Treatment of Oil Sands Process-affected Water (OSPW) Using Integrated Fixed-film Activated Sludge (IFAS) Reactors

by

Chunkai Huang

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ABSTRACT

The oil sands process-affected water (OSPW) generated from bitumen extraction of oil sands, is currently stored in tailings ponds due to its toxicity to the aquatic organisms. The primary toxic constituents of OSPW are a complex mixture of alicyclic and aliphatic compounds containing carboxyl radicals known as naphthenic acids (NAs). Cost-effective removal of NAs from OSPW is a key determinant for OSPW remediation. Thus, appropriate OSPW treatment processes are urgently needed to achieve the demand for extensive remediation of OSPW. To address the above need, the current study investigated the application of integrated fixed-film activated sludge (IFAS) reactor for OSPW treatment by applying different modes of operation (continuous and batch) for both raw and ozonated OSPWs. After 11 months of start-up, 12.1% of the acid extractable fraction (AEF) and 43.1% of the parent NAs were removed in the raw OSPW IFAS, while 42.0% AEF and 80.2% of parent NAs were removed in the ozonated OSPW IFAS. UPLC/HRMS analysis showed that NA biodegradation significantly decreased as the NA cyclization number increased. After 283 days of cultivation, the biofilm in the ozonated OSPW IFAS was significantly thicker than that in the raw OSPW IFAS. The quantitative polymerase chain reaction (*q*-PCR) revealed that the abundance proportions of both nitrifier genes (*aomA*, *NSR* and *Nitro*) and denitrifier genes (*narG*, *nirS*, *nirK* and *nosZ*) within total bacteria were significantly higher in biofilms than in flocs in the raw OSPW IFAS system, but a different trend was observed in the ozonated OSPW IFAS system.

Further study investigated the microbial communities of OSPW, the seed sludge, both flocs and biofilms from two IFAS systems during the start-up period. Bacterial community in the seed sludge (activated sludge from Gold Bar Wastewater Treatment Plant (AS from GBWTP)) of two IFAS systems showed the greatest richness and evenness. Chao 1 value and Shannon diversity

index results showed that the bacterial richness and microbial diversity of biofilms were significantly higher than those in flocs in both IFAS systems. The microbial community analysis from 454 sequencing revealed that *Proteobacteria*, *Nitrospirae*, *Acidobacteria* and *Bacteroidetes* were dominant phyla in both flocs and biofilms of IFAS reactors. However, the phyla and classes distribution of flocs and biofilms were significantly different. Principal Coordinate Analysis (PCoA) indicated that there were obvious differences in the microbial community between the biomass in IFAS systems and the seed sludge (AS from GBWTP) used for the start-up of IFAS systems.

The batch experiments evaluated the roles of suspended flocs and attached biofilms from IFAS systems in their overall contribution toward organic compounds removal in OSPW. Compared to the biofilms, the flocs demonstrated considerably higher removal rates for chemical oxygen demand (COD) and ammonium, whereas, biofilms had better performance on the AEF removal than flocs. Meanwhile, the results also revealed that the biodegradation was the principal removal mechanism, whereas the biosorption contributed little to the OSPW organic compounds and the ammonium removals in the IFAS system.

The optimization of IFAS systems evaluated the effects of hydraulic retention time (HRT) and the COD/N ratio on the OSPW treatment performance. After 11 months of HRT and ammonium optimization, 54.56% of the COD and 30.20% of the AEF were removed in raw OSPW IFAS, and 56.83% of the COD and 51.51% of the AEF were removed in ozonated OSPW IFAS. Extension of the HRT in the IFAS had no significant effect on the removal of COD and nitrogen, whereas a lower COD/N ratio increased the removal of organics and total nitrogen. The quantitative polymerase chain reaction (*q*-PCR) indicated that the abundance of nitrifier and denitrifier genes decreased during HRT optimization, and increased significantly after

ammonium optimization.

Further study compared microbial characteristics and OSPW treatment performance of five types of microbial biomass (moving bed biofilm reactor (MBBR)-biofilm, IFAS-biofilm, IFAS-flocs, membrane bioreactor (MBR)-aerobic-flocs, and MBR-anoxic-flocs) cultivated from three types of bioreactors (MBBR, IFAS, and MBR) in batch experiments. MBR-aerobic-flocs and MBR-anoxic-flocs demonstrated COD removal rates higher than microbial aggregates obtained from MBBR and IFAS. MBBR and IFAS biofilm had AEF removal capacities higher than that of flocs. MBBR-biofilm demonstrated the most efficient NAs removal from OSPW. NA degradation was highly dependent on the carbon number and Z value according to UPLC/HRMS analysis.

PREFACE

All of the research conducted in this thesis was designed and planned by myself and supervised by Professors Yang Liu and Mohamed Gamal El-Din at the University of Alberta. Some of the research conducted for this thesis was done in collaboration with Dr. Yijing Shi with Professor Yang Liu being the lead collaborator at the University of Alberta. All the research work was conducted by myself except the following contribution from collaborators and coauthors:

Chapter 3:

- Professors Yang Liu and Mohamed Gamal El-Din contributed to the research planning and manuscript edits.
- Miss Nian Sun performed samples analysis using the Ultra pressure liquid chromatography-high resolution mass spectrometry (UPLC/HRMS).
- Dr. Yijing Shi was a postdoctoral fellow at the University of Alberta and contributed to the research planning.

Chapter 4:

- Professors Yang Liu and Mohamed Gamal El-Din contributed to the research planning and manuscript edits.
- Dr. Zhiya Sheng provided us with her Quantitative Insights into Microbial Ecology (QIIME) software to analyse pyrosequencing data.
- Dr. Yijing Shi and Dr. Zhiya Sheng contributed to the manuscript edits.

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- Professors Yang Liu and Mohamed Gamal El-Din and Dr. Yijing Shi contributed to the research planning and manuscript edits.
- Dr. Pamela Chelme-Ayala and Dr. Yanyan Zhang contributed to the manuscript edits.

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- Professors Yang Liu and Mohamed Gamal El-Din contributed to the research planning and manuscript edits.
- Dr. Yijing Shi was a postdoctoral fellow at the University of Alberta and contributed to the research planning.
- Dr. Yanyan Zhang contributed to the manuscript edits.

Chapter 7:

- Professors Yang Liu and Mohamed Gamal El-Din contributed to the research planning and manuscript edits.
- Dr. Yijing Shi was a postdoctoral fellow at the University of Alberta and contributed to the research planning.
- Dr. Rongfu Huang performed samples analysis using the Ultra pressure liquid chromatography-high resolution mass spectrometry (UPLC/HRMS).
- Dr. Yanyan Zhang and Mr. Jinkai Xue provided us the activated sludge from their MBR systems.

All the research work was conducted by myself except the above mentioned contribution from collaborators and coauthors.

DEDICATION

I dedicate my thesis work to my family. A special gratitude to my loving parents, Xiangguan Huang and Liangzhan Wang, who had a great influence on my life, and taught me everything with their hard work, and were always there for me when I needed them. This thesis work is dedicated to my sister, Yu Huang, who always supported and encouraged me to overcome all challenges during my gradate school life, but unfortunately is not here now to see my achievement.

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LIST OF ABBREVIATIONS

AEF	acid extractable fractions
AMO	ammonia monooxgenase
AOB	ammonia oxidizing bacteria
AOPs	advanced oxidation processes
APHA	American public health association
AS-SBR	activated sludge-sequencing batch reactor
BOD	biochemical oxygen demand
BTEX	benzene, toluene, ethylbenzene, and xylenes
CAS	conventional activated sludge
CFS	coagulation/flocculation/sedimentation
CLSM	confocal laser scanning microscope
COD	chemical oxygen demand
COD/N	chemical oxygen demand/nitrogen
DCM	dichloremethane
DEN	denitrifying bacteria
DGGE	denatured gradient gel electrophoresis
DNA	deoxyribonucleic acids
DO	dissolved oxygen
EOF	extractable organic fraction
EPS	extracellular polymeric substances
ESI-MS	negative ion electrospray ionization-mass spectrometry
FBBR	fluidized bed biofilm reactors

FISH	fluorescence *in situ* hybridization
F/M ratio	food to microorganism ratio
FT-IR	Fourier transform infrared
GAC	granular activated carbon
GC-MS	gas chromatography-mass spectrometry
GBWTP	gold bar wastewater treatment plant
HDMS	high definition mass spectrometry
HPLC	high-performance liquid chromatography
HRT	hydraulic retention time
IFAS	integrated fixed-film activated sludge
ISBRs	immobilized soil/sediment bioreactors
MBBR	moving bed biofilm reactor
MBR	membrane bioreactor
MFT-SBR	mature fine tailings-sequencing batch reactor
MLE-MBR	modified Ludzack-Ettinger membrane bioreactor
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids
NAs	naphthenic acids
NCBI	national center for biotechnology information
NOB	Nitrite oxidizing bacteria
OLA	organic loading rate
OSPW	oil sands process-affected water
OTU	operational taxonomic unit

PAHs	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PC	petroleum coke
PCoA	principal coordinate analysis
PE	polyethylene
PU	polyurethane
PyNAST	python nearest alignment space termination
q-PCR	quantitive polymerase chain reaction
RA	relative abundance
RAS	return activated sludge
RO	reverse osmosis
rRNA	ribosome ribonucleic acids
SEM	scanning electron microscope
SRT	solid retention time
TDS	total dissolved solids
TN	total nitrogen
T-RFLP	terminal restriction fragment length polymorphism
TSS	total suspended solids
UPLC/HRMS	ultrahigh pressure liquid chromatography/high resolution mass spectrometry

CHAPTER 1. GENERAL INTRODUCTION AND RESEARCH OBJECTIVES

1.1 Background and Motivation

The oil sands in Alberta are abundant, accessible, and affordable sources of crude oil. The oil sands industry focuses on oil sands exploration, development, and production due to the continuing decrease of conventional crude oil reserves in North America. Oil sands are crude deposits that are more viscous than the crude oil. Oil sands are made of sand, bitumen, other rock materials, and water. They are near solid state at room temperature, and require upgrade to produce synthetic crude oil.

The oil sands deposits in the Athabasca region of Alberta are the third largest oil reserve in the world. According to the Oil & Gas Journal and the US Energy Information Administration, Canada has the third largest oil reserves (about 180 billion barrels), accounting for 15% of world reserves, just after Saudi Arabia and Venezuela. Over 170 billion barrels of oil are found in Alberta's oil sands among these reserves. With today's technology, about 170 billion barrels of oil are recoverable, and 2.5 trillions of barrels in total (Kannel and Gan, 2012). These deposits are separated into three regions: Peace River, Athabasca (Fort McMurray area), and Cold Lake (north of Lloydminster). However, around 7% of them are extractable economically using current technology, and most of the oil exaction industry is located in Fort McMurray in the Athabasca region. The bitumen production reached 2.3 million barrels per day (bpd) in 2014 due to the rapid expansion of oil sands industry and the daily bitumen production is expected to achieve 4 million bpd by 2024 (Alberta Energy, 2015).

There are two main methods to extract the bitumen from the oil sands, *in-situ* and surface mining. *In-situ* or "in place" methods are mainly applied for the underground deposits that are

too deep (more than 100 m deep) for excavation (Smith et al., 2008). In addition, it needs to drill specific well into the deposit area and inject some high-temperature or high-pressure carriers into the deposits to extract the bitumen continuously and intermittently. Compared to the surface mining, *in-situ* methods are less land disturbing. In contrast, overburden layers should be removed to expose the oil sands in the surface mining, leading to serious and significant damage to the landscape.

Currently, the surface mining is the dominant method to extract the bitumen from the oil sands. In particular, about 55% of the bitumen production from the Alberta oil sands is through surface mining operations (Alberta Energy, 2015). In the surface mining operation, oil sands are moved through trucks and shovels, and an alkaline hot water extraction process is utilized to separate bitumen from the sand and clay (Chelsea Leishman et al. 2013). More specifically, mixing the hot water with the oil sands form a slurry. As a result, air flotation is necessary to remove the bitumen from water, because the density of bitumen is extremely close to the water in the regular operating temperatures. During this process, a diluent is essential to reduce the viscosity of bitumen and remove water and remaining fine solids. After that, the remaining water and solids from the previous treatment processes are sent to a primary tailings treatment unit. Coagulants and flocculants are used to accelerate the solids settling in this stage. The underflow stream will be pumped to large settling basins, which are well known as tailing ponds by the general public.

However, large volume of fresh water is demanded to support oil sands surface mining, extraction, and upgrading processes. On average each extracted barrel of bitumen consumes at least 3 barrels of fresh river water during the extraction process (Scott et al., 2008). Approximately 359 million m^3 (less than 3% of the average annual water flow) of fresh water is

currently taken from Athabasca river every year (Hooshiar et al., 2012). The average water flow in the winter time is 10 times less than spring or summer water flow, thus, the government made additional limits on the fresh water withdraw from the Athabasca river due to the lower water flow rate during the winter time. Therefore, these limitations may eventually limit the further increase of the oil sands production (Allen, 2008). Meanwhile, a considerable volume of fine tailings was generated from the bitumen production processes, which contain clays, sand particles, and residual bitumen. However, water management is critical for the sustainable development of oil sands in Northern Alberta. A large amount of oil sands process-affected water (OSPW) is accumulated and temporarily stored in the tailing ponds due to the a zero-discharge approach in the environmental maintained by Alberta's regulatory framework (Huang et al., 2015), which is eventually expected to be remediated for reuse or safe release into the receiving environments.

OSPW contains high concentration of petroleum hydrocarbons, organic acids, oilfield chemicals, salts, suspended solids, and heavy metals. OSPW is alkaline, slightly brackish (total dissolved solids = 2000-2500 mg/L), and acutely toxic to aquatic biota. The toxicity of OSPW is attributed to organic acids released from bitumen during the extraction process. Therefore, its discharge without treatment can cause considerable environmental problems. In the future, these tailing ponds need to be remediated according to their agreement with the Alberta Government. Water treatment strategies are urgently required to allow tailings water to be recycled or to be released safely to the environment, and to reduce the need to withdraw fresh water from the Athabasca River (Hwang et al., 2013). Therefore, appropriate OSPW treatment processes are urgently needed to achieve the need for extensive remediation of oil sands landscape.

1.2 Scope of Research and Objectives

The major constituents of concern in OSPW are suspended and dissolved solids, hydrocarbons, salts, metals and organic acids such as naphthenic acids (NAs), which are considered to be one of the primary toxic components in OSPW (Scott et al., 2005). In order to reuse or safely release OSPW, individual treatment processes may not be effective or economical for the OSPW remediation. Literature has reported that combined water treatment processes such as coagulation-flocculation, filtration, adsorption and biological degradation, as well as ozonation and membrane filtration, are possible candidates for OSPW treatment. Our previous studies have showed that OSPW has a low biodegradability (biological oxygen demand (BOD)/chemical oxygen demand (COD) ratio < 0.1), and OSPW generally contains a low COD concentration (250-350 mg/L). Recently, both suspended growth and attached growth bioreactors have been tested for OSPW remediation. However, to date, none of the studies have considered the application of bioreactors combining biofilms and activated sludge (hybrid bioreactors) processed for OSPW treatment. Previous studies have showed that ozone treatment might be used as a pretreatment option for bioreactor treatment to break down recalcitrant NAs and enhance OSPW biodegradability (Dong et al., 2015; Islam et al., 2014). Therefore, ozonation combined with hybrid bioreactor might be a promising process for OSPW treatment.

The integrated fixed-film activated sludge (IFAS) reactor represents a novel reactor design, consisting of a suspended growth system and an attached biofilm system in one bioreactor. The IFAS configuration utilizes synergetic interactions between biofilms on carriers and suspended biomass in activated sludge, thus increasing microbial population diversity and rates of contaminant degradation. IFAS reactors promote the growth of both suspended and attached biomass. Moreover, the mobile IFAS media provide large areas for biofilm development and

growth, and enable the treatment of wastewater with low biodegradability with a lower reactor volume (Germain et al., 2007). Compared to similar technologies, IFAS reactors generally have higher effective biomass (Stricker et al., 2007), a higher nitrification rate (Kim et al., 2011), greater resistance toward organic and hydraulic shock loading (Mendoza-Espinosa and Stephenson, 2001), and lower sludge production (Sriwiriyarat et al., 2008). The hybrid nature of the IFAS system makes them an ideal candidate for OSPW treatment. Thus, there is a significant research gap with respect to the investigation of the applicability and effectiveness of IFAS treatment technology for OSPW remediation. The combination of ozonation followed by an IFAS reactor may improve the overall degradation and mineralization of acid extractable fraction (AEF) and NAs as well as organic contaminants.

Based on the above hypotheses, the overall objective of this Ph.D. research was to investigate the application of a combined ozonation and integrated fixed-film activated sludge system (IFAS) for OSPW treatment. To achieve this overall objective, bench-scale IFAS systems were designed to simulate full-scale wastewater treatment processes; the systems were applied and optimized, and the effectiveness and treatment mechanism of combined ozonation and biological processes was investigated.

It was hypothesized that the optimal biomass growth and microbial community structure would lead to maximal organic carbon removal efficiency in OSPW. The selected media (polyethylene) in the IFAS system has high surface area, which would enhance the biodegradation in the reactor.

Ozonation can decrease the recalcitrance and toxicity of organic components in raw OSPW, and increase the biodegradability of NAs. Ozone reactions with long chain organic molecules are based preferentially on aromatic ring number, molecular weight, and side chains.

Therefore, the combination of ozonation and IFAS could enhance the degradation and mineralization of NAs and other organic compounds. It was hypothesized that aerobic treatment followed by ozonation will increase the degradation of NAs.

The objectives were achieved in four phases described below:

Phase I. Experimental set-up and start-up of IFAS reactors

The IFAS reactors were filled with raw or ozonated OSPW. Synthetic wastewater was used as the nutrient source and the activated sludge from a municipal wastewater treatment plant (Gold Bar Wastewater Treatment Plant, Edmonton, Canada) was used as the reactor seed. Phase I goals included:

- Identify and characterize OSPW constituents before and after ozone treatment.
- Understand the mechanisms and assess the performance of the IFAS reactors, characterize NAs removal mechanisms, and characterize floc and biofilm microbial communities during the bioreactor start up stage.
- Establish thriving biofilms and flocs in the bioreactors.
- Compare the start-up performance of IFAS between raw OSPW and ozonated OSPW.

Phase II. Degradation and adsorption batch experiment set-up

To understand and identify the roles of flocs and biofilms in the overall performance in the IFAS reactors, batch IFAS reactors were created after the continuous IFAS systems were stabilized. Phase II goals included:

- Characterize the physico-chemical properties of activated sludge flocs and biofilms from two IFAS batch systems.
- Identify the individual roles of suspended-phase and attached-phase processes in the IFAS system to the overall performance for the OSPW treatment.

- Investigate the adsorption and biodegradation performance of activated sludge flocs and biofilms on COD, ammonium, AEF, and NAs removals.
- Characterize the NA removal mechanisms and the microbial communities structure of activated sludge flocs and biofilms in the IFAS systems.

Phase III. Operation and optimization of continuous IFAS reactors in raw and ozonated OSPW treatments

Factors affecting floc and biofilm properties in both suspended and attached microbial populations and their biodegradation activities in IFAS reactors were evaluated. Phase III goals were:

- Optimize operational parameters of the IFAS system to obtain the most effective OSPW treatment.
- Elucidate the factors that affect floc and biofilm properties in both suspended and attached microbial populations in the IFAS reactors.
- Evaluate factors that affect the removal efficiency of NAs and other organic contaminants in OSPW.
- Evaluate IFAS reactor performance in removing the COD, AEF and NAs.
- Characterize NA removal mechanisms and the floc and biofilm microbial communities after optimization.

Phase IV. Performance comparison of five types of biomass from MBBR, IFAS and MLE-MBR reactors for OSPW treatment

To compare the OSPW treatment performance of the flocs and biofilms from moving bed biofilm reactor (MBBR), IFAS and modified Ludzack-Ettinger membrane bioreactor (MLE-

MBR), batch reactors were created after MBBR, IFAS and MLE-MBR reactors were stabilized after optimization. Phase IV goals included:

- Investigate the biodegradation performance of MBBR-biofilms, IFAS-biofilms, IFAS-flocs, MBR-aerobic flocs and MBR-anoxic flocs on COD, ammonium, AEF and NA removals.
- Evaluate the effects of the electron accepter condition on the performance for the OSPW treatment.
- Characterize the classical and oxidized NA removal mechanisms and the microbial communities structure of five types of biomass from three types of bioreactor.

1.3 Thesis Organization

The structure of this dissertation is presented below.

Chapter 1 introduces the general background of the oil sands bitumen extraction processes and defines the scope of research and objectives.

Chapter 2 reviews previous research in three sections: (1) the OSPW characteristics; (2) its potential environmental issues; (3) possible water treatment technologies for OSPW treatment, such as physico-chemical treatment, biological treatment, and combined treatment processes.

Chapter 3 evaluates the performance of IFAS systems in the treatment of raw and ozonated OSPW at the start-up phase by measuring the COD, AEF, and NAs. Activated sludge floc and biofilm samples from the reactors were characterized in terms of their physiochemical properties, morphological features, and microbial community composition. The impact of IFAS treatment on the degradation of NAs based on carbon and Z numbers from the reactor was also analyzed. The thickness of biofilm on the polyethylene (PE) carriers was characterized by confocal laser scanning microscope (CLSM).

Chapter 4 characterizes the microbial communities during the start-up phase of IFAS systems through applying 454 high-throughput pyrosequencing techniques. The microbial community evolution of suspended flocs and attached biofilms during the start-up period of IFAS systems was revealed. The biodiversities of dominant bacterial communities of activated sludge flocs and biofilms in the IFAS systems was investigated. The dissimilarity of microbial communities composition between tailings pond microorganisms, aerobic activated sludge from Gold Bar Wastewater Treatment Plant (GBWTP), suspended flocs and attached biofilms in the IFAS systems were identified.

Chapter 5 compares and elucidates the performance and mechanisms of suspended and attached biomass in IFAS systems for the OSPW treatment. Batch IFAS reactors were operated using flocs and biofilms, taken from stabilized continuous IFAS systems, for both raw and ozonated OSPW treatments. The roles of the two main removal mechanisms (biodegradation and biosorption) on OSPW remediation were evaluated. Microbial community structures in suspended and attached biomass was also characterized.

Chapter 6 investigates the effect of the hydraulic retention time (HRT) and COD/N ratio on the treatment performance of IFAS system for the treatment of OSPW. The treatment performance and microbial community structure of raw and ozonated OSPW IFAS systems during the optimization stage were compared. The influences of HRT and COD/N ratio on the microbial communities composition in the IFAS systems were evaluated.

Chapter 7 compares the OSPW treatment performance and microbial characteristics of five kinds of biomass (MBBR-biofilms, IFAS-biofilms, IFAS-flocs, MBR-aerobic flocs and MBR-anoxic flocs). The reactors treatment performance of OSPW including total COD, AEF, and NAs

biodegradation were evaluated. Microbial communities structures in biomass of batch reactors were also characterized.

Chapter 8 presents the general conclusions and recommendations for future work.

The Appendix contains experimental methodologies, figures, and tables.

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CHAPTER 2. LITERATURE REVIEW

2.1 OSPW Characteristics

Oil sands process-affected water (OSPW) consists of water, unrecoverable bitumen, sands, silts, clays, heavy metals, organic, and inorganic compounds (Kannel and Gan, 2012; Herman et al., 1994; Scott et al., 2005; Mikula et al., 1996). The characteristics of OSPW depend on various parameters, such as the extraction and upgrading processes, the mined ore quality, source, and the duration of storage (Kannel and Gan, 2012; Ityokumbul et al., 1994). Generally, OSPW contains 1-3% wt. of residual bitumen, 20-30% wt. of solids, and the remaining portion (70-80% wt.) as water (Allen, 2008a; Kannel and Gan, 2012). Most of settleable particles and solids are well separated from the OSPW in the tailings ponds, which lead to the low amounts of solids in the surface water layer of tailing ponds and fine particles with around 20% wt. solids in the suspension layer (Ityokumbul et al., 1994).

2.1.1 Inorganic Compounds

The concentrations of inorganic contaminants in OSPW are relatively higher compared to the surface water of the Athabasca region (Allen, 2008a; Zubot et al., 2012). Typically, OSPW is slightly alkaline (ranged from 7.8 to 8.7) (Gamal El-Din et al., 2011) and considered relatively hard water due to the existence of calcium (15-25 mg/L) and magnesium (5-10 mg/L) (Allen, 2008a). The total dissolved solids (TDS) concentrations of OSPW are in the range of 2000 to 2500 mg/L and are dominated by sodium, bicarbonate (>500 mg/L each), chloride, and sulfate (~300 mg/L each) (Allen, 2008a; Choi et al., 2014). OSPW has extremely high conductivity (3150-3750 mS/cm) (Gamal El-Din et al., 2011; Pourrezaei et al., 2011). In addition, the concentration of ammonia in OSPW typically ranges from 4.4 to 22 mg/L (Pourrezaei et al., 2011), which is higher than that in the surface water in the Athabasca River (0.6 mg/L)

(MacKinnon and Boerger, 1986). The concentrations of some trace metals including aluminum, nickel, arsenic, copper, zinc, and chromium in OSPW exceed the Athabasca River values (Allen, 2008a).

2.1.2 Organic Compounds

The organic fractions in OSPW are made of insoluble organic compounds (unrecovered bitumen, measured as oil and grease) and soluble organic compounds. Various types of dissolved organic compounds are detected in OSPW including polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, ethylbenzene, and xylenes (BTEX), phenols, fulvic and humic acids, and naphthenic acids (NAs) (Allen, 2008a; MacKinnon et al., 1993; Mohamed et al., 2011; Rogers et al., 2002a). The concentration of bitumen in OSPW ranges from 9 to 92 mg/L in Suncor and 25 mg/L in Syncrude tailings ponds (Allen, 2008a). The concentrations of total PAHs in OSPW are less than 0.01 mg/L (Allen, 2008a; Rogers et al., 2002b). The concentrations of BTEX in OSPW are below detection levels in Syncrude tailing ponds, and the total phenol concentrations in OSPW ranges from 3.3 to 5.4 mg/L (Mah and Kotecha, 2011). The typical concentration of extractable organic fraction (EOF) in OSPW ranges from 40 to 70 mg/L, which is significantly higher than that present in the Athabasca River (< 1mg/L) (Holowenko et al., 2002; Headley et al., 2004). It has been also reported that the concentration of dissolved organic matter (DOM) ranges from 50 to 100 mg/L, which is mostly composed of organic acids of which 80% are NAs (Nelson et al., 1993).

Naphthenic acids (NAs) are alkyl-substituted cyclic and aliphatic carboxylic acids that are removed from bitumen in the extraction process. Previously, Fourier transform infrared spectroscopy (FT-IR) had been used to quantify the concentration of NAs in OSPW. However, this concentration is currently expressed as acid extractable fraction (AEF), since the FT-IR

instrument can detect NAs and oxidized NAs, as well as other acid extractable fractions due to its advances in analytical chemistry (Zubot et al., 2010; Grewer, et al., 2010). The AEF includes non-NAs, such as organics compounds with –COOH, -CHO and >CO- groups. The NA concentrations are variable, depending on the ore quality, the age of OSPW and the extraction process (Quagraine et al., 2005). It has been reported that the AEF concentration in OSPW ranges from 40 to 120 mg/L (Holowenko et al., 2001; Holowenko et al., 2002), whereas the concentrations of NAs in OSPW are in the range of 7.1 to 47 mg/L (Grewer et al., 2010). Previous studies have shown that NAs show high resistance to bioremediation. It is believed that NAs are associated with the majority of the toxicity of OSPW to aquatic organisms. Therefore, the remediation technologies for NAs in OSPW have drawn significant attention due to its specifical chemical characteristics and toxicity (Allen, 2008b).

2.2 Naphthenic Acids

The components of NAs are variable in different OSPW sources and include a complicate mixture of alkyl-substituted acyclic and cycloaliphatic acids with the general chemical formula $C_nH_{2n-Z}O_x$, where n is the carbon number and Z is zero or a negative even number representing the hydrogen deficiency due to ring formation (*e.g.*, 0, no rings; -2, 1 ring; -4, 2 rings, etc.), and x indicates the number of oxygen atoms (x = 2 for classical NAs and x > 2 for oxidized NAs) (Clemente and Fedorak, 2005; Grewer et al., 2010; Barrow et al., 2010). In the fresh OSPW, the dominant NAs have a variable carbon number ranging from 13 to 16, while in aged OSPW, the carbon number of dominant NAs increases to higher values (*i.e.*, C_{22} to C_{30}). As for the classical NAs, the carboxylic acid group (COOH) is always attached to a side alkyl chain rather than to the rings (Mohamed et al., 2011; Headley et al., 2004).

NAs are present in variable concentrations in crude oil, depending on the geographical location of the oil sands reservoir (Brient et al., 1995). It has been reported that about 380 mg NAs/kg oil sand are present in Suncor samples, whereas around 125 mg NAs/kg oil sand are present in TrueNorth Energy samples. More variability of NAs (51 to 504 mg NAs/kg oil sand) is detected in Syncrude samples, as compare to Suncor and TrueNorth Energy. The possible reason is the variation in the number of samples analyzed from each company.

NAs are non-volatile, chemically stable organic acids that work like surfactants in the extraction process (Headley and McMartin, 2004). The non-polarity and non-volatility of NAs increase with the increase of molecular weight, which results in the variable chemical, toxicological, and physical properties of individual NAs (Headley et al., 2002). The solubility of NAs in water is variable depending on the pH; however, they are soluble in the organic solvents, such as dichloremethane (DCM) (Quagraine et al., 2005). NAs are present in the form of water-soluble salts in OSPW due to the alkaline pH of OSPW. Moreover, the solubility of NAs can vary from soluble (low molecular weight or fewer cyclic rings) to insoluble (high molecular weight or highly cyclic) (Quagraine et al., 2005).

2.2.1 Toxicity of NAs

Previous toxicological studies showed that OSPW has toxicity to a variety of aquatic organisms, such as zooplankton, fish, rats, bacteria, and plants (Clemente and Fedorak, 2005). Since NAs are quite soluble in slight alkaline water, NAs are believed to be one of the main responsible of OSPW toxicity (Frank et al., 2008; Scott et al., 2008) and the degree of toxicity depends on the molecular structures, concentrations and compositions of NAs (Frank et al., 2008; Headley and McMartin, 2004). It has been reported that the NAs from OSPW with low molecular weight showed higher toxicity, as compare with the high molecular fractions

(Clemente and Fedorak, 2005; Frank et al., 2008). It also has been found that the linear NAs showed higher toxicity in OSPW and the increase of rings significantly decreased the toxicity (Frank et al., 2009). Jones et al. (2011) also found that the increases of the solubility of NAs could significantly increase the toxicity of NAs.

NAs are classified as surfactants with hydrophobic ends, which can cause toxicity through the penetration of organisms' cell membrane (Smith et al., 2008; Rogers et al., 2002b; Mackinnon et al., 1986). Due to the surfactant characteristics of NAs and their relative small molecular size, the probable mode of acute toxicity of NAs to aquatic organisms is the narcosis, and NAs have been proposed potential carcinogens and endocrine disruptors (Frank et al., 2008; Frank et al., 2009; Scarlett et al., 2012). It has been reported that the lethal NAs dose for human beings is 11 g/kg (Rockhold, 1955). There is limited information about the consequences of exposure to commercial NAs on mammals (using rat and mouse models) (Khanna et al., 1971), fish (Dokholyan and Magomedov, 1984), plants (Wort et al., 1973) and zooplankton and phytoplankton (Harris, 2001; Leung et al., 2001,2003). Garcia-Garcia et al. (2011) reported that OSPW NAs had adverse effects on the immune system of mice, which lead to a poor immune ability of their organs against diseases. Harris (2001) found that the exposure to OSPW has a much less positive effect on perch embryos. Teratogenic effects, such as deformities of the eyes, spine, head, and tail, were found in embryos exposed to different dilutions of OSPW. NAs were through to contribute to these phenomena. Studies with plants have generated conflicting results depending on the test species, methodology and NA source (Wort and Patel, 1970; Wort et al., 1973). Wort and Patel (1970) investigated the exposure effects of Eastman potassium naphthenates and surrogate NAs to plant species including bush bean, wheat, maize, sugar beet, and radish. The results showed that an 8 to 12% increase in the growth of foliage when 11.5 g/L

of potassium naphthenates was sprayed onto the leaves of 14 days old plants. However, Kamaluddin and Zwiazek (2002) observed the decreases of leaf growth, photosynthetic activity, root respiration and leaf chlorophyII concentrations occurred when the aspen seedlings were grown in sodium naphthenates medium. Harris (2001) studied the effects of Syncrude OSPW on zooplankton and phytoplankton communities, it was found that OSPW exposure had the detrimental influence on zooplankton and phytoplankton communities.

Microtox toxicity assay is rapid, simple, sensitive, and cost-effective with large sample throughput capabilities, which uses luminescent bacterium *Vibro fischeri* as an indicator, as compare to traditional, complex, and expensive whole-animal testing with invertebrates and fish (Blaise and Ferard, 2005). Microtox has been widely used for the toxicity identification evaluations in OSPW industry. However, there are some limitations of the Microtox system. More exactly, the Microtox is too sensitive to some organic compounds that can easily cross the cell walls of the bacteria, whereas, it is less sensitive to some metals due to the salt present in the medium. Moreover, various factors including temperature, improper handing of luminescent bacteria, inaccurate dilutions, and exposure time, and other human errors can significantly affect the results.

2.2.2 Analytical Techniques of Naphthenic Acids Analysis

The analysis and quantification of NAs in environmental samples have been a significant challenge because NAs are a complex mixture present in environment (Grewer et al., 2010). To date, several analytical techniques have been developed to analyze and quantify NAs, such as Fourier transform infrared (FT-IR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), negative ion electrospray ionization-mass spectrometry (ESI-MS), and high performance

liquid chromatography (HPLC). However, there is no specific analytical technique for identification or quantification of individual NAs in the mixture due to the complexity of NAs.

FT-IR spectroscopy is a common method for the quantification of NAs, which was developed by Syncrude Canada Ltd (Jivraj et al., 1995). However, it is currently expressed as AEF, since the FT-IR instrument can quantify NAs and oxidized NAs, as well as other acid extractable fractions (Zubot et al., 2010; Grewer, et al., 2010). To conduct FT-IR analysis, OSPW samples are filtered (0.45 μm), acidified to pH 2.0, and NaCl is added to each sample and dissolved (salt is used to prevent emulsion formation during shaking). Liquid-liquid extraction of organics from the samples is performed with a separation funnel using dichloromethane (DCM). DCM is evaporated and the extracted AEF is dissolved with a known mass of DCM and analyzed by FT-IR. The sample absorbance is then monitored at 1743 cm^{-1} and 1706 cm^{-1}, absorption bands characteristic of monomeric and dimeric carboxylic groups (Clemente and Fedorak, 2005). The detection limited of FT-IR for AEF measurement is typically 1 mg/L, which depends on the volume of samples extracted (Jivraj et al., 1995).

Ultra-pressure liquid chromatography high-resolution mass spectrometry (UPLC-HRMS) is currently used to quantify the mass of each NA component, which was developed by Martin et al. (2010). More exactly, the structure of each NA component in this method can be presented in a 3D plot in which X-axis represents the carbon number n, Y-axis indicates the Z number, and Z-axis shows the intensity.

To quantify the NAs using UPLC-HRMS, first chromatographic separations are run on a Waters UPLC Phenyl BEH column (150 × 1 mm, 1.7 μm) using a gradient mobile phase of (A) 10 mM ammonium acetate solution prepared in Optima-grade water and (B) 10 mM ammonium acetate in 50% methanol 50% acetonitrile, both Optima-grade. Gradient elution is as follows: 1%

B for the first 2 min, then ramped to 60% B by 3 min, to 70% B by 7 min, to 95% B by 13 min, followed by a hold until 14 min and finally returned to 1% B, followed by a further 5.8 min equilibration time. The flow is maintained at 100 µL/min and column temperature is constant at 50°C, while samples are kept at 4°C.

Detection is employed by a high resolution Synapt G2 HDMS mass spectrometer equipped with an electrospary ionization source operating in negative ion mode. The system is controlled using MassLynx® ver. 4.1, tuning and calibration is performed using standard solutions, leucine enkephalin and sodium formate respectively, provided by Waters Corporation (Milford, MA, USA). TargetLynx® ver. 4.1 is used for data analysis of the target compounds, and the relative ratio of each analyte's chromatographic peak area to the internal standard is calculated for subsequent analysis.

2.3 Possible Water Treatment Processes for OSPW Treatment

Large amount of fresh water is required for the extraction of bitumen from oil sands, and approximately 3 m^3 of fresh water are needed for the extraction of bitumen from each m^3 of oil sands, which is generally referred to as OSPW (Schramm et al., 2000). The OSPW are temporarily stored in the tailing ponds due to the zero-discharge approach in the environment maintained by the Alberta's regulatory framework (Allen, 2008b). Currently, the stored OSPW is continuously recycled back into the extraction process; however, large volume of fresh water ultimately ends up as OSPW. Approximately 1 billion m^3 of OSPW is currently stored in the Mildred Lake Settling Basin at Syncrude (MacKinnon et al., 2005). The accumulation of the OSPW in the tailings ponds causes considerable environmental concerns (Zubot et al., 2010). In the future, the oil sands companies need to remediate OSPW for a safe discharge into the environment to minimize the intake of fresh water from the Athabasca River (Allen, 2008a).

For the safe release of OSPW to the environment, the quality of treated OSPW should meet the standard guidelines; therefore, the removal of NAs should be considered as the highest treatment priority due to the greatest risk to the aquatic life (Allen, 2008a). In order to remove the contaminants of major concern including NAs, sulphate, chloride, ammonia, dissolved organic compounds, and insoluble hydrocarbons, a series of treatment processes is needed (Zubot et al., 2010). In the recent years, the application of various treatment processes for OSPW treatment has been investigated, such as, coagulation-flocculation, sedimentation, adsorption, membrane filtration, chemical oxidation and biological treatment. The choice of treatment process applied is highly dependent on the constituents of OSPW, release regulations, operational cost and treatment demands. Therefore, there is significant work to be done to develop the effective and applicable treatment technologies for the OSPW treatment at industrial scale. The advantages, disadvantages, and possibilities of those water treatment technologies are discussed below.

2.3.1 Physical and Chemical Processes

Coagulation/flocculation/sedimentation (CFS) is a common pre-treatment process for suspended solids, colloidal particles and insoluble hydrocarbons removal in municipal and industrial wastewater treatment (Duan and Gregory, 2003; Tansel and Regula, 2000). It was found that CFS with polyaluminum chloride, alum, and ferric sulfate was an effective treatment technique for petroleum refining wastewaters treatment (Santo et al., 2012). Pourrezaei et al. (2011) observed 37% classical NAs and 86% oxidized NAs removals for the OSPW treatment using an enhanced coagulation treatment process with chemical alum and polymer. Therefore, CFS is a potential pretreatment option for the removal of suspended solids and colloidal particles from OSPW. In addition, the enhanced CFS also is effective for the removal of AEF and NAs

from OSPW.

Adsorption has been widely applied to remove a variety of contaminants (e.g., soluble organic carbon compounds; oil and grease, and heavy metals) associated with oilfield-produced waters. Materials used as adsorbents to remove NAs from OSPW include petroleum coke (PC), granular activated carbon (GAC), cyclodextrin (CD)-based porous materials and soils (Janfada et al. 2006, Gamal El-Din et al. 2011). Zubot et al. (2012) used petroleum coke as an adsorbent to remove total acid-extractable organics (TAO) from OSPW with adsorption capacities between 0.1 to 0.46 mg/g when TAO concentration was 60 mg/L. Mohamed et al. (2011) investigated the adsorption of NAs using GAC and three types of synthetic materials. The sorption range for GAC was 100 to 160 mg NAs/g of GAC, while the CD based polymeric materials sorbed 20 to 30 mg NAs/g.

In the past two decades, polymer and ceramic membranes have been investigated by the oil industry for the potential removal of oil, suspended solids, and other contaminants from process waters (Farnand and Sawataky, 1985; Zenon Environment 1987). Membrane filtration has shown great performance for reducing the total dissolved solids (TDS) concentration in OSPW (Kim et al., 2011). Membrane filtration was used for dewatering and deoiling of OSPW sludge, but the associated problem was bitumen attachment on the polymeric parts of the pump (Farnand and Sawataky, 1985). A modified ceramic-supported polymer (CSP) membrane performed well for the removal of oil-water emulsions because it reduced the number of functional groups on the active surface layer of the membrane. Reverse osmosis (RO) was employed by Mehrotra and Banerjee (1986) for oil sands *in-situ* produced water treatment with 95% total organic carbon (TOC) and 98% TDS removals. However, membrane fouling issue decreases permeate flux, increases transmembrane pressure, and eventually raises the operating costs. It is well known

that the low flux rates, membrane durability, and flux decline are the major technological concerns with respect to the full-scale application of membrane for OSPW treatment.

Advanced Oxidation Processes (AOPs) have been extensively recognized as effective treatment processes for the treatment of recalcitrant wastewaters (Mantzavinos and Psillakis, 2004). For OSPW, the degradation of recalcitrant NAs using AOPs can lead to increased biodegradability of these compounds. However, using chemical oxidation for the complete mineralization of compounds leads to unacceptably high operational costs (Marco et al., 1997). Ozone treatment is a useful pre-treatment to break down NAs in OSPW to enhance the biodegradability of OSPW (Scott et al., 2008; Martin et al., 2010; Wang et al., 2013). This treatment leads to a decrease in OSPW toxicity by decreasing the concentration of NAs (Scott et al., 2005). The OSPW effluents were shown to be non-toxic to *Vibrio fisheri* after 50 minutes ozonation, and more than 95% NAs were removed after 130 minutes of ozonation (Scott et al. 2008).

2.3.2 Biological Treatment Processes

Biological treatment has been extensively used in the oil industry for the removal of organic carbon and nitrogen compounds. Tellez et al. (2002) found that 98%-99% of total petroleum hydrocarbons can be removed from the produced water, but the microbes need to acclimatize to optimized conditions before the high removal efficacy can be achieved. In contrast, other studies have shown poor treatment performance due to the toxic effects of produced water (Tellez et al., 2002). For example, only 14%-30% of phenols and 70%-73% total organics were removed from the process water by activated sludge treatment (Hansen and Davies, 1994).

Bioreactor technology has been successfully used in the wastewater treatment field for many years since it is an effective, economical, and energy efficient method for industrial wastewater treatment (DeFilippi and Lewandowski, 1998). The combination of an activated sludge system and microbial biofilm system in bioreactors helps to improve the removal of the toxic and recalcitrant organic compounds because the support of biofilm promotes microbial colonization where compounds could be degraded. Innovative bioreactor technologies have been used widely in the oil industry for the removal of the specific organic pollutants. Maerienssen and Simon (1996) reported that the TOC removal efficiency increased from 70% to 96% after adding powdered activated carbon (PAC) to a fluidized bed reactor to treat an oil-in-water emulsion. Hayes and Arthur (2004) reported that 98% BTEX can be removed by GAC-fluidized bed reactor (GAC-FBR) process at influent TDS concentrations as high as 15000 mg/L. Scholz and Fuchs (2000) used a membrane bioreactor to remove surfactants from oil contaminated water with results showing 99.9% of oil, 98% of surfactants, and 93% to 95% of COD removals.

The potential for using the bioreactor treatment process for OSPW has only recently been proposed (Hwang et al., 2013; Islam et al., 2014; Choi et al., 2014; Shi et al., 2015; Huang et al., 2015; Xue et al., 2016). For example, 41.0 % of COD, 18.5% NAs and 13.8% of the AEF were removed from OSPW using a laboratory scale 1L continuous biofilm reactor operated with an average hydraulic retention time (HRT) of 24 hours (Huang et al. 2013). Using a 0.3 L fluidized bed biofilm reactor (FBBR) with GAC as support media, Islam et al. (2014) demonstrated 62% of COD, 88% of AEF and 99.9% of classical NAs removal with HRT of 8.5 hours. Recently, Choi et al. (2014) evaluated the potential application of activated sludge flocs for OSPW treatment. It was observed that COD removal reached 12% and 20% in the activated sludge-sequencing batch reactor (AS-SBR) and the mature fine tailings-sequencing batch reactor (MFT-

SBR). The maximum removal of AEF was 8.7% and 16.6% in the AS-SBR and the MFT-SBR, respectively. Shi et al. (2015) observed the removal of 18.3% of the AEF and 34.8% of the NAs without OSPW ozonation and removals of 41% of the AEF and 78.8% of the NAs with OSPW ozonation. Huang et al. (2015) demonstrated that 12.1% of the AEF and 43.1% of parent NAs were removed in IFAS treatment of raw OSPW, while 42.0% of the AEF and 80.2% of parent NAs were removed in IFAS treatment of ozonated OSPW. Xue et al. (2016) evaluated the feasibility of OSPW treatment using a modified Ludzack-Ettinger membrane bioreactor (MLE-MBR) with a submerged ceramic membrane under both nitrifying and nitrate-reducing conditions. It was found 51% of total COD and 24.7% of NAs were removed after 361 days of operation.

Bioreactors that contain biofilms and activated sludge flocs might have a significantly higher NA removal efficiency than the conventional activated sludge system due to the increased microbial populations being able to increase the rate of pollutant degradation. In addition, the attached biofilm system can maintain a high bacteria retention time, so, it can support the development of slowly growing NAs degraders. Therefore, a more suitable bioreactor should be selected for OSPW treatment due to the low biodegradability of OSPW.

2.3.3 Combined Treatment Processes

None of the individual cost-efficient water treatment processes are effective for the removal of all contaminants from OSPW to achieve the requirements for recycle and safe discharge to the receiving environment. Therefore, it is necessary to develop an efficient, economical and feasible strategy for the OSPW remediation through combining different water treatment processes.

