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Methane Cycling and Methanotrophic Bacteria in Base Mine Lake, a Model End-Pit Lake in the Alberta Oilsands

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Methane Cycling and Methanotrophic Bacteria in Base Mine Lake, a Model End-Pit Lake in the Alberta Oilsands

by

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Abstract

We studied methanotrophic bacteria over three years (2015 - 2018) in Base Mine Lake, Fort McMurray, Canada. The lake represents the first large scale demonstration of end-pit lake technology in the Alberta oilsands. 16S rRNA gene amplicon sequencing and measurement of methanotrophic rates were applied to evaluate the effect of seasonal changes on methanotrophic diversity and activity, and to understand the biogeochemical cycling of methane and oxygen. Based on 16S rRNA gene sequence relative abundance, the predominantly detected methanotrophic genera were *Methylobacter/Crenothrix* in the winter and *Methylocaldum* in the summer. Methanotrophs were most abundant in winter throughout the water column, and in summer at the bottom of the lake near the fluid fine tailings interface. Potential methanotrophic rates decreased over three years from 2015-2018.

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I am endlessly thankful to my parents, brothers, and sisters for their profound love, patience, and understanding throughout my studying abroad. Thank you for giving me the chance to be where I am. I hope I made you proud.

Dedication

This master thesis is modestly dedicated to my late grandmothers and grandfathers whose were wise, far sighted and owned the courage to open the door for my education regardless of their lack of literacy. It is also dedicated to my parents, brothers, and sisters for their endless love, patience, and encouragement. Above all, to God almighty our savior.

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List of Symbols, Abbreviations and Nomenclature

AER	Alberta Energy Regulator
AOB	Ammonia Oxidizing Bacteria
AOM	Anaerobic Oxidation of Methane
AOSR	Athabasca Oil Sands Region
BCR	Beaver Creek Reservoir
BLAST	Basic Local Alignment Search Tool
BML	Base Mine Lake
BOD	5 - day Biological Oxygen Demand
BTEX	Benzene, Toluene, Ethylbenzene, and Xylenes
CCA	Canonical Correspondence Analysis
CEAA	Canadian Environmental Assessment Agency
CH_2O	Formaldehyde
CH_3OH	Methanol
CH_4	Methane
CO_2	Carbon Dioxide
CSS	Cyclic Steam Stimulation
DW	Distilled Water
ECCC	Environmental and Climate Change Canada
EIP	East In Pit
EPEA	Environmental Protection Enhancement Act
EPL	End Pit Lake
EPR	Electron Paramagnetic Resonance
FASAT	FAST-all (text based format)
FFT	Fluid Fine Tailings
FISH	Fluorescence In Situ Hybridization
GC	Gas Chromatography
GHGs	Greenhouse Gases
GTC	Guanidinium Thiocyanate
GWP	Global Warming Potential
H_2	Hydrogen
HCOO	Formate
HFCs	Hydrofluorocarbons
JOSM	Joint Oil Sands Monitoring
LARP	Lower Athabasca Regional Plan
masl	Meters Above Sea Level
MFT	Mature Fine Tailings
MLSB	Mildred Lake Settling Basin
MMO	Methane Monooxygenase
MOB	Methane Oxidizing Bacteria

N_2O	Nitrous Oxide
NGS	Next-Generation Sequencing
NMEPL	North Mind End Pit Lake
NMS	Nitrate Mineral Salts
NMSPS	North Mine South Pond Storage
ODSs	Ozone-Depleting Substances
OSPW	Oil Sands Process- affected Water
OSTP	Oil Sands Tailings Pond
OTU	Operational Taxonomic Units
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PFCs	Perfluorocarbons
pMMO	Particulate Methane Monooxygenase
QIIME	Quantitative Insight Into Microbial Ecology
qPCR	Quantitative Polymerase Chain Reaction
RuMP	Ribulose Monophosphate RuMP
SAGD	Steam Assisted Gravity Drainage
SF_6	Sulphur Hexafluoride
sMMO	Soluble Methane Monooxygenase
SWIP	Southwest In Pit
SWSS	Southwest Sand Storage
T-RFLP	Terminal Restriction Fragment Length Polymorphism
WBEA	Wood Buffalo Environmental Association
WCTT	Water Capped Tailings Technology
WIP	West In-Pit

CHAPTER ONE: INTRODUCTION

One challenge facing oil sands operators in the Athabasca Oil Sands Region (AOSR) is reclaiming the area that is affected by oilsands process - affected water (OSPW) and fluid fine tailings (FFT) due to mining activities [1]. Surface mining or open - pit mining is one of several oil recovery methods that have contributed greatly to the Canadian economy, but recently they have also received negative attention because of environmental concerns. Extracting oil from the bitumen through mining has some lasting impacts on the environment such as destruction of wildlife, disrupted landscape, use of large amounts of fresh water to extract oil, greenhouse gas emissions [2] and toxic chemical pollution from tailings [3,4].

Oil sands industries are responsible for monitoring, tracking, documenting, and developing their strategies and technologies during the operation in the AOSR. Alberta's government, the Canadian Environmental Assessment Agency (CEAA), the Alberta Energy Regulator (AER), Joint Oil Sands Monitoring (JOSM), and the Wood Buffalo Environmental Association (WBEA), through legislation such as the Water Act, Public Lands Act and Oil Sands Conservation Act, Oil & Gas Conservation Act, Lower Athabasca Regional Plan (LARP), all are participating and working together with the Canadian oil sands companies to ensure that the environmental health is maintained, lands are protected, air quality is high, and improved strategies are applied to hydrocarbon resources contributing greatly to Canada's economy (http://www.canadasoilsands.ca). For instance, the Environmental Protection Enhancement Act (EPEA) has mandated that oil sands industries must reclaim land areas affected by surface mining activity, and the oil sands industries should obtain certificates to demonstrate restoration and reclamation [5].

At present, there are over twenty tailings ponds around the oil industries near Fort McMurray that have been constructed to store tailings water. Most of these ponds were constructed between 1977 and 2010 [3]. Syncrude is one of several oil sands companies who is managing and operating ponds. Some of tailings ponds that are operated and managed by Syncrude are Mildred Lake Settling Basin (MLSB), Base Mine Lake (BML), Aurora Settling Basin [5], East In Pit (EIP), Southwest In Pit (SWIP), Southwest Sand Storage (SWSS), North Mine South Pond Storage (NMSPS), North Mine Center Pond Storage (NMCPS), and North Mine End Pit Lake (NMEPL) [6].

MLSB was constructed in 1977. It is one of the oldest and the largest tailings dams ever built in the world. It is located 40 km from Fort McMurray, Alberta, Canada. [7]. The construction of this pond took two years [8]. The water surface at this pond is over 9.5 km^2 [2,3]. The pond was established to receive fluid fine tailings (FFT) from ponds Aurora North and Mildred Lake [9]. Early studies reported that aerobic and anaerobic heterotrophs, sulfate-reducing bacteria, and methanogens were present [9,10].

BML is a pond previously known as West In Pit (WIP). This end pit lake (EPL) configuration is referred to as water capped tailings technology (WCTT), which involves the placement of a layer of water over a deposit of fluid fine tailings and oil sands process- affected water (OSPW) to form a lake. Over time, this technology should support healthy communities of flora and fauna. Based on intensive research with other tailings ponds [7,11-13], the prediction for WCTT over time is that water quality should improve because the tailings sediments (FFT) will be insulated below the freshwater cap. Infrastructure has been built-up to pump fresh water in from Beaver Creek Reservoir (BCR) and pump water out to the tailings recycle water system (RCW) until more substantial upstream surface watershed is remediated (from tailings materials)

and connected to BML, and outflow is established into the Athabasca River (east side of BML pond). This engineered process enhances dilution of the BML water cap over time.

In this study, we monitored methane oxidizing bacteria (MOB), and their demand for O_2 to oxidize methane at BML between 2015 to 2017. We investigated microbial communities at BML over two years.

1.1 Research Objectives

The major aims of this study were to:

1. Monitor the Biological Oxygen Demand (BOD), and the contribution of MOB to BOD (methane - dependent BOD) at 0 m to 10 m depth at three fixed platforms over a period of two years.
2. Examine how overall microbial communities, and more specifically MOB change within BML water samples during four seasons a year.

1.2 Hypotheses

1. We hypothesized that the MOB cluster underneath the ice surface (0 m) to oxidize methane during winter where methane bubbles are trapped by an ice cover. In contrast, we hypothesize that MOB during summer stratification cluster and localize to oxidize CH_4 at the transition zone at the metalimnion (6 m to 8 m) where dissolved oxygen and methane gradients cross.
2. We hypothesized that MOB demand for DO to oxidize CH_4 varies seasonally due to turnover of the BML water column (0 m -10 m).
3. We hypothesized that the microbial community compositions vary during four seasons due to changes in environmental conditions such as ice cover, ambient temperature, and DO within the 10 m water column at BML.

1.3 Study Site

Syncrude Canada currently is developing BML, an EPL, based on work experience of more than two decades. Syncrude is using WCTT to improve the BML water quality and store the FFT. There were no more sediments transferred into BML (previously WIP) after the placement of tailings was completed in late 2012. BML is located in the former West In-Pit (WIP) tailings pond, Alberta (57° 0' 39.3012" N111° 37' 16.3092" W). Water cap thickness was approximately 8.5 m initially, with a maximum depth of 10 m. It is anticipated that the consolidation of tailings sediments FFT in the BML pond should deepen the lake over years and increase the depth up to 18 m (Syncrude Canada Ltd). The pond was filled with ~ 185 million m^3 of tailings (MFT) from the Mildred Lake Settling Basin (MLSB) through a pipeline in 2012. These MFT were mixed with the sediments that had formed in WIP between 1995 and December

2012 **[Figure 1. 1]**. The fresh water cap was pumped from two natural reservoirs: the Mildred Lake Reservoir (MLR) and Beaver Creek Reservoir (BCR) to fill up the BML to the level of 308.7 m above sea level (masl). Variation in physical parameters of BML revealed that depth, temperature, specific conductivity, pH, dissolved oxygen, and turbidity ranged from 0 to 10 m; 0.0 to 21.0 °C; 2664.5 to 3013.33 mS/cm; 3.69 to 8.99; 2.4 to 9.26 mg/L; 2.56 to 66.9 NTU **[Table 1. 1]**.

The temperature of transferred tailings sediments to WIP was between 11 °C to 19 °C [14]. In October 2012, it was estimated that tailings sediments had reached a maximum thickness 48 m and was covered with 52 million of water (6.5 m the average depth) [15].

Table 1. 1 Seasonal variation in physical parameters of Base Mine Lake during two years (2016-2017).

	Summer 2016					Fall 2016				
Depth (m)	Temp (°C)	Sp. Cond (mS/cm)	pH	DO (mg/L)	Turbidity (NTU)	Temp (°C)	Sp. Cond (mS/cm)	pH	DO (mg/L)	Turbidity (NTU)
0	21.42 ± 1.46	2727.33 ± 89.25	7.746 ± 0.46	7.03 ± 0.10	21.42 ± 1.46	6.56 ± 0.77	2720.33 ± 14.88	8.67 ± 0.12	8.99 ± 0.16	13.5 ± 3.76
1	19.72 ± 0.53	2716.66 ± 83.31	7.83 ± 0.44	6.68 ± 0.13	19.72 ± 0.53	6.66 ± 0.72	2730.33 ± 16.50	8.65 ± 0.12	8.90 ± 0.11	13.33 ± 3.29
2	18.82 ± 1.13	2737.33 ± 59.22	7.73 ± 0.44	6.36 ± 0.19	18.82 ± 1.13	6.66± 0.72	2730.33 ± 16.66	8.64 ± 0.11	8.89 ± 0.10	13.4 ± 3.49
3	18.39 ± 1.29	2736.66 ± 61.72	7.69 ± 0.55	6.19 ± 0.30	18.39 ± 1.29	6.66 ± 0.72	2730.33 ± 16.66	8.63 ± 0.11	8.88 ± 0.10	13.2 ± 3.47
4	16.87 ± 1.07	2752.33 ± 65.68	7.65 ± 0.56	5.75 ± 0.38	16.87 ± 1.07	6.66 ± 0.72	2730.33 ± 16.66	8.62 ± 0.11	8.87 ± 0.10	13.5 ± 3.65
5	15.60 ± 0.36	2755.66 ± 67.35	7.36 ± 0.85	5.19 ± 0.24	15.60 ± 0.36	6.7 ± 0.70	2730.66 ± 16.83	8.65 ± 0.11	8.84 ± 0.09	12.8 ± 3.44
6	15.08 ± 0.04	2756.66 ± 68.15	7.22 ± 0.98	4.98 ± 0.16	15.08 ± 0.04	6.7 ± 0.70	2730.66 ± 16.83	8.64 ± 0.11	8.84 ± 0.09	13.36 ± 3.50
7	15.17 ± 0.73	2760.66 ± 64.24	6.91 ± 1.27	4.09 ± 0.20	15.17 ± 0.73	6.73 ± 0.73	2730.66 ± 16.83	8.62 ± 0.11	8.84 ± 0.09	13.46 ± 3.71
8	13.56 ± 0.36	2767.33 ± 63.31	6.47 ± 1.61	2.15 ± 0.73	13.56 ± 0.36	6.7 ± 0.75	2731.55 ± 17.00	8.61 ± 0.11	8.836 ± 0.09	13.63 ± 3.49
9	12.93 ± 0.32	2774.66 ± 65.34	6.05 ± 1.97	0.84 ± 0.62	12.93 ± 0.32	6.7 ± 0.75	2730.66 ± 16.83	8.60 ± 0.10	8.83 ± 0.09	13.66 ± 3.63
10	12.48 ± 0.23	2664.5 ± 194.5	4.19 ± 3.39	0.44 ± 0.36	12.48 ± 0.23	-	-	-	-	-
	Winter 2017					**Spring 2017**				
Depth (m)	Temp (°C)	Sp. Cond (mS/cm)	pH	DO (mg/L)	Turbidity (NTU)	Temp (°C)	Sp. Cond (mS/cm)	pH	DO (mg/L)	Turbidity (NTU)
0	0.1 ± 0.1	3013.33 ± 7.26	8.52 ± 0.18	9.26 ± 0.26	2.56 ± 0.37	5.2 ± 2.60	2828.66 ± 125.75	8.41 ± 0.1	8.56 ± 0.07	7.53 ± 1.75
1	0 ± 0	3016.33 ± 3.38	8.53 ± 0.19	9.26 ± 0.25	2.36 ± 0.54	4.9 ± 2.45	2830.66 ± 121.34	8.45 ± 0.10	8.64 ± 0.15	7.5 ± 2.08
2	0 ± 0	3014.33 ± 2.33	8.54 ± 0.19	9.23 ± 0.23	2.2 ± 0.6	4.43 ± 2.24	2849.66 ± 114.16	8.37 ± 0.12	8.25 ± 0.23	8.46 ± 2.79
3	0 ± 0	3012.33 ± 3.52	8.53 ± 0.18	9.22 ± 0.23	2.3 ± 0.66	3.7 ± 1.85	2880.66 ± 98.87	8.36 ± 0.12	7.88 ± 0.37	9.83 ± 3.52
4	0.06 ± 0.03	3009.33 ± 4.33	8.53 ± 0.18	9.17 ± 0.24	2.53 ± 0.73	3.53 ± 1.79	3261.66 ± 366.59	8.35 ± 0.12	7.7 ± 0.50	12.3 ± 5.04
5	0.13 ± 0.08	3003.33 ± 5.56	8.53 ± 0.18	9.12 ± 0.23	2.83 ± 0.81	3.43 ± 1.53	2910.66 ± 72.08	8.23 ± 0.01	7.46 ± 0.42	13.13 ± 4.78
6	0.43 ± 0.08	2987.33 ± 4.91	8.48 ± 0.13	8.92 ± 0.25	3.93 ± 1.25	3.3 ± 1.33	2928.66 ± 59.20	8.11 ± 0.09	7.06 ± 0.51	16.93 ± 7.53
7	0.86 ± 0.03	2977.66 ± 1.45	8.25 ± 0.07	8.32 ± 0.39	6.56 ± 2.66	3.16 ± 1.10	2952.66 ± 37.97	7.97 ± 0.21	6.75 ± 0.48	69.1 ± 50.7
8	1.4 ± 0.15	2965.33 ± 3.66	7.89 ± 0.39	7.28 ± 0.25	14 ± 5.64	2.96 ± 0.73	2979.66 ± 11.93	7.32 ± 0.83	5.48 ± 0.10	37.7 ± 11.02
9	2.56 ± 0.32	2977.66 ± 22.39	6.22 ± 1.85	2.4 ± 1.02	57.33 ± 4.21	3.33 ± 0.36	2979.66 ± 12.58	5.67 ± 2.48	3.69 ± 1.49	54.13 ± 5.01
10	3.11 ± 0.01	3007.25 ± 26.57	5.38 ± 2.55	0.54 ± 0.18	665.9 ± 4.34	3.42 ± 0.3	2992.66 ± 23.20	4.1 ± 4.02	2.40 ± 2.32	73.75 ± 7.25

1.4 Seasonal Temperature and Dissolved Oxygen Profiles

BML has an active methane cycle. Methane bubbles were observed on the surface of BML during a site visit on 10 August 2017. It was reported that CO_2 and CH_4 emissions from MLSB were higher (52.85-26.22 t/ha/y) than in BML (previously known as WIP) (88.07-3.32 t/ha/y) between 2010 to 2011 [3].

BML is typically covered by ice from late December to late March. During winter under the ice, the water column is poorly mixed because the surface of the water is not exposed to wind **[Figure 1. 1]**. CH_4 is produced due to biological activity, biodegradation of low molecular weight alkanes (C_6 - C_{10}) and high molecular weight of n-alkanes ($C_{14,}$ C_{16}, and C_{18}) and benzene, toluene, ethylbenzene, and xylene (BTEX) [16] by archaeal methanogenesis in FFT [10,16-18]. CH_4 and CO_2 are transported via diffusion or as bubbles through MFT and BML water column and may accumulate below the ice cover during winter [19]. Water temperature on the surface is generally colder than the bottom of the lake while the dissolved oxygen concentration is higher on the surface than the bottom of the lake **[Figure 1. 3] [Figure 1. 4].**

We hypothesized that temperature can be a strong controlling factor on oxidation of CH_4 by MOB below the ice surface. Some studies suggest that the metabolism of CH_4 by MOB can be slow during winter in cold waters [20,21]. As the ice on the surface of BML starts melting, trapped GHGs can escape to the atmosphere **[Figure 1. 1]**. The temperature gradients are between 0 to 3 °C in the whole water column (10m) while the DO is between 8.2 to 5.75 mg/ L across the 10m **[Figure 1. 3] [Figure 1. 4]**.

BML is a dimictic stratified lake **[Figure 1. 2]**. During summer stratification, the sun heats the surface of the lake forming the epilimnion layer (0 m - 6 m). The bottom layer of the BML, the hypolimnion (8m-10m) does not receive sunlight and therefore remains cold. Since the

epilimnion is less dense, it stays on top of the hypolimnion, so the two layers do not mix. The metalimnion or thermocline (6m - 8m) is a transition zone between the upper zone and lower zone of the lake. The temperature and dissolved oxygen are higher on the surface than the bottom **[Figure 1. 3] [Figure 1. 3]**. We hypothesized that MOB localize at the thermocline zone to oxidize CH_4 **[Figure 1. 2]**.

During fall, the sunlight is not strong and the nights become cooler in BML. This change in season allows the epilimnion to cool off. As the BML water in the epilimnion (0-6 m) cools, the water becomes denser and sinks to the hypolimnion, mixing both layers. This mixing event allows oxygen to be mixed to the bottom of the lake. The temperature is between 7 to 15 °C across the whole water column and the dissolved oxygen concentrations are between 6 to 9mg/L across the whole column **[Figure 1. 3] [Figure 1. 4]**.

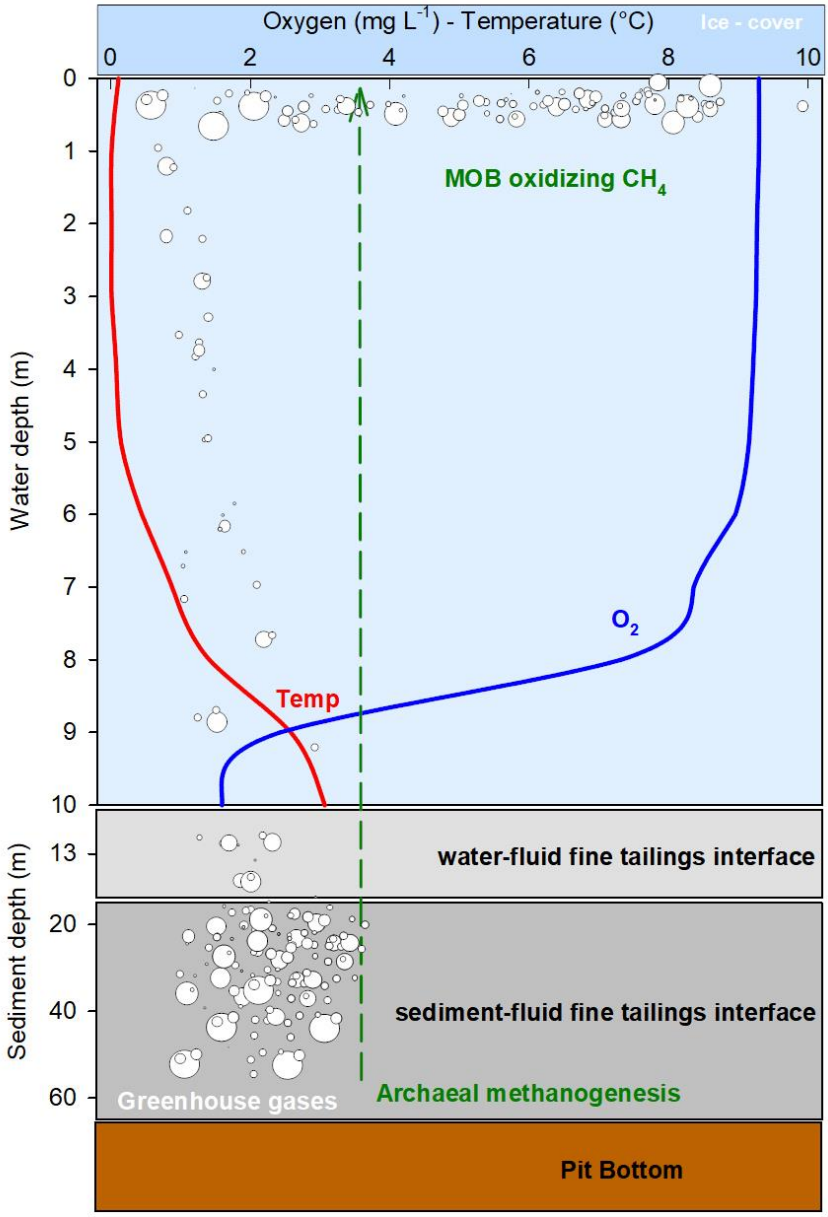

Figure 1. 1 Schematic cross-section of Base Mine Lake (BML) representing water column (10 m), water-FFT tailings, and sediment-FFT interface during the winter season. Within the BML system, GHG (CH_4 and CO_2) bubbles are formed and released from anoxic environment (FFT) by methanogenesis and GHGs are accumulated underneath the ice cover in BML. We hypothesized that MOB may concentrate under the ice if methane bubbles accumulate there.

