Genotoxicity and Mutagenicity of Extractable Organics from Oil Sands Process-Affected Water

by

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ABSTRACT

Oil sands process-affected water (OSPW) is produced in the surface mining industry during the hot-water extraction of bitumen from oil-sands ore. Its acid extractable organic (AEO) fraction is known to be acutely toxic, but few studies have addressed its genotoxicity or mutagenicity. Here, the *in vitro* SOS-Chromo genotoxicity test and Ames test (TA98 and TA100) were used to evaluate these endpoints for the whole AEO mixture and two chemical sub-fractions (acid and neutral extractable), with and without S9 enzymes. Whole AEO showed increased genotoxicity at concentrations above 7x (SOS response of 9.63±2.11SD) where S9 fraction decreased genotoxicity by 70%. AEO mutagenicity in the TA 98 strain was similar to the TA100 strain and S9 generally decreased the mutagenicity. Genotoxicity of the acid extractable subfraction was lower than whole AEOs, with or without S9, while the neutral extractable subfraction SOS-response was the lowest and S9 presence did not significantly decrease genotoxicity. The acid extractable subfraction mutageniticy was also similar in TA98 and TA100, and S9 decreased mutagenicity by approximately half, with the TA 100 strain showing the highest mutagenicity. OSPW AEOs are genotoxic and mutagenic in the in vitro assays used here, but the mechanisms are in need of clarification and further studies examining the carcinogenic potential of OSPW are warranted.

Keywords: OSPW, genotoxicity, mutagenicity, SOS Chomo-test and AMES

PREFACE

Chapter 2 and 3 of this thesis will be submitted for publication as *Zetouni NC, Siraki A, Weinfeld M, Pereira, A and Martin, JW. (2015). Screening of Genotoxicity and Mutagenicity in Extractable Organics from Oil Sands Process-Affected Water. Environmental Toxicology and Chemistry.* I was responsible for the data collection and analysis as well as the draft manuscript. All co-authors contributed to the research ideas and the manuscript edits, and Alberto Pereira contributed with the analytical chemistry of OSPW and its fractions.

DEDICATION

This research is dedicated to many people. Each one of them had a different role in my time as a graduate student, but all of them are special in many ways.

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GLOSSARY OF TERMS

2-AA: 2-Amino-anthracene, a genotoxic/mutagenic chemical that needs bioactivation.

4-NQO: 4-Nitroquinoline 1-oxide, a genotoxic chemical that does not need bioactivation.

Ames: A test created in 1973 that uses a genetic engineered bacteria to detect mutations on the histidine producer gene

C.F.U.: Colony forming units

Carcinogenesis: A multistage process involving initiation (gene changes that supress tumor activity), promotion (a build-up of mutations making cells less susceptible to apoptosis and prone to cell division) and progression (where cells become tumor becomes malign due invasiveness). The final stage is cancer.

CSS: Cyclic steam stimulation

Cytotoxicity: forced cell death via an agent (toxic or toxicant).

DCM: Dichloromethane

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

EC: Extraction control.

F1-NE: Fraction 1, Neutral extract

F2-AE: Fraction 2, Acid extract

S9 fraction / liver enzymes: A mix of liver enzymes (including p450)

Genotoxicity: A physical DNA damage. It can be repaired.

Genotype: The genetic makeup of a cell/individual usually with reference to a specific characteristic under consideration.

Hist-: Histidine deficient bacteria

Hist+: Histidine producer bacteria.

Histidine: An important amino acid that participates on DNA replication.

Mutagenicity: A permanent change in a gene that can be caused by genotoxicity.

NAs: Naphthenic acids

OSPW AEO: Oil sands process-affected water extractable organics

OSPW: Oil sands process-affected water

Phenotype: Composition of species characteristics like morphology, development, biochemical and physiological properties.

pNPP: para-Nitrophenylphosphate (pNPP) is a chromogenic substrate for acid and alkaline phosphatase in ELISA assays.

RNA: Ribonucleic acid

SADG: Steam-assisted gravity drainage

SOS chromo-test: A test created in 1975 to measure genotoxicity via a colorimetric reaction

SOS: A DNA gene repair system that is activated when general damage occurs in the DNA.

SOSIF: SOS inducing factor

UMU: Another DNA repair system that is only activated when the SOS response is not enough to repair the DNA.

Xenobiotics: a foreign chemical substance found within an organism that is not naturally produced by or expected to be present within that organism.

X-gal: X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is an organic compound consisting of galactose linked to a substituted indole

β-gal: Beta-galactosidase. A hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides.

CHAPTER 1: INTRODUCTION

1.1 The Oil Sands Regions of Northern Alberta

The Athabasca region in Alberta, Canada, contains one of the largest bitumen reserves in the world, where approximately 3 billion barrels of crude oil are recoverable, currently supplying 90% of all oil demand in Canada (1). The bitumen is present in the form of oil sands (i.e. bituminous sands), and is generally located in underground reservoirs underneath a layer of overburden. Oil sands are a complex mixture of bitumen, water, sand, clay and silt. The bitumen is a form of heavy oil that is viscous and sticky and cannot be pumped out of the ground like conventional oil. When present close enough to the surface, oil sands can be recovered by surface mining operations.

Such activity is highly concentrated north of Fort McMurray in the Athabasca oil sands region, close to the Athabasca River. Deeper reservoirs of oil sands are present in the Cold Lake (22,000 km^2) and Peace River regions (8,000 km^2) (*Figure 1*) which cannot be economically recovered by surface mining (2), and are instead recovered by *in situ* processes (Section 1.2).

Figure 1: Canada's oil sands regions. Source: ERCB 2009 (3), Figure 2.1, p. 2-1

Total bitumen production in Northern Alberta was 180 billion barrels (1 barrel = 119 L) per year in 2006 (4), increasing to 213 billion barrels in 2013-2014 (5,6). Aside from being a profitable industry, government of Alberta royalties were $15.5 billion in 2009, and the oil sands industry employed more than 15,000 people between 2008 and 2010 (7).

1.2 Oil sands recovery and extraction

1.2.1 In situ extraction

The *in situ* extraction method is used to obtain bitumen in reservoirs deeper than 150 meters (8). *In situ* extraction activity is present in all three regions, but mainly in the Peace River and Cold Lake regions. This method involves steam injection surrounding the bitumen reservoir,

heating it and thereby making it less viscous, allowing the bitumen to be extracted from the ore and be pumped to the surface; together with condensed water from the injected steam (4). This general method can be divided in two distinct processes: cyclic steam stimulation (CSS) and steam-assisted gravity drainage (SAGD).

CSS requires a cap rock and overburden of more than 300–400 metres to withstand the high pressure created by the steam. Steam is injected inside the reservoir for several hours, and bitumen can be pumped to the surface where it is collected, and the cycle repeated (9).

SAGD is used to extract oil in areas where open mining is not possible and the bitumen is between 150 and 75 meters deep. In this process, steam is continuously injected underneath the reservoir for three to six months through one set of pipes, and once the reservoir is thoroughly heated it can be pumped to the surface through another set of pipes, where the bitumen is recovered (1). Both processes are water intensive, needing an average of 3.5 cubic meters of water to recover 1 cubic meter of bitumen. The steam condensed water in both processes contains hydrocarbons from the bitumen but can be recycled and re-used (9).

1.2.2 Open-pit surface mining

This method is used in the Athabasca region where bitumen is present as oil sands at depths less than 75 meters. This method is the simplest and most economical technique to obtain the oils sands, but also very damaging due a complete removal of the natural landscape. To access the bitumen it is necessary to remove the muskeg and overburden above the reservoir. If the oil sands are below wetlands, the drainage of the water is also necessary before extraction

operations can begin (10). Reclamation of the natural landscape has begun, but takes long periods of time.

1.2.3 Bitumen extraction process in surface mining operations

After surface mining of the oil sands, it is necessary to separate the bitumen from sand, silt and clay in the raw ore. The process used is called the Clark hot water process, which mixes the oil sands with warm alkaline water (pH~ 8.5, adjusted with sodium hydroxide) in large mixing tanks, where air injection is used to make the bitumen (in a froth form) float to the surface (11).

The bitumen froth is skimmed off the top, sent to treatment, and upgrading. The underlying oil sands process-affected water (OSPW) contains residual bitumen, suspended clay, and a high concentration of dissolved polar organic compounds which cannot be disposed into the environment due to its acute toxicity. At the bottom of the extraction vessel are the settled sands and silts which are used to build large dykes of the tailings ponds, which contain the fluid fine tailings and OSPW (7), which will be described further in this chapter.

This method is water intense, using approximately 2.5 barrels of fresh water to extract 1 barrel of bitumen (7), and the ultimate source of this water is the Athabasca River. The resulting OSPW is continuously recycled to decrease freshwater intake, but must sit in tailings ponds for months prior to reuse to allow the fine suspended clay particles to settle before the water can be used again in the extraction process (3).

1.3 Oil Sands Process-Affected Water (OSPW)

OSPW is the aqueous mixture containing fine clay, heavy metals, dissolved inorganic ions (such as Na^+,Cl^-,SO_4^{2-}, and HCO_3^-) and a super complex organic fraction which is known to be toxic to aquatic organisms (12–15). The vast majority of the toxic dissolved organic chemicals in OSPW are natural bitumen-derived substances whose structures are largely unknown.

Low concentrations of polycyclic aromatic hydrocarbons (PAHs) can be detected in OSPW, present predominately as the alkylated series of PAHs (3). Nevertheless, the bulk of the organic fraction is an undefined mixture of acidic and polar substances containing carbon, hydrogen, oxygen, sulfur or nitrogen atoms in various proportions. One of the more prominent groups of compounds in OSPW are the naphthenic acids (NAs) (16–18).

NAs are simple carboxylic acids having the molecular formula $C_nH_{2n+Z}O_2$, where n represents carbon number and *Z* represents the hydrogen deficiency due to rings or unsaturated bonds (19). An *Z* equal to -2 means that the NA molecule lost 2 hydrogen molecules. OSPW NAs structures were classically defined by generalized schematic alicyclic structures (*Figure 2*) having 9 to 20 carbon atoms (20–22) and cyclopentyl or cyclohexyl rings (23). A few specific NA structures have only recently been identified (24), and despite the general structures shown in *Figure 2*, NAs are now accepted to contain aromatic, and olefinic moieties (25), as well as adamantane carbon cores (26). Nevertheless, the total number of NAs structures in OSPW is likely in the hundreds of thousands, or millions (27), and complete characterization is not likely to be achieved.

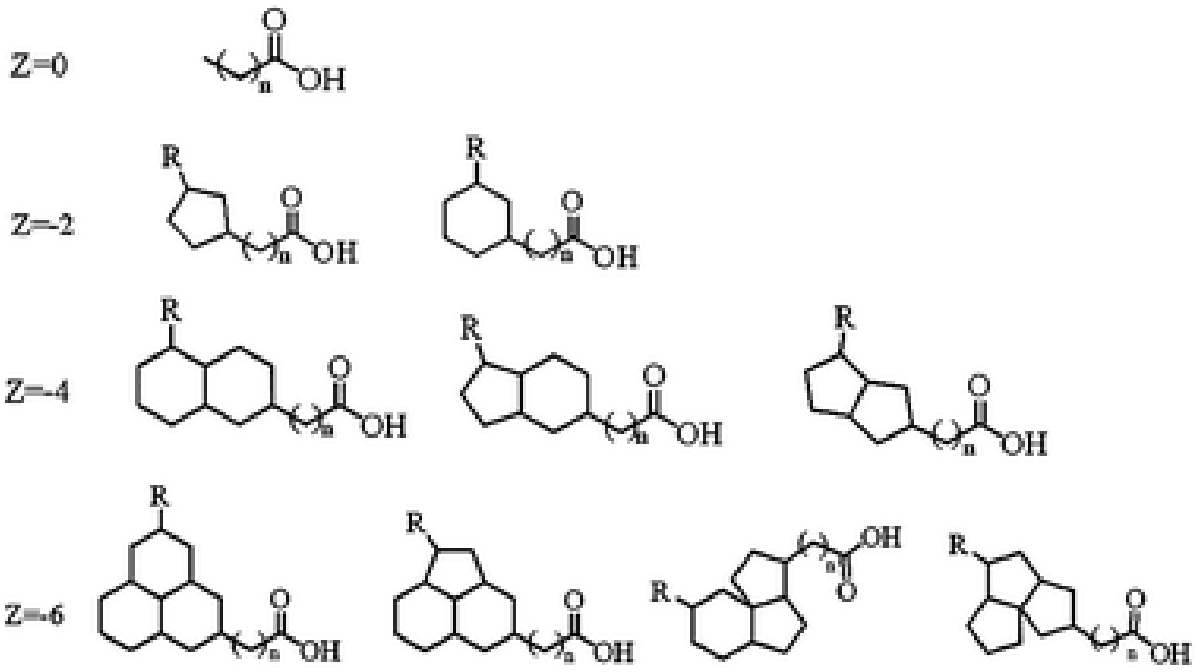


Figure 2: General hypothetical structures of NAs.

Currently, there is no remediation process that has been proven in the field to remove all the toxicity from OSPW, and it was estimated that natural degradation of the bitumen-derived chemicals in OSPW could take up to 150 years (28). Han *et al.* (2009) reported that the half-life of NAs in experimental ponds was at least 13 years (29). The oil industries does not release the OSPW into the environment due its toxicity, and the government of Alberta also have to a zero discharge policy for tailings (30), thus tailing ponds continue to grow in volume and in number.

Most tailings ponds are above grade dyked structures, acting as large settling basins, to temporarily store OSPW for recycling back into the extraction during the operational phase of the mine, but eventually the toxic OSPW stored in tailings ponds must be remediated. To date no OSPW has been detoxified under field conditions. Due the high fresh water use, bitumen

extraction and OSPW production, the tailing ponds now cover 182 km^2 (3), having a physical volume of 840 m^3 (1) and stocking 720 million m^3 of OSPW (31,32).

The primary long-term OSPW remediation strategy involves end pit lakes. End pit lakes are below-grade artificial lakes, where OSPW is mixed with fresh water (and in some cases fine tailings) and left to age with the hope that it detoxifies due to natural processes, including microbial biodegradation via α and β oxidation (33). Eventually, OSPW stored in end pit lakes will flow back into the Athabasca River. The only current end pit lake is called Base Mine Lake (BML) at Syncrude Canada Ltd. BML was commissioned in November 2012 (34). This lake no longer receives fresh OSPW from the extraction and is currently being monitored. By 2014, the landscape around BML is largely revegetated and is slowly being reclaimed (*Figure 3)*, but the more challenging objective has yet to be demonstrated; to remediate the contained OSPW.

Figure 3: Base Mine lake photos taken in 2014. Source: Author's personal pictures, 2014

Although there is great uncertainty in the numbers, The David Suzuki Foundation (2008) (35) estimated that OSPW from all tailings ponds is seeping at a cumulative rate of 11 million L/day (11,000 m^3/d). An Environment Canada study, Frank *et al.* (2014), used various environmental forensic analytical methods to suggest that OSPW from a tailings pond was reaching the Athabasca River, although no quantitative estimates of the flux were made (36). This environmental issue becomes a possible health risk to downstream aquatic ecosystems and human communities, including Fort Mackay and Fort Chipewyan.

1.3.1 OSPW toxicological effects

The toxicological effects of OSPW are generally well documented. In aquatic species OSPW causes inflammation and necrosis of liver and gills (12,13,37), endocrine disruption (17,33,38–40), increase in pro apoptotic, oxidative stress and immune function gene expression (41), dermal erosion (42) and immune impairment (13). Amphibian effects include metamorphosis delay, decrease of snout-vent length and larval deformities (43,44). Exposure to avian species shows increased levels of blood potassium, thyroid hormones and decrease eosinophils (45). In Wistar rats, OSPW can cause behavioral changes, liver pericholangitis, myocardial necrosis and altered biochemistry (46).

Kavanagh *et al.* (2012) (17) assessed the toxicity of OSPW acid extractable organics (AEO) on fathead minnow fecundity exposed to several concentrations for 96 hours (5 mg/L – 100 mg/L). The results showed that fish exposed to concentrations higher than 10 mg/L AEO showed decreased fecundity, number of spawns, lower concentrations of both androgens and estrogens, and increased liver size. The conclusion drawn was that AEO exposure could impair

reproductive physiology of fathead minnows due its estrogenic activity which can decrease male sperm quality.

Van Den Heuvel *et al.* (2012) (47) assessed the reproductive development of yellow perch exposed chronically to OSPW (and related fine tailings) in several experimental ponds where the approximate ratio of OSPW and freshwater was 50/50. Their results suggested a two-fold testis size reduction in male fish, and decreased ovary size in female fish, although adverse influence on fecundity was not proven.

Young *et al.* (2011) (48) demonstrated the distribution of OSPW AEO in tissues of 4 species of wild fish (lake whitefish, northern pike, walleye and white sucker) from near the oil sands operation area. Their results showed small concentration of NAs in the liver (from 0.2 to 1.1 μg/g) in all fish, but no significant concentration in heart, kidney and muscle tissue.

Peters *et al.* (2007) (16) studied the embryonic development in fish eggs from yellow perch and *Japanese medaka*, using commercial NAs (1.25-20 mg/L) and OSPW from Mildred Lake settling basin [concentrations ranging from 0.0016 – 1× (i.e. full strength) OSPW]. For the yellow perch group, many samples on day 6 showed deformities, as optic-cephalic abnormalities, spinal malformations and even little or no tail development. The threshold was 0.08× for OSPW and 1.5 mg/L for the commercial NA. For the *Japanese medaka* group, teratogenic effects on the heart were shown for both commercial NAs and OSPW, culminating in circulatory distress, such as sluggish circulation, blood pooling at the tail, blood islands over the yolk, pericardial edema, tube-shaped heart, decreased optic cup pigmentation anisophthalmia and microphthalmia, and mandible malformations. For these effects, the OSPW threshold was 0.09× and for commercial NAs was 1.5 mg/L. The conclusion was that OSPW from Mildred Lake settling basin can cause

deformities in yellow perch and *Japanese medaka* fish, but the toxicity of the commercial NAs was apparently higher than OSPW samples, suggesting that refined commercial NAs are not a perfect model for OSPW.

Nero *et al.* (2006) (37) assessed the effects of OSPW AEOs and commercial refined NAs on yellow perch, focusing on gills and liver histopathology, and they demonstrated a high mortality for both samples. AEO showed 100% mortality at a concentration of 6.8 mg/L in 96 hours of exposure. By comparison, commercial NAs caused 100% mortality at 3.6 mg/L in 96 hours of exposure. The histological analysis in gills showed proliferative, inflammatory, degenerative and cellular structure alterations compared to negative controls. The liver histopathology demonstrated slightly higher levels of degenerative, inflammatory and structural alterations compared to the negative controls, but not significantly different ($p > 0.05$). The high morbidity was explained by the yellow perch sensitivity to the NA, nevertheless it also demonstrates the toxic effects of the NAs on organs.

Rogers *et al.* (2006) (46) conducted an experiment with OSPW AEOs in Wistar rats to evaluate the 14 day acute toxicity (single dose; 3, 30 and 300 mg/kg) and 90 day sub-chronic toxicity (5 doses per week; 0.6, 6 and 60 mg/kg/d) of OSPW organics. The acute group behavioral changes included lethargy and mild ataxia (the loss of full control of bodily movement), a small decrease in food consumption and a small but significant decrease in body weight (medium and high dose). Histology showed pericholangitis, mild myocardial necrosis and brain hemorrhage for the medium and high dose group. Only the gonads, heart and spleen showed an increased organ weight in the high dose group. The authors evaluated behavior, food and water consumption, body weight, organ weights and histology, biochemistry and hematology for the sub-chronic group.

The sub-chronic behavioral findings were seizures after the 40th day, decreased water consumption for the low dose and increased for the high, but decreased food consumption for the high dose group and lower body weight in the high dose group. The organ weights from the high dose groups showed a significant increase for liver (36%), kidney (12%) and brain (9.1%). The biochemistry findings on the high dose group were increased cholesterol levels (43%) and decreased amylase (33%). A minor decrease in hemoglobin and hematocrit was found in the hematology assays in the high dose group. Histology demonstrated a low prevalence of pericholangitis, compared with the acute groups, but a positive correlation was found between dose and glycogen accumulation. All the alterations point towards toxicity to mammals for both acute and chronic exposure.

1.3.2 OSPW genotoxicity

The only study that assessed the mutagenicity potential of authentic OSPW was Madill *et al* (1999) (49). By using two different mutagenicity assays, Ames and Mutatox, the authors exposed bacteria to fractions of OSPW porewater (i.e. OSPW present in the pores of fine tailings) from Mildred Lake settling basin, a tailings pond at Syncrude Canada Ltd. While the Ames assay detects mutagenicity and will be described in the methodology chapter of this thesis, the Mutatox assay (another reverse mutation assay) uses a dark mutated strain of *Photobacterium phosphoreum* (incapable of luminescence) to detect mutagenicity via genotoxicity. This strain is sensitive to mutations caused by DNA damaging agents, DNA intercalators, direct mutagens which either cause base substitution or are frame-shift agents, and DNA synthesis inhibitors. If the used sample is mutagenic, the bacteria will return to its wild type and become luminescent. A

modified Beckman Microtox Model 2055 analyzer can measure the reverted bacteria light emission. Their results demonstrated that the porewater had 2.6 µg/L of identified polycyclic aromatic compounds. Ames testing showed no significant mutagenicity for all OSPW fractions in the presence or absence of S9 liver enzyme fraction at concentrations from 1,000× to 10,000×, and the authors concluded that OSPW AEO has no mutagenic properties with this assay. The Mutatox assay demonstrated a dose-dependent positive response for all OSPW fractions in terms of genotoxic damage, with and without S9 extract, at concentrations of the extract equivalent to 1,000× to 10,000× relative to the original porewater. It was concluded overall that the organic compounds in the pore water sample had small mutagenic potential.

Lacaze *et al.* (2014) (50) used a C18 extract (i.e. hydrophobic compounds) of "synthetic OSPW", 4 different model NAs compounds (Z= -2, -4, -6 and -8) and a commercial refined NAs mixture to investigate genotoxicity in rainbow trout hepatocytes with the Comet assay. The cells were exposed to OSPW and NAs for 18 hours at 15ºC, where OSPW concentrations were 0.1×, 0.5× and 1×, and model/commercial NAs concentrations were 0.66, 1.55, 5 and 15 mg/L. NAs with Z = -2 and -4 did not cause significant genotoxicity, but the NAs with Z = -6 showed a genotoxic response at concentrations of 1.55 mg/L (22.4 ± 2.4 DNA damage, arbitrary units), 5 mg/L (28.5 ± 5.1) and 15 mg/L (28.3 ± 5). The model NAs with Z = -8 showed genotoxicity at concentrations 1.55 mg/L (23.5 ± 2.3), 5 mg/L (18.6 ± 1.7) and 15 mg/L (19.9 ± 2.1). Commercial NAs genotoxicity was noted at 5 (19.9 ± 2.8) and 15 mg/L (30.6 ± 3.3). The simulated OSPW extract showed increased genotoxicity at 0.5× (39.4 ± 6.9) and 1× (68.7 ± 10.2); the relevance of this simulated OSPW is not known.

Since only 2 studies have tried to identify OSPW genotoxicity or mutagenicity, and none of these used a highly relevant OSPW sample, a better understanding of OSPW genetic

toxicology is warranted. As discussed above, the importance of this is compounded by evidence that OSPW may be seeping into the Athabasca River today, and because end pit lakes will eventually discharge OSPW back to the natural environment. Although no causal link exists, a report by the Alberta Cancer Board (2009) (51) showed that the downstream community of Fort Chipewyan had a higher than normal cancer incidence for liver (cholangiocarcinoma, a rare and lethal type of liver cancer where the mutated cells arise from the bile ducts), soft tissue (sarcomas) and blood (leukemia) cancer.

1.4 Genetic toxicology

1.4.1 DNA, RNA and the phenotype

Deoxyribonucleic acid (DNA) is the molecule encoding all instructions for development and functioning of all living organisms. DNA molecules are formed by two complementary strands connected together, forming a double-helix structure. It is stored inside the nucleus for eukaryotes, balled in the form of chromatin (52).

