different terminology. Consistency in reporting as well as language will promote collaboration and progress in this area of research.

5.3. Benefits of DNA tracers

The BTCs demonstrated that the behaviour of the DNA was solute-like and may be useful for obtaining solute transport information about streams such as advective and dispersive behaviour. Similar conclusions were noted in the literature (Dahlke et al., 2015; Foppen et al., 2013).

The sensitive detection technique of qPCR allows for very low concentrations of DNA to be detected in water samples. The lowest concentrations of DNA detected in the field water samples ranged from about $5.04*10^{-10}$ ng/µL (Cq: 35) to $1.23*10^{-9}$ ng/µL (Cq: 33) (about 20 copies to 50 copies per 5 µL sample). The range of the lowest limits of detection reported in the literature for other naked DNA surface water tests ranged from 1 copy to 1000 copies per 4 µL sample volume (Bovolin et al., 2014; Dahlke et al., 2015; Foppen et al., 2013; McCluskey et al., 2021; Pang et al., 2020; A. N. Sharma et al., 2012). Due to the sensitivity of the analysis, the 1.5 mL samples that were taken in the field contained a sufficient signal of the target DNA for the BTCs. Several other authors reported using small quantities of field water samples ranging from about 0.5 mL to 1 mL (Bovolin et al., 2014; Foppen et al., 2011, 2013). Other DNA tracer tests utilized larger water sample volumes: 15 mL (Dahlke et al., 2015), 100 mL (A. N. Sharma et al., 2012), 1 L (McCluskey et al., 2021), and 2 L

(Pang et al., 2020). During PCR plate preparation for this project, field samples were directly pipetted into the PCR plate similar to some reported studies (Bovolin et al., 2014; Foppen et al., 2013). Other studies included some additional steps during PCR preparation such as filtration (Foppen et al., 2011), dilution if the samples were brown in colour and showed the presence of organic matter (Foppen et al., 2013), or filtration and extraction (McCluskey et al., 2021; Pang et al., 2020). In terms of qPCR analysis, when DNA tracing is conducted in clean creeks, such as the Washington Creek field site, low volumes of water can be collected, and field water can be directly applied for qPCR. The direct analysis in the qPCR saves considerable preparation effort, but caution must be taken due to issues associated with handling of low concentration samples as discussed above.

The main utility of DNA tracers is being able to use multiple tracers at the same time, often referred to as multitracing (Dahlke et al., 2015), multipoint tracing (A. Sharma et al., 2021), or multipoints tracing (Liao et al., 2018). For this project, T11 and T22 were simultaneously released into the creek and both tracers exhibited nearly identical behaviour in terms of their BTCs. Theoretically, many unique DNA tracers could be input into the creek based on these findings. The signal from most conventional tracers, such as salt, cannot be separated, whereas DNA tracers can easily be distinguished in a single low volume water sample. In addition, using multiple types of conventional tracers could be costly and different types of analysis may be required (Foppen et al., 2011).

Another function for DNA tracers is to use them as a surrogate for eDNA fate and transport. Utilizing a surrogate for the fate and transport of eDNA to be analyzed without having to analyze eDNA directly. A surrogate for eDNA can provide insight to biological and ecological questions. Conventional salt and dye tracers cannot be compared directly to eDNA. The benefits to using synthetic DNA tracers are notable and they hold a lot of potential, especially in terms of research applications. In summary, the results of the tracer test showed that in clean environments, relatively short-term (span of a day) use of synthetic DNA as an environmental tracer can be effective.

5.4. Limitations of DNA tracers

DNA tracers have several benefits but also have limitations that must be addressed to plan a successful tracer test. Planning for sampling, analysis, and preventing contamination and tracer loss are some factors that must be accounted for when conducting a DNA tracer test.

DNA tracers require a manually intensive sampling and analysis processes. Compared to salt tracing, DNA tracing is more complex in terms of obtaining sample data. Salt tracing can be directly measured in the field with an electrical conductivity meter. To simplify field sampling, a multichannel pipette to collect multiple samples (e.g., triplicates) could be used. This would cut down on sampling time and allow for enough samples to be collected to conduct statistical analysis. None of the previously conducted surface water tracer tests conducted statistics to compare water samples (Bovolin et al., 2014; Dahlke et al., 2015; Foppen et al., 2011, 2013; McCluskey et al., 2021; Pang et al., 2020; A. N. Sharma et al., 2012). Two studies reported using duplicates during qPCR but it is likely that these were technical replicates (Bovolin et al., 2014; Dahlke et al., 2015). In another study, error bars were presented on the sample BTC's (Foppen et al., 2013), but it is unclear if the error bars are for technical replicates or biological replicates. For qPCR, duplicate or triplicate technical replicates are included for each sample on the plate (technical replicates are obtained from one biological replicate). In the qPCR technique, the common practice is to only report one value, which would be the average of the technical replicates included for that qPCR run (Taylor et al., 2019). In terms of this research, a biological replicate is an independent field water sample and true replication is desirable. In the future it may be possible to use improved methods and instrumentation that streamline the DNA sample collection and analyses and improve coverage and replication. In the past year, widespread COVID-19 related PCR work has occurred across laboratories globally, including at the University of Waterloo. The PCR technique has become more common in laboratories due to demands from the pandemic and this may be a benefit in terms of advancements in PCR technology.

In terms of sampling technique, it did not appear as though any other surface water tracer studies reported if they were in or out of the water body during sampling. This current research involved collecting samples from wooden boards to not disturb the streambed. Although set up required an extra process and more equipment needed to be brought to the field, this technique maybe have contributed to clean downstream water samples. Clean samples ensured that stream water could be used directly in the PCR plate without additional extraction or dilution steps to account for PCR inhibition or changes in the partitioning of the DNA in the river.

In general, turbidity was not reported for other surface water naked DNA tracer tests. High turbidity were noted for the proglacial stream where a DNA tracing test was conducted (Dahlke et al., 2015). Turbidity of the groundwater fed stream in New Zealand was 6 FNU for the first experiment, then 13 FNU for the second experiment. This study involved using both naked and encapsulated

DNA. In the Washington Creek tracer test, the turbidity was relatively low and was unlikely to have contributed to significant mass losses. However, this parameter is important to consider and even small changes could be a limitation during naked DNA tracer tests.