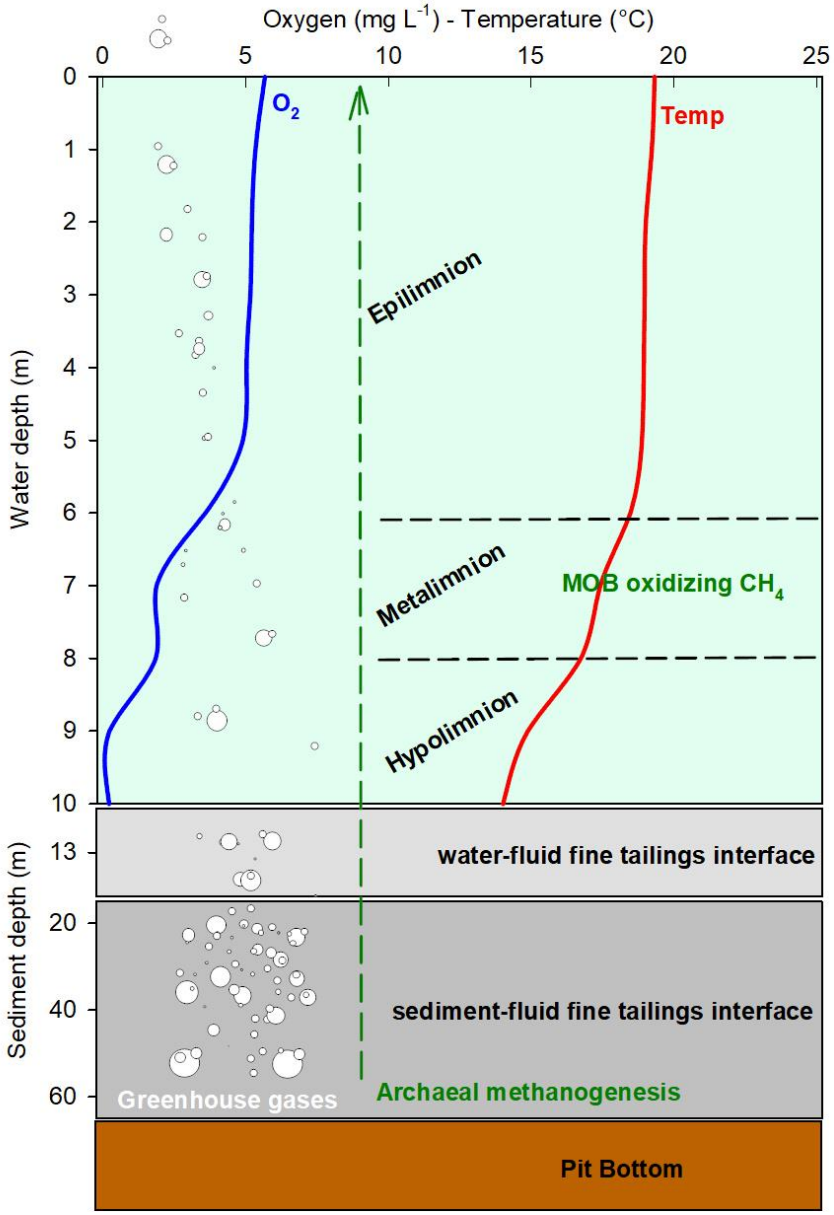

Figure 1. 2 Schematic cross-section of Base Mine Lake (BML) representing water column (10 m), water-FFT tailings, and sediment-FFT interface during summer stratification. Within the BML system, GHG (CH_4 and CO_2) bubbles are formed and released from anoxic environment (FFT) by methanogenesis. GHGs are transported through the water column bypassing CH_4 oxidation in BML bottom. We hypothesized that MOB may localize in the metalimnion if CH_4 and DO are both present.

Figure 1. 3 illustrates the average temperatures of BML in three platforms within 10 m deep water from 2016 until 2017 (Syncrude Canada Ltd). During January and February, the lowest temperatures were recorded on the surface at 0 °C but that increased gradually to reach up to 3 °C at the bottom of the lake. During May, the temperature increased to 7.5 °C on the surface with a slight decline to less than 5 °C in the bottom. During summer 2016, June and August temperatures on the surface were 19 - 22 °C, and the temperatures declined to less than 15 °C for both months at the bottom. In September and October, the lake temperature started to cool down and temperatures were constant in the entire water column.

Figure1. 4 illustrates the average dissolved oxygen concentration of BML in three platforms within 10 m deep water from 2016 until 2017. The dissolved oxygen concentrations vary by month and depth from 5.8 mg/L (surface, summer) to 0.5 mg/L (10 m, late summer), with full lake turnovers in spring and fall equalizing DO levels around 6 - 9 mg/L for all depths.

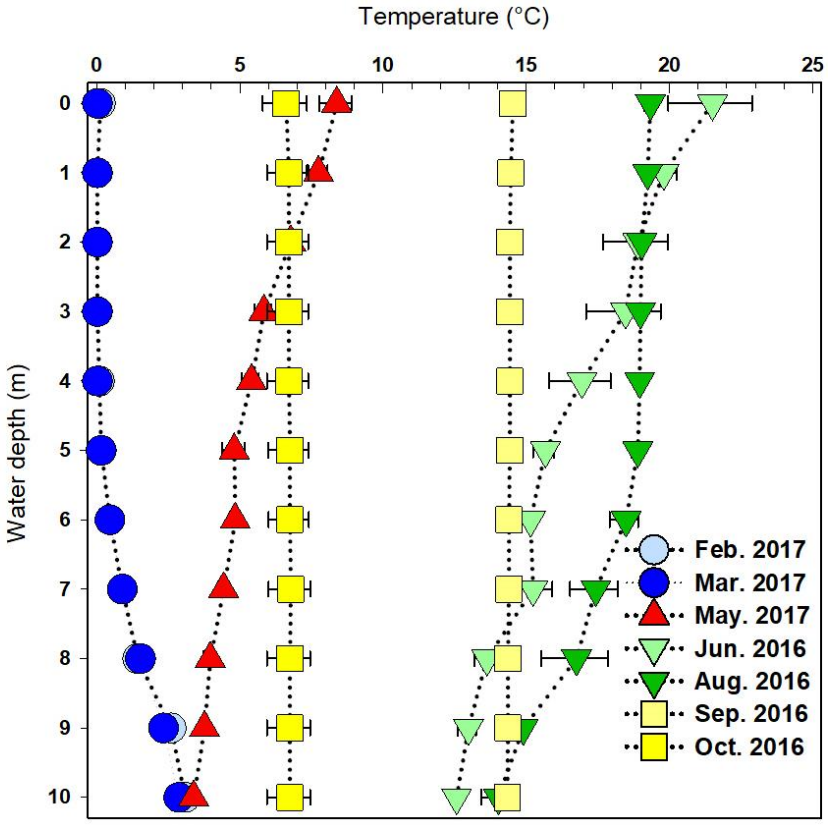

Figure 1. 3 Field measurements of average temperatures with depth. between 2016 to 2017 at Base Mine Lake (BML). Data are means of measurements at three fixed platforms. The temperature profiles were recorded on 13-15 February 2017; 13-15 March 2017; 9-11 May 2017; 27-29 June 2016; 22-24 August 2016; 19-20 September 2016; 11-17 October 2016. Temperature profiles are field measurements. Provided by Syncrude Canada.

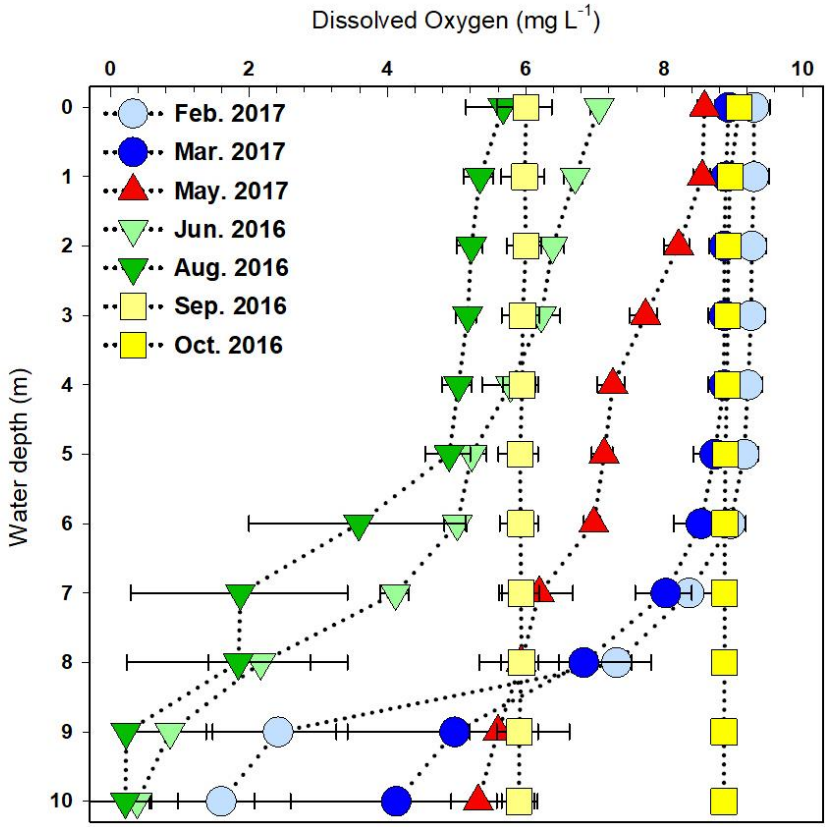

Figure 1. 4 Field measurements of average dissolved oxygen with depth between 2016 to 2017 at Base Mine Lake (BML). Data are means of measurements at three fixed platforms. The dissolved oxygen profiles were recorded on 13-15 February 2017; 13-15 March 2017; 9-11 May 2017; 27-29 June 2016; 22-24 August 2016; 19-20 September 2016; 11-17 October 2016. Temperature profiles are field measurements. Provided by Syncrude Canada.

CHAPTER TWO: LITERATURE REVIEW

2.1 The Canadian Oil Sands

The Canadian oil sands are made up of sands, silts, and clays (~85 wt%) water (~5 wt%), and a slick heavy oil called bitumen (~12 wt%) [22]. It is estimated that the oil sands reserves contain approximately 140 billion barrels of bitumen, and the oil sands deposit covers over 140 000 km^2. The geological formation and the geographic location of oil sands in Alberta are deposited at three major oil sands areas: the Athabasca River, Cold Lake, and Peace River [11].

There are many technologies that are applied by oil sands operators to extract oil from bitumen in Alberta. These methods are applied based on how deep the oil sands reserves are deposited. The most known methods to extract oil from bitumen are Open-pit mining, Steam Assisted Gravity Drainage (SAGD), and Cyclic Steam Stimulation (CSS). In 2016, Alberta's oil sands proven reserves were 165.4 billion barrels. Out of this volume, 80% is recoverable through in-situ while 20% is recoverable through surface mining [23].

Open-pit mining (surface mining) is used to recover oil sands that lie within 75 m of the surface. At least 46% of oil production was through this method in 2015 [24]. In this technique, large shovels scoop sands saturated with bitumen into huge trucks. Trucks take loaded oil sands to crushers where clumps are broken down into a small size. This mixture is then mixed with hot water and chemical solvents to remove the sands, mineral fines and separate the oil. Once the bitumen extraction process is completed, the product is ready to be pumped via hydrotransport for further refining or upgrading [25]. By law, most mining areas that are disturbed after mining by oil sands operators must be fully reclaimed and returned to a self -sustaining ecosystems. Reclamation is progressive: it begins in mined-out areas while mining continues in other areas [26].

Surface mining process are illustrated and described in detail in eight steps, starting from removing vegetation and ending with reclaiming the mining site (http://www.oilsandsmagazine.com/technical/mining/surface-mining).

The disadvantage of the surface mining method is that large amounts of tailings water and fine sands are produced. Recent advancement in technologies have allowed oil sands industries to increase their oil production and bitumen recovery through two common techniques termed in-situ (in position): Steam Assisted Gravity Drainage and Cyclic Steam Stimulation. Both techniques SAGD and CSS are comprised of four basic components: well pads, steam generation, bitumen production, and water treatment. However, the differences between SAGD and CSS lies in the number of wellheads and the position of wellheads (horizontal and/ or vertical) [23]. These two techniques typically target bitumen that are localized at depths between 75 to 300 m [27].

SAGD is a thermal production technology which employs two main horizontal wells (L-shaped). Each well can be up to 1000 m long and can vary from 150 to 450 m in depth [28]. One well is used to inject high -temperature steam continuously into the oil -producing reservoir. After the steam permeates the oil sand, the heavy oil becomes less viscous. The melted oil now is ready to flow easily and is pumped to the surface through the second well. Then, the bitumen is sent to the plant for further processing while the separated water is recycled back into the process (http://www.oilsandsmagazine.com/).

CSS is also known as Huff n' Puff. The method involves a single well, and it consists of three main stages: injection, soaking, and production. The CSS process has three stages. First, the oil sands operator drills a well into deep ground, where the oil sands deposit is localized.

Then enough steam should be injected under high-pressure to break up the heavy-oil reservoir and high-temperature (350 °C) to melt the bitumen deposit. Second, the well is shut down after enough steam is injected into oil reservoir. This stage is called the soaking stage, and it is basically done to liquify the bitumen and decrease the viscosity of extra heavy-oil. Third, the melted bitumen below the surface is pumped out via a production well to the surface for further process [23,29]. Both technologies are determined by the geological locations of oil reservoirs. For example, SAGD technology is performed better in the Athabasca region while the CSS performs better at Cold Lake. An advantage of using these methods is water usage: water can be recycled and recovered and there are no additional tailings ponds generated. The disadvantage of thermal production technology is lower bitumen recovery compared to surface mining and more GHG emission due to burning fossil fuel.

2.1.1 Alberta's Oil Sands Tailings

With rising global demand for oil and increasingly stringent environmental regulations in Alberta, oil sands industries that are extracting bitumen through mining are facing two main obstacles: a) obtaining and utilizing large volumes of fresh water to extract bitumen through mining. b) finding a place to store large volumes of tailings water that are produced during the process of bitumen extraction, which poses environmental stresses. Each barrel of oil sands extracted via mining results in 1.5 barrels of tailings water which need to be kept in tailings ponds, and every day the oil industries produce at least 25 million liters of tailings water. Today, scientists estimate that the total volume of tailings water has reached more than 1.18 trillion liters [30].

At present there are many tailings ponds in Alberta that are fully managed and operated by oil sands industries. However, all oil sands mining and tailings ponds are under regulation, evaluation, and even site inspection by AER. In addition, there are also more industrial wastewater treatment technologies that have been applied to remediate oil sands tailings by oil industries, such as consolidated tailings process [11,22], adsorption [31-33], membrane processes [34-37], advanced oxidation processes [38-44], freeze separation [23], reverse osmosis [45], and entrapped bioaugmented cells system [46]. However, all the treatment technologies listed above are not considered here in detail except the biological process [47-51].

The production of crude oil via open-pit mining requires large volumes of fresh water and recycled water during the bitumen extraction process, resulting in large quantities of tailings water or oil sands process-affected water (OSPW) [11,52]. The OSPW is alkaline water with a mixture of 70-80 wt% water, 20-30 wt% solids (sands, clays, and silt), 1-3 wt% residual bitumen [11], inorganic and organic compounds, and diluents [3]. Tailings water is highly enriched with a

variety of chemical compounds. The chemicals compositions of OSPW at different tailings ponds are different and depend on several factors, such as mineralogy, the age of oil sands, bitumen extraction techniques performed during bitumen recovery, and the addition of chemicals during bitumen processing [53]. During bitumen processing some additives are added to enhance or increase the oil recovery rate, such as caustic soda, diluents, and sodium citrate. Initially, bitumen can be separated from clay and sands by using the Clark Hot Water Separation Process. The caustic soda is mixed with slurry and hot water to allow for the separation of clays, dissolved metals, and organic compounds [54,55]. Diluents are generally used for two main purposes: a) to remove the residual water and solids from the extracted bitumen [56]; and b) to decrease the viscosity of the bitumen for efficient transportation through pipeline [10]. Sodium citrate is used to increase the bitumen extraction efficiency [57,58]. Furthermore, more additives are added to the tailings water for treating purposes and storage. For example, gypsum is used for coagulation and densification of mineral fines [59] while anionic polyacrylamides are added to produce thickened tailings, as well as to accelerate the dewatering tailings [60]. Moreover, OSPW is highly concentrated with salts, dissolved and suspended solids, and toxic compounds. OSPW is highly enriched with naphthenic acids (NAs), BTEX, polycyclic aromatic hydrocarbons (PAHs), heavy metals and ions, phenols [11,61,62]. NAs are considered as a major toxic compound in tailings water [11,63]. The biodegradation of commercial NAs either aerobically or anaerobically depends on the molecular mass of NAs. For instance, low molecular mass NAs ($C \leq 17$) were more biologically degradable within 14 days than those of the high molecular mass ($C \geq 18$) [63]. In addition to NAs, BTEX, PAHs, heavy metals, dissolved iron, and other organic compounds may participate in to the OSPW toxicity [64-68].

Tailings ponds (tailings storage facilities) naturally consist of four different layers: sands, MFT, FFT, and OSPW. As tailings water discharges into the pond, sands and solid fines settle quickly to the bottom forming different anoxic layers of tailings waste (sands, MFT, FFT). MFT contains of at least 70 wt% water and 30 wt% solids and behave as a viscous fluid while FFT consist of 20-80 wt% clays and silts. A recyclable surface water layer at the upper layer is re-used for further extraction, and this process makes tailings water enriched in organic and inorganic contaminants from the mined ore [11]. Methane and carbon dioxide are formed and produced from tailings sediments, where methanogenesis occurs. In an early study, methane emissions from MLSB was estimated to be at least 43,000 m^3 CH_4 day^{-1} [69,70].

2.1.2 Reclamation and End Pit Lakes

Bitumen recovery via open-pit surface mining is still the major method used in Alberta. However, with surface mining the size of the disrupted areas near to boreal forest is growing rapidly and the number of tailings ponds are increasing [13]. Recently, it was estimated that the total land influenced by surface mining was approximately 900 km^2 in 2013 [2]. As a part of their licensing agreement with Alberta's Environmental Protection and Enhancement Act (EPEA), oil sands industries must reclaim mining affected areas to diverse self-sustaining ecosystems [71]. To demonstrate the best practices and successful reclamation, the Cumulative Environmental Management Association (CEMA) outlined criteria in detail [72,73]. The reclamation plans include two options, 'dry' and 'wet'. For the dry landscaping option, tailings fines and sediments must be trafficable, and this requires physical and/or chemical manipulation of tailings sediments (MFT) to increase solids content after the excess water is removed. For the wet landscaping option, one option is placing tailings sediments into a basin and capping the entire tailings pond with freshwater to form an 'end pit lake' (EPL), where tailings solids and fines can be compressed.

Also, the wet landscaping reclamation provides a viable solution to reclaim CT and OSPW and remediate hydrocarbons and NAs that are present. Both options require the presence of microbial community to achieve the main goal [7,11,13]. An EPL is defined as *"An engineered water body, located below grade in oil sands post-mining pits. An EPL may contain oil sands byproduct materials and will receive surface and groundwater from surrounding reclaimed and undisturbed landscapes. EPLs will be permanent features in the final reclaimed landscape, discharging water to the downstream environment."* [74].

The importance of biological processes in oil sands tailings has been recognized. The onset of CH_4 production from the MLSB pond coincided with the depletion of sulfate from the pore waters in the MFT that contain anaerobic microorganisms including sulfate reducing bacteria (SRB) [70,75,76]. CH_4 emission from the largest oil sands tailings pond (MLSB) was estimated to be 43,000 m^3 day^{-1} [70]. In another study, it reported that CH_4 emission from MLSB pond was higher in deep sediments (30 mbs) than surface sediments (6 mbs) [10]. Anaerobic methanogenesis was confirmed in residual hydrocarbons of MFT. Samples from MFT also have been tested in the laboratory to investigate methanogenesis of long chain of *n*-alkanes, short-chain *n*-alkanes, NAs, BTEX, and monoaromatic hydrocarbons [16-18].

Archaeal methanogens are strictly anaerobic microorganisms, while bacterial methanotrophs live at anoxic/oxic interface of different environments [77]. Thus, we hypothesized that fresh water capping from BCR and MLR into BML will result in a change in the community composition over years as they respond and adapt to new (oxic) conditions. Consequently, we also hypothesized that methanotrophs may oxidize a large proportion of methane at the oxic/anoxic transition zone (metalimnion) during summer stratification, where the temperature and DO

concentration decreases rapidly. During winter, methanotrophs should prefer to grow underneath the ice surface, where methane bubbles are blocked due to the ice layer.

Methane emissions have been already measured or estimated from at least 19 tailings ponds in Fort McMurray. CH_4 emission from WIP was eight times lower compared to an active tailings pond (MLSB) [3]. Surface water samples from two tailings ponds (WIP and MLSB) were collected to examine methane oxidation and methanotroph populations. Both ponds are mostly anoxic and produce CH_4; however, both ponds have oxic surface layers. Potential methane oxidation via methanotrophic bacteria in these samples was measured in the laboratory (ex-situ) [78]. The potential methane oxidation rates from both ponds were recorded to be approximately from 75-150 nmol CH_4 $ml^{-1}d^{-1}$, and this range of rates can be seen in both natural lake and ocean waters. These data suggested that methanotrophs were highly active where O_2 was present. Based on laboratory experiments these data suggested that ~17% of methane formed is oxidized aerobically via methanotrophs, which therefore help to mitigate greenhouse gas emissions [78]. The methanotroph species compositions were investigated via pyrotag sequencing of amplified 16S rRNA genes. The analysis indicated that methanotrophs were present in both ponds (WIP and MLSB). Type I methanotrophs predominated in WIP, with *Methylococcus/Methylocaldum* (OTU 12103) making up on average 1.5% of total reads. Other methanotrophs were also detected in the same pond but at lower relative abundance, such as *Methylobacter, Methylosoma, Crenothrix,* and *Methylomonas.* In WIP, the genera detected were *Methylococcus* and *Methylomicrobium*. Further analysis was also performed to identify most active methanotroph communities in tailings environments via DNA stable isotope probing (SIP). The analysis showed that *Methylomonas* spp. and *Methylocaldum* spp. were the predominant ^{13}C-labeled OTUs, followed by *Methylomicrobium* and *Methylobacter* [78].

After the placement of fluid fine tailings was completed at the end of 2012, BML was officially commissioned as of 31 December 2012. No additional tailings solids are added, transferred from the MLSB, and/or removed. In 2013, fresh water was added from BCR and MLR to the existing OSPW inventories to achieve a final water elevation of approximately 308 masl [15].

We monitored the potential methane oxidation in BML samples collected from three platforms at three depths (0, 4, and 8 m) in 2014 [79]. This measurement was performed *ex-situ* (*not in situ*). The potential methane oxidation rates (at 25^{o}C) were highest during spring and late summer and lowest during early summer. Methane oxidation in sub-ice samples from March 2015 was also measured at 4^{o}C which is near to *in-situ* temperature. The potential methane oxidation rates at five depths (0 -8 m) ranged from 3.86 to 85.84 nmol mL^{-1} d^{-1}. However, the highest rate of methane oxidation was observed just below the ice (0 m) and decreased with depth. The results suggest that methane oxidation occurs at low temperature (methanotrophs are psychrophilic) in the BML water column [79].

The biological oxygen demand (5d-BOD) with 10% methane (methane-dependent) and without methane (independent BOD) was measured in BML water samples taken from three platforms at five depths (0, 2, 4, 6, and 8 m). The potential methanotrophic oxygen demand (MOD) was defined as the difference of the two measurements. The BOD and MOD results showed seasonal variations in 2015 exhibiting highest values during late winter, late spring, and late summer and lowest during early summer and early fall [79].

