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Mutagenicity and Dioxin-like Activity of Biodiesel Emissions

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Mutagenicity and Dioxin-Like Activity of Biodiesel Emissions

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Thesis submitted to the
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Abstract

Diesel emissions have been shown to elicit a variety of toxicological effects, and alternative fuels (i.e., biodiesel) are currently being assessed to determine their ability to reduce the risks of adverse health effects. Exhaust emissions were generated using ULSD and biodiesel blended fuels and extracts of diesel PM (i.e., filters and PUFs) were separated into polar aromatic and non-polar neutral compounds. Mutagenic activity was assessed using the Salmonella mutagenicity assay, and Ah-receptor agonism was assessed using the DR-CALUX assay. Results indicate that organic extracts of diesel/biodiesel particles contain direct- and indirect-acting polar aromatic mutagens as well as polar and non-polar Ah-receptor agonists. The mutagenicity of direct-acting compounds decreases with increasing concentrations of biodiesel in the fuel; however, there is no change in the indirect-acting mutagenicity. Furthermore, the ability of polar and non-polar compounds to induce the Ah-receptor increases with increasing concentrations of biodiesel in the fuel. These results provide an initial framework for evaluating the toxicological hazards of biodiesel emissions.

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List of Abbreviations

µg	microgram(s)
µl	microlitre(s)
µm	micrometre(s)
Ah-R	aryl-hydrocarbon receptor
Amp	Ampicillin
ARNT	aryl-hydrocarbon receptor nuclear translocator
ASE	accelerated solvent extraction
bhp	brake horse-power
BXX	biodiesel blend where XX is the percent content (v/v) of biodiesel
CEPA	Canadian Environmental Protection Act
cm	centimetre
cnr	classic nitroreductase
CO	carbon monoxide
C_x	hydrocarbon with x number of carbons
CYP/P450	cytochrome P450 enzymes
DCM	dicholoromethane
DMSO	dimethylsufoxide
DNA	deoxyribonucleic acid
DOC	diesel oxidation catalyst
DPS	diesel particulate sampling
DR-CALUX	dioxin-responsive chemically activated luciferase expression
DRE	dioxin-response element
EC	Environment Canada
EGR	exhaust gas recirculation
ERMD	Environmental Research and Measurement Division
EROD	ethoxyresorufin-O-deethylase
FTP	Federal testing procedure
g	gram
GVWR	gross vehicle weight rating

HC	hydrocarbon
HCCI	homogeneous charge compression ignition
HCHO	formaldehyde
HSP90	heat shock protein 90 kD
IARC	International Agency for Research on Cancer
kD	kilodalton
kg	kilogram
km	kilometre
L	litre
LAFY	Los-Angeles freeway
LANF	Los-Angeles non-freeway
lb	pound
LSD	low sulphur diesel
M	molar
mg	milligram
ml	millilitre
mM	millimolar
mm	millimetre
MMVT	mouse mammary viral tumour
Mpa	megapascal
NA	not applicable
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NIOSH	National Institute for Occupational Safety and Health
NIST	National Institute of Standards and Technology
NM	not measured
nm	nanometre
NMHC	non-methane hydrocarbons
NO_x	oxides of nitrogen
NRC	National Research Council of Canada
NTP	National Toxicology Program
NYNF	New-York non-freeway

OAT	O-acetyltransferase
°C	degrees Celcius
PAC(s)	polycyclic aromatic compound(s)
PAH(s)	polycyclic aromatic hydrocarbon(s)
PCB(s)	polychlorinated biphenyl(s)
PM	particulate material
ppm	parts per million
PUF(s)	polyurethane foam plug(s)
rfa	deep rough mutation
RLU	relative light units
SCR	selective catalytic reduction
SOF	soluble organic fraction
Tet	tetracycline
ULSD	ultra low sulphur diesel
US	United States
US EPA	United States Environmental Protection Agency
uv	ultra-violet
v/v	volume/volume
W	Watt
w/v	weight/volume
XAP2	immunophilin-like protein hepatitis B virus X-associated protein 2

Chapter 1: Introduction

The diesel engine was invented by Rudolph Diesel in the late 1800s. This internal combustion engine, which is operated with diesel fuel, was the most efficient engine in its time and still is today. Because of its ability to convert energy more efficiently than other engines, the diesel engine is responsible for moving a large portion of the world's goods via trains, commercial vehicles and marine vessels [1]. However, diesel is one the largest contributors to airborne environmental pollution, and is known to be responsible for adverse health effects in humans [2-8]. A vast array of research has shown that diesel emissions contribute to the development of cancer in animal studies [9], to cardiovascular and respiratory heath effects in humans [8,10], and to the pollution of air, water and soil [11,12].

Organizations such as the US Environmental Protection Agency (US EPA), the European Commission, Environment Canada (EC) and Transport Canada are responsible for regulating diesel emissions in the USA, Europe and Canada, respectively. Emissions of criteria air pollutants including carbon monoxide (CO), particulate material (PM), hydrocarbons and nitrogen oxides (NO_x) are all regulated by these agencies in order to safeguard human health and reduce pollution. In order to meet stringent guidelines, it has been necessary to implement changes in fuel composition (i.e., ultra-low sulphur diesel (ULSD), biodiesel, etc.), engine design (e.g., homogeneous charge compressions ignition (HCCI)) and after-treatment technologies (i.e., particle traps, oxidation catalysts, exhaust gas recirculation (EGR)), all of which have resulted in lower emissions of regulated substances [13-16]. The continued implementation of these regulations and the development of new technologies (i.e., fuel, engine design, and after-treatment) will allow for greater reductions of air pollution and the risk of adverse health effects in humans, while permitting the use of the more efficient diesel engine.