The functional unit of DNA is the nucleotide, composed of a carbohydrate (deoxyribose for most organisms), a phosphate group and a nucleobase (nitrogenous base). The phosphate groups act as connectors of adjacent nucleotides via phosphodiester bonds, linking the deoxyriboses. Nucleobases have two functions. The first function is to non-covalently bond the two complementary strands together to give stability to the DNA. The second is to act as a code for protein production, via ribonucleic acid (RNA) synthesis. There are only four nucleobases in the DNA, the purines: adenine (A) and guanine (G), and the pyrimidines: cytosine (C) and

thymine (T). In double stranded DNA, A is always paired with T and C is always paired with G (52).

RNA has a similar structure to DNA, but RNA differs from DNA in that the former is only present as single strands, the carbohydrate is ribose instead of deoxyribose, and in RNA, T is replaced by uracil (U). The RNA can be classified according to its function, where messenger RNA (mRNA) carries the DNA information to encode a protein, and transfer RNA (tRNA) brings an amino acid from the cell cytoplasm to build polypeptides based on the mRNA sequence (53).

The definition of a gene is a molecule that codes a protein that has a specific function in an organism, and can be inherited via reproduction. In other words, genes hold the information to build and maintain an organism's cells and pass its traits to offspring. The whole collection of an organism's genes is called the genome (53). In order to express a gene, the DNA must produce an mRNA in a process called transcription. The mRNA will bind to a ribosome, which will read three nucleobases at a time; this triplet is called a codon (*Table 1*). Each codon will have a correspondent amino acid molecule and the sequence of all codons in an mRNA generally creates a protein in a process called translation. Translation of proteins results in an expressed phenotype (54).

Second Letter

First Letter	U	C	A	G	Third Letter
U	**UUU** Phe **UUC** **UUA** Leu **UUG**	**UCU** **UCC** Ser **UCA** **UCG**	**UAU** Tyr **UAC** **UAA** STOP **UAG**	**UGU** Cys **UGC** **UGA** STOP **UGG** Trp	**U** **C** **A** **G**
C	**CUU** **CUC** Leu **CUA** **CUG**	**CCU** **CCC** Pro **CCA** **CCG**	**CAU** His **CAC** **CAA** Gln **CAG**	**CGU** **CGC** Arg **CGA** **CGG**	**U** **C** **A** **G**
A	**AUU** **AUC** Ile **AUA** **AUG** Met	**ACU** **ACC** Thr **ACA** **ACG**	**AAU** Asp **AAC** **AAA** Lys **AAG**	**AGU** Ser **AGC** **AGA** Arg **AGG**	**U** **C** **A** **G**
G	**GUU** **GUC** Val **GUA** **GUG**	**GCU** **GCC** Ala **GCA** **GCG**	**GAU** Asp **GAC** **GAA** Glu **GAG**	**GGU** **GGC** Gly **GGA** **GGG**	**U** **C** **A** **G**

Table 1: The genetic code, showing codons and the amino acid, or instructions, they encode.

Cells have an intricate machinery to avoid DNA replication errors but, if it happens, mechanisms can be activated to repair it, such as the excision repair mechanism. This prevents, for instance, protein malformation and gene mis-expression. Nevertheless, due the complexity of the DNA and the large amount of information encoded, errors do happen during replication, and those changes could alter translation and protein synthesis. DNA errors may happen naturally, or be induced by various mechanisms (52).

1.4.2 Genotoxicity

DNA damage occurs naturally and is a fundamental problem for organisms, happening randomly and often as a result of cell metabolism. It is estimated that mammalian DNA can be naturally damaged 60,000 times per day per cell, for example, via oxidation from cells own metabolic products (55).

Xenobiotics also are known to cause DNA damage, whereby nucleotide structure may be damaged (i.e. the deoxyribose, phosphate group or the bases). Common mechanisms of DNA-reactive damage include oxidative processes, hydrolysis of phosphodiester bonds, or covalent modification by alkylating reagents. A xenobiotic may also cause damage by intercalating between base pairs of double-stranded DNA (56). Increased genotoxicity can become an issue when cells are exposed to environmental DNA damaging substances like PAHs, alcohol, asbestos, or vinyl chloride among others (57). Any type of cell can have DNA damage, like proliferative cells (such as epithelial cells or hematopoietic cells) or non-dividing cells (like neurons or muscles).

The most common cell metabolites that can oxidize the DNA are reactive-oxygen species and anions such as O_2^-, H_2O_2, $OH^\bullet$. Reactive nitrogen species (for example $NO^\bullet$, $OONO^-$, NO^-, NO^+ and NO_2) can cause DNA hydrolysis. Other chemicals capable of causing genotoxicity are reactive carbonyl species, including glyoxal and malondialdehyde and lipid peroxidation metabolites, acting as a source of reactive carbonyls and free radicals (58). Hydroxyl radical ($OH^\bullet$) can add to double bonds of heterocyclic DNA bases, or abstract a hydrogen atom from the methyl group of thymine, or any of the five carbon atoms of deoxyribose (59). On addition reactions, $OH^\bullet$ can yield OH-adduct on DNA bases, whereas the alkyl radical of thymine and carbon-centered carbohydrates radicals are formed from abstraction reactions. If present, molecular oxygen adds to carbon-centered radicals at diffusion-controlled rates to yield peroxyl radicals (60). Further reactions of base and pentose radicals generate a variety of base-free sites, strand breaks, and DNA-protein cross-links (59). Spontaneous hydrolytic damage of the DNA can happen but it is a slow process, althought genotoxicants can increase the speed of it; including lanthanide and transition metal ions (61).

Other oxidative damage examples are widely explored in the literature and these are just a few examples of how it can happen, but further explanation is beyond this thesis' scope.

DNA alkylation can happen anywhere in the DNA since alkylating agents are very electrophilic. The reaction can happen on the phosphodiester bonds, breaking the DNA, while base alkylation can cause base mispairing when the DNA is being transcribed to produce mRNA, and it can be very damaging to the cell because protein synthesis can be affected. If alkylated DNA undergoes replication, permanent mutations could result. The preferred sites of base-pair alkylation are the nitrogen atoms of guanine bases, cytosines and adenosines, or the oxygen atoms of guanines, but this varies according to the alkylating agent (58).

Even with several other mechanisms of natural DNA damage, the most common effects are oxidation, depurination, depyrimidination, single or double strand breaks, and O^6 - methylguanine and cytosine deaminations (55,58,59). This damage only rarely causes a mutation because they happen at a rate that the cells can repair, unless the damage is enhanced by genotoxicants.

Every cell has repair system genes that are responsible for repairing the DNA when damage occurs. Some of these built-in repair mechanisms are the SOS and UMU repair systems. Nevertheless, genotoxicants can overload the repair system, and the DNA damage can build up in cells without being repaired. When non-proliferative cells suffer unrepaired DNA damage, that cell ages, or undergoes apoptosis if the damage is extensive enough. When the damage is extensive, chronic, escapes the repair system, or physically changes the DNA and is inherited by the daughter cell, a mutation may be created (54).

It is an important concept that genotoxicity can lead to mutagenicity, but not all genotoxic chemicals are mutagens, owing to various repair systems and apoptosis. Furthermore, not all mutations occur via genotoxicity. For example, an enhanced rate of cell division increases the probability of natural DNA replication errors, leading to mutation. Thus, some estrogen-like xenobiotics, growth factors and hormones are considered as non-genotoxic carcinogens.

1.4.3 Mutagenicity

Mutagenicity is the ability of chemicals to cause permanent changes in the genetic material in the nucleus of cells in ways that allow the changes to be transmitted during cell division (56). Certain mutations can lead to cancer, but only if they occur in specific genes, to be discussed later. Natural mutations (via natural genotoxicity or cell replication errors) can happen but is not common since the cells have intricate repair mechanisms (62). But DNA replication is not a perfect process and errors caused by DNA polymerase can lead to natural mutations. These events are rare, occurring at frequencies of 1 error per 1,000,000, to 1 error per 1,000,000,000 replicated bases (63). Mutations can be neutral, good or adverse, but all may play a role in evolution. Here, mutagenicity caused by genotoxic events will be the focus.

There are several mutagens in the environment such as synthetic chemicals that pose a risk to human health, although quantifying this risk is difficult. Some examples are UV radiation, heterocyclic aromatic amines, alcohol, viruses, molecules that can promote cellular growth (growth factors), air pollution, tobacco smoke, and acridine dyes (54,56,64,65).

There are three main mechanisms of mutation. When DNA carrying a damaged base is replicated, an incorrect base can often be inserted opposite the site of the damaged base in the new complementary strand, and this can become a mutation in the next round of replication. Repair mechanisms can also fail when repairing double strands breaks, inserting different and non-matching bases in the same *loci*. In addition, a double strand break can cause rearrangements of the chromosome structure (possibly disrupting at least one gene, or causing a gene to come under abnormal regulatory control). If this rearrangement is not fixed by the repair mechanisms

and is replicated, a mutation is formed. In any case, DNA repair is a crucial protective process blocking cells from proceeding towards mutagenesis and carcinogenesis (56).

Mutations can be the result of a single altered base pair, span several base pairs, or even span multiple genes. They can happen anywhere in the DNA but there are some standards on how they occur. The base mutation patterns are substitutions, deletions, insertions, inversions. Substitutions are mutations where a number of base pairs is replaced by other base pairs. Deletions are mutations where a number of base pairs are erased from the DNA. Insertion mutations happen when a number of new base pairs are inserted into the DNA. Inversion mutations are when a number of bases exchange with the posterior or anterior bases on the DNA molecule. Illustration of the mutation types are shown in *Figure 4*.

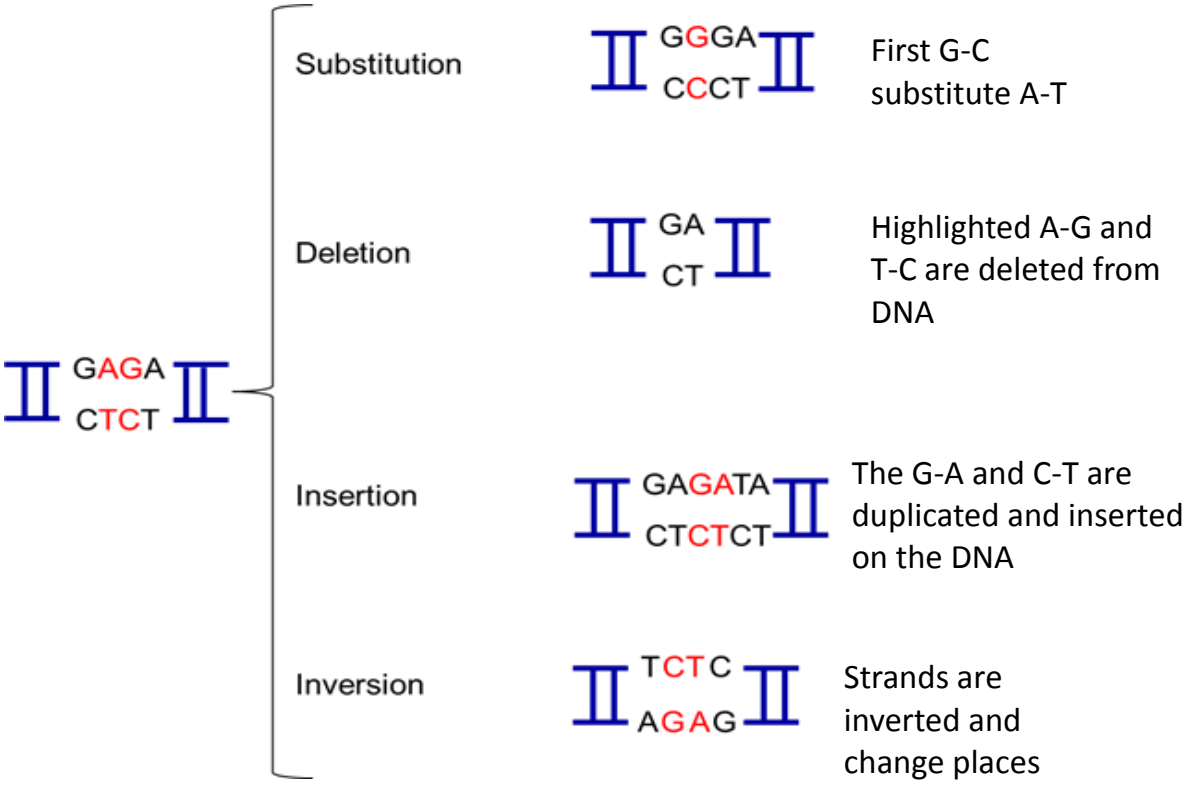


Figure 4: Types of mutations in DNA.

Mutations can alter a protein's structure and function but, depending on how the DNA was altered, the effect could be neutral and therefore not damaging to the cell. As shown in Table 1, several codons can generate the same amino acid. For example, the amino acid Valine can be produced by the following codons: GUU, GUC, GUA and GUG, thus a point mutation of the last base in this codon would result in a "silent" mutation. Alternatively, a missense mutation is another form of point mutation that results in a different amino acid being inserted into the polypeptide chain. The effect of this can be very severe, or have no major effect, depending on the position of the amino acid (53).

A more damaging type of mutation is the non-sense mutation that can be caused by substitutions and inversions. These mutations encode stop codons (*Table 1*) in the mRNA and therefore truncate the resulting protein, often resulting in an incomplete and non-functional molecule (63).

Deletion and insertion mutations can also cause frameshift mutations. Due to the triplet nature of the codon reading, these mutations can alter all downstream codons, either changing the functionality of the resultant protein, or leading prematurely to a stop-codon. Such mutations are the cause of some severe genetic diseases, such as Tay-Sachs disease and Cystic Fibrosis (66).

If enough mutations occur in specific genes, such as tumor suppressors or proto-oncogenes genes like p53, Ras, Raf, the cell will lose its capacity to control its growth or replication (67–69). When cells start to replicate without control, a carcinogenesis process may begin to occur.

1.4.4 Carcinogenesis

Carcinogenesis is a multi-stage and life-long process where normal cells begin to divide without control, and without a specific function. There are three main stages, initiation, promotion and progression. The result of the last phase of carcinogenesis will be a tumor, and if it invades adjacent tissue or spreads to distant tissues, the tumor is considered cancerous.

The initiation phase requires that the DNA suffers one or more mutations from any source (via genotoxicity, viruses, growth factor, among others), as described above.

The promotion phase is the build-up of mutations in proto-oncogenes and tumor suppressor genes, slowly causing a cell to take on uncontrolled growth and division, and loss of apoptosis. The daughter cells will have the same mutations and the resulting damage becomes more and more severe. When the mass of cells reaches a certain size, a benign tumor can be detected (62) and may cause symptoms in the tissue where it originated. For example, a liver tumor could make the liver less efficient at bile production and excretion, which could eventually disrupt the biochemistry of blood and other organs that depend on the liver (60).

Finally, in the progression phase, the tumor becomes malignant after building up further mutations on, for example cell adhesion genes, such as e-cadherin, making the cells less attached to the tumor mass and more likely to invade neighbouring tissues or migrate to distant tissues via metastasis. If metastasis occurs, the migrating cells will create a new tumor at a distant site. *Figure 5* shows the carcinogenetic process (62).

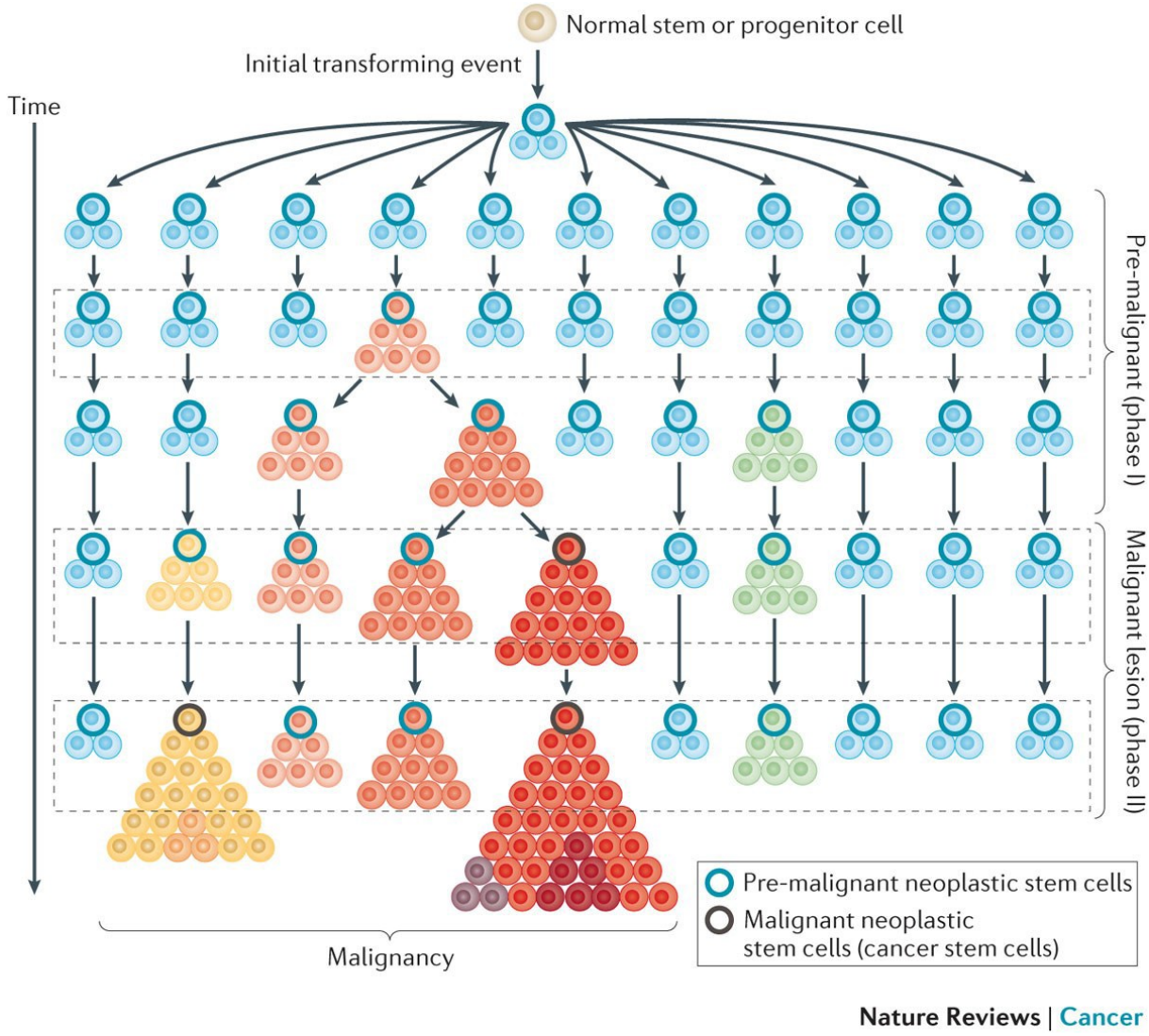


Figure 5: The carcinogenetic process. Pre-malignant phase is considered initiation and promotion. Malignant phase is considered progression. Source: Valent *et al.* (2012) (70)

The terms tumor and cancer are often misused, but both arise from the carcinogenic process. A benign tumor is a mass of cells that grows without control inside a specific tissue and does not invade neighbouring tissue or spread through the body. When a tumor starts to spread, or leaves its original tissue (via metastasis), it becomes a malignant tumor, or cancer (62). For most types of cancer, a benign tumor can evolve to a malign form if not treated. The only exceptions are blood cancer, like lymphoma, where the malignant cells are inside the circulatory system and constantly spreading through the body (71).

Characteristics of cells in benign tumors include insensitivity to internal or external signals that regulate the cell cycle, bypassing apoptotic pathways, not differentiating or going through de-differentiation, and secreting growth factors to spur angiogenesis, thus providing more nutrients to the tumor which furthermore increases cellular growth and division (72). Malignant cells behave like benign cells but are more genetically unstable due to lack of cell cycle checkpoints, thus avoiding apoptosis even more, having increased cell motility (turning into metastatic cells where they can invade surrounding tissues), surviving and proliferating in secondary sites (73).

There are several genes that, when mutated, can contribute to carcinogenesis in several different ways. Some examples are p53 and its gene family (such as p19, p63), Ras, Rac, Bax, Fas, Sco2, Bad, FADD, Rho, e-cadherin and TGF-β. In order to better understand how a carcinogenic process works, the roles of p53 (a tumor suppressor gene), Ras (a proto oncogene) and e-cadherin (a gene involved on cell adhesion) will be discussed (55).

The p53 gene is known as the guardian of the genome, preventing cells from undergoing carcinogenesis. Its main role is to control the cell cycle and division by activating DNA damage

repair, preventing the cell from division or arresting the cell cycle by interacting with several other molecules, like p21 and Bcl2 proteins (74). In the nucleus, p53 regulates transcription, promoting apoptosis by activating pro-apoptotic genes such as Puma, Noxa, p53AIP, Bax and Apaf-1, all halting DNA replication and homologous recombination (68). In the cytoplasm, it controls centromere duplication, apoptosis via MOMP if DNA damage is detected and also inhibits autophagy, thereby promoting apoptosis (75). The mutation of p53 impairs several of the most important pathways to prevent cell proliferation and cell cycle arrest. Mutations on p53 are common in almost all types of cancer, at rates ranging from 38 to 50% in ovarian, esophageal, colorectal, head and neck, larynx, and lung cancers, to approximately 5% in primary leukemia, sarcoma, testicular cancer, and melanoma (69,76)

The Ras gene family is responsible for signaling the DNA to replicate, promoting cytoskeletal integrity, differentiation, cell adhesion, apoptosis and cell migration (54). When Ras is inactive, the cell does not replicate. This gene family is responsible to activate cell cycle promoters, such as MAPK, Myc, Cyclin D and E2F. Ras is activated by GTP hydrolysis (thus this family is known as small GTPases), a process that directly interacts with E2F and Rb, both cell cycle promoters. When these are activated, they stimulate DNA replication, making the cell leave G1 and enter the S phase. The most common Ras mutation makes the encoded protein to be constantly in its active state, whereby the DNA constantly receives a signal to enter the S phase and divide. Like with p53, mutations in the Ras family are common, being found in 20% to 30% of all human tumours (67).

Cadherin molecules are transmembrane proteins, playing an important role in cell adhesion. Its name derives from the calcium dependency to create bonds between cells. This molecule is responsible to form belt desmosomes (or intermediate junctions), which are complex

protein structures that also play a role on cell architecture and cytoskeleton (77). In its wild-type form, this protein prevents metastasis and interacts with neighbor cell cadherin deactivating Ras genes via p120 cascade. Furthermore, as stated before, when Ras is inactive, the cell cycle stops at G1, making this molecule to prevent not only metastasis but also cell division. The most common mutation on cadherin genes renders the encoded proteins not functional, preventing cell adhesion or inactivating its own or other cells Ras. The mutation rates of cadherin genes are controversial and varies to each type of tumor, but they range from 3 to 50% and are highly associated with metastasis (78).