Biological oxygen demand (BOD) is a test of how much easily biodegradable organic matter is present in water, and is one of the most widely used criteria for monitoring water quality [80]. The BOD measurement was first selected in 1908 as an indicator of organic pollution of rivers

by the United Kingdom Royal Commission on River Pollution [81]. This parameter was adopted by the American Public Health Association Slandered Methods Committee in 1936 as a reference indicator to assess the biodegradation of chemicals and hazardous compounds [82].The principle of 5d-BOD and its fundamental strength is that it estimates the amount of dissolved oxygen that will be utilized in sample via aerobic microorganisms during the incubation period to degrade organic compounds that are present in sample (e.g. water or sludge) when the sample is incubated at a specified temperature (20 ℃) for a specified period (typically 5 days: 5d-BOD and /or BOD_5) [83].

The 5-d BOD has three applications: a) it is used as an indicator of the compliance with standards of wastewater discharge and the waste treatment procedure to applicable regulations and laws; b) in wastewater treatment plants (WWTPs), a ratio between 5-d BOD and COD (chemical oxygen demand) indicates the biodegradable fraction of an effluent; and c) the ratio 5-d BOD/COD is an indicator of the size of a wastewater treatment plant required for a specific location. Comprehensive references cover all aspects of water and wastewater analysis techniques, and the standard methods and best technical practice for monitoring water and/or wastewater are available online at the American Public Health Association (APHA), the American Water Works Association (AWWA), and the Water Environment Federation (WEF) websites. Alternative methods for BOD assessment include photometric methods, manometric methods, biosensors based on bioluminescent bacteria, microbial fuel cells, BOD biosensors with redox-mediators, biosensors with entrapped bacteria, biosensors based on the bioreactor/chemostat technology), and other approaches [82].

The BOD_5 values in natural lakes, rivers, and creeks typically have a lower BOD ranging from 0.2 to 2.9 mg/L, which is considerably less than the saturation value of 8.5 mg/L at room

temperature [84]. The BOD is also used to measure the strength of pollutant loads such as spills, sewages. The BOD values in wastewater treatment is ranged from 110 (weak) to 400 (strong) mg/L while the BOD value in sewage is ranged from 100 to 270 mg/L while the BOD value in sewage is ranged from 100 to 270 mg/L [85,86].

2.1.3 Lake Stratification and Turnover

Lake stratification is a separation of lakes into three layers: epilimnion, metalimnion, and epilimnion. The metalimnion (thermocline) acts as transition zone between the epilimnion and the hypolimnion, and during stratification the oxygen sources are confined only to the epilimnion, and must move to the hypolimnion by molecular diffusion. Oxygen that is consumed for respiration by living organisms in the hypolimnion layer causes a depletion. Another reason for the depletion in lake DO during stratification is the oxidation of large quantities of reduced substances that have accumulated in the hypolimnion during the summer. The level of DO and the duration of low DO affect the survival and growth of living organisms [87].

Lake environments contain physical, chemical, and biological gradients. Water density in lakes is usually affected by temperature. As a consequence, lake stratification can be formed during summer season if the water column is deep enough [88]. During summer stratification, warmer and less dense surface layer (the epilimnion), is separated from the colder and denser bottom layer (the hypolimnion). The epilimnion layer usually higher in temperature, dissolved oxygen concentration, and light intensity while the hypolimnion layer can experience lower or no dissolved oxygen due to much slower process of diffusion. The thermocline layer is a transitional layer where temperature rapidly shifts (greatest water temperature change) [89]. Dissolved oxygen

(DO) concentration, the availability of nutrients, biological productivity, and fish are some parameters that are highly controlled by temperature changes.

A study also showed that microbial communities and microbial compositions change during stratification and mixing periods in lakes. Some differences, such as microbiological, chemical, and physical parameters were observed between two layers (epilimnion and hypolimnion). The RNA: DNA ratios were higher in the hypolimnion ($P < 0.05$) but not in the epilimnion layer. The study suggested that microorganisms were more active at low temperature, low dissolved oxygen concentration, and high TN/TP [90-94].

2.1.4 Methane Dynamics in Lakes

Earlier studies have investigated global and local methane emissions. Most methane production occurs in sediments, where the O_2 is mostly absent [95,96]. One study at 11 North American and 13 Swedish lakes developed a model to estimate methane emissions in lakes. The method works by predicting methane emissions from open water though monitoring and measuring different variables in lake, such as the concentration of dissolved organic carbon, the concentration of dissolved methane, the concentration of total phosphorus , and water depth [97]. Methane is formed and produced at the bottom of the lake (sediments). Produced methane is released as bubbles and this gas released to the atmosphere after passing the water column. Methane emission at natural lakes is controlled by both methanogens (methane producers) and methanotrophs (methane oxidizers) [98]. Also, methane bubbles can be trapped underneath ice cover during winter [21]. Methane can move though different pathways in water column, ebullition flux, diffusive flux, storage flux, and flux through aquatic vegetation. These four pathways of methane make it difficult to estimate gas emissions from lakes and reservoirs.

Methane can be released from anoxic (sediments) to oxic (water column) by either ebullition or by diffusion. Ebullition results in direct flux of methane from anoxic environment to the atmosphere, with limited effect of methane oxidation in oxic environments. For this reason, the ebullition flux component is closely related to the net methane production rate in anoxic environments (sediments) (i.e., the gross methane production rate minus potential methane oxidation) and to the hydrostatic pressure which has to be overcome for the bubbles to leave the sediment [99,100].As a result of the diffusive flux from anoxic sediment, a large amount of methane that is produced in anoxic sediment can be oxidized by MOB as soon as methane reaches an oxic environment [101,102]. Most of the methane that is not oxidized in the water column will be emitted by diffusive flux. The flux component depends on the difference in methane concentration between the water and the atmosphere and the physical rate of exchange in water and air [103]. During lake stratification, methane can accumulate in the anoxic environment, causing methane storage in the water column. This stored methane can be released rapidly by diffusion during spring and fall when the lake turns over [104]. The potential flux component includes plant-mediated emission in littoral zones with vegetation. This flux component depends on vegetation characteristics [105].

2.1.5 Methane and Global Warming

Greenhouse gases (GHGs) are gases that warm earth's climate by absorbing energy in the atmosphere and trapping the heat from the sun, such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), ozone-depleting substances (ODSs), hydrofluorocarbons (HFCs), perfluorocarbons (PFCs), and sulphur hexafluoride (SF_6) [106]. CH_4 plays an important role in the

Earth's atmosphere. One of several greenhouse gases, the more CH_4 is released to the atmosphere, the hotter the average temperature becomes. Methane is the second most important greenhouse gas after carbon dioxide, and it contributes to the enhancement of the greenhouse gas effect with a portion of approximately 20% [107]. The higher potential of CH_4 for absorbing infrared radiation was recently newly calculated as 28 to 34 times of that of CO_2 per g over an integrated period of 100 years [108]. The atmospheric CH_4 concentration has increased from 0.7 to 1.8 ppmv since pre-industrial times [109]. Unlike other chemical pollutants that remain in the atmosphere for days or weeks, the mean global atmospheric lifetime of CH_4 in the atmosphere was estimated to be 8.4 years [107]. However, it is important to note that the value of GWP can change due to updated scientific estimates of the energy absorption or lifetime of the gases. It is also clear that methane does not remain (localized) at certain zones in which it released, and it spreads through the global atmosphere. Thus, its effects will be felt globally in the future.

The global atmospheric CH_4 budget is determined by several terrestrial and aquatic sources that can be generally grouped into three categories: thermogenic, pyrogenic, and biogenic. Thermogenic CH_4 has been formed from fossil fuel due to a geological process during millions of years. Some examples of thermogenic CH_4 sources are oil, natural gas, marine seeps, mud volcanoes, and terrestrial seeps. Pyrogenic CH_4 is produced by combustion of either biofuels and fossil fuels or the incomplete combustion of biomass and soil carbon during wildfires. Biogenic CH_4 is produced anaerobically due to archaeal methanogenesis in natural wetlands, rice paddies, dissolved oxygen-poor digestive systems [110], and organic waste deposits (sewage, landfills, manure). The three types of CH_4 emissions have different isotopic δ13C

signature: -13 to -25‰ for pyrogenic emissions, -25 to -55‰ for thermogenic emissions, and -55 to -70‰ for biogenic emissions [111,112].

CH_4 sinks globally are: the earth's atmosphere layers (troposphere, stratosphere), ocean, and soil. The most effective sink of atmospheric CH_4 is the hydroxyl radical at the lowest portion of earth's atmosphere [113], which accounts to approximately 90% of the global methane sink [114]. Additional oxidation sinks include reaction with atomic oxygen and chlorine radicals in the stratosphere ~3% [115], reaction with chlorine radicals from ocean in the marine boundary layer ~3% [113], and methane oxidizers (aerobic methanotroph) in aerated soil [114,116].

Alberta's oil and gas sectors are the largest producer of fossil fuel resources in Canada. Also, it is the largest emitter of methane gas and volatile organic compounds (air pollutants), some of which increases the global GHG and negatively affects human health. In December 2015, Canada signed the Paris Agreement to fight the climate change crisis. The new agreement aims to reduce global GHG emissions to limit the rise in global average temperature rise to less than 2 °C and pursue efforts to limit the temperature increase to 1.5 °C. As a part of the Paris Agreement, Canada has promised to reduce GHG emissions by 30% below 2005 levels by 2030, and this includes developing regulations and addressing methane emissions from oil and gas sectors [117,118].

In March 2017, Alberta's government announced new plans to develop industrial regulations to cut down methane emissions from the oil and gas sector. The plans include: a) applying and designing high standards for detecting methane emission in the region; b) improving CH_4 emissions and repairing all leak in methane emissions, as well as reporting methane emissions more accurately; and c) developing a joint initiative on methane reduction

and verification for existing facilities to achieve the target by 2025. Most applications of the new standards for oil and gas industry will be in collaboration between the Alberta Energy Regulator (AER), Alberta Energy, and the Alberta Climate Change Office. [119].

In October 2017, a new report has shown that methane emissions from the natural gas and other sectors in Alberta need to be reduced. The report pointed that methane emission from oil and gas was 60 per cent higher than had previously been reported from oil industries. Scientists at Carleton University published a new paper proving that methane emissions from the Albertian oil and gas industry are at least 25-50% higher than the government is estimating and/ or the companies are reporting. The study was done over Red Deer and Lloydminster, Alberta, Canada. In this study, an airborne technique was used to quantify regional methane emissions at the two regions, and all collected data compare top-down and bottom-up methane emissions estimates. Measured CH_4 fluxes near Red Deer was more than 17 times higher than reported data while measured CH_4 fluxes near Lloydminster was five times greater than reported emissions from flaring and venting [120]. Today, climate scientists believe that heroic efforts by the global community are necessary to reduce greenhouse gases emissions, so the global warming may be limited to 1.5 °C above pre-industrial levels [121]. It is also very important to reduce methane emissions from oil and gas sectors, which would greatly contribute to decelerate and tackle the impact of climate change and avoid the economic influence of climate change events [118].

2.2 Aerobic Methanotrophs

Methanotrophs or MOB have been known for one century; but it is only in the last three decades that we have begun to study their biochemistry and physiology [122]. Methanotrophic

bacteria were first isolated from aquatic plants by Sohngen in 1906, and named *Bacillus methanicus.* In 1908, the same bacterium which grew on only CH_4 or CH_3OH was renamed to *Methanomonas methancia* by Orla Jensen. In 1956, Dworkin and Foster described and classified the bacterium again. They considered and renamed the *Methanomonas methancia* to *Pseudomonas methanica* (Shongen) nov. comb [123]. Nowadays, *Pseudomonas methanica* is known as *Methylomonas methanica.* Also, more methanotrophs are described as obligate aerobic to CH_4 or CH_3OH; these included *Methanomonas methanooxidans* [110,124] and *Methylococcus capsulatus* [125]. Methanotrophs have been reviewed by Hanson and Hanson in 1996 and Semrau *et al in* 2010. Aerobic methanotrophs are a subset of bacteria known as methylotrophs. The bacteria can oxidize CH_4 aerobically to methanol as their sole carbon and energy source. The MOB is involved in mitigating, global atmospheric emissions of methane from different habitats. They convert methane to methanol, and then oxidize it to formaldehyde, and then to formate then to carbon dioxide at the last step of conversion [126].

Methanotrophs contribute to specific steps in the carbon, nitrogen, and oxygen cycles as well as in the biodegradation of organic material substances [127], and they also play a crucial role in regulating the atmospheric CH_4 [128]. Methanotrophs are widespread in environments such as natural wetlands, soil, landfills, sediments, wetlands, lake, ocean, paddy rice, and petroleum waste water [126]. Most methanotrophs are mesophilic and grow at pH (5-8); however, there are also species which can grow at different temperature and pH: psychrophilic, thermophilic, alkaliphilic, and acidophilic. There are also an extremely acidophilic methanotrophs which belong to the phylum *Verrucomicrobia,* and can grow at very low pH (1.0) [129].

2.2.1 Methane Oxidation

Methanotrophs convert CH_4 to CO_2 through four main steps: methane (CH_4) to methanol (CH_3OH), then formaldehyde (CH_2O), formate (HCOOH), and the final product is carbon dioxide (CO_2). The oxidation of methane to methanol is performed by two forms of methane monooxygenase. The soluble methane monooxygenase (sMMO) (iron-dependent) is located inside the bacterial cytoplasm, and only a few species express this enzyme [130]. The membrane-associated or the particulate methane monooxygenase (pMMO) (copper-dependent) is located on the cytoplasmic membrane, and most methanotrophic species can express this enzyme [131] **[Figure 2. 3]**.

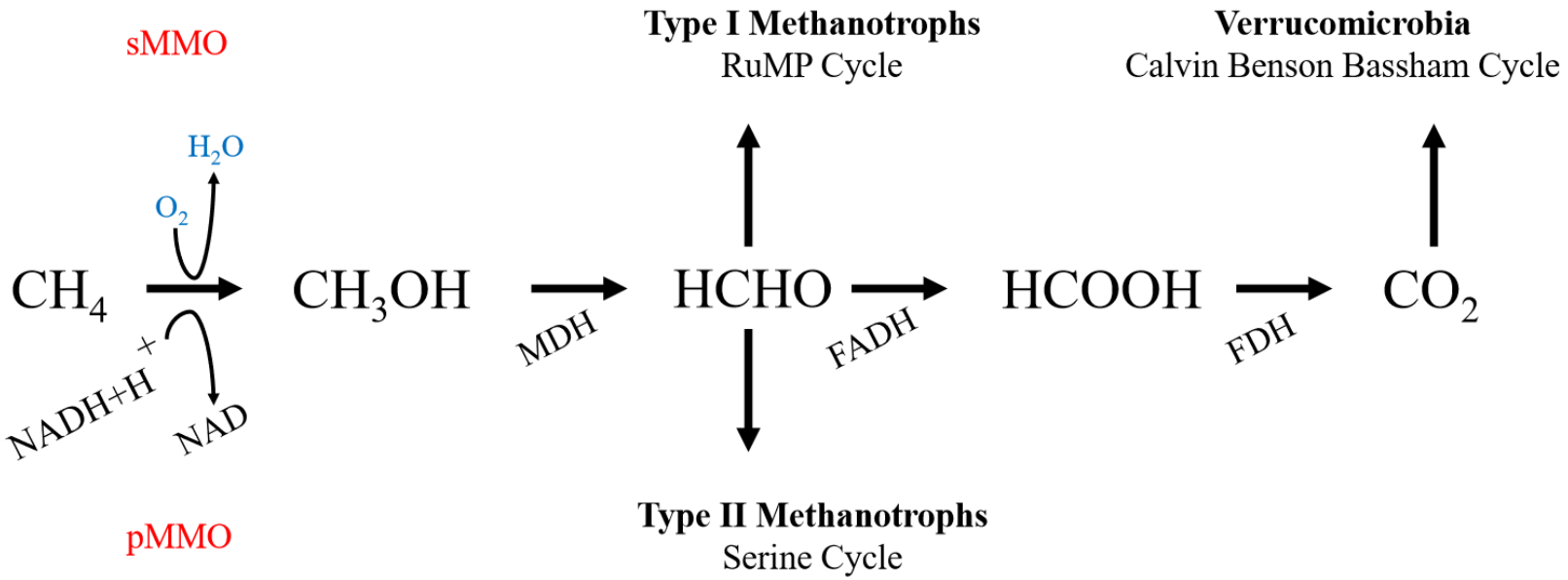

Figure 2. 1 Methane oxidation pathways in methanotrophs. The two distinct forms of methane monooxygenase, pMMO and sMMO, use CH4 as electron donors. Carbon is assimilated at the level of formaldehyde and proceeds via the Ribulose Monophosphate cycle (type I), or Serine cycle (type II), and/or Calvin Benson Bassham cycle (Verrucomicrobia).

However, certain species of methanotrophs contain genes for both enzymes, whose expression are regulated by the copper (Cu) concentration in the medium [127]. It has been well known for over three decades that the presence of Cu in the environment plays a crucial role on methanotroph physiology and response. For instance, the sMMO enzyme oxidizes CH_4 when Cu is below 1 µM while the pMMO oxidizes CH_4 when the Cu is above 5 µM [127,132,133]. Recently, it was found that large quantities of Cu can be stored in Csp1 protein, and this potentially delivers the metal to pMMO [134].

2.2.2 Factors that Affect Methane Oxidation in the Environment

Methane producers and methane oxidizers in freshwater lake ecosystems drive global methane emissions and terrestrial sinks. The majority of methane formation occurs in anoxic sediment by a specific group of archaea (methanogens) as a part of anaerobic respiration of organic matter [115]. The methane thus produced can be exported from the anoxic sediment by either ebullition or diffusion, and then ultimately enter the water column [135]. If a methane bubble passes anoxic sediment it escapes the methanotrophic oxidation process and is emitted to the upper layer of the water column and then to atmosphere [97]. However, dissolved methane that diffuses down a concentration gradient can be oxidized aerobically by methanotrophs [136].

Methane production and methane consumption in lakes can be influenced by some environmental factors [137].. The activity of methanotrophic bacteria depends on the availability of both O_2 and CH_4 [137]. The highest methane oxidation rates take place at the oxic-anoxic interface where CH_4 diffuses toward the surface and O_2 diffuses into the water layer from the atmosphere. In some freshwater lakes, this zone can be found in the upper layer of sediment with direct contact with oxic water of the water column during summer stratification. A large amount of

CH_4 diffusing from sediment (deep bottom) is oxidized aerobically and/or anaerobically near the sediment surface (66-95%) and in the water column (45% - 100%) [101,138]. In other lakes, the activity of methanotrophic bacteria was recorded to be highest at the bottom of the oxycline layer, where O_2 concentration is low and CH_4 concentrations is high [101]. Previous study shows that CH_4 can accumulate in the hypolimnion zone during summer stratification and CH_4 is mixed through the water column during spring and fall seasons (turnover). At that time, methane oxidation rates were recorded to be high throughout the water column due to high concentrations of CH_4 and O_2 [139].

Second, temperature is known to be a strong controlling factor for the biological and chemical process of methane oxidation. In a study, it suggested that CH_4 production and CH_4 oxidation rates were strongly correlated together with temperature. In one study, surface sediments and water samples were collected from Xiangxi Bay of the Three Gorges Reservoir, China. They measured methane formation and methane oxidation rates. The results suggested that CH_4 efflux decreased when temperature increased due to increasing oxidation rates. CH_4 oxidation in surface sediments samples were 51.8% of the total production and reached to more than 75% at 35℃. Methane oxidation rates in water samples (30 m deep at 35℃) were between 1.26 to 4.65 mg/(m^2h), of which the average and greatest rate accounted for 46.7% and 73.9% of CH_4 production. [140].

A few early studies suggest that the metabolism by model methanotroph species can slow down in cold water or in winter [20,21]. For microbial processes mediated by multiple species with different adaptations, this pattern does not necessarily hold, as certain populations and their enzymes may feature contrasting temperature optima [141]. Some methanotrophic species such as *Methylobacter psychrophilus, Methylosphaera hansonii, Methylocella palustris, Methylocella*

silvestris, Methylocella tundrae, Methylocapsa acidiphila, and *Methylomonas scandinavica* are able to oxidize methane at temperatures and are psychrophilic or psychrotolerant [126,142-144].

Third, nitrogen input also affects methanotrophs. The influence of nitrogen on methanotrophic bacteria and methane oxidation process were examined in freshwater lakes with regards to anthropogenic-quickened eutrophication [138]. Addition of ammonium (NH_4^+) inhibits the methane oxidation process and incorporation of methane into cellular compounds [145]. The degree of inhibition depends on the concentration, and metabolic features of specific methanotrophs species that are present in an environment. The inhibition of methane oxidation can be either short-term (NH_4^+ blocks access of CH_4 to the active site of MMO) or long term (toxic NH_4^+ oxidation products, particularly nitrite and hydroxylamine) [146,147]. For instance, Murase *et al.* (2005) found that CH_4 oxidation can be inhibited when NH_4^+ concentration is 200 µM while Bosse *et al.* (1993) observed that CH_4 oxidation can be inhibited when NH_4^+ concentration is above 4 mM [138,148]. In contrast, Liikanen *et al.* (2003) did not found any significant change in methane emission from sediment of a eutrophic lake, even at 15 mM of NH_4^+ [149]. Also, methanotrophs and ammonia oxidizers share many similarities. For example, methanotrophs are capable of nitrification, and ammonia oxidizers are capable of CH_4 oxidation since their MMO enzymes both have a common evolutionary history [146]. The addition of ammonium can also enhance methane oxidation rates when NH_4^+ (is utilized as nitrogen source by methanotrophs) is limiting [150].

Fourth, top-down regulation of methanotrophy can occur depending on local and seasonal conditions in freshwater lakes. Methane oxidation in water represents a substantial carbon transformation pathway, and evidence shows that methane may be a potential source of carbon for pelagic food webs (planktonic and /or benthic biomasses). In one study, researchers

investigated the primary production (PP), heterotrophic bacterial production (HBP), methanotrophic bacterial growth efficiency (MBGE), methanotrophs bacterial production (MBP), and the relative contribution of methanotrophic bacteria to total bacterial biomass in three various lakes during two seasons (winter and summer) [151]. In addition, they also measured stable isotope carbon ratio in particulate organic matter (POM), zooplankton, surface sediments, and methane. MBP was 3-120% of HBP during winter while the MBP was 0.5-17% of HBP, and 0.3-7% of the organic carbon production (OCP) by primary producers during the summer. MBP dominated the HBP at greater depths. Methanotrophic bacteria biomass was between 3 to 11% of the overall bacterial biomass on depth-integrated basis. POM were generally less depleted in ^{13}C than zooplankton. If phytoplankton $\delta^{13}C$ signatures were between -30 to -35%, like the POM signals, observed zooplankton signatures could be explained by a fraction of 5-15% methanotrophic bacteria in their diets. The results suggest that methanotrophic bacteria can be a food source for zooplankton, and that methane oxidation by methanotrophs represents an important benthic -pelagic carbon and energy link in many lakes during winter [151]. Grazing pressure may impact methane oxidation rates and the methanotrophic community in a lake. In one study, it was hypothesized that methanotrophic bacteria activity may be inhibited by the presence of *Daphnia longispina.* They measured methanotrophic bacteria population densities in water, and they found that methanotrophic activity and the proportion of methanotrophs reduced significantly at higher *Daphnia* densities. This experiment was performed ex-situ (not in situ), but it did suggest an impact of grazers on methanotrophic activity and methane effluxes of lakes [152].

