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Molecular Methods for the Identification and Quantification of Cyanobacteria in Surface Water Sources

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Molecular Methods for the Identification and Quantification of
Cyanobacteria in Surface Water Sources

Treyton Michael Moore

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

Molecular Methods for the Identification and Quantification of Cyanobacteria in Surface Water Sources

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Geosmin is a strong musty-flavored organic compound that is responsible for many taste-and-odor events in surface drinking water sources like lakes and reservoirs. The taste threshold of geosmin for humans is lower than 10 ng/L. Traditional treatment methods will not remove geosmin to this level. Additional water treatment methods must be implemented to successfully remove the geosmin and its associated flavor and odor from drinking water. Furthermore, geosmin is produced by cyanobacteria somewhat sporadically, so it is difficult to predict when taste-and-odor events are going to occur. The difficulty involved with predicting geosmin events has led most water treatment facilities to adopt reactive approaches towards geosmin treatment; these facilities typically treat for geosmin in response to complaints of an earthy off-flavor in the drinking water. This reactive approach causes issues with consumer confidence, as the flavor of the water is one of the only metrics a consumer has for judging the safety of his or her water. To enable proactive treatment of geosmin from water, more sensitive methods for geosmin detection or taste-and-odor event prediction must be developed.

This study investigates the use of quantitative Polymerase Chain Reaction (qPCR) for the early detection of geosmin-producing cyanobacteria. qPCR can detect geosmin-producing cyanobacteria via their DNA. I developed a qPCR assay for this study that is capable of sensitively detecting multiple strains of the geosmin-producing *Nostoc* genus. The developed assay showed high sensitivity, demonstrating the possibility for its use in detecting low concentrations of geosmin-producing cyanobacteria before detectable levels of geosmin have been produced and released into the water. Through further sequencing of more geosmin-producing genera and species, the methodology outlined in this research could be applied to develop the tools necessary to predict taste-and-odor events caused by geosmin-producing cyanobacteria.

Keywords: geosmin, cyanobacteria, qPCR

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Lastly, I would like to thank my wife, Sarah Moore, and my daughter, Ruby Moore, for their support and patience with me. They made this whole endeavor worth the effort.

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1 INTRODUCTION

1.1 Cyanobacterial Production of Odorous Metabolites

According to the most recent USGS water use report, 63% of public water supply comes from surface-water sources (USGS 2017). Though these surface-water sources are susceptible to contamination from a variety of causes, cyanobacterial contamination has been a major source of concern in recent years. Cyanobacteria create many issues with the water quality of surface water sources. One of the many water quality concerns caused by cyanobacteria is the production of taste-and-odor compounds such as geosmin. Though there are multiple compounds that cause off-flavor issues with water, the research presented in this document focused on geosmin-producing cyanobacteria. Geosmin is a volatile organic compound (VOC) that has a distinct musty/earthy flavor. Because humans can taste geosmin in concentrations lower than 10 ng/L (Hobson et al. 2010), traditional treatment methods will not produce water without objectionable taste and odor characteristics. Furthermore, the extra treatment required for effective geosmin removal is often too expensive to be implemented year-round, so most treatment plants treat on an as-needed basis.

Unfortunately, geosmin events cannot currently be predicted, so most water treatment facilities treat reactively: they treat in response to complaints of off-flavor water received from their consumers. This treatment practice harms the public opinion of the safety of their drinking water, as water exhibits taste-and-odor before it is treated. Though the consumption of geosmin

has no known negative health implications, its off-flavor causes concern in consumers.

Consumers are typically only capable of judging the quality of their water via two criteria: flavor and clarity (lack of turbidity). If either of these criteria is compromised, the consumer has no way of knowing that their water is still safe to drink. For this reason, methods for either economical early detection of geosmin events or prediction of geosmin events have been investigated (Jiang et al. 2008; John et al. 2018; Jüttner and Watson 2007). Recent advances in molecular biology have provided promising technology that may be adequately sensitive and economical to use for the detection of geosmin-producing cyanobacteria. The purpose of this research is to investigate one of these molecular methods, quantitative Polymerase Chain Reaction (qPCR), and its potential for early detection or prediction of geosmin events.

1.2 Molecular Methods for the Detection of Cyanobacteria

qPCR is a method that allows the molecular detection and quantification of DNA. qPCR uses polymerase chain reaction (PCR) to amplify a targeted DNA sample via duplication and fluorescent markers to measure the amount of product created via PCR. Because PCR duplicates the DNA exponentially, it can take a minute sample of DNA and amplify it until there are billions of copies of the DNA. Due to the sensitivity of qPCR, we investigated it for its ability to detect trace amounts of cyanobacteria for geosmin-producing potential before the organisms have released enough geosmin into the water to cause off-flavor issues. This research investigated the full methods required for developing the tools necessary to use qPCR to detect and quantify geosmin-producing cyanobacteria. In doing so, we identified species capable of producing geosmin through literature review, we found a successful method for properly extracting DNA from cyanobacteria samples, and we learned the steps required to develop a successful qPCR assay. We then implemented these methods to develop a qPCR assay that could

accurately and sensitively detect a variety of strains belonging to the *Nostoc* genus, a genus known for its geosmin-producing potential.

2 LITERATURE REVIEW

2.1 Current Methods for Geosmin Treatment

Conventional water treatment (coagulation, sedimentation, and chlorination) does not effectively remove taste-and-odor compounds like geosmin (Bruce et al. 2002). The difficulty involved in treating geosmin is tied to its low detection threshold in humans. Humans are capable of detecting geosmin in concentrations lower than 10 ng/L (Hobson et al. 2010). Multiple methods have been investigated to test their efficacy in removing geosmin and other taste-and-odor compounds from drinking water sources. In 2011, Srinivasan and Sorial reviewed the use of various methods and found adsorption by powdered activated carbon (PAC) to be the most effective method for the treatment of water contaminated with taste and odor compounds (Srinivasan and Sorial 2011). They also found that ozone and other advanced oxidation processes (AOPs), like UV and H₂O₂ can also be very effective, but their implementation would require a significant capital investment. Because taste-and-odor events caused by cyanobacteria are seasonal, the implementation of expensive infrastructure is not practical, making PAC treatment especially attractive due to its flexibility in implementation (Srinivasan and Sorial 2011). Zamyadi, Henderson et al. also confirmed that PAC treatment is effective at removing dissolved geosmin from drinking water (Zamyadi et al. 2015). Though adequate treatment methods are available that can be implemented in a cost-effective manner, proactive treatment of geosmin is not implemented due to the difficulties involved with predicting geosmin events. Watson et al.

(2016) outline a number of the difficulties involved with developing proactive treatment approaches to removing geosmin. Current methods that could be implemented proactively are too resource intensive to be practically implemented, there is a lack of effective diagnostic tools, and these taste-and-odor events are unpredictable. The currently available methods for identifying cyanobacteria and examining them for potential geosmin producers involves the collection of a highly concentrated sample that can then be taxonomically analyzed. This process is both time and resource intensive. Furthermore, because taxonomic analysis requires a concentrated sample, some sort of taste-and-odor (T&O) event is likely already occurring in the water source by the time it can be effectively analyzed. The development of sensitive molecular methods like PCR and qPCR have provided a more cost-effective option that could prove capable of identifying problematic taxa before T&O events occur. Recent research into which taxa are problematic and their associated sequences have made the use and development of these new molecular methods feasible for the detection of geosmin producers (Graham et al. 2010).

2.2 Known Geosmin Producing Cyanobacteria

Though both 2-Methylisoborneol (MIB) and geosmin are known to cause taste and odor problems in drinking water, Graham et al. (2010) found during their study that geosmin is more commonly produced. In all of the blooms that they sampled and analyzed throughout their study, they detected geosmin in 87% of the analyzed blooms, but they only detected MIB in 39% of their analyzed blooms. For this reason, this study has been focused on geosmin and geosmin-producing cyanobacteria. A great deal of research has already been performed that identifies cyanobacteria as a major contributor to the production of geosmin in surface waters (Graham et al. 2010; Jüttner and Watson 2007). To properly develop molecular methods to detect problematic cyanobacteria, the individual species responsible for geosmin production needed to

be identified. Jüttner and Watson (2007) confirmed the role of cyanobacteria as geosmin producers and also identified the following genera as producers: *Phormidium*, *Anabaena*, *Aphanizomenon*, and *Planktothrix*. There were other genera included in their list, but they are not common to the Utah Valley area, so we did not take them into consideration for this study. The additional genera they outlined can be viewed in Table 2 of the referenced document. In addition, Jüttner and Watson (2007) identified a number of specific species amongst these genera, including *Anabaena circinalis*, *Aphanizomenon flos-aquae*, and *Aphanizomenon gracile*, to name a few. Graham et al. (2010) also confirmed *Anabaena*, *Aphanizomenon*, and *Planktothrix* as genera with geosmin producing capabilities. In addition to the species identified above, Suurnäkki et al. (2015) successfully identified a number of different species in the *Nostoc* genus as geosmin producers. Researchers Watson et al. (2016) compiled a comprehensive list of genera and species that produce various VOCs, including geosmin. Table 1 in the referenced document lists these producers by species and includes additional *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* strains in addition to new genera like *Lyngbya*, *Calothrix*, and *Cylindrospermum*, to name a few. It is worth noting that various species in the same genus may produce different VOCs. For example, Watson et al. (2016) show that *Lyngbya aestuarii* produces MIB, whereas *Lyngbya subtilis* produces geosmin. Furthermore, diversity in VOC production was noted even amongst different strains of cyanobacteria. Watson et al. (2016) show in their Table 1 that one strain of *Phormidium limosum* was found to produce only MIB, while a different strain of *Phormidium limosum* produced both MIB and geosmin.

