Microbial Methylation of Mercury in the North Harbor of Lake Superior

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Abstract

Mercury is a known contaminant in the North Harbour of Lake Superior due to the decommissioned "Thunder Bay Fine Papers" pulp and paper mill. The cause for concern for organisms in the lake and for humans occurs when mercury undergoes a transformation to methylmercury (MeHg). Methylmercury is a neurotoxin that has the ability of bioaccumulation and biomagnification- making it a primary concern for human health. The methylation of mercury can occur by a variety of mechanisms, including both biotic and abiotic, however methylation via microorganisms is considered the primary mechanism in most aquatic systems. Microorganisms that can carry out this reaction do so to lessen the toxic effects of mercury on themselves. There are several known microbes that will methylate mercury, such as sulfate reducing bacteria, however the diversity of microorganisms that can carry out this reaction goes beyond what researchers currently know. Recent studies have identified a gene correlated to methylation known as the *hgcAB* gene cluster. This research aims to answer two main questions: 1) are mercury-methylating microorganisms present in sediment samples from the North Harbour and if so, 2) which microorganisms are present? Amplification of the *hgcA* gene by PCR shows that the gene is present in sediment samples from the North Harbour. Sequencing experiments were inconclusive, and the identities of the microorganisms that contain the *hgcA* gene are unknown at this time.

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Abbreviations

MeHg- Methylmercury

SRB- Sulfate-reducing bacteria

IRB- Iron-reducing bacteria

AOC- Area of Concern

SEL- Severe Effect Level

THF- Tetrahydrofolate

DMSP- Dimethylsulphoniopropionate

CFeSP- Corrinoid iron-sulfur protein

PCR- Polymerase chain reaction

NCBI- National Center for Biotechnology Information

DNA- Deoxyribonucleic acid

RNA- Ribonucleic acid

qPCR- Quantitative PCR

DOC- Dissolved organic matter

ICP- Inductively coupled plasma

CVAFS- Cold Vapour Atomic Fluorescence Spectroscopy

BLAST- Basic Local Alignment Search Tool

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1.0 Introduction

Mercury is a heavy metal that is known to be toxic. It occurs naturally in the environment; however human activities have increased its presence (Rice et. al, 2014). Mercury will cycle through the atmosphere, land and water while undergoing various physical and chemical transformations. According to Díez (2009), the four main sources of mercury in the environment are:

> "1) natural sources; 2) current anthropogenic releases from mobilization of mercury impurities in raw materials; 3) current anthropogenic releases resulting from mercury used intentionally in products and processes; and 4) re-mobilization of historically-deposited anthropogenic mercury releases worldwide".

It can be present in various forms, but the most toxic form is methylmercury (MeHg), which is commonly found in aquatic environments (Díez, 2009).

MeHg is considered especially toxic due to its ability to bioaccumulate and biomagnify within the aquatic food chain, causing detrimental neurological damage to humans (Díez, 2009). Figure 1 depicts how bioaccumulation occurs. It is absorbed in the blood when ingested and has the ability to interact and bind to the sulfhydryl protein group, which can be found in cysteine. Once in the blood, MeHg can be distributed throughout the body and will accumulate in specific areas such as the brain, liver, kidneys and placenta. (Bernhoft, 2012). MeHg can easily cross the blood-brain barrier in humans, subsequently causing cellular functions within the body to fail, such as: enzymes, cell membrane function, and neuron delivery materials (Hong, Kim & Lee, 2012). Methylmercury can cause birth defects in fetuses, such as cerebral palsy. Postnatal effects include: paresthesia, visual and auditory impairments and chronic seizures (Bernhoft, 2012).

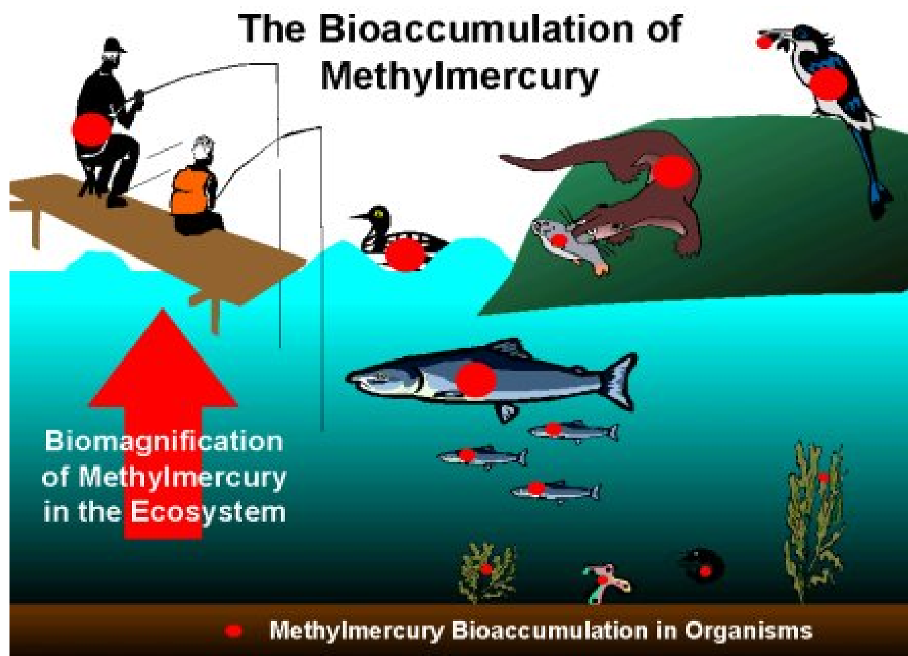

Figure 1: Bioaccumulation and biomagnification of MeHg (Government of Canada, 2013)

While there are many theories about the mechanism of the methylation of mercury, the processes are complex and not completely understood. The two main mechanisms of methylation occur through either abiotic or biotic methylation. Abiotic methylation is also known as chemical methylation, and the mechanism involves a methyl donor. These donors can be smaller organic molecules like methyliodide or organic components of dissolved organic matter, such as fulvic and humic acids. The transmethylation reaction involves the transference of either carbocationic Me+, carbanionic Me- or radical Me, depending on the methylating agent. Biotic methylation occurs as part of microbial metabolism. There are a variety of known microorganisms that are able to methylate mercury, including sulfate-reducing bacteria (SRB) and iron-reducing bacteria (IRB). Within biotic methylation, there are various mechanisms. A gene that is known to methylate mercury in microorganisms has been discovered and researched over the past few years, and it is known as the *hgcAB* gene cluster. Microbial methylation is considered to be the main mechanism in aquatic environments (Segade, Dias & Ramalhosa, 2011). The complexity and depth of potential biotic methylation mechanisms, including the important discovery of the *hgcAB* gene cluster, will be further addressed in the literature review.

In aquatic environments, methylation and demethylation of mercury are both occurring and to understand how much MeHg is present, the net amount must be determined. Similar to methylation mechanisms, demethylation and the factors affecting it are not well understood. Demethylation can occur through abiotic and biotic mechanisms, similar to methylation. Demethylation in the sediment is thought to be caused biotically, while demethylation in the water column is more commonly caused abiotically. Demethylation occurs naturally and is an inherent mechanism that can remove toxic MeHg from aquatic environments. In most studies, MeHg has a net-positive concentration, which leads researchers to assume that demethylation is occurring at a slower rate than methylation (Li & Cai, 2013).

There are a variety of routes of exposure of mercury and methylmercury, including the air, food and beverages, and some dental procedures. A very common source is through the consumption of fish and seafood from contaminated aquatic systems (Hong, Kim & Lee, 2012). Mercury and methylmercury contamination are an issue in Thunder Bay due to an old, decommissioned paper mill that once dumped pulp waste into the lake. The North Harbour of Lake Superior in Thunder Bay is considered a Great Lakes Area of Concern (AOC). An AOC is defined by Environment Canada as: “a location where environmental quality has been degraded compared to other areas in the Great Lakes, and beneficial uses of the aquatic ecosystem are impaired” (Environment Canada, 2010).

A paper mill, known by multiple names including Thunder Bay Fine Papers and Cascades Fine Papers, was built in 1918 and operated between 1920 and 2008. Operation was intermittent in its later years but was permanently closed in 2008. The mill was producing book paper and coated litho-paper (Winch et. al, 2013). Mercury contamination arose from the processes used within the pulp mill in its early years. The mill used a process known as Chlor-Alkali Manufacturing, also referred to as chlor-alkali mercury cells. The cells include an electrolyzer and a denuder, involving cathodes, made from mercury, and anodes. Throughout the electrolysis process, a brine produces sodium and chloride ions. When they reach the mercury cathode, the sodium ion combines with mercury (NaHg). This then leaves the cell and goes to the denuder, which is present next to the electrolyzer. Once it is here, water is added to free the mercury, which is recycled. Even though the Hg was recycled, it was still present in effluents,

emissions and solid waste from these types of plants, causing contamination (Paine, 1994). Water from Lake Superior was used in this paper-making processes and discharged back into the lake after waste-water treatment. The amount of contaminated sediment from these processes is 350,000 to 400,000 m^3, covering an area of around 22 hectares. The pulp matter that remains is up to 4 m deep in some areas (Milani & Grapentine, 2011).

It has been known that there is contamination in the North Harbour of Lake Superior for many years. Throughout this time, several environmental assessments have been performed on the area by Environment Canada and independent organizations (Milani & Grapentine, 2011; Winch et. al, 2013). The focus of these studies has varied, but the studies done by Environment Canada have been focused on the toxicity of sediment to benthic invertebrates. There are a number of chemicals that are present in the sediment, including lead, zinc and copper, however, mercury is the main concern. The topic of these studies is more specifically looking at the effects of mercury on fish and wildlife. Many of the studies have focused solely on mercury, however it is known that MeHg is of greater concern to human health. Understanding the levels of mercury can help in the understanding of the methylation of mercury, while also taking into account other factors involved in the process.

In Canada, there are maximum safe levels of mercury and methylmercury in aquatic environments, and these values offer a guideline for Environment Canada and other independent companies that provide assessments of the North Harbour. The Severe Effect Level (SEL) for mercury is 2 µg/g- values above this level indicate heavy contamination and the potential for negative health affects in aquatic organisms (Milani & Grapentine, 2011; Persaud, Jaagumagi and Hayton, 1993). In a 2011 assessment completed by Milani and Grapentine, they found levels of up to 11 µg/g of mercury in the North Harbour, as seen in Figure 2. This implies that the mercury present in high amounts could be transformed into high levels of methylmercury. There is less information the maximum safe levels of methylmercury in aquatic environments, however the average daily intake of methylmercury that could cause health effects is 0.23 µg per kilogram of body weight per day (Health Canada, 2004). This intake is often through fish living in contaminated aquatic environments. In the same study completed by Milani and Grapentine (2011), they found levels of methylmercury ranging from 4.18 ng/g to 111.14 ng/g as seen in

figure 3. This study, as well as several others have shown that the North Harbour is contaminated, and the contamination needs to be addressed and dealt with.

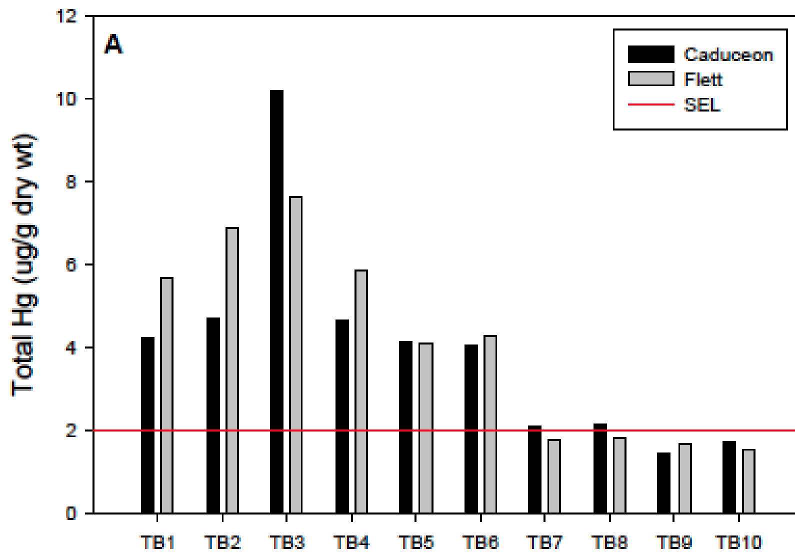

Figure 2: Levels of Mercury in the North Harbour of Lake Superior (Milani & Grapentine, 2011)

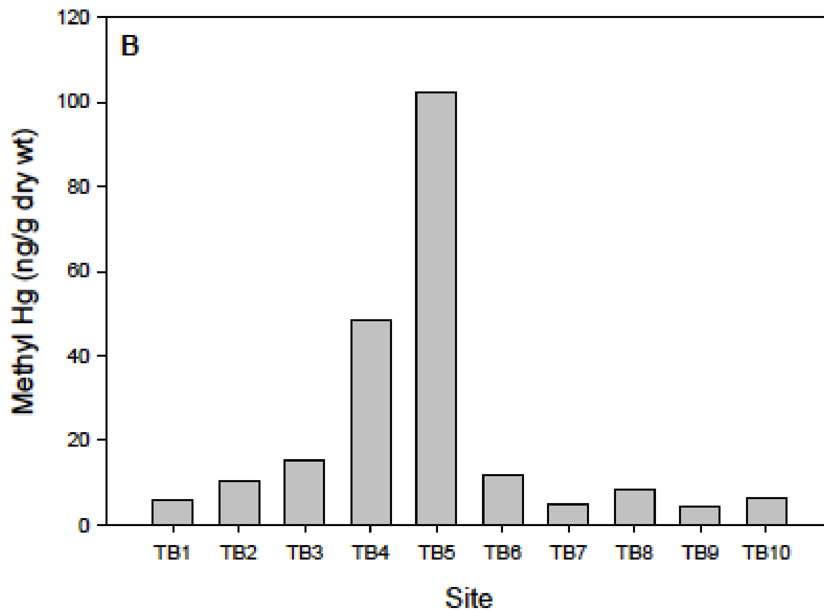

Figure 3: Levels of Methylmercury in the North Harbour of Lake Superior (Milani & Grapentine, 2011)

It is clear that there needs to be a solution for the contamination, however, the city of Thunder Bay and the Governments of Canada and Ontario have been attempting to develop a solution for years. There is debate whether or not clean-up is necessary. Dredging or capping the area are the main proposed clean-up solutions- however, no one has been willing to cover the expenses. Leaving the area as is could cause further issues and potential detrimental health effects. On the other hand, experts think that if methylation is not occurring at high rates, the danger is not as serious. Since the majority of methylation comes from microorganisms, the presence of methylating microbes can indicate levels of methylation. The goal of this thesis project is to determine if mercury methylating microorganisms are present in the North Harbour and causing methylation.

