Spatial and temporal investigation of taste and odor-producing microorganisms in Lake Saugahatchee

by

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Abstract

Geosmin is one of the most prevalent taste and odor compounds found in drinking water and is produced by a variety of algae. Quantitative PCR (qPCR) can be used to identify and monitor the growth of geosmin producers by targeting the gene encoding for geosmin synthase. However, the accuracy of qPCR is dependent on the assays used. In this study, two newly developed assays and three from the literature were tested using environmental samples collected from 4 water bodies located in and around Auburn, Alabama. Sequencing revealed that the CGeo-1F/CGeo-1R primer set was specific to the cyanobacterial geosmin synthase gene and had a linear calibration curve from 6.15 $x10^2$ to 6.15 x 10^5 gene copies/mL. The other primers used in this study struggled with specificity as well as quantification ability. The 288AF/288AR, SGF1/JDR1, AMgeo-F/AMgeo-R, and ActGeo-2F/ActGeo-2R primer sets amplified the geosmin synthase gene across genera in different phyla. Furthermore, the 288AF/288AR primer set required two rounds of PCR before enough amplicon was generated for sequencing. The CGeo-1F/CGeo-1R primer set showed a good positive correlation between gene copies and geosmin in the water column (r^2 = 0.47). Also, the ActGeo-2F/ActGeo-2R primer set showed a good correlation between gene copy number and geosmin concentrations measured by gas chromatography-mass spectrometry (r^2 = 0.79). The use of these primers may be used by water management facilities to characterize geosmin episodes and choose management options accordingly.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

T&O	Taste and Odor
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
OTU	Operational Taxonomic Unit
VOC	Volatile Organic Compound
FISH	Fluorescence In Situ Hybridization
LMB	Lanthanum modified bentonite
C_t	Cycle Threshold

Introduction

In recent decades, anthropomorphic activities have resulted in the eutrophication of water bodies and subsequent cyanobacterial and algal blooms. Through the production of toxins and volatile secondary metabolites, these blooms can cause major deterioration of water quality (Lurling et al., 2020). One of the most pervasive metabolites produced by cyanobacteria is geosmin. Actinobacteria, which inhabit soil, are also a major producer of geosmin. The presence of geosmin in water results in an earthy or musty smell and taste that is often interpreted by consumers to mean that their water is unsafe to drink. This is problematic for water utilities as geosmin can be detected by humans at an extremely low concentration of less than 10 ng/L (Watson et al., 2016). Because of the low odor threshold, many conventional water treatment processes, such as coagulation, sedimentation, sand filtration, and chlorination are unable to remove geosmin from drinking water (Dzialowskia et al., 2009). This means that additional treatment methods, such as activated charcoal or ozone must be implemented (Watson et al., 2007). These additional treatment methods are often too costly for continuous application. As a result, it is imperative that predictive methods be developed to determine when T&O events may occur. Reservoir management practices can also be an important tool in controlling geosmin outbreaks but require knowledge of the types of organisms responsible for geosmin production.

At present, many water utilities rely on microscopy to identify and quantify geosmin-producing cyanobacteria (John. 2018). To date, nearly 70 cyanobacteria species have been identified that produce geosmin, but they often are hard to differentiate morphologically from non-producers (Devi et al., 2021). Even if the cyanobacteria species can be properly identified through microscopy, geosmin production can vary within strains of a species and environmental factors (Wang & Li, 2015; Watson et al., 2016). Thus, microscopy is time-intensive and cannot

accurately discern producers from non-producers. As a result, there has been a great effort by researchers to develop methods to predict nuisance geosmin levels. In an effort to find a more rapid and universal method for predicting nuisance geosmin levels, many researchers have instead taken a biomolecular approach (Giglio et al., 2008; Su et al., 2013; Tsao et al., 2014). For most, this means targeting the geosmin synthase gene.

Polymerase Chain Reaction (PCR) and quantitative PCR (qPCR) are two such biomolecular approaches that were developed after the identification of a geosmin synthase gene in cyanobacteria (Giglio et al., 2008). Both methods have seen a considerable increase in interest due to their ability to detect low cell numbers and ability to analyze a large number of samples at once and their short analysis time (Devi et al., 2021). However, only 62 geosmin synthase gene regions have been published in the NCBI database. This makes the development of a universal primer difficult. As a result, multiple primer pairs with unique amplification protocols must be used to capture all possible geosmin producers within a waterbody. Another major drawback for PCR and qPCR is that of the 11 primer sets published targeting the cyanobacterial geosmin synthase gene, none of them were able to fully align with the target region of all identified geosmin-producing cyanobacteria (Devi, 2021). The development of accurate primers would allow water utilities to determine when water will require additional treatment, improving customer satisfaction and potentially reducing operational costs. The objective of this study was to develop new primer tools targeting the geosmin synthase gene in cyanobacteria and actinobacteria and compare their specificity and efficiency with primers that are already in use by the research community.

In this study, five molecular assays have been evaluated for their ability to identify and quantify the geosmin synthase gene in cyanobacteria and actinobacteria. To properly evaluate

primer specificity and efficiency, water and sediment samples containing a wide range of geosmin concentrations were obtained. Samples were collected from Lake Saugahatchee in Lee County, Alabama, United States during late Spring to early Fall of 2020. However, geosmin levels never exceeded the odor threshold limit, so additional samples from regional lakes and ponds were used to supplement the sample set. Following extraction of genetic material, An Analytic Jena qTower3 qPCR instrument was used to quantify the geosmin synthase present in each sample. Two annealing temperatures were used to determine optimal thermal cycling conditions. This was done for each primer set. Gel electrophoresis was then carried out on the PCR products to validate each primer's ability to produce a standard product size. Flow cell genetic sequencing was then performed, revealing the genes amplified by each primer, and these were mapped to the geosmin-producing taxa.

Literature Review

Recently, algae blooms have become a problem around the globe. Eutrophication and pollution have caused a significant increase in the frequency and duration of algae blooms. These blooms are not only aesthetically displeasing, but they also greatly reduce water quality through the production of volatile compounds. Some of the volatile compounds produced by algae include a range of terpenoids, sulfur compounds, carotenoid derivatives, and other volatile organic compounds (VOCs) (Lee et al., 2017; Rui-Peng et al., 2020). Of the VOCs produced by algae, geosmin is one of the most problematic. Although geosmin does not pose a health concern, it is detectable by humans at a very low concentration of 10 ng/L or less and is resistant to degradation in water (Cook et al., 2001; Kutovaya & Watson, 2014). The earthy/musty smell of geosmin is often interpreted by consumers to mean that their drinking water is unsafe. These geosmin episodes are often attributed to cyanobacteria, however, actinobacteria are also known to synthesize geosmin in lake sediments (Zaitlin & Watson, 2006). Accordingly, the present monitoring and control methods for geosmin events rely on the identification and quantification of problem organisms. However, these methods often have a long turnover time and various levels of accuracy. This inhibits the ability of water utilities to quickly employ the additional water treatment methods required to remove geosmin. The recent identification of the geosmin synthase pathway by Giglio et al. (2008) has led to the development of biomolecular methods, specifically PCR and qPCR, for the detection of geosmin episodes. This review aims to provide a summary of the overarching issue of taste and odor (T&O) episodes, focusing on the factors that

proliferate algae blooms, current methods employed to control blooms and geosmin levels in drinking water, and the biomolecular tools that have been developed to detect and predict oncoming T&O episodes.

Driving Factors and Geographical Distribution of Geosmin Events

Geosmin taste and odor events are a worldwide problem, lowering drinking water quality in six of the seven continents (Xu et al., 2015; Barros et al., 2019; Konopka & Brock; Tsao et al, 2014; Jones & Korth, 1995; Wnoroski & Scott, 1992). Descriptions of these episodes reveal a range of geosmin-producers and factors that proliferate their growth. Excessive nutrients loading, specifically nitrogen and phosphorus, is the most important factor related to the acceleration of cyanobacterial growth. This is especially true when N:P ratio is low. Some cyanobacteria are capable of fixing atmospheric nitrogen, giving them an advantage over other algae species (Wu et al., 1991). Barros et al. (2019) conducted a study of 20 drinking water reservoirs located in northeastern Brazil. Their study found that cyanobacteria accounted for 99% of the total biovolume and that nitrogen was inversely proportional to cyanobacteria growth, while phosphorus was directly related to cyanobacteria growth. Similarly, Kramer et al. (2018) reported a dominance of cyanobacteria when N:P ratios were low. When conducting a study on a eutrophic lake in southern Sweden, Li et al. (2018) found that the N:P ratio of 50 was required to stop cyanobacterial blooms. The availability of phosphorus was also found to be a limiting factor in the growth of cyanobacteria in Lake Taihu, China (Xu et al., 2015). To further complicate the matter, some species of cyanobacteria have been shown to raise water pH. In aerobic sediments, this can facilitate the release of phosphorus (Shan et al., 2019). It has been hypothesized that geosmin synthesis is a stress response (Churro et al., 2020). Not only do low N:P ratios increase

cyanobacterial algae blooms, but they also favor the production of secondary metabolites. Wu et al. (1991) found that *Anabaena viguieri* produced higher amounts of geosmin when grown under nitrogen-limited conditions.

Although not as important as nutrient concentrations, water temperature is another main factor affecting the growth and proliferation of cyanobacteria algae blooms. The speed of chemical reactions increases as temperatures increase. Photosynthesis is no exception. Higher rates of photosynthesis result in higher rates of reproduction. This is why algae blooms are typically season with a peak in frequency and intensity during the summer (Johnk et al, 2008). However, the degree to which temperature affects photosynthesis is dependent on algae species. Konopka & Brock (1978) studied the photosynthetic rates of three cyanobacteria strains isolated from a bloom in Lake Mendota, Wisconsin. They found that although all the cultures had about the same optimal temperature, Anabaena photosynthesized at approximately the same rate within the temperature range of 20 ^{o}C to 30 ^{o}C, while *Aphanizomenon* and *Microcystis* experienced a 30% decrease in the photosynthetic rate at a temperature of 25^{o}C. Compared to green algae and diatoms, cyanobacteria have a relatively high optimal growth rate, exhibiting peak growth at 25^{o}C (Chorus & Bartram, 1999). A 6-year study conducted from 1997 to 2002 on the Liptovska Mara Reservoir in Slovakia showed that cyanobacteria concentrations were highest at temperatures above 20^{o}C (Onderka, 2007). Johnk et al. (2007) studied the effects of temperature on cyanobacterial growth in Lake Nieuwe Meer in Sweden. They found that increased temperatures not only increased the growth rates of cyanobacteria, but it also stabilized the water column. This reduced turbulent vertical mixing, thereby allowing cyanobacteria to stay buoyant at the water's surface. Temperature can also affect geosmin production. A study by Alghanmi et

al. (2018) found that the cyanobacteria *Phormidium retzii* reached peak geosmin production at 25^{o}C as well.

Algae Bloom Mitigation and Geosmin Removal Methods

Algae blooms and their production of VOCs have become such a nuisance that some water management facilities have been forced to dam entire areas of the lake. In one instance, 5 ha of De Gouden Ham Lake (Netherlands) were dammed to stop the spread of a cyanobacteria bloom (Lurling & Van Oosterhout, 2013). Rather than taking such drastic measures, many alternative physical, chemical, and biological methods have been used to mitigate algae blooms, and in turn, reduce the frequency of geosmin episodes. Each of these methods has varying levels of success. Superficial vacuuming is one such method that aims to remove the surface scums that are sometimes formed by cyanobacterial blooms (Stroom & Kardinaal, 2016). However, superficial vacuuming is limited to small lakes where the scum is located close to shore (Lurling & Mucci, 2020). Furthermore, the efficacy of vacuum trucks for the mitigation of algae blooms has yet to be determined (Lürling & Mucci, 2020). The use of artificial mixers, such as fountains and surface aerators has been used to inhibit the accretion of cyanobacteria in surface waters. Artificial aeration is typically only effective in deep lakes where stratification can cause lower light availability and in turn decrease algae growth (Heo & Kim, 2004). In shallow lakes, aeration can actually favor cyanobacteria growth by stirring up sediment, thereby facilitating the release of phosphorus (Visser et al., 2016).

Dredging and excavation are more suitable for shallow lakes. Dredging and excavation work to mitigate T&O episodes in two ways: by the removal of benthic VOC producers and by

the removal of phosphorus-rich sediment (Cooke et al., 2016). In 1970, dredging successfully mitigating cyanobacterial blooms occurred in Lake Trummen in Vaxjo, Sweden (Bjork et al., 2010). A reduction in cyanobacterial blooms in Lake Trummen was possible due to the limitation of external nutrient loading prior to dredging (Bjork et al., 2010). In instances where no effort was made to limit nutrient loading, only a short-term reduction in algae blooms occurs. The removal of 330,000 m^2 of sediment from Vajgar fishpond in the Czech Republic resulted in the temporary nonappearance of *Microcystis* sp. blooms (Pokorny & Hauser, 2002). However, external nutrient loadings quickly restored the phosphorus concentration to pre-dredging levels and the blooms returned two years later (Pokorny & Hauser, 2002). Not only does dredging require the identification and control of both point and non-point pollution sources, but dredging can also potentially cause adverse environmental effects. Dredging can result in the smothering of benthic flora and fauna and the reduction of habitat complexity (Burton & Johnston, 2010). Furthermore, dredged sediments can potentially be toxic (Peterson, 1981). As a result, permits often must be obtained for their disposal, and discharge from the disposal area may require further treatment (Peterson, 1981).

Instead of removing nutrient-rich sediment, phosphate immobilizers have been used extensively to inhibit phosphorus release. Lanthanum modified bentonite (LMB) is one such phosphate immobilizer and has been applied to nearly 200 lakes (Copetti et al., 2016). A large-scale application of LMB to a river in Perth, Australia suffering from eutrophication showed that LMB was capable of reducing dissolved inorganic phosphorus in the water (Robb et al., 2003). Additionally, a sediment core incubation study by Waajen et al. (2016) demonstrated the ability of LMB to greatly reduce the release of phosphorus from the sediment. The capacity of LMB to reduce cyanobacterial blooms was demonstrated by Epe et al. (2017). Their study found that an

initial large application of LMB followed by two smaller doses prevented cyanobacterial blooms in a lake for 6 years (Epe et al., 2017). The effects of LMB on *Actinobacteria* are less documented. It was hypothesized that the increased phosphorus in the sediment would increase *Actinobacteria* growth, but the results of Yin et al. (2021) showed that the application of LMB and subsequent increase in phosphorus in the sediment had little effect on the reduction of *Actinobacteria* (Dai et al., 2021).

Rather than try to prevent algae blooms from occurring, many management strategies aim to kill algae cells. Since 1904, copper-based algaecides have commonly been employed to control algae blooms and are considered to be some of the most effective means of killing algae (Moore & Thomas, 1905; Iwinski et al., 2016). Copper algaecides can be applied at low concentrations, but repeated applications are required. This is predominantly due to the fact that more than 80% of the copper is in particulate form just 2 hours after application (Haughey et al., 2000). Studies have also shown that algae can build a tolerance to copper algaecides through repeated exposure (García-Villada et al., 2004). Not only does this reduce the treatment efficacy, but it also increases the exposure of non-target organisms to the algaecides. This can result in die-offs of aquatic organisms that have similar or lower EC50 levels than algae (Jancula & Marsalek, 2011).