The molecular interactions and pathways that are important to carcinogenesis, such as the 3 previously cited, can be found in *Figure 6.*

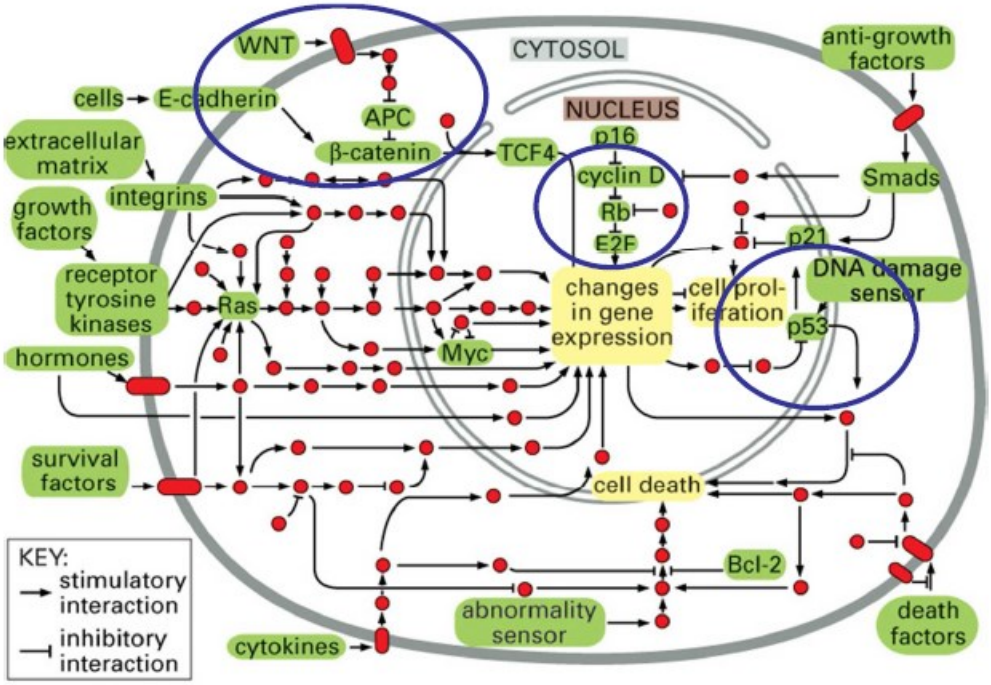


Figure 6: Cellular pathways that can contribute to carcinogenesis. Source: The Molecular Biology of the Cell, figure 23-31(54).

1.4.5 SOS-Chromo Test

The SOS-Chromo test is a bacterial test to screen for genotoxicity, developed in 1982 by Quillardet *et al.* (79) as an alternative for mutagenicity testing. The innovation of this assay was to examine genotoxicity directly instead of the cellular responses to the genotoxicant, such as altered protein production. The rationale was, to quantitatively measure, expression of bacteria DNA repair genes, specifically the SOS genes, which are activated when DNA damage occurs.

The SOS repair system generates a global response to DNA damage in which the cell tries to repair the damage. If the damage is too extensive, the cycle can be arrested or DNA damage may lead to a mutation. The system involves the RecA and the LexA proteins. The RecA protein, stimulated by single-stranded DNA, is involved in the inactivation of the LexA repressor thereby inducing the repair response (80). LexA gene negatively regulates the SOS box, preventing SOS gene expression and the start of the repair mechanism, although some of the SOS genes are expressed at low levels.

Activation of the SOS genes occurs after DNA damage by the accumulation of single stranded regions in the DNA, generated at replication forks, where DNA polymerase is blocked. RecA is activated, forming filaments around these damaged regions. RecA filaments interacts with the LexA repressor, facilitating the LexA repressor's self-cleavage. Once the pool of LexA decreases, the repression of the SOS genes ceases and the SOS box genes are activated. In this way LexA can sequentially activate different mechanisms of repair. Genes having a weak SOS box (such as lexA, recA, uvrA, uvrB, and uvrD) are fully induced in response to even weak SOS-inducing treatments. Thus the first SOS repair mechanism to be induced is nucleotide

excision repair, whose aim is to fix DNA damage without commitment to a full-fledged SOS response.

Since genotoxicity measurements can be influenced (i.e biased) by cytotoxicity, the SOS-Chromo test also monitors the bacterial production of alkaline phosphatase as a measure of cell viability via the conjugation of para-nitrophenylphosphatase (pNpp), which generates a colorimetric reaction.

The bacterial strain selected for this assay is *E. coli* PQ37 which has the genotype F- thr leu his-4 pyrD thi galE galK or galT lacAU169 srl300::TnJO rpoB rpsL uvrA rfa trp::Muc' sftA::Mud(Ap, lac)cts. It is constitutive for alkaline phosphatase synthesis (79). This genotype shows the lacZ operon (galE, gal K or galT and LacAU169, genes that control the metabolism of lactose and its cleavage into galactose). An operon is a functioning unit of genomic DNA containing a cluster of genes under the control of a single promoter, in this case the SOS gene sfiA (an SOS gene involved in cell division inhibition).

Direct quantification of the expression of a repair gene was beyond 1982 technology, but the SOS expression could be measured indirectly. The fusion of the lacZ operon with the β-galactosidase gene (β-gal) under control of the sfiA gene, allowed the cell to co-express β-gal every time that genotoxic damage activates the SOS system. The first version of this assay used o-nitrophenyl galactoside, a chemical that can conjugate with β-gal to generate a colorimetric reaction that can be monitored by simple spectroscopy. Cell viability is measured in the same fashion, by conjugating bacterial alkaline phosphatase with p-nitrophenyl phosphate (pNPP), also generating a colorimetric reaction.

After obtaining a viable colony of the strain and adding it to LB media (Luria Bertani) in a test tube, ampicillin is mixed to prevent other bacterial contamination, and Z and Tris buffer to make the pH stable at 8.8, creating a favorable environment for bacterial growth. After 12 hours of incubation, the optical density readings of the broth should be approximately 0.4. If desirable, S9 fraction (a liver homogenate containing of phase I and II enzymes) could be used in order to evaluate genotoxicant bioactivation or detoxification. This mixture is incubated for 2-4 hours with the desired samples. To start the reading after this first incubation, cell membranes need to be disrupted by sodium dodecyl sulfate solution and chloroform, where β-gal and alkaline phosphatase will be released from the cell into the test tube. The tubes are then incubated for 2-4 hours, permitting the colorimetric reaction to proceed. β-gal colorimetric reaction is read at 605 nm, while alkaline phosphatase colorimetric activity is measured at 420 nm (79).

Compared to other genotoxic assays at its time, the SOS-chromo test had several advantages, such as requiring only one bacterial strain to have reliable data, providing simple and readily measurable colorimetric reactions using non-toxic conjugates, and its rapidity, requiring only hours to obtain the results. Some improvements were later achieved by making results faster to read, and multiplexing the exposures while shortening the assay time. These improvements happened in 1987 when Fish *et al.* (81) created the SOS-Chromo test kit. The stability of the reagents and the standardization of the micro titration allowed a decrease of the reagents volume by 80%, allowing this version of the assay to be made in 96-well plates, and the results obtained by a plate reader, where cell lysis is no longer needed to obtain the results. The same bacterial strain is used but instead of obtaining it from frozen cultures of plates, it is lyophilized, permitting longer storage at -10ºC without damaging the cells. The genotoxicity chromogen changed to permit genotoxicity and cell viability readings at the same time; 5-bromo-4-chloro-3-

indolyl-β-D-galactopyranoside (or X-gal) replaced o-nitrophenyl galactoside, but pNPP is still used to measure cell viability (81). The incubation time decreased to 1-2 hours, and the colorimetric reaction incubation to 30 min-1 hour. The commercial kit also comes with several positive controls, which vary depending on the company manufacturing the kit. The total time of the assay in kit format is between 4 to 6 hours, compared to 8 hours or more with the original version.

The SOS Chromotest results are highly reliable when testing carcinogens. Quillardet *et al.* (1993) (80) evaluated 65 confirmed class 1 carcinogens and 44 suspected class 2 carcinogens by the SOS Chromotest. The results, 41 of 65 Class 1 carcinogens, and 30 of 44 suspected Class 2 carcinogens had positive results in the SOS assay and in other assays such as the Ames. The specificity (ability to discriminate between carcinogens and non-carcinogens) of the SOS Chromotest is higher than for other genotoxic/mutagenicity tests, such as Ames, where the SOS assay had a specificity of 100% when testing 73 carcinogenic or non-carcinogenic chemicals, while the Ames specificity was only 62% (82).

The details of this assay will be well described in Chapter 2.

1.4.6 Ames Test

The mutatest, commonly known as the Ames test due to its inventor Bruce Ames, was developed in 1972 as an initial screen to detect potential mutagenic / carcinogenic activity of new and uncharacterized compounds or complex mixtures. This came at a time when several new chemicals were being produced with little information on carcinogenic properties and when cancer diagnosis was becoming more common among people.

The goal of the assay was to be a quick and cheap screen that could be used in a daily laboratory routine with everyday laboratory tools. In its first years of use, the assay demonstrated good reliability, whereby 135 out of 158 known carcinogens demonstrated positive results in the assay (83), and several validations followed. Ames and collaborators tested 300 chemicals, most of which were known carcinogens, and obtained a positive correlation for the majority of them, also validated by the Imperial Chemical Industries, the National Cancer Center Research Institute in Tokyo, and the International Agency for Research on Cancer (84). Nearly 90% of the carcinogens tested were mutagenic in these studies, but the assay has a flaw when trying to detect a few classes of carcinogens, such as polychlorinated pesticides because they are not direct alkylating agents, thus not genotoxic (85).

Furthermore, a considerable number of mutagens first detected by the Ames assay have subsequently been shown to be carcinogenic in animal tests (85). These results made the assay well recognized by the scientific community, as well as by government agencies and corporations. There are international established guidelines for the Ames test (such as the Organisation for Economic Co-operation and Development, and International Commission on Harmonization) to assure uniformity of testing procedures prior to submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides (84).

The Ames test is considered a reversion assay, with the rationale being to expose a mutated (and genetically engineered) bacteria of salmonella lineage (derived from salmonella LT2) containing a mutated and non-functional histidine producer gene to the chemical of interest in an environment containing trace histidine concentrations. If a mutation on the histidine producer gene occurs, the bacteria may revert to its wild type, thereby regaining the ability to

synthesize its own supply of histidine. Thus mutated cells form colonies on histidine-depleted media, whereas all non-mutated cells die and are not observed as colonies. The number of revertant colonies can be visually quantified and compared to well-known mutagens. There are several types of salmonella engineered for this assay, each strain designed with specific mutations that can therefore detect different types of revertant mutations. *Table 2* summarizes the strains and their characteristics. The strains that contain mutations on *hisC207* were discontinued (85), thus are not shown. The strains marked with an asterisk are the strains that are more sensitive to mutagens and most used today. For example, TA97 replaced TA1537 and TA2637; TA98 and TA100 replace TAI535, TAI537 and TAI538. These two strains are more sensitive and can detect 2 different types of mutations (TA 98 detects frameshift mutations, TA100 detects single base pair mutations). The use of these two strains together to evaluate the mutagenic potential of a chemical is most reliable (84).

Summarizing Table 2, the strain traits are (83–86):

- *Defect in repair mechanism and biotin dependency:* A deletion mutation through the uvrB-bio genes (except TA102) eliminates the accurate excision repair mechanism, allowing more DNA lesions to not be repaired. But by deleting this gene, the bacteria also loses the biotin genes, making the bacteria biotin dependent.
- *Lipopolysaccharide (LPS) layer removal:* A mutation on rfa gene leads to a defective LPS layer that coats the bacterial membrane, making the bacteria more permeable to large chemicals.

- *Plasmid introduction:* Plasmid pKM101 enhances chemical and UV-induced mutagenesis via an increase in the error-prone recombinational DNA repair pathway, also conferring ampicillin resistance.

The classical Ames has not had significant changes with time and its methodology is described in the methodology chapter of this thesis.

Mutation / Strain	**LPD defect**	**Plasmid insertion**
hisG46		
TA1535	rfa	
TA100*	rfa	pKM101
TA92	+	
TA1530	Δgal	
TA1950	+	
TA1975	rfa	
TA2410	+	
TA2631	Δgal	
hisD3052		
TA1538	rfa	
TA94	+	
TA98*	rfa	pKM101
TA2420	+	
TA1534	+	
TA1964	Δgal	
TA1978	rfa	
TA2420	+	
TA2641	Δgal	
hisC3076		
TA1537	rfa	
TA1952	+	
TA1977	rfa	
TA1532	Δgal	
TA2637	rfa	
hisO1242		
TA97*	+	
hisG428		
TA104	rfa	
TA102	rfa	pKM101 and pAQ1
hisD6610 and *his01242*		
TA90	rfa	
TA97*	rfa	
TA110	+	
TA89	Δgal	

+: wild type. Δ: gene deletion.

Table 2: Ames assay salmonella strains.

The original Ames method, today known as classical Ames, was not able to detect the mutagenicity in some types of samples, such as urine metabolites, gases or water insoluble compounds, samples where the metabolites could interact with other reagents from the assay and some antibiotics. To address this issue, the authors modified the classical Ames assay to detect mutagenicity in those specific cases (85), creating the Urine, desiccator, pre-incubation and spot assay. Since these modified assays were not used in this research, they will not be described.

The details of this assay will be well described in Chapter 2.

When used to investigate possible genotoxic and mutagenic effects of substances, the correlation of SOS and Ames tests provides a validation that any mutagenicity is indeed caused by genotoxicity. Additionally, the SOS results are at least, or even more, sensitive than Ames testing due to SOS specificity being less prone to false positives or negatives (79,81).

1.5 **Rationale and Justification**

The current volume of stored OSPW is increasing daily. The rate of OSPW xenobiotic natural degradation is slow, and currently there is no water treatment method being applied to decrease OSPW toxicity. The temporary storage of OSPW in tailings ponds, and recycling of OSPW for further bitumen extraction does not solve the issue, but delays inevitable treatment and safe release. The water will need to be detoxified, as it will eventually flow back into the natural environment from end pit lakes.

Current data on OSPW toxicological effects include effects on species of fish, amphibian, bird and mammals, with endpoints ranging from acute toxicity (i.e. mortality), to growth, reproduction, immune and development. To date, no studies have examined OSPW carcinogenicity, and only very few studies to data have examined the genotoxicity or mutagenicity of oil sands related samples; but even these were not highly relevant OSPW samples. Given the chemical complexity of OSPW, it would furthermore be of interest to know what types of chemicals are causing any genotoxicity or mutagenicity. When these are identified, a focused treatment could be done, where the genotoxic/mutagenic chemicals are removed so that the water can be returned to the natural environment safely.

1.6 **Hypothesis and objectives**

The main hypothesis of this thesis is that neutral extractable OSPW organics (i.e. those that are most hydrophobic, and able to enter cells) will be genotoxic mutagens, and that endogenous mammalian enzymes will have a bioactivating effect. In order to better understand the possible effects of OSPW on DNA, the primary objective of this study was to investigate the genotoxic and mutagenic effects of OSPW AEOs by the SOS Chromo-test and classical Ames, respectively. A wide range of concentrations was studied, and the effect of S9 liver enzyme fraction was systematically evaluated. A secondary objective was to apply the same tests to 2 sub-fractions of OSPW dissolved organics, to determine what types of compounds (neutral extractable or acid extractable) were most important in causing the observed effects.

CHAPTER 2: METHODS

2.1 OSPW collection and organic extraction

OSPW was collected in fall of 2013 in a 20 L high-density polyethylene pail from a floating barge in Base-Mine Lake, located north of Fort McMurray. The sample was obtained from shallow depts (low sediment), and it has been aging for two years. OSPW from Base-Mine Lake has been mixed with freshwater twice, which could had decreased the water toxicity, when if compared to OSPW from the tailing ponds. The water was stored at 4°C. Prior to extraction, the sample was vacuum filtered (1.2 mm glass fiber filter – Fisher Brand, Ottawa, Ontario) then centrifuged for 5 minutes at 6000 rpm (12074 g) to exclude suspended sediment. One litre of the centrifuged supernatant was transferred to a separatory funnel.

For the primary objective of this work, dissolved organics were isolated by extraction into organic solvent under acidic conditions, consistent with most previous toxicological studies. Specifically, 1 L batches of OSPW were adjusted to pH 1.0 using ~2 mL of concentrated sulfuric acid (98%). This was extracted 3 times with 200 mL of dichloromethane (99.5%). The combined solvent extract was dried by rotary evaporation and weighed (Table 3) prior to creating stock solutions. This extract is referred to here as whole acid-extractable organics (whole AEO).

For the secondary objective, other 1 L batches of OSPW were extracted sequentially at pH 7 (F1), then at pH 1.0 (F2), to obtain distinct organic chemical fractions termed neutral extractable (F1-NE) and acid extractable (F2-AE). Specifically, F1-NE was obtained by adjusting the pH to 7.0 with concentrated sulfuric acid, extracted, dried and weighed (*Table 3*). For F2-AE fraction, the same residual water was further acidified to pH 1.0, extracted, dried and

weighed (*Table 3*). The dissolved organics from each of the fractions are shown on *Figure 7*. The appearance of each extract is found on *Figure 8*. The residual chemicals left in OSPW can be extracted at alkaline pHs and is called basic fraction. The weight of this fraction is 51 mg/L and testing it on both assays generated no genotoxic or mutagenic response, reason why the data is not shown in this thesis.

Extract type	**Weight (grams)**	**SOS Chromo-test (10 µL)**	**Classical Ames (50 µL)**
Whole AEO	0.31939 g	0.0031939 g	0.0159695 g
F1-NE	0.179805 g	0.00179805 g	0.00899025 g
F2-AE	0.24521 g	0.0024521 g	0.0122605 g

Table 3: 2L of OSPW AEO and its sub fractions, weight of and assay concentration

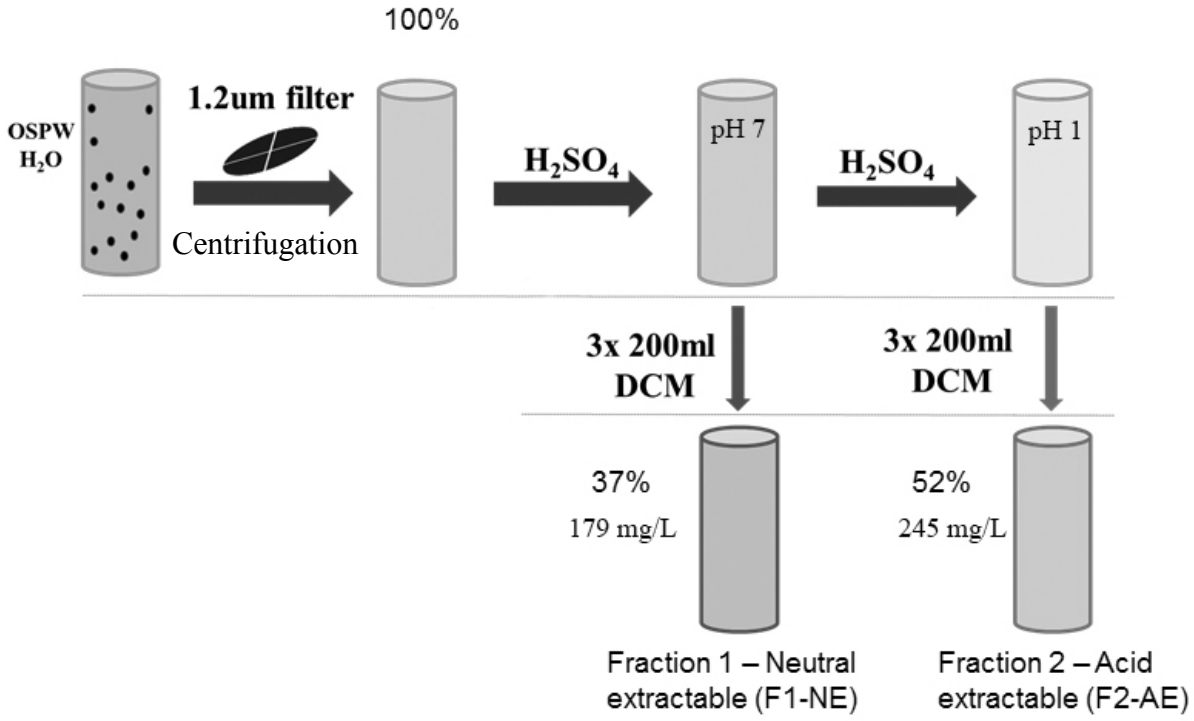
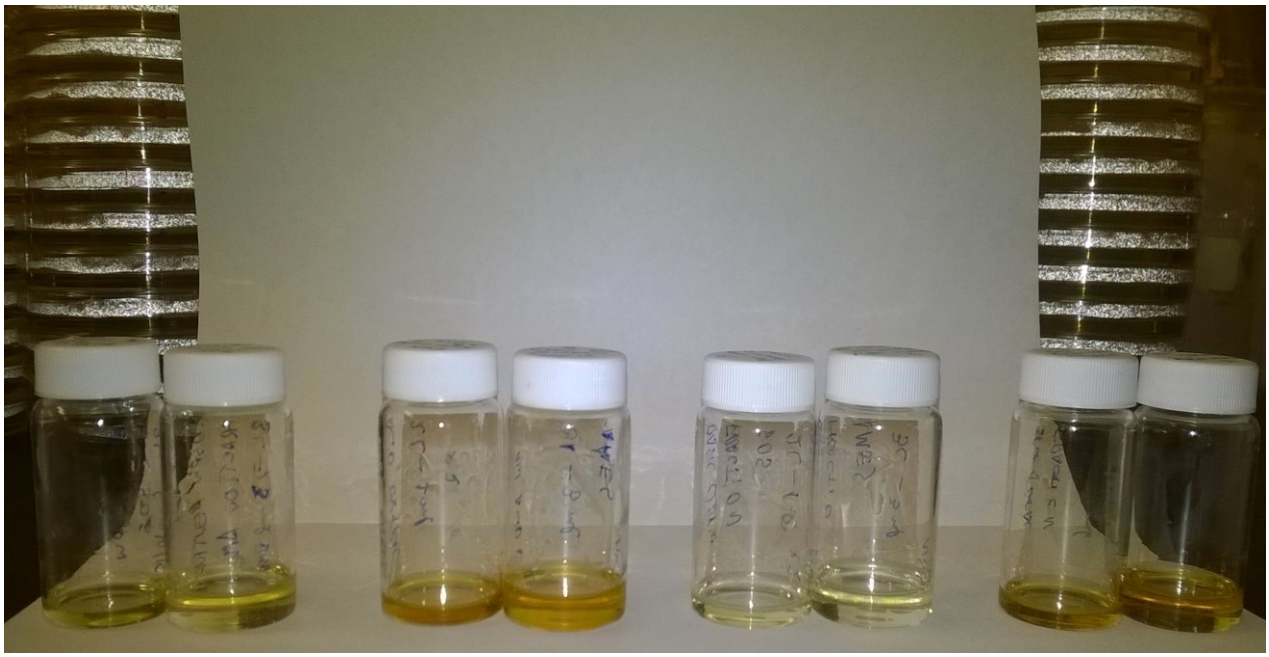


Figure 7: Schematic of OSPW organics sub fractionation into F1-NE and F2-AE.



F1 - NE F2 - AE Residual organics (alkaline extract) Whole AEO

Figure 8: OSPW AEO and its sub fractions.

Distilled water was processed through the liquid-liquid extraction steps to create extraction controls (EC) to account for any possible contamination by reagents and residual solvents that might be genotoxic or mutagenic.

For the SOS Chromotest, the organic extracts (whole AEO, F1-NE, F2-AE) from 2 L of OSPW were dissolved in sterile 2 mL solutions [55% dimethylsulfoxide (DMSO), 45% water (0.85% NaCl)] to create stock solutions of organics that were 1000 times (1000×) more concentrated than the original OSPW. For the Ames test, the organic extracts (whole AEO, F1-NE, F2-AE) from 1 L of OSPW were dissolved in sterile 2 mL solutions (55% DMSO), 45% water (0.85% NaCl) to create stock solutions of organics that were 500× more concentrated than the original OSPW. Ampicillin (25 µl, 50 mg/mL; Sigma Aldrich, Missouri, US) was added to the stock solutions and all subsequent dilutions to prevent possible bacterial contamination in the assays.

2.2 OSPW AEO analytical analysis on HPLC−LTQ-Orbitrap−MS

Reversed-phase liquid chromatography was paired with a linear ion trap-orbitrap mass spectrometer (Orbitrap ELITE, Thermo Fisher Scientific, San Jose, CA). The Orbitrap XL mass spectrometer was operated with nominal resolving power of 100,000 at m/z 400. Separate injections were made for characterization in negative ionization mode (i.e. for characterization of organic acids), and positive ionization mode (i.e. for polar organic neutrals and organic bases). The chromatographic separation was performed using a HPLC Accela System (Thermo Fisher

Scientific, San Jose, CA), consisting of a degasser, a 600 bar quaternary pump, an auto sampler, and a column oven. Separation was performed on a C18 gold column (100 × 2.1 mm, 1.9 μm particle size, Thermo Fisher Scientific, San Jose, CA) at 40 °C. A flow rate of 0.5 mL/min and an injection volume of 3 μL were used in all analyses. The mobile phases consisted of (A) 0.1% acetic acid in water and (B) 100% methanol. The mobile phase composition was 5% B for 1 min, followed by a linear gradient ramp to 90% B at 9 min, to 99% B over 5 min, and returning to 5% B in 1 min, followed by a 4 min hold prior to the next injection (87).