The specific objectives of this study include:

1. Determining the presence of methylating microorganisms in sediment from the contaminated area of the North Harbour of Lake Superior by amplifying the *hgcA* gene
2. Identifying the microorganisms that are present in the sediment samples through DNA sequencing

2.0 Literature Review

2.1 Biotic Methylation

Biotic methylation is considered the main mechanism of mercury methylation in aquatic environments. This is facilitated by certain microorganisms, including sulfate-reducing bacteria (SRB), iron-reducing bacteria (IRB) and sulfide and sulfur-oxidizers (Segade, Dias & Ramalhosa, 2011). Early work done by Jensen and Jernelöv (1969) was the first to demonstrate that methylation of mercury occurs in sediments that contain microorganisms. Since then, extensive research has been done into the potential mechanisms of methylation, factors that affect the process, and reasons why methylation occurs. This literature review aims to cover these topics and offer an in-depth analysis on the mercury methylating capabilities of microorganisms.

2.1.1 Why does biotic methylation occur?

Methylation of mercury occurs as part of the natural mercury cycle in the environment when oxidized mercury (Hg^{2+}) reacts with a methyl group (Figueiredo et. al, 2018). Methylation can either be enzymatically or non-enzymatically catalyzed (Ullrich, Tanton, and Abdrashitov, 2001). While the reasons for methylation are not fully understood, research on this topic showed evidence of bacterial mercury resistance as a mechanism for the methylation of mercury. There are a variety of mechanisms in which microorganisms can be resistant to mercury, including mercury methylation. While methylmercury is more toxic to humans, it is not as toxic as mercury to certain microbes, making it favourable for them to produce MeHg. The MeHg is less toxic because the microorganisms are able to either sequester the methylated form or the MeHg is volatilized from the cells of the microbe (Osborn et. al, 1997).

2.1.2 Where does biotic methylation occur?

Mercury methylation can occur in a variety of locations within aquatic environments. In the early work done by Jensen and Jernelöv (1969), the researchers were able to show that

methylation occurs in the sediment. As the research on this topic has continued and expanded, there is a better understanding of where methylation is occurring.

2.1.2.1 Sediment

Sediment methylation is considered a main source of methylmercury in aquatic environments, including freshwater lakes and marine environments (Paranjape & Hall, 2017). Early work completed by Rudd et. al (1983) demonstrated that methylation of mercury was mostly occurring in the surface sediment and water column of the Wabigoon River. Research done by Korthals and Winfrey (1987) and Matilainen (1995) confirmed this conclusion with their work. More recent studies have also shown that methylation occurs at greater rates at surface sediments compared to deeper sediment. For example, Liu et. al (2015) demonstrated that the rate of methylation decreases with increased depth into the sediment of the Northern Gulf of Mexico, suggesting higher microbial activity at the surface. Sediment is often studied when looking at methylation of mercury because of the high probability that methylating microbes are present, based on many years of research on this topic.

2.1.2.2 Water Column

Methylation also occurs in the water column of aquatic environments. While this methylation is happening to a lesser extent, it is potentially more important because of the much larger volume of water compared to sediment in aquatic environments (Ullrich, Tanton, and Abdrashitov, 2001). Methylation in the water column is occurring in marine environments, as well as fresh-water environments. Soerensen et. al (2016) determined that mercury methylation is occurring in the water column of the Arctic Ocean, and the majority of MeHg production is occurring 20-200m below the surface. In Canada, research has been done at several lakes to look at methylation potential in the water column. Eckley and Hintelmann (2005) determined that the greatest methylation potential in these lakes was at the hypolimnetic region, where anaerobic microorganisms were more prevalent. In addition, methylation rates varied depending on the time of year. It is clear that methylation is occurring in the water columns of aquatic environments, however, there is significant variation in different systems due to varied

microbiological communities, time of year, type of environment and more (Ullrich, Tanton, and Abdrashitov, 2001).

2.2 Proposed mechanisms of biotic methylation

2.2.1 Mechanisms associated with the acetyl CoA pathway

In 1969, Jensen and Jernelöv discovered the methylation of mercury via microorganisms in lakes and coastal regions of Sweden. They thought the transfer of the methyl group to Hg^{2+} came from methylcobalamin, a B12 derivative (methylcorrinoid). This methyl group is a part of the carbon monoxide dehydrogenase pathway in methanogens and other microorganisms. This process was believed to be a non-enzymatic transfer. Methylcobalamin was considered the most likely source of CH_3 due to its natural occurrence in aquatic environments (Ullrich, Tanton, and Abdrashitov, 2001). Around the same time as Jensen and Jernelöv, Bertilsson and Neujahr (1971) determined that methylcobalamin produced through metabolism of microorganisms can spontaneously methylate mercury (II) in aqueous solution, however, the mechanism of the methylation in natural environments was still largely unknown.

Methylation of mercury was thought to occur by different types of microorganisms, including aerobes, anaerobes and facultative anaerobes, however, anaerobes were thought to be the main methylating population. In 1985, Compeau and Bartha reported their findings after a series of inhibition-stimulation experiments. They determined that sulfate-reducing bacteria were the principal methylators of mercury. They were able to demonstrate the continued increased methylation of mercury when a methanogen inhibitor was used in HgCl-spiked sediment. When an inhibitor of sulfate reducers was added to the spiked sediment, the methylation of mercury significantly decreased (95%) (Compeau and Bartha, 1985). These results paved the way for further research into the mercury methylation mechanism using sulfate-reducing bacteria.

In 1994, Choi, Chase and Bartha studied the sulfate-reducing bacteria, *Desulfovibrio desulfuricans,* in order to determine the mechanism of mercury methylation. Their research determined that the source of the methyl group originated from either the serine C3 or formate

that are involved in the acetyl-CoA pathway. Figure 4 depicts a proposed pathway for the mechanism, adapted from the work of Choi et. al (1994). Within this process, the methyl group attached to tetrahydrofolate (THF) is transferred to the corrinoid protein (methylcobalamin) via methyltransferase I and finally, methyltransferase II enzymatically methylates Hg. The question of whether or not mercury methylation occurs spontaneously or enzymatically is seemingly answered by Choi, Chase and Bartha, and this proposed mechanism became the most well-studied and universally accepted regarding mercury methylation. The mechanism and involvement of the acetyl CoA pathway was confirmed through continued research, including enzyme-inhibition experiments (Ekstrom, Morel, & Benoit, 2003).

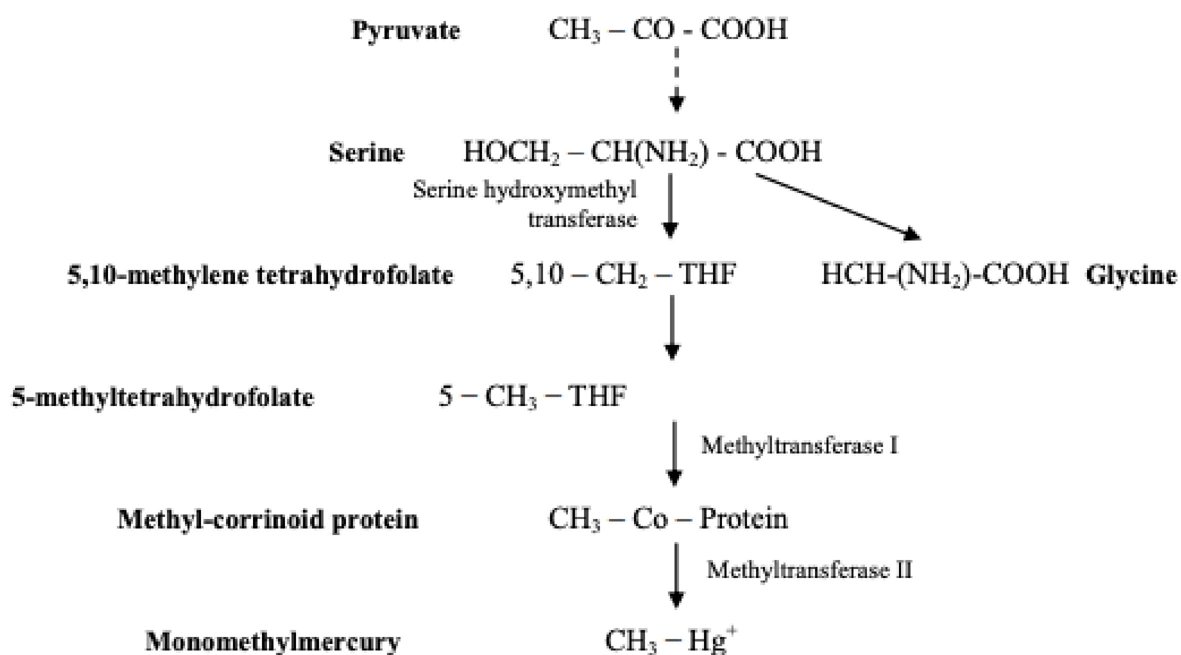

Figure 4: Proposed pathway of mercury methylation in *Desulfovibrio desulfuricans* (Segade, Dias & Ramalhosa, 2011)

2.2.2 Mechanisms independent of the acetyl CoA pathway

The biochemistry and mechanisms proposed by the Bartha group have been widely accepted and studied in-depth. Through the further study of mercury methylation, other findings have been made and mechanisms have been proposed. There are two different strains of SRB-complete oxidizing SRB and incomplete oxidizing SRB. Complete oxidizing SRB utilize the acetyl CoA pathway to convert acetate into carbon dioxide (CO) and vice versa (Ma, Du &

Wang, 2019). *D. desulfuricans* is thought to use a methyl group from this pathway for mercury methylation. Interestingly, this specific microorganism is an incomplete oxidizing SRB, meaning it does not use the acetyl CoA pathway for major carbon metabolism. However, *D. desulfuricans* can utilize the acetyl CoA pathway for metabolism of abnormal carbon sources and/or minor biosynthetic purposes (Ekstrom, Morel & Benoit, 2003). This insight suggests that perhaps the acetyl CoA pathway is not responsible for donating a methyl group to Hg^{2+}. In the research completed by Ekstrom, Morel and Benoit (2003), five strains of incomplete oxidizing SRB as well as two strains of *Desulfobacter* that do not use the acetyl CoA pathway for carbon metabolism were assayed by analyzing methylmercury formation. Three of the SRB strains were exposed to an acetyl CoA inhibitor (chloroform) and did not produce any methylmercury. Four strains of incomplete oxidizing SRB were found to methylate mercury independently of the acetyl CoA pathway. A comparison of acetyl CoA metabolism and methylmercury formation can be seen in Table 1.

Table 1: Connection between mercury methylation and acetyl CoA metabolism (adapted from Ekstrom, Morel and Benoit (2003)).

Species	Result for:	
	Hg methylation	**Acetyl-CoA pathway**
Incomplete oxidizer		
D. africanus	Yes	<DL
D. desulfuricans LS	Yes (14)	Yes (14)
D. desulfuricans subsp. *desulfuricans*	<DL	<DL
D. vulgaris subsp. *vulgaris* Marberg	<DL	<DL
Desulfobulbus propionicus 1pr3	Yes	<DL
D. propionicus MUD	Yes	<DL
Complete oxidizer		
D. multivorans 1be1	Yes	Yes
D. curvatus	<DL	<DL

[a]The presence of the acetyl-CoA pathway was determined based on positive CODH activity. <DL, less than detection limit.