High and low power ultrasound is another method employed by some facilities to kill surface-level algae cells. Ultrasound is considered low-frequency at 20-200 Hz and high-frequency above 10 MHz (Ahmadi et al., 2012). When used in water, ultrasound radiation generates a succession of compression and rarefaction, resulting in the formation of cavitation bubbles (Rajasekhar et al., 2012). The implosion of cavitation bubbles can produce temperatures upwards of 5,000^{o}C, pressures of 100 MPA, and free radicals (Suslick, 1990). Studies have

shown that ultrasound can cause the collapse of gas vesicles, disrupt the cell wall, inhibit photosynthetic activity, and prevent cell division (Rajasekhar et al., 2012). However, many studies demonstrating the ability of ultrasound to reduce cyanobacterial growth use high-power ultrasound (Hao et al., 2004; Lee et al., 2010). Higher frequencies are more effective because they are closer in resonance to gas vesicles in cyanobacteria, meaning they are more likely to cavitation and collapse (Hao et al., 2004). Unfortunately, the production of higher frequencies requires the use of more power, representing an increased cost for water utilities (Lurling & Mucci, 2020). Furthermore, the efficacy of high-frequency ultrasound is reduced when applied to lakes and ponds because less energy is transmitted over large volumes (Rajasekhar et al., 2012). Also, high-frequency ultrasound is non-specific and can kill zooplankton and might damage fish skin or may adversely affect macrophytes (Carstensen et al., 1990; Holm et al., 2008; Wu & Wu, 2006).

Natural and synthetic coagulants applied with a ballast are another method used to mitigate cyanobacterial blooms. This approach can immediately remove cyanobacteria from the waterbody but is more effective in stratified lakes where the cyanobacteria can sink to colder and darker sediments. A study conducted by Pan et al. (2011) found that a dose of 25-31 mg/L of a combined coagulant and ballast mixture was capable of clearing a 1-cm thick cyanobacteria bloom in Lake Taihu, China. In more shallow water bodies, the flocs may be resuspended and repopulate the water column (Lurling et al., 2020). Metal-based coagulants, such as aluminum sulfate and poly-aluminum sulfate, are the most cost-effective coagulants on the market (Lurling et al., 2020) However, the hydrolysis of these coagulants results in the production of hydrogen ions (Cooke et al., 2016). Because of this, buffers must often be used with metal-based coagulants. Although more expensive, chitosan is an environmentally friendly and biodegradable

alternative to metal-based coagulants (Lurling & Mucci, 2020). Furthermore, local soils can be used as a ballast (Lurling et al., 2020). However, chitosan can damage the cell membrane of many types of cyanobacteria (Mucci et al., 2017). Methods that damage the cell membrane or kill algae often fail to prevent T&O episodes as geosmin can be released into the water column after cell lysis (Watson et al., 2016). Also, many of the aforementioned methods fail to remove geosmin-producing actinobacteria in the sediment. As a result, water utilities often have to use additional treatment methods to remove geosmin from drinking water.

Activated carbon is one of the most simple and effective practices used by water treatment facilities to remove geosmin from drinking water (Matsui et al., 2015). One disadvantage of activated carbon is the requirement of continued applications during a T&O episode. Furthermore, 5-50 mg/L of activated carbon is required to absorb just 10-100 ng/L of geosmin (Cooke et al., 2001). Not only is the absorbent-to-absorbate ratio incredibly high, the presence of other oxidants, such as chlorine, can further reduce the effectiveness of activated carbon (Yuan et al., 2020). Chlorine itself has been used to degrade geosmin, but with little success. Lin et al. (2009) found that the use of chlorine actually ruptured *Aanbaena* cells, exacerbating the release of geosmin. Alternatively, ozonation has been found to effectively remove geosmin from water through the formation of hydroxyl radicals (Schrader et al., 2010). Westerhoff et al. (2006) found that the application of 1.5 mg/L of ozone resulted in a 95% reduction in geosmin concentrations after just 10 minutes. Terashma et al. (1988) found that a dosage of 5 mg/L resulted in a 100% reduction of geosmin. Though, the overuse of ozone should be cautioned against as it can oxidize bromide ions to form carcinogenic bromate ions (Mizuno et al., 2011).

Identification and Quantification of Geosmin Producers

Although geosmin is such a widespread problem, the ability to quantify and identify geosmin-producers in water has eluded researchers. Many methods have been developed that aim to detect and monitor geosmin events include gas chromatography-mass spectrometry (GC-MS), fluorescence in situ hybridization (FISH), and microscopy. While GC-MS can detect geosmin levels well below the odor threshold, it cannot identify the sources of geosmin. Klausen et al. (2005) attempted to use FISH to identify a possible relationship between actinomycetes and geosmin levels. They found that FISH was neither able to characterize geosmin producers from non-producers or quantify actinobacteria abundance. Microscopy is one of the most rapid and cost-effective methods used to identify organisms that may be capable of producing geosmin. Accordingly, it is also one of the most widespread methods (John et al., 2018). In freshwater, *Dolichospermum, Aphanizomenon, Phormidium, Tychonema,* and *Anabaena* are the most common cyanobacterial producers of geosmin (Churro et al., 2020, Tsao et al., 2014). Typically found in sediment, *Streptomyces* is a common geosmin-producing actinobacteria (Li et al., 2010; Blevins et al., 1995). Although the number of common geosmin producers is quite low, there are over 20 known geosmin producers (Churro et al., 2020). As a result, a detailed understanding of taxonomy is required for the accurate use of microscopy for the monitoring of geosmin. An alternative to microscopy is to develop models that can predict when geosmin levels will surpass the odor threshold (Harris & Graham, 2017; Chung et al., 2016; Chong et al., 2018). These models often rely on easy-to-measure environmental and physiological factors, making them a possible cost-effective solution for water management facilities. However, these models are often specific to a single lake or regional area, meaning a new model would need to be developed for each water utility (Rousso et al. 2020).

Biomolecular approaches, such as real-time polymerase chain reaction (PCR) and quantitative PCR (qPCR) can process a large volume of samples affordably and rapidly. However, PCR and qPCR could not be used to monitor geosmin and its sources without knowledge of the metabolic pathway or genes involved in geosmin synthesis. Although Gerber and Lechevalier identified geosmin in 1965, it would be decades until the geosmin metabolic biosynthesis pathway was identified (Gerber & Lechevalier, 1965). At first, it was suggested that geosmin was synthesized using the mevalonate isoprenoid pathway or the 2-methylerythritol-4-phosphate pathway (Juttner & Watson, 2007). These theories were proven to be incorrect when in 2006, Jiang et al. discovered that the conversion of farnesyl diphosphate to geosmin was facilitated by a single enzyme known as germacradienol/germacrene D synthase. Another breakthrough was achieved in 2008 when Giglio et al. discovered that a single gene encoded the geosmin synthase enzyme in cyanobacteria, known simply as *geo*. This discovery meant that PCR and qPCR procedures could be developed for the identification and quantification of geosmin producers in water bodies, specifically drinking water sources. However, further research revealed that the geosmin synthase gene is highly variable. Wang et al. (2019) isolated the geosmin synthase gene in 16 cyanobacterial strains and found that they varied in lengths from 2235 bp, encoding 744 amino acids to 2313 bp, encoding 770 amino acids. The cyanobacteria included in the study had a gene similarity of only 72% (Wang et al., 2019). Wang et al. (2019) also reported that Cyanobacterial *geo* shared DNA identities of 0.49–0.72 with homologous genes in actinomycetes. Although this makes the development of a universal primer difficult, these differences between phyla can be exploited to create primers that are specific to each phylum, but general to species within that phylum.

A majority of the primers in the literature target the cyanobacterial geosmin synthase gene (Devi et al., 2021). Giglio et al. (2008) published the first PCR assay as a diagnostic tool for cyanobacterial geosmin producers. This initial protocol was able to detect the presence of geosmin producers in water, but it relied on melt curve analysis to identify the organisms responsible for geosmin synthesis, making it largely inconclusive. Additionally, no clear relationship between gene copy number and geosmin concentration failed to be established. Su et al. (2013) developed and tested two primer sets, one to quantify *Anabaena* strains and another to quantify the geosmin synthase gene in *Anabaena*. They were successfully able to quantify *Anabaena* and detect the geosmin synthase gene at 0.02 pg DNA and quantify it at 0.2 pg DNA (Su et al., 2013). Furthermore, when their approach was used on field samples, Su et al. (2013) reported a positive correlation ($r^2 = 0.742$) between gene copy number and intracellular geosmin levels. However, a correlation between gene copy number and extracellular geosmin levels failed to be established ($r^2 = 0.253$). Kutovaya and Watson (2014) created a primer that successfully amplified the geosmin synthase gene of a wide variety of benthic and planktonic cyanobacteria in lab cultures and environmental samples. The quantification ability of the primer was undetermined. In 2014, Tsao et al. developed SYBR Green (SGF1/JDR1) and TaqMan qPCR (3139F/3245R) assays based on the geosmin synthase gene of *Anabaena ucrainica* CHAB2155. The SGF1/JDR1 primer set only failed to amplify one of six geosmin producers, while the 3139F/3245R primer set successfully amplified only and all of the geosmin producers. When applied to a cyanobacterial bloom in the Myponga Reservoir, South Australia, both assays showed a good positive relationship (r^2=0.87 and 0.78 for the 3139F/3245R and SGF1/JDR1 primer sets, respectively) between gene copies and geosmin levels. The SGF1/JDR1 primer set was used in a study by Gaget et al. (2020) to monitor the production of geosmin in benthic mats.

Poor correlation between the *geoA* copy number and geosmin concentrations was reported, indicating that cyanobacteria are likely not the major producers in benthic mats (Gaget et al., 2020).

Actinobacteria, such as *Streptomyces*, are also capable of synthesizing geosmin (Gerber, 1979). Auffret et. al (2011) developed qPCR primers AMgeo-F/AMgeo-R based on a portion of the *geoA* gene in *S. avertmitilis.* Analysis using four individual *geoA* sequences representing a wide range of large diversity of the geosmin synthase gene showed that the AMgeo-F/AMgeo-R primer set had high amplification efficiencies ranging from 0.9 to 1.1 and a significant linear relationship between cycle threshold and gene copies (Auffret et al., 2011). Additionally, when the four geoA sequences were combined into a single standard to simulate environmental samples, the primer set was capable of quantifying the geosmin synthase gene within the range of 4×10^2 to 4×10^8 *geoA* copies (Auffret et al., 2011). However, further testing by Kutovaya and Watson (2014) revealed that the AMgeo-F/AMgeo-R primer set failed to generate any PCR product in both culture and environmental samples. Kutovaya and Watson (2014) instead used the alignment from Auffret et al. (2011) to develop degenerate primers for the *actinomycete* geosmin synthase gene referred to as *geoA_act.* Field validation of the *geoA_act* primer set failed to amplify the actinomycete geosmin synthase gene in environmental samples and further improvement of the primer was not pursued (Kutovaya and Watson, 2014). Lukassen et al. (2017) developed a TaqMan-based qPCR approach to quantify non-cyanobacterial geosmin producers and applied their approach to recirculating aquaculture systems. Although, the studied systems showed the presence of *Actinobacteria* they were unable to either quantify *Actinobacteria* abundance or detect Actinobacterial geosmin producers (Lukassen et al., 2017). However, the primers developed by Lukassen et al. (2017) did amplify an abundance of *geoA*

genes belonging to uncultured bacteria. This suggests that there are likely many more geosmin-producing bacteria than are currently listed in the NCBI database.

Conclusions

Methods to control algae blooms and their associated geosmin episodes are species-specific and often require multiple treatment applications. Not only do these repeated applications represent an increase in operational cost for water management facilities, but in some cases, they can reduce treatment efficacy. Therefore, it is imperative that early detection of geosmin and its producers be developed. Biomolecular approaches, such as PCR and qPCR, are promising alternatives to present methods of monitoring geosmin. PCR and qPCR are sensitive, rapid, allow for a large number of samples to be analyzed at once,e and can identify known and unknown geosmin producers. However, the accuracy of PCR and qPCR is dependent on the assay. The inability of primers to detect all geosmin producers, non-specific amplification,n, and low efficiencies inhibit the widespread use of PCR and qPCR to monitor geosmin episodes in drinking water utilities.

Methods

3.1 Sample Collection

Samples with medium to high levels of geosmin were needed in order to test the quantification ability of each primer set. Samples were collected during the late Spring and early Fall of 2020, as Lake Saugahatchee (32.6712° N, 85.4305° W) taste and odor episodes typically occur during this period. Water and sediment samples were taken from seven locations within Lake Saugahatchee and two tributaries.

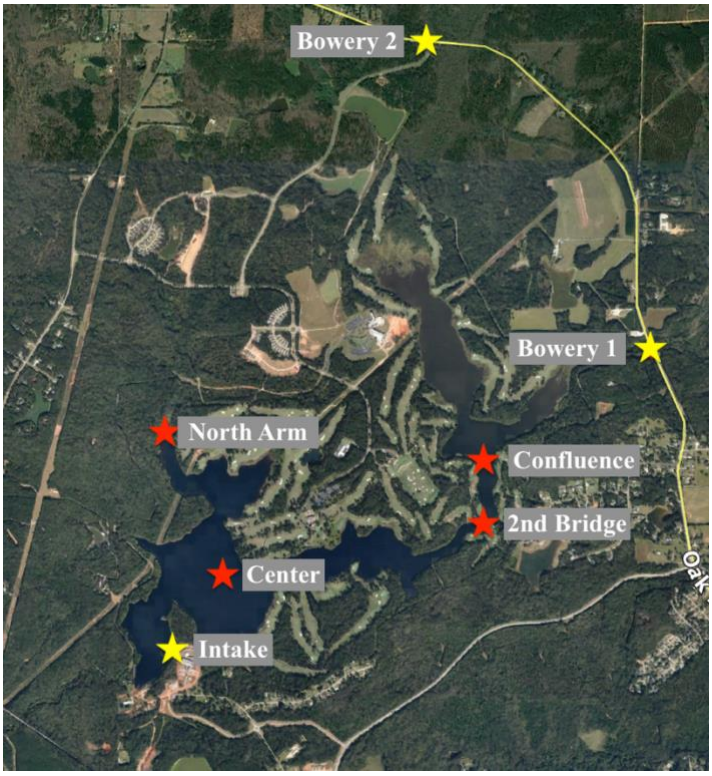

Figure 1. Sampling locations in Lake Saugahatchee and two tributary creeks. Yellow stars denote areas of interest.

All samples were taken at surface level, except at the “Intake” and “Center” where a horizontal water sampler was used to take additional samples at a depth of 10ft. 250 ml water samples and 15 ml sediment samples were collected for DNA extraction. A complementary set of 40 mL water samples were collected in glass vials for geosmin analysis by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. All water samples were taken in duplicate. To prevent the loss of geosmin through volatilization, the samples were checked for air bubbles and then sealed immediately with parafilm upon collection. Using a sediment scoop, sediment was collected at all locations, except for the “Intake” and “Center” locations, where the water was too deep to allow for easy sample collection.

