Combined Adsorption and Biodegradation Processes for Oil Sands Process-Affected Water Treatment

by

Md. Shahinoor Islam

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ABSTRACT

The oil sands process-affected water (OSPW) generated from bitumen extraction of oil sands by industries in Northern Alberta, Canada, is a great environmental concern because of the OSPW toxicity in the environment. This toxicity has been attributed to a group of alicyclic and aliphatic compounds containing carboxyl radicals known as naphthenic acids (NAs). Thus, appropriate OSPW treatment approaches are urgently needed to permit the safe discharge of treated OSPW to the receiving environment. By realizing the above need, the current study treats oil sands process-affected water (OSPW) using engineered granular activated carbon (GAC) biofilm reactors by applying different modes of operation (continuous, semicontinuous and batch) for both raw and ozonated OSPW treatments. After 120 days of continuous operation of a GAC fluidized bed biofilm reactor (FBBR), it was shown that the GAC-FBBR process (adsorption and biodegradation) removed more than 86% of classical NAs from raw OSPW. Generally, ozonation is known to be effective in the removal of NAs from raw OSPW. Using a combined 80 mg/L utilized ozone dose followed by GAC-FBBR treatment of ozonated OSPW resulted in the removal of > 99% of classical NAs. In addition, the overall removal of the acid extractable organic-fraction (AEF) and chemical oxygen demand (COD) were 88% and 62%, respectively from this combined process. Further analysis of the microbial community using polymerase chain reaction (PCR) - denatured gradient gel electrophoresis (DGGE) from both raw and ozonated OSPW and their respective GAC-FBBR biofilms showed that there was reduced diversity of the microbial structure in the GAC biofilm with the majority of the bacteria being carbon degraders.

Further study investigated the synergistic removal of NAs (classical and oxidized) and toxicity from a simultaneous GAC adsorption and biodegradation treatment of raw and ozonated

OSPW (20 mg/L utilized ozone dose). At a GAC dose of 0.4 g GAC/L OSPW, this process removed 93% and 96% of classical NAs, and 74% and 77% of oxidized NAs from raw and ozonated OSPW, respectively. As well, this process reduced the toxicity of both raw and ozonated OSPW. There was higher removal of COD, AEF and NAs from the simultaneous GAC adsorption and biodegradation treatment of OSPW compared to the biodegradation or adsorption only treatments which indicated the enhanced bioregeneration in the GAC biofilm process. The lower utilized ozone dose used in these experiments (as compared with 80 mg/L previously) had little impact on the removal of both classical and oxidized NAs in the raw OSPW as compared to the 80 mg/L utilized ozone dose used in previous experiments.

The analysis of the microbial community using PCR-DGGE cannot reveal the comprehensive and complete characterization of microbial community in OSPW. Therefore, a high throughput 454-pyrosequencing was performed for analyzing the microbial community in the GAC biofilm, where biofilm was allowed to form in another set of experiments using a semicontinuous process (batch process with continuous change of OSPW). Frequent observations of the biofilm growth (every six days) on the GAC surface was performed using a confocal laser scanning microscope, real time PCR, and heterotrophic plate count with results showing an effective growth of biofilm. The dominant microbial composition in the GAC biofilm was *Proteobacteria* and further analysis of *Proteobacteria* revealed that the GAC biofilm was rich with carbon degrading orders of bacteria namely *Myxococcales, Pseudomonadales,* and *Burkholderiales*. The GAC biofilm microbial community was able to remove over 66% of NAs in the ozonated OSPW treatment after a short contact time. Overall the study demonstrated an effective removal of NAs (classical and oxidized) and toxicity from OSPW, which indicated that the GAC biofilm treatment approach is a promising technology for OSPW remediation.

DEDICATION

I dedicate my thesis work to my family. A special gratitude to my loving parents, Md. Abu Bakkar Biswas and Kulsum Begum, for their invaluable support throughout my life and encouraging me for higher study. This thesis work is dedicated to my wife, Sonia Ferdaus, who always supported and encouraged me to overcome all challenges during my graduate school life. And finally my elder brother, Dr. Shah Alamgir, who encouraged me to pursue higher education.

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LIST OF ABBREVIATIONS

AEF	acid extractable organic fractions
APHA	American public health association
BLAST	basic local alignment search tool
BOD	biochemical oxygen demand
CDC	centre for disease control
CLSM	confocal laser scanning microscope
COD	chemical oxygen demand
DCM	dichloremethane
DNA	deoxyribonucleic acids
DOC	dissolved organic carbon
ESI-MS	electrospray ionization-mass spectrometry
FBBR	fluidized bed biofilm reactors
FT-IR	Fourier transform infrared
FWHM	full width at half maximum
GAC	granular activated carbon
GC-MS	gas chromatography-mass spectrometry
HDMS	high definition mass spectrometry
HLR	hydraulic loading rate
HPLC	high-performance liquid chromatography
MEGA	molecular evolutionary genetics analysis
NAs	naphthenic acids
NCBI	national center for biotechnology information

OLR	organic loading rate
OSPW	oil sands process-affected water
OTU	operational taxonomic unit
PCR-DGGE	polymerase chain reaction-denatured gradient gel electrophoresis
PE	polyethylene
PVC	polyvinyl chloride
RNA	ribonucleic acids
SEM	scanning electron microscope
TDS	total dissolved solids
TS	total suspended solids
TOC	total organic carbon
UPLC-HRMS	ultrahigh pressure liquid chromatography-high resolution mass spectrometry
WIP	West in-Pit

CHAPTER 1. GENERAL INTRODUCTION AND RESEARCH OBJECTIVES

1.1. Background and Motivation

The bitumen reserves in the oil sands regions of northern Alberta, Canada, are the second largest in the world containing an estimated 1.7 trillion barrels of bitumen (Barrow et al., 2010; Martin et al., 2008). Because of rapid expansion of the oil sands industry, the bitumen production reached 1.6 million barrels per day (bpd) in 2012 (Flury et al., 2014) and the anticipated daily production is expected to grow to 3 million bpd by 2018 (Hooshiar et al., 2012). The compositions of oil sands deposits are 4–18 wt% bitumen, 55–80 wt% inorganic materials and 2–15 wt% water depending on the types of ores (Osacky et al., 2013). Currently, surface mining and steam assisted gravity drainage (SAGD) are applied commercially for the extraction of bitumen from oil sands. The surface mining method is applied to shallow oil sand ores with a hot alkaline water (50-80 ^{0}C) process being used to recover the bitumen (Holowenko et al., 2002; Rao and Liu, 2013). A high bitumen recovery approaching 90% can be achieved by using this process (Hooshiar et al., 2012). For deep deposits, an in situ SAGD technology is used with steam injected through the upper horizontal wells to reduce the viscosity of bitumen by heating the oil sands. The heated bitumen drains into lower horizontal wells where the fluid is recovered as a mixture of bitumen, water and mineral solids, called bitumen emulsion (Nguyen et al., 2013; Rao and Liu, 2013). The bitumen recovery by the SAGD technology is typically less than 60% (Hooshiar et al., 2012).

Currently, about 55% of the oil production from the Alberta oil sands is through surface mining operations (Alberta Energy, 2010). The surface mining extraction process is a water intensive process, however, 80-85% of the required water is recycled from the generated oil

sands process-affected waters (OSPW) (Allen, 2008). Despite the high reuse, approximately 3 to 4 barrels of fresh river water is still required per barrel of bitumen production (Barrow et al., 2010; Scott et al., 2008). Thus, each m^3 of bitumen extracted using this process produces approximately 4 m^3 of oil sands tailings water slurries (Holowenko et al., 2002; Scott et al., 2008). Oil sands companies do not currently release the generated OSPW into the receiving environment (Clemente and Fedorak, 2005). Instead, OSPW is transported to constructed tailings ponds for long-term storage (Holowenko et al., 2002; Pereira et al., 2013b). Thus, tailings ponds are growing in volume and in number, and in 2011 covered approximately 170 km^2 despite the heavy recycling of OSPW for reuse in the extraction process (Pereira et al., 2013a). The current accumulated amounts of OSPW are estimated to be more than 1 billion m^3 (Del Rio et al., 2006; Martin et al., 2008; Wiseman et al., 2013).

As the oil sands mining production increases in volume, more water will be needed to be taken as fresh water intake from the Athabasca River which can negatively impact the aquatic environment. Oil sands projects are approved to withdraw < 3% (359 million m^3) of the average annual water flow of the Athabasca River (Hooshiar et al., 2012). Although less than 2% of the annual river flow is currently taken from the Athabasca River, it is of concern whether the low winter flow will be adequate enough to supply the required water in the future (Allen, 2008; Grant et al., 2009). During winter time, the average water flow is 10 times less than spring or summer flow, thus oil sands projects withdraw a significantly greater proportion of the Athabasca River's flow at a time, which makes limiting conditions for fish and other aquatic organisms (Grant et al., 2009; Hooshiar et al., 2012). Thus, specifically in the winter season, the government put additional limits on the water withdrawal because of the lower water flow rate. The typical withdrawal limit in the winter season for oil sands mining is capped around 10% of

the natural water flow, therefore this limitation may eventually impact the further increase of oil sands production (Allen, 2008).

The generated tailings slurries are composed of OSPW, unrecovered bitumen, sands, silts, clays, heavy metals, organic, and inorganic compounds (Allen, 2008; Holowenko et al., 2002; Wiseman et al., 2013). The tailings compositions depend on the source of the ore, the extraction and upgrading processes, and its age. Generally, the slurries are around 70 to 80% (by weight) of water, 20 to 30% of solids (i.e., sand, silt and clays) and 1–3% of residual bitumen (Allen, 2008; Kannel and Gan, 2012). Once delivered to the tailings ponds, the slurries settle into 3 layers: (i) the bottom layer includes rapidly settled particles such as sand (ii) the middle layer is an aqueous suspension with fine particles including silt and clay; and (iii) the clarified top surface water layer contains total suspended solids of 15–70 mg/L and residual bitumen (ppm level) and is the source of the recycle water (OSPW). The fine silt and clay fractions in the middle layer take several years of settling to form matured fine tailings (30-35% solids, ~3% bitumen and water), while settling they release additional OSPW to the top surface waters (Allen, 2008; Han et al., 2009). In the future, these tailings ponds need to be remediated according to lease agreements with the Alberta Government. The oil sands industries have to reclaim their tailings ponds after closing of operations for restoring them into sustainable aquatic and terrestrial ecosystems (Allen, 2008; Clemente and Fedorak, 2005). Given this need for extensive remediation of the oil sands landscape, appropriate OSPW treatment approaches are urgently needed to extend OSPW recycling, to reduce the need for raw water withdrawal from the Athabasca River and to permit the safe discharge of treated OSPW to the receiving environment.

1.2. Scope of Research and Objectives

The majority of the studies have been carried out using the planktonic biodegradation technique for commercial NAs and OSPW NAs. Commercial NAs was faster biodegraded in aerobic cultures compared to OSPW NAs (Biryukova et al., 2007; Han et al., 2008; Scott et al., 2005; Smith et al., 2008). Literature reported that biofilm reactor have higher potential for the removal of organics compared to planktonic biodegradation. However, to date, only three studies have been carried out on the application of biofilm reactors for the treatment of OSPW. None of these studies have considered the use of a support media (i.e., GAC) despite the potential benefits of using GAC biofilm reactors for the treatment of drinking and industrial wastewaters (Baban et al., 2010; Liao et al., 2013). Thus, there is a significant research gap with respect to the investigation of the applicability and effectiveness of a GAC-biofilm reactor treatment technology for OSPW remediation. The combination of ozonation followed by a GAC-biofilm reactor may enhance the overall degradation and mineralization of NAs as well as other organic compounds.

Based on the above hypothesis, the overall objective of this Ph.D. research was to investigate the feasibility of the engineered GAC-biofilm reactors for OSPW treatment. To achieve the overall objective, the research was carried out in different phases through the application of GAC-biofilm reactors using continuous flow, batch, and semicontinuous reactors for the treatment of raw and ozonated OSPW. The reactors performance was evaluated by measuring the organic compounds (COD, AEF and NAs) removal from the treatment and the impact of ozonation on the performance of the reactors was assessed.

In phase I (Chapter 3), continuous GAC-FBBR was applied for the treatment of raw OSPW. The main objective of this phase of study was to investigate the effectiveness of the

GAC-FBBR treatment for the combined (adsorption and biodegradation) removal of the organic compounds from OSPW by measuring COD, AEF and NAs from OSPW under different operating conditions (i.e., various hydraulic loading rates (HLR) and organic loading rates (OLR) of the reactor). The impact of microbial community in the GAC-biofilm treatment was also investigated.

In phase II (Chapter 4), continuous GAC-FBBR was applied for the treatment of ozonated OSPW. The research objective from this phase was to investigate the impact of ozonation of raw OSPW on the organic compound removal and microbial community structure dynamics in GAC-FBBR.

In phase III (Chapter 5), GAC was applied for the treatment of raw and ozonated OSPW with and without the application of endogenous bacteria using batch treatments. The principle objective of this study was to investigate the role of adsorption (GAC), biodegradation, and the synergetic effect of a combined system on the removal of organics (COD, AEF and NAs) in raw and ozonated OSPW. The impact of NAs chemical structure such as carbon number, cyclicity and oxygen number on the performance of GAC adsorption, biodegradation and simultaneous GAC adsorption and biodegradation treatments for both raw and ozonated OSPW was also investigated.

In phase IV (Chapter 6), a semicontinuous process was applied in a similar manner as the batch mode with GAC and endogenous bacteria (phase III), with the OSPW changed once every second day to help promote biofilm growth on the GAC surface. The main objective from this study was to investigate the biofilm growth affinity on the GAC surface, and to investigate the impact of microbial community (using 454 pyrosequencing analyses) in the GAC biofilm in raw and ozonated OSPW treatment.

By applying a GAC-biofilm treatment process using different modes of operation of biofilm reactors and by achieving the above research objectives, the study will provide a unique opportunity for the potential treatment of the high volume of OSPW generated by different oil sands industry currently. The study will present the impact of ozonation on the biodegradability of OSPW compounds prior to biofilm reactors. The study will provide information regarding the removal of oxidized NAs from OSPW, which was not feasible using the individual ozonation and biodegradation techniques alone. Moreover, the study will address the effective toxicity removal from OSPW via the Microtox toxicity bioassay. Finally, the study will provide insightful information on the GAC biofilm by providing information regarding the biofilm growth capability and bacterial population density on the GAC surface, microbial community structure in the biofilm and their function in the OSPW treatment.

1.3. Thesis Organization

The thesis consists of seven chapters. Chapter 1 contains a general overview of the oil sands bitumen extraction processes and scope of research and objectives. In Chapter 2, the OSPW characteristics and its potential environmental impacts, and the applied treatment processes for OSPW treatment has been presented. The currently considered processes for OSPW treatment, the experimental methodologies and the results and discussion of the current treatment processes are presented in Chapters 3-6.

The performance of the GAC-FBBR in the treatment of raw OSPW was evaluated at different operating conditions by measuring the COD, AEF and NAs as presented in Chapter 3. The system parameters, such as the HLR and OLR, were varied to assess the FBBR performance. The impact of GAC biofilm treatment on the degradation of NAs based on carbon

and Z numbers from the reactor was also analyzed. The biofilm adhesion on the GAC media was visualized by confocal and scanning electron microscope imaging. The biofilm was characterized by measuring the biofilm thickness, dry biomass concentration and identifying the microbial community structure using the polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE) method.

Chapter 4 includes an analysis of a combined ozonation and GAC-FBBR treatment for OSPW. The impact of ozonation on the biodegradability of OSPW was assessed by measuring BOD_5 before and after ozonation. An optimum ozone dose (based on BOD_5 increment) was selected for the treatment of OSPW at different operating conditions (varying HRT and OLR) by measuring the COD, AEF and NAs. The biofilm adhesion and development on the GAC media was characterized by confocal and scanning electron microscope imaging, the biofilm thickness, the microbial density and by identifying the microbial community structure using the PCR-DGGE method.

Chapter 5 includes methodologies and results used for the investigation of the batch treatment of raw and ozonated OSPW using GAC. The batch experiments were carried out in the following modes of operation: biodegradation only, adsorption only and simultaneous adsorption and biodegradation. Through comparison of COD, AEF and NAs removals, the impact of the combined treatment of the OSPW was determined. The impacts of carbon and Z numbers in NAs structure on the performance of all treatment processes have been presented. The biofilm adhesion on the GAC media was visualized using CLSM and the bacteria quantity in the biofilm per gram of GAC was determined using qPCR. The toxicity of both raw and ozonated OSPW was assessed by Microtox toxicity.

Chapter 6 represents the experimental methodologies and results used for the investigation for biofilm characterization and organics (COD, AEF and NAs) removal in raw and ozonated OSPW using a semicontinuous process. The biofilm characterization was performed by CLSM, plate counting, qPCR and pyrosequencing analysis of extracted DNA based on16sRNA. Detailed discussion of the microbial community structure obtained in biofilms of raw and ozonated OSPW treatments has been provided in Chapter 6. The impact of biofilm on the treatment is discussed according to the functional metabolisms of different bacteria found in the microbial community developed on the GAC.

General conclusions and recommendations for future work are presented in Chapter 7. The Appendix contains experimental methodologies, figures and tables.

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CHAPTER 2. LITERATURE REVIEW

2.1. OSPW Characteristics

2.1.1. Inorganic Compounds

OSPW contains higher concentrations of inorganic contaminants compared to the natural surface waters of the Athabasca region (Allen, 2008a; Zubot et al., 2012). It is categorized as brackish water because of high total dissolved solids (TDS) concentrations in the range of 1800 to 2800 mg/L (Allen, 2008a; Mah and Kotecha, 2011) and is slightly alkaline with pH range 7.8-8.7 (Gamal El-Din et al., 2011; Quagraine et al., 2005). Among the dissolved solids present in OSPW, the major components are bicarbonate 700-1000 mg/L, sodium 500-890 mg/L, sulfate 200-310 mg/L, and chloride 75-590 mg/L (Allen, 2008a; Choi et al., 2014; Mah and Kotecha, 2011). OSPW has very high conductivity ranging from 3150 to 3750 mS/cm (Gamal El-Din et al., 2011; Pourrezaei et al., 2011; Zubot et al., 2012). In addition, OSPW has ammonia concentrations in the range of 4.4-22 mg/L (Choi et al., 2014; Pourrezaei et al., 2011). Many of the trace metals concentrations such as aluminum, nickel, arsenic, copper, zinc, and chromium in OSPW are in exceedance of the levels in the Athabasca River (Allen, 2008a; Baker et al., 2012).

2.1.2. Organic Compounds

In addition to inorganic compounds, various types of organic compounds are detected in OSPW including residual bitumen (measured as oil and grease), polyaromatic hydrocarbons (PAHs), benzene, toluene, ethylbenzene, and xylenes (BTEX), phenol and naphthenic acids (NAs) (Allen, 2008a). A wide range of bitumen concentrations (5-145 mg/L) have been detected in OSPW (Mah and Kotecha, 2011). The total PAHs concentrations in OSPW are below 0.01

mg/L (Allen, 2008a; Rogers et al., 2002b).The BTEX and phenol content in the tailings ponds water are 0.01- 4.3 mg/L and 3.3 - 5.4 mg/L, respectively (Allen, 2008a; Mah and Kotecha, 2011).

Overall, the NAs account for around 50% of the total organics present in the OSPW (Garcia-Garcia et al., 2011a) and it is believed that majority of the toxicity in OSPW is associated with NAs (Madill et al., 2001). Moreover, NAs are the most biopersistent compounds found in the OSPW. Thus, the remediation of the NAs present in OSPW is a major concern due to their high concentrations, biopersistence and toxicity to aquatic organisms. Previously, the acid extractable organic fraction (AEF) has been used by the oil sands industries as an equivalent measure to the NAs concentration. The use of AEF as equivalent to NAs concentrations leads to the overestimation of the NAs concentrations since the AEF includes non-NAs such as organic compounds having –COOH, -CHO and >CO- groups. Recent studies reported that the actual NAs concentrations in OSPW are in the range of 7.1-47 mg/L (Grewer et al., 2010), whereas the AEF concentration in the OSPW is in the range of 40-120 mg/L (Holowenko et al., 2000; Holowenko et al., 2001; Holowenko et al., 2002). The concentrations of NAs, AEF and other parameters such as dissolved organic carbon (DOC), chemical oxygen demand (COD) and biochemical oxygen demand (BOD) are higher in OSPW than in the Athabasca River (Grewer et al., 2010; Zubot et al., 2012). Historically, the parameters including COD and BOD have been used as guidelines for the assessment of water quality in the treatment and disposal of water and wastewaters (i.e., industrial and municipal sources). However, there are no specific guidelines currently in use for the maximum concentrations of NAs, DOC, COD and BOD in OSPW effluents for their safe release into the receiving environments.

2.2. Naphthenic Acids

NAs are natural constituents of bitumen, and are produced from incomplete microbial degradation of petroleum hydrocarbons (Headley and McMartin, 2004; Whitby, 2010). It has been established that the NAs are a complex mixture of aliphatic acyclic, cyclic, or polycyclic alkyl-substituted carboxylic acids and there are potentially more than 200,000 individual NA structures associated with the oil sands (Rowland et al., 2011). The carboxylic acids group (-COOH) is usually attached to a side alkyl chain rather than directly to the aromatic rings. The general chemical formula of the NAs is $C_nH_{2n+Z}O_x$, where n is the number of carbon atoms which may vary from 7 to 30, Z is either zero or a negative even integer representing the number of hydrogen atoms lost because of ring formation which range from 0 to -12, and x represents the number of oxygen atoms ($x = 2$ for classical NAs and $x \geq 3$ for oxidized NAs) (Barrow et al., 2010; Grewer et al., 2010; Wang, 2013). Approximately, 200 mg of NAs are present in 1 kg of oil sands ore (Clemente et al., 2004) and 15% of these acids are partitioned into the OSPW during extraction (Scott et al., 2008). Figure 2.1 shows the general NAs structures.

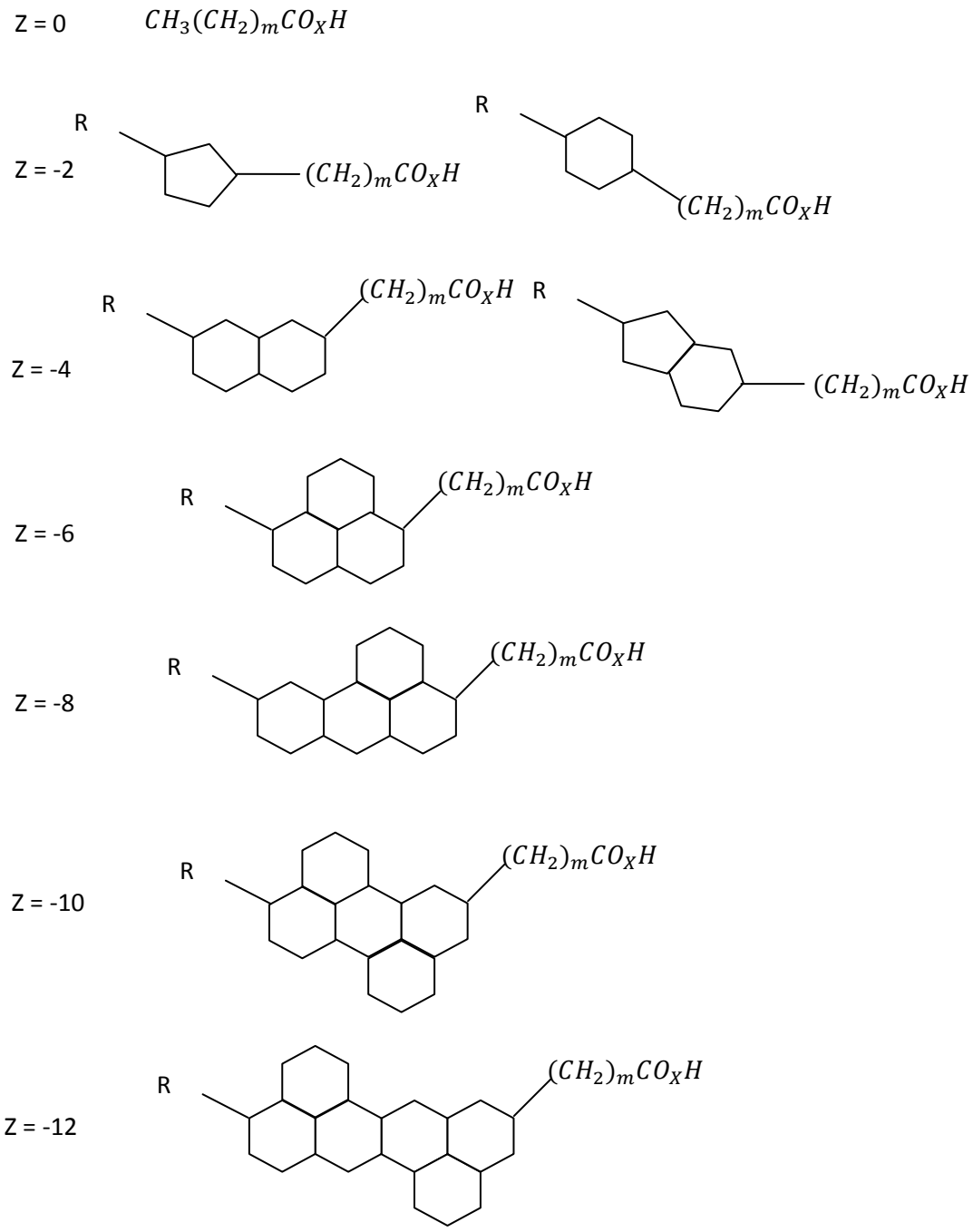

Figure 2.1. General NAs structures for various *z*-families. R represents an alkyl group and *m* represents the length of the alkyl chain, x represents number of oxygen atoms (x = 2 for classical NAs and x ≥ 3 for oxidized NAs).

2.2.1. Analytical Techniques of Naphthenic Acids Analysis

OSPW is a complex mixture that is analytically challenging for accurate quantification, identification of structures and characterization of NAs. To date, a number of analytical techniques have been developed for quantification of NAs, with some methods used to quantify the total NAs concentrations from the aqueous phase, while others can reveal information in regards to structural composition based on carbon and Z numbers which may be useful for further characterization (Zhao et al., 2012).

Fourier transform infrared (FT-IR) spectroscopy has been used in the earliest stages of method development for NAs quantification. However, the method determines the total AEF which includes all -CO, -CHO and -COOH groups, thus the method overestimates the NAs concentrations (Grewer et al., 2010). In this method, the OSPW organic fraction is extracted and concentrated using HPLC grade dichloromethane (DCM) and analyzed by measuring the absorbance of the monomeric and dimeric form of the carboxylic groups at 1743 and 1706 cm^{-1}, respectively (Gamal El-Din et al., 2011).

Targeted separation of the NAs compounds has been established by applying comprehensive gas chromatography (GC) and liquid chromatography (LC) techniques. Both GC and LC are currently used to separate the NAs into isomer groups and used in combination with various detection methods for qualitative, semi-quantitative and quantitative assessments. Characterization of the NAs components by a relative response of each mass (i.e., m/z value) corresponding to a particular combination of n and Z is possible by applying mass spectrometry (MS) in NAs samples (Martin et al., 2008). Recently, some other detection methods coupled with an MS have been applied to increase analytical sensitivity, to enhance the accuracy of determination of NAs, to facilitate the partial separation of NAs components into isomer groups

and to reduce the sample matrix effects (Zhao et al., 2012). These MS analytical techniques include ionization couple with unit mass resolution and high resolution methods that have been utilized for the analysis of NAs in aqueous solutions (Zhao et al., 2012). The major ionization techniques are electron ionization (EI) (Holowenko et al., 2002), chemical ionization (CI) (Lu et al., 2004), atmospheric pressure chemical ionization (APCI)- (Lo et al., 2006), electrospray ionization (ESI) (Rogers et al., 2002a), atmospheric pressure photoionization (APPI) (Grewer et al., 2010), and fast atom bombardment (FAB) (Fan, 1991). In unit mass resolution, a quadruple (Q) is used as a selective mass detector since it allows only one mass at a time to reach the detector. The mass that has a stable trajectory between four rods in the mass analyzer will reach the detector. The lower and higher mass organics/particles, other than the selected mass particle, will have unstable trajectories and will filter out from the detector (Zhao et al., 2012). Currently used unit mass resolution techniques are GC-MS of N-methyl-N-(*t*-butyldimethylsilyl)-trifluoroacetamide MTBSTFA derivatized NAs (Clemente and Fedorak 2004, Merlin et al. 2007, Young et al. 2010) and high performance liquid chromatography (HPLC)-MS/MS (Wang and Kasperski, 2010).

Recent technological advances allow for coupling LC and GC with high-resolution mass spectrometer (HRMS) or tandem mass spectrometer (i.e., quadruple time of flight MS) for compositional analyses of NAs (Zhao et al., 2012). The recent applications of these techniques include HPLC-HRMS (Bataineh et al., 2006; Han et al., 2009), two-dimensional gas chromatography/time of flight mass spectrometry (GC x GC/TOF-MS) (West et al., 2013), liquid chromatography coupled to a dual pressure linear ion trap Orbitrap MS (Headley 2011), high performance liquid chromatography- quadruple time of flight- mass spectrometry (HPLC-QTOF-MS) (Frank et al., 2006; Hao et al., 2005) and ultrahigh performance liquid

chromatography- quadruple time of flight- high resolution mass spectrometry (UPLC-QTOF-HRMS) (Wang et al., 2013).

2.3. Toxicity of OSPW

Fresh OSPW has been shown to have acute, sub-chronic and chronic toxicity to a variety of aquatic organisms (Herman et al., 1994; Toor et al., 2013). Previous toxicological investigations suggested that OSPW is toxic to zooplankton (Frank et al., 2009), phytoplankton (Leung et al., 2003), invertebrates (Anderson et al., 2012), fish (Hagen et al., 2014; Peters et al., 2007), mammals (Garcia-Garcia et al., 2011a; Rogers et al., 2002b), birds (Gentes et al., 2006), bacteria (Frank et al., 2009) and plants (Armstrong et al., 2010).

The majority of OSPW toxicity has been attributed to NAs (Frank et al., 2008; Goff et al., 2013) and the degree of toxicity depends on the target species, molecular structures, concentrations and composition of NAs (Frank et al., 2008; Headley and McMartin, 2004). Previously it has been reported that extracted fractions of OSPW NAs with low molecular weight showed higher toxicity compared with the high molecular fractions (Clemente and Fedorak, 2005; Frank et al., 2008). For instance, Frank et al., (2009) reported that the effective concentration (EC_{50}; toxicity increases with decreasing EC_{50}) of *V. fischeri* bioluminescence bacteria (via Microtox assay) was 41.9 ± 2.8 mg/L for lower molecular weight NAs and 64.9 ± 7.4 mg/L for higher molecular weight NAs. In contrast, recent work by Jones et al. (2011) reported that NAs toxicity is associated with hydrophobicity with lower carbon number NAs (less hydrophobic) being less toxic to *V. fischeri* than NAs with higher carbon numbers (more hydrophobic) (Frank et al., 2009; Jones et al., 2011). OSPW NAs have endocrine-disrupting effects to fish with exposures decreasing concentrations of testosterone (T) and estradiol (E2) in

the plasma of yellow perch (*Perca flavescens*) (van den Heuvel et al., 1999) and goldfish (*Carassius auratus*) (He et al., 2012) and causing decreased synthesis of T and E2 by explants of ovarian and testicular tissue from goldfish (Lister et al., 2008). Significant acute hepatoxic effects were observed during the exposure of rats to OSPW with brain hemorrhage in high-dosed males, and cardiac periarteriolar necrosis and fibrosis in female rats (Rogers et al., 2002b). Moreover, excessive liver weight, blood amylase, and hepatic glycogen accumulation were observed as chronic effects (Rogers et al., 2002b). The organic fraction of OSPW has been shown to have immunotoxic properties in vitro and in vivo, affecting various macrophage microbicide functions and immune gene expression in different organs (Garcia-Garcia et al., 2012; Garcia-Garcia et al., 2011b).

2.4. OSPW NAs Remediation Technology

Oil sands process industries are operated under a no-release policy for OSPW, which leads to a continually increasing volume of the OSPW inventory in tailings ponds (Allen, 2008a; Pereira et al., 2013). The quality of OSPW in operational tailings ponds continually deteriorates due to the increasing concentrations of inorganic and organic constituents created through OSPW recycle. Considering the reclamation or environmental discharge of OSPW, the contaminants of major concern include NAs, sulphate, chloride, ammonia, bitumen, aromatic hydrocarbons, and trace metals (Allen, 2008a; Quagraine et al., 2005). Among these chemicals, NAs pose the greatest risk to aquatic life as outlined previously. Generally, to achieve the sustainability of the oil sands operations, appropriate OSPW treatment technologies are urgently needed to decrease its toxicity, to facilitate its reclamation for safe release into the receiving environment and to reduce the need for fresh water from the Athabasca River (Allen, 2008a; Alpatova et al., 2014).

Research on the effective treatment of OSPW has been done using processes including chemical (oxidation) (Pereira et al., 2013; Scott et al., 2008; Wang, 2013), physical (adsorption and membranes) (Gamal El-Din et al., 2011; Kim et al., 2013; Mohamed et al., 2011), physicochemical (coagulation-flocculation-sedimentation) (Pourrezaei et al., 2011), constructed wetlands (Allen, 2008b; Quagraine et al., 2005), and biological (suspended and attached) processes (Han et al., 2008; Hwang et al., 2013). Investigations of these processes have been and are currently being carried out in order to develop an efficient performance and cost effective strategy for OSPW remediation (Alpatova et al., 2014). However, there is a significant research gap considering the effectiveness and applicability of the proposed treatment technologies for the OSPW remediation as they are scaled up to the industrial scale.

2.4.1. Physical and Chemical Process

Coagulation/flocculation/sedimentation (CFS) has been a common pretreatment process in municipal and industrial wastewaters for the removal of suspended solids, colloidal particles and insoluble hydrocarbons (Pourrezaei et al., 2011; Verrna et al., 2010). It has been an effective treatment process for petroleum refining wastewaters in large-scale applications (Santo et al., 2012; Verrna et al., 2010). Recently, Pourrezaei et al. (2011) applied an enhanced coagulation technique using chemical alum and polymer to increase the rate of flocculation and sedimentation for the treatment of OSPW. After this CFS treatment, NAs and oxidized NAs were removed by 37% and 86%, respectively. As well, CFS is a useful pretreatment process for removal of suspended solids and insoluble hydrocarbons given their potential to negatively impact other treatment processes including membrane filtration, biological treatment, and ultraviolet light (UV)-based advanced oxidation processes (Demirci et al., 1998; Santo et al.,

2012). For example, it was reported that the addition of coagulant and polymer enhanced membrane permeability by decreasing the binding of flocs on the membrane surface from alteration of membrane physicochemical properties (surface hydrophilicity, zeta potential, and morphology) (Kim et al., 2011).

For more than two decades, synthetic polymer and ceramic membranes have been used for the treatment of water and wastewater to remove oil, suspended solids, and other pollutants (Allen, 2008b). For OSPW, the use of membrane separation can offer both economic and technical benefits to meet water quality requirements for release to receiving waters (Alpatova et al., 2013). Although successful removal of organics and inorganics have been achieved with the application of membrane, nanofiltration (NF) and reverse osmosis (RO), they also have limitations. For example, severe fouling of polymeric membranes and rapid decrease in permeate flux was observed due to high concentration of solids, organic compounds and ionic species found in OSPW (Kim et al., 2011, 2012; Peng et al., 2004). Concentration polarization, cake layer formation, pore blocking and adsorption contributed to the membrane fouling (Alpatova et al., 2014). In addition, it was reported that chemical cleaning of polymeric NF and RO membranes was not capable of restoring the initial permeate flux (Kim et al., 2012).

Granular activated carbon (GAC) adsorption is one of the most effective methods for removing contaminants from water and wastewater, including even the most recalcitrant organics (Simpson, 2008). However, few studies have investigated the effectiveness of adsorption of NAs using various media for the treatment of OSPW. These studies include the use of organic rich soil (Janfada et al., 2006), raw petroleum coke (Gamal El-Din et al., 2011; Zubot et al., 2012), activated petroleum coke (Small et al., 2012), cyclodextrin-based polymers (Mohamed et al., 2011) and activated carbon (Mohamed et al., 2011). GAC has a very high adsorption capacity for

NAs as compared to the other media due to its large specific surface area and well-developed porous structure (Putz et al., 2005; Simpson, 2008). Previously, the enhanced adsorption of carboxylic acid based surfactants was observed on the GAC surface (Wu and Pendleton, 2001). However, the GAC eventually became saturated with organic matter and regeneration was needed to allow for further use of the GAC (Aktas and Cecen, 2007b; Gibert et al., 2013). The adsorption characteristics and performance of GAC for the degradation of the NAs found in OSPW has not yet been investigated.

Advanced oxidation processes (AOPs) are widely recognized as efficient processes for recalcitrant wastewater treatment (Mantzavinos and Psillakis, 2004). Ozonation has been utilized for the treatment of OSPW with results indicating increased biodegradability (Martin et al., 2010), reduced toxicity (Garcia-Garcia et al., 2011a; He et al., 2011), and greater oxidation of the more persistent fraction of NAs (Perez-Estrada et al., 2011). However, the rate of increase of biodegradability of OSPW is not substantial from ozonation unless applied at unrealistically high doses that are not economically feasible (Scott et al., 2008; Wang, 2013). Scott et al. (2008) found that OSPW did not reach a highly biodegradable region, as defined by a BOD/COD > 0.3, even after more than 95% NAs had been removed and after production of a non-toxic effluent based on Microtox bioassay results (Scott et al., 2008). However, after ozonation the BOD_5 was increased from 2 mg/L to 15 mg/L which indicates a general increase in biodegradability (Scott et al., 2008). Despite the benefits of degradation, the ozonation of NAs in OSPW can also lead to the formation of several by-products such as aldehydes, ketones and peroxides and other carboxylic constituents (Kannel and Gan, 2012; Pereira et al., 2013). Moreover, some of these by-products may even be more toxic than the original NAs components in OSPW (Kannel and Gan, 2012). In addition, the use of these chemical oxidation processes for the complete

mineralization of organics is expensive (Marco et al., 1997). Thus, other treatment options need to be investigated for achieving the complete mineralization of NAs and other organic compounds in OSPW.

2.4.2. Constructed Wetlands

In a recent review, Allen et al. (2008b) reported that constructed wetlands have been utilized successfully for industrial wastewaters treatment including oil fields produced water, contaminated groundwater, and refinery and petrochemical plant wastewaters. Regarding OSPW treatment, dry and wet landscape techniques have been considered by oil companies for bioremediation and reclamation of OSPW (Allen, 2008b). In the dry landscape approach, the water content in the pores of the fine tailings is reduced (i.e., consolidated tailings), leaving a solid deposit and thus, the process has a capability to be reclaimed as either a land surface or a wetland. The major processes available to produce consolidated tailings are evaporation and freeze-thaw, and the addition of calcium sulfate (Quagraine et al., 2005). However, there may be an issue with the NAs contaminated OSPW draining or leaching from the reclamation area (Quagraine et al., 2005).

In the wet landscape approach, the fine tailings are transferred into an abandoned mined out pit, and a fresh water layer is established over the fine tailings base. It has been reported that the wet landscape treatment approach was used to successfully reduce the suspended solids, organic carbon and nitrogen compounds, aromatic compounds, and trace metals from other wastewaters (Allen, 2008b). However, the application of this process for oil sands fine tailings was not considered as environmentally viable because of the extremely slow settling rate of fine particles in the tailings (decades to centuries) (Madill et al., 2001; Quagraine et al., 2005).

However, this process releases biopersistent and toxic contaminants, including the NAs, to the water layer from the pores of fine tailings, and through the biodegradation of residual bitumen which may complicate the remediation process (Madill et al., 2001; Quagraine et al., 2005).

2.4.3. Biological Treatment Processes

Biodegradation is a potentially economical, energy-efficient, and environmentally sound approach for OSPW reclamation. Generally, biodegradation techniques are applied in two different approaches including suspended growth and attached growth (Metcalf and Eddy, 2004). Microorganisms are maintained in suspension in suspended-growth bioreactors such as sequencing batch reactors (SBRs) and activated sludge processes (Metcalf and Eddy, 2004). Air is provided in these processes using pneumatic aeration or mechanical agitation systems. The microorganisms used in the reactor for the treatment of contaminants form flocs of diameter range 50 - 200 μm by conglomerating and bridging through polysaccharides and proteins (Kuhn et al., 2010). In attached growth systems, biofilms are developed on various support media such as GAC, sands, glass beads, polyethylene, polypropylene, polyvinyl chloride, ceramics, and rocks (Loupasaki and Diamadopoulos, 2013). The systems where the media are used for the treatment of water are referred to as biofilm reactors. Biofilm reactors are utilized in both up flows (e.g. fluidized bed biofilm reactors, air lift biofilm reactors) and down flows (e.g. packed bed biofilm reactors, trickling filters) modes with either batch or continuous processes (Burghate and Ingole, 2014). In the case of the packed bed biofilm reactors (PBBR) and trickling filters (TF), it is very difficult to control the biofilm thickness, oxygen levels, the bed clogging from foreign particles, the channeling of the bed and excess biomass sloughing (Metcalf and Eddy, 2004). The use of fluidized bed biofilm reactors (FBBR) for the treatment of wastewater can

eliminate the disadvantages associated with PBBR and TF (Rodgers and Zhan, 2003). However, there are high costs associated with pumping recycle and feed through the reactor to keep the bed in fluidized state (Burghate and Ingole, 2014).

Overall, the biofilm reactors have some advantages over the suspended-growth wastewater treatment systems such as: (a) less energy required; (b) simple operation and less equipment maintenance; (c) no problems of sludge bulking and better sludge thickening properties; (d) compactness due to the availability of the biofilm media with high specific surface area; (e) co-existence of aerobic and anoxic microorganisms within the same ecosystem; and (f) lower sensitivity and better recovery from shock loadings (Rodgers and Zhan, 2003). However, the majority of the biodegradation research on OSPW reported to date has focused on the application of planktonic cells using batch bioreactors. Only a few studies have been carried out on biofilm-based bioreactors for the treatment of OSPW as discussed below.

