Characterization and Attempted Isolation of Bacteria from the Marine Myxobacterial Clade

Bahar Pakseresht

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By: Bahar Pakseresht

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complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

Dr Yves Gélinas_____ Chair

Dr David Walsh_____ Examiner

Dr Cameron Skinner_____ Examiner

Dr Brandon Findlay_____ Supervisor

Approved by Dr Yves Gélinas_____
Chair of Department or Graduate Program Director

July 7th 2020 Pascale Sicotte_____
Dean of Faculty

ABSTRACT

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Antibiotic resistant infections are caused by antibiotic resistant microorganisms. According to World Health Organization (WHO) antibiotic resistance is one of the major threats to global health, food and development. The increasing rate of mortalities caused by antibiotic resistant infections has highlighted the need to find a way to tackle these resistant microbes. One of the ways to solve this problem is to introduce novel antibiotics, the likes of which bacteria have not encountered before. Most of the antibiotics are natural products derived from bacteria. Among these natural product producers, myxobacteria have proven themselves as one of the main sources of antibiotics. These bacteria carry large numbers of gene clusters that can express different secondary metabolites for various purposes. Most of these gene clusters can encode secondary metabolites that not only help the bacteria to survive but also can be biologically active. Marine myxobacteria, in particular, produce biologically active natural products that are different from the ones terrestrial myxobacteria make. Therefore in this study we looked into an environment with unique conditions from terrestrial or marine, Gulf of Saint-Lawrence, to find novel strains from the marine myxobacterial clade. Sediment samples were extracted from six stations in Gulf of Saint-Lawrence. Based on the studies conducted on the DNA content of the sediments we learned that the primers that were previously designed to specifically target MMC were also detecting other strains of bacteria closely related to MMC. Furthermore, we isolated the RNA content of the sediment samples to get an insight into their metabolic activity. For this purpose we employed qPCR techniques to measure their abundance and ribosome content. Furthermore, in an attempt to cultivate marine myxobacterial clade (MMC) we isolated the bacteria present in the sediment samples to use them as prey for marine myxobacterial clade. Based on qPCR studies we were able to conclude that the MMC were growing actively under estuary conditions. However, the attempt to cultivate the MMC on the bait plates led to emergence of vancomycin resistant Bacillus strains along with other saprophytes on the plates. These findings suggest that members of the MMC are

active under the Estuary condition and can be cultivated if subjected to the same condition as present in the Estuary of St-Lawrence.

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CONTRIBUTIONS

This research project was carried out in collaboration with Dr. David Walsh and Dr. Yves Gelinas research groups.

This study was designed by Dr. Brandon Findlay and Dr. David Walsh. All the sediment samples were collected by Anic Imfled from Dr. Gelinas' research lab. All the DNA content of the sediment samples were extracted by Susan McLatchie. Part of the qPCR data and most of the sequences used to make the phylogeny tree were retrieved from Susan McLatchie.

Table of Contents

List of Figures

List of Tables

Chapter 1. Introduction

1.1 Antibiotics, the force against infections

The word "antibiotic" refers to compounds produced by microbes that can kill other microbes or stop them from growing and was first used by Selman Waksman.[1] The first antimicrobial compound was discovered by Ehrlich in 1911. This compound was considered as a "magic bullet" that could cure Syphilis without harming the patient.[2] The "magic bullet" was actually arsphenamine and being the 606th compound that was tested by Ehrlich, it was also called 606.[3] Later this compound was called Salvarsan and it was widely used as an effective cure for syphilis until the 1940s, when penicillin became accessible.[3]

In 1928 penicillin was serendipitously discovered by Alexander Fleming.[4] Although the discovery of penicillin offered an effective solution to cure infections, it was not purified and produced for medical use until 1940.[5–7] By late 1940, a team of scientists in Oxford University led by Howard Florey designed a method to produce penicillin in large quantities.[8] The discovery of penicillin and its healing effects on infections prompted the research into the identification of many other new antibiotics. This marked the beginning of an era, 1950 to 1960, known as golden age of antibiotic discovery.[9,10]

Although the antibiotics were effective in curing infections, the battle against infections didn't end. Unfortunately, soon after antibiotics were discovered, the bacteria grew resistance against the antibiotics that were meant to kill them.[10] However, while antibiotic-resistant bacteria have increased in prevalence since the 1940's, metagenomic studies have demonstrated that many resistance-conferring genes clusters are millions of years old.[11,12] These gene clusters might have appeared due to early use of natural products with antibiotic activity in ancient times.[11–13]

Antibiotic resistant infections cause problems in many ways. They take lives, hinder healing processes from different diseases and increase costs. According to the Centers for Disease Control and Prevention (CDC) every year over 2.8 million people are affected by antibiotic-resistant infections, leading to over 35,000 deaths in United States.[14] Even when they do not kill, antibiotic-resistant infections can cause longer stays at the hospital which in turn makes the patients more prone to acquiring other infections.[15] According to a study published in Health Affairs, these prolonged hospital stays increase medical costs by 2.2 billion dollars every year in the United States.[18] Antibiotic resistant infections have been listed as major threats to human health.[16] As shown in Figure 1, it is postulated that antibiotic resistant infections will be the leading cause of death by year 2050.[16]

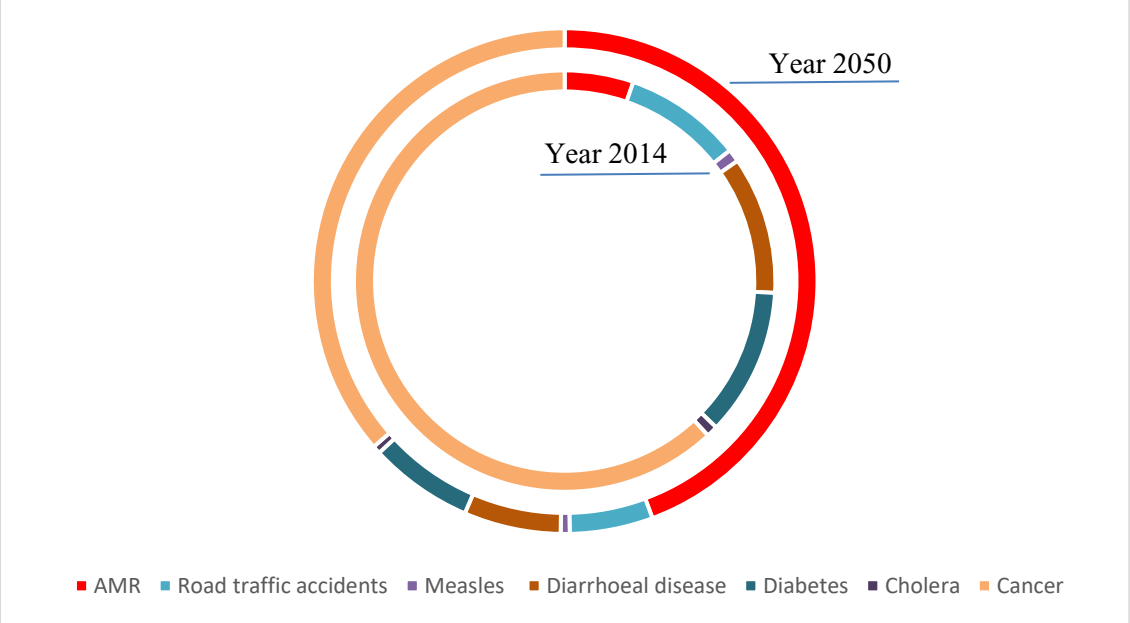

Figure 1. Deaths projected in 2014. Based on information retrieved from Review on Antimicrobial Resistance 2014.[17] Antimicrobial resistant (AMR) infections (shown in red) are expected to be the leading cause of death in year 2050.

The unremitting problem of antibiotic resistance has led to the development of many tactics to combat antibiotic resistance. One very efficient and immediate solution to antibiotic resistance is finding novel antibiotics from natural resources.[18]

1.2 Natural products are a key source of new antibiotics

Natural products are compounds made by living organisms.[19] Secondary metabolites, compounds synthesized by organisms that aren't directly needed for growth, form the bulk of bioactive natural products. Secondary metabolites can help organisms to adapt to their environment better, protect themselves against any external threats, and be used as a means of communication.[20,21]

The process to find a new bioactive natural product includes screening and testing the extracts collected from the natural resource for biological activity at every level of purification, which makes this process time consuming and costly.[22] However, natural products possess the complexity and diversity that makes them more preferred than synthetic drugs in drug discovery.[23] Another reason that makes natural products more desired than synthetic drugs in drug discovery is the fact that bioactive secondary metabolites possess good bioavailability which makes them good drug candidates despite not following the Lipinski rule. Lipinski rule states that synthetic drugs in order to be active have to have a molecular weight less than 500 Da, 5 hydrogen bond donor, 10 hydrogen bond acceptors and log P of 5 to have good bioavailability.[24] Unlike synthetic drugs, the bacterial secondary metabolites that are already bioactive when extracted don't need refinements and modifications to penetrate the cell membrane, they reach their target within the cell through transmembrane transporters. This comes as an advantage for natural products, since they don't need modifications to enable them to pass through the membrane.[24]

These bioactive compounds can have a wide range of actions, one of which is antibiotic activity. These compounds can be retrieved from many natural product resources and bacteria have proven themselves as good resources for potent antibiotics. They account for more than 75% of the antibiotics found in the years between 1981 and 2006.[25] These compounds produced by bacteria can act through different mechanisms for instance shutting down the protein synthesis "factory", preventing their target from completing the DNA replication, etc.[26] This provides an advantage, since using different mechanisms to fight off the bacteria can decrease the chance of bacteria growing resistance against that particular class of antibiotics.[26]

Bioactive secondary metabolites isolated from different strains of bacteria like the species from *Streptomyces* have been successfully used in medicine for many years.[27] Although there are many known bioactive secondary metabolite producers that can be used as sources of natural products, finding other resources offer discovering compounds with different scaffolds which appears as an advantage in the battle against antibiotic resistant infections.[28]

Myxobacteria in particular are a group of predatory bacteria that have established themselves as significant producers of bioactive secondary metabolites with new chemical scaffolds.[29] They prey upon other microorganisms to get their essential nutrients. These bacteria secrete secondary metabolites which provide myxobacteria with strategies to incapacitate the prey.[29] Their ability to make biologically active secondary metabolites has put myxobacteria amongst the greatest natural product producers like *Actinomycetes spp.*[30,31]

1.3 Myxobacteria are potent bioactive natural product producers

Myxobacteria are rod-shaped Gram-negative proteobacteria capable of moving and preying on other microorganisms present in the environment. They have been found ubiquitously in soils from all seven continents[32–34]and were first described in 1892 by Thaxter, an American mycologist.[35] In this article, he described finding samples resembling fungi in New England and the Southern United States which, given the samples lacked hyphae, Thaxter assumed that they were dried fungi in the course of forming fruiting bodies.[35] His work was followed by additional seminal studies on myxobacteria by researchers such as Baur, Kofler, Jahn, and Kühlwein.[36] Myxobacteria, apart from being a prolific natural product resource, also exhibit interesting social behaviour.[29] The small molecules that are transferred within the extracellular matrix that myxobacteria have secreted, are responsible for their communication.[37] The matrix is also used as a platform for their group movement towards the prey. As a result, when grown on agar myxobacteria form large swarms that can be used to visually identify members of this group.[37]

The survival of myxobacteria in harsh conditions depends on myxobacteria adopting a more resilient form. Therefore they have evolved a strategy in which they form fruiting bodies that

harbor dormant cells, myxospores.[37] Fruiting body formation is triggered by nutrient limitation, and allows cells to survive until conditions improve.[37] Fruiting bodies are visible to the naked eye, and can be used to distinguish myxobacteria from other bacterial species.[37]

Natural products that have been isolated from these bacteria so far not only exhibit antibiotic effects but also are potent antifungal, antimalarial, immunosuppressant, antioxidative and anticancer agents.[38] So far 100 core structures and at least 500 derivatives have been identified from different strains of myxobacteria.[29] These metabolites are from different classes of natural products including polyketides, phenyl-propanoids, and alkaloids. These secondary metabolites have their own biosynthetic gene clusters in the myxobacterial genome along with many other gene clusters that encode novel bioactive secondary metabolites.[30] The natural products produced by myxobacteria have diverse structures, mechanism of action and effects on microorganisms. It is reported that about 54% of these compounds are known to act as antifungal agents. They can interfere with electron flow in the respiratory chain of mitochondria. Around 29% of the secondary metabolites produced by myxobacteria are antibacterial.[39] Thuggacin is one of these secondary metabolites that targets the electron transport chain and can act against *Mycobacterium tuberculosis.*[40] While other compounds like corallopyronin[41], etnangien[42], myxopyronin[43], ripostatin, and sorangicin[44]are known to inhibit RNA polymerase.

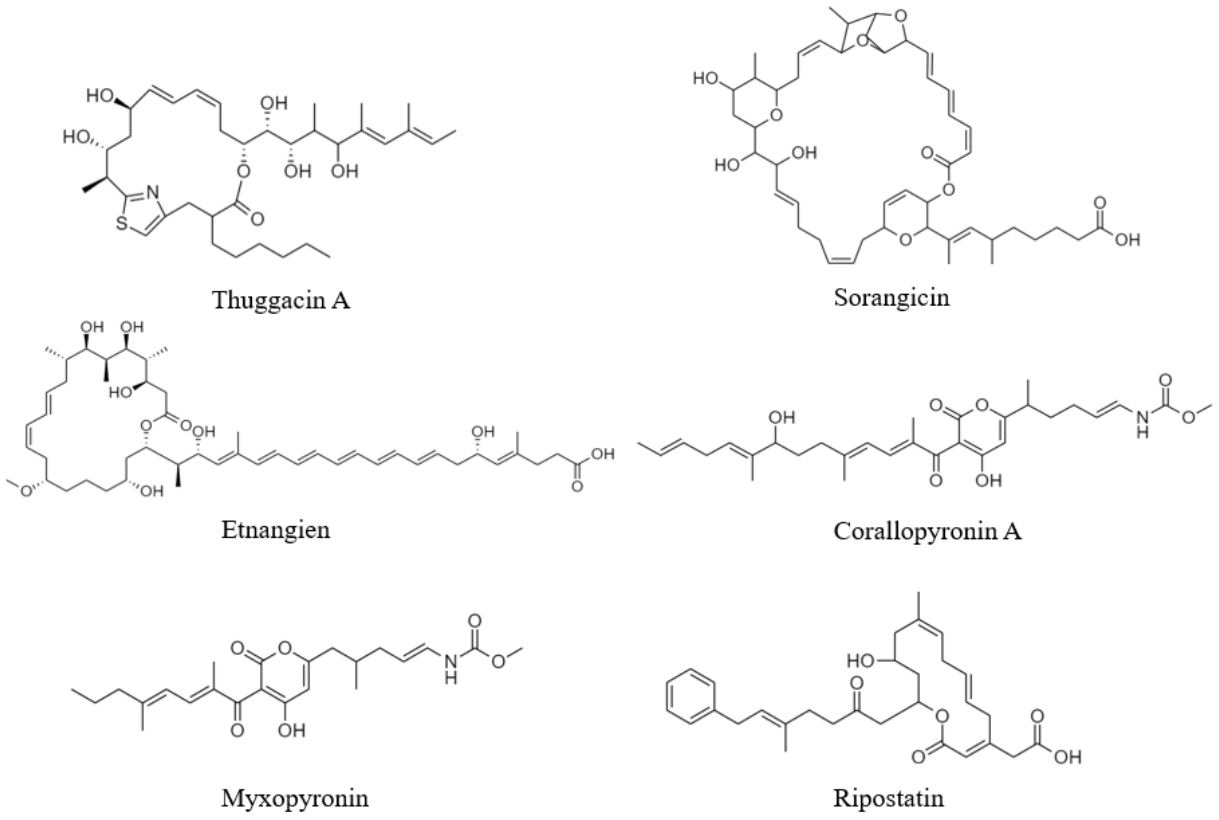

Figure 2. Chemical structure of the secondary metabolites isolated from Myxobacteria.