The sampling frequency required for DNA tracing can be laborious. Downstream sampling frequency was high for this study: every minute for 30 min, then every 5 min for 20 min. In general, the sampling frequency was 5 min or less for other surface water DNA tracer tests (Bovolin et al., 2014; Dahlke et al., 2015; Foppen et al., 2011, 2013; A. N. Sharma et al., 2012). The exact reported frequencies are outlined in Table 11. A high enough sampling frequency is required to capture the BTC, especially in fast flowing water. When sampling is not frequent enough, the breakthrough curve can easily be missed (A. N. Sharma et al., 2012).

This current research also differed from other reported DNA tracer studies as sampling was conducted in three locations across the stream width. No record of sampling across the stream width was noted for other similar surface water tracer tests (Bovolin et al., 2014; Dahlke et al., 2015; Foppen et al., 2011, 2013; McCluskey et al., 2021; Pang et al., 2020; A. N. Sharma et al., 2012). Although this style of sampling was time consuming, the results allowed for confirmation of the assumption that the DNA would be well mixed by the time it reached the downstream sampling sites.

Inter-plate variability in the qPCR was notable from the field results, despite no mention of this issue in the literature (Bovolin et al., 2014; Dahlke et al., 2015; Foppen et al., 2011, 2013; McCluskey et al., 2021; Pang et al., 2020; A. N. Sharma et al., 2012). Variation between plates is normal for qPCR and considerable effort must be applied to account for this. In addition, greater variability is expected when working with low concentrations of DNA, especially samples with less than 100 copies or where the Cq is greater than 29. A method to partially address this variation is to include more technical replicates during qPCR to ensure the biological replicate is well represented (Taylor et al., 2019). However, this solution may result in increased time and cost during analysis. The qPCR analysis may introduce a high degree of artificial variability if QA/QC procedures are not strictly applied.

Contamination of samples is a risk when working with DNA tracers. Contaminated samples may introduce biases during analysis. Therefore, numerous controls are required to ensure sample cross-contamination has been avoided (blanks, NTC, etc.). Careful aseptic techniques must also be used at every step of the way to ensure sample vials are not contaminated before, during, or after field work. Sample analysis must be conducted in a facility that supports molecular biology work (e.g., PCR

hoods and biosafety cabinets). Separate rooms for qPCR plate preparation and amplification are highly recommended to prevent contamination. Frequent decontamination techniques should be implemented such as using a UV light, changing gloves frequently, and using surface decontaminants, such as ethanol. qPCR is sensitive and can detect even the smallest levels of contamination. NTCs should be run on every plate and clear criteria applied for acceptance of data, especially when working with low DNA concentrations.

5.5. Applications

DNA tracers are a technology that promote interdisciplinary research and encourages collaboration among various fields including earth sciences, ecology, and molecular biology. Research into an area that merges multiple fields opens opportunities for new questions to be resolved. The two main areas of application for DNA tracers are focused on hydrology (Foppen et al., 2011; Pang et al., 2017), and eDNA biomonitoring.

Considerable uncertainty with regards to the downstream transport of eDNA remains. Conducting a DNA tracer test at sites where eDNA sampling is to be conducted may help fill this gap in knowledge. The additional unique DNA tracers that were designed for the second objective of this research can be applied in stream environments. Their uniqueness and ability to be detected was tested with water samples from Washington Creek. The DNA design and optimization steps can be used to develop additional sequences, that are unique to their field site.

In terms of hydrology, encapsulated DNA tracers can be designed with a functionalized coating to control their fate. Application of DNA in various forms may be highly effective at tracing multiple pathways and help to interpret the fate of other compounds (e.g., nutrients, contaminants, and particles) (A. Sharma et al., 2021). Future method developments may allow for more rapid testing in the field or multiple endpoints simultaneously (e.g., multiplexing, sequencing). The possible applications for DNA tracers are endless, although considerable research is needed to operationalize this potential.

5.6. Future directions

Based on the results of this research, there appear to be many potential future research opportunities to enhance the application of DNA tracers. In the bigger picture, the next steps for DNA tracer research will likely entail increasing the durability of DNA tracers. This could be done either by uncovering which factors lead to mass losses or by encapsulating synthetic DNA to alter its fate.

Increasing the stability of DNA tracers will enhance the hydrological applicability of this technology and potentially expand their use beyond the current narrow research applications. Further down the road, DNA tracer field data could be paired with modelling to predict contaminant pathways.

Although the current study involved real-time qPCR technology, additional PCR technologies could potentially be used to increase efficiency and flexibility of the methods. Multiplexing is a PCR approach that could reduce the number of reactions and therefore plates required. This could reduce the variability as well as cost. Multiplexing may be suitable if multiple DNA tracers are present in one sample. The effectiveness of multiplexing with synthetic DNA tracers was demonstrated in a controlled experimental setting (Liao et al., 2020). As advancements are made in terms of qPCR technologies, additional more efficient routes of analysis may emerge to make analysis less laborious. The emergence of rapid, low-cost instruments for DNA detection will greatly enhance the ability to use DNA tracers. New sequencing technologies may also allow for many endpoints to be rapidly assessed simultaneously.

The primary objective of this research was to assess the ability for DNA to be used as an environmental tracer. A simple way to validate this hypothesis would be to compare the behaviour of a DNA tracer to a traditional conservative tracer such as a salt or dye. Due to the nature of the Washington Creek field site, a salt tracer test could not be conducted concurrently. However, in future tracer tests, a salt tracer test (or other conservative tracer) could be released before hand to plan for downstream sampling. In addition, comparing the mass recovery of a conservative tracer to a DNA tracer could provide a useful means of comparison. Mass balance calculations using analytical methods such as linear regression techniques through Excel or R would provide more accuracy than manual approaches. If using the salt tracer method, the salt solution should be injected well before the DNA tracer as salinity may partially inhibit DNA detection under some conditions (Cristescu & Hebert, 2018; Díaz-Ferguson & Moyer, 2014; Wolkersdorfer & LeBlanc, 2012). Other tracers may be applied simultaneously to directly compare and help understand and validate the fate of DNA tracers.

