Application of Fixed-Bed Biofilm Reactors for the Treatment of Oil Sands Process Water

by

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ABSTRACT

The accumulation of huge amount of oil sands process water (OSPW) in tailing ponds has shown a serious threat to the local public health for their potential to contaminate surface and groundwater. Naphthenic acids (NAs) are considered to be one of the most important toxic compounds present in OSPW. Current physical and chemical NA degradation processes are still too expensive to be piloted; therefore, full-scale applications have not been assessed so far. With the advantages of ease construction, low energy input and robustness, the biofiltration process has been considered a promising approach for OSPW treatment.

In this study, with the application of gravity as an important part of energy source, indigenous microorganisms based fixed-bed biofilm reactors were developed by using raw OSPW as influent process water and pure sand as media. Ultra performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOFMS) detection showed that 21.8% of classical NAs could be removed from raw OSPW after 8 cycles of circulation through the established fixed-bed biofilm reactor with an equivalent hydraulic retention time (HRT) of 16 hours. With the combination of mild ozonation (with utilized ozone dose of 30 mg/L) pretreatment, the classical NAs in OSPW could be removed by 92.7% after the circulation of raw OSPW for eight times (with an equivalent HRT of 16 hours). Although both ozonation and biofiltration alone did not show degradation of oxidized NAs from raw OSPW, the combined process led to a 52.9% and 42.6% removal for O_3-NAs and O_4-NAs which were the dominant oxidized NA species in raw OSPW. With the advantage of high NA removal efficiency, the ozonation-biofiltration process is a promising approach for NA degradation.

An innovative biofiltration-ozonation-biofiltration process was also developed and applied for the treatment of OSPW. By using the established fixed-bed biofilm reactor, a biofiltration pretreatment (with an equivalent HRT of 8 hours) showed consistent NA removal effect on raw OSPW as before, and it was found to benefit the following ozonation process for NA removal substantially. Through the biofiltration pretreatment, the ozonation (with utilized ozone dose of 30 mg/L) removal of classical NAs from OSPW improved from 32.1% to 84.8%, and the NA degradation efficiency improved from 0.1 mg NAs/mg O_3 to 0.3 mg NAs/mg O_3. Compared with the biofiltration pretreatment, the post-biofiltration process showed higher degradation effect on oxidized NAs (removal ratio: 22.9% vs. 3.3%; removal rate: 0.4 mg/L/h vs. 0.1 mg/L/h). With biofiltration pretreatment, the utilized ozone dose is expected to be reduced to achieve the same NA removal as the ozonation of raw OSPW. With the high possibility to reduce the cost of ozonation, the biofiltration-ozonation-biofiltration process shows improved potential to be used in industrial applications.

It is known that bacteria are the main players for petroleum hydrocarbons degradation and fungi are also potential organisms. 16S rRNA and 18S rRNA genes targeted sequencing revealed that *Proteobacteria* and *Cryptomycota* were the dominant bacterial and fungal phyla in the established biofilter. The dominant bacterial class was *Alphaproteobacteria*, the abundance of which decreased from 34.9% to 18.3% with depth, but the abundance of *Anaerolineae* increased from 1.5% to 8.3%. The changes in bacterial community structure along the biofilter suggested that the bioremediation of OSPW was achieved by aerobic and anaerobic combined degradation processes. Metatranscriptomic sequencing analysis showed that the functional abundance of aromatic compounds metabolism and organic acids degradation pathways in indigenous microbial community improved from 0.05% and 0.29% to 0.76% and 0.39% in the biofilter,

respectively. The diverse microbial community structures and transcriptomic profiles might explain the effective degradation of NAs from different OSPW by biofiltration.

Fixed-bed biofilm reactors were successfully developed and used for OSPW treatment in this study. The mild ozonation combined biofiltration process showed high efficiency on the removal of recalcitrant NAs from OSPW. Biofiltration pretreatment was observed to improve the ozonation efficiency on NA removal remarkably. The biofiltration-ozonation-biofiltration process also removed cyclic and aliphatic NAs from OSPW effectively. The diverse microbial communities along the fixed-bed biofilm reactors could alter their transcriptomic profiles for the effective degradation of NAs from different OSPW. With the advantages of cost-effective and high NA removal efficiency, the established biofiltration system is promising to be scaled up for OSPW treatment.

PREFACE

All of the research described in this thesis was designed and performed by myself with the supervision from Dr. Mohamed Gamal El-Din in the Department of Civil and Environmental Engineering at the University of Alberta. Some of the experiments conducted in this study were completed through the collaboration with Dr. Yanyan Zhang who was a postdoctoral fellow in Dr. Gamal El-Din's research group. The contribution from the collaborators and the coauthors of the manuscripts are described below.

Chapter 2 has been published as "Zhang L, Zhang YY, Gamal El-Din M. Degradation of recalcitrant naphthenic acids from raw and ozonated oil sands process-affected waters by a semi-passive biofiltration process. Water Res. 2018; 133: 310-318". Dr. Mohamed Gamal El-Din contributed to the research conduction and manuscript edits. Dr. Yanyan Zhang and Dr. Pamela Chelme-Ayala contributed the edit of the manuscript. Dr. Rongfu Huang conducted the naphthenic acids (NAs) detection by using the ultra pressure liquid chromatography-high resolution time-of-flight mass spectrometry (UPLC-TOFMS).

Chapter 3 will be submitted to Science of the Total Environment as "Zhang L, Zhang YY, Gamal El-Din M. Removal of cyclic and aliphatic naphthenic acids from oil sands process water by a biofiltration-ozonation-biofiltration process with enhanced ozonation efficiency". Dr. Mohamed Gamal El-Din contributed to the research conduction and manuscript edits. Dr. Yanyan Zhang and Dr. Pamela Chelme-Ayala contributed the edit of the manuscript. Dr. Rongfu Huang did NA detection by using UPLC-TOFMS.

Chapter 4 will be submitted to the Nature Communications as "Zhang L, Zhang YY, Patterson J, Gamal El-Din M. Metagenomic and metatranscriptomic analysis of the microbial

community in fixed-bed biofilm reactors for oil sands process water (OSPW) reclamation" Dr. Mohamed Gamal El-Din contributed to the research conduction and manuscript edits. Dr. Yanyan Zhang contributed the edit of the manuscript. Jordan Patterson contributed by assisting with next generation sequencing data analysis and editing the manuscript. Dr. Rongfu Huang conducted NA detection by using UPLC-TOFMS.

All the work associated with this study was performed by myself except for the contributions from the collaborators and co-authors described above.

DEDICATION

I dedicate my thesis work to my wife who gives me tremendous support for the completion of the study, and to my parents who always care about me thousands of miles away. I would also like to dedicate the thesis to my friends who stayed with me when I was facing difficulties during the completion of this study.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AEF	Acid Extractable Fraction
AOPs	Advanced Oxidation Processes
BOD	Biochemical Oxygen Demand
CLSM	Confocal Laser Scanning Microscope
COD	Chemical Oxygen Demand
DCM	Dichloromethane
DNA	Deoxyribonucleic Acids
DO	Dissolved Oxygen
EBCT	Empty Bed Contact Time
EPS	Extracellular Polymeric Substances
FT-IR	Fourier-transform Infrared Spectroscopy
GAC	Granular Activated Carbon
HRT	Hydraulic Retention Time
IFAS	Integrated Fixed-film Activated Sludge
IMS	Ion Mobility Spectrometry
MBBR	Moving Bed Biofilm Reactor

MBR	Membrane Bioreactor
MS	Mass Spectrometry
NAs	Naphthenic Acids
OSPW	Oil Sands Process Water
OTU	Operational Taxonomic Unit
PAHs	Polycyclic Aromatic Hydrocarbons
PCoA	Principal Coordinate Analysis
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitive Polymerase Chain Reaction
RNA	Ribonucleic Acids
rRNA	Ribosome Ribonucleic Acids
TOC	Total Organic Carbon
UPLC/TOFMS	Ultra-high Pressure Liquid Chromatography/Time-of-Flight Mass Spectrometry

CHAPTER 1. GENERAL INTRODUCTION

1.1 Background of the study

The accumulation of huge amounts of toxic oil sands process water (OSPW) in tailing ponds have shown a serious threat to contaminate local surface and groundwater quality (Pramanik, 2016). Canada has been recognized as the country with the third largest oil reserves in the world, with more than 169 billion barrels (27×10^9 m^3) of recoverable bitumen deposit in the Athabasca region, northern Alberta (Brown and Ulrich, 2015; Teare et al., 2012). More than 1.53 billion m^3 bitumen has been produced and the bitumen production is supposed to reach 121 million m^3 per year by 2023 (Huang et al., 2016; Teare et al., 2014). However, with the production of bitumen by alkaline hot water extraction technology, a huge amount of OSPW is produced at the same time (Brown and Ulrich, 2015; Gamal El-Din et al., 2011). OSPW is currently stored in local tailings ponds as required by the zero-discharge approach (Brown and Ulrich, 2015). It is estimated that more than 10^9 m^3 of OSPW has been accumulated and the volume keeps on increasing with the expansion of the bitumen production (Klamerth et al., 2015). OSPW is a complex mixture of inorganic and organic compounds, showing toxic effects to a wide range of organisms such as algae, fishes, amphibians, birds, and mammals (Gamal El-Din et al., 2011; Klamerth et al., 2015). The continuous reuse of the tailing water leads to the OSPW toxicity increase which is caused by the accumulation of toxic compounds (Brown and Ulrich, 2015; Klamerth et al., 2015). The accumulation of toxic OSPW poses a great risk to the local environment and public health. Therefore, technically practicable and

economically feasible OSPW remediation methods need to be established urgently (Brown and Ulrich, 2015; Pramanik, 2016).

OSPW is a mixture of suspended solids, various inorganic ions, and organic compounds (Wang et al., 2016b). Naphthenic acids (NAs) are part of the organic fraction of the OSPW (Fig. 1.1) (Whitby, 2010). NA is a mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids, with the general formula of $C_nH_{2n+Z}O_x$, where n is the number of carbon atoms ($7 \leq n \leq 26$), Z specifies the hydrogen deficiency due to the formation of ring or double bond structure ($0 \leq -Z \leq 18$), and x indicates the number of oxygen atoms present in the NAs (Huang et al., 2016; Wang et al., 2016b). NAs have been considered the primary toxic constituents of OSPW (Wang et al., 2016b). Classical NAs (O_2-NAs) has been determined to be one of the most toxic compounds from OSPW (Morandi et al., 2015). The oxidized NAs (O_x-NAs, $x \geq 3$) which possess more oxygen atoms show less toxicity due to its hydrophilicity nature (He et al., 2010; Wang et al., 2016b). The removal of classical NAs is the most critical step for OSPW remediation, and the classical NA removal efficiency has been widely used for the evaluation of different OSPW treatment method (Huang et al., 2015a).

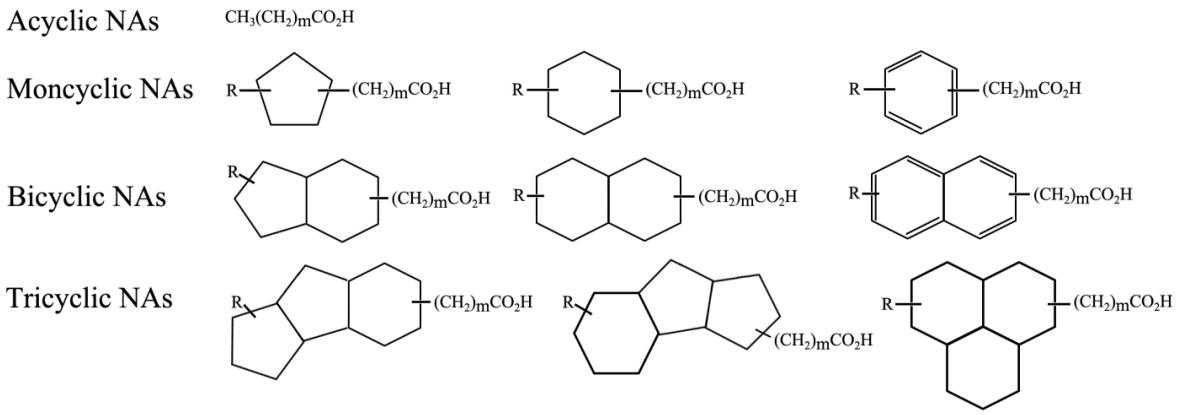

Figure 1.1. Sample NA and aromatic NA structures, where R is alkyl chain and m is the number of CH_2 units (Whitby, 2010).

Various methods have been developed for the treatment of OSPW(Choi and Liu, 2014; Huang et al., 2015a; Shi et al., 2015; Wang et al., 2016a; Zhang et al., 2016). However, there is still no acceptable OSPW remediation method to be used by the industry at a full-scale level (Xue et al., 2016). As we know, current physical and chemical treatment methods are still too expensive and complicated to be piloted, therefore, full-scale applications have not been evaluated so far. The removal of NAs through biological degradation is cost-effective but shows the disadvantage of low NA removal efficiency. How to increase the NA removal efficiency of the biological treatment process has become one of the most important areas of OSPW treatment studies (Xue et al., 2018).

By using the microorganisms survived in OSPW and activated sludge collected from a municipal wastewater treatment plant as seed, different biological treatment methods have been developed for NA degradation (Fig. 1.2) (Xue et al., 2018). However, previous studies reported that NAs could not be significantly removed by the suspended growth biological treatment process (Zhang et al., 2018). With the addition of carrier into OSPW, it was

interesting to find that the attached growth biological treatment approach showed improved NA removal efficiency (Huang et al., 2015a). As described above, due to the cooperation of microorganisms with metabolic diversity, the attached growth process is supposed to benefit microorganisms for better adaption to harsh conditions and faster degradation of organic compounds than the suspended growth process (Zhang et al., 2018). It is also known that the attached growth biological treatment processes can be divided into moving bed and fixed-bed treatment processes. Because the shear force from influent wastewater in fixed-bed treatment reactor is considered to be lower than that of moving bed reactor, it can probably benefit microorganisms for easier attachment and higher growth rate on the fixed-bed media, and which may further improve the degradation efficiency on organic compounds by the microorganisms in the biofilm (Rochex et al., 2008).

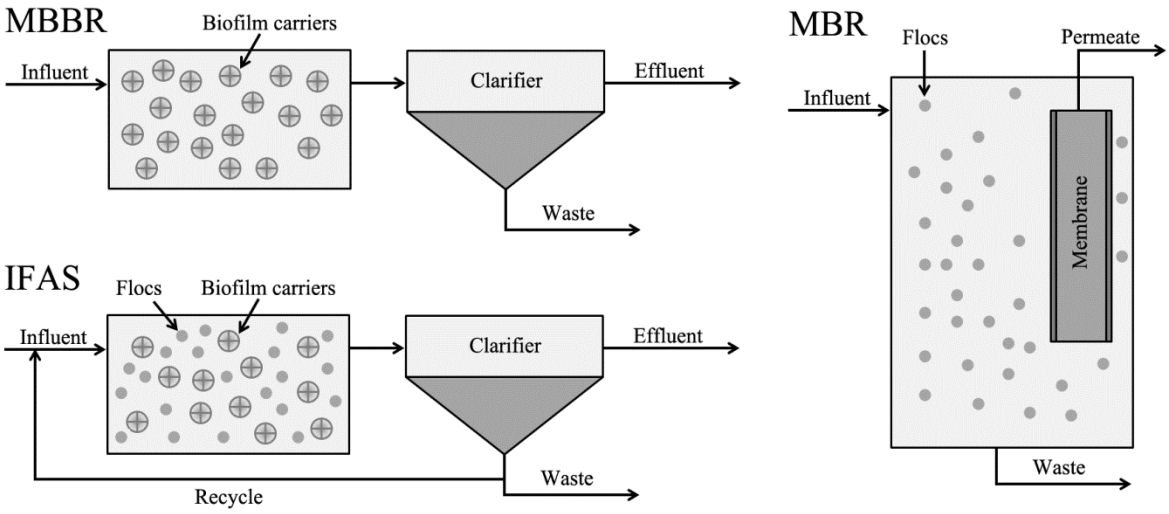

Figure 1.2. Schematics of established moving bed biofilm reactor (MBBR), membrane bioreactor (MBR), integrated fixed-film activated sludge reactor (IFAS) for OSPW treatment (Xue et al., 2018).

Due to robustness, the simplicity of construction and low energy input, fixed-bed attached growth biological process is hypothesized to have higher NA degradation efficiency than the established OSPW remediation bioreactors, which shows high potential to be scaled up for the treatment of OSPW *in situ*. However, the biodegradation efficiency of fixed-bed attached growth biological treatment processes on NA removal has not been investigated so far.

1.1.1 Toxicity of OSPW

The OSPW with high salinity and alkalinity contains various organic compounds and inorganic ions shows toxic effects to a wide range of organisms (Garcia-Garcia et al., 2011; Goff et al., 2013; He et al., 2012). Heavy metals, NAs, residual bitumen, and other organic compounds are all considered the toxic compounds present in OSPW (Brown and Ulrich, 2015). However, NAs have been regarded as the major toxicity contributors. Goff *et al*. investigated the toxicity of NAs to algae and found that NAs were able to alter the cell wall of algae, which can further lead to the formation of palmelloid and reduction of growth rate (Goff et al., 2013). Similar to surfactants, NAs can change the cell wall proteins of algae, which could probably result in the NA toxicity to algae (Goff et al., 2013). Anderson *et al*. determined that both short-term and long-term exposures to untreated OSPW could lead to the toxic effect on benthic invertebrate *C. dilutes* by reducing larvae production (Anderson et al., 2012). The toxicity of OSPW to zebrafish has been analyzed after the exposure to OSPW extract and it was observed that the concentration for OSPW extract to produce 50% mortality of fish was 8 mg/L (Scarlett et al., 2013). Amphibian larvae were found susceptible to NA exposure as well. Melvin *et al*. revealed that embryos of *Lithobates pipiens* and *Silurana tropicalis* suffered significant reductions (32% and 25%, respectively)

in growth and development upon hatching after the exposure to 2-4 mg/L of commercial NA blend and all the embryos could be killed following the exposure to NAs at a concentration of 6 mg/L (Melvin and Trudeau, 2012). Exposure to OSPW can also lead to great changes in transcripts at all levels of the brain-gonad-liver axis in male and female fathead minnows (*Pimephales promelas*) (He et al., 2012). The toxic effect of NAs to avian species has been studied as well. Following the exposure to NAs, the tree swallow showed an increase in extramedullary erythropoiesis in the liver, though the nestling growth, organ weights have not been affected significantly (Gentes et al., 2007). OSPW also shows a toxic effect on mammalian macrophage (mouse bone marrow-derived macrophage) by altering immune gene expression, such as inducing of the expression of pro-inflammatory cytokine genes (Garcia-Garcia et al., 2011).

1.1.2 NA analysis

Robust NA detection and quantification methods are important for OSPW toxicity assessment and for investigating the performance of different OSPW treatment processes. However, because NAs refer to various organic compounds, including alkyl-substituted acyclic and cycloaliphatic carboxylic acids, it is difficult to quantify and characterize NAs accurately. Several studies have been done on the development of a useful method for NA analysis (Ajaero et al., 2017; Huang et al., 2015b; Sun et al., 2014). The Fourier transform infrared (FTIR) spectroscopy method developed by Syncrude Canada Ltd. has been used as a standard method to measure the acid extractable fraction (AEF) from OSPW, and the results can be used for the assessment of NA concentration (Clemente and Fedorak, 2005). Briefly, OSPW is acidified and then the dichloromethane is used for the extraction of AEF, which contains the majority of NAs in OSPW. FTIR is used to measure the absorbance of

the monomeric and dimeric forms of the carboxylic groups from the concentrated AEF (at 1743 and 1706 cm^{-1}, respectively), and the AEF concentration can be determined by fitting the value of the whole absorbance to the calibration curve (Holowenko et al., 2001).

The extracted NAs can also be derivatized to form their methyl esters, which can be detected by gas chromatography with a flame ionization detector (Jones et al., 2001). NAs can also be extracted by using the solid extraction method, which uses a divinylbenzene support sorbent as media. The NAs can be concentrated through the elution by acetonitrile and can then be analyzed by the negative ion electrospray ionization mass spectrometry (Lo et al., 2003). High performance liquid chromatography has been studied for the carboxylic acids detection as well (Miwa, 2000). With the further requirement for NA analysis, besides the measurement of NA concentration, molecular structures, and compositions of NAs need to be addressed as well. Mass spectrometry (MS) technology has been considered one of the most useful tools for NA analysis (Clemente and Fedorak, 2005).

Recently, ultra-performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOFMS), Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS), and ion mobility spectrometry (IMS) based methods have been developed for the NA analysis (Sun et al., 2014). It is known that MS alone cannot distinguish isomers and ultrahigh resolution MS is needed for the detection of some isobaric species. However, the separation and detection of isomeric and isobaric NA species have been considered critical for the improved understanding of the NA composition in OSPW, which can help for further determination of specific toxic NA species and identification of NA byproducts after different treatment processes (Huang et al., 2015b). Through the integration of IMS to UPLC-TOFMS, the UPLC-IMS-TOFMS detection can provide additional information on

NA composition such as isomer separation (Gabelica and Marklund, 2018). It was found that by using UPLC-IMS-TOFMS technique, a two-dimensional (2D) separation (drift vs. retention times) of NAs can be achieved, and isobaric and isomeric NA species were observed in classical NAs (Huang et al., 2015b). With the capacity to separate both isomeric and isobaric NA species, the established UPLC-IM-TOFMS has been considered an upgraded analytical method for the separation and characterization of different NA species in OSPW.

1.1.3 Physical and chemical OSPW treatment

Serial physical and chemical OSPW remediation methods have been developed and evaluated, which usually show high NA removal efficiency (Islam et al., 2015; Wang et al., 2016a; Wang et al., 2016b; Zhang et al., 2017). The application of petroleum-coke for the adsorption of NAs from OSPW has been studied. Gamal El-Din *et al*. found that 91% total acid-extractable organics and 84% NAs can be removed by petroleum-coke adsorption which can improve the ozonation removal of NAs as well (Gamal El-Din et al., 2011). Granular activated carbon (GAC) has been widely used as an adsorbent for the removal of organic compounds. The GAC adsorption and biodegradation combined OSPW treatment method has been developed and showed 93.3% and 73.7% removal efficiency on the classical and oxidized NAs from OSPW, respectively (Islam et al., 2015). Peng *et al*. found that nanofiltration can remove 95% of NAs from OSPW (Peng et al., 2004).

Chemical oxidation has also been considered an important process for the degradation of recalcitrant organic compounds (Meshref et al., 2017). Hydroxyl radicals (·OH) based organic compounds destruction technologies, which are known as advanced oxidation

processes, have been investigated for their performance on OSPW treatment (Wang et al., 2016b). Oxidants like ozone and hydrogen peroxide can be used separately or combined with UV irradiation as advanced oxidation methods for OSPW remediation. By using a semi-batch ozonation system, more than 76% total acid-extractable organics from OSPW can be degraded (Gamal El-Din et al., 2011). And the pretreatment of OSPW by ozonation showed significant improvement for the post-biological remediation processes (Huang et al., 2015a; Shi et al., 2015; Zhang et al., 2016). Wang *et al*. found that the toxicity of OSPW to *Vibrio fischeri* can be removed completely after the ozonation and biodegradation combined treatment, and ozonation treatment can reduce the OSPW toxicity to the mammalian immune system as well (Wang et al., 2013). Other advanced oxidation processes such as UV/H_2O_2, UV/chlorine based NA degradation methods have been investigated, and also showed promising to be used for OSPW treatment (Xu et al., 2017).

1.1.4 Biological OSPW treatment

With the advantages of low cost and minimal waste generation, biological treatment method has been widely used for the remediation of different wastewater (Di Iaconi, 2012; Reungoat et al., 2011). Aerobic and anaerobic microorganisms can be used as suspended and attached growth biological wastewater treatment processes. The aerobic process has been considered the main NA biodegradation process, and the aerobic biodegradation of model NAs can occur within a short time of incubation (Whitby, 2010). Although the biodegradation of crude oil by methanogenesis has been determined and associated metabolic pathways have been investigated, the study on anaerobic NA biodegradation is rare (Jones et al., 2008).

A consortium of indigenous algae and bacteria was used for NA biodegradation *in situ*, the results of which showed that NAs can be biodegraded mainly by aerobic bacteria and the toxicity of OSPW can be reduced significantly after bioremediation (Mahdavi et al., 2015). Considering the tailings ponds are mainly under anoxic even anaerobic condition, research on anaerobic NA biodegradation by using indigenous microorganisms has been investigated, which showed that simple surrogate NAs can be biodegraded by anaerobic biological treatment (Clothier and Gieg, 2016).

Based on different biological treatment processes, serial bioreactors have been developed for the remediation of OSPW (Xue et al., 2018). Moving bed biofilm reactor has been established by using the activated sludge from the wastewater treatment plant as seed, and showed 34.8% of NA removal from raw OSPW (Shi et al., 2015). Integrated fixed-film activated sludge bioreactor has also been developed and showed 43.1% of NA removal from raw OSPW (Huang et al., 2015a). Xue *et al*. developed a membrane bioreactor, which can remove 24.7% NAs from OSPW (Xue et al., 2016).

Through the combination of physical and chemical treatment processes, the bioremediation of OSPW can be improved significantly (Islam et al., 2015; Zhang et al., 2016). With the pretreatment of ozonation, the NA removal ratio of moving bed biofilm reactor, integrated fixed-film activated sludge bioreactor can be remarkably improved (Huang et al., 2015a; Shi et al., 2015). Mild ozonation pretreatment can accelerate the biodegradation of OSPW and has no apparent impact on the organic matter degradation capacity of indigenous microorganisms (Brown et al., 2013). Mild ozonation pretreatment coupled with biodegradation treatment shows high potential to be applied for OSPW remediation.

1.1.5 Biofiltration of wastewater

With the attachment of biomass on the bed media surface, biofilter can be used for air, water and wastewater treatment as a biofiltration process. The trickling filter was the first application of biofilter for wastewater treatment. Microorganisms going through biofilter can attach to supporting media and form biofilm there. Biofiltration often refers to a fixed film process, which can be used for the biodegradation of pollutants by the microorganisms in the biofilm (Chaudhary et al., 2003).

Biofiltration has been applied for the treatment of different industrial wastewater successfully (Kang et al., 2007; Kornaros and Lyberatos, 2006; Reungoat et al., 2011). By using peat and perlite as filter media, trickling biofilter has been established and used for the removal of pollutants from cheese industry wastewater which contains a high volume of organic pollutants, solids, and nutrients. After biofiltration treatment, the quality of the wastewater can be significantly improved. More than 99% of chemical oxygen demand (COD) and biological oxygen demand (BOD) can be removed, and the total suspended solids were reduced by 95% (Saminathan et al., 2013). The application of sand biofiltration on the treatment of turkey processing wastewater has been investigated as well. Bioreactors using sand as bed media were set for the biofiltration of wastewater at different operation conditions. It was found that sand biofiltration can also be a feasible treatment method for the turkey processing wastewater, and the hydraulic loading of bioreactor was found critical for the lifespan of biofiltration process (Kang et al., 2007). The biological trickling filter can also be applied for the treatment of dye manufacturing wastewater. By using hydraulic loading from 0.6 $m^3/m^2/day$ to 1.1 $m^3/m^2/day$, the COD removal efficiency of the system was determined as 80-85% to 60-70%. The biofilm developed on the trickling filter were

found to be able to remove COD levels up to 36,000 mg/L, with the aerobic operation condition at pH values between 5.5 and 8.0 (Kornaros and Lyberatos, 2006).

Biofiltration has also been used to remove the recalcitrant organics such as pharmaceuticals and personal care products from the effluent of wastewater treatment plant (Reungoat et al., 2011). Sand and granular activated carbon were used as different filter media for the comparison of removal efficiency on pollutants. The impacts of ozonation pretreatment process and empty bed contact time (EBCT) on the performance of wastewater treatment by biofiltration have been analyzed as well. It was found that biological activated carbon can remove pharmaceuticals, personal care products, and other toxicity compounds effectively at a very low cost.

Filter media has been considered an important impact factor for the biofiltration process, which can impact the organic compounds removal efficiency and the cost of construction (Chaudhary et al., 2003). The mesquite wood chips were used as a novel media for the development of biofilter, and the established biofilter system could effectively remove the pollutants from municipal wastewater. And it was found that the treated wastewater could even be used for irrigation (Sosa-Hernandez et al., 2016). In order to further reduce the cost of filter media, a study has been done on the application of agave fiber as biofilter media. It was determined that the agave fiber based biofilter can also be effectively used for municipal wastewater treatment. After the biofiltration treatment, the quality of treated wastewater can meet all the requirements from the Mexican and US EPA standards for agricultural irrigation and green spaces, except for the number of coliforms. This research found that agave fiber can be a favorable choice to be used as media for biofiltration processes (Vigueras-Cortes et al., 2013). Biofiltration is hypothesized as a

promising process for OSPW treatment. However, no study has been performed on the application of fixed-bed biofilm reactor for OSPW remediation until now.

1.2 Significance of the research

The huge volume of toxic OSPW stored in tailing ponds has shown a serious threat to the local environment and ecosystem (Pramanik, 2016). In recent years, various studies have been conducted at a bench-scale level to investigate the performance of OSPW treatment methods. However, large-scale applications have not been assessed so far (Xue et al., 2016).

The OSPW treatment methods can basically be divided into physical, chemical and biological treatments. For physical treatment methods, petroleum-coke adsorption was found to remove 91% of total acid-extractable organics and 84% of NAs from OSPW, and the acute toxicity to *Vibrio fischeri* could be reduced from 4.3 to 1.1 toxicity units (Gamal El-Din et al., 2011). Chemical OSPW treatment methods such as ozonation can effectively remove the fluorescing aromatics, sulfur NAs, and classical NAs at a dose of 2.0 mM (Wang et al., 2016b). However, the established physical and chemical treatment methods are either high cost or too complicated to be applied at a large-scale.

Biological treatment processes have been considered as effective, economical, and energy efficient approaches for wastewater reclamation in various industries, which can potentially be used as a technically practical and environmentally sound approach for tailings water reclamation (Pramanik, 2016). Previous studies have shown that sediment microorganisms like *Pseudomonas putida* and *Pseudomonas fluorescens* had the capability of degrading a commercial mixture of NAs and *Pseudomonads* could reduce the toxicity of

OSPW through the biodegradation of aromatic compounds (Del Rio et al., 2006; Zhang et al., 2015). Biological treatment processes can be further divided into two types: suspended growth (e.g., activated sludge) and attached growth (e.g., fixed-bed biofilm reactor) process. Previous studies have shown that NAs cannot be significantly removed by the suspended growth biological treatment method (Zhang et al., 2015). However, with the addition of a carrier into OSPW, the attached growth biological treatment approach can remove the NAs significantly (Huang et al., 2015a). Compared with suspended growth, the attached growth is supposed to benefit microorganisms for the better adaption to harsh conditions and the faster degradation efficiency on organic compounds in wastewater through the cooperation among microorganisms in the biofilm (Zhang et al., 2015). Furthermore, the attached growth biological treatment processes can be divided into moving bed and fixed-bed treatment processes. As a result of low shear force from wastewater, the fixed-bed treatment processes are supposed to benefit microorganisms for easier attachment and higher growth efficiency, which can further improve their performance on organic compounds degradation. As a fixed-bed attached growth biological treatment process, biofiltration has been widely used for water and wastewater treatment due to their robustness, ease, and simplicity of construction and low energy input (Reungoat et al., 2011). However, the improvement of NA biological degradation efficiency by using biofiltration processes has not been investigated until now.

Ozonation has been widely studied as a pretreatment method for the biological treatment of OSPW (Huang et al., 2015a; Shi et al., 2015; Zhang et al., 2016). It has been found that ozonation pretreatment can improve the bioremediation of OSPW by indigenous microorganisms through the degradation of recalcitrant NA fraction selectively (Martin et

al., 2010). Through the mild ozonation (with utilized ozone dose of 30 mg/L) pretreatment, the acid-extractable fraction (AEF) and NA removal by moving bed biofilm reactors (MBBR) can be improved from 18.3% to 41% and from 34.8% to 78.8%, respectively (Shi et al., 2015). Mild ozonation pretreatment can improve the removal of AEF and NAs by integrated fixed-film activated sludge reactors (IFAS) and membrane bioreactors (MBR) as well (Huang et al., 2015a; Xue et al., 2016).

According to the description above, it is hypothesized that fixed-bed biofilm reactors (i.e., biofilters) can be developed efficiently by using indigenous microorganisms from OSPW. The biofiltration treatment would probably show a higher NA removal effect from OSPW than the biological OSPW treatment processes established previously. In addition, mild ozonation (with utilized ozone dose of 30 mg/L) pretreatment is expected to improve the biodegradation of NAs by biofiltration. With clear cost and environmental advantages, developing a mild ozonation combined biofiltration treatment process is promising and can be easily scale-up and applied by the oil and gas industry.

1.3 Research objectives

The main purpose of this research project was to develop indigenous microorganisms based fixed-bed biofilm reactors which could be used as a fixed-bed attached growth biological treatment approach for the degradation of NAs from OSPW. With the application of established biofilters for OSPW treatment, the NA degradation efficiency was aimed to be determined. The effect of ozonation treatment on the performance of NA biodegradation by biofiltration was also investigated. The dynamics of microbial

community structures and the changes in the functional pathway abundance was aimed to be illuminated as well.

To achieve the main research objective, the project was divided into three sub-objectives according to the research progress. Stage Ⅰ: Develop indigenous microorganisms based sand biofilters, and apply them for the treatment of raw and ozonated OSPW; Stage Ⅱ: Investigate the impact of biofiltration pretreatment on the removal of NAs by mild ozonation, and determine the efficiency of the mild ozonation integrated biofiltration process on OSPW treatment; Stage Ⅲ: Investigate the key microorganisms and determine the critical biodegradation pathways for NA degradation in the biofilter by using next generation sequencing.

Stage Ⅰ: Establishment of fixed-bed biofilm reactors for raw and ozonated OSPW treatment.

The biofilters were developed by using pure sand as media and raw OSPW as influent wastewater. The established fixed-bed biofilm reactors were then used for the treatment of raw and ozonated OSPW. The mild ozonation (with utilized ozone dose of 30 mg/L) treatment was used as a pretreatment process for the improvement of NA biodegradation efficiency of the established fixed-bed biofilm reactor.

The aims of this stage include:

A: Detect the growth of bacteria and the formation of biofilm on the sand media surface of the biofilters.

B: Monitor the removal of organic compounds from raw OSPW during the biofilters start-up period.

C: Investigate the characteristics of the indigenous microbial community.

D: Determine the performance of the established fixed-bed biofilm reactor on the treatment of OSPW and investigate the impact of hydraulic retention time on the removal of NAs from OSPW.

E: Characterize the microbial community structure in the biofilm after the biofiltration of OSPW.

F: Assess the effect of ozonation pretreatment on the removal of NAs from raw OSPW.

G: Determine the effect of ozonation pretreatment on the improvement of NA removal efficiency of the established fixed-bed biofilm reactor, and investigate the impact of hydraulic retention time on the removal of NAs from the ozonated OSPW.