2.2.3 Methanotroph Taxonomy

Methanotrophs are specialized to utilize CH_4. Most known MOB belong to the phylum *Proteobacteria,* and they are classified into three main major groups based on carbon assimilation, phylogeny, and bacterial cell structure. Methanotrophs assimilate carbon through three different cycles: ribulose monophosphate (RuMP), serine, and Calvin-Benson-Bassham (CBB). Most known methanotrophs that belong to the *Gammaproteobacteria* (type I) (*Methylobacter, Methylomonas, Methylosoma, Methylomicrobium, Methylothermus, Methylosarcina, Methylosphera, Methyloglobulus, Methylomarinum, Methylosphera, Methylovulum, Methylogaea, Methylomagnum, and Methyloparacoccus*) [153-159] and (type X) (*Methylococcus* and *Methylocaldum*) assimilate one-carbon compounds through the ribulose monophosphate (RuMP) pathway whereas the methanotrophs that belong to *Alphaproteobacteria* (type II) (*Methylosinus, Methylocystis, Methylocella,* and *Methylocapsa,*and *Methyloferula*) assimilate C_1 through the serine pathway [126,127,160,161].The phylum *Verrucomicrobia* (acidophilic methanotrophs) share some genetic similarity to the proteobacterial methanotrophs, and they assimilate carbon through CBB pathway [129]. Methane can be also oxidized anaerobically, and the anaerobic-oxidation of methane is known as AOM. The NC10 is a candidate phylum connected -with a novel bioprocess - (AOM) coupled to nitrate reduction or denitrification [162 163]. In another study related to the candidate phylum NC10 bacteria, it shows that the AOM coupled to NO reduction [163-166]. Remarkably, the NC10 phylum candidatus *Methylomirabilis oxyfera* utilizes O_2 that is produced from nitric oxide dismutation [167].

There are many critical review papers covering the oxidation of methane via methanotrophs [122,126,168-171]. In this section will briefly describe the three main carbon fixation

pathways that are used by methanotrophs. The gammaproteobacteria use the RuMP pathway. The RuMP is more energy efficient than the serine cycle. In the RuMP pathway, formaldehyde is condensed with monophosphate to create a hexulose phosphate, which is then converted to fructose-6-phospahte through a key enzyme hexulosephosphate synthase. Two routes are possible to convert the fructose-6-phosphate to pyruvate through either the Embden-Meyerhof-Parnas (EMP) or the Entner-Douderoff (EDD) pathways [172]. The alphaprotobacteria use the Serine pathway. In this pathway, CH_4 is catabolized to formate by the tetrahydromethanopterin pathway, which is converted later to methylene tetrahydrofolate (H_4F). Methylene H_4F condenses with glycine to generate serine. The rest of the cycle is involved in reproducing of the glycine acceptor [173]. The verrucomicrobial methanotrophs and type X use the CBB pathway for energy and carbon fixation [174].

The alpha subunit of pMMO, encoded by *pmoA* gene, is conserved [169] , and it has been often used as functional marker in molecular ecology studies of aerobic methanotrophs [175]. As the *pmoA* gene phylogeny is highly conserved and largely comparable to the 16S rRNA gene phylogeny from which the gene sequences were retrieved [176,177]. This gene is also present in most known methanotrophs with the exception of *Methyloferula* and *Methylocella* [153,154]. This a group-specific biomarker has resulted in thousands of sequences representing "unknown aerobic methanotrophs." This limits data interruption due to limited available information about uncultured methanotrophs. The *pmoA*-specific primers set is A189F/mb661R widely used to detect aerobic methanotrophs and useful tool for obtaining functional and taxonomic inventories of aerobic methanotroph in environment [178,179].

2.2.4 Particulate Methane Monooxygenase (pMMO)

The interest in pMMO and its biological methane oxidation and Cu dependent regulation in methanotrophs has been increasing over the last decade [169]. Although pMMO is much more widespread then sMMO in nature, the biochemistry of pMMO is more difficult to characterize [180]. The pMMO is composed of three main subunits, α, β, γ, and they are encoded by the genes *pmoA, pmoB,* and *pmoC* [168,181]. pMMO is characterized into three possible models of the metal center(s). Five trinuclear copper centers have been suggested by Chan *et al*. on the basis of the hyperfine splitting pattern of an electron paramagnetic resonance (EPR) signal at g = .2.06, and have been hypothesized to fall into catalytic (C) and electron transfer (E) functional classes 17 [182]. Other researchers who are working on the same subject observed a type two mononuclear Cu(II) EPR signal only [132,183,184]. DiSpirito and co-workers have proposed two copper and two iron ions with 6-8 additional copper ions bound to methanobactin, a siderophore-like molecule [185], based on pMMO metal content and the isolation of methanobactin [132]. The pMMO from *Methylococcus capsulatus* (Bath) has been crystallized and reported with a resolution structure of 2.8-Å [186,187]. The pMMO is a metalloenzyme produced by most known methanotrophs and this included *M. capsulatus*. The three subunits *pmoA, pmoB, and pmoC* are arranged in a trimeric α_3, β_3, γ_3 complex [169]. The pMMO in *Methylosinus trichosprium* OB3b contains two distinct forms of copper (di-copper and mono-copper) centers beside metal. These are located in the soluble regions of the PmoB subunit. A dicopper center is observed in pMMO from *M. trichosprium* OB3b [188].

2.2.5 Soluble Methane Monooxygenase (sMMO)

The sMMO has been a favored target for industrial applications, such as bioremediation applications [189]. The enzyme has a wide substrate specificity, oxidizing alkanes, alkenes, and aromatics [190]. sMMO has been purified from two types of methanotrophs (type II and type X), including *Methylococcus capsulatus* and *Methylosinus trichosorium*. The sMMO has been characterized into three main components based on the variety of kinetic, spectroscopic, and structural techniques: a) hydroxylase (MMOH) contains a non-heme di-iron site and is an oligomer of three different subunits (α, β, γ) to stimulate di-oxygen activation and methane hydroxylation. b) reductase (MMOR) contains flavin adenine dinucleotide and Ferredoxins (Fe_2S_2) cofactors. c) a regulator protein (MMOB) in which affects the kinetics of the dioxygen-activation reaction [191]. The cofactor of the sMMO contains two irons- a 'di-iron cluster' that reacts with oxygen (O_2) and oxidizes methane (CH_4) to methanol (CH_3OH) [192].

2.2.6 Methanotrophs in Stratified Lakes

Natural lakes are important contributors to the global methane budget. Methane from lakes contributes 16% of the total methane emissions into the earth's atmosphere [193]. Aerobic methanotrophs are present in lakes to regulate methane emissions. The process of methane oxidation via aerobic methanotrophs is linked with the availability of molecular oxygen (electron acceptor) [194]. Methanotrophs are widely present in water and sediments to mitigate methane emissions [179,195-197]. In early study, it was reported that methanotrophs are more diverse in lakes compared to other aquatic environments, such as marine environments [179]. At present time, most of the studies of aerobic methanotrophs have focused on lake sediments as this functional guild is highly abundant in such systems. The *pmoA* copies genes range was between 10^4 to 10^6 copies

of *pmoA* per gram fresh sediments [198]. Information about methanotroph abundance through the quantitative polymerase chain reaction (qPCR) technique in lake water column is still rare; however, there are different methods have been already used to demonstrate the presence of methanotrophs in previous studies: enrichment cultures [196,199], fluorescence in situ hybridization (FISH) [200], proteomics [201], metagenomic sequencing [202,203].

In one study, qPCR and terminal restriction fragment length polymorphism (T-RFLP) were performed to determine methanotroph diversity, abundance, community composition, and spatial and temporal distribution of freshwater at five temperate lakes: Ekoln, Erken, Långsjön, Siggeforasjön, and Ramsen. Lake waters were collected from surface, middle, and bottom during summer and winter. These five lakes are in the eastern part of south-central Sweden. Methanotroph population was between 10^5 to 10^6 cells per ml throughout surface and bottom water, comprising less than 1.3% of the total microbial community. Methanotroph abundance was significantly lower in summer than winter and consistently decreased with depth in lakes. T-RFLP results of the target gene *pmoA* showed significant differences in methanotroph community during summer and winter as well as between lakes, but there were no differences between water layer [204].

In another study, DNA -based stable isotope probing (SIP), qPCR, pyrosequencing and enrichment cultures were used to determine and characterize most active methanotrophs and methanotrophs communities in water columns (oxic) and sediments (anoxic) from two lakes. Samples (waters and sediments) were collected from an arctic tundra lake, north Alaska (Lake Killarney) and a subarctic lake, Alaska (Lake Qalluuraq). They found that methanotrophs of type II, *Methylocystis*, were more dominant in an enrichment culture from the water columns. The methanotrophs of type I, *Methylobacter, Methylosoma,* and *Methylomonas* were most active in

utilizing methane at the sediment water interface (0-1 cm), while the type I *Methylobacter* and/or type II *Methylocystis* contributed substantially to methane oxidation in the deeper (15-20 cm) sediments [196].

Another study investigated if methane oxidation is coupled to photosynthesis in anoxic waters or not. Water samples were from Lago di Cadagno, Switzerland. In this study, Milucka *et al*. (2015) used several techniques to investigate methane oxidation in water samples. The addition of oxygen enhanced the oxidation of methane in anoxic water samples. Interestingly, anaerobic methanotrophs (methanotrophs archaea) were not detected. Instead, they found that the abundance of aerobic alpha - gamma methanotrophs comprised over 1% of the total cell counts. The oxidation of methane by methanotrophs in water samples was due to O_2 generation by photosynthetic algae [205].

In another study, methanotroph richness and abundance were investigated along with their oxygen demand to oxidize methane at BML, Alberta, Canada BML. Water samples were collected from March 2015 to June 2015 from three fixed platforms. In this study, the 5d-BOD and 5d- Methanotrophs Oxygen Demand (MOD) were performed to monitor the standard BOD and to determine methanotrophic oxygen demand to oxidize methane in water samples. The potential methane oxidation rate was estimated through gas chromatography (GC) to determine methane decline in the headspace, and 16S rRNA gene amplification via PCR followed by Illumina next-generation sequencing (NGS) was used to identify microbial communities with emphasis on type I and type II methanotrophs during four seasons of one year [79]. In this study, the BOD and MOD suggested that methane oxidation by methanotrophs was active during spring at all depths (0 m - 8 m). The minimum BOD value was 0.5 mg L^{-1} and the maximum BOD value was 4 mg L^{-1}. In contrast, the highest MOD value was 5.9 mg L^{-1} and the lowest MOD

value was 3.5 mg L^{-1} during mixing time or turnover. However, during summer stratification, the minimum BOD value was greater than 2 mg L^{-1} at the metalimnion zone, and it was lower on surface and bottom (1.5 mg L^{-1}). In contrast, the MOD value was greater (3 mg L^{-1}) at the metalimnion zone, where methanotrophs cluster to oxidize methane and lower on both surface and bottom (epilimnion and hypolimnion) zones (2 mg L^{-1}). The potential methane oxidation was measured during spring and summer 2014. The potential methane oxidation rates in May and August were high (60.3±2.4 nmol $ml^{-1}d^{-1}$ and 52.9±5.0 nmol $ml^{-1}d^{-1}$), but in June and July were low (25.5±0.2 nmol $ml^{-1}d^{-1}$ and 23.5±10.2 nmol $ml^{-1}d^{-1}$). Type I methanotrophs are more dominant during March and May (less than 5% of total reads) but in June there was a significant decline in type II methanotrophs (less than 0.2% of all reads). In contrast, the type I methanotrophs were less than 0.1% in March, but that started to increase slightly to 0.5% in May and June. Also, methanotroph composition based on relative abundance within 16S rRNA gene reads results showed that the type I methanotrophs (*Methylobacter Crenothrix, Methylocaldum,* and *Methylobacter*) were more dominant and abundant during March and May compared to the type II (*Methylocystis* and *Methylosinus*). However, the total community of methanotroph type I declined in June, while *Methylocystis* and *Methylosinus* abundances increased.

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1.8 Conclusions

In summary, extraction of bitumen through open-pit mining remain a major challenge to Alberta's oil industry because it requires large quantities of freshwater, and it results environmental issues including air and ground contaminations. However, it also provides great

opportunities for researchers and scientists who are working in this field to investigate the role of microbes present in this massive engineered pond and complex ecosystems. The program focuses on monitoring the dissolved oxygen concentration in the BML because the O_2 is essential for a healthy aquatic ecosystem and aerobic respiration, Syncrude began to monitor the dissolved oxygen levels at the three platforms through the water column. and found that dissolved oxygen concentration is increasing over time while the turbidity is decreasing through the whole water column. Methanotrophs have been studied at various tailings environments including MLSB, WIP, and BML, and may be greatly contributing to limit Water capped tailings technology (WCTT) keeps the sediment -fluid fine tailings interface below an aerobic water cap and the EPL water quality should improve over time. Furthermore, this technology should support aerobic methanotrophs and enhance methane oxidation through the entire water column during four seasons with variation in methane oxidation rates during summer stratification and turnover. It is therefore valuable to understand methane oxidizing bacteria which are present in the oxic water cap.

CHAPTER THREE: BIOCHEMICAL CYCLING IN BASE MINE LAKE

*BOD and MOD are Combined data from Evan Haupt (March-July 2015) and Emad Albakistani (August 2015-October 2017).

3.1 Introduction

One of several goals of the BML project is to develop a better understanding of O_2 and CH_4 cycles in the first commercial-scale oilsands end-pit lake (EPL) in the AOSR. We hypothesized that aerobic methanotrophs are present through the capped water layer (10 m deep), and they are actively oxidizing methane in the lake when O_2 is supplied from the air, contributing to the BML's biological oxygen demand (BOD). We also hypothesized that the process of methane oxidation by aerobic methanotrophic bacteria is influenced by seasonal changes in temperature gradients and dissolved O_2 concentrations over the year. Aerobic methanotrophs may thrive underneath the ice surface during winter, and at the metalimnion zone during summer stratification. Methane oxidation through the water column (10 m) was detected during spring and fall seasons. Since the Base Mine Lake (BML) program started in late 2012, the Water Capped Tailings Technology (WCTT) has greatly increased the concentration of O_2 and decreased the biological oxygen demand (BOD_5) through the water column of the lake. Seasonal variations were observed at three platforms in both DO and BOD_5 (Syncrude Canada unpublished annual report, 2016). The average of DO concentrations are increased while the average of BOD_5 at three platforms seems to be decreasing over time since the monitoring program has begun in 2013 (Syncrude Canada unpublished annual report, 2016). Also, the annual and seasonal DO and BOD_5 profiles from 2013 to 2016 showed increase (DO) and decrease (BOD_5). The minimum and maximum DO concentrations during fall 2013 to 2015 were

reported to be 1.6 and 7.73 mg/L^{-1} while the minimum and maximum DO concentration during fall 2016 were reported to be 5.1 and 9.85 mg/L^{-1}. In contrast, the minimum and maximum BOD_5 during fall 2013 to 2015 were reported to be <2 and 5.9 mg/L^{-1} while the minimum and maximum BOD_5 during fall 2016 were reported to be 2.9 and 3.7 mg/L^{-1} (Syncrude, unpublished data 2016). However, it is important to note that the DO concentration in the lake will vary and depends on water temperature through the whole column.

3.2 Methods

3.2.1 Sampling Tailings Water and Tailings Sediments

Samples were obtained from the top 10 m BML water at three permanent platforms at BML. The first platform is located in the center: platform 1_(C), the second platform is located in the Northwest: platform 2_ (NW), and the third platform is located in the Southwest platform 3_ (SW) **[Figure 1. 1]**. Sample were collected from three platforms at depths of 0 m, 1 m, 2 m, 3m, 4 m, 5 m, 6 m, 7 m, 8 m, 9 m, and 10 m (eleven depths, including surface) during BML thermal stratification, and at 0 m, 2 m, 4 m, 6 m, 8 m, and 10 m when thermally mixed. Samples were collected with a Kemmerer water sampler and transferred directly into Thermo Scientific 2-L Nalgene bottles and shipped to the University of Calgary in plastic coolers with ice packs.

Three water quality monitoring stations installed on floating platforms record key parameters in the water profile: pH, temperature (°C), DO (mg/L), specific conductivity (µS/m). Field measurements of pH, temperature, DO, and specific conductivity were recorded before collecting samples using YSI 556 Multi-Meter, and they were sent to us monthly by Syncrude.

On May 01, 2016, an unprecedented wildfire began in a forested area at the Horse River, Wood Buffalo, AB, [206]. It was estimated that the wildfire at the first day was two-hectares. After

two days, the wildfire continued to burn and swept toward the northern Alberta city of Fort McMurray and went on to threaten nearby communities, oil and gas facilities, and other important sites, forcing the largest emergency evacuation and worst wildfire in Alberta's history. On May 05, 2016, the fire spread quickly across the Athabasca River, and burning continuously through the city with growth to ~ 157,000 ha. In June 13, 2016, it was estimated the total size of wildfire was approximately 590,000 ha before it was under control. The wildfire destroyed at least 2400 homes and buildings forcing 88,000 people to leave their residents, resulting in $ 3.6 billion of insured losses [207]. BML water samples during that time were not taken due to the wildfire that resulted in the complete suspension of the BML program from May 4 to June 26, 2016.

BML water turbidity has been decreasing at all platforms since the monitoring program started in 2013. The water turbidity during summer 2013-2015 was 4.7 to 630 Nephelometric Turbidity Unit (NTU) while during summer 2016 it was 19 to 130 NTU. The BML water turbidity during fall 2013-2015 was 16 to 290 NTU. However, the water turbidity in BML was decreased, and this was noticed during fall 2016. The BML water turbidity was 6.6 to 23 NTU. This decline was notable after Syncrude decided to apply alum to reduce water turbidity in BML. A dosage of 25 mg liquid alum per liter of BML was applied with a total of 1520 ton of alum to the entire lake. Also, to ensure that the alum treatment had the maximum contact with solids suspended in lake water, alum was added during fall turnover to take the advantage of mixing in water column. The addition of alum started on September 1, 2016, and was on September 28, 2016 (Syncrude unpublished report, 2016).

3.2.3 Biological Oxygen Demand

3.2.3.1 Tailings Water Samples Preparation

In 2016, BML water samples were collected monthly at ten depths (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 m) in early summer and six depths (0, 2, 4, 6, 8, and 10 m) during fall turnover from the three platforms. 5d-BOD tests were performed on consecutive days for each platform (three days for the three platforms). 400 mL of each sample were shaken in a 1-L Pyrex media bottle (volume 1.2 L) at 120 rpm for 15 min at (23°C ±2) to saturate it with O_2. Each sample was then poured into a 50 mL Falcon tube to monitor the DO in mg/L using a dissolved oxygen probe (see below). After stabilization of the DO probe, a 100-ml Pyrex bottle (volume 135 ml) was filled to the brim with BML sample water, then capped with a butyl rubber stopper pierced by two syringe needles and finally capped securely with a screw ring, squeezing out some excess sample and removing any bubbles **[Figure 3. 1]**.

In addition, we performed a second BOD_5 incubation with methane added to the water, referred to as "BOD (+)". It assumed that methane in BML water was lost during sampling and shipping, and this assay would measure potential O_2 demand by methanotrophs. Each 1-L Pyrex media bottle (with ~200 ml of BML water sample remaining at the 1-L Pyrex media bottle from the previous step) was recapped with a rubber stopper and screw cap. Using a BD 60 ml syringe attached to a VWR sterile syringe filter 0.2 µm cellulose acetate, 100 ml of headspace was removed from each 1-L bottle, and it replaced with 100 ml of filtered CH_4 (100 ml gas represents 10% v/v of the 1 L of headspace). Each 1-L Pyrex media bottle containing at least 200 ml of BML water was shaken for 30 seconds by hand then set aside for 20 minutes to equilibrate the methane in the gas and liquid phases. After equilibration, 10 ml water samples were taken through 10 ml syringe and injected into a 30 ml chemglass tube for GC measurement (BML

samples in 2016). The DO was measured in a VWR 50-ml Falcon tube and 100-ml Pyrex bottles were filled as before. The standards and protocols for performing this experiment was followed as designed by Evan Haupt (2016).

After five days of stationary incubation at 20°C (4°C±2 in case of February and March 2017), incubation bottles were uncapped and DO concentration was measured again using the RDO probe directly in the 100-ml Pyrex bottles. In order to avoid exposing water sample to O_2, which may influence on the DO reads and/or the 5d-BOD results, the DO reads were performed within 2 minutes (maximum) for each sample. However, two dates followed a slightly different protocol. A 5d-BOD and 5d-BOD (NH_4Cl) were monitored with the BML samples collected on August 2016, while a 5d-BOD, 5d-BOD (CH_4), 5d-BOD (NH_4Cl), and 5d-BOD (nitrapyrin) were monitored with the BML samples collected on February 2017. The assays were performed with these substances to examine the oxygen demand uptake in BML water potentially due to the process of nitrification (a biological process by microorganisms in which ammonia (NH_3) or ammonium (NH_4^+) is oxidized to nitrite (NO_2^-) and further followed by the oxidation of the nitrite to nitrate (NO_3^-). The NH_4Cl solution was prepared as follows: 9.5 mg of NH_4Cl was dissolved in about 250 ml autoclaved distilled water (DW). Then 1000 µl of dissolved stock solution was added through micropipette into each 1-L Pyrex media bottle which contains about 200 ml of water sample. All bottles were incubated at room temperature for one hour before measuring the initial DO. The nitrapyrin solution was dissolved as follows: 0.52 mg of nitrapyrin was heated in 20 ml of autoclaved DW at 65° C. After the nitrapyrin was melted well, 100 µl of stock solution was pipetted into a 100-ml Pyrex bottle. Then, the 100-ml Pyrex bottle filled with water sample and recapped as described previously. In this experiment, nitrapyrin was applied to

reduce the activity of nitrifying bacteria and block NH_4^+ to NH_2OH step of ammonia oxidation [208].

BOD and BOD (+) values were calculated by subtracting the final DO concentration measured on day 5 in (mg/L^{-1}) from the initial DO concentration measured on day 0. This yields 5d-BOD or 5d-BOD (+), which have units of mg $L^{-1}5d^{-1}$ of O_2. Subtracting the 5d-BOD from the 5d-BOD (+) gives a value for MOD, or the "methanotrophic oxygen demand.". In order to oxidize 1 mol of CH_4 methanotrophic bacteria require 2 mol of O_2, so methanotrophic oxygen demand can be converted to a methane oxidation rate. This is accomplished by calculating moles of O_2 utilized by methanotrophic bacteria to oxidize methane over the 5 days (mmol L^{-1} d^{-1}), then dividing by 2 to get moles CH_4 consumed (mmol CH_4 L^{-1} d^{-1}). This value is divided by incubation time which is maximum 5 days (mmol CH_4 L^{-1} d^{-1}). Then the value from (mmol CH_4 $L^{-1}d^{-1}$) is converted to (nmol mL^{-1} d^{-1}) to obtain moles CH_4 consumed per ml per day. Methane oxidation rates were also measured directly via GC as described in the next section.

Two sensors were employed to detect the initial and final DO. The "Thermo Scientific Orion Star A323 Dissolved Oxygen/Portable Meter" was employed from August 2015 to February 2017, while the "HACH HQ440 Portable LDO Meter" was employed from June to October 2017. The calibrations for both probes were in water-saturated air (www.thermofisher.com) (www.hach.com) according to the manufacturer's instructions at constant temperature and wiped with Kimberly-Clark Kimtech Science Kimwipes between subsequent reads. DO concentration was measured in unit of mg L^{-1} with a resolution of 0.01 mg L^{-1} at room temperature. However, winter samples (February-March 2017) were the only samples that are incubated for five days at 5°C (±1) and to reduce errors with the DO probe and increase accurate BOD results, sample temperature was adjusted at room temperature (re-

incubated for three hours) before monitoring the final DO concentration through the DO probe. In addition to the initial calibration, the DO probe was also tested with tap water to obtain reproducible results and reads.

