Evaluating the Persistence of Dissolved Heteroatomic Organic Chemicals in

Oil Sands Process-Affected Water by Non-Targeted Analysis

by

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Abstract

Over 1 billion m^3 of wastewater in tailings ponds currently awaits remediation as a result of the extraction of bitumen – the Canadian oil sands ore. Relative to the Athabasca River and regional tributaries, oil sands process-affected water (OSPW) has increased levels of salts, metals and a complex mixture of toxic organic chemicals including naphthenic acids (NAs). One current strategy for treating OSPW employs the use of end pit lakes (EPLs). In this method, OSPW will be placed on top of a layer of fluid fine tailings (FFT) for long term storage in evacuated mines, while the FFT dewaters, and the toxicity of the overlying cap water is reduced through natural or managed processes. However, one requirement for future approvals of EPLs is the effective removal of chemicals in OSPW which are known to be hard to degrade. Syncrude's Base Mine Lake (BML) is the only existing oil sands EPL. Using BML OSPW, the persistence of thousands of dissolved organic species was monitored here, for the first time. The **central null hypothesis** was that all organics in OSPW have equal rates of biodegradation. The hypothesis was tested using three distinct strategies: a field study of OSPW of different ages, a laboratory study monitoring *in situ* biodegradation of BML OSPW dissolved organics, and the metabolism of toxic components in BML OSPW by subcellular fractions of model fish species.

The organic profile of BML was compared to field samples representing fresh and aged OSPW (<1 year to 31 years). Principal components analyses distinguished OSPW of different ages based on relative contribution of chemical classes. Aged samples had the lowest concentrations of organics and larger proportions of oxidized heteroatomic chemicals. Overall, the most acutely toxic OSPW chemicals degraded in aged OSPW (i.e., O_2^- species), but the residual toxicity of aged OSPW may be explained by persistent degradation products, possibly due to photolysis and microbial degradation.

In situ microbial biotransformation of BML OSPW was monitored in laboratory microcosms containing BML OSPW alongside positive (containing commercial NAs) and negative (autoclaved, chemically treated) controls for 424 days. Samples were analyzed using high pressure liquid chromatography-Orbitrap mass spectrometry in negative ionization mode. After 272 days, the profile of organics in BML OSPW showed no difference compared with negative controls. Following a series of treatability experiments, nitrogen and phosphorus were added on day 389 to support microbial growth. By the end of the experiment (day 424), chemicals in the O_2^- class had decreased by 60% as did one other chemical class (O_2S^-). Changes were not observed in any other heteroatomic class.

Rainbow trout are native to the Athabasca Oil Sands region and thus will eventually be exposed to reclaimed OSPW. To study the ability for these fish to metabolize OSPW organics, *in vitro* biotransformation was monitored in sub-cellular fractions from rainbow trout liver homogenate. Phase I hydroxylation and phase II glucuronidation were assessed. Microsomes and S9 fractions each demonstrated trout have some capacity to metabolize dissolved toxic organics, but most dissolved organics were unchanged. All products generated in the assays were generated were only from phase I metabolism; no glucuronide products were observed.

This thesis investigated the biodegradability and degradation kinetics of the dissolved organics in OSPW. It is the first application of non-targeted analysis to compare OSPW of different ages. Here, I demonstrate that NAs can be slowly degraded over time and show that other heteroatomic classes are more persistent. The use of trout subcellular fractions has never before been applied to assess the biotransformation potential of trout. Biotransformation observed in microsomes suggests trout have some capacity to metabolize toxic components in OSPW and provide a starting point for the targeted analysis of metabolites in bile or urine.

Collectively, these results suggest that NAs may be degraded in EPLs, and therefore remove the acute toxicity, but other dissolved organics may contribute to chronic toxicity.

Preface

In chapter 2, I performed sample preparation, Orbitrap sample analysis, data interpretation, and manuscript composition. Trace metal and major ion analyses were performed at Syncrude Canada Limited (Edmonton, Alberta) through Warren Zubot. Comprehensive statistical analyses were performed by Sushmitha Thirumalaivasan under the supervision of Dr. Vinay Prasad. This collaboration with Sushmitha Thirumalaivasan and Dr. Prasad was facilitated by Dr. Ania Ulrich, who also contributed to data interpretation. Dr. Monika Keelan contributed to revising the manuscript. Dr. Jonathan Martin contributed to concept development, and data interpretation.

The BML microcosm project for Chapter 3 was conceived by Drs. Ania Ulrich and Jonathan Martin. I was responsible for project oversight including experimental set-up, sampling, data collection. I completed all chemical analysis including pH, dissolved oxygen, gas chromatography, ion chromatography, dissolved organic carbon analysis, and Orbitrap MS and data interpretation. Dr. Ania Ulrich advised on the strategy for DNA extraction, treatability studies, and subsequent microcosm intervention. I performed microbial DNA extraction, but DNA amplification and Illumina DNA sequencing were performed by RTL Genomics (Lubbock, TX). Bioinformatics (microbial community characterization and Bubble plot) were completed by Ken Zhao under the supervision of Dr. Bin Ma (Zhenjiang University). Dr. Matthew Ross advised on the use of heat maps to present changes occurring in microcosms.

The study in Chapter 4 expanded upon preliminary assays conducted by Joey Scheff and overseen by Dr. Matthew Ross. I was responsible for concept development, experimental design, sample preparation, OSPW fractionation, chemical analysis, and the development of the workflow for non-targeted analysis in Compound Discoverer™. The fractionation method used for extraction of BML OSPW in was developed by Dr. Chenxing Sun. Dr. Monika Keelan provided guidance for the assays.

<u>To those still to come:</u>

The spark began long ago among the stars. The ethereal wisdom of your ancestors has endured the ages. Their light has spanned generations and in this epoch is passed on to you.

This is your time.
You were born equipped with tools to build your dreams.

Find your calling.
While the path is at times unclear, let curiosity be your guide.
You will falter and you will fall. Do not fret.
The dance of a flickering flame is beguiling. It is energy adapting.
Steps and missteps in the Great Lesson.

Your intelligence is innate.
In times of doubt, look inward. A wealth of knowledge stirs deep within your pit.
Fed by hope, it gets hotter. Stoked by your experience, it grows stronger.
Let intuition ignite the passion in your heart.
Trust the fire within.

You will be suppressed and repressed but still you will smoulder.
No man can extinguish the empyreal energy that burns inside of you.
Your wondrous Soul cannot be stamped out.
You are Black, brilliant, and beautiful.
This world needs you.
Love yourself and shine for all to see.

For my King and Queen,
Kwame Asiedu and Esther Owusu

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I began this journey under the tutelage of Dr. Jon Martin: renowned scientist and leader in the field of oil sand environmental toxicology. The concepts for the projects in my PhD were largely conceived by Dr. Martin. With his support, I gained access to world-class instrumentation, attended several national conferences, and was introduced to new lifelong colleagues. Our paths diverged early in my program, but our interactions have left a lasting impact. I am cracked open. Now exposed in my mind are places previously unknown. I am not the person who moved to Edmonton in 2013, and for that Dr. Martin is partially to thank.

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Table of Contents

Chapter 3   **Biodegradation in Base Mine Lake: a comprehensive analysis of the *in situ* biodegradation of dissolved organic acids in the first oil sands end pit lake**

Chapter 4   Characterizing Phase I &, II biotransformation of BML OSPW toxic fractions by Rainbow Trout (Oncorhynchus mykiss) microsomes and S9

Appendix A Supporting information for Chapter 2

Appendix B Supporting information for Chapter 3

Appendix C Supporting Information for Chapter 4

List of Tables

List of Figures

List of Equations

List of Abbreviations

AEO	acid extractable organics
AER	Alberta Energy Regulator
amu	atomic mass unit
AOS	Alberta Oil Sands
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
BAF	bioaccumulation factor
BCF	bioconcentration factor
BHM	Bushnell Haas media
BML	Base Mine Lake
BSA	bovine serum albumin
CT	composite/consolidated tailings
CYP	cytochrome P450
Da	Dalton
DBE	double bond equivalent
D_{MW}	Distribution ratio of an organic compound between a membrane and water
Dow	Apparent Kow, coefficient used to describe the distribution of a compound between octanol and water
EC50	concentration of a compound which induces half of the maximum response (effect) after a specified time in the population examined
EDA	effects directed analysis
EP	experimental (reclamation) pond
EPL	end pit lake
EROD	7-ethoxyresorufin-O-deethylase
ESI	electrospray ionization
FFT	fluid fine tailings
FHM	fathead minnows
FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometry
FTIR	Fourier transform infrared spectroscopy

G6P	glucose 6-phosphate
G6PD	glucose 6-phosphate dehydrogenase
GC	gas chromatography
GST	glutathione S-transferase
HPLC	high pressure (or performance) liquid chromatography
HRMS	high resolution mass spectrometry
HRT	hydraulic retention time
Ka	acid dissociation constant
Kd	dissociation constant
K_{OC}	organic carbon (soil) adsorption coefficient
K_{OW}	octanol-water partition coefficient
LARP	Lower Athabasca Regional Plan
LC50	concentration of a compound which is lethal for 50% of the population examined
LLE	liquid-liquid extraction
m/z	mass to charge ratio
mbs	metres below surface
MeOH	methanol
MFT	mature fine tailings
MLSB	Mildred Lake Settling Basin
MLX	Mildred Lake Extension Project
Mm^3	Million cubmic metres
MS	mass spectrometry
MW	molecular weight
NADP	β-nicotinamide adenine dinucleotide phosphate
NADPH	reduced β-nicotinamide adenine dinucleotide 2′-phosphate
NAFC	naphthenic acid fraction components
NAs	naphthenic acids
NAT	N-acetyl transferase
NTA	non-targeted analysis

OECD	Organisation for Economic Co-operation and Development
OSPW	oil sands process-affected water
OTU	operational taxonomic unit
PAH	polyaromatic hydrocarbon
PDMS	polydimethylsiloxane
pH	power of hydrogen, $-\log_{10}$(Concentration of H^+)
PSC	primary separation cell
QTOF	quadrupole time of flight
RP	resolving power
SAGD	steam assisted gravity drainage
SCL	Syncrude Canada Limited
SFS	synchronous fluorescence spectroscopy
S/N	Signal to noise ratio
SPE	solid phase extraction
SULT	sulfotransferase
TIE	toxicity identification evaluation
TMF	tailings management framework
TMS	t-butyldimethylsilyl
TP	tailings pond
Tris	tris(hydroxymethyl)aminomethane hydrochloride
UDPGA	uridine-diphosphate-glucuronic acid
UGT	uridine-diphosphate-glucuronosyl-S-transferase
UHPLC	ultrahigh performance liquid chromatography

Chapter 1   Introduction

1.1   Alberta oil sands (AOS)

The Western Canada Sedimentary Basin contains much of Canada's oil sources and natural gas. The geological formation spans across British Columbia, Saskatchewan, and Alberta. The oil sands represent 99% of Alberta's oil reserves. The deposits are classified into three regions covering 142 000 km^2.[1,2] The ore is found in three generalized areas designated as the Athabasca, Peace River, and Cold Lake deposits (**Figure 1-1**). In 2019, 171 000 m^3 of bitumen was mined in Canada and 25 billion m^3 of bitumen remains yet undeveloped in those reserves.[3] Depending on the geology where it is found, oil can be produced in different ways. Conventional crude oil is liquid at atmospheric pressure and temperature can be removed by standard methods (i.e., drilling and pumping). When oil cannot be extracted by these standard methods it is termed non-conventional. The oil sands represent one form of non-conventional oil.

1.1.1   Bitumen: The Oil sands ore

After they die, plants and animals can re-enter the life cycle in a different form. Over millions of years, organic matter can be converted to fossil fuels under pressure. Coal, natural gas, and oil are all forms of fossil fuels – resources which can be burned and converted to energy. Oil is a liquid made up of a mixture of organic compounds called hydrocarbons.[4] Generally, the chemical structures of hydrocarbons are made of hydrogen and carbon. The hydrocarbons in conventional crude oil tend to be "light" or "medium" in molecular weight (<500 Da).[5] While light/medium oil can flow, bitumen is so viscous that it cannot.[6] The hydrocarbons in bitumen have higher molecular weights (500 - 800 Da)[5,7] and more compounds with nitrogen and sulfur which decrease the value of the oil. As such, bitumen requires alternative strategies to be produced (removed from the ground) and refined into a form that can be sold as synthetic crude oil.

Figure 1-1 Alberta oil sands areas: Peace River, Athabasca, and Cold Lake.
(http://history.alberta.ca/energyheritage/sands/origins/the-geology-of-the-oil-sands/the-location-of-oil-sands.aspx)

The ore is up to 14 wt% bitumen and 3 – 5 wt% water,[5] approximately 3% clay and the remainder is silt and sand. In fact, "oil sand" is casual term for bituminous sand which speaks to the physical nature of the ore (See Section 1.2.3). Different methods are used to mine bitumen depending how close the oil sands deposit to the surface of the earth.

1.2   Mining

Oil sands mining occurs are by two general methods: *in situ* methods and surface mining.

1.2.1   In situ

In situ bitumen recovery occurs "in place" when a deposit is more than 75 metres below surface (mbs). *In situ* is Latin for 'in place', which is a scientific description for this method of mining. Most oil sands deposits (80%) are located between 350 – 600 mbs. Thermal energy (heat) is used to make bitumen flow. One method is steam-assisted gravity drainage (SAGD):[8]

1.2.1.1 Steam Assisted Gravity Drainage

SAGD (pronounced "sag-D") uses two horizontal wells 200 mm in diameter and up to 1000 m in length. These wells are drilled with the lower well between 1 – 2 m above base of the formation.[5] The upper well runs parallel to the first well and is about 5 m above the formation. Steam at 250°C and 9600 kPa is continuously injected into the upper well to create a "steam chamber" which heats bitumen to a temperature at which it can flow into the lower (production) well.[8] A mixture of bitumen and steam (i.e., condensate) is brought to the surface (wellhead) by the pressure created in the initial process, and then by the application of a high pressure gas (gas lift).

1.2.2   Surface mining

Surface (or "open pit") mining is the most economically feasible strategy for bitumen removal occurring no more than of 75 m below earth's surface.[8] About 20% of deposits contain bitumen which is recoverable by this method [9]. In 2013, it was estimated that the surface mineable area of the oil sands was 895 km^2 – a small fraction of the total oil sands area.[10] Open pit mining occurs primarily in the Athabasca deposit with the heart of activity occurring in Fort McMurray, Alberta. In 2019, 64 Mm^3 of bitumen was produced from both mining and *in situ* methods.[3]

1.2.3   Bitumen extraction

Bitumen extraction makes use of the physical chemistry of the ore. Each grain of sand has a film of water which is encased in bitumen (**Figure 1-2**). The commercial development of oil sands required an inexpensive way to heat bitumen enough to flow. Working at the University of Alberta, Dr. Karl Clark developed a method for the separation of bitumen from "bituminous sand" in the 1920s.[11] This was a critical improvement for oil sands technology and the Clark Caustic method (or Hot Caustic method) of bitumen extraction is the strategy is still used today.

Sodium hydroxide (caustic soda) is a base used to release natural chemicals from bitumen which allow it to be separated from sand particles.[2] Surfactants are chemicals that lower the surface tension at the interface of two phases.[12,13] These compounds lower the surface tension between bitumen and water, allowing for effective bitumen recovery.[13]
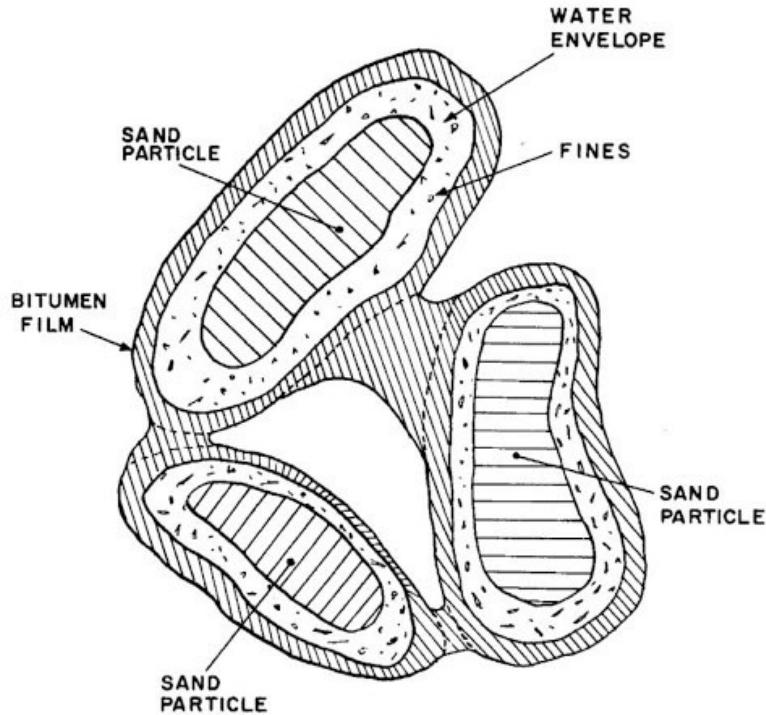
Figure 1-2 In the ore, each sand grain is enveloped by a layer of water and encased by a film of bitumen.[14]

1.2.3.1 Bitumen froth treatment

Bitumen slurry is generated by the addition of hot water (40°C – 55°C) and sodium hydroxide or sodium carbonate are added to reduce the viscosity of bitumen enough for it to flow.[12,13] This liquid form of bitumen can then travel through hydrotransport pipelines where the speed of the flow helps to separate the slurry into different components and liberate bitumen from the sand grains. The slurry pipeline ends at the primary separation cell (PSC) or primary separation vessel (PSV), where the bitumen is separated from solid material by gravity. In the PSC, air bubbles covered in bitumen are skimmed off at the top, while heavier particles including sand are removed from the bottom. Bitumen froth contains about 60 wt% bitumen, 30 wt% water and 10 wt% fine solids.

The addition of diluents (organic solvents) lowers the viscosity of bitumen, and by solubilizing bitumen in the froth, diluents increase the separation bitumen from water.[5,13] Dilbit is a mixture of diluent and bitumen which must then undergo upgrading and/or refining prior to being sold to market.

1.2.4 Upgrading and Refining

Dilbit is sent to either a high conversion refinery or to an upgrader depending on its quality.[5,15,16] If the bitumen has less than 0.5% water and solids, it is sent to a high conversion refinery (~65% of bitumen product), otherwise it must go to an upgrader (~35% of bitumen product).[16]

1.3 Water usage

In situ mining requires just a half barrel of freshwater and 2.5 barrels of recycled and non-potable water to produce one barrel of synthetic crude oil, thus water is an integral part of this system. Surface mining is more water intensive, requiring 2 – 3 barrels of freshwater and 8 – 10 barrels of recycled water per barrel of oil.[17] The Athabasca River and its tributaries are the primary source of freshwater for operators. Currently, restrictions on water withdrawals are set through the provincial government by Alberta Environment and Parks (AEP),[18] who works closely with the Alberta Energy Regulator (AER) as part of the Lower Athabasca Regional Planning program (LARP).[5] The withdrawal limits are set weekly and are influenced by the seasonal variability in river flow. Less than 1% of annual flow (137.9 Mm^3) was used by operators in 2017.[19]

For years, the Government of Alberta has attempted to balance the competing interests of the economic development of the oil sands and the protection of the environment. The AER is a non-government organization funded by industry to review energy development in the province. In 2016 Directive 085 was established to "minimize fluid tailings accumulation which may reduce environmental effects such as the reduction of seepage, and occurrences of wildlife contact with tailings ponds."[20] The provincial government and industry agree that wastewater should not be directly released into the environment. This understanding, known as the "Zero Discharge Policy",[21] produced storage units known as tailings ponds (TPs). Following bitumen extraction, oil sands process-affected water (OSPW) and suspended solid byproducts (i.e., tailings) are sent to TPs.[22]

1.3.1 Tailings ponds in the oil sands industry

Tailings ponds are a critical component of the oil sands industry, growing both in number and size alongside the expansion of commercial oil sands development. In 2016, the total active

tailings area was 257 km^2 of which 103 km^2 was due to the surface area of TPs.[23] At that time the total volume of tailings was 1.21 billion m^3 (**Figure 1-3**).[24] And while recycling practices have greatly improved in the last decade, TPs and the contained OSPW remain a major issue plaguing the oil sands industry.

TPs are built either in evacuated mines or above ground on cleared land. Sediments (sand, silt) settle to the bottom of TPs, and clarified water is recycled back into the extraction process to optimize bitumen recovery.[25] These impoundments enable the settling of heavier components passively (through gravity) or actively via a number of processes including centrifugation, flocculation and coagulation.[26] Fines are tiny particles ≤ 44 µm in diameter found in the semi-solid phase of TPs referred to as fluid fine tailings (FFT).[27] FFT is a byproduct of bitumen extraction and contains $< 8 - 15$ wt% solids.[28] Clays in tailings are charged, and particles repel each other resulting in a suspension of these particulates in water in a matrix with yogurt-like consistency.[29]
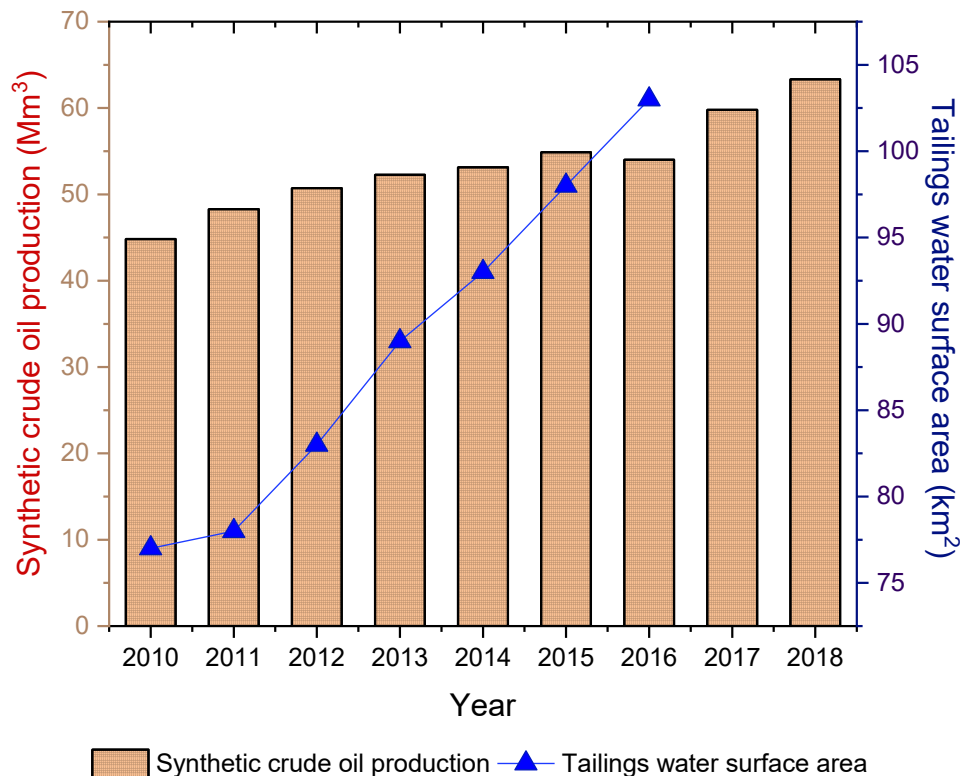
Figure 1-3 Synthetic crude oil production and tailings water surface area between 2010 and 2019.[3, 26]

Mature fine tailings (MFT) are aged fine tailings (> 2 years) found at the bottom of TPs containing less water than FFT and a higher solid content (≥ 30 wt%).[30] Composite/consolidated tailings (CT) have yet a higher solids content (up to 80 wt%).[27] MFT becomes CT following the addition of chemical coagulants which force porewater out of the matrix. In the CT process, tailings are treated with chemicals such as gypsum or alum to decrease solids volume.[31] Remaining porewater is found at the surface (within to top 2 – 10 m of a TP).[27]

Tailings water chemistry is greatly influenced both by the ore extracted, and the processes used in extraction.[26,32] OSPW has trace levels of metals such as arsenic, chromium, copper, lead, mercury, manganese, nickel, and vanadium.[33] OSPW also contains minerals like dolomite, illite, and kaolinite present in suspended clays, and ions like calcium, sodium, magnesium, chloride, and sulfate.[5] TPs are slightly basic with a pH between 8.0 – 8.4. Residual bitumen contains polar and saturated hydrocarbons and some polyaromatic hydrocarbons (PAHs).[27] While most aromatic organics are not soluble in water, sodium salts of phenols, cresols, and thiophenols are dissolved. Dissolved organic compounds are the primary drivers of OSPW toxicity to aquatic biota.

Between 80 to 95% of water is recycled through the extraction process,[17,25] however continued recycling of OSPW increases concentrations of salts which can decrease efficiency of bitumen recovery and can damage machinery used in extraction. For this reason, OSPW cannot be recycled forever.[33,34] Hydrocarbons from diluent used in the treatment of bitumen froth are used as substrates for degradation by microorganisms.[26,35] In TPs, these microbes generate greenhouse gases (GHGs) and contribute considerably to GHG emissions of in the AOS region.[26,36] Total carbon dioxide and methane emitted from TPs rose from 1.1 million tonnes in 2011 to 1.7 million tonnes in 2013, and fell to 1.3 million tonnes in 2015.[37] OSPW volumes will continue to grow until a strategy of detoxification has been identified and employed. Many studies have been conducted with the aim identifying which dissolved organic chemicals are most responsible for OSPW toxicity.

1.4 Identification of toxic fractions in OSPW

In 1986, two industry scientists studied active and passive treatments of TP OSPW. MacKinnon and Boerger postulated that inherent toxicity was due to "relatively low molecular weight (200 to 500 amu), polar organic compounds with properties similar to naphthenic acid

(NA)".[38] An infrared (IR) spectrum of this extract confirmed the presence of carboxylic acids and thereby also confirmed their hypothesis. Shortly thereafter in 1993, Verbeek *et al.* conducted one of the first toxicity identification evaluations (TIE) on OSPW.[39] Before and after each step of fractionation, toxicity of OSPW (initially pH of 8) was assessed using the Microtox bioassay. Fractions which appeared to have high acute toxicity in Microtox were then re-tested using higher level organisms such as *Daphnia magna* and rainbow trout (*Oncorhynchus mykiss*). Polar organic acids accounted for at least 55 – 60% of toxicity in all samples from a Suncor TP. Nearly 25 years later conducting a TIE, Hughes *et al.* determined that NAs could be used to quantitatively assess acute toxicity of OSPW in rainbow trout.[40] Their results aligned with many others confirming polar organic acids as the primary toxic component of OSPW.

In 2015, using three successive rounds of fractionation Morandi *et al.* aimed to separate NAs from other chemicals in OSPW. Toxicity of the components was assessed using effects directed analysis (EDA) by sequential exposure of fathead minnow (FHM) embryos to the various fractions. They determined OSPW toxicity was apportioned to two fractions: one comprised mainly of NAs, the other was a mixture of polar neutral organic classes detected in positive ion mode including species in the O_2^+, O^+, ON^+ and OS^+ classes. NAs remain of primary focus for toxicity studies.

1.4.1 Naphthenic Acids

Naphthenic acids (NAs) are chemicals naturally found in bitumen which are concentrated during extraction. These chemicals are released into the process water and known to be highly persistent.[41–44] Generally NAs are described as a complex group of aliphatic and cyclic carboxylic acids fitting the formula $C_nH_{2n+z}O_2$, where *n* defines the number of carbons and *Z*, an even negative integer, represents the number of double bond equivalents (DBE) within the structure. NAs have a wide range of molecular weights and structures.[45,46] As analytical techniques have improved over time, NAs having 3 to 5 rings (tri-, tetra-, and pentacyclic NAs) have been identified. [47–49] Carboxylic acids are defined by a carboxyl group: a carbon which is both double bonded to an oxygen and bonded to a hydroxyl group.[50] The introduction of each double bond replaces two hydrogen atoms in the structure. NAs with no double bonds (besides the one in the carboxyl group) can be said to have Z = 0. So, Z describes the subsequent removal of hydrogens upon introduction of a DBE (e.g., 2 DBEs - Z = -2, 3 DBEs – Z = -4 etc., **Figure 1-**

4). The number of carbons tend to range between from 7 – 20, while the number of double bond equivalents (DBE) typically ranges from 2 to 12.[43]

NAs are only a small proportion of the total organics in OSPW.[47] In literature, naphthenic acid fraction components (NAFCs) and/or acid extractable organics (AEOs), generally refer to the dissolved organics which are extracted at an acidic pH along with NAs. To be specific, those species with two oxygens (i.e., O_2^-) in their structures are sometimes referred to as "classical NAs" to distinguish them from species which have more oxygens and/or other heteroatoms (i.e., an atom that is not carbon or hydrogen).[40,47,51–54]

	Z = 0	DBE = 1
	Z = -2	DBE = 2
	Z = -4	DBE = 3
	Z = -6	DBE = 4
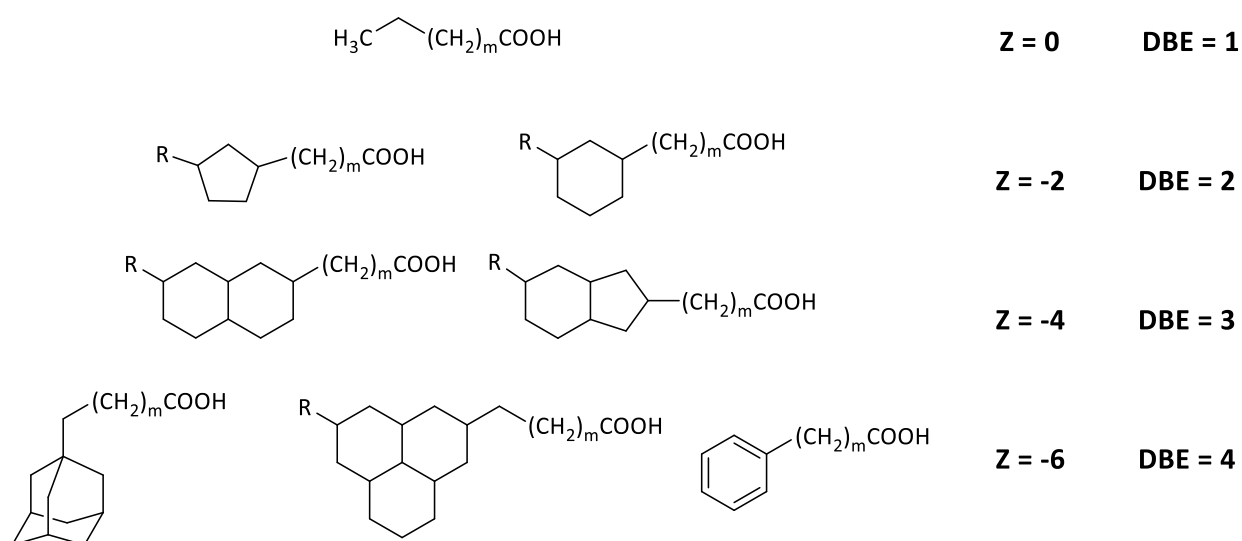
Figure 1-4 Some example structures of NAs in OSPW. Adapted from Frank *et al.*[60]

1.4.1.1 Properties and environmental distribution

The physical properties of a chemical can be used to predict where it is found in the environment. Distribution coefficients are ratios expressing the expected amount of a given chemical between two phases or compartments. The summary of the properties of NAs is summarized in **Table 1-1**.

Table 1-1 Physical properties of naphthenic acids

Property	Description	Value	Reference
Molecular weight (Da)		140-600	[61,62]
K_a (unitless)	Acid dissociation constant	5-6 (at pH~7)	[61]
K_{oc} (mL/g)	Organic carbon adsorption coefficient	0.2 (at low pH)	[13]
		17.8 (at pH 8)	[63]
K_{ow} (unitless)	Octanol-water partition coefficient	2.4 (At pH~7)	[61]
		3.5	[64]
K_H (atm m^3/mol)	Henry's law constant air-water partition coefficient	8.56 x 10^{-6}	[65]
Solubility (g/mL)		50 mg L^{-1} at pH ~7	[66]

As with all weak acids, the pH of the environment in which NAs are found will influence whether it will be in neutral or charged form. NAs have an acid dissociation constant (K_a) comparable to a weak acid with a K_a in the range of 10^{-5} to 10^{-6}, meaning that above a pH of 5 – 6, NAs will be ionized.[56] NAs have a low solubility at a neutral pH (50 mg L^{-1} at pH ~ 7)[61] but under the alkaline conditions of extraction (pH > 7), NAs will be ionized, making these chemicals more hydrophilic and increasing their solubility in water. Young *et al.* estimated that 99% of NAs will be ionized in both OSPW and the Athabasca River.[62,63] Concentrations of NAs in the Athabasca River have been measured to range from 0.001 – 0.1 mg/L,[43] while concentrations in OSPW range from 20 – 120 mg/L.[57] Overall, NAs are largely partitioned into the water column at the environmentally relevant pH of 7 – 8, some fraction of which will be bioavailable.[61]

1.5 OSPW Toxicity

1.5.1 Animal studies

The following reviews toxicity studies by species and focuses on exposures to unextracted OSPW, NAs and/or NAFCs.

1.5.1.1 Fish

The Athabasca and Peace Rivers are the two largest in Alberta. They are home to several fish including burbot, goldeye, whitefish, pike, and walleye.[5] First Nations communities in the area observe the right to hunt and fish. These fish are important for traditional subsistence lifestyles and thus their health is of utmost importance to local Indigenous communities.[64] Studies of fish have examined a few endpoints including (but not limited to) reproductive health, immunotoxicity, and mutagenicity.

Fish gills are important for respiration and ionic homeostasis of the organism, as well as the exchange of important ions with the surrounding environment.[65] Van den Heuvel *et al.* (2000) hypothesized that survival rates of these fish were impacted by OSPW exposure.[65] To test this theory, yellow perch (*Perca flavenscens*) were stocked into Demonstration Pond (Demo Pond)- an experimental pond containing OSPW. Exposures of up to 10 months produced aneurysms on the gills, and erosions and lesions on fins resembling dermal sarcoma. The researchers in this study postulated that because water soluble compounds can be absorbed by fish through their gills, this might explain gill erosion observed in yellow perch exposed to NAs extracted from OSPW. Despite these physical changes, the general survival of yellow perch (related to immunoregulation) did not appear to be compromised.

Interestingly, FHM (*Pimephales promelas,* FHM) were accidentally added to Demo Pond and this fish population thrived for years to follow.[66] However, a 2013 field study of FHM in Demo Pond also observed cell proliferation in the gills.[67] Furthermore, gonads in exposed fish were larger than in controls – potentially indicative of a delay sexual development (related to endocrine disruption). Adverse effects on reproduction were also studied in goldfish (*Carassius auratus*) caged in Demo Pond for 19 days.[68] Higher levels of testosterone, and lowered levels of 17β-estradiol (E2) were observed in the plasma of juvenile female goldfish earlier in their vitellogenin (oocyte maturation) cycle compared to mature female fish. By impacting oocyte development, lowered amounts of sexual steroids could disrupt or impair reproduction in the population. Previous studies reported significant decreases in E2 and testosterone in yellow perch exposed to OSPW aged 2 years in experimental ponds (< 50%) compared to off-site reference lakes.[65,69] Rowland *et al.* identified structures of NAs and suggested some polycyclic monoaromatic naphthenic acids that resemble steroids, and therefore had the possibility to generate ED effects in animals.[70]

Exposure to NAs has negative effects on the early-life stages of fish.[71] The embryos of wild yellow perch and lab-raised Japanese medaka (*Orizias latipes*) were incubated in OSPW from Mildred Lake Settling Basin (MLSB – a TP at Syncrude Canada Limited, SCL). The unextracted MLSB OSPW treatment exposed both fish to serial dilutions which ranged from 0.16% - 100%. These resulted in exposure concentrations of dissolved NAs at 0.5 – 84.6 mg/L in yellow perch tests and 0.20 – 71.0 mg/L in medaka tests. The deformity threshold effect concentration for yellow perch exposed to OSPW was 7.52 mg/L, about 4.5 times higher than this threshold in commercial exposures at 1.67 mg/L. A similar effect was seen in medaka. The number of deformities were positively correlated, and larval length at hatch was negatively correlated with NA concentration.

Another important consideration is whether fish can be consumed following exposure to OSPW. Lake whitefish, northern pike and walleye are often eaten by northern Indigenous communities. Tolton *et al.* reviewed studies in which panelists from the community assessed the smell/taste of fish from exposure studies.[72] They describe fish taint as "a foreign flavour or odor in the organism induced by conditions in water to which the organism is exposed". While fish toxicity studies will remain of great importance to all stakeholders, qualitative research on the perceptions and experiences of community members can be used to create a more detailed picture on the impact to the area.

1.5.1.2 Amphibians

Metamorphosis is the transition in amphibians from tadpole to adult."[73] The northern Canadian toad (*Bufo boreas*) and wood frogs (*Lithobates sylvaticus,* previously named *Rana sylvatica*), both native to AOS region, were exposed to dyke seepage and CT OSPW.[74] Metamorphosis was delayed by 3 days compared to reference sites and a 63% decrease in survival before the completion of metamorphosis. Differences in sensitivity to exposure was also observed: frog tadpoles held in OSPW impacted wetlands completed metamorphosis, but toad tadpoles were unable to complete development. Further, delayed metamorphosis was observed in tadpoles of wood frogs held in wetlands for 75 days, which had been recently impacted by solid and liquid tailings of bitumen extraction (≤ 7 years earlier) compared to old reference wetlands (impacted by tailings >7 years prior to the study).[73] Newly impacted wetlands were postulated to be more toxic compared to reference wetlands as toxicity in OS waste materials

appears to decrease with age. The former group had a 54.7% higher rate of mortality. The Western clawed frog (*Silurana, Xenopus, tropicalis*) is a good proxy for human early developmental stages.[50] Gutierrez-Villagomez reported a positive relationship in the concentrations of AEOs and teratogenic effects on Western clawed frogs. Concentrations of 0.0 – 55.0 mg/ L NAs produced increased abnormalities (ex. cranial abnormality, edema rate, face edema, etc.) in frogs exposed as tadpoles.[75] So, the greatest harm on amphibians will be to their reproductive activity – a recurring observation across many animals.

1.5.1.3 Avian studies

To date, few studies have reviewed toxicity of OSPW in birds. Having a diet which largely consists of aquatic insects, tree swallows (*Tachycineta bicolor*) were speculated to have a future risk associated with reclamation ponds.[76] In 2007, 10 groups of nestlings were assigned to treatments of either saline solution or varied dilutions of NAs (in water). The environmentally relevant concentration of exposure was estimated to be about 0.15 mg NA/day. To simulate a worst-case scenario, Gentes *et al.* looked at the effects of a concentration ten times greater than this estimated daily dose. Finally, the examination of major organs such as kidneys, spleen, liver, and heart revealed no inflammation, necrosis, or parasites, suggesting that tree swallows chronically exposed to NAs would not suffer adverse effects.[77] Although these findings are hopeful, more comprehensive studies are needed bolster this hypothesis.

1.5.1.4 Mammalian studies

The US EPA deduced the LD_{50} for a single oral dose of commercial NAs to rats was 3.0 g/kg. Rogers *et al.* (2002) produced the first mammalian study assessing both the acute and subchronic toxicity of oral exposures to NAs from OSPW. [78] To assess acute toxicity, single oral doses of NAs (3, 30, 300 mg/kg) were administered to Wistar rats (*Rattus norvegicus*) at concentrations estimated to be 0.5, 5 and 50 times a single worst case exposure scenario assuming drinking water were at levels comparable to TPs. The rats were monitored for 14 days. Observations of a dose-dependent incidence of pericholangitis led to the hypothesis that the liver was a target organ of OSPW exposure. Inflammation of bile ducts in the liver, indicative of chronic liver disease, was seen in 6 out of 10 high dose males and 7 out of 10 high dose females. Over 90 days, the subchronic study delivered lower doses (0.6, 6, 60 mg/kg) to rats for 5 days per week.[78] Increased liver weight was observed in both acute and subchronic studies. The

conclusion of this study was that although environmental concentrations of NAs may not be acutely toxic to small mammals, a low dose daily exposure may prove to be more detrimental. In a later study, Rogers *et al.* found that upon exposure to NAs, the reproductive success of Wister rats was decreased to 7%.[79] Plasma cholesterol and were reduced compared to controls (37% and 58%, respectively). The researchers confirmed NA exposure resulted in hypocholesterolemia which in turn decreased the production of the sex steroid, progesterone, which ultimately resulted in reproductive toxicity.[79]

Following Rogers, few other mammalian studies have been conducted. Garcia-Garcia (2012) measured the levels of phagocytosis (one cell consuming another cell/particle) and expression of pro-inflammatory genes to assess immune response of mice exposed to NAs.[42] This study revealed a general downregulation of genes in both the liver and spleen upon exposure to both OSPW and commercial NAs.[42] These organs were chosen as they have relevant function in gastrointestinal absorption of xenobiotics (foreign chemicals). Down-regulation of a gene translate to a delayed immune response. This study ultimately agreed with Rogers's first study in showing that a low dose acute exposure of OSPW may produce detrimental effects in mammals.

1.5.2   Microtox toxicity assay

The Microtox toxicity assay measures the response of the marine bacteria, *Vibrio Fisheri*, to exposed toxicants. This non-pathogenic bacterium is bioluminescent, meaning it naturally produces light.[95] Exposure to toxic chemicals disrupts the natural processes, reducing luminescence. In this test, bacteria are exposed to increasing concentrations of the chemical(s) under investigation. The more toxic a chemical is, the greater the reduction in light intensity. This test is quick, cost-effective and requires little training, so for this reason it is widely used as a crude estimate for acute toxicity in many studies. An early study by Mackinnon and Boerger reported OSPW aged by $1 - 2$ years had Microtox $IC_{50} > 100\%$ (v/v) and 96 hr LC_{50} values of $>100\%$ in bioassays with *Daphnia magna* and rainbow trout.[39] This study became a benchmark for toxicity studies for years to come. Not only did it prove OSPW could indeed be detoxified, but also that reduction in toxicity could be measured consistently across multiple taxa.

Species differences must be considered when conducting OSPW toxicity studies.[41] Since Microtox is used so frequently in OSPW toxicity research, there is value in comparison of

Microtox results across studies. However, researchers must be aware of the limited ability for this test to be extrapolated to fish, and perhaps to other animals.

1.5.3 Mechanisms of toxicity

The exact mechanisms of action for the toxicity of dissolved organics in OSPW is unknown. A myriad of effects including oxidative stress, narcosis, cell death and endocrine disruption are often cited as methods of toxic action by organics in OSPW.[55,81–87] Electrophilic reactivity and enzyme inhibition may be an alternative mechanism of action for AEOs. It is likely, that some combination of these is operative in the toxicity of OSPW.

1.6 Accumulation, biotransformation, and metabolism of organic fractions in fish

Bioaccumulation is defined as the uptake of a compound passively by an organism which results in a higher concentration of that compound inside the organism compared to the surrounding environment. Bioconcentration is typically measured in the lab to estimate bioaccumulation. Our current understanding of the bioaccumulation potential of dissolved organics in OSPW comes from studies which have measured concentrations of NAs in fish tissue. Additionally, surrogate materials which approximate both fat and cellular membranes of fish have been used to estimate the octanol-water partition coefficient, or K_{ow}, of compounds. K_{ow} can be used to estimate bioaccumulation in the absence of bioaccumulation or bioconcentration values.[88] A substance is bioaccumulative if it has a bioconcentration factor (BCF) > 5000 or $\log_{10}K_{OW}$ > 1.[88] For mixtures, bioaccumulation can be estimated by the apparent octanol-water coefficient, D_{OW}.

The most comprehensive work on assessing NAs in fish was conducted by Rozlyn Young, nearly 10 years ago. She exposed rainbow trout to 3 mg/L Merichem NAs in the lab for 10 days.[89] NAs were detected in various tissues in fish, with the highest amounts found in gills and liver – organs known to have detoxification enzymes.[89] The BCF of NAs at pH 7.6 was ~4. Related to this study, Zhang *et al.* (2015) estimated that neutral chemical species had a high ability to bioconcentrate in fish using polydimethylsiloxane (PDMS, a surrogate for fish storage lipids). Species which partitioned to the highest degree (i.e. had the highest D_{OW}) included those of SO^+ and NO^+ heteroatomic classes detected using ultrahigh resolution mass spectrometry in positive ion mode.[90] To give a broader picture, Zhang *et al.* later used the TRANSIL^{XL} membrane affinity kit to estimate partitioning of ionizable compounds into membranes (K_{MW},

membrane-water partition coefficient).[91] Fish phospholipid bilayer membrane was modeled by silica beads coated in phosphatidylcholine. Less than 10% of species detected in positive ion mode had $logD_{MW}$ higher than 1 and these included species belonging to O^+, O_2^+, SO^+ and NO^+ classes –previously been found to have the highest D_{OWS}.[91] A higher proportion of species detected in negative ion mode (14%) had $logD_{MW}$ higher than 1, consisting of SO_2^- and O_2^- species. Interestingly, the researchers observed that O_2^-, NO^+ and SO^+ species were the only ones detected in the lipids of laboratory exposed Japanese medaka. Their *in vivo* BCF calculations showed good agreement with *in vitro* calculations, except for SO^+ and NO^+ species which may have implied that these compounds may have been biotransformed *in vivo*. A recent review by Scott *et al.* summarized all studies of bioaccumulation of NAs in OSPW.[92] The review highlighted that NAs did not concentrate in tissues of fish exposed to OSPW.[62]

Our current understanding of the bioaccumulation potential of organics in OSPW is limited. Persistence of these compounds may pose a risk to aquatic organisms if a) they are toxic or b) they cannot be metabolized. Biotransformation plays a key role in understanding chemical persistence and remains understudied. There is a need to investigate the capabilities of native fish to naturally metabolize and excrete these compounds.

1.7 OSPW characterization

1.7.1 Analytical methods of profiling dissolved organic compounds in OSPW

In 2009, Panda *et al.* addressed the complexity of the organic components of fossil fuels, including in oil sands.[93] They described how and why it is difficult to identify individual components in the mixture of compounds in crude oils. The more atoms a compound has in its molecular formula, the higher the number of potential structural arrangements. Thus, the compounds in petroleum were described as 'supercomplex'. Furthermore, highly biodegraded ores, like bitumen, will be enriched in compounds with large and complicated structures with atoms like nitrogen and sulfur. [5,47] Researchers have sought to elucidate which organics are dissolved in OSPW.[94] The way in which these chemicals are identified depends on the sample preparation and analytical methods used.

1.7.2   Spectroscopic methods

Fourier Transform Infrared Spectroscopy (FTIR) is frequently used in industry to measure NA concentrations.

Table 1-2 Comparison of FTIR and SFS methods for NA detection

Method	Principle	Advantages	Disadvantages	References
Infrared Spectroscopy	Measures absorbance at carboxylic acid peaks	Fast Cheap User-friendly	Cannot distinguish between carboxylic acids and NAs Overestimates NA concentrations	111 49 112 113
Synchronous Fluorescence spectroscopy	Spectral peaks of PAHs are measured to estimate aromatic NAs	*Ibid* Can confirm presence of aromatic NAs	Cannot detect other structures of NAs	114 115 79

1.7.3   Low and high resolution methods

Resolution is the ability of a mass spectrometer to distinguish between ions of two distinct mass-to-charge ratios. Resolution (R, also called resolving power, RP) is described by the Equation 1-1:

$$R = \frac{m}{\Delta m}$$

There are a few definitions for resolution, described in **Figure 1-5**. The definition generally used by low resolution mass spectrometry describes that for a peak at the exact mass, m, the full width at half the maximum (FWHM) height of the peak is Δm. Low resolution instruments have 'unit resolution' meaning masses are separated by integers (e.g., m/z 50 is separated from m/z 51). High resolution mass spectrometry has two definitions for resolution: 1) Δm is the resolving power or the difference of the measurements (in m/z units) between two successive peaks and is the exact mass of the first peak, and 2) Δm considers the spacing of two peaks of equal intensity

with the valley between at 10% or 50% of the peak height. The resolution of various mass spectrometers is described in **Table 1-3**. Low resolution methods have mass resolution less than 10 000.

Methods developed for analysis of OSPW often use a hybrid quadrupole-time of flight detector (QTOF). Ions pass through the quadrupole – an arrangement of four rods to which an electric field is applied. An alternating current voltage is applied to two rods and a direct current voltage is applied to the other two rods. Only ions of a certain *m/z* pass through the magnetic field applied in the quadrupole. The ions will then pass through the time of flight mass analyzer whereby the *m/z* for each ion is related to the time it takes for an ion to reach the detector. Lighter ions with low charges travel fastest.

Table 1-3 Comparison of mass analyzers in LC-MS instruments within the range of *m/z* ~ 300-400. Adapted from Strauss *et al.* 2010

Analytical method	Resolution (FWHM)	Mass accuracy (ppm)
Triple quadrupole (QqQ)	Unit resolution	50
Quadrupole ion trap	10 000	50
Quadrupole time of flight (TOF)	10 000	20
Orbitrap	100 000	2
Fourier transform ion cyclotron resonance (FT-ICR)	1 000 000	≤ 1
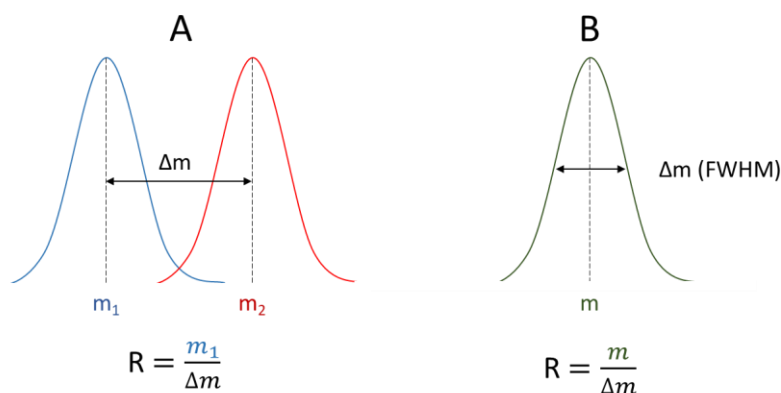
Figure 1-5 Two descriptions of mass spectral resolution. In panel A, Δm is defined by the difference in *m/z* units between two successive peaks. Panel B displays an alternate definition with Δm as the full width of the peak at half the maximum (FWHM) of the peak corresponding to *m/z* unit *m*.

The mass accuracy of an instrument is a way to know how well it can assign a molecular weight to an ion. It describes the error of mass assignments in parts per million by Equation 1-2:

$$Mass\ error = \frac{m_{experimental} - m_{theoretical}}{m_{theoretical}} \times 10^6$$

Chromatography allows for the separation of chemicals in a mixture related to their structural features and their interactions with adsorbing material (known as the stationary phase,\), and a solvent (called a mobile phase). Several analytical methods now use a form of chromatographic separation (usually gas chromatography, GC, or liquid chromatography, LC) followed by some form of mass spectrometry to NA analysis.[99] LC is discussed in more detail below.

1.7.3.1 Liquid chromatography (LC) and high pressure liquid chromatography-mass spectrometry (HPLC-MS)

Separation efficiency of a column of length, L, can be described by Equation 1-3:

$$H = \frac{L}{N}$$

As the theoretical plate height decreases (H), theoretical plate number (N) increases. Thus, chromatographic resolution increases with N. In early HPLC methods, NAFCs were derivatized in an acidic solution of 2-nitropheylhydrazine and neutralized with potassium hydroxide.[100] Derviatized analytes eluted as collection of unresolved peaks (a hump) whose area could be integrated to estimate concentrations of NAFCs. A modified method by Yen *et al.* demonstrated that day-to-day changes in microcosm studies could be tracked by HPLC, but no standard could be used to accurately and reproducibly quantify NA concentrations in mixtures.[101] Nevertheless, the HPLC method offered advantages over both FTIR (and GC-MS) methods in that sample preparation and data analysis time were reduced.

Panda *et al.* (2009) recommended the use of LC with high resolution mass spectrometry (HRMS) to identify the chemicals in petroleum.[93] In 2006, a HPLC-QTOF method for detection of NAs sought to increase mass resolution of the then commonly used GC-MS method.[45] This method improved peak shapes and increased the sensitivity by 40 to 350 times for detection of NAs by the direct injection MS method (unit resolution). Later, Martin *et al.* compared characterization and quantification of NAs by low and high resolution methods.[102] The low resolution quadrupole-MS method demonstrated a high response by NAs with Z = 0 (~15% of

total) and Z = -2 (19% of total), for which the response was absent or low (3.7% of total response) using high resolution HPLC-QTOF MS. Low resolution mass spectrometry resulted in many misclassifications of NAs (due to polyphenols, phenolic acids and other compounds), while HRMS had a higher selectivity (greater ability to detect NAs, specifically). Both low and high-resolution methods correlated well with FTIR quantification of NAs (p-value of regressions <0.05) and ultimately the researchers suggested that either method could be useful for the semi-quantitative profiling of NAs.

1.7.4   Ultrahigh resolution Mass Spectrometry (UHRMS)

Ultrahigh resolution mass spectrometry (UHRMS) methods can achieve resolution of >200 000. UHRMS enable the distinction of isobaric ions (i.e., different ions with the same nominal mass). The instruments discussed below generate a signal which must be separated into frequencies for unique chemical species by Fourier transform. The ions travel in "packets" and are detected as an interferogram (or transient) – an image of many sine waves with different frequencies. Fourier transform converts the generated signal from time-related data into useable frequency-related data.[116,131] These instruments have a large dynamic range, low signal-to-noise ratio, and high mass accuracy.[131] The high resolution allows for increased confidence in chemical formulae assignments.[132] These benefits come at a high cost: maintenance, operation by skilled analysts, and the instrument itself are all factors which contribute to the limited accessibility.

1.7.4.1 Fourier Transform Ion Cyclotron Resonance MS (FT-ICR MS)

In Fourier Transform Mass spectrometers, ions are detected in a Penning trap. The trap is formed by three sets of parallel plates used for trapping, excitation, and detection. A strong magnetic field is applied to the trapping plates. A weak electric field (which is perpendicular to the magnetic field) forces the trapped ions to travel in a circular motion and generate a rotational frequency in the cell. Cyclotron frequency is inversely proportional to the *m/z*. In this way, coherent ions have a unique frequency which enables ions to be resolved. The rotational frequency (ω) of an ion in a magnetic field of magnitude (B) can be given by Equation 1-4:

$$\omega = \frac{zB}{m}$$

This relationship demonstrates the inverse relationship between *m/z* and frequency (i.e., smaller ions with low charges will have a higher frequency). These mass spectrometers can perform many functions in the ion trap (i.e., excite, analyze, decrease energy) which it is later counterpart, the Orbitrap, cannot.[105] While FT-ICR instruments demonstrate the highest resolution and have sub-ppm mass accuracies (< 1 ppm).[99]

FT-ICR MS has long been the preferred characterization method in petroleomics, where both high resolving power and high mass accuracy are required. [106–111] Using this instrumentation, OSPW has been differentiated from other natural water samples (interceptor wells, ground water, river water) by its relative contributions of sulfur-containing organics.[51] Similarly, studies by Huang *et al.* (2018) suggested chemical species having O_2^- and/or O_4^- could be used for this purpose.[112,113]

Grewer *et al.* (2010) reported less than 20% of the observed peaks could be attributed to NAs and emphasized the larger proportion of dissolved organics that were not yet studied at that time.[47] Offline fractionation followed by the use of FT-ICR MS greatly increased the number of compounds identified- including two- and eight-fold increases in the detection of sulfur-and nitrogen-containing AEOs.[114,115] Fractionation prior to injection decreased the matrix effects which would decrease the number of species observed and therefore identified.

1.7.4.2 Orbitrap MS

As new methods of FT-ICR MS were being developed, one other ultrahigh resolution mass spectrometer was simultaneously being explored for its utility in oil sands analytical chemistry. In this mass analyzer, ions oscillate around a "spindle-like" central electrode which is encompassed by two outer electrodes. Ions are trapped by the electric field generated between the electrodes. The ions injected into the Orbitrap have a speed which generates a centrifugal force which opposes the applied electric field.[105] Ions then radially and axially separated in the orbitrap with a frequency which is given by Equation 1-5:

$$\omega = \sqrt{\left(\frac{z}{mk}\right)}$$

Where *k*, is the field of curvature. Comparison of Equations 1-4 and Equation 1-5 indicate the differences between Orbitrap and FT-ICR MS instruments: i) resolution in FT-ICR is dependent on a magnetic field as opposed to the electrostatic field applied in the Orbitrap ii) frequency is

inversely proportional to *m/z* in FT-ICR MS but it is inversely proportional to the square root of *m/z* in the Orbitrap. This translates to a threshold for the resolving power which is dependant on *m/z*. Above 300 *m/z*, the resolution of the Orbitrap performs better that FT-ICR. The two instruments also differ with respect to size: where the in the Orbitrap a smaller ion trap improves axially resolution, in FT ICR bigger is better! More space in the latter case decreases space-charge effects. Ultimately, Orbitraps tend to be smaller and less expensive than FT-ICR instruments.

Headley *et al.* were the first to apply Orbitrap MS to the analysis of NAFCs from OSPW, reporting similar results to those obtained using FT-ICR MS analysis.[116] The addition of HPLC to Orbitrap analysis enabled the distinction of co-eluting compounds in OSPW (especially O_2^- to O_5^- chemical species with carbons <12 and DBE 1 – 4), thereby greatly improving chromatographic resolution.[9]

Fingerprinting methods to differentiate natural, bitumen-derived, and industrial effluent samples are now possible through UHRMS methods. To complement the 2015 study by Barrow *et al.*, Sun *et al.* (2017) used HPLC-Orbitrap MS (in both positive and negative ion modes) to profile OSPW and environmental water samples in the AOS region.[117] The analysis of the Athabasca River mainstem samples allowed the researchers to conclude that TP seepage was not a major concern at that time. A study by Rowland *et al.* was one of the few to use both FT-ICR MS and Orbitrap MS to analyze OSPW.[118] They discovered SO_3 and SO_5 chemical species detected in negative ion mode with aromatic structures.

1.7.5 Ionization Techniques

The charge of a compound also plays a part in its detection. In negative-ion mode, acidic compounds are detected as these compounds tend to have on overall negative charge due to loss of a positively charged hydrogen atom during ionization. Whereas, in positive ion mode basic compounds are detected due to the addition of a positively charged hydrogen atom during ionization. Therefore NA, and all acids, are best detected in negative ion mode.[111]

The most popular ionization method by far, is electrospray ionization (ESI). In ESI, the sample is introduced into the source as a mist (or spray) through a charged capillary.[119] The droplets in the spray carry charged molecules. As droplets evaporate, they decrease in size, and the charge density increases. When the charge density becomes greater than surface tension, the

droplet breaks into smaller sizes. This process is called ion evaporation. ESI is influenced by a few molecular properties including the solvent, Ka, and hydrophobicity.[120]

Atmospheric pressure photoionization (APPI) is an alternative technique which can be used for less polar (or non-polar) molecules detected as radical, protonated, and deprotonated ions. [108,121] Here, UV light can directly ionize analytes in the sample, or a dopant (such as toluene) can be ionized first and then transfer charge to dissolved analytes.[111] This method is different than atmospheric pressure chemical ionization (APCI) which relies on both ion evaporation and collision of molecules to ionize analytes. In the APCI source, the corona discharge emits electrons which ionize needle gas molecules. In series of reactions, the ionized gas transfers charge to solvent molecules in the sample and eventually to the analytes. In APCI and APPI, compounds are ionized in the gas phase, unlike ESI where compounds are ionized in the liquid phase.[111] Both APCI and APPI can explore a wider range of molecules than ESI. APCI[54,94,117,122] and APPI [123] are used less frequently than ESI. APCI, however, has the advantage of being able to detect more compounds than just ionic and polar neutrals which are often detected in ESI.[118,122] APCI is also relatively softer than ESI and results in less fragmentation of molecules. For this reason, I chose to use APCI in the analysis of OSPW in my thesis work.

1.8   Non-targeted analysis (NTA)

With every advancement in technology emerges new and unforeseen challenges. The challenges of NTA in environmental chemistry are the result of the increasing power of technology (i.e., instrumentation). HRMS now allows for the analysis of complicated organic matrices. In their 2017 critique, Schymanski *et al.* promoted the development of reliable online tools which scientists can used to interrogate their data.[124]

1.8.1   Levels of confidence in reporting "unknown" unknowns

Still in its infancy, NTA methodologies are quickly evolving as they aim to become more standardized in both approach to (i.e., workflows), and reporting of newly discovered chemicals. Language is an important part of communication, and terminology is still being defined. While 'suspect screening' involves comparing putative analytes against an *a priori* shortlist found in chemical library/database,[125] 'non-targeted analysis' is distinct in that both data acquisition *and* analysis are agnostic.[124,126–128] **Figure 1-6** is provided to help to clarify these ideas.
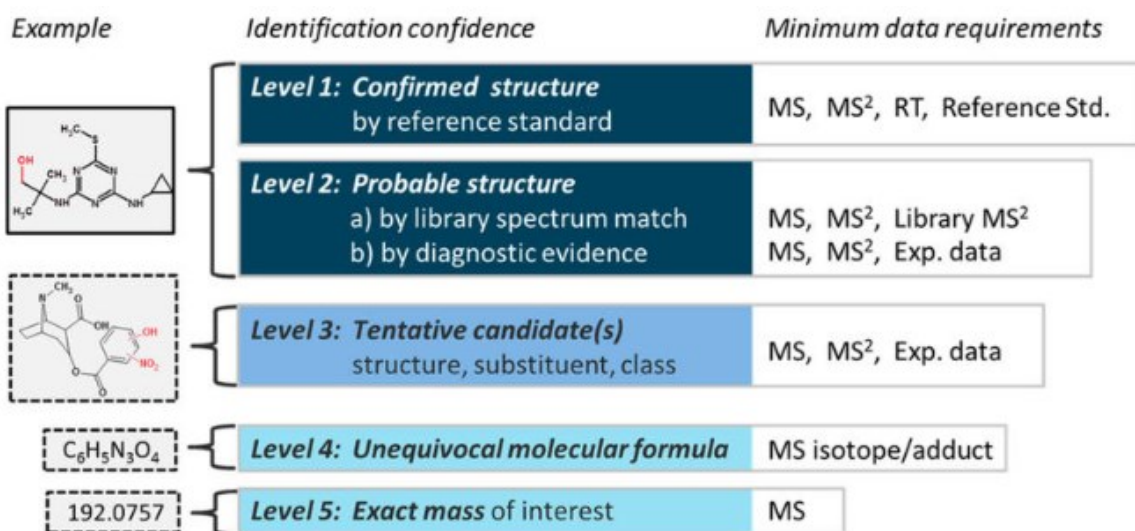
Figure 1-6 Description of matrix of identification approach vs identification confidence by Schymanski *et al*.[129] RT – retention time. License 4800030029948.

Emma Schymanski proposed a system that scientist can use to describe the confidence level achieved in identifying unknowns.[130] These efforts reveal a respect for accuracy, and the pursuit for openness and clarity in scientific communication. The levels were suggested as an improvement to previous work by the Metabolomics Standards Initiative[131] and Jeon *et al*. (2013) [132] Five levels of decreasing confidence: level 5 indicating an exact mass from mass spectrometry and level 1 reporting an unequivocal structure and validation using orthogonal (other analytical) methods. [129] The methods used in this thesis are a level 4, at best. Level 3 or higher requires the use of MS/MS experiments and/or references to confirm a tentative structure. The authors acknowledged that at times, identification of all components is neither feasible, nor logical – and this certainly holds true for dissolved organics in OSPW.

1.8.1.1 Formulae assignment in oil sands analytical chemistry

Many studies have taken to the use of HRMS and describe the composition of analytes by chemical class. By this method, each species is assigned a molecular formula and categorized ("binned") by the heteroatom content. In this way, a species with the formula $C_{10}H_{18}O_3^-$ would be assigned to the O_3^- chemical class.[9,46,47,104,111,117] Few studies have gone further to confirm the structures of species in OSPW. Using standards and MS/MS experiments, bicyclic and diamondoid NAs have been confirmed in OSPW.[48,133] Other heteroatomic compounds have also

been confirmed by these methods.[118,134,135] Generally, the approach has been to assess the toxicity of the mixture (whole effluent toxicity, WET), and eventually identify key components which may need to be monitored before/during OSPW treatment and reclamation.