From their research, Ekstrom, Morel and Benoit (2003) demonstrated that mercury methylation can be independent of the acetyl CoA pathway, suggesting that there is an alternative mechanism that exists in SRB, and potentially in other mercury methylators.

The participation of L-methionine in mercury methylation was suggested after research done by Landner (1971) showed that adding L-methionine to culture medium decreased the production of MeHg, while the addition of D, L-homocysteine and L-cysteine increased the production. The author suggested that the biosynthesis of L-methionine was involved in mercury methylation. In 1973, Birgersson et. al confirmed that L-methionine creates a complex with Hg (II) chloride, acetate and nitrate. Unfortunately, research into the relationship between methylmercury formation and L-methionine has not been extensively done since the 1970's. Additional research is required to fully understand the biochemical mechanisms of mercury methylation that is independent of the acetyl CoA pathway in SRB and other microorganisms.

2.2.3 Other potential mechanisms

Another suggested biochemical pathway for the methylation of mercury is the degradation of dimethylsulphoniopropionate (DMSP), reported by Larose et. al (2010) and. DMSP is produced by some species of marine algae and salt-tolerant plants as a way to regulate their internal osmotic environments. It is a tertiary sulfonium compound and has the following formula: $(CH_3)_2S^+CH_2CH_2COO^-$ (Yoch, 2002). During the metabolism or decomposition of DMSP, the transfer of a methyl group to bioavailable Hg (II) could occur suggesting the involvement of DMSP in mercury methylation (Larose et. al, 2010). However, there has been no confirmed detailed mechanism, as DMSP metabolism and degradation is complicated.

The complex nature of biomethylation is clear based on the work outlined. There are a variety of potential mechanisms, however, one clear, universal pathway has not been determined. The types of microorganisms that have shown methylation capabilities range from aerobes to anaerobes, and include bacteria, algae, fungi and methanogens. This review so far has focused mostly on bacteria, and more specifically anaerobic bacteria, because they are thought to be primary methylators in aquatic environments. However, the reality is much more complex and diverse and requires more extensive research (Paranjape & Hall, 2017).

2.3 Discovery of the genetic basis for mercury methylation

A major impact in this area of research came from the discovery of a two-gene cluster, *hgcA* and *hgcB,* that is required for mercury methylation. The research completed by Parks et. al (2013) was ground-breaking in further understanding how mercury methylation occurs. The 2013 study focused on the analysis of bacterial genomes from methylating and non-methylating microorganisms, specifically focusing on the biochemical pathways that involve single-carbon metabolism. The researchers recognized the role of the well-characterized corrinoid iron-sulfur protein (CFeSP) that is able to transfer methyl groups in acetyl CoA synthase. They used this knowledge to hypothesize that a corrinoid protein involved in the acetyl CoA pathway may be a requirement of methylation. The researchers thought perhaps a similar protein could transfer a methyl group to Hg (II) and that they could locate genes encoding the required protein in the genomes of known mercury methylators.

Analysis of the genomes of known Hg-methylators resulted in the discovery of a gene that is similar to the putative corrinoid protein-encoding gene mentioned previously, as well as a ferredoxin-like gene located downstream. The location of the genes in relation to each other suggest that they could be co-expressed and work together in the methylation of mercury. The researchers were able to locate orthologs of these two genes in confirmed methylators as well as confirm the absence of the gene sequences in confirmed non-methylators. The genes were also located in 46 additional microorganisms that have not been analyzed for Hg-methylation. The two genes, also known as *hgcA* and *hgcB,* are thought to work together as key facilitators of Hg-methylation. The gene cluster is depicted in figure 5. It was hypothesized that *hgcA* encodes a corrinoid protein that facilitates the methyl transfer and that *hgcB* encodes ferredoxin that will facilitate corrinoid reduction (Parks et. al, 2013).

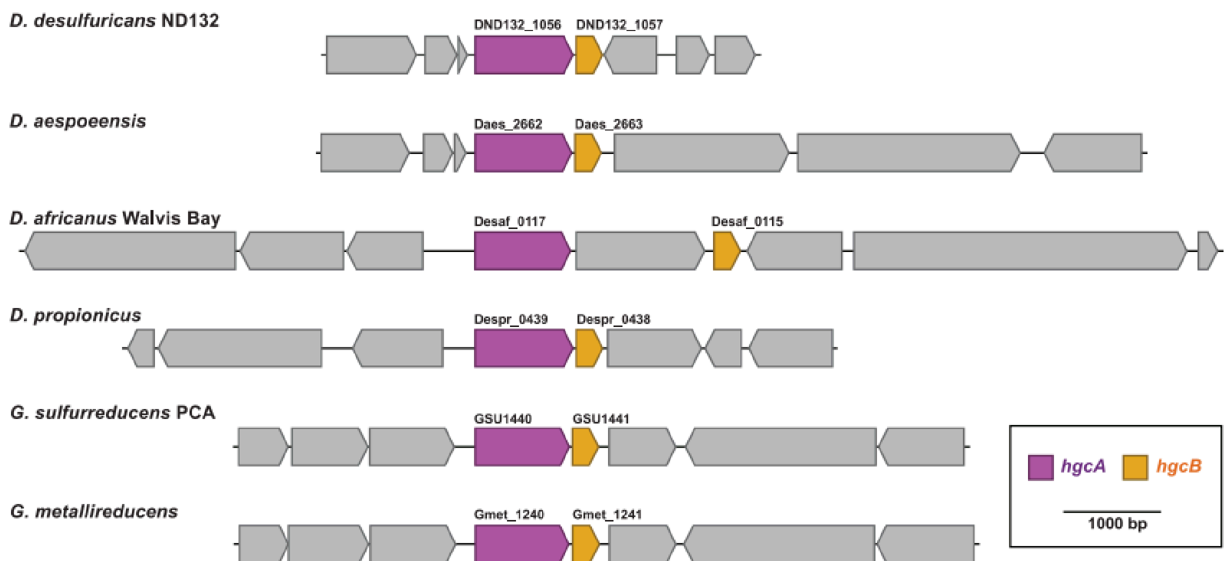

Figure 5: *hgcA* and *hgcB* gene cluster in confirmed, sequenced mercury methylators (Parks et. al, 2013).

In the same paper, Parks et. al (2013) continued to further confirm their findings. They deleted the *hgcA* and *hgcB* genes together and individually in *D. desulfuricans* ND132 and the gene orthologs in *G. sulfurreducens* PCA. Results showed that the formation of MeHg was reduced by more than 99% compared to the wild-type strains. Aiming to prove that deletions of these genes did not impact general cell metabolism and growth, they prepared comparative growth curves that showed no major growth impediment. This confirms that issues with growth were not responsible for lower MeHg production. This research also confirms the previous research completed by Bartha and Choi (1994) such that the methyl groups seems to originate from the acetyl CoA pathway (Parks et. al, 2013).

The research completed by Parks et. al (2013) was an important breakthrough in understanding the biomethylation of mercury. They were able to discover the *hgcAB* gene cluster in multiple microorganisms, including those that have been sequenced with and without further Hg-assays to confirm methylation capabilities. Not all mercury-methylating microorganisms have been sequenced, so they were not able to fully generalize these findings; however, based on the work that was done they reached a strong conclusion that this gene cluster is involved in mercury methylation (Parks et. al, 2013). The *hcgAB* gene cluster was found in a variety of

microorganisms in this study, as well as other studies that have been completed since. Table 2 offers an example of those microorganisms that contain the gene cluster.

Table 2: Microorganisms that possess the *hgcAB* gene cluster

Kingdom	Group	Genus & Species	Reference
Archaea	Methanomicrobia	*Methanofollis liminatans*	Parks et al, 2013
		Methanospirillum hungatei	Yu et al, 2013
		Methanolobus tindarius	Gilmour et al, 2013
Bacteria	Chloroflexi	*Dehalococcoides mccartyi*	Yu et al, 2013
		Desulfomonile tiesjei	Liu et al, 2014
Bacteria	Firmicutes	*Syntrophobotulus glycolicus*	Parks et al, 2013
		Desulfosporosinus orientis	
		Desulfosporosinus acidiphilus	Yu et al, 2013
		Dehalobactr restrictus	Podar et al, 2015
		Ethanoligenens harbinense	
		Desulfitobacterium dehalogenans	
Bacteria	Deltaproteobacteria	*Desulfomicrobium baculatum*	Parks et al, 2013
		Geobacter sulfurreducens	
		Deferrisoma camini	
		Syntrophus aciditrophicus	Gilmour et al, 2013
		Desulfonatronum lacustre	Liu et al, 2014
		Desulfovibrio desulfuricans	Podar et al, 2015
		Geobacter metallireducens	
		Desulfovibrio Africans	

2.4 Experimental applications using the *hgcAB* gene cluster

The discovery of the *hgcAB* gene cluster allows for the genomic study of mercury-methylating microorganisms. Researchers now have the ability to perform genomic and metagenomic experiments using the findings from Parks et. al (2013). This section will review some of the work that has been done following the discovery of the gene cluster, showcasing the possibilities for continued research in this field.

Shortly after the identification of the *hgcAB* gene cluster, Gilmour et. al (2013) aimed to determine the reliability of the *hgcAB* gene orthologs in predicting microorganism methylation capabilities. The researchers first used biotechnology modelling techniques to screen for the *hgcAB* gene among available microbial genomes. Figure 6 demonstrates the different microorganisms that contain *hgcA* orthologs. Next, they performed mercury methylation assays on a variety of microorganisms that contain and some that do not contain the *hgcAB* gene. Total amounts of Hg and MeHg were determined after the assays. They were able to report that mercury methylation was occurring in 15 microorganisms that were previously untested and contained the *hgcAB* gene. They were also able to find novel methylators in new environments. Overall, the researchers concluded that Hg-methylating microorganisms are much more diverse, phylogenetically and environmentally, than originally thought (Gilmour et. al, 2013).

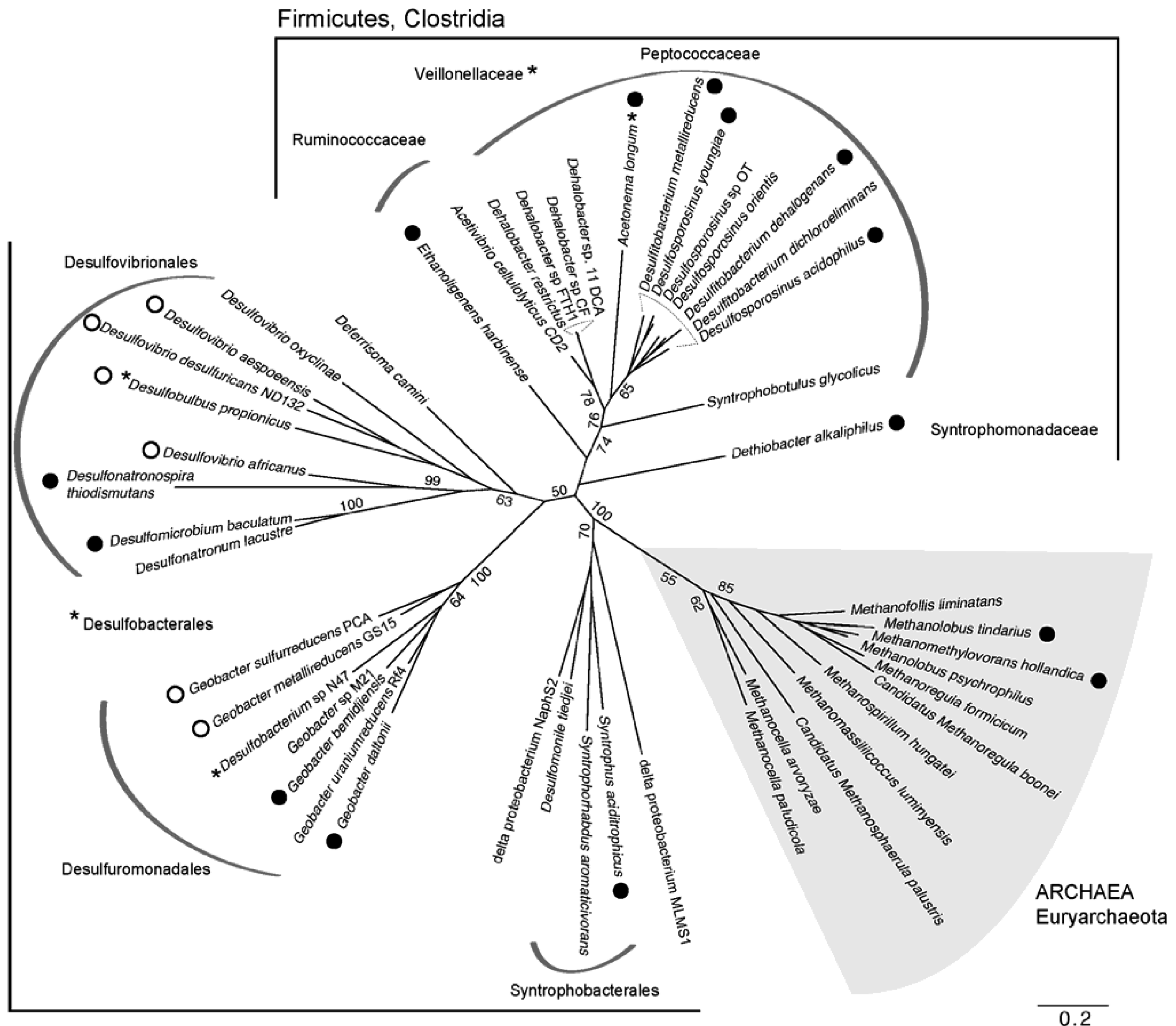

Figure 6: Phylogenetical tree of *hgcAB*-containing microorganisms. White dots have been previously identified as Hg-methylators; black dots show newly established methylators based on the work done in this paper (Gilmour et. al, 2013).