A large-scale experiment tracking synthetic DNA through multiple pathways is a possible future direction for this research. The results of this project showed that using multiple tracers in one stream concurrently was successful. Extending this work to a larger area or for a longer term may shed light on the reliability of this technology. Larger scale experiments could involve releasing DNA tracers in a watershed and tracking its downstream detection through a network of different streams. Multitracing through a fractured system of an aquifer could also be an application area involving

multiple pathways. In addition, the use of encapsulated DNA may limit mass losses and allow for further downstream detection. The benefits and limitations of encapsulated DNA versus naked DNA could be compared through this type of contrasting experiment. Laboratory experiments testing how environmental variables impact DNA degradation showed that high temperature, high salinity, DNase (deoxyribonuclease, an enzyme that degrades DNA) degradation, and UV radiation would destroy naked DNA, whereas the encapsulated forms were resistant to degradation (Liao et al., 2020). A recent study revealed a biodegradable alginate and chitosan encapsulation technology that is suitable in sensitive environments like Washington Creek (Pang et al., 2020). Ecotoxicological tests have also shown that silica encapsulation of DNA tracers presents a small risk to low trophic level species (Koch et al., 2021).

A significant knowledge gap in terms of DNA tracers is their sources of mass loss. In the context of the Washington Creek case study, only about 70% of the original DNA mass was detected 350 m downstream. In comparison, a naked DNA tracer experiment reported in the literature demonstrated an 87% recovery rate at the 300 m downstream sampling site (Bovolin et al., 2014). These contrasting results show the potential to control for or reduce the factors that could be contributing to mass losses. These differing results also have implications for better understanding eDNA transport in different stream environments. Determining which environmental variables play a role in mass removal could be pursued in future research.

Field results can often be put toward creating useful predictive models. Breakthrough curve information could be fitted to models to improve advective and dispersive information (Foppen et al., 2013). Modelling could therefore be a future direction for DNA tracer research. If the fate of DNA tracers is or can be manipulated to be like other compounds of interest, models based on DNA tracers could be used as a surrogate. Examples include small contaminants that may exhibit a similar fate and behaviour as DNA (or encapsulated DNA). Most DNA tracer studies have been laboratory or field based but once their behaviour becomes more well understood and predictable, modelling may be a good route to support risk assessment (Liao et al., 2018).

There are many research opportunities to work with DNA tracers as most applications have been limited to case studies testing their robustness in different environments (Liao et al., 2018). The factors contributing to the mass loss of DNA and their long-term behaviour remain uncertain (Foppen et al., 2011, 2013). Large scale experiments with synthetic DNA are also very limited and provide an opportunity for this area to be explored (Liao et al., 2018). The future directions for DNA tracers

relate to better understanding their fate and improving sampling and analysis. If questions related to their robustness can be settled, then DNA tracers have the potential to be used beyond the confines of research.

The Washington Creek tracer test demonstrated the proof-of-concept of DNA tracing. The T11 and T22 tracers, which were previously used in European naked DNA tracing tests (Dahlke, 2014; Foppen et al., 2013), were able to be detected downstream in this unique Canadian environment. Some mass losses of the DNA occurred but it did not interfere with the ability to characterize the creek and its flow patterns. The results of the tracer test show promise in terms of better predicting and potentially improving eDNA sampling as well. Although some challenges were encountered in terms of PCR work, solutions were developed to ensure results were not compromised. For future DNA tracing, careful planning is vital to eliminate potential errors or biases during sampling and analysis. The time-consuming nature of DNA tracing is another challenge, but it does not greatly interfere with the ability to reliably conduct DNA tracer tests. The benefits of using DNA tracers outweigh their limitations as a large number of unique tracers can be used simultaneously and can be detected downstream through qPCR analysis. The applications for DNA tracing in the short term is likely to be limited to research but there remain many future research opportunities to apply this relatively novel technology. Understanding how environmental factors impact their fate and assessing their long-term and large-scale tracing abilities are areas in need of further exploration. Although synthetic DNA tracers may not yet be an alternative to using dye or salt tracers, as DNA tracers lack the simplicity of conventional tracers, they may become a routine tool in the future.

6. Conclusion

This research demonstrated that DNA tracers work and that the proof-of-concept has been validated. The water sample data shows that naked DNA tracers can be detected at very low concentrations and the results can be used to gather hydrological information. Some mass loss was detected and shows that naked DNA in a cold-water creek environment such as Washington Creek may not persist for long periods. With regards to the second objective, the DNA tracers that were designed demonstrate the methodology of designing and preparing DNA tracers for field studies. These methodologies could be followed to design a multitude of tracers for various applications. More research is needed to explore the fate of these DNA tracers in various environments and conditions.

Recommendations for improving DNA tracer research are centred around techniques to make sampling and analysis less time consuming. Other important improvements include taking additional measures to monitor quality control and minimize variability in the qPCR methods. When working with low concentrations of DNA there is more variation introduced. The DNA tends to sorb to surfaces and it can be difficult to accurately quantify the sample, especially at low concentrations. Techniques to minimize quantification bias must be implemented such as using a carrier and low-adhesion pipette tips and tubes. Samples may also be sensitive to degradation therefore storing samples in a buffer solution is recommended.

DNA tracers currently are not an alternative to salt and dye tracers, but they open new opportunities and questions to be answered. This research has provided an opportunity for interdisciplinary research to advance an emerging tool for environmental processes: synthetic DNA tracers.

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Appendix

Synthetic DNA designed at the University of Waterloo

Out of the 10 unique DNA sequences and primer/probe sets that were designed, six sequences were concluded to not be suitable tracers. These unsuitable tracers are listed in Table A - 1. Dye-based qPCR optimization was a better approach than using a probe due to the cost involved, although probes should make the detection more specific. The following DNA and primers sets were only able to achieve the following maximum efficiencies: S9 (86%), S15 (84%), and S19.1 (83%). NTC's for S5, S20 and S26 runs exhibited fluorescence as early as cycle 37 in some cases therefore the primers may have been forming secondary structures. These hypotheses could be verified on a gel to confirm how many products were created from the reactions; however, this was not explored further.

Table A - 1. DNA sequences and primer sets (in bold) designed at the University of Waterloo but were not selected as tracers due to unideal amplification efficiency, amplification of non-template controls (NTCs), and/or melt-curve analysis potentially indicating multiple products when only 1 was expected.