The secondary metabolites produced by myxobacteria have a wide range of actions, while some are capable of acting as antimicrobial agents others display anticancer activity.[45] Some of these compounds that have shown promising activity against tumor cells are disorazol, tubulysin[45], and epothilone.[46] They target the tubulin structure of the tumor cells and they interfere with the cell division.[47] Since these secondary metabolites have shown promising biological effects, studying myxobacteria as natural product producers is important in drug discovery.

Myxobacteria were initially thought to inhabit terrestrial environments exclusively[48], but halotolerant species have later been identified in marine sediments and saline lakes.[32] These microorganisms are a rich source of bioactive lead compounds.[49]

Marine Myxobacteria are bacteria related to terrestrial myxobacteria and their existence was first hypothesized in 1999 by Hans Reichenbach.[48] They differ in many ways from their terrestrial

counterparts, but to date all marine myxobacteria are still able to prey upon other microorganisms, form fruiting bodies and glide over solid surfaces.[50–52] However some of the secondary metabolites that have been isolated from these bacteria are different from the ones terrestrial myxobacteria produce.[53] The explanation for this behavior is that in response to environmental changes the bacteria are provoked to produce secondary metabolites that help them survive the new conditions.[54] For instance, to cope with the tremendous amount of pressure that is subjected upon bacteria living on the ocean floor, they develop strategies to survive. These strategies can be through synthesizing proteins and secondary metabolites or structural changes.[55,56] For instance in *P. profundum* SS9 the cell membrane has become more fluid due to the ratio of unsaturated to saturated lipids in the membrane.[57]

Some of these secondary metabolites that have been produced in response to the stress have various biological effects . Therefore strains of bacteria that are already known to be potent producers of bioactive compounds and are found in a different environment than their original habitat can be interesting targets for natural product studies.[53]

Although marine myxobacteria have not been as thoroughly studied as terrestrial myxobacteria, there have been papers published on the potent bioactive secondary metabolites derived from these strains.[58,59] For instance haliangicin, is a potent antifungal agent produced by *Haliangium luteum* that was isolated by Ryosuke Fudon et al. in 2000.[58] These compounds not only can be used as treatments for diseases but also studying these structures provides scientists with insights into antibiotic biosynthesis and their mechanism of action. To isolate and investigate on these small molecules it is necessary to cultivate these strains and isolate the metabolites they produce.

The marine myxobacterial clade (MMC) is a recently discovered cluster of myxobacteria that are distantly related to both the currently cultured marine myxobacteria and terrestrial myxobacteria.[60] Like the currently cultured marine myxobacteria, bacteria from the MMC inhabit environments with salinity ranging from brackish to marine.[60] Metagenomic studies suggest these bacteria may also produce a multitude of secondary metabolites, with biosynthetic gene clusters responsible for the production of type I polyketides, highlighting the importance of continuous effort to isolate and cultivate these natural product producers.[61–63]

Isolating novel secondary metabolites from marine myxobacteria can help address the ongoing problem of antibiotic resistance. However, this requires pure cultures of each strain of interest, and despite several attempts, to date no MMC strains have been cultured in the lab.[64]

1.4 Assessing marine myxobacterial activity using qPCR methods

Real time quantitative polymerase chain reaction, abbreviated as qPCR, is a technique in which the amplification of a target DNA can be monitored throughout the PCR process.[65] In this process a fluorescent dye is introduced to the mixture containing the DNA template. After being excited by ultraviolet light, the dye bound to double-stranded DNA fluoresces, producing a visible signal that is detected by a conventional UV-Vis spectrometer.[65] As the template is amplified the signal increases in intensity, and this increase is plotted against the corresponding cycle number. This is then plotted against a standardized exponential curve, revealing the starting copy number for the DNA of interest. In cases where the starting material is RNA, an additional step of generating the cDNA is conducted prior to qPCR.[66] One of the most popular applications of qPCR is to quantify the expression level of a target gene. This goal can be reached by using the specific primers to target the desired fragment of RNA.[67]

Like all known organisms, bacteria produce ribosomes to translate RNA into protein.[68,69] The small ribosomal subunit, 30S, contains 16S rRNA that is commonly used in phylogenetic studies. What makes 16s rRNA a favored target in these kind of studies is that it can be used to distinguish different bacterial strains.[70] 16s rRNA is composed of both hypervariable and conserved regions. The conserved regions are common among all the bacterial strains while the hypervariable regions are specific to each strain.[70] These sequences can be used to assess their similarity to the other strains of bacteria that have been already discovered.[70] Targeting the conserved region of 16S rRNA helps to identify the different strains of bacteria present in the sample, while targeting the hypervariable region assists studies conducted on a specific strain of bacteria.[70]

Furthermore, 16S rRNA can be used to measure the 16S rRNA copies of a particular strain. Knowing that 16S rRNA is a part of the ribosome scientists can relate the amount of 16S rRNA

transcripts to the metabolic activity of the bacteria.[71,72] This idea has been used in different studies to measure the activity of the bacteria or to measure their abundance in different samples.[73] For instance, Axel Fey and his colleagues sought to quantify the titre of pathogenic bacteria in environmental water samples. They determined the expression level of the genes using qPCR and the specific primers designed to identify them.[74] Since 16S rRNA also contains a fragment that is common amongst all strains of bacteria, it can also be used to reflect the total amount of bacteria present in the sample. An example for this case is a study conducted by Axel Schippers and Lev N. Neretin. They used universal primers for 16S rRNA gene to quantify the total amount of bacteria in their sediment samples.[72]

It's been suggested that quantifying 16S rRNA might not reflect the accurate information on bacterial community and their state of activity.[75] Other ways to determine bacteria's level of activity includes measuring their consumption of organic sources like oxygen.[75] However the sediment samples consist of many different strains of bacteria and measuring the oxygen content of the sediments might not give insights into MMC level of activity.

As a conclusion, by comparing the amount of MMC 16S rRNA to MMC 16S rRNA gene we can learn more about their metabolic activity and come up with ways to grow these bacteria under laboratory conditions to extract secondary metabolites.

1.5 Previous attempts to cultivate members of the marine myxobacterial clade

There are an estimated 5×10^{30} bacterial species in the world[70], but only approximately 2 % of these are culturable.[71] This discrepancy, the difference between the number of culturable bacteria and the total bacteria present in the sample, has been termed the Great Plate Count Anomaly.[76] Some strains of myxobacteria, like the members of marine myxobacterial clade are good examples for this concept. There can be several reasons to explain why they cannot be cultivated. Some of the

reasons refer to the media the bacteria is being seeded in. The physical condition present in the lab can also hinder the growth of bacteira.[71] Myxobacteria are slow growing microorganisms in nature, and this gives the other saprophytes present in the environment the chance to over grow myxobacteria.[77]

Although many members of the marine myxobacterial clade are unculturable, many terrestrial strains have been successfully cultivated in the lab.[78] A classic way to isolate terrestrial myxobacteria is to grow them on rabbit dung and if cultivated in a moist chamber at room temperature the colonies will start to form within a few days.[79] Rabbit dung provides a nutritious source for myxobacteria and stimulates them to form fruiting bodies.[80] These fruiting bodies can form in different colors and they are used to visually distinguish terrestrial myxobacteria.[80] This method of cultivation seems to work fine for isolation of bacteriolytic myxobacteria.[80] There are also other ways to cultivate terrestrial myxobacteria, like using prey species as bait in their culture.[81] Almost all of the myxobacterial strains would grow with the presence of a bait, in most cases *E. coli*, in the media.[81] This method is called baiting method and includes suspending live prey bacteria, *E. coli,* in a nutrient poor media as the sole nutrient source for myxobacteria and incubate them at room temperature for a month.[29] Although this method will provoke myxobacteria into forming swarms and fruiting bodies to make them easier to identify, the growth of myxobacteria is hindered by contamination with amoeba and other bacteria.[29]

Some species within myxobacterial clade would grow easier if provided with more specific nutrients to grow. For instance, *Sorangium* strains that are cellulose degraders would grow easier if cultivated on filter paper pads. These species grow slowly, and in case of *Sorangium,* it can take 12 to 14 days for them to grow into visible colonies.[79]

The marine myxobacteria strains that have been cultivated heretofore are mostly classified in *Nannocystineae, Haliangium*, *Enhygromyxa*, and *Plesiocystis* suborders.[64] Furthermore these strains are found to be halophiles and they need salts in their media to grow.[82] After a few years since the discovery of marine myxobacteria suborders a novel clade was discovered that represented a range of myxobacteria strains relating to marine myxobacteria. Attempts to cultivate, this newly discovered marine myxobacteial clade were unsuccessful . Based on previous studies[60,83], the strains of marine myxobacteria clade discovered so far seem to exclusively inhabit

environments with salinity of about 3.5 %.[83,84] In research studies conducted so far the samples were isolated from seawater and sediment samples.[60,84,85]

This finding indicates that some of the MMC strains may need fastidious conditions to grow, including the salinity and the elements present in their original habitat.[82] The ability of MMC to grow in saline environment serves a few benefits to the study, one of them being able to isolate MMC from the bacteria that can't survive saline conditions. Terrestrial myxobacteria cannot tolerate a salinity percentage of 1 or above, which in turn can lead to members of the MMC being able to grow in environments that restrict the growth of terrestrial myxobacteria.[79]

Previous attempts to isolate members of the MMC led to the conclusion that the condition under which the MMC were being cultivated should be similar to the conditions in which the bacteria were initially isolated from.[60,82] This idea of subjecting the sediment samples to ocean like condition to grow MMC was confirmed by the studies conducted in the same manner to cultivate marine myxobacterial strains.[77,85] There have been attempts to cultivate MMC, in a study conducted by Till F. Schaberle et al. They isolated members of the marine myxobacteria from beach sediments and cultivated them under ocean-like conditions using artificial sea water.[77] In this study it was stated that if cultivated in rich media cell density would be low. In order to screen for marine myxobacteria, samples retrieved from the beach were spotted on *E.coli* in agar containing an antifungal agent: cycloheximide.[77]

The members of the MMC appear to be very difficult to cultivate and no one was able to cultivate them to this date. In the previous sections we discussed using qPCR techniques to verify MMC metabolic activity. By using the information collected from the assessment of MMC metabolic activity we will be able to comprehend their living needs better. In other words, we surmised that the MMC could be cultivated if subjected to the same condition as their original habitat.

If the MMC are active in the estuary we can mimic some of the conditions present there, including the salt concentration and their food source, bait bacteria, in the sediment, to cultivate MMC. In order to apply exactly the same salt concentration we collected the water samples from the Gulf of St-Lawrence to provide the bacteria with the same concentrations of salts and other organic matters

they would need. Also, we tried to isolate the other strains of bacteria present in the sediment samples to prepare the MMC with the prey it would need to grow.

This study focuses on the assessment of MMC metabolic activity and using that information to cultivate MMC.

Chapter 2. Experimental

2.1. Materials

2.1.1. Reagents and kits

Here the reagents and kits used in this study are listed in regard to the provider and their catalog number.

Table 1. The list of important reagents and kits used in this study.

Product	*Manufacturer*	*CAT#*
RNeasy® PowerSoil® Total RNA Kit	QIAgen, Germantown, MD, United States	12866-25
EZ-10 Spin Column Plasmid DNA Miniprep Kit	Bio Basic, Markham, ON, Canada	BS614
Milli-Q® IQ 7003/05/10/15 Water Purification Systems	Sigma-Aldrich, St. Louis, MO, United States	
MBI evolution EvaGreen qPCR master mix	Montreal Biotech, Dorval, QC, Canada	MBI-E250
Low-retention aerosol filter tips (10µl, 20µl, 100µl, 1000µl)	VWR, Radnor, PA, United States	89174-520 (10µl) 89174-524 (20µl) 10126-388 (100µl)

		89174-530 (1000µl)
Phusion® High-Fidelity DNA Polymerase	New England Biolabs, Ipswich, MA, United States	M0530S
Falcon® 96-Well Cell Culture Plates, Corning®	VWR, Radnor, PA, United States	CA15705-064
GeneJET PCR Purification Kit	Thermo Fisher, Waltham, MA, United States	K0702
Eco qPCR Thermo resistive sealing film	Montreal Biotech, Dorval, QC, Canada	EC-200-1003
Eco qPCR 48 well custom reaction plate	Montreal Biotech, Dorval, QC, Canada	EC-200-1002-10
Taq DNA Polymerase with ThermoPol® Buffer	New England Biolabs, Ipswich, MA, United States	M0267L
TaqMan™ Reverse Transcription Reagents	Applied Biosystems, Foster City, CA, United States	N8080234
Proteinase K Solution	Bio Basic, Markham, ON, Canada	PB0451
Difco™ Marine Broth 2216, BD	VWR, Radnor, PA, United States	CA90004-006
VWR® Vacuum Filtration Systems, Standard Line	VWR, Radnor, PA, United States	10040-436
T4 DNA Ligase	New England Biolabs, Ipswich, MA, United States	M0202S
PCR clean up for DNA sequencing	BioBasic, Markham, ON, Canada	BT5100
CloneJET PCR Cloning Kit	Thermo Fisher, Waltham, MA, United States	K1232

RNaseZAP™	Sigma-Aldrich, St. Louis, MO, United States	R2020-250ML
Phenol	TCI chemicals, Portland, OR, USA	P1610
Disposable Inoculating Loops and Needles, Sterile, BD Difco™	VWR, Radnor, PA, United States	220217
EZ-10 Spin Column Bacterial Genomic DNA Miniprep Kit	BioBasic, Markham, ON, Canada	BS624

2.1.2. Bacterial strains

Escherichia coli DH5-α cells were used for transformation. *E. coli* DH5-α has the genotype:

fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17.[86]

The strain of *E. coli* used to produce the qPCR standard curve was MG1655.

The strains, provided in Table 2, used for preparing bait plates were retrieved from Zachary Schiffman, a former undergraduate student in the lab. He isolated these strains from bait plates in his attempts to cultivate marine myxobacteria.

Also some unidentified strains (A, B and C) were retrieved from the undergraduate volunteers working in the lab, Seydee Bien-Aime and Harman Warraich.

Table 2. Strains of bacteria used as bait. The list of bacteria isolated by Zachary Shiffman, that were used as bait in this study. These strains were selected from a list of bacteria that were present in the sediment samples of the Gulf of St-Lawrence.

Sample ID	*Station*	*Closest relative*	*Identity*	*Accession number*
18B-C2	S19	*Paenibacillus taiwanensis*	99 %	NR_044007.1
25B-B2	S25	*Psychrobacter cryohalolentis*	82 %	NR_075055.1
22B-B2	S19	*Pseudoalteromonas nigrifaciens*	99 %	NR_114188.1

2.1.3. Plasmids

The plasmid used for transformation was pJET1.2/blunt. This plasmid bears an ampicillin resistant gene and a lethal restriction enzyme gene. The gene encoding the restriction enzyme is Eco47I, and is responsible for production an endonuclease that cleaves DNA at GGWCC sites. During ligation, the insert is placed in between this gene, disrupting its function. As a result the colonies that emerge on the plate are the ones that hold the insert in their plasmids.