Toxic and recalcitrant wastewaters can be resistant toward biodegradation using the conventional biological processes due to specific structures of the recalcitrant compounds. Ozonation has been applied as a pretreatment for a variety of industrial wastewaters, such as pulp and paper wastewater (Oeller et al., 1997; Helble et al., 1999), petrochemical wastewater (Lin et al., 2001), oil field drilling wastewater (Wang et al., 2004), and olive oil production (Amat et al., 2003; Bettazzi et al., 2006). It was found that ozonation was able to improve the biodegradability through the following phenomena: (i) ozonation can change the form of organic compounds to be more biodegradable for microorganisms; (ii) ozonation can remove some compounds that show toxicity to the microorganisms; (iii) ozonation is able to breakdown the high molecular weight organic compounds into low molecular weight compounds.

It has been reported that OSPW NAs are highly resistant toward biodegradation due to the extensive cyclical nature of their molecular structures. Previous studies showed that ozone treatment might be used as a pretreatment option for bioreactor treatment to break down recalcitrant NAs and enhance OSPW biodegradability (Dong et al., 2015). Ozonation can break down highly branched and/or highly cyclic fractions of NAs. In addition, previous studies also showed that ozone treatment helps to reduce OSPW toxicity toward *Vibrio fischeri* (Martin et al., 2010; Gamal El-Din et al., 2011). Based on the evaluation of ozonation and bioreactor treatment technologies, the ozone-bioreactor combined treatment process might be an ideal candidate for the OSPW remediation.

2.4 Integrated Fixed-Film Activated Sludge (IFAS) Reactor

The integrated fixed-film activated sludge (IFAS) reactor represents a novel reactor design, consisting of a suspended growth system and an attached biofilm system in one bioreactor. The IFAS configuration utilizes synergetic interactions between biofilms on carriers and suspended

biomass in activated sludge, thus increasing microbial population diversity and the rates of contaminant degradation. The hybrid nature of the IFAS increases the surface area of the media, allowing larger areas for attachment and growth of microorganisms and enabling the treatment of wastewater with low biodegradability with lower reactor volume (Germain et al., 2007).

Compared to similar technologies, IFAS reactors generally have higher effective biomass (Stricker et al., 2007), a higher nitrification rate (Kim et al., 2011), greater resistance towards organic and hydraulic shock loading (Mendoza-Espinosa and Stephenson, 2001), and lower sludge production (Sriwiriyarat et al., 2008). The hybrid nature of the IFAS system makes them an ideal candidate for OSPW treatment.

IFAS is ideal option to upgrade existing wastewater treatment plant, because new tanks are not necessary for the upgrading (Di Trapani et al., 2010). Compare to the traditional suspended growth systems, IFAS system has 50% more biomass (Stricker et al., 2007), and IFAS system improves the nitrification and denitrification performance due to the formation of anaerobic and anoxic conditions in the deeper layers (Germain et al., 2007). A reduced food to microorganism ratio (F/M ratio) and higher solid retention time (SRT) result in reduced sludge production (Sridiriyarat et al., 2008). However, IFAS has its limitations, such as: (i) more energy input than conventional activated sludge (CAS) processes due to free moving media; (ii) more complex operations and operating appurtenances than CAS processes, such as relocating media; and (iii) potential odor issue when the tank is dewatered.

IFAS technology has been commonly used to upgrade and expand existing municipal wastewater treatment plants. In the past few decades, a large number of IFAS full-scale facilities are in operation in the United States, Canada and Europe (Jackson et al., 2007; Mahendran et al., 2012).

There is a definite link between the media type and the performance of IFAS. Generally, the types of media in the IFAS system could be divided into fixed media and free-floating media. It has been reported that the properties of media can significantly influence the formation of biofilm, the quantity of attached biomass, the microorganism community composition and treatment performance (Dong et al., 2011). A wide range of biofilm carriers, including polyethylene (PE), polyurethane (PU), GAC and inorganic carrier (sands, graystone, limestone, and slag), had been used in IFAS systems. Therefore, the selection of proper biofilm carrier plays the key role in the IFAS operation for the wastewater treatment.

Successful treatment using IFAS has been achieved for a large variety of recalcitrant organic compounds removed from industrial wastewaters and oilfield produced wastewaters (Jou et al., 2003). Moreover, the hybrid nature of the IFAS increases the surface area of the media, allowing larger areas for attachment and growth of microorganisms and supports the growth of slow NAs degraders. The application of ozonation combined IFAS system could potentially be an effective technology for the OSPW remediation.

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CHAPTER 3. TREATMENT OF OIL SANDS PROCESS-AFFECTED WATER (OSPW) USING OZONATION COMBINED WITH INTEGRATED FIXED-FILM ACTIVATED SLUDGE (IFAS)[1]

3.1 Introduction

The oil sands deposits in Alberta, Canada are the second largest oil reserve (over 169 billion barrels of recoverable bitumen) in the world (Teare et al., 2012). The caustic hot water extraction process used to separate bitumen from associated sands and clays results in a considerable volume of fine tailings—clay, sand particles, and residual bitumen—which are temporarily stored due to a policy of zero-discharge in the environment maintained by Alberta's regulatory framework. A large amount of oil sands process-affected water (OSPW) accumulates in tailings ponds because three volumes of fresh water are utilized to produce one volume of synthetic crude oil during the mining process (Allen, 2008b). OSPW contains high concentrations of petroleum hydrocarbons, organic acids, oilfield chemicals, salts, suspended solids, and heavy metals, therefore its discharge without treatment can cause considerable environmental problems. Water treatment strategies are urgently required to allow tailings water to be recycled or to be released safely to the environment, and to reduce the need to withdraw fresh water from the Athabasca River (Hwang et al., 2013).

Raw OSPW contains about 2000 to 2500 mg/L of inorganic and organic dissolved solids

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(Allen, 2008a). The primary toxic constituents of OSPW are naphthenic acids (NAs) (Scott et al., 2005), comprising about 50% of the acid extractable organic fraction (AEF) in OSPW (Garcia-Garcia et al., 2011; Pourrezaei et al., 2011). Classical NAs are a complex mixture of alicyclic and noncyclic alkyl-substituted carboxylic acids with the general chemical formula $C_nH_{2n+Z}O_2$, where n indicates the carbon number and Z shows the number of hydrogen atoms lost in the formation of rings or double bonds. Normally, Z is zero or negative due to ring formation (Clemente and Fedorak, 2005; Afzal et al., 2012). NAs are concentrated to 20–120 mg/L in the hot water bitumen extraction process, depending on the nature of the extraction process, the age of the tailings, and NA detection methods (Clemente and Fedorak, 2005; Allen, 2008a). Cost-effective removal of NAs from OSPW is a key determinant of OSPW remediation (Kim et al., 2013).

Biodegradation using bioreactor technology is an effective, economical, and energy efficient industrial wastewater treatment (Di Iaconi et al., 2003; Abd-Elsalam and EL-Hanafy, 2009). However, our previous studies showed that OSPW has a low biodegradability (Biological Oxygen Demand (BOD)/Chemical Oxygen Demand (COD) ratio < 0.1), and OSPW generally contains a low COD concentration (250–350 mg/L). Therefore, the keys for successful bioreactor operation are (1) to encourage the growth of carbon and NA degrading microorganisms and (2) to enhance the biomass concentration. Recently, both suspended growth and attached growth bioreactors have been tested for OSPW remediation. For example, 41.0 % of COD, 18.5 % NAs and 13.8 % of the AEF were removed from OSPW using a laboratory scale 1L continuous biofilm reactor operated with an average HRT of 24 hours (Hwang et al., 2013). Using a 0.3 L fluidized bed biofilm reactor (FBBR) with granular activated carbon (GAC) as support media, Islam et al. (2014a) demonstrated 62% of chemical oxygen demand (COD), 88% of AEF and

99.9% of classical NAs removal with HRT of 8.5 hours. Recently, Choi et al. (2014) evaluated the potential application of activated sludge flocs for OSPW treatment. It was observed that COD removal reached 12% and 20% in the activated sludge-sequencing batch reactor (AS-SBR) and the mature fine tailings-sequencing batch reactor (MFT-SBR), respectively, and the maximum removal of AEF was 8.7% and 16.6% in the AS-SBR and the MFT-SBR, respectively. McKenzie et al. (2014) investigated the treatment of NAs in OSPW using two immobilized soil/sediment bioreactors (ISBRs) operated continuously for 16 months with OSPW, and they found 38 ± 7% NA removals were consistently achieved at a residence time of 160 h at a removal rate of 2.32 mg NAs L^{-1} d^{-1}. It should be noted that attached and suspended bioreactors differ not only in their reactor configuration (with and without clarifier). Other factors, such as dominant microbial population, substrate uptake rates, specific microbial growth rates and yield, as well as the oxygen transfer efficiency in attached and suspended growth systems are all different. Hereby, certain groups of compounds may be easily removed from biofilms systems but cannot be impacted by the suspended flocs, and vice versa. For instance, the attached biofilm system maintains a high bacteria retention time, preventing biomass loss and thus supporting the growth of slow NAs degraders. However, microorganisms such as denitrifiers tend to stay in the suspended phase in activated sludge and do not form biofilms well. (Mazumder et al., 2010)

The integrated fixed-film activated sludge (IFAS) reactor represents a novel reactor design, consisting of a suspended growth system and an attached biofilm system in one bioreactor. The IFAS configuration utilizes synergetic interactions between biofilms on carriers and suspended biomass in activated sludge, thus increasing microbial population diversity and rates of contaminant degradation. IFAS reactors promote the growth of both suspended and attached

biomass. The mobile IFAS media provide large areas for biofilm development and growth, and enable the treatment of wastewater with low biodegradabiliy with a lower reactor volume (Germain et al., 2007). Compared to similar technologies, IFAS reactors generally have higher effective biomass (Stricker et al., 2007), a higher nitrification rate (Kim et al., 2011), greater resistance toward organic and hydraulic shock loading (Mendoza-Espinosa and Stephenson, 2001), and lower sludge production (Sriwiriyarat et al., 2008). The hybrid nature of the IFAS system makes them an ideal candidate for OSPW treatment. To date, no study has been reported on the application of bioreactors combining biofilms and activated sludge processed for OSPW treatment.

Previous studies showed that ozone treatment might be used as a pretreatment option for bioreactor treatment to break down recalcitrant NAs and enhance OSPW biodegradability (Dong et al., 2015; Islam et al., 2014b). Ozonation can break down highly branched and/or highly cyclic fractions of NAs. Previous studies also showed that ozone treatment helps to reduce OSPW toxicity toward *Vibrio fischeri* (Martin et al., 2010; Gamal El-Din et al., 2011). Ozonation combined with IFAS might be a promising process for OSPW treatment.

The present study tested the effects of ozonation in an IFAS system used to remediate OSPW. Two IFAS systems were operated in parallel—one to treat raw OSPW and one to treat ozonated OSPW. Aerobic activated sludge taken from the Gold Bar Wastewater Treatment Plant (Edmonton, Canada) was applied to degrade and remove NAs from both ozonated and raw OSPW. Polyethylene (PE) media was employed in the IFAS systems. Biodegradation of OSPW and AEF, including NAs, in IFAS systems were evaluated. Activated sludge floc and biofilm samples from the reactors were characterized in terms of their physiochemical properties, morphological features, and microbial community composition. Significant changes in the

biofilm microbial community and its density and thickness were also investigated. This study will benefit the modelling and optimization of IFAS system as we know of no other study that characterized flocs and biofilms taken from an IFAS system treating OSPW.

3.2 Materials and Methods

3.2.1 Source Water Information

OSPW was obtained from the West In-Pit water pumping station at Syncrude Canda Ltd. Fort McMurray, AB, Canada, in September 2013. OSPW characteristics are listed in Table B1 (Appendix-B). OSPW samples were stored in 200 L polyvinyl chloride barrels in a cold room (4 °C) and were well mixed before being used for testing.

The 0.1% (v/v) trace nutrient solution contained: $MgCl_2 \cdot 6H_2O$, 3 g L^{-1}; $CaCl_2$, 1.5 g L^{-1}; $FeSO_4 \cdot 7\ H_2O$, 0.28 g L^{-1}; $MnCl_2 \cdot 4\ H_2O$, 0.13 g L^{-1}; $ZnSO_4 \cdot 7\ H_2O$, 0.12 g L^{-1}, $CuSO_4 \cdot 5\ H_2O$, 0.0074 g L^{-1}. The pH was 7.3 ± 0.3. All chemicals and supplies were obtained from Thermo Fisher Scientific.

3.2.2 IFAS System Operation

The bench-scale IFAS reactor used in these experiments had an area of 0.15 m × 0.35 m and a height of 0.3 m (Fig. C1) (Appendix-C). The reactor was separated into two cells, the aeration tank (working volume 8.5 L) and clarifier. Two IFAS systems were employed in this study, one with raw OSPW as influent and the second with ozonated OSPW (30 mg/L ozone dose) as influent. A 60% volume fraction of polyethylene (PE) carriers (Bioflow 9, Rauschert, Steinwiessen, Germany) with specific biofilm growth areas of 800 m^2/m^3 were employed in the IFAS reactors.

During the acclimation stage of the IFAS system, the carbon source in the influents of the IFAS systems were raw or ozonated OSPW, commercial NAs (naphthenic acid, SIGMA-

ALDRICH, Belgium) and sodium acetate. Reactor operation was initiated by inoculating 2 L of activated sludge. Initially, 10% (volume ratio) raw or ozonated OSPW was introduced to the IFAS reactors and operation continued at room temperature (20 °C). Air was diffused from the bottom of the reactor using an aeration tube to supply oxygen to the biomass and mix with the carriers. After aeration for 24 h, one-half of the mixed liquid was drained off, the same volume of influent was introduced, and the system was left to operate for another 24 h. This procedure was repeated during the first week of startup. After that, influent was fed continuously for 320 days. During the reactor startup stage, we increased the volume ratios of raw and ozonated OSPW step by step (from 10% OSPW to 100% OSPW). From the 10% to 60% phase, commercial NAs (30 mg/L COD) were provided to help the growth of NA degraders in IFAS systems. During the entire startup stage, extra carbon (sodium acetate) as well as nitrogen (NH_4Cl, 30.0 ± 3.0 mg N/L), phosphorus (KH_2PO_4, 3.0 ± 0.2 mg P/L) and other necessary nutrients (mentioned in section 2.1) were provided to maintain the growth of biomass in the IFAS systems. The average activated sludge solid retention time (SRT) was 43 days, which was maintained through manually wasting sludge from the clarifier. The detailed start-up strategies are listed in Table 3.1. The hydraulic retention time (HRT) of two IFAS reactors was 48h. The dissolved oxygen (DO) concentration was in the range of 6–7 mg/L during the operation.

Table 3.1 The start-up strategies of IFAS systems (from day 0 to day 320)

The volume ratio of OSPW (%)	Total COD of raw OSPW IFAS influent (mg/L)	Total COD of ozonated OSPW IFAS influent (mg/L)	COD from raw OSPW (mg/L)	COD from ozonated OSPW (mg/L)	COD from commercial NAs (mg/L)	COD from sodium acetate (mg/L)
10	247.6	246.2	17.6	16.2	30	200
20	245.2	242.4	35.2	32.4	30	180
40	240.4	234.8	70.4	64.8	30	140
60	245.9	238.1	115.9	108.1	30	100
60	245.9	238.1	115.9	108.1	0	130
80	284.5	274.2	154.5	144.2	0	130
100	323.2	310.2	193.2	180.2	0	130

3.2.3 Ozonation of OSPW

A sufficient volume of OSPW was ozonated before the experiment. An ozone dose of 30 mg/L was applied based on our previous study (Dong et al., 2015). An AGSO 30 Effizon ozone generator (WEDECO AG Water Technology, Herford, Germany) was used to produce ozone gas from extra-dry, high-purity oxygen, which was sparged into the liquid phase by a ceramic bubble gas diffuser at the bottom of the container. The ozone concentration in the feed gas was continuously monitored by a high-concentration ozone monitor (HC500, WEDECO, Charlotte, NC, USA). The residual ozone concentration was monitored utilizing the indigo method (APHA, 2005). Ozonated OSPW was purged with nitrogen for 10 minutes to strip oxygen and excess ozone.

3.2.4 Measurement of Organic Contaminants

Acid extractable fraction (AEF). A Fourier transform infrared spectrometer (Spectrum 100, PerkinElmer Ltd, Bucks, UK) was employed to measure the AEF. Samples (25 mL) from influent and effluent of each IFAS system were filtered (0.45 µm), acidified to pH 2.0, and 3.759 g NaCl was added to each sample and dissolved (salt was used to prevent emulsion formation during shaking). Liquid-liquid extraction of organics from the samples was performed with a separation funnel using dichloromethane (DCM). DCM was evaporated and the extracted AEF was dissolved with a known mass of DCM and analysed by Fourier transform infrared spectrometry (FTIR). The sample absorbance was monitored at 1743 cm^{-1} and 1706 cm^{-1}, absorption bands characteristic of monomeric and dimeric carboxylic groups (Clemente and Fedorak, 2005).

HRMS analysis of NAs. The mass of each NA component was measured using ultra pressure liquid chromatography/high resolution mass spectrometry (UPLC/HRMS) according to

the method developed by Martin et al. (2010). Prior to analysis, 10 mL samples of influent and effluent from each reactor were filtered (0.22 µm nylon). Filtered samples (500 µL) were transferred to 2 mL glass vials containing 450 µL methanol and 50 µL tetradecanoic-1-13C acid (internal standard). Separation of parent NAs and oxidized products was performed by UPLC using a Waters Acquity UPLC@ System (Milford, MA, USA). More details of the UPLC/HRMS analyses are provided in the section A-3 (Appendix-A).

3.2.5 *Vibrio fischeri* Bioassay

Toxic effects of samples from the IFAS systems toward *Vibrio fischeri* were determined using a Microtox@ 500 analyzer (Azur Environmental, Carlsbad, USA) operated with 15 min exposure time and the 81.9% Basic Test Protocol (AZUR Environmental, Microtox Omni Software). Inhibitory effects toward the luminescent bacteria were calculated from the change in luminescence intensity. Phenol toxicity was measured as a quality control prior to the analysis of samples from IFAS systems.

3.2.6 Microbiological Analysis

Genomic DNA was extracted from activated sludge flocs and biofilms in raw and ozonated OSPW IFAS systems. To obtain samples of activated sludge flocs, 4 mL of reactor liquid were centrifuged at 10,000 g for 2 min; supernatants were discarded. To gather microorganisms from the biofilms formed on the carriers, the carriers were immersed in phosphate buffered saline (PBS) to remove nonadhearent bacteria, placed in 5 mL sterile PBS in a 20 mL sterile glass bottle (Falcon, CA, USA), and sonicated for 10 min. The glass bottle was then vortexed for 1 min until the biofilm was completely removed from the carrier. The mixed liquid in the glass bottle was used for biofilm DNA extraction. After sampling the carriers, the same number of new carriers was placed into in each reactor.

To observe changes in nitrifier and denitrifier genes abundance in the suspended phase (activated sludge flocs) and the attached phase (biofilms) versus the percentage of OSPW during the startup period, *q*-PCR assays were performed using a CFX96TM Real-Time Detection System (Bio-RAD, California, USA). Details of the *q*-PCR experiments are provided in the section A-4 (Appendix-A).

3.2.7 Biofilm Thickness and Surface Morphology Characterization

Biofilm images were acquired and biofilm thickness was determined with a confocal laser scanning microscope (CLSM, Zeiss LSM 710, Carl Zeiss Micro Imaging GmbH, Germany). Biofilm samples were directly stained by using LIVE/DEAD® BacLightTM Bacterial Viability Kits. The thickness of the biofilm on the inner part of the carrier media was monitored using CLSM. Details of biofilm thickness measurement are provided in the section A-5 (Appendix-A).Further, biofilm structure and morphology were characterized using a scanning electron microscope (S-2500, Hitachi, Japan). Details of the pre-treatment of biofilm samples for SEM are provided in the section A-6 (Appendix-A).

3.2.8 Statistical Analysis

A statistical analysis was performed to evaluate the IFAS treatment performance. An analysis of variance (ANOVA) was carried out using Microsoft Excel® software. Correlations were considered statistically significant at the 95% confidence interval ($p < 0.05$).

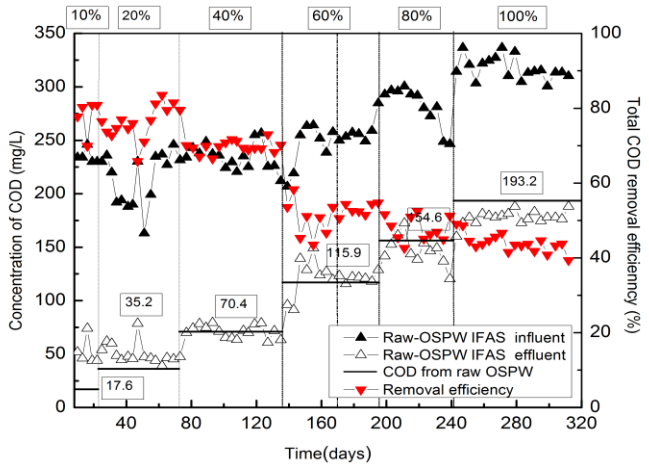
3.3 Results and Discussion

3.3.1 Performance of Treatment Systems

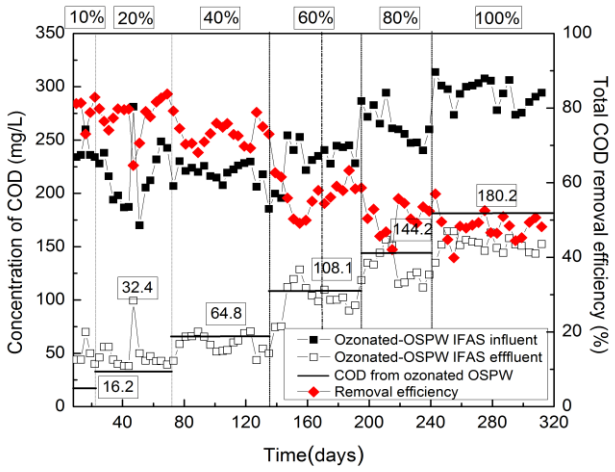
3.3.1.1 Biodegradation of COD

As shown in Fig. 3.1(A) and 3.1(B), at an OSPW volume percentage of 10%, the average

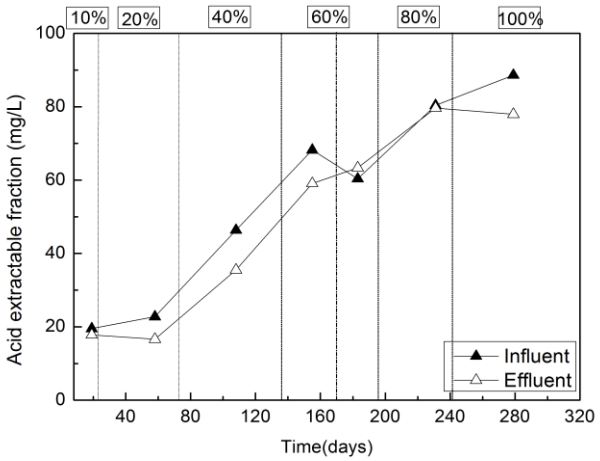
(A) **Raw-OSPW IFAS**



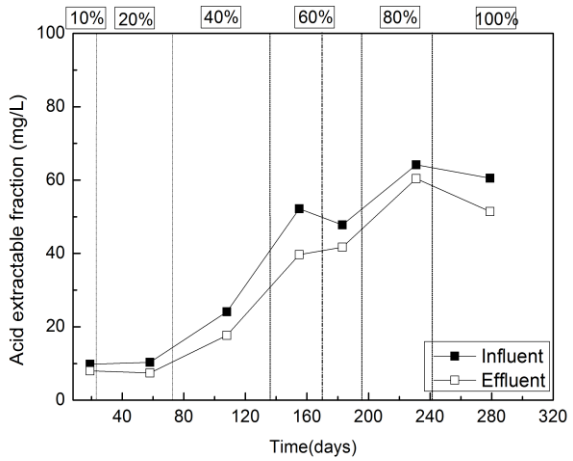
(B) **Ozonated-OSPW IFAS**



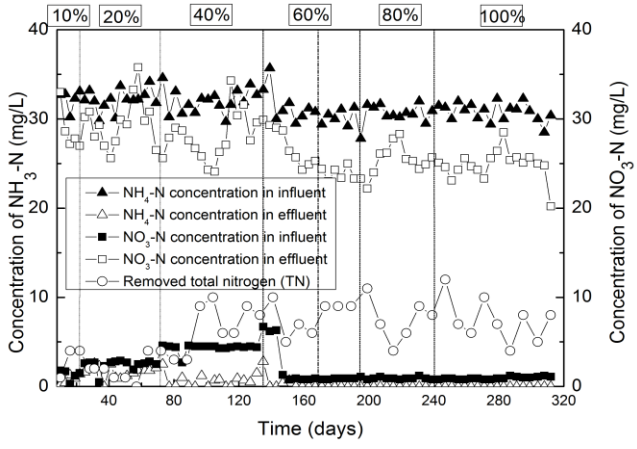
(C)



(D)



(E)



(F)

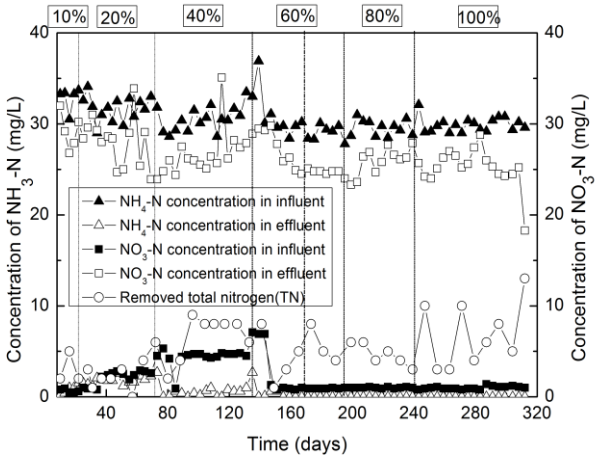


Figure 3.1. Profile of COD concentration in influent and effluent and COD removal efficiency over reactor operation time in raw (A) and ozonated (B) OSPW IFAS systems, Profile of AEF concentration in influent and effluent in raw (C) and ozonated (D) OSPW IFAS systems at different periods, Profile of NH_4-N and NO_3-N concentration in influent and effluent and removed total nitrogen over reactor operation time in raw (E) and ozonated (F) OSPW IFAS system.

efficiency of COD removal was around 82% in each reactor. There was no significant difference in COD removal between raw and ozonated OSPW. When the volume percentage of OSPW increased from 10% to 20%, however, the COD removal efficiencies decreased at the beginning. After a period of adaptation, when the volume percentage of OSPW increased to 20%, the COD removal efficiencies recovered to previous levels in both reactors. This observation suggests that the bacteria need time to get used to the increase percentage of OSPW. When the volume ratio of OSPW was further increased from 20% to 40%, the average COD removal efficiencies of the two reactors again exhibited a downward trend. The COD removal efficiencies decreased from an average value of about 78% to 70% in raw OSPW and from 80% to 75% in ozonated OSPW. The average COD removal during this period was slightly higher ($p < 0.05$) in the ozonated OSPW IFAS than in the raw OSPW IFAS. When the volume percentage of OSPW was increased from 40% to 60%, the COD removal efficiencies further decreased from an average value of about 70% to 51% in raw OSPW and from 75% to 59% in ozonated OSPW. Eventually, when the OSPW volume percentage was increased to 100%, the COD removal efficiencies were 43% ± 2.4% in raw OSPW, and 51% ± 3.5% in ozonated OSPW. The total COD removal efficiency was found to be higher in the ozonated OSPW IFAS than in the raw OSPW IFAS ($p < 0.05$),

which indicates that ozonation treatment enhanced COD removal efficiency. The observed enhancement is thought to be a result of the oxidation of organic compounds, which facilitates their biodegradation (Hwang et al., 2013, Islam et al. 2014a).

3.3.1.2 AEF Removal

The AEF was commonly used by the oil sands industry to evaluate the extent of biodegradation of OSPW. Fig. 3.1(C) and 3.1(D) show the AEF reduction in raw and ozonated reactors. The AEF fraction in the influent and effluent of the IFAS reactors, including parent NAs ($C_nH_{2n+Z}O_2$), oxy-NAs ($C_nH_{2n+Z}O_x$, x = 3–5), and other organics containing carboxylic groups, was monitored over reactor operation time by FTIR (Grewer et al., 2010). AEF concentrations in the influent of the ozonated OSPW IFAS system were significantly lower than AEF concentrations in the influent of the raw OSPW IFAS system over the time of reactor operation, which indicate that ozone treatment can significantly reduce the AEF level. When the volume ratio of OSPW increased from 10% to 60% (Fig. 3.1(C) and 3.1(D)), removed AEF increased from 1.7 to 9.1 mg/L in raw IFAS and from 1.8 to 12.5 mg/L in ozonated IFAS, indicating that AEF removal increased when the OSPW percentage increased. This observation may be attributed to the development of AEF degraders at higher OSPW percentage. Microorganisms in bioreactors began to utilize oxidized the branched alkyl groups, ethanoate groups, or alicyclic rings of AEF compounds when OSPW percentage increased in the reactors.

It was noted that AEF removal was significantly reduced when we stopped adding commercial NAs to reactor influent on day 172. This observation indicates that in the presence of commercial NAs, microorganisms in bioreactors mainly oxidized commercial AEF. When the volume ratio of OSPW was increased from 60% to 80%, only 0.74 and 3.81 mg/L AEF was removed in raw and ozonated OSPW, respectively. However, when the volume percentage of

OSPW was increased from 80% to 100%, 10.69 and 9.09 mg/L of the AEF were removed from the influent in raw and ozonated OSPW IFAS, respectively, indicating microorganisms in bioreactors are capable of utilizing OSPW AEF for their metabolism after an extended acclimation time.

The removal efficiency of COD was observed to be higher than the removal efficiency of AEF. For instance, when the volume ratio of OSPW was 100%, AEF removal percentages were 12.06% for raw OSPW and 15.02% for ozonated OSPW, whereas COD removal percentages were 43% for raw OSPW and 51% for ozonated OSPW IFAS systems. The higher removal percentages of COD compared to AEF can be attributed to (i) by-products of IFAS biodegradation such as oxidized (O_3 or higher) NAs and sulphur/nitrogenated (NAs + S/N) NAs which contributed to the AEF in the effluents, and (ii) the fast removal rate of non-AEF compounds such as sodium acetate and polycyclic aromatic hydrocarbons from OSPW (Choi et al. 2014). The removed AEF showed a significant upward trend with an increase in the volume percentage of OSPW in both IFAS reactors before we stopped adding commercial NAs (Fig. 3.1(C) and 3.1(D)). A paired samples t-test showed that AEF removal efficiency in the ozonated OSPW IFAS was significantly higher than that in the raw OSPW IFAS ($p < 0.05$). This supports the hypothesis that ozonation enhances organic contaminant removal through direct oxidation which facilitates biodegradation.

3.3.1.3 NAs Removal

UPLC/HRMS provides information about NA molecular weights and structures. Fig. C2 and Fig. C3 (Appendix-C) show multiple-dimension plots of NA concentration versus carbon number and Z value before and after ozonation and biological treatment. Ozone (30 mg/L) treatment decreased parent NA concentrations in OSPW from 27.69 to 14.97 mg/L and removed

more than 45% of the parent NAs in OSPW. As was shown in Fig. C2 (Appendix-C), when the volume percentage of OSPW was 60%, IFAS biological treatment decreased parent NA concentrations in raw OSPW from 17.47 to 13.28 mg/L (removal efficiency = 23.98%). In ozonated OSPW, parent NA concentrations declined from 5.85 to 3.69 mg/L (removal efficiency = 36.92%). Parent NA profiles before and after IFAS biodegradation were similar in both raw and ozonated OSPW IFAS systems.

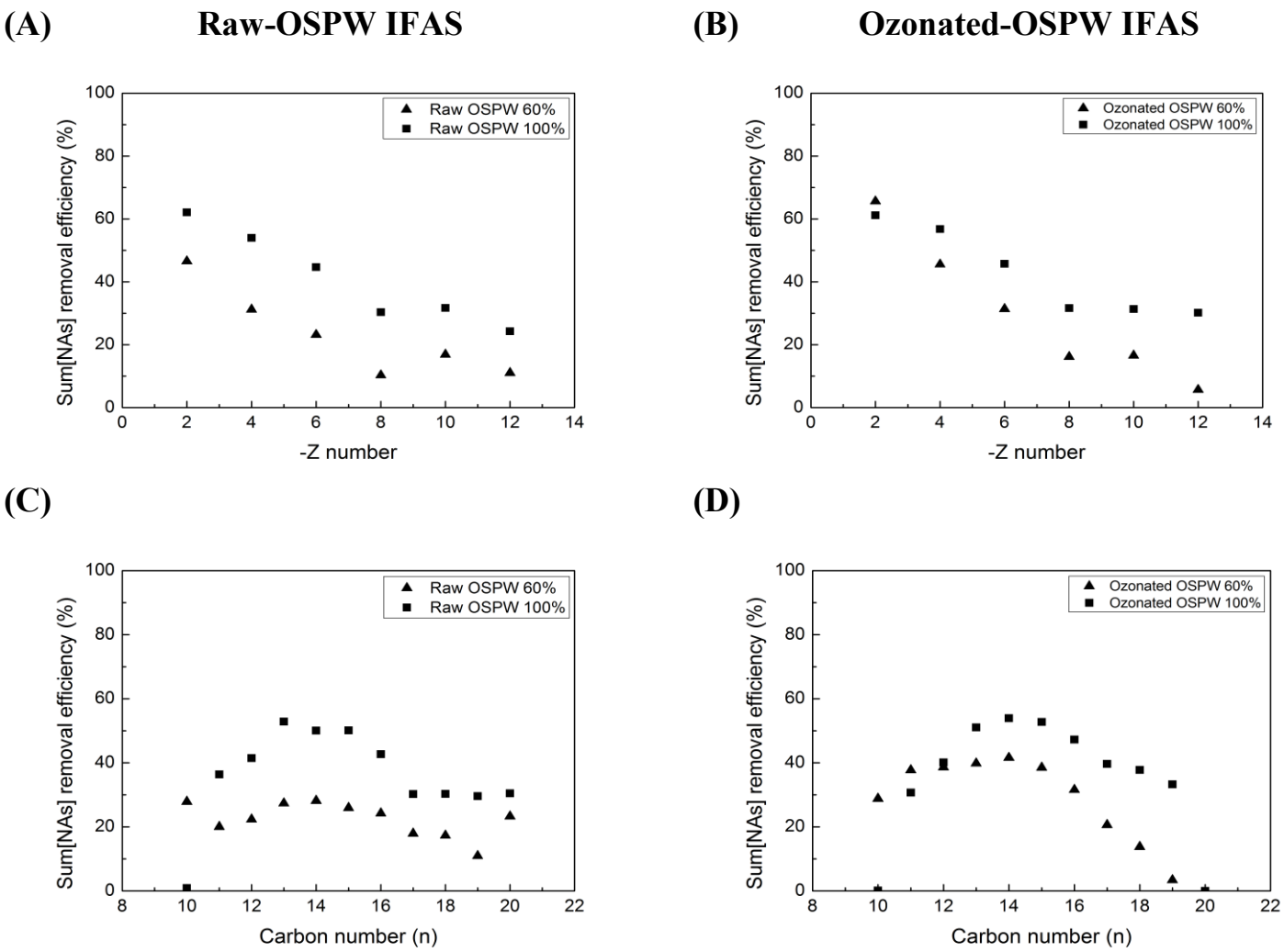


Figure 3.2. Profile of Sum [NAs] removal efficiency versus Z value (A) raw-OSPW IFAS, (B) ozonated-OSPW IFAS, profile of Sum [NAs] removal efficiency versus carbon number n (C) raw-OSPW IFAS, (D) ozonated-OSPW IFAS.

Fig. C3 (Appendix-C) shows that when the volume percentage of raw OSPW was 100%, in raw-OSPW the parent NA concentrations decreased from 25.13 to 14.29 mg/L (removal efficiency = 43.14%) after IFAS biodegradation. In a 100% volume percentage of ozonated OSPW, parent NA concentrations declined from 9.49 to 4.98 mg/L (removal efficiency = 47.52%) after IFAS biodegradation. Parent NA removal efficiencies in both IFAS systems exhibited a significant upward trend when the volume percentage of OSPW increased from 60% to 100%; that is, parent NAs biodegradation increased with an increase in influent OSPW volume ratio, indicating the increased NAs biodegradability of microorganisms from bioreactors during the acclamation process. .

Fig. 3.2 shows the standardized NA removal efficiency, Sum [NAs] removal efficiency based on Z number or carbon number in both IFAS systems after biodegradation. Our results showed that the removal of parent NAs decreased with the increase of Z number for any carbon number. For instance, the standardized NA removal efficiency for 100% OSPW treatment decreased from 62.1% at z = -2 to 24.3% at z = -12 for raw OSPW IFAS and 61.1% at z = -2 to 30.2% at z = -12 for the ozonated-OSPW IFAS. The enhanced parent NAs removal with decreasing Z number has been observed in previous studies (Clemente and Fedorak, 2005; Martin et al., 2010; Hwang et al., 2013). The biodegradability and the rate of biodegradation of NAs are related to the molecular structure (Misiti et al., 2013). Under the aerobic condition, the rate and degree of biodegradation was lower for NAs with higher cyclicity (i.e., high Z number) and alkyl branching (Smith et al., 2008). Moreover, the decrease of NA biodegradation rates is likely associated with the actual number of rings and possibly the number and size of alkyl side chains (i.e., alkyl branching) (Toor et al., 2013).

There is no obvious regularity in the effect of carbon number (n) on the parent NAs removal as shown in Fig. 3.2 (C) and 3.2(D). However, the highest removal was observed for parent NAs with carbon number ranging between 13 and 16. The low parent NAs removal with carbon number less than 13 may be attributed to the breaking down of large molecular weight organics into low carbon number organics after IFAS biodegradation. Similar results have been reported previously (IsIam et al., 2014a). It is also noted that high carbon number NA molecules have higher nonpolarity and hydrophobicity as compared to low carbon number molecules (Zubot et al., 2012), which should result in a higher affinity of larger molecules to absorb onto the surface of activated sludge flocs and biofilm and enhanced biodegradability. However, NAs with higher carbon number ($n > 16$) were more persistent than those with lower carbon numbers ($n < 15$), this trend was consistent with previous reports (Toor et al., 2013). In general, studies investigating the influence of NA structure on biodegradability have shown that the more complex NAs, i.e., those with higher cyclicity, branching and molecular weight, are the most recalcitrant (Han et al., 2008; Smith et al., 2008).

Parent NAs profiles before and after the IFAS biodegradation are shown in Fig. C2 and C3 (Appendix-C). It was noted that the highest removal was observed for parent NAs with the smallest Z number for $n = 13$ to $n = 16$ in both raw and ozonated OSPW IFAS systems. It indicates that compounds with low Z numbers were preferentially degraded. It is well know that NAs with fewer rings are more inclined to biodegradation. Our results are in agreement with the previous studies on biological treatment of OSPW parent NAs (Clemente and Fedorak, 2005; Martin et al., 2010; Hwang et al., 2013). Therefore, Z series numbers had more effect on the NA biodegradation than carbon number, which was consistent with the conclusion from Han et al. (2008). When the volume percentage of OSPW increased from 60% to 100%, the parent NAs

removal efficiency increased from 23.98% to 43.14% in raw OSPW IFAS, and from 36.92% to 47.52% in ozonated OSPW IFAS. It is also observed that parent NAs with higher initial concentrations were degraded with higher removal efficiencies, as compared to NAs with lower initial concentration. Similar results have been observed previously (Hwang et al., 2013; Martin et al. 2010).

3.3.1.4 Impact of Ozone Pretreatment

After 11 months operation, when the volume of OSPW reached 100%, 12.06% of the AEF and 43.14% of the NAs were removed by direct biodegradation in IFAS without ozone pretreatment, while 41.97% of the AEF and 80.18% of NAs were removed in the combined ozonation IFAS process. In the combined system, 31.72% of AEF and 62.23% of NAs removal was achieved due to ozone oxidation (ozone dose, 30 mg/L) and 10.25% of AEF and 17.95% of NAs removal was achieved due to the following IFAS biodegradation. Ozone pretreatment played a significant role in removing AEF and NAs from OSPW.

It should be noted that although the supplemented sodium acetate supported the microbial growth in the bioreactors, microbial population dynamics might be significantly affected. Without carbon supplements, NA degraders might slowly but steadily grow, and eventually dominate the microbial community in these reactors. The impact of a carbon source supplement deserves further study.

3.3.1.5 Nitrogen Removal

The time course of NH_4-N oxidation to NO_3-N is plotted in Fig. 3.1(E) and 3.1(F). During the entire treatment process, the NH_4-N concentration in the influent of both IFAS systems was maintained at approximately 32 mg/L. Both reactors performed well regarding ammonia oxidation. Most of the NH_4-N was oxidized to NO_3-N by nitrification. Evidence for nitrification

was obtained by measuring effluent nitrate production (Fig. 3.1 (E) and 3.1(F)). More than 95% of the initial NH_4-N was removed at all stages of IFAS operation. NH_4-N can be removal from wastewater in three ways: (a) assimilation into biomass, (b) volatilization, and (c) biological nitrification under aerobic condition and denitrification under anaerobic or anoxic condition (Gálvez et al., 2003).

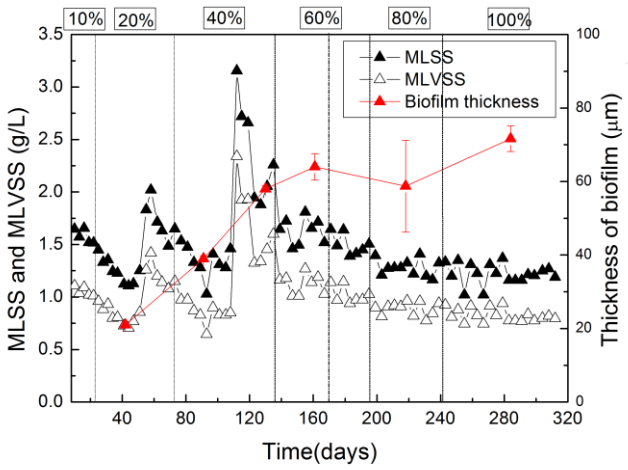
The concentration profile of total nitrogen at different stages of reactor operation is shown in Fig. 3.1(E) and 3.1(F); measurements were obtained after the IFAS systems had attained a steady state during the startup stage. The percentage of total nitrogen (TN) (NH_4^+-N, NO_2^--N, NO_3^--N) removal in the two IFAS systems at the beginning of the reactor operation (in the first ~100 days) was about 7–11%, which can be explained by the process of assimilation in the biomass. NH_4^+-N is the preferable source of nitrogen for assimilation (Wu et al., 2009). The TN removal percentage slow increased, and after day 120, the percentage of TN removal in the two IFAS systems was above 30 ± 2.5%, indicating the maturation of the biofilm and floc communities in the bioreactors and the establishment of the denitrification community. This is supported by the biofilm thickness results (Fig. 3.3 (A) and 3.3(B)) described in section 3.3.2.1.

3.3.2 Biomass Characteristics in IFAS Systems

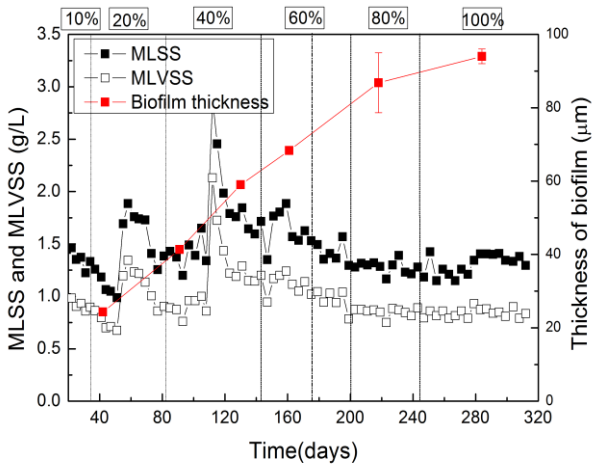
3.3.2.1 Suspended Activated Sludge

To determine the operational behaviour and biomass concentration in the IFAS system, mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were used to quantify the suspended biomass in the IFAS system. Fig. 3.3 (A) and 3.3(B) show MLSS and MLVSS variations during the startup operation. The sudden increase in MLSS/MLVSS was attributed to the reinoculation of the bioreactors with aerobic activated sludge from the Gold Bar Wastewater Treatment Plant (Edmonton, Canada). MLSS

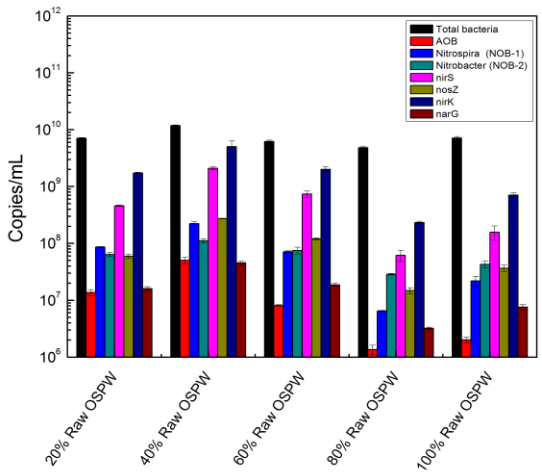
(A) **Raw-OSPW IFAS**



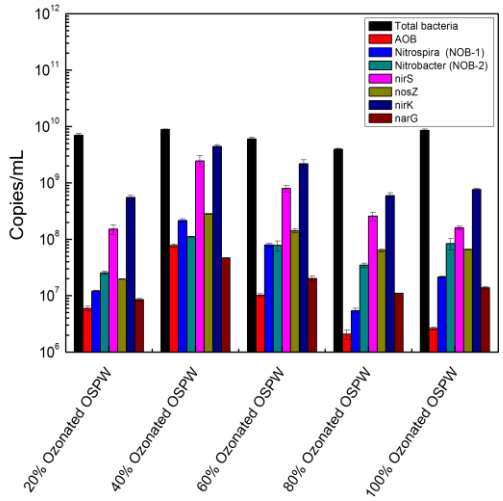
(B) **Ozonated-OSPW IFAS**



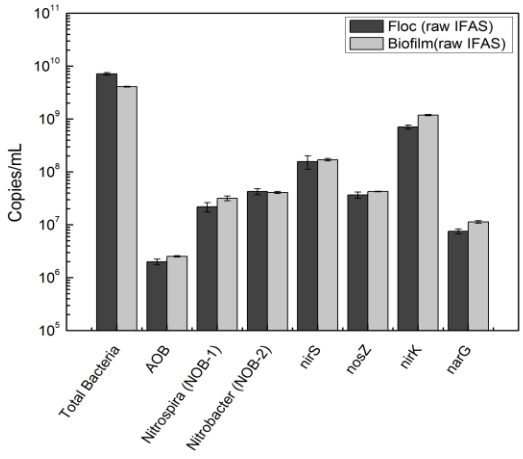
(C)



(D)



(E)



(F)

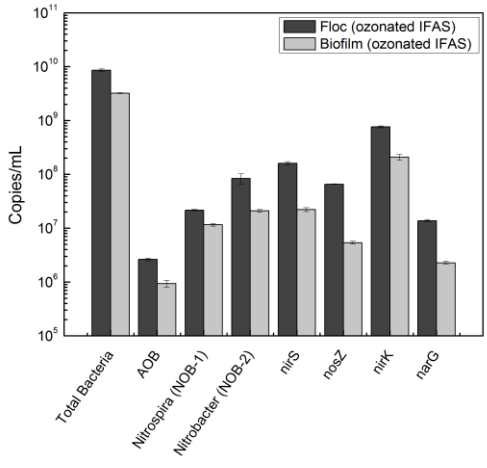


Figure 3.3. Variation of MLSS and MLVSS concentration and biofilm thickness during experimental operation in raw (A) and ozonated (B) OSPW IFAS systems, changes in copies per mL of the total bacteria, AOB, Nitrospira, Nitrobacter and the denitrifying bacteria (nirS, nosZ, nirK and narG) in the activated sludge flocs at different stages in raw (C) and ozonated (D) OSPW IFAS systems, the gene copies per mL of the total bacteria, AOB, Nitrospira, Nitrobacter and denitrifiers (DEN) between the activated sludge flocs and biofilm in raw (E) and ozonated (F) OSPW IFAS systems (100% phase).

concentrations in both IFAS systems were determined to be 1.6–1.7 g/L when the two IFAS systems were inoculated. The MLSS and MLVSS concentrations in both IFAS systems decreased with an increase in OSPW percentage in the influent, which may be attributed to the reduced biodegradable COD and increased toxicity at higher OSPW percentage conditions. When the percentage of OSPW was above 80%, MLSS concentrations in both IFAS systems tended to stabilize, and were maintained in a relatively constant range of 1.2–1.4 g/L.