2.4.3.1. Planktonic Biodegradation of NAs

Numerous studies have been carried out using the planktonic biodegradation technique for commercial NAs and OSPW NAs. Overall, rapid biodegradation was observed in aerobic cultures of commercial NAs compared to much slower biodegradation of OSPW NAs (Biryukova et al., 2007; Han et al., 2008; Scott et al., 2005; Smith et al., 2008). The biodegradability of NAs is related to their molecular masses (Headley et al., 2010a; Scott et al., 2005; Whitby, 2010) and chemical structures (i.e., cyclicity, linearity, *cis*- and *trans*- isomers) (Han et al., 2009; Han et al., 2008; Smith et al., 2008). Researchers suggested that the exposure of commercial NAs and OSPW NAs solutions to aerobic microbial processes selectively removed lower molecular mass NAs resulting in an increase in the concentration of branched,

cyclic and higher molecular weight NAs that are more recalcitrant to biodegradation (Biryukova et al., 2007; Clemente et al., 2004; Holowenko et al., 2002; Scott et al., 2005). Carbon number (*n*) was shown to have little impact on the rate of biodegradation, whereas the biopersistence was positively correlated with the increase of cyclization (Z/2) in both commercial and OSPW NAs mixtures (Han et al., 2009). The half-life for commercial NAs was 1 to 8 days among all NAs in the mixture, whereas the half-life for OSPW NAs ranged from 44 to 240 days. The least cyclic fraction (i.e. Z = 0 and Z = -2 homologues) in both commercial and OSPW NAs underwent relatively rapid biodegradation (Han et al., 2009; Han et al., 2008). Some studies have found that mixed bacterial populations can increase the degradation of higher cyclic and methyl substituted cyclic NAs in other wastewaters (Del Rio et al., 2006; Headley et al., 2002; Herman et al., 1993; Smith et al., 2008). However, although a mixed bacterial community has been observed in OSPW (Golby et al., 2012; Quagraine et al., 2005), the half-life for the recalcitrant fractions during aerobic biodegradation of OSPW NAs was found to be 12.8 to 13.6 years (Han et al., 2009). Toxicity of OSPW is not removed easily by native microbial populations found within in situ planktonic treatments (i.e., tailings ponds), and residual toxicity remains even after decades of storage in experimental ponds containing OSPW (Pereira et al., 2013b).

2.4.3.2. Biofilm Treatment of NAs

Engineered bioreactors employing microbial aggregations as biofilms might provide a significantly higher NAs removal efficiency than planktonic bioreactors. The microbial community can be retained in biofilm reactors which have low amounts of suspended solids and a limited biomass wash out (Allen, 2008b). Success has been achieved using engineered biofilm-based bioreactors for the treatment of a large variety of recalcitrant organic compounds in

municipal and industrial wastewaters (Liu et al., 2005; Ulson de Souza et al., 2008; Zhao et al., 2006). Paslawski et al. (2009) found that the degradation of a model NA *trans*-4-methy-1-cyclohexane carboxylic acid was almost two orders of magnitude higher for an immobilized PBBR versus a continuous stirred tank bioreactors.

Golby et al. (2012) showed that a biofilm can be cultured on media under both aerobic and anaerobic growth conditions by indigenous microorganisms found in OSPW. However, few studies have addressed the potential application of these endogenous microorganisms in engineering biofilm-based bioreactors for OSPW treatment (Choi et al., 2014; Headley et al., 2010a; Hwang et al., 2013). Headley et al. (2010a) investigated the degradation of Athabasca OSPW NAs and Fluka (commercial) NAs using biofilms created using surface water bacteria (i.e., non-endogenous bacteria of OSPW) in continuous rotating annular biofilm reactors. Fluka NAs degradation were found to be dependent on the NAs molecular structure and molecular weight. However, no degradation of the OSPW NAs was observed over the experimental duration (24 days). In constrast, Hwang et al. (2013) observed NAs removal (18.5%) using continuous flow biofilm reactors with biofilms developed using endogenous OSPW microorganisms, thus indicating that biofilm reactors have a potential to be used for OSPW treatment. More recently, Choi et al. (2014) showed that a continuous biofilm reactor produced a higher degradation rate (14% AEF removal) as compared to that of a batch biofilm reactor (6.2% AEF removal). Thus far, a complete assessment of a variety of media based biofilm reactors (e.g., adsorbent media such as GAC and coke; and non-adsorbent media such as sand and plastic) has not been considered previously for the treatment of OSPW. Therefore, additional research work is needed to investigate these various media based bioreactors for their performance for treating OSPW NAs.

2.5. GAC-Biofilm Reactor

The GAC is a widely used media for the treatment of recalcitrant organics in water and wastewater (Aktas and Cecen, 2007b). However, there is an issue with the GAC becoming exhausted with prolonged contact times in the GAC filter and thus it must be either replaced or regenerated (Nath and Bhakhar, 2011). Researchers have suggested that the presence of a biofilm on the GAC surface increases the biofilter operational lifetime for the removal of problematic compounds (Gibert et al., 2013; Wang et al., 2007). The bacteria in these reactors are immobilized on the GAC surface when they produce an active biofilm where they are able to remove recalcitrant compounds via biological degradation (biodegradation) processes (Gibert et al., 2013; Wang et al., 2007) and regenerate the GAC surface for further adsorption (Aktas and Cecen, 2007a, b; Ng et al., 2009).

The use of the adsorbent GAC has the following advantages versus non-adsorptive media as a bioreactor supporting medium: (a) high microbial colonization on the GAC surface is observed due to high surface roughness and large macropores, which provide excellent shelter to attached bacteria from fluid shear forces and from the high adsorptive capacity of GAC that enriches substrates, nutrients and oxygen concentration on its surface (Aktas and Cecen, 2007b; Yapsakli and Cecen, 2010); (b) has the capability to remove high or toxic influent concentrations of pollutants and can maintain constant effluent quality because of its adsorptive capacity (Allen, 2008b); (c) gradual desorption of adsorbed compounds from shock loads at nontoxic concentrations allows for pollutant biodegradation (Herzberg et al., 2003); and (d) adsorbed substrate biodegradation within the biofilm and desorption from GAC surface because of the concentration gradient between the GAC surface and the liquid phase allows the regeneration of

the GAC versus the biofilm of an inert media (Aktas and Cecen, 2007a, b; Herzberg et al., 2003; Nath and Bhakhar, 2011).

Successful treatment using GAC-biofilm reactors (e.g. FBBR, PBBR) has been achieved for a large variety of recalcitrant organic compounds found in industrial wastewaters (Baban et al., 2010; Lei et al., 2010; Rao et al., 2005), synthetic recalcitrant and toxic wastewaters (Caldeira et al., 1999; Wang et al., 2007) and oilfield produced wastewaters (Campos et al., 2002; Seybold et al., 1997; Zhao et al., 2006). Moreover, GAC has been shown to have a very high adsorption affinity for NAs because of the various types of pores on the GAC surface (Small et al., 2012). The use of the GAC-biofilm process for the treatment of OSPW could potentially be an effective technology given the use of endogenous microorganisms in OSPW for the growth of the biofilm on this adsorptive media (Golby et al., 2012; Hwang et al., 2013).

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CHAPTER 3. MICROBIAL COMMUNITY STRUCTURE AND OPERATIONAL PERFORMANCE OF A FLUIDIZED BED BIOFILM REACTOR TREATING OIL SANDS PROCESS-AFFECTED WATER[1]

3.1. Introduction

Water treatment and management is a burgeoning problem in the Alberta oil sands industry. For every barrel of bitumen produced, two to four-and-a-half barrels of water is required (Griffiths et al., 2006). Oil sands process-affected waters (OSPWs) generated from bitumen extraction are currently stored in tailings ponds surrounding mining sites because of their toxicity to aquatic organisms such as fish (He et al., 2012), bacteria (Gamal El-Din et al., 2011), benthic invertebrates (Anderson et al., 2012) and mammalian species (Garcia-Garcia et al., 2011). The primary toxic constituents of OSPW are a group of organic acids, collectively known as naphthenic acids (NAs) (Anderson et al., 2012). NAs are alicyclic or noncyclic alkyl-substituted carboxylic acids with a general chemical formula $C_nH_{2n+Z}O_x$, where n is the carbon number, Z is zero or a negative even integer related to a hydrogen deficiency due to ring formation, and x represents the number of oxygen atoms (x = 2 for classical NAs and 3 to 5 for oxidized NAs) (Barrow et al., 2010; Grewer et al., 2010; Wang et al., 2013). Nitrogen and sulfur species (Barrow et al., 2010; Wang et al., 2013) as well as aromatic (Reinardy et al., 2013) and multiple-carboxylated compounds (Headley et al., 2011) are also found in OSPW. While natural

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surface water in the Athabasca oil sands region contains less than 0.04 mg NAs/L measured by gas chromatography-mass spectroscopy (GC-MS), the concentration of classical NAs in OSPW ranges from 7.1 to 47 mg/L (Gamal El-Din et al., 2011; Grewer et al., 2010; Pourrezaei et al., 2011; Wang et al., 2013). Appropriate OSPW treatment approaches are urgently needed for safe discharge of treated OSPW to the receiving environment.

Biodegradation is potentially an economical, energy-efficient and environmentally sound approach for tailings water reclamation. However, previous studies have shown that oil sands classical NAs are persistent toward biodegradation because of their extensive cyclical molecular structures (Hwang et al., 2013). The majority of biodegradation research on OSPW reported to date has focused on the application of planktonic cells using batch bioreactors. Engineered bioreactors that employ microbial aggregations (i.e., biofilms or biological flocs) might provide a significantly higher NAs removal efficiency. Success has been achieved using engineered biofilm-based bioreactors for the treatment of a large variety of recalcitrant organic compounds in industrial wastewaters (Baban et al., 2010; Rao et al., 2005). Other research has demonstrated that biofilm-based biological treatment can effectively degrade organic components in conventional oil field wastewater (Zhao et al., 2006). Moreover, although previous studies suggest that there is a wealth of microbial diversity in oil sands tailings (Golby et al., 2012a; Siddique et al., 2011), few studies have addressed the potential application of these endogenous microorganisms in engineered biofilm-based bioreactors for OSPW treatment. To date, there were only two reports about bioreactor-based OSPW treatment. Headley et al. (2010) showed that the biological treatment of NAs in OSPW by lake biofilms in a rotating annular bioreactor was dependent on the compositions of NAs. More recently, Hwang et al. (2013) observed ~18.5% NAs removal using continuous biofilm reactors seeded with endogeneous

microorganisms (bacteria in the raw OSPW), indicating that biofilm reactors have a potential to be used for OSPW biological treatment. However, additional research is needed to improve the bioreactors performance and to enhance NAs treatability.

Activated carbon has been widely used in drinking and wastewater treatment for the removal of organics (Aktas and Cecen, 2007). The irregular shape and porous structure of granular activated carbon (GAC) particles can protect and shield microorganisms from high fluid shear forces and thus can promote microbial colonization (Herzberg et al., 2006). In addition, its high adsorption capacity, due to its large specific surface area, increases the availability of organic substrates and nutrients to microorganisms at the media surface and, therefore, can enhance the biodegradation efficiency (Herzberg et al., 2006). It is also suggested that GAC can be bioregenerated in a biofilm reactor from microbial oxidation of adsorbed organics on the GAC surface from exoenzymatic reactions and biodegradation of organics in the liquid phase, which enhances organics removal beyond the point at which adsorption capacity would normally be exhausted (Aktas and Cecen, 2007).

The objective of this study was to determine the degradation of organic compounds and the fate of classical NAs in a fluidized bed biofilm reactor (FBBR) treating OSPW with GAC as support media. The microbial communities in the biofilms were also investigated to illustrate their roles in the biodegradation of organic compounds in OSPW.

3.2. Materials and Methods

3.2.1. Source of OSPW

Raw OSPW was received in 200 L polyvinyl chloride barrel from one of the OSPW recycle pond sites in Fort McMurray, Alberta, Canada in October 2010. The raw OSPW samples

were preserved at 4 °C before subjected to any treatment. The chemical characterization of OSPW is shown in Table B1 (Appendix-B).

3.2.2. Configuration and Operation of FBBR

A single cylindrical bioreactor as shown in Figure 3.1 was utilized to treat raw OSPW. The reactor diameter was 2.5 cm and the fixed bed packing height was 25 cm. The reactor was made of clear acrylic plastic with a 50 mesh stainless steel grid at the bottom to hold the GAC media inside the reactor. Aquasorb® 1500, a high activity GAC manufactured by steam activation from selected grades of bituminous coal (50 g) was used as a carrier media for biofilm support in the FBBR. The total pore volume for the GAC was 9.9×10^{-7} m^3/g and the specific surface area was 1050 m^2/g. Endogenous microorganisms in OSPW were used as the only bacterial inoculums for the bioreactor. Peristaltic pumps with masterflex tubing were used to transfer the feed and recycle OSPW into the reactor from a feed tank. To facilitate OSPW microbial colonization, the bioreactor was initially operated for three days under a semi-batch condition with a recycle flow rate of 260 mL/min. After an initial three days in batch mode operation, the reactor was operated in a continuous mode with raw OSPW for 120 days.

The bioreactor was operated in six phases, each having different feed and recycle flow rates (Table 3.1). The bioreactor organic loading rate (OLR) was varied by changing the feed flow rates. The bioreactor hydraulic loading rate (HLR), which influences the bioreactor flow rate (i.e., the summation of recycle and feed flow rates) and the fluidization of the media particles in the bioreactor, was varied by changing the recycle flow rate. For COD, pH and dissolved oxygen (DO) analysis triplicate samples were taken daily and for AEF analysis

triplicate samples were taken every 2 days. The pH and DO of the OSPW during the bioreactor operation were in the range of 8.9 to 9.0 and 5.9 to 6.2 mg/L, respectively.

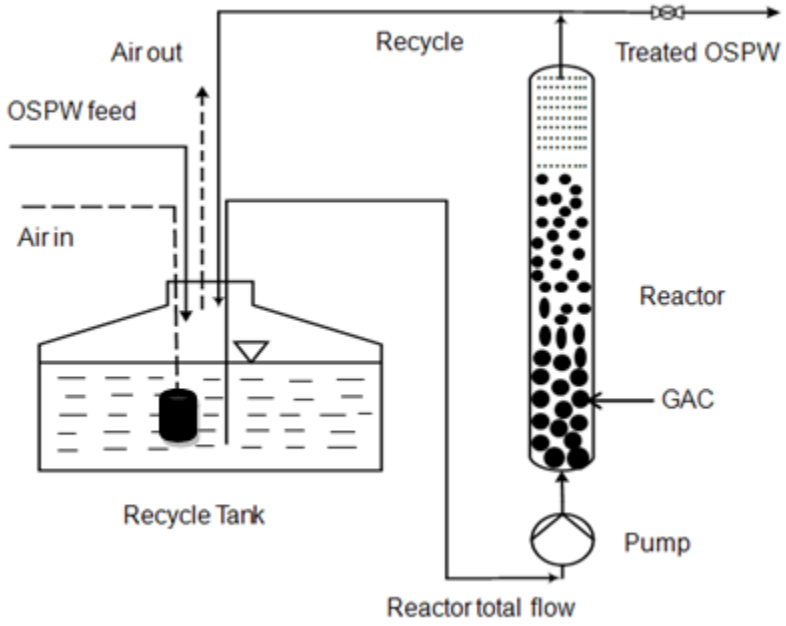

Figure 3.1. Schematic diagram of the experimental set-up.

3.2.3. Water Chemistry Analysis and Reactor Performance Evaluation

Analyses of basic water chemistry, including pH, DO, as well as its physical properties (COD, 5-day biochemical oxygen demand (BOD_5), acid-extractable fraction (AEF), and classical NAs) were performed prior to and after bioreactor treatment. COD and BOD_5 were measured according to standard methods (APHA, 2005). A detailed procedures for the measurement techniques of AEF, COD and BOD_5 have been provided in Appendix-A. Fourier transform infrared (FT-IR) spectroscopy (PerkinElmer®precisely, PerkinElmer Life and Analytical Sciences, ON, CA) was used to measure the AEF for carbonyl stretch equivalents in OSPW.

AEF was measured following the standard protocol described elsewhere (Gamal El-Din et al., 2011) and a detailed procedure has been provided in Appendix-A. In brief, OSPW samples were filtered using a 0.45 μm nylon filter, acidified to pH 2.0–2.5, and extracted twice with dichloromethane. The extracted layer was separated, evaporated to dryness, and then redissolved in a known mass of high performance liquid chromatography (HPLC)-grade dichloromethane for FT-IR analysis. The absorbance of monomeric and dimeric AEF for carbonyl stretch equivalent was measured at 1,743 and 1,706 cm^{-1} respectively. A calibrated absorbance curve was prepared using a Fluka (Sigma-Aldrich, ON, CA) NAs mixture. For each sampling occasion, triplicate samples (n = 3) for COD and AEF were analyzed from influent and effluent. One-way analysis of variance (ANOVA) was applied to determine reliability of analysis and the differences of reactor performance between phases at a significance level (α) of 0.05.

Further, the classical NAs concentrations in OSPW before and after bioreactor treatment were measured (as a function of carbon and Z numbers) using ultra performance liquid chromatography/high resolution mass spectroscopy (UPLC/HRMS) (Gamal El-Din et al., 2011; Hwang et al., 2013). OSPW samples were centrifuged for 5 min at 10,000 rpm and applied to a Waters Acquity UPLC® System (Milford, MA, USA) for the separation of NAs. 500 μL of centrifuged sample was mixed with 100 μL of 4 mg/L internal standard (myristic acid -^{13}C) in methanol and 400 μL methanol (Fisher Scientific, ON) and injected into the UPLC-HRMS system for the analysis of OSPW NAs. Chromatographic separations were performed using a Waters UPLC Phenyl BEH column (1.7 μm, 150 mm × 1 mm). The mobile phase through the Phenyl BEH column consists of a 10 mM ammonium acetate solution prepared in Optima-grade water (A), and a 10 mM ammonium acetate in 50% methanol and 50% acetonitrile (B). Optima-grade methanol and acetonitrile were used for the preparation of solution B. The gradient elution

throughout the column was as follows: 1% B was passed through the column for the first 2 min which then ramped to 60% B for 3 min followed by 70% B for 7 min, and 95% B for 13 min, and hold for another 14 min followed by 1% B for 5.8 min to reach the equilibrium. Constant flow rate at 100 µL/min was maintained through the column with column temperature at 50 °C, while samples were maintained at 4 °C.

Classical NAs were detected with a high resolution (40,000 FWHM) Synapt G2 HDMS mass spectrometer (m/z from 0 to 600) equipped with an electrospray ionization source operating in negative ion mode. Standard solutions of leucine enkephalin and sodium formate (Waters Corporation, Milford, MA, USA) were used for tuning and calibration, respectively. Data analyses of target compounds were performed using TargetLynx® ver. 4.1 software. The ratio of chromatographic peak area of each analyte to that of the internal standard was calculated for estimating the concentration of analyte.

3.2.4. Microbial Characterization in the Bioreactor

3.2.4.1. Polymerase Chain Reaction-Denatured Gradient Gel Electrophoresis

16S rRNA gene-based polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE) was applied to analyze the biofilm microbial communities involved in the biological treatment of raw OSPW in duplicate. Microorganisms in the raw OSPW and from GAC biofilms were isolated according to the protocol described by Hwang et al. (2013). In brief, total genomic DNA was isolated from the samples using a PowerSoil® DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA). Otherwise, extractions were performed following the manufacturer's protocol. 200-300 µL samples were added to a bead beating tube and homogenized by vortex. Cell lysis was performed mechanically, by grafting the tubes in a

MOBIO vortex adapter tube holder, and chemically by the addition of PowerSoil® DNA isolation chemicals. A silica membrane in a spin column was used to capture total genomic DNA. DNA was then washed using an ethanol solution and eluted from the membrane using a 100 µL sterile elution buffer. The quality and quantity of DNA suspensions were evaluated by electrophoresis on a 0.8% agarose gel followed by staining with ethidium bromide.

PCRs were carried out in an Eppendorf thermal cycler using a GoTaq® PCR kit (Promega Corporation, WI, USA). The primer sets 907r, with sequence 5'-CCG TCA ATT CMT TTG AGT TT-3', and 341f-GC, having 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3', were used for amplification of extracted DNA before performing DGGE. Each PCR mixture consisted of 5 µL of 5× PCR buffer, 1.5 µL of $MgCl_2$ at 25 mM, 0.5 µL of dNTP at 10 mM, 0.5 µL of forward (341f-GC) and reverse (907r) primer mixture at 25 mM, 0.125 µL of Taq DNA polymerase at 5 units/µL, 1.0 µL of extracted DNA at 20 ng/µL, and water in a total volume of 25 µL. The PCR was conducted using the following conditions: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 10 min; PCR products were stored at 4 °C until visualization. The amplifications were separated with electrophoresis on a 0.8% agarose gel, stained with ethidium bromide solution, and visualized with a transluminometer (Vilber Lourmat, Marne La Vallee-Cedex 1, France). The amplified PCR products were purified with a mixture of ExoSap exonuclease I (10 U/µL) and shrimp alkaline phosphatase (SAP) (1 U/µL) and sequenced.

DGGE was conducted using BIORAD D-Code™ (Bio-Rad Laboratories, CA, USA). A 6.5% acrylamide gel with a 30–70% gradient solution of urea and formamide was prepared with 40% (v/v) formamide and granular urea. During DGGE, PCR products (40 µL) were loaded on

the acrylamide gel at a constant voltage of 180 V at 60 °C for 6 h in 0.5×TAE buffer. The gel was stained for 30 min in SYBR green solution (30 µL SYBR green solution in 300 mL demineralised water) and the background was reduced by destaining in demineralised water for 20 min. The DGGE fingerprints were visualized with a transluminometer (Vilber Lourmat, Marne La Vallee-Cedex 1, France). Sharp bands were selected under UV light, cut out with razor blades, placed in tubes, and mashed. 40 µL of diffusion buffer was added to the tubes and the tubes were stored at 4 °C overnight before sequencing.

The NCBI nr nucleotide database was used for matching the sequenced data using the nucleotide BLAST program. CLUSTALW was used to build multiple sequence alignment. Mega 5.2.2 software was used for calculating and constructing a neighbor-joining phylogenetic tree where known strains were included. Phylogenetic distances between the strains were calculated based on the p-distance substitution model with pairwise deletion treatment of gaps. Bootstrap re-sampling (n = 500) was performed to test the robustness of the tree topologies.

3.2.4.2. Confocal Laser Scanning Microscope (CLSM) Imaging

Imaging of biofilm samples was performed using a confocal laser scanning microscope (CLSM). The GAC biofilms were stained with SYTO 9 (BacLight Live/Dead bacterial viability kit, Molecular Probes, USA) and Concanavalin A (ConA, Molecular Probes, Eugene, OR) lectin conjugated with Texas Red (Jefferson et al., 2005), for the probing of total bacterial cells and extracellular polymeric substances (EPS), respectively. Aliquots (3 µL) of SYTO 9 and ConA were added into 1 mL distilled water for fluorescent staining separately. 100 µL of diluted SYTO 9 was added to each biofilm sample and samples were incubated in the dark at room temperature for 1 h. Unbound SYTO 9 was rinsed carefully with distilled water. In the same manner as the

SYTO 9 staining, ConA solution was added to the SYTO 9-stained biofilm coupons, which were then incubated and the excessive ConA solution was removed. Fluorescence stained biofilm samples were placed in relevant OSPW before obtaining images. Biofilm image observation, acquisition, and biofilm thickness measurements were performed with a confocal laser scanning microscope (CLSM) (Zeiss LSM 710, Carl Zeiss Micro Imaging GmbH, Germany). For monitoring the stained cells, the confocal laser scanning microscope was equipped with a spectral detector with a PMT (photon multiplier tube) array and optical grating elements: SYTO 9 (excitation wave length (ex) = 488 nm; emission wave length (em) = 522/32 nm); ConA conjugated with Texas Red (ex = 568 nm; em = 605/32 nm). Biofilm images were observed and scanned randomly at 4 or 5 positions with a lens (20× 0.8NA Plan-Apochromat). A series of z-axis images were generated through optical sectioning at a slice thickness of 1 μm. CLSM z-axis images were stacked and presented. The image analysis was performed by using ImageJ software. The mean biofilm thickness was automatically calculated based on the ratio of total biovolume and surface area at each z-stack by the software.

3.2.4.3. Scanning Electron Microscope (SEM) Imaging

Scanning electron microscope (S-2500, Hitachi Ltd., Japan) was also used to analyze the morphology of biofilms formed on the GAC in the bioreactor. Biofilm/GAC samples were fixed with 2.5% (v/v) glutaraldehyde ($C_5H_8O_2$) and 1.0% (v/v) osmium tetraxide (OsO_4) (Ted Pella Inc., Redding, CA, USA) in phosphate buffered saline (PBS) for 24 h. After fixation, the samples were dehydrated with 50, 70, and 100% ethanol (C_2H_5OH) (Sigma-Aldrich, St. Louis, MO, USA) and dried completely in a critical point dryer. GAC samples were attached to an aluminum sample holder with carbon tape and sputter coated with gold for 30 s prior to imaging.

3.2.4.4. Biomass Concentration

The dry biomass on GAC in the FBBR was determined using a protocol described by Vainberg et al. (2002) and the dry biomass density was determined using the equation 3-1 from previous literature (Mulcahy and Shieh, 1987) . Samples of GAC were taken from reactors and dried at 105–110 °C for at least 48 h in preweighed crucibles. Crucible weights were recorded when they achieved a constant weight after confinement in a dessicator for at least 90 min. NaOH (4 N) was added at double the volume of the GAC and the mixture was mildly agitated for at least 18 h to detach the biomass from the GAC. The NaOH solution was decanted and the carbon particles were washed using deionized distilled water (Millipore) to free them from any remaining digested biomass. After 48 h of drying, the crucibles were desiccated and reweighed. Dry biomass density was calculated based on the following equation (Mulcahy and Shieh, 1987):

$$X = X_f(1-\varepsilon)\left[1-\left(\frac{d_m}{d_p}\right)^3\right] \qquad (3-1)$$

Where, X = dry biomass, g/L GAC;

X_f = dry biomass density, g/L;

ε = bed porosity;

d_m = media diameter, mm;

d_p = bioparticles diameter, mm;

3.3. Results and Discussion

3.3.1. Organic Compounds Removal during FBBR Operation

3.3.1.1. COD Removal

The bioreactor operational time was divided into six phases (I-VI) to study the effect of OLR and HLR on the performance of bioreactor. At feed flow rates of 0.6, 2.0, and 5.0 mL/min, OLRs for the bioreactor treating OSPW were 0.75, 2.46, and 6.15 kg COD/m^3·d, respectively.

During bioreactor operating phases I, II and III, HLRs were kept at a constant value (31.8 m/h) and the OLRs were varied by changing the feed flow rate (Table 3.1). In phase I (0–38 days), the bioreactor OLR was 2.46 COD/m^3·d and greater than 70% COD removal was obtained in the first 5 days (Figure 3.2A). GAC adsorption was considered to be the major mechanism for COD removal in this phase because of the low bacterial number (Figure C1, Appendix-C) and the high adsorption capacity of GAC. Based on Figures 3.2A and 3.2C, it could be assumed that the COD removal via adsorption started to decrease from day 5, which might indicate that the GAC adsorption sites were becoming exhausted. The COD removal rate became steady after 30 days and the steady-state COD removal efficiencies in the bioreactor were 44 ± 3% for raw OSPW with COD mass removal rate at 314 mg COD/d (Table 3.1).

In phase II of the bioreactor operation (39–50 days), OLR was reduced to 0.75 kg COD/m^3·d by reducing the feed flow rate from 2 mL/min to 0.6 mL/min. Subsequently, COD removal efficiency quickly increased and eventually reached a steady-state condition within 4 days. The percent COD removal efficiencies in this stage with reduced OLRs increased to 51 ± 3% for raw OSPW; whereas the COD mass removal rate was decreased to 108 mg COD/d at reduced OLR (Table 3.1). The COD removal efficiencies increased significantly ($p = 2.05 \times 10^{-4}$) by comparing with the ones in phase I.

Table 3.1. Bioreactor operational conditions and organic compounds (COD, AEF, and classical NAs) removal efficiencies ([a] feed flow rate + recycle flow rate; [b] hydraulic loading rate; [c] organic loading rate).

Operating phase	Operating time (day)	Feed flow rate (mL/min)	Recycle flow rate (mL/min)	Reactor flow rate[a] (mL/min)	HLR[b] (m/h)	OLR[c]			Removal efficiency (%)			Mass removal rate (mg/d)		
						(kg COD/m^3·d)	(kg AEF/m^3·d)	(kg NAs/m^3·d)	COD	AEF	NAs	COD	AEF	NAs
I	0-38	2	258	260	31.8	2.46	0.65	0.30	44	45	92	314	88	75
II	38-50	0.6	259.4	260	31.8	0.75	0.20	0.11	51	56	96	108	35	31
III	50-60	5	255	260	31.8	6.15	1.65	0.84	24	24	38	430	125	114
IV	60-70	2	128	130	15.9	2.46	0.65	0.30	28	29	72	202	63	83
V	70-80	2	58	60	7.3	2.46	0.65	0.30	24	27	68	196	60	89
VI	80-120	2	258	260	31.8	2.46	0.65	0.30	31	33	86	225	62	106

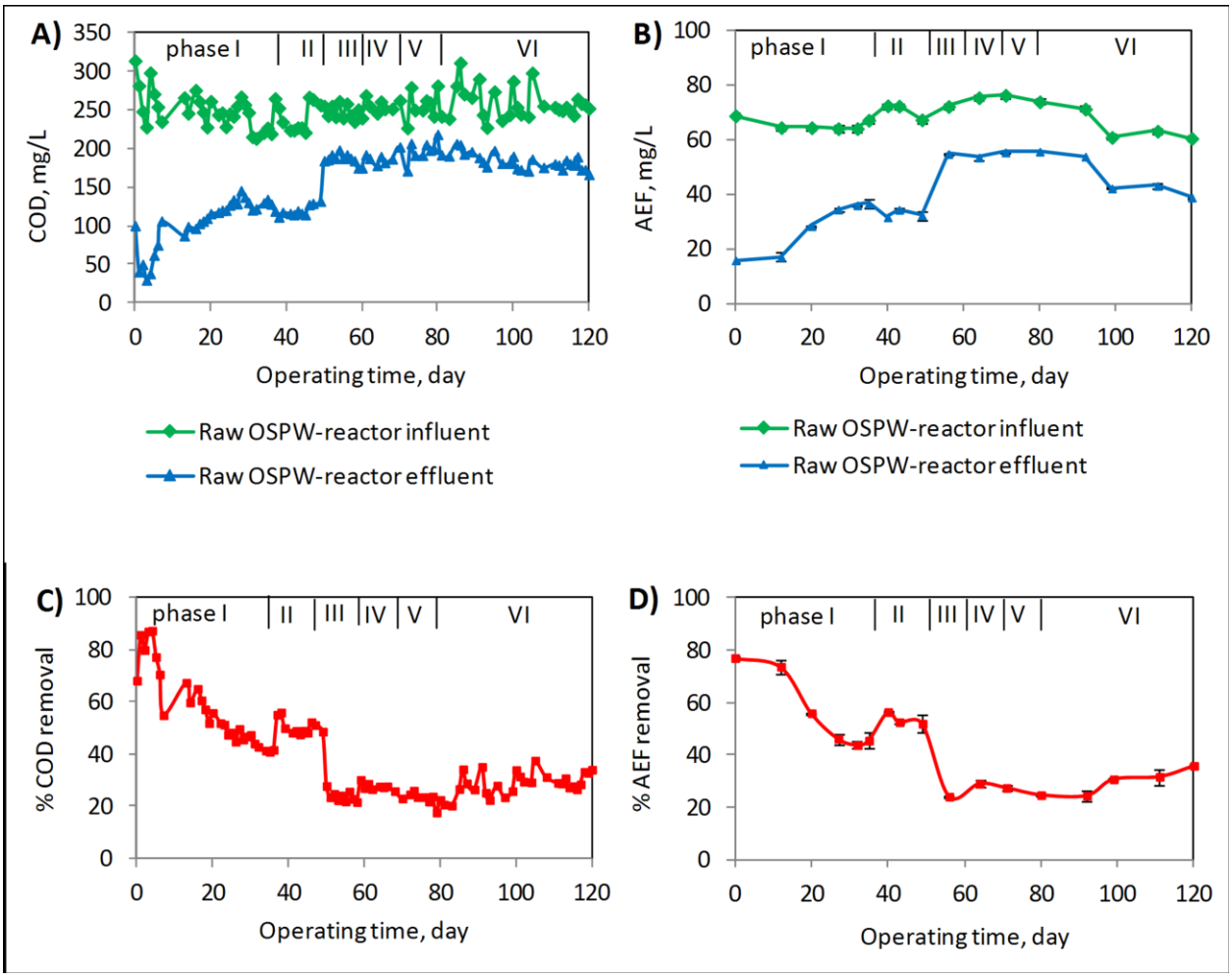

Figure 3.2. Influent and effluent concentrations of (A) COD and (B) AEF in FBBR. Percent removal of (C) COD and (D) AEF versus days of reactor operation. Phase I: feed flow rate 2 mL/min and reactor flow rate 260 mL/min, phase II: feed flow rate 0.6 mL/min and reactor flow rate 260 mL/min, phase III: feed flow rate 5 mL/min and reactor flow rate 260 mL/min, phase IV: feed flow rate 2 mL/min and reactor flow rate 130 mL/min, phase V: feed flow rate 2 mL/min and reactor flow rate 60 mL/min, phase VI: feed flow rate 2 mL/min and reactor flow rate 260 mL/min. Triplicate samples (n = 3) were taken on each sampling occasion with standard deviations indicated in error bars. Note: error bars smaller than symbols are not visible.

To examine the impact of high OLRs on the bioreactor operation, OLRs were increased in phase III of the bioreactor operation (50–60 days) to 6.15 kg/m^3·d. COD removal efficiencies in this stage decreased significantly ($p = 6.75 \times 10^{-11}$) to 24 ± 2% for raw OSPW. Although the COD removal efficiency was decreased at the increased OLR, the COD mass removal rate was increased to 430 mg/d (Table 3.1). In phases IV (60–70 days), V (70–80 days), and VI (80–120 days), OLRs were kept constant at 2.46 kg COD/m^3·d. The HLRs through the bioreactor were 15.9, 7.3, and 31.8 m/h for phases IV, V, and VI, respectively. COD removal efficiencies obtained from the start of the fourth phase until the end of the sixth phase were 28 ± 4% for raw OSPW. The average COD mass removal rate from these three phases was 208 ± 15 mg COD/d (Table 3.1). Overall, the results showed that COD removal efficiency did not change significantly with the change in bioreactor HLRs while it decreased with the increase of OLRs.

3.3.1.2. AEF Removal

AEF values are commonly used by the oil sands industry to evaluate NAs concentrations, including classical and oxidized NAs and other organic compounds with functional groups containing carboxylic acids, ketones and aldehydes (Jivraj et al., 1995). In the present study, AEF loading rates in the bioreactor treating raw OSPW, were 0.2, 0.65, and 1.62 kg/m^3·d for 0.6, 2, and 5 mL/min feed flow rates, respectively. The maximum steady-state AEF removal efficiency (56 ± 1%) was obtained at the lowest AEF loading rate tested in this study (0.2 kg AEF/m^3·d). Meanwhile, at the highest AEF loading rate (1.62 kg/m^3·d), the removal efficiency of AEF in the bioreactor was 24 ± 1% (Figures 3.2.B and 3.2.D). In general, higher AEF removal efficiency was observed compared with the removal of COD. Similar results have been reported previously on bioreactor treatment of OSPW (Hwang et al., 2013), which can be attributed to the

conversion of components of the AEF to smaller organic compounds that can still contribute to COD. The AEF mass removal rate was increased with the increase of AEF loading rate in the bioreactor as we observed for COD mass removal rate from the bioreactor. The lowest AEF mass removal rate (35 mg/d) was observed at 0.2 kg AEF /m^3·d, whereas the highest AEF mass removal rate was 125 mg/d at the highest AEF loading rate of 1.62 kg AEF /m^3·d (Table 3.1).

3.3.1.3. Removal of Classical NAs

Table 3.1 shows the bioreactor's removal efficiencies and mass removal rate (mg/d) of classical NAs from OSPW. Bioreactor influent classical NAs concentration was 39.2 ± 5.7 mg/L as it fluctuated between 28.4 to 42.9 mg/L during the bioreactor operation. The maximum removal efficiency of classical NAs from raw OSPW was 96% at a loading rate of 0.1 kg NAs/m^3·d (the lowest OLR), whereas the minimum classical NAs removal efficiency was 38% at a loading rate of 0.84 kg NAs/m^3·d (the highest OLR), as shown in Table 3.1. The mass removal rate (mg/d) of classical NAs was increased with the increase of classical NAs loading rate although the percentage removal decreased as shown in Table 3.1. The highest mass removal rate of classical NAs (114 mg/d) was observed at highest loading rate. Compared with loading rate, recycle flow rate affected the mass removal rate to a less extent (Table 3.1). The fluctuation between 83 mg/d and 106 mg/d might be caused by the variation of NAs concentration in different barrels as we mentioned above.

The percent removal of classical NAs based on each individual Z and carbon number is shown in Table B2 (Appendix-B). For the bioreactor treating raw OSPW, the concentration of classical NAs was 42.6 mg/L in the bioreactor influent (Figure 3.3.A) and 5.9 mg/L in the bioreactor effluent (Figure 3.3.B) on day 120. The overall classical NAs removal in raw OSPW

was 86.1%, whereas the removals based on Z number were 86.9, 87.3, 82.2, 87.1, and 88.6% for Z = -4, -6, -8, -10 and -12, respectively. Compared with the classical NAs removal of 18.5% in a biofilm reactor (Hwang et al., 2013), the high removal efficiency of classical NAs having all Z and n values in our study might be attributed to (a) the continuous GAC adsorption of classical NAs with high Z numbers (Gamal El-Din et al., 2011); and (b) the highly available surface area of GAC allowing for better biofilm attachment, the latter contributing to biodegradation of classical NAs and hence regeneration of the GAC surface for further adsorption (Hwang et al., 2013). Biodegradation leading to bioregeneration of the GAC surface and to an increase in its service life has been reported elsewhere (Nath and Bhakhar, 2011). Compared with the removal of COD and AEF, the higher fractional NAs removal can be attributed to the high adsorption affinity of classical NAs. Classical NAs are a very large group of organic compounds having high molecular weight, long chain and more cyclic compounds with branches, which may increase the overall hydrophobicity of classical NAs compared to other organic compounds present in OSPW responsible for AEF and COD (Nguyen et al., 2013).

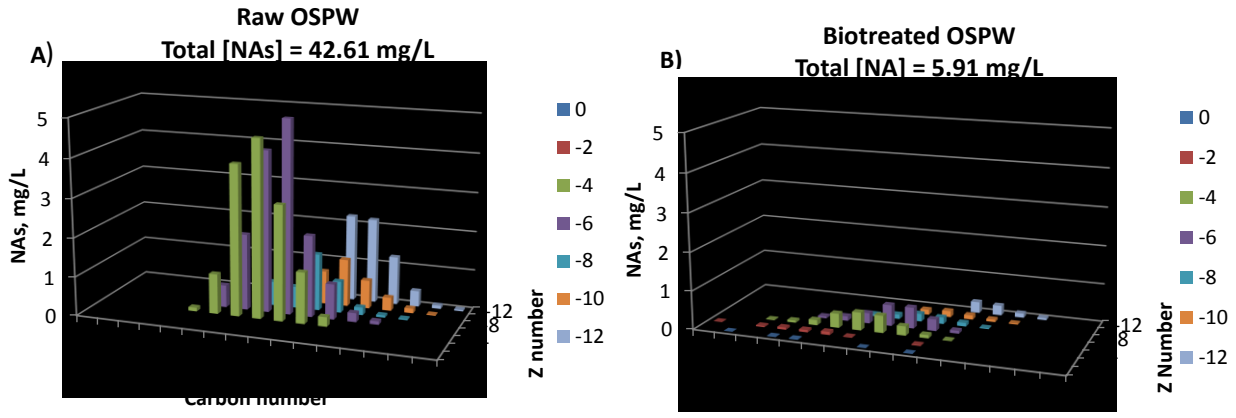

Figure 3.3. Profiles of classical NAs concentrations in reactor influent and effluent in OSPW on sampling day 120; (A) Reactor influent (Raw OSPW); (B) Reactor effluent (Biotreated OSPW).

From Table B2 (Appendix-B), the removal of classical NAs increased with the increase of carbon number for any Z number. For example, the classical NAs species with Z = −6, the removal of NAs was increased from 79.3% to 100% for n = 11 to n = 19. The increase of classical NAs removal with n can be attributed to the increase of hydrophobicity and nonpolarity of NAs with higher number of the carbons, and resulting in a higher affinity of larger molecules to adsorb onto the GAC surface (Zubot et al., 2012). There is no obvious regularity in the effect of Z number on classical NAs removal as shown in Table B2 (Appendix-B) in this study. However, the highest removal was observed for classical NAs with the smallest Z number when the range of n number was between 13 and 22. It should be noted that the increase of cyclicity in the structure of classical NAs can increase the hydrophobicity of the compound (Zubot et al., 2012) which could improve the adsorption of classical NAs. However, our results showed that compounds with low Z numbers were preferentially degraded that indicate classical NAs removal was controlled by both GAC adsorption and biodegradation mechanisms. It is well known that NAs with fewer rings are more inclined to biodegradation. These results are in agreement with previous studies on biological degradation of OSPW NAs (Clemente and Fedorak, 2005; Hwang et al., 2013; Martin et al., 2010).

3.3.2. Microbial Community Analyses

After 120 days of bioreactor operation, biofilms developed on GAC in the bioreactor treating OSPW were examined using SEM and CLSM (Figures 3.4.A and 3.4.B). High biofilm coverage was observed on GAC surfaces in the bioreactor. CLSM images showed that the average thickness of biofilm was 34 ± 5 μm on the GAC of the bioreactor (Figure 3.4B) and dry biomass densities (X_f), calculated using biofilm thicknesses and dry biomass, were 94.2 ± 13.6

g/L (Table B3, Appendix-B). These values are supported by a previous study on FBBR using GAC as support media (Ro and Neethling, 1991). Compared with the biofilm formed on the PE and PVC coupon of CDC bioreactor (Hwang et al., 2013), the reduced biofilm thickness on GAC may be attributed to low biodegradable organic loading caused by the larger surface area on the GAC. The phylogenic tree of bacterial members is shown in Figure 3.5.B. At the phylum level, microorganisms identified in the present study were in agreement with the dominant species in OSPW reported in previous studies (Golby et al., 2012; Siddique et al., 2011). The two *Polaromonas* species (band 1 and 13) from raw OSPW were clustered together with 100% support value while two strains which belong to bacteroidetes (band 2 and 5) also form a cluster with a 100% bootstrap value. *Methylobacterium fujisawaense* strain (band 19) and *Desulfotomaculum sp.* (band 20) were clustered, but have the larger genetic distances with other strains. Low bootstrap values in several places of this tree indicate some uncertainties in the tree structure.