Target DNA	DNA Oligo sequence (5' to 3')	Length	GC (%)
S5	TCCG**AGACGGTCGACCATACCTT**CGATTATCGCGG CCACTCTCGCATTAGTCGGCAGAGGTGGT**AGTAAC GGTGTTGCGATAGC**CCAGTAT	91	55
S9	GTTAGA**ACTGGACGTGCCGTTTC**TCTGCGAAGAAC ACCTCGAGCTGTAGCGTTGTTGCGCTG**CCTAGATGC AGTGTTGCTCATA**TCACAT	90	51
S15	GCAA**ACGCAGAACAATGGTTACTATTTC**GATACGT GAAACATGTCCCACGGTAGCTCCAAAGACT**TAAGA GTCTATCACCCGGTGT**CTG	89	46
S19.1	A**AATCCGCATGTTAGGGATTT**CTTATTCATCGTGA GGAAATTCACAGCGGATCTTAATGGATGCG**CTATC GGGAGTACAGGTGGTA**TGGAAGCTAA	96	44
S20	CGCGGGTGA**GAGGGTAATCAGCCGTGTTC**ACCTA CACAACGCTAACGGGCGATTCTATAAG**ATTCCGC ATTGCGTCTACTTATA**AGATGTCTC	93	49
S26	TGA**GCCTTGAACTCCAGCAACT**CGGGCGACAACT CTTCATACGCATACCAGAGCAAGGGCGTCGAACG GTCGT**GAAACCGTCTTAGTACCGCAC**GTAC	98	55

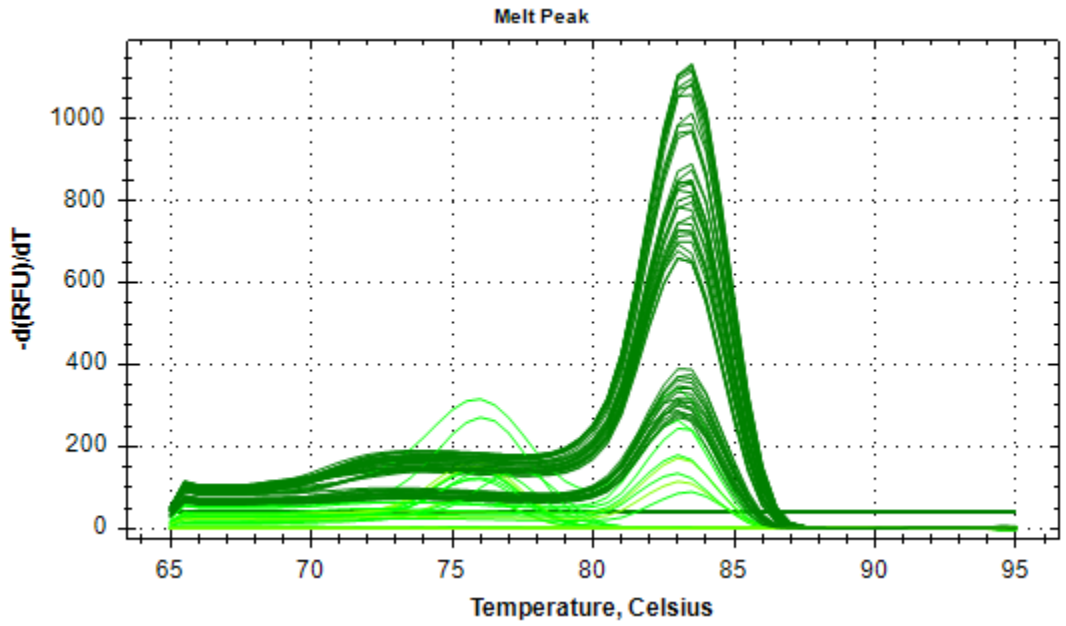

Figure A - 1. S26 melt curve output shows multiple peaks due to possible secondary structures. An ideal melt-curve should show a single peak.

qPCR optimization of T11 and T22 DNA tracers

Significant efforts were put into optimizing T11 and T22 and their primers. Figure A - 4 to Figure A - 7 show the result of qPCR optimization, where the standard curves showed acceptable efficiencies and melt-curve analysis illustrated the formation of a single product.

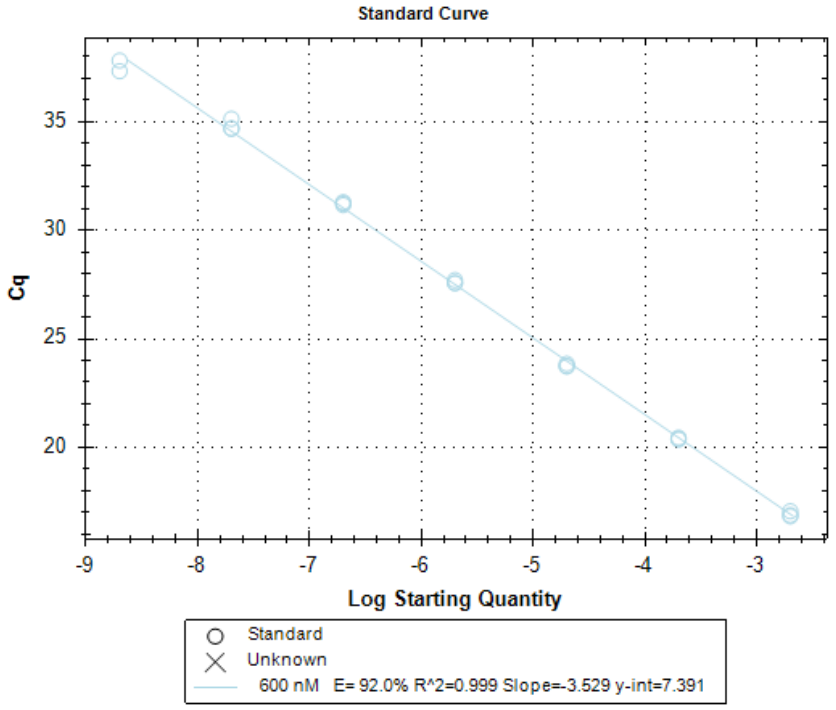

Figure A - 2. Ten-fold dilution standard curve for T11, displaying an efficiency (E) of 92%.