Water samples were refrigerated for no more than three days prior to filtration. Sediment was aliquoted into 2 ml tubes and frozen at -20 °C. Samples with medium to high levels of geosmin were required to evaluate primer assay performance. However, geosmin concentrations never exceeded the odor threshold during the sampling period. In order to obtain a wide variety of samples with medium to high levels of geosmin, additional samples were obtained from fishpond samples collected by Dr. Alan Wilson at Auburn University and by staff at the City of Auburn and Columbus Water Works.

3.2 Microbial Biomass Harvesting and DNA Extraction

Microbial biomass was harvested by vacuum filtering lake water through 0.2 μm nitrocellulose filters. Nitrocellulose filters were proven to be among the most effective for DNA extraction from environmental water samples (Kaevska & Slana, 2015). Filters were soaked for 5 minutes in NanoPure water before filtering lake water to leach and remove residual nitrate in the filters. Water was added to the filter in 25 ml increments until it clogged. The filters were cut

into pieces and stored at -20 ºC in 2 ml tubes. A Qiagen DNeasy PowerSoil Pro Kit was used to extract genetic material per the manufacturer's instructions. The PowerSoil Kit has been shown to effectively extract DNA from both sediment and water samples (Kaevska & Slana, 2015).

3.3 qPCR and DNA Quantification

An Analytic Jena qTower3 qPCR instrument was used to quantify geosmin synthase in DNA extracts of the different samples. SYBR Green detection was used. Five primers targeting the geosmin synthase gene in cyanobacteria and actinobacteria were used in this study (Table 1). Three of these primer sets were developed by others and they were selected because they have been used by at least one additional research group (besides the developers) since their publication. The other two primer sets, CGeo1 and ActGeo2 were developed in our lab. Alignment of geosmin synthase genes from a range of cyanobacteria (*Aphanizomenon, Nostoc, Planktothrix, Oscillatoria*, and *Dolichospermum* (*Anabaena)*) was used to develop the CGeo1 primer. Regions that were conserved among the cyanobacteria were identified visually and roughly 20 bp sequences were extracted. These sequences were run through NCBI's BLAST tool to check for specificity toward geosmin synthase. The CGeo1 forward primer is almost exclusively mapped to gene sequences associated with cyanobacteria taxa that are known to produce geosmin (e.g. *Cylindrospermum, Aphanizomenon, Oscillatoria, Planktothrix, Nostoc*). The E-value was 0.19 for hits with perfect coverage and identity matches, reflecting that there was a low probability these hits were obtained by random chance. The CGeo1 reverse primer yielded top hits matching the germacradienol synthase (geoA) gene in a range of uncultured bacteria, *Oscillatoria*, and *Planktothrix*, all with E values less than or equal to 0.19. Finally, these primers were analyzed for melt temperature, hairpin, homodimer, and heterodimer

structure using IDT's OligoAnalyzer tool. Low propensity for dimerization is important for achieving efficient and meaningful qPCR results. A similar procedure was used for the development of the ActGeo2 primer set except that synthase genes were aligned from two species of *Streptomycetes, Kibdelosporangium, Kitasatospora,* and *Nocardia*. Identifying conserved regions of ~20 bp length was more challenging for the actinobacteria than it was for the cyanobacteria. The top BLAST hits for the ActGeo2 primers were to *Streptomycetes* with E-values of 0.035 for the forward primer and 0.55 for the reverse primer.

Table 1. Primer used in qPCR and genetic sequencing analysis and their expected product lengths.

Primer Set	**Forward Primer**	**Reverse Primer**	**Expected Length (bp)**	**Target Organisms**	**Source**
CGeo-1F/CGeo-1R	GATCACTTCCTGGAAATCTAT	ACTCCATCAGCAAATGTGTC	180	Cyanobacteria	This Study
SGF1/JDR1	CATCGAATACATCGAGATGCG	TCGCCTTCATCTTCCACTTC	200	Cyanobacteria	Tsao et al., 2014
288AF/288AR	AACGACCTGTTCTCCTA	GCTGGAGCGCATGTGCC	288	Cyanobacteria	Giglio et al., 2008

ActGeo-2F/ActGeo-2R	GACTGGTACGTGTGGGTGTTCT	CGGCGCATCTGATGTACTC	324	Actinobacteria	This Study
AMgeo-F/AMgeo-R	GAGTACATCGAGATGCGCCGCAA	GAGAAGAGGTCGTTGCGCAGGTG	167	Actinobacteria	Auffret et al., 2011

Samples from a variety of sources with moderate to high geosmin levels were amplified using each primer set. The thermal cycling conditions were as follows: an initial denaturing step at 95ºC for 5 minutes, followed by 45 cycles of 95ºC for 15 seconds, the desired annealing temperature for 30 s (Table 2), and 72ºC for 45 seconds, and a final extension at 72ºC for 5 minutes. For each primer set, three different annealing temperatures were tested and are displayed in the below table. Generally, a higher annealing temperature should lead to better binding specificity but with a potential tradeoff in PCR efficiency.

Table 2. Annealing temperatures used for each assay.

Primer set	**Low annealing temp. (ºC)**	**T_m anneal temp. (ºC)**	**High annealing temp. (ºC)**
CGeo-1F/CGeo-1R	48	50	52
SGF1/JDR1	52.3	54.3	57
ActGeo-2F/ActGeo-2R	58	59.8	62
AMgeo-F/AMgeo-R	60.5	62.5	64.5
288AF/288AR	48.4	50.4	52.4

Next, primer efficiency was tested. For each primer set, a standard was created by purifying the products from a previous PCR run using the QIAquick PCR Purification Kit (Qiagen). The DNA in each standard was quantified using the Promega QuantiFluor dsDNA System per the manufacturer's instructions. A 10-fold serial dilution was performed on the standard material to create a curve. PCR was then run again using this curve to determine primer efficiency.

3.4 Gel Electrophoresis

Gel electrophoresis was conducted on the PCR products to verify that the primers were amplifying genes of the correct length. 25X TAE Buffer was prepared by dissolving 60.5 g Tris-base and 14.275 mL acetic acid in DI water. NaOH was added to the solution to bring the pH to about 8.5. Then, 25 mL of 0.5M sodium EDTA was dissolved into the Tris-solution. The TAE Buffer then underwent a 1X dilution before being used. To prepare the gel, 0.5 g of agar were dissolved in 50 mL of 1X TAE Buffer. The agar solution was then poured into a gel tray and cooled until it solidified. Next, 10 μL of PCR product were combined with 5 μL of DI water and 5 μL of loading dye on parafilm. The resulting 20 μL of each sample were then loaded into a well on the gel. The gel was run at 125V for about 20 minutes. The gel was then soaked in a solution of 0.5 μg/mL EtBr buffer solution on a shaker table at 60 rpm and 20 ^{o}C for 20-30 minutes. The gel was then transferred to water and allowed to de-stain for 5 minutes. After de-staining, the gels were viewed under UV light.

3.5 Genetic Sequencing

Twelve samples showing high levels of geosmin were selected for sequencing. These samples underwent at least one round of PCR with 12.5µl Hotstar Taq DNA Polymerase (Qiagen), 2.0 µl $MgCl_2$, 0.2 µl bovine serum albumin, 6.8 µl microbial DNA-Free water, 1 µl of sample, and 1.25 µl each of the 0.5 µM forward and reverse primers. The thermal cycling conditions included an initial holding time of 5 minutes at 95°C; 35 cycles at 95°C for 30 seconds, 53°C for 40 seconds and 72 °C for 1 minute; and a final extension at 72 °C for 10 minutes. This process was for each primer set. PCR results were then analyzed using 2% agarose gel. PCR products that were considered "very weak" and "negative" were then purified using AMPure XP beads. 1 µL of the purified sample then underwent another round of PCR using the same protocol described above. A final PCR reaction was carried out to add adapters to each sample. Each well contained 9µl microbial DNA-free water, 2.5µl 5µM unique adapter, 12.5µl Hotstar Taq DNA Polymerase (Qiagen), and 1µl PCR product for each sample. The thermal cycling conditions included an initial holding time of 5 minutes at 95°C; 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72 °C for 30 seconds; and a final extension at 72 °C for 5 minutes. Samples were again analyzed on 2% agarose gel. Samples were then pooled together and purified using AMPure XP beads. The purified pool was analyzed on Agilent 2100 Bioanalyzer to determine the final library size for sequencing dilutions. Dilutions were made following Illumina's Miseq protocol.

Results

4.1 Geosmin Concentrations

Geosmin levels within Lake Saugahatchee were below the threshold for human detection throughout the warm season in 2020 (Figure 2).

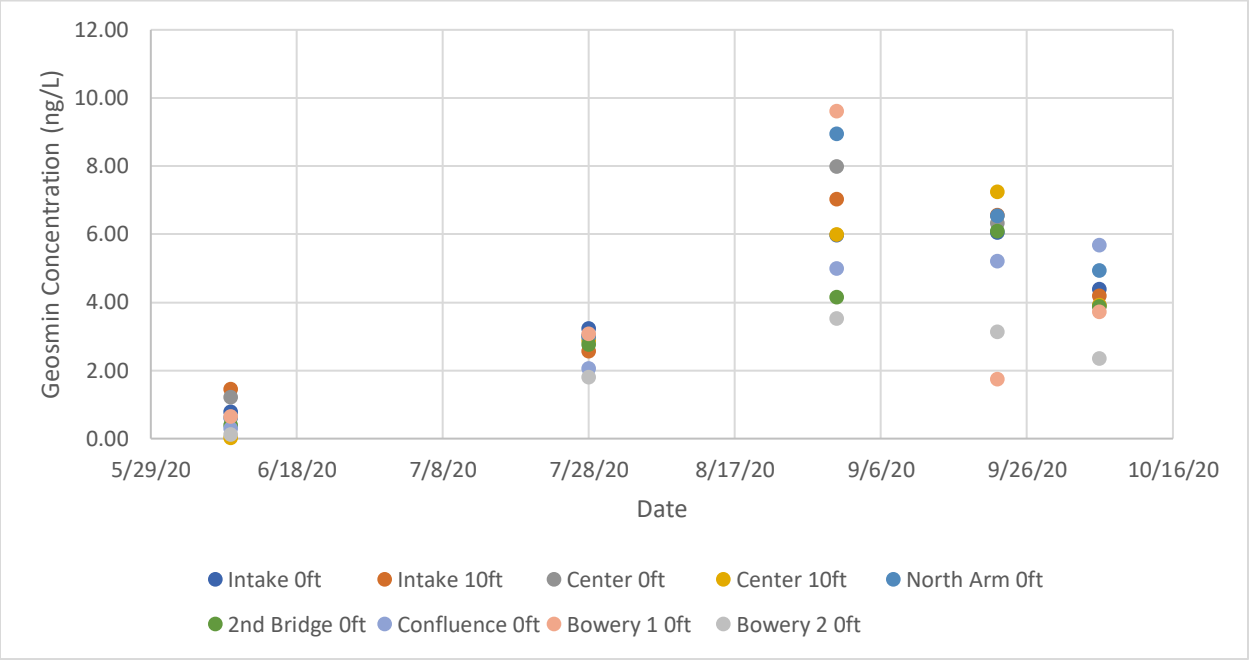

Figure 2. The geosmin concentrations measured in Lake Saugahatchee using GC-MS. At no point during the sampling period did the geosmin concentrations exceed the odor threshold of 10 ng/L.

The geosmin concentrations did follow the expected temporal pattern with levels being very low in late Spring and steadily increasing before hitting a peak in August. The geosmin levels then continued to decline. No one area of the lake or either of the tributary creeks seems to be responsible for geosmin synthesis. Many researchers have reported an inverse relationship

between chlorophyll-a and geosmin synthesis (Blevins et al., 1995; Giglio et al., 2011; Zhang et al., 2009). Geranyl pyrophosphate and farnesyl pyrophosphate are precursors for both geosmin and chlorophyll-a synthesis (Giglio et al. 2018; Espinosa et al., 2021). It is thought that this inverse relationship is due to the competition over these substrates (Wang & Li, 2015). This theory is supported by Cai et al. (2017) who reported that lower light intensities reduced the chlorophyll-a synthesis by *O. limosa* CHAB7000, while simultaneously stimulating the release of intracellular geosmin into the environment. Additionally, Tsao et al. (2014) found that *Anabaena* cells at a depth of 20 m had higher geosmin cell quotas than those at the surface. However, geosmin levels at the surface were due to higher overall biomass (Tsao et al., 2014). This trend was not observed in the present study. At locations "Intake" and "Center", depth does not seem to affect geosmin concentrations, with the levels being higher at the surface one sampling period, then higher at a depth of 10ft the next. This may be because a bloom never occurred during the sampling period and overall biomass remained low. As previously stated, the odor detection threshold by humans for geosmin is 10 ng/L. Even during its peak, geosmin levels failed to reach a level high enough to constitute a T&O event. In order to properly evaluate the primer sets, the sample set was supplemented with samples from regional water bodies that showed high levels of geosmin. A list of the samples used for primer evaluation as well as their geosmin concentrations can be found below.

Table 3. Samples used for primer evaluation. An "X" indicates that the sample was used in the analysis, while an "NA" means that the sample was not used for analysis.

Sample	**Waterbody**	**Type**	**Geosmin Concentration (ng/L)**	**Flow Cell Sequencing**	**qPCR**
A-Lower 5/5/20	Ogletree	Water	26	X	X
A-Upper 5/5/20	Ogletree	Water	41.8	X	X
A-Upper 3/10/20	Ogletree	Water	21.4	X	X
OP-5ft 5/6/20	Saugahatchee	Water	23.1	X	X
A-Upper 4/20/20	Ogletree	Water	19.1	X	X
C-BOAT 4/1/20	Oliver	Water	6.6	X	X
OP-5ft 4/22/20	Saugahatchee	Water	19.4	X	X
Museum sample	Museum retention pond	Water	1000	X	NA
Bowery 2 Sed. 7/28/20	Saugahatchee	Sediment surface	1.79	X	NA
Confluence sed. 6/9/20	Saugahatchee	Sediment surface	0.28	X	NA
NA sed. 6/9/20	Saugahatchee	Sediment surface	0.6	X	NA
Bowery 1 sed. 7/28/20	Saugahatchee	Sediment surface	3.06	X	NA

The sample set consisted of 8 water samples and 4 surface sediment samples from three water bodies. Geosmin concentrations varied from 0.6 ng/L to 1000 ng/L. Due to an insufficient amount of material, some of the samples could not be used in both analyses. The samples used in each analysis can be found in Table 3.

4.2 Gel Electrophoresis

To evaluate whether or not the primers amplified a gene of the expected target length, gel electrophoresis was performed. Figure 3 shows the PCR products amplified using the CGeo-1F/CGeo-1R primer set at two annealing temperatures of 48^{o}C and 52^{o}C.

