Studies on Phosphate Solubilizing Bacteria from two lakes in central southern Ontario.

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Abstract

It has been well documented that phosphorus pollution has been one of the most significant factors inhibiting aquatic health on Lake Simcoe. However, reports on the impacts of microbial contribution to phosphorus levels are sparse. Phosphate solubilizing bacteria (PSB), are a group of bacteria that are known to release inorganic phosphate from sediments into a bioavailable form of phosphorus under specific conditions and are the focus of the present study. Sediment samples were collected once monthly from June-September 2017 from three nearshore locations along the northwestern shore of Lake Simcoe and compared to three nearshore locations along Sparrow Lake, ON. The phosphate solubilizing bacteria were isolated from the sediment, and the abundances between each lake were compared to see if anthropogenic influence was a factor on their abundance and distribution. After the bacterial isolates were counted, they were subjected to a series of laboratory tests in order to find out which of the isolates were the most efficient ones at utilizing inorganic phosphate. This test was first completed on Pikovskaya's agar plates, and then the isolates that had the best results were tested again in Pikovskaya's broth. The ten isolates that utilized the most inorganic phosphate were further classified on their abilities to grow at various temperatures, pH levels, and inorganic phosphate concentrations. Results indicated that there were significant differences on the abundance of PSBs based on their lake of origin (three-factor ANOVA, $p<0.05$). However, the higher abundance was observed in Sparrow Lake, which did not agree with the hypothesis that PSBs would be more abundant in areas that had high nutrient concentrations. Results from a laboratory screening test showed that the isolate incubation period had a significant impact on how efficient the isolates were at utilizing inorganic phosphate on the Pikovskaya's agar plates (repeated measures ANOVA, $p<0.05$). They also showed that some of the isolates were significantly better at utilizing the inorganic phosphate in the broth than others (1-factor ANOVA, $p<0.05$), and that of the ten isolates that were characterized, only three were significantly different from each other (discriminant function analysis, $p<0.001$). The phosphate solubilizing bacteria were found to be most abundant and grew the best when they originated from areas that had water total phosphorus concentrations of less than 2mg/L. Thus, indicating that this group of microorganisms may not be useful as indicators of phosphorus pollution.

Lay Summary

The mission statement of Lakehead University's Biology Department is "Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms." This study focuses on the isolation and characterization of phosphate solubilizing bacteria within the northern portion of Lake Simcoe, and as such it contributes to two of the central themes identified in the Department's mission statement. Those being the fit between organisms and their ecological functions and the distribution and abundance of organisms. This study advances our understanding of phosphate solubilizing bacteria in nearshore freshwater environments by identifying some of the abiotic factors that influence their growth rates and abundance. Understanding the habitat conditions that phosphate solubilizing bacteria find preferable could help to understand further how these microorganisms contribute to internal phosphorus loading in lakes. The primary research questions that were investigated in this study were: 1. Are phosphate solubilizing bacteria more prevalent in freshwater environments that have an excess amount of phosphorus is present? 2. What are some of the growth conditions needed for these organisms to thrive? 3. Can phosphate solubilizing bacteria be used as an indicator of phosphorus pollution? Results showed higher abundances of phosphate solubilizing bacteria in locations that did not have an excess amount of nutrients present. These results suggest that phosphate solubilizing bacteria may not be an indicator of phosphorus pollution if used alone. However, phosphate solubilizing bacteria could potentially be used as an additional indicator of aquatic health. This study provides a preliminary step for classifying phosphate solubilizing bacteria within nearshore sites along the northwestern part of Lake Simcoe and could be expanded upon in future studies.

Acknowledgements

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Chapter 1: Introduction and Review of Pertinent Literature

1.1 Introduction

It has been well documented that phosphorus is a limiting factor in freshwater systems (Winter et al., 2007; Nürnberg et al., 2013; Dodds & Whiles, 2010). This is so well known that the concentration of phosphorus is commonly one of the first problems addressed when freshwater water bodies, such as inland lakes, are under environmental duress (Winter et al., 2007; Young & Jarjanazi, 2015). When studying phosphorus pollution in lakes, the emphasis is primarily put on the anthropogenic factors that may be the cause of the pollution (this usually is defined as point source and non-point source pollution) (Dodds & Whiles, 2010). While some studies may elude to internal phosphorus loading, there has yet to be extensive research done on the internal processes that may contribute to it. Often, it is merely stated that 'microbial processes' contribute to internal phosphorus loading, but specifics as to what these processes are is often lacking (Winter et al., 2007). The following study is focused on phosphate solubilizing bacteria, a group of microorganisms that can externally secrete various organic acids which can help unbind phosphate from the compounds to which it was previously bound to (Mohammadi, 2012). This study aims not only to isolate these microorganisms from a local water source but to characterize these microbes as well because they have been understudied in freshwater environments until fairly recently (Paul & Sinha, 2017). These microorganisms will provide insights into the water quality and phosphorus pollution levels of their respective habitats. Determining if these microorganisms could be a suitable indicator of phosphorus pollution in a particular location was one of the research goals of this study.

1.2 Freshwater Environments

It is common knowledge that freshwater is a valuable resource. So much so that economists have included water resources in ecosystem goods and services reports since at least the mid-1990s (Dodds & Whiles, 2010). A report published in 2004 states that ecosystem services provided by "open freshwater " had an estimated annual value of $66 million U.S. dollars in New Jersey alone (Costanza et al., 1997). Around the same time, it was reported that on a global scale, "open freshwater" later defined as rivers and lakes, had an estimated annual value of nearly $1.7 trillion U.S. dollars (Dodds & Whiles, 2010). These dollar values would likely

be much higher today, due to inflation and due to how sensitive these ecosystems are to anthropogenic pollution (Dodds & Whiles, 2010).

Nutrient levels in freshwater ecosystems are known to impact primary production (Schindler, 1971; Carpenter, 2008). Phosphorus, in particular, has been proven to be correlated with chlorophyll *a* amounts (Schindler, 1977). Studies have proven that total phosphorus levels are the leading contributor to eutrophication. However, the lack of other nutrients such as nitrogen and carbon, amplify the effect of eutrophication (Carpenter, 2008; Schindler, 1977; Schindler, 1971; Elser et al., 2007). These excess concentrations of phosphorous enter freshwater ecosystems naturally through rock weathering. However mining, agricultural runoff, industrial, and municipal waste discharges cause for phosphorus to be found more frequently (Carpenter, 2008). In 2000, the global influx in phosphorus increased from ~10-15 tonnes/year in pre-industrial times to ~33-39 tonnes/year (Carpenter, 2008). Carpenter's study goes on to discuss the impacts of phosphorus pollution on a global scale, heavily implying that phosphorus levels are so high because of anthropogenic stress.

A study by Winter et al. (2007) observed phosphorus inputs to Lake Simcoe from 1990-2003. This study looked at the statistical relationships between total phosphorus levels and chlorophyll *a*, as well as chlorophyll *a* and dissolved oxygen depletion rates; indicating that by way through photosynthetic processes, total phosphorus levels impact dissolved oxygen concentrations (Winter et al., 2007). In their 2014 Lake Simcoe monitoring report, the Ontario Ministry of the Environment and Climate Change (now the Ontario Ministry of the Environment, Conservation and Parks) identified that phytoplankton biomass was most abundant in Cook's Bay. This also happens to be where the highest total phosphorus concentrations were found during the same monitoring period which could be significant as a study completed by Dillon and Rigler (1974) infers that there is a relationship between chlorophyll *a* and total phosphorus concentrations. There was enough evidence to support this hypothesis that they were able to create a model that would estimate the summer chlorophyll *a* concentrations based on total phosphorus concentrations collected from the spring (Dillon & Rigler, 1974).

Dodds and Whiles (2010) define eutrophication as “an increase in nutritive factor or factors that lead to greater rates of whole-system heterotrophic or autotrophic metabolism” (Dodds & Whiles, 2010). The causes of eutrophication were not widely understood until Schindler et al. (1977) who completed a five-year study on the impacts that nutrient levels on a lake-wide scale in the Experimental Lakes system in Ontario. During these studies, it was documented that controlling the addition of other nutrients (carbon and nitrogen) did not remedy the eutrophication issue. Schindler found that reducing the ratios of total nitrogen to total phosphorus simply changed the most prominent algal composition to nitrogen-fixing cyanobacteria of the genus *Anabaena* (Schindler, 1977). It was concluded that this was not an appropriate solution to the eutrophication issue from a water quality standpoint and that the most efficient way to combat eutrophication was to limit the amount of total phosphorus that was in the waterbody.

Researchers tend to agree that preventing eutrophication from occurring is a better action plan than remediating eutrophic lakes once they have become eutrophic. A lake is typically classified as eutrophic once its phytoplankton population has shifted to being predominately cyanobacteria (Smith, et al., 1999).

Phosphorus is the key limiting factor for primary production in freshwater habitats (Dodds & Whiles, 2010). It is usually found in its inorganic state, phosphate. Phosphate is known to precipitate with some metals, including calcium (commonly found in calcareous soils and substrates) and ferric iron (in more acidic soils and substrates) in the presence of oxygen (Correll, 1998). This leads to the phosphate settling into the sediment when the surface water is oxygenated (Dodds & Whiles, 2010). Anoxic zones cause the inorganic phosphates to disassociate due to the absence of oxygen, and processes such as eddy diffusion (the process of moving particles from one place to another in a circular motion) then move the now disassociated phosphate (Dodds & Whiles, 2010). This process explains how phosphorus is brought back to the surface, which commonly occurs in the autumn when seasonal mixing is known to break down the anoxic hypolimnion (Dodds & Whiles, 2010). That being said, temperature, redox reactions, pH, dissolved oxygen concentrations, nitrates, sulfates, and bacterial activity have all been suggested as of major factors that affect phosphate release from

sediments as well (Kim, et al., 2003; Jin, et al., 2006; Ribeiro, et al., 2008).These factors can impact what substances the phosphate binds to (e.g., phosphate will bind with calcium in alkaline conditions (Dodds & Whiles, 2010)), as well as contribute to how the phosphate will disassociate (e.g., phosphate will disassociate from ferric iron when oxygen levels are depleted (Dodds & Whiles, 2010)). The process of phosphate re-entering the water column from sediments is called 'internal phosphorus loading' (Qian et al., 2010; Nürnberg et al., 2013).

1.3 Microbial Communities

A few of the most dominant culturable bacteria include the following genera: *Arthrobacter*, *Pseudomonas*, and *Bacillus.* These microbes thrive in aerobic conditions and are heterotrophic*. Arthrobacter spp.* are gram-variable bacteria that may comprise up to 40% of the culturable bacteria with their primary functions being nutrient cycling and biodegradation (Pepper, et al., 2014). Members of the *Pseudomonas* genus comprise 10-20% of culturable bacteria, possess multiple enzyme systems and are gram-negative. They too assist with nutrient cycling and biodegradation*, some Pseudomonas* spp. can also be used as a biocontrol agent (Pepper, et al., 2014). The *Bacillaceae* family is comprised of rod-shaped, spore-forming and gram-positive bacteria. This family only makes up 2-10% of culturable bacteria. The functions of *Bacillus* include carbon cycling, biodegradation, and can be used as a biocontrol agent (*Bacillus thuringiensis*) (Pepper, et al., 2014). Due to the frequency at which phosphate solubilizing bacteria have been tested in terrestrial environments, it is important to note that the bacteria previously mentioned are commonly found in terrestrial environments. Often in literature, these are the genera of phosphate solubilizing bacteria that are discussed (see phosphate solubilizing bacteria section for a list of commonly found phosphate solubilizing bacteria in terrestrial environments).

As algae and cyanobacteria are the most abundant microbes in the planktonic community in the littoral zone, primary production levels in this zone of a lake are typically quite high (Pepper, et al., 2014). However, due to the availability of nutrients in the benthic zone, the bacterial community is often much more abundant in this portion of the lake than throughout the rest of the water column (Pepper, et al., 2014). In a review completed by Capone and Kiene (1988), it was identified that the density of viable bacteria in shallow

sediments typically ranged between 10^9 and 10^{10} cells cc^{-1} of sediment regardless if the waterbody was marine or freshwater. Their findings were in contrast to the viable cells found throughout the water column in the same area which they determined was typically around 10^6 cells cc^{-1} (Capone & Kiene, 1988). Due to these previous studies, it is expected to find the highest abundance of bacteria within the first few centimetres of sediment.

Cyanobacteria, commonly referred to as blue-green algae, are the commonly known side effect of eutrophication. These algae blooms have been listed as one of the main concerns with water quality on Lake Simcoe (Young & Jarjanazi, 2015). Factors that impact cyanobacterial bloom formation, density, and genus include light intensity, temperature, nutrient concentrations, and presence/ absence of gas vesicles in cyanobacteria cells (Martins, et al., 2011). “They can produce different metabolites which include alkaloids, lipopolysaccharides, polyketides, and peptides that may act as toxins on other bacteria, and eukaryotes” (Huisman, et al., 2005).

Phosphate solubilizing bacteria are a group of bacteria that are known to be one of the most significant contributing sources of microbes that release inorganic phosphate from sediment (Sanjotha & Manawadi, 2016). This group of microorganisms has been well studied in terrestrial environments as they have been proven to have symbiotic relationships with various flora. Phosphate solubilizing bacteria are used in industry as a biofertilizer in sustainable agriculture practices as their presence can eliminate the need for chemical fertilizers to be used on crops (Correll, 1998). In terrestrial environments, some common bacterial genera that have phosphate solubilizing capabilities include *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium* and *Erwinia*. (Rodríguez & Fraga, 1999).

Prior studies have determined that phosphate solubilizing bacteria are commonly found to have both aerobic and anaerobic strains (Sanjotha G & Manawadi, 2016; Qian, et al., 2010; Rodríguez & Fraga, 1999), while aerobic phosphate solubilizing bacteria have been found most prevalently in submerged soil (Rodríguez & Fraga, 1999).

These bacteria produce organic acids which are generally described by Qian et al. (2010) as "...low molecular weight organic acids which are produced in the periplasmic space of some Gram-negative bacteria through a direct glucose oxidation pathway". It is these inorganic acids that convert inorganic phosphate compounds into a bioavailable form of phosphorus (Ponmurugan & Gopi, 2006). Some of these organic acids include monocarboxylic acid (acetic, formic), monocarboxylic hydroxy (lactic, glucenic, glycolic), monocarboxylic, ketoglucenic, decarboxylic (oxalic, succinic), dicarboxylic hydroxy (malic, maleic) and tricarboxylic hydroxy (citric) acids (Ponmurugan & Gopi, 2006). The organic acids allow for the solubilization of inorganic phosphate by either directly dissolving rock phosphate or chelating (binding to) calcium ions (Kucey, 1983).

1.4 Lake Simcoe

Lake Simcoe (44°25'N, 79°20'W), the largest inland lake in central southern Ontario other than the Great Lakes, has a surface area of 722km², a mean depth of 14m, a maximum depth of 42m, and a shoreline perimeter of 303km (Palmer, et al., 2011). Lake Simcoe has a residence time of 7.5 years due to its single outflow by way of the Trent Severn Waterway into Lake Couchiching (Crossman, 2013). When discussed in various other scientific papers, Lake Simcoe is generally broken up into three different parts- the Main Basin, Kempenfelt Bay, and Cook's Bay (Figure 1.1). The Main Basin, which includes the largest, northern portion of the lake has an area of 643km^2, a mean depth of 14m, and a maximum depth of 33m. Kempenfelt Bay, which is the deepest part of the lake, located to the west, has the city of Barrie located along its shoreline; it has an area of 34km^2, mean depth of 20m and a maximum depth of 42m. Finally, Cook's Bay, the southernmost part of the lake, which is quite shallow when compared to the rest of the lake, has an area of 44km^2, a mean depth of 13m, and a maximum depth of 15m (Young & Jarjanazi, 2015; Palmer et al., 2011). The Lake Simcoe Watershed has 35 tributary rivers (the majority of which are in the southern region of the watershed) and consumes a total area of 2899km² (Palmer, et al., 2011). Over 450,000 people are living around the watershed with most of the population situated around the cities of Newmarket, Aurora, Barrie, Orillia and Keswick (LSRCA, 2016).

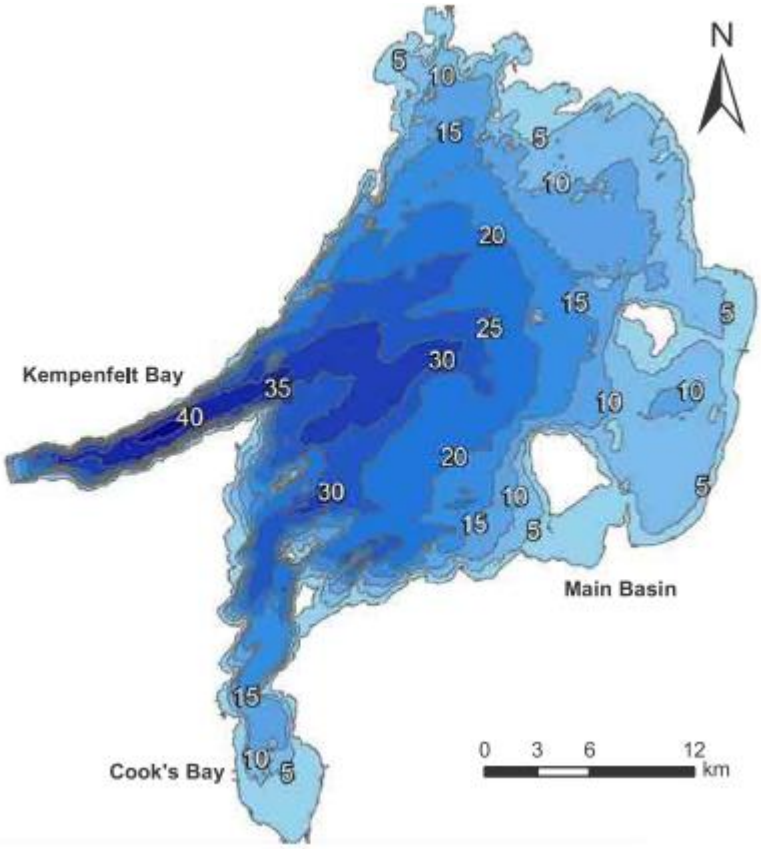


Figure 1.1. Bathymetric chart of Lake Simcoe with the depth contours labelled in meters. The three main parts of the lake (Main Basin, Kempenfelt Bay, and Cook's Bay) are labelled as well (Young & Jarjanazi, 2015).

Presently, the Lake Simcoe watershed is made up of 45% agricultural land, 7% urban areas, and 35% natural cover (Young & Jarjanazi, 2015). This is below the Lake Simcoe Protection Plan's target of 40% natural cover (Young & Jarjanazi, 2015). The Lake Simcoe Protection Plan (LSPP) identifies natural cover as "woodlands, swamps, non-treed wetlands, grasslands, alvars, prairie grasslands, sand barrens, and savannah" (Young & Jarjanazi, 2015). As of 2008/2009, only the Maskinonge River, Hewitts Creek, and Barrie Creek subwatersheds were reported to have less than 20% natural cover (Young & Jarjanazi, 2015). At this time, only the Hawkstone and Ramara Creeks, Black and Talbot River, and Georgina, Snake, and Thorah Island sub-watersheds met the minimum standard of having 10% interior forest cover (Young & Jarjanazi, 2015). The subwatersheds that were more heavily agricultural based (Hewitts Creek, West and East Holland Rivers, and Beaver River) all had less than 5% interior forest cover while Barrie Creeks subwatershed had none (Young & Jarjanazi, 2015).

In 1993, it was reported that 523km^2 of land in the watershed was cultivated while 739km^2 of land was used as pasture (LSRCA, 1995). At this time, the most common crops planted within the watershed were grain, maize, hay, and soy while there was a decline in livestock production throughout the watershed (LSRCA, 1995). The Holland River system is the largest cultivated polder in Ontario and contributes a substantial portion of the market for lettuce, carrots, onions, and celery in the province (LSRCA, 1995). At this time, large areas of land were being set aside for industrial, commercial, and residential use. In 1993, the City of Barrie added 663ha of the commercial, industrial area while Newmarket added 597ha (LSRCA, 1995). The town of Aurora's residential land use doubled from 450 to 1074ha, while Bradford's residential quadrupled from 150ha in 1981 to 665ha in 1991 (LSRCA, 1995).

The impacts that both agriculture and urbanization have on Lake Simcoe have been the base of many studies. The most notable results of both the deforestation and agriculturalization have had on Lake Simcoe include an increase in phosphorus loading and an increased mass sedimentation rate of soil particles (Evans, et al., 1996). The inputs of nutrients and organic matter likely caused by sewage increased along Lake Simcoe in proportion to the increase in urbanization and permanent residents to the watershed (Evans, et al., 1996). In 2015, it was reported that there were nearly 3700 septic systems all within 100 metres of Lake Simcoe (MOECC, 2015). These adverse impacts imposed on the lake by anthropogenic practices are the reason behind the movement to improve the water quality of Lake Simcoe.

While there are many areas of concern surrounding the health of this lake and its watershed, one area of concern for Lake Simcoe that is discussed frequently is the cold-water fishery. There has been much study on the decline of *Salvelinus namaycush* (lake trout), *Coregonus clupeaformis* (lake whitefish), and *Coregonus artedi* (lake herring) due to their economic significance. It has been hypothesized that the decline of these fishes may be due to the lower than necessary dissolved oxygen levels that are present primarily due to eutrophication (Winter et al., 2007). As recreational fishing on Lake Simcoe contributes over $200 million yearly to the local economy, any adverse impacts seen on these fish species need to be thoroughly investigated (LSRCA, 2016).

The Lake Simcoe Protection Plan was put in place by the province to "protect, improve or restore the elements that contribute to the ecological health of the Lake Simcoe watershed, including, water quality,[and] hydrology….."(Ontario Ministry of the Environment, 2009). This plan was to provide legislation for the provincial government, municipalities, and other stakeholders such as the Lake Simcoe Regional Conservation Authority to complete more research on the lake. This raises the importance of perhaps the most critical problem that Lake Simcoe has been facing, which is phosphorus pollution. Figure 1.2 shows which areas on Lake Simcoe have the highest concentrations of total phosphorus (Young & Jarjanazi, 2015).

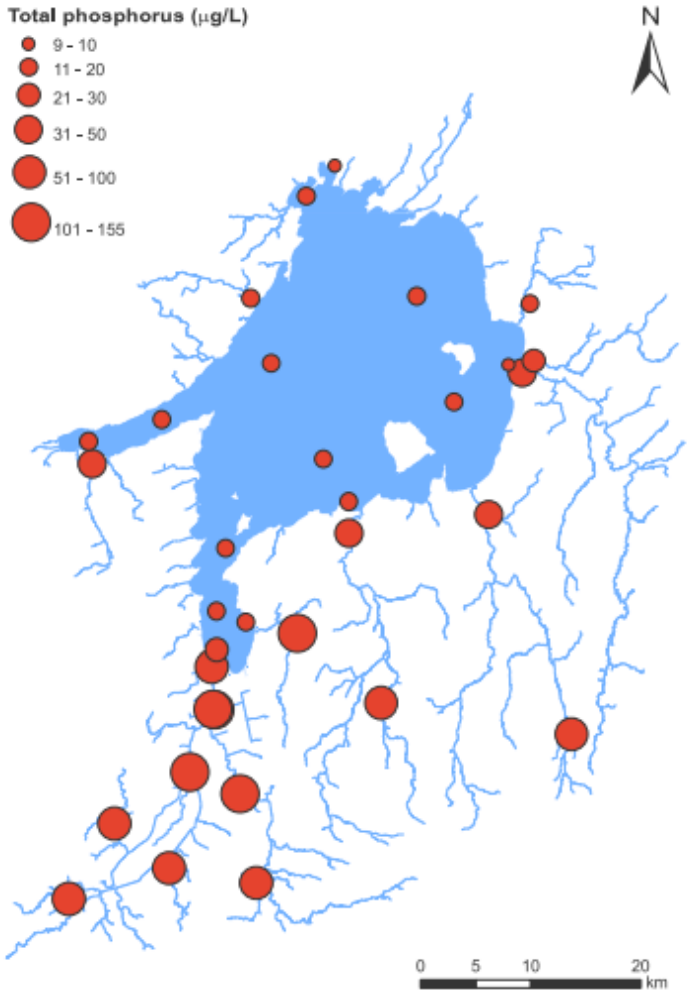


Figure 1.2. This map of Lake Simcoe and the tributary rivers shows the locations within the lake that had the highest concentrations of total phosphorus in 2014 when the most recent monitoring report on the lake was completed (Young & Jarjanazi, 2015).

The Lake Simcoe Protection plan created in 2009, identifies phosphorus pollution as one of the most significant factors impacting the water quality of Lake Simcoe. The average phosphorus loading from 2002-2007 was 72 tonnes/year, while the average from 2005-2009 increased to 86 tonnes/ year (MOECC, 2009; Young & Jarjanazi, 2015). Meanwhile, the average from 2010-2015 remained quite similar to the previous five years at 85.5 tonnes/year (LSRCA, 2017). The Lake Simcoe Protection Plan aimed to limit external sources of phosphorus from entering the lake by limiting phosphorus discharges from sewage treatment plants, preventing new sewage treatment plants from discharging effluent into the lake, and updating stormwater drainage systems. Figure 1.3 shows the five leading causes of phosphorus pollution in Lake Simcoe as well as which of these causes contribute the most to phosphorus pollution.

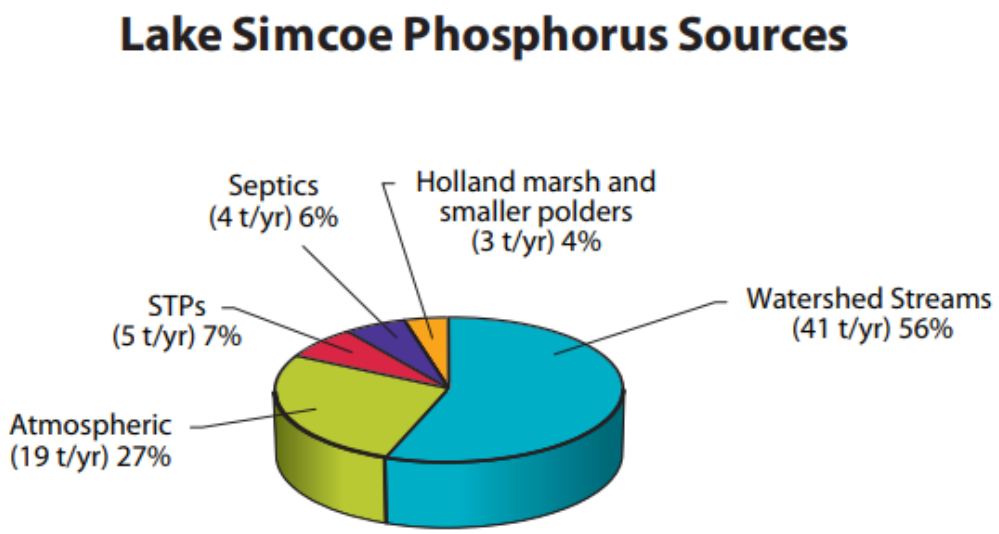


Figure 1.3. This pie graph from the Lake Simcoe Protection Plan identifies the most common phosphorus sources that are entering Lake Simcoe (MOECC, 2009). STP was the short form used for sewage treatment plants within the Lake Simcoe Protection Plan.

The lake is mesotrophic and has been monitored for external phosphorous input since the 1970s due to the multitude of impacts that nutrient loading would have on the lake (Nürnberg et al., 2013). The impacts that nutrient loading has had on Lake Simcoe have been the subject of much study and efforts to attempt to lessen these adverse impacts have been underway since the 1970s (Winter et al., 2007). In 2010, the Lake Simcoe phosphorus reduction strategy was created by the Ministry of the Environment and Climate Change in conjunction

with the Lake Simcoe Region Conservation Authority to aid in the remediation of Lake Simcoe and its surrounding watershed. This was in response to the Clean Water Act that was announced in 2006 which promoted pursuing solutions across an entire watershed (OMECC, 2010). The goals of the reduction strategy are to bring dissolved oxygen levels up to 7mg/L or an annual phosphorus load of 44 T/year (OMECC, 2010).

The Lake Simcoe Phosphorus reduction strategy proposes some ways that the involved stakeholders plan to try to remove some of the phosphorus pollution in the lake. One of these methods is a product called Phoslock. This product is composed of lanthanum modified clay and was found to significantly reduce the amount of phosphorus when compared to the control tank in a laboratory experiment conducted by Reitzel et al. (2013). Reitzel et al. (2013) also found that the addition of Phoslock did not have any negative impacts on the burrowing benthic invertebrates that they tested. The company claims that Phoslock has no impacts on the pH or alkalinity concentrations within the lake (Reitzel et al. 2013). This product was already being tested on some locations throughout Lake Simcoe when the phosphorus reduction strategy was published in 2010 (OMECC, 2010).

Other methods used to try to combat external phosphorus loading within Lake Simcoe include using red sand filtration chambers on urban stormwater runoff and reusing treated wastewater effluent and stormwater within the watershed (new systems in homes, businesses, and industries, and public facilities to re-use water for non-potable water usages; and irrigation for golf courses, lawns, and sod farms) (OMECC, 2010).

The City of Orillia is located on the western side of the northern end of Lake Simcoe. This area, where Lake Simcoe meets Lake Couchiching is commonly referred to as the Atherley Narrows and is recorded having the lowest concentrations of phosphorus within the entire lake (Young & Jarjanazi, 2015). When the Lake Simcoe Phosphorus Reduction Strategy was published, the Orillia Wastewater treatment plant had an annual phosphorus load of roughly 1,274 kg/year, which was not in compliance with their baseline phosphorus load of 996 kg/year (OMECC, 2010). However, since 2009, the Orillia wastewater treatment plant managed to stay under that the phosphorus limit. The Minister of the Environment's 5-year report on Lake

Simcoe reported that the WWTP in Orillia reported annual phosphorus loads of 600 kg/yr., 504 kg/yr., 501 kg/yr., 471 kg/yr., and 510 kg/yr. In the years 2010, 2011, 2012, 2013, and 2014, respectively (MOECC, 2015).

The wastewater treatment facility is currently in the process of installing a $4 million tertiary filtration system as they currently operate a conventional secondary system using activated sludge (OMECC, 2010).

1.5 Sparrow Lake

Sparrow Lake (N 44°60'46.4", W 079°28'47.8"), indirectly receives the outflow of Lake Simcoe by way of the Trent-Severn Waterway via the Severn River and a man-made canal travelling from Lake Couchiching to Sparrow Lake (Charron, et al., 2013). This small lake has a surface area of 11.4km^2, a mean depth of 16m, and a watershed area of 145.28km^2 (The District Municipality of Muskoka, 2016). This lake is part of the quaternary watershed of the Severn River watershed and the communities of Severn Bridge, Killworthy, and Port Stanton surround the lake (The District Municipality of Muskoka, 2016). Approximately 7% of the area of this lake can be classified as a provincially significant wetland. The Sparrow Lake wetland is 224ha with 86% of the wetland residing on crown land. This wetland includes both the Ellison Bay wetland (75ha), and McLean Bay wetland (65ha) (The District Municipality of Muskoka, 2004).

A wetland is considered provincially significant if it scores highly in the four principal components that the Ontario Ministry of Natural Resources and Forestry has deemed important. These components are biological, social, hydrological, and special features(Schulte-Hostedde, et al., 2007). The Sparrow Lake wetland received this status because the McLean Bay wetland is a neutral mesotrophic shallow open water community, which is not common elsewhere in Muskoka. The only other wetland which is classified the same is the Ellison Bay wetland which is also located within Sparrow Lake; this wetland also contains the largest wild rice community within Muskoka (The District Municipality of Muskoka, 2004). The McLean Bay wetland also provides habitat for *Carpinus caroliniana* (Blue Beech), *Platanthera flava* (pale green orchis), *Scripus fluviatilis* (river bulrush), and *Scripus heterochaetus* (slender bulrush); all of which are rare vascular plant species, with an additional 15 species of regionally-uncommon

plants being identified (The District Municipality of Muskoka, 2004) . Both of these wetlands are recognized as migratory staging areas in the spring and fall for waterfowl and are considered important spawning and nursery habitat for sport fish (The District Municipality of Muskoka, 2004). The wild rice beds in Ellison Bay are noted for their intactness, and 15 species of *Potamogeton spp.* (pondweed) have been identified in this bay (The District Municipality of Muskoka, 2004). Also, as the entirety of the lake is classified as part of the Severn River system, which has been identified as a corridor of high scenic value. Due to these factors, the Sparrow Lake wetland in all of its parts currently is recommended to become a heritage area, and as a result, McLean Bay has been labelled a provincially significant wetland (The District Municipality of Muskoka, 2004).

The protected wetlands and relatively low permanent human population are the main contributing factors as to why this lake was chosen in comparison to Lake Simcoe. The sites along Lake Simcoe Atherley Narrows form a gradient from the inlet of Mills Creek to the outlet of Lake Simcoe, encompassing effluent from the Orillia wastewater treatment plant and landfill as well as runoff from the City of Orillia. The sites along Sparrow Lake follow a similar pattern (forming a gradient from an inlet to an outlet). However, there are no known sources of pollution into this system. This has allowed for the comparison of phosphate solubilizing bacteria abundance between areas of point source and non-point source pollution. Unpublished water quality data was provided by the District of Muskoka. This raw data allowed for a preliminary analysis of the quality of Sparrow Lake and how it may have changed over time. Chemical data has been completed by the District of Muskoka sporadically over the past 20 years. Some water quality parameters have been tested more frequently and for a longer period than some others. Due to this provided data, the averaged annual total phosphorus trends (µg/L) for Sparrow Lake from 2002 onwards can be viewed in Figure 1.4. This data also shows that Sparrow Lake has always remained under that provincial total phosphorus standard of 2.0mg/L (20µg/L) (MOECC, 2003).

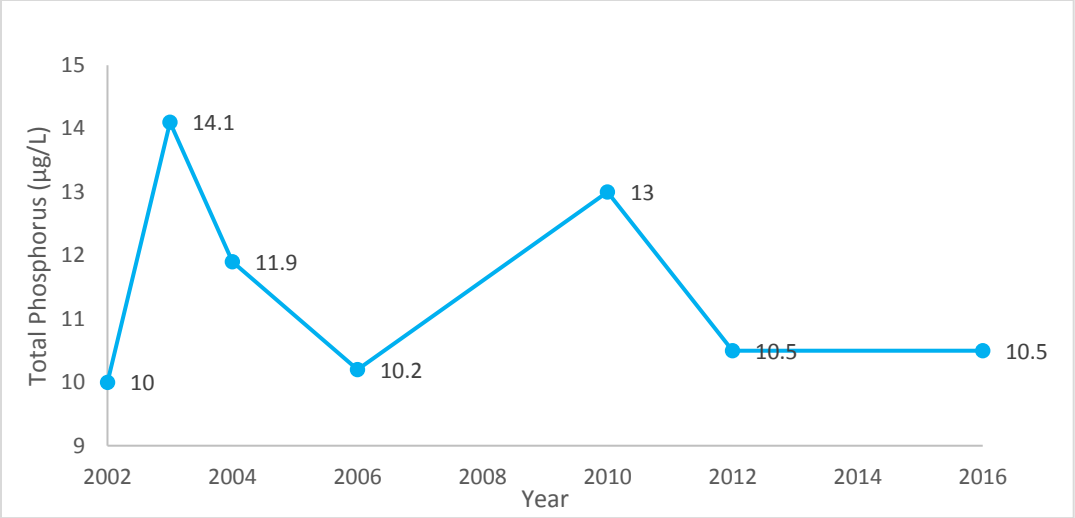


Figure 1.4. Sparrow Lake mean total phosphorus (µg/L) levels by year. There was not a significant correlation between the amount of phosphorus collected and the year it was collected in (r=-0.244, n=7, p=0.598). However, a negative trend can be seen, and the amount of total phosphorus observed has remained stagnant.

1.6 Study Significance

Studies on phosphate solubilizing bacteria in freshwater systems are relatively new; only a few investigations have been completed. Previous studies on phosphate solubilizing bacteria have heavily focused on their presence in terrestrial habitats. Specific strains of *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Enterobacter* have been found to be the most 'powerful' phosphate solubilizers (Mohammadi, 2012). Studies on phosphate solubilizing bacteria that have been completed so far in freshwater environments have focused heavily on the phosphorus uptake by aquatic plants (Qian, et al., 2010). The identification of the phosphate solubilizing bacteria in freshwater habitats has yet to be studied in detail, although there are recent studies focused on the phosphate solubilizing abilities of specific strains of phosphate solubilizing bacteria that originated from freshwater habitats (Paul & Sinha, 2017; Sanjotha & Manawadi, 2016). However, these current studies typically only have one sampling event to collect phosphate solubilizing bacteria. This has eliminated the potential to study any seasonal variation that may impact the density of phosphate solubilizing bacteria in a given location.

With all of the work put in place to reduce the phosphorus concentrations entering Lake Simcoe, and to return dissolved oxygen concentrations back to averaging around 7mg/L (which

is needed in order to support a self sustaining cold water fishery (Young & Jarjanazi, 2015)), more resources should be put into studying internal phosphorus loading and the factors that may contribute to it (eg. Phosphate solubilizing bacteria).

The following parts of this thesis are divided into four additional chapters. Chapter 2 focuses on the collection, extraction, and isolation of the phosphate solubilizing bacteria collected from the Lake Simcoe and Sparrow Lake sites. Chapter 3 explains the two main tests that were completed to screen out some of the collected isolates by testing the abilities that the phosphate solubilizing bacteria had to utilize inorganic phosphate on both a solid media and in a broth. Chapter 4 examines some of the different growing conditions (temperature, pH, and inorganic phosphate concentrations) in controlled settings in order to try to characterize some of the phosphate solubilizing bacteria. Finally, chapter 5 consists of general discussions, suggestions for future research, and conclusions.