Figure 3. 1 (A-B) A) BOD bottles are prepared and ready for five-day incubation at room temperature in the dark. B) 30-ml chemglass tubes contained 10 ml of water samples taken from the BOD bottles (final day). These chemglass tubes were prepared for measuring the potential methane oxidation though gas chromatography (GC).

3.2.2 Methane Oxidation Potential (ex-situ)

To determine the methane oxidation potential of BML water samples directly, 10 ml samples of water from the BOD_5 experiment were taken at day 0 and day 5 and the methane concentrations measured by gas chromatography (GC). Samples of 10 ml of the BML water used to set up the BOD (+) experiments (duplicated) were taken through a BD 10-ml syringe and injected into 30-ml chemglass bottles that were already fitted with butyl rubber stoppers and autoclaved before this injection. While injecting 10 ml of water sample into the 30 ml chemglass bottles, a second needle was attached to avoid over pressure that can be caused during the injection. This step can cause a loss of methane in each tube and therefore the second needle was removed immediately after each injection. The same step was repeated for measuring the final

methane concentrations (day 5), with 10 ml of water (duplicated) taken from BOD bottles incubated for five days **[Figure 3. 1]**. Duplicated samples of 30 ml chemglass tubes containing BCR water was used as a positive control and distilled water (DW) was used as a negative control for this experiment.

CH_4 in the gas phase of the 30-ml chemglass tubes was determined via gas chromatography (GC) equipped with a flame ionization detector (FID) in the laboratory to track the decline of methane for 5 days incubation. For the GC measurement, at least 10 ml of air was injected in the GC sampling loop (0.1 ml sample volume) between sample injection to remove the residual gases. A BD Microlance 3 Hypodermic Needle 22G × 1 ½ (0.7mm × 40mm) was attached to a BD 3-ml syringe to take gas sample from the headspace and inject into a gas chromatograph (GC) (Model 8610C, SRI Instruments) equipped with a flame ionization detector (FID) (Column T 190 °C, detector T 300 °C, and N_2 as carrier gas). The potential methane oxidation rates were calculated as follows: The differences between the initial and final CH_4 mixing ratio (v/v) in the headspace was calculated to determine the total volumes of CH_4. Methane in each sample was detected from the GC peak (area) with reference to a 0.5% v/v methane standard. The GC peak areas for methane were converted to mol fractions by multiplying by 0.005 then dividing by the area of CH_4 standard peaks. The differences between two days were determined by subtracting the reads at day 0 (mmol fraction/L/5d) on day 5 (mmol fraction/L/5d). Then, all obtained reads were divided by five days to determine methane oxidation rate in (mmol ml^{-1} d^{-1}), then multiplied by 1000 to determine the potential methane oxidation rate in nmol ml^{-1} d^{-1}. Also, the standard error of three platforms at certain depth are considered in this calculation.

3.3 Results and Discussion

3.3.2 Biological Oxygen Demand and Methanotrophic Oxygen Demand

Means of 5d-BOD and 5d-BOD (CH_4) for water samples for each depth (averaged over the three platforms) are presented in **[Fig 3. 2 (A-N)]** and means of two depths only for 2017 are presented in **[Fig 3. 2 (O)]**. Means of 5d-BOD (NH_4CL) and 5d-BOD (nitrapyrin) for water samples for each depth (averaged over the three platforms) are presented in **[Fig 3. 2 (K) (N)]**. The values of both 5d-BOD and 5d-BOD (+) varied from 0.30 to 6.30 mg/L, a difference of 6 mg/L, throughout the period of study.

Our results suggested that there was no depth effect in both 5d-BOD and 5d-BOD (CH_4) of BML through the water column at three platforms. The 5d- BOD (+) were higher near the surface than the rest of the depths during winter 2015. However, the 5d-BOD and 5d-BOD (+) either increased or decreased with depths during the study period. For example, the 5d-BOD and 5d-BOD (+) were higher during winter 2015 near the surface than the bottom while the 5d-BOD and 5d-BOD (+) were much higher near the bottom compared to the surface during 2017. In general, there were no consistent depth patterns. As observed, the 5d- BOD (+) were higher at the metalimnion layer during summer stratification in June 2015, but we did not observe that again at the epilimnion layer during summer stratification in 2016. It is possible that the water samples collected on June 2016 were during early summer when the lake started to form three different layers (the beginning of BML summer stratification).

BML water samples that were collected during late summer (August 2016) were tested with the 5d-BOD standard and NH_4Cl instead of CH_4. The obtained result does not show big differences with other treatments through the column depths. However, the 5d-BOD values were slightly higher near the bottom of the lake than the rest of the depths. In addition to that, 5d-BOD

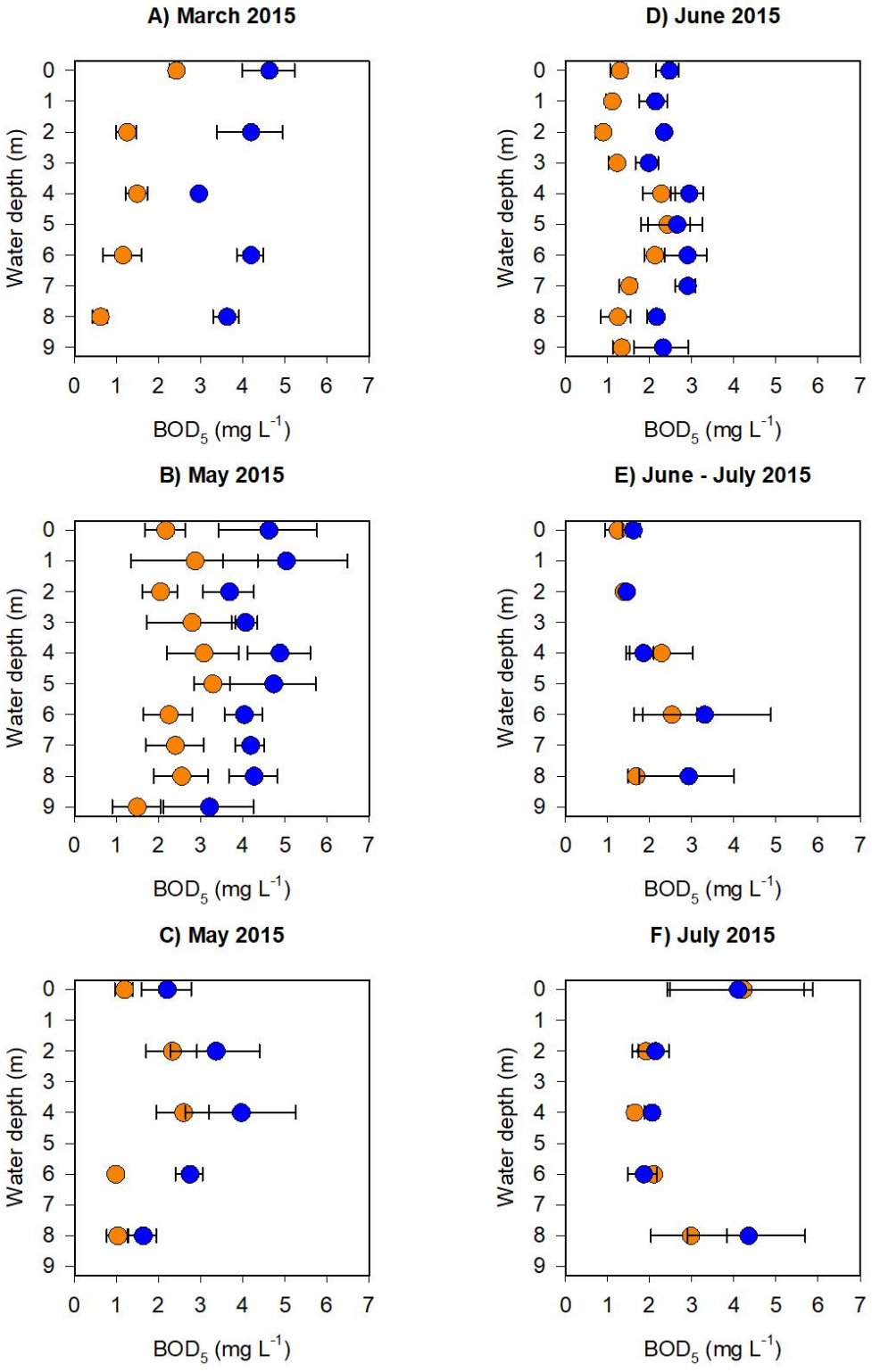
and 5d-BOD (+) patterns were mostly constant during spring and fall seasons (BML turnover) through the whole water column **[Fig 3. 2 (A-O)]**. We also tested the 5d- BOD (+) with different treatments during summer 2016 and winter 2017. We found that the 5d- BOD were higher near the bottom (9 m) compared to the other depths during summer (August 2016) and winter (February 2017). The main point of using NH_4Cl in this experiment is to compare it with the BOD and BOD (+) while the nitrapyrin was added to test the effect of this inhibitor on nitrification process. However, there was little effect of either treatment, suggesting that nitrification rates were very low.

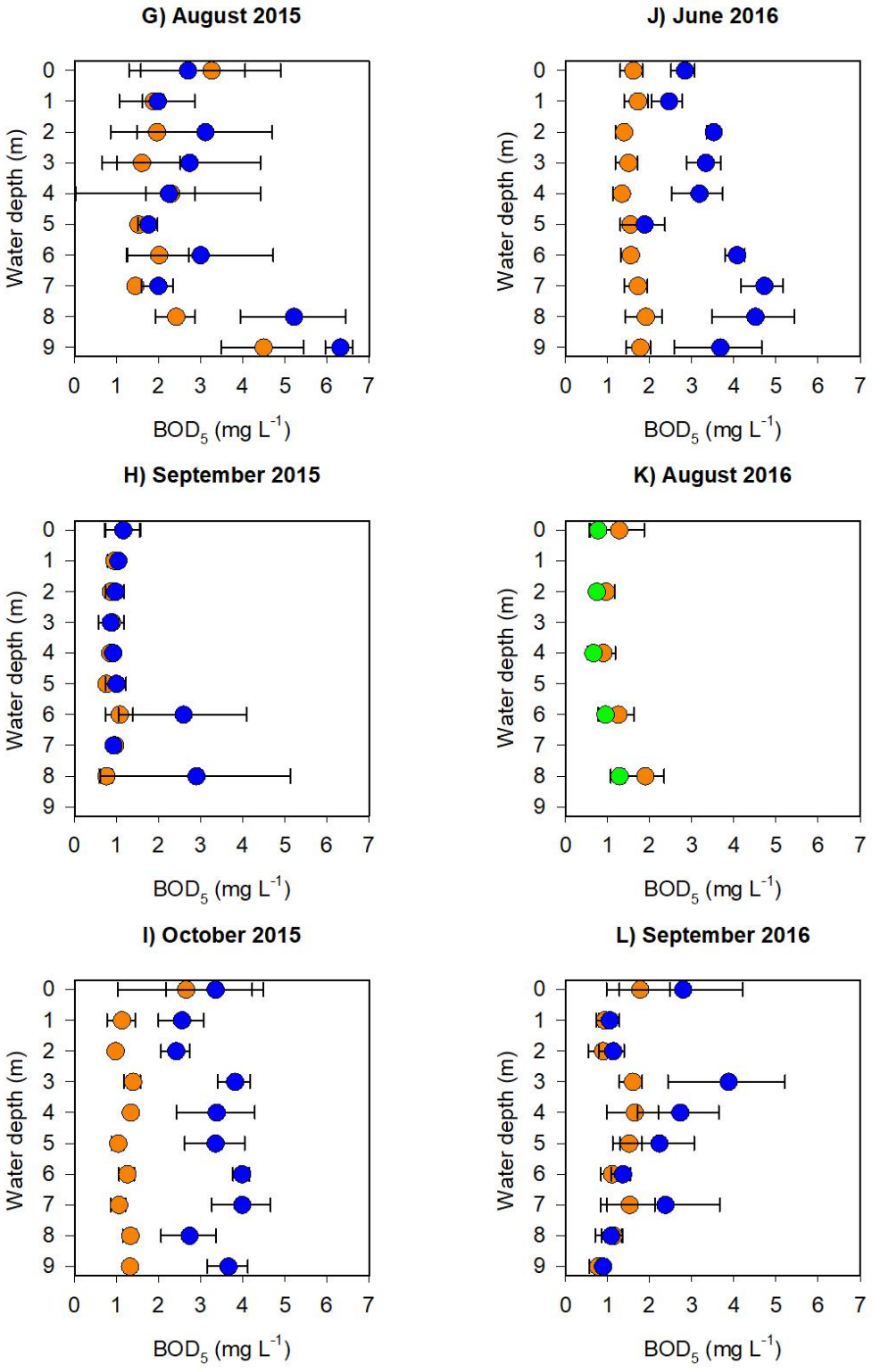
In general, the values of both BOD and MOD also showed considerable variations among different seasons over the three years. As observed, the 5d-BOD and 5d-MOD were much higher in 2015 and 2016, but both declined in 2017. Also, the patterns for both assays were inconsistent over the year, month, and season. In 2015, the highest BOD and MOD values were observed in March and May. Intermediate BOD and MOD values were observed in August and October. The lowest BOD and MOD values were observed in June, July, and September. In 2016, the BML water samples were collected from the three platforms and analyzed during two seasons (winter and spring). Sampling time points were disrupted twice due to budget constraints (winter) and wildfire (summer). However, the monitoring program and water sample collection started again at the mid of the year. The highest BOD and MOD values were observed in June. The lowest BOD and MOD values were observed in August. BOD was monitored in September, but the MOD was not monitored for this month. In 2017, the highest BOD and MOD values were observed in July and August. Intermediate BOD and MOD values were observed in May, June, September, and October. The lowest BOD and MOD values were observed in February and March. The MOD for both months were higher than the BOD for these months **[Figure 3. 3]**

although this probably just shows random measurement error. BOD and MOD were low during May and June, and there were not big differences between the surface and bottom samples **[Figure 3. 2]**. It is important to note that the BOD and MOD values (March 2015-Feburary 2017) represent the average of three platforms from five depths while (March - October 2017) are represent the average of three platforms from surface and bottom.

A decrease in BOD and MOD were observed with samples collected in 2017 compared to 2015 and 2016. In addition, the water turbidity shows a decrease while the dissolved oxygen concentration shows an increase through the water column (Syncrude unpublished annual report 2016). These effects could be due to the alum treatment when it applied to the entire lake on September 2016. Alum reacts with bicarbonate, solids, and fines and forms a gelatinous precipitate [209-211]. This floc reacts with other particles and suspended material across the whole water column, and then finally settles to the bottom of the water column near to the water-FFT interface. It is possible that after alum treatment, methanotrophic bacterial cells settled down at the bottom of the lake as well as other compounds. Or it might be most of organic matter present in the water column settled down, which can cause less BOD through the whole water column but high BOD at the bottom **[Figure 3. 3]**.

Methane oxidation rates estimated from the MOD measurements over three years (2015-2017) ranged between 7.89 and 1.26 nmol mL^{-1} d^{-1} in 2015 **[Fig 3. 4]**. In general, there were no consistent patterns with the BOD and BOD (+) over the study period.


A) March 2015
D) June 2015
B) May 2015
E) June - July 2015
C) May 2015
F) July 2015
Water depth (m)
BOD5 (mg L-1)
0 1 2 3 4 5 6 7 8 9
0 1 2 3 4 5 6 7


G) August 2015
J) June 2016
H) September 2015
K) August 2016
I) October 2015
L) September 2016
Water depth (m)
BOD5 (mg L-1)
0
1
2
3
4
5
6
7
8
9

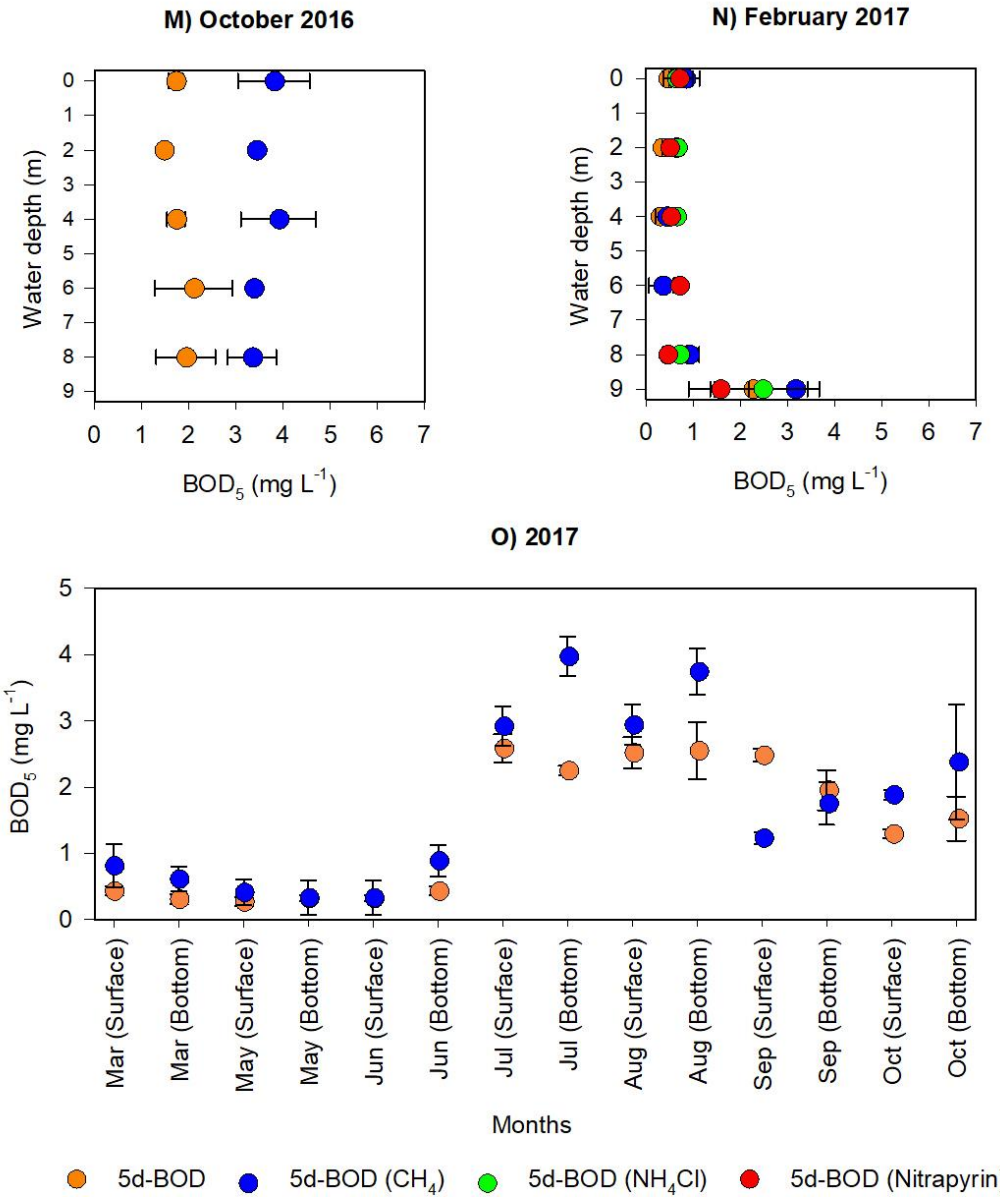


Figure 3. 2 (A-O) Five-day Biological Oxygen Demand and Biological Oxygen Demand with methane added for BML samples collected A) 5-7 March 2015; B) 5-8 May 2015; C) 20-21 May 2015; D) 2-3 June 2015; 27-28 July 2015; E) 29 June -02 July 2015; F) 27-28 July 2015; G) 10-11 August 2015; H) 08 September 2015; I) 05 October 2015 J) 27-29 June 2016; K) 08-10 August 2016; L) 20 September 2016; M) 17 October 2016; N) 11-13 February 2017; O) 13-14 March, 2017; 29-30 May 2017; 20 June 2017; 4-7 July 2017; 21-26 August 2017; 4-8 September 2017; 2-6 October 2017. Error bars represent standard error across three sampling platforms.

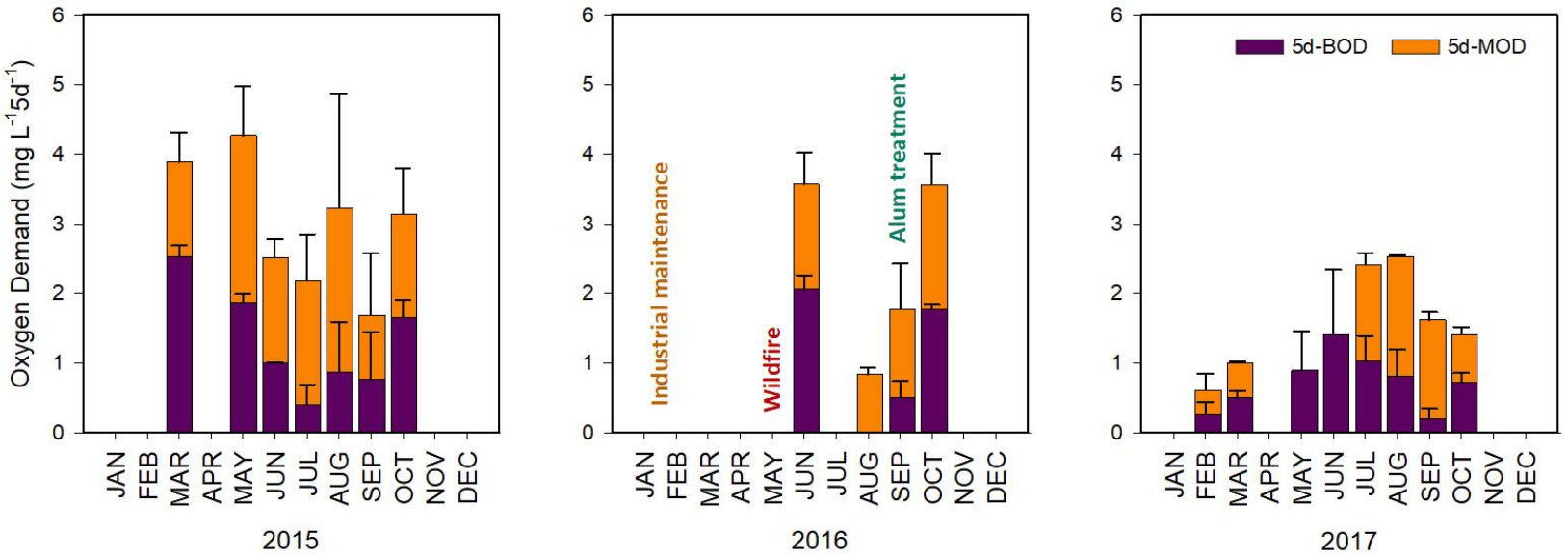


Figure 3. 3 Five-day oxygen demand of Base Mine Lake samples from March 2015 to October 2017. Figure shows both standard BOD and MOD, which is the difference of the BOD (+) and BOD bars. Monthly values are averages of five depths and three platforms (March 2015 - February 2017) while (March 2017 to October 2017) are averages of surface and bottom and three platforms. BML samples of August 2016 were performed without and with (NH_4Cl). BML samples of February 2017 were performed without and with methane (CH_4), (NH_4Cl), and nitrification inhibitor (Nitrapyrin). Combined data from Evan Haupt (March - July 2015) and Emad Albakistani (August 2015-October 2017).