2.3 SOS Chromotest

SOS Chromotest (EBPI, Mississauga, Ontario) methodology has been reported elsewhere [24, 25]. Aseptic techniques were used in sample preparation and during the assay. On the night before the assay, *E. coli* PQ37 was incubated at 37°C for 16 hours in 24 mL of LB media. The next day, the bacteria broth was diluted using LB media to obtain an optical density at 600 nm (OD_{600}nm) of 0.05. In a 96 well micro plate, 10 µL of OSPW organics were mixed with 100 µL of diluted bacteria broth, and 900 µL of 1.0 M sodium phosphate buffer (pH 7.4). For metabolic activation, 500 µL of Sprague Dawley rat S9 liver fraction (20 mg/mL, Environmental Bio-detection Products, Mississauga, Ontario) was mixed per well in the presence of a NADPH generating system (0.5 M NADP+, 0.5 M glucose-6-phosphate and 5 M $MgCl_2$), with 400 uL of phosphate buffer. The final concentrations of OSPW organics in the wells ranged from 1× to 10×, relative to the original OSPW sample, and final concentrations of DMSO were <10%. All samples were run in triplicate.

Incubation times were always 4 hours at 37°C. In initial experiments, incubation of bacteria with OSPW organic extracts for 2 hours did not show any genotoxicity, while the positive controls did show an increasing dose-response. We hypothesized that this might be due to the charged and/or polar nature of OSPW organics, which would cause them to be more slowly absorbed by the cells than the neutral positive controls, thus incubation time was increased to 4 hours before addition of the chromogens.

After incubation, 100 µL of a mix of blue chromogen (X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and the alkaline phosphatase substrate (pNPP, p-nitrophenyl phosphate) was added to the wells and the plate was incubated for 2 more hours at 37 °C. As a

marker of genotoxicity, X-gal (a lactose analogue) is metabolized by β-galactosidase to generate a blue coloured product. For cytotoxicity measurement, alkaline phosphatase metabolizes pNPP to generate a yellow coloured product. A micro plate reader measured absorbance at 610 nm for genotoxicity and at 405 nm for cytotoxicity. Vehicle control wells were always run (10% DMSO). Although DMSO is not genotoxic on its own, and does result in false positives or false negatives, as shown for other genotoxicants it can have a modulating effect on the quantitative SOS response [26]. Extraction controls (EC, water that was liquid-liquid extracted like OSPW samples) were also run to ensure that any trace contaminants in the reagents were not a source of genotoxicity or cytotoxicity.

To account for the effect of cytotoxicity on the genotoxic response, the SOS Inducing Factor (SOSIF) was also calculated, as previously suggested (81). The SOSIF formula is:

$$\frac{OSPW\ genotoxicity\ absorbance\ /\ OSPW\ cytotoxicity\ absorbance}{Vehicle\ control\ genotoxicity\ absorbance\ /\ Vehicle\ control\ cytotoxicity\ abosrbance}$$

The quantitative result from SOSIF calculations can generally be interpreted as:

- Greater than 2: indicates genotoxicity that might require further testing;
- Between 1.5 and 2: is inconclusive and new sample dilutions should be tested;
- Less than 1.5: the sample is not genotoxic.

When samples show high cytotoxicity, cells will quickly die, not producing enough beta-gal to cause significant genotoxicity, making the SOSIF low. Nevertheless, when cells survive, they constantly suffer DNA, activating the SOS gene repair system thus the beta-gal producer gene. In the later scenario, the SOSIF will be higher, accusing a genotoxic sample.

A schematic of the SOS methodology can be seem on *Figure 9*.

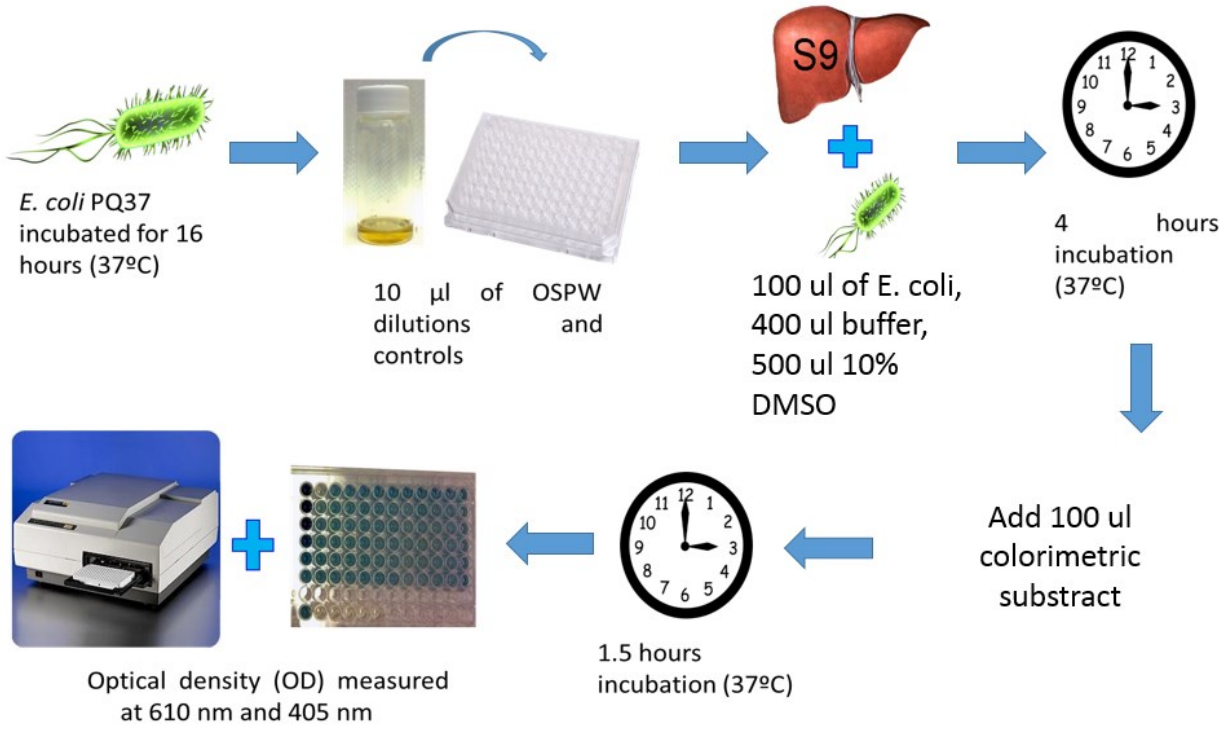


Figure 9: SOS Chromo-test methodology flowchart

Positive control wells were run to validate each batch of OSPW organics exposures, including 4-nitroquinoline 1-oxide (4-NQO, a genotoxicant that does not need bioactivation, at 10, 5, 2.5 and 1.25 µg/mL) which is a direct acting genotoxicant, and 2-aminoanthracene (2-AA, a genotoxicant that needs bioactivation, at 100, 50, 25 and 12.5 µg/mL) which is genotoxic after metabolic bioactivation and was therefore used to validate the metabolic activation by S9 incubations.

2.4 Ames test

Ames test methodology is reported elsewhere [22, 27–29]. Each sample was incubated in triplicate, and aseptic techniques were used during the assay and in sample preparation. Two histidine dependent salmonella strains were used: TA 98, which detects frame shift mutations, and TA100, which detects base pair mutations. Final exposure conditions were always < 10% DMSO.

Both strains (EBPI, Mississauga, Ontario) were inoculated into 25 mL of Oxoid Broth #2 (containing trace histidine), incubated overnight in 50 mL plastic tubes for 16 hours at 37°C. The OD600 the following morning was between 0.1 and 0.2. In 15 ml plastic tubes, 2 mL of a melted top agar (45°C) containing trace histidine (45 μM) was mixed with 100 μL of the bacteria broth (TA 98 or TA 100), 50 μL of the OSPW organic extracts, and 500 μL of sodium phosphate buffer (pH 7.4, with or without rat S9). The tubes were then shaken to ensure homogeneity and the content poured into minimal glucose plates (10%) where it was spread evenly. After the top agar had solidified, the plates were inverted and incubated at 37°C. OSPW AEO exposures were incubated for 2 days, but this was not sufficient time for the subfractions which were therefore incubated for 5 days before counting. The final concentration of OSPW organics during the exposures ranged from 0.003× to 25× of the original OSPW samples. Resulting colonies were counted manually, and recorded as colony forming units (CFUs). A schematic of this process can be seem on *Figure 10*.

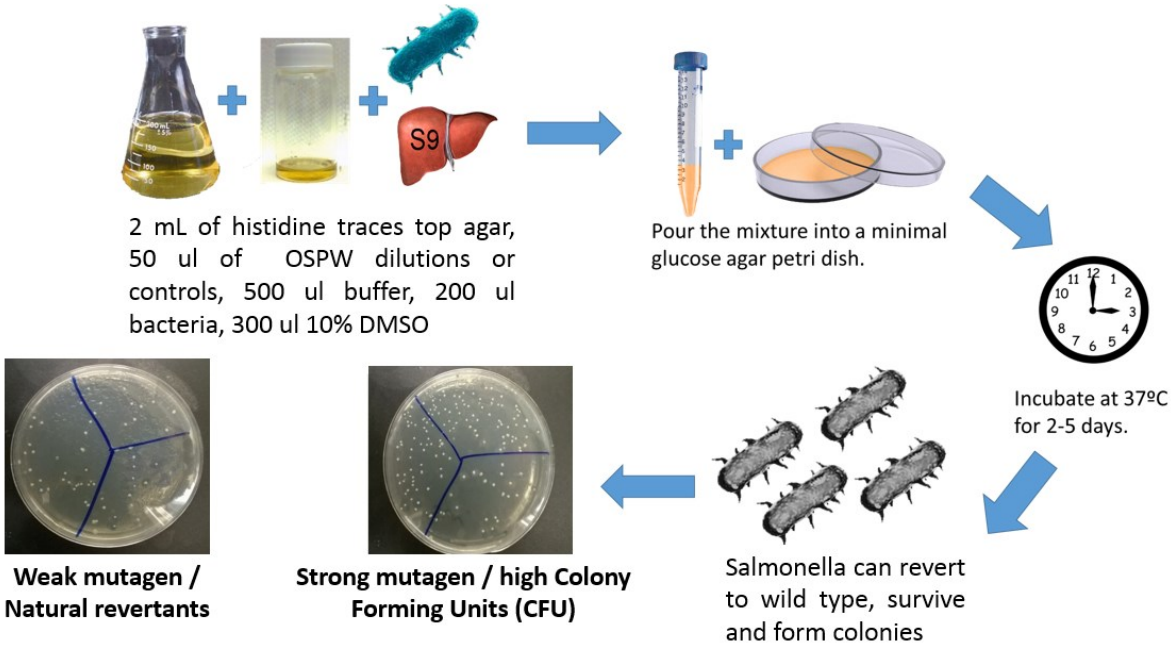


Figure 10: Classical Ames methodology flowchart.

Positive controls were also run, including substances that are direct acting mutagens (5 µg 4-nitro-o-phenylenediamine per plate for TA 98, and 0.25 µg sodium azide per plate for TA 100) and a mutagen requiring bioactivation by S9 (2-AA, 2.5 µg per plate for both strains). Vehicle controls, blanks (no chemicals, to determine natural revertants), and extraction controls were also run with each batch.

Cytotoxicity in the Ames assay was estimated by the alkaline phosphatase pNPP method. As above, both strains were inoculated into 25 ml of Oxoid broth #2 and incubated overnight at 37ºC in 50 ml tubes, but for 8 hours instead of 16 hours so that histidine had not yet depleted. The OD600 the following day was approximately 0.1. The exposure to OSPW extracts occurred in a 96 well microplate containing 100 µL of both bacterial broth, 500 µL of sodium phosphate

buffer (with and without S9), and 50 µL of diluted OSPW organics. The exposure time was 2 hours at 37ºC, after which time 100 µL of pNPP (1 mg/mL) was added, and samples were incubated for another 1.5 hours at 37ºC for the colorimetric reaction to occur. Absorbance was read at 405 nm.

2.5 Statistics

Statistical analyses were performed with Sigma Plot 12.5 (Systat Software Inc.). One-way ANOVA was used to test for significant differences among samples and the vehicle control. The Shapiro-Wilk test was used to verify normality. The Holm-Sidak test was used for multiple comparisons when normality passed, or Dunn's test when the assumption of normality was not met. Significance was always set to 0.05.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 OSPW analysis by HPLC−LTQ-Orbitrap−MS

OSPW is a complex mixture and the main goal of this analysis was to have a qualitative understanding of the classes of compounds present in both subfractions (*Figure 11*).For F1-NE, the data shows that O_2 species (i.e. all organics containing all combinations of carbon, hydrogen and exactly 2 oxygen atoms) were among the three most abundant empirical formula classes in both ionization modes, followed by ON and OS in positive mode, and OS and O in negative mode. In negative mode, the O_2 species are the classical NAs, and the O_3 group (also considered oxidized NAs) were also present at high abundance. Notably, the O_2 species detected in positive mode are known to be chemically distinct from NAs, being polar neutral compounds, likely with dihydroxyl, diketo, or keto-hydroxyl substitution (88).

F2-AE data in positive mode shows a predominance of O_3, O_3S, O_2 and ON in similar abundance. Negative data of F2-AE shows a predominance of the same compounds. Interestingly, the relatively high abundance of NAs in F2-NE demonstrates that these compounds have a hydrophobic core structure that allows them to migrate into neutral organic solvent, even when ionized, possibly due to an ion-pairing phenomenon.

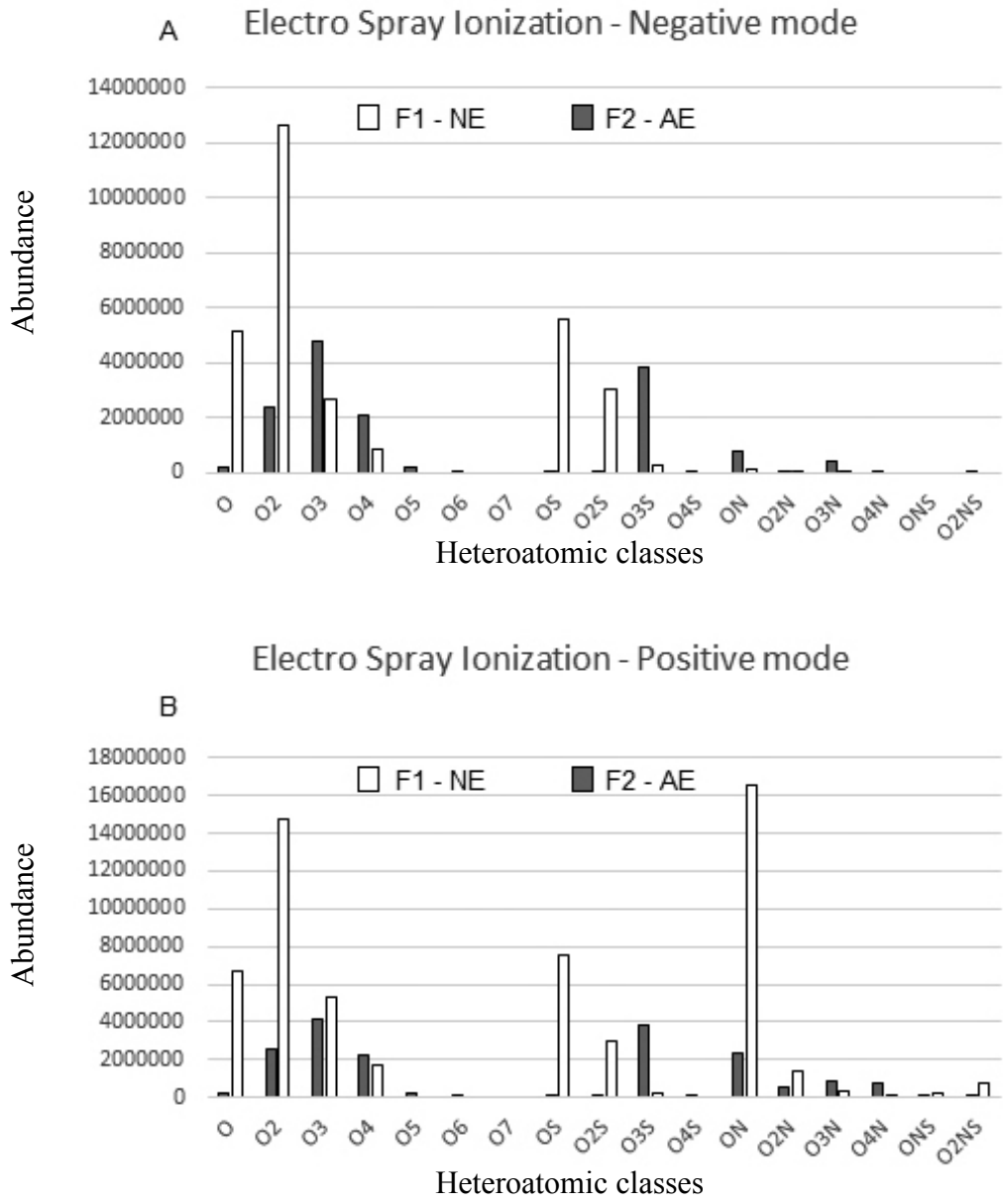


Figure 11: Empirical formula class distribution of OSPW fractions in (A) negative and (B) positive mode, determined by HPLC-Orbitrap.

3.1 SOS response to OSPW exposure

The colorimetric reactions of the SOS assay can be seen in *Figure 12* (OSPW AEO 1× to 5×) and *Figure 13* (OSPW AEO 6× to 10×). The yellow colored wells demonstrate little genotoxicity but significant cell viability due to alkaline phosphatase activity. Conversely, in *Figure 13*, some wells show increased genotoxicity at the highest concentrations (7× to 10×), shown by a deeper blue colour. The first column in *Figure 12* and the seventh row in *Figure 13* represent the positive control (4-NQO) and, in both figures, the eighth row represents the vehicle control.

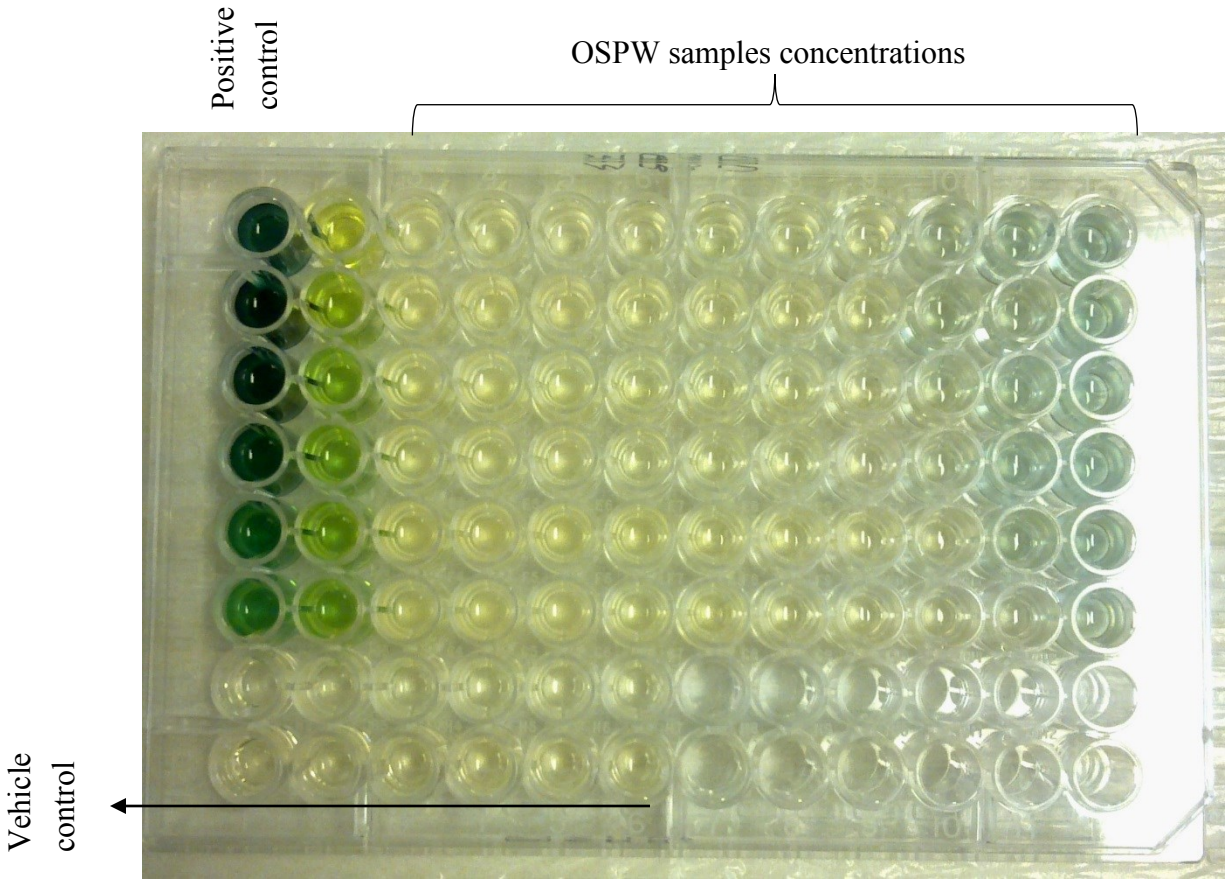


Figure 12: SOS-Chromo test visual results for OSPW AEO concentrations 1× to 5×.

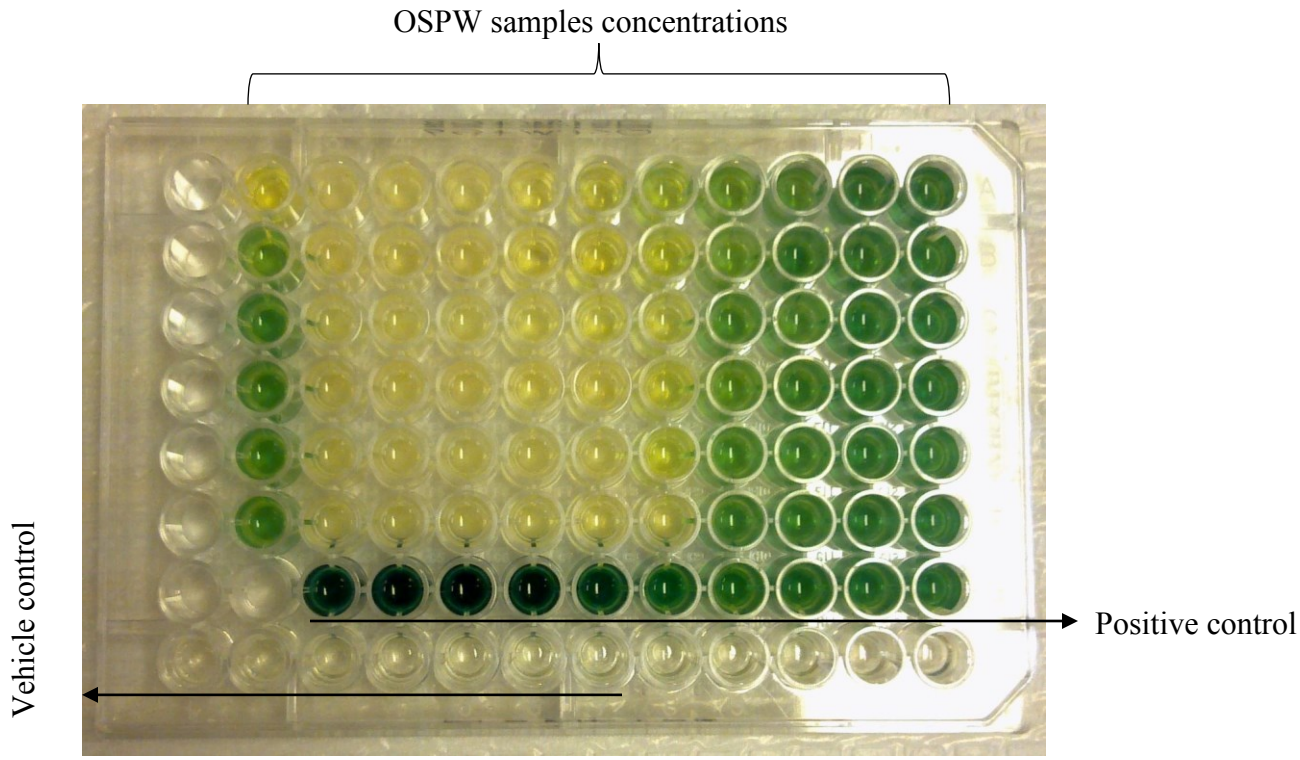


Figure 13: SOS-Chromo test visual results for OSPW AEO concentrations 6× to 10×.