1.9   OSPW reclamation

Reclamation is "the process of reconverting disturbed land to its former or other productive uses"[5], however there are many concerns about oil sands reclamation. In 2017, Directive 085 was approved by the AER under the Oil Sands Conservation Act. This directive is meant to describe how the large volumes of tailings will be managed during mining and after the mine has closed. Specifically, it outlines how the organization will keep operators accountable. Prior to mine closure, operators must reclaim land and tailings disturbed prior to applying for a reclamation certificate (**Figure 1-7**). Reclamation certificates are issued when the disturbed area is self-sustaining i.e., when it can support the surrounding ecosystem.[10] One example of a certified area is Wapiskew (Cree name for 'swan') Lookout. This dry landscape was previously Suncor's oldest TP, Pond 1, which received tailings from 1967 – 1997.[27] The pond was decommissioned in 2001,[136] and reclaimed by 2010. In 2016, Wapisiw lookout represented the only area to have received a reclamation certificate in AOS. More than 99% of disturbed area still needs to be reclaimed (**Figure 1-8**).[137]
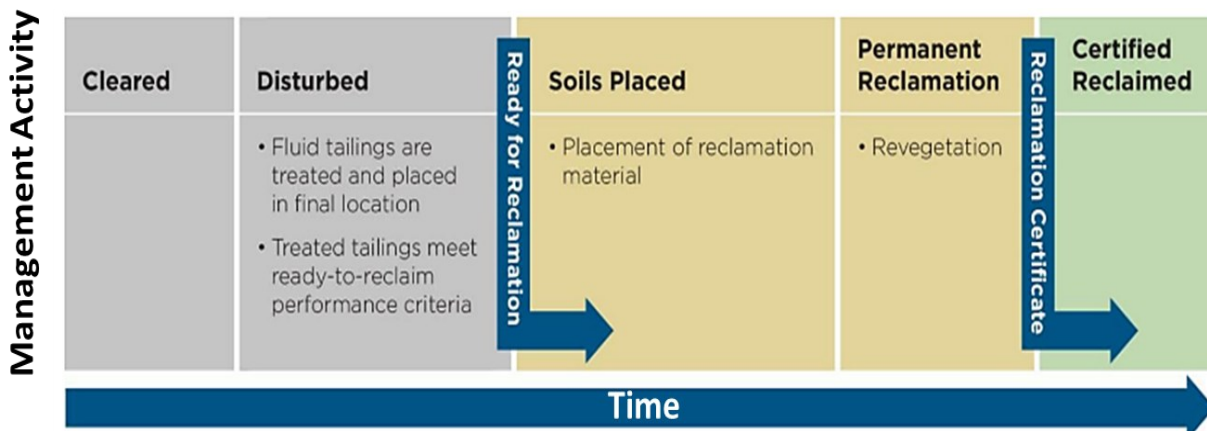
Figure 1-7 Schematic describing general process by which operators obtain a reclamation certificate by the AER.[23]
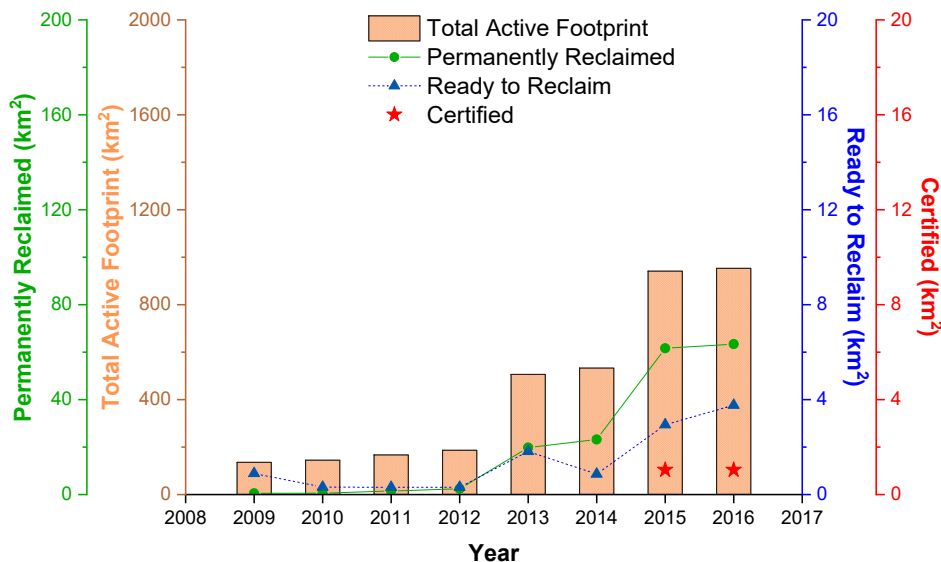
Figure 1-8 Summary of reclamation in the Alberta oil sands region between 2008 and 2016 (km^2). The "total active footprint" (▦) describes all land currently associated with oil sands mining operations excluding those which have received a reclamation certificate and including land cleared, disturbed, ready to reclaim, and lands both temporarily and permanently reclaimed. Land is "permanently reclaimed" (●) when reclamation material has been placed and revegetation has begun. Lands are "ready to reclaim" (▲) when they are no longer used for mining or plant activities. A "certified" (★) area has received a reclamation certificate from the AER.[3,23]

1.9.1 Active strategies

Active methods for remediation of OSPW have focused on the use advanced oxidation processes (AOPs) which are chemical treatments to remove organics in wastewater. These treatments may use ozonation (addition of ozone, O_3);[138–140] ultraviolet (UV) irradiation;[141,142] and addition of chemicals like persulfate (S_2O_8)[143] or titanium dioxide (TiO_2).[144–146] These strategies have been shown to effectively remove recalcitrant organics in OSPW, however they are expensive. Passive strategies are often cheaper and therefore preferred in industry for the treatment of tailings.

1.9.2 Passive strategies

1.9.2.1 Bioremediation

Both lab and field studies have a place in the assessment in the potential of bioremediation. Alexander *et al.* 1999 outlined requirements for feasible bioremediation of contaminated environments including (but not limited to) the necessity for biotransformation products to be less toxic than their parent compounds, and for microbes to grow and survive under the environmental conditions (including oxygen, nutrients, temperature, and organic substrates).[147] Important nitrogen-containing nutrients include ammonia and nitrate, while orthophosphate can supply phosphorus. Furthermore, the cost of bioremediation must be no more than that of active strategies.

1.9.2.1.1 Aerobic bioremediation of dissolved organics in OSPW

Aerobic microbial biodegradation has been proposed as a cost-effective method of OSPW bioremediation, and over the years, many studies have supported this idea.[148] The 1986 study at Syncrude was the first to assess this strategy for OSPW remediation.[38] The storage of TP water in shallow aerated pits saw a decrease in toxicity (as measured by Microtox, *Daphnia magna* and rainbow trout toxicity tests) after 12 months. The findings suggested that toxic acidic components could be slowly removed over time. In the following 3 decades, many others studied the capacity for microbes in OSPW to degrade dissolved organics.

For example, Herman *et al.* (1994) enriched cultures from oil sands tailings were monitored to assess the capability of endogenous bacteria to degrade extracted organic acids from OSPW (21 and 50 mg/L) and commercial mixture of NAs (50 mg/L).[149] Closed 60 mL

microcosms for observed for 24 – 30 days. Mineralization of organics was measured by CO_2 using GC. While 48% of the commercial mixture was converted to CO_2, only 20% of the extracted OSPW organics were mineralized to the same level. Commercial NAs generally, have simpler structures than those found in OSPW and thus, they are more labile (more easily removed). The Clemente study was one of many to indicate differences in biodegradability of the two mixtures, while also showing the capacity for endogenous bacteria to remove organic compounds in OSPW. Soon after using aerobic microcosms, Clemente *et al.* confirmed the ability for endogenous microbes from MLSB to degrade commercial NAs with a corresponding decrease in toxicity (an increase in Microtox EC50 values).[150]

Many aerobic microcosm studies have focused on the use of commercial NAs to understand the biodegradation potential of endogenous microbes. The potential of these microbes has been confirmed, but so too has the relative biodegradability of commercial compared to OSPW-derived NAs. NA concentrations in OSPW which had aged 9 and 17 years (Pond 9 and CT Pond, respectively) was used to review true rates of *in situ* biodegradation in the field.[60] Assuming first order kinetics, Han *et al.* estimated biodegradation had occurred slowly and the half-lives of NAs in ageing ponds were between 12.8 – 13.6 years. The discrepancies in estimated half-lives of dissolved organics in OSPW reflect changes in methodology and experimental design due to improved technology with time.

1.10 Microbial communities present in bitumen, OSPW and related environments.

1.10.1 Microbial communities present in OSPW

The primary sources of microbial communities in OSPW are bitumen ore and freshwater used in the extraction process.[27,151] The Athabasca River runs through the oil sands region and in the north, it is flanked by industry on either side.[117,152] In some areas, the river cuts through bitumen deposits. So, years of interaction between the river and bitumen has likely contributed to the development of diverse communities which are tolerant of hydrocarbons.[153] Meckenstock *et al.* suggested that microbes can exist in the film of water which surrounds oil sands grains.[154] Facultative and anaerobic microbes have been shown to exist in deposits greater than 75 m deep, so it is not surprising that said species may be found in the anoxic zones of TPs.

Microbial community analyses have largely focused on prokaryotic species, but eukaryotes are also present in TPs. Aguilar *et al.* identified eukaryotic organisms in sediments

and surface water from West-in-Pit (WIP, a TP at SCL established 1997) in 2011. Many of these organisms had some ability to metabolize substrates both aerobically and anaerobically.[155] Whitby *et al.* (2010),[44] Penner and Foght (2010),[36] Siddique *et. al.* (2018),[156] Skeels *et al.* (2018),[157] have reviewed such microbial communities living in oil sands TPs.[27] Their findings (and others) are included in **Table 1-4**. The following sections, however, will focus on prokaryotic microbial species.

Table 1-4 Hydrocarbon degrading bacteria in OSPW and other environmental sources

Source	Phylum, (Class), Genus*, spp.	Ref
Biodegraded oil-bearing sediments	*Deferribacteres, (Deferribacterales), Deferribacteraceae** *Firmicutes* *Thermotogae (Kosmotoga)* *Thermotogae (Petrotoga)* *Proteobacteria (mainly Gammaproteobacteria)*	[158]
Various hydrocarbon-containing environments	*Actinobacteria, (Actinobacteria), Mycobacterium** *Brevibacterium* *Proteobacteria, (Betaproteobacteria) Achromobacter** *Actinobacteria, (Actinobacteria),Corynebacterium** *Actinobacteria, (Actinobacteria), Rhodococcus** *Proteobacteria, (Gammaproteobacteria), Acinetobacter** *Proteobacteria, (Betaproteobacteria), Alcaligenes** *Bacteriodes, (Flavobacteriia),Flavobacterium** *Proteobacteria, (Gammaproteobacteria), Moraxella*.* *Actinobacteria, (Actinobacteria), Micrococcus** *Firmicutes, (Baciili), Bacillus** *Actinobacteria, (Actinobacteria), Acinetobacter** *Proteobacteria, (Gammaproteobacteria), Pseudomonas**	[44]
OSPW and oil sands	*Proteobacteria, (Gammaproteobacteria), Pseudomonas*.* *Proteobacteria, (Gammaproteobacteria), Alcaligenes** *Actinobacteria, (Actinobacteria), Acinetobacter** *Firmicutes, (Baciili), Kurthia**	[158]

Gasoline polluted groundwater	*Actinobacteria, (Actinobacteria), Mycobacterium**	159
Groundwater well near TPs	*Firmicutes* *Chloroflexi* *Bacteroidetes* *Actinobacteria* *Fusobacteria* *Euryarchaeota*	160
Soil/Sediment bioreactor experimental studies	*Proteobacteria, (Betaproteobacteria),* Nitrosomonas* *Proteobacteria, (Gammaproteobacteria),* Arenimonas* *Proteobacteria, (Alphaproteobacteria),* Mesorhizobium* *Proteobacteria, (Alphaproteobacteria),* Bradyrhizobium* *Proteobacteria, (Betaproteobacteria),* Nitrosospira* *Actinobacteria, (Actinobacteria),* Mycobacterium* *Proteobacteria, (Betaproteobacteria),* Limnobacter*	161
Oil sands TPs	*Proteobacteria, (Gammaproteobacteria), Pseudomonas** *Proteobacteria, (Betaproteobacteria), Hydrogenophaga** *Proteobacteria, (Betaproteobacteria), Acidovorax** *Proteobacteria, (Gammaproteobacteria), Luteibacter** *Proteobacteria, (Alphaproteobacteria),* *Enhydrobacter*Firmicutes, (Baciili), Paenibacillus** *Firmicutes, (Baciili), Exiguobacterium** *Firmicutes, (Baciili), Bacillus*.*	162,163

1.10.1.1   Aerobes

NA degradation is mainly an aerobic process.[47] Microbial community analyses of microbes in produced waters will likely have a mixture of microbes endogenous to the subsurface, and those found in the waters. Most of these bacteria are aerobic heterotrophs and are native to the natural waters in the region.[6] Yergeau *et al.* (2013) used 16s rRNA gene sequencing to study microbial communities in TPs and identified *Pseudomonas, Thauera, Rhodoferax, Acidocorax, Thiobacillus* and *Branchymonas*.[169] They determined that communities in TPs were less diverse than those in unaffected waters due to limited sources of carbon. However,

Mehrabad *et al.* (2013) found a mixture of obligate aerobes, obligate anaerobes, and facultative anaerobes in TP OSPW.[170] The presence of a large anaerobic community in OSPW has now been well studied.

1.10.1.2   Anaerobes

Anaerobic processes are primarily found in the deep subsurface and methanogenesis (the formation of CH_4 by microorganisms) is an important process found in the oil sands TPs.[6] Methanogens can reduce CO_2 to CH_4. Hydrogen is required for this to occur and is likely derived from water, mineral hydrolysis, or maturation of organic matter. Oxygen levels decline with depth from the surface of a TP resulting in aerobic communities found in just a narrow region.[171] The remainder of species are largely anaerobic in both lower depths of TPs, FFT and MFT. Facultative anaerobes have been found in Syncrude OSPW.[27] Iron-, nitrate-and sulfate-reducers have also been reported in Syncrude OSPW.

Wilson *et al.* (2016) previously reported that the core community of prokaryotes in WIP which largely included methanogens.[177] Mehrabad *et al.* (identified facultative anaerobes *Comamonadaceae* and *Clostridia* were found both at the surface and lower depth of TPs, stating their presence was justified in the oxygen-depleted TPs.[170] Sulfate-reduction also increases with depth and is inversely correlated to concentrations of sulfate (highest at surface of water).[167,179] Some dissolved organics, including NAFCs, have been shown to be degraded by sulfate-reducing bacteria in TPs[180] but these are unlikely substrates for methanogens.[28]

1.10.2 Factors affecting microbial communities

In 1990, Leahy and Colwell reviewed the microorganisms which have been identified in hydrocarbon-bearing environments and variables which may impact the efficiency of microbial growth.[181] Almost 3 decades later, Kinley *et al.* (2016) conducted a study which confirmed much of the same: temperature, pH, dissolved oxygen, and nutrients are important factors influencing bacterial communities.[182] Moreover, the nature of the oil sands ore and processing chemistry used for extraction have large impacts on bacterial community structure.[177] Additionally, few studies have observed a decrease in microbial diversity with depth in a TP.[165,179] Penner *et al.* (2010) maintain that carbon sources, available electron acceptors, and presence of toxic byproducts can also impact community dynamics.[36]

1.10.3 Microbial community characterization

Research has focused on the use of 16S rRNA to study microbial community structures. A nucleotide is an essential building block used to make DNA (double stranded – found in eukaryotes), and the related RNA (singe stranded - found in prokaryotes). In prokaryotes, there are 4 different nucleotides defined by their chemical structures: adenine, cytosine, guanine, and uracil. The 16S rRNA sequence (1542 nucleotides) has not changed much for millions of years, but small deviations in the sequence are used to determine phylogenetic the relationships between bacteria.

Phylogeny makes use variable regions in the DNA/RNA. Microorganisms can be differentiated by sections of the DNA that are not conserved (the same).[184] In other words, the sequence of nucleotides at the beginning and end of the sequence are the same, and variable regions within the sequence are unique to microbial species. This gene sequence is short which makes it easy to extract, copy and amplify. Following amplification, the sequences can be compared to online libraries which scientists have built up over many years for this very purpose. Bacteria are categorized by operational taxonomic units (OTUs). Typically, a group of sequences which are 97% similar are one OTU, or members of the same genus. By increasing the threshold of similarity to 98% or greater, the species of a microbe can be identified.

Illumina sequencing is an accessible and reliable method for community analyses and for this reason is frequently used to study microbial communities in bitumen, oil sands sediment, OSPW and cores from the deep subsurface.[160,164,189,190]

1.11 The End pit lake strategy

1.11.1 Pit lakes reclamation

One approach to wetland landscape reclamation proposes large man-made lakes to store and detoxify tailings and OSPW.[30] In oil sands end pit lakes (EPLs), FFT and heavier tailings will be placed in a pit and then topped with water from the Athabasca River, its tributaries or surface runoff. The idea is that over time, the EPL will be naturally detoxified via bioremediation. Ultimately successful bioremediation must render OSPW and tailings non-toxic to allow for the safe re-integration into the environment.[32] EPLs will therefore be permanent fixtures of the AOS region.[30]

While pit lakes have been used in coal and diamond industries, much uncertainty remains regarding their efficacy in the treatment of waste produced from the oil sands industry.[231] The complicated nature of EPLs is part of what raises concerns for many, but much work has been done to prove this concept. The earliest of which began in the late 1980s at SCL.

1.11.1.1   Experimental ponds reclamation facility

In the late 1980s a series of experimental ponds (EPs) were established at SCL (57°05.0500N,111°41.5050W) informed by the findings of MacKinnon and Boerger (1986).[39] Testing various sizes and physical compositions, the goal was to used EPs to understand how water-capping technology could transform OSPW to water capable of supporting a healthy aquatic ecosystem.[98] The research program established 11 scaled-up ponds to test MFT-capped with water (based on prior laboratory testing). The EPs ranged between 2000 to 140 000 m^3 in volume and up to 7 m in depth.[193] The MFT (taken MLSB) had been densifying for 8 years prior to its use in these EPs. All ponds were closed systems (no inflow or outflow of water). The first ponds were built in September 1989, and an additional 3 were built in 1993 as scaled-up experiments of some the early ponds. These ponds now hold OSPW which has aged for decades and can be studied as a model of future EPLs. After more than 2 decades monitoring EPs, the next step was to assess the strategy in real-time. On December 31st, 2012, Base Mine Lake (BML) was commissioned as a pilot scale EPL.

1.11.2 Base Mine Lake

Base Mine Lake (57°038.88N, 111°37' 22.44'W) is the first oil sands EPL established to test the feasibility of the EPL remediation strategy. BML formerly went by the name West-In-Pit (WIP) and was used to store tailings. FFT from MLSB was transferred into WIP from 1995 to 2012 (W. Zubot 2017, Personal Communication, April 4, 2018). As it is no longer a TP, BML has received no new input of OSPW, but freshwater from Beaver Creek Reservoir is added periodically and recycled into a temporary reservoir. The primary purpose of dilution is to decrease concentrations of toxic components within. Freshwater addition also increases aeration and introduces microorganisms into BML. Both these processes are implemented to increase the effectiveness of *in situ* aerobic biodegradation.[170] Initially, BML had a cap water volume of 25 Mm^3 (depth of 5 – 10 m). Underlying MFT was a volume of about 175 Mm^3 (depth of ~28.5 m), and with a surface area of 800 ha (M. MacKinnon 2017, Personal Communication, March

29.2018) this pilot is easily at least 1000-fold larger than any EP. Ultimately BML will be connected to the Athabasca River as part of the closure landscape.[194]

In July 2019, the AER published a report reviewing SCL tailings management plan as part of the Mildred Lake Extension Project (MLX).[194] MLX will include two new surface mines at SCL, with projected start dates in 2024 and 2028. Syncrude was to provide a back-up treatment plan to AER by September 30, 2020 (as an alternate if EPL strategy is not deemed viable). The 2027 commissioning of North Mine Lake has been proposed. This EPL will be even larger than BML (i.e., 250.5 Mm^3 of FFT), but its approval was stayed at least until September 30, 2020. Syncrude has requested that BML be reassessed in 2023, allowing 10 years to prove the feasibility water-capping strategy. At that time, they will be required to submit an updated tailings management plan to AER.

Challenges at BML include turbidity and residual bitumen and oil sheens at the surface. Currently, there are 32 EPLs proposed,[35] and the future of OSPW reclamation is riding on the success of BML. Scientists have worked since 2013 to decipher the complicated system in BML. The limnology,[195] biogeochemistry,[196–200] microbiology,[155,201,202] analytical chemistry[203,204] and toxicology[85,90,91,205] of BML have been reviewed and yet, many unknowns remain. The extent to which endogenous microbes will be able to contribute to the aerobic biodegradation of dissolved organics is not well understood.

1.12   Research Objectives and Proposed studies

The End Pit Lake strategy relies on the premise that toxicity of OSPW will be remedied naturally through biodegradation with time. There is a pressing need to evaluate the efficacy of this strategy and for this reason **the persistence of dissolved organic chemicals therein must be better understood**. The future risk of exposure to aquatic wildlife and humans is partially influenced by the ability of fish to metabolize toxic chemicals in OSPW**. A better understanding of the metabolism of these toxic chemicals by native species is required** to get a true picture of the risk posed by persistent chemicals. To this end, I completed the following three projects:

1. **To estimate the chemical profiles of future oil sands end pit lakes:** Samples representing "aged" OSPW were taken from three experimental ponds at SCL and their chemical profiles were compared against four TP samples representing "fresh" OSPW.

This provides first estimations of the relative persistence of chemicals using samples from the field.

2. **To investigate rates of *in situ* aerobic biodegradation by endogenous microbes in BML OSPW using laboratory microcosms**: Using BML OSPW from 2014, this study provides first estimates of half-lives for all compounds detected in negative ion mode. These may improve expectations for rates of biodegradation in EPLs.

3. **To characterize biotransformation products of phase I and phase II metabolism by rainbow trout subcellular assays:** S9 and microsomes from a native fish species were used to review the biotransformation potential of toxic organics in BML OSPW. This *in vitro* study provides information which can be used to improve our predictions of EPL chronic toxicity.

Water from EPLs will eventually be released to the natural watershed. Downstream communities, such as Fort McKay and Fort Chipewyan rely on healthy ecosystems in the Athabasca River for traditional foods and as a source of clean water. Effective remediation of OSPW is therefore important, not only for aquatic organisms, but also for the protection of the health of Canadians who will continue to use this watershed in the future. The results of this thesis provide a better understanding on the persistence of dissolved organic chemicals in OSPW. Furthermore, the results may inform stakeholders in their continued assessment of the feasibility of the EPL reclamation strategy.

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Chapter 2   Predicting the future of oil sands end pit lakes by chemical profiling in aged and fresh process-affected waters

2.1   Introduction

Analytical characterization of the complex mixture of bitumen-derived organics in oil sands process affected water (OSPW) has been improved by enhanced chromatography[1,2] and high resolution mass spectrometry.[3–5] It is now understood that naphthenic acids (NAs, but more specifically O_2^- species) are only a minor proportion (~11% mass) of total extractable organics in OSPW.[6,7] In negative ionization mode, NAs and several other organic acid classes containing sulfur and oxygen can be detected,[3] while organic bases containing nitrogen, and a broad range of polar organics (i.e. non-acid) containing oxygen, sulfur or nitrogen can be detected in positive ion mode.[4,8] Though we now have a good understanding of OSPW's chemical composition and toxicity, the relative environmental persistence of all the toxic chemical classes has not been well studied.

To predict the future toxicity of end pit lakes (EPLs), the relative environmental persistence of each chemical class must be understood. MacKinnon and Boerger monitored OSPW stored in outdoor pits (3 – 5 m deep) under aerobic conditions. They reported increased EC_{50} using Microtox after 10 months, and increased EC_{50} for 96-hr static assays with Daphnia and rainbow trout (*Oncorhynchus mykiss*) after one year.[9] Continued observation for an additional 12 months demonstrated that the acute toxicity of fresh OSPW had further decreased (EC_{50} and LC_{50} for all acute toxicity tests >100%).[9] Larger-scale experimental ponds were then created at Syncrude in 1989 and 1993,[10] and in one study of these waters, NAs were estimated to slowly degrade, with half-lives of ~13.6 yrs,[11] but analytical methods at this time were not powerful enough to resolve other chemical classes of toxicological relevance. These experimental ponds are now 27 – 31 years old, and analytical methods are now available to semi-quantitatively characterize the relative abundance of thousands of OSPW chemical species.

The aim of the current work was to infer the relative persistence of OSPW chemical classes through a cross-sectional survey of OSPW of different 'ages' (experimental ponds = aged, tailings ponds = fresh, and BML) analyzed by HPLC-Orbitrap mass spectrometry, and thus to predict the future toxicity of EPLs.

2.2 Methods

2.2.1 Chemicals and Reagents

Acetic acid, methanol (MeOH), isopropanol and Optima® water were purchased from Thermo Fisher Scientific (Edmonton, AB). The internal standards, D_{23}-lauric acid (tetradecanoic acid, Aldrich, St. Louis, MO) and D_3-progesterone (CIL laboratories, Andover, MA) were used in negative and positive mode, respectively. Refined commercial naphthenic acids were purchased from Merichem (Houston, Texas, USA).

2.2.2 OSPW Sampling.

In this cross-sectional study design, OSPW was sampled at Syncrude Canada Ltd. from three sources in 2014: i) active tailings ponds, herein described as "fresh" OSPW; ii) experimental ponds, established 27 – 31 years ago and herein described as "aged" OSPW, and iii) BML, the only oil sands end pit lake. Tailings ponds (TP) sampled included Aurora, Southeast, Southwest, and Mildred Lake Settling Basin, defined hereafter as TP1, TP2, TP3, and TP4, respectively. Experimental ponds sampled were Pond 5 (EP5), Pond 9 (EP9) and Pond 11 (EP11). The experimental ponds had been created at different times using different combinations of OSPW, freshwater and FFT (**Figure 2-1**). In 1989, EP5 was established with 1000 m^3 of FFT capped with an equivalent volume of OSPW from MLSB.[12] Four years later, EP9 was created from 50,000 m^3 of OSPW, and with no underlying FFT.[10] In the same year, EP11 was established with 70,000 m^3 of FFT and 80,000 m^3 of overlying freshwater.[6] BML was sampled approximately 1.6 years after its commissioning in December 2012.

All samples were taken 50 – 100 cm below the surface with a Van Dorn water sampler deployed from a floating barge and stored in 1 L brown polyethylene bottles. BML samples were collected in 20 L white high-density polyethylene pails in August 2014 that were submerged approximately 0.5 to 1 m below the surface from a floating barge. All OSPW was stored at 4°C until analysis.
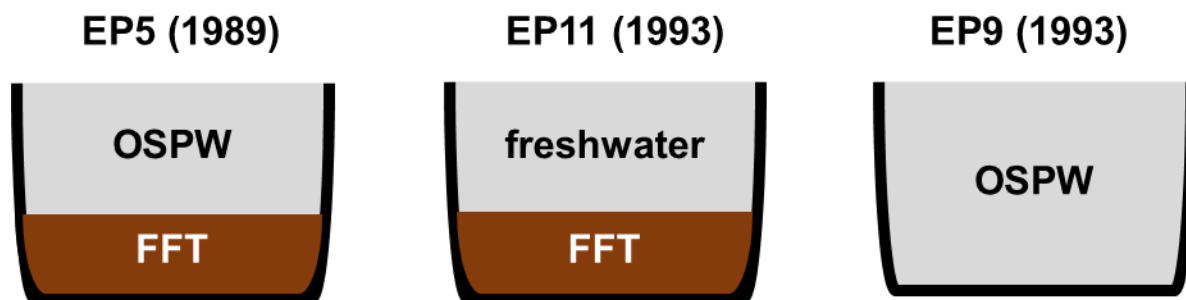
Figure 2-1 Schematic cross-section showing construction of the three experimental ponds and (year of establishment). Fluid fine tailings (FFT) are the densified suspension of the components of bitumen extraction including residual bitumen, clay, and sand (up to 30 wt% solids).

2.2.3   Sample Preparation

Suspended solids were removed from OSPW samples by syringe filtration (0.45 µM nylon, Thermo Scientific, Rockwood, TN), and 1 mL aliquots of filtrate were spiked with 100 µL of the combined internal standard solution. Internal standard concentrations in the vialed samples were 1 ppm D_{23}-lauric acid (for negative ionization) and 0.01 ppm D_3-progesterone (for positive ionization). The ultrapure water blanks were subjected to the same treatment as the samples.

2.2.4   Instrumental Analysis

Reversed-phase HPLC was paired with a hybrid linear ion trap-Orbitrap mass spectrometer (Orbitrap Elite, Thermo Scientific, San Jose, CA) and a modified version of the analytical method of Pereira *et al.* was used.[5] HPLC separation was performed on a Hypersil Gold C18 Selectivity Column (Thermo Scientific, 50 × 2.1 mm, particle size 1.9 µm) using an Accela HPLC (Thermo Scientific). The column was maintained at 40°C and a flow rate of 0.5 mL/min was used with 20 µL injection volumes. Initial mobile phase composition was 95% A (0.1% acetic acid in water) and 5% B (100 % MeOH) for 30 s, followed by linear gradient ramp to 90% B over 10 min, and a final ramp to 99% B over 5 min. Percent composition of B was then decreased to 5% over 2.5 min and held for 2 min for re-equilibration. Atmospheric pressure chemical ionization (APCI) was used in both positive ($^+$) and negative ($^-$) modes. Nominal resolution of the Orbitrap was set to 240 000 at *m/z* 400, and full scan mass spectral data were acquired between 100 and 500 *m/z*. This high resolving power was not always sufficient for the separation of isobaric components. Capillary temperature was 300 °C, corona discharge current

was 4.5 μA, and sheath, auxiliary and capillary gas flow were 20, 15, and 3 arbitrary units (au) respectively. For information on quality assurance and quality control, see **Appendix A.1**.

2.2.5   Qualitative and Quantitative Data Analysis

Spectra were extracted from each chromatogram in the broad retention window over which all OSPW analytes eluted (7 – 13 min in positive mode, 3 – 13 min in negative mode). Only those species with a response at least 3 times greater than a procedural blank (ultrapure water) were included in subsequent analyses. Empirical formulas were assigned in Xcalibur™ software from the exact mass of all detected species in both negative and positive modes (i.e., $C_vH_wO_xN_yS_z^{\pm}$) with the following restrictions: minimum (C – 5, H – 10), maxima (N – 1, S – 1, O – 6) and maximum error tolerance of 5 ppm. The number of features for which a formula was confidently assigned ranged among samples from 147 – 544 in negative ionization mode, and from 798 – 1575 in positive ionization mode. These chemical species were then binned into heteroatomic chemical 'classes' in each ionization mode: $O^{\pm}$, $O_2^{\pm}$, $O_3^{\pm}$, $O_4^{\pm}$, $O_5^{\pm}$, $O_6^{\pm}$, $SO^{\pm}$, $SO_2^{\pm}$, $SO_3^{\pm}$, $SO_4^{\pm}$, $SO_5^{\pm}$, $SO_6^{\pm}$, $NO^{\pm}$, $NO_2^{\pm}$ $NO_3^{\pm}$, $NO_4^{\pm}$, $NO_5^{\pm}$, $NO_6^{\pm}$, $(N)S^{\pm}$, $N^{\pm}$, and $O_xNS^{\pm}$. Although data analyses were generally based on total spectra rather than chromatographic peaks, chromatograms were examined in some cases for purposes of characterizing the complexity of isomers that may have been present for any given species. With exception of a few NAs, standards do not exist for dissolved organics found in OSPW. To estimate NA concentrations, a 100 mg/L Merichem NA stock solution was prepared in isopropanol and a 6-point standard curve was constructed from 0.5 to 50 mg/L (**Figure 2-2**); each standard solution (1 mL) was spiked with 100 μL of the internal standard. Double bond equivalents (DBEs) were calculated for the neutral species detected as an anion in negative mode by $\frac{(2C+2)-(H+1)}{2}$, or as a cation in positive mode by $\frac{(2C+2)-(H-1)}{2}$; whereby C and H are the number of carbon and hydrogen atoms in the assigned chemical formula of each charged species.
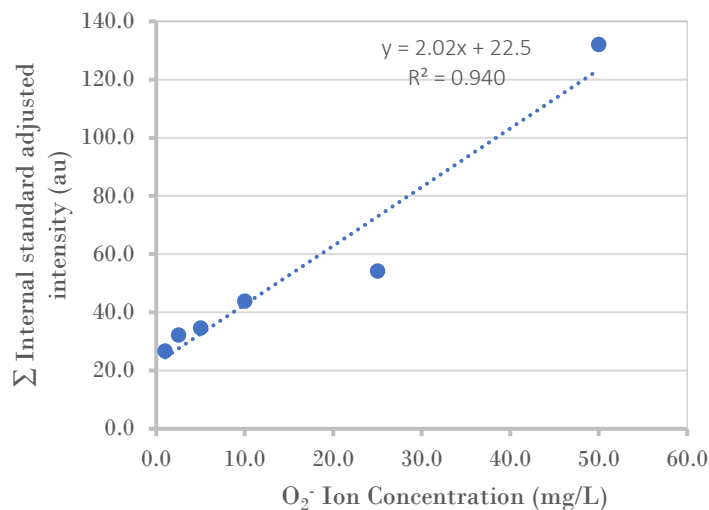
Figure 2-2 O$_2^-$ ion standard curve. The sum of internal standard adjusted intensity of O$_2^-$ ions in standards prepared from Merichem naphthenic acids.

To evaluate possible matrix effects, dilutions of fresh OSPW that ranged from full strength to 20% were injected into the instrument in triplicate (n=3,). These experiments showed lower accuracy and matrix effects when not diluted, thus all 'fresh' tailings pond OSPW samples were diluted by 50% with clean water prior to analysis. A blind test was also conducted to assess accuracy whereby samples of OSPW (n=8) were diluted by unknown factors by volunteers and spiked with the same concentrations of internal standard (100 uL in 1 mL of unknown sample). Based on total response of all species in the O$_2^-$ class, the estimated dilution factors ranged from 16 to 104%, giving a good correlation with true dilution factors ($R^2 = 0.79$, slope $= 1.04$), particularly for those samples that had been diluted by 50% or more ($R^2 = 0.83$, slope $= 1.39$), thereby validating the semi-quantitative approach.

Samples from this project were included in a run with other OSPW samples (Chapter 3), in a randomized-batch design. The run began with 3 instrumental blanks (50/50 v/v UHPLC water and MeOH), followed by 5 – 10 procedural blank injections to equilibrate the column and then one quality control sample (QC). As no true OSPW samples exist, the QC used in this project was a sample of BML OSPW diluted to 50% and spiked with internal standard to allow for within-run comparison and (generalized) longitudinal comparisons between runs over time. To test the reproducibility of analyses, profiles of aged and fresh samples were compared between two analysts to assess consistency (data not shown).
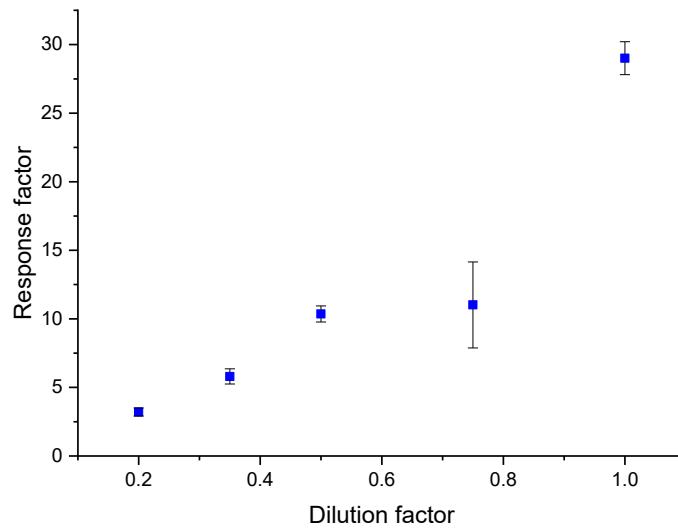
Figure 2-3 Matrix effect at different dilution factors with mean (blue box) and standard deviation of 5 replicate samples.
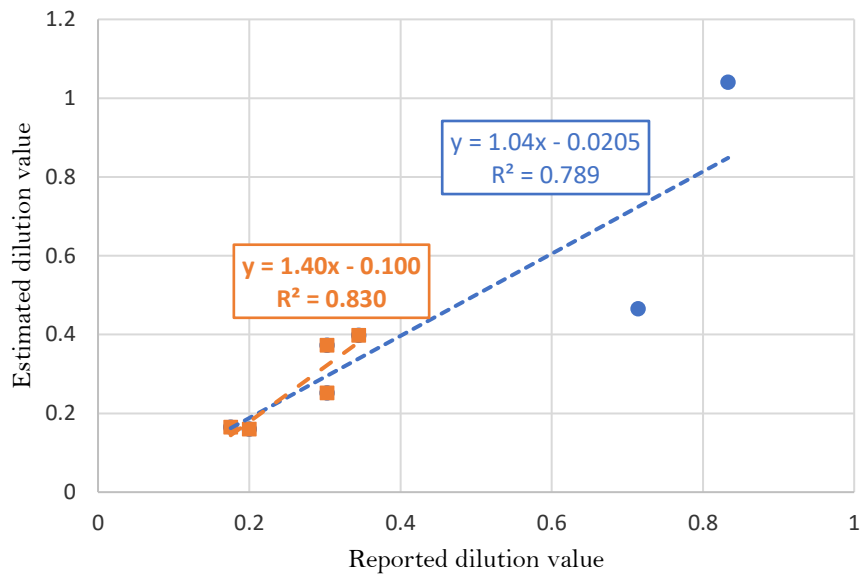
Figure 2-4 Estimated and true dilution factors for blind-test, and linear regression of the associations. Dilutions between 20 and 50 % (shown in orange) showed a better correlation, thus samples of OSPW were diluted for analysis.

2.2.6   Data Interpretation and Statistical Analyses

Two principal component analyses (PCA) were performed to identify differences among samples based on either: (I) class profiles, or (II) species profiles. Each PCA analysis was repeated separately for negative and positive ionization data. In PCA-I, the percent relative contributions of heteroatomic classes were input as variables (i.e., forced groupings of chemical species), with all samples (4 fresh, BML, 3 aged) as observations using the XLStat®2016 plug-in for Microsoft Excel 2013. PCA-II was performed by Sushmitha Thirumalaivasan under the supervision of Dr. Vinay Prasad. Here, the absolute intensities of each chemical species were entered in a matrix, wherein the 8 different samples were assigned to columns (as variables) and the individual intensities of each species were assigned in rows (as observations). Normalization was performed column-wise using the Normc function with the equation $\sqrt{\frac{intensity^2}{\Sigma intensity^2}}$. If a species was not detected in a sample it was assigned as 'not a number' (NaN) in the matrix, and all PCA analyses were performed in MATLAB® 2018b.

To broadly compare species profiles of fresh and aged OSPW, the $\log_{10}$ ratio of the mean relative response in fresh and aged OSPW was calculated as per Equation 2-1:

$$\log_{10}\left(\frac{mean\ \%\ relative\ response_{FRESH\ OSPW\ (n=3)}}{mean\ \%\ relative\ response_{AGED\ OSPW\ (n=4)}}\right)$$

For this calculation, we first included only those species that were detected in all fresh OSPW samples, and an arbitrary response near detection limits was substituted in aged OSPW samples for species which were not detectable. To also consider chemicals that may be formed as degradation products over time, we calculated the ratio for additional species if they were detected in all aged OSPW samples but were not detected in any or all fresh OSPW samples; again, by substituting an arbitrary response value for non-detects in fresh OSPW samples.

2.3   Results and Discussion

2.3.1   Chemical Profiling in Negative Ionization Mode

All oxygen-containing classes detected in negative ionization mode had lower absolute intensities ($p \leq 0.05$) in aged OSPW compared to fresh OSPW (**Figure 2-5**, **Table 2-1**). Moreover, total NA (i.e., O_2^- class) concentrations in aged OSPW (<1 mg/L) were significantly lower ($p < 0.01$, **Table 2-2**) than in fresh OSPW or BML (8.3 to 21.2 mg/L). This has previously been noted for NAs in fresh OSPW compared to these same experimental ponds sampled in 2006,[11] but has not previously been described for the other chemical classes. Chemical classes in BML OSPW were in a similar intensity range as fresh OSPW, except for O_2^- which had the highest intensity in BML OSPW. This may be due to the increased oxidation experienced in BML which has periodic dilution and periodic outflow into a recycle pond. Intensities of the O_5^- and O_xSN^- classes were in a similar range among all OSPW types, but these were minor contributors to the profiles of all samples **Figure 2-5A**).

Considering relative profiles of the chemical classes, total oxygen-containing acid classes (i.e., O_x^-, where $x = 1 - 6$) made the largest contribution to spectral response across all OSPW types in negative mode (**Figure 2-5B**), on average accounting for 92.9% of total signal in fresh OSPW, 98.7% in BML and 95.8% in aged OSPW. More specifically, O_2^- was the dominant chemical class in all samples (**Figure 2-6**), typically accounting for more than half of total O_x^- signal in each sample. The O_4^- class had the second-highest relative contribution in fresh OSPW (24.0 %, $p = 0.02$, **Table 2-3**) whereas in aged OSPW it was the O_3^- class (21.3%, $p = 0.03$). Sulfur- and nitrogen-containing organic acids made only minor contributions to the total signal in all OSPW samples (**Figure 2-5B**). Sulfur-containing organics (i.e., SO_x^-) contributed similarly to the total signal in aged and fresh OSPW (7.1% and 6.8% , respectively), and less in BML (1.2%); of these, the SO_2^- class made the highest contribution to fresh OSPW (0.8 – 6.8% of total response), compared to 0.3% in BML and up to 0.1% in aged OSPW.
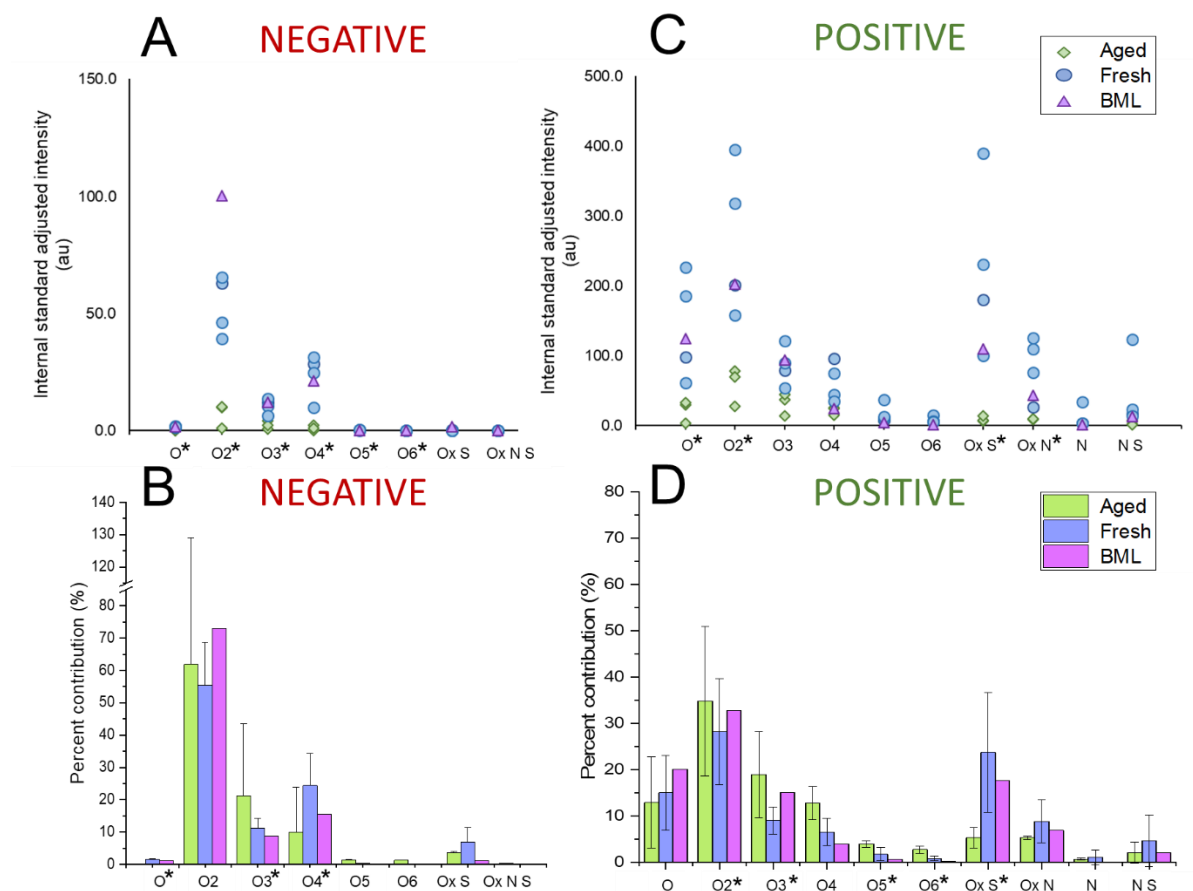
Figure 2-5 Absolute and relative intensities of heteroatomic chemical classes. Absolute intensities of heteroatomic chemical classes, adjusted for internal standard, in (A) negative and (C) positive ionization modes, organized by OSPW type. The same data are also plotted as relative intensities (mean % contribution + standard error of mean) in negative (B) and positive (D) ionization modes. Classes which were significantly different between fresh and aged OSPW (p < 0.05) are indicated by (*).

Table 2-1 Internal standard adjusted absolute intensities of heteroatomic classes in aged and fresh OSPW

Mode	Class	Aged OSPW Mean ± SEM	Fresh OSPW Mean ± SEM	p-value
NEG	O^-	0.01 ± 0.002	1.61 ± 0.11	<0.01
	O_2^-	6.96 ± 3.00	53.5 ± 6.39	<0.01
	O_3^-	2.40 ± 1.00	10.7 ± 1.57	0.01
	O_4^-	1.12 ± 0.62	23.6 ± 4.73	0.01
	O_5^-	0.15 ± 0.01	$0.31 \pm 0 .04$	0.02
	O_6^-	0.15 ± 0.004	0.02 ± 0.002	<0.01
	$S\ O_x^-$	0.42 ± 0.02	6.78 ± 2.2	0.06
	$O_x\ SN^-$	0.05 ± 0	0.09 ± 0.02	0.12
POS	O^+	21.6 ± 9.53	142.5 ± 38.2	0.05
	O_2^+	58.3 ± 15.6	267.5 ± 54.3	0.02
	O_3^+	31.8 ± 9.01	108.3 ± 27.6	0.07
	O_4^+	21.4 ± 3.43	62.4 ± 13.9	0.06
	$O5^+$	6.69 ± 0.64	16.9 ± 6.78	0.26
	O_6^+	4.68 ± 0.76	8.04 ± 2.40	0.30
	$S\ O^+$	0.75 ± 0.16	72.2 ± 24.9	0.16
	$S\ O_2^+$	3.88 ± 0.93	79.7 ± 14.3	<0.01
	$S\ O_3^+$	2.96 ± 0.98	63.3 ± 20.2	0.05
	$N\ O^+$	3.11 ± 0.33	62.3 ± 19.1	0.05
	$N\ O_2^+$	5.16 ± 0.14	5.16 ± 3.52	0.04
	$N\ O_3^+$	0.56 ± 0.11	4.15 ± 1.53	0.10

Results of t-test comparisons of the average values of the internal standard adjusted intensities of heteroatomic classes detected in aged (EP5, EP9, EP11), BML, and fresh (TP1, TP2, TP3, TP4) OSPW. Comparisons of classes between OSPW types with p-values ≤ 0.05 were considered significantly different are highlighted in red (NEG) or green (POS).

Table 2-2 Estimated concentrations of the O_2^- class (naphthenic acids) based on calibration by external standard curve composed of commercial Merichem naphthenic acids

Fresh OSPW		BML OSPW		Aged OSPW	
Sample	$[O_2^-]$	Sample	$[O_2^-]$	Sample	$[O_2^-]$
TP1	20.1 mg/L	EPL	23.1 mg/L	EP5	<1.00 mg/L
TP2	8.3 mg/L			EP9	<1.00 mg/L
TP3	21.2 mg/L			EP11	<1.00 mg/L
TP4	11.8 mg/L				

Table 2-3 Relative contributions of heteroatomic classes in aged and fresh OSPW

Mode	Class	Aged OSPW Mean ± SEM	Fresh OSPW Mean ± SEM	p-value
NEG	O^-	0.001 ± 0.001	0.02 ± 0.003	0.01
	O_2^-	0.56 ± 0.09	0.56 ± 0.04	0.99
	O_3^-	0.23 ± 0.03	0.11 ± 0.02	0.02
	O_4^-	0.08 ± 0.03	0.24 ± 0.03	0.02
	O_5^-	0.03 ± 0.02	0.004 ± 0.001	0.15
	O_6^-	0.03 ± 0.02	0 ± 0	0.12
	$S\ O_x^-$	0.07 ± 0.04	0.07 ± 0.02	0.95
	$S\ O^-$	0.063 ± 0.038	0.01 ± 0.002	0.16
	$S\ O_2^-$	0.001 ± 0	0.04 ± 0.012	0.05
	$S\ O_3^-$	0.006 ± 0.004	0.018 ± 0.012	0.45
	$O_x\ S\ N^-$	0.05 ± 0	0.09 ± 0.02	0.12
POS	O_x^+	0.85 ± 0.03	0.62 ± 0.03	<0.01
	O^+	0.11 ± 0.04	0.14 ± 0.02	0.41
	O_2^+	0.34 ± 0.02	0.28 ± 0.01	0.02
	O_3^+	0.19 ± 0.01	0.10 ± 0.01	0.01
	O_4^+	0.14 ± 0.02	0.07 ± 0.02	0.08
	O_5^+	0.04 ± 0.01	0.02 ± 0.004	0.03
	O_6^+	0.03 ± 0.01	0.008 ± 0.001	0.01
	$S\ O_x^+$	0.059 ± 0.014	0.229 ± 0.019	<0.01
	$S\ O^+$	0.01 ± 0	0.071 ± 0.012	0.01
	$S\ O_2^+$	0.024 ± 0.004	0.09 ± 0.004	0.00
	$S\ O_3^+$	0.02 ± 0.01	0.063 ± 0.01	0.03
	$N\ O_x^+$	0.062 ± 0.017	0.101 ± 0.041	0.47
	$N\ O^+$	0.02 ± 0.004	0.08 ± 0.04	0.25

Results of t-test comparisons of the average values of the relative contributions of heteroatomic classes detected in aged (EP5, EP9, EP11), BML, and fresh (TP1, TP2, TP3, TP4) OSPW. Means represent the proportion out of 1, where 1 is the sum of all heteroatomic classes. Comparisons of classes between OSPW types with p-values ≤ 0.05 were considered significantly different are highlighted in red (NEG) or green (POS).
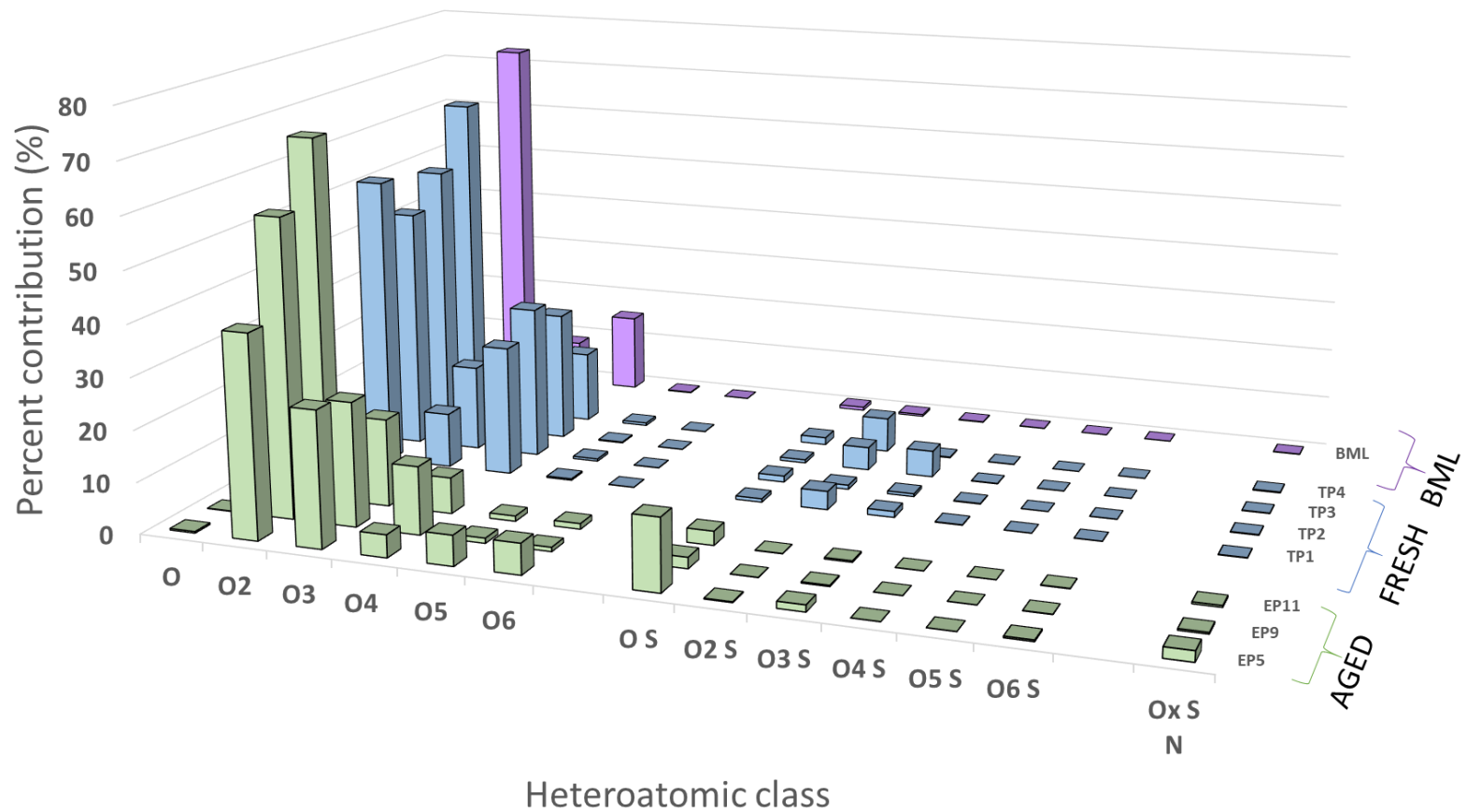
Figure 2-6 Summary of OSPW profiles detected in negative ion mode by percent relative contribution.

The lower absolute intensities of the major organic classes in aged OSPW have a few possible explanations. One possibility is that the experimental ponds may have been diluted over time, but long-term trends in chloride concentrations (a conservative tracer[13]) provide little evidence for (**Figure 2-7**). Minor dilution was only evident in EP9, as chloride significantly decreased from 273 to 240 mg/L over 15 years (**Figure 2-7**, p=0.043), but this 11% change cannot explain the 77% difference in intensities of O_2^- species between OSPW types (**Figure 2-5A**). A second consideration is that tailings ponds may contain more concentrated OSPW today than in the past because of increased amounts of OSPW reuse in the extraction of oil sands ore. At the time of establishment in 1993, EP9 contained 100% fresh OSPW and had a measured chloride concentration of 273 mg/L (**Table 2-4**), which can be compared to fresh OSPW in 2014 from TP1, TP2, TP3 and TP4 (283, 481, 683 and 300 mg/L, respectively). Thus, differences in starting concentrations of the experimental ponds do not explain the much lower concentrations of organics in aged samples, which for example, in the O_2^- class was in the range of at least 8 – 20-fold lower in aged OSPW (**Table 2-5**). As discussed later, a third and most likely explanation is that biotic or abiotic degradation processes were active in the experimental ponds that selectively degraded the organics. Biodegradation of OSPW organics by bacteria has received much of the historic attention, but a recent laboratory study has shown that photolysis may also be significant.[14]
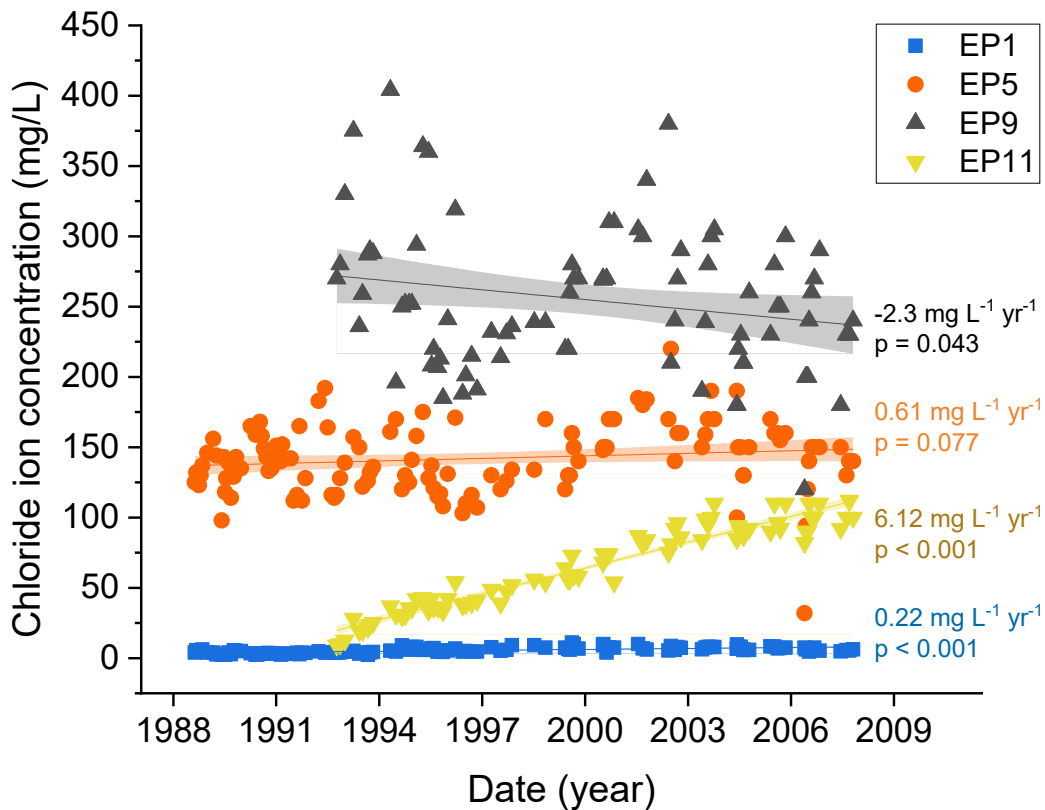
Figure 2-7 Chloride ion concentration in a control pond (EP1) and in experimental ponds containing OSPW, freshwater and/ tailings (EP5, EP9, EP11) between 1989 and 2008. Slope and significance (p) of the linear regression of chloride ion concentration over time are shown for each pond. In 1989, EP1 was established as a control pond with surface water from a nearby river (Muskeg) and no oil sands process materials. From 1989 to 2008, chloride increased in EP1 from 3.9 mg/L to 6.2 mg/L at rate of 0.22 mg L^{-1}year^{-1} ($p < 0.05$). EP5 had a marginal increase in chloride concentrations, from 125 mg/L to 140 mg/L ($p = 0.077$) at a rate of 0.61 mg L^{-1}year^{-1}. EP11 had a unique and significantly increasing trend of chloride (6.12 mg L^{-1}year^{-1}, $p < 0.001$) from near background levels (EP1) up to 100 mg/L. The 10-fold change was a result of the consolidation of clays and the complimentary expression of porewater from fluid fine tailings (FFT containing OSPW) at the base of the pond into the freshwater cap. This has been previously reported by Hogan *et al.* 2018. This mechanism was also likely operative in EP5, although it was masked by the presence of OSPW cap water. Possible dilution was evident in EP9, as the overall chloride significantly decreased ($p = 0.043$) from 273 mg/L to 240 mg/L, but this does not explain the large difference in intensities of organics between fresh and aged OSPW (**Figure 2-5A**, **Figure 2-5B**).

Table 2-4 Comparison of chloride ion concentration of experimental ponds at time of establishment (year) and of tailings ponds in 2014

Experimental Pond		Fresh OSPW	
Sample (year)	$[Cl^-]$ (mg/L)	**Sample** (year)	$[Cl^-]$ (mg/L)
EP5 (1989)	125	**TP1** (2014)	283
EP9 (1993)	273	**TP2** (2014)	481
		TP3 (2014)	683
		TP4 (2014)	300

Table 2-5 Estimated concentrations of the O_2^- class (naphthenic acids) based on calibration by external standard curve composed of commercial Merichem naphthenic acids

Fresh OSPW		BML OSPW		Aged OSPW	
Sample	$[O_2^-]$	**Sample**	$[O_2^-]$	**Sample**	$[O_2^-]$
TP1	20.1 mg/L	EPL	23.1 mg/L	EP5	<1.00 mg/L
TP2	8.3 mg/L			EP9	<1.00 mg/L
TP3	21.2 mg/L			EP11	<1.00 mg/L
TP4	11.8 mg/L				

To visualize all classes and species most broadly, and to highlight those which were most degraded in fresh OSPW(or most enhanced in aged OSPW), the ratio of relative proportions of each species in aged and fresh OSPW was plotted (**Figure 2-8A**, for negative mode). These data can be interpreted such that species above 0 (i.e., $\log_{10}1$) had a higher relative abundance in fresh OSPW and are likely degraded over time, while species below 0 had higher relative abundance in aged OSPW and may be persistent or formed as degradation products. Of the 200 species plotted across 9 classes in **Figure 2-8A**, most species in the O_2^-, SO^- classes, and many SO_2^- species were relatively diminished in aged samples, whereas the O_3^-, O_4^-, O_5^-, O_6^- and SO_3^- classes became relatively enhanced in aged OSPW. This lends to the hypothesis that the acutely toxic NAs (O_2^- species) may be among the most labile organic acid classes, and that these may be oxidized to more persistent classes (e.g., O_3^-) over time. Han *et al.* previously suggested that higher proportions of O_3^- *and* O_4^- in the aged OSPW sample EP5 may be a marker of NA biodegradation.[11] Similarly, that the relatively labile SO_2^- class may be oxidized to a more persistent SO_3^- class over time.

Species profiles within each major chemical class in negative mode were then plotted for each sample to visualize patterns by carbon number and DBE (**Figure 2-9** – **Figure 2-14**), and typical profiles for NAs (O_2^- class) are shown in x15 top panels) for a fresh OSPW, BML, and an aged OSPW. In fresh OSPW, the O_2^- species profiles were similar among fresh samples, showing typical right-skewed Gaussian distributions across carbon numbers,[15] and a bimodal distribution of DBEs with apexes between 2 – 8 and 9 – 16 (TP1 in **Figure 2-15**, and TP1 – TP4 in **Figure 2-9**). In the O_2^- class, the major species contained C_{13} – C_{14} and 2 – 3 DBEs. For species with greater than 9 DBEs, the major species were distributed around C_{18} and 12 DBEs. The O_2^- profiles of fresh OSPW were similar to BML – unsurprising because BML OSPW had only aged 2 years at time of sample collection. In samples of EP9 (**Figure 2-15**) and EP11 (**Figure 2-9**), the remaining O_2^- species had 2 – 3 DBEs, similar to fresh OSPW and BML (**Figure 2-9**) . EP5 had non-detectable concentrations of most O_2^- species. Overall, these NA profiles show the great extent to which all species disappear over time yet provide little evidence of any structure-activity in the loss process. Under aerobic laboratory conditions, the recalcitrance of OSPW O_2^- species to microbial biodegradation was correlated with DBE, whereby recalcitrance increased with the number of DBEs.[16] Thus, we had hypothesized that if oxidative microbial biodegradation were a fate pathway for O_2^- species in the field, an enhancement of species with DBE greater than 5 would be observed in aged water samples. There was no evidence for this.