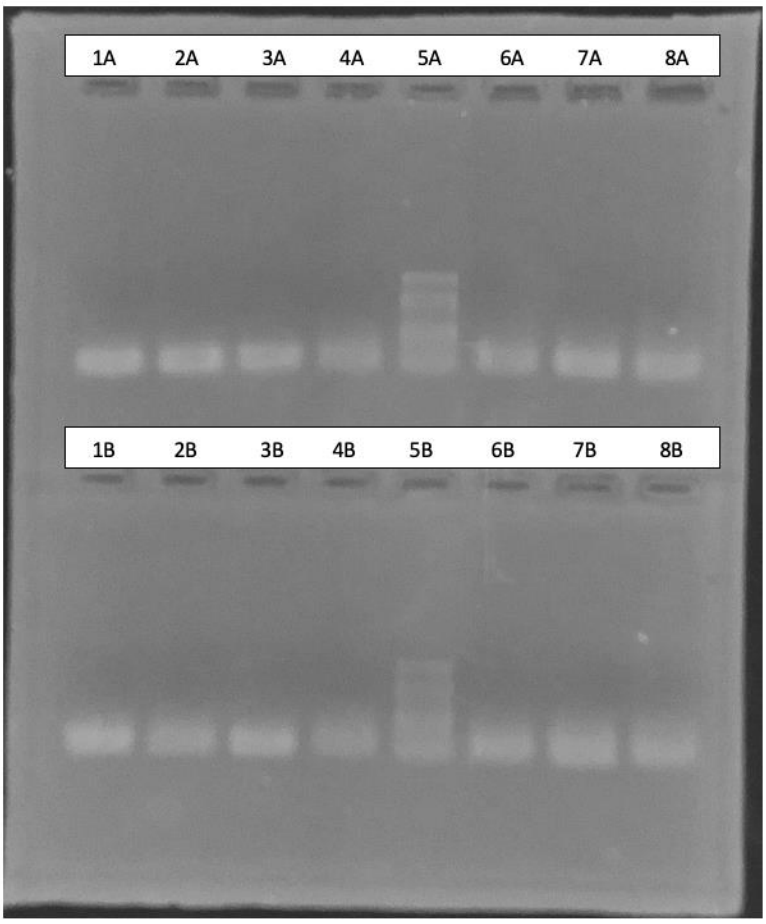

Figure 3. Product length after one round of PCR using the CGeo-1F/CGeo-1R primer set. The expected product length was 180 bp.

All lanes resulted in lengths of about 200 bp, which is in agreement with the expected length of 180 bp. Additionally, there was very little dimer formation even at the lower annealing temperature, indicating that the CGeo-1F/CGeo-1R primer set is highly specific.

SGF1/JDR1 primer set, which also was designed to target geosmin synthase in cyanobacteria, was also evaluated. The gel indicates some challenges with primer specificity at both the low and high annealing temperatures of 52.3ºC and 57ºC, respectively. (Figure 4). However, in most cases, there was a band around 200 bp long which was the expected product length.

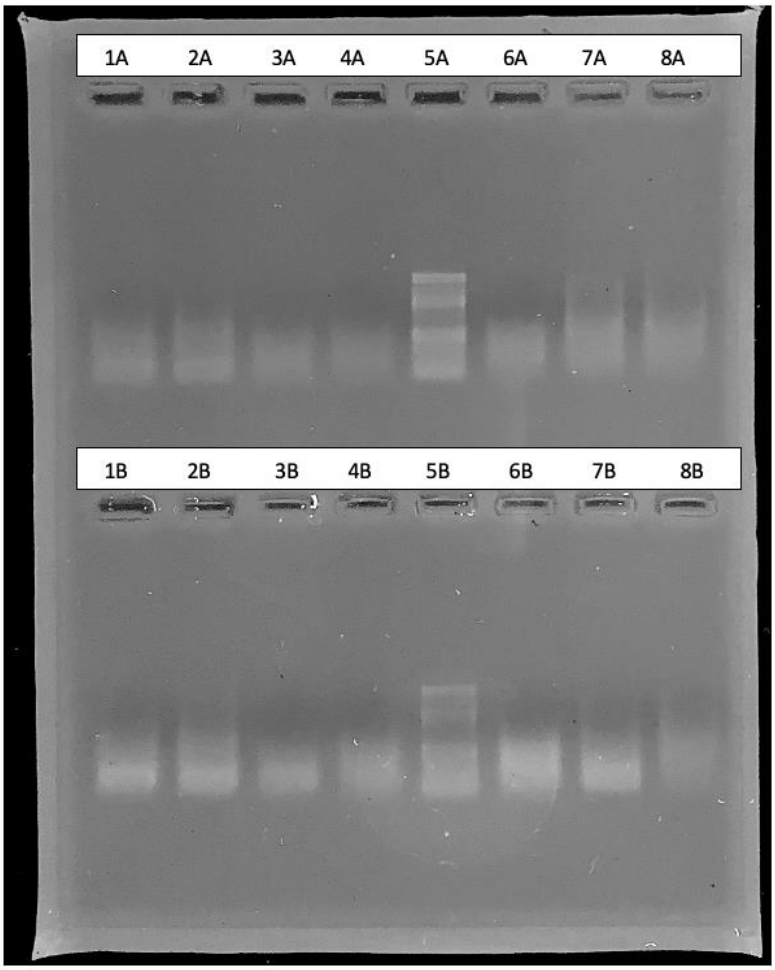

Figure 4. Product length after one round of PCR using the SGF1/JDR1 primer set. The expected product length was 200 bp.

In lanes with weak product formation, there was evidence of primer dimerization. At the higher annealing temperature, products appeared brighter, indicating stronger amplification. Lanes 1B, 2B, and 7B resulted in strong smudges around the expected length of 200bp. Lane 3B

resulted in a product that was predominantly around the desired length, yet there was still quite a bit of dimerization and the resulting smudge was weak.

Of the three primers targeting the cyanobacteria geosmin synthase gene, the 288AF/288AR primer set exhibited the least specificity. Nearly all lanes resulted in weak smudging with the low annealing temperature resulting in stronger smudges than the high annealing

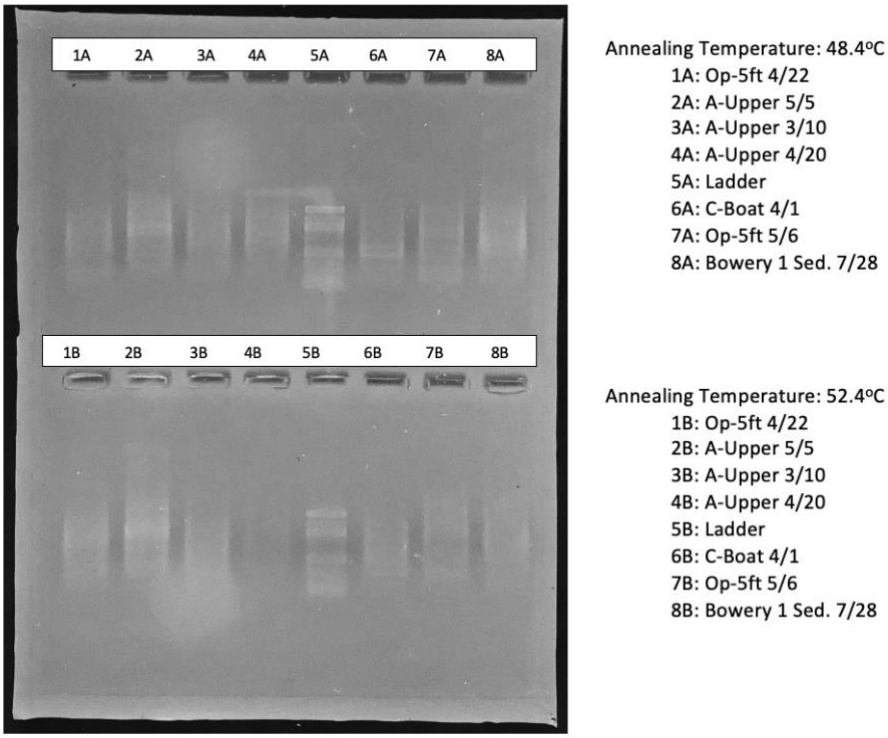

Figure 5. Product length after one round of PCR using the 288AF/288AR primer set. Expected product length was 288 bp.

Lanes 2A, 6A, and 2B were the only lanes to form weak bands. Lanes 2A and 2B showed weak bands of about 700bp, while the weak band in lane 6A had a length of 500bp. With each of the previously mentioned lanes, the weak bands were much larger than the expected 288bp length.

The ActGeo-2F/ActGeo-2R primer set resulted in similar patterns between the low and high annealing temperatures of 58°C and 62°C, respectively. The results of the gel can be seen in Figure 5.

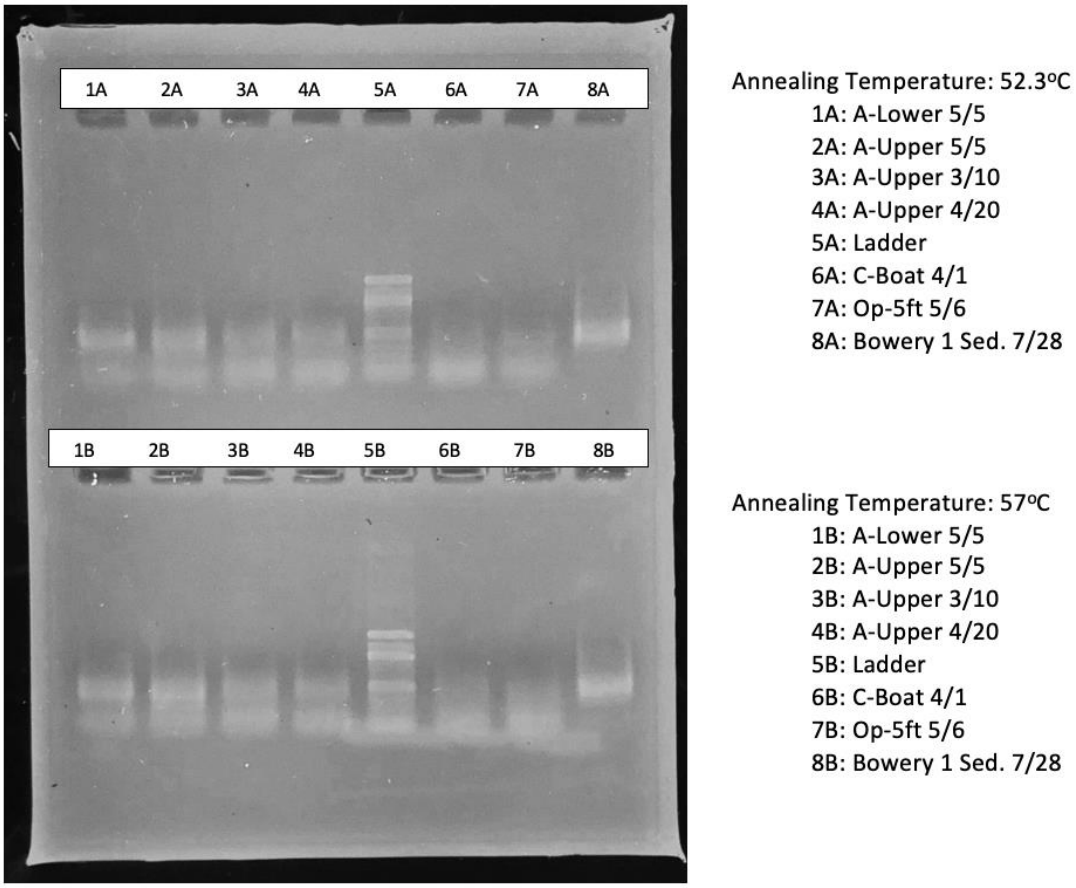

Figure 6. Product length after one round of PCR using the ActGeo-2F/ActGeo-2R primer set. The expected product length was 324 bp.

The higher annealing temperature resulted in brighter bands. However, the difference is hardly discernible. Lanes 1A, 2A, 1B, and 2B achieved a medium band formation at the expected band length of about 400bp yet there was also a large amount of dimer formation around the 100 bp marker. The dimer band is stronger than the expected product band for lanes 3A, 4A, 6A, 7A, 3B, 4B, 6B, and 7B. Lanes 8A and 8B (sediment samples) were the only lanes that achieved

strong band formation at the desired length. Both lanes had little dimer formation. This result is somewhat expected – sediment is expected to harbor large quantities of actinobacteria whereas lower levels would be expected in the water column. Indeed, with the exception of the C-Boat sample, the sediment sample had 4 to 106 times more gene copies than the water column samples based on threshold cycle analysis. The C-boat sample appeared to have a large number of gene copies (similar to the sediment samples), but it is apparent from the gel that much of the amplified product was dimers. These results suggest that ActGeo2 primers likely can amplify the correct product and can be useful for sequencing analysis, but dimerization may complicate their deployment in qPCR assays.

The AMgeo primer set was designed to target actinobacteria geosmin producers. The expected product length for the AMgeo-F/AMgeo-R primer set was 167 bp however, nearly all lanes resulted in a strong smudge ranging in size from 500bp to about 200bp.

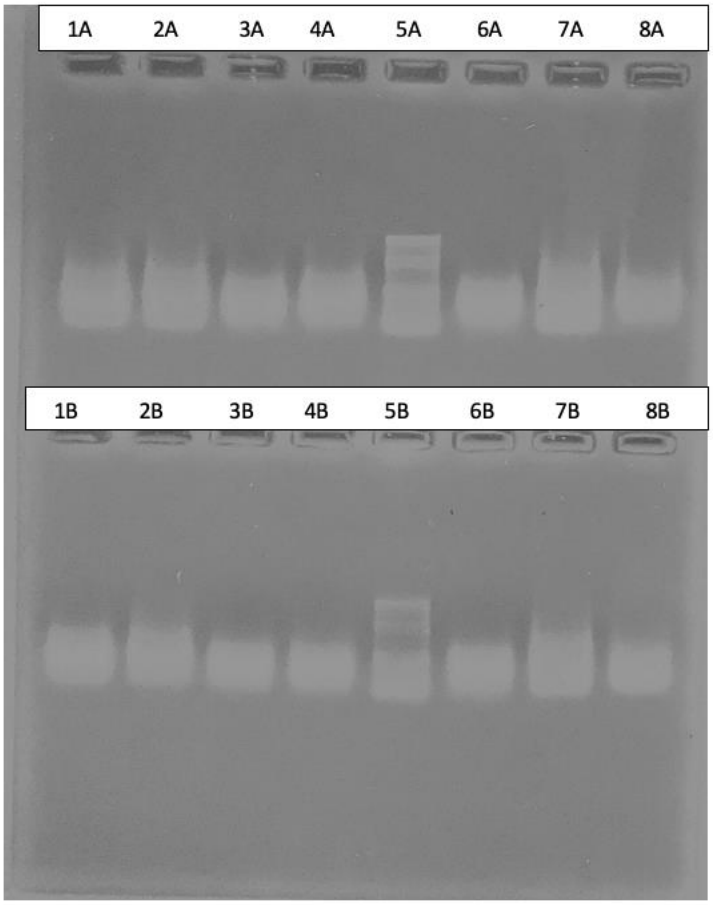

Figure 7. Product length after one round of PCR using the AMgeo-F/AMgeo-R primer set. Expected product length was 167 bp.

Even at the higher annealing temperature of 64.5ºC no individual bands formed. Variation in gene length is often observed among different taxa and sequencing is necessary to better understand if these products match to geosmin synthase.