2.1.4. Primers

Primers were synthesized on demand by Integrated DNA Technologies, Inc. (IDT), USA. The lyophilized primers were dissolved in TE buffer upon arrival. The final concentration of the stock solution for each primers was 100µM and the samples were stored at -20 °C.

The primers used in this study are listed along with their sequences in Table 3.

Table 3. The list of primers used in this study.

Primer	*Sequence (5'-3')*
MMC 4F	AATGGAGAGGGTGGCGGAA
MMC 155R	CGTGGACTACCAGGGTATC
341F	CCTACGGGAGGCAGCAG
518R	ATTACCGCGGCTGCTGGCA
1492R	TACGGYTACCTTGTTACGACTT
27F	AGAGTTTGATCMTGGCTCAG
pJET F	CGACTCACTATAGGGAGAGCGGC
pJET R	AAGAACATCGATTTTCCATGGCAG
MMC 655F	AGTAATGGAGAGGGTGGC
MMC 841R	GGCACAGCAGAGGTCAAT

2.1.5. Culture media

Marine broth was used in this research project to cultivate the bait bacteria. The composition of this media is explained in Table 4.

Table 4. Composition of Difco™ Marine Broth 2216. The formula is per Liter.

Compound	*Amount (grams)*

Peptone	5.0 g
Yeast Extract	1.0 g
Ferric Citrate	0.1 g
Sodium Chloride	19.45 g
Magnesium Chloride	5.9 g
Magnesium Sulfate	3.24 g
Calcium Chloride	1.8 g
Potassium Chloride	0.55 g
Sodium Bicarbonate	0.16 g
Potassium Bromide	0.08 g
Strontium Chloride	34.0 mg
Boric Acid	22.0 mg
Sodium Silicate	4.0 mg
Sodium Fluoride	2.4 mg
Ammonium Nitrate	1.6 mg
Disodium Phosphate	8.0 mg

Marine broth was diluted in half strength sea water that was isolated from the Gulf of St-Lawrence. In order to prepare the solid media agar (1.5 %) was added. In order to sterilize the liquid media, the final mixture was autoclaved at 121℃ for 30 minutes.

For transformation Lysogeny Broth (LB) was used.

Table 5. Composition of LB broth. The formula is per Liter.

Compound	*Amount (grams)*
Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g

To mitigate the risk of contamination ampicillin (final concentration of 100 µg/mL) was added to the LB media after the media was autoclaved and cooled to room temperature.

2.1.6. Gel electrophoresis

All the PCR products were run on 0.8% agarose gel. The dye used for detection was ethidium bromide. The running buffer used in this study was Tris-Acetate-EDTA (TAE) 1X (Table6). The buffer was stored at room temperature after preparation.

Table 6. Composition of 1X TAE buffer.

Compound	*Concentration*
Tris	40 mM
Acetic acid	20 mM
EDTA	1 mM

2.1.7. Software:

Phylogenetic trees were built with Mega-X version 10.0.5.[87]

qPCR data was analyzed with the Eco study software supplied with the EC-101-1001 qPCR thermocycler.

Primers were designed with the help of the primer-blast tool (NCBI).[88–90]

2.2. Methods

2.2.1. Study sites and sampling acquisition

The sediment samples were collected from six different stations by Anic Imfled from Dr. Gelinas research group during a cruise to Gulf of St-Lawrence in the summer of 2017. The samples were collected using a box-core in Stations 19, 21, 23 and 25 and Van Veen grabs in Station DE aboard the RV Coriollis II. The top 10 cm of sediment were collected from the box core and distributed in Eppendorf tubes and stored at -20°C until further treatment.

2.2.2. Isolating bait bacteria

The strains used to prepare bait plates were retrieved from Zachary Schiffman, a former undergraduate student in the lab. Strains A, B and C, that were used as bait were obtained by the volunteers working in the lab, Seydee Bien-Aime and Harman Warraich. As shown in Figure 3, the sediment samples were spread on top of the marine broth agar plate and incubated in a dark place at room temperature, overnight.

The colonies that had formed on nutrient-rich agar plates were isolated and streaked to purity on nutrient rich media. For long-term storage of the bait bacteria, -80 ℃ stock of the strains were

prepared by adding 60 % glycerol (final concentration of 30 %) to 1mL of liquid cultures, then the samples were frozen using liquid nitrogen.

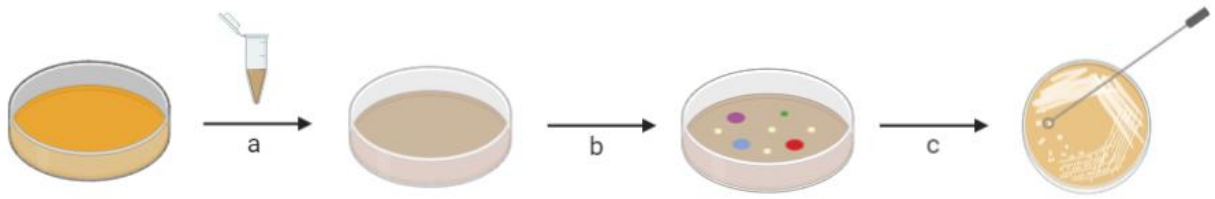

Figure 3. Isolating bait from the sediment samples. The initial culture contains nutrient rich media (marine broth) with seawater. a) The sediment samples that were dispersed in seawater by sonication are then spread over the plate and b) the plate was incubated at room temperature in the dark. c) the colonies that had grown on the plate were isolated and streaked to purity.

2.2.3. Preparation of bait plates

As illustrated in Figure 4, the isolated strains were inoculated initially in 5 mL seed cultures containing marine broth and autoclaved seawater. The liquid cultures were incubated at room temperature in a rotary shaker at 225 rpm for approximately 24 hours.[91] Then 1 mL of the seed cultures were inoculated in 1 L of marine broth liquid media and incubated at room temperature in the shaker for approximately 18 hours. Later on the cells were harvested by two tandem steps of first centrifugation at 5000 rpm for 20 min and then washing with distilled water. The cells were then suspended in agar along with vancomycin (20 µg/mL) and cycloheximide (100 µg/mL).

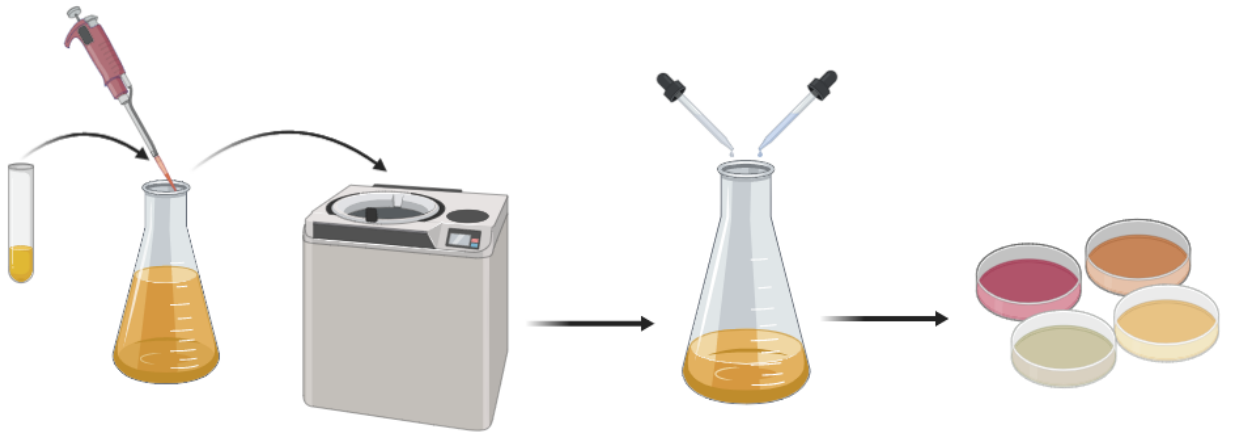

Figure 4. Preparation of bait plates. The seed culture contains marine broth and seawater. The bacteria are initially cultivated in the 5 ml seed culture in the shaker at 225 rpm adjusted to room temperature. Then 1 mL of the seed culture is inoculated in 1 L of marine broth and incubated in the shaker at 225 rpm and room temperature. Later the culture is spun down to harvest the cells. The cell pellets were then suspended in agar media along with vancomycin and cycloheximide and then poured into petri-dishes.

2.2.4. Cultivation, purification and extraction of putative marine myxobacterial clade members

About 1-2 mg of the sediment samples were dispersed in milliQ water via micropipette. Working close to the flame, approximately 100 µL of the mentioned solution was spread on each bait plate. The plates were then stored in the dark at room temperature to grow. Visible colonies started forming after 3-4 weeks of incubation. Representative colonies were then isolated and streaked to purity on separate agar plates containing marine broth, vancomycin (20 µg/mL) and cycloheximide (100 µg/mL). After 2-3 subcultures the bacteria were suspended in 30 % glycerol, and frozen using liquid nitrogen, for long term storage.

2.2.5. Genomic DNA extraction

Genomic DNA from strains speculated to be from the MMC were extracted with EZ-10 Spin Column Bacterial Genomic DNA Miniprep Kit. The 16S rRNA fragments of the genome were then amplified with universal primers 27F and 1492R. The amplified fragments were harvested using a PCR clean up kit for DNA sequencing. Then the fragments were blunt-end ligated into pJET 1.2/ blunt and transformed into *E. coli* DH5-α.

2.2.6. Transformation

E. Coli DH5-α cells were transformed using the pJET 1 2/blunt vector kit. For transforming E .coli DH5-α, approximately 1 µL of the ligation mixture was added to 100 µL of chemically competent DH5-α and mixed by pipetting. The mixture was then left to incubate on ice for about 10 minutes followed by a 45 second heat shock at 42 ℃.[92] Later the mixture was put on ice for 3 minutes. In order to let the bacteria grow the mixture was inoculated in 1 mL of LB media and then incubated in 37 ℃ incubator for about 1 hour. Then using the benchtop centrifuge, that can hold 1.5 mL to 2 mL tubes, the cells were harvested by centrifugation at maximum speed (15000 rpm) for 3 minutes.[92] The transformed DH5-α cells were then suspended in 200 µL of Lysogeny Broth (LB) and spread on top of LB ampicillin plates while working close to the flame. The plates were sealed with parafilm and incubated on the bench for 5 minutes and then transferred to a 37 ℃ incubator for 14 hours incubation. The colonies formed on the plates were then isolated for plasmid extraction.[92]

2.2.7. Plasmid purification

The pJET 1 2/blunt plasmids were extracted from *E. Coli* DH5-α using the GeneJET plasmid purification kit. Using Nandrop (ND-1000, Thermo Fisher) the concentration of the extracts were measured. In addition, the 260 nm/230 nm and 260 nm/280 nm UV absorption ratios were obtained to check for presence of protein or other contaminants. The cut off value for 260/280 was approximately 1.8 to ensure the integrity of the DNA samples.

2.2.8. DNA extraction from the sediment samples

The DNA content of the sediment was extracted by Susan McLatchie using the Powersoil DNA isolation kit. For long term storage all the DNA extracts were stored at -80°C.

2.2.9. Detecting MMC

The primers used to amplify the 16S rRNA using PCR were derived from a previous study on the MMC.[60] The forward and reverse primers are as follows: MMC655f (5-AGT AATGGAGAGGGTGGC-3)/MMC841r (5-GGCACAG CAGAGGTCAAT-3). The PCR products were generated using the 50 µL mixture detailed in Table 7.

Table 7. PCR reaction mixture to amplify MMC 16S rRNA. The primers designed by Bronkhoff et al[60]were used to identify and amplify MMC 16S rRNA. The total volume of the reaction mixture is 50 µL.

Reagent	*Amount*
5X Phire reaction buffer	10 µL
dNTP (10mM)	1 µL
Forward primer (final concentration of 0.5µM)	2.5 µL

Reverse primer(final concentration of 0.5µM)	2.5 µL
Phire Hot Start DNA Polymerase	1 µL
MilliQ	31.75 µL
Template DNA	1.25 µL

Fragments were amplified in a Applied Biosystems® 2720 Thermal Cycler under these conditions: 1 denaturation step at 98 ℃ for 30 seconds followed by 30 cycles of 5 seconds at 98 ℃, 5 seconds at 55 ℃, an 10 seconds at 72 ℃. The last step consists of polymerization at 72 ℃ for 1 minute. The PCR products were kept at 4 ℃ upon the completion of PCR. In order to check the integrity of PCR products the fragments were cast into 0.8 % agarose gel and electrophoresed for 60 minutes under 70 V.

2.2.10. Phylogenetic tree analysis

To construct a phylogenetic tree for the strains found in the sediment the sequences were aligned with their closest relatives using the MUSCLE software. Phylogenetic trees were constructed using the maximum likelihood method and Tamura-Nei model[93]. The initial trees for the heuristic were constructed by applying Neighbor-Join and BioNJ algorithms to a matrix pairwise distance that was estimated using the maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value in MEGAX software.[87]

2.2.11. RNA extraction from the sediment samples

The sediment samples collected from all the stations were weighed, then extracted with the RNeasy power soil total RNA kit according to manufacturer's instructions. The mass of the sediment samples ranged from 0.64 to 2.35 g. RNA extracts were diluted in 100 µL RNase free TE (Tris-EDTA) buffer and stored at -80°C. Aliquots of 10 µL were prepared and kept at -20°C for further experiments. RNA concentrations of the samples were measured using a Nanodrop ND-1000. Their 260/230 and 260/280 were measured to check the purity of the extracts. The 260/230 ratio for RNA samples varied between 0.83 and 1.24, and the 260/ 280 ratio was between 1.57 and 1.72.

2.2.12. cDNA synthesis

The cDNA samples were prepared from the RNA extracts on October 30th of 2019, with TaqMan™ Reverse Transcriptase according to manufacturer's instructions. The amount of RNA in the 100 µL cDNA synthesis cocktail was maintained at a maximum of 2 µg according to manufacturer's instructions [51,94]. The mixture was transferred to the thermocycler for cDNA synthesis. The mixture was first kept at 48 °C for 30 min and then the temperature was elevated to 95 °C and maintained for 5 min. The products were kept at 4 °C for further experiments.

2.2.13. qPCR assay

MMC-specific primers 4F and 155R were used to quantify the amount of MMC DNA/RNA in the samples. Standards for quantifying MMC RNA and DNA copy numbers were obtained from Susan McLatchie. The qPCR master mix consisted of Eva green super mix, forward and reverse primers and 1 µL of the cDNA or DNA samples.

To measure the abundance of MMC in the samples the total amount of bacteria was quantified using universal primers (341F/518R). In addition, the standards for universal primers were made using a pure culture of *E. coli* MG1655. The qPCR standard curve was generated on March 11th, 2020 for measuring the total amount of 16S rRNA and 16S rRNA gene in the sediment samples.

The total amount of 16S rRNA was measured on October 31st 2019, and the amount of 16S rRNA gene was measured through qPCR on October11th 2019.

The amount of the MMC 16S rRNA was measured on October 31th 2019. And the data used for the MMC 16S rRNA gene was retrieved from Susan McLactchie back in 2017 (August and September).

The values reported by the qPCR machine for total 16S rRNA gene standards were normalized by dividing the values by the standard concentrations.