2.3 Polymerase Chain Reaction

According to the National Institute of Health, PCR “is a fast and inexpensive technique used to ‘amplify’ – copy – small segments of DNA” (Health 2015). In order to do so, the sample

of DNA is heated until the DNA denatures, or “unzips,” into two single strands of DNA. Once the DNA is denatured, the Taq polymerase enzyme uses the original strands of DNA as templates to build the complementary strands, resulting in a copy of the original DNA segment. At this point there are now two complete segments of DNA; each copy contains a new strand of DNA and the old strand of DNA. This process can be repeated multiple times, allowing a small sample of DNA to be exponentially amplified. Figure 1 below shows how this process works. Please note that the temperatures listed in Figure 1 are not absolute and may differ depending on the primers and thermal cycler that are used.

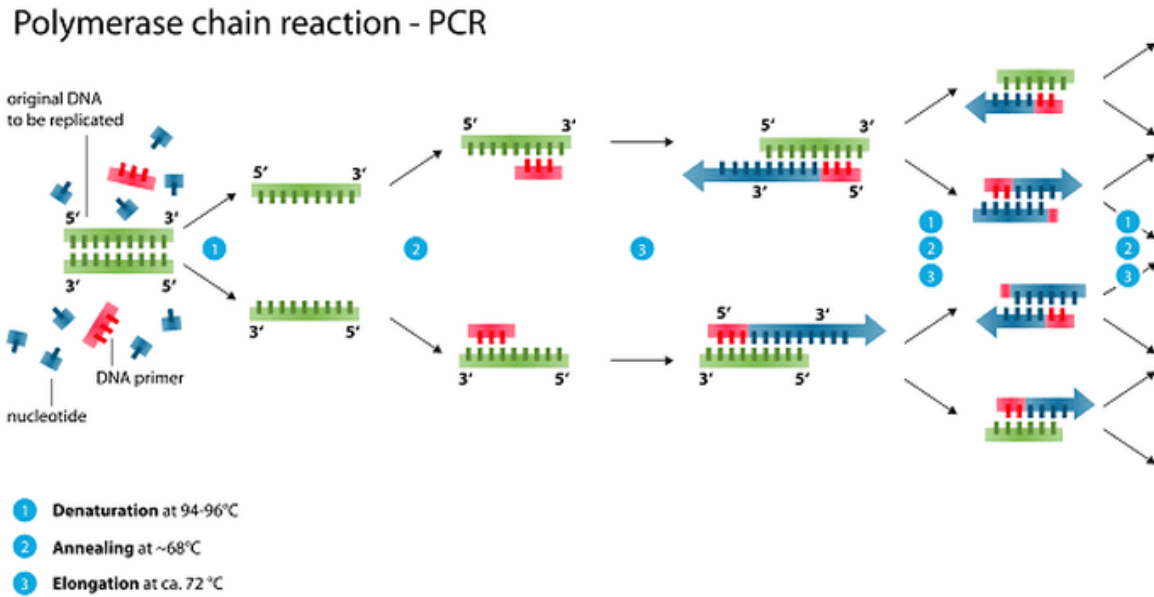


Figure 1: PCR Process (Phillips, T. (n.d.))

The resulting PCR product (DNA amplified after multiple PCR reactions) can then be analyzed to determine if the desired gene was present. The amplification procedure is facilitated by a thermal cycler. The thermal cycler heats the DNA to the proper denaturing temperature (about 94°C), cools down so that the primers can anneal to the denatured DNA (typically

between 50°C and 56°C), then heats back up to about 72°C to allow the polymerase to extend and copy the strands of DNA. These three steps are then repeated 35-40 times, and each step lasts between 15 and 30 seconds, so each cycle will typically last for 1-2 minutes.

For the Taq polymerase enzyme to begin its chain reaction, primers are required. Primers are short strands of DNA that provide starting points for the reaction to start, as enzymes like Taq polymerase can only add new nucleotides to an existing strand of DNA. Use of these primers is what permits PCR to target specific genes. Both a forward primer and a reverse primer are used. The forward primer is a short strand of DNA that matches the DNA at the beginning of the target gene sequence. The reverse primer is a short strand of DNA that matches the complement to the DNA at the end of the target sequence. With the use of carefully designed primers, only the target sequence of a given gene will be amplified. The target gene and associated PCR product can be as short as 75 base pairs long or longer than 900 base pairs depending on the desired application. In addition to the use of primers, master mixes are used to provide free nucleotides that can be used as “building blocks” in the polymerase chain reaction. These master mixes typically contain the enzymes that facilitate polymerase chain reaction, free nucleotides, and buffers to promote reaction stability.

We investigated PCR due to the advantages it can offer in molecular detection. In his book *Advanced Topics in Forensic DNA Typing: Methodology*, Butler (2011) outlines a number of the advantages that PCR can offer. Though his book is written with forensic science in mind, the advantages can still be applied to detecting cyanobacteria. John M. Butler lists advantages of PCR to be among the following: “very small amounts of DNA template may be used from as little as a single cell,” contaminant DNA will not amplify as long as care has been taken in primer design, and “large numbers of copies of specific DNA sequences can be amplified

simultaneously with multiplex PCR reactions” (Butler 2011). Because the taste threshold for geosmin can be lower than 10 ng/L (Watson et al. 2016), very few cyanobacteria are required to cause off-flavor issues in water. The sensitivity of PCR has potential to detect off-flavor producers before taste-and-odor events occur. The last advantage listed above will be important due to the vast variety found amongst the genes that code for geosmin production in cyanobacteria. According to John et al. (2018), the gene responsible for the production of geosmin “is variable at the nucleotide level and potential geosmin producers represent a broad taxonomic distribution.” This broad range of potential producers facilitates the need for multiple PCR primer pairs with different amplification targets to properly encompass the range of problematic species. Through proper design of multiple primer pairs that are compatible, a multiplex PCR reaction could be developed that targets and amplifies the entire spectrum of geosmin-producing cyanobacteria.

Multiple researchers have shown that PCR can successfully detect cyanobacteria (Jiang et al. 2008; Kataoka et al. 2013; Suurnäkki et al. 2015). Researchers have developed primers that can detect 76% of the *Microcystis* sequences currently available in GenBank (Kataoka et al. 2013). Jiang et al. (2008) successfully developed a PCR primer pair that amplified the entire geosmin synthase gene of *Nostoc punctiforme*. The resulting PCR product was around 700 base pairs long. In addition, these researchers were also able to use their primer pair to amplify sequences on *Anabaena laxa*, a different *Nostoc* strain, and *Phormidium calcicola* (Jiang et al. 2008). Suurnäkki et al. (2015) developed a pair of PCR primers that amplified the geosmin synthase gene on a number of known geosmin producing cyanobacteria. Their PCR product was just over 900 base pairs long and was successful in identifying a range of species, including a

strain of *Aphanizomenon*, two *Oscillatoria* strains, two *Planktothrix* strains, and a number of *Nostoc* strains (Suurnäkki et al. 2015).

Though there are many advantages to using PCR, it is not without its disadvantages. According to John M. Butler, “the target DNA template may not amplify due to the presence of PCR inhibitors in the extracted DNA” (Butler 2011). Many PCR inhibitors such as humic compounds and polysaccharides can be present in nature (Butler 2011), so care must be taken when collecting and preparing samples to avoid contamination as much as possible. The largest drawback to using conventional PCR in identifying cyanobacteria lies in its inability to quantify the DNA that is amplified. Though various types of end-point analysis could be used after a PCR reaction to quantify the amount of available DNA, PCR inhibitors, reagent limitation, or “accumulation of pyrophosphate molecules, the PCR reaction eventually ceases to generate template at an exponential rate (i.e., the plateau phase) making the end point quantitation of PCR products unreliable” (Arya et al. 2005). This lack of reliability stems from the fact that these issues cause duplicate reactions to produce differing amounts of PCR product instead of exponential duplication. Without the ability to quantify the target gene sequence in a given sample, it becomes difficult to develop tools or protocol for forecasting taste-and-odor events. For this reason, we investigated the use of quantitative Polymerase Chain Reaction (qPCR) and its potential for early, sensitive detection of geosmin-producing cyanobacteria.

2.4 Quantitative Polymerase Chain Reaction

QPCR, also known as real-time PCR, differs from PCR in that it quantifies the amount of amplified DNA after every amplification cycle using fluorescent markers. There are two main methods behind qPCR that are generally used in practice today: SYBR Green qPCR and TaqMan

qPCR. The SYBR Green method uses the SYBR Green intercalating dye to measure amplification. This dye binds to double stranded DNA. The SYBR Green dye emits very little fluorescence when unbound to DNA, but when bound to DNA, it emits a much stronger fluorescent signal that can be detected by the thermal cycler. The amount of fluorescence can then be correlated to the amount of DNA in the product. The TaqMan probe technique relies on the design of a gene-specific probe. The probe behaves in a manner analogous to a primer in that it binds to a target region on the desired gene during the annealing process. Attached to one end of the probe is a fluorescent reporter dye, and the other end of the probe has a quencher dye. While the probe is still attached to the gene sequence, the quencher dye prevents the reporter dye from emitting fluorescence. During extension, the probe is broken down and removed, or cleaved, from the DNA sequence, which separates the reporter dye from the quencher dye, allowing the fluorescence to be emitted. The fluorescence is then measured after every cycle by the thermal cycler and can be correlated to the quantity of PCR product (Bassler et al. 1995; Liu et al. 2006; Livak et al. 1995).

Though both qPCR methods can be effective when used properly, neither is without disadvantages. When using the SYBR Green method, care must be taken during primer design so that genes other than the desired sequence are not amplified, as SYBR Green will bind to any double-stranded DNA. The lack of specificity offered by SYBR Green qPCR leads to the possible generation of false positive signals, which can occur in the presence of primer-dimers or nonspecific products. TaqMan Probe qPCR, though more specific than SYBR Green, is much more expensive to both set up and run. Part of the expense involved in its set-up is the design of the probes. Probes are typically a few base-pairs longer than the primers, as their melting temperature needs to be at least 5-10°C higher than the primer melting temperature, which

requires a larger conserved region on the target gene. Furthermore, checks must be made to ensure that the designed probe will not have a higher affinity towards binding with either of the primers than it will towards binding with the target DNA. TaqMan Probe qPCR is still very useful, as its selectivity ensures that only the specific PCR product is able to emit fluorescence (Cao and Shockey 2012).