4.3 Genetic Sequencing

The distribution of the amplified genes by the five primer sets were analyzed in order to better understand what products were amplified. Genetic sequencing confirmed the gel results for the CGeo-1F/CGeo-1R primer set, revealing it to be highly selective for cyanobacteria geosmin synthase. In all cases, the CGeo-1F/CGeo-1R primer set only amplified cyanobacteria. Additionally, the CGeo-1F/CGeo-1R primer set successfully amplified the geosmin synthase gene in seven genera: *Oscillatoria, Phormidium, Dolichospermum, Aphanizomenon, Planktothrix, Nostoc,* and *Tychonema.* All seven of the genera have been shown to contain geosmin-producing species (Devi et al., 2021). In all cases, the mapping identity of sequence reads to a reference database of known-geosmin synthase gene sequences was >90%.

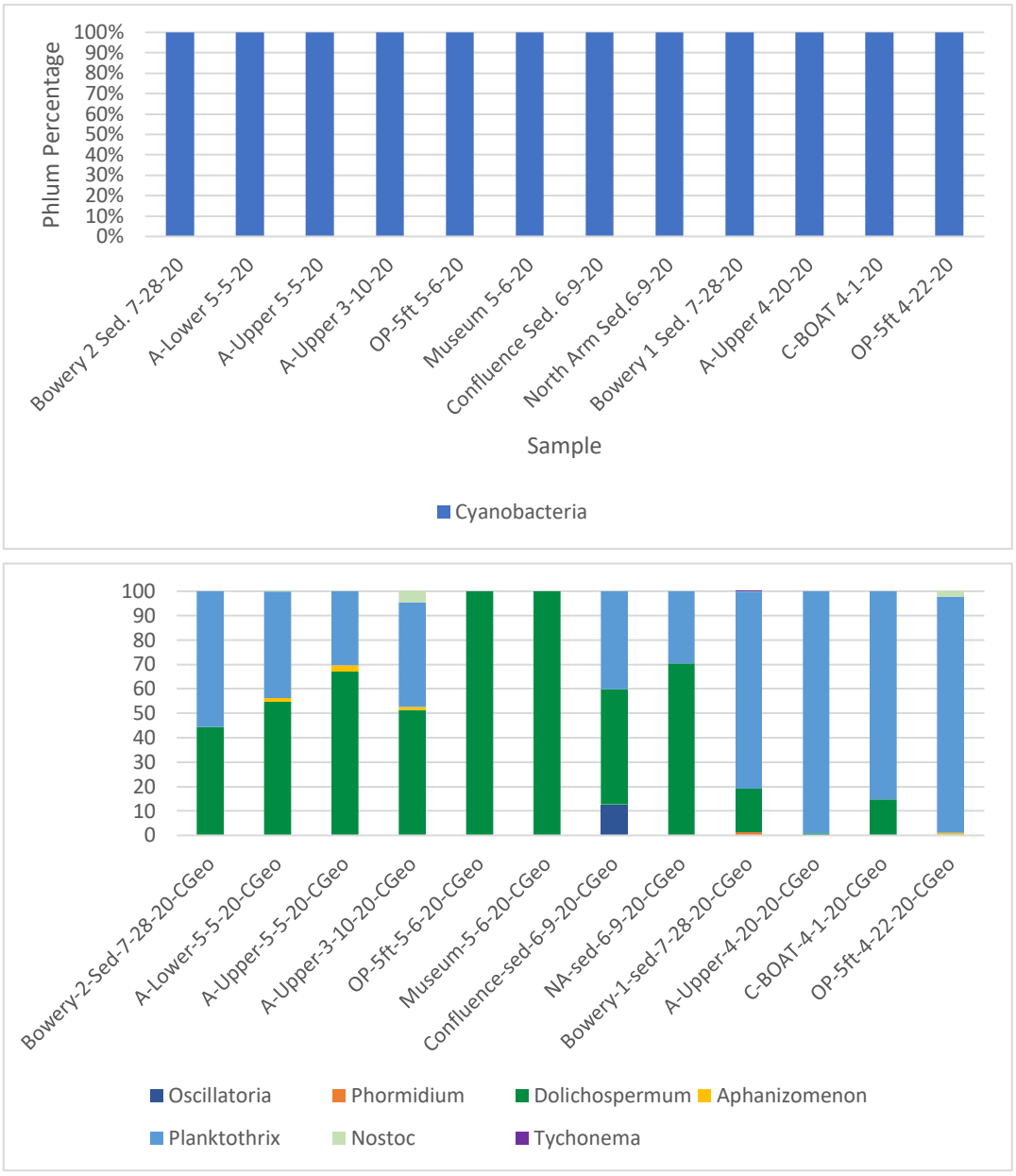

Figure 8. Genetic sequencing results following amplification using the CGeo-1F/CGeo-1R primer set.

In contrast, the SGF1/JDR1 primer set was much less selective. Seven of the samples analyzed did amplify a majority of cyanobacteria with one sample amplifying only the cyanobacterial geosmin synthase gene. However, many of the reads from the SGF1/JDR1 primer

set mapped to actinobacteria, proteobacteria, and Bacteriodetes. Amplification of both cyanobacteria and actinobacteria is not necessarily a problem – a universal primer would be useful as long as the target truly is geosmin synthase. Overall, the sequences mapping to cyanobacteria also mapped to the geosmin synthase gene, and actinobacteria sequences generally mapped to geosmin synthase in *Streptomyces*. While this was encouraging, the high abundance of proteobacteria and Bacteroidetes raise questions about fidelity to geosmin synthase. The proteobacteria reads mapped predominantly to Acinetobacter and *Sorangium* (a myxobacteria). Acinetobacter are not known to produce geosmin and the sequence associated with this OTU did not map to a cyclase gene in the NCBI database. Strains of *Sorangium* are known to contain a terpene cyclase with similarity to geosmin synthase (Schneiker et al., 2007) and may be capable of producing geosmin (Lukassen et al., 2019). Here too, however, the sequence associated with this OTU did not map well to the cyclase gene for *Sorangium* in the NCBI database (~66% match).

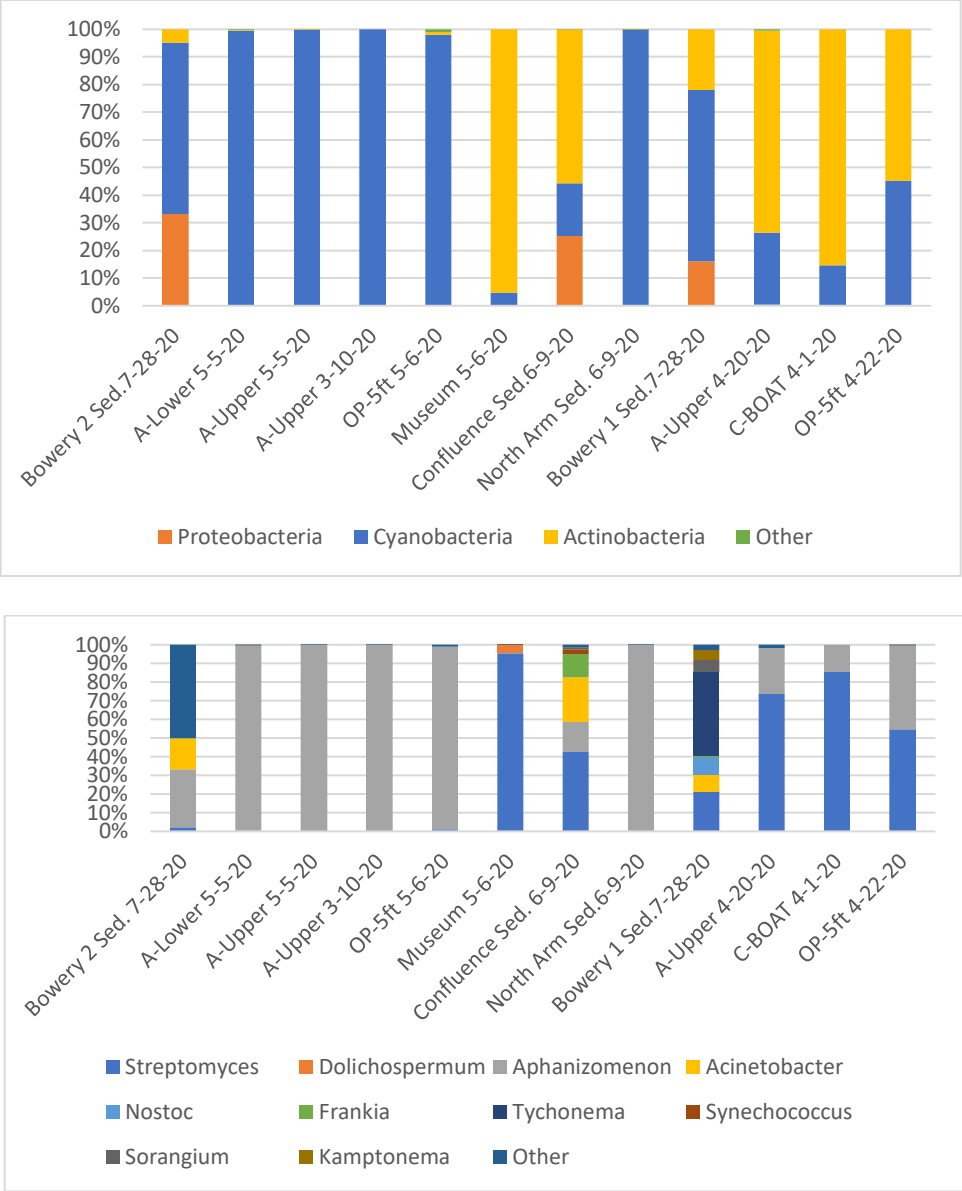

Figure 9. Genetic sequencing results following amplification using the SGF1/JDR1 primer set.

The 288AF/288AR primer set exhibited high levels of dimerization. As a result, two rounds of PCR prior to sequencing were required. Once sufficient amplification could be achieved, sequencing revealed that the 288AF/288AR primer set was not selective, amplifying cyanobacteria as well as actinobacteria and Bacteroidetes.

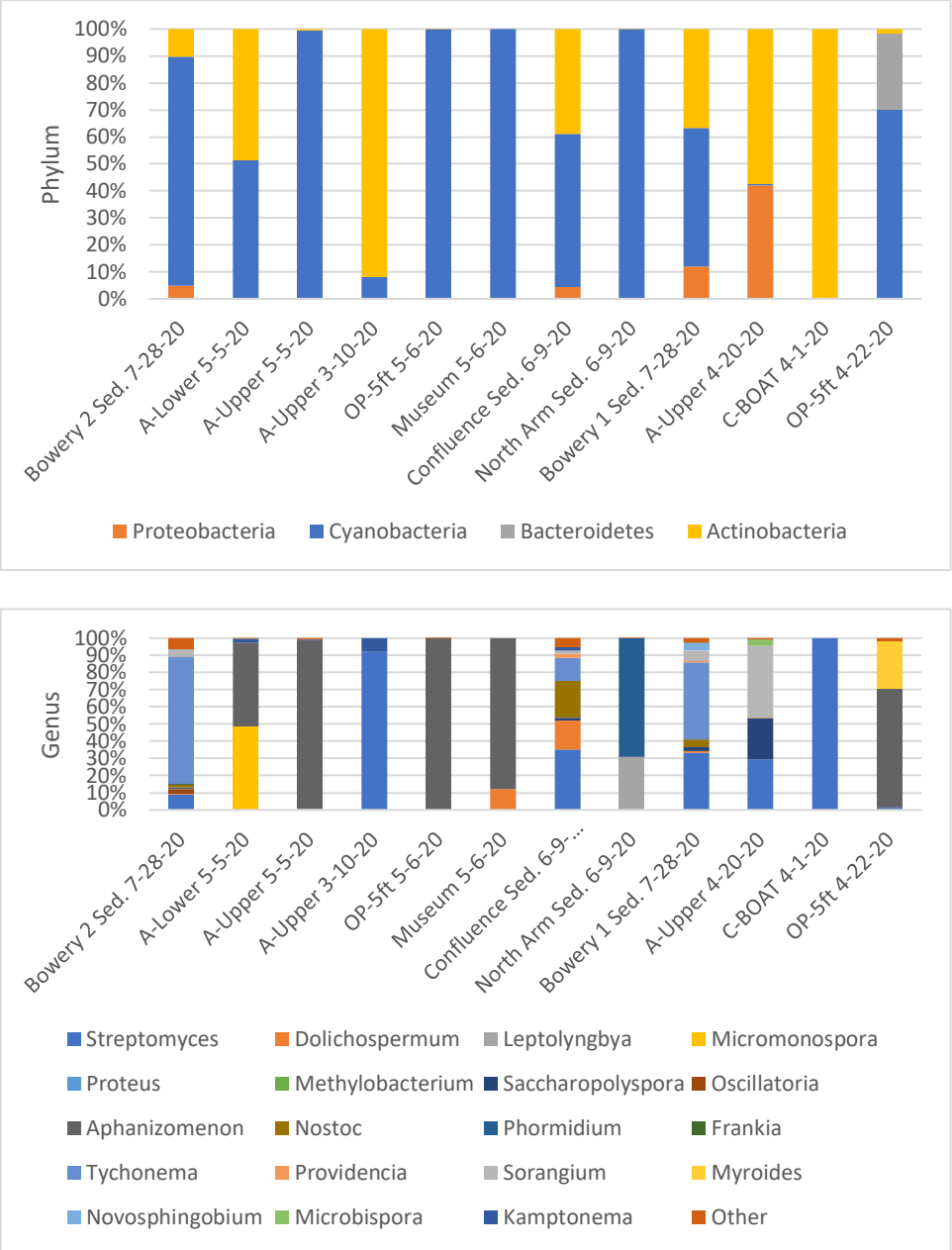

Figure 10. Genetic sequencing results following two rounds of amplification using the 288AF/288AR primer set.

Though all eight of the cyanobacterial genera amplified have been shown to produce geosmin and the sequences generally matched to the geosmin synthase gene (Devi et al., 2014; Melo et al., 2020). Additionally, *Streptomyces* is a well-documented producer of geosmin and is

commonly responsible for T&O episodes (Zuo et al., 2010; Asquith et al., 2018). The other actinobacteria amplified here include *Microbispora, Saccharopolyspora, Micromonospora*, and *Frankia*. *Microbispora hainanensis* strain CSR-4 has been shown to contain a gene with 100% similarity to the geosmin synthase gene cluster from *Nostoc punctiforme* PCC 73102 (Thawai et al., 2020). Four gene clusters of *Micromonospora* spp. are identical to ones known to produce geosmin (Carro et al., 2018). Three strains of *Frankia* have been shown to contain the geosmin synthase gene (Udwary et al., 2011) and *Saccharopolyspora spinosa* has been predicted to contain the geosmin synthase gene (Guo et al., 2020). Similar to the SGF1/JDR1 primer set, a majority of the sequences mapping to proteobacteria were for *Sorangium,* which as previously stated, may be capable of producing geosmin (Lukassen et al., 2019). Interestingly, *Methylobacterium* sp. and strains of *Novosphingobium* are potential geosmin degraders (Xue et al., 2011; Churro et., 2020). Although non-specific to the geosmin synthase gene, the 288AF/288AR primer set may be suitable for the detection of geosmin producers

None of the primers were selected for actinobacteria exclusively. The ActGeo-2F/ActGeo-2R primer set amplified actinobacteria as well as cyanobacteria and proteobacteria. However, the cyanobacteria amplified have been shown to produce geosmin and the sequences (Devi et al., 2014; Melo et al., 2020). For the cyanobacteria, the mapping identity of sequence reads to a reference database of known-geosmin synthase gene sequences was >87%. A majority of the sequences mapping to actinobacteria belonged to *Streptomyces*. These sequences had an identity match >70% when compared to the *Streptomyces* geosmin synthase genes in the NCBI database. Although Frankia has been shown to contain the geosmin synthase gene the sequences mapping to *Frankia* spp. did not code for the geosmin synthase gene (Udwary et al., 2011). Furthermore, the ActGeo-2F/ActGeo-2R primer set mapped with sequences identities of >70%

with the geosmin synthase genes of uncultured bacteria. These sequences did however map to the geosmin synthase gene in *Saccharopolyspora* with a 74% identity match. The majority of proteobacteria amplified was again *Sorangium*. Again, the regions amplified did not code for the geosmin synthase gene.