The R^2 cut off value for the standard curve was 0.99, which ensured the precision of the experiment.

Chapter 3. Results and Discussion

This section is divided into two sections: one that focuses on arguments towards the presence and prevalence of MMC in the sediment and another that studies their cultivation.

3.1. Presence of MMC in Saint-Lawrence estuary

In the Summer of 2017 the research vessel Coriolis II did a tour of the Estuary and Gulf of St. Lawrence. During this time 30 cm core samples of St. Lawrence sediments were collected by Anic Imfled. The samples were collected from six different stations starting from the estuary going farther into the Gulf of St Lawrence.

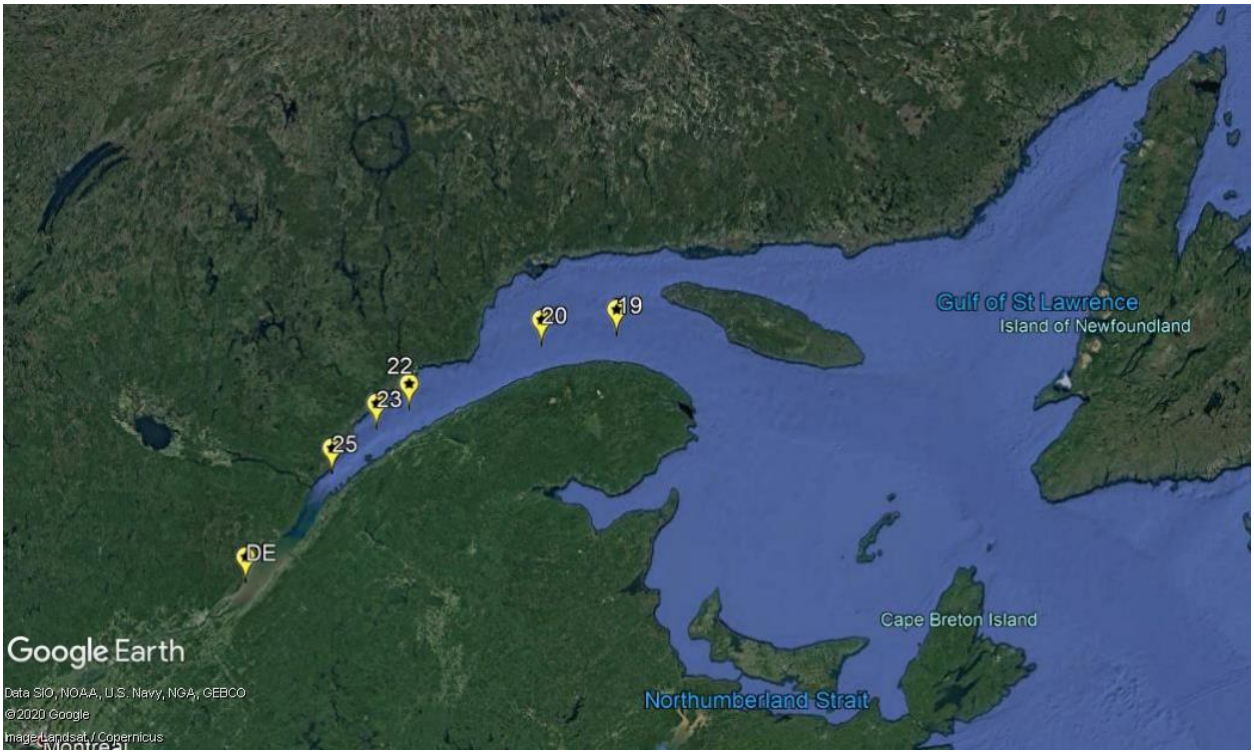

Figure 5. Sampling sites in the Gulf of Saint Lawrence. Six different stations were chosen for collecting sediment samples. The sampling started from Station DE located in the estuary and going farther into the Gulf to Station 19.

The sediment samples were collected from different depths of the sediment, from surface to 10 cm deep. The sampling was conducted by Anic Imfled in the Summer of 2017.

The sampling starts from station DE, deep in the estuary and represents a semi-saline environment. By sampling environments with same salt concentrations as the Estuary we could determine the impact of salt concentration on MMC prevalence, and potential increase the diversity of isolated/isolatable myxobacterial strains.

Strains from the marine myxobacterial clade (MMC) can be detected by qPCR. Therefore, prior to my arrival on the project the samples were weighed and their DNA content was extracted by Susan McLatchie. To verify the presence of MMC in the samples, the 16S rRNA gene fragment of the DNA extracts were amplified using MMC specific primers designed by Brinkhoff et al.[60] The successful amplification of the fragments was verified by gel electrophoresis, demonstrating the right amplification of the amplicon in all stations (DE, 25, 23, 22, 20 and 19) and depths of sediment tested (surface, 3 to 5 cm and 5 to 10 cm).

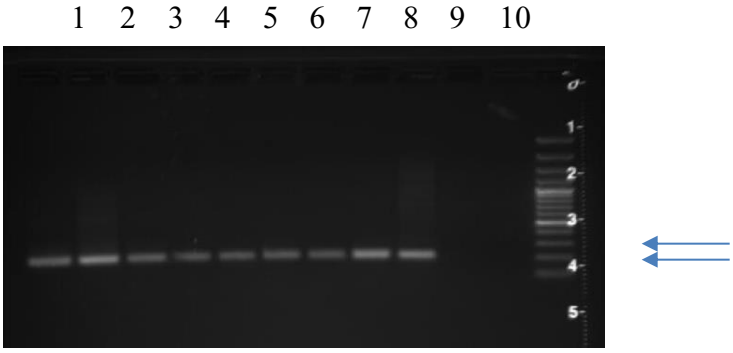

Figure 6. Gel electrophoresis of amplified MMC 16S rRNA fragments present in Station 19. Lanes 1 to 3 represent the MMC 16S rRNA fragments obtained from sediment samples in 3-5 cm depth. Lanes 4-6 show the samples taken from 5-10cm of sediment depth. Lanes 7-9 display the 16S rRNA fragments taken from the sediment samples collected from the surface of the ocean floor. Lane 10 represents the negative control, no DNA template was added. The DNA ladder is 100bp plus and the amplified fragment is 186bp long. The upper arrow on the DNA ladder shows 200bp and the lower arrow shows 100bp.

To further study the sequences of these fragments and create a phylogenetic tree, these fragments were inserted into pJET vectors and transformed into *E. coli* DH5α. The DNA of the cells lacking the insert in their vector were cut upon activation of endunoclease-1, greatly reducing the rate of

false-positives. The *E. coli* cells that had not absorbed the vector were susceptible to ampicillin, and so their growth was inhibited by streaking onto LB-amp plates. Thus the colonies that had formed on the plate were thought to harbor the vector with the insert. Antibiotic activity was verified after each transformation with a negative control, which had *E. coli* cells "transformed" with nanopure water. Positive colonies had their plasmids extracted and sent to Eurofins for sequencing. The identity of strains with 16S sequences close to these metagenomic samples were then obtained with the blastn tool.[95–102] To construct a phylogenetic tree the retrieved sequences were aligned using MUSCLE tool, MEGAX software. The final phylogenetic tree was then constructed using MEGA (Figure 7).

Strains sequenced as part of this work fell into several distinct clades. Based on the results retrieved from the phylogenetic tree (Figure 7), we can see that the strains that were identified by the MMC primers designed by Brinkhoff et al[60]fall into two separate clades closely related to each other the,MMC and Nb1-J. These observations led to the conclusion that the previously designed MMC specific primers are not exclusively targeting MMC strains. The bootstrap values shows the certainty of the position of the clades, and we can see that Nb1-J has fallen apart from MMC in the tree with the bootstrap value of 87. However based on the data presented in the phylogenetic tree we can see that this clade, Nb1-J, is closely related to marine, Plesiocystis and Enhygromyxa, and terrestrial myxobacteria, Sorangiineae and Cystobacterineae.

In the phylogenetic tree shown in Figure 7 the out groups were chosen based on their proximity to delta proteobacteria class. The out-group strains; *Bacillus mycoids*, *Bacillus subtilis*, and *Bacillus thuringiensis*, were selected from Firmicutes phylum to represent the root of the tree.

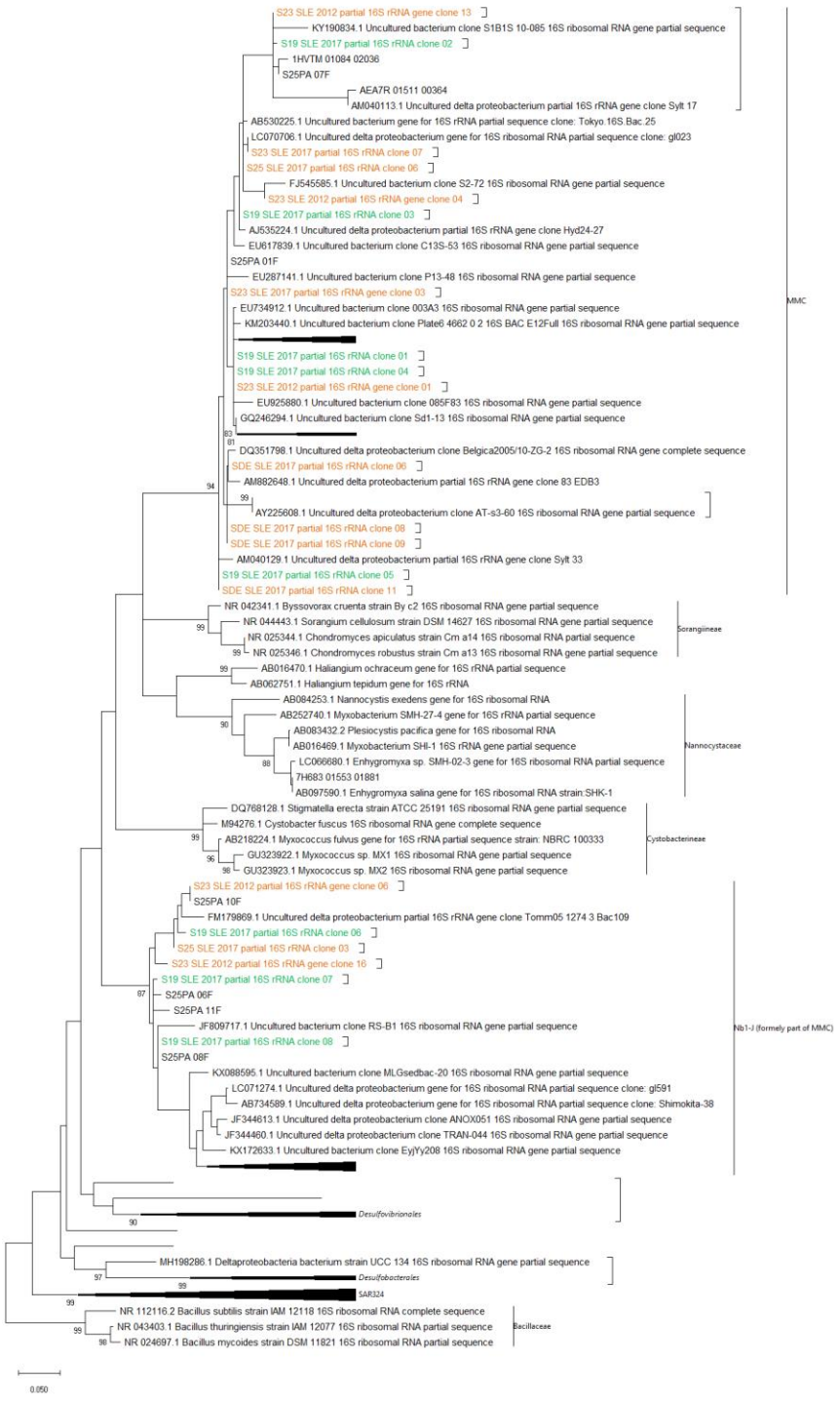

Figure 7. The phylogenetic tree of MMC strains isolated from Gulf of St. Lawrence. The strains isolated from the sediment samples were extracted and identified through 16S rRNA gene sequencing. The bootstrap values below 80 are not shown. The strains shown in orange were isolated by Susan McLatchie[103], and the strains shown in green were isolated by me. Some of the branches have been compressed due to lack of space.

As shown in the phylogenetic tree, the MMC specific primers that were previously designed by Brinkhoff et al[60]are also identifying some other closely related strains to marine myxobacteria. This in turn suggests that using these primers for qPCR may lead to errors in the quantification of strains from the MMC clade.

Based on the results obtained from the sequencing studies and the phylogenetic tree, we were able to conclude that strains from the MMC are present in some of the sediment samples, regardless of depth. These findings encouraged us to study MMC abundance and metabolic activity. Investigating their abundance and metabolic activity can give us insights into methods of cultivating them.

3.2. Abundance of MMC in Gulf of Saint-Lawrence

The qPCR studies were conducted using MMC primers designed by Susan McLatchie, to measure the amount of bacteria from the MMC present in the sediment, and universal primers, to measure the total amount of bacteria present. Both primers were designed to amplify a small fragment of the 16S rRNA (about 150bp) to guarantee efficient qPCR amplification.[104]

Table 8. The list of the primers used in these qPCR studies. The MMC specific primers were used to quantify MMC abundance and the universal primers were used to measure the total amount of bacteria in sediment samples.

Targeted group	*Type of extract*	*F primer*	*R primer*	*Amplicon size*
MMC	DNA	4F	155R	151bp
	RNA	4F	155R	151bp
Total bacteria	DNA	341F	518R	177bp
	RNA	341F	518R	177bp

As previously isolated marine myxobacteria prefer saline environments[60,82], we hypothesized that that their abundance would be low in the sediment samples with low salinity, Station DE, and their abundance would increase as we move farther into the sea, through Stations 25, 23, 22, 20 and 19. Furthermore, we hypothesized that metabolic activity would increase as the environmental condition becomes more suitable for marine myxobacteria.

To measure the abundance of MMC in the sediment, their 16S rRNA gene were amplified and quantified through qPCR using specific primers (4F and 155R) by Susan MacLatchie. I then adjusted these copy numbers to the total mass of the sediment sample (Table 9).

The limitation of this experiment was that the melt curve generated by Eco software showed broad peaks along the curve (Figure 8). Based on these observations it is possible that the designed primers (4F and 155R) were not exclusively targeting MMC, because of the broad peak we see in Figure 8. However, further studies need to be conducted to elucidate the possibility of targeting other bacterial strains than the MMC. The melt curve generated from Station 19 in Figure 8, is shown as an example.