3.3.2.2 Attached Biofilm

To visualize the presence of biofilm on the surface of carriers in the IFAS systems, SEM images of biofilm-containing carriers were prepared as shown in Fig. C4 (Appendix-C). Biofilm thickness at different cultivation time in the two IFAS systems is depicted in Fig. 3.3 (A) and 3.3(B). The biofilms in both raw and ozonated OSPW IFAS systems grew constantly from day 42 to 91 and 130, respectively. The biofilm thickness in both of the IFAS systems exhibited a significant upward trend. After 130 days of cultivation, the biofilm thickness was 60 ± 0.8 µm in both IFAS systems. After 283 days of cultivation, the biofilm in the ozonated OSPW IFAS was significantly thicker (94 ± 1.6 µm) than the biofilm in the raw OSPW IFAS (72 ± 2.8 µm) ($p <$

0.05). This implies that the ozone pretreatment of OSPW significantly influenced the biofilm thickness in IFAS. The thicker biofilm observed in ozonated IFAS might be attributed to the different biofilm morphology and structure in these two systems. Fig. C4 (Appendix-C) shows that the biofilms in raw OSPW IFAS are denser, while the biofilms in ozonated OSPW IFAS have porous structure.

3.3.2.3 Comparison Between Suspended and Attached Biomass Concentration

To observe the attached biomass concentration in the IFAS system, MLSS were used to quantify the attached biomass in the IFAS system. However, the MLSS measurement procedures applied for the biofilms might underestimate the attached biomass due to the residual biofilms existing on carriers after sonication treatment. After about 320 days of cultivation, the MLSS concentration of attached biofilm in raw and ozonated OSPW IFAS systems were 0.377 ± 0.12 g/L and 0.534 ± 0.19 g/L, respectively. There was again no significant difference between the two reactors ($p > 0.05$). The attached biomass was significantly lower than suspended biomass (1.2-1.4 g/L) in the IFAS system. This trend was consistent with the quantification of the gene copies of total bacteria of flocs and biofilm per mL in the Fig. 3.3 (E) and 3.3(F). It should be noted that the biofilm biomass was recovered by sonicating the biofilms samples to dissociate the attached biomass from the biofilm carriers. Although high biomass recovery has been observed in this study, our MLSS measurement procedure might underestimate the attached biomass concentration. In addition, the MLSS concentration of attached biofilm in ozonated OSPW IFAS systems was relatively higher than the raw OSPW IFAS system, which agreed with the biofilm thickness measurement in the Fig. 3.3 (A) and 3.3(B).

3.3.3 Quantification of Bacterial Populations by he Quantitative Polymerase Chain Reaction

3.3.3.1 Activated Sludge Flocs Bacterial Number

Fig. 3.3 (C) and 3.3(D) show that in all samples the total bacterial population in activated sludge flocs ranged from $4.84 \times 10^9 \pm 3.02 \times 10^8$ to $1.17 \times 10^{10} \pm 3.82 \times 10^8$ copies/mL in the raw OSPW IFAS system and from $3.97 \times 10^9 \pm 1.45 \times 10^8$ to $8.89 \times 10^9 \pm 1.67 \times 10^8$ copies/mL in the ozonated OSPW IFAS system. When the OSPW percentage increased from 40% to 100%, in raw OSPW IFAS system, the abundance of *amoA*, *NSR and Nitro* present in activated sludge flocs significantly decreased and similar trend has been observed in ozonated OSPW IFAS system. It has been reported that nitrifying organisms are sensitive to a wide range of organic toxic compounds at low concentration (Blum and Speece, 1991). The abundance decrease of ammonia oxidizing bacteria (AOB) relevant gene did not significantly impact ammonia removal in IFAS systems, possibly because the AOB population in bioreactors was sufficient to remove the ammonia in the systems.

When the OSPW volume increased from 40% to 100%, in the raw OSPW IFAS system, the abundance of denitrifier genes (*narG*, *nirS*, *nirK* and *nosZ*) present in activated sludge flocs significantly decreased and a similar trend was observed in the ozonated OSPW IFAS system. The intense competition for limited nutrients and higher toxicity due to the increased of OSPW led to the decrease of denitrifying bacteria relevant genes in the activated sludge flocs, which is supported by the toxicity result (Fig. 3.4) described in section 3.3.4. The detection of denitrifying bacteria in activated sludge flocs of IFAS systems could be explained by the existence of anoxic zones within the biological floc or biofilm (Liu et al. 2014). Dissolved oxygen and dissolved substrates outside the floc or biofilm diffuse into the aerobic zone. Oxygen may be depleted due

to the nitrification and organic substrates oxidation within the floc and biofilm. Thus, the DO cannot penetrate the entire floc and biofilm, the denitrification occurs at the inner or deep anoxic zone.

3.3.3.2 Comparison of Raw and Ozonated OSPW IFAS

The total bacteria population of activated sludge flocs and biofilms in ozonated OSPW IFAS system was comparable with raw OSPW IFAS system when the volume ratio of OSPW reached to 100%, in which the differences of the total bacterial number were all within one log unit. More specifically, the population of total bacteria of flocs was $7.16 \times 10^9 \pm 4.21 \times 10^8$ copies/mL in raw OSPW IFAS system and $8.60 \times 10^9 \pm 4.83 \times 10^8$ copies/mL in ozonated OSPW IFAS system, the population of total bacteria of biofilms was $4.09 \times 10^9 \pm 6.86 \times 10^7$ copies/mL in raw OSPW IFAS system and $3.21 \times 10^9 \pm 5.69 \times 10^7$ copies/mL in ozonated OSPW IFAS system. The abundance of four denitrifier genes (*narG*, *nirS*, *nirK*, and *nosZ*) in biofilms was $1.14 \times 10^7 \pm 6.15 \times 10^5$ copies/mL, $1.71\times 10^8 \pm 9.44 \times 10^6$ copies/mL, $1.19 \times 10^9 \pm 3.37 \times 10^7$ copies/mL, and $4.27 \times 10^7 \pm 5.76 \times 10^5$ copies/mL, respectively, in the raw OSPW IFAS system, and $2.28 \times 10^6 \pm 1.45 \times 10^5$ copies/mL, $2.22 \times 10^7 \pm 1.87 \times 10^6$ copies/mL, $2.09 \times 10^8 \pm 2.62 \times 10^7$ copies/mL, and $5.38 \times 10^6 \pm 3.42 \times 10^5$ copies/mL, respectively, in the ozonated OSPW IFAS system, indicating that biofilm denitrification bacteria grew better in the raw OSPW IFAS system. This observation could be attributed to the low nutrients transfer efficiencies in the ozonated OSPW IFAS biofilm due to the thicker and denser biofilm.

3.3.3.3 Comparison of Flocs and Biofilms Bacterial Number

The gene abundance of total bacteria, nitrifying (AOB, NOB), and denitrifying (DEN) bacteria between the IFAS biofilms and flocs are compared in Fig. 3.3 (E) and 3.3(F). The abundance of total bacteria in flocs were $7.16 \times 10^9 \pm 4.21 \times 10^8$ and $8.06 \times 10^9 \pm 4.83 \times 10^8$

copies/mL in raw and ozonated OSPW IFAS, respectively. The abundance of total bacteria in biofilms were $4.09 \times 10^9 \pm 6.86 \times 10^7$ and $3.21 \times 10^9 \pm 5.69 \times 10^7$ copies/mL in raw and ozonated OSPW IFAS, respectively. The results indicate that there was no significant difference ($p > 0.05$) in the total bacteria population in raw and ozonated OSPW IFAS. The total bacteria population of activated sludge flocs was comparable with that in the attached biofilms in both raw and ozonated OSPW IFAS systems.

In raw OSPW IFAS, the gene copies of nitrifiers were higher in biofilms than that in flocs, except for the *Nitro* ($4.27 \times 10^7 \pm 5.69 \times 10^6$ copies/mL in flocs and $4.10 \times 10^7 \pm 1.80 \times 10^6$ copies/mL in biofilms). The abundance of all the four denitrifier genes (*narG*, *nirS*, *nirK* and *nosZ*) were higher in biofilms compared to that in flocs. The results indicate that both activated sludge flocs and biofilms play significant roles in nitrification and denitrification in the raw OSPW IFAS system. Fig. C6 (A) (Appendix-C) shows that the abundance proportions of both nitrifier genes (*aomA*, *NSR* and *Nitro*) and denitrifier genes (*narG*, *nirS*, *nirK* and *nosZ*) within total bacteria are significantly higher in biofilms than in flocs in the raw OSPW IFAS system. These results may be attributed to the extended SRT of biofilm bacteria that favours the growth of slow growing nitrifying organisms (Kim et al., 2010). The higher abundance proportion of denitrifier genes in biofilms than in flocs might be a result of the anoxic condition that develops as the biofilm thickness increases.

In contrast, in the ozonated OSPW IFAS system, the gene copies of both nitrifying and denitrifying bacteria were greater in the flocs as compared to that in the biofilms. Fig. C6 (B) (Appendix-C) shows that the gene abundance proportions of nitrifying and denitrifying bacteria within total bacteria are greater in flocs than in biofilms in ozonated OSPW IFAS system. Ozone pretreatment can enhance the biodegradability of OSPW and decrease the OSPW toxicity effect

toward *V. fischeri*, which may promote the heterotrophic bacterial growth. However, the absolute gene copies of nitrifying and denitrifying bacteria in biofilms were sufficient to maintain the treatment performance in ozonated OSPW IFAS system. Fig. 3.3(A) and 3.3(B) show that the biofilm thickness in the ozonated OSPW IFAS was significantly higher than that in the raw OSPW IFAS. Thus, oxygen mass transfer efficiency in biofilms of ozonated OSPW IFAS might have been restricted due to the thicker biofilm.

3.3.4 Toxicity of OSPW to *V. fischeri*

A microbe toxicity assessment can provide information about wastewater quality before and after treatment to remove toxins. The Mirotox method is widely used to measure the toxicity of naphthenic acids in OSPW samples (Clemente and Fedorak, 2005). The levels of *V. fischeri* inhibition in OSPW influent and effluent were monitored with a Microtox bioassay at different stages of OSPW treatment with ozonation combined with integrated fixed-film activated sludge.

The toxicity of ozonated OSPW IFAS influents toward *V. fischeri* was significantly lower than the toxicity of raw OSPW IFAS influents in all phases. These results support a previous report (Scott et al., 2008) that ozonation decreased the toxicity of OSPW toward *V. fischeri*. The toxic effects of influent and effluent in raw and ozonated OSPW IFAS systems on *V. fisheri* after 15 min of exposure at different stages are shown in Fig. 3.4. Before we stopped adding commercial NAs to the influent (OSPW volume percentage from 10% to 60%), the inhibitory effects of reactor influent toward *V. fischeri* significantly decreased after biodegradation. This observation could be attributed to the toxicity reduction of commercial NAs by IFAS degradation.

After we stopped adding the commercial NAs to the IFAS influent (OSPW volume percentage from 60% to 100%), inhibitory effects of effluents toward luminescent bacteria in

both IFAS systems exhibited a significant upward trend—from 5.41% to 22.58% for the raw OSPW IFAS system and from 0% to 5.82% for the ozonated OSPW IFAS system during the 60% OSPW stage. When the volume ratio of OSPW was further increased from 60% to 80%, the effluent inhibition of luminescent bacteria increased to 29.20% for the raw OSPW IFAS system

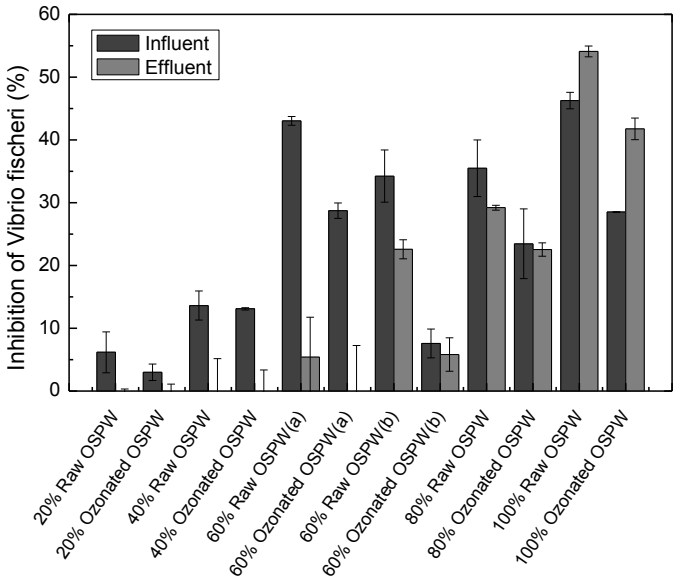


Figure 3.4. Toxic effects of influent and effluent of raw and ozoated-OSPW IFAS systems on *Vibrio fisheri* after 15 min of exposure at different stages.

and to 22.55% for the ozonated OSPW IFAS system. When volume ratio of OSPW reached 100%, the effluent inhibition of luminescent bacteria in both IFAS systems was even higher than the influent inhibition. Specifically, at an OSPW volume ratio of 100%, influent and effluent inhibition of luminescent bacteria was 46.26% and 54.09%, respectively, in the in raw OSPW IFAS system and 28.51% and 41.75%, respectively, in the ozonated OSPW IFAS system. As the volume percentage of OSPW increased, the concomitant increase in NAs might exhaust the ability of biofilm and flocs to break down molecules toxic to luminescent bacteria. Jones et al. (2011) found links between toxicity and the structure and carbon number of NAs, with

concomitant increases in toxicity and carbon number of the acids (Jones et al., 2011). Previous studies showed that by-products of NAs oxidation such as hydroxyl- or keto-NAs (Martin et al., 2010) produced after ozonation can still contribute to the toxic effects of OSPW toward *V. fischeri* (Wang et al., 2013). At high OSPW percentages, oxidized NAs such as hydroxyl- or keto- NAs would also accumulate after biodegradation and contribute to the toxic effects (Wang et al., 2013). Therefore, we can conclude that ozonation can significantly decrease the toxicity of OSPW toward *V. fischeri*. However, IFAS treatment was not effective in reducing the toxicity toward *V. fischeri*.

3.4 Conclusions

This study utilized ozonation combined with IFAS to remediate OSPW. After 11 months operation, when the volume percentage of OSPW reached to 100%, 12.06% of the AEF and 43.14% of the NAs were removed by direct biodegradation in IFAS without ozone pretreatment, while 41.97% of the AEF and 80.18% of NAs were removed in ozonation combined IFAS process. The biofilm in the ozonated OSPW IFAS was significantly thicker (94 ± 1.6 μm) than the biofilm in the raw OSPW IFAS (72 ± 2.8 μm) after 283 days of cultivation. NA biodegradation significantly decreased as the NA cyclization number increased. *q*-PCR revealed that the gene abundances of total bacteria and nitrogen removal relevant bacteria significantly decreased in two IFAS systems during the start-up stage. The gene abundance proportions of nitrogen removal relevant bacteria within total bacteria are significantly higher in biofilms than in flocs in the raw OSPW IFAS system. The combined ozonation and IFAS system showed the promise for OSPW treatment.

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CHAPTER 4. CHARACTERIZATION OF MICROBIAL COMMUNITIES DURING START-UP OF INTEGRATED FIXED-FILM ACTIVATED SLUDGE (IFAS) SYSTEMS FOR THE TREATMENT OF OIL SANDS PROCESS-AFFECTED WATER (OSPW)[1]

4.1 Introduction

Development and growth of the Athabasca oil sands industry in northern Alberta, Canada, has been rapid in recent decades (Choi and Liu, 2014). The region of north Alberta produce 1.3 million barrels of bitumen per day (CAPP 2010), the oil production is projected to reach over 3.3 million barrels per day by 2020 (Schindler, 2010). The waste by-products of the oil sands industry are collected in the large artificial settling ponds called tailing ponds (Nix and Martin, 1992). The aqueous hot water extraction process to separate the bitumen from associated sands and clays produces large quantities of aqueous tailings, known as oil sands process-affected water (OSPW) which contain water, sand, clay, residual bitumen, heavy metal, naphtha diluent and naphthenic acids (NAs). It has been reported that OSPW shows toxicity to a range of aquatic organisms, which lead to a "zero discharge policy" (Quagraine et al., 2005; Nero et al., 2006; Armstrong et al., 2009; Hagen et al. 2014). A limit for the extension of future tailings ponds and aggressive timelines for OSPW reclamation have been mandated by the Alberta government (Ramos-Padrón et al., 2010). The primary toxicity of OSPW to the aquatic organisms is

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attributed to a group of low molecular weight organic acids, which knows as naphthenic acids (NAs) (Anderson et al., 2012), which constituting about 50% of the acid extractable organic fraction (AEF) in OSPW (Pourrezaei et al., 2011). Reported concentration of the parent NAs in OSPW ranged from 20 to 120 mg/L (Toor et al., 2013). Currently, appropriate treatment technologies of OSPW are being studied to recycle the OSPW, in order to reduce the demand for fresh water intake from the Athabasca River. In addition, proper OSPW treatment methods are urgently needed for safe discharge of treated OSPW to the receiving environments.

Biodegradation techniques have been extensively investigated for their ability to treat OSPW (Golby et al., 2012; Johnson et al., 2013). It was demonstrated that bioreactor technology is an economical, environmental friendly and energy-efficient method for the OSPW remediation (Abd-Elsalam and EL-Hanafy, 2009). Bioreactors employing suspended or attached aggregations (activated sludge flcos or attached biofilms) have been studied for OSPW treatment (Choi and Liu, 2014; Hwang et al., 2013; Islam et al., 2014; Shi et al., 2015; Huang et al., 2015). It has been reported previously that ozone treatment might be used as a pre-treatment option for bioreactor treatment to break down recalcitrant NAs and improve the biodegradability of OSPW (Dong et al., 2015; Islam et al., 2014). The lower ozone doses employed for a partial degradation of target recalcitrant compounds in OSPW would beneficial for downstream bioreactors treatment (Islam et al., 2015). Our recent study applied the combined ozonation with integrated fixed-film activated sludge (IFAS) process for OSPW treatment (Huang et al. 2015). It was observed that 12.1% of the AEF and 43.1% of the parent NAs were removed in the raw OSPW IFAS, while 42.0% AEF and 80.2% of parent NAs were removed in the ozonated OSPW IFAS. Given these positive results, microbial communities in the IFAS systems need to be

characterized to investigate the complex interaction between the suspended and attached biomass and to improve the design and understanding of the operation of IFAS reactors.

Among the microorganisms in bioreactors, including bacteria, eukaryote, archaea and virus (Wu and Liu, 2009), bacteria play important roles in wastewater treatment processes (Zhou et al., 2014). It is quite clear that bacteria community composition is very sensitive to the changes of operational parameters and nutrients composition of the bioreactors. Since microbiological parameters indicate stress or changes in the system, characterization of microbial communities composition in the bioreactor will be critical for assessing biological limiting factors related to removal efficiency of contaminants and further improving the bioreactor performance (Ding et al., 2008). The conventional molecular biological techniques had been extensively used in wastewater treatment systems in the last two decades, such as, denatured gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), quantitative polymerase chain reaction (*q*-PCR), and fluorescence *in situ* hybridization (FISH) (Wang et al., 2012). However, the conventional molecular biology approaches cannot provide sufficient sequences to capture comprehensive information of the diverse bacterial communities (Kiely et al., 2010; Lu et al., 2012). The main limitation of PCR-based fingerprinting techniques (DGGE and T-RFLP) is their low resolution (Dighton et al., 2005). More sensitive techniques are needed to employ to achieve a more precise and complete characterization of microbial communities of IFAS systems.

In the recent years, high-throughput sequencing technologies have been used to study the microbial communities in soil (Lauber et al., 2009), raw sewage (McLellan et al., 2010), activated sludge (Zhang et al., 2012) and bioreactors (Choi and Liu, 2014; Zhou et al., 2015). These technologies can be used to investigate the microbial community of the environmental

samples, and can generate hundreds of thousands of short sequences from hyper-variable regions in the rRNA genes (Sogin et al., 2006; Huber et al., 2007). This powerful technique has the full potential for investigating at deeper level and obtaining a comprehensive coverage of microbial communities (Polka et al., 2015).

The present study focuses on the utilization of ozonation combined with IFAS to treat OSPW. Through applying 454 high-throughput pyrosequencing techniques, this study aims (i) to reveal microbial community evolution of the suspended flocs and attached biofilms during the start-up period of IFAS systems, (ii) to investigate the biodiversities of dominant bacterial communities of activated sludge flocs and biofilms in the IFAS systems, and (iii) to identify the dissimilarity of microbial communities composition between tailings pond microorganisms, aerobic activated sludge from the Gold Bar Wastewater Treatment Plant (GBWTP) (Edmonton, Canada), suspended flocs and attached biofilms in the IFAS systems. This is the first report of high-throughput pyrosequencing of microbial communities in IFAS reactors treating oil sands tailings water.

4.2 Materials and Methods

4.2.1 Source Water and Ozonation

Fresh OSPW was collected from tailings ponds in Northern Alberta in September 2013, and stored in a cold room (4 °C) prior to use. An ozone dose of 30 mg/L was applied for OSPW pretreatment based on our previous studies (Dong et al., 2015). Ozonated OSPW (ozone pretreated) was produced using an AGSO 30 Effizon ozone generator (WEDECO AG Water Technology, Herford, Germany). The stable concentration ozone gas from the ozone generator was introduced into the liquid phase through a ceramic bubbles gas diffuser at the bottom of the

container. Two ozone monitors (HC500, WEDECO, Charlotte, NC, USA) were applied to monitor the ozone concentration in the feed-gas, off-gas, and the residual ozone concentration in the container was monitored using the indigo method (APHA, 2005). Ozonated OSPW was purged with nitrogen for 10 minutes to strip oxygen and residual ozone.

4.2.2 IFAS System Operation

Two parallel laboratory-scale IFAS reactors (Huang et al., 2015) provided by Napier-Reid Ltd. (Markham, Canada) were start up. One of the IFAS reactors was used for raw OSPW treatment, and the other was applied for ozonated OSPW treatment. Reactor operation was initiated by inoculating 2 L of activated sludge from GBWTP. During the reactor start-up stage, we increased the volume ratio of raw and ozonated OSPW step by step (from 10% to 100%). From 10% phase to 60% phase, commercial NAs (30mg/L COD) (naphthenic acid, SIGMA-ALDRICH, Belgium) were provided to help the growth of NAs degraders in IFAS systems.

Table 4.1 The start-up strategies of IFAS systems

Start-up phase	The volume ratio of OSPW (%)	Total COD of raw OSPW IFAS influent (mg/L)	Total COD of ozonated OSPW IFAS influent (mg/L)	COD from raw OSPW (mg/L)	COD from ozonated OSPW (mg/L)	COD from commercial NAs (mg/L)	COD from sodium acetate (mg/L)
I	10	247.6	246.2	17.6	16.2	30	200
II	20	245.2	242.4	35.2	32.4	30	180
III	40	240.4	234.8	70.4	64.8	30	140
IV	60	245.9	238.1	115.9	108.1	30	100
V	60	245.9	238.1	115.9	108.1	0	130
VI	80	284.5	274.2	154.5	144.2	0	130
VII	100	323.2	310.2	193.2	180.2	0	130

During the entire start-up stage, extra carbon (sodium acetate) as well as nitrogen (NH_4Cl, 30.0 ± 3.0 mg N/L) and phosphorus (KH_2PO_4, 3.0 ± 0.2 mg P/L) were provided to maintain the growth of biomass in the IFAS systems (Huang et al., 2015). The hydraulic retention time (HRT) of two IFAS reactors was 48h. The suspended solid retention time (SRT) was maintained 43

days through manually discharging sludge from the clarifier. The dissolved oxygen (DO) concentration was in the range of 6-7 mg/L during the operation. The detailed start-up strategies are listed in Table 4.1.

4.2.3 DNA Extraction

Genomic DNA was extracted from activated sludge flocs and biofilms in raw and ozonated OSPW IFAS systems. The total DNA was extracted from 5 mL of the activated sludge flocs using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's protocol. To obtain microorganisms from the biofilms formed on the carriers, 5 carriers from each reactor were immersed in phosphate buffered saline (PBS) to remove non-adhearent bacteria, placed in 5 mL sterile PBS in a 20 mL sterile glass bottle (Falcon, CA, USA), and sonicated for 10 min. The glass bottles were then vortexed for 1 min until the residual biofilm was completely removed from the carrier. The mixed liquid in the glass bottle was used for biofilms DNA extraction. DNA was extracted in duplicate from each sample.

4.2.4 454 High-throughput 16S rRNA Gene Pyrosequencing

To determine the diversity and composition of the microbial communities in tailing ponds endogenous microorganisms (source waters), aerobic activated sludge from GBWTP (inocula), suspended flocs and attached biofilms from the IFAS systems, paired-end sequencing of the extracted DNA samples were performed on a 454/Roche GS-FLX platform by the Research and Testing Laboratory (Lubbock, TX, USA). The V1-V3 regions of the 16S rRNA genes were amplified by PCR using primer pairs of 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3') (Campbell and Kirchman，2013).

After read quality checking and denoising, the microbial diversity analysis was performed using the pipeline Quantitative Insights Into Microbial Ecology (QIIME), version 1.9.0

(Caporaso et al., 2010). Raw reads were filtered by QIIME quality filters. Effective reads were normalized for the following analysis. Sequences with more than 97% similarity were clustered into the same operational taxonomic units (OTUs) using the UCLUST algorithm. Then representative OTUs were picked based on the most abundant sequences, and the taxonomic assignment were performed with UCLUST consensus taxonomy classifier. After that, the OUT sequences were aligned with the Python Nearest Alignment Space Termination (PyNAST) tool. Eventually, the communities were summarized by taxonomic composition. In order to compute alpha diversity, QIIME calculate four diversity metrics: Chao 1 metric (estimating the richness of species), observed OTUs metric (the count of unique OTUs found in the sample), Phylogenetic diversity metric and the Shannon Index. Alpha rarefaction curves were created base on these four metrics. In addition, the explicit comparison of microbial communities based on their composition was explained by beta diversity. Beta diversity metrics were used to assess the difference between microbial communities and reflect the dissimilarity between the samples. Hierarchical cluster analysis was performed using Unweighted Pair Group Method with Arithmetic mean. Both weighted and unweighted UniFrac were calculated using QIIME, which were used to measure beta diversity phylogenetically.

4.2.5 Statistical Analysis

Data were analysis using Microsoft Excel® software. The level of statistical significance was determined using a t-test. Correlations were considered statistically significantly at the 95% confidence interval ($p < 0.05$).

4.3 Result and Discussion

4.3.1 Overall Performance of Labortory-scale IFAS Reactors

Table 4.2 Mean values for operating conditions and performance of IFAS reactors after start-up

Parameters	Raw-OSPW IFAS		Ozonated-OSPW IFAS	
	Influent	Effluent	Influent	Effluent
Influent flow (L/day)	2.0		2.0	
HRT (h)	48		48	
RAS flow (L/day)	4.0		4.0	
DO (mg/L)	6-7		6-7	
Biofilm thickness (μm)	71.67 ± 3.51		94.00 ± 1.6	
Reactor MLSS (suspended) (mg/L)	1.226 ± 0. 095		1.298 ± 0.091	
Reactor MLSS (attached) (mg/L)	0.377 ± 0.12		0.534 ± 0.19	
COD (mg/L)	317.5 ± 10.5	177.7 ± 6.1	294.15 ± 12.0	151.9 ± 7.5
Ammonium (mg/L)	30.8 ± 0.9	0	29.9 ± 0.8	0
Nitrate (mg/L)	0.94 ± 0.14	24.90 ± 1.58	1.00 ± 0.16	25.35 ± 2.10
AEF (mg/L)	88.62	77.93	60.51	51.43
Classical NAs (mg/L)	25.13	14.29	9.49	4.98

During the start-up stage, two laboratory-scale IFAS reactors were operated for 320 days, one with raw OSPW influent and the second with ozonated OSPW influent, typical operating conditions, influent and effluent quality of start-up stage was presented in Table 4.2. IFAS removed 43% of the total chemical oxygen demand (COD) and 43.14% of the parent naphthenic acids (NAs) from raw OSPW and 51% of total COD and 47.52% of parent NAs from ozonated OSPW. As for the AEF, 12% and 15% of the AEF were removed from influent in raw and ozonated OSPW IFAS, respectively. Both IFAS reactors performed an excellent ammonia oxidation as indicated by the low ammonia concentration in the effluents. The hydraulic retention time (HRT) of two reactors was 48h. Mixed liquor suspended solids (MLSS) concentration in both IFAS systems tended to stabilize, and were maintained in a relative constant range of 1.2-1.4 g/L. After 283 days of cultivation, the biofilm in the ozonated OSPW

IFAS was slightly thicker (94 ± 1.6 μm) than the biofilm in the raw OSPW IFAS (72 ± 2.8 μm). In addition, the MLSS concentration of attached biofilm in ozonated OSPW IFAS systems was relatively higher than the raw OSPW IFAS system, which was agree with the biofilm thickness measurement.

4.3.2 Richness and Diversity Analysis of Microbial Communities

A total 20468 effective sequences were obtained from the seven kinds of biomass samples (AS from GBWTP: 1708, raw OSPW: 3863, ozonated OSPW: 4498, flocs in raw OSPW IFAS: 2956, biofilms in raw OSPW IFAS: 2319, flocs in ozonated OSPW IFAS: 2368, biofilms in ozonated OSPW IFAS: 2756) by 454-pyrosequencing (Table 4.3). The effective sequences were normalized for the following analysis. The rarefaction curves based on the OTUs (Fig. 4.1) show that AS from GBWTP did not reach the plateau, while other six samples tended to approach the saturation plateau. The total number of OTUs determined by Chao 1 estimator was 427 (AS from GBWTP), 399 (raw OSPW), 240 (ozonated OSPW), 326 (flocs in raw OSPW IFAS), 351 (biofilms in raw OSPW IFAS), 278 (flocs in ozonated OSPW IFAS), 379 (biofilms in ozonated OSPW IFAS), respectively (Table 4.3). It indicates that activated sludge from GBWTP had the greatest richness, while endogenous microorganisms from ozonated OSPW had the lowest richness.

Table 4.3 Diversity statistics for seven types of biomass samples

Sample ID	Effective Sequences	97% similarity		
		OTU	Chao 1	Shannon
AS from GBWTP *	1708	427	631	7.46
Raw OSPW	3863	399	474	6.61
Ozonated OSPW	4498	240	296	4.36
Flocs in raw OSPW IFAS	2956	326	423	4.91
Biofilms in raw OSPW IFAS	2319	351	495	6.22
Flocs in ozonated OSPW IFAS	2368	278	432	4.98
Biofilms in ozonated OSPW IFAS	2756	379	483	6.16

* AS from GBWTP - Activated sludge sample from Gold Bar Wastewater Treatment Plant.

Richness diversity (Table 4.3) was calculated by estimating the number of OTUs based on the Chao 1 values at the 97% similarity levels. Among the seven types of biomass samples, the Chao 1 value indicated that the bacterial richness in AS from GBWTP was higher than the richness in other biomass samples. The Chao 1 value in raw OSPW was significantly higher than the value in ozonated OSPW, which indicates that the bacterial richness of endogenous microorganisms in OSPW was significantly reduced after ozone treatment. Moreover, the Chao 1 values in biofilms samples were higher than the values in flocs samples in both IFAS systems, which demonstrated that the microbial richness in biofilms samples was higher than the richness in the flocs samples in two IFAS systems.

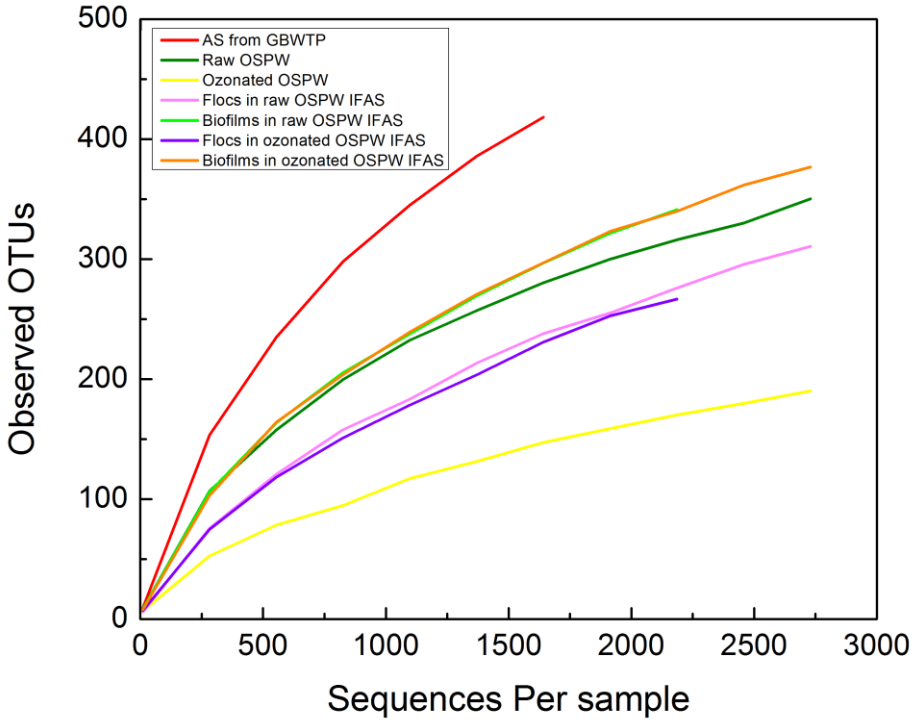


Figure 4.1. Rarefaction analysis of different biomass samples. Rarefaction curves of OTUs clustered for a dissimilarity of 3%.

The Shannon diversity index demonstrates both species richness and evenness (Luo et al., 2013). The AS from GBWTP had the highest diversity (Shannon = 7.46) among the seven types of biomass. The Shannon diversity index in raw OSPW was significantly higher than that in ozonated OSPW ($p < 0.05$, Table 4.3). The result revealed that the bacterial diversity of OSPW was significantly decreased after ozone pretreatment. In addition, the Shannon diversity indices in biofilms samples were significantly higher than the indices in flocs samples in both IFAS systems, the results suggested that the bacterial diversity in the biofilms samples was higher than the diversity in flocs samples of IFAS system. The Chao 1 values (community richness) and Shannon indices (community diversity) confirmed that the richness and diversity of activated sludge flocs and suspended biofilms samples were both less than those of the AS samples from GBWTP (Table 3). This decreased richness and diversity may be attributed to the change of the culturing environment and the toxicity of NAs from OSPW, which allows survival only of bacteria with high tolerances to the NAs (Islam et al., 2015).

Microbial community evenness was found to link with the resistance of microorganisms to environmental stress, such as, high salinity and pH stress, toxicity (Wittebolle et al., 2009). This community evenness can be illustrated by the Shannon diversity index (Zhou et al., 2015). Both types of biomass (suspended activated sludge flocs and attached biofilms) of IFAS systems had the lower Shannon diversity indices than AS from GBWTP (Table 4.3), the results indicate that the microbial community evenness in AS from GBWTP was significantly higher than flocs and biofilms from IFAS systems. This can be explained by the specific characteristics of OSPW, which are low biodegradability, high salinity and high toxicity mainly due to NAs, the primary toxic constituents in OSPW (He et al., 2012).

4.3.3 Bacterial Community Structure Analysis

To identify the phylogenetic diversity of microbial communities in seven types of biomass (AS from GBWTP, endogenous microorganisms from raw and ozonated OSPW, flocs and biofilms in raw OSPW IFAS, flocs and biofilms in ozonated OSPW IFAS), the DNA samples of them were analyzed by 454-pyrosequencing, effective reads were assigned to phyla, classes, and orders (Fig. 4.2 and Fig. 4.3).

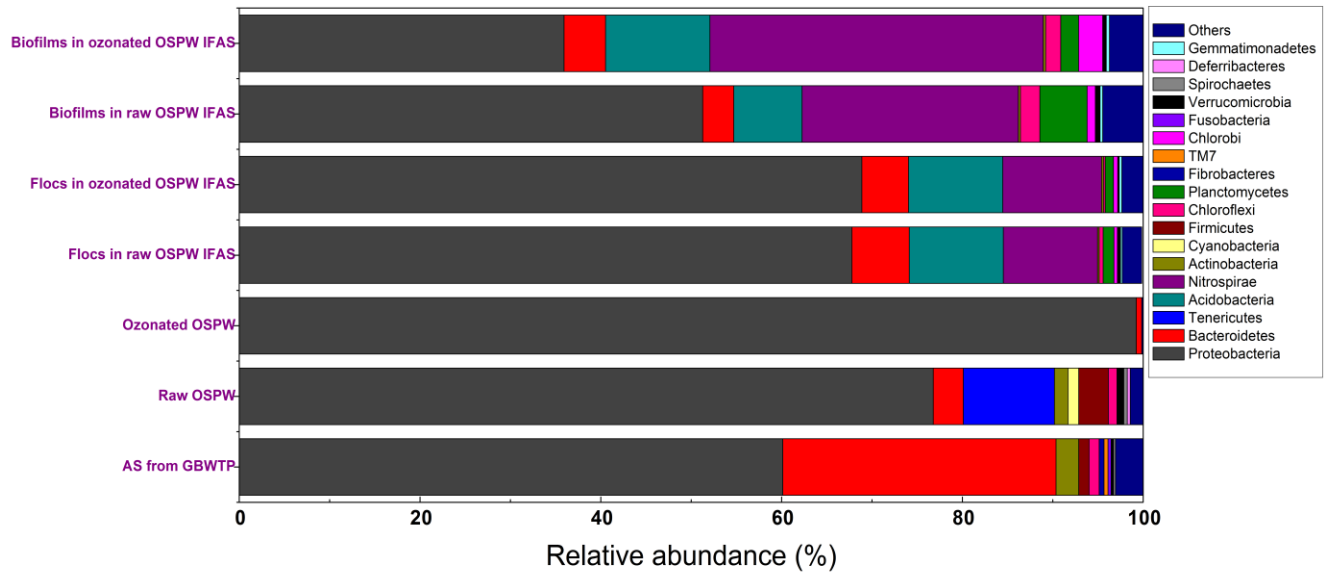


Figure 4.2. Relative read abundance of different bacterial phyla in different biomass samples above a cutoff value of 0.2%. Some phyla at relative abundance < 0.2% was grouped as "others".

The phylum level identification of the bacterial communities is illustrated in Fig. 4.2. Based on an abundance cutoff of 0.2%, *Proteobacteria* and *Bacteroidetes* were the predominant phylum, making up 60.13% and 30.23% of the reads in AS from GBWTP (Fig. 4.2). Compare to the AS from GBWTP, the abundances of *Proteobacteria* in both raw (76.77%) and ozonated

(99.23%) OSPW were significantly higher ($p < 0.05$). Conversely, the abundances of *Bacteroidetes* in both raw (3.33%) and oznated (0.60%) OSPW were significantly lower than the abundance in AS from GBWTP ($p < 0.05$). After ozone pretreatment (30 mg/L), the phylum diversity of OSPW was significantly reduced, which is consistent with Chao 1 value and the Shannon diversity index results in the section 4.3.2. In addition, it was found that *Tenericutes* accounted for 10.08% of total reads in raw OSPW, which did not exist in ozonated OSPW.

The microbial community structures of flocs and biofilms after start-up at phylum level are shown in Fig. 4.2. At the phylum level, good similarities were found between the flocs and biofilms of IFAS systems. It appeared that *Proteobacteria*, *Nitrospirae, Acidobacteria* and *Bacteroidetes* were dominant phyla in both flocs and biofilms samples in two IFAS systems. The total of these four phyla accounted for 94.94% and 86.18% of total reads in flocs and biofilms of raw OSPW IFAS, 95.39% and 88.93% of total reads in flocs and biofilms of ozonated OSPW IFAS. The abundances of *Proteobacteria* in biofilms of two IFAS systems were significantly lower than the abundances in flocs ($p < 0.05$). In contrast, the abundances of *Nitrospirae* in biofilms of two IFAS systems were significantly higher than the relative abundance (RA) in flocs ($p < 0.05$). In addition, the abundances of *Chloroflexi* and *Chlorobi* in biofilms of two IFAS syetems were higher than their abundance in flocs ($p < 0.05$).

To evaluate the impact of ozonation on the microbial community composition of IFAS system, we compared the abundances of different phyla in both flocs and biofilms between raw and ozonated OSPW IFAS systems. It was found that there was no significant difference in phyla abundances in flocs between raw and ozonated OSPW IFAS. However, *Proteobacteria* in biofilms of ozonated OSPW IFAS was found to be more abundant compared with raw OSPW IFAS, whereas the RA of *Nitrospirae* in biofilms of ozonated OSPW IFAS was significantly

higher than their abundance in raw OSPW IFAS. It can be concluded that ozonation of OSPW has more influence on the microbial community composition of biofilms as compared to that of flocs.

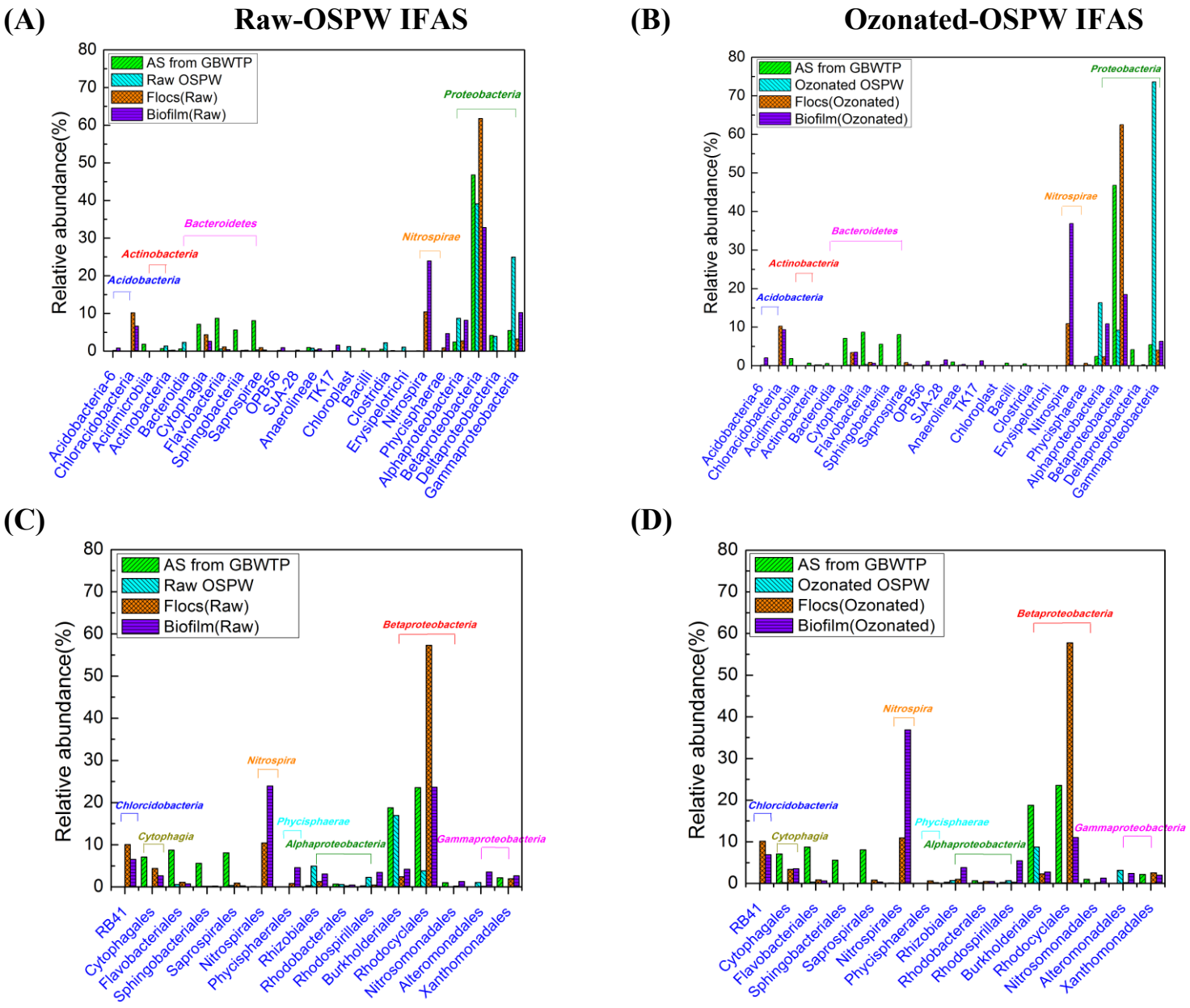


Figure 4.3. Taxonomic classification of pyrosequencing at class and order level. (A) raw OSPW IFAS at class level; (B) ozonated OSPW IFAS at class level; (C) raw OSPW IFAS at order level; (D) ozonated OSPW IFAS at order level.