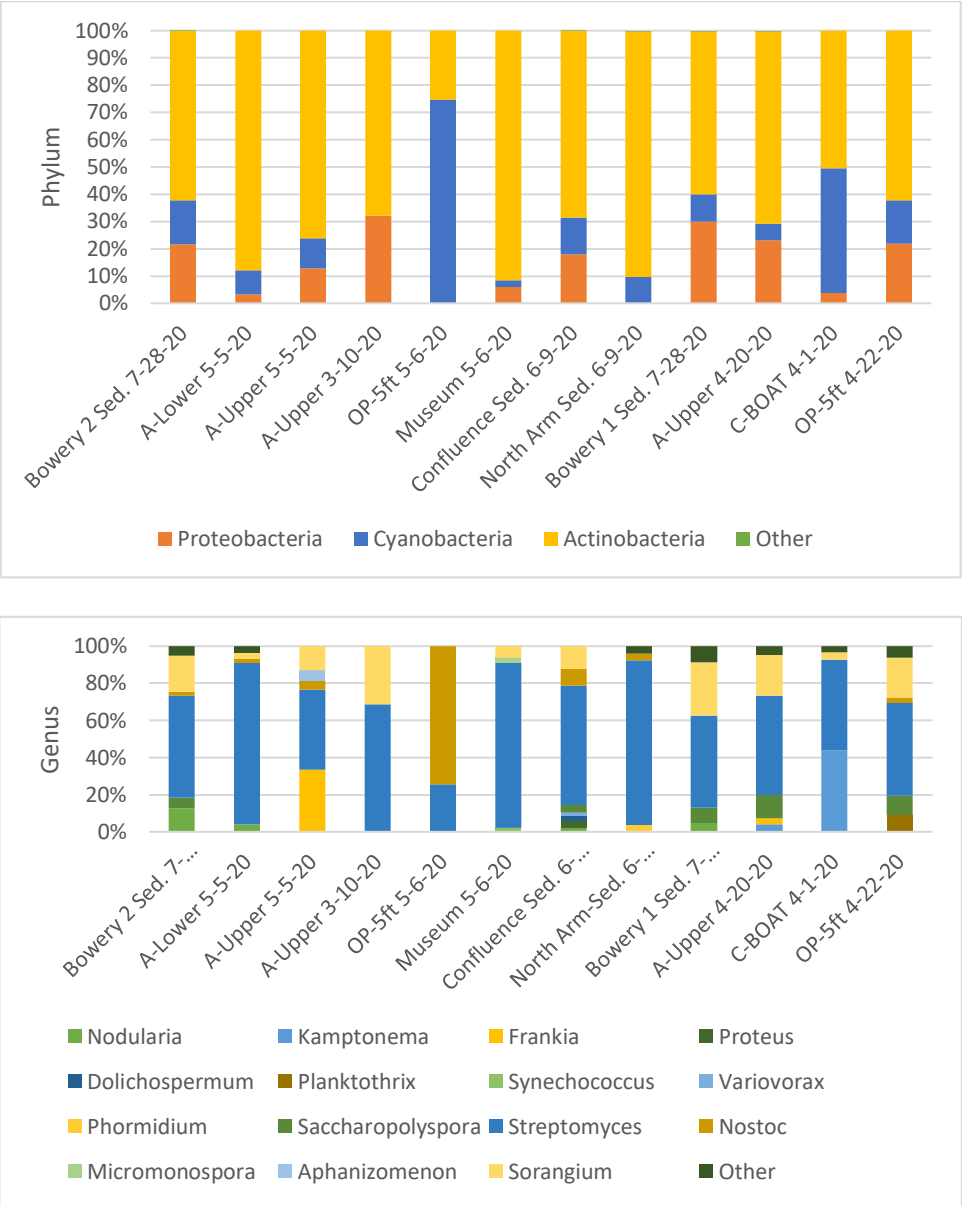

Figure 11. Genetic sequencing results following amplification using the ActGeo-2F/ActGeo-2R primer set.

Genetic sequencing of the PCR products following amplification using the AMgeo-F/AMgeo-R primer set depicted a similar microbial composition as the ActGeo-2F/ActGeo-2R primers with actinobacteria being the dominant phylum in every sample, except Op-5ft 5-6-20. The dominant proteobacteria amplified by both primers was *Sorangium.* Again, the sequences mapping to *Sorangium* did not code for the geosmin synthase gene. Also, both primers amplified a majority of *Streptomyces*. This was expected because the primers were designed using the *Streptomyces* geosmin synthase gene as a reference.

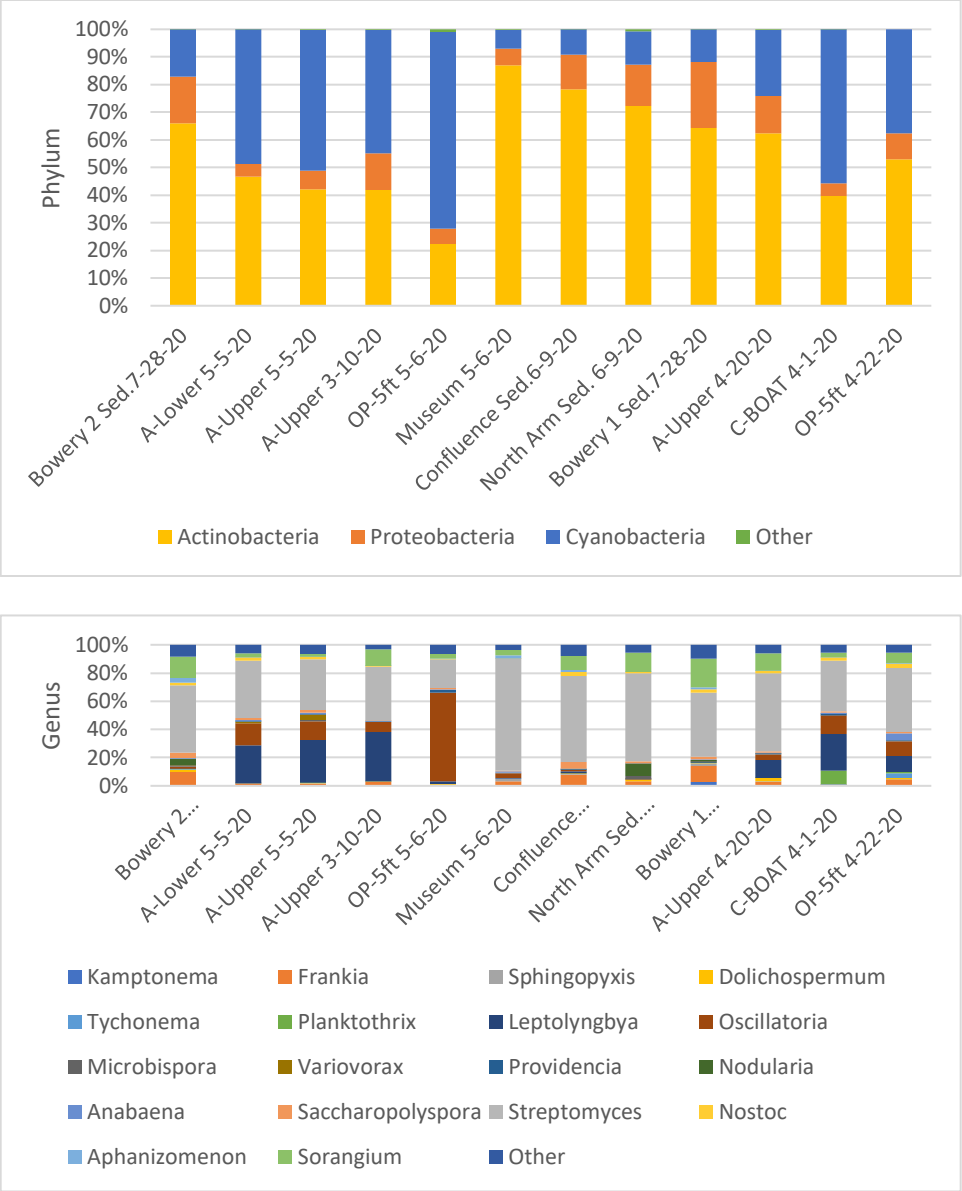

Figure 12. Genetic sequencing results following amplification using the AMgeo-F/AMgeo-R primer set.

However, with sample Op-5ft 5-6-20, the AMgeo-F/AMgeo-R primer set indicated that *Oscillatoria* was the dominant phyla, while ActGeo-2F/ActGeo-2R showed *Nostoc* as the dominant species. The binding sites for the ActGeo-2F/ActGeo-2R primer sets could potentially be more conserved in *Nostoc* spp. than in *Oscillatoria* spp. The reverse could be said of the

AMgeo-F/AMgeo-R primer set. Furthermore, the actinobacteria amplified by both primers were similar. However, there were some differences in non-actinobacterial organisms' identity between the two primer sets. The difference in abundance of these organisms was less apparent. Furthermore, the AMgeo-F/AMgeo-R primer set successfully amplified a significant number of geosmin synthase genes belonging to uncultured bacteria with identity matches >80%. It may be beneficial for the dual use of these primers to identify potential geosmin producers.

4.4 Amplification Efficiency

To determine the analytical characteristics and optimal thermocycling conditions of the assays, a ten-fold dilution was run at two annealing temperatures. These temperatures were specific to each primer set. However, using the lower concentrations often resulted in poor efficiencies because of low copy numbers. Table 4 shows the efficiencies of amplification using the full range of all standards with at least 10 gene copies per mL.

Table 4. Range of quantification and primer efficiencies at each primer set's respective high and low annealing temperatures.

Primer Set	**Annealing Temperature (ºC)**	**Efficiency**	**Range of Quantification (Gene copy number/mL)**
CGeo-1F/CGeo-1R	48	90%	$6.15x10^{1}$-$6.15x10^{5}$
	52	89%	$6.15x10^{1}$-$6.15x10^{5}$
SGF1/JDR1	52.3	184%	$1.12x10^{1}$ – $1.12x10^{6}$
	57	192%	$1.12x10^{1}$ – $1.12x10^{6}$
288AF/288AR	48.4	220%	$3.17x10^{1}$- $3.17x10^{5}$
	52.4	209%	$3.18x10^{1}$ - $3.40x10^{5}$
ActGeo-2F/ActGeo-2R	58	158%	$1.38x10^{1}$ –$1.38x10^{5}$
	62	158%	$1.38x10^{1}$ -$1.38x10^{5}$
AMgeo-F/AMgeo-R	60.5	211%	$1.22x10^{1}$ – $1.22x10^{5}$
	64.5	161%	$1.22x10^{1}$ – $1.22x10^{5}$

In order to better understand amplification efficiency, standard curves were truncated to exclude standards with low copy numbers to produce efficiency within or as close to the range of 90% to 110%. This is referred to here in this paper as the optimal range for qPCR but results in a loss of dynamic range. A summary of the results can be found in Table 5.

Table 5. Optimized Range of quantification and primer efficiencies at each primer set's respective high and low annealing temperatures.

Primer Set	**Annealing Temperature (°C)**	**Efficiency**	**Optimal Range of Quantification (Gene copy number/mL)**
CGeo-1F/CGeo-1R	48	99%	$6.15x10^{2}$- $6.15x10^{5}$
	52	100%	$6.15x10^{2}$- $6.15x10^{5}$
SGF1/JDR1	52.3	166%	$1.12x10^{5}$ – $1.12x10^{6}$
	57	138%	$1.12x10^{5}$ – $1.12x10^{6}$
288AF/288AR	48.4	111%	$3.17x10^{4}$ - $3.17x10^{5}$
	52.4	111%	$3.18x10^{4}$ - $3.18x10^{5}$
ActGeo-2F/ActGeo-2R	58	109%	$1.38x10^{3}$- $1.38x10^{5}$
	62	103%	$1.38x10^{3}$- $1.38x10^{5}$
AMgeo-F/AMgeo-R	60.5	154%	$1.22x10^{3}$ – $1.22x10^{5}$
	64.5	115%	$1.22x10^{3}$ – $1.22x10^{5}$

For the CGeo-1F/CGeo-1R primer set, standard curve analysis shows a linear range of quantification stretching from $6.15x10^{2}$ to $6.15x10^{5}$ gene copies/mL and an efficiency of 99% for when the annealing temperature was 48°C. Within this range, a linear relationship between the $\log_{10}$ of the gene copies and cycle threshold is defined as $\log_{10}(\text{gene copies}) = -0.2978C_t + 9.6479$ ($r^2 = 0.99$). For all quantification formulas, C_t is the cycle threshold. An increase in annealing temperature to 52°C had a negligible effect on assay performance. The linear relationship between the log of the gene copies and cycle threshold was $\log_{10}(\text{gene copies.}) = -$

$0.3016C_t + 9.708$ (r^2=0.99) with an efficiency of 100%. These efficiencies indicate that at both annealing temperatures the amplicon quantity almost doubled every cycle and that the assay is acceptable for quantification use. When expanding the quantification range to $6.15x10^1$ to 6.15x105 gene copies/mL, efficiencies were 111% and 110% at the low and high annealing temperatures, respectively. There were still linear relationships however, they were not as significant (r^2>0.88).