It was hypothesized above that the NAs may be oxidized over time, thus the O_x^- profiles were examined (**Figure 2-10** – **Figure 2-12**). For O_3^- species these displayed Gaussian distributions across carbon number in both fresh OSPW and BML (**Figure 2-10**), with major species having C_{13} – C_{15} and 3 – 5 DBEs. Intensities of O_3^- species in aged OSPW were typically less than half of that in fresh OSPW (**Table 2-1**, p = 0.01) or BML. A cluster larger O_3^- species with 6 or more DBEs were effectively absent in the aged OSPW, but otherwise there was also little evidence of structure activity in their loss (or formation) processes. O_4^- species profiles (**Figure 2-11**) had lower absolute intensities in aged samples (**Table 2-1**, p = 0.01), in fact EP9 was the only aged sample with a detectable profile and had a similar profile to fresh OSPW. The O_5^- profiles were more variable (**Figure 2-12**) and some aged OSPW had higher abundances of these compared to fresh OSPW, but smaller carbon numbers (n≤ 20) than in some of the fresh OSPW samples.
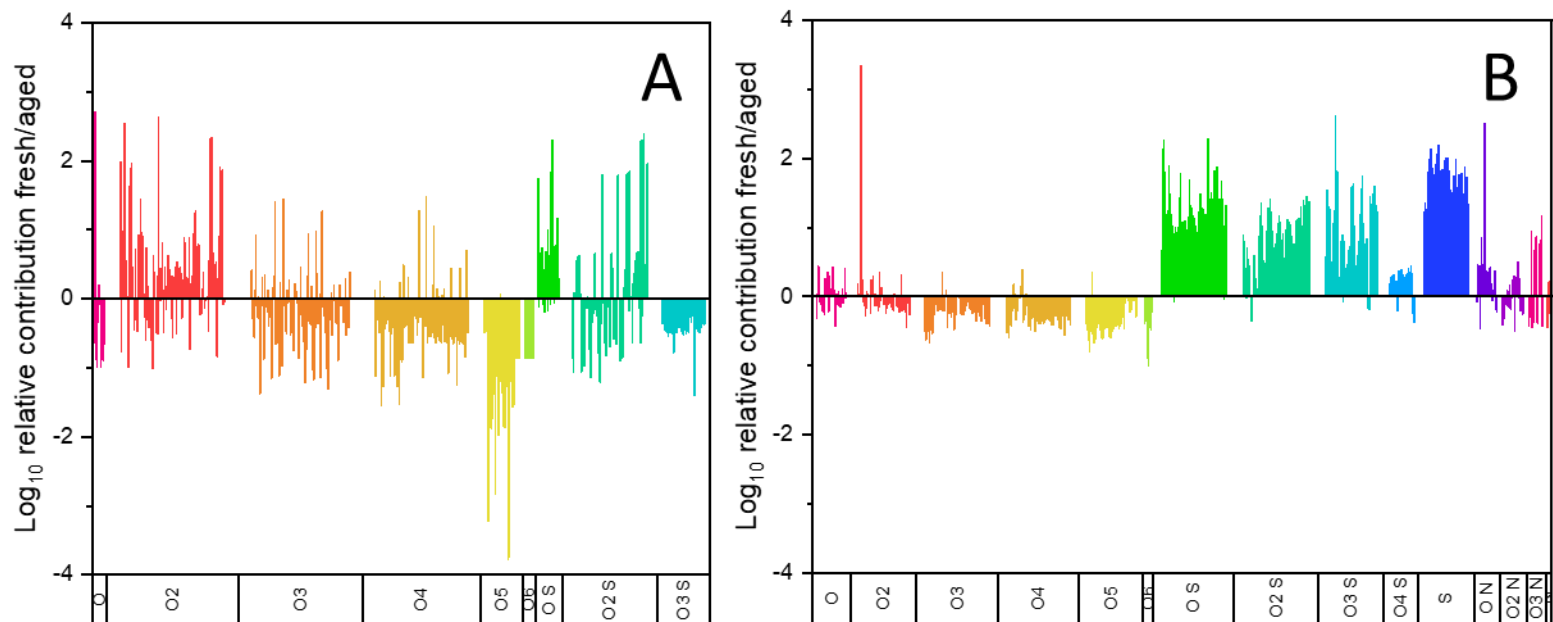
Figure 2-8 The log$_{10}$ ratio of mean relative species abundance in fresh and aged OSPW for (A) 200 species in negative mode, and (B) 700 species in positive ionization mode. Numbers above zero represent species with a higher relative proportion in fresh OSPW, while numbers below 0 represent species with higher relative proportion in aged OSPW. Species are colour coded by heteroatomic class, and within each class are organized left-to-right by primary sorting on DBE, and then secondary sorting on carbon number. All species plotted were detected in all fresh OSPW samples, or in all aged OSPW samples.
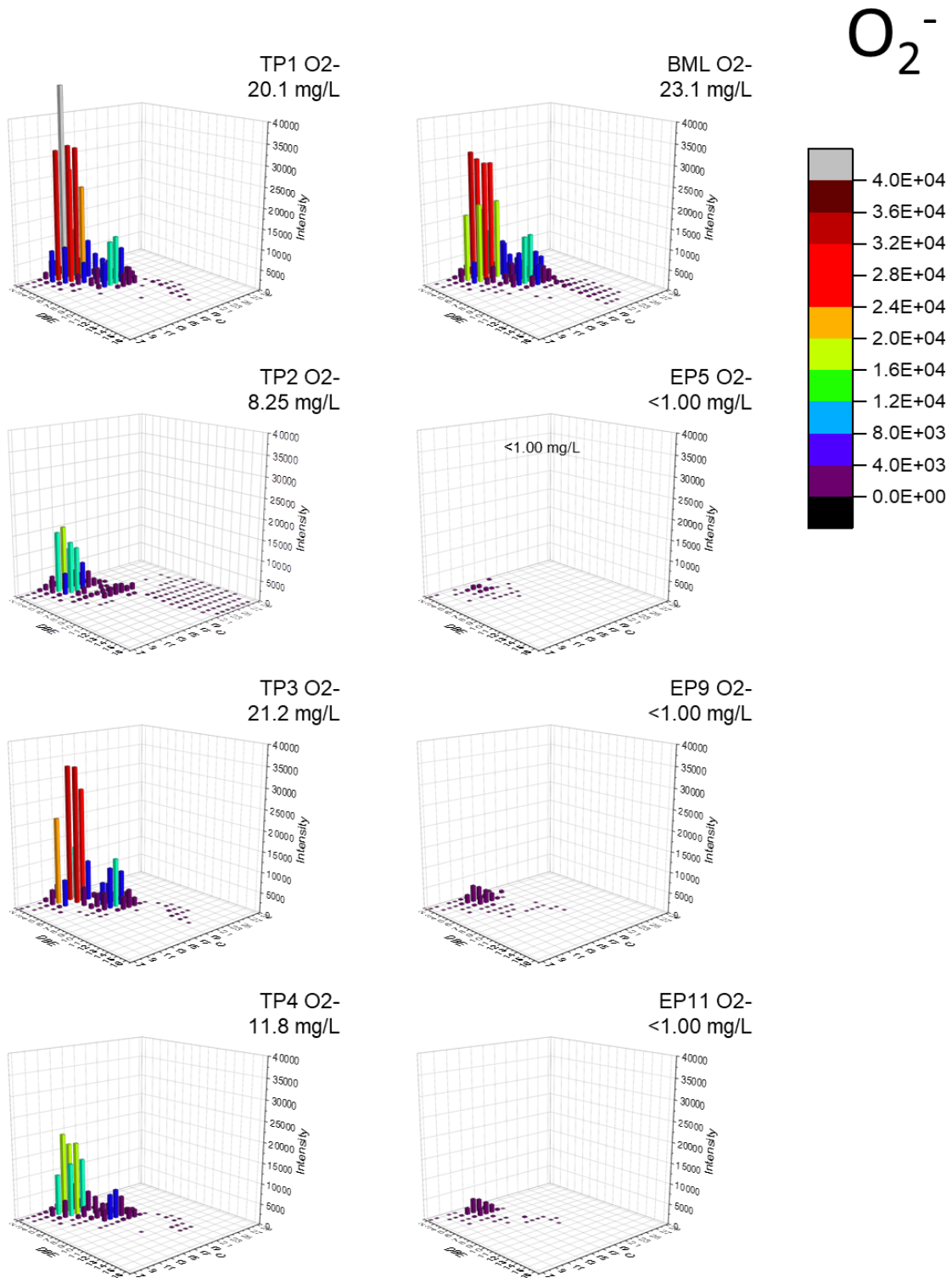
Figure 2-9 O$_2^-$ profiles for fresh (TP1, TP2, TP3, TP4), BML, and aged OSPW (EP5, EP9, EP11). Intensity arbitrary units: 0 - 4E4 (bottom to top); DBE (back to front) 2 – 16; C (7 – 29) and concentrations (mg/L).
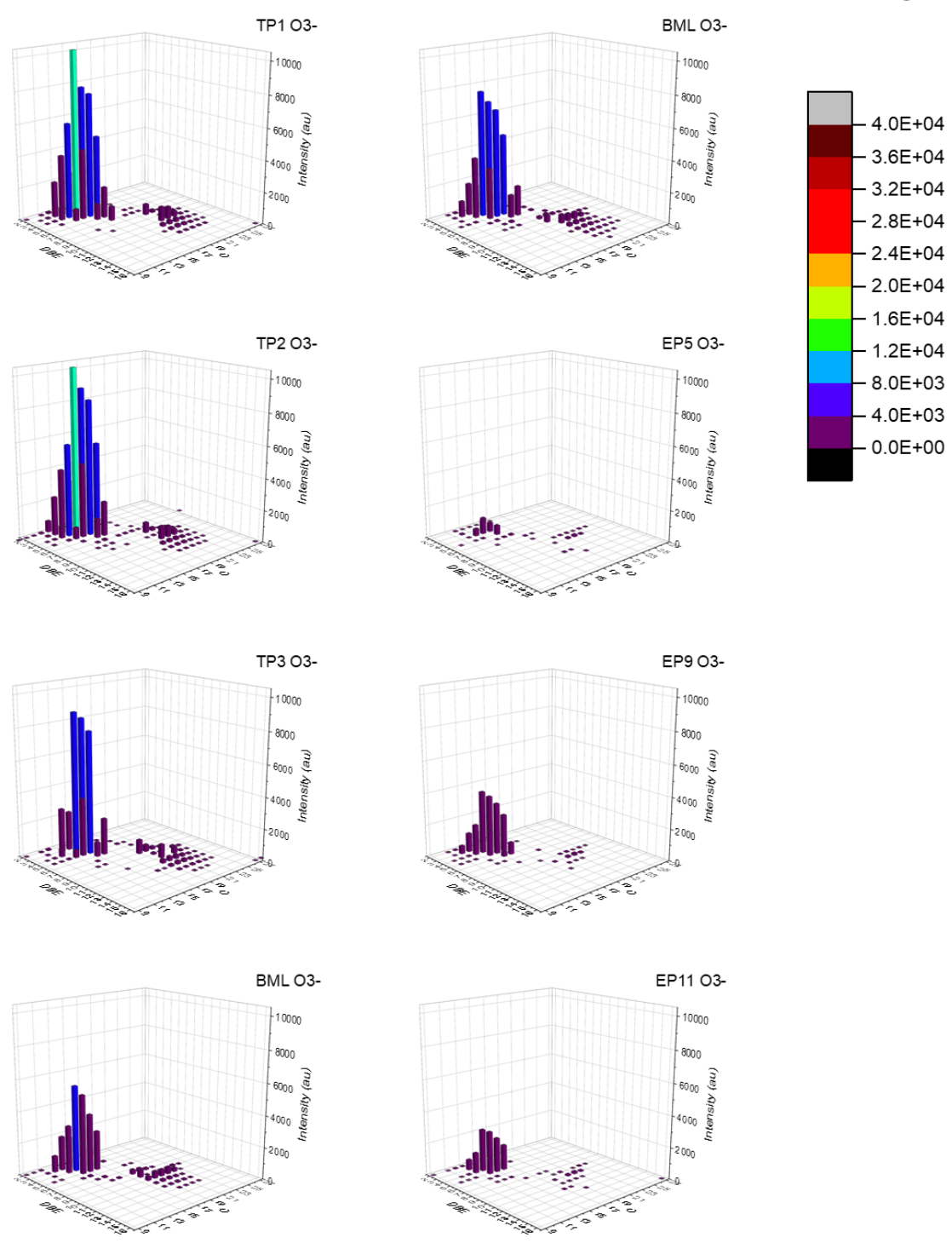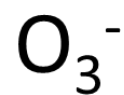
Figure 2-10 O₃⁻ profiles for fresh (TP1, TP2, TP3, TP4), BML, and aged OSPW (EP5, EP9, EP11). Intensity: 0 – 2 E4 au (bottom to top); DBE (back to front) 2-16; C 7-29 (front to back).
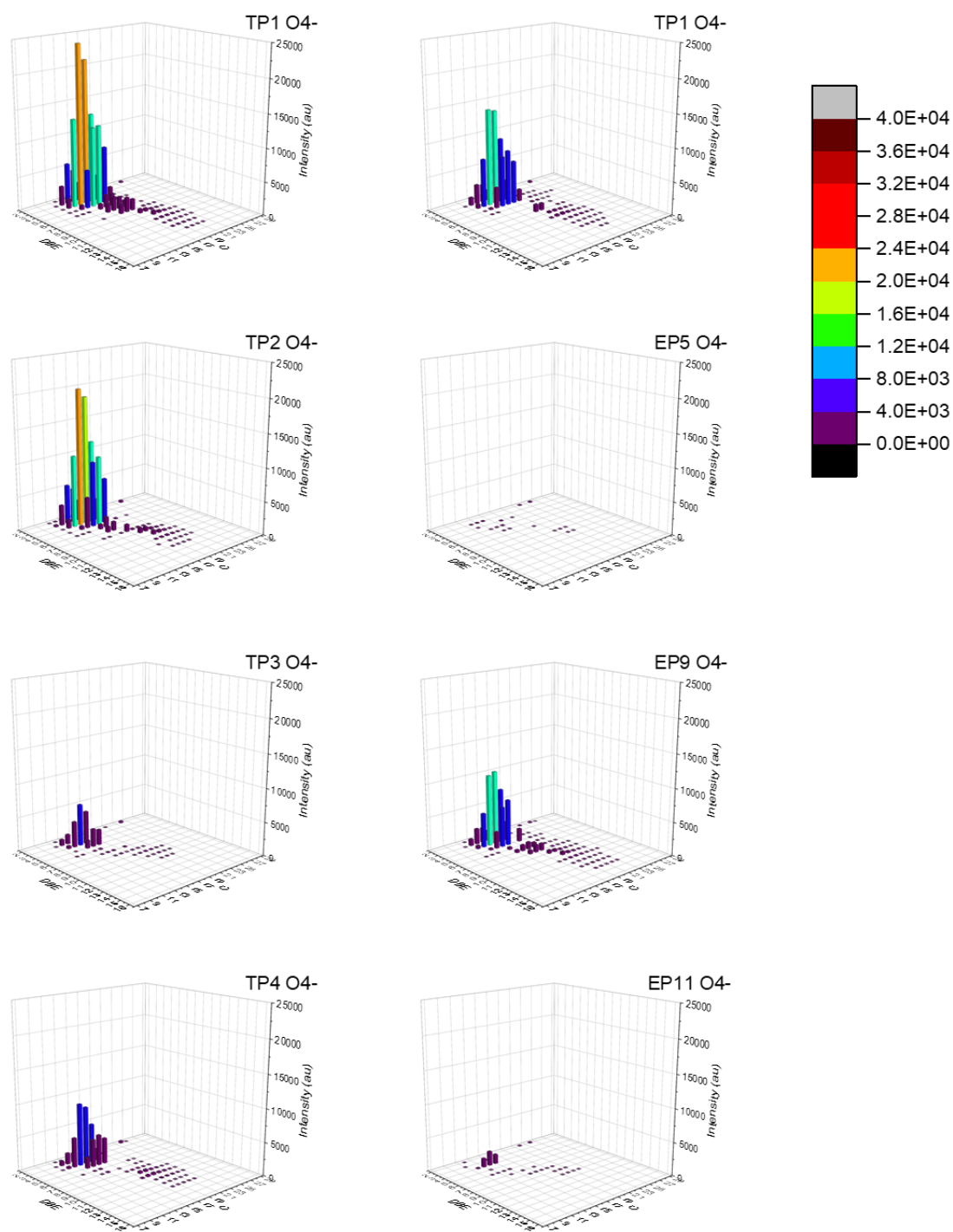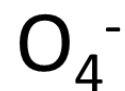
Figure 2-11 O$_4^-$ profiles for fresh (TP1, TP2, TP3, TP4), BML, and aged OSPW (EP5, EP9, EP11). Intensity: 0 – 2 E4 au (bottom to top); DBE (back to front) 2-16; C 7-29 (front to back).
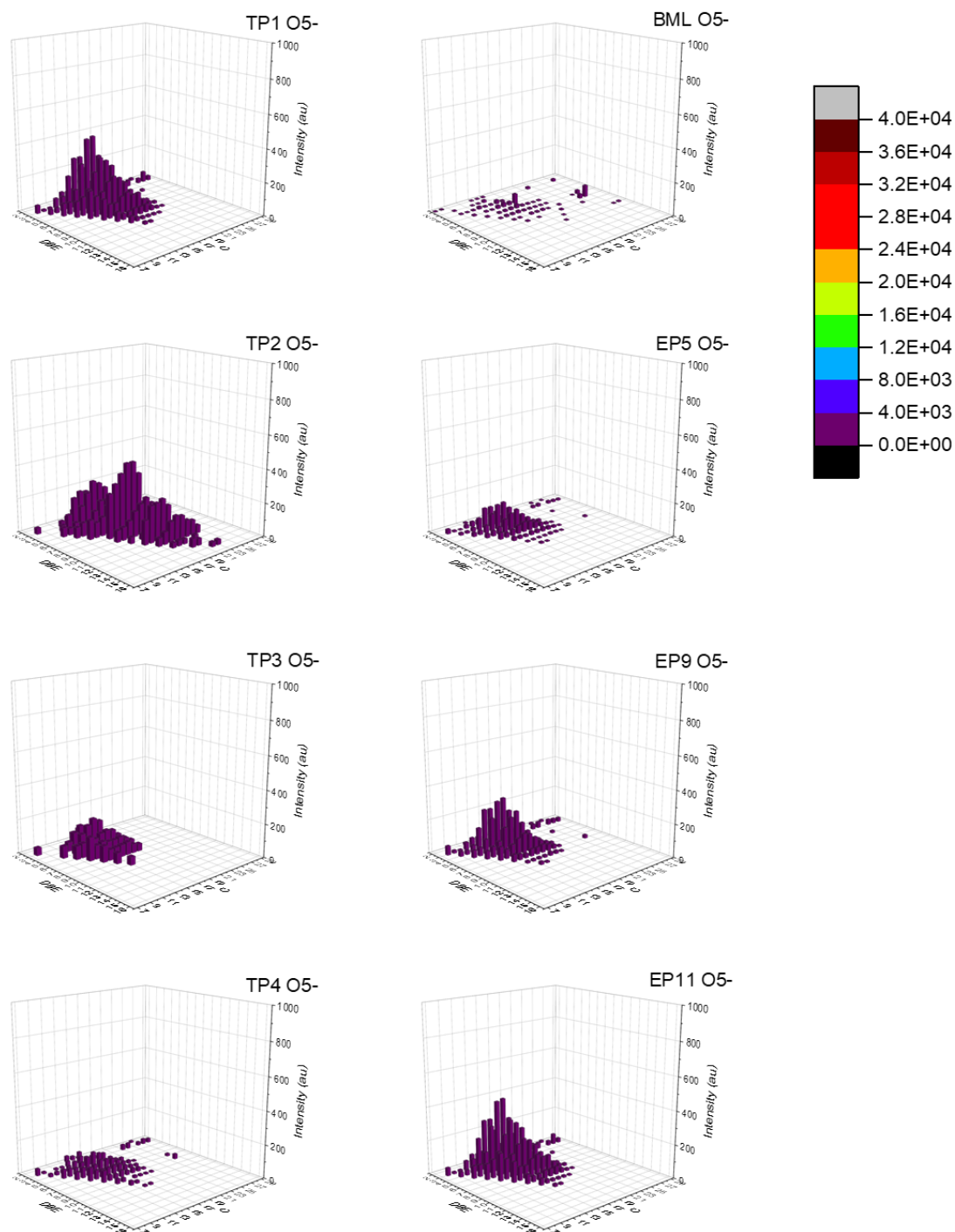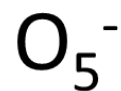
Figure 2-12 O$_5^-$ profiles for fresh (TP1, TP2, TP3, TP4), BML, and aged OSPW (EP5, EP9, EP11). Intensity: 0 – 2 E4 au (bottom to top); DBE (back to front) 2-16; C 7-29 (front to back).
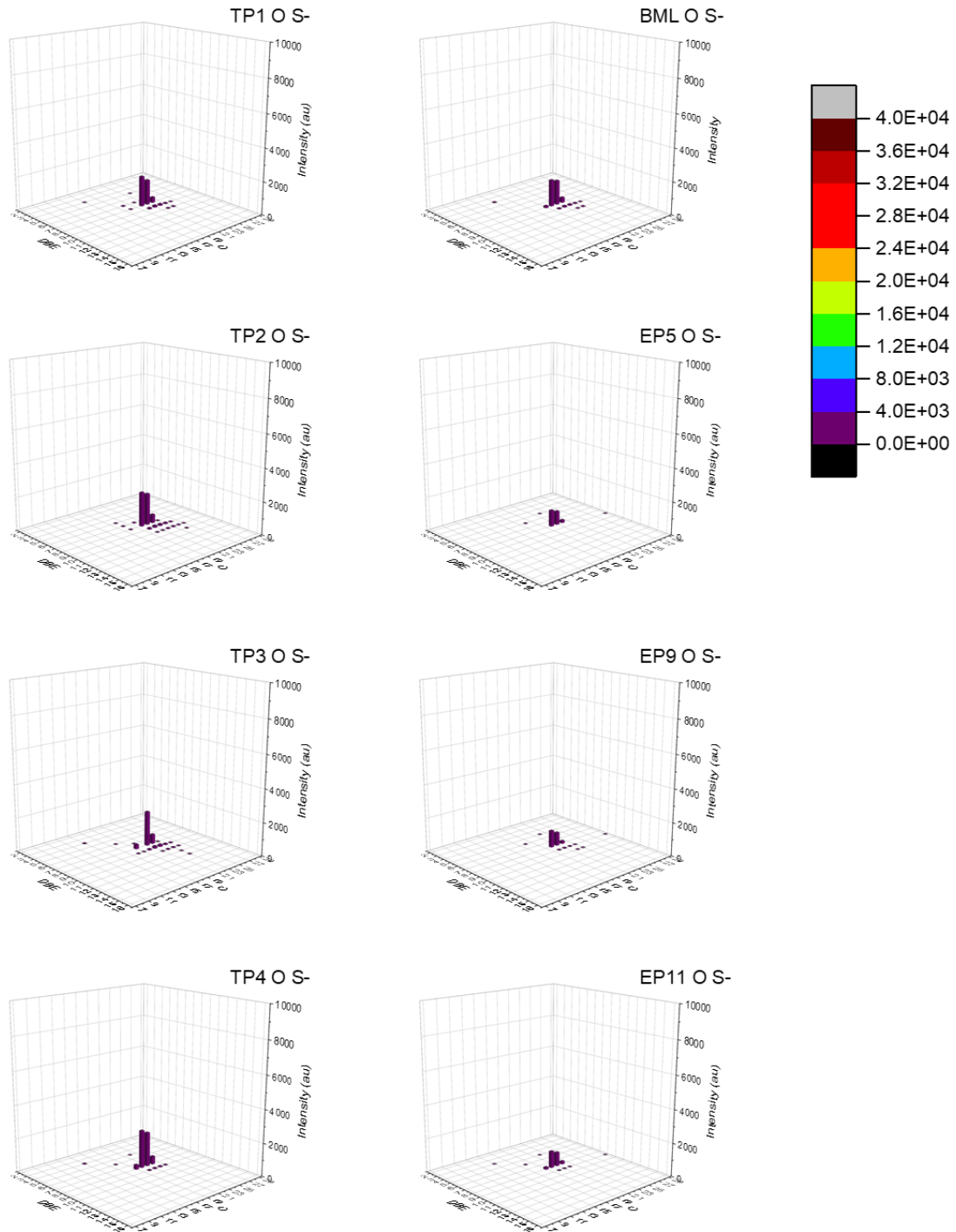
Figure 2-13 O S⁻ profiles for fresh (TP1, TP2, TP3, TP4), BML, and aged OSPW (EP5, EP9, EP11). Intensity: 0 – 1 E4 au (bottom to top); DBE (back to front) 2-16; C 7-29 (front to back).
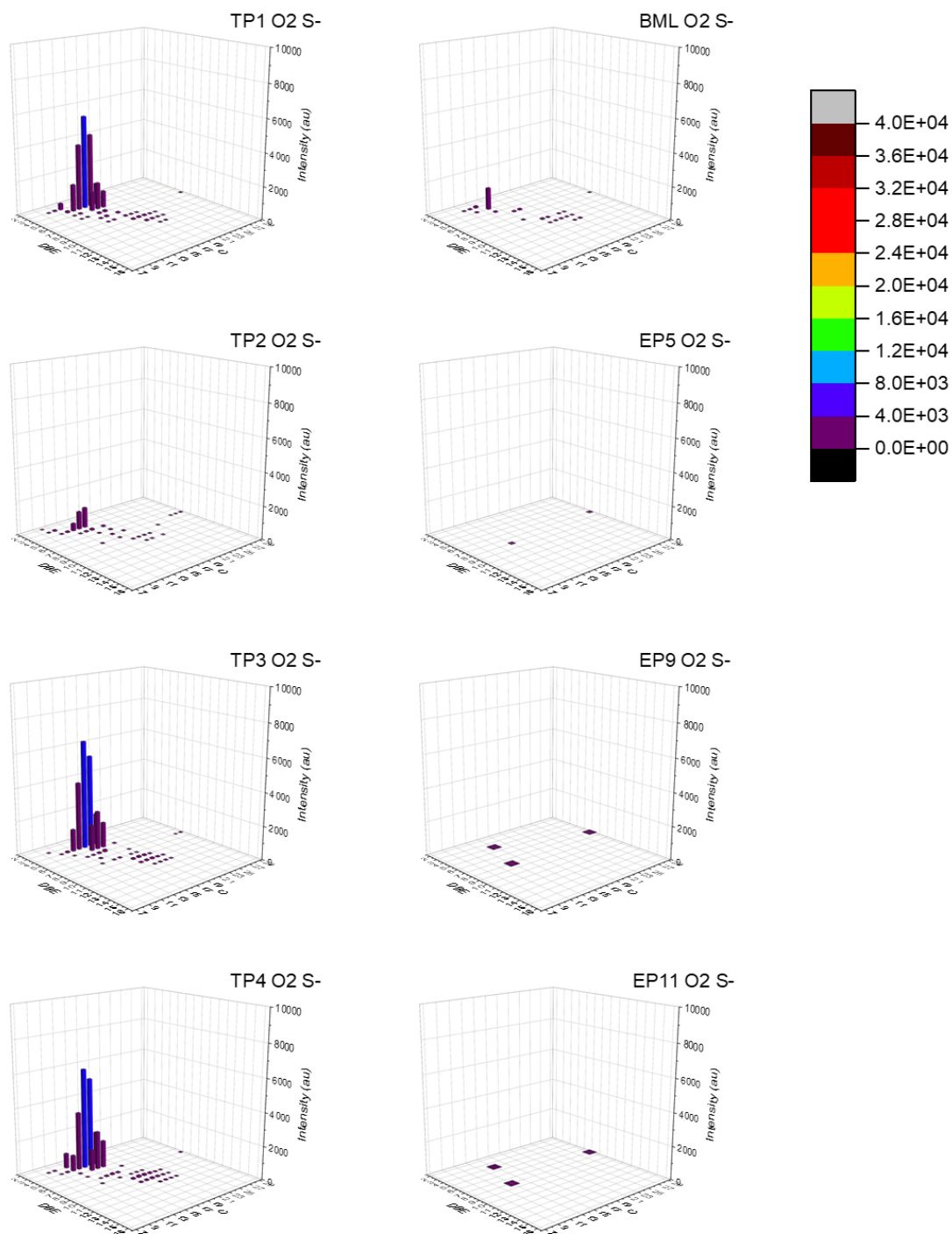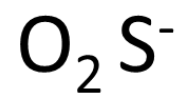
Figure 2-14 O₂ S⁻ profiles for fresh (TP1, TP2, TP3, TP4), BML, and aged OSPW (EP5, EP9, EP11). Intensity: 0 – 1 E4 au (bottom to top); DBE (back to front) 2-16; C 7-29 (front to back).
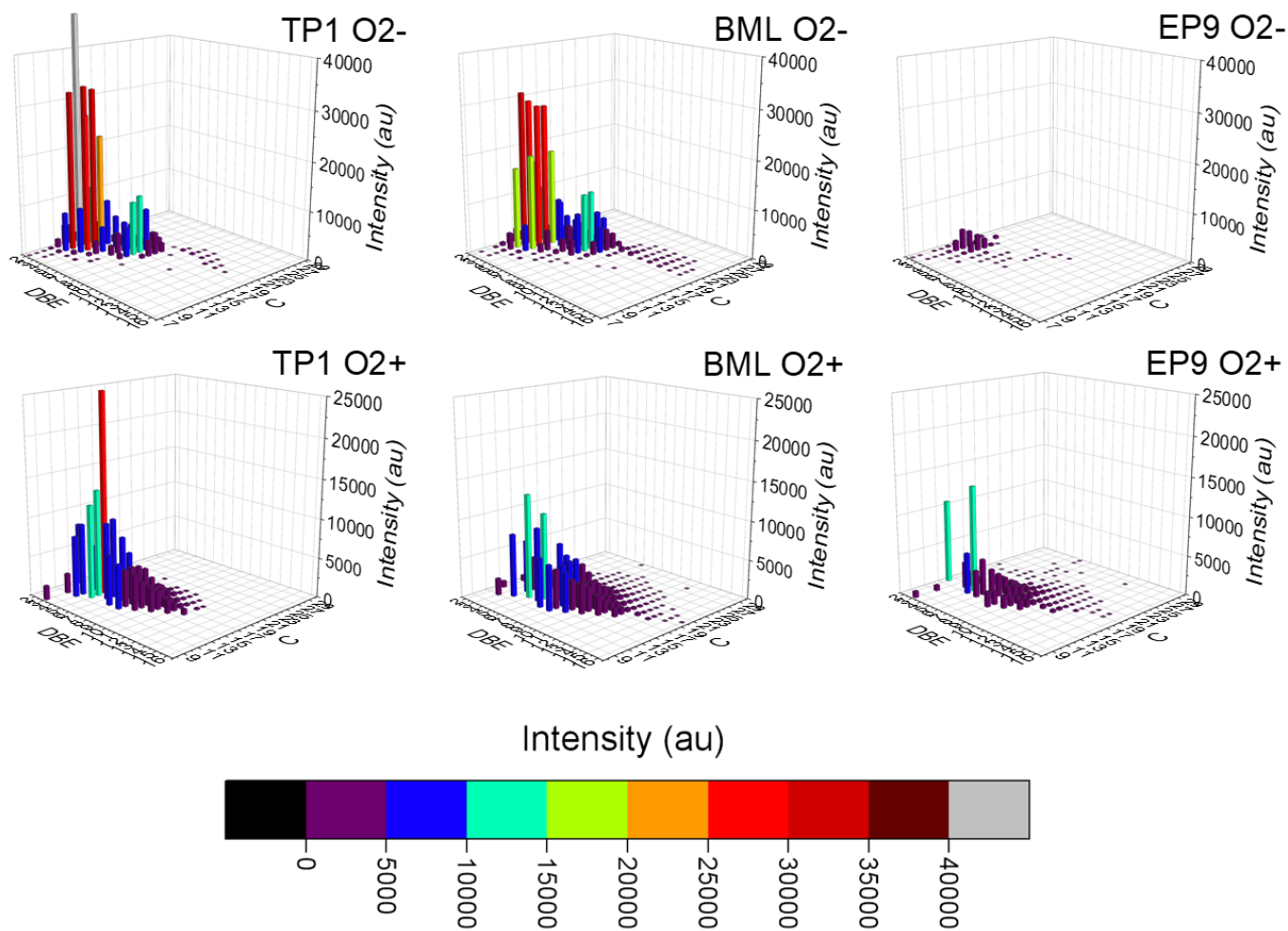
Figure 2-15 Species profiles for the O₂⁻ (top) and O₂⁺ (bottom) classes in fresh (left), BML (middle), and aged (right) OSPW samples. Each plot shows distribution by carbon numbers, DBE (double bond equivalents), and intensity (arbitrary units, au) for fresh OSPW (TP1), Base-Mine Lake (BML), and an aged OSPW (EP9). Colours correspond to the range of intensity (au).

2.3.2   Chemical Profiling in Positive Ionization Mode

Absolute intensities in positive ion mode were also generally higher in fresh OSPW than aged OSPW (**Figure 2-5C**, **Table 2-1**), and this was statistically significant for the O^+ ($p = 0.05$) and O_2^+ ($p = 0.02$) classes, as well as for several sulfur- and nitrogen-containing classes, including the SO_2^+ ($p < 0.01$), SO_3^+ ($p = 0.05$), NO^+ ($p = 0.05$) and NO_2^+ ($p = 0.04$) classes. In positive mode, the major class in all fresh and aged OSPW samples was O_2^+ (**Figure 2-5D**), contributing 32.0 – 37.5% of the relative response in fresh OSPW, 32.8% in BML, and 26.4 – 31.2% in aged OSPW (**Figure 2-16**). In fresh OSPW and BML, O^+ and O_3^+ classes had the second and third largest relative contributions among O_x^+ classes, respectively, whereas in aged OSPW the O^+, O_3^+ and O_4^+ classes all had similar relative contributions (**Figure 2-16**, **Table 2-3**). As in negative mode, sulfur- and nitrogen-containing species in positive mode had lower responses than other classes of organics in all samples. This is not surprising because the mass contribution of sulfur and nitrogen in bitumen are around only 4.0 and 0.42%, respectively.[17] In addition to NAs, the SO^+ and NO^+ classes have been identified in the acutely toxic fraction of BML OSPW,[3] and are also among the more bioaccumulative chemicals in OSPW,[18,19] thus the evidence that these classes can degrade over time (**Figure 2-5**, **Figure 2-16**) is an important observation with long-term environmental significance.

To visualize all classes and species in positive ion mode more broadly, and to highlight those substances which were most degraded in fresh OSPW, thus most enhanced in aged OSPW, the ratio of relative proportions of the 700 most abundant species (of 2090) in aged and fresh OSPW were plotted (**Figure 2-8B**, for positive mode). These data revealed a strong pattern whereby most O_x^+ species plotted below 0 (i.e., had a higher relative abundance in aged OSPW), suggesting that these are relatively persistent chemical classes, or formed as degradation products. It is notable that only two O_2^+ species deviated strongly from this trend (C_{11} and C_{12} with 4 DBEs), suggesting that some O_2^+ species can degrade. In strong contrast to the O_x^+ species, the sulfur- and nitrogen-containing species (SO_x^+, NO_x^+) had lower relative contributions in aged OSPW, suggesting that these heteroatomic classes are more easily degraded over time. A secondary observation was observed within sulfur-containing classes, such that for SO^+, SO_2^+, SO_3^+, and SO_4^+ there appeared to be consistent structure activity, whereby those chemicals with greater DBE were more easily degraded (i.e., visualized by increasing trends from right to left, **Figure 2-8B**).

Consistent with the suggestive evidence in **Figure 2-5** that the O_x^+ classes are relatively persistent, the species profiles within all oxygen-containing classes (O_{1-6}^+) were generally similar among all OSPW types (**Figure 2-17** – **Figure 2-20**, O^+ and O_6^+ not shown). Profiles were skewed to lower carbon numbers (C_{10} – C_{12}) and lower DBEs (4 – 6). Notably, the profiles of the O_2^+ class are clearly different from those of the corresponding O_2^- class (**Figure 2-15**), consistent with the fact that these are different molecules with different retention times, as proven by Pereira *et al.*[4] The resemblance of corresponding O_x^+ species profiles in fresh and aged OSPW (**Figure 2-17** – **Figure 2-20**), despite differences in intensity, suggests a similar rate and mechanism of degradation, removal, or dilution by carbon numbers and DBEs. However, chromatographic peaks (i.e. unresolved humps representing thousands of unresolved isomers[4]) for individual species were different in aged and fresh OSPW samples. For example, the extracted ion chromatograms of $C_{15}H_{24}O_2^+$ and $C_{16}H_{18}O_2^+$ in BML and a fresh OSPW were different than in all aged OSPW samples (**Figure 2-21**). Of particular interest is that the latest eluting isomers in BML and fresh OSPW (i.e., retention times ~10.5) were selectively depleted in aged samples. This general isomer trend was similar in nearly all O_2^+ species examined (n=28), suggesting biotic or abiotic degradation or removal process that depends on physical or chemical properties.
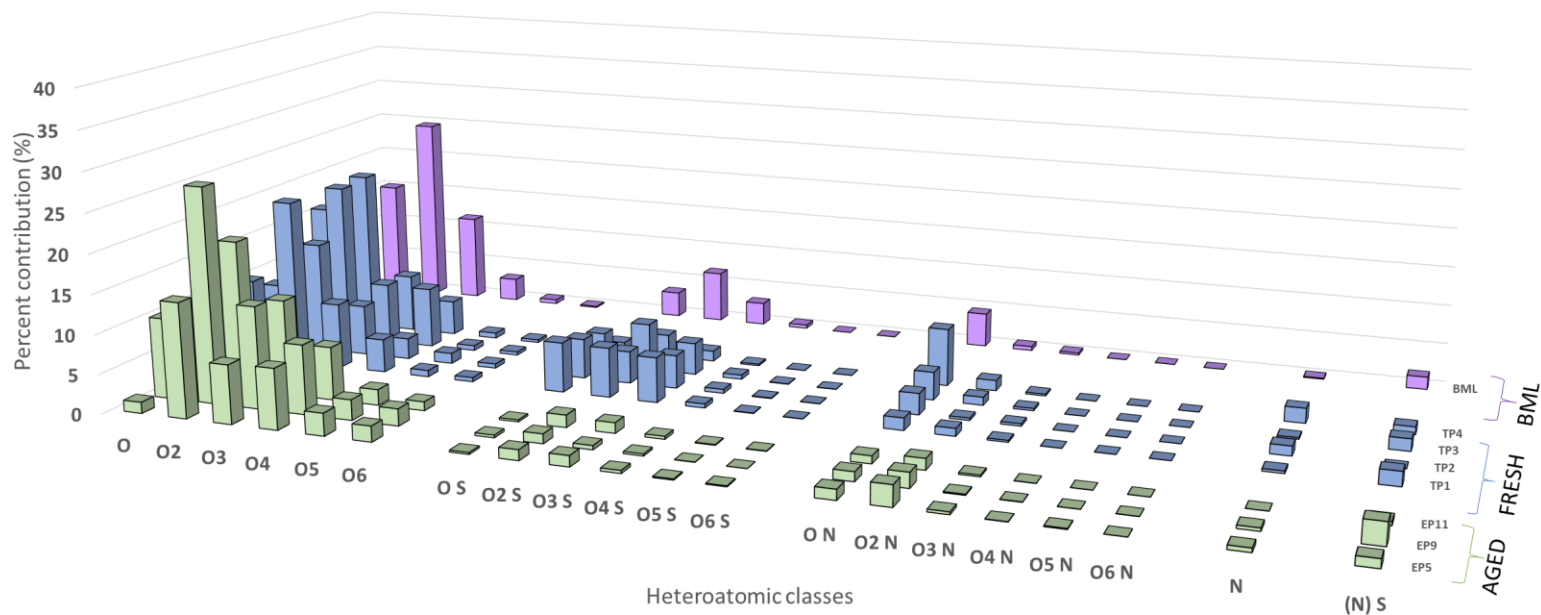
Figure 2-16 Summary of OSPW profiles detected in positive ion mode by percent contribution.
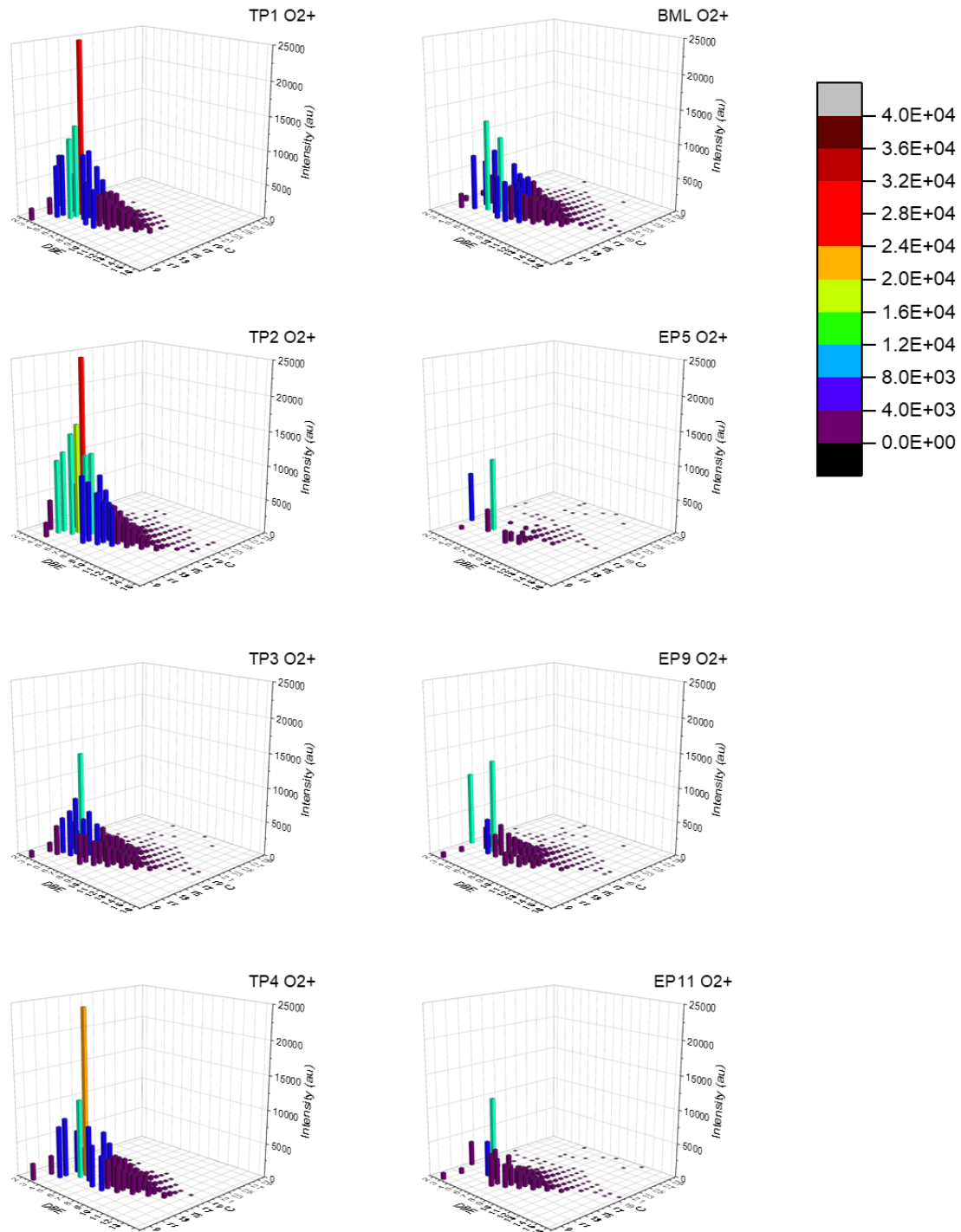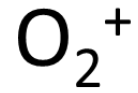
Figure 2-17 O₂⁺ profiles for fresh (TP1, TP2, TP3, TP4), BML, and aged OSPW (EP5, EP9, EP11). Intensity: 0 – 4 E4 au (bottom to top); DBE (back to front) 2-16; C 7-29 (front to back).
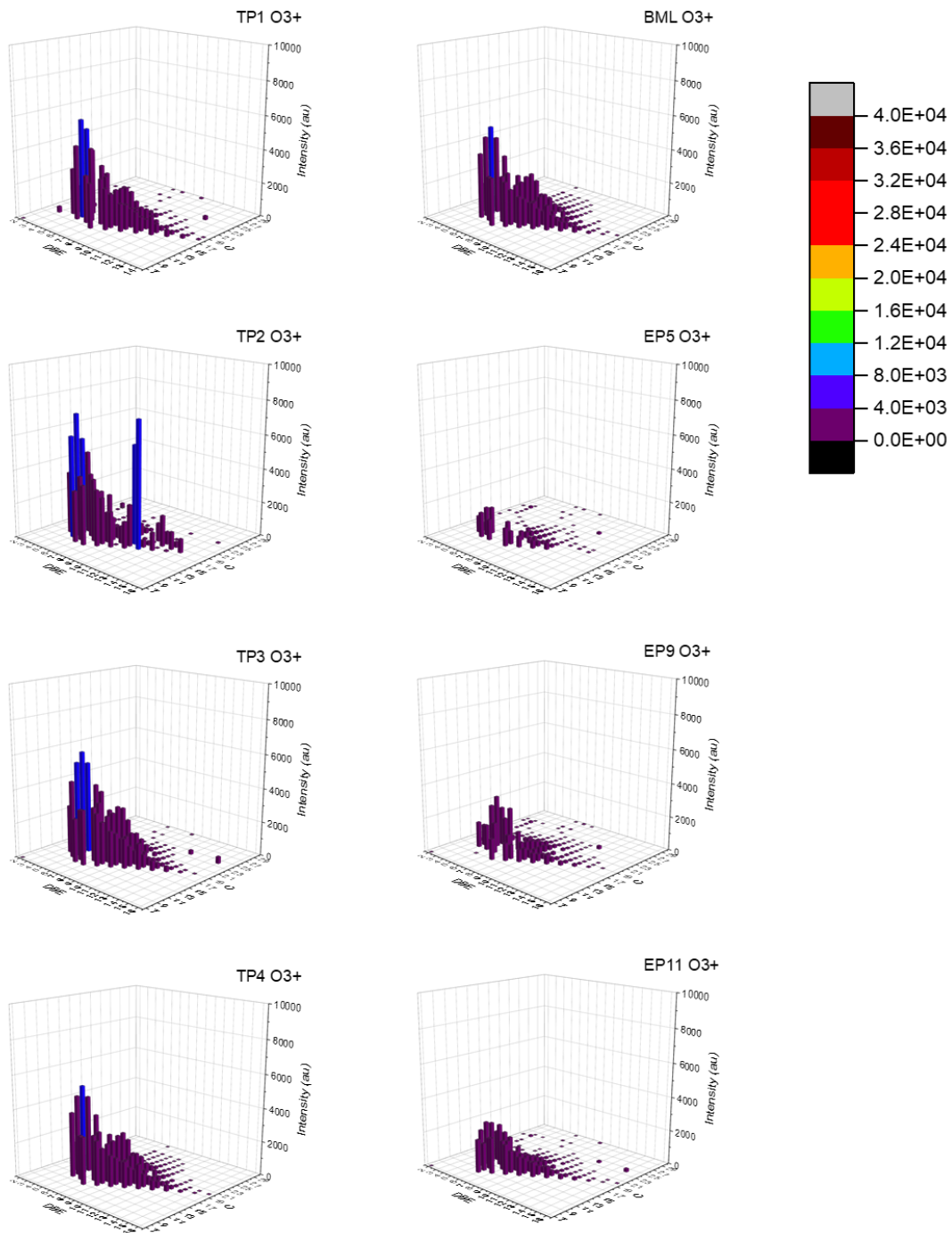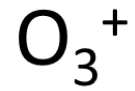
Figure 2-18 O$_3^+$ profiles for fresh (TP1, TP2, TP3, TP4), BML, and aged OSPW (EP5, EP9, EP11). Intensity: 0 – 4 E4 au (bottom to top); DBE (back to front) 2-16; C 7-29 (front to back).
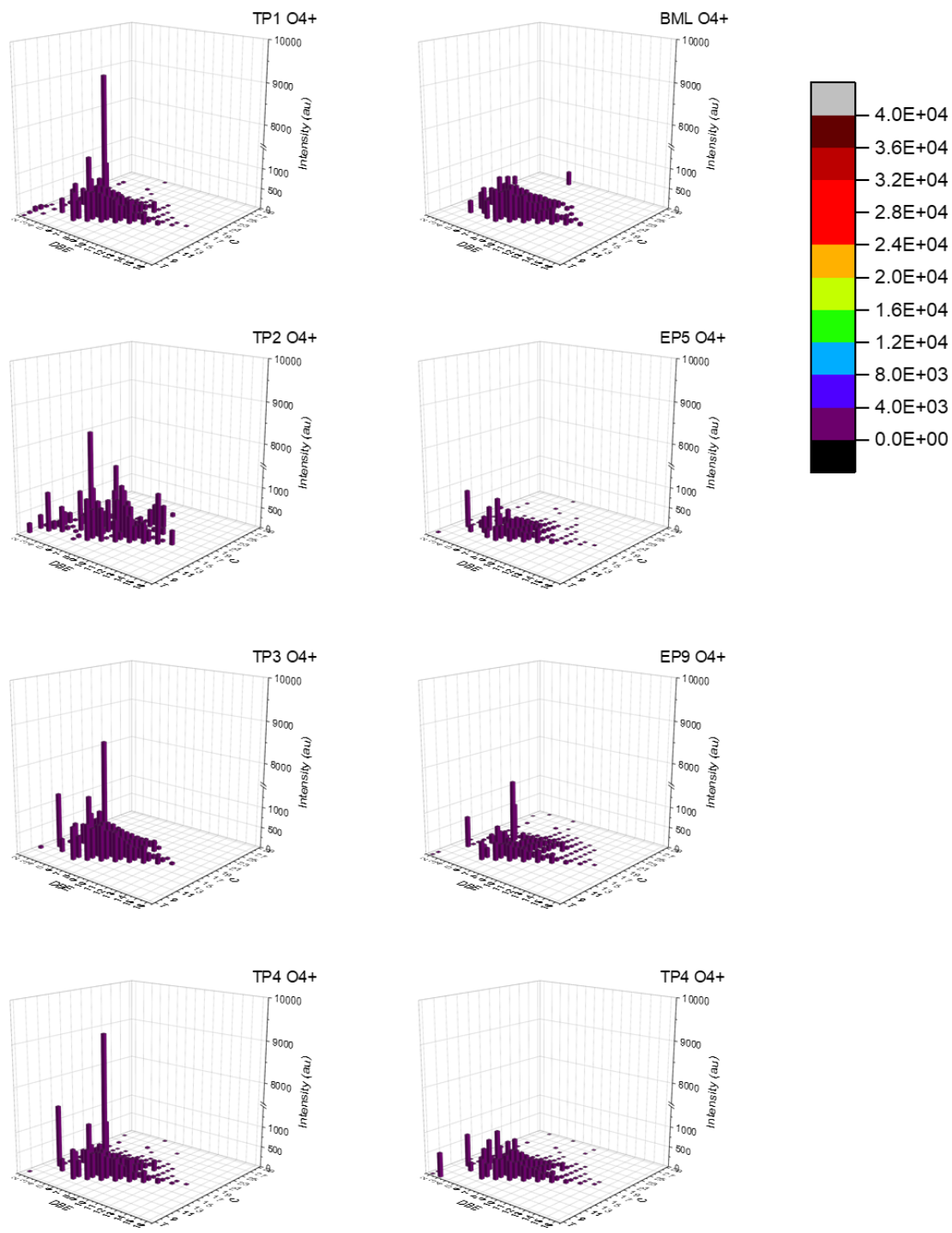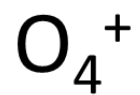
Figure 2-19 O₄⁺ profiles for fresh (TP1, TP2, TP3, TP4), BML, and aged OSPW (EP5, EP9, EP11). Intensity: 0 – 1 E4 au (bottom to top); DBE (back to front) 2-16; C 7-29 (front to back).
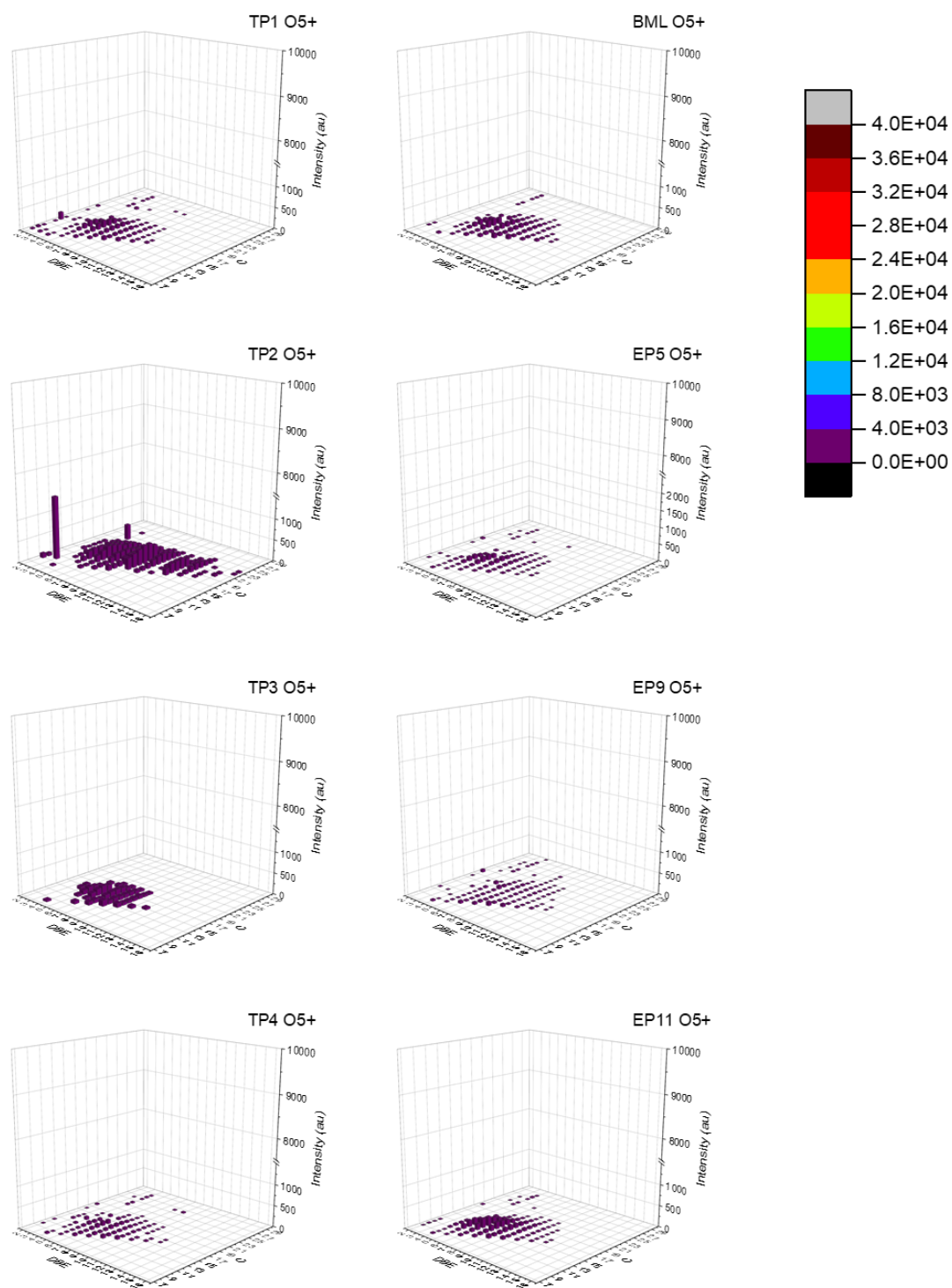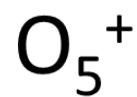
Figure 2-20 O$_5^+$ profiles for fresh (TP1, TP2, TP3, TP4), BML, and aged OSPW (EP5, EP9, EP11). Intensity: 0 – 1 E4 au (bottom to top); DBE (back to front) 2-16; C 7-29 (front to back).
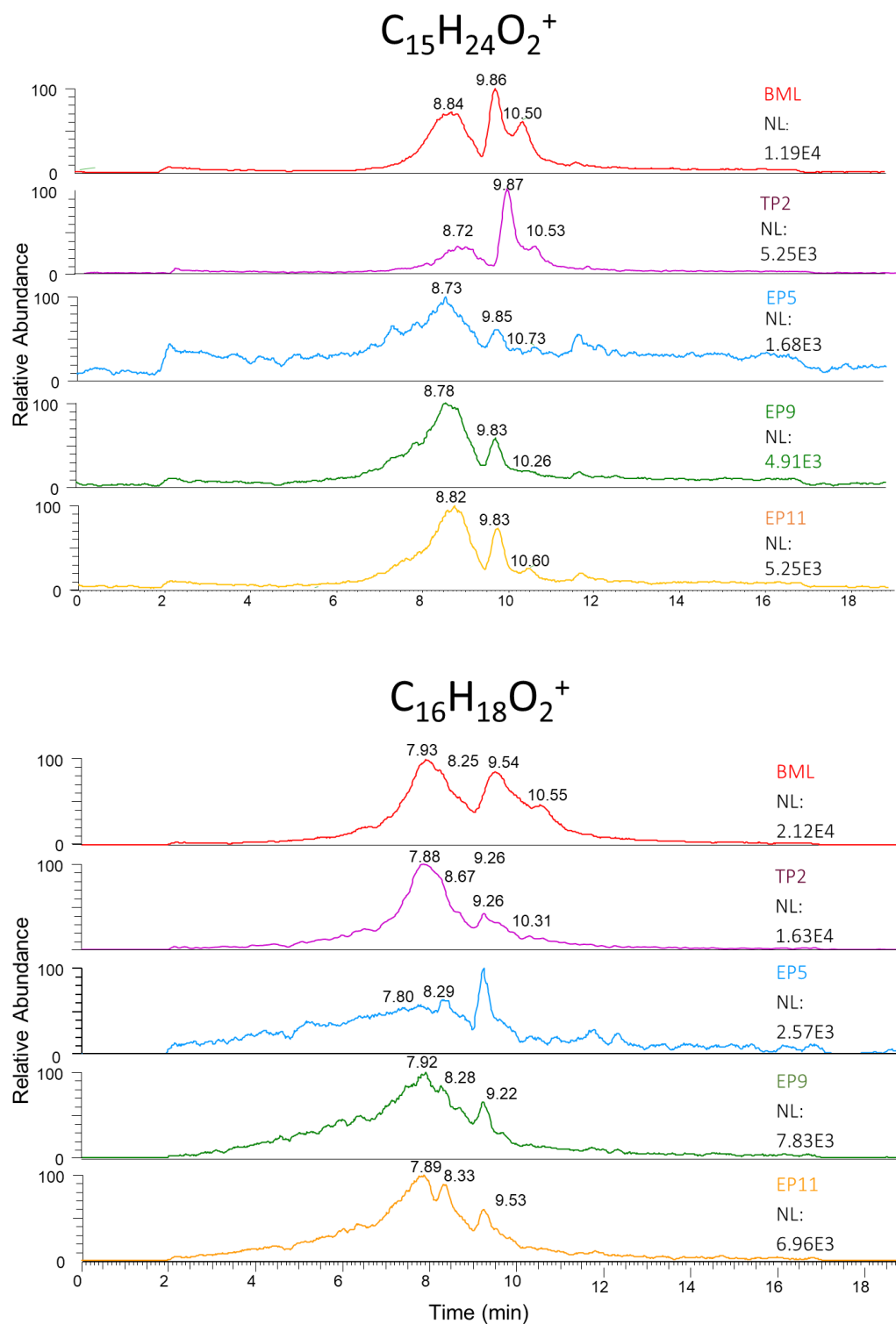
Figure 2-21 Comparison of $C_{15}H_{19}O_2$ and $C_{16}H_{19}O_2$ chromatograms showing isomer distributions of this species in OSPW detected in positive ion mode. BML (red), three aged OSPW samples (EP5 in blue, EP9 in green and EP11 in gold, and one fresh OSPW sample (TP2 in purple).

2.3.3 Principal Components Analyses (PCA)

In addition to the visual observations and simple statistical differences described above, multivariate approaches were also used to identify chemical differences between fresh and aged OSPW. The first PCA (PCA-I) was performed with the aim of identifying the heteroatomic classes whose proportional response differed between fresh and aged OSPW. With negative mode data, principal component 1 (PC1, 65.1%) and principal component 2 (PC2, 20.3%) together accounted for 85.4% of variability between samples (**Figure 2-22A**), and aged and fresh OSPW types were separated on PC1. With strong positive loadings on PC1, the O_3^-, O_5^-, O_6^- and O_xSN^- classes co-varied and were strongly correlated with aged OSPW, while the O^-, O_2^-, and O_4^- classes co-varied and were strongly correlated with fresh OSPW. The scores plot further indicated that EP5 was unlike any other sample, mainly because the SO_x^- classes (**Figure 2-6**) were relatively higher in EP5 (13.9%) than in EP11 (2.7%) or EP9 (2.2%). The O_3^-, O_5^-, and O_6^- classes were positively associated with PC1, consistent with their higher relative contribution in aged OSPW (**Figure 2-5A**). In contrast, O_2^- was negatively associated with PC1 due to its high relative contributions in fresh OSPW (**Figure 2-22B**).

Performing PCA-I with positive ionization mode data (**Figure 2-22B**), PC1 (47.6%) and PC2 (31.9%) together accounted for 79.5% of the variability between samples. Aged and fresh OSPW were again separated on PC1, although a lower proportion of variability was accounted for by PC1 (47.6%) compared to the same analysis with negative mode data (65.1%). Here, the covariance of oxygen-containing classes was pronounced. O_2^+ through O_6^+ co-varied, with negative loadings on PC1, while O^+, SO_x^+, N^+ and $(N)S^+$ classes co-varied and had positive loadings on PC1. This reflects the higher proportions of multi-oxygenated classes in aged OSPW (i.e. O_2^+, O_3^+, O_5^+ O_6^+) as shown in **Figure 2-5D** and **Figure 2-8B**. Higher contributions of O_2^+ and O_3^+ classes also separated EP9 (55.3%) and EP11 (54.6%) from EP5 (48.3%) on PC2, and from all fresh OSPW samples which had lower combined contributions of O_2^+ and O_3^+ classes (32.4 – 43.1%). TP2 was distinguishable from other tailings ponds due to its higher relative contributions of $(N)S^+$ classes (8.25%) compared to other fresh samples (2.3 – 2.4%) and BML (2.13%, **Figure 2-16**). Furthermore, the contribution of O^+ was lowest in EP5 (3.10%) but highest in BML (20.1%), pushing these samples to opposite domains of the scores plot (**Figure 2-22B**).
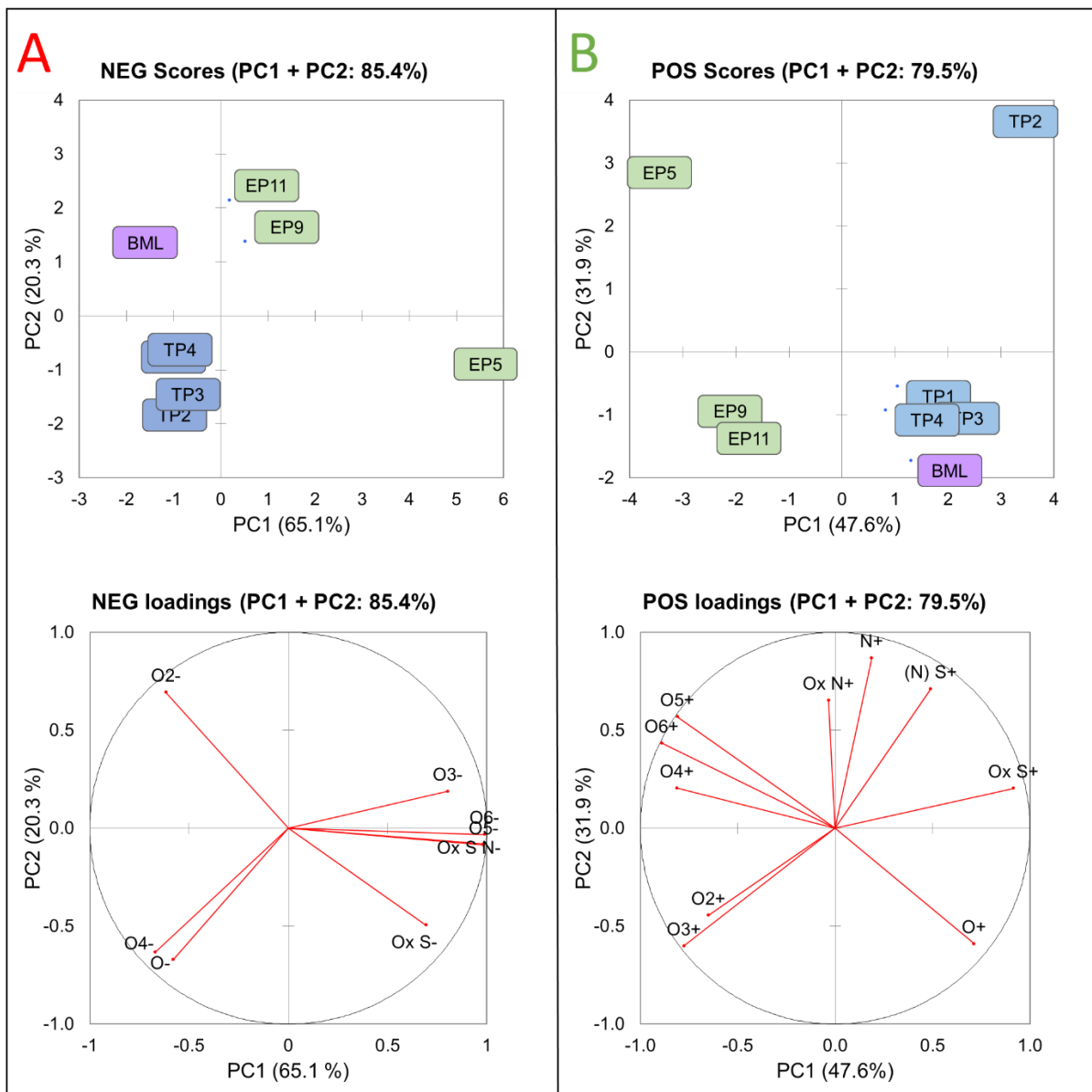
Figure 2-22 Scores and loadings plots for principal components analysis I, based on heteroatomic classes in negative ionization mode (A) and positive ionization mode (B).

In the second PCA analysis (PCA-II) we examined differences at the chemical species level, by including all species from negative (706 species) and positive mode (2090 species) in separate models. As in PCA-I (**Figure 2-22**), aged and fresh OSPW were separated on the scores plots in both models, but curiously not on PC1 which explained most of the variability between samples (i.e., 71 – 75%). Rather, separation between fresh and aged OSPW samples was primarily on PC2 (**Figure 2-23**), which explained only 11 – 14% of the variability between samples. Specifically, in negative mode, PC1 and PC2 explained 74.9% and 11.3% of the variability, respectively (**Figure 2-23A**). The separation of aged and fresh OSPW on PC2 was such that BML and fresh OSPW had positive scores on PC2, while all aged samples had negative scores on PC2. Thus, to identify individual chemical species that contributed most to the variance among aged and fresh OSPW, we focused on species with loadings higher than 0.1, or less than -0.1 on PC2 (i.e., outside the red or green exclusion zones plotted in **Figure 2-23**). Among these 25 chemical species (**Table A-1**), only 2 contained sulfur heteroatoms (i.e., $C_{20}H_{26}$ SO^-, $C_{20}H_{28} SO^-$), and the remainder belonged to O_x^- classes. All O_3^- species had negative loadings on PC2, while all O_4^- species and many O_2^- species had positive loadings on PC2. The O_3^- species identified had C_{14} – C_{15} and 4 or 5 DBEs. Species belonging to the O_4^- class also had C_{14} – C_{15} and 4 or 5 DBEs. The results for PCA-II expand on those of PCA-I in identifying the O_4^- species which distinguish fresh OSPW, and O_3^- species which are conspicuous in aged OSPW.