Chapter 2: A comparison study of the phosphate solubilizing bacterial abundances between the littoral zones of a lake that is subject to anthropogenic stress and a less disturbed lake

Chapter 2 Abstract

This study is the first to monitor phosphate solubilizing bacteria (PSB) in the Lake Simcoe watershed. Thus, the objective was to determine if this group of bacteria could be used as an indicator of phosphorus pollution based on their abundance. Sediment and water samples were collected from three locations in Sparrow Lake and along the northwestern shoreline of Lake Simcoe (Atherley Narrows) once a month from June- September 2017. Bacterial strains were isolated from these samples and plated onto Pikovskaya's agar to determine if any of the bacterial isolates had phosphate solubilizing capabilities. A three-factor ANOVA and a nested ANOVA were completed to determine whether any statistically significant ($p > 0.05$) differences existed in the PSB abundances between the two lakes. A Pearson's correlation analysis was completed to see if there was a relationship between the amount of total phosphorus in the water column and the abundance of PSB present. Negative correlations were observed. The southern portion of Lake Simcoe, Cook's Bay, was sampled in September 2017 for abundances of PSB. It was hypothesized that there would be a higher abundance in Cook's Bay opposed to the Atherley Narrows as there have consistently been higher concentrations of total phosphorus in Cook's Bay. A one-way ANOVA found that there was a statistically significant ($p<0.05$) difference between the abundances of PSB in Cook's Bay and the Atherley Narrows locations. Another preliminary study was carried out to determine how the abundance of phosphate solubilizing bacteria varied with the amount of total reactive phosphorus in the sediment was completed simultaneously. A regression analysis showed that there was not a significant relationship between PSB abundance and the amount of total reactive phosphorus in the sediment. However, this experiment should be completed again with more replicates to confirm this result. Overall, these field studies suggest that higher abundances of phosphate solubilizing bacteria are found when there are lower concentrations of total phosphorus present. This could imply that phosphate solubilizing bacteria abundance would be better suited as an additional measurement of aquatic health.

2.1: Introduction

Phosphorus pollution has been a cause for concern in Lake Simcoe since the 1970s (Nürnberg et al., 2013) and is still considered one of the most significant factors that impact water quality on that (LSRCA, 2017). As phosphorus is the limiting factor in most freshwater ecosystems, phosphorus pollution is one of the most frequently studied types of pollution that greatly impact lake health. Many of these studies have identified anthropogenic sources to be the greatest cause of pollution (Young & Jarjanazi, 2015; Nürnberg et al., 2013; Dodds & Whiles, 2010). However, reports on the impacts of microbial contribution to phosphorus levels are sparse.

As of 2015, general microbial tests were not included in the yearly monitoring programs that the Ontario Ministry of Environment and Climate Change, the Ontario Ministry of Natural Resources and Forestry, or the Lake Simcoe Region Conservation Authority completes. When these groups conduct their yearly programs the aquatic life that is assessed includes aquatic plants and algae, phytoplankton, zooplankton, benthic invertebrates, and fish populations (Young & Jarjanazi, 2015). The Simcoe Muskoka District Health Unit screens for various strains of *E. coli* at all of the public beaches throughout the region once weekly during the summer months (Simcoe Muskoka District Regional Health Unit, 2017). While the results are made public when *E. coli* concentrations are too high for public health, if any long-term data has been analyzed, it has not been made available to the public.

A few different studies looking at various ways that microorganisms can be used as ecological health indicators of Lake Simcoe have been completed by faculty members and other graduate students at Lakehead University-Orillia Campus. Using algal biofilms as water quality indicators (Kanavillil, et al.,2012), comparing total coliforms and caffeine concentrations as suitable indicators of anthropogenic waste (Kurissery, et al., 2012), and using periphyton as a potential indicator of water quality (Kanavillil & Kurissery, 2013) have been some of the main topics covered by Dr. Kurissery, Dr. Kanavillil, and their students since 2012.

A preliminary study on phosphate solubilizing bacteria in Lake St. John was completed by Dr. Kurissery and Gzi Chow in 2017. This study followed the methods of Pérez, et al., (2007)

and served as a pilot project for this study. Besides the current study, phosphate solubilizing bacteria have not previously been studied in Lake Simcoe or the Lake Simcoe watershed.

The phosphate solubilizing bacteria used for this study were collected from an area in Lake Simcoe that is suspected to be anthropogenically stressed and then compared to an area in a different lake (Sparrow Lake) that does not have nearly the same level of anthropogenic influence. The phosphate solubilizing bacteria studied were collected from three sites along the northwestern shore of Lake Simcoe once monthly from June-September 2017. These sites represented a gradient between an effluent outlet from a wastewater treatment plant in Orillia and Lake Simcoe. They were then compared to bacteria abundances collected from three sites along the eastern shore of Sparrow Lake, ON. Sparrow Lake is surrounded by privately owned lots and crown land that contains provincially significant wetlands (The District Municipality of Muskoka, 2004). The communities that are surrounding this lake are small and without a sewer system. Figure 2.1 is a map of the sampling locations for each lake.

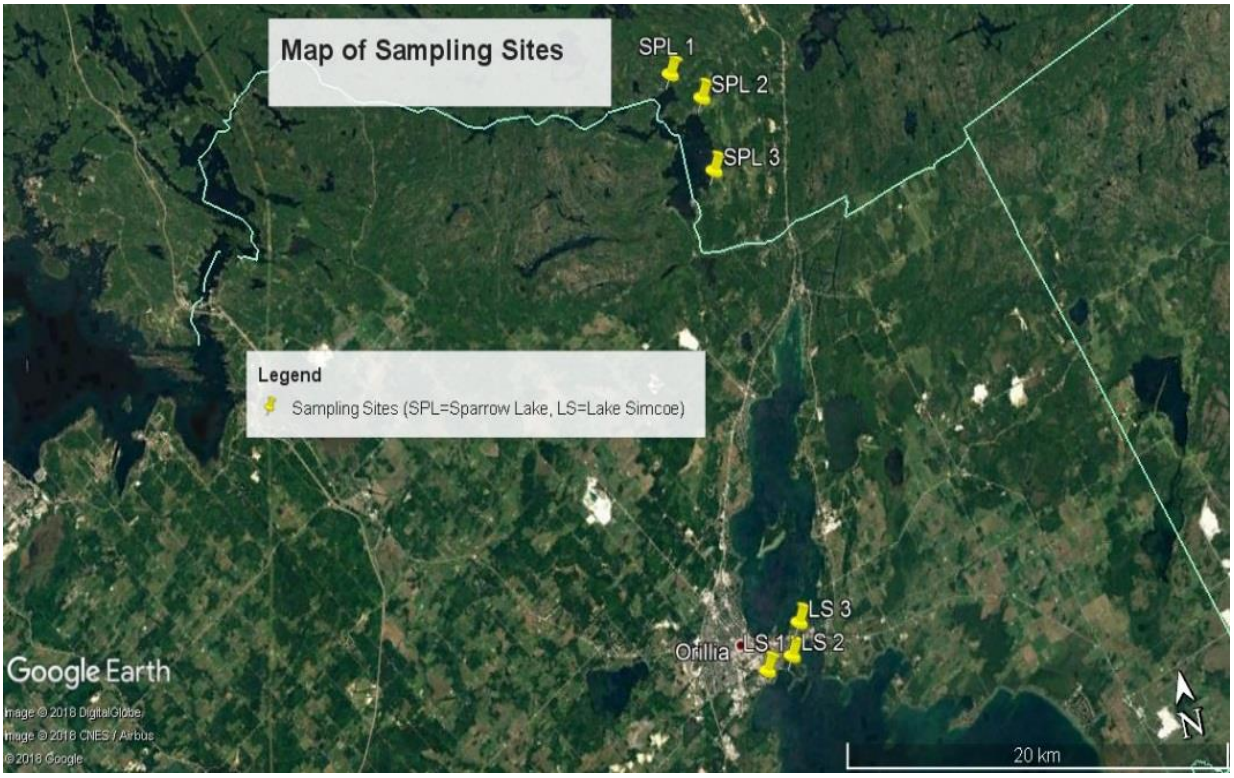


Figure 2.1. A map showing the sampling locations for Lake Simcoe (Atherley Narrows) and Sparrow Lake.

The objectives of this study were to estimate and isolate the phosphate solubilizing bacteria that were present in the sediment and water column in nearshore sites on the northwestern part of Lake Simcoe (where the city of Orillia is located) and Sparrow Lake. The addition of Sparrow Lake was to assess the impact that the presence of anthropogenic stressors would have on the abundance of phosphate solubilizing bacteria. Both lakes were sampled once monthly throughout the growing season (June-September) to determine if seasonality should be studied in future to characterize further the phosphate solubilizing bacteria that may be present in near-shore environments. It was hypothesized that a greater abundance of phosphate solubilizing bacteria would be found at locations that were under a great deal of anthropogenic stress resulting in higher concentrations of total phosphorus in those locations.

2.2 Materials and Methods

2.2-1 Sampling Site Descriptions

The sampling sites in Lake Simcoe (Atherley Narrows) were chosen based on their proximity to anthropogenic stressors. The sites in Lake Simcoe were centred around the effluent site from Orillia's wastewater treatment plant (WWTP). The opposite can be said for the sites selected on Sparrow Lake. The Sparrow Lake sites canvased the eastern shore of this lake, travelling from near one of the primary inlets towards the lake's outlet, the northernmost site (site 1) on this lake was along the protected wetlands on the lake. All the sites sampled were nearshore sites, and samples were collected at a depth of approximately 0.5m.

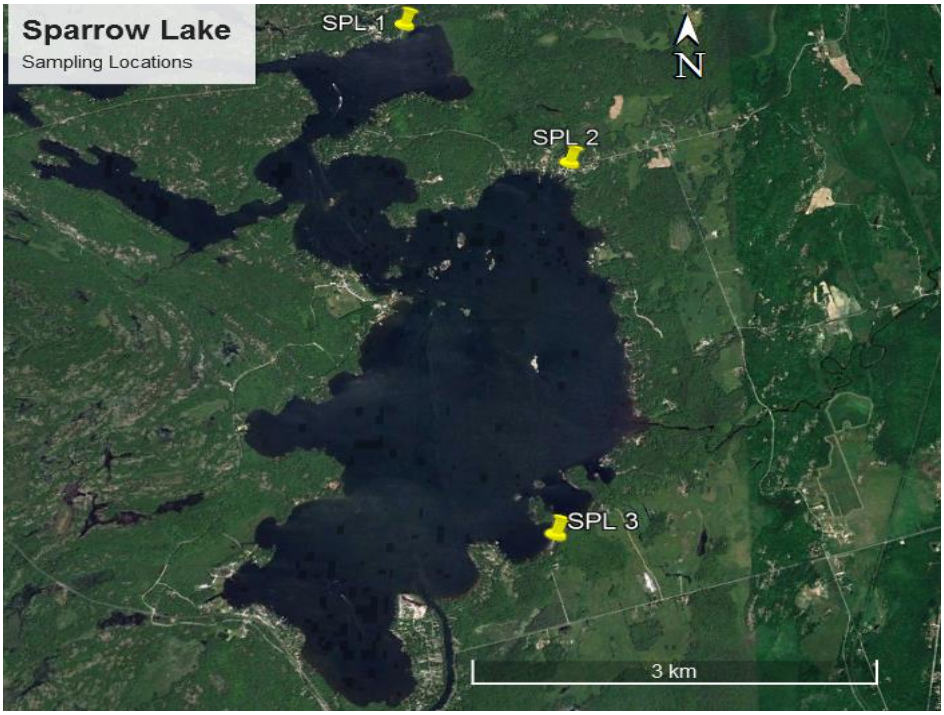


Figure 2.2. The Sparrow Lake Sampling locations.

Figure2.3. The Lake Simcoe sampling locations were all along the northwestern shoreline, where the City of Orillia is located.

Site 1(LS-1). Kitchener Park

Mills Creek runs right next to Orillia's WWTP and municipal landfill and then discharges into Lake Simcoe directly. The outflow point of this creek was selected for Lake Simcoe-Atherley Narrows site 1 (LS-1). This site was located at N 44°35'21.1", W 079°28'86.1" (Figure 2-4). This site was considered to be the most polluted site and therefore might contain the largest abundance of phosphate solubilizing bacteria.

Figure 2.4. LS-1 in Kitchener Park. This photo was taken from shore looking towards the mouth of Mill's Creek. This picture was taken on September 26th, 2017.

Site 2 (LS-2) Gill Street

This site was located approximately 500m downstream from LS-1. Lake Simcoe-Atherley Narrows site 2 (LS-2) was located N 44°35'87.8", W 079°28'40.8" at the end of Gill Street, Orillia, ON (Figure 2-5).

Figure 2.5. LS-2 was located alongside Gill Street in Orillia. This photo was taken from the side of the street facing the lake and was also taken on September 26th, 2017.

Site 3 (LS-3) Tudhope Launch

Site 3 (LS-3) was located as close to the Atherley Narrows as one could safely wade in on the Lake Simcoe side of the Narrows at N 44°35’20.7”, W 079°28’07.9” (Figure 2-6). This area was located next to a boat launch that saw high levels of boat traffic in the summer months.

Figure 2.6. LS-3. This site was located next to the public boat launch at Tudhope park. This was approximately 500m away from the Atherley Narrows that connect Lake Simcoe and Lake Couchiching.

Site 4 (SPL-1) McLean Bay

Site 4 (SPL-1) was located at the northern end of Sparrow Lake in McLean Bay (N 44°51’10.1”, W 079°23’35.9”). This site was located adjacent to the wetland area which is a known area of significance as it is a neutral mesotrophic shallow open water community (Figure 2-7). Rare vascular plant species have been recorded here, and the bay is recommended to become a protected heritage site of Municipality of Muskoka (The District Municipality of Muskoka, 2016).

Figure 2.7. SPL-1. This was part of McLean Bay looking across the bay rather than down to the rest of the lake.

Site 5 (SPL-2) Franklin Park

Site 5 (SPL-2) was located at a public boat launch at Franklin Park (N 44°49’59.4”, W 079°22’48.8”) (Figure 2-8). This park was located downstream from a local resort and restaurant and was one of the most anthropogenically stressed sites on Sparrow Lake.

Figure 2.8. Site 2 on Sparrow Lake, this site had the most obvious source of anthropogenic impact on this lake.

Site 6 (SPL-3) Wenona Lodge Rd

Site 6 (SPL-3) was located at a bay alongside Wenona Lodge Rd (N 44° 48’05.6”, W 079°22’49.2”) (Figure 2-9). This bay is one of the first bays after the lake’s inlet from both the man-made canal that connects Lake Couchiching directly to Sparrow Lake and the Severn River which travels north, before returning to Sparrow Lake after leaving Kahshe Lake (Charron, et al., 2013).

Figure 2.9. Site 3 on Sparrow Lake was the closest to the lake’s inlet, this bay located next to Wenona Lodge road is the most southern site sampled on this lake.

2.1-2 Core Experiment

A preliminary experiment was conducted to explore the possibility of occurrence of phosphate solubilizing bacteria in anaerobic conditions. A sediment core was collected at the site on each lake (Lake Simcoe-Atherley Narrows and Sparrow Lake) that appeared to be the most anthropogenically impacted locations. These sites were LS-1 (N 44°35’21.1”, W 079°28’86.1”) and SPL-2 (N 44°49’59.4”, W 079°22’48.8”). Both cores were collected on September 26th/17, and each core of 25cm deep. The sediment cores were then compared to see how far into the sediment phosphate solubilizing bacteria would be found and whether their abundance varies with sediment depth. Subsamples of each core were sent to Lakehead University’s Environmental Laboratory for nutrient analysis. Total reactive phosphorus and

nitrogen levels were analyzed and then compared to the abundance of phosphate solubilizing bacteria.

2.1-3 Cook's Bay Experiment

In 2014, the Ministry of the Environment and Climate Change, partnered with the Ministry of Natural Resources and Forestry and the Lake Simcoe Regional Conservation Authority, published a water quality monitoring report on Lake Simcoe. They identified total phosphorus as still being one of the most prominent factors affecting water quality in the Lake Simcoe watershed. Agricultural runoff was reported as one of the main sources of phosphorus pollution. According to Young and Jarjanazi (2015), the southern end of Lake Simcoe, especially the Holland River area, still has the highest concentrations of total phosphorus within Lake Simcoe. Sampling was conducted here to compare the abundances of phosphate solubilizing bacteria at a highly polluted (regarding total phosphorus µg/L) area with the other parts of Lake Simcoe and Sparrow Lake.

The phosphate solubilizing bacteria sampling took place on September 27th/17, and three sites at Cook's Bay, Lake Simcoe, were sampled: site 1 (CB 1) (N 44°14'88.6", W 079°29'28.4) was located on the eastern side of the bay, site 2 (CB 2) (N 44°12'00.9", W 079°28'49.0") on the southern side of the bay near the Holland River, and site 3 (CB 3) (N 44°18'48.5", W 079°81'48.8") was on the western side of the bay. Figure 2.10 is a map showing the location of each site.

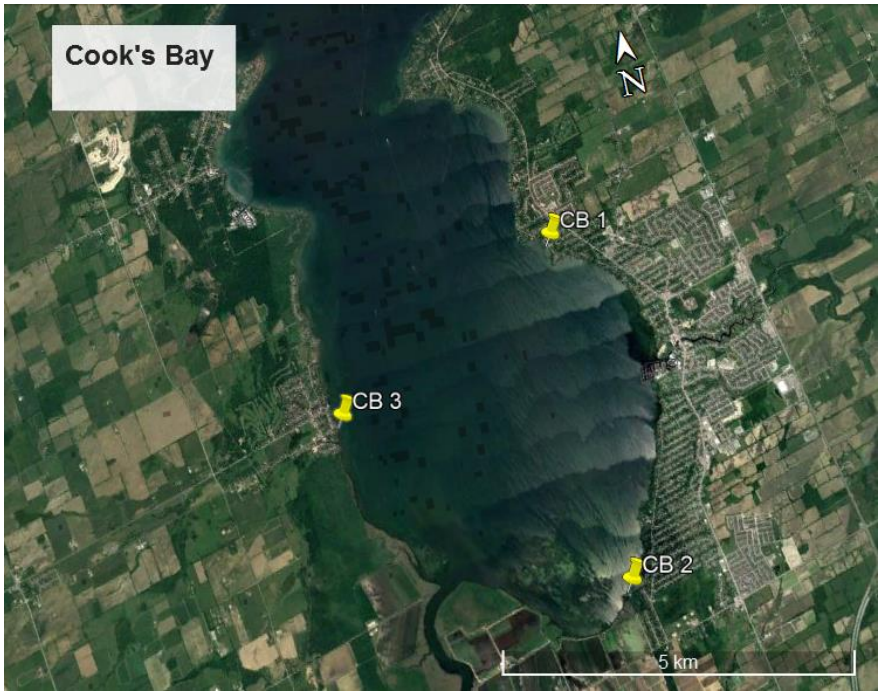


Figure 2.10. The sampling locations in Cook's Bay. Cook's Bay is the southernmost portion of Lake Simcoe and is heavily impacted by the farming that occurs around the Holland Marsh (Young & Jarjanazi, 2015).

2.2-1 Hydrological Parameters

2.2-1.1 Field Collection

The following hydrolab units; HACH HQ40D, VWR SympHony SB90M5, and VWR SympHony SB70P, were used to gather water temperature (°C), dissolved oxygen (mg/L), pH, and conductivity(µS/cm) readings on site. All hydrolab units were calibrated prior to use on each sampling day.

The ambient temperature was taken upon arrival at each site, and each site was scanned visually to determine if algae were present prior to sampling. 1L water samples were collected in triplicate and brought back to the laboratory for chlorophyll *a* analysis. The same was completed for a total suspended solids measurement. 500ml samples were collected in triplicate and brought back for nutrient analyses.

2.2-1.2 Laboratory Analyses

Chlorophyll a Analysis

Chlorophyll *a* analysis was completed immediately upon return to the laboratory after field sampling. The method used was adapted from Aminot and Rey's (2002) spectroscopic method. One thousand ml of the sample was filtered through glass fibre filters (47 mm, 0.1µ pore size). After the filtration, the filter paper was placed in a darkened conical tube containing 12ml of 90% acetone and the contents were mechanically mulched, then placed in the dark for 16 hours at 4°C. After 16 hours, the samples were centrifuged at 4200 RPM for 15 minutes. Three ml of the supernatant from each sample was transferred into separate cuvettes, and the absorbance of the sample was measured in the spectrophotometer at 750, 664, 647, and 630nm against the 90% acetone blank. The concentration of chlorophyll *a* was calculated using the following equation:

$$(11.85*(E_{664}-E_{750})-1.54*(E_{647}-E_{750})-0.08(E_{630}-E_{750}))*V_e/L*V_f$$

Where:
L= Cuvette light-path in centimetres
V_e=Extraction volume in millilitres
V_f= filtered volume in litres
Concentrations are in unit mg m^{-3} (Aminot & Rey, 2002).

Total Suspended Solids Analysis

The total suspended solids were determined by first weighing dry glass fibre filters (47mm) followed by filtering 1000ml of water sample through the filter. Once the filtration was done, the filters were kept in an oven at 60°C for 48 hours to remove all the moisture from the sample. The dried filters were then weighed. The original weight of the filter paper was then subtracted from the dried weight to get the total suspended solids in milligrams per litre.

Total Phosphorus Analysis

Most of the total phosphorus samples were analyzed at Lakehead University's Environmental Laboratory in Thunder Bay, ON. However, 14 of the 81 total phosphorous samples were analyzed in Lakehead University's Orillia Laboratory. The total phosphorus procedure used was adapted from the American Public Health Association's (1995) standard

methods for the examination of water and wastewater. All glassware was acid washed using 10 % HCl and allowed to air dry for 24 hours prior to use. The procedure began by digesting the orthophosphate. All analyses were completed in duplicate. One drop of phenolphthalein was added to 50ml of each sample and blank sample (deionized water). This was followed by the addition of 1ml of 30% sulfuric acid (H_2SO_4) and 0.4g of ammonium persulfate ($(NH_4)_2S_2O_8$). Once the samples had been reduced to 10ml each by heating on a hot plate, distilled water was added to make it to 30ml. As per the APHA methodology, an additional drop of phenolphthalein was added to the digested sample to confirm that the samples were at the proper pH. Ten ml of the final digested sample was pipetted into acid washed 25ml test tubes. One Hach Phos-Ver 3 pillow was added to each sample and shook for 15 seconds after which time 3ml was pipetted into a clean; acid washed cuvette. The absorbance of each sample was measured in the spectrophotometer at 880nm. A standard curve was prepared by following the same procedure, and the unknown samples were plotted along this curve to get the total phosphorus amount in milligrams per litre (Appendix I) (APHA, 1995). A few samples were tested for total phosphorus both by the above method and by an auto analyzer at Lakehead University's Environmental Laboratory to verify the accuracy. Both analyses did yield very similar and comparable results.

Nitrate Analysis

Sixty-seven out of the 81 samples collected throughout the sampling period were analyzed for total nitrogen at Lakehead University's Environmental Laboratory in Thunder Bay, ON in an autoanalyzer.

Fourteen samples for nitrate concentration were analyzed at Lakehead University's Orillia laboratory. The procedure outlined by the American Public Health Association's (1995) standard methods for the examination of water and wastewater was used for this analysis. The samples were analyzed in duplicate, and the blank was prepared by using distilled water. 15ml of the sample was transferred into an acid washed (10% HCl) test tube followed by the addition of the contents from one Hach Nitra-Ver 6 pillow. The samples were shaken for 3 minutes and kept idle for 2-minutes. After this, 10ml of the supernatant was transferred into a new test tube, and the contents of a Hach Nitri-Ver 3 pillow were added. The samples were shaken for

30 seconds and then allowed to stand for 15 minutes to complete the reaction. Finally, 3ml of the sample was transferred into a cuvette, and the absorbance was measured in a spectrophotometer at a wavelength of 507nm. A standard curve was made by following the same procedure and used to calculate the nitrate concentrations of the unknown samples in milligrams per litre (Appendix I) (APHA, 1995).

2.2-2 Collection of Microbial Samples

Sediment samples were collected in triplicate randomly from the littoral zone (water depth was approximately 0.5m) from three different sites along the northwestern shore of Lake Simcoe and the eastern shore of Sparrow Lake. Three water samples were collected from each of the sites at the same time at a depth of approximately 25cm above the benthic layer. These samples were collected by opening a sterile, 50ml conical tube at the desired depth. Sediment samples were collected via a Russian peat borer that was sterilized with 75% ethanol before each use. Samples were aseptically transported back to the laboratory in sterile conical tubes and kept at a temperature of ~4°C until they were analyzed, which occurred within 8 hours of collection.

2.2-3 Isolation of Microbes

The isolation of the phosphate solubilizing bacteria was completed by following the method described by Sanjotha and Manawadi (2016). A subsample (0.1g) of the sediment sample was suspended in 10ml of distilled water. An aliquot (1ml) from the serially diluted samples was inoculated onto a Pikovskaya's agar plates (yeast extract 0.5g/L, dextrose 10g/L, $Ca_3(PO_4)_2$ 5g/L, $(NH_4)_2SO_4$ 0.5g/L, KCl 0.2g/L, $MgSO_4$ 0.1g/L, $MnSO_4$ 0.0001g/L, $FeSO_4$ 0.0001g/L, and agar 15g/L) by way of the pour plate method and incubated at 30°C (±2°C) for seven days. Each of the serially diluted samples was analyzed in triplicate. As the samples were collected from the top 5cm of the sediment, the bacteria were grown in aerobic conditions, as per the methodology described by Sanjotha and Manawadi (2016).

Once the plates were incubated for seven days, all the viable bacteria were counted and expressed as colony forming units. The average colony forming units (CFUs) per site were calculated using the following formula:

$$CFU = 2\left(\frac{a + B + c}{Mu} * \ 10^{-n}\right)$$

Where:

a= replicate 1
B=replicate 2
c= replicate 3
Mμ=mean of replicates
10^{-n}= dilution factor

The number of microbes that had a phosphate solubilizing zone was also calculated using the same formula. The microbes that had a phosphate solubilizing zone around them were purified by transferring them to other Pikovskaya's agar plates by streak plate method and incubated at 30°C (±2°C) for another seven days. After this point, the isolates were transferred onto Pikovskaya's agar slants and stored for further study.

2.2-4 Core Experiment

The sediment core samples were collected using a sterilized Russian peat borer. The 30cm cores were aseptically transported back to the lab where they were divided into 5cm sections. After different sections were made, 0.5mg from each section was used for microbial isolation, and the procedure above was completed to calculate CFU/g. An additional 5g sediment from each section was dried and sent to Lakehead University Environmental Laboratory for total phosphorus analysis.

2.2-5 Statistical Analysis

2.2-5.1 Hydrological Parameters

Total suspended solids, chlorophyll *a*, total phosphorus, and total nitrogen data were all collected in triplicate. Conductivity, pH, dissolved oxygen, ambient and water temperature were all collected in situ at each site once per sampling period.

Data were analyzed using the statistical program R version 3.4.3 (R Core Team, 2017) with the packages 'nortest' (Gross & Ligges, 2015), 'car' (Fox & Weisberg, 2011), 'lattice' (Sarkar & Deepayan,2008), and 'scatterplot 3D' (Ligges & Mächler,2003) to complete a multiple

regression. This type of analysis was completed to determine if any of the following parameters: chlorophyll *a*, total suspended solids, dissolved oxygen, conductivity, pH, water temperature, or total heterotrophic bacteria, had any statistical impact on the abundance of phosphate solubilizing bacteria. The algebraic model used to this analysis is:

$$y_i = \beta_0 + \beta_1 X_{i1} + \beta_2 X_{i2} + \cdots + \beta_j X_{ij} + \cdots + \beta_p x_{ip} + \varepsilon_i$$

Where:

β_0= value of y when all other x equal zero
p= 11 (the number of parameters being analyzed)
x_1= predictor 1 (example chlorophyll α)
x_2=predictor 2 (example total phosphorus)
…
ε=random error (Quinn & Keough, 2002).

Initial analyses showed that the raw data did not meet the assumptions of normality and homogeneity, so all the parameters (except site pH levels which were already in logarithmic form) were $\log_{10}$ transformed. The null hypotheses tested was that the abundance of phosphate solubilizing bacteria not related to any of the hydrological parameters. While the alternative hypothesis tested was that at least one of the hydrological parameters tested had a significant relationship with the abundance of phosphate solubilizing bacteria.

2.2-5.2 Comparison Study

Microbial data was collected from the field in triplicate. The serial dilutions of each sample were also completed in triplicate. From these values the means of the plates that showed viable total heterotrophic cell counts between 30-300 were recorded.

Three-Factor ANOVA

The statistical program R version 3.4.3 (R Core Team, 2017) with the packages 'nortest' (Gross & Ligges, 2015) and 'car' (Fox & Weisberg, 2011) were used to perform a three-factor analysis of variance (ANOVA) to compare the means of the phosphate solubilizing bacteria abundances that were isolated from sediment samples. The lake, month, and site sampled were modelled as fixed effects as those variables were orthogonal contrasts. This was completed to determine if either the lake, months or sites sampled impacted the total abundance of the

phosphate solubilizing bacteria or if any interactions between the fixed effects would impact the abundance of phosphate solubilizing bacteria. The algebraic model that was used for the three-factor ANOVA was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkl}$$

Where:
α= the lake sampled
β= the site sampled
γ= the month sampled
ε= random error (Quinn & Keough, 2002).

The statistical hypotheses tested were: H_{01}: no statistically significant difference in PSB abundance between lakes, site, and months exists. H_{A1}: there is a significant difference in PSB abundance between lakes, the abundance of PSBs, site, and months. H_{02}: no interaction between lake and site, lake and month, or site and month on the abundance of PSBs occurred. H_{A2}: interactions between lake and site, lake and month, or site and month on the abundance of PSBs do exist.

Initial inspection of diagnostic plots showed that the data deviated from homoscedasticity and normality. Exploding variance was observed on the residuals vs. fitted plot, and the residuals deviated drastically from the normal q-q plot (Figure 2.11). After the data transformation to $\log_{10}$ values, the visual inspection of the diagnostic plots revealed that the data met the assumptions of normality and homoscedasticity. While some exploding variance was observed on the residuals vs. fitted plot, the residuals were much straighter on the normal q-q plot (Figure 2.12). After the diagnostic plots were inspected a series of post hoc tests were completed to ensure that the results obtained from the three-factor ANOVA were statistically correct. The *A posteriori* (Tukey HSD) test was completed to verify the significance between the factors (lake, month, site) that were tested, while an Anderson Darling Normality test was completed to ensure the data is normally distributed, and a Levene's test was completed to test for homogeneity of variance.

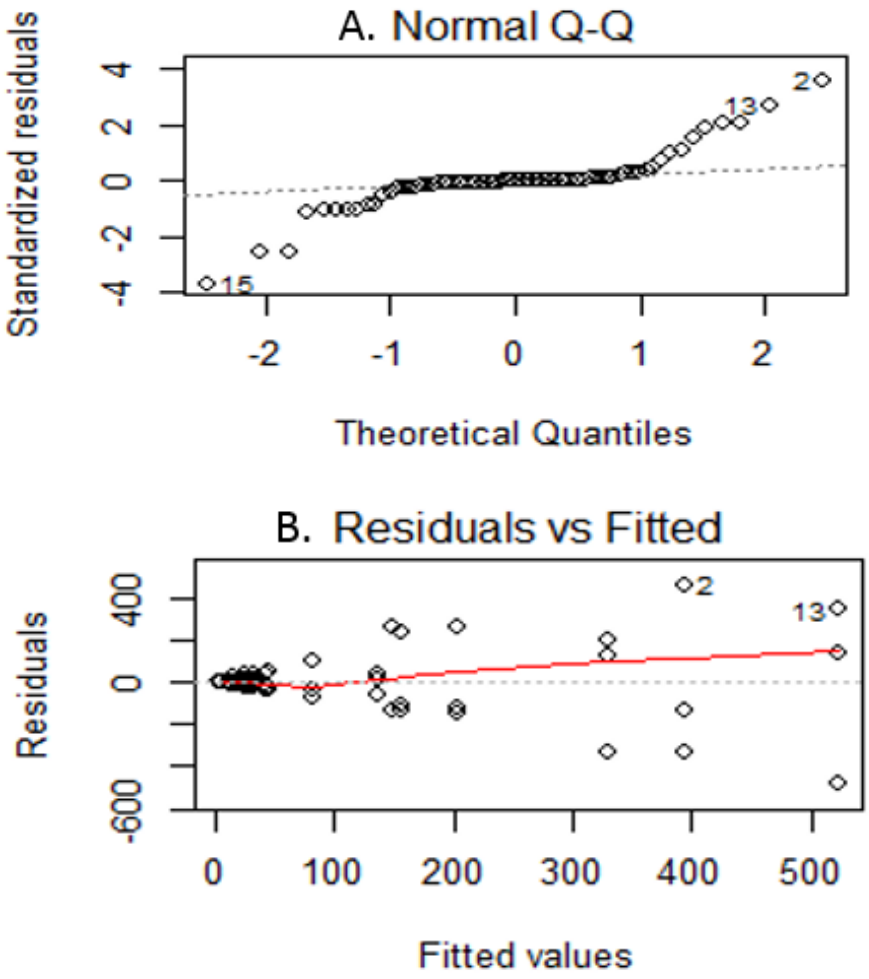


Figure 2.11. Diagnostic plots of the original data. Plot A demonstrates that the data is not normally distributed, and Plot B has an exploding variance pattern, which indicates that there is too much variance between the residual data points for there to be a significant result.

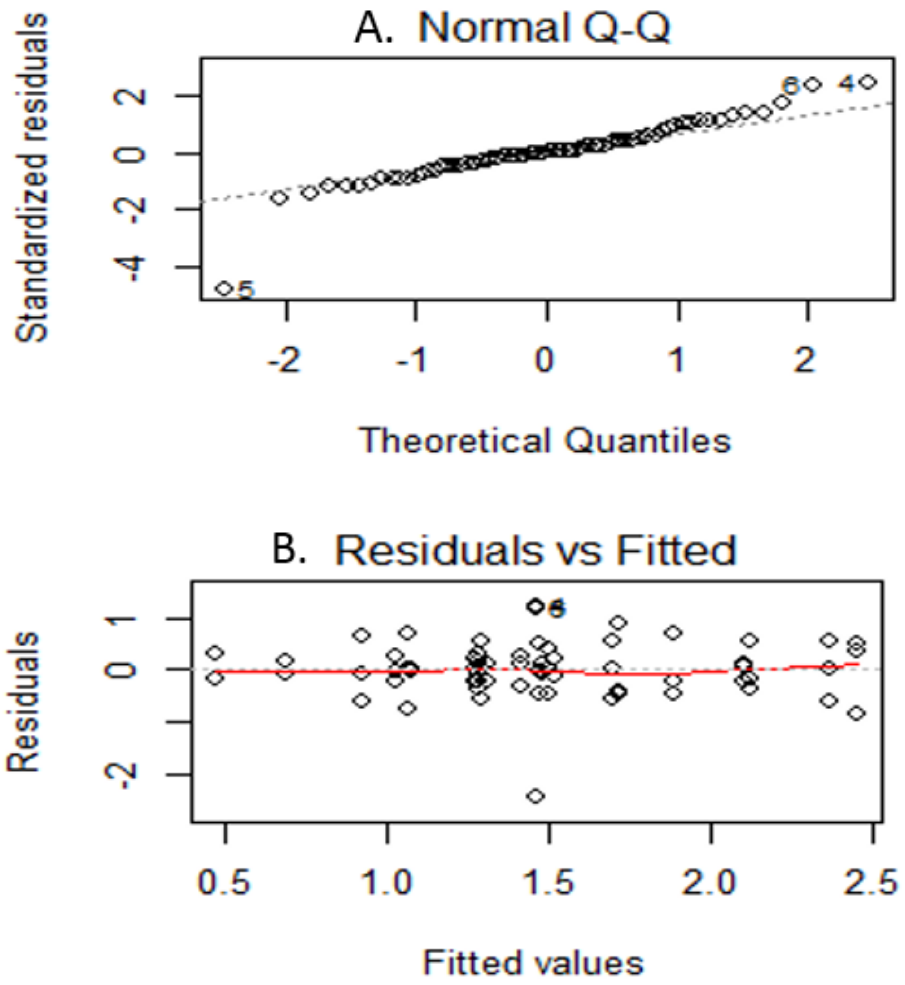


Figure 2.12. These diagnostic plots are visually illustrating that the $\log_{10}$ transformation corrected the issues observed with normality and homoscedasticity. Plot A shows that the data are closer to being normally distributed and plot B show that the exploding variance has been resolved due to the transformation. This was also confirmed by the non-significant results obtained by the Anderson Darling (A=0.585, p= 0.123) and Levene's tests ($F_{23,48}$=0.628, p=0.885).

Nested ANOVA

The statistical program R version 3.4.3 (R Core Team, 2017) with the packages 'nortest' (Gross & Ligges, 2015) and 'car' (Fox & Weisberg, 2011) were used to perform a nested analysis of variance (ANOVA) to compare the means of the phosphate solubilizing bacteria abundances that were isolated from sediment samples for each lake. Time, or the months sampled, was used as the repeated measure while the sites were nested within the lake sampled which was used as the fixed effect. The algebraic model used for the nested ANOVA was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + [(\alpha\beta)]_{ij} + \varepsilon_{ijk}$$

Where:
Y_{ijk} = PSB Abundance
μ= the population mean
α_i = the lake sampled
β_j = time or months sampled
[αβ]= the random interaction between month and lake (it is assumed to be zero)
ε_{ijk} = random error (Quinn & Keough, 2002).

The statistical hypotheses tested were H_0: phosphate solubilizing bacteria abundance for each lake did not change monthly. H_A: monthly variability was observed for at least one of the lakes for each month sampled. Preliminary analysis showed that the data deviated from normality and homoscedasticity. This was confirmed by the significant Anderson Darling normality test (A= 4.2391, p= 1.218×10^{10}) and Bartlett's test for homogeneity of variance (K^2= 33.444, df = 1, p= 7.335×10^{9}). The data underwent a $\log_{10}$ transformation, and upon further inspection, it became apparent that this transformation resolved the deviations from normality and homoscedasticity.

Correlation of PSBs and Total Phosphorus

The statistical program R version 3.4.3 (R Core Team, 2017) was used once again to test for any possible correlations that may exist between the abundance of phosphate solubilizing bacteria and total phosphorus within both Lake Simcoe (Atherley Narrows) and Sparrow Lake. As neither the phosphate solubilizing bacteria nor the total phosphorus data were normally distributed for either lake, all the data were $\log_{10}$ transformed. Due to the data being normally distributed after transformation a Pearson's correlation was performed. The algebraic formula used to calculate the Pearson's correlation coefficient was:

$$r_{Y1Y2} = \frac{\sum_{i=1}^{n}(y_{i1} - \bar{y}_1)(y_{i2} - \bar{y}_2)}{\sqrt{\sum_{i=1}^{n}(y_{i1} - \bar{y}_1)^2 \sum_{i=1}^{n}(y_{i2} - \bar{y}_2)^2}}$$

Where:

Y_1= Phosphate Solubilizing Bacteria (PSB) Abundance (CFU/g)
Y_2= Total Phosphorus concentration (mg/L) (Quinn & Keough, 2002).

The following statistical hypotheses were tested by this analysis: H_0: There was no correlation between PSB abundance and total phosphorus concentrations in either the lake. H_A: A significant correlation between PSB abundance and total phosphorus concentrations exists in at least one of the lakes.