The SOS inducing factors for whole OSPW AEOs, and its two subfractions, are shown in *Figure 14, 15* and *16*. Whole OSPW AEO exposures (*Figure 14*) showed no significant elevation in SOS inducing factor below 6× concentrations, but at higher exposures (7× - 10×) a statistically significant increase was observed. The SOS inducing factor was significantly higher for samples without S9 at 7×, 8×, and 9×, suggesting that S9 was able to detoxify genotoxic substances to some extent. Nevertheless, even with S9 present, OSPW AEO concentrations between 7× and 10× were statistically elevated, compared to vehicle controls ($p < 0.01$).

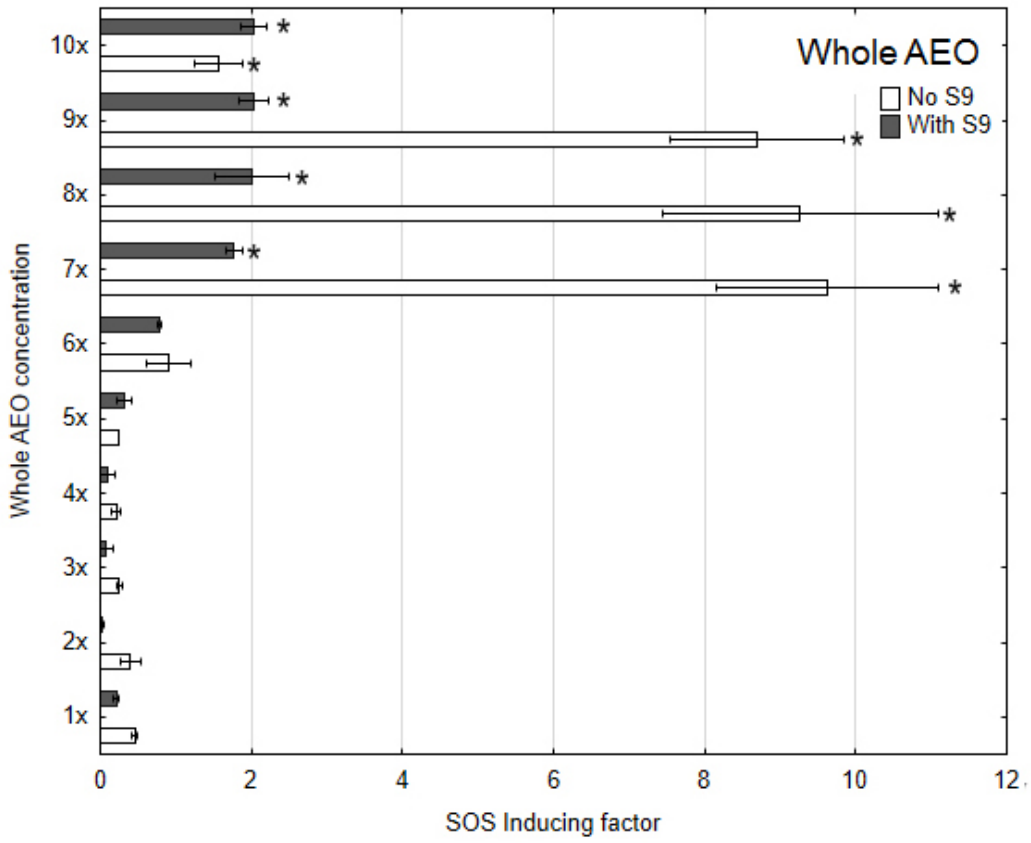


Figure 14: OSPW whole AEO SOSIF results (average ± SD). Gray represents samples with S9 and white without it. Increased SOS response can be noticed starting at 7× with and without S9. S9 is protecting the cells since the SOSIF response is lower in samples with S9. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

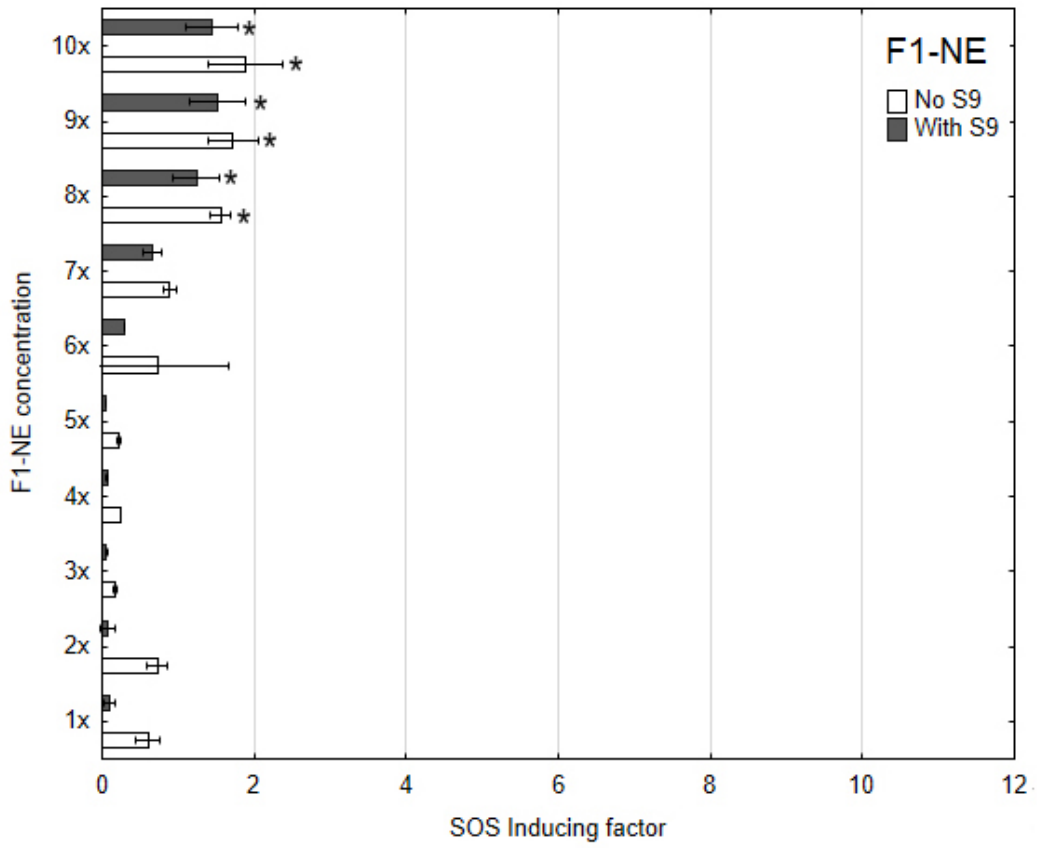


Figure 15: OSPW F1-NE SOSIF results (average ± SD). Gray represents samples with S9 and white without it. Increased SOS response can be noticed starting at 8× with and without S9. S9 protective effects are not seem here, since samples with and without S9 had similar results. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

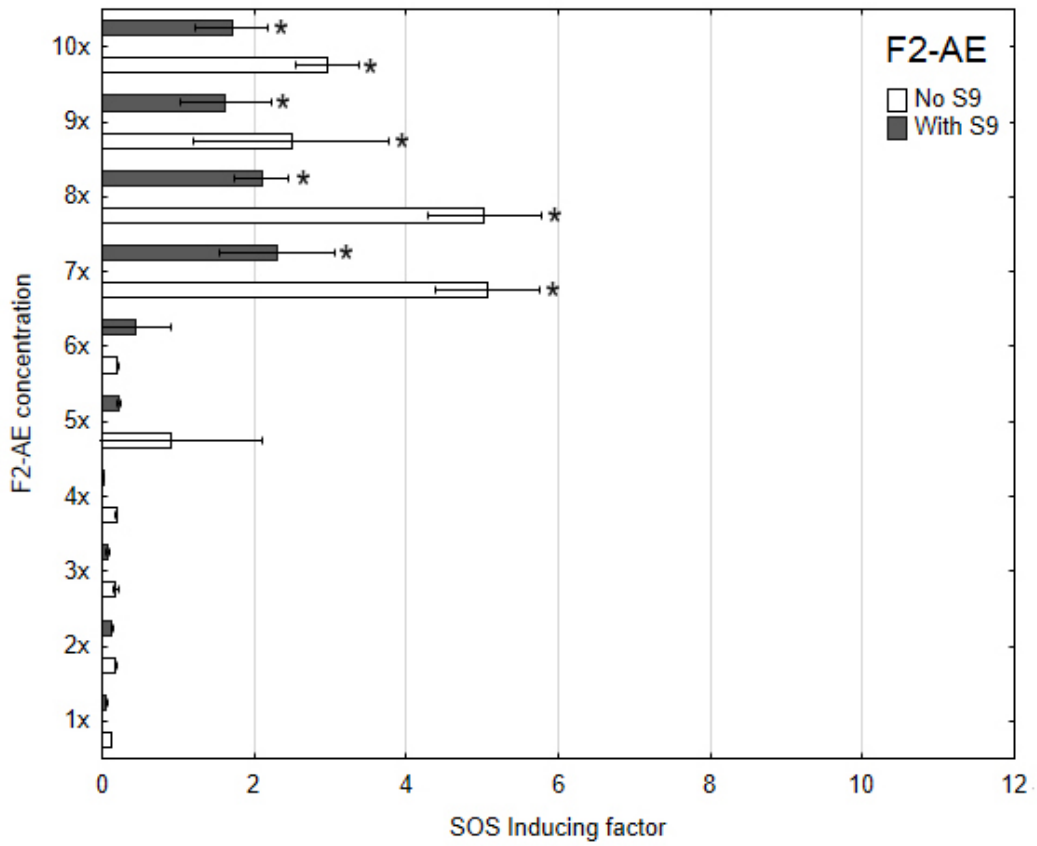


Figure 16: OSPW F2-AE SOSIF results (average ± SD). Gray represents samples with S9 and white without it. Increased SOS response can be noticed starting at 7× with and without S9. S9 is protecting the cells since the SOSIF response is lower in samples with S9. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

The raw absorbance data showed that the genotoxic reponse for OSPW AEOs was similar across most concentrations (*Figures 17, 18* and *19*), but that cell survival was low above 1× concentrations with no S9 (*Figures 20, 21* and *22*). The presence of S9 provided significant protection to cell survival, except at the highest doses (*Figures 17, 18* and *19*). This latter result is why the SOS inducing factors are significantly different between S9 and no S9, as displayed in *Figures 14* to *16*.

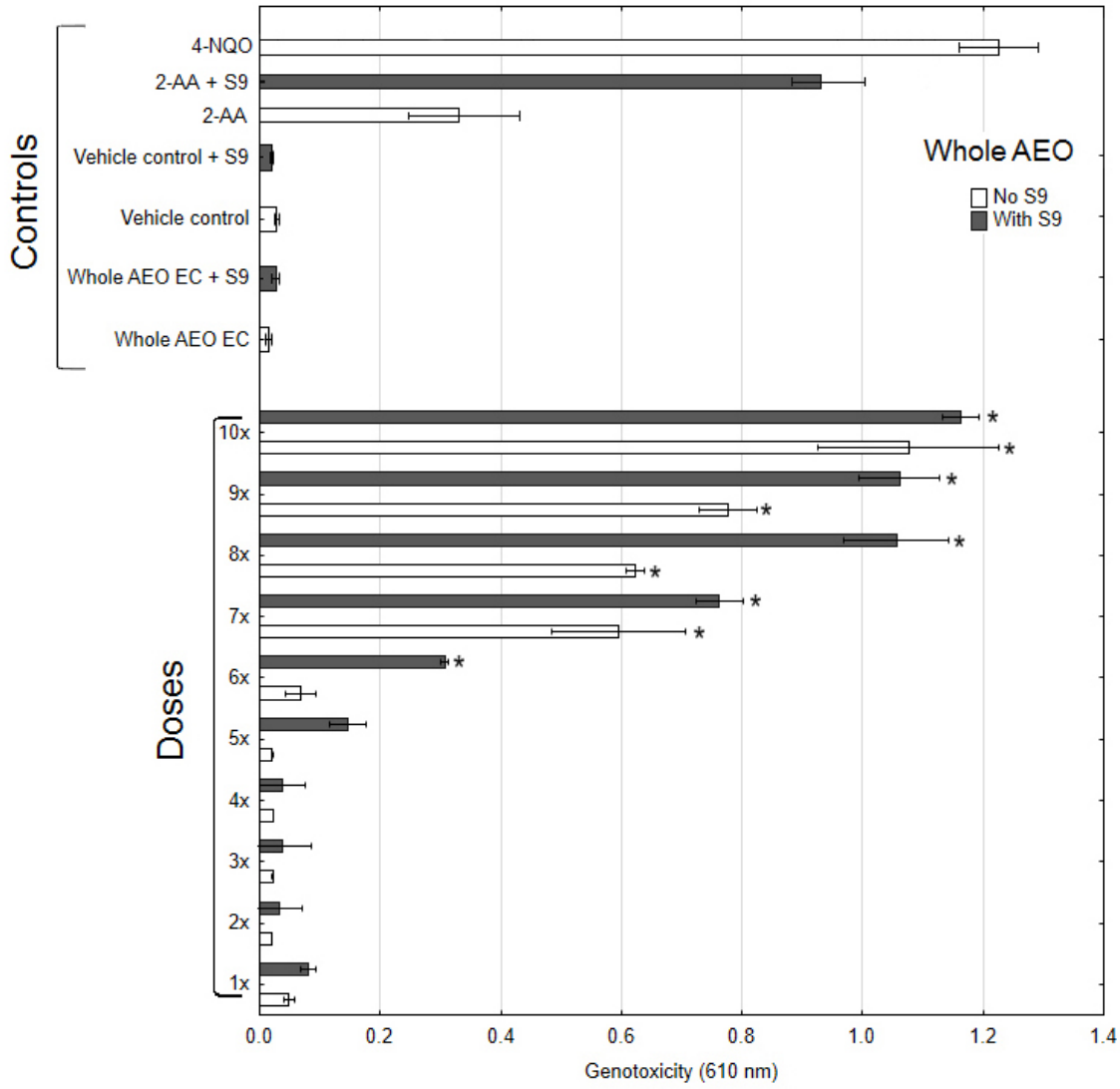


Figure 17: OSPW whole AEO genotoxicity (absorbance at 610 nm) results (average ± SD). Gray represents samples with S9 and white without it. Genotoxicity increases significantly at 6× for samples with S9 and 7x without it. S9 does not seem to be protecting cells since both results are similar. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

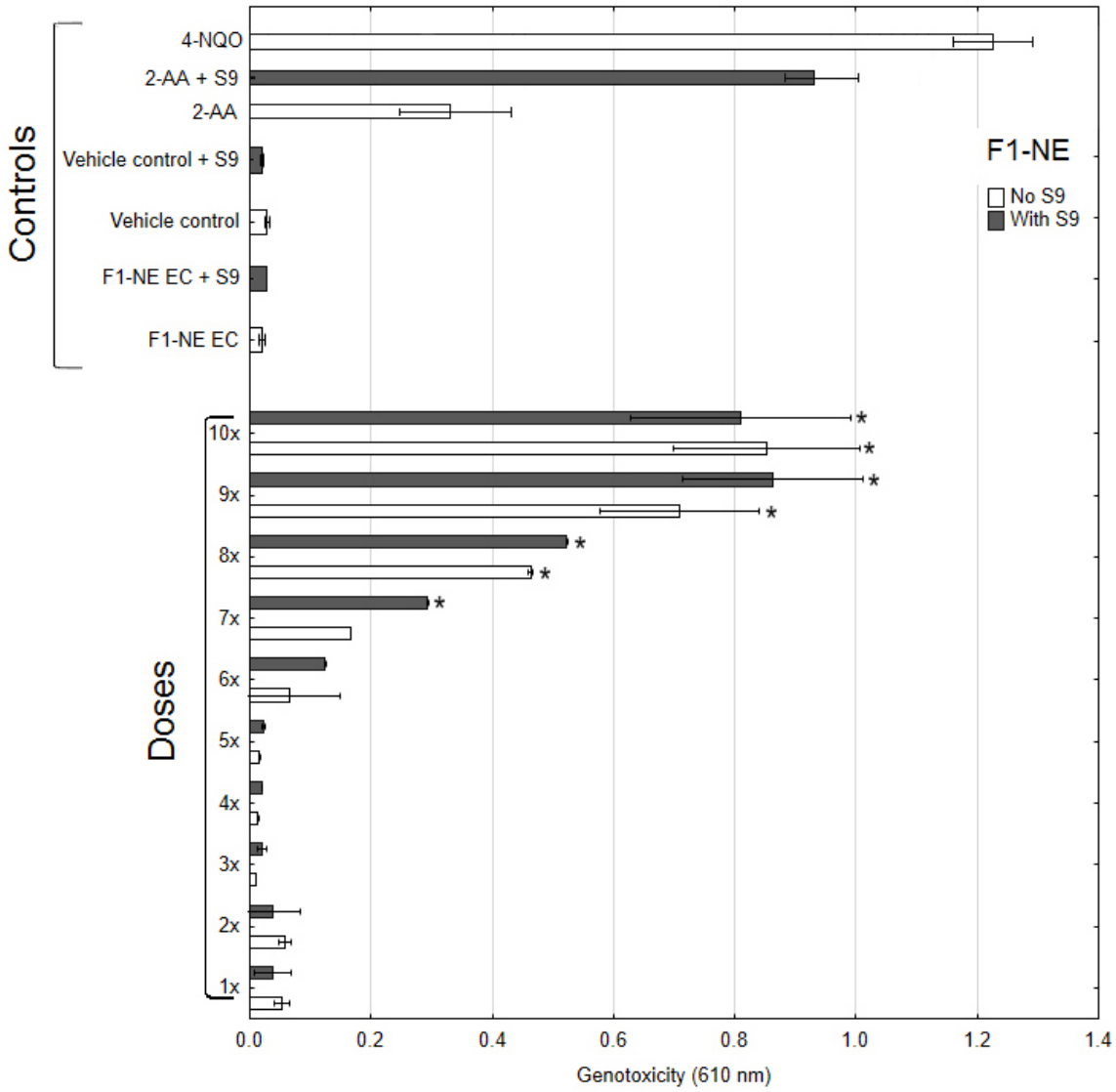


Figure 18: OSPW F1-NE genotoxicity (absorbance at 610 nm) results (average ± SD). Gray represents samples with S9 and white without it. Genotoxicity increases significantly at 7× for samples with S9 and 8x without it. S9 does not seem to be protecting cells since both results are similar. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

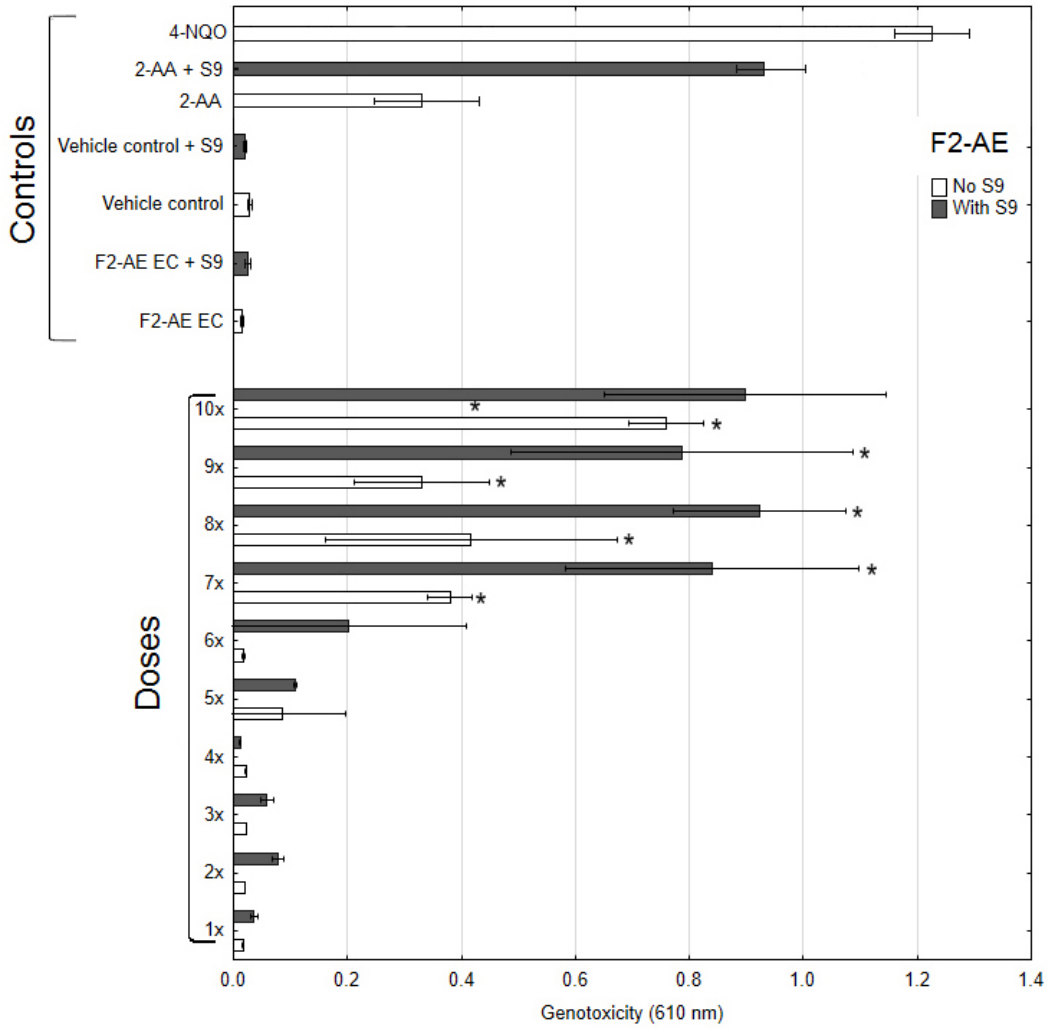


Figure 19: OSPW F2-AE genotoxicity (absorbance at 610 nm) results (average ± SD). Gray represents samples with S9 and white without it. Genotoxicity increases significantly at 7× for samples with without S9. At concentrations 7x - 9x, S9 seems to increase the genotoxic response, where at 10x, genotoxicity is similar with and without S9. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

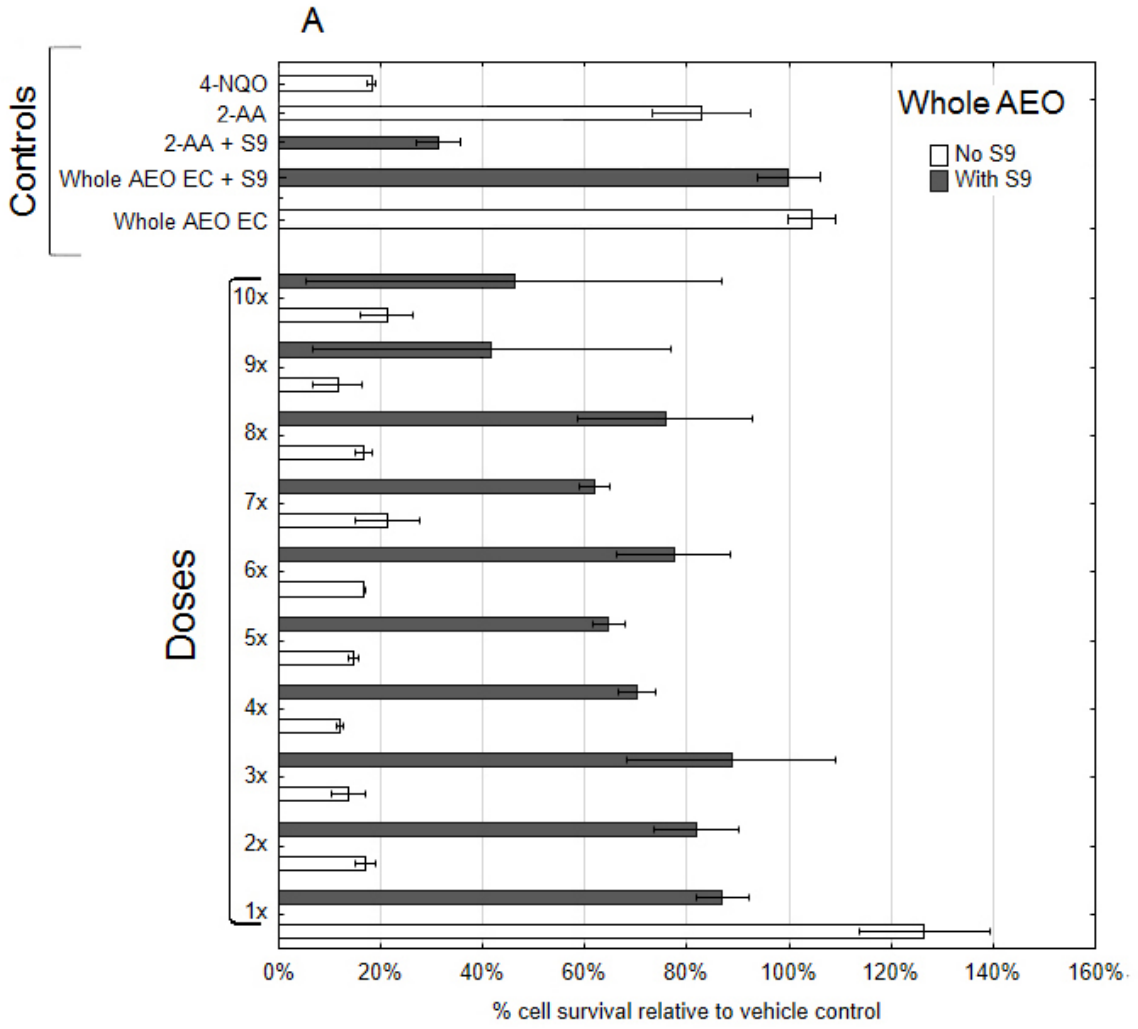


Figure 20: OSPW whole AEO cytotoxicity relative to negative control results (average ± SD). Gray represents samples with S9 and white without it. Cytotoxicity is evident on samples where S9 was absent (cell survival approximately 20% for all concentrations). When S9 was present, the cytotoxicity decreased, and cell survival was between 60 to 80%.

