The impact of various ozone pretreatment doses on the performance of endogenous microbial communities for the remediation of oils sands process-affected water

by

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ABSTRACT

In this study, the effects of different ozone pretreatment doses on the performance of endogenous microbial populations in degrading naphthenic acids (Nash and Traver) for the treatment of oil sands process-affected water (OSPW) were evaluated. The results showed that ozonation enhanced the biodegradability of OSPW and the maximum COD removal through biodegradation which occurred at utilized ozone dose of 50 mg/L. After pretreatment with the highest utilized ozone dose of 200 mg/L and bioreactor operation for 73 days, the batch bioreactor removed more than 80% of the chemical oxygen demand (COD), it also removed greater than 95% of the acid extractable fraction (AEF) from the OSPW. High-resolution mass spectrometry analysis showed complete degradation of NAs with specific degrees of cyclization (Z= -2 and -4) after combined treatment of ozonation and biodegradation. Furthermore, with increasing the utilized ozone, the total bacterial number increased while specific bacterial strains such as *Sphingomonadaceae*and *Azoarcus* have shown to improve the bioreactor performance. At high pretreatment utilized doses of ozone (116 and 200 mg/L), the biodegradation completely eliminated the acute toxicity of OSPW towards *Vibrio fischeri* (IC_{20}> 100% v/v). In summary, increasing the ozone dose used for pretreatment of OSPW showed clear benefits regarding the removal of organic compounds, the growth of bacteria, and the reduction of toxicity.

PREFACE

This thesis is an original work by Tao Dong. No part of this thesis has been previously published.

DEDICATION

This work is dedicated to my wife, Miss Xuejiao Yang, for her love and encouragement keeping me motivated in the whole process of my education; to my mom, who always supports and stands behind me during the difficult time; to my mother and father in law, I can feel their great love always with me in my whole life; and to my adorable son, I will continue to strive for the better future to you.

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List of Symbols, Nomenclature and Abbreviations

·OH	Hydroxyl radicals
AMO	Ammonia Monooxygenase
AOB	Ammonia Oxidizing Bacteria
AOPs	Advanced Oxidation Processes
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
DI Water	Deionized Water
DGGE	Denatured Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
AEF	Acid Extractable Organic Fraction
EPA	Environmental Protection Agency (United States)
GC-MS	Gas Chromatography – Mass Spectrometry
HPC	Heterotrophic plate count
HRMS	High Resolution Mass Spectrometer
IC	Ion Chromatography
NAs	Naphthenic Acids
NCBI	National Center for Biotechnology Information
NF	Nanofiltration
NOB	Nitrite Oxidizing Bacteria
OSPW	Oils Sands Process-affected Water
PCR	Polymerase Chain Reaction
qPCR	Real Time Polymerase Chain Reaction

RO	Reverse Osmosis
rpm	round per minute
TOC	Total Organic Carbon
TSS	Total Suspended Solids
UF	Ultrafiltration
UPLC	Ultra Performance Liquid Chromatography
v/v	volume per volume

CHAPTER 1 INTRODUCTION

With the decline in conventional light oil reserves worldwide, the development of alternative resources for oil production is becoming more attractive. One of the world's largest single accumulations of oil sands was discovered in the shallow reservoirs of Canada. It is estimated that there are 173.2 billion barrels of recoverable bitumen within the oil sands of the Athabasca Basin (Frank, 2008). However, most oil sands refineries use large volumes of hot, alkaline water when extracting the bitumen(Schramm et al., 2000; Hadwin et al., 2006) which generates large amounts of wastewater, known as oil sands process-affected water (OSPW) (Hadwin et al., 2006), that may lead to acute and chronic aquatic toxicity if the untreated OSPW is released into the environment. As a result, OSPW is currently retained in large tailings ponds. On a very conservative estimate, there will be 1 billion m^3 of tailings pond water accumulated over the next 15–20 years (MacKinnon 1989; Herman et al., 1994; Lo et al., 2003; Del Rio et al., 2006).

The major constituents of concern in OSPW are suspended and dissolved solids, hydrocarbons, salts, metals and organic acids (Nash and Traver) (Zubot, 2012). Naphthenic acids (NAs) and naphthenates have long been considered as the primary toxic components of tar sands deposits and OSPW (Schramm et al., 2000), Whitby (2010) demonstrating both acute and chronic toxicity to a variety of organisms including plants, fish, amphibians, zooplankton, phytoplankton, mammals (e.g., rats and guineapigs), and bacteria (Vibriofischeri). Except for highly toxic to organisms, there are several problems associated with NAs. First of all, NAs are corrosive and can also form metal naphthenate precipitates, which block pipelines. Secondly, due to the presence of carboxylic acids

within NAs mixtures the increased total acid number in petroleum product eventually affects the rate of oil and the commercial value. Therefore, removal of carboxylic acids from heavy oil sand the super heavy oils in tar sands is of great economic interest. Consequently, the industry faces the large costs to reduce NA concentrations during petroleum-refining processes as well as reducing NA concentration and toxicity in NA-contaminated environments to acceptable levels (Holowenko et al., 2002; MacKinnon, 1989). In order to reuse or safely discharge tailings pond water, a single water treatment method may not be efficient or economical to remove all the contaminants in OSPW. Certain combined water treatment processes like coagulation-flocculation, sedimentation, filtration, adsorption and biological degradation, as well as advanced treatment options such as ozonation and biological treatment, are possible candidates for OSPW treatment.

1.1 Hypothesis

- ✧ We hypothesize that ozonation is capable of breaking the most bio-persistent NA fraction (such as highly branched and cyclic carboxylic fraction of NAs) and mitigating the toxicity of OSPW that are benefit for the subsequent biological process. Therefore, the combined treatment is a promising application in reclamation of OSPW.

1.2 Objectives of the whole project

The final goal of current study is to establish an effective and efficient process for the treatment of OSPW.

- ✧ To evaluate the effect of utilized ozone dose on the detoxification and biodegradation of OSPW.

- ✧ To differentiate the role of ozonation and biodegradation in removal of organic compounds in OSPW.
- ✧ To investigate the change of microbial communities structure and reduction of OSPW toxicity.

CHAPTER 2 LITERATURE REVIEW

2.1 Alberta's Oil Sands

Oil sands are a mixture of sand, clay, water and bitumen. Oil sands deposits in Alberta, Canada, are estimated to be the second largest oil reserves in the world. They are located in three geological regions: Athabasca, Cold Lake, and Peace River. It is estimated that there are 173.2 billion barrels of recoverable bitumen within the oil sands of the Athabasca Basin (Frank et al., 2008), enough to satisfy the world's demand for petroleum for the next century.

Unlike conventional crude oil, bitumen in Alberta's oil sands is a thick, tar-like mixture of hydrocarbons that contains high levels of sulphur and nitrogen (Strausz, 2003). Further, bitumen cannot be piped to the surface unless it is heated or diluted. Meanwhile, bitumen must also be upgraded into crude oil which is used to produce gasoline, aviation fuel, or other products by further refineries (Timoney, 2001). Currently, two tonnes of oil sands produce one barrel of oil based on current techniques. Oil sands production is increasing drastically. Oil sands production stands at around 1 million barrels per day; by 2020 oil production will increase to 3 million barrels per day; and 5 million barrels per day are estimated by 2030 (Kelly et al., 2010). The oil sands industry is and will continue to be an important driver of economic development in Alberta, Canada.

Oil sands development provides significant economic benefits to Canada including employment, economic stability, government revenue, and investment in research and development. However, the oil sands industry also brings with numerous environmental and social impacts associated to their mining and purification processes. These include river water consumption for the oil sands extraction; the contamination on the quality of

surface and groundwater; greenhouse gases and other air emission; land remediation and reclamation, and the impacts on wildlife. To minimize these environmental impacts, solutions are needed to ensure sustainable development of oil sands reserves.

2.2 Oil Sands Process-Affected Water and Environmental Issues

Large volumes of water are needed to support oil sands mining and subsequent processes. Data shows that for every cubic meter of mined oil sands, a volume of 3 m^3 of water is required; and approximately 4 m^3 of slurry waste consisting of sands, clays, organic residual bitumen, and process-affected water is produced as a by-product of oil sands production - mainly from the bitumen extraction process (Giesy, 2010). Currently, approximately 85% of the water taken from the river can be recycled to satisfy bitumen extraction, process cooling and hydro-transport requirements. Under zero discharge policy, no oil sands tailings are allowed to be released into ground or surface water supplies. These tailings ponds will eventually be treated for reuse or safe release in the future. Water treatment options for increasing reuse and recycle achieving to lower imports from river water, as well as remediation of current tailings pond water for safe discharge are required.

The major constituents of concern in the OSPW are suspended and dissolved solids, hydrocarbons, salts, metals and organic acids such as NAs (Smith, 2008). Although currently more than 85% of OSPW is recycled, the quality of OSPW has impacts on some operation processes and equipment. It is known that divalentions such as calcium and magnesium impact the ability of the extraction process to recover bitumen. Therefore, higher quality water is still needed to be imported from the river to feed the boiler, cooling tower, firewater, potable water and various miscellaneous uses (Zubot, 2007).The

oil sands industry's goal is to minimize the amount of water withdrawn from the river, to reduce water usage, and to reuse process-affected water more effectively. The objective of OSPW treatment is to improve water quality to efficiently recycle in the production processes; to reduce the amount of river water withdrawals by reuse OSPW for utilities and otherequipment uses; to remove toxicities to meet the guidelines for the protection of aquatic ecosystems; and to release treated water in the future to avoid largest orage on-site (Allen, 2008).

2.3 Naphthenic Acids and its Toxicity

The oil sands process-affected water (OSPW) is of wide concern due to its toxic property and relative environmental problems. The acute and chronic aquatic toxicity of OSPW has been attributed primarily to a relatively persistent group of carboxylic acids known as naphthenic acids (Nash and Traver). NAs can enter environments from a number of different sources such as effluent discharge, crude oil spills and erosion of riverbank oil deposits (Headley and McMartin, 2004). These recalcitrant compounds have been reported to be potential carcinogenic and mutagenic health hazards on humans, animals and plant life (Frank, 2008; van Gestel, 2001). Consequently, high concentrations of NAs in a wide range of aquatic systems have been found including the Athabasca River (0.1–0.9 mg/L; Schramm et al., 2000) and groundwater aquifers (>55 mg/L; Conrad, 1998). NA concentrations in OSPW may reach to120 mg/L, and long-term storage of OSPW causes severe environmental risks (Clemente, 2003; Quagraine et al., 2003). Therefore, due to the highly toxic and recalcitrant properties of naphthenic acids, it is important to develop efficient remediation strategies to decrease both their abundance and toxicity in the environment.

The NAs found in crude oils (which typically contain thousands or tens of thousands of components) contain impurities with various levels of unsaturation and aromaticity (Mohamed et al., 2008), and generally contain small amounts of acyclic aliphatic (paraffinic or fatty) acids with aromatic olefinic, hydroxyl, and dibasic acids as minor components (Hsu et al., 2000) (Dzidic et al., 1988). The NAs are essentially complex mixture of predominantly alkyl-substituted acyclic and cyclo-aliphatic saturated carboxylic acids (Clemente and Fedorak, 2005; Smith et al., 2008). They are carboxylic acids, with a -COOH group which is usually bonded as a side chain instead of directly to the cyclo-aliphatic ring (Headley and McMartin, 2004), represented by a chemical formula of R-$(CH_2)_m$-COOH, where 'R' represents saturated, single or multi ring structures predominantly containing 5 or 6 carbon atoms in various combinations and the $(CH_2)_m$ is the carbonyl chain (Holowenko et al., 2001). The structure of NAs could also include an aliphatic alkyl group (Clemente and Fedorak, 2005) and their acyclic components are highly branched (Rudzinski et al., 2002). The parent NAs are most frequently represented by a general formula $C_nH_{2n+z}O_2$, where n is the carbon numbers and z represents zero or negative even integers that specify homologous series of the compound (Frank et al., 2009; Scott et al., 2005; Smith et al., 2008). The value of z reflects the number of lost hydrogen atoms resulted from the ring formation (Rogers et al., 2002) and its absolute value divided by 2 gives the number of rings (i.e., number of saturated rings = z/2) that may be fused or bridged. The parent NAs can be categorized into acyclic, monocyclic, bicyclic and polycyclic families based on their z-groups and number of rings (Brient et al., 1995). Figure 1 shows structural diagrams of typical NAs for z = 0 (acyclic), −2 (monocyclic), −4 (bicyclic), −6 (tricyclic).NAs found in OSPW are

in the range of n=7–30; Z = 0 to <-12 (Gabryelski and Froese, 2003; Hao et al., 2005). Although, the general formula $C_nH_{2n+z}O_2$ implies that NAs contain a single carboxyl group (Bataineh et al., 2006; Clemente and Fedorak, 2005), studies have considered NAs to also include those compounds which have more than one carboxyl group (Lutnaes et al., 2007; Smith et al., 2007).

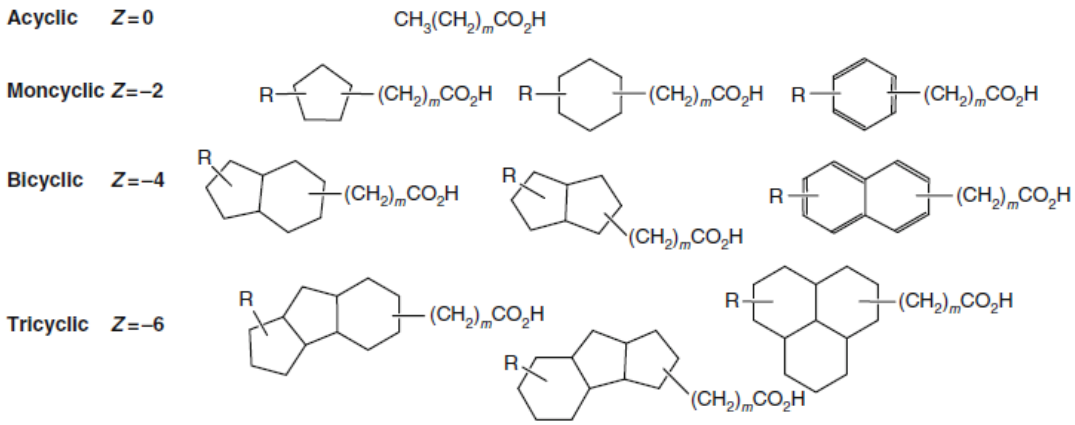


Figure 1. *Examples of aromatic and nonaromatic NA structures. R: is a small aliphaticgroup such as methyl; Z is loss of hydrogen atoms due to ring formation; m is thenumber of CH_2 units (Modified from Whitby, 2010).*

NAs and naphthenates have significant detrimental effects in aquatic environments (Peters et al., 2007). NAs are toxic to fish at concentrations >2.5–5 mg/L, and high concentrations of NAs (2.8 mg/kg) have been detected in rainbow trout (Young et al., 2007). NAs are also toxic to aquatic algae and other microorganisms (Frank et al., 2008).The degree of toxicity is generally related to NA molecular weight, with higher molecular weight acids often demonstrating acute toxicity in the MicroTox test (Holowenko et al., 2002). This may be due to higher molecular weight NAs having an increased carboxylic acid content, which decreases their hydrophobicity and thus become more bioavailable and bio-accumulate in the cells (Frank et al., 2008). Acute toxicity of

NAs may also be related to the surfactant properties of NAs whereby the NA compounds penetrate the cell wall, disrupting the membrane lipid bilayer, or change the membrane properties (Frank et al., 2009; Schramm et al., 2000). As compared to aquatic organisms, there is limited information about the toxicity of NAs on mammals. For human, the lethal dosage of NAs reported is 11 g/kg (Rockhold, 1995). Similarly as rats, the oral LD_{50} (lethal dose of a substance that results in 50% mortality) value is 3.0–5.2 g/kg of bodyweight for commercial NAs, with death caused by gastrointestinal disturbances (Pennisi et al., 1977). The observed symptoms on mammals were increased vascular permeability in capillaries, effects on the formation of red and white blood cells, efflux of potassium from cells causing first stimulation then inhibition of cellular respiration, central nervous system depression, convulsion, gastrointestinal disturbances, hepatoxicity and respiratory arrest (Rogers et al., 2002).