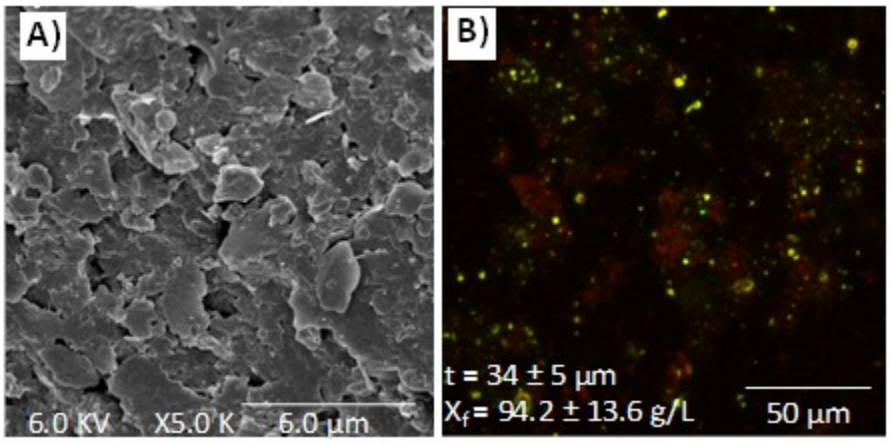

Figure 3.4. (A) SEM and (B) CLSM images of biofilms formed on the GAC. t: biofilm thickness, µm; X_f: dry biomass concentration in biofilm, g/L of biofilm.

Based on the DGGE bands, a notable similarity between the bands for planktonic microorganisms from raw OSPW and the bands obtained for biofilm was observed (Figure 3.5. A). This might be caused by the application of raw OSPW microorganisms during the start-up periods of bioreactor, which also showed the resilience of the biofilm communities during the bioreactor operation, as it was also demonstrated by a previous study (Hwang et al., 2013). Based on the DGGE bands, a total of 18 bacterial strains were identified. Membership was found to comprise *β-Proteobacteria* (7 bands), *α-Proteobacteria* (4 bands), *Bacteroidetes* (3 bands), *γ-Proteobacteria* (1 band), *Acidobacteria* (1 band), *Firmicutes* (1 band), and unclassified bacterial strains (3 bands). As shown in (Table B4 Appendix B), a total of 11 bands were associated with raw OSPW planktonic microorganisms and 13 bands were associated with biofilm in the reactor. Among the bacteria identified in raw OSPW, *Polaromonas jejuensis* NBRC 106434 (band 1), (Weon et al., 2008) the genus *Algoriphagus* (band 5), (Alegado et al., 2011) *Chelatococcus sp.* E1 (band 6), (Jeon and Kim, 2013) and *Methylobacterium fujisawaense* (band 15) (Madhaiyan et al., 2006) can utilize a wide range of carbon sources.

Among the other bands in raw OSPW, the genus *Limnobacter* (band 9) is a thiosulfate oxidizer which uses carboxylic and amino acids as energy and carbon source (Lu et al., 2011). *Rhodoferax ferrireducens* T118 (band 3) (facultative anaerobe) is an iron (III) reducer, (Finneran et al., 2003) and *Desulfotomaculum sp.* ECP-C5 (band 16) is a microbe that can reduce sulfate (Pikuta et al., 2000). Both of the bacterial communities listed above utilize organic carbon as an electron donor. Further, the genus *Desulfotomaculum* forms endospores to protect the cells from heat, oxygen, and desiccation.

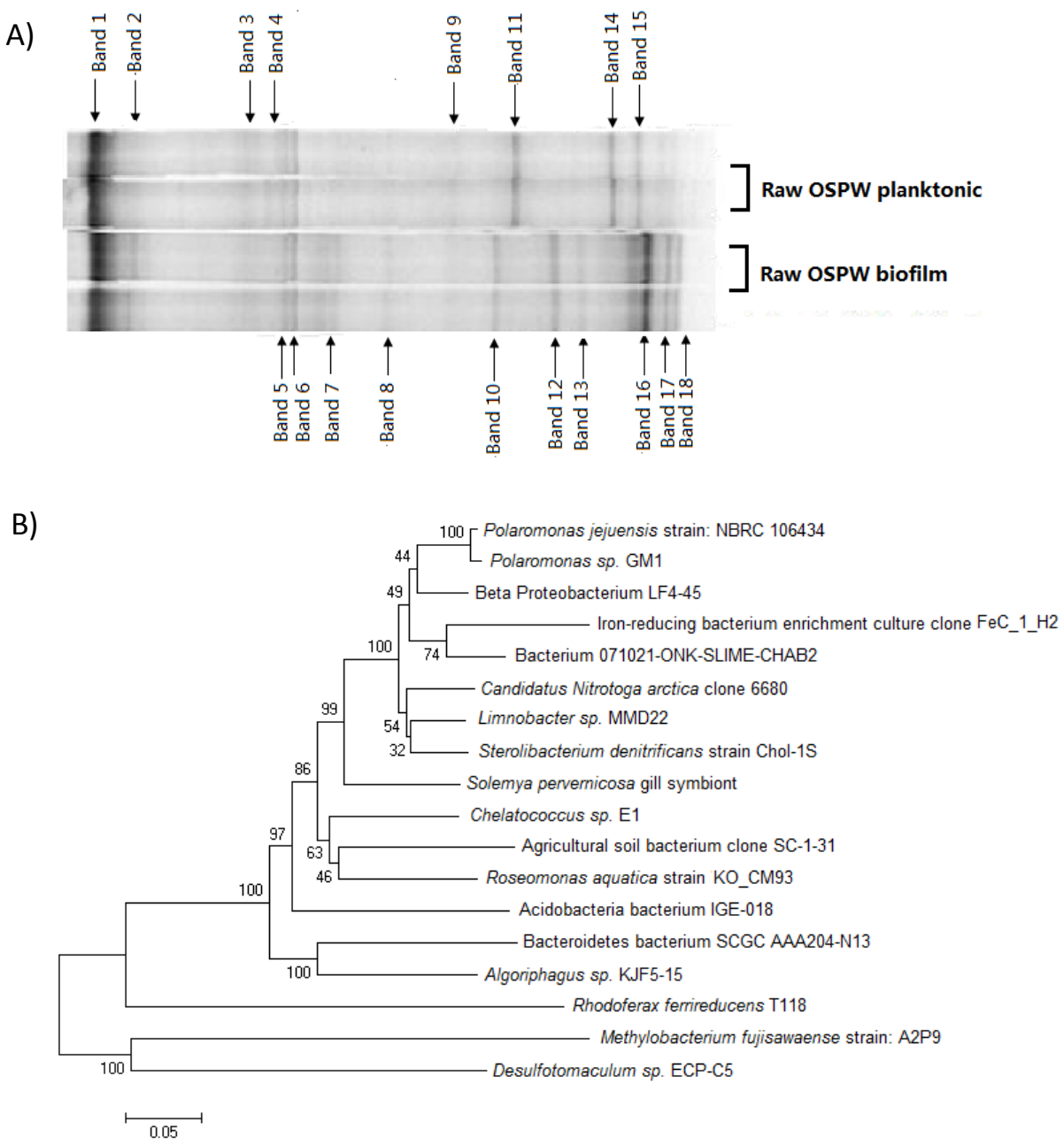

Figure 3.5. Microbial community analysis: (A) DGGE profiles; (B) Neighbour-joining tree (with pairwise deletion treatment of gaps and p-distance substitution model) of 16S rRNA gene sequences of strains. Numbers (%) indicated at the nodes are bootstrap values based on 500 replicates. Scale bar: substitution per nucleotide.

Interestingly, seven new bands were identified in the biofilm in the bioreactor that were not detected in raw OSPW planktonic community, including several strains that can metabolize simple to complex organic carbons (Jiang et al., 2008; Kleinsteuber et al., 2008; Nedashkovskaya et al., 2005; Nishiyama et al., 2010). For instance, *S. denitrificans* Chol-1S (band 10) is a facultative cholesterol-degrading bacteria which can use oxygen or nitrate as the terminal electron acceptor for metabolism (Tarlera and Denner, 2003). This indicates that the environment in the bioreactor facilitated the enrichment of certain carbon degrading strains on the GAC support media. No information was available for *Bacteroidetes bacterium* SCGC AAA204-N13 (band 2), *Acidobacteria bacterium* IGE-018 (band 7), Bacterium 071021-ONK-SLIME-CHAB2 (band 12) and *Beta-proteobacterium* LF4-45 (band 13). However, it has been reported that the class *Acidobacteria* can metabolize simple to complex organic carbons (Kleinsteuber et al., 2008).

It should be noted that although PCR-DGGE is widely employed to assess microbial communities in environmental samples to determine the community dynamics in response to environmental variations, the method has several drawbacks. First, preferential amplification of rRNA genes with the PCR may lead to missing some original members of the community. Second, if multiple rRNA copies of a single species are displayed on the DGGE fingerprint, the community might be overestimated. Third, the DGGE band distribution might be affected by the formation of chimeric, or heteroduplex, molecules. Fourth, comigration of DNA fragments with identical melting behaviour can make it difficult to separate DGGE bands. Fifth, the separation of live and dead cells is not feasible with DGGE (Ercolini, 2004; Hwang et al., 2013).

3.3.3. Batch Studies on GAC Adsorption

To estimate the adsorption capacity of the GAC used in our bioreactor, a batch isotherm test was conducted. Freundlich-type COD isotherm for OSPW samples in our experiments is shown in Figure C2 (Appendix-C) and the Freundlich constant K_f and slope 1/n were 3.5 and 0.62, respectively. Based on the adsorption equilibrium isotherm parameters, the maximum COD removal was estimated to be 120.85 ± 3.01 mg/g of GAC for OSPW using Freundlich equation as described in Appendix-A1 (Scharf et al., 2010). From Figure 3.2A, the cumulative COD removal in 11^{th} day (6209.6 mg) was already above the maximum COD adsorption for the GAC column (6042.5 ±150.5 mg), which suggested that bioregeneration and biodegradation played important roles in FBBR system. From Figure 3.3, new classical NAs species with lower negative integer of Z number (Z = 0 and -2) were observed in the effluent of the bioreactor, indicating the microbial activity in the bioreactor. Several studies have demonstrated that biodegradation can lead to the production of new fatty acids or derivatives (Johnson et al., 2010; Whitby, 2010). In addition, classical NAs component with Z = - 4 (n = 11) has 43.2% more than its concentration in raw OSPW after treatment, suggesting that biofilms in bioreactor have the capability to decompose organic compounds with high molecular weights into those of lower molecular weight through the processes of α-, β- oxidation and aromatization (Johnson et al., 2011; Quagraine et al., 2005; Rontani and Bonin, 1992). Additional experiments are needed to investigate the dynamic biodegradation and bioregeration in the bioreactor.

3.3.4. Overall Impacts of FBBRs Operational Conditions on Organic Compounds Removal from OSPW

In the bioreactor, a change in feed flow rate (associated with the OLR) had a higher impact on the removals of COD, AEF, and classical NAs than a change in the recycle flow rate (associated with the HLR) (Table 3.1). Changes in OLRs can be used to evaluate a bioreactor's capacity to handle shock loading and its capacity to degrade and remove organic pollutants. A low OLR can cause a deficiency of organic substrates for microorganism growth; a decrease in microbial growth rate will reduce the biological treatment efficiency. A high OLR can decrease the efficiency (%) of organic compound removal (Aygun et al., 2008; Mann et al., 1999); however, it can increase the mass removal rate of organics. In our study, optimal organic removal efficiency was observed at the lowest OLRs with the least mass removal rate of organics. The fact that a high removal efficiency of organic compounds was not maintained under a high OLR although higher mass removal rate of organics was observed at high OLR might be attributed to the low biodegradability of OSPW components.

Changes in the superficial flow rate can also impact bioreactor performance by affecting the shear stress on GAC surfaces causing biomass density variations (Liu and Tay, 2002). In our study, we observed biofilm formation on the GAC surfaces in phase V by naked eye when the reactors were operated at a low flow rate (60 mL/min). However, the biofilm on the GAC surface was not visible (by naked eye) for other modes of operation of the biofilm reactor. When the bioreactor flow rate increased from 60 (phase V) to 260 mL/min (phase VI), a considerable amount of biofilm was detached from the GAC causing high turbidity in the bioreactor effluents. This indicated that at low shear stress (phase V) thicker, heterogeneous, porous, and weaker structured biofilm with less bacteria concentration was developed on the solid surfaces (GAC)

when the applied shear force was weak (Liu and Tay, 2002), which (weaker structured biofilm) detached from the carrier with the increase of shear stress in phase VI (Herzberg et al., 2006; Liu and Tay, 2002). Therefore, changes in operational conditions in our study led to alterations in biofilm thickness, biomass density, and even microbial diversity; resulting in a different extent of organic compound removal.

It should also be noted that the raw OSPW bioreactor performance in phase VI was lower than the performance in phase I (Table 3.1), although the reactor operational conditions were the same in the two phases. The reduced COD removal rate in phase VI might have been caused by enhanced sheer forces imposed during the change of reactor operational conditions, which caused biofilm sloughing and thus a reduced biomass concentration in phase VI as compared to phase I. In addition, the organic adsorption capacity of GAC in phase VI might have been lower than that in phase I. It is reported that the adsorption capacity of GAC may decrease with bioreactor operating time due to the accumulation of metabolic by-products and extracellular polymeric substances on GAC surfaces (Aktas and Cecen, 2007) which have an impact of reduced simultaneous adsorption and biodegradation compared to fresh GAC in phase I.

3.4. Conclusions

The FBBR reactor employed in the present study removed classical NAs and other organic pollutants in OSPW through the simultaneous GAC adsorption and biodegradation/GAC regeneration mechanisms. Endogenous populations of microorganisms in OSPW can quickly form biofilms on GAC surfaces. Different carbon degraders observed in the biofilms and the production of new classical NAs species confirm the presence of microbial activity in the GAC-bioreactor. Further UPLC/HRMS analysis showed that classical NAs removal increased as the

classical NAs carbon number increased in the FBBR with GAC as media. With a maximum reduction of classical NAs by 96%, the GAC-biofilm seems to be a promising treatment method for OSPW remediation.

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CHAPTER 4. IMPACT OF OZONATION PRE-TREATMENT OF OIL SANDS PROCESS-AFFECTED WATER ON THE OPERATIONAL PERFORMANCE OF A GAC-FLUIDIZED BED BIOFILM REACTOR[1]

4.1. Introduction

The large amount of wastewater produced during the surface mining extraction of bitumen used by the Alberta oil sands industry has become an emerging problem (Allen, 2008). This wastewater is known as oil sands process-affected water (OSPW) and is currently retained in large tailings ponds in the oil sands region. Naphthenic acids (NAs) are a group of naturally-occurring acyclic, monocyclic, and polycyclic carboxylic acids that have been historically considered as the primary toxic compound of OSPW (He et al., 2010). The NAs have a general formula of $C_nH_{2n+Z}O_x$, where n is the carbon number, Z is zero or a negative even integer, and x represents the total oxygen atoms (classical NAs are x = 2; oxidized NAs are x = 3 to 5) (Barrow et al., 2010; Grewer et al., 2010; Wang et al., 2013). The NAs have a known toxicity to a variety of organisms including zooplankton (Clemente and Fedorak, 2005), phytoplankton (Leung et al., 2003), fish (He et al., 2012), bacteria (Gamal El-Din et al., 2011), invertebrates (Anderson et al., 2012), birds (Gentes et al., 2006), plants (Armstrong et al., 2010) and mammalian species (Garcia-Garcia et al., 2011). Although many different treatments have been investigated for their ability to remove NAs from OSPW, most of the approaches have shown poor efficiency for

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removal of the most recalcitrant compounds. Additionally, the relatively high costs of many of these treatments may limit their applications in large-scale industrial treatments (Kim et al., 2011).

Ozonation has been shown to be capable of breaking the bonds of highly branched and cyclic carboxylic fraction of NAs and of reducing the toxicity of OSPW (Pérez-Estrada et al., 2011; Scott et al., 2008). Recent studies have shown that ozone can increase the overall biodegradability of OSPW and accelerate the biodegradation of NAs present in OSPW (Gamal El-Din et al., 2011; Hwang et al., 2013; Martin et al., 2010; Wang et al., 2013). In addition, ozonated OSPW was less toxic than raw OSPW to mice (Garcia-Garcia et al., 2011), fathead minnows (He et al., 2012), *Vibrio fischeri* (Gamal El-Din et al., 2011), and larvae of *Chironomus dilutus* (Anderson et al., 2012). However, due to the recalcitrance of many OSPW NAs there is residual toxicity that must be removed from the OSPW to allow its eventual release to the environment. Treatment using biodegradation can be an economical, energy-efficient, and environmentally protective approach for tailings water remediation. However, the pre-treatment of raw OSPW, using a process such as ozonation, may be required to improve the biodegradability of its constituents before entering a biological treatment process (Scott et al., 2005).

Combinations of ozonation and biofilm-based engineered bioreactors (biofilters) have been successfully used to treat drinking water and industrial wastewaters resulting in the reduction or elimination of a large variety of recalcitrant organic compounds (Reid et al., 2007; Yapsakli et al., 2010). Previous studies suggest that there is a wealth of microbial diversity in oil sands tailings (Siddique et al., 2011; Golby et al., 2012), however, the majority of combined ozonation and biodegradation research specifically addressing NAs in OSPW has focused on the

application of planktonic cells via batch bioreactor studies (Gamal El-Din et al., 2011; Martin et al., 2010; Wang et al., 2013a). So far, two studies (Hwang et al., 2013; Choi et al., 2014) have attempted to address the potential application of endogenous OSPW microorganisms by applying centre for disease control (CDC) biofilm reactors. These biofilm reactors provided NAs removal of less than 20% (Hwang et al., 2013; Choi et al., 2014) at high hydraulic retention times (19 h), which indicated their inapplicability for the treatment of OSPW. However, the use of ozonation followed by media-based engineered bioreactors, as in the current study, has not been considered previously for OSPW treatment.

The use of granular activated carbon (GAC) as the media for a biofilm process has been widely used for the removal of organics for both drinking water and wastewater treatment (Liang et al., 2007). In addition to allowing for a biofilm formation, the GAC also acts an adsorptive media for removal of organic compounds. The GAC provides a suitable surface for microbial colonization given the availability of organic substrates and nutrients at the surface due to its high adsorption capacity (Gibert et al., 2013). In addition, the microbial colonization is further increased from the rough and pitted surface of GAC which provides shelter to microorganisms from fluid shear forces (Yapsakli and Cecen, 2010). Microorganisms use sorbed organics on the GAC surface as a source of food and energy which allows for the regeneration of the GAC for further adsorption (Nath and Bhakhar, 2011). The ability of GAC to be bioregenerated in biofilm reactors acts to enhance organics removal well beyond only the adsorption capacity which would normally be exhausted and need to be regenerated in the absence of this bioregeneration (Aktas and Cecen, 2007).

The objective of this study was to determine the degradation of organic compounds and the fate of NAs in fluidized bed biofilm reactors (FBBR) with GAC as support media.

Additionally, the role of ozonation on the removal of organic compounds prior to the FBBR treatment process, the general investigation of the changes in the microbial community structures during various operating conditions, and the overall performance of FBBR for overall organic compound removal were also investigated.

4.2. Materials and Methods

4.2.1. Source of OSPW

The OSPW samples were stored at 4 $^{\circ}$C before subjected to any treatment as received in 200 L polyvinyl chloride barrels from an individual OSPW recycle pond site in Fort McMurray, Alberta, Canada in October 2010.

4.2.2. Ozonation of OSPW

OSPW was ozonated following the procedure described elsewhere (Wang et al., 2013b). OSPW was ozonated with an ozone generator (C, GSO-40, Herford, Germany) using extra dry and high purity oxygen. A 200 L plastic barrel was equipped with a ceramic fine bubble gas diffuser located at the bottom of the reactor which was used to sparge the feed gas into the OSPW. The schematic of ozonation of raw OSPW is shown in Figure 4.1. During OSPW ozonation, the ozone concentrations in the feed and off-gas lines were continuously monitored by two identical ozone monitors (model HC-500, PCI-WEDECO). The utilized ozone dose was calculated using the following equation (Gamal El-Din et al., 2011):

$$\Delta O_3 = \int_0^t \frac{\left(Q_{G,in}C_{G,in} - Q_{G,out}Q_{G,out}\right)}{V_L} dt - C_L \qquad (4-1)$$

where ΔO_3 is the ozone concentration (mg/L) in the ozonated product; $C_{G,in}$ is the ozone concentration (mg/L) in the feed gas; $C_{G,out}$ is ozone concentration (mg/L) in the off gas; C_L is the residual ozone concentration (mg/L); V_L is the effective reactor volume (L); $Q_{G,in}$ is the feed-gas flow rate (L min); $Q_{G,out}$ is the off-gas flow rate (L/min); and t is the ozone contact time (min). After treatment with ozone, the residual ozone in the OSPW was purged using pure nitrogen and the ozone reactor outlet monitor reading was recorded. The Indigo method (APHA, 2005) was used to estimate the residual ozone remaining in the OSPW after purging. The effect of ozone dose on the water quality of OSPW and its biodegradability was evaluated in batch studies prior to full-scale experiments. In order to enhance the performance of FBBR, an optimal utilized ozone dose was determined to ozonate raw OSPW as a pre-treatment prior to biodegradation. The chemical characterization of raw and ozonated OSPW are shown in Table B1 (Appendix B).

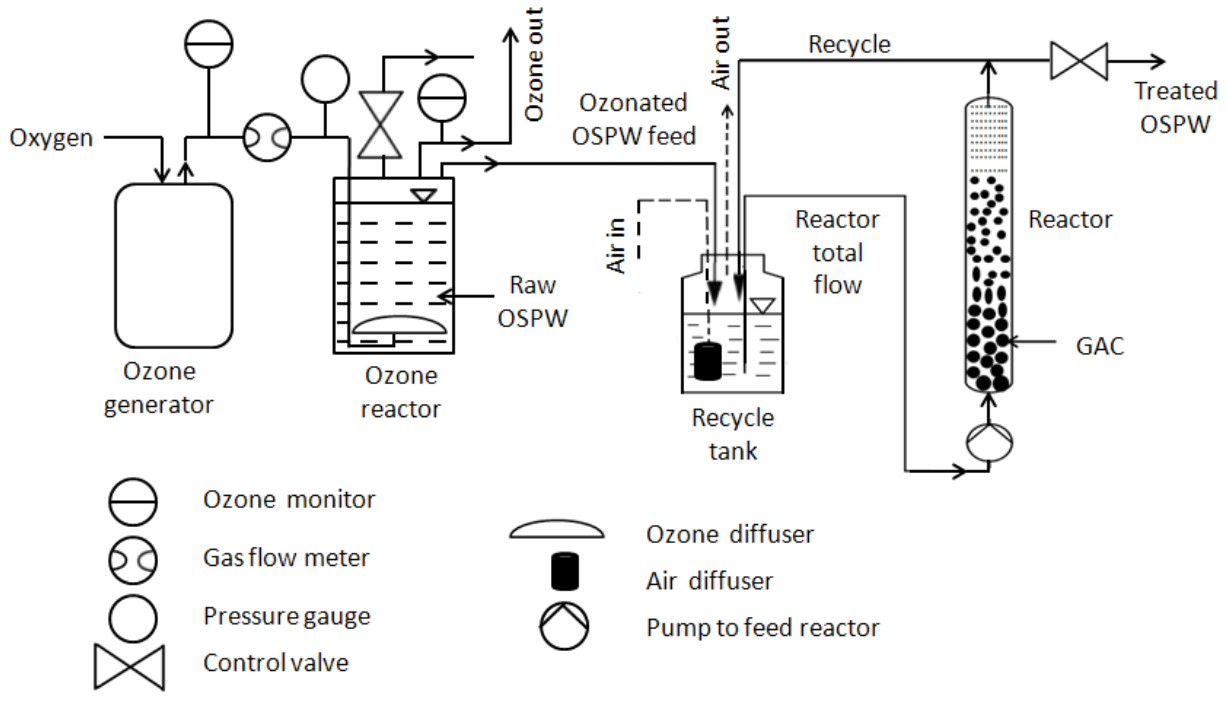

Figure 4.1. Schematic of combined ozonation and GAC-FBBR system.

4.2.3. Configuration and Operation of FBBR

A clear acrylic cylindrical bioreactor of diameter 2.5 cm (Figure 4.1) and height 70 cm was applied to treat ozonated OSPW. A 50 mesh stainless steel grid was set at the bottom to evenly distribute the influent and to hold GAC media inside the reactor. 50 g of a high activity steam activated GAC (Aquasorb® 1500) was used having a total pore volume of 0.99 cm^3/g and specific surface area of 1,050 m^2/g.

The reactor was inoculated with endogenous OSPW microorganisms using the following protocol. A 2.5 L of ozonated OSPW was centrifuged (Multifuge3S/3S-R, Heraeus) at 3700 RPM for 30 min to remove ozonated OSPW microbes, and the supernatant was collected. Raw OSPW microorganisms were then collected as pellets from the 2.5 L of raw OSPW after centrifuging at the same operating condition. The raw OSPW microorganisms were inoculated in the bacteria-free ozonated OSPW supernatant. The bioreactor was operated for three days in batch mode circulating the inoculated ozonated OSPW. After this initial period, the bioreactor was operated continuously with ozonated OSPW (without centrifugation or inoculation) for 120 days at six different phases by changing organic loading rate (OLR) and hydraulic loading rate (HLR) as shown in Table 4.1.

4.2.4. Water Chemistry Analysis and Reactor Performance Evaluation

Physicochemical analysis including pH, DO, chemical oxygen demand (COD), 5 day biochemical oxygen demand (BOD_5) and acid-extractable fraction (AEF) were performed on raw OSPW, ozonated OSPW, and biotreated effluents. Standard methods (APHA, 2005 were followed to measure COD and BOD_5. Detailed procedures for the measurement of AEF, COD and BOD_5 have been included in Appendix-A. Carbonyl stretch equivalents, known as AEF, was

measured using Fourier transform infrared (FT-IR) spectroscopy (PerkinElmer® precisely, PerkinElmer Life and Analytical Sciences, ON, CA) following the standard protocol described in Appendix-A. Triplicate samples (n=3) were analyzed from influents and effluents during the reactor operation. The NAs concentrations in OSPW before and after both ozonation and bioreactor treatment were estimated (as a function of carbon and Z numbers) using ultra performance liquid chromatography-high resolution mass spectroscopy (UPLC-HRMS) Chapter 3.

4.2.5. Microbial Characterization in Bioreactor

Microorganisms in the raw and ozonated OSPW and from GAC biofilms were isolated according to the protocol described by Islam et al., (2014) (Chapter 3). A PowerSoil® DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA) was used to extract total genomic DNA from the samples by following manufacturer's protocol prior to PCR amplification. The bacterial 16S rRNA gene was amplified using GC-clamped forward primer 341f and reverse primer 907r, followed by denatured gradient gel electrophoresis (DGGE) to analyze the biofilm microbial communities. PCR products were loaded onto a 6.5% acrylamide gel with a 30–70% gradient solution of urea and formamide for gel electrophoresis at 180 V.

The sequenced data was analyzed using the protocol described in Chapter 3 and the protocol has been described below. Nucleotide BLAST program was applied for matching the sequenced data from the NCBI nr nucleotide database. Multiple sequence alignment was constructed using CLUSTALW. A neighbor-joining phylogenetic tree was built using identified strains by applying Mega 5.2.2 software. A p-distance substitution model with pairwise deletion

treatment of gaps was applied for calculating the phylogenetic distances between the strains. Tree topologies robustness was verified using bootstrap re-sampling (n = 500).

At the end of bioreactor operation, the GAC was sampled and fluorescent stained with SYTO 9 and Concanavalin A lectin conjugated with Texas Red. A confocal laser scanning microscope (CLSM) (Zeiss LSM 710, Carl Zeiss Micro Imaging GmbH, Germany) with a 20× 0.8NA Plan-Apochromat objective lens was used to take a fluorescent image of the biofilm. The biofilm structure and morphology after treatment was visualized by scanning electron microscope (SEM, S-2500, Hitachi Ltd., Japan) after fixation, dehydration and critical point drying.

For further details regarding DNA isolation, PCR-DGGE, CLSM and SEM methods have been provided in Chapter 3.

4.3. Results and Discussion

4.3.1. Impact of Ozonation on the Organic Compounds Removal from OSPW

The COD, NAs, and AEF concentrations varied among OSPW sample barrels (n = 4), and were in the ranges of 276 ± 33 mg/L, 39.2 ± 5.8 mg/L, and 68.1 ± 5.1 mg/L (avg. ± SD), respectively (Table 4.1). These results are comparable to the OSPW matrix characteristics reported in the literature (Gamal El-Din et al., 2011). Figure 4.2A shows that an increase in ozone dose led to a sharp rise followed by a slow increase in the removal of organic compounds as indicated by COD, NA and AEF. Ozonation also substantially enhanced the biodegradability of OSPW as indicated by an increasing BOD_5/COD ratio in response to an increasing ozone dose (Figure 4.2B) (Wang et al., 2013). Although the ratio continued to increase with the higher utilized ozone dose, the rate of increase of the BOD_5/COD ratio was much slower at utilized

ozone doses greater than 68 mg/L. The current ozonation results are consistent with those previous reported for the ozonation of phenolic pollutants (Amat et al., 2003).

Given the ozonation results and economic cost considerations, an ozone dose of 80 mg/L was chosen to improve the biodegradability of OSPW while avoiding redundant ozonation. After ozone treatment using 80 mg/L, the BOD_5/COD ratio increased from 0.01 to 0.07, indicating that the biodegradability of OSPW increased after ozonation (Wang et al., 2013). It should be noted that, even after ozonation, the OSPW was still not considered to be easily biodegradable as a BOD_5/COD ratio less than 0.3 is which is considered as the minimum easily biodegradable ratio (Alvarez-Vazquez et al., 2004). This utilized ozone dose of 80 mg/L resulted in the removal of approximately 33% of COD and 75% of AEF from raw OSPW (Figure 4.2) and 96.5% of NAs (Figure 4.4) which are comparable to reported ozonation removal values of 64% of AEF and 90% of NAs, respectively (Gamal El-Din et al., 2011). NAs with Z = 0 became the dominant fraction in ozonated OSPW (Figure 4.4B) although this fraction of NAs was absent in raw OSPW (Figure 4.4A). The results suggest that ozone is highly reactive to cyclic NAs with higher Z numbers, and that NAs with a linear structure (Z = 0) are formed during the ozonation process in agreement with results reported previously (Gamal El-Din et al., 2011; Martin et al., 2010; Wang et al., 2013). A higher percent removal of NAs and AEF in comparison to that of COD indicates that there is incomplete degradation of NAs and other compounds measured as AEF into smaller organic compounds that still contribute to the overall COD.

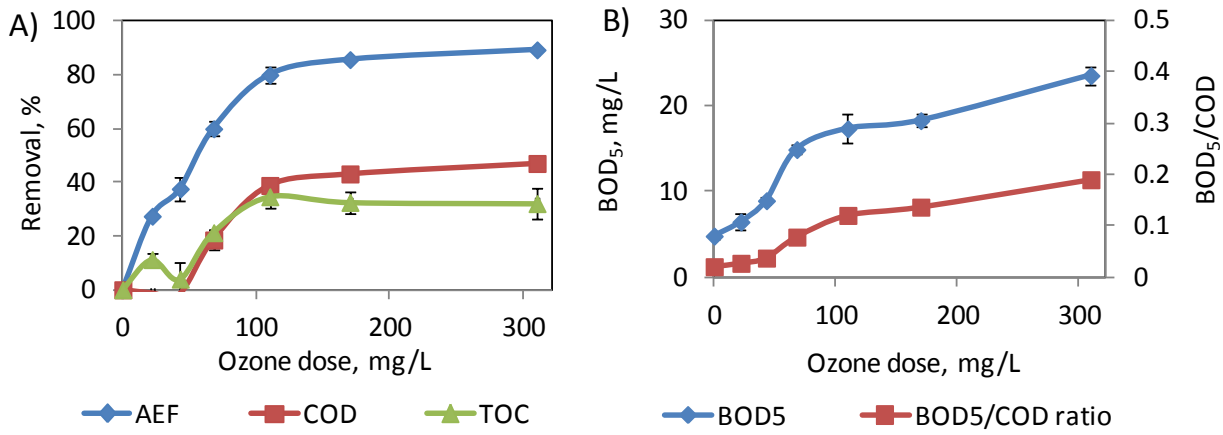

Figure 4.2. Effect of ozone dose on (A) the removal of organic compounds as indicated by AEF, COD and TOC; and (B) the biodegradability of raw OSPW as indicated by BOD_5 and BOD_5/COD ratio.

4.3.2. Organic Compounds Removal during FBBR Operation

4.3.2.1. The Effect of OLR on the Bioreactor Performance

The bioreactor performance was evaluated at different HLRs and OLRs by operating the bioreactor into six phases as shown in Table 4.1. The HLR (31.8 m/h) was constant during the bioreactor operational time for phases I, II and III, and the OLRs were changed by adjusting the feed flow rates of 0.6, 2.0, and 5.0 mL/min, OLRs for the bioreactor used to treat the ozonated OSPW were 0.48, 1.61, and 4.12 kg COD/m^3.d, respectively. Accordingly, AEF loading rates in the bioreactor used to treat ozonated OSPW were 0.06, 0.2, and 0.51 kg AEF/m^3.d, respectively.

The bioreactor was operated at OLR of 1.61 kg COD/m^3.d in phase I (0–38 days) and a more than 70% COD removal rate was obtained in the first 5 days (Figure 4.3C). GAC adsorption would be the major mechanism for COD removal in this phase because of low bacteria concentrations on the GAC and subsequent lack of biodegradation by an underdeveloped biofilm (Wang et al., 2007). The COD removal rate became steady after 20 days

for ozonated OSPW with the COD removal rate in the bioreactor of 41 ± 3% (Figure 4.3C). By using a Freundlich isotherm of COD removal in a batch adsorption trial experiment, the theoretical maximum COD removal was estimated via the y-axis intercept (Figure 4.7). Using this isotherm, the theoretical maximum COD removal for the bioreactor experiments was estimated to be 127 ± 3.2 mg/g of GAC for the ozonated OSPW. Based on Figure 4.3A, the cumulative COD removal by day 21 (6516 mg) was already above the theoretical maximum COD adsorption for the GAC column (6333 ± 161 mg), which suggested that bioregeneration and biodegradation were starting to play an important role in the FBBR system prior to day 21.

The performance of the bioreactor was inspected at low OLR in phase II of the bioreactor operation (39–50 days). The OLR was reduced to 0.48 kg COD/m^3.d by reducing the bioreactor feed flow rate to 0.6 from 2 mL/min in (phase I) for the ozonated OSPW. Consequently, a spike in the bioreactor COD removal efficiency was observed, which reached a steady state condition within 4 days of 45 ± 4%. The impact of high OLR on the performance of the bioreactor was inspected in phase III of the bioreactor operation (50–60 days) by increasing the bioreactor OLR to 4.12 kg/m^3.d. As a result, the bioreactor performance was decreased with the COD removal efficiency declining to 21 ± 2%. Overall, the highest COD removal efficiency was achieved at the lowest OLR in Stage II (0.48 kg COD/m^3.d), and the overall COD removal (ozonation followed by bioreactor treatment) was 62 ± 4% (Table 4.1).

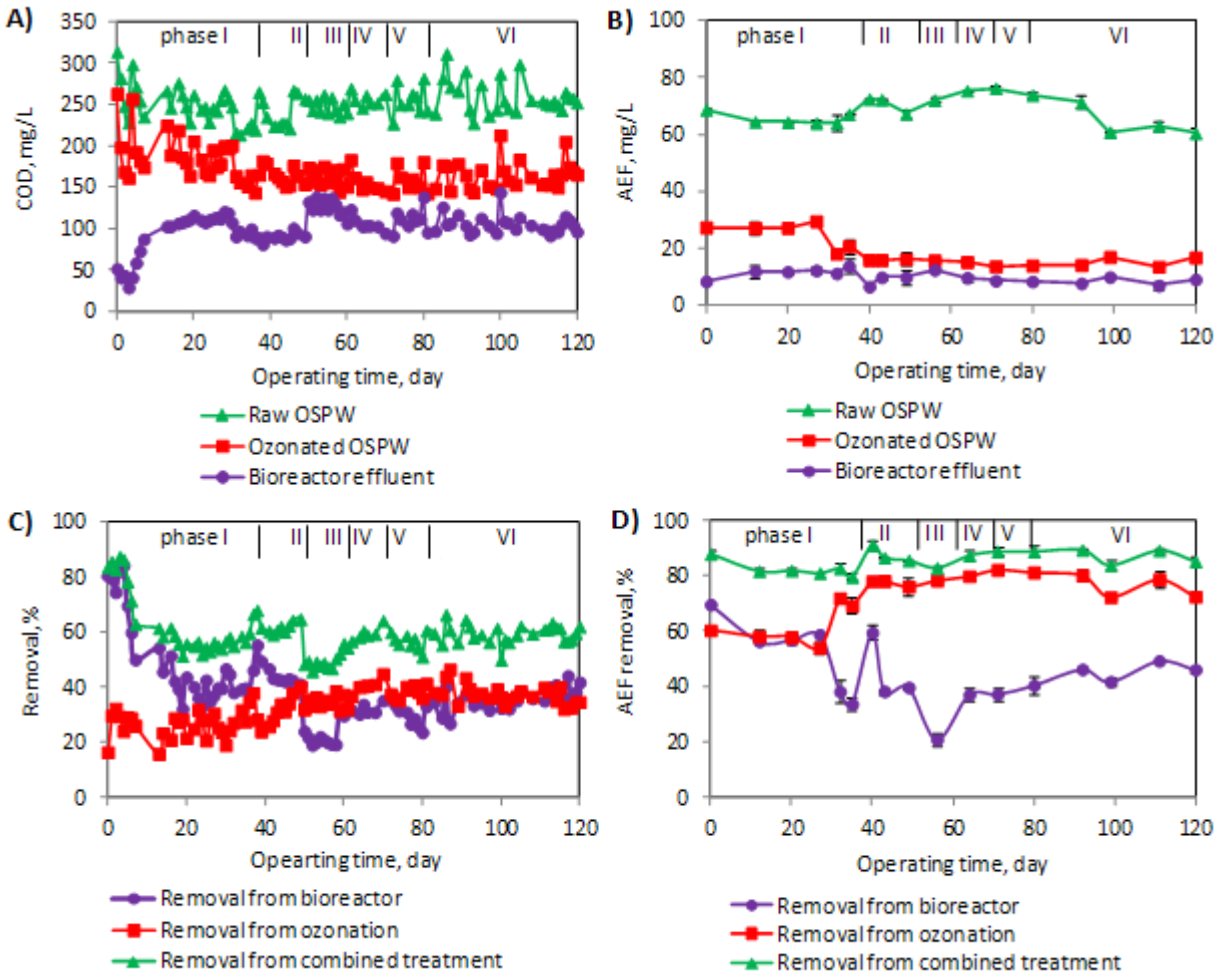

Figure 4.3. The concentrations of (A) COD and (B) AEF for raw, ozonated and biotreated OSPW; and percent removal of (C) COD and (D) AEF by ozonation, bioreactor (ozonated OSPW) and combined treatment during the 120 day experiment including six phases (I-VI). Triplicate samples (n = 3) were taken from the column and error bars indicate standard deviations.

AEF measurements include classical and oxidized NAs and other organics containing carboxylic acids, ketones, and aldehydes functional groups. Despite the non-specificity of this measure, AEF is commonly used by the oil sands industry to estimate the NAs concentrations (Jivraj et al., 1995). Currently, the highest AEF removal efficiency of 59 ± 7 % was achieved at the lowest AEF loading rate of 0.06 kg AEF/m^3.d (Table 4.1). The AEF removal efficiency in the bioreactor was decreased to 21 ± 6% at the highest AEF loading rate (0.51 kg/m^3.d in phase III). The highest AEF removal efficiency was achieved with ozonation followed by GAC-bioreactor treatment was 88 ± 2%. Overall, the AEF removal was higher than the COD removal which may be explained by the fact that biodegradation converted organic compounds with carbonyl group (C=O) to other forms not measured by AEF, but still contribute to COD since they are not fully mineralized.

Table 4.1 shows the bioreactor removal efficiencies of NAs of ozonated OSPW. The influent NAs concentration ranged from 0.04 to 1.48 mg/L. The lowest removal efficiency of NAs (20%) occurred at the highest OLR while the highest removal (98%) observed at the lowest OLR during the bioreactor operation (Table 4.1). After the ozonation followed by bioreactor treatment, the combined removal efficiency of NAs was 99.5–99.9%.

Based on these findings, it is apparent that the removal of organic compounds for ozonated OSPW decreased with increasing OLRs in the bioreactor which might be attributed to the generally low biodegradability of OSPW components.