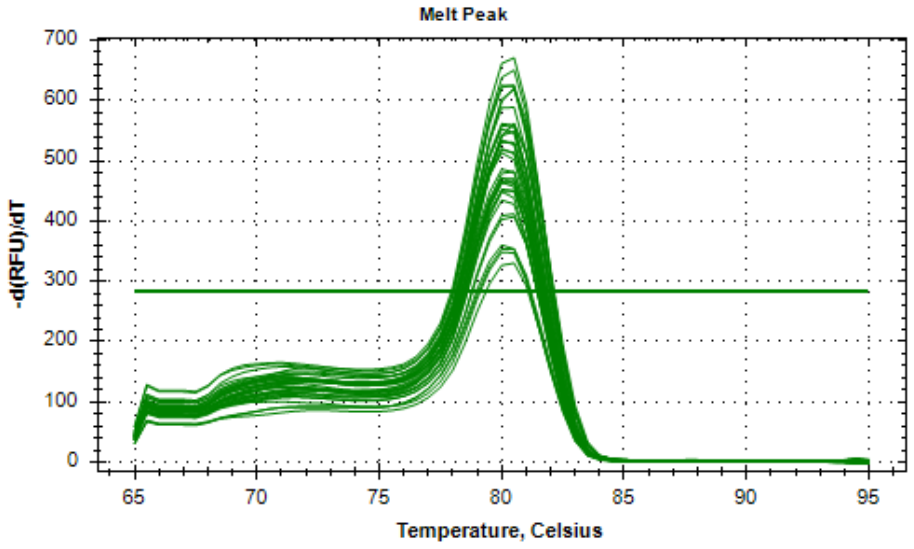

Figure A - 3. Melt curve output for T11, showing a melt temperature range of 80.0 – 80.5ºC.

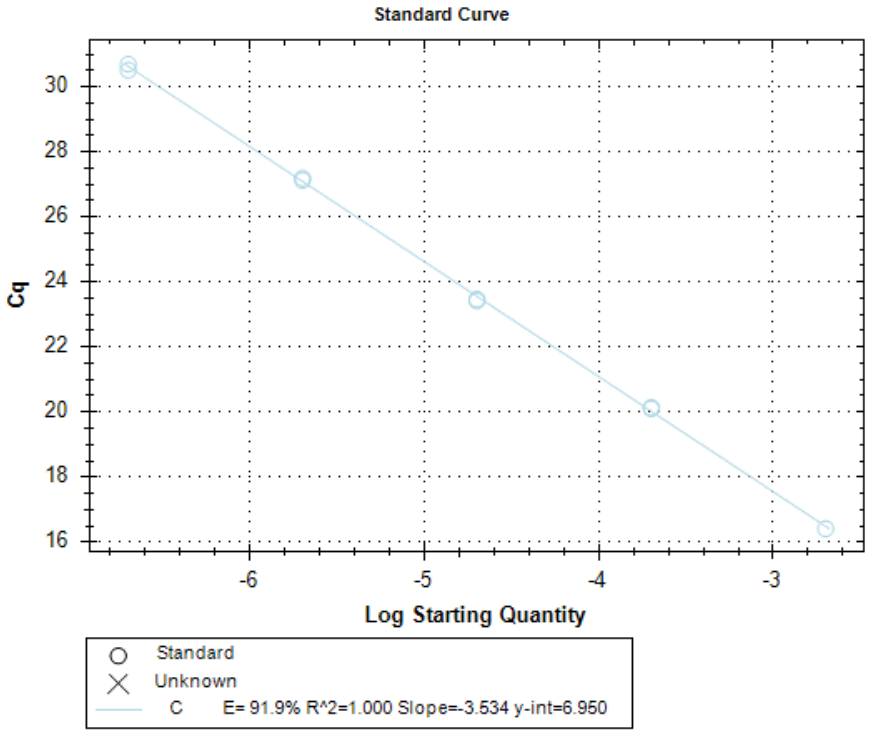

Figure A - 4. Ten-fold dilution standard curve for T22 which had an efficiency (E) of 91.9%.

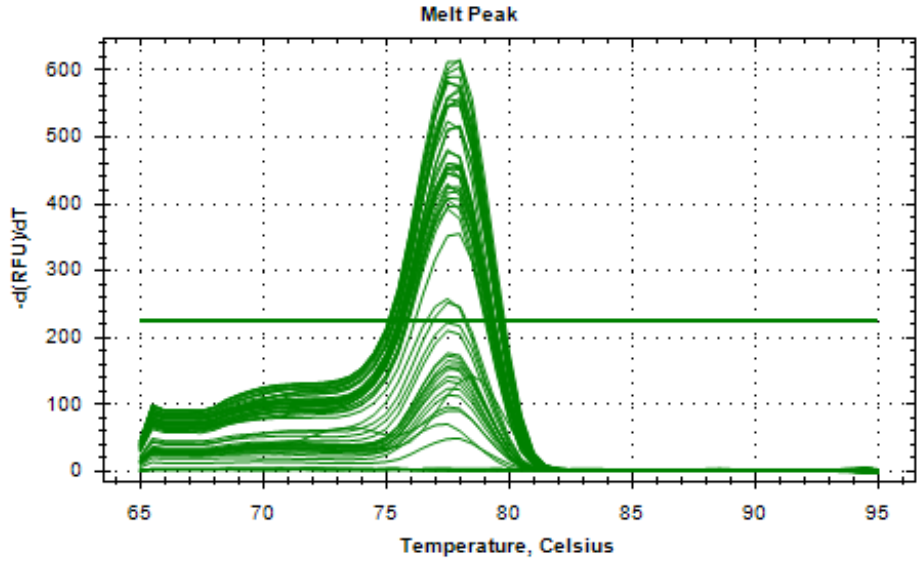

Figure A - 5. Melt curve output for T22, showing a melt temperature range of 77.5 – 78.0ºC.

Case study: Washington Creek tracer experiment

Washington Creek water samples were analyzed through qPCR then quantified with an average standard curve. Each qPCR plate contained 7 sets of diluted standards to create a standard curve, however due to variations between qPCR plate runs, there were large differences in quantification. The standard curve on each plate was expected to be identical but this was not the case. The high variability of each standard curve was due to inter-plate variability. The qPCR is sensitive to changes and different plates led to slightly different curves. Table A - 2 to Table A - 5 list the absolute concentration values for each water sample, and demonstrates the variation between sample duplicates, despite the sample being obtained nearly simultaneously. To account for this variability, an average standard curve was created for each tracer (Figure A - 6 and Figure A - 7). The individual curves on plates could not be used as it meant the quantification of samples was not comparable. Creating one standard curve for quantification allowed for field samples to then be comparable. The concentration values were adjusted by comparing qPCR Cq output values to an average standard curve. The normalized T11 standard curve included 8 separate standard curves from water sample PCR plates run over the course of several months. The normalized T22 standard curves included 12 separate standard curves with each standard curve including three technical replicates.