1.1 Diesel fuel

1.1.1 Origin and use

Petroleum is a naturally occurring non-renewable resource buried deep in the Earth's crust. This black liquid consists of a mixture of hydrocarbons of various chain lengths (i.e., C_5 to C_{18}) [17]. It also contains other elements such as oxygen, sulphur and nitrogen. Petroleum is separated by distillation into a variety of hydrocarbon fractions [18]. These distillates are used as fuels and starting materials for a variety of industrial purposes. Table 1.1 illustrates the various fractions obtained by petroleum distillation.

Table 1.1 Fractions of petroleum distillates with their approximate boiling temperatures and approximate number of carbon atoms per molecule.

Crude Oil Refining		
Distillate Fraction	**Boiling Temperature (°C)**	**Carbon Atoms per Molecule**
Gases	Below 30	1-4
Gasoline	30-210	5-12
Kerosene	150-250	11-13
Diesel	160-400	13-17
Heavy-oil Fuel	315-540	20-45

Diesel fuel is a middle distillate of petroleum, and separates at approximately 300 °C [17]. It is used in conventional diesel internal combustion engines as a power source. Diesel engines work by compressing air at high pressure and temperature to ignite the fuel-air mixture. They have the ability to compress air at a ratio (i.e., volume of air before compression compared to the volume of air after compression) between 15:1 and 20:1, whereas gasoline engines compress the air at a ratio of approximately 10:1 [19]. Because diesel fuel has higher energy content per litre, it is optimal for powering heavy-duty vehicles [19].

1.1.2 Physical-chemical composition

Diesel fuel is a viscous liquid, usually dark brown in color. It is primarily composed of saturated alkanes, but also contains dozens of other hydrocarbon compounds [20]. The chemical composition of diesel fuel greatly depends on the location from which the petroleum was mined. Aside from alkanes, diesel contains alkenes, aromatic compounds, and sulphur. Diesel fuel also contains criteria pollutants highlighted under the Canadian Environmental Protection Act (CEPA), including polycyclic aromatic hydrocarbons (PAHs), toluene, benzene, ethyl-benzene and xylene [17,21]. Although concentrations of these chemicals are usually low, their presence in diesel fuel is closely monitored.

1.1.3 Alternative fuels

In order to meet emerging diesel emission regulations, new fuels are being marketed as alternatives to standard diesel fuels, and others are being explored as possible future alternatives. Fuels such as ULSD, biodiesel, and synthetic diesel (e.g., gas-to-liquid or GTL) are currently being explored as alternatives. Although some of these alternatives have been shown to reduce the volume/concentration of regulated emissions [13,14,16], little is known regarding their effect on the composition of the soluble organic fraction (SOF) associated with diesel emission particles.

1.1.3.1 Low sulphur diesel and ultra-low sulphur diesel

The terms low sulphur diesel and ultra-low sulphur diesel refer to the concentration of sulphur (mg/kg) in diesel fuels. Sulphur is a naturally occurring element found in petroleum and can only be removed by a chemical process called hydrodesulphurization [22,23]. This simple process, carried out at high temperature and elevated pressure, involves a hydrogenation process whereby a sulphur atom is replaced by a hydrogen atom. This results in the formation of a sulphur-free fatty acid and hydrogen sulphide.

Many fuel properties can impact the toxicity of diesel emissions (e.g., cetane index, aromatic content), and sulphur content has been shown to positively correlate with the emission rate of both PM and sulphur dioxide [17]. Consequently, sulphur content in fuel is strictly regulated in North America. As of June 1, 2006, low sulphur diesel (i.e., 500 ppm) sold for on-road vehicles is being phased out in North America, and is being replaced by ultra-low sulphur diesel (i.e., 15ppm) that will be mandatory by 2010 [24]. ULSD is cleaner-burning, producing less particulate emissions in both older and new engines. The use of this fuel will also allow the use of improved exhaust treatment devices to reduce emissions of particulates and smog-forming nitrogen oxides (NO_x) The effectiveness of these devices can be adversely impacted by the sulphur in conventional diesel fuel [17].

1.1.3.2 Biodiesel and biodiesel blends

Biodiesel is a relatively clean burning, non-toxic, renewable fuel prepared from vegetable oils, animal fats, or waste cooking oils via a process known as transesterification [25]. Figure1.1 illustrates the chemical reaction resulting in the formation of biodiesel. Essentially, the oil reacts with an alcohol (e.g., methanol or ethanol) in the presence of a catalyst (e.g., sodium hydroxide) to produce an alkylester (i.e., the biodiesel) and glycerol [25]. Biodiesel itself contains no petroleum products, but can be blended with diesel fuel (e.g., ULSD) at any concentration to produce a biodiesel blend. Blends up to 20% have been shown to be compatible with existing diesel engines, often with little or no modifications. Compared to conventional diesel, biodiesel has a high combustion efficiency, presumably resulting from the higher cetane rating, and produces fewer regulated emissions (i.e., CO, PM, HC, NO_x) [26]. The use of biodiesel and biodiesel blends is thought to be safer for the environment and economically beneficial since it supports local farmers, and reduces reliance on fossil fuel imports.