To reveal microbial community evolution of IFAS systems for OSPW treatment, the taxonomic was broken down at class level. The class level identification of the bacterial communities is illustrated in Fig. 4.3. At the class level, more differences were observed among them. As shown in Fig. 4.3(A) and 4.3(B), among the *Proteobacteria* phyla, *beta-Proteobacteria* was the dominant class in both flocs and biofilms samples from IFAS systems. The RA of *beta-Proteobacteria* in the biofilms was 32.8% and 18.5% of raw and ozonated-OSPW IFAS reactors, and 61.8% and 62.5% in the flocs from two types of IFAS reactors. After acclimation, the *delta-Proteobacteria* abundance in flocs and biofilms from IFAS reactors were relatively small (< 1%) as compared to the abundance in AS from GBWTP and OSPW. Moreover, the abundances of *beta-Proteobacteria* in biofilms of two IFAS systems were significantly lower than the abundances in flocs ($p < 0.05$), while *alpha-* and *gamma-Proteobacteria* in biofilms were found to be more abundant compared with flocs in two IFAS systems ($p < 0.05$). After the ozone pretreatment, the relative abundance of *beta-* and *delta- Proteobacteria* in OSPW experienced a significant downward trend, while *alpha-* and *gamma-Proteobacteria* in ozonated OSPW were found to be more abundant than raw OSPW.

As for the *Nitrospira* class, which belongs to the *Nitrospirae* phyla. It was found that the relative abundances of *Nitrospira* in both flocs and biofilms samples from two IFAS systems were significantly higher than the abundances in AS from GBWTP and OSPW, which means *Nitrospira* become more abundant after acclimation in two IFAS systems. The RA of *Nitrospira* in the biofilms was 23.9% and 36.9% of raw and ozonated-OSPW IFAS reactors, and 10.4% and 10.9% in the bioflocs from two types of IFAS reactors, it demonstrated that the RA of *Nitrospira* in the biofilms were more abundant than those in the flocs ($p < 0.05$).

Among the *Acidobacteria* phyla in flocs and biofilms of IFAS systems, *Chloracidobacteria* was the dominant class. As shown in Fig. 4.3(A) and 4.3(B), the relative abundances of *Chloracidobacteria* in both flocs and biofilms samples from two IFAS systems were significantly higher than the abundances in AS from GBWTP and OSPW, which indicates *Chloracidobacteria* become more abundant after acclimation in two IFAS systems. It was found that some members of Chloracidobacteria are associated with the degradation of a group of aromatic hydrocarbons and bio-resistant organic compounds (Roling et al., 2002). In raw OSPW IFAS, the relative abundance of *Chloracidobacteria* was 10.2% and 6.6% in flocs and biofilm, respectively, and 10.3% and 9.4% in flocs and biofilms of ozonated OSPW IFAS, respectively.

Conversely, the RA of *Cytophagia*, *Flavobacteriia*, *Sphingobacteriia* and *Saprospirae* significantly decreased after acclimation in two IFAS systems, the populations of *Flavobacteriia*, *Sphingobacteriia* and *Saprospirae* in both flocs and biodilms in two IFAS systems after start-up were relatively small.

Fig. 4.3(A) and 4.3(B) shows that *Chloracidobacteria, Cytophagia, Nitrospira, alpha-Proteobacteria, beta-Proteobacteria* and *gamma-Proteobacteria* were the dominant classes in both flocs and biofilms in two IFAS systems. To further investigate the microbial evolution of the biomass during the start-up period of IFAS systems, the dominant classes were further identified at the order level of classification for seven types of biomass (AS from GBWTP, endogenous microorganisms from raw and ozonated OSPW, flocs and biofilms in raw OSPW IFAS, flocs and biofilms in ozonated OSPW IFAS) (Fig. 4.3(C) and 4.3(D)). It was found that the relative abundances of *Cytophagales, Flavobacteriales, Sphingobacteriales* and *Saprospirales* orders significantly decreased after acclimation in two IFAS systems.

The *Nitrospira* class was further identified at order level of classification for seven types of biomass (Fig. 4.3(C) and 4.3(D)). It had been reported that the members of *Nitrospirales* are involved in nitrite oxidization, sulfur oxidation and sulphate reduction (Huang et al., 2016). As shown in Fig. 4.3(C) and 4.3(D), *Nitrospirales* order represented 10.41% and 23.92% of total bacteria in flocs and biofilms of raw OSPW IFAS, respectively; whereas it accounted for 10.93% and 36.87% of total bacteria in flocs and biofilms of ozonated OSPW IFAS, respectively. The relative abundances of *Nitrospirales* in both flocs and biofilms samples from two IFAS systems were significantly higher than the abundances in AS from GBWTP and OSPW, which indicates *Nitrospirales* become more abundant after acclimation in two IFAS systems. It had been reported that some lithotrophic nitrite-oxidizing bacteria had synergetic interaction with various organic compounds (such as, pyruvate, yeast extract and peptone) degradation due to the mixtrophical growth (Waston et al., 1986). Therefore, the significant increase of *Nitrospirales* order after start-up in IFAS systems might be beneficial for the organic compounds removal from OSPW. In addition, the relative abundances of *Nitrospirales* in biofilms were much higher than the abundances in flocs in two IFAS systems, which indicates that the slow growing *Nitrospirales* orders preferred to gathering in the attached phase in biofilm.

Among the *alpha-Proteobacteria* class, *Rhizobiales, Rhodobacterales*, and *Rhodospirillales* were the dominant order in the seven types of biomass (Fig. 4.3(C) and 4.3(D)). It had been reported that the members of *Rhizobiales* and *Rhodobacterales* are responsible for the degradation of sulfur- and nitrogen-containing organic compounds, respectively (Carvalho et al., 2010). The members of *Rhodospirillales* are associated with the oxidation of carbohydrates and alcohols (Gupta and mok, 2007).

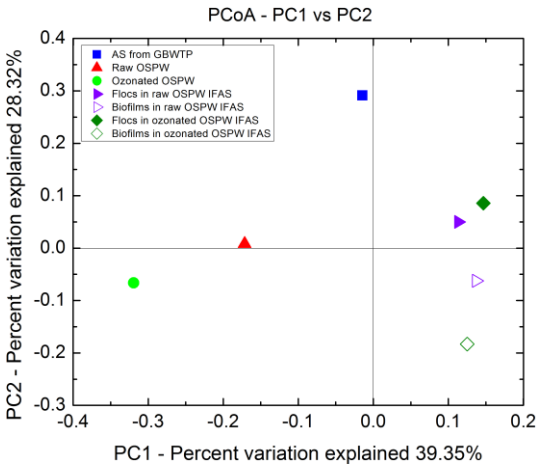
The major orders observed in *beta-Proteobacteria* class were *Burkholderiales, Rhodocyclales*, and *Nitrosomodales* in all biomass samples (Fig. 4.3(C) and 4.3(D)). The relative abundances of *Burkholderiales* in both flocs and biofilms samples from two IFAS systems were significantly lower than the abundances in AS from GBWTP and OSPW, which indicates *Burkholderiales* become less abundant after start-up in two IFAS systems. It had been reported that *Burkholderiales* were able to degrade various aromatic compounds, such as, naphthalene and phenanthrene (Pérez-Pantoja et al., 2012). However, the RAs of *Rhodocyclales* in flocs from two IFAS systems were significantly higher than the abundances in AS from GBWTP and OSPW. The members of *Rhodocyclales* are reported to be involved in the degradation of aliphatic and aromatic compounds (Hesselsoe et al., 2009). The members of *Nitrosomodales* are involved in ammonia oxidizing and fix carbon autotrophically from carbon dioxide (Garcia et al., 2013). The dominant orders of *gamma-Proteobacteria* class were *Alteromonadales* and *Xanthomonadales* in seven types of biomass, which belong to polycyclic aromatic hydrocarbon (PAH) degraders (Lamendella et al., 2014).

4.3.4 Comparative Analysis of Microbial Communities

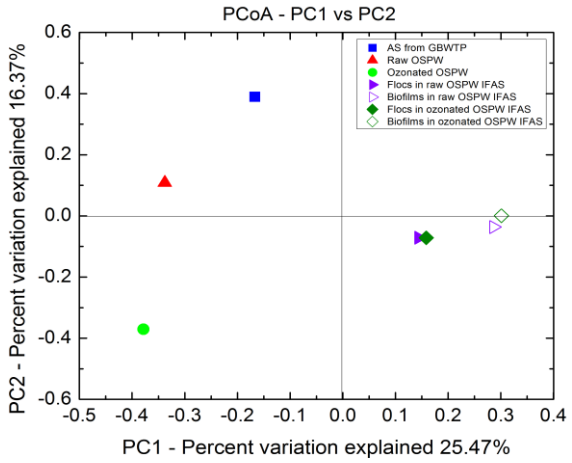
Based on the unweighted and weighted UniFrac distance metric, Principal Coordinate Analysis (PCoA) was calculated (Fig. 4.4). PCoA was performed to reveal the relationships among the different biomass samples.

Based on the weighted UniFrac distance metric, as shown in Fig. 4.4(A), the principal components 1 and 2 explained 39.35% and 28.32% of the total community variations, respectively. The flocs and biofilms samples after acclimation from two IFAS systems were grouped on the right-hand side of the graph and well separated from AS from GBWTP, raw and ozonated OSPW, which was the first principal components axis (PC1), accounted for 39.35% of

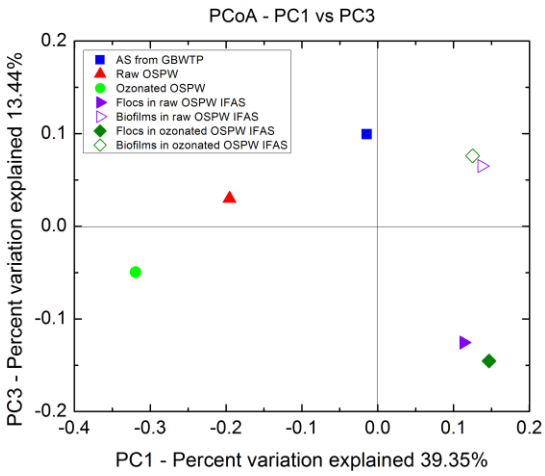
(A) **Weighted**



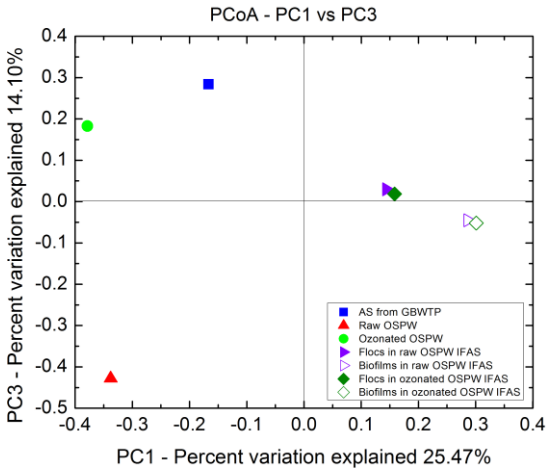
(B) **Unweighted**



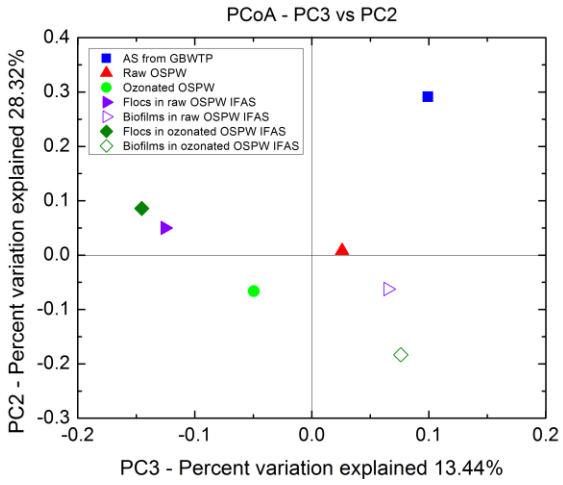
(C)



(D)



(E)



(F)

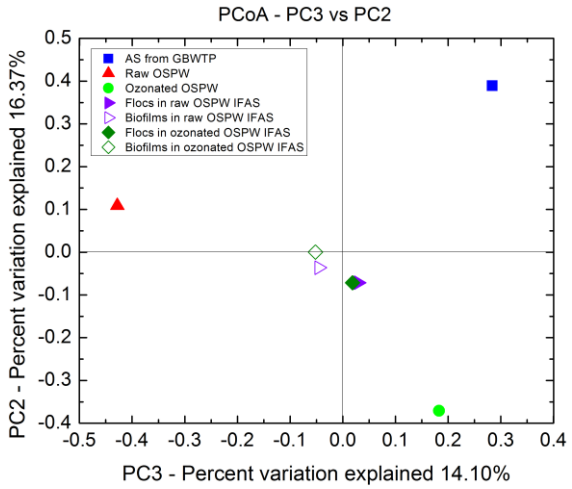


Figure 4.4. Principal coordinates analysis (PCoA) of weighted and unweighted UniFrac distance of *16S rRNA* genes. (A) and (B) principal components 1 and 2; (C) and (D) principal components 1 and 3; (E) and (F) principal components 2 and 3.

the total variations. They were also separated distinctly by the second principal component (PC2) rather than by PC1. PC2 accounted for 28.32% of the variation in the bacterial communities. Overall, the PC1 and PC2 axes could explain 67.67% of the variations between the different bacterial communities. The similar result was found in unweighted PCoA (Fig. 4.4(B)). These results indicate that there were obvious differences in microbial community between the biomass in IFAS systems and the seed sludge (AS from GBWTP) used for the start-up of IFAS systems. There are several possible reasons for this result. Firstly, the water chemistry of our influents for IFAS systems is significantly different from the municipal wastewater of GBWTP. Zhou et al. (2015) demonstrated that different electron donors had significant effects on the microbial communities of bioreactors. The electron donors of our influents for IFAS systems are sodium acetate and organic compounds of OSPW, which are totally different with municipal wastewater. Secondly, the configuration of IFAS systems is significantly different from the aerobic treatment process in GBWTP, which combined suspended and attached biomass. Previous studies showed that the microbial communities could vary significantly depending on the bioreactor configuration (Chio et al., 2014). Thirdly, The operational parameter of IFAS systems is significantly different from the aerobic treatment process in GBWTP, such as, hydraulic retention time (HRT), carbon/nitrogen (C/N) ratio, dissolved oxygen (DO), solid retention time (SRT) and return activated sludge (RAS) rate.

It was found that the raw and ozonated OSPW were separated distinctly by both second and third principal components axis (PC2 and PC3) based on the weighted UniFrac distance

metric (Fig. 4.4(E)). They accounted for 28.32% and 13.44% of the variation in the bacterial communities, respectively. The similar results could be found in weighted PCoA (Fig. 4.4(F)). It demonstrates that the bacterial community of endogenous microorganisms in the raw OSPW significantly changed due to the ozone oxidation.

To assess the impact of ozonation on the microbial community composition of flocs and biofilms in IFAS system, we did the principal coordinate analysis of flocs and biofilms samples between raw and ozonated OSPW IFAS. Two groups were distinguished, two flocs samples from both raw and ozonated OSPW IFAS system, two biofilms samples from two IFAS systems (Fig. 4.4). These results indicate that the ozone pretreatment of OSPW has no significant effects on the microbial communities of two types of biomass in IFAS system. It can be explained by the similar operation conditions and influent water quality between raw and ozonated OSPW IFAS.

To compare the microbial communities differences between activated sludge flocs and biofilms in IFAS systems. It was found that two groups (flocs and biofilms group) formed in Fig. 4.4. The flocs and biofilms samples after start-up from two IFAS systems were grouped on the right-hand side of the first principal components axis (PC1) (Fig. 4.4(A) and 4.4(B)). It indicates that there are limited dissimilarities of microbial communities between flocs and biofilms in IFAS systems. However, these two groups were separated distinctly by second and third principal component (PC2 and PC3) in weighted PCoA (Fig. 4.4(C) and 4.4(E)), and they were also well separated by the third principal component (PC3) in unweighted PCoA (Fig. 4.4(D) and 4.4(F)). These results suggested that there were some differences of bacterial communities existing between flocs and biofilms in two IFAS systems, which can be explained by the difference of physical factors between flocs and biofilms, such as, flow velocity, mixed liquid

shear. Obviously, the distance between flocs and biofilms samples in ozonated OSPW IFAS was greater than the distance in raw OSPW IFAS, which indicates that the more dissimilarities of microbial communities exist between flocs and biofilms in ozonated OSPW IFAS as compared to raw OSPW IFAS.

4.4 Conclusions

Bacterial community in the seed sludge (AS from GBWTP) of two IFAS systems showed the greatest richness and evenness. Chao 1 value and Shannon diversity index results showed that the bacterial richness and microbial diversity of biofilms were significantly higher than flocs in both two IFAS systems. The microbial communities analysis from 454-pyrosequencing revealed that *Proteobacteria*, *Nitrospirae*, *Acidobacteria* and *Bacteroidetes* were dominant phyla in both flocs and biofilms of IFAS reactors. However, the phyla and classes distribution of flocs and biofilms were significant different. Principal Coordinate Analysis indicated that there were obvious differences in microbial community between the biomass in IFAS systems and the seed sludge (AS from GBWTP) used for the start-up of IFAS systems. Then, the ozone pretreatment of OSPW has no significant effects on the microbial communities of flocs and biofilms in IFAS system. What's more, there was some variation of bacterial communities existing between flocs and biofilms in two IFAS systems.

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CHAPTER 5. PERFORMANCE OF FLOCS AND BIOFILMS IN INTEGRATED FIXED-FILM ACTIVATED SLUDGE (IFAS) SYSTEMS FOR THE TREATMENT OF OIL SANDS PROCESS-AFFECTED WATER (OSPW)[1]

5.1 Introduction

Development and expand of the Athabasca oil sands industry in Northern Alberta, Canada, has been rapid during the past few decades (Choi et al., 2014a). The Athabasca oil sands of Northern Alberta is one of the world's largest oil reserves, containing over 168 billion barrels of recoverable bitumen (Teare et al., 2012). The mined bitumen is separated from associated sands and clays using the caustic hot water extraction process (Hwang et al., 2013), resulting in significant volume of oil sands process-affected water (OSPW), which are current stored in tailings ponds following a "zero discharge policy" maintained by the Alberta's regulatory framework (Allen, 2008). Oil sands tailings contain a mixture of sand, clay fines, slits, dissolved salts, heavy metals and organic compounds, which has demonstrated toxicity to a number of aquatic organisms such as algae (Gamal El-Din et al., 2011), fish (He et al., 2012), benthic invertebrates (Anderson et al., 2012) and mammalian species (Garcia-Garcia et al., 2011). The primary toxic constituents of OSPW are a group of organics, collectively known as naphthenic acids (NAs) (Scott et al., 2005). Reported concentrations of parent NAs in OSPW are in the range of 20-120 mg/L (Toor et al., 2013). Appropriate OSPW treatment methods that allow maximum reuse or safe discharge of treated OSPW would decrease the amount of freshwater

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withdraw from the Athabasca River and reduce the potential environmental threat of the OSPW.

Biodegradation utilizing bioreactor technology is an economical, energy-efficient and environmentally sound approach for the OSPW reclamation (Abd-Elsalam and EL-Hanafy, 2009). Microbial aggregates are employed in biological wastewater treatment processes to remove organic compounds and nutrients (nitrogen and phosphorus). Microbial aggregates are complex ecosystems consisting of highly stratified microbial communities embedded in a matrix of extracellular polymeric substances (EPS). The two types of aggregates are suspended flocs (e.g., in the activated sludge process) and attached biofilms. Promise has been shown from both suspended and attached growth processes for their application on OSPW treatment (Choi et al., 2014a; Hwang et al., 2013; Choi et al., 2014b; Islam et al., 2014, Islam et al., 2015; Shi et al., 2015; Huang et al., 2015).

Suspended and attached biomass differs in many aspects in bioreactors. Attached growth systems (i.e., biofilms) maintain a high microbial retention time, eliminating microbial wash out and encouraging the growth of slow growing microbes such as nitrifying bacteria and recalcitrant organics degraders. The improved endogenous metabolism of the biofilm biomass under longer SRT allow development of microbial communities, which are efficient for the biodegradation of poorly degradable contaminants (Liu and Tay, 2001). Suspended systems, on the other hand, favour greater substrate uptake rate and specific microbial growth rate as compared to attached growth systems, and are suitable for the easily biodegradable organic substrates removal. The difference of microbial population and hydrodynamic conditions in suspended and attached growth bioreactors may also lead to the significantly different biomass physical structure (e.g., biomass surface areas and density) and physicochemical characteristics (e.g., hydrophobicity and surface charge) between suspended and attached biomass. Under identical substrate loading

condition, the biofilms structure tends to be smooth, dense and less porous, while the flocs have highly porous structure (Loosdrecht et al., 1995). This observation is often attributed to the faster suspended biomass growth rate and higher extracellular polymeric substances (EPS) production rates as compared to the attached biomass (Droppo et al., 2001). Previous studies also showed that suspended biomass has greater negativity and higher hydrophobicity than attached biomass (Mahendran et al., 2012). The difference of physical structure and surface propreties between attached and suspended biomass may thus result in different substrate and oxygen diffusion rates in these structures.

Our recent study applied integrated fixed film activated sludge (IFAS) reactors (i.e., combined suspended and attached growth systems). This study showed that ozonation combined with IFAS is a promising technology for OSPW treatment (Huang, et al., 2015). The coexistence of suspended and attached biomass leads to the degradation of a wide range of contaminants. In IFAS systems, biofilm detachment occurs naturally, and can enable attached biomass to spread and attach to the flocs, thus enriching flocs with slow growing biomass (Chambless et al., 2007). Suspended and attached biomass may also compete for available organic substrates and oxygen (Chen et al., 1997). To further optimize the bioreactor design and operation, information on the diversity and functionality of the microbial biomass in suspended and attached phases is needed because bioreactor performance is determined by the diversity and density of the bacterial population present in activated sludge or biofilm.

Our current study aims at comparing and elucidating the performance and mechanisms of suspended and attached biomass in IFAS systems for the OSPW treatment. Batch IFAS reactors were operated using flocs and biofilms, taken from stabilized continuous IFAS systems, for both raw and ozonated OSPW treatment. The roles of the two main removal mechanisms

(biodegradation and biosorption) on OSPW remediation were evaluated. Microbial community structures in suspended and attached biomass were also characterized. This study will benefit the modelling and optimization of IFAS systems.

5.2 Materials and Methods

5.2.1 Source Water Information

The fresh OSPW was collected from oil sands tailings ponds in Fort McMurray, AB, Canada, in September 2013. OSPW samples were preserved in 200 L polyvinyl chloride barrels in a cold room (4 °C) and were well mixed prior to use.

Synthetic wastewater was prepared by dissolving sodium acetate, NH_4Cl, KH_2PO_4, and trace metals in distilled, deionized (DI) water. The 0.1% (v/v) trace nutrient solution contained: $MgCl_2 \cdot 6H_2O$, 3 g L^{-1}; $CaCl_2$, 1.5 g L^{-1}; $FeSO_4 \cdot 7\ H_2O$, 0.28 g L^{-1}; $MnCl_2 \cdot 4\ H_2O$, 0.13 g L^{-1}; $ZnSO_4 \cdot 7\ H_2O$, 0.12 g L^{-1}, $CuSO_4 \cdot 5\ H_2O$, 0.0074 g L^{-1}. The pH was 7.3 ± 0.3. All chemicals and supplies were obtained from Thermo Fisher Scientific.

5.2.2 Batch IFAS Reactor Set-up and Operation

Batch reactor (biodegradation and biosorption) set-up and operation. The batch reactors were performed using suspended activated sludge flocs and attached biofilm carriers from the continuous IFAS systems (both raw and ozonated OSPW IFAS). The detailed information of IFAS operational conditions is shown in Table 5.1. Three types of biomass inoculants were evaluated and compared in the batch tests, including: (1) suspended activated sludge (IFAS-flocs), (2) attached biofilm carriers (IFAS-biofilms), and (3) suspended activated sludge and attached biofilm carriers (IFAS-hybrid).

Table 5.1 Mean values for operating conditions and performance of continuous IFAS systems

Parameters	Raw-OSPW IFAS		Ozonated-OSPW IFAS	
	Influent	Effluent	Influent	Effluent
Influent flow (L/day)	2.0		2.0	
HRT (h)	96		96	
RAS flow (L/day)	2.0		2.0	
DO (mg/L)	6-7		6-7	
Reactor MLSS (suspended) (g/L)	0.976 ± 0. 146		1.254 ± 0.153	
Reactor MLSS (attached) (g/L)	0.386 ± 0.14		0.545 ± 0.17	
COD (mg/L)	293.7 ± 14.1	161.3 ± 7.4	277.1 ± 14.3	143.5 ± 8.2
Ammonium (mg/L)	29.7 ± 0.9	0	29.4 ± 0.9	0
Nitrate (mg/L)	0.46 ± 0.07	22.76 ± 1.39	0.48 ± 0.08	23.42 ± 1.38
Total Nitrogen (mg/L)	31.84 ± 1.79	23.76 ± 2.04	31.23 ± 1.96	23.92 ± 2.89
AEF (mg/L)	93.15	79.36	57.30	54.02

A sludge concentration of ~1000 mg TSS/L, and/or colonized biofilm carriers (volume fraction is 60%) (The same with that in continuous IFAS reactors) was applied. Amber bottles containing duplicates of individual treatments were applied. After the influents and biomass were introduced, the bottles were shaken and immediately sampled to obtain initial pollutant concentrations at t = 0 h. Samples were shaken at 150 rpm on a platform shaker (InnovaTM 2100, Platform Shaker, New Bruns Wick Scientific, USA) for 21 days at room temperature (20 ± 1 ˚C). Samples (about 5 mL) were taken at different times by means of plastic syringes, filtered through a nylon membrane filter (0.45 μm pore) and stored at 4 ˚C for subsequent analyses. The schematic diagram of batch reactor was shown in Fig. 5.1.

Biosorption only batch reactor set-up and operation. For biosorption only experiments, the inhibition of biological activities was carried out to distinguish pure biosorption onto activated sludge flocs and biofilms from biodegradation. The inactivation of activated sludge and biofilm were performed with the addition of sodium azide (NaN_3) at a concentration of 0.2g

NaN_3/g TSS. Adsorption experiments were conducted in duplicate using amber bottles with 500 mL OSPW. The detailed strategies of batch experiments are shown in Table 5.2.

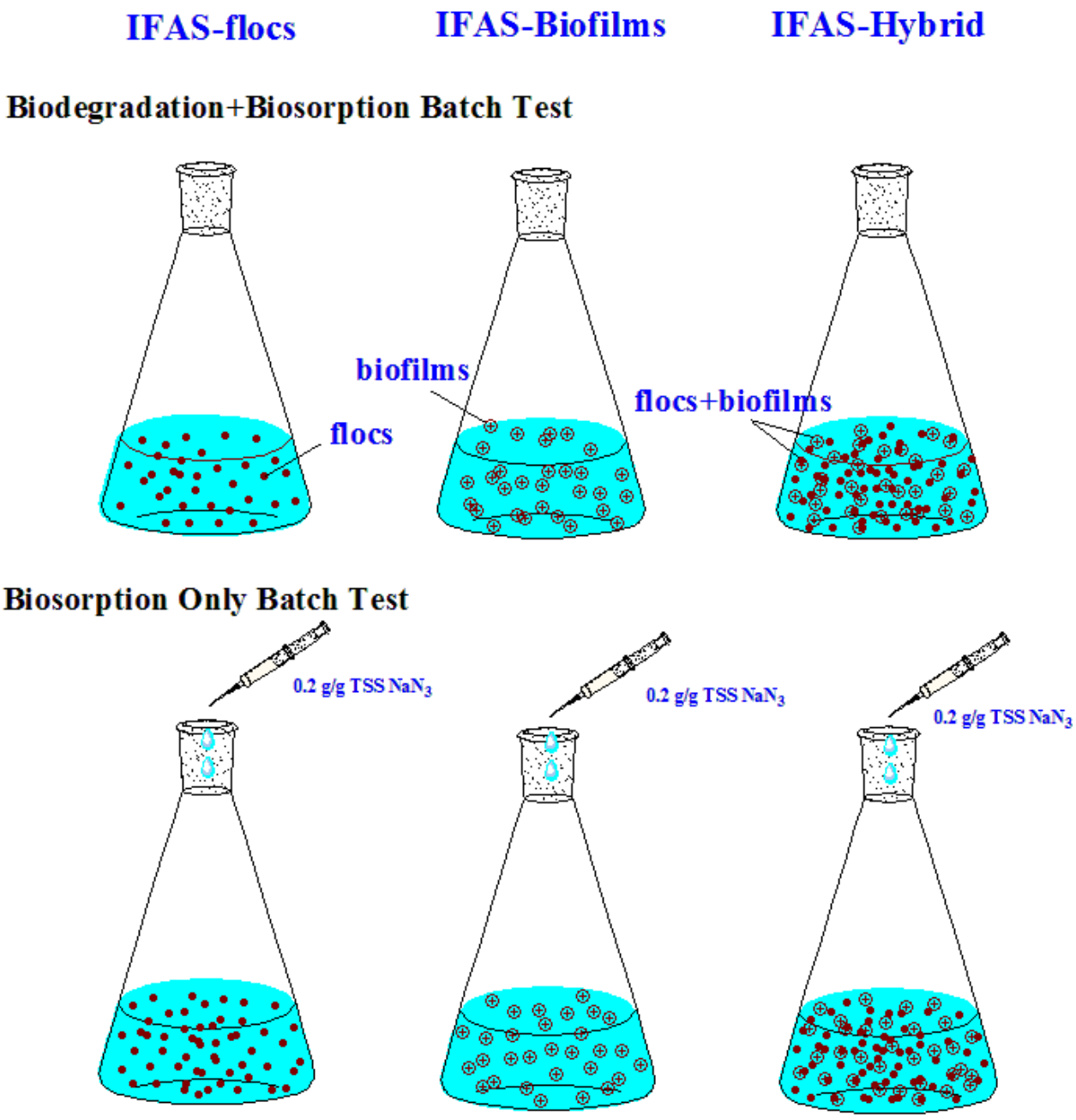


Figure 5.1. The schematic diagram of batch IFAS reactors.

Table 5.2 The strategies of batch IFAS reactor set-up and operation. Batch reactors were prepared by adding 30mg/L NH_4-N and 300 COD mg/L sodium acetate to raw and ozonated (30 mg/L ozone dose) OSPW.

Batch test phase	Biorption and biodegradation test	Biomass concentration (mg TSS/L)	Volume ratio of carrier (%)
Control[a]	-	0	0
Flocs	Biosorp. only	1000	0
	Biodegrad. + Biosorp.	1000	0
Biofilms	Biosorp. only	0	60 (**220**[b])
	Biodegrad. + Biosorp.	0	60 (**220**[b])
Hybrid	Biosorp. only	1000	60 (**220**[b])
	Biogegrad. + Biosorp.	1000	60 (**220**[b])

* All the experiments above described were performed in duplicate.

a We set up control reactor without flocs and biofilms addition.

b The number of carriers in the batch tests.

5.2.3 Ozonation of OSPW

Raw and ozonated OSPW (30 mg/L ozone dose) samples were used in those batch experiments. Ozone was generated with extra-dry, high-purity oxygen fed into an AGSO 30 Effizon ozone generator (WEDECO AG Water Technology, Herford, Germany). Ozone was sparged into OSPW samples through a ceramic fine bubble gas diffuser located at the bottom of a PVC container. The ozone concentration in the feed gas was continuously monitored by a high-concentration ozone monitor (HC500, WEDECO, Charlotte, NC, USA). The residual ozone concentration was monitored utilizing the indigo method (APHA, 2005). Ozonated OSPW was purged with nitrogen for 10 minutes to strip oxygen and excess ozone before use.

5.2.4 Measurement of Organic Contaminants

COD concentrations of influents and effluents from batch IFAS reactors were determined using DR3900 Benchtop Spectrophotometer (DR3900, HACH, Germany) according to the manufacturer's instruction (8000 TNTplus LR) with HACH kits (Chemical Oxygen Demand

(COD) Reagent, TNT821, LR, Germany). AEF in batch reactors was measured using a Fourier transform infrared (FTIR) spectrometer (Spectrum 100, PerkinElmer Ltd, Bucks, UK). Samples (25 mL) from influent and effluent of each IFAS batch reactor were filtered (0.45 μm), acidified to pH 2.0 and organics were extracted by liquid-liquid extraction with dichloromethane (DCM) in a separation funnel. After the extraction, the DCM was evaporated and the AEF remained in the glass container. The extracted AEF was dissolved with a known mass of DCM and analysed by FTIR. The sample absorbance was monitored at 1743 cm^{-1} and 1706 cm^{-1}, absorption bands characteristic of monomeric and dimeric carboxylic groups (Clemente and Fedorak, 2005).

5.2.5 Microbiological Analysis

Total genomic DNA was extracted from activated sludge flocs and biofilms in raw and ozonated OSPW batch IFAS reactors. The total DNA was extracted from 5 mL of the activated sludge flocs using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. To gather microorganisms from the biofilms formed on the carriers, 5 carriers from each reactor were immersed in phosphate buffered saline (PBS) to remove non-adherent bacteria. Subsequently, those carriers were placed in a 20 mL sterile glass vial with 5 mL of sterile PBS (Falcon, CA, USA), followed by sonication for 10 min. The glass vial was then vortexed for 1 min before the mixed liquid in the glass bottle was used for biofilms DNA extraction. DNA was extracted in duplicate from each sample.

To determine the amounts of the total bacteria, nitrifying bacteria and denitrifying bacteria, quantitative polymerase chain reaction (*q*-PCR) assays were conducted using a CFX96TM Real-Time Detection System (Bio-RAD, California, USA). Details of the *q*-PCR experiments are provided in the section A-4 (Appendix-A), and detailed description of *q*-PCR primers are shown in Table B2 (Appendix-B).

To determine the diversity and structure of the bacterial communities in activated sludge flocs and biofilms samples, paired-end sequencing of the extracted DNA samples were performed on the Illumina MiSeq platform (Caporaso et al., 2012) by Research & Testing Laboratory (Lubbock, Texas, USA). Bacterial 16S rRNA variable regions V1-V3 were targeted using the primer pairs of *28F* (5'-GAGTTTGATCNTGGCTCAG-3') and *519R* (5'-GTNTTACNGCGGCKGCTG-3') (Campbell et al., 2013).

All further data analysis was performed using the pipeline Quantitative Insights Into Microbial Ecology (QIIME), version 1.9.0 (Caporaso et al., 2010). Raw reads were filtered by QIIME quality filters. Effective reads were normalized for the next to come analysis. Sequences with more than 97% similarity were clustered into the same operational taxonomic units (OTUs) using the UCLUST algorithm. Then, representative OTUs were picked based on the most abundant sequences, and the taxonomic assignment were performed with UCLUST consensus taxonomy classifier. After that, the OUT sequences were aligned with the Python Nearest Alignment Space Termination (PyNAST) tool. Eventually, the communities were summarized by taxonomic composition.

5.2.6 Statistical Analysis

Data were analysed using Microsoft Excel® software where the level of the statistical significance was determined using a t-test. Correlations were considered statistically significantly at the 95% confidence interval ($p < 0.05$).

5.3 Results and Discussion

5.3.1 Overall Performance of Bench Scale IFAS Reactors

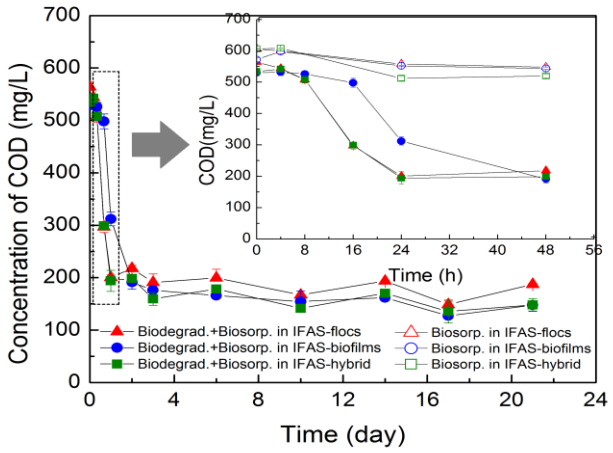
The batch tests were carried out using flocs and biofilms (taken from the two stabilized continuous-flow bench-scale IFAS reactors) for both raw and ozonated OSPW treatments. Two-bench scale IFAS reactors with hydraulic retention time (HRT) of 96 h were operated for 450 days. Their typical operating conditions as well as influent and effluent quality were presented in Table 5.1. The total COD removal efficiencies were 45.1% and 48.2% in raw and ozonated-OSPW IFAS reactor, respectively. The reactors performed an excellent ammonia oxidation as indicated by the low ammonia concentrations in the effluents. Total nitrogen concentrations in effluent were lower than those in influent and this is possibly due to the denitrification occurring within the aeration tank and/or the second clarifier. The MLSS concentrations of attached biofilms in both raw and ozonated-OSPW IFAS reactors were significantly lower than suspended biomass (Table 5.1).

5.3.2 Performance of Batch IFAS Reactors

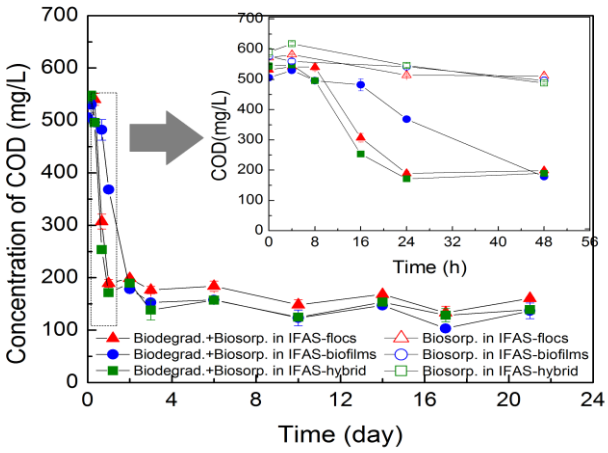
5.3.2.1 Organic Compounds Removal

The COD removal by flocs and biofilms in batch reactors was evaluated for both biosorption only condition (with NaN_3 addition) and biosorption and biodegradation condition. It has been reported that NaN_3 can significantly alter biomass viscosity, and the addition of NaN_3 result in a respiration inhibition of biomass (Barbot et al., 2010). As shown in Fig. 5.2(A) and 5.2 (B), without NaN_3 addition, the COD biodegradation proceeded extremely quickly and the biodegradation process did not stop after 24h. The COD removal efficiencies due to the combination of biosorption and biodegradation were 65.0%, 39.1% and 66.4% in raw-OSPW

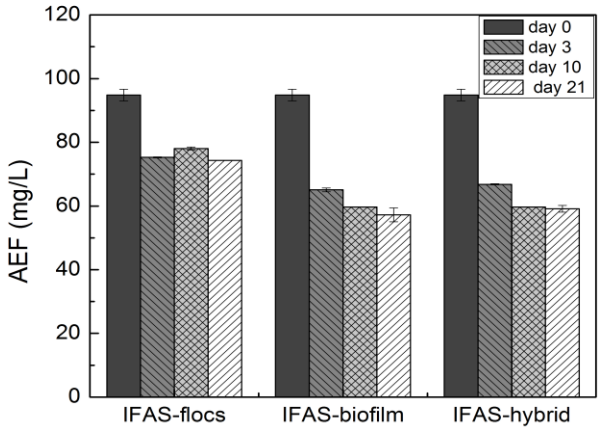
(A) **Raw-OSPW IFAS**



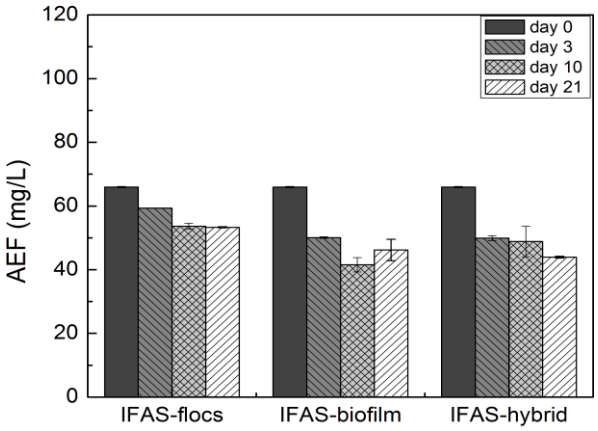
(B) **Ozonated-OSPW IFAS**



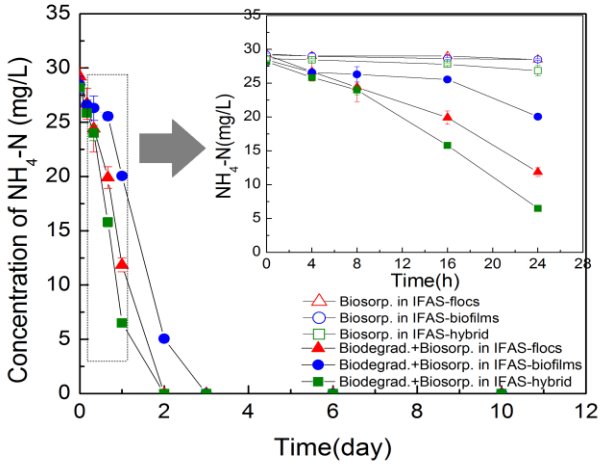
(C)



(D)



(E)



(F)

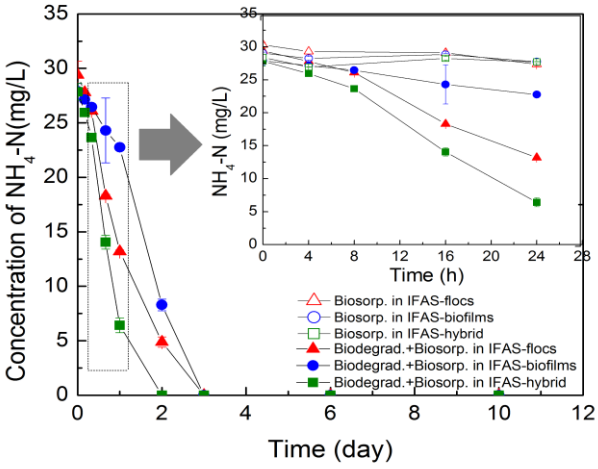


Figure 5.2. Profile of COD concentration changes over batch reactor operation time in raw (A) and ozonated (B) OSPW IFAS reactors; Profile of AEF concentration changes in raw (C) and ozonated (D) OSPW IFAS reactors at different periods; Profile of NH_4-N concentration changes over reactor operation time in raw (E) and ozonated (F) OSPW IFAS reactors.

reactor and 65.7%, 27.2% and 68.7% in ozonated-OSPW reactor respectively in three systems (IFAS-flocs, IFAS-biofilms and IFAS-hybrid).

In comparison, the biosorption of COD caused by the non-respiring flocs and biofilms obtained in the presence of NaN_3 occurred quickly and achieved the maximum adsorptive capacity in 24h. As shown in Fig. 5.2(A) and 5.2(B), it appears that the removal of the organic compounds in OSPW in the IFAS system is characterized by a quick biosorption on the biomass and a slow biodegradation process. In three NaN_3 inhibited systems (IFAS-flocs, IFAS-biofilms and IFAS-hybrid), the total COD removal efficiencies due to the biosorption in raw-OSPW reactors reached 7.9%, 3.5% and 15.5%, respectively, while the removal efficiencies in ozonated-OSPW reactors were 10.1%, 5.9% and 7.5%, respectively.

This observation revealed that the biodegradation was the principal removal mechanism and biosorption contributed little to the OSPW organic compounds removal in the IFAS system. One possible explanation is that the diffusion processes in the biomass reached equilibrium as the diffusion resistance increased (Shi et al., 2011), resulting in the limited biosorption capacity of flocs and biofilms. In the bioreactor treatment processes, the substrates, oxygen and nutrients diffuse across the stagnant liquid layer from the bulk liquid to the floc and biofilm, this process was regarded as *film diffusion transport*, which was a principal step in the biosorption process

(Tchobanoglous. et al., 2003). So the biosorption plays an important role in the promotion of biodegradation of organic compounds in OSPW.