2.2-5.3 Core Experiment

The statistical program R version 3.4.3 (R Core Team, 2017) with the package 'boot' (Canty & Ripley (2017), Davison & Hinkley (1997)) was used to complete the non-parametric bootstrap test. This data was bootstrapped due to the sparse number of total reactive phosphorus (TRP) replicates that were collected from each core. Bootstrapping allows for the random drawing of numbers from a portion of a data set with replication in order to simulate repeated sampling of the same variables (Quinn & Keough, 2002). The sediment core and PSB data from each lake underwent bootstrapping, and then a regression was completed to determine if a relationship between phosphate solubilizing bacteria abundance and total reactive phosphorus existed. The following were the hypotheses that were tested: H_0: no relationship between total reactive phosphorus and phosphate solubilizing bacteria exists for either lake. H_A: there is a relationship between total reactive phosphorus and phosphate solubilizing bacteria exists for either lake.

2.2-5.4 Cook's Bay Study

The statistical program R version 3.4.3 (R Core Team, 2017) with the packages 'nortest' (Gross & Ligges, 2015) and 'car' (Fox & Weisberg, 2011) were used to complete a single factor analysis of variance (ANOVA). This test was completed in order to compare the means of the phosphate solubilizing bacteria abundances that were collected from the Atherley Narrows and Cook's Bay sites that were sampled on Lake Simcoe in September 2017. The statistical hypotheses tested were: H_0: There was not a significant difference in the abundance of phosphate solubilizing bacteria between the northern and southern ends of Lake Simcoe. H_A: A significant difference was observed within the abundance of phosphate solubilizing bacteria

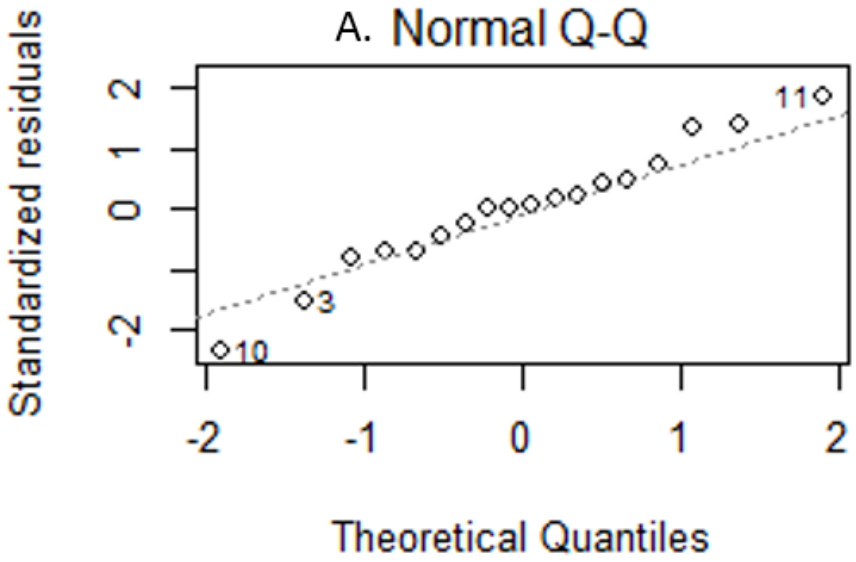
found in the northern and southern ends of Lake Simcoe. The algebraic model used for the single factor ANOVA was:

$$y_{ij=}\mu + \alpha_i + \varepsilon_{ij}$$

Where:

μ= portion of lake sampled
α= the site sampled
ε= random error (Quinn & Keough, 2002).

Initial inspection of diagnostic plots showed that the data deviated from homoscedasticity and normality. After the data underwent a $\log_{10}$ transformation, visual inspection of the diagnostic plots, along with an Anderson-Darling Normality test and a Levene's test revealed that the data met the assumptions of normality and homoscedasticity. No patterns were observed on the residuals vs. fitted plot, and the residuals were much straighter on normal q-q plot (Figure 2.13). The *A posteriori* (Tukey HSD) test was completed to verify significance between the Atherley Narrows and Cook's Bay sites.



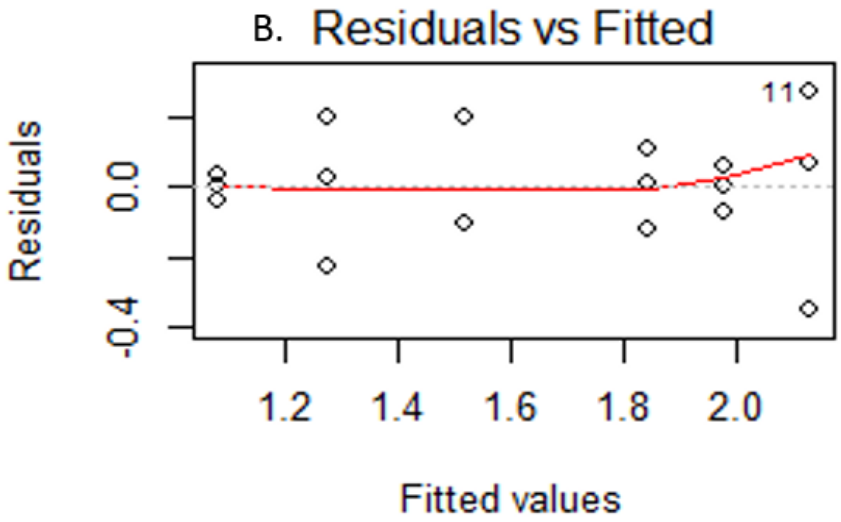


Figure 2.13. Diagnostic plots of the $\log_{10}$ transformed data providing a visual representation to the results obtained by the Anderson Darling, Bartlett's and Levene's tests all confirming that the data meet the assumptions of normality and homoscedasticity after the transformation occurred. Plot A shows that the data is reasonably close to being normally distributed while plot B shows that there are no patterns to the residuals plot.

2.3 Results

2.3-1 Hydrological Parameters

While significant variations in water temperature were not observed between the Sparrow Lake sites ($F_{3,8}$= 2.599, p=0.1245), significant variations could be seen in the water temperature between the Lake Simcoe Atherley Narrows sites ($F_{3,8}$=6.433, p=0.015). The highest recorded temperature was 26.7°C in both Atherley Narrows sites 2 and 3 in September, and the lowest recorded temperature was 18.3°C in Atherley Narrows site 1 in June. The lowest recorded temperature in the Sparrow Lake sites was 19.5°C which was recorded at site 1 in August while the highest recorded water temperature was 23.4°C, which was recorded at site 1 in September.

Significant variations in dissolved oxygen were not observed for either lake (SPL: $F_{3,8}$=2.877, p=0.103; LS: $F_{3,8}$=1.396, p=0.3129). The lowest dissolved oxygen (DO) concentration for Sparrow Lake was recorded at site 1 in July (6.83 mg/L) while the highest DO concentration in the lake was at site 1 in August (10.42 mg/L). In the Atherley Narrows sites, the lowest DO

concentration was at site 1 in June (6.18 mg/L), and the highest DO concentration was at the same site in September (16.81 mg/L).

There was significant variation observed between the pH levels in the Sparrow lake sites ($F_{3,8}$= 12.1408, p=0.0023), but not with the Atherley Narrows sites ($F_{3,8}$= 0.7643, p=0.5418). The lowest pH concertation in Sparrow Lake was recorded at site 3 in September (6.88) while the highest concentration was recorded at the same site in June (8.17). For the Atherley Narrows sites, the lowest pH was recorded at site 1 in June (7.35) while the highest concentration was recorded at site 2 in September (8.71).

Even though statistically significant variation did not observe between sites and months for the conductivity, much variation was observed between months for each lake. In Sparrow Lake, conductivity ranged from 88.2µS/cm in site 1 in July to 391 µS/cm in site 3 in September. For the Atherley Narrows sites, conductivity ranged from 421 µS/cm in site 3 in August, to 1030 in site 1 in June.

Chlorophyll *a* concentrations in Sparrow Lake varied from below detectable limits (BDL) in site 1 in September, to 6.68µg/L (SD ±12.238) in site 2 in June. In the Atherley Narrows sites chlorophyll *a* concentrations ranged from 47.84 µg/L (SD ±3.23) in site 1 in July to 1.05 µg/L (SD ±2.33) in site 2 in September. For the Atherley Narrows sites, site 1 consistently had the highest chlorophyll *a* concentration except for in September when site 3 had the highest concentration of chlorophyll *a*. Sparrow Lake site 1 consistently had the lowest concentrations of chlorophyll *a* out of the Sparrow Lake sites.

Total suspended solids in the Atherley Narrows sites was highest in site 1 in September (127.33mg/L, SD ±107.353) and lowest at site 2 in July (6.9mg/L, SD ±4.357). The highest concentration for Sparrow Lake was at site 3 in September (69.4mg/L, SD ±71.963), while the lowest concentration in site 2 also in September (1.033mg/L, SD ±1.429). Total Nitrogen concentration in the Atherley Narrows sites was highest at site 1 in June (4.24 mg/L, SD ±0.69) and lowest at site 2 in July (0.12 mg/L, SD ±0.079). Nitrate concentration in August was highest at site 1 (0.139mg/L, SD±0.025), and lowest at site 3 (0.008 mg/L, SD ±0.00). In Sparrow Lake,

the lowest total nitrogen concentration was at site 3 in June (0.143, SD ± 0.08), and highest at site 2 in July (0.713, SD ± 0.343).

Table 2.1. Monthly Total Nitrogen concentrations (mg/L) for the Sparrow Lake (SPL) and Lake Simcoe-Atherley Narrows (LS) sites. The standard deviation is given in parenthesis.

	June	*July*	*Aug*	*Sept*
SPL 1	0.19 (0.075)	0.50 (0.059)	0.37 (0.035)	0.31 (0.016)
SPL 2	0.33 (0.113)	0.71 (0.343)	0.43 (0.031)	0.35 (0.07)
SPL 3	0.14 (0.08)	0.41 (0.013)	0.51 (0.041)	0.39 (0.057)

	June	*July*	*Aug*	*Sept*
LS 1	4.24 (0.269)	0.73 (0.075)	NO3	NO3
LS 2	0.29 (0.263)	0.12 (0.079)	NO3	0.62(0.285)
LS 3	0.19 (0.108)	0.19 (0.145)	NO3	0.50(0.044)

Table 2.2. Nitrate concentrations (mg/L) for the Lake Simcoe-Atherley Narrows sites. The standard deviation is given in parenthesis.

	Aug	*Sept*
LS 1	0.14 (0.025)	0.38 (0.093)
LS 2	0.01 (0.002)	TN
LS 3	0.008 (0.00)	TN

Total phosphorus concentrations at the Atherley Narrow sites was highest at site 1 in September (0.086mg/L, SD ±0.118), and lowest at site 3 in June (0.013mg/L, SD±0.0072). While the lowest total phosphorus concentration in Sparrow Lake was found at site 3 in June (0.018mg/L, SD ± 0.0056) and the highest was found at site 2, also in June (0.095mg/L, SD ±0.133). The total phosphorus results that were collected from the sites in the north part of Lake Simcoe were all higher than the 9µg/L that was reported in the OMECC's 2014 monitoring report (Young & Jarjanazi, 2015). Between the three sites on Lake Simcoe that were continuously sampled, June had the lowest amount of TP (15.4µg/L, SD ±0.0018) while the highest concentration of total phosphorus was recorded in September (52.11. 4µg/L, SD

±0.0297). Site 1 consistently had the highest concentrations of total phosphorus and only met the provincial standard of 2µg/L or less (Ontario Ministry of the Environment, 2013) in June and July. The average monthly total phosphorus concentrations are given in Table 2.3.

Table 2.3. Monthly mean total phosphorus concentrations for all sites on Atherley Narrows and Sparrow Lake. The standard deviations are given in parentheses.

Month	Atherley Narrows (µg/L)	Sparrow Lake (µg/L)
June	15.4 (±0.001)	48.5 (±0.053)
July	28.1 (±0.01)	51.8 (±0.012)
August	45.2 (±0.014)	43.2 (±0.014)
September	52.1 (±0.029)	36.2 (±0.014)

The highest abundance of total heterotrophic bacteria was recorded in the sediment from Sparrow Lake site1 in July ($2.96x10^5$, SD±159904.1). The lowest total heterotrophic bacteria abundance in Sparrow Lake was collected from site 2 in September ($1.96x10^2$, SD±90.42). The highest abundance of total heterotrophic bacteria collected from the Lake Simcoe Atherley Narrows sites was in site 1 in June ($7.35x10^3$, SD±11135.24). Meanwhile, the lowest total heterotrophic bacteria abundance was also recorded in site 1, but in September ($2.12x10^2$, SD±151.26).

The highest abundance of phosphate solubilizing bacteria in Sparrow Lake was recorded from site 2 in July ($5.22x10^2$, SD±429.94). Meanwhile, the lowest phosphate solubilizing bacteria abundance in Sparrow Lake was observed in site 2 in September ($2.2x10^1$, SD ±12.7). The highest abundance of phosphate solubilizing bacteria from the Lake Simcoe Atherley Narrows sites was found in site 2 in June ($1.57x10^2$, SD± 210.39), and the lowest abundance was found in site 1 in August ($5x10^0$, SD ±1.73). The number of bacterial colonies counted at each site for each of the sampling months can be seen in Table 2.4.

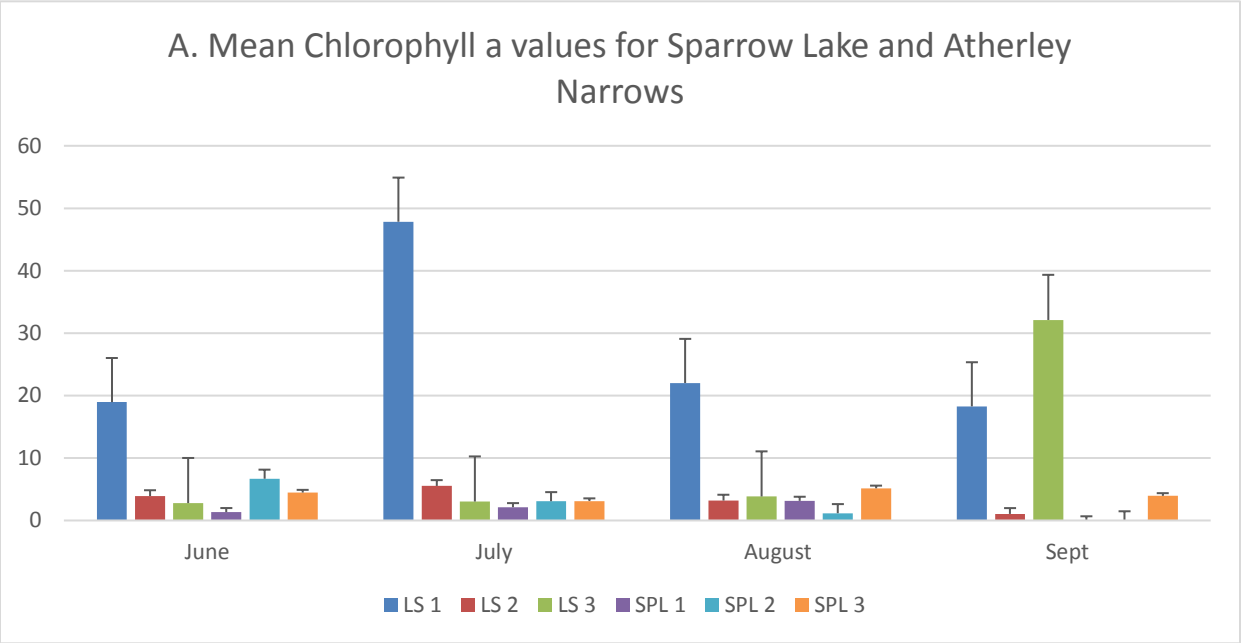
Table 2.4. The number of phosphate solubilizing bacteria colonies counted each month at Atherley Narrows Site 1 and Sparrow Lake Site 1. The standard deviations are given in parenthesis.

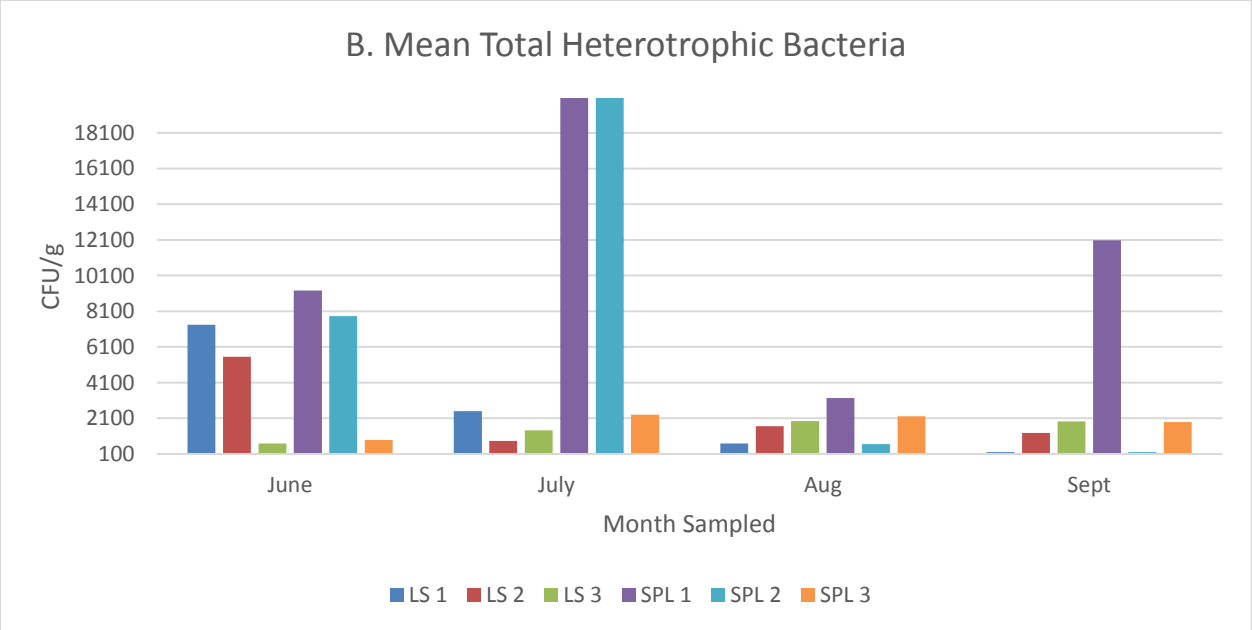
Month	Atherley Narrows Site 1 (CFU/g)	Sparrow Lake Site 1 (CFU/g)
June	$1.2x10^1$ (±7.21)	$3.94x10^2$ (±416.3)
July	$4.6x10^1$ (±48.07)	$2.04x10^2$ (±227.2)
August	$5x10^0$ (±1.73)	$3.1x10^1$ (±7.0)
September	$2.1x10^1$ (±9.504)	$8.2x10^1$ (±87.35)

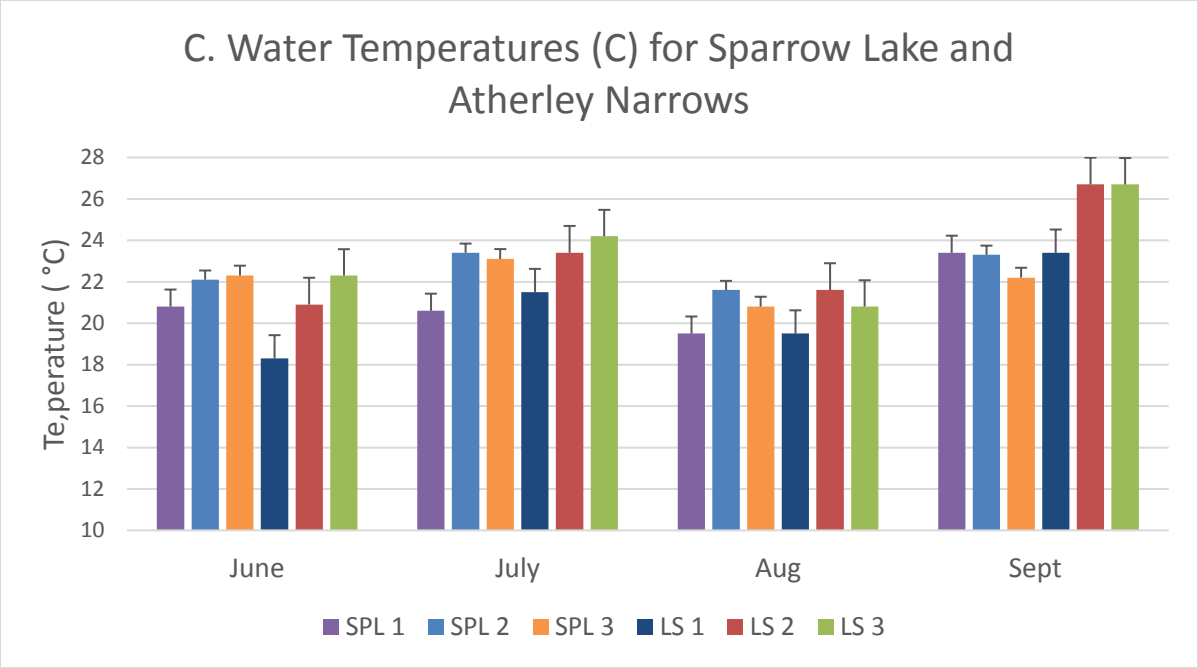
2.3-1.1 Hydrological Parameters Statistical Testing Results

Quinn and Keough (2002), define multiple regression as "the impact of correlated predictor variables on the estimates of parameters and hypotheses tests." As per the guidelines set out by Zuur, Leno, and Elphick (2010), all of the hydrological parameters that had a variance inflation factor (VIF) greater than two were removed from the model in order to avoid results that may have been skewed by multicollinearity. As dissolved oxygen (VIF= 4.548), pH (VIF=4.509), and conductivity (VIF= 2.059) all had variance inflation factors that were higher than the recommended amount, they were not included in the final analysis and were sequentially removed from the equation. As chlorophyll *a* (VIF= 1.098), total suspended solids (VIF=1.609), water temperature (VIF= 1.272), and total heterotrophic bacteria (VIF=1.314), all had low VIFs, they were included in the final analysis and graphs showing the distribution of these parameters can be seen in Figure 2.14.

The final model was statistically significant ($F_{4,19}$=4.96, p=0.0065), with chlorophyll *a*, total suspended solids, water temperature, and total heterotrophic bacteria abundance explaining 51.08% of the variance in phosphate solubilizing bacteria abundance. Due to this the null hypothesis stating that none of the hydrological parameters would explain any of the variance observed in the abundance of phosphate solubilizing bacteria was rejected.


A. Mean Chlorophyll a values for Sparrow Lake and Atherley Narrows
60
50
40
30
20
10
0
June
July
August
Sept
LS 1
LS 2
LS 3
SPL 1
SPL 2
SPL 3


B. Mean Total Heterotrophic Bacteria
CFU/g
18100
16100
14100
12100
10100
8100
6100
4100
2100
100
June
July
Aug
Sept
Month Sampled
LS 1
LS 2
LS 3
SPL 1
SPL 2
SPL 3



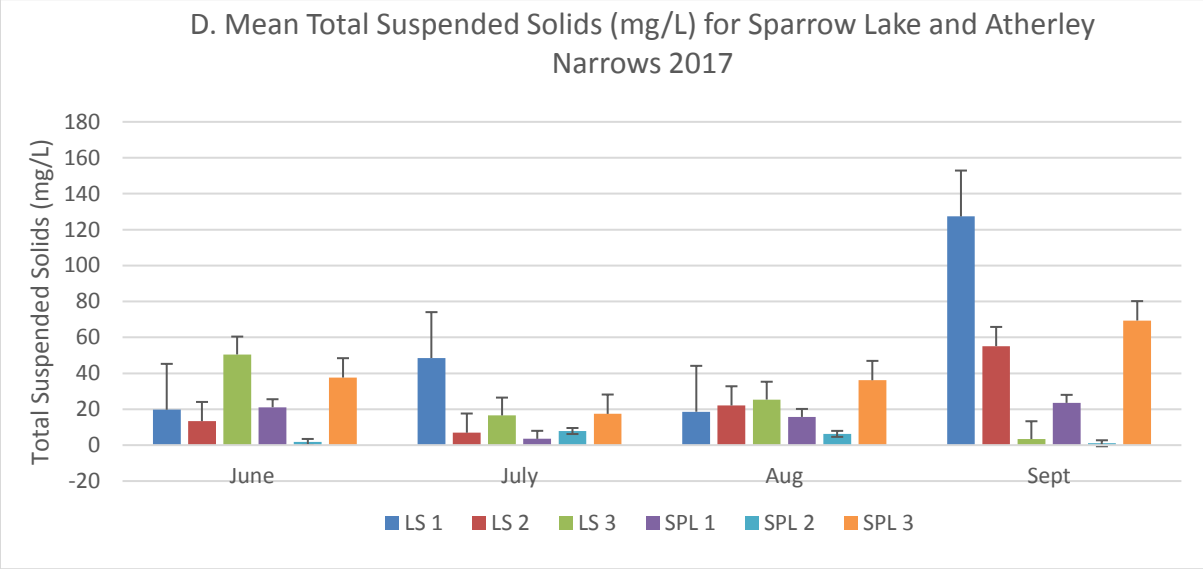


Figure 2.14. These bar graphs are showing the mean average results for the significant hydrological parameters that were included in the multiple regression.

Plot A showed the Chlorophyll *a* concentrations at the Sparrow Lake and Lake Simcoe sites each month they were sampled.

Plot B shows the mean amounts of total heterotrophic bacteria (CFU/g) at each site for each month.

Plot C shows the water temperatures for each site for every sampling event.

Plot D shows the mean total suspended solids for the Sparrow Lake and Lake Simcoe sites for each month they were sampled.

2.3-2 Comparison Study

Three-Factor ANOVA

Significance was found with the interactions between lake and site ($F_{2,48}$=4.068, p=0.023) and with the interaction between site and month ($F_{6,48}$=2.783, p=0.021) but not between any of the other factors or interactions. Due to the results expressed above, the first null hypothesis (H_{01}) stating that there is no significant difference between lakes is accepted. However, the second null hypothesis is rejected, and the second alternative hypothesis stating that at least one interaction exists between lake and site, lake and month, or site and month on the abundance of PSBs is accepted.

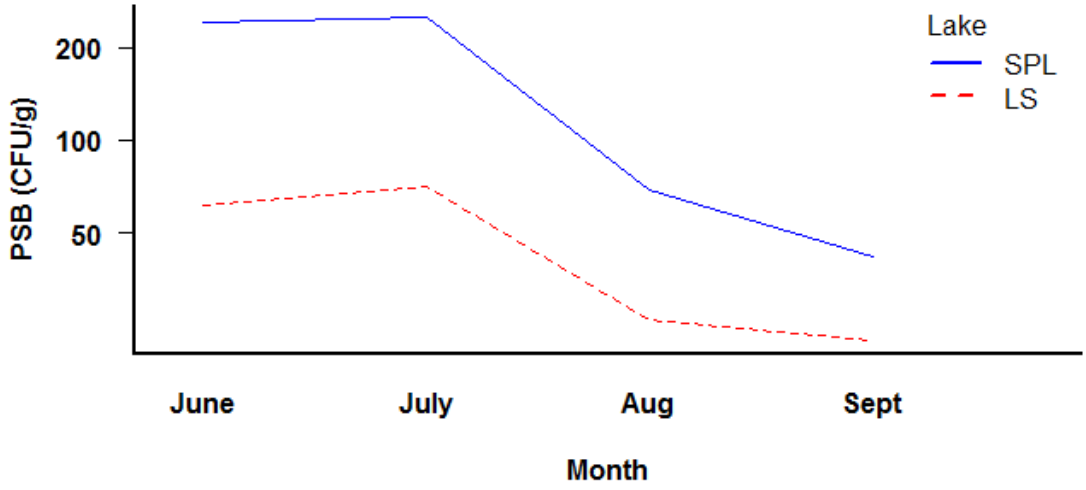


Figure 2.15. Interaction plot showing that no interactions occurred between Sparrow Lake and Lake Simcoe Atherley Narrows during the sampling period. SPL is the short form used for the Sparrow Lake sites while LS was used for the Lake Simcoe Atherley Narrows sites.??

Interaction plots showed no evidence of interactions occurring between Sparrow Lake and Lake Simcoe (Atherley Narrows) (Figure 2.15). Boxplots of the raw data showed some evidence of skewness, particularly in Sparrow Lake where high abundances of the phosphate solubilizing bacteria were found in June and July (Figure 2.16). The Anderson-Darling normality test that was performed on this data provides evidence that transforming the data did fix the distribution problems that were seen with the original data set. The Anderson-Darling test proved to be insignificant (A=0.585, p= 0.123). Levene's test for homogeneity of variance also

indicated that the variance within the transformed data was manageable since Levene's test also had an insignificant result ($F_{23,48}$=0.628, p=0.885).

Tukey's HSD test identified that the significant interaction between lake and site occurred between Sparrow Lake site 1 and Atherley Narrows site 1 (p=0.031). The only significant interaction that Tukey's test identified between the site and month interaction was between site 2 in July and site 3 in June (p=0.003), there was an almost significant difference between site 1 in August and site 2 in July (p=0.066).

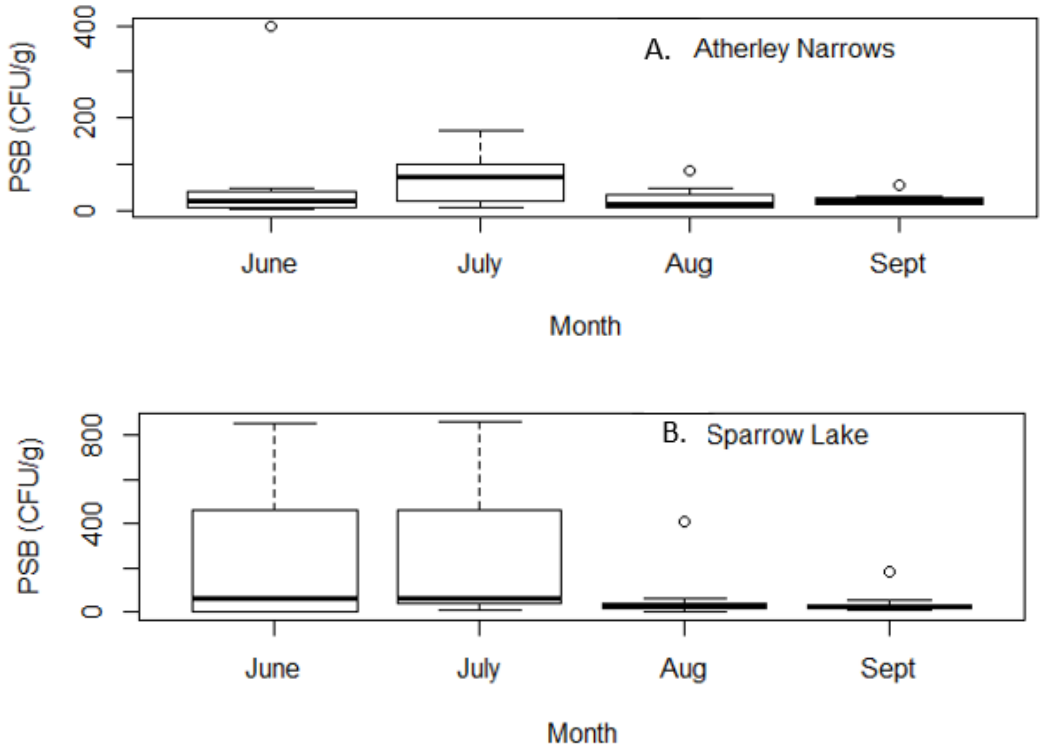


Figure2.16. Boxplots of the raw PSB abundance for both Atherley Narrows (Plot A) and Sparrow Lake (Plot B). Plot A shows that the amount of PSBs stayed relatively consistent throughout the sampling events. Plot B showed that a much higher abundance of PSBs in June and July. Note that the dark line in the center of each plot represents the median of the data while the upper and lower lines show the 95% confidence intervals.

Nested ANOVA

Significant differences were not found between the abundance of phosphate solubilizing bacteria and the month sampled ($F_{3,19}$= 1.062, p=0.389), As the data met the assumptions of normality (A=0.3691, p=0.3991) and homoscedasticity (K^2=2.6437, df=1, p=0.104), the null hypothesis is accepted,

and no further testing was completed. No interactions were observed between lakes, which is clearly observed in Figure 2.17.

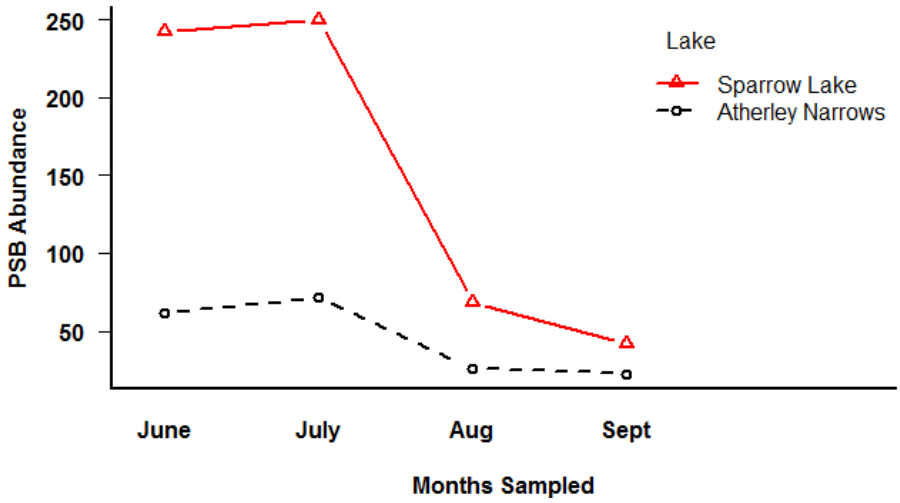


Figure 2.17. This interaction plot is showing the phosphate solubilizing bacteria abundances on both Sparrow Lake and Lake Simcoe Atherley Narrows.

Correlation between PSBs and Total Phosphorus

A significant correlation between phosphate solubilizing bacteria abundance and total phosphorus concentration was observed on Sparrow Lake (r = -0.362, t_{33} = -2.231, p = 0.032) but not on Lake Simcoe (Atherley Narrows) (r = 0.053, t_{34}= 0.312, p= 0.75). As a significant correlation was observed in Sparrow Lake, the null hypothesis is rejected, and the alternative hypothesis is accepted.

2.3-3 Core Experiment

The water temperature at the Sparrow Lake core site (site 2) was 21.8°C when the core was collected. Atherley Narrows, site 1 was 20.3°C when the core was collected. Algae was present at both locations, the water pH for Sparrow Lake was 7.44 while it was 7.48 for the Lake Simcoe Core. The conductivity was 385µS/cm at the Sparrow Lake site while it was 1127µS/cm at the Lake Simcoe site. There was 9.8mg/L of dissolved oxygen at the Sparrow Lake site, while there was 11.59mg/L at the Lake Simcoe site. At the Sparrow Lake site, the chlorophyll *a* concentration was 1.19µg/L (SD ±0.377), while the chlorophyll *a* concentration was 66.24µg/L (SD ± 19.39) at the Lake Simcoe site. While the amount of total suspended solids averaged 4.66g/L (SD ±5.35) at the Sparrow Lake site and averaged 120.06mg/L (SD ±73.92) at

the Lake Simcoe site, the nutrients and abundance of bacteria found at each core site are expressed in Table 2.5 and Table 2.6.

Table 2.5. Lake Simcoe Atherley Narrows core data collected September 26th, 2017.

Core Depth	Total Reactive Phosphorus (mg/g)	Percent Nitrogen	Phosphate Solubilizing Bacteria (CFU/g)	Total Heterotrophic Bacteria (CFU/g)
50mm	0.19	0.02	$2x10^1$	$1.62x10^2$
100mm	0.36	2.86	$3x10^1$	$3.8x10^1$
150mm	0.46	2.04	$1.1x10^1$	$3x10^2$
200mm	0.44	2.83	$1.1x10^1$	$8.1x10^1$

Table 2.6. Sparrow Lake core data collected September 26th, 2017.

Core Depth	Total Reactive Phosphorus (mg/g)	Percent Nitrogen	Phosphate Solubilizing Bacteria (CFU/g)	Total Heterotrophic Bacteria (CFU/g)
50mm	0.21	0.02	$2.8x10^1$	$3x10^2$
100mm	0.24	0.02	$3x10^1$	$1.52x10^2$
150mm	0.31	0.02	$8x10^0$	$2.52x10^2$
200mm	0.39	0.01	$4x10^0$	$1.52x10^2$

As the bootstrapped estimate of the relationship between total reactive phosphorus and phosphate solubilizing bacteria for the Sparrow Lake core was 169.28, with a 95% confidence interval of 50-314.28, there is a significant positive relationship between total reactive phosphorus and phosphate solubilizing bacteria for Sparrow Lake. However, the linear regression that was completed to complement the bootstrapped design was not significant (r^2=0.803, $F_{1,2}$= 13.29, p=0.067).

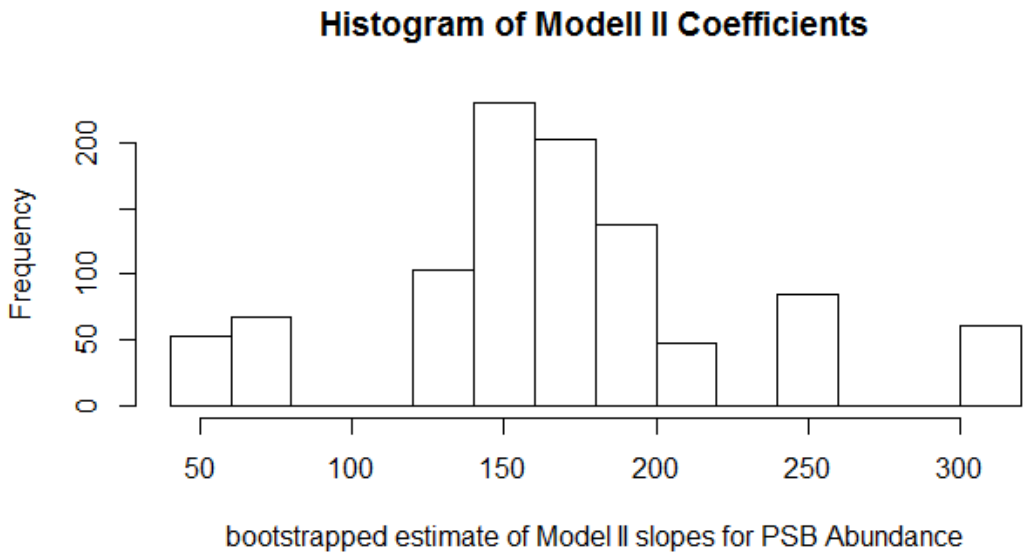


Figure 2.18. Histogram showing the bootstrapped result for PSB abundance for the Sparrow Lake core.