Table 4.1. Bioreactor operational conditions and COD, AEF, and NAs removal efficiencies ([a] feed flow rate + recycle flow rate; [b] hydraulic loading rate; [c] organic loading rate; [d] performance of bioreactor for ozonated OSPW; [e] performance of combined ozonation and bioreactor treatment for raw OSPW)

Operating phase	Operating time (days)	Flow rates (mL/min)			HLR[b] (m/h)	OLR[c] (kg COD/m^3-d)	Bioreactor treatment efficiency[d] (%)			Combined (ozone + bioreactor) treatment efficiency[e] (%)		
		Feed	Recycle	Reactor[a]			COD	AEF	NAs	COD	AEF	NAs
I	0-38	2.0	258	260	31.8	1.61	41	36	82	56	81	99.9
II	38-50	0.6	259.4	260	31.8	0.48	45	59	98	62	88	99.9
III	50-60	5.0	255	260	31.8	4.12	21	21	32	48	83	99.9
IV	70-80	2.0	128	130	15.9	1.61	32	37	50	58	86	99.9
V	60-70	2.0	58	60	7.3	1.61	32	37	20	56	85	99.9
VI	80-120	2.0	258	260	31.8	1.61	38	46	84	60	86	99.5

4.3.2.2. The Effect of HLR on the Bioreactor Performance

The HLR influences the bioreactor flow rate (i.e., the summation of recycle and feed flow rates) and the fluidization of the media particles in the bioreactor. Currently, the HLR was varied by changing the recycle flow rate. For phases I, II and III the HLR was kept constant at 31.8 m/h. Phases IV (60–70 days), V (70–80 days), and VI (80–120 days), OLRs were operated at a fixed OLR of 1.61 kg COD/m^3.d, while the HLRs were varied to 15.9, 7.3, and 31.8 m/h for phases IV, V, and VI, respectively. The results showed that COD and AEF removal did not change markedly when HLR decreased to 7.3 m/h from 15.9 m/h. In contrast, when the HLR was increased to 31.8 m/h the removal of both COD and AEF increased by approximately 10%, respectively. NA removal in the bioreactor was considerably improved with the increase of HLR to 31.8 m/h from 7.3 m/h. The improved organic removal at high HLR might be caused by the increased recycle flow rate, which could facilitate the adsorption of organic compounds on GAC surfaces and the nutrient uptake by biofilm microorganisms. The NAs are a group of organic compounds having high molecular weight, long chains and more cyclic compounds with branching, which make the NAs more hydrophobic as compared to other organic compounds present in OSPW measured as part of the AEF and COD concentrations. Therefore, NAs are more sensitive to the change of HLR as compared with the general compounds measured as AEF and COD. In addition, more than 96.5% of the NAs were removed through ozonation and the reactor performance was reported based on the remaining NAs (1.48 mg/L). Thus, a small change in concentration of NAs in the bioreactor indicated a higher percentages NAs removal which might not be a true indicator of the impact of the OLR and HLR on bioreactor performance. However, our overall results indicate a change in the recycle flow rate (associated

with the HLR) had less impact on the COD removals than a change in feed flow rate (associated with the OLR).

4.3.2.3. The Fate of NAs in Bioreactor

The summation of influent and effluent NAs concentrations in the bioreactor treating ozonated OSPW on day 120 were 1.48 mg/L (Figure 4.4B) and 0.24 mg/L (Figure 4.4C), respectively. The percent removal of NAs in ozonated OSPW treated by the GAC-bioreactor was 84.1%. Formation of newly oxidized NAs having small carbon numbers (n= 7-13) resulted from the biodegradation of NAs in ozonated OSPW (Figure 4.4C). However, complete removal of NAs with Z = -10 and -12 was observed in the bioreactor which might be the result of continuous adsorption and biodegradation. No further removal of NAs with Z = -6 and -8 was observed following the bioreactor process, which may indicate a conversion via biodegradation of NAs with Z = -10 and -12 to oxidized NAs with Z = -6 and -8 having lower carbon numbers (Figure 4.4C). The removal of NAs with Z = 0, -2 and -4 were 94.5, 55.1, and 31.3%, respectively, suggesting that NAs with low cyclicity were more biodegradable as suggested previously (Gamal El-Din et al., 2011; Martin et al., 2010).

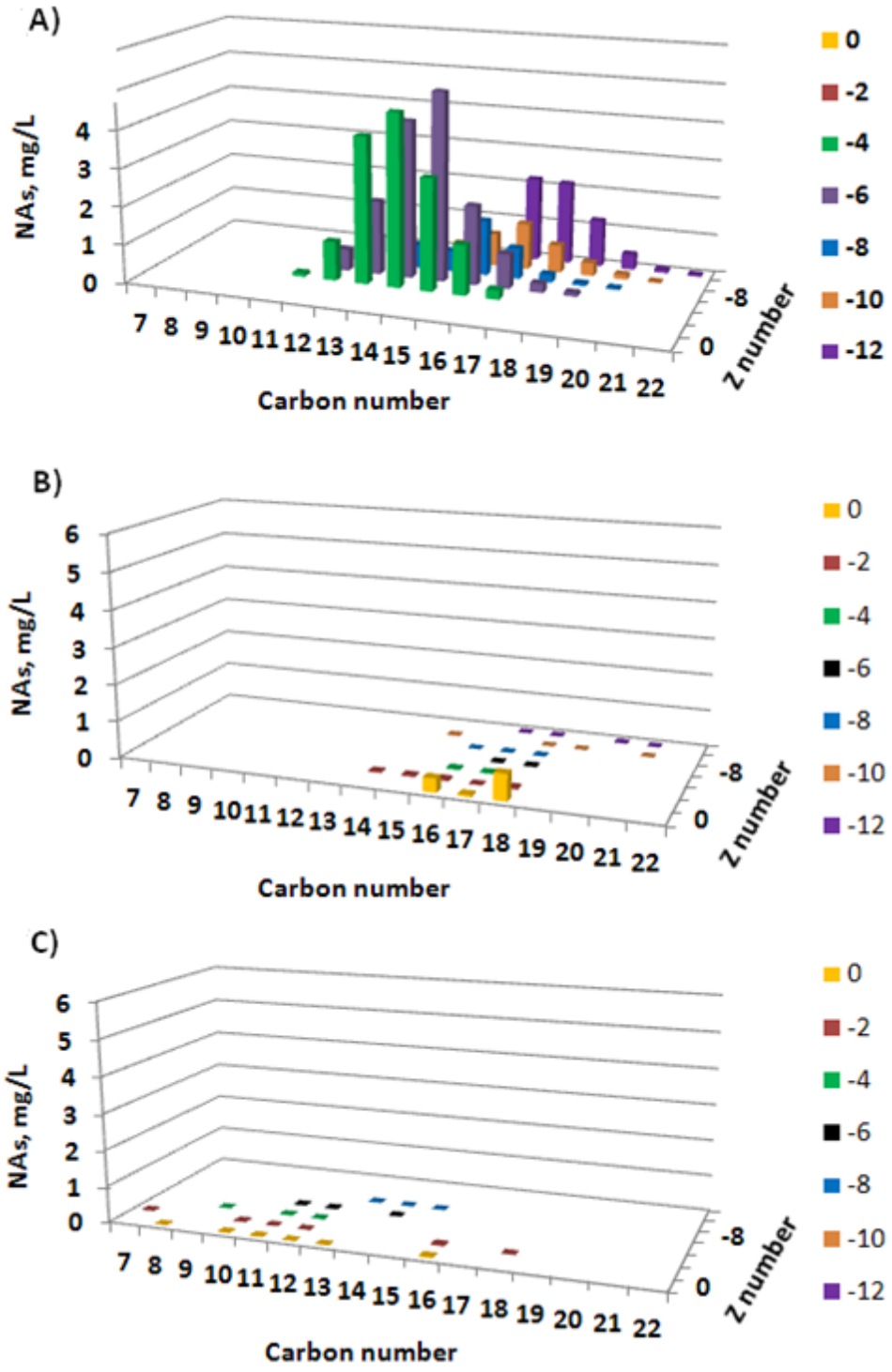

Figure 4.4. NAs concentration profiles of (A) raw OSPW; (B) ozonated OSPW; and (C) bioreactor effluent on sampling day 120.

4.3.3. Microbial Community Analyses

The biofilm developed on the GAC surface was examined by using SEM (Figure 4.5) and CLSM (Figure C3, Appendix-C) after 120 days of bioreactor operation. Compared with clean GAC (Figure 4.5A), there was high biofilm coverage on GAC surfaces (Figure 4.5B). The average biofilm thickness was 19 ± 2 μm with 8.5 mg dry biomass per gram GAC. Using these biofilm thicknesses and dry biomass, the dry biomass density (X_f) was calculated as 87.2 ± 8.6 g/L, which is in the range of values previously reported (Ro and Neethling, 1991).

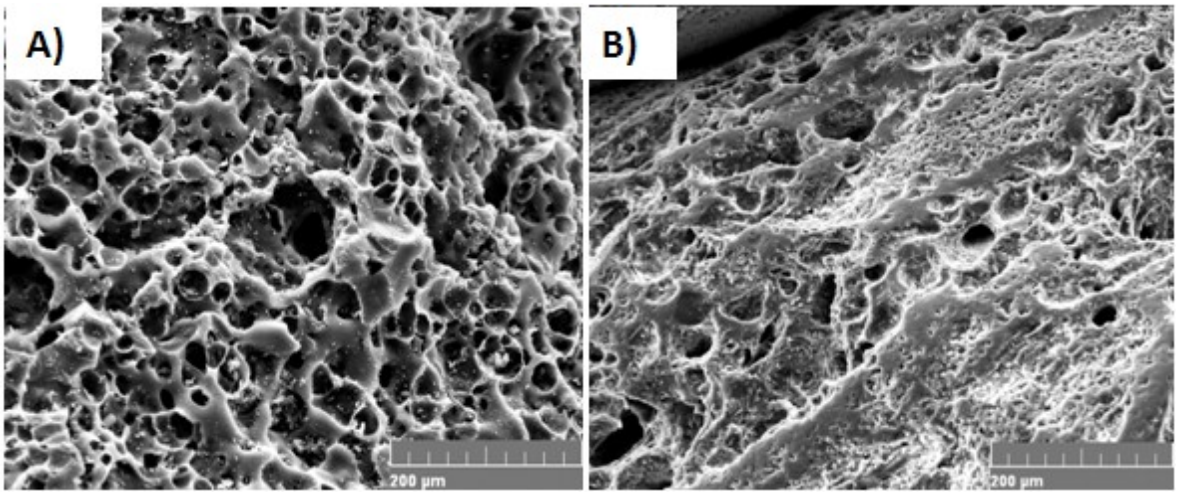

Figure 4.5. Scanning electron microscope (SEM) images of GAC before (A) and after (B) biofilm formation.

Based on the fingerprints of DGGE bands, planktonic microorganisms from ozonated OSPW were markedly different from raw OSPW planktonic organisms (Figure 4.6A), indicating that ozonation caused a noticeable change in the microbial community. Interestingly, a high similarity between the bands for planktonic microorganisms from raw OSPW and the bands

observed for biofilms in the bioreactor treating ozonated OSPW was observed. The use of raw OSPW microorganisms as inoculants during the bioreactor start up for the treatment of ozonated OSPW might be the plausible reason for this similarity. It is demonstrated previously that the similarity between the bands for planktonic microorganisms from raw OSPW and the bands observed for biofilms in the bioreactor shows the resilience of the biofilm communities during the bioreactor operation (Hwang et al., 2013).

A total of 21 bacterial strains were identified from DGGE bands (Figure 4.6B). The bacterial community comprised of *β-Proteobacteria* (8 bands), *α-Proteobacteria* (5 bands), *Bacteroidetes* (3 bands), *γ-Proteobacteria* (1 band), *Acidobacteria* (1 band), *Firmicutes* (1 band), and unclassified bacterial strains (2 bands). Among the strains, the dominant bacterial fractions were carbon degraders with the ability to degrade a wide range of organic compounds (discussed below). The phylogenic tree of bacterial members is shown in Figure 4.6B, which was supported by high bootstrap values (most nodes received bootstrap values of 100%). At the phylum level, microorganisms identified in the present study were in agreement with the dominant species in OSPW reported in previous studies (Golby et al., 2012; Siddique et al., 2011). An excellent cluster with a 100% bootstrap value were observed among the three *Polaromonas* species (band 1, band 4 and 13) while the two *Roseomonas* species (band 17 and 18) strains belonging to the α-*Proteobacteria* also form a cluster with a 100% bootstrap value. Although, *Methylobacterium fujisawaense* strain (band 19) and *Desulfotomaculum sp.* (band 20) were clustered, they showed larger genetic distances than those observed for the other stains.

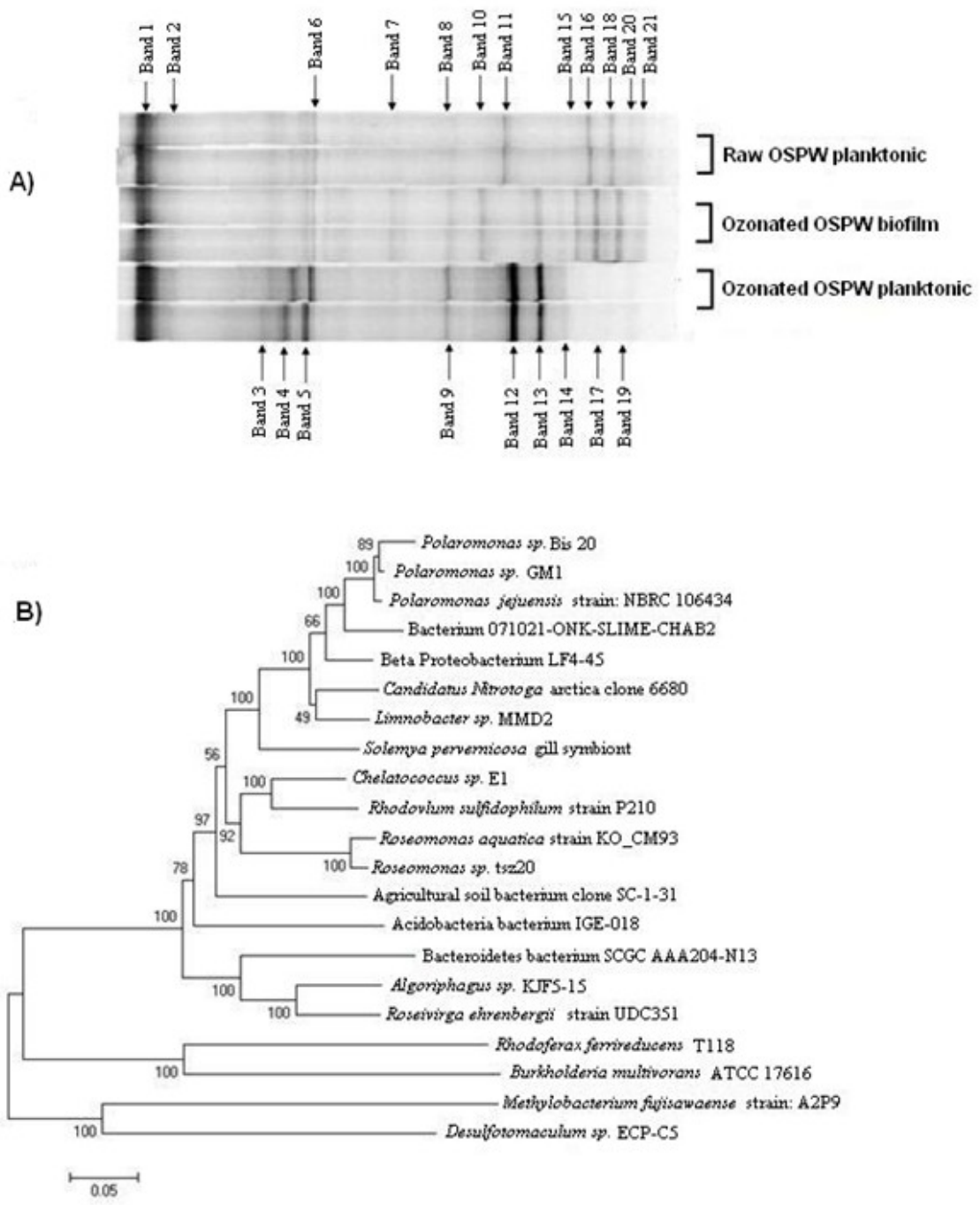

Figure 4.6. Microbial community analysis: (A) DGGE profiles; (B) Neighbour-joining tree (with pair wise deletion treatment of gaps and p-distance substitution model) of 16S rRNA gene sequences of strains. Numbers (%) indicated at the nodes are bootstrap values based on 500 replicates. Scale bar: substitution per nucleotide.

As shown in Table B5 (Appendix-B), a total of 15 bands were identified for planktonic microorganisms, of which 11 bands were associated with raw OSPW planktonic microorganisms and 9 bands were associated with ozonated OSPW planktonic microorganisms. The raw OSPW bacteria *Polaromonas jejuensis* NBRC 106434 (band 1) (Weon et al., 2008), the genus *Algoriphagus* (band 5) (Alegado et al., 2011), *Chelatococcus sp.* E1 (band 6) (Jeon and Kim, 2013) and *Methylobacterium fujisawaense* (band 19) (Madhaiyan et al., 2006) can utilize a wide range of carbon sources. *Polaromonas sp.* (band 4) was demonstrated to have the capability of biodegrading petroleum hydrocarbons and chlorinated solvents (Mattes et al., 2008). The genus *Limnobacter* (band 9) is a thiosulfate oxidizer which uses carboxylic and amino acids as energy and carbon sources have been reported earlier (Lu et al., 2011). *Candidatus Nitrotoga* arctica clone 6680 (band 12) was identified as nitrite oxidizing bacteria active at low temperature (Alawi et al., 2007). Both iron (III) reducer strain *Rhodoferax ferrireducens* T118 (band 3) (facultative anaerobe) (Finneran et al., 2003) and sulfate reducer *Desulfotomaculum sp.* ECP-C5 (band 20) (Pikuta et al., 2000) can utilize organic carbon as an electron donor.

Four bands were identified as planktonic microorganisms in the ozonated OSPW that were not observed in influent raw OSPW. *Polaromonas sp*. Bis 20 (band 13) is able to utilize alkane, chloroalkane, and aromatic and polycyclic compounds, (Mattes et al., 2008) but no information was available in the literature regarding the other three bacterial strains: *Bacteroidetes bacterium* SCGC AAA204-N13 (band 2), Bacterium 071021-ONK-SLIME-CHAB2 (band 14), and *Beta proteobacterium* LF4-45 (band 15). Bands that disappeared after ozonation of OSPW were identified as *Chelatococcus sp.* E1 (band 6), *Candidatus Nitrotoga* arctica clone 6680 (band 12), *R. aquatica* strain KO_CM93 (band 17), *Methylobacterium fujisawaense* A2P9 (band 19), *Desulfotomaculum sp.* ECP-C5 (band 20), and agricultural soil

bacterium clone SC-I-31 (band 21). The findings from raw and ozonated OSPW planktonic community analyses suggest that ozonation can selectively kill microbial strains with the resultant reduction in bacterial diversity. However, ozonation can increase OSPW biodegradability, which might enhance the growth of certain strains that degrade ozonation by-products which may not be dominant in raw OSPW.

As shown in Figure 4.6 and Table B5 (Appendix-B), a total of 13 strains were identified in the ozonated OSPW biofilm. Interestingly, six new bands were identified that were not detected in the raw and ozonated OSPW planktonic communities. This indicates that the environment in the ozonated OSPW bioreactor facilitated the enrichment of these strains on the GAC support media. Among the new bands, *Solemya pervernicosa* gill symbiont (band 8) and *Rhodovulum sulfidophilum* P210 (1) (band 11) are sulfur, sulfide, and thiosulfate oxidizers (Appia-Ayme et al., 2001; Stewart and Cavanaugh, 2006). *Roseivirga* sp. (band 9) is a Gram-negative, strictly aerobic bacterium that can decompose organic carbons (Nedashkovskaya et al., 2005). *Burkholderia multivorans* ATCC 17616 (band 16) is a three chromosome strain that can utilize a wide variety of carbon sources (Nishiyama et al., 2010) and *Roseomonas* sp. (band 18) can degrade organophosphorus compounds (Jiang et al., 2008). No information was available for *Acidobacteria bacterium* IGE-018 (band 7). However, the class *Acidobacteria* can metabolize simple to complex organic compounds (Kleinsteuber et al., 2008).

4.3.4. Overall Impact of Ozonation on FBBR Performance

Ozonation breaks down complex compounds into simpler compounds that are easier to degrade with subsequent biological processes. Ozonation at a utilized dose of 80 mg/L removed 96.5% of the total NAs, and the removals for specific *Z* values were 89%, 99.6%, 99.8%, 99.5%,

99.3% and 99.0% for *Z*= –0 (0 ring), –2 (1 ring), –4 (2 rings), –6 (3 rings), –8 (4 rings), −10 (5 rings) and −12 (6 rings), respectively (Figure 4.4B). However, the NAs with lower Z values (0 and -2) were formed during ozonation with concentrations of 1.14 and 0.05 mg/L, respectively (Figure 4.4B). The inclusion of the bioreactor further removed NAs with Z = 0, -2 and -4 by 94.5%, 55.1%, and 31.3% with no removal for NAs with $Z \leq -6$. Therefore, ozonation can enhance biodegradation by reducing the cyclicity of NAs, thus creating more easily biodegradable NAs species (Gamal El-Din et al., 2011). In addition, ozonation has been shown to break the highly branched and cyclic carboxylic fractions of NAs which helps to mitigate the toxicity of OSPW (Pérez-Estrada et al., 2011; Scott et al., 2008).

Ozone is a strong oxidant which attacks organic contaminants either directly or indirectly, through generation of free radical intermediates (Hoigne and Bader, 1983). Ozonation could directly impact GAC adsorption by altering the adsorbate properties which influences the performance of FBBR. For example, the larger molecules in raw OSPW might be excluded from some pores of GAC, thus ozonation degrades these compounds into smaller sizes which can enhance the adsorption to GAC and the migration into GAC pores (Moreno-Castilla, 2004).

The theoretical maximum adsorption capacity of the GAC was estimated using a batch isotherm test for raw and ozonated OSPW samples. The Freundlich COD isotherms for the raw and ozonated OSPW samples are shown in Figures 4.7A and 4.7B. The Freundlich constant K_f and slope 1/n were 3.5 and 0.62, respectively, for raw OSPW; and were 3.2 and 0.66, respectively, for ozonated OSPW. Based on the isotherm parameters, the maximum COD removal was estimated to be 121 ± 3.0 mg/g of GAC for raw OSPW and 127 ± 3.2 mg/g of GAC for ozonated OSPW. Clearly, the adsorption capacity for the ozonated OSPW did not increase as expected based on creation of smaller molecules as discussed previously. In contrast, the

unchanged adsorption capacity may be attributed to the generation of smaller size organic compounds which are hydrophilic, thus, the increase of adsorbate hydrophilicity would led to the reduction of adsorption of these new compounds (Carlson and Silverstein, 1997). Clearly further research is needed to determine whether the smaller molecules created after the ozonation process would result in increased or decreased adsorption capacity and the related impacts on the biodegradation efficiency of the biofilm.

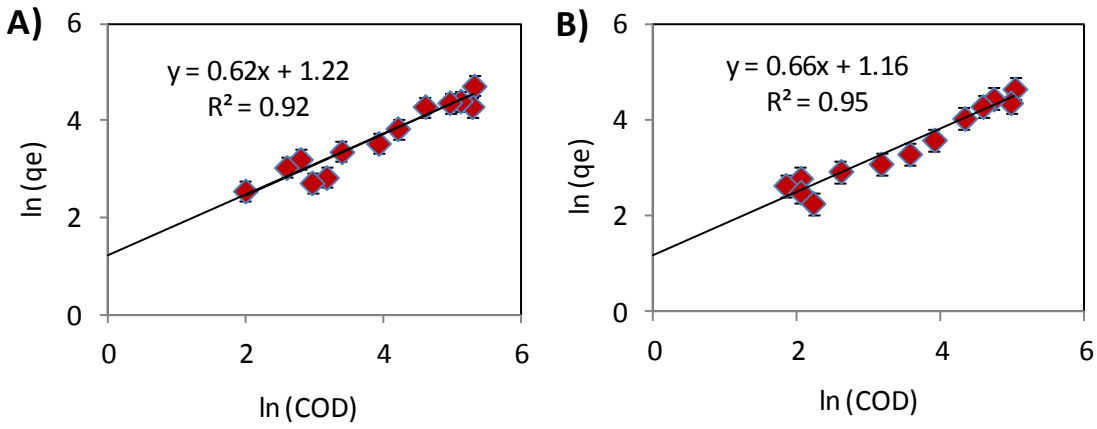

Figure 4.7. Freundlich COD isotherms for GAC from batch adsorption OSPW experiments; A) raw OSPW and B) ozonated OSPW.

4.4. Conclusions

In this study, we investigated the degradation of OSPW through the combination of ozonation following by a FBBR bioreactor using GAC media. Overall, ozonation not only enhanced the direct reduction of organic compounds, but also improved the biodegradability of OSPW organic compounds for subsequent biological treatment. The performance of the bioreactor (as indicated by NAs, COD and AEF removals) was most dependent on the organic loading rate while being less impacted by the hydraulic loading rate. Endogenous populations of

microorganisms in OSPW can form biofilms on GAC surfaces with the different carbon degraders observed currently in the OSPW biofilms confirming the presence of microbial activity in the GAC-bioreactor. Simultaneously, bacterial metabolism in the GAC biofilms and strong adsorption on the GAC surface enabled the removal of NAs and other organic contaminants from OSPW. Overall reductions of NAs by greater than 99.5%, COD by greater than 48% and AEF by at least 81%, indicated that the combined treatment process stream of ozonation followed by GAC-biofilm of the FBBR is a promising treatment method for OSPW remediation.

4.5. References

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CHAPTER 5. GRANULAR ACTIVATED CARBON FOR SIMULTANEOUS ADSORPTION AND BIODEGRADATION TREATMENTS OF OIL SANDS PROCESS-AFFECTED WATER[1]

5.1. Introduction

Crude oil deposits in the form of bitumen are estimated to be around 176.8 billion barrels (Pereira et al., 2013) in the oil sands (consisting of a mixture of bitumen, sand, and fine clay) of Northern Alberta, Canada. Extraction of bitumen from the oil sands using hot alkaline water generates a large volume of oil sands process-affected water (OSPW) (Garcia-Garcia et al., 2012; He et al., 2012). OSPW is stored in cement-lined tailings ponds in compliance with Alberta's zero discharge policy for polluted water (Del Rio et al., 2006) with the current volume of tailings ponds being greater than 10^9 cubic meters (Hagen et al., 2014). The major toxicity in OSPW is attributed to a group of organic carboxylic surfactants known as naphthenic acids (NAs) (Anderson et al., 2012; Garcia-Garcia et al., 2011). The general chemical formula of NAs is $C_nH_{2n+Z}O_x$, where n is the number of carbon atoms, Z is either zero or a negative even integer representing the number of hydrogen atoms lost because of ring formation, and x represents the number of oxygen atoms (x = 2 for classical NAs and 3 to 5 for oxidized NAs) (Barrow et al., 2010; Grewer et al., 2010; Wang et al., 2013b). Appropriate OSPW treatment technologies are currently being investigated to extend OSPW recycling to reduce the need for fresh water from Athabasca River and to permit the safe discharge of treated OSPW to the aquatic environment.

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[1]A version of this chapter has been submitted previously: Islam, M. S., Y. Zhang, K. N. McPhedran, Y. Liu, and M. Gamal El-Din, Granular activated carbon for simultaneous adsorption and biodegradation of oil sands process-affected water organic compounds. Bioresource Technology, 19 July, 2014

Biofilm treatment has been shown to be an environmentally sound and economical approach for municipal and industrial wastewater treatment. The high solids retention in biofilms, which harbours the growth of slow growing microorganisms, allows for the degradation of recalcitrant organics. Despite the benefits of the biofilm process, few biofilm based OSPW treatments have been reported. Headley et al. (2010) showed that NA degradation in OSPW using lake biofilm bacteria in a rotating annular bioreactor was ineffective. Hwang et al. (2013) observed ~ 18.5% NA removal using endogeneous microorganisms and a 19 hour hydraulic retention time in a biofilm reactor. Although Choi et al. (2014) observed that NA removal from OSPW was faster in a continuous biofilm reactor than in batch biofilm reactors, their study also showed that oil sands NAs are very resistant toward biodegradation. Such resistance to degradation can be attributed to the extensive cyclical nature of their molecular structures being difficult for microorganisms to metabolize.

The GAC-biofilm combination has been applied successfully to treat industrial wastewaters (Baban et al., 2010; Lei et al., 2010; Rao et al., 2005) and oil field produced water (Campos et al., 2002; Zhao et al., 2006) that contain a large variety of recalcitrant organic compounds. A high surface area derived from interconnected micropores, mesopores, and macropores (Sulaymon et al., 2013) gives GAC a high adsorption capacity However, prolonged contact of GAC with organics diminishes the available sites for adsorption of organic pollutants, and the adsorbent must be either replaced or regenerated (Nath and Bhakhar, 2011). The irregularity, roughness, and porosity of the GAC surface provides an excellent environment for bacterial growth (Yapsakli and Cecen, 2010); in turn, bacterial growth can help to regenerate the GAC surface by biodegrading adsorbed organics (Aktas and Cecen, 2007).

Our recent studies showed that GAC, as a biofilm supporting medium, can effectively assist in the degradation of NAs in OSPW (Islam et al., 2014). Islam et al. (2014) showed ~ 86% NA removal which was much greater than the NA removals achieved in previous OSPW treatment studies (Hwang et al., 2013). The improved treatment may be associated with synergetic effects of GAC and the attached biofilm. GAC adsorbs organic compounds in OSPW as well as supporting the growth of a biofilm. Biofilms growing on GAC particles degrade NAs and other organics through biodegradation, which helps to regenerate the GAC surfaces and increases the GAC adsorption capacity. Synergetic effects may also be caused by concentrated NAs and organics adsorbed on GAC surfaces triggering specific microbial metabolism mechanisms which can preferentially accelerate the biodegradation efficiency. The biofilm growth on GAC surfaces increases the contact time between microorganisms and the organic compounds adsorbed on the GAC, which may help to improve the treatment efficiency. The synergistic removal mechanisms have not been investigated previously, thus further study is needed. These studies may include OSPW treatments including GAC only, biodegradation only, and a combination of GAC adsorption and biodegradation which will lead to better understanding of the synergetic mechanisms, and allow for bioreactor optimization for OSPW treatment.

This study investigated the role of adsorption (GAC), biodegradation only, and the synergetic effect of a combined (simultaneous) adsorption and biodegradation system on the removal of NAs in OSPW. The impact of NAs chemical structure, such as carbon number, cyclicity, and oxygen number, on the performance of a adsorption and biodegradation system are assessed. It is reported that ozone can degrade classical NAs that exhibit high cyclization and long chains and thus increase their biodegradability (Martin et al., 2010; Wang et al.,, 2013b).

Although ozonation cannot reduce the oxidized NA concentrations in OSPW (Pereira et al., 2013), the combined adsorption and biodegradation system might enhance NA removal in ozonated OSPW. Thus the efficacy of combined adsorption and biodegradation treatment of ozonated OSPW was also tested.

5.2. Materials and Methods

5.2.1. Materials

Selected grade bituminous coal based steam-activated granular carbon (GAC) (SGL 8×30) (Calgon Carbon Corporation, Pittsburgh, PA, USA) was used to conduct the experiments. The mean diameter of the GAC was 1.5–1.7 mm. OSPW was sampled from the oil sands tailings ponds in Northern Alberta, November, 2011, and stored in a cold room at 4 °C before use.

5.2.2. Methods

5.2.2.1. Ozonation of Raw OSPW

OSPW was ozonated with an ozone generator (PCI-WEDECO, GSO-40, Herford, Germany) using extra-dry, high-purity oxygen. A 4 L vacuum flask equipped with a ceramic fine bubble gas diffuser located at the bottom of the reactor was used for ozonation. The ozone dose was calculated by reading the ozone concentrations at the reactor inlet and outlet with identical ozone monitors (Model HC-500, PCI-WEDECO). The detailed ozonation procedures have been described in Chapter 4.

5.2.2.2. Simultaneous Adsorption and Biodegradation of OSPW

Amber bottles containing 0.2 g GAC and 500 mL OSPW were shaken at 150 rpm on a horizontal shaker (Innova^{TM} 2100, Platform Shaker, New Brunswick Scientific, USA) for 28

days at room temperature. Figure 5.1 shows the experimental set up. For comparison, sterilized OSPW, OSPW without GAC, and sterilized OSPW with sterilized GAC were treated as described above as experiments of blank, biodegradation only, and adsorption only, respectively. The sterilized GAC and OSPW were prepared using a vacuum/gravity autoclave (Model 733LS, Getinge group Inc., NY, USA) at 121 °C for 30 minutes. All the experiments were carried out in triplicate. For biodegradation experiments, raw OSPW with endogenous bacteria was used without pretreatment, whereas ozonated OSPW was inoculated with endogenous bacteria extracted from raw OSPW by centrifugation using the following protocol. A defined volume of ozonated OSPW was centrifuged (Multifuge 3S/3S-R, Heraeus, Houston, USA) at 3700 rpm for 10 min to remove ozonated OSPW microbes, and the supernatant was collected. Raw OSPW microorganisms were then collected as pellets from the same volume of raw OSPW after centrifuging at the same operating condition. Subsequently, raw OSPW microorganisms were inoculated in the bacteria-free ozonated OSPW supernatant.

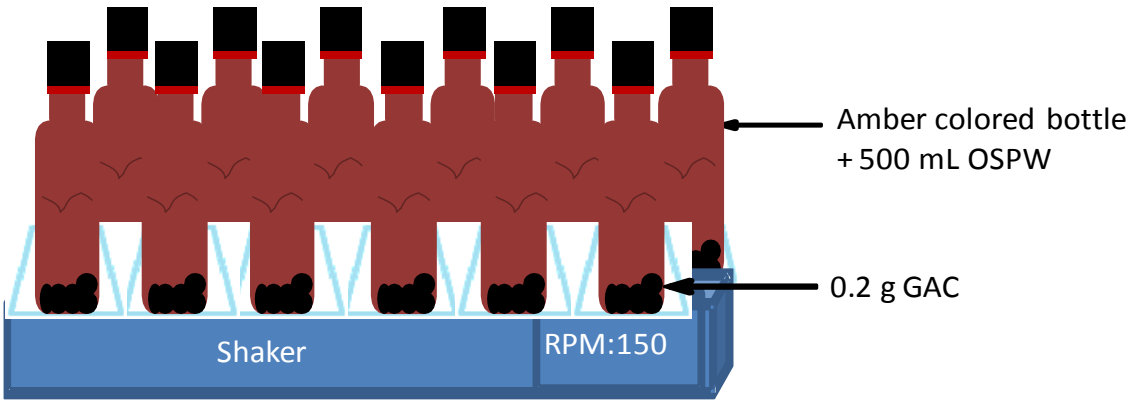

Figure 5.1. Experimental set up for carrying out GAC adsorption, biodegradation and combined treatment mechanisms.

5.2.3. Analysis of Water Chemistry

Basic water chemistry, including chemical oxygen demand (COD), dissolved organic carbon (DOC), biochemical oxygen demand (BOD_5), and total solids (TS) were analyzed in triplicate in raw and ozonated OSPW according to standard methods (APHA, 2005). Fourier transform infrared (FT-IR) spectroscopy (PerkinElmer®, Woodbridge, ON, CA) was used to measure the acid extractable fraction (AEF) concentration in OSPW prior to and after treatment, as described elsewhere (Gamal El-Din et al., 2011). A detailed description is provided in Appendix-A.

Aliquots (2 mL) of sample from each replicate were collected on days 5, 14, and 28 and pooled for NA analysis (single replicate for each sample) using UPLC-HRMS methods. Myristic acid was used as internal standard (ISD) and the concentration of classical and oxidized NAs were estimated using the equation 5-1 by assuming that the ionization efficiency of the ISD was similar to the NAs species during the ion evaporation process. Ion mobility spectra (IMS) analyses were performed using a Waters Acquity ultrahigh performance liquid chromatography (UPLC) system (Milford, MA, USA) and a high resolution Synapt G2 HDMS (40,000 FWHM) as described in a previous study (Wang et al., 2013b). The Synapt G2 HDMS system was integrated with an ion-mobility cell between the electrospray ionization source operating in negative ion mode and a TOF MS detector. An instrumental description and detailed procedures are described in Chapter 3.

$$C_{(NAs)} = \frac{Area_{(NAs)}}{Area_{(ISD)}} \times C_{(ISD)} \qquad (5-1)$$

Where, $C_{(ISD)}$ is the concentration of internal standard in mg/L;

$C_{(NAs)}$ is the concentration of classical and oxidized NAs in mg/L.

The acquisition of ion mobility spectra (IMS) have been described previously by Wang et al. (2013b). In brief, a Tri-Wave® ion-mobility cell of 15 cm long was used to conduct the ion mobility spectra (IMS) using nitrogen (purity > 99%) as the drift gas. A transfer cell in the IMS gathered a defined amount of ions (number of ions not known) and a helium gate released the ions into the ion-mobility cell. Ions were moved counter-current to the gas flow using an electric field (T-wave) which drifted the ions based on the cross collision section (CCS).

5.2.4. Microbial Characterization in the Bioreactor

5.2.4.1. DNA Extraction and Real Time PCR

A PowerSoil® DNA isolation kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) was used to isolate DNA from bacterial cells. Planktonic bacteria were isolated as a pellet by centrifuging (Multifuge3S/3S-R, Heraeus, rotor with 4×750 mL water/bottle, Thermo Scientific, USA) raw or ozonated OSPW at 3700 rpm for 10 min. The collected pellet was added to power soil bead tubes to isolate total genomic DNA. For isolating DNA from biofilm on the GAC, a weighed amount of GAC with biofilm was added to power soil bead tubes directly. The manufacturer's protocol for isolating total genomic DNA was followed. Real time PCR was carried out in a CFX 96 Touch™ Real Time PCR System (Bio-Rad Laboratories Inc., USA) containing 1 × SsoFast™ EvaGreen® Supermix, 0.5 μM of each primer (Integrated DNA Technologies, Coralville, IA), and 5 μL of diluted DNA in a 25 μL total reaction volume.

The PCR amplification program consisted of 5 min at 95 °C, 40 cycles of 60 s at 94 °C, 60 s at 56 °C. All experiments were performed in triplicate per sample, and all PCR runs included plasmid standards and control reactions without the DNA template. The threshold cycle (C_t) of each real-time PCR reaction was automatically determined by detecting the cycle at

which the fluorescence exceeded the calculated threshold. Gene copies were calculated by comparison of threshold cycles obtained in each PCR run from known standard plasmid DNA concentrations. The standard plasmids containing 16S rRNA were constructed by a PCR4-TOPO TA Cloning kit (Invitrogen Corporation, Carlsbad, CA). The primer sets 907r, with sequence 5'-CCG TCA ATT CMT TTG AGT TT-3', and 341f, having 5'- CCT ACG GGA GGC AGC AG -3', were used for amplification of extracted DNA during qPCR.

5.2.4.2. Confocal Laser Scanning Microscopy (CLSM) Imaging

Confocal laser scanning microscopy (CLSM) (Zeiss LSM 710, Carl Zeiss Micro Imaging GmbH, Germany) was used to observe and acquire biofilm images, and measure biofilm thickness with a protocol described by Hwang et al. (2013). Biofilms formed on the GAC in raw and ozonated OSPW were stained with SYTO 9 (BacLight Live/Dead Bacterial Viability Kit, Molecular Probes, USA) and concanavalin A (ConA, Molecular Probes, Eugene, OR), a lectin conjugated with Texas Red for the probing of live cells and extracellular polymeric substance (EPS). Images were observed and scanned randomly at 4 positions with a lens (20 × 0.8 NA Plan-Apochromat). The protocol has been described in Chapter 3.

5.2.5. *Vibrio Fischeri* Bioassay

An 81.9% screening test using a marine fluorescence bacterium was used to assess microtoxicity using a protocol described in Wang et al. (2013b). The toxic effects of raw and treated OSPW samples toward *Vibrio fischeri* were measured in triplicate using a Microtox 500 Analyzer (AZUR Environmental, Carlsbad, USA) and the 81.9% screening test protocol (AZUR Environmental, Microtox Omni™ Software). All materials were purchased from AZUR

Environmental (Carlsbad, CA, USA). The percentage inhibition was calculated after 5 and 15 min incubations. A phenol standard (100 mg/L) was used as a toxicity control to verify the sensitivity of the luminescent bacteria prior to the analyses.

5.3. Results and Discussion

5.3.1. Characterization of Raw and Ozonated OSPW

A low utilized ozone dose of 20 mg/L was applied to OSPW before simultaneous adsorption and biodegradation treatment of OSPW to make the combined treatment more economical. As well, even after a high ozone dose (80 mg/L utilized) the ozonated OSPW was not easy biodegradable ($BOD_5/COD < 0.3$) even with removal of 96.5% of the NAs as shown previously (Chapter 4)

The chemical characterizations of the raw and ozonated OSPW were performed as shown in Table 5.1. The average concentrations of classical and oxidized NAs in raw OSPW were 17.4 ± 0.8 mg/L and 27.6 ± 1.0 mg/L, respectively. No acyclic classical NA species (aliphatic NAs with Z = 0) were observed in raw OSPW. Only 2.5% of classical NAs with a single ring (Z = -2) were observed. The dominant fractions of classical NAs based on Z- numbers were species with Z = -4 (25.5%), Z = -6 (32.2%), and Z = -12 (18.6%). The average concentrations of classical and oxidized NAs in ozonated OSPW were 12.6 ± 0.7 mg/L and 27.3 ± 0.9 mg/L, respectively. The relative concentrations of classical NA species with Z = -8 to Z = -12 were reduced and concentrations of NA species with Z = -2 to Z = -6 were increased in ozonated OSPW versus raw OSPW.

The concentrations of total classical NAs and the AEF in ozonated OSPW were 28% and 21% lower than raw OSPW, respectively. The total oxidized NA (Table 5.1) concentration did

not decrease substantially (< 1.7%) after ozonation of OSPW. Specifically, the concentrations of O_2-NAs ($C_nH_{2n+Z}O_4$) and O_3-NAs ($C_nH_{2n+Z}O_5$) decreased by 1.6% and 16.7%, respectively, while there was a 5.9% increase in O-NAs ($C_nH_{2n+Z}O_3$). These results can be explained given the rate of formation of O-NAs from degradation of classical NAs was greater than the rate of conversion of O-NAs to O_2-NAs and O_3-NAs (Pereira et al., 2013). Thus classical NAs appear to be more reactive to ozone than the oxidized NAs (Pereira et al., 2013).

The pH, total solids, COD and DOC measurements in raw and ozonated OSPW were not significantly different (Table 5.1). The BOD_5 concentration increased from 4.3 ± 1.7 mg/L to 8.6 ± 2.1 mg/L after ozonation of OSPW, however, the BOD_5/COD ratio increased only slightly from 0.02 to 0.04 indicating that ozonated OSPW was not easily biodegradable as defined by a ratio ≥ 0.3 (Alvarez-Vazquez et al., 2004) . The higher percentage of classical NAs and the AEF removal compared to COD and DOC removal indicated that the conversion of classical NAs and the AEF to oxidized NAs and other smaller organic compounds still contributed to COD and DOC concentrations.