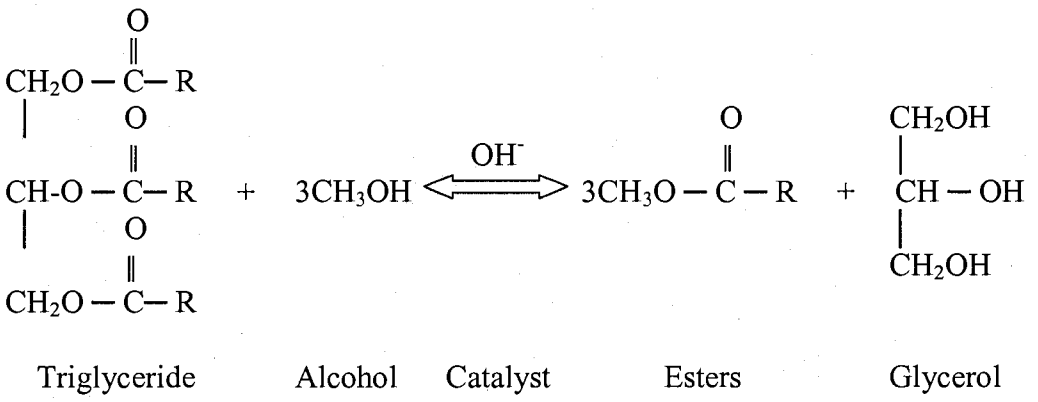


Figure 1.1 Illustration of the chemical reaction resulting in the formation of biodiesel (methyl-ester) and glycerol. In the presence of a catalyst, the triglyceride reacts with an alcohol (e.g., methanol or ethanol) to produce a methyl ester and glycerol. The resulting methyl-esters are termed biodiesel and the glycerol is used for other comestible products such as soap.

Biodiesel is currently produced in North America and around the world. According to Alberta Agriculture and Food (agric.gov.ab.cal), the European Union is the world's largest producer of biodiesel with countries like Germany, France, and Italy responsible for approximately 70% of production. There are currently three biodiesel production facilities in Canada and one under construction. Milligan BioTech (Foam Lake, SK), BIOX (Hamilton, ON) and Rothsay (Montreal, QC) have the capacity to annually produce biodiesel fuel from 1 million litres of canola oil, 66 million litres of tallow, and 30 million litres of animal fats/yellow grease, respectively [27]. A new facility currently under construction in Alberta is projected to have the capacity to process 225 million litres of canola. Canada has established a biodiesel production target for 2010 of 500 million litres. According to Canada Clean Fuels (www.canadacleanfuels.com), biodiesel production in the US has risen from 500 000 gallons (approximately 1.9 million litres) in 1999 to 25 million gallons (approximately 94 million litres) in 2004, roughly a 5000 % increase. US citizens are given incentives to

purchase biodiesel blended fuels in order to promote the cleaner burning fuels. Pure biodiesel (i.e., 100% biodiesel) in the US is regulated and must meet ASTM-D 6751 standards and be registered with the US EPA. Unfortunately, biodiesel is not currently regulated in Canada. Consequently, there are outstanding quality control and quality assurance issues.

1.2 Diesel emissions

Diesel exhaust refers to any substance, gas or particulate, released from the exhaust pipe or chimney of a machine/vehicle using an internal combustion engine operating on diesel fuel. Diesel emissions are the result of both complete and incomplete combustion of the diesel fuel. Diesel exhaust is a complex mixture of organic and inorganic compounds found in both solid and gaseous phases. It includes over 40 cancer-causing chemicals, and various organizations have classified diesel emissions as a potential carcinogen. In 1988, the US National Institute for Occupational Safety and Health (NIOSH) classified diesel exhaust a potential occupational carcinogen, whereas in 1989 IARC (International Agency for Research on Cancer) classified it as 2A, "probable human carcinogen"[28]. In 2000, the US National Toxicology Program (NTP) listed diesel exhaust particles as "reasonably anticipated to be a human carcinogen", and in 2002, the US EPA considered diesel emissions "likely to be carcinogenic to humans" [29].

1.2.1 Gaseous phase

The gaseous phase of diesel emissions is primarily composed of carbon dioxide, oxygen, nitrogen and water vapour [17]. However, hydrocarbons, carbon monoxide, aldehydes, nitrogen oxides, and sulphur dioxide are also present in small quantities. Hydrocarbons, carbon monoxide, and aldehydes are produced from the incomplete

combustion of the diesel fuel. Hydrocarbons in the gaseous phase can include chemicals such as formaldehyde, benzene, toluene, PAHs, and dioxins [17]. Nitrogen oxides are generated from nitrogen and oxygen at the high pressure and temperature conditions in the combustion chamber. Nitrogen oxides are mainly composed of nitric oxide, but also contain some nitrogen dioxide, a highly toxic compound [30]. Sulphur dioxide is generated from the sulphur present in the fuel, and its emissions are highly dependent on the sulphur concentration in the fuel.