The bootstrapped estimate of the relationship between total reactive phosphorus and phosphate solubilizing bacteria for the Atherley Narrows core was 96.03, with 95% confidence intervals of 0-237.5. This result indicates that there was also a significant relationship between total reactive phosphorus and phosphate solubilizing bacteria for Atherley Narrows. Once again, the accompanying linear regression that was completed did not produce significant results (r^2=0.229, $F_{1,2}$=0.596, p=0.5206).

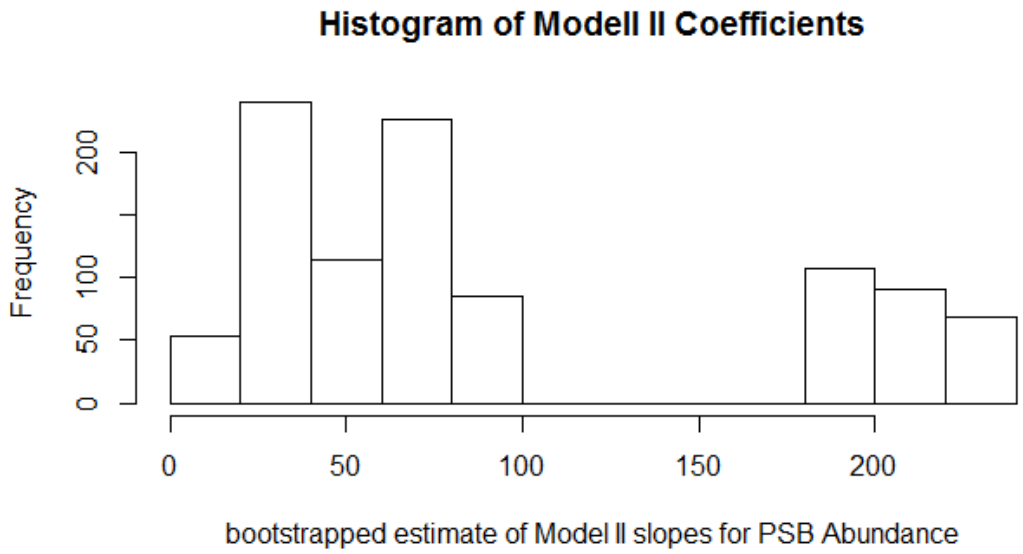


Figure 2.19. Histogram showing bootstrapped result for PSB abundance for the Atherley Narrows core. The bootstrapped data still appears to be binomial, even after bootstrapping.

2.3-4 Cook's Bay Study

The water temperatures at the Cook's Bay sites were 23.4°C, 24.4°C, and 22.5°C for sites one, two, and three respectively. The dissolved oxygen concentrations were 8.65mg/L at site one, 8.46mg/L at site two, and 8.49mg/L at site three. The pH levels at the sites were 7.65, 7.69, and 7.52 at sites one, two, and three respectively. The conductivity was 477µS/cm at site one, 506 µS/cm at site two, and 463 µS/cm at site three. The chlorophyll *a* concentrations were 1.19µg/L (SD ±0.617) at site one, 2.85µg/L (SD ±1.023) at site two, and 0.79µg/L (SD ±0.31) at site three. The concentration of total suspended solids was 1.53 mg/L (SD ±0.61) at site one, 7.93 (SD ±1.87) at site two, and 35.00 (SD ±21.94) at site three. There was 0.033mg/L (SD ±0.022) of total phosphorus at site one, 0.038mg/L (SD ±0.017) of total phosphorus at site two, and 0.03mg/L (SD ±0.014) at site three. Cook's Bay site one had a nitrate average of 0.017mg/L (SD ±0.011). These results are compared to the total phosphorus results from the Atherley Narrows sites in September in Table 2.7. Cook's Bay sites two and three had average total nitrogen concentrations of 0.49 (SD ±0.012), and 0.33mg/L (SD ±0.025) respectively.

Table 2.7. Total Phosphorus concentrations (mg/L) for Atherley Narrows and Cook's Bay, collected Sept 26th and 27th, 2017. Standard deviations are in parenthesis.

Site	Atherley Narrows (mg/L)	Cook's Bay (mg/L)
1	0.086 (±0.118)	0.033 (±0.033)
2	0.037 (±0.008)	0.037 (±0.017)
3	0.032 (±0.032)	0.03 (±0.014)

Cook's Bay site one had a mean total heterotrophic bacteria count of $8.08x10^3$ CFU/g (SD ±5826.56), site two had a mean heterotrophic bacteria count of $1.22x10^3$CFU/g (SD ±273.65), and site three had a mean heterotrophic bacteria count of $1.42x10^3$CFU/g (SD ±311.12). The Cook's Bay sites had a mean phosphate solubilizing bacteria count of $1.6x10^2$ CFU/g (SD ±141.42), $9.5x10^1$ CFU/g (SD ±20.5), and $7.1x10^1$ CFU/g (SD ±26.16) for sites one, two, and three respectively. The Cook's Bay and Atherley Narrows PSB abundances per site are listed in Table 2.8.

Table 2.8. Phosphate solubilizing Bacteria abundance (CFU/g) for Atherley Narrows and Cook's Bay. Collected Sept 26th and 27th, 2017. The standard deviations are in parenthesis.

Site	Atherley Narrows (CFU/g)	Cook's Bay (CFU/g)
1	$2.1x10^1$ (±9.5)	$1.6x10^2$ (±141.42)
2	$1.2x10^1$ (±1.07)	$9.5x10^1$ (±20.5)
3	$3.5x10^1$ (±15.58)	$7.1x10^1$ (±26.16)

2.3-4.1 Cook's Bay Statistical Analysis

A significant difference in PSB abundance was found between the Atherley Narrows and Cook's Bay sites ($F_{1,16}$=19.12, p=0.000473). Due to this, the null hypothesis is rejected, and the alternative hypothesis that there is a significant difference between the abundance of phosphate solubilizing bacteria between the northern and southern parts of Lake Simcoe is accepted. The boxplot of the raw data provides a visual representation of the distribution of the phosphate solubilizing bacteria and depicts which sites had the highest abundances (Figure 2.20).

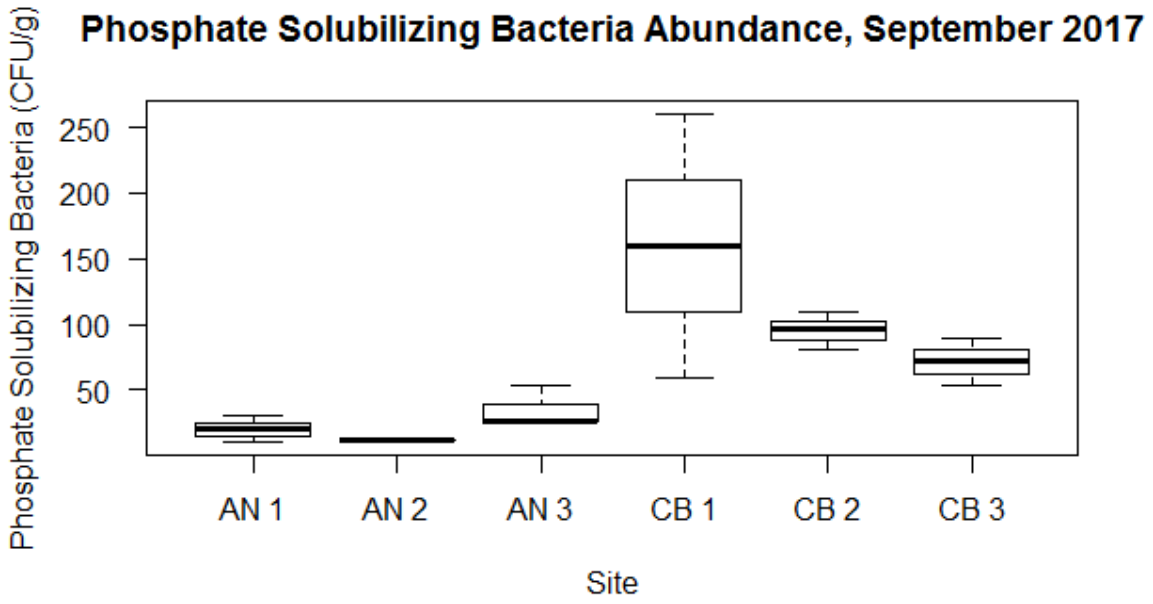


Figure 2.20. Boxplot graph showing the abundance of PSBs found in Lake Simcoe Atherley Narrows (AN 1, 2, &3) and in Cook's Bay (CB 1, 2, & 3) in September 2017. Note that the dark line in the center of each plot represents the median of the data while the upper and lower lines show the 95% confidence intervals.

The Anderson-Darling Normality test that was performed on the transformed data provides evidence that the $\log_{10}$ transformation did make the data normally distributed as the residual test had an insignificant result (A=0.4245, p= 0.2834). The Levene's test for homogeneity of variance also indicated that the variance between the sites was within an acceptable limit as well, as this residual test also had an insignificant result ($F_{5,12}$=0.8529, p=0.5388). These tests served the purpose to confirm that the data collected followed the assumptions of normality after the data was transformed.

Tukey's HSD test showed that there were statistically significant differences occurring between Atherley Narrows site 1 and Cook's Bay site 1 (p=0.001), Atherley Narrows site 1 and Cook's Bay site 2 (p=0.0054), Atherley Narrows site 1 and Cook's Bay site 3 (0.023), Atherley Narrows site 2 and Cook's Bay site 1 (p=0.00016), Atherley Narrows site 2, and Cook's Bay site 2 (p=0.00071), Atherley Narrows site 2 and Cook's Bay site 3 (p=0.0027), and Atherley Narrows site 3 and Cook's Bay site 1 (p=0.0148). These results are visually displayed in Figure 2.21.

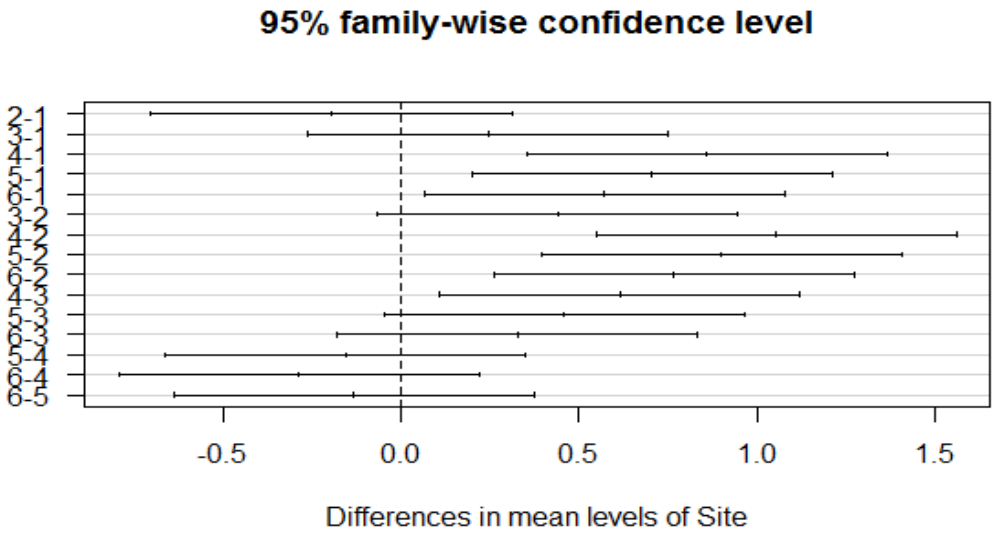


Figure 2.21. The results of the Tukey HSD test between all the sites sampled in September. 1= Atherley Narrows Site 1, 2= AN 2, 3=AN 3, 4= Cook's Bay 1, 5=Cook's Bay 2, 6= Cook's Bay 3. This graph shows that Atherley Narrows sites 1 and 2 are statistically different from all of the Cook's Bay sites.

2.4 Discussion

2.4-1 Hydrological Parameters

The significant relationships between the abundance of phosphate solubilizing bacteria and total heterotrophic bacteria, total suspended solids, and chlorophyll *a* that were observed in this study were similar to results observed in other studies (Gholizadeh, et al., 2016; Dodds & Whiles, 2010). Relationships between the previously mentioned hydrological parameters and phosphate solubilizing bacteria could indicate that these microbes thrive in similar conditions to microalgae and other heterotrophic microbes in the littoral zone of a lake. This theory is supported by various studies that also imply that temperature, redox reactions, pH, dissolved oxygen concentrations, nitrates, and sulphates have all been suggested as controlling factors that affect phosphorus release from sediments (Kim, et.al, 2003; Jin, et.al, 2006; Ribeiro, et.al,

2008). All of these factors also contribute to the density and species richness of microalgae (Pepper, et al., 2014).

It was observed that the highest abundance of bacteria that demonstrated phosphate solubilizing capabilities were collected from both sampling locations in July. As the water temperature was above 20°C at every site, this result correlates with those of previous studies. Mohammadi (2012) demonstrated that temperature was a growth limiting factor for phosphate solubilizing bacteria in terrestrial environments. As other studies (Qian et al., 2010; Manawadi, et al., 2016; and Paul & Sinha, 2017) used higher temperatures (30^{o}C) to grow PSBs in incubators, one would expect to see this group of mesophilic organisms to thrive at temperatures between 20-40°C. The laboratory component of this study explored further the role of temperature as a growth limiting factor of phosphate solubilizing bacteria.

2.4-2 Comparison Study

The low numbers of phosphate solubilizing bacteria that were found throughout this study were similar to the results of Sanjotha and Manawadi (2016). They collected 35 different phosphate solubilizing bacterial isolates between 15 sediment samples that were all collected at the same time from a coastal region (Sanjotha and Manawadi, 2016).

Both the three-factor ANOVA and the nested ANOVA were completed in this study in order to understand the abundance of phosphate solubilizing bacteria in the study area and their variability. Previous studies on phosphate solubilizing bacteria in freshwater systems did not involve compassion of two waterbodies differing in water quality and exposure to anthropogenic activities. The three-factor ANOVA analyzed the differences among sampling locations on each lake while the nested ANOVA tested any significance that might have existed between Sparrow Lake and Lake Simcoe (Quinn & Keough, 2002).

The significance observed in the Tukey's HSD test in the three-factor ANOVA between Atherley Narrows site 1, and Sparrow Lake site 1 was initially thought to support the hypothesis that phosphate solubilizing bacteria would be more abundant in areas that had obvious signs of anthropogenic stress. In Atherley Narrows site 1, filamentous algae and anthropogenically produced trash were present (there was a tire, plastic bags, plastic water bottles, and other

particles of trash in the sediment). Sparrow Lake site 1 was located within McLean Bay, which is a part of the protected wetland within Sparrow Lake (The District Municipality of Muskoka, 2016). However, there was a higher abundance of phosphate solubilizing bacteria at Sparrow Lake site 1 as opposed to Atherley Narrows site 1. This disproves the hypothesis that the opposite would be true due to the impact that the Orillia wastewater treatment facility and the Orillia landfill may have had on that location. The insignificant result observed in the nested ANOVA suggests that the differences observed between the two lakes was random, and more testing would have to be completed to make any significant conclusions.

The high abundance of phosphate solubilizing bacteria in Sparrow Lake in June 2017 could potentially be explained by the weather that occurred the week preceding June 25th when sampling took place. According to the historical weather data collected at the Muskoka Airport, 19.1mm of precipitation accumulated in the region between June 19th-June 25th, 2017; while 22mm of precipitation accumulated in the region between July 16th -23rd (Pelmorex Weather Networks,2017). The historical data collected in Orillia has no precipitation recorded between June 18th-24th, 2017, nor between July 15th-22nd (Pelmorex Weather Networks,2017). As the water temperature ranged between sites (in both lakes) from 18.8-22.3°C in June, and 20.6-24.4°C in July, the amount of precipitation could be one of the explanations for the increased phosphate solubilizing bacteria abundances in those months. As previously mentioned, the study completed by Mohammadi (2012) goes into detail about how temperature is a key growth factor for the presence and abundance of phosphate solubilizing bacteria.

The variation in precipitation could have also played an impact on the slightly negative correlation that was observed between total phosphorus (mg/L) and the abundance of phosphate solubilizing bacteria in Sparrow Lake. Both total phosphorus concentrations and phosphate solubilizing bacteria abundance saw a decreasing trend with time. The precipitation in June and July could have impacted the total phosphorus concentrations in the lake as well (Dodds & Whiles, 2010). While there were no studies explicitly discussing the impact that precipitation has on the presence of phosphate solubilizing bacteria, it is well documented that

runoff caused by lots of precipitation can lead to higher levels of nutrients in freshwater systems and the higher amount of available nutrients can contribute to an increase in microorganism populations (Dodds & Whiles, 2010 ; Pepper et al., 2014; Conley et al., 2009; Dillon & Rigler, 1974).

2.4-3 Core Experiment

The results that were obtained in this study were similar to those of Sanjotha and Manawadi (2016). Sanjotha and Manawadi (2016), had a low abundance of phosphate solubilizing bacteria and collected their samples randomly from sediment depths between 5-17cm. This current study tried to enumerate the phosphate solubilizing bacteria in sediment cores of similar depth range (5-20cm). However, raw results obtained from this quick experiment support the insignificant result obtained from the bootstrapped test. It can be seen in tables 2.5 and 2.6, that the mean amount of phosphate solubilizing bacteria did not change much within the first 20cm of sediment. If this study was expanded upon in future, it would be useful to collect larger sediment cores to assess the abundance of bacteria well into the sediment; more sediment core replicates would be needed as well as to get a better assessment of the results.

2.4-4 Cook's Bay Comparison

The comparison between the Cooks Bay and Atherley Narrows sites on Lake Simcoe was completed as another experiment designed to try to determine if the abundance of phosphate solubilizing bacteria could be correlated with the amount of total phosphorus that was present in the water column at the sampling site. The intent was to complement the study that compared the abundance of phosphate solubilizing bacteria between Lake Simcoe-Atherley Narrows and Sparrow Lake. Atherley Narrows recorded the lowest levels of total phosphorus within the entire lake while Cook's Bay recorded the highest (Young & Jarjanazi, 2015). Knowing that historically Cook's Bay had the highest levels of total phosphorus, it was hypothesized that Cook's Bay would have a higher abundance of phosphate solubilizing bacteria and that these microorganisms contributed to the elevated levels of total phosphorus in the area (Nürnberg et al., 2013). During the week prior to sampling, no precipitation was recorded in Orillia, ON, while only 0.1mm of precipitation was recorded in Newmarket, ON (Pelmorex Weather

Networks,2017) which indicated that precipitation might not have had a significant influence over the phosphate solubilizing bacteria abundance that was observed.

However, during the Atherley Narrows sampling period, there was a northward wind stirring up the sediment along the shoreline where the sampling sites were located, this would explain why the total suspended solids, and chlorophyll *a* results were higher during the September sampling event than they were throughout the rest of the sampling events in this location. Due to this anomaly, these results were omitted from statistical analysis due to their high levels of variance (Quinn & Keough, 2002). It is plausible that the differences caused by these meteorological conditions could be one of the contributing factors that resulted in the difference in phosphate solubilizing bacteria abundances between the Cook's Bay and Atherley Narrows sites.

A higher abundance of phosphate solubilizing bacteria was observed in Cook's Bay than in Atherley Narrows while total phosphorus levels remained relatively consistent. The total phosphorus levels were consistent apart from Atherley Narrows Site 1 which was located closest to the Orillia wastewater treatment plant. This site had the lowest levels of total phosphorus in September (0.019 mg/L) and was just below the provincial limit of < 0.02mg/L of total phosphorus in the wastewater effluent (LSRCA, 2017).

The isolates were collected following the same procedure as Sanjotha and Manawadi, (2016), and Qian et al., (2010), and although the results were similar to those observed in previous studies, many specific questions relating to Lake Simcoe were left unanswered. A more intense study on Lake Simcoe would need to be done to determine if phosphate solubilizing bacteria contribute to the internal phosphorus loading in this lake. As only aerobic, heterotrophic bacteria were isolated in this study it is likely that a significant number of microbes were missed in the collection process. Species richness was not calculated at this time as various heterotrophic microbes grew on the Pikovskaya's agar that were not phosphate solubilizing bacteria.

2.5 Conclusion

In general, higher abundances of phosphate solubilizing bacteria were observed after weather events, and this could be the response this group of microbes have to the land runoff. This requires further study. Generally, phosphate solubilizing bacteria were found more abundant in warm water when there was not an excessive amount of total phosphorus in the water column. This could imply that the locations that had lower amounts of total phosphorus are better suited for phosphate solubilizing bacteria. Due to the inorganic acids that this group of microorganisms possess, it does make sense that they would thrive in locations that did not have a bioavailable form of phosphorus (Qian et al., 2010).

The results obtained by the multiple regression analysis support the alternative hypothesis that at least one of the hydrological parameters observed throughout the sampling period had a significant influence on the abundance of phosphate solubilizing bacteria in both Sparrow Lake and Lake Simcoe. Total suspended solids (TSS), chlorophyll *a*, water temperature and total heterotrophic bacteria (THB) abundance were statistically proven to have the lowest variance inflation factors, and due to this, had the most substantial impact on the abundance of the phosphate solubilizing bacteria. A more in-depth study would be needed to determine the impacts that seasonality may have had on the abundance of phosphate solubilizing bacteria in the studied sites. More vigorous sampling for an extended period will help to limit the sources of error that occurred due to precipitation would be needed to omit some of the sources of error that were observed during this study.

The results obtained in the comparison study lead to the rejection of the hypothesis that a higher abundance of phosphate solubilizing bacteria would be found in the Atherley Narrows rather than in Sparrow Lake. While a significant difference was observed between Atherley Narrows site 1 and Sparrow Lake site 1, Sparrow Lake site 1 had the higher abundance of PSBs, which is not what was hypothesized. Due to the proximity to the Orillia Wastewater Treatment Plant, and Orillia Landfill, it was expected that Atherley Narrows Site 1 would have the highest abundance of PSBs, this was not what was observed. Sparrow Lake site 1 was found to have a significantly higher abundance of phosphate solubilizing bacteria when compared to Lake Simcoe Atherley Narrows Site 1.

More sampling would be required in the core experiment in order to determine if the insignificant relationship in the linear regression that was performed had enough statistical power to be a viable result. There was little difference in the abundance of phosphate solubilizing bacteria and the total reactive phosphorus between the sections of the cores. There did not seem to be much difference between the cores collected at each lake. However, a more robust data set would be needed in order to determine if this was significant or not.

Cook's Bay was found to have a significantly higher abundance of phosphate solubilizing bacteria than the Atherley Narrows sites when they were both sampled in September. However, only Atherley Narrows sites 1 and 2 had a statistically significantly lower abundance of phosphate solubilizing bacteria than the three Cook's Bay sites. The total phosphorus concentrations were reasonably consistent between the six sites that were sampled on Lake Simcoe in September.

Chapter 3: Testing the phosphate solubilizing capabilities and efficiencies of bacterial isolates from two lakes.

Chapter 3 Abstract

Measuring a bacterial isolate's ability to solubilize inorganic phosphate is a common way to characterize different phosphate solubilizing bacteria (PSB). The most common test completed to estimate the phosphate solubilization efficiencies are the solubilization index measurements. The tests were completed on the bacterial strains (435 isolates) that showed phosphate solubilizing capabilities that were isolated from the water and sediment samples from Lake Simcoe and Sparrow Lake. Of those 435 isolates, only 394 grew once they were transferred to Pikovskaya's agar slants. These were the isolates that were used for the phosphate solubilization index test. The average colony and halo diameter were measured every 24hrs for 9 days, and the solubilization index was calculated by using the Edi-Premono et.al (1996) formula. A total of 98 isolates showed halo development throughout this test. The 60 isolates that had the highest solubilization index numbers were used in the inorganic phosphate test. The isolates were grown in Pikovskaya's broth in triplicate for 72 hours with sterilized uninoculated broth serving as the control. The cultured broth was sterilized and filtered, and the phosphate concentration was measured using the spectrophotometric method. Only 20 of the tested isolates showed phosphate reducing capabilities during this experiment. Of these isolates, statistically significant (p<0.05) differences between the phosphate reducing capabilities of each isolate were observed, specifically between isolates 196 and 198, both of which originated from Lake Simcoe.

3.1 Introduction

The methods used to screen phosphate solubilizing bacteria from soil were applied to aquatic environments. Studies completed by Zhou et al. (2011), and Paul & Sinha (2017) both focused on the isolation, characterization, and identification of phosphate solubilizing bacteria in freshwater environments. Both of these studies collected water and sediment samples from their respective locations. After the isolates had been transported back to the lab, they were screened on Pikovskaya's agar plates which uses tri-calcium phosphate as the inorganic

phosphate source. Paul and Sinha (2017) then used a solubilization index test and an inorganic phosphate test in order to quantitatively estimate the phosphate solubilizing capabilities of each isolate that exhibited the ability to use inorganic phosphate (this was noted by the visual presence of a halo or clear zone around the colony on the Pikovskaya's media).

Water and sediment samples were collected from two sampling locations from June to September 2017 (a third location (Cook's Bay) was only sampled in September). Three replicate samples were collected from each sampling site (Sparrow Lake, Atherley Narrows (Lake Simcoe), and Cook's Bay (Lake Simcoe)) during each sampling event. Five representative colonies were isolated from each replicate, leading to a total of 15 isolates from each site every time a site was sampled. Thus, altogether 480 representative colonies were isolated from the sediment and water samples between June and September 2017. A colony was considered to be a representative if two or more morphologically similar ones were present on the Pikovskaya's agar plates when grown for seven days at 30^{o}C. The presence of a clear zone surrounding the microbial colony was another mandatory criterion followed, as this was the indication of the phosphate solubilizing capability of the microorganism (Chen et al., 2006). In instances where there were fewer than five different types of colonies that had a clear zone or halo, a duplicate of the most common type was isolated. The rationale behind this was to continue to have equal sample sizes for statistical analysis.

The phosphate solubilization test was followed by a test which measured the concentration of total phosphorus that was left in Pikovskaya's broth after the phosphate solubilizing bacteria had grown in the broth for 72 hours (Mehta & Nautiyal, 2001). The 60 bacterial isolates that had the highest average solubilization index number were selected for this test (the solubilization index for each of the isolates was calculated using the Edi-Premono et al. (1996) formula). The rationale behind phosphate solubilization and inorganic phosphate tests was to screen the phosphate solubilizing bacteria that were collected from the three sampling locations. These tests were completed in an attempt to determine which of the isolates were the most efficient ones at reducing the inorganic phosphate that was found in the Pikovskaya's media (yeast extract 0.5g/L, dextrose 10g/L, $Ca_3(PO_4)_2$ 5g/L, $(NH_4)_2SO_4$ 0.5g/L, KCl 0.2g/L, $MgSO_4$ 0.1g/L, $MnSO_4$ 0.0001g/L, and $FeSO_4$ 0.0001g/L and 15 g/L agar).

The objectives of these tests were to monitor the rate of phosphate solubilization of the phosphate solubilizing bacteria as well as to test them to see which of the phosphate solubilizing bacteria are the most efficient at releasing phosphorus. It was hypothesized that the isolates collected from the Lake Simcoe Atherley Narrows sites would be the most efficient at phosphate solubilization in the media as there were typically higher concentrations of total phosphorus in the water column in those locations.

3.2 Materials and Methods

3.2-1 Phosphate Solubilization Index Test

The methodology for the phosphate solubilization index test was taken from Sanjotha and Manawadi (2016), and Paul and Sinha (2017). This test was used as it is a straightforward way to estimate the abilities of the phosphate solubilizing bacteria. Of the 480 isolates, the first 45 isolates were isolated from Lake Couchiching, but since that lake was not able to be tested after June due to time restraints, those isolates were omitted from testing. Out of the 435 isolates, 394 isolates that were healthy and growing well in the Pikovskaya's agar slants were finally used for the phosphate solubilization index test.

Individual Pikovskaya's agar plates were made for each isolate. A circle with a diameter of 6mm was drawn in the center of each Pikovskaya's agar plate to ensure that the inoculation site was the same for each bacterium. The inoculated plates were inverted and incubated at 30°C (±2°C) for 216 hours (Paul & Sinha, 2017). After the bacteria had been in the incubator for 48 hours, the average diameter of the colony and any halo formation was measured daily (every 24 hrs) up to 216 hrs (9 days) using the Edi-Premono et al. (1996) formula:

$$SI = \frac{(CD + HD)}{CD}$$

Where:SI= Phosphate Solubilization Index
CD= Colony Diameter (mm)
HD= Halo Diameter (mm) (Paul & Sinha, 2017).

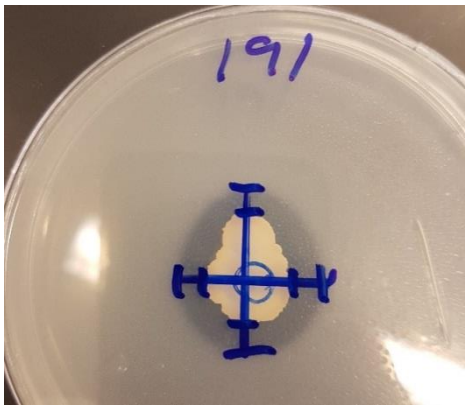


Figure 3.1. Example of the measurements taken to get the SI number for each isolate. The culture was inoculated inside the 6mm circle while the lines going to the edge of the colony and the halo indicate where the measurements were taken for the colony and halo.

Each bacterial colony and halo were measured twice, and an average was taken to determine both the colony diameter and the halo diameter. Sixty-one isolates (29 from Lake Simcoe-Atherley Narrows, 29 from Sparrow Lake, and 3 from Lake Simcoe-Cook's Bay) that had the largest average solubilization index number during the phosphate solubilization index test were selected to continue with the next screening test. The isolates were selected based on how quickly they began to solubilize the calcium phosphate, and how high their solubilization index number was after the 216-hour timeframe. This was determined by the measurements that were taken once every 24 hours during the testing period (when the isolates were between 48 and 216 hours old).

3.2-2 Inorganic Phosphate Test

The sixty isolates that showed the highest solubilization index (28 from Atherley Narrows - the 23 isolates that formed halos from this lake plus 5 isolates that had a clear ring around the isolate that was too small to measure; 29 from Sparrow Lake; and 3 isolates from Cook's Bay) were selected for the second screening test. Eight of the isolates were from June (2 from Lake Simcoe, and 6 from Sparrow Lake), 15 from the July isolates (8 from Lake Simcoe, 7 from Sparrow Lake), 16 from the August isolates (10 from Lake Simcoe, 6 from Sparrow Lake), and 22 from the isolates collected in September (9 from Lake Simcoe-Atherley Narrows, 10 from Sparrow Lake, and 3 from Cook's Bay). All isolates were grown in Pikovskaya's broth (yeast extract 0.5g/L, dextrose 10g/L, $Ca_3(PO_4)_2$ 5g/L, $(NH_4)_2SO_4$ 0.5g/L, KCl 0.2g/L, $MgSO_4$ 0.1g/L,

$MnSO_4$ 0.0001g/L, and $FeSO_4$ 0.0001g/L) for 24 hours as mentioned in Mehta and Nautiyal (2001). After this time, the test tubes were vortexed for 10seconds, and 50µl of inoculum of the young culture was transferred into 10ml of sterile Pikovskaya's broth in triplicate. Autoclaved uninoculated broth was used as control (Mehta & Nautiyal, 2001; Islam et al., 2007). The cultures were incubated for 72 hours at 30°C on a shaker plate set at 180rpm as per Mehta and Nautiyal (2001). After this time, the colonies were destroyed (autoclaved at 121°C for 90 minutes), and then filtered through a 47mm (0.45µm pore size) membrane filter under vacuum (Islam et al., 2007).

The filtered, sterile broth was used to measure the phosphate concentration by spectrophotometric method (Mehta & Nautiyal, 2001). Sterile deionized water was added to any samples that had less than 10ml of broth to complete this assay. One Hach Phos Ver 3 pillow was added to each sample. The samples were then shaken for 15 seconds (APHA, 1995). 3ml of this solution was pipetted into an acid washed cuvette, and the absorbance was measured in the spectrophotometer at 880nm. The samples were compared to the previously made standard curve via linear regression to get the amount of total phosphorus in micrograms per millilitre (APHA, 1995). This method is still efficient at measuring the inorganic phosphate that was present in the broth media as tri-calcium phosphate is the only source of phosphorus in the Pikovskaya's broth.

3.2-3 Statistical Analyses

3.2-3.1 Phosphate Solubilization Index Test

The statistical program R version 3.4.3 (R Core Team, 2017) with the packages 'nortest' (Gross & Ligges, 2015) and 'car' (Fox & Weisberg, 2011) was used to perform a repeated measures analysis of variance (ANOVA) to statistically compare the solubilization index from all of the representative isolates that visibly utilized the calcium phosphate that was in the Pikovskaya's agar. Time, in hours (observation time, i.e., from 48 hrs to 210 hrs at every 24-hr interval), was used as the repeated measure while the isolates sampled were used as the fixed effect. The algebraic model used for the repeated measures ANOVA was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + [(\alpha\beta)]_{ij} + \varepsilon_{ijk}$$

Where:
μ= the solubilization index
α_i = the bacterial isolate
β_j = time (hours)
[αβ]= the random interaction between the isolates and time in hours (it is assumed to be zero)
ε_{ijk} = random error (Quinn & Keough, 2002).

An Anderson Darling Normality Test conducted on the data revealed the Solubilization Index test data was not normally distributed (A= 2.7608, p= 5.87×10^{7}). Transforming the data did not resolve this issue, and therefore Friedman's non-parametric test was used. The algebraic model used for the Friedman's test was:

$$F_r = \frac{12}{ba(a+1)} \sum R_i^2 - 3b(a+1)$$

Where:

a=no. of treatments
b=no. of blocks(time(hours))
$\sum R_i^2$= group rank sum over all isolates(Quinn & Keough, 2002).

The following hypotheses were tested: H_0: Time (hours) did not have a significant impact on the solubilization index of the tested isolates. H_A: Time (hours) did have a statistically significant impact on the tested isolates.

3.2-3.2 Inorganic Phosphate Test

The statistical program R version 3.4.3 (R Core Team, 2017) with the packages 'nortest' (Gross & Ligges, 2015), 'car' (Fox & Weisberg, 2011), and 'agricolae' (De Mendiburu, 2017) was used to perform a one-way analysis of variance (ANOVA) in order to determine if statistically significantly different results were observed between the isolates that showed reductions in the amount of phosphate present in the broth after the experiment compared to the control (initial concentration of phosphate). The isolates were the independent variable, while total phosphorus (μg/L) readings were used as the dependent variable. The algebraic model used for the one-way ANOVA was:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

Where:

μ= Total Phosphorus concentration (μg/L)

α_i = the bacterial isolate

ε= random error (Quinn &Keough, 2002).

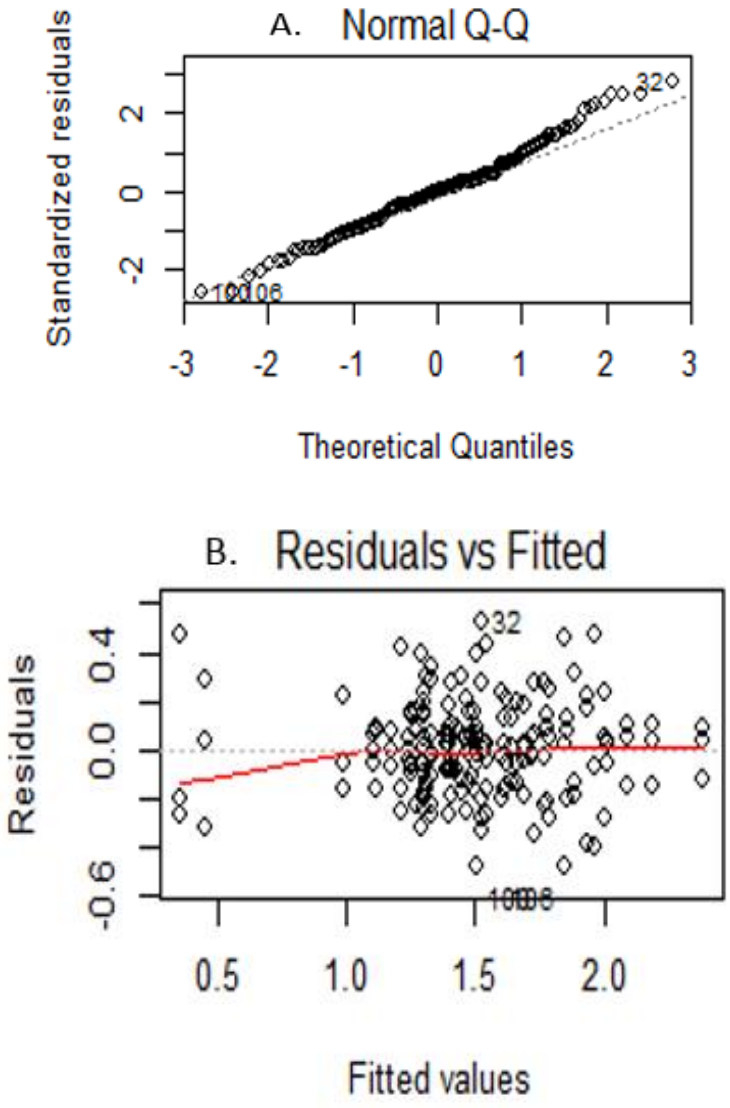


Figure 3.2. Diagnostic plots illustrating that the raw data meet assumptions of normality. Plot A shows how normally distributed the data is while Plot B shows that there are no patterns to the residual data plots.

The statistical hypotheses tested were H_o: the means of the total phosphorus values for the tested isolates were not significantly different. H_A: At least two isolate means were

significantly different from each other. The Anderson Darling normality test and Levene's test for homogeneity of variance were conducted on the data used for the one-way ANOVA. These tests revealed that the data met the assumptions of normality and homoscedasticity. No transformations were needed in order to interpret the results. Fisher's least significant difference (LSD) test was completed as the post-hoc test as this test allowed for the comparison between isolates, highlighting which isolates were significantly different from each other.

3.3 Results

3.3-1 Phosphate Solubilization Index Test

As the solubilization index was a rank-based test performed on every isolate that appeared to develop an area of solubilization upon extraction from the sediment, and not every isolate that was screened developed a zone of inhibition, not every isolate was given a solubilization index (SI) number. These isolates, along with some of the isolates that had a solubilization index of 1.5 or less after 216 hours were eliminated from statistical analysis. This parameter allowed for a slightly smaller sample size and a more robust data set, leading to more impactful results. The data was further split according to which sampling location the isolates originated from in an attempt to remove variance that may have occurred due to differences in habitat conditions. The complete data set from the 480 isolates that underwent the solubilization index test is given in Appendix II. However, Table 3.1 shows the colony and halo averages and standard deviations that were used to calculate the solubilization index for the halo forming isolates.