2.4 Analytical Techniques of Naphthenic acids

Initially, NAs quantification was achieved using Fourier transform infrared (FT-IR) spectroscopy method. However, it is the major problem that FT-IR method estimates total extractable organic acids in the samples instead of NAs. Mass spectrometry (MS) is the most common tool for analyzing the NAs mixtures, which characterize the NAs by a relative response of each mass (i.e., m/z value) corresponding to a particular combination of n and Z (Martin et al., 2008). These methods require extensive physical work including liquid-liquid extraction and solid-phase extraction (SPE) for detecting NAs (Bataineh et al., 2006). The major MS techniques are gas chromatography (GC)-MS, electron ionization (EI)-MS, liquid secondary ion (LSI)-MS, GC-electron impact mass spectrometry (EIMS), fast atom bombardment (FAB)-MS, chemical ionization (CI)-MS,

atmospheric pressure chemical ionization (APCI)-MS, electrospray ionization (ESI)-MS, ultra pressure liquid chromatography (UPLC)-high resolution mass spectroscopy (HRMS), and high performance liquid chromatography (HPLC)-HRMS (Holowenko et al., 2000; Barrow et al., 2004; Bataineh et al., 2006; Lo et al., 2006; Martin et al., 2008)

2.5 Possible Water Treatment Technologies for OSPW

Several conventional water treatment technologies such as physical-chemical treatment including coagulation-flocculation, sedimentation, adsorption, membrane filtration, chemical oxidation and biological treatment have been recently studied as possible OSPW treatment technologies for the oil sands industry. The choice of the treatment method utilized is heavily dependent on the constituents of OSPW, industry processes, cost, and reuse or release regulations and demands. It is potential that a combination of conventional and advanced treatment technologies will achieve these challenging goals. The advantages, limitations and possibilities of these water treatment technologies are discussed as follows.

2.5.1 Physical-Chemical Treatment

Basically, physical-chemical treatments such as coagulation-flocculation and sedimentation, adsorption, ion-exchange, filtration, and chemical oxidation, have been widely used in municipal and industrial water and wastewater treatment. These processes can be used to effectively remove suspended and dissolved solids, to oxidize inorganic species and organic compounds, and to decrease the toxicity of the wastewater to meet the needs of high quality effluent (AWWA, 1999).

2.5.1.1 Coagulation-flocculation

Coagulation-flocculation is a low cost and easy-to-operate water and wastewater treatment process. It is used primarily for the removal of tiny particles or colloids, which are too small to settle by gravity in a reasonable time. Small particles in water are also difficult to settle down because they have negative charges on the surface that create repelling forces, and therefore prevent agglomeration and settling - making it a stable system. Pourrezaei (2011) performed as a pre-treatment for remediation of oil sands process-affected water by use of alum and cationic polymer poly DADMAC under Coagulation/flocculation (CF) process. The results indicated that CF process significantly reduced concentrations of naphthenic acids (Nash and Traver) and oxidized NAs by 37 and 86%, respectively, demonstrating the applicability of CF pre-treatment to remove a persistent and toxic organic fraction from OSPW.

2.5.1.2 Adsorption Process

Due to its high carbon content, petroleum coke can be activated to produce activated carbon which is a common adsorbent in water and wastewater treatment (DiPanfilo and Egiebor, 1996). Zubot (2010) studied the adsorption of petroleum coke (PC) (a carbon-based material >80 wt. % and sulphur >6 wt. %) to remove NAs from OSPW and their associated toxicity. Results showed the PC was effective at adsorbing the more structurally complicated NAs (i.e., $12 \geq n \geq 18$, $z=-10$ and -12), which is similar to the results (i.e., preferential sorption of NAs with n range of approximately 13 and 17) observed by Janfada et al., (2006).

Besides, activated carbon (such as GAC) based on porous materials was studied as an adsorbent to remove NAs from OSPW (Janfada et al., 2006; Gamal El-Din et al., 2011).

Martienssen and Simon (1996) studied the effect of activated carbon on the biological treatment of oil-water emulsions which contained high concentrations of emulsified mineral oil, stabilizers and different additives. It showed that about 60% of the influent TOC was reduced during the first activated carbon treatment process. Mohamed et al. (2008) studied the sorption of NAs in solution using GAC and three types of synthetic materials. The sorption range for GAC was 100 to 160 mg NAs/g of GAC.

2.5.1.3 Membranes Filtration

Membrane filtrations including nanofiltration (Janfada et al., 2006), reverse osmosis (RO), ultrafiltration (Ledakowicz et al., 2006), and microfiltration (van Gestel, 2001) are able to remove a wide range of constituents such as suspended solids, colloidal organic matters, hydrocarbons, NAs, dissolved solids, salts, trace metals, and hardness. However, since it requires membrane replacement, this technology is more costly than other techniques.

In water and wastewater treatment, membranes technologies are the most effective desalination methods (Greenlee et al., 2009).It was reported that NF had been applied successfully for treatment of oil sands waters particularly for water softening and the removal of toxic components such as NAs (Peng et al., 2004). A bench-scale flat sheet membrane system with several commercially available NF membranes was applied to remove hardness and NAs from both imported and potential discharge waters. It was found that after membrane filtration more than 95% of water hardness and NAs were reduced.NAs, hardness, TDS, aromatic hydrocarbons can also be removed by applying reverse osmosis (RO) but the associated problem with these processes is fouling from oil, dissolved organics (Allen, 2008). Sierka and King (1986) investigated four membrane

types: cellulose acetate, poly-ether amide, poly-ether urea, and non-cellulose on a poly-sulfone base, with a two-stage RO process for wastewater treatment from the oil sands extraction process. In the RO process, a maximum of 98% organic rejection and 97% of inorganic rejections were achieved after a sequence of pretreatments that include: coagulation, activated carbon adsorption and ozonation.

2.5.2 Chemical Oxidation

Chemical oxidation is used to: reduce inorganic species, reduce hazardous organic compounds, destroy taste- and odor-causing compounds, and eliminate color. The most common chemical oxidants used in water treatment are chlorine, chlorine dioxide, ozone, peroxide and permanganate. Advanced oxidation processes (AOPs) are based on the generation and the use of highly reactive hydroxyl radicals (·OH) to oxidize organic and inorganic substances, which are otherwise very recalcitrant to conventional oxidation processes (AWWA, 1999). Chemical oxidation technologies are capable of degrading petroleum contaminants.

Ozonation is one of the several methods to significantly degrade the recalcitrant OSPW NAs within a practical time period (Scott et al., 2005). However, due to the complex nature of the wastewaters, organic substances cannot be completely mineralized by O_3, which can also result in the formation of several by-products, such as aldehydes, ketones, peroxides, carboxylic organics (Petala et al., 2008). Scott et al. (2008) tested the effectiveness of ozonation of the Athabasca OSPW. With 50 minutes exposure of OSPW to ozonation, the resulting effluent was effectively non-toxic (based on the microtox bioassay) with decreased NA concentrations by approximately 70%.With 130 minutes of ozonation, higher molecular weight NAs ($n \geq 22$) decreased by over 95 % together with

increase in the proportion of lower molecular weight NAs (n = 5–13) by over 75 %. He et al. (2010) examined the effect of ozonated OSPW on sex steroid production (known as steroidogenesis). Results indicated that ozonation could be an effective treatment to reduce NA concentrations in OSPW without increasing affects on steroidogenesis.

2.5.3 Biological Treatment

Aerobic biological treatment is a process used to provide secondary and (in some cases) tertiary treatment of effluent (Mara and Horan, 2003). After primary treatment via liquid-solid separation, dissolved and some suspended organic matter is still present in effluent. As microorganisms consume the organic matter, they also consume oxygen or create an oxygen demand. The resulting low oxygen or hypoxic conditions negatively affect the receiving environment (Chan et al., 2011). Additional oxygen demand is exerted by other constituents in wastewater. As organic nitrogen (N) is broken down to the ammonium (NH_4^+) form which creates a demand for oxygen as microorganisms convert the ammonium form to nitrate (NO_3^-) through nitrification (Bassin et al., 2011). Thus, aerobic treatment systems reduce oxygen demand in effluent by providing naturally-occurring microorganisms with sufficient dissolved oxygen to consume organic matter and convert ammonium nitrogen to the nitrate form. The efficiency of processes primarily depends on the biomass concentration and specific conversion rate of the microorganisms (Manem and Sanderson, 1996). In most basic form, biological treatment systems are divided into two categories: suspended growth and attached growth systems (Stephenson et al., 1993).

2.5.3.1 Suspended Growth - Biological Treatment

Suspended growth treatment systems freely suspend microorganisms in water and wastewater (Vesilind, 2003). They use biological treatment processes in which microorganisms are maintained in suspension within the liquid. They also comprise aggregates of microorganisms generally growing as flocs in wastewater which are responsible for the removal of polluting material and comprise a wide range of microbial species (Mara and Horan, 2003). A typical suspended growth aerobic treatment system includes aeration basins into which air is injected. Air injection mixes and brings the suspended microorganisms into contact with the organic matter and dissolved oxygen. Suspended growth treatment systems permit the exploitation of the full range of microbial metabolic capabilities. The microbes oxidize the organic matter into carbon dioxide, new microbes and insoluble matter (*residuals*) (Ramothokang et al., 2004). The most prevalent and important of microorganisms are the bacteria, the protozoa and the metazoan. Microbes complete their life cycle while suspended in the effluent (Spellman, 2013). Meanwhile, the full spectrum of redox environment from aerobic, through anoxic to anaerobic can be found within the floc itself, but they can also be created by appropriate process reactor design (Asenjo, 1994).

A suspended growth wastewater treatment process is a biological reactor which has been engineered to encourage the growth of specific types of microorganisms that are able to undertake the reactions necessary to achieve purification of the wastewater influent. Suspended growth treatment can be accomplished in a variety of ways. The primary difference among the optional configurations is how effluent flows through the component and how the biomass is managed. Each option may incorporate additional

design modifications to achieve nutrient reduction. Thus, four basic configurations for suspended growth aerobic treatment systems are included: Complete-mix suspended growth; Sequencing batch reactors (SBR); Membrane bioreactors (MBR) and Integrated fixed-film/activated sludge (IFAS).

2.5.3.2 Attached Growth - Biological Treatment

The biological treatment of industrial wastewater by suspended growth process, as activated sludge, may encounter in several cases poor bioflocs and, as a result, a high concentration of biosolids in the effluent, leaving for a relatively low amount of biomass. Therefore, attached growth systems are considered to be of good performance and good resistance under effects of high organic load conditions (Grady et al., 2011). Like suspended growth biological wastewater treatment systems, attached growth systems use microorganisms to remove organic matter from a wastewater stream (Loupasaki and Diamadopoulos, 2013). The primary difference is that, in an attached growth system, the microorganisms are retained and grown on inert support mediums. Attached growth could create the biofilm on the support media to provide better treatment efficiency due to accumulation of high microbial population in the presence of large surface area (Rittmann and McCarty, 2012; Kermani et al., 2008). The microorganisms secrete sorts of natural polymer to facilitate firmly adhesion on support matrix for biofilm development and bio-oxidation mechanism (Wang et al., 2005; González-Martínez and Duque-Luciano, 1992). In addition, biofilm systems have higher effluent quality with limited biomass washed out and lower maintenance and equipment costs than suspended growth systems (Jiang et al., 2009; MetCalf and Eddy, 2003; Jianlong et al., 2000). The shape and size of biomass-supporting media can also play a significant role in the design

of biofilm processes in order to meet an obligatory surface area for microbial growth (Nabizadeh et al., 2008; Moore et al., 2001). Based on the above advantages, attached growth aerobic treatment systems have been widely used for the treatment of domestic and industrial wastewaters in large and small scale systems (Khanh et al., 2011; Hu and Gagnon, 2006; Zhao and Wang, 1996).

2.5.3.3 The Mechanism of NAs degradation By Biological Treatment

Microbial degradation of NAs in aerobic conditions is generally the most cost-effective way of reducing both the toxicity and other undesirable characteristics of NAs from wastewaters (Scott et al., 2008). However, little is known about the mechanism by which NAs degradation in OSPWs takes place (Holowenko et al., 2002; Whitby, 2010). Recent technological advances have enabled the quantification and characterization of NAs found in contaminated environments (Headley et al., 2009; Quagraine et al., 2005); and developments in molecular ecology mean more sophisticated analysis of NA-degrading microorganisms and their processes is now possible. During NA degradation, an increase in the formation of oleic, linoleic, palmitic, and steric acids has been widely concerned (Biryukova et al., 2007; Clemente et al., 2004), because these fatty acids are common constituents of prokaryotic and some eukaryotic membranes and predominate particularly in NA degrading microorganisms (Clemente et al., 2004; Lechevalier 1988). However, the recent analysis of crude oils from different sources also showed an increase in the formation of recalcitrant hopanoic acids, which is thought to be mainly derived from the bacteria that are responsible for the biodegradation of crude oils (Meredith et al., 2000).

Several papers showed that microorganism including *Acinetobacteranitratum*, *Alcaligenesfaecalis*and *Pseudomonas putida*have capability of metabolizing model NAs

such as the cyclohexane-carboxylic-acid (CCH) through β-oxidation pathway (Blakley, 1974; Blakley et al., 1982; Rho and Evans, 1975). So, various mechanistic pathways have been proposed for the biodegradation of aliphatic and alicyclic carboxylic acids including β-oxidation (Quagraine et al., 2005), combined α- and β- oxidations (Rontani et al., 1992) and aromatization (Taylor and Trudgill, 1978). For all the three pathways, the β-oxidation pathway is the preferred route by which most microorganisms degrade aliphatic and alicyclic carboxylic acids (including NAs) (Herman et al., 1994; Quagraine et al., 2005), which involves the formation of new carboxylic acids with two carbons fewer than its predecessor.

In more recent findings, it has been suggested that biodegradation rates of NAs are affected primarily by their chemical structures. The more recalcitrant NAs are those with higher degree of alkyl substituted aliphatic chains (Han et al., 2008; Whitby, 2010), 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; Whitby, 2010), increased cyclicity (Han et al., 2008), evenness of carbon side chain, and *cis*-isomerism in alicyclic acids (Holowenko et al., 2002; Headley et al., 2002). Generally, lower molecular weight acids are more readily degraded than their higher molecular weight counterparts (Biryukova et al., 2007).