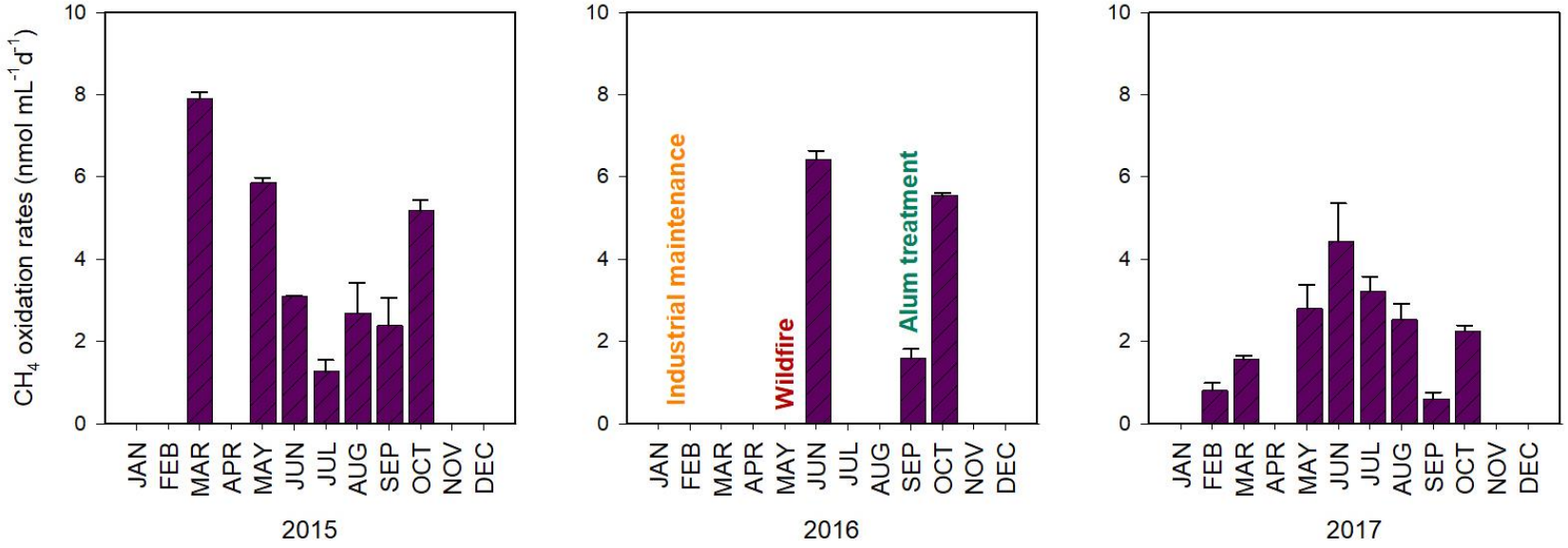


Figure 3. 4 Monthly averages of potential methane oxidation rates from Base Mine Lake water samples. Samples were collected from March 2015 to October 2017. All samples were that received during spring, summer, and fall were incubated at 20 °C while samples that received during winter (March 2015, February 2017, and March 2017) were incubated at 4 °C. Error bars represent standard error of samples from the three sampling platforms and five depths (0, 2, 4, 6, and 8m) while (March 2017 to October 2017) are averages of samples from the three sampling platforms and two depths (surface and bottom). Combined data from Evan Haupt (March - July 2015) and Emad Albakistani (August 2015 - October 2017).

3.3.1 Potential Methane Oxidation

In addition to the BOD and MOD measurements, gas chromatography (GC) was used to measure rates of methane oxidation in BML samples taken from June to October 2016 **[Figure 3. 5]** to verify the results obtained via the MOD assay. The GC measurements were mostly similar with the BOD (+) through the whole water column **[Figure 3. 2 (J, L, M)] [Figure 3. 5 (A-C)]**. This verifies the value of the MOD procedure.

It is important to note that these experiments and measurements were in the laboratory not in the field, and the values represent potential oxidation rates. Methane oxidation rates were generally constant over different depths during two seasons (summer and fall), and season was observed to have a stronger impact on methane oxidation rates rather than depths or platform. For this reason, the potential methane oxidation rates for each platform was calculated as the average rate of 10 or 6 depths.

During BML summer stratification (from June to August) water temperature decreased with depth. The epilimnion warmed while the hypolimnion was cold. Water temperature ranged from 21.4 °C at the surface to 12.5 °C at 10 m, a difference of 8.9 °C **[Figure 1. 3]**. Near the surface, the DO concentrations reached to 7.03 but that started to decrease near the bottom to 0.37 mg L^{-1} **[Figure 1. 4]**. These changes in temperature gradient and DO reflect thermal stratification in BML during summer. Unfortunately, the temperature and DO profile are not available for July 2016. During early fall, lake temperature ranged from 14.5-6.5 °C with all depths, a difference of 8.0 °C **[Figure 1. 3]**. while the DO concentrations ranged from 14.4-6.5. mg L^{-1}, a difference of 7.9 mg L^{-1} **[Figure 1. 4]**. As BML surface water cools in the fall, it becomes denser, causing it to sink. This dense water forces the water of the hypolimnion to rise,

"turning over" the three layers. During BML fall turnover (September to October), water temperature was lower and constant through the whole water column.

We hypothesized that methanotrophs are present through the whole water column, but they may be more active at the metalimnion zone during summer stratification. To test this, we measured the potential methane oxidation rate during the three months in 2016. We found that the potential methane oxidation rates were relatively high in June but decreased in September before rebounding in October **[Figure 3. 5 (D)]**. However, in June the potential methane oxidation rates varied dramatically with depth, exhibiting low rates at 0-4 m and high rates at 5-9 m. In September and October, the lake started to enter fall season (turnover) the potential methane oxidation rates were similar at all depths.

The lake was thermally stratified during summer 2015 and 2016, and the temperature and oxygen concentration profiles clearly showed three different layers [**Figure 1. 3] [Figure 1. 4].** This lack of a clear metalimnion peak for methane oxidation is surprising given previous research, such as the study by Kankaala (2006) on the methanotrophic activity during lake stratification and turnover. In this study they found that methanotrophs oxidized 3-5 times more CH_4 in the water column than was released to the atmosphere, and the highest methanotrophic activity was found in the metalimnion layer where the O_2 concentration was at the detection limit or below (<6 mmol m^{-3}). Unfortunately, we were not able to measure methane in the water profiles.

The potential methane oxidation rate was measured in WIP (the tailings pond on this site before BML was made) from 2010-2011. Estimated rates ranged from 75-152 nmol $mL^{-1}d^{-1}$. Also, Haupt (2016) measured the potential methane oxidation in BML water samples in 2014. He found that the potential methane oxidation rates ranged from 60-22 nmol $mL^{-1}d^{-1}$. The

methane oxidation rates were much lower through the whole water column in BML samples collected in June, September, and October 2016. Overall, methane oxidation rates were much higher in 2015 and 2016, but then dropped in 2017.

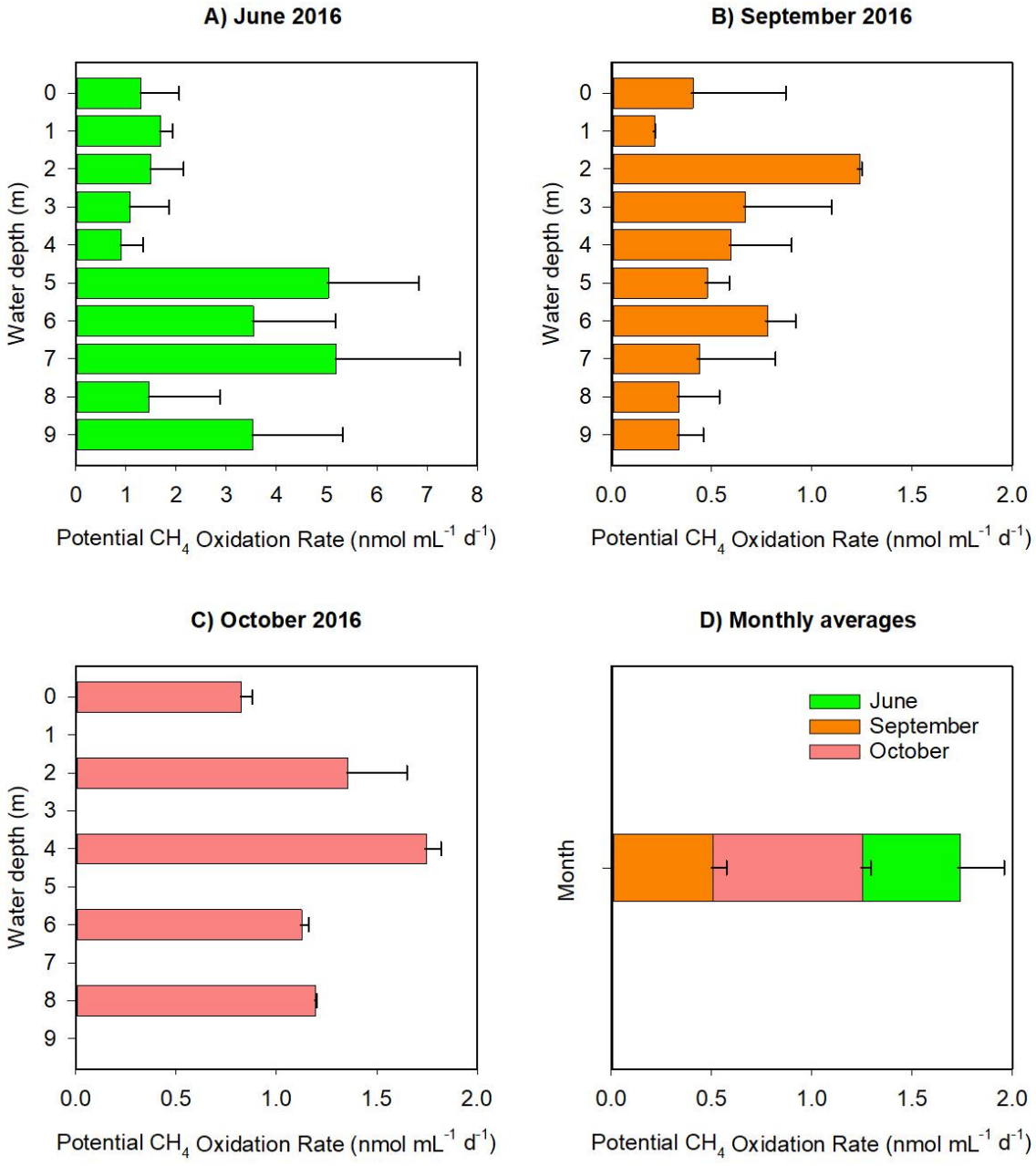


Figure 3. 5 (A-C) Monthly averages of potential methane oxidation rates from water samples collected from 5 and/or 10 depths in BML from June to October 2016 (BML water samples incubated at 20°C). Rates were calculated from liner regression of methane decline in the headspace of chemglass. Error bars represent standard errors of duplicated samples from the three sampling. D) Monthly averages of potential methane oxidation rates during three months of 2016.

3.4 Conclusions

In this study, our goal was to determine the MOD associated with the BOD in the first pilot EPL during seasonal changes and over time. In addition, we determined the potential aerobic methane oxidation through the water column (10 m deep) directly via measuring methane consumption. Our results suggest that (1) the BOD and MOD varied during four seasons, but not in consistent patterns, and (2) they were high in the first two years, but much lower in 2017. This could be due to alum treatment when was applied on September 2016. (3) methanotrophs in summer samples were most active near the sediment-water interface.

It is possible that the declining in BOD in 2017 was caused by alum addition. Most organic compounds present in the water column may have settled to the bottom of the lake after alum addition, as well as microbial cells including methanotrophs. We noticed that the water turbidity was much higher at depth 9 m then the rest of depth 0-8 m during February 2017.

However, the BOD and MOD values might increase again during spring and fall turnover next coming years if settled fines and solids are re-suspended through the whole water column. This might result in an increase of both BOD and MOD. It also could be possible that large proportions of organic compounds have been degraded since the BML was filled with freshwater. Therefore, the BOD and MOD are gradually decreasing, and the lake is becoming similar to a natural lake over time. The historical data profile shows that water turbidity from 2013 to 2016 has been decreasing through the whole depths (Syncrude unpublished annual report, 2016). A study by Huser *et al*. (2011) on the effect of alum (aluminum sulfate) was investigated in four lakes of the Minneapolis Chain of lakes (MN, USA). Researchers found that alum treatment with different doses improved water quality over time in four lakes, and reduced internal P release from deeper sediments by approximately 85% within two years (Lake of the

Isles, Cedar Lake, Calhoun, and Lake Harriet) [212]. In another study, alum (aluminum sulfate) was applied in oily wastewater to reduce the BOD. The BOD value was reduced by over 90% in 6 min [213].

The MOD was observed to be high underneath the ice surface in 2015; however, this pattern was not observed again during winter 2017. This could be due alum treatment which impacted on organic compounds present through the whole water column as well as methanotrophic bacteria.

The BOD and MOD measurements were higher during BML summer stratification in June 2015, but this was not observed again in June - July 2016. In general, methanotrophic oxygen demand and methane oxidation decreased in 2017. Methane oxidation potential was noticed to be higher underneath the ice surface March 2015, which could be possibly due to methane production at deep bottom sediments providing a constant input of carbon and energy sources that encouraged methanotrophs growth more rapidly at this zone and actively oxidize methane [214,215]. In natural lakes, during winter ice-covered period, methane bubbles are trapped underneath the ice and methanotrophs consume the dissolved CH_4 that comes from the bubbles. During summer, methanotrophs are present where O_2 and CH_4 are available, but methane in form of bubbles escape to atmosphere [216]. In addition, greater methane oxidation was noticed through water column during summer stratification in 2015 near the metalimnion zone compared to surface water; however, this trend was not observed again next summer in 2016. It could be possible that the lake was at early stage of summer stratification (shifting from spring to summer) [79,214].

The potential methane oxidation rates were lowest through the water column during early fall (turnover) in each year. In a natural lake, methane accumulates at the hypolimnion layer

during summer stratification, but not all methane can be oxidized by methanotrophs in water due to O_2 limitation. Mixing through the entire water column in the fall results in gradual mixing of CH_4 and O_2. Consequently, aerobic methanotrophs re-spread through the water column, and oxidize methane, as shown in other studies [139].

CHAPTER FOUR: MICROBICAL COMMUNITY ANALYSES

*Illumina samples were prepared for sequencing by Evan Haupt (March -July 2015), Joong-Jee Kim (August 2015-June 2016), Emad Albakistani (August 2016 - May 2017).

4.1 Introduction

One aim of this study was to characterize microbial communities and how they change over time. We characterized microbial communities at different depths, time points, and sampling platforms on BML. Bacterial diversity over three years might be used as an indicator for the reclamation status of the first end-pit lake (EPL) of the Alberta oilsands tailings, Base Mine Lake (BML). We particularly examined methane oxidizing bacteria, and their relative abundance over time. The Polymerase Chain Reaction (PCR) was performed on the V3-V4 regions of the 16S rRNA gene using universal primers, and then amplicons sequenced on the Illumina platform. QIIME (Quantitative Insights Into Microbial Ecology) was applied to support a wide range of microbial community analyses and visualizations.

4.2 Methods

4.2.1 Sample Collection

Samples for this section was discussed in chapter three section 3.2.1. In 2016, the MLSB water samples were collected during three months (Aug, Sep, and Oct). In addition to that samples from the water-FFT interface and sediment-FFT interface were taken with a fixed interval sampler (Syncrude Canada unpublished annual report, 2016), at 10-cm intervals during late summer (August 2016), fall (October 2016), and winter (March 2017) (Syncrude

unpublished data). At least sixty samples were taken from the three platforms in August and sixty in October 2016 **[Figure 3. 1]**. These samples were taken to examine microbial community compositions before alum treatment and after alum treatment applied. Both water-FFT interface and sediment-FFT interface samples were collected through a fixed-interval sampler, at 10-cm intervals and extruded into labeled 250 ml transparent bottles for laboratory analysis. Samples were stored at 4°C ±1 to minimize any physical, chemical, and biological changes until analysis. However, all sample temperatures were adjusted before beginning the required analysis to room temperature (20°C ±2).

4.2.1.1 Sample Centrifugation, DNA Preparation, and DNA Extraction

As soon as samples received, water samples (1000 mL) were centrifuged at 8,000 × g in a Beckman Coulter Avanti® J-E centrifuge at 4°C in a JA-14 rotor (Beckman Coulter). The desired volume of water was reached by centrifuging 200 ml of water sample in a 250-mL Nalgene HDPE bottle for 10 minutes, then an additional 200 mL of sample water was added after discarding the supernatant e.g. a total of 1-L water was centrifuged. After the final centrifugation, approximately 500 µL of supernatant was retained along with the pellet to resuspend the cells for transfer to 2-mL Lysing Matrix E tubes (MP BIO) for DNA extraction.

Water-FFT interface (20 mL) samples were centrifuged at 8,000 × g in a Beckman Coulter Avanti® J-E centrifuge at 4°C in a JA-14 rotor (Beckman Coulter), in 50-mL Falcon tubes for 10 minutes. After the centrifugation, approximately 500 µL of supernatant was retained along with the pellet to resuspend the cells for transfer to 2-mL Lysing Matrix E tubes (MP BIO) for DNA extraction. In contrast, sediment-FFT interface samples were centrifuged at 8,000 × g

in Prism R™ Refrigerated Micro Centrifuge in a 24 x 1.5 / 2.0 mL rotor. A volume of 0.5 g from sediment-FFT interface samples from each platform and depth were selected for centrifugation and DNA extraction. Then, the extracted DNA transferred into a 2-mL Lysing Matrix E tubes (MP BIO).

DNA was extracted using the FastDNA® SPIN Kit for Soil according to manufacturer's instructions (http://www.mgp.cz/files/kity/FastDNASPINkit.PDF) [217-220]. A wash step with 5.5 M guanidine thiocyanate (GTC) was applied in order to increase DNA purity. The extracted DNA was recovered in 50 µL of DNase/Pyrogen free water (DES). All DNA extracts were stored at -20°C in sterile 1.5 or 2 mL microcentrifuge tubes. The measurement of nucleic acid and protein in each sample was determined by using a NanoVue ™ Plus spectrophotometer.

4.2.1.2 PCR Amplification and Paired-end Illumina Sequencing

PCR was performed to amplify the 16S rRNA gene. The hypervariable region V3-V4 of the 16S rRNA gene was amplified using modified primers: 341F (5′ CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) [221]. PCR reactions were carried out in a total volume of 25.15 µl reactions with 0.25 µl of Top Taq DNA Polymerase (QIAGEN), 5.0 µl of 1 µM forward primer (341), 5.0 µl of 1 µM reverse primer (785), 5.0 µl 10× TopTaq PCR Buffer, 1.0 µl of 10mM dNTP mix, 6.35 µl of RNase-free water, and 2.5 µl of template DNA. PCR amplification was initiated by a denaturation cycle of 94 °C for 30 min, followed by 30 cycles of initial denaturation at 94 °C for 30 s, annealing at 60 °*C* for 30 *s*, extension at 72°C for 60 s, and final extension at 72°C for 10 min. After all PCR products were amplified, 5 µl of each reaction was loaded onto a 2% agarose gel for size

verification. Next, samples were purified using Agencourt AMPure XP magnetic beads (Becker Coulter, Brea, CA, USA). The second round of PCR amplification was implemented to introduce Illumina sequencing adapters and barcodes to differentiate each individual sample after 16S rRNA gene amplicons are pooled. Each PCR reaction mixture contained 5µl of purified PCR, 10 µl of both forward and reverse barcoded primers (0.2 µM), and Qiagen master mix, 2× (25 µl HotStarTaq) and 10 µl RNase-free water (Qiagen) in a total reaction volume of 50 µl. PCR cycling conditions comprised pf a pre-denaturation step of 95 °C for 15 min, followed by 8 cycles of initial denaturation at 94 °C for 1 min, annealing at 68 °C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. After PCR products were amplified, all products were quantified using a Qubit Fluorometer (dsDNA BR) and the size of each library was estimated using the Agilent 2100 Bioanalyzer (DNA BR). The PCR libraries were then normalized to 5 nM, pooled and sequenced using Illumina MiSeq following the16S Metagenomic Sequencing Library Preparation protocol. (https://support.illumina.com/sequencing/protocols).

4.2.3 QIIME Analysis

Sequence data were processed using Quantitative Insights into Microbial Ecology (QIIME v1.9.1), an open-source software used to pair raw sequence-read data and to support a wide range of microbial community analyses and visualizations [222]. The obtained sequences reads were then clustered into OTUs (operational taxonomic units) at 97% similarity by using the open-reference-based OTU-picking workflow in QIIME v1.9.1 based on UCLUST in order to generate high quality clusters [223]. Water samples were rarified to 500 reads to standardize

diversity statistics while sediments and water-interface samples were rarified to 1000 reads. This is because the number of obtained reads varied among sample platforms, depths, months, and layers. QIIME was used to analyze both alpha diversity (within samples) and beta diversity (among samples). In alpha diversity analysis, five metrics were calculated: Phylogenetic Diversity, Chao1, Observed species, Shannon index, and Simpson index. Phylogenetic Diversity measures the differences in the total branch lengths of a tree constructed of the OTUs [224]; Chao1 estimates the total richness of microbial communities ; Observed species estimates the amount of OTU's in each sample; Shannon index and Simpson index estimates are based on the number of species present in sample as well as their relative abundance [225]. Based on these metrics, rarefaction curves were generated. In beta diversity analysis, the QIIME software package was also used to calculate Bray Curtis, Euclidean, Unweigted Unifrac, and Weighted Unifrac distance matrices, which were used to generate PCoA or NMDS plots.

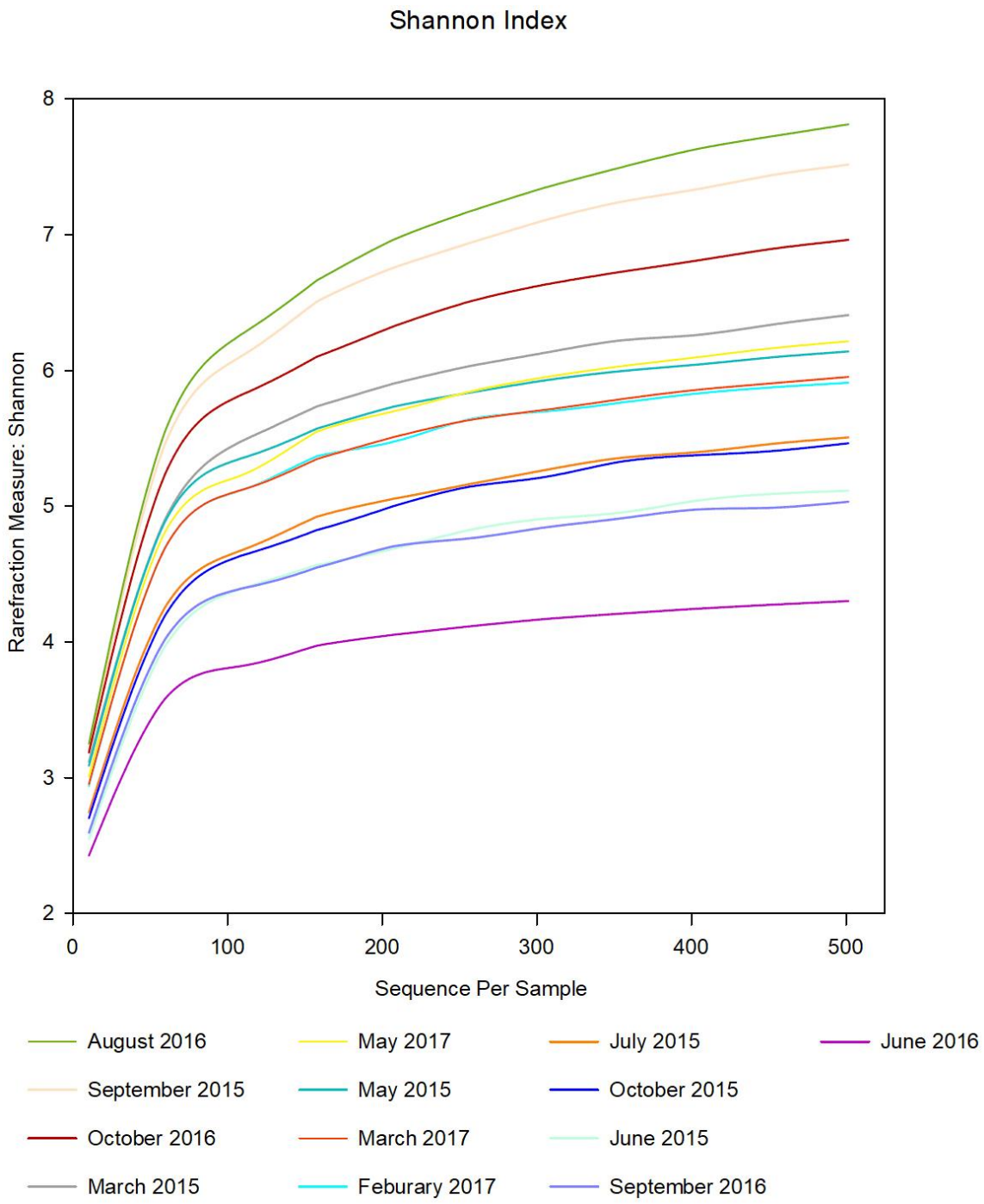


Figure 4. 1 Shannon diversity rarefaction curves of 16S rRNA gene sequences of BML samples collected from 2015 to 2017. Curves were built calculating the average Shannon index on samples that were collected from three platforms from March 2015 to May 2017. Combined data from Evan Haupt (March-June 2015) and Emad Albakistani (July 2015-May 2017).