In positive ion mode, PC1 and PC2 captured 71.2% and 13.8% of the variability, respectively. The separation of aged and fresh OSPW on PC2 (**Figure 2-23B**) was such that BML and fresh OSPW samples had negative scores on PC2, while all aged samples had positive scores on PC2. Only 3 nitrogen-containing species were identified (Table A6), while most of the species were either O^+ species (6 of 22) with 4 – 8 DBEs, or O_2^+ species (10 of 22) with 2 – 8 DBEs. Unlike in the loadings plot for the negative ions, here, the species from all classes were mixed on positive and negative domains of PC2 (**Figure 2-23B**). Taken together, results of both PCA suggest that O^+ species may be oxidized to oxygenated species (e.g., O_2^+) over time.
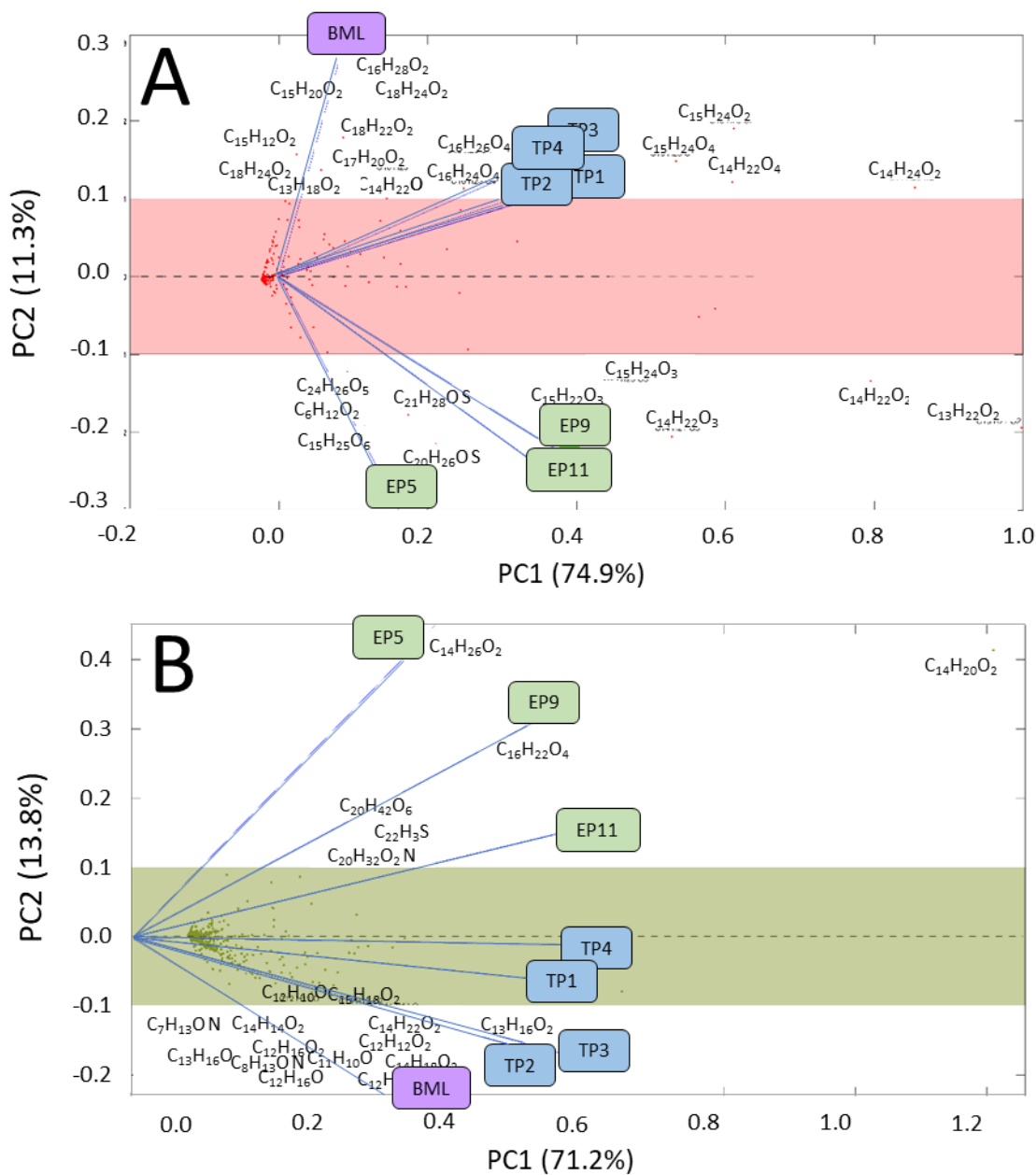
Figure 2-23 Separation of OSPW types by scores and loadings plots of principal components analysis II (PCA-II), based on all species detected in negative (A) and positive (B) ion mode. OSPW types were separated primarily on PC2, thus we focused attention on species with greatest loadings (between -0.1 to 0.1) on the PC2 axis, with the exclusion zone shown as red (negative mode) or green (positive mode). Chemical formulas shown are for the neutral species.

2.4   Limitations and Significance

In an ideal study designed to understand the potential of oil sands EPLs to detoxify OSPW, several experimental ponds containing OSPW would be constructed in replicate and would be monitored over decades, with all physical (including volume added/removed, evaporation/dilution etc.), chemical, and toxicological parameters monitored in a comprehensive, and systematic manner. Such an ideal study was never established and would be too slow to serve any purpose if established now. The current study therefore has limitations but has high environmental relevance at a time when full-scale EPLs are now being established. The major limitations include the cross-sectional design, and the small sample size of experimental ponds which were all created using different combination of OSPW and fine tailings. I have also acknowledged that today's fresh OSPW may be more concentrated than the fresh OSPW assessed here, and that seepage and dilution in fresh OSPW are possible factors that were not controlled here. Nevertheless, I used NA concentrations as a reference and used chloride ion as a conservative tracer which demonstrated that at least, dilution had minimal influence on results presented here.

Here, for the first time, the organic profiles of field samples of various ages were compared using ultrahigh resolution HPLC-Orbitrap mass spectrometry. Samples representing fresh and aged OSPW were compared with the aim of predicting how the chemistry of oil sands EPLs, including the most acutely toxic chemicals, may change in the future. Despite the limitations, this study provides important evidence that NAs and some of the toxic sulfur- and nitrogen-containing chemical classes can slowly degrade under environmentally relevant conditions. Real-world monitoring of BML and future EPLs should confirm that these classes decline at a rate which will allow reclamation of these waters in a reasonable timeframe. Nevertheless, the other implication is that residual toxicity that has been detected in these same experimental ponds is likely due to other chemical classes which degrade more slowly,[21] for example polar oxygenated species such as the O_2^+ class. Ajaero *et al.* (2019) also suggested O_2^+ species had slower rates of removal compared to O_2^- species in constructed wetlands.[22] Moreover, previously studies showed that the O_2^+ class is present in the acutely toxic fraction of BML.[7]

Future consideration and experiments must be given to the mechanisms by which NAs and other toxic organics were degraded in these experimental ponds. As discussed above,

microbial biodegradation of OSPW NAs has received much historic attention, but a recent laboratory study has shown that photolysis at environmentally relevant wavelengths could be a significant fate process of most organics in OSPW, particularly if water is not turbid and EPLs are not too deep.[14] Notably, the laboratory photolysis experiments showed selective degradation of NAs, with a concomitant relative increase in the abundance of oxidized NAs. The study also recorded that while most organics in positive mode were relatively persistent to photolysis, particularly the O_x^+ classes; all these characteristics are hallmarks of what was observed here in aged OSPW. The experimental ponds monitored in this study are relatively shallow and have high water clarity compared to BML, which is very deep and has seasonal variations in turbidity. These physical differences mean that the fate of OSPW in BML, or of other future full-scale EPLs, will not necessarily follow the same chemical or toxicological trajectories observed in these relatively small experimental ponds. [14] As suggested by Challis *et al*. (2020),[14] the design of future EPLs should consider maximizing the potential of natural photolysis by minimizing lake depth and maximizing factors that contribute to water clarity.

2.5   References

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Chapter 3   Biodegradation in Base Mine Lake: a comprehensive analysis of the *in situ* biodegradation of dissolved organic acids in the first oil sands end pit lake.

3.1 Introduction

The two main methods by which oil sands are recovered include open-pit mining (ores $\leq$ 70 m below ground surface) and one type of *in situ* method called steam assisted gravity drainage (SAGD). Although only 20% of reserves are accessible from the surface, open-pit mining is currently the primary method of bitumen recovery.[1] The physical structure of the ore includes a layer of oil that encases sand grain and is enveloped in a film of water. Extraction occurs by decreasing the viscosity of the bitumen and increasing the pH by adding hot 'caustic' or basic water (pH > 8, 40 – 55°C). This step makes use of the natural surfactant-like properties of bitumen. By ionizing endogenous organics in the ore, these surfactants become water soluble carboxylates and sulphonates which attach to air bubbles and enable the separation of bitumen from the sand.[2] The air bubbles accumulate in a froth which is skimmed, diluted, and then separated from the water. From the froth, a series of upgrading and refining steps are required to produce the synthetic crude oil which is sold to market.

The Athabasca River and its tributaries provide the water used for bitumen extraction. However, following extraction the resultant water is sent to containment units called tailings ponds to be recycled. Oil sands process-affected water (OSPW) is not released directly into the environment due to toxicity from its elevated concentrations of salts, metals, and organic chemicals. To date, an estimated 1 billion m^3 of OSPW is held in tailings ponds to reduce of exposure to native animals and the environment.[3]

Toxicity of OSPW in animals has been thoroughly reviewed. [4–7] Animal studies have demonstrated OSPW produces toxic effects in vertebrates (amphibians[8,9], birdss[10], fishes[11–18], mammals[19,20]) and invertebrates.[21,22] Exposure to OSPW presents a diverse array of effects and toxicity occurs through various modes of action including endocrine disruption, membrane disruption, and oxidative stress.[23–30] To better understand the root causes, studies have narrowed their focus to assess the impact dissolved organic species alone. MacKinnon and Boerger were the first to attribute the primary toxicity of OSPW to polar organic carboxylic acids and other dissolved organics extracted at a low pH following a report by Zennon Environmental in 1986.[31] Thereafter naphthenic acids (NAs) became the topic of focus for OSPW toxicity studies. In

performing a toxicity identification evaluation (TIE), Verbeek *et al.* found that most of the acute toxicity in OSPW could be eliminated by removing components of OSPW which precipitated at a pH of 2.5.[32] In recent years, studies of OSPW toxicity (including effects directed analysis, EDA, using a variety of organisms) have been hindered by the inability to efficiently extract large volumes and/or reproducibly generate toxicologically relevant fractions.[33] To this end, Bauer *et al.* compared 5 resin types to decide which was best used in their large scale SPE method. Their preparative fractionation method enabled the extraction of 200 L of OSPW in two stages which isolated the basic fraction (pH = 12) from the acidic fraction (pH = 2) with large columns and associated apparatus (plungers, hand-mixers, etc.). Three concentrated fractions of basic neutral (F1), basic polar (F2), and acidic polar (F3) organics were generated with a high recovery (96.2%). Mass spectra (ESI-MS) overlapped well with those of previous studies since for each fraction, majority of compounds were between 200 to 400 *m/z*. They noted a pH and polarity dependence on the oxygen-content of the fractions, where Fraction 3 (polar acids) had the highest contributions of multi-oxygenated dissolved organics.[34–36] Morandi *et al.* were the first to describe two toxic fractions in OSPW.[37] The EDA determined OSPW toxicity was apportioned to two fractions: one comprised mainly of NAs, the other was a mixture of polar neutral organic classes including species in the O_2^+, O^+, ON^+ and OS^+ classes.[37]

In 1989, a series of pilot ponds were set up to study ways in which tailings could be naturally remediated. The results of this study revealed that in 1 – 2 years the acute toxicity of OSPW had been naturally attenuated.[31] This study was the foundation for the "wet landscape" approach of OSPW reclamation. The leading strategy employs end pit lakes (EPL) whereby OSPW will be stored in evacuated mines until toxicity is naturally attenuated. Base Mine Lake (BML) is the first (and only) oil sands EPL. This pilot scale project was commissioned in 2013 and founded on two premises: 1) the progressive release of tailings pond water after treatment using EPL technology and 2) the subsequent approval of approximately 30 EPLs.[38] Although there are questions about what reclamation in this region looks like, some consensus lies in the idea of decreased aquatic toxicity over time.[39]

Tailings ponds have distinct microbial communities which may drive biodegradation of organics. The biodegradation of NAs is largely an aerobic process,[40–42] and using oxygen as an electron acceptor is the least energy intensive for microbes. An early study reported evolution of CO_2 in experimental microcosms at room temperature and suggested 50% and 70% removal of

extracted OSPW organic acids and commercial naphthenic acids (c-NAs) after 24 and 16 days, respectively.[40] Han *et al.* estimated the half-life of OSPW NAs (44 – 240 days) to be > 5× longer than c-NAs.[43] Although there is an understanding of the persistence of NAs, knowledge about the biodegradation of sulfur and/or nitrogen-containing organics in OSPW is limited. Few studies have reviewed the ability for endogenous microbes in tailings ponds to degrade the complex mixture of chemicals in OSPW. In 2016, the total active tailings area was 257 km^2 (including 103 km^2 of tailings pond structures) - a 10-fold increase in just 30 years. Without sufficient treatment and subsequent release of these waters, these numbers will continue to grow at a steady rate. Thus, the objective of this project was to assess the feasibility of the EPL strategy by examining *in situ* microbial biodegradation of dissolved organics in by endogenous microorganism in BML OSPW.

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3.2 Methods

3.2.1 Chemicals and reagents

Unless otherwise stated, all materials were obtained from Fisher Scientific (Edmonton, AB) including solvents such as acetic acid, methanol (MeOH) and water. The internal standards, D$_{23}$-lauric acid (tetradecanoic acid, Aldrich, St. Louis, MO) and D$_3$-progesterone (CIL laboratories, Andover, MA) were used in negative and positive mode, respectively. Refined Merichem oil was purchased from Merichem (Houston, Texas, USA).

3.2.2 Description of BML

The formal description of an oil sands end pit lake is "an area where overburden and oil sand has been removed and is then filled with fluids prior to closure. {It} contains water from oil sands extraction or freshwater and may or may not contain {tailings}" (Personal communication, Mark MacKinnon). Base Mine Lake replaced what was previously the West-in-pit tailings pond at Syncrude Canada Limited. From 1995 to 2012, approximately 186 million m^3 of fluid fine tailings (FFT) from Mildred Lake Settling Basin (MLSB) was transferred into the enclosure by pipeline.[44] A pump was then installed to bring water freshwater from Beaver Creek Reservoir which would slowly dilute the water cap over time. BML was commissioned on December 31st of 2012. At this time, BML comprised 40 million m^3 of OSPW (depth ~ 5 m) overlaying 200

million m^3 of fine tailings (depth ~ 40m). The total surface area of BML is approximately 8 km^2.[45] The upper layer of the water cap in BML is aerobic, but dissolved oxygen levels decrease progressively from 1 m to the interface of FFT.[46,47] Winds, waves, and natural turnover contribute to aeration in the oxic zone in the summer. Ice cover in the winter limits aerobic processes.[44]

3.2.3 Sample collection

BML OSPW was collected by Syncrude Canada Limited in August of 2013. Samples were taken 50 - 100 cm below the surface with a Van Dorn water sampler deployed from a floating barge and placed into 20 L white high density polyethylene pails (Personal Communication, Warren Zubot). OSPW was stored in sealed pails at 4°C until transferred to microcosm experimental flasks in November 2015.

3.2.4 Microcosm incubations

Three treatments were set up in triplicate to assess the aerobic biodegradation of all detectable dissolved organic compounds by endogenous microbes in BML OSPW: (1) untreated BML OSPW (BML), (2) BML OSPW with 50 mg/L Merichem NAs added (BML + Merichem), and (3) chemically-treated negative control (control). Microcosms were established in 500 mL Pyrex glass baffled flasks (Sigma-Aldrich, Oakville, Canada). Before use, flasks and foam plugs were sterilized in an autoclave at 200°C for 60 min. All treatments began with an initial volume of 300 mL BML OSPW. The Merichem-added treatment served as a positive control. [40,41] In these flasks 300 μL of a 50 mg/L Merichem stock solution was added.

Negative (killed) control flasks were established with BML OSPW which was both autoclaved (twice at 121°C for 45 min each with 24 hours in between) and chemically treated with 0.02% sodium azide (NaN$_3$). This aggressive protocol was applied to controls due to observations in the initial trial of the experiment (Fall 2014, data not shown). The same study design was followed for 90 days but negative controls had only been heat treated by autoclaving. [42,48,50]

Microcosms were placed on an orbital shaker at 200 rpm at room temperature (~21°C) throughout the course of the experiment). Foam plugs were used to close the flasks in order ensure sufficient levels of oxygen would be dissolved in OSPW.[42,43] Dissolved oxygen

concentration averaged 6.7 mg/L on day 0 of the experiment. All flasks were covered with aluminum foil to minimize abiotic biodegradation (by UV radiation). Evaporative loses were measured gravimetrically and were calculated to be 8.6×10^{-3} % day^{-1} (0. 42 g day^{-1}). It was estimated that at least 20% of microcosm volume would be lost due to evaporation after 52 weeks. Due to the limited volumes used in this lab setting (300 mL), no more than 4 mL was withdrawn per week for any flask in the study.

      Microcosm studies often follow two regimes for sampling: a period where aliquots are removed regularly and often (when microcosms are first established), followed by a decrease in the frequency of sampling and greater intervals between time points.[52] Aliquots (maximum of 4 mL: 1 mL for Orbitrap analysis; 3 mL for Microtox Toxicity tests) were removed once per week for the first 49 days; biweekly until day 77; then monthly until day 272. The sampling regime in this study was designed after an initial 90 day assessment of the rate of change of dissolved organics in BML OSPW (2014 - data not shown). Sampling frequency can be reviewed in (**Figure 3-1**). Samples were stored preserved with methanol (50/50 proportions) at 4°C until analysis.
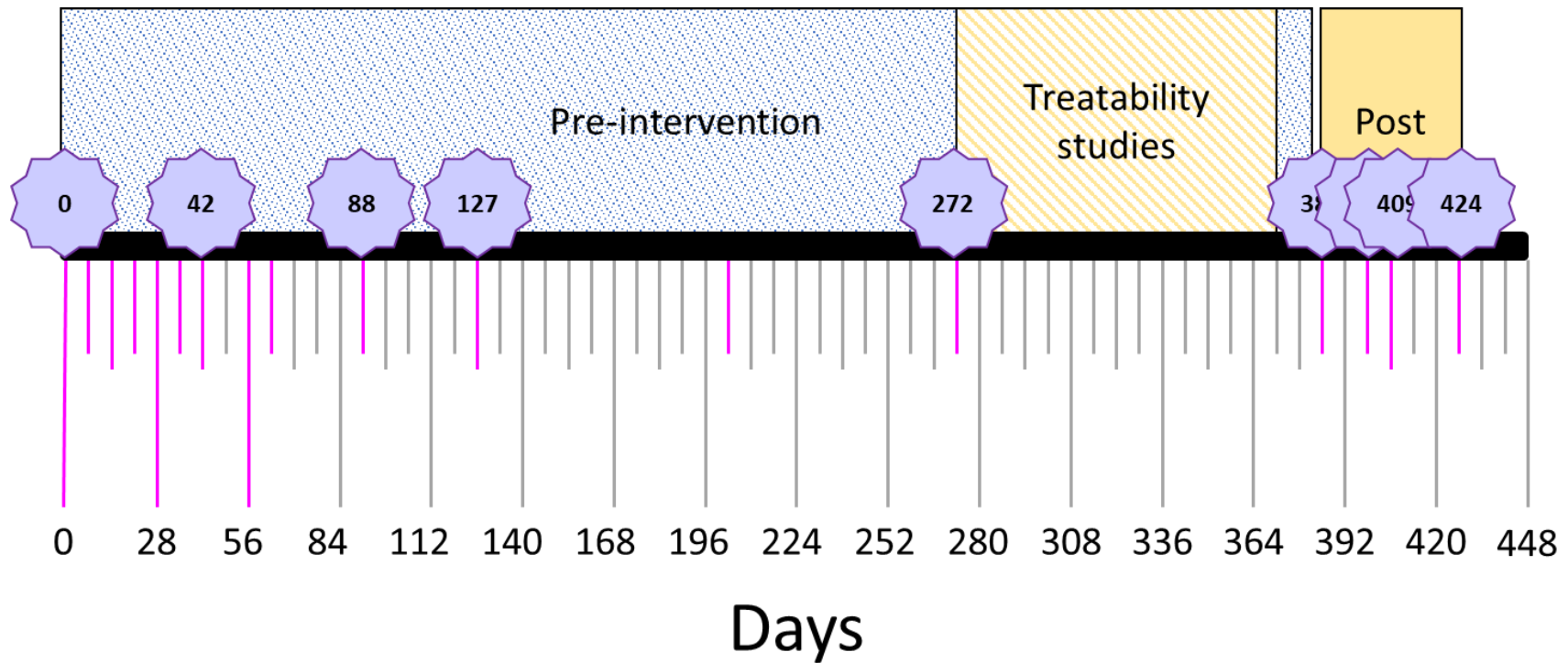
Figure 3-1 Timeline for microcosm sampling schedule showing days on which aliquots were taken as magenta lines and days on which no sampling occurred as faint grey lines. All lines are spaced at weekly intervals (7 days) with 28-day increments labelled. Phases of the experiment are labelled as pre-intervention (Day 0 to 389); treatability studies (Day 272 to Day 371) and post-intervention (Day 389 to 242). Days chosen for Orbitrap analyses are indicated in purple stars.

3.2.5 Treatability studies

Initial screening of biodegradation in microcosms revealed little change in intensities of NA species. Thus, tests were established to evaluate factors limiting biodegradation in BML OSPW following day 272. Experiments were conducted in 500 mL duplicate/triplicate bottles per treatment for up to 14 days on a rotary shaker (T ~ 21°C, 200 rpm). The initial volume in all bottles was 300 mL. Bottle caps were fitted with rubber septa to allow for both headspace and liquid sampling. Dissolved oxygen concentrations were measured in each treatment before day 0, and ranged between 6.10 to 7.84 mg/L – enough for aerobic biodegradation (i.e., > 4.76 mg/L).[53] Earlier microcosm experiments indicated initial dissolved oxygen concentrations in microcosms using OSPW from West-In-Pit decreased from 6.8 mg/L and reached equilibrium at 5.4 mg/L.[54]

Liquid samples (4 mL) were collected via syringe to track dissolved organic carbon (DOC) using the Shimadzu Model TOC-L$_{CPH}$ ASI-L Shimadzu autosampler. The non-purgeable organic carbon method was used for DOC analysis. Headspace CO_2 tracked mineralization of dissolved organics. A series of studies were established with various combinations of the following treatments (**Figure 3-2**): Untreated BML OSPW (BML); BML OSPW with mature-fine tailings (MFT) added (Bioaugmentation); BML OSPW with 5% (v/v) Bushnell Haas media (BHM, Biostimulation); BML OSPW with added 5% (v/v) BHM and 98 mg/L acetate (Acetate); and BML with added MFT which was autoclaved and chemically treated with 0.2% sodium azide (killed control). BHM was modified from that of Brown *et al*. [50] and contained 12.7 mM of each N and P due to the addition of each of the following in 1 L of MilliQ water: 0.2 g $MgSO_4$; 0.02g $CaCl_2$; 1.0 g KH_2PO_4; 1.0 g Na_2HPO_4; 1.0 g K_2HPO_4; 0.5 g $(NH_3)NO_3$; 0.002g $FeCl_3$; 0.0018 g $MnSO_4 \cdot H_2O$; and 0.5 g $(NH_3)_2SO_4$. For Acetate treatments, 28 mL of a 1083.9 mg/L stock solution were added to 280 mL of BML OSPW. The stock solution was prepared by the addition of 0.625 g of $NaCH_3COO \cdot 3H_2O$ to 25 mL of Optima water. Changes in acetate concentration were monitored by ion chromatography. All parameters measured here (DOC, CO_2, Acetate) are described in the **Appendix B**. The results of these tests were applied as interventions to microcosms on day 389.
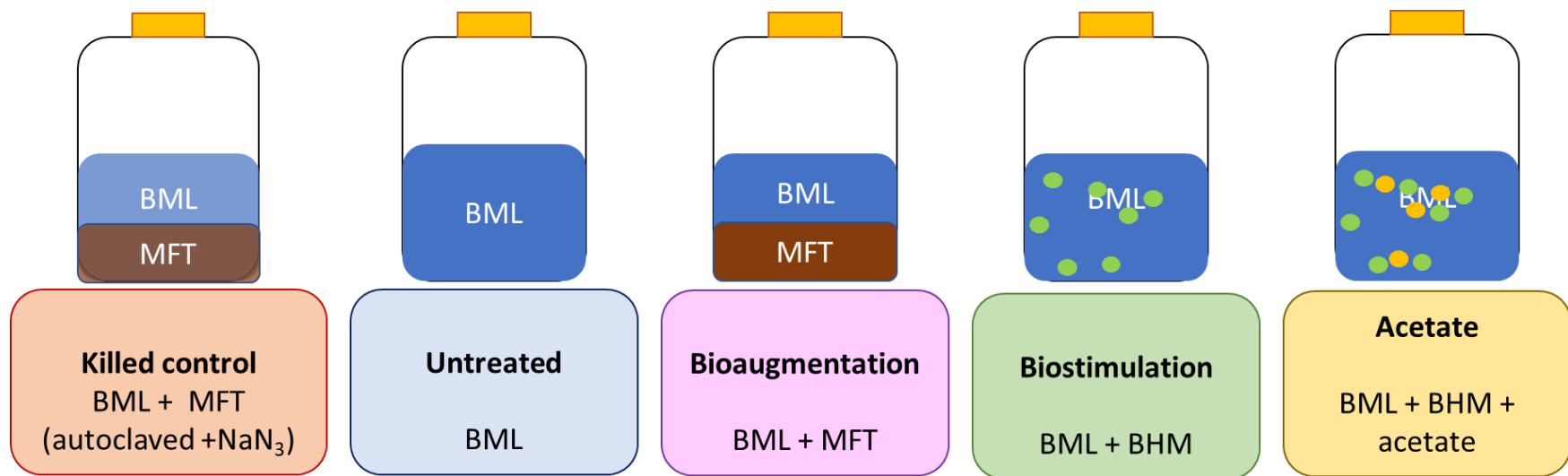
Figure 3-2 Schematic describing the various treatments used for the treatability studies (between day 272 and 389 of the experiment). All treatments were performed in duplicate or triplicate. Three studies were performed in series with the displayed treatments: Killed control (autoclaved and chemically-treated with 0.02% sodium azide); untreated BML OSPW; Bioaugmentation treatment- BML OSPW with added mature fine tailings (MFT); Biostimulation treatment- BML OSPW with 5% v/v added Bushnell Hass nutrient media (BHM); and an acetate treatment with 5% v/v added BHM and 98 mg/L acetate.

3.2.6 Orbitrap sample preparation and analysis

From an aliquot (2 mL) suspended solids were removed by syringe filtration (0.45 μm nylon) and added to an equivalent volume of Optima grade MeOH to prevent further biodegradation prior to analysis. Each sample was then transferred to 2 mL glass vials and spiked with 100 μL of the combined internal standard solution. Internal standard concentrations in the vialed samples were 1 ppm D_{23}-lauric acid and 0.01 ppm D_3-progesterone.

Reversed-phase chromatography was paired with a hybrid linear ion trap-Orbitrap mass spectrometer (Orbitrap Elite, Thermo Fisher Scientific, San Jose) and operated using a modified version of the method of Pereira *et al.*[55] with atmospheric pressure chemical ionization in negative ionization (-) mode. Samples were also run in positive mode but do to sampling error the results could not be interpreted and reported. Nominal resolution was set to 240 000 at *m/z* 400 and mass spectral data was acquired in full scan between 100 and 500 *m/z*. Capillary temperature was 300 °C, discharge current was set to 4.5 μA, and sheath, auxiliary and capillary gas flow was set to 20, 15, and 3 (arbitrary units), respectively. Chromatographic separation was performed on a Hypersil Gold C18 Selectivity Column (Thermo Scientific, Edmonton, AB, 50 × 2.1 mm, particle size 1.9 μm), using an Accela HPLC system (Thermo Scientific, San Jose, CA). The column was maintained at 40°C and a flow rate of 0.5 mL/min was used with 10 μL injection volumes. Initial mobile phase composition was 95% A (0.1% acetic acid in water) and 5% B (100 % MeOH) for 30 seconds followed by linear gradient ramp to 90% B over 10 min, and a final ramp to 99% B over 5 min. Percent composition of B was then decreased to 5% over 2 min 30 s and held for 2 min for column re-equilibration.

The O_2^- class was selected as a subclass of organics to allow a quick and focused review of bacterial biodegradation. It was estimated that following changes in O_2^- species could enable the confirmation of an active bacteria community in BML OSPW. Given constraints in resources (namely instrument and data analysis time) O_2^- was chosen to allow for informative and timely review of the status of the microcosms.

3.2.7 Quantitative analysis

Xcalibur™ software was used for data acquisition, and mass spectral peak intensities were for the semi-quantitative review of the concentrations of dissolved organic species. The total spectrum was generated in the retention window in which most species eluted (7-13 min in

negative mode). To assign empirical formulas to detected ions by exact mass, the following restrictions were set: minimum (C-5, H-10), maxima (N-1, S-1, O-6) and the maximum error tolerance for formula prediction was 5 ppm. Using this method, all compounds of a given exact mass are termed 'species' and defined by a distinct chemical formula, $C_vH_wO_xN_yS_z$. Each sample from Xcalibur™ was exported to Excel ® for data processing thereafter. The peak intensity of the internal standard was found (manually in Xcalibur™) and used to adjust the intensities of all other species detected. Species were compared to those in the blank using the VLOOKUP function. Only those species with an intensity at least 3 times greater than the blank were included in subsequent analyses. Intensities were also adjusted for evaporation by gravimetric mass according to the loss on a given day. Gravimetric evaporative loses were applied to the assessment of mass spectral intensities as a percent. Prior to removing an aliquot from each microcosm, the mass of that flask was taken. Later, based on the expected mass, the intensities were adjusted.

Separation of species by heteroatomic class was done manually: Using the 'Text to Columns' function, formulae were split at a 'Fixed Width' and any atom which was not Carbon or Hydrogen was retained. Species were grouped into 'classes' based on heteroatom content and ionization mode: O^-, O_2^-, O_3^-, O_4^-, O_5^-, O_6^-, SO^-, SO_2^-, SO_3^-, (N) S^-, and O_x N S^-. To compare species detected between treatments on a given day, all species were compiled, duplicates removed, and the VLOOKUP function was applied. Three treatments (three replicates per treatment, n = 3) were analyzed across 9 experimental days resulting in a total of 81 samples to be processed manually in Xcalibur™.

3.2.8 Statistical analyses

All statistical analyses were performed in OriginPro 2019b (Student version). For each heteroatomic class, the differences between the average internal standard adjusted intensity values on Day 0 (only) were compared amongst treatments using ANOVA with Bonferroni post-hoc pairwise analyses. Rate of change over time was analyzed by using the internal standard adjusted intensities for the sum of all species in heteroatomic classes. Linear regression of intensities over time was analyzed assuming first order kinetics. Intercepts and slopes were used to calculate the percent change per day for each heteroatomic class/species by Equation 3-1 below:

$$(3-1)\ln\frac{C_0}{C_t} = -kt \ x \ 100\% \ [1]$$

Changes in Carbon number and double bond equivalents (DBE) for a given heteroatomic class was summarized by heat maps. Species across all samples were aligned to create Pivot Tables in Microsoft ® Excel ® for Office 365. The Pivot tables were exported into Origin2019b for heat map generation.

3.2.9 Microbial community analysis

Microcosms were vacuum filtered onto pre-sterilized 0.2 µM Nylon filters (Merck Millipore Ltd, Etobicoke, ON). DNA extractions were performed using the protocol suggested in the FastDNA™ Spin Kit for Soil (MP Biomedicals).

DNA library prep and sequencing were performed by RTL Genomics (Lubbock, TX). The V4 hypervariable regions of microbial 16S rRNA genes were amplified using the forward primer constructed with the (5'-3') Illumina i5 sequencing primer (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and the 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') primer.[56] The reverse primer was constructed with the (5'-3') Illumina i7 sequencing primer (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) and the 806R (5'-GGACTACVSGGGTATCTAAT-3') primer.[57] Amplifications were performed in 25 µl reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1µl of each 5µM primer, and 1µl of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, California) under the following thermal profile: 95°C for 5 min, then 35 cycles of 94°C for 30 s, 54°C for 40 s, 72°C for 1 min, followed by one cycle of 72°C for 10 min and 4°C hold.

Products from the first stage amplification were added to a second PCR based on qualitatively determined concentrations. Primers for the second PCR were designed based on the Illumina Nextera PCR primers as follows: Forward (5'- 3') – AATGATACGGCGACCACCGAGATCTACAC [i5index]TCGTCGGCAGCGTC and Reverse (5'- 3') – CAAGCAGAAGACGGCATACGAGAT[i7index]- GTCTCGTGGGCTCGG. The second stage amplification was run the same as the first stage except for 10 cycles. Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was size selected in two rounds using SPRIselect Reagent (Beckman Coulter, Indianapolis, Indiana) in a 0.75 ratio for both rounds.

Size selected pools were then quantified using the Qubit 4 Fluorometer (Life Technologies) and loaded on an Illumina MiSeq (Illumina, Inc. San Diego, California) 2x300 flow cell at 10 pM. Sequences were clustered into operational taxonomic units (OTUs) and the OTUs were then assigned to taxa via the UPARSE pipeline.[50] The RDP training set v16 was used to perform taxonomy annotations (with the SINTAX algorithm).[51] The community matrix was normalized with the DESeq package.[52]

3.3 Results and Discussion

3.3.1 Pre-intervention aerobic degradation screening

The use of commercial NAs enabled the review of the biodegradation capabilities of bacteria endogenous to tailings.[61–64] Early studies revealed that these mixtures were more labile than the NAs found in tailings and OSPW,[40,48,65] therefore the treatment with BML and added Merichem (hereafter Merichem) treatment served as positive control. It should be mentioned here that while Merichem NAs serve as a good reference for cross-study comparison, they O_2^- species contained therein are not identical to those in BML OSPW. At the time of this study, Merichem NAs were the best positive control available as they have been tested in multiple studies. Summary of all changes in intensities for the sum of all heteroatomic classes can be seen in Appendix B, **Figure B-1** – **Figure B-7**). Initial concentrations of O_2^- species in BML OSPW were 23.1 mg/L (**Figure B-7**). The addition of 100 mg/L Merichem NAs increased the concentration by about 4 times in the positive control treatments. The elevated intensities of species with $C_{11} – C_{14}$ and 2 or 3 DBEs is typical of commercial NAs (**Figure 3-3A**) and consistent with previous reports.[66] ANOVA with Bonferroni post-hoc analysis determined of the sum of intensities of O_2^- species on day 0 confirmed the Merichem treatments were significantly higher than in both BML and killed controls ($p < 0.01$, $F < 0.01$, $df = 2$). Although the control appeared to have a higher intensity than BML, these treatments were not different ($p = 0.37$).

In the first 389 days, the Merichem treatment had a significantly change of -0.10%/day (Table B-1, $R^2 = 0.68$, $p = 0.04$). During the same timeframe BML ($p= 0.15$) and negative control treatments ($R^2 = 0.28$) showed non-significant decreases (-0.04% and -0.11% per day, respectively, $p > 0.05$). Linear regression analyses were then performed for those 55 unique species detected in all flasks on all pre-intervention days in Merichem treatments.
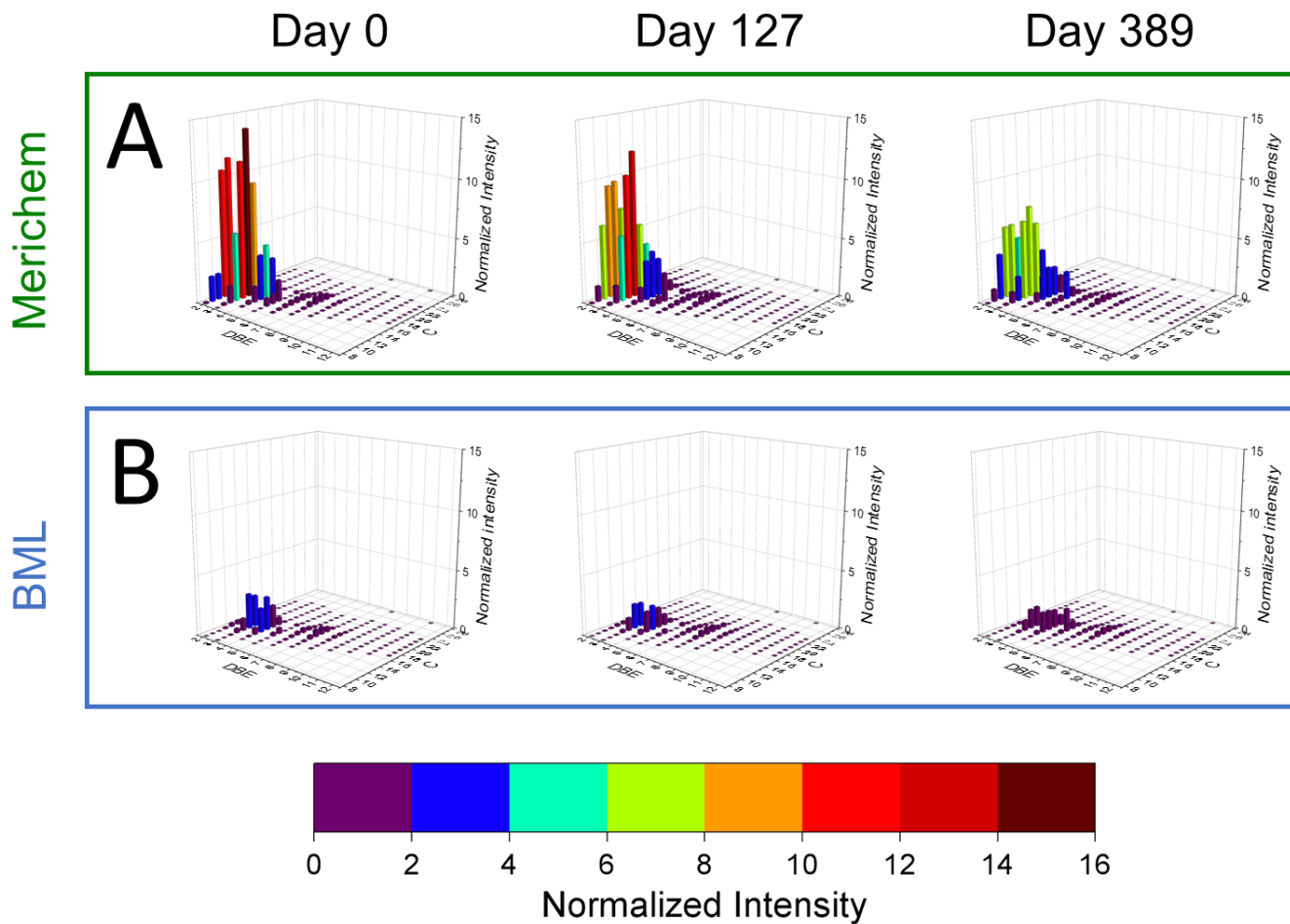
Figure 3-3 Species profiles for the O₂⁻ in BML (bottom) and Merichem (top), for the average of day 0, 127 and 389. Each plot shows distribution by carbon numbers (8 to 30), DBE (2 to 12), and the internal standard adjusted intensity.

Table 3-1 Summary of simple linear regression analyses of all heteroatomic classes detected in negative ion mode for untreated BML (n = 3).

Sample	Class	Pre-intervention slope	p-value	Daily change (%)	Post-intervention slope	p-value	Daily change (%)
BML	O^-	6.63E-04	0.49	0.07%	-1.20E-02	0.47	-1.20%
	O_2^-	-3.71E-04	0.42	-0.04%	-3.24E-02	0.03	-3.24%
	O_3^-	-4.15E-04	0.39	-0.04%	-1.45E-02	0.32	-1.45%
	O_4^-	-1.06E-04	0.85	-0.01%	-1.01E-03	0.95	-0.10%
	O_5^-	2.05E-04	0.77	0.02%	7.71E-03	0.55	0.77%
	O_6^-	1.38E-03	0.26	0.14%	7.13E-03	0.66	0.71%
	$S\,O^-$	4.57E-03	0.04	0.46%	-2.16E-02	0.14	-2.16%
	$S\,O_2^-$	-5.61E-04	0.32	-0.06%	-3.29E-02	0.05	-3.29%
	$S\,O_3^-$	1.76E-04	0.63	0.02%	-6.64E-03	0.61	-0.66%
Merichem	O_2^-	-1.00E-03	0.04	-0.10%	-3.08E-02	0.15	-0.26%

Analyses were performed on the sum of the internal standard adjusted mass spectral intensities of species pre- and post-intervention. Pre-intervention regression analysis was performed using days 0, 42, 88, 127, 272 and 389; post-intervention regression analysis was performed using days 389, 402, 409 and day 424. An F-test compared the pre- and post-intervention regressions for each heteroatomic class to review if the models were different (Prob > F, 0.05). Classes with significant slopes ($p \leq 0.05$) are highlighted in blue. Heteroatomic classes which returned a significant p-value for the comparison of pre and post-intervention regression analyses are labeled as responsive to intervention (Y), otherwise (N).

Only 3 species with had significantly decreasing slopes ($p \leq 0.05$): C_8 3 DBE, C_{11} 2 DBE, and C_{12} 4 DBE (data not shown). The prevalence of simpler chemical structures of commercial O_2^- species are also known to lend in faster rates of removal by indigenous microbes compared to bitumen-derived organics.[49,67,68] Commercial mixtures have unsaturated species, and these are easily degraded.[67] They are generated from petroleum and typically have not undergone significant amounts of biodegradation.[69] Degree of unsaturation, aromaticity and alkyl substitution also impart recalcitrance to dissolved organic compounds. It was therefore not surprising that no changes were observed in the pre-intervention timeframe.

Heat maps enable a clear and simplified visualization of structure-activity relationships in the microcosms (**Figure 3-4**). In Merichem treatments by day 127 a group of species decreased between $C_6 - C_{16}$ and 2 – 7 DBEs in the centre of the grid, while species $C_{17} - C_{21}$ and 2 - 10 DBEs increased. The greatest decreases were seen in those species with lower MW ($\leq C_{16}$) which continued to day 389 (- 75%). Meanwhile, species which increased (+0 – 25%) were those higher MW species found in BML OSPW. Species which increased >25% had $C_{15} - C_{21}$ and 2 – 10 DBE. With the removal of lower MW species, the intensities of higher MW species increased.[59]

Overall, degradation between day 0 and 389 in positive control treatments occurred more slowly and to a lesser extent than expected (**Figure 3-3A**) – especially in species >C_{13}. In a similar study, a >98% decrease in Merichem NAs was observed in just 28 days.[43] A major difference in experimental design was the supplementation of nutrients. Nitrogen and phosphorous were intentionally excluded in the microcosms to evaluate the capabilities of indigenous microbes as they are found in BML. Tailings ponds are known to have low concentrations of nitrogen and phosphorous and previous studies consistently supplemented nutrient media to aerobic microcosms containing OSPW.[41,43,48,49] To test the factors inhibiting biodegradation in microcosm, a series of treatability studies were established. The aim was not only to assess the effect of nutrient addition on the microcosms, but also to understand the best method by which biodegradation here could be improved.
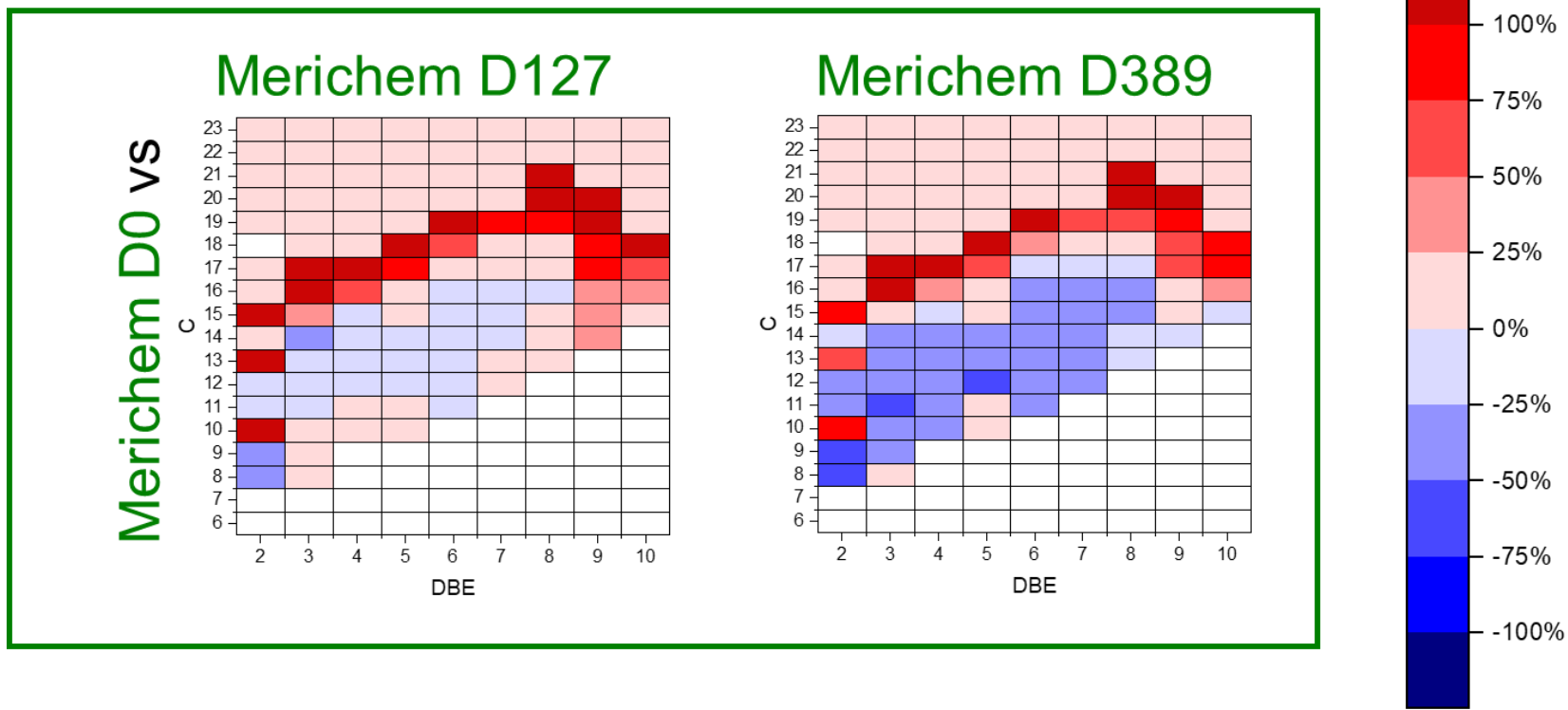
Figure 3-4 Heat map analyses representing the changes between Merichem the average intensity of treatments (n = 3) on day 127 and day 389 compared to day 0. The graduated colour bar indicates the change as percent difference (%) relative to Merichem on day 0 for the days shown. White spaces on the grid denote no species was detected.

3.3.2 Treatability studies

A variety of factors affect the biodegradability of dissolved organics including pH, DO, nutrients, temperature and salinity,[53,70] however, given the slow changes in Merichem treatments, the viability of the microbial community was questioned. Related to this was the question of whether enough bacteria were present in BML surface OSPW to remove dissolved organics in the microcosms. Microbial cells can be found at surface of clays and sediment in tailings ponds.[71] BML is a dimictic lake[72] and surface OSPW (< 1 m) used in microcosms here was sampled in late August 2015 (prior to fall turnover) - hence had low turbidity (22.9 NTU). The objective of Study 1 was to assess if an increase in microbial density (Bioaugmentation) and/or nutrient addition (Biostimulation) would improve the rate of biodegradation by endogenous microbes.

3.3.2.1 Test of biostimulation vs bioaugmentation

It has been estimated that heterotrophs can degrade dissolved organic carbon and release 50% CO_2 as a product,[49] and CO_2 evolution is commonly used to measure rates of mineralization in biodegradation studies.[40,49,56,63] CO_2 evolution in untreated BML (22.9 NTU) was compared to BML + MFT (103 – 109 NTU), and BML supplemented with BHM (**Figure 3-5**). After 13 days, untreated BML OSPW produced CO_2 just above background levels (1.01 mg/L), and bioaugmentation doubled CO_2 evolution to 2.06 mg/L. The largest difference was in the nutrient added treatments. Biostimulation increased CO_2 evolution up to 8.38 mg/L after 2 weeks. The results suggested nutrient concentrations in microcosms were more limiting than levels bacteria in the microcosms. This was expected because it had previously been reported that bioaugmentation alone does not necessarily increase rates of biodegradation, however, it may change community dynamics by the addition of organisms that can serve in other roles like production of biosurfactants or the consumption of secondary metabolites.[73,74] Additionally, this test confirmed that a viable microbial community was indeed present in BML OSPW and bioaugmentation was not required for the microcosms.
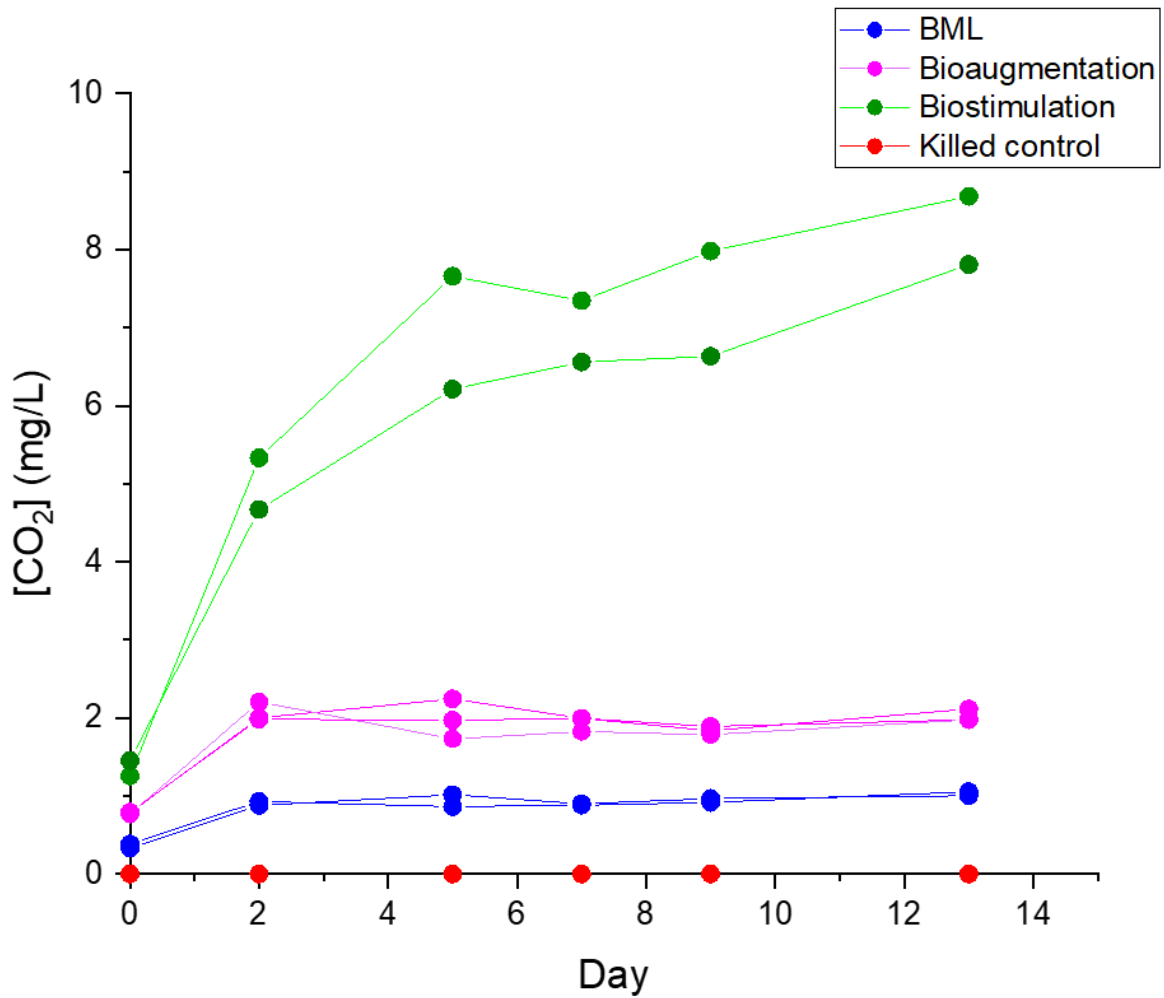
Figure 3-5 CO₂ evolution was tracked to measure the mineralization of dissolved organics in 4 treatments in treatability study 1. Untreated BML (BML, n = 2), BML + MFT (Bioaugmentation, n = 3); BML + BHM (Biostimulation, n = 2); autoclaved and chemically treated BML + MFT (Killed control) were tested over 13 days.

3.3.2.2 .Test of acetate addition

Acetate plays an important role in the microbial growth. The "acetate switch" is the metabolic process whereby acetate is excreted or taken in depending on the phase of growth and the environment. In the presence of excess carbon sources (ex. glucose), acetate is produced and secreted as a metabolite. Conversely, in the absence of carbon sources, acetate will be assimilated by microbes. This critical biomolecule is related to many metabolic pathways including the Tricyclic Acid (Kreb's cycle) – which generates energy in the cell. I postulated that the addition of acetate to microcosms could improve the ability for microbes to metabolize labile carbon sources and that, perhaps, the metabolism of acetate itself could give an indication of the potential of endogenous microbes to degrade a simple carbon source.[76,77]

Study 2A compared CO_2 evolution in nutrient supplemented BML OSPW with and without added acetate (5% v/v BHM and ~100 mg/L acetate, **Figure 3-6**) over 13 days. Without acetate, up to 5.21 mg/L CO_2 was produced – nearly 8 times lower than that of treatments with acetate (up to 38.2 mg/L). Biostimulation treatments in this iteration, produced slightly less CO_2 than in Study 1 (up to 3.8 mg/L less) but otherwise aligned well.

In order track changes in both DOC and acetate alongside CO_2 evolution, Study 2B repeated the treatment of BML with added acetate and nutrients in Study 2A (**Figure 3-7**). Acetate was completely removed within just 2 days, by which point about 15 mg/L CO_2 was produced. By day 7, CO_2 appeared to plateau at 17.6 and 17.3 mg/L, much less than in the previous experiment. However, an additional data point was taken (for interest's sake) one week later. On day 14 these bottles produced 33.4 and 34.0 mg/L CO_2. DOC decreased from 96.1 mg/L on day 0 to 45.3 mg/L on day 7, but these concentrations also appeared to plateau on day 2 (decreased to 47.1 mg/L). DOC was not expected to decrease to 0 due to persistent bitumen-derived species in BML OSPW. Concentrations of CO_2 did not increase after this day demonstrating that either the labile fraction had been completely removed, or that more nutrients were required for further mineralization.
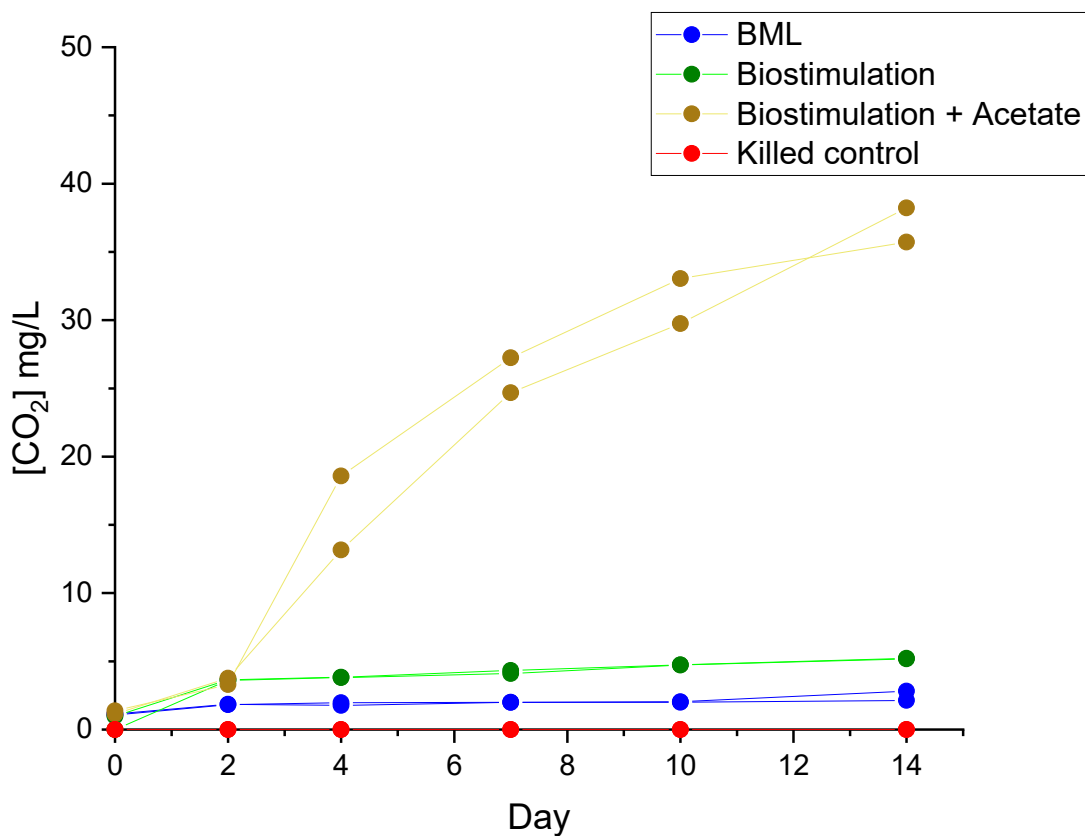
Figure 3-6 Summary of treatability Study 2A showing the evolution of CO$_2$ up to 38.2 mg/L in the Biostimulation+Acetate treatment after 14 days. Untreated BML (BML), BML + BHM (Biostimulation); autoclaved, BML + BHM + 100 mg/L acetate (Biostimulation+Acetate) and chemically treated BML + MFT (Killed control) All treatments were performed in duplicate.
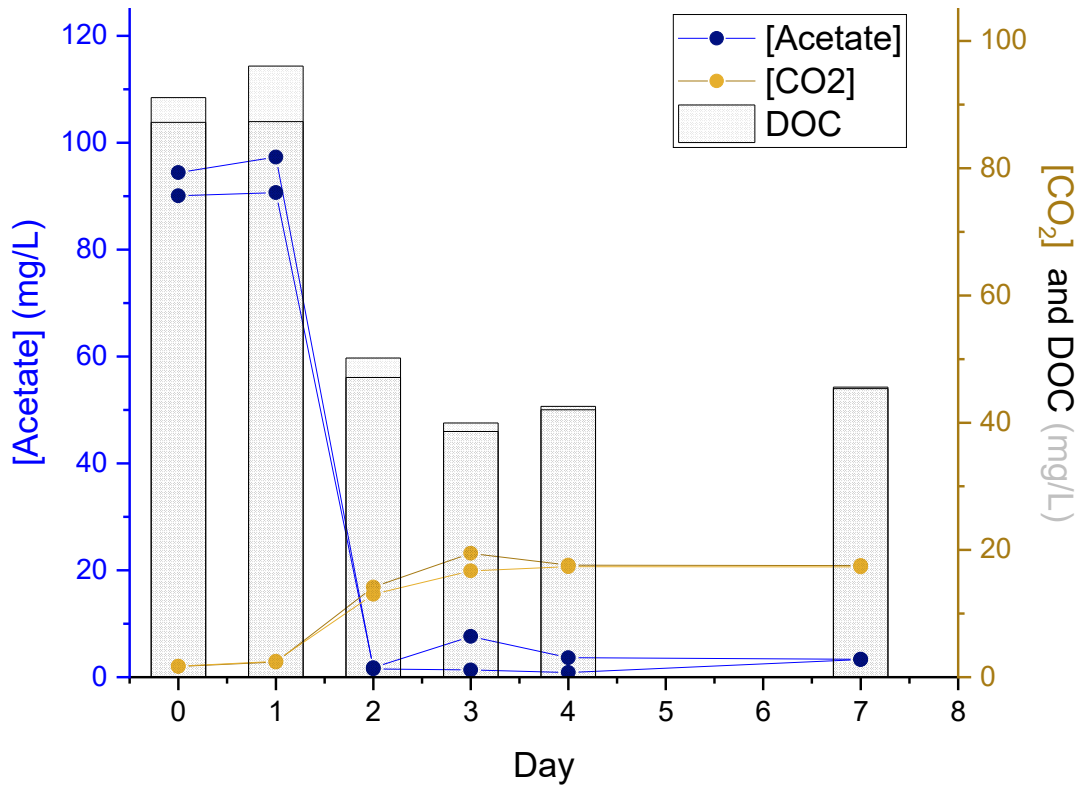
Figure 3-7 Summary of treatability Study 2B showing duplicate treatments of BML + 5% v/v BHM + up to 95 mg/L acetate. Acetate was depleted within just 2 days and up to 17.55 mg/L of CO_2 had evolved after one week.

3.3.2.3 Test of acetate addition without nutrient supplementation

The objective of the final treatability study, study 3, was to review if endogenous microbes could metabolize acetate without the addition of nutrients. To this end, treatments like those in to study 2B were established but without BHM (**Figure 3-8**). Treatments were monitored for 7 days, prior to the addition of BHM on day 8. In the following 5 days acetate was degraded, and a corresponding increase in CO_2 (up to 19.7 mg/L) was observed.

The cumulative observations from these studies indicated viable microbes exist in BML OSPW and that addition of nutrients to microcosms could enable removal of simple dissolved organics.
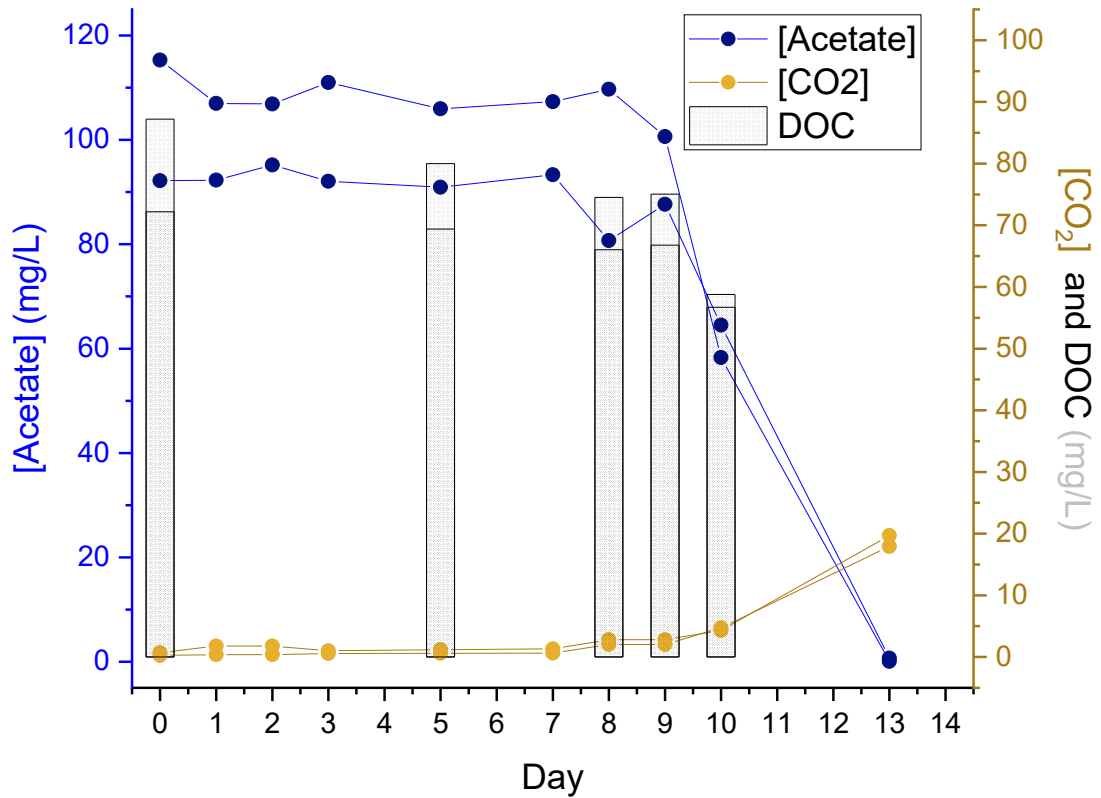
Figure 3-8 Summary of treatability Study 3. 100 mg/L acetate was added to duplicate flasks on day 0. In the first 7 days no nutrients were added. After the addition of nutrients (BHM) on day 8, acetate concentrations began to decrease, while CO_2 concentrations increased. On day 13 values for CO_2 were 19.7 and 17.9 mg/L, and acetate were 0.68 mg/L and 0.05 mg/L.