Table 3.1. Colony and halo diameter measurements for each halo forming isolate in millimetres with standard deviations. The measurements were completed in duplicate, and the average was used to calculate the solubilization index. These were the measurements for all of the halo forming isolates that had been growing for 216 hours.

ISOLATE NUMBER	COLONY AVG. (MM)	COLONY S.D.	HALO AVG. (MM)	HALO S.D.	S.I.
49	16.5	0.71	31.5	0.71	2.90
51	15.5	0.71	28.5	0.71	2.84
52	9.5	0.71	12	1.41	2.26
55	15.5	3.53	27	1.41	2.74
57	15	1.41	22.5	0.71	2.5

59	12	2.83	19	1.41	2.58
75	29.5	20.50	46.5	12.02	2.58
77	18	0	24.5	0.71	2.36
86	15.5	4.95	22.5	3.53	2.45
93	12.5	0.71	17.5	0.71	2.4
121	11.5	0.71	28	0	3.43
122	31	24.04	34.5	20.51	2.11
123	15	2.83	22	1.41	2.47
124	9	1.41	16.5	0.71	2.83
126	11	1.41	35.5	4.95	4.23
128	12	2.83	16.5	2.12	2.37
129	11	0	25.5	0.71	3.32
143	10	2.83	12.5	0.71	2.25
149	7.5	2.12	12.5	2.12	2.77
156	15	1.41	17	1.41	2.13
160	10.5	0.71	17.5	0.71	2.77
161	15.5	7.78	16	5.66	2.03
178	13	1.41	16.5	0.71	2.37
179	9	0	12.5	0.71	2.39
189	17	2.83	30.5	2.12	2.79
191	15.5	2.12	24	1.41	2.54
196	3	0	14.5	0.71	5.83
198	6	1.41	18.5	0.71	4.08
216	15	0	19.5	0.71	2.3
217	14	0	20.5	0.71	2.46
219	6.5	0.71	15.5	2.12	3.38
220	7.5	2.12	16	0	3.13
243	10.5	4.95	17.5	0.71	2.66
244	10.5	0.71	15.5	0.71	2.47
250	9.5	2.12	19	1.41	3.00
255	13	2.83	20.5	2.12	2.58
256	11	1.41	20	2.83	2.81
277	7.5	0.71	11	0	2.47
283	6.5	0.71	10.5	0.71	2.61
285	16.5	0.71	18	1.41	2.09
287	11	0	16	1.41	2.45
288	14.5	0.71	16.5	0.71	2.14
289	15.5	3.53	17	2.83	2.09
302	27	24.04	41	18.381	2.52
303	13.5	2.12	19.5	0.71	2.44
304	9.5	0.71	25	0	3.63
305	8.5	0.71	21.5	0.71	3.53
307	8	2.83	13.5	0.71	2.69
307	21.5	6.36	24	0	2.12

308	13.5	0.71	19	1.41	2.41
312	9.5	0.71	16.5	0.71	2.74
316	14.5	2.12	19.5	0.71	2.34
318	17	1.41	20	0	2.18
325	16.5	3.53	21.5	4.95	2.30
328	12	4.24	15	2.83	2.25
332	9	1.41	13.5	0.711	2.5
335	15	2.83	19.5	2.12	2.3
337	13.5	0.71	26.5	3.53	2.96
338	17	0	35.5	0.71	3.08
343	11	0	13.5	0.71	2.23
345	15	2.83	19	1.41	2.26
348	13.5	3.54	18	2.83	2.33
363	12	1.41	14	2.83	2.16
364	12	1.41	15	1.41	2.25
366	14	1.41	16.5	0.71	2.17
374	14	4.24	16.5	2.12	2.17
376	16	2.83	18.5	2.12	2.15
377	11	1.41	14	2.83	2.27
381	11.5	0.71	15	0	2.31
386	9.5	0.71	12.5	0.71	2.31
390	10.5	2.12	11	1.41	2.04
391	13	1.41	14	0	2.08
398	8	2.83	10	0	2.25
399	7	0	10.5	0.71	2.5
400	8	2.83	11.5	0.71	2.44
403	11	1.41	15.5	2.12	2.41
408	7.5	2.12	21.5	0.71	3.86
410	12.5	2.12	37	0	3.96
411	8.5	2.12	31.5	2.12	4.71
412	16.5	0.71	34.5	2.12	3.09
414	11.5	0.71	28	1.41	3.43
415	15.5	7.78	27	5.66	2.74
417	14	2.83	30	2.83	3.14
422	24	1.41	25.5	0.71	2.06
423	16.5	2.12	19	1.41	2.15
425	23	2.83	23.5	0.71	2.02
426	14.5	7.78	20.5	0.71	2.41
427	15.5	0.71	17.5	2.12	2.12
429	15.5	2.12	37.5	0.71	3.42
430	21.5	2.12	24.5	2.12	2.14
432	10.5	0.71	13.5	0.71	2.28
436	9	1.41	11	1.41	2.22
440	9	0	10.5	0.71	2.16

441	9	4.24	12.5	3.53	2.38
443	10	1.41	14.5	0.71	2.45
444	13.5	0.71	16	0	2.18
445	14.5	0.71	16	0	2.10
446	16.5	0.71	16.5	0.71	2
451	17	2.83	19	4.24	2.12
452	7.5	2.12	17	1.41	3.26
453	12	2.83	14.5	3.53	2.21
454	16	0	17	0	2.06
456	15	0	16.5	0.71	2.1
459	13	4.24	17	2.83	2.31
461	12.5	3.53	13.5	2.12	2.08
462	14	1.41	15	0	2.07
464	15	1.41	18	1.41	2.20
466	17.5	2.12	21.5	0.71	2.23
467	19.5	0.71	16.5	2.12	1.85
470	9	0	13.5	0.71	2.5
473	10	0	11	0	2.1
475	11.5	2.12	17.5	2.12	2.52
477	10.5	0.71	12.5	2.12	2.19
478	14	1.414	17	0	2.21
481	14.5	3.53	16.5	4.95	2.14
483	11.5	2.12	14.5	0.71	2.26
486	11.5	0.71	13.5	0.71	2.17

It was observed that the largest number of bacterial isolates that were capable of solubilizing the inorganic phosphate in the Pikovskaya's agar plates were collected from Sparrow Lake with 68 halo-forming isolates. Only 23 isolates formed halos during the solubilization index test from Atherley Narrows while Cook's Bay had 7 halo-forming isolates when it was sampled in September. The five isolates that had the largest solubilization index number at the end of 216 hours were 196, 411, 126, 198, 410 with the respective solubilization index numbers being 5.833, 4.705, 4.227, 4.083, and 3.96. The rest of the isolates can be found in Appendix II. Isolate 196 came from the water sample from Atherley Narrows site 1 in July, isolate 411 came from Sparrow Lake site 1 in September, isolate 126 came from Sparrow Lake site 1 in July, isolate 198 came from the water sample from Atherley Narrows site 1 in July, and isolate 410 came from Sparrow Lake site 1 in September. Meanwhile, isolate 467 which came

from Atherley Narrows site 1 in September, had the lowest solubilization index number out of the isolates that were included in the statistical analysis (1.846).

The null hypothesis was rejected for the Atherley Narrows isolates as the Friedman rank sums test determined that time of observation did have a significant impact of the solubilization index of the isolates ($\chi^2_6 = 62.756, p = 1.238\text{x}10^{11}$). The non-parametric post-hoc Nemenyi's rank sums test was completed to determine which time frames showed significant differences. Significant observations were made between measurements taken at 48 hours and 120 hours (p=0.00026), 48 hours and 144 hours (p= $4.3\text{x}10^{05}$), 48 hours and 168 hours (p = $1.5\text{x}10^{05}$), 48 hours and 216 hours (p =$2.7\text{x}10^{07}$), 72 hours and 120 hours (p =0.00683), 72 hours and 144 hours (p= 0.00156), 72 hours and 168 hours (p =0.00065), 72 hours and 216 hours (p = $2.1\text{x}10^{05}$), and 96 hours and 216 hours (p = 0.00775). Nemenyi's rank sum test results can be seen in Table 3.2. Growth rate trends between the isolates from Atherley Narrows that had the highest SI numbers can be seen in Figure 3.3.

Table 3.2. Pairwise comparisons of each timeframe tested on the Atherley Narrows isolates using Nemenyi rank sums test with q approximation for un-replicated blocked data. There was no *p*-value adjustment method. The significant results are mentioned in the text.

	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs	168 hrs
72 hrs	0.98	n/a	n/a	n/a	n/a	n/a
96 hrs	0.32	0.82	n/a	n/a	n/a	n/a
120 hrs	0.01	0.01	0.29	n/a	n/a	n/a
144 hrs	4.3^{05}	0.01	0.13	0.99	n/a	n/a
168 hrs	1.5^{05}	0.01	0.07	0.99	0.99	n/a
216 hrs	2.7^{07}	2.1^{05}	0.01	0.84	0.96	0.99

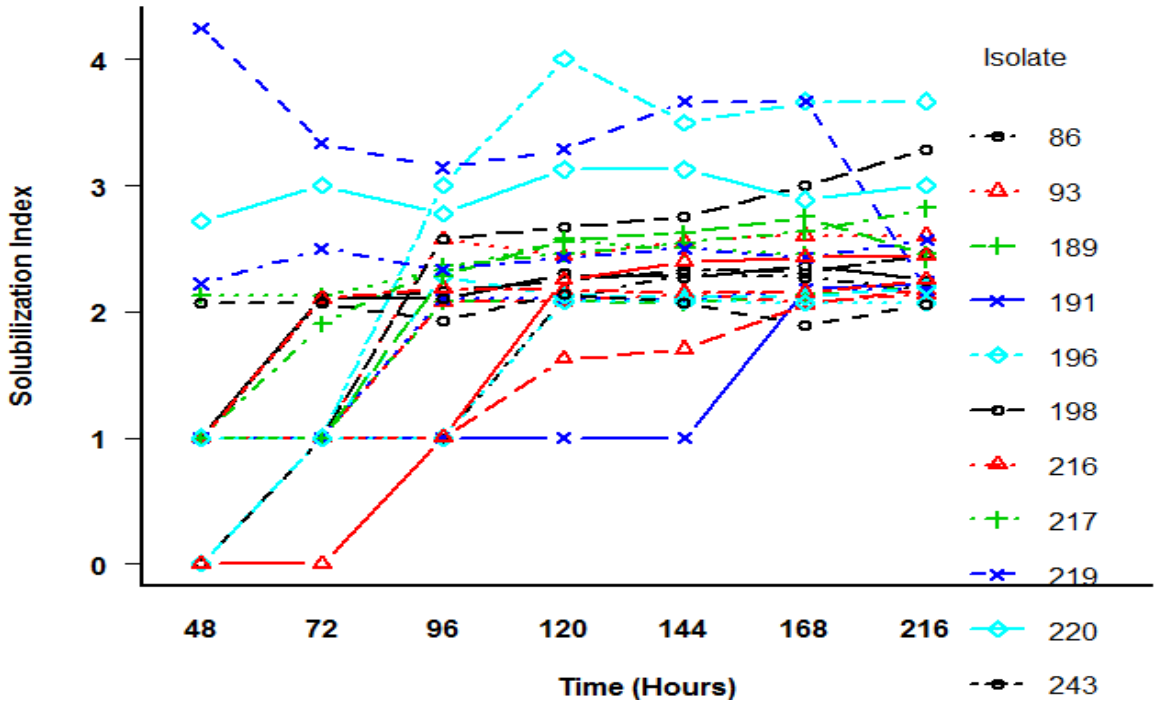


Figure 3.3. This interaction plot is showing the growth rates of the 11 isolates from the Atherley Narrows that had the highest solubilization Index after 216 hours.

The null hypothesis was also rejected for the Sparrow Lake isolates as the Friedman rank sums test determined that observation time also had a significant impact on the solubilization index of these isolates ($\chi^2_6 = 111.4$, $p = 2.2\text{x}10^{16}$). Nemenyi's rank sums test was used once again as the post-hoc test. Significant differences were once again seen between measurements taken at 48 hours and 120 hours (p=0.00351), 48 hours and 144 hours (p= $1.8\text{x}10^{05}$), 48 hours and 168 hours (p = $4.8\text{x}10^{10}$), 48 hours and 216 hours (p =$6.8\text{x}10^{013}$), 72 hours and 120 hours (p =0.01611), 72 hours and 144 hours (p= 0.00014), 72 hours and 168 hours (p =$8\text{x}10^{09}$), 72 hours and 216 hours (p = $1.6\text{x}10^{11}$), 96 hours and 168 hours (p= 0.00193), 96 hours and 216 hours (p = $3.2\text{x}10^{05}$), and 120 hours and 216 hours (p=0.00218). Table 3.3 shows the Nemenyi's rank sums

test results. Figure 3.4 shows these trends observed between eight representative isolates from Sparrow Lake.

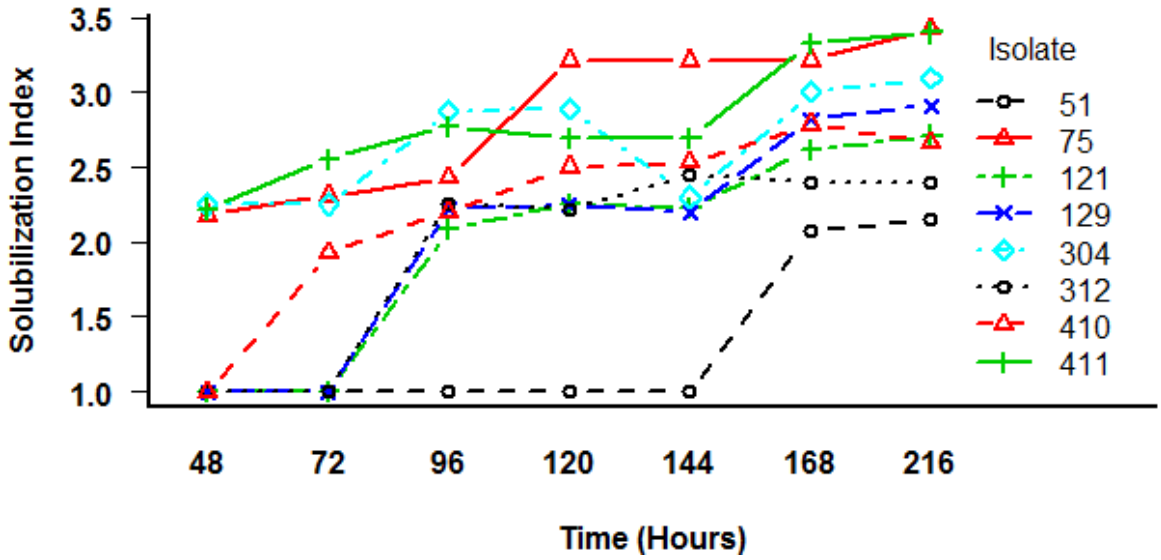


Figure 3.4. The interaction plot is showing the growth rates of eight representative halo forming isolates from Sparrow Lake. This plot allows for the comparison of how the isolates grew compared with each other over the same timeframe. Isolates 411 and 410 had the highest Solubilization Index after 216 hours.

Table 3.3 Pairwise comparisons of each timeframe tested on the Sparrow Lake isolates using Nemenyi rank sums test with q approximation for un-replicated blocked data. There was no *p*-value adjustment method. The significant results are mentioned in the text.

	48 Hrs	72 Hrs	96 Hrs	120 Hrs	144 Hrs	168 Hrs
72 Hrs	0.99	n/a	n/a	n/a	n/a	n/a
96 Hrs	0.07	0.21	n/a	n/a	n/a	n/a
120 Hrs	0.01	0.02	0.96	n/a	n/a	n/a
144 Hrs	1.8^{05}	0.01	0.33	0.89	n/a	n/a
168 Hrs	4.8^{10}	8.0^{09}	0.01	0.05	0.57	n/a
216 Hrs	6.8^{13}	1.6^{11}	3.2^{05}	0.01	0.11	0.97

3.3-2 Inorganic Phosphate Test

At the end of the experiment, isolates such as 244, 196, 198, 243, and 149 all had lower concentrations compared to the control (Table 3.4). The difference in phosphate concentration between the control and the above isolates were 1.46, 1.12, 1.02, 0.49, and 0.36 mg/L respectively. Isolate 149 originated from Sparrow Lake site 2 in July, isolates 196 and 198 originated from the water sample collected at Atherley Narrows site 1 in July and isolates 243 and 244 originated from Atherley Narrows site 1 in August. All results from the insoluble phosphate test can be seen in Table 3.4.

The alternative hypothesis was accepted for the one-way ANOVA conducted for the inorganic phosphate test. This indicates that significant differences were obtained between the total phosphorus concentrations among the sixty-one isolates that were tested ($F_{26,54}$= 4.421, p=2.02x10^{6}). As the data met the assumptions of normality (A= 0.44548, p= 0.2764) and homogeneity of variance ($F_{26,54}$=0.4627, p=0.9826), this analysis was accepted as being statistically significant (Figure 3.5, Table 3.5).

Table 3.4. Phosphate concentrations (mg/L) for the experimental control and 60 isolates that were tested are expressed in this chart. The average total phosphorus concentration with standard deviation was used to determine the differences in concentrations between the sterile control and the isolate. NR was used to indicate which isolates showed no reduction in the phosphate concentration after the experiment.

Isolate Number	Avg PO_{43-}	SD	Diff from Control
Control	1.47	0.21	
49	1.33	0.28	-0.13
51	NR		
55	1.41	0.09	-0.06
57	NR		
59	1.41	0.23	-0.25
75	NR		
86	1.39	0.08	-0.08
93	1.39	0.07	-0.07
121	1.45	0.26	-0.02
123	1.24	0.05	-0.23
124	NR		
126	1.21	0.36	-0.26

129	NR		
149	1.11	0.06	-0.36
160	NR		
189	1.31	0.26	-0.15
191	NR		
196	0.35	0.41	-1.12
198	0.45	0.31	-1.02
216	1.29	0.36	-0.17
217	1.46	0.25	-0.01
219	NR		
220	NR		
243	0.99	0.19	-0.48
244	0.01	0	-1.46
250	1.17	0.07	-0.30
255	1.30	0.14	-0.17
256	1.25	0.14	-0.22
277	1.33	0.06	-0.14
283	NR		
285	1.33	0.29	-0.14
287	1.31	0.18	-0.17
289	1.40	0.24	-0.07
302	NR		
304	NR		
305	NR		
307	NR		
312	NR		
332	NR		
337	1.42	0.09	-0.05
338	NR		
366	1.31	0.22	-0.15
374	1.27	0.21	-0.18
377	1.25	0.12	-0.22
408	NR		
410	NR		
411	NR		
412	NR		
414	NR		
415	NR		
417	NR		
429	NR		

443	NR		
452	NR		
464	1.11	0.14	-0.36
466	NR		
470	NR		
475	1.13	0.19	-0.33
483	1.21	0.18	-0.26
486	1.31	0.05	-0.17

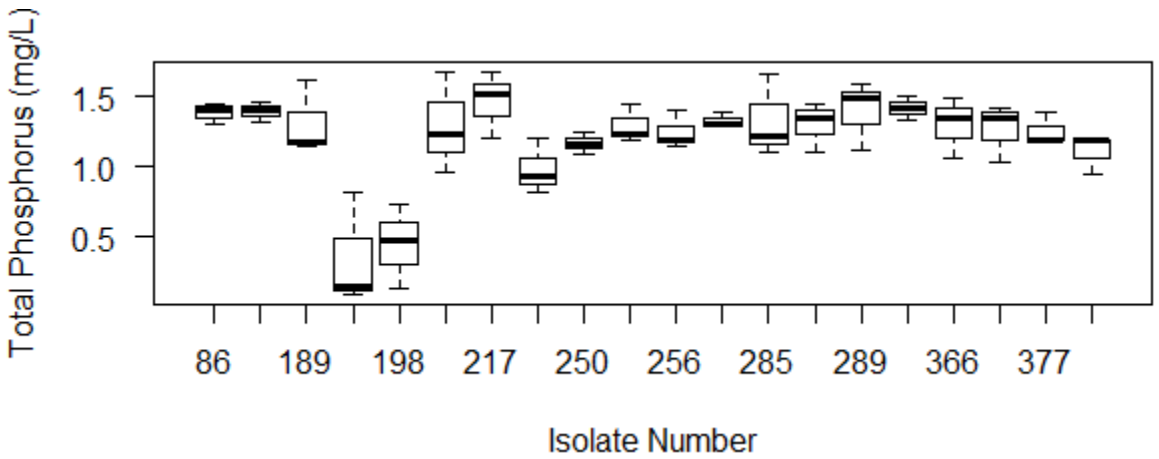


Figure 3.5. The boxplot is illustrating the distribution of the total phosphorus concentrations that were measured for each of the isolates that had viable results. Note that the dark line in the center of each plot represents the median of the data while the upper and lower lines show the 95% confidence intervals.

Table 3.5. Results of a one-way ANOVA on the reduction in total phosphorus concentrations between 26 isolates when compared to control. This was measured to determine if the qualitative trends observed during the inorganic phosphate test had any statistical significance.

	DF	SS	MS	F Value	P value
Isolates	26	5.46	0.21	4.42	$< 2^{06}$
Residuals	54	2.57	0.04		

Fisher's Least Significant Difference (LSD) test was completed to determine precisely which available phosphate concentrations (mg/L) of the sixty isolates were significantly

different from each other (Williams & Abdi, 2010). The Fisher's least significant difference t-test showed the mean available phosphate concentration for all of the sixty isolates and had statistically significant results (α=0.05, DF_{error}=54, t=2.01, LSD= 0.356). Isolates 198 and 196 were statistically different from every other isolate. The detailed results are presented in Appendix III.

The isolates were separated according to sampling location and analyzed separately to see if the lake that the isolate originated from had an impact on the results from the previous one-way ANOVA. Statistically significant changes between the remaining amount of available phosphate in the media were not observed between the six from Sparrow Lake that had a viable result to the inorganic phosphate test ($F_{6,14}$=0.932, p=0.502). The ANOVA results can be seen in Table 3. 6. The Sparrow Lake isolates did meet assumptions of normality (A= 0.57492, p=0.118) and homogeneity of variance ($F_{6,14}$=0.5791, p=0.7412). A scatterplot illustrating how the available phosphate concentrations were distributed between these isolates is given in Figure (3.6).

Table 3.6. Results of a one-way ANOVA analyzing the reduction of total phosphorus between the isolates from Sparrow Lake and the sterile control sample.

	DF	SS	MS	F value	P value
Isolates	6	0.2841	0.04735	0.932	0.502
Residuals	14	0.7116	0.05083		

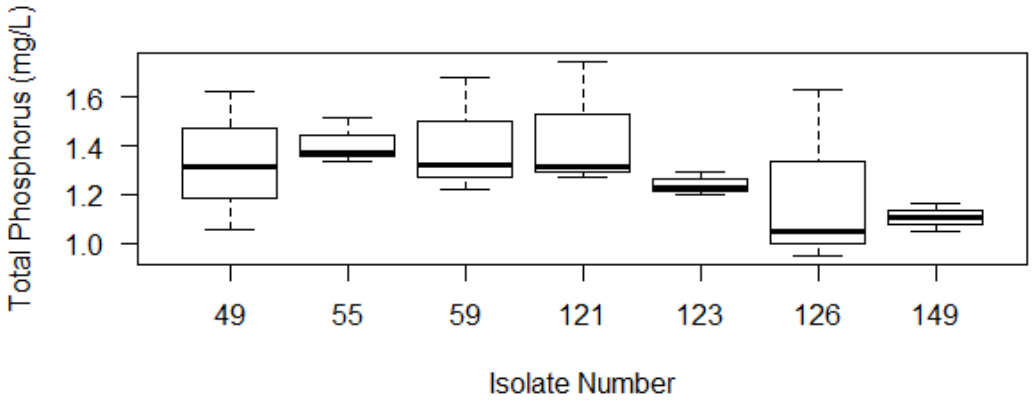


Figure 3.6. Boxplot showing the amounts of total phosphorus that were observed in the Sparrow Lake Isolates that had viable results in the inorganic phosphate test.

Fisher's least significant difference test was also used to compare the means of the Sparrow Lake isolates. The following test statistics were used to compare the means of available phosphate concentration between isolates: α=0.05, DF_{error}=14, t= 2.145, LSD=0.3948. None of these Sparrow Lake isolates had significantly different amounts of total phosphorus remaining in the Pikovskaya's broth after the experiment concluded.

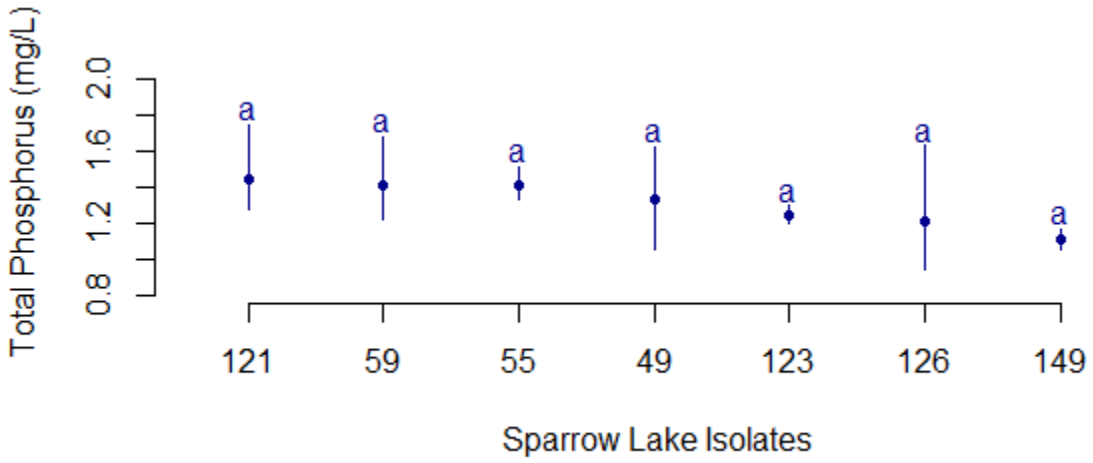


Figure 3.7. Fisher's Least Significant Difference test results for the isolates from Sparrow Lake. It is important to note that isolates that have the same letter are not significantly different.

Significant results were also obtained for the one-way ANOVA that was completed on the 28 isolates that came from Lake Simcoe-Atherley Narrows ($F_{19,40}$=5.69, p= 1.93x10^{06}). The Atherley Narrows isolates also met the assumptions of normality (A=0.24914, p=0.7367) and homogeneity of variance ($F_{19,40}$=0.4476, p=0.9688). Figure 3.8 is a boxplot illustrating how the available phosphate concentrations were distributed between the Atherley Narrows Isolates. Fisher's Least Significant Difference test was once again used to compare the means of the Atherley Narrows isolates. The following test statistics were used to compare the means of available phosphate concentration between isolates: α=0.05, DF_{error}=40, t= 2.021075, LSD=0.3554178. While isolates 198 and 196 were not different from each other, they were

different from the rest of the tested isolates. Figure 3.9 depicts the Fisher's LSD test results for the Atherley Narrows isolates.

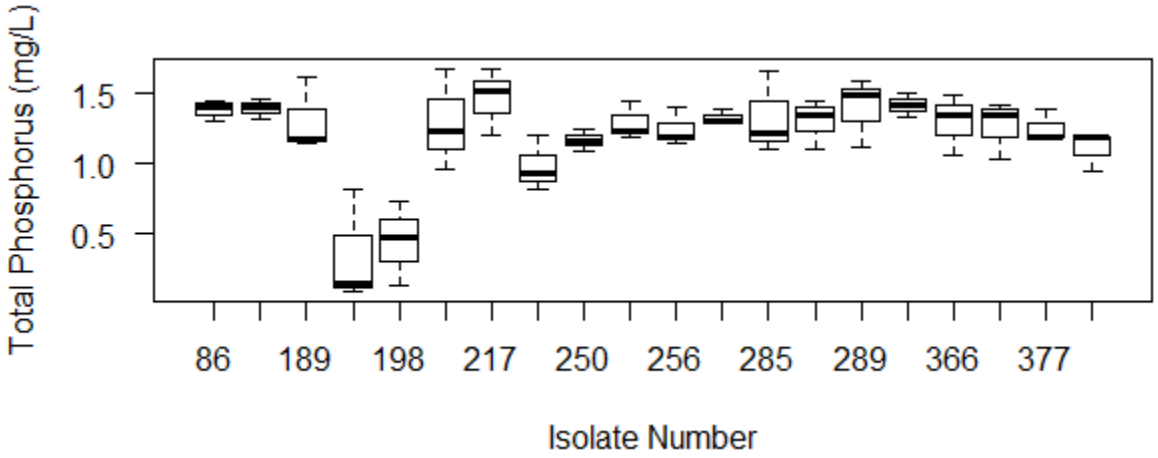


Figure 3.8. Boxplot of the Atherley Narrows isolates that had viable results in the inorganic phosphate test. This plot shows the variation in the amount of total phosphorus that was recorded for each of the isolates. Note that the dark line in the center of each plot represents the median of the data while the upper and lower lines show the 95% confidence intervals.

Table 3.7. The results of a one-way ANOVA between the reduction of total phosphorus among the isolates from Atherley Narrows and the sterile control sample.

	DF	SS	MS	F Value	P Value
Isolates	19	5.016	0.26399	5.691	1.93^{06}
Residuals	40	1.856	0.04639		

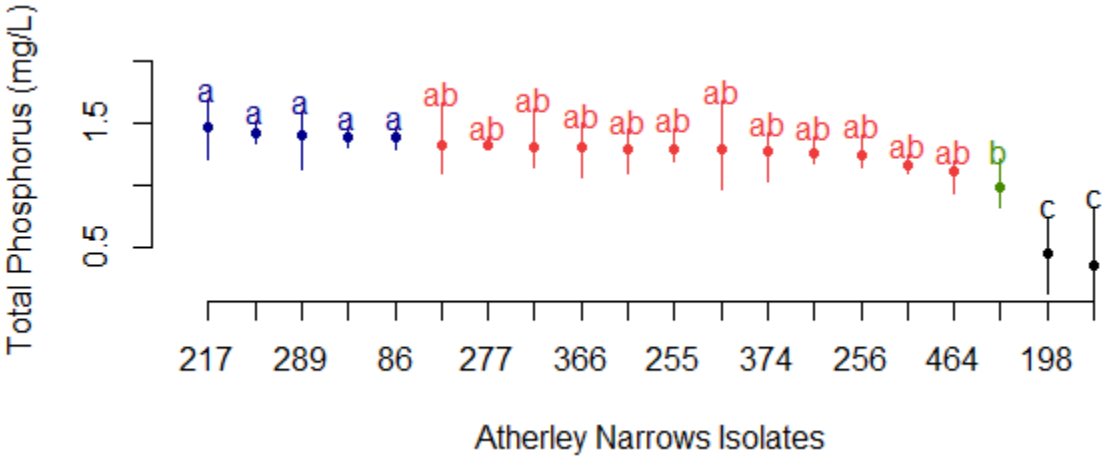


Figure 3.9. Fisher's Least Significant Difference test results for the isolates from Atherley Narrows. It is important to note that isolates that have the same letter are not significantly different.

3.4 Discussion

3.4-1 Phosphate Solubilization Index Test

The solubilization index results for this test appeared to be consistent with those found in the literature. Paul and Sinha (2017) tested the phosphate solubilizing capabilities of a known *Pseudomonas sp.* which had a solubilization index of 2.85 after 48 hours (Paul & Sinha, 2017). Sanjotha and Manawadi (2016) had five microbial isolates that had a solubilization index ranging from 2-2.63 after 15 days (360 hours) of incubation (Sanjotha & Manawadi, 2016). The halo zone formed on the Pikovskaya's agar plates is because of the microorganism's ability to produce phosphatase enzymes, or organic acid production (Paul & Sinha, 2017). This research helps to confirm that metabolic processes were occurring during the incubation process and the presence of a halo was not random error. 95 of the halo forming isolates that were tested had a solubilization index within the range of 2-2.96 while 19 isolates had a solubilization index that was higher than 3.0. Just under half (8) of these isolates with higher than normal solubilization index numbers came from Sparrow Lake site 1 in September. Correll (1998), found that repeated sub-culturing of phosphate solubilizing bacteria caused the microbes to lose their ability to solubilize inorganic phosphate. Correll's findings could help explain why so many isolates collected in September had high solubilization index numbers. The isolates collected in September had been sub-cultured four times and were only stored for two months prior to this test.

June had the lowest number of isolates that produced a halo during the solubilization index test between the Atherley Narrows and Sparrow lake sites (10 isolates) while July, August, and September all had 27 isolates that produced a halo during this test. This could have been due to the cooler temperatures that were observed in June, or as June is at the beginning of the growing season, it is possible the phosphate solubilizing bacteria were not as numerous as they would be during the peak of the growing season. This could have been part of the reason, so few bacterial isolates from June formed a visible halo (these isolates had been regrown and transferred onto slants in order to test all isolates at the same time). This could also explain why only 98 isolates formed a halo out of the 395 isolates that were tested for the solubilization index test. As there was no previous literature comparing the growth of various

phosphate solubilizing bacteria collected from the same location throughout a growing season, the above speculations are based solely upon the results that were collected for this experiment. Typically, the phosphate solubilization index test is used as a preliminary test to ensure that the bacterial isolates moving forward have the capabilities to utilize inorganic phosphate in some capacity and this test is used along with others in order to characterize the bacterial isolates (Paul & Sinha, 2017; Sanjotha & Manawadi, 2016; Mehta & Nautiyal, 2001).

Mehta and Nautiyal (2001) discuss the shortcomings of this particular test. They found that while the solubilization indices can be quantified, it is qualitative by nature in the sense that in order to determine that the bacterium is utilizing the inorganic phosphate a visual change in the media must be observed (the formation of a clear zone around the bacterial isolate). During their study, Mehta and Nautiyal (2001) discovered that many isolates that did not have a halo on the agar plates still showed reduced rates of phosphate in the broth assay. They suggest that microbes should be screened using a Pikovskaya's or NBRIP broth assay to quantitatively identify the isolates that are the most efficient phosphate solubilizers. This was why it was accepted that the phosphate solubilization index test was used as a preliminary test to screen all of the isolates saved from the field study and the inorganic phosphate test was used to screen further the isolates that were collected from Atherley Narrows, Sparrow Lake, and Cook's Bay.

The results from this test further disprove the hypothesis that the majority of the phosphate solubilizing bacteria would be collected from the Atherley Narrows sites and as such may be able to use as an indicator species of phosphorus pollution. Instead, the opposite appears to be true. The largest number of these bacteria were not only observed in Sparrow Lake, but those bacteria were also able to utilize the most inorganic phosphorus within the 216-hour timeframe.

3.4-2 Inorganic Phosphate Test

While the one-way ANOVA indicated that there was significant variation between the phosphate concentrations remaining in the media that the viable isolates that were tested grew on, however, the Fisher's least significant difference results indicated that only a few of the

isolates were significantly different from each other. This can be seen in Appendix III. Paul and Sinha (2007) found that their *Pseudomonas spp*. had 219 µg/ml of available phosphate in the broth after the isolate had been incubated for 96 hours. Islam et al. (2007) found that after 48 hours *Acinetobacter spp.* had 387 µg/ml, *Klebsiella spp* had 395 µg/ml, *Enterobacter spp.* had 206 µg/ml, *Pseudomonas spp.* had 132 µg/ml, *Microbacterium spp* had 97 µg/ml, and their unknown isolates ranged from 2-94 µg/ml concentrations of available phosphate. While Paul and Sinha (2007) did not explicitly state what their control concentration was, they also used Pikovskaya's broth, which was the same media used for this study. Meanwhile, the isolates that showed phosphate reduction capabilities for this study had available phosphate concentrations ranging from -1.464-0.003mg/L (-1464-3 µg/mL). This variability could be due to a few different scenarios as Rodríguez and Fraga (1999), discussed in their article which was about how phosphate solubilizing bacteria can help promote plant growth.

> *"Changes in P concentration could be a consequence of P precipitation of organic metabolites and/or the formation of organo-P compounds with secreted organic acids, which are subsequently used as an energy or nutrient source, this event being repeated several times in the culture. An alternative explanation could be the difference in the rate of P release and uptake. When the rate of uptake is higher than that of solubilization, a decrease of P concentration in the medium could be observed, when the uptake rate decreases (for instance as a consequence of decreasing growth or entry into stationary phase), the P level in the medium increases again. More probably, a combination of two or more phenomena could be involved in this behaviour"* (Rodríguez & Fraga, 1999).

Rodríguez and Fraga conclude by saying that the explanations as mentioned earlier are the often-found limitations to studying the phosphate solubilizing capability using a liquid medium. Either of these actions may have affected the bacterial isolates that were tested and could potentially contribute to why so many of the isolates did not show any reduction capabilities. The results from the inorganic phosphate test were compared to the experimental time when halos started to form during the solubilization index test for each of the sixty isolates to see if the unexpected inorganic phosphate test results could hypothetically be due to the growth phase that the isolate may have been in during the end of this study. In Table 3.8 it can be seen that 17 of the 29 isolates that showed no phosphate reduction activity first began to show a

visible halo by the time that the isolate was 72 hours old during the solubilization index test. Isolates 196, 198, didn't form a halo until after they had been growing for 96 hours, isolate 243 only began to form a halo once it had reached 72 hours, and 283 did not form a halo until it had been incubating for 144 hours. Meanwhile, isolates 408 and 410 had their first recorded halo once they reached 72 hours and isolate 411 had a halo with a solubilization index of 2.22 when it was first measured at 48 hours.

This theory is supported by the research of Mehta and Nautiyal (2001). They stated that the only reason a halo or clear zone would form on a Pikovskaya's plate was due to the organic acids that contribute to the microorganism's ability to solubilize inorganic phosphate. Mehta and Nautiyal (2001) used Bromophenol blue as a pH indicator. This allowed them to qualitatively see if organic acids had been released from the microorganisms to help them to interpret their results. However, this is a source of error for this study as the pH indicator was not included as part of the experiment.

Table 3.8. This table compares the amount of available phosphate present in the supernatant of the Pikovskaya's broth after 72 hours to the time that the first halo was recorded for the isolate during the phosphate solubilization index test.