COD degradation rate in the first 48 hours of the experiment was presented in the top right inserts in Fig. 5.2(A) and 5.2(B). For the reactors with activated sludge flocs (IFAS-flocs and IFAS-hybrid) for both raw and ozonated OSPW treatment, the total COD removal experienced a sharp decrease between 8h and 48h, while the reactors with biofilms only (IFAS-biofilms) showed a slight decrease during this period. The higher COD removal rate in the reactors with flocs may be contributed to three reasons: (i) the higher biomass concentration of the suspended flocs, (ii) the lower transport rates within the biofilm which extended the time for the soild-water equilibriums to be reached (Falas et al., 2012), or (iii) the different microbial population present in these aggregations. It was noticed that the COD in IFAS-flocs and IFAS-hybrid batch reactors decreased in a similar way, the COD degradation kinetics of these two conditions overlapped for both raw and ozonated OSPW treatment. Based on our observation, it can be demonstrated that the flocs play a more significant role for the COD removal in the IFAS system. For the easily biodegradable organic substance, the suspended biomass is essential. Compared to the attached biofilms, the suspended flocs have the ability to utilize substrate as well as oxygen in a short period of time (Chen et al., 1997).

The AEF was commonly applied by the oil sands industry to evaluate the degree of biodegradation of naphthenic acids (NAs) in OSPW. FTIR was applied to monitor the AEF fraction in both influents and effluents of the IFAS batch reactors, the AEF fraction contains parent NAs ($C_nH_{2n+Z}O_2$), oxidized NAs ($C_nH_{2n+Z}O_x$, x = 3–5), and other organics containing carboxylic groups (Grewer et al., 2010). Fig. 2(C) and 2(D) show the AEF reduction in raw and ozonated batch reactors at different operation time. The initial AEF concentrations in raw and

ozonated OSPW were 95.8 and 65.9 mg/L, respectively. On day 3, the AEF removal efficiencies of raw-OSPW in IFAS-flocs, IFAS-biofilms and IFAS-hybrid reactors reached 20.6%, 31.4% and 29.5%, respectively, while the removal efficiencies of ozonated-OSPW reactors were 9.98%, 23.9% and 24.2%, respectively. In order to provide a long HRT for the OSPW degradation, the experiments were carried out for 21 days. After 21 days operation, the AEF removal efficiencies of raw-OSPW in IFAS-flocs, IFAS-biofilms and IFAS-hybrid reactors reached 21.6%, 39.6% and 37.6%, respectively, and reached 19.1%, 29.9% and 33.3%, respectively, for ozonated-OSPW treatment. It was noted that AEF removal efficiencies in IFAS-flocs reactors were significantly lower ($p < 0.05$) than the reactors with biofilms (i.e., IFAS-biofilms and IFAS-hybrid). This observation indicates that the biofilms in the IFAS system have better performance on the AEF removal, and the AEF degraders preferred to gathering in the form of attached biofilms.

Our previous studies showed that OSPW has a low biodegradability (BOD/COD ratio < 0.1), and the AEF fractions show high resistance to the biodegradation. Two possible reasons for the higher AEF removal with the attached biomass compared to the suspended biomass are, i) slow growing AEF degrading microorganisms may benefit from a higher solid retention time in the attached biofilms as compared to the suspended flocs, and ii) the different oxygen and nutrients gradients between the flocs and biofilms may lead to the highly stratified and diversified microbial community in the biofilms. Biofilms may harbour microorganisms adapted to the slowly biodegradable organic substrates (Falas et al., 2012).

5.3.2.2 Nitrogen Removal

The NH_4-N removal by the biodegradation and biosorption was also evaluated. As shown in Fig. 5.2(E) and 5.2(F), with NaN_3 addition (biosorption only), the NH_4-N removal percentage

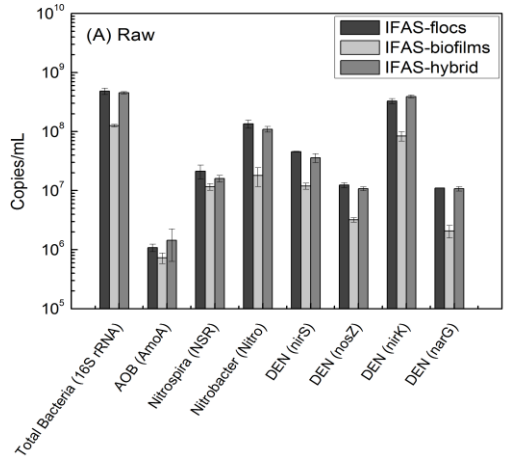
in IFAS-flocs, IFAS-biofilms and IFAS-hybrid reactors reached 2.7%, 2.4% and 6.3%, respectively for raw OSPW, and 9.7%, 4.5% and 2.5%, respectively, for ozonated OSPW after 24h operation. However, the ammonia oxidation process occurred rapidly without the NaN_3 inhibition. After 24h operation, in three types of reactors, the NH_4-N removal efficiencies in IFAS-flocs, IFAS-biofilms and IFAS-hybrid achieved 59.4%, 29.5% and 76.9%, respectively in raw-OSPW reactors, and 55.1%, 18.5% and 77.0% respectively, in ozonated-OSPW reactors. Therefore, it demonstrated that the nitrification was the main removal mechanism and biosorption contributed very little to the NH_4-N removal in the IFAS system.

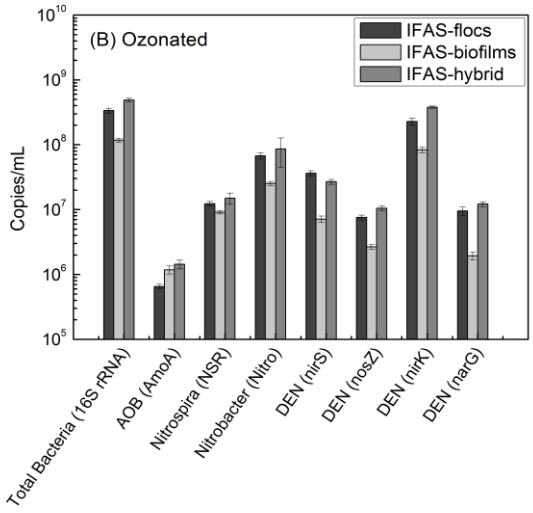
The NH_4-N degradation kinetics for the batch reactors was shown in Fig. 5.2(E) and 5.2(F). Within 3 days, the NH_4-N was completely oxidized in all the reactors. For the reactors with flocs, the NH_4-N concentration experienced a sharp decrease between 4h and 24h, while the reactors with carriers showed a slight decrease during this time. It indicates that both kinds of biomass form IFAS system show good performance of nitrification and IFAS-flocs reactors have significantly higher nitrification rate compared to the IFAS-biofilms reactors. Among those three types of reactors, IFAS-hybrid reactors had the most outstanding performance on the ammonia oxidation. The possible causes that the reactors with flocs had higher nitrification rate than biofilms are i) the significantly higher biomass concentration in IFAS-flocs reactors as compared to the IFAS-biofilms reactors enhanced ammonia oxidation rate in reactors with flocs, and ii) the difference of surface properties of flocs and biofilms may lead to the different nutrients and oxygen transfer efficiency. It was reported that biofilms possesses a higher biomass density and lower surface negativity and hydrophobicity as compared to flocs in bioreactors (Mahendran et al., 2012). Hereby, flocs may facilitate faster molecular oxygen diffusion and ammonia oxidation

(Chen et al., 1997). Therefore, the nitrification rate in reactors with IFAS-flocs was higher than IFAS-biofilms reactors.

5.3.3 Quantification of Bacterial Population by the Quantitative Polymerase Chain Reaction

The gene abundances quantification of total bacteria, AOB, *Nitrospira*, *Nitrobacter* and denitrifiers were performed by determining 16S rRNA gene copies using quantitative real-time PCR. Fig. 5.3(A) and 5.3(B) compared the gene abundance of total bacteria, *AmoA*, *NSR*, *Nitro* and the denitrifier relevant genes (*nirS*, *nosZ*, *nirK* and *narG*) measured within IFAS-flocs, IFAS-biofilms and IFAS-hybrid batch reactors. In three types of IFAS batch reactors (IFAS-flocs, IFAS-biofilms and IFAS-hybrid), the total bacterial population in raw-OSPW reactors was around 4.84×10^8, 1.26×10^8, and 4.54×10^8 copies/mL, respectively, and 3.38×10^8, 1.17×10^8 and 4.89×10^8 copies/mL, respectively, in ozonated-OSPW reactors. It indicates that the total bacteria population in flocs is significantly larger than biofilms ($p < 0.05$) in the IFAS systems, which was consistent with the MLSS concentration (Table 5.1) of the suspended and attached biomass in the IFAS systems. Further, the gene abundance of AOB was determined using q-PCR quantification methods targeting the ammonia monooxygenase α-subunit gene (*AmoA*) of *Nitrosomonas oligotropha*, an AOB species. As shown in Fig. 5.3(A) and 5.3(B), in three types of IFAS batch reactors (IFAS-flocs, IFAS-biofilms and IFAS-hybrid), the abundance of *AmoA* in raw-OSPW reactors was 1.08×10^6, 7.28×10^5, and 1.44×10^6 copies/mL, respectively, and in ozonated-OSPW reactors, was 6.53×10^5, 1.18×10^6 and 1.44×10^6 copies/mL, respectively. These results help to explain why the IFAS-flocs reactors have better performance on the COD and NH_4-N removal as compare to the IFAS-biofilms reactors.


(A) Raw
IFAS-flocs
IFAS-biofilms
IFAS-hybrid
Copies/mL
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
Total Bacteria (16S rRNA)
AOB (AmoA)
Nitrospira (NSR)
Nitrobacter (Nitro)
DEN (nirS)
DEN (nosZ)
DEN (nirK)
DEN (narG)


(B) Ozonated
IFAS-flocs
IFAS-biofilms
IFAS-hybrid
Copies/mL
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
Total Bacteria (16S rRNA)
AOB (AmoA)
Nitrospira (NSR)
Nitrobacter (Nitro)
DEN (nirS)
DEN (nosZ)
DEN (nirK)
DEN (narG)

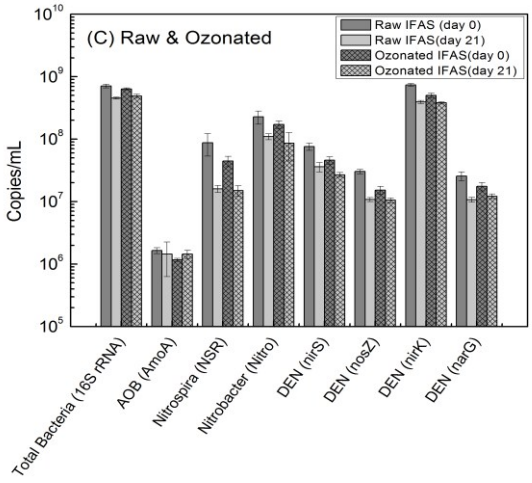

(C) Raw & Ozonated
Raw IFAS (day 0)
Raw IFAS(day 21)
Ozonated IFAS(day 0)
Ozonated IFAS(day 21)
Copies/mL
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
Total Bacteria (16S rRNA)
AOB (AmoA)
Nitrospira (NSR)
Nitrobacter (Nitro)
DEN (nirS)
DEN (nosZ)
DEN (nirK)
DEN (narG)

Figure 5.3. The gene copies per mL of total bacteria, AOB, *Nitrospira*, *Nitrobacter* and denitrifying bacteria (*nirS*, *nosZ*, *nirK* and *narG*) in flocs, biofilms and hybrid samples in raw (A) and ozonated (B) OSPW IFAS batch reactors (day 21), and on day 0 and day 21 in raw and ozonated IFAS-hybrid samples (C).

We also studied the *Nitrospia* and *Nitrobacter* genera (*NSR* and *Nitro*) for NOB, and *narG*, *nirS*, *nirK*, and *nosZ* genes of denitrifying bacteria. It was noted that the gene abundance of *NSR* in all of the batch reactors was significantly lower than that of *Nitro*. One possible explanation was that *Nitrospia* were far more sensitive to the toxicity of OSPW than *Nitrobacter*. The abundance of *NSR*, *Nitro*, and denitrifier genes (*narG*, *nirS*, *nirK* and *nosZ*) in IFAS-flocs, IFAS-biofilms and IFAS-hybrid are compared in Fig. 5.3(A) and 5.3(B). It indicates that the gene abundances of *NSR* and *Nitro* in IFAS-flocs reactors were higher than those in IFAS-biofilms reactors, which was consistent with the nitrification activity of those two types of batch reactors. Regarding the denitrifier genes (*narG*, *nirS*, *nirK* and *nosZ*), in three kinds of batch reactors, the abundance of denitrifying genes in IFAS-flocs was significantly higher than those in IFAS-biofilms.

As was shown in Fig. 5.3(C), the gene abundance changes of the total bacteria, AOB, *Nitrospira*, *Nitrobacter* and the denitriers in IFAS-hybrid reactors was not significant ($p > 0.05$) after 21 days batch reactor operation. The gene abundance changes at the beginning and the end of batch tests operation are within one log unit.

5.3.4 Microbial Community Analysis

To assess the microbial communities, comparison was made between the IFAS-flocs and IFAS-biofilms. The DNA samples of IFAS-flocs and IFAS-biofilms were analysed by Illumina

sequencing.

The microbial community structures of flocs and biofilms (relative abundance, RA > 0.2%) at the phylum level are shown in Fig. 5.4. At the phylum level, similar distributions were found between the flocs and biofilms of IFAS reactors. It appeared that *Proteobacteria*, *Acidobacteria*, *Nitrospirae* and *Bacteroidetes* were dominant phyla in both flocs and biofilms of IFAS reactors. These four phyla accounted for 92.6% and 89.3% of total reads in flocs and biofilms of raw-OSPW reactors, respectively, and 93.9% and 91.2% of total reads in flocs and biofilms from ozonated-OSPW reactors, respectively. Fig. 5.4 also shows that *Nitrospira*, *Planctomycetes*, *Cyanobacteria*, *SBR 1093*, *Verrucomicrobia*, and *Chloroflexi*, were found to be more abundant in the biofilms compared with flocs, whereas the RA of *Acidobacteria, Bacteroidetes* and *Gemmatimonadetes* in the flocs were significantly higher than those in the biofilms.

The taxonomic classifications at the class level are shown in Fig. 5.5. At the class level, more differences were observed between flocs and biofilms. Among *Proteobacteria* in the flocs and biofilms of IFAS reactors (Fig. 5.5), *alpha-*, *beta-* and *gamma-Proteobacteria* were the dominant classes, the *delta-Proteobacteria* populations in both flocs and biofilms from IFAS reactors were relatively small (< 1%), which was consistent with previous studies of an Alberta oil sands tailing pond (Golby et al., 2012). The relative percentages of them were 2.2%, 33.1% and 3.2% in the flocs, 11.0%, 14.0% and 7.5% in the biofilms of raw-OSPW IFAS reactors, while 5.5%, 26.4% and 4.6% in the flocs, 8.7%, 25.6% and 4.4% in the biofilms of ozonated-OSPW IFAS reactors, respectively. This finding indicates that the biofilms contained more *alpha-Proteobacteria* than flocs, however, the populations of *beta-* and *gamma-Proteobacteria* were comparable between flocs and biofilms.

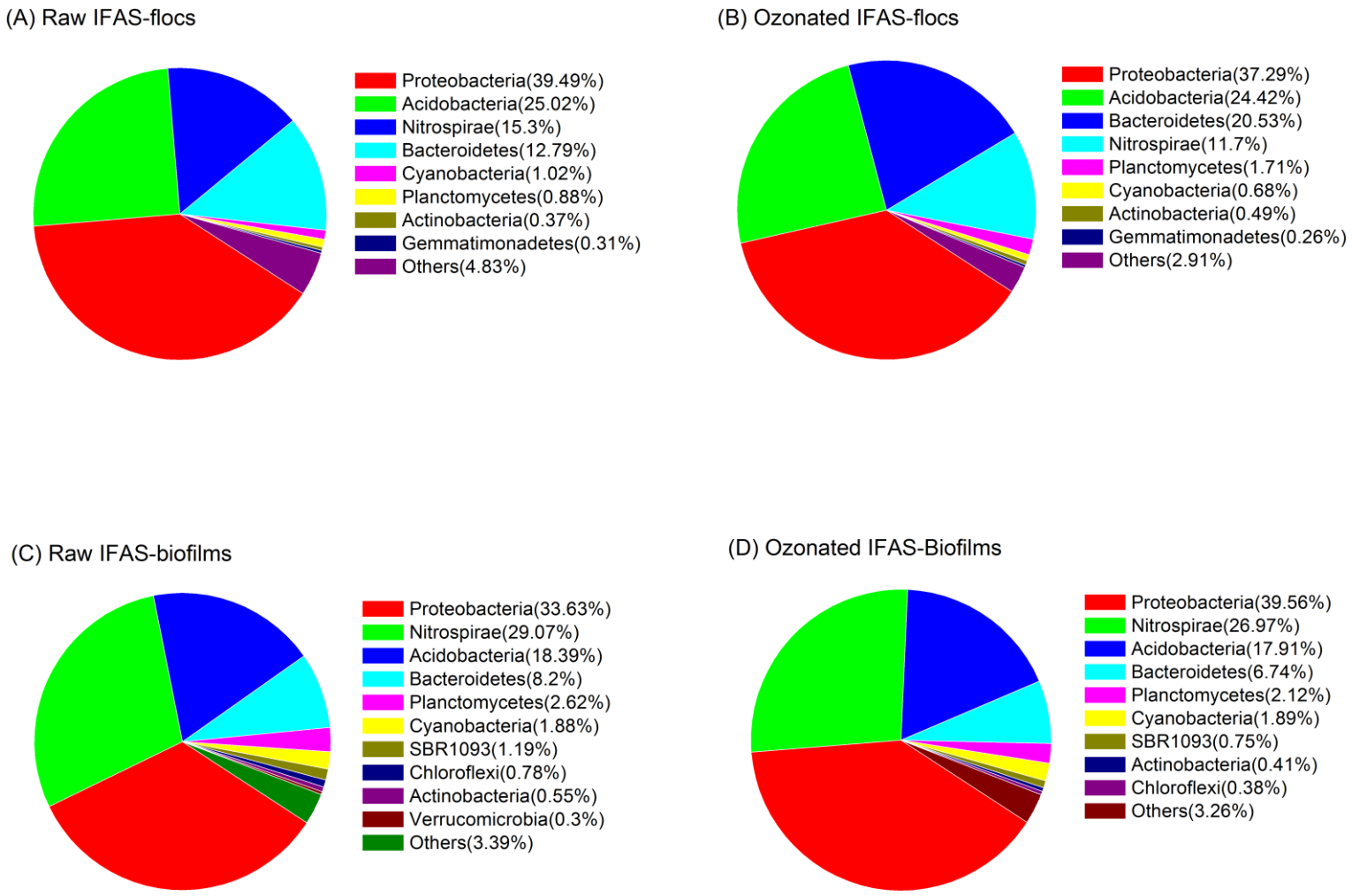


Figure 5.4. Microbial community structures of the flocs in raw (A) and ozonated (B) OSPW IFAS, and biofilms in raw (C) and ozonated (D) OSPW IFAS at phylum levels on day 21. Phyla at relative abundance < 0.2% were grouped as "others".

The nitrite oxidizing bacteria in the flocs and biofilms were *Nitrospira*, which belongs to the *Nitrospira* class. Fig. 5.5 shows that there is a significant difference of the *Nitrospira* class population between biofilms and flocs of IFAS reactors. The RA of *Nitrospira* in the biofilms

were 29.1% and 27% of raw and ozonated-OSPW IFAS reactors, respectively, and 15.3% and 11.7% in the flocs from these two types of IFAS reactors, respectively. Our results demonstrated that the RA of *Nitrospira* in the biofilms was more abundant than that in the flocs (in the previous section). It has been previously reported that *Nitrospira* were involved in denitrification, sulphur oxidation, and sulphate reduction (Lin et al., 2014a).

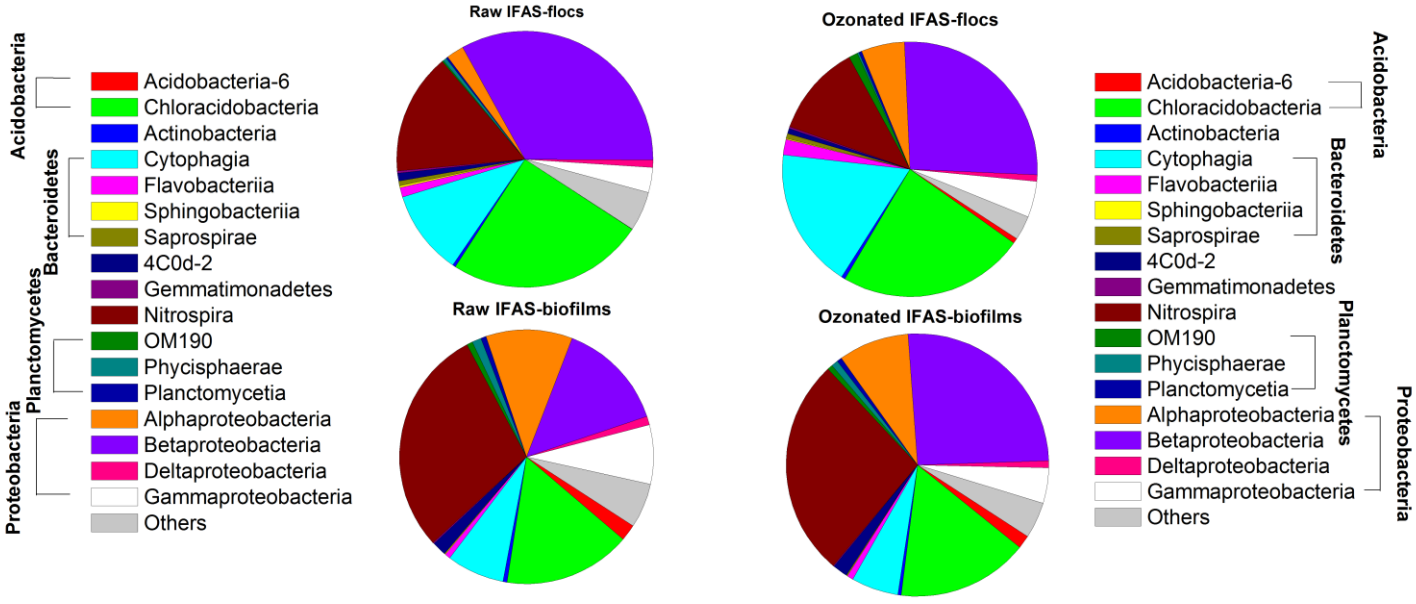


Figure 5.5. Taxonomic classification at class level in flocs and biofilms of raw and ozonated OSPW IFAS on day 21. Minor bacterial class accounting for < 0.2% of total sequences were summed up as "others".

Further, among the *Acidobacteria* in the flocs and biofilms of IFAS batch reactors, the members of *Acidobacteria* are important to the degradation of carbohydrates in boreal forest peat land, and they are able to well adapt to acidic, nutrient-poor conditions (Lin et al., 2014b). The dominant class was *Chloracidobacteria*, and the relative percentages of *Acidobacteria-6* in both

flocs and biofilms were relatively low (< 2%). It was found that the distribution of *Chloracidobacteria* was more abundant in flocs than in biofilms. Regarding the *Bacteroidetes*, the members of *Bacteroidetes* play an important role in mineralization of complex organic compounds including polysaccharides and proteins in the marine realm (Kabisch et al., 2014). *Cytophagia* was the dominant class in both flocs and biofilms samples, and the RA of *Cytophagia* in the flocs was significant higher than those in biofilms. It has been found that *Cytophagia* linked to the degradation of hydrocarbons (Roling et al., 2002). The populations of *Flavobacteria*, *Sphingobacteriia* and *Saprospirae* in both flocs and biofilms were relatively small.

5.4 Conclusions

This study evaluated the role of suspended flocs and attached biofilms toward organic compounds and ammonium removal in OSPW, and compared the role of the two main removal mechanisms (biodegradation and biosorption) on the overall OSPW remediation. Compared to the biofilms, the IFAS-flocs demonstrated considerably higher removal rates for COD and NH_4-N, whereas, IFAS-biofilms had better performance on the AEF removal than IFAS-flocs. Meanwhile, our study also revealed that the biodegradation was the principal OSPW contaminant removal mechanism, and biosorption had limited contribution to the OSPW organic compounds and ammonium removal. Microbial analysis from *q*-PCR showed that the abundances of nitrifier and denitrifier genes were significantly higher in the flocs than those in the biofilms in both raw and ozonated-OSPW IFAS reactors. The microbial communities analysis from MiSeq sequencing revealed that *Proteobacteria*, *Acidobacteria*, *Nitrospirae* and *Bacteroidetes* were dominant phyla in both flocs and biofilms of IFAS reactors. However, the

microbial community composition of flocs and biofilms at the class level were significantly different. All these results support the distinct role of the IFAS flocs and biofilms in the contaminant removal of OSPW.

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CHAPTER 6. OPTIMIZATION OF INTEGRATED FIXED-FILM ACTIVATED SLUDGE (IFAS) IN THE TREATMENT OF OIL SANDS PROCESS-AFFECTED WATER (OSPW)[1]

6.1 Introduction

Water associated with bitumen extraction in the Athabasca oil sands region of northern Alberta contains a mixture of suspended solids, residual bitumen, heavy metals, inorganic compounds, and naphthenic acids (NAs) (Sun et al., 2014) and is acutely and chronically toxic to aquatic organisms, invertebrates, and mammals (Gamal El-Din et al., 2011; He et al., 2012; Anderson et al., 2012; Garcia-Garcia et al., 2011). Due to its toxicity, this oil sands process-affected water (OSPW) is currently being stored in tailings ponds following Alberta's zero discharge policy (Speight, 2000). A conservative estimate suggests that the accumulated volume of OSPW will reach 1 billion m^3 in the next 15–20 years (Lo et al., 2003; Del Rio et al., 2006). The concentration of classical naphthenic acids (NAs) in OSPW ranges from 7.1 to 47 mg/L (Grewer et al., 2010; Gamal El-Din et al., 2011;Wang et al., 2013) and presents a major threat to aquatic organisms (Anderson et al., 2012). Adequate OSPW treatment technologies will reduce the accumulation of OSPW in the tailings ponds and enable OSPW recycling to reduce the demand for fresh water intake from the Athabasca River (Islam et al., 2015a).

Bioreactor technology exploits the degradation of organic compounds through reactions mediated by microbial aggregates (i.e., flocs and biofilms) formed in activated sludge, and

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has been used effectively for OSPW reclamation (Hwang et al., 2013; Choi et al., 2014; Shi et al., 2015; Huang et al., 2015). Hwang et al. (2013), using attached biofilms, observed that 41.0% of chemical oxygen demand (COD), 18.5% NAs, and 13.8% of the AEF were removed from OSPW in a 1L continuous flow biofilm reactor operated in batch mode for 24 hours. Islam et al. (2014a) observed 62% of COD, 88% of AEF and 99.9% of classical NAs removal with HRT of 8.5 hours using a 0.3L fluidized bed biofilm reactor (FBBR) with granular activated carbon (GAC) as support media. However, FBBR requires high upflow velocity and thus has high energy requirements. Choi et al. (2014) demonstrated that 12% of COD and 8.7% of the AEF were removed in an activated sludge-sequencing batch reactor (AS-SBR), and 20% of COD and 16.6% of the AEF were removed in a mature fine tailings-sequencing batch reactor (MFT-SBR). Using 8.5 L moving bed biofilm reactors (MBBRs) with polyethene carriers as support media, Shi et al. (2015) observed the removal of 18.3% of the AEF and 34.8% of the NAs without OSPW ozonation and removal of 41% of the AEF and 78.8% of the NAs with OSPW ozonation.

An IFAS reactor comprising combined suspended and attached growth in a single reactor can achieve significant microbial population diversity and the degradation of a wide range of contaminants. Our recent study (Huang et al., 2015) demonstrated that 12.1% of the acid extractable fraction (AEF) and 43.1% of parent NAs were removed in IFAS treatment of raw OSPW, while 42.0% of the AEF and 80.2% of parent NAs were removed in IFAS treatment of ozonated OSPW. Most published work on OSPW remediation using engineering bioreactors focus on the potential feasibility and start-up treatment performance. To date, no bioreactor optimization study has been conducted for OSPW treatment using IFAS.

To develop an efficient OSPW bioremediation system, the hydraulic retention time (HRT) and influent COD/N ratio must be optimized. The HRT contributes to the biomass characteristics

and the microbial community composition (Huang et al., 2011). A high HRT benefits the removal of recalcitrant organics but an increase in reactor volume is required to achieve higher removal performance. Low HRT values result in higher organic loading rates (OLR) (Fallah et al., 2010), which can lead to more active biomass and faster degradation rates. However, a high OLR might also lead to incomplete organics removal. The chemical oxygen demand (COD) measures the amount of organic compounds in water, which can significantly influence microbial community structures (Feng et al., 2012). As microbial growth requires nitrogen (N), an optimal COD/N ratio can facilitate the growth of degraders leading to a higher OSPW treatment efficiency. Nitrifiers such as *Nitrosomonas europaea* catalyze the oxidation of ammonia to nitrite. Ammonia is first converted to hydroxylamine by the enzyme ammonia monooxygenase (AMO), followed by the oxidation of hydroxylamine to nitrite by hydroxylamine oxidoreductase. Previous studies showed that AMO may catalyze the oxidation of various organics, including hydrocarbons and halogenated hydrocarbons (Rasche et al., 1990). The investigation of the effects of HRT and COD/N ratio on treatment performance and microbial community structure are necessary for OSPW bioremediation strategy development. Studies are needed to evaluate these parameters for OSPW treatment using ozonation combined with IFAS.

OSPW has low biodegradability, a relatively low COD (250-350 mg/L), and a biological oxygen demand (BOD)/COD ratio < 0.1. The high toxicity of OSPW leads to a high resistance to biodegradation of NAs. As ozonation breaks down highly branched, highly cyclic NA fractions (Martin et al., 2010; Gamal El-Din et al., 2011), pretreatment of OSPW with ozone has been tested to break down recalcitrant NAs and enhance OSPW biodegradability (Dong et al., 2015; Islam et al., 2014b). In this study, the ability of microbial community structures in IFAS to

detoxify raw and ozonated OSPW was tested, and the impacts of HRT and the COD/N ratio on microbial community dynamics in the IFAS systems were evaluated.

6.2 Materials and Methods

6.2.1 Source Water Information

Raw (untreated) OSPW was obtained from oil sands tailings ponds in Fort McMurray, AB, Canada, in September 2013. OSPW samples were stored in 200 L polyvinyl chloride containers in a step-in cold room (4 °C) prior to use.

A 0.1% (v/v) trace nutrient solution was prepared containing: $MgCl_2 \cdot 6H_2O$, 3 g L^{-1}; $CaCl_2$, 1.5 g L^{-1}; $FeSO_4 \cdot 7\ H_2O$, 0.28 g L^{-1}; $MnCl_2 \cdot 4\ H_2O$, 0.13 g L^{-1}; $ZnSO_4 \cdot 7\ H_2O$, 0.12 g L^{-1}, $CuSO_4 \cdot 5\ H_2O$, 0.0074 g L^{-1}. The pH was 7.3 ± 0.3. All chemicals and supplies were obtained from Thermo Fisher Scientific.

6.2.2 Reactor Operation

Two identical bench-scale IFAS reactors provided by Napier-Reid Ltd (Markham, Canada) were operated in parallel, one to treat raw OSPW and the other to treat ozonated (30 mg/L ozone dose) OSPW under the same conditions. The IFAS reactors had a working volume of 8.5 L (15 cm × 35 cm base, 30 cm height) and a clarifier (Fig. C1) (Appendix-C). Air diffusers were installed for aeration and to circulate the support carriers. The dissolved oxygen (DO) concentration was maintained at 6-7 mg/L during operation. A 60% volume fraction of polyethylene (PE) carriers (Bioflow 9, Rauschert, Steinwiessen, Germany) with specific biofilm growth areas of 800 m^2/m^3 were applied in the IFAS reactors. The reactors were operated continuously at room temperature (20 ± 1 ˚C).

Table 6.1 The optimization strategies of IFAS systems. The influent were prepared by adding 130 COD mg/L sodium acetate and ammonium chloride to raw or ozonated (30 mg/L ozone dose) OSPW

Optimization phase	Operation days	HRT (h)	Total COD of raw OSPW IFAS influent (mg/L)	Total COD of ozonated OSPW IFAS influent (mg/L)	The concentration of NH_4-N (mg/L)	COD/N ratio of raw OSPW IFAS influent	COD/N ratio of ozonated OSPW IFAS influent
I	283-365	48	323.2	310.2	30	10.8	10.3
II	366-439	72	323.2	310.2	30	10.8	10.3
III	440-520	96	323.2	310.2	30	10.8	10.3
IV	521-553	96	323.2	310.2	45	7.2	6.9
V	554-607	96	323.2	310.2	60	5.4	5.2

Both reactors were inoculated with seed sludge taken from the Gold Bar Wastewater Treatment Plant (Edmonton, Canada). The bioreactor start-up strategies and reactor performance results (from 0 - 280 days) have been published previously (Huang et al., 2015). After reactor reached stable operation, reactor optimization started (from day 280). During the optimization stage, the carbon sources in the influents of the IFAS systems were raw or ozonated OSPW and sodium acetate, the HRT was increased from 48 to 72 to 96 h. To maintain a stable HRT, a peristaltic pump (Masterflex L/S, Gelsenkirchen, Germany) was used to control the flow rate of the influent. Ammonium chloride was added to increase the ammonium concentration of the influent from 30 to 45 to 60 mg/L in three phases resulting in COD/N ratios of 10.8, 7.2, and 5.4 in raw OSPW IFAS and 10.3, 6.9, and 5.2 in ozonated OSPW IFAS (Table 6.1). During the entire optimization stage, extra carbon (sodium acetate, 130 mg COD/L), nitrogen (ammonium chloride, 30, 45, and 60 mg N/L), phosphorus (KH_2PO_4, 3.0 ± 0.2 mg P/L), and other necessary nutrients (see section 6.2.1) were provided to maintain the growth of biomass in the IFAS systems. The average activated sludge solid retention time (SRT) was 43 days, which was

maintained by manually wasting sludge from the clarifier. The detailed optimization strategies are listed in Table 6.1.

6.2.3 Ozonation of OSPW

A sufficient experimental volume of OSPW was ozonated before the experiment. An utilized ozone dose of 30 mg/L was applied according to our previous research (Dong et al., 2015). Ozone pretreatment of raw OSPW was performed using an AGSO 30 Effizon ozone generator (WEDECO AG Water Technology, Herford, Germany), which can produce ozone gas using extra dry, high purity oxygen. A detailed description of the ozonation procedure is reported in Wang et al. (2013). Briefly, ozone was introduced into the liquid phase by a ceramic fine bubble gas diffuser installed at the bottom of the 200 L polyvinyl chloride container. Ozone concentrations in the feed-gas (110-120 mg/L) and off-gas (varied from 0 to 80 mg/L) were continuously monitored during the process by two identical ozone monitors (HC-500, PCI-WEDECO, USA), With the ozone contact time of 15-17 min gas flowrate of 10 L/min, the cumulative consumption of ozone was approximate 6000 mg for 200 L OSPW. The Indigo method was used to estimate the residual ozone (APHA, 2005). The ozonated OSPW was purged with nitrogen for 10 min to strip oxygen and excess ozone.

6.2.4 Measurement of Organic Contaminants

The acid extractable fraction (AEF) was measured with a Fourier transform infrared (FTIR) spectrometer (Spectrum 100, PerkinElmer Ltd, Bucks, UK). Samples (25 mL) from influent and effluent of IFAS reactors were filtered (0.45 μm), acidified to pH 2.0, and organics were extracted with a separation funnel using dichloromethane (DCM). After DCM evaporation, the extracted AEF was dissolved in a known mass of DCM and analysed by FTIR. The sample

absorbance was monitored at 1743 cm^{-1} and 1706 cm^{-1}, absorption bands characteristic of monomeric and dimeric carboxylic groups, respectively (Clemente and Fedorak, 2005).

6.2.5 Microbiological Analysis

DNA extraction. Genomic DNA was extracted from activated sludge flocs and biofilms obtained from raw and ozonated OSPW IFAS systems. Activated sludge floc samples were obtained by centrifuging (10,000 g) 4 mL of mixed liquid from each reactor for 1 min; supernatants were discarded and the total DNA in activated sludge flocs was extracted with a Power Soil DNA Isolation Kit (MO BIO Laboratories, CA, USA) according to the manufacturer's protocol. Biofilm on the support media was obtained from 2 carriers in each reactor. Carriers were immersed in phosphate buffered saline (PBS) to remove unattached biomass, placed in 5 mL sterile PBS in a 20 ml sterile glass bottle (Falcon, CA, USA), sonicated for 10 min, and vortexed for 1 min to remove residual biofilm. The biomass in the glass bottle was used for biofilm DNA extraction.

q-PCR (quantitative polymerase chain reaction). To investigate changes in nitrifier and denitrifier gene abundance in the suspended phase (activated sludge flocs) and the attached phase (biofilms) of IFAS systems during the optimization stage, *q*-PCR assays were conducted using a CFX 96TM Real-Time Detection System (Bio-RAD, California, USA) to quantify the *bacterial 16S rRNA* gene for total bacteria, *amoA* gene for ammonia oxidizing bacteria (AOB), two *16S rDNA genes* (*Nitrospira* spp. and *Nitrobacter* spp.) for nitrite-oxidizing bacteria (NOB), and *narG*, *nirS*, *nirK*, and *nosZ* genes for denitrifiers. As shown in Table B1 (Appendix-B), the total *bacterial 16S rRNA gene* was amplified using primers (*341f* and *907r*) (Muyzer et al., 1993). To determine the amount of *amoA* gene, primers (*amoA-1F* and *amoA-2R*) (McTavish et al., 1993) were used. Primers (*NSR 1113f/NSR 1264r*) (Dionisi et al., 2002) and (*Nitro 1198f/Nitro 1423r*)

(Graham et al., 2007) were used to determine amount of *Nitrospira* (*NSR*) and *Nitrobacter* (*Nitro*) *16S rRNA genes*, respectively. In addition, primers (*narG 1960m2f/narG 2050m2r*) (Lo´pez-Gutie´rrez et al., 2004), (*nirS 1f/nirS 3r*) (Braker et al., 1998), (*nirK 876/nirK 1040*) (Henry et al., 2004) and (*nosZ 2f/nosZ 2r*) (Henry et al., 2006) were used to determine amount of denitrifying functional genes (*narG*, *nirS*, *nirK*, and *nosZ* genes).

Reactions (20 µl) were performed in 96-well microplates. Each tube was separately loaded with 2 µl of genomic DNA (14-26 ng/µl) or plasmid standard, followed by the forward and reverse primers (1 µl) together with 10 µl SsoFast™ EvaGreen® Supermix (Bio-RAD, Hercules, California) and 6 µl PCR grade sterile water. All experiments were performed in triplicate per sample and control reactions were performed for all PCR runs without the template. Detailed descriptions of the *q*-PCR experiments and the primers are provided in the section A-4 (Appendix-A) and Table B2 (Appendix-B).

454 high-throughput 16S r RNA gene pyrosequencing. To determine the microbial community composition in suspended activated sludge flocs and attached biofilms in the IFAS systems, DNA samples were diluted to 20 ng/µL. Amplicon libraries were constructed for 454 pyrosequencing using bacterial pairs of 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3') (Dowd et al., 2008) for the V1-V3 regions of the 16S rRNA gene by Research & Testing Laboratory (Lubbock, Texas, USA).

Further data analyses were performed using the Quantitative Insights Into Microbial Ecology (QIIME), version 1.9.0 (Caporaso et al., 2010). Raw reads were filtered by QIIME quality filters. Effective reads were normalized for the following analysis. Sequences with more than 97% similarity were clustered into the same operational taxonomic units (OTUs) using the UCLUST algorithm. Then representative OTUs were picked based on the most abundant

sequences, and the taxonomic assignment were performed with UCLUST consensus taxonomy classifier. The latest version of GreenGenes database (v13.8) was used for taxonomy assignment (greengenes.lbl.gov). After that, the OUT sequences were aligned with the Python Nearest Alignment Space Termination (PyNAST) tool. Eventually, the communities were summarized by taxonomic composition. Raw pyrosequencing data that obtained from this study were deposited to the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/Traces/sra_sub/sub.cgi) with accession no. SRP069842.

6.2.6 Statistical Analysis

Statistical analyses were performed to evaluate IFAS treatment performance. Data were analysed by Microsoft Excel® software. The level of statistical significance was determined using a t-test. Correlations were considered statistically significant at the 95% confidence interval ($p < 0.05$).

6.3 Results and Discussion

6.3.1 Physicochemical Properties of Raw and Ozonated OSPW

The pH of raw OSPW (8.28 ± 0.26) and the pH of ozonated OSPW (8.31 ± 0.24) were not significantly different ($p > 0.05$) and remained essentially constant throughout the experiments. Table B1 (Appendix-B) summarizes the major chemical compositions of raw and ozonated OSPW. As shown in Table B1 (Appendix-B), most chemical properties remained constant after ozone pretreatment. However, the COD and AEF of ozonated OSPW (180.2 ± 0.8 mg/L and 56.11 ± 3.51 mg/L, respectively) were significantly lower ($p < 0.05$) than their concentrations in raw OSPW (193.2 ± 0.5 mg/L and 82.76 ± 8.09 mg/L, respectively). These results are comparable to previous OSPW ozonation studies (Gamal El-Din et al., 2011; Hwang et al.,

2013). Decreases in COD and AEF of OSPW after ozonation could have been caused by oxidation and mineralization of organic compounds. Compounds with specific functional groups (aromatic rings, unsaturated hydrocarbons, etc.) are susceptible to ozone attack (Dong et al., 2015). The concentration of ammonia in both raw and ozonated OSPW was extremely low making ammonia a limiting nutrient component for microbial growth in OSPW (Hwang et al., 2013).

6.3.2 Performance of IFAS Systems

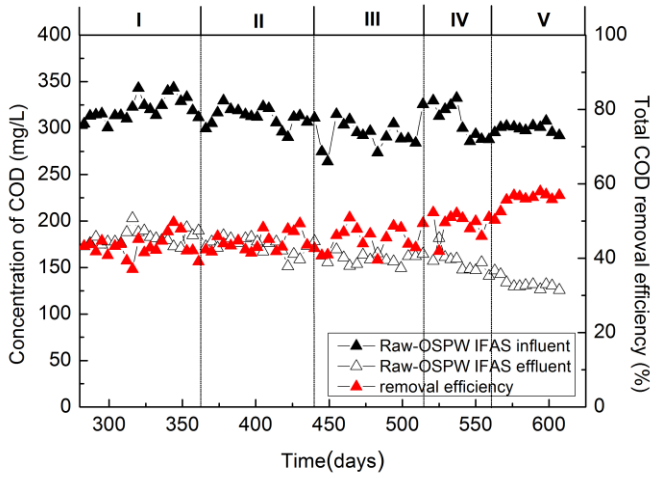
6.3.2.1 Biodegradation of COD

At constant influent COD concentrations of 323.2 and 310.2 mg/L in raw and ozonated OSPW IFAS, respectively, Fig. 6.1(A) and 6.1(B) show that at 48 h HRT (phase I), the average COD removal efficiencies were ~ 43.12% and 47.25% in raw and ozonated OSPW IFAS, respectively, at 72 h HRT (phase II), the average COD removal was ~ 44.42% and 46.21% in raw and ozonated OSPW IFAS, respectively, and at 96 h HRT (phase III), the average COD removal efficiencies were 45.33% and 48.55% in raw and ozonated OSPW IFAS (Table 6.2), respectively. Therefore, extension of the HRT in the IFAS systems from 48 h to 96 h had no significant effect on the COD removal in raw or ozonated OSPW.