For each sampling site, e.g., Downstream 1 Left, a set of two duplicates were obtained, however some values were omitted due to degradation from an extra freeze-thaw cycle or due to potential contamination noted by NTC amplification. For each water sample, it could only be thawed once otherwise the sample would degrade and no longer be usable. One sample contained two target DNA's therefore two separate plates and mixes at a minimum would be required once one water sample was thawed. It was not initially clear that one extra cycle of freeze-thaw would make some samples completely unusable. At the initial stages of sample analysis, several samples underwent two cycles of freeze-thaw and this was enough to require omittance of these samples. For T11, the sets of samples that were omitted from the BTC reporting were DS1, left bank, 1st duplicate; DS1, centre, 1st duplicate; and DS2, centre, 1st duplicate. For T11, three sets of samples (a time series of water samples from a specific downstream site's segment) were omitted. For the T22 tracer, the samples omitted were DS1, centre, 1st duplicate; and DS2, centre, 1st duplicate. For T22, two sets of water samples were omitted therefore two breakthrough curves could not be created and both centre site breakthrough curves for DS1 and DS2 do not include averaged values like the DS1 and DS2 left and right breakthrough curves for T22. The centre water samples were analyzed first therefore several of those ones underwent extra freeze-thaw at the early stages of the analysis process. Initially, T11 and

T22 were not analyzed on the same day and that led to only one of the samples having a usable BTC for that sample. A single freeze-thaw would lead to a rapid decline in concentration and a BTC could not be created. Additional freeze-thaw cycles did not lead to complete losses of the DNA and the target DNA was still detectable in the samples, but the samples are not useful for creating BTCs.

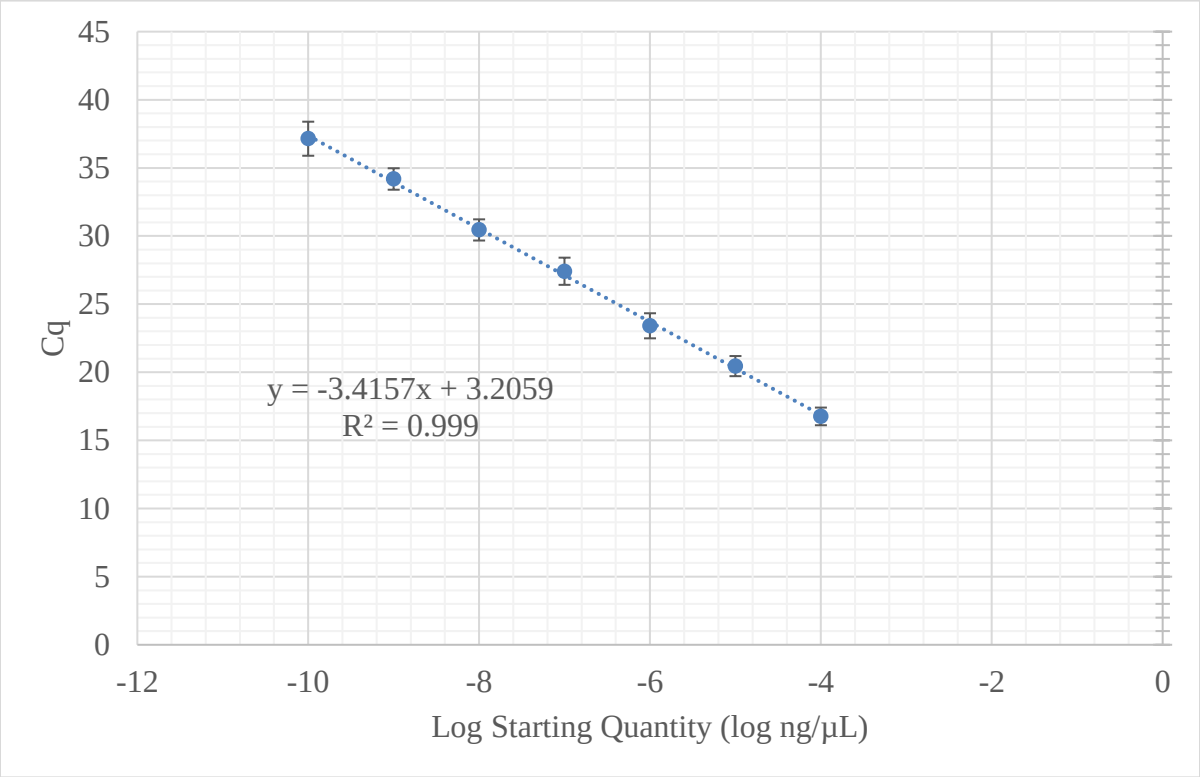

Figure A - 6. T11 average standard curve with efficiency 96.23% that was used to quantify water samples. The efficiency is calculated from the slope value, according to Equation 1. The slope (*s*) is based on the equation in the figure and is -3.4157.