3.3.3 Post-intervention analyses

3.3.3.1 Linear regression analyses of heteroatomic classes and unique chemical species

Following termination of the experiment, linear regression models were compared to review the impact of intervention on all heteroatomic classes (O^-, O_2^-, O_3^-, O_4^-, O_5^-, O_6^-, SO^-, SO_2^-, SO_3^-) within each treatment. Pre-intervention analyses of BML revealed that no heteroatomic class decreased significantly before day 389, but two heteroatomic classes (O_2^- and SO_2^-) displayed detectable decreases post-intervention (**Figure 3-9**). Intensities of O_2^- species were similar across all treatments shortly after the addition of nutrients (**Figure 3-10**). BML and killed controls appeared to diverge on day 409 – the day on which acetate was added – after which BML decreased steadily until the end of the experiment. The rate of biodegradation of O_2^- species in BML (previously -0.04%/day) greatly increased to -3.24%/day. Decreases demonstrated by unique O_2^- species were not significant, but collectively, the changes were observed. Only 1 species, C_{13} 2 DBEs, decreased significantly (p = 0.02, =0.28%/day). The precise reason for why this species would be preferentially degraded cannot be confirmed here, but compared to the range of O_2^- compounds, this relatively small and simple structure is likely more labile than larger species with more DBEs.

Similarly, the rate of removal had increased in Merichem treatments following intervention (-0.10 %/day to -0.26 %/day), and the post-intervention slope was non-significant (p = 0.15). The largest post-intervention decrease in the Merichem treatment was between days 389 and 402 (**Figure 3-9**). After day 402, endogenous microbes were unable to further degrade dissolved organics, including more labile species. Thus, Merichem microcosms appeared to have depleted the supplemented nutrients more quickly than in BML treatments, which may have curbed degradation and limited further removal. An ANOVA on the intensities of O_2^- species on day 424 demonstrates that there was no difference between treatments by the end of experiment (p < 0.05, F = 0.129, df = 2). The O_5^- and O_6^- classes underwent a non-significant increase by the end of the experiment, while species in the O_3^- and O_4^- presented non-significant decreases In comparison, the non-significant decrease of the SO_2^- class in BML (-0.06%/day, p = 0.09, **Figure 3-11**) appeared to be boosted following intervention reflected in a significant increase in the rate to -0.22%/day (p = 0.03). No species reviewed had a significantly decreasing slope
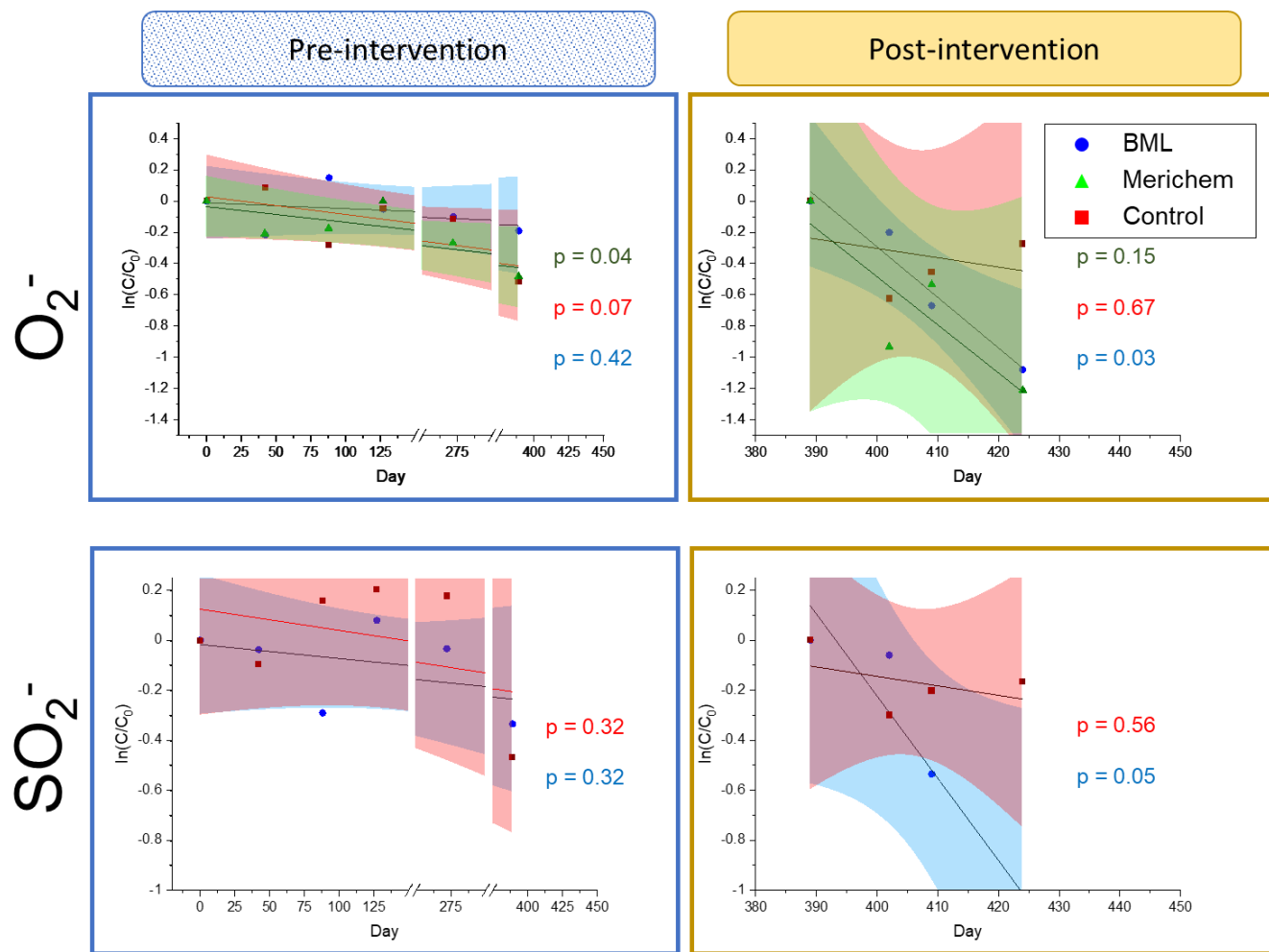
Figure 3-9 Linear regression analyses for the sum of all species in the O_2^- and SO_2^- classes (with standard deviations and confidence intervals). Pre-intervention analyses considered the average of aliquots taken on days 0, 42, 88, 127, 272, and 389, and post-intervention analyses considered aliquots for samples taken on days 389, 402, 409, and 424. Untreated BML, Merichem (positive control) and killed control treatments are displayed in blue, green and red, respectively (n = 3 each).
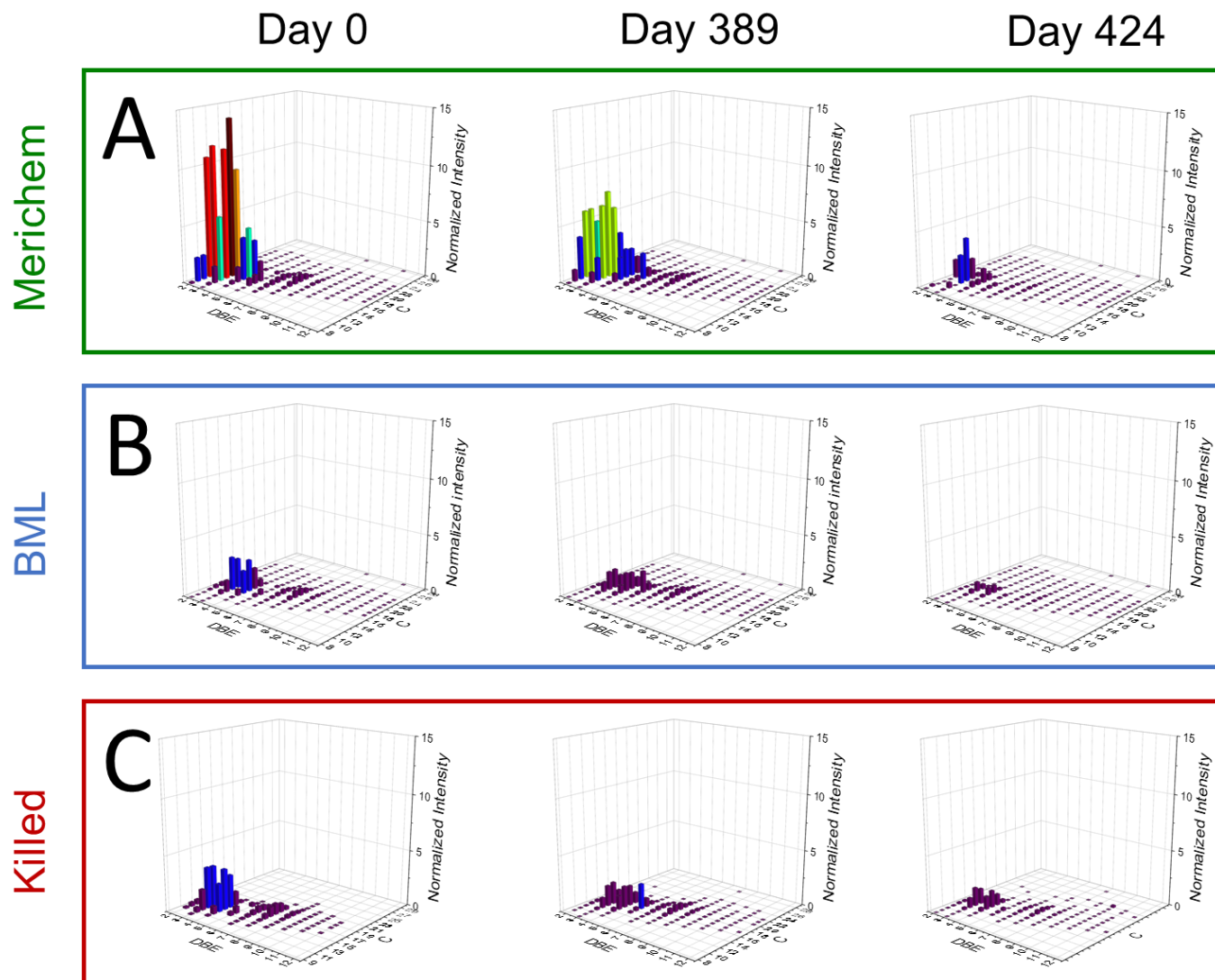
Figure 3-10 Species profiles for the O₂⁻ in Merichem (A), BML (B) and Killed controls (C) for the average intensities on day 0, 389 and 424. Each plot shows distribution by carbon numbers (8 to 30), DBE (2 to 12), and the internal standard adjusted intensity.

(p<0.05). One interesting result was in the observed increase of the SO- class pre-intervention (**Table 3-1**), but this increase was also observed in the killed control treatments (**Table B-1**).The response of the SO$^-$ heteroatomic class was highly variable across all treatments (Figure B-7) and appeared to stabilize in the post-intervention phase of the experiment. Pre-intervention changes in untreated BML showed a rate of 3.04%/day, followed by a decrease of -0.22%/day following the intervention (Figure B-9). The cause for this increase is unknown, however, the SO$^-$ class also demonstrated a significant increase in killed controls prior to intervention.

3.3.3.2 Changes in the species profiles in BML OSPW pre- and post-intervention

Differences in the distributions of O_2^- species across all treatments is shown in **Figure 3-10**. The bimodal distribution of species across DBE (characteristic of OSPW[1,2]) can be seen in untreated BML and killed controls **Figure 3-10B** and **C**, respectively). The species with the largest intensities by day 424 having $C_{13} – C_{14}$ and 3 – 4 DBEs were the same as those with greatest intensities on day 0. The bimodal distribution is not evident on day 424, however highly unsaturated species $C_{15} – C_{17}$ and 7 DBEs were still present at low intensities. Species in SO_2^- class had a different distribution than their O_2^- counterparts. The greatest intensities were in $C_{14} – C_{16}$ with 3 – 4 DBEs, demonstrating that species in this class had similar degrees of unsaturation but had higher MWs (i.e., greater carbon numbers) than the largest contributing species in the O_2^- class. Furthermore, the distribution of these were not bimodal, and species had a maximum of 9 DBEs (compared to up to 12 in the O_2^- class).
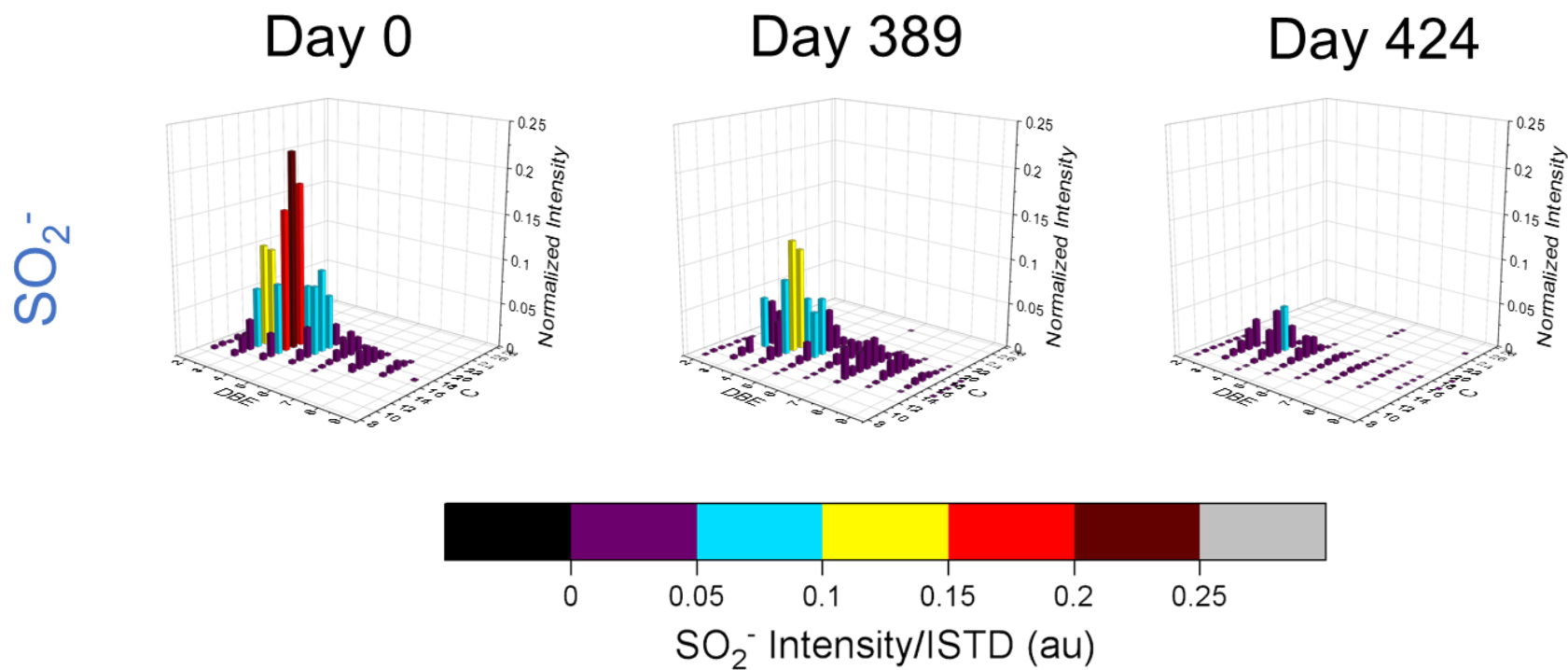
Figure 3-11 Species profiles for the average intensities of SO₂⁻ species in BML on days 0, 389 and 424. Each plot shows distribution by carbon numbers (7 to 30), DBE (2 to 12), and the internal standard adjusted intensity.

Using ESI-FTICR MS, Headley *et al.* demonstrated that depending on the industry, SO_2^- (or SO_3^-) had the greatest percent contribution to the total signal of all sulfur-containing classes.[79] Similar distributions of species classes were observed in BML OSPW, here. To my knowledge, only two studies have commented on the biodegradability of sulfur-containing organics: in one, the bioremediation potential of cattails (*Typha latifola)* demonstrated after 30 days there was a greater decrease in NAs compared to chemicals which contained sulfur or nitrogen.[51] In contrast, a more recent study using photosynthetic algae described a decrease in sulfur-containing organics but not NAs and a simultaneous decrease in toxicity (yeast assay).[80] These results implicate a different group of chemicals in OSPW is also amenable to biodegradation.

3.3.3.3 Percent relative changes in species distributions in O_2^- and SO_2^- heteroatomic classes

Heat maps reveal by the end of the experiment, BML and Merichem largely saw differences in the same O_2^- species (**Figure 3-12**). The removal of larger O_2^- species ($> C_{16}$) was seen in all treatments, including in the killed control. Species with C_{15} changed between -25% and 0.25% in the killed controls compared to Merichem and BML treatments and species.

Despite the overall decrease pre-intervention, there was an increase in SO_2^- species with at least C_{16} and 2 – 3 DBEs (**Figure 3-13**). More interestingly, species $< C_{16}$ and 7 – 9 DBE had begun to decrease. Changes in these larger, more complex species (observed in O_2^-) were also observed in the analyses of SO_2^- species. Save for a few species with 2 DBE (and one lone species C_{10} 5 DBEs), all species decreased because of the addition of nutrients and/or acetate. These findings are the first to report of decreases in high molecular weight and highly unsaturated species in OSPW.
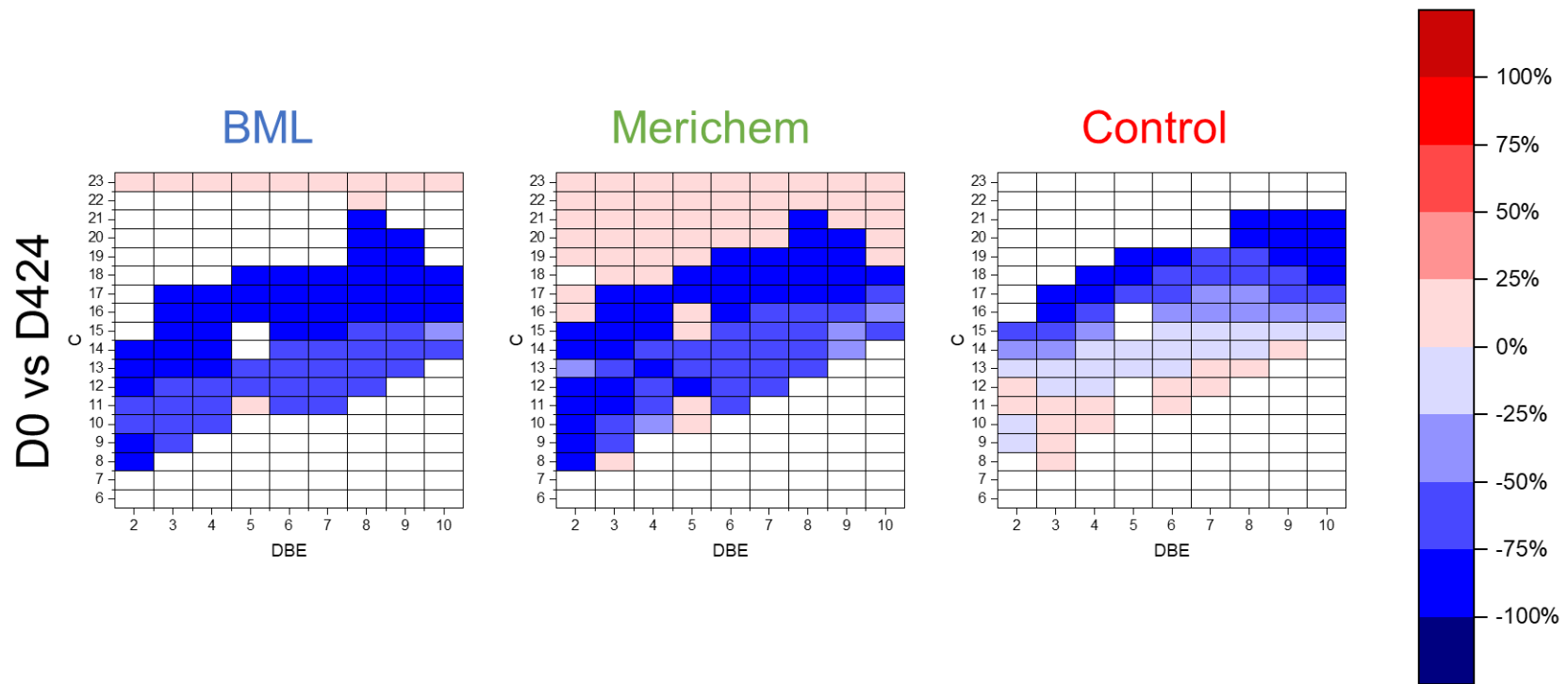
Figure 3-12 Heat map analyses depicting changes in the average intensity of O₂⁻ species within untreated BML, Merichem-added and killed control treatments (n = 3 each). The graduated colour bar indicates changes as percent difference. White spaces on the grid denote no species was detected.
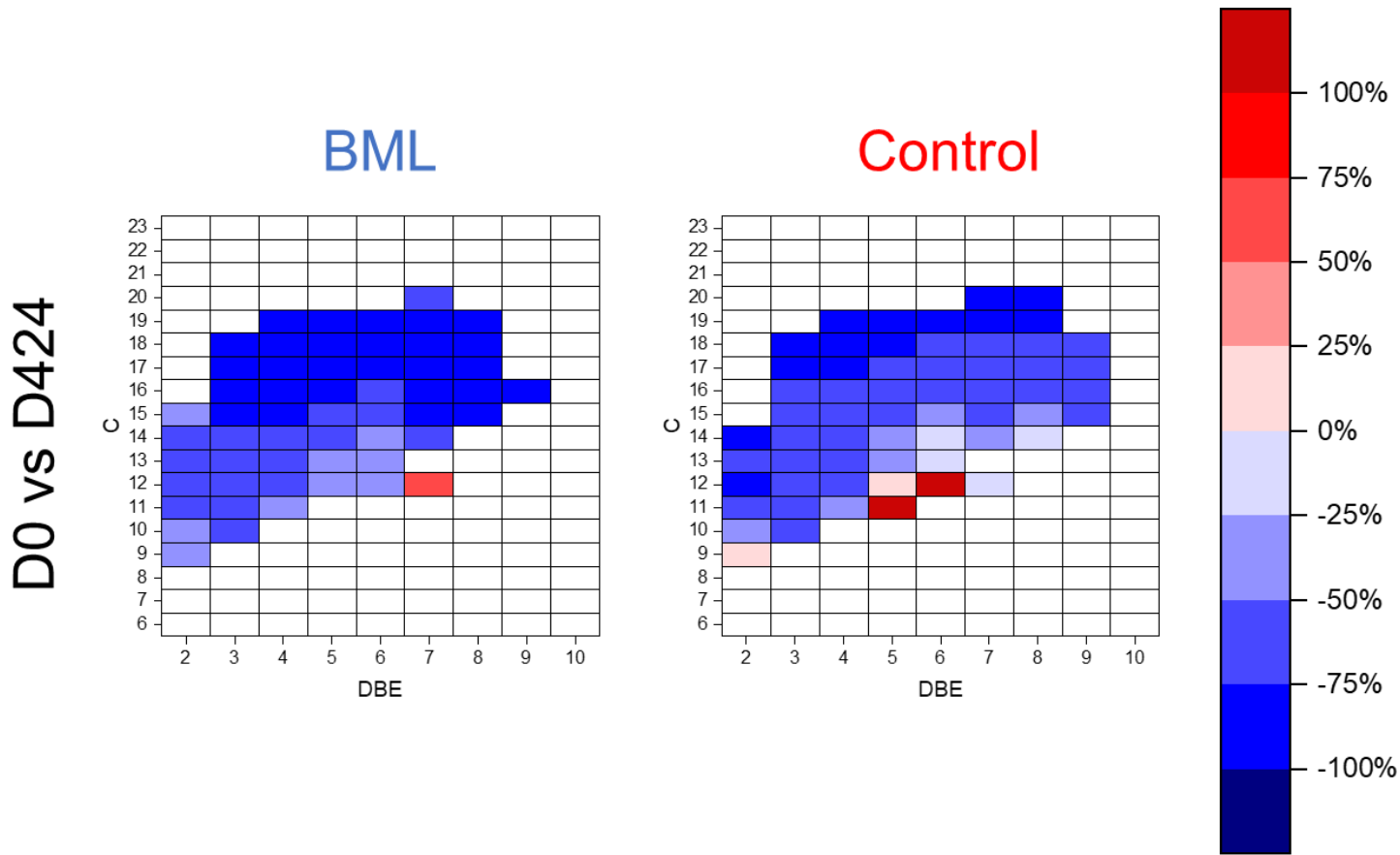
Figure 3-13 Heat map analyses representing the changes in SO₂⁻ species in BML and killed control treatments on day 424 compared to day 0. The graduated colour bar indicates the change as percent difference (%). White spaces on the grid denote no species was detected.

3.3.3.4 Changes in killed controls

To assess the variability in the killed controls, these treatments were repeated. Abiotic changes in killed controls saw adsorption and desorption of O_5^- and SO^- heteroatomic classes. **Table B-1** summarizes the changes in heteroatomic classes in killed controls and reveals that killed controls were a dynamic system. The current study sought to monitor abiotic factors contributing to changes in microcosm using killed controls. While NAs have low adsorption to coarse sands ($K_d = 0.2$), they are known to adsorb to other sediment materials ($K_d \sim 2.5$).[81] Residual bitumen on the surface of particles may also contribute to adsorption processes occurring in MFT.[56,82] In their simulated wetlands study, Toor *et al.* also observed an initial decrease, then a slight increase in NAs.[65] They hypothesized an initial adsorption occurred and once binding sites on the soils were saturated the system became more stable. Adsorption studies in OSPW have been limited to NAs and have yet to determine the K_d for other dissolved organics. Perhaps adsorption contributed to the observations in controls here.

Changes occurring in the heteroatomic classes may present an alternative consideration for negative controls: perhaps endogenous microbes were not all killed in control microcosms. In their study, Lai *et al.* studied biodegradation potential of endogenous microbes in MLSB.[42] The researchers noted an increase in NA degradation in negative controls, despite triple autoclaving and withholding nutrients from these microcosms. Therefore, there is precedent for the resilience of endogenous microbes in OSPW. As a known bacteriostat, NaN_3 was added to negative controls to inhibit growth of any microbes which may survived sterilization (by double autoclaving). Other biodegradation studies have also added NaN_3 at various concentrations as a microbial inhibitor.[51,83,84] Mercuric chloride ($HgCl_2$) and formalin are chemical alternatives to NaN_3.[85] The killed controls in this study did not emulate the environment identical to that in untreated BML OSPW, however, it was the best attempt given the circumstances.

3.3.4 Bacterial community analyses.

The V4 region of 16s rRNA is often used to study hydrocarbon-degraders in oil and oil contaminated environments.[73] Microbial community analyses present a diverse community of microbial species dominated by gram-negative Proteobacteria. Many species here are like those identified by Misiti e*t al.* in the inoculum from oil refinery activated sludge,[63] and Wilson *et. al* in their review of prokaryotes in OSPW.[86] Using a series of bioassays, Misiti *et al.* found 80% of

bacteria in the microcosm were hydrocarbon-degrading species of *Gammaproteobacteria* including (but not limited to) the genera *Aeromonas*, *Microbulbifer, Pseudomonas putida, Pseudomonas fluorescens*, and *Xanthomonas*. Bacterial communities identified in BML OSPW also included *Pseudomonas* (**Figure 3-14**). Other phyla common to both studies included *Acidobacteria*, *Actinobacteria*, *Rhizobium* (*Alphaproteobacteria*) and *Methylophilus* (*Betaproteobacteria*). A subclass of the *Alphaproteobacteria* genus *Caulobacter*, was previously identified in the metagenome of bacteria found in surface water of a tailings pond. An *et al.* estimated this and other aerobic species were hydrocarbon degraders.[87]

Aerobic biodegradation will occur in a narrow region of BML as it is reported to be anoxic below 1 m.[88] Therefore, it is not be unusual for some anaerobes to have been detected. Most of the total volume of tailings ponds is anoxic.[44] Several anaerobes were identified in BML OPSW. The ammonium- and/or nitrate-oxidizers *Mesorhizobium* (*Alphaproteobacteria*) and *Limnobacter* (*Betaproteobacteria*) were identified in the current study. Previously, McKenzie *et al.* observed these genera in a study of an anaerobic biofilm reactor in which NAs in OSPW were degraded.[89] In the analysis of microbial communities in the Utah oil sands, Lewis *et al.* revealed genera of gram-positive *Actinobacteria* dominated followed by (in order of decreasing relative contribution) *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria* and *Acidobacteria*.[90] Interestingly, these classes of aromatic hydrocarbon-degraders in the Utah oil sands ore were similar to those found in BML OSPW, but the bacterial community compositions were not identical.

Firmicutes only became prominent in BML microcosms at the end of the experiment on day 424. Foght *et al.* have described their role as integral to a syntrophic community of microbes which metabolized hydrocarbons under methanogenic conditions over time, rendering the crude oil severely biodegraded.[93] Indeed, *Fusibacter* (a member of the phylum *Firmicutes*) later demonstrated the ability to anaerobically degrade the aromatic compound 2-methylnapthelene.[94] Yergeau *et al.* identified this genus in sediments from tailings ponds,[95] and in a follow-up study, these researchers noted *Firmicutes* species were unable to survive at high concentrations of dissolved bitumen-derived organics.[96] To add, Collins *et al.* suggested fixed nitrogen and a labile carbon source enabled *Trichococcus* (a genus of *Firmicutes*) to become dominant in anaerobic communities.[97] The introduction of nutrients (including NO_3^-) and readily-degradable acetate during intervention may have lent to the increase in the numbers of *Firmicutes*. Unfortunately,

dissolved oxygen concentrations were not measured on day 424, however this likely impacted the aerobic biodegradation of O_2^- and SO_2^- species, fostering an environment in which *Firmicutes* could thrive. This is reflected in the proliferation of *Lysinbacillus* and *Bacillus* at the end of this microcosm experiment.

Between 20 - 40% of species were not identified by UPARSE and were categorized as "Other". The species found here can be hypothesized from the findings of other studies. Golby *et al.* used a tailings sample to culture species biofilms. Aerobic conditions promoted the growth of *Thauera* in a biofilm, but without nutrients the population was distributed between *Rhodoferax*, *Acidovorax*, (*Betaproteobacteria*), *Acinetobacter*, *Pseudomonas* (*Gammaproteobacteria*) and some others.[98] Prokaryotes in West-In-Pit (former name of BML) which largely included methanogens like *Methanosaeta* (*Euryarchaeaota*) but also other taxa *Comamonadaceae* (*Betaproteobacteria*) and *Anaerolineaceae (Chloroflexi)*.[86] *Chloroflexi* and other phototrophs have been postulated to increase during reclamation, in syntrophic relations with chemotrophs.[99] A different study of biofilms found *Azoarcus* (a monoaromatic hydrocarbon-degrading species of *Betaproteobacteria*) *and* the strict aerobe, *Roseomonas aquatica* (organophosphorus degrading species of *Alphaproteobacteria*) in fresh OSPW.[100,101] Together, these studies present mixed species of bacteria which are tolerant to NAs and other dissolved organics in OSPW, which may have been present in untreated BML OSPW.
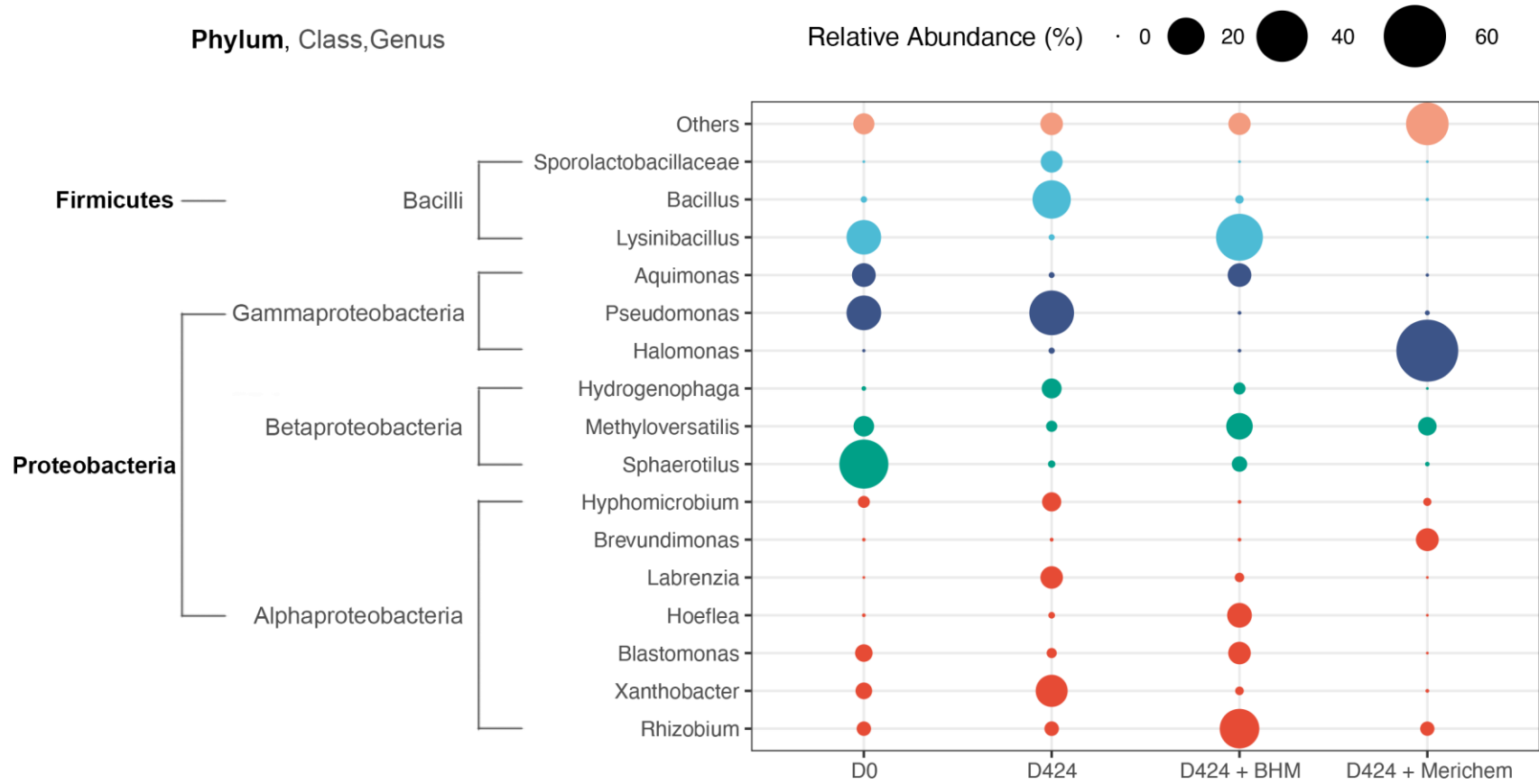
Figure 3-14 Microbial community analysis of BML OSPW.

3.4 Limitations and Significance

Here for the first time, I report rates of biodegradation of O_2^- species in the first (and currently the only) oil sands end pit lake. Pre-intervention approximations confirm the recalcitrance of organics in OSPW and suggest that without dilution (and perhaps other management strategies) biodegradation in EPLs would require many years. Without intervention, half-lives of dissolved O_2^- species were estimated to be around 3.5 years (1250 days to 50% at a rate of -0.04%/ day) but half-lives decreased to ~ 7 months following the introduction of nutrients and acetate. Except for species in the SO^- class, no species demonstrated significant changes prior to intervention. The post-intervention rate falls in line with the half-lives suggested by Han *et al.*[82] In their study, around 217 days were required to reach 50% of the initial sum of intensities for O_2^- species. This study showed that despite being the most toxic chemical class, OSPW NAs appear to be among species which are the most responsive to intervention. Furthermore, I showed another class of organics, SO_2^-, was also amenable to bioremediation.

Aerobic biodegradation in BML is limited to the top 1 m of the pond,[46,47] and seasonal changes largely restrict these processes to warmer months.[44] While many aerobic species were present, several anaerobic bacteria were also identified. The community dynamics in this matrix are quite complex- not only due to the dissolved organics, but also because of the electron acceptors and the process by which they are recycled. Biogeochemistry of BML has been reviewed and will influence biodegradation processes.[88,102–106] Although aerobic biodegradation of microcosms is the most favoured thermodynamic process for microbes, the limnology of BML suggests anaerobic processes will contribute and impact processes occurring at the surface. Oil degrading microbes often exist in syntrophy, with many organisms surviving due to niche ecological roles.[73] This seems to be true of the bacterial community present in BML.

Rates of hydrocarbon removal are often slower in the field compared to laboratory studies, since the latter are typically performed at elevated temperatures (~21°C).[73] It is germane to mention that the microcosms here depicted a simplified representation of the complex and dynamic system which exists in BML. The impacts of dilution, photolysis, and anaerobic degradation – which all would decrease concentrations of O_2^- species at a minimum - were not reviewed. While samples were preserved to assess toxicity, they were not adjusted for evapotranspiration prior to the Microtox tests. These samples were concentrated over the duration of the experiment, and as I did not account for this prior to testing, the results of toxicity

analyses could not be reported. Nonetheless, the findings are promising and support the previous microcosm studies. More importantly, they bolster the hypothesis of MacKinnon and Boerger.[31] The removal of NAs, will likely be associated with a corresponding reduction in toxicity.

Unfortunately, issues with instrumentation and data processing (i.e., computational power and processing time) prevented the reporting of rates of biodegradation in polar neutrals and other non-acid species found in BML OSPW which are detected in positive ionization mode. Future work would benefit from reviewing changes in these species and would improve predictions for the implication of biodegradation on toxicity of BML OSPW.

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Chapter 4   Characterizing Phase I & II biotransformation of BML OSPW toxic

fractions by Rainbow Trout (Oncorhynchus mykiss) microsomes and S9.

4.1   Introduction

Mechanisms of action for toxic components in OSPW have been postulated to range from endocrine disruption, to oxidative stress and narcosis. One important consideration to assess the hazard a chemical may pose to animals is its ability to bioaccumulate. A bioaccumulation factor (BAF) describes the extent to which a toxicant is taken up into an organism relative to the exposure media. BAF is generalized term which considers the uptake of a chemical by respiration and/or diet described by the ratio in Equation 4-1:

$$Bioaccumulation\ factor = \frac{concentration\ of\ toxicant\ in\ fish}{concentration\ of\ toxicant\ in\ water}$$

A BAF is determined from examination of an organism in the field. Attempts to understand the risk of bioaccumulation of OSPW organics in fish have been addressed in different ways in the lab by estimating either the bioconcentration factor (BCF) or the octanol-water coefficient (K_{OW}). Measurements of BCFs and K_{OW} offer a reliable alternative to BAF estimations. A chemical is bioaccumulative when its BAF is greater than or equal to 5000 or a log K_{OW} greater than 5.

The earliest estimate of bioaccumulation potential of organics in OSPW (15 mg/L NAs) observed rainbow trout (*Oncorhynchus mykiss*) held in Syncrude's experimental Pond 9 for 96 hours in 9 L aquaria.[1] An unresolved chromatographic peak (GC-MS) indicated NAs were present in the tissue, but the amounts were not quantified. One single species, $C_{13}H_{22}O_2$, was selected to estimate all NAs in the tissue. A follow-up study measured this species in rainbow trout exposed to Merichem NAs for 9 days and reported a BCF of approximately 2 L/kg.[2] Young *et. al* later exposed rainbow trout to Merichem NAs (3 mg/L) for 10 days to estimate accumulation in various tissues, and NA concentrations in exposed fish (mean ± SD µg/g wet wt) were higher in gills (56 ± 41) and in livers (88 ± 54) than in muscle (11 ± 2), likely because these organs are responsible for a good proportion of xenobiotic transformation.[3,4] However, no NAs were measured in the tissues of wild fish gathered close to oil sands mining and extraction operations.[5] Background concentrations of NAs in the Athabasca River have been detected at 11.8 to 20.0 µg/L.[6] The inability to detect organics in fish tissue suggested that these components

do not accumulate in fish. While this is hopeful, chemical analyses at the time of the first bioaccumulation studies (2008) were limited by methods which were less sensitive and had higher limits of detection.

In their first assessment of bioaccumulation potential of OSPW organics in fish, Zhang *et al.* used polydimethylsiloxane (PDMS) as a surrogate material for lipids.[7] The log D_{OW} (or apparent log K_{OW}) is an estimate of octanol-water partition coefficient for chemical mixtures. A PDMS-coated stir bar was spun in OSPW from West-In-Pit (now BML) for up to 144 hrs. The stir bar was removed, placed into clean water, and depurated for 24 hrs. Only 3.6% of acidic species detected in negative ion mode, including most O_2^- species, had a log $D_{OW} > 1.0$. This not surprising since these acidic species would be ionized (and less bioavailable) at an environmentally relevant pH. More species detected in positive ion mode appeared to have higher bioaccumulation potential. Three heteroatomic classes, O^+, NO^+ and SO^+ had log D_{OW}s of 2.5 ± 1.5 and 1.0 ± 1.7, respectively. In a second study, the researchers improved their estimations by measuring the phospholipid membrane-water distribution coefficient of mixtures, log D_{MW}.[8] This time, phosphatidylcholine was used as surrogate material. At the same they estimated BCFs in Japanese Medaka (*Oryzias latipes*) held in OSPW (replenished every 24 hours) for up to 288 hrs.[8] Log D_{MW} correlated well with Log D_{OW} for O^+, O_2^+, NO^+ and SO^+ species. Interestingly, only SO^+, NO^+ (BCFs from 0.6 to 28 L/kg) and O_2^- (BCFs from 0.7 to 53 L/kg) species were detected in the fat of fish. The *in vitro* BCF values were plotted against *in vivo* values and showed good correlation in both positive in negative ion modes ($R^2 = 0.835$, p < 0.001; $R^2 = 0.74$, p < 0.001, respectively). Discrepancies between the predictions were hypothesized to be due to metabolism of SO^+ and NO^+ species, but this has yet to be studied.

While K_{OW} and D_{OW} are good estimates for BCF, some multi-compartment models have considered adsorption, distribution, metabolism, and excretion as these processes may impact bioaccumulation potential within organisms.[9] Among these, metabolism is most often studied as it enables more accurate predictions for the clearance of a compound from an organism. Metabolism (or biotransformation) occurs in two phases which convert non-polar (hydrophobic) lipophilic compounds to more polar (water-soluble) forms that are more readily excreted. Cytochrome P450 monooxygenases (CYPs) are a superfamily of enzymes which are predominantly responsible for oxidation of xenobiotics in phase I metabolism.[3] They have diverse capabilities including hydrocarbon hydroxylation, epoxidation, deamination,

desulfurization, and dehalogenation. EROD (7-ethoxyresorufin-*O*-deethylase), is an enzyme which removes an alkyl group from a substrate. The activity of this enzyme is often measured to assess exposure of fish to polyaromatic hydrocarbons (PAHs) and other xenobiotics in the environment.[10] In phase II, enzymes conjugate large polar functional groups to xenobiotics. This process usually increases water solubility, but this is dependent on the conjugate added.

Some fish toxicity studies have studied metabolism of PAHs by measuring EROD activity[11–14] or directly measuring PAH metabolites in fish[11,15,16] These studies present evidence for the ability for native fish to detoxify a narrow subset of chemicals in OSPW but have focused on non-polar aromatic compounds. Currently there is a lack in our understanding of the biotransformation potential of NAs and other dissolved organics in OSPW. This project was established with the aim of assessing the metabolism of toxic dissolved organic compounds in OSPW by using rainbow trout liver subcellular fractions. Native to the region, rainbow trout have been used in a variety of other toxicity studies and therefore serve an excellent choice for a model fish species. Enzyme activity in rats is higher than in fish. For this reason, rat microsomes were studied as a reference animal species to contextualize the results.

Metabolism can be studied using subcellular fractions of hepatocytes (liver cells).[4] Microsomes are small pieces of the endoplasmic reticulum isolated by centrifugation of homogenized liver tissue at 100 000 x g.[17] This fraction includes a high concentration of the enzymes responsible for phase I metabolism. The equivalent subcellular fraction generated at 9000 x g is called S9. It includes a larger array of enzymes, including phase II enzymes which are found in the cytosol of hepatocytes. Each subcellular fraction presents its own advantages, and both were chosen for review in this study.

The goals were simple and two-fold: 1) to assess if toxic fractions in OSPW would be metabolized by trout liver enzymes, and 2) to review the products of metabolism and infer impacts on OSPW toxicity and/or bioaccumulation. The chemical profiles of metabolites can shed light on biotransformation potential in fish. [9]

4.1 Methods

4.1.1 Chemicals and reagents

Acetic acid, ammonium hydroxide(NH_4OH), dichloromethane (DCM), methanol (MeOH), UHPLC water (H_2O), sulfuric acid (H_2SO_4) and sodium hydroxide (NaOH) were purchased from were purchased from Thermo Fisher Scientific (San Jose, California). Pooled male rat (Sprague-Dawley), rainbow trout liver microsomes (20 mg/mL, each), and rainbow trout S9 (20 mg/mL) were purchased from directly from Thermo Fisher Scientific and stored at -80°C. All other reagents were purchased from Sigma-Aldrich, unless otherwise stated. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), glucose 6-phosphate sodium salt (G6P•Na) were stored at room temperature (~21°C). β-Nicotinamide adenine dinucleotide phosphate disodium salt (NADP•Na$_2$), anhydrous β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH•Na$_4$), glucose 6-phosphate dehydrogenase (1000 U G6PD lyophilized powder) were stored in the fridge (4°C). Uridine-diphosphate-glucuronic acid trisodium salt (Na$_3$ UDPGA), resorufin and 7-ethoxyresorufin (7-ER) were stored at -20°C.

The addition of pore-forming alamethicin (5 mg/mL in DMSO) was meant to increase the speed of the reaction by allowing UDPGA (the cofactor) to pass more easily into the microsomes. The channels formed by this detergent allow for reagents and substrates to penetrate microsomes, access catalytic domains, and thereby increase enzyme activity.[18]

One litre of 0.05 M Tris-HCl (pH adjusted to 7.4) was prepared bi-weekly. Stock 25 mM $MgCl_2$, 29.2 mM G6P, 2.5 mM NADP, 2.0 mM NADPH and 100 U/mL G6PD were prepared using Tris-HCl buffer and stored at 4°C for up to two weeks prior to use in any assay.

Reference compounds testosterone ($C_{19}H_{28}O_2$, 1 mg/mL in acetonitrile), methylbenzodioxolylbutanamine hydrochloride (MBDB•HCl, $C_{12}H_{17}NO_2$•HCl, 1 mg/mL in MeOH), and isotopically labelled ^{13}C-lauric acid ($C_{11}H_{23}{}^{13}CO_2H$) were stored at -20°C. Refined commercial naphthenic acids were purchased from Merichem Chemicals and Refinery Services (Houston, Texas, USA) and stored at 4°C.

The Quick Start ™ Bradford Protein Assay was purchased from Bio-Rad Laboratories, Inc., (Mississauga, ON).

4.1.1.1 Rat microsomes as a reference assay

Activity of enzymes in rats are consistently reported to be higher than in fish. The first report of this kind comparing the biotransformation capabilities of the two animals used hepatocytes of fish and rats to compare activities for several enzymes (substrates) including CYP1A (7ER), CYP3A (testosterone), CYP4A (lauric acid) and glutathione-S-transferase (1-chloro-,2,4-dinitrobenzene).[19–21] As they are known to be more active, rat microsomes were used in this merely for comparison against trout – the animal model of focus.

4.1.2 Sample description

BML OSPW was collected at Syncrude Canada Limited in August of 2014. Samples were taken 50 – 100 cm below the surface with a Van Dorn water sampler deployed from a floating barge and placed into 20 L white high density polyethylene pails. OSPW was stored in sealed buckets at 4°C until use.

4.1.3 Solid phase extraction

The extraction method used here was slightly modified a method originally developed by Dr. Chenxing Sun during her time as a research associate at the University of Alberta.[22] This method increased the efficiency of a method developed by Dr. Alberto Dos Santos Pereira.[23] The objective was to isolate two dissolved organic fractions: one containing O_2^- species, and the other comprised of polar and neutral organic classes O^+, O_2^+, SO^+ and NO^+. First, BML OSPW was vacuum filtered (1.2 μm) to remove solid particulate. Oasis HLB SPE columns (33 cc, 6 g sorbent, 60 μm particle size, Waters Limited, Ontario, Canada) were affixed to a manifold (Waters Limited, Ontario) which was connected to vacuum through a series of 4 L rubber stoppered Erlenmeyer flasks. Prior to extraction, each cartridge was first conditioned with 20 mL MeOH, then 20 ml water (gravity).

Through a filter inlet, 2 L of OSPW was drawn from a plastic storage container to each SPE cartridge (fitted with stoppers). A total of 6 L of BML OSPW was extracted. Columns were washed with 40 mL of MeOH:H_2O (50/50 v/v), followed by 20 mL MeOH:2% acetic acid (55/45 v/v). Washes were discarded.

This basic extract (F1) was generated in the following way: The column was washed with 40 mL MeOH:5% NH_4OH (65/35 v/v). This wash was maintained, the pH was increased to 12

using NaOH, and was then extracted three times using 40 mL of DCM (liquid-liquid extraction). Next, 25 mL of MeOH was used to wash the column and combined with the 120 mL from the basic extraction. The acidic fraction, (F2) was then generated by adding H_2SO_4 to the unextracted aqueous phase. At a pH of 2, a second liquid-liquid extraction was performed using DCM (three times, 40 mL each). Extracts were evaporated to dryness, weighed, transferred to MeOH, and stored at 4°C.

4.1.4   Protein quantification

Protein concentrations in prepared enzymes were quantified using the Quick Start™ Bradford Assay.[24] In this assay, proteins are measured spectrophotometrically upon binding with reagent dye, Coomassie Blue G-250 at the absorbance wavelength of 595 nm. The anionic form of the dye is stabilized when bound to basic and aromatic amino acids.[27] Seven standards (0.125 – 2.0 mg/mL) of bovine serum albumin (BSA) are used to create a calibration curve from which proteins in unknown samples are measured.

4.1.5   EROD enzyme activity assays

Enzyme activity was measured using a modified protocol for the EROD assay by Hasbi *et al*.[26] Briefly, NADPH regenerating system (NGS) was prepared with the following reagents and concentrations in solution: $MgCl_2$ (10 mM), KCl (20 mM), G6P (6 mM), NADPH (1.25 mM) and 100 U G6PD. BSA from the Bradford assay kit (2.0 mg/mL standard) was used. Tris-HCl (10 µM) was prepared at pH 7.4. The final concentration of 7-ER in reactions was 10 µM and mass of microsomal protein in reactions was 30 µg. The volumes and sequence of addition to each reaction well is shown in **Table 4-1**. Reagents were added on ice to a 96 Corning™ Costar™ 96-well flat-bottom microplates. Incubations were initiated with the addition of 7-ER (1 µM), the plate was covered with aluminum foil and then placed in a shaker-incubator (200 rpm, 30°C) for 10 min. The final reaction volume of 150 µL in each well. Test and control reactions, resorufin calibration standards, and blanks (Tris-HCl only) and were prepared in triplicate (n = 3, each). Reactions were terminated with the addition of 150 µL of cold MeOH. Resorufin standards (0.1 – 2.5 nM) were prepared in Tris-HCl. Following reaction termination, standards were added to wells containing microsomes. To account for matrix affects, an equivalent mass (30 µg) of microsomes were also added to blanks. Resorufin fluorescence was measured on the

Filtermax™ F5 microplate reader using SoftMax Pro® Software (Molecular Devices, San Jose, CA) at excitation wavelength of 530 nm and emission wavelength of 585 nm.

Table 4-1 Reagents and procedure of EROD assay

Order	Reagent	Test Volume (µL)	Control Volume (µL)
1	BSA	15.0	15.0
2	NGS	37.5	37.5
3	Tris-HCl	74.1	89.1
4	microsomes	8.4	8.4
5	7-ER	15.0	0
	V_{total} (µL)	150	150

4.1.6   Logistical progression of methodologies

Enzyme activity in fish is known to be low compared to that in rats.[20] Therefore, rat microsomes served as a biological reference for comparison against all assays performed with trout microsomes. The detailed descriptions of those assays can be found in Appendix C.

A series of enzyme assays were executed - each one increasing the complexity of the organic chemical fraction applied. Phase I and phase II metabolism of reference compounds were studied first, followed by the phase I metabolism of Merichem NAs. The final assays were performed using the acidic and basic extracts from BML OSPW. In the final assays, testosterone and ^{13}C-lauric acid were added to reactions as positive control substrates for assays using the basic and acidic fractions, respectively.

4.1.6.1 Phase I and Phase II metabolism of reference compounds

Reference compounds testosterone ($C_{19}H_{28}O_2$, 1 mg/mL in acetonitrile) and MBDB•HCl, ($C_{12}H_{17}NO_2$•HCl, 1 mg/mL in MeOH) were selected for detection in positive ion mode (**Figure 4-1**), and ^{13}C-lauric acid ($C_{11}H_{23}{}^{13}CO_2H$, 1 mg/mL in MeOH) selected for detection in negative ion mode. Assays were designed from a modified protocol by Wu and McKown.[29] Phase I metabolism was performed in both trout and rat microsomes according to procedure in section

4.1.7. Phase II metabolism was performed in trout microsomes only according to the procedure in section 4.1.8. Final concentrations of all reference compounds in reactions were 1 ug/mL.

4.1.6.2 Phase I metabolism of Merichem NAs

Stock Merichem NAs were prepared at 1000 mg/mL in isopropanol. Phase I metabolism was performed according to procedure in section 4.1.7. Final concentrations of Merichem NAs in reactions was 10 ug/mL. For assays using the acidic fraction, ^{13}C-lauric acid was used as a positive control substrate,

4.1.7   Phase I biotransformation

The volumes and sequence of reagent addition is shown **Table 4-2**. Test and control incubations were performed in triplicate (n = 3, each). To subcellular components was added 10 or 20 μL of substrate (0.5 to 12.9 μg/mL in well). Reactions were pre-incubated for 15 min on a rotary shaker at 150 rpm at 15°C for trout and 37°C for rat assays. Initially, controls were performed without NADPH. However midway through the project, ThermoFisher discontinued production of trout microsomes. Thus, to save on this reagent, control incubations were subsequently performed with NADPH but without microsomes. The differences between the two negative control matrices were not assessed. This is another reason why the thresholds to detect the differences between test and control reactions were set high. Blanks consisted of all reagents except the substrate of interest which was substituted with Tris-HCl. Reactions were initiated by the addition of NADPH (210 μL), and aliquots of 150 μL were removed throughout at 0, 15, 45, 105 and 225 min. Upon removal, aliquots were quenched with an equivalent volume of pre-cooled MeOH. Samples were snap frozen at in a bath of dry ice/MeOH and stored in a freezer at -80°C until analysis.
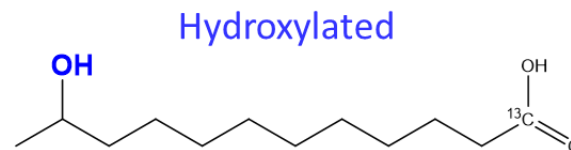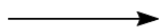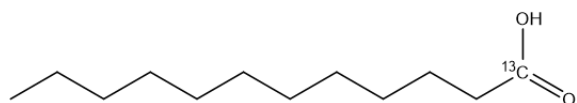
Figure 4-1 Measured phase I reactions of reference compounds ^{13}C-Lauric acid, testosterone and methylbenzodioxolylbutanamine (MBDB). Hydroxylations are shown in blue, the demethylation of MBDB is shown in red.

144

Table 4-2 Reagents and procedure of Phase I biotransformation reactions

Order	Reagent	Test Volume (μL)	Control Volume (μL)	Incubation Concentration
1	$MgCl_2$	200	200	5 mM
2	G6PNa	200	200	6 mM
3	G6PD	10	10	2 U
4	microsomes	50	50	1 mg/mL
5	substrate	20	20	--
6	Tris-HCl	310	520	--
7	NADPH	210	0	0.4 mM
	V_{total} (μL)	1000	1000	--

4.1.8 Phase I + II Biotransformation

The volumes and sequence of reagent addition is shown in **Table 4-3.** Blanks were prepared with all reagents, but Tris-HCl was substituted for microsomes and substrate. Alamethicin (membrane-disrupting detergent) was added to increase the activity of UDP-glucuronosyltransferases (UGTs) as they are found in the lumen of the endoplasmic reticulum (and therefore microsomes).[21,30] Test and control incubations were performed in triplicate (n = 3, each). To subcellular components was added 10 or 20 μL of substrate (0.5 to 12.9 μg/mL). Reagent mixtures (1 mL) were pre-incubated for 15 min on a rotary shaker at 150 rpm at 15°C for trout and 37°C for rat assays. Reactions were initiated with the addition of a mixture of UDPGA (stock concentration 2.5 mM) and NADP. Aliquots of 150 μL were removed at 0, 15, 45, 105 and 225 min. Upon removal, aliquots were quenched with an equivalent volume of pre-cooled MeOH. Aliquots were snap frozen in a bath of dry ice/MeOH and stored at in a freezer at -80°C.

Table 4-3 Reagents and procedure of Phase II biotransformation reactions

Order	Reagent	Test Volume (μL)	Control Volume (μL)	Incubation Concentration
1	MgCl$_2$	200	200	5 mM
2	G6PNa	200	200	6 mM
3	G6PD	10	10	2 U
4	microsomes	50	0	1 mg/mL
5	substrate	20	20	--
6	alamethicin	20	20	10 μg/ml
7	Tris-HCl	100	150	--
8	NADP	200	200	0.5 mM
8	UDPGA	200	200	0.5 mM
	V$_{total}$ (μL)	1000	1000	--

4.1.9   Post-assay sample preparation

Aliquots were thawed and centrifuged at 10 000 x g for 10 min. From the reaction tubes, 200 µL of the supernatant was transferred into autosampler vials with inserts (300 µL) and spiked with 20 µL of the combined internal standard solution. Internal standard concentrations in the vialed samples were 1 mg/L D_{23}-lauric acid (for negative ionization mode) and 0.01 mg/L D_3-progesterone (for positive ionization mode).

4.1.10   Instrumental analysis

Reversed-phase HPLC was paired with a hybrid linear ion trap-Orbitrap mass spectrometer (Orbitrap Elite, Thermo Scientific, San Jose, CA) and a modified version of the analytical method of Pereira *et al.* was used.[29] HPLC separation was performed on a Hypersil Gold C18 Selectivity Column (Thermo Scientific, 50 × 2.1 mm, particle size 1.9 µm) using an Accela HPLC (Thermo Scientific). The column was maintained at 40°C and a flow rate of 0.5 mL/min was used with 10 µL injection volumes. Initial mobile phase composition was 95% A (0.1% acetic acid in water) and 5% B (100 % MeOH) for 1 min, followed by linear gradient ramp to 90% B over 5 min, and a final ramp to 99% B over 5 min. Percent composition of B was then decreased to 5% over 2.5 min and held for 75 s for re-equilibration. Atmospheric pressure chemical ionization (APCI) was used in both positive ($^+$) and negative ($^-$) modes. Nominal resolution of the Orbitrap was set to 240 000 at *m/z* 400, and full scan mass spectral data were acquired between 100 and 500 *m/z*. Capillary temperature was 300 °C, discharge current was 4.5 µA, respectively.

4.1.11   Summary of Non-targeted data analysis workflow

Non-targeted analyses were applied to the metabolism assays of Merichem NAs, Acidic fraction and Basic fraction. A full description of the method (C.1) and figure of the workflows (**Figure C-1** and **Figure C-2**) are in the supplementary information, Section C. For these experiments, raw data files were imported into Compound Discoverer 2.1 SP1 (Thermo Fisher Scientific, San Jose, CA). In the software, features are defined as any species for which both the exact mass and retention time is defined. Alignment of chromatographic peaks was performed in the software such that for a feature identified within more than one sample (raw file), the maximum chromatographic retention time shift was 0.2 minutes and mass spectral error

tolerance was ≤ 5 ppm. Further restrictions included identification of species which met a signal to noise (S/N) Threshold > 3, minimum peak intensity of 1000. Minimum element counts for molecular formulae assignments were C5 H5 and maximum element counts were C50 H100 N2 O6 S2 for phase I and C50 H100 N2 O8 S2 for phase I + II. To check if they had previously been identified elsewhere, all features were also screened using online databases *m/z* Cloud and *m/z* Vault. Some features which did not have a formula assigned but met the criteria for further review (chromatographic peak area > 1000, p-value 0.05, $\log_2$Fold-Change > 2) were searched in the free online PubChem online database by exact mass with the precision (mass error tolerance) set to 10 ppm. For a given *m/z*, the list was manually search and the most probable formula was selected considering how many PubChem molecules were in the database, the mass error and the elements included. Blank subtraction was performed using the 'Mark Background Compounds' node. Features hereafter will be referred to as compounds.

4.1.12 Statistical analysis

Sample groups were established to compare test and control reactions at the defined time points (0, 15, 45, 105 and 225 min). The 'Differential analysis' node in Compound Discoverer was applied to generate volcano plots. Volcano plots have been increasingly applied to the visualization of statistical analyses of complex mixtures.[30–33] In these plots, the $\log_2$(Fold-Change) is plotted on the x-axis and the $-\log_{10}$(p-value, t-test) is plotted on the y-axis for all detected species in all samples. The software did not allow for internal standard normalization of samples. To screen the largest changes in the assay, high thresholds were set to compare test to control reactions at 225 min (last time point). Differences were determined to be significant if the peak areas between the test and control reactions were significantly different ($p < 0.05$) and demonstrated a $\log_2$(Fold-Change) of at least 2 (decreased or increased 4 fold). Compounds with a maximum peak area below 1000 were excluded. Peak areas for compounds of interest were exported from Compound Discoverer to Microsoft Excel®. Subsequent statistical tests were completed using OriginPro 2019b and Origin 2020b (Student Versions). Paired t-tests compared the weighted average of the molecular weight at reaction initiation (t = 0 min) and upon reaction termination (t = 225) for test reactions. Calculation for the weighted average can be found in **Appendix C.2**.

4.2 Results and Discussion

4.2.1 Solid phase extraction

The concentration of organics in BML OSPW (2014) was 80.0 mg/L. This is in line with the reported decrease in concentration from >150 mg/L in 2012 to <42 mg/L in 2015.[22] The SPE technique applied here did not separate basic from acidic components in F2 (**Figure 4-2**). Generation of the basic extract was successful overall as the basic and polar neutral organic species presented a distribution similar that of Dr. Sun.[22] The three largest contributing heteroatomic classes in the basic fraction were O^+ (43.64%), O_2^+ (22.12%), and O_3^+ (18.61%). Some O_2^- (10.82%) and O_3^- (3.19%) species could be detected in this fraction, but these were also present in the fraction by Dr. Sun (exact percentages not given).[22] The acidic extract was largely comprised of O_2^- species. The three heteroatomic classes with the highest responses were O_2^- (15.1%), O^- (4.44%), and O^+ (5.39%). There was also some contribution from O_3^- (5.04%) and O_2^+ (4.44%). Ultimately, naphthenic acids were not completely isolated from basic organic components, but, with the highest contribution in the acidic extract this was good enough for the current study.

The SPE method involved a series of steps which included (but were not limited to) proper column conditioning, the monitoring of system pressure, careful monitoring of column fraction elution, accurate pH adjustment, in addition to standard liquid-liquid extraction. While SPE is not a new technique,e it not been widely applied to the fractionation of dissolved organics in OSPW. Frank *et. al* was the first to report the use of similar technology by development of a large column (75 g of diethylaminoethylcellulose sorbent) to fractionate OSPW.[34] This method was slightly improved by Bauer *et al.* who aimed to optimize large volume extraction. The effect of solvents and SPE resin, the sequence of fractionation, and flow rates were assessed. Ultimately ENV+ as the best material for SPE extraction and successfully fractionated 180 L of OSPW. A few years earlier in 2015, a method for the extraction of naphthenic acids fraction components (NAFCs, organics that co-eluent with NAs) was developed using activated carbon as a sorbent.[35] Similarly, the various ways in which solvent and sorbent type effect the extraction of OSPW was recently reviewed by Alharbi *et al.*[36] While activated carbon was not reviewed in the later study, they recommend the use of a hydrophilic balance sorbent to profile organics in OSPW. Thus, there are several ways in which OSPW has been (and can be) extracted. My results

demonstrate the complexity of both the sample and the method. Requiring 8 – 12 hrs, this SPE is much faster than the previous online fractionation method which required many days - weeks for the same volume.[23] However application of the SPE method requires an art that cannot be described by the reported procedure.

4.2.2   Protein Quantification

The Bradford Assay was used to quantify purchased S9 and microsomal fractions. Calibration curves can be found in Appendix C.3 (**Figure C-3**). The concentration of proteins in microsomal fractions was reported by the manufacturer as 20 mg/mL. Protein concentrations in trout microsomes and rat microsomes were determined to be 15.8 and 14.5 mg/mL, respectively. The trout S9 fraction had a significantly lower amount at 1.0 mg/mL.

4.2.3   Enzyme Activity

Enzyme activity is measured by the EROD assay. When PAHs enter a cell, they initiate a cascade, or series, of reactions in the cell leading to the synthesis of detoxification enzymes.[37] CYPs are located not only in the liver, but in the gills and gastrointestinal tract of fish too.[9] These enzymes expose/add a polar substituent to the toxic chemical which will increase its ability to be excreted from the organism.