1.2.2 Particulate phase

Diesel particles are a complex mixture of both solid and liquid phase compounds. They consist primarily of solid carbon spheres known as soot or black carbon, to which organic compounds are adsorbed. The volatile component of diesel particles consist of sulphates appearing as hydrated sulphuric acid [17]. Primary diesel particles range in size from 0.01 to 0.08 μm, but these can agglomerate to form particles of up to 1 μm in diameter [29]. The SOF consists of organic material, primarily polycyclic aromatic compounds (PACs), adsorbed to the carbon spheres. The organic content of diesel particulates can range from 10-64% for light duty cars, and 14-90% for heavy-duty vehicles [31]. Figure 1.2 illustrates an agglomerated diesel emission particle.

Although the gaseous phase of diesel emissions contributes to environmental pollution and adverse effects in humans, it is generally accepted that the carcinogenic properties of diesel emissions can be attributed to the particulate phase [9]. Indeed, many of the PACs adsorbed onto the surface of diesel emission particles are classified by IARC, the US EPA, and under CEPA, as potential human carcinogens [9,21,28]. However, the organic composition and percent extractable mass of diesel emission particles are not regulated.

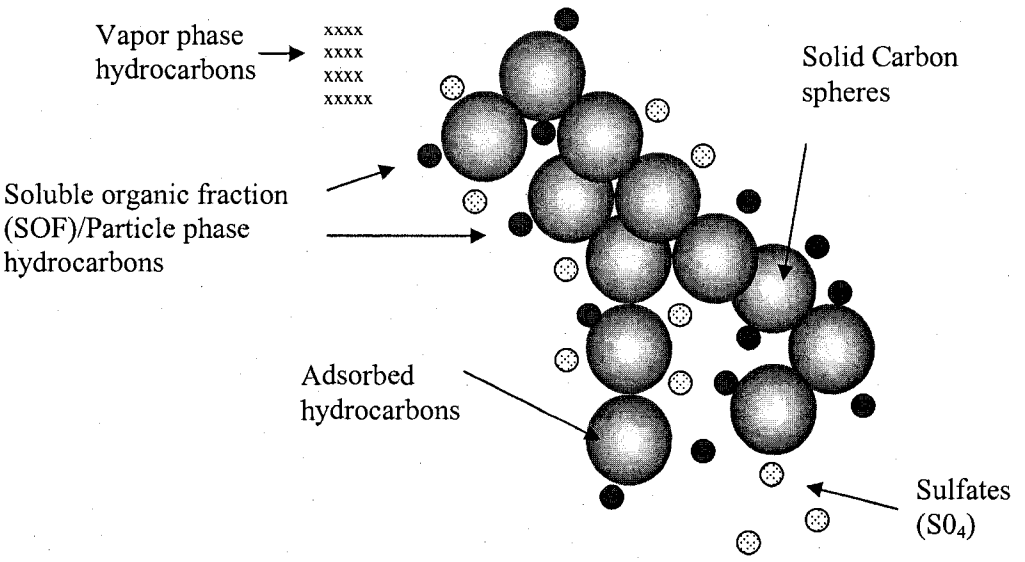


Figure 1.2 Illustration of an agglomerated diesel emission particle. Primary diesel particles consist of solid carbon spheres to which hydrocarbons are adsorbed. Primary diesel particles range in size from 0.01 to 0.08 μm, but can agglomerate to form particles of up to 1 μm. Modified from Johnson et al [32].

1.2.3 Standard Reference materials

Standard reference materials (SRMs) of diesel particulates can be obtained from the US National Institute of Standards and Technology (NIST). Diesel particulate SRMs are materials (e.g., PM) collected under several engine/fuel combinations, and can be purchased in various pre-weighed amounts. Many SRMs are available, but two were selected for the purposes of this study: SRM 1650b and SRM 2975.

1.2.3.1 SRM 1650b

NIST SRM 1650b was prepared by collecting the particulate material from a dilution tunnel receiving emissions from a heavy-duty four-cycle diesel engine operating under a variety of engine conditions (200 hours of accumulation). The material is not intended to

represent diesel emissions from a particular engine operating on a particular cycle, rather, it is intended to represent heavy-duty emissions in general [33]. The concentration of various PAHs and nitro-PAHs in SRM 1650b were measured and are listed in Table 1.2. The percent extractable mass of these particles (i.e., SOF), as measured by Soxhlet extraction with dichloromethane (DCM), is approximately 20.2% ± 0.4% (mass fraction). The specific surface area is 108 m^2/g, and the mean particle diameter is 0.18 μm (volume distribution).

1.2.3.2 SRM 2975

NIST SRM 2975 was originally obtained from M.E. Wright of the Donaldson Company, Inc., Minneapolis, MN. Essentially, particulate material was collected from a filtering system designed specifically for diesel-powered forklifts [34]. The concentrations of various PAHs and nitro-PAHs have been measured and are listed in Table 1.2. The percent extractable mass of these particles, as measured by Soxhlet extraction in DCM, is approximately 2.7% ± 0.2% (mass fraction). The calculated specific surface area, assuming solid spherical particles, is 0.538 m^2/g, and the mean particle diameter is 31.9 μm (volume distribution).

Table 1.2 Certified concentrations of selected PAHs and nitro-PAHs in SRM 1650b and SRM 2975. Priority PAHs are marked with an asterisk. For more information regarding methods, see the Certificates of Analysis available at: http://ts.nist.gov/MeasurementServices/ReferenceMaterials/232.cfm.