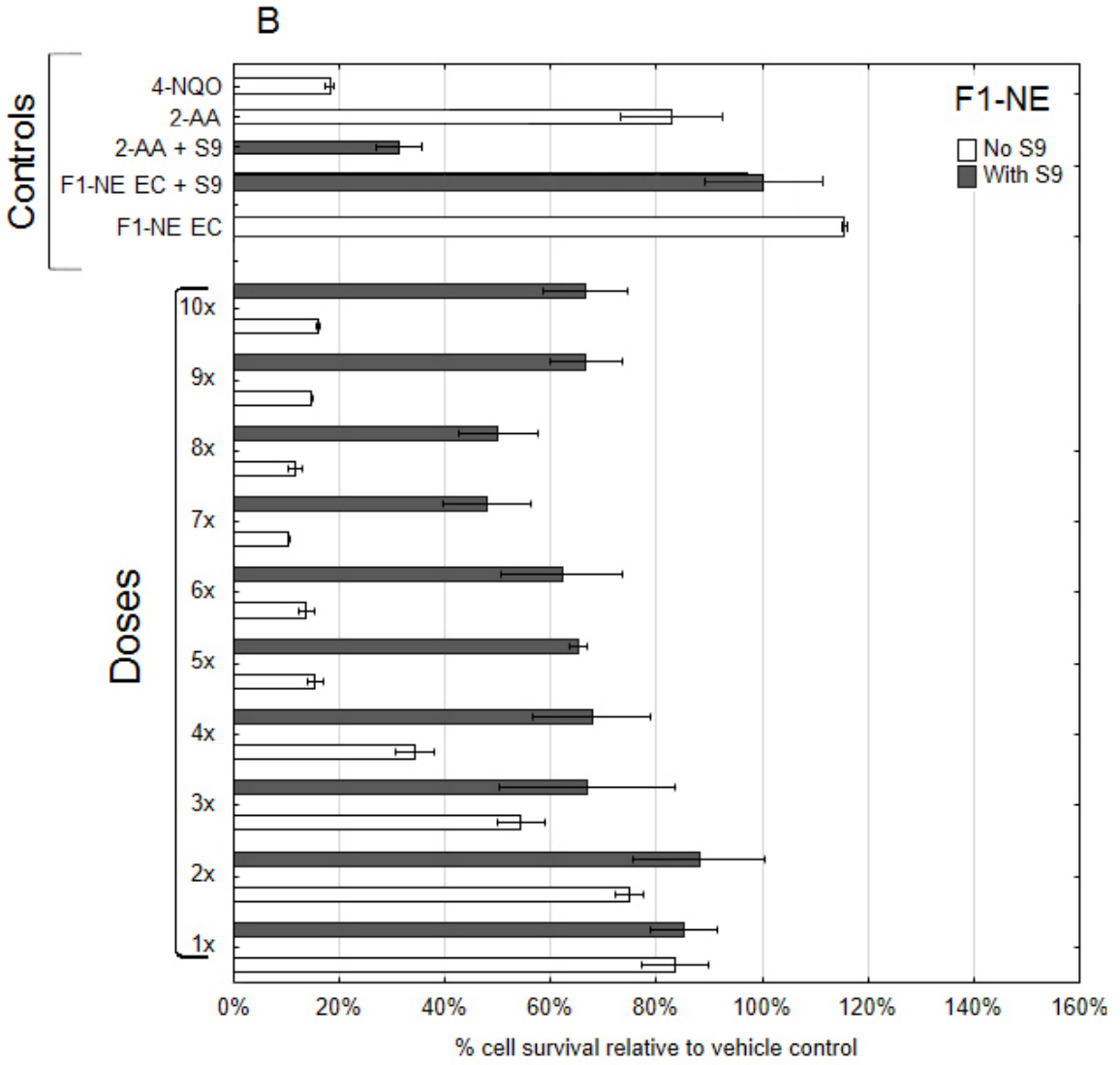


Figure 21: OSPW F1-NE cytotoxicity relative to negative control results (average ± SD). Gray represents samples with S9 and white without it. Cytotoxicity is evident on samples where S9 was absent (cell survival approximately 20% for all concentrations) at concentrations 5× and above. Lower concentrations (1 to 4x) show a slow increase in cytotoxicity. When S9 was present, the cytotoxicity decreased, and cell survival was between 60 to 80%.

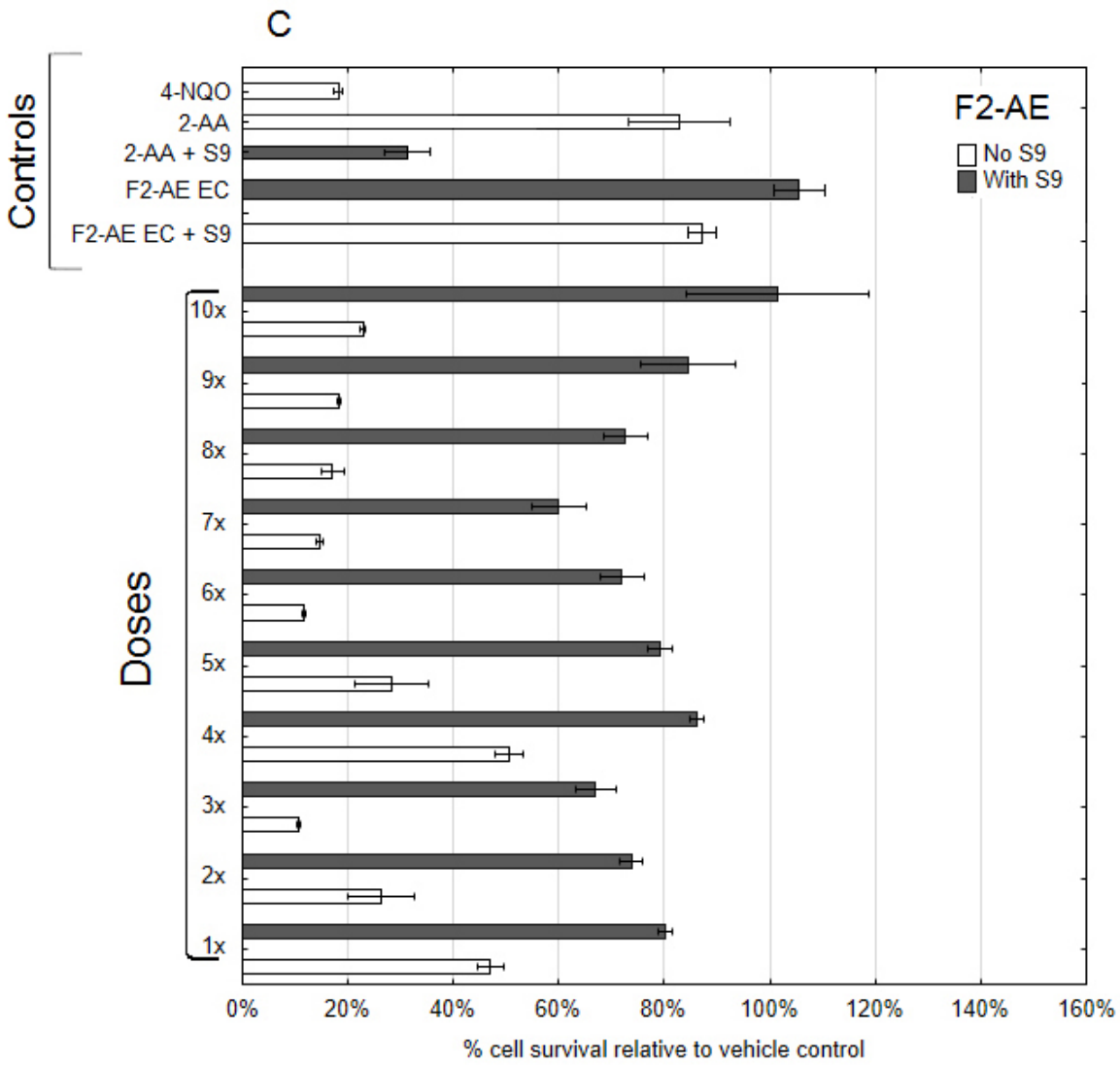


Figure 22: OSPW F2-AE cytotoxicity relative to negative control results (average ± SD). Gray represents samples with S9 and white without it. Cytotoxicity is evident on samples where S9 was absent (cell survival approximately between 20% and 40% for all concentrations). When S9 was present, the cytotoxicity decreased, and cell survival was between 80 to 100%.

Positive controls (4-NQO, and 2-AA with S9) showed genotoxicity (absorbance at 610 nm) at a concentration of 5 µg/mL (*Figures 17, 18* and *19*). This response declined slightly at the highest doses (~ 10 ug/mL), likely owing to increasing cytotoxicity (*Figures 20, 21* and *22*). As expected, 2-AA without S9 showed no genotoxicity, nor any cytotoxicity over the doses studied

(*Figures 17* to *22*). The vehicle and extraction controls, nor the presence of S9, showed any genotoxicity or cytotoxicity in the absence of test chemicals (*Figures 17* to *22*).

By examination of the raw absorbance data for the subfractions, F1-NE and F2-AE, the acute cytotoxicity of OSPW AEOs without S9 was predominantly, but not exclusively, attributable to F2-AE (i.e. comparing *Figures 20* and *22*). Consistent with results for whole AEO, the presence of S9 lowered the cytotoxicity for both of these subfractions. In both subfractions, the threshold for increasing genotoxic response (i.e. absorption at 610 nm) was similar, between 7× and 8× (*Figures 17* to *19*), as was the maximum response at the highest doses. Overall, the pattern of SOS inducing factors for F2-AE (*Figure 19*) was similar to whole AEO (*Figure 17*), whereby genotoxicity was highest in samples between doses 7× and 8×, and the presence of S9 alleviated this effect to some extent. Nevertheless, both subfractions showed significantly elevated SOS inducing factors at the highest doses, thus the genotoxic response cannot be attributed to only one group of substances.

The use of the SOS inducing factor is a common metric that controls for cytotoxicity when measuring genotoxicity by the current method. It is generally regarded that an SOS inducing factor of 2 and above can be considered a positive result for genotoxicity in this test, while a result of 1.5 or less is considered not genotoxic (79,81,89–91). The SOS inducing factor was as high as 8-10× in whole OSPW AEOs, but the concomitant high cytotoxicity at these doses nevertheless raises some questions about the relevance of the result. In the presence of S9, the maximum SOS inducing factor was approximately 2.0 for whole AEOs and its two subfractions, but never significantly exceeded 2.0. As a screening test, the overall SOS Chromotest results provide some evidence that OSPW extractable organics have a genotoxic potential, but these results – at high doses and for short exposures – are difficult to extrapolate to

a real world scenario where exposure will be lower, but possibly longer term (i.e. chronic) exposure.

Lacaze *et al.* (2014) showed evidence for genotoxicity in rainbow trout hepatocytes by the COMET assay at concentrations as low as 0.1× simulated OSPW for longer exposures (18 hours) (50). The authors suggested that PAHs in their samples could be the main culprit for the genotoxicity observed. We did not analyze PAHs in the current samples, but they are likely low because the OSPW was filtered. PAHs on OSPW can be found attached to bitumen particles and, since bitumen is water insoluble, filtering the samples in a regular glass fiber filter (1.2 mm) will remove the majority of PAHs. Furthermore, because of their hydrophobicity, the PAHs remains would be concentrated in the neutral extractable fraction (F1-NE). The fact that we demonstrated genotoxicity in both F1-NE and F2-AE suggests that PAHs are not entirely responsible for the effects.

The SOS system per se is absent in humans but human orthologs of the bacterial genes LexA, RecA, Rad6 and Rad18, among others, promote a repair mechanism that is similar to the bacterial SOS response (92). Thus the current results are not irrelevant to vertebrates. It is also important to consider that the SOS chromotest might produce false-negative results for intercalating genotoxicants. Intercalation is a process whereby molecules are inserted between DNA base pairs. Chemicals like 9-aminoacridines can cause genotoxicity via intercalation in a SOS-dependent manner, where the excision repair system response will detect and try to fix the intercalation but creating a mutation via deletion (loss of the base pair adjacent to the intercalated molecule) or addition (reading 9-aminoacridine as another base) instead of removing 9-aminoacridines (93–96). OSPW is a complex mixture of xenobiotics that could cause genotoxicity in several different ways, including intercalation, covalent adduct formation or

through DNA oxidation. Further investigation of such mechanisms in relevant cells could be valuable.

3.2 Ames response to OSPW exposure

Positive control CFU counts did not change with increasing incubation time between 2 and 5 days. The rate of natural revertants in vehicle controls and blanks (with and without S9) were consistent with the literature: for TA98, 20 to 75 CFUs, and for TA100, 100 to 250 CFUs) (83–86). The lowest dose CFU was also consistent with the literature and the highest dose CFU was higher than the background levels as shown on *Figure 23*.

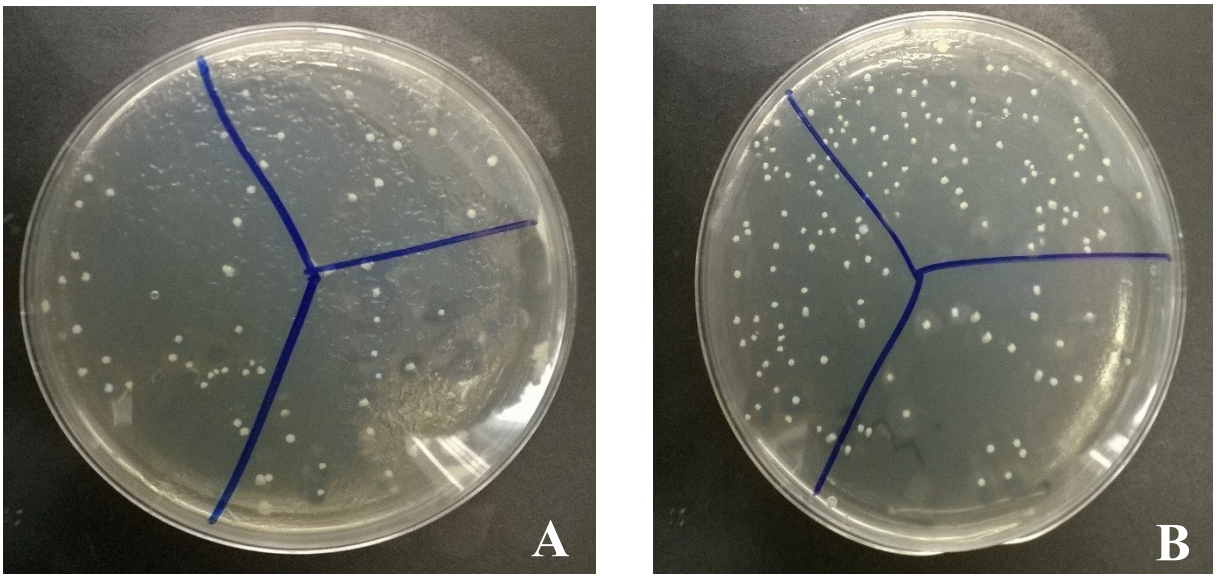


Figure 23: Revertant colonies in the Ames test on plates for different samples. “A” shows 0.003× F1-AE, where the mutagenic response was similar to vehicle control. This natural background is also known as natural revertants. “B” shows whole AEO at 25×.

For TA98 exposed to whole OSPW AEO without S9 (*Figure 24*), a gradual increasing rate of revertants was observed beginning at approximately 12.5×, reaching a maximum at the highest dose of 25×. Co-incubation with S9 showed a significant increase in the mutagenicity at low doses between 1.5× and 6.3×, with all other doses not being above control. Interestingly, at the lowest doses without S9 (10× and below), CFUs were statistically higher than in vehicle ($p < 0.01$). We suggest that this is not an indication of highly potent frame-shift mutagens, but could rather be attributable to a proliferative effect of the complex mixture of organics on the bacteria. The complex mixture of organic compounds in OSPW AEO (88) might provide additional nutrients to the bacteria, allowing them to proliferate more than the controls during early stages of the exposure when trace histidine was still present. In fact, this was observable in the alkaline phosphatase assay for OSPW AEO without S9 (*Figures 25* and *26*), whereby a higher response was measured at doses between 0.3× to 13.7×. The presence of S9 eliminated this effect. A similar effect was seen in the F2-AE exposures (*Figures 27* and *28*), but not in the F1-NE exposures (*Figures 29* and *30*), suggesting that organic acids (e.g. trace acetic acid) might have contributed to cell proliferation. We analyzed the OSPW AEOs for traces of histidine, and none was detected.

For TA100 exposed to OSPW AEO without S9 (*Figure 31*), a similar maximal response in mutagenicity was observed compared to TA98. The threshold for significantly increased mutagenicity was 12.5× ($p<0.01$), the same as for TA98, with an increasing rate of CFUs up to the highest dose of 25×. Co-incubation with S9 did not eliminate the mutagenicity at high doses (21× - 25×), but the threshold for mutagenicity was higher in the presence of S9, showing some potential for S9 to detoxify the mixture.

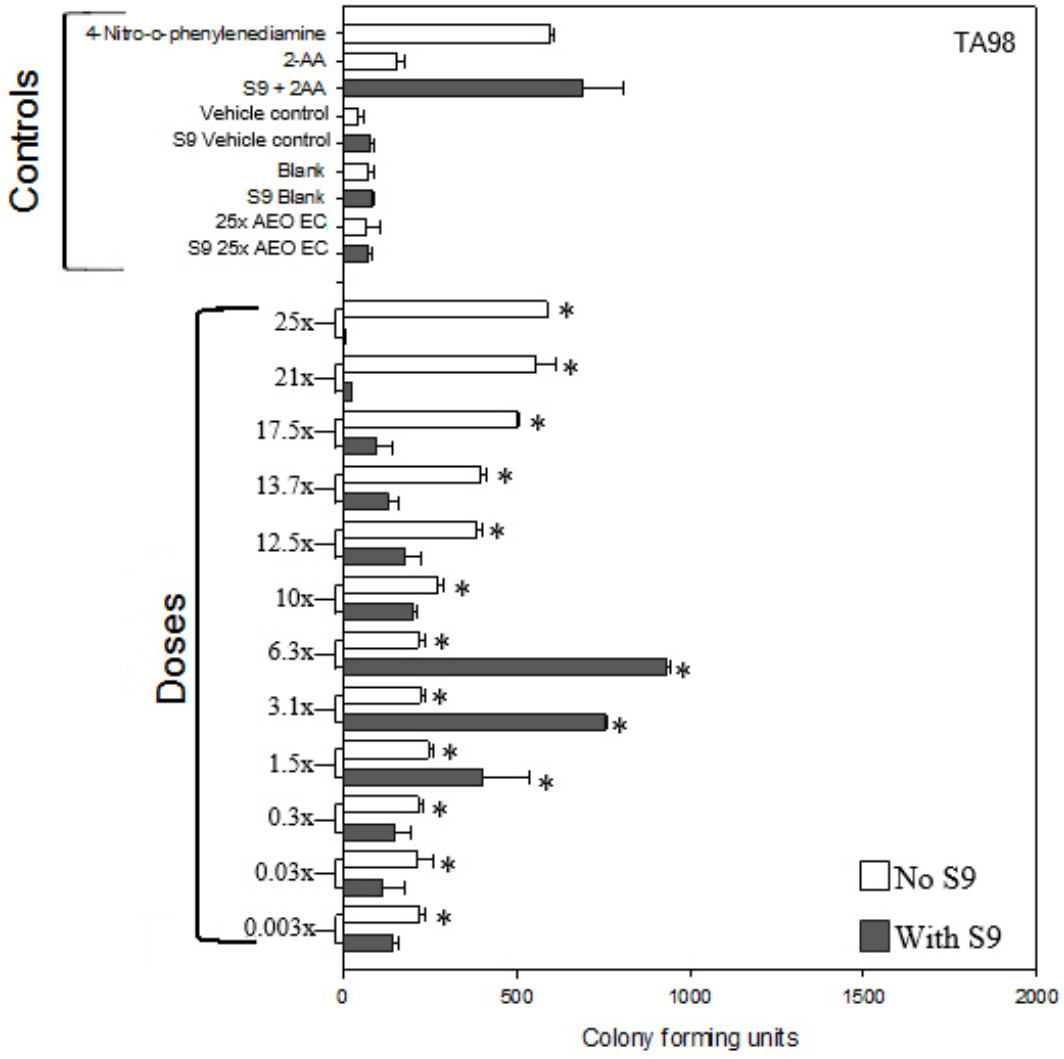


Figure 24: Ames TA98 whole AEO mutagenicity (average ± SD). Gray represents samples with S9 and white without it. For samples without S9, significantly increased mutagenicity is noticed at 10x and above. When S9 is present, a shift in the mutagenicity happens, where whole AEO seems to be bioactivated, showing mutagenicity between 1.5× to 6.3× * represents a statistically significant increase relative to vehicle control ($p<0.05$).