Isolate	Available Phosphate(mg/L)	Incubation time at 1st visible halo
49	-0.14	48hrs
51	NR	168hrs
55	-0.06	72hrs
57	NR	72hrs
59	-0.25	48hrs
75	NR	48hrs
86	-0.08	120hrs
93	-0.07	96hrs
121	-0.02	96hrs
123	-0.23	120hrs
124	NR	144hrs
126	-0.26	96hrs
129	NR	96hrs
149	-0.36	96hrs

160	NR	168hrs
189	-0.15	96hrs
191	NR	168hrs
196	-1.12	96hrs
198	-1.02	96hrs
216	-0.17	96hrs
217	-0.01	48hrs
219	NR	48hrs
220	NR	48hrs
243	-0.48	72hrs
244	-1.46	120hrs
250	-0.30	72hrs
255	-0.17	96hrs
256	-0.22	96hrs
277	-0.14	96hrs
283	NR	144hrs
285	-0.13	72hrs
287	-0.17	72hrs
289	-0.07	72hrs
302	NR	48hrs
304	NR	48hrs
305	NR	48hrs
307	NR	120hrs
312	NR	96hrs
332	NR	216hrs
337	-0.05	96hrs
338	NR	72hrs
366	-0.16	72hrs
374	-0.19	48hrs
377	-0.212	72hrs
408	NR	72hrs
410	NR	72hrs
411	NR	48hrs
412	NR	96hrs
414	NR	72hrs
415	NR	72hrs
417	NR	72hrs
429	NR	72hrs
443	NR	96hrs
452	NR	72hrs

464	-0.36	120hrs
466	NR	48hrs
470	NR	120hrs
475	-0.33	72hrs
483	-0.26	120hrs
486	-0.16	72hrs

It is interesting to note that most of the isolates that did not show any phosphate reduction capabilities in this experiment were collected from Sparrow Lake in September. Meanwhile, the isolates that did show phosphate reduction capabilities all originated from the Atherley Narrows sites, two of which were from July, with the third being from August. This could be due to how many times the isolate had been sub-cultured as Correll (1998) suggests, or it could potentially have something to do with seasonal variation. However, the latter claim is harder to justify as there are no previous studies completed on the seasonal variability of phosphate solubilizing bacteria within a benthic environment of a lotic system.

If the inorganic phosphate tests were to be completed again, it should be completed by following the methods of Mehta and Nautiyal (2001) exactly. This would include having a larger amount of Pikovskaya's broth to start with and by taking aliquots of the isolate sample once daily, similar to the timeframe of the solubilization index test, along with using a pH indicator and allowing the isolates to grow in the broth for an extended period. If the inorganic phosphate test included these small changes, further explanations as to why roughly half of the tested isolates did not show phosphate reduction abilities could potentially be made.

Due to the variability observed in the inorganic phosphate test, five isolates from Lake Simcoe Atherley Narrows and Sparrow Lake were selected to be characterized and potentially identified. These isolates were selected based on having an average solubilization index of over 2.0. The concentration of available phosphate at the end of the inorganic phosphate test became a selection factor for the selection of isolates for further study. As the reason for the variability seen in the phosphate solubilization test was unknown at the time, five the isolates with the highest amount of available phosphate and five the isolates with the lowest amount of

available phosphate were selected, as long as their solubilization index was over 2. These isolates along with their test results and lake and month of origin can be seen in Table 3.9.

Isolate	Avg SI	TP(µg/L)	Lake	Month
126	4.22	NR	SPL (Site 1)	July
149	2.66	-0.36	SPL (Site 2)	July
196	5.83	-1.12	LS (Site 1)	July
198	4.08	-1.02	LS (Site 1)	July
243	2.66	-0.48	LS (Site 1)	August
256	2.81	-0.22	LS (Site 1)	August
283	2.61	NR	LS (Site 3)	August
408	3.86	NR	SPL (Site 1)	September
410	3.96	NR	SPL (Site 1)	September
411	4.71	NR	SPL (Site 1)	September

Table 3.9. This table shows the ten isolates that were chosen for characterization studies based on their avg SI number. SPL is the short form assigned to the isolates that originated from Sparrow Lake while LS was the short form assigned to the isolates that were from Lake Simcoe-Atherley Narrows.

3.5 Conclusion

The phosphate solubilization index test and the inorganic phosphate test were found to be successful screening tests for the phosphate solubilizing bacteria that were collected from Lake Simcoe- Atherley Narrows, Lake Simcoe- Cook's Bay, and Sparrow Lake. Of the 480 isolates that were saved and screened from the various sites on Lake Simcoe and Sparrow Lake, a total of 98 isolates had a clear zone develop during the solubilization index test. More isolates that rated higher than 1 on the solubilization index came from the Sparrow Lake sites rather than from the Lake Simcoe sites, which disproved the hypothesis that phosphate solubilizing bacteria originating from Lake Simcoe would be more efficient at reducing inorganic phosphate. However, this result supports the conclusion found in chapter 2 which suggests that phosphate solubilizing bacteria are more prevalent when they originate from locations that could have had more inorganic phosphate in the sediment rather than from locations that had higher amounts of total phosphorus in the water column.

General trends seen among the various isolates help confirm that time is a significant factor in relation to how high upon the solubilization index an isolate will end up. The present study showed that generally, the solubilization index of an isolate tended to grow larger the longer the isolate was incubated. The inorganic phosphate test aided in further reducing the number of isolates by examining exactly how much inorganic phosphate was present in the broth media. This test found that differences in the amount of total phosphate remaining in the broth after the isolate had been growing for 72 hours did vary slightly between isolates. The results were variable between some of the isolates. A number of the isolates showed no phosphate reduction capabilities even though they produced halos during the phosphate solubilization index study.

This study showed that the phosphate solubilizing bacteria collected were relatively slow-growing microorganisms. The solubilization index test showed great success as a method of screening through multiple isolates to determine which isolates were capable of utilizing inorganic phosphate and how quickly each isolate was capable of doing so. The inorganic phosphate test was not as conclusive however it was a relatively straightforward method for quantitatively determining how much inorganic phosphate was used by each isolate. As such, both of these tests provided useful information for preliminary testing of the collected phosphate solubilizing bacteria.

Chapter 4: Characterizing ten phosphate solubilizing bacterial isolates

Chapter 4 Abstract

In this study, phosphate solubilizing bacteria from freshwater environments are tested on their ability to solubilize inorganic phosphate. Few studies have been completed on characterizing the different strains of them. After both the solubilization index measurements and the inorganic phosphate solubilization tests, 10 isolates were chosen to be characterized based on their growth at various temperatures, pH concentrations, and inorganic phosphate concentrations. The results from the solubilization index test were compared with the results of the inorganic phosphate solubilization test in order to select 5 isolates that showed high phosphate reducing potential. Previous research indicates that phosphate solubilizing bacteria as a group would be more mesophilic as all of the isolates screened for this test thrived when they were incubated at 30° C (Sanjotha & Manawadi, 2016). The isolates also grew best when they were in media that varied from neutral to only slightly alkaline pH levels although the isolates grew when they were in more acidic media as well. The isolates also grew best when only 5g/L of tri-calcium phosphate was present in the media. A discriminant function analysis was completed on the results of these tests, and the overall model indicated that ultimately these isolates were not statistically significantly different from each other. However, the discriminant function analysis showed that isolates 126, 283, and 256 were different from the others. A simple gram stain confirmed that these isolates were different from the other seven. Isolate 126 originated from Sparrow Lake site 1 in July. Isolate 283 originated from Atherley Narrows site 3 in August, and isolate 256 originated from Atherley Narrows site 1, also in August.

4.1 Introduction

Phosphate solubilizing bacteria have been commonly studied in terrestrial environments, with an intent to use them in sustainable agriculture as a potential bio-fertilizer (Correll, 1998; Kucey, 1983; Mohammadi, 2012; Rodríguez & Fraga, 1999). However, the impact they may pose on aquatic freshwater systems has been understudied until quite

recently (Sanjotha G & Sudheer Manawadi, 2016; Paul & Sinha, 2017; Pérez, et al., 2007). Of the few studies that have isolated and characterized phosphate solubilizing bacteria, very few characterized these isolates in varying growing conditions that these isolates thrive in. This chapter aims to further classify ten bacterial isolates from Sparrow Lake and Lake Simcoe-Atherley Narrows that were deemed to be the strains that utilize inorganic phosphate most efficiently. This selection for these ten isolates was made considering the solubilization index result for each of the isolates as well as from the inorganic phosphate test results. These tests were compared in order for these ten isolates to be selected. Some of the selected isolates did not show any phosphate reduction in the inorganic phosphate test and were characterized because they had promising results when tested on Pikovskaya's agar plates. The primary objective of this study was to characterize the phosphate solubilizing bacteria based their ability to grow at various temperatures, pH concentrations, and various tri-calcium phosphate concentrations. One of the secondary objectives of this study was to determine if it could be explained why some of the isolates did not show any phosphate reduction during the inorganic phosphate test, which is why the 10 isolates were grown in media that had varying concentrations of inorganic phosphate.

4.2 Materials and Methods

4.2-1 Temperature Range Test

The ten isolates were grown at various temperatures to try to determine at which temperature these bacteria would thrive the best. As previous studies reported (Pérez et al., 2007; Paul & Sinha, 2017) phosphate solubilizing bacteria are known to be mesophilic in terrestrial environments (Mohammadi, 2012), and it was hypothesized that these isolates would grow best around 24°C.

Each isolate was plated on the center of a Pikovskaya's agar plate, once again a 6mm diameter circle was drawn on the center of each plate in order to ensure the cells were only transferred into the desired location. The isolates in triplicate were incubated in controlled environments at the following temperatures: 4°C, 15°C, 24°C, 30°C, 37°C, and 47°C. The isolates were left at these temperatures for seven days, and after this time Edi-Premono, et al. (1996) formula was used to calculate the solubilization index of each isolate.

4.2-2 pH Range Test

Mehta and Nautiyal, (2001) used pH as an indicator for an isolate's ability to solubilize inorganic phosphate. Thus, pH was used as another way to classify the collected isolates. 1M NaOH and 1M HCl were used to change the pH of the Pikovskaya's agar before the medium being sterilized in the autoclave. The VWR SympHony SP70P probe was calibrated and used to read the pH concentrations of the liquid agar. Before the addition of any NaOH or HCl, the pH of the Pikovskaya's agar was 6.66. No more than 0.5ml of either the HCL or NaOH was added to the agar at any time, and slowly brought to one of the following concentrations: 3, 5, 7, 9, 11, and 13. After each of the 1L agar flasks at the various concentrations had been autoclaved, they were poured into plates.

Each isolate had plated in triplicate on individual Pikovskaya's agar plates at each pH concentration and were all incubated at 30°C (±2°C, the temperature was selected after studying the growth rate at various temperatures as described above) for seven days. After the seven days had passed, the (Edi-Premono et al., 1996) formula was used to calculate the solubilization index for each of the isolates.

4.2-3 Calcium Phosphate Range Test

The top isolates were also tested on their ability to solubilize inorganic phosphate at various concentrations. This test was completed due to the unexpected results from the inorganic phosphate test (Chapter 3). As some of the isolates appeared better at solubilizing inorganic phosphate within a short amount of time, this test was completed to see how much each of the isolates could solubilize Calcium phosphate within seven days. Pikovskaya's agar has a $Ca_3(PO_4)_2$ concentration of 5g/L, this was used as the mid-point in the calcium phosphate range. Nutrient agar (peptones from meat 5g/L, meat extract 3g/L, and agar 12g/L) with calcium phosphate tribasic powder was used to create the low concentrations of this range while additional calcium phosphate tribasic powder was added to the Pikovskaya's agar to increase the concentration of calcium phosphate in this range. This allowed for the creation of a range of plates that had 0, 3, 5, 8, 10, and 20g of calcium phosphate per litre.

Each isolate had three replicates plated on individual Pikovskaya's agar plates at each calcium phosphate concentration and were all incubated at 30°C (±2°C) for seven days. After the seven days had passed, the Edi-Premono, et al. (1996) formula was used to calculate the solubilization index for each of the isolates.

4.2-4 Gram Stain

The ten isolates were Gram stained following the procedure set out by Claus (1992) using BD BBL Gram crystal violet (crystal violet 3g/L, isopropanol 50ml/L, ethanol/methanol, 50ml/L, distilled water, 900ml/L), stabilized Gram iodine(iodine crystals, 3.3g/L, potassium iodide 6.6 g/L, and distilled water, 1L), Gram decolorizer (acetone, 250ml/L, and isopropanol 750ml/L), and Gram safranin (safranin O powder 4g/L, ethanol/methanol 200ml/L, and distilled water 800ml/L).

The ten bacterial isolates were streaked onto new Pikovskaya's agar plates and grown for 24 hours at 30°C (±2°C). A few cells from each isolate were smeared onto individual sterile glass slides and fixed in place by being passed over the flame from a Bunsen burner three times (Claus, 1992). After the cells had cooled, they were flooded with Gram crystal violet for 60 seconds then rinsed with water. Next, the stained isolate was flooded with stabilized Gram iodine for 60 seconds then rinsed. Immediately following this, the isolate was flooded with Gram decolourizer which was only allowed to sit on the isolate for 10 seconds (Claus, 1992). Finally, the isolate was flooded with Gram safranin for 60 seconds and then rinsed. After the slides had air dried, they were placed under a microscope and identified as either Gram-positive or negative (Claus, 1992).

4.2-5 Statistical Analysis

IBM SPSS statistics version 20 was used to complete a discriminant function analysis to determine which of the ten tested isolates are statistically different from each other based on how they grew at the various temperatures, pH, and calcium phosphate concentrations (Manly, et al., 2002). A Canonical analysis was completed as part of the discriminant function analysis so that the temperature, pH, and the calcium phosphate tests would not have to be combined into additional groups (Manly, et al., 2002). The null hypothesis that was tested was that there

would be no discriminating differences between the ten tested isolates, while the alternative hypothesis stated that there would be significant differences between some of the isolates based on the different growth quality parameters that were tested.

A Box's M test was completed to determine if the covariance matrices between the results for the temperature, pH, and calcium phosphate tests were homogeneous (Lachenbruch, et al., 1973). The alternative hypothesis that the observed covariance matrices for the variables would not be equal across all groups was accepted (Box's M = 210.589, $F_{48, 34118}$= 4.070, p<0.01). Transforming the data did not resolve this issue. However, due to the sensitivity of the Box's M test the discriminant function analysis was still used for the following analysis as the deviations from normality and homogeneity of variance were accounted for by using the Pillai's Trace statistic in place of the Wilks' Lambda statistic (Lachenbruch, et al., 1973).

4.3 Results

4.3-1 Temperature Range Test

Isolate 196 had the largest solubilization index number after growing at 4°C for seven days (SI 2.849, SD ±0.2855), while isolates 283, and 126 did not grow at all at this temperature. Isolate 196 also had the largest solubilization index after growing at 15°C for seven days (SI 2.944, SD ±0.2795), and while colonies grew for isolates 243, 411, and 408, these isolates did not form a halo during the seven-day incubation period and isolate 283 did not grow at all. Isolate 411 had the largest solubilization index after growing at 24°C for seven days (SI 3.293, SD ±0.22) and isolate with the lowest solubilization index was isolate 410 (SI 2.40, SD±0.229). After the isolates had been incubated at 30°C for seven days, isolate 196 had the highest solubilization index (SI 4.13, SD±0.367), and isolate 126 had the smallest solubilization index (SI 0.757, SD±1.312). Isolate 198 had the highest solubilization index after the isolates had been incubated at 37°C for seven days (SI4.149, SD 0.4338), while isolates 283 and 126 were tied with the lowest solubilization index numbers (SI 0.333, SD ±0.577 for each). Finally, isolate 411 had the highest solubilization index number after the isolates had been incubated for seven days at 47°C (SI 1.807, SD 0.699), while isolates 198 and 408 did not grow at this temperature.

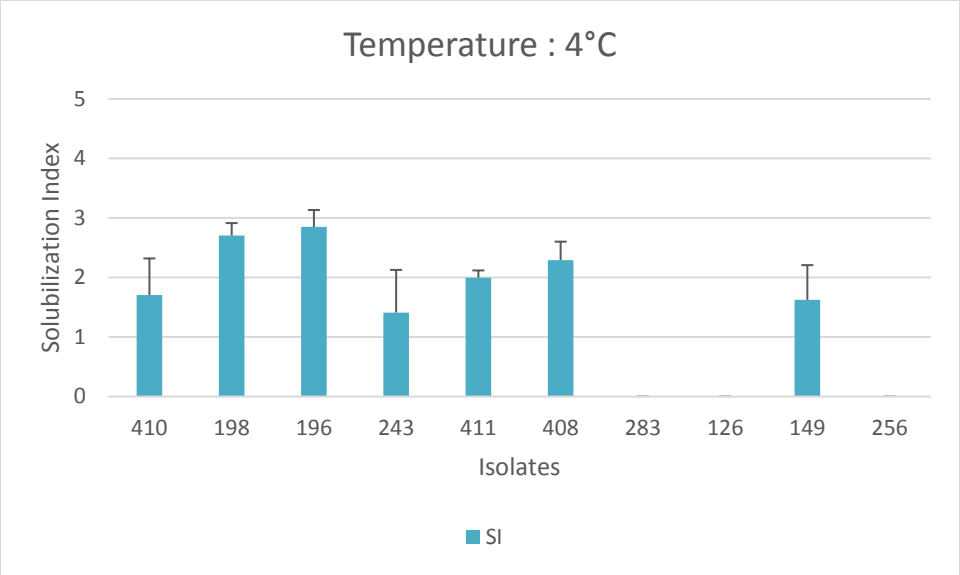


Figure 4.1. The solubilization indices (SI) for the 10 isolates that grew at 4°C for seven days. This graph shows the solubilization index number for each isolate with standard deviations.

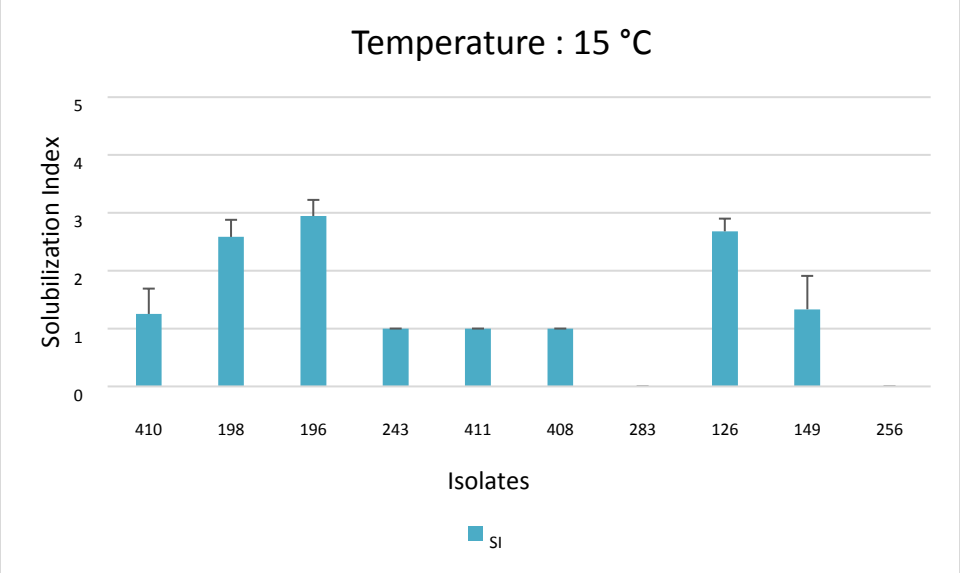


Figure 4.2. The solubilization indices (SI) for the 10 isolates that grew at 15°C for seven days. This graph shows the solubilization index number for each isolate with standard deviations.

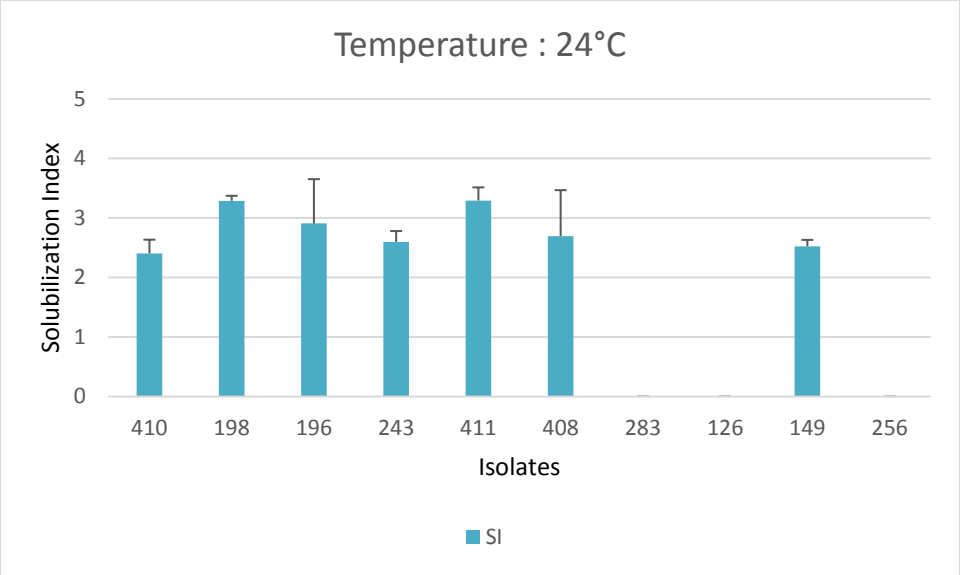


Figure 4.3. The solubilization indices (SI) for the 10 isolates that grew at 24°C for seven days. This graph shows the solubilization index number for each isolate with standard deviations.

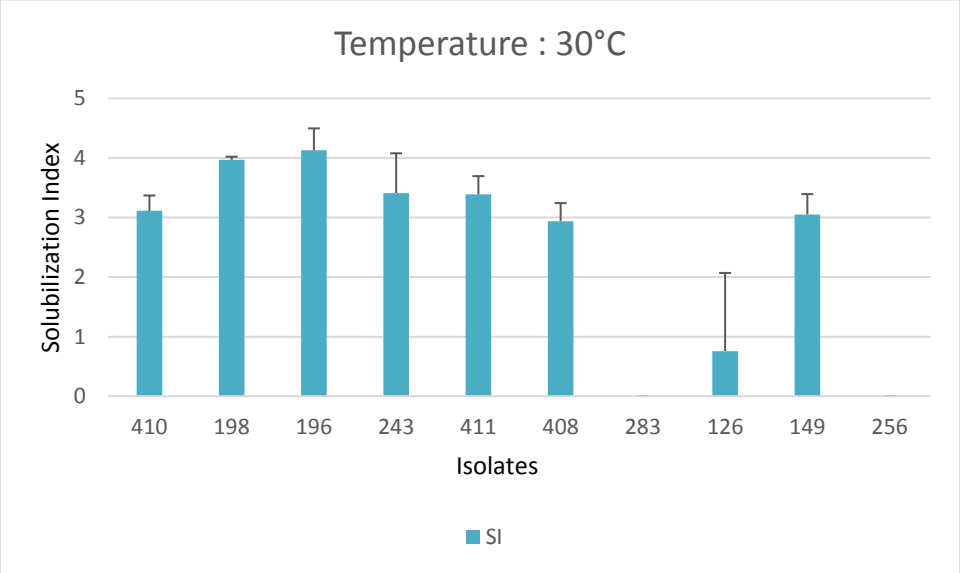


Figure 4.4. The solubilization indices (SI) for the 10 isolates that grew at 30°C for seven days. This graph shows the solubilization index number for each isolate with standard deviations.

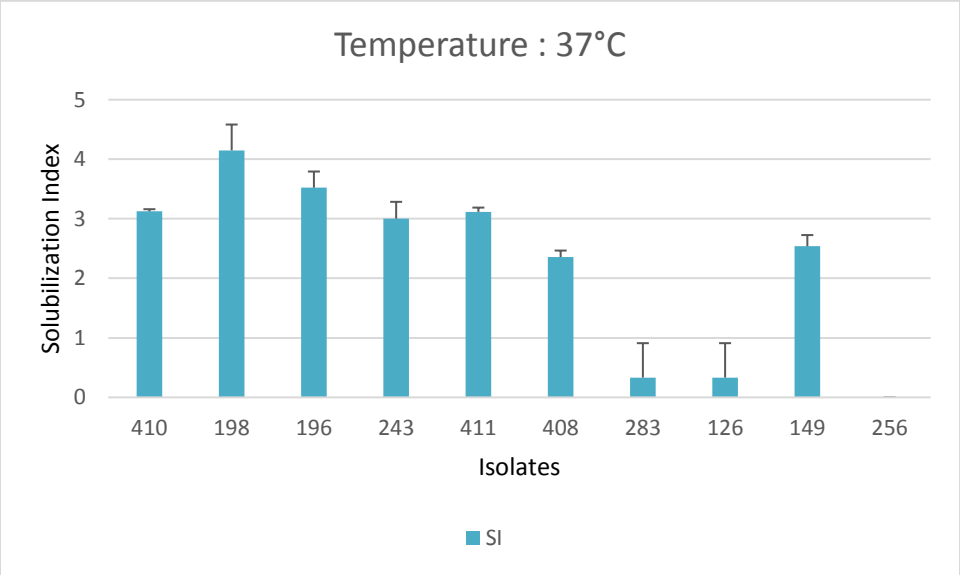


Figure 4.5. The solubilization indices (SI) for the 10 isolates that grew at 37°C for seven days. This graph shows the solubilization index number for each isolate with standard deviations.

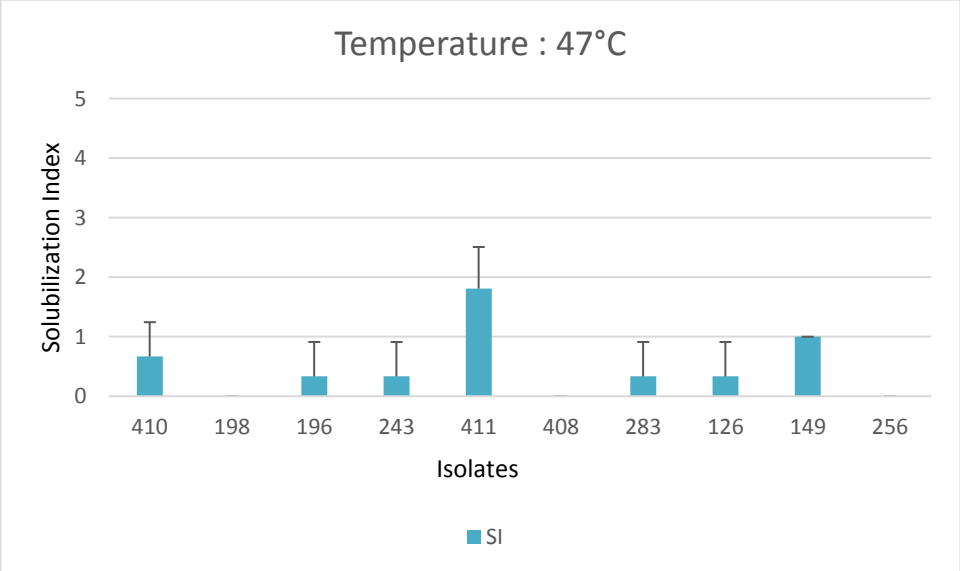


Figure 4.6. The solubilization indices (SI) for the 10 isolates that grew at 47°C for seven days. This graph shows the solubilization index number for each isolate with standard deviations.

4.3-2 pH Range Test

Isolate 198 had the largest solubilization index when grown on Pikovskaya's medium that had a pH of 3 for seven days (SI 3.658, SD ±0.115), while isolate 256 had the lowest solubilization index at the same pH (SI 1.70, ±SD 0.606). When the isolates grew on Pikovskaya's agar plates that were at a pH of 5 for seven days isolate 196 had the largest solubilization index (SI 4, SD ±1.309), and isolate 256 had the lowest solubilization index (SI 1.965, SD ±0.511). There was a much smaller range in numbers when the isolates were grown on the media that was at a pH of 7. Isolate 198 had the largest solubilization index (4.05, SD ±0.354) and isolate 283 had the smallest solubilization index (2.09, SD ±0.046). The isolates all grew well when the media became slightly basic as well. When the media's pH was at 9 isolate 196 had the largest SI number with an index number of 4.4 (SD ±0.27) and isolate 283 had the lowest index number which was 2.23 (SD ±0.166). When the isolates were grown at a pH of 11 isolate 196 still had the largest index number (4.05, SD ±0.62), while isolate 283 remained with the smallest solubilization index (2.08, SD± 0.021). When the isolates were grown at a pH of 13, only isolates 410, 408, 283, 126, 149, and 256 had colonies that grew, but none of the isolates developed a visible halo during the seven days that they were grown. The tables below show how each of the isolates grew at the previously mentioned pH ranges.

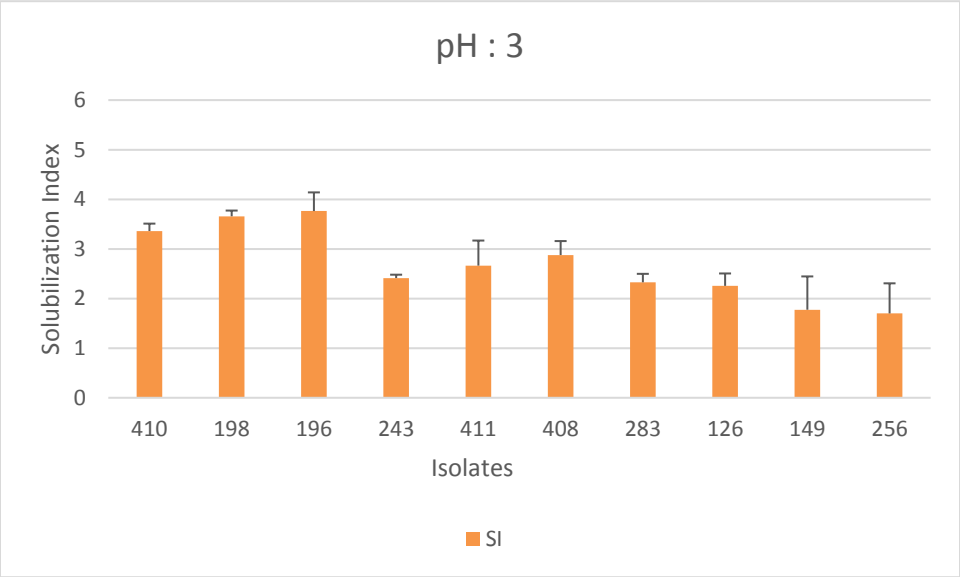


Figure 4.7. The solubilization indices (SI) for the 10 isolates that were grown at a pH of 3. This graph shows the solubilization index number for each isolate with standard deviations.

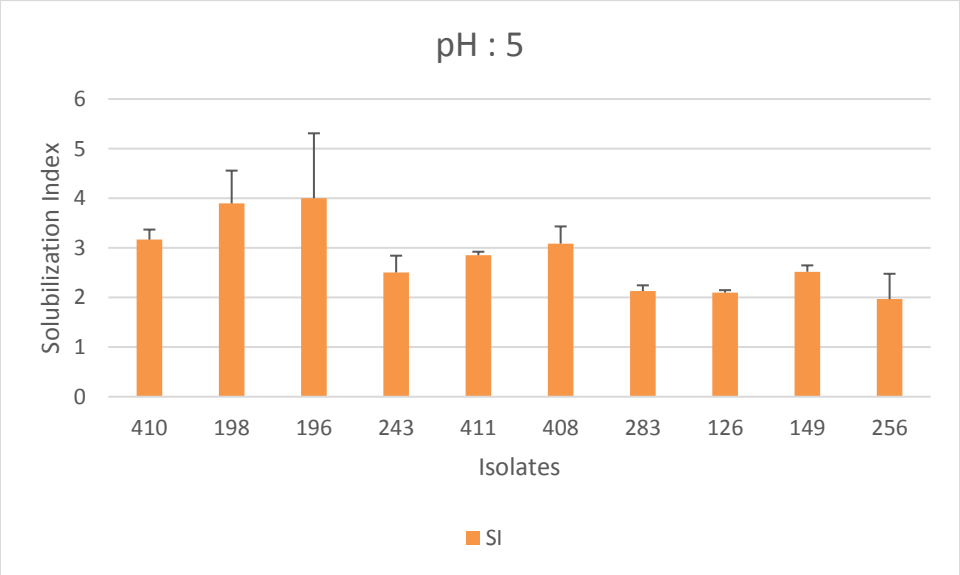


Figure 4.8. The solubilization indices (SI) for the 10 isolates that were grown at a pH of 5. This graph shows the solubilization index number for each isolate with standard deviations.

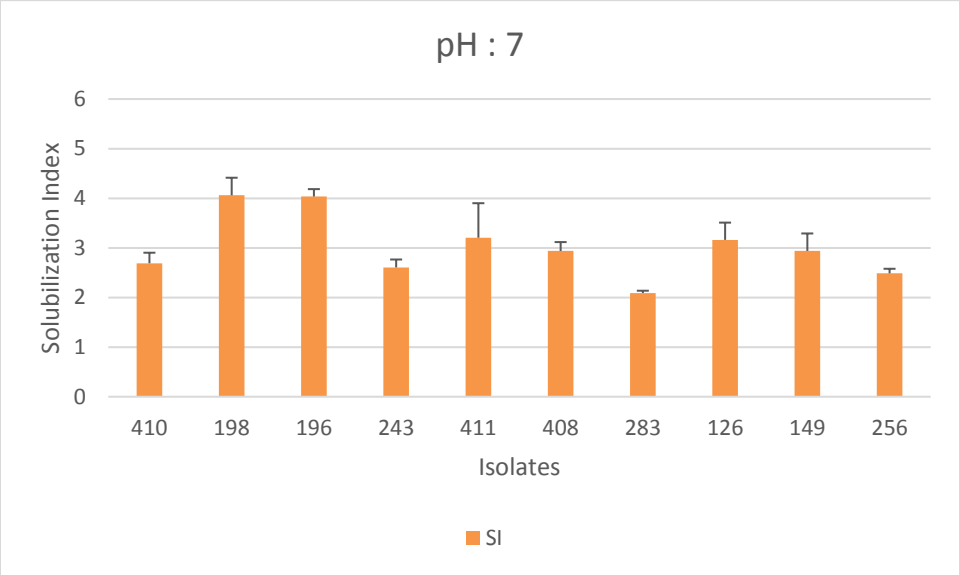


Figure 4.9. The solubilization indices (SI) for the 10 isolates that were grown at a pH of 7. This graph shows the solubilization index number for each isolate with standard deviations.

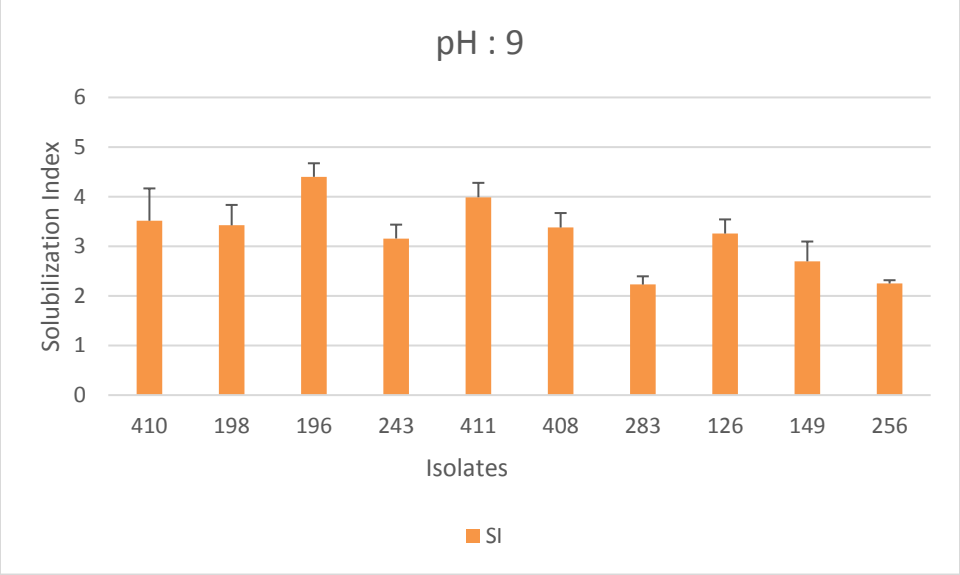


Figure 4.10. The solubilization indices (SI) for the 10 isolates that were grown at a pH of 9. This graph shows the solubilization index number for each isolate with standard deviations.

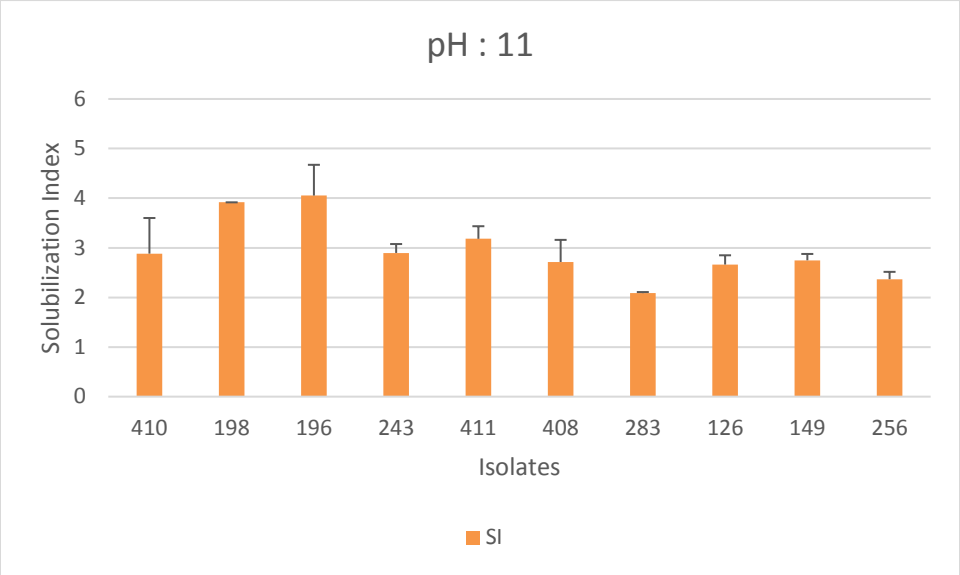


Figure 4.11. The solubilization indices (SI) for the 10 isolates that were grown at a pH of 11. This graph shows the solubilization index number for each isolate with standard deviations.

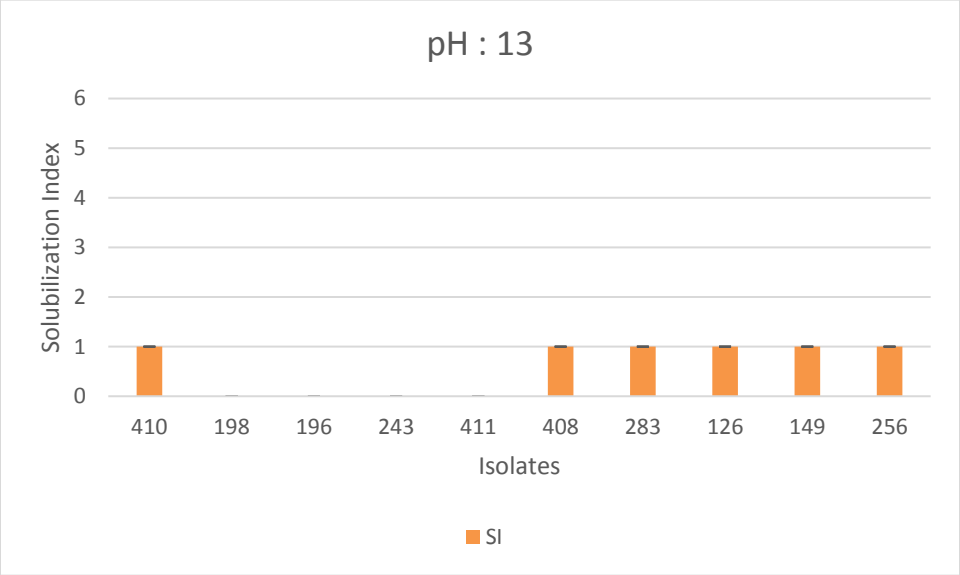


Figure 4.12. The solubilization indices (SI) for the 10 isolates that were grown at a pH of 13. This graph shows the solubilization index number for each isolate with standard deviations.