A number of other studies have shown success with developing qPCR assays capable of detecting cyanobacteria and other geosmin-producing organisms (Auffret et al. 2011; Su et al. 2013; Tsao et al. 2014). Marc Auffret and his fellow researchers successfully developed a qPCR assay capable of detecting the geosmin producing actinobacteria *Streptomyces*. Their assay targeted the *geoA* gene that codes for geosmin synthesis, and Auffret et al. (2011) used SYBR Green as their fluorophore for quantifying their PCR product. Su et al. (2013) applied qPCR technology to develop a SYBR Green assay that was capable of detecting most strains of the known geosmin-producing cyanobacteria *Anabaena sp.* Their assay targeted the *Anabaena rpoC₁* gene, making their qPCR very specific to *Anabaena*. Su et al. (2013) then tested their assay on 15 *Microcystis* strains and 2 *Cylindrospermopsis* strains via conventional PCR to test for specificity of their primers. Their PCR resulted in no amplification for any of the *Microcystis* strains and no amplification for any of the *Cylindrospermopsis* strains, showing the specificity of the designed primers (Su et al. 2013). Another group of researchers, Tsao et al. (2014), looked to target *Anabaena sp.* like Su et al. (2013), but their method targeted the species via its geosmin producing gene, *geoA*, instead of through the *Anabaena rpoC₁* gene. Their qPCR assay was also able to detect numerous strains of *Anabaena sp.*, but it was limited to that genus (Tsao et al. 2014). Though their focus was on 2-Methylisoborneol (2-MIB), Chiu et al. (2016) were able to successfully design a working probe and primer set that successfully detected 17 different strains

of cyanobacteria. Their work showed the possibility for developing a qPCR assay that works on fields samples of cyanobacteria instead of pure laboratory cultures. They tested their qPCR approach on 29 different reservoirs in Taiwan and found high correlations between their qPCR results and actual 2-MIB concentrations in each reservoir (Chiu et al. 2016). This work showed that the technology holds great promise for developing a working qPCR assay that can successfully predict geosmin events in reservoirs and lakes found in Utah. Up until this point, successful qPCR approaches to detecting and quantifying geosmin-producing cyanobacteria have not been able to detect a broad range of genera. The purpose behind this study is to develop a qPCR assay, or the tools leading to a potential qPCR assay, that can successfully detect a majority of geosmin producers, or at least geosmin producers in Utah, so that methods can be developed for predicting these off-flavor episodes in the drinking water supply.

3 MATERIALS AND METHODS

3.1 Primer Design Preparation

Before designing primers for the PCR/qPCR assays, taxonomy data on cyanobacteria genera commonly found in Deer Creek reservoir were analyzed. The data were gathered by Rushforth Phycology and supplied by Erik Cram from the Central Utah Water Conservancy District. The data supplied listed genera and species found in Deer Creek reservoir for the years 2011 through 2017. The most common geosmin-producing genera observed in Deer Creek reservoir were *Anabaena* and *Aphanizomenon*, though many other problematic geosmin-producing genera were observed, including *Nostoc*, *Dolichospermum*, and *Cylindrospermopsis*, to name a few. Sequences for the geosmin synthase gene of geosmin-producing cyanobacteria were found in the National Center for Biotechnology Information (NCBI) GenBank, which is a sequence database that contains all publicly available DNA sequences. At the time of this study, the *Nostoc* genus was the most documented in GenBank. In addition, *Nostoc sp.* ATCC 53789, one of the strains whose geosmin synthase gene was sequenced in GenBank, had cultures available for purchase through the American Type Culture Collection (ATCC). Though other genera are historically more prolific than *Nostoc* in Deer Creek Reservoir, this strain was selected as the control species for this study due to the availability of its sequence and the ability to easily acquire a pure laboratory culture.

With the control strain selected, we ran its sequence through a basic local alignment search tool (BLAST) to find other sequenced strains of cyanobacteria that would align well with the control, and we used the online BLAST provided by NCBI for preliminary analysis. After some review, the following sequences showed the highest overlap, so we chose them as good candidates for primer design: *Nostoc sp.* UK2, *Nostoc sp.* 268, *Nostoc sp.* UK3, *Nostoc sp.* UK4, *Nostoc punctiforme* PCC 73102 NPUNMOD, *Nostoc punctiforme* PCC 73102 NJS, and *Nostoc sp.* ATCC 53789. Table 1 below shows the results received from the alignment check for the aforementioned strains.

Table 1: Nostoc Alignment Results

Strain	Query Cover	E value	% Identical	Accession
<i>Nostoc sp.</i> ATCC 53789	100	0	100%	KJ658368.1
<i>Nostoc punctiforme</i> PCC 73102 NPUNMOD	100	0	96%	FJ010203.1
<i>Nostoc punctiforme</i> PCC 73102 NJ2	100	0	96%	FJ010202.1
<i>Nostoc sp.</i> UK1	99	0	91%	KJ658372.1
<i>Nostoc sp.</i> 268	99	0	91%	KJ658369.1
<i>Nostoc sp.</i> UK3	99	0	90%	KJ658371.1
<i>Nostoc sp.</i> UK4	99	0	90%	KJ658370.1

3.2 Primer Design

In order to visualize the alignment from the BLAST, we loaded the genes listed in Table 1 above into CLC Sequence Viewer. CLC Sequence Viewer is a free software provided by QIAGEN Bioinformatics. This software allows the user to visualize alignments so that primers can be more easily designed. Figure 2 below shows part of the output provided by CLC Sequence Viewer after the target genes were loaded and aligned.

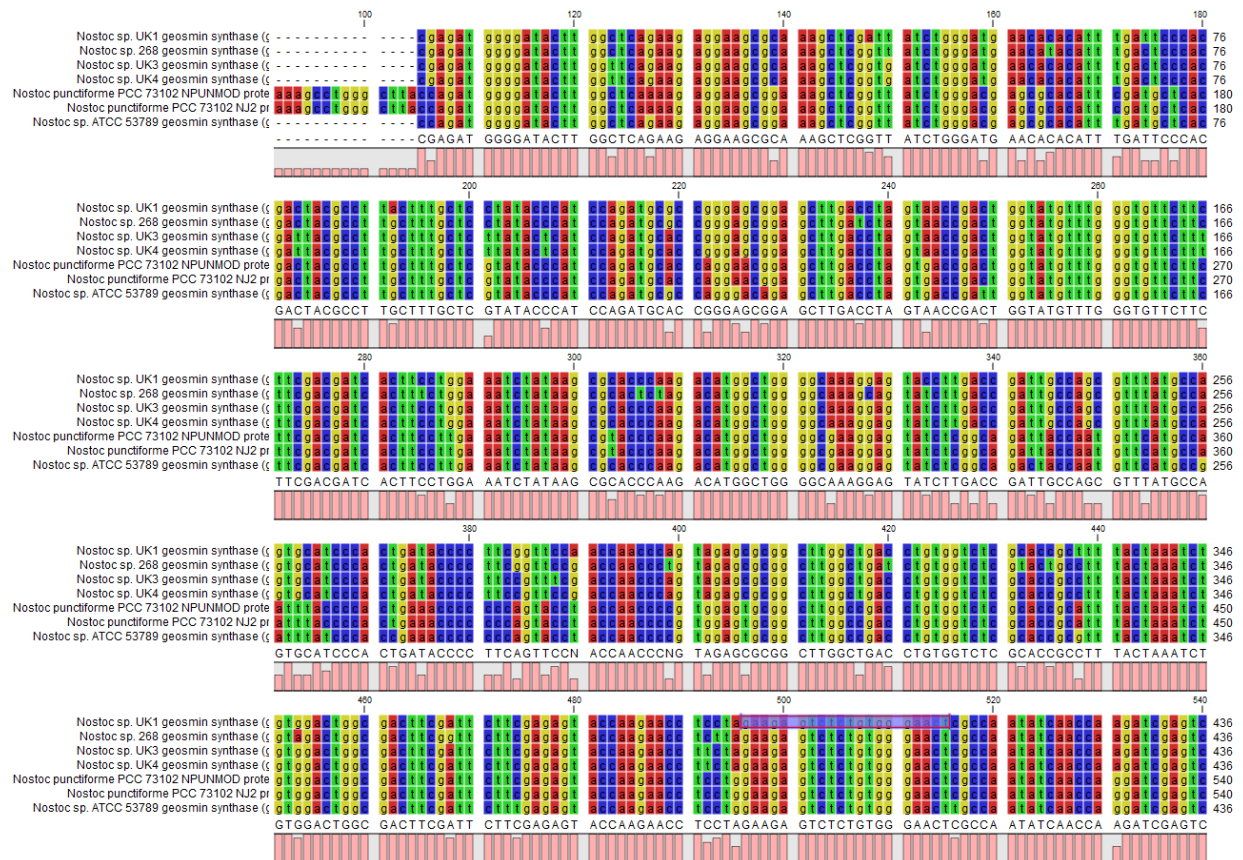


Figure 2: Example of Aligned Genes as Viewed in CLC Sequence Viewer

As viewed in Figure 2, even amongst the most similar of strains of the same genus, dissimilarity is common. To properly design primers that could detect all the selected strains, we identified a number of different conserved regions between 15 and 24 base pairs. To promote the success of the designed primers, numerous criteria were considered. We designed all primers with lengths between 15 and 24 base pairs. In addition, the GC content of the designed primers was kept between 30% and 70%. Hairpin melting temperatures were limited to at least 10°C lower than designed primer melting temperature, and the ΔG values for both hetero-dimers and self-dimers were kept above -7.0 if possible. In addition, the PCR product resulting from the design was kept between about 75 base pairs and 250 base pairs when designing primers that were to be used with a TaqMan probe. Dr. Robison of the Microbiology Department at Brigham

Young University (BYU) and his graduate student, Olivia Brown supplied us with these criteria. Integrated DNA Technologies (IDT) has an online tool (OligoAnalyzer Tool) that we used to check the parameters of our primer design. We input our designed primer sequences in their OligoAnalyzer Tool and checked the various parameters listed above for compatibility. We designed the forward primers first. If a proposed sequence did not meet any of the criteria listed above, we adjusted it by adding base pairs, subtracting base pairs, shifting the entire sequence inside of the conserved region, or testing different conserved regions on the sequences that were aligned for primer design. We iterated through this method until multiple forward primer candidates met the proper criteria. We designed multiple forward primers so that more options would be available later during probe design. Primers are inexpensive and more flexible to design than probes, so having multiple options available for probe design is highly beneficial.