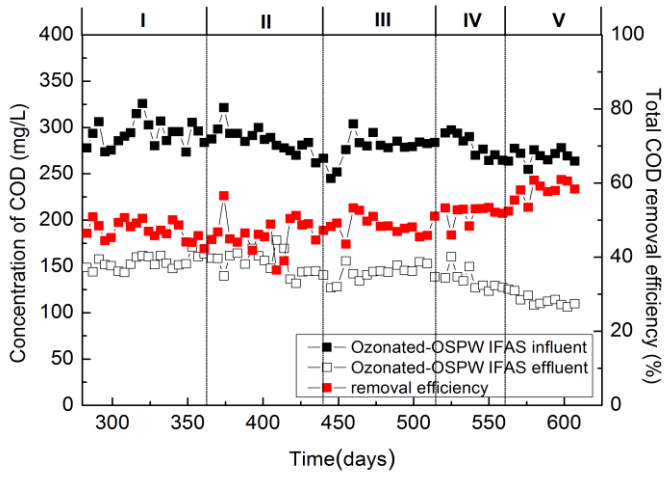
Table 6.2 Mean values for treatment performance of IFAS systems at different phases

Optimization phase	Raw-OSPW IFAS			Ozonated-OSPW IFAS		
	Removal efficiency (%)			Removal efficiency (%)		
	COD	TN	AEF	COD	TN	AEF
I	43.12 ± 2.13	23.37 ± 6.33	12.06 ± 2.24	47.25 ± 2.04	23.30 ± 7.21	13.71 ± 1.28
II	44.42 ± 2.82	30.10 ± 5.58	23.32 ± 3.68	46.21 ± 4.13	26.18 ± 8.05	28.93 ± 1.75
III	45.33 ± 3.26	26.71 ± 7.04	17.29 ± 1.76	48. 55 ± 2.57	24.96 ± 7.02	22.42 ± 2.43
IV	49.45 ± 3.12	34.25 ± 9.55	22.37 ± 2.97	51.62 ± 2.64	34.04 ± 9.36	36.99 ± 2.54
V	54.56 ± 3.48	31.15 ± 3.76	30.20 ± 3.86	56.83 ± 3.32	28.42 ± 6.02	51.51 ±3.32

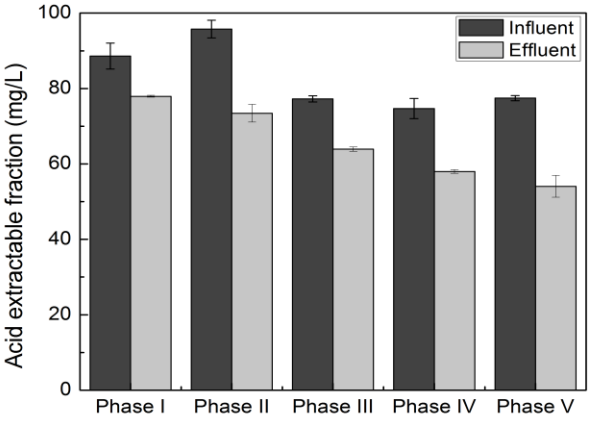
(A) **Raw-OSPW IFAS**



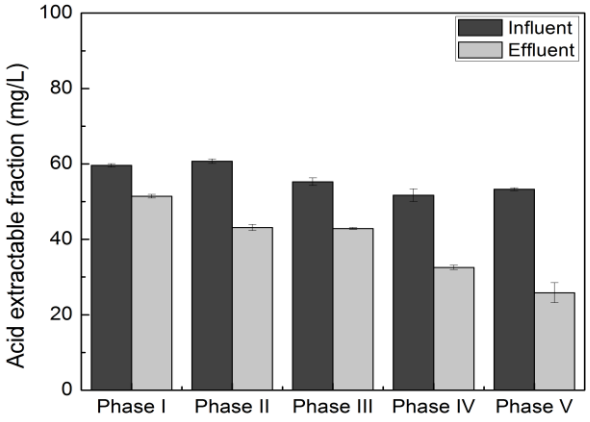
(B) **Ozonated-OSPW IFAS**



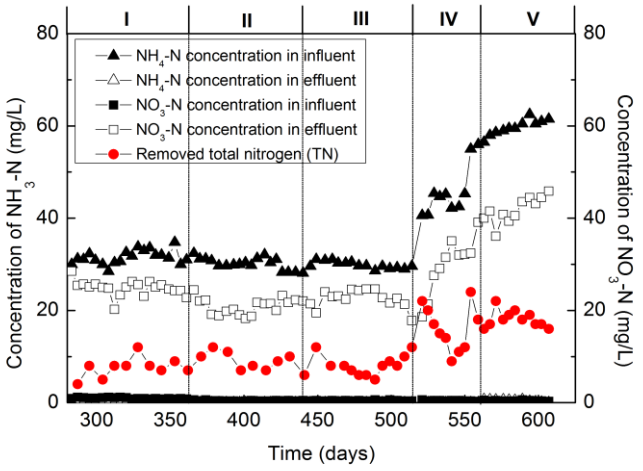
(C)



(D)



(E)



(F)

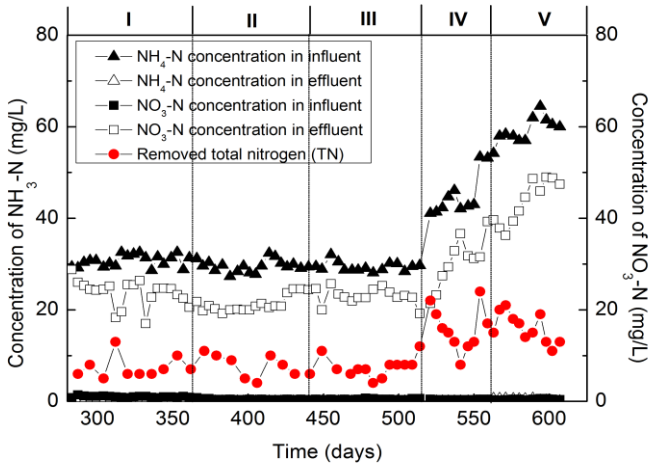


Figure 6.1. Profile of COD concentration in influent and effluent and COD removal efficiency over reactor optimization stage (from day 280 to day 607) in raw (A) and ozonated (B) OSPW IFAS systems, Profile of AEF concentration in influent and effluent in raw (C) and ozonated (D) OSPW IFAS systems at different phases, Profile of NH_4-N and NO_3-N concentration in influent and effluent and removed total nitrogen over reactor optimization stage (from day 280 to day 607) in raw (E) and ozonated (F) OSPW IFAS system.

During the ammonium optimization stage, a HRT of 96 h was maintained in the two IFAS systems and the ammonium concentration in the influent was increased from 30 to 45 to 60 mg/L in phases I, II, and III, respectively; respective COD/N ratios were 10.8, 7.2, and 5.4 in raw OSPW IFAS and 10.3, 6.9, and 5.2 in ozonated OSPW IFAS. COD concentrations in influent and effluent and average total COD removal efficiencies during the ammonium optimization stage with different COD/N ratios are shown in Fig. 6.1(A) and 6.1(B). In phase III (days 440–520) the average COD removal was 45.33% and 48.55% in raw and ozonated OSPW IFAS, respectively. In phase IV (days 521–553) the COD removal efficiencies increased to 49.45% in raw OSPW IFAS and to 51.62% in ozonated OSPW IFAS. In phase V (days 554–607) the COD removal efficiencies further increased ($p < 0.05$) and ammonia nitrogen loading resulted in a further increase in the removal of COD (54.56% in raw OSPW IFAS and 56.83% in ozonated OSPW IFAS) (Table 6.2). Thus an increase in ammonia nitrogen loading is beneficial to organics removal, which might be explained by an enhanced growth of nitrifiers (see section 6.3.3). Previous studies suggest that AMO may oxidize recalcitrant substrates, including hydrocarbons (Tran et al., 2013). Similar observations have been reported for OSPW biodegradation (Shi et al., 2015).

6.3.2.2 AEF Removal

AEF values are widely used to evaluate the concentration of classic and oxidized NAs and other organics with carboxylic acid, ketone, and aldehyde functional groups (Jivraj et al., 1995). The AEF includes classical NAs ($C_nH_{2n+Z}O_2$), oxidized NAs ($C_nH_{2n+Z}O_x$, with x = 3–5), and other organics, particularly those containing carboxylic groups (Grewer et al., 2010). The AEF was monitored by FTIR. Fig. 6.1(C) and 6.1(D) show a reduction in the AEF in raw and ozonated OSPW IFAS systems at different phases of optimization. The AEF concentration in reactor influent was lower in ozonated OSPW IFAS than in raw OSPW IFAS, indicating that ozone pretreatment reduced the AEF. When the HRT was increased from 48 to 72 h (phase I to phase II, Fig. 6.1(C) and 6.1(D)), the AEF removal efficiency increased from 12.06% to 23.33% ($p < 0.05$) in raw OSPW IFAS and from 13.71% to 28.93% ($p < 0.05$) in ozonated OSPW IFAS (Table 6.2). Thus a longer HRT enhanced a decrease in the AEF. As there was no significant change in COD removal when the HRT was increased from 48 to 72 h, the AEF might have been converted to other organic compounds that also contribute to COD. However, when the HRT was further increased from 72 to 96 h (phase II to phase III), the AEF removal efficiency decreased from 23.33% to 17.29% ($p < 0.05$) in raw OSPW IFAS and from 28.93% to 22.42% ($p < 0.05$) in ozonated OSPW IFAS (Table 6.2). This observation might be explained by a reduced food to microorganism ratio during this stage, which limited microorganism metabolism and thereby decreased AEF biodegradation.

During the COD/N ratio optimization stage, from phase III to IV, the AEF removal efficiency increased from 17.29% to 22.37% ($p < 0.05$) in raw OSPW IFAS and from 22.42% to 36.99% ($p < 0.05$) in ozonated OSPW IFAS (Fig. 6.1(C) and 6.1(D)). In phase V, a further decrease in the COD/N ratio resulted in an increased removal ($p < 0.05$) of AEF (30.20% in raw

OSPW IFAS and 51.51% in ozonated OSPW IFAS) (Table 6.2), indicating that AEF removal significantly increased when the COD/N ratio decreased, which is consistent with the COD removal trend reported above.

6.3.2.3 Nitrogen Removal

During the ammonia optimization stage, NH_4-N (provided as ammonium chloride) was added in the influent of both IFAS systems to maintain the biological nitrogen level and improve the growth of ammonia oxidizing bacteria (AOB). It has been reported that the AMO enzyme produced by AOB was beneficial for the removal of slow biodegradable compounds in wastewater (Tran et al., 2013). The time course of NH_4-N and NO_3-N variation is plotted in Fig. 6.1(E) and 6.1(F). The nitrate levels in the effluent indicated that both IFAS reactors performed well regarding ammonia oxidation. The ammonium removal efficiencies in the two IFAS systems in all phases were higher than 98%. The total nitrogen loss could be attributed to microbial assimilation and biological denitrification. The results shown in Fig. 6.1(E) and 6.1(F) indicate that extending the HRT in the IFAS systems from 48 h to 96 h had no significant effect on the total nitrogen removal. The removed total nitrogen was 8.22 ± 2.10 and 7.55 ± 2.25 mg/L in raw and ozonated OSPW IFAS, respectively. From phases III to IV, the total nitrogen removed increased to 15.00 ± 4.18 and 14.75 ± 4.05 mg/L in raw and ozonated OSPW IFAS. In phase V, a decreased COD/N ratio resulted in an increased removal ($p < 0.05$) of total nitrogen (18.54 ± 2.24 mg/L in raw OSPW IFAS and 16.70 ± 3.54 mg/L in ozonated OSPW IFAS), indicating that the removal of total nitrogen significantly increased when the COD/N ratio decreased, and indicating that denitrifing activities were highly improved by an increase in ammonia nitrogen loading. Therefore, the ammonia nitrogen loading had a strong effect on the organic matters utilization pathways, and the routes of COD consumption could diversify with

increased nitrogen loading. This might explain why COD and AEF removal efficiencies in both IFAS systems significantly increased when ammonia nitrogen loading increased.

6.3.3 Quantification of Bacteria Populations by the Quantitative Polymerase Chain Reaction

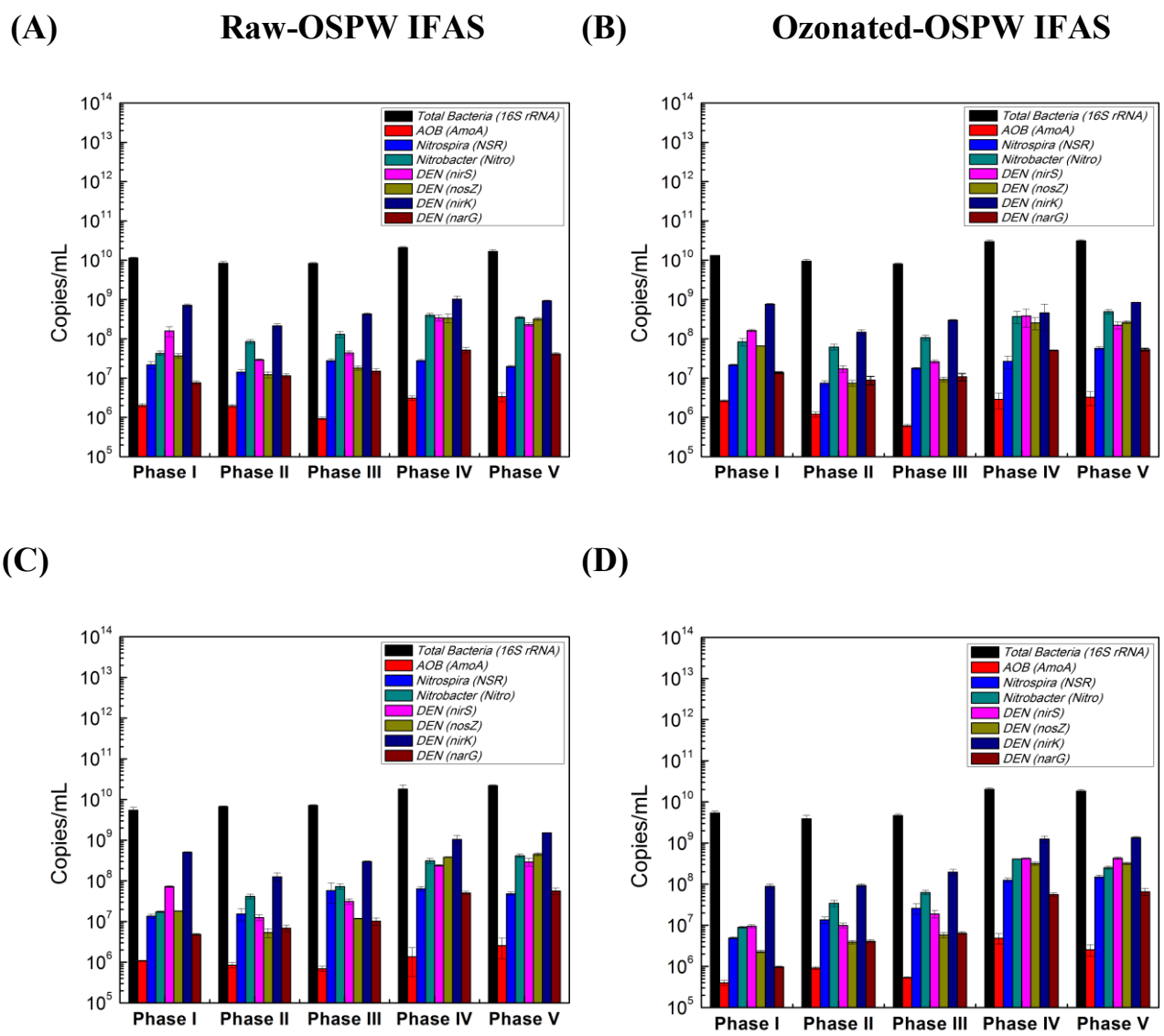


Figure 6.2. Gene abundance changes of the total bacteria *16S rRNA* gene, nitrifier relevant genes (*AomA*, *NSR* and *Nitro*) and the denitrifier relevant genes (*nirS*, *nosZ*, *nirK* and *narG*) in activated sludge flocs at different phases in raw (A) and ozonated (B) OSPW IFAS systems, the

gene copies per mL of the total bacteria *16S rRNA* gene, *AmoA*, *NSR*, *Nitro* and denitrifier relevant genes (*nirS*, *nosZ*, *nirK* and *narG*) in attached biofilms at different phases in raw (C) and ozonated (D) OSPW IFAS systems.

Changes in total bacteria, nitrifier, and denitrifier gene abundance in activated sludge flocs and attached biofilms in IFAS systems were measured by quantitative real-time PCR. Fig. 6.2 showed the gene abundance changes in the *bacterial 16S rRNA* gene, nitrifier genes (*aomA*, *NSR*, and *Nitro*), and denitrifier genes (*nirS*, *nosZ*, *nirK*, and *narG*) in activated sludge flocs and attached biofilms in different phases of optimization of raw and ozonated OSPW IFAS systems. Fig. 6.2 shows that the *16S rRNA* gene abundance in activated sludge flocs and biofilms ranged from $8.32 \times 10^9 \pm 4.26 \times 10^8$ to $2.09 \times 10^{10} \pm 1.31 \times 10^9$ and $5.45 \times 10^9 \pm 9.51 \times 10^8$ to $2.20 \times 10^{10} \pm 1.00 \times 10^9$ copies/mL, respectively, in raw OSPW IFAS and from $8.01 \times 10^9 \pm 3.80 \times 10^8$ to $3.12 \times 10^{10} \pm 2.22 \times 10^9$ and $3.88 \times 10^9 \pm 7.70 \times 10^8$ to $2.02 \times 10^{10} \pm 1.73 \times 10^9$ copies/mL, respectively, in ozonated OSPW IFAS. The data indicate that HRT optimization had no significant effect on the total bacteria population in flocs and biofilms in the two IFAS systems. However, abundance of the *16S rRNA* gene in flocs and biofilms in the two IFAS systems significantly increased after ammonium optimization (Fig. 6.2).

During HRT optimization (phase I to phase III), it was noted that the abundance of the gene *aomA* in flocs decreased from $2.01 \times 10^6 \pm 2.43 \times 10^5$ to $9.27 \times 10^5 \pm 7.17 \times 10^4$ copies/mL ($p < 0.05$) in raw OSPW IFAS, and from $2.64 \times 10^6 \pm 1.43 \times 10^5$ to $6.12 \times 10^5 \pm 4.88 \times 10^4$ copies/mL ($p < 0.05$) in ozonated OSPW IFAS. Changes in of *aomA* gene abundance in biofilms in the two IFAS systems were not significant. Possibly, *aomA* genes in flocs were more sensitive to HRT changes than were *aomA* genes in biofilms. When the ammonium concentration in

influent increased from 30 to 60 mg/L (phase III to phase V), the abundance of *aomA* genes in flocs and biofilms in the two IFAS systems significantly increased (Fig. 6.2). It has been found that ammonia-oxidizing archaea (AOA) are important and quite diverse in many natural environments, such as in marine sediment, soil, estuary and seawater (Zhang et al., 2009). Hereby it will be important to evaluate the presence of AOA in IFAS systems. However, our current study only focused on the bacterial community analysis. Further studies should be performed to evaluate the impact of AOA group. *NSR* and *Nitro* gene abundance in flocs and biofilms also showed an upward trend after ammonium optimization. These results help to explain why ammonium oxidation increased in both IFAS systems during ammonium optimization.

When the HRT of IFAS systems was extended from 48 to 96 h, the abundance of denitrifier genes (*narG*, *nirS*, *nirK* and *nosZ*) present in activated sludge flocs and biofilms significantly decreased ($p < 0.05$) in both IFAS systems (Fig. 6.2), possibly because the increase in HRT led to a decrease in the food to microorganism (F/M) ratio. The decrease in denitrifier genes did not significantly impact nitrogen removal (denitrification) in the IFAS systems (Fig. 6.1(E) and 6.1(F)). However, the abundance of denitrifying genes in flocs and biofilms in the two IFAS systems significantly increased ($p < 0.05$) during ammonium optimization (phase III to phase V), demonstrating that an increase in nitrogen loading had a significant effect on denitrifier gene abundance in both IFAS systems. Thus, denitrifying activities improved with an increase in ammonia nitrogen loading (Fig. 6.1(E) and 6.1(F)).

6.3.4 Microbial Community Diversity

To assess the effects of the HRT and the COD/N ratio on the microbial community in IFAS reactors, comparisons were made between flocs and biofilms in different optimization

phases (phase I, III, and V). DNA samples from flocs and biofilms in the IFAS systems were analysed by 454 high-throughput *16S rRNA* gene pyrosequencing.

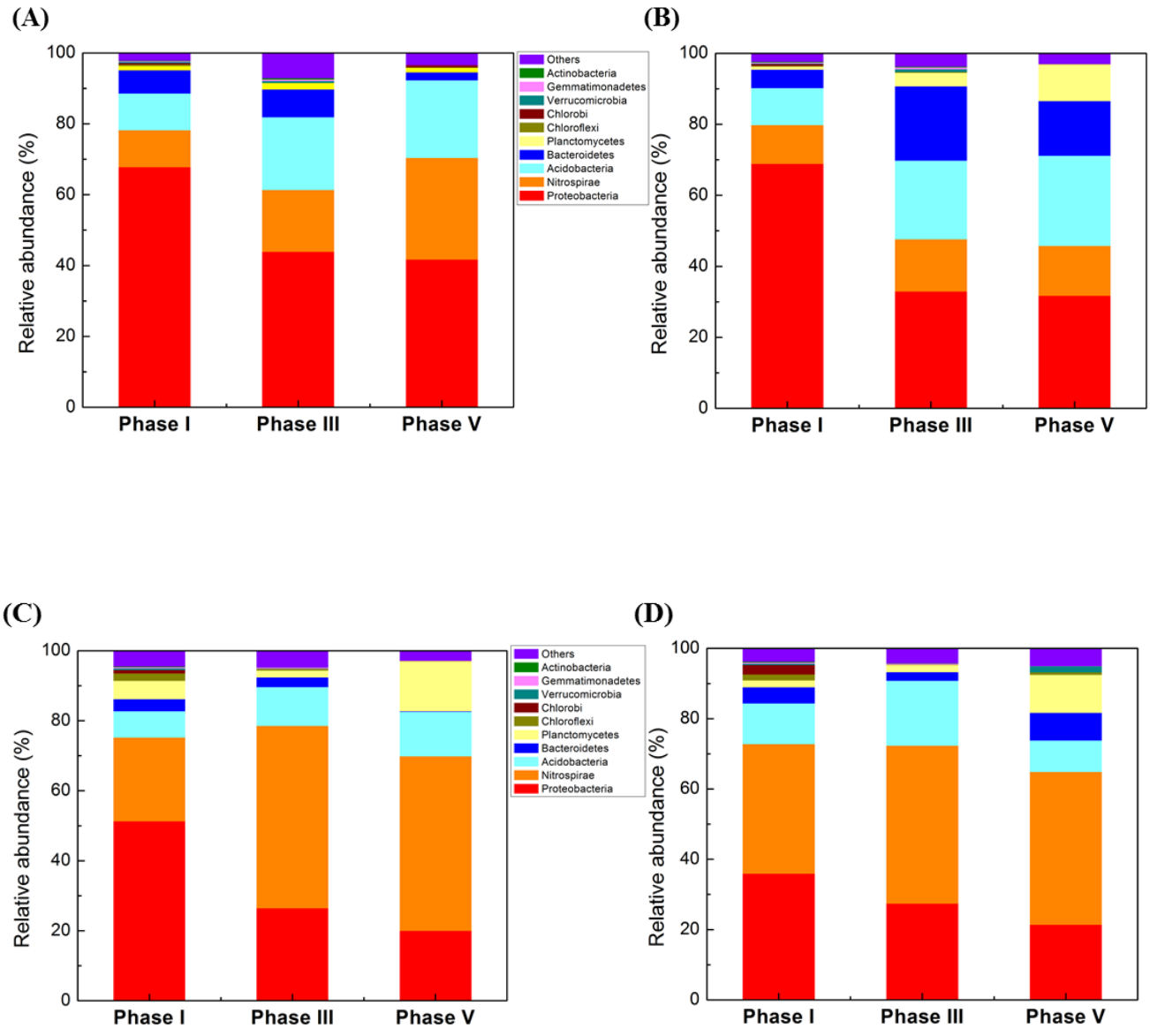


Figure 6.3. Microbial community structures of the flocs in raw (A) and ozonated (B) OSPW IFAS, and biofilms in raw (C) and ozonated (D) OSPW IFAS at phylum levels from phase I, III and V. Phyla at relative abundance < 0.2% were grouped as "others".

The phylum level identification of bacterial communities in the IFAS systems is illustrated in Fig. 6.3. Based on an abundance cutoff of 0.2%, *Proteobacteria*, *Nitrospirae*, *Acidobacteria*, *Bacteroidetes*, and *Planctomycetes* were dominant phyla in both flocs and biofilms in IFAS systems during the entire optimization stage. *Proteobacteria* were the most abundant in all samples, with 67.77% and 68.87% of total bacteria in flocs in raw and ozonated OSPW IFAS, respectively, and 51.27% and 35.93% of total bacteria in biofilms in raw and ozonated OSPW IFAS respectively, in phase I. *Proteobacteria* were reported to be the dominant phyla in raw and ozonated OSPW and in granular activated carbon (GAC) biofilms during OSPW treatment (Islam et al. 2015b), and the dominant microbes of the Athabasca watershed and sediments belong to *Proteobacteria* were involved in the degradation of recalcitrant bituminous compounds (Yergeau et al., 2012). During HRT optimization (phase I to phase III), the relative abundance of *Proteobacteria* decreased dramatically in all biomass measurements in both IFAS systems (Fig. 6.3), which might be attributed to the low NA loading rate due to the extended HRT. Yergeau et al. (2012) noted that *Proteobacteria* abundance was positively correlated with the concentration of NAs because this phylum showed high resistance to NA toxicity. The abundance of *Proteobacteria* showed a slight decrease in all flocs and biofilms in both IFAS systems during ammonium optimization (phase III to phase V) (Fig. 6.3), possibly because the increase in nitrogen loading might favour other bacteria.

Nitrospirae phyla were relatively abundant in all biomass measurements in both IFAS systems (Fig. 6.3). It had been reported that the members of *Nitrospirae* phyla associated with nitrite oxidation, sulphur oxidation, and sulphate reduction (Lin et al., 2014), the existence of *Nitrospiae* was supported by the presence of heteroatomic compounds with nitrogen and sulfur in OSPW samples, which had been reported previously (Pereira et al., 2013). *Nitrospirae* phyla

represented as 10.41% and 10.93% of total bacteria in flocs in raw and ozonated OSPW IFAS, respectively, and 23.92% and 36.87% of total bacteria in biofilms in raw and ozonated OSPW IFAS, respectively, in phase I. The slow growth rate of *Nitrospirae* phyla might favour the formation of biofilms rather than flocs in IFAS systems. During HRT optimization (phase I to phase III), *Nitrospirae* increased significantly ($p < 0.05$) in all biomass measurements in both IFAS systems (Fig. 6.3), but the increase was greater in biofilm samples where *Nitrospirae* increased to 52.09% and 44.95% of the total bacteria in biofilms in raw and ozonated OSPW IFAS, respectively, in phase III. It indicates that the extension of the HRT in the IFAS systems is beneficial for the accumulation of the members of *Nitrospirae* phyla.

Members of the *Acidobacteria* phyla can adapt to acidic, poor nutrient conditions (Islam et al., 2015b) and play an important role in carbohydrate degradation in boreal forest peat. The relative abundances of *Acidobacteria* were 10.41% and 10.43% in flocs from raw and ozonated OSPW IFAS, respectively, and 7.55% and 11.54% in biofilms from raw and ozonated OSPW IFAS, respectively, in phase I. During HRT optimization (phase I to phase III), the abundances of *Acidobacteria* presented a significant upward trend ($p < 0.05$) in all biomass measurements in both IFAS systems (Fig. 6.3), which might help to explain why both IFAS systems displayed higher AEF removal after HRT optimization (Fig. 6.1(C) and 6.1(D)). Extension of the HRT in the IFAS systems decreased the food to microorganism (F/M) ratio leading to a higher competition for nutrients.

To reveal the influence of HRT and ammonium optimization on the microbial community structure of flocs and biofilms in the IFAS systems, the microbial taxonomy was broken down at the class level as shown in Fig. 6.4. Among the *Proteobacteria* phyla, *beta-Proteobacteria* was the dominant class in both flocs and biofilms in two IFAS systems. Relative to total bacteria,

beta-Proteobacteria were 61.75% and 62.48% in flocs in raw and ozonated OSPW IFAS, respectively, and 32.81% and 18.45% in biofilms in raw and ozonated OSPW IFAS, respectively, in phase I. After HRT optimization (phase I to phase III), *beta-Proteobacteria* showed a significant decrease ($p < 0.05$) in all biomass measurements in both IFAS systems (Fig. 6.4), consistent with the changing trend of *Proteobacteria* phyla. *Beta-Proteobacteria* members found in oil sands tailing pond sediments have been observed to perform effective bitumen degradation (Yergeau et al., 2012). Extension of the HRT in both IFAS systems caused a significant decrease in bitumen contaminants loading, leading to a decreased abundance of *beta-Proteobacteria*.

The *Nitrospira* class increased significantly in flocs and biofilms—but the increase was greater in biofilms ($p < 0.05$)—after HRT optimization. Fig. 6.4 shows that *Chloracidobacteria*, the dominant class of *Acidobacteria* phyla in in flocs and biofilms of both IFAS systems, were 10.18% and 10.27% in flocs in raw and ozonated OSPW IFAS, respectively, and 6.62% and 9.38% in biofilms in raw and ozonated OSPW IFAS, respectively, in phase I. From phase I to phase III, *Chloracidobacteria* increased to 20.43% and 21.40% in flocs in raw and ozonated OSPW IFAS, respectively, and increased to 9.61% and 18.01% in biofilms in raw and ozonated OSPW IFAS, respectively, consistent with changes in *Acidobacteria* phyla. It demonstrated that the extension of HRT significantly increased the relative abundance of *Chloracidobacteria* in all biomass samples from IFAS systems. Some members of *Chloracidobacteria* are responsible for degradation of a variety of aromatic hydrocarbons and bio-recalcitrant organic compounds (Roling et al., 2002), which might explain the increased AEF removal after HRT optimization.

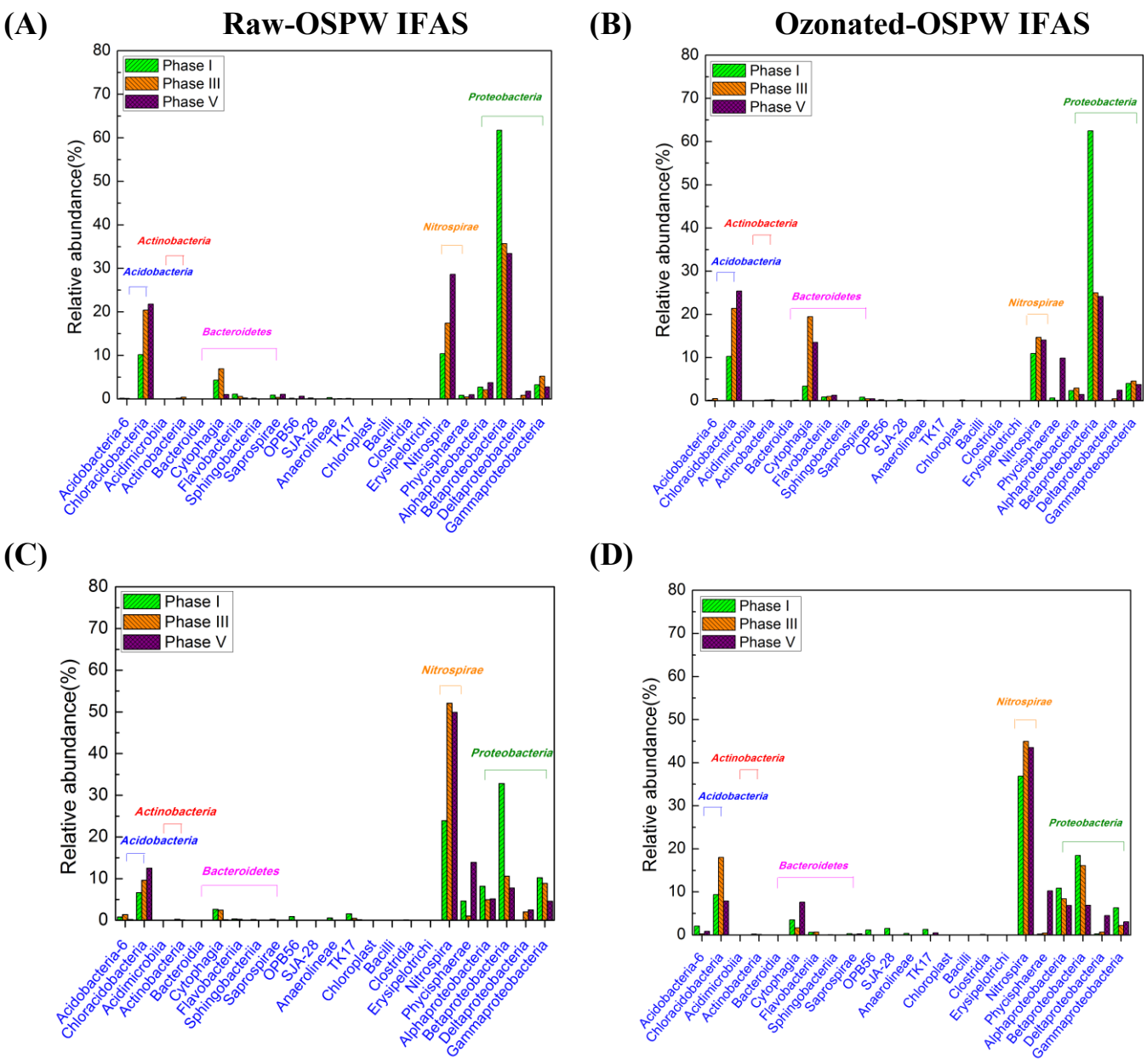


Figure 6.4. Taxonomic classification of pyrosequencing at class level in flocs and biofilms samples from phase I, III and V. (A) Flocs in raw OSPW IFAS, (B) Flocs in ozonated OSPW IFAS, (C) Biofilms in raw OSPW IFAS, (D) Biofilms in ozonated OSPW IFAS.

Fig. 6.4 shows that *Chloracidobacteria*, *Cytophagia*, *Nitrospira*, *Phycisphaerae*, *alpha-Proteobacteria*, *beta-Proteobacteria* and *gamma-Proteobacteria* were the dominant classes in

both flocs and biofilms in two IFAS systems. To evaluate the affect of HRT and ammonium optimization on the microbial community composition of IFAS systems, the dominant classes were further identified at the order level of classification for both flocs and biofilm samples from two IFAS systems (Fig. 6.5).

The class *Nitrospira* was further identified at order level of classification for both floc and biofilms from IFAS systems (Fig. 6.5). The members of Nitrospirales are associated with nitrite oxidization, sulphur oxidation and sulphate reduction (Watson et al., 1986; Ehrich et al., 1995; Henry et al., 1986). As shown in Fig. 6.5, *Nitrospirales* order represented 10.41% and 10.93% of total bacteria in flocs in raw and ozonated OSPW IFAS, respectively; whereas it accounted for 23.92% and 36.87% of total bacteria in biofilms in raw and ozonated OSPW IFAS, respectively, in phase I. Elongated HRT (from phase I to phase III) increased *Nitrospirales* order significantly ($p < 0.05$) in both IFAS systems (Fig. 6.5). After HRT optimization (phase III), the relative abundance *Nitrospirales* increased to 17.41% and 14.68% in flocs of raw and ozonated OSPW IFAS, respectively, and 52.09% and 44.95% in biofilms of raw and ozonated OSPW IFAS, respectively. It demonstrated that the extension of HRT significantly raised the abundance of *Nitrospirales* in IFAS systems. The growth rates of *Nitrospirales* members were much lower than those heterotrophic organisms, the extension of HRT provided longer retention time, which lead to the significantly increase of the abundance in IFAS systems (Fig. 6.5). However, some lithotrophic nitrite-oxidizing bacteria are able to grow mixotrophically, Watson et al. (1986) observed that the growth rate of *Nitrospira marina* was much higher in a complex medium containing nitrite, pyruvate, yeast extract and peptone than in mineral salts nitrite medium, which attributed to the chemolithotrophical and mixotrophical growth (cometabolism) of nitrite

oxidizing bacteria. Therefore, the significantly increase of *Nitrospirales* order might lead to the higher AEF removal after HRT optimization.

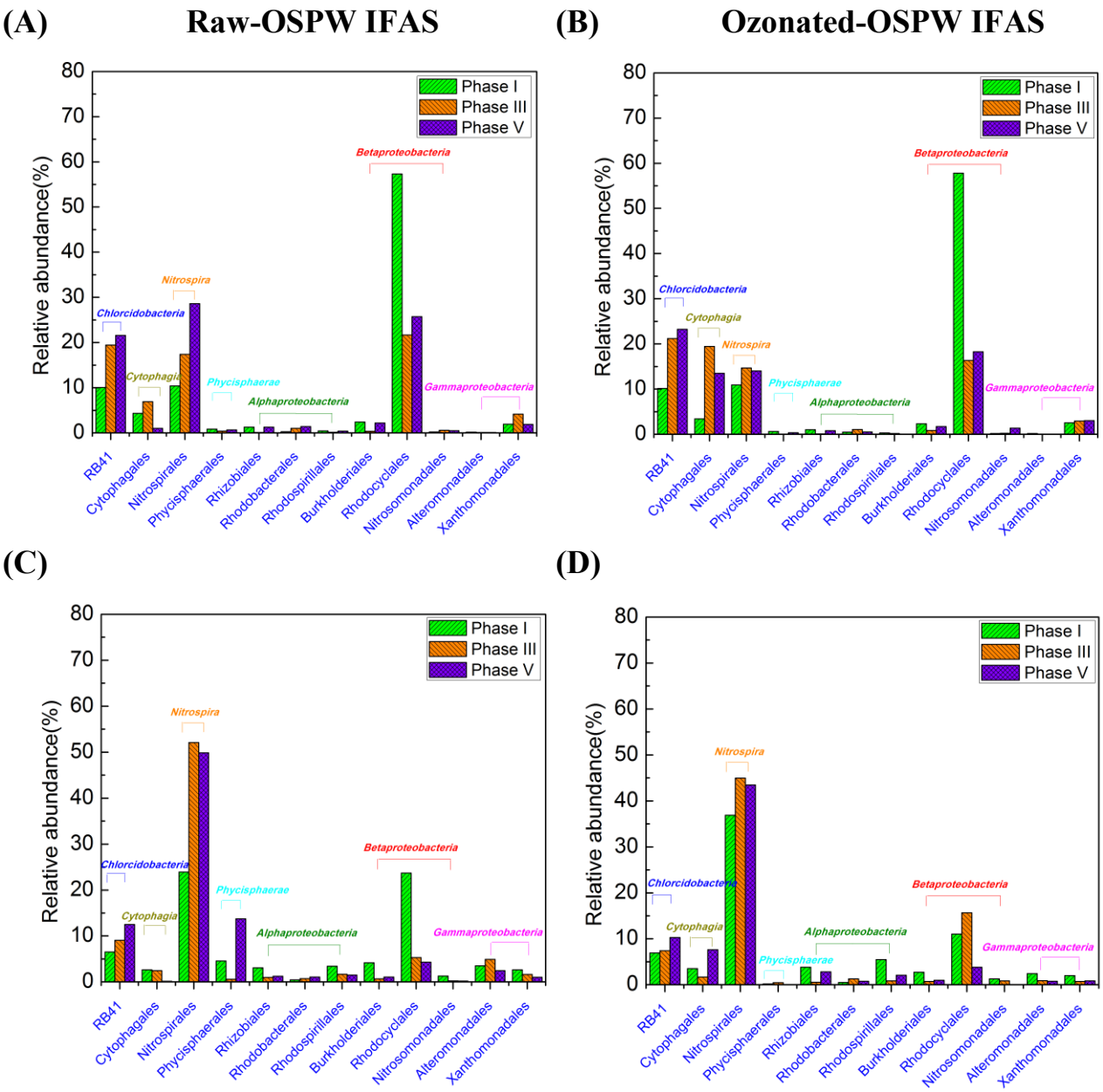


Figure 6.5. Taxonomic classification of pyrosequencing at order level in flocs and biofilms samples from phase I, III and V. (A) Flocs in raw OSPW IFAS, (B) Flocs in ozonated OSPW IFAS, (C) Biofilms in raw OSPW IFAS, (D) Biofilms in ozonated OSPW IFAS.

The *alpha-Proteobacteria* class plays an important role in environmental processes. Among the *alpha-Proteobacteria*, *Rhizobiales*, *Rhodobacterales*, and *Rhodospirillales* were the dominant order in both flocs and biofilms in two IFAS systems (Fig. 6.5). The members of *Rhizobiales* are involved in nitrogen fixation (Carvalho et al., 2010). Some members of *Rhodobacterales* are chemoorganotrophs, which associate with the degradation of sulfur-containing organic compounds. The members of *Rhodospirillales* are involved in the partial oxidation of carbohydrates and alcohols (Gupta et al., 2007). The existence of *Rhizobiales* and *Rhodobacterales* demonstrates that the degradation of sulfur- and nitrogen-containing organic compounds.

The *beta-Proteobacteria* class has been reported to be involved in the degradation of bitumen (Yergeau et al., 2012). The major orders found currently in this class of bacteria include *Burkholderiales*, *Rhodocyclales*, and *Nitrosomodales* in all biomass samples from IFAS (Fig. 6.5). The most abundant order of *beta-Proteobacteria* was *Rhodocyclales* in all samples. The members of *Rhodocyclales* are reported to be capable for aliphatic and aromatic compounds degradation and nitrogen and phosphorus removal (Hesselsoe et al., 2009). It was shown that the abundances of *Rhodocyclales* presented a significant downward trend ($p < 0.05$) in all biomass measurements in both IFAS systems after HRT increasement from Phase I to Phase III, which might be explained by the decreased organic loading due to the extension of HRT. As for the *gamma-Proteobacteria*, the dominant orders in this class were *Alteromonadales* and *Xanthomonadales* in IFAS syetems, which are polyaromatic hydrocarbon (PAH) degraders (Lamendella et al., 2014).

6.4 Conclusions

This study evaluated the effects of HRT and the COD/N ratio on raw and ozonated OSPW treatment performance of IFAS systems. After 11 months of HRT and ammonium optimization, Ozonation combined IFAS showed better OSPW treatment performance. Extending the HRT in the IFAS systems had no significant effect on the removal of COD and nitrogen, whereas a lower COD/N ratio increased the removal of organics and total nitrogen. The *q*-PCR and 454 sequencing results indicate that the HRT and the COD/N ratio had significant influence on the microbial community composition in IFAS systems.

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CHAPTER 7. COMPARISON OF BIOMASS FROM INTEGRATED FIXED-FILM ACTIVATED SLUDGE (IFAS), MOVING BED BIOFILM REACTOR (MBBR) AND MEMBRANE BIOREACTOR (MBR) TREATING RECALCITRANT ORGANICS: IMPORTANCE OF ATTACHED BIOMASS[1]

7.1 Introduction

The rapidly developing oil industry in Northern Alberta, Canada, generates large volumes of oil sands process-affected water (OSPW) containing petroleum hydrocarbons, chemicals, salts, suspended solids, heavy metals, organic, and inorganic constituents (Scott et al., 2005). OSPW is stored in large manufactured settling basins called tailings ponds (Nix and Martin, 1992), which are estimated to hold 1 billion m^3 in the next 15 to 20 years (MacKinnon, 1989; Dong et al., 2015). OSPW is slightly alkaline (pH 7.8–8.7) (Gamal El-Din et al., 2011) and acutely toxic to aquatic life, plants, birds, and mammals (Lo et al., 2006; Pereira et al., 2013; Garcia-Garcia et al., 2011). The zero discharge policy of the Alberta Government has put pressure on the industry to develop efficient and cost-effective water treatment technologies to remediate OSPW (Xue et al., 2016) for future and safe release of the reclaimed OSPW leading to the reduction of the adverse environmental impacts on the receiving environment (Choi et al., 2014a).

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[1]A version of this chapter has been submitted previously: Huang, C., Shi, Y., Xue, J., Zhang, Y., Gamal El-Din, M., Liu, Y., Comparison of biomass from integrated fixed-film activated sludge (IFAS), moving bed biofilm reactor (MBBR) and membrane bioreactor (MBR) treating recalcitrant organics: importance of attached biomass. Journal of Hazardous Materials, 28 April, 2016.

Naphthenic acids (NAs) are thought to be the primary toxic constituents of OSPW (Anderson et al., 2012) because they are quite soluble in slightly alkaline water (Frank et al., 2008). NAs constitute a mixture of alicyclic and noncyclic alkyl-substituted carboxylic acids represented by $C_nH_{2n+Z}O_x$, where n indicates the carbon number, x is the number of oxygen atoms, and Z is zero or negative, representing the hydrogen deficiency resulting from ring or double bond formation. The variable number of oxygen atoms categorizes NAs as classical (x = 2) or oxidized ($x \geq 3$) (Islam et al., 2014a). Classical NAs are more recalcitrant than commercial NAs to biodegradation (Han et al., 2008). Depending on the ore position, the age of tailings, the extraction process, and NAs measurement methods, the NA concentration in OSPW varies from 20 to 120 mg/L (Clemente and Fedorak, 2005; Allen, 2008). The in situ biodegradation half-life of NAs in tailings ponds has been estimated to be about 13 years (Han et al., 2009). Therefore, efficient and cost-effective removal approaches of NAs from OSPW is essential for OSPW reclamation (Kim et al., 2013).

Biodegradation technologies (i.e., bioreactors) are considered to be efficient, cost-effective, and energy-efficient approaches for industrial wastewater remediation (Di Iaconi et al., 2003), and microbial aggregates (suspended floc and/or attached biofilm) are utilized in bioreactors treatments to remove organic contaminants in OSPW (Hwang et al., 2013; Choi et al., 2014a; Xue et al., 2016; Shi et al., 2015; Huang et al., 2015). Previous studies showed that the treatment performance and microbial population dynamics in OSPW bioreactor can be significantly affected by ozone pretreatment doses (Dong et al., 2015), bioreactor flow conditions, e.g., continuous-flow or batch systems (Huang et al., 2012; Choi et al., 2014b), biofilm supporting media types (Hwang et al., 2013), bioreactor seeding types (Choi et al., 2014a), and bioreactor operational conditions, e.g., organic loading rates (Islam et al., 2014b), ozone pretreatment

conditions (Dong et al., 2015) and electron acceptor conditions, i.e., aerobic and anoxic condition (Xue et al., 2016). In particular, bioreactor configuration can significantly affect the OSPW treatment effectiveness (Choi et al., 2014b). IFAS, MBBR, and MBR bioreactors have been proven to be promising for OSPW treatment (Xue et al., 2016; Shi et al., 2015; Huang et al., 2015). These three bioreactor types can help to enhance the biomass density in bioreactors, and also decouple the hydraulic retention time and solid retention time. Thus, these bioreactors can allow the growth of slow growing microorganisms that are essential for the degradation of recalcitrant organic compounds. However, no studies have been carried out so far to compare these bioreactor types.

In the present study, we compared the OSPW treatment performance and microbial characteristics of five types of biomass (MBBR-biofilm, IFAS-biofilm, IFAS-floc, MBR-aerobic-floc, and MBR-anoxic-floc) from three types of bioreactor: MBBR (Shi et al., 2015), IFAS (Huang et al., 2015), and MBR (Xue et al., 2016). To gradually acclimatize the microorganisms in these three bioreactors, the similar start-up strategies (the volumetric ratio of OSPW increased stepwise from 10% to 100% in the influents) were employed during the acclimation stage. Polyethylene (PE) media was applied in both MBBR and IFAS systems. MBR system with a modified Ludzack-Ettinger (MLE) configuration with internal recirculation from the aerobic tank to the anoxic tank was applied for OSPW treatment under nitrificantion and denitrification conditions. The total chemical oxygen demand (COD), the acid extractable fraction (AEF), and NA biodegradation were evaluated. Microbial community structures in the biomass of the batch bioreactors were characterized.