	Mass Fraction (mg/kg)	
PAH, nitro-PAH	**SRM 1650b**	**SRM 2975**
Napthalene*	5.07 ± 0.43	17.0 ± 2.8
1-Methylnaphthalene	1.51 ± 0.12	NM
2-Methylnaphthalene	3.05 ± 0.56	NM
Phenanthrene*	69.5 ± 1.9	NM
Anthracene*	7.67 ± 0.47	NM
1-Methylphenanthrene	28.3 ± 1.5	NM
2-Methylphenanthrene	70.7 ± 2.7	NM
3-Methylphenanthrene	55.1 ± 1.9	NM
9-Methylphenanthrene	35.1 ± 1.9	NM
Fluoranthene*	47.3 ± 0.8	26.6 ± 5.1
Pyrene*	43.4 ± 1.6	0.90 ± 0.24
Benzo[*ghi*]fluoranthene	10.8± 1.0	NM
Benzo[*c*]phenanthrene	2.51 ± 0.29	NM
Benz[*a*]anthracene*	6.18 ± 0.30	0.317 ± 0.066
Chrysene*	13.3 ± 1.1	4.56 ± 0.16
Triphenylene	9.17 ± 0.94	5.22 ± 0.20
Benzo[*a*]fluoranthene	0.370 ± 0.029	NM
Benzo[*b*]fluoranthene*	6.77 ± 0.84	NM
Benzo[*j*]fluoranthene	3.24 ± 0.42	0.82 ± 0.11
Benzo[*k*]fluoranthene*	2.37 ± 0.21	0.678 ±0.076
Benzo[*a*]pyrene*	1.17 ± 0.09	0.0522 ± 0.0053
Benzo[*e*]pyrene	6.30 ± 0.50	1.11 ± 0.10
Perylene	0.165 ± 0.032	NM
Benzo[*ghi*]perylene*	5.91 ± 0.18	0.498 ± 0.044
Indeno[*1,2,3-cd*]pyrene*	4.44 ± 0.28	NM
Dibenz[*a,c*]anthracene	0.438 ± 0.043	NM
Dibenz[*a,h*]anthracene*	0.365 ± 0.071	NM
Dibenz[*a,j*]anthracene	0.387 ± 0.051	NM
Benzo[*b*]chrysene	0.290 ± 0.020	NM
Picene	0.499 ± 0.061	NM
1-Nitropyrene	18.2 ± 0.20	36[a]
9-Nitroanthracene	5.89 ± 0.31	NM
7-Nitrobenz[*a*]anthracene	0.967 ± 0.042	NM
6-Nitrochrysene	0.0455 ± 0.0019	NM
6-Nitrobenzo[*a*]pyrene	1.39 ± 0.1	NM
1,6 Dinitropyrene	0.084 ± 0.003	NM

NM=not measured

[a] The concentration for 1-nitropyrene is a concentration of all nitro-substituted PAHs in the sample. See certificate of analysis for further details [33,34].

1.2.4 Emission standards

On-road diesel emissions in Canada are regulated by Environment Canada and Transport Canada under CEPA 1999. Tailpipe emissions are regulated based on two factors: vehicle weight and vehicle model year. Newer vehicles have more stringent regulations, whereas older vehicles are less restricted. Although heavier vehicles (i.e., heavy-duty trucks) generally produce more emissions per distance travelled than lighter vehicles (i.e., light-duty cars), they are subject to less stringent regulations and/or extended periods of time to meet emerging regulations. Table 1.3 summarizes the various vehicle weight ratings, and Table 1.4 summarizes the levels of regulated emissions for heavy-duty diesel trucks. Emissions regulated by Environment Canada and Transport Canada include: non-methane hydrocarbons (NMHC), CO, NO_x, PM and formaldehyde (HCHO).

Table 1.3 Class of vehicle by weight rating as determined by Environment Canada and Transport Canada. The heavy-duty vehicle/heavy-duty engine is the focus of this study. Table adapted from: http://www.dieselnet.com/standards/ca/#hdv.

Class	**Gross Vehicle Weight Rating (kg (lb))**
Motorcycle	≤793 (1749)
Light-Duty Vehicle	≤3856 (8500)
Light-Duty Truck -light light-duty truck -heavy light-duty truck	≤3856 (8500) ≤2722 (6000) >2722 to 3856 (6000 to 8500)
Medium-Duty Passenger Vehicle	3856 to<4536 (8500 to 10000)
Complete Heavy-Duty Vehicle (Otto cycle only)	3856 to 6350 (8500 to 14000)
Heavy-Duty Vehicle/Heavy-Duty Engine -light heavy-duty engine -medium heavy-duty engine -heavy heavy-duty engine	>3856 (8500) <8847 (19500) 8847 to 14971 (19500 to 33000) >14971 (33000)

Table 1.4 Heavy-duty engine emission standards (g/bhp-hr) for Canada. Note that phase 2 standards will be phased in from 2008, with 100% of vehicles meeting standards by 2010. There will be no emission averaging options for heavy-duty vehicles. Table adapted from: http://www.dieselnet.com/standards/ca.

Year	GVWR kg (lb)	NOx	NMHC	NOx + NMHC	CO	PM
Pre-2005	≤6350 (14000)	4.0	1.1	-	14.4	-
	>6350 (14000)	4.0	1.9	-	37.1	-
Phase 1 (2005)	≤6350 (14000)	-	-	1.0	14.4	-
	>6350 (14000)	-	-	1.0	37.1	-
Phase 2 (2008-2010)	≤3856 (8500)	0.2	0.14	-	14.4	0.01

g/bhp-hr = grams/brake horsepower-hour; GVWR = Gross Vehicle Weight Rating; NOx = Oxides of nitrogen, NMHC = non-methane hydrocarbons, CO = carbon monoxide, PM = particulate matter.