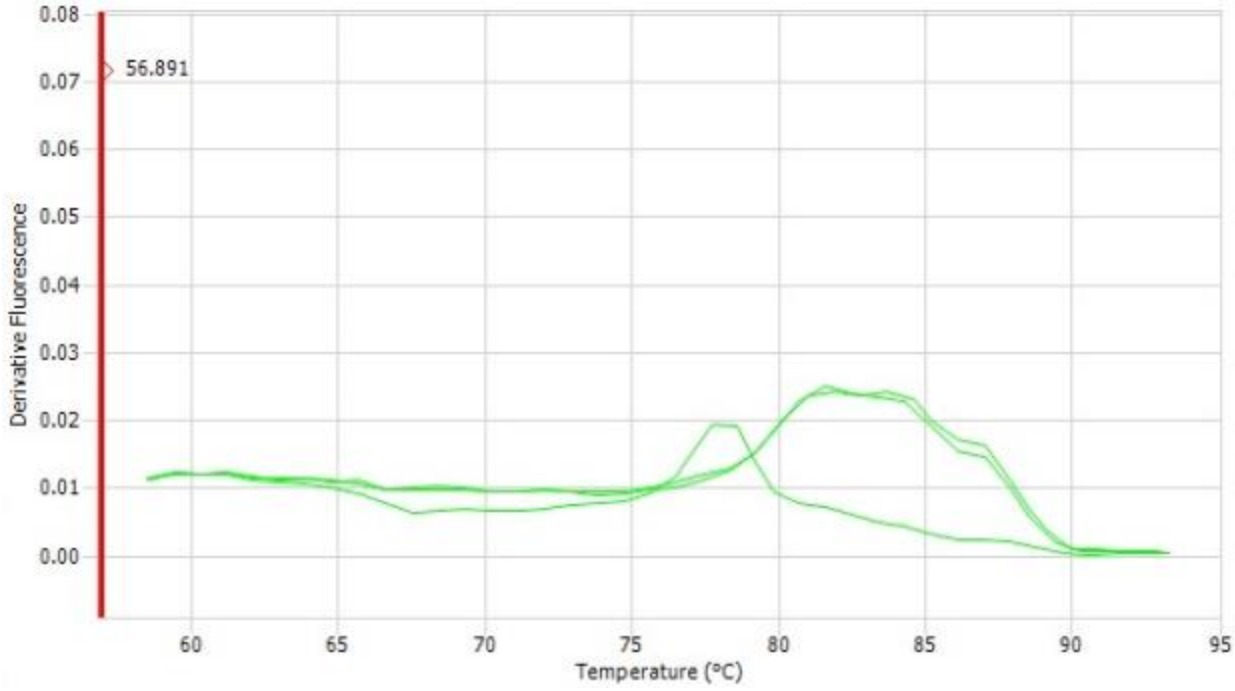

Figure 8. The melt curve generated from Station 19. The melt curve shows broad peaks starting from approximately 77 ℃ to 90 ℃. The curves are triplicates of the sample from Station 19. One of the curves does not overlap with the other two curves, that might be due to an error in the experiment.

Table 9. Amount of MMC present in the sediment samples. 16S rRNA gene copies were measured through qPCR using MMC specific primers. The amount of MMC strains was calculated by dividing 16S rRNA gene copies by 16S rRNA copy number in *E. salina (Enhygromyxa salina* ASM299463v1*)*[105]and *P. pacifica (Plesiocystis pacifica* SIR-1)[106]gnome (6 copies in the genome). This data was generated by Susan McLatchie.

Station	*Sediment depth interval*	*Abundance (16S rRNA gene copies/g)*	*Abundance (amount of bacteria/ g)*
DE	Surface	$5.98\times10^{7}\pm 9.43\%$	$9.96\times10^{6} \pm 9.44\%$
25	Surface	$3.57\times10^{7} \pm 10.33\%$	$5.95\times10^{6} \pm 10.33\%$
	3-5 cm	$4.11\times10^{6} \pm 4.94\%$	$6.85\times10^{5} \pm 4.94\%$
	5-10 cm	$9.56\times10^{6} \pm 54.39\%$	$1.59\times10^{6} \pm 54.52\%$

23	Surface	$5.95\times10^{7}\pm 7.56\%$	$9.92\times10^{6} \pm 7.56\%$
	3-5 cm	$4.87\times10^{7} \pm 8.7\%$	$8.12\times10^{6} \pm 8.7\%$
	5-10 cm	$4.09\times10^{7} \pm 18.33\%$	$6.82\times10^{6} \pm 18.32\%$
22	Surface	$6.31\times10^{7} \pm 4.56\%$	$1.05\times10^{7} \pm 4.57\%$
	3-5 cm	$4.58\times10^{7} \pm 2.59\%$	$7.63\times10^{6} \pm 2.59\%$
	5-10 cm	$2.41\times10^{7} \pm 20.12\%$	$4.02\times10^{6} \pm 20.12\%$
20	Surface	$3.95\times10^{7} \pm 3.24\%$	$6.59\times10^{6} \pm 3.24\%$
	3-5 cm	$3.39\times10^{7} \pm 4.69\%$	$5.65\times10^{6} \pm 4.69\%$
	5-10 cm	$2.52\times10^{7} \pm 13.09\%$	$4.19\times10^{6} \pm 13.09\%$
19	Surface	$5.95\times10^{7} \pm 6.84\%$	$9.91\times10^{6} \pm 6.84\%$
	3-5 cm	$3.19\times10^{7} \pm 11.56\%$	$5.31\times10^{6} \pm 11.56\%$
	5-10 cm	$1.86\times10^{7} \pm 12.47\%$	$3.10\times10^{6} \pm 12.47\%$

As seen above in Table9, the number of bacteria decreases as we go deeper into the sediment. These findings suggest that the MMC and the strains identified by primers are mostly aerobic, as their abundance decreases with drop in oxygen availability. In the surface layers of the sediment more oxygen is available compared to the lower layers.[108] Furthermore, myxobacteria are generally known as aerobic microbes.[32] However, this doesn't overrule the possibility of the presence of anaerobic strains of marine myxobacteria[109]in the Gulf of St. Lawrence.

The amount of the MMC and other strains identified by the primers, slightly increases in the surface layer of the sediment samples as we move farther into the sea, where the salinity along

with other characteristics of marine environment is more preferable for marine myxobacterial strains.[60,82] However, analysis of the melt curve suggests that these primers may be amplifying non-myxobacteria (vide supra). It is possible that Table9 tracks the activity of these strains in addition to the MMC.[110]

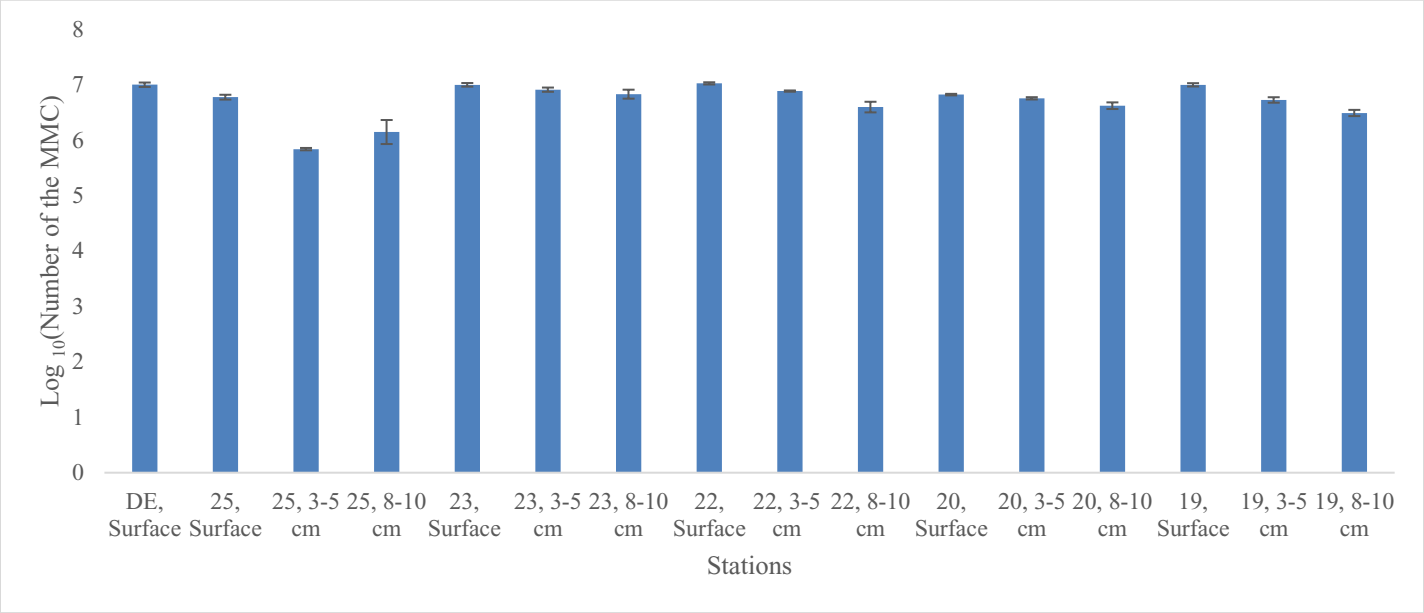

Figure 9. The prevalence of MMC 16S rRNA genes in Stations DE, 25, 23, 22, 20 and 19. The ordinate shows the logarithm base 10 of the number of the MMC and strains identified along them. Abscissa shows the stations and the sediment depth.

As they readily form spores in marginal environments[32,111], the presence of myxobacterial DNA isn't a guarantee that these strains are active. To determine the activity of the MMC and other strains identified along them in the St. Lawrence Gulf and Estuary I determined the copy numbers of the concentration of MMC 16s rRNA fragments in the sediment samples by qPCR, then compared to these values to the concentration of MMC 16s rRNA genes.

Table10. MMC 16S rRNA copies. The amount of MMC 16S rRNA was measured using MMC specific primers through qPCR.

Station	*Sediment depth interval*	*Abundance (16S rRNA copies/g)*

DE	Surface	$1.68\times10^{6} \pm 8.15\%$
25	Surface	$1.79\times10^{8} \pm 18.10\%$
	3-5 cm	$6.77\times10^{6} \pm 24.51\%$
	5-10 cm	$1.84\times10^{6} \pm 4.40\%$
23	Surface	$3.33\times10^{8} \pm 0.73\%$
	3-5 cm	$5.21\times10^{7} \pm 5.66\%$
	5-10 cm	$3.09\times10^{6} \pm 13.62\%$
22	Surface	$4.33\times10^{8} \pm 18.75\%$
	3-5 cm	$1.33\times10^{7} \pm 23.53\%$
	5-10 cm	$2.79\times10^{7} \pm 20.39\%$
20	Surface	$1.98\times10^{8} \pm 16.51\%$
	3-5 cm	$8.73\times10^{7} \pm 26.11\%$
19	Surface	$2.26\times10^{8} \pm 24.55\%$
	3-5 cm	$7.58\times10^{7} \pm 20.84\%$

As shown above (Table10), the concentration of 16S rRNA decreases as we go deeper into the sediment. This is in agreement with the 16S RNA gene data, suggesting that the MMC strains and/or their close relatives aren't as prevalent deeper in the sediment.

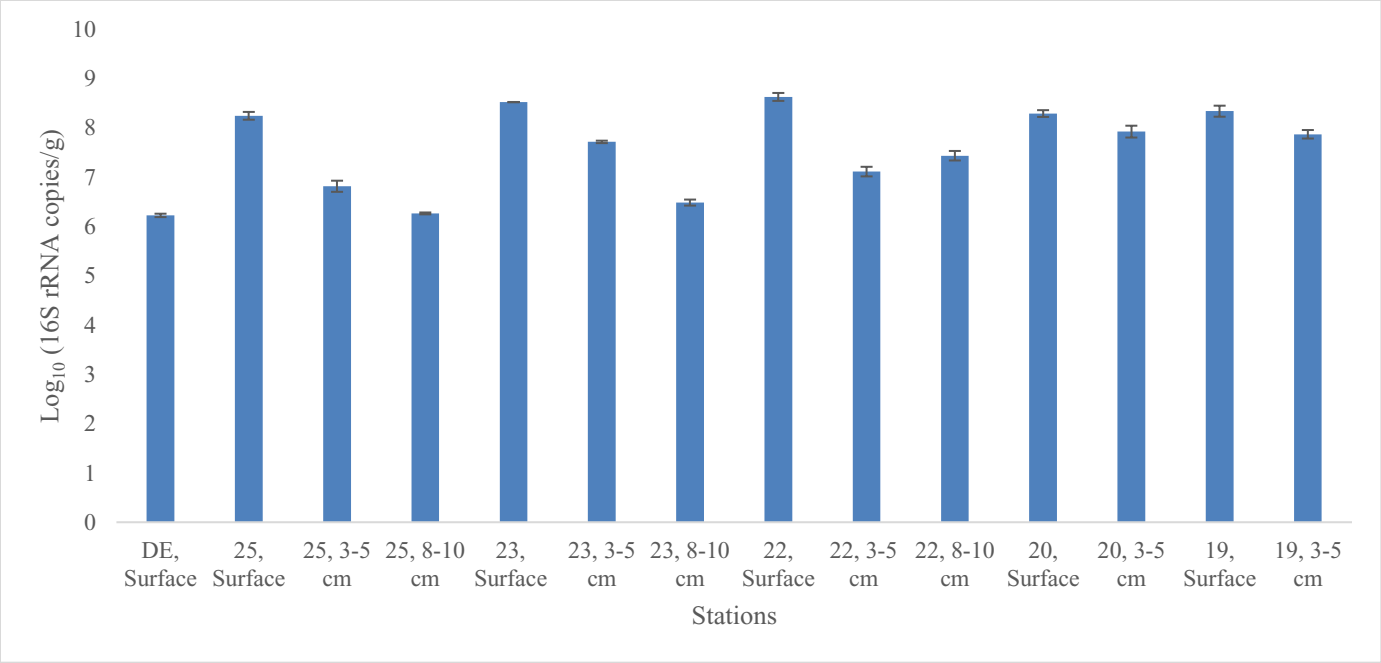

Figure 10. Bar chart of the MMC 16S rRNA copies in stations DE, 25, 23, 22, 20 and 19. The ordinate represents the logarithm base 10 of the values reported in Table10. Abscissa shows the stations and the depth of the sediment studied for the amount of MMC 16S rRNA. The standard deviation of the logarithm base 10 of the values varies between 0.003 and 0.120.

There are several factors that are different between the surface layer and the layers underneath. One the most important factors is the availability of oxygen. In the shallow parts of the ocean, for instance the parts closer to land, oxygen cannot penetrate the sediment below 10mm depth[112], and the samples taken from any depth lower than that would be lacking oxygen. This trend can be seen in Station 25, where the surface holds more bacteria and their abundance decreases by 10-fold in the lower layer (3-5 cm), and 3.67-fold in 5-10 cm depth.

However in the deeper regions of the ocean depending on the salinity the oxygen content of the sediment increases in comparison to the deep sediment samples isolated from estuary.[113] The reason for increased oxygen availability is that deep sea sediments are low in nutrients, therefore less organisms are present to consume the oxygen at hand.[113] Hence the oxygen remains available for bacteria inhabiting the layers down to 10 cm deep.[113] This change can be seen starting from Station 23, the amount of active bacteria in 3-5 cm sediment sample is approximately 10 times as much as the bacteria present in the same layer in Station 25. Furthermore this amount is to some extent sustained in Stations 22, 20 and 19.

The other factor affecting the bacterial community is salinity, which increases as we go farther into the sea. It is hypothesized that bacteria from the MMC prefer environments with higher salinities than brackish water[82], thus we would expect their abundance to increase with higher salt concentrations.[82] Our data supports this conclusion, with the prevalence of the MMC and its closely related strains, increases starting from Station DE to Station 25 (Table10).

The ratio of 16S rRNA copies to 16S rRNA gene copies were calculated and reported in Table 11. This ratio is a common means of determining bacterial activity.[103,110]

Table 11. Metabolic activity of MMC strains in the Gulf and Estuary of St. Lawrence. The 16s rRNA to 16s rRNA gene ratio in the St. Lawrence sediments.