H: Characterize the microbial community structure in the fixed-bed biofilm bioreactor after the ozonation combined biofiltration OSPW treatment process.

Stage Ⅱ: Development of ozonation integrated biofiltration process (biofiltration-ozonation-biofiltration) for OSPW treatment.

The mild ozonation treatment (with utilized ozone dose of 30 mg/L) was used as an integrated treatment process for the improvement of OSPW treatment efficiency of the biofiltration process. The aims of this stage include:

A: Investigate the effect of biofiltration pretreatment on the removal of NAs by mild ozonation.

B: Assess the effect of biofiltration-ozonation combined treatment on the removal of NAs from OSPW.

C: Evaluate the effect of post biofiltration treatment on the removal of NAs from OSPW.

D: Characterize the microbial community structure in the fixed-bed biofilm bioreactor after the biofiltration-ozonation- biofiltration OSPW treatment process.

Stage III: Metagenomic and metatranscriptomic analyses.

The bed media samples were collected from the top, middle and bottom of the fixed-bed biofilm reactors, and also collected after the biofiltration treatment of different OSPW for the next generation sequencing analysis. The aims of this stage include:

A: Exam the characteristics of the microbial community structures from the different depth of the established fixed-bed biofilm reactors.

B: Investigate the variance of microbial community structures and functional pathway abundance in the fixed-bed biofilm reactors to explore the microbial ecological interactions between the microbial community and different OSPW.

C: Determine the key microorganisms and critical metabolic pathways in the microbial community for the degradation of NAs from raw and ozonated OSPW.

1.4 Thesis organization

The thesis is logically organized according to the research stages presented above.

Chapter 1 is the general introduction of the thesis-based research. Briefly, it contains the contents of the research background and motivation description, research significance analysis as well as a list of research objectives.

Chapter 2 shows the research findings on the development of indigenous microorganisms based fixed-bed biofilm reactors and the application of biofilters on the treatment of raw and ozonated OSPW. By using raw OSPW as influent wastewater and pure sand as media, the fixed-bed biofilm reactors were developed and operated to a stabilized stage. The growth of bacteria on the sand media surface in the fix-bed biofilm reactors was monitored by using qPCR detection method. The removal of organic compounds in OSPW during the development of the fixed-bed biofilm reactors system was measured through the COD and AEF detection analysis. With the application of fluorescent staining method, the growth of biofilm on the sand media surface in the fixed-bed biofilm reactors was observed by using confocal laser scanning microscopy (CLSM) and the thickness of biofilm was measured as well. The performance of the fixed-bed biofilm reactors on the treatment of raw and ozonated OSPW was investigated based on the NA degradation analysis. The NAs in OSPW were detected by the UPLC-TOFMS. In addition, the Illumina Miseq platform based next generation sequencing was used for the characterization of the dynamics of microbial community structure in biofilm during the treatment of different OSPW.

Chapter 3 presents the research results for the application of biofiltration-ozonation-biofiltration process on the treatment of OSPW. The beneficial effect from biological pretreatment on the removal of NAs by following mild ozonation was investigated. In addition, the performance of OSPW treatment by different treatment processes, including mild ozonation-biofiltration and biofiltration-ozonation-biofiltration was compared, aiming to find the most effective and industrial applicable OSPW treatment process. With the detection by UPLC-TOFMS, the removal of NAs including classical and oxidized NAs by different treatment processes was tested and compared. The impact of circulation times (hydraulic retention time) on the removal of NAs from OSPW was investigated and compared. Based on the 16S rRNA gene targeted next generation sequencing analysis, the diversity of the microbial community structure in fixed-bed biofilm reactors was characterized and the functional pathway abundance was predicted based on the established bioinformatic pipelines.

Chapter 4 describes the research findings on the investigation of microbial ecosystem. By using the Illumina Miseq platform based 16S/18S rRNA genes targeted next generation sequencing analysis, the variance of bacterial and fungal community structures on the top, middle, and bottom of the established fixed-bed biofilm reactor was investigated. With the application of fixed-bed biofilm reactors for the treatment of the OSPW with a higher concentration of NAs than that in the OSPW used before, the adaption of the biofilters to different OSPW was confirmed. Through the detection of NA removal by UPLC-TOFMS, the efficiency of the fixed-bed biofilm reactors on the treatment of raw and ozonated OSPW was analyzed. After the treatment of different OSPW by using the same hydraulic retention time, the dynamics of the microbial community structure in the fixed-bed biofilm

reactor was investigated and compared. In addition, after the treatment of different OSPW, the changes in functional pathway abundance of the microbial community were analyzed by using the Illumina Hiseq platform based metatranscriptomic sequencing. The dominant bacteria and fungi in the fixed-bed biofilm reactor were investigated and the changes of functional pathway abundance were illustrated.

Chapter 5 contains the thesis overview and general conclusions of these stages. In addition, the recommendations for future studies have been discussed as well.

Besides, the appendix section contains the supplementary figures and detailed description of some of the experimental methods.

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CHAPTER 2. DEGRADATION OF RECALCITRANT NAPHTHENIC ACIDS FROM RAW AND OZONATED OIL SANDS PROCESS WATER BY A SEMI-PASSIVE BIOFILTRATION PROCESS[1]

2.1 Introduction

Canada has been recognized as the country with the third largest oil reserves in the world, which has more than 169 billion barrels of recoverable bitumen deposit in Athabasca, northern Alberta (Brown and Ulrich, 2015; Teare et al., 2012). It was estimated that 5.4 percent of the initial established crude bitumen reserves have been produced and the bitumen production is supposed to reach 4.1 million barrels per day by 2023 (Teare et al., 2014). The caustic hot water extraction process is applied for the extraction of bitumen from the oil sands, and the process water generated from the extraction and following upgrading process is referred as the oil sands process water (OSPW) (Brown and Ulrich, 2015; Wang et al., 2016a). A large amount of toxic OSPW has been generated and the volume is keeping on increasing with the expansion of the bitumen production. The accumulation of huge amount of OSPW in tailing ponds has shown a serious threat to local surface and groundwater quality through the contamination of toxic compounds (Hodson, 2013; Pramanik, 2016). OSPW is a complex mixture of inorganic and organic compounds,

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which shows the toxic effect to a wide range of organisms such as algae, fishes, amphibians, birds and mammals (Garcia-Garcia et al., 2011; Gentes et al., 2007; Goff et al., 2013; He et al., 2012; Melvin et al., 2013). Reuse of the tailing water for bitumen extraction can reduce the demand for fresh water, but can also lead to an increase of OSPW toxicity due to the accumulation of toxic compounds (Allen, 2008). Therefore, technically practicable and economically feasible OSPW remediation methods need to be established urgently (Brown and Ulrich, 2015; Pramanik, 2016).

Naphthenic acids (NAs) are considered one of the main toxic constituents in OSPW (Zhang et al., 2011). NAs are a mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids with a general chemical formula of $C_nH_{2n+Z}O_x$, where n is the number of carbon atoms ($7 \leq n \leq 26$), Z specifies the hydrogen deficiency due to the formation of ring or double bond structure ($0 \leq -Z \leq 18$), and x indicates the number of oxygen atoms present in the NAs (Huang et al., 2016a; Wang et al., 2016b). Classical NAs, which possesses two oxygen atoms (O_2-NAs), has been regarded as the most toxic NAs from OSPW (Morandi et al., 2015). Because the increase of hydrophilicity, the oxidized NAs which possess more oxygen atoms show less toxicity (He et al., 2010). Therefore, the removal of classical NAs is considered critical for the OSPW remediation. As such, the removal of classical NAs has been used as one of the parameters for the evaluation of the OSPW treatment methods (Huang et al., 2015).

In recent years, studies have been conducted to develop OSPW treatment methods, though none of them have been tested at the full-scale level (Huang et al., 2015; Shi et al., 2015; Xue et al., 2016b). The OSPW treatment can basically be divided into physical, chemical, biological methods. As an example of physical treatment method, petroleum-

coke adsorption can remove 91% of acid-extractable fraction (AEF) and 84% of NAs from OSPW, and its toxicity toward *Vibrio fischeri* can be reduced from 4.3 to 1.1 toxicity units (Gamal El-Din et al., 2011). Chemical OSPW treatment methods such as ozonation can effectively remove the fluorescing aromatics, sulfur-containing NAs, and classical NAs at the dose of 2.0 mM (Wang et al., 2016b). Compared with physical and chemical processes, biological processes are usually less effective for OSPW treatment. Nevertheless, they are economical, energy-efficient, and environmentally sound approaches for large-scale applications. Sediment microorganisms such as *Pseudomonas putida* and *Pseudomonas fluorescens* showed high degradation effect on a commercial mixture of NAs, and they could also reduce the toxicity of OSPW through the biodegradation of aromatic compounds (Del Rio et al., 2006; Zhang et al., 2015). However, our previous studies showed that NAs cannot be considerably removed by suspended growth biological treatment processes (Zhang et al., 2015). Compared with suspended growth systems, the attached growth systems are supposed to benefit microorganisms for the better adaption to the harsh conditions and faster degradation of organic compounds due to the cooperation of microorganisms with metabolic diversity. By adding biofilm carriers into the OSPW, the attached-growth biological treatment approach can remove the NAs by 34.8% to 43.1% (Huang et al., 2015; Shi et al., 2015).

Previous research has demonstrated the biopersistence of NAs with extensive cyclical molecular structures (Han et al., 2008), indicating that pretreatment is necessary to improve the biodegradability of OSPW. It has been demonstrated that low-dose ozone could improve the biodegradability of OSPW by breaking NAs with high cyclicity and long carbon chains (Martin et al., 2010). Ozonation combined biological treatment has been

successfully developed and applied for OSPW remediation (Huang et al., 2015; Shi et al., 2015; Zhang et al., 2016). Through the ozonation pretreatment, the AEF and NA removals in a moving bed biofilm reactor (MBBR) were improved from 18.3% to 41.0% and from 34.8% to 78.8%, respectively (Shi et al., 2015). Similar results were also observed by using integrated fixed-film activated sludge reactors (IFAS) and membrane bioreactors for OSPW treatment (Huang et al., 2015; Xue et al., 2016b). However, it was observed that the ozonation pretreatment itself contributed to the most NA removal while it did not substantially facilitate the NA removal rate in the bioreactors. Additionally, long hydraulic retention time (HRT) requirement and high aeration cost of those bioreactors also limit their applications at large scale.

With the advantages of robustness, ease, and simplicity of construction, and low energy input, biofiltration has been widely used for water and wastewater treatment (Kang et al., 2007; Reungoat et al., 2011; Saminathan et al., 2013). Reungoat et al. (2011) found that biofiltration can remove pharmaceuticals and personal care products by more than 90% and reduce the toxicity of wastewater by 28-68%. In another study, circulation biofilter was determined as a robust process for the degradation of pharmaceutically active compounds with the removal of 92-99%, 89-99% and 62-92% for ibuprofen, naproxen, and gemfibrozil, respectively (Krkosek et al., 2014). Recently, a fixed-bed biofilm reactor showed high efficiency on the biodegradation of methyl tertiary-butyl ether which is a recalcitrant organic compound causing groundwater contamination (Alfonso-Gordillo et al., 2016). Fixed-bed biofilm reactors have shown higher efficiency on the removal of various compounds compared to MBBR (Choi et al., 2012). However, the remediation of OSPW by using biofiltration process has not been investigated until now.

With the application of low-cost natural materials and consuming low energy, mild ozonation pretreatment (moderate ozone dose) followed by biofiltration may be a promising approach for OSPW treatment, which can be easily scaled up and applied by the industry. In this research, circulating fixed-bed biofilters were established by using indigenous microorganisms from OSPW. In order to avoid unnecessary expenditure of chemicals and energy, thereby lowering the operating cost, an ozone dose of 30 mg/L was used for the pretreatment of OSPW prior to biofiltration. Our previous study has demonstrated that the NAs showed a sharp decrease after ozonation up to about 50 mg/L, and ozone dose of 30 mg/L led to the maximal toxicity reduction (Dong et al., 2015; Wang et al., 2013). And ozone dose of 30 mg/L could also result in the minimal mineralization during the pretreatment stage. The main objective of this study was to confirm the feasibility of the biofiltration process in OSPW remediation and to investigate the benefit of mild ozonation pretreatment on the biofiltration performance. The NA removal efficiency of the biofiltration and the combined processes was investigated. The impact of circulation times on the removal of classical and oxidized NAs was also analyzed. Dominant bacteria related to NA degradation were determined through the analysis of the microbial community by using the next-generation sequencing technology.

2.2 Materials and methods

2.2.1 Source of OSPW

OSPW samples were taken from an active oil sands tailings pond in Fort McMurray, Alberta, Canada. OSPW was stored in 200 L polyvinyl chloride barrels at 4 °C prior to being used in this study within one year of collection. Characteristics of OSPW were

analyzed periodically to ensure the representativeness of OSPW after storage. The physicochemical parameters of raw OSPW were shown in Table 2.1.

Table 2.1. Physicochemical measurements for raw OSPW

Parameters	Raw OSPW
TOC (mg/L)	90.3 ± 7.3
DOC (mg/L)	50.9 ± 0.8
TSS (mg/L)	523 ± 11
DO (mg/L)	5.32 ± 0.02
BOD_5 (mg/L)	0.26 ± 0.02
COD (mg/L)	128.6 ± 3.7
Ammonia (mg/L)	0.6 ± 0.1
Nitrite (mg/L)	0.11 ± 0.0
Nitrate (mg/L)	0.65 ± 0.1
Total Kjedahl Nitrogen (TKN)	1.72 ± 0.1
Alkalinity (mg/L)	314
Turbidity (NTU)	1860
Redox (mV)	117
Conductivity (µS)	1671
pH	9.60

2.2.2 Ozonation

Briefly, high-purity oxygen was used for the production of ozone by an ozone generator (GSO-40, WEDECO, Herford, Germany). By using a gas diffuser and stir mixer, ozone could be well mixed within the water sample with the volume of 4 L. Ozone monitors (HC500 Ozone monitor, WEDECO, USA) were used to monitor the ozone

concentrations in feed and off-gas lines, and a wet test meter (Precision Scientific Petroleum Instruments, Texas, USA) was used to continuously measure the flow rate of feed and off-gas lines. The concentration of ozone residual in the water samples was monitored by using the indigo method. After the ozonation treatment, the ozone residual in the water was removed by purging with purified nitrogen gas for 5 minutes (Gamal El-Din et al., 2011; Huang et al., 2015). The utilized ozone dose of 30 mg/L was calculated through the integration of the amount of used ozone minus the amount of the residual ozone.

2.2.3 Configuration and operation of the biofilters

Duplicate glass columns (25 mm internal diameter and 15 cm bed depth) were served as biofilters. The polyethylene terephthalate (PET) disk was set at the bottom to hold the media inside the bioreactor. Pure sand (Acros Organics, New Jersey, USA) with the size of 40-100 mesh was used as media. In order to support the colonization of indigenous microorganisms in the biofilters, the reactors were fed with raw OSPW at a flow rate of 0.61 mL/min. The biofilters were operated at room temperature.

Biodegradation of raw and ozonated OSPW was implemented by circulating OSPW through the biofilter with an empty bed contact time of 120 minutes. The treated OSPW samples were taken for water quality analyses after 4 and 8 circulation times. The untreated raw OSPW was collected and used as a control.

2.2.4 Water chemistry analyses

Raw, ozonated and treated OSPW samples were collected for water chemistry analyses. Chemical oxygen demand (COD) was measured by using the HACH TNT test

tube method (TNT821, Hach, Colorado, USA). AEF was quantified using a Fourier transform infrared (FTIR) spectroscopy method (Jivraj et al., 1995). Briefly, 25 mL of water sample was filtered by using a nylon membrane filter with the pore size of 0.45 μm and acidified to pH 2 - 2.5. Optima grade dichloromethane (DCM) was used for the extraction. After evaporation, the extracted AEF was then dissolved in DCM with a known mass. FTIR spectrometry (PerkinElmer, ON, CA) was used to measure AEF by recording the absorbance of the monomeric and dimeric forms of the carboxylic groups at 1743 and 1706 cm^{-1}. The COD and AEF tests were all performed in duplicate. The profiles of the NAs present in different OSPW samples were analyzed using ultra performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOFMS) (Huang et al., 2015; Xue et al., 2016b). In brief, 10 mL of water sample was collected and filtered by using nylon membrane filter with the pore size of 0.22 μm. 0.5 mL of filtered water sample was mixed with 0.4 mL of methanol and 0.1 mL of myristic acid-1-^{13}C (4.0 mg/L), which was used as an internal standard. Waters UPLC Phenyl-BEH column (1.7 μm, 150 mm × 1 mm) was used for the liquid chromatographic separation. 10 mM ammonium acetate in water (A) and 10 mM ammonium acetate in a 50:50 methanol/acetonitrile mixture (B) were used as the mobile phases, and the elution gradient was 0 to 2 min, 1% B; 2 to 3 min, 60% B; 3 to 7 min, 70% B; 7 to 13 min, 95% B; 13 to 14 min, back to 1% B and hold to 20 min to equilibrate the column at a flow rate of 100 μL/min. The temperature of the column and the sample was set at 50 °C and 10 °C. With the electrospray ionization (ESI) source operating in negative ion mode and TOF analyzer in high-resolution mode (mass resolution of ~40000 at m/z 1431), the samples were further analyzed with a high-resolution time-of-flight mass spectrometer (Synapt G2, Waters, Milford, MA, USA) (Huang et al., 2016a).

As an integrated separation and detection process for UPLC-TOFMS, the ion-mobility spectrometry (IMS) was conducted in a Tri-Wave® ion-mobility cell by using pure nitrogen (> 99%) as the drift gas to separate and identify different NA species. TargetLynx® Ver. 4.1 software was used to analyze the data for target compounds (Huang et al., 2016b).

2.2.5 Microbial characterization

The microbial samples for confocal laser scanning microscopy (CLSM) and quantitative polymerase chain reaction (qPCR) analyses were collected from the top of the fixed-bed biofilm reactor, which was within 1 cm of the depth of the sand media, and tested for three times. After staining the sand samples with SYTO 9 and ConA conjugated with Texas Red (Molecular Probes, Invitrogen), CLSM (Zeiss LSM 710, Carl Zeiss Micro Imaging GmbH, Germany) was performed to characterize the distribution of deposited microbial cells and extracellular polymeric substance (EPS) on sand media (Xue et al., 2016a). PowerSoil® DNA isolation kit (Mo-Bio Laboratories, Inc., CA, USA) was used for bacterial DNA extraction from sand media (0.25 g) according to the manufacturer instructions. The extracted DNA samples were then used for the qPCR analysis to quantify the total bacteria on the sand media (Zhang et al., 2016). Moreover, Illumina MiSeq sequencing was used to further understand the microbial communities related to OSPW biodegradation on the sand media as described previously (Huang et al., 2017). After the raw and ozonated OSPW treatment, the sand samples for microbial community analysis were also collected from the top level of the fixed-bed biofilm reactor and used for 16S metagenomic sequencing analysis after DNA extraction.

2.3 Results and discussion

2.3.1 Development of biofilm in the biofilters

Raw OSPW was used as feed for the development of indigenous-microorganisms based biofilm on the sand media of the biofilter. Through monitoring the growth of total bacteria on the sand by qPCR, it was found that the biofiltration system reached a relatively steady state after 23 days of operation with $3.3 \pm 0.8 \times 10^9$ copies/g total bacteria on the sand media (Fig. 2.1A). These results showed that indigenous microorganisms from raw OSPW were able to attach to the surface of the sand media and form biofilm. Our previous study showed that a bacteria density of $\sim 10^9$ copies/g granular activated carbon (GAC) could be reached after 30 days of operation in fed-batch biofilm reactors (Islam et al., 2015). Considering the much lower surface area and higher specific gravity of sand media compared with GAC, denser biofilm was formed on the unit area of sand media, which may benefit the OSPW degradation. The results also indicated that the microorganisms could form biofilm more efficiently on the fixed-bed media compared to moving bed media (suspended GAC in the fed-batch reactors), which was potentially associated with the lower sheering force (Rochex et al., 2008). After 87 days of operation, the thickness of the biofilm was 35.8 ± 0.9 μm (Fig. 2.1B) with the bacterial density of $3.0 \pm 0.2 \times 10^9$ copies/g. The biofilm images indicated that the bacteria did not fully cover the sand surface even after 87 days. There was no significant COD and AEF removal in the stage of biofilm development, which indicates that the sand biofilter could not absorb or trap the organic compounds present in the OSPW (Fig. 2.2 A-B).

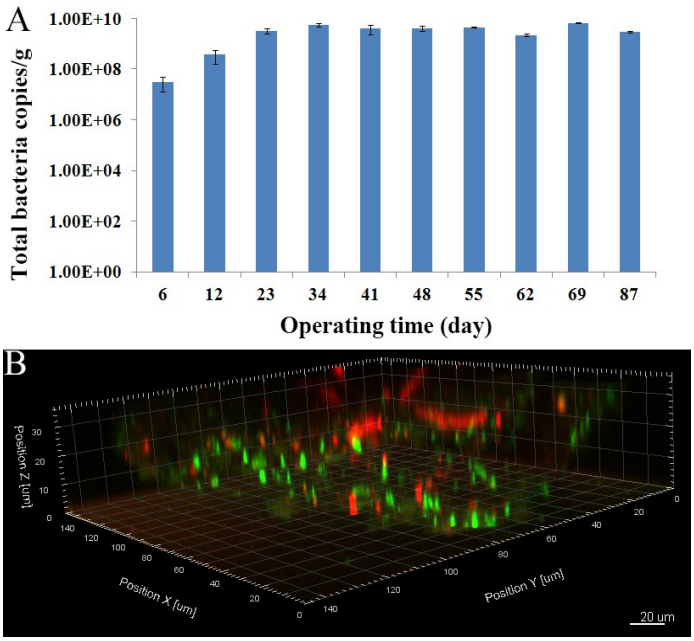

Figure 2.1. Development of biofilm in the fixed-bed reactors. A: Total bacterial quantification by qPCR; B: Biofilm detection and thickness measurement by CLSM. Biofilm was composed of bacterial cells (green) and extracellular polymeric substance (red).

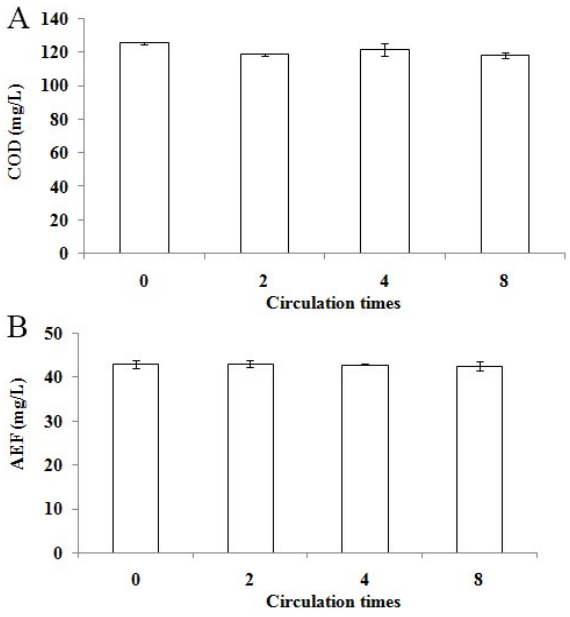

Figure 2.2. Performance of biofilter for raw OSPW treatment. A: Chemical oxygen

demand (COD) in effluent OSPW after different circulation times; B: Concentration of acid-extractable fraction (AEF) in effluent OSPW after different circulation times.

2.3.2 Effect of ozonation

Ozonation has been widely used as a pretreatment method for OSPW biodegradation (Shi et al., 2015; Zhang et al., 2016). Ozonation can promote the biodegradability of OSPW by preferentially breaking the most bio-persistent NAs into species with less cyclicity and shorter carbon chains (Martin et al., 2010). In our study, with a utilized ozone dose of 30 mg/L, the classical NAs decreased from 13.06 mg/L to 8.88 mg/L with the removal of 32.1% (Fig. 2.3A-B). However, the concentration of oxidized NAs was not remarkably reduced (Fig. 2.3A-B). Particularly, the concentrations of O_3-NAs, O_5-NAs, and O_6-NAs were relatively stable, but the concentration of O_4-NAs decreased by 8.0% (Fig. 2.3A-B). This finding suggested that the oxidized NAs were probably less sensitive to mild ozonation compared with classical NAs, the result of which was consistent with a previous study (Zhang et al., 2016).

The degradation of classical NAs by ozonation was further analyzed according to the carbon number and -Z number (Fig. 2.4, 2.5A, C). Based on Fig. 2.4, it is interesting to note that there was a steady descent in the NAs removal with increased -Z number of 2-12, whereas a sharp rise of NAs removal was observed with increasing -Z number of 14-18. Our previous study also proved that ozonation has a capacity of decomposing highly branched and cyclic carboxylic NAs, which led to the improvement of NA removal in the following bioreactors (Zhang et al., 2016).

The effect of ozonation on raw OSPW was analyzed by using IMS as well. The result showed that the NAs from the OSPW could basically be separated into three clusters, including oxidized NAs, heteroatomic NAs, and classical NAs (Fig. 2.6). It can also be observed that the classical NAs from raw OSPW were reduced. However, the concentrations of the oxidized NAs from the OSPW remained unchanged (Fig. 2.6A, C). It was reported that the oxidized NAs can be generated by the oxidation of classical NAs (Sun et al., 2014). Thus, the limited removal of oxidized NAs may be attributed to the generation of new oxidized NAs from classical NAs. Moreover, the concentration of O_3-NAs increased after ozonation, which may reveal that it was easier to oxidize the classical NAs to O_3-NAs than to O_4-NAs. Compared with classical NAs, oxidized NAs containing more -OH groups were less hydrophobic which may result in reduced toxicity (Wang et al., 2013).

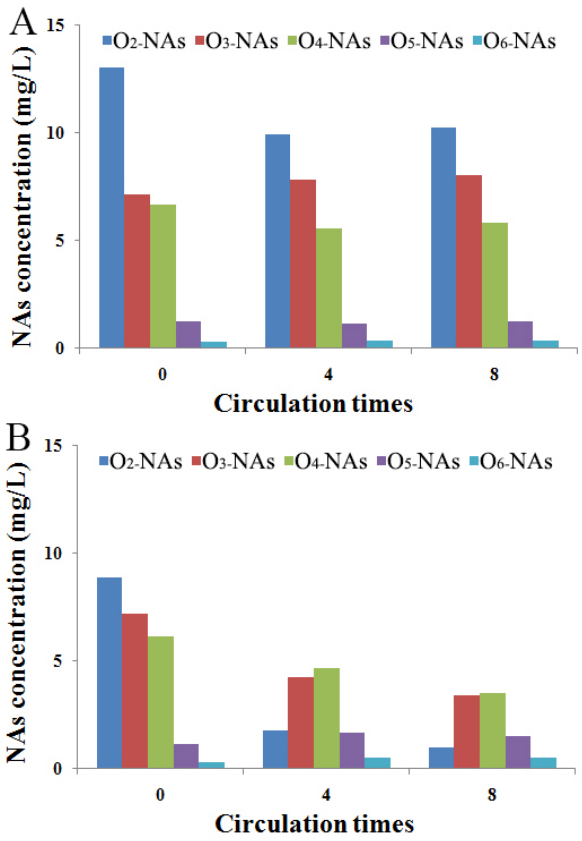

Figure 2.3. Performance of the fixed-bed biofilm reactors on the OSPW treatment. A: Biodegradation of NAs from raw OSPW; B: Biodegradation of NAs from ozonated OSPW.

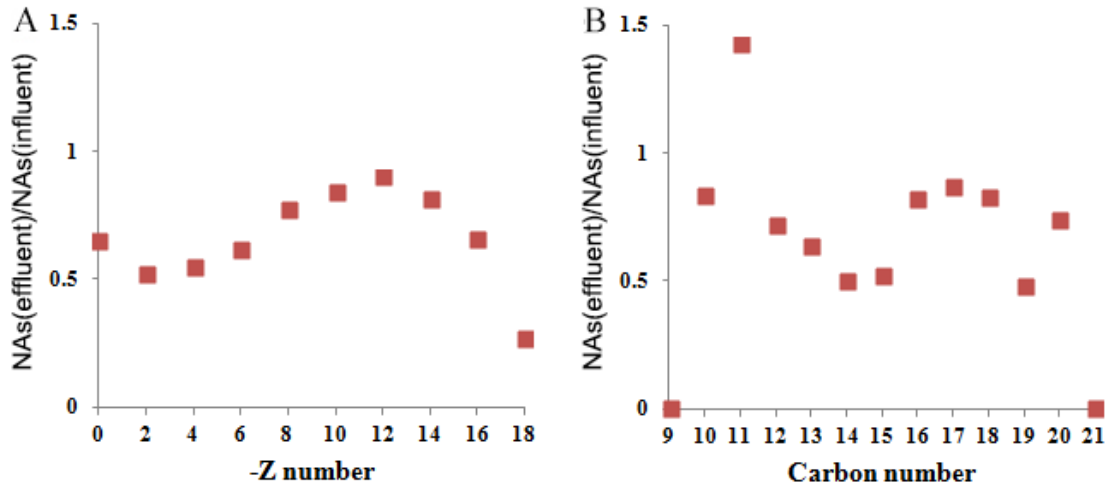

Figure 2.4. Relative removal of classical NAs after ozonation treatment: A: Impact of -Z number; B: Impact of carbon number.

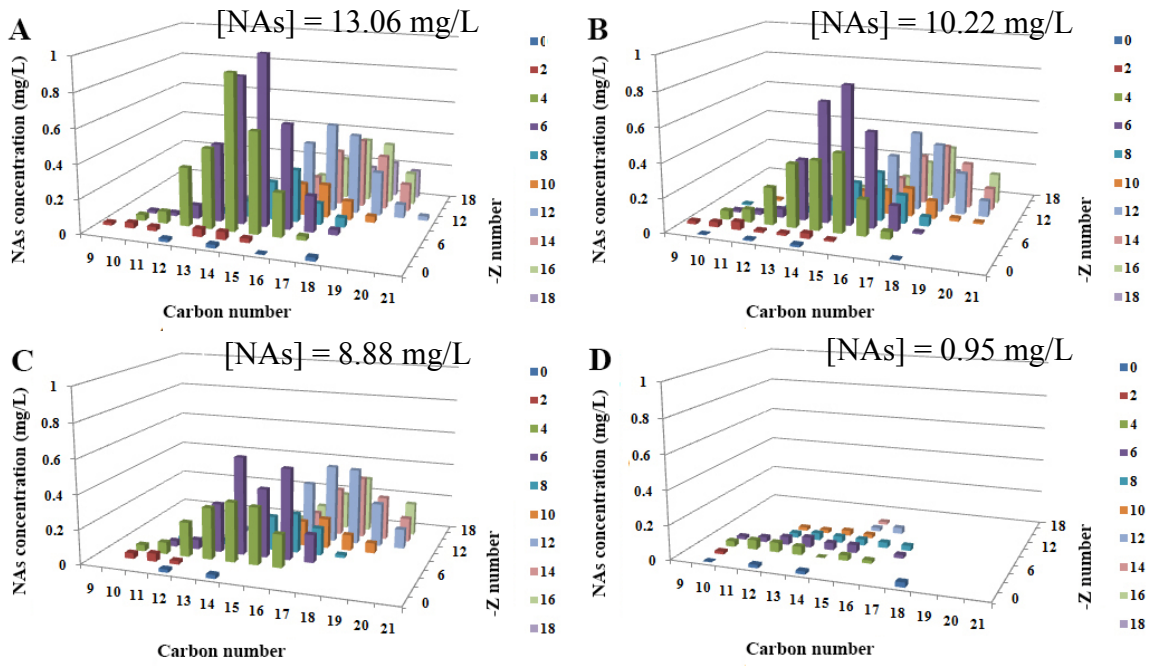

Figure 2.5. Profiles of classical NAs for different OSPW. A: Raw OSPW; B: Biofiltered OSPW; C: Ozonated OSPW; D: Biofiltered ozonated OSPW.

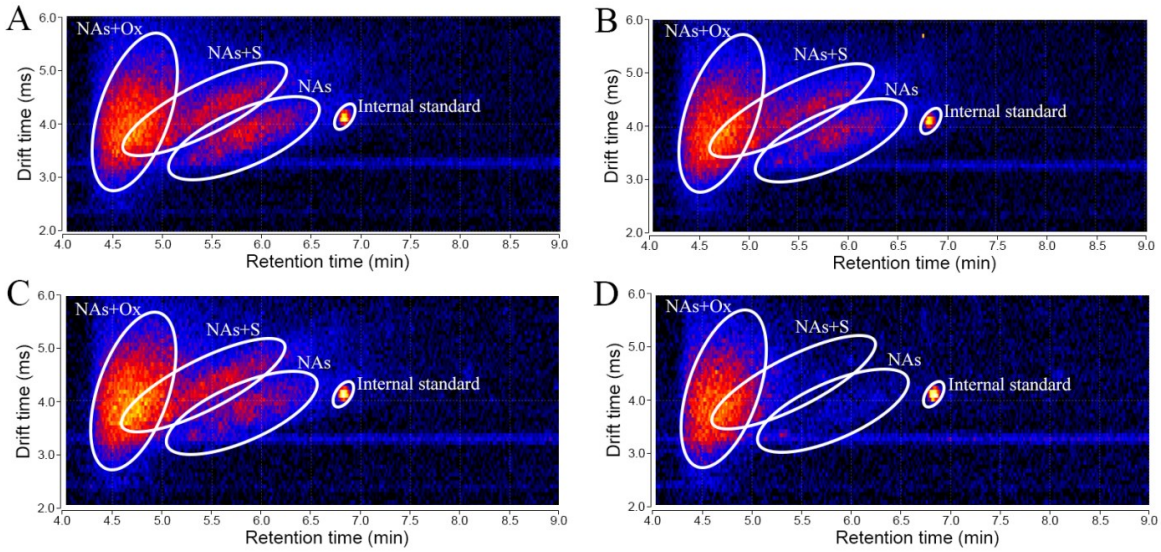

Figure 2.6. Profiles of ion mobility separation spectra for different OSPW samples. A: Raw OSPW; B: Biofiltered OSPW; C: Ozonated OSPW; D: Biofiltered ozonated OSPW.

2.3.3 Biodegradation of raw and ozonated OSPW in the biofilters

2.3.3.1 Removal of NAs from raw OSPW

Bioremediation of OSPW by biofiltration was investigated by circulating OSPW through the biofilters. After 8 times of circulation with a total HRT of 16 h, the NA removal ratio for raw OSPW was 21.8% (Fig. 2.3A, 2.5A-B). Although 34.8% and 43.1% of classical NA removals were achieved in MBBR and IFAS reactor with an HRT of 48 h, biofiltration led to lower classical NA concentration in the effluent with much shorter start-up and treatment time (Huang et al., 2015; Shi et al., 2015). Particularly, the NA removal rate for the biofiltration process was 0.18 mg/L/h, which is higher than that for MBBR (Table 2.2).