In 2014, Liu et. al developed novel polymerase chain reaction (PCR) primers to amplify the *hgcA* gene in paddy soil samples in a mining area of China. The *hgcA* gene alone is sufficient for analysis because it encodes the main protein involved in methylation. The primers were designed using known HgcA orthologs in the NCBI database. The researchers were able to successfully amplify and quantify the *hgcA* gene from their samples. The PCR gene products were then transformed into clone libraries and positive clones were sequenced with subsequent

phylogenetic analysis. There were a number of microorganisms identified as having the Hg-methylating gene, including *Proteobacteria, Firmicutes,* and *Euryachaeota.* This study demonstrated that the direct amplification of the *hgcA* gene from environmental samples was possible and could be used to establish further links between the production of MeHg and the presence of Hg-methylating microorganisms in the environment (Liu et. al, 2014).

The discovery of the *hgcAB* gene cluster has created an opportunity for increased research into the various environments in which Hg-methylating microbes reside, allowing for a deeper understanding on MeHg contamination and how it can affect humans. Podar et. al (2015) used biotechnological techniques and scanned 3500 publicly available genomes and metagenomes for *hgcAB* presence, in order to fully encompass the diversity and distribution of the Hg-methylating gene. They were able to find the gene in expected microorganisms from typical environments, however, they also discovered the gene in potentially new methylation habitats, including invertebrate digestive tracts, thawing permafrost soils and the sediment and soil of extreme environments, such as hypersaline and hypersulfidic waters. Additionally, they identified novel taxonomic groups that have Hg-methylation capabilities and have yet to be cultured. Figure 7 depicts the phylogeny and diversity of microbes containing the HgcA protein. These researchers were able to demonstrate that using information of the *hgcAB* gene cluster can lead to the discovery of newly identified Hg-methylating microorganisms and the environments in which they reside. This allows for further research into how MeHg contamination can occur and potentially affect human health (Podar et. al, 2015).

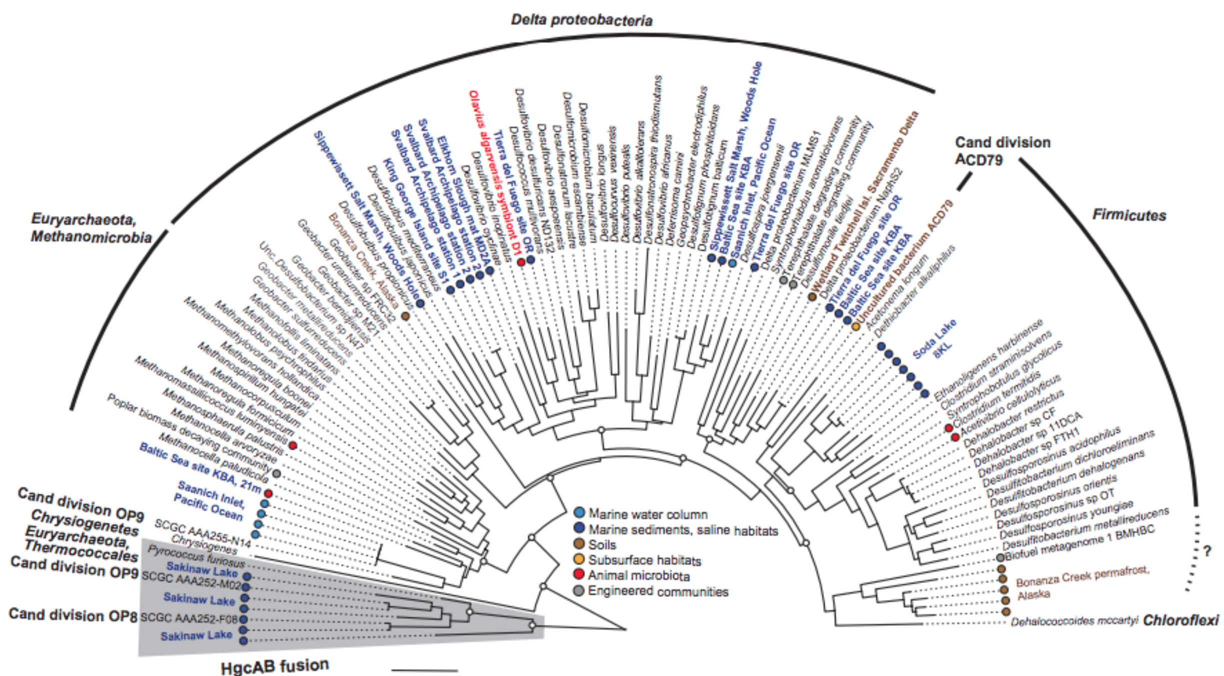

Figure 7: Phylogenetic tree of HgcA proteins found in complete genomic and metagenomic publicly available sequences (Podar et. al, 2015).

The *hgcAB* has been proven to be a good biomarker for Hg-methylators on a broad scale, where the goal of the research is to discover novel microorganisms and environments for Hg-methylation. This biomarker can also be used on a smaller scale to identify responsible methylators in specific environments. In the research done by Bravo et. al (2017), the microbial communities of sediments that have been impacted by waste water contamination were analyzed for Hg-methylation. DNA was extracted from the sediment samples and a general primer for the bacterial 16S rRNA gene was amplified. Specific primers targeting the *hgcA* gene were then used for PCR and subsequent sequencing and phylogenetic analysis. The results showed that the microbial community included SRB, methanogens and other microbes. SRB was found to only be a minor contributor to Hg-methylation, while *Geobacteraceae* dominated the community of microbes that contain *hgcA,* likely due to the presence of iron in this environment. This research offered insight into the microbial communities of certain environments, and how that can affect Hg-methylation (Bravo et. al, 2017).

There are numerous factors that affect the methylation of mercury, some of which will be covered later in this review. In the work of Liu et. al (2018), *hgcA* is used as a biomarker for Hg-methylation and specifically used to analyze the effect of certain factors on Hg-methylation. DNA was extracted from soil samples taken from old mining sites in China. The expression of the *hgcA* gene was analyzed using a specific primer pair and quantitative PCR (qPCR), which allows for the abundance of the gene to be obtained. The DNA was sequenced and subsequently sequenced. The findings showed that the abundance of the *hgcA* gene varied based on environmental factors such as organic matter, pH and chemical composition of the soil. The results also indicated that there is a diverse community of Hg-methylating microorganisms within the various sampling sites. Overall, this research demonstrated that microbial communizes and environmental factors must be studied together in order to fully understand the impact of Hg-methylation on human health (Liu et. al, 2018).

So far, research using *hgcAB* as a biomarker for Hg-methylation has proven to be useful and innovative. Results have shown novel methylating microorganisms, new environments for methylation and helped identify Hg-methylation in specific regions of contamination. The impact of the work done by Parks et. al (2013) is significant and will continue to be imperative to the future work on Hg-methylation.

2.5 Factors affecting mercury methylation

As mentioned previously, the methylation of mercury is a complex process that is not fully understood. A better understanding of the mechanisms that control methylation has developed over the past decade, however these mechanisms are affected by biotic and abiotic factors in the environment. There are a variety of factors that affect methylation, including the microbiology of the aquatic system which can be affected by temperature, pH, salinity and more. These factors do not work independently, but together as a complex system (Ullrich, Tanton, and Abdrashitov, 2001). This section will cover some of the factors that affect the methylation of mercury in aquatic systems.

2.5.1 Microbiology and bioavailability

It has been established that microbial methylation of mercury is responsible for the majority of methylation in aquatic systems (Segade, Dias & Ramalhosa, 2011). The microorganisms present in the water and the sediment can directly affect whether or not methylation is occurring and the amount of methylation. SRB have been determined to be the principal methylators in aquatic environments, however there is also evidence that iron-reducing bacteria and methanogens can methylate mercury as well. For SRB, the strength of methylating capabilities varies based on phylogenetic group. The methylating abilities of IRB and methanogens vary based on the strain type (Ma, Du & Wang, 2019). Not only does the general microbial population matter for methylation, but the specific types of microorganisms are crucial for Hg-methylation and how they interact with each other. In addition, for these microorganisms to thrive they must have acceptable nutrient availability (Ullrich, Tanton, and Abdrashitov, 2001).

Methylation also depends on the bioavailability of the Hg (II). It is believed that newly deposited Hg (II) is more bioavailable than Hg (II) that has been present in an aquatic environment for a long time. The bioavailability of mercury is determined by the distribution of mercury between the solid and aqueous phases and the specific Hg species in water (Li & Cai, 2013). Hg can only be transported through a cell membrane when it is dissolved. The adsorption and desorption of Hg also affects the bioavailability. Additionally, the species of Hg is important for bioavailability. Hg ions are not free ions in an aquatic environment and will complex with various ligands. Neutral complexes such as $HgS(0)_{(aq)}$ are the major available species of mercury, as they can passively diffuse into microorganisms to be methylated. Active transport of Hg^{2+} complexes has also been reported (Li & Cai, 2013). Figure 8 depicts the different mercury and methylmercury species that are available for methylation and demethylation.

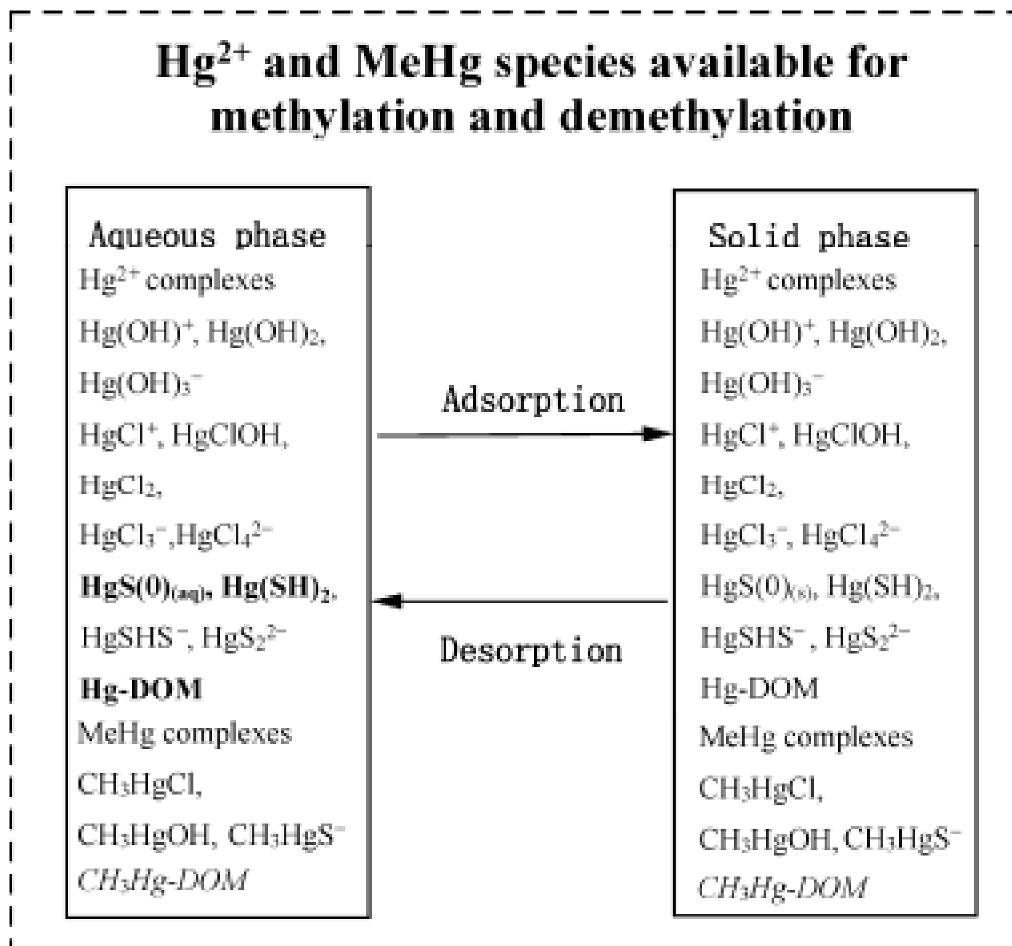

Figure 8: The available Hg (II) and MeHg species available for absorption and desorption

2.5.2 Temperature and pH

In many reports, methylation rates are often higher in the summer months, due to the warmer temperatures affecting the microbial populations (Ullrich, Tanton, and Abdrashitov, 2001). In 1982, Wright and Hamilton reported a significant decrease in methylation rates at 4°C compared to 20°C, due to an overall decrease in microbial activity in the winter months. This trend is seen in multiple reports for the methylation of mercury (Ullrich, Tanton, and Abdrashitov, 2001).