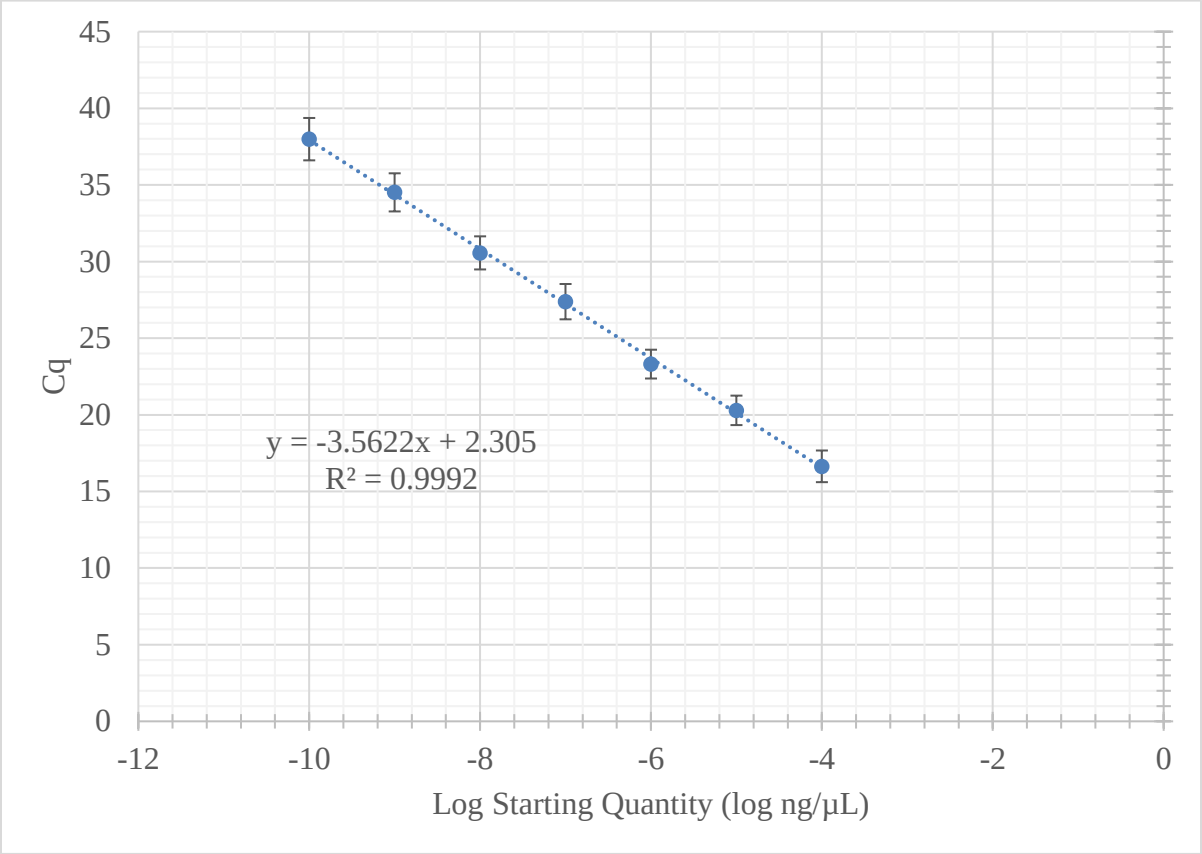

Figure A - 7. T22 standard curve with efficiency 90.86% that was used to quantify water samples. The efficiency is calculated from the slope value, according to Equation 1. The slope (*s*) is based on the equation in the figure and is -3.5622.

Table A - 2. Cq and concentration for T11 Downstream 1 samples.

Time after injection (min)	T11 Downstream 1 Left		T11 Downstream 1 Centre		T11 Downstream 1 Right			
	Second duplicate		Second duplicate		First duplicate		Second duplicate	
	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)
6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	0.00	0.00	0.00	0.00	39.54	2.31E-11	38.51	4.63E-11
8	32.85	2.09E-09	31.02	7.21E-09	30.47	1.05E-08	28.86	3.08E-08
9	28.36	4.33E-08	27.68	6.85E-08	27.22	9.32E-08	27.10	1.01E-07
10	27.06	1.04E-07	26.82	1.22E-07	26.22	1.83E-07	25.84	2.36E-07
11	25.32	3.36E-07	26.00	2.12E-07	25.57	2.84E-07	25.18	3.68E-07
12	25.57	2.83E-07	25.59	2.79E-07	25.62	2.74E-07	25.48	3.01E-07
13	26.65	1.37E-07	27.19	9.52E-08	26.09	2.00E-07	26.14	1.93E-07
14	26.78	1.26E-07	28.03	5.41E-08	27.61	7.15E-08	27.99	5.54E-08
15	26.98	1.10E-07	27.31	8.76E-08	28.00	5.51E-08	27.88	5.98E-08
16	27.87	6.01E-08	28.60	3.67E-08	29.62	1.85E-08	28.19	4.83E-08
17	28.40	4.19E-08	29.35	2.21E-08	29.31	2.28E-08	28.97	2.87E-08
18	29.46	2.06E-08	29.97	1.46E-08	30.50	1.02E-08	29.91	1.52E-08
19	29.89	1.54E-08	30.66	9.15E-09	30.57	9.76E-09	30.21	1.24E-08
20	30.78	8.44E-09	31.03	7.14E-09	31.78	4.30E-09	30.76	8.57E-09
21	31.56	5.01E-09	31.95	3.83E-09	31.60	4.88E-09	31.66	4.67E-09
22	32.21	3.24E-09	33.11	1.76E-09	32.67	2.36E-09	32.80	2.17E-09
23	32.35	2.93E-09	33.36	1.49E-09	33.30	1.55E-09	33.53	1.33E-09
24	33.00	1.90E-09	33.42	1.43E-09	34.62	6.36E-10	33.64	1.23E-09

Table A - 3. Cq and concentration for T11 Downstream 2 samples. Blanks represent samples that were not analyzed.

Time after injection (min)	T11 Downstream 2 Left				T11 Downstream 2 Centre		T11 Downstream 2 Right			
	First duplicate		Second duplicate		Second duplicate		First duplicate		Second duplicate	
	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)
20					0.00	0.00				
21	0.00	0.00	38.93	3.49E-11	38.66	4.17E-11	40.09	1.59E-11	39.18	2.94E-11
22	36.22	2.16E-10	36.10	2.34E-10	35.65	3.16E-10	36.68	1.59E-10	35.08	4.66E-10
23	33.16	1.70E-09	33.53	1.33E-09	32.53	2.61E-09	33.75	1.14E-09	32.47	2.7E-09
24	31.34	5.78E-09	31.52	5.13E-09	31.53	5.11E-09	30.79	8.41E-09	30.69	9.01E-09
25	30.24	1.21E-08	29.46	2.07E-08	29.83	1.61E-08	30.01	1.42E-08	29.85	1.58E-08
26	29.66	1.80E-08	28.07	5.25E-08	28.53	3.85E-08	29.71	1.73E-08	27.90	5.89E-08
27	28.38	4.28E-08	27.91	5.86E-08	28.57	3.75E-08	29.27	2.33E-08	27.61	7.16E-08
28	27.49	7.77E-08	27.70	6.76E-08	28.14	5E-08	28.00	5.50E-08	27.76	6.49E-08
29	27.31	8.79E-08	27.30	8.83E-08	27.86	6.07E-08	27.84	6.15E-08	27.73	6.59E-08
30	27.35	8.54E-08	26.79	1.25E-07	27.24	9.17E-08	27.71	6.71E-08	27.10	1.01E-07
35	28.70	3.43E-08	27.83	6.18E-08	28.21	4.79E-08	28.89	3.02E-08	27.46	7.94E-08
40	29.92	1.51E-08	30.11	1.33E-08	31.24	6.2E-09	30.20	1.25E-08	30.19	1.26E-08
45	32.23	3.18E-09	31.98	3.76E-09	32.97	1.94E-09	32.41	2.83E-09	32.72	2.29E-09
50	34.44	7.15E-10	34.23	8.25E-10	34.39	7.43E-10	34.38	7.48E-10	34.15	8.73E-10