7.2 Materials and Methods

7.2.1 Source Water

Fresh OSPW was obtained from oil sands tailings ponds in Fort McMurray, AB, Canada, in September 2013. OSPW samples were stored in 200 L polyvinyl chloride containers in a step-in cold room (4 °C) prior to use.

The 0.1% (v/v) trace nutrient solution contained: $MgCl_2 \cdot 6H_2O$, 3 g L^{-1}; $CaCl_2$, 1.5 g L^{-1}; $FeSO_4 \cdot 7\ H_2O$, 0.28 g L^{-1}; $MnCl_2 \cdot 4\ H_2O$, 0.13 g L^{-1}; $ZnSO_4 \cdot 7\ H_2O$, 0.12 g L^{-1}, $CuSO_4 \cdot 5\ H_2O$, 0.0074 g L^{-1}. The pH was 7.3 ± 0.3. All chemicals and supplies were obtained from Thermo Fisher Scientific.

7.2.2 Batch Reactor Set-up and Operation

Continuous MBBR and IFAS reactors provided by Napier-Reid Ltd. (Markham, Canada) were separated into two cells: an aeration tank (working volume 8.5 L) and a clarifier. Aeration tubes were installed for aeration and for the free movement of support carriers. A 60% volume fraction of polyethylene (PE) carriers (Bioflow 9, Rauschert, Steinwiessen, Germany) were employed in the MBBR and the IFAS systems. A return activated sludge pumping system maintained the suspended biomass concentration in the IFAS. Details of MBBR and IFAS systems are described in Shi et al. (2015) and Huang et al. (2015). The MBR comprised a feed tank, an anoxic tank, an aerobic tank with an immersed ceramic microfiltration membrane (0.1 μm pore) module (MEIDENSHA, Japan) in the central position of the tank, and a permeate tank. A detailed operational description of the MBR is presented in Xue et al. (2016). Similar start-up and optimization strategies were applied in the three types of bioreactor.

Five types of biomass (MBBR-biofilm, IFAS-biofilm, IFAS-floc, MBR-aerobic-floc, and MBR-anoxic-floc) were used to carry out batch experiments; operational conditions are shown in

Table 7.1. Five types of biomass inoculants were compared in the batch tests: (1) MBBR attached biofilm carriers (MBBR-biofilm); (2) IFAS attached biofilm carriers (IFAS-biofilm); (3) IFAS suspended activated sludge (IFAS-floc); (4) MBR aerobic activated sludge (MBR-aerobic-floc); and (5) MBR anoxic activated sludge (MBR-anoxic-floc).

Table 7.1 Mean values for operating conditions and performance of the bench-scale MBBR, IFAS and MLE-MBR systems

Parameters	MBBR		IFAS		MLE-MBR	
	Influent	**Effluent**	**Influent**	**Effluent**	**Feed**	**Permeate**
Influent flow (L/day)	2.0		2.0		3.2	
HRT (h)	96		96		12	
RAS flow (L/day)	-		2.0		-	
DO (mg/L)	6-7		6-7		6-7	
Reactor MLSS (suspended) (g/L)	-		1.442 ± 0. 062		3.155 ± 0.085[a] 3.515 ± 0.025[b]	
Reactor MLSS (attached) (g/L)	1.014 ± 0.057		0.843 ± 0.051		-	
COD (mg/L)	412.26 ± 13.4	172.31 ± 8.12	297.86 ± 5.67	135.22 ± 8.45	518.87 ± 5.13	240.93 ± 4.13
NH_4^+ (mg/L)	60.1 ± 3.9	0	59.08 ± 2.15	0	26.55 ± 0.99	0.18 ± 0.18
NO_3^- (mg/L)	0.64 ± 0.1	55.5 ± 5.2	0.43 ± 0.08	40.87 ± 3.55	28.11 ± 0.84	11.8 ± 1.26
AEF (mg/L)	75.78 ± 5.26	56.71 ± 5.47	77.45 ± 0.69	54.06 ± 2.90	-	-
Classical NAs (mg/L)	-	-	-	-	44.7	27.9

a The MLSS concentration in aerobic tank of MLE-MBR.
b The MLSS concentration in anoxic tank of MLE-MBR.

A biomass concentration of ~ 500 mg total suspended solids/liter (TSS/L) (in suspended activated sludge floc or biofilm on supporting media) was applied during the batch tests. To observe the attached biomass concentration in the MBBR and IFAS systems, the attached biofilms were detached from the supporting media by sonication. Amber bottles containing duplicates of individual treatments were utilized. After OSPW with nutrients and biomass were introduced, the bottles were shaken and immediately sampled to obtain initial pollutant concentrations at t = 0 h. Batch bioreactors were operated on a platform shaker at 150 rpm

(InnovaTM 2100, Platform Shaker, New Brunswick Scientific, USA) for 14 days at room temperature (20 ± 1 ˚C). Aliquots (about 5 mL) of samples were taken periodically by means of plastic syringes, filtered through a nylon membrane filter (0.45 µm pore) and stored at 4 ˚C for subsequent analyses. During the entire operation of the batch bioreactors, extra carbon (NaAC, 300 COD mg/L) as well as nitrogen (NH_4Cl or $NaNO_3$, 60.0 ± 2.6 mg N/L), phosphorus (KH_2PO_4, 3.0 ± 0.2 mg P/L) and other necessary nutrients (mentioned in section 7.2.1) were added to OSPW. A schematic diagram of the batch bioreactors is shown in Fig. 7.1. The detailed strategies of batch experiments are shown in Table 7.2.

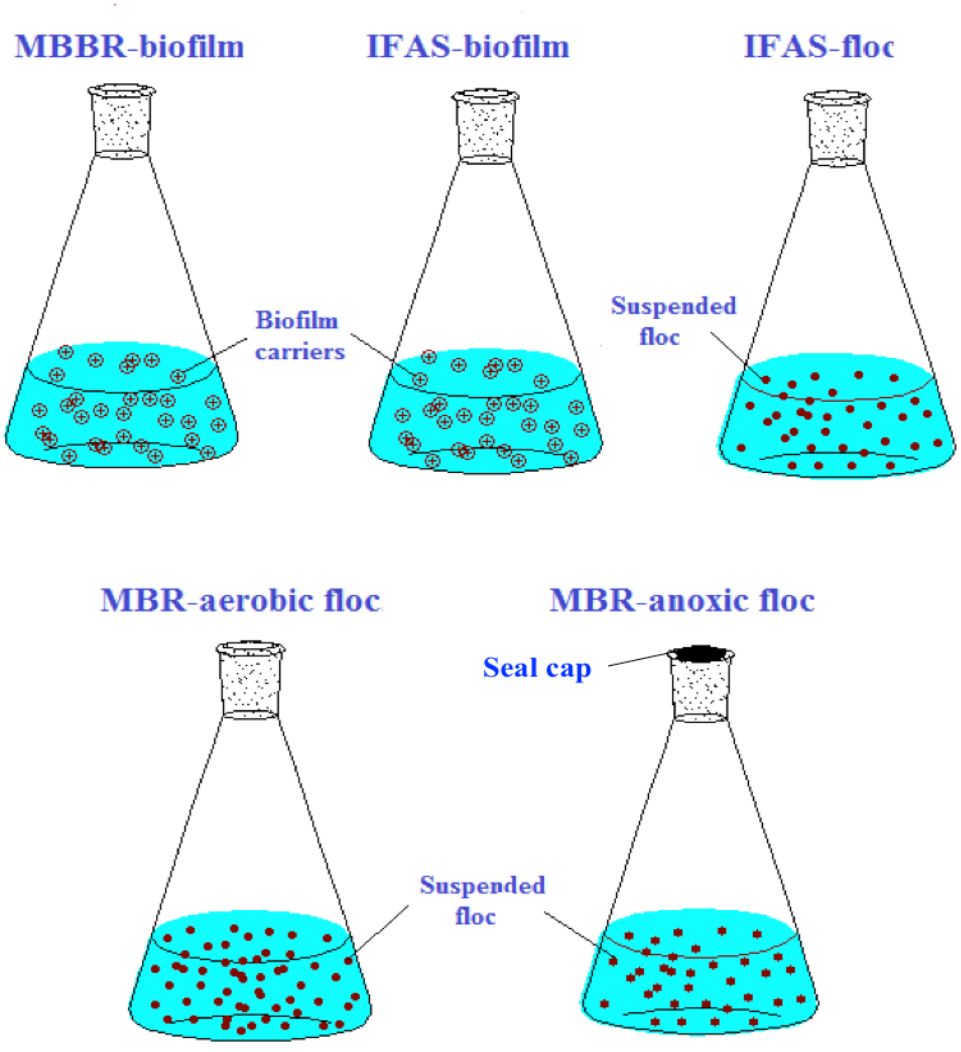


Figure 7.1. The schematic diagram of batch reactors

Table 7.2 The strategies of batch reactors set-up and operation*

Batch test	The type of bioreactor	Initial biomass con. (mg TSS/L)	The number of carrier	Total COD of influent (mg/L)	COD from OSPW (mg/L)	COD from sodium acetate (mg/L)	The con. of NO_3-N (mg/L)	The con. of NH_3-N (mg/L)
MBBR-biofilm	MBBR	500	105	508	208	300	-	60
IFAS-biofilm	IFAS	500	126	508	208	300	-	60
IFAS-floc		500	-	508	208	300	-	60
MBR-aerobic floc	MLE-MBR	500	-	508	208	300	-	60
MBR-anoxic floc		500	-	508	208	300	60	-

* All the experiments above described were performed in duplicate.

7.2.3 Measurement of Organic Contaminants

Chemical oxygen demand (COD). The COD concentration of the samples from the batch bioreactors was monitored with a DR3900 Benchtop Spectrophotometer (DR3900, HACH, Germany) according to the manufacturer's protocol (8000 TNTplus LR) with HACH kits (Chemical Oxygen Demand (COD) Reagent, TNT821, LR, Germany).

Acid extractable fraction (AEF). A Fourier transform infrared (FTIR) spectrometer (Spectrum 100, PerkinElmer Ltd, Bucks, UK) was used to measure the reactor AEF. Influent and effluent samples (25 mL) from the batch bioreactors were filtered (pore size of 0.45 μm) and acidified to pH ~ 2.0. Organics were extracted by liquid-liquid extraction with dichloromethane (DCM) in a separation funnel and the extracted AEF was monitored at 1743 cm^{-1} and 1706 cm^{-1}, adsorption bands characteristic of monomeric and dimeric carboxylic groups (Clemente and Fedorak, 2005).

HRMS analysis of NAs. Ultra performance liquid chromatography/high-resolution mass spectrometry (UPLC/HRMS) system was used to measure NA concentration profiles. 10 mL samples from batch reactors were filtered (pore size of 0.22 μm) before analysis. Separation of classical NAs and oxidized NAs was performed by UPLC using a Waters Acquity UPLC System

(Milford, MA, USA). Targetlynx 4.1 software was used for data analysis according to the method described in Martin et al. (2010). More detailed information about HRMS analysis is described in section A-3 (Appendix-A).

7.2.4 Characterization of Microbial Communities

DNA extraction. After 14 days of operation, genomic DNA was extracted from activated sludge floc and biofilm in the batch bioreactors with a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). To gather microorganisms from biofilms formed on the carriers, five carriers from each bioreactor were immersed in 5 mL sterile phosphate buffered saline (PBS) to remove nonadherent bacteria in a 20 mL sterile glass bottle (Falcon, CA, USA) and sonicated for 10 min. The glass bottle was then vortexed for 1 min and DNA was extracted in duplicate from the supernatant.

q-PCR (quantitative polymerase chain reaction). To investigate nitrifier and denitrifier gene abundance in the five types of biomass, *q*-PCR assays in a CFX 96TM Real-Time Detection System (Bio-RAD, California, USA) were used to quantify the bacterial *16S rRNA* gene in terms of total bacteria, *amoA* gene for ammonia oxidizing bacteria (AOB), two *16S rDNA genes* (*Nitrospira* spp. and *Nitrobacter* spp.) for nitrite oxidizing bacteria (NOB), and *narG*, *nirS*, *nirK*, and *nosZ* genes for denitrifiers. Reactions (20 µl) were performed in 96-well microplates; each tube was separately loaded with 2 µl of diluted sample or plasmid standard, followed by 1 µl each of forward and reverse primer, 10 µl SsoFast™ EvaGreen® Supermix (Bio-RAD, Hercules, California), and 6 µl PCR grade sterile water. All *q*-PCR experiments were performed in triplicate per sample and all PCR runs used plasmid standards for quantification; reactions without the DNA template were negative controls. Detailed descriptions of the *q*-PCR

amplification program and the primers can be found in section A-4 (Appendix-A) and Table B2 (Appendix-B).

7.2.5 Statistical Analysis

Statistical analyses were applied to compare the performance of the different biomasses with respect to OSPW remediation. Data were analysed using Microsoft Excel® software. The level of statistical significance was determined using a t-test. Correlations were considered to be statistically significant at the 95% confidence interval ($p < 0.05$).

7.3 Results and Discussion

7.3.1 Bench Scale Performance of MBBR, IFAS, and MLE-MBR Systems

Batch experiments to evaluate OSPW treatment were carried out using suspended activated sludge flocs and attached biofilm (taken from stabilized continuous running bench scale MBBR, IFAS, and MBR bioreactors). Similar start-up and operational parameter optimization strategies were employed for the three types of bioreactor during the acclimation and optimization stages. Typical operating conditions and influent and effluent water quality are presented in Table 7.1. The average total COD removal efficiencies were 58.2 ± 3.7%, 54.6 ± 3.4%, and 53.5 ± 4.5% in the MBBR, IFAS, and MLE-MBR bioreactors, respectively. The three bioreactors demonstrated efficient ammonia oxidation as evidenced by the low ammonia concentrations in effluents and permeate. Hydraulic retention times (HRTs) were 96 h, 96 h, and 12 h in the MBBR, IFAS, and MLE-MBR bioreactors, respectively. Mixed liquor suspended solids (MLSS) were utilized to quantify the concentrations of both suspended and attached biomass in the bioreactors. MLSS concentrations in the suspended floc in both aerobic and anoxic MBR tanks were significantly higher than that in the IFAS flocs.

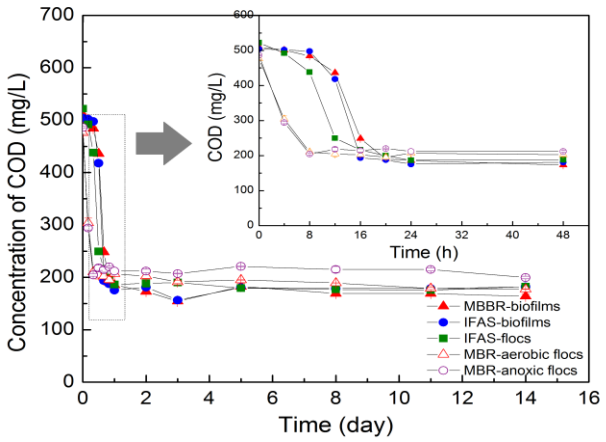
7.3.2 OSPW Treatment Performance of Batch Reactors

7.3.2.1 Biodegradation of COD

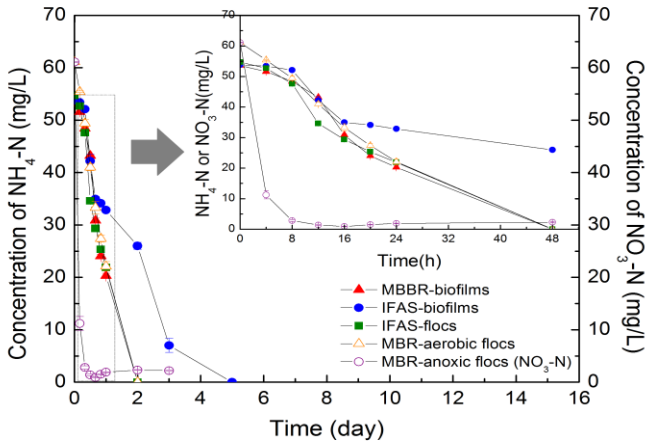
Initial COD concentrations (consisting of OSPW and the external substrate NaAC) were maintained at 508 mg/L in the batch bioreactors. An external carbon source (NaAC) was added to improve microbial density and AEF and NA removals following previous studies (Liu et al., 2015). As shown in Fig. 7.2(A), COD biodegradation proceeded quickly in the first 24 hours. The total COD removal efficiencies after 24 hours of operation were 63.6%, 65.2%, 64.3%, 56.5%, and 56.4%, respectively in MBBR-biofilm, IFAS-biofilm, IFAS-floc, MBR-aerobic-floc, and MBR-anoxic-floc. After 14 days of operation, the COD removal efficiencies due to biodegradation were 67.8%, 65.8%, 65.2%, 62.7%, and 58.9% respectively in the MBBR-biofilm, IFAS-biofilm, IFAS-floc, MBR-aerobic-floc, and MBR-anoxic-floc.

The COD degradation kinetics in the first 48 hours of the experiment is shown in the top right insert in Fig. 7.2(A). The total COD removal efficiencies in batch bioreactors with suspended activated sludge flocs (IFAS-flocs, MBR-aerobic-flocs, and MBR-anoxic-flocs), decreased sharply from 0 h to 12 h, but decreased only slightly in the bioreactors with attached biofilm (MBBR-biofilm, IFAS-biofilm) during this period. The higher COD degradation rate in the bioreactors with flocs compared to those with biofilm might be attributed to (i) the more porous floc structure which would foster higher nutrient and oxygen transport rates in the flocs than in the biofilm (Falas et al., 2012) and (ii) although we maintained the same initial biomass concentration in all batch bioreactors (Table 7.2), the more abundant denitrification relevant gene copies supported by floc compared to biofilm (as shown in the *q*-PCR results, section 7.3.3), which would allow floc to be more efficient than attached biofilm on COD removal.

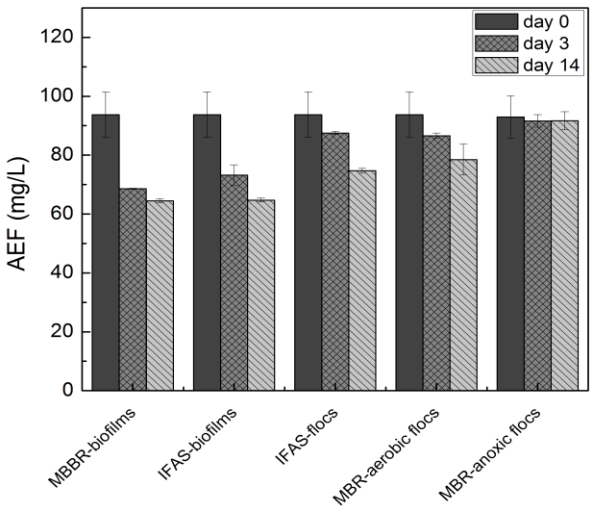
(A)


Concentration of COD (mg/L)
Time (day)
COD (mg/L)
Time (h)
MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs

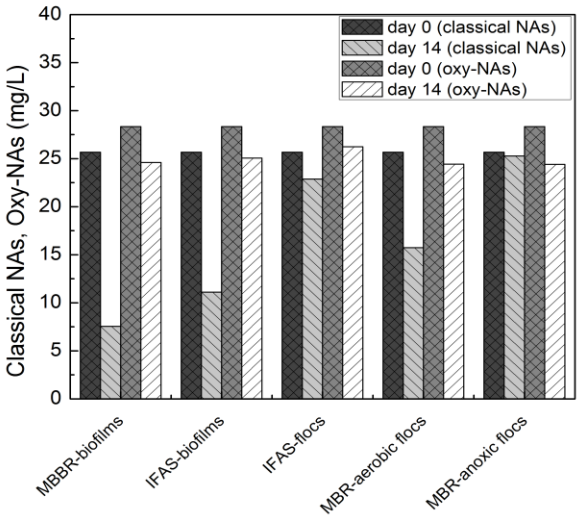
(B)


Concentration of NH4-N (mg/L)
Concentration of NO3-N (mg/L)
Time (day)
NH4-N or NO3-N(mg/L)
Time(h)
MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs (NO3-N)

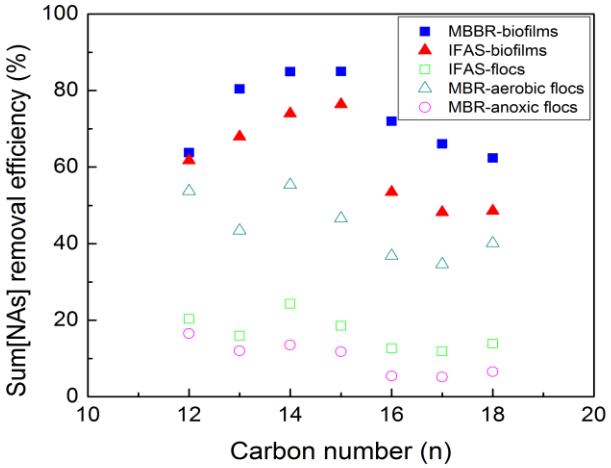
(C)


AEF (mg/L)
day 0
day 3
day 14
MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs

(D)


Classical NAs, Oxy-NAs (mg/L)
day 0 (classical NAs)
day 14 (classical NAs)
day 0 (oxy-NAs)
day 14 (oxy-NAs)
MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs

(E)


Sum[NAs] removal efficiency (%)
Carbon number (n)
MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs

(F)

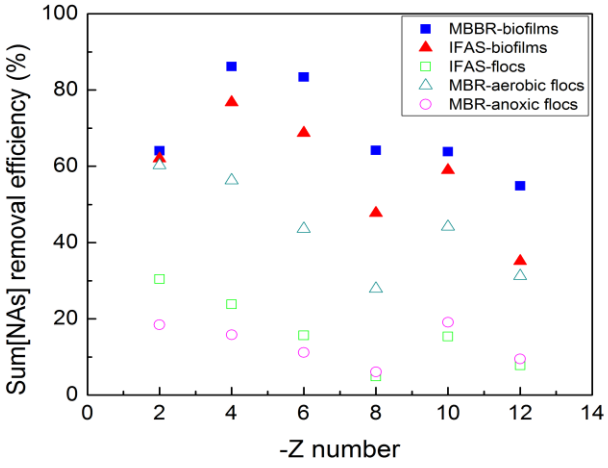

Sum[NAs] removal efficiency (%)
-Z number
MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs

Figure 7.2. Profile of COD concentration changes (A) along the batch bioreactors operation time, Profile of NH_4-N or NO_3-N concentration changes (B) along the batch bioreactors operation time, Profile of AEF concentration (C) changes in different batch bioreactors at different operation times (day 0, 3, and 14), Classical and oxidized NAs removal (D) in different batch bioreactors, Profile of Sum [classical NAs] removal efficiency versus carbon number n (E) and Z value (F) in different batch bioreactors.

Within the first 8 hours, MBR-aerobic-flocs and MBR-anoxic-flocs batch bioreactors achieved the highest COD removal rate. The continuously running bench scale MLE-MBR system, which was supported by a higher MLSS concentration in both aerobic and anoxic tanks in the MLE-MBR system (Table 7.1), provides stronger substrate and oxygen assimilation, and thus a low food to microorganism (F/M) ratio. This might account for the much higher COD removal rate by MBR floc than by the IFAS floc. The COD in IFAS-flocs batch bioreactor decreased much more rapidly than the COD in the MBBR-biofilm and IFAS-biofilm bioreactors. Further, MBBR and IFAS biofilms perform similarly with respect to COD removal in from OSPW. The similar MBBR-biofilm and IFAS-biofilm bioreactors performance might be explained by (i) the similar reactor configuration, which can significantly affect biofilm formation (Choi et al., 2014b); (ii) the same type of biofilm support media, which can directly influence the biofilm formation and adhesion to the support media, the biomass density, and the treatment performance (Dong et al., 2011); (iii) a similar active microbial population ($p > 0.05$) (as observed in the *q*-PCR analysis, section 7.3.3).

7.3.2.2 Nitrogen Removal

During batch bioreactors operation, NH_4-N (provided as ammonium chloride) was added in the influents of the MBBR-biofilm, IFAS-biofilm, IFAS-flocs, and MBR-aerobic-flocs bioreactors. NO_3-N (provided as sodium nitrate) was introduced into the MBR-anoxic-floc bioreactor influent to maintain a biological nitrogen level of ~ 60 mg/L and to improve the growth of nitrifying and denitrifying bacteria (Table 7.2). Previous studies demonstrated that the enzyme ammonia monooxygenase (AMO) produced by ammonia oxidizing bacteria (AOB) may catalyze the oxidation of many slow biodegradable compounds, such as hydrocarbons and halogenated hydrocarbons, in industrial wastewater (Rasche et al., 1990; Tran et al., 2013). It was reported that a higher biodegradation rate of naphthenic acids was coupled to nitrate reduction under anoxic (denitrifying) conditions but not under aerobic conditions (Gunawan et al., 2014). The NH_4-N degradation kinetics in MBBR-biofilm, IFAS-biofilm, IFAS-floc, and MBR-aerobic-floc batch bioreactors and NO_3-N reduction in the MBR-anoxic-floc batch bioreactor are shown in Fig. 7.2(B). Within three days, the NH_4-N was completely oxidized in all reactors except the IFAS-biofilm bioreactor. NH_4-N concentrations underwent a sharp decrease between 4 h and 16 h; the IFAS-flocs bioreactor showed the highest ammonia oxidation rate, while the IFAS-biofilm bioreactor achieved the lowest NH_4-N degradation rate during this period. These results indicate that flocs play a more significant nitrification role than the biofilm in the IFAS bioreactors. Due to the different surface properties, flocs may promote faster molecular oxygen diffusion and leading to faster ammonia oxidation than in biofilm systems (Chen et al., 1997). In the bioreactor containing NO_3-N, nitrate reduction (denitrification) proceeded extremely quickly in the first 24 hours, demonstrating the high denitrification performance of the MBR-anoxic-floc bioreactor.

7.3.2.3 AEF Removal

AEF values are commonly used by the oil sands industry to evaluate the degree of biodegradation of naphthenic acids (NAs) and other organic compounds containing carboxylic groups in OSPW (Grewer et al., 2010). FTIR was employed to measure the AEF fraction in the batch bioreactor during biological treatment. Previous studies reported that AEF fractions resisted biodegradation (Huang et al., 2015; Shi et al., 2015). Fig 7.2(C) shows the reduction in the AEF in the five types of batch bioreactors at different operation times. The initial AEF concentration in the bioreactors was 93.7 ± 7.8 mg/L. On day 3, the AEF removal efficiencies in the MBBR-biofilm, IFAS-biofilm, IFAS-floc, MBR-aerobic-floc, and MBR-anoxic-floc reactors were 26.83%, 21.87%, 6.64%, 7.65%, and 1.44%, respectively. After 14 days of operation, the AEF removal efficiencies in the MBBR-biofilm, IFAS-biofilm, IFAS-floc, MBR-aerobic-floc, and MBR-anoxic-floc bioreactors reached 31.24%, 30.95%, 20.32%, 16.31%, and 1.46%, respectively. Evidently, AEF removal efficiencies were significantly higher ($p < 0.05$) in the batch bioreactors with attached biofilm (MBBR-biofilm and IFAS-biofilm) than in the bioreactors with suspended activated sludge flocs (IFAS-flocs, MBR-aerobic-flocs, and MBR-anoxic-flocs). The benefits of biofilm versus floc to slow growing AEF degraders can be rationalized by the longer solid retention time and the higher stratified and diversified microbial communities in attached biofilm. Microorganisms adapted to slowly biodegradable organic compounds apparently prefer to accumulate in attached biofilm rather than in flocs (Falas et al., 2012).

AEF removal efficiencies in batch bioreactors with aerobic flocs (IFAS-flocs and MBR-aerobic-flocs) were significantly higher ($p < 0.05$) than in the bioreactor with anoxic floc (MBR-anoxic-floc) on day 14. Aerobic (nitrifying) flocs degraded AEF more efficiently than anoxic

(nitrate-reducing) floc, possibly due to the preferred to oxygen-dependent pathways for AEF degradation. OSPW-AEF might be better suited in the aerobic pathway.

7.3.2.4 NAs Removal

UPLC/HRMS was applied to obtain information about NA molecular weights and structure. Fig. 7.2(D) shows the degradation of classical NAs and oxidized NAs (oxy-NAs) in the five types of batch bioreactors before and after 14 days of operation. The initial concentrations of classical and oxidized NAs in the influents were 25.68 and 28.34 mg/L, respectively. After 14 days of operation, the removal efficiencies of classical NAs in the MBBR-biofilm, IFAS-biofilm, IFAS-flocs, MBR-aerobic-flocs, and MBR-anoxic-flocs bioreactors were 70.59%, 56.70%, 10.85%, 38.69%, and 1.52%, respectively, while the removal efficiencies of oxidized NAs in the MBBR-biofilm, IFAS-biofilm, IFAS-flocs, MBR-aerobic-flocs, and MBR-anoxic-flocs bioreactors were 13.17%, 11.54%, 7.38%, 13.77%, and 13.83%, respectively. The removal efficiencies of oxidized NAs were significantly lower ($p < 0.05$) than the removal efficiencies of classical NAs in the aerobic batch bioreactors, which might be attributed to the following: (i) oxidized NAs (O-NAs, O_2-NAs, O_3-NAs and O_4-NAs) are simply more persistent than classical NAs towards biodegradation (Han et al., 2009) and (ii) some of the classical NAs were converted to oxidized NAs after aerobic biodegradation, as supported by the oxidized NAs results presentd in Fig. 7.3(A). Han et al. (2008) observed classical NAs being converted to oxidized NAs during aerobic microbial biodegradation of commercial NAs. It has been also reported that oxidized NAs were produced as intermediates during aerobic biodegradation of various NA model compounds (Alexander, 1999).

The classical NAs removal efficiencies in the bioreactors with attached biofilm (MBBR-biofilm and IFAS-biofilm) were significantly higher ($p < 0.05$) than in the bioreactors with

suspended activated sludge flocs (IFAS-floc and MBR-aerobic-floc). The MBBR-biofilm batch bioreactor biodegraded classical NAs more efficiently than the other bioreactors. The batch bioreactor with aerobic biomass degraded NAs more efficiently than the bioreactor with anoxic biomass. The classical NAs biodegraders tend to preferr to exist under aerobic condition. However, under anoxic conditions, suspended flocs degraded oxidized NAs better than classical NAs; probably because oxidized NAs biodegradation is more favourable than biodegradation of classical NAs due to the poor enzyme-oxygen affinity and co-biodegradation with nitrate reduction and sulfide bio-oxidation (Nemati et al., 2001a,b).

Fig. 7.2(E) and 7.2(F) show the classical NA removal efficiency based on carbon number and Z value, respectively, in the five types of batch bioreactors after 14 days of operation. Classical NAs with carbon numbers 13–15 were most efficiently removed in the batch bioreactors with attached biofilm, and classical NAs with a carbon number of 14 were most efficiently removed in the bioreactors with suspended floc (Fig. 7.2(E)). Some classical NAs with carbon numbers less than 14 may have been produced by bioconversion of larger molecular weight NAs, as previously reported (Islam et al., 2014b; Huang et al., 2015; Xue et al., 2016).

Classical NAs with absolute Z values of 4–6 were removed most efficiently in the batch bioreactors with attached biofilm, and NAs with an absolute Z value of 2 were removed most efficiently in the bioreactors with suspended floc (Fig. 7.2(F)). The low removal efficiency of classical NAs with larger Z values (absolute Z values > 8) in the biofilm batch bioreactors and absolute Z values > 4 in the flocs batch bioreactors indicates that NAs with higher cyclicity and branching were more persistent towards biodegradation than those with fewer cyclic rings. Our results are in agreement with the previous studies on biological treatment of OSPW classical NAs (Xue et al., 2016; Shi et al., 2015; Huang et al., 2015). Han et al. (2008) observed that the

biodegradation rate of NAs decreased with the increase of cyclicity (larger Z values) in commercial and OSPW NAs.

Fig. 7.3(A) shows the oxidized NAs (oxy-NAs) degradation in the five types of batch bioreactors before and after 14 days of operation. The proportions of O-NAs, O_2-NAs, O_3-NAs, and O_4-NAs within the total oxy-NAs are 47.05%, 52.95%, 10.49%, and 4.31%, respectively, indicating that mono- and dioxidized NAs (i.e., $C_nH_{2n+Z}O_3$ and $C_nH_{2n+Z}O_4$) are the dominant oxy-NA species in OSPW samples. The concentrations of monooxidized NAs (O-NAs) in all aerobic batch bioreactors were even significantly higher than those in the influents after 14 days of operation, which might be explained if some classical NAs were converted to O-NAs after aerobic biodegradation. However, dioxidized NAs removal efficiencies in the MBBR-biofilm, IFAS-biofilm, IFAS-flocs, MBR-aerobic-flocs, and MBR-anoxic-flocs bioreactors were 28.50%, 30.29%, 22.15%, 33.11%, and 22.80%, respectively. The removal efficiencies of O_3-NAs and O_4-NAs in all batch bioreactors were significantly lower than those of O_2-NAs (Fig. 7.3(A)), indicating that oxidized NAs with larger numbers of oxygen atoms were more resistant to biodegradation than those with fewer oxygen atoms.

The sum of the concentrations of oxy-NAs/sum of the concentrations of classical NAs revealed an interesting trend that may be a good indicator of NA biodegradability. After 14 days of operation, the total concentration of mono- and di-oxidized NAs divided by the total concentration of classical NAs, decreased in the following order: MBBR-biofilm > IFAS-biofilm > MBR-aerobic-flocs > IFAS-flocs > MBR-anoxic-flocs (Fig. 7.3(B)). This result suggested that the lowest OSPW NAs biodegradability was found after MBBR-biofilm treatment, as compared to other batch bioreactors. The concentrations of oxidized NAs did not change much after 14 days of bioreactor operation; indicating that: (i) oxidized NAs are more resistant to

biodegradation than classical NAs or (ii) the formation and degradation rates of oxidized NAs reached equilibrium (Han et al., 2009).

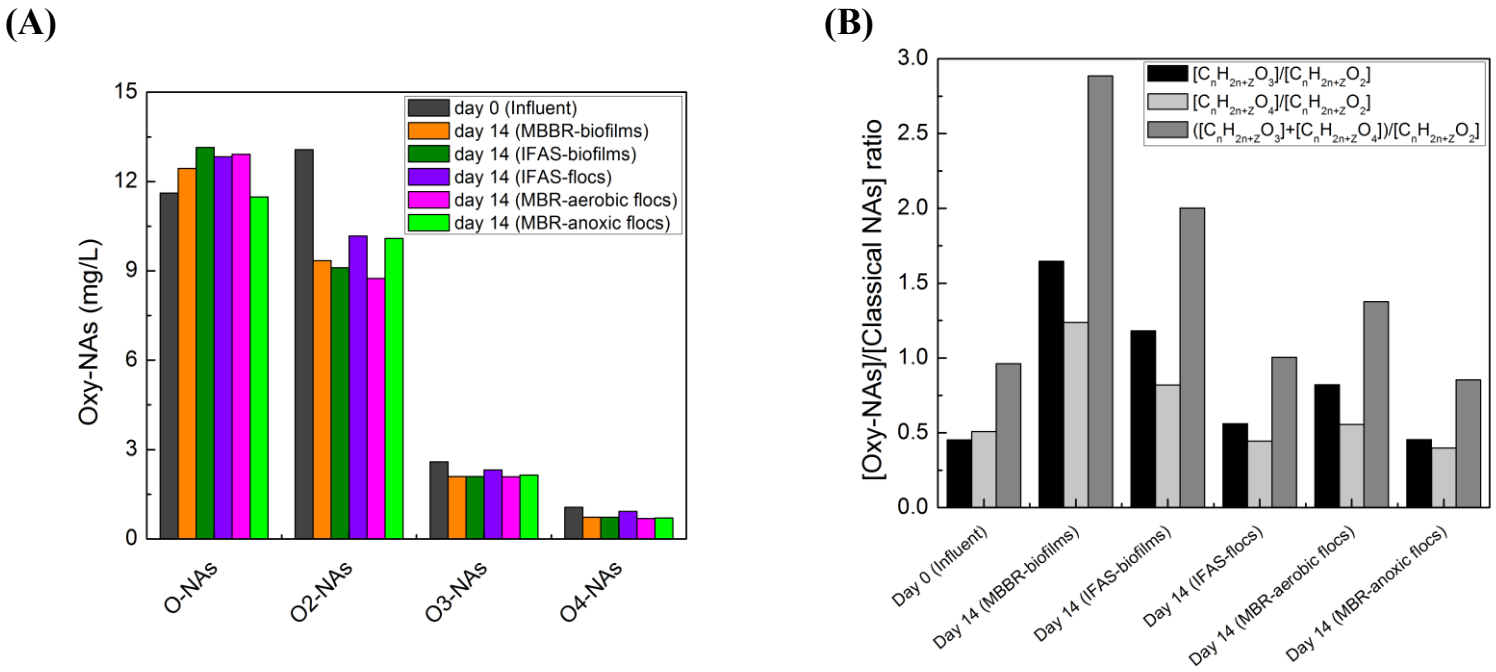
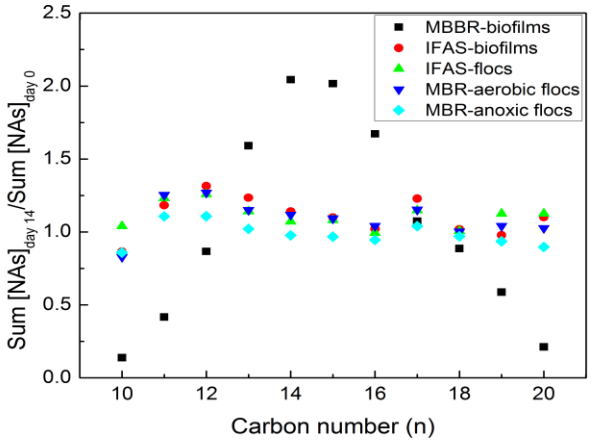


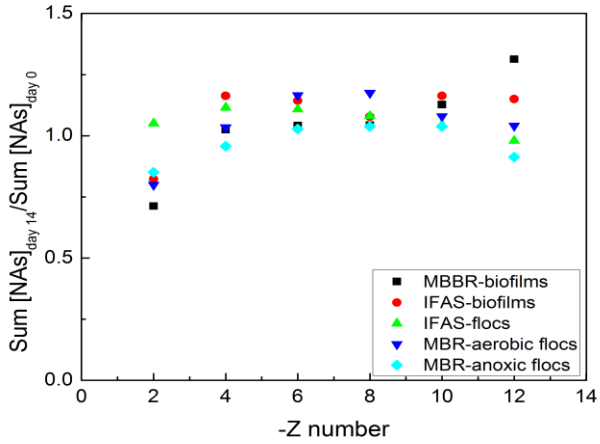
Figure 7.3. Oxy-NAs (O-NAs, O_2-NAs, O_3-NAs, and O_4-NAs) removal (A) in different batch bioreactors, Total HRMS abundance of mono- (O-NAs) and/or di-oxidized (O_2-NAs) NAs relative to total HRMS abundance of classical NAs in influents (day 0) and effluents (day 14) in the different batch bioreactors.

Fig. 7.4(A) and 7.4(B) shows the Sum [O-NAs] (total O-NA concentration) removal in the five types of batch bioreactors based on carbon number and Z value after 14 days of operation. In the MBBR-biofilm bioreactor, the ratio Sum $[O\text{-}NAs]_{day\ 14}$/Sum $[O\text{-}NAs]_{day\ 0}$ based on carbon numbers 13-16 was significantly larger than 1.0 (Fig. 7.4(A)), indicating that the formation rates were significantly higher than the degradation rates for the O-NAs with carbon numbers 13-16 . However, for O-NAs with carbon numbers $n < 13$ and $n > 16$, the formation rates were lower than the degradation rates.

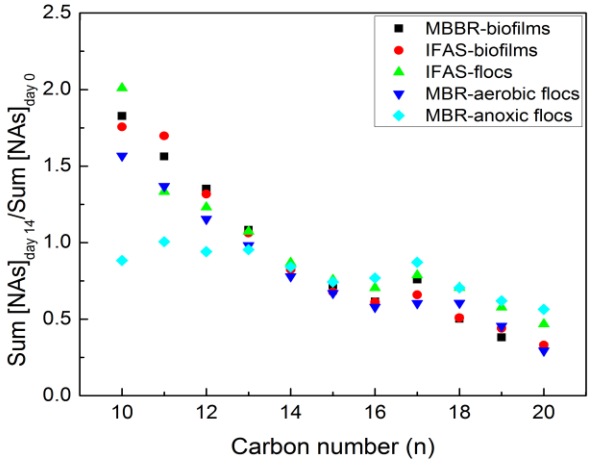
(A)


MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs
Sum [NAs]day 14/Sum [NAs]day 0
Carbon number (n)

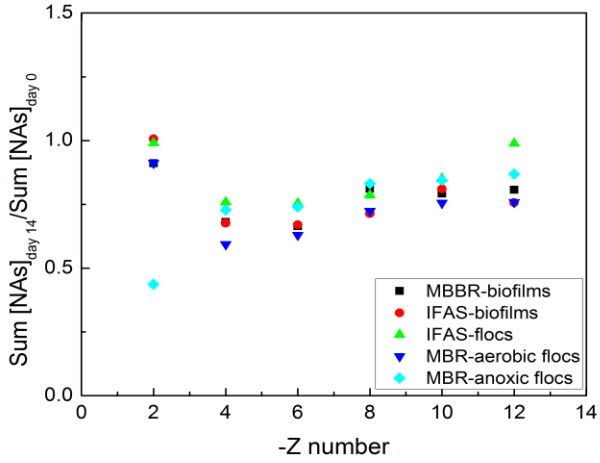
(B)


MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs
Sum [NAs]day 14/Sum [NAs]day 0
-Z number

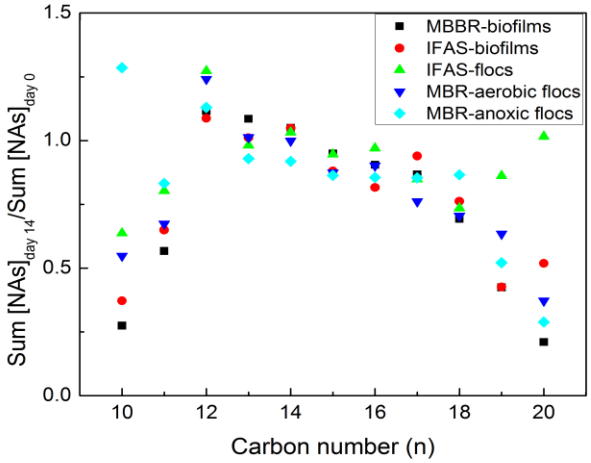
(C)


MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs
Sum [NAs]day 14/Sum [NAs]day 0
Carbon number (n)

(D)


MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs
Sum [NAs]day 14/Sum [NAs]day 0
-Z number

(E)


MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs
Sum [NAs]day 14/Sum [NAs]day 0
Carbon number (n)

(F)

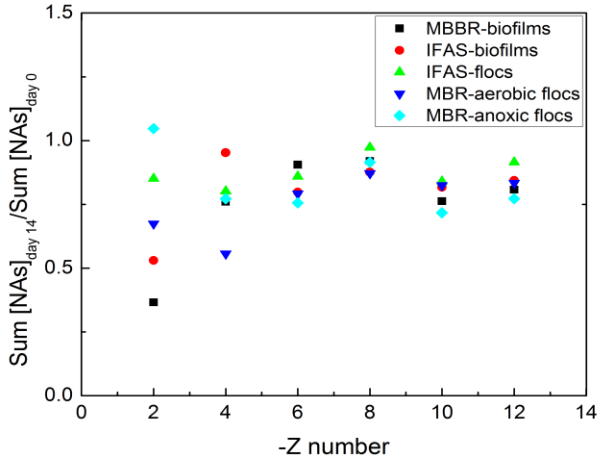

MBBR-biofilms
IFAS-biofilms
IFAS-flocs
MBR-aerobic flocs
MBR-anoxic flocs
Sum [NAs]day 14/Sum [NAs]day 0
-Z number

Figure 7.4. Profile of Sum $[O\text{-}NAs]_{day\ 14}$/ Sum $[O\text{-}NAs]_{day\ 0}$ ratio versus carbon number n (A) and Z value (B) in the different batch bioreactors, Profile of Sum $[O_2\text{-}NAs]_{day\ 14}$/ Sum $[O_2\text{-}NAs]_{day\ 0}$ ratio versus carbon number n (C) and Z value (D) in the different batch bioreactors, Profile of Sum $[O_3\text{-}NAs]_{day\ 14}$/ Sum $[O_3\text{-}NAs]_{day\ 0}$ ratio versus carbon number n (E) and Z value (F) in the different batch bioreactors.

In the other batch bioreactors, the ratio Sum $[O\text{-}NAs]_{day\ 14}$/Sum $[O\text{-}NAs]_{day\ 0}$ of O-NAs with all carbon numbers fluctuated around 1.0 (Fig. 7.4(A)), demonstrating that O-NAs with all carbon numbers were being formed and degraded at equivalent rates. The ratio Sum $[O\text{-}NAs]_{day\ 14}$/Sum $[O\text{-}NAs]_{day\ 0}$ increased slightly with increasing Z value (Fig. 7.4(B)), indicating that the rate of O-NA biodegradation decreased with the increase in the cyclicity (larger Z value).

The Sum $[O_2\text{-}NAs]$ removal in all batch bioreactors is shown in Fig. 7.4(C) and 7.4(D). The ratio Sum $[O_2\text{-}NAs]_{day\ 14}$/Sum $[O_2\text{-}NAs]_{day\ 0}$ in all batch bioreactors decreased with an increase in the carbon number (Fig. 7.4(C)), indicating that the formation rates of O_2-NAs decreased as carbon number increased. Regarding O_2-NAs with more cyclic rings (absolute Z value > 4), the ratio Sum $[O_2\text{-}NAs]_{day\ 14}$/Sum $[O_2\text{-}NAs]_{day\ 0}$ increased slightly with the increase in Z value (Fig. 7.4(D)). The O_3-NAs removal in all batch bioreactors, as shown in Fig. 7.4(E) and 7.4(F), demonstrated that the removal of O_3-NAs varied significantly with carbon number and Z value.