The toxicity (inhibition) of raw OSPW toward *Vibrio fischeri* decreased from 40.7% to 28.9% after ozonation (Table 5.1) with similar results reported previously (Wang et al., 2013b). A low dose of ozone, such as the presently used 20 mg/L, reduces but does not eliminate the toxicity of raw OSPW toward *Vibrio fischeri* because oxidized NA byproducts such as hydroxy- or keto-NAs can still contribute to OSPW toxicity (Martin et al., 2010; Wang et al., 2013b).

Ion mobility spectra of raw and ozonated OSPW showed three characteristic regions (Figure 5.2), which represent classical NAs, oxidized NAs (O_x-NAs, where x = 1, 2, 3), and NAs with sulfur and nitrogenated species. After ozonation, the intensity of classical NAs and

NAs containing sulfur (S) and nitrogen (N) decreased (Figure 5.2B) whereas the intensity of total oxidized NAs did not decrease markedly.

Table 5.1. Characterization of raw and ozonated OSPW

Parameters	Raw OSPW	Ozonated OSPW
pH	8.6 ± 0.1	8.7 ± 0.1
TS (mg/L)	2721 ± 52	2673 ± 59
AEF (mg/L)	67.45 ± 0.6	53.1 ± 2.3
Classical NAs (mg/L)	17.4 ± 0.8	12.6 ± 0.7
Total oxidized NAs (mg/L)	27.6 ± 1.0	27.3 ± 0.9
COD (mg/L)	209 ± 4.3	214 ± 5.3
DOC (mg/L)	56.6 ± 3.3	56.5 ± 1.7
BOD_5 (mg/L)	4.3 ± 1.7	8.6 ± 2.1
Toxicity, 15 min incubation (%)	40.7 ± 3.6	28.9 ± 2.1

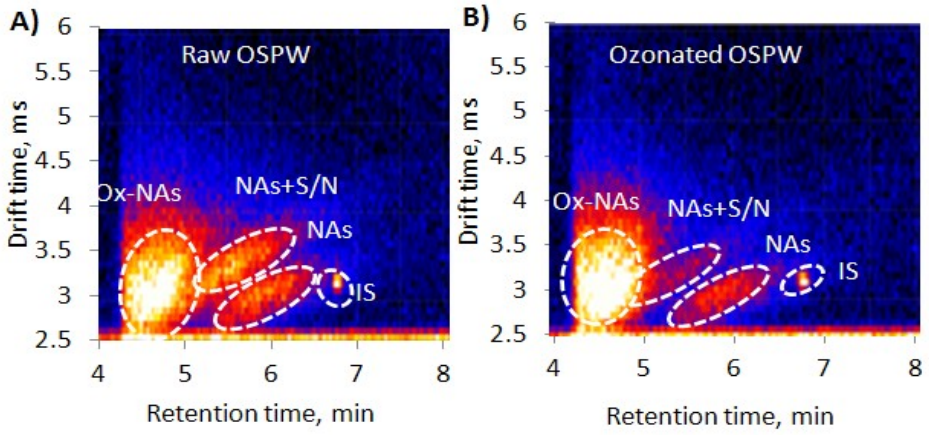

Figure 5.2. Ion mobility separation spectra of raw and ozonated OSPW before GAC adsorption and biological treatment. (A) untreated raw OSPW; and (B) ozonated OSPW.

5.3.2. Organics Removal Characterization

Based on a preliminary GAC adsorption experiment with different GAC doses (Figure D1; Appendix-D), an optimized dose of 0.4 g GAC/L OSPW was selected for the current experiment. Figure 5.3 shows the characteristic removal of COD and classical NAs in raw and ozonated OSPW during 28 days of treatment of control (sterilized OSPW), biodegradation only (OSPW without GAC), GAC adsorption only (sterilized OSPW with GAC), and simultaneous GAC adsorption and biodegradation (OSPW plus GAC) experiments.

As shown in Figures 5.3A and 5.3B, COD removals increased in raw and ozonated OSPW with an increase in reactor operating time in all treatment conditions. Unexpected COD removal (< 7%) was observed from the sterilized raw and ozonated OSPW which has been reported earlier (Kargi and Pamukoglu, 2003, 2004). However, the possible reasons of removal of COD from abiotic control experiment (sterilized wastewater) have not been reported previously. The probable reason might be an impact of photodegradation of organics from the light although brown coloured bottles were used to conduct the experiment. The biodegradation only treatment had a low impact on COD removal in raw and ozonated OSPW after 28 days of treatment (< 20%). The GAC adsorption only and simultaneous GAC adsorption/biodegradation had a higher impact on COD removal in raw and ozonated OSPW of > 40%.

Classical NA concentration changes versus time in raw and ozonated OSPW treatments are shown in Figures 5.3C and 5.3D. A similar photodegradation may also be attributed to the reduction of classical NAs by 5% from the treatment of sterilized raw and ozonated OSPW which has been stated in earlier paragraph for COD removal. The biodegradation only treatments had a minimal impact on the removal of classical NAs in both raw and ozonated OSPW (< 20%). The GAC adsorption only and simultaneous GAC adsorption/biodegradation

had a higher impact on NAs removal in raw and ozonated OSPW of > 90%. Classical NA concentrations varied significantly in the presence of GAC. NA concentration changes reached a plateau within 5–10 days in raw and ozonated OSPW (GAC adsorption only and combined treatment) which can be attributed to the high adsorption capacity of GAC in the first 5–10 days of the experiment.

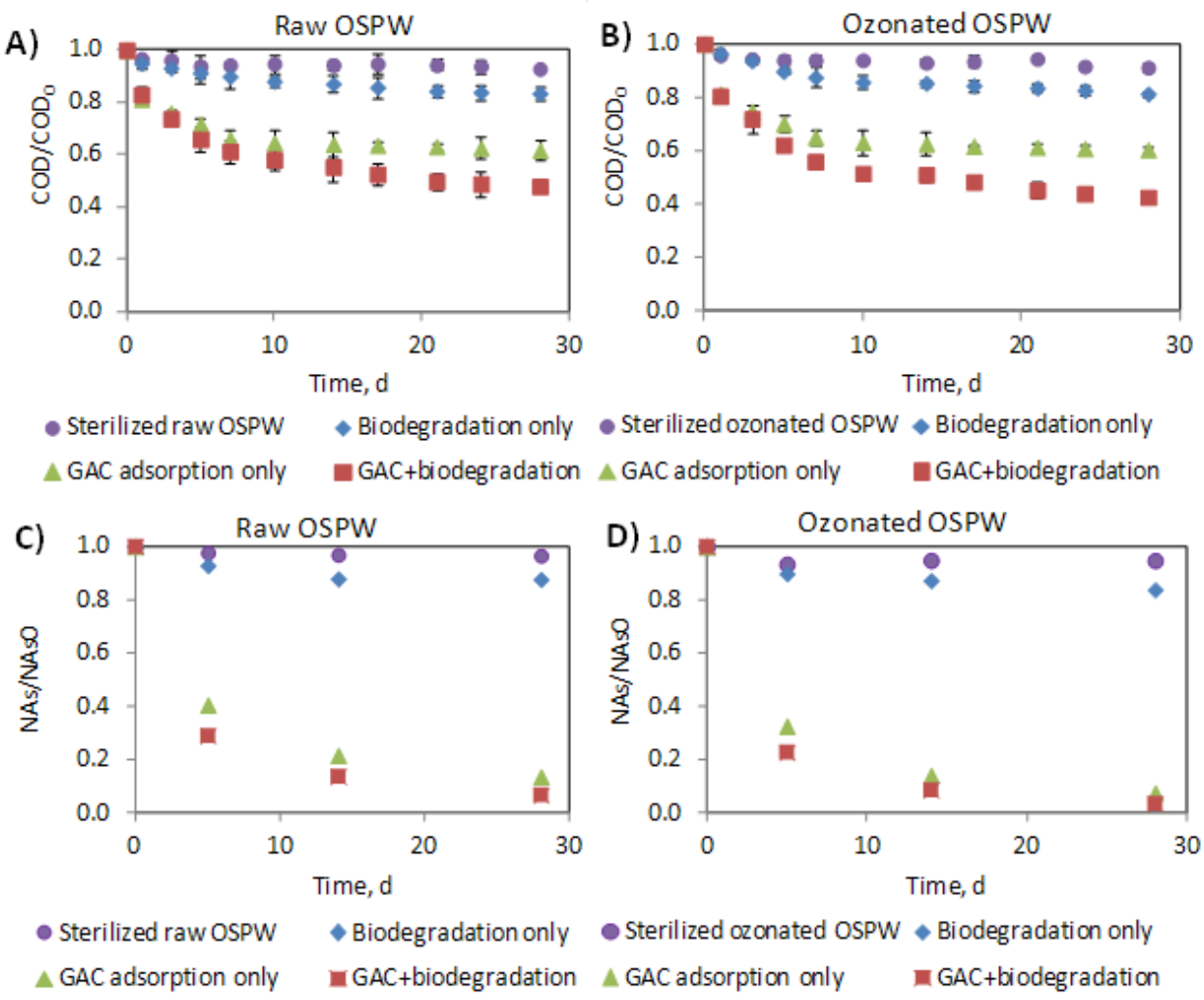

Figure 5.3. Relative concentration of COD and classical NAs in raw and ozonated OSPW with time; (A) COD in raw OSPW; (B) COD in ozonated OSPW; (C) classical NAs in raw OSPW; and (D) classical NAs in ozonated OSPW.

Table 5.2 shows the removal of COD, AEF, classical NAs, and total oxidized NAs after biodegradation only, GAC adsorption only, and simultaneous GAC adsorption and biodegradation experiments in both raw and ozonated OSPW after 28 days of treatment. The percentage removal values in Table 5.2 were calculated based on the relative organic concentration in each treatment compared to the organic concentration in control experiments (comprising sterilized raw and ozonated OSPW without GAC).

Table 5.2. Removal of COD, AEF, and classical and oxidized NAs from raw and ozonated OSPW after 28 days of treatment‡.

Parameters	Raw OSPW, % removal			Ozonated OSPW, % removal		
	Biodegradation only	Adsorption only	Combined*	Biodegradation only	Adsorption only	Combined*
COD	9.4	31.1	44.9	9.8	30.7	48.5
AEF	4.2	62.8	69.9	6.7	64.4	75.7
Classical-NAs	2.6	92.2	93.3	9.2	94.5	96.2
Oxy- NAs†	3.2	67.9	73.7	5.3	71.0	77.1

* simultaneous GAC adsorption and biodegradation

‡The percentages removal in the Table was calculated based relative organic concentration from each treatment compared with organic concentration remained in control experiment (experiment with sterilized raw and ozonated OSPW without GAC) after 28 days of treatment.

† Oxy-NAs (total oxidized NAs) concentration (∑(O-NAs, O_2-NAs and O_3-NAs) has been shown in Table B6 in Appendix-B.

The highest removal for GAC adsorption and simultaneous adsorption and biodegradation treatments was observed for classical NAs followed by removals of oxidized NAs, AEF, and COD in both raw and ozonated OSPW (Table 5.2). Classical NAs are hydrophobic branched cyclic organic compounds with high molecular weights and long carbon chains, which might account for their high GAC adsorption. In comparison, oxidized NAs are less hydrophobic containing more –OH groups (Wang et al., 2013a). Thus, a reduced GAC adsorption affinity of oxidized NAs compared to classical NAs might be expected.

The simultaneous GAC adsorption and biodegradation process removed greater amounts of COD, AEF, and total oxidized NAs than the summed amounts of the biodegradation only and GAC only processes, indicating the existence of synergistic effects in the biodegradation and GAC adsorption processes (Combarros et al., 2014; Nath and Bhakhar, 2011). This observation can be attributed to biodegradation of COD, AEF, and total oxidized NAs on the GAC surface which helped to release new adsorption sites on the GAC surface. These synergistic effects led to decreased COD, AEF, and total oxidized NAs concentrations in the liquid phase which creates an increased capacity for desorbing the organics on the GAC surface. The GAC surface is bioregenerated by the biodegradation of adsorbed organics (exoenzymatic biodegradation mechanism) and desorption of adsorbed organics due to the decreasing concentration gradient between the GAC surface and the bulk liquid which is attributed to the biodegradation occurring in the liquid phase (Aktas and Cecen, 2007; Kim et al., 1997; Nath and Bhakhar, 2011; Oh et al., 2012; Xiaojian et al., 1991). Furthermore, synergetic effects may be caused by the concentrated organics on GAC surfaces which might accelerate the biodegradation efficiency through the specificity of microorganism metabolic processes. A previous study demonstrated that NAs

biodegradation was limited due to their concentrations being a minimum threshold substrate concentration, which supported our observation (Misiti et al., 2013).

The removal of classical NAs in the simultaneous GAC adsorption and biodegradation process was similar to the summed values of classical NA removals from GAC only and biodegradation only processes (Table 5.2). Compared with oxidized NAs, classical NAs have increased hydrophobicity due to fewer hydroxyl groups (Wang et al., 2013a), which may cause higher toxicity. Acute toxicity of monocarboxyl NAs increases with an increase in carbon number (hydrophobicity), and decreases with an increase in carboxyl groups in the structure (Frank et al., 2009), an observation that is supported by the narcosis theory that hydrophobic compounds can more easily permeate the lipid bilayer and destroy cell membranes (Könemann, 1981). It was beyond the scope of this study to identify what categories (monocarboxylic/polycarboxylic) of NA species were present in the OSPW samples. Thus, the high (> 92%) GAC adsorption of classical NAs might increase the bacterial toxicity on the GAC surface which would decrease biodegradation and thus reduce the bioregeneration of GAC in the simultaneous GAC adsorption and biodegradation treatment. Thus, a lower synergetic effect might be attributed to the toxicity caused by high hydrophobicity and adsorption affinity for GAC of classical NAs.

As shown in Figure 5.4, ion mobility spectra (IMS) analysis indicated that the biodegradation only condition did not considerably reduce the intensity of any type of NA in both raw and ozonated OSPW, which indicates the ineffectiveness of the process for NA removals under the experimental conditions tested (also refer to Figure 5.2). However, 0.4 g GAC/L OSPW plus biodegradation was effective in removing spectra associated with both

classical NAs and sulfur/nitrogenated NA species, as well as the dominant fraction of oxidized NAs in raw and ozonated OSPW (Figure 5.4, B and D).

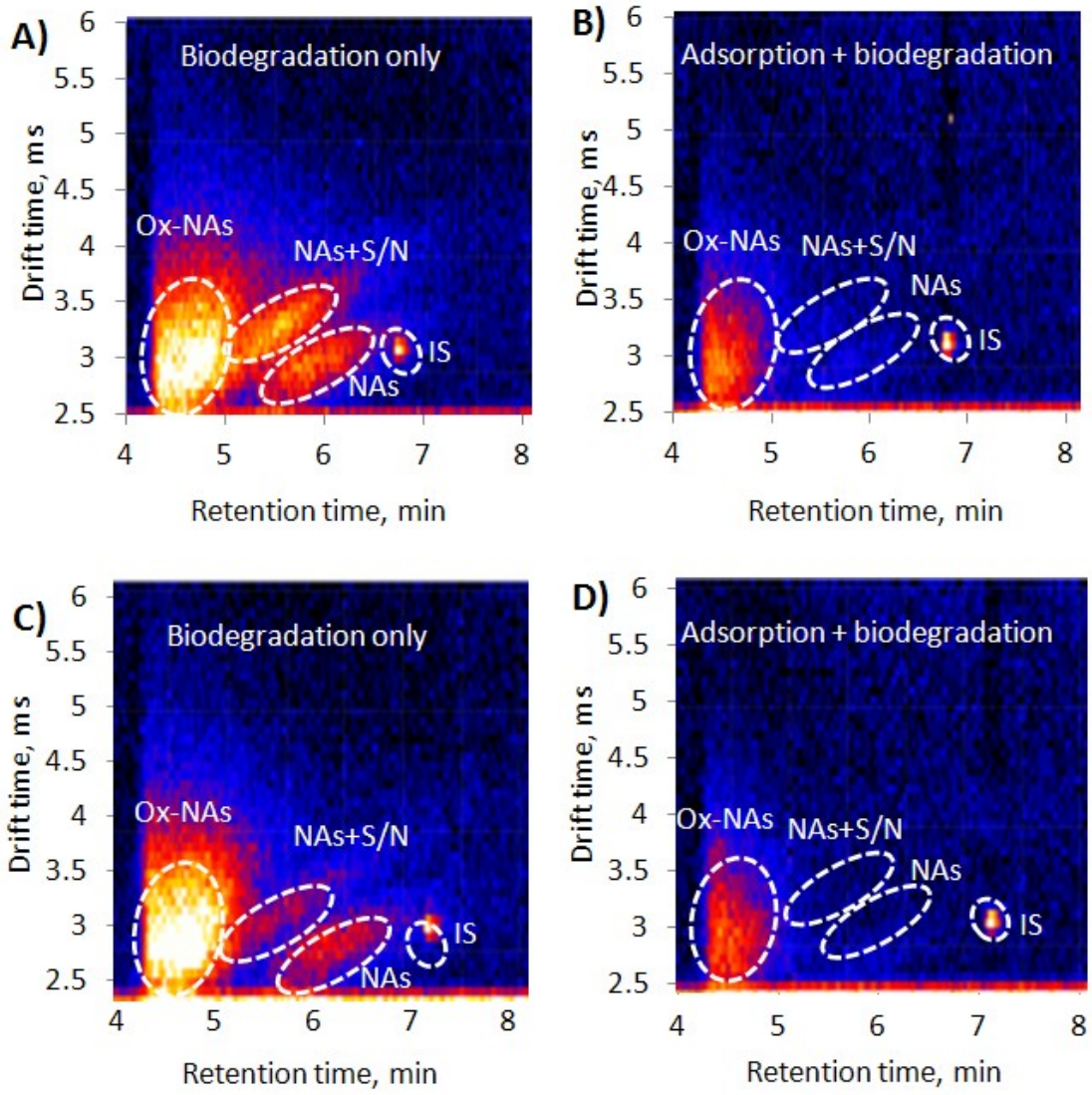

Figure 5.4. Ion mobility separation spectra of raw and ozonated OSPW after treatment; (A) biodegradation only treatment of raw OSPW; (B) simultaneous GAC adsorption and biodegradation treatment of raw OSPW; (C) biodegradation only treatment of ozonated OSPW; and (D) simultaneous GAC adsorption and biodegradation treatment of ozonated OSPW.

5.3.3. Characterization of NA Removals

The classical and oxidized NAs removal were characterized based on summation of carbon numbers $[NAs]_{\Sigma ni}$ and summation of Z numbers $[NAs]_{\Sigma Zi}$ from biodegradation only, GAC adsorption only and combined treatments as discussed in the following subsections 5.3.3.1 and 5.3.3.2. The three dimensional UPLC-HRMS raw data for both untreated and treated raw and ozonated OSPW are provided in Appendix-C (Figures C4 -C11) based on carbon and Z numbers.

5.3.3.1. Impact of Z Number on NA Removals

The relative concentrations of classical and oxidized $[NAs]_{\Sigma ni}$ in raw and ozonated OSPW based on Z number after 28 days of treatment is shown in Figure 5.5. Variation in the relative concentrations of classical and oxidized $[NAs]_{\Sigma ni}$ from biodegradation only experiments in raw and ozonated OSPW suggest that the least cyclic fraction (Z = -2) undergoes relatively rapid biodegradation (Figure 5.5). These results agree with those of previous studies that reported that classical NAs with lower molecular weights and fewer rings are more biodegradable (Han et al., 2009; Han et al., 2008). Higher NA removals were observed in ozonated OSPW compared to NA removals in raw OSPW in the biodegradation only experiment, which can be attributed to the higher biodegradability of oxidized NAs.

Compared with classical and oxidized $[NA]_{\Sigma ni}$ removals in raw and ozonated OSPW with biodegradation only, higher removal of classical and oxidized $[NAs]_{\Sigma ni}$ was observed in the presence of GAC (i.e., both adsorption only and simultaneous adsorption and biodegradation experiments) (Figure 5.5). Considering NA removals versus Z number from GAC adsorption only, lower removals were observed for Z = -4 and Z = -6 groups of classical and oxidized NAs. Zubot et al. (2012) observed an increased removal of classical NAs with an

increase in Z number using a petroleum coke adsorbent. An increase in Z number increases the hydrophobicity and decreases the solubility of NAs which increases their adsorption affinity to GAC. The lower percentage removals for Z = -4 and Z = -6 groups of classical and oxidized NAs_i might be due to their higher concentrations saturating binding sites on the GAC. Further, the lower removal of oxidized $[NAs]_{\Sigma ni}$ compared to classical $[NAs]_{\Sigma ni}$ indicated that the adsorption affinity for GAC of oxidized $[NAs]_{\Sigma ni}$ was less than that of classical $[NAs]_{\Sigma ni}$.

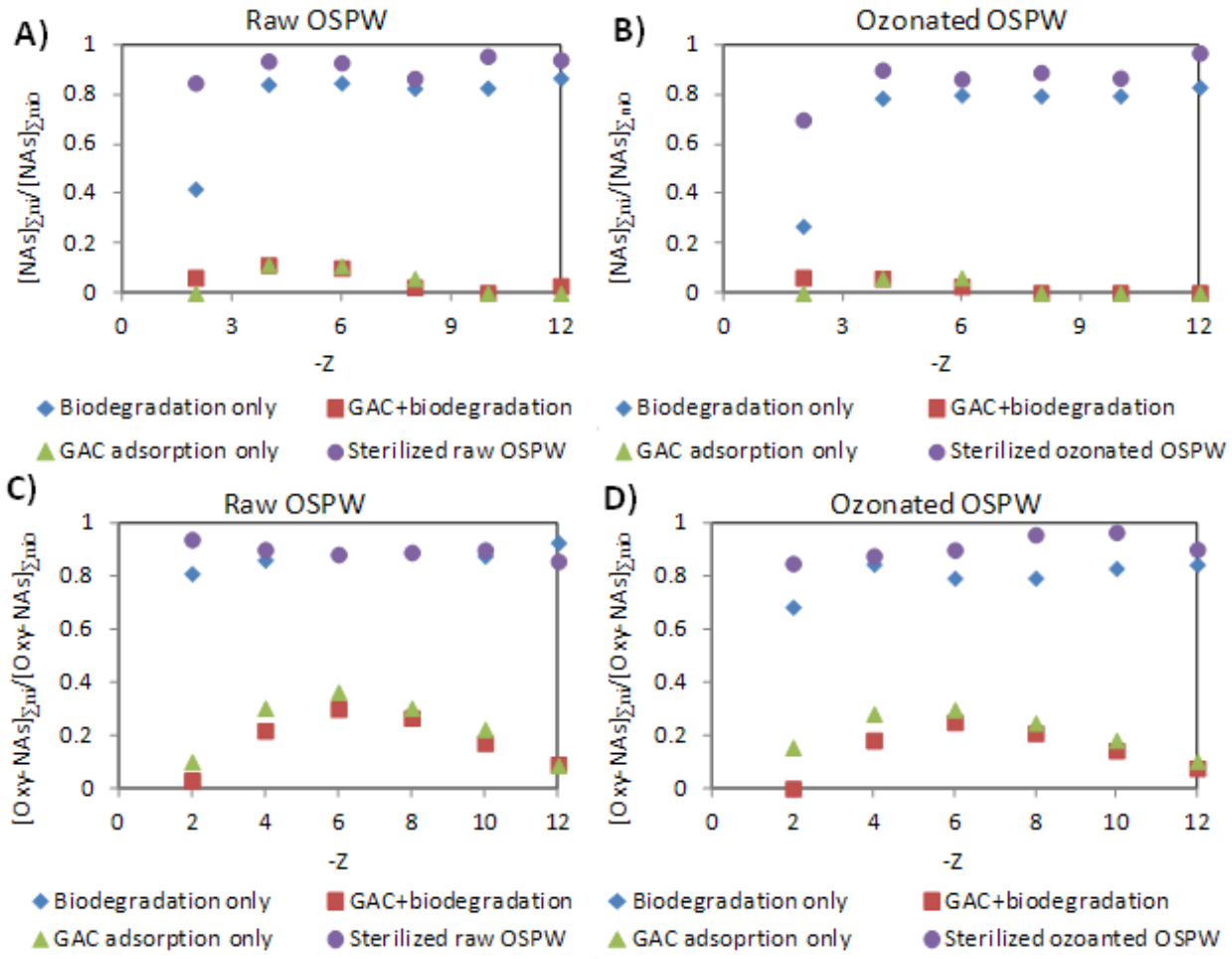

Figure 5.5. Relative concentration of NAs with different Z numbers in raw and ozonated OSPW after 28 days treatment. (A) classical $[NAs]_{\Sigma ni}$ in raw OSPW; (B) classical $[NAs]_{\Sigma ni}$ in ozonated OSPW; (C) total oxidized $[NAs]_{\Sigma ni}$ in raw OSPW; and (D) total oxidized $[NAs]_{\Sigma ni}$ in ozonated OSPW.

The removal trend of classical and oxidized $[NAs]_{\Sigma ni}$ shown in Figure 5.5 in simultaneous adsorption and biodegradation experiments was similar to that in the GAC adsorption only experiments which indicates that adsorption was the predominant removal mechanism. Classical NAs were removed more efficiently than oxidized NAs in the simultaneous adsorption and biodegradation treatment as discussed in section 5.3.2.

5.3.3.2. Impact of Carbon Number on NAs Removal

Carbon number had a minimal impact on classical and oxidized NA biodegradation over 28 days in raw and ozonated OSPW in the biodegradation only and sterilized raw OSPW treatments (Figures 5.6A-D). The removal of classical and oxidized $[NAs]_{\Sigma Zi}$ increased with an increase in n in raw and ozonated OSPW treated for 28 days with GAC adsorption only and simultaneous GAC adsorption and biodegradation (Figure 5.6). The increase in GAC adsorption of NAs with an increase in n can be attributed to increases in hydrophobicity and nonpolarity of NAs with an increase in n (Scarlett et al., 2012). Longer molecules can be adsorbed more readily on the GAC surface (Zubot et al., 2012) and can more easily approach the GAC surface to reach GAC pores.

Figure 5.6 shows that based on carbon number (n), the removal of classical and oxidized $[NAs]_{\Sigma Zi}$ from OSPW through simultaneous GAC adsorption and biodegradation was predominantly due to adsorption. For instance, there was 100% removal of classical $[NAs]_{\Sigma Zi}$ at $n \geq 16$ for raw OSPW and at $n = \geq 14$ for ozonated OSPW after both GAC adsorption only and simultaneous GAC adsorption and biodegradation experiments.

We did not observe synergetic effects on classical NA removals in simultaneous adsorption and biodegradation for different Z and n values (Figures 5.5A, 5.5B and 5.6A, 5.6B,

respectively), compared to GAC adsorption only treatments. The plausible reasons for this observation have been discussed in section 5.3.2. However, the greater removal of all types of NAs (classical, oxidized $[NAs]_{\Sigma Zi}$, oxidized $[NAs]_{\Sigma ni}$) observed after simultaneous GAC adsorption and biodegradation treatment compared with removal by biodegradation only or GAC adsorption only indicates the benefits of applying the combined process for removal of both classical and oxidized NAs.

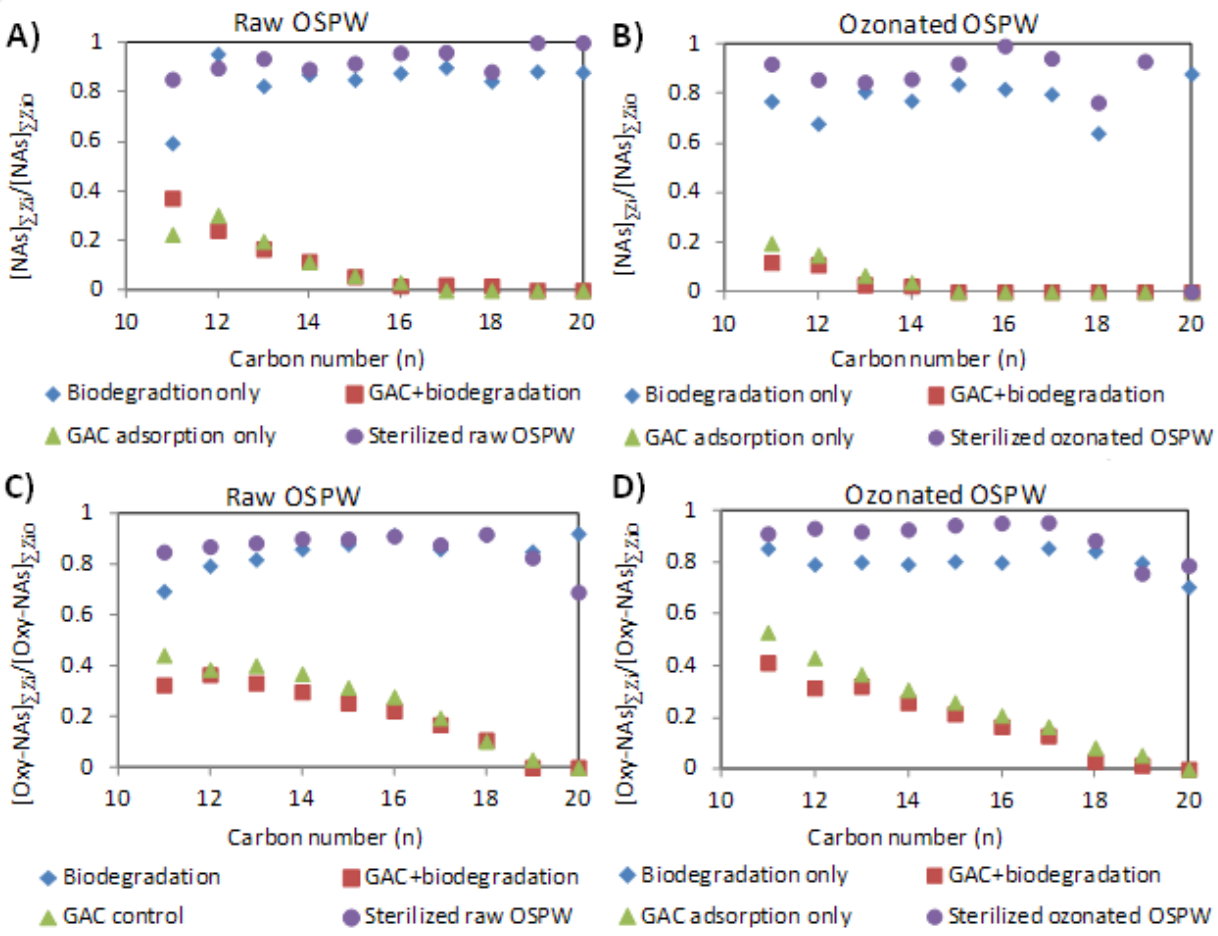

Figure 5.6. Relative concentration of total oxidized NAs at different carbon numbers for raw and ozonated OSPW after 28 days treatment. (A) classical $[NAs]_{\Sigma Zi}$ for raw OSPW; (B) classical $[NAs]_{\Sigma Zi}$ for ozonated OSPW; (C) total oxidized $[NAs]_{\Sigma Zi}$ for raw OSPW; and (D) total oxidized $[NAs]_{\Sigma Zi}$ for ozonated OSPW.

5.3.4 Biological Parameters Characterization

The biofilm developed on the GAC surface was visualized using a confocal laser scanning microscope after 14 and 28 days of simultaneous GAC adsorption and biodegradation treatment of raw and ozonated OSPW (Figure 5.7) Marginally higher biofilm thickness (39 ± 18 μm) was observed on the GAC surface used to treat ozonated OSPW (Figure 5.7B) than on the GAC surface used to treat raw OSPW (34 ± 7 μm) (Figure 5.7A) after 28 days of treatment. Similar biofilm thicknesses were observed on GAC surfaces in the treatment of synthetic phenolic wastewater using fluidized bed biofilm reactors (Herzberg et al., 2006).

After 28 days of biodegradation, DNA was extracted from raw and ozonated OSPW (biodegradation only), and GAC biofilms and supernatants in raw and ozonated OSPW (GAC treated). Real time PCR (qPCR) was performed for all samples to quantify bacteria from extracted DNA. Initial bacteria concentrations in raw and ozonated OSPW were similar because the ozonated OSPW was inoculated with raw OSPW. After 28 days of biodegradation, the concentration of total bacteria doubled in both raw and ozonated OSPW reactors (Table 5.3). The OSPW supernatants had bacterial concentrations of 4.4×10^5 bacteria/mL for raw OSPW and 3.6×10^5 bacteria/mL for ozonated OSPW. The GAC concentrations were 1.67×10^8 bacteria /g GAC for raw OSPW and 6.85×10^8 bacteria/g GAC for ozonated OSPW. Velten et al. (2007) observed 7.9×10^9 cells/g GAC using ATP analysis of GAC biofilms at the top of the bed in a biofilter after continuous treatment of ozonated lake water.

The bacteria copy numbers on the GAC surface and in the suspension for the combined treatment are shown in Table B7 (Appendix-B). The total bacteria number (sum of biofilm bacteria on the GAC and bacteria in OSPW) in the bioreactor (Table 5.3) did not increase substantially in the combined adsorption and biodegradation treatment compared to the

biodegradation only treatment of both raw and ozonated OSPW. The use of a negligible mass of GAC (4×10^{-4} times the total mass of OSPW) or limited biodegradable carbon source in the combined (GAC adsorption and biodegradation) treatment might be the reason for the lack of increase in the total bacteria concentrations in combined treatments versus biodegradation only treatments

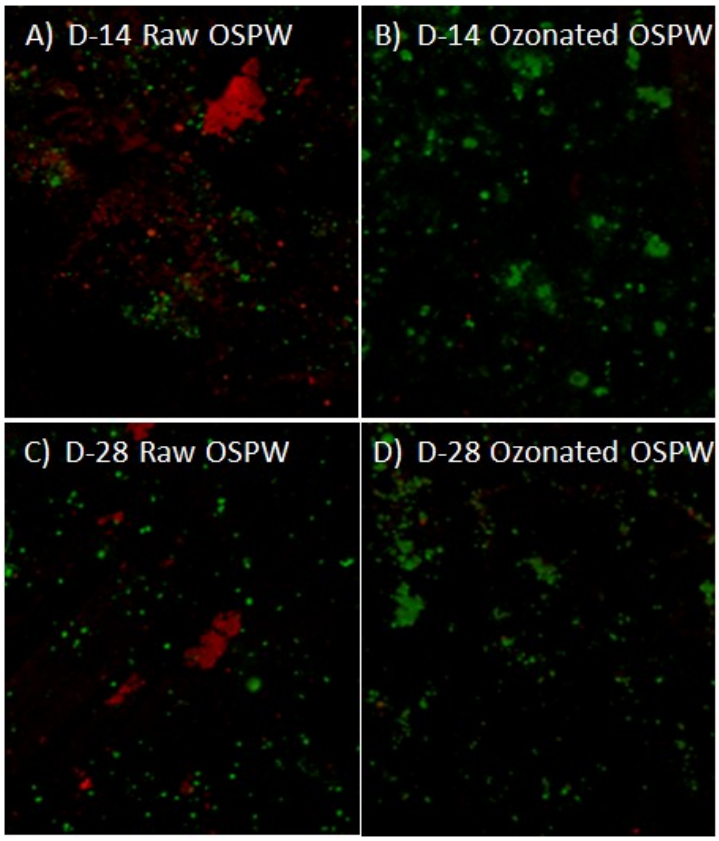

Figure 5.7. Confocal images of raw and ozonated OSPW after 14 and 28 days treatment with simultaneous GAC adsorption and biodegradation.

The microbial attachment to the GAC surface is accomplished by: (a) the irregular shape, rough surface, and high porosity of the GAC surface provides microorganisms protection from high fluid forces. The GAC surface allows microorganisms to accumulate and thus promotes microbial colonization (Shen et al., 2012); (b) the high adsorption capacity of GAC increases the

availability of substrates, oxygen, and nutrients to microorganisms at the media surface, enhancing microbial attachment efficiency (Yapsakli and Cecen, 2010); and (c) the GAC surface contains many bacterial binding sites (e.g., mesopores and macropores) for microbial colonization (An and Friedman, 1998; Herzberg et al., 2006; Hijnen et al., 2010).

Table 5.3. % Inhibitory effect using 81.9% Microtox test and bacteria copy number from qPCR in OSPW before and after 28 days of treatment.

Parameters	OSPW	Before treatment	Biodegradation only	Adsorption only	Combined treatment‡
Toxicity (%)	Raw	40.7 ± 3.6	32.6 ± 2.2	9.2 ± 5.3	1.9 ± 2.9
	Ozonated	28.9 ± 2.1	17. 0 ± 1.1	14.1 ± 6.5	6.0 ± 1.2
qPCR (Bacteria copy number $\times 10^8$/reactor)	Raw	1.3 ± 0.1	2.5 ± 0.0	-	2.7 ± 0.0
	Ozonated	1.4 ± 0.1	2.8 ± 0.0	-	2.9 ± 0.1

‡ Combined treatment: simultaneous GAC adsorption and biodegradation. The total bacteria concentration per reactor in the combined treatment was calculated by summing the bacteria copy in the biofilm on the GAC surface (as bacteria copy number/g GAC) and in the 500 mL OSPW (as bacteria copy/mL OSPW).

Bioregeneration has been demonstrated in other wastewater treatment processes, which involves desorption and biological removal of adsorbed organics on biologically activated carbon (BAC) surface (Aktas and Cecen, 2007; Oh et al., 2011). However, quantifying the

bioregeneration during BAC treatments of wastewater has difficulties because of simultaneous biodegradation, adsorption, and desorption of sorbates (Aktas and Cecen, 2007). Compared to the sum of organics (COD and AEF) removal in adsorption only and biodegradation only experiments, the higher removal of organics (COD and AEF) in OSPW from the simultaneous adsorption and biodegradation indicates synergistic effects in the combined system. Confocal images (Figure 5.7) show an active biofilm developed on the GAC surface which contributed both biodegradation and GAC bioregeneration. However, a quantitative estimate of the biofilm role was beyond the scope of the current study. The higher removal of COD and AEF in ozonated OSPW compared to the removal of COD and AEF in raw OSPW corresponds with the higher biological activity on the GAC surface used in the treatment of ozonated OSPW. qPCR results (Table 5.3) and confocal images (Figure 5.7) showing higher bacteria concentration on the GAC surface used to treat ozonated OSPW confirms these results.

5.3.5. Toxicity Assessment

The Microtox® bioassay is a simple and fast test for determining toxicity and it has been utilized previously to determine the acute toxicity of OSPW (Holowenko et al., 2001; MacKinnon & Boerger, 1986). As well, this assay has been shown to have toxicity results that show good correlation to other animal-based toxicity assays (MacKinnon & Boerger, 1986). Currently, the EC_{50} values were not measured due to the low overall toxicity (< 50% from 15 min inhibition) of raw and ozonated OSPW using the Microtox® bioassay (e.g., 40.7% for raw OSPW from 15 min inhibition). Therefore, the 15 min inhibition values were currently used to exhibit the relative toxicity changes for the various experiments (Table 5.3). The biodegradation only treatment was not as effective in reducing raw OSPW toxicity as we observed from

combined treatment in agreement with previous study findings (Gamal El-Din et al., 2011; Wang et al., 2013a). The toxicity removals for raw and ozonated OSPW were 77.5% and 51.4% from GAC adsorption only, and 95.3% and 79.4% from combined treatments, respectively. The combined toxicity removal from ozonated OSPW was less compared to the combined removal from raw OSPW which was unexpected. Thus, despite the microtox bioassay is fast and promising technology for evaluating the toxicity in the lab using *V. fischeri*, a more comprehensive studies including tests such as fish toxicity assays should be carried out to assess the impact of combined treatment on OSPW toxicity removal.

5.4. Conclusions

Biodegradation alone was not an effective process for complete degradation of classical and oxidized NAs in raw and ozonated OSPW. The GAC adsorption alone removed 92% and 95% of classical NAs and 68% and 71% of oxidized NAs in raw and ozonated OSPW, respectively. Simultaneous GAC adsorption and biodegradation removed 93% and 96% of classical NAs and 74% and 77% of oxidized NAs, respectively. A large number of bacteria (> 10^8 bacteria/g GAC) on the GAC surface were observed by qPCR and CLSM. Adsorption played a vital role in the removal of NAs in simultaneous adsorption and biodegradation treatment of both raw and ozonated OSPW. A low ozone dose (20 mg/L) had little impact on the removal of NAs (2–3%) in OSPW compared to the impact of simultaneous GAC adsorption and biodegradation treatments. Our findings indicate that simultaneous GAC adsorption and biodegradation treatment can expedite the removal of toxicity due to the presence of classical and oxidized naphthenic acids in OSPW, and is thus a promising technology to reclaim reusable water from OSPW generated by the oil sands industry.

5.5. References

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CHAPTER 6. GRANULAR ACTIVATED CARBON BIOFILM MICROBIAL COMMUNITY ANALYSIS AND IMPACT OF MICROBIAL COMMUNITIES ON OIL SANDS PROCESS-AFFECTED WATER QUALITY

6.1. Introduction

The open-pit mining of Canada's Athabasca oil sands extracts the mined bitumen using the "Clark hot water process" where 1 m^3 of oil sands bitumen extracted generates 4 m^3 of oil sands process-affected water (OSPW) (Anderson et al., 2012; Goff et al., 2013; Holowenko et al., 2002). This OSPW is contaminated with a large number of inorganic and organic chemicals during the extraction process that are known to be toxic to aquatic and terrestrial life (Allen, 2008). Thus, the water use associated with the extraction of oil sands bitumen is a great environmental issue that needs to be addressed. Currently, over a billion m^3 of OSPW are stored in active settling basins given that the oil sands companies have been operating under a no release policy of OSPW until suitable treatment is available (Del Rio et al., 2006; Han et al., 2009). These volumes are continually increasing as the oil sands bitumen extraction remains in the Athabasca region (Anderson et al., 2012).