1.2.5 Control technologies

Diesel exhaust emissions can be controlled by modifying the engine design and/or using after-treatment technologies. The use of conventional after-treatment technologies such as catalytic converters and particle traps have helped to reduce emissions of regulated substances [16,35]. However, due to more stringent regulations, new technologies are being developed and are currently emerging. Devices employing selective catalytic reduction (SCR) and exhaust gas recirculation (EGR) are being investigated for their ability to further reduce regulated emissions [36,37]. Exhaust gas recirculation, which is used to reduce the emissions of NO_x, involves circulating exhaust air back into the combustion chamber. Cool or uncooled exhaust gas is mixed with new air for a second combustion cycle, reducing the combustion temperature and therefore reducing the amount of excess oxygen. Research indicates that using exhaust gas recirculation reduces emissions of NO_x, however, it increases the emissions of particulate material [14]. Nonetheless, it is possible to use

multiple after-treatment devices such as an EGR system coupled with a particle trap to reduce several components of the emissions.

1.2.6 Adverse health effects

The toxic substances contained in diesel emissions enter the body primarily through inhalation of vehicle exhaust. Upon breathing, the gases and particles of diesel exhaust are drawn into the lungs and have the potential to reach deep into the alveolar regions [38]. Diesel exhaust, which is a complex mixture of hundreds of chemical compounds (i.e., CEPA criteria air pollutants such as benzene, formaldehyde, arsenic, PAHs, etc.), can induce the formation of DNA mutations that have the potential to initiate the formation of neoplastic lesions (i.e., cancer) [39,40]. In 2002, the US EPA released a health assessment document for Diesel Engine Exhaust and concluded that "long-term inhalation exposure is likely to pose a lung cancer hazard to humans as inferred from epidemiologic and certain animal studies"[12].

To a lesser extent, exposure to diesel exhaust can have immediate non-life threatening health effects. Other than a displeasing odour, diesel exhaust can cause eye, nose, throat, and lung irritations as well as cause coughs and nausea [13]. Exposure to diesel exhaust can also cause inflammation in the lungs and increase the severity of asthma attacks in the elderly and in young children who are especially sensitive to fine particle air pollution [41]. Research has shown that particle concentrations in the air are directly related to increased hospital admissions, emergency room visits and premature deaths among those who are more susceptible to the effects of fine particles [42]. In a recent study conducted by Health Canada, it was estimated that just under 6000 Canadians die prematurely every year due to the adverse health effects of air pollution [43].

1.3 PACs, PAHs, and nitro-PAHs

PACs are a family of environmental pollutants including PAHs, N-, O- or S-heterocyclics, oxy-PAHs, nitroarenes, and aromatic amines. These chemicals are organic compounds comprised of two or more fused aromatic rings [21]. PACs are formed mainly as a result of pyrolytic processes, especially the incomplete combustion of organic materials during industrial and other human activities, including the processing and combustion of coal and crude oil, combustion of natural gas, and operation of internal combustion vehicles (both off- and on-road) [21]. PAHs contain only hydrogen and carbon, whereas nitro-PAHs posses one or more nitro groups. Heterocyclic amines contain an aromatic amino group.

PACs are organic compounds that have a high potential for adsorption to particulate matter such as dust and soil, and they can be transported by wind over long distances [44]. PAHs can be found in air, drinking water, food and soil, and a variety of studies have shown that PAH exposure is related to a variety of adverse effects (e.g., immunotoxicity, genotoxicity, carcinogenicity, and reproductive toxicity affecting both male and female offspring) [21]. The US EPA has identified 16 unsubstituted PAHs as priority pollutants, some of which are considered to be probable human carcinogens [45]. Figure 1.3 illustrates the chemical structure of the 16 PAHs identified as priority pollutants. Furthermore, certain nitro-substituted PAHs such as 1-nitropyrene are classified as possible human carcinogens, and thus are also problematic. Research indicates that 1-nitropyrene induces DNA damage and sister chromatid exchange in rodents, DNA damage, mutations and transformation in cultured human cells, and DNA damage, sister chromatid exchange, chromosomal aberrations, mutation and transformation in cultured animal cells [9]. Furthermore, the evidence available to date largely attributes the carcinogenic properties of diesel emissions to

nitro-PAHs [17]. Thus, monitoring the abundance of both PAHs and nitro-PAHs in diesel emissions is crucial for human health risk assessment.