Table 2.2. Comparison of NA biodegradation in various biofilm reactors for the treatment of raw and ozonated OSPW

Biodegradation process (Reference)	HRT	OSPW	Initial NA concentration (mg/L)	NA concentration after biodegradation (mg/L)	NA biodegradation rate (mg/L/h)	NA removal by biodegradation (%)
Biofilter (This study)	16 h	Raw	13.06	10.22	0.18	21.75
		Ozonated	8.88	0.95	0.50	89.30
MBBR (Shi et al., 2015)	48 h	Raw	19.80	12.90	0.14	34.80
		Ozonated	6.40	4.20	0.05	34.40
IFAS (Huang et al., 2015)	48 h	Raw	25.13	14.29	0.23	43.14
		Ozonated	9.49	4.98	0.10	47.52

*Notes: A utilized ozone dose of 30 mg/L was used when ozonation was applied prior to the bioreactors.

The degradation of NAs by biofiltration was analyzed based on the -Z number and carbon number (Fig. 2.7A-B). Based on the -Z number, the NAs present in the OSPW was clustered into two main groups, with -Z number of 4, 6 and 12, 14 (Fig. 2.8A). Through the biofiltration, the dominating NAs concentration were all decreased, and the NA with the lowest cyclicity (-Z=4) showed the highest removal ratio (31.2%) (Fig. 2.7A), which is consistent with previous biodegradation study (Zhang et al., 2016). It was indicated that the classical NAs with fewer rings are easier to be degraded (Hwang et al., 2013). The NAs with carbon number of 14, 15 and 16 are the most abundance NAs species in OSPW (Fig. 2.9A). Among them, the NAs with carbon number of 14 showed the highest removal (33.5%) after biofiltration (Fig. 2.7B). To better understand how molecular carbon number and hydrogen deficiency (-Z) affected the degradation of classical NAs in the biofilter, Figs. 2.8A and 2.9-2.10 were generated based on the NA concentrations profiles. It was observed that the NAs with less cyclic rings were relatively easier to be biodegraded when the carbon number was lower than 15 (Fig. 2.9B), which was consistent with the research findings from other studies (Han et al., 2008; Xue et al., 2016b). The cyclicity (-Z) did not affect the removal of NAs with long carbon chains (n number $\geq$ 16). Thus, adsorption of biofilter might play a role in the removal of NAs with a large molecular weight (Krkosek et al., 2014). Through the analysis of NAs with same -Z number, the NAs with higher carbon number were found easier to be biodegraded as long as -Z $\leq$ 10 (Fig. 2.8B). NAs with less carbon number even increased after the treatment, the reason of which can probably be the biotransformation of NAs with higher carbon number. Other studies also found that the

biodegradation was lower for NAs with higher -Z number and lower carbon number (Xue et al., 2016b). For the recalcitrant NAs, such as the NAs with high -Z numbers (Z >10), the similar high removal effect can potentially be caused by the sorption of the biofilter as well (Krkosek et al., 2014).

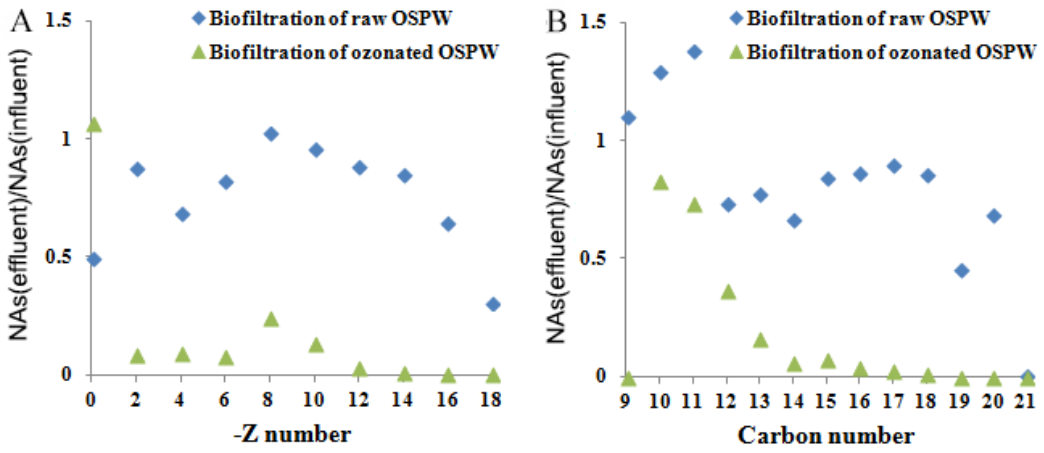

Figure 2.7. Relative removal of classical NAs after biofiltration, ozonation and combined treatment process. A: Effluent NAs/influent NAs ratio of biofilters based on -Z number; B: Effluent NAs/influent NAs ratio of biofilters based on carbon number.

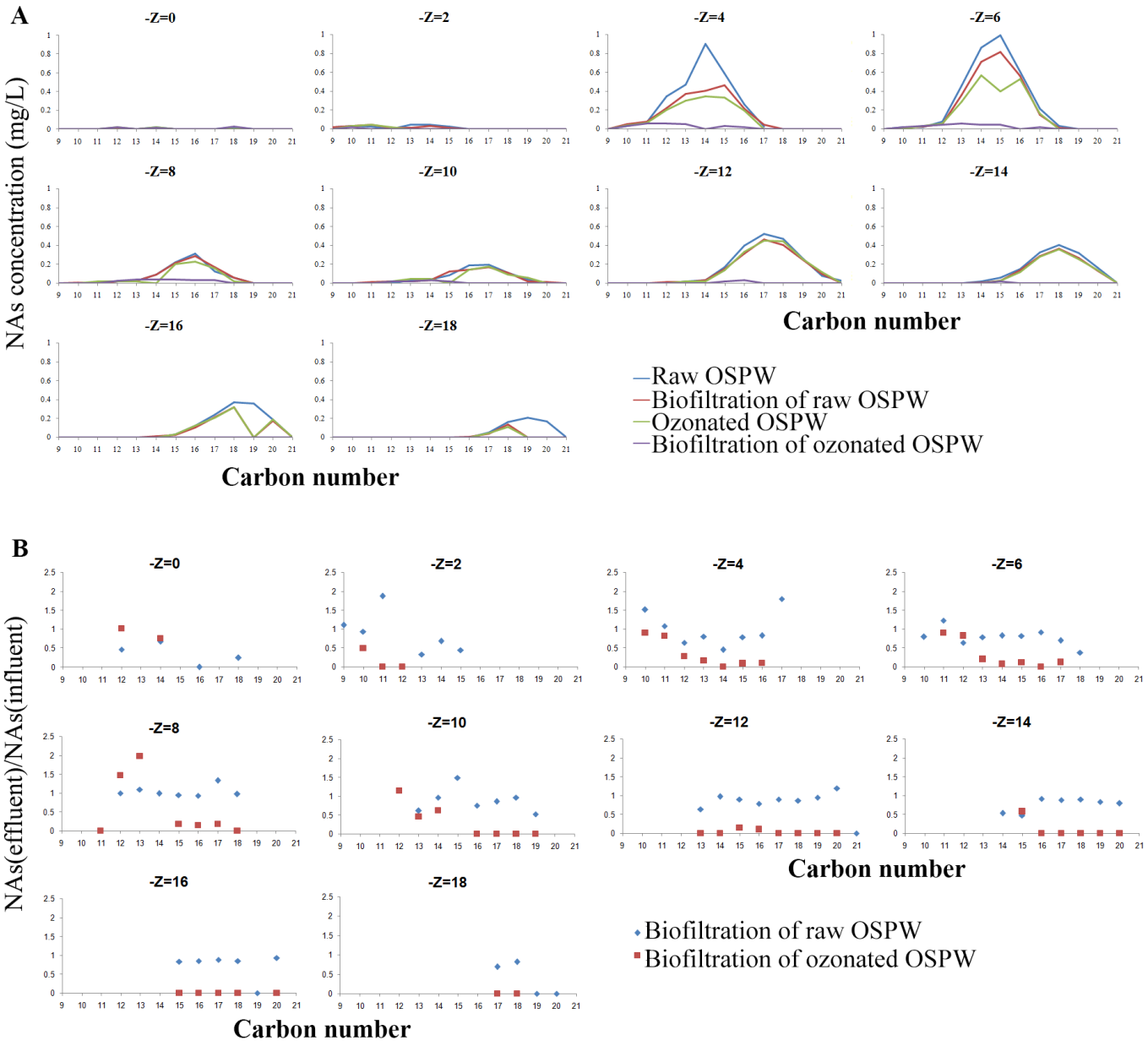

Figure 2.8. Concentration of classical NAs (A) and relative removal (B) after biofiltration, ozonation and combined treatment: impact of carbon number.

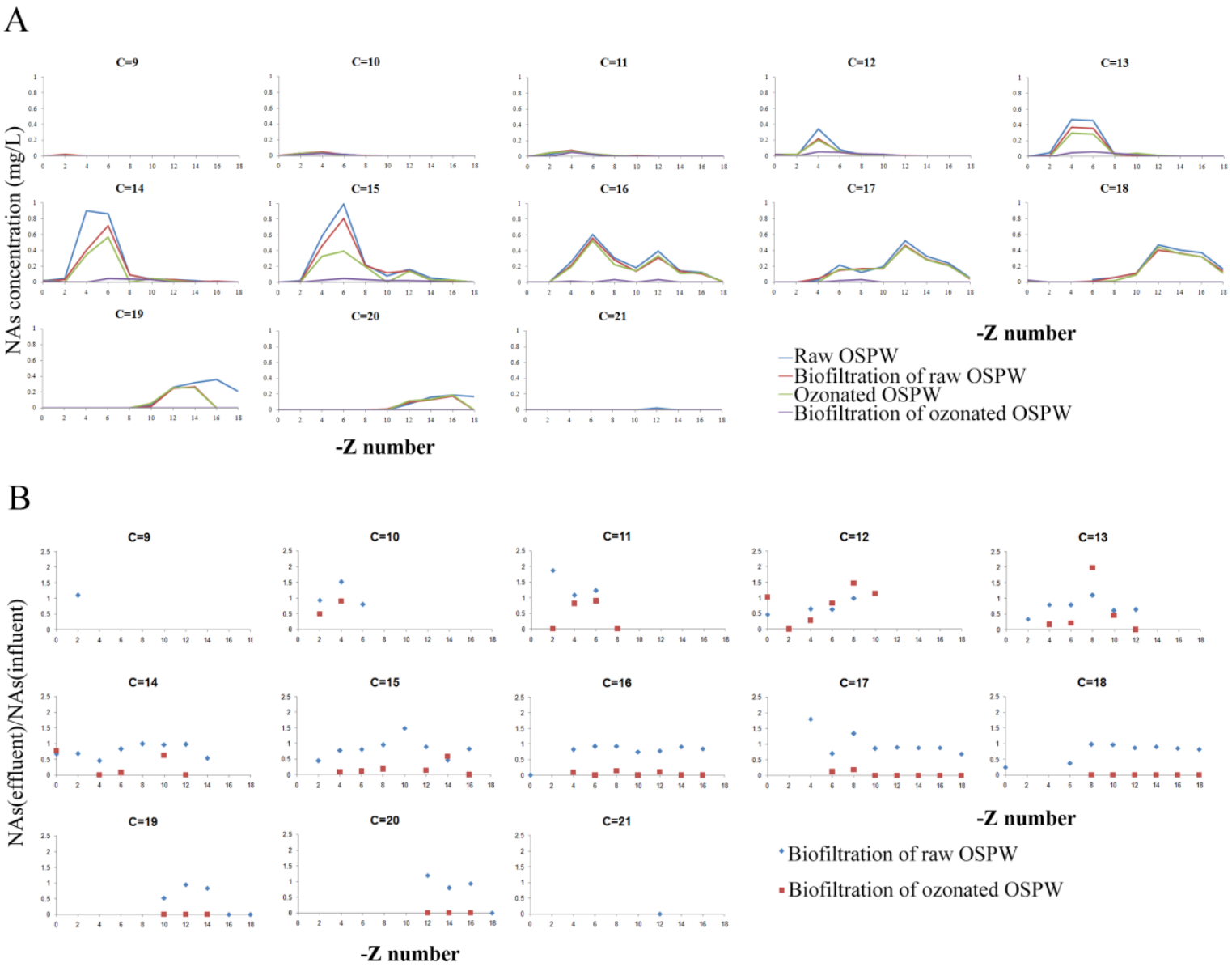

Figure 2.9. Concentration of classical NAs (A) and relative removal (B) after biofiltration, ozonation and combined treatment: impact of -Z number.

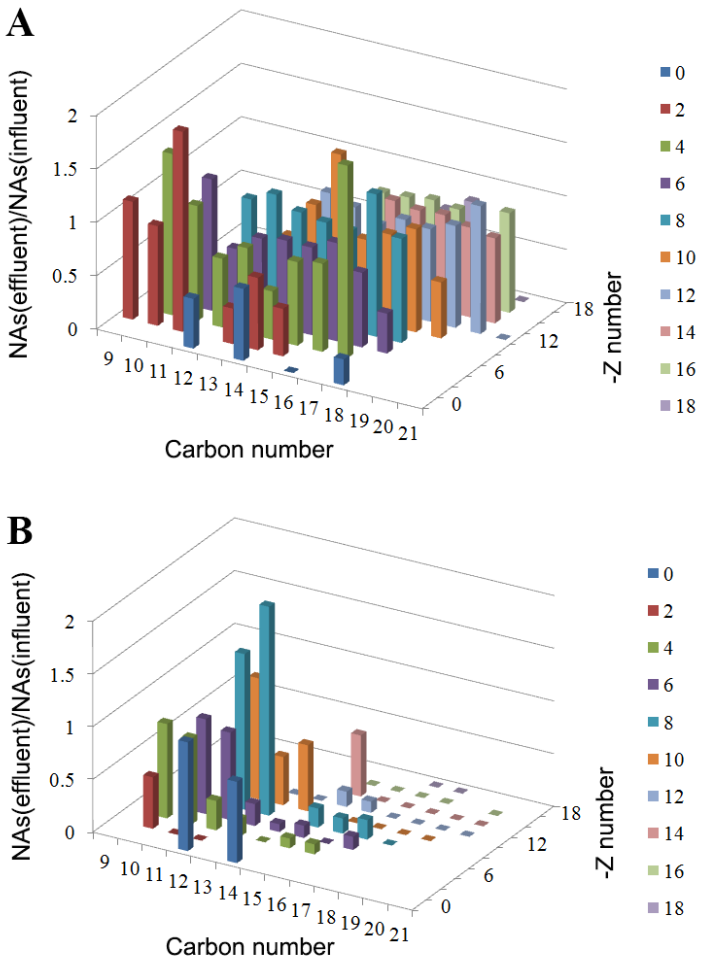

Figure 2.10. Effluent/influent ratio for classical NA species. A: Removal of different NA species from raw OSPW. B: Removal of different NA species from ozonated OSPW.

The removal of oxidized NAs was investigated as shown in Fig. 2.3A. Compared with classical NAs, the concentration of different oxidized NAs in OSPW was noticeably lower. O_3-NAs and O_4-NAs are the most abundant oxidized species present in the OSPW. It was interesting to find that the concentration of O_3-NAs was increased by 12.3% after biofiltration. Oxidized NAs were considered as the intermediates for the biodegradation of classical NAs (Han et al., 2009), which was probably the reason for the increase of O_3-NAs. The concentration of O_4-NAs decreased in this study with removal of 12.9%, which is

consistent with our previous study (Zhang et al., 2016). Because of the high cyclicity of most species of O_5-NAs and O_6-NAs, no obvious degradation was observed as described previously (Zhang et al., 2016). Through biofiltration, the concentration of the overall oxidized NAs did not change, which suggested that the oxidized NAs were more resistant to the biodegradation compared with classical NAs.

The IMS result showed that the signal density of classical and heteroatomic NAs clusters became less intensive after biofiltration. It confirmed that the biofiltration substantially reduced the concentration of classical and heteroatomic NAs in raw OSPW. However, no obvious change of the oxidized NAs was observed after biofiltration. It was found that the COD and AEF from the effluent of biofiltration did not decrease remarkably (Fig. 2.2). It suggested that the NA removal was caused by the biotransformation instead of biomineralization, which is consistent with NA biodegradation in a membrane bioreactor (Xue et al., 2016b). Due to low removal through biofiltration, physical or chemical pretreatment is needed to enhance the treatment efficiency.

2.3.3.2 Removal of NAs from ozonated OSPW

Biodegradation of ozonated OSPW by biofiltration was investigated by circulating OSPW through the biofilter as well. It was interesting to find that the biofiltration could remove 89.3% of classical NAs from the ozonated OSPW (Fig. 2.3B). Through the combination of mild ozonation and biofiltration, 92.7% of the classical NAs from the raw OSPW were removed (Fig. 2.5A, D). The NA removal was further analyzed according to the carbon and -Z numbers (Fig. 2.7). Similar with the treatment of raw OSPW by using biofilter, the removal trend for ozonated OSPW also showed that NAs with lower -Z

number and higher carbon number had relatively higher removal ratio when carbon number and -Z number was smaller than 15 and 12, respectively (Fig. 2.8-2.9). Similar high removals (>90%) were observed for NAs with a large molecular weight (carbon number ≥15 and -Z ≥ 12), which might be attributed to biofilter adsorption. Although ozonation or biofiltration alone was not able to remove oxidized NAs, the ozonation followed by biofiltration reduced the total oxidized NAs by 41.7%. It can also be noticed that the removal of dominating O_3-NAs and O_4-NAs species in OSPW were 52.6% and 47.2%, respectively (Fig. 2.3B). The molecular structure of NAs was associated with the biodegradability and the rate of biodegradation (Misiti et al., 2013; Smith et al., 2008). It was reported that the internal structure of NAs could contribute to the recalcitrance of NAs substantially, including a higher degree of alkyl-substituted aliphatic chains (Han et al., 2008; Smith et al., 2008), tertiary substitution at positions other than the β-position to the carboxylic acid of the main carbon chain, methyl substitution on the cycloalkane rings (Herman et al., 1993; Smith et al., 2008), increased cyclicity (Han et al., 2008), evenness of the carbon side chain, and cis-isomerism in alicyclic acids (Headley et al., 2002; Holowenko et al., 2002). Our study showed that ozone had the capacity to decompose long carbon chains and cyclic carboxylic fractions of NAs, which were also the most recalcitrant fractions in OSPW. In addition, compounds with specific functional groups (aromatic rings, unsaturated hydrocarbons, etc.) are prone to ozone attack, while other compounds (saturated hydrocarbons, alcohols, aldehydes, etc.) which are readily degradable by microorganisms, are considered to be resistant to ozone attack (Glaze, 1987). Since there was still a substantial amount of classical NAs with the same carbon numbers and cyclicity after the ozonation, it indicated that mild ozonation pretreatment might also change the

biodegradability of NAs through internal structure transformation. Previous studies demonstrated that the molecular structure of NAs was associated with the biodegradability and the rate of biodegradation as well (Misiti et al., 2013; Smith et al., 2008). The internal structure change of NAs can be explored further in future studies through the functional group analyses by using Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), and so on. The IMS detection also showed that only minimal classical and heteroatomic NAs remained after combined treatment. Furthermore, the signal density of oxidized NAs also decreased after biodegradation, showing the advantages of the combined treatment (Fig. 2.6C-D).

Through the pretreatment of OSPW by mild ozonation, it was interesting to find that the NA removal percentage of the biofiltration process could increase from 21.8% to 89.3% while the removal rate of which could improve from 0.18 to 0.50 mg/L/h (Table 2.2). As in previous publications, this study further confirmed that the ozonation pretreatment can improve and accelerate the biodegradation of OSPW (Shi et al., 2015; Zhang et al., 2016). With the circulation of OSPW on the biofilter, there was no NA removal after 4 circulation times for raw OSPW (Fig. 2.3A). However, there was additional NA removal for ozonated OSPW. It was indicated that more biodegradable NA species were generated by the ozonation, although more contact time was needed for the degradation of those new generated NA species. Based on the -Z number, the biofiltration of ozonated OSPW can degrade the cyclic NAs by more than 95% (Fig. 2.7A). Based on the carbon number, the biofiltration showed high removal for NAs with carbon number from 14 to 21 but showed less removal ratio to NAs with carbon number from 10 to 13 (Fig. 2.7B). Compared with the treatment of raw OSPW, biofilter showed higher removal ratio to the NAs with higher

carbon number and cyclicity in ozonated OSPW (Fig. 2.8-2.9, 2.10B,). It suggested that the ozonation pretreatment could probably benefit the biodegradation of NAs with highly branched and cyclic carboxylic structures.

Previous studies showed that NA removal in OSPW increased from 34.8% to 78.8% when ozone was used as the pretreatment for the MBBR (Shi et al., 2015). For the IFAS, an NA removal of 80.2% was reached when combined treatment process was applied (Huang et al., 2015). In this study, more than 92% classical NAs were removed with an ultimate classical NA concentration of 1.0 mg/L by ozonation followed by biofiltration. Furthermore, the NA removal rates for different biofilm-based treatment processes were compared in Table 2.2. It was found that the biofilter established in this study showed the highest NA removal rate, which was 10 times and 5 times of the NA removal rate of MBBR and IFAS, respectively (Huang et al., 2015; Shi et al., 2015). It was confirmed that ozone pretreatment accelerated the NA removal in the sand-based biofilter compared to MBBR and IFAS. It was reported that the sheering force of the bioreactor could impact the microbial community structure, reduce the biofilm diversity and slow down the maturation of biofilm (Rochex et al., 2008). The result of this study indicated that the microbial community in the biofilm formed under lower sheering force (e.g. on the biofilter) could degrade the NAs from the ozonated OSPW more effectively.

2.3.4 Microbial community analysis

Target fragment on 16S rDNA from the bacteria was amplified and sequenced by using MiSeq technology and then applied for microbial community investigation. From the phylum level, the dominant phyla were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* in

indigenous microbial community with the percentage of 62.9%, 10.5%, and 4.7%, respectively (Fig. 2.11A-M1). After the biofiltration of raw OSPW, the dominating phyla on the biofilter was *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* with the percentage of 36.6%, 33.7%, 10.7%, respectively (Fig. 2.11A-M2). Biofiltration of ozonated OSPW resulted in the same dominating bacteria phyla in the biofilter with the percentage of 50.4%, 14.2%, 12.2%, respectively (Fig. 2.11A-M3). The dominating *Proteobacteria* was also observed in an MBBR and membrane bioreactor for the treatment of OSPW (Islam et al., 2015; Shi et al., 2015; Xue et al., 2016b). In addition, *Rhodococcus* was the most dominant bacterial genus in biofilters treating both raw and ozonated OSPW, which accounted for 30.3% and 11.6% of the total bacterial genus in biofilters, respectively (Fig. 2.12A-B). In contrast, only 0.7% of *Rhodococcus* was detected in the indigenous microbial community of OSPW (Fig. 2.12A-B). Compared with the abundance of *Rhodococcus* in the indigenous microbial community, it was interesting to find that the abundance of *Rhodococcus* on the biofilter increased more than 40 times after the treatment of raw OSPW, and more than 10 times after the treatment of ozonated OSPW. *Rhodococcus* has been regarded as an active oil degrader and can be used for the biodegradation of other organic compounds such as alkanes and trichloroethene (Sharma and Pant, 2000; Suttinun et al., 2010). The remarkable increase of *Rhodococcus* after biofiltration might indicate that the *Rhodococcus* played a key role in the biodegradation of the NAs during the biofiltration. Previously, the relative abundance of *Rhodocyclales* in a membrane bioreactor also showed a positive correlation with classical NAs (Xue et al., 2017). In addition, dominating of *Rhodococcus* may be also associated with its ability of biofilm formation on the filter media and their capacity of using other organic matters in OSPW (Orr et al., 2004;

Schreiberová et al., 2012). However, further study needs to be done to confirm the correlation between *Rhodococcus* and NA degradation. Furthermore, the biodegradation of the NAs from the OSPW should be the result of the synergistic effect of the whole microbial community. With the investigation of the metatranscriptomics of the microbial community by using next-generation sequencing technology will probably give further detailed mechanisms for the NA biodegradation.

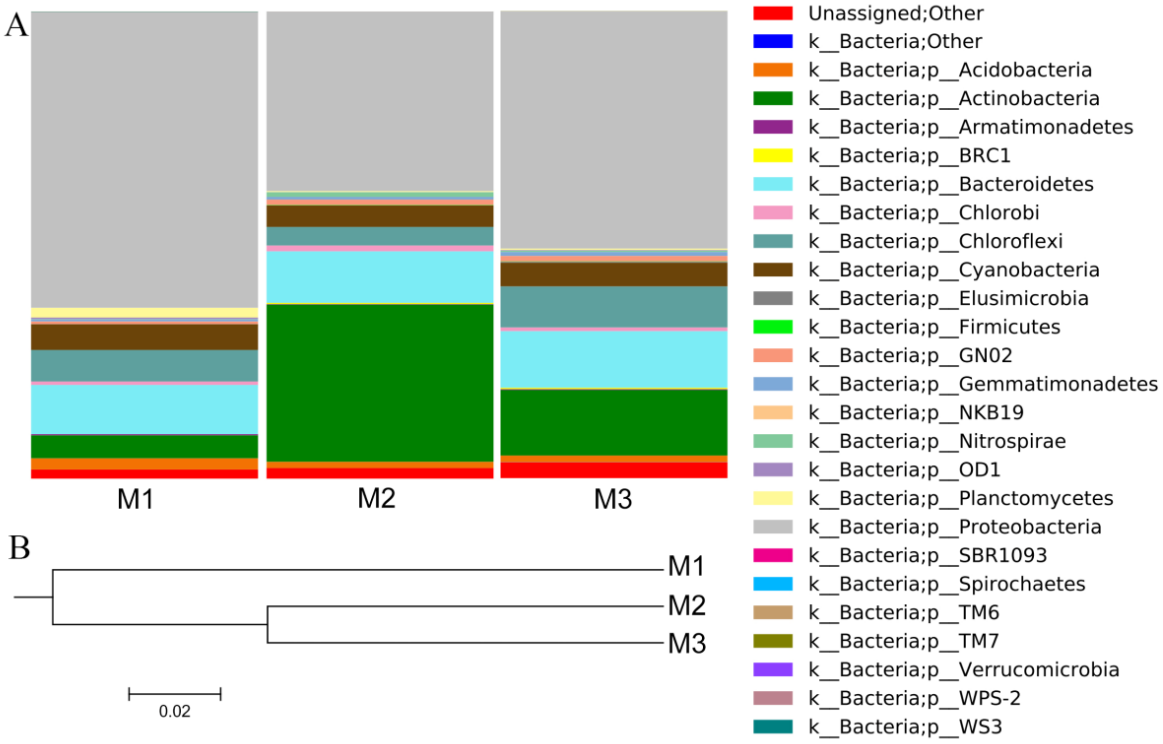

Figure 2.11. Abundance of bacterial phyla from different microbial communities and phylogenetic analysis. A: Abundance of bacterial phyla from the microbial community in raw OSPW (M1), from microbial community on the biofilter after raw OSPW treatment (M2), and from microbial community on the biofilter after ozonated OSPW treatment (M3); B: Hierarchical clustering of different microbial communities based on UPGMA.

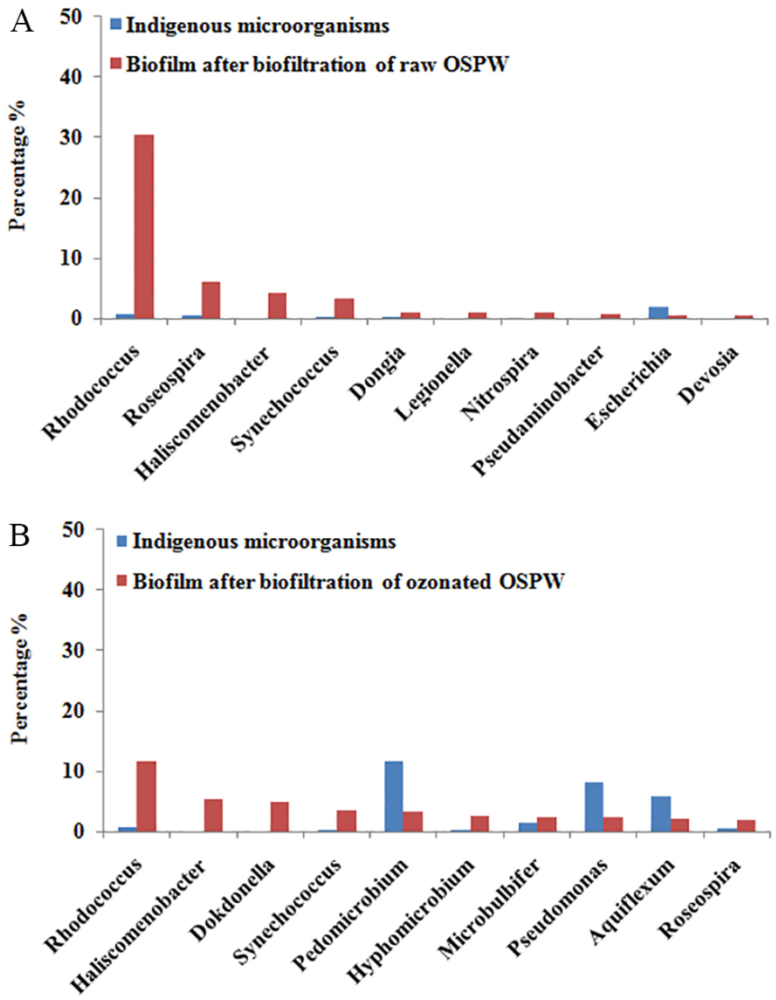

Figure 2.12. Dominant bacterial genera. A: Comparison of dominant bacterial genera in the biofilter after raw OSPW treatment with the indigenous microbial community in OSPW; B: Comparison of dominant bacterial genera in the biofilter after ozonated OSPW treatment with the indigenous microbial community in OSPW.

The phylogenetic analysis showed that the microbial communities in the biofilters after raw and ozonated OSPW treatment had the shortest genetic distance, whereas the indigenous microbial community was located in a separated branch (Fig. 2.11B). The diversity analysis showed that the Shannon indices for the indigenous microbial community, microbial communities after raw OSPW treatment and combined OSPW

treatment were 7.31, 6.33 and 7.15, respectively. The higher Shannon diversity may contribute to the higher efficiency on the bioremediation of the ozonated OSPW. The Chao1 richness index of those microbial communities was 3643.9, 2495.4 and 1888.6, respectively. Compared with the indigenous microbial community, the decreased richness indicated that only bacterial species that could adapt to the new environment of the biofilter survived ultimately.

2.4 Conclusions

Biofiltration system which utilized indigenous microorganisms was developed successfully for the treatment of OSPW. Through the combination of ozonation and biofiltration, 92.7% of the classical NA removal was achieved with less than 1 mg/L of NAs in the treated water. Compared with other biofilm reactors established previously, ozonation substantively accelerated the NA removal in our fixed-bed biofilm reactors (biofilters), especially for those recalcitrant NAs with high molecular weight. The advantage of the combined treatment also consisted in the removal of oxidized NAs compared with ozonation or biofiltration alone. The *Rhodococcus* was the dominant bacterial genus on the biofilter after OSPW treatment, which might play a critical role in the NA biodegradation. Considering that the biofiltration uses gravity as part of energy source and readily available sand as media, the biofiltration possesses remarkable advantages such as low energy cost and low capital demand. The fixed-bed biofilm reactor shows high potential to be scaled up and applied for the *in-situ* treatment of OSPW.

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CHAPTER 3. REMOVAL OF CYCLIC AND ALIPHATIC NAPHTHENIC ACIDS FROM OIL SANDS PROCESS WATER BY A BIOFILTRATION-OZONATION-BIOFILTRATION PROCESS WITH ENHANCED OZONATION EFFICIENCY[1]

3.1 Introduction

Hundreds of billion barrels of recoverable bitumen have been found deposited in Alberta, Canada (Teare et al., 2014). A large volume of oil sands process water (OSPW) which contains dissolved organic matter as principle pollutants have been generated with the production of a huge amount of bitumen (Mahaffey and Dubé, 2017). As a result of lacking proper treatment methods, OSPW is temporarily stored in local tailings ponds and has shown a serious threat to the local environment and public health (Pramanik, 2016). Among all the contaminants in OSPW, naphthenic acids (NAs) have been found to be the main toxic constituent (Garcia-Garcia et al., 2011; Goff et al., 2013; Zhang et al., 2011). NAs are one of the main organic compounds present in OSPW, which are referred to as alkyl-substituted acyclic and cycloaliphatic carboxylic acids (Jones et al., 2013; Wang et al., 2016). The general chemical formula of NAs is $C_nH_{2n+Z}O_x$, where n is the number of carbon atoms, Z is related to the hydrogen deficiency caused by the formation of the ring or double bond structure, and x refers to the number of oxygen atoms present in the NAs.

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[1] A version of this chapter will be submitted to a peer-review journal: Zhang, L., Zhang, Y. and Gamal El-Din, M. Removal of cyclic and aliphatic naphthenic acids from oil sands process water by a biofiltration-ozonation-biofiltration process with enhanced ozonation efficiency. 2019, Science of the Total Environment.

Classical NAs (O_2-NAs, x = 2) have been considered the most toxic organic compound from the SPW (Morandi et al., 2015). Compared with classical NAs, oxidized NAs ($x \geq 3$) are supposed to show less affinity to cell membrane due to their increased hydrophilicity, which may contribute to their reduced toxicity (Wang et al., 2013; Zhang et al., 2016). Because of their toxicity, the removal of NAs has always been considered as one of the most important targets of OSPW treatment (Huang et al., 2015).

With clear advantages of low cost and little environmental impact, biological wastewater treatment methods have been studied for the degradation of NAs in OSPW. Moving bed biofilm reactors (MBBR) were developed and used for NA degradation (Shi et al., 2015). After the operation of MBBR for 210 days, the OSPW treatment process removed 18.3% of acid-extractable fraction (AEF) and 34.8% of NAs from raw OSPW. Besides, integrated fixed-film activated sludge (IFAS) reactors were established and applied for OSPW treatment as well. With the continuous operation of the IFAS for 11 months, 12.1% of the AEF and 43.1% of the NAs were removed by the IFAS process (Huang et al., 2015). Research on the application of membrane bioreactor for OSPW treatment has also been conducted. The membrane bioreactor was run for 425 days and achieved 24.7% of NA removal (Xue et al., 2016b). According to the results of those biological treatment processes, it can be observed that the start-up of bioreactors often took a long time and their ability to remove NAs was limited, which is attributed to the biopersistence of the NAs from OSPW. Apparently, the efficiency of biological OSPW treatment is required to be further improved before it can potentially be used by the industry.

Ozonation pretreatment was found to be able to improve the bio-removal of NAs from OSPW (Martin et al., 2010). It was confirmed that mild ozonation (utilized ozone dose of 30 mg/L) followed by MBBR treatment process could remove the AEF and NAs from OSPW by 41.0% and 78.8%, respectively (Shi et al., 2015). By using mild ozonation followed by IFAS treatment process, 42.0% of AEF and 80.2% of NAs were removed from OSPW (Huang et al., 2015). In addition, mild ozonation combined membrane bioreactor treatment achieved 70% of NA removal from OSPW (Zhang et al., 2016). According to our previous study, the mild ozonation combined biofiltration process can remove 92.7% of classical NAs from raw OSPW (Zhang et al., 2018).