Abiotic factors such as nutrient availability, temperature, oxygen concentration, pH, salinity, redox potential, and sunlight should also be consider because they may influence NA degradation as well. The biodegradation of NAs by microorganisms is crucial, especially for the removal of recalcitrant higher molecular weight NAs in polluted

environments (Lai et al., 1996). Therefore, a better understanding of NA-degrading microorganisms and their processes is required.

2.5.4 Evaluation of treatment technologies for OSPW

The applicability of possible physical, chemical and biological treatment technologies for OSPW is summarized in Table 1. The advantages and limitations are listed in Table 2.

Table 1: Applicability of various water treatment technologies for OSPW (Crittenden, 2005)

Class	Unit treatment process	Typical application in water treatment
Physico-chemical	Coagulation-flocculation-sedimentation	Removal of suspended solids, some colloidal organic matters, color, metals
	Activated carbon Adsorption	Removal of dissolved organic dissolved solids
	Ozonation	Decomposition and destruction of hydrocarbons, organic acids;control of odors; removal of ammonia
	Nanofiltration	Removal of dissolved submicrometer particles (0.001 to 0.01μm) by size exclusion, removal of natural organic matter, NAs, hardness, ammonia, bacteria

		and protozoa in OSPW
	Reverse osmosis	Removal of dissolved submicrometer particles (0.0001 to 0.005μm) by size exclusion, removal of salts, everything except water in OSPW
Physico-biological	Biological activated carbon filtration	Dual purpose of particle removal by filtration and removal of biodegradable organic matter by biological oxidation; removal of hydrocarbons, naphthenic acids, ammonia in OSPW
Biological	Microbiological degradation	Decomposition and destruction of organic matter; degradation oforganics and NAs in OSPW

Table 2: Advantages and limitations of various water treatment technologies (Sorgini, 2007)

Technology	Advantages	Limitations
Coagulation-flocculation-sedimentation	Removes major suspended solids, turbidity and color Easy to operate Low cost	Chemicals required Temperature affected More labor needed

Media filtration	Simple, easy to operate Cheap few labor required Media can last longer time	Effluent quality varies Feed water conditions affected Large volumes of wastewater during backwash
Membrane filtration	Physical barrier to pathogens, biosolids and turbidity Simple and automated operation Few labor required	Requires pretreatment Higher cost than granular media filters Will require membrane replacement at some point
Reverse osmosis	Reduces total dissolved solids, salts Provides water suitable for disinfection High-quality water treatment method	Requires pretreatment Fouling by suspended solids costly
Ozonation	Chemical-free method No labor required	Health, safety, and environmental issues with chemical handling Produces disinfection by-products
Biodegradation	Low cost Easy to operate	Slow degradation rate incomplete degradation

2.5.5 Combined Ozonation and Biological Processes

Ozone preferentially breaks down complex compounds into simpler compounds that are easier to degrade for the subsequent biological process. Compared with other advanced oxidation process, ozonation does not leave chemicals to the water and the high amount of oxygen release in ozonated water could greatly benefit the microbial community. Gamal et al. (2011) shows ozone decreased the amount of NA in OSPW while increasing its biodegradability. Martin et al. (2010) found ozonation followed by the inoculation of native microbes result in a significant acceleration of toxicity removal and NA biodegradation in batch reactors. More recently, Hwang, Dong et al. (2013) observed over 99.9% of parent NAs removal using ozonation followed by continuous bio-film reactors, indicating combined ozonation and biological treatment have a good potential for OSPW treatment. However, to our knowledge, low dosage of ozone utilized may not be enough to break up the recalcitrant NAs while high dosage of ozone applied doesn't seem an economical way. Recent study shows that prolonged 5h ozonation of oil contaminated sand and peat did not substantially improve the subsequent biodegradability and its application can be considered senseless given the high ozone consumption. Furthermore, high dose of ozone could be disadvantageous to biodegradation. With the increase of ozone dose, a sharp rise followed by a slow decrease of the BOD was observed for the treatment of phenolic pollutants, indicating ozonation must be carefully controlled before bio-treatment is encouraged because redundant ozonation leads to low molecular weight carboxylic acids that do not show enhanced biodegradability. Moreover, by-products of ozonation could be even more toxic than the parent compounds. For

instance, the ozonated naphthalene sulphonic acid derivative (<1 kDa) caused increased toxicity towards the marine algae *Phaeodactylumtricornutum*. In many cases, the toxicity of by products depends on ozone dose. Previous study shows the toxicity of ozonated resin acids solutions decreased with increasing applied ozone dose up to about 0.3 - 0.5 mg O_3/mg COD, thereafter increased. Therefore, the ozone dose should be optimized in order to maximize the subsequent biodegradability and avoid the increase of toxicity.

2.5.6 Knowledge Gap and Trends in OSPW Research

To our knowledge, little research has been undertaken to determine the effect of differing ozone doses on the degradation of parent NAs and on the subsequent biologically mediated NAs degradation using endogenous microorganisms in OSPW. For instance, a utilized ozone dose that is too low may not adequately break down the recalcitrant NAs, while a utilized ozone dose that is too high would be unfeasible economically. Previous study showed that prolonged, five-hour ozonation of oil-contaminated sand and peat did not substantially improve its biodegradability (Goi et al., 2006). A high dose of utilized ozone could actually be disadvantageous to biodegradation, as seen in one study where an increase in ozone dose led to a sharp rise followed by a slow decrease in the biochemical oxygen demand (BOD) during the treatment of phenolic wastewater (Amat et al., 2003). This finding indicated that ozonation before bio-treatment must be carefully controlled, to avoid redundant ozonation which generates carboxylic acids of low molecular weight that do not show enhanced biodegradability (Amat et al., 2003). Moreover, by-products of ozonation can be even more toxic than the parent compounds, such as when an ozonated naphtalenes ulphonic acid derivative (<1 kDa) was shown to exhibit increased toxicity toward the marine algae *Phaeodacty lumtricornutum* (Germirli Babuna et al.,

2009). Previous study investigating ozonated resin acid solutions showed a limit of 0.3 - 0.5 mg O_3/mg COD before toxicity increased (Ledakowicz et al., 2006). Another study related to the degradation of an azo dye, observed the increase in toxicity after 150 of the total 360 minute ozone treatment (Wang et al., 2003). For the treatment of OSPW, where biodegradability and toxicity are both of paramount concern, there is still need for more work to determine the optimal utilized dose of ozone for pre-treatment prior to biodegradation.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

Raw OSPW was obtained from Syncrude's West Inpit Pond (WIP) located in Fort McMurray, Alberta, Canada. OSPW samples were fresh and surface water collected at the WIP tailings pond by Syncrude's staff. The samples were then delivered to the Natural Resources Engineering Facility (NREF) at the University of Alberta.

All chemicals were of analytical reagent grade conforming to specifications of the Committee of Analytical Reagents of the American Chemical Society. De-ionized (DI) water was used in all the experiments.

3.2 Experimental Methods

3.2.1 Sample Collection and Storage

All the raw and ozonated OSPW samples were reserved in barrels (200 L) or small glass barrels (20 L) in a cold room maintained at 4 °C. The samples in 200 L barrels were mixed evenly using a LIGNTNIN Model L mixer before being transferred to 20L glass barrels. The samples were then moved out of

the cold room one night prior to conducting experiments, so the OSPW could reach room temperature. The OSPW samples were mixed again before being transferred to each bioreactor. All standard sampling methods were used in this research. Lab wares were rinsed with DI water and dried before use.

3.2.2 Batch experiment overview

Six Erlenmeyer flasks of 2,000mL capacity were designed and operated as bioreactors to degrade and remove NAs from OSPW. Each reactor containing 1,000mL of OSPW was autoclaved to inactivate endogenous microorganisms. Ozone was then injected into each of the six reactors for varying lengths of time and the final utilized ozone concentrations were calculated following the procedure described by Gamal El-Din, Fu et al. (2011). To achieve standardized levels of endogenous bacterial strains in each bioreactor, separate 1,000mL batches of raw OSPW were centrifuged at 4000 × g for 20 minutes and the pellets were collected and inoculated into the test bioreactors. The reactors were operated at room temperature (20 ± 1.0^{o}C) and were rotated at 150 rpm on a shaker. Water samples were collected periodically for the analysis of the residual organic compounds, using measurement of the chemical oxygen demand (COD), acid-extractable fraction (AEF), and NAs. The biomass in the reactors was also collected to identify the structure and population of the microbial communities. All batch experiments were conducted in duplicate.

In the second study, the effect of different nutrient conditions on the survival and activity of NAs-degrading strains in OSPW and ozonated OSPW was investigated experimentally. Batch reactors of NAs biodegradation were designed and operated. Each reactor contains 1000 ml of OSPW which has been previously autoclaved to inactivate any

microorganisms. Ammonium Chloride was added into the reactors at two different levels, reaching to 30 and 130N-mg/L (total nitrogen concentration in aqueous phase). Endogenous bacterial strains were obtained by centrifuging 1000 ml OSPW under 4000g for 20 min, and then inoculated into each reactor.The reactors were operated at room temperature (20°C) and rotated at 150 rpm on shaker.Water was sampled and analyzed for residual organic contents and NAs by chemical methods, and the quantity, composition and diversity of bacteria was monitored by PCR-DGGE and Real time PCR technology. The study of the effect of nutrient addition on these mixture cultures during NAs degradation is of interest to optimize their activity and to elucidate the mechanism of co-metabolism. Therefore, water chemical analysis and NAs concentration tests were set up to examine the response of indigenous strains under different inorganic nutrient supplements. To assess the reproducibility of the results, batch experiments were repeated.

3.2.3 Ozonation of OSPW

To evaluate the effect of ozone dose on OSPW biodegradation, each batch reactor except for one (0 concentration for "raw OSPW" as a control), was ozonated for different times at the beginning of the experiment in order to reach the desired utilized ozone doses. An ozone generator (GSO-40, Herford, Germany) was used to produce ozone gas using extra dry, high purity oxygen. Each 2,000 mL-glass reactor was equipped at the bottom with a ceramic fine-bubble gas diffuser, enabling the feed gas to be sparged into the liquid phase with a flow rate of 1 L/min. The ozone concentrations in the feed and off-gas lines were continuously monitored during the process by two identical ozone monitors (model HC-

500, PCI-WEDECO, USA), while the ozone residual in the reactor was measured using the Indigo method(APHA, 2005).

The utilized ozone dose for this system was calculated using the following equation (Gamal El-Din and Smith 2002):

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

where ΔO_3 is the amount of utilized ozone (mg/L), $C_{G,in}$ and $C_{G,out}$ are the ozone concentrations (mg/L) in the feed and off-gas respectively (both obtained from the first ozone monitor), C_L is the residual ozone concentration in the liquid phase (mg/L), V_L is the effective reactor volume (L), $Q_{G,in}$ is the feed-gas flow rate (L/min), $Q_{G,out}$ is off-gas flow rate (L/min), and t is ozone contact time (Balkwill, Fredrickson et al.). Based on this calculation, the final ozone concentrations for each bioreactor were 0, 18, 50, 78, 116 and 200 mg/L, respectively.

3.2.4 Water chemistry analysis

3.2.4.1 pH

The pH of the OSPW was measured daily using an Accumet® AR 20 pH/conductivity meter (Cole-Parmer, Il, USA) connected to a digital pH scale (Orion 720A, Thermo Fisher Scientific, Inc, MA).

3.2.4.2 Dissolved oxygen (DO)

Dissolved oxygen (DO) was measured daily by a Hach model dissolved oxygen meter (Hach Incorporated, USA). The COD was determined every three days according to the Standard Methods (APHA, 2005).

3.2.4.3 Ion chromatography(IC)

Concentrations of anions and cations were determined by ICS-2500 and ICS-2000 Ion Chromatography (Diones, Sunnyvale, CA, USA). Aliquots (10 mL) of each sample were filtered through a 0.2µm nylon filter and placed in vials of the auto sampler. The flow rate of the effluent was 1 mL/min with an injection volume of 25 µL.

3.2.4.4 Chemical Oxygen Demand (COD)

COD is used as a nonspecific measure of the organic matter content of a liquid sample (APHA 2005).The closed reflux COD method was used in this project. 3.5mL of digestion reagent (35mM potassium dichromate and 3.0 M sulfuric acid) and 2mL of Micro-COD reagent (add Ag_2SO4 to concentrated sulfuric acid at the rate of 5.5 gAg_2SO4/kg H_2SO4) (APHA, 2005) were added to a 10mL sealed test tube with 2mL of the sample. The tightly closed test tubes including samples, standards and blanks were mixed and then placed in the COD reactor for 2-hour-digestion at 140^{o}C. After samples were cooled, the spectrophotometer was used to measure the absorbance at 600 nm. Standards and blanks were used to prepare a Standard Curve to determine COD of the sample. All the samples were analyzed in duplicates and their average was recorded as the final result.

3.2.4.5 Acid extractable organic fractions (AEF)

The AEF of OSPW includes parent NAs ($C_nH_{2n+Z}O_2$), oxy-NAs ($C_nH_{2n+Z}O_x$, with x = 3 to 5), and other organics containing carboxylic groups. The residual concentrations of the AEF in the OSPW were determined every six days using Fourier Transform Infrared (FT-IR) spectroscopy (Nicolet 8700, Thermo Electron Corporation, Waltham, USA) which is widely applied to characterize the functional groups present in OSPW (Han et al., 2008).

Briefly, 50mL aqueous samples were acidified with sulfuric acid to pH 2–2.5 and then extracted twice with a total volume of 50mL dichloromethane. The extract was combined, evaporated to dryness, and reconstituted in a known volume of dichloromethane for FT-IR analysis. The absorbance of the monomeric and dimeric forms of the carbonyl groups, represented by peak values at 1,743 and 1,706 cm^{-1} respectively, were measured (Grewer et al., 2010). Fluka NAs mixture (Fluka, Sigma-Aldrich Canada, Inc., Oakville, ON, Canada) served as a standard.

3.2.4.6 NAs analysis

High-resolution mass spectrometry was used to identify and quantify individual NAs. A 10mL aqueous sample was filtered through 0.22 μm syringe filters (Millex GS, Millipore) and 50mL of 0.2μg/mL tetra-decanoic acid-1-^{13}C ($C_{14}H_{28}O_2$) was added as an internal standard to correct for sensitivity and retention time drift. The parent NAs and their oxidized products were separated by a Waters Acquity UPLC System (Waters, MA) based on carbon number (n) and degree of cyclization (Z). In-line measurements were performed with a high resolution (~10,000) QSTAR

Pulsar i mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystem/MDS Sciex, Concord, ON, Canada) operated in a negative mode. To determine the profile and concentrations of the NAs, a relative response to the internal standard for each NA isomer class was plotted over time.

3.2.5 Microbiological analysis

3.2.5.1 Bacterial enumeration using heterotrophic plate count (HPC)

Bacterial enumeration was performed by HPC using the drop plate method (Zelver et al., 1999; Liu et al., 2007).A series of 10-fold dilutions were performed and 10 mL of each

dilution was plated in triplicate on R2A agar (Voigt Global Distribution Inc., KS, USA). Plates were incubated at 31°C for 24 hours and held at room temperature for another three days. Counting was performed after 24 hours (for fast-growing bacteria) and again after a four-day period (for total number of bacteria). The lower detection limit was 10^2 CFU/mL. Welch's t-tests (two-sample unequal variance) were performed in Microsoft Excel™ 2007 to examine the statistical significance of the results. P values less than 0.05 were indicative of a statistically significant difference.