The research done on how pH affects methylation of mercury is extensive, due to concerns of lake acidification and acid rain. Overall, the results have shown the correlation

between a decreased pH in both water and sediment with an increase in methylmercury production. This could be due to a low pH helping to release heavy metals from sediments or that an acidic environment can increase microbial activity. It is not clear whether or not pH is causing a direct effect on methylation rates, or if it affects other factors, such as the aquatic system's microbiology (Ullrich, Tanton, and Abdrashitov, 2001).

2.5.3 Organic matter

Organic matter is thought to be an important factor in the methylation of mercury, however not much is known as organic matter can greatly vary in its composition (Jiang et. al, 2018). The results obtained from various research groups have been contradictory. In some cases, higher concentrations of dissolved organic matter (DOC) inhibit methylation due to the reduction of Hg (II) bioavailability when Hg forms a complex with DOC, inhibiting entrance into cells. In other cases, an increase in methylation was seen when a higher amount of organic matter was present, perhaps due to the increased microbial activity when more carbon sources from organic matter were available (Li & Cai, 2013). It is still unclear if DOC or organic matter in sediment has a direct effect on methylation, and it is complicated to make assumptions due to different compositions of organic matter in different environments and its interactions with other factors affecting mercury methylation.

2.5.4 Sulfur

The levels of sulfur in an aquatic environment can have strong effects on mercury methylation because the activity of SRB relies on the presence of sulfate (Paranjape & Hall, 2017). In the work of Benoit et. al (1999), it was determined that low sulfide concentrations increased methylation potential because neutral mercury-sulfide complexes could be formed and are able to diffuse through cell membranes. The study also demonstrated that higher concentrations of sulfide created charged mercury complexes which reduced the bioavailability of mercury. The sulfur levels in aquatic environment have an indirect effect on methylation, as it firstly affects the bioavailability of Hg which then affects over methylation rates.

Overall, there are many factors involved in creating methylmercury. These factors tend to interact and affect each other, making this aspect of mercury methylation especially complicated. Each individual aquatic environment is unique in its composition and this will also affect the rates of methylation. It is important to understand that Hg-methylation is very complex and involves a complicated system of factors that will regulate the amount of methylation that occurs.

2.6 Demethylation

Much of this review has been focused on the methylation of mercury, however Hg-methylation is not independent of demethylation. Demethylation is the reverse reaction of methylation and can also occur biotically or abiotically. Biotic demethylation appears to be the dominant mechanism in sediments, while abiotic photo-demethylation typically occurs more often in the water column (Li & Cai, 2013). Anaerobic bacterial demethylation appears to occur via the enzymatic cleavage of the carbon-mercury bond yielding methane and Hg^{2+}, then mercuric reductase enzyme reduces the Hg^{2+} to Hg^0. These enzymes come from the *merA* and *merB* genes, which are part of the *mer* operon that is present in mercury-resistant bacteria (Ullrich, Tanton, and Abdrashitov, 2001). Aerobic demethylation can also occur in some environments, and the major product of that pathway is carbon dioxide (CO_2). This suggests an oxidative pathway for demethylation and inhibition studies have shown that both SRB and methanogens are capable of oxidative demethylation (Ullrich, Tanton, and Abdrashitov, 2001). Abiotic demethylation can occur through a variety of suggested pathways, including UV radiation photodegradation, free oxygen radicals, oxygen and transfer of electrons. The exact is pathway is unknown, but there are numerous studies into likely mechanisms (Li & Cai, 2013). Demethylation of mercury is just as complex and complicated as Hg-methylation and requires future research independent of and in-tandem with methylation in order to fully understand how both processes are related and to accurately determine net MeHg levels in aquatic environments.

There is still a lot that is unknown about the methylation of mercury, allowing for extensive and exciting future studies to be completed. With the discovery of the *hgcAB* gene cluster, opportunities for new research into microbial methylation are possible. With the research

that has been done so far, new and novel microorganisms have been determined using genomic and metagenomic techniques. Based on this, it is clear that there is a possibility of many unknown methylating microorganisms that could be found and characterized to ultimately aid in the further understanding of mercury methylation and the potential for remediation and innovation in protecting aquatic environments.

3.0 Materials and Methods

3.1 Sediment Samples

3.1.1 Sampling Area

All samples were taken within the North Harbour of Lake Superior, in close proximity to the decommissioned paper mill. The approximate amount of contamination in this area is 350,000 to 400,000 m^3. The area of contamination can be seen in Figure 9.

Figure 9: Approximate Area of Contaminated Sediment in the North Harbour (Milani & Grapentine, 2011)

3.1.2 Sediment Collection

Sediment samples were collected by PhD candidate Nathan Wilson from the Department of Geography at Lakehead University. Sediment was collected at five sites in the North Harbour using a handheld dredging device, taken at a depth of between 10-14 feet. Two samples from each site were collected and placed into double Ziploc bags before subsequent storage at 4°C and -20°C for each site. At site 5, only one sample could be taken due to the sampling device malfunctioning. A total of nine samples were collected. A final weight of about 2 lbs was collected for each sample.

3.2 Nutrient Analysis

All nutrient analysis experiments were completed by the team at the Lakehead University Environmental Laboratory (LUEL).

3.2.1 Sample Preparation

Five samples were prepared for nutrient analysis by drying, grinding and sieving.

3.2.2 pH

pH was determined by titration using a Mettler Toledo DL53 titrator and autosampler. The protocol is done is four steps, including: titrant addition, titration reaction, signal acquisition and evaluation.

3.2.3 Total Nitrogen

Total nitrogen analysis was completed using a Nitrogen Analyzer. Samples were dried at <40°C and analyzed on an autoanalyzer SKALAR with built in UV digestion and acid hydrolysis system.

3.2.4 Total Phosphorus

Total phosphorus analysis was completed by microwave assisted acid digestion and measurement by inductively coupled plasma (ICP).

3.2.5 Total Carbon

Total carbon was analyzed using a carbon analyzer via combustion. The samples were placed in a high temperature chamber, and then the combustion products were reduced down to N_2 and CO_2 and detected with a thermal conductivity detector.

3.2.6 Total Potassium

Total potassium was analyzed using ICP.

3.3 Direct Methylmercury Analysis

Analysis of methylmercury (MeHg) present in the sediment samples was completed by the team at the Lakehead University Environmental Laboratory (LUEL). Samples from all five sites were

used for this analysis. Samples were first distilled and ethylated before introduction to MeHg system. Analysis was completed using a modified method of EPA 1630: Methyl Mercury in Water by Distillation, Aqueous Ethylation, Purge and Trap, and Cold Vapour Atomic Fluorescence Spectroscopy (CVAFS) (EPA, 1998).

3.4 DNA Extraction

DNA extraction was completed using the Invitrogen PureLink Microbiome DNA Extraction Kit with a modified protocol for extraction of soil. The first steps are to prepare the lysate. In the fume hood, ~0.25 g of sediment sample was added to the provided bead tube containing 600 µl of lysis buffer solution. 100 µl of lysis enhancer is added to the mixture, vortexed and incubated at 95°C for 10 minutes. Homogenization by bead beating was completed using a Bead Mill 4 at maximum speed for 10 minutes. The tubes were centrifuged at 14,000 x g for 5 minutes. 400 µl of the supernatant was transferred to a clean microcentrifuge tube. 250 µl of cleanup buffer was added to this and vortexed immediately. The tubes were incubated on ice for 10 minutes, followed by centrifugation at 14,000 x g for 2 minutes. 500 µl of supernatant was transferred to a clean microcentrifuge tube. The next steps are to bind the DNA to the column. 900 µl of binding buffer was added to the tubes and vortexed. 700 µl of this mixture was added to a provided spin column-tube assembly and centrifuged at 14,000 x g for 1 minute. The flow-through was discarded and the centrifugation was repeated with the remaining mixture. The final steps are to wash and elute the DNA from the column. The spin column was placed into a clean collection tube and 500 µl of wash buffer was added to the assembly and centrifuged at 14,000 x g for 1 minute. The flow-through was discarded and the column assembly was centrifuged again for 30 seconds. The column was placed in a clean tube and 100 µl of elution buffer was added, and the tubes were incubated for 1 minute at room temperature. The spin column assembly was centrifuged at 14,000 x g for 1 minute and the column was discarded. The flow-through was the purified DNA. The tubes were stored at -20°C. Specific buffer compositions were not disclosed by Invitrogen.

3.5 Qubit Fluorometric DNA Quantification

The concentration of DNA extracted from the five samples was quantified using Qubit Fluorometric Quantification. The device and all required supplies were utilized in the Paleo

DNA Laboratory at Lakehead University. To analyze the samples, a master mixture working solution was prepared. The Quant-it reagent was diluted 1:200 in Quant-it buffer by mixing 995 µl buffer and 5 µl reagent, vortexing and quick-spinning. 195 µl of the working solution was added to five microcentrifuge tubes, and 5 µl of each sample was added to the corresponding tube. The tubes were vortexed and quick-spun, then incubated at room temperature for 2 minutes. During this time, the Qubit device was set up using the previously completed calibration and the Quant-it dsDNA HS setting. Once incubation was complete, the tubes were placed in the Qubit device one at a time, and the function "calculate sample concentration" was chosen. The concentration of DNA in µl/ml was recorded for each sample.

3.6 PCR

All Polymerase Chain Reaction (PCR) experiments were carried out at the Paleo DNA Laboratory at Lakehead University under the supervision and guidance of Mr. Stephen Fratpietro.

3.6.1 Initial PCR

The first trial PCR was completed under standard protocol for the Paleo DNA Laboratory. A 25 µl PCR reaction mixture contained the following: 12.5 µl AccuStart II PCR SuperMix (2X reaction buffer containing 3 mM $MgCl_2$, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), AccuStart II Taq DNA Polymerase and stabilizers) or 12.5 µl Thermo Scientific PCR Master Mix (2X) (Taq DNA polymerase (0.05 U/µL), reaction buffer, 4 mM $MgCl_2$, and 0.4 mM of each dNTP), 0.25 µl 10 µM forward primer (*hgcA*F; 5′-GGNRTYAAYRTCTGGTGYGC-3′), 0.25 µl 10 µM reverse primer (*hgcA*R; 5′-CGCATYTCCTTYTYBACNCC-3′), 5 µl DNA template (1-10 ng) and 7 µl sterile water. Primers used are those designed and reported by Liu et. al (2014). Two master mixtures for seven reactions each were prepared, then vortexed and quick-spun. 20 µl of the master mixes were added to seven 0.2 ml thin-walled microcentrifuge tubes each. 5 µl of the DNA samples were added to their respective tubes for a total reaction volume of 25 µl. A blank sample and negative control were also prepared. The mixtures were vortexed and quick-spun, then transferred to the BioRad C1000 Thermal Cycler. The thermal cycler parameters were as follows: 94°C for 2 minutes; 94°C for 30 seconds; 60°C for 1 minute; 72°C for 2 minutes (30 cycles); 7°C forever.

3.6.2 Optimization PCR

A gradient PCR reaction was performed on one DNA sample in order to optimize the annealing temperature. A 25 µl PCR Reaction mixture contained the following: 12.5 µl AccuStart II PCR SuperMix, 0.5 µl 10 µM forward primer (*hgcA*F), 0.5 µl 10 µM reverse primer (*hgcA*R), 6.5 µl and 5 µl of Sample 5 DNA. A total of six reactions were prepared. The tubes were transferred to the MJ Research PTC-225 Gradient Thermal Cycler. The thermal cycler parameters were as follows: 94°C for 5 minutes; 94°C for 1 minute; 60°C, reduced by 0.5°C per cycle, for 1 minute; 72°C for 1 minute (10 cycles); 94°C for 1 minute; 50°C to 60°C for 1 minute (gradient); 72°C for 1 minute (30 cycles); 7°C forever.

3.6.3 PCR for Sequencing Analysis

Once the optimal annealing temperature was determined, a second PCR was prepared with all five DNA samples, plus a negative control and a blank sample. A 25 µl PCR reaction mixture contained the following: 12.5 µl AccuStart II PCR SuperMix, 0.5 µl 10 µM forward primer (*hgcA*F), 0.5 µl 10 µM reverse primer (*hgcA*R), 6.5 µl and 5 µl of template DNA (1 ng). A total of seven reactions were prepared. The reaction tubes were transferred to MJ Research PTC-225 Gradient Thermal Cycler and the thermal cycling parameters were as follows: 94°C for 5 minutes; 94°C for 1 minute; 60°C, reduced by 0.5°C per cycle, for 1 minute; 72°C for 1 minute (10 cycles); 94°C for 1 minute; 52°C for 1 minute; 72°C for 1 minute (30 cycles); 7°C forever. All additional PCR reactions followed this methodology.