Previous studies reported that ozone preferentially reacts with the NAs with higher -Z and carbon number (Martin et al., 2010; Zhang et al., 2016). It was interesting to find that NAs with lower -Z number were preferentially biodegraded (Xue et al., 2016b; Zhang et al., 2016). Removing the NAs with low cyclicity and other biodegradable organic compounds in OSPW through biodegradation pretreatment may enhance the removal efficiency of NAs by ozonation. Hence, biological pretreatment followed by ozonation process may potentially provide a promising alternative to reduce the ozone dose, maintaining the same NA removal efficiency. However, no study has been conducted yet to investigate the effectiveness of biodegradation followed by ozonation treatment for OSPW reclamation.

Biological treatment followed by advanced oxidation processes (AOPs) treatment processes has been studied for the treatment of contaminants of emerging concerns (CECs) (Li et al., 2015; Sirtori et al., 2009). It was reported that micropollutants such as antibiotics and endocrine-disrupting chemicals were significantly removed from municipal wastewater

by a denitrification biofilter, ozonation and biological aerated filtration treatment process (Li et al., 2015). Immobilized biomass reactor followed by solar photo-Fenton AOP was also evaluated for the treatment of pharmaceutical wastewater. It was observed that nalidixic acid in the wastewater was totally degraded and the toxicity of the wastewater to *Vibrio fischeri* was decreased (Sirtori et al., 2009). It was also reported that high removal of organic carbon content from the wastewater of dye house was achieved through the biodegradation-ozonation-biodegradation treatment process (Ledakowicz et al., 2017). The above studies indicate the potential of biofiltration followed by ozonation for OSPW treatment.

During our previous study on the application of biofiltration for OSPW treatment, the biofilter system was developed (Zhang et al., 2018). The main objective of this study was to evaluate the effect and efficiency of a biofiltration-ozonation-biofiltration process on the NA removal from OSPW. The impact of biofiltration pretreatment on the ozonation of NAs was explored. In addition, the performance of the biofiltration processes as a pretreatment and post-treatment of ozonation for the degradation of NAs was investigated. The microbial community structure and functional profiles in the biofiltration system were investigated by using metagenomic sequencing method, the results of which can be used to elucidate the potential mechanisms for NA biodegradation.

3.2 Materials and methods

3.2.1 Source of OSPW

Raw OSPW samples were collected from an active oil sands tailings pond in Fort McMurray, Alberta, Canada. Prior to being used in this research, the OSPW samples were stored in 200 L polyvinyl chloride barrels in a cold room at 4 °C (Huang et al., 2015; Zhang et al., 2016). The characteristics of raw OSPW are shown in Table 3.1.

Table 3.1. Characterization of OSPW before and after biofiltration-ozonation-biofiltration

Parameter	Raw OSPW	Treated OSPW
Total organic carbon (mg/L)	37.6 ± 0.8	25.7 ± 0.4
Chemical oxygen demand (mg/L)	114.5 ± 3.5	91.1 ± 4.0
Turbidity (NTU)	27.9 ± 0.8	0.0 ± 0.0
Classical NAs (mg/L)	13.1	1.4
Oxidized NAs (mg/L)	15.3	10.1
pH	8.3	8.8
Na^+ (mg/L)	246.2 ± 3.2	280.1 ± 0.3
K^+ (mg/L)	12.3 ± 0.2	15.0 ± 0.1
Mg^{2+} (mg/L)	11.3 ± 0.2	12.8 ± 0.0
Ca^{2+} (mg/L)	22.8 ± 0.3	17.8 ± 0.4
SO_4^{2-} (mg/L)	167.5 ± 0.3	175.3 ± 1.8
Cl^- (mg/L)	108.1 ± 0.3	110.9 ± 1.1
NO_2^--N (mg/L)	6.4 ± 0.0	6.8 ± 0.0
NO_3^--N (mg/L)	6.2 ± 0.0	5.8 ± 0.0
Acute toxicity towards *Vibrio fischeri*	32.9 ± 1.4	29.4 ± 2.6

3.2.2 Ozonation

Pure oxygen was applied to generate ozone by an ozone generator (GSO-40, WEDECO, Herford, Germany). In this study, ozone was fed into a 4L OSPW in a glass reactor with the flow rate at 1 L/min. The wet test meter (Precision Scientific Petroleum Instruments, Texas, USA) was applied to control the flow rate of ozone. Ozone monitors (HC500 Ozone monitor, WEDECO, USA) were used for the detection of feeding and off letting ozone concentration. Based on the quantity of ozone measured in the feed and off-gas lines, utilized ozone dose could be calculated. Ozone was well mixed and interacted with OSPW water sample through the gas diffuser and stir mixer. After ozonation treatment, purified nitrogen gas was used for the removal of residual ozone in OSPW (Gamal El-Din et al., 2011; Huang et al., 2015; Wang et al., 2016).

3.2.3 Biofilter system development and operation

The biofilter system was developed as described previously (Zhang et al., 2018). Briefly, pure sand (40-100 mesh) (Acros Organics, New Jersey, USA) was used as the media of biofilter. Raw OSPW was applied to go through the bioreactor for the development of indigenous microorganisms based biofilter at room temperature. The empty bed contact time of the biofilter was 120 minutes with influent flow rate at 0.61 mL/min. As our previous study showed that the established biofilter could complete NA biodegradation after 4 times of circulations, the biofiltration of OSPW in this study was performed by circulating (4 times) OSPW through the established biofilter at the same flow rate (Zhang et al., 2018).

3.2.4 Biofiltration-ozonation-biofiltration process

In order to investigate the impact of biofiltration pretreatment on the ozonation of OSPW, the biofiltrated OSPW samples were collected and used for ozonation with an utilized ozone dose of 30 mg/L. After that, the same biofiltration process was applied for the ozonated OSPW, the results of which were used to confirm the benefit of ozonation as an intermediate process and compare the performance of biofiltration as a pretreatment against a post-treatment process.

3.2.5 NA analysis

The NAs present in OSPW sample were further analyzed by using ultra performance liquid chromatography/time-of-flight mass spectrometry (UPLC-TOFMS) (Sun et al., 2014). Briefly, 0.5 mL filtered water sample was mixed with 0.4 mL methanol and 0.1 mL myristic acid-1-^{13}C (4.0 mg/L), which was used as an internal standard before injection. The LC-MS data were analyzed by using the TargetLynx® Ver. 4.1 software for the quantification of NA species (Xue et al., 2016b; Zhang et al., 2016).

3.2.6 Microbial characterization

The triplicate sand media samples for microbiological analyses were collected from the top layer of the fixed-bed biofilm reactor, which was within 1 cm of the depth of sand media. The thickness and distribution of the biofilm on sand media surface were characterized by using fluorescent staining (SYTO 9 for bacterial cells and Texas red conjugated Concanavalin A for polysaccharides, Molecular Probes, USA) in conjunction with confocal laser scanning microscopy (CLSM, LSM 710, Carl Zeiss Micro Imaging GmbH, Germany) (Xue et al., 2016a). According to manufacturer instructions, bacterial

DNA was extracted from sand media by using PowerSoil® DNA isolation kit (Mo-Bio Laboratories, Inc., CA, USA). The extracted DNA samples were then applied for the quantification of total bacteria copies on sand media by qPCR test (Zhang et al., 2016). In addition, Illumina MiSeq sequencing was performed for the metagenomic analyses of microbial communities in the biofiltration systems (Huang et al., 2017). Quantitative Insights Into Microbial Ecology (QIIME, http://qiime.org) pipeline was used for metagenomic sequencing data analysis. Briefly, by using an open-reference clustering algorithm, operational taxonomic units (OTUs) were picked. The representative sequence for each OUT was picked and applied for taxonomic assignment (Huang et al., 2017; Zhang et al., 2016). The uclust consensus taxonomy classifier was used by QIIME to assign taxonomy. After taxonomic composition analysis, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was applied for the prediction of functions of microbial community (Langille et al., 2013).

3.3 Results and discussion

3.3.1 Biofilter development and biofiltration pretreatment

As described previously (Zhang et al., 2018), indigenous microorganisms based biofilter was established by passing raw OSPW through the bioreactor for three weeks. With the detection of total bacterial copies on sand media by qPCR and the observation of biofilm thickness on the media surface by using confocal microscopy, it was confirmed that indigenous microorganisms from raw OSPW could attach to the sand media surface and form biofilm on it (Fig. 3.1). After the conditions of bioreactor reached a steady state with

total bacterial copies of 2.9 × 10^9 /g sand, it was applied for the treatment of OSPW by circulating method (Fig. 3.1A).

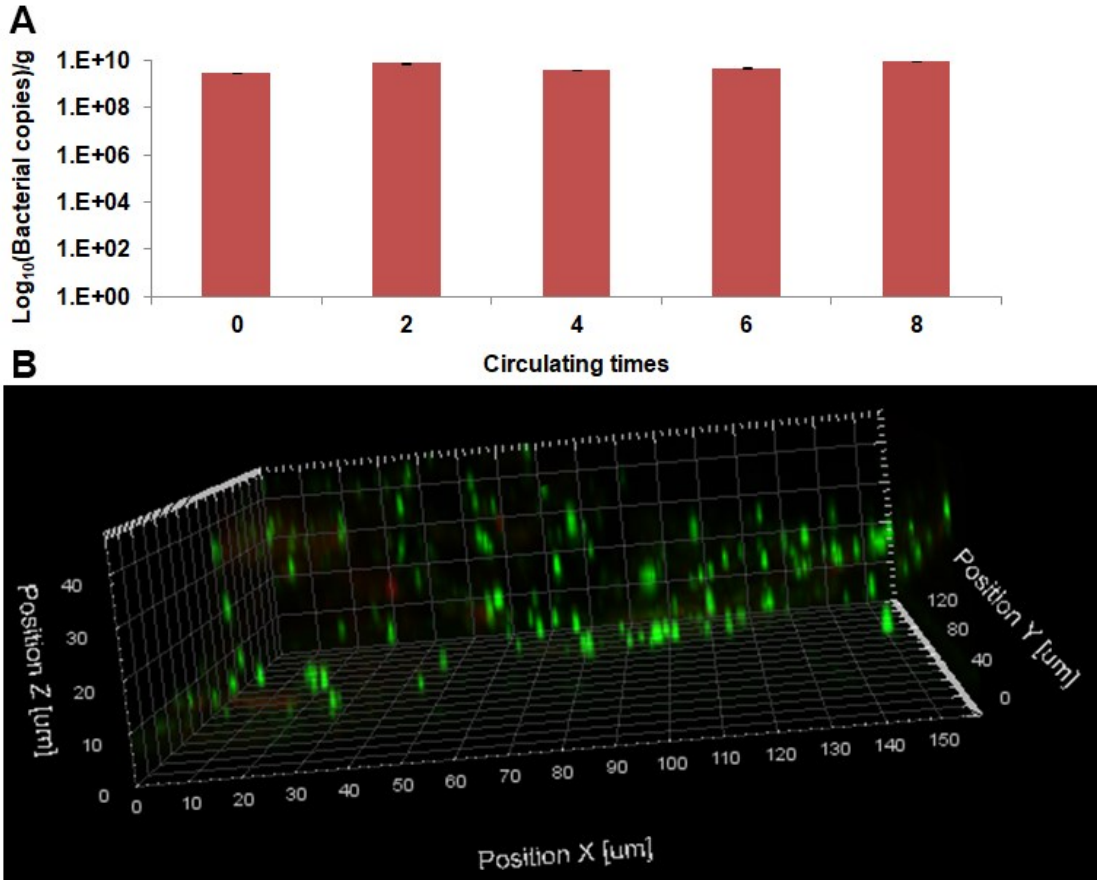

Figure 3.1. Biofilm thickness and total bacterial copy on the sand media of the biofilter. A: Total bacterial quantification by qPCR; B: Biofilm detection and thickness measurement by CLSM.

Previously, it was reported that the NAs from OSPW were persistent to biodegradation (Xue et al., 2018). By circulating OSPW through the established biofilter with an equivalent hydraulic retention time (HRT) of 8 hours, 24.4% of classical NAs could be removed from raw OSPW (Table 3.2). The classical NA removal rate for the biofiltration

process was 0.4 mg/L/h, which was higher than that for MBBR (0.1 mg/L/h) and IFAS (0.2 mg/L/h) (Huang et al., 2015; Shi et al., 2015).

Table 3.2. Removal of NAs by biofiltration-ozonation-biofiltration treatment

NA species	Biofiltration (1st stage) (HRT = 8h)		Ozonation (utilized dose =30 mg/L)		Biofiltration (2nd stage) (HRT = 8h)	
	Removal ratio (%)	Removal rate (mg/L/h)	Removal ratio (%)	Removal efficiency (mg NAs/mg O_3)	Removal ratio (%)	Removal rate (mg/L/h)
O_2-NAs	24.4	0.4	84.8	0.3	6.7	0.0
O_3-NAs	-9.9	-0.1	41.0	0.1	8.7	0.1
O_4-NAs	16.4	0.1	10.7	0.0	24.0	0.2
O_5-NAs	8.3	0.0	-109.1	0.0	34.8	0.1
O_6-NAs	0.0	0.0	-300.0	0.0	50.0	0.1
Oxidized NAs	3.3	0.1	11.5	0.1	22.9	0.4

*Notes: - = Increased.

As shown in Fig. 3.2, with the biofiltration pretreatment, the classical NAs from raw OSPW were decreased from 13.1 mg/L to 9.9 mg/L. The biodegradation of classical NAs was further analyzed according to carbon number and -Z number. NAs with -Z number of 4, 6 and 12 were found as the dominant groups in OSPW (Fig. 3.3A). Among them, NAs (–Z = 4) showed the highest removal (34.7%) and the highest degradation rate (0.12 mg/L/h) by the first round of biofiltration (Fig. 3.3B, 3.4A). It was suggested that the classical NAs with fewer rings are more biodegradable (Hwang et al., 2013). The NAs with carbon number of 14, 15 and 16 were observed as the most abundance NA species (Fig. 3.3C). The biofiltration showed the highest removal (34.1%) and the highest degradation rate (0.09

mg/L/h) for the NAs with carbon number of 14 (Fig. 3.3D, 3.4B). The results were consistent with previous OSPW bioremediation study (Zhang et al., 2018).

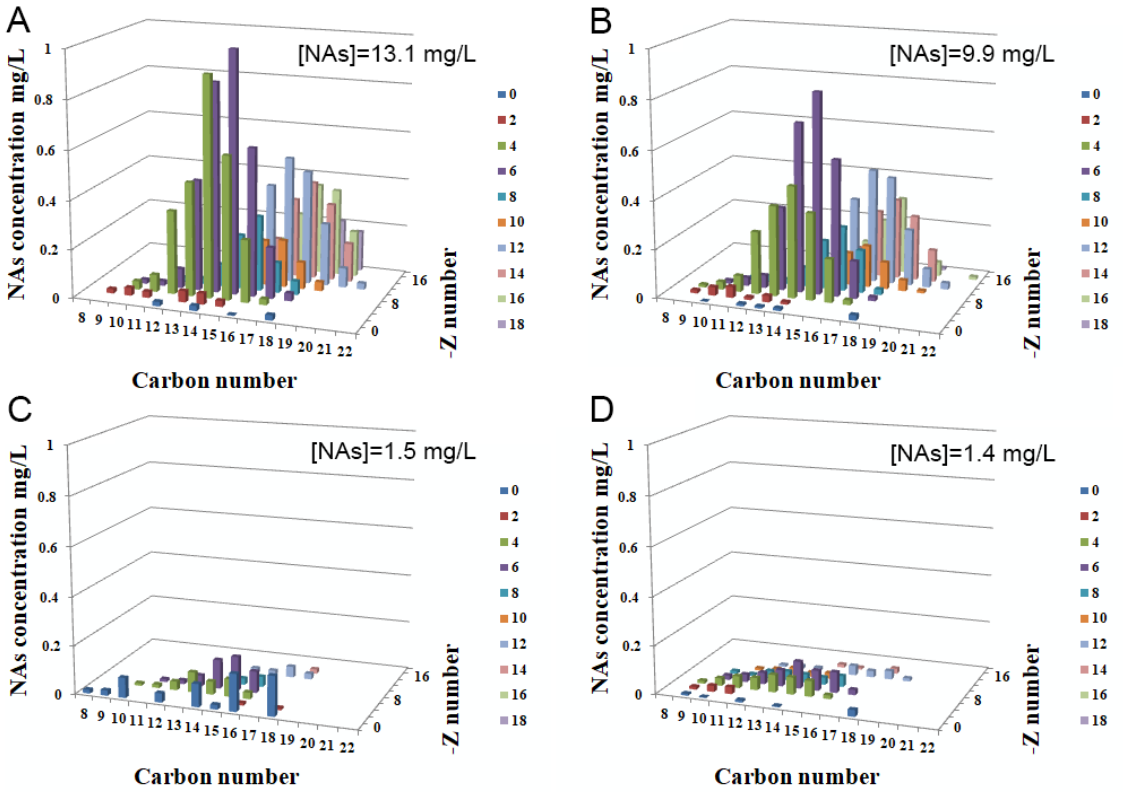

Figure 3.2. Profiles of classical NAs from different OSPW. A: Profiles of classical NAs from raw OSPW; B: Profiles of classical NAs from biofiltrated OSPW; C: Profiles of classical NAs from biofiltration-ozonation treated OSPW; D: Profiles of classical NAs from biofiltration-ozonation-biofiltration treated OSPW.

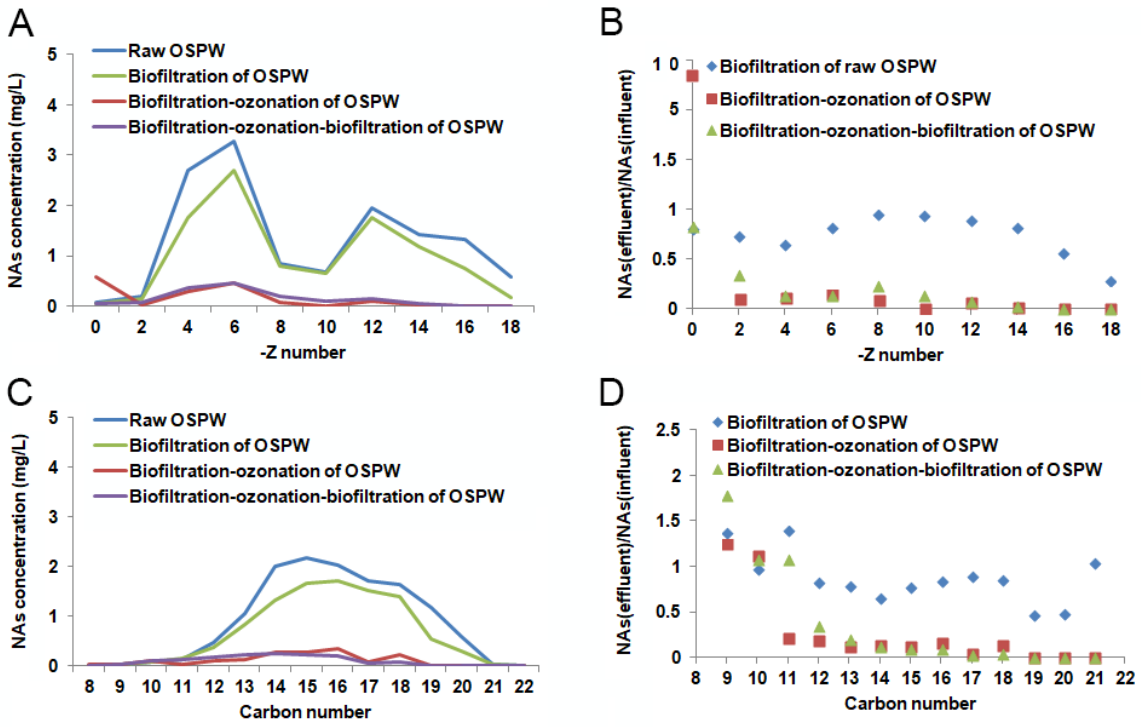

Figure 3.3. Concentration of classical NAs and relative removal after biofiltration, biofiltration-ozonation, and biofiltration-ozonation-biofiltration treatment. A: NA degradation based on -Z number; B: Effluent NAs/influent NAs ratio of biofilters based on -Z number; C: NA degradation based on carbon number; D: Effluent NAs/influent NAs ratio of biofilters based on carbon number.

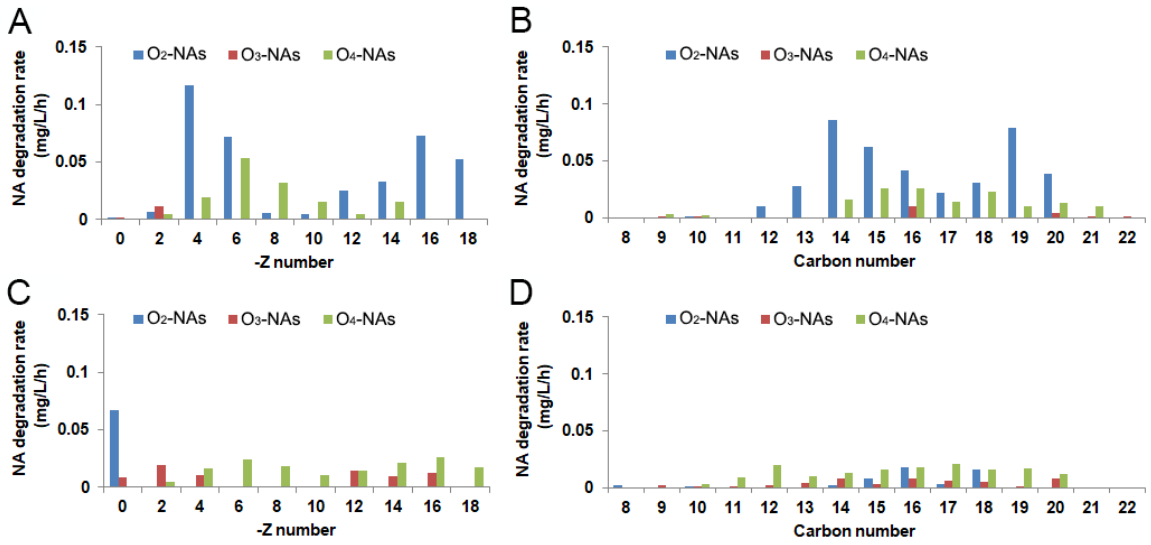

Figure 3.4. NA degradation rate of the biofiltration pretreatment and post biofiltration of OSPW. A: NA degradation rate of biofiltration pretreatment based on -Z number; B: NA degradation rate of biofiltration pretreatment based on carbon number; C: NA degradation rate of post biofiltration treatment based on -Z number; B: NA degradation rate of post biofiltration treatment based on carbon number.

The oxidized NAs showed high resistance to the biofiltration process as the concentration of overall oxidized NAs were stable (Fig. 3.5-3.8). Particularly, the concentration of O_3-NAs even increased by 9.9% after biofiltration (Table 3.1). It was reported that O_3-NAs and O_4-NAs were probably the biodegradation products of classical NAs (Ajaero et al., 2017; Xue et al., 2018). The biotransformation of classical NAs into oxidized NAs can be the reason for the increase of O_3-NAs concentration, and low bioremoval of other oxidized NAs.

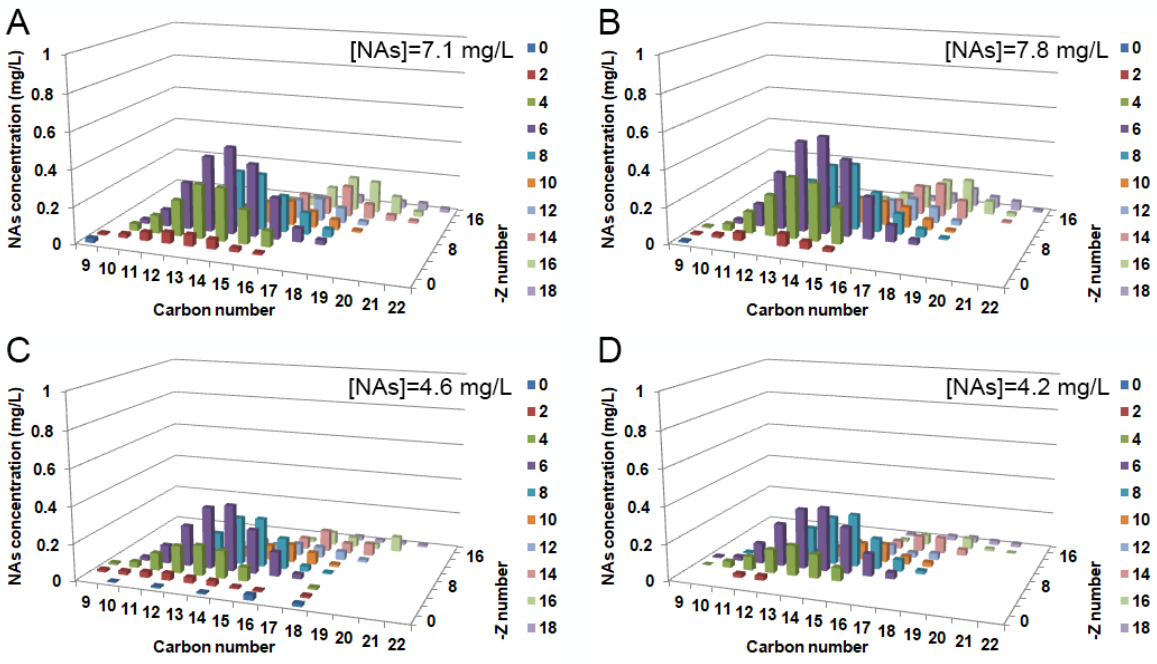

Figure 3.5. O_3-NAs concentration profiles of different OSPW. A: Profiles of O_3-NAs from raw OSPW; B: Profiles of O_3-NAs from biofiltrated OSPW; C: Profiles of O_3-NAs from biofiltration-ozonation treated OSPW; D: Profiles of O_3-NAs from biofiltration-ozonation-biofiltration treated OSPW.

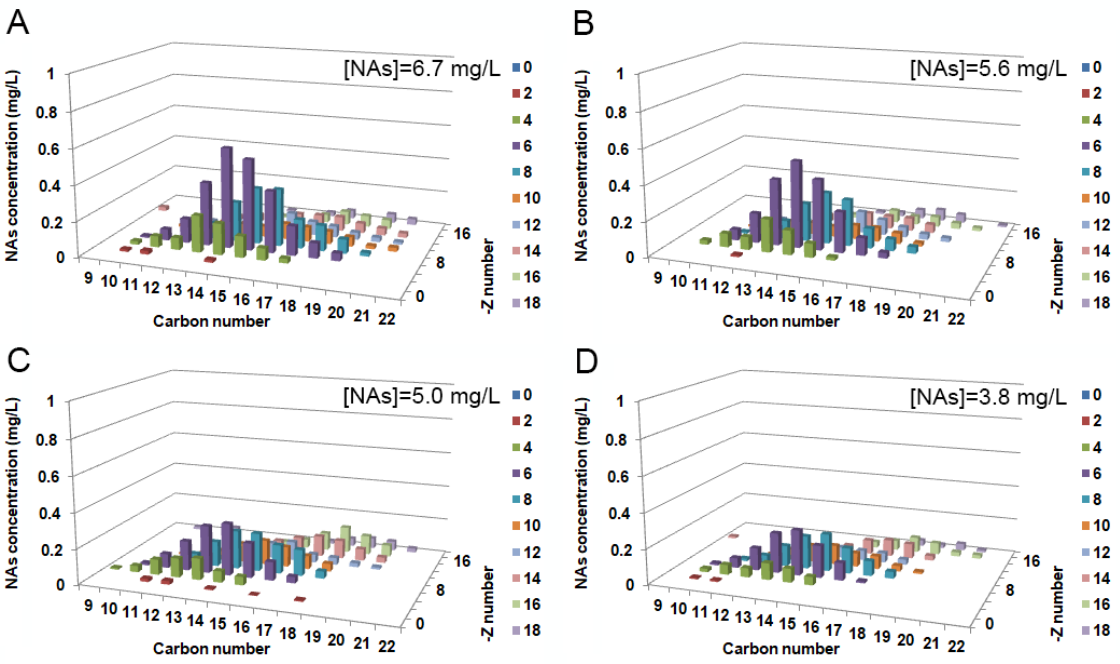

Figure 3.6. O_4-NAs concentration profiles of different OSPW. A: Profiles of O_4-NAs from raw OSPW; B: Profiles of O_4-NAs from biofiltrated OSPW; C: Profiles of O_4-NAs from biofiltration-ozonation treated OSPW; D: Profiles of O_4-NAs from biofiltration-ozonation-biofiltration treated OSPW.

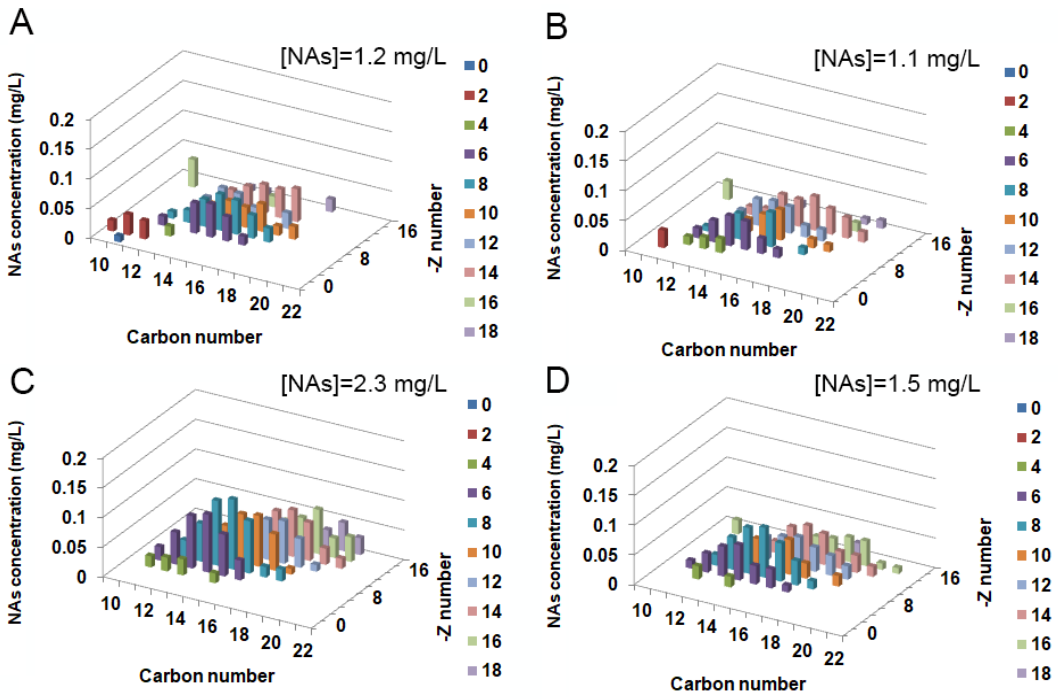

Figure 3.7. O_5-NAs concentration profiles of different OSPW. A: Raw OSPW; B: OSPW after biofiltration; C: OSPW after biofiltration-ozonation treatment; D: OSPW after biofiltration-ozonation-biofiltration treatment.

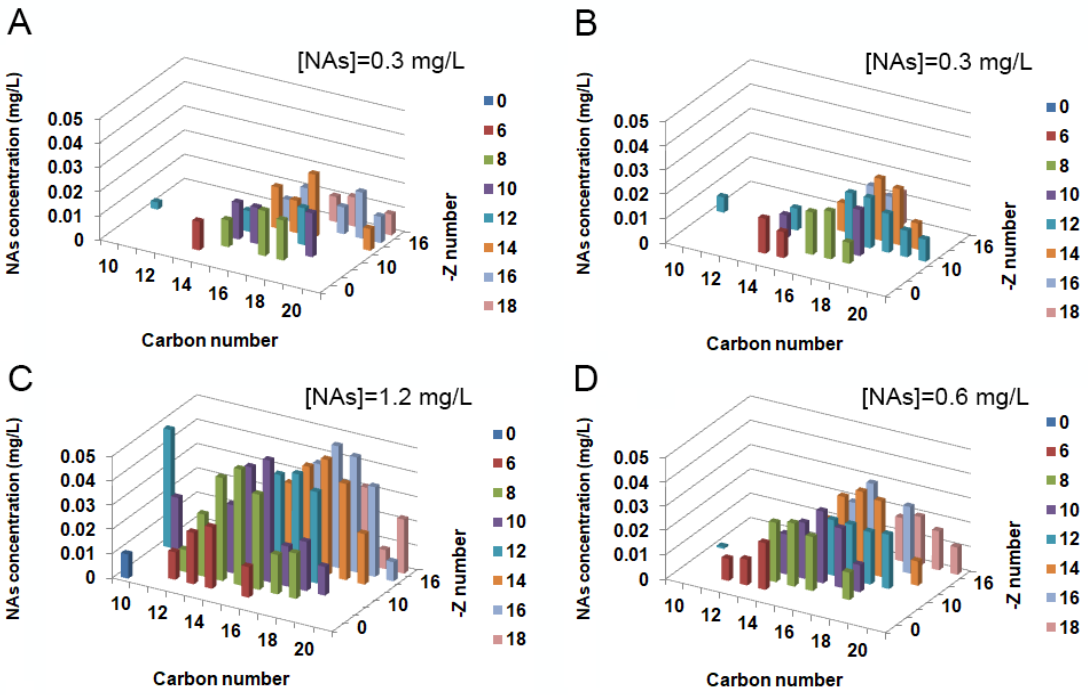

Figure 3.8. O_6-NAs concentration profiles of different OSPW. A: Raw OSPW; B: OSPW after biofiltration; C: OSPW after biofiltration-ozonation treatment; D: OSPW after biofiltration-ozonation-biofiltration treatment.

The reactive species from ozone were postulated to selectively attack the hydrogen atoms of the tertiary carbons which exist mainly in alkyl-branched and ring-bearing structures (Xu et al., 2017). After the biodegradation occurred in the biofilter, the structure and reduction state of some carbon compounds in OSPW changed, which may reduce their competition with NAs for hydroxyl radicals during ozonation and improve the degradation of NAs by ozonation (Buffle et al., 2006).

3.3.2 Ozonation of biofiltrated OSPW

After applying mild ozone with an utilized dose of 30 mg/L for the biofiltrated OSPW, it was interesting to find that the concentration of classical NAs was decreased

from 9.9 mg/L to 1.5 mg/L (Fig. 3.2). The ozonation efficiency reached 0.3 mg classical NAs/mg O_3 (Table 3.2), which was higher than the ozonation efficiency (0.1 mg classical NAs/mg O_3) for raw OSPW (Table 3.3). Besides, the concentration of the main oxidized NAs from OSPW was reduced as well. Specifically, the concentration of O_3-NAs and O_4-NAs was decreased from 7.8 mg/L to 4.6 mg/L, 5.6 mg/L to 5.0 mg/L, respectively (Fig. 3.5-3.6). However, though O_5-NAs and O_6-NAs were not the main NAs in OSPW, the concentration of O_5-NAs and O_6-NAs were increased from 1.1 mg/L to 2.3 mg/L and 0.3 mg/L to 1.2 mg/L, respectively (Fig. 3.7-3.8). It was probably caused by the oxidation of the NAs with less oxygen number.