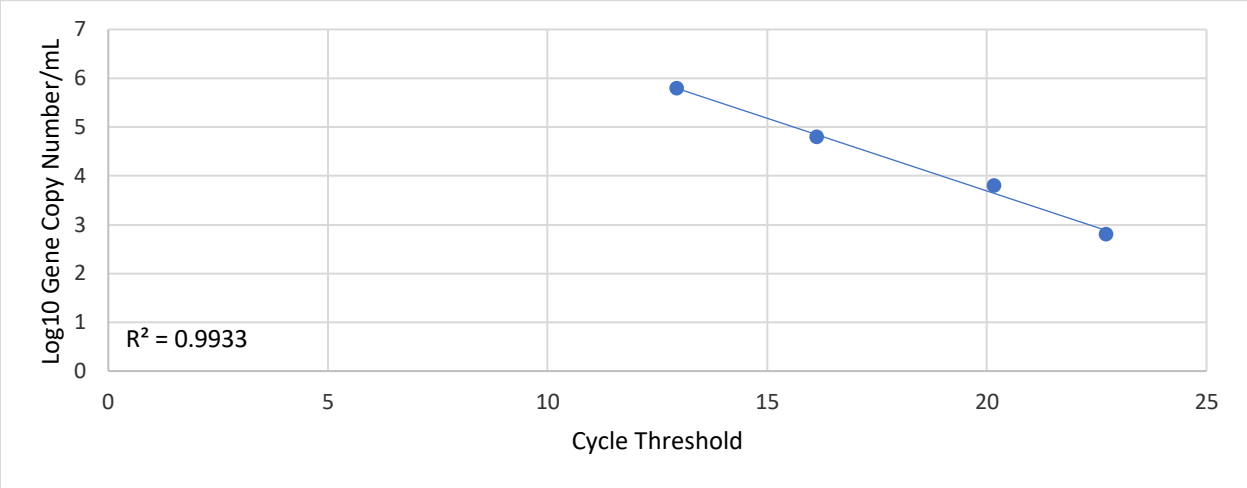

Figure 10. Optimized standard curve using the CGeo-1F/CGeo-1R primer set when annealing temperature was 48°C.

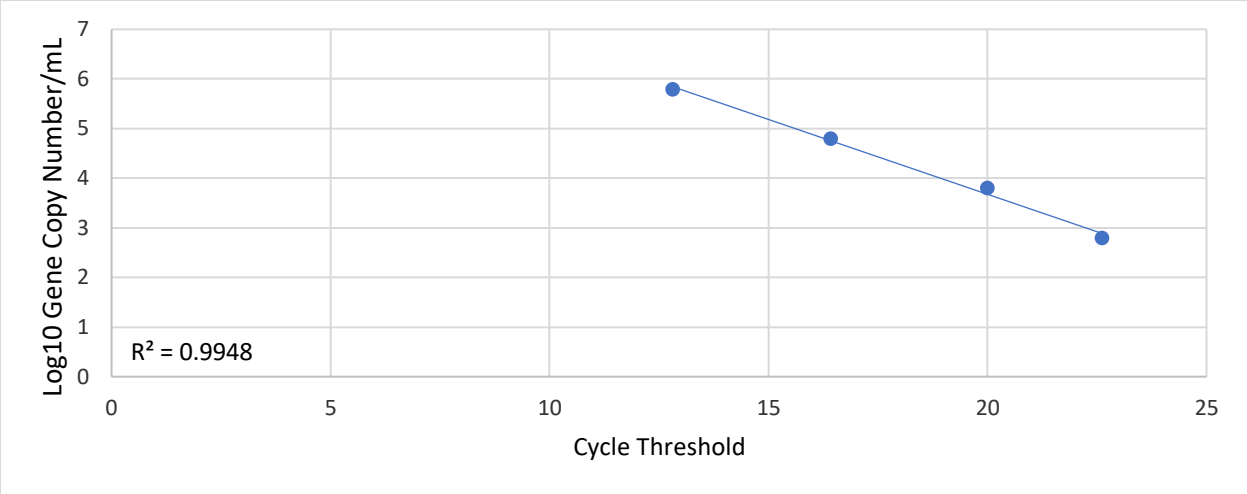

Figure 11. Optimized standard curve using the CGeo-1F/CGeo-1R primer set when annealing temperature was 52°C.

Tsao et al. (2014) reported a quantification range of 2 $x10^2$ -$2x10^6$ copies/mL and an efficiency of 109% with good linear correlation (r^2 =0.999). In this study, however, the SGF1/JDR1 primer set exhibited poor efficiencies at both annealing temperatures tested. At the lower annealing temperature of 52.3°C, the linear range of quantification was $1.12x10^1$ – $1.12x10^6$ copies/mL with an efficiency of 184%. An increase in annealing temperature to 57°C reduced the assay performance resulting in an efficiency of 192%.

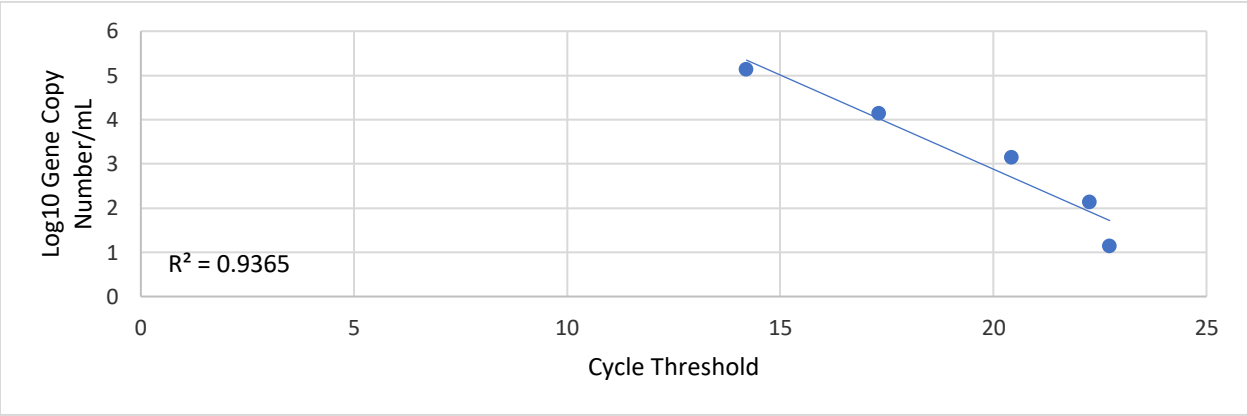

Figure 12. Standard curve using the SGF1/JDR1 primer set when the annealing temperature was 52.3°C

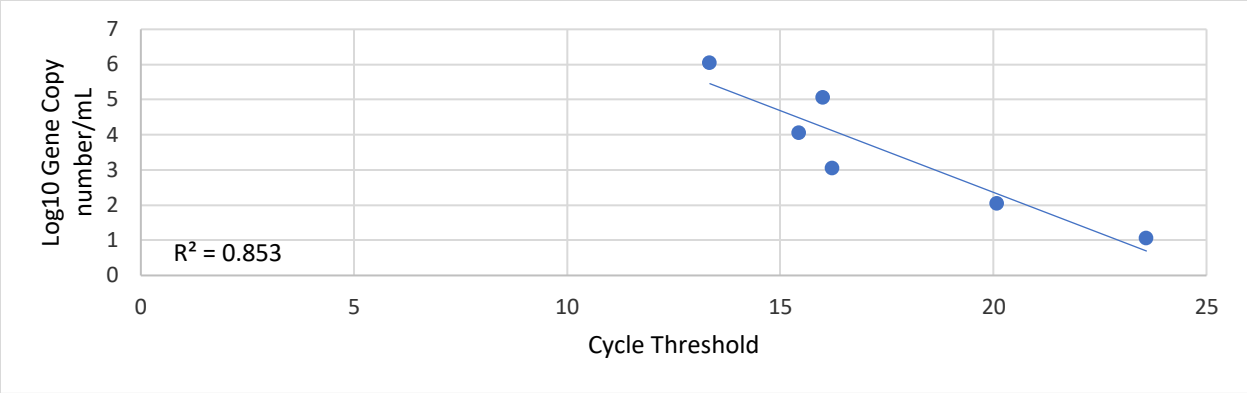

Figure 13. Standard curve using the SGF1/JDR1 primer set when the annealing temperature was 57°C.

The best efficiency achieved in this study using the SGF1/JDR1 primer set was 138% and was only achieved by reducing the quantification range to $1.12x10^5$ – $1.12x10^6$ copies/mL. The SGF1/JDR1 primer set was originally designed as a TaqMan assay. TaqMan qPCR is a tri-oligonucleotide system where the probe is the third oligonucleotide. The inclusion of a third oligonucleotide creates an additional level of sequence specificity, virtually eliminating reads of non-specific products (Nagy et al., 2017). In contrast, SYBR Green can emit a signal for the desired product as well as dimers and non-specific products (Kubista et al., 2006). In this study, the SGF1/JDR1 primer set was used as a SYBR Green assay and therefore did not use the probe designed by Tsao et al. (2014). The lack of a probe coupled with the presence of a non-specific product (Figure 20) may have resulted in the poor primer efficiencies observed in this study. The low levels of amplicon would further reduce primer efficiency (Figure 20).

In the study conducted by Giglio et al. (2008), the 288AF/288AR primer set was used only to detect geosmin producers, not quantify them. The results of this study indicate that the 288AF/288AR is not suitable for the quantification of geosmin producers at low concentrations.

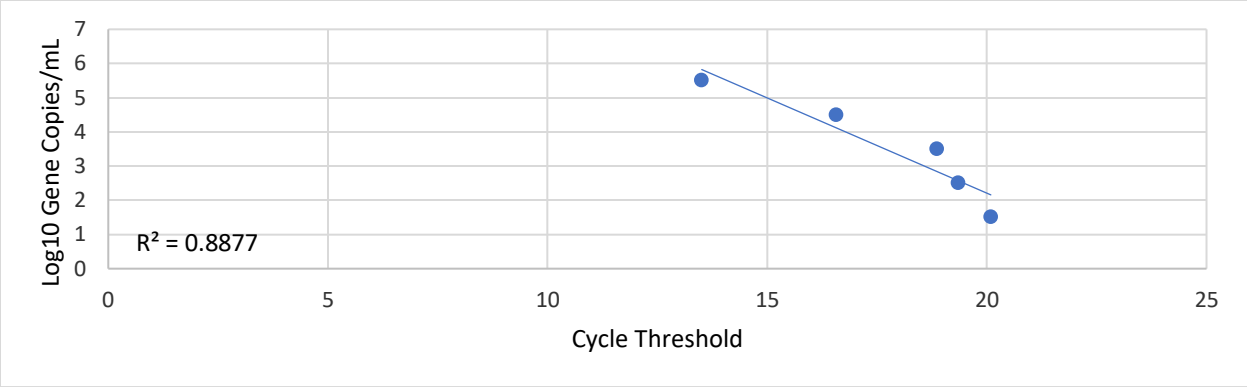

Figure 14. Standard curve using the 288AF/288AR primer set with an annealing temperature of 48.4°C.

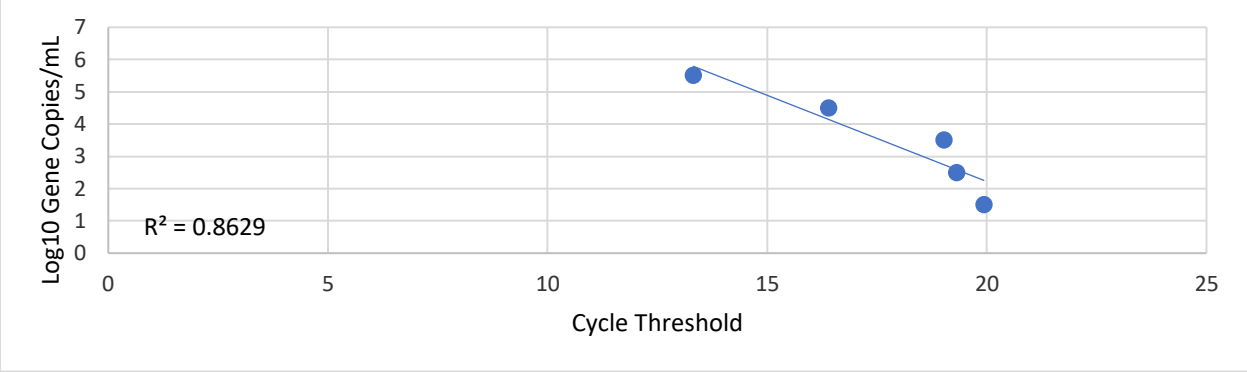

Figure 15. Standard curve using the 288AF/288AR primer set with an annealing temperature of 52.4°C.

Although the 288AF/288AR primer set showed a correlation between the log of the gene copies and the cycle threshold at both annealing temperatures (r^2>0.86), primer efficiencies were greater than 200%. A concentration of $3.17x10^4$ copies/mL must be reached before quantification is possible. A long amplicon length may result in poor amplification efficiency (Tsao et al., 2014) When compared to the other primers in this study targeting the cyanobacterial geosmin

synthase gene, the 288AF/288AR primer set had a relatively large amplicon length, which may have resulted in the poor efficiencies observed in this study.

The ActGeo-2F/ActGeo-2R primer set had poor efficiencies at both the high and low annealing temperatures.

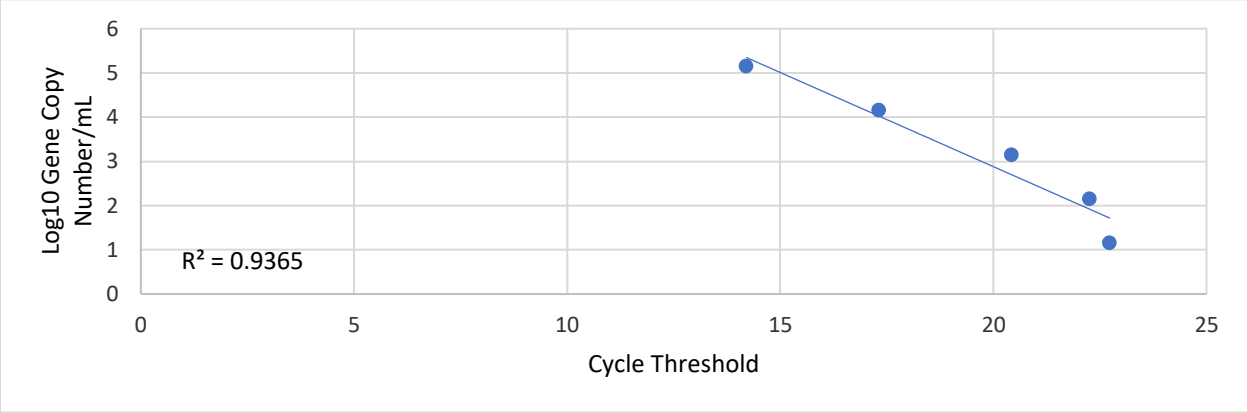

Figure 16. Standard curve using the ActGeo-2F/ActGeo-2R primer set with an annealing temperature of 58°C.

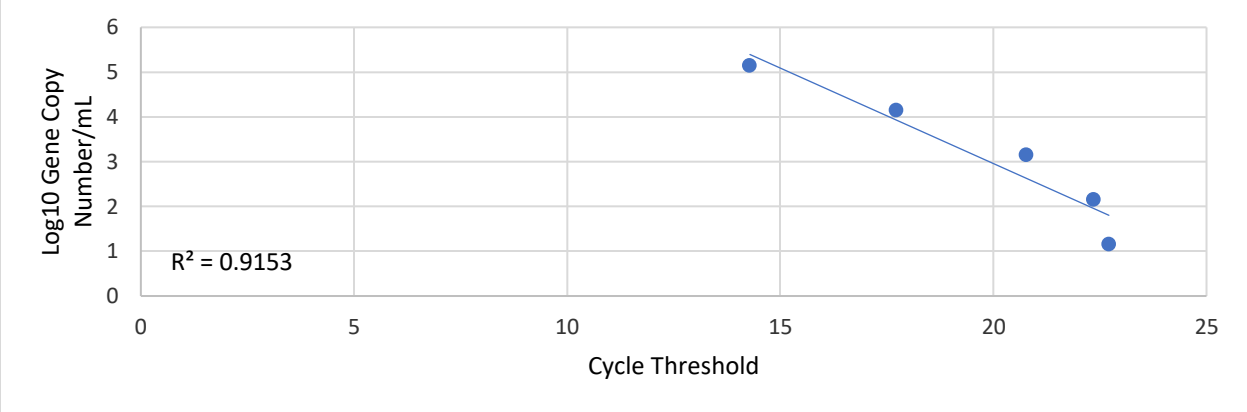

Figure 17. Standard curve using the ActGeo-2F/ActGeo-2R primer set with an annealing temperature of 57°C.