The fresh OSPW has acute, sub-chronic and chronic toxicity to aquatic organisms with the majority having been attributed to the high concentrations of naphthenic acids (NAs) (Anderson et al., 2012; Garcia-Garcia et al., 2012). NAs are a mixture of organic surfactants containing carboxyl groups and it has been estimated that there exists more than 200,000 individual NA structures associated with oil sands bitumen (Rowland et al., 2011). The general chemical formula of classical NAs is $C_nH_{2n+Z}O_2$, where n is the number of carbon atoms, and Z is either zero or a negative even integer representing the number of hydrogen atoms lost because

of ring formation (Clemente and Fedorak, 2005; Toor et al., 2013). Currently, the use of wetlands treatment technology by oil sands industry for treatment of OSPW is not effective in eliminating toxicity given that many NAs are recalcitrant to natural biodegradation. Therefore, establishment of an adequate OSPW treatment technology is urgently needed to help eliminate the need for continual accumulation of OSPW in tailing ponds, as well as reduce the current OSPW storage by safe discharge of treated OSPW to the receiving environments. In addition, the improved treatment of OSPW may also lead to the reduction of the fresh water withdrawal from the Athabasca River by extending the recycling capacity of the OSPW.

Granular activated carbon (GAC) has recently been considered for use in OSPW treatment (Mohamed et al., 2011) because of its high adsorption capacity for OSPW organics due to its high surface area as a result of interconnected micropores, mesopores and macropores (Sulaymon et al., 2013). However, despite its high adsorption capacity, the GAC adsorption sites become exhausted by bound organic pollutants within a short time for high organic wastewater loadings leading to the need for regeneration of adsorption sites to allow for further capacity (Aktas and Cecen, 2007; Nath and Bhakhar, 2011; Oh et al., 2011). This regeneration can be accomplished via degradation of adsorbed organics from bacteria found within a developed microbial biofilm on the GAC surface (termed bioregeneration). Thus, the combined adsorption-degradation of a GAC-biofilm treatment requires less frequent chemical/thermal regeneration of the carbon resulting in lower energy requirements and operating costs (Aktas and Cecen, 2007; Nath and Bhakhar, 2011; Walker and Weatherley, 1999). The biofilm technology is very promising for removal of recalcitrant and toxic organic compounds, such as NAs, due to the high retention time of organics and high biomass concentration of the biofilm (Combarros et al., 2014; Frascari et al., 2014). However, the biofilm performance is reliant on the effective

transfer and degradation of organics within the biofilm that is dependent on the biofilm morphology including thickness, porosity, distribution on the surface, and microbial communities (Choi et al., 2014; Taherzadeh et al., 2012). Thus, an improved understanding of this morphology and the biofilm microbial communities can provide important guidance for biofilm reactor design, stable operation and improvement of reactor performance in wastewater treatment systems (Luo et al., 2013).

To date, only one study has considered the use of a GAC biofilm reactor for treatment of OSPW NAs reporting that more than 86% of OSPW NAs were removed after the treatment process (Islam et al., 2014). Given these positive results, there is a clear need for further investigation of the efficacy of a GAC biofilm reactor for the treatment of OSPW including characterization of the biofilm bacteria (e.g., pyrosequencing) is necessary to understand the effectiveness of the GAC biofilm system. Denatured gradient gel electrophoresis (DGGE) has been utilized for the analysis of the biofilm microbial community in OSPW treatment on various surfaces such as polyethylene (Choi et al., 2014; Golby et al., 2012; Hwang et al., 2013), polyvinyl chloride (Choi et al., 2014; Hwang et al., 2013), and GAC (Islam et al., 2014). However, this conventional molecular biological method does not provide comprehensive and systematic information on the various microbial communities (Lu et al., 2012). A more accurate and complete characterization of the microbial community can be assessed using high throughput pyrosequencing (Luo et al., 2013). This method has been recently utilized for the analysis of the microbial community in biofilm for raw water distribution (Luo et al., 2013), drinking water (Liao et al., 2013) and wastewater biofilm treatment (Hu et al., 2012). However, no relevant study has been conducted to investigate how biofilm formed on GAC could affect the performance of OSPW treatment so far.

Thus, the main objective of the present study was to evaluate the biofilm growth on the GAC surface and microbial community using high throughput 454-pyrosequencing in GAC biofilm applied in OSPW treatment. Ozone can degrade classical NAs with high cyclization and long chains into lower molecular weight oxidized NAs compounds, which increases their biodegradability (Martin et al., 2010; Wang et al., 2013b). Thus, the impact of ozonation on the biofilm growth, biofilm community structure and NAs removal were also assessed for comparison to the raw OSPW treatment process. The biofilm development on the GAC was performed using a batch study with continuous change of the raw and ozonated OSPW (i.e., a semicontinuous process). The impact of the bacteria community of the biofilm on the OSPW quality was also investigated.

6.2. Materials and Methods

6.2.1. Source of OSPW

Aurora process water from an oil sands tailing pond in Fort McMurray, AB, Canada was sampled and shipped in October 2012. Raw OSPW was received at the University of Alberta in 200 L barrels and preserved at 4 °C in a cold storage room prior to use in experiments.

6.2.2. Source of GAC

Selected grade bituminous coal based steam-activated granular carbon (GAC) (SGL 8×30) was purchased from Calgon Carbon Corporation (Pittsburgh, PA, USA). GAC was sterilized at 121 °C for 30 min (Model 733LS vacuum/gravity system sterilizer, NY, USA), dried at 104 °C for ~72 h and allowed to cool in a dessicator before being used in GAC experiments.

6.2.3. Ozonation of OSPW

The detailed descriptions of the ozonation procedure and calculation of the utilized ozone dose have been reported previously in Chapter 4. Briefly, ozonation of raw OSPW was performed by generating ozone from extra-dry and high-purity oxygen using an ozone generator (PCI-WEDECO, GSO-40, Herford, Germany). The gas mixture (ozone and oxygen) containing an ozone concentration ~150 mg/L was sent through a ceramic fine bubble gas diffuser located at the bottom of a 200 L plastic barrel containing raw OSPW. The ozone concentrations in the feed and off-gas lines were continuously monitored by two identical ozone monitors (model HC-500, PCI-WEDECO) during OSPW ozonation. The residual ozone (unreacted, physically absorbed in the OSPW) was purged using pure nitrogen and the off-gas line ozone concentration was recorded. The Indigo method was used to estimate the residual ozone as per standard methods (APHA, 2005).

6.2.4. Biofilm Growth Experimental Methods

The culture of a biofilm on the GAC surface was carried out at room temperature (21 ± 1 ^{0}C) at 150 rpm on horizontal shaker using a 1 L amber bottle containing 2 g GAC in 500 mL OSPW (Innova^{TM} 2100, platform shaker, New Brunswick Scientific, USA). The schematic for the experimental set-up has been provided in Chapter 5 (Figure 5.1). A control GAC adsorption only (i.e., sterilized OSPW and sterilized GAC) experiment was carried out in parallel. For biofilm growth experimental start up, raw OSPW was used without addition of any inoculants, whereas ozonated OSPW was inoculated with endogenous raw OSPW bacteria culture (5 mL) to promote biofilm growth in the absence of endogenous bacteria which may have been reduced or eliminated during the ozonation process. Initially, the reactors were run for 4 days in batch mode

without water change to allow for the attachment of bacteria on the GAC surface. Subsequently, the reactors were operated in a semicontinuous mode with continuous renewal of raw and ozonated OSPW every second day until day 48.

6.2.5. Water Chemistry Analysis

Chemical oxygen demand (COD) in both raw and ozonated OSPW was measured according to standard methods (APHA, 2005). The acid extractable organic fraction (AEF) was analyzed every 6 days using the protocol developed and used by the oil sands industry and described by (Gamal El-Din et al., 2011). Briefly, raw and ozonated OSPW were filtered through a 0.45 μm nylon filter and the pH adjusted to 2.4-2.5 using sulfuric acid. 50 mL of this acidified OSPW was extracted with HPLC grade DCM, dried and reconstituted in Optima grade DCM prior to analysis. Fourier transform infrared (FT-IR) spectroscopy (PerkinElmer, ON, CA) was used to measure the AEF for carbonyl stretch equivalents in OSPW using a KBr cell. It should be noted that the AEF measures all compounds with functional groups containing carboxylic acids, ketones, and aldehydes; it cannot be used as a direct measure of NAs concentrations. However, AEF values are meaningful as a surrogate measure for NAs as commonly used by the oil sands industry.

Classical NAs concentrations were determined using ultra-high pressure liquid chromatography (UPLC)-high resolution mass spectrometry (HRMS). The detailed methodology has been detailed previously (Hwang et al., 2013; Islam et al., 2014). Briefly, centrifuged (10,000 rpm for 10 min) OSPW samples were passed through a Waters Acquity UPLC® System (Milford, MA, USA) for the separation of NAs using myristic acid -^{13}C as an internal standard. A high resolution (40,000 FWHM) Synapt G2 HDMS mass spectrometer (m/z from 0 to 600)

equipped with an electrospray ionization source operating in negative ion mode and quadruple time-of-flight (QTOF) was used for detecting the classical NAs. TargetLynx® ver. 4.1 software was used to analyze the data for target compounds.

6.2.6. Biological Parameters Characterization

6.2.6.1. Bacterial Enumeration using Heterotrophic Plate Counting (HPC)

Bacterial enumeration was performed by heterotrophic plate counting (HPC) using the drop plate method (Zelver et al., 1999) for both raw and ozonated OSPW and the biofilms developed on the GAC surfaces. A mass (0.5-1.0 g) of GAC was placed into 2 mL of a phosphate buffer solution (PBS) buffer in a 15 mL sterile tube and mixed using a vortex for 1 minute. A series (7 serial) of 10-fold dilutions were performed and 10 µL of each dilution was plated in triplicate on R2A (Difco) agar culture plates. Plates were incubated at 37 °C with counting performed at 24, 48 and 72 h. For biofilm samples, the counts were converted into bacteria colony/g GAC of biofilm.

6.2.6.2. CLSM Imaging

GAC biofilm samples were taken from both raw and ozonated OSPW treatments every 6 days. The GAC biofilms were stained with SYTO 9 (BacLight Live/Dead bacterial viability kit, Molecular Probes, USA) and Concanavalin A (ConA, Molecular Probes, Eugene, OR) lectin conjugated with Texas Red, for the probing of live cells and EPS, respectively (Jefferson et al., 2005). Biofilm image observation, acquisition, and biofilm thickness measurements were performed with a confocal laser scanning microscope (CLSM) (Zeiss LSM 710, Carl Zeiss Micro Imaging GmbH, Germany). The images were observed and scanned randomly at 4 to 5

positions with a lens (20 × 0.8NA Plan-Apochromat). The detailed procedure has been described previously by Hwang et al. (2013).

6.2.6.3. DNA Extraction, Real Time-PCR & 454 Pyrosequencing

A PowerSoil® DNA isolation kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) was used for isolating DNA from bacteria cells in duplicate. Planktonic bacteria were isolated as a pellet by centrifuging raw and ozonated OSPW at 3,700 rpm for 10 min (Multifuge3S/3S-R, Heraeus, Thermo Scientific, USA). The pellet was collected and added to power soil bead tubes to isolate total genomic DNA. For isolating DNA from the GAC biofilm, a mass of GAC (0.5-1.0 g) was added directly to the power soil bead tubes. The manufacturer's protocol was followed for isolating the total genomic DNA. Real-time PCR was carried out using a CFX 96 Touch™ Real Time PCR Systems (Bio-Rad Laboratories Inc., USA) containing 1 × SsoFast™ EvaGreen® supermix, 0.5 μM of each primer (Integrated DNA Technologies, Coralville, IA), and 5 μL of diluted DNA within a 25 μL total reaction volume. The protocol for qPCR has been provided in Chapter 5.

After DNA extraction, the V1–V3 regions of the 16S rRNA genes were amplified by PCR using 28F and 519R primer pairs (GAGTTTGATCNTGGCTCAG and 519R GTNTTACNGCGGCKGCTG, respectively) and a Qiagen hotstart Taq mastermix. The DNA was denatured at 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 54 °C for 45 s, 72 °C for 60 s. Finally, an extension reaction was performed at 72 °C for 10 min. The amplified DNA was sequenced and analyzed using a 454/Roche GS-FLX Instrument by the Research and Testing Laboratory in Lubbock, Texas as previously described (Smith et al., 2010). An automated pipeline was used to process raw 454 sequence data. After de-noising (USEARCH

application) and chimera removal (UCHIIME in *de novo* mode), the sequences ware clustered into operational taxonomic units (OTU) clusters using USEARCH for taxonomic identification.

Trimmed sequences were also processed through the Ribosomal Database Project (RDP) pyrosequencing pipeline (Cole et al., 2009). The sequences of each sample were further aligned using the RDP aligner tool before the RDP Clustering function was applied. The resulting clusters were submitted to calculate the Chao1 estimator, Shannon-Weaver (H') index, the evenness, and the rarefaction curves at the level of 3% dissimilarity, which was considered to be approximately related to species level. The RDP abundance statistics tool was also used to calculate the differences between samples based on the Jaccard method (Chao et al., 2006) and construct a distance matrix at 3% dissimilarity by using the unweighted pair group method with arithmetic mean (UPGMA).

6.3. Results and Discussions

6.3.1. Characterization of Biofilm

6.3.1.1. Confocal Imaging

Figure 6.1 shows confocal image of biofilms developed on the GAC surfaces in both raw and ozonated OSPW during the 48 day experimental duration. Bacteria attachment was observed on the GAC surface on day 6 (first sample day) in both raw and ozonated OSPW treatments as indicated by the green and red areas of the images. The observed state of the bacterial attachment on the GAC surface is called spotty aggregation stage (Bogino et al., 2013). The biofilm growth reached steady state after 30 days and the final biofilm thicknesses on day 48 were around 50 μm for both raw and ozonated OSPW (Figure 6.1). The biofilm images indicate that the bacteria did not fully cover the GAC surface even after 48 days. This is expected as the bacteria form a

complex structure of aerobic biofilms formed by discrete aggregates of densely packed cells and interstitial voids as reported previously (Herzberg et al., 2006; Herzberg et al., 2003). The ozonated OSPW treatment showed faster initial attachment of bacteria (by day 18), however, had a similar biofilm thickness after 48 days (Figure 6.1). Ozonation can increase the biodegradability and reduce toxicity of OSPW (Martin et al., 2010), which might be a plausible reason of faster attachment of bacteria on the GAC surface in ozonated OSPW. The GAC biofilm thicknesses were higher than the previously observed biofilm thickness (34 ± 5 μm) for the treatment of OSPW using fluidized bed biofilm reactors (Islam et al., 2014).

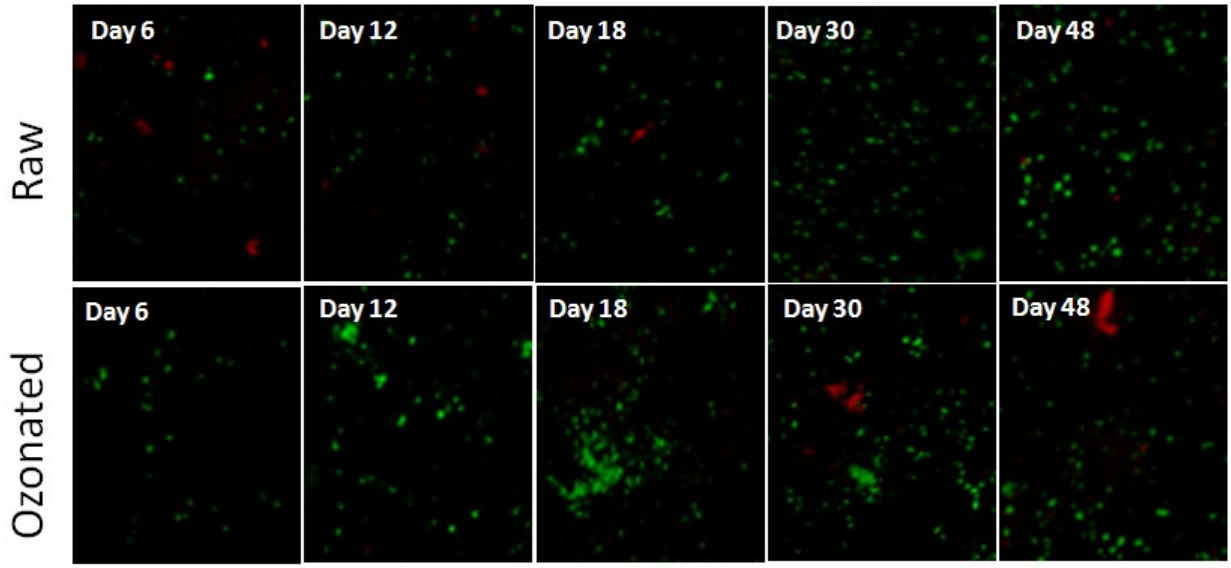

Figure 6.1. Confocal images of GAC surfaces developed during biofilm growth over 48 days in raw OSPW and ozonated OSPW.

6.3.1.2. Quantification of Microbial Community in the GAC Biofilm

Figure 6.2 shows the results for bacterial growth for qPCR and HPC analyses of raw and ozonated OSPW over the 48 day experimental duration. The bacterial attachment on the GAC surface was observed on day 6 for both raw and ozonated OSPW. For both analyses the bacterial growth increased in the GAC biofilm until reaching a plateau on day 30. The final bacterial concentrations using qPCR were ~10^9 copies/g GAC for raw and ozonated OSPW (Figure 6.2A). This results can be supported by a previous study by Velten et al. (2007) showed 7.9×10^9 cells/g GAC in GAC biofilms at the top of the bed in a biofilter from continuous treatment of ozonated lake water using ATP analysis.

The HPC bacterial colony growth increased over time and reached a plateau on day 30 for both raw and ozonated OSPW (Figure 6.2B). The growth patterns trends were similar over time for both raw and ozonated OSPW. The initial number (Day 0) of cultivable bacteria were 2×10^3 CFU/mL and 3×10^3 CFU/mL for raw and ozonated OSPW, respectively. At day 30 and day 45 there were ~10^7 CFU/g GAC in both raw and ozonated OSPW (Figure 6.2B). As compared with qPCR results, approximately 1-5% of bacterial colonies were detected by conventional cultivation dependent microbiological methods. Similar percentages of HPC bacterial growth (1-10%) was reported on a polystyrene surface used in an activated sludge under aerobic condition (Iffat et al.,2013).

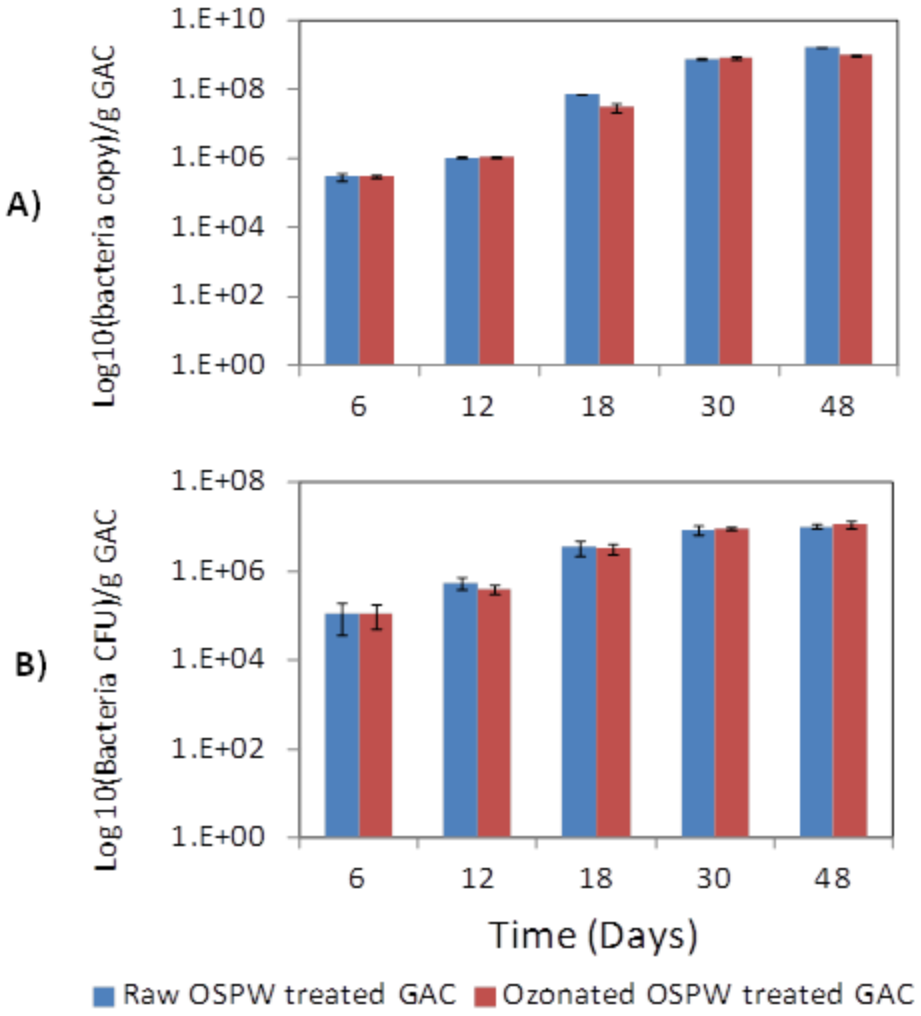

Figure 6.2. Bacteria growth on the GAC surface over 48 days for raw and ozonated OSPW treated GAC; (A) qPCR analysis, and (B) heterotrophic plate counting method.

The bacterial growth on the GAC surfaces for both raw and ozonated OSPW treatments was done without the addition of an external organic food source to the reactor. Therefore, the microbial community metabolic needs were being met by the oxidation of adsorbed organics on the GAC surface and the continuous supply of easy biodegradable OSPW organics from fresh OSPW provided every second day. Similarly, Hwang et al. (2013) concluded that the easily biodegradable fraction of OSPW organics in raw and ozonated OSPW could be useful in the

development of a biofilm on the GAC surface (Hwang et al., 2013). The ability of the GAC surface to achieve a biofilm containing a high bacteria concentration might be accounted for by: (a) the surface morphology which is irregular, rough, and highly porous which may enhance bacterial colonization on the surface by protecting from high fluid forces (Shen et al., 2012); (b) the high adsorption capacity of GAC which increases the availability of substrates, oxygen, and nutrients which attracts bacteria to the GAC surface (Yapsakli and Cecen, 2010); and (c) the high surface area of the GAC due to the presence of different pores on its surface (An and Friedman, 1998; Herzberg et al., 2006; Hijnen et al., 2010).

6.3.2. Microbial Community Structure

The advantage of pyrosequencing analysis is its ability to determine more sequences than conventional cloning and sequencing methods (Liao et al., 2013). This ability makes this analysis useful in the elucidation of the bacterial community structure in complex water and wastewater treatments such as the current GAC biofilm reactors.

Table 6.1 shows the overall bacterial diversity statistics of the various OSPW samples. Rarefaction curves were generated based on OTUs and sequences counted at a 3% cutoff (i.e., equivalent to species level) to make a comparison of species richness among different samples. Figure C12 (Appendix-C) shows the rarefaction curves of the four samples suggesting that the overall microbial communities in the GAC biofilm treatments were less diverse compared with those in the OSPW treatments. As well, the Chao1 (community richness), Shannon (community diversity) index and evenness (community diversity) confirmed that the richness and diversity of GAC biofilm samples were less diverse than the OSPW samples (Table 6.1). This decreased diversity may be attributed to the toxicity of NAs accumulated on the GAC which will only

allow survival of bacteria with high tolerances to the high concentrations of adsorbed NAs. Moreover, the higher diversity observed for the ozonated versus raw OSPW GAC biofilm samples (Table 6.1) confirms the impact of accumulated NAs as the ozonated OSPW has much lower NAs concentrations versus raw OSPW (see Section 5.3.3). The phylogenetic distance cladogram based on the Jaccard index for the bacterial communities found in the different OSPW samples is shown in Figure C13 (Appendix-C). The bacterial communities of the raw and ozonated OSPW were clustered together while the two types of GAC biofilm were also grouped. GAC biofilm samples in ozonated OSPW have the largest genetic distances with other samples indicating that GAC adsorption significantly affected the structure of microbial community.

The relative abundance of phyla identified in OSPW and GAC biofilm samples are shown in Figure 6.3. The sequenced bacterial phyla mainly consisted of bacteria including *Proteobacteria*, *Nitrospirae*, *Acidobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, and some other bacteria (Figure 6.3A). *Proteobacteria* were most abundant in all samples with approximately 40, 60, 70 and 90% of overall bacterial abundances for ozonated OSPW, raw OSPW, ozonated GAC biofilm and raw GAC biofilm, respectively. The *Proteobacteria* sequences decreased by 10% in the ozonated treatments versus the analogous raw treatments. As well, *Acidobacteria* and *Bacteroidetes* sequences were increased to 18% and 13%, respectively in the ozonated OSPW samples compared to 8% and 5% in raw OSPW samples, respectively.

The increase in the *Acidobacteria* for ozonated OSPW samples can be attributed to the formation of biodegradable acidic components from ozonation of raw OSPW leading to preferential selection of this phyla (Hwang et al., 2013). More than 92% of the total bacteria abundances were *Proteobacteria* in the raw OSPW GAC biofilm which may result from resistance to the toxicity of NAs which would be abundant on the GAC surfaces of the raw

OSPW. Generally, differences in microbial communities between two biofilms could be due to changes in organic substrate composition, planktonic microbial community diversity, and bacterial surface polymer (e.g., LPS) physicochemical properties (Bogino et al., 2013). .

Table 6.1. Bacterial diversity statistics from different OSPW samples (clustered at 97% sequence identity)

OSPW sample	Reads	OTUs	Chao	Shannon	Evenness
raw	2 006	897	2 424	5.75	0.845
	2 419	1 020	2 464	5.95	0.859
ozonated	5 109	1 458	3 292	5.86	0.805
	1 992	776	1 830	5.44	0.817
GAC biofilm - raw	892	314	663	4.73	0.823
	2 033	563	1 249	4.77	0.753
GAC biofilm -	1 621	563	1 301	5.23	0.826
ozonated	1 731	548	1 009	5.24	0.831

OTUs – operational taxonomic units.

Chao – community richness. A higher number represents more richness.

Shannon – community diversity. A higher number represents more diversity.

Evenness – A higher number represents more evenness.

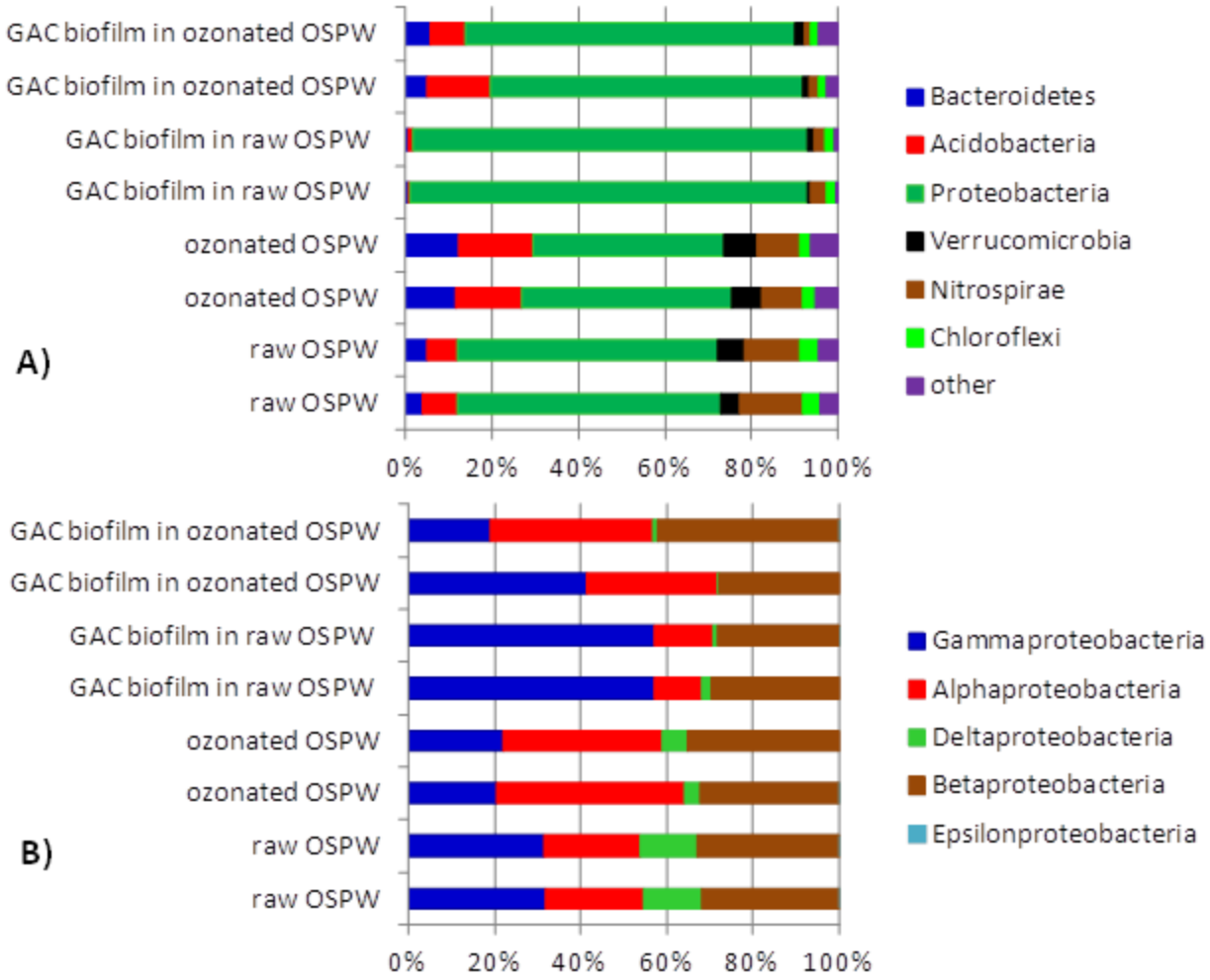

Figure 6.3. Relative abundance of bacterial community composition in raw and ozonated OSPW and GAC biofilms (including duplicates); (A) Relative abundance of total bacteria grouped by phyla and (B) Relative abundance of the *Proteobacteria* classes.

The phylum *Proteobacteria* accounts for more than 40% of the prokaryotic genera which are known to have extreme metabolic diversity (Kersters et al., 2006). Many bacteria of this order are ecologically important because they play key roles in the carbon, sulfur and nitrogen cycles (Kersters et al., 2006). Moreover, it has been reported that the dominant microorganisms of the Athabasca watershed and sediments belong to *Proteobacteria* and many of them are

known to degrade recalcitrant bituminous compounds (Yergeau et al., 2012).Thus, given this ecological importance and their overall abundances in the current samples further analysis of the *Proteobacteria* classes were considered (Figure 6.3B). The *Proteobacteria* abundances mainly composed of the orders *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria* and *δ-Proteobacteria*. The ozonated GAC biofilm and raw and ozonated OSPW samples exhibited similar abundances of these groups other than the *δ-Proteobacteria* abundances being marginal in the ozonated GAC biofilm sample. The dominant composition in raw OSPW GAC biofilm was *γ-Proteobacteria* with almost 60% of the total abundance. Our results differed with previously reported *Proteobacteria* compositions in the aerobically grown biofilm using rotating annular biofilm reactor using Athabasca river sediments as inoculants (Yergeau et al., 2013). Ozonation of OSPW led to an increase in the *α-Proteobacteria* relative abundance, whereas *γ-* and *δ-Proteobacteria* relative abundance decreased in the ozonated OSPW samples. It is clear that the *δ-Proteobacteria* were not able to grow on the GAC biofilm of both raw and ozonated OSPW (Figure 6.3B).

6.3.2.1. Bacterial Diversity in Order Level

The microbial order structures of the four most abundant orders of *Proteobacteria* were further determined at the order classification (Figure 6.4). The *α-Proteobacteria* class thrives in widely divergent habitats and plays a significant role in the biosphere of our planet (Kersters et al., 2006). The major orders in *α-Proteobacteria* were *Rhizobiales, Rhodospirillales, Sphingomonadales, Rickettsiales, Rhodobacterales,* and *Caulobacterrales* (Figure 6.4A). The dominant order was *Rhizobiales* in all four samples ranging from 45 to 75% of total abundances (Figure 6.4A). The *Rhizobiales* species have diverse functions such as atmospheric nitrogen

fixation through symbiosis with roots of leguminous plants, are known pathogens and are active carbon degraders in the bioremediation of contaminated soils (Carvalho et al., 2010; Gupta and Mok, 2007). The *Rhodobacterales* include species of photosynthetic bacteria and species that can metabolize organics containing sulfur compounds (Gupta and Mok, 2007). The major functions of *Rhodospirillales* species are taking part in plant photosynthesis and flower and fruit formation. However, it is reported that some of the *Rhodospirillales* species are chemoorganotrophs and can actively biodegrade carbohydrates and alcohols (Gupta and Mok, 2007; Kersters et al., 2006). *Caulobacterriales* are usually chemoorganotrophs, whereas *Sphingomonadales* have phototrophic and chemoorganotrophic species (Gupta and Mok, 2007; Kersters et al., 2006). *Rickettsiales* are known to be pathogenic for humans and animals (Darby et al., 2007).

The *β-Proteobacteria* are very heterogeneous with regard to metabolism, morphology and ecology (Kersters et al., 2006). This class has previously been shown to be bitumen degraders found in the sediments of the Athabasca River, its tributaries, and oil sands tailings ponds (Yergeau et al., 2012). The major orders observed currently in this class of bacteria include *Rhodocyclales, Burkholderiales, Nitrosomadales, Hydrogenophilales,* and *Neisseriales* (Figure 6.4B). However, *Rhodocyclales, Burkholderiales* and *Nitrosomadales* were the most abundant orders with > 95% of total abundances in all four samples. The dominant order was *Rhodocyclales* in raw OSPW (~ 40%), whereas, in ozonated OSPW and GAC biofilm samples (raw and ozonated OSPW), *Burkholderiales* was the dominant order (40 to 70%). *Rhodocyclales* can degrade a diverse group of carbons including aliphatic and aromatic compounds as well as nitrogen and phosphorous (Hesselsoe et al., 2009). *Burkholderiales* has been reported as the main contributor to the microbial bioremediation treatments for aromatic decontamination (Perez-Pantoja et al., 2012). Bacteria in this order can degrade a diverse group of aromatic

compounds including polychloro by-phenyls, naphthalene, and phenanthrene (Perez-Pantoja et al., 2012). The bacteria of the order *Nitrosomonadales,* are chemolithotropes which oxidize ammonia and fix carbon autotrophically from carbon dioxide (Garcia et al., 2013). This bacteria can adapt to low ammonium concentrations and can use carbon dioxide produced from bacterial metabolism (Kersters et al., 2006).

The *γ-Proteobacteria* are a diverse order of bacteria with currently observed orders including *Pseudomonadales, Xanthomonadales, Chromatiales, Alteromonadales, Oceanospirillales, Legionellales, Methylococcales* and unclassified *γ-Proteobacteria* (Figure 6.4C). For raw OSPW, the dominant order was *Pseudomonadales* (> 50%). The most abundant order in ozonated OSPW was *Alteromonadales* (> 40%). In raw and ozonated OSPW GAC biofilms, the dominant orders were *Chromatiales* (> 48%) and unclassified *γ-Proteobacteria* (> 40%). Among the orders, *Pseudomonadales, Xanthomonadales, Alteromonadales*, and *Oceanospirillales* are PAH degraders, whereas *Methylococcales* are methane degraders (Kersters et al., 2006; Lamendella et al., 2014). The order *Chromatiales* is known as phototrophic purple sulfur bacteria, able to perform photosynthesis and store elemental sulfur in cells (Tank et al., 2009). *Legionellales* is a gram negative chemoorganotorph pathogenic bacteria which survives in protozoan hosts (use amino acids as carbon and energy source) in natural environments and are responsible for pneumonia and influenza in humans (Kersters et al., 2006).

Figure 6.4D shows the *δ-Proteobacteria* compositions and the major orders were *Desulfuromonadales*, *Myxococcales, Desulfovibrionales, Desulfurellales,* and *Bdellovibrionales*. The *Desulfovibrionales* abundances were highest in the raw and ozonated OSPW samples with

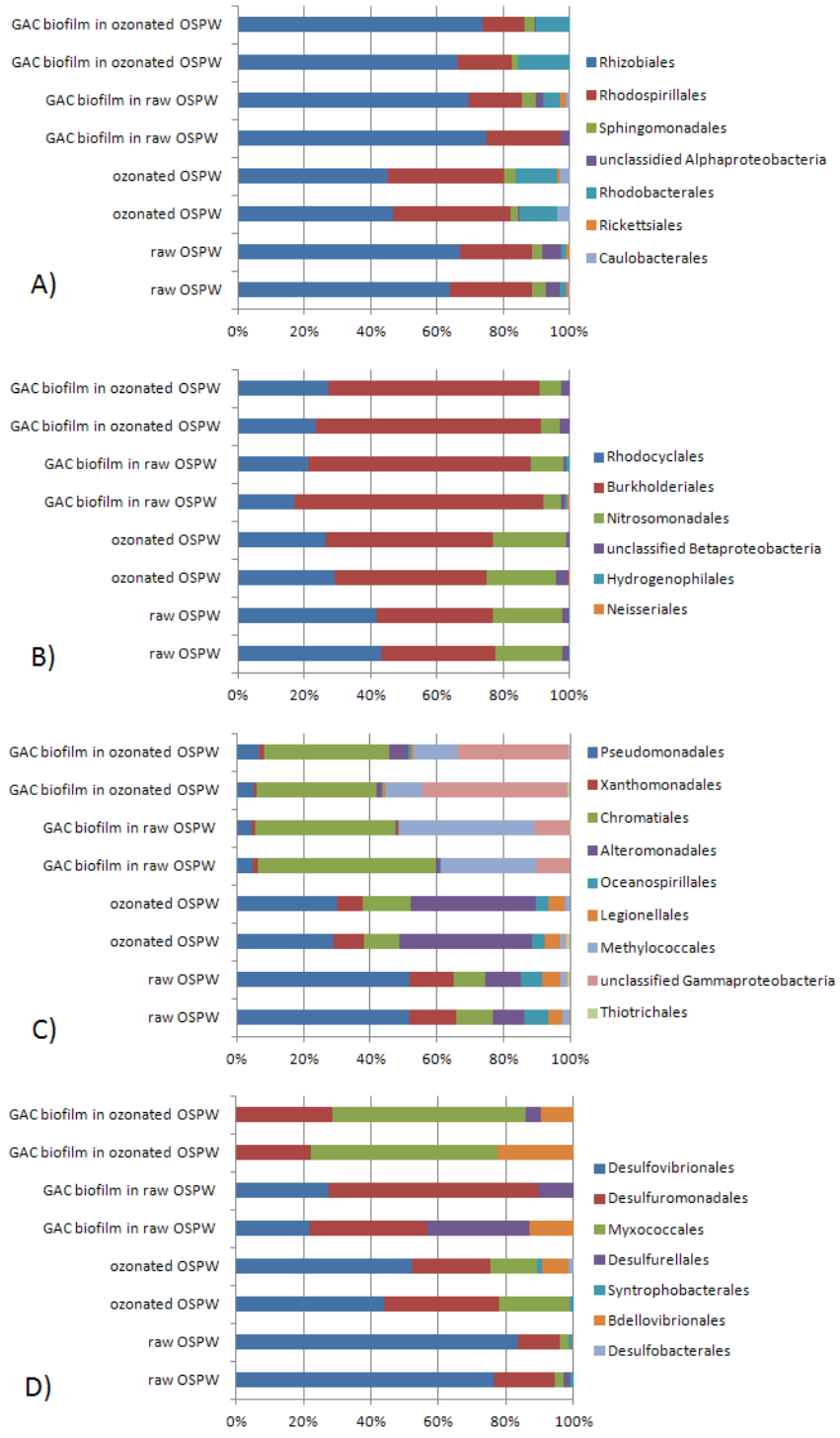

Figure 6.4. Relative abundance of different orders of *Proteobacteria* in raw and ozonated OSPW and GAC biofilms for the following classes; (A) *Alpha-*, (B) *Beta-*, (C) *Gamma-* and (D) *Delta-proteobacteria.*

45% and 80%, respectively. The *Desulfuromonadales* were most abundant in the raw GAC biofilm and the *Myxococcales* in the ozonated GAC biofilm. Among these orders, *Bdellovibrionales* and *Myxococcales* are aerobes and carbon degraders (Acosta-Gonzalez et al., 2013; Thomas et al., 2008; Velicer and Vos, 2009), whereas the sulfate and sulfur-reducers including *Desulfuromonadales, Desulfovibrionales* and *Desulfuromonas* are strictly anaerobic (Miroshnichenko et al., 1998b; Thomas et al., 2008; Ye and Zhang, 2013). *Desulfurella* order bacteria species is capable of oxidizing acetate and saturated fatty acids via sulfur reduction (Miroshnichenko et al., 1998a; Miroshnichenko et al., 1998b).

6.3.3. Water Quality Change

Table 6.2 shows the removal of COD, AEF, and NAs from GAC adsorption only (i.e., sterilized GAC) and GAC biofilm treatments for both raw and ozonated OSPW. An improved removal of COD and AEF was observed by the GAC biofilm treatment compared to GAC adsorption only from both raw OSPW and ozonated OSPW (Table 6.2). The COD removals were 7.1% and 9% higher after combined treatment as compared to GAC adsorption only for raw and ozonated OSPW, respectively. Similarly, AEF removals were 6.0% and 8.4% higher in combined versus adsorption only treatments for raw and ozonated OSPW, respectively (Table 6.2). However, there appeared to be no differences in NAs removal in the raw OSPW treatments with 28.3% due to adsorption and 30.7% in the combined treatment NAs removal (Table 6.2). In contrast, 50.7% and 66.0% of classical NAs (Table 6.2) were removed from adsorption only and combined treatments of ozonated OSPW, respectively.

Considering the GAC adsorption only treatment of both raw and ozonated OSPW (Table 6.2), the highest removal rate was observed for the classical NAs as compared to COD and AEF.

The higher removal of the classical NAs may be attributed to the higher hydrophobicity of the NAs compounds as compared to the general compounds measured via COD and AEF. It has been reported that classical NAs include mostly highly branched, cyclic compounds, with high molecular weights and long carbon chains which lead to high hydrophobicity (Zubot et al., 2012). AEF includes classical and oxidized NAs and other organic compounds with functional groups containing carboxylic acids, ketones and aldehydes (Jivraj et al., 1995) and has medium adsorption affinity to GAC. COD represents the oxidation of all organic and inorganic compounds/ions and has the lowest adsorption affinity to GAC.