CYP1A1 converts 7-ethoxyresorufin to the fluorescent product, resorufin. In this way, the activity of the CYP1A1 enzyme can be detected fluorometrically using resorufin and allows for a sensitive method to determine fish exposure to PAHs and other planar aromatic compounds. The linear regression of fluorescence intensity and resorufin concentration is shown in **Figure 4-3**. Former tests indicate EROD activities were 4.5-fold higher in rat compared to trout.[20] The EROD activity in trout and rat microsomes generated 0.15 and 0.17 nM mg^{-1} min^{-1}. Rat enzymes produced concentrations only slightly higher than that of fish, contrary to what was expected. Due to time restrictions, both assays were conducted simultaneously at 30°C in the fluorometer, whereas Han *et al.* conducted experiments at the optimal conditions for each species (10°C for trout, 37°C for rats).[19] The difference in temperature likely impacted the outcome reported here by increasing and decreasing the activities of enzymes in trout and rat assays, respectively. Nonetheless, the results confirm the presence of functional CYP1A enzymes in both reference species.
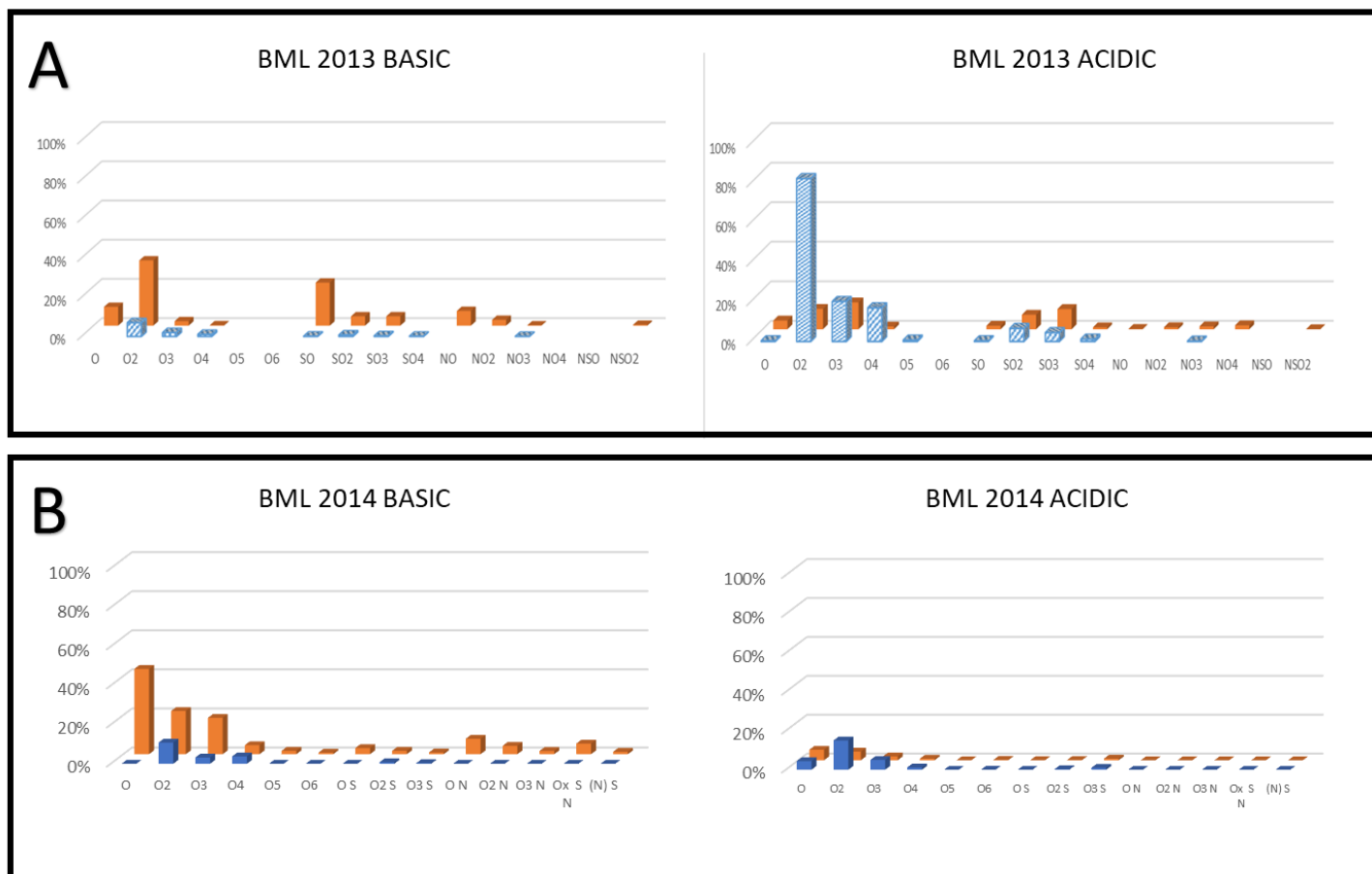
Figure 4-2 Basic (left) and acidic (right) extracts from BML OSPW from the years (A) 2013 and (B) 2014. Solid phase extraction method was developed by Dr. Chenxing Sun at the University of Alberta. Extraction of BML 2013 OSPW and MS analysis was performed on the Orbitrap using Electrospray ionization (ESI). I extracted BML OSPW from the following year to be used for this project and extracts were analyzed by atmospheric pressure ionization (APCI).
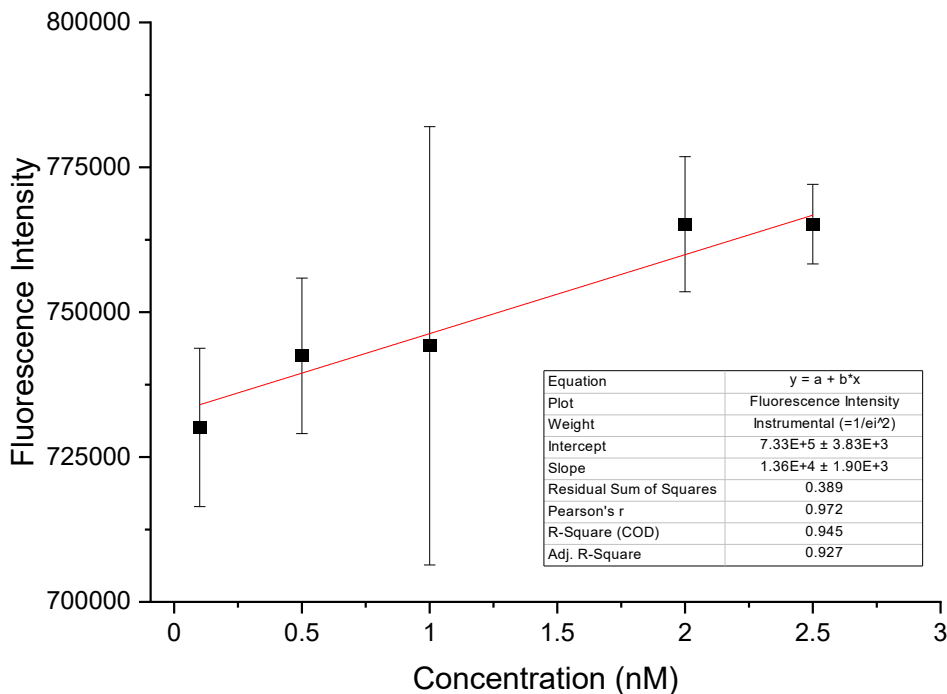
Figure 4-3 Linear regression of fluorescence intensity vs. resorufin concentration formed in the EROD assay. Regression values were used to quantify enzyme activities in purchased trout and rat microsomes.

4.2.4   Logistic progression of methodologies

4.2.4.1 Phase I metabolism of reference compounds

The reference compounds were chosen as they have been well-studied in both animals studied here. Although not surrogates for all the chemicals found in OSPW, they present an array of structural diversity (**Figure 4-1**). [13]C- Lauric acid represented a simple carboxylic acid whose metabolism could be detected in negative ionization mode. Neutral compounds to be analyzed in positive ion mode were testosterone and MBDB. Testosterone was chosen as an oxygen-containing polycyclic hydrocarbon (steroid) and MBDB selected as an example of a nitrogen-containing aromatic compound. Previous studies strongly suggest the formation of hydroxylated products for these compounds. No standards were used in this study to confirm the identities of the products, instead the exact masses (*m/z*) provided a good indication of the products.

Hydroxylation was reported by a mass shift of 16 amu representing the replacement of a hydrogen atom with a hydroxyl group (i.e. -H + OH = +O).

CYP1 is a well studied family of enzymes in fish with the most studied form being CYP1A. These enzymes oxidize endogenous and environmental chemicals. Oxidation by CYP enzymes in the presence of molecular oxygen (O_2) is essentially the most frequent reaction which occurs in phase I metabolism.[4] Hydroxylation of lauric acid occurs at different positions. The terminal (ω) carbon is typically catalyzed by CYP4A isoforms in mammals, and the second last carbon (ω -1) can be hydroxylated by a larger variety of enzymes including (but not limited to) CYP1A1, CYP2A1, and members of the CYP2C subfamily. In trout, CYP2K1 and CYP2M1 were previously isolated and have been proven to hydroxylate lauric acid, but other isozymes also contribute. While the $\omega - 1$ (subterminal) carbon atoms are most reactive, $\omega - 2$ to $\omega - 6$ products have all been detected in trout liver microsomes.[39]

Phase I metabolism of ^{13}C-lauric acid (*m/z* 201.31) generated two products with *m/z* 217.17 which eluted at 6.10 and 6.30 minutes in trout microsomes (**Figure 4-4A**). The chromatographic peak detected for this product in the rat assays (**Figure 4-4B**) barely had a valley potentially reflecting two closely related structural isomers were generated. Buhler *et al.* reported the ω-1 hydroxylated product of ^{13}C-hydroxydodecanoic acid is formed at a higher rate,[20] and that this metabolite eluted earlier than products on a Zorbax ® HPLC chromatographic column – a C18 column like the one used here.[39] Both trout and rat microsomes exhibited a time-dependant linear formation of products (**Figure 4-5**). Trout microsomes increased steadily over 2 hr of the experiment, but rat biotransformation appeared to reach saturation after 30 minutes. Further biotransformation may have required additional cofactor (NADPH) to be added. The lowered activity of trout subcellular fractions compared to that of rats was also observed by Han *et al.*[21]
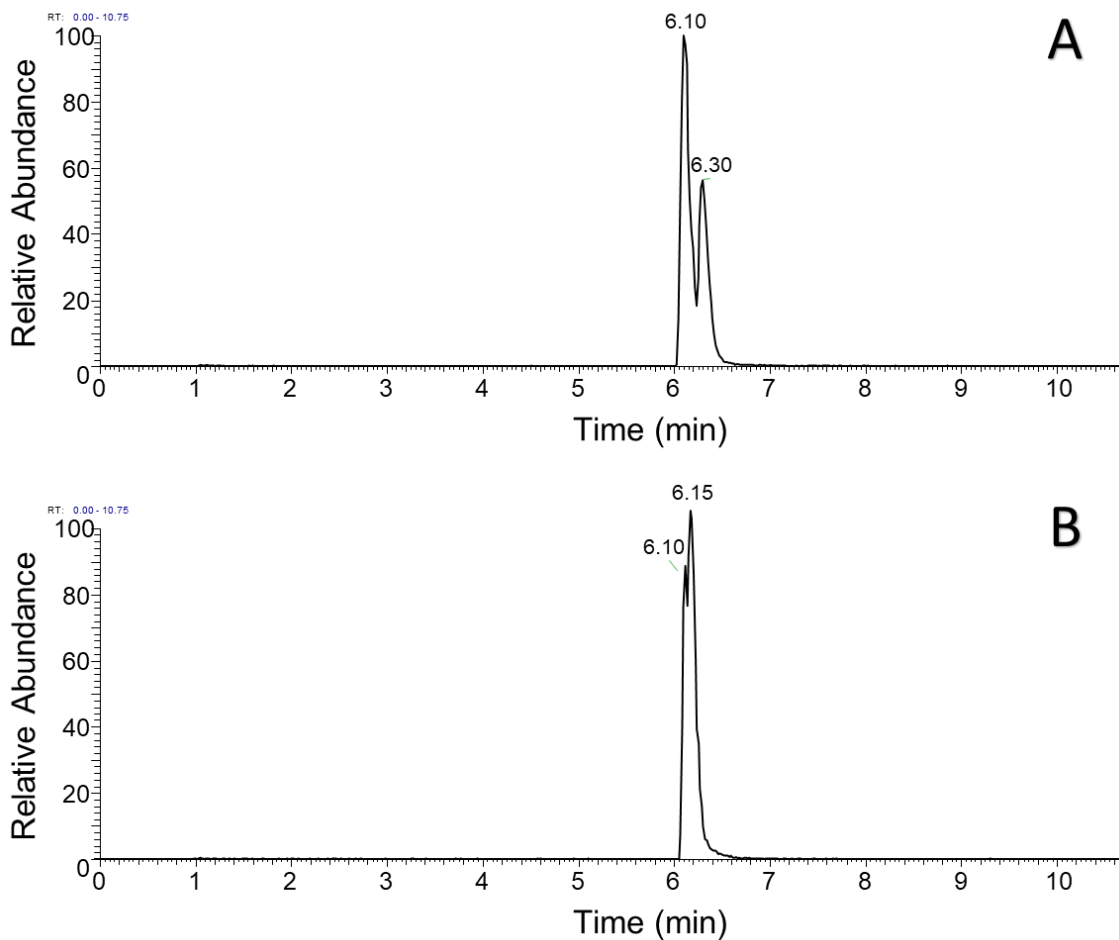
Figure 4-4 Chromatogram of the phase I hydroxylated products of ^{13}C-Lauric acid in (A) trout and (B) rat microsomes after 120 min. Two products eluting at 6.10 and 6.30 min may represent the ω and ω-1 hydroxylated products in the trout assay, while it is likely that two structural isomers are presented at 6.10 and 6.15 min in the rat assay.
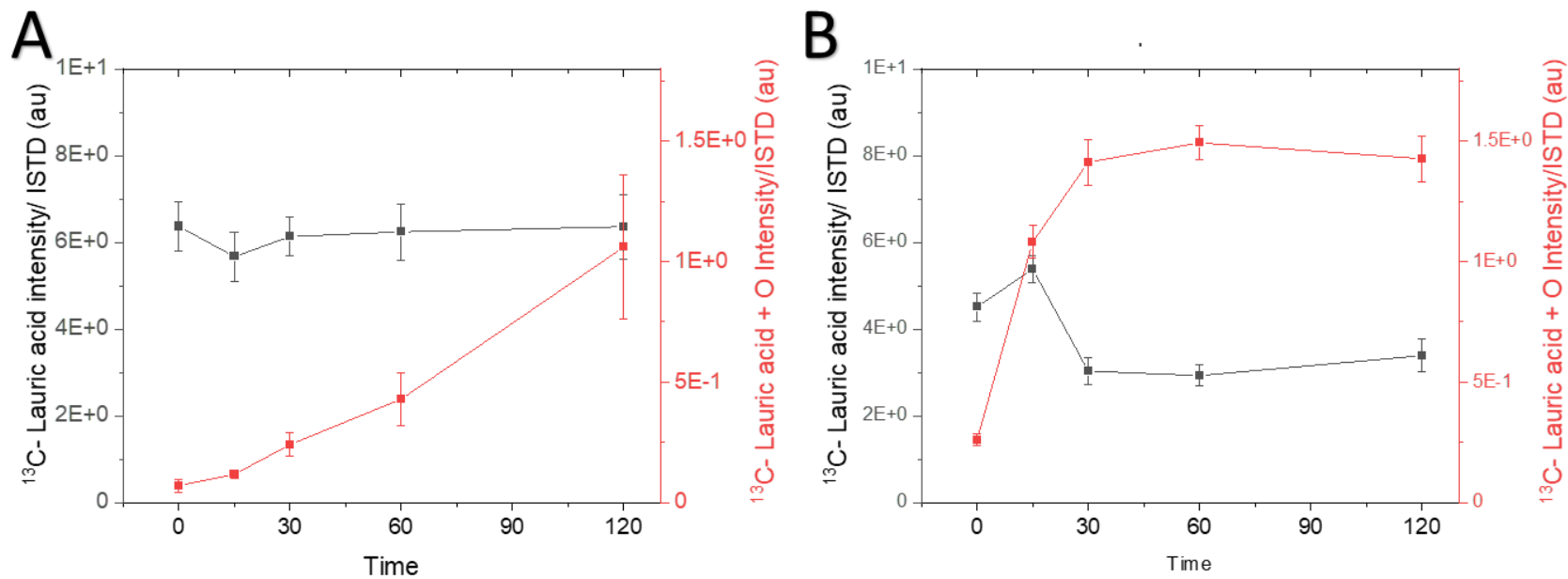
Figure 4-5 Plots of mass spectral peak intensity vs time for (A) trout and (B) rats. Mass spectral peak intensities of ^{13}C-lauric acid values are shown in black; mass spectral peak intensity of the hydroxylated product is shown in red. Evolution of the product increased linearly in trout but appeared to reach saturation in rats after 30 min.

The metabolism of MBDB and testosterone (m/z 207.12) were assessed simultaneously. Due to an error, no time point was recorded at initiation (time 0 min) in rat incubations for these compounds. To ensure all studies had an equal number of time points, an additional aliquot was taken (for interest's sake) one hour past t = 225 min.

Testosterone (m/z 288.21) was metabolized to the greatest extent compared to the other reference compounds studied. The hydroxylated product (m/z 304.20) was detected at intensities up to two orders of magnitude greater in rats than in trout (**Figure 4-6**). Activity ratios have previously been reported to be 3.4 to 14.4 times higher in rats and the activity ratio for hydroxylation of testosterone to 6β-hydroxytestosterone reported at 4.5.[19] This result is in line with previous findings by Nabb *et al.* who reported the rat to trout activity ratio of the corresponding enzyme (CYP3A) to be 11.8 in hepatocytes.[20] Only one chromatographic peak was detected in all assays (data not shown). The 6β-hydroxylated product is most often detected, testosterone can also be hydroxylated at the 2β- and 16β- positions (**Figure 4-7**).[3] The product concentrations in the rat assay appeared to be highly variable but hydroxylation appeared to steadily increase over the duration of 2 hours. This may have been due higher matrix effects in rat assays compared to fish assays.

MBDB (m/z 207.12) is a controlled psychoactive drug which had been previously studied in human liver microsomes.[40] Two products were detected in both trout and rat microsomes in low amounts (**Figure 4-8**). The highest intensity of the hydroxylated product was at least 2 orders of magnitude lower than the testosterone in trout and over 3 orders of magnitude lower in rats. The hydroxylated (+O, m/z 223.118) and demethylated products (-CH_2, m/z 193.107) appeared to evolve linearly in trout but highest amounts in rat assays were achieved after 1 hour. The demethylated (-CH_2) product decreased in the rat assay beyond 1 hour. The limited ability of microsomes to metabolize MBDB ruled this compound out as a reference for the future experiments.

The results align well with what had been observed in literature and demonstrated generally, rat microsomes had higher activities (produced more product mg^{-1} min^{-1}, **Figure 4-9**). Furthermore, the results demonstrated that the assays worked well. Thus, the conditions of these assays were carried forward to the next test experiment using a reference mixture of organics. Given their success in these initial assays, I decided to add ^{13}C-Lauric acid and testosterone as references to all incubations of BML OSPW toxic fractions.
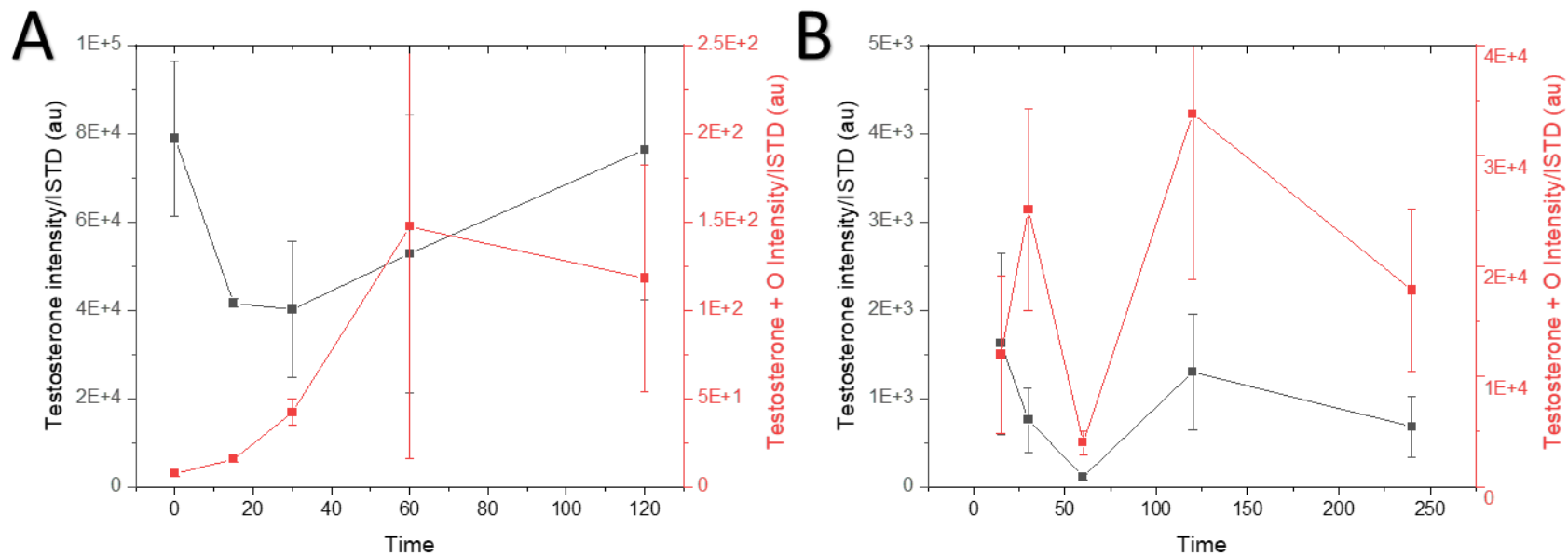
Figure 4-6 Plots of mass spectral peak intensity vs time for (A) trout and (B) rats. Mass spectral peak intensities for testosterone are shown in black; mass spectral peak intensities for hydroxylated product shown in red. Mass spectral peak intensities of the product in rats was at least 2 orders of magnitude higher in rats compared to trout.
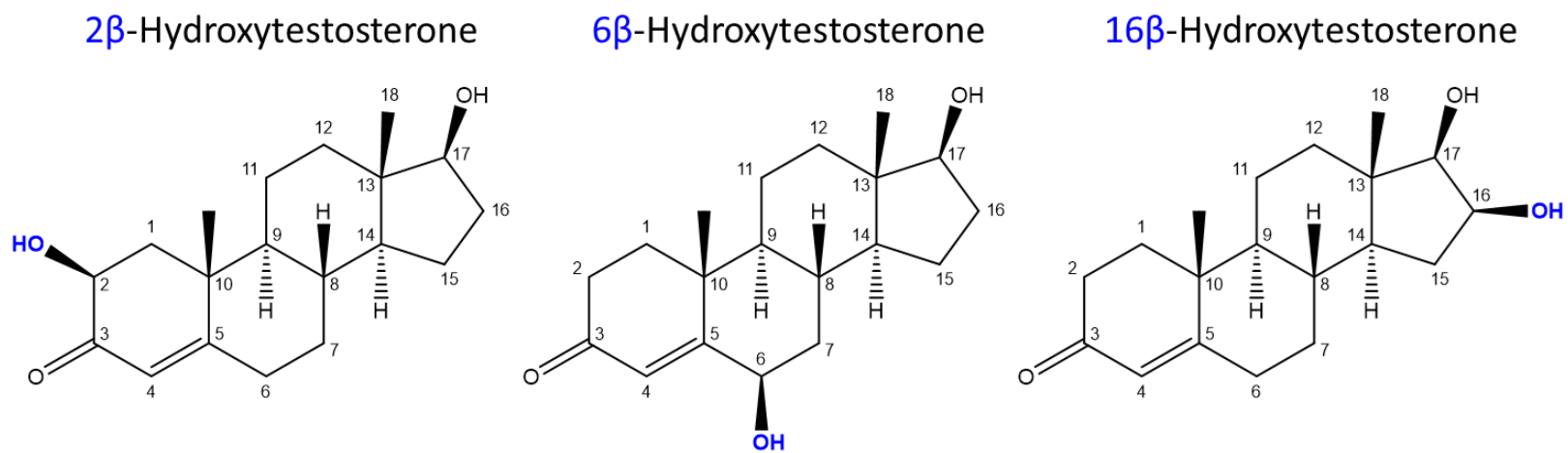
Figure 4-7 Common products of the phase I hydroxylation of testosterone.
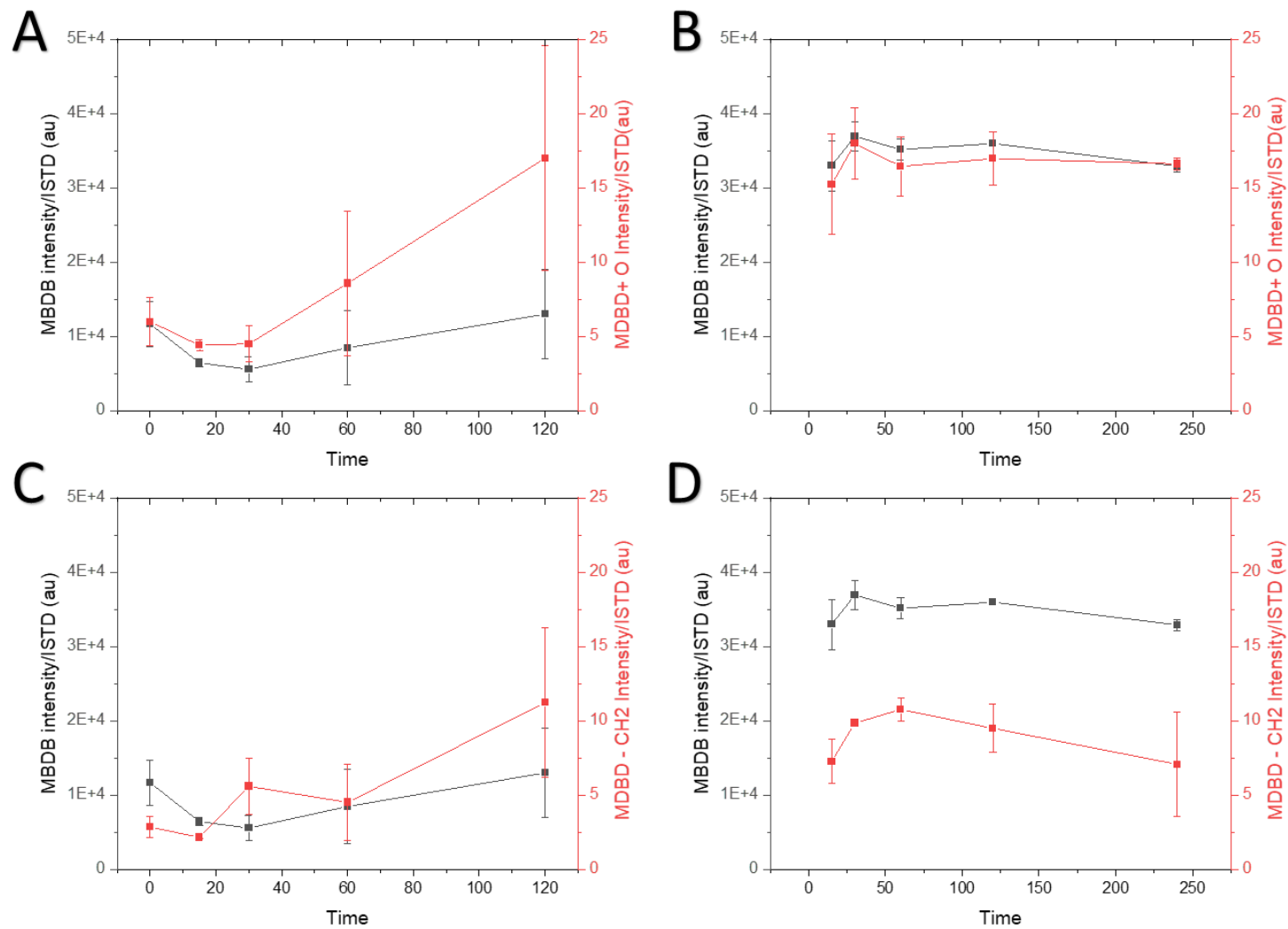
Figure 4-8 Plots of mass spectral peak intensity vs time for trout (A, C) and rats (B, D). Mass spectral peak intensities for MDBD are shown in black; mass spectral peak intensities for hydroxylated (A, B) and demethylated (C,D) products are shown in red .
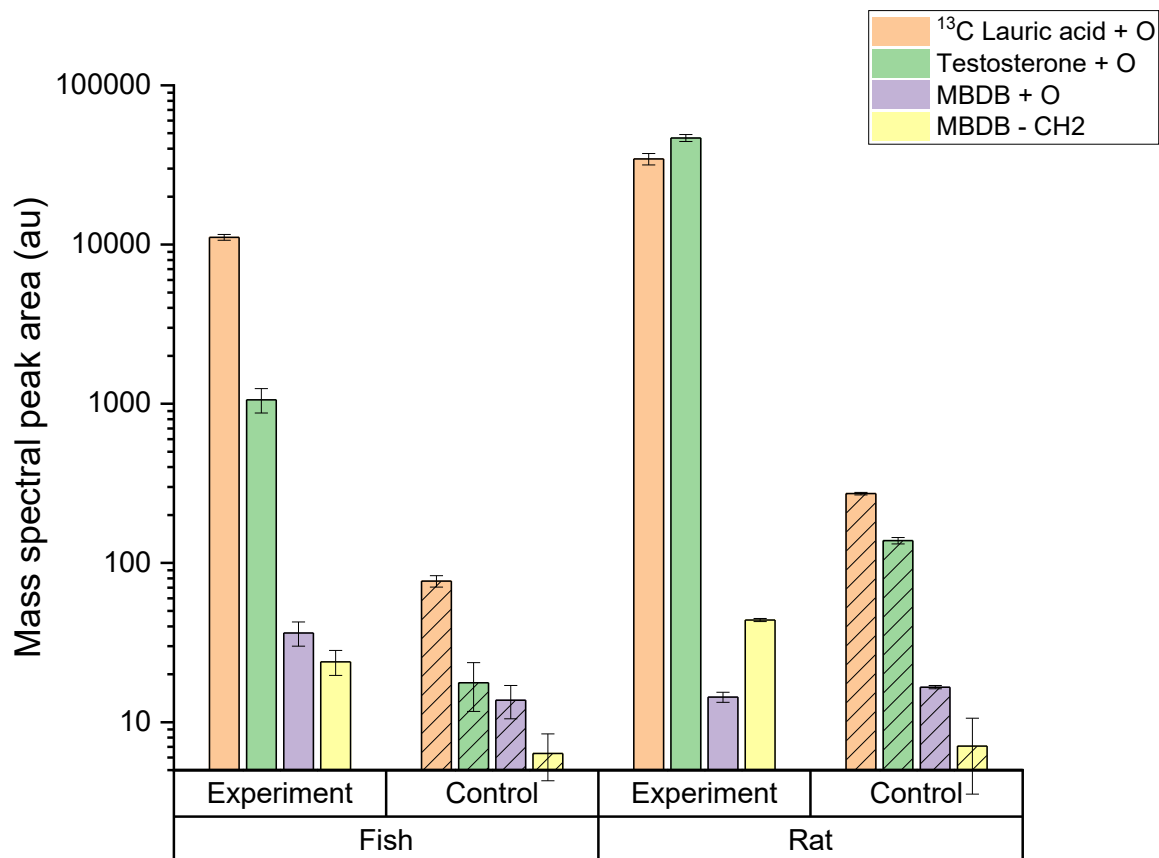
Figure 4-9 Products of phase I metabolism of reference compounds in trout (120 min) and rat microsomes (240 min). Hydroxylated ^{13}C-Lauric acid (pink), hydroxytestosterone (green), hydroxylated MBDB (purple), and demethylated MBDB (yellow) are shown for trout (left) and rats (Right). Control reactions were performed without NADPH and the products of these incubations are denoted by hashed bars.

4.2.4.2 Phase I metabolism of Merichem NAs

The workflow used to analyze the phase I metabolism of Merichem NAs included the node "Search mass list" (**Figure C-2**). The mass list was created in Excel and included 130 O_2^- species (NAs) identified in BML OSPW by research associate, Dr. Alberto Dos Santos Pereira in 2014. The parameters of the list included a generalized name (identified by carbon number and DBE ex. 'C$_{12}$ DBE 3'), the formula of the molecular ion (ex. $C_{12}H_{17}O_2$) and the *m/z* ratio for each species. Using the non-targeted metabolomics workflow, 322/618 and 303/431 unique features were identified by the Predict compositions node in rat and trout assays, respectively. This indicated the mass list was not properly utilized – likely because the list required molecular weight of the neutral molecule and not that of the ion. Mass lists were only used for this experiment in the project.

One objective of this experiment was to assess the ability of Compound Discoverer software to accurately integrate the peaks of Merichem NAs. Typically, the total ion chromatograms of organic mixtures from OSPW are viewed as 'humpograms' due to many co-eluting, overlapping unresolved peaks. Commercial mixtures of NAs are simpler than those found in OSPW, so were chosen as an example to review how peaks of individual species would be picked and integrated. To answer this question, the results were sorted by those which had a formula which contained O2 and did not contain N. Species identified were compared to the previously described mass list. O_2 species were accurately integrated by the software (130 and 110 species in trout and rat assays, respectively). This demonstrated the CD workflow could in fact be trusted to detect and integrate unique species in a mixture, and likely would be useful for OSPW.

After 225 minutes, 53 species had increased in trout assays, but no species reached the p-value threshold (**Figure 4-10**). On the other hand, 6 species significantly increased in rat assays by the same time (**Table C-1**, **Figure C-5**). It is worth mentioning more than half (29) of these species contained at least 3 oxygen atoms which suggested oxidation was occurring. The differences between O_2^- species in OSPW and those in the Merichem commercial mixture has been discussed elsewhere.[41,42] The biggest difference being that Merichem NAs tend to have fewer DBEs than do the bitumen derived organics found in OSPW, but both are found in mixtures. Therefore. I hypothesized that enzymes in fish or rat microsomes could metabolize the dissolved acidic species in OSPW.
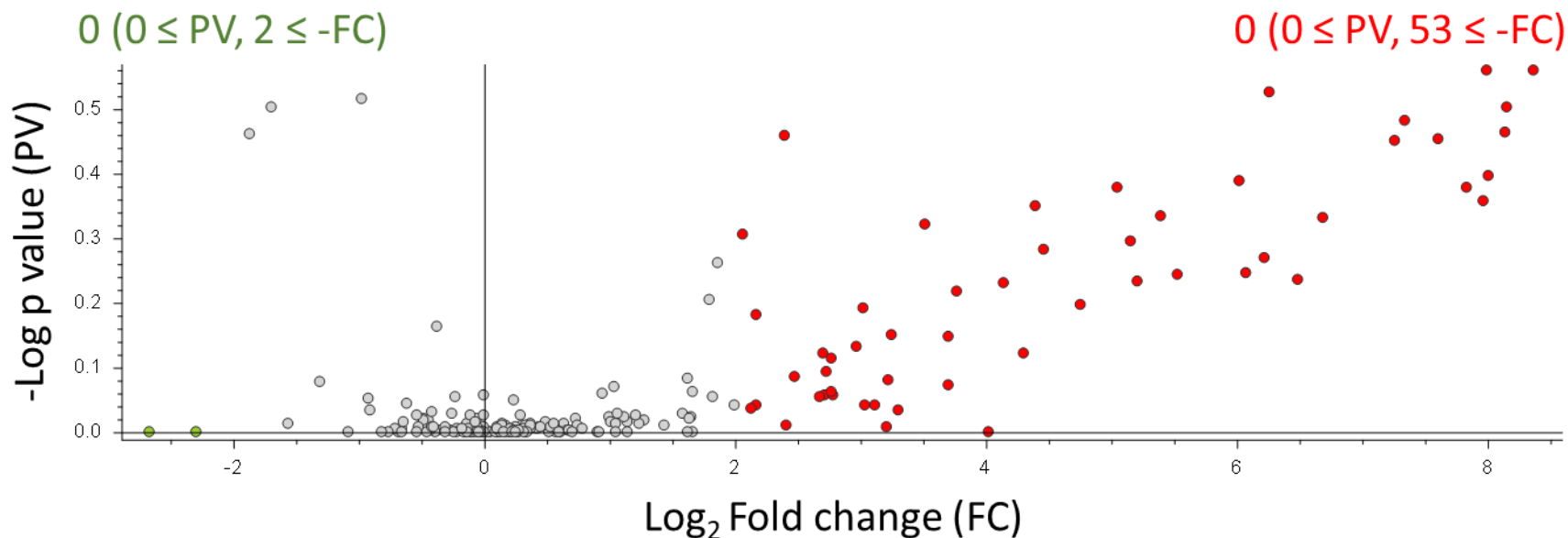
Figure 4-10 Volcano plot of phase I metabolism of Merichem NAs in trout microsomes. Log_2(Fold Change) is shown on the x-axis and p-value difference between test and control (n = 3 each) is shown on the y-axis. Species within the shaded boxes represent those which have met both the p-value (p < 0.05) and log_2(Fold-Change> 2) cut-off and have significantly decreased (green) or increased (red). Species which have met the Fold-Change cut-off but were not statically different in tests compared to controls fall outside of the shaded boxes but are coloured accordingly. No species had significantly changed in these assays, but 53 species had increased by at least 4 times ($log_2 > 2$).

4.2.5   Phase II metabolism of reference compounds

UGTs are an important class of phase II enzymes.[3] Glucuronide conjugation ($+C_6H_8O_6$) to testosterone was observed in low amounts (maximum peak area 486, data not shown) in trout microsomal reactions. Products formed in with testosterone appeared to be produced linearly (first order) with time. This transformation was not observed in any other reaction using reference compounds (data not shown). Given that glucuronidation was not observed using most reference compounds, I hypothesized that glucuronidation of OSPW dissolved organics would not occur in trout microsomes.

4.2.6   Phase I metabolism of BML dissolved organics in Trout microsomes

4.2.6.1 Phase I metabolism of Basic extract

In the trout microsomal assay, 1531 species were detected in the phase I transformation of polar neutral and basic dissolved organics (**Figure 4-11**). Only 1 species decreased significantly in this assay: $C_{18}H_{34}O_2$. However, 52 species did increase (**Figure 4-12**). Three of which were aliphatic compounds (not shown) and 1 compound containing 5 nitrogen atoms and 6 DBE ($C_5H_5N_5$). Over 1/3 of the species which increased belonged to the O_2^+ heteroatomic class of organics. Many of the products of this reaction high molecular weights (> 268 amu) and anywhere from 17 to 30 carbon atoms. These compounds appeared to represent a diverse array of structures having between 1 to 7 DBEs. The species evolved provide evidence for the generation of oxygenated compounds. The weighted average of all species detected showed no significant difference test assays at reaction initiation compared to termination ($p > 0.05$, **Table C-3**) which confirms metabolism by trout microsomes did not change the profile of basic and neutral compounds to a considerable degree.
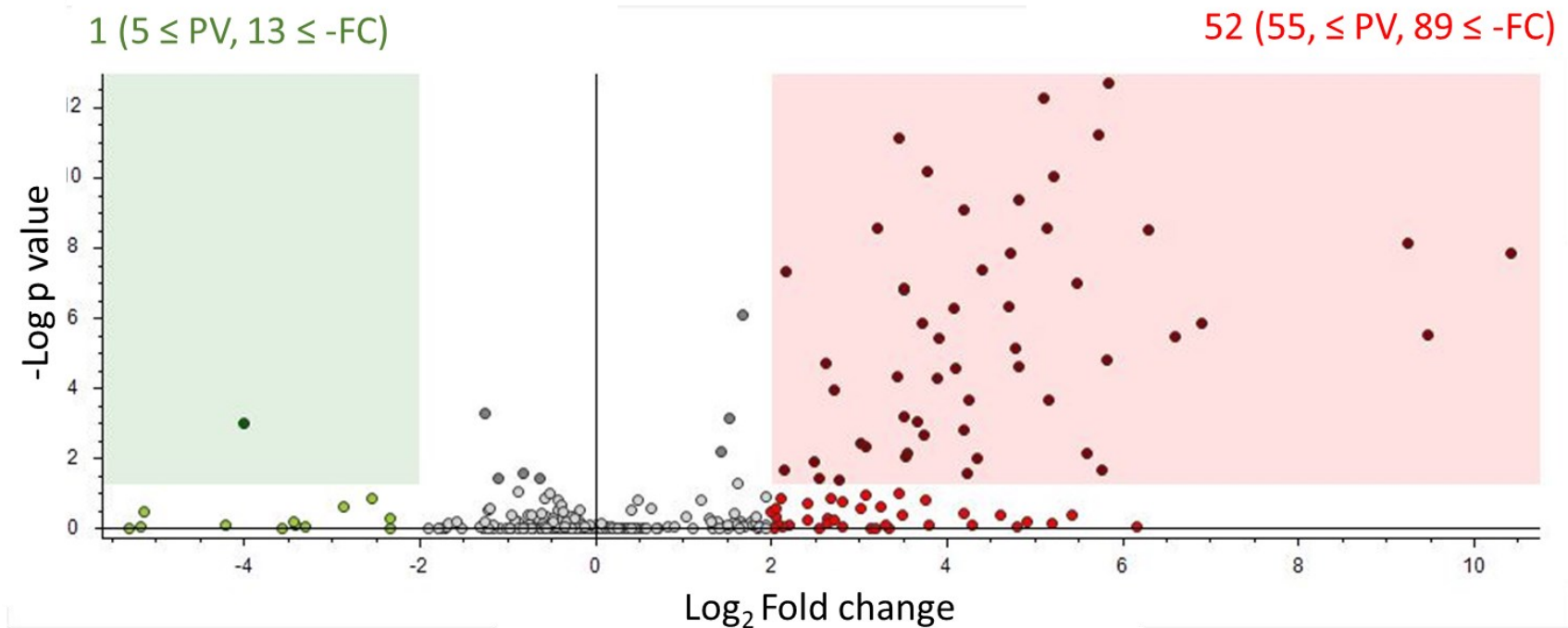
Figure 4-11 Volcano plot of phase I metabolism of BML basic extract in trout microsomes. Log_2 (Fold-Change) is shown on the x-axis and p-value difference between test and control (n = 3 each) is shown on the y-axis. Species within the shaded boxes represent those which have met both the p-value (p < 0.05) and log_2(Fold-Change > 2)cut-off and have significantly decreased (green) or increased (red). Species which have met the Fold-Change cut-off but were not statically different in tests compared to controls fall outside of the shaded boxes but are coloured accordingly. At 225 min, 52 species had increased and 1 decreased by at least 4 times (log_2 > 2).
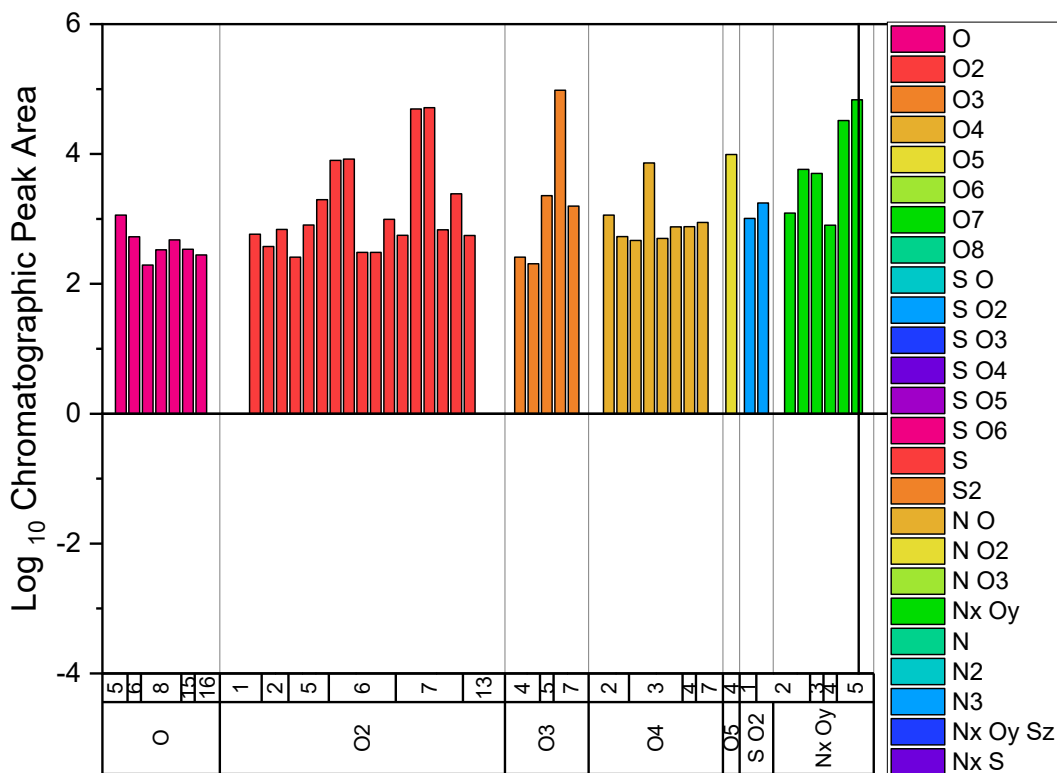
Figure 4-12 Summary of species evolved (and transformed) in phase I metabolism of BML basic extract in trout microsomes. Species displayed met both the p-value ($p < 0.05$) and $\log_2$(Fold-Change >2) cut-off. Species are organized by heteroatomic class, and the number of double bond equivalents on the x-axis. The $\log_{10}$Chromatographic peak area is on the y-axis. Any species which increased is shown above 0, while those which decreased are shown below 0. All species evolved had at least one oxygen atom.

Plots of peak area vs. time followed three general trends: some species appeared to increase linearly with time, others appeared to have reached their maximum concentration early on, while a few appeared to be generated and then transformed (**Figure 4-13**). A fast initial formation of a first product, and the subsequent drop (at 15 min) suggested the product was undergoing an additional transformation. Three species ($C_{19}H_{26}$, $C_{19}H_{28}O$ and $C_{19}H_{30}O_2$) followed this profile. The evolution and biotransformation of this species may reflect the affinity of enzymes present for compounds like this – with 6 DBE, it is likely to be an aromatic or cyclic substrate which could be biotransformed by CYP1A isoforms.
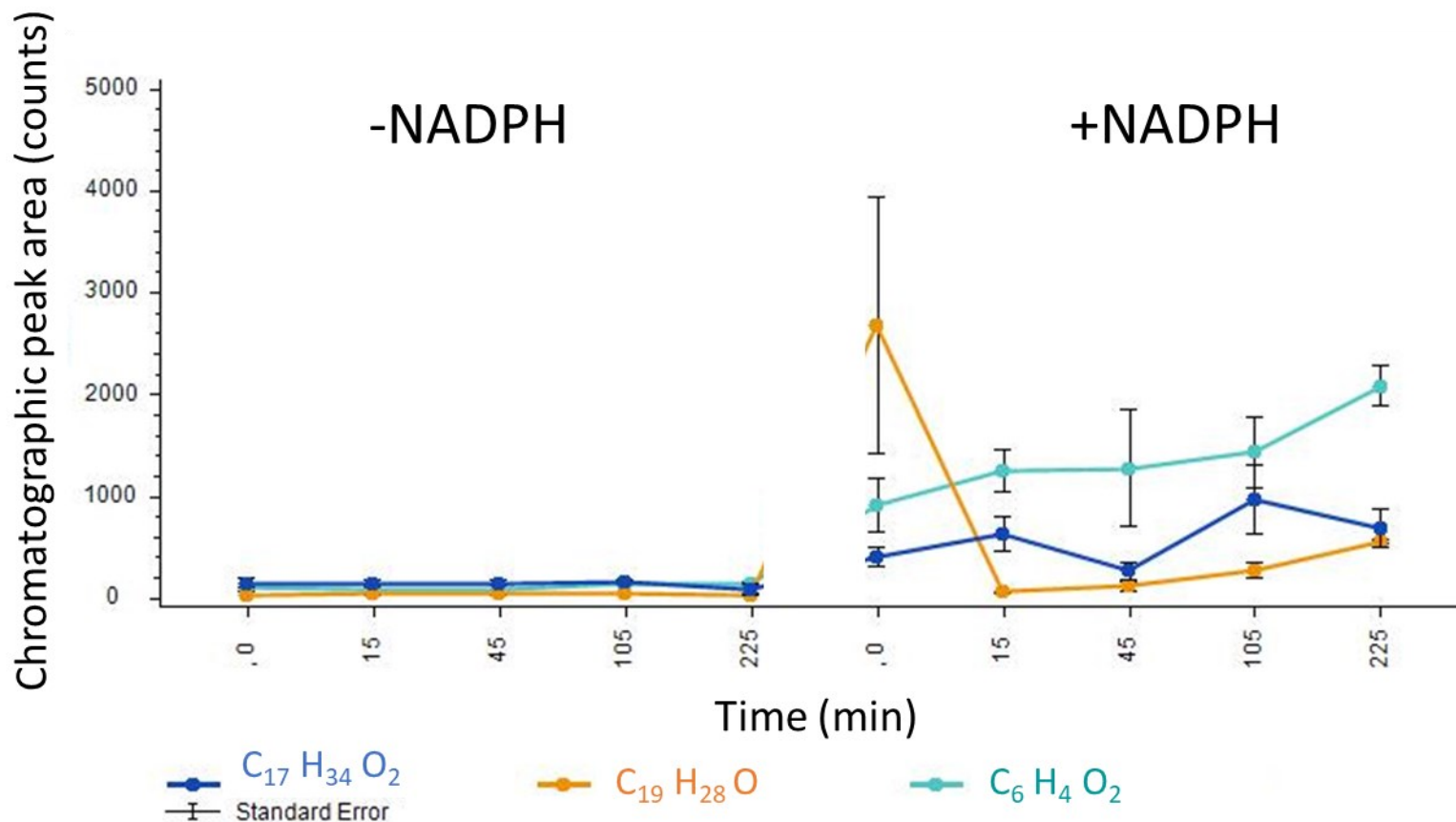
Figure 4-13 Negative controls (- NADPH) compared to the test (+ NADPH) for three generalized reactions profiles observed in the phase I metabolism of basic dissolved organic fraction of BML OSPW in trout microsomes. Steep decrease followed by a slow increase (ex. $C_{19}H_{28}O$); progressive increases and decreases (ex. $C_{17}H_{34}O_2$); and a steady increase (ex. $C_6H_4O_2$).

Most species appeared to undergo series of increases and decreases like $C_{17}H_{34}O_2$. These species appear to have reached saturation after 15 minutes of the reaction. Finally, the profile of $C_6H_4O_2$ is the profile of the smallest molecules which evolved (<150 amu).

4.2.6.2 Phase I metabolism of Acidic extract

In this experiment, 935 unique compounds were detected using the applied workflow. Relative to controls, just 6 of these compounds had increased and 2 decreased after 225 min (Figure 4-14). Two simple $C_8H_{16}O_2$ and $C_{16}H_{30}O_3$ (1 and 2 DBEs, respectively) decreased, but those which increased were more structurally diverse as they had 2 to 7 DBEs. Overall, the species which evolved followed first order kinetics (R2 of 0.66 - 0.93, Table C-2, Figure C-6) but only two species ($C_{16}H_{32}O_3$ and $C_{16}H_{30}O_4$) had significant linear slopes ($p < 0.05$,

Figure **4-15**). These species had 1 and 2 DBEs. This contrasts with earlier findings where hydroxylated 13C-lauric acid (1 DBE) which showed a linear increase with time (zero-order kinetics). The 4 other species which larger and more complex structures with $C_{20} - C_{22}$ and 6 or 7 DBEs. All species which evolved had O_3 or O_4. Surely, this reflects oxidation which occurred in the mixture.

The weighted average of all species detected show no significant difference between test at the beginning and end of the experiment ($p > 0.05$, **Table C-3**). The data suggests a greater proportion of products were generated in the metabolism of basic (2%) compared to acidic compounds (<< 1%) following phase I metabolism. Ultimately, most compounds in both mixtures appear relatively unchanged but these preliminary findings suggest that trout microsomes have the capacity to hydroxylate some organics in toxic fractions in BML OSPW.
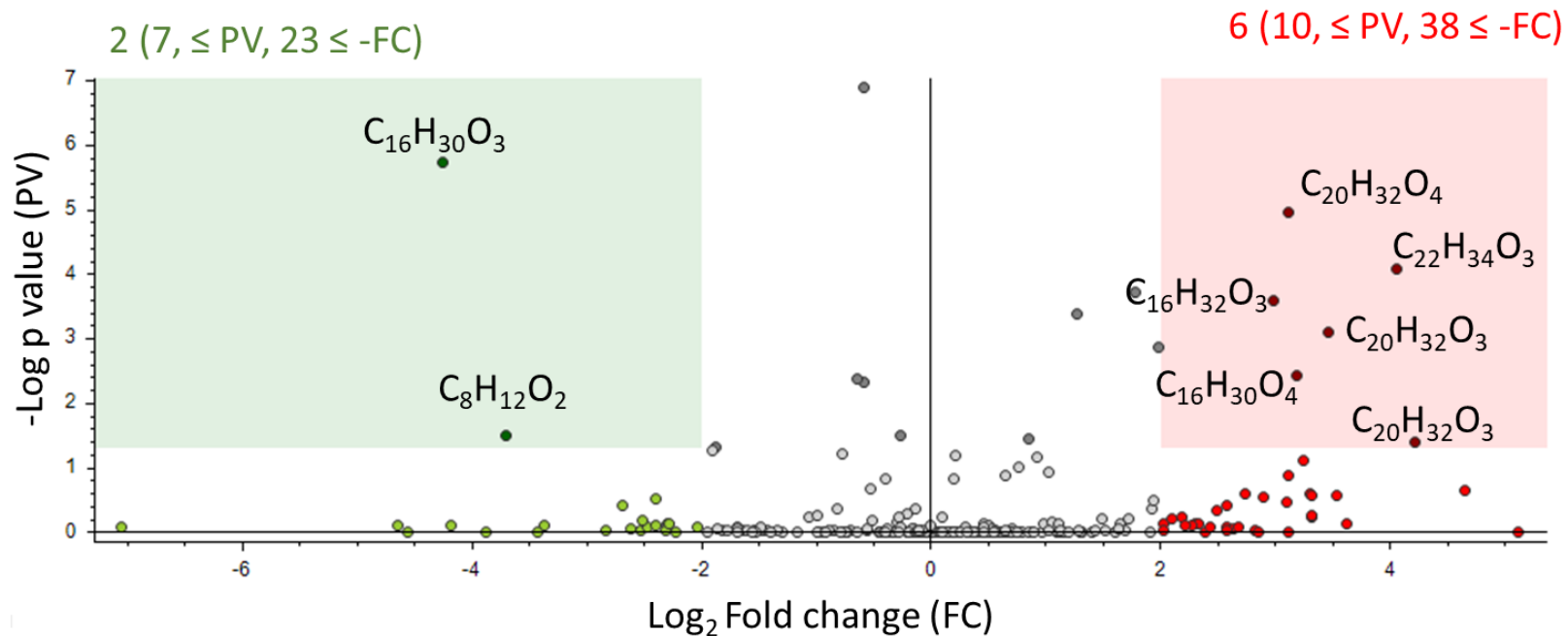
Figure 4-14 Volcano plot of phase I metabolism of BML acidic extract in trout microsomes. Log_2Fold Change is shown on the x-axis and p-value difference between test and control (n = 3 each) is shown on the y-axis. Species within the shaded boxes represent those which have met both the p-value ($p < 0.05$) and log_2(Fold-Change > 2)cut-off and have significantly decreased (green) or increased (red). Species which have met the Fold-Change cut-off but were not statically different in tests compared to controls fall outside of the shaded boxes but are coloured accordingly. Only 6 species had significantly increased, and 2 species decreased after 225 minutes.
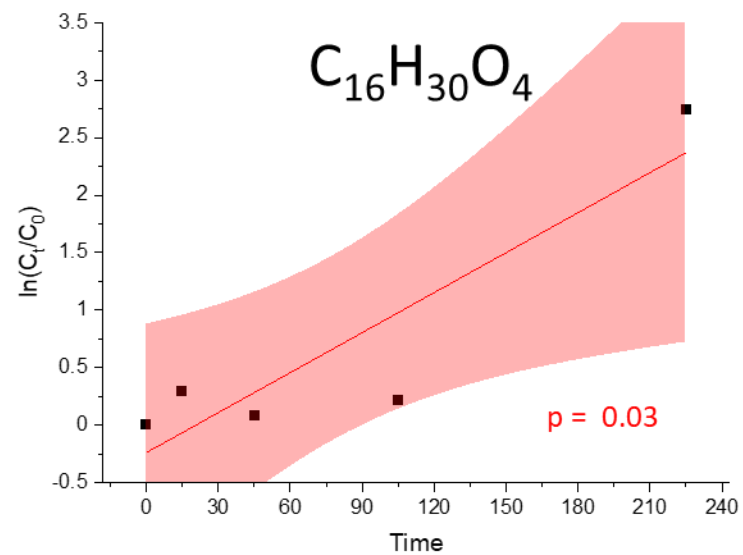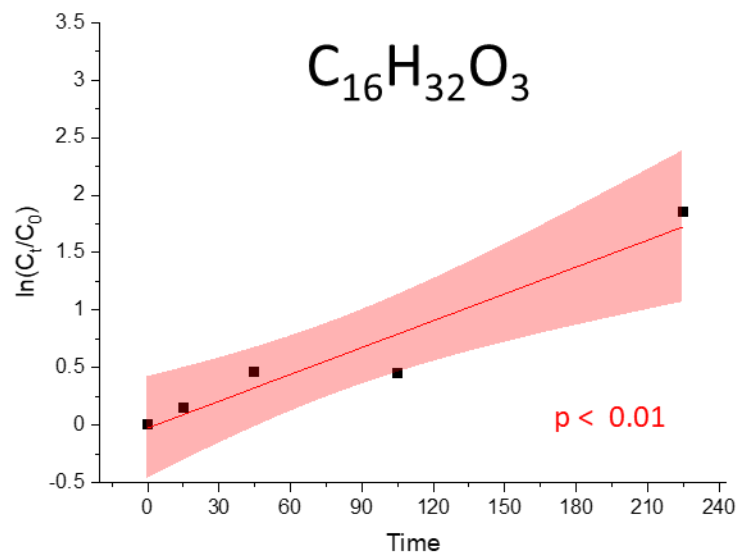
Figure 4-15 First order kinetic plots of $C_{16}H_{32}O_3$ and $C_{16}H_{30}O_4$ produced in phase I biotransformation of BML acidic fraction in trout microsomes.

4.2.7   Phase I + II metabolism of BML Dissolved organics

4.2.7.1 Phase I + II metabolism Basic fraction

For simplicity, this study limited observations to glucuronidation in microsomes as it is one of the most common phase II reactions. UDP was also added to S9 but given that endogenous levels of other cofactors may be present, other phase I + II reactions could (theoretically) have occurred. UDP-glucotransferases (UGTs) are found primarily in the liver and intestines but are also located in gills and kidneys of fish.[3] These enzymes are bound to the membranes of the endoplasmic reticulum (and therefore microsomes) with the active site facing inwards (towards the lumen).[28] UGTs are reported to work together with CYPs by using the hydroxyl-products of phase I metabolism and converted to their glucuronide equivalents.[28] Depending on size, glucuronide products are then excreted into to urine (<350 amu) or bile (> 350 amu). Alcohols, phenols, and carboxylic acids may be converted to glucuronides via an oxygen atom (O-glucuronidation), while amides, amines, and carbamates are reacted at nitrogen atom (N-glucuronidation).[3] It is not only useful for metabolism of xenobiotics but endogenous compounds (testosterone, estradiol, and bilirubin) are also substrates for UGTs in fish.[3,44] Research on the biotransformation by UGTs in mammals has been greatly studied because of their usefulness in understanding human metabolism of drugs. These reactions in fish (and other organisms) only gained interest in the late 2000s.[3]

At termination, 42 of 1177 had increased and just 1 decreased (**Figure 4-16**). Glucuronic acid ($C_6H_{10}O_7$) is a sugar molecule and with many electronegative oxygen atoms, its conjugation greatly increases the solubility of toxicants lending to facile elimination from the organism. The conjugation of glucuronide ($+C_6H_8O_6$) should result in products with at least 6 oxygen atoms, but it is evident that products detected had a maximum of 4 oxygen atoms (**Figure 4-17**). To add, a similar number of products were generated following just phase I metabolism of the basic organic fraction of BML OSPW (52 in Phase I vs 42 in Phase I + II). As seen in phase I, paired t-test for the weighted average of all species detected in test assays showed the molecular weight did not change in the experiment ($p > 0.05$, Table C-3). This evidence suggests, the products of this experiment are due to phase I metabolism.
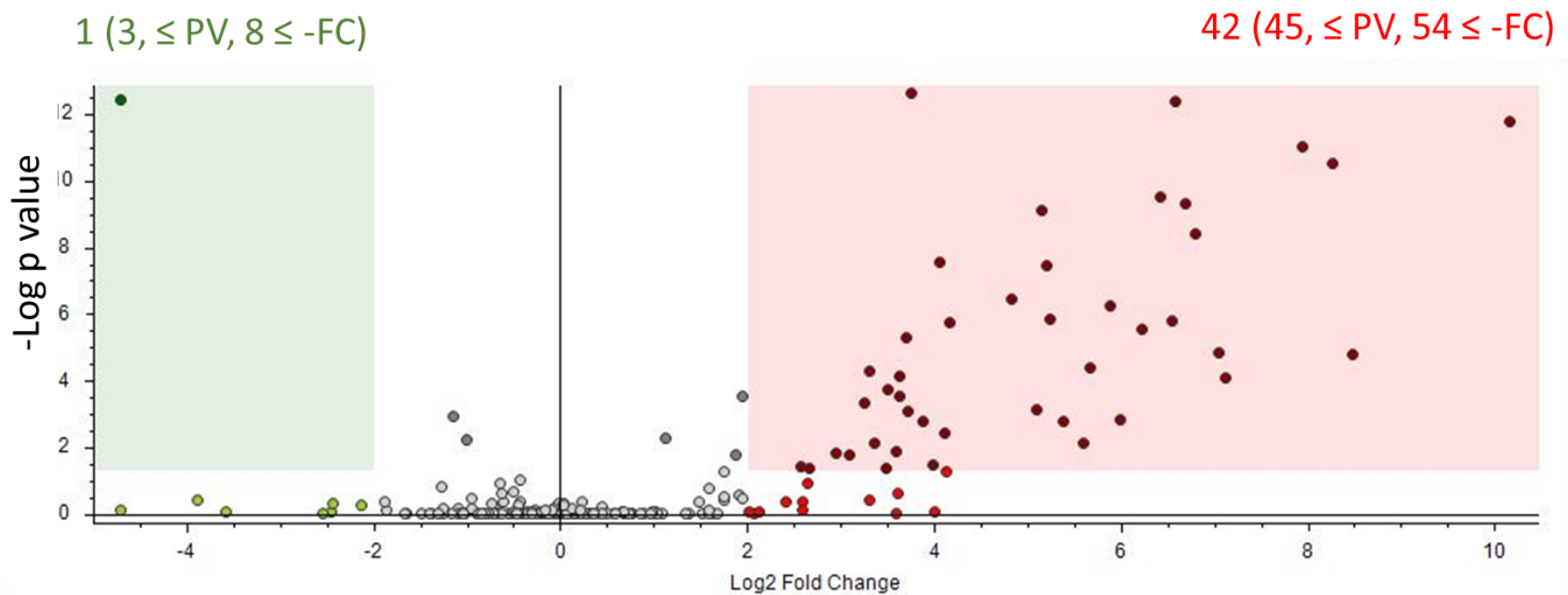
Figure 4-16 Volcano plot of phase I + II metabolism of BML basic extract in trout microsomes. Log₂Fold Change is shown on the x-axis and p-value difference between test and control (n = 3 each) is shown on the y-axis. Species within the shaded boxes represent those which have met both the p-value (p < 0.05) and $\log_2$(Fold-Change >2) cut-off and have significantly decreased (green) or increased (red). Species which have met the Fold-Change cut-off but were not statically different in tests compared to controls fall outside of the shaded boxes but are coloured accordingly. At 225 min, 52 species had increased and 1 decreased by at least 4 times ($\log_2 > 2$).
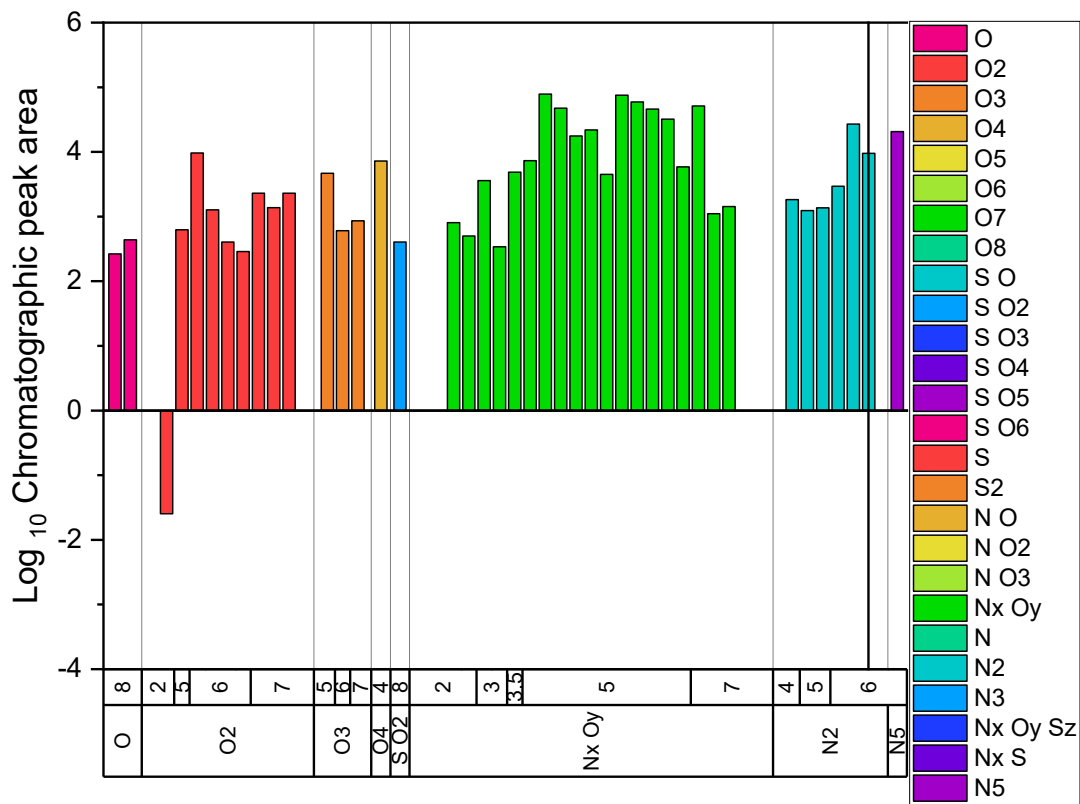
Figure 4-17 Summary of species evolved (and transformed) in phase I + II metabolism of BML basic extract in trout microsomes. Species are organized by heteroatomic class, and then the number of double bond equivalents on the x-axis and the Log_{10} Chromatographic peak area on the y-axis. Any species which increased is shown above 0, while those which decreased are shown below 0. Species displayed met both the p-value ($p < 0.05$) and log_2(Fold-Change > 2) cut-off.