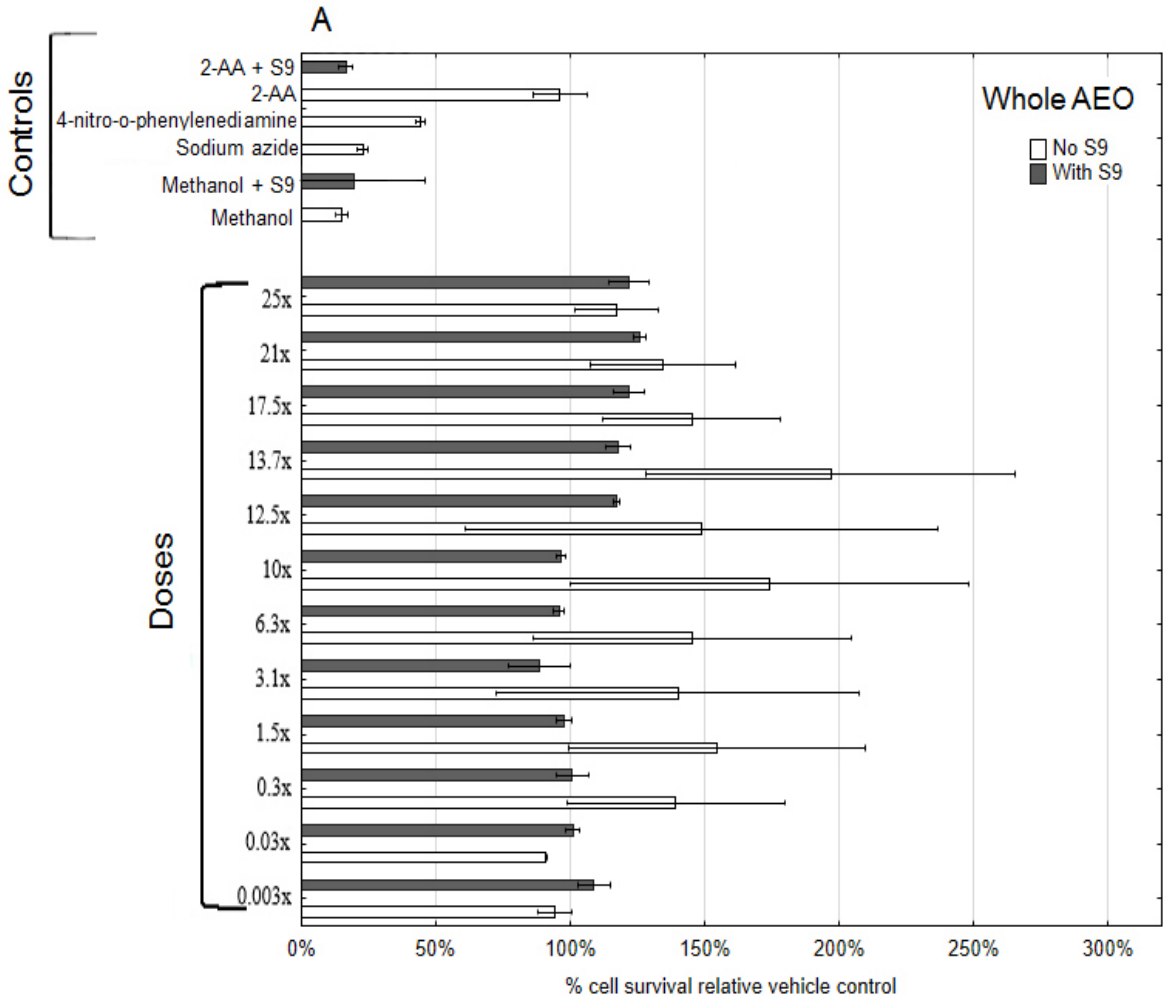


Figure 25: Ames TA98 whole AEO cytotoxicity (average ± SD). No evident cytotoxicity is noticed, but an increased cell growth is noticed when S9 is absent (more than 100% survival if compared to vehicle control) at 0.3× to 25×. Since there are several unknown chemicals on OSPW whole AEO, it is possible to infer that the increased growth can be caused by chemicals used as nutrient by the bacteria.

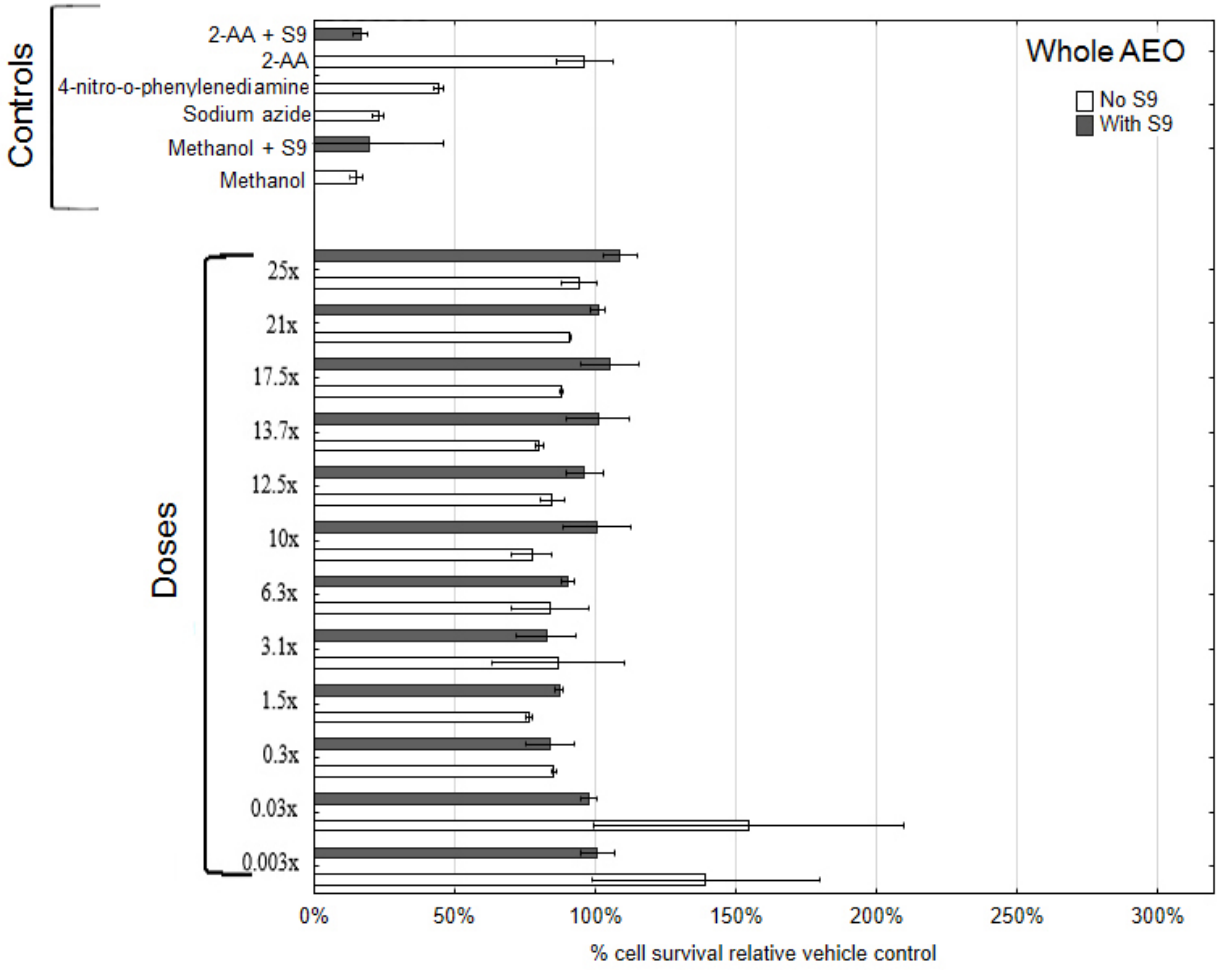


Figure 26: Ames TA100 whole AEO cytotoxicity (average ± SD). Gray represents samples with S9 and white without it. No evident cytotoxicity is noticed for any concentrations. Samples without S9 showed increased cell growth at the lowest concentrations (0.03× and 0.003×).

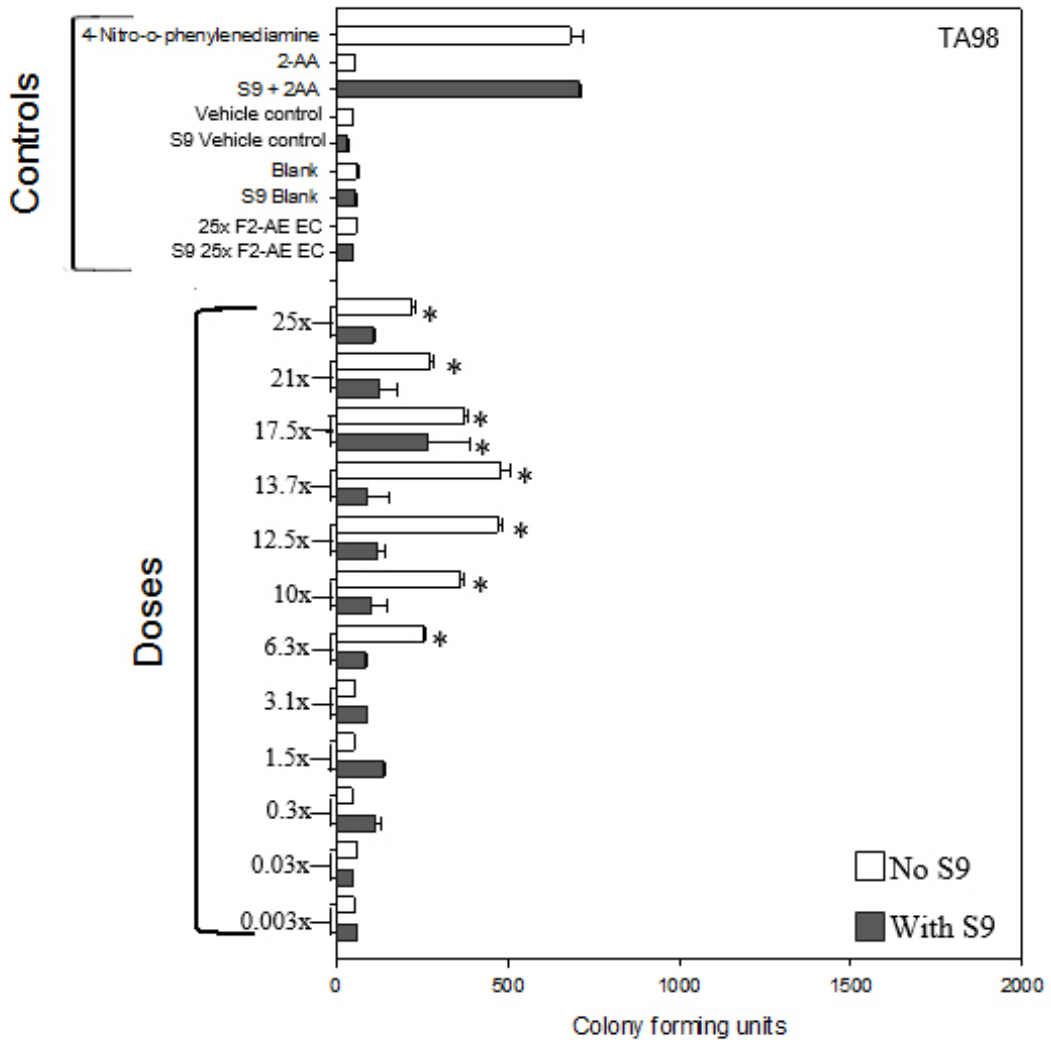


Figure 27: Ames TA98 F2-AE mutagenicity (average ± SD). Gray represents samples with S9 and white without it. Increased mutagenicity is noticed from 6.3× to 25× when S9 was absent. 17.5× showed increased mutagenicity when S9 was present, but lesser than the same concentration with S9. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

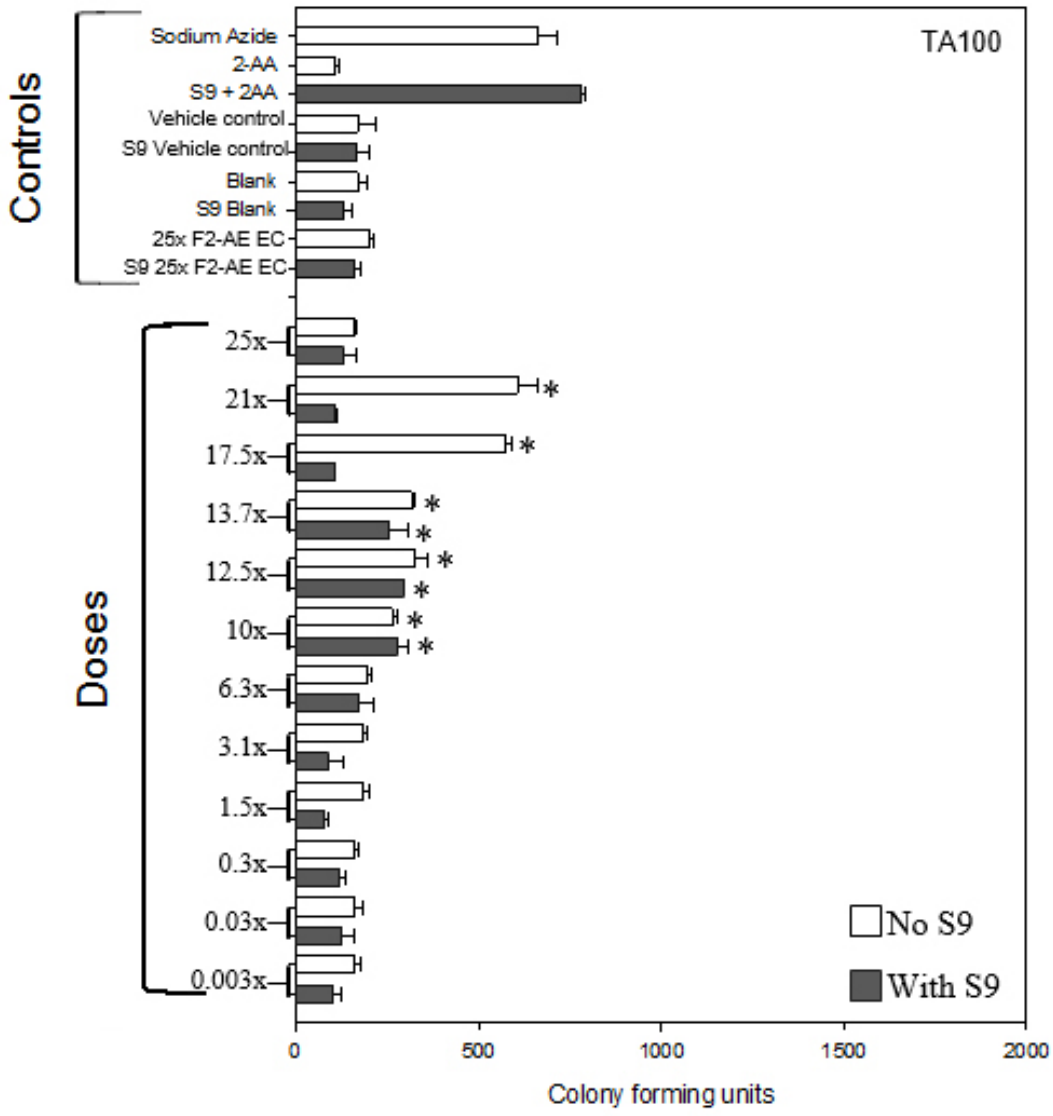


Figure 28: Ames TA100 F2-AE mutagenicity (average ± SD). Gray represents samples with S9 and white without it. Increased mutagenicity for samples without S9 is noticed from 10x to 21×, where when S9 is absent the mutagenicity is seen from 10× to 13.7×. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

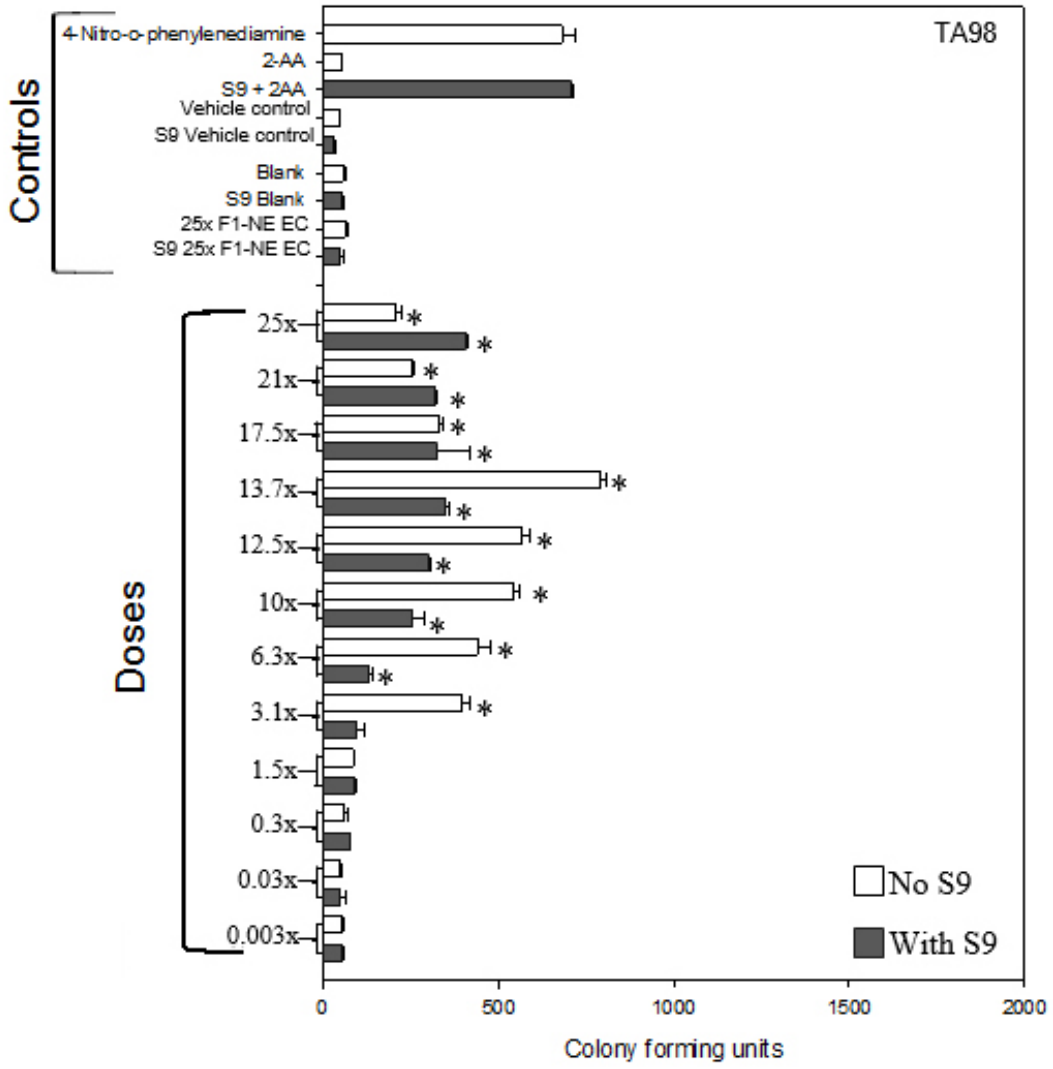


Figure 29: Ames TA98 F1-NE mutagenicity (average ± SD). Gray represents samples with S9 and white without it. Increased mutagenicity is noticed from 3.1× to 25× for samples without S9 and from 6.3× to 25× with S9. From 6.3 to 13.7, S9 had a protective effect but at 17.5× to 25× bioactivation increases mutagenicity. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

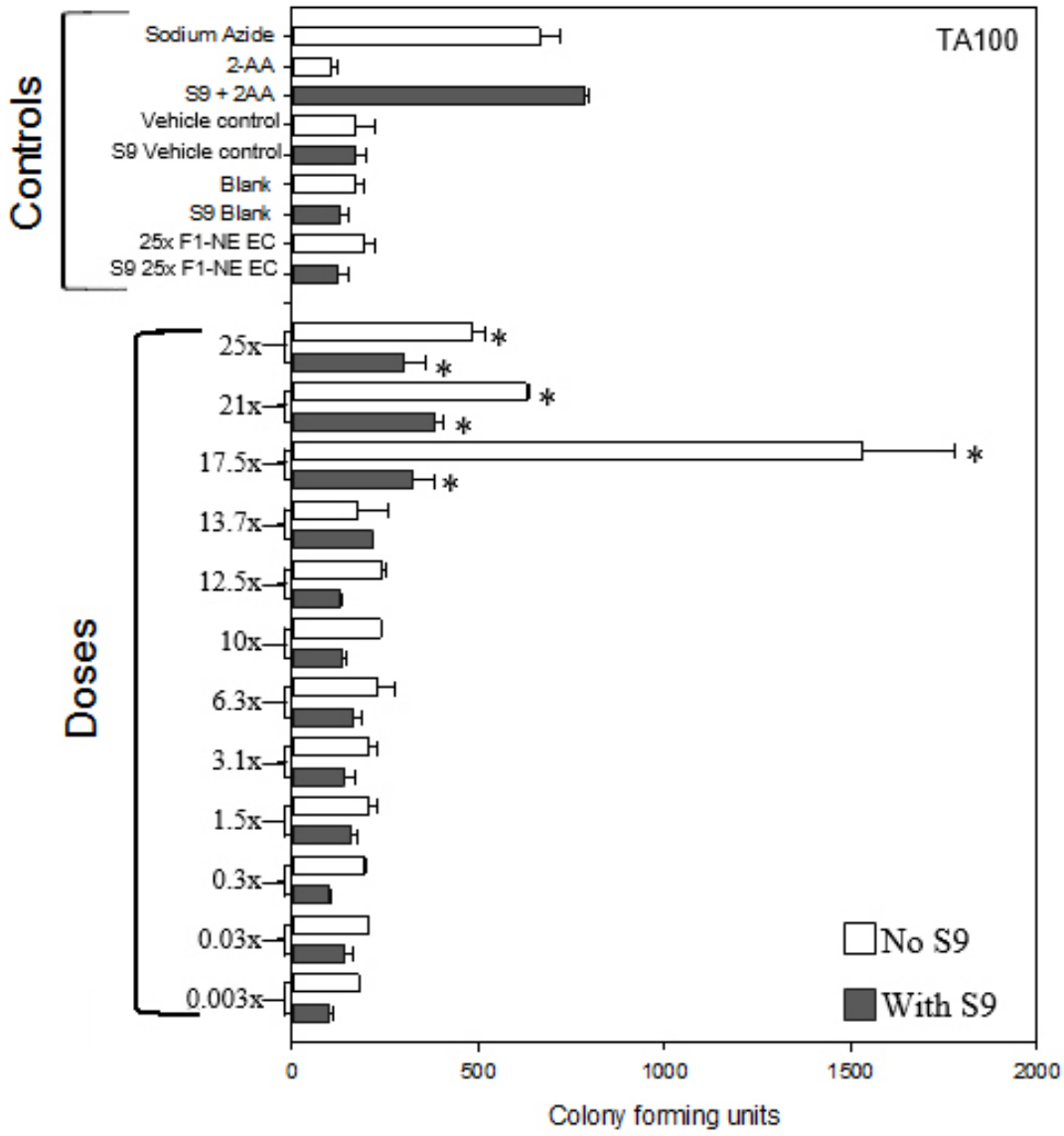


Figure 30: Ames TA100 F1-NE mutagenicity (average ± SD). Gray represents samples with S9 and white without it. Increased mutagenicity is noticed starting at 17.5× to 25×. A slight decrease of mutagenicity occurs when S9 is present. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

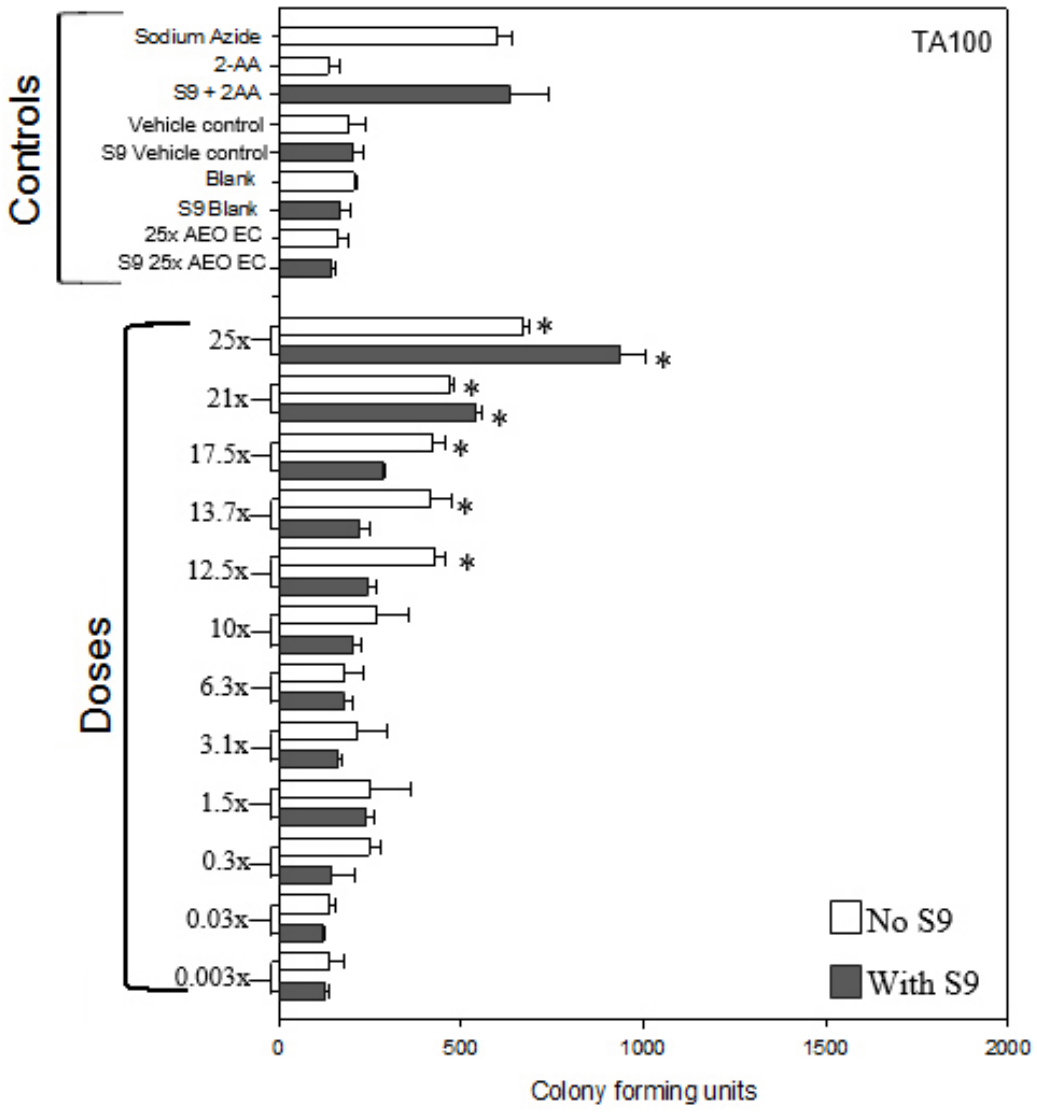


Figure 31: Ames TA100 whole AEO mutagenicity (average ± SD). Gray represents samples with S9 and white without it. Increased mutagenicity is noticed starting at 12.5× for samples without S9, where only 21× and 25× showed increased mutagenicity when S9 was present and some degree of bioactivation since the results are higher than samples without S9. * represents a statistically significant increase relative to vehicle control ($p<0.05$).