3.7 Gel Electrophoresis

All gel electrophoresis experiments were carried out at the Paleo DNA Laboratory at Lakehead University under the supervision and guidance of Mr. Stephen Fratpietro.

5 µl of each DNA sample from the PCR was mixed with 3 µl of 6x gel loading dye (10 mM Tris-HCl (pH 7.6) 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol 60 mM EDTA). A 6% polyacrylamide gel was used. The gel was placed in the gel rig and covered with 1X TBE buffer (Tris-borate-EDTA). 1 µl of Thermo Scientific GeneRuler Low Range DNA Ladder was pipetted into the first well of the gel. In the subsequent wells, 8 µl of the DNA-dye mixture was added. The gel rig was connected to the power box. The gel was run for 45 minutes at 118 V.

Once the run was complete, the power box was turned off and the gel was removed from the rig. In a dark room, the gel was removed from its mold, placed in a small container and covered with 0.5µg/ml Ethidium Bromide (EtBr) and then incubated for 15 minutes. After incubation, the gel was placed on a UV Transilluminator and imaged with a camera box.

3.8 DNA Sequencing

All DNA sequencing experiments were carried out at the Paleo DNA Laboratory at Lakehead University under the supervision and guidance of Mr. Stephen Fratpietro.

3.8.1 Purification of PCR Products

3.8.1.1 QIAQuick PCR Purification Kit

The PCR products of the five DNA samples were purified using QIAQuick PCR Purification kit. 5 volumes of Buffer PB (guanidine hydrochloride and isopropanol) was added to 1 volume of the PCR reaction and vortexed. The buffer/DNA mixture was added to the QIAquick column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and 700 µl of Buffer PE (ethanol based) was added to the column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the empty column was centrifuged at 13,000 rpm for 1 minute to remove any excess ethanol. The column was placed in a clean microcentrifuge tube and 30 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) was added to the column to elute the DNA. This was incubated for 3 minutes then centrifuged at 13,000 rpm for 1 minute. The column is discarded, and the purified DNA is stored appropriately before the next step. Some buffer compositions are confidential as per Qiagen.

3.8.1.2 Exo I/Shrimp Alkaline Phosphatase (SAP) Purification

Exo I/SAP purification was used for the purification of PCR products in later experiments. 4 µl of SAP and 2 µl of *Exo I* were added to each 20 µl PCR product. This was mixed thoroughly, then incubated for 37°C for 15 minutes, then 80°C for 15 minutes to inactivate the enzymes. The purified PCR products were stored at 4°C until the next step.

3.8.2 Preparation and Running of the Sequencing Reaction

A 10 µl sequencing reaction mixture contained the following: 0.5 µl BigDye Terminator Reaction Mixture v3.1, 0.3 µl of one of the 10 µM forward or reverse primers, 3.0 µl purified DNA sample, 2.0 µl 5x Sequencing buffer and 4.2 µl sterile water. Two master mixtures were prepared for 8 reactions each- one containing the forward primer and the other containing the reverse primer. 7 µl of the master mixture was added to 0.2 ml thin-walled PCR tubes and 3 µl of each sample were added to their respective tubes. All solutions were vortexed and quick-spun. The tubes were transferred to the BioRad S1000 Thermal Cycler. The thermal cycler parameters were as follows: 96°C for 1 minute; 96°C for 10 seconds; 50°C for 5 seconds; 60°C for 75 seconds (15 cycles); 96°C for 10 seconds; 50°C for 5 seconds; 60°C for 90 seconds (5 cycles); 96°C for 10 seconds; 50°C for 5 seconds; 60°C for 120 seconds (5 cycles); hold at 7°C.

3.8.3 Ethanol/ Sodium Acetate Precipitation

A premade ethanol/sodium acetate solution was prepared by S. Fratpietro. For 90 µl of solution, the composition was as follows: 3.0 µl 3M sodium acetate, pH 5.4, 62.5 µl 95% ethanol, 24.5 µl sterile water. 90 µl of this solution was added to a 0.5 ml microcentrifuge tube, the 10 µl sequencing reactions were added to respective tubes and then vortexed and incubated at room temperature for 15 minutes. The tubes were then placed in an Eppendorf 5424 microcentrifuge with their orientations marked and centrifuged at 13,000 rpm for 15 minutes. The supernatants were carefully aspirated, ensuring no disruption of the pellet. 250 µl of 70% ethanol was added to each tube and vortexed for 20-30 seconds. The tubes were then placed back into the microcentrifuge in the same orientation and centrifuged at 13,000 rpm for 5 minutes. The supernatants were again carefully aspirated. The samples were placed in a vacuum centrifuge for 10 minutes to be fully dried. The dried DNA was stored appropriately.

3.8.4 Sequence Loading

Sequence loading was done on an ABI 3130xl sequencer machine. 15 µl of Hi-Di Formamide was added to each tube of dried DNA. The tubes were vortexed for 1 minute then heated at 95°C for 3 minutes and chilled on ice for 2 minutes. The samples were again vortexed and quick-spun and placed on ice until loading. In the provided 96-well plate, all 15 µl of the samples were added to the wells. Any wells without sample were filled with 11µl Hi-Di Formamide. The plate

was centrifuged at 1000 rpm for 1 minute before loading on the machine. All sequencing parameters were set by S. Fratpietro.

3.9 Alternate Primer Design

New primers were designed in order to run further PCR and sequencing experiments. The basis of the design was to make the previously utilized primers (*hgcA*F and *hgcA*R) more specific with less non-specific base-pairs. Using BLAST analysis, primers of known sequences of the *hgcA* gene were generated and compared to the original primers. Primers that had sequence similarity to the original primer pairs were noted. In the end, two primer pairs were identified to have similarity to the original primers while being more specific. In addition to the newly designed primers, a primer that is specific for the *hgcA* gene in *Desulfovibrio desulfuricans* (ND132) was ordered. The sequences of the three new primer pairs are outlined in table 3.

Table 3: Alternate Primers Used for PCR

Forward and Reverse Primer Pair Names	Primer Sequence	Reference
*hgcA1*F and *hgcA1*R	F: 5'-CTCTTGCTGCCTATCCTCGG-3' R: 5'-GCTTACGACTAGCATCGGCT-3'	Designed using BLAST Analysis
*hgcA2*F and *hgcA2*R	F: 5'-CTGAAGCCGATGCTAGTCGT-3' R: 5'-GAGAAACGCCGCAATGGATG-3'	Designed using BLAST Analysis
ND132F and ND132R	F: 5'-GCCAACTACAAGCTGACCTTC-3' R: 5'-CCCGCCGCGCACCAGACGTT-3'	Christensen et. al (2016)

3.10 Sequencing with Alternate Primers

Sequencing reactions were prepared using the three new primer sets and previously purified PCR products. The sequencing reaction was done using the same protocol as outlined in section 3.8.2. The subsequent ethanol/sodium acetate precipitation and sequence loading were completed using the same protocols as outlined in sections 3.8.3 and 3.8.4, respectively.

3.11 PCR with Alternate Primers

After completing the sequencing experiments with the original PCR products and the new primer pairs, it was clear that a new PCR reaction was required for sufficient results. A PCR reaction was prepared using the same protocol as outlined in 3.6.3. In order to visualize the amplification of the DNA, gel electrophoresis was completed as outlined in section 3.7.

4.0 Results and Discussion

4.1 Sample collection

Sediment collection was done using a hand-held dredging device. Two sets of samples were taken from each site and placed into double Ziploc bags. The map of the collection sites can be seen in figure 10. The sites were relatively close in proximity to the shore and the old paper mill. In this area, there is pulp waste that is white, and in some areas the pulp is 4 m deep (Milani & Grapentine, 2011). Each of the five samples taken were relatively similar in terms of physical characteristics. Table 4 outlines the visual appearance of each sample. Samples 2, 4 and 5 were very similar and contained comparable debris such as small sticks and rocks. Sample 5 was slightly drier than the other samples. Sample 1 sediment particles were similar to those of 2, 4 and 5 but contained more wood fibres and larger sticks. Sample 3 was the most different, as it was predominantly pulp waste that was obtained. Site 3 was the closest in proximity to the old mill and therefore it is the most likely region to have significant pulp waste deposits.

Figure 10: Map of the 5 sediment collection sites in the North Harbour of Lake Superior

Table 4: Physical characteristics of the sediment samples

Sample	Visual Appearance
1	Dark brown fine sand with organic debris (wood fibres)
2	Dark brown fine sand with organic debris
3	White pulp waste with little organic debris
4	Brown fine sand with organic debris
5	Dark brown sand with organic debris; drier

4.2 Nutrient and MeHg sediment analysis

As mentioned in the literature review, environmental factors can have a significant effect on the methylation of mercury. It is important to consider these factors when analyzing mercury methylation in aquatic environments. In this study, some factors were analyzed, as well as the total levels of methylmercury (MeHg) in the 5 samples. The scope of this research unfortunately did not include studying the rates of methylation or demethylation, therefore an extremely in-depth analysis into how the environmental factors of Lake Superior affect the net-methylation of mercury could not be done. However, this section aims to outline the overall sediment nutrient profile in relation to the amount of MeHg present at each site and the results can be seen in table 5.

The moisture content of the samples was fairly consistent at all sites, except site 5. The sample was considerably less "wet", as mentioned in section 4.1. This parameter is not mentioned in literature as having an effect on methylation, however there is a possibility it could affect microbial growth.

Total organic carbon seems to vary from site to site and is considerably lower at site 5. The values do not seem to correlate to an increase or decrease in MeHg concentration. Similar to the effects of organic matter on methylation, there are contradictory findings. Sometimes a higher level of organic matter can inhibit methylation, other times it increases methylation (Li & Cai, 2013).

The levels of potassium and phosphorus vary between the sample sites. MeHg is able to bind to phosphorus and create a complex (Segade, Dias & Ramalhosa, 2011). This could affect

how MeHg is distributed in sediment and water, as well as its uptake into fish and humans. Based on the results in table 5, it does not appear that the total phosphorus in the sediment samples has an effect on the total MeHg. The two highest amounts of phosphorus at sites 1 and 4 are associated with the highest and lowest concentrations of MeHg. In terms of potassium, there does not seem to be any correlation between total K and total MeHg. Potassium has not been reported in literature as being a major factor in the methylation of mercury, however it may have an overall effect on microbial growth. More research is required for a full understanding of the direct effects of phosphorus and potassium on MeHg.

Literature has shown that pH has an inverse relationship with Hg-methylation (Ullrich, Tanton, and Abdrashitov, 2001). These results show that the pH does slightly vary between sites, however there is no clear correlation between pH and MeHg concentration. At a pH of 6.03 and 5.60, the MeHg concentrations are high, however at a pH of 5.34- the lowest recorded pH in this study- the MeHg concentration is significantly lower at 2.22 ng/g.

In some environments, nitrogen availability has been correlated with an increase in MeHg concentration, suggesting the potential that methylation is stimulated by nitrogen (Braaten et. al, 2014). These results do not appear to have any increase in MeHg concentration with increased nitrogen. For example, the highest and lowest MeHg concentration sites had the same percentage of nitrogen.

The concentrations of MeHg at each site are variable, with the highest being at site 4. Results from previous assessments of the North Harbour of Lake Superior reported a range of 4.2 ng/g to 102.3 ng/g (Milani & Grapentine, 2011). These results show that MeHg is in fact present in the North Harbour at this time, and that the subsequent experiments in this study can be completed. The nutrients that were tested in this study do not appear to have any correlation with MeHg concentration. These results were obtained on a small scale, however, to fully understand the complex nature of the affecting factors on Hg-methylation, a more in-depth study is required to account for more nutrients and factors, as well as the changing flow in water that can shift MeHg concentrations.

Table 5: Nutrient and MeHg analysis of sediment samples

Description	MDL	Units	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
% Moisture Content	0.00	%	87.89	78.04	84.03	77.67	33.83
%Total Organic Carbon	0.20	%	13.92	14.79	22.60	13.39	1.21
Total Recoverable Potassium	20.00	µg/g	2348.09	1401.93	338.92	1732.07	2056.65
Total Recoverable Phosphorus	16.00	µg/g	1187.12	723.41	601.53	1240.14	467.31
pH 1:1 water to soil ratio	0.00	unit	6.45	6.03	5.34	5.60	7.59
N sediment	0.01	%N	0.67	0.32	0.24	0.67	0.09
MeHg	0.1	ng/g	0.335	7.85	2.22	9.7	4.29

4.3 DNA Extraction

The DNA extraction process involved trial and error in order to obtain high enough concentrations of dsDNA to continue with downstream experiments. A DNA extraction kit was used; however, the protocol was optimized for this specific type of sample. The original protocol from the Invitrogen PureLink Microbiome DNA Extraction Kit called for around 0.2g of sediment but based on numerous trials, 0.25-0.3 g of sediment resulted in higher DNA concentrations. Incubation times and temperatures were also adjusted for optimum extraction. Once the protocol was optimized, final dsDNA concentrations were obtained using a Qubit Fluorometer. The results in figure 11 show similar concentrations in all five samples except the sample from site 2. It had a significantly higher concentration of 9.75 µg/ml. This could be due to variations in the extraction protocol, or it could mean that site 2 had more DNA. This could also relate to a higher number of microbes and potential microbial activity. Additionally, the dynamic aspect of the lake can cause change in location and concentration of microbes and DNA over time.