Each experimental model has its advantages and disadvantages. S9 offers a more diverse array of phase II enzymes, but the concentrations of proteins in this matrix are much lower than they are for microsomes.[19] Higher enzyme concentrations in microsomes lend to faster clearance rates within a study. Metabolism by S9 has been said to provide a more complete picture of metabolism.[45] Sulfotransferase (SULT), glutathione-S-transferase (GST), and N-acetyl transferase (NAT) enzymes are present in the cytosol and contribute to phase II metabolism. For this reason, the metabolism of BML extracts in S9 were also studied.

When S9 was compared to the phase II microsomal metabolism, the biggest difference was the greater number of species generated in the former (**Figure 4-18**). When the two were compared, 243 compounds had higher chromatographic peak areas in S9. More sulfur- and nitrogen-containing metabolites were evolved in S9 (**Figure 4-19**). Another notable observation was that the metabolites of the S9 fraction spanned a larger range of molecular weights from 100 to 425 *m/z*. To add, the species which were higher in trout microsomes had no more than 4 DBEs which suggested fewer aromatic compounds were metabolized by microsomes compared to S9. Most importantly, no O_6 species were observed following phase I + II metabolism of the basic extract of BML OSPW in either microsomes or S9.
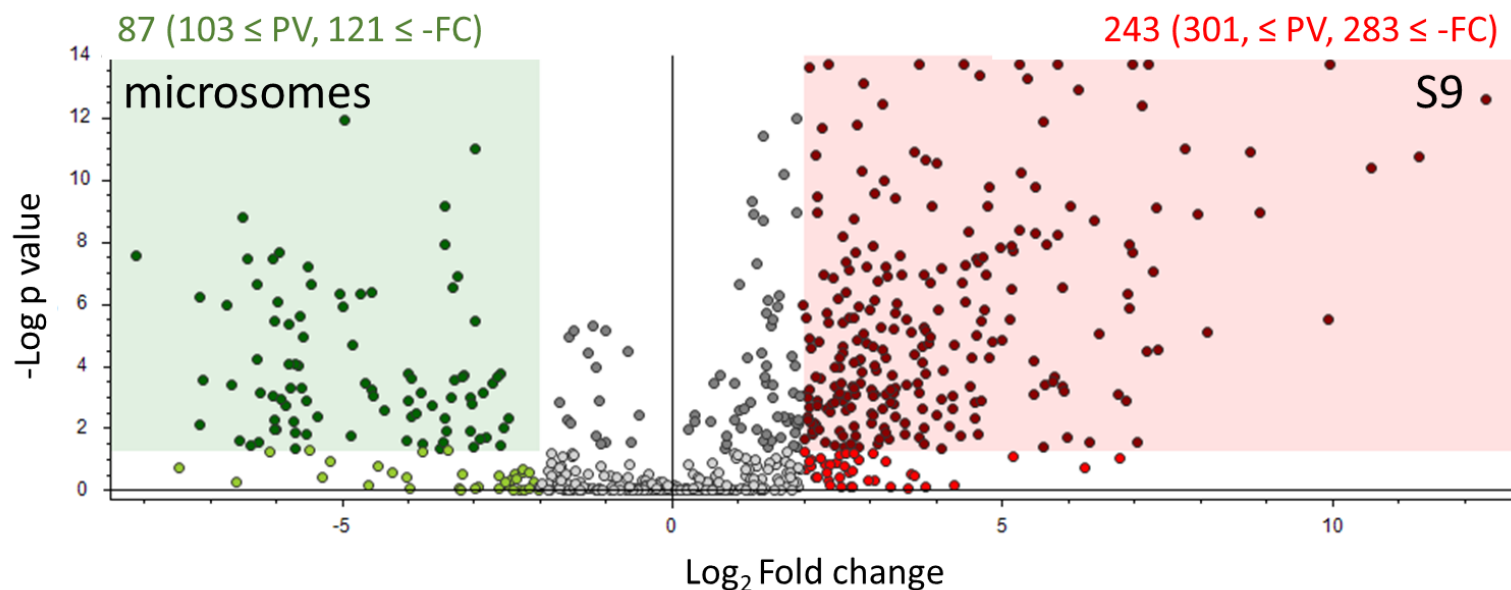
Figure 4-18 Volcano plot of phase I + II metabolism of BML basic extract in trout S9 and microsomes. Log_2(Fold-Change) is shown on the x-axis and p-value difference between test and control (n = 3 each) is shown on the y-axis. Species within the shaded boxes represent those which have met both the p-value ($p < 0.05$) and log_2(Fold-Change > 2) cut-off and have significantly increased in microsomes relative to S9 (green) or increased in S9 relative to microsomes (red). Species which have met the Fold-Change cut-off but were not statically different in tests compared to controls fall outside of the shaded boxes but are coloured accordingly. At 225 min, 243 species were higher in S9 and 87 were higher in microsomes.
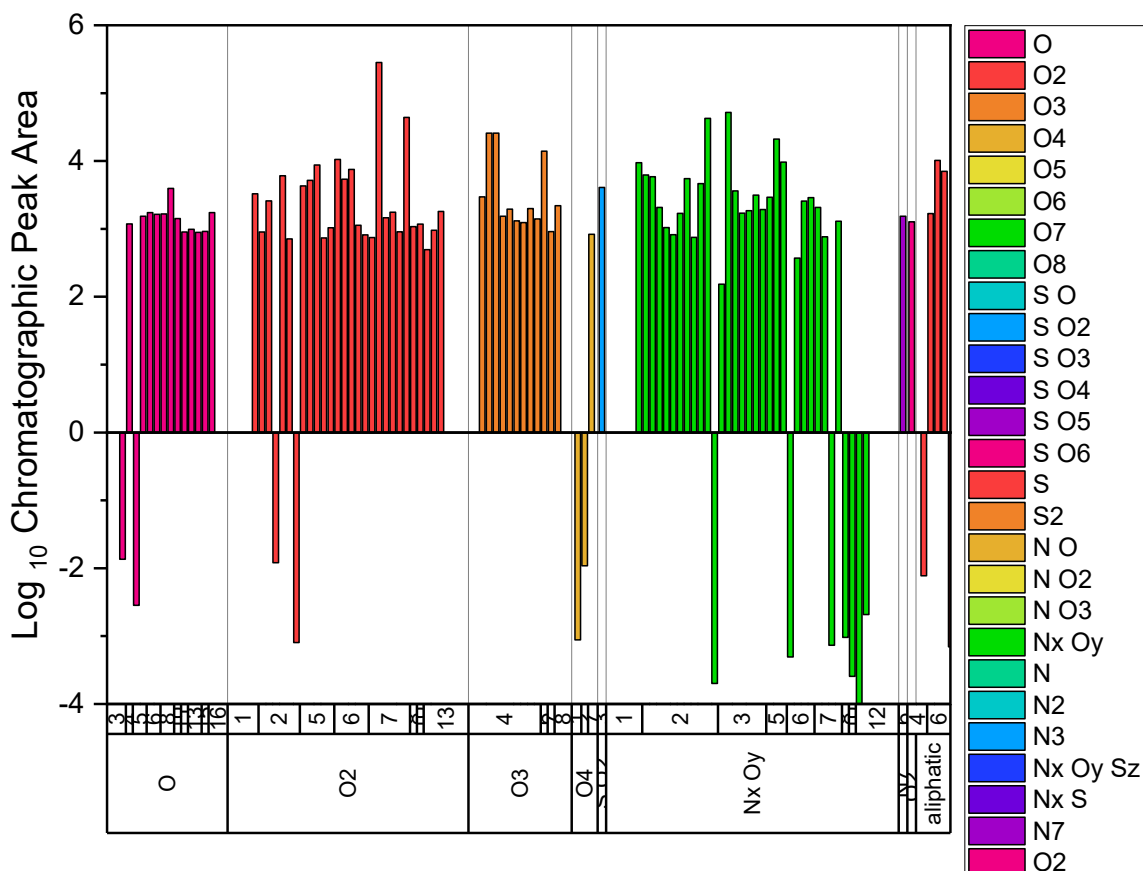
Figure 4-19 Summary of species evolved (and transformed) in phase I + II metabolism of BML basic extract in trout S9. Species are organized by heteroatomic class, and then the number of double bond equivalents on the x-axis and the Log_{10}Chromatographic peak area on the y-axis. Any species which increased is shown above 0, while those which decreased are shown below 0. Species displayed met both the p-value ($p < 0.05$) and log_2(Fold-Change > 2) cut-off.

4.2.7.2 Phase I + II metabolism Acidic Extract

Compared to phase I (935 species), just 9 more species were detected following phase II experiments with fish microsomes (**Figure 4-20**). A ten-fold increase in the number of products was observed here – 63 products detected in phase II vs. just 6 phase I reaction products. Nearly all newly generated products contained at least one oxygen atom (except $C_{14} H_{22} N_2$ or $C_{15} H_9 N_3$, **Figure 4-21**). The bulk of products were O_2^- species with 1 to 8 DBEs. Still, the average molecular weights of test reactions did not change during the experiment ($p = 0.97$) – showing again, limited change of the total species profile.
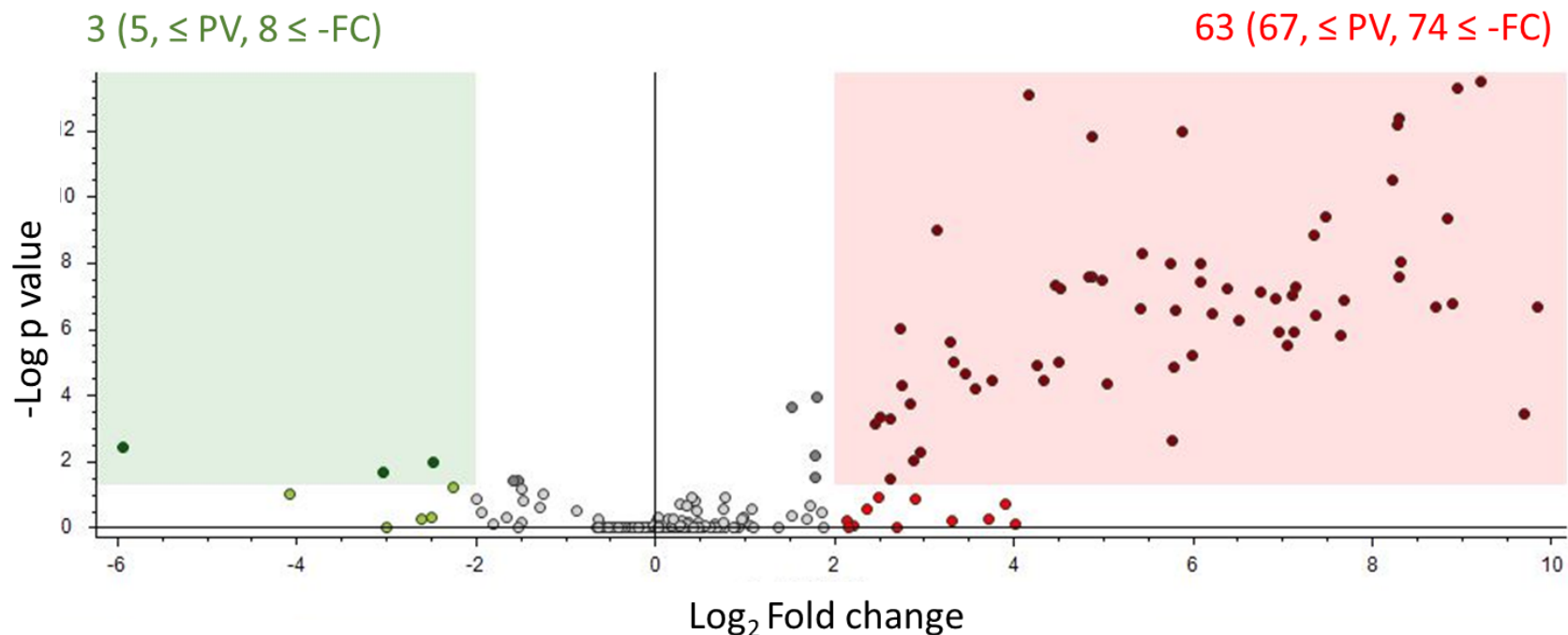
Figure 4-20 Volcano plot of phase I + II metabolism of BML acidic extract in trout microsomes. Log_2Fold Change is shown on the x-axis and p-value difference between test and control (n = 3 each) is shown on the y-axis. Species within the shaded boxes represent those which have met both the p-value (p < 0.05) and log_2(Fold-Change > 2) cut-off and have significantly decreased (green) or increased (red). Species which have met the Fold-Change cut-off but were not statically different in tests compared to controls fall outside of the shaded boxes but are coloured accordingly. At 225 min, 63 species had increased and 3 decreased by at least 4 times ($log_2 > 2$).
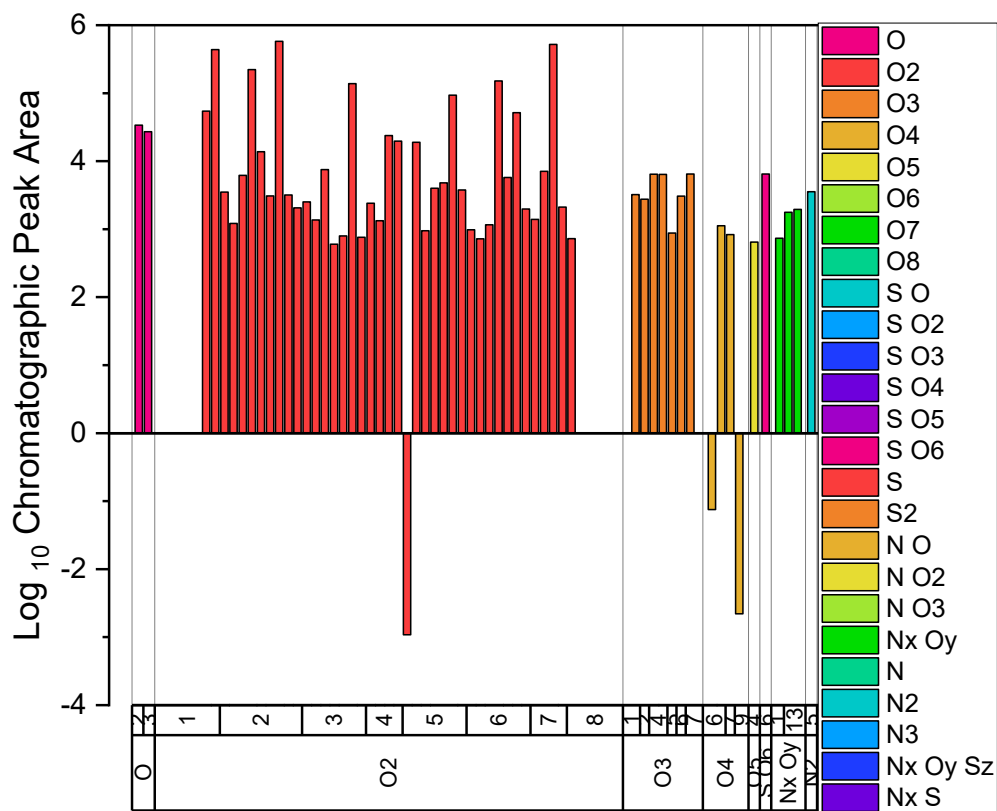
Figure 4-21 Summary of species evolved (and transformed) in phase I + II metabolism of BML acidic extract in trout microsomes. Species are organized by heteroatomic class, and then the number of double bond equivalents on the x-axis and the Log_{10}Chromatographic peak area the y-axis. Any species which increased is shown above 0, while those which decreased are shown below 0. Species displayed met both the p-value ($p < 0.05$) and log_2(Fold-Change >2) cut-off.

Two species stood out: $C_{16}H_{22}SO_6$ and $C_{18}H_{26}SO_8$ reached their maximum concentrations in just 15 minutes (**Figure 4-22**). This might suggest the production of a sulfur-containing glucuronide product. MS/MS experiments detect these products by the neutral loss of *m/z* 176 related to the anhydroglucuronide unit (i.e., glucuronide which has lost a water molecule: $-C_6H_8O_6$). This type of reaction (single reaction monitoring) can be detected in both ionization modes, but positive mode has been reported to be more diagnostic. Due to limits on time, MS^n experiments were not performed here so speculations, therefore, cannot be confirmed.

Aa total of 67 unique species were detected in both S9 and microsomes. Those exclusive to microsomes (21) were mainly species belonging to O_2^- to O_5^- classes, while those found only in S9 had more sulfur-and nitrogen atoms including $C_{10}H_{13}N_5O_3S$ – the species with the highest number of heteroatoms. All metabolites which contained a sulfur atom also contained at least 3 oxygen atoms (**Figure 4-23**). This may be reflective of the greater number of reactions which can occur in S9 due to a variety of cofactors present in the cytosol. $C_6H_{12}S_2O_4$ and $C_5H_{10}S_2O_4$, with 6 heteroatoms atoms each, were also unique to S9 metabolism and members of the same homologous series. Conjugation of taurine ($+C_2H_5O_2NS$) and glutamine ($+C_5H_7O_3N$) could both result in a large increase in heteroatom content of a molecule. However, amino acid conjugations occur much less frequently than the most common phase II reactions of glucuronidation ($+C_6H_8O_6$), sulfation ($+SO_3$) and glutathione ($+C_{10}H_{15}O_6N_3S$) conjugation. $C_{16}H_{22}SO_6$ and $C_{18}H_{26}SO_8$ were produced by both microsomes and S9, thereby providing further evidence that these were products of phase II metabolism. At this same time, it highlights the general absence of glucuronidation of BML acidic extract by trout subcellular fractions.

Glucuronide products of xenobiotics have been detected in bile and urine of fish, but there was little evidence of glucuronidation of BML dissolved organics by trout, here. Han *et al.* hypothesized that phase II metabolism did not occur to a considerable degree in subcellular fractions of trout.[21] Later, they suggested CYP1A may be responsible for the bulk of the observed metabolism.[19] My results echo findings by Han *et al.* in demonstrating that organics from BML OSPW appear to be metabolized largely by phase I metabolism in trout microsomes and S9. Glucuronidation and other phase II pathways may not contribute to the metabolism of these species *in vivo*.

All in all, while there appeared to be some capacity for xenobiotic transformation of toxic organic compounds in BML OSPW, my study suggested this may not happen to a considerable degree.
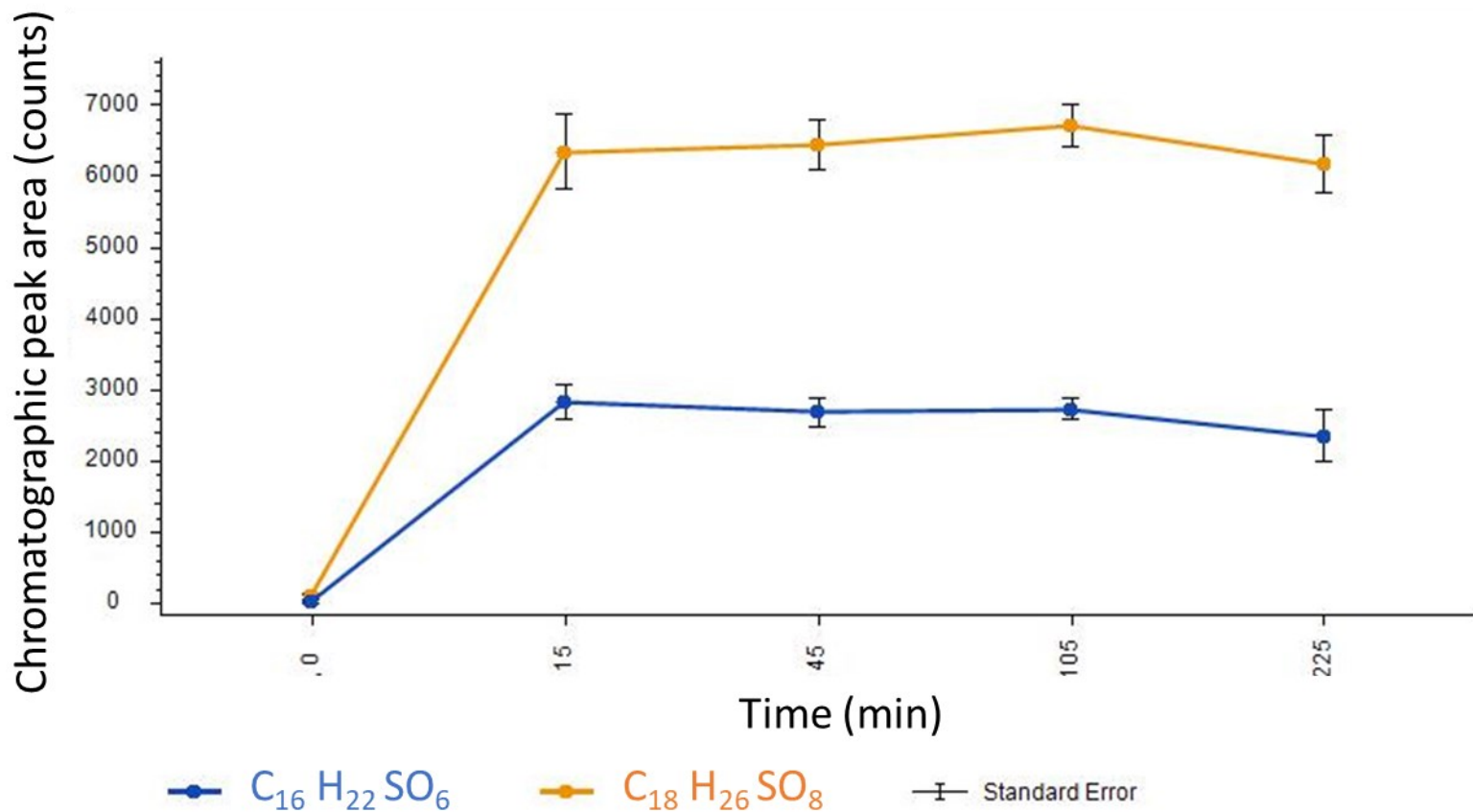
Figure 4-22 Evolution of two sulfur-containing organics in BML acidic fraction following phase I + II metabolism in fish microsomes. These two were the only species which may have been generated by glucuronidation.
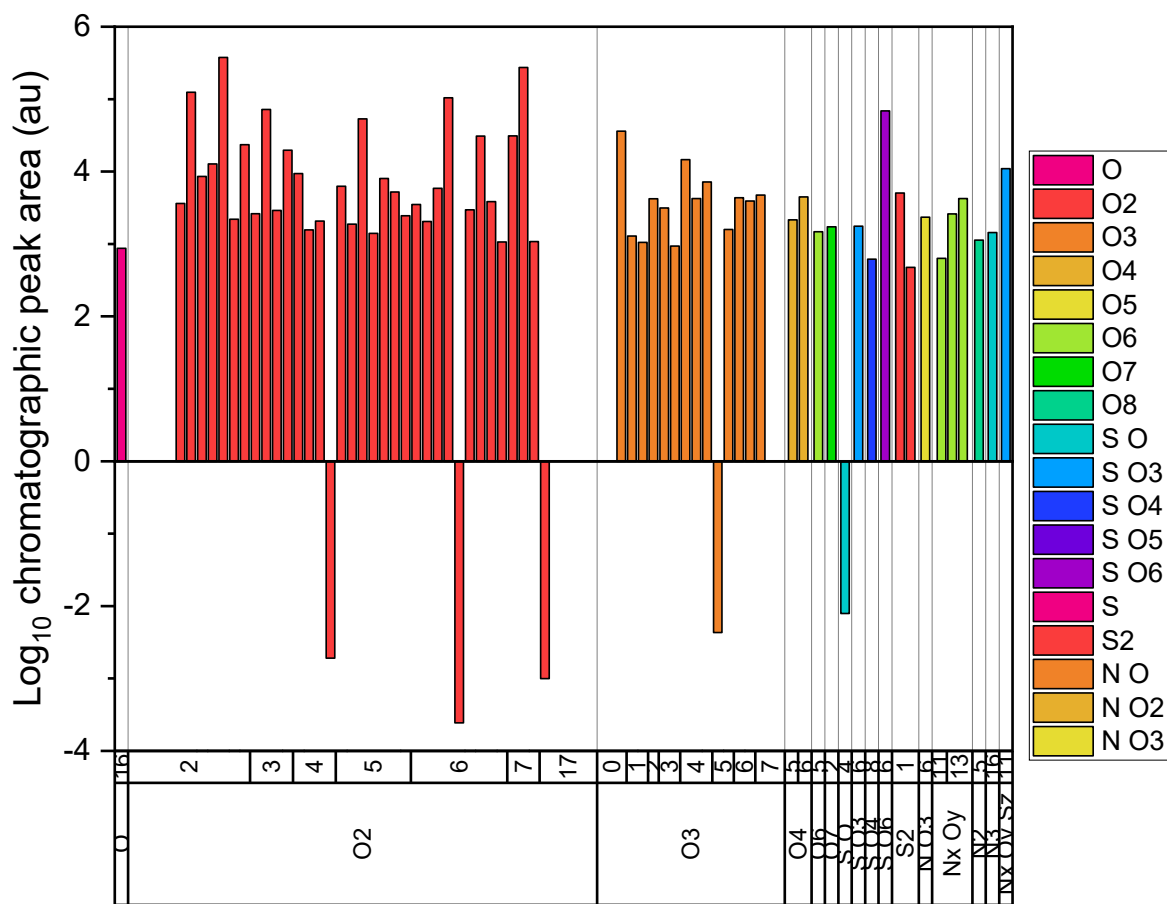
Figure 4-23 Summary of species evolved (and transformed) in phase I + II metabolism of acidic in trout S9. Species are organized by heteroatomic class, and then the number of double bond equivalents on the x-axis and the Log_{10}Chromatographic peak area the y-axis. Any species which increased is shown above 0, while those which decreased are shown below 0. Species displayed met both the p-value ($p < 0.05$) and log_2(Fold-Change >2) cut-off.

4.3 Limitations and Significance

Xenobiotics which have been biotransformed can be found in the bile prior to their elimination through feces. Bile fluorescence of native fish has been well-studied in oil sands toxicology.[12,15] An early study of yellow perch exposed to extracted NAs observed a number of physiological changes at concentrations as low as 1.7 mg/L.[46] Gill cells appeared to proliferate in response to exposure but interestingly, the liver was unaffected.[46] Later, NA metabolites were found in the bile of yellow perch held in experimental ponds (as measured by LC-MS).[41] The analytical method derivatized organics for detection and only a few NAs were identified. The study concluded that NAs were excreted via biliary route and did not accumulate in the tissue of fish. Other studies which measured concentrations of NAs in fish tissue also saw little evidence of bioaccumulation of NAs and corroborate the former hypothesis.[1]

This study is the first to use trout microsomes and S9 to directly measure metabolism of OSPW dissolved organics by a model fish species. Statistical analyses of the biotransformation assays showed that, in fact, many reactions occurred in the incubations. However, considering the applied thresholds ($p < 0.05$, $\log_2$Fold-Change > 2), very few of the observed changes were deemed to be significant. Here, the generation of $O_x^{\pm}$ species provided evidence for phase I metabolism of commercial NAs, and the acidic and basic extracts of BML OSPW. The results are in line with previous studies in showing that O_2^- species can be metabolized in fish, but my results expand further to suggest that metabolism occurs mainly in phase I. More species evolved in the phase I metabolism of the basic extract compared to the acidic extract, perhaps due to differences in toxicity. There was little evidence of glucuronidation by trout microsomes and S9 studied here. However, other phase II enzymes (ex. SULT, GST, NATs) may have higher affinities for BML dissolved organics. Ultimately, I found that toxic dissolved organics in BML OSPW can be biotransformed in trout subcellular fractions, but the extent of this metabolism may be limited.

Considering the low percentage of metabolites formed, future studies should consider the use of hepatocytes which have been described as a more complete *in vitro* model.[21] Furthermore, the assays here were not optimized with respect to reagent concentrations or time. Adjusting the proportions of these reagents and the reaction time of these incubations may yield different results.

4.4   References

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Chapter 5   Conclusions and Synthesis

5.1   Overview and Current Knowledge Gaps

The Canadian federal elections in fall of 2019 pushed tensions felt across the country to the surface. At the forefront of topics important to Canadians were environment, climate change, pipelines, and the Carbon tax. Natural resources in the West had provided wealth and prosperity for the middle-class nationwide. However, these provinces were hit the hardest by the recession after the collapse in oil prices in 2014.[1] "The engine" driving the Canadian economy had suffered in the last decade and the effects were being felt throughout. Still, in 2018, 11% of the GDP was due to the energy sector and mostly occurring in Alberta.[2] Liberal incumbent Justin Trudeau had a difficult track to re-election. In his first term, the Liberals had abandoned one major pipeline project (Northern Gateway Pipeline) and scaled back plans for another (Energy East). Between these and several political scandals, the Trudeau campaign was hard-pressed to retain leadership. But October 10, 2019, the Liberals scraped by and were elected as leaders of a minority government with 157 seats. The provinces of Saskatchewan, Alberta (save one constituency in Edmonton) were solidly blue. They, along with much of British Columbia, elected the 121 Conservative MPs which established the largest Official Opposition in Canadian history. Differing ideas on how Canadians could balance economic growth with environmental sustainability had forced a deep divide between citizens and at the heart of the issue is the energy sector.

The Alberta oil sands development had come a long way since the 1940s. In 2009, bitumen production was 86.7 million m^3 and by 2019 this production had nearly doubled to 171 million m^3.[3] Due to the prohibition on release, this time saw a corresponding increase in tailings volume. The volume of tailings ponds rose from 781 million m^3 in 2009[4] to 1252.7 million m^3 in 2018.[5] While tailings ponds are critical structures to operations during the lifetime of a mine, the accumulating volumes pose a major challenge for industry. Operators must present a plan for reclamation of all tailings generated upon mine closure to be approved by AER.[6] Currently, the leading strategy proposes that tailings ponds be treated naturally. The hypothesis of the EPL strategy is that biodegradation by endogenous microorganisms and dilution with freshwater will eliminate toxic organics such that these bodies of water may become permanent fixtures which can safely be re-integrated into the environment.

The criteria for reclamation will be evaluated on a case-by-case basis and while that presents its own host of challenges, science has worked to answer two main questions: 1) what makes OSPW toxic and 2) how long does it take for these components to degrade? To answer the latter question, a variety of studies have worked to understand how exposure elicits toxicity in a variety of animals including benthic invertebrates[7–9], fish[10–21], frogs[22–25] and rats.[26,27] Tests of whole OSPW, or fractionated components have determined the main mechanisms of toxicity to be endocrine disruption,[15,16,28,29] membrane disruption,[30] oxidative stress,[8,31] narcosis,[32–34] and the capacity to act as teratogens.[15,16,19,25,35] Naphthenic acids (NAs) have been the toxicants of interest in OSPW in the late 1980s. Improvements in analytical instrumentation and the advent of ultrahigh resolution mass spectrometry later revealed a variety of other organic compounds co-exist in this matrix but ultimately have re-enforced the notoriety of NAs. Strides have been made in understanding toxicity, but our understanding of persistence has lagged.

The toxic dissolved organics in OSPW are slow to degrade. While OSPW is described as a supercomplex mixture,[36] biodegradability of chemicals has largely focused on NAs. The rates of biodegradation for most compounds in OSPW are largely unknown. Other toxic heteroatomic classes such as the O_2^+, SO^+ and NO^+ classes have also been found to be toxic.[6] Their persistence, and those of all species in OSPW, beyond NAs, is therefore worth investigating.

To test this remediation strategy, BML was commissioned as the first oil sands EPL in December 2012. The future success of EPLs as a water management and remediation tool is contingent on proven remediation in BML. To assess the feasibility of the EPL strategy, my research used 3 diverse strategies: In the first, I reviewed samples from the field including tailings pond samples and experimental pond samples which had aged >20 years. I report the results of this study in Chapter 2 and therein describe how the profile of OSPW changes following natural exposure. In my laboratory study, microcosms containing BML OSPW with and without nutrient supplementation were monitored for over 1 year. In Chapter 3, I report the rates of *in situ* aerobic biodegradation for thousands of dissolved organics in BML and additionally I review resultant changes in the microbial community structure. My final research project shifted from *in situ* biodegradability of dissolved organics in the environment to the persistence of these species in a native fish species. Chapter 4 describes the *in vitro* biotransformation of toxic fraction of BML OSPW by enzymes of rainbow trout. This thesis presents the first investigation of the persistence of dissolved organics in the first (and currently

only) end pit lake using a variety of experimental designs and HPLC coupled to ultrahigh resolution Orbitrap mass spectrometry.

5.2 Advances in Knowledge

5.2.1 Profiling of OSPW at various stages of ageing and identification of persistent compounds

With mounting volumes and accelerated production, oil sands operators began testing for tailings treatments strategies as early as 1970s. Early tests considered physical processes to treat OSPW which focused coagulation and flocculation of fine suspended particles.[37] This is at the crux of the consolidated tailings treatment strategy which is still in use today.[38] In the late 1980s the experimental reclamation pond facility was established at Syncrude to explore various natural treatment strategies for OSPW reclamation by the wet landscape approach.[39] To this end, 11 shallow ponds (up to 5 metres deep) were established with various permutations of OSPW, surface run-off water, and MFT. For the next 2 decades the toxicity of OSPW in these experimental reclamation ponds were monitored through the impact of exposure to aquatic organisms. First tests of this strategy revealed that natural aeration for $1 - 2$ years could render OSPW habitable for aquatic biota.[40] Later, the study of exposures of yellow perch to aged waters demonstrated the physiology of these fish was not negatively affected, and that effects observed could not be associated with dissolved organic compounds.[41] It bears noting, however, that the analytical techniques at the time were not sensitive enough to differentiate between different chemicals in OSPW. While one study demonstrated survival or growth of fathead minnows were not significantly impacted upon exposure of aged OSPW, other studies reveal reproductive health of these fish may suffer.[7,15,19,42,43] Overall, research showed that acute toxicity is efficiently removed by natural biodegradation of OSPW. Toxicity reduction in aged OSPW is largely associated with the decreased concentrations of NAs, and the oxidation of these compounds to less toxic components.[44] The question of chronic toxicity imparted by residual organics remains. To my knowledge, no study has done comprehensive investigation on the chemical profile of aged OSPW until now.

In this project, samples of experimental ponds aged 27-31 years were grouped to represent aged OSPW and compared to 4 samples of fresh OSPW, and water from the first oil sands EPL. This cross-sectional study is the first review all detectable chemical species using ultrahigh resolution mass spectrometry in negative ($^-$) and positive ($^+$) ionization modes. The

results show oxygen-containing classes (O_x^-) have the largest overall contribution to samples irrespective of age and furthermore, O_2^- class was the foremost of these. Shown in Table A-1, the intensities of all detected species were lower in aged OSPW including the O_2^- class for which intensities (and concentrations) of species in aged OSPW (< 1.0 mg/L) were significantly lower than in fresh OSPW (8.25 – 20.1 mg/L). Interestingly, this class had the highest relative contribution (**Figure 2-6**) and the highest concentrations in BML (23.1 mg/L) which was reflective of increased levels of oil sands development in more recent years. Decreases in concentration could not be explained by dilution (**Figure 2-7**) which indicated degradation processes (photolysis and biodegradation) likely contributed to the observed differences. Profiles of species in positive ion mode were also reported here. Intensities of O^+, O_2^+, SO_2^+, SO_3^+, NO^+ and NO_2^+ were all significantly lower in aged OSPW compared to fresh OSPW (**Table A-1**). The log_{10} relative ratio of contributions of species was helpful to identify global trends in the data. For the top 200 species studied, O_3^-, O_4^-, O_5^-, O_6^- and SO_3^- classes were enhanced in aged samples (**Figure 2-8A**). Other sulfur-containing classes were relatively enhanced in fresh OSPW. Similar observations were seen for the top 700 abundant species in OSPW detected in positive ion mode (**Figure 2-8B**).

Distinctions in the percent relative distributions between samples were revealed by principal components analysis (PCA). For classes detected in negative ion mode, O_2^- separated BML from all samples (**Figure 2-22B**) . Fresh OSPW was correlated with O^-, O_2^- and O_4^- classes, while the remaining O_x^- classes were correlated with aged OSPW. For species in positive ion mode, $O_2^+ – O_6^+$ were correlated with aged OSPW and sulfur-containing organics were associated with fresh OSPW (**Figure 2-22B**). When the separation of samples was interrogated at a species level, O_3^- and O_4^- were associated with aged and fresh OSPW, respectively (**Figure 2-23**).

This cross-sectional study is the first to scrutinize OSPW samples in this way. The juxtaposition of these samples enabled inferences about the effect of natural ageing on OSPW. Differences in the distributions between aged and fresh confirm oxidation as an important process occurring in OSPW over time. Besides this, no other structure-activity relationships (related to Carbon number or double bond equivalents and age) could be inferred. Also, this study is the first to produce an in-depth profile of thousands of unique species found in OSPW including those of experimental ponds samples. My results suggest that the acutely toxic OSPW

chemicals (O_2^-, O_2^+ SO^+, NO^+) degraded in these small-scale ponds, and bolster the premise of the end pit lake reclamation strategy. Therefore, my results also suggest that residual toxicity of aged OSPW may be explained by persistent degradation products. Ultimately this research expands upon knowledge about the effectiveness of wet landscape reclamation and provides evidence for which chemicals may persist in future end pit lakes.

The largest challenge of this project was the limited sample set. The analysis could be made more robust with the inclusion of additional experimental pond and tailings pond samples. Furthermore, I recognize that mass balance was not accounted for in this study. While chloride ion data was used to infer evaporation, seepage and dilution were not assessed. Future work may seek to review how depth impacts transformation processes, and furthermore, a long-term study may review the degree to which photolysis contributes to degradation. There is value too, in understanding the difference in the microbial communities in fresh and aged samples to glean which microorganisms thrive and understand the factors necessary for their proliferation.

5.2.2 Assessment of rates of *in situ* biodegradation in BML OSPW

No other study has reviewed rates of *in situ* aerobic biodegradation of dissolved organics in BML. This information is necessary to understand lifetimes of EPLs and manage expectations by all stakeholders. To address this, I established laboratory microcosms containing OSPW and monitored rates of biodegradation with and without nutrient amendments. Previous microcosm studies (2004 to 2008) which reviewed biodegradation potential of endogenous microbes, had focused on degradation of commercial NAs.[45–47] In positive control microcosms, Merichem commercial NAs were added and used as a reference as their relative biodegradability has previously been established. A series of tests were performed to assess how intervention may be applied to expedite rates of removal prior to applying amendments in the final stage of the experiments.

At the onset of the study, I proposed a simple testable hypothesis: rates of sulfur-containing organics in BML OSPW were equal to that of NAs (O_2^- species). Except for species of the SO^- class (0.46% d^{-1}), all heteroatomic classes experienced non-significant changes (assuming first order kinetics, slope $p > 0.05$) prior to intervention. The values of these pre-intervention changes in heteroatomic classes ranged from -0.06% d^{-1} to 0.14% d^{-1} (Table 3-1). Following the addition of nutrients and acetate, only two of the studied heteroatomic classes

decreased: O_2^- and SO_2^-. These two classes had similar decreasing rates at -3.24% d^{-1} and -3.29% d^{-1}, respectively. I conducted an F-test to compare the models and found these rates of decrease are not statistically different (F = 0.157, p = 0.85). The post-intervention rate of removal of O_2^- species here translates to a half-life of 214 d and falls within the range of previously observed microcosm studies (**Table 5-1**).[47,48] Furthermore, as no other heteroatomic class changed, the relative recalcitrance of remaining species in BML OSPW was confirmed here.

The pre-intervention rates for O_2^- (–0.04%) suggested a half life of 1868 d (~5.12 yrs). My results present a shorter timeframe for biodegradation compared to previous studies of aged OSPW which suggested lifetimes of 12.8 – 13.6 yrs.[44] The latter estimate was derived using data from a long timescale which compared concentrations from point of establishment to measured concentrations at the time of study (15 years). In contrast, the estimate here was generated from a study of 389 d. Also, BML has a greater proportion of MFT than any of the previously established experimental ponds. The differences in analytical methods used to assess the ponds surely contributed to this apparent discrepancy. Han *et al.* used HPLC QTOF MS[44] while Orbitrap MS was used here. Ultimately the pre-intervention estimates for O_2^- species reveal that without amendment, natural biodegradation in BML would occur on the scale of several years. This study is the first to report any rates of aerobic biodegradation occurring in BML OSPW.

There is some disagreement in the field about how chemical structures of O_2^- species influence rates biodegradation in OSPW. The chemical profiles of O_2^- (**Figure 3-10**) and SO_2^- species (**Figure 3-11**) differ both in species intensities and distributions, but similar rates of removal may suggest structural similarities between the two. Heat map analyses support this hypothesis as the greatest post-intervention changes were observed in species with $>C_{15}$ (**Figure 3-12** and **Figure 3-13**). Qualitative review of these heat maps does not suggest preferential degradation of species with fewer DBEs as postulated by Han *et al.* They were the first to refute a significant influence of carbon number and rate of biodegradation, suggesting instead that cyclization and branching had inhibitory effects.[47] Later, Toor *et al.* confirmed these observations.[48] These two microcosm studies analyzed changes using a similar HPLC/QTOF-MS method. However, more recently Ajaero *et al.* used a constructed non-aerated wetland treatment and reported the quick rates of removal for O_2^- species using Orbitrap ESI-MS.[49] The study used plants (sedges) and sand with OSPW circulated in a closed flow-through system and no nutrients were added. Despite many differences between the studies, the study by Ajaero and co-workers

also observed appreciable decreases in NAs with $>C_{15}$ and concluded longer carbon lengths were more effectively removed.[49] My study also shows there is an inverse relationship between carbon number and rate of biodegradation of O_2^- species.

Table 5-1 Reported rates of biodegradation for the O_2^- class by recent microcosm studies

Study	Design	OSPW Source	Reported $t_{1/2}$ O_2^- (d)
Han 2008	300 mL aerobic microcosms	Syncrude	40 - 240
Toor 2013	20.8 L flow-through microcosm	Syncrude	186 - 294
Ajaero 2018	Non-aerated constructed wetland treatment	Shell	9 - 23
My study	300 mL aerobic microcosms (pre-intervention)	Syncrude	1868
My study	300 mL aerobic microcosms (post-intervention)	Syncrude	213

Many of the bacteria found in BML OSPW have been previously reported.[50–54] Aerobic hydrocarbon degrading species such as *Pseudomonas* and *Caulobacter* were found as were several anerobic species such as *Mesorhizobium* and *Limnobacter*. Following nutrient and acetate addition, populations of the aerobic hydrocarbon degrader, *Xanthobacter*, increased. *Firmicutes* increased by the largest margin towards the end of the experiment. These have previously been identified to degrade simpler hydrocarbons including short-chain alkanes ($C_6 - C_{10}$) and are capable of nitrate reduction.[54] It was likely the addition of nitrogen which enabled these bacteria to thrive and perhaps contribute to the removal of some O_2^- and SO_2^- species.

5.2.3   Metabolism of dissolved organics in BML OSPW by native fish species

Under the Canadian Environmental Protections Act, federal agencies must evaluate substances which may pose harm to humans and the environment.[55–57] Chemicals are assessed for their persistence, bioaccumulation potential and toxicity.[58] Laboratory exposures of fish to OSPW have reported low concentrations of organics in tissues .[59–61] Following extraction and analysis, researchers suggested biotransformation processes may account for the lower than expected concentrations observed in tissue of Japanese medaka (*Oryzias latipes*).[62] Previous

work reviewing the biotransformation potential of fish focused on the metabolism of PAHs.[12,42,63,64] Many such studies measured the activity of prominent phase I enzymes CYP1A or EROD. Until now, none have directly measured the biotransformation of dissolved organic acids, polar neutrals, or basic species in aquatic organisms. To this end, I reviewed the *in vitro* metabolism of toxic organic chemicals in BML OSPW using liver subcellular fractions of rainbow trout. Fractionation of OSPW has proved helpful in understanding the toxic properties of OSPW,[32,40,65,66] so a previously developed SPE method was applied here to generate two simplified extracts: one basic and one acidic. Non-targeted analytical software provided an efficient way to monitor changes in these mixtures throughout the experiments (225 min).

In my study, phase I metabolism of Merichem NAs by trout microsomes saw 53 species increase by at least 4 -fold compared to the control, but the differences were not statistically significant ($p > 0.05$). Products generated belonged to the O_3^- and O_4^- heteroatomic classes. The phase II glucuronidation was not detected in the metabolism of Merichem NAs by trout microsomes. All observations of the commercial mixtures were used to generate hypotheses for similar reactions in the BML acidic extract. In this experiment, the 6 species which were generated ($p < 0.05$, $\log_2$(Fold-Change ≥ 2) had 1 or 2 DBEs which indicated that the substrates with the simplest structures were preferred. While they did not reach the threshold for critical review, 38 other species were also formed. Similarly, most products of the phase I metabolism of the BML basic extract had at least 2 oxygen atoms. Glucuronidation was not observed in any permutation of the assay using trout microsomes or S9, but the limited contribution of phase II enzymes in trout S9 has been previously reported.[67] Interestingly, mores species were formed in phase I + II metabolism by S9 compared to microsomes, perhaps due to the availability of other cytosolic enzymes. Ultimately, my results indicate there is some capacity for the biotransformation of OSPW species by phase I enzymes in trout liver cells. The products generally suggest formation of oxidized compounds with decreased toxicity relative to the substrates (ex. in the case of O_2^- to O_3^- species). It is germane to note a maximum of 2% of species changed significantly in OSPW extracts. This calls into question the overall impact on estimates of bioaccumulation potential for these compounds. Contributions of other phase II reactions and other organs (i.e., gut, gills) should be considered to further the understanding of fate of these persistent compounds in aquatic biota.

5.3   Inefficiency of data analysis

Data analysis of environmental samples is complex, and this does not exclude the review of OSPW. The power of the Orbitrap has led to a better understanding of what is in OSPW, and in the process it produces extremely large datasets. Thermo's Xcalibur™ software can be used for the processing of simple samples with targeted workflows. In my opinion, it is not suitable for processing complicated samples, like the ones included in my research. Data analysis time was a limiting factor as it required over 8 months to manually process the data. In fact, much of the PhD thesis of Kevin Kovalchik centred on the development software to increase processing speed of the .raw files generated by Xcalibur™ .[68–70] This goes to show the great effort and mental fortitude required to endure the task of OSPW non-targeted analysis. Of the many issues incurred throughout my program, the time-intensive labour which was required to do my research is foremost. Surely, someone well-versed in a coding a language like R or Python may have been better equipped to handle the datasets which were generated from my projects. Without a previous computing science degree, it took many years to develop and execute a strategy to review the data from these research projects through the notorious Microsoft Excel – yet I emerged (not unscathed, very much scathed) to tell the tale through this thesis.

5.4   Conclusion

Until a treatment strategy is approved, volumes of OSPW will continue to accumulate. Pit lakes have been used for remediation of waste in other industries. Silkstone and Lovett lakes in Edson, Alberta were coal mines prior to 1986.[56,71] Syncrude has led research by being the only operator to establish a large scale oil sands end pit lake. The removal of toxic components is critical for the eventual release of OSPW and integration of EPLs into the surrounding environment. Using OSPW from BML, my PhD work investigated the environmental persistence of thousands of dissolved organic species in different ways. Non-targeted analysis provided a data-driven approach to observe trends and generate hypotheses for future work.

Significant research has reviewed the physical chemistry of BML,[72–74] and new evidence suggest that natural light may be an important abiotic process in the degradation of dissolved compounds.[75] My findings from Chapter 2 corroborate the latest results and reaffirm that the composition and physical construction of EPLs will have significant impacts on *in situ* biodegradation process. The successful removal of organics in experimental ponds seen in

previous studies (and here) was in part due to their shallow depths which allowed for sufficient aeration. Experimental ponds were $1 - 2$ m deep, but the total depth of BML is over 40 m. Fluid fine tailings are at the base, and the free water zone is the uppermost 11 m.[5] These proportions are much different than in any experimental ponds studied and will likely result in different rates of biodegradation.

The inorganic constituents are important as they influence the community dynamics of microbial populations. Nutrient limitation in OSPW is not a new fact.[45,77] As observed in other OSPW samples,[50,51,53,78,79] my results report a diverse co-operative community of microorganism in BML including both aerobes and anaerobes. Aerobic biodegradation was studied here as it is fast and more energetically favourable in hydrocarbon degradation. However, contributions of anaerobic microbes are not to be ignored. Nitrogen-fixation is one of many critical biogeochemical reactions executed by anaerobes in OSPW.[52,80,81] The addition of nitrogen and phosphorous shifted microbial community dynamics and greatly accelerated the rates of *in situ* biodegradation. In recent years, alum (coagulant) has been added to manage turbidity and has effectively improved clarity in BML. Concentrations of all additives must be carefully managed as they also can have toxic effects on aquatic animals. Furthermore, as FFT densifies and porewater is expressed, the ecology and dissolved organic chemistry of BML will be affected.

The recalcitrance of organics in BML OSPW was confirmed here. Without nutrient amendments, lifetimes of the most labile of the organics were estimated to be at least half a decade. Interestingly, my results show of all heteroatomic classes detected in negative ion mode, that O_2^- (and SO_2^-) species were the most responsive to intervention. Comparison of profiles suggest O_2^- compounds are converted to more oxidized compounds. Thus, lowered concentrations in BML since 2013 (i.e. 25% decrease in total concentrations from 2013 to 2018, data not shown) are due in large part to dilution by freshwater.[82] For reasons unknown, polar neutral organics (O_2 , O_3 , O_4, O_5 , O_6 species detected in positive ion mode) have increased contributions to the profile of OSPW over time and appear to be the most persistent. Naphthenic acids in OSPW consist of thousands of individual compounds. My experiments suggest structures with $>C_{15}$ are removed more quickly than those $<C_{15}$.

My work has been the first to evaluate the ability for a native fish species to metabolize chemicals in OSPW, and furthermore to use non-targeted analysis to describe the non-aromatic products which may be detected as bile or urine metabolites. Aquatic biota may have some

ability to transform toxic organics and excrete them as oxidized metabolites, but numerous toxic species may remain unchanged by liver enzymes. Phase I metabolism may be integral to oxidizing dissolved organic species, but there was little evidence for phase II glucuronidation in assays using subcellular fractions of rainbow trout liver cells. Currently, predictions for the bioaccumulation potential of most chemicals in OSPW are not worrisome, but discrepancies between *in vitro* and *in vivo* BCFs could benefit from further investigation.

Persistent chemicals are not environmentally relevant if they pose no risk to organisms which are exposed. So, it will be important for regulators to focus on persistent compounds that are also toxic and/or bioaccumulative in OSPW in EPLs. My thesis work has demonstrated that despite the recalcitrant nature of chemicals in bitumen-impacted waters, there is some capacity of these organics to be changed by a variety of processes. In the environment, the most acutely toxic compounds (O_2^- species) can be degraded aerobically by microorganisms, and photolysis may lend to the removal of these chemicals at the surface of tailings ponds. When considering processes within an organism, phase I biotransformation may lend to the removal of these organics. Overall, the lifetimes of these chemicals are on the order of years to decades. Remediation strategies will need to consider these timeframes and expectations for OSPW treatment and release must be managed accordingly.

5.5   Future research

The methods used to interrogate the persistence of chemicals in OSPW in the thesis were diverse. In Chapter 2, the chemical profiles of field samples were analyzed to generate hypotheses on the effect of natural ageing. A succeeding study would benefit from a larger data set. A power analysis suggests a sample set of 50 would be sufficient to detect an effect in a t-test comparing aged and fresh OSPW samples at a significance level of 0.95. The samples used in my study represented tailings ponds at Syncrude – the operator which holds the largest volume of tailings. The increase in sample size can be done in a few ways: 1) samples of OSPW from different operators (Suncor, Canadian Natural Resources Limited, Canadian Natural Upgrading Limited, and Imperial) could collected or 2) using one company, multiple samples could be taken from the same set of tailings ponds. Increased number of samples would make predictions generated by PCA more powerful. Finally, it may be worthwhile to review the differences in the microbial community composition in fresh and aged OSPW. We should know what microbes

survive over the years and how the diversity shifts given the physical construction of the ponds. I found that the O_3^-: O_2^- ratio may be used as an indicator for age of OSPW. Follow-up studies may further investigate this using a larger number of experimental pond samples.

Many microcosm studies using OSPW were conducted prior to my thesis work,[45–47,77,83,84] but mine is the first to use BML OSPW to study the aerobic biodegradation of dissolved organic compounds. In Chapter 3, I showed that some bitumen-derived organics can still degrade. While most chemicals studied were recalcitrant, the notorious NAs (O_2^- species) were one of the only heteroatomic classes which decreased following intervention. This group of organics also had the fastest pre-intervention changes, on the scale of many years. Unfortunately, changes in the heteroatomic classes detected in positive ion mode were not reported here. The data about this group of chemicals is still necessary to build the full picture on the fate of these chemicals in EPLs. In a subsequent (improved) iteration of this work, a persistent reference compound can be used to adjust for evaporation. I planned to use perfluorohexanesulfunate (PFHxS) for this purpose, but later found this compound was not efficiently ionized in APCI. To add, concentrations were too low and by the end of the experiment could not be detected even by ESI.

Microcosm studies are strengthened by concurrent toxicity analysis. A review by Mahaffey and Dube demonstrates that while the two go together, few studies have effectively presented both toxicity and chemical analysis together.[85] In their 2017 review, Penner and Foght note that unique differences in EPLs will influence their endogenous microbial communities.[54] They described the need to identify the toxic byproducts created by incomplete metabolism of dissolved organics. My samples were collected and analyzed by Microtox, but results were not reported as they were not evaporation-adjusted (diluted to reflect original sample concentrations) prior to analysis. These values would create a more complete picture of how persistence is impacting toxicity in BML OSPW.

It would be interesting to assess how biogeochemical processes in BML impact this remediation strategy in real time. Alum was added to BML in 2015 to increase sedimentation. As fines consolidate, porewater is forced out. This process together with the ebullition of biogenic gases from FFT, increases concentration of dissolved organics in surface water and curb overall rates of detoxification in EPLs.[72,80,86] To simulate this, a subsequent study may feature the

controlled addition of methane to mesocosms with proportions of MFT and OSPW like BML. The impact on microbial community dynamics should also be observed.

The metabolism of dissolved OSPW organics and its effect on bioaccumulation in aquatic organisms is still largely unstudied. Yellow perch (*Perca flavescens*) exposed to aged OSPW confirmed low concentrations of NAs in muscle tissue (<0.1 mg kg^{-1} via GC-MS and revealed statistically significant correlation between NA and fluorescent bile metabolite concentrations (r = 0.86, phenanthrene wavelengths).[61] Not withstanding the less sensitive instrumentation used at that time, the results indicated that perhaps, some NA species could be metabolized and warranted further studies. In Chapter 4, I demonstrated that some toxic chemicals in BML OSPW can be substrates for enzymes found in trout livers. MS^n can confirm structures of metabolites found here.[87,88] Furthermore, these products may be used in the targeted screening of bile and urine of fish to assess exposure.

The conditions of the assays used in Chapter 4 can be improved by optimizing reagent concentrations and incubation times. Besides the structure of each chemical, biotransformation can be influenced by water temperature, exposure route, enzyme competition, and inhibition.[89] Future work may consider including heat-treated negative control incubations to eliminate any reactions by endogenous levels of cofactors and identify decreases due to non-specific binding.

It has now been almost 8 years since BML was first commissioned but end pit lake technology is not yet been approved for OSPW treatment. Research continues to review ways improve the removal of dissolved hydrocarbons in this matrix. The persistence of these chemicals requires further investigation towards the goal of reducing toxicity of OSPW to aquatic fauna and humans upon the imminent release into our environment.[5]

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Appendix A Supporting Information for Chapter 2

A.1 Quality assurance and quality control for OSPW analyses

In this study, samples were grouped into OSPW types, whereby each member of the 'fresh' and/or 'aged' sample group served as a pseudo-replicate. This study was opportunistic i.e., given the samples available, the project sought to draw conclusions about samples in the field. No field blank sample was provided; however, ultrapure water was subject to the same processes as OSPW samples, so underwent filtration with 0.45 µm syringe and spiked with the combined internal standard. This blank was assigned as the 'procedural blank'. Instrumental blanks included unfiltered ultrapure water spiked with internal standard. Replicates in chapters 3 and 4 were included in the experimental design (laboratory studies).

Each sequence began with 5 – 10 instrumental blank injections to ensure the system was equilibrated. Sample randomization was assigned with an online random number generator. Prior, to sample injections a quality control standard was run, flanked by the procedural blank. As no standards exist in the oil sands analytical chemistry field, the chosen quality control (QC) sample for the thesis projects was a 0.5 X dilution of Base Mine Lake OSPW from 2014, spiked with the combined internal standard. Randomized-block design was applied to sample order in the sequence such that 'fresh' and 'aged' samples were mixed to reduce systematic bias over the duration of the run. The response of the QC sample was monitored throughout the duration of the run in two ways by reviewing: i) the mass spectral response of the internal standard and ii) the sum of the response of all O_2^- species. As the sequence progressed, a Shewhart chart was generated to assess the average response of the internal standard. The coefficient of variation (i.e., standard deviation / average) was used to assess blocks (portions of the sequence) which required re-analysis. If the response of the internal standard in a QC sample was greater than the coefficient of variation, the system was washed, re-calibrated, and the block was reanalysed. The sum of the response of all O_2^- species, was evaluated as a secondary method to evaluate the overall system's performance throughout the duration of the run.

Table A-1 Chemical formula (neutral formula shown) and DBE for species that contributed most to distinguishing fresh from aged OSPW (i.e., PCA-II along PC2).

Negative			Positive		
Species	DBE	PC2 (above/below)	Species	DBE	PC2 (above/below)
$C_{14}H_{22}O$	4	above	$C_{11}H_{16}O$	4	below
			$C_{12}H_{16}O$	5	below
			$C_{13}H_{18}O$	5	below
			$C_{13}H_{16}O$	6	below
			$C_{11}H_{10}O$	7	below
			$C_{12}H_{10}O$	8	below
$C_6H_{12}O_2$	1	below	$C_{14}H_{26}O_2$	2	above
$C_{13}H_{22}O_2$	3	above	$C_{12}H_{18}O_2$	4	below
$C_{14}H_{24}O_2$	3	above	$C_{14}H_{22}O_2$	4	below
$C_{16}H_{28}O_2$	3	above	$C_{12}H_{16}O_2$	5	below
$C_{14}H_{22}O_2$	4	below	$C_{14}H_{20}O_2$	5	above
$C_{15}H_{24}O_2$	4	below	$C_{13}H_{16}O_2$	6	below
$C_{13}H_{18}O_2$	5	above	$C_{14}H_{18}O_2$	6	below
$C_{16}H_{24}O_2$	5	above	$C_{12}H_{12}O_2$	7	below
$C_{15}H_{10}O_2$	6	above	$C_{15}H_{18}O_2$	7	below
$C_{18}H_{24}O_2$	7	above	$C_{14}H_{14}O_2$	8	below
$C_{17}H_{20}O_2$	8	above			
$C_{18}H_{22}O_2$	8	above			
$C_{15}H_{12}O_2$	10	above			
$C_{14}H_{22}O_3$	4	below			
$C_{15}H_{24}O_3$	4	below			
$C_{15}H_{22}O_3$	5	below			
$C_{14}H_{22}O_4$	4	above	$C_{16}H_{22}O_4$	6	above
$C_{15}H_{24}O_4$	4	above			
$C_{16}H_{26}O_4$	4	above			

$C_{16} H_{24} O_4$	5	above			
$C_{24} H_{26} O_5$	12	below			
$C_{15} H_{26} O_6$	3	below	$C_{20} H_{42} O_6$	0	above
$C_{20} H_{26} O S$	8	below			
$C_{21} H_{28} O S$	8	below			
			$C_{22} H_{39} S$	0	above
			$C_8 H_{13} O N$	2	below
			$C_7 H_{13} O N$	1	below
			$C_{20} H_{31} O_2 N$	5	above

Species included had loadings ≥ 0.1 or ≤ -0.1 on PC2 in negative ionization (left panel) or positive ionization (right panel) mode. Shading used to separate heteroatomic classes.

Appendix B Supporting information for Chapter 3

B.1.1 Methods in the analyses of treatability studies

All methods used in the closed bottle experiments can be described previously by *Yu et al.* but are re-iterated here: [1]

B.1.1.1 CO_2 analysis

100 uL was sampled from the headspace for CO_2 measurement. The method has been described elsewhere: Briefly, an Agilent 7890A gas chromatograph with a thermal conductivity detector (GC-TCD) was used with an AgilentHP-PLOT/Q column (30 m x 320 µm x 0.2 µm). The oven temperature method began at 50°C for 2 min; increased to 150°C at a rate of 30 °C/min; held for 2 min. The flow rate for Helium (carrier gas) was held at 8.83 mL/min for 2 min and then decreased to 5.67 mL/min until the end of the method. Total run time was 7.33 min. The detector was maintained at 200°C, and the injection port at 300°C.

B.1.1.2 Acetate analysis

Aliquots (1 mL) from each bottle were removed using a 22 gauge syringe and transferred to 1 mL glass vials and analyzed the Dionex ICS 2100 equipped with Dionex™ IonPac™ AS18 IC columns. Eluent flow rate was 0.25 mL/min and began with 10 mM KOH for 7 min followed by a gradient increase from 10 to 32 mM KOH for 2 min. This concentration was held for 11 min followed by a decrease in the KOH concentration from 32 to 10 mM over 2 min and a return to the initial concentration of 10 mM KOH in 1 min. Oven temperature was maintained at 30°C. Conductivity detector temperature was maintained at 35°C and conductivity was suppressed by the anion self-regenerating suppresser (ASRS; 2 mm, AutoSuppression mode, 20 mA current). Samples were diluted (five-fold) such that they were within the range of the calibration curve (1–20 mg L^{-1}, $R^2 > 0.99$).

Table B-1 Summary of simple linear regression analyses of all heteroatomic classes detected in negative ion mode for killed control (n = 3).