Table A - 4. Cq and concentration for T22 Downstream 1 samples. Blank cells represent samples that were not analyzed.

Time after injection (min)	T22 Downstream 1 Left				T22 Downstream 1 Centre		T22 Downstream 1 Right			
	First duplicate		Second duplicate		Second duplicate		First duplicate		Second duplicate	
	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)
6	40.85	1.51E-11	0.00	0.00			0.00	0.00	0.00	0.00
7	0.00	0.00	0.00	0.00	38.38	7.45E-11	0.00	0.00	36.96	1.86E-10
8	33.22	2.10E-09	31.89	4.96E-09	30.89	9.46E-09	30.80	9.99E-09	27.91	6.48E-08
9	28.80	3.65E-08	27.49	8.49E-08	26.82	1.31E-07	26.71	1.41E-07	26.41	1.71E-07
10	26.89	1.25E-07	26.18	1.98E-07	26.30	1.84E-07	25.23	3.68E-07	24.54	5.74E-07
11	25.38	3.32E-07	24.50	5.89E-07	26.56	1.55E-07	25.45	3.17E-07	24.54	5.74E-07
12	24.95	4.40E-07	24.64	5.36E-07	25.74	2.64E-07	25.50	3.09E-07	24.49	5.93E-07
13	26.77	1.36E-07	25.88	2.41E-07	26.89	1.25E-07	26.55	1.56E-07	25.16	3.85E-07
14	26.98	1.18E-07	25.75	2.63E-07	27.79	7.03E-08	27.72	7.35E-08	27.31	9.55E-08
15	27.36	9.24E-08	26.01	2.22E-07	27.13	1.07E-07	28.54	4.33E-08	26.79	1.33E-07
16	27.36	9.25E-08	26.82	1.31E-07	28.33	4.95E-08	29.03	3.15E-08	27.18	1.04E-07
17	28.12	5.68E-08	27.92	6.44E-08	28.84	3.56E-08	29.30	2.65E-08	28.03	6.01E-08
18	29.92	1.77E-08	28.23	5.27E-08	29.20	2.81E-08	30.43	1.27E-08	29.51	2.31E-08
19	29.35	2.56E-08	28.91	3.39E-08	30.45	1.25E-08	30.67	1.09E-08	29.15	2.92E-08
20	29.90	1.79E-08	29.67	2.07E-08	30.86	9.65E-09	33.12	2.24E-09	29.90	1.79E-08
21	30.60	1.14E-08	30.38	1.31E-08	31.77	5.34E-09	32.34	3.70E-09	30.76	1.03E-08
22	31.57	6.11E-09	31.04	8.59E-09	32.35	3.67E-09	32.89	2.60E-09	31.88	4.98E-09
23	32.07	4.40E-09	31.12	8.16E-09	33.17	2.17E-09	33.85	1.39E-09	32.09	4.35E-09
24	32.58	3.17E-09	32.08	4.37E-09	33.54	1.71E-09	34.75	7.80E-10	32.81	2.73E-09

Table A - 5. Cq and concentration for T22 Downstream 2 samples. Blank cells represent samples that were not analyzed.

	T22 Downstream 2 Left				T22 Downstream 2 Centre		T22 Downstream 2 Right			
	First duplicate		Second duplicate		Second duplicate		First duplicate		Second duplicate	
Time after injection (min)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)	Cq	Concentration (ng/uL)
18					40.53	1.85E-11				
19					39.31	4.10E-11				
20					40.39	2.03E-11				
21	0.00	0.00	40.11	2.45E-11	37.92	1.00E-10	38.91	5.28E-11	40.14	2.40E-11
22	35.98	3.53E-10	36.41	2.67E-10	35.20	5.82E-10	35.84	3.84E-10	36.05	3.36E-10
23	32.63	3.08E-09	32.64	3.05E-09	31.68	5.68E-09	33.32	1.96E-09	32.55	3.23E-09
24	30.79	1.01E-08	30.93	9.24E-09	30.53	1.19E-08	30.87	9.59E-09	30.96	9.06E-09
25	29.72	2.02E-08	29.80	1.92E-08	29.54	2.26E-08	29.71	2.03E-08	29.93	1.76E-08
26	29.27	2.68E-08	28.87	3.50E-08	27.96	6.28E-08	29.59	2.19E-08	28.16	5.53E-08
27	27.83	6.83E-08	27.52	8.37E-08	27.51	8.38E-08	28.91	3.41E-08	28.37	4.81E-08
28	26.86	1.28E-07	27.50	8.45E-08	27.20	1.03E-07	28.08	5.81E-08	28.06	5.89E-08
29	26.87	1.27E-07	27.61	7.85E-08	27.21	1.02E-07	27.81	6.93E-08	28.23	5.27E-08
30	26.66	1.46E-07	27.15	1.06E-07	26.62	1.49E-07	28.35	4.88E-08	28.14	5.61E-08
35	28.11	5.70E-08	27.63	7.76E-08	27.42	8.91E-08	28.19	5.42E-08	28.49	4.45E-08
40	29.79	1.93E-08	29.80	1.91E-08	30.43	1.27E-08	30.56	1.17E-08	30.53	1.19E-08
45	32.60	3.13E-09	32.52	3.30E-09	32.68	2.97E-09	33.15	2.19E-09	31.74	5.44E-09
50	34.95	6.87E-10	34.70	8.04E-10	34.04	1.23E-09	35.42	5.04E-10	34.31	1.04E-09