3.2.5.2 DNA Sample extraction

DNA extraction was conducted on each batch reactors every six days; 50mL of samples were centrifuged at 4,000g for 20 minutes.Total 16s RNA gene was extracted with the Power Soil Kit (MO-BIO, Carlsbad, CA, USA) according to the manufacturer's instructions. Extracted DNA was eluted to a final volume of 100 µL. The quality and quantity of the DNA extraction were evaluated by nanodrop.

3.2.5.3 Real-time polymerase chain reaction (q-PCR) assay

Biomass samples were collected from each batch bioreactor every six days by centrifuging 50 mL water samples at 4,000 × g for 20 minutes. Microbial genomic DNA was extracted from the pellets with a Power Soil Kit (MO-BIO, Carlsbad, CA, USA) according to the manufacturer's instructions and eluted to a final volume of 100 µL. The quality and quantity of the DNA extracts were evaluated by nanodrop before the real-time PCR and PCR-DGGE assays. Real-time PCR was carried out in a CFX 96 Touch™ Real Time PCR Systems (Bio-Rad Laboratories Inc., USA). The reaction mixtures contained 0.5µM of each primer for *nar*G, and 0.3 µM for 16S rDNA amplification, 12.5 µl of SYBR Green PCR master mix, including HotStarTaq™ DNA Polymerase, Quanti

Tec SYBR Green PCR Buffer, dNTP mix with dUTP, SYBR Green I, ROX and 5mM $MgCl_2$ (QuantiTect^{TM} SYBR$^{®}$ Green PCR Kit, QIAGEN, France), 2.5µl of DNA diluted template corresponding to 25ng of total DNA, and RNase-free water to complete the 25µl volume.

The conditions of 16S rDNA real-time PCRs were 180 s at 95 °C for enzyme activation and 35 cycles of 10 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C for denaturation, annealing and extension steps, respectively.

The conditions of ammonia monooxygenase were 5 min at 95 °C; then 35 cycles consisting of 30 s at 94 °C (denaturation), 40 s at 47 °C (annealing), and 40 s at 72 °C (elongation); and a final cycle of 2 min at 72 °C. The conditions of nitrite oxidoreductase were 3 min at 94 °C followed by 35 cycles of 94 °C for 30 s (denaturation), annealing at 55 °C for 45 s and elongation at 72 °C for 45 s with terminal elongation at 72 °C for 5 min.

Fluorescence produced by the binding of the SYBR Green fluorochrome to the double-strand DNA was measured at the end of each data acquisition step for narG and at each extension step for 16S rRNA gene (excitation 450–495 nm, emission 505–537 nm). A ten-fold dilution series of a purified 16S rRNA PCR product gene with known concentration were used for standard curve creation. The negative controls (without DNA template) were included in all qPCR reactions.

3.2.5.4 Polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE)

After DNA extraction, 16s rRNA gene-based polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE) was applied to analyze microbial communities

involved in the biological treatment of raw and ozonated OSPW. Fragments of 16s rRNA genes having with the sizes of ~550 bps from each DNA sample were amplified from each DNA sample. An equal amount of PCR product (600ng) from each sample was loaded on the DGGE gel. Selected bands were retrieved from the DGGE gel and sequenced before each sequence was matched against the NCBI nr nucleotide database using the nucleotide BLAST program. A neighbor-joining phylogenetic tree was calculated and constructed using TREECON (Van De Peer and De Wachter, 1994).Known strains were also included in the tree for reference. More detailed information about PCR-DGGE assay is included in the Supplementary Data.

3.2.6 Toxicity measurements

The acute toxicity of the OSPW samples toward *Vibrio fischeri* was measured using a Microtox® 500 Analyzer (Azur Environmental, Carlsbad, USA) with an incubation time of 5 minutes following the 81.9% Basic Test protocol (Azur Environmental, Microtox Omni™ Software manual). All materials were purchased from Azur Environment (Carlsbad, CA, USA). The percentage inhibition after incubation and the toxicological parameter IC_{20} (effective volume percent concentration required to cause 20% inhibition of bacterial luminescence) were calculated from the change in luminescence intensity. The toxicity of phenol standard (100 mg/L) was used as quality control to verify the level of sensitivity of the luminescent bacteria prior to the analyses of the OSPW samples.

CHAPTER 4 RESULTS AND DISCUSSION

4.1. Chemical properties of raw and ozonated OSPW prior to biodegradation

4.1.1 pH and inorganic chemical composition

Table 3 summarizes the pH and concentrations of anions and cations in the raw and ozonated OSPWs. When the concentration of ozone was above 78 mg/L, the pH values of the OSPW increased slightly (from 8.98 to 9.24) after ozonation; the lower concentrations of ozone had less impact on the pH value. The pH increase of OSPW after ozonation can be attributed to the direct reaction of ozone with some inorganic and organic compounds through redox reactions leading to the formation of OH^- (Beltran, 2003; Rivas et al., 2003).

Table 3. The initial[a] and final[b] concentrations (mg/L) of chemical constituents in each reactor

Composition	Fresh OSPW		Ozonated OSPW 18mg/L		Ozonated OSPW 50mg/L		Ozonated OSPW 78mg/L		Ozonated OSPW 116mg/L		Ozonated OSPW 200mg/L	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
PH	8.98 ± 0.15	8.76 ±0.24	8.96 ± 0.18	8.73 ±0..27	8.98 ± 0.13	8.72 ± 0.05	9.24 ± 0.16	8.73 ± 16.56	9.28 ± 0.14	8.61 ± 0.17	9.24 ± 0.06	8.66 ±0.22
Na^{+}	833.57 ±15.43	829.76 ±14.34	854.26 ±11.78	851.33 ± 12.57	874.69 ± 12.13	871.32 ± 17.03	886.54 ± 17.76	877.41 ± 16.56	898.78 ±13.26	887.41 ±14.47	923.64 ±17.36	917.41 ±13.32
Ca^{2+}	12.75 ± 0.41	12.51 ±0.72	14.80 ± 0.11	14.04 ± 0.35	15.43 ± 0.21	15.07 ± 0.01	16.88 ± 0.35	16.06 ± 0.24	18.48 ± 0.65	18.06 ± 0.14	22.63 ± 0.82	22.70 ± 0.61
K^{+}	17.98 ± 0.48	17.41 ± 0.22	17. 81 ± 0.18	17.31 ± 0.32	17. 34 ± 0.26	17.02 ± 0.44	17. 04 ± 0.17	16.82 ± 0.06	17.21 ± 0.88	16.71 ± 0.54	17.01 ± 0.72	17.46 ± 0.74

Mg^{2+}	12.47 ± 0.41	11.68 ± 0.90	12.60 ± 0.45	12.78 ± 0.30	12.66 ± 0.25	12.68 ± 0.12	12.63 ± 0.80	12.91 ± 0.72	12.69 ± 0.70	12.72 ± 0.33	12.70 ± 0.50	12.94 ± 0.32
NH_4^+	3.35 ± 0.39	n.d[c]	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Li^+	0.22 ± 0.01	0.19 ± 0.01	0.21 ± 0.02	0.20 ± 0.01	0.24 ± 0.03	0.18 ± 0.02	0.23 ± 0.01	0.21 ± 0.02	0.24 ± 0.04	0.20 ± 0.02	0.24 ± 0.02	0.21 ± 0.03
Cl^-	527.00 ± 6.05	541.14 ± 6.25	537.60 ± 8.05	545.33 ± 6.25	539.77 ± 8.05	555.33 ± 7.45	538.61 ± 5.74	574.31 ± 8.91	540.61 ± 4.37	576.22 ± 6.64	544.84 ± 5.17	571.12 ± 8.34
SO_4^{2-}	419.03 ± 6.81	436.68 ±6.24	421.54 ± 4.81	436.14 ± 7.71	423.47 ± 5.67	445.54 ± 5.44	426.66 ± 4.98	468.05 ± 3.00	429.39 ± 7.81	453.47 ± 8.23	431.72 ± 4.81	478.46 ± 6.83
NO_3^-	33.73 ± 0.02	28.48 ±0.59	34.14 ± 0.18	26.29 ± 0.49	35.44 ± 0.08	25.79 ± 0.39	35.72 ± 0.42	23.34 ± 0.87	36.64 ± 0.46	22.66 ± 0.34	37.04 ± 0.36	21.38 ± 0.89
F^-	2.44	2.55	2.39	2.49	2.42	2.63	2.33	2.60	2.50	2.65	2.48	2.66

	±0.14	±0.27	±0.08	± 0.17	±0.16	± 0.24	± 0.03	± 0.04	± 0.13	± 0.05	± 0.12	± 0.08
Br^-	0.23 ± 0.04	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

[a]Initial values were determined after ozonation.

[b]Final values were determined after 73 days operation time in bioreactors.

[c]n.d denotes not determined.

The inorganic chemicals existing in OSPW after surface mining are mainly from ore digestion (leaching of ions) and the extraction and tailings processes (recycling from settling basins) (Allen, 2008; Han et al., 2008). Concentrations of sodium, calcium, chloride, and sulphate increased slightly after ozonation. No significant change in concentration was observed for potassium, magnesium, lithium, nitrate, and fluoride ions after ozonation (p value > 0.05). Additionally, it was noticeable that the concentrations of ammonium and bromide reduced to below the detection limit, while the concentration of nitrate increased, indicating that ammonium was likely oxidized by ozone to nitrate.

4.1.2 Chemical oxygen demand

Compared with the COD of raw OSPW (266.5 mg/L), the COD of ozonated OSPW decreased to 244.0 ±1.0 mg/L (8.4% decrease), 230.5 ±1.1 mg/L (13.5%), 224.0 ± 2.1 mg/L (15.9%), 210.5 ± 2.2 mg/L (21.0%) and 165.0 ± 2.4 mg/L (38.1%) when the dose of ozone was 18, 50, 78, 116 and 200 mg/L, respectively (Table 4). These results are comparable to previous OSPW ozonation studies (Gamal El-Din et al., 2011; Pérez-Estrada et al., 2011; Hwang et al., 2013), confirming that as the applied ozone dose increases, a higher proportion of organic compounds in OSPW is oxidized and mineralized.

Table 4 Effect of ozone doses on the removal of organic compounds (mg/L)

Ozone (mg/L)		**0**	18	50	78	116	200
COD Removal	Ozonation	0	22.5 (8.4%)[a]	36 (13.5%)	42.5 (15.9%)	56 (21.0%)	101.5 (38.1%)
	Biodegradation	104.5 (39.2%)	121 (49.6%)[b]	130.5 (56.6%)	130 (58.0%)	122.5 (58.2%)	120.5 (73.0%)

	Combined	104.5 (39.2%)	143.5 (53.8%)[c]	166.5 (62.5%)	172.5 (64.7%)	178.5 (67.0%)	222 (83.3%)
	Ozonation	0	7 (11.0%)	21.3 (33.6%)	27.6 (43.5%)	37.8 (59.6%)	54.1 (85.3%)
AEF Removal	Biodegradation	12.8 (20.2%)	15.7 (27.8%)	5 (11.9%)	7 (19.6%)	5 (19.5%)	6.5 (69.9%)
	Combined	12.8 (20.2%)	22.7 (35.8%)	26.3 (41.5%)	34.6 (54.6%)	42.8 (67.5%)	60.4 (95.3%)
	Ozonation	0	N/A	6.05 (55.7%)	N/A	8.46 (77.9%)	N/A
NAs Removal	Biodegradation	2.41 (22.2%)	N/A	1.29 (26.8%)	N/A	0.86 (35.8%)	N/A
	Combined	2.41 (22.2%)	N/A	7.34 (67.6%)	N/A	9.32 (85.8%)	N/A

a The removal rate caused by ozonation

b The removal rate caused by biodegradation

c The removal rate caused by the combined treatment ozonation and biodegradation c=1-(1-a)(1-b)

It is well known that ozonation leads to either direct reactions, through ozone attacking branching points of readily ozone-reacting compounds, or to indirect reactions where recalcitrant pollutants react with formed hydroxyl radicals (•OH) (Hoigne and Bader, 1983). Any difference in COD removal efficiency can be caused by diverse reaction constants of complex compounds with ozone, which can vary by many orders of

magnitude. Generally, the difference in susceptibility for different organic molecules originates from the electronic configuration of the ozone molecule, which promotes oxidation-reduction, dipolar cycloaddition, and electrophilic substitution reactions in water and wastewater (Beltran, 2003). 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. The unsaturated compounds presenting π electrons, such as in olefinic compounds, are readily available for addition reactions with electrophilic compounds. The second (indirect) type of ozone reaction can also play an important role, although the extent will also depend on the concentration of fast ozone-reacting compounds and •OH, the way they are generated, the presence of inhibiting substances, and the water's pH. It has been demonstrated that •OH radicals play a leading role in the degradation of aliphatic carbon chain while molecular ozone is only reactive with readily oxidized compounds such as aldehyde and ketone (Glaze, 1987).

4.1.3 Acid-extractable Orgaanic fraction

AEF values are commonly used by the oil sands industry to evaluate the concentrations of classic and oxidized NAs and other compounds with carboxylic acid, ketone, and aldehyde functional groups (Jivraj et al., 1995). Ozone pre-treatment reduced the initial AEF concentrations by 7.0 mg/L (11.0% reduction), 21.3 mg/L (33.6% reduction), 27.6 mg/L (43.5% reduction), 37.8 mg/L (59.6% reduction), and 54.1 mg/L (85.3% reduction) (Table 4). The FT-IR spectroscopy results showed absorbance at 1,746 and 1,711 cm^{-1} (monomeric and dimeric forms of the carbonyl groups), indicating that ozone broke the

carbonyl group (C=O) present in the OSPW (Han, Scott et al. 2008). The carbonyl groups may have undergone the direct reactions with ozone as described above; some research has suggested that ozone as an electrophilic agent can attack a ketone or aldehyde (nucleophilic position of organic molecule) through an electrophilic reaction (Riebel et al., 1960; White and Bailey, 1965; Erickson et al., 1969). The formed •OH radicals can also react with the carbonyl group (Whiting et al., 1968; Denisov et al., 1975). The higher percent removals of AEF compared to COD was also observed by other researchers (Gamal El-Din et al., 2011; Hwang et al., 2013) and indicates the conversion of components of the AEF to smaller organic compounds that can still contribute to COD.

4.1.4 Naphthenic acids

In this study, only the parent NAs (not oxidized NAs or also referred to as oxy-NAs) adhering to the formula $C_nH_{2n+Z}O_2$ were quantified. Fig. 2 shows the three-dimensional plots of NAs concentration versus carbon number (n) andspecific degrees of cyclization (*Z* value) after ozonation (Figs. 2A, C and E). For raw OSPW (Fig. 2 A) the remaining NAs were those with n from 9 to 21 and *Z* values from 0 (acyclic) to −12 (hexacyclic).Analyzing the distribution of NAs in raw OSPW, the most representative NAs were those with n from 12 to 19 and *Z* values from −4 (bicyclic) to −12 (hexacyclic), similar to other reports (Merlin et al., 2007).