4.3 Results and Discussion

4.3.1 Illumina Sequence Data

A total of 97317 OTUs were obtained from 305 BML water samples collected from March 2015 to May 2017 by clustering of sequences at 97% similarity in all water samples. Samples were then rarefied to 500 sequences and used for alpha and beta diversity analysis. The Shannon diversity rarefaction curves showed that 500 reads were adequate for all BML samples **[Figure 4. 1].**

4.3.2 Species Diversity

Five different alpha diversity metrics were calculated in BML samples: Phylogenetic Diversity, Shannon, Simpson, Chao 1, and Observed species. The obtained results from these metrics showed different values among eight months (February - October) and are shown in **[Table 4. 1]**. Values given are averages of all depths and three platforms. These numbers are calculated based on averages of three platforms at 10m, 5m, and/or surface or bottom. A notable increase in diversity was noticed during late summer and fall each year (Sep 2015, Aug 2016) meanwhile a notable decrease in diversity was observed with June samples.

Non-metric multidimensional scaling (NMDS) was used to measure the dissimilarity among the bacterial community composition among three platforms, different depths, seasons, and years. The distance matrices between water samples were calculated using the Bray-Curtis measure of dissimilarity [226] and were used for plotting NMDS [227-229] using the QIIME v 1.9.1 platform.

There were no clear depth or platform effects, as also noted previously (Haupt, 2015). NMDS plots and results clearly identified four different community types during four seasons (winter, spring, summer, and fall) **[Figure 4. 2]**. This analysis indicated seasonal changes in bacterial community composition as depicted by formation of season-specific clusters of BML communities within NMDS plots. The analysis also indicated a strong effect of alum addition. The bacterial community shifted gradually during fall 2015 (September to October), but shifted dramatically during fall 2016 (September to October 2016) after alum addition. In other words, the bacterial communities were not the same in the same season between two years. Bacterial communities during October 2016 were similar to winter communities. It could be that organic particles and bacterial cells settled down near the water-FFT interface (oxic-anoxic) after alum was applied, resulting in changed communities. The water turbidity of the lake through the water column during winter 2017 was much lower than in fall 2016, but it was ten times higher at the bottom of the lake during winter **[Table 1. 1]**.

Bacterial community analysis from the BML water samples that were collected on June 2016 were not considered in this NMDS analysis. We found a very unusual community in June 2016 water samples. As mentioned earlier in the previous chapter, these samples were collected during a wildfire (Albert's Wildfires 2016). Smoke and ash could have affected airborne microbes and and/or bacterial community structure in the water during this month **[Figure 4. 2]** **[Figure 4. 3 (A)]**.

Table 4. 1 Alpha diversity parameters results (Phylogenetic Diversity, Chao1, OTUs, Shannon, Simpson) of BML.

Month Year	Phylogenetic Diversity	SEM	Chao1	SEM	OTUs	SEM	Shannon	SEM	Simpson	SEM	Simpson_e	SEM
March.2015	30.975	4.233	670.980	279.702	196.833	34.100	6.409	0.286	0.966	0.007	0.164	0.041
May.2015	31.819	2.607	370.053	70.694	159.996	19.717	6.141	0.364	0.968	0.011	0.212	0.044
June.2015	23.216	4.468	503.844	212.919	157.619	44.633	5.116	1.123	0.854	0.136	0.072	0.041
July.2015	32.575	2.626	573.999	166.282	171.169	25.798	5.508	0.713	0.897	0.077	0.090	0.052
September.2015	43.108	2.881	1364.235	187.050	299.874	20.520	7.517	0.305	0.985	0.013	0.283	0.093
October.2015	32.682	4.131	665.631	210.262	180.489	34.675	5.465	0.833	0.887	0.075	0.076	0.039
June.2016	7.930	8.373	236.364	259.046	94.554	82.656	4.303	1.688	0.853	0.105	0.173	0.123
August.2016	45.834	5.429	1677.717	301.438	333.370	28.194	7.814	0.280	0.989	0.005	0.324	0.081
September.2016	24.211	4.625	470.168	173.845	139.780	44.736	5.034	1.186	0.871	0.109	0.108	0.075
October.2016	35.361	4.872	965.414	389.042	242.925	58.831	6.963	0.584	0.979	0.009	0.229	0.050
March.2017	30.357	4.576	517.836	180.549	173.135	40.535	5.954	0.607	0.946	0.036	0.144	0.068
Feburary.2017	29.494	2.075	450.197	83.962	160.824	16.997	5.911	0.214	0.953	0.015	0.148	0.048
May.2017	28.652	0.713	661.431	100.663	197.333	12.243	6.216	0.278	0.956	0.015	0.131	0.041

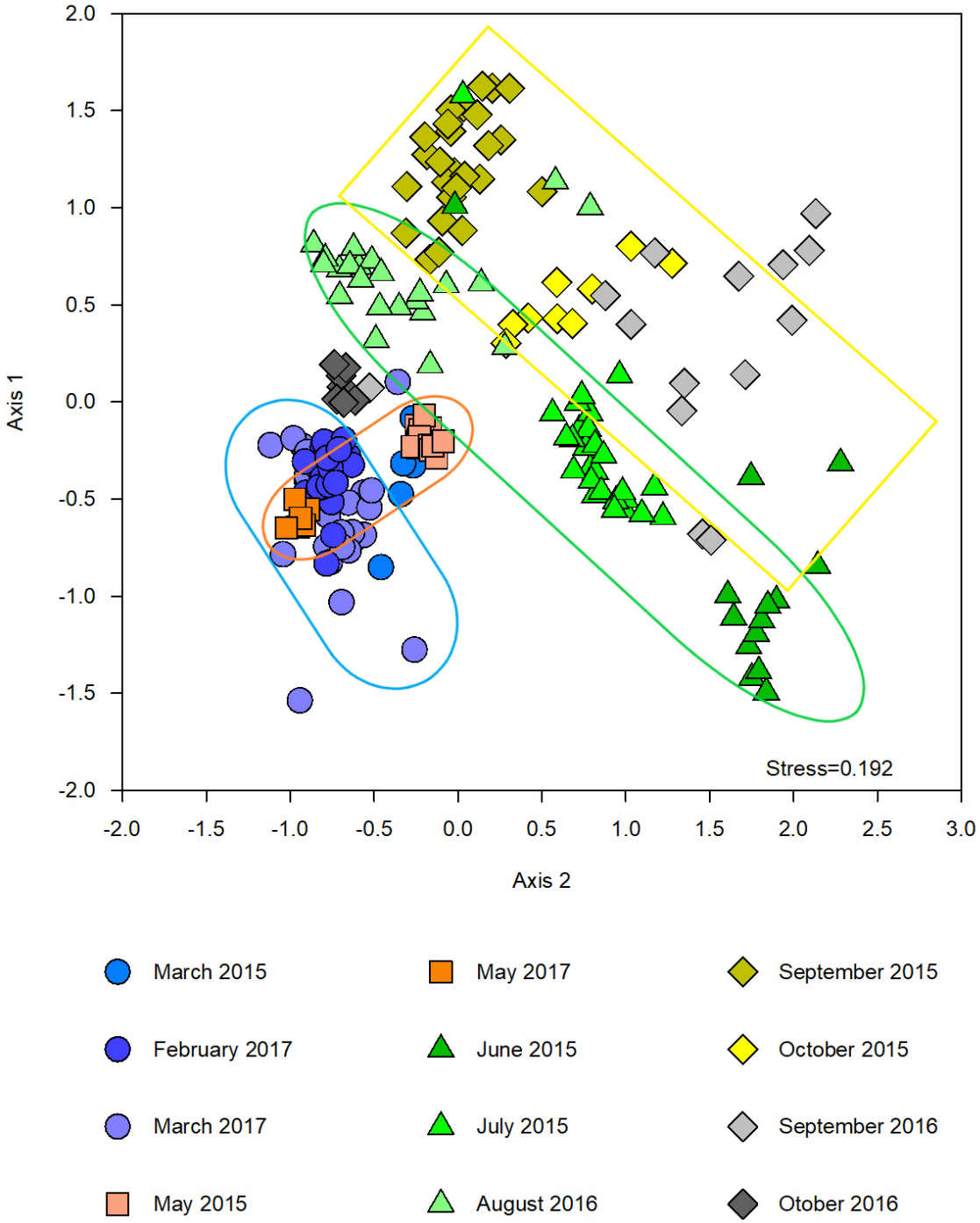


Figure 4. 2 Two-dimensional non-metric multidimensional scaling (NMDS) plot of the 16S rRNA gene-based bacterial community compositions at different depths and seasons in BML: winter samples (blue circles), spring samples (red squares), summer samples (green triangles), fall 2015 (yellow diamonds), and fall 2016 (gray diamonds). Combined data from Evan Haupt (March-June 2015) and Emad Albakistani (July 2015-May 2017).

4.3.3 Community Dynamics

In both winter and spring, the dominant phyla in BML were *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* **[Figure 4. 3 (A)]**. The abundances of *Proteobacteria* and *Bacteroidetes* in winter and spring were higher than in summer and fall, whereas the *Actinobacteria* was much higher in summer and fall than winter and spring. The abundance of *Firmicutes* was higher in summer and fall both years. The *Chloroflexi* appeared during fall 2015 (September). It is expected that physical conditions such as temperature and dissolved oxygen showing distinct seasonal variations in BML through the entire water column influencing on the microbial community changes **[Figure 1. 3]** and **[Figure1. 4]**. These changes in lake temperature (22 - 0 °C) and dissolved oxygen concentration (average 5.5 - 9 mg/L) from summer to winter seems to have strongly impacted (shifted) the composition of the bacterial communities. These observations are quite similar to findings of Diao *et al.* (2017), who studied bacterial community composition in a stratified lake (Lake Vechten, Utrecht, Netherlands) [230]. They showed that the lake hypolimnion was anoxic during summer stratification and the whole lake become oxic during turnover. However, the BML is an oxic through 10 m water capped during the whole year **[Figure1. 4]**. The bacterial community compositions through the water column were similar to sediment samples. Previous study also showed that bacterial communities in river waters may shift from season to season in predictable patterns and these shifts are strongly correlated to bacterial response and adaption to environmental changes [231]. Overall, these results showed large spatio-temporal changes in bacterial community dynamics, especially during summer stratification and fall turnover.

At the phylum level, the communities in active tailings pond MLSB were very different from those in BML. They were dominated by *Proteobacteria*, averaging 80% of the observed reads, followed by *Firmicutes* at 15% in August samples, and *Chloroflexi* at 19% in September sample **[Figure 4. 3 (B)].**

At the phylum level in water-FFT interface and sediment-FFT interface most samples were dominant by 9 phyla; namely *Proteobacteria, Firmicutes, Bacteroidetes, Cyanobacteria, Chloroflexi, Gammatimonadetes, Planctomycetes, Actinobacteria Verrucomicrobia*. *Proteobacteria, Firmicutes,* and *Chloroflexi* **[Figure 4. 4 (A-F)]**. *Proteobacteria* were much lower in August samples and much higher with October samples. *Firmicutes* were much higher in August samples compared to October samples. Samples collected during winter shows that *Actinobacteria* were more dominant than any other phylum **[Figure 4. 4 (E-F)]**.

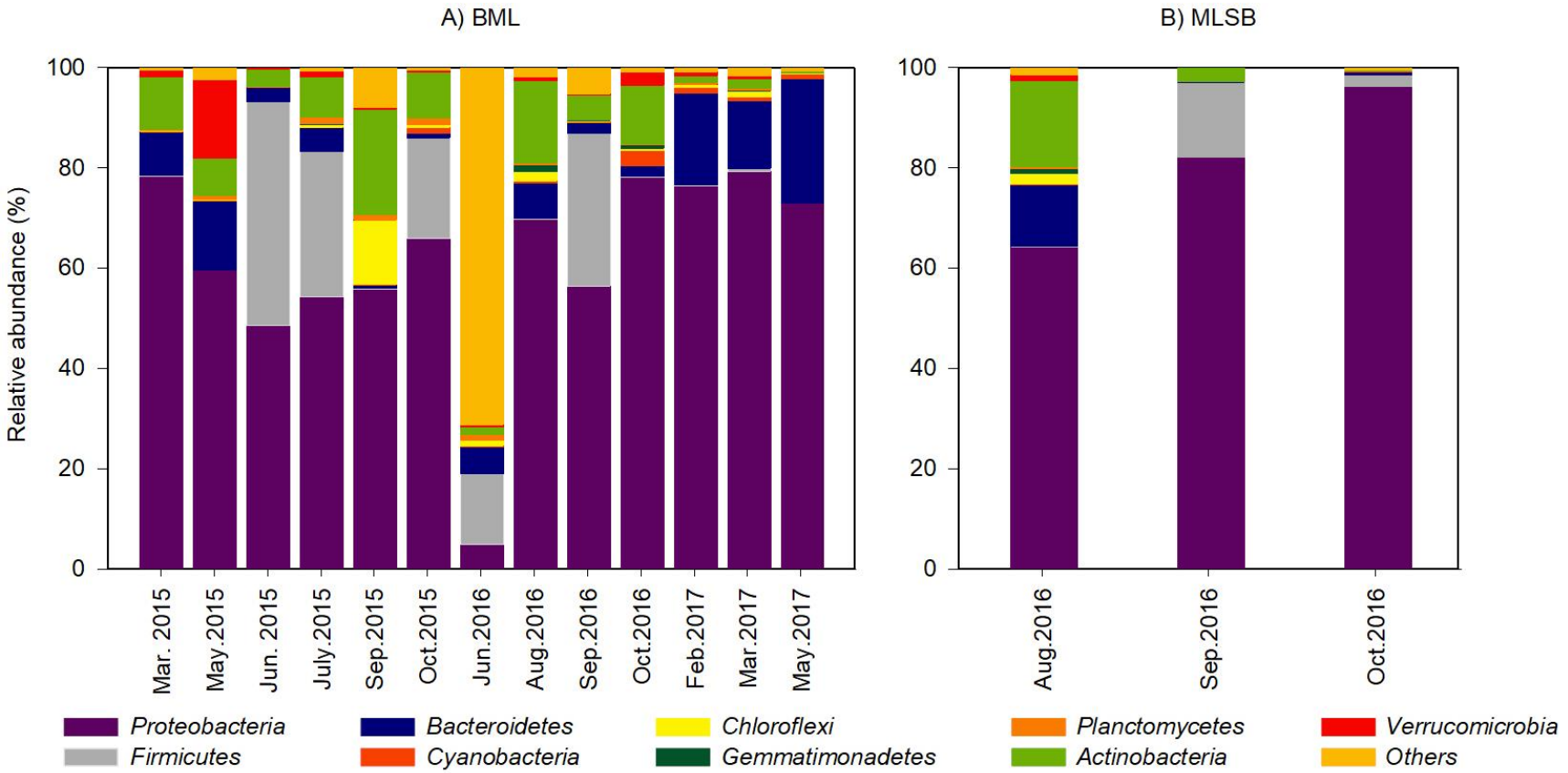


Figure 4. 3 A) The relative abundance of different phyla in Base Mine Lake (2015-2017). B) The relative abundance of different phyla in Mildred Lake Settling Basin during three months of 2016. These data represent the averages of three platforms during the study period, and some samples were ignored due to low reads of OTU's during the sequencing.

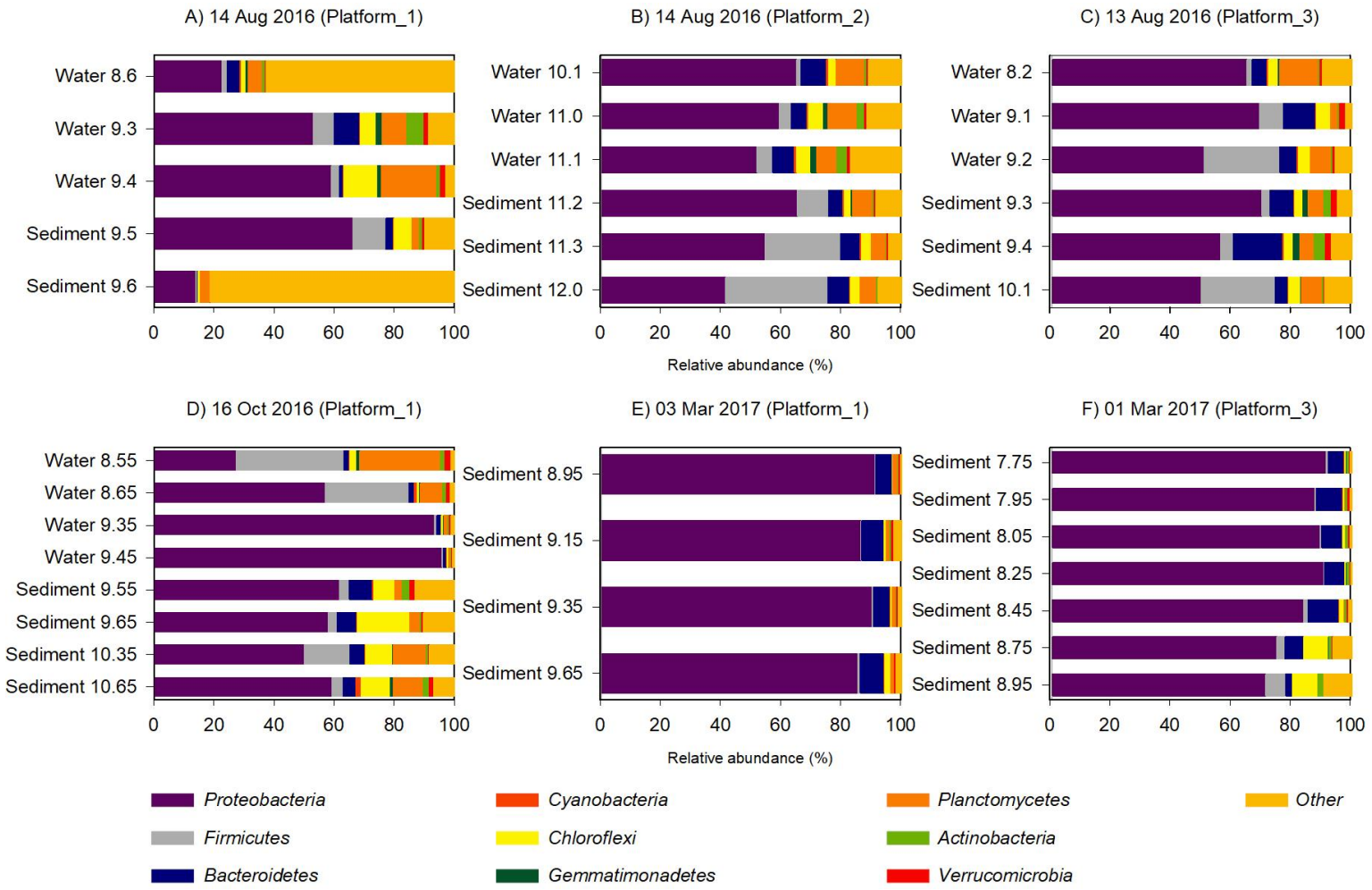


Figure 4. 4 (A-F) The relative abundance of different phyla at water-FFT interface and sediment-FFT interface of in Base Mine Lake. Samples were collected on (Aug and Oct 2016 and Mar 2017).

4.3.4 Methanotrophic Bacteria Dynamics in BML

The abundance of type I and type II methanotrophs in water columns was investigated in BML (2015-2017) and MLSB (2016). In this study, we identified methanotrophs and their relative abundance in the water columns. Our findings showed minor shifts in dominant methanotroph genera from season to season. The type I methanotrophs dominated over the type II methanotrophs during all sampling times. The type II appeared during summer and fall (June and September 2015) **[Figure 4. 7]**.

The type I methanotrophs *Methylobacter/Crenothrix and Methylobacter* were present through the entire water column and reached the highest abundances during winter of 2017. Methanotrophs made up large percentages of the entire community (10-20%,) in winter. In winter 2015 methanotroph abundances through the water columns were much lower than observed in 2017. However, methanotrophs made up small percentages of the entire community (less than 5 %) through the water columns during spring. During summer, their abundances through the water column varied over the two years but generally made up small percentages of the entire community (2%) at the epilimnion. We hypothesized that methanotrophs cluster at the metalimnion, but this was only seen once. In August of 2016, there was an increase in the metalimnion and hypolimnion (10 and 30% respectively). High abundances were also detected at the water-FFT interface (transition zone between water capped and tailings sediments) at this time. **[Figure 4. 6 (A-C)]**. However, methanotrophs made up small percentages of the entire community (up to 6 %) through the water columns during fall.

During winter, methanotrophs were observed to be abundant through the entire water column. The methanotrophs were also similarly distributed (but less abundant) through the water

columns during dimitic turnover (spring and fall turnover). As observed, methanotrophs were high at the surface (0 m), and at the bottom. These samples were collected during late summer, when the lake started shifting to fall season **[Figure 1. 3]** and **[Figure 1. 4]**. Similar patterns with depth were observed with the biological oxygen demand **[Figure 3. 2 (I, J)]** and methane oxidation rate **[Figure 3. 5 (A)]**.

In disagreement with our two hypotheses, (1) methanotrophs were similarly abundant throughout the water columns (10 m), they were not clustering underneath the ice surface where methane bubbles accumulate. However, methanotroph in winter were very high throughout the entire water column, suggesting that methane does accumulate below the ice cover during winter and methanotrophs oxidize CH_4 through the water column (2) methanotrophs were not clustering at the metalimnion during summer stratification. They were higher at hypolimnion and near the bottom of the lake at the water-FFT interface in August 2016. This month during the three years was the only month (late summer) that we detected the highest methane oxidation near the sediments.