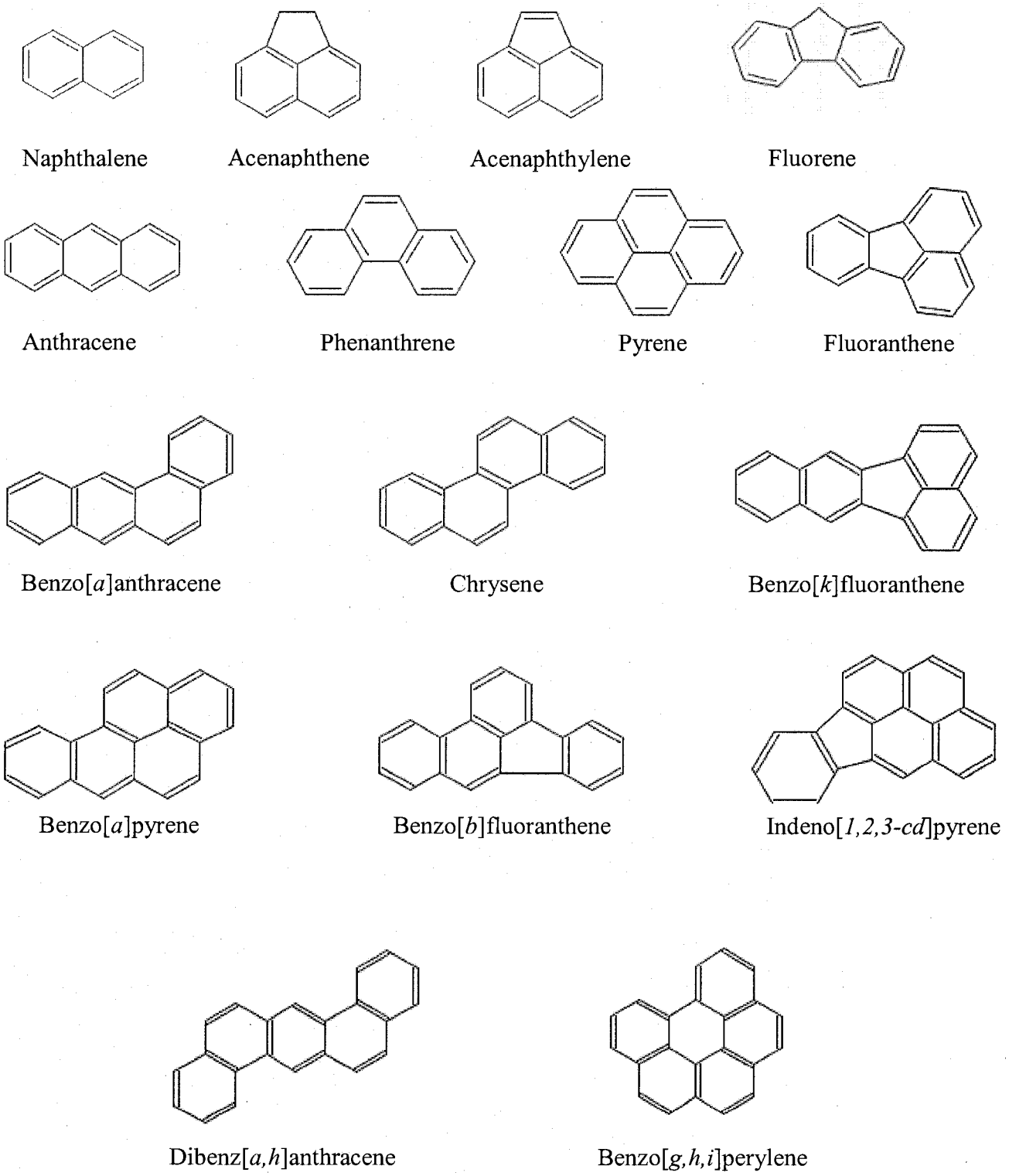


Figure 1.3 Chemical structures of 16 PAHs often referred to as priority PAHs

1.4 Metabolism and toxicity of PACs

Polycyclic aromatic compounds can be classified as either direct- or indirect-acting mutagens. Parent compounds with the ability to induce gene mutations without being metabolically transformed are generally referred to as *direct-acting*. However, PACs, such as PAHs, that cannot react with DNA to form adducts until they are metabolically transformed to reactive species are often referred to as *indirect-acting* or *promutagenic*. Metabolism of PAHs to reactive species requires oxidation by cytochrome P450 isozymes to produce epoxides. Figure 1.4 illustrates the metabolism of benzo[*a*]pyrene, a representative PAH, into a reactive metabolite and the DNA (i.e., deoxyguanosine) adduct (+)trans-*anti*-10-(deoxyguanosin-N2-yl)-7-8-9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. During the oxidative metabolism by P450 isozymes (e.g., CYP1A1, CYP1A2), PAHs are transformed into epoxides and phenols, and the primary metabolites are then transformed into secondary metabolites by various enzymes [46]. For example, epoxide hydrolase catalyzes the formation of dihydrodiols from ring epoxides, and dihydrodiols can be further metabolised by P450 isozymes to form dihydrodiol esters, which can covalently bind to DNA bases to form adducts [47]. However, reactive metabolites can also be conjugated to glutathione, glucuronic acid or sulphate during Phase II metabolism, and excreted from the body through the bile [46].