	Class	Pre-intervention slope	p-value	Daily change (%)	Post-intervention slope	p-value	Daily change (%)
Control	O^-	-1.08E-03	0.10	-0.11%	7.27E-03	0.35	0.73%
	O_2^-	-1.14E-03	0.07	-0.11%	-5.97E-03	0.67	-0.60%
	O_3^-	-8.14E-04	0.37	-0.08%	1.07E-02	0.40	1.07%
	O_4^-	-3.30E-04	0.45	-0.03%	7.43E-03	0.21	0.74%
	O_5^-	-1.65E-03	0.04	-0.17%	1.13E-02	0.29	1.13%
	O_6^-	-6.89E-04	0.51	-0.07%	4.61E-03	0.87	0.46%
	$S\ O^-$	2.43E-03	0.05	0.24%	4.71E-03	0.57	0.47%
	$S\ O_2^-$	-8.49E-04	0.32	-0.08%	-3.78E-03	0.56	-0.38%
	$S\ O_3^-$	3.14E-04	0.68	0.03%	9.45E-03	0.22	0.95%
Merichem	O_2^-	-9.66E-02	0.02	-0.08%	-1.21E+00	0.38	0.23%

Analyses were performed on the sum of the internal standard adjusted mass spectral intensities of species pre- and post intervention. Pre-intervention regression analysis was performed for days 0, 42, 88, 127, 272 and 389; post-intervention regression analysis was performed for days 389, 402, 409 and day 424. An F-test compared the pre- and post-intervention regressions for each heteroatomic class to review if the models were different (Prob > F, 0.05). Simple linear regression analysis for O_2^- species in the Merichem treatment is also displayed (n = 3).
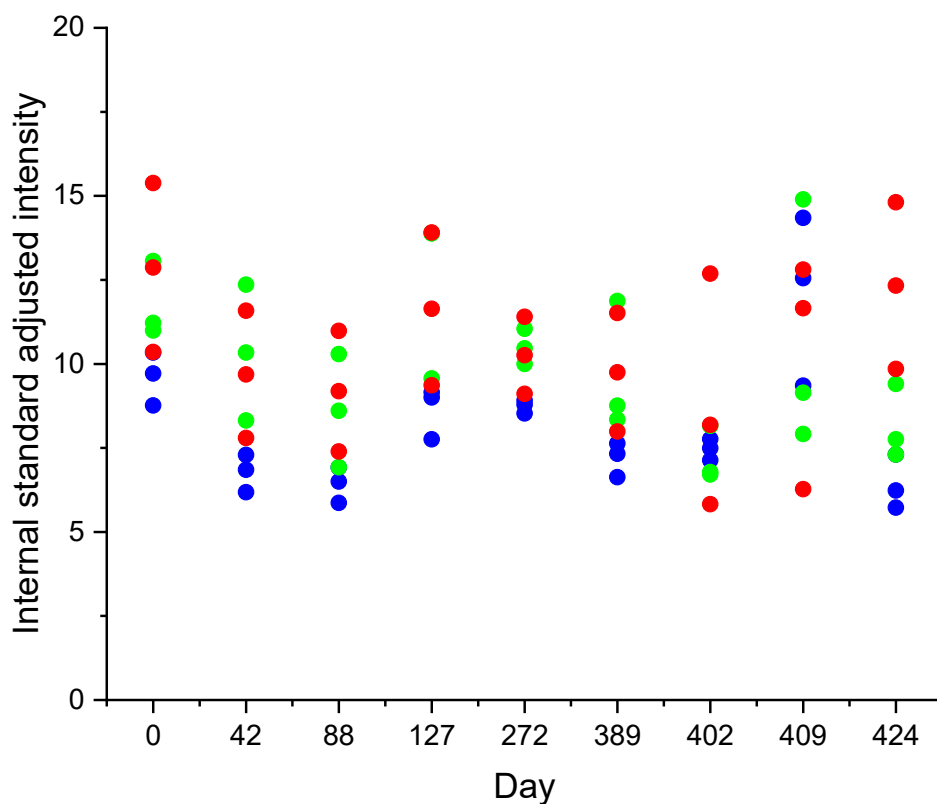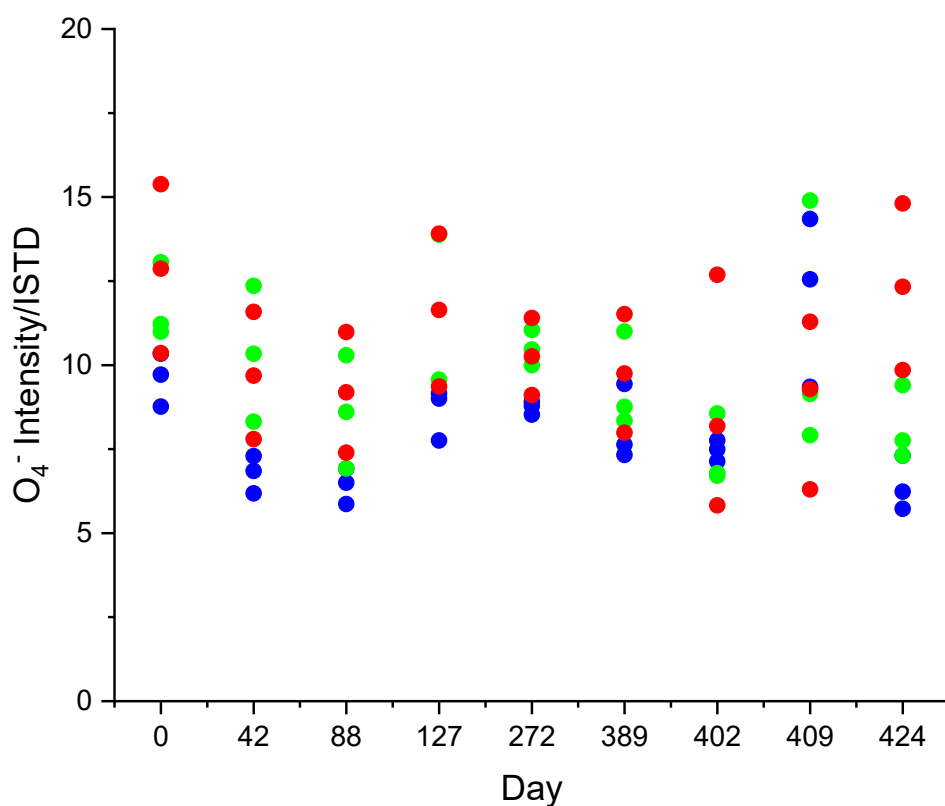
Figure B-1 Scatter plot summarizing the sum of species in the O⁻ class (i.e., Σ CxHyO⁻) in microcosm experiments. Untreated BML, Merichem (positive control) and killed control are displayed in blue, green and red, respectively. Internal standard adjusted mass spectral intensities as measured by the Orbitrap mass spectrometer are reported (arbitrary units, au). Triplicates for each treatment were sampled and analyzed for days 0, 42, 88, 127, 272, and 389.
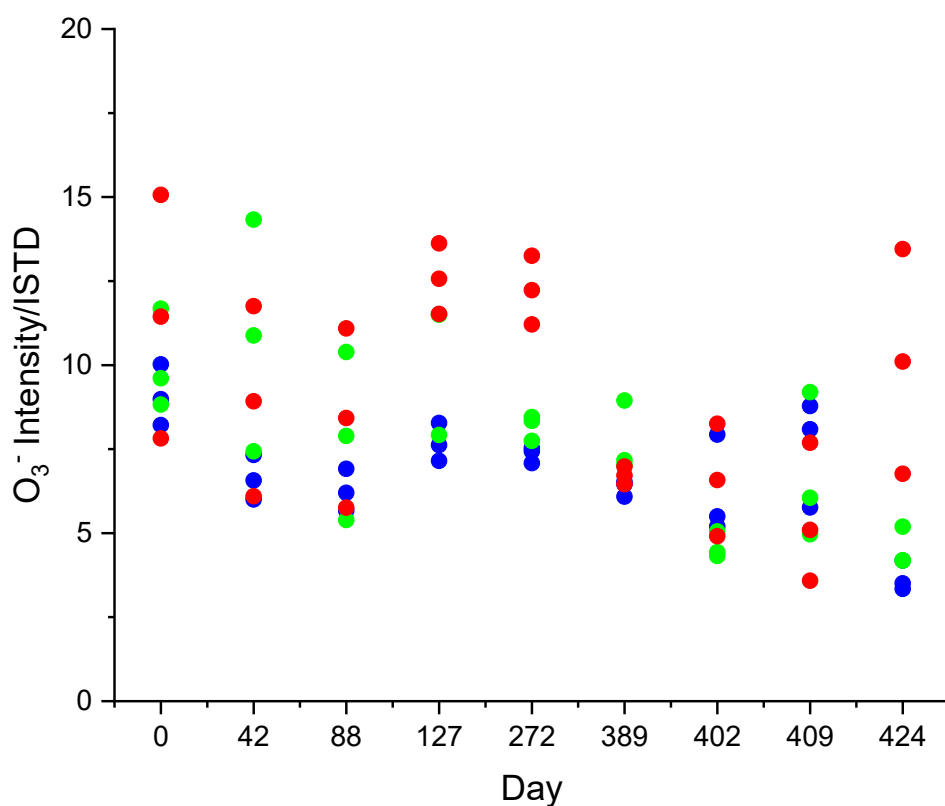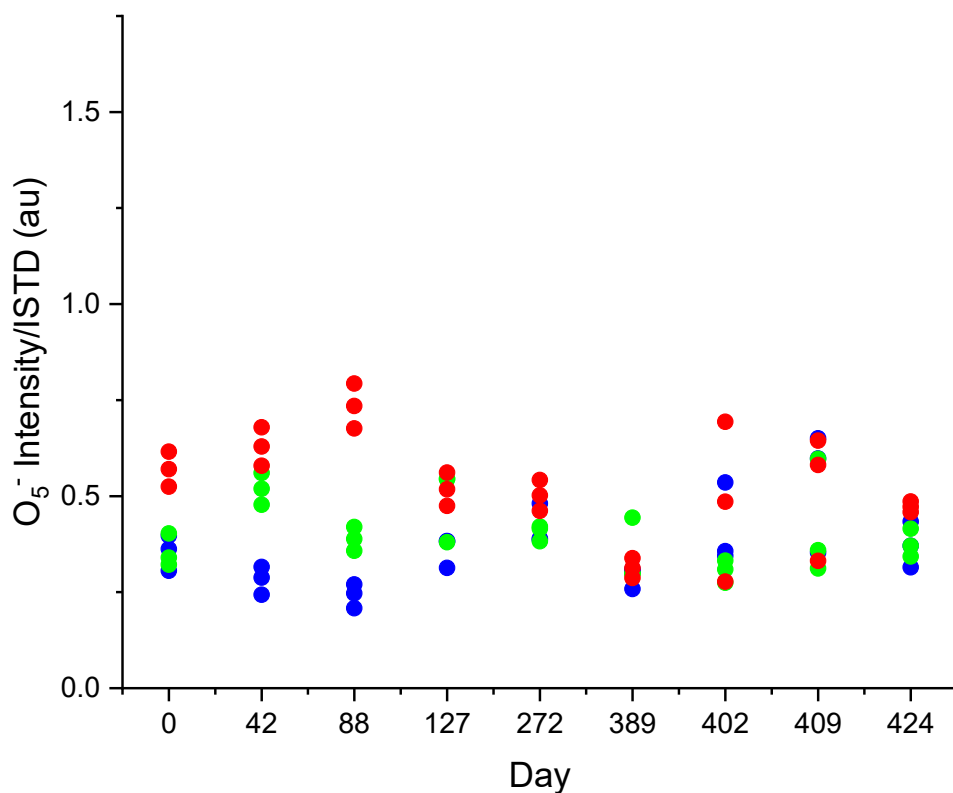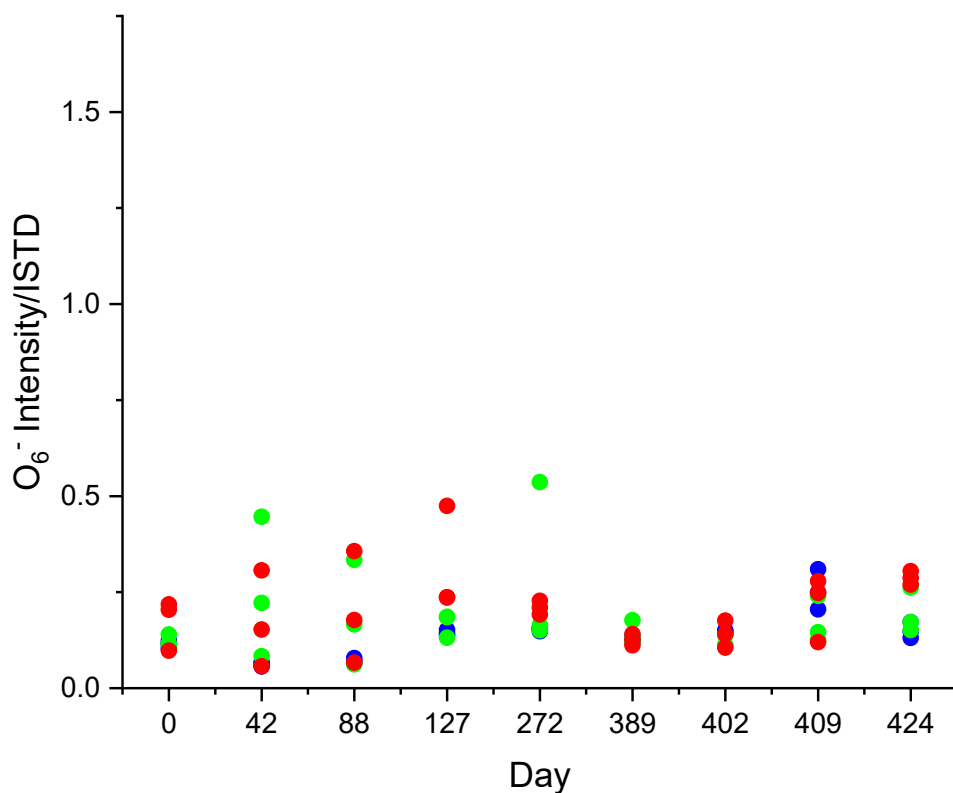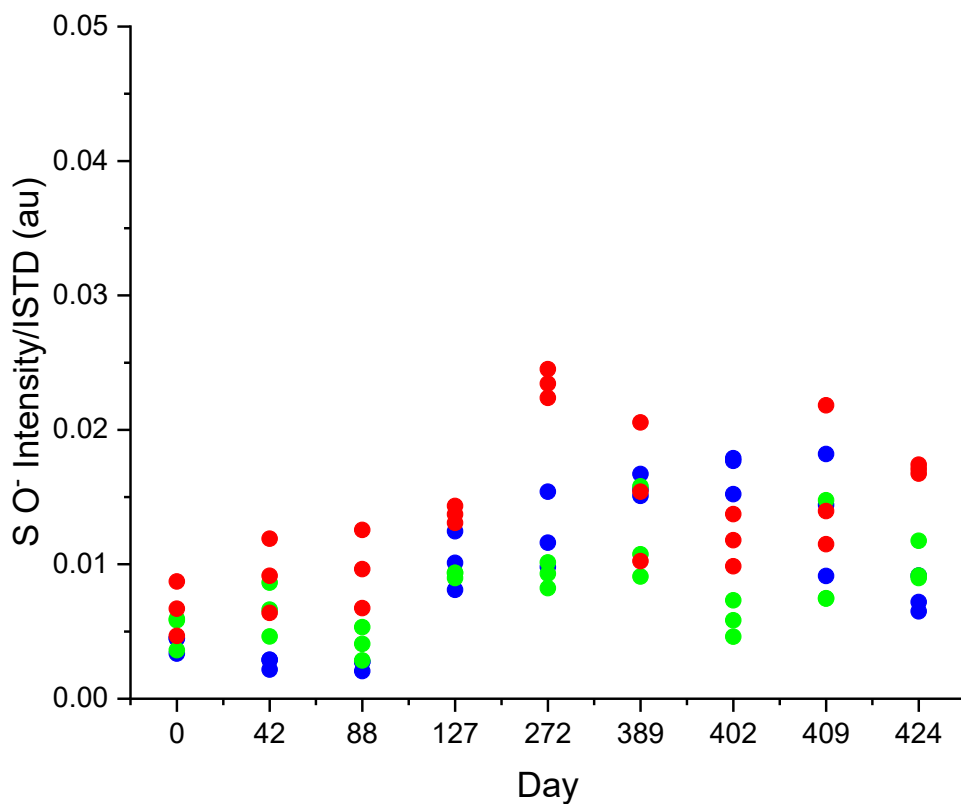
Figure B-2 Scatter plot summarizing the sum of species in the O$_3^-$ class (i.e., Σ CxHyO$^-$) in microcosm experiments. Untreated BML, Merichem (positive control) and killed control are displayed in blue, green and red, respectively. Internal standard adjusted mass spectral intensities as measured by the Orbitrap mass spectrometer are reported (arbitrary units, au). Triplicates for each treatment were sampled and analyzed for days 0, 42, 88, 127, 272, and 389.
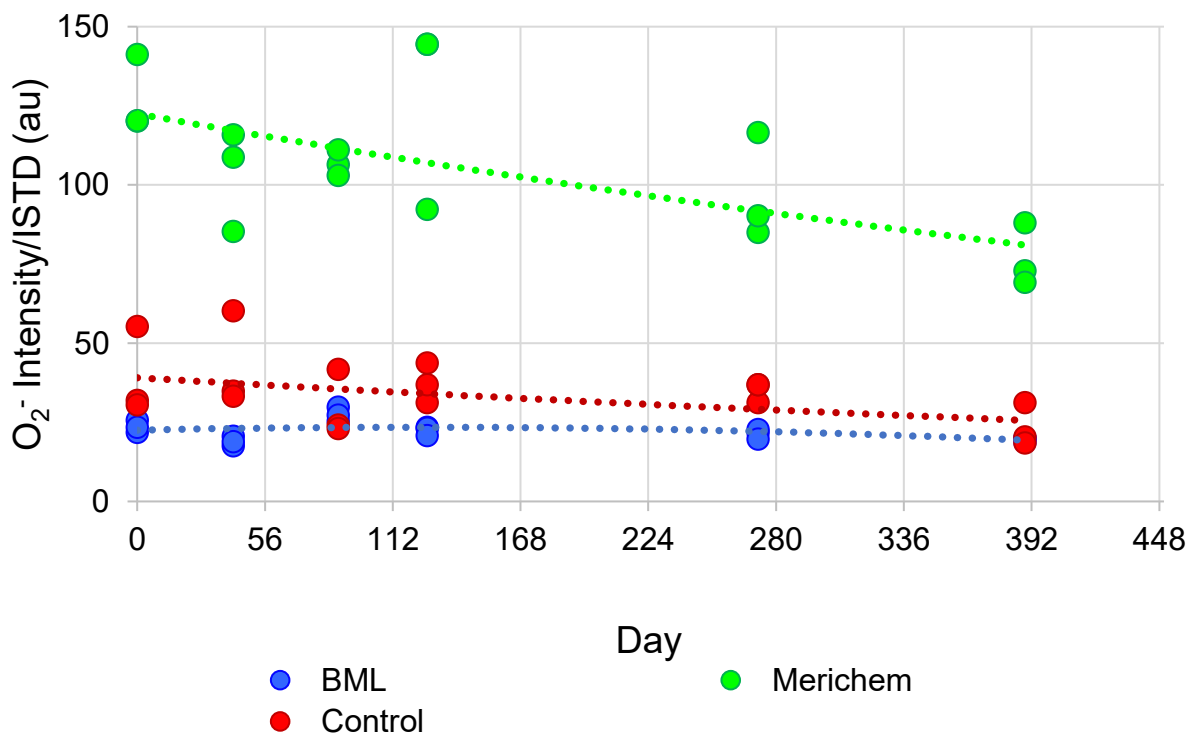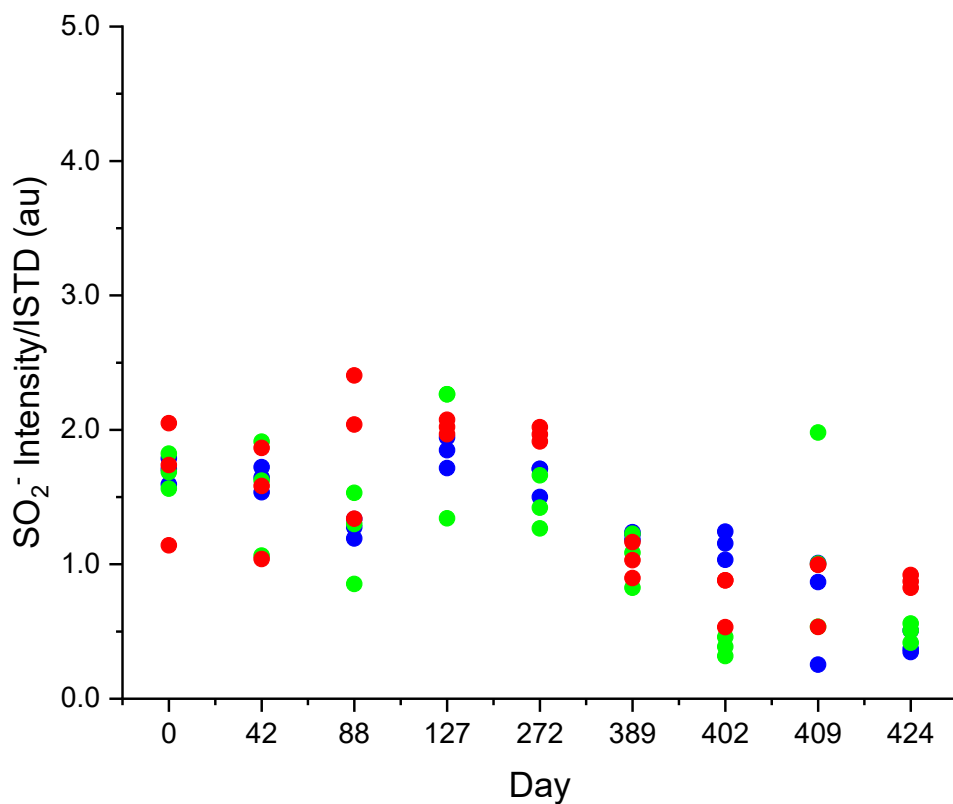
Figure B-3 Scatter plot summarizing the sum of species in the O$_2$$^-$ class (i.e., Σ C$_x$H$_y$O$^-$) in microcosm experiments. Untreated BML, Merichem (positive control) and killed control are displayed in blue, green and red, respectively. Internal standard adjusted mass spectral intensities as measured by the Orbitrap mass spectrometer are reported (arbitrary units, au). Triplicates for each treatment were sampled and analyzed for days 0, 42, 88, 127, 272, and 389.

Figure B-4 Scatter plot summarizing the sum of species in the O$_4^-$ class (i.e., Σ CxHyO$_3^-$) in microcosm experiments. Untreated BML, Merichem (positive control) and killed control are displayed in blue, green and red, respectively. Internal standard adjusted mass spectral intensities as measured by the Orbitrap mass spectrometer are reported (arbitrary units, au). Triplicates for each treatment were sampled and analyzed for days 0, 42, 88, 127, 272, and 389.

Figure B-5 Scatter plot summarizing the sum of species in the O$_5^-$ class (i.e., Σ C$_x$H$_y$O$_5^-$) in microcosm experiments. Untreated BML, Merichem (positive control) and killed control are displayed in blue, green and red, respectively. Internal standard adjusted mass spectral intensities as measured by the Orbitrap mass spectrometer are reported (arbitrary units, au). Triplicates for each treatment were sampled and analyzed for days 0, 42, 88, 127, 272, and 389.
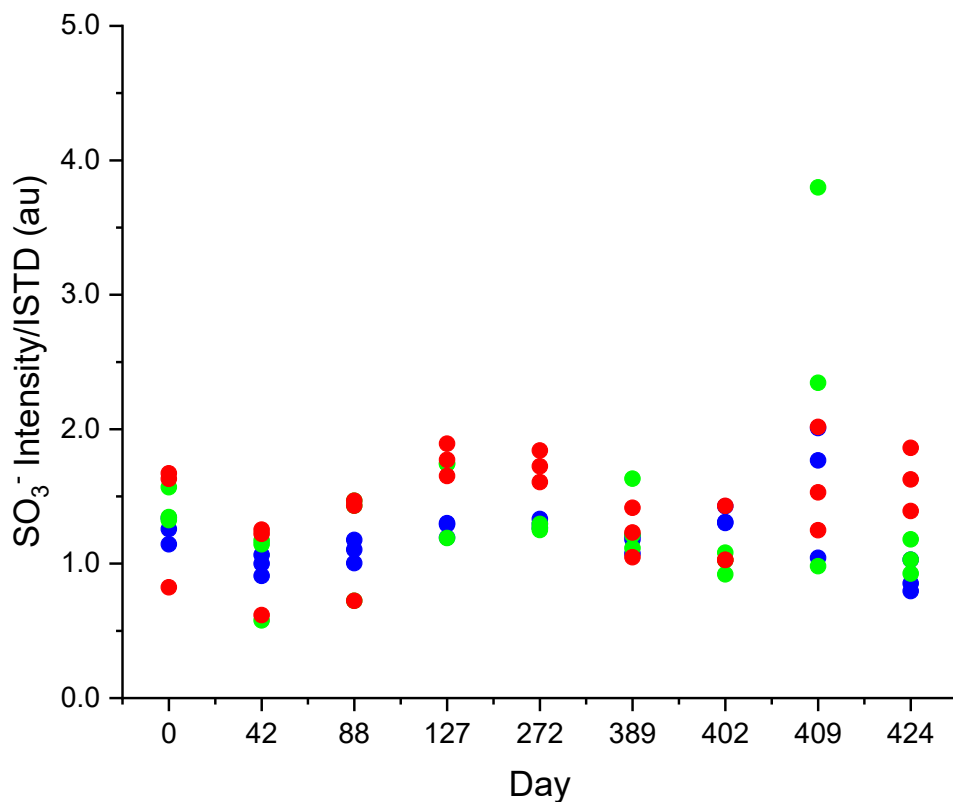
Figure B-6 Scatter plot summarizing the sum of species in the O_6^- class (i.e., Σ $C_xH_yO_6^-$) in microcosm experiments. Untreated BML, Merichem (positive control) and killed control are displayed in blue, green and red, respectively. Internal standard adjusted mass spectral intensities as measured by the Orbitrap mass spectrometer are reported (arbitrary units, au). Triplicates for each treatment were sampled and analyzed for days 0, 42, 88, 127, 272, and 389.
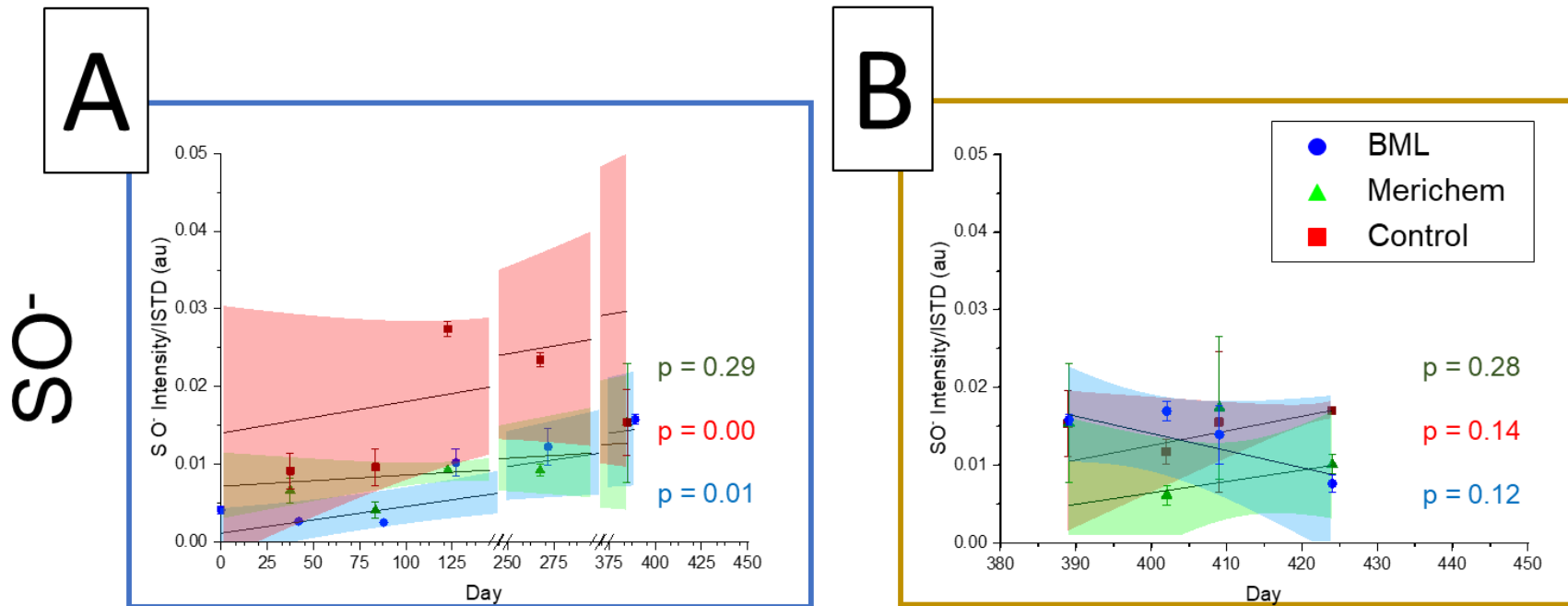
Figure B-7 Scatter plot summarizing the sum of species in the S O⁻ class (i.e., Σ CxHySO⁻) in microcosm experiments. Untreated BML, Merichem (positive control) and killed control are displayed in blue, green and red, respectively. Internal standard adjusted mass spectral intensities as measured by the Orbitrap mass spectrometer are reported (arbitrary units, au). Triplicates for each treatment were sampled and analyzed for days 0, 42, 88, 127, 272, and 389.

Figure B-8 Scatter plot summarizing the sum of species in the SO₂⁻ class (i.e., Σ CxHySO₂⁻) in microcosm experiments. Untreated BML, Merichem (positive control) and killed control are displayed in blue, green and red, respectively. Internal standard adjusted mass spectral intensities as measured by the Orbitrap mass spectrometer are reported (arbitrary units, au). Triplicates for each treatment were sampled and analyzed for days 0, 42, 88, 127, 272, and 389.

Figure B-9 Scatter plot summarizing the sum of species in the SO₃⁻ class (i.e., Σ CxHySO₃⁻) in microcosm experiments. Untreated BML, Merichem (positive control) and killed control are displayed in blue, green and red, respectively. Internal standard adjusted mass spectral intensities as measured by the Orbitrap mass spectrometer are reported (arbitrary units, au). Triplicates for each treatment were sampled and analyzed for days 0, 42, 88, 127, 272, and 389.

Figure B-10 Linear regression analyses for BML microcosm experiments with standard deviations and confidence intervals of triplicates. The sum of internal standard adjusted mass spectral intensities as measured by HPLC-Orbitrap-MS are displayed (arbitrary units, au). Pre-intervention analyses considered aliquots taken on days 0, 42, 88, 127, 272, and 389, and post-intervention analyses considered aliquots for samples taken on days 389, 402, 409, and 424. Pre-and post-intervention regression plots for all species in the SO⁻ (A and B, respectively).

Appendix C Supporting Information for Chapter 4

C.1 Description for non-targeted analysis workflow

Non targeted analyses were applied to the metabolism assays of Merichem, F1 and F2. For these experiments raw data files were imported into Compound Discoverer 2.1 SP1 (Thermo Fisher Scientific, San Jose, CA). Spectrum properties filter (S/N Threshold > 3), peak alignment (RT < 0.2 min and mass tolerance 5 ppm). A feature is chemical for which an exact *m/z* value and a retention time have been defined. That feature is described here as a species if its molecular formula and retention time have been confirmed. The feature is described as compound if its molecular formulae, retention time and structure are confirmed. The 'Detect Unknown Compounds Node' is used to find chromatographic peaks for species. This node was set to a mass error tolerance <5 ppm, relative intensity tolerance (used for isotope search) of 30%, S/N Threshold > 3, minimum peak intensity of 1000. Ion (adduct) detection was set to [M+H] + 1, for positive ionization and [M- H] – 1 for negative ionization modes. Settings in both the 'Detect Unknown Compounds' and 'Predict Compositions' nodes had minimum element counts of C5 H5 and maximum element counts to C50 H100 N2 O6 S2 for phase I and C50 H100 N2 O8 S2 for phase II. The 'Group Unknown compounds' node combines unknown species across sample files. Parameters in this node were set to a maximum mass and retention time difference of 5 ppm and 0.2 min, respectively. Mass tolerance and relative mass tolerance in the 'Merge Features' node was set to 0.5 and 5 ppm, respectively. All compounds were also screened using the 'Search mzVault' and 'Search mzCloud' nodes using the HighChem HighRes Search Algorithm. These nodes search the mzCloud database for matching fragmentation spectra and set compound classes (ex. endogenous metabolites, illegal additives, natural toxins etc.) to be used in the search. The 'Fill Gaps' node finds chromatographic peaks detected by the 'Detect Unknown Compounds' node in one sample file that may be missing in others. Parameters were set to mass tolerance <5 ppm and S/N >1.5. Any feature which did not have a formula assigned was searched in the free online. Some features which did not have a formula assigned, but met the criteria for further review (chromatographic peak area > 1000, p-value 0.05, log$_2$Fold Change > 2) were searched in the free online PubChem online database by exact mass with the precision (mass error tolerance) set to 10 ppm. For a given *m/z*, the list was manually search and the most probable formula was selected considering how many PubChem molecules were in the database, the mass error and the elements included. 'Mark Background Compounds' identified compounds

which were found in blank samples. Max Sample/Blank ratio was set to 3.0. Anything below this threshold was considered as background. Full workflows can be seen in **Figure C-1** and **Figure C-2** below.

C.2 Calculation of the weighted average for species

All detected species were converted to a percentage of the total response. The converted intensities were then used as a proportional weight (w_i) for the molecular weight (*MW*) of each species. The product of the molecular weight of all species and their respective proportional weights were summed to give the weighted average. It can be described by equation C-1:

$$\sum w_i \; x \; MW \; \text{(C-1)}$$

C.3 Protein Quantification calibration curves

The 7-point standard curve was first tested to determine the range of linearity and a 5-point curve was then established between the concentrations of 100 to 1000 ug/mL of protein (**Figure C-3**).

C.3.1 Phase I biotransformation of Merichem NAs in rat microsomes

Just 6 species significantly increased at after 225 minutes in the experiment of phase I metabolism using rat microsomes (**Figure C-4**). These species had at least 3 oxygen atoms indicating they were the products of phase I metabolism (**Table C-1**). This experiment demonstrated enabled a generalized estimation for what could be expected for the metabolism of dissolved organics in OSPW.

C.3.2 Phase I metabolism of BML dissolved organics

C.3.2.1 Phase I metabolism of BML basic extract

The rat microsomal assay saw 107 metabolites formed and a decrease in 8 species (**Figure C-6**). More than double the number of products were formed in rat compared to fish microsomes by phase I biotransformation (108 vs 50 species). One notable difference between fish and rat assays was in the number of heteroatomic products that were generated: only 6 species in the products of fish metabolism contained a nitrogen atom, whereas all but 17 of metabolites of the rat assay contained nitrogen atoms. Also, the chromatographic peak areas were much higher in rat compared to fish metabolites. The five species which displayed the greatest increase in rat assays were $C_{18}H_{31}N_5O_5$, $C_{26}N_{34}N_6SO$, $C_{13}H_{20}N_2O_5$, $C_{15}H_{32}N_2O_6$ and $C_{22}H_{12}N_4O_2$. All had *m/z*

at least 300 and chromatographic peak areas were between 2925 – 7809 greater when metabolized by rat enzymes. These compounds (except $C_{13}H_{20}N_2O_5^+$) have at least 5 DBEs. In fact, 80% of metabolites in rats had 4 or more DBEs. This may reflect that enzymes in rat microsomes have a greater ability to metabolize larger compounds in OSPW, or perhaps, that these enzymes have an affinity for aromatic substrates.

One interesting difference in fish assays compared to rats was that many isomers in the rat assays were detected for the same species. One example is in the isomers for hydroxytestosterone, $C_{19}H_{28}O_3$ (**Figure C-7**), a metabolite of phase I biotransformation of reference compound, testosterone. Other metabolites with two or more isomers include $C_{10}H_{12}O_3$, $C_{16}H_{34}N_2O_4$, $C_{18}H_{30}O$, $C_{20}H_{30}O$, and $C_{28}H_{27}N$. Some products are likely derivatives of the compound. For example, $C_{19}H_{26}O_3$ may have resulted from a methylation followed by successive hydrogenation reaction and it is conceivable that $C_{21}H_{34}O_2^+$ could be the result of a series of methylations.

C.3.2.2 Phase I biotransformation of BML acidic extract

Failed. Must repeat experiment.

C.3.3 Phase I + II Biotransformation

C.3.3.1 Phase I + II metabolism of BML basic extract

Previous studies of GST activity monitored in trout S9 (97.2 pmol/min/mg protein) compared to rats (1400 pmol/min/mg protein) has led researchers to suggest phase II conjugation in trout liver may not be as active as in rats.[7] Here, glucuronidation of basic species was not observed in either trout or rat microsomes. The metabolite with the highest chromatographic peak area in rat microsomes was $C_{15}H_{32}N_2O_4$ (area max 274398). After 225 minutes, members of the same homologous series, $C_{16}H_{34}N_2O_4$ and $C_{17}H_{36}N_2O_4$, were at maximum peak areas 2% and 1% of $C_{15}H_{32}N_2O_4$. Interestingly, $C_{14}H_{28}N_2O_4$ was the only species of this heteroatomic class that was evolved to a higher degree in fish microsomes (**Figure C-8**). After 15 minutes the species was completely depleted in rats. Species of the N_2O_4 heteroatomic class appeared to be substrates of the enzymes in rat microsomes but not in fish.

C.3.3.2 Phase I + II metabolism of BML acidic extract

The acidic fraction of BML OSPW did not appear to have increased products of glucuronidation in rats compared to fish. Fish and rat microsomal assays produced a maximum of two products which may have been the result of glucuronidation: $C_{16}H_{22}SO_6$ and $C_{19}H_{28}SO_8$ (6 DBEs) in fish, and $C_{19}H_{18}O_6$ and $C_{21}H_{22}O_8$ (11 DBES) in rats. Within a given animal, the metabolites had the same number of DBEs and differed by two oxygen atoms. Some compounds can be detected in both positive and negative ion mode depending on their ability to be protonated and/or deprotonated in the gas phase. The evolution of $C_{15}H_{32}N_2O_4$ was a product in the phase I reactions in rat microsomes with the BML acidic fraction. Evolution of this species in a second assay suggests $C_{15}H_{32}N_2O_4$ (like many metabolites in this assay) was a formed due to phase I metabolism, and not phase II.

Table C-1 Chromatographic peak areas and retention times (RT) of metabolites which significantly increased (p < 0.05) following of phase I biotransformation of Merichem NAs in rat microsomes.

Product (*m/z*)	RT (min)	Area Max.	Product formula
226.1558	5.771	8144	$C_{13}H_{22}O_3$
230.1510	6.084	12295	$C_{13}H_{24}O_3$
238.1556	5.864	2614	$C_{14}H_{22}O_3$
240.1713	6.061	9134	$C_{14}H_{24}O_3$
266.1867	6.446	2061	$C_{16}H_{26}O_3$
316.2600	6.676	1018	$C_{18}H_{36}O_4$

Table C-2 Summary for first order kinetic plots of all species which increased in phase I biotransformation of the acidic fraction in trout microsomes.

Species	m	SE	Prob>|t|	LCL	UCL	R^2
$C_{16}H_{32}O_3$	0.008	0.001	0.008	0.004	0.012	0.93
$C_{16}H_{30}O_4$	0.012	0.003	0.033	0.002	0.021	0.68
$C_{20}H_{32}O_3$	0.011	0.004	0.093	-0.003	0.025	0.66
$C_{20}H_{32}O_4$	0.010	0.004	0.095	-0.003	0.022	0.66
$C_{22}H_{32}O_3$	0.013	0.006	0.105	-0.005	0.031	0.64
$C_{22}H_{34}O_3$	0.018	0.006	0.061	-0.002	0.038	0.74

Linear regression of C_t/C_0 vs t was performed and plotted as $ln\ C_t/C_0 = mt + b$.
Slope for the line (m)
standard error (SE) of the average chromatographic peak area (n = 3)
lower confidence level (LCL) and the upper confidence level (UCL) for the 95% confidence interval

Table C-3 Summary of paired t-test reviewing the weighted average molecular weight of species at reaction initiation and termination in trout microsomal assays.

BML extract	Phase	MW at 0 min mean	SD	MW at 225min mean	SD	Prob>|t|
BASIC	I	158.06	2.08	170.38	16.45	0.34
	II	127.50	1.00	129.05	0.32	0.10
ACIDIC	I	239.19	9.45	250.01	1.04	0.21
	II	243.99	0.82	244.24	11.15	0.97
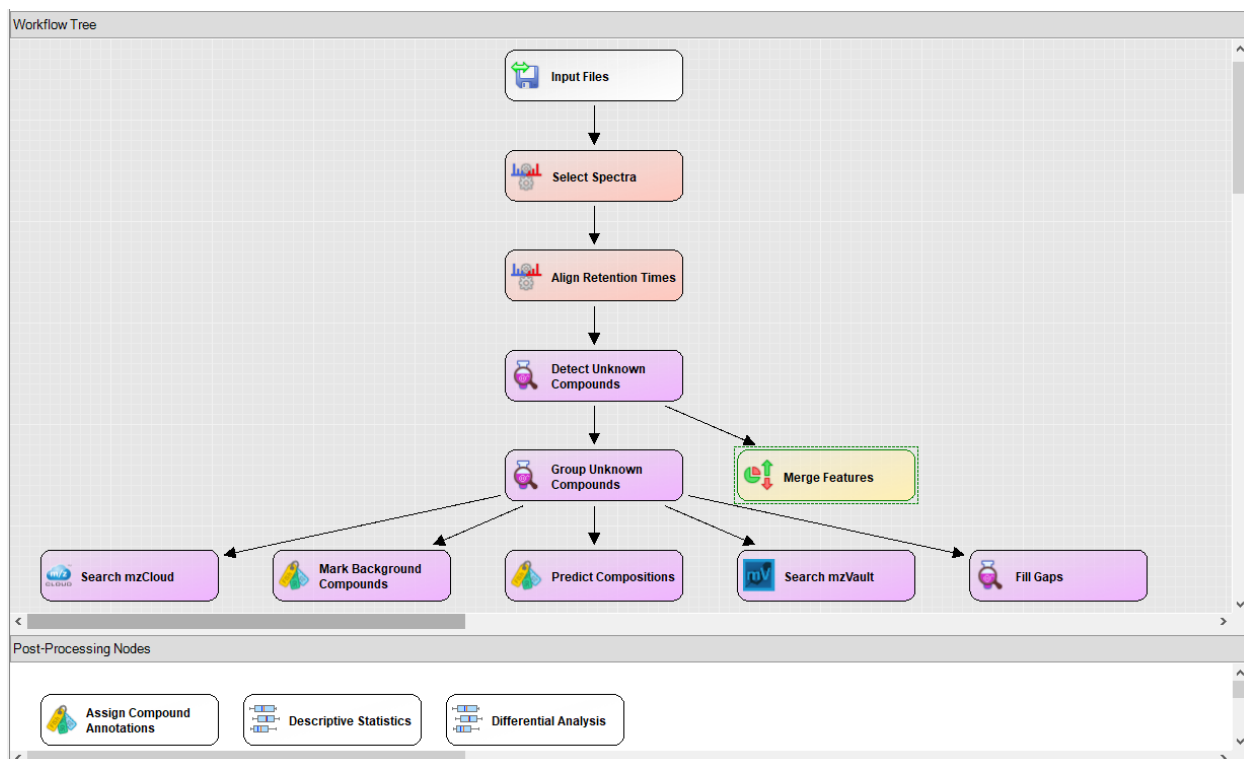
Figure C-1 Compound Discoverer 2.1 non-targeted analysis workflow used in study of biotransformation of toxic OSPW fractions by fish and rat microsomes and S9.
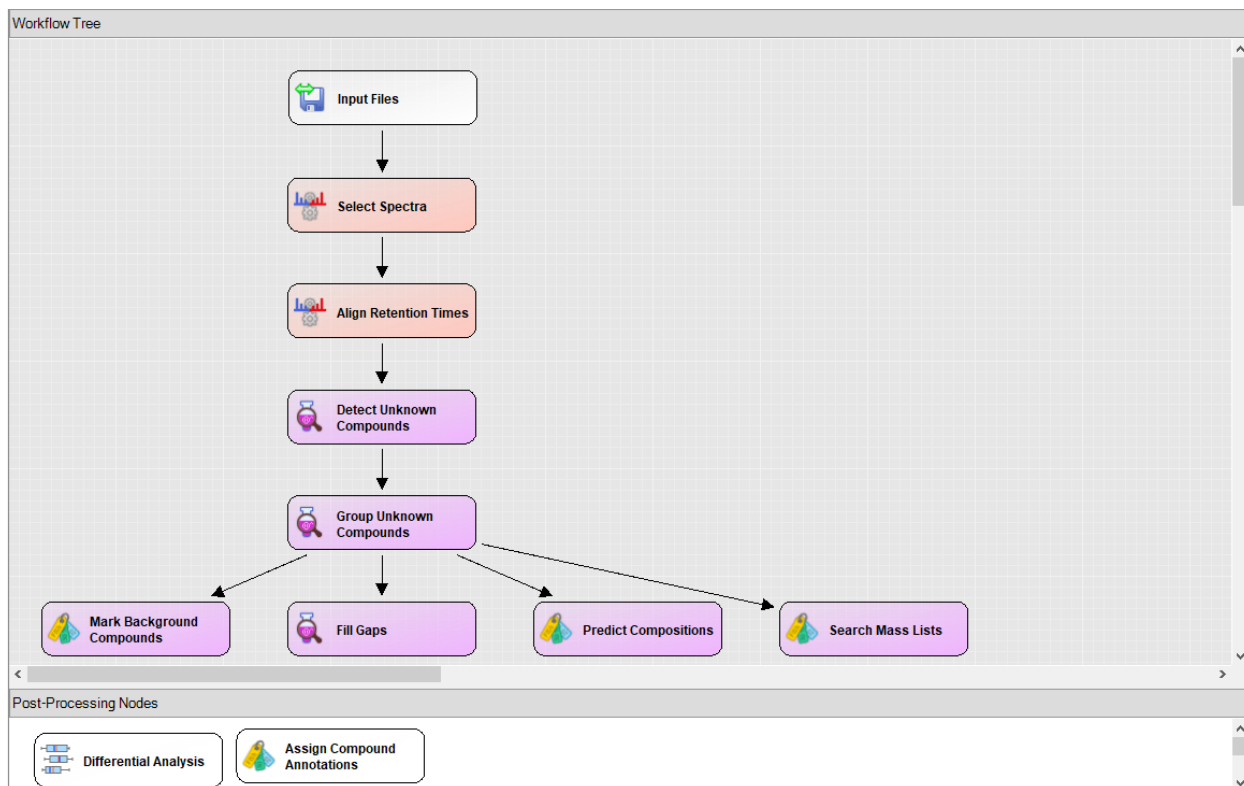
Figure C-2 Compound Discoverer 2.1 non-targeted analysis workflow used in study of Merichem NAs by fish and rat microsomes.
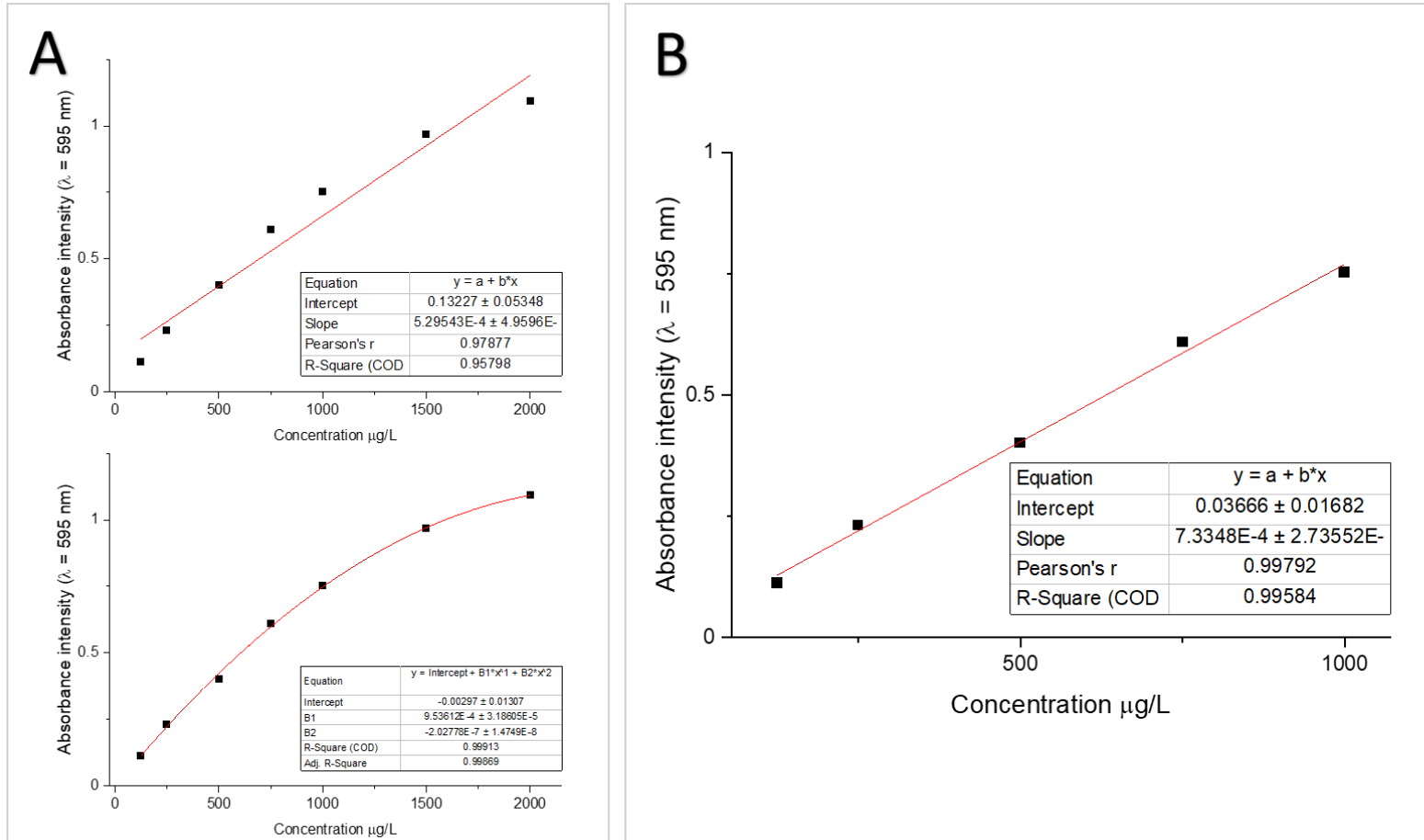
Figure C-3 Calibration curve from the Bradford assay used for protein quantification. The 7 point curve (A) was plotted using linear (top) and polynomial (bottom) functions. The 5-point curve (B) was used for quantification of proteins in trout microsomes, rat microsomes and trout S9.
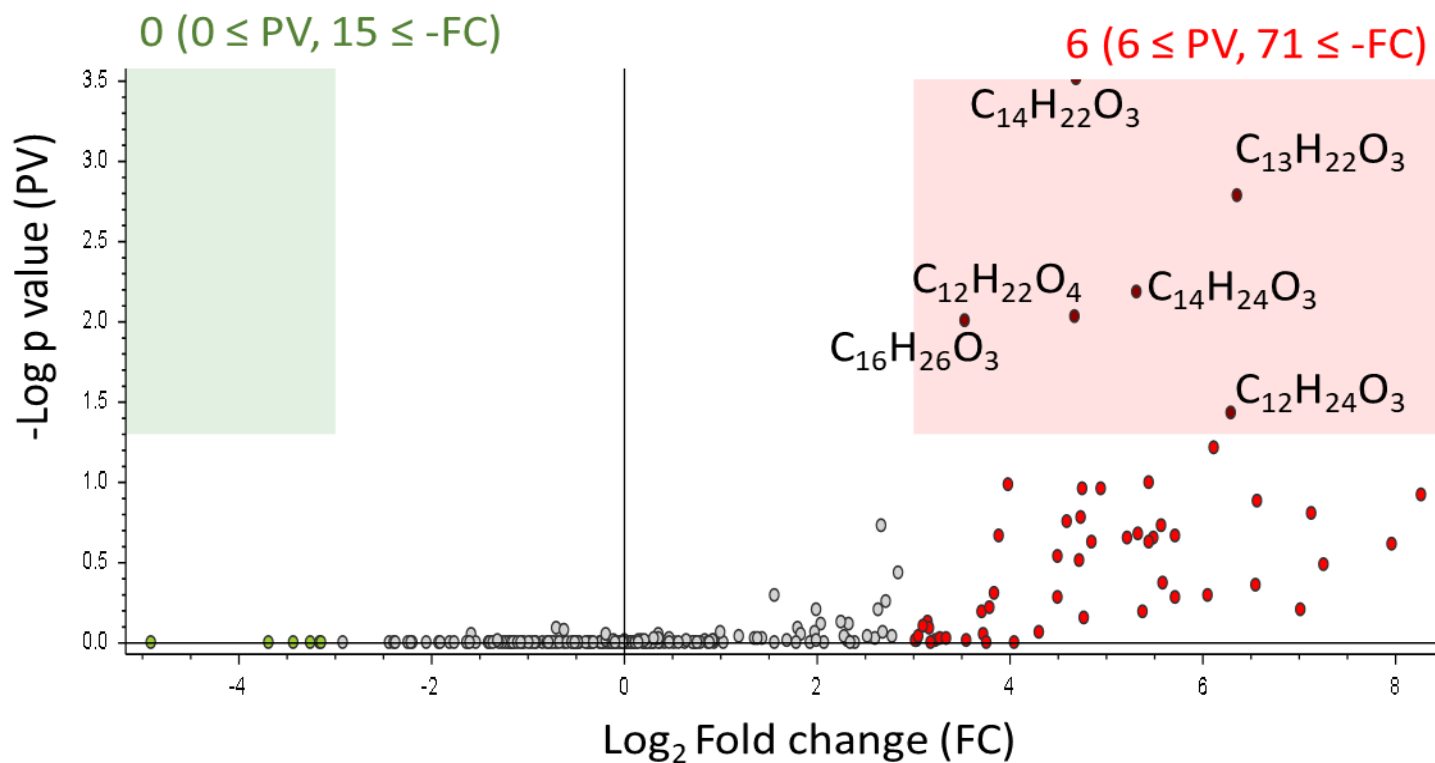
Figure C-4 Volcano plot of phase I metabolism of Merichem NAs in rat microsomes. Log_2Fold Change is shown on the x-axis and log p-value (PV) on the y-axis for the difference between test and control (n = 3 each). All species displayed have a chromatographic peak area ≥ 1000. Species within the shaded boxes represent those which have met both the p-value (p < 0.05) and log_2Fold Change (x 4) cut-off and have significantly decreased (green) or increased (red). Species which have met the Fold Change cut-off but were not statically different in tests compared to controls fall outside of the shaded boxes but are coloured accordingly. Only 6 species had significantly increased after 225 minutes.
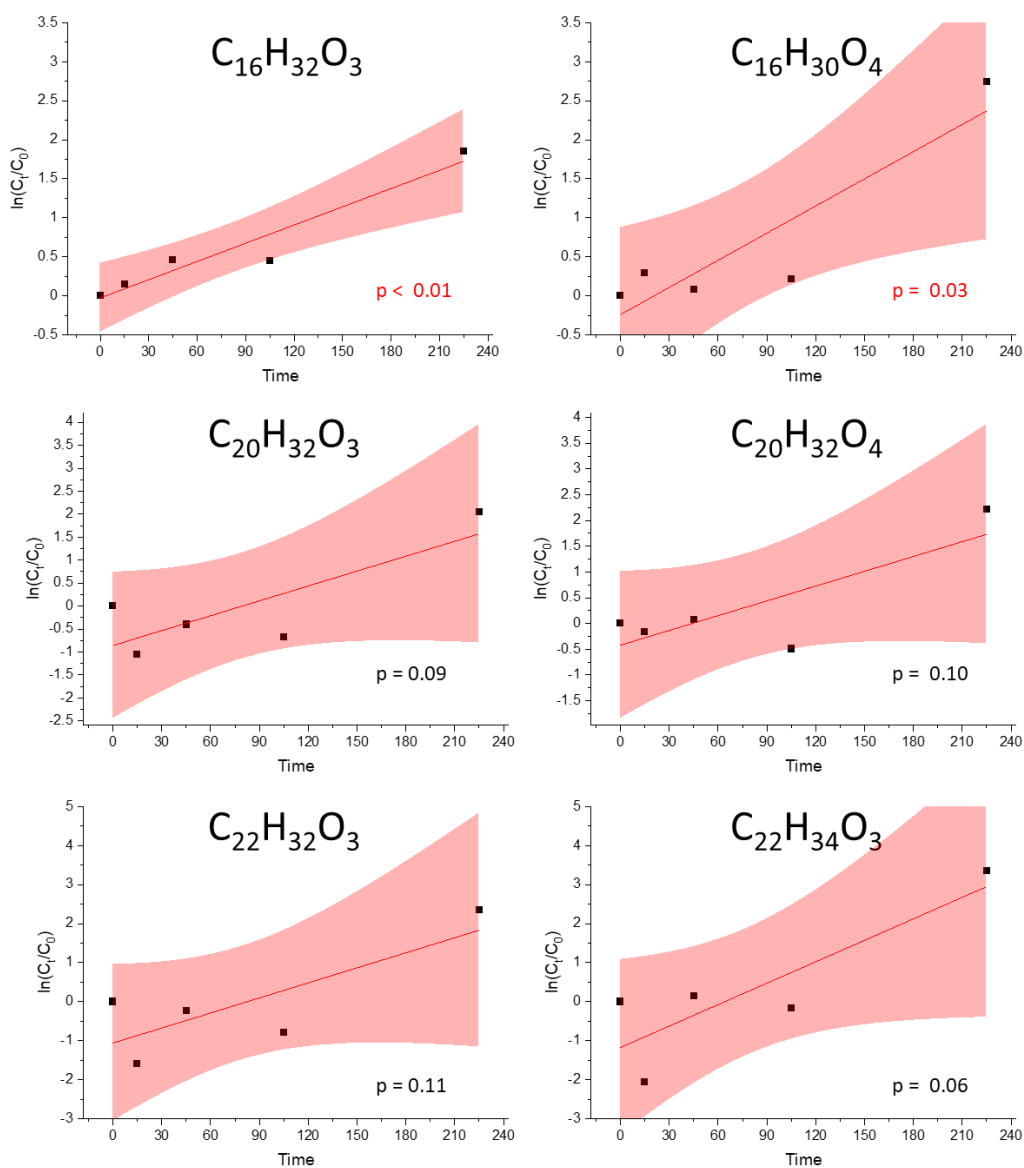
Figure C-5 First order kinetic plots of all species which increased in phase I biotransformation of BML acidic fraction in trout microsomes.
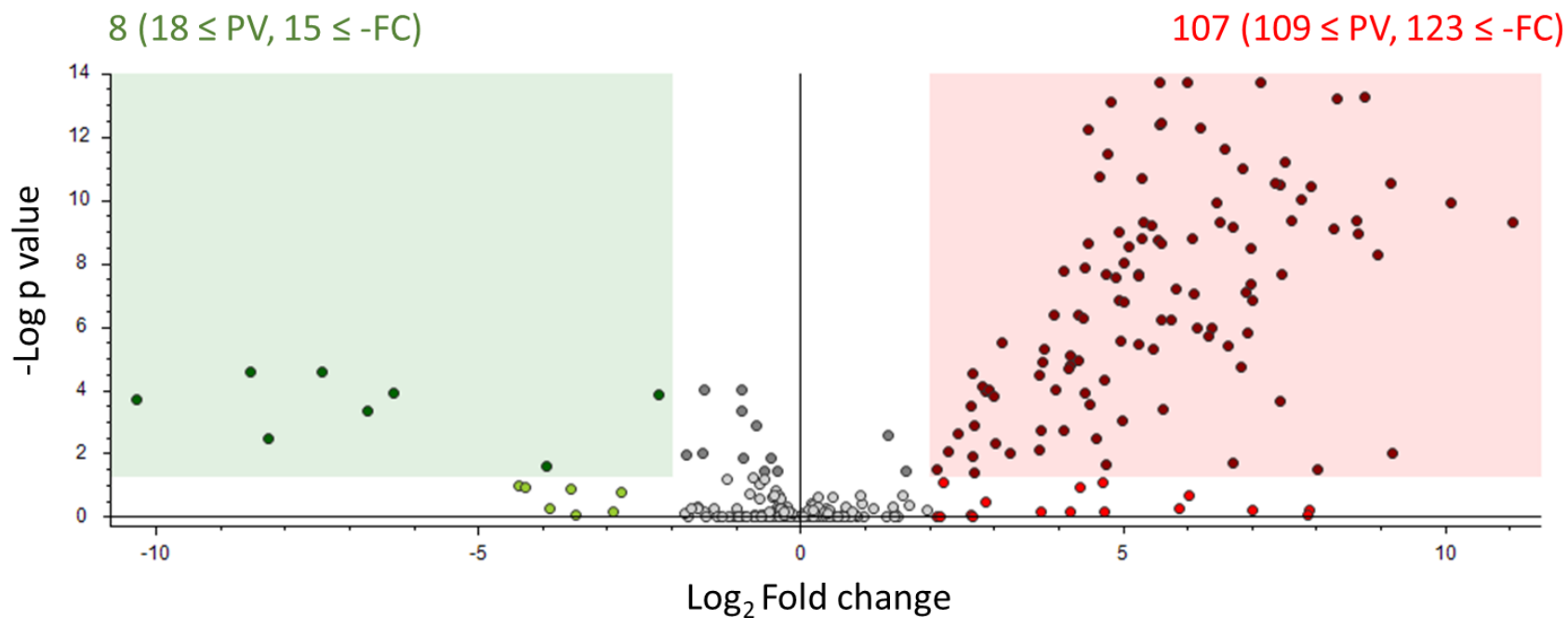
Figure C-6 Volcano plot of phase I metabolism of BML basic extract in rat microsomes. Log_2Fold Change is shown on the x-axis and log p-value (PV) on the y-axis for the difference between test and control (n = 3 each). All species displayed have a chromatographic peak area $\geq$ 1000. Species within the shaded boxes represent those which have met both the p-value ($p < 0.05$) and log_2Fold Change (x 4) cut-off and have significantly decreased (green) or increased (red). Species which have met the Fold Change cut-off but were not statically different in tests compared to controls fall outside of the shaded boxes but are coloured accordingly.
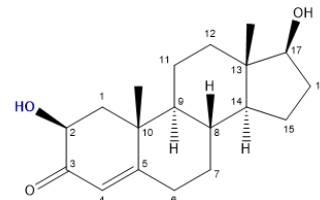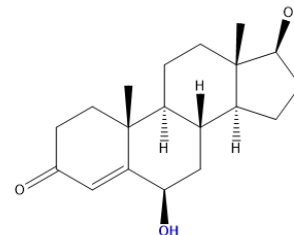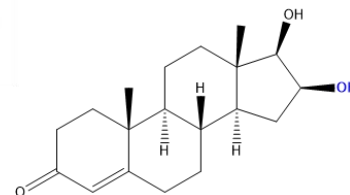
Figure C-7 Chromatogram of hydroxytestosterone (C₁₉H₂₈O₃, *m/z* 304.203) showing 3 peaks. Multiple isomers detected in rat microsomal assays which were not detected in trout microsomal assays. The most common isomers in rats include 2-, 6- and 16β-hydroxytestosterone.
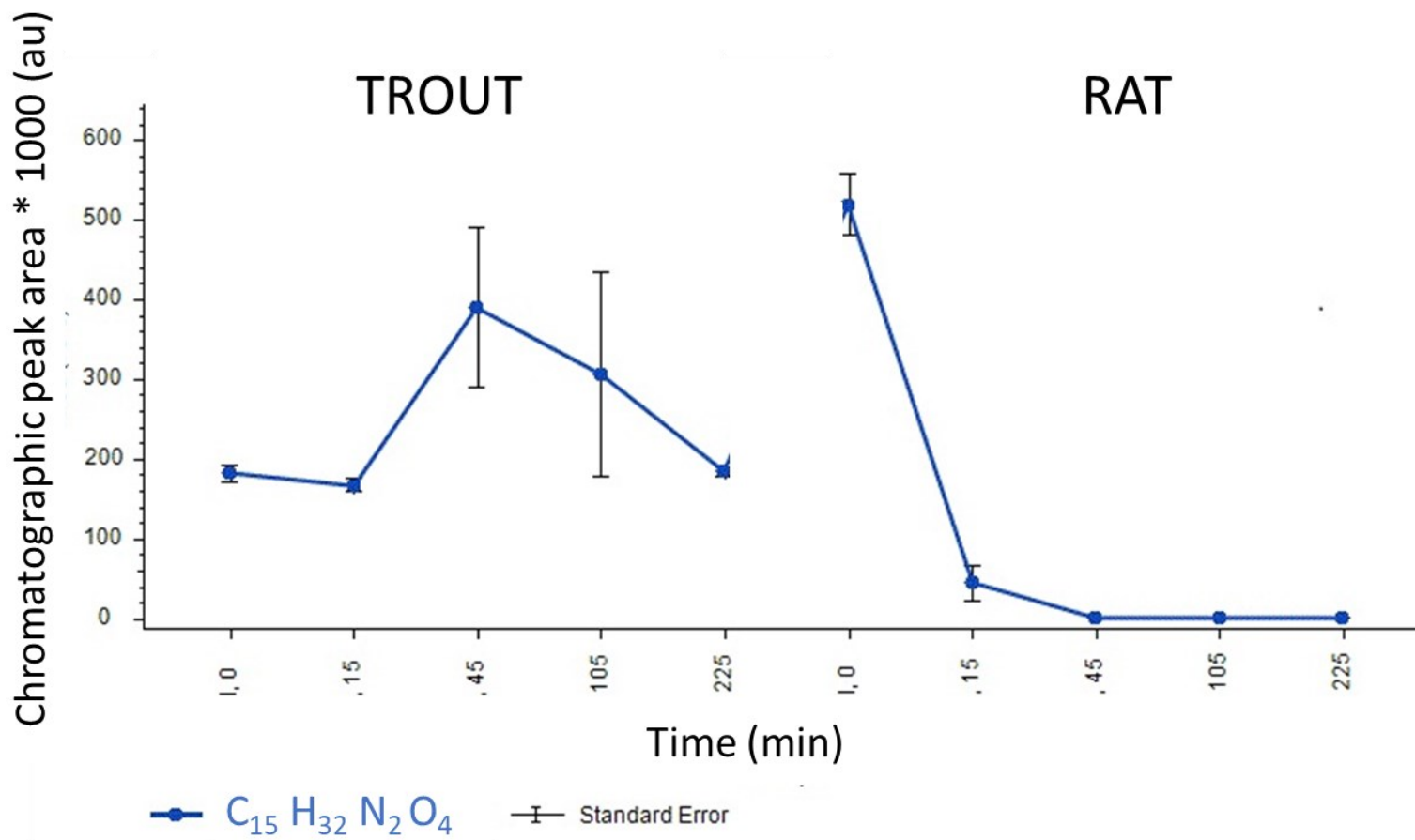
Figure C-8 $C_{15}H_{32}N_2O_4$ was the only metabolite in F1 which was detected at higher levels in trout compared to rat microsomes at 225 min.

C.4 References

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