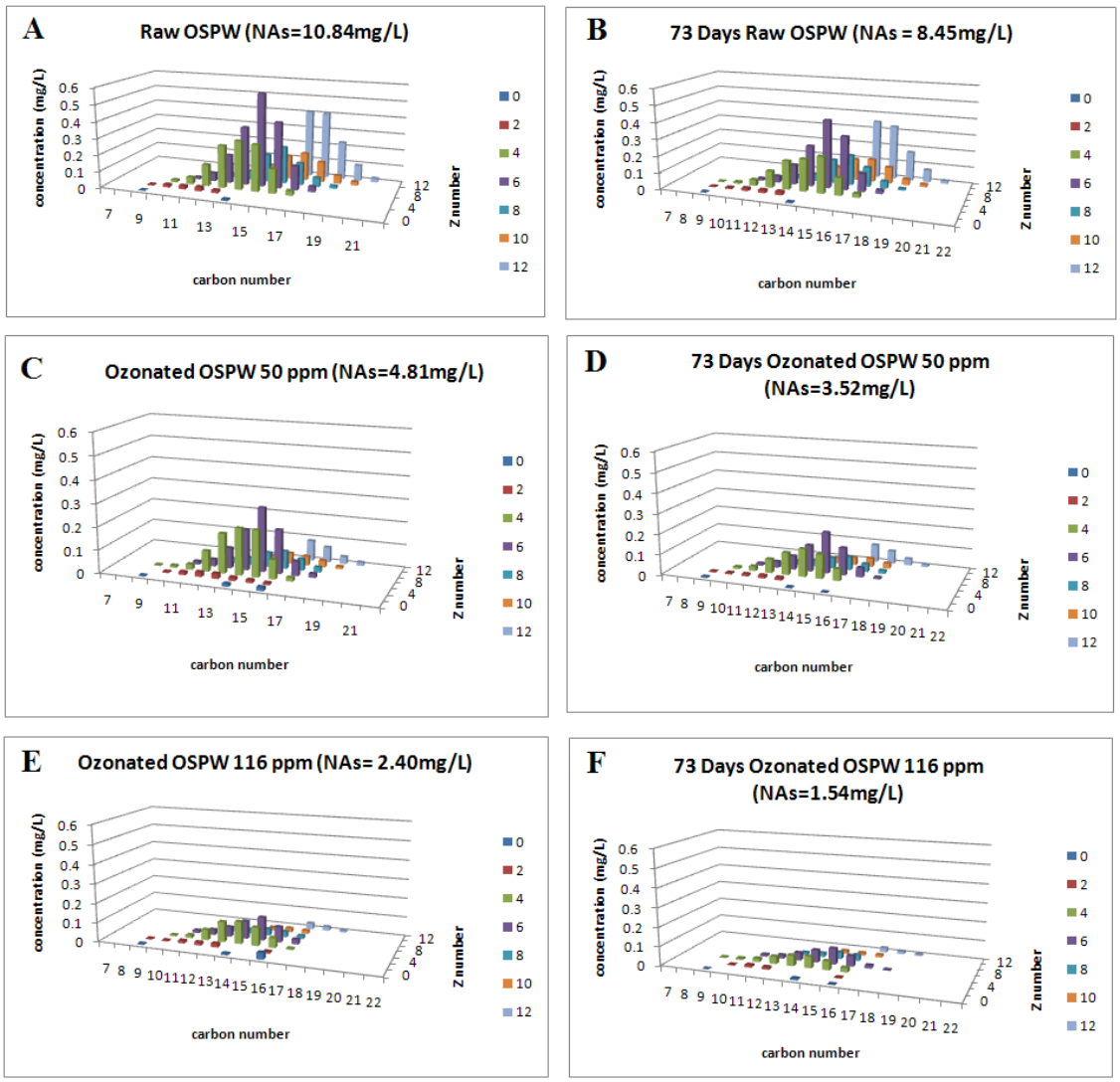


Fig. 2 Three dimensional plots showing NAs concentrations versus carbon number (n) and Z number (A) NAs in raw OSPW, (B) NAs in raw OSPW after biodegradation, (C) NAs in ozonated OSPW with the dose of 50mg/L, (D) NAs in ozonated OSPW with the dose of 50mg/L after biodegradation, (E) NAs in ozonated OSPW with the dose of 116mg/L, and (F) NAs in ozonated OSPW with the dose of 116mg/L after biodegradation.

When ozonation was applied at a utilized ozone dose of 50 mg/L, it removed 55.7% of the parent or unoxidized NAs, and the removals for specific *Z* values were 32.5, 50.1,

61.4, 73.7, and 82.6% for Z=–4 (2 rings), –6 (3 rings), –8 (4 rings), −10 (5 rings) and −12 (6 rings) respectively (Fig. 2C). This indicates that the degradation of NAs during ozonation is dependent on Z value, such that compounds with higher Z values were more readily degraded. The removal percentage of NAs was also enhanced with the increase of n, especially for C_{21} for which there was 100% removal. The molecular formulae of NAs which showed to be removed completely (by 100%) by ozonation were $C_{19}H_{30}O_2$, $C_{20}H_{30}O_2$ and $C_{21}H_{30}O_2$. In general, ozonation had the overall effect of diminishing the majority of NAs in OSPW. However, a trend of increasing concentrations of NAs with C_9-C_{16} and Z = 0 to −2 (1 ring or double bond equivalence) was observed. At the same time, ozonation led to the formation of some low molecular weight products such as $C_{14}H_{28}O_2$ (Z = 0), $C_{14}H_{26}O_2$ (Z = –2) and $C_{16}H_{30}O_2$ (Z = –2) which were absent in the raw OSPW. This shift in the distribution of NAs toward a greater proportion of low molecular weight compounds possibly represents branch decomposition or ring opening during the oxidation of NAs (Wang et al., 2013). These results confirm that ozone has the capacity to decompose long chains and break highly branched and cyclic carboxylic fractions of NAs as previously reported (Gamal El-Din et al., 2011; Wang et al., 2013).

The profile of NAs pre-treated with an ozone dose of 116 mg/L was very similar to that treated with 50 mg/L, although some distinct differences were observed particularly in the range of higher carbon numbers ($18 \leq n \leq 21$) having Z= −6 to −12. As a result, the total concentration of NAs decreased by 77.9%; NAs in the C_{20} and C_{21} range with Z=−10 and −12 were completely eliminated, while NAs with smaller n values and Z= –4, –6, –8, –10 and −12 decreased by 62.6, 76.2, 82.4, 90.9, and 94.9%, respectively. Moreover, the higher ozone doses (116 mg/L) completely removed NAs with high

molecular weight and cyclization such as $C_{21}H_{30}O_2$ (Z = –12), $C_{20}H_{28}O_2$ (Z = –12), $C_{20}H_{30}O_2$ (Z = –10) and $C_{19}H_{28}O_2$ (Z = –10). These results show that ozone preferentially reacts with NAs having a higher carbon numbers and Z values. As shown above, an increasing concentration was observed for some NAs with Z = 0, –2 and –4 (low molecular weight compounds). Overall, the results suggest that the degradation of NAs can be enhanced by increasing the ozone dose. Due to the complexity of OSPW matrix and the uncertainty about the structure of some of the NAs, the mechanisms of NA degradation are still not clear. In previous studies, the addition of •OH radical scavengers confirmed that both molecular ozone and •OH radicals had an effect on the degradation of NAs (Pérez-Estrada et al., 2011).

4.2Biodegradation of organic compounds after ozonation

4.2.1 Removal of COD

Fig. 3a illustrates the variation of COD concentrations in the samples of raw and ozonated OSPW during 73 days of biodegradation. The COD reduction can be characterized by two stages of degradation rates. In the first stage (0 - 30 days), the endogenous microorganisms reduced the COD at a faster rate as compared to that in the second stage (30 - 73 days). Endogenous microorganisms in the OSPW samples which were pre-treated with 18, 50, 78, 116, and 200 mg/L utilized ozone doses had an additional 45.4, 49.0, 48.8, 46.0, and 45.2%, respectively, of COD removal during biodegradation compared to the COD removal percentages achieved during the ozone pre-treatment. For the raw OSPW that received no ozone pre-treatment, only 39.2% of the COD was depleted after biodegradation for 73 days. These results are consistent with previous research (Martin et al., 2010; Gamal El-Din et al., 2011), suggesting that higher

ozone doses convert more recalcitrant organic compounds to more easily biodegradable forms. For the combined treatment of ozonation followed by biodegradation, the total COD removal efficiencies were 39.2, 53.8, 62.5, 64.7, 67.0, and 83.3%, with 18, 50, 78, 116 and 200 mg/L utilized ozone doses respectively, indicating a potentially feasible approach to effectively degrading recalcitrant organic compounds in OSPW.

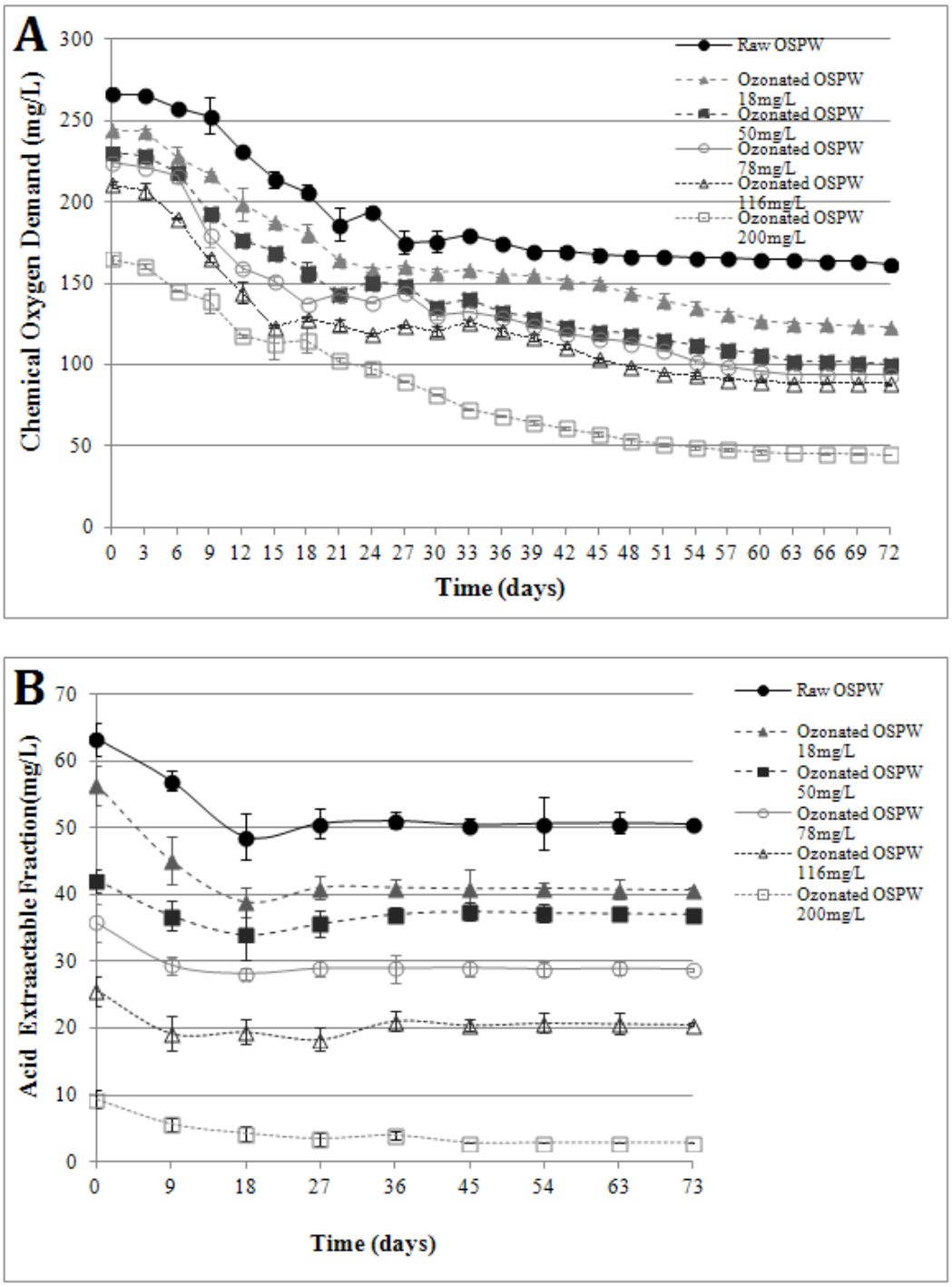


Fig. 3 The effect of different ozone doses on the (A) COD and (B) AEF.

For the OSPW without ozonation, COD removal efficiency achieved after the 73-days of biodegradation was 39.2% (corresponding to a total reduction in COD concentration of

104.5 mg/L) which represents the total amount of COD in OSPW which is degradable by endogenous microorganisms. The presence of recalcitrant compounds could be one of the main factors inhibiting further biodegradation (Taylor and Trudgill, 1978; Rontani, 1992; Quagraine et al., 2005). In addition, current research indicated that the performance of biodegradation could be improved by increasing biomass concentration and microbial activity (Headley et al., 2010; Misiti et al., 2013). Therefore, besides carbon source, inorganic macronutrients such as nitrogen (N) and phosphorus (P) are also needed to facilitate bacterial growth. The OSPW with the limiting nutrient components deserves further study (Table 3).

Ozone pre-treatment contributed minimally to the COD removal efficiency compared to the 104.5 mg/L removed from raw OSPW. There was 121, 130.5, 130, 122.5, and 120.5 mg/L COD removed in the reactors having utilized ozone doses of 18, 50, 78, 116 and 200 mg/L, respectively. Our results indicate that ozonation mainly removed the non-biodegradable portion of the COD from raw OSPW, and that ozonation could increase the biodegradability of some organic compounds. Furthermore, considering that the amount of COD removed by biodegradation was the greatest amount after ozonation with 50 and 78 mg/L utilized ozone doses, it is possible that more organic compounds can be completely mineralized after applying high-dose ozonation as a pre-treatment. It is known that high ozone doses applied in an alkaline aquatic environment, leads to the formation of very reactive •OH radicals which may react with degradation intermediates. Further analysis of the NAs content confirmed that NAs with higher molecular weights (i.e., higher carbon number and *Z* number) were readily decomposed by ozone into simpler organic compounds which could have contributed to the COD of samples.

Similar results have been reported in previous research (Gilbert, 1988; Pérez-Estrada et al., 2011).

4.2.2 Removal of AEF

Fig. 3b shows the AEF removal from raw and ozonated OSPW during the 73 days of biodegradation. Biodegradation of AEF-causing compounds mainly occurred in the first 20 days, after which the residual AEF concentration in the bioreactor tended to stabilize. This pattern suggests that biodegradation could only remove part of the AEF even after pre-treatment using ozonation. Moreover, because of the incomplete oxidation by the biological degradation, an amount of carbonyl stretching equivalents remained in the reactor system during the second period.