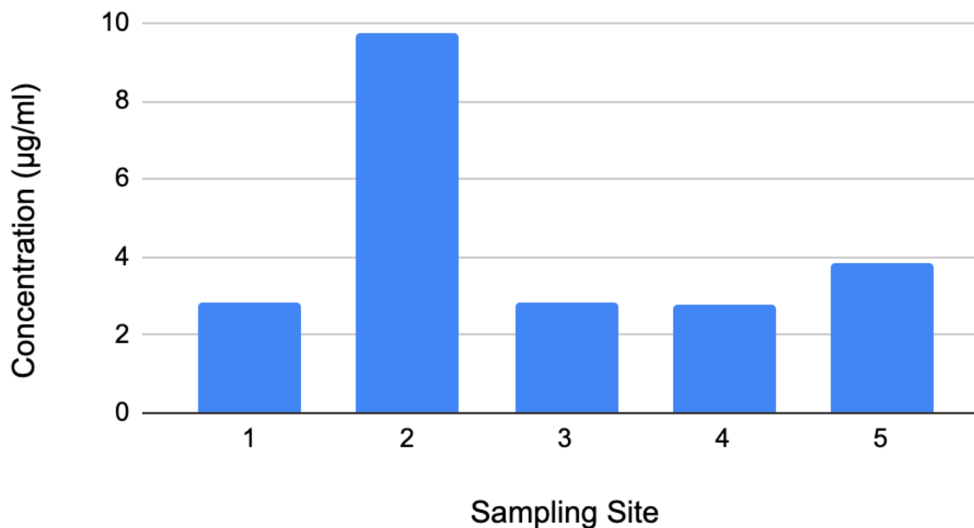

Figure 11: Total dsDNA concentrations from North Harbour sample sites

4.4 PCR with Original Primers

4.4.1 Initial PCR

The first PCR that was completed used a standard PCR protocol and the specific primers to isolate the *hgcA* gene, as reported by Liu et. al (2014). The PCR products were run on an agarose gel to visualize the DNA bands. In this first trial, the PCR was unsuccessful, and the gel electrophoresis did not produce any visible bands (Fig. 12). In this gel run, 14 lanes were loaded as seen in table 6. Two different PCR master mixtures were used; AccuStart Supermix and Thermo Fisher MasterMix. This was done to determine any differences between the two mixtures and decide on which one to use for future PCR experiments. For each master mixture, a blank and a control sample were used. The blank used sterile water in place of a DNA sample in the PCR set-up, and the negative control was a DNA extraction sample using only water. The purpose of these two samples was to ensure that DNA was only coming from the extracted samples and that no contamination was present during the DNA extraction or the PCR experimental set up. Based on the results seen in figure 11, this PCR did not amplify any DNA from the samples. This indicated a need for PCR optimization because even if the specific *hgcA* gene fragment was not amplified, some DNA would be visible on the gel.

Table 6: First PCR gel run loading pattern

Lane Number	Sample
1	Low Range DNA Ladder
2	AccuStart PCR Supermix Control
3	AccuStart Blank
4	AccuStart Site 1
5	AccuStart Site 2
6	AccuStart Site 3
7	AccuStart Site 4
8	AccuStart Site 5
9	Thermo Fisher PCR MasterMix Control
10	MasterMix Blank
11	MasterMix Site 1
12	MasterMix Site 2
13	MasterMix Site 3
14	MasterMix Site 4
15	MasterMix Site 5

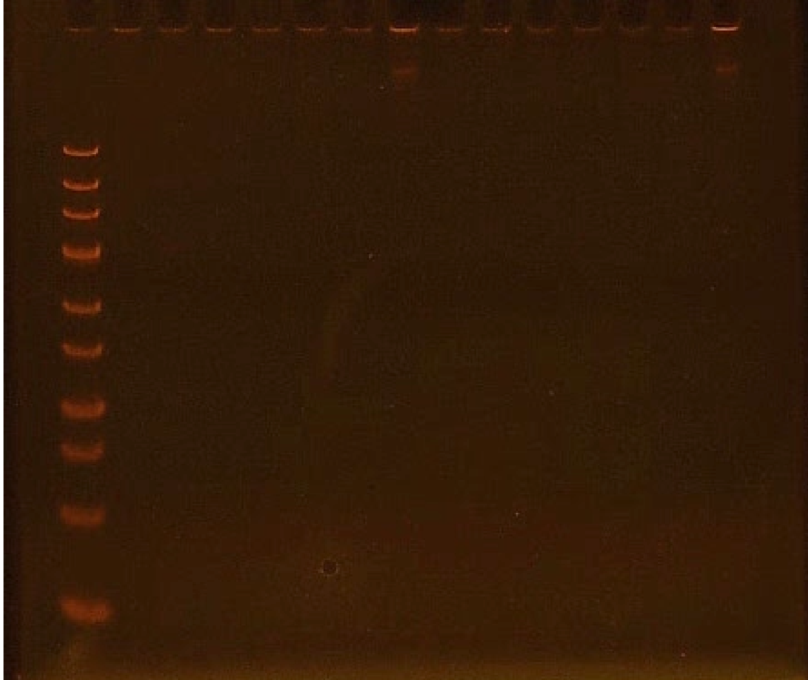

Figure 12: Gel electrophoresis of the initial PCR results

4.4.2 Gradient PCR for Optimization

PCR involves many steps and solutions, making it easy to get negative results if one step is not done correctly or is not optimal for the specific DNA and primers. The first step in optimization was evaluating the PCR protocol. In this case, the paper by Liu et. al (2014) from which the primers were based on, reported a different protocol than the standard on used for PCR. Next, the annealing temperature needed to be optimized for these primers. In order to accomplish this, a gradient PCR was the best option to determine the optimal annealing temperature. One sample of DNA was used (from site 5) and 6 identical PCR reactions were prepared using the AccuStart PCR Supermix. The new protocol used followed the one outlined in the report from Liu et. al (2014) and is detailed in section 3.6.2. The annealing step was made into a gradient, in which the temperature varied from 50°C to 60°C based on the PCR tube location in the thermal cycler. The results show DNA bands at around 620 bp (based on the DNA ladder) at varying intensities (Fig. 13). The annealing temperatures for each band are outlined in table 7. The gel electrophoresis results show the strongest bands in lanes 1,2 and 3, while lanes 4-6 show weaker bands. This demonstrates that the best annealing temperature for these specific primers is between 50.0°C and 52.8°C. Moving forward, all PCR experiments were done using an annealing temperature of 52°C, based on these results.

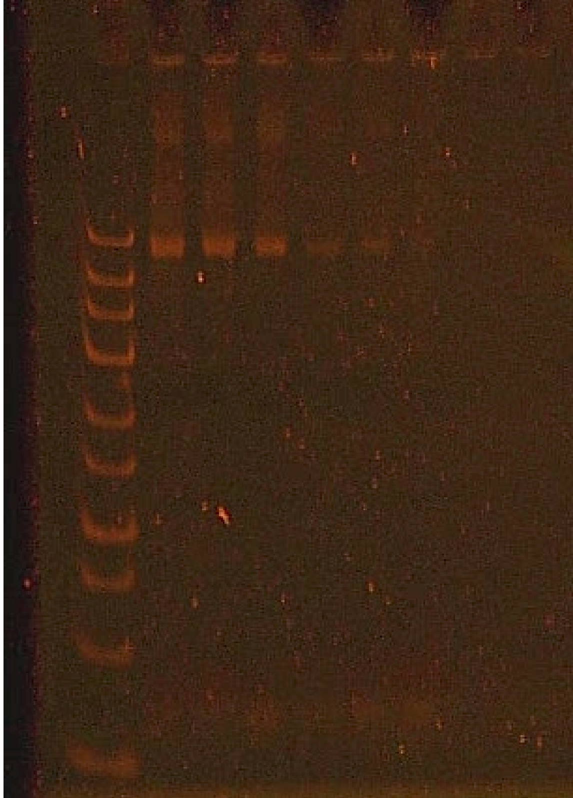

Figure 13: Gel electrophoresis of the gradient PCR optimization trial

Table 7: Annealing temperatures of gradient PCR

Lane	Annealing Temperature (°C)
1	50.0
2	50.9
3	52.8
4	56.0
5	58.5
6	59.8

4.4.3 Optimized PCR Results

Once the optimized PCR protocol was determined, more PCR experiments were run in order to produce DNA that could be used for sequencing. The first PCR that was successfully completed with the new optimal procedure showed *hgcA* gene amplification for all 5 DNA

samples (Fig. 14). Lanes 1 and 2 were the blank and negative control. No bands are seen here, meaning there is no DNA contamination and the DNA that is shown is coming directly from the extracted samples. Lanes 3 and 4 were samples 1 and 2 and produced very strong bands at the expected base pair size (~620). There was a high amount of DNA in this samples, as seen from the smearing on the agarose gel. Lane 5 contained the low range DNA ladder. Lanes 6-9 were samples 3, 4 and two different site 5 DNA samples. The results show that amplification is occurring for samples 3, 4 and 5a, but there is little to no amplification of sample 5b. Sample 5b was a DNA extraction sample that was completed at the beginning of the extraction experiments, and was included here to determine if the sample had degraded at all during its storage. It is clear that the fresher DNA samples provided better results. Overall, this gel electrophoresis run of the PCR products from the first optimized PCR show that the *hgcA* gene is in fact being amplified and that this gene is present at all five sample sites of the North Harbour. While these results answer the question of the first objective, it is still necessary to determine which microorganisms are present and if the gene amplification is in-fact coming from Hg-methylating microorganisms.

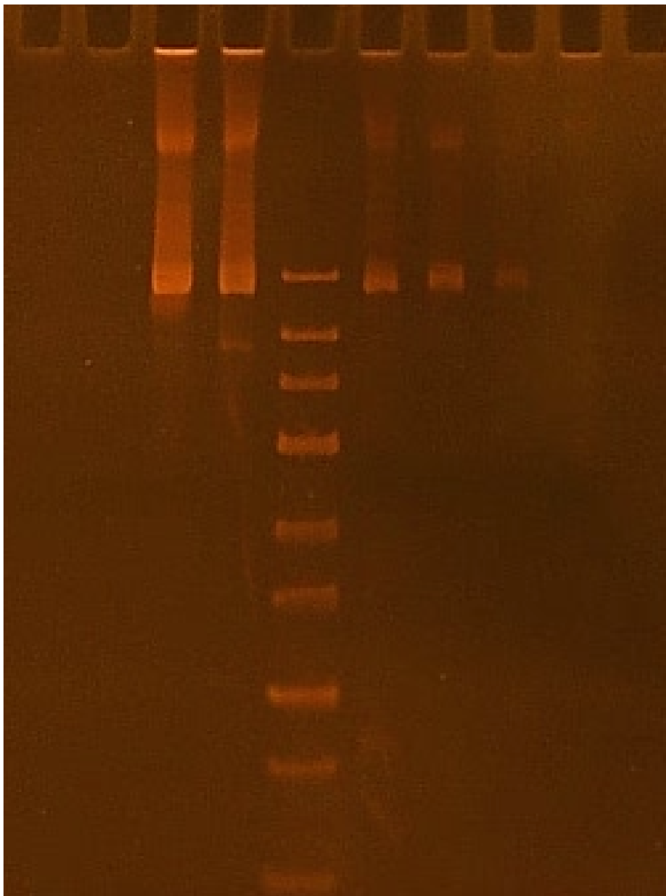

Figure 14: First PCR gel electrophoresis for downstream sequencing

The previous PCR products (Fig. 14) were used for sequencing reactions, as outlined in section 3.8. The sequencing results will be discussed later in this section, but based on those results, it was necessary to run another PCR experiment in order to produce more amplified DNA for additional sequencing reactions. Figure 15 shows the gel electrophoresis of the second optimized PCR reaction run. In this reaction, a blank and a negative sample were run in lanes 1 and 2. Lanes 3-7 were samples 1-5 and the final lane, 8, was the LR DNA ladder. Similar to the previous results, all five samples produced amplified *hgcA* DNA. Samples 1, 3 and 5 produced large amounts of DNA, as seen by the smearing on the agarose gel. Samples 2 and 4 produced enough DNA to move forward with downstream sequencing. Again, these results confirm the previous results (Fig. 14), demonstrating the presence of the *hgcA* gene in sediment samples from the North Harbour of Lake Superior.