Table 6.2. COD, AEF and NAs removal in raw and ozonated OSPW treatment after 2 days treatment including both adsorption only and combined (adsorption and biodegradation) treatments.

Parameters	Raw OSPW, % removal		Ozonated OSPW, % removal	
	Adsorption	Combined	Adsorption	Combined
COD	10.5	17.6	11.5	20.5
AEF	17.4	23.4	19.6	28.0
Classical NAs	28.3	30.7	50.7	66.0

The removal of classical NAs in GAC biofilm process was comparable to the removal from GAC only processes in raw OSPW (Table 6.2). Previously, it was found that the higher concentrations of bituminous compounds in the sediments of the Athabasca river reduced the biofilm bacterial activity in an annular biofilm reactor because of inhibitory effects (Yergeau et al., 2013). Thus, it is postulated that the reduced degradation of NAs on the GAC surface may

be a result of the acute toxicity of the concentrated classical NAs on the GAC surface inhibiting the bacterial biodegradation of the NAs. In comparison, the GAC biofilm in the ozonated OSPW treatment was able to remove around 15% more NAs than the GAC adsorption only treatment. This increased biodegradation of NAs may be attributed to the reduced toxicity of the GAC surface due to the reduced NAs concentrations after ozonation. It has been reported that ozonation produces oxidized NAs (Pereira et al., 2013) and oxidized NAs contain more –OH groups in their structure which caused less hydrophobicity (Wang et al., 2013a), and thus have less toxicity (Jones et al., 2011) compared to classical NAs.

Concentrations of individual classical NAs groups in untreated and treated raw and ozonated OSPW are shown in Figure C14 (Appendix-C). The removal of NAs species based on carbon and Z numbers are shown in Figure 6.5. The removal of NAs increased with an increase in carbon and Z numbers in raw OSPW from both GAC adsorption only and GAC biofilm treatments which indicates that the removal of NAs from raw OSPW was dominated by adsorption only (Figure 6.5A and 6.5C). For the ozonated OSPW, the NAs removal showed a similar trend, however, the GAC biofilm treatment had increased removal versus adsorption only (Figure 6.5B). As well, the removal of NAs from GAC biofilm treatment in ozonated OSPW did not follow any trend with Z number (Figure 6.5D). Higher removal of NAs was observed at low carbon numbers (n = 11 to 15) and Z numbers (Z = -2, -4) from GAC biofilm treatment compared to GAC adsorption only treatment. For example, the removal of Z = -2 class NAs was 82% from GAC biofilm treatment compared to 33% from GAC adsorption only treatment of ozonated OSPW. Higher biodegradability of lower molecular weight NAs has been reported previously (Han et al., 2009; Han et al., 2008). The increase of NAs adsorption with an increase of n and Z can be attributed to increases in hydrophobicity and nonpolarity of NAs with an

increase in n and Z (Scarlett et al., 2012; Zubot et al., 2012). Longer molecules can be adsorbed more readily on the GAC surface (Zubot et al., 2012) and can more easily approach the GAC surface to reach GAC pores.

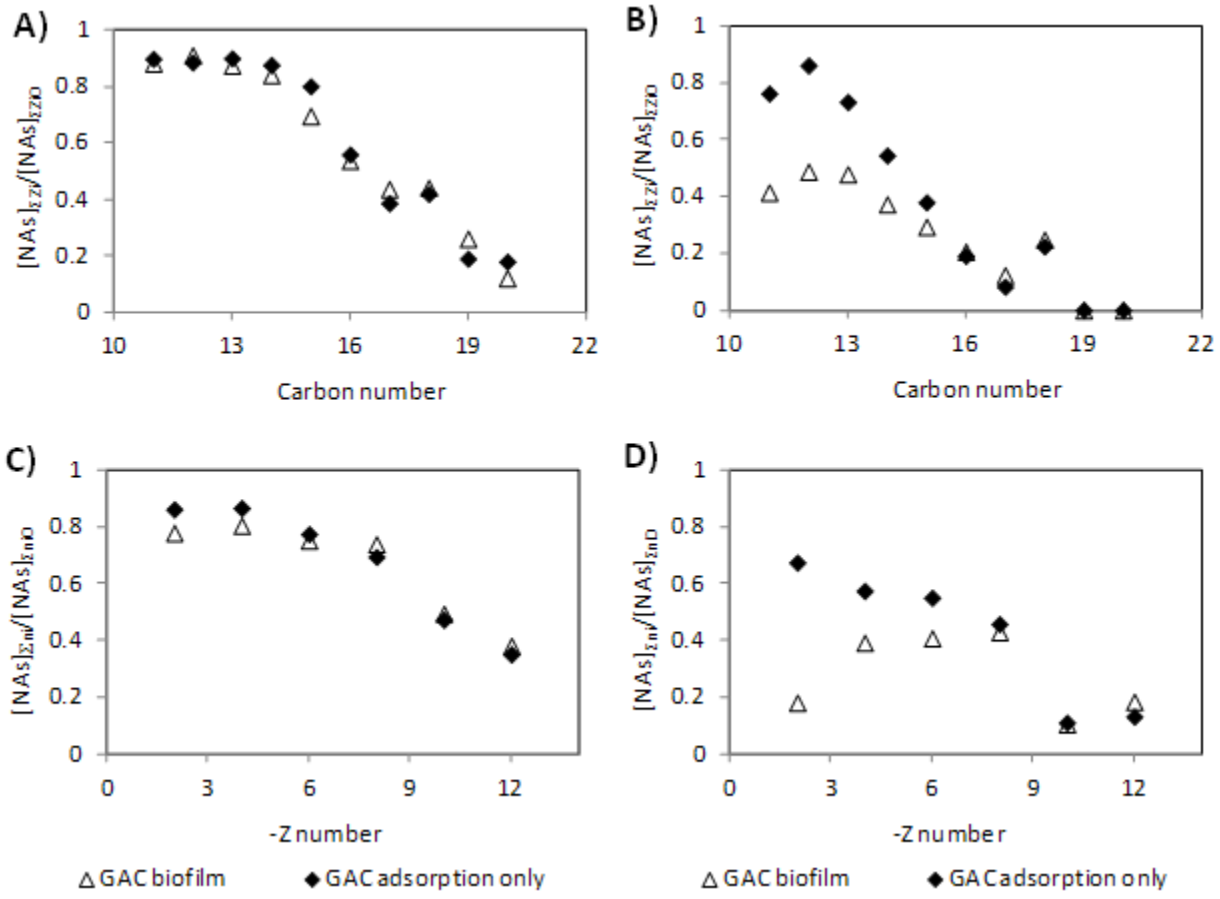

Figure 6.5. NAs relative concentrations in raw and ozonated OSPW before and after 2 days of treatment. (A) impact of carbon number for raw OSPW; (B) impact of carbon number for ozonated OSPW; (C) impact of Z number for raw OSPW; (D) impact of Z number for ozonated OSPW.

Overall, the GAC biofilm was more efficient in treatment of OSPW versus the GAC sorption only for both raw and ozonated OSPW treatments because of the presence of carbon

degrading orders in the reactors. The 454 pyrosequencing analysis suggested diverse microbial orders dominanted by carbon degraders that can utilize simple carbons and more complex polyaromatic carbons (e.g. *Myxococcales*, *Pseudomonadales,* and *Burkholderiales*). Numerous studies have reported that GAC with biofilm exhibited a combination of adsorption and biodegradation mechanisms for the removal of organic compounds from wastewater (Gibert et al., 2013; Moore et al., 2001; Putz et al., 2005). This combination indicates the bioregeneration of GAC from the biodegradation activity of microorganisms colonized on the external surface and macropores of the GAC (Xing et al., 2008). The combined effect (adsorption and biodegradation) on contaminant removal rates is dependent on the microbial abundance and composition, the retention time and their metabolic rates. A higher microbial degradation observed from ozonated OSPW compared to raw OSPW indicated higher biological activity on the GAC surface with ozonated OSPW. The plausible reasons are: (a) more diversity and richness of microbial community was observed in the GAC biofilm due to the supply of more biodegradable organic compounds in ozonated OSPW (section 6.3.2); and (b) reduced toxicity on GAC surface because of the decreased concentration of classical NAs in ozonated OSPW. The current study revealed a wide diversity of carbon degrading orders (e.g. *Burkholderiales*) had grown on the GAC biofilm surface which is useful for consideration of application of biofilm reactors for the treatment of OSPW. Previously, exogenous bacteria were applied to enhance the biofilm reactor performance for the treatment of oil field produced water (Zhao et al, 2006). Thus, pyrosequencing results may be helpful in the application of exogenous bacteria in bioreactors to promote the increase in carbon degraders' concentrations and to optimize the bioreactor performance.

6.4. Conclusions

This study demonstrated that an effective microbial colonization was observed on the GAC surface in raw and ozonated OSPW based on the observation from CLSM image, HPC, and qPCR. The biofilm developed on the GAC surface was a patchy biofilm. The microbial community structure analysis using 454 high-throughput pyrosequencing demonstrated the core population in OSPW and biofilms was *Proteobacteria* and ozonation changed the microbial diversity in OSPW and biofilms. A diverse carbon degrading orders of bacteria namely *Myxococcales*, *Pseudomonadales,* and *Burkholderiales* were present in biofilm samples indicating the microbial activity in the GAC biofilm. However, the less removal of NAs from raw OSPW (31%) compared to the NAs removal from ozonated OSPW (66%) indicated less microbial activity in the GAC biofilm from higher toxicity of raw OSPW NAs. Based on the biofilm growth affinity on the GAC, presence of carbon degraders, and water quality results, it can be demonstrated that the GAC biofilm treatment is a promising technology for the treatment of ozonated OSPW.

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CHAPTER 7. GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1. Thesis Overview

Water and wastewater management is an enormous concern for the oil sands industry in Northern Alberta, Canada. The OSPW generated from the surface mining bitumen extraction process is a complex matrix comprised of suspended and dissolved particles, unrecovered bitumen, trace metals and many other toxic compounds including the NAs. The NAs are biopersistent organic compounds, which contribute more than 50% of the OPSW toxicity to aquatic organisms. Thus, the oil sands companies operate based on a zero discharge policy with OSPW stored in tailings ponds near the mining sites. Recycling of OSPW in the extraction process results in the concentration of contaminants which are released to tailings ponds. Thus, an appropriate OSPW contaminant remediation technique is urgently needed to extend OSPW recycling and to permit the safe discharge of treated OSPW to the natural environments. The extended recycling will also allow for the reduced intake of fresh water from the Athabasca River which will limit the aquatic ecosystem impacts.

Up to date, various treatment techniques such as coagulation-floculation-sedimentation, adsorption, membrane filtration, nanofiltration and reverse osmosis, constructed wetlands, advanced oxidation processes, and biological treatment have been investigated for their viability for remediation of contaminants from OSPW. However, a large-scale and cost effective treatment technology for OSPW has yet to be developed. Biological treatment is an economic, environmentally friendly and potentially viable process for OSPW. Most of the recent studies for the biological treatment of OSPW have been carried out using the planktonic batch treatment approach. To date, only three studies have utilized the biofilm treatment approach for the treatment of OSPW, but none of them were specifically media based biofilm reactors. This is unexpected given the

effective use of media based engineered biofilm reactors for the treatment of other industrial and toxic wastewaters in the literature. Therefore, there is a significant research gap related to the evaluation of the applicability and performance of biofilm reactors for the remediation of OSPW at both small and large scales.

The present research focused on the treatability of OSPW using an engineered GAC-biofilm process. The biofilm reactors were tested for both raw and ozonated OSPW using batch and continuous treatment reactors. Evaluations included the impact of ozonation on the biodegradability of OSPW (by measuring BOD_5), the characterization of the microbial community structure in both OSPW and GAC-biofilm (using PCR-DGGE and 454 pyrosequencing), the assessment of the biofilm growth on the GAC surface (by analyzing qPCR and HPC), and the overall reactor performance (by measuring COD, AEF and NAs concentration). The characterization of NAs removal based on carbon number, Z number and oxygen number were also investigated.

7.2. Conclusions

The following conclusions were drawn based on our experimental results and analysis:

1. After 120 days of bioreactor operation, the COD, AEF and NAs removals from the GAC-FBBR were 31%, 33% and 86%, respectively, at the organic loading rate (OLR) of 2.46 kg COD/m^3.d for the treatment of raw OSPW. The impact of changing of OLRs and the hydraulic loading rate (HLRs) demonstrated that the reactor performance was mainly dependent on the OLRs and, to a lesser extent, on HLRs during the operation of the reactor. Maximum removals of 51%, 56% and 96% for COD, AEF and NAs, respectively, were observed at the lowest OLR (0.75 kg COD/m^3.d). The GAC-biofilm seems to be a promising treatment method for OSPW remediation by removing 96% of classical NAs.

2. New NAs species with lower Z numbers (Z = 0 and -2) in the bioreactor effluents were confirmed using UPLC-HRMS. Moreover, PCR-DGGE results demonstrated that a variety of carbon degraders namely *Polaromonas jejuensis*, *Algoriphagus sp.*, *Chelatococcus sp.* and *Methylobacterium fujisawaense* were in the GAC-biofilms. The production of new NAs species and presence of carbon degraders in the biofilm confirmed that there was microbial activity in the GAC-FBBR.
3. The combined removal from ozonation and GAC biofilm treatment (adsorption and biodegradation) process removed more than 62 % COD, 88 % AEF and 99.9 % NAs under optimized operational conditions (phase VI; Chapter 4) indicates that the GAC-FBBR is effective for the treatment of pre-ozonated OSPW.
4. A less diverse planktonic bacterial community in ozonated OSPW was observed compared to raw planktonic OSPW suggesting that ozonation selectively kills microbial strains. However, more diverse microbial communities (with polyaromatic carbon degrader namely *B. multivorans*) were found in GAC biofilms used for the treatment of ozonated OSPW after 120 days indicating that ozonated OSPW facilitated more strains to grow on the GAC support media in the GAC-FBBR.
5. The removal of COD, AEF and total oxidized NAs was higher from simultaneous adsorption and biodegradation compared to the sum of removal of COD, AEF and total oxidized NAs from GAC adsorption only and biodegradation only indicated the removal synergy from the combined process (simultaneous adsorption and biodegradation) because of GAC regeneration from biodegradation.
6. Biodegradation only treatment was not efficient for degrading any NAs in either raw or ozonated OSPW. However, by using 0.4 g GAC/L OSPW, the removals of classical and

total oxidized NAs from simultaneous GAC adsorption and biodegradation were 74% and 93% for raw OSPW, respectively, and 77% and 96% for ozonated OSPW, respectively. These results indicate that a simultaneous GAC adsorption and biodegradation treatment is a useful process for the treatment of OSPW NAs.

7. Based on the removal of different classes of classical and total oxidized NAs, the NAs removal increased with increasing carbon number for the adsorption and simultaneous adsorption/biodegradation treatments. In contrast, no trends of NAs removal were observed with ring number. The highest biodegradation was observed for the single ring (Z = -2) NAs (classical and oxidized) for raw and ozonated OSPW from the biodegradation only treatment.
8. A Microtox bioassay demonstrated that a higher effective toxicity removal (> 97.5%) was observed from simultaneous GAC adsorption and biodegradation compared to GAC adsorption only (> 81.5%), which indicated that GAC biofilm process is a promising treatment technology for the elimination of OSPW toxicity due to the presence of classical and oxidized naphthenic acids in OSPW.
9. An effective biofilm growth on the GAC surface was monitored from a semicontinuous process in both raw and ozonated OSPW based on CLSM, HPC and qPCR results. The total bacteria copy number in the biofilm on one gram GAC was more than 10^9 in both raw and ozonated OSPW treatment at steady state indicated a high microbial population can grow on the GAC surface from OSPW treatment.
10. The 454-pyrosequencing results demonstrated that the *Proteobacteria* was the dominant phylum in all samples. Analysis of the *Proteobacteria* community structure found that the major orders of *Proteobacteria* were carbon degraders namely *Sphingomonadales,*

Burkholderiales, Pseudomonadales, Rhodocyclales, Bdellovibrionales and *Myxococcales* indicated a highly active biofilm for the degradation of organic carbon in the biofilm treatment.

11. The NAs removals from raw and ozonated OSPW were 31% and 66%, respectively, which demonstrated that the GAC biofilm process would be a good option for secondary treatment of pre-ozonated OSPW. The reduced toxicity and higher biodegradability of ozonated OSPW, in addition to the more diverse microbial community in the GAC biofilm in ozonated OSPW, resulted in the increased of NAs removal.

7.3. Recommendations

Based on the obtained results and conclusions, the following recommendations can be addressed for future research:

1. Higher NAs removal was observed from the GAC-FBBR treatment even after long time operation (120 days) indicated the significance of the GAC biofilm for the removal of toxic NAs from OSPW. However, the removal mechanisms of biodegradation and adsorption could not be determined separately. Thus, further research on the GAC biofilm treatment is necessary for the identification of the removal mechanisms from the GAC biofilm by each individual adsorption and biodegradation contribution.

2. The combined ozonation and GAC-biofilm treatment was highly efficient for the removal of NAs. Moreover, we observed that the GAC biofilm treatment removed NAs effectively from raw OSPW without any pre-treatment. Thus, it is suggested that OSPW can be treated effectively and economically by applying GAC-biofilm treatment followed by ozonation

and GAC-biofilm treatment (combined-ozone-combined), which will help in reducing the ozone dose and overall cost.

3. Exogenous biofilm forming carbon degrading strains (e.g. strains of *Burkholderia* order) can be applied for the OSPW treatment. The reactor with GAC inside can be inoculated with the exogenous bacteria strains to grow biofilm on the GAC surface using batch mode of operation. Once biofilm developed on the GAC surface in the reactor, the OSPW can be treated continuously. This process will increase the concentration of carbon degrading bacteria on the GAC surface and may play a key role in increasing the combined removal by biodegradation of adsorbed NAs.

APPENDIX A: Experimental Methods and Analysis

A-1. Batch Adsorption Isotherm

A batch equilibrium adsorption experiment was carried out to evaluate the adsorption capacity of granular activated carbon (GAC) based on chemical oxygen demand (COD) removal from oil sands process-affected water (OSPW). The study was carried out at ambient temperature (approximately 21 °C) for 3 days at 300 rpm on a horizontal shaker at GAC dose from 0.33 to 20 g GAC/L raw OSPW. Prior to study, the GAC was washed and sterilized (heat killed of bacteria at 121°C) for 30 minutes using a sterilizer (Model 733LS vacuum/gravity system sterilizer, NY, USA). A series of 500 mL conical flask was used for the study. In each flask, a specified amount of GAC was added with 300 mL OSPW. Based on the adsorption data, the Freundlich linear isotherm model was plotted and Freundlich parameters (n, K_f) were determined. The linear form of the Freundlich equation (A-1) is:

$$\mathrm{Ln} q_e = \frac{1}{n} \mathrm{Ln}\, C_e + \mathrm{Ln} K_f \qquad (A-1)$$

where q_e is the amount adsorbed at equilibrium in terms of (mg organics adsorbed/g GAC), C_e is the equilibrium COD concentration, K_f (L/g) is the Freundlich constant and is related to the capacity of the adsorbent for the adsorbate, and 1/n is related to the strength of adsorption driving force and the degree of nonlinearity between solution concentration and adsorption. The maximum adsorption was estimated by using $q_{max} = K_f C_i^{1/n}$ (Scharf et al., 2010).

A-2. Calibration of Ozone Generator Outlet Monitors

The ozone generator (C, GSO-40, Herford, Germany) outlet ozone concentration monitors (model HC-500, PCI-WEDECO) were calibrated (wt% to mg/L) according to manufacturer's protocol. Two ozone monitors were connected in parallel with the ozone

generator outlet gas before calibration. For calibration, the ozone generator outlet gas was passed through wet test meter in series connected with 2.5 wt% KI (potassium iodide) solution containing wash bottle for a fixed volume of generated gas mixture. The detailed procedures are listed below.

1. Ozone generator outlet 3-way valve were opened to the exhaust and outlet monitors were plugged on with power supply.
2. Ozone generator was started according to operating procedures (provided by manufacturer) and the ozone concentration knob was adjusted to a certain value (range used for calibration was 0.2 - 1.0 mV).
3. 400 mL of 2.5 wt% KI solution was transferred into a wash bottle and then connected after the wet test meter in series.
4. Exhaust valve at the rotameter outlet was opened and adjusted the rotameter flow rate at 8.0 LPM.
5. After getting a stable reading from the outlet monitors, calibration was started by passing the generator outlet gas through the wet test meter and wash bottle. The time was recorded for each revolution (equivalent to 3 L) of big dial in wet test meter. The monitor readings were recorded at every 5 seconds while the gas was passing through the wet test meter.
6. The KI solution from the wash bottle was transferred into a 500 mL volumetric flask. Wash bottle was ringed several times using milliQ water and all of the washed solution was transferred into the volumetric flask. Additional milliQ water was used to fill the solution to 500 mL and mixed thoroughly.

7. Steps 5 and 6 were repeated for four times and the ozone generator knob was changed to four different positions (to change the ozone concentration in the gas) to produce four volumetric flasks of reacted KI solution in total.
8. 50 mL solution from each volumetric flask was transferred into a 100 mL Erlenmeyer flask, and 2 mL of 2M sulphuric acid (H_2SO_4) was added in the flask. The solution was then titrated against 0.1 N sodium thiosulfate ($Na_2S_2O_3$) solution until appears light yellow color. 1 mL starch indicator was added to the flask and titrated until the violet color disappeared. The titrated volume of the used $Na_2S_2O_3$ from the burette was recorded and the titration was performed in triplicate.
9. Calibration of 0.1 N $Na_2S_2O_3$ solution was performed by titration against a solution prepared by adding 1 mL concentrated H_2SO_4, 10 mL 0.1N $KH(IO_3)_2$, 1 g KI and water to 80 mL total volume (APHA, 2005). The normality of $Na_2S_2O_3$ was calculated as:

$$\text{Normality of } Na_2S_2O_3(N) = \frac{1}{\text{mL } Na_2S_2O_3 \text{consumed}} \qquad (A-2)$$

10. Ozone concentration was calculated using the following equation:

$$O_3 \frac{mg}{L} = \frac{\text{normality of } Na_2S_2O_3(N) \times \text{volume of } Na_2S_2O_3 \text{as titrant (mL)} \times 24}{\text{wet meter reading(L)} \times \frac{(P - P_w)}{760} \times \frac{293.15}{273.15 + T}} \quad (A-3)$$

Where, P is the barometric reading + wet test meter outlet pressure drop, mm Hg, P_w is the vapor pressure of water at the temperature (T) of the gas in the wet test meter, mm Hg.

11. The weight percent of the ozone concentration was calculated by using the following formulae:

$$O_3(\text{wt\%}) = \frac{100 \times O_3\left(\frac{\text{mg}}{\text{L}}\right)}{1308 \times \left(1 + 0.00255 \times O_3\left(\frac{\text{mg}}{\text{L}}\right)\right)} \qquad (A-4)$$

12. Calibrate the ozone monitor by using the calculated data and monitor readings.

A-3. Residual Ozone Calculation using Indigo method

Standard methods (APHA 2005) for indigo method was used to measure the residual ozone after purging the ozonated OSPW with nitrogen for 15-20 minutes. The basic principle of measuring ozone concentration in indigo method is based on the reaction of ozone with potassium indigo trisulfonate in acidic solution. Usually, indigo reagent I and indigo reagent II are prepared from indigo stock solution based on ozone concentration in the solution. In the current study, indigo reagent II was used for determining residual ozone in the ozonated OSPW.

A.3.1. Preparation of Indigo Stock Solution

- Around 500 mL ODF water, 1 mL concentrated phosphoric acid and 770 mg potassium indigo trisulfonate ($C_{16}H_7N_2O_{11}S_3K_3$) were added to 1L volumetric flask.
- ODF water was used to fill the 1L mark and stirred the mixture in the volumetric flask for 1 hour after placing stir bar in the flask.
- The resultant solution was stored in the refrigerator at 4^0 Cby covering with aluminum foil as indigo stock solution.

A.3.2. Preparation of Indigo Reagent II

- 100 mL indigo stock solution, 10 g sodium dihydrogen phosphate (NaH_2PO_4) (or 11.5 g $NaH_2PO_4 \cdot H_2O$) and 7 mL concentrated phosphoric acid (H_3PO_4) were added to a 1 L volumetric flask, mixed and diluted to 1L using ODF water.

A.3.3. Procedures for Concentration Range 0.05 to 0.5 mg O_3/L

- 10 mL indigo reagent II was added to each of two 100-mL volumetric flasks.
- One flask was marked for the blank and 90 mL raw OSPW was added.
- The other flask was marked for sample and 90 mL of ozonated OSPW was added.
- The absorbance of both raw and ozonated OSPW was taken at 600 nm using a 10 mm cell.

A.3.4. Calculation of Ozone Concentration

The residual ozone concentration after purging of ozone from ozonated OSPW was calculated using the following equation (APHA 2005):

$$\mathrm{mg}\frac{O_3}{L} = \frac{100 \times \Delta A}{f \times b \times V} \qquad (A-5)$$

Where, 100 is the flask volume, mL;

ΔA is the difference of absorbance between the blank and the sample (blank absorbance – sample absorbance);

f is 0.42

b is the path length of the cell, cm; and

V is the sample volume, mL.

A-4. Analysis of Water Chemistry

A.4.1. COD

COD was measured according to standard methods (APHA, 2005). 2 ml sample was added to a mixture of 3.5 mL H_2SO_4 reagent and 2 mL micro-COD digestive solution in a 10 mL test tube. The mixture was mixed uniformly and digested the solution for 120 min at 140^0C using an accu-TEST™ COD reactor blocks (Bioscience Inc, ON, CA).. All samples were cooled at room temperature after digestion and measured the absorbance at 600 nm using a UV-Vis spectrophotometer (UV-2100PC, thermo scientific, ON, CA). H_2SO_4 reagent (5.5 g Ag_2SO_4/kg H_2SO_4) and micro-COD digestive solution (5 g dried $K_2Cr_2O_7$ + 167 mL concentrated H_2SO_4 + 33.3 g $HgSO_4$ + miliQ water to attain a total volume of 1000 mL) were prepared accordingly. Potassium hydrogen phthalate ($KHC_8H_4O_4$, MW=204) was used to prepare COD standard curve.

A.4.2. Biochemical Oxygen Demand (BOD_5)

The BOD_5 of raw and all ozonated samples were measured according to the Standard Methods (APHA, 2005). In brief, four kinds of solutions including phosphate buffer solution (PBS), $MgSO_4$, $CaCl_2$, and $FeCl_3$ were prepared accordingly for the BOD test. All samples were tested for four replicates. All the glass wares used were sterilized before the analysis carried out. The method consists of filling of an airtight bottle of the specified size (300 mL) with sample, seed and buffer sequentially until the bottle is over flown. Dissolved oxygen (DO) was measured just after preparation of BOD testing bottles (D_0) and after 5 d incubation at room temperature in dark (D_5). BOD_5 was computed according to equation A-6. Seed test and blank test were carried out in parallel accordingly.

$$\mathrm{BOD_5, mg/L} = \frac{(D_0 - D_5) - (B_0 - B_5)}{P} \qquad (A-6)$$

Where,

D_0 = DO of diluted sample immediately after preparation, mg/L,

D_5 = DO of diluted sample after 5 d incubation at 20°C, mg/L,

B_0 = DO of the blank immediately after preparation, mg/L,

B_5 = DO of the blank after 5 d incubation at 20°C, mg/L,

P = Dilution factor

Acclimated microorganisms to OSPW were used as inoculums in the bioreactor. The inoculums culture was prepared according to Wang et al. (2013) (supporting information). Briefly, tailings ponds sludge was used in Bushnell-Haas Broth (BHB) medium for the preparation of seed for the BOD_5 tests. The composition of the BHB medium was 1 g K_2HPO_4, 1 g KH_2PO_4, 1 g NH_4NO_3, 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 0.05 g $FeCl_3$ in one litre miliQ water. A 10 mL of sludge was added into 90 mL BHB medium with 10 mg of glucose in a flask and aeration was continued with a small air pump for one week. Another flask was made ready with 90 mL BHB medium, 45 mL OSPW and 10 mg glucose where into 15 mL of cultured solution and aeration was continued for one week. The concentrations of glucose and OSPW in the flask were 66.7 mg/L and 30%, respectively. Then 15 mL of culture (from 2nd flask) was added to another flask with the same BHB, OSPW and glucose mixture again. The grown inoculums were ready for use in the biodegradation tests after one week of aeration. The seed preparations were performed at room temperature (20 ±1 ^{0}C).

A.4.3. Acid Extractable Organic Fraction (AEF)

About 160 mL of OSPW for each sample was filtered through 0.45 μm Nylon filter (diam. 47 mm) (Sigma-Aldrich, ON, CA). Triplicate samples of 50 mL each was taken in biker and the pH of the samples were then adjusted to pH 2.5 using 2M H_2SO_4. Each replicate of sample was transferred into a 250 mL separatory funnel and 25 mL of DCM was added to the sample and mixed well by shaking the separatory funnel for 2 min. The funnel was opened frequently to let all the generated gases go out. The mixture was then allowed to settling for around 4 min and the bottom layer (DCM extract) was separated into a 50 mL glass vial. After separation of bottom layer another 25 mL of DCM was added to the separatory funnel (top layer) and mixed by shaking for 2 min and allowed to settle for 4 min. The bottom layer was transferred into the 50 mL glass vial and kept overnight to dry under the fume hood by using an evaporation unit with low air flow until DCM is completely dried out. A beaker on an analytical balance with the 50 mL glass vial (containing dried sample inside the vial) was set to zero. The dried samples were dissolved with around 5.0 -7.0 g of the DCM and the exact weight of the added DCM was recorded for the calculation purpose. A 3 mm calibrated KBr cell was used for the FT-IR analysis using FT-IR spectrometer (PerkinElmer: Spectrum 100, ON, CA). The absorbance of monomeric and dimeric AEF for carbonyl stretch equivalent was measured at 1,743 and 1,706 cm^{-1} respectively. A calibrated absorbance curve was prepared using a Fluka (Sigma-Aldrich, ON, CA) NAs mixture for the cell. The actual concentration of AEF in the sample was calculated based on the mass of DCM optima, total height of peaks (peaks at 1,743 and 1,706 cm^{-1}) and the volume of the original sample.

APPENDIX B: Supporting Tables

Table B1. Chemical characterization of raw and ozonated OSPW (n = 4)

Parameters	Raw OSPW	Ozonated OSPW
pH	8.4 ± 0.06	8.5 ± 0.04
TS (mg/L)	2681 ± 61	2513 ± 44
AEF (mg/L)	68.1 ± 5.1	18.6 ± 5.5
NAs (mg/L)	39.2 ± 5.8	0.72 ± 0.7
COD (mg/L)	276 ± 33	227 ± 26
TOC (mg/L)	75 ± 15	68 ± 12
BOD_5 (mg/L)	3.3 ± 1.5	16.5 ± 1.5

Table B2. Percent removal of classical NAs from raw OSPW based on Z and carbon numbers on day 120.

Z	-4	-6	-8	-10	-12
n =11	-43.2				
n =12	64.0	79.3			
n =13	87.9	86.3			
n =14	90.1	86.8	81.3		
n =15	91.9	89.0	63.4	73.9	
n =16	93.6	85.6	88.4	81.4	
n =17	93.3	92.0	88.5	91.0	87.1
n =18		100	87.5	91.2	88.3
n =19		100	100	92.4	91.2
n =20			100	100	87.1
n =21				100	100
n =22					100

Table B3. Biomass density in biofilms and biofilm thickness.

Type of biofilm	Biofilm thicknes, μm	Dry biomass, mg/g of GAC	Biomass volume (cm^3) /g of GAC	Dry biomass concentration in biofilm, X_f g/L of biofilm	Biomass concentration in reactors bed, X, (g/L fluidized bed)
Raw OSPW biofilm	34 ± 5	16.5	0.18 ± 0.03	94.2 ± 13.6	5.5

Table B4. Bacterial strains in raw OSPW and in GAC biofilms.

Sample	Raw OSPW	Raw OSPW biofilm
Band number	B-1, B-3, B-4, B-5, B-6, B-9, B-12, B-17, B-19, B-20, B-21	B-1, B-2, B-5, B-6, B-7, B-11, B-14, B-15, B-19, B-20, B-21, B-22, B-23
Total band	11	13
α-*Proteobacteria*	*Chelatococcus sp.* E1(B-6), *Roseomonas aquatica* strain KO_CM93(B-17), *Methylobacterium fujisawaense* strain: A2P9(B-19)	*Chelatococcus sp.* E1(B-6), *Methylobacterium fujisawaense* strain: A2P9(B-19)
β-*Proteobacteria*	*Polaromonas jejuensis* strain: NBRC 106434(B-1), *Rhodoferax ferrireducens* T118(B-3), *Polaromonas sp.* GM1(B-13), *Limnobacter sp.* MMD22(B-9), *Candidatus Nitrotoga* arctica clone 6680(B-12)	*Polaromonas jejuensis* strain: NBRC 106434, *Sterolibacterium denitrificans* strain Chol-1S(B-11), *Betaproteobacterium* LF4-45(B-15)
γ-*Proteobacteria*		*Solemya pervernicosa* gill symbiont(B-7)
Acidobacteria		*Acidobacteria* bacterium IGE-018(B-22)
Bacteroidetes	*Algoriphagus sp.* KJF5-15(B-5)	*Bacteroidetes* bacterium SCGC AAA204-N13(B-2) *Algoriphagus sp.* KJF5-15(B-5)
Firmicutes	*Desulfotomaculum sp.* ECP-C5(B-20)	*Desulfotomaculum sp.* ECP-C5(B-20)
Unclassified bacteria	Agricultural soil bacterium clone SC-I-31(B-21)	Bacterium 071021-ONK-SLIME-CHAB2(B-14), Agricultural soil bacterium clone SC-I-31(B-21), Iron-reducing bacterium enrichment culture clone FeC_1_H2(B-23)

Table B5. Bacterial strains in raw and ozonated OSPW and in GAC biofilm

Sample	Raw OSPW	Ozonated OSPW	Ozonated OSPW biofilm
Band number	B-1, B-3, B-4, B-5, B-6, B-9, B-12, B-17, B-19, B-20, B-21	B-1, B-2, B-3, B-4, B-5, B-9, B-13, B-14, B-15	B-1, B-3, B-5, B-6, B-7, B-8, B-10, B-14, B-15, B-16, B-18, B-20, B-22
Total band	11	9	13
α-*Proteobacteria*	*Chelatococcus sp.* E1(B-6), *Roseomonas aquatica* strain KO_CM93(B-17), *Methylobacterium fujisawaense* strain: A2P9(B-19)		*Chelatococcus sp.* E1(B-6), *Roseomonas sp.* tsz20(B-18)
β-*Proteobacteria*	*Polaromonas jejuensis* strain: NBRC 106434(B-1), *Rhodoferax ferrireducens* T118(B-3), *Polaromonas sp.* GM1(B-4), *Limnobacter sp.* MMD22(B-9), *Candidatus Nitrotoga* arctica clone 6680(B-12)	*Polaromonas jejuensis* strain: NBRC 106434(B-1), *Rhodoferax ferrireducens* T118(B-3), *Polaromonas sp.* GM1(B-4), *Limnobacter sp.* MMD22 (B-9), *Polaromonas sp*. Bis 20(B-13), *Betaproteobacterium* LF4-45(B-15)	*Polaromonas jejuensis* strain: NBRC 106434(B-1) *Rhodoferax ferrireducens* T118(B-3) *Rhodovulum sulfidophilum* strain P210(1)(B-10) *Burkholderia multivorans* ATCC 17616(B-16) *Beta-proteobacterium* LF4-45(B-15)
γ-Proteobacteria			*Solemya pervernicosa* gill symbiont(B-7)
Acidobacteria			Acidobacteria bacterium IGE-018(B-22)
Bacteroidetes	*Algoriphagus sp.* KJF5-15(B-5)	*Algoriphagus sp.* KJF5-15(B-5) Bacteroidetes bacterium SCGC AAA204-N13(B-2)	*Algoriphagus sp.* KJF5-15(B-5) *Roseivirga ehrenbergii* strain UDC351(B-8)
Firmicutes	*Desulfotomaculum sp.* ECP-C5(B-20)		*Desulfotomaculum sp.* ECP-C5(B-20)
Unclassified bacteria	Agricultural soil bacterium clone SC-I-31(B-21)	Bacterium 071021-ONK-SLIME-CHAB2(B-14)	Bacterium 071021-ONK-SLIME-CHAB2(B-14)

Table B6. Oxidized NAs concentration change from treatment using different mechanisms.

OSPW	Day	O-NAs, mg/L			O_2-NAs, mg/L			O_3-NAs, mg/L			Total oxy-NAs, mg/L		
		0	28	Δ	0	28	Δ	0	28	Δ	0	28	Δ
Raw	Control	11.04	10.01	1.03	11.75	9.89	1.86	4.68	4.55	0.13	27.47	24.45	3.02
	Biodegradation only	11.07	9.78	1.29	11.83	10.1	1.83	4.87	4.4	0.47	27.77	23.68	3.59
	Adsorption only	11.04	2.17	8.87	11.75	4.6	7.15	4.68	1.08	3.79	27.47	7.85	19.81
	Combined	11.07	1.91	9.16	11.83	3.73	8.1	4.87	0.8	3.88	27.77	6.44	21.14
Ozonated	Control	10.32	9.85	0.47	10.86	9.89	0.97	3.67	3.44	0.23	24.85	23.18	1.67
	Biodegradation only	11.72	9.14	2.58	11.64	9.49	2.15	3.9	3.32	0.58	27.26	21.95	5.31
	Adsorption only	10.32	2.04	8.28	10.86	3.54	7.32	3.67	1.14	2.53	24.85	6.72	18.13
	Combined	11.72	1.64	10.08	11.64	2.96	8.68	3.9	0.72	3.18	27.26	5.32	21.94

Combined: simultaneous adsorption and biodegradation

Table B7. The copy of bacteria in suspended growth and biofilm in combined treatment bioreactor.

	GAC biofilm Bacteria, copy	Suspended bacteria, copy	Total bacteria, copy
Raw OSPW	3.33E+07	2.16E+08	2.7 E+08
Ozonated OSPW	1.37E+08	1.49E+08	2.9 E+08

APPENDIX C: Supporting Figures

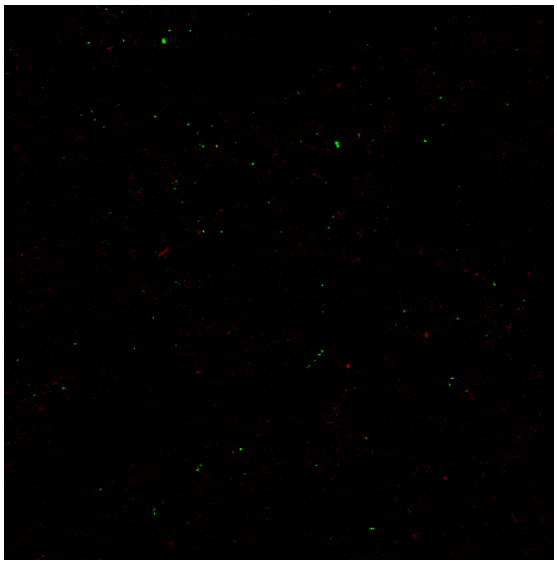

Figure C1. CLSM images of bacteria on the GAC on day 6 for raw OSPW treatment using GAC-FBBR.

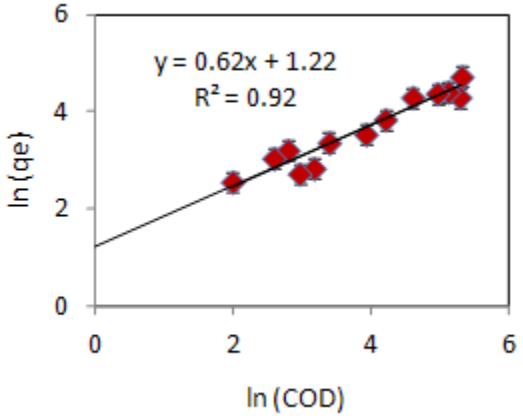

Figure C2. Freundlich type COD isotherms for GAC from batch adsorption of organics in OSPW. q_e: amount organic adsorbed per g GAC, mg.

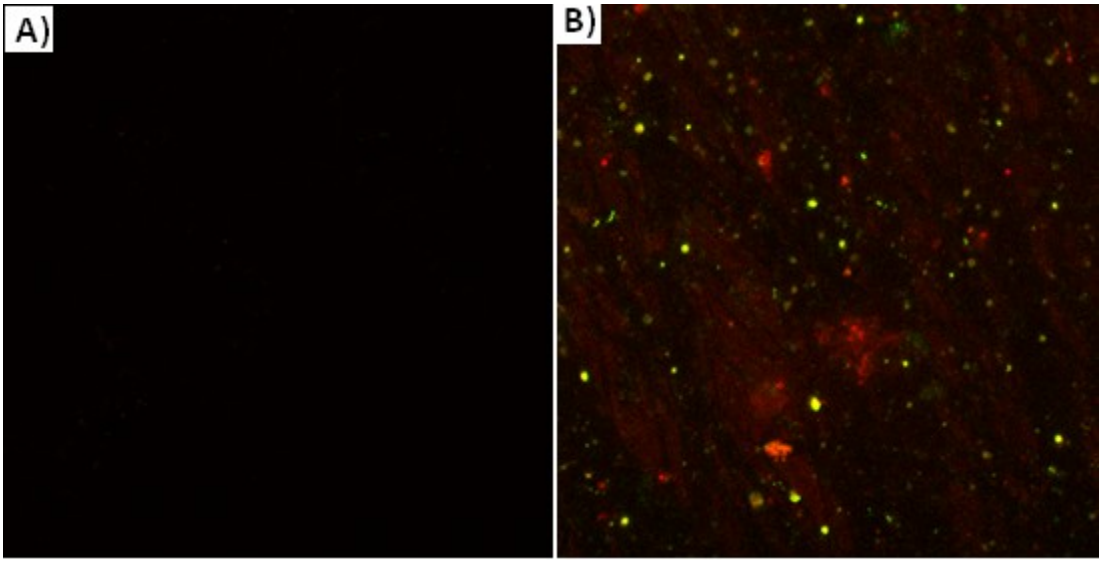

Figure C3. CLSM images of biofilms formed on GAC in phase I (A); and phase VI (B).