After 73 days of bioreactor operation, total removal of the AEF after both ozonation followed by biodegradation was 20.2, 35.8, 41.5, 54.6, 67.5, and 95.3% in the raw and ozonated OSPW samples (Table 4). The amounts of AEF removed after 73 days of biodegradation were 12.8, 15.7, 5, 7, 5 and 6.5mg/L for the OSPW pre-treated with 0, 18, 50, 78, 116, and 200 mg/L utilized ozone doses, respectively. With the exception of the 18 mg/L utilized ozone dose condition, AEF removal was lower in ozonated than in raw OSPW. This result differs from our COD biodegradation results, and may be explained by the fact that the AEF only represents the total amount of carbonyl groups (C=O) and that carbonyl groups could be formed during the biodegradation process. Several studies have demonstrated that biodegradation can lead to the production of new fatty acids or derivatives with carboxylic acid groups (Johnson et al., 2010; Whitby, 2010). For instance and under the β-oxidation pathway (the preferred route by most microorganisms), aliphatic and alicyclic carboxylic acids will be oxidized to form new carboxylic acids

with two carbons fewer than its predecessor. In the present study, although the ozone could oxidize a large portion of the organic compounds with functional groups (e.g. aromatic rings and unsaturated hydrocarbons) (Beltran, 2003) and increase the proportion of biodegradable compounds, biodegradation pathways involving enzymes such as monooxygenase and dioxygenase may not mineralize the organic compounds completely (Van Der Meer, 2006; Uyttebroek et al., 2007). Hereby, it was difficult to evaluate the extent of biodegradation through the AEF changes.

4.2.3 Removal of NAs

For raw OSPW, biodegradation decreased the NAs concentration from 10.86 mg/L to 8.45 mg/L (removal efficiency of 22.2%) after 73 days operation of the bioreactor (Fig. 2B). Thus, the total amount of biodegradable NAs in OSPW was 2.41 mg/L and the corresponding non-biodegradable part of NAs in raw OSPW was 8.45 mg/L. Endogenous microorganisms removed NAs over the range of all carbon numbers except when n = 9 and Z = 0. The results also showed that endogenous microorganisms preferentially degrade NAs with low cyclicity (i.e., Z= 0 and Z= –2) and with lower molecular weight (such as carbon number n≤15) (Fig.2 B). Moreover, the concentration of NAs with the molecular structure of $C_9H_{18}O_2$ (Z = 0) increased after biodegradation, suggesting that endogenous microorganisms have the capability to decompose organic compounds with high molecular weights into those of lower molecular weight through the processes of α-, β- oxidation and aromatization (Rontani, 1992, Quagraine et al., 2005; Johnson et al., 2010). Likewise, previous research has demonstrated that biodegradation changes the composition of NAs and has revealed that NAs with lower molecular weight and cyclization are more susceptible to biodegradation (Scott et al., 2005; Han et al., 2008).

Compared with raw OSPW, ozonated water samples had better biodegradability with NAs removals of 26.8 and 35.8% after 73 days of bioreactor operation when 50 and 116 mg/L of ozone were utilized, respectively (Table 4). Accordingly, total removal rates of NAs with combined ozone and biodegradation were 67.6and 85.8%. However, only 1.29 and 0.86 mg/L of NAs were removed through biodegradation and the increases in ozone dose did not increase the amount of NAs available for biodegradation (Table 4). This observation may be the result of ozonation, having already eliminated some biodegradable NAs through direct and indirect oxidation pathways. It is interesting that high molecular weight compounds (n=17 and 18) with specific *Z* values of -2 or -4 could be totally removed by biodegradation (Figs. 2D and 2F). Other studies have reported that the molecular weight (Scott et al., 2005; Biryukova et al., 2007; Whitby, 2010) and chemical structure (Headley et al., 2002; Han et al., 2008; Smith et al., 2008) of NAs can influence their biodegradation. For instance, determinants of recalcitrant NAs can include the following: *(i)* a higher degree of alkyl-substituted aliphatic chains (Han et al., 2008; Smith et al., 2008), *(ii)* tertiary substitution at positions other than the *β*-position to the carboxylic acid of the main carbon chain, *(iii)* methyl substitution on the cycloalkane rings (Herman et al., 1993; Smith et al., 2008), *(iv)* increased cyclicity(Han et al., 2008), *(v)* evenness of the carbon side chain, and *(vi)cis*-isomerism in alicyclic acids (Headley et al., 2002; Holowenko et al., 2002). Ozonation could change the molecular weight and chemical structure of NAs by decomposing long chains and by breaking highly-branched and cyclic carboxylic fractions, thereby improving their biodegradation. In this study, ozonation appeared to remove the majority of NAs and also to accelerate biodegradation to some extent. However, there was a small amount of residual NAs after biodegradation

even after pre-treatment with an ozone dose of 116 mg/L. It has been reported that bacteria do not utilize compounds in low concentrations which could not satisfy their needs for energy production, and that this scarcity can trigger enzyme systems of the microbial communities to pursue other available carbon sources (Riser-Roberts, 1998).

4.3 Suspended microbial growth in bioreactors

Fig. 4A shows the growth curve of culturable heterotrophic bacteria in raw and ozonated OSPW over the 73 days of operation. Compared with ozonated OSPW, bacterial growth in raw OSPW was inhibited in the first 15 days which may be caused by the toxicity of NAs. In contrast, no significant lag phase was observed when ozonation was used prior to biodegradation. Moreover, the specific growth rate of bacteria increased with increasing ozone dose in the first few days, indicating that ozonation could improve the growth of endogenous bacteria. Although bacterial communities seemed to acclimatize within raw OSPW (indicated by grow after the lag phase), these reactors still had the lowest bacterial number after 73 days of operation. In all conditions, the microbial population reached a steady state after 60 days with a microbial density of approximately 10^6 CFU/mL. The positive effect of ozone on the bacterial concentrations may be due to ozone's ability to break down the toxic high-molecular weight organic compounds.

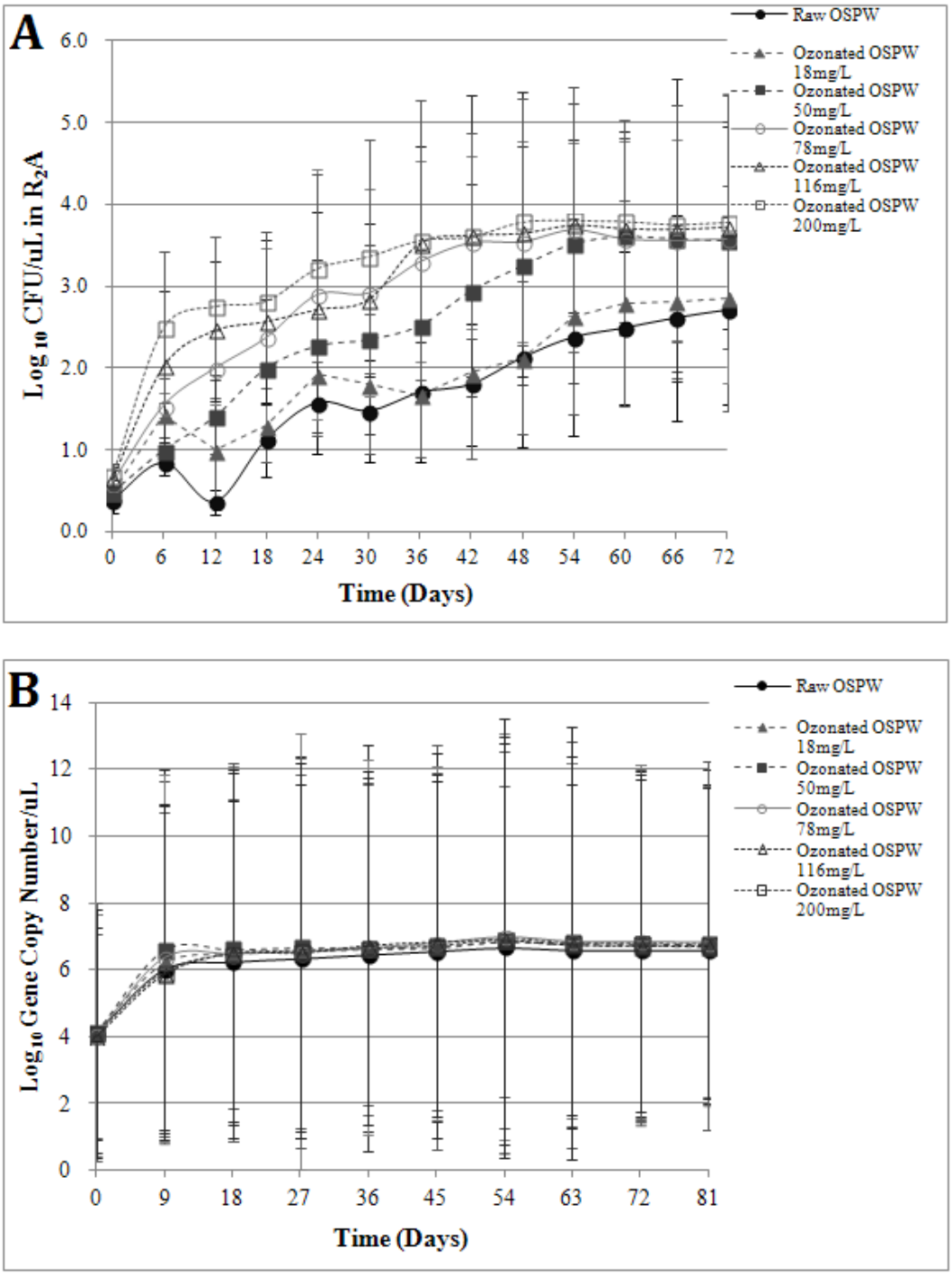


Fig. 4 The effect of different ozone doses on the microbial growth based on: (A) Plate counting and (B) Real-time PCR.

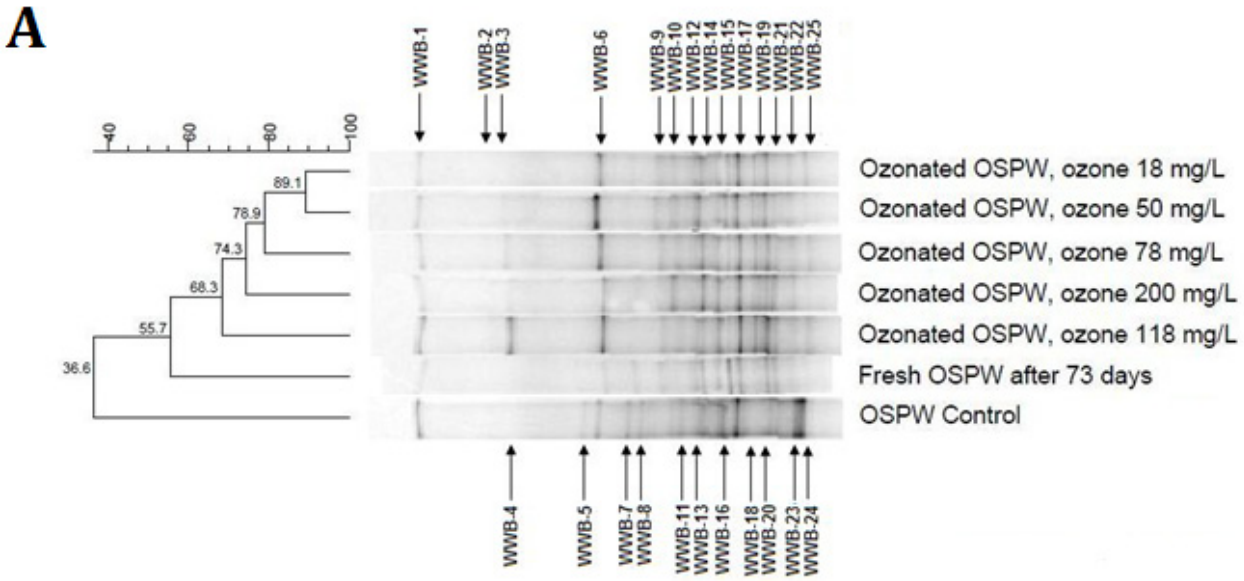
Fig. 4B shows the total biomass in raw and ozonated OSPW over the operation time as determined by real-time PCR. The amount of total bacteria grew gradually in the first few day followed by a short period of rapid growth which may have resulted from acclimatization. Peak values of biomass were reached at day 54, and then after a significant drop of the DNA quantities in all reactors reached a steady state with approximately 10^6 copy number per μL. This drop and then the steady state that has been reached by all the reactors can possibly be attributed to bacterial decay triggered by the limited biodegradable carbon, ammonia and phosphate sources in the OSPW (Table 3). OSPW without ozonation had the lowest bacteria numbers over the whole biodegradation process, consistent with the results obtained from HPC.

By comparing the two series of data, it is not difficult to ascertain that the numeration of total bacteria monitored by real-time PCR was higher than the amount of heterotrophic bacteria counted on agar plates, since it is well known that these plate counts underestimate the total number of bacterial cells (Glaze, 1987; Paslawski et al., 2009). However, similar growth trends were seen between the two methods, indicating high reliability of the experiments.

4.4 Microbial community in bioreactors

Fig. 5 shows the DGGE profiles and taxonomy of microorganisms in raw and ozonated OSPW. As shown in Fig. 5A, microbial diversity and quantity decreased with increasing ozone concentration. Additionally, there was some reduction in microbial diversity and quantity over the operating time (raw OSPW after 73 biodegradation). These are suggestive of the generation of by-products and terminal products which switched the

nutrition source of the microorganisms, thus altering the original microbial community structure. A total of 26 strains were successfully sequenced based on DGGE bands.



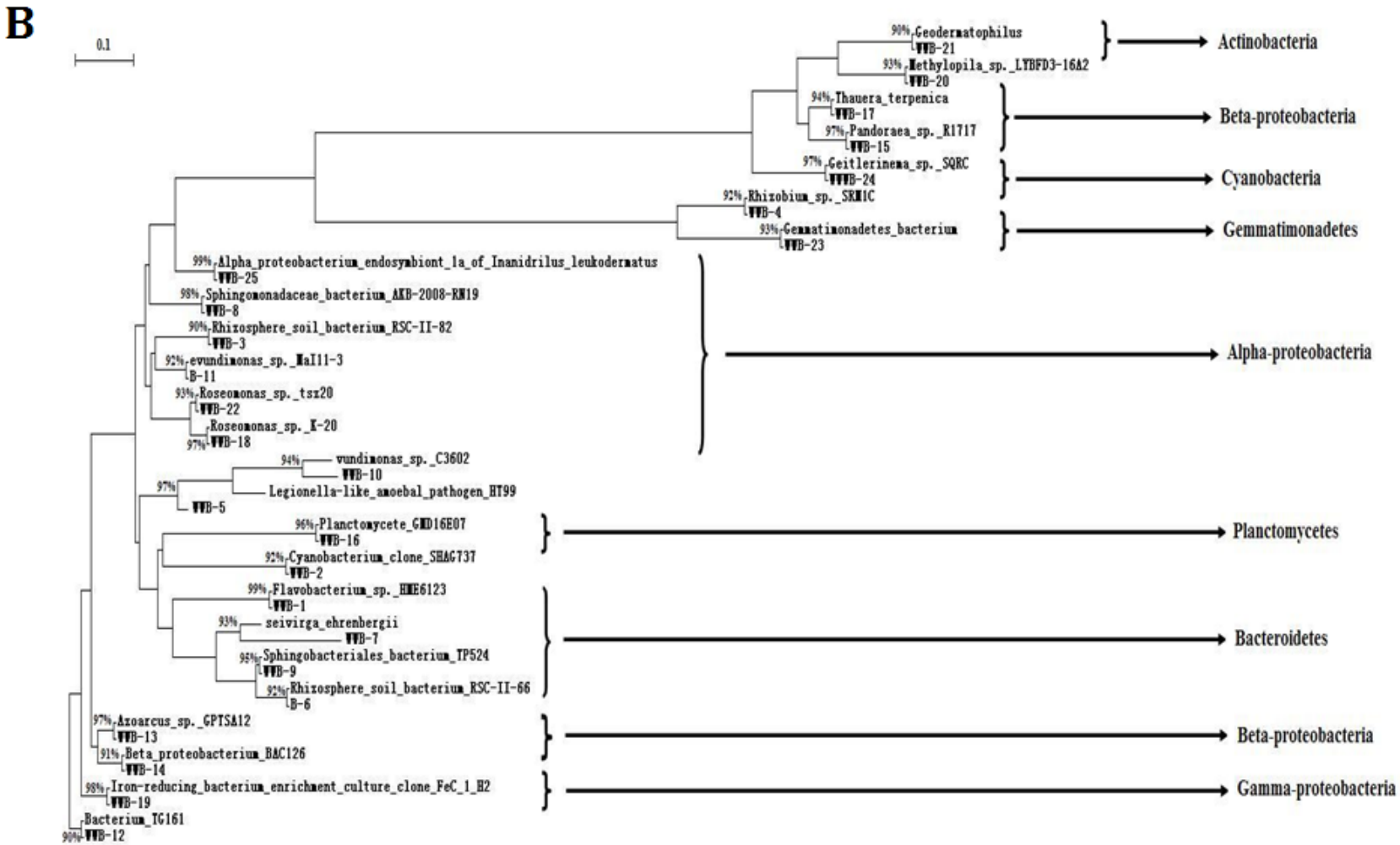


Fig. 5 Microbial community profiles in OSPW: (A) DGGE and Cluster profiles of the indigenous microorganisms (B) phylogenetic tree based on DGGE bands