We then designed reverse primers for each of the forward primers based on the same criteria supplied by Dr. Robison. In addition to these criteria, each primer pair was designed so that the resulting PCR product spanned a conserved region that could be suitable for probe design. We ordered all of our designed primers from IDT using the standard desalting option. Once the primers arrived, we rehydrated them with 1x TE buffer according to the provided instructions to create 100 μ M solutions. As a final preparation step, we prepared 10% dilutions of the primers to create working stocks. We prepared each 100 μ L working stock with 10 μ L of forward primer, 10 μ L of reverse primer, and 80 μ L of 1x TE (1x Tris EDTA) buffer.

3.3 DNA Extraction

We used the Qiagen QIAamp DNA Mini kit to extract the DNA from both the control species and field samples. The steps used can be found in Appendix A. Please note that while the

instructions state that the final 2 elution steps ought to be performed with Buffer AE, 1x TE Buffer was used in this study instead as it better preserves the extracted DNA, according to Olivia Brown. We extracted DNA from the *Nostoc* sp. ATCC control sample and used a NanoDrop[®] ND-1000 spectrophotometer to estimate the DNA concentration of the extracted sample. The first extracted DNA control sample had a very low concentration of DNA: less than 1 ng/ μ L. The low concentration is likely due to the fact that most of the cells from the first control culture may have died during culturing. If this was the case, and the cells had lysed upon death, then a lot of the DNA would have been lost in solution and would not have been efficiently extracted. A protocol for ethanol precipitation of DNA was found online through MRC-Holland. This protocol allows the user to precipitate the DNA out of solution, centrifuge the DNA into a mass, and decant excess buffer from the sample, which in turn increases the concentration of DNA in the sample when done properly (MRC-Holland 2008).

We used the following procedures to precipitate the DNA. First, we prepared 10 mL of a 3-molar solution of sodium acetate. The extracted DNA had a volume of 320 μ L, so we added about 32 μ L of the sodium acetate solution to the extracted DNA sample, as the protocol stated to add about 10% by volume of sodium acetate solution. We then added 1000 μ L (2.5X-3X the volume of DNA solution plus sodium acetate solution) of 100% ethanol to the resulting solution and incubated the solution in the freezer overnight because the initial spectrophotometer reading was very low. After incubation, we centrifuged the solution at high speed for 30 minutes, discarded the supernatant, and rinsed the walls of the tube and the DNA pellet with 70% ethanol. We then centrifuged the resulting solution on high speed for 15 minutes, discarded the supernatant, and dissolved the resulting DNA pellet in 1x TE buffer, resulting in a final solution volume of about 150 μ L. The final DNA concentration read by the spectrophotometer read at

43.6 ng/ μ L: a much higher DNA concentration than the starting concentration. Later DNA extractions of the control sample yielded better results and did not require ethanol precipitation, though some field sample extractions did require ethanol precipitation.

3.4 PCR Protocol

Basic PCR reactions typically use the following compounds to facilitate the process: master mix, ddH₂O, template (extracted) DNA, and primers. For the basic PCR used in this study, AmpliTaq Gold™ 360 Master Mix, purchased from ThermoFisher Scientific, was used as the master mix. For most of the reactions performed, we used a mixture of 13 μ L of master mix, 7 μ L of ddH₂O, 3 μ L of extracted DNA sample, and 2 μ L of the primer working stock. In situations where the extracted DNA concentration was very low, we replaced some of the ddH₂O with more of the DNA sample, ranging from a 5 μ L to 5 μ L split to replacing all of the ddH₂O with extracted DNA. In all cases, the total volume of PCR reactants was 25 μ L. All PCR reactants were mixed underneath an AirClean® 600 PCR Workstation DNase/RNase free molecular hood. We mixed all of our reactions in 0.2 mL PCR strip tubes with 8 wells. Each instance of our PCR was run in a ProFlex PCR System thermal cycler by Applied Biosystems®, following the AmpliTaq_360 protocol, which started at a 95°C melting phase for 3 minutes. This 3-minute melting phase was followed by 35 cycles of the following steps: a 30-second melting period at 95°C, a 30-second annealing period at the melting temperature of the primer being used, and then a 1-minute extension period at 72°C. After the 35th cycle, a 7-minute extension period at 72°C was used.

3.5 Gel Electrophoresis

To analyze the PCR product and make sure the right gene was amplified, we used gel electrophoresis. We prepared a 1% agarose gel by mixing 1g of agarose into 100 mL of 1x TAE buffer. We heated and stirred the solution until the agarose was completely dissolved, then added 10 μ L of ethidium bromide to the solution. This quantity of 1% agarose gel was typically enough to analyze 2-3 runs worth of PCR product. The gels were prepared in a gel electrophoresis unit. We loaded the first and last wells with 5 μ L of GeneRuler #SM0333 DNA ladder, then mixed 5 μ L of each tube of final PCR product with 2 μ L of 5x GelPilot DNA Loading Dye loading dye. We loaded the resulting solution into its own well in the agarose gel. Each gel was run at 100 volts, using an EC703 Microprocessor Controlled Electrophoresis machine, for about 30 minutes, with care taken to not run the gels for so long that the DNA migrated through the gel and back into solution. We monitored the progress of the DNA as it traveled through the gel via the bands created by the dye as it traveled through the gel.

Once the gel was finished, we analyzed it using an Amersham Imager 600 fluorescent imager to check for bands at the correct locations along the gel. Figure 3 below shows an example of an agarose gel that we analyzed for PCR amplification. The bars created by the DNA ladder on the sides of the gel act as a benchmark. Each bar represents a different length of DNA. We compared the bars created by the PCR product to the DNA ladder to make sure that the amplified PCR product was the same length as the target sequence. This step allowed a control to make sure that the primers were working as intended. Figure 4 below is from ThermoFisher Scientific's product catalog for the GeneRuler #SM0333 DNA Ladder. It provides the key for the DNA ladder so that PCR product length can be properly compared.

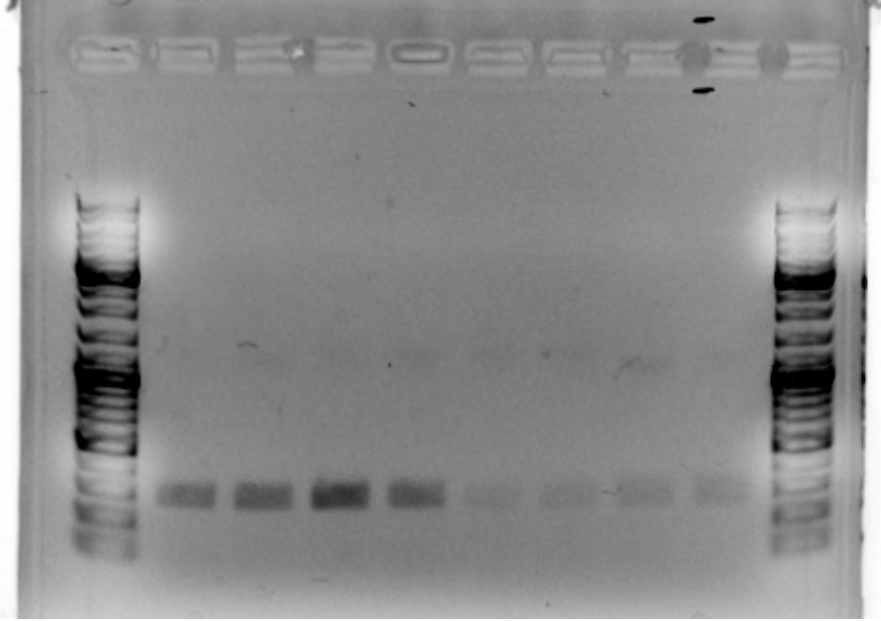


Figure 3: Agarose Gel with Amplified PCR Product and DNA Ladder

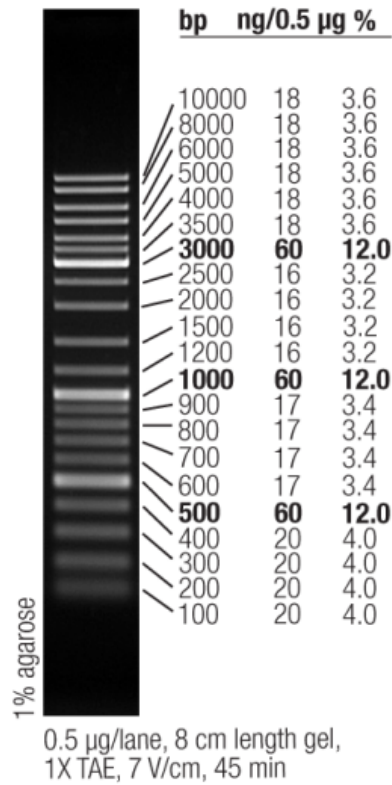


Figure 4: DNA Ladder Key for GeneRule #SM0333

3.6 PCR Optimization

Before designing the probe, optimization of primers was necessary. Interactions between the primers and other compounds used in PCR can affect the efficiency of the reaction. Furthermore, though primer melting temperatures are initially calculated during design, the actual temperature can vary in practice for various reasons, including interactions between forward and reverse primers, interactions between the primers and the PCR ingredients, and interactions between the primers and the extracted DNA itself, to name a few. We used SYBR Green qPCR to optimize the working primers. To determine how efficiently the primers were working in the qPCR, we ran multiple different reactions with different parameters and compared the cycle threshold (CT) values for each set of parameters. The CT value corresponds to the cycle when the amount of fluorescence read by the thermal cycler rises high enough to be statistically significant. In optimization, the lower the CT value, the more efficient the reaction. Outside of optimization, low CT values can also correspond to high starting amounts of template DNA.