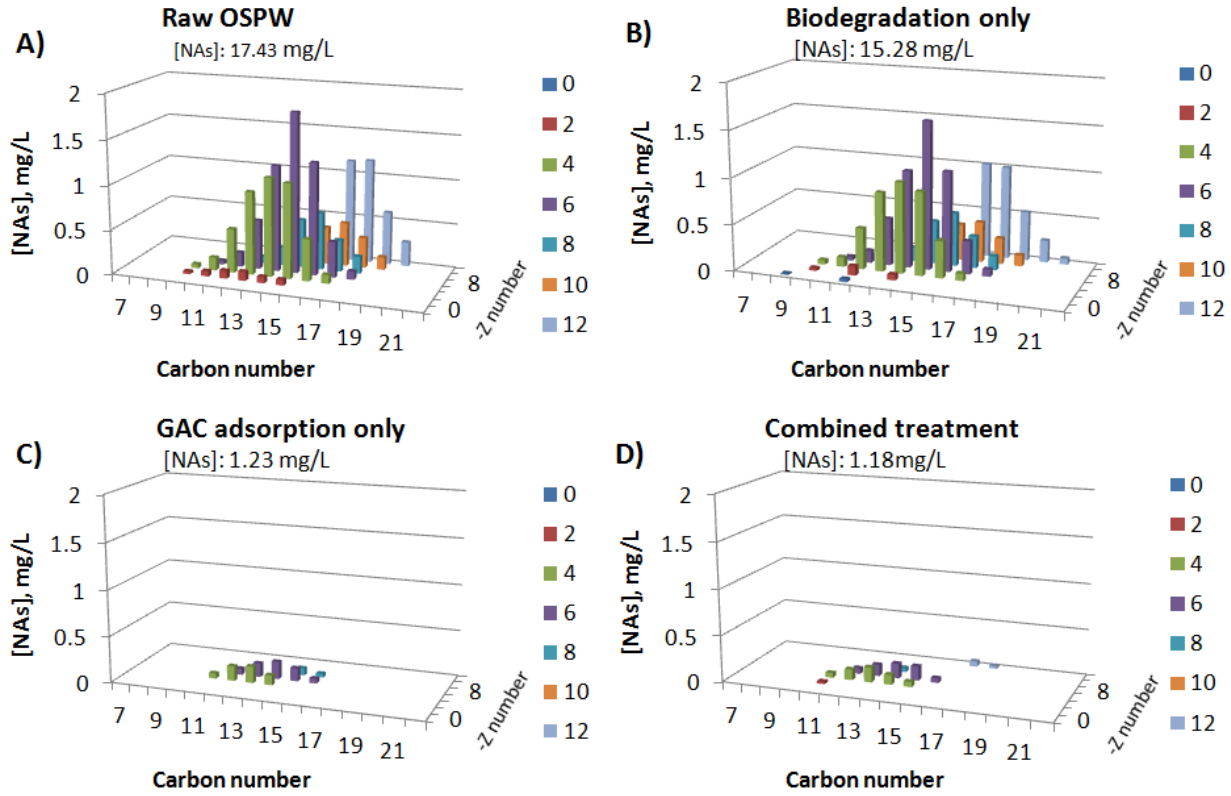

Figure C4. UPLC-HRMS 3-dimensional classical NAs concentrations in raw and treated raw OSPW before and after 28 days of treatment.

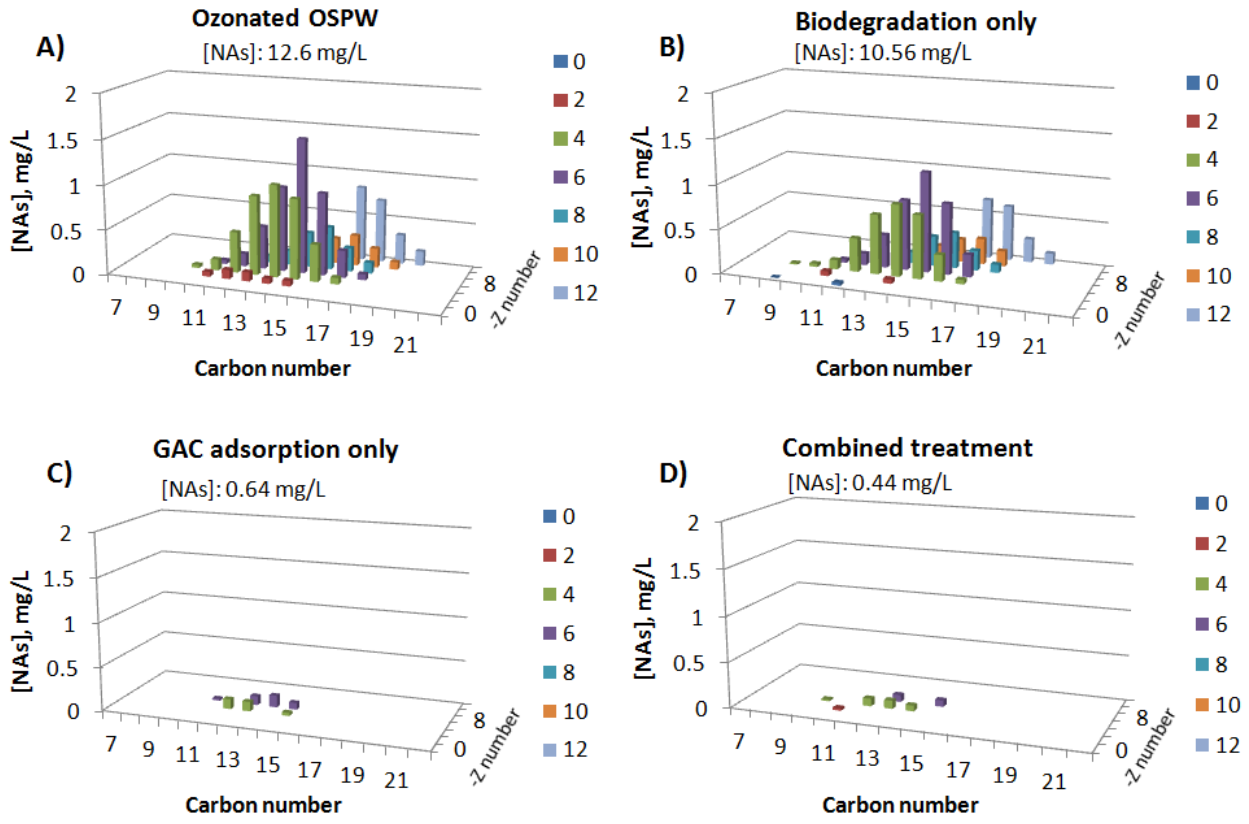

Figure C5. UPLC-HRMS 3-dimensional classical NAs concentrations in ozonated and treated ozonated OSPW before and after 28 days of treatment.

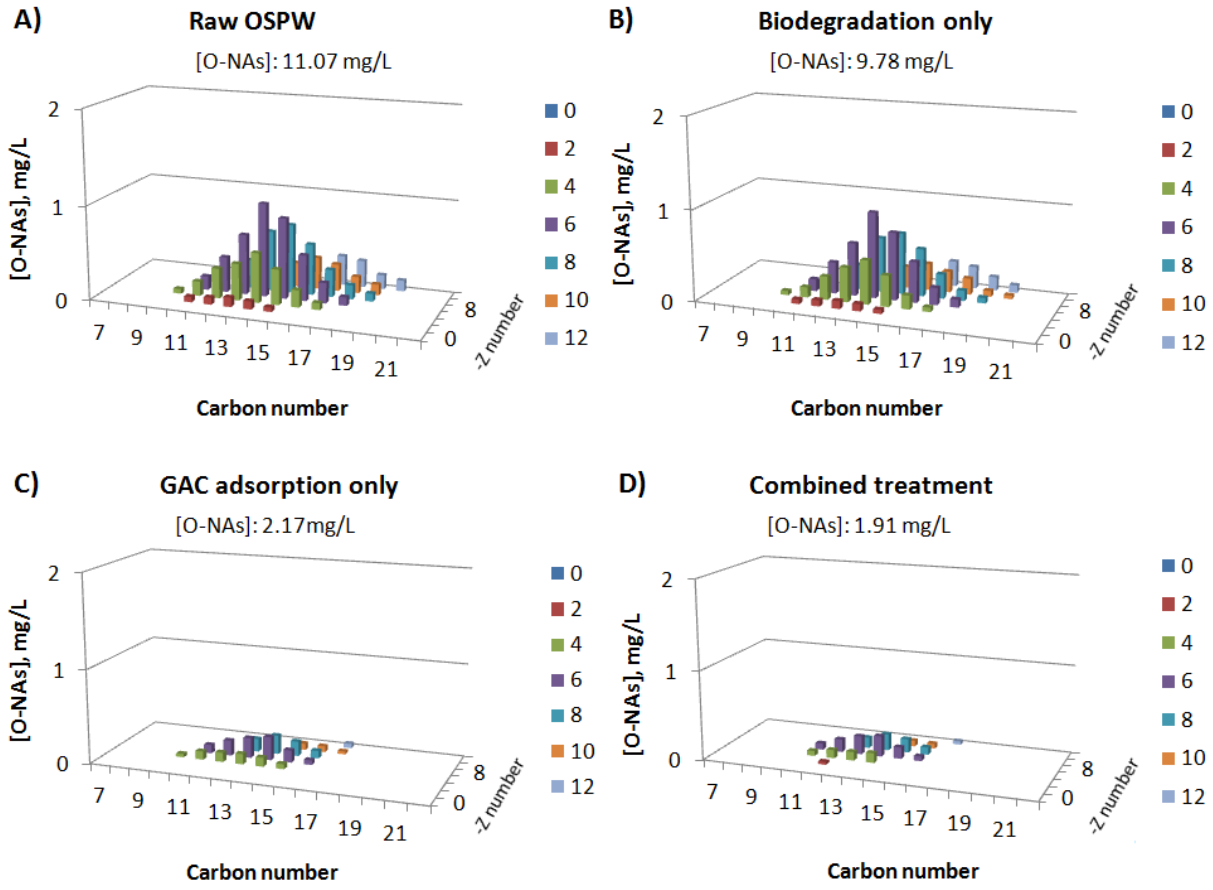

Figure C6. UPLC-HRMS 3-dimensional O-NAs concentrations in raw and treated raw OSPW before and after 28 days of treatment.

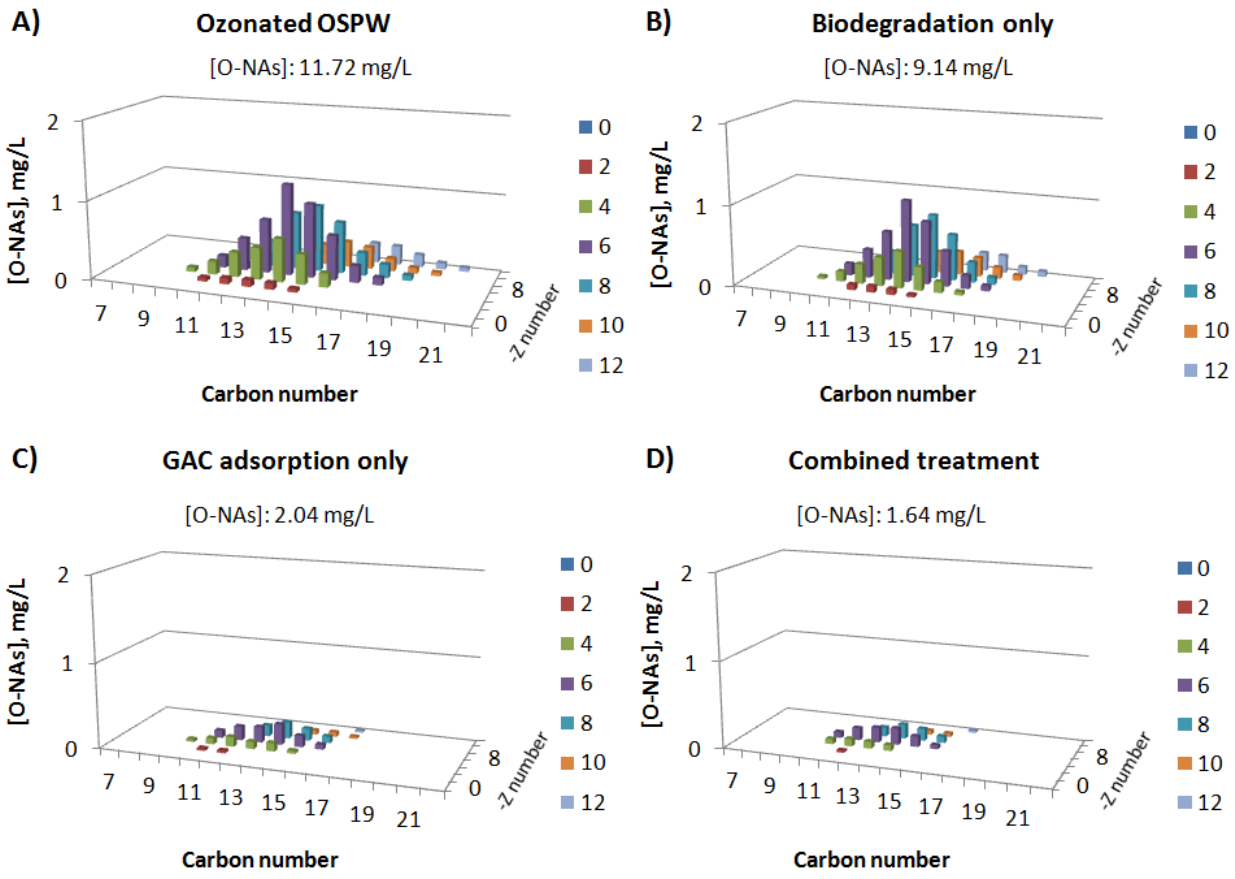

Figure C7. UPLC-HRMS 3-dimensional O-NAs concentrations in ozonated and treated ozonated OSPW before and after 28 days of treatment.

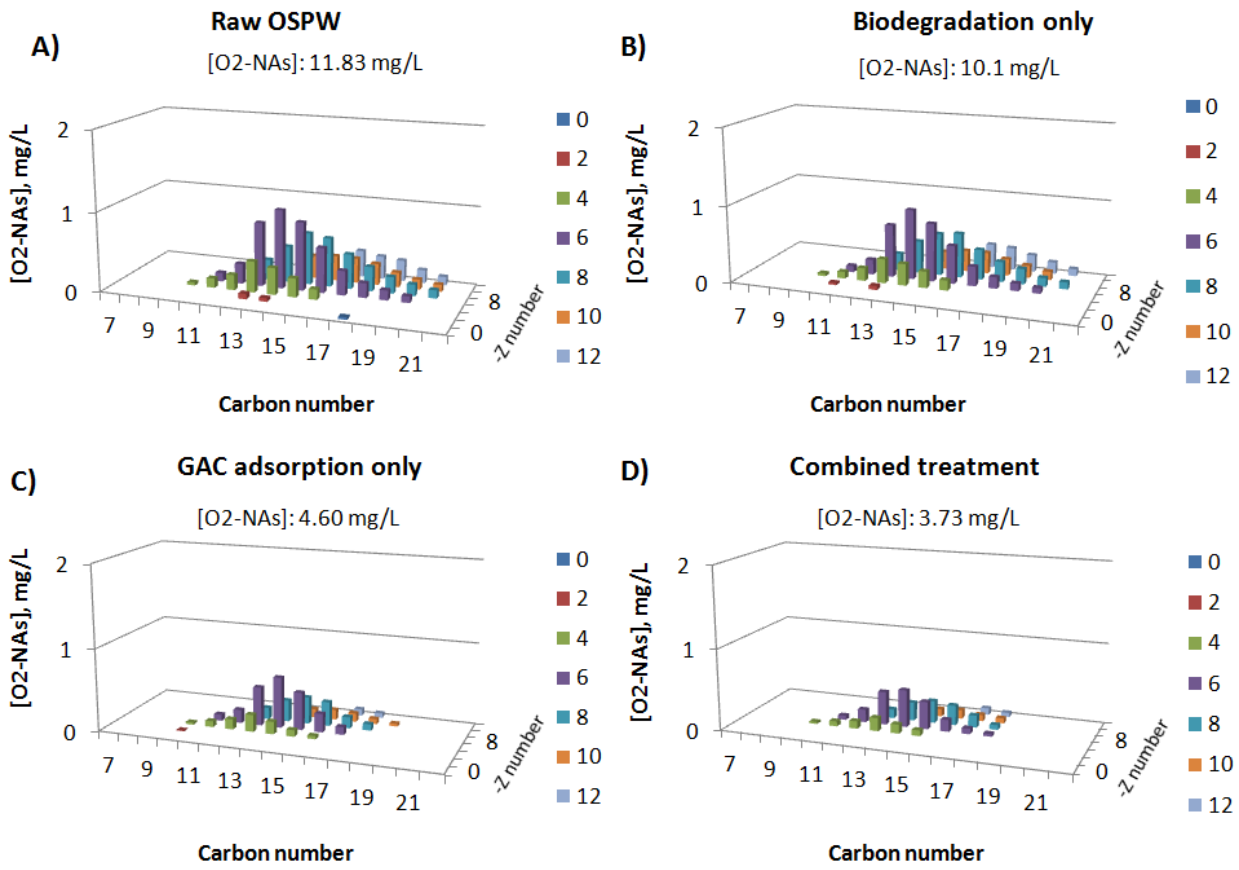

Figure C8. UPLC-HRMS 3-dimensional O_2-NAs concentrations in raw and treated raw OSPW before and after 28 days of treatment.

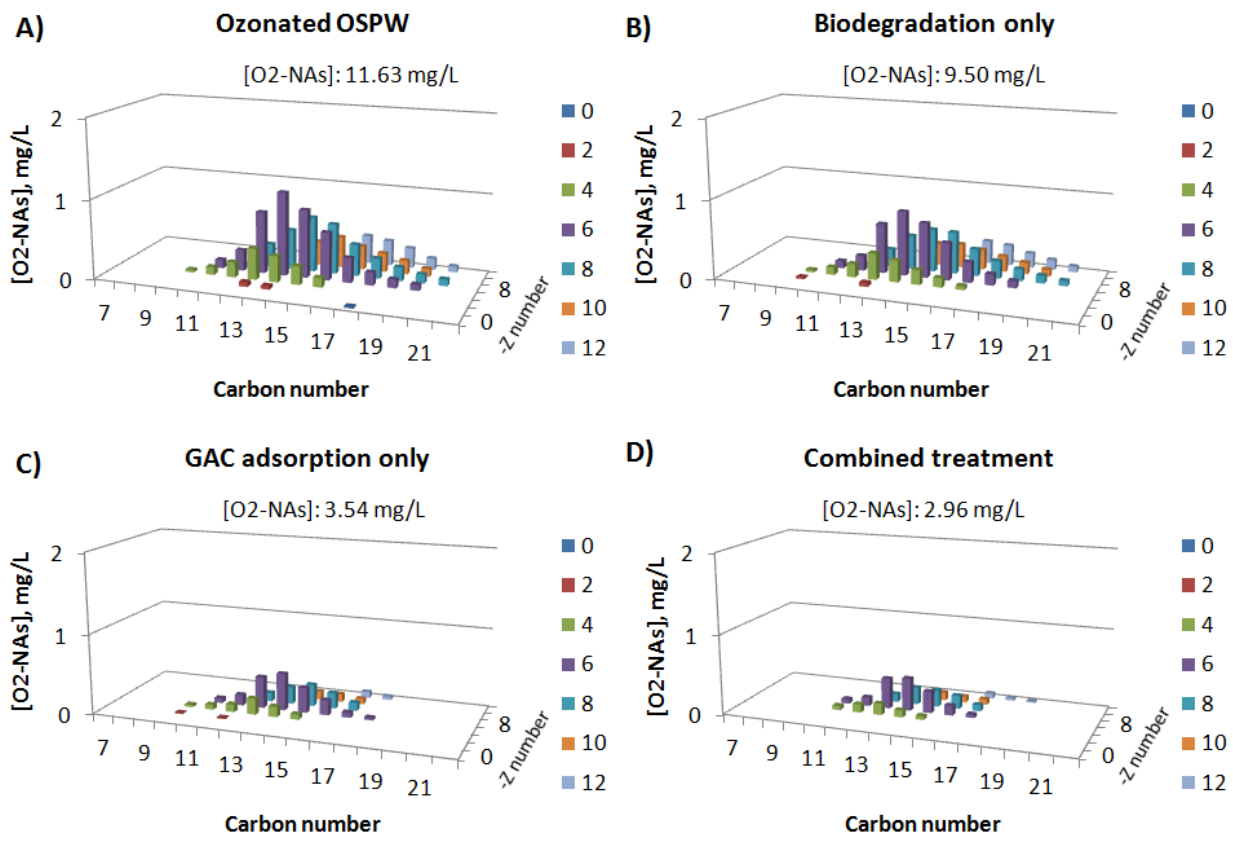

Figure C9. UPLC-HRMS 3-dimensional O_2-NAs concentrations in ozonated and treated ozonated OSPW before and after 28 days of treatment.

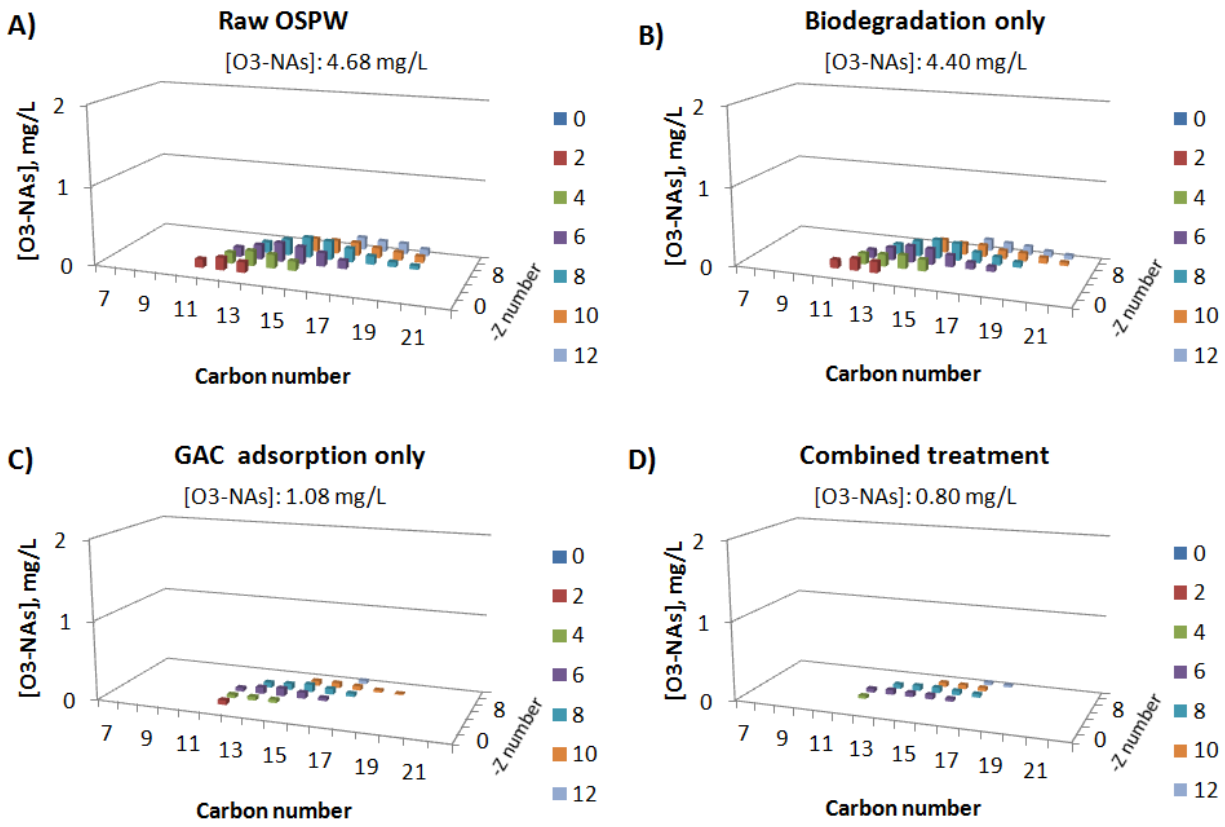

Figure C10. UPLC-HRMS 3-dimensional O_3-NAs concentrations in raw and treated raw OSPW before and after 28 days of treatment.

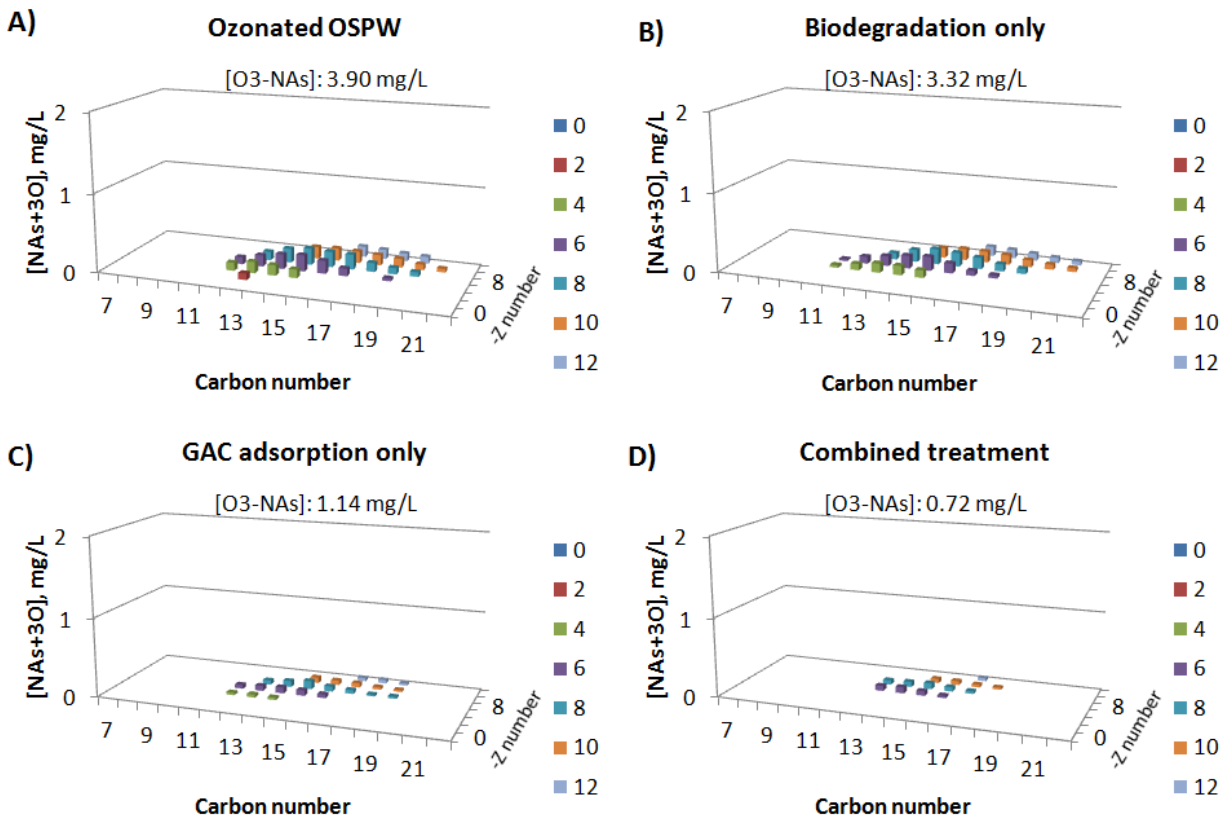

Figure C11. UPLC-HRMS 3-dimensional O_3-NAs concentrations in ozonated and treated ozonated OSPW before and after 28 days of treatment.

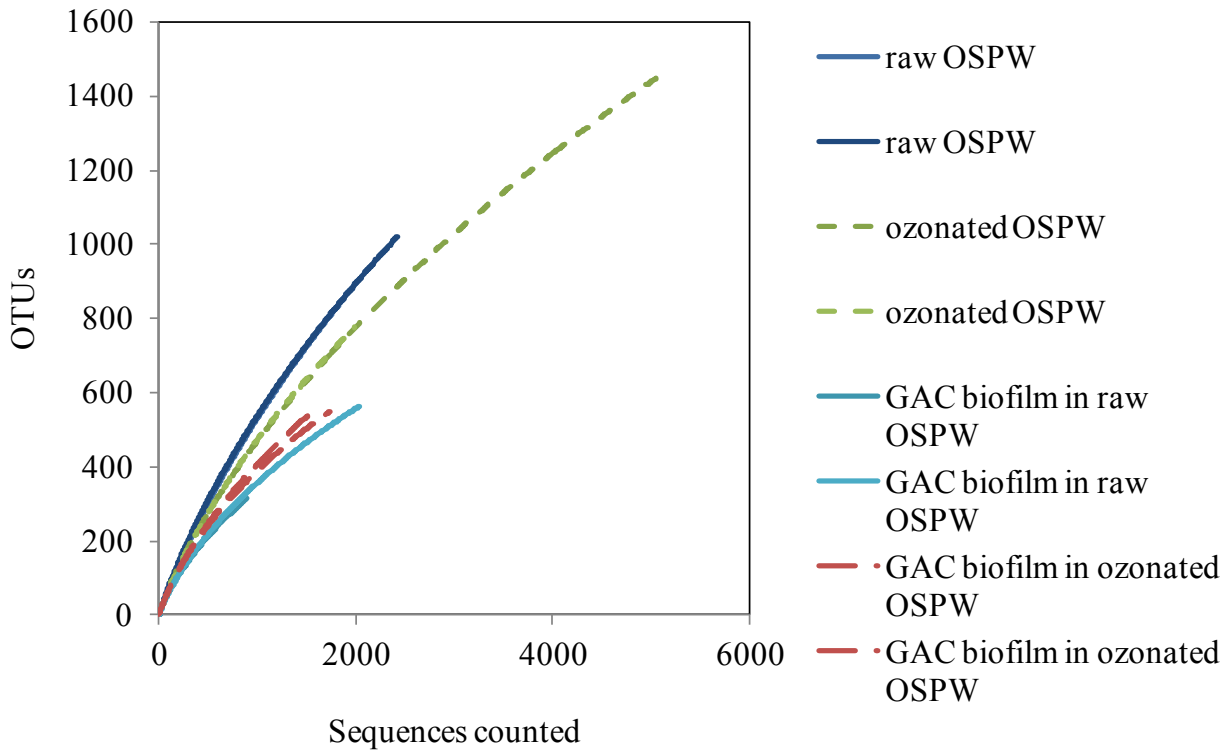

Figure C12. Rarefaction curves of OTUs clustered at 0.03 distance across different samples.

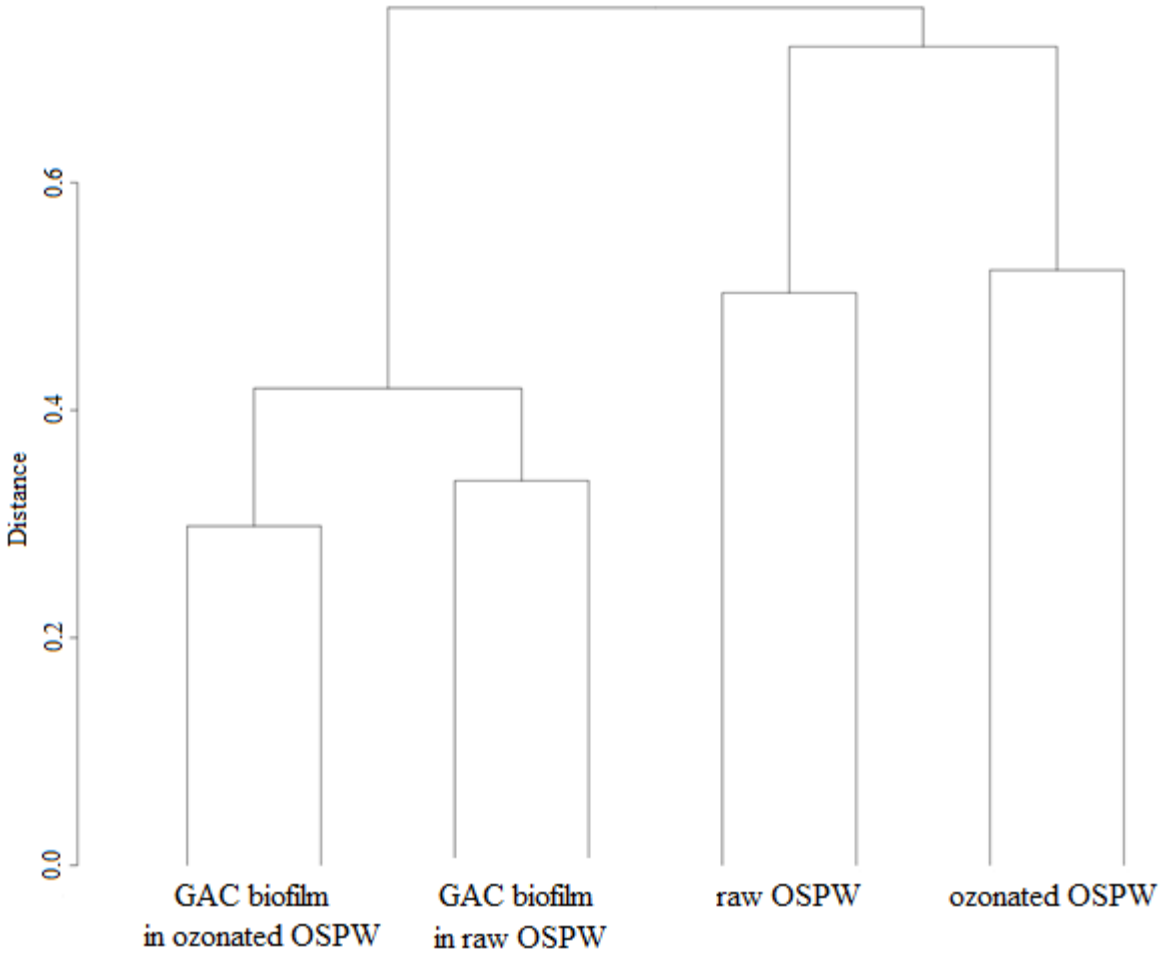

Figure C13. Cladogram of the bacterial communities based on Jaccard distance (3% dissimilarity).

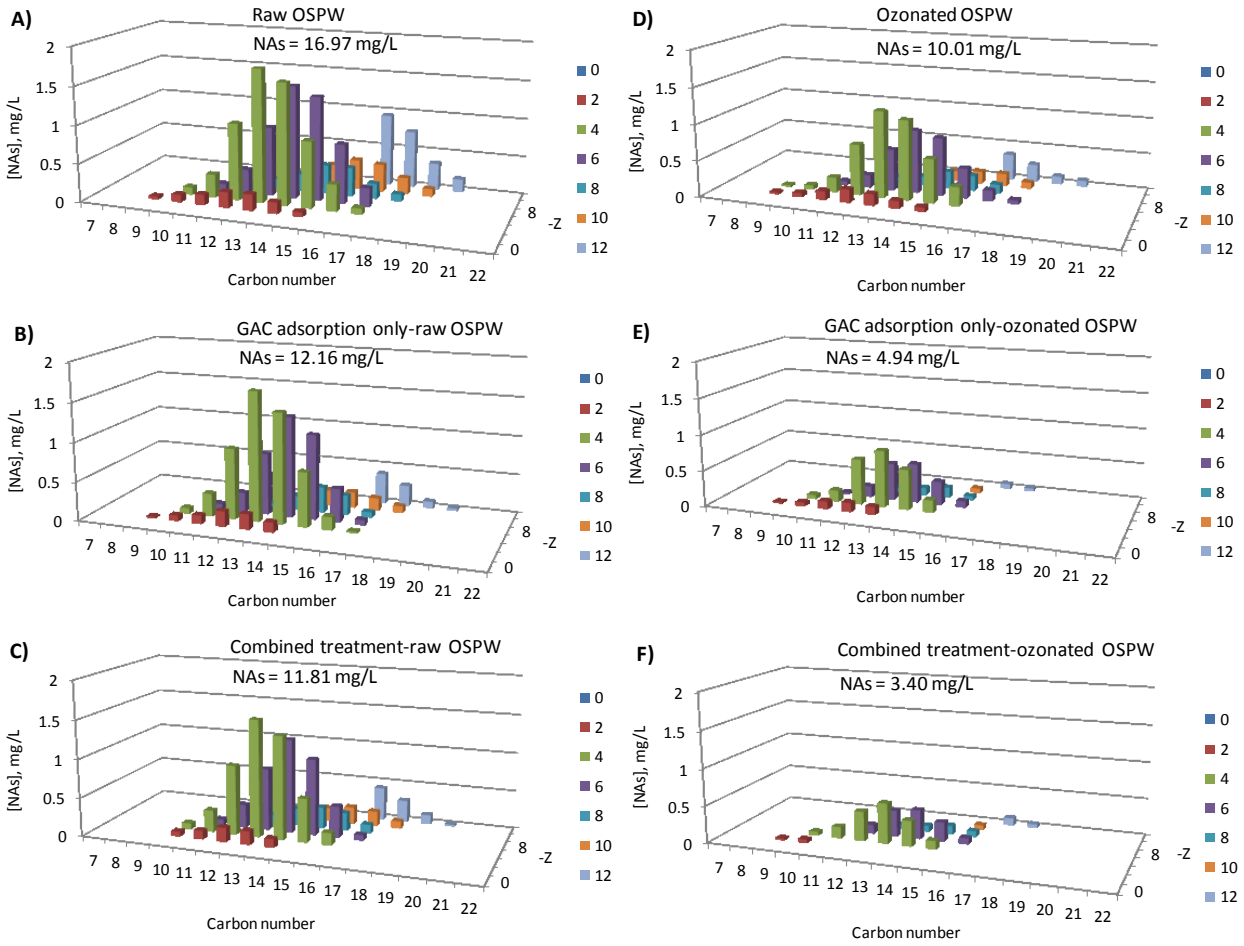

Figure C14. NAs concentrations in raw and ozonated OSPW before and after 2 days of treatment. (A) raw OSPW; (B) GAC adsorption only treatment of raw OSPW; (C) combined treatment of raw OSPW; (D) ozonated OSPW; (E) GAC adsorption only treatment of ozonated OSPW; (F) combined treatment of ozonated OSPW

Appendix-D. Preliminary GAC Adsorption Experiment for GAC Dose Optimization

D-1. Experimental Methods

Preliminary studies were carried out to characterize the GAC adsorption of organics and to optimize a suitable GAC dose for a simultaneous GAC adsorption and biodegradation experiment. The study was carried out in triplicate at ambient temperature (approximately 21 ± 1 °C) for 10 days at 150 rpm on a horizontal shaker (Innova^{TM} 2100, Platform Shaker, New Brunswick Scientific, USA) at doses of 0.2–10 g GAC/L OSPW. Prior to study, the GAC was washed and sterilized at 121 °C for 30 minutes using a vacuum/gravity sterilizer (Model 733LS, Getinge group Inc., NY, USA). OSPW and the bottles used as batch reactors for the study were sterilized.

D-2. GAC Dose Selection

The initial concentration of classical NAs in raw OSPW was 17.43 mg/L, which decreased to 5.38 mg/L (69% removal) after 10 days treatment with 0.4 g GAC/L raw OSPW (Figure D1A). The corresponding total oxidized NAs, AEF, and COD removals were 47.40%, 44.40%, and 30.04%, respectively, in raw OSPW. The concentration of classical NAs in ozonated OSPW was 12.60 mg/L which decreased to 2.19 mg/L (82.62% removal) after 0.4 g GAC/L ozonated OSPW (Figure D1B). The corresponding total oxidized NAs, AEF, and COD removals were 56.85%, 53.36%, and 31.1% in ozonated OSPW, respectively. However with 1 g GAC/L raw and ozonated OSPW treatment, the concentration of NAs in raw and ozonated OSPW decreased to 0.54 mg/L (97.7% removal) and 0.006 mg/L (99.9% removal), respectively. Thus, based on the classical NAs removal from GAC adsorption in both raw and ozonated OSPW; and the biodegradability of raw and ozonated OSPW (Table 5.1), 0.4 g

GAC/L OSPW treatment was selected to carry out the simultaneous adsorption and biodegradation experiment.

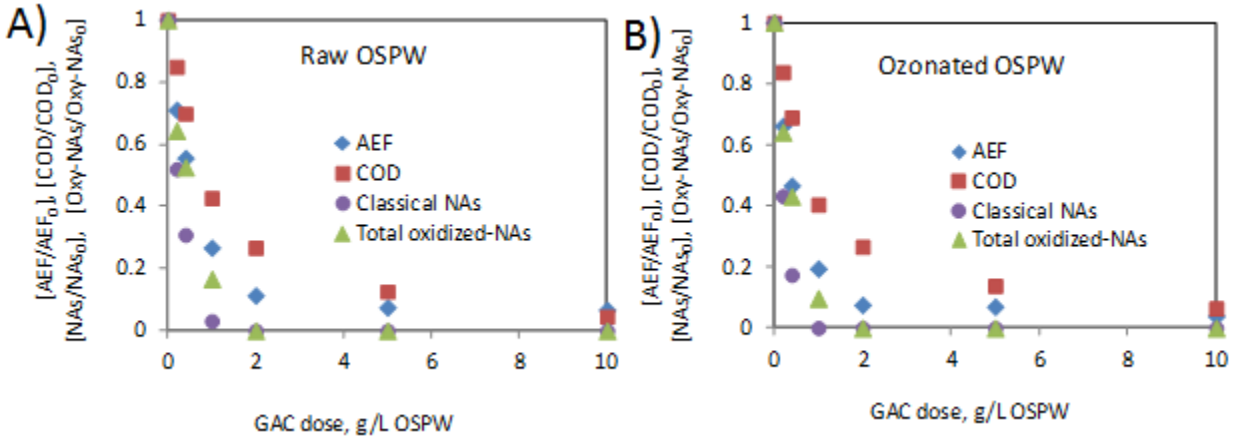

Figure D1. Relative removal of AEF, COD, classical-NAs and oxidized-NAs from both raw and ozonated OSPW after preliminary GAC treatment (Raw OSPW: AEF_0 = 67.48 mg/L, COD_0 = 214 mg/L, Classical NAs_0 =17.43 mg/L, Total oxy NAs_0 = 27.58 mg/L; Ozonated OSPW: AEF_0 = 53.05 mg/L, COD_0 = 222 mg/L, Classical NAs_0 = 12.59 mg/L, Total oxy-NAs_0 = 27.26 mg/L).

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