Station	*Sediment depth interval*	*16S rRNA copies/amount of bacteria from the MMC*
DE	Surface	17.872 ± 0.117
25	Surface	24.631 ± 0.270
	3-5 cm	20.098 ± 0.372
	5-10 cm	18.194 ± 0.014
23	Surface	25.506 ± 0.004
	3-5 cm	22.844 ± 0.075
	5-10 cm	18.772 ± 0.185
22	Surface	25.851 ± 0.272
	3-5 cm	20.847 ± 0.321
	5-10 cm	21.980 ± 0.333
20	Surface	24.7744 ± 0.229

	3-5 cm	23.570 ± 0.394
19	Surface	24.896 ± 0.370
	3-5 cm	23.398 ± 0.277

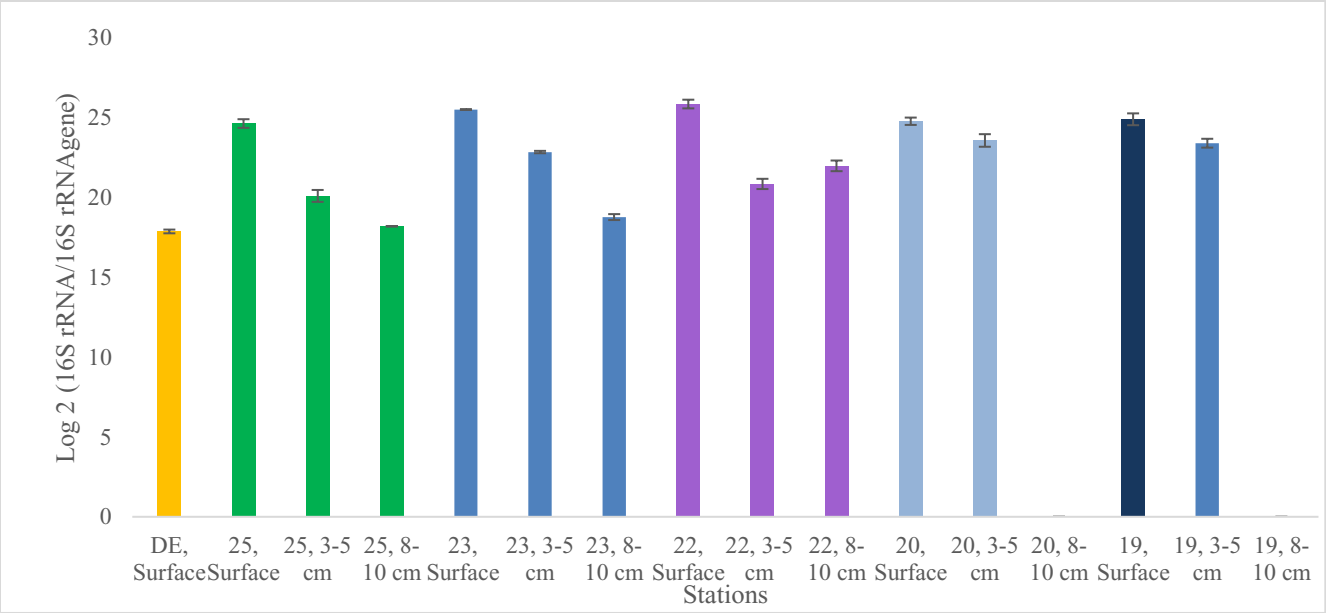

Figure 11. The ratio of MMC 16S rRNA copies over the MMC 16S rRNA gene copies. Stations are arranged by colors. Yellow represents Station DE, values from Station 25 are shown in green, Station 23 is marked as blue, values from Station 22 are shown in purple, Station 20 is shown in light blue and values from Station 19 are shown in black. The ordinate represents the logarithm base two of the values in Table 11 and the abscissa shows the stations and the depth of the sediment. The standard deviation ranges from 0.046 to 0.570.

As shown in Figure 11, the ratio of 16S rRNA copies over the 16S rRNA gene copies are highest in surface sediment samples from Station 25, 23, 22, 20 and 19. And the values start to decrease in deeper layers of the sediment, which suggests lower activity of the MMC and the strains identified along them. In Station 25 the ratio of the 16S rRNA copies over the 16S rRNA gene copies, as shown in Table 11, is 5.14 and this amount decreases to 1.64 and 0.24 in the 3-5 cm and

5-10 cm samples respectively. This suggests that the MMC and other bacteria identified along with them are more active in the surface were the oxygen is more available.[60] The same trend can be observed in the other stations, Station 23, 22, 20 and 19. It is worth mentioning that the ratio varies between 3.79 and 6.93 in surface sediment samples in all the stations except for Station DE (Table 11). In order to compare the amount of the MMC to other bacteria present in the sediment samples, I measured the total amount of bacteria present in the sediment through qPCR. The primers used in these measurements were universal primers (341f/ 518r)[114], previously used for the same purpose by Kamarisima et al.[115]

Table 12. Total amount of bacteria present in different sediment samples collected from each station. Universal primers 341f/ 518r were used to quantify 16S rRNA gene copies. The copy numbers were normalized to ng DNA of the standards. Data shown in the Tablerepresent the 16S rRNA gene copies in one gram of the sediment. The fourth column represents the number of bacteria per gram of the sediment. The 16S rRNA gene copies values were divided by 5.5, which is the average 16S rRNA gene repetitions in bacteria.[116]

Stations	*Sediment depth interval*	*Abundance (16S rRNA gene copies/g)*	*Number of bacteria per gram of the sediment (16S rRNA copies/ 5.5)*
DE	Surface	$1.88\times10^{10} \pm 4.49\%$	$3.41\times10^{9} \pm 4.49\%$
25	Surface	$1.04\times10^{10} \pm 2.63\%$	$1.89\times10^{9} \pm 2.63\%$
	3-5 cm	$3.52\times10^{9} \pm 2.89\%$	$6.39\times10^{8} \pm 2.89\%$
	5-10 cm	$3.43\times10^{9} \pm 9.63\%$	$6.24\times10^{8} \pm 9.63\%$
23	Surface	$1.08\times10^{10} \pm 6.24\%$	$1.96\times10^{9} \pm 6.24\%$
	3-5 cm	$1.12\times10^{9} \pm 4.52\%$	$2.04\times10^{8} \pm 4.52\%$
	5-10 cm	$1.24\times10^{9} \pm 1.90\%$	$2.26\times10^{8} \pm 1.90\%$
22	Surface	$1.27\times10^{10} \pm 6.81\%$	$2.32\times10^{9} \pm 6.81\%$
	5-10 cm	$5.22\times10^{9} \pm 9.15\%$	$9.49\times10^{8} \pm 9.15\%$

20	3-5 cm	$6.89\times10^{9} \pm 7.94\%$	$1.25\times10^{9} \pm 7.94\%$
	5-10 cm	$5.85\times10^{9} \pm 2.84\%$	$1.06\times10^{9} \pm 2.84\%$
19	Surface	$9.47\times10^{9} \pm 2.59\%$	$1.72\times10^{9} \pm 2.59\%$
	3-5 cm	$7.49\times10^{9} \pm 4.40\%$	$1.36\times10^{9} \pm 4.40\%$
	5-10 cm	$5.07\times10^{9} \pm 3.55\%$	$9.21\times10^{8} \pm 3.55\%$

The data presented in Table 12 shows that the total amount of 16S rRNA gene copies varies between 1.12×10^{9} and 1.88×10^{10} copies/g, which is close to the amount calculated by other papers, 10^{8}–10^{10} copies/cm^{-3}.[73] These values should be corrected against the 16S rRNA copy number in genome to reflect the amount of bacteria in the sediment, which is represented in the fourth column in Table 12. The average 16S rRNA gene copy number is around 5.5 in soil.[116] The fourth column in Table 12 represents the corrected values for each station. We can conclude that the total number of bacteria in the sediment is 3.4×10^{9} cells/g.

To measure the total amount of 16S rRNA copies, universal primers were used (Table 13). This will give us insights into bacterial community present in the sediment of Gulf of St. Lawrence. However, this can not be the sole representative of the bacterial activity, since the values obtained from qPCR studies using universal primers will measure the 16S rRNA in dormant and active cells altogether. To gain an understanding of the full bacterial community the primers specific to each strain of bacteria in the estuary should be designed and tested.

Table 13. Total amount of bacterial 16S rRNA copies. The 16S rRNA fragments extracted from the sediment samples were quantified using universal primers (341f/ 518r) through qPCR. These values reflect the total amount of metabolically active bacteria present in the sample.

Stations	*Sediment depth interval*	*Abundance (16S rRNA copies/g)*
DE	Surface	$1.02\times10^{9} \pm 15.19\%$

25	Surface	$5.57\times10^{9} \pm 15.45\%$
	3-5 cm	$1.34\times10^{9} \pm 11.64\%$
	5-10 cm	$7.63\times10^{8} \pm 4.33\%$
23	Surface	$8.01\times10^{9} \pm 7.87\%$
	3-5 cm	$2.32\times10^{9} \pm 38.53\%$
	5-10 cm	$8.58\times10^{8} \pm 13.75\%$
22	Surface	$8.38\times10^{9} \pm 9.90\%$
	3-5 cm	$1.57\times10^{9} \pm 11.59\%$
	5-10 cm	$2.38\times10^{9} \pm 44.95\%$
20	Surface	$4.54\times10^{9} \pm 28.19\%$
	3-5 cm	$2.80\times10^{9} \pm 41.78\%$
19	Surface	$8.81\times10^{9} \pm 10.22\%$
	3-5 cm	$4.09\times10^{9} \pm 2.64\%$

The increasing amount of 16S rRNA fragments in surface layers of the sediment samples starting from Station DE indicates the increasing bacterial level of activity. Based on the values obtained from qPCR (presented in Table 13), the amount of 16S rRNA is higher in the surface layers compared to the layers underneath. This suggests that most of the bacteria present in the sediment samples are aerobic.

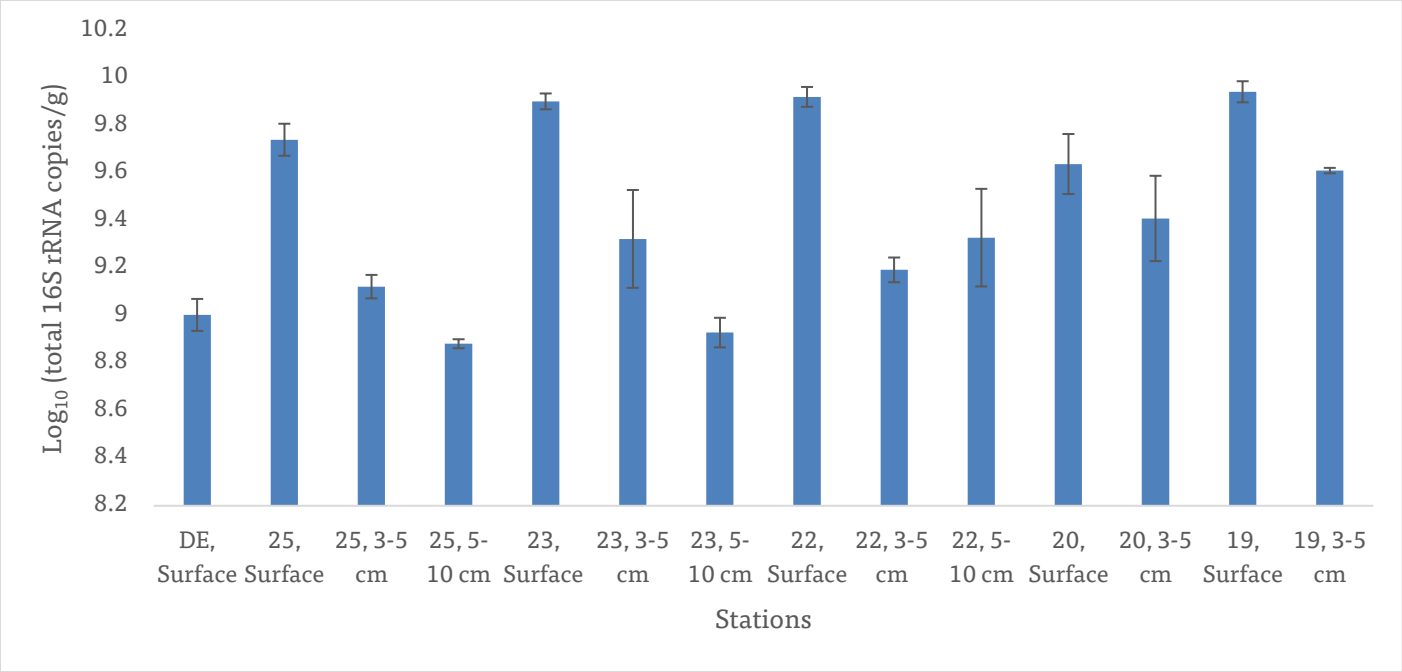

Figure 12. Bar chart representation of the total amount of 16S rRNA fragments in stations DE, 25, 23, 22, 20 and 19. The values shown on the ordinate are the logarithm base 10 of the values presented in Table 13. The standard deviation varies between 0.0189 and 0.2059.

To understand the abundance of MMC and closely related strains in comparison to other bacteria present in the sediment, I then calculated the ratio MMC over total amount of bacteria present in the sediment (Table 14).

Table 14. Abundance of MMC per gram of the sediment per ng of the DNA. The amount of MMC present in the sediment is compared to the total amount of bacteria by getting the ratio of the abundance of MMC to the total amount of bacteria.

Stations	*Depth*	*MMC /Total amount of bacteria (percentage)*
DE	Surface	0.296 ± 0.0409
25	Surface	0.311 ± 0.0350
	3-5 cm	0.106 ± 0.00728

	5-10 cm	0.244 ± 0.133
23	Surface	0.516 ± 0.0680
	3-5 cm	3.89 ± 0.320
	5-10 cm	3.01 ± 0.577
22	Surface	0.454 ± 0.0259
	5-10 cm	0.438 ± 0.117
20	3-5 cm	0.444 ± 0.0283
	5-10 cm	0.399 ± 0.0545
19	Surface	0.580 ± 0.0531
	3-5 cm	0.390 ± 0.0291
	5-10 cm	0.331 ± 0.0348

Based on the qPCR results, we can conclude that MMC are active in all the stations and their abundance decreases slightly with depth. This suggests that the MMC is well adapted to growth in an estuary environment. To determine the metabolic potential of these strains I then set out to cultivate them from the sediment samples.

3.3. Cultivation of representatives of the MMC

Cultivating an unculturable strain of bacteria entails mimicking conditions in which the bacteria grows well, usually their original habitat.[117] Therefore, to isolate strains from the MMC we need to subject them to the representative conditions present in the Saint-Lawrence Estuary. Since the fresh water coming from the land is mixed with seawater in the estuary, the salinity is low in the parts of the estuary that are closest to land. The brackish water contains a salinity range of 0.5 to 35 ppt.[82]

To mimic the concentration of salt and other minerals in to the estuary, the water used in media preparation was the water extracted from the Gulf of St. Lawrence, diluted two fold to approximate the semi-saline environment present in the estuary. The other factor that is of importance in cultivating marine myxobacteria is to acknowledge their ecological behavior. Since a few known halophile myxobacteria, *H. ochraceum, H. tepidum*, and *Enhygromyxa niigatensis*, are able to lyse and consume live cells, marine myxobacteria are expected to be bacterial predators and presence of prey bacteria in their habitat is anticipated.[82] Hence to mimic the environment in which marine myxobacterial cluster existed, it is necessary to also isolate their prey.