7.3.3 Quantification of Bacterial Populations by the Quantitative Polymerase Chain Reaction

The gene abundance of total bacteria, nitrifiers, and denitrifiers was determined by counting 16S rRNA gene copies using quantitative real-time PCR. Fig. 7.5 compares the

abundance of the bacterial *16S rRNA* gene, nitrifier genes (*aomA*, *NSR*, *Nitro*), and denitrifier genes (*nirS*, *nosZ*, *nirK*, *narG*) in each of the five batch bioreactors. The MBBR-biofilm, IFAS-biofilm, IFAS-floc, MBR-aerobic-floc, and MBR-anoxic-floc contained $5.47 \times 10^{11} \pm 5.92 \times 10^{10}$, $5.62 \times 10^{11} \pm 1.42 \times 10^{10}$, $1.27 \times 10^{12} \pm 7.86 \times 10^{10}$, $2.26 \times 10^{12} \pm 2.03 \times 10^{11}$, and $2.92 \times 10^{12} \pm 2.93 \times 10^{11}$ bacteria counts, respectively. Although the initial biomass was almost uniform in all the five batch bioreactors, the bacteria population was significantly larger in the bioreactors with suspended flocs ($p < 0.05$) than in the bioreactors with attached biofilm. These results support the phenomenon that the bioreactors with suspended flocs removed COD more efficiently than reactors with attached biofilm. Fig. 7.5 shows that in the batch bioreactors MBBR-biofilm, IFAS-biofilm, IFAS-flocs, MBR-aerobic-flocs, and MBR-anoxic-flocs, the abundance of the *amoA* gene of ammonia oxidizing bacteria (AOB) was $5.68 \times 10^{8} \pm 8.27 \times 10^{7}$, $1.17 \times 10^{9} \pm 2.67 \times 10^{7}$, $2.23 \times 10^{9} \pm 1.33 \times 10^{8}$, $2.64 \times 10^{9} \pm 2.61 \times 10^{8}$, and $5.46 \times 10^{9} \pm 1.38 \times 10^{9}$ copies, respectively. Although the abundance of *amoA* in the MBBR-biofilm bioreactor was significantly lower than in other bioreactors ($p < 0.05$), ammonia oxidation in the MBBR-biofilm bioreactor was similar to that in the other bioreactors; apparently there was a sufficient AOB population for ammonia removal in the MBBR-biofilm bioreactor.

The abundance of *NSR*, *Nitro*, and denitrifier genes (*narG*, *nirS*, *nirK*, *nosZ*) in the MBBR-biofilm, IFAS-biofilm, IFAS-flocs, MBR-aerobic-flocs, and MBR-anoxic-flocs bioreactors are compared as shown in Fig. 7.5. The *NSR* gene abundance in all batch bioreactors was significantly lower than the Nitro gene abundance ($p < 0.05$), consistent with previous studies (Shi et al., 2015; Huang et al., 2015). The *NSR* gene abundance in the MBR-anoxic bioreactor was significantly lower than that in the aerobic batch bioreactors. One possible explanation was that *Nitrospia* spp. could assimilate inorganic carbon and pyruvate only under aerobic conditions.

However, there was no significant difference in *Nitro* gene abundance between the MBR-anoxic bioreactor and other aerobic bioreactors except for the MBBR-biofilm bioreactor, probably because *Nitrobacter* spp. can utilize pyruvate under anoxic conditions (Kim and Kim, 2006). The abundance of denitrifying genes *narG*, *nirS*, *nirK,* and *nosZ* in batch bioreactors with suspended floc was significantly higher than in bioreactors with attached biofilm, which indicates that denitrifiers tend to gather in the suspended flocs.

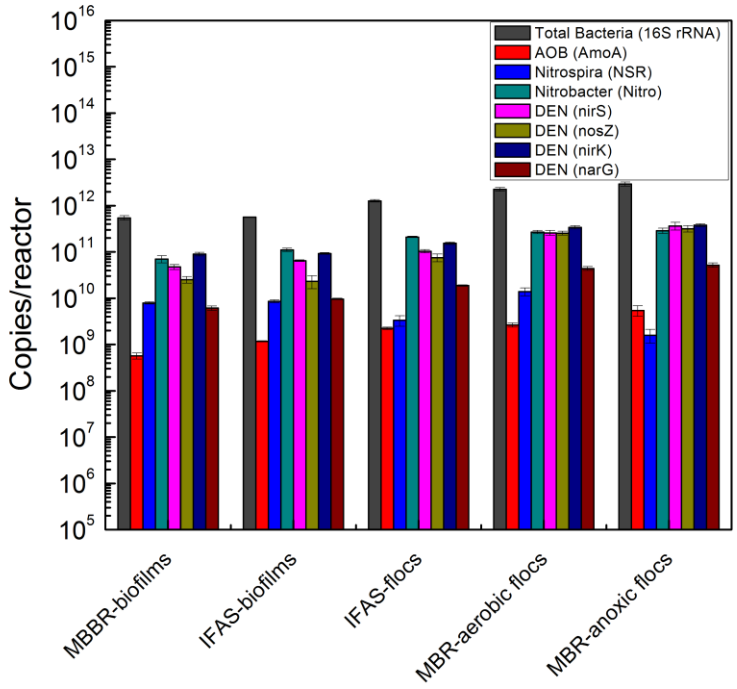


Figure 7.5. The gene copies per bioreactor of the total bacteria, AOB, *Nitrospira*, *Nitrobacter* and the denitrifying bacteria (*nirS*, *nosZ*, *nirK* and *narG*) in flocs and biofilms samples from the five types of batch bioreactors (day 14).

7.4 Conclusions

This study compared the ability of five types of biomass (MBBR-biofilm, IFAS-biofilm, IFAS-flocs, MBR-aerobic-flocs and MBR-anoxic-flocs) to reclaim OSPW for recycling and safe

release to the receiving environment. MBR-aerobic-flocs and MBR-anoxic-flocs bioreactors demonstrated the highest COD removal rates, whereas the bioreactors with biofilms achieved considerably higher AEF removal efficiency compared to the bioreactors with flocs. MBBR-biofilm was the most successful in removing NAs from OSPW. UPLC/HRMS analysis showed that NAs degradation depended highly on the carbon number and Z value of the NA species. Mono- and dioxidized NAs were the dominant oxy-NAs species in our OSPW samples. The bacterial *16S rRNA* gene was significantly higher in the bioreactors with suspended flocs than in the bioreactors with biofilm, the *NSR* gene abundance in the MBR-anoxic bioreactor was significantly lower than that in the aerobic batch bioreactors, and denitrifiers tended to stay in the suspended phase.

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CHAPTER 8. GENERAL CONCLUIONS AND RECOMMENDATIONS

8.1 Thesis Overview

The OSPW generated during the extraction of surface mining bitumen from oil sands in Northern Alberta, Canada, which is a complex matrix comprised of petroleum hydrocarbons, organic acids, oilfield chemicals, salts, suspended solids, heavy metals and many other toxic compounds including NAs. Therefore, its discharge without treatment can cause considerable environmental problems. Currently, OSPW is being stored in tailings ponds until environmental-safe and economically feasible treatment processes are available prior to its release. Water treatment strategies are urgently required to allow tailings water to be recycled or to be released safely to the receiving environment, and reduce the demand to withdraw fresh water from the Athabasca River.

Up to date, various treatment technologies such as coagulation-flocculation, adsorption, membrane filtration, advanced oxidation processes, and biological treatment have been studied for their efficiency in treating OSPW. However, a large industrial scale and cost-effective treatment technologies for the remediation of OSPW has not yet to be developed. Biodegradation using bioreactor technology is an effective, economic, and energy efficient for OSPW remediation. Recently, both suspended growth and attached growth bioreactors have been studied for OSPW treatment. However, to date, none of the studies have considered the application of bioreactors combining suspended activated sludge flocs and attached biofilms (hybrid bioreactors) process for OSPW remediation. Therefore, there is a significant research gap Related to the evaluation of the feasibility and treatment performance of hybrid reactors for the biodegradation of OSPW at both small and large scales.

The research herein, focused on the treatability of OSPW using an engineering IFAS reactor. The bench-scale IFAS reactors were tested for both raw and ozonated OSPW using continuous and batch treatment reactors. The impact of ozonation on the IFAS biodegradation of OSPW was evaluated. The overall reactor treatment performance was evaluated by the measurement of COD, AEF, and NAs concentration. The assessment of the biofilm growth on the biofilm carrier was evaluated by CLSM and *q*-PCR. The characterization of the microbial community structure in both suspended flocs and attached biofilms in the IFAS systems were investigated by *q*-PCR and pyrosequencing. The NAs removal based on carbon number, Z number, and oxygen number were also investigated.

8.2 Conclusions

The following conclusions were drawn as a result of this research:

1. After 11 months start-up operation, when the volume percentage of OSPW reached to 100%, 12.06% of the AEF and 43.14% of the NAs were removed by direct biodegradation in IFAS without ozone pretreatment, while 41.97% of the AEF and 80.18% of NAs were removed in ozonation combined IFAS process. The biofilm in the ozonated OSPW IFAS was significantly thicker (94 ± 1.6 μm) than the biofilm in the raw OSPW IFAS (72 ± 2.8 μm) after 283 days of cultivation. NA biodegradation significantly decreased as the NA cyclization number increased.
2. The gene abundances of total bacteria and nitrogen removal relevant bacteria significantly decreased in two IFAS systems during the start-up stage. The gene abundance proportions of nitrogen removal relevant bacteria within total bacteria are significantly higher in biofilms than in flocs in the raw OSPW IFAS system.

3. Bacterial community in the seed sludge (AS from GBWTP) of two IFAS systems showed the greatest richness and evenness. Chao 1 value and Shannon diversity index results showed that the bacterial richness and microbial diversity of biofilms were significantly higher than flocs in both two IFAS systems. The microbial communities analysis from 454-pyrosequencing revealed that *Proteobacteria*, *Nitrospirae*, *Acidobacteria* and *Bacteroidetes* were dominant phyla in both flocs and biofilms of IFAS reactors.
4. The phyla and classes distribution of flocs and biofilms were significantly different. Principal Coordinate Analysis indicated that there were obvious differences in microbial community between the biomass in IFAS systems and the seed sludge (AS from GBWTP) used for the start-up of IFAS systems. Then, the ozone pretreatment of OSPW has no significant effects on the microbial communities of flocs and biofilms in IFAS system. What's more, there was some variation of bacterial communities existing between flocs and biofilms in two IFAS systems.
5. Compared to the biofilms, the IFAS-flocs demonstrated considerably higher removal rates for COD and NH_4-N, whereas, IFAS-biofilms had better performance on the AEF removal than IFAS-flocs. Meanwhile, our study also revealed that the biodegradation was the principal OSPW contaminant removal mechanism, and biosorption had limited contribution to the OSPW organic compounds and ammonium removal.
6. The abundances of nitrifier and denitrifier genes were significantly higher in the flocs than those in the biofilms in both raw and ozonated-OSPW IFAS reactors. *Proteobacteria*, *Acidobacteria*, *Nitrospirae*, and *Bacteroidetes* were dominant phyla in both flocs and biofilms of IFAS reactors. However, the microbial community composition of flocs and biofilms at the class level were significantly different.

7. After 11 months of HRT and ammonium optimization, 54.56% of the COD and 30.20% of the AEF were removed in raw OSPW IFAS, and 56.83% of the COD and 51.51% of the AEF were removed in ozonated OSPW IFAS. Extending the HRT in the IFAS systems had no significant effect on the COD and total nitrogen removal from raw or ozonated OSPW, whereas a lower COD/N ratio increased organics removal and thus total nitrogen removal.
8. The abundances of nitrifer and denitrifer genes decreased during HRT optimization (phase I to III), and increased significantly after ammonium optimization (phase III to V). *Proteobacteria*, *Nitrospirae*, *Acidobacteria*, and *Bacteroidetes* were dominant phyla in both flocs and biofilms in both raw and ozonated IFAS systems. During optimization, *Proteobacteria* decreased significantly and *Nitrospirae* and *Acidobacteria* increased significantly in both flocs and biofilms in raw and ozonated IFAS systems. The results indicate that the HRT and the COD/N ratio have the significant influence on the microbial community composition in IFAS systems.
9. MBR-aerobic-floc and MBR-anoxic-floc reactors demonstrated the highest COD removal rates, whereas reactors with biofilms achieved considerably higher AEF removal compared to reactors with floc. MBBR-biofilm was the most successful in removing NAs from OSPW. NA degradation depended highly on the carbon number and Z value of the NA species. Mono- and dioxidized NAs were the dominant oxy-NAs species in our OSPW samples.
10. The bacterial *16S rRNA* gene was significantly higher in reactors with suspended floc than in reactors with biofilm, the *NSR* gene abundance in the MBR-anoxic reactor was significantly lower than that in aerobic batch reactors, and denitrifiers tended to stay in the suspended phase.

8.3 Recommendations

Based on the results obtained from this study, the following recommendations can be addressed for future research:

1. The economical evaluation of ozonation combined IFAS process for the real industrial application is recommended. Estimation the overall operational cost of the combination process is important for up-scaling the system.
2. The modeling and evaluation of the performance of IFAS systems in OSPW treatment are recommended. The establishment of a mathematical model based on the successful operation of IFAS reactors for the OSPW remediation is necessary to understand the biological, chemical, and physical characteristics of microbial structures (flocs and biofilms) and their behaviors in bioreactors.
3. The novel biofilm-supporting medium in the IFAS process for OSPW remediation is recommended. Only one type of biofilm substrate material (polyethylene) was employed in this research. For instance, use of activated carbon (AC)-assisted IFAS for ozonated OSPW treatment combines adsorption, biodegradation, and advanced oxidation. This might lead to the advance removal of recalcitrant contaminants and completely mineralization of OSPW.

APPENDIX A: Experimental Methods and Analysis

A-1 OSPW Chemistry Analysis

OSPW was obtained from the West In-Pit water pumping station at Syncrude Canda Ltd. Fort McMurray, AB, Canada, in September 2013. OSPW characteristics are listed in Table B1. Concentrations of anions and cations were monitored in raw and ozonated OSPW samples. Anions were determined by a Dionex ICS-2500 including an AS50 autosampler, a CD025 conductivity system, a CSRS 300 suppressor, an Ionpac® 14A analytical column for anion separation, and a GP50 gradient pump. Cations were determined with a Dionex ICS-2000 consisting of an AS50 autosampler, a CSRS 300 suppressor, a DS6 heated conductivity cell, an Ionpac® CS12A analytical column for cation separation, and an ICS-2000 pump. Dionex anion and cation standard solutions were used for anion and cation analyses. 10 mL of each sample was filtered (0.2 μm nylon) and transferred in a vial without headspace for autosampling; the injection volume was 25 μL and the flow rate was 1 mL/min. Eluents for anion and cation analyses were 8.0 mM sodium carbonate/1.0 mM sodium bicarbonate and electrolytically generated (RFICTM eluent generator cartridge) methanosulfonic acid (20 mM), respectively. Millipore water was used in the experiment for dilution and other purposes.

A-2 Chemical Oxygen Demand (COD)

COD concentrations of IFAS influents and effluents were determined using DR3900 Benchtop Spectrophotometer (DR3900, HACH, Germany) according to the manufacturer's instruction (8000 TNTplus LR) with HACH kits (Chemical Oxygen Demand (COD) Reagent, TNT821, LR, Germany).

A-3 UPLC/HRMS Analysis of NAs

Chromatographic separations were run on a Waters UPLC Phenyl BEH column (150 × 1 mm, 1.7 μm) using a gradient mobile phase of (A) 10 mM ammonium acetate solution prepared

in Optima-grade water and (B) 10 mM ammonium acetate in 50% methanol 50% acetonitrile, both Optima-grade. Gradient elution was as follows: 1% B for the first 2 min, then ramped to 60% B by 3 min, to 70% B by 7 min, to 95% B by 13 min, followed by a hold until 14 min and finally returned to 1% B, followed by a further 5.8 min equilibration time. The flow was maintained at 100 µL/min and the column temperature was constant at 50°C, while samples were kept at 4°C.

Detection was employed by a high-resolution Synapt G2 HDMS mass spectrometer equipped with an electrospray ionization source operating in negative ion mode. The system was controlled using MassLynx® ver. 4.1, tuning and calibration was performed using standard solutions, leucine encephalin, and sodium formate respectively, provided by Waters Corporation (Milford, MA, USA). TargetLynx® ver. 4.1 was used for data analysis of the target compounds, and the relative ratio of each analyte's chromatographic peak area to the internal standard was calculated for subsequent analysis.

A-4 Quantitative Polymerase Chain Reaction

To determine the amounts of the total bacteria, nitrifying bacteria and denitrifying bacteria, quantitative polymerase chain reaction (*q*-PCR) assays were conducted using a CFX96TM Real-Time Detection System (Bio-RAD, California, USA). Eight independent *q*-PCR assays were conducted by quantifying total bacterial 16S rRNA gene, ammonia oxidizing bacterial *amoA* gene, the 16S rDNA genes of two nitrite-oxidizing bacteria (NOB) (*Nitrospira* spp. and *Nitrobacter* spp.), and four types of genes from the enzymes of denitrifiers including nitrate reductase (*narG* gene), nitrite reductase (*nirS* and *nirK* gene), and t nitrous oxide reductase (*nosZ* gene) (Table B2). Reaction (20 µl) were performed in 96-well microplates, where each micro-well was separately loaded with 2 µl of genomic DNA (14-26ng/µl) or plasmid standard,

followed by the forward and reverse primers (1µl), together with 10µl 2×SsoFast EvaGreen Supermix (Bio-RAD, Hercules, California) and 6 µl PCR grade sterile water.

Standard plasmids containing target genes were constructed using a TOPO TA Cloning kit (Invitrogen Corporations, Carlsbad, California). The products obtained were sequenced to confirm identity. The plasmids containing target genes were used as the *q*-PCR standard to generate a standard curve with 10-fold serial dilutions.

The total bacterial 16S rRNA gene was amplified using the primer (341f and 907r) (Muyzer et al. 1993). The *q*-PCR program was 3 min at 95 °C; 35 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C. To determine the amount of *amoA* gene, the forward primer (*amoA-1F*) targets a stretch corresponding to positions start from 332 to 349 and the reverse primer (*amoA-2R*) targets a stretch corresponding to positions start from 802 to 822 of the open reading frame published previously for the *amoA* gene sequence of *Nitrosomonas europaea* (McTavish et al. 1993). The *q*-PCR program for *AmoA* gene quantification includes 5 min at 94 °C; 42 cycles of 90 s at 60 °C, 90 s at 72 °C and 60 s at 94 °C and a final cycle consisting of 90 s at 60 °C and 10 min at 72 °C. Primers (*NSR 1113f/NSR 1264r*) (Dionisi et al., 2002) were used to determine the amount of *Nitrospira* (*NSR)* 16S rRNA gene. *q*-PCR amplification consisted of 2 min at 50°C, 10 min at 95 °C; 50 cycles of 30 s at 95 °C, 60 s at 60 °C. The *Nitrobacter* (*Nitro)* 16S rRNA gene was amplified using primers (*Nitro 1198f/Nitro 1423r*) (Graham et al., 2007). The *q*-PCR program used for amplification was 2 min at 50 °C, 10 min at 95 °C; 50 cycles of 20 s at 94 °C, 60 s at 58 °C, and 40 s at 72 °C.

Meanwhile, the denitrifying functional genes were quantified with the *narG* gene (Lo´pez-Gutie´rrez et al., 2004), *nirS* gene (Braker et al., 1998), *nirK* gene (Henry et al., 2004) and *nosZ* gene (Henry et al., 2006). The qPCR amplification consisted of 30 s at 95 °C; 35 cycles of 15 s at

95 °C, 30 s at 58 °C, and 31 s at 72 °C. The *nirS* gene qPCR amplification with primers of *nirS 1f and nirS 3r* consisted of 30 s at 95 °C; 30 cycles of 15 s at 95 °C, 20 s at 60 °C, and 31 s at 72 °C. The PCR program for *nirK* gene included 30 s at 95 °C; 30 cycles of 15 s at 95 °C, 30 s at 58 °C, and 31 s at 72 °C. The program used for amplification consisted of 30 s at 95 °C; 30 cycles of 15 s at 95 °C, 30 s at 60 °C, and 31 s at 72 °C.

All *q*-PCR were performed in triplicate per sample and all *q*-PCR runs had control reactions without the template. The gene copy numbers were calculated through a comparison of threshold cycles values obtained in each *q*-PCR run with plasmid DNA standards with known concentrations. The amplification efficiency (E) of *q*-PCR was estimated using the slope of the standard curve through the following formula: $E = (10^{-1/slope}) - 1$ (Kim, Y.M. et al. 2011). The efficiency of PCR amplification for each gene should be between 90% and 100%.

A-5 Confocal Laser Scanning Microscopy (CLSM)

Biofilms grown on the PE carriers obtained from the IFAS systems were immediately stained with SYTO9. Samples (approximately 5 x10 mm) were generated by cutting the interior of the carrier with a sterile surgical scalpel. Samples were directly stained by using LIVE/DEAD ® BacLightTM Bacterial Viability Kits. The distribution of live and dead bacteria was determined using fluorescent. With an appropriate mixture of the SYTO9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO9 stain and 490/635 nm for propidium iodide. The biofilm samples were immersed in PBS to remove nonadherent cells. Approximately 250μL of the appropriate dilution dye mixture (3 μL of the dye mixture for each mL) was applied to the biofilm samples. The samples were incubated at room temperature in the dark room for 60 min. After staining, the

samples were carefully rinsed with 0.85% NaCl three times to remove the residual dye mixture solution. Immediately after staining, biofilm samples were analyzed using a confocal laser scanning microscope (CLSM).

Biofilm samples were observed at a 20x objective under condition optimized green and red fluorescence. Three view positions were randomly chosen to evaluate each sample. The thickness of biofilm was determined by first scanning on the surface of media and marking this stage position as the origin. The stage was moved until the top surface of a cell cluster came into focus. The thickness was taken as the difference between the stage positions. The biofilm thickness was measured for three view fields.

A-6 Scanning Electron Microscopy (SEM)

Carriers were taken from each IFAS system and inner part of the carrier was cut into small flat pieces (5 x10 mm). Prior to analyze, the biofilm samples were immersed in PBS to remove nonadherent cells, and fixed by submerging the small piece in a 2.5% glutaraldehyde (w/w) solution for 2h (Ahmed Eldyasti et al., 2013). Then, the fixed samples were sequential exposed in an ethanol dehydration series (35%, 50%, 70%, 95%, 100% ethanol (EtOH(aq)) (v/v)). At each step, the biofilm samples were submerged for 10 min. Biofilm samples were stored in 100% ethanol. After critical point drying with absolute ethanol and coating with gold, each piece was observed at the different magnification ranging from 1000x to 5,000x under low vacuum mode SEM.

A-7 References

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APPENDIX B: Supporting Tables

Table B1. Characteristics of Raw and Ozonated OSPW

Parameters	Raw OSPW	Ozonated OSPW
Physicochemical property		
pH	8.28 ± 0.2	8.31 ± 0.2
Total dissolved solids (TDS), mg/L	1980 ± 2.8	2120 ± 2.5
Total suspended solids (TSS), mg/L	420.0 ± 0.2	280.0 ± 0.3
Chemical oxygen demand (COD), mg/L	176.0 ± 0.6	162.0 ± 0.8
Acid-extractable fraction (AEF), mg/L	68.8 ± 2.3	41.0 ± 2.5
Total Nitrogen (TN), mg/L	22 ± 0.8	20 ± 0.6
Total Phosphorus (TP), mg/L	0.396 ± 0.04	0.283 ± 0.03
Cations		
Sodium, mg/L	966.90 ± 7.2	971.20 ± 6.8
Magnesium, mg/L	14.10 ± 0.3	14.16 ± 0.5
Potassium, mg/L	16.13 ± 0.2	18.42 ± 0.1
Calcium, mg/L	28.66 ± 0.8	29.38 ± 0.7
Silicon, mg/L	3.37 ± 0.2	3.15 ± 0.3
Ammonium, mg/L	0.2	0.1
Typical trace metal		
Barium, µg/L	232.0 ± 9.2	199.5 ± 8.3
Arsenic, µg/L	9.7 ± 1.3	6.3 ± 1.5
Iron, µg/L	51.1 ± 4.2	152.2 ± 7.2
Chromium, µg/L	26.9 ± 2.3	13.9 ± 2.8
Rubidium, µg/L	52.3 ± 7.2	45.7 ± 6.7
Cobalt, µg/L	1.5 ± 0.2	0.7 ± 0.3
Cadmium, µg/L	0.1	0.2
Cesium, µg/L	0.3	0.1
Anions		
Chloride, mg/L	472 ± 16.8	680 ± 17.5
Nitrite, mg/L	0.003	0.006
Nitrate, mg/L	9.6 ± 0.4	10.2 ± 0.3
Sulphate, mg/L	515 ± 12.3	520 ±14.3

Table B2. Primers used in *q*-PCR

Target	Primer	Sequence (5'-3')	Reference
Bacterial 16S rRNA	*341f*	5'-CCTACGGGAGGCAGCAG-3'	Muyzer et al. 1993
	907r	5'-CCGTCAATTCCTTTRAGTTT-3'	Muyzer et al. 1995
amoA gene	*amoA-1F*	5'-GGGGTTTCTACTGGTGGT-3'	McTavish et al. 1993
	amoA-2R	5'-CCCCTCKGSAAAGCCTTCTTC-3'	McTavish et al. 1993
NSR 16S rRNA	*NSR 1113f*	5'-CCTGCTTTCAGTTGCTACCG-3'	Dionisi et al., 2002
	NSR 1264r	5'-GTTTGCAGCGCTTTGTACCG-3'	Dionisi et al., 2002
Nitro 16S rRNA	*Nitro 1198f*	5'-ACCCCTAGCAAATCTCAAAAAACCG-3'	Graham et al., 2007
	Nitro 1423r	5'-CTTCACCCCAGTCGCTGACC-3'	Graham et al., 2007
narG gene	*narG 1960m2f*	5'-TAYGTSGGGCAGGARAAACTG-3'	Lo´pez-Gutie´rrez et al., 2004
	narG 2050m2r	5'-CGTAGAAGAAGCTGGTGCTGTT-3'	Lo´pez-Gutie´rrez et al., 2004
nirS gene	*nirS 1f*	5'-TACCACCCSGARCCGCGCGT-3'	Braker et al., 1998
	nirS 3r	5'-GCCGCCGTCRTGVAGGAA-3'	Braker et al., 1998
nirK gene	*nirK 876*	5'-ATYGGCGGVCAYGGCGA-3'	Henry et al., 2004
	nirK 1040	5'-GCCTCGATCAGRTTRTGGTT-3'	Henry et al., 2004
nosZ gene	*nosZ 2f*	5'-CGCRACGGCAASAAGGTSMSSGT-3'	Henry et al., 2006
	nosZ 2r	5'-CAKRTGCAKSGCRTGGCAGAA-3'	Henry et al., 2006

APPENDIX C: Supporting Figures

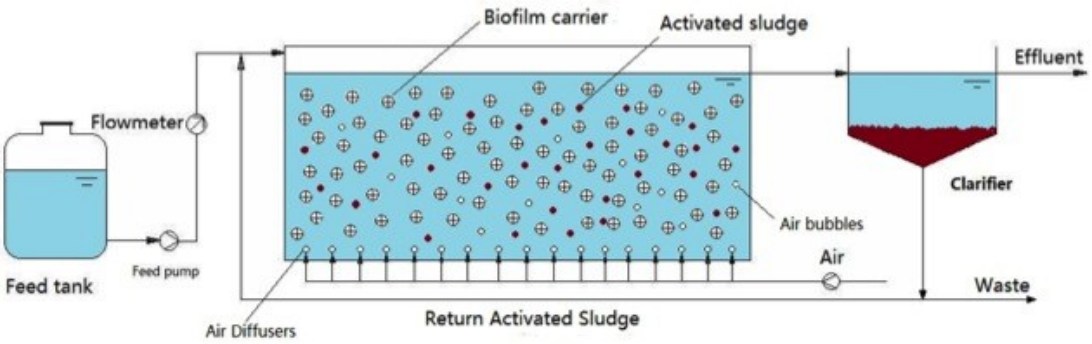


Figure C1. The schematic diagram of IFAS reactors

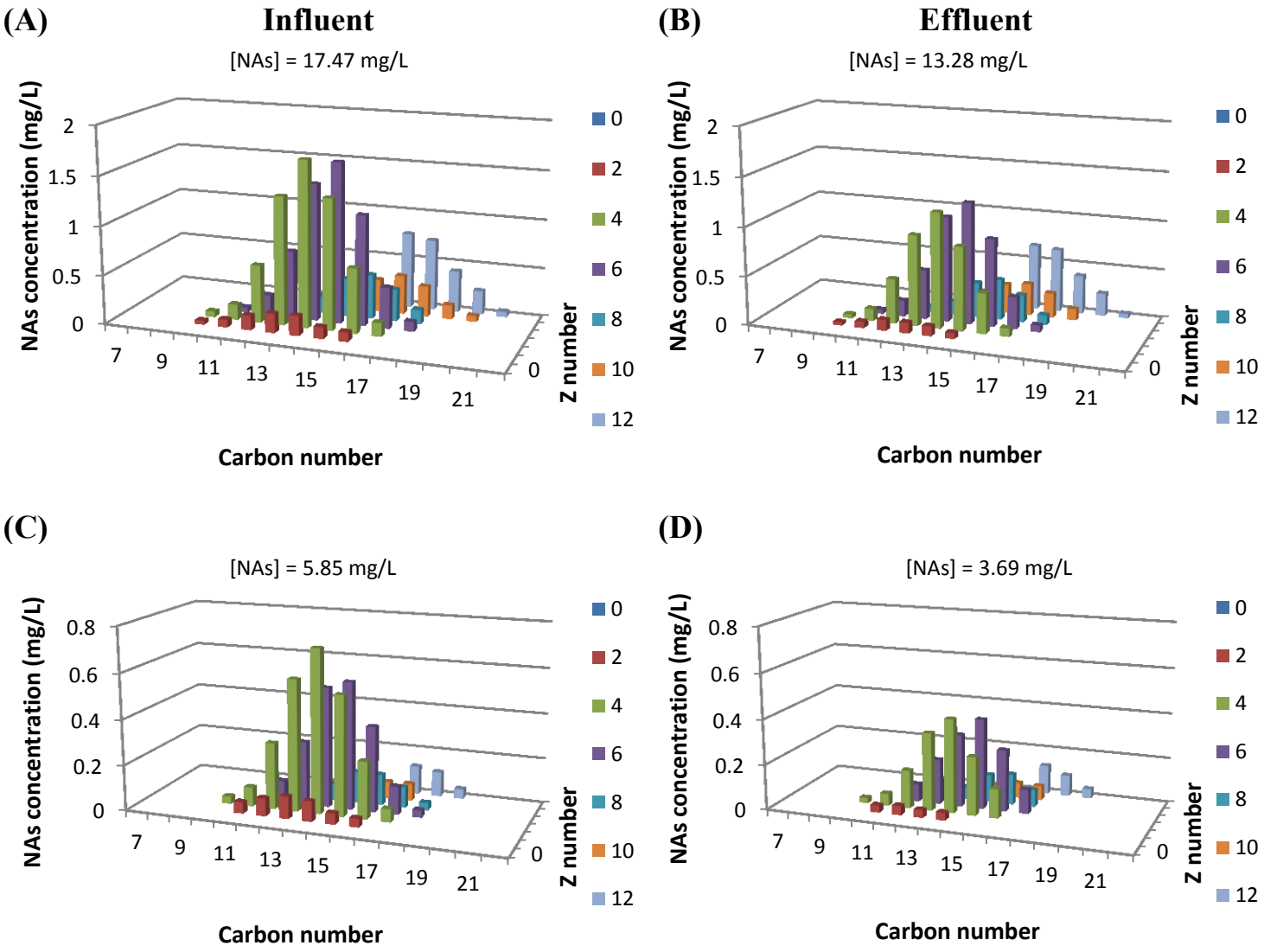


Figure C2. Three-dimensional plots of NA concentration versus carbon number (n) and Z values (A) before biodegradation in raw-OSPW IFAS (60% phase), (B) after biodegradation in raw-OSPW IFAS (60% phase), (C) before biodegradation in ozonated-OSPW IFAS (60% phase), (D) after biodegradation in ozonated-OSPW IFAS (60% phase).

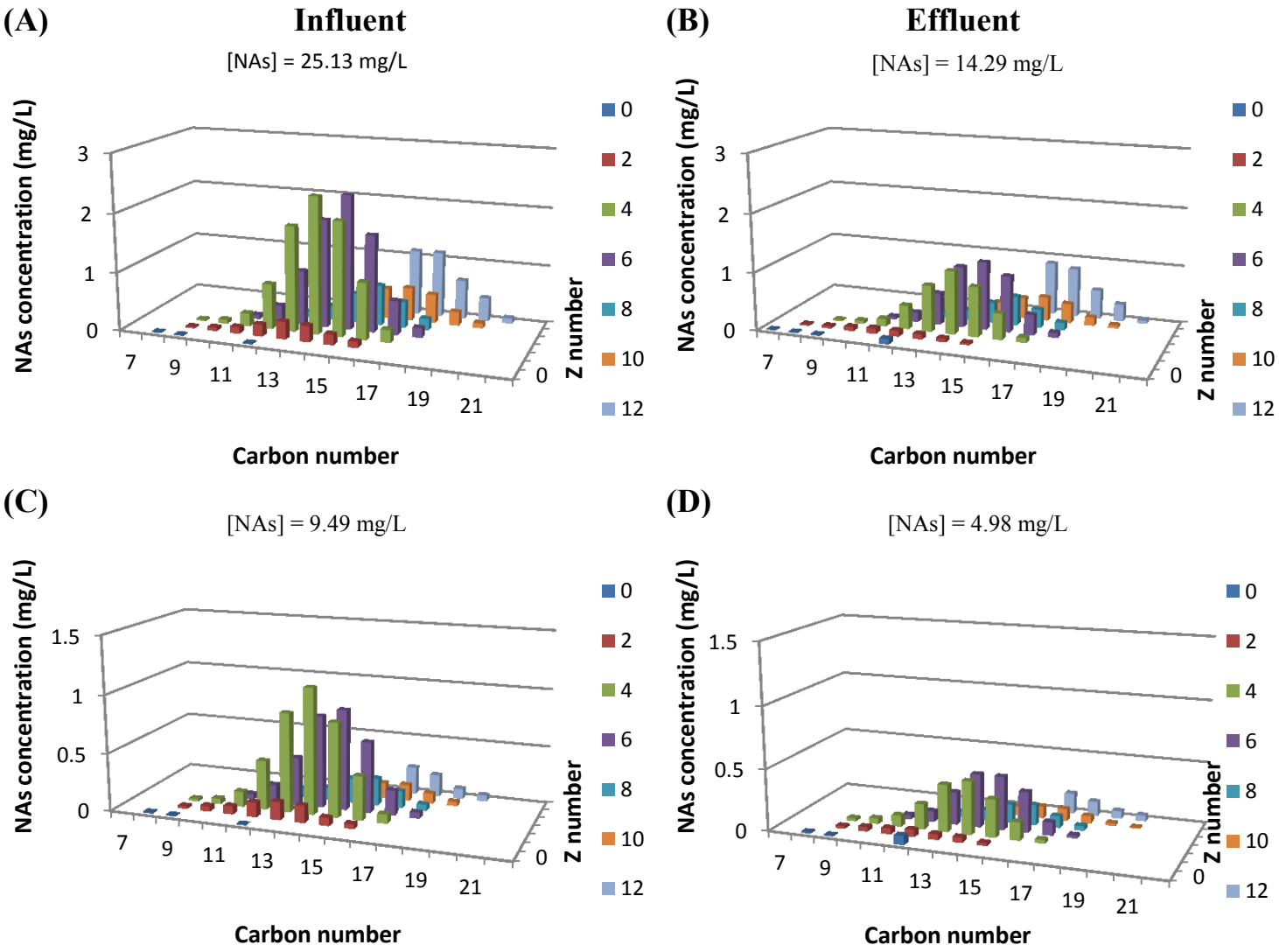
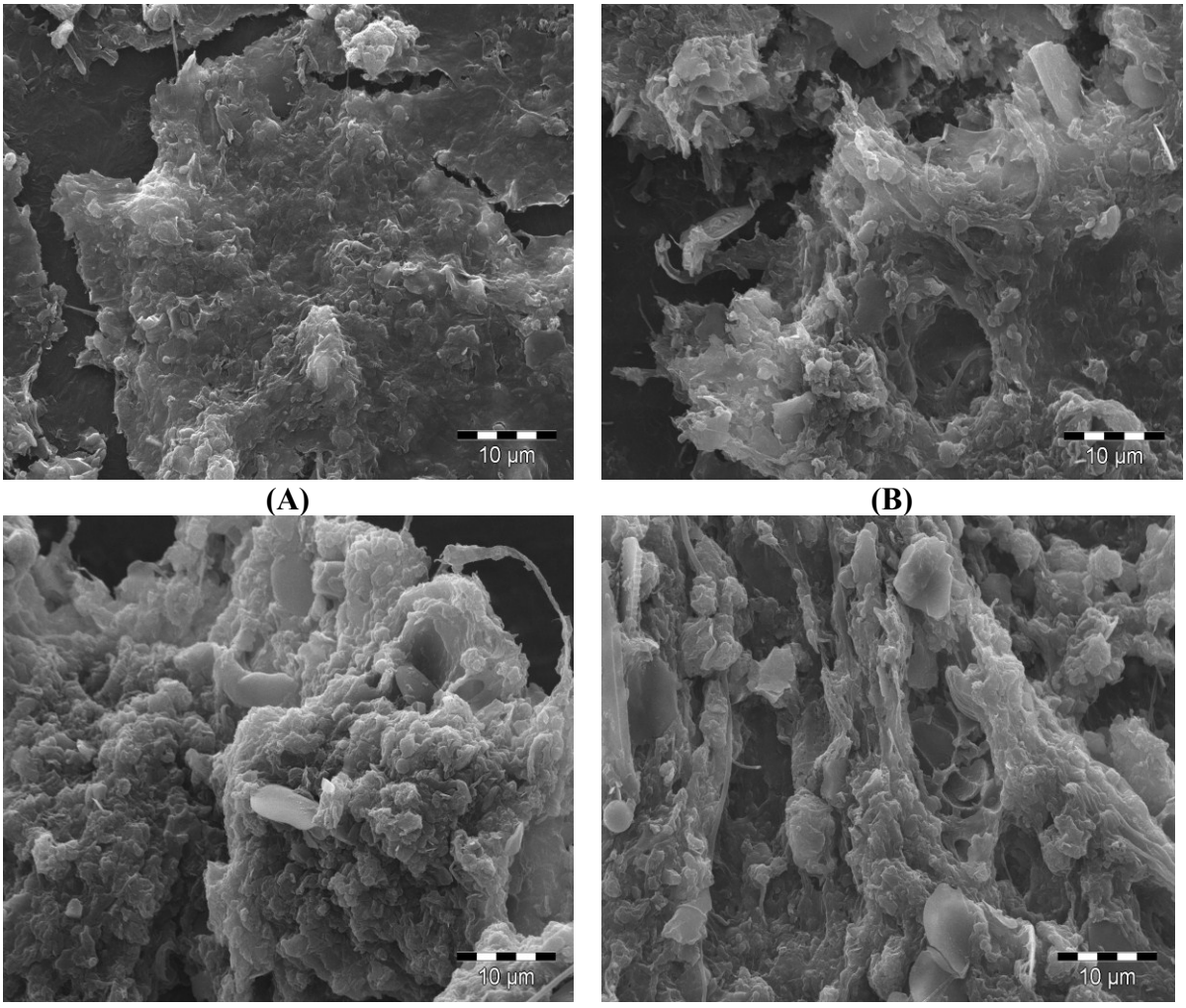


Figure C3. Three-dimensional plots of NA concentration versus carbon number (n) and Z values (A) before biodegradation in raw-OSPW IFAS (100% phase), (B) after biodegradation in raw-OSPW IFAS (100% phase), (C) before biodegradation in ozonated-OSPW IFAS (100% phase), (D) after biodegradation in ozonated-OSPW IFAS (100% phase).



(A) (B)

(C) (D)

Figure C4. SEM micrographs of biofilm formed on biocarriers during the start up operation of two IFAS systems: (A) carrier cultivated for 82 days of raw-OSPW IFAS system; (B) carrier cultivated for 130 days raw-OSPW IFAS system; (C) carrier cultivated for 82 days of ozonated-OSPW IFAS system; (D) carrier cultivated for 130 days ozonated-OSPW IFAS system.

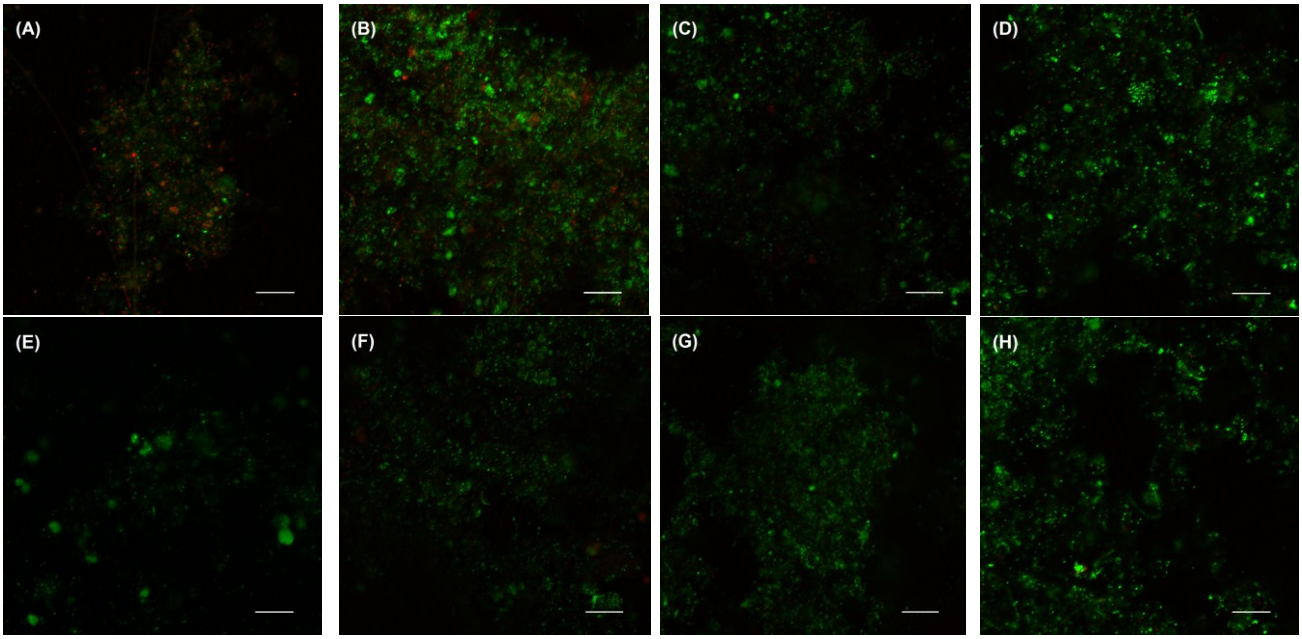
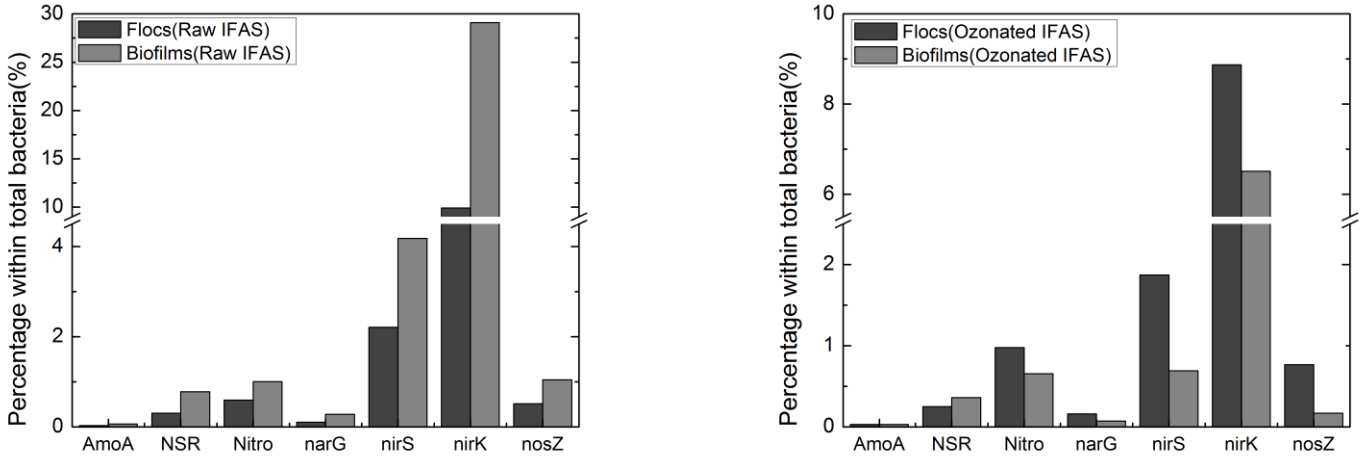


Figure C5. CLSM micrographs of biofilm formed on support media during the start up operation of two IFAS systems: (A), (B), (C) and (D) are the carrier cultivated for 42, 91, 130 and 161 days of raw-OSPW IFAS system; (E), (F), (G) and (H) are the carrier cultivated for 42, 91, 130 and 161 days of ozonated-OSPW IFAS system. Bar equal to 50μm.



(A) **(B)**

Figure C6. The abundance percentage of nitrifying genes (*amoA*, *NSR* and *Nitro*) and denitrifying genes (*narG*, *nirS*, *nirK* and *nosZ*) within total bacteria in flocs and biofilms of raw (A) and ozonated (B) IFAS systems.

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