During optimization, we ran multiple qPCR reactions at various temperatures and recorded the different resulting CT values. Table 2 below shows the different temperatures that we used when attempting to optimize the primers. After optimization, we chose a melting temperature of 60°C, as it tended to yield a very reasonably low CT value when compared to the other temperatures.

Table 2: PCR Optimization Temperatures

Temperature (°C)	CT Value (Cycles)
54	21.13
55	17.31
56	17.86
57	17.48
58	17.29
59	16.86
60	16.86
61	19.07

3.7 Probe Design

We chose the Taqman Probe qPCR method as the principal qPCR method investigated for this research because of the specificity it enables. Taqman Probe qPCR would also enable the use of a multiplex qPCR assay if necessary. A multiplex qPCR assay allows the use of multiple primer/probe sets simultaneously, allowing a broader coverage of taxa. We designed all probes using similar methods to those used in the design of primers, though we considered some additional criteria. We used the same criteria for self-dimers and hairpins during the design of all probes. In addition, we checked hetero-dimers between the designed probes and both the forward and reverse primers to make sure that their ΔG values were greater than about -7.0 kcal/mole. Furthermore, we designed the final probe so that its melting temperature was at least 5-10°C higher than the design melting temperature of the corresponding primer pair.

3.8 QPCR Protocol and Optimization

For all TaqMan Probe reactions in this study, we used a Cepheid™ SmartCycler® II thermal cycler. The quantities of qPCR reactants used in a typical qPCR run were as follows:

13 μL of TaqMan Multiplex master mix, 6 μL of ddH₂O, 3 μL of extracted DNA, 2 μL of primer working stock, and 1 μL of the TaqMan probe. We prepared all of our qPCR reactions under the molecular hood in 25 μL Cepheid Smartcycler qPCR tubes. The protocol began with a 95°C melting phase for 120 seconds. This melting phase was followed by 40 cycles of the following steps: a 15-second melting period at 95°C, a 30-second annealing phase at the optimized melting temperature, and finally a 30-second extension period at 72°C.

3.9 DNA Standard Curve

To determine the quantity of DNA in the extracted control samples, we created a standard curve using the fluorometer. The standard curve method, though tedious and time intensive, can be used to accurately calculate the concentration of extracted DNA in a control sample. If multiple control samples of varying concentrations are quantified, a qPCR protocol could be calibrated. Calibration of the qPCR assay allows for the development of a correlation between fluorescent levels and DNA concentrations. Though a correlation was not developed for the assay in this study, we explored the methodology for developing a standard curve so that future calibration could be done if necessary. A correlation was not developed for this research because we later determined that a direct correlation will likely not be necessary for the prediction of geosmin events; qPCR fluorescence readings can be correlated directly to geosmin event likelihood. Instructions for developing a standard curve were supplied by Dr. Robison and can be found online (Robisonlab888 2011).

3.10 Sensitivity Check

To check the sensitivity of the designed probe, or in other words, to test how small of a concentration of DNA could be detected in a sample, we created 6 serial dilutions of the control

DNA, ranging from 60.35 ng/ μ L to 6.035E-4 ng/ μ L. We ran all 6 of the dilutions through the qPCR assay to test how low of a concentration could be detected after 40 cycles of qPCR.

4 RESULTS

4.1 Designed Primers

We designed four different working primer pairs that were compatible with the aligned *Nostoc* strains, and we designed one additional reverse primer for Geo2F. We designed the additional reverse primer because its conserved region was large, making the design simple, and the design of the extra primer provided a “backup” in case the first Geo2 reverse primer did not work. Information about each designed primer can be viewed in Table 3 below. Table 4 includes the sequences used for each of the respective primers and the length of their associated PCR product. The ΔG for Geo2.1R was -8.09 kcal/mole, so we designed two reverse primers to complement Geo2F so that another option was available should Geo2.1R not work properly.

Table 3: Primer Design Specifications

Primer	Forward/Reverse?	GC Content	Melting Temp. (°C)	Self-Dimer ΔG (kcal/mole)	Hetero-Dimer ΔG (kcal/mole)
Geo1F	Forward	50%	53.6	-4.77	
Geo1R	Reverse	48%	53.2	-3.61	-4.77
Geo2F	Forward	40%	51.8	-1.47	
Geo2R	Reverse	47%	52.6	-4.77	-1.47
Geo2.1R	Reverse	50%	52.1	-4.77	-8.09
Geo3.1F	Forward	45%	50.8	-3.61	
Geo3.1R	Reverse	39%	51.5	-5.71	-5.34

Table 4: Designed Primers and their Associated Product Length

Primer	Sequence	Product Length (Base Pairs)
Geo1F	GAA GAG TCT CTG TGG GAA CT	145
Geo1R	CGG AAT CTC TAT AAA CAC GGC	
Geo2F	TGG TAT GTT TGG GTG TTC TT	266
Geo2R	AGT TCC CAC AGA GAC TCT T	
Geo2.1R	AGT TCC CAC AGA GAC TCT	265
Geo3.1F	CGC CGT GTT TAT AGA GAT TC	246
Geo3.1R	GTG TTA TCA AAC TGG TAT AAC CG	

Though we designed many more primers in this study than those tabulated above, most of them did not work. Several primers did not work due to the inexperience of those designing them. Early attempts at primer design were met with failure because the primers were designed based off gene sequences that had been reverse translated from amino acid sequences. This process did not work because there are degenerate codons that do not code for amino acids but are still included in the genetic sequence; any sequences derived from amino acid sequences would have been incomplete. Once corrected, subsequent designs yielded varying amounts of amplification. Figure 5 shows the gel electrophoresis for the primer pairs listed in Table 4. We used gel electrophoresis on all ordered primers to check for proper PCR amplification. As seen in Figure 5, the first two bands, which correspond to the primer named Geo1, are the darkest. Darker bands tend to correlate to higher amounts of amplified PCR product, so we chose Geo1 for optimization in preparation for probe design.

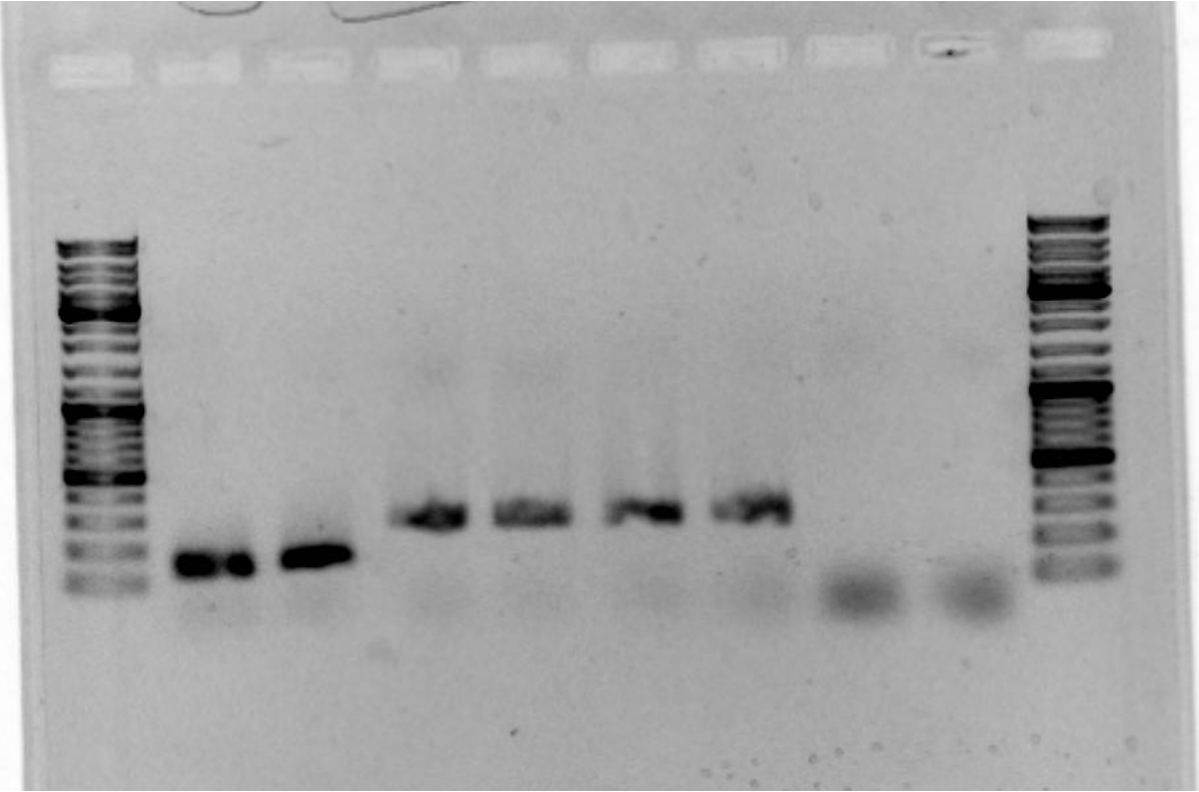


Figure 5: PCR Gel Showing Strong Amplification for the Primer Pair Geo1

The middle four bands corresponded to the Geo2 and Geo2.1 primer pairs; the left two were from Geo2 and the right two were from Geo2.1. Though the bands from these reactions are in the correct location along the ladder, meaning that the gene sequence that was amplified was indeed the sequence we were targeting, these bands are lighter than those created by the Geo1 PCR. These bands are likely less dark for several reasons. First, the gel tore slightly when removing the comb from the wells, which accounts for the “holes” in the bands. Second, the primers likely were not as effective as the first primer pair, Geo1, resulting in lower over amplification. The last pair of bands, which correspond to PCR product from the Geo3.1 primer pair, are very faint. Furthermore, they are not in the correct location on the ladder. Their location corresponds to a PCR product of less than 75 base pairs in length, but the designed product ought to have been 246 base pairs long. This suggests that some type of secondary structure was formed during the

reaction. Primer dimers could have formed, which are structures caused by primers annealing to themselves or each other, or hairpin structures (structures created when the primer bends back and anneals to itself) could have been created.