Table 3.3. Ozonation removal of NAs from raw and biofiltrated OSPW

	NA removal ratio (%)		**NA ozonation efficiency (mg NAs /mg O_3)**	
NA species	**Ozonation of raw OSPW**	**Ozonation of biofiltrated OSPW**	**Ozonation of raw OSPW**	**Ozonation of biofiltrated OSPW**
O_2-NAs	32.1	84.8	0.1	0.3
O_3-NAs	-1.4	41.0	-0.0	0.1
O_4-NAs	9.0	10.7	0.0	0.0
O_5-NAs	8.3	-109.1	0.0	-0.0
O_6-NAs	0.0	-300.0	0.0	-0.0
Oxidized NAs	3.9	11.5	0.0	0.1

*Notes: - = Increased; A utilized ozone dose of 30 mg/L was used when ozonation was applied prior to the bioreactors.

Compared with the ozonation of raw OSPW with the same utilized ozone dose, the degradation of the classical NAs from OSPW was remarkably improved from 32.1% to 84.8% (Table 3.3). Besides, the degradation of the main oxidized NAs (O_3-NAs and O_4-

NAs) from OSPW was improved as well. The removal ratio for O_3-NAs and O_4-NAs was increased from no removal to 41.0% and from 9.0% to 10.7%, respectively (Table 3.3). With the pretreatment of raw OSPW by biofiltration, the degradation efficiency of mild ozonation on NAs was remarkably improved, confirming that biodegradation can benefit NA degradation by mild ozonation.

Through the pretreatment of biofiltration, the ozonation efficiency on the removal of dominant NAs with the same cyclicity (-Z = 4, 6, 12) reached 0.05, 0.08, and 0.06 mg NAs/mg O_3 (Fig. 3.9). Correspondingly, the removal ratio for those NAs reached 83.0%, 83.4%, and 94.0%, respectively (Fig. 3.10). However, the ozonation of raw OSPW could only remove 45.0%, 37.9%, and 9.7% of those NAs with the ozonation efficiency of 0.04, 0.04, 0.01 mg NAs/mg O_3, respectively (Fig. 3.9-3.10). On the other hand, through the ozonation of biofiltrated OSPW, the ozonation efficiency on the removal of dominant NAs with the same carbon number (n = 14, 15, 16) was 0.04, 0.05, and 0.05 mg NAs/mg O_3, respectively (Fig. 3.9). Correspondingly, the removal ratio for those NAs was increased to 79.7%, 83.3%, and 80.0%, respectively (Fig. 3.10). In comparison, with the ozonation of raw OSPW, those NAs were only degraded by 49.6%, 48.0%, 18.0%, and the ozonation efficiency was 0.03, 0.03, 0.01 mg NAs/mg O_3, respectively (Fig. 3.9-3.10). It can be clearly observed that the biofiltration pretreatment can benefit the removal of NAs by mild ozonation. However, it was noticed that biofiltration pretreatment did not benefit the removal of NAs with less cyclicity and carbon numbers, there is possibility that the NAs with higher –Z number and carbon number were degraded to the NAs with less –Z number and carbon number, which has also been observed by other studies (Xue et al., 2016a).

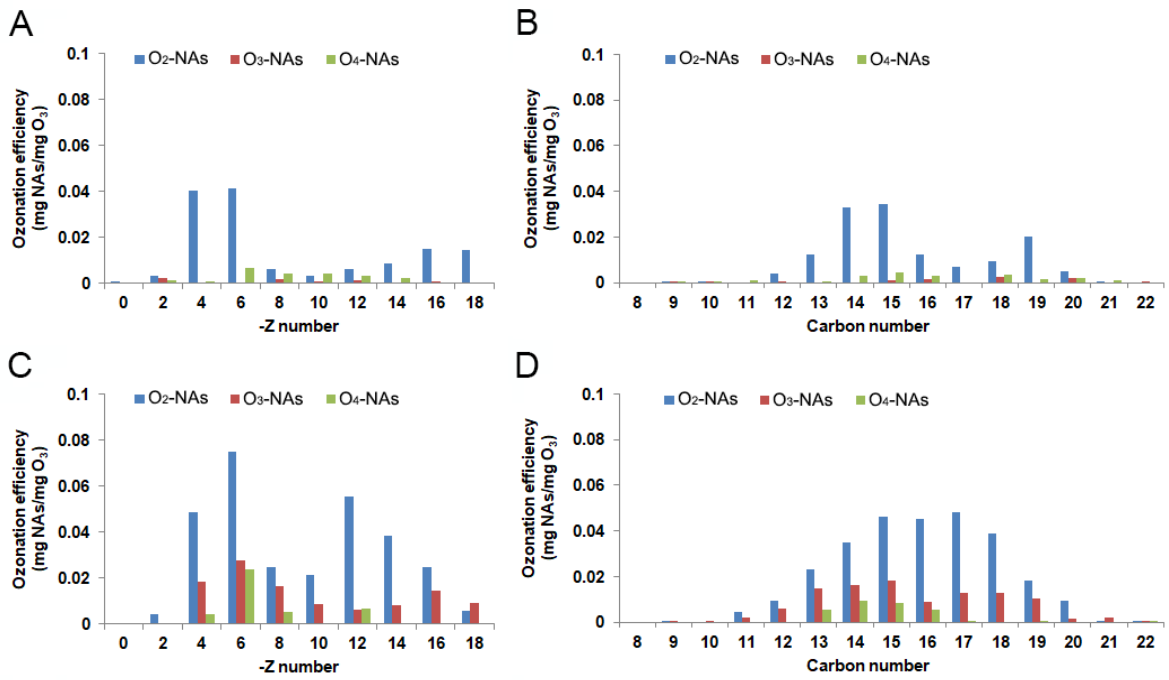

Figure 3.9. Ozonation efficiency on the degradation of NAs from raw and biofiltrated OSPW. A: NA degradation efficiency on raw OSPW based on -Z number; B: NA degradation efficiency on raw OSPW based on carbon number; C: NA degradation efficiency on biofiltrated OSPW based on -Z number; D: NA degradation efficiency on biofiltrated OSPW based on carbon number.

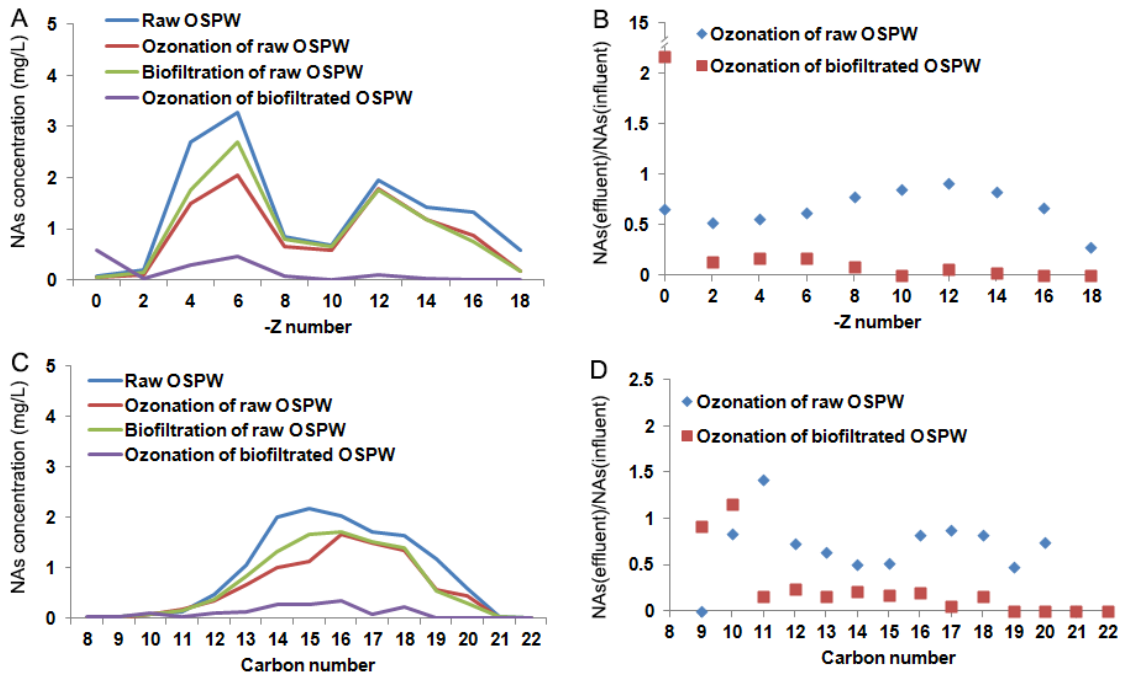

Figure 3.10. Comparison of the classical NA concentrations and removal ratios of raw OSPW and biofiltered OSPW after ozonation. A-B: Concentration and effluent NAs /influent NAs ratio of ozonation based on -Z number; C-D: Concentration and effluent NAs /influent NAs ratio of ozonation based on carbon number.

The degradation of individual O_3-NAs was also investigated based on their carbon number and -Z number (Fig. 3.9, 3.11-3.12). With the treatment by biofiltration followed by mild ozonation process, the ozonation efficiency on dominant O_3-NAs (-Z number from 4 to 8) was 0.02, 0.03, 0.02 mg NAs/mg O_3, respectively (Fig. 3.9). Based on carbon number, the O_3-NAs with n number from 13 to 15 were the most abundant in OSPW, and the ozonation efficiency of which was 0.02, 0.02, 0.02 mg NAs/mg O_3, respectively (Fig. 3.9). It was observed that the biofiltration pretreatment could also improve the removal of O_3-NAs except for the ones with the low -Z and carbon numbers (-Z = 0 and 2; n = 9 and 10) by mild ozonation (Fig. 3.12). With the pretreatment of biofiltration, O_3-NAs with high

-Z number and n number were more sensitive to ozone attack (Fig. 3.12). The results also confirmed that mild ozonation treatment showed the capacity to degrade highly branched and cyclic carboxylic NAs (Zhang et al., 2016). O_3-NAs with higher carbon number showed higher removal ratio after mild ozonation treatment, however, the decomposition of the O_3-NAs with higher carbon number may contribute to the lower removal ratio of O_3-NAs with less carbon number.

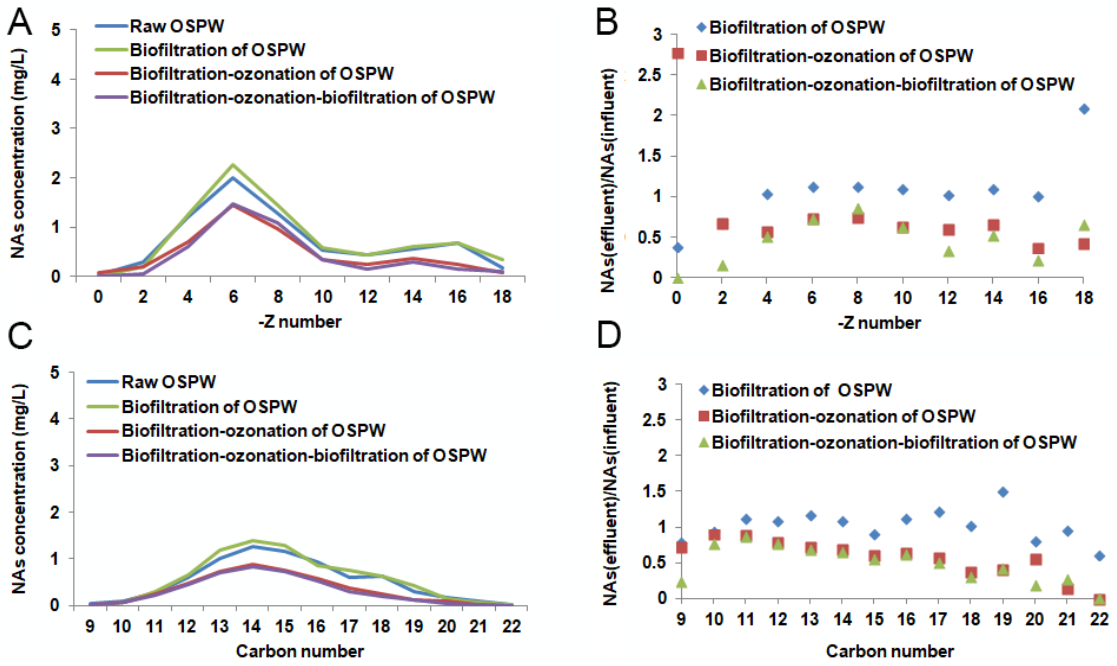

Figure 3.11. Concentration of O_3-NAs and relative removal after biofiltration, biofiltration-ozonation, and biofiltration-ozonation-biofiltration treatment. A: NA degradation based on -Z number; B: Effluent NAs/influent NAs ratio of biofilters based on -Z number; C: NA degradation based on carbon number; D: Effluent NAs/influent NAs ratio of biofilters based on carbon number.

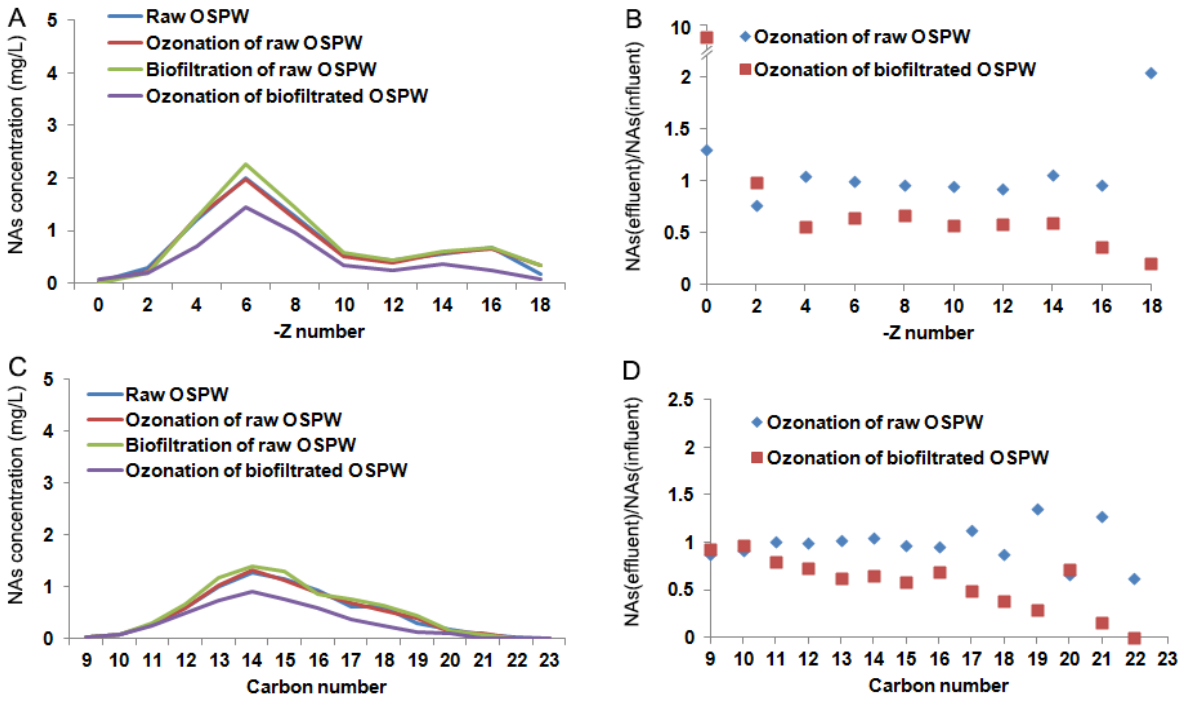

Figure 3.12. Comparison of the O_3-NAs concentrations and removal ratios of raw OSPW and biofiltered OSPW after ozonation. A-B: Concentration and effluent NAs /influent NAs ratio of ozonation based on -Z number; C-D: Concentration and effluent NAs /influent NAs ratio of ozonation based on carbon number.

The degradation of O_4-NAs was also compared based on carbon number and –Z number (Fig. 3.9, 3.13-3.14). It was observed that the NAs with –Z number of 6 and 8 and carbon number from 14 to 16 were observed as the dominant O_4-NAs from OSPW (Fig. 3.13). With the treatment by the biofiltration-ozonation process, the ozonation efficiency of those O_4-NAs (–Z number = 6 and 8) was 0.02 and 0.01 mg NAs/mg O_3, respectively (Fig. 3.9). And the ozonation efficiency of those O_4-NAs (n number from 14 to 16) all reached 0.01 mg NAs/mg O_3 (Fig. 3.9). It was also found that the ozonation removal ratio for those dominant O_4-NAs were all increased with the pretreatment of OSPW by biofiltration (Fig. 3.14). However, it was observed that the improvement of biofiltration on the removal of

O_4-NAs by ozonation was not as much as that of O_3-NAs (Table 3.3). The oxidization of O_3-NAs to O_4-NAs may be the reason for that the ozonation treatment can generate oxidized NA species, which was also found in other studies (Sun et al., 2014; Zhang et al., 2016).

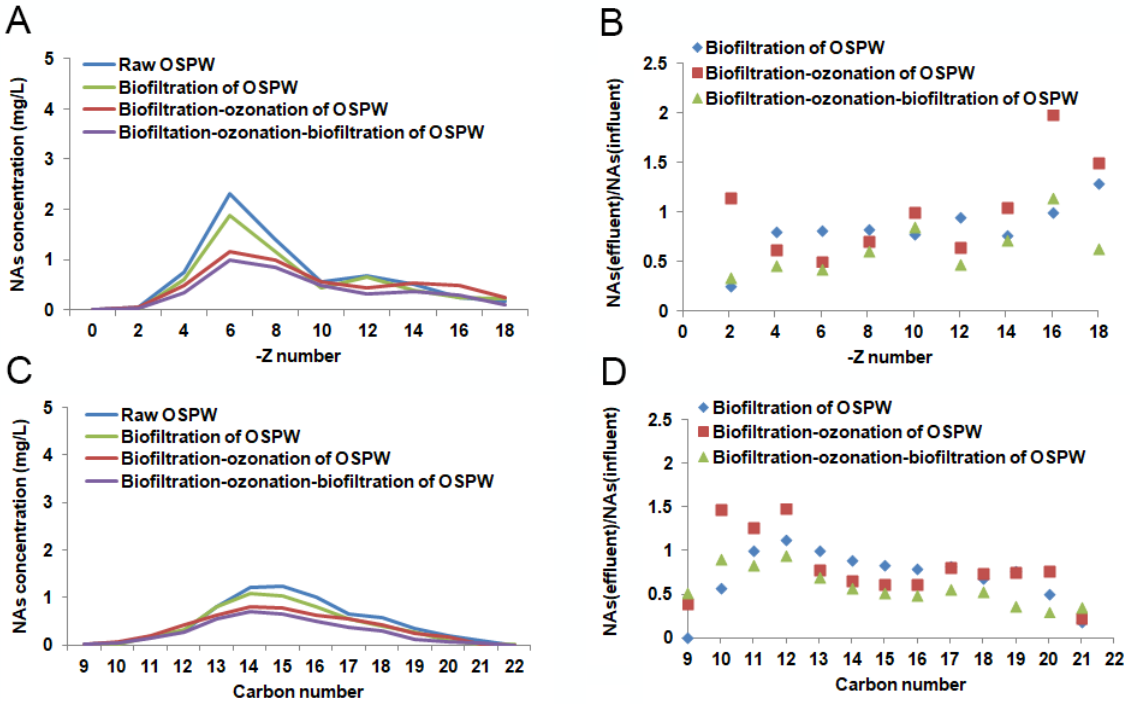

Figure 3.13. Concentration of O_4-NAs and relative removal after biofiltration, biofiltration-ozonation, and biofiltration-ozonation-biofiltration treatment. A: NA degradation based on -Z number; B: Effluent NAs/influent NAs ratio of biofilters based on -Z number; C: NA degradation based on carbon number; D: Effluent NAs/influent NAs ratio of biofilters based on carbon number.

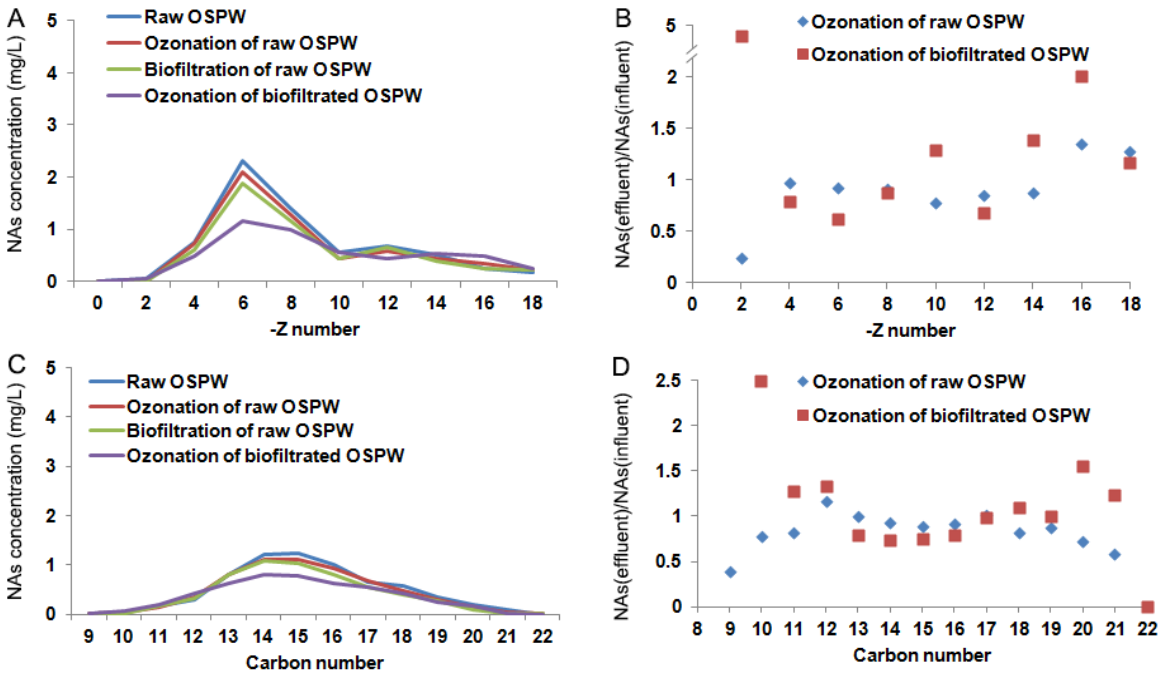

Figure 3.14. Comparison of the O_4-NAs concentrations and removal ratios of raw OSPW and biofiltered OSPW after ozonation. A-B: Concentration and effluent NAs /influent NAs ratio of ozonation based on -Z number; C-D: Concentration and effluent NAs /influent NAs ratio of ozonation based on carbon number.

3.3.3 Post biofiltration treatment

As described previously, mild ozonation pretreatment of OSPW can benefit the removal of NAs by biodegradation (Martin et al., 2010). In this study, after the biofiltration-ozonation treatment, the OSPW was further treated through another four times of circulation through the bioreactor to assess the benefit of ozonation as the intermediate process. It was interesting to find that classical NAs could be further biodegraded even at such a low concentration. The NA detection results showed that the classical NAs from OSPW were decreased further from 1.5 mg/L to 1.4 mg/L (Fig. 3.3). Interestingly, the concentration of O_3-NAs, O_4-NAs, O_5-NAs, and O_6-NAs was decreased from 4.6 mg/L to

4.2 mg/L, 5.0 mg/L to 3.8 mg/L, 2.3 mg/L to 1.5 mg/L, and 1.2 mg/L to 0.6 mg/L, respectively (Figure 3.5-3.8).

Compared with the biofiltration of raw OSPW by four circulating times, the classical NA removal ratio of the post biofiltration process was decreased from 24.4% to 6.7%, and the removal rate was decreased from 0.4 mg/L/h to 0.0 mg/L/h (Table 3.2). The biodegradation rate of dominant classical NAs was decreased to 0.0 mg/L/h as well (Fig. 3.4). It was probably caused by the low influent NA concentration, as a threshold level of NAs exists for microorganisms to initiate the biodegradation process (Misiti et al., 2013b). Long HRT might be needed to biodegrade the classical NAs at low concentration. A recent study showed that pilot-scale hybrid constructed wetland can degrade NAs effectively from OSPW with the HRT of 16 days (Hendrikse et al., 2018).

Conversely, it was interesting to observe that the removal ratio and removal rate of all oxidized NAs increased for the post biofiltration process when compared with the biofiltration process as the pretreatment (Table 3.2). Specifically, the removal ratio of the O_3-NAs and O_6-NAs was increased to 8.7% and 50.0%, and the removal ratio of O_4-NAs and O_5-NAs was increased from 16.4% and 8.3% to 24.0% and 34.8%, respectively (Table 3.2). The biofiltration of raw OSPW showed negligible removal effect on oxidized NAs, and ozonation of biofiltrated OSPW only showed high removal effect on O_3-NAs and O_4-NAs (Table 3.2, 3.4). It was noticed that all the oxidized NAs were decreased after the post biofiltration (Table 3.2). The low biodegradation rate was similar to biofiltration pretreatment, indicating long HRT might be needed to biodegrade the oxidized NAs as well (Fig. 3.4). According to previous studies, oxidized NAs are recalcitrant organic compounds in OSPW, which are considered as intermediates of the biodegradation. During the post

biofiltration process, the initial classical NA concentration was as low as 1.5 mg/L, and oxidized NAs became the main components of NAs. It was probably because little classical NAs can be used or bioconverted, the microorganisms had to use oxidized NAs as the carbon source. Further studies need to be conducted to clarify the removal mechanisms.

Table 3.4. Comparison of total NA removal ratio by ozonation based treatment processes

	NA removal ratio (%)			
NA species	**Ozonation - biofiltration (4 times)**	**Biofiltration (4 times) - ozonation**	**ozonation - biofiltration (8 times)**	**Biofiltration (4 times) - ozonation - Biofiltration (4 times)**
O_2-NAs	86.3	88.5	92.4	89.3
O_3-NAs	40.8	35.2	52.1	40.8
O_4-NAs	29.9	25.4	47.8	43.3
O_5-NAs	-41.7	-91.7	-25.0	-25.0
O_6-NAs	-66.7	-300.0	-66.7	-100.0
Oxidized NAs	27.5	14.4	41.8	34.0

*Notes: - = Increased; A utilized ozone dose of 30 mg/L was used when ozonation was applied prior to the bioreactors.

3.3.4 Performance of biofiltration-ozonation-biofiltration process

The quality characteristics of treated OSPW after the treatment of OSPW by the whole biofiltration-ozonation-biofiltration process are shown in Table 3.1. It can be observed that the total organic carbon (TOC) and chemical oxygen demand (COD) of raw OSPW were reduced by 31.6% and 20.4%, and the biofiltration pretreatment showed 11.7% removal of TOC from raw OSPW. The removal of TOC was found to improve the following ozonation treatment by removing ozone scavenger (de Wilt et al., 2018). It can probably the reason

for the ozonation showed improved efficiency on NA removal from biofiltration pretreated OSPW. The turbidity of raw OSPW decreased from 27.9 NTU to 0.0 NTU. The concentrations of inorganic ions were relatively stable. The acute toxicity of OSPW to *V. fischeri* was reduced by 10.6%. Importantly, the biofiltration-ozonation-biofiltration process showed 89.3% and 34.0% removal of classical and oxidized NAs from OSPW, which was substantially higher than that of the biofiltration of raw OSPW (22.1% and -2.0%) (Zhang et al., 2018). It was confirmed that the ozonation-integrated biofiltration process had improved NA removal efficiency.

Compared with ozonation-biofiltration (8 times of circulation) process, the biofiltration-ozonation-biofiltration treatment established in this study showed less classical NA removal (89.3% vs. 92.4%) and oxidized NA removal (34.0% vs. 41.8%) (Table 3.5) (Zhang et al., 2018). However, biofiltration (4 times of circulation)-ozonation process showed higher classical NA removal (88.5% vs. 86.3%) but less oxidized NA removal (14.4% vs. 27.5%) than ozonation-biofiltration (4 times of circulation) process (Zhang et al., 2018). For the treatment of OSPW with low NA concentration, such as the OSPW used in this study, the ozonation integrated biofiltration process showed similar NA removal as ozonation combined biofiltration process. Compared with biofiltration of raw OSW, the post-biofiltration process showed less classical NA removal efficiency, but enhanced oxidized NA removal efficiency (Table 3.2), the reason probably was little classical NAs can be used or bioconverted in the second biofiltration process (Misiti et al., 2013a). It can be assumed that the biofilter would show similar NA removal efficiency to ozonated OSPW if there was enough NA concentration in ozonated OSPW (Huang et al., 2015; Shi et al., 2015). Concentrations of NAs in tailings ponds ranged from 40 to 70 mg/L and can

reach to 130 mg/L in fresh tailings water (Allen, 2008). If OSPW with higher NA concentration would be used, the ozonation-integrated biofiltration would probably show higher NA removal efficiency than ozonation-biofiltration for higher ozonation efficiency on OSPW. More importantly, as biofiltration (4 times of circulation) pretreatment can improve ozonation efficiency on the removal of NAs from OSPW, it suggested that with biofiltration pretreatment, the utilized ozone dose can be reduced to achieve the same NA removal effect by the established ozonation combined biofiltration process, so the total capital cost of this process can be decreased which can further improve its potential for industrial applications (Zhang et al., 2018).

Table 3.5. Comparison of total NA removal rate by ozonation based treatment processes

	NA removal rate (mg/L/h)			
NA species	**ozonation - biofiltration (4 times)**	**Biofiltration (4 times) - ozonation**	**ozonation - biofiltration (8 times)**	**Biofiltration (4 times) - ozonation - Biofiltration (4 times)**
O_2-NAs	1.4	1.5	0.8	0.7
O_3-NAs	0.4	0.3	0.2	0.2
O_4-NAs	0.3	0.2	0.2	0.2
O_5-NAs	-0.1	-0.1	0.0	0.0
O_6-NAs	0.0	-0.1	0.0	0.0
Oxidized NAs	0.5	0.3	0.4	0.3

*Notes: - = Increased; A utilized ozone dose of 30 mg/L was used when ozonation was applied prior to the bioreactors.

3.3.5 Microbial community analysis

16S rDNA gene fragment based microbial community analysis was done by using Illumina MiSeq technology. With advantages of high efficiency and low DNA sample demand, amplicon sequencing method has been widely used for microbial community investigation. Based on gene sequence comparison and abundance analysis, the microbial community can be investigated on different taxonomic levels, such as class, order, family and so on. After the biofiltration-ozonation-biofiltration OSPW treatment process, the dominating bacterial phyla present on the biofilter were *Proteobacteria*, *Actinobacteria,* and *Bacteroidetes*, the abundance of which was 47.21%, 13.24%, and 12.59%, respectively (Fig. 3.15A). And it was interesting to find that the abundance of dominating bacterial phyla present in the biofilter after the biofiltration-ozonation-biofiltration and ozonation-biofiltration OSPW treatment were similar (Zhang et al., 2018). It was indicated that both of the OSPW treatment processes had a similar effect on the shaping of the microbial community structure in the biofilter. However, the abundance of those dominating bacterial phyla in raw OSPW was remarkably different, which was probably caused by the formation of biofilm and the treatment of ozonated OSPW (Fig. 3.15A) (Zhang et al., 2018). Comparatively, after the treatment of OSPW by using moving bed biofilm reactor and membrane bioreactor, *Proteobacteria* was found to be the most dominant phylum as well (Shi et al., 2015; Xue et al., 2016b), which suggests that *Proteobacteria* phylum bacteria can survive in OSPW and probably played a key role in NA biodegradation.

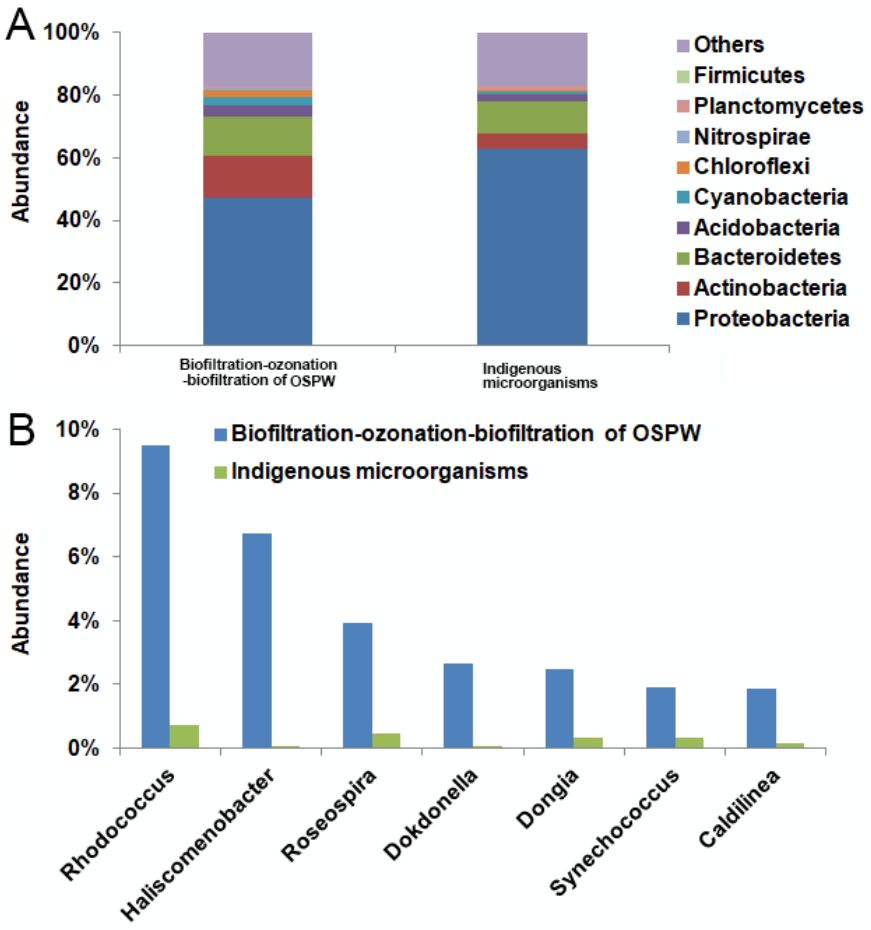

Figure 3.15. Investigation of the dynamics of microbial community structure. (A): Composition and abundance of bacterial phylum in the biofilter after biofiltration-ozonation-biofiltration, and in the indigenous microbial community; (B): Abundance of dominant bacterial genera in the biofilter after biofiltration-ozonation-biofiltration and in the indigenous microbial community.