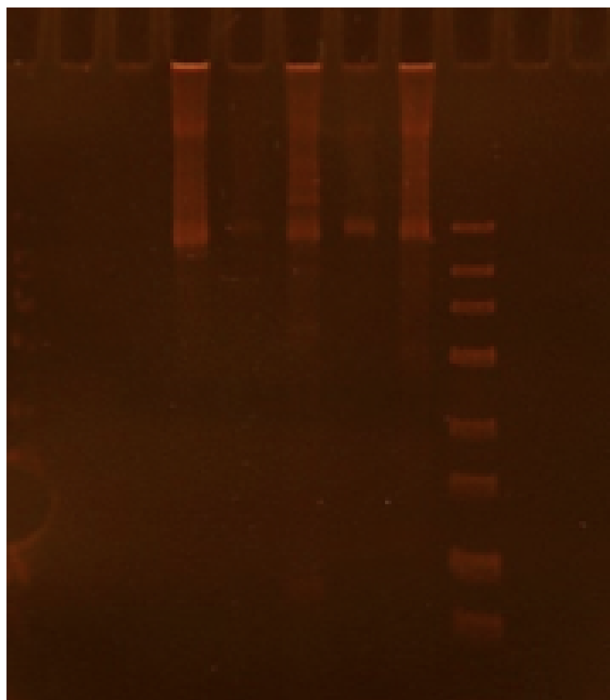

Figure 15: Second PCR gel electrophoresis for downstream sequencing

4.5 Sequencing with original primers

After the successful PCR experiments, the next step was sequencing the DNA that was amplified to confirm that it was originating from Hg-methylating microorganisms and determining the identity of those microbes. The sequencing completed used the original primers in the sequencing reaction. The final sequencing reaction products were run through and ABI

3130xl sequencing machine. The expected results will show a long chromatograph with peaks of different colours associated with one of the four base pairs (Adenine (A), Guanine (G), Cytosine (C), and Thymine (T)) for the entire length of the DNA fragment. Any base pairs that are not recognized by the system will show up as a black peak and the base pair "N", which could be any of the four established base pairs. The results from the sequencing that was completed were not reportable and did not sequence any clear DNA fragment. Many of the samples produced results that were only N-base pairs. Other samples produced some peaks of known base pairs, but there were too many unknown spots in the fragment, making it difficult to identify full sequences for further analysis. In order to find some useful results from this sequencing experiment, the chromatographs that had long enough stretches of readable results were identified and the sequences were recorded, including the N-base pairs. Nine short sequences between 50-100 base pairs were recorded and entered into the BLAST (basic local alignment search tool) data base. The BLAST database has sequences from all different organisms, including various microorganisms. The search tool aligns the sequence of interest with other similar sequences and produces results of microorganisms that the sequence of interest may originate from. A BLAST search of one sequencing result for sample 3 provided results for a "somewhat similar" sequence in the BLAST database (Fig. 16). The first result is that of a *Geobacteraceae* culture that contains the *hgcA* gene. Other queries show similar results, all showing partial sequence alignment with the sequence obtained from DNA sample 3. Some of the results specify that the microorganism is one that can methylate mercury.

Alignments Download GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Geobacteraceae bacterium enrichment culture clone B2_C-14 HgcA (hgcA) gene, partial cds	59.0	59.0	80%	3e-05	72.29%	KJ021069.1
Uncultured Geobacteraceae bacterium clone B2-28 HgcA (hgcA) gene, partial cds	56.3	56.3	66%	3e-04	75.36%	KJ021136.1
Geobacteraceae bacterium enrichment culture clone B2_FE-06 HgcA (hgcA) gene, partial cds	56.3	56.3	66%	3e-04	75.36%	KJ021090.1
Geobacteraceae bacterium enrichment culture clone B2_FE-02 HgcA (hgcA) gene, partial cds	56.3	56.3	66%	3e-04	75.36%	KJ021087.1
Uncultured microorganism clone 2-1-16 mercury methylating protein (hgcA) gene, partial cds	54.5	54.5	80%	0.001	71.08%	KJ184744.1
Uncultured Geobacteraceae bacterium clone B7-04 HgcA (hgcA) gene, partial cds	52.7	52.7	69%	0.004	73.61%	KJ021147.1
Geobacteraceae bacterium enrichment culture clone B2_FE-17 HgcA (hgcA) gene, partial cds	52.7	52.7	69%	0.004	73.61%	KJ021099.1
Geobacteraceae bacterium enrichment culture clone B2_C-13 HgcA (hgcA) gene, partial cds	52.7	52.7	69%	0.004	73.61%	KJ021068.1
Uncultured microorganism clone MNGP8 hgcA gene, partial sequence	50.0	50.0	66%	0.014	72.06%	MH809276.1
Uncultured microorganism clone MNGP4 hgcA gene, partial sequence	50.0	50.0	66%	0.014	72.06%	MH809273.1
Uncultured microorganism clone MCQS23 hgcA gene, partial sequence	49.1	49.1	67%	0.048	72.86%	MH809215.1
Uncultured Geobacteraceae bacterium clone B7-09 HgcA (hgcA) gene, partial cds	48.2	48.2	69%	0.048	72.22%	KJ021151.1
Uncultured microorganism clone 4-3-11 mercury methylating protein (hgcA) gene, partial cds	48.2	48.2	69%	0.048	72.22%	KJ184721.1
Uncultured microorganism clone 4-3-12 mercury methylating protein (hgcA) gene, partial cds	43.7	43.7	55%	2.1	73.68%	KJ184720.1

Figure 16: BLAST results for somewhat similar sequences to DNA sample 3

The BLAST results provide a partial answer to the second objective, identifying some microorganisms that may be present in the sediment samples from the North Harbour. However, these results are not sufficient to make any conclusions. The BLAST results are only those of somewhat similarity to the sequence in question and are not highly similar. The sequence used for this analysis was a partial sequence that still contained many unknown base pairs (N). Under the advice of Mr. S. Fratpietro, who assisted with all DNA experiments, none of these sequencing results can be presented due to the high amount of unknown base pairs in all sequences. The BLAST results are presented only to demonstrate the possibility of sequence similarity and microorganism identification. They provide insight into the fact that the experiments completed so far are on the right track for identification of microorganisms that contain the *hgcA* gene.

4.6 BLAST Analysis for new primers

The previously unenxpected sequencing results could be the product of insufficient primer binding during the sequencing reaction. The primers used were very general in order to amplify the *hgcA* gene from as many microorganisms as possible. Unfortunately, it appears that the primers are too general for the sequencing reaction. Therefore, new primers were designed to be more specific. To design the new primers, BLAST was utilized again as there is a built-in primer designer in the system. The sequence of the previous BLAST results (Fig. 15) for the *Geobacteraceae* enrichment culture were used to design the primers. This was done because the results showed that this microorganism could be present in the North Harbour samples and could potentially be isolated using new primers. The primer design tool in BLAST was used and the proposed primers were compared to the original primers. Since the original primers provided some sequencing results, it was the goal to create a primer that was similar but more specific. The second half of the original primers had similarity to two BLAST-created primer pairs. These two primer pairs covered the first and second halves of the *hgcA* gene, respectively. Next, to confirm the ability of these primers to amplify the *hgcA* gene of interest, they were searched in BLAST for highly similar sequences. As expected, the results indicated microorganisms containing the *hgcA* gene. The specific primer pairs, *hgcA1*F&R and *hgcA2*F&R are outlined in table 3, section 3.9. Additionally, a primer pair reported by Christensen et. al (2016) specifically

for the *hgcA* gene in *D. Desulfuricans* (ND132F&R) was purchased and utilized for further sequencing experiments.

4.7 PCR with new primers

A PCR reaction was prepared using the two new primer pairs, *hgcA1*F&R and *hgcA2*F&R. The protocol used for the PCR was the same as in section 4.4.3. The LR DNA ladder was loaded first, followed by a blank, then all five samples using the *hgcA1* primer pair, and finally all five samples using the *hgcA2* primer pair (Fig. 17). The agarose gel electrophoresis showed that many DNA bands are present, including bands at the expected size of around 620 bp. The gel results appear to be warped, due to a loss of power during the gel electrophoresis run. This did not impact the results in any way. This gel demonstrates that the amplification was not specific enough, compared to other gels in this study. Ideally, one strong band would be visible in each lane. There is a possibility for optimization of the PCR with these primers, however due to time constraints this could not be completed in this study. The PCR products were not sufficient for further downstream sequencing reactions due to the lack of specificity and the amplification of other DNA that is not of interest.

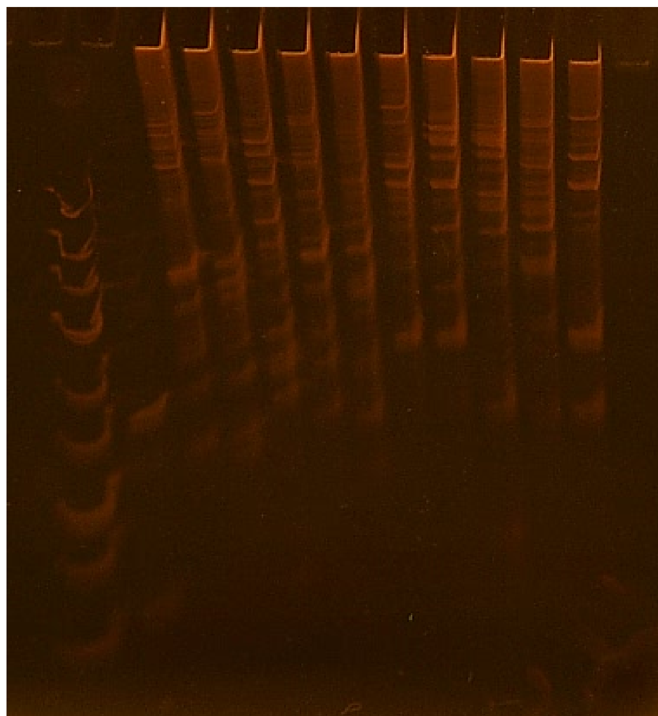

Figure 17: Gel electrophoresis of PCR with new primers

4.8 Sequencing with new primers

The PCR products from figure 16 could not be used for further sequencing, however, the new primers were used with the original PCR products (section 4.4.3) for sequencing. The original primers amplified DNA that was too general and could not be sequenced with high specificity for the *hgcA* gene. This means that the general PCR products could potentially be sequenced using more specific primers in the sequencing reaction. All three new primer pairs (*hgcA1*F&R, *hgcA2*F&R and ND132F&R) were utilized in the sequencing reaction with the originally amplified DNA. The results for this reaction were unexpected, as they were completely unsuccessful; no readable sequences were produced, including partial sequences. There are a variety of reasons this could have been happened, as sequencing is a complex procedure that involves many steps where mistakes may occur. One reason is that the DNA was degraded. The original amplified DNA could have degraded over the 2-week period that it was stored; however, this is unlikely as DNA can be frozen for much longer periods of time without degradation. Another reason is that the DNA amplified was not as general as originally thought. If the PCR products were actually more specific, then these primers would not work and there would be an alternative reason for the original sequencing reaction failure. The primer design could have been flawed, causing insufficient binding in the sequencing reaction and providing unreadable results. There are a number of reasons this could have failed, but it was not within the scope of this research to be able to troubleshoot and fully optimize the sequencing reaction.

5.0 Conclusions

Mercury methylation can occur via microorganisms that reside in aquatic environments. More specifically, microorganisms that contain the *hgcAB* gene cluster have been confirmed as Hg-methylators. The primary goal of this study was to determine the presence of microorganisms that contain the *hgcA* gene, and to identify the microorganisms present. The presence of MeHg in the North Harbour of Lake Superior was confirmed through analytical testing. The presence of the *hgcA* gene was confirmed using DNA experimental techniques, including PCR. The gene of interest was amplified from sediment samples taken from the North Harbour, establishing the presence of microorganisms containing the *hgcA* gene, and the potential presence of Hg-methylating microorganisms. However, without significant sequencing results, the presence and identity of Hg-methylating cannot be confirmed. The BLAST analysis of one sequencing result indicated a microorganism that had somewhat similarity to the partial sequence. The indicated that *Geobacteraceae* microorganisms could be present in the sediment samples, but the results were not convincing enough to reach any conclusions about the microbial community present in the samples. New, more specific primers were designed to potentially amplify and sequence the *hgcA* gene with more specificity, however those results were unsuccessful and inconclusive. DNA work can be difficult to master and optimize. There is potential for entire theses to be completed on the primer design and PCR optimization alone. Given the scope of the research, completing optimization for all aspects of the DNA experimentation was not possible. The future of this research is exciting and there are many possibilities for continued study of the microbial community of the North Harbour in Lake Superior. Due to the complex nature of MeHg research, opportunities to study various aspects of mercury methylation are present and necessary. In conclusion, *hgcA*-containing microorganisms are present in the North Harbour, however the identification of these microorganisms was not possible and deserves continued investigation. A final decision on the best way to handle the contamination in the North Harbour will depend on a thorough analysis of all factors involved in Hg-methylation from a large number of samples points

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