Fourteen dominant strains were identified in raw OSPW. Among them, WWB-1, 7, 8, 10, 11, 17, 20, and 24 were closely related to *Flavobacterium* sp., Rhizosphere soil bacterium, *Roseivirgaehrenbergii*, *Sphingobacteriales*, *Brevundimonas*sp., *Planctomycete*, Iron-reducing bacterium, and *Gemmatimonadetes*, respectively (see Fig. 5B). Strains WWB-5, 6, 13, 16, 21, and 23 may be related to *Rhizobium* sp., Legionella-like amoebal pathogen, Bacterium TG161, *Pandoraea* sp., *Methylopila* sp., and *Roseomonas* sp., respectively. Some strains could not be identified with high confidence due to their relatively low sequence comparability with templates in the database. Certain *Flavobacterium* strains, which are yellow-pigmented, motile, Gram-negative rods in the family *Flavobacteriaceae* (one of the families within the *Bacteroidetes*group) can degrade organic compounds such as pentachlorophenol (Saber and Crawford, 1985). Rhizosphere soil bacterium includes bacteria that can utilize a variety of organic compounds and some of them also have the capability of nitrogen fixation. *Brevundimonas* sp. can degrade some organophosphate compounds (Deshpande et al., 2004). After 73 days of operation, the intensity of many bands was significantly reduced in raw OSPW; WWB-5 was missing and the intensities of WWB-23 and 24 were also significantly reduced. This indicates that this experimental condition may not be suitable for the growth of several endogenous microbial species, probably due to the difference in temperature and oxygen concentration in the laboratory compared with that in the tailings ponds.

After ozonation, there were also significant changes in the microbial communities in OSPW; for instance, WWB-7 and 8 were missing in all ozonated samples. When the utilized ozone concentration was as high as 200 mg/L, the intensity of all bands reduced and the diversity of microbial communities diminished. This may have been caused by

the decreased concentration of organic compounds which can be used for the growth of endogenous bacteria. However, certain bands only appeared in ozonated samples. For example, WWB-6 presented at high intensity especially after the ozone dose of 50 mg/L; WWB-3 showed up when the ozone concentration was increased to 78 mg/L; and WWB4 appeared when the ozone concentration increased to and beyond 116 mg/L. There were also some other bands (WWB-9, 12, 14, 15, 18, 19, 22, 25 and 26) that were barely visible in raw OSPW but easily identified in ozonated samples. From these data, we infer that the oxidation of organic compounds in OSPW by ozone provided some molecules that were readily degraded and thus enhanced the growth of certain bacteria. WWB-9 was closely related to the family of *Sphingomonadaceae*, in which some species have shown to degrade aromatic compounds (Balkwill et al., 2003), so it should also have good potential to degrade NAs. WWB14 was closely related with *Azoarcus* sp., which has also been associated with aromatic compound and hydrocarbon degradation in anoxic waters and soils (Rabus et al., 2005). The enrichment of these strains may have facilitated NAs degradation after ozonation.

Our results indicate that ozonation changed the microbial communities in OSPW and that microbial communities in the bioreactors also varied with increasing ozone dose. Ozonation appeared to enrich certain strains which are known to degrade aromatic compounds and hydrocarbons, thus potentially improving COD removal and NAs degradation. Previous studies have also shown that NAs-degradation preferences were dependent on the microbial community (Del Rio et al., 2006).

4.5 Microtoxicity of the OSPW after different ozone doses

Many factors contribute to the toxicity of OSPW, but NAs have been identified as one of the main components responsible for the acute toxicity in OSPW. NAs with surfactant characteristics could easily penetrate the cell membrane to alter membrane function, ultimately causing cell death (Quagraine et al., 2005; Frank et al., 2008).The major mechanisms for the toxicity of NAs can be summarized as contributing to necrosis, which is recognized as the disruption of the cell cytoplasmic membrane structure by the simple physical presence of hydrophobic chemicals in the lipid bi-layer which could alter membrane properties including fluidity, thickness, and surface tension (Klopman et al., 1999; Kannel and Gan, 2012).

Our IC_{20} results indicate that OSPW becomes less toxic towards *Vibrio fischeri* after ozonation (Fig. 6). Increased ozone dose had clear benefits regarding the reduction of toxicity. The toxicity (IC_{20}) of raw OSPW was 20% v/v and the values rose to 32.3% v/v, 48.6% v/v, 60.8% v/v, 78.6% v/v and 93.5% v/v with increasing utilized ozone doses, indicating large decreases in the degree of toxicity. This outcome may be attributed to the destruction of NAs with higher molecular weights and ring structures during ozonation. Previous studies have reported that a utilized ozone dose of 150 mg/L eliminated the toxicity of OSPW (Scott et al., 2008; Gamal El-Din et al., 2011). Although our IC_{20} value of 93.5% v/v at a utilized ozone dose of 200 mg/L approaches this published result, some toxicity still remained which may be from the presence of the residual NAs in the ozonated OSPW as well as inorganic compounds in OSPW that could also contribute to the toxicity (such as the high salinity values) (McCarty and McKinney, 1961; Dolfing and Bloemen, 1985; Nero et al., 2006). It has been reported that the level of salts in

OSPW could increase the toxicity of NAs by inducing osmotic stress (Quagraine et al.; 2005). The formation of intermediate products, with more complex structures or hydrophobic properties, after NAs or other organic compounds reacted with ozone may also have potentially contributed to the toxicity. In addition, ozone could not eliminate from OSPW the inhibitory effects of a high concentration of salts on microorganisms.

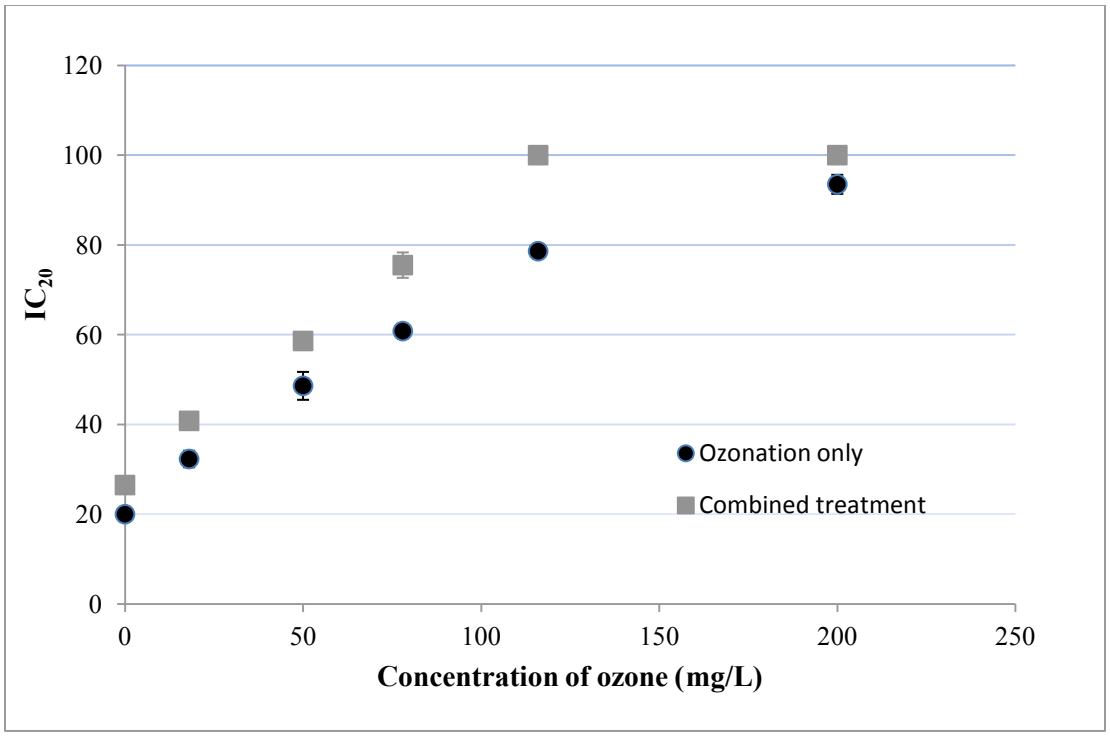


Fig.6 Effect of ozone dose on the toxicity of OSPW inferred as Microtox (IC_{20})[b](%v/v) in each reactor (Arrow indicated the value of IC_{20} is above 100)

During the biodegradation process, toxicity decreased over time in all OSPW samples. This additional reduction in toxicity during biodegradation was enhanced with increased ozone dose; IC_{20} values were increased by more than 21.4% v/v with the utilized ozone dose of 116 mg/L, while only a 6.5% v/v increase was found in the raw OSPW. This also indicates that the endogenous microorganisms had limited capability to degrade the

recalcitrant NAs. Ozonation did not only affect the initial toxicity of OSPW, but also facilitated the endogenous microorganisms to mitigate toxicity during the bioremediation. After 73 days of biodegradation, the water was completely detoxified, IC_{20}> 100% (v/v) after the high levels of ozone pre-treatment (116 and 200 mg/L) under the conditions tested. This result suggests the combination of ozone and biodegradation eliminated the OSPW toxicity by altering chemical structure, molecular size, and hydrophobicity of NAs which are the primary factors that affect the toxicity (Protic and Sabljic, 1989; Lo et al., 2006; Frank et al., 2009). Therefore, our results testify to a greatly reduced toxicity of OSPW after combined ozone and biological treatment.

4.6 Overall performance/implication of combined ozonation and biological treatment

OSPW has been reported to be recalcitrant for biological treatment (Scott et al., 2005) and biodegradation may benefit from OSPW pre-treatment with ozone. Inadequate amounts of ozone cannot react with organic compounds in OSPW, while an overdose of ozone may result in intermediates with low molecular weight which are not readily degradable (Amat et al., 2003). Consequently, the ozone dose for pre-treatment of OSPW should be optimized in order to maximize the subsequent biodegradability while avoiding any increase of toxicity. In this study, maximal biodegradation in terms of COD removal occurred at the ozone dose of 50 mg/L (Table 4). For the combination of ozonation and biological treatment, the removals of COD and NAs were enhanced with increasing ozone doses up to 50 mg/L, but thereafter it reached a steady phase until ozone dose of 116 mg/L was reached (Figure 2). Although much higher COD and AEF removals were observed after pre-treatment with the highest ozone concentration (200mg/L),

biodegradation resulted in hardly any further COD removal. Therefore, 50 mg/L was identified as the most suitable ozone treatment dose for biodegradation. Based on the toxicity test, by-products of ozonation did not cause increased toxicity and the combined treatment eliminated the toxicity of OSPW when the ozone dose was above 116 mg/L (Fig. 6).

This study confirms that ozonation can be used as a pre-treatment method to improve the biodegradability of OSPW. However, further study is warranted to improve the biodegradation efficiency. It has been reported that microbial communities from environments that have a history of NAs contamination have the capability of degrading recalcitrant NAs with more speed (Herman, et al., 1993; Scott et al., 2005; Del Rio et al., 2006); this seems to indicate a necessity of sludge acclimation. Some other factors which could affect the growth of NA-degrading microorganism have also been identified, such as nutrient conditions, aeration and pH level (Whitby, 2010). Biodegradation performance could also be improved by increasing biomass concentration in the reactor. Biofilm reactors can maintain the high biomass productivity by immobilizing bacterial cells on the supporting materials. An immobilized cell reactor with established biofilm was shown to enhance the biodegradation rate of a model NA up to two orders of magnitude when compared to a reactor with suspended cells (Paslawski et al., 2009). More recently, a continuous biofilm reactor showed to remove 99% of parent NAs from ozonated OSPW (Hwang et al., 2013). Membrane bioreactors are another potential option for biological treatment of OSPW since they use a permeable membrane to retain a high amount of bacterial cells in the reactor.

CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS

In this study, we investigated the effect of different doses of ozone pre-treatment on OSPW biodegradation. Increased ozone doses not only enhanced the reduction of the studied organic compounds, via chemical reactions, but also improved the biodegradability of OSPW for subsequent biological treatment. Our results also indicate that ozone pre-treatment decreases the toxicity of OSPW and provides extra biodegradable carbon sources to accelerate the growth of microbial populations. Moreover, the increasing bacterial number and the appearance of emergent microorganisms could further mitigate the toxicity of OSPW and the degradation of the recalcitrant organic compounds including NAs. Therefore, combining ozone and biodegradation is a promising technology for OSPW treatment.

Through this study several factors affected NA bioremediation should be considered due to the persistence and toxicity of NAs. Typically for biodegradation, microbial communities from environments that have a history of NA contamination such as those found in oil sands and OSPW can degrade NAs, including recalcitrant NAs. Thus, prior exposure to NAs could induce and/or select for microorganisms capable of more effective NA degradation. Furthermore, previous studies have indicated that mixed consortia of microorganisms possibly have more competence than pure cultures in NAs degradation. Meanwhile, studies on factors that stimulate the growth of NA-degrading microorganisms (e.g., addition of nutrient, aeration, trace metal, etc.) has important implications for the development of more effective NA bioremediation strategies.

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Appendix A

Supplementary Information

Table S1. The primer information and the optimized condition for PCR and DGGE.