The microbial community structure was analyzed based on genus level as well. After the biofiltration-ozonation-biofiltration OSPW treatment process, the dominating bacterial genera present on the biofilter were *Rhodococcus*, *Haliscomenobacter,* and *Roseospira*, the abundance of which was 9.50%, 6.73%, and 3.91%, respectively (Fig. 3.15B). Consistently, after the ozonation-biofiltration OSPW treatment process, *Rhodococcus* was also

determined as the most dominant bacterial genera in the biofilter (Zhang et al., 2018). However, the abundance of those bacterial genera in raw OSPW only account for 0.72%, 0.01% and 0.47% of the bacterial community, respectively (Fig. 3.15B). It is obvious that the abundance of bacterial genus changed significantly between the microbial community in raw OSPW and in the biofilter. It was further verified that both of the OSPW treatment processes had a similar impact on the microbial community structure in the biofilter. Only selective bacterial groups which could form biofilm and utilize organics in OSPW as a carbon source could be retained in the biofilter. Previous studies have shown that suspended growth biological treatment method cannot degrade NAs significantly (Del Rio et al., 2006). With the generation of biofilm, the microorganisms in biofilm can benefit from the protection of extracellular polymer matrix. Hence, microorganisms in biofilm can adapt to the harsh environment and start using the recalcitrant organic compounds like NAs as a carbon source (Chaudhary et al., 2003). As a result, biofilm reactors showed higher NA removal efficiency compared with a suspended growth treatment method. Through the abundance comparison of those bacterial genera from the microbial community after the treatment of ozonated OSPW by 8 times of circulations, it was found that the abundance of *Rhodococcus* was reduced by 2.07%, but the abundance of *Haliscomenobacter* and *Roseospira* was increased by 1.33% and 2.04%, respectively (Zhang et al., 2018). The results indicated that microbial community structure fluctuated when using different OSPW as influent.

As *Rhodococcus* was determined as the most abundant bacterial genus in the biofilter after both of the biofiltration-ozonation-biofiltration and ozonation-biofiltration OSPW treatment processes, it indicated that *Rhodococcus* may play a key part in NA

biodegradation. *Rhodococcus* has been determined as an active oil degrader in other research, and it showed high biodegradation efficiency on other recalcitrant organic compounds like alkanes and trichloroethene (Sharma and Pant, 2000; Suttinun et al., 2010). In the future, *Rhodococcus* can probably be used as model bacteria to elucidate the molecular mechanisms for NA biodegradation.

Functional profiling analysis showed the dynamics of the abundance of different metabolic pathways from the microbial community in the biofilter (Fig. 3.16A). It can be easily observed that the predictive functional profiling of the microbial community in the biofilter after OSPW treatment was different from the predictive functional profiling of the indigenous microbial community. After the biofiltration-ozonation-biofiltration OSPW treatment process, the abundances of the metabolism of amino acid, carbohydrate, lipid, terpenoids, and polyketides increased. Particularly, the abundance of xenobiotics biodegradation and metabolism pathway increased from 4.4% to 5.5% (Fig. 3.16A). The increase of xenobiotics biodegradation and metabolism pathway associated genes abundance may benefit the biodegradation of NAs by the biofilter. According to the previous study, *Cupriavidus gilardii* strain CR3 has the ability to use NA as a sole carbon source and many of its protein-coding genes from *Cupriavidus gilardii* strain CR3 were related with xenobiotics biodegradation and metabolism, which can probably confer *Cupriavidus gilardii* strain CR3 the ability to use NA as a sole carbon source (Wang et al., 2015). The abundance of the functional profile of xenobiotics biodegradation and metabolism is shown in Fig. 3.16B. It can be noted that the functional abundance of most observed xenobiotic degradation pathways was increased after OSPW treatment. More importantly, it was interesting to find that the functional pathways which showed increased

abundance after OSPW treatment were all associated with alicyclic and aromatic hydrocarbons. For instance, the abundance of the functional pathway for polycyclic aromatic hydrocarbon degradation was increased from 0.1% to 0.3% (Fig. 3.16B). While the cytochrome P450 enzyme degradation pathway which is critical for alkane degradation showed deceased functional abundance after the OSPW treatment (Peixoto et al., 2011). It indicated that after biofiltration-ozonation-biofiltration treatment, aromatic NAs may become the main component of NAs in OSPW. As *Pseudomonads* has been found efficient on aromatic NA degradation, it can probably be used for the post-treatment of the OSPW treated by a biofiltration-ozonation-biofiltration process for safe discharge (Zhang et al., 2015). With the dynamics of the microbial community structure in the biofiltration system, different functional abundance changed accordingly. As the biodegradation of NAs was coordinated by genes in different cell components rather than 16s rRNA only, whole genomic sequencing will be necessary for the investigation of the complicated mechanisms used for NA biodegradation. Metatranscriptome and proteome analysis can be also applied for the comprehensive analysis of the degradation of NAs by microbial community.

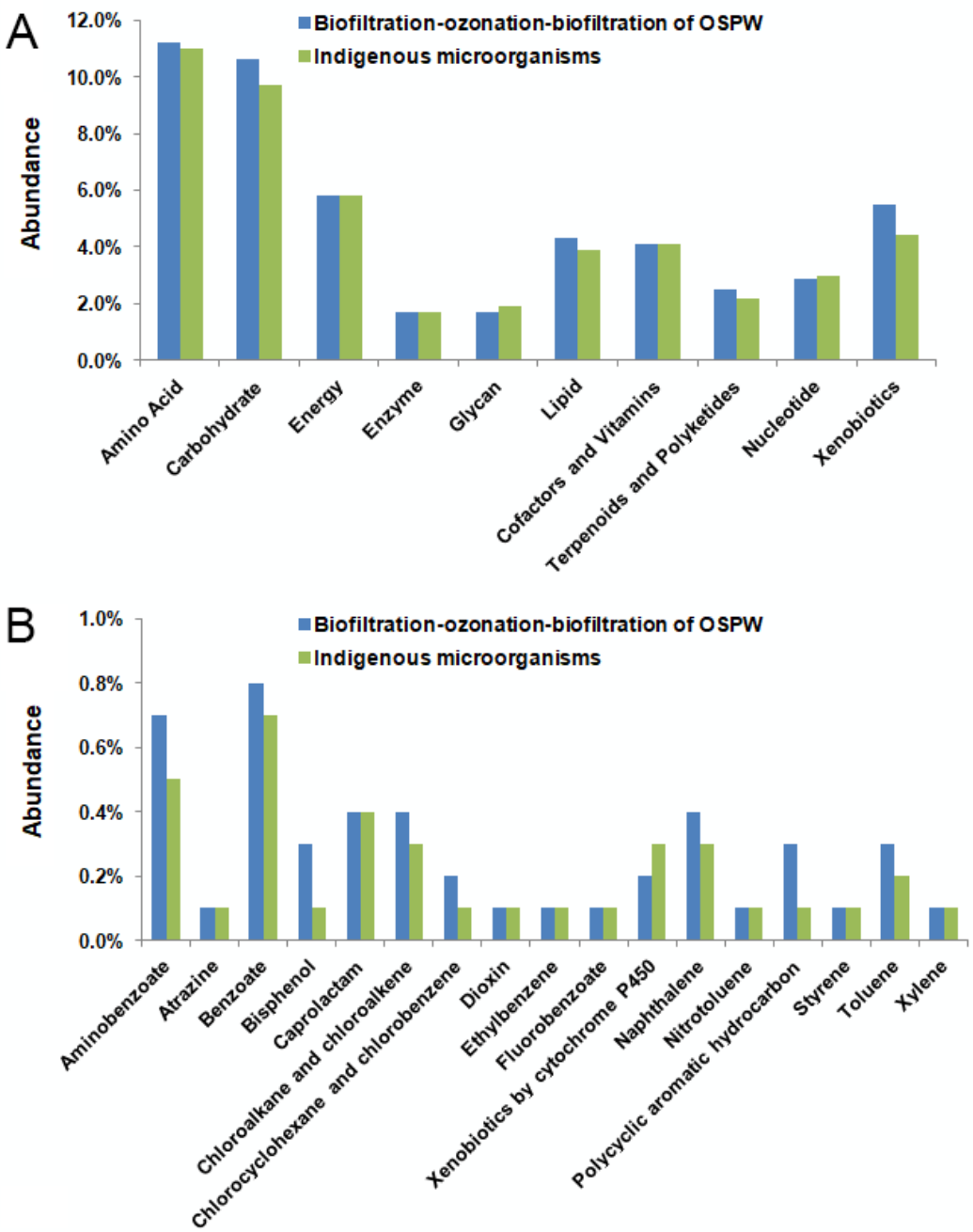

Figure 3.16. Abundance of different metabolic categories and functional pathways. A: Abundance of metabolic categories in the biofilter after biofiltration-ozonation-biofiltration and in the indigenous microbial community; B: Abundance of different functional pathways for xenobiotics biodegradation in the biofilter after biofiltration-ozonation-biofiltration and in the indigenous microbial community.

3.4 Conclusions

Biofiltration and mild ozonation showed complementary advantages to the degradation of NAs. The biofiltration-ozonation-biofiltration process showed higher NA removal than the biofiltration of raw OSPW. The biofiltration pretreatment can improve the ozonation removal of NAs while the post biofiltration process showed its contribution to the improved removal of the oxidize NAs from OSPW. With biofiltration pretreatment, the utilized ozone dose is expected to be reduced to achieve the same NA removal effect as the ozonation of raw OSPW. With a possible reduced cost of ozonation treatment, the biofiltration-ozonation-biofiltration process would show improved potential to be used by the industry. After OSPW treatment, *Rhodococcus* was determined as the most dominant bacterial genus on the biofilter which may contribute to NA removal considerably.

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CHAPTER 4. METAGENOMIC AND METATRANSCRIPTOMIC ANALYSIS OF THE MICROBIAL COMMUNITY IN A BIOFILTER FOR OIL SANDS PROCESS WATER RECLAMATION[1]

4.1 Introduction

With billions of barrels of recoverable bitumen deposit, the Athabasca region in northern Alberta, Canada contains the majority of Canada's oil reserves (Teare et al., 2012). By using hot water extraction process, bitumen can be separated from the oil sands. However, oil sands process water (OSPW) with naphthenic acids (NAs) as the primary toxic contaminants is generated at the same time (Shell, 2009). NAs refer to a series of alkyl-substituted acyclic and cycloaliphatic carboxylic acids with a general chemical formula of $C_nH_{2n+Z}O_x$, where n represents the number of carbon atoms, Z number indicates the hydrogen deficiency related with the formation of ring or double bond structure, and x stands for the number of oxygen atoms (Holowenko et al., 2001). The local environment and public health are at high risk to be negatively impacted by OSPW tailing ponds (Hodson, 2013; Pramanik, 2016). Importantly, the local government has decided to control

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[1] A version of this chapter will be submitted to a peer-review journal: Zhang, L., Zhang, Y. Patterson, J. and Gamal El-Din, M. Metagenomic and metatranscriptomic analysis of the microbial community in a biofilter for oil sands process water (OSPW) reclamation. 2019, Nature Communications.

the extension of tailing ponds and reclaim the OSPW according to a settled timeline (Alberta, 2015). OSPW remediation methods for industrial application on a large scale are urgently needed.

Low-cost biological wastewater treatment methods have been widely used for the remediation of different wastewater (Xue et al., 2018). However, NAs are resistant to biodegradation and are difficult to be removed from OSPW by biodegradation only. Some bacterial species such as *Pseudomonas putida* and *Pseudomonas fluorescens* have been found to degrade a mixture of commercial NAs (Del Rio et al., 2006). However, when those bacteria were applied to the bioremediation of raw OSPW, no significant NA removal was observed (Zhang et al., 2015). Mixed bacterial populations from the environment have been used to degrade recalcitrant NAs, indicating that a mixed consortium of microorganisms may have an advantage in the biodegradation of NAs (Smith et al., 2008). Our previous study showed that an indigenous microorganism based biofilter can remove 21.8% of NAs from raw OSPW (Zhang et al., 2018).

Though bacteria are considered the main players for the degradation of petroleum hydrocarbons, fungi also participate in the biodegradation process (Van Hamme et al., 2003). Many ligninolytic and nonligninolytic fungi have been found to possess polycyclic aromatic hydrocarbons (PAH) oxidation capacity (Krauss et al., 2011). The PAH degradation exoenzymes from fungi showed higher efficiency on the degradation of immobile high-molecular-weight PAHs than the intracellular PAH-degradation enzymes from bacteria, which may be attributed to higher PAH bioavailability of exoenzymes through diffusing in the environment (Johnsen et al., 2005). Though few bacteria possess the capacity to degrade PAHs with a high number of aromatic rings, bacterial enzymes may

improve the mineralization of PAH that has been oxidized by fungal exoenzymes. The combination of biofilm, planktonic bacterial, and fungal communities have been found to be able to degrade recalcitrant PAH from OSPW (Folwell et al., 2016).

Depending on molecular communications, bacteria and fungi can form various physical associations which can result unique biological processes such as the degradation of monoaromatic compounds in petroleum-contaminated soil through cooperative metabolism (Frey-Klett et al., 2011). It was also verified that hydrophilic fungal hyphae can be used as vectors for the dispersion of pollutant degrading bacteria which can improve the degradation efficiency on target contaminants (Kohlmeier et al., 2005; Wick et al., 2007). Investigation of the microbial community in biofilter is important to assess biological limiting factors related to the performance on NA removal (Huang et al., 2017b). However, there has been no report on the study of fungal communities in bioreactors used for OSPW treatment (Huang et al., 2015; Xue et al., 2016a). The microbial community structures at a different depth of biofilter have not been investigated either (Zhang et al., 2018).

Previous studies have revealed that ozone preferentially reacts with NAs possessing higher -Z and carbon number which are the most bioresistant species (Martin et al., 2010; Zhang et al., 2016). The ring structures present in NAs can be destroyed with ozonation treatment, which can remarkably reduce their bioresistance, because NAs with less –Z number can be preferentially biodegraded (Zhang et al., 2018; Zhang et al., 2016). Our biofilter removed 92.7% of NAs by a mild ozonation combined treatment process and the NA bioremoval ratio increased from 21.7% to 89.3% (Zhang et al., 2018). However, the interaction between the microbial community in biofilter and different OSPW has not been systematically characterized.

Metagenomic sequencing analysis has been widely used for the investigation of microbial community structure in bioreactors for OSPW treatment. For moving bed biofilm reactor (MBBR) OSPW treatment systems, the *Proteobacteria*, *Nitrospirae*, and *Acidobacteria* bacterial phyla have been observed as the dominant phyla in the bioreactor after raw and ozonated wastewater treatment. In sequencing batch OSPW treatment reactors, the three dominant phyla observed were *Proteobacteria*, *Planctomycetes*, and *Bacteroidetes* (Shi et al., 2015). For integrated fixed-film activated sludge (IFAS) OSPW treatment systems, *Proteobacteria*, *Nitrospirae*, *Acidobacteria*, and *Bacteroidetes* were the dominant phyla observed in both flocs and biofilms in the IFAS reactor (Huang et al., 2017b). The microbial community structure in different OSPW treatment systems is different, which can lead to a difference in functional pathways. It has been reported that the richness and diversity of microbial community present in biofilm was significantly higher than that in flocs, which may be the reason for the biofilm showed high acid extractable fraction (AEF) removal ability and the flocs showed high chemical oxygen demand (COD) removal ability (Huang et al., 2017a). However, no study has been performed to investigate the variation in the functional pathway abundances of microbial communities in the biofilter for OSPW treatment. Through metatranscriptomic sequencing analysis, the interaction between the microbial community in biofilter and OSPW may be illuminated.

The main objective of this study was to investigate the microbiota responsible for NA biodegradation in the biofilter. Specifically, 16S/18S genes targeted metagenomic sequencing was used to determine the differences of the microbial community structures from the top, middle, and bottom of the biofiltration system. Metagenomic sequencing was

also performed to investigate the variance in bacterial and fungal community structures in the biofilter after raw and ozonated OSPW treatment. Finally, metatranscriptomic sequencing was performed to investigate the change of gene expression profiles in the microbial community in biofilter after different OSPW treatment.

4.2 Materials and methods

4.2.1 Source of OSPW

OSPW samples were collected from an active oil sands tailings pond that is located at Fort McMurray, Alberta, Canada. The OSPW samples were then stored in 200 L polyvinyl chloride barrels in a cold room at 4 °C before they were used in this study (Xue et al., 2017).

4.2.2 Ozonation treatment

An ozone generator (GSO-40, WEDECO, Herford, Germany) was used to produce ozone from pure oxygen. A wet test meter (Precision Scientific Petroleum Instruments, Texas, USA) was used for the control of ozone flow rate. A 4L glass flask was used as the reactor for ozonation and ozone was fed into the reactor at the flow rate of 1 L/min. With the application of a gas diffuser and stir mixer, the ozone could be well mixed and homogenously interact with the constituents in OSPW sample. The feeding and offletting ozone concentration were measured using ozone monitors (HC500 Ozone monitor, WEDECO, USA). Based on the decrease in concentration between the feeding and offletting ozone, the utilized ozone dose (30 mg/L) was calculated. After the completion of ozonation treatment, purified nitrogen gas was fed in for the removal of residual ozone (Gamal El-Din et al., 2011; Huang et al., 2015).

4.2.3 Biofiltration system development and operation

A 20-cm cylindrical borosilicate glass column, which allows a three-fourths packing height, was used as the container for the bioreactor. The biofilter was established using pure sand (40-100 mesh) (Acros Organics, New Jersey, USA) as bed media. At the bottom of the reactor, a polyethylene terephthalate (PET) disk was equipped to hold the sand media. Glass wool was added to prevent clogs on the PET disk. Raw OSPW was used as influent process water during the period of biofilter development. The biofilter was operated at room temperature and empty bed contact time was set at 120 minutes. Our previous research has shown that the density of the bacterial copies on bed media can reach a steady state after three weeks of operation (Zhang et al., 2018). Sand media samples were collected from the top, middle, and bottom of the biofilter for microbial community structure analysis. Raw and ozonated OSPW was then circulated through the biofilter for the treatment of OSPW with the same flow rate as before. The media samples were also collected for metagenomic and metatranscriptomic analysis after OSPW treatment and the biodegradation of NAs from raw and ozonated OSPW was tested.

4.2.4 NA analysis

The NAs from OSPW samples were detected using ultra performance liquid chromatography/time-of-flight mass spectrometry (UPLC/TOFMS) (Sun et al., 2014). Briefly, using the 0.22 μm pore size nylon filter, 10 mL of water sample was filtered prior to being further used. Before the analysis, 1 mL of sample mixture was prepared, which included 0.5 mL filtered water sample mixed with 0.4 mL methanol and 0.1 mL myristic acid-1-^{13}C (4.0 mg/L). Among them, myristic acid-1-^{13}C was used as the internal standard.

A Waters UPLC Phenyl BEH column (1.7 μm, 150 mm × 1 mm) was used for chromatographic separations. The mobile phases included 10 mM ammonium acetate in water (A) and 10 mM ammonium acetate in 50/50 methanol/acetonitrile (B). After elution, the sample was then analyzed by the high-resolution time-of-flight mass spectrometer (Synapt G2, Waters, Milford, MA, USA). The NA detection data was further analyzed by using the TargetLynx® Ver. 4.1 software (Wang et al., 2013; Xue et al., 2016b).

4.2.5 Metagenomic analysis

To characterize the microbial communities on media samples, PowerSoil® DNA Isolation Kit (Mo-Bio Laboratories, CA, USA) was used for deoxyribonucleic acid (DNA) extraction (Huang et al., 2017c) from 0.25 g of sand media. The DNA samples were stored in a freezer at -20 °C before metagenomic analyses (Islam et al., 2015).

Illumina Miseq sequencing was performed at the Applied Genomics Core at the University of Alberta for the investigation of microbial community structure. V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified through the conventional polymerase chain reaction (PCR) by using specific primers (Forward primer: 5'-CCTACGGGNGGCWGCAG-3'; Reverse primer: 5'-GACTACHVGGGTATCTAATCC-3') (Fadrosh et al., 2014). The V4 hypervariable regions of the 18S rRNA gene were specifically amplified by using the conventional PCR with locus-specific primer sequences 5'-CCAGCA(G/C)C(C/T)GCGGTAATTCC-3' and 5'-ACTTTCGTTCTTGAT(C/T)(A/G)A-3' (Stoeck et al., 2010). The amplicon library was prepared according to the protocol specified for Illumina Miseq System (Illumina, CA, USA).

After the completion of the sequencing test, the Quantitative Insights Into Microbial Ecology (QIIME2, http://qiime2.org) pipeline was applied to the metagenomic sequencing data (Caporaso et al., 2010). Briefly, quality control was done on the input reads and identical reads were clustered together. Alpha diversity analysis was performed for the evaluation of the microbial community structure within each sample. Beta diversity analysis was used to compare the composition of microbial communities from different samples. The taxonomy classifiers used were trained on the Greengenes 13_8 99% operational taxonomic units (OTUs) and Silva 119 99% OTUs datasets and were applied to the taxonomic analysis of the unique 16S and 18S rRNA gene target sequences (Zabat et al., 2018). The microbial community taxonomic composition can be summarized based on a series of taxonomic levels, such as phylum, class and so on.

4.2.6 Metatranscriptomic analysis

The metatranscriptome sequencing analysis was performed at the BGI Company (BGI, Shenzhen, China). Briefly, total ribonucleic acid (RNA) was extracted from the indigenous microorganism sample and the sand media samples after 8 circulation cycles of raw and ozonated OSPW. The mRNA from prokaryotes was obtained by depleting rRNA from total RNA. After fragmentation, first-strand cDNA synthesis and second-strand cDNA synthesis, the reverse transcription products were purified, underwent end repair and had poly(A) sequence ligated on. With a connection to the sequencing adapter, the second-strand of the cDNA was degraded and then purified again before PCR amplification. After library preparation, metatranscriptome sequencing was performed on the Illumina HiSeq2000 platform (Illumina, CA, USA). The adapter and low-quality reads were filtered from raw sequencing data and contaminant and rRNA sequences were removed by the

kneaddata 0.6.1 tool (Li et al., 2009). The filtered reads were run through the SAMSA 2 pipeline for functional abundance analysis (Westreich et al., 2016).

4.3 Results and discussion

4.3.1 Distribution of the microbial community structure along the biofilter

Bacteria have been considered the main agents for the degradation of petroleum pollutants, and fungi have also been reported to play an important role in petroleum hydrocarbon pollutant degradation (Das and Chandran, 2011; Varjani, 2017). Based on the 16S and 18S rRNA gene fragments, metagenomic sequencing was applied for the investigation of the bacterial and fungal community structures from the top, middle, and bottom of the established biofilter.

At the bacterial phylum level, we found that the proportions of some bacterial phyla decreased with the depth of the biofilter (Fig. 4.1A). For instance, after the development of the biofilter, it was observed that the dominant bacterial phylum on the top level of the biofilter was *Proteobacteria*, with 61.3%. The dominant bacterial phylum at the middle and bottom level of the biofilter was *Proteobacteria* as well but decreased to 41.1% and 34.0%, respectively. *Proteobacteria* has also been observed as the dominant bacterial phylum in other bioreactors that were used for OSPW treatment.

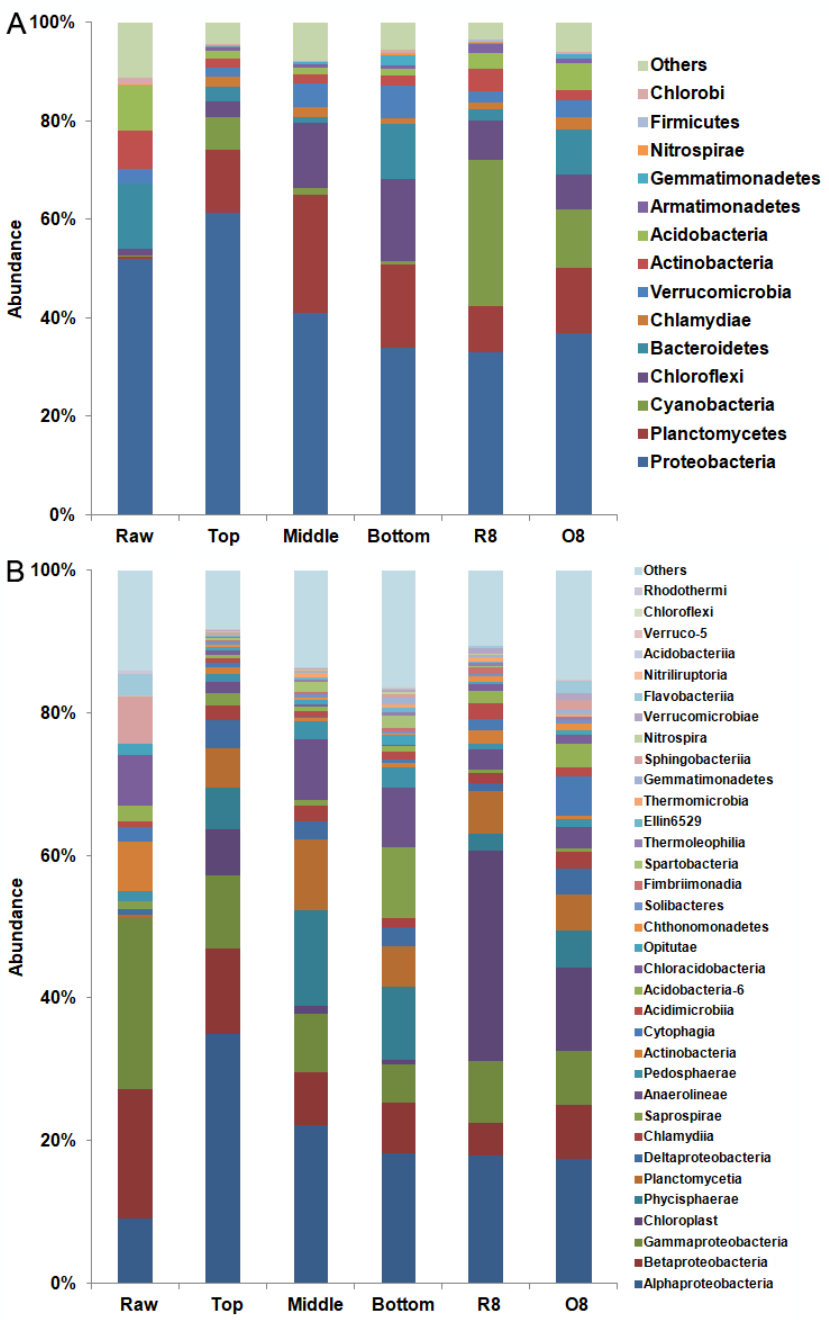

Figure 4.1. Abundance of bacterial phyla and classes from different microbial communities. A: Abundance of different bacterial phyla from raw OSPW (Raw), from the top, middle and bottom of the biofilter (Top, Middle, Bottom), from the biofilter after raw OSPW treatment with 8 cycles of circulation (R8), and from the biofilter after ozonated

OSPW treatment with 8 cycles of circulation (O8). B: Abundance of bacterial classes from the microbial communities.

In the MBBR, *Proteobacteria* was determined as the dominant bacterial phylum in the bioreactor for treatment of raw and ozonated OSPW, with 35.1% and 46.4%, respectively (Shi et al., 2015). *Proteobacteria* has also been observed as the dominant bacterial phylum in the membrane reactor (MBR) and IFAS systems for OSPW treatment (Huang et al., 2017b; Zhang et al., 2016). Bacteria from the *Proteobacteria* phylum have been found to degrade petroleum hydrocarbon compounds such as alkyl benzenes and cycloalkanes (Varjani, 2017). This indicates that *Proteobacteria* adapted well to the condition in the biofilter and may play an important role in OSPW remediation. We also found bacterial phylum which increased from top to bottom along the biofilter (*Chloroflexi*), or had increased abundance in the middle but decreased abundance in the bottom (*Planctomycetes*) (Fig. 4.1A). In short, the microbial community structures on the top, middle, and bottom of the biofilter varied substantially, which may be attributed to the difference in environmental conditions (e.g. dissolved oxygen, available nutrients' concentration) in the top, middle and bottom sections of the filter column.

The distribution of dominant bacterial class along the biofilter is shown in Fig. 4.1B. The dominant bacterial class on the top, middle and bottom level of the biofilter was *Alphaproteobacteria*, which occurred at 34.9%, 22.2%, and 18.3%, respectively (Fig. 4.1B), decreasing with depth. Conversely, the percentage of *Saprospirae* and *Anaerolineae* class increased from 1.8% to 9.9% and from 1.5% to 8.3%, from the top to bottom, respectively (Fig. 4.1B). The abundance of bacteria which lives in anaerobic conditions such as *Anaerolineae* increased at the bottom of the biofilter indicating the bottom of the

biofilter was somewhat anaerobic. *Anaerolineae* was also found as dominant microbial populations in other anaerobic digesters (Xia et al., 2016). With the establishment of oxygen gradients along the biofilters, both anaerobic and aerobic species can coexist in biofilm, which could improve the biodegradation of organic compounds (Davey and O'Toole G, 2000). The aerobic process has been considered played the main role for NA biodegradation because decreases in oxygen concentrations could result in a decrease in NA degradation (Whitby, 2010). It was verified that both the anoxic and aerobic conditions showed a degradation effect on classical and oxidized NAs from OSPW with the aerobic condition demonstrating higher NA removal efficiency (Xue et al., 2016b). However, based on the study on the biodegradation of a surrogate naphthenic acid (trans-4-methyl-1-cyclohexane carboxylic acid), continuous stirred tank reactor and biofilm reactor operated under anoxic conditions (denitrifying conditions) showed significantly higher NA removal rates than that in the aerobic continuous stirred tank reactor and biofilm reactor, indicating that some NAs can be better degraded by using other electron acceptors instead of oxygen (Gunawan et al., 2014). With the coexistence of anaerobic and aerobic bacteria in the biofilter, the efficient biodegradation of OSPW by the biofilter might also be enhanced by the combined degradation processes.

The dominant bacterial orders at the top and middle of the biofilter were *Rhodospirillales* (20.6%) and *Rhizobiales* (6.9%), from the *Alphaproteobacteria* class (Fig. 4.2A). *Saprospirales* (9.9%), from the *Saprospirae* class, was observed as the dominant bacterial order at the bottom of the biofilter (Fig. 4.2A). Further, the dominant bacterial family on the top and middle of the biofilter was *Rhodospirillaceae,* from the *Rhodospirillales* order, which made up 20.3% and 6.4% of those communities (Fig. 4.2C).

Members of *Rhodospirillaceae* have been reported as key players for the degradation of oil hydrocarbons in the marine environment (Berry and Gutierrez, 2017). However, *Saprospiraceae,* from the *Saprospirales* order, was determined as the dominant bacterial family at the bottom of the biofilter, with 8.7% (Fig. 4.2C). Bacteria from the *Saprospiraceae* family have also been found to hydrolyze complex carbon sources and degrade recalcitrant organic compounds (Xia et al., 2008). Alpha diversity analysis was performed for investigating the species richness of the microbial communities on the top, middle, and bottom of the biofilter. Based on the phylogenetic tree, the value of faith's phylogenetic diversity (Faith_PD) was calculated for measuring alpha diversity (Faith et al., 2009). As shown in Fig. 3A, the microbial community on the top of biofilter had the highest alpha diversity. According to the characteristics of the biofilter, nearly all suspended matter in influent water is trapped at the schmutzdecke level, which could be the reason why the microbial community on the top layer had the highest species richness (Ellis and Wood, 2009). The bacterial diversity was lower in the middle and bottom of the biofilter, but they still had more diversity than the microbial communities after different OSPW treatment (Fig. 4.3A).

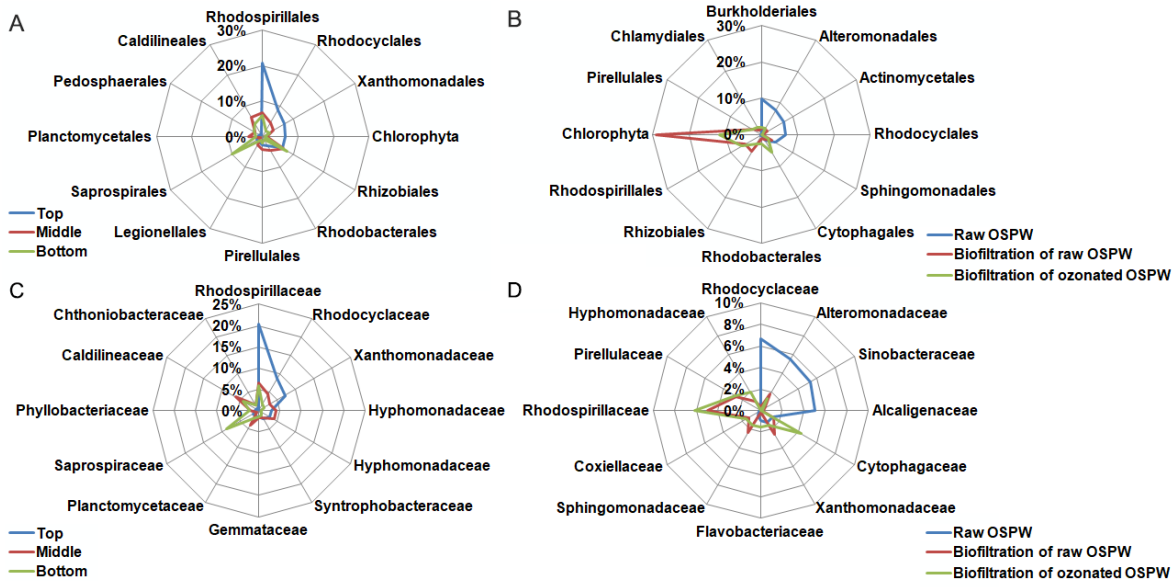

Figure 4.2. Abundance of the dominant bacteria in the biofilter at the order and family level. A and B: Abundance of bacterial orders from the top, middle and bottom level of the biofilter, and from the biofilter after raw and ozonated OSPW treatment with 8 cycles of circulation, respectively. C and D: Abundance of bacterial families from the top, middle and bottom level of the biofilter, and from the biofilter after raw and ozonated OSPW treatment with 8 cycles of circulation, respectively.