Similarly, the AMgeo-F/AMgeo-R primer set performed poorly with an efficiency of 267% when the annealing temperature was 60.5°C. Increasing the annealing temperature greatly improved primer efficiency, but at 192% accurate quantification is unlikely.

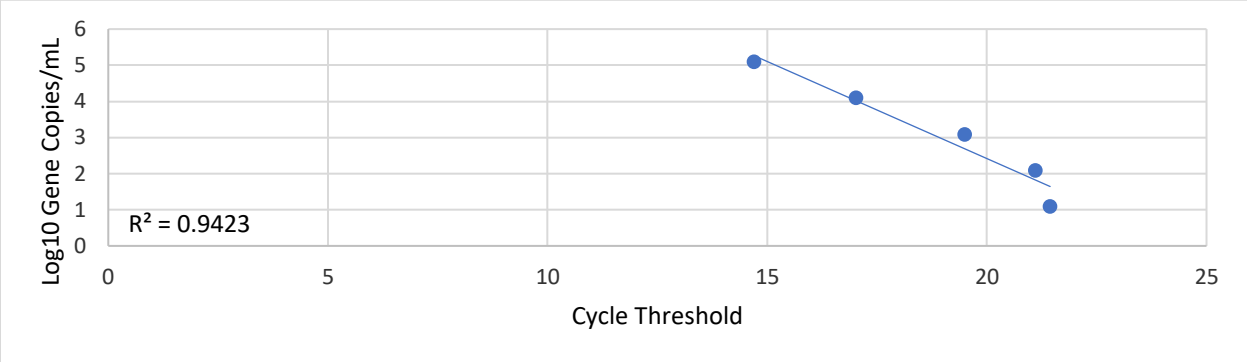

Figure 18. Standard curve using the AMgeo-F/AMgeo-R primer set with an annealing temperature of 60.5°C.

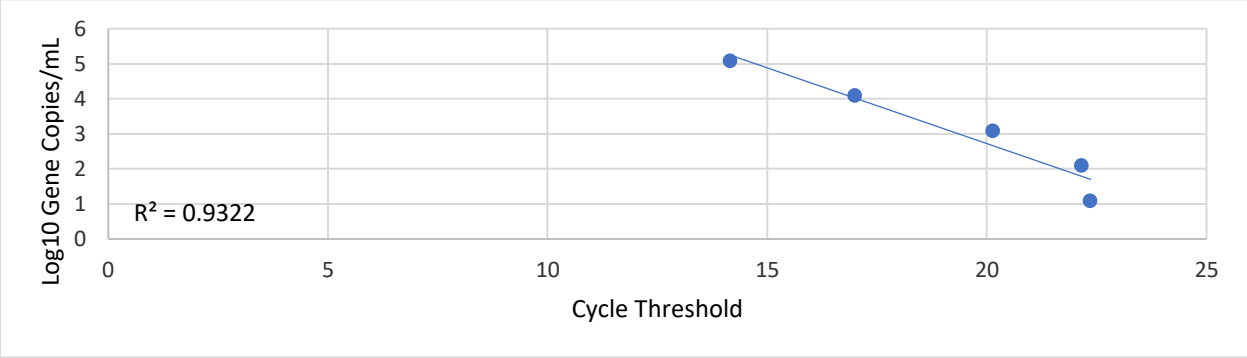

Figure 19. Standard curve using the AMgeo-F/AMgeo-R primer set with an annealing temperature of 64.5°C.

With the ActGeo-2F/ActGeo-2R primer set, the amplicon quantity failed to double every cycle with efficiencies 158% at both annealing temperatures of 58°C and 62°C. A reduction in the dynamic range from $1.38x10^{1}$ –$1.38x10^{5}$ copies/mL to $1.38x10^{3}$-$1.38x10^{5}$ copies/mL resulted in efficiencies of 109% and 103% for the low and high annealing temperatures, respectively.

At low template concentrations, primers have a higher incidence of primer dimer formation. This mainly is the result of primers binding to each other. These primer dimers are often slightly shorter than double the length of the primers themselves. This phenomenon is especially apparent in the gel electrophoresis on the 10^5 and 10^9 diluted standards of the ActGeo-2F/ActGeo-1F and AMgeo-F/AMgeo-R primers (Figure 20).

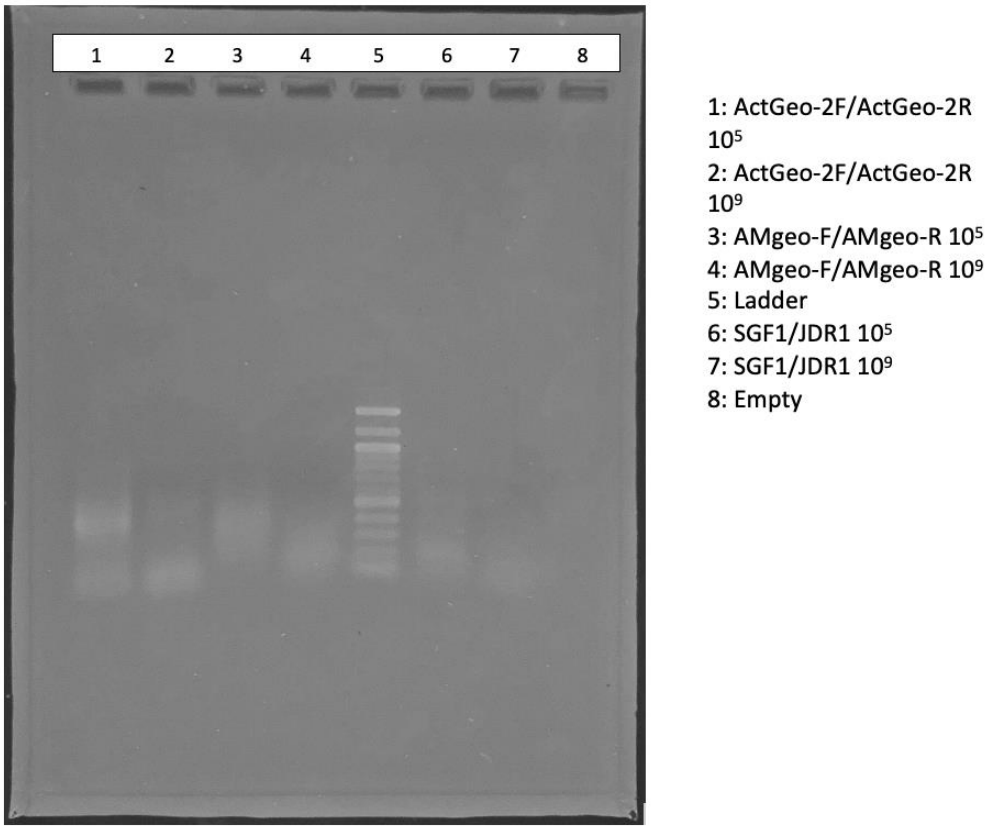

Figure 20. Product length of the 10^5 and 10^9 diluted standards. The expected lengths for the ActGeo-2F/ActGeo-2R, AMgeo-F/AMgeo-R and SGF1/JDR1 primer sets were 324 bp, 167 bp and 200 bp, respectively.

Cycle threshold is proportional to the amount of target gene only, so when there is little to no target DNA, cycle threshold is dependent on the amount of primer present in the standard.

This is why the cycle thresholds are very similar at the lower concentrations. In the case of SGF1, there is a fairly high tendency toward dimerization, hence C_t never gets very high.

4.5. Quantification of geosmin synthase in sequenced samples

In order to evaluate the quantification ability of each primer set, the qPCR approach was applied to a subset of environmental water samples that were also subjected to the aforementioned sequencing. These samples were obtained from three separate lakes and contained geosmin concentrations ranged from 1.79 ng/L to 41.8 ng/L. The optimized standard curves of each primer's high annealing temperature were used to calculate the geosmin synthase gene abundance for each primer set.

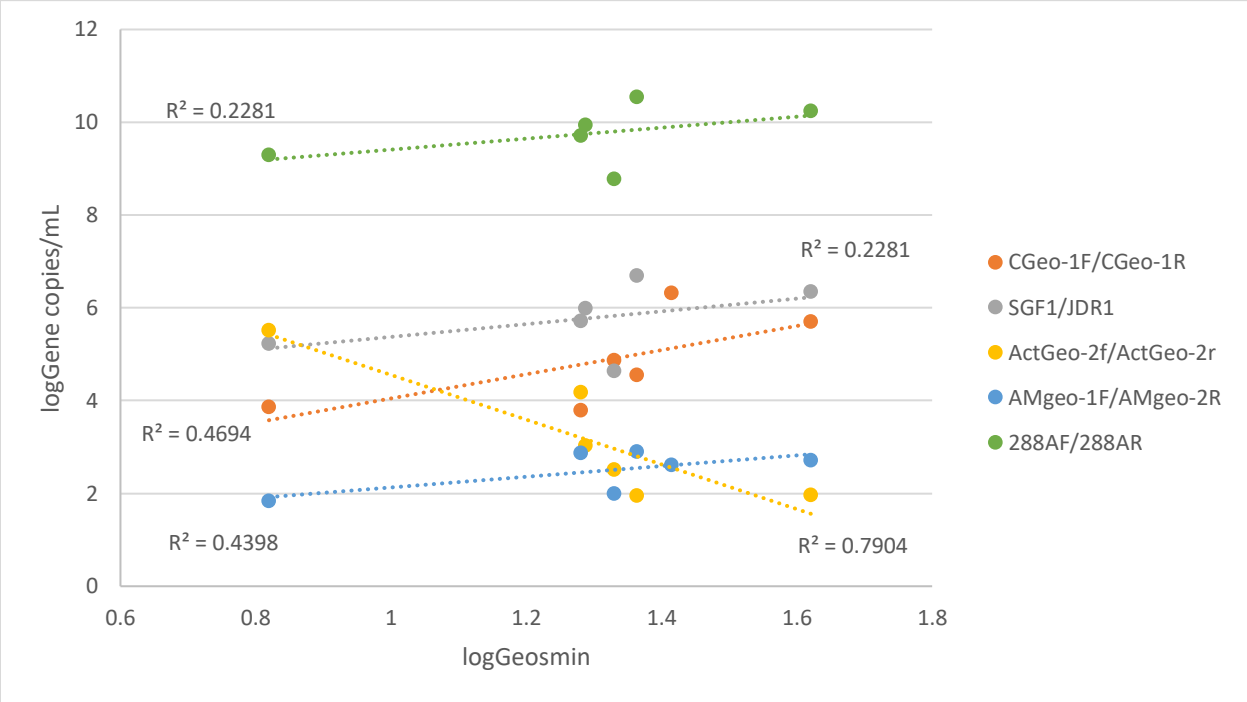

Figure 21. Correlation between geosmin levels measured via GC-MS and gene copy number amplified by each primer set. The optimized standard curve corresponding to each primer's high annealing temperature was to calculate gene copy abundance.

The SGF1/JDR1 and 288AF/288AR primer sets exhibited weak positive correlations to geosmin concentrations ($r^2 = 0.23$). The poor correlation between the gene copy numbers amplified by the SGF1/JDR1 primer set was somewhat expected because the primer only achieved an efficiency of 138%, which is considered inadequate for proper quantification. Gaget et al. (2020) used the SGF1/JDR1 primers to amplify the cyanobacterial geosmin synthase gene in benthic mats, but it was not well-correlated to the geosmin concentration in the water column. Our results show that this primer set also amplifies actinobacteria and proteobacteria geosmin producers in addition to cyanobacteria producers. A poor correlation between gene abundance amplified by the 288AF/288AR primer set was also expected due to its low specificity to the geosmin synthase gene. The CGeo-1F/CGeo-1R primer set more closely targeted the cyanobacterial geosmin synthase gene with high specificity and yielded a good correlation between gene copies and geosmin ($r^2 = 0.47$). The ActGeo2F/ActGeo-2R primer set had a strong but negative relationship between gene copies and geosmin concentrations ($r^2 = 0.79$). The majority of sequences amplified by the ActGeo-2F/ActGeo-2R primer set mapped to actinobacteria, which are typically benthic and likely were not a major source of geosmin in the water column. Although this trend was not observed with the AMgeo-F/AMgeo-R primer set, this primer set exhibited very low specificity and also amplified specific cyanobacterial taxa. Taken together, these results suggest that cyanobacteria were the organisms primarily responsible for the geosmin events observed in these water bodies. This is consistent with other research suggesting that cyanobacteria are the predominant geosmin producers in freshwater systems (Tsao et al., 2014; Lee et al., 2020). Primer sets that amplified both cyanobacteria and actinobacteria geosmin producers (SGF1/JDR1, AMgeo-F/AMgeo-R) yielded a weaker correlation with geosmin which would be expected for a primer that amplifies both the actual

producer as well as organisms that are not actively producing geosmin. This suggests that qPCR or sequencing with multiple complementary primers may be necessary to fully understand a particular taste and odor episode. Further study using these primer sets using a wider range of samples should be done in order to evaluate their joint ability to characterize T&O episodes.

Conclusions

Five primer sets targeting the geosmin-synthase gene in cyanobacteria and actinobacteria were evaluated for their ability to identify and quantify geosmin-producers in water and sediment samples. Three of the primer sets were adopted from the literature, while two primer sets were newly developed for this study. The CGeo-1F/CGeo-1R primer set successfully targeted only cyanobacteria, all of which have previously been shown to produce geosmin. The CGeo-1F/CGeo-1R primer set is also capable of quantifying the cyanobacterial geosmin synthase gene, having a qPCR efficiency of 110% with an annealing temperature of 52^{o}C. The SGF1/JDR1 and 288AF/288AR primer sets lacked the abilities to target or quantify the cyanobacterial geosmin synthase gene, having poor efficiencies at the tested annealing temperatures. The two primer sets targeting the actinobacterial geosmin synthase gene failed to amplify actinobacteria exclusively. The AMgeo-F/AMgeo-R primer sets exhibited poor quantification abilities with efficiencies over 200%. The ActGeo-2F/ActGeo-2R primer set performed better, although still poorly, with an efficiency of 165% when the annealing temperature was 58^{o}C. The CGeo-1F/CGeo-1R primer set exhibited a positive, though the weak correlation between gene copy numbers and geosmin concentration was exclusive to the cyanobacterial geosmin synthase gene. The ActGeo-2F/ActGeo-2R primer set exhibited strong relationships between geosmin concentrations and gene copy numbers and target the two main groups of geosmin producers. Further study using these primer sets should be done in order to evaluate their joint ability to characterize T&O episodes.

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