In order to isolate bait from the sediment, the samples were spread on Marine agar plates. After overnight incubation at room temperature in the dark, colonies were observed on the plates. A subset of these colonies were then streaked to purity on separate nutrient rich plates, then cultivated in 5 mL of liquid marine broth media. For the preparation of bait plates this seed culture was then inoculated into 1 L of marine broth liquid media and left to grow overnight. The cells were then harvested by centrifugation, washed with either saltwater or distilled water, and then suspended in agar diluted with half strength saline water from the Gulf of St. Lawrence.

The bait plates are considered nutrient poor media, since the only source for bacterial growth is expected to be the prey bacteria. However, agar itself can serve as a carbon source which would let other contaminants like fungi grow.[118] To suppress the growth of fungi on the bait plates, cycloheximide was added. Moreover, to limit the risk of contamination we added vancomycin to the media. Vancomycin is a bactericidal antibiotic that has little to no effect on Gram negative

bacteria but can block the growth of Gram positive bacteria.[88] Considering that marine myxobacteria are Gram negative, vancomycin was chosen to be added to the bait plates.

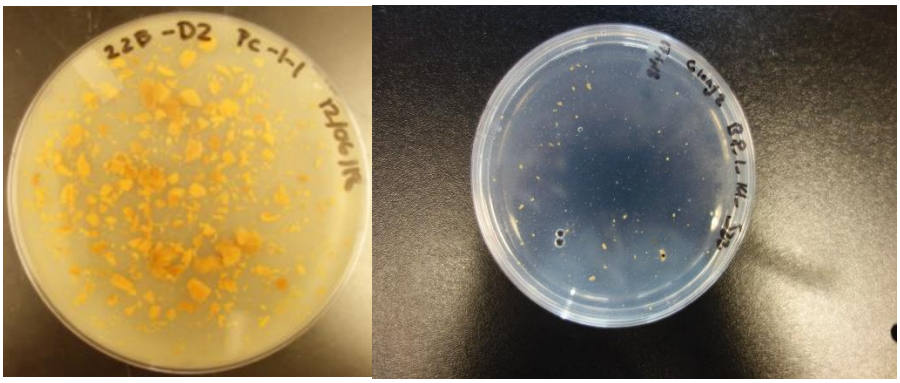

Figure 13. Sample bait plates used to isolate representatives of the MMC. Bait plates were prepared by suspending prey bacteria along with cycloheximide (100µg/mL) and vancomycin (20 µg/mL) in a liquid media composed of agar and filter sterilized sea water.

Over the Summer of 2018, 184 bait plates were prepared with the assistance of two CEGEP students, Safiya Soullane and Pascale Coulombe. The bait bacteria used in these plates included strains most similar to *Pseudoalteromonas nigrifaciens, Paenibacillus taiwanensis, Psychrobacter cryohalolentis* (isolated from SLE), which were provided by a former undergraduate student, Zachary Schiffman, and three unknown marine strains by undergraduate students volunteering in the lab, Seydee Bien-Aime and Harman Warraich.

The sediment samples were then spread on top of the bait plates to let the MMC strains grow. Following 1-2 weeks of incubation at room temperature colonies began to emerge on the plates. Many of these colonies were brightly colored, including bright orange, black and red, while others formed structures similar to terrestrial fruiting bodies, and others appeared to move over the agar in swarms. All three of these features are found in terrestrial myxobacteria[32], and so colonies with these features, as shown in Figure 14, were streaked to purity on nutrient-rich agar plates for further analysis.

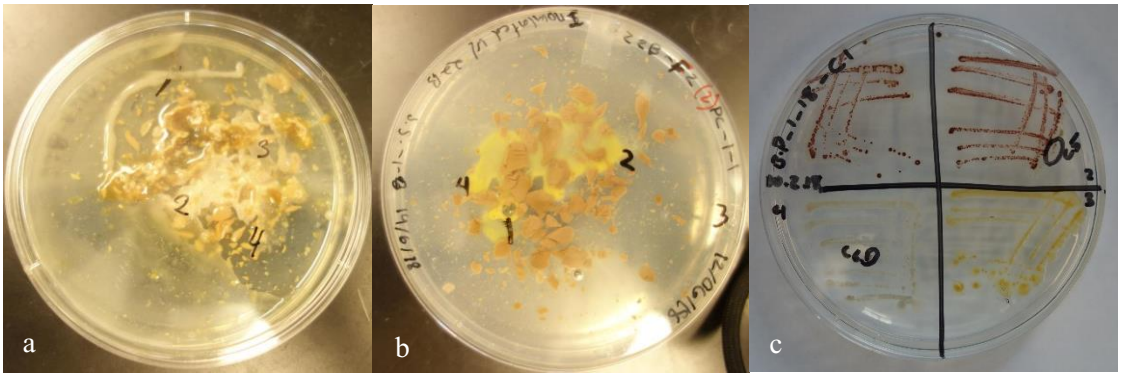

Figure 14. Putative MMC strains. Colorful colonies, red and yellow, emerged on the plate after one week of incubation (a and b). The brown clumps are bait bacteria suspended in agar along with cycloheximide and vancomycin. Colonies with characteristics typical of myxobacteria were streaked to purity on a marine agar (half strength) with sea water media, Figure c.

Based on my observations, the plates containing the bait bacteria most similar to *Pseudoalteromonas nigrifaciens* had the most colonies growing on them. As shown in Figure 15, 74 plates out of 129 plates that had colonies growing on them had *Pseudoalteromonas nigrifaciens* bait. Furthermore11 plates had *Paenibacillus taiwanensis* as the prey bacteria, and 23 of the bait plates had *Psychrobacter cryohalolentis*. Also, 9 of the plates were supplied with strain A (provided by the undergraduate volunteers, Seydee Bien-Aime and Harman Warraich), 4 plates with strain B, 3 plate with strain C, 4 plates with strain D (strains A, B, C and D have not been identified, but were isolated from sediments found in the Gulf of St. Lawrence).

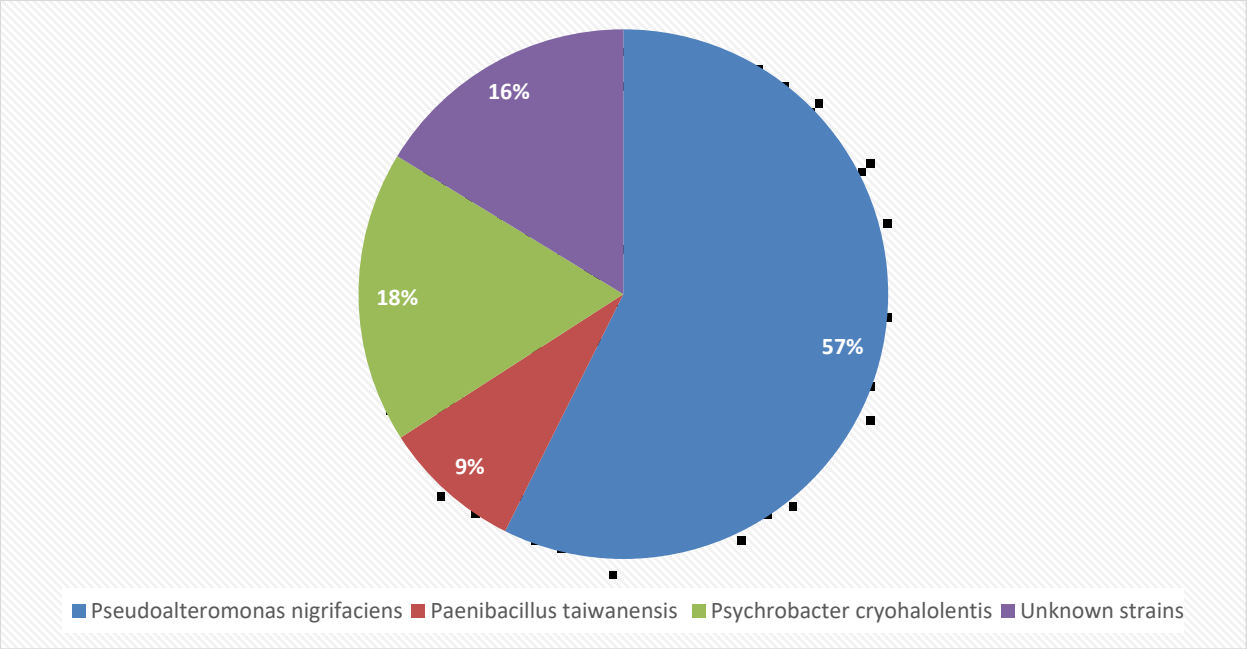

Figure 15. The type of bait plates prepared. In total 129 plates were prepared. About 74 plates had *Pseudoalteromonas nigrifaciens*, 11 plates had *Paenibacillus taiwanensis,* 23 plates had *Psychrobacter cryohalolentis,* and 20 had unknown strains (A, B,C and D) as bait.

The 131 strains that were chosen for further studies were grown on separate petri-dishes containing half strength marine broth and half strength filter sterilized seawater (about 17.5 ppt). These strains were then streaked to purity for 3 times. All 131 strains underwent genomic DNA extraction using a EZ-10 Spin Column Bacterial Genomic DNA Miniprep Kit . Their 16S rRNA fragments were amplified using 27F and 1492R universal primers. The 1500 bp PCR products were run on 0.8 % agarose gel to verify the correct amplification of the fragments. Representative amplicons are shown in the Figure below (Figure 16).

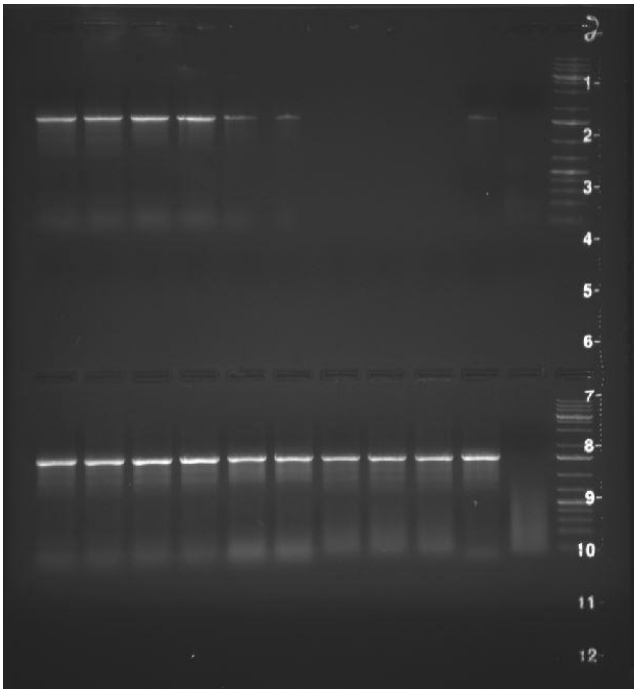

Figure 16. The 16S rRNA fragments amplified using the universal primers. The DNA ladder used here is 1kb plus. The fragments projected in this gel are 1500bp long. Each lane represents the different colonies isolated from the bait plates.

In this step the 16S rRNA fragment of approximately 95 strains were successfully amplified and then passed through PCR cleanup in preparation for transformation. About 60 strains were chosen to be transformed into DH5-α cells using pJET vector. The PCR products were inserted into pJET vector and then transformed into *E. coli* DH5-α. Colony PCR confirmed the correct insertion of the fragments into the vector. After approximately 14h of incubation at 37 ℃, the plasmids were extracted from the DH5-α cells and sent for sequencing by Eurofins.

Around 50 strains were successfully sequenced. The sequences were then aligned with other strains of bacteria from the NCBI database using the blastn tool. Largest fraction of the strains, 22 strains out of 50 strains, found on the bait plates were related to *Pseudomonas* spp (44%). Around 19 strains were closely related to *Bacillus* spp, 38 %. And around 9 strains out of 50 strains sequenced were closely related to *Stenotrophomonas* spp (Figure 17). The list of the stains successfully sequenced are presented in Appendix.

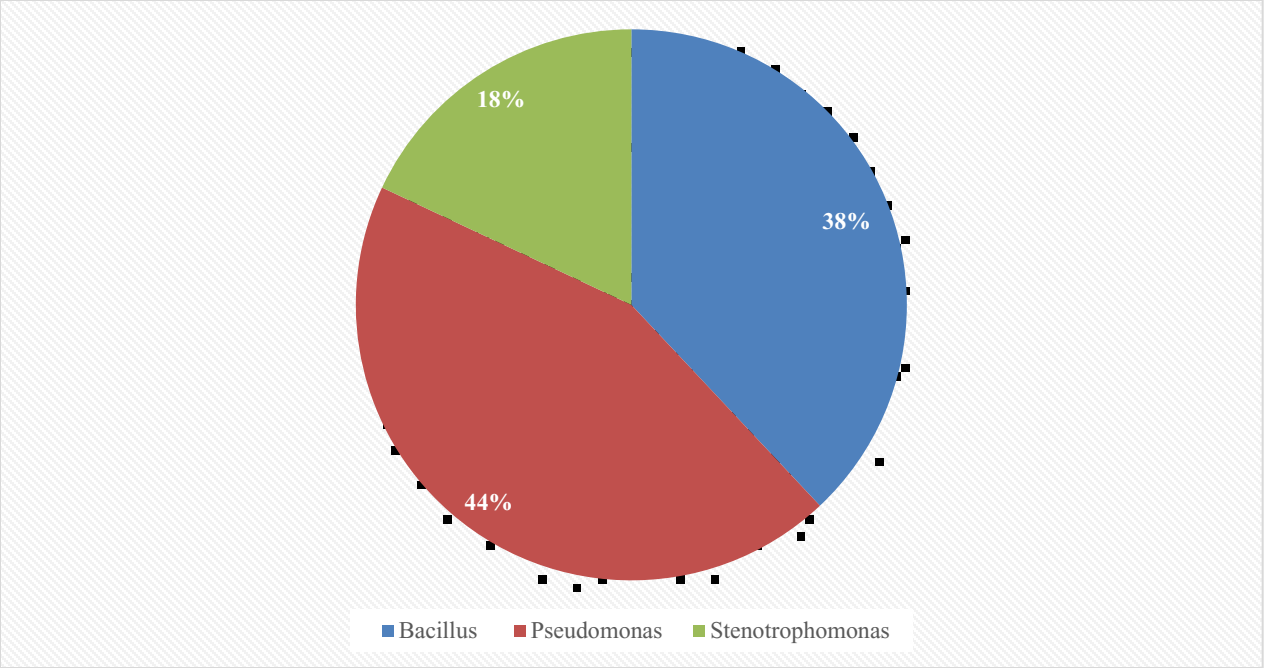

Figure 17. The pie chart illustration of the isolates extracted from the bait plates. Most of the isolates extracted from the bait plates are related to either Pseudomonas spp. or Bacillus spp. 22 strains were related to *Pseudomonas spp,* 19 strains were related to *Bacillus spp,* and 9 strains were related to *Stenotrophomonas spp.*

As mentioned above largest fraction of the plates had Pseudomonas strains growing on them. Most of these strains were closely related to *Pseudomonas lactis, Pseudomonas azotoformans, Pseudomonas plecoglossicida* or *Pseudomonas taeanensis.* Pseudomonas strains are known saprophytes, able to grow on any decaying matter[119]and here it seems that the prey bacteria and the agar provided enough nutrient sources for the Pseudomonas strains to grow.