The mutagenicity of F1-NE (*Figures 29* and *30*) and F2-AE (*Figures 27* and *28*) were generally lower than for whole OSPW AEO, which is reasonable since the latter is composed of the former. In TA98, without S9, both fractions resulted in significantly elevated mutagenic responses for doses above 3.1× (F1-NE, *Figure 29*) and ≥ 6.3× (F2-AE, *Figure 27*). Unlike for OSPW AEO, where there was significant bioactivation at medium doses in TA 98 (*Figure 29*, 1.5× - 6.3×), there was no evidence for bioactivation in either fraction.

For TA100, without S9, both fractions resulted in significantly elevated mutagenic responses for doses above 17.5× (F1-NE, *Figure 30*) and ≥ 10× (F2-AE, *Figure 28*). Only the highest dose of F1-NE (25×) showed significant bioactivation in TA100, comparing incubation with S9 to no S9 ($p < 0.05$). Both sub-fractions had similar mutagenic potencies to each other, thus the mutagenic response of whole OSPW AEO could not be attributed to only one fraction.

Cytotoxicity was low at all doses in both strains for whole OSPW AEOs and both fractions (*Figures 25-36* and *32-35*).

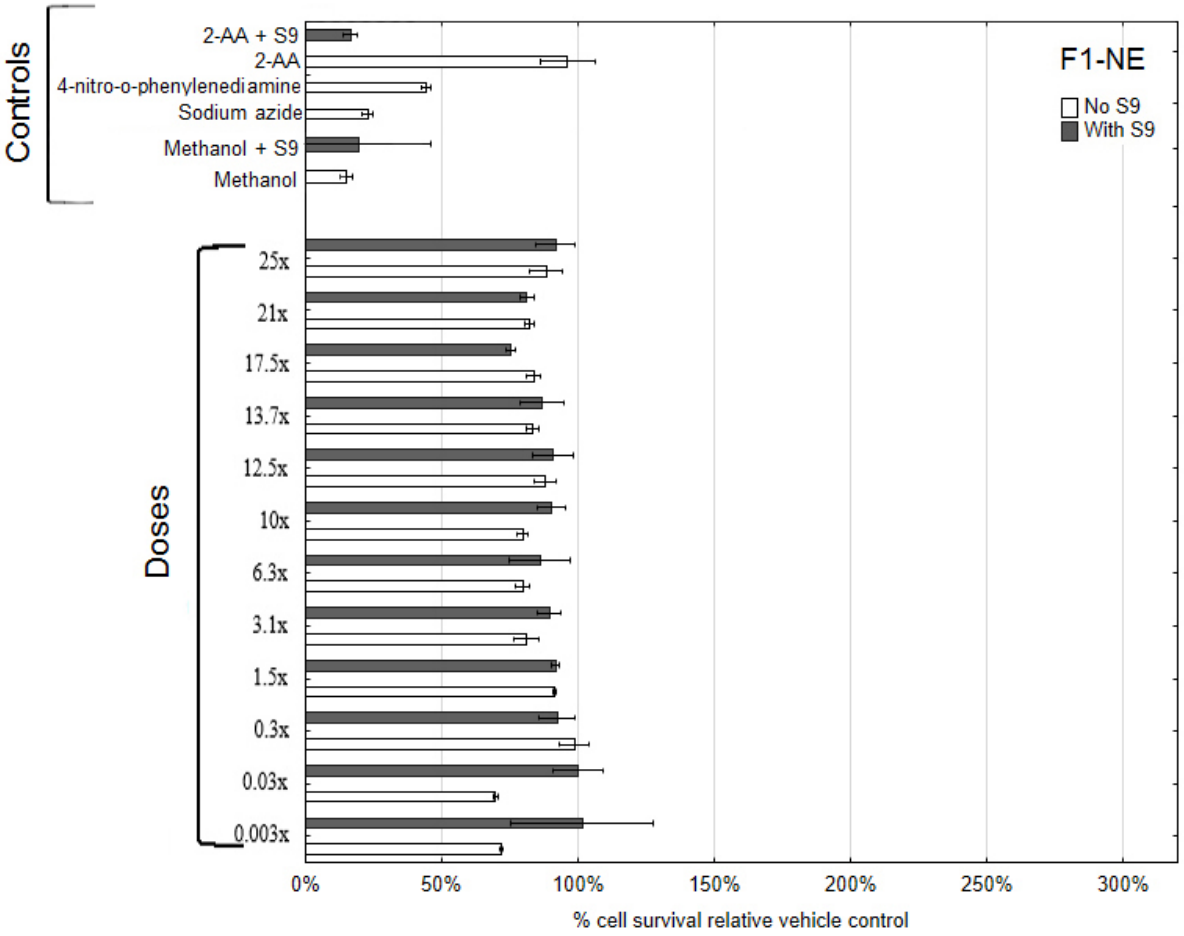


Figure 32: Ames TA98 F1-NE cytotoxicity (average ± SD). Gray represents samples with S9 and white without it. A small cytotoxicity is noticed at all concentrations, where the maximum cytotoxicity is 21% at the lowest dose (0.03×)

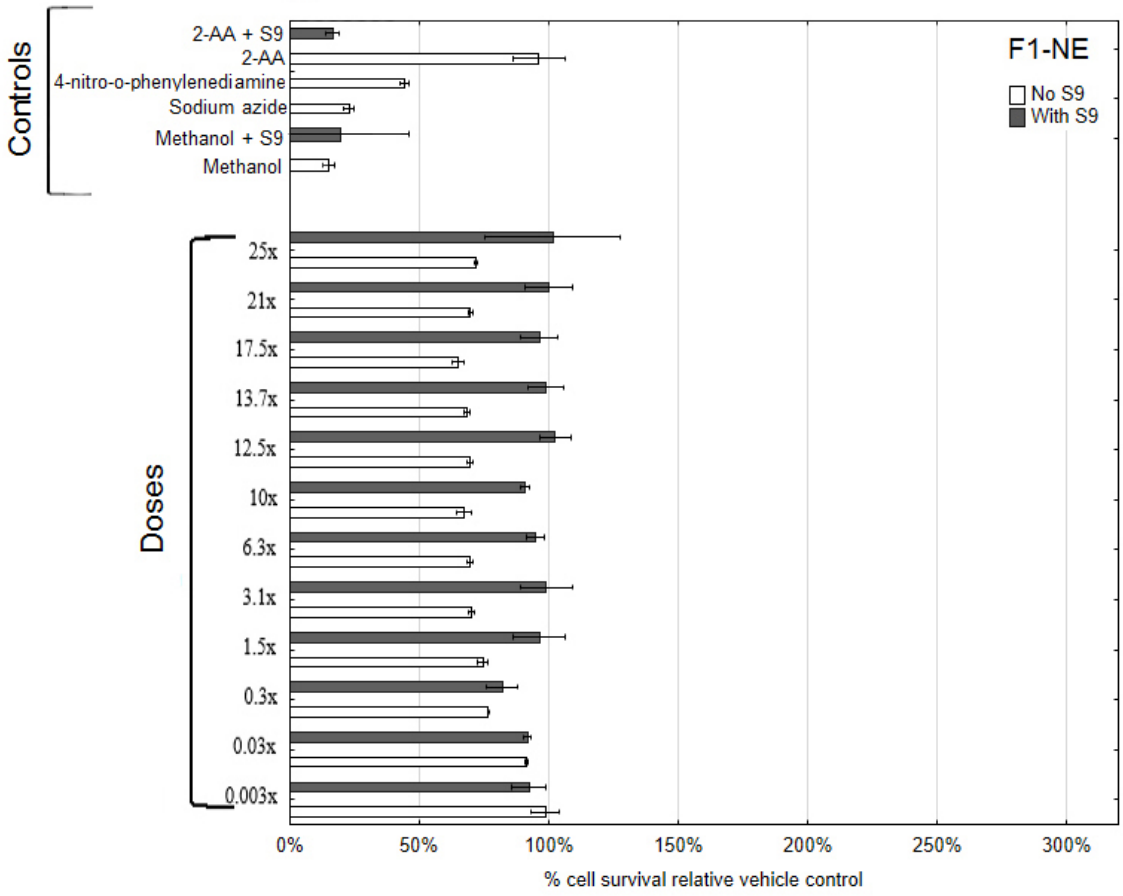


Figure 33: Ames TA100 F1-NE cytotoxicity (average ± SD). Gray represents samples with S9 and white without it. Cytotoxicity is noticed at all concentrations, where the maximum cytotoxicity is 35% at 10×.

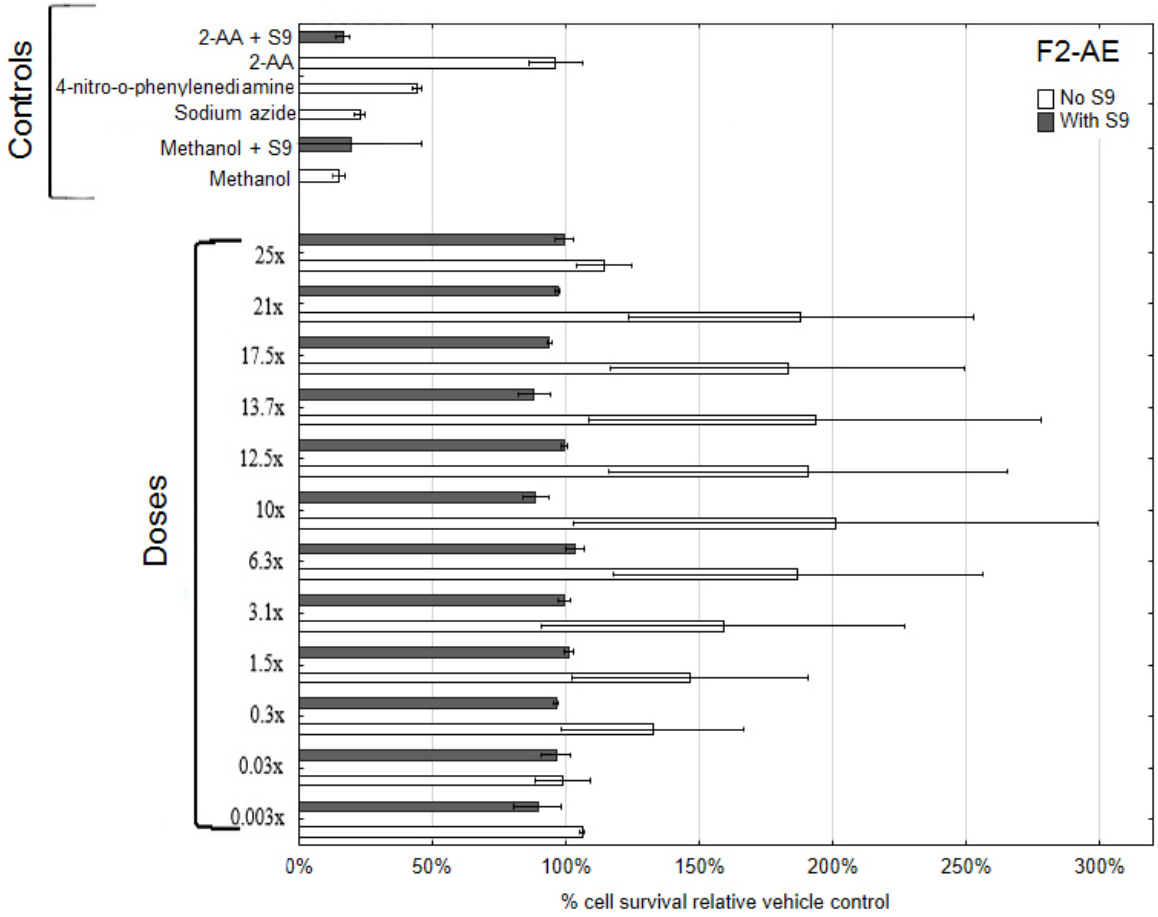


Figure 34: Ames TA98 F2-AE cytotoxicity (average ± SD). No evident cytotoxicity is noticed, but an increased cell growth is noticed when S9 is absent (more than 100% survival if compared to vehicle control) at 0.3× to 25×. It is possible that all chemicals used by the bacteria on whole AEO are present in F2-AE but not F1-NE.

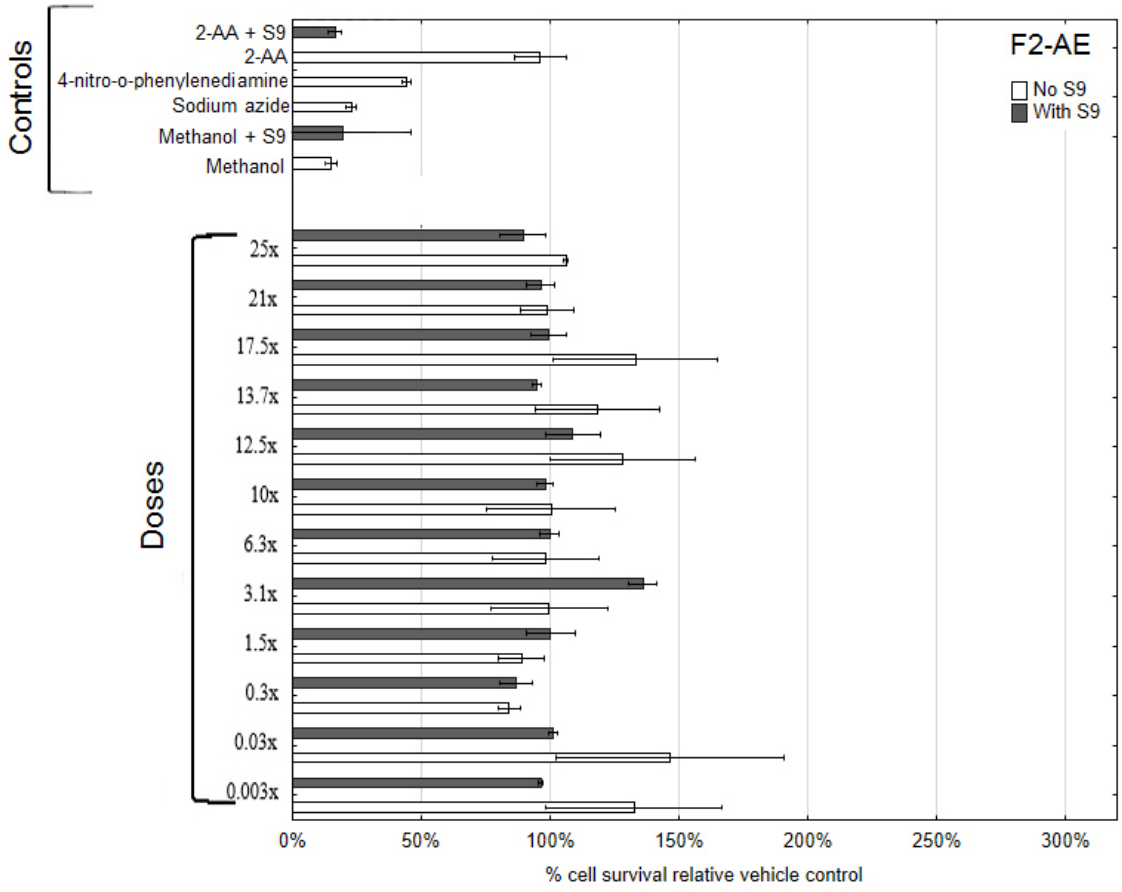


Figure 35: Ames TA100 F2-AE cytotoxicity (average ± SD). No evident cytotoxicity is noticed, but an increased cell growth is noticed when S9 is absent (more than 100% survival if compared to vehicle control) at 0.03×, 0.03×, 12.5×, 13.7× and 17.5×.

CHAPTER 4: CONCLUDING REMARKS

4.1 Environmental significance

Overall, the results from both genotoxicity testing (SOS Chromotest) and mutagenicity testing (Ames) support that OSPW extractable organics may be genotoxic mutagens. Moreover, the threshold for each response in these in vitro tests were similar, with concentrations from 7× to 10× of whole AEO showing a genotoxic response in *E. coli* PQ37, and concentrations from 1.5× to 25× showing mutagenic potential in two Salmonella strains. Although Madill *et al.* (1999) formerly showed genotoxicity for OSPW porewater (49), curiously they reported no mutagenic response in TA98 and 100 strains. We suggest that the concentrations they used may have been too high (1,000× to 10,000×), which likely caused cytotoxicity, resulting in a false negative results.

Although no clear mutagenic response was detected at an environmentally relevant concentration here (i.e. all significant effects occurred above 1× concentrations), the current results do indicate a potential for mutagenicity at environmentally relevant doses. First, because of the short exposure time here (i.e ~12 hours before histidine is depleted), the thresholds might be lower in assays with longer exposure time. Second, it was recently predicted that the bioconcentration factors for organic compounds in OSPW can be as high as 100,000 [38], thus the highest dose tested here (25×) is not necessarily irrelevant, and may reflect the concentration of xenobiotics that could be accumulated in aquatic organisms exposed chronically. Also notable is that significant mutagenicity was observed at 1.5× with S9 in TA98, very close to the environmentally relevant concentration. When testing the whole OSPW from Base Mine Lake (without extracting the organic fraction), no genotoxicity or cytotoxicity was detected in the SOS

Chromo-test (short term exposure, 4 hours), but for Ames the cytotoxicity was 100% (long exposure scenario, 2 days – data not shown) preventing mutagenicity being conclusively tested. This result is to be expected due the high concentration of salts and metals in OSPW [1-5].

Comparing the potency of OSPW to cause mutagenicity with other well-studied mutagenic toxicants is difficult because OSPW is a mixture of several unknown chemicals that may interact. One way to put OSPW mutagenicity into perspective is to compare the mass of extract required to induce 100 C.F.U.s above control in the Ames test, as done by Meselson *et al.* for a range of mutagens and known rodent carcinogens (97), such as benzo[a]pyrene, benzidine and aflatoxin B1). For example, 0.3 μg of benzo[a]pyrene, 0.007 μg of aflatoxin B1, and 10 ug of benzidine are required in the test for each 100 C.F.U.s. For whole AEO from OSPW in the current work, between 288 and 5280 μg of whole extract was required to induce the same response, depending on the presence or absence of S9.

The current study focused on OSPW from the world's first end pit lake, Base Mine Lake. Approximately 30 other end pit lakes are projected as a legacy to the mining operations, and the long-term detoxification of OSPW in these man-made-structures will need to be closely monitored. Taking into account the current in vitro results, as well as the weight of evidence from other studies of OSPW genotoxicity and mutagenicity [22, 23], further testing of the carcinogenic risks of OSPW is warranted.

4.2 Summary

The detection of genotoxicity and mutagenicity of OSPW AEO is very relevant due the increasing growth of the oil industry and the tailing water production, instigating further research

on more relevant *in vivo* models to better elucidate if OSPW should be considered a carcinogenic threat.

4.3 Concluding remarks

OSPW concentrations from 1.5× to 25× can cause genotoxicity and mutagenicity. Due to toxicological interactions, AEO is more genotoxic and mutagenic than other fractions and S9 liver enzymes can decrease genotoxicity and mutagenicity for most fractions, except NE in SOS-Chromo test and TE in Ames TA98 strain. Further studies aiming to investigate other genes or fractions *in vivo* are needed. This is the first study that investigated the OSPW AEO genetic toxicology, and results are only for bacterial models thus caution should be used when extrapolating the data to humans, and the data should be primarily viewed as justification for further testing.

4.4 Future directions

One of the objectives of this study was to find what fraction could be mainly responsible for the genotoxicity. Both subfractions show a similar genotoxic and mutagenic response, but the neutral fraction, by operational definition has more hydrophobic chemicals, meaning that these might accumulate in organisms to a greater extent. The aim of this future investigation is to show what specific class of chemicals might be causing F1-NE toxic effects, especially because NAs (historically the compound responsible for OSPW toxicity) can be found in this fraction.

Currently, the characterization of these new F1-NE fractionations is being made (31,87,88), but testing *in vitro* or *in vivo* is still warranted.

The assays used in this research are well accepted screens used in the first steps to detect the early stages of carcinogenesis via genotoxicity, but not provide information on how the damage occurs or how the genes behave towards the damage. Although very reliable and correlated with carcinogenesis, both assays are screens and prone to false positives. Only 2 studies tried to investigate gene expression to OSPW in fish (41,98), and similar models focused on human relevant species are needed.

Since the use of animals requires ethics approval, requiring constant care and extensive funding, the use of human cells could provide more insight into how humans would react acutely and chronically if exposed to OSPW. Nevertheless, the cells can be used to investigate the genetic response via microarray or real time PCR, which can be interlinked with pathways analysis. The combination of the genetic data with a pathway analysis software could provide information if OSPW can activate/mutate proto-oncogenes or tumor suppressor genes.

Even though Rogers *et al.* (46) described OSPW exposure effects in Wistar rats, carcinogenic data is missing and research done using similar models, or even the 2-year rodent assay, could identify if OSPW exposure can pose a threat as human carcinogens.

The bitumen extraction is important for Canadian economy and growth, but its environmental and health effects are also cause for concern, and a better elucidation of how the extraction by-products, such as OSPW, can pose a threat to life is needed.

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