4.2 Designed Probes

Because the designed Geo1 primer optimized well, we designed a TaqMan Probe to work with the Geo1 primer pair. Of the probes designed for this study, the Geo1P probe looked the most promising. Information about it can be found in Table 5.

Table 5: Specifications of the Geo1 Probe

Probe	GC Content	Melting Temp. (°C)	Self-Dimer ΔG (kcal/mole)	Worst Hetero-Dimer ΔG (kcal/mole)	Sequence
Geo1P	56%	60.8-62.7	-6.76	-4.19	TGG TCA GCG GAT CTC RTC GAA CAC

There is a range in possible melting temperatures for the final probe because we used a nonspecific nucleotide in the probe design. Nonspecific nucleotides refer to sequences where a certain nucleotide could vary depending on the specific gene in the aligned sequences. For example, in the aligned sequences used, space 607 contained a mixture of mostly guanine, but one sequence contained an adenine, as viewed in Figure 6.

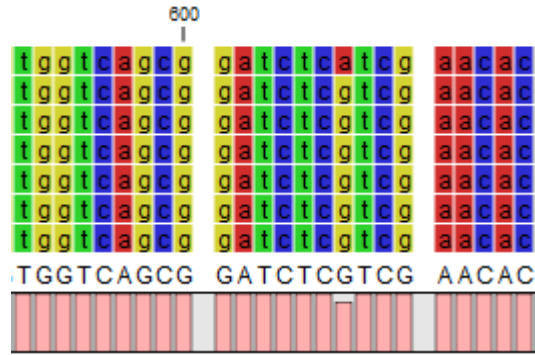


Figure 6: Aligned Sequences Used for Probe Design

Note that the melting temperature of the designed probe varies; the melting temperature varies because the final probe had a mixture of the two different sequences viewed in Figure 6. This mixture of adenine and guanine is represented by the R in the designed probe sequence. The lower temperature corresponds to the part of the probe mixture that has an adenine nucleotide in place of the R, while the higher temperature corresponds to the part of the probe mixture that has a guanine nucleotide in place of the R. Initially, we ran all qPCR reactions with a melting temperature of 60°C, as that was close to the design melting temperature for the probe, and it was the optimized melting temperature for the working primers. At this temperature, the probe worked relatively well; its CT values were consistently between 19.3 and 19.4. These values are notably higher than those for the SYBR Green, but this is to be expected when using a probe instead of the SYBR Green. Though the 60°C melting temperature worked reasonably well, we optimized the probe so that the CT value could be lowered. Lower CT values are desirable because a lower CT corresponds to a more sensitive probe (i.e. a probe that can reliably amplify DNA from lower starting concentrations).

During optimization attempts, we used a melting temperature range from 60°C to 64°C. Though the CT value did not change substantially with changes in temperature, we used 64°C as

the optimized melting temperature as it yielded slightly lower CT values. Table 6 shows the different CT values at the tested melting temperatures.

Table 6: Probe Optimization Results

Melting Temp. (°C)	Ave. CT (Cycles)
60	19.31
61	19.38
62	19.39
63	19.36
64	19.13

To calculate the concentration of the control DNA so that the sensitivity of the qPCR assay could be tested, we generated a standard curve. As seen in Figure 7 below, the generated standard curve was nearly linear, as intended, with an R^2 value of 0.995, so the data gathered can safely be used to calculate the DNA concentrations of the extracted samples.

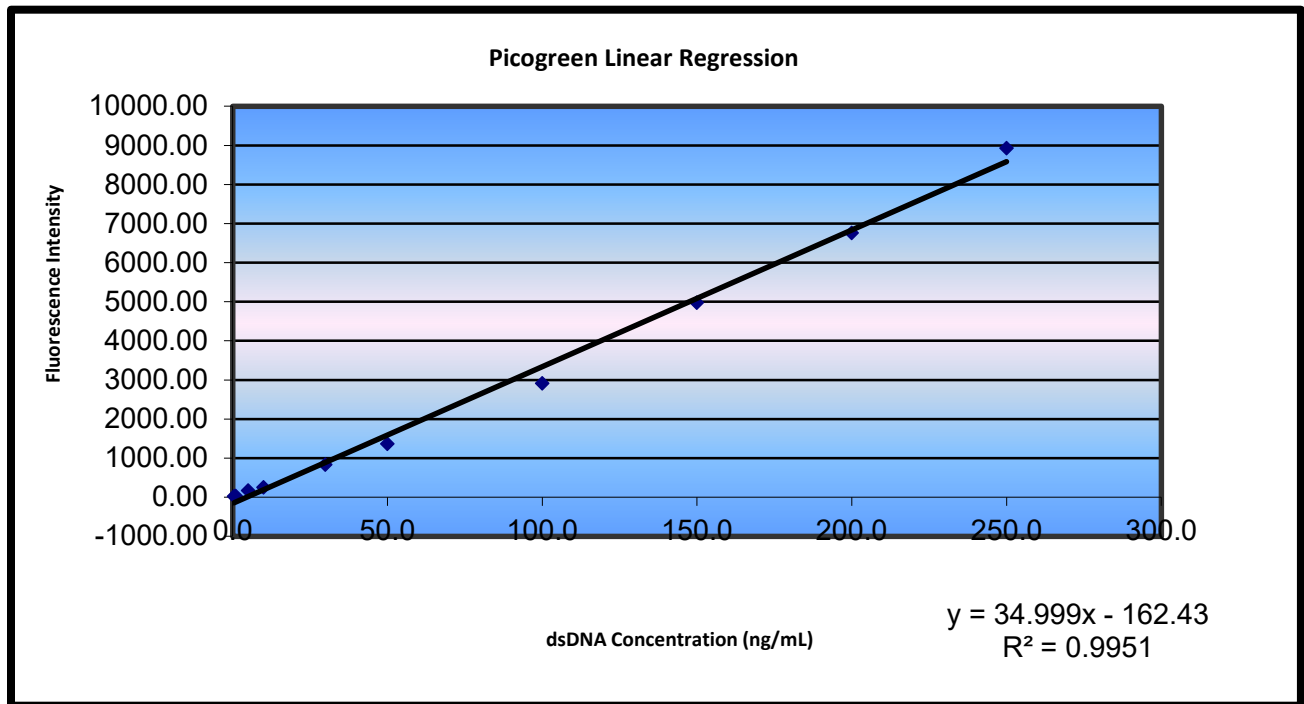


Figure 7: DNA Concentration Standard Curve

Because we had to extract DNA from the control sample twice, we used the standard curve to calculate the amount of DNA in both samples. Using the equation from the linear regression, we calculated the DNA concentration from the first extraction of the *Nostoc* control to be 9.14 ng/μL, and we calculated the DNA concentration from the second batch of the *Nostoc* control to be 60.35 ng/μL. We used the second batch of extracted control DNA to create serial dilutions that would be used for the sensitivity analysis.

We ran the six serial dilutions, ranging from 60.35 ng/μL to 6.035E-4 ng/μL, through the optimized qPCR assay. Figure 8 below shows the output from the thermal cycler during the reaction for each of the serial dilutions of the *Nostoc sp.* control DNA, while Table 7 gives the CT for each of the different DNA concentrations that we tested.

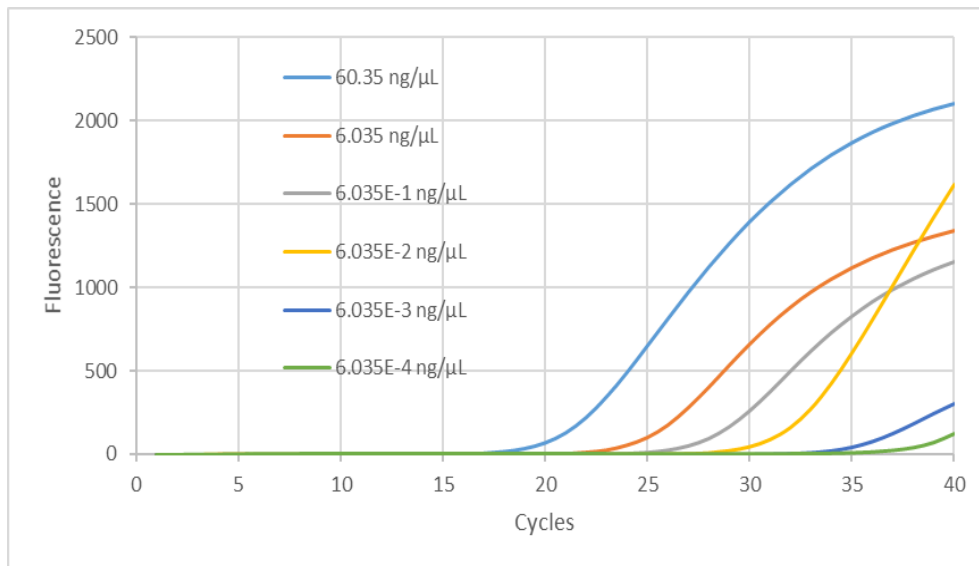


Figure 8: QPCR Assay Sensitivity Analysis

Table 7: qPCR Sensitivity Analysis Results

DNA Concentration (ng/ μ L)	Ave. CT (Cycles)
60.35	18.92
6.035	23.37
6.04E-01	26.21
6.04E-02	30.09
6.04E-03	34.07
6.04E-04	36.91

Once we finished optimizing the qPCR assay, we analyzed 5 field samples for *Nostoc sp.* The concentrations of their extracted DNA can be found in Table 8. These concentrations were measured using the spectrophotometer for convenience, so the actual concentrations may vary. Because the Strawberry sample did not extract very efficiently, we used ethanol precipitation to increase the concentration of the sample. Before ethanol precipitation, the Strawberry sample had a concentration of 2.9 ng/ μ L. The quantity depicted in Table 8 is the concentration achieved in the Strawberry sample after the ethanol precipitation process was completed.