4.3-3 Calcium Phosphate Range Test

All of the isolates grew on the nutrient agar that did not have any tri-calcium phosphate added to it, but none of the isolates formed a visible halo. The same was true to the isolates that were grown on the nutrient agar that had 3g/L of calcium phosphate added to it except isolates 196 and 126 did not grow. The isolates grew and formed halos on the Pikovskaya's agar which had 5g/L of tri-calcium phosphate. Isolate 196 had the largest solubilization index number (SI 5.16, SD ±1.22), while isolate 256 had the lowest solubilization index number (2.15, SD ±0.036). Only six of the isolates formed halos when there was 8g/L of calcium phosphate within the media. Isolate 198 had the largest solubilization index number (4.09, SD ±0.27), while isolates 243, 408, and 283 did not form halos at all. When there was 10g/L of tri-calcium phosphate in the media, isolate 198 still had the largest solubilization index number (4.18, SD ±0.868) while isolates 410, 243, 408, 283, and 256 did not form visible halos during the seven days that the isolates were incubated. When 20g/L of tri-calcium phosphate was present only isolates 198 and 196 formed halos and had a solubilization index number, and they were 2.61 (SD ±0.26), and 3.08 (SD ±0.22) respectively. Isolates 410, 408, and 126 did not grow during the incubation period, and the others all had colonies that had an average diameter less than 10mm. The tables below show how these ten isolates grew at each calcium phosphate concentration.

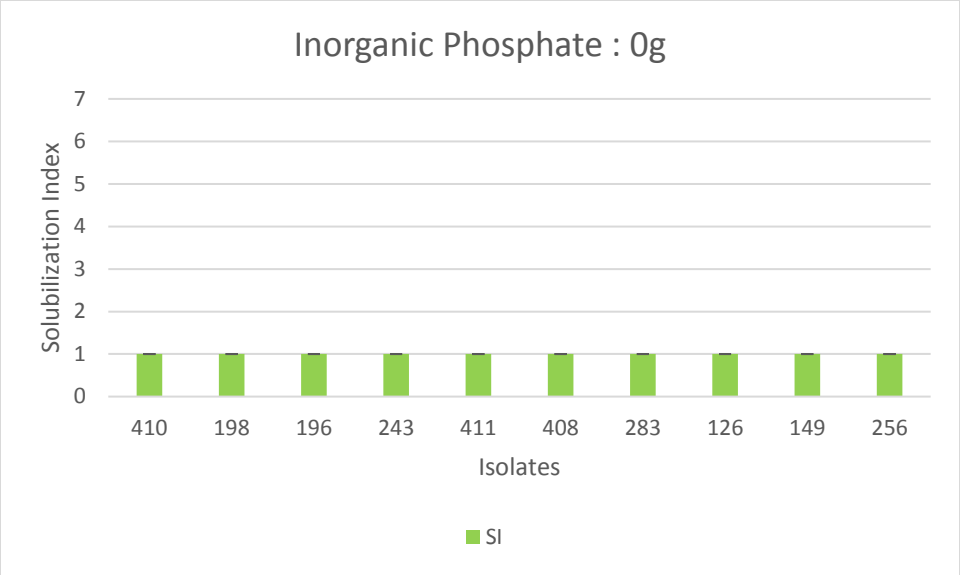


Figure 4.13. The solubilization indices (SI) for the 10 isolates that were grown with 0g/L of tri-calcium phosphate. This graph shows the solubilization index number for each isolate with standard deviations.

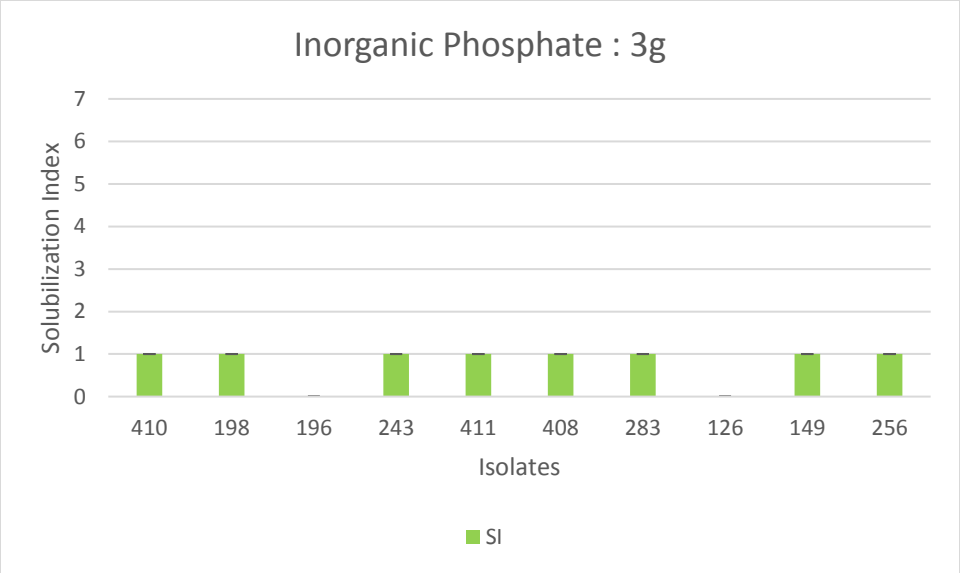


Figure 4.14. The solubilization indices (SI) for the 10 isolates that were grown with 3g/L of tri-calcium phosphate. This graph shows the solubilization index number for each isolate with standard deviations.

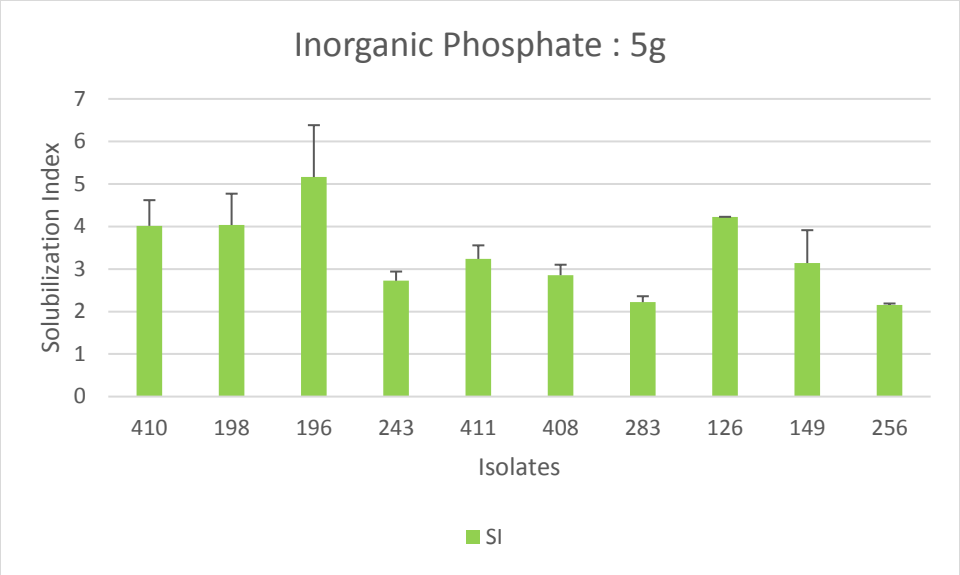


Figure 4.15. The solubilization indices (SI) for the 10 isolates that were grown with 5g/L of tri-calcium phosphate. This graph shows the solubilization index number for each isolate with standard deviations.

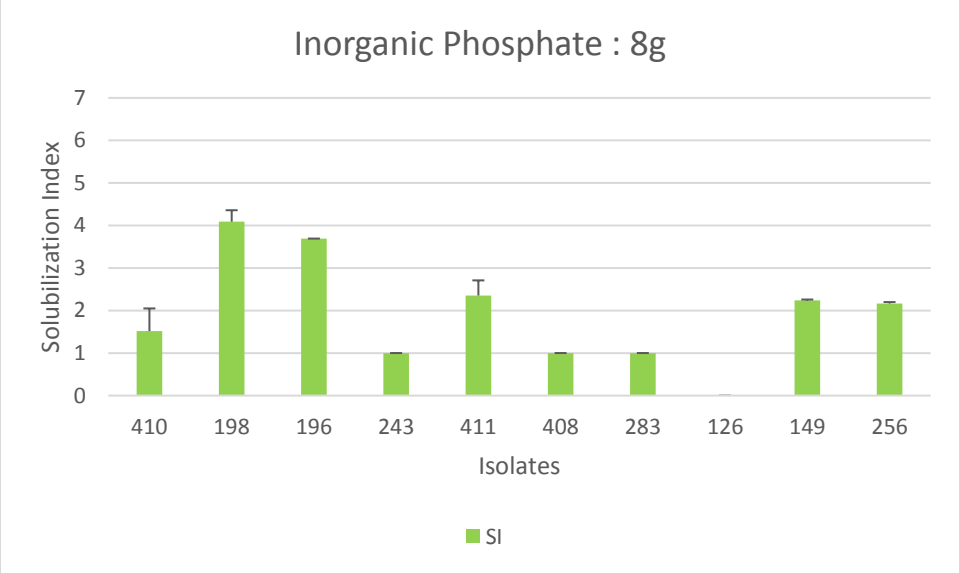


Figure 4.16. The solubilization indices (SI) for the 10 isolates that were grown with 8g/L of tri-calcium phosphate. This graph shows the solubilization index number for each isolate with standard deviations.

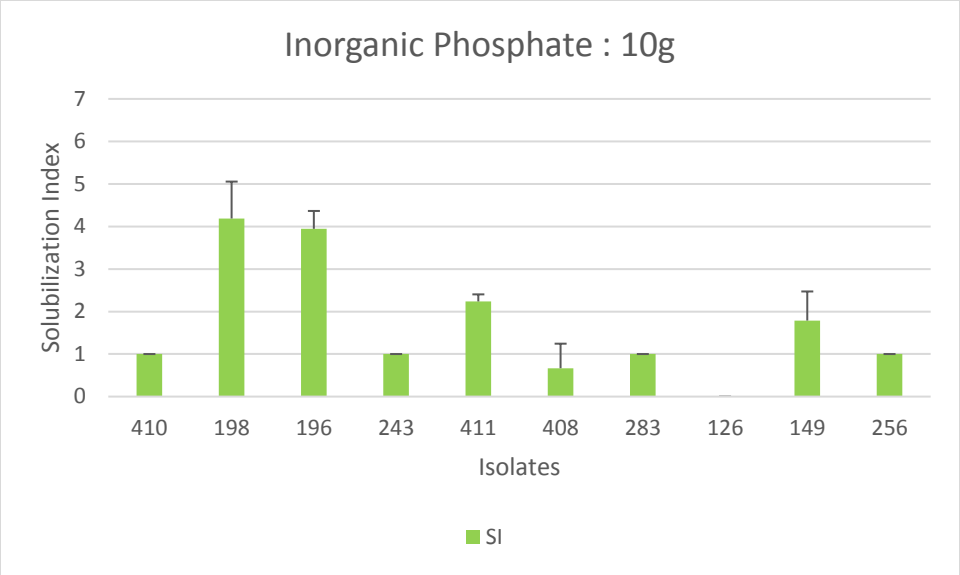


Figure 4.17. The solubilization indices (SI) for the 10 isolates that were grown with 10g/L of tri-calcium phosphate. This graph shows the solubilization index number for each isolate with standard deviations.

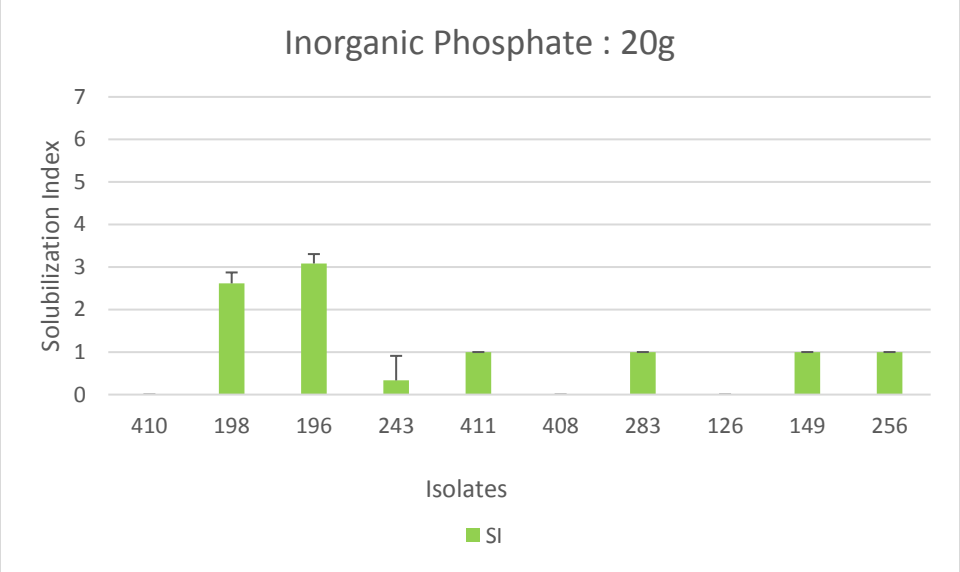


Figure 4.18. The solubilization indices (SI) for the 10 isolates that were grown with 20g/L of tri-calcium phosphate. This graph shows the solubilization index number for each isolate with standard deviations.

4.3-4 Gram Stain

Isolate 126 had Gram-negative coccus cells. Isolates 149, 196, 198, 243, 408, 410, and 411 all had Gram-positive rod-shaped cells. Isolate 283 had Gram-positive coccus cells and isolate 256 had Gram-negative rod-shaped cells. Table 4.19 summarizes these results.

Table 4.19 Gram stain results for the 10 isolates that were tested.

Isolate	Gram Stain	Morphology
126	negative	cocci
149	positive	rod
196	positive	rod
198	positive	rod
243	positive	rod
283	positive	cocci
256	negative	rod
408	positive	rod
410	positive	rod
411	positive	rod

4.3-5 Statistical Analysis

A significant Pillai's Trace statistic was observed (Pillai's trace= 0.856, $F_{27,510}$=7.542, p<0.001, observed power= 1.0) indicating that the null hypothesis that the isolates are not significantly different from each other should be accepted. However, the linear discriminant function analysis results were still used to analyze the growing conditions data further to see if some of the isolates may be significantly different. The three-function model that was used showed that functions 1 through 3 were significant (χ^2= 194.49, p<0.001), along with functions 2 through 3 (χ^2= 62.57, p<0.001). Function 1 (discriminated between the temperature range test results and the combined pH and inorganic phosphate test results) accounted for 73.6% of the entire variance within the model; function 2 (discriminated between the pH and inorganic phosphate test results) accounted for 21.7% of the variance and function 3 (solely the inorganic phosphate test results) accounted for 4.7% of the variance within the model. The functions had canonical correlations of 0.731, 0.503, and 0.261 respectively. Since 95.3% of the total variance was accounted for by the first two functions, these were the functions used to create the

canonical plot that is seen in Figure 4.1. Table 4.2 shows the correlations between the discriminating variables and the standardized discriminant functions while Table 4.3 shows the Fisher's linear discriminant function coefficient for each isolate with each of the discriminating variables. Ultimately, the results from this test support the null hypothesis that most of these isolates are not significantly different from each other. However, isolates 126, 283, and 256 had negative Fisher's linear discriminant function coefficients with one or more of the discriminating variables; this indicates that these three isolates are significantly different from the other isolates. These trends can be seen in Figure 4.19 as the group centroids for isolates 126, 283, and 256 are separated from the remainder of the group centroids.

Table 4.2. Pooled within-group correlations between the discriminating variables (Temperature, pH, and inorganic phosphate) and the standardized canonical discriminant functions. The variables were ordered by size of correlation within each function.

	1	2	3
Temp	0.925	-0.067	0.373
Phosphate	0.466	0.882	-0.075
pH	0.314	0.148	0.938

Table 4.3. Fisher's linear discriminant functions were used to classify the function coefficient of each isolate for each test that was completed. These are the numerical function coefficients for each of the 10 isolates and were the values used to determine which isolates were statistically different from each other and during which tests these differences were observed. It can be seen that Isolate 126 was shown to have negative coefficients for both the phosphate and temperature tests while isolates 256 and 283 both had negative coefficients for the temperature range test.

Isolate	126	149	196	198	243	256	283	408	410	411
pH	2.876	1.414	2.294	1.971	1.432	2.915	2.826	2.101	2.099	1.545
Phosphate	-0.157	0.924	1.733	1.629	0.447	1.361	1.145	0.397	0.659	0.828
Temp.	-1.177	0.684	0.554	0.807	0.799	-2.433	-2.186	0.298	0.362	1.046

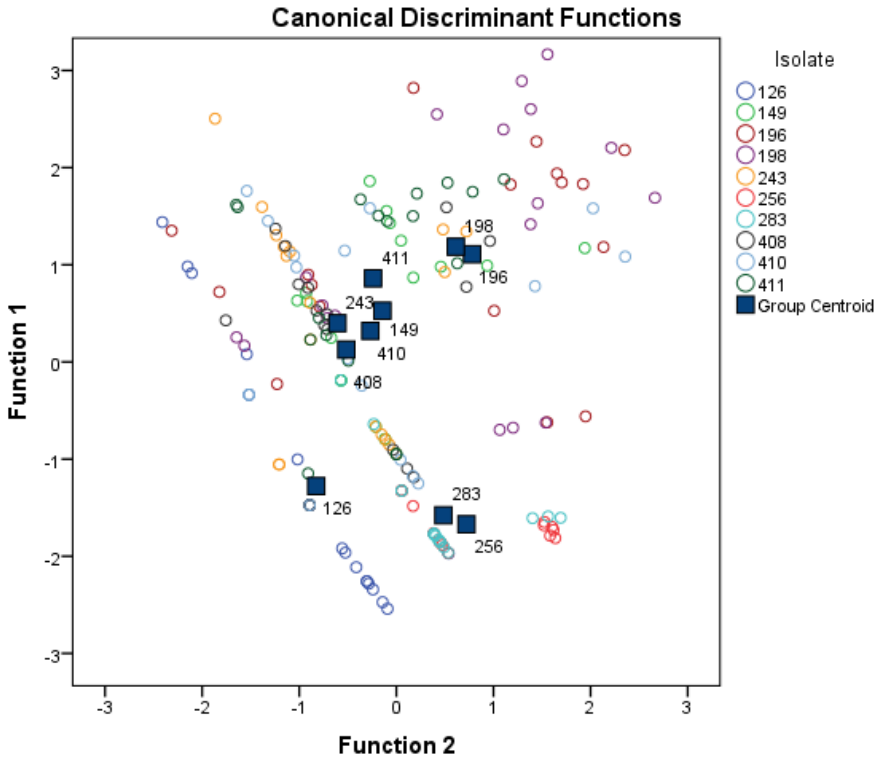


Figure 4.19. Canonical discriminant function plot to differentiate the ten isolates that were classified as being the most effective at utilizing inorganic phosphate. This plot was based on a linear discriminant function analysis of the isolate's responses the growth conditions that were tested. There are 18 replicates for each isolate indicated by the colour coded circles. The group centroids are indicated by the solid squares that are labelled with the isolate number.

Another discriminant function analysis was completed. This time, the lake and site that these 10 isolates originated from were pooled together and used as the independent variable. The solubilization index results from the inorganic phosphate range test were removed from the model as they accounted for the least amount of variance from the original model. A significant Box's M result was observed (Box's M= 118.37, $F_{18,\ 13455}$=6.23, p<0.001) which indicates that the covariance matrices were not homogenic, however once again, due to the robustness of the discriminant function analysis the results were used as is. This model showed that functions 1 through 3 were significant (χ^2= 58.57, p<0.001), along with functions 2 through 3 (χ^2= 25.32, p<0.001), but function 3 was not (χ^2= 3.85, p=0.05). In this model, Function 1 (discriminated between the temperature test results and the combined pH test and inorganic phosphate test results) accounted for 57.8% of the total variance within the model, function 2 (discriminated between the pH range test results and the inorganic phosphate test results) accounted for 36.1% of the variance within the model, while function 3 (solely the inorganic phosphate test results) accounted for 6.2% of the variance within the model. The functions had canonical correlations of 0.415, 0.339, and 0.147 respectively. Since Functions 1 and 2 accounted for 93.8% of the variance within the entire model, they were used to create the axes

for the canonical plot seen in Figure 4.20. Table 4.4 shows the correlations between the discriminating variables and the standardized discriminant functions while Table 4.5 shows the Fisher's linear discriminant function coefficient for each site with each of the discriminating variables. Ultimately the results for this analysis show that the location that the isolates originated from did impact how the isolates grew during the temperature, pH, and inorganic phosphate range tests.

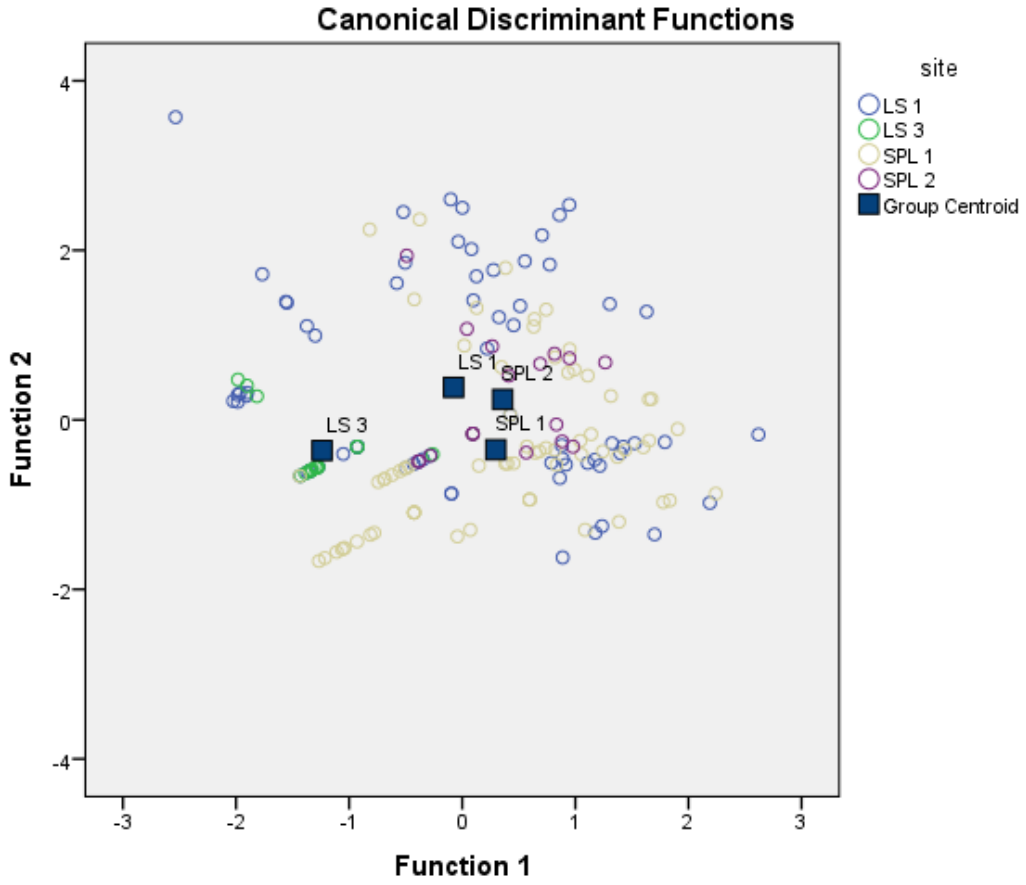
Table 4.4. Pooled within-group correlations between the discriminating variables (Temperature, pH, and inorganic phosphate) and the standardized canonical discriminant functions. The variables were ordered by size of correlation within each function.

	1	*2*	*3*
pH	-0.37	-0.25	1.22
Temperature	1.33	0.19	-0.44
Phosphate	-0.62	0.96	0.007

Table 4.5. Fisher's linear discriminant functions were used to classify the function coefficient of each site that each of the isolates tested originated. These are the numerical function coefficients for each of the sites and were the values used to determine which sites were statistically different from each other as we all as during which tests these differences were observed. For this table LS 1= Lake Simcoe (Atherley Narrows) Site 1, LS 3= Lake Simcoe (Atherley Narrows) site 3, SPL 1= Sparrow Lake site 1, and SPL 2= Sparrow Lake site 2. These are the same site locations that were used in Chapter 2. It can be seen in this table that temperature consistently had negative results with all of the sites except for Sparrow Lake site 2.

Site	*LS 1*	*LS 3*	*SPL 1*	*SPL 2*
pH	2.26	2.62	2.26	1.61
Temperature	-0.58	-1.82	-0.31	0.005
phosphate	0.97	0.98	0.21	0.64

Figure 4.20. Canonical discriminant function plot to differentiate the different sites that the ten isolates that were classified as being the most effective at utilizing inorganic phosphate originated from. This plot was based on a linear discriminant function analysis of the isolate's responses the growth conditions that were tested. There are 18 replicates for each isolate indicated by the colour coded circles. The group centroids are indicated by the solid squares that are labelled with the isolate number.



4.4 Discussion

The results for the temperature range test appeared to be consistent with those found in the previous studies. Studies completed by Mohammadi (2012), Sanjotha and Manawadi (2016), and Paul and Sinha (2017), all indicate that phosphate solubilizing bacterial isolates should be grown ideally at 30°C. The highest solubilization index observed was when one of the isolates had grown at 37°C (isolate 198, SI= 4.15 SD ±0.433). However, when the isolates were grown at 30°C for seven days, six of the ten isolates had a solubilization index over 3.0, this is consistent with the results of the other studies. A study completed by Johri, et al. (1999), tested the ability of 856 phosphate solubilizing bacterial isolates from a root-free terrestrial environment to grow under conditions of varying temperature, pH and salts. The study

completed by Johri et al. (1999) also found that the phosphate solubilizing bacteria produced the largest halos when the isolates were grown at 30°C, once again confirming that the results obtained in this study were similar to those found in previous research.

The isolates grew well at every pH concentration except for when the agar was at pH 13 before autoclaving. All of the isolates had a solubilization index over 2.0 when they grew on Pikovskaya's agar plates that had pH concentrations of 7 and 9. This result was consistent with the results of studies completed by Mohammadi (2012), and Sanjotha and Manawadi (2016). Both of those studies found that their phosphate solubilizing bacterial isolates grew best when they were grown on agar that was neutral or slightly alkaline pH. The study completed by Johri et al. (1999) also found that the phosphate solubilizing bacterial isolates that they tested grew best when the pH of the media was slightly alkaline.

The isolates had the most success growing on the regular Pikovskaya's agar which had 5g/L of tricalcium phosphate. Once again, this was consistent with the methods and results obtained by other studies (Mohammadi, 2012; Sanjotha and Manawadi, 2016; Paul and Sinha, 2017). Typically, PSB isolates are not tested on media that contained anything other than 5g/L of tricalcium phosphate ($Ca_3(PO_4)_2$) (Johri, et al., 1999; Mohammadi, 2012; Sanjotha and Manawadi, 2016; Paul and Sinha, 2017). The results of the study completed by Johri, et al. (1999) suggest that the addition of calcium salts compliments the solubilization efficiency of the phosphate solubilizing isolates that were isolated from the root-free soil.

Discriminant function analyses have been used in previous studies to confirm that differences between groups of microorganisms are significantly different from each other (Farr, et al., 1989; Jarvis & Goodacre, 2004; Leung, et al., 2004). The results from this study failed to do so as the bacterial isolates that were tested were too similar. That being said, the results from this discriminant function analysis were consistent with the results obtained from the Gram stain results that were completed on these ten isolates. Isolates 126, 283, and 256 were proved to be different from the other isolates which were again indicated by the different results that were obtained by these isolates when they were Gram stained. Isolate 283 was the only isolate that originated from Lake Simcoe (Atherley Narrows) site 3, and this bacterial

isolate was isolated from the sediment in August. Isolate 126 was collected from Sparrow Lake site 1 in July and isolate 256 was collected from Lake Simcoe (Atherley Narrows) site 1 in August. The second discriminant function analysis completed showed that the site that these 10 isolates originated from did not provide any additional variation as to how the isolates performed during the temperature, pH, and inorganic phosphate range tests.

The low variation in phosphate solubilizing bacteria could be due to a few factors. Firstly, as Correll (1998) demonstrated in his study, phosphate solubilizing bacteria tend to lose their phosphate solubilizing activity after repeated sub-culturing, with only a few exceptions. As the isolates were isolated from the sediment once and repeatedly sub-cultured since that time, it could be that only specific strains of phosphate solubilizing bacteria maintain phosphate solubilizing activity over time, and that is why seven out of the ten isolates that were tested here are not statistically different from each other, are all rod-shaped and were Gram-positive bacteria. Also, Sanjotha and Manawadi (2016) found that of the five-phosphate solubilizing bacterial isolates that they got genetically sequenced were all rod-shaped, and three of the five were Gram-negative. Paul and Sinha (2017) found that most of their bacterial isolates were Gram-negative rod-shaped bacteria as well. Meanwhile, in Pal's (1998) study, it was found that there were more Gram-positive phosphate solubilizing bacteria than Gram-negative, but once again, the majority of the isolates were rod-shaped. Gaind and Gaur (1991), also tested ten phosphate solubilizing bacterial isolates in their study and found that 8 of the 10 isolates were Gram-positive. However, their study was a little more varied as only four of the ten isolates were rod-shaped. That being said, this study was completed on terrestrial phosphate solubilizing bacteria. These studies all indicate that while there may be some variation as to the Gram staining result of the phosphate solubilizing bacteria, it is quite common to find rod-shaped isolates. This could be another factor explaining why the majority of these isolates were not statistically different from each other.

4.5 Conclusion

The ten phosphate solubilizing bacterial isolates that were previously shown to be the most effective at solubilizing inorganic phosphate were characterized based on their ability to

grow at various temperatures, pH concentrations, tri-calcium phosphate concentrations, Gram stain, and cell morphology. The results observed by these isolates supported the work done by previous research. The previous research indicates that phosphate solubilizing bacteria as a group would be more mesophilic as all of the isolates screened for this test thrived when they were incubated at 30° C (Sanjotha & Manawadi, 2016). The isolates also grew best when they were in media that varied from neutral to only slightly alkaline pH levels although the isolates did grow when they were in more acidic media as well. The isolates also grew best when only 5g/L of tri-calcium phosphate was present in the media.

The discriminant function analysis supported the null hypothesis that the ten isolates were not statistically different from each other. Analyzing the 10 isolates with respect to their location of origin did not change this result. However, trends that were seen within the discriminant function analysis of the isolates and the Gram staining test showed that isolates 126, 283, and 256 were the only three that were significantly different from the others. These isolates originated from Sparrow Lake site 1 in July, Lake Simcoe (Atherley Narrows) site 3 in August, and Lake Simcoe (Atherley Narrows) site 1 in August respectively. The other seven isolates were all Gram-positive, rods, and all had positive function coefficients(Table 4.3) with the temperature, pH, and calcium phosphate tests. These tests were good preliminary tests that could be used to classify phosphate solubilizing bacteria. In future work, other factors such as the time spent allowing the isolates to grow, the concentration of salts, and continued sub-culturing of the phosphate solubilizing bacteria may be factors of interest that could be used along with other biochemical tests to classify the phosphate solubilizing bacteria.

Chapter 5: General Discussion and Conclusions.

Eutrophication and its associated consequences are some of the most detrimental factors that impact the health of freshwater lakes (Dodds & Whiles, 2010). Internal phosphorus loading has been identified as being an area of particular concern, especially on lakes where total phosphorus has been added to the system for decades, a local example of a lake like this is Lake Simcoe (Winter et al., 2007; Nürnberg et al., 2013; Palmer, et al. 2011). Lake Simcoe is both an ecologically and economically important lake in central southern Ontario, and as such, many measures have been put in place to try to limit the amount of phosphorus that enters the lake. However, these strategies on Lake Simcoe fail to address how to mitigate the effects that internal phosphorus loading has on the Lake. This could be due to the lack of research that has been done on internal phosphorus loading. Phosphate solubilizing bacteria are known as a group of bacteria that contribute to internal phosphorus loading due to various metabolic processes (Ponmurugan & Gopi, 2006).

In this study, phosphate solubilizing bacteria were studied in an attempt to understand if these microorganisms could potentially be used as an indicator of phosphorus pollution. This study was completed on the northern part of Lake Simcoe to understand the microorganisms that potentially contribute to internal phosphorus loading in this lake, as well as to classify phosphate solubilizing bacteria in this freshwater system.

In order to understand where phosphate solubilizing bacteria would be most abundantly present, three sites were selected along the northwestern shore of Lake Simcoe, and three sites along the shoreline of Sparrow Lake. The three sites along Lake Simcoe were within the City of Orillia, and as such, it was thought that these sites would be under lots of anthropogenic stress due to the presence of wastewater treatment plants, the presence of anthropogenic structures (i.e., waterfront housing and infrastructure), and fishing and water sporting activities. While there was some evidence of anthropogenic impact at the Sparrow Lake sites, one of the sites was along the edge of a protected wetland, and it was thought that these sites are far less impacted than their Lake Simcoe counterparts. The sampling was carried out four times to see

if the abundances of the phosphate solubilizing bacteria would change with the season. Representative bacterial isolates were tested in the lab to assess their abilities to solubilize inorganic phosphate on solid agar and in a liquid broth. The isolates that appeared to utilize inorganic phosphate were characterized on their ability to grow at various temperatures, pH levels, and the amount of calcium phosphate present.

The objectives of this study were to isolate and characterize the phosphate solubilizing bacteria that were present in the sediment and water column in the nearshore sites on Lake Simcoe and Sparrow Lake; along with monitoring the rate of phosphate solubilization of these bacteria; and test to see which isolates were the most efficient ones at utilizing inorganic phosphate. All of these objectives were addressed over three chapters; the field study (Chapter 2), and laboratory experiments (Chapter 3 and Chapter 4).

Firstly, the four-month field study was completed to compare any changes in the abundances of phosphate solubilizing bacteria between the two lakes and to see if seasonality should be studied on these microorganisms in the future. Ultimately, a more in-depth study would be needed to assess any changes in the abundance of phosphate solubilizing bacteria at any of the sampled locations as it was found that the abundance of these microorganisms could be correlated with weather events that took place a week or so before sampling. It was also found that positive relationships existed between the abundance of phosphate solubilizing bacteria and the water temperature, amount of total suspended solids, chlorophyll *a*, and abundance of total heterotrophic bacteria. However, the results that were obtained during this study reject the hypothesis that a higher abundance of phosphate solubilizing bacteria at the anthropogenically stressed sites. Throughout the four-month sampling period, it was found that the site on Sparrow Lake that was adjacent to the protected wetland had the highest abundances of phosphate solubilizing bacteria.

A preliminary study was completed on a sediment core that was collected from each lake to compare the amount of total reactive phosphorus in the sediment to the abundance of phosphate solubilizing bacteria. The results from this portion of this study indicated that there was little difference between the abundance of phosphate solubilizing bacteria in relation to

the amount of total reactive phosphorus that was in the sediment. However, further study would be required in order to either reject or accept the hypothesis that more of these microbes would be present when there was a fair amount of phosphorus available in the sediment as the samples that were collected were too sparse to make any significant conclusions.

In September 2017, phosphate solubilizing bacteria isolates were collected from the Lake Simcoe Atherley Narrows sites, as well as three additional sites from the most southern portion on the lake (Cook's Bay). When these different locations within Lake Simcoe were compared against each other, it was found that Cook's Bay had a significantly higher abundance of phosphate solubilizing bacteria than others. However, this cannot be correlated with a higher amount of total phosphorus present in the water at this time as the total phosphorus amounts were reasonably consistent between the sites when they were sampled. A more in-depth study on Lake Simcoe would be useful to understand the reasons for the higher abundance of phosphate solubilizing bacteria in Cook's Bay.

Secondly, after the phosphate solubilizing bacteria had been isolated and purified, they were subjected to two laboratory experiments to assess their ability to utilize inorganic phosphate. The first experiment was a 216-hour growth test to create a phosphate solubilization index for each of the isolates. For this, each isolate was aseptically transferred to the center of a Pikovskaya's agar plate and incubated at 30°C. After 48 hours, the colony and halo diameters of each isolate were measured daily. From these readings, phosphate solubilization index was calculated. An index number of zero indicated no growth of the isolate and any number higher than one indicated larger size of the halo than the size of the bacterial colony (Paul & Sinha, 2017). The 61 isolates that had the highest phosphate solubilization index were used for the inorganic phosphate test.

The inorganic phosphate test was completed in 72 hours in Pikovskaya's broth. After this time the isolates were destroyed, and the amount of total phosphorus that was in the broth for each isolate was measured. The results were more variable in this experiment than expected, but this could have been due to the timeframe of the experiment or due to how

many times the isolates were sub-cultured prior to this experiment (Correll, 1998). While there may have been a few errors in this test, it was still relatively straightforward to quantitatively determine how much inorganic phosphate was used by each isolate. Both the phosphate solubilization index and the inorganic phosphate test were efficient tools for preliminary screening on the bacterial isolates for their phosphate-solubilizing ability.

The final series of laboratory tests that were completed were to characterize the ten isolates that recorded the highest solubilization index and showed promising results on the inorganic phosphate utilization test. These ten isolates were grown at various temperatures, pH, and inorganic phosphate concentrations to characterize the isolates with respect to varying growth conditions. The present study results supported the previous studies. The results obtained supported the theory that these microorganisms do contribute to internal phosphorus loading in this area as they grew reasonably well in cooler temperatures.

A discriminant function analysis compared the growth conditions (temperature, pH, inorganic phosphate) on these isolates. A second discriminant function analysis compared these isolates responses to varying growth conditions to the sites that these isolates originated from. The results of the first discriminant function analysis showed that three of the isolates were significantly different from the others. This result was further supported by the results of gram stain and cell morphology and helped to explain why Lake Simcoe (Atherley Narrows) site 3 was the only site that appeared different than the others on the second discriminant function analysis that was completed. These isolates originated from Isolate 126 originated from Sparrow Lake site 1 in July, isolate 283 originated Lake Simcoe (Atherley Narrows) site 3 in August (it was also the only isolate out of the ten that were characterized that originated from that site), and isolate 256 originated from Lake Simcoe (Atherley Narrows) site 1 in August. The next step in this research would be to get these ten isolates genetically sequenced in order to determine which species they belong.