Also *Stenotrophomonas rhizophila* were observed in some of the bait plates. These bacteria are known to inhabit terrestrial environments and the fact that they appeared on the bait plates suggests that they were brought to the estuary from land. However there have been reports of some of the strains of Stenotrophomonas being isolated from marine invertebrates and this suggests that the Stenotrophomonas strains growing on the plate also might have originated from marine environment.[120] Considering their Gram negative nature[121], vancomycin could not have stopped them from growing. It is worth mentioning that these strains were growing on the plates that had *Pseudoalteromonas nigrifaciens* as bait. In addition, the sediment samples that were spread on

these bait plates were mostly from Station 25 and DE which are the closest stations to land, suggesting that these strains were washed from the land and brought to the Estuary.

The inspection of the plates revealed that most of the strains closely related to *Bacillus spp* were growing on the plates that had the unidentified strains (B and C) or *Pseudoalteromonas nigrifaciens* as bait. The strains found on plates with strain B as bait, were related to *Bacillus thuringiensis, Bacillus toyonensis* and *Bacillus pacificus.* Hence we can conclude that using strain B as the prey will mostly result in emergence of the strains closely related to Bacillus spp on the plate. The plates containing strain C as prey, selected for strains closely related to *Bacillus cereus* and *Bacillus wiedmannii.* Plates that contained *Pseudoalteromonas nigrifaciens* as bait, had strains related to *Bacillus nitratireducens, Bacillus tropicus, Bacillus thuringiensis, Bacillus toyonensis, Bacillus proteolyticus, Bacillus wiedmannii* and *Bacillus cereus* growing on them. These observations suggest that using *Pseudoalteromonas nigrifaciens* as prey will create an opportunity for *Bacillus* spp to grow on the bait plates. The reason that Bacillus strains showed up on the bait plates might be due to them developing resistance against vancomycin. Also myxobacteria are slow growing bacteria, that gave Bacillus strains, which mostly are known as saprophytes[122,123], the chance to grow on the bait plates.

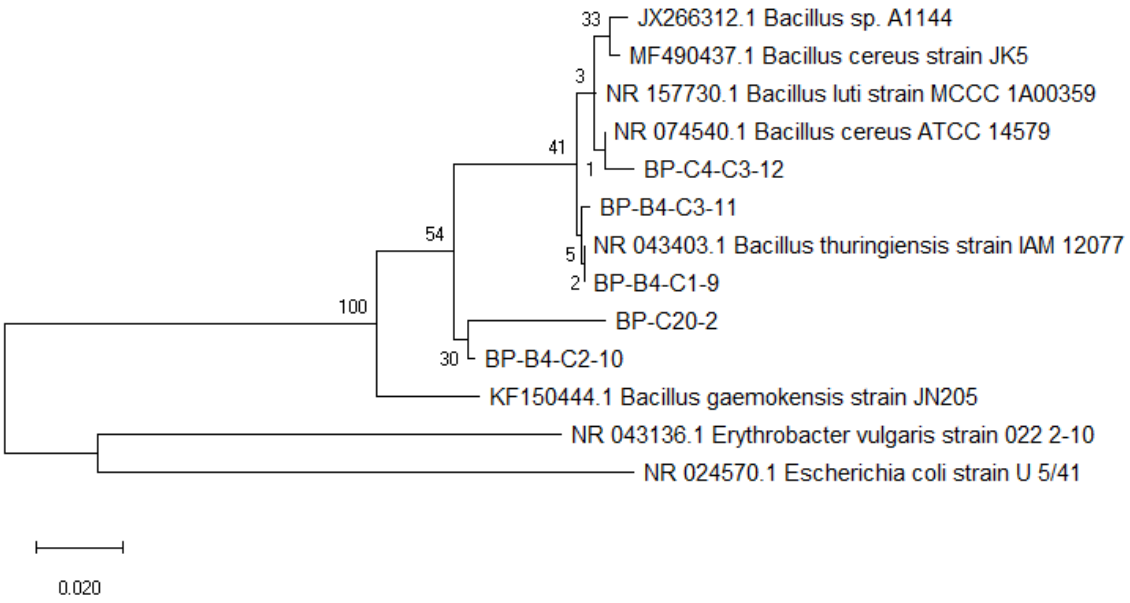

Figure 18. Phylogenetic tree of isolated strains most similar to bacillus *spp*. The isolates extracted from bait plates that showed most similarity to Bacillus strains were chosen to build the phylogenetic tree. *E. coli* strain U 5/41 serves as the outgroup.

Some of the isolates most similar to *Bacillus* strains were aligned with a subset of well-known *Bacillus* species to construct a phylogenetic tree (Figure 18). Some of the strains found on the plate showed high similarity to *Bacillus cereus*, *Bacillus luti*, *Bacillus thuringiensis* and *Bacillus gaemokensis*. About two of the strains found on the bait plates were closely related to *Bacillus cereus. Bacillus cereus* have been known to inhabit terrestrial environments.[124] However, it has been reported that the strains from this bacteria have been isolated from the marine environment.[125] Four strains isolated from the bait plates were closely related to *Bacillus thuringiensis,* which are also considered soil-dwelling bacteria. Since the sediment samples were extracted from the estuary, it is probable that some of these strains were washed from the land and brought to the estuary.

Bacillus spp. are not well known for vancomycin resistance,[126,127] but vancomycin resistance has been observed in some Bacillus strain like *B. popilliae*. *B. popilliae* and several other isolates from this strain were found to be vancomycin resistant.[128] This in turn led Rippere et al. to speculate the possibility of vancomycin resistant genes being transferred to other Gram positive bacteria.[128]

While it is possible that the strains we isolated are also vancomycin resistant, that is not the only potential reasons for these results. Vancomycin can only be stored at room temperature for up to 10 days before it begins to degrade in buffer.[93] The agar plates that were prepared had to be stored at room temperature for weeks for myxobacteria to grow, allowing plenty of time for the vancomycin stored in the plates to become ineffective.[129] To determine if the marine *Bacillus* isolates were vancomycin resistant I performed a minimum inhibitory concentration test (MIC).[130]

3.4. MIC test

To determine the vancomycin susceptibility of strains isolated from the bait plates the strains were treated with different concentrations of the aforementioned antibiotic. The concentration at which vancomycin is known to inhibit *Bacillus subtilis* is 4 µg/mL.[131] The susceptibility of the bacillus strains were tested against different concentrations of vancomycin ranging from 128 µg/mL to 0.025 µg/mL . The incubation was carried out for 18 hours.

Table15. MIC values for the isolates retrieved from bait plates. Isolate Z6A-A-B1 is most similar to *B. zhangzhouensis* and was isolated by Zachary Schiffman. Isolates C4 and B4 were provided by Seydee Bien-Aime and Harman Warraich, volunteers working in the lab. Isolate C4 showed the greatest 16S rRNA sequence identity to *B. cereus* and isolate B4 was most similar to *B. pacificus*.

Isolate	*Vancomycin mg/L*										*negative control*	*positive control*
	128	64	32	16	8	4	2	1	0.5	0.025		
Z6A-A-B1	_	_	+	+	+	+	+	+	+	+	_	+
C4	_	_	_	_	+	+	+	+	+	+	_	+
B4	_	_	_	_	_	_	+	+	+	+	_	+

Isolate Z6A-A-B1, most closely related to *B. zhangzhouensis,* showed resistance against vancomycin up to 32 mg/L of the antibiotic, beyond the clinical breakpoint for this antibiotic.[132] While Isolate C4, closely related to *B. cereus*, did not show the same level of resistance as Z6A-A-B1, it was able to grow at 8 mg/L, close to the 10 mg/L concentration found in the bait plates. Isolate B4, most similar to *B. pacificus*, did not grow until the concentration of vancomycin was lowered to 2 mg/L. These findings showed that some of the isolates like Z6A-A-B1 and C4 are resistant to vancomycin while some other strains like isolate B4 are still susceptible to vancomycin.

Chapter 4. Conclusion

In this study we aimed to isolate the MMC strains from the sediment samples collected from Gulf of St. Lawrence. Based on the results retrieved from 16S rRNA gene sequencing, we found out that the previously designed MMC specific primers were also targeting other strains closely related to MMC.

The presence of MMC strains in the sediment samples of Gulf of St. Lawrence raised the question about their state of activity. In order to obtain information about their abundance and their ribosome content, we conducted qPCR studies on 16S rRNA gene and 16S rRNA content of the sediment. By measuring MMC 16S rRNA gene and 16S rRNA copy numbers we were able to conclude that MMC strains are metabolically active under the conditions present in the estuary. This in turn revealed that MMC strains present in the sediment samples might grow if subjected to same condition as the estuary.

To mimic the conditions present in the estuary prey bacteria, *Pseudoalteromonas nigrifaciens*, *Psychrobacter cryohalolentis* and *Paenibacillus taiwanensis* were retrieved from Zachary Schiffman. The prey bacteria were cultivated in liquid media and then harvested and suspended in nutrient poor media along with seawater to replicate the MMC strains original habitat. After two weeks of incubation the strains that grew on the plates containing the prey bacteria were isolated and their genomic content was extracted. The 16S rRNA gene sequencing studies on the strains isolated from the bait plates revealed that none of the mentioned strains were MMC and most of them were either *Bacillus* or *Pseudomonas* strains. This led us to speculate that the *Bacillus* strains were resistant to vancomycin.

Vancomycin was added to the bait plates to decrease the risk of contamination with Gram positive bacteria like Bacillus. Since Bacillus strains started to grow on the plate we suspected that these bacillus strains were resistant to vancomycin. The MIC test showed that the bacillus strains we isolated from the bait plates were resistant to vancomycin.

Chapter 5. Future work

In this study we found out that the primers designed by Brinkhoff et al[60] were not exclusively detecting the MMC strains. Therefore, a new set of primers need to be designed to specifically target the MMC. Since the primers designed for qPCR studies were not specific either, the 16S rRNA copies should be measured again with the new primers that are going to be designed.

In addition, in our qPCR studies the amount of 16S rRNA fragments were measured on separate days than the samples used to construct the standard curve. To minimize potential variation these experiments should be repeated on the same day.

On a separate note, since MMC presents as a potent natural product producer the attempts to cultivate the MMC should be continued by modifying the cultivation conditions and the antibiotics added to the bait plates. In addition, the strains that were isolated from bait plates might be resistant to other antibiotics as well. Therefore more studies are needed to unravel the possibility of their resistance to other antibiotics.

These experiments can lead to discovery of novel bioactive secondary metabolites that can help us tackle the ongoing problem of antibiotic resistance. In addition, the strains found in the Gulf of Saint-Lawrence are from different recently discovered bacterial clades which makes this environment interesting for further studies on the bacterial community. This can help in discovery of more novel bacterial strains that are capable of producing new biologically active secondary metabolites.

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Appendix

Table 16. The list of strains isolated from the bait plates.

Sample ID	*Closest relative*	*Bait*	Station
C4_C3_12	*Bacillus cereus*	*C*	Unknown
C4_C2_8	*Bacillus wiedmannii*	*C*	Unknown
B4_C1_9	*Bacillus toyonensis*	*B*	Unknown
B4_C2_10	*Bacillus thuringiensis*	*B*	Unknown
B4_C2_8	*Bacillus mobilis*	*B*	Unknown
22B-A2-2	*Bacillus cereus*	*pseudoalteromonas nigrifaciens*	19
22B-B2-2	*Bacillus wiedmannii*	*pseudoalteromonas nigrifaciens*	19
22B-A2-1	*Bacillus proteolyticus*	*pseudoalteromonas nigrifaciens*	19
22B-C2-2	*Bacillus toyonensis*	*pseudoalteromonas nigrifaciens*	19

22B-A2-7	*Bacillus thuringiensis*	*pseudoalteromonas nigrifaciens*	19
22B-F2-6	*Bacillus tropicus*	*pseudoalteromonas nigrifaciens*	19
22B-G2-3	*Bacillus nitratireducens*	*pseudoalteromonas nigrifaciens*	19
22B-H2-7	*Bacillus thuringiensis*	*pseudoalteromonas nigrifaciens*	19
22B-H2-1	*Bacillus proteolyticus*	*pseudoalteromonas nigrifaciens*	19
22B-F2-2	*Bacillus thuringiensis*	*pseudoalteromonas nigrifaciens*	19
22B-G2-5	*Bacillus nitratireducens*	*pseudoalteromonas nigrifaciens*	19
22B-E2-6	*Bacillus nitratireducens*	*pseudoalteromonas nigrifaciens*	19
B4-C3-11	*Bacillus gaemokensis*	*B*	Unknown
22B-C2(2)-Col4	*Pseudomonas azotoformans*	*pseudoalteromonas nigrifaciens*	19
22B-E2(8)-Col3	*Pseudomonas gessardii*	*pseudoalteromonas nigrifaciens*	19
22B-A2(1)-Col2	*Pseudomonas reinekei*	*pseudoalteromonas nigrifaciens*	19

22B-A2(1)-Col1	*Pseudomonas reinekei*	*pseudoalteromonas nigrifaciens*	19
22B-F2(1)-Col3	*Pseudomonas lactis*	*pseudoalteromonas nigrifaciens*	19
22B-F2(6)-Col3	*Pseudomonas lactis*	*pseudoalteromonas nigrifaciens*	19
22B-E2(8)-Col2	*Pseudomonas azotoformans*	*pseudoalteromonas nigrifaciens*	19
22B-E2(8)-Col1	*Pseudomonas azotoformans*	*pseudoalteromonas nigrifaciens*	19
18B-B2(6)-Col3	*Pseudomonas gessardii*	*pseudoalteromonas nigrifaciens*	19
18B-B2(4)-Col2	*Pseudomonas libanensis*	*pseudoalteromonas nigrifaciens*	19
18B-B2(6)-Col2	*Pseudomonas synxantha*	*pseudoalteromonas nigrifaciens*	19
18B-B2(4)-Col1	*Pseudomonas libanensis*	*pseudoalteromonas nigrifaciens*	19
25B-A2-A(4)-Col2	*Pseudomonas monteilii*	*Psychrobacter cryohalolentis*	25
25B-A2-B(4)B-Col4	*Pseudomonas plecoglossicida*	*Psychrobacter cryohalolentis*	25
25B-A2-B(5)-Col2	*Pseudomonas plecoglossicida*	*Psychrobacter cryohalolentis*	25

25B-A2-A(4)-Col4	*Pseudomonas monteilii*	*Psychrobacter cryohalolentis*	25
25B-A2-B(2)-Col1	*Pseudomonas guariconensis*	*Psychrobacter cryohalolentis*	25
22B-C2(2)-Col3	*Pseudomonas azotoformans*	*pseudoalteromonas nigrifaciens*	19
22B-C2(3)-Col2	*Pseudomonas azotoformans*	*pseudoalteromonas nigrifaciens*	19
22B-E2(6)-Col3	*Pseudomonas azotoformans*	*pseudoalteromonas nigrifaciens*	19
22B-E2(5)-Col1	*Pseudomonas monteilii*	*pseudoalteromonas nigrifaciens*	19
22B-E2(3)-Col2	*Pseudomonas azotoformans*	*pseudoalteromonas nigrifaciens*	19
18B-D2(2)-Col2	*Stenotrophomonas rhizophila*	*pseudoalteromonas nigrifaciens*	19
18B-D2(2)-Col1	*Stenotrophomonas rhizophila*	*pseudoalteromonas nigrifaciens*	19
18B-D2(4)-Col2	*Stenotrophomonas maltophilia*	*pseudoalteromonas nigrifaciens*	19
18B-C2(3)-Col3	*Stenotrophomonas rhizophila*	*pseudoalteromonas nigrifaciens*	19
18B-D2(4)-Col1	*Stenotrophomonas maltophilia*	*pseudoalteromonas nigrifaciens*	19