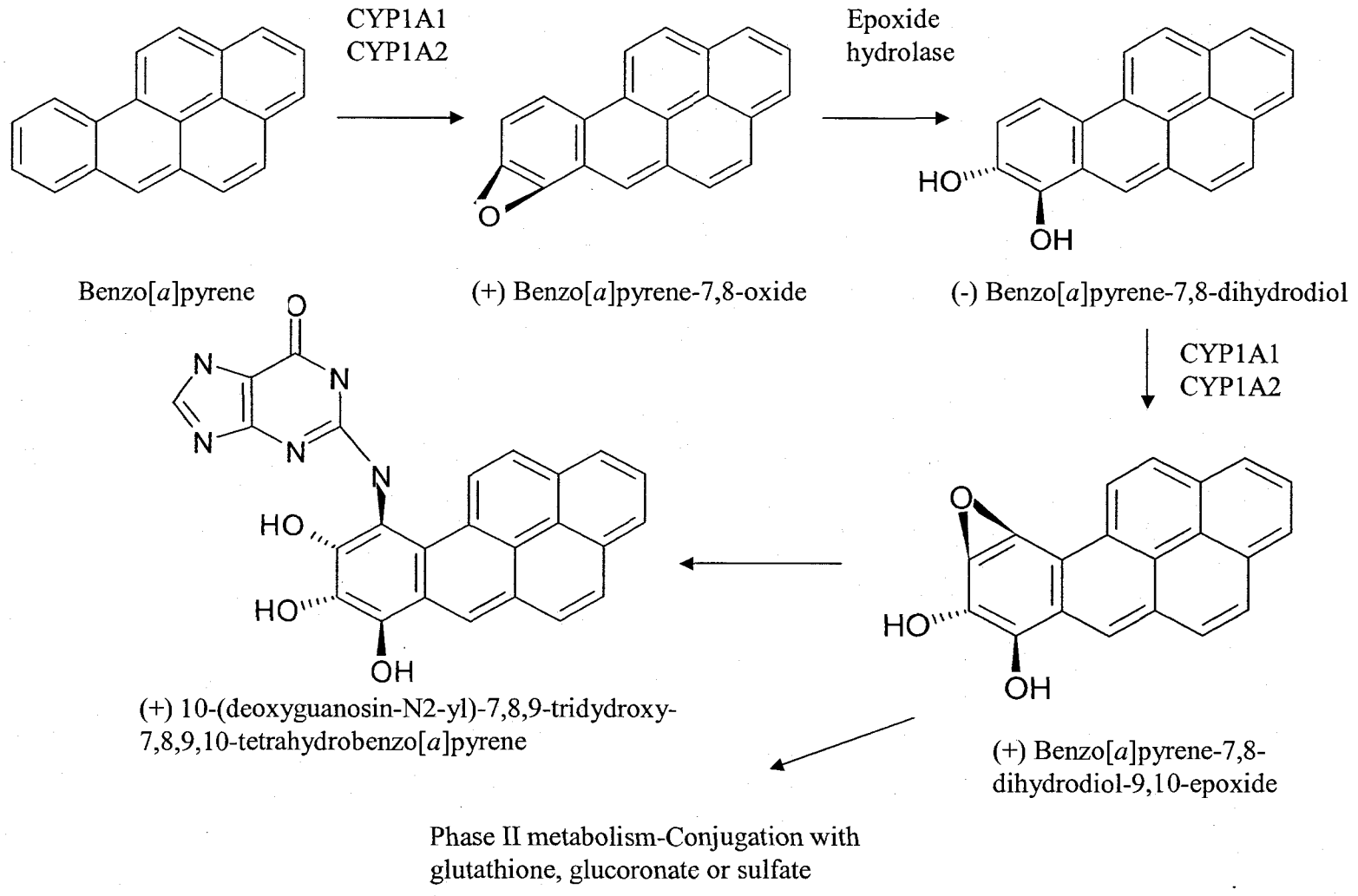


Figure 1.4 Metabolism of benzo[*a*]pyrene into an active metabolite capable of forming the DNA adduct (+) 10-(deoxyguanosin-N2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. In Salmonella, oxidation of PAHs into reactive metabolites is facilitated by the addition of S9 (an exogenous metabolic activation mixture containing a post-mitochondrial supernatant from rat liver).

Nitro-PAHs exert a mutagenic effect in a similar way as PAHs. They can undergo ring oxidation through the same process as PAHs; however, they can also exert a mutagenic effect via reduction of the nitro moiety [17]. Although the metabolism of nitro-PAHs is more complex than that of PAHs, it is thought that there are five pathways through which DNA adducts can be formed: ring oxidation, ring oxidation and nitroreduction, ring oxidation followed by nitroreduction and subsequent esterification, nitroreduction, and nitroreduction followed by esterification [17]. Amino-PAHs are also metabolized in a similar way; however, the amino moiety must first be oxidized by P450 enzymes. Figure 1.5 illustrates the metabolism of 1-nitropyrene to produce the 1-nitropyrene-C^8-deoxyguanine adduct. Briefly, nitroreduction enzymes (e.g., NAD(P)H:quinone oxidoreductase, aldehyde oxidase, etc.) reduce the nitro-PAH to a nitrosoarene that is subsequently reduced to an arylhydroxylamine. Esterification of the arylhydroxylamine by N-acetyltransferases to an N-acetoxyarylamine can ultimately lead to the formation of highly reactive (i.e., electrophilic) nitrenium or carbenium ions that can readily react with DNA [17,46]. However, not all metabolites contribute to the formation of DNA adducts. Some are conjugated to glutathione, sulfate or glucuronic acid to form phase II metabolites which are excreted from the bile and urine.

In *Salmonella*, an organism frequently used to monitor mutagenic activity *in vitro*, nitroreductase activity is conferred by the classical nitroreductase (*cnr*) and the major nitroreductase (*Snr*). Furthermore, acetyltransferase activity is conferred by O-acetyltransferase (*OAT*).