Methane concentration was measured during summer 2015 (between June to August) and 2016 (between June to August) through the water column at BML. Methane concentration seems to be declining in the lake over time, and methane oxidation appears to be mostly active near the water-FFT interface rather than water column [232]. We have seen similar trends with the BOD and MOD **[Figure 3. 2 (K)]** and we have also seen similar trends with the potential methane oxidation **[Figure 3. 5]**. During fall, methanotrophs of both types spread out through the water columns, but their abundances were low through the water columns. We have also found that they were much lower in 2016 than 2015 **[Figure 4. 5].**

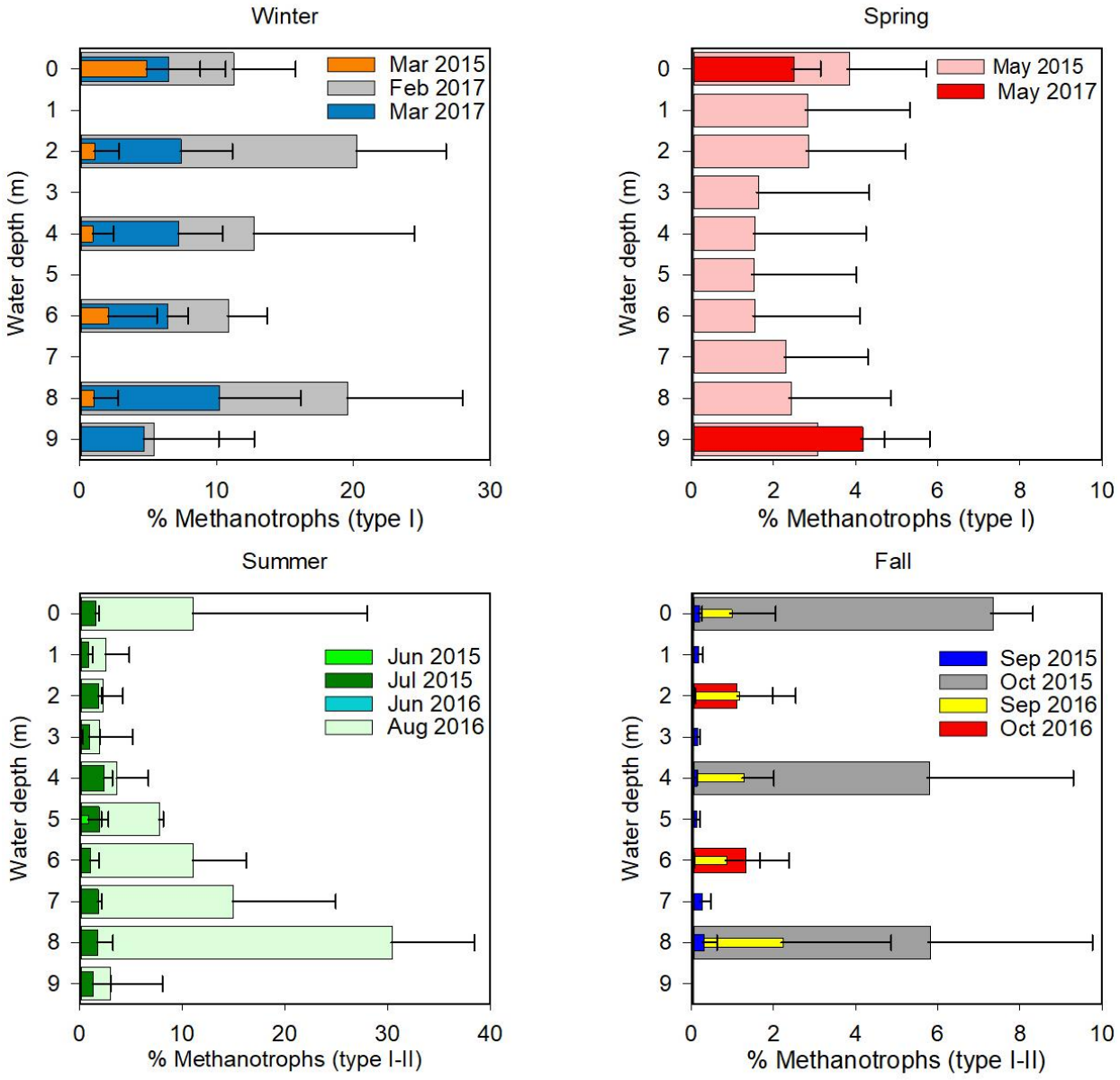


Figure 4. 5 Percentage of total 16S rRNA gene reads accounted for by methanotrophs in Base Mine Lake samples collected from March 2015-May 2017. Bars represent the standard error across three platforms.

High abundances were detected at the water-FFT interface (transition zone between water cap and tailings sediments) and sediment-FFT interface samples. In all samples that were collected from sediments during the three months (2016-2017), *Methylobacter* and *Methylocaldum* were the dominant genera **[Figure 4. 6 (A-D)]**, except March samples, *Crenothrix* showed up again in these samples **[Figure 4. 6 (E-F)]**. The relative abundances of type I and type II methanotrophs in BML water varied in samples at different timepoints **[Figure 4.7 (A)]**. The most abundant bacterial genera in winter samples (February and March) were the type I methanotrophs *Methylobacter/Crenothix* (the OTU could not be assigned with certainty to either genus) and *Methylobacter* with average relative abundances of 62.4 and 15.9% of all methanotrophs respectively; other genera were *Methylocaldum* and *Methylovulum.* It appears that the most dominant methanotrophs in winter were psychrophilic. Some *Methylobacter* strains are able to oxidize methane at cold temperatures [144]. The most abundant bacterial genus in spring samples (May) was *Methylobacter/Crenothix* with an average relative abundance of 84.6% of all methanotrophs. *Methylomonas* was also present. The most abundant bacterial genus in summer samples (June, July, and August) was the type I methanotroph *Methylocaldum* with an average relative abundance of 78.9%. Additionally, type II *Methylocystis* were detected in June and September 2015, but not in 2016 during the same seasons. The most abundant bacterial genus in fall samples (September and October) was *Methylocaldum* with an average relative abundance of 89.7%, followed by *Methylocystis* (6.7%). The type I methanotroph *Methylocaldum* was predominant with an average relative abundance of 93.1% in MLSB during the three months of 2016. In August and September of 2016, other type I methanotrophs (*Methylobacter/Crenothix*) made up to 12.2 and 0.8% respectively **Figure 4. 7 (B)]**. These observations were mostly similar

to previous findings of Saidi-Mehrabad et al. (2013), who studied methanotrophs diversity in WIP and MLSB for two years. They also observed that *Methylocaldum* was predominant in both ponds [78]. These observations were similar to recent findings of Diao et al. (2017), who studied seasonal bacterial communities in seasonal stratified lake (Lake Vechten, Netherlands). In this study, they found that methanotrophs were higher at the bottom than at the surface during summer stratification. The results from the relative abundance of bacterial genera showed that *Methylobacter* were much higher in sediments rather than water columns (2.6% and 1.2% respectively) during summer stratification while the *Methylobacter* were higher in 10 m than in the sediments (2.1% and 1.5% respectively) during fall [230].

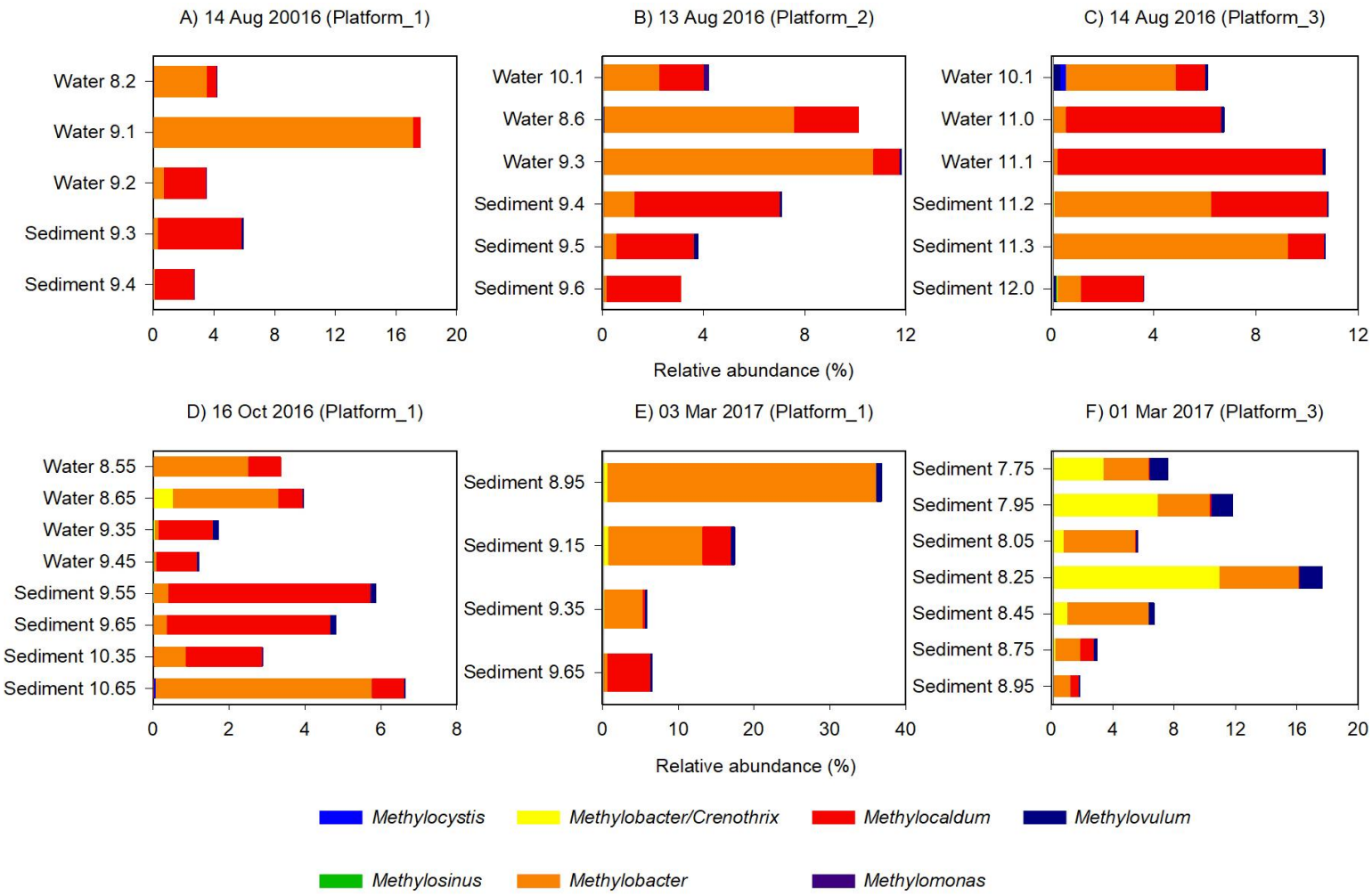


Figure 4. 6 (A-F) The relative abundance off different methanotrophs at genus level in water-FFT interface and sediments-FFT interface in Base Mine Lake. Samples were collected on (Aug and Oct 2016-Mar 2017).

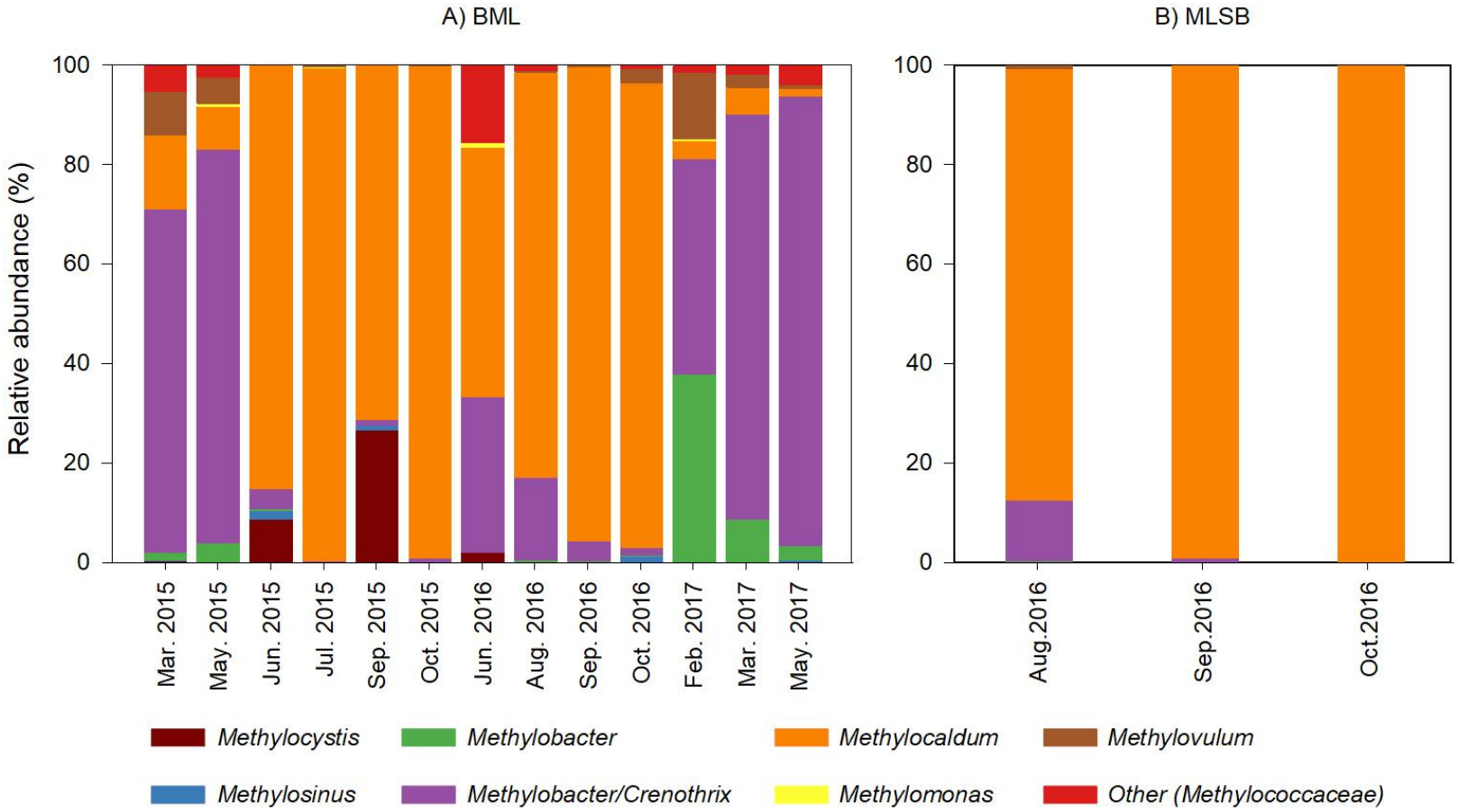


Figure 4. 7 The relative abundance of methanotrophs at genus level in Base Mine Lake (2015-2017). B) The relative abundance of methanotrophs in Mildred Lake Settling Basin during three months in 2016.

4.4 Conclusions

Beta diversity analysis showed that the bacterial community composition in the water column was more diverse than in water-FFT interface and sediment-FFT interface, but the MLSB and WIP were less diverse than BML. The NMDS analysis showed a shift in bacterial community during four seasons. There are similarities in bacterial community compositions among winter and spring samples, and in spring and fall samples over the three years. Alum treatment appeared to alter the bacterial community in 2016.

CH_4 emission has probably become lower from BML pond due to WCTT, and methanotrophs are actively oxidizing methane through the water column and mostly near the water-FFT interface. The genera *Methylobacter/Crenothix* were abundant during winter and spring, while *Methylocaldum* was abundant during summer and fall through the water columns and in the sediments-water interface. The type II methanotrophs (*Methylocystis* and *Methylosinus)* were detected only during summer and fall 2015 through the water column but not in the sediment water-interface. The water-FFT interface samples showed high abundances of *Methylobacter* and *Methylocaldum*, which means that methane oxidation may be maximal at the deep bottom of the lake rather than in the water column. The activity of methanotrophs in BML water appears to be limited, although they are more abundant during winter.

CHAPTER FIVE: CONCLUSIONS

This study is part of a larger program examining the first commercial-scale demonstration of the end pit lake (EPL) in Alberta's Oilsands tailings. The placement of fluid fine tailings in WIP was completed in 2012, and the Base Mine Lake was commissioned in late December 2012. The use of water capped tailings technology (WCTT) has increased the dissolved oxygen concentration, light penetration, and water clarity through the water column. The depth of water column has increased from 10 m to 12 m deep.

In this study, we monitored and examined:

(1) the biological oxygen demand and methanotrophic oxygen demand through the water column at Base Mine Lake over time. Our data suggested that the methanotrophic oxygen demand is associated with the biological oxygen demand. The methanotrophic oxygen demand was relatively high, but this has decreased over time. In general, the MOD and BOD were much higher at the first two years (2015-2016), and a gradual decrease was noticed through the water columns in 2017. Overall, the BOD and MOD through the water column were highest during winter, spring, and fall seasons, but they were much lower during summer stratification. There was a decline in BOD and MOD when alum treatment was added to the entire lake. We found that the MOD and BOD were clearly decreased through the water column at the beginning of the 2017. We propose that this may be a continued effect of alum addition. The BOD and MOD might increase again through the water column if tailing fines and solids at the bottom of the lake are re-suspended during spring and fall turnover in the next coming years. It depends how alum remains active and keeps fine particles and solids at deep bottom of the lake. An

increase in BOD and MOD might be prevented if alum treatment is applied again through the lake.

The potential methane oxidation (*ex-situ*) rates measured directly via GC were similar to rates measured via oxygen demand. Aerobic methane oxidation was lower in BML compared to other tailings ponds, such as WIP and MLSB. Overall, monthly methane oxidation rates in the water column were highest during the first year (2015), especially during winter, and during summer at the hypolimnion layer. However, the lowest CH_4 oxidation rates were during spring and fall. Generally, aerobic CH_4 oxidation is mostly active at the oxic and suboxic zone in lake [233,234]. In lakes, methanotrophs are present where CH_4 and O_2 are. During late summer (August 2016), the concentration of O_2 was very limited at hypolimnion, but the methanotrophs represented a major proportion of bacteria at that layer (see Fig. 1. 5 and Fig. 4. 9). In addition, water-FFT interface and sediment-FFT interface samples collected at the same month and year contained a larger portion of methanotrophs than water columns, which means that methanotrophs are most abundant at the bottom of the lake (water-FFT interface). The 16S rRNA analysis showed very high methanotroph abundances in the water-FFT interface during summer and winter (see Fig. 4. 9).

CH_4 and NH_4^+ as key oxygen consuming constituents (OCC) was studied by Risacher (2018) *et al.* in Base Mine Lake. They found that CH_4 was the only OCC related to water cap oxygen concentration during summer 2015; however, NH_4^+ emerged as an important OCC related to water cap oxygen concentration during summer 2016. The process of CH_4 and NH_4^+ oxidation require O_2 to oxidize either CH_4 or NH_4^+ via methane and/or ammonia oxidizers. It appears that methanotrophs were actively using O_2 as electron acceptor to oxidize methane. In

this study, CH_4 and NH_4^+ concentrations were measured *in-situ* through the water columns during summer (2015-2016) when the lake was thermally stratified. They found that CH_4 concentration was 0 µM at the epilimnion and metalimnion, and it was 50 µM at the hypolimnion. In contrast, NH_4^+concentration was 25- 35 µM in the epilimnion and metalimnion, and it increased gradually at the hypolimnion 50 µM. However, both compounds were much higher in 2015 than 2016 through the water columns. Most CH_4 oxidation occurred at the water-FFT interface, but not in the water columns [232].

It is also important to note that we found the BOD and MOD were very low near the surface, but they were high near the bottom during summer stratification of 2015 and 2016. Although we hypothesized that the methanotrophs may be most active in the hypolimnion, we never saw this. Evidence indicates that in fact the methanotrophs may be most active at the FFT interface.

(2) the microbial community composition in tailings water and sediments. The characterization of bacterial community in BML was very different than the active MLSB pond. The bacterial community were dominated by *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* in water column, water-FFT interface, and sediment-FFT interface. The bacterial community was dominated mostly by *Proteobacteria* in MLSB. The NMDS analysis also revealed a clear seasonal pattern of bacterial community during four seasons. It suggests that the bacterial community are mostly similar during winter and spring, and they are dissimilar during summer and fall through the water columns (10 m).

(3) methanotrophic bacteria in tailings water and sediments. There was a difference in bacterial composition of BML water compared to other tailings ponds. Furthermore, there

were large differences in methanotroph diversity at BML pond compared to other tailings ponds, such as WIP and MLSB [78,79]. In this study, we investigated seasonal variation of methanotrophs through the water column. During winter and spring, type I methanotrophs dominated, especially the genera *Methylobacter/Crenothrix* and *Methylobacter.* In addition to these dominant methanotrophs, small numbers of *Methylomonas* and *Methylovulum* were detected in BML. During summer and fall, the type I methanotroph *Methylocaldum* was dominant, and type II genera *Methylocystis* and *Methylosinus* were detected only in 2015. Moreover, methanotrophs in BML vary greatly with season and depth through the water column into the sediment-water interface. Methanotroph abundance in BML through the water column accounted for up to 20% of the total microbial community during winter and summer, but they were much less of total microbial community during spring and fall turnover. Furthermore, their abundance was highest during winter 2017, but decreased during spring 2017. It appears that methanotrophs are mostly abundant at water and sediment-FFT interface (summer 2016 and winter 2017). In contrast, the bacterial community in the water-FFT interface (below 10 m water column) and sediment-FFT interface (below the water-FFT interface) of BML showed much less seasonal variation during three seasons (summer, fall, and winter) of two years (2016-2017) than that in the water column. The most dominant methanotrophs detected in these samples (water and sediment FFT interface) belonged to the gammaprotobacteria or type I methanotroph genera *Methylobacter*, *Methylocaldum*, and *Methylomonas*. The majority of methanotrophs during winter and summer were found deep at the water-FFT interface. This suggests that most of produced methane is actively oxidized by a strong layer of methanotrophs before it reaches the water column.

In the future, we are planning to:

a) Run stable isotope probing (SIP) experiments to link methanotrophs with their function in BML. For this experiment, we better collect samples starting from 8 m to the bottom of the water column, and the water-sediment interface. This will use $^{13}CH_4$ as a model compound to label microbes followed by PCR amplification of 16S rRNA genes and genes encoding specific methane oxidation functions from the ^{13}C-labelled DNA This would be useful to understand how different methanotrophs are responsible for driving the biogeochemical cycling of methane in BML.

b) Measuring potential CH_4 and NH_4^+ oxidation with water and sediment-FFT interface samples during winter and summer. Obtaining samples from the water and sediment-FFT interface would allow us to calculate CH_4 and NH_4^+ oxidation rates, which could help us to understand oxygen consumption to oxidize both compounds in lake. Most activity may be at the interface rather than the water, where we were measuring to date.

c) Using more software analysis that could help us to understand the relationship between bacterial community dynamics, environmental parameters, and operational parameters in BML. For example, canonical correspondence analysis (CCA) would be useful to explore how the bacterial community including methanotrophs and nitrifying bacteria are associated with water temperature, dissolved oxygen, biological oxygen demand, pH, water turbidity at BML water and sediment-water interface.

d) Using a cultivation-independent method targeting the particulate methane monooxygenase (*pmoA*) as functional gene marker for detecting methanotrophs in the water column and FFT-sediments interface. This gene is present in most methanotrophs, and it would

be useful to build a phylogeny of methanotrophs based on *pmoA* and compare it to the 16S rRNA gene sequence. The 16S rRNA gene provides valuable information about the phylogenetic placement of methanotrophs detected in tailings environment, but it does not identify methanotrophs beyond the well-known families. In addition, qPCR can be used later to connect methane metabolism and evaluate methanotroph population sizes more quantitatively.

Capped Tailings Technology (WCTT) has greatly contributed to limit methane emission from BML pond, and it increased the chances for methanotrophs to oxidize CH_4 aerobically through the water column and at the water-FFT interface. The dissolved oxygen concentration has increased, and the biological oxygen demand decreased through the water column. The microbial community has changed and become more diverse than other tailings ponds.

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