Gene	Fragment length (nbs)	Name	Sequence
16S rRNA	~550	341FGC	5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G CCT ACG GGA GGC AGC AG-3′
		341F	5′-CCT ACG GGA GGC AGC AG-3′
		907R	5′-CCG TCA ATT CMT TTG AGT TT-3′

Gene	PCR system		PCR program	DGGE condition
16S rRNA	Volume	25μL	Initial denaturation of the DNA at 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 1 min at 53 °C and 1 min at 72 °C. The reaction was completed after 10 min at 72 °C.	7.0%PAA, 20-80% 10h, 120 V, 60 Hz, 1×TAE 1:10000 (v/v) SYBR Gold, 30 min
	PCR buffer	1×		
	$MgCl_2$	1.5mM		
	dNTPs	200μM		
	Taq	1.25U		
	primer	1.0mM		
	Temp.	10-100ng		
	BSA	600ng/μL		

Appendix B

Original Data

Table S2. The Original Data of COD (Chemical Oxygen Demand) (mg/L)

	Fresh OSPW		Ozonated OSPW 18mg/L		Ozonated OSPW 50mg/L		Ozonated OSPW 78mg/L		Ozonated OSPW 116mg/L		Ozonated OSPW 200mg/L	
Day	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**
0	266.50	0.98	244.00	1.01	230.50	1.14	224.00	2.14	210.50	2.21	165.00	2.44
3	266.48	0.59	243.49	1.45	228.84	0.93	221.12	0.56	207.04	4.97	160.58	1.1
6	257.85	1.51	227.99	5.60	218.63	0.76	216.18	4.75	190.13	0.81	145.15	0.43
9	253.25	10.75	217.50	1.64	193.93	0.91	180.05	8.13	165.21	2.01	139.10	7.69
12	231.83	1.37	198.69	10.39	177.02	2.21	159.54	2.12	144.52	7.01	117.81	1.09

15	214.03	4.90	187.52	0.74	169.54	2.48	151.25	2.39	123.34	0.94	113.24	10.19
18	206.68	3.70	180.69	5.65	157.39	6.01	137.86	0.16	127.99	1.70	115.29	7.41
21	186.32	9.87	164.21	1.05	143.84	0.31	143.21	0.59	124.58	3.63	102.32	1.94
24	193.67	2.73	158.49	1.80	151.19	3.84	137.91	0.54	118.59	0.59	97.59	3.51
27	175.29	6.64	160.22	0.91	148.52	3.05	144.63	1.15	123.84	0.83	90.03	0.67
30	175.58	6.78	156.44	2.28	135.92	0.59	130.2	2.42	120.59	2.90	81.74	0.26
33	179.44	0.71	158.70	0.73	140.54	2.39	132.51	0.16	126.00	2.20	72.60	0.17
36	174.66	2.78	155.34	0.98	132.48	1.92	129.75	2.12	120.46	2.43	68.34	0.45

39	169.33	3.43	155.04	1.24	128.84	2.12	124.32	1.32	116.48	1.89	64.46	1.34
42	169.43	2.76	151.44	1.56	123.55	1.86	119.38	1.44	110.78	1.76	60.75	0.65
45	167.28	3.85	149.82	2.47	121.08	0.93	116.24	1.68	103.32	1.46	56.94	1.44
48	166.56	3.48	143.87	3.33	118.76	1.73	113.56	0.84	98.67	1.09	52.84	2.08
51	166.32	2.87	139.44	4.12	115.54	0.34	108.76	0.32	94.34	0.84	50.98	0.88
54	165.36	1.23	135.55	3.36	112.42	1.86	101.85	1.34	93.24	2.19	48.76	0.98
57	165.44	1.46	131.28	1.08	109.46	0.43	98.76	1.82	90.88	1.08	47.66	1.32
60	164.65	0.87	127.36	0.46	106.49	1.68	95.36	1.01	89.91	0.88	46.38	1.44

63	164.37	1.09	125.44	1.48	102.25	0.88	94.13	0.24	88.76	0.46	45.56	0.24
66	163.96	0.24	124.76	0.56	102.06	0.33	94.10	0.62	88.47	0.21	45.34	0.33
69	163.66	0.33	123.92	0.44	101.56	0.55	94.05	0.14	88.25	0.11	44.98	0.21
72	162.00	0.10	123.00	0.40	100.00	0.40	94.00	0.10	88.00	0.30	44.50	0.20

Table S3. The Original Data of AEF (Acid Extractable Fraction) (mg/L)

	Fresh OSPW		**Ozonated OSPW 18mg/L**		**Ozonated OSPW 50mg/L**		**Ozonated OSPW 78mg/L**		**Ozonated OSPW 116mg/L**		**Ozonated OSPW 200mg/L**	
Day	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**
0	63.40	2.46	56.40	2.88	42.10	1.72	35.80	2.77	25.60	2.27	9.30	1.34
9	57.10	1.48	45.10	3.57	36.90	2.34	29.40	1.39	19.30	2.65	5.60	1.02
18	48.70	3.45	38.90	2.14	34.10	3.96	28.10	0.88	19.50	1.78	4.20	1.08

27	50.70	2.21	41.10	1.69	35.70	2.02	28.90	1.24	18.40	1.63	3.40	0.98
36	51.10	1.22	41.10	1.33	37.10	1.08	28.90	2.13	21.10	1.59	3.90	0.65
45	50.50	0.98	40.90	2.99	37.50	1.37	29.00	1.12	20.50	0.97	2.80	0.00
54	50.70	3.86	41.00	0.78	37.30	1.41	28.80	1.07	20.80	1.43	2.80	0.00
63	50.80	1.55	40.80	1.53	37.20	1.01	28.90	0.99	20.70	1.57	2.80	0.00
73	50.60	0.10	40.70	0.40	37.10	0.20	28.80	0.20	20.60	0.30	2.80	0.00

Table S4. The Original Data of Naphthenic acids (mg/L)

Table S4-A. The Concentration of Naphthenic acids in Raw OSPW (10.84mg/L)

Hydrogen Deficiency / Carbon Number	**0**	**2**	**4**	**6**	**8**	**10**	**12**
7	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0
9	0.002808	0.00605	0	0	0	0	0
10	0	0.016319	0.014371	0	0	0	0

11	0	0.020413	0.042097	0.011674	0	0	0
12	0	0.024199	0.13113	0.049185	0	0	0
13	0	0.01672	0.257517	0.17233	0.021975	0	0
14	0.008918	0	0.295304	0.353053	0.051462	0	0
15	0	0	0.28251	0.56245	0.166386	0.073159	0
16	0	0	0.146843	0.398436	0.223801	0.142626	0
17	0	0	0.027593	0.145854	0.13492	0.168439	0.407813
18	0	0	0	0.031452	0.054724	0.122686	0.401607
19	0	0	0	0	0.0156	0.048799	0.229684
20	0	0	0	0	0	0.020266	0.094328
21	0	0	0	0	0	0	0.022124
22	0	0	0	0	0	0	0

Table S4-B. The Concentration of Naphthenic acids in Raw OSPW after 73 days (8.45mg/L)

Hydrogen Deficiency	**0**	**2**	**4**	**6**	**8**	**10**	**12**

Carbon Number							
7	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0
9	0.003348	0.003836	0.00338	0	0	0	0
10	0	0.008687	0.010682	0	0	0	0
11	0	0.016679	0.03632	0.012808	0	0	0
12	0	0.018255	0.101024	0.038704	0	0	0
13	0	0.020408	0.173908	0.120675	0.019144	0	0
14	0.004913	0	0.196282	0.247057	0.040696	0	0
15	0	0	0.221924	0.411628	0.14352	0.070084	0
16	0	0	0.106159	0.323332	0.181366	0.129767	0
17	0	0	0.022783	0.110134	0.118042	0.137098	0.351119
18	0	0	0	0.021475	0.043019	0.099308	0.329048
19	0	0	0	0	0.00934	0.035664	0.175743
20	0	0	0	0	0	0.015248	0.073649

21	0	0	0	0	0	0	0.016385
22	0	0	0	0	0	0	0

Table S4-C. The Concentration of Naphthenic acids in Ozonated OSPW 50 ppm (4.81mg/L)

Hydrogen Deficiency / Carbon Number	**0**	**2**	**4**	**6**	**8**	**10**	**12**
7	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0
9	0.003912	0	0.004482	0	0	0	0
10	0	0.005167	0.009139	0	0	0	0
11	0	0.012816	0.023269	0.013516	0	0	0
12	0	0.020555	0.090086	0.030528	0	0	0
13	0	0.022626	0.174185	0.089538	0.017626	0	0
14	0.011998	0.01428	0.204342	0.177974	0.030548	0	0
15	0	0.012332	0.203197	0.279489	0.062039	0.027503	0

16	0.015006	0.008777	0.083745	0.191132	0.076593	0.045818	0
17	0	0	0.015372	0.062703	0.049331	0.039825	0.088042
18	0	0	0	0.016254	0.021718	0.028263	0.066554
19	0	0	0	0	**0**	0.010086	0.031872
20	0	0	0	0	0	0	0.014385
21	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0

Table S4-D. The Concentration of Naphthenic acids in Ozonated OSPW 50 ppm after 73 days (3.52mg/L)

Hydrogen Deficiency / Carbon Number	**0**	**2**	**4**	**6**	**8**	**10**	**12**
7	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0
9	0.004259	0.004863	0	0	0	0	0
10	0	0.007511	0.00991	0	0	0	0

11	0	0.010086	0.022716	0.011586	0	0	0
12	0	0.013624	0.068498	0.029955	0	0	0
13	0	0.013055	0.10976	0.067534	0.015773	0	0
14	0.004379	0	0.137557	0.13118	0.029286	0	0
15	0	0	0.119596	0.204292	0.05253	0.027247	0
16	0.000982	0	0.057698	0.135436	0.065907	0.03772	0
17	0	0	0	0.045801	0.038214	0.040885	0.086567
18	0	0	0	0.007803	0.014434	0.02573	0.063347
19	0	0	0	0	0	0	0.032053
20	0	0	0	0	0	0	0.011979
21	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0

Table S4-E. The Concentration of Naphthenic acids in Ozonated OSPW 116 ppm (2.40mg/L)

Hydrogen Deficiency	**0**	**2**	**4**	**6**	**8**	**10**	**12**

Carbon Number							
7	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0
9	0.004972	0.004661	0	0	0	0	0
10	0	0.005235	0.006115	0	0	0	0
11	0	0.013817	0.015405	0.013593	0	0	0
12	0	0.014911	0.05435	0.027546	0	0	0
13	0	0.019037	0.105381	0.051105	0.0194	0	0
14	0.007673	0	0.115039	0.091423	0.020586	0	0
15	0	0	0.095	0.120887	0.033049	0.015286	0
16	0.0337	0.009661	0.050276	0.08017	0.028265	0.020154	0
17	0	0	0.005939	0.024948	0.016171	0.016688	0.029788
18	0	0	0	0	0	0	0.020368
19	0	0	0	0	0	0	0.008885
20	0	0	0	0	0	0	0

21	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0

Table S4-F. The Concentration of Naphthenic acids in Ozonated OSPW 116 ppm after 73 days (1.54mg/L)

Hydrogen Deficiency / Carbon Number	**0**	**2**	**4**	**6**	**8**	**10**	**12**
7	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0
9	0.000558	0	0.00438	0	0	0	0
10	0	0.003883	0.00875	0	0	0	0
11	0	0.012778	0.017519	0.010449	0	0	0
12	0	0.012065	0.038659	0.017147	0	0	0
13	0	0	0.055227	0.040228	0.013162	0	0
14	0.007789	0	0.059856	0.064898	0.020001	0	0
15	0	0	0.046489	0.08383	0.035075	0.015521	0

16	0.001376	0.003188	0.022346	0.055731	0.030075	0.013716	0
17	0	0	0	0.015595	0	0.012978	0.022332
18	0	0	0	0.003272	0	0	0.014075
19	0	0	0	0	0	0	0.006152
20	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0

Table S5. The Original Data of Log $_{10}$ CFU/uL in R_2A (Heterotroph Plate Counting)

	Fresh OSPW		**Ozonated OSPW 18mg/L**		**Ozonated OSPW 50mg/L**		**Ozonated OSPW 78mg/L**		**Ozonated OSPW 116mg/L**		**Ozonated OSPW 200mg/L**	
Day	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**	**Value**	**STD**
0	0.39	-0.15	0.48	0.15	0.48	0.15	0.60	-0.15	0.65	0.15	0.69	0.15
6	0.85	0.15	1.43	0.45	1.00	-0.15	1.54	0.15	2.02	0.93	2.50	0.93
12	0.37	-0.15	1.00	0.63	1.41	0.45	2.00	0.45	2.45	0.85	2.76	0.85

18	1.12	0.45	1.30	0.45	2.00	0.85	2.37	1.10	2.56	1.00	2.82	0.85
24	1.58	0.63	1.92	0.75	2.27	1.05	2.90	1.53	2.71	1.19	3.22	1.15
30	1.48	0.63	1.80	0.85	2.35	1.15	2.92	1.26	2.83	0.93	3.37	1.43
36	1.70	0.85	1.67	0.75	2.52	0.93	3.30	1.23	3.51	1.19	3.56	1.71
42	1.80	0.75	1.94	1.05	2.95	1.30	3.54	1.05	3.61	1.72	3.61	1.26
48	2.12	1.10	2.12	0.93	3.26	1.45	3.55	1.23	3.64	1.73	3.79	1.51
54	2.37	1.19	2.63	1.19	3.53	1.70	3.69	1.05	3.74	1.05	3.81	1.62
60	2.48	0.93	2.80	1.26	3.61	1.19	3.59	1.19	3.69	1.19	3.80	1.23
66	2.61	1.26	2.82	0.93	3.59	1.63	3.57	1.23	3.69	1.84	3.76	1.45
72	2.70	1.15	2.85	1.38	3.56	1.45	3.58	1.76	3.71	1.23	3.79	1.54

Table S6. The Original Data of Log $_{10}$ Gene Copy Number/uL (Real-time PCR)

	Fresh OSPW	**Ozonated OSPW 18mg/L**	**Ozonated OSPW 50mg/L**	**Ozonated OSPW 78mg/L**	**Ozonated OSPW 116mg/L**	**Ozonated OSPW 200mg/L**

Day	Value	STD	Value	STD	Value	STD	Value	STD	Value	STD	Value	STD
0	4.09	3.56	4.14	3.85	4.10	3.19	4.05	3.61	4.01	3.03	4.09	3.74
9	6.01	4.91	6.21	5.42	6.60	5.39	6.37	5.48	5.88	4.86	5.94	5.01
18	6.24	4.79	6.48	4.61	6.61	5.27	6.49	5.61	6.51	5.66	6.49	5.51
27	6.34	5.19	6.57	5.29	6.67	5.72	6.54	6.51	6.52	5.83	6.58	5.60
36	6.46	5.28	6.62	4.98	6.65	6.07	6.66	5.61	6.74	4.81	6.66	5.28
45	6.57	5.09	6.66	6.05	6.72	5.76	6.81	5.29	6.84	5.06	6.72	5.14
54	6.67	6.13	6.85	4.64	6.85	6.10	6.99	6.08	6.89	5.63	6.94	6.59
63	6.60	4.97	6.76	5.43	6.81	5.57	6.86	5.33	6.75	6.08	6.81	6.47
72	6.59	5.12	6.74	5.39	6.79	5.17	6.85	5.27	6.74	5.24	6.80	5.06
81	6.59	5.39	6.70	4.75	6.77	4.80	6.84	4.65	6.71	5.51	6.79	4.65

Table S7. The Original Data of Microtoxicity of the OSPW after different ozone doses

Ozone Dose (mg/L)	Ozone Treatment IC_{20}		Combined Treatment IC_{20}	
	Value	STD	Value	STD
0	20	0.71	26.5	1.41
18	32.3	1.84	40.8	1.70
50	48.6	3.11	58.6	0.85
78	60.8	1.13	75.5	2.83
116	78.6	1.56	100	N/A
200	93.5	2.12	100	N/A