Table 8: DNA Concentrations from Extracted Field Samples

DNA Sample	Final Concentration (ng/ μ L)
Deer Creek 10/14/2017	20.3
Strawberry	43.6
Utah Lake	5.8
Deer Creek Upper 10/25/2017	18.5
Deer Creek Mid-Upper 10/25/2017	98

We ran every field sample for 40 cycles in the optimized qPCR assay. The resulting CT values from the field samples can be found in Table 9.

Table 9: Results from Field Samples Tested for Nostoc with the QPCR Assay

Sample	Ave. CT (Cycles)
Deer Creek 10/14/17	0
Strawberry South East Bloom 10/23/17	38.31
Deer Creek Upper 10/25/17	38.34
Deer Creek Mid-Upper 10/25/2017	39.26
Utah Lake Lincoln Marina 09/04/2018	0

5 DISCUSSION

5.1 Value of Working Probe

As seen from our results, this qPCR has great potential for the proactive detection of geosmin-producing cyanobacteria. The sensitivity analysis showed that the developed qPCR assay can detect *Nostoc sp.* when its DNA concentration is extremely low, even as low as 1e-5 ng/ μ L. High sensitivity will be essential when attempting to predict geosmin events.

The developed primer and probe set detected small amounts of *Nostoc sp.* in the 10/25/2017 Deer Creek samples, even though none of the taxonomy reports for that year identified *Nostoc sp.* in any of their analyzed samples. There could be a few different reasons for this phenomenon. It is possible that the *Nostoc sp.* was not identified by the taxonomists because they were a minority in the overall sample and were not physically seen during analysis. A different, though related, reason for the lack of *Nostoc sp.* in the taxonomy report could be that the sample analyzed in this study was not also analyzed by the taxonomists. Taxonomy data for different samples for the same year were analyzed, but the microbiota in Deer Creek Reservoir could have changed significantly between the time the taxonomists sampled the reservoir and the time the sample used in this study was taken. This second reason helps to outline the value qPCR could provide. Because qPCR assays are fast and inexpensive to run, a more frequent sampling regimen could be implemented. A frequent sampling regimen with accompanying qPCR analysis could shed further light on how the cyanobacteria populations change throughout the year.

5.2 Probe Shortcomings

Though it is impressive that the designed primer and probes were able to identify trace amounts of *Nostoc sp.* in the analyzed samples, it can be noted that most of the extracted DNA was likely not *Nostoc sp.* In the two Deer Creek samples from 2017 that tested positive for *Nostoc sp.*, the total DNA concentration ranged between 18.5 ng/ μ L and 98 ng/ μ L, as seen in Table 8, but the CT for these two samples suggests that the concentration of *Nostoc sp.* detected was much lower: likely less than 6E-4 ng/ μ L. This confirms that there are other genera of cyanobacteria that are more common to Deer Creek Reservoir than *Nostoc sp.* that the designed probe is not detecting. While it could be possible that the unidentified DNA is from cyanobacteria that are not capable of producing geosmin, the taxonomy report suggests otherwise. The taxonomy report showed that various species of *Anabaena* were abundant during 2017, and *Anabaena* is well-known for its ability to produce geosmin. For this reason, it is important to develop a primer/probe set, or multiple primer/probe sets, that can detect a broader range of potential geosmin producers.

During this study, attempts were made at developing a probe that could detect more prolific genera, like *Anabaena* and *Aphanizomenon*. These design attempts, unfortunately, proved unsuccessful. Currently, *Aphanizomenon* is not well documented in the gene databases, so we could not find enough sequence data to allow the design of primers targeting geosmin-producing *Aphanizomenon*. Though there was more sequence information available for *Anabaena*, the data were still sparse. Furthermore, the sequences of *Anabaena* that were available did not align well, suggesting that the gene coding for geosmin synthesis on *Anabaena* can vary significantly from strain to strain. We made several preliminary attempts at designing *Anabaena*-based primers using a few degenerate codons, but none of these primers could

successfully amplify the *Anabaena sp.* control sample nor any of the previously extracted field samples.

One way to shore up the shortcomings of the assay designed in this study could be through multiplexing. Multiplexing is a process that allows multiple probes and primers to be used in one single reaction. Though the geosmin synthase gene varies significantly from genus to genus, and sometimes even from strain to strain, multiple primer/probe sets that target smaller regions could be designed and then run simultaneously. Difficulties can arise when using this method, as the different primers and probes will have some amount of affinity towards one another: the primers and probes can have tendencies to preferentially bond with one another instead of the target DNA. The odds for this tendency increase as more primers and probes are introduced to the reaction, so a multiplex reaction with more than 4 primer/probe sets is not seen to be feasible, according to Olivia. Difficulties aside, it could be possible to use the already designed *Nostoc sp.* assay in tandem with other primer/probe sets in a multiplex qPCR assay that could amplify a much broader spectrum of geosmin-producing cyanobacteria.

6 CONCLUSION

6.1 Method Feasibility

We verified qPCR as a feasible method for sensitive detection of geosmin-producing cyanobacteria. The methods outlined in this study showed that qPCR can detect even trace amounts of specific cyanobacteria species in field samples. The designed primer/probe set was shown to be capable of detecting and amplifying DNA concentrations from *Nostoc sp.* that were lower than 0.001 ng/ μ L from a field sample that contained multiple nanograms of other cyanobacterial DNA. Unfortunately, there is not currently enough sequence information available to develop a TaqMan Probe qPCR assay capable of targeting *all* geosmin-producing cyanobacteria. For the research to be furthered, the gene sequences for many more geosmin-producing cyanobacteria will need to be discovered so that more comprehensive primers and probes can be developed.

7 FUTURE RESEARCH

The eventual goal behind this research is to develop a methodology via qPCR that permits the prediction of geosmin events in local lakes and reservoirs that serve as sources for drinking water so that proactive treatment for geosmin can be implemented at the water treatment facilities. If the proper primers/probes are developed, a frequent, consistent sampling regimen could be established to develop a correlation between qPCR results and geosmin events. Unfortunately, there is not yet enough sequence information available for the major geosmin-producing species in Utah County and the surrounding areas to permit the design of working primers and probes. The next step in this research needs to be the development of more gene sequences for geosmin-producing cyanobacteria. Unfortunately, obtaining the entire gene sequence for an organism is very expensive. Obtaining the entire gene sequences for all the most common species in Deer Creek Reservoir is not economically feasible at this time.

Regarding this roadblock, Dr. Robison was consulted for guidance. He brought up the idea to use PCR to clone out just the *geoA* sequence from field samples and have the resulting DNA sequenced. The *geoA* gene is typically only around 900 base pairs long, so it would not be expensive to sequence such a short gene. Furthermore, Suurnäkki et al. (2015) developed primers that are reported to be capable of targeting the entire *geoA* gene and cloning it through PCR. Their primers could be tested on the field samples used in this study. If they were capable of successfully amplifying the *geoA* gene of the various species contained in those samples, then

those samples could be sent to the BYU DNA Sequencing Center (hereafter “the Sequencing Center”) for gene sequencing. Because there are likely multiple different species of geosmin-producing cyanobacteria in those field samples, the different sequences received from the Sequencing Center could be run through the BLAST and analyzed for conserved regions, allowing the design of new primer/probe sets. A multiplex qPCR assay could then be developed using these primer/probe sets and the *Nostoc sp.* set developed in this research. The resulting qPCR assay could then be used to develop a correlation between qPCR fluorescence and geosmin events at Deer Creek Reservoir. Local lakes and reservoirs could be sampled regularly for cyanobacteria in coordination with the operators at the Jordan Valley Water Treatment Plant (JVWTP). The collected samples could then be analyzed with the qPCR for geosmin-producing cyanobacteria. Because the JVWTP already samples for geosmin, the results from the qPCR assay could then be compared to the results from the JVWTP geosmin tests so that a correlation could be developed and tested. This methodology could provide for the first feasible approach to predicting geosmin events so that proactive treatment of the water can be implemented.

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APPENDIX A: ISOLATION OF GENOMIC DNA FROM BACTERIAL

SUSPENSION CULTURE

1. Pipet 1 ml of bacterial culture into a 1.5 ml microcentrifuge tube, and centrifuge for 5 min at 5000 x g (7500 rpm).
2. Calculate the volume of the pellet or concentrate and add Buffer ATL (supplied in the QIAamp DNA Mini Kit) to a total volume of 180 μ l.
3. Add 20 μ l proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform. Note: Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL. Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. To ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.
4. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
5. If RNA-free genomic DNA is required, follow step 5a. Otherwise, follow step 5b.
Transcriptionally active tissues, such as liver and kidney, contain high levels of RNA

which will copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR.

- a. First add 4 μ l RNase A (100 mg/ml), mix by pulse-vortexing for 15 s, and incubate for 2 min at room temperature (15–25°C). Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid before adding 200 μ l Buffer AL to the sample. Mix again by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid. It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL. In most cases it will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.
 - b. Add 200 μ l Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid. It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.
6. Add 200 μ l ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid. It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. It is

essential to apply all of the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application. Do not use alcohols other than ethanol since this may result in reduced yields.

7. Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.* Close each spin column to avoid aerosol formation during centrifugation. It is essential to apply all of the precipitate to the QIAamp Mini spin column. Centrifugation is performed at 6000 x g (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through. * Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach.
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.*
9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 μ l Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.
12. Repeat step 11. A 5 min incubation of the QIAamp Mini spin column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield. A third elution step with a further 200 μ l Buffer AE will increase yields by up to 15%. Volumes of more than 200 μ l should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation. Elution with volumes of less than 200 μ l increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 5, page 25). Eluting with 4 x 100 μ l instead of 2 x 200 μ l does not increase elution efficiency. For long-term storage of DNA, eluting in Buffer AE and placing at -30 to -15°C is recommended, since DNA stored in water is subject to acid hydrolysis. Yields of DNA will depend both on the amount and the type of tissue processed. 25 mg of tissue will yield approximately 10–30 μ g of DNA in 400 μ l of water (25–75 ng/ μ l), with an A260/A280 ratio of 1.7–1.9 (Qiagen 2016).

APPENDIX B: RAW SENSITIVITY DATA FOR GEO1P

Table 10: Raw QPCR Fluorescence Readings from the Geo1P Sensitivity Analysis

Cycles	60.35 ng/μL	6.035 ng/μL	603.5 pg/μL	60.35 pg/μL	6.035 pg/μL	603.5 fg/μL
1.0	0.745	0.250	-0.372	-4.408	-0.227	-7.878
2.0	-0.145	0.500	-1.821	-3.003	-0.446	-6.344
3.0	0.964	0.750	-1.270	-2.599	-0.666	-4.810
4.0	0.073	1.000	-1.719	-1.195	-0.886	-4.276
5.0	0.182	1.250	-1.168	-0.791	-0.106	-2.742
6.0	0.291	0.500	0.382	-1.386	-0.326	-1.208
7.0	0.400	0.750	-0.067	-0.982	0.455	-0.674
8.0	0.509	0.000	0.484	-0.578	0.235	-0.140
9.0	-0.382	-0.750	0.035	-0.173	0.015	1.394
10.0	-0.273	-0.500	-0.414	0.231	-0.205	0.928
11.0	-1.164	-0.250	0.137	-0.365	-0.425	0.462
12.0	-1.055	-1.000	0.688	1.040	0.356	0.996
13.0	0.055	-0.750	1.239	0.444	0.136	0.529
14.0	0.164	-0.500	0.789	0.848	-0.084	1.063
15.0	1.273	-0.250	0.340	1.252	-0.304	0.597
16.0	3.382	0.000	-0.109	1.657	0.477	0.131
17.0	8.491	-0.750	-0.558	1.061	0.257	0.665
18.0	18.600	-0.500	-1.007	1.465	0.037	0.199
19.0	36.709	0.750	-1.456	0.870	-0.183	0.733
20.0	70.818	2.000	-0.905	0.274	0.597	0.267
21.0	129.927	5.250	-0.354	-0.322	0.378	-0.199
22.0	222.036	14.500	0.196	-0.918	0.158	0.335
23.0	346.145	27.750	1.747	-0.513	-0.062	-0.131
24.0	492.255	57.000	5.298	-2.109	-0.282	-0.597
25.0	652.364	103.250	12.849	-0.705	-0.501	-0.063
26.0	814.473	177.500	26.400	-0.300	-0.721	-0.529
27.0	973.582	281.750	51.951	2.104	-0.941	-0.996
28.0	1124.691	404.000	95.502	7.508	-1.161	-0.462
29.0	1265.800	535.250	166.053	20.912	-0.381	-0.928
30.0	1396.909	662.500	261.604	44.317	0.400	-0.394

Cycles	60.35 ng/ μ L	6.035 ng/ μ L	603.5 pg/ μ L	60.35 pg/ μ L	6.035 pg/ μ L	603.5 fg/ μ L
31.0	1514.018	779.750	376.154	87.721	2.180	-0.860
32.0	1620.127	884.000	499.705	160.125	4.960	-0.326
33.0	1715.236	975.250	620.256	272.530	11.740	0.208
34.0	1798.345	1052.500	729.807	424.934	22.521	1.742
35.0	1870.455	1119.750	827.358	606.338	43.301	4.276
36.0	1933.564	1178.000	914.909	804.742	77.081	9.810
37.0	1986.673	1228.250	988.460	1011.147	123.861	19.344
38.0	2032.782	1271.500	1052.010	1218.551	180.641	35.878
39.0	2071.891	1308.750	1106.561	1419.955	241.422	66.412
40.0	2105.000	1342.000	1153.112	1615.360	298.202	118.945

APPENDIX C: RESULTS FROM QPCR TESTS ON FIELD SAMPLES

Table 11: Deer Creek Mid-Upper 10/25/2017 Nostoc QPCR Results

<u>Cycles</u>	<u>Fluorescence</u>
1	-4.62
2	-4.98
3	-4.35
4	-0.71
5	-0.08
6	-0.45
7	-0.81
8	-0.18
9	-0.54
10	0.09
11	-0.27
12	-0.64
13	-0.01
14	0.63
15	0.26
16	-0.10
17	-0.47
18	0.16
19	0.80
20	1.43
21	1.07
22	0.70
23	0.34
24	0.97
25	0.60
26	0.24
27	-0.13
28	-0.49
29	0.14
30	-0.23

Cycles	Fluorescence
31	-0.59
32	-0.96
33	-1.32
34	-1.69
35	-0.05
36	1.58
37	5.21
38	11.85
39	24.48
40	46.12

Table 12: Deer Creek Upper 10/25/2017 Nostoc QPCR Results

Cycles	Trial 1 Fluorescence	Trial 2 Fluorescence
1	-3.426	-2.943
2	-2.809	-1.166
3	-3.193	-0.389
4	-1.577	-0.611
5	-1.960	-0.834
6	-1.344	-1.057
7	-0.727	-0.280
8	-1.111	-0.503
9	-0.495	0.274
10	0.122	0.051
11	0.738	-0.172
12	1.354	-0.395
13	1.971	0.382
14	0.587	0.159
15	1.204	0.936
16	0.820	0.714
17	1.436	0.491
18	0.053	1.268
19	-0.331	1.045
20	0.286	0.822
21	0.902	0.599
22	0.518	0.376
23	0.135	0.153
24	-0.249	-0.070
25	0.367	-0.293

Cycles	Trial 1 Fluorescence	Trial 2 Fluorescence
26	-0.016	-0.516
27	-0.400	-0.739
28	-0.783	-0.961
29	-1.167	-1.184
30	-0.551	-1.407
31	-1.934	-0.630
32	-1.318	-0.853
33	-0.701	0.924
34	-0.085	1.701
35	0.531	4.478
36	2.148	10.255
37	5.764	20.032
38	13.380	37.809
39	26.997	66.587
40	51.613	109.364

Table 13: Deer Creek 10/14/2017 Nostoc QPCR Results

Cycles	Trial 1 Fluorescence	Trial 2 Fluorescence
1	-5.977	-1.964
2	-6.343	-2.142
3	-4.710	-1.320
4	-4.077	-0.498
5	-4.443	-0.676
6	-2.810	-0.854
7	-1.176	-0.032
8	-1.543	-0.211
9	-0.909	0.611
10	0.724	0.433
11	0.357	0.255
12	-0.009	0.077
13	1.624	-0.101
14	2.258	0.720
15	1.891	0.542
16	1.525	0.364
17	2.158	0.186
18	1.791	0.008

Cycles	Trial 1 Fluorescence	Trial 2 Fluorescence
19	0.425	-0.170
20	1.058	-0.348
21	0.692	-0.527
22	1.325	0.295
23	0.959	0.117
24	0.592	-0.061
25	1.225	-0.239
26	-0.141	-0.417
27	0.492	0.405
28	0.126	0.226
29	-0.241	0.048
30	-0.607	-0.130
31	-0.974	-0.308
32	-1.341	-0.486
33	-1.707	-0.664
34	-1.074	0.157
35	-1.440	-0.021
36	-0.807	0.801
37	-1.173	0.623
38	-0.540	0.445
39	-0.907	0.267
40	-1.273	0.089

Table 14: Utah Lake Lincoln Marina 09/04/2018 Nostoc QCPR Results

Cycles	Trial 1 Fluorescence	Trial 2 Fluorescence
1	-1.837	-2.549
2	-0.943	-1.773
3	-1.049	-1.996
4	-1.155	-1.220
5	-1.261	-1.443
6	-0.367	-0.667
7	-0.474	-0.890
8	0.420	-1.113
9	-0.686	-0.337
10	0.208	-0.560
11	0.102	0.216

Cycles	Trial 1 Fluorescence	Trail 2 Fluorescence
12	-0.004	-0.007
13	-0.110	-0.231
14	0.784	0.546
15	0.677	0.323
16	0.571	1.099
17	0.465	0.876
18	0.359	0.652
19	0.253	1.429
20	0.147	1.205
21	0.041	-0.018
22	-0.065	0.759
23	-0.172	0.535
24	-0.278	0.312
25	-0.384	0.088
26	0.510	0.865
27	0.404	0.641
28	0.298	0.418
29	0.192	0.195
30	0.086	-0.029
31	-0.021	-0.252
32	-0.127	-0.476
33	-0.233	-0.699
34	-0.339	-0.922
35	-0.445	-1.146
36	-0.551	-1.369
37	-0.657	-1.593
38	-0.763	-1.816
39	-0.870	-1.040
40	0.024	-1.263

Table 15: Strawberry South East Bloom 10/23/2017 Nostoc QPCR Results

Cycles	Fluorescence
1	-10.430
2	-7.981
3	-7.533
4	-6.084
5	-4.635
6	-4.186

<u>Cycles</u>	<u>Fluorescence</u>
7	-2.737
8	-2.289
9	0.160
10	0.609
11	1.058
12	1.506
13	1.955
14	2.404
15	2.853
16	2.302
17	2.750
18	2.199
19	1.648
20	1.097
21	1.546
22	0.994
23	1.443
24	-0.108
25	-0.659
26	-1.210
27	-0.762
28	-0.313
29	-0.864
30	-1.415
31	-1.967
32	-1.518
33	-2.069
34	-0.620
35	0.829
36	4.277
37	9.726
38	22.175
39	44.624
40	83.073