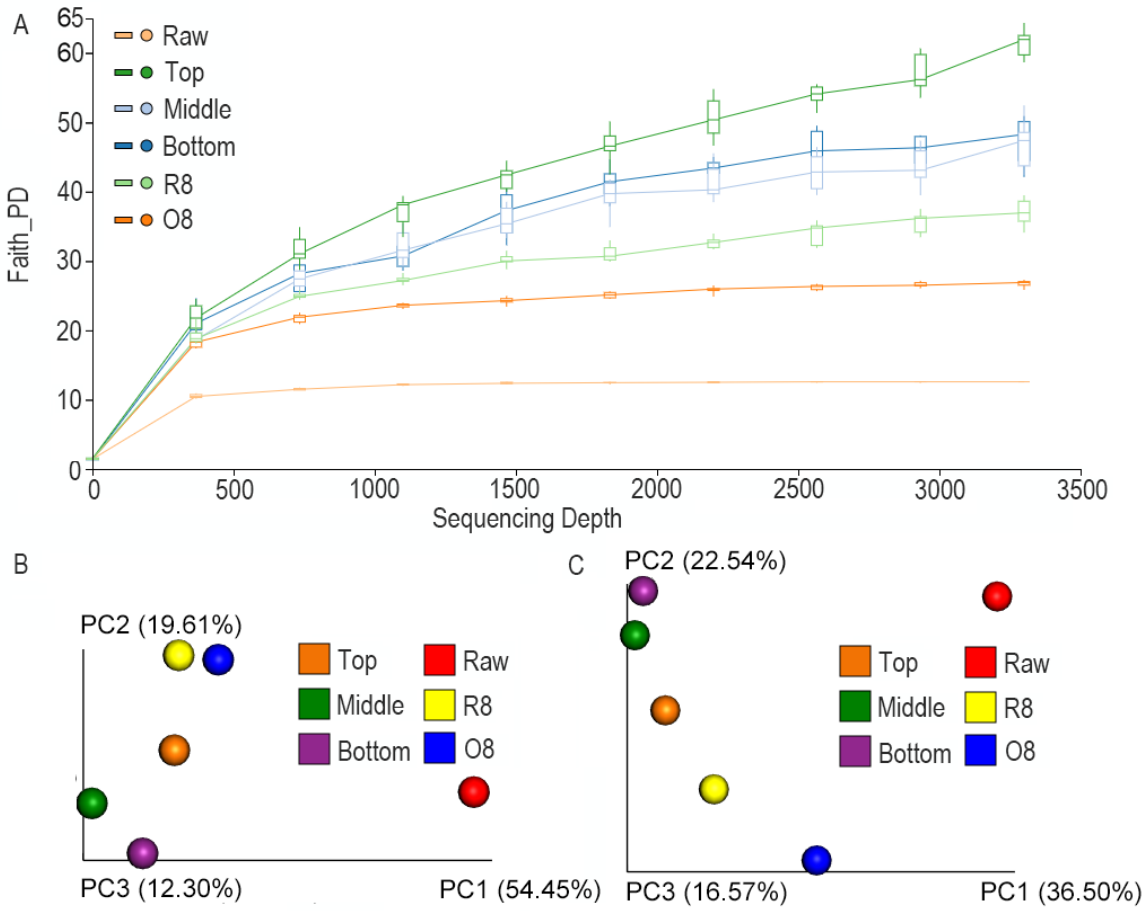

Figure 4.3. 16S rRNA gene segments based alpha and beta diversity analysis results. A: alpha diversity of the microbial community from raw OSPW (Raw), from the top, middle and bottom of the biofilter (Top, Middle, Bottom), from the biofilter after raw OSPW treatment with 8 cycles of circulation (R8), from the biofilter after ozonated OSPW treatment with 8 cycles of circulation (O8). B: Weighted beta diversity analysis of the microbial communities. C: Unweighted beta diversity analysis of the microbial communities.

Beta diversity analysis was performed to assess the differences among those microbial communities in the biofilter and feeding water. Two principal coordinates analyses (PCoA) were performed using the weighted and unweighted UniFrac distance

metrics (Fig. 4.3B-C). After 8 cycles of treatment on raw and ozonated OSPW, the microbial communities were more similar to the community at the top of the established biofilter than to the microbial community in raw OSPW.

Moving to fungi, we show the phylum level communities at the top, middle, and bottom of the biofilter in Fig. 4.4A. *Cryptomycota* was the dominant fungi observed in all three sections (top, middle, and bottom) of the biofilter, at 27.7%, 41.9%, and 41.3%, respectively (Fig. 4.4A). *Cryptomycota* has also been detected in the anoxic soft sediments from OSPW tailings ponds in another study (Aguilar et al., 2016). The proportion of *Cryptomycota* increased towards the bottom of the biofilter, suggesting that the anoxic condition in the middle and bottom of the biofilter benefited the growth of *Cryptomycota*. At the class level, *Incertae Sedis,* from the *Cryptomycota* phylum, was the dominant fungal class in the biofilter (Fig. 4.4B). As shown in Fig. 4.5A, the fungal communities in the middle and bottom of the biofilter had the highest alpha diversity indicating that more fungal species can adapt to the condition at the middle and bottom of the biofilter than at the top. Similarly, fungal community based beta diversity analysis confirmed that the fungal communities in the middle and bottom of biofilter were more closely related (Fig. 4.5B-C). The fungal communities were not as diverse as the bacterial, with only four fungal phyla observed in the biofilter in this study (Fig. 4.4). 18S rRNA gene-targeted amplicon sequencing has also been used for fungal metagenomics analysis in another study (Aguilar et al., 2016). However, our results showed that fungi can only be well determined at the phylum and class levels. In the future, more specific classification of fungal communities could be done using internal transcribed spacer (ITS) targeted sequencing (Schoch et al., 2012).

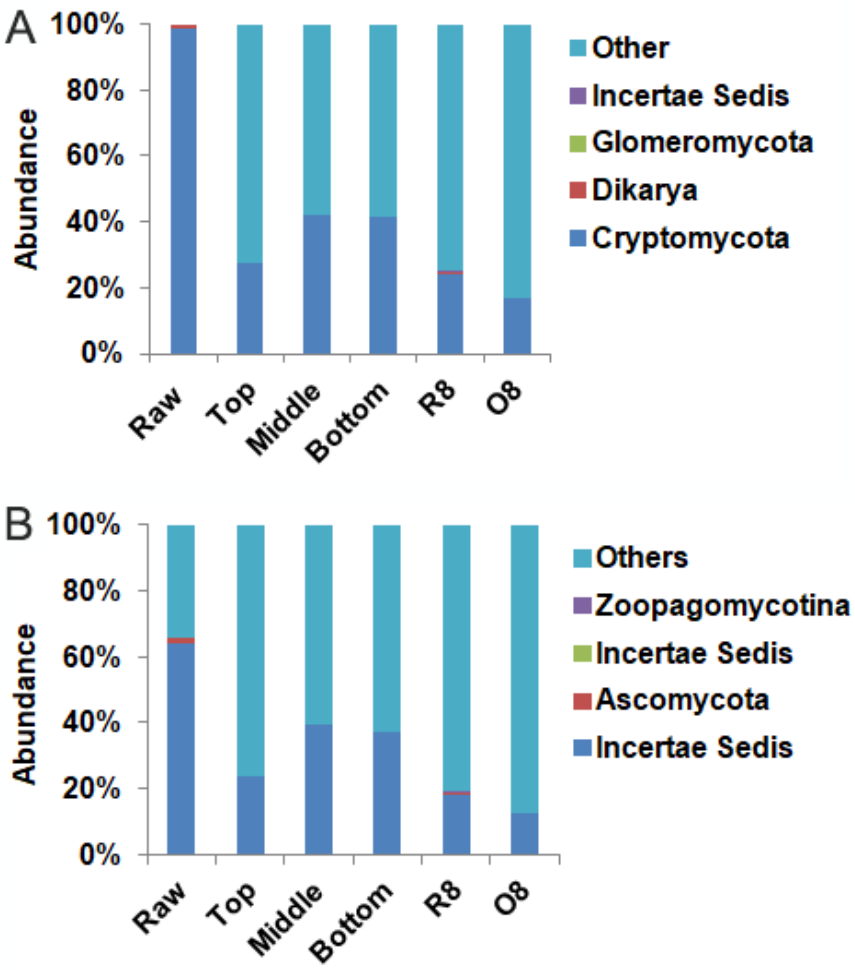

Figure 4.4. Abundance of fungal phyla and classes from different microbial communities. A: Abundance of different fungal phyla from raw OSPW (Raw), from the top, middle and bottom of the biofilter (Top, Middle, Bottom), from the biofilter after raw OSPW treatment with 8 cycles of circulation (R8), and from the biofilter after ozonated OSPW treatment with 8 cycles of circulation (O8). B: Abundance of fungal classes from the microbial communities.

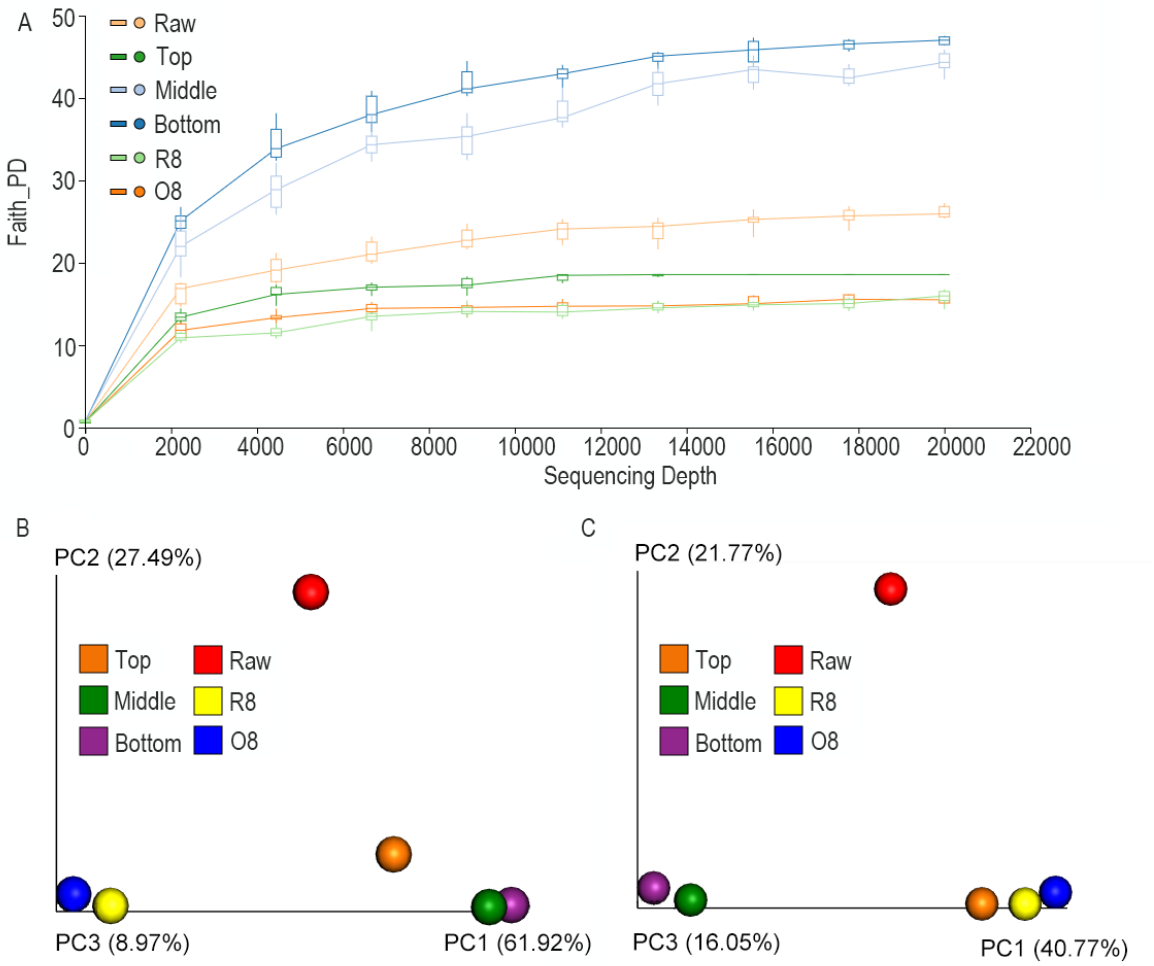

Figure 4.5. 18S rRNA gene segments based alpha and beta diversity analysis results. A: alpha diversity of the fungal community from raw OSPW (Raw), from the top, middle and bottom of the biofilter (Top, Middle, Bottom), from the biofilter after raw OSPW treatment with 8 cycles of circulation (R8), and from the biofilter after ozonated OSPW treatment with 8 cycles of circulation (O8). B: Weighted beta diversity analysis of the fungal communities; C: Unweighted beta diversity analysis of the fungal communities.

4.3.2 Removal of NAs from raw and ozonated OSPW

After the biofilter was established for the treatment of OSPW, biodegradation of NAs was investigated with the circulation of OSPW through the biofilter. After 8 cycles of

circulation, the concentration of NAs was decreased from 20.1 mg/L to 12.9 mg/L (Fig. 4.6A-B). With a total hydraulic retention time (HRT) of 16 h, the NA removal ratio reached 35.8%. The NA removal rate for the biofiltration process was 0.45 mg/L/h (Table 4.1).

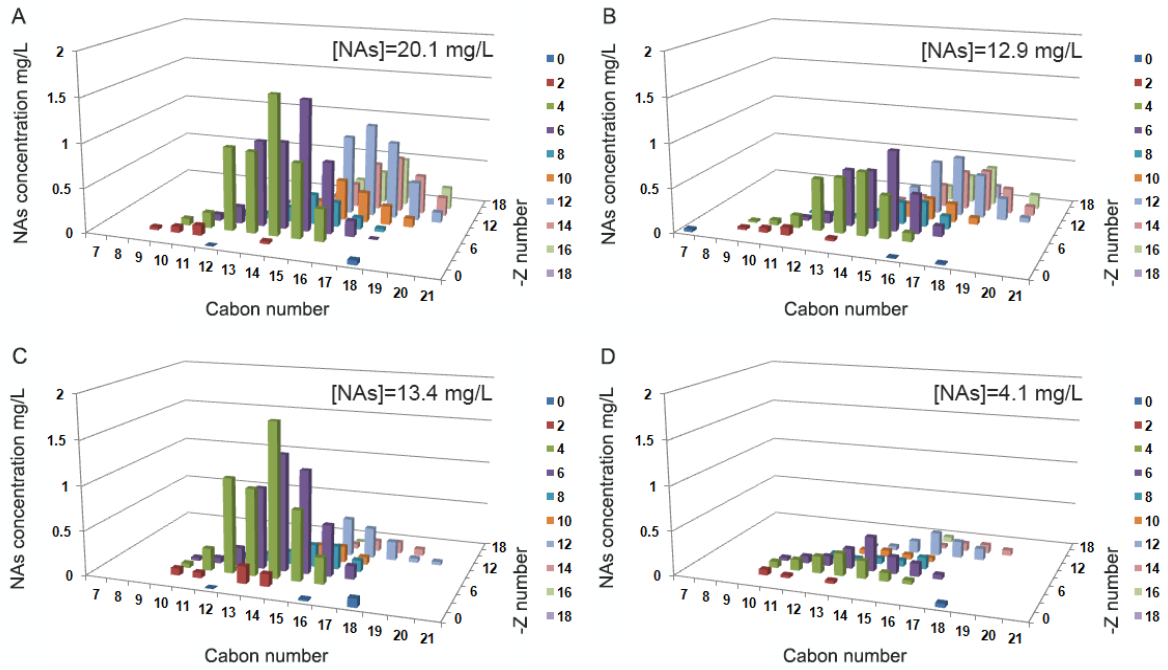

Figure 4.6. Profiles of classical NAs from different OSPW. A: Raw OSPW B: Biofiltered OSPW. C: Ozonated OSPW. D: Biofiltration of ozonated OSPW.

Table 4.1. Comparison of NA biodegradation in different bioreactors for OSPW treatment

Biodegradation process (Reference)	HRT	OSPW	Initial NA concentration (mg/L)	NA concentration after biodegradation (mg/L)	NA biodegradation rate (mg/L/h)	NA removal by biodegradation (%)
Biofilter (This study)	16 h	Raw	20.1	12.9	0.45	35.82
		Ozonated	13.4	4.1	0.58	69.40
MBBR (Shi et al., 2015)	48 h	Raw	19.8	12.9	0.14	34.85
		Ozonated	6.4	4.2	0.05	34.38
IFAS (Huang et al., 2015)	48 h	Raw	25.1	14.3	0.23	43.03
		Ozonated	9.5	5.0	0.09	47.37
MBR (Zhang et al., 2016)	48h	Raw1	18.1	17.1	0.02	5.52
		Ozonated1	10.4	5.9	0.09	43.27
		Raw2	25.5	19.5	0.13	23.53
		Ozonated2	13.4	7.8	0.12	41.79

*Notes: A utilized ozone dose of 30 mg/L was used when ozonation was applied prior to those bioreactors.

The impact of ozonation treatment on OSPW is shown in Fig. 5. After ozonation, the concentration of the NAs was decreased from 20.1 mg/L to 13.4 mg/L, with a removal ratio of 33.3% (Fig. 4.6A, C). In order to compare the reaction preference of the mild ozonation to different species of NAs, the removal ratio of different NAs was further investigated based on the carbon number and -Z number (Fig. 4.7). With the increase of -Z number, the NAs showed increased removal ratio accordingly (Fig. 4.7). It verifies that the ozonation preferentially reacts with the highly branched and cyclic carboxylic NAs, which is consistent with our previous studies (Zhang et al., 2018).

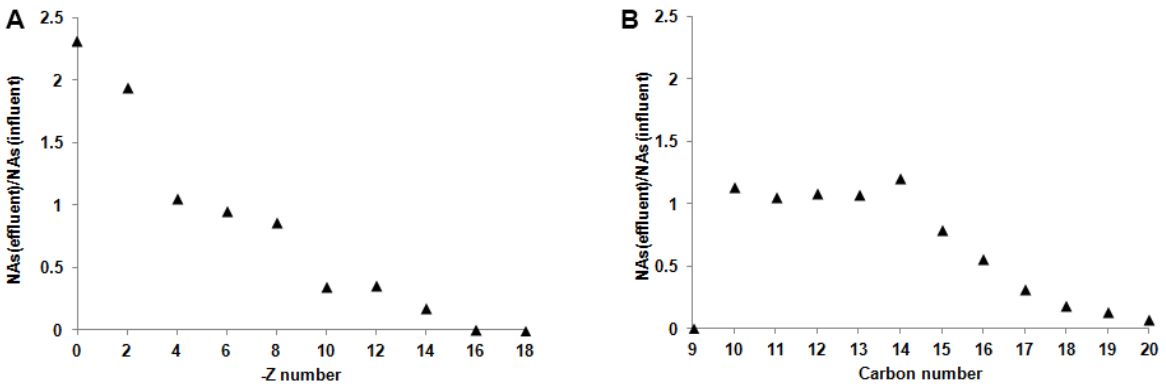

Figure 4.7. Relative removal of classical NAs after ozonation treatment: A: Impact of -Z number; B: Impact of carbon number.

After circulating through the biofilter, the biodegradation of NAs from ozonated OSPW was investigated as well. After 8 cycles of circulation, the concentration of NAs from OSPW was decreased from 13.4 mg/L to 4.1 mg/L (Fig. 4.6C-D). Biofiltration removed 69.4% of NAs from the ozonated OSPW, and the combined treatment can remove 79.6% of NAs from raw OSPW (Fig. 4.8). Compared with the biofiltration of raw OSPW, the biofiltration of ozonated OSPW showed a 33.6% higher NA removal ratio (Fig. 4.8). This verifies that the ozonation pretreatment can improve the biodegradation of NA from OSPW (Martin et al., 2010).

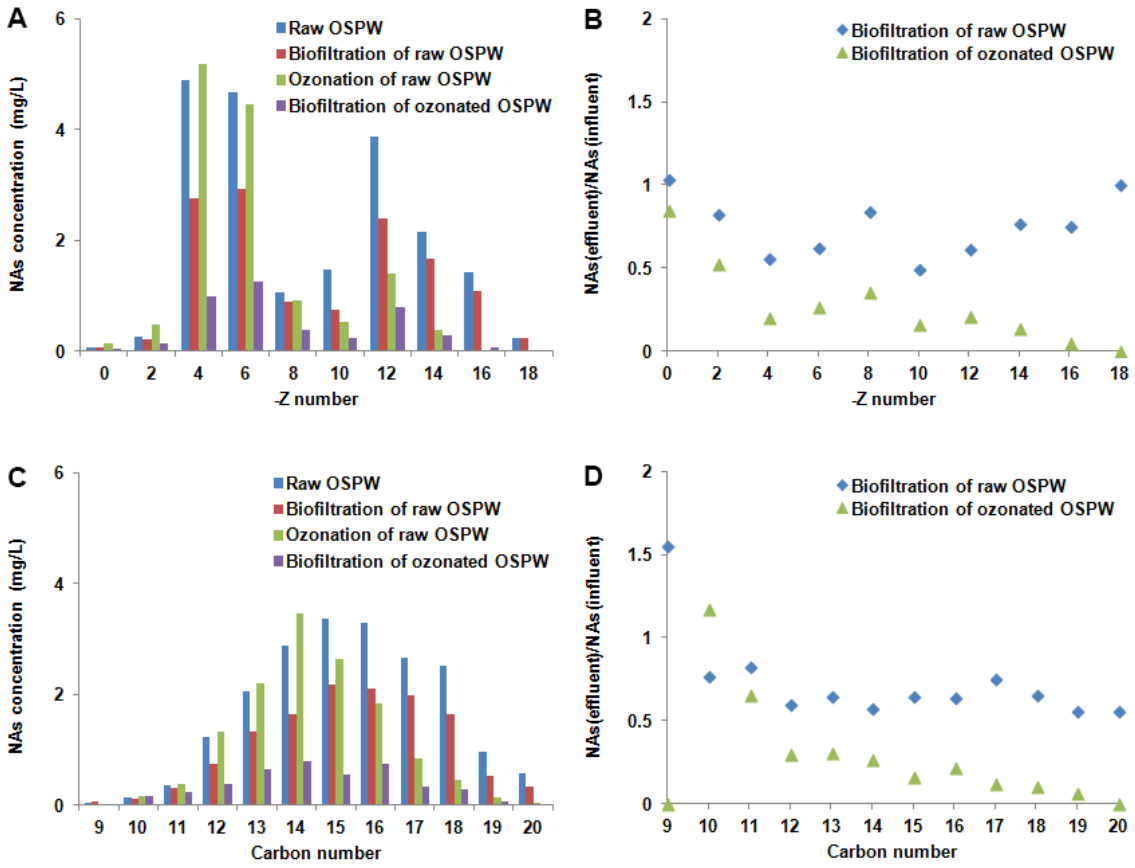

Figure 4.8. Concentration of classical NAs and relative removal after biofiltration, ozonation and combined treatment. A: NA degradation analysis based on -Z number; B: Effluent NAs/influent NAs ratio of biofilters based on -Z number; C: NA degradation analysis based on carbon number; D: Effluent NAs/influent NAs ratio of biofilters based on carbon number.

More importantly, the biofilter was established much quicker and showed higher NA removal efficiency than the MBBR, IFAS, and MBR reactors (Table 4.1). It has been found that biofilters showed superior performance on NA biodegradation when compared with other OSPW treatment reactors (Huang et al., 2015; Shi et al., 2015; Xue et al., 2016a), which may be attributed to the distinctive microbial communities in the biofilter.

4.3.3 Change of microbial community structure after OSPW treatment

After the treatment of raw and ozonated OSPW, the sand media samples from the top layer of the biofilter were collected for systematic bacterial community analysis because of the high microbial activities on the top layer of biofilter (Ellis and Wood, 2009). *Proteobacteria* was observed as the dominant bacterial phylum in the biofilters, with 33.0% and 36.9% for biofilters treating raw and ozonated OSPW (Fig. 4.1A). Compared with the microbial community in the biofilters before OSPW treatment, the proportion of *Proteobacteria* decreased (Fig. 4.1A). The abundance of *Cyanobacteria* and *Chloroflexi* phylum increased after raw and ozonated OSPW treatment (Fig. 4.1A). In addition, the abundance of *Bacteroidetes* phylum increased after ozonated OSPW treatment, and the abundance of *Actinobacteria* phylum increased after raw OSPW treatment (Fig. 4.1A). This shows that the microbial community structures adapted differently to the different OSPW (Fig. 4.3). Consistently, our previous study also found that ozone pretreatment significantly impacted the microbial community structure in MBR (Zhang et al., 2016).

As shown in Fig. 4.1B, *Gammaproteobacteria* (24.0%), *Betaproteobacteria* (18.2%), and *Alphaproteobacteria* (9.0%) were observed as the dominant bacterial classes in the indigenous microbial community. After OSPW treatment, the abundance of *Gammaproteobacteria* and *Betaproteobacteria* decreased, but the proportion of *Alphaproteobacteria* increased remarkably (Fig. 4.1B). The high abundance of *Alphaproteobacteria* class has also been observed in other bioreactors for OSPW treatment (Xue et al., 2016a; Zhang et al., 2016). It has been reported that *Alphaproteobacteria* played an important role in the degradation of recalcitrant hydrocarbon compounds in oil at an oil-contaminated beach (Kostka et al., 2011).

At the order level, the change of microbial communities after OSPW treatment can also be observed (Fig. 4.2). The abundance of bacterial classes such as *Chlorophyta*, *Rhodospirillales*, *Rhizobiales* increased remarkably, but the proportions of *Burkholderiales*, *Alteromonadales*, and *Actinomycetales* decreased after circulating OSPW through the biofilter (Fig. 4.2B). The abundance of *Cytophagales* increased only after circulating the ozonated OSPW, at 5.6% (Fig. 4.2B). *Chlorophyta* has been regarded as beneficial to the biofiltration treatment process by producing soluble and biodegradable organics in water (Ellis and Wood, 2009). *Rhizobiales* has been reported to biodegrade sulfur-containing organic compounds and *Rhodospirillale* possesses the ability to degrade carbohydrates and alcohols (Carvalho et al., 2010; Gupta and Mok, 2007).

At the family level, *Rhodocyclaceae* (6.6%) was dominant in the indigenous microbial community, whereas *Rhodospirillaceae* was dominant in the biofilters after OSPW treatment, the percentages of which were 5.0% and 6.2% for biofilters treating raw and ozonated OSPW, respectively (Fig. 4.2D). *Rhodospirillaceae* has also been considered an important microorganism for polycyclic aromatic hydrocarbon-degradation in the deep sea (Cui et al., 2008). Besides, the abundance of *Cytophagaceae* increased to 4.3% after ozonated OSPW treatment. Bacteria from *Cytophagaceae* have been isolated from hexachlorocyclohexane-contaminated soil indicating that bacterial from *Cytophagaceae* family may degrade recalcitrant hydrocarbons (Singh et al., 2014).

Alpha and beta diversity analysis were performed for the bacterial communities in the biofilter after OSPW treatment. As shown in Fig. 4.3, the bacterial communities in the biofilters had higher alpha diversity than the bacterial community in raw OSPW, indicating the enrichment of bacteria in the biofilter, which is consistent with our previous study

(Zhang et al., 2016). Given that the circulation of OSPW resulted in the reduced amount of carbon source for the microbial community in the biofilter, lower richness was observed, as expected, compared with the microbial community during the biofilter development stage. During the treatment of OSPW with HRT of 0, 8 and 16 hours, the alpha and beta diversity of bacterial communities in the biofilters were relatively stable, indicating that the bacterial community structures changed slowly with the degradation of OSPW (Fig. 4.9). After raw and ozonated OSPW treatment, the bacterial communities appeared different than that in OSPW, appearing more similar to the communities in the established biofilter. This confirms that the bacterial community structure in the biofilter can be impacted by the feed of different OSPW (Shi et al., 2015; Zhang et al., 2018; Zhang et al., 2016).

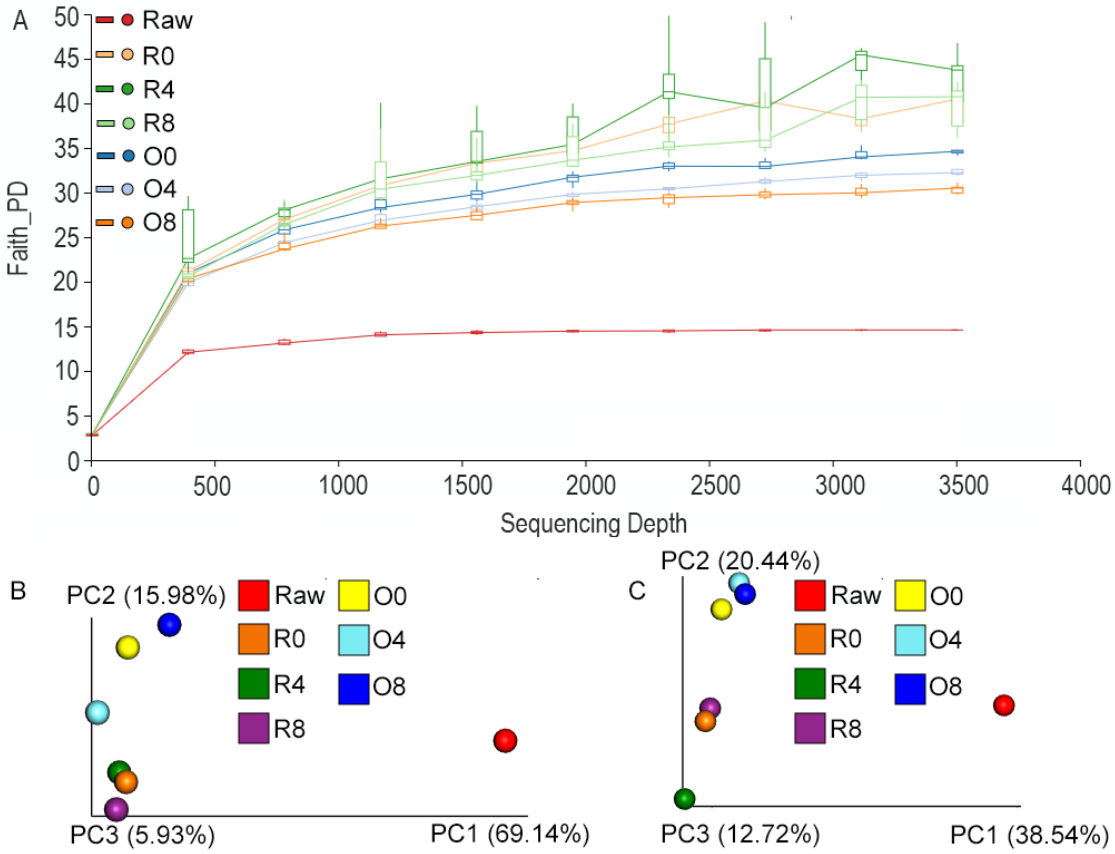

Figure 4.9. Alpha and beta diversity analysis for bacterial communities in the biofilter during different OSPW treatment. A: alpha diversity of the bacterial community from raw OSPW (Raw), from the biofilter after raw OSPW treatment with 0, 4, 8 cycles of circulation (R0, R4, R8), from the biofilter after ozonated OSPW treatment with 0, 4, 8 cycles of circulation (O0, O4, O8). B: Weighted beta diversity analysis of the bacterial communities. C: Unweighted beta diversity analysis of the bacterial communities.

Based on our previous studies, it was observed that different bioreactors (MBBR, IFAS, MBR, and Biofilter) showed different NA degradation efficiency (Huang et al., 2015; Shi et al., 2015; Xue et al., 2016a; Zhang et al., 2018; Zhang et al., 2016). Because the origin of microorganisms and the operation conditions of those bioreactors were different, the microbial community in the bioreactors under steady state condition may be

impacted, which may further affect the NA removal efficiency of different bioreactors. In MBBR and IFAS, *Proteobacteria*, *Nitrospirae*, and *Acidobacteria* have been determined as the dominant bacterial phyla after OSPW treatment (Huang et al., 2017b; Shi et al., 2015); *Proteobacteria*, *Bacteroidetes*, and *Planctomycetes* have been determined as the dominant bacterial phyla in MBR after OSPW treatment (Xue et al., 2016a); while *Proteobacteria*, *Planctomycetes*, and *Cyanobacteria* were revealed as dominant bacterial phylum in a biofilter in this study. *Proteobacteria* has always been observed as a dominant phylum in the bioreactors for OSPW treatment, indicating that bacteria from this phylum may play an important role in NA biodegradation. Further, *Betaproteobacteria* has been found as a dominant bacterial class in MBR and IFAS, but *Alphaproteobacteria* was found as a dominant bacterial class in the biofilter, which may have distinctive properties in terms of NA degradation (Huang et al., 2017c; Xue et al., 2016a). In addition, *Nitrospira*, *Rhodocyclales*, and *Chlorophyta* have been observed as the dominant bacterial order in the IFAS, MBR, and biofilter, respectively (Huang et al., 2017c; Xue et al., 2016a). The microbial community structure in biofilters had high diversity and unique microbial community structure, which may contribute to its high performance on NA degradation.

For the fungal community, *Cryptomycota* was determined as the dominant fungal phylum in the indigenous microbial community (98.5%) and in biofilter after raw (24.3%) and ozonated (16.6%) OSPW treatment (Fig. 4.4A). The growth of unclassified fungi during the development of biofilter can be observed (Fig. 4.4A). The dominant fungal class was *Incertae Sedis* in the indigenous microbial community (64.2%), and its abundance became 18.3% and 12.4% in biofilter used for treating raw and ozonated OSPW, respectively (Fig. 4.4B).

As shown in Fig. 4.5A, the indigenous fungal community had higher alpha diversity than that in the biofilter, suggesting that only fungal species which could adapt to the condition in the biofilter can survive. The bacteria in the biofilter also competed with fungi for available carbon source and nutrients. Thus, the impact of the bacterial community in the biofilter may also help explain the dynamics of fungal community structure. Beta diversity analysis also showed that the fungal community in the biofilter can be impacted by the feed of different OSPW (Fig. 4.5B-C). During the treatment of OSPW with HRT of 0, 8 and 16 hours, the alpha and beta diversity of fungal communities in the biofilters were relatively stable too, which confirmed that the microbial community structures kept on changing as a slow process (Fig. 4.10).

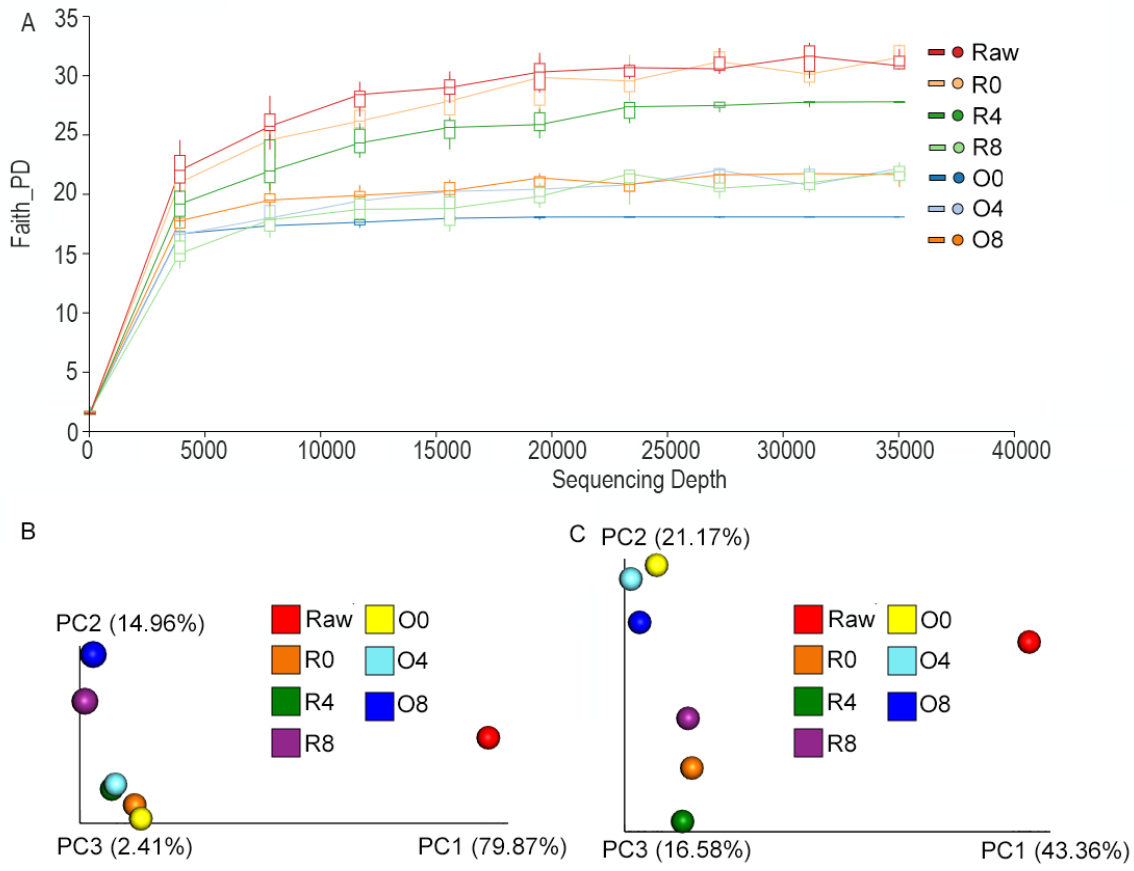

Figure 4.10. Alpha and beta diversity analysis for fungal communities in the biofilter during different OSPW treatment. A: alpha diversity of the fungal community from raw OSPW (Raw), from the biofilter after raw OSPW treatment with 0, 4, 8 cycles of circulation (R0, R4, R8), from the biofilter after ozonated OSPW treatment with 0, 4, 8 cycles of circulation (O0, O4, O8). B: Weighted beta diversity analysis of the fungal communities. C: Unweighted beta diversity analysis of the fungal communities.