Generally, the highest abundances of these bacteria were observed in the locations that had the lowest total phosphorus concentrations. This could be indicative of there being higher amounts of inorganic phosphate in the sediment at those locations rather than higher

abundances of soluble phosphate, which may have contributed to the lower abundances of phosphate solubilizing bacteria at the Lake Simcoe Atherley Narrows sites. If this experiment were to be completed again in the future, it might be a good idea to look at the amount of inorganic phosphate in the sediment at the site locations as opposed to the total phosphorus concentrations that were in the water column. The results observed suggest that phosphate solubilizing bacteria could potentially be used as an additional indicator of aquatic health. However, this study suggests that phosphate solubilizing bacteria would not be a useful indicator of phosphorus pollution in the water column if used alone. The data collected in this study contributes to the understanding of phosphate solubilizing bacteria in these aquatic habitats. Further study into these microorganisms would be needed to confirm these observations.

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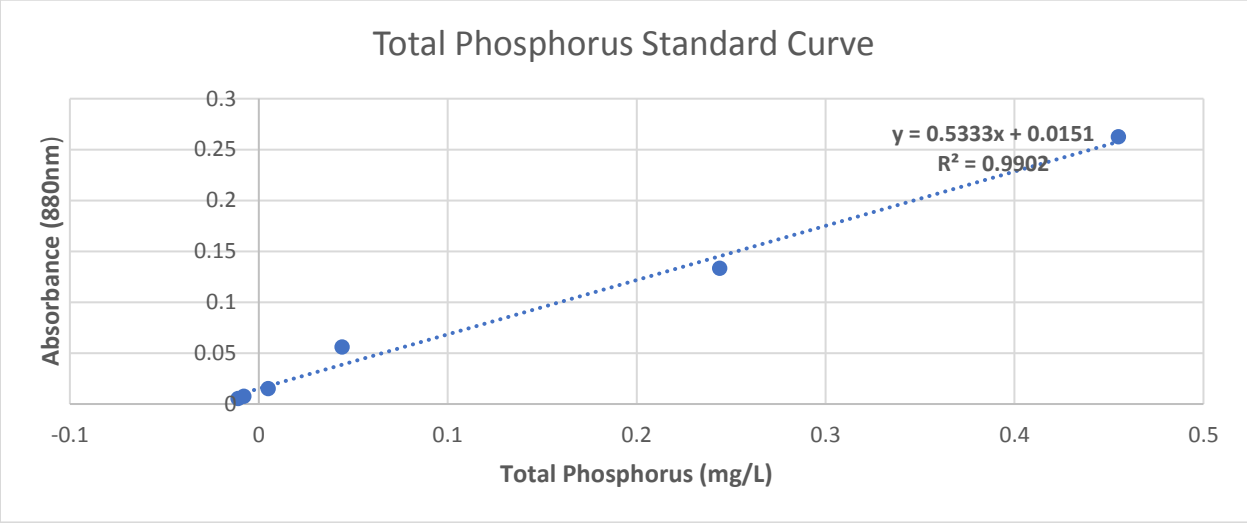
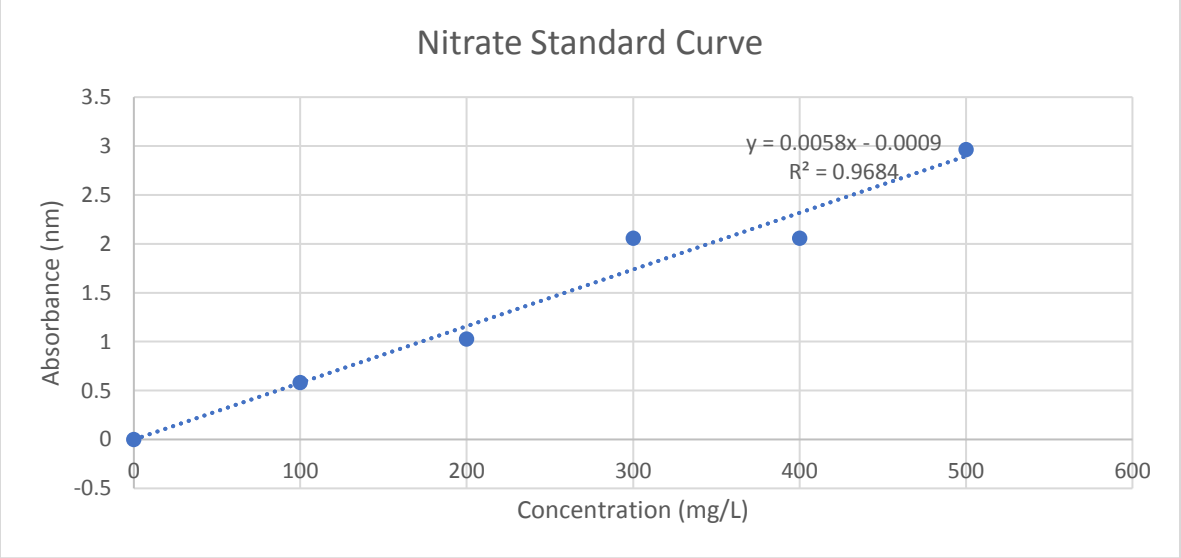
Appendix I

The total phosphorus standard curve was created using the methodology from the American Public Health Association (1995). The stock solutions that were used were 0, 40,80,100, 400, 800, and 1000mg/L.



The nitrate standard curve was created using the methodology from the American Public Health Association (1995). The stock solutions that were used were 0, 100,200,300, 400, and 500mg/L.



Appendix II

The Solubilization Index for each isolate that underwent the solubilization index test. The month, lake, and site that each isolate was collected from is also indicated on the following chart. The solubilization index indicates the isolate's ability to use the inorganic phosphate if the SI number is larger than 1. The codes for the lakes are LS=Lake Simcoe-Atherley Narrows, SPL= Sparrow Lake, and CB= Lake Simcoe- Cook's Bay. The letter accompanying the site number indicates from which replicate the isolate came from.

Isolate	Month	Lake	Site	48hrs	72hrs	96hrs	120hrs	144hrs	168hrs	216 hrs
46	June	SPL	1A	0	0	0	0	0	0	0
47	June	SPL	1A	0	0	0	0	0	0	0
48	June	SPL	1A	0	0	0	0	0	0	0
49	June	SPL	1A	2.1	2.045455	2.083333	2.08111	2.07333	2.066667	2.071429
50	June	SPL	1A	0	0	0	0	0	0	0
51	June	SPL	1B	1	1	1	1	1	2.076923	2.153846
52	June	SPL	1B	1	1	1	1	1	2.181818	2.3
53	June	SPL	1B	0	0	0	0	0	0	0
54	June	SPL	1B	0	0	0	0	0	0	0
55	June	SPL	1B	1	2.357143	2.4	2.433331	2.5555	2.647059	2.352941
56	June	SPL	1C	0	0	0	0	0	0	0
57	June	SPL	1C	1	2.055556	2.055556	2.080808	2.166666	2.230769	2.2
58	June	SPL	1C	0	0	0	0	0	0	0
59	June	SPL	2A	2.1	2.090909	2.090909	2.181818	2.211111	2.25	2.142857
60	June	SPL	2A	0	0	0	0	0	0	0
61	June	SPL	2A	0	0	0	0	0	0	0
62	June	SPL	2A	1	1	1	1	1	1	1
63	June	SPL	2C	1	1	1	1	1	1	1
64	June	SPL	2C	1	1	1	1	1	1	1
65	June	SPL	2C	1	1	1	1	1	1	1
66	June	SPL	2C	1	1	1	1	1	1	1
67	June	SPL	2C	1	1	1	1	1	1	1
69	June	SPL	3A	1	1	1	1	1	1	1

70	June	SPL	3A	1	1	1	1	1	1	1
71	June	SPL	3B	1	1	1	1	1	1	1
72	June	SPL	3B	1	1	1	1	1	1	1
73	June	SPL	3B	0	0	0	0	0	0	0
74	June	SPL	3B	0	0	0	0	0	0	0
75	June	SPL	3C	2.181818	2.307692	2.428571	2.818181	3.090909	3.214286	3.428571
76	June	SPL	3C	0	0	0	0	0	0	0
77	June	SPL	3C	1	1	3	3.181818	3.818181	3.666667	3
78	June	SPL	3C	0	0	0	0	0	0	0
80	June	LS	1A	0	0	0	0	0	0	0
81	June	LS	1A	1	1	1	1	1	1	1
82	June	LS	1A	1	1	1	1	1	1	1
83	June	LS	1A	1	1	1	1	1	1	1
84	June	LS	1A	1	1	1	1	1	1	1
85	June	LS	1B	0	0	0	0	0	0	0
86	June	LS	1B	1	1	1	2.1	2.3	2.272727	2.166667
87	June	LS	1B	0	0	0	0	0	0	0
88	June	LS	1B	0	0	0	0	0	0	0
89	June	LS	1C	1	1	1	1	1	1	1
90	June	LS	1C	0	0	0	0	0	0	0
91	June	LS	1C	1	1	1	1	1	1	1
93	June	LS	1C	1	1	2.571429	2.444444	2.555556	2.6	2.6
94	June	LS	2A	1	1	1	1	1	1	1
95	June	LS	2A	1	1	1	1	1	1	1
96	June	LS	2A	1	1	1	1	1	1	1
97	June	LS	2A	1	1	1	1	1	1	1
98	June	LS	2B	1	1	1	1	1	1	1
99	June	LS	2B	1	1	1	1	1	1	1
100	June	LS	2B	1	1	1	1	1	1	1
101	June	LS	2B	1	1	1	1	1	1	1
102	June	LS	2C	0	1	1	1	1	1	1

103	June	LS	2C	0	1	1	1	1	1	1
104	June	LS	2C	0	1	1	1	1	1	1
105	June	LS	2C	0	1	1	1	1	1	1
106	June	LS	2C	0	1	1	1	1	1	1
107	June	LS	2C	0	1	1	1	1	1	1
108	June	LS	3A	0	1	1	1	1	1	1
109	June	LS	3A	0	1	1	1	1	1	1
110	June	LS	3A	0	1	1	1	1	1	1
111	June	LS	3A	0	0	0	0	0	0	0
112	June	LS	3A	0	0	0	0	0	0	0
113	June	LS	3B	0	0	0	0	0	0	0
114	June	LS	3B	1	1	1	1	1	1	1
115	June	LS	3B	1	1	1	1	1	1	1
116	June	LS	3B	1	1	1	1	1	1	1
117	June	LS	3B	1	1	1	1	1	1	1
118	June	LS	3C	0	0	0	0	0	0	0
119	June	LS	3C	1	1	1	1	1	1	1
120	June	LS	3C	0	0	0	0	0	0	0
121	July	SPL	1A	1	1	2.083333	2.25	2.230769	2.615385	2.714286
122	July	SPL	1A	0	1	1	1.222222	1.333333	1.377778	2.130435
123	July	SPL	1A	1	1	2.071429	2	2.133333	2.133333	2.176471
124	July	SPL	1A	0	0	1	1	2.222222	2.111111	2.333333
125	July	SPL	1A	0	0	0	0	1	1	1
126	July	SPL	1B	1	1	2.222222	2.333333	2.444444	2.3	2.5
127	July	SPL	1B	1	1	1	1	1	1	1
128	July	SPL	1B	0	0	1	1	1.769231	2.25	2.230769
129	July	SPL	1B	1	1	2.222222	2.4	2.8	2.818182	2.818182
130	July	SPL	1B	3.666667	3.515151	3.4	3.454545	3.818182	3.461538	4.083333
131	July	SPL	1C	2.666667	2.818181	3.3	3.7	3.727273	3.909091	4.090909
132	July	SPL	1C	2.9	2.9	3.416667	3.5	2.444444	3.571429	3.785714
133	July	SPL	1C	1	1	1	1	1	1	1

134	July	SPL	1C	1	1	1	1	1	1	1
135	July	SPL	1C	2.7	2.7	3.454545	3.5	3.692308	3.571429	3.785714
136	July	SPL	2A	1	1	1	1	1	1	1
137	July	SPL	2A	1	1	1	1	1	1	1
138	July	SPL	2A	1	1	1	1	1	1	1.375
139	July	SPL	2A	1	1	1	1	1	1	1
140	July	SPL	2A	0	0	0	0	0	0	0
141	July	SPL	2B	0	0	0	0	0	0	0
142	July	SPL	2B	0	1	2.875	3.333333	3.5	4.1	3.909091
143	July	SPL	2B	1	1	1	1	2.142857	2.25	2.25
144	July	SPL	2B	0	0	0	0	0	0	0
145	July	SPL	2B	0	0	0	0	0	0	0
146	July	SPL	2C	0	0	0	0	0	0	0
149	July	SPL	2C	0	0	2.25	2.25	2.375	2.428571	2.5
151	July	SPL	3A	0	0	1	1	1	1	1
152	July	SPL	3A	0	0	0	0	0	0	0
153	July	SPL	3A	1	1	1	1	1	1	1
154	July	SPL	3A	1	1	1	1	1	1	1
156	July	SPL	3B	1	1	2.153846	2.153846	2.230769	2.333333	2.133333
157	July	SPL	3B	0	0	1	1	1	1	2.076923
160	July	SPL	3B	0	0	1	1	1	2.222222	2.222222
161	July	SPL	3C	1	1	1.588235	1.611111	2.05	2.1	2.1
162	July	SPL	3C	0	0	0	0	0	0	0
163	July	SPL	3C	0	0	0	0	0	0	0
164	July	SPL	3C	1	1	1	1	1	1	1
165	July	SPL	3C	0	0	0	0	0	0	0
166	July	SPL	1W	1	1	1	1	1	1	1
167	July	SPL	1W	0	0	0	0	0	0	0
168	July	SPL	1W	1	1	1	1	1	1	1
171	July	SPL	2W	0	0	0	0	0	0	0
172	July	SPL	2W	1	1	1	1	1	1	1

173	July	SPL	2W	1	1	1	1	1	1	1
174	July	SPL	2W	1	1	1	1	1	1	1
175	July	SPL	2W	0	0	0	0	0	0	0
176	July	SPL	3W	1	1	1	1	1	1	1
177	July	SPL	3W	0	0	0	0	0	0	0
178	July	SPL	3W	1	1	2.2	2.181818	2.25	2.230769	2.142857
179	July	SPL	3W	1	1	2.2	2.4	2.5	2.428571	2.375
180	July	SPL	3W	0	0	0	0	0	0	0
181	July	LS	1A	0	0	0	0	0	0	0
182	July	LS	1A	1	1	1	1	1	1	1
183	July	LS	1A	1	1	1	1	1	1	1
187	July	LS	1B	0	0	0	0	0	0	0
188	July	LS	1B	1	1	1	1	1	1	1
189	July	LS	1B	1	1	2.083333	2.076923	2.066667	2.133333	2.2
190	July	LS	1B	0	0	1	1	1	1	1
191	July	LS	1C	0	0	1	1	1	2.176471	2.235294
192	July	LS	1C	0	0	0	0	0	0	0
194	July	LS	1C	1	1	1	1	1	1	1
196	July	LS	1W	0	1	3	4	3.5	3.666667	3.666667
197	July	LS	1W	0	0	0	0	0	0	0
198	July	LS	1W	0	0	2.571429	2.666667	2.75	3	3.285714
199	July	LS	1W	0	0	0	0	0	0	0
200	July	LS	1W	0	0	0	0	0	0	0
201	July	LS	2A	0	1	1	1	1	1	1
202	July	LS	2A	0	0	0	0	0	0	0
203	July	LS	2A	0	1	1	1	1	1	1
204	July	LS	2A	1	1	1	1	1	1	1
205	July	LS	2A	0	0	0	0	0	0	0
206	July	LS	2B	1	1	1	1	1	1	1
207	July	LS	2B	0	1	1	1	1	1	1
208	July	LS	2B	0	0	0	0	0	0	0

209	July	LS	2B	1	1	1	1	1	1	1
210	July	LS	2B	1	1	1	1	1	1	1
212	July	LS	2C	0	0	0	0	0	0	0
213	July	LS	2C	1	1	1	1	1	1	1
214	July	LS	2C	1	1	1	1	1	1	1
215	July	LS	2C	0	0	0	0	0	0	0
216	July	LS	2W	1	1	2.083333	2.076923	2.142857	2.066667	2.133333
217	July	LS	2W	2.125	2.181818	2.333333	2.555556	2.5	2.454545	2.416667
219	July	LS	2W	4.25	3.333333	3.142857	3.285714	3.666667	3.666667	2.176471
220	July	LS	2W	2.714286	3	2.777778	3.125	3.125	2.888889	3
221	July	LS	3A	1	1	1	1	1	1	1
222	July	LS	3A	1	1	1	1	1	1	1
223	July	LS	3A	1	1	1	1	1	1	1
224	July	LS	3A	0	0	0	0	0	0	0
225	July	LS	3A	1	1	1	1	1	1	1
226	July	LS	3B	1	1	1	1	1	1	1
229	July	LS	3B	1	1	1	1	1	1	1
230	July	LS	3B	1	1	1	1	1	1	1
231	July	LS	3C	1	1	1	1	1	1	1
232	July	LS	3C	1	1	1	1	1	1	1
233	July	LS	3C	0	0	0	0	0	0	0
234	July	LS	3C	0	0	0	0	0	0	0
235	July	LS	3C	1	1	1	1	1	1	1
236	July	LS	3W	1	1	1	1	1	1	1
237	July	LS	3W	0	0	0	0	0	0	0
238	July	LS	3W	1	1	1	1	1	1	1
241	AUG	LS	1A	0	1	1	1	1	1	1
242	AUG	LS	1A	0	1	1	1	1	1	1
243	AUG	LS	1A	1	2.090909	2.166667	2.25	2.333333	2.307692	2.461538
244	AUG	LS	1A	1	1	1	1.625	1.705882	2.058824	2.176471
245	AUG	LS	1A	1	1	1	1	1	1	1

246	AUG	LS	1B	1	1	1	1	1	1	1
247	AUG	LS	1B	1	1	1	1	1	1	1
248	AUG	LS	1B	1	1	1	1	1	1	1
249	AUG	LS	1B	1	1	1	1	1	1	1
250	AUG	LS	1B	1	1.9	2.363636	2.454545	2.545455	2.636364	2.818182
253	AUG	LS	1C	1	1	1	1	1	1	1
254	AUG	LS	1C	1	1	1	1	1	1	1
255	AUG	LS	1C	1	1	2.1	2.1	2.090909	2.181818	2.166667
256	AUG	LS	1W	1	1	2.285714	2.125	2.111111	2.111111	2.2
257	AUG	LS	1W	1	1	1	1	1	1	1
258	AUG	LS	1W	1	1	1	1	1	1	1
259	AUG	LS	1W	1	1	1	1	1	1	1
260	AUG	LS	1W	0	1	1	1	1	1	1
265	AUG	LS	2A	1	1	1	1	1	1	1
269	AUG	LS	2B	1	1	1	1	1	1	1
270	AUG	LS	2B	0	0	1	1	1	1	1
271	AUG	LS	2B	1	1	1	1	1	1	1
272	AUG	LS	2C	1	1	1	1	1	1	1
273	AUG	LS	2C	0	0	0	0	0	0	0
274	AUG	LS	2C	0	0	0	0	0	0	0
275	AUG	LS	2C	0	0	0	0	0	0	0
276	AUG	LS	2W	0	0	0	0	1	1	1
277	AUG	LS	2W	1	1	2	2	1.125	2.25	2.5
278	AUG	LS	2W	0	0	0	0	1	1	1
279	AUG	LS	2W	0	0	0	0	1	1	1
280	AUG	LS	2W	0	0	0	0	1	1	1
281	AUG	LS	3A	0	1	1	1	1	1	1
282	AUG	LS	3A	1	1	1	1	1	1	1
283	AUG	LS	3A	1	1	1	1	2.333333	2.333333	2.5
284	AUG	LS	3A	1	1	1	1	1	1	1
285	AUG	LS	3A	1	2.1	2.2	2.272727	2.363636	2.454545	2.333333

286	AUG	LS	3B	0	0	0	0	0	0	0
287	AUG	LS	3B	1	2.111111	2.1	2.3	2.272727	2.363636	2.25
288	AUG	LS	3B	1	2.111111	2.1	2.2	2.363636	2.363636	2.25
289	AUG	LS	3B	1	2.1	2.181818	2.166667	2.153846	2.153846	2.142857
291	AUG	LS	3C	0	0	0	0	0	0	0
292	AUG	LS	3C	0	0	0	0	0	0	0
293	AUG	LS	3C	1	1	1	1	1	1	1
294	AUG	LS	3C	1	1	1	1	1	1	1
295	AUG	LS	3W	1	1	1	1	1	1	1
296	AUG	LS	3W	1	1	1	1	1	1	1
297	AUG	LS	3W	1	1	1	1	1	1	1
298	AUG	LS	3W	0	0	0	0	0	0	0
299	AUG	SPL	1A	0	0	0	0	0	0	0
300	AUG	SPL	1A	0	0	0	0	0	0	0
301	AUG	SPL	1A	1	1	1	1	1.56	1.538462	1.576923
302	AUG	SPL	1A	2.026316	2.090909	2.113636	2.155556	2.222222	2.195652	2.142857
303	Aug	SPL	1A	1	1	2.090909	2.083333	2.153846	2.142857	2.266667
304	Aug	SPL	1B	2.25	2.5	2.885	2.888889	2.3	3	3.1
305	Aug	SPL	1B	2.375	2.414141	2.5	2.5	2.7	2.7	2.9
306	AUG	SPL	1B	0	0	0	0	0	0	0
307	AUG	SPL	1B	1	1	1	2.222222	2.3	2.4	2.5
308	AUG	SPL	1B	1.1	2.1	2.181818	2.25	2.230769	18	2.058824
309	AUG	SPL	1C	0	0	0	0	0	0	0
310	AUG	SPL	1C	1	1	1	1	1	1	1
311	AUG	SPL	1C	1	1	1	1	1	1	1
312	AUG	SPL	1C	1	1	2.25	2.222222	2.444444	2.4	2.4
315	AUG	SPL	1W	1	1	1	1	1	1	1
316	Aug	SPL	1W	1	1	2.076923	2.071429	2.214286	2.2	2.25
317	AUG	SPL	1W	0	0	0	0	0	0	0
318	AUG	SPL	1W	1	1	2.333333	2.363636	2.363636	2.363636	2.333333
323	AUG	SPL	2A	1	1	1	1	1	1	1

324	AUG	SPL	2A	1	1	1	1	1	1	1
325	Aug	SPL	2B	1	1	2.214286	2.2	2.333333	2.235294	2.235294
326	AUG	SPL	2B	1	1	1	1	1	1	1
327	AUG	SPL	2B	1	1	#DIV/0!	1	1	1	1
328	AUG	SPL	2B	1	1	1	1	1.846154	2.071429	2.133333
331	AUG	SPL	2C	0	0	1	1	1	1	1
332	AUG	SPL	2C	1	1	1	1	1	1	2.142857
333	AUG	SPL	2C	0	0	0	0	0	0	0
334	AUG	SPL	2W	0	0	0	1	1	1	1
335	AUG	SPL	2W	1	1	2.181818	2.076923	2.153846	2.071429	2.142857
336	AUG	SPL	2W	0	0	0	0	0	0	0
337	AUG	SPL	2W	1	1	2.285714	2.571429	2.625	2.75	2.454545
338	AUG	SPL	2W	1	2.25	2.25	2.444444	2.555556	2.4	2.333333
339	AUG	SPL	3A	1	1	1	1	1	1	1
340	AUG	SPL	3A	1	1	1	1	1	1	1
341	AUG	SPL	3A	0	0	0	0	0	0	0
343	AUG	SPL	3A	1	1	1	2.2	2.166667	2.125	2.111111
344	AUG	SPL	3B	0	0	0	0	0	0	0
345	Aug	SPL	3B	1	1	2.272727	2.166667	2.142857	2.2	2.2
346	AUG	SPL	3B	1	1	1	1	1	1	1
347	AUG	SPL	3B	1	1	1	1	1	1	1
348	AUG	SPL	3B	1	1	1	1	1.6875	2.133333	2.125
349	AUG	SPL	3C	1	1	1	1	1	1	1
350	AUG	SPL	3C	1	1	1	1	1	1	1
351	AUG	SPL	3C	0	0	0	0	0	0	0
352	AUG	SPL	3C	0	0	0	0	0	0	0
353	AUG	SPL	3C	0	0	0	0	0	0	0
356	AUG	SPL	3W	0	0	0	0	0	0	0
357	AUG	SPL	3W	1	1	1	1	1	1	1
358	AUG	SPL	3W	1	1	1	1	1	1	1
359	AUG	SPL	3W	1	1	1	1	1	1	1

360	AUG	SPL	3W	1	1	1	1	1	1	1
361	Sept	LS	1W	1	1	1	1	1	1	1
362	Sept	LS	1W	1	1	1	1	1	1	1
363	Sept	LS	1W	1	1	1	1	2.181818	2.181818	2.166667
364	Sept	LS	1W	1	1	2.1	2.090909	2.166667	2.25	2.333333
365	Sept	LS	1W	1	1	0	1	1	1	1
366	Sept	LS	2A	1	2.111111	2.2	2.3	2.272727	2.363636	2.333333
367	Sept	LS	2A	1	1	1	1	1	1	1
368	Sept	LS	2A	1	1	1	1	1	1	1
369	Sept	LS	2A	1	1	1	1	1	1	1
370	Sept	LS	2A	1	1	1	1	1	1	1
371	Sept	LS	2B	1	1	1	1	1	1	1
372	Sept	LS	2B	0	0	0	0	0	0	0
373	Sept	LS	2B	1	1	1	1	1	1	1
374	Sept	LS	2B	1.909091	2	2.083333	2.166667	2.153846	2.142857	2.142857
375	Sept	LS	2B	1	1	1	1	1	1	1
376	Sept	LS	2C	1.75	2.428571	2.222222	2.2	2.4	2.181818	2.25
377	Sept	LS	2C	1	2.111111	2.1	2.090909	2.181818	2.090909	2.181818
378	Sept	LS	2C	1	1	1	1	1	1	1
379	Sept	LS	2C	1	1	1	1	1	1	1
380	Sept	LS	2C	0	0	0	0	0	0	0
381	Sept	LS	2w	1	2.166667	2.111111	2.1	2.181818	2.090909	2.272727
382	Sept	LS	2w	1	1	1	1	2.083333	2	2.066667
383	Sept	LS	2W	1	1	1	1	1	1	1
384	Sept	LS	2W	1	1	1	1	1	1	1
385	Sept	LS	2W	1	1	1	1	1	1	1
386	Sept	LS	3A	1	1	1	2.1	2.2	2.2	2.090909
388	Sept	LS	3A	0	0	0	0	0	0	0
389	Sept	LS	3A	1	1	1	1	1	1	1
390	Sept	LS	3A	0	1	1	1	2.2	2.090909	2.083333
391	Sept	LS	3B	1	1	1	1	2.272727	2.083333	2.272727

392	Sept	LS	3B	0	0	0	0	0	0	0
393	Sept	LS	3B	0	0	0	0	0	0	0
394	Sept	LS	3B	0	0	0	0	0	0	0
395	Sept	LS	3B	0	0	0	0	0	0	0
396	Sept	LS	3C	0	0	0	0	0	0	0
397	Sept	LS	3C	1	1	1	1	1	1	1
398	Sept	LS	3C	1	2	2.125	2.125	2.222222	2.111111	2.2
399	Sept	LS	3C	2.222222	2.5	2.333333	2.428571	2.5	2.428571	2.571429
400	Sept	LS	3C	0	2.2	2.2	2.3	2.3	2.2	2.2
401	Sept	LS	3W	0	1	1	1	1	1	1
403	Sept	LS	3W	2.375	2.5	2.25	2.333333	2.333333	2.333333	2.333333
404	Sept	LS	3W	1	1	1	1	1	1	1
405	Sept	LS	3W	1	1	1	1	1	1	1
407	Sept	SPL	1A	1	1	1	1	1.333333	2.142857	2.25
408	Sept	SPL	1A	1	2.111111	2.222222	2.222222	2.3	2.444444	2.9
410	Sept	SPL	1A	1	1.928571	2.2	2.5	2.533333	2.785714	2.666667
411	Sept	SPL	1B	2.222222	2.555556	2.777778	2.7	2.7	3.333333	3.4
412	Sept	SPL	1B	1	1	2.363636	2.2	2.333333	2.4375	2.666667
413	Sept	SPL	1B	1	1	1	1	1	1	1
414	Sept	SPL	1B	1	2.083333	2.076923	2.230769	2.142857	2.214286	2.384615
415	Sept	SPL	1B	1	2.05	2.5	2.1	2.1	2.25	2.190476
417	Sept	SPL	1C	1	1.714286	2.142857	2.266667	2.333333	2.25	2.3125
418	Sept	SPL	1C	1	1	1	1	1	1	1
420	Sept	SPL	1C	1	2.1	2.090909	2.083333	2.166667	2.153846	2.071429
421	Sept	SPL	1W	1	2.125	2.111111	2.2	2.083333	2.166667	2.181818
422	Sept	SPL	1W	2.090909	2.181818	2.166667	2.166667	2.133333	2.2	2.1875
423	Sept	SPL	1W	1	1	1.909091	2.090909	2.090909	2.166667	2.076923
424	Sept	SPL	1W	1	1	1	2.1	2.2	2.181818	2.166667
425	Sept	SPL	1W	2.111111	2.2	2.076923	2.133333	2.133333	2.058824	2.055556
426	Sept	SPL	2W	1	2.166667	2.428571	2.333333	2.181818	2.076923	2.071429
427	Sept	SPL	2W	1	2.25	2.111111	2.1	2.090909	2.181818	2.181818

428	Sept	SPL	2W	2.142857	2.2	2.076923	2.142857	2.0625	2.058824	2.052632
429	Sept	SPL	2W	1	2.111111	2.1	14	2.181818	2.272727	2.153846
430	Sept	SPL	2W	2.142857	2.111111	2.222222	2.181818	2.153846	2.142857	2.2
431	Sept	SPL	3A	1	1	1	1	1	1	1
432	Sept	SPL	3A	1	1	2.1	2.1	2.1	2.2	2.2
433	Sept	SPL	3A	1	1	1	1	1	1	1
434	Sept	SPL	3A	1	1	1	1	1	1	1
435	Sept	SPL	3A	1	1	1	1	1	1	1
436	Sept	SPL	3B	1	1	1	1	2.090909	2.181818	2.3
437	Sept	SPL	3B	0	1	1	1	1	1	1
438	Sept	SPL	3B	0	0	1	1	1	1	1
439	Sept	SPL	3B	1	1	1	1	1	1	1
440	Sept	SPL	3B	1	1	2	1	2.25	2.25	2.222222
441	Sept	SPL	3C	1	1	1.909091	1.818182	2.25	2.083333	2.272727
442	Sept	SPL	3C	1	1	1	1	1	1	1
443	Sept	SPL	3C	1	1	2.111111	2.1	2.3	2.3	2.454545
444	Sept	SPL	3C	1	1	1	1	1	1	2.083333
445	Sept	SPL	3C	0	1	2.083333	2.076923	2.071429	2.142857	2.0625
446	Sept	SPL	3W	1	1	2.090909	2.181818	2.090909	2.076923	2.142857
447	Sept	SPL	3W	1	2.142857	2.166667	1.9	2.1	2.090909	2.090909
448	Sept	SPL	3W	1	1	1	1	1.363636	1	1.5
449	Sept	SPL	3W	0	0	0	1	1	1	2.14287
450	Sept	SPL	3W	0	0	1	1	1	1	2.25
451	Sept	SPL	2A	1	1	1.733333	2.058824	2.058824	2.058824	2.111111
452	Sept	SPL	2A	1	2.142857	2.375	2.75	3	3	3
453	Sept	SPL	2A	1	1	2.111111	2.1	2.090909	2.166667	2.153846
454	Sept	SPL	2A	1	1	1	1	2.071429	2.071429	2.142857
455	Sept	SPL	2B	0	1	1	1	1	1	2
456	Sept	SPL	2B	1	1	1	1	2.0625	2.125	2.058824
457	Sept	SPL	2B	1	1	1	1	1	1	1
458	Sept	SPL	2B	0	0	0	0	0	0	0

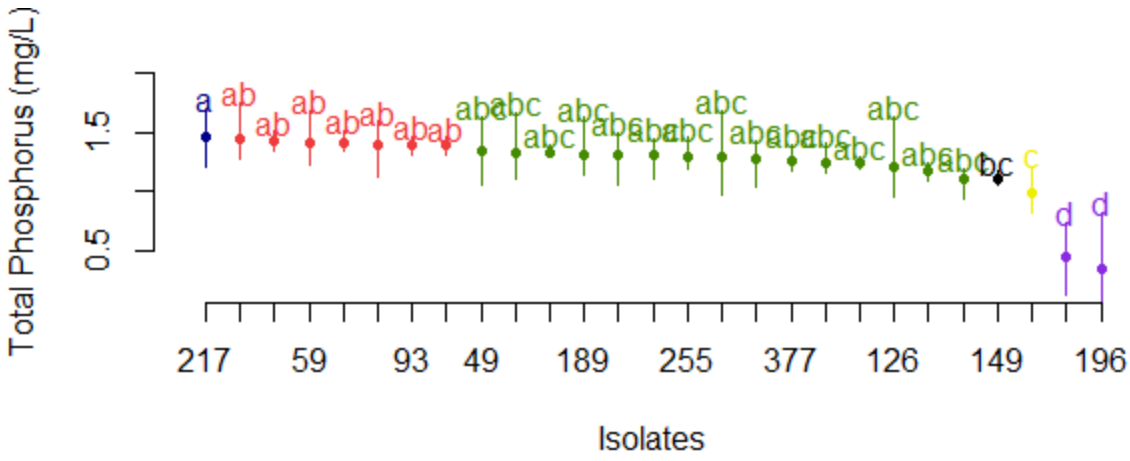
459	Sept	SPL	2C	1	1	1.692308	2.071429	2.133333	2.133333	2.25
460	Sept	SPL	2C	0	0	0	1	1	1	1
461	Sept	SPL	2C	1	1.083333	1	1	2.066667	2.066667	2.133333
462	Sept	SPL	2C	1	1	2.090909	2.076923	2.071429	2.071429	2.142857
463	Sept	LS	1A	0	1	1	1	1	1	1
464	Sept	LS	1A	1	1	1	2.076923	2.076923	2.066667	2.066667
465	Sept	LS	1A	0	0	0	0	0	0	0
466	Sept	LS	1B	2.071429	2.066667	1.928571	2.142857	2.066667	1.888889	2.055556
467	Sept	LS	1B	1	1	1	1.9375	1.941176	2.066667	2.052632
468	Sept	LS	1B	0	0	0	0	0	0	0
469	Sept	LS	1C	0	0	0	0	0	0	0
470	Sept	LS	1C	0	0	1	2.25	2.4	2.428571	2.444444
471	Sept	LS	1C	1	1	1	1	1	1	1
472	Sept	CB	1W	0	0	0	0	0	0	0
473	Sept	CB	1W	1	1	1	1	1	1	2.111111
474	Sept	CB	1W	1	1	1	1	1	1	1
475	Sept	CB	1A	1	2.1	2.181818	2.166667	2.166667	2.083333	2.076923
476	Sept	CB	1A	1	1	1	1	1	1	1
477	Sept	CB	2A	1	2.125	2	2.1	1	2.181818	2.272727
478	Sept	CB	2A	1	2.153846	2.076923	2.071429	2.066667	2.066667	2.125
479	Sept	CB	2W	1	1	1	1	1	1	1
480	Sept	CB	2W	1	1	1	1	1	1	1
481	Sept	CB	2W	1	1	2.090909	2.1	1.866667	2.066667	2.066667
482	Sept	CB	3A	0	0	0	1	1	1	1
483	Sept	CB	3A	1	1	1	2.083333	2.071429	2.153846	2.153846
484	Sept	CB	3A	0	1	1	1	1	1	1
485	Sept	CB	3W	0	1	1	1	1	1	1
486	Sept	CB	3W	1	2	2.125	2.2	2.2	2.083333	2.181818
487	Sept	CB	3W	0	0	0	0	0	0	0

Appendix III

Fisher's Least Significant Difference t-test results for the inorganic phosphate test, comparing the means of all 61 isolates that were tested in the inorganic phosphate test.

Isolates, TP means and individual (95 %) Confidence Intervals for each isolate. LCL=lower confid ence limit, UCL=upper confidence limit.

Isolate	T.P.	std	r	LCL	UCL	Min	Max
121	1.45	0.27	3	1.20	1.70	1.27	1.75
123	1.24	0.05	3	0.99	1.49	1.19	1.29
126	1.21	0.36	3	0.95	1.46	0.94	1.63
149	1.11	0.06	3	0.85	1.36	1.05	1.16
189	1.31	0.26	3	1.06	1.57	1.14	1.62
196	0.35	0.41	3	0.10	0.60	0.09	0.82
198	0.44	0.30	3	0.19	0.69	0.13	0.73
216	1.29	0.36	3	1.04	1.54	1.14	1.62
217	1.46	0.24	3	1.21	1.72	1.20	1.68
243	0.98	0.19	3	0.73	1.23	0.82	1.20
250	1.17	0.73	3	0.91	1.42	1.09	1.24
255	1.29	0.13	3	1.04	1.55	1.19	1.45
256	1.25	0.13	3	0.99	1.50	1.15	1.40
277	1.33	0.05	3	1.07	1.58	1.29	1.39
285	1.33	0.29	3	1.08	1.58	1.10	1.45
287	1.30	0.17	3	1.04	1.55	1.10	1.44
289	1.40	0.24	3	1.14	1.65	1.12	1.58
337	1.42	0.08	3	1.17	1.67	1.33	1.50
366	1.30	0.22	3	1.05	1.56	1.06	1.49
374	1.27	0.21	3	1.02	1.52	1.04	1.42
377	1.27	0.12	3	1.01	1.21	1.18	1.39
464	1.11	0.14	3	0.86	1.36	0.94	1.19
49	1.33	0.28	3	1.08	1.58	1.06	1.62
55	1.04	0.09	3	1.15	1.65	1.33	1.51
59	1.41	0.24	3	1.15	1.65	1.33	1.51
86	1.38	0.08	3	1.13	1.64	1.30	1.45
93	1.39	0.07	3	1.14	1.65	1.31	1.46



This figure visually demonstrates that the data that is shown in the above table. It visually illustrates that isolates 198 and 196 are the only isolates that were significantly different from the rest.