4.3.4 Metatranscriptomic analysis

In order to understand the NA biodegradation at the transcriptomic level, the gene expression profiles of the indigenous microbial community in the biofilter after raw and ozonated OSPW treatment was investigated by metatranscriptome sequencing. It was

observed that genes associated with detoxification, oxidative stress, organic acid degradation and metabolism of fatty acids and aromatic compounds were expressed in all those microbial communities (Fig. 4.11). With the treatment of AEF from OSPW, a previous study also found that the xenobiotic detoxification pathway associated genes were up-regulated in *Arabidopsis thaliana* and the stress-responsive pathway genes were activated (Widdup et al., 2015). Genes associated with xenobiotic biodegradation have also been identified in *Cupriavidus gilardii* which showed NA biodegradation capacity (Wang et al., 2015).

Through gene expression abundance comparison, we found that the functional abundance related to the metabolism of aromatic compounds was improved from 0.05% in the indigenous microbial community to 0.76% after raw OSPW treatment (Fig. 4.11). In addition, the functional abundance related to organic acids degradation increased from 0.29% to 0.39%, indicating that the pathways of organic acids degradation and aromatic compounds metabolism were critical for the OSPW remediation by biofiltration. The functional abundance of organic acids degradation and aromatic compounds metabolism was 0.08% and 0.10% in the microbial community after ozonated OSPW treatment (Fig. 4.11). Given that the ozonation pretreatment probably had degraded most aromatic compounds in OSPW, the decreased functional abundance of aromatic compounds and organic acid metabolism observed in the microbial community was expected. This was probably also due to the fact that few NAs were present in the biofiltrated ozonated OSPW.

In addition, the organic acids and aromatic compounds metabolism pathways had more associated genes expressed in microbial community in the biofilter for raw OSPW treatment than that for ozonated OSPW treatment and the microbial community in raw

OSPW (Table 4.2-4.3). With the formation of biofilm on the sand media surface, the attached growth process of the microbial community in biofilter was different from the suspended growth process in raw OSPW, which may be the reason for the microbial community in biofilter had higher functional abundance of aromatic compounds and organic acids metabolism pathway. The NA concentration in raw OSPW was higher than ozonated OSPW, which can probably be resulted in the regulation of gene expression profiles in the microbial community and lead to the expression of more types of enzymes in the biofilter for raw OSPW treatment.

It is well known that most microorganisms degrade aliphatic and alicyclic carboxylic acids via β-oxidation pathway (Whitby, 2010). The abundance of β-oxidation pathway associated acyl dehydratase gene product was 0.02% in indigenous microbial community and the expression level was the same after raw OSPW treatment. The previous study also found that benzoate metabolism pathway was important for alicyclic carboxylic degradation. For instance, 3-cyclohexanecarboxylic acid was mainly biodegraded by benzoate pathway (Blakley and Papish, 1982). It was interesting to find that the abundance of gene products associated with benzoate transport and degradation pathway increased from 0.04% to 0.64% in the indigenous microbial community after raw OSPW treatment (Table 4.2). NAs with cyclic have been considered the main NAs in OSPW (Huang et al., 2018a; Huang et al., 2018b). It suggested that the NAs with alicyclic may mainly be degraded by benzoate degradation pathway in the biofilter. It was reported that the methylcitrate cycle is critical for the degradation of propionyl-CoA which is the intermediate product of fatty acids biodegradation (Upton and McKinney, 2007). The abundance of methylcitrate cycle in the indigenous microbial community was improved

from 0.12% to 0.22% after raw OSPW treatment (Table 4.3), indicating that methylcitrate cycle may also play an important role during the NA biodegradation process. Our results show that the microbial community changed expression profiles in response to OSPW with different NA concentrations, which is consistent with the metagenomic sequencing analysis results described above.

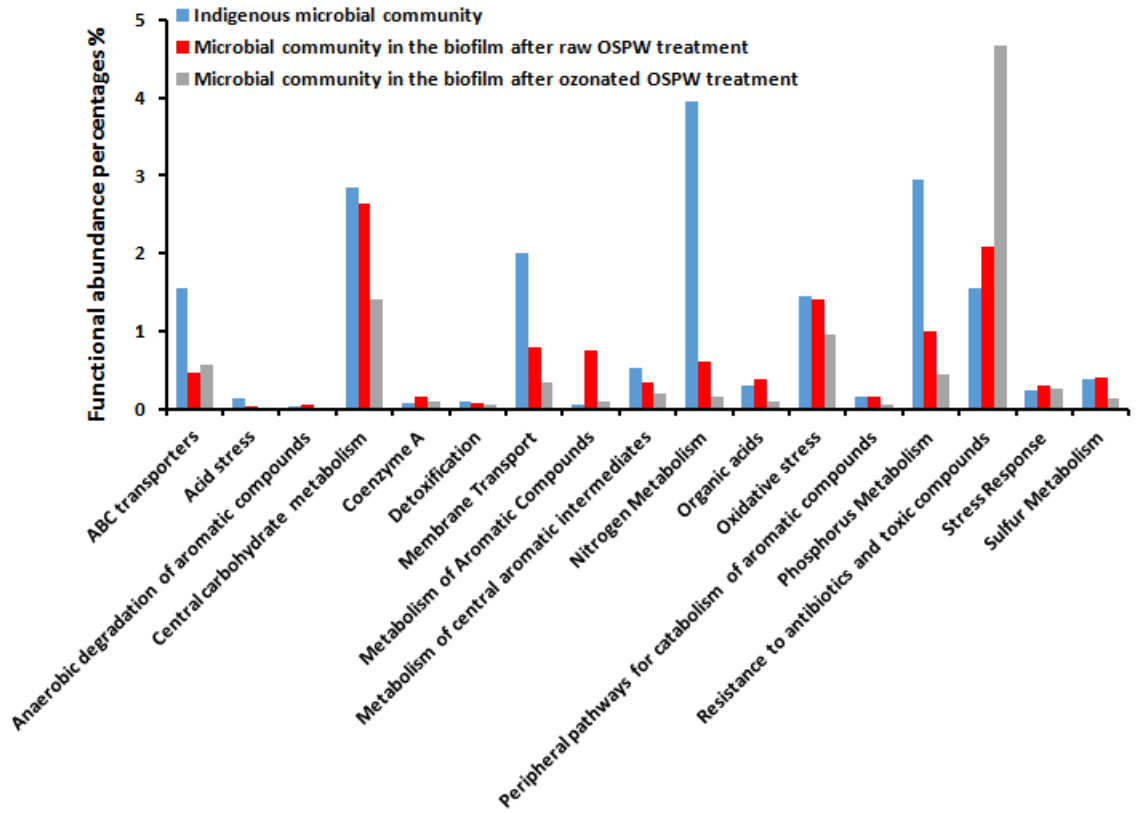

Figure 4.11. Relative functional abundance of metabolic pathways from the microbial communities.

Table 4.2. Functional abundance for the metabolism of aromatic compounds

	Percentages	Gene products	Pathway
Indigenous microbial community	0.02	Benzoyl-CoA-dihydrodiol lyase (BoxC)	Benzoate_transport_and_degradation_cluster
	0.02	Benzoate transport, inner-membrane translocator	Benzoate_transport_and_degradation_cluster
	0.01	2,3-dihydroxy-p-cumate-3,4-dioxygenase (CmtC)	p-cymene_degradation
Microbial community in the biofilter after raw OSPW treatment	0.31	Benzoyl-CoA oxygenase component B	Benzoate_transport_and_degradation_cluster
	0.22	Benzoyl-CoA-dihydrodiol lyase (BoxC)	Benzoate_transport_and_degradation_cluster
	0.08	Benzoyl-CoA oxygenase component A	Benzoate_transport_and_degradation_cluster
	0.02	Benzoate-CoA ligase (EC 6.2.1.25)	Benzoate_transport_and_degradation_cluster
	0.01	Toluene-4-monooxygenase, subunit TmoE	Toluene_4-monooxygenase_(T4MO)
	0.01	Aldehyde dehydrogenase (EC 1.2.1.3), PaaZ	Aromatic_Amin_Catabolism
	0.01	Toluene-4-monooxygenase, subunit TmoA	Toluene_4-monooxygenase_(T4MO)
	0.01	4-oxalocrotonate decarboxylase (EC 4.1.1.77)	Benzoate_transport_and_degradation_cluster
Microbial community in the biofilter after ozonated OSPW treatment	0.03	4-hydroxyphenylacetate 3-monooxygenase (EC 1.14.13.3)	Aromatic_Amin_Catabolism
	0.02	Benzoate degradation ring-cleavage hydrolase	Benzoate_transport_and_degradation_cluster
	0.02	Benzoyl-CoA oxygenase component B	Benzoate_transport_and_degradation_cluster
	0.02	4-cresol dehydrogenase [hydroxylating] flavoprotein subunit (EC 1.17.99.1)	Cresol_degradation
	0.01	Benzoate transport, extracellular ligand-binding receptor	Benzoate_transport_and_degradation_cluster

Table 4.3. Functional abundance for the metabolism of organic acids

	Percentages	Gene products	Pathway
Indigenous microbial community	0.07	2-hydroxy-3-oxopropionate reductase (EC 1.1.1.60)	Glycerate_metabolism
	0.05	2-methylcitrate synthase (EC 2.3.3.5)	Methylcitrate_cycle
	0.05	Pyruvate kinase (EC 2.7.1.40)	Glycerate_metabolism
	0.02	Propionyl-CoA carboxylase biotin-containing subunit (EC 6.4.1.3)	Propionyl-CoA_to_Succinyl-CoA_Module
	0.02	Methylisocitrate lyase (EC 4.1.3.30)	Methylcitrate_cycle
	0.02	2-methylcitrate dehydratase (EC 4.2.1.79)	Methylcitrate_cycle
	0.02	Predicted L-lactate dehydrogenase, Iron-sulfur cluster-binding subunit YkgF	Lactate_utilization
	0.02	Propionate--CoA ligase (EC 6.2.1.17)	Methylcitrate_cycle
	0.01	L-lactate permease	Lactate_utilization
	0.01	2-methylisocitrate dehydratase (EC 4.2.1.99)	Methylcitrate_cycle
	0.01	L-lactate dehydrogenase (EC 1.1.2.3)	Lactate_utilization
Microbial community in the biofilter after raw OSPW treatment	0.05	2-methylisocitrate dehydratase (EC 4.2.1.99)	Methylcitrate_cycle
	0.05	2-methylcitrate dehydratase FeS dependent (EC 4.2.1.79)	Methylcitrate_cycle
	0.05	2-methylcitrate synthase (EC 2.3.3.5)	Methylcitrate_cycle
	0.03	Propionate--CoA ligase (EC 6.2.1.17)	Methylcitrate_cycle
	0.03	Glyoxylate carboligase (EC 4.1.1.47)	Glycerate_metabolism
	0.03	Pyruvate kinase (EC 2.7.1.40)	Glycerate_metabolism
	0.03	Methylisocitrate lyase (EC 4.1.3.30)	Methylcitrate_cycle
	0.02	L-lactate dehydrogenase (EC 1.1.2.3)	Lactate_utilization
	0.02	2-hydroxy-3-oxopropionate reductase (EC 1.1.1.60)	Glycerate_metabolism
	0.01	Predicted L-lactate dehydrogenase, Iron-sulfur cluster-binding subunit YkgF	Lactate_utilization
	0.01	Hydroxypyruvate isomerase (EC 5.3.1.22)	Glycerate_metabolism
	0.01	PrpF protein involved in 2-methylcitrate cycle	Methylcitrate_cycle
	0.01	Tartrate decarboxylase (EC 4.1.1.73)	Glycerate_metabolism
	0.01	Propionyl-CoA carboxylase biotin-containing subunit (EC 6.4.1.3)	Propionyl-CoA_to_Succinyl-CoA_Module
Microbial community in the biofilter after ozonated OSPW treatment	0.03	Methylmalonyl-CoA epimerase (EC 5.1.99.1)	Propionyl-CoA_to_Succinyl-CoA_Module
	0.02	Hydroxypyruvate isomerase (EC 5.3.1.22)	Glycerate_metabolism
	0.01	Propionate--CoA ligase (EC 6.2.1.17)	Methylcitrate_cycle
	0.01	L-lactate dehydrogenase (EC 1.1.2.3)	Lactate_utilization
	0.01	Predicted L-lactate dehydrogenase, Fe-S oxidoreductase subunit YkgE	Lactate_utilization
	0.01	D-glycerate 2-kinase (EC 2.7.1.-)	Glycerate_metabolism

4.4 Conclusions

Biofiltration process is a promising approach for removing NAs from OSPW. 35.8% and 69.4% of NAs were removed from raw and ozonated OSPW by biofiltration with an equivalent hydraulic retention time of 16 hours. *Proteobacteria* and *Cryptomycota* were the dominant bacterial and fungal phyla in the established biofilter. The dominant bacterial class was *Alphaproteobacteria*, the abundance of which decreased from 34.9% to 18.3% with depth, but the abundance of *Anaerolineae* increased from 1.5% to 8.3%. The dynamics of bacterial community structure along the biofilter indicate that the treatment of OSPW was achieved by aerobic and anaerobic combined degradation processes. The abundance of aromatic compounds metabolism and organic acids degradation pathways in indigenous microbial community improved from 0.05% and 0.29% to 0.76% and 0.39% in the biofilter. Benzoate transport and degradation pathway may play an important role in NA degradation. The diverse microbial community structure and transcriptomic profiles can probably be the reason for the effective degradation of NAs from different OSPW by biofiltration.

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CHAPTER 5. GENERAL CONCLUSIONS AND RECOMMENDATIONS

5.1 Thesis overview

Canada has large oil reserves with billions of cubic meters of recoverable bitumen found deposited in the Athabasca region, northern Alberta. Bitumen is produced by using the alkaline hot water extraction technology, as a result of which, OSPW is generated at the same time. OSPW has been found to be toxic to a wide range of aquatic organisms and NAs have been considered the most important toxic organic compounds in OSPW. Due to the zero-discharge approach, OSPW is stored in the local tailing ponds and its volume keeps on increasing to such a large amount that it has posed a great threat to the local environment and public health. Technically practicable and economically feasible OSPW treatment processes are urgently demanded to be developed.

During the last few years, several studies have been performed to establish and assess different OSPW treatment methods. Generally, physical, chemical, biological and combined treatment processes have been evaluated for the remediation of OSPW. Physical and chemical treatment methods such as coagulation/flocculation, UV/H_2O_2, ozonation treatment processes are either high cost or too complicated to be applied at large scale, though those methods show high NA removal efficiency from OSPW. With clear advantage of cost-effective, biological treatment process has shown a high potential to be developed as an economical, energy-efficient, and environmentally friendly approach for OSPW reclamation. FBBR, MBBR, IFAS, MBR biological treatment processes have been developed for the treatment of OSPW. With the combination of mild ozonation OSPW

pretreatment, the NA removal efficiency of those treatment processes can be significantly improved.

The biofilm present in bioreactors has been found critical for efficient NA degradation. The attached growth biological treatment processes are supposed to benefit microorganisms for better adaption to harsh conditions and show faster NA degradation rate in the biofilter. However, the application of fixed-bed attached growth biological treatment process for OSPW treatment has not been studied previously. For the reason of robustness, ease, and simplicity of construction and low energy input, the biofilter has been widely used for wastewater treatment. It is important to fill the research gap to evaluate the application of fixed-bed attached growth biological treatment process for OSPW remediation. The research is expected to provide a better approach for the OSPW treatment for industrial applications.

Indigenous microorganisms based fixed-bed film reactors were developed in this study. By using qPCR and CLSM detection, the growth of microorganisms on the sand media was determined. With the application of the established fixed-bed biofilm reactors for OSPW treatment, the removal of NAs from OSPW was investigated using UPLC/TOFMS detection method. In addition, the impact of circulation times (i.e., hydraulic retention time) on NA removal was analyzed. With the combination of mild ozonation pretreatment, the improvement of NA biodegradation in the fixed-bed biofilm reactor was verified. The beneficial effect of biofiltration treatment on the mild ozonation removal of NAs was also investigated. The OSPW remediation efficiency by different biofiltration based processes (biofiltration; ozonation-biofiltration; biofiltration-ozonation-biofiltration) was systematically compared. By using next generation sequencing, the

dynamics of the microbial community structures in raw OSPW and in the fixed-bed biofilm reactors after different OSPW treatment were investigated. In addition, the metatranscriptomic sequencing was also performed for the investigation of the critical pathways in the microbial community for NA degradation.

5.2 Conclusions

Based on fixed-bed attached growth biological process, this study successfully developed a novel OSPW treatment method which is a promising approach for NA degradation and shows high potential to be scaled up for *in-situ* OSPW treatment. In this research, fixed-bed biofilm reactors were established by using indigenous microorganisms from OSPW. The performance of the biofiltration process on the treatment of OSPW was evaluated. With the combination of mild ozonation pretreatment, the improvement of OSPW treatment by biofiltration was investigated. In addition, the beneficial impact of biofiltration pretreatment on the ozonation removal of NAs from OSPW was analyzed as well. After different OSPW treatment, the changes of microbial community structures and functional pathway abundance in the biofilters were elucidated by next generation sequencing test. The findings from this study can be an important reference for future research on the biological treatment of OSPW.

The conclusions generated from the results of each study are listed:

5.2.1 Degradation of NAs from OSPW by a semi-passive biofiltration process

- With the assistance of qPCR and CLSM, indigenous microorganisms from OSPW were confirmed to attach to the surface of sand media and form biofilms. The number of total bacteria on the biofilter media reached a steady state (10^9/g) after 23 days of operation.

- UPLC/TOFMS analysis showed that 21.8% of the classical NA removal was achieved through the circulation of raw OSPW on the biofilter for 8 times (equivalent to a hydraulic retention time of 16 hours). When ozonation with utilized ozone dose of 30 mg/L was applied as pretreatment, the classical NAs in raw OSPW were removed by 92.7% with an accelerated biodegradation rate of 0.5 mg/L/h. Ozonation pretreatment improved the removal of NAs from OSPW by biofiltration.
- Compared with other biofilm reactors such as MBBR, ozonation pretreatment could benefit the biodegradation of NAs in the biofilter more (classical NA removal: 89.3% vs. 34.4%), especially for those with high carbon number and cyclicity. The ozonation combined treatment improved the removal of oxidized NAs from OSPW. Although both ozonation and biofiltration alone did not show degradation of oxidized NAs from raw OSPW, the combined process led to a 52.9% and 42.6% removal for O_3-NAs and O_4-NAs, respectively, which were the dominant oxidized NA species in OSPW.
- Metagenomic sequencing analysis showed that *Rhodococcus* was the dominant bacterial genus on the sand media, which may play a crucial role in the NA biodegradation.
- With the advantage of high NA removal efficiency, the combined ozonation-biofiltration process is a promising approach for NA degradation and shows high potential to be scaled up for *in-situ* OSPW treatment.

5.2.2 Removal of NAs from OSPW by ozonation integrated biofiltration

- Biofiltration showed higher degradation efficiency on classical NAs than oxidized NAs. With the biofiltration pretreatment, the classical NAs from raw OSPW

decreased from 13.1 mg/L to 9.9 mg/L. However, the oxidized NAs showed high resistance to the biofiltration process as the concentration of overall oxidized NAs was stable.

- Biofiltration pretreatment can improve the degradation efficiency of mild ozonation on classical NAs. Compared with the ozonation (with utilized ozone dose of 30 mg/L) of raw OSPW with the same utilized ozone dose, the degradation of classical NAs from biofiltrated OSPW remarkably improved from 32.1% to 84.8%. The ozonation efficiency reached 0.3 mg classical NAs/mg O_3, which was higher than the ozonation efficiency (0.1 mg classical NAs/mg O_3) on raw OSPW.
- Biofiltration pretreatment could improve the degradation efficiency of mild ozonation on oxidized NAs. Compared with the ozonation of raw OSPW, the ozonation removal ratio for O_3-NAs and O_4-NAs from biofiltrated OSPW increased from no removal to 41.0% and from 9.0% to 10.7%, respectively.
- With the pretreatment of raw OSPW by biofiltration, the TOC in OSPW decreased indicating that the ozone scavenger may be removed which can probably be the reason for the improved efficiency of the following ozonation step on NA degradation.
- Compared with the biofiltration pretreatment, the classical NA removal ratio of the post biofiltration process was decreased from 24.4% to 6.7%, and the removal rate was decreased from 0.4 mg/L/h to 0.0 mg/L/h. It was probably caused by the low influent NA concentration, as a threshold level of NAs exists for microorganisms to initiate the biodegradation process. However, the post biofiltration process showed

higher degradation effect on oxidized NAs (removal ratio: 22.9% vs. 3.3%; removal rate: 0.4 mg/L/h vs. 0.1 mg/L/h).

- Microbial community structure analysis showed that *Proteobacteria* and *Rhodococcus* were dominant bacterial phylum and genus in the biofiltration system.
- With biofiltration pretreatment, the utilized ozone dose is expected to be reduced to achieve the same NA removal effect as the ozonation of raw OSPW, so the capital cost of the whole process may be reduced which could further improve the potential for the ozonation combined biodegradation process to be applied in industry.

5.2.3 Systematic analysis of the microbial community in biofilters for OSPW reclamation

- 16S rRNA and 18S rRNA gene segments targeted metagenomic sequencing analysis showed that *Proteobacteria* and *Cryptomycota* were the dominant bacterial and fungal phyla in the established biofilter. The microorganisms from those phyla may be the main player for the degradation of NAs from OSPW. The coexistence of bacteria and fungi may benefit NA removal through catabolic cooperation.
- The bacterial community at the top layer of biofilter had the highest species richness. According to the characteristics of the biofilter, nearly all suspended matter in influent water was trapped at the schmutzdecke level, which could be the reason for the microbial community on the top layer had the highest alpha diversity.
- The dominant bacterial class on the top, middle and bottom level of the biofilter was *Alphaproteobacteria*, which occurred at 34.9%, 22.2%, and 18.3%, respectively, decreasing with depth. Conversely, the percentage of *Saprospirae* and *Anaerolineae* class increased from 1.8% to 9.9% and from 1.5% to 8.3%, from the top to bottom,

respectively. Besides the aerobic and anaerobic combined conditions in biofilm, the distribution of the aerobic and anaerobic microorganisms along the biofilter also indicated that OSPW in biofilter was biodegraded by aerobic-anaerobic combined treatment processes.

- The bacterial communities in the biofilter had higher alpha diversity than the indigenous bacterial community, indicating the enrichment of bacteria in the biofilter. Beta diversity analysis showed that after raw and ozonated OSPW treatment, the bacterial communities the bacterial communities appear different which confirms that the bacterial community structure in the biofilter can be impacted by the feed of different OSPW. The indigenous fungal community had higher alpha diversity than the fungal community in the biofilter, suggesting that only fungal species which could adapt to the condition in the biofilter can survive. Beta diversity analysis confirmed that the fungal community in the biofilter can also be impacted by the feed of different OSPW.
- Metatranscriptomic sequencing analysis revealed that the functional abundance of aromatic compounds metabolism and organic acids degradation pathways in the indigenous microbial community were improved from 0.05% and 0.29% to 0.76% and 0.39% in the biofilter. In addition, the abundance of benzoate metabolism pathway for aromatic compounds metabolism increased from 0.04% to 0.64% in the indigenous microbial community after raw OSPW treatment indicating that this pathway played a critical role in NA biodegradation.
- The diverse microbial community structure and transcriptomic profiles may be the reason for the effective degradation of NAs from different OSPW.

5.3 Recommendations

According to the progress of this research on the development of fixed-bed biofilm reactors for OSPW treatment, recommendations for future study on OSPW biofiltration treatment are proposed here:

- The overall capital cost for the establishment and operation of an OSPW treatment process is one of the main concerns for its application by the industry. By using petroleum-coke or native soil materials as bed media, the capital cost for the establishment of biofilter *in situ* is expected to be reduced. The biofilters using petroleum-coke or native soil materials as media is recommended to be developed and applied for OSPW treatment. The performance of those new biofilters on NA degradation is recommended to be evaluated and compared with the sand biofilter established in this study.
- It has been verified that ozonation pretreatment can improve the removal of NAs from OSPW by biofiltration. The ozonation combined biofiltration process showed high efficiency on NA removal, the cost for ozonation pretreatment is expected to be further reduced though. Solar-based AOPs can potentially be an alternative to ozonation to be combined with biofiltration for developing a cost-effective process for OSPW reclamation. It is recommended to establish solar-based AOPs combined biofiltration processes for OSPW treatment. The performance of the novel OSPW treatment processes is recommended to be evaluated and compared with the ozonation combined biofiltration processes established in this study.
- Temperature can impact the performance of the biofiltration process, and biofilter is normally recommended to be operated at mesophilic temperature. The local

temperature will be an important impact factor for the application of biological OSPW treatment process *in situ*. Therefore, it is recommended to evaluate the performance of NA degradation by the biofiltration process at low temperature (4 ^{o}C). As the biofiltration process operated at low temperature is expected to have reduced efficiency on NA removal, it is also recommended to optimize the development and establishment of OSPW biofiltration treatment process at 4 ^{o}C. The impact of HRT and nutrients addition on the performance of OSPW biofiltration operated at 4 ^{o}C is recommended to be evaluated and optimized.

- With 16S/18S rRNA sequencing and metatranscriptomic sequencing analysis, the specific microorganisms and functional pathway adopted by microorganisms which play important role in NA biodegradation were investigated from nucleic acid level. However, the main players for NA degradation in the biofilter are expected to be determined at a deeper level. Metagenomic sequencing which has higher taxonomical resolution than 16S/18S rRNA sequencing is recommended to be used to investigate the microbial community structure in bioreactors.
- *Rhodococcus* was found as the most dominant bacterial genus in biofilters after raw and ozonated OSPW treatment indicating that bacteria from *Rhodococcus* genus played a key role in the biodegradation of NAs. It is recommended to evaluate the NA degradation performance by using potential bacterial species from *Rhodococcus* genus. It is expected to determine the bacterial species from *Rhodococcus* genus which may show efficient NA degradation capacity. Based on the model of NA degradation by *Rhodococcus*, it is also recommended to use metabolomic and proteomic analysis to further elucidate the main functional enzymes for NA

degradation from protein level. The pathway for NA degradation is expected to be clarified as well.

Indigenous microorganisms based biofiltration system has been successfully developed for the treatment of OSPW. Mild ozonation combined biofiltration process showed high efficiency on NA degradation which verified that ozonation treatment could degrade recalcitrant NA fraction and improved the biodegradation of NAs from OSPW. Mild ozonation integrated biofiltration process could also remove NAs from OSPW effectively. And it was found that biofiltration treatment could improve ozonation efficiency on NA degradation through the removal of ozone scavenger from OSPW. *Rhodococcus* was determined as the dominant bacterial genus in the biofiltration system, and benzoate metabolism pathway may play a critical role in NA degradation. In our future study, we will investigate the performance of other media (e.g., native soil) based biofiltration systems on the degradation of NAs from OSPW. The beneficial impact of other AOPs (e.g., solar-based photocatalytic oxidation) treatment on the biofiltration of OSPW will be evaluated. We will also try to identify the microorganisms and enzymes which play the main role in NA degradation. In a word, the biofiltration process is a promising method for the treatment of OSPW which can potentially be scaled up by the establishment of engineered wetlands.

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APPENDIX A. EXPERIMENTAL METHODS

A-1 DNA extraction and qPCR detection

PowerSoil® DNA isolation kit (Mo-Bio Laboratories, Inc., CA, USA) was used for bacterial DNA extraction from sand media (0.25 g) according to the manufacturer instructions. The DNA sample was stored at -20 °C prior to qPCR analysis. qPCR was conducted with a Bio-Rad CFX96 real-time PCR system with a C1000 Thermal Cycler (Bio-Rad Laboratories, ON, CA). Forward primer 341F (CCTACGGGAGGCAGCAG) and the reverse primer 534R (ATTACCGCGGCTGCTGG) were applied to quantify the copy number of total bacteria (He et al., 2007). A total reaction volume of 20 μL was used for each reaction including 10 μL of 2 × SsoFast EvaGreen Supermix, 6 μL of sterile water, 1 μL of each primer with a concentration of 10 μM, and 2 μL of diluted sample DNA. The qPCR program consisted of an initial 3 min denaturation at 95 °C, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 45 s, and extension at 72 °C for 30 s was used to amplify the 16s rDNA genes of total bacteria. All PCR runs used plasmid standards for quantification. Standard plasmids containing target genes were constructed using a TOPO TA Cloning kit (Invitrogen Corporation, Carlsbad, California, USA). Reactions without the DNA template were used as negative controls.

A-2 CLSM detection

Confocal laser scanning microscopy (CLSM, Zeiss LSM 710, Carl Zeiss Micro Imaging GmbH, Germany) was performed to characterize the deposited microbial cells and extracellular polymeric substance (EPS) on sand media which were taken from the top of the filter. SYTO 9 (excitation wavelength (ex) = 488 nm; emission wavelength (em) =

522/32 nm) and ConA conjugated with Texas Red (ex = 568 nm; em = 605/32 nm) were used to stain bacterial cells and EPS, respectively. 1 mL of distilled water containing 3 μL each of SYTO 9 and ConA were prepared as staining solution to immerse the sand media for 20 minutes. Unbound fluorescent dyes were rinsed carefully with distilled water before CLSM observation. Biofilm images were acquainted and scanned randomly at 4 or 5 positions with a lens (20× 0.8NA Plan-Apochromat). Biofilm thickness measurements were performed under Z-stack mode. A series of z-axis images were generated through optical sectioning at a slice thickness of 1 μm.

A-3 Next generation sequencing

Illumina MiSeq sequencing was used to further understand microbial communities related to OSPW biodegradation on the sand media. All sequencing was performed at the Research and Testing Laboratory (Lubbock, TX, USA). Samples were sequenced following the manufacturer's guidelines using Illumina MiSeq sequencing instruments and reagents. Raw data, FASTA data, and analysis data were provided by the company along with a methodology to analyze the data. Briefly, after DNA extraction, the V1-V2 hypervariable regions of 16S rRNA genes were amplified for sequencing using a forward primer 28F and reverse fusion primer 338 R prior to being loaded on an Illumina MiSeq (Illumina, Inc. San Diego, California). An automated pipeline was used to process raw MiSeq data. After denoising (USEARCH application) and chimera removal (UCHIIME in de novo mode), the sequences were clustered into operational taxonomic units (OTU) clusters with 100% identity (0% divergence) using USEARCH for taxonomic identification. The trimmed sequence data were also processed with Quantitative Insights Into Microbial Ecology (QIIME, http://qiime.org) pipeline. After quality checks and open reference picking, OUT

table was used to perform diversity analysis, such as Chao1 index, Shannon index, and Observed species. A distance matrix was constructed by using the unweighted pair group method with arithmetic mean (UPGMA) to interpret the relationships among different samples.

A-4 Calculations for HRT

Glass columns (25 mm internal diameter and 15 cm bed depth) were served as fixed-bed biofilm reactors. The EBCT of the fixed-bed biofilm reactors were set for 120 minutes.

Volume of sand media: $V = 1/4\pi d^2 h = 1/4 \times 3.14 \times (2.5\ cm)^2 \times 15\ cm = 73.58\ ml$

Influent flow rate of OSPW: $Q = V/T = 73.58\ ml / 120\ min = 0.61\ ml/min$

HRT = n × EBCT; n: number of circulating times; EBCT = 120 min.

APPENDIX B. SUPPORTING FIGURES

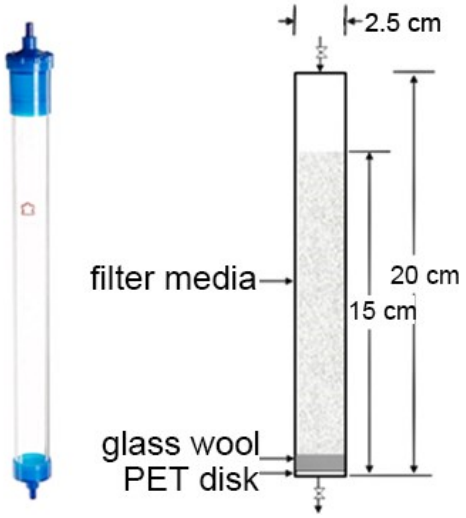

Figure B1. The schematic diagram of the fixed-bed biofilm reactor.

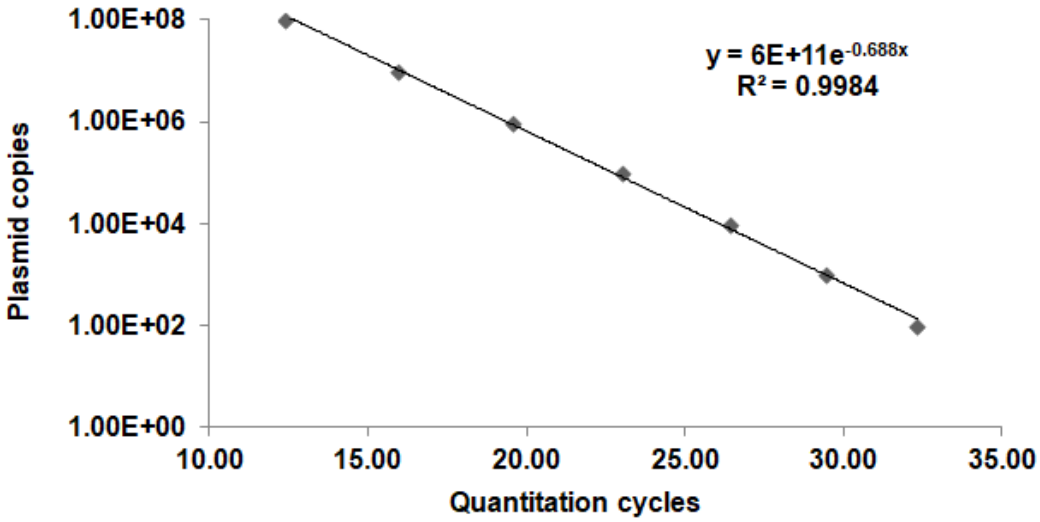

Figure B2. Standard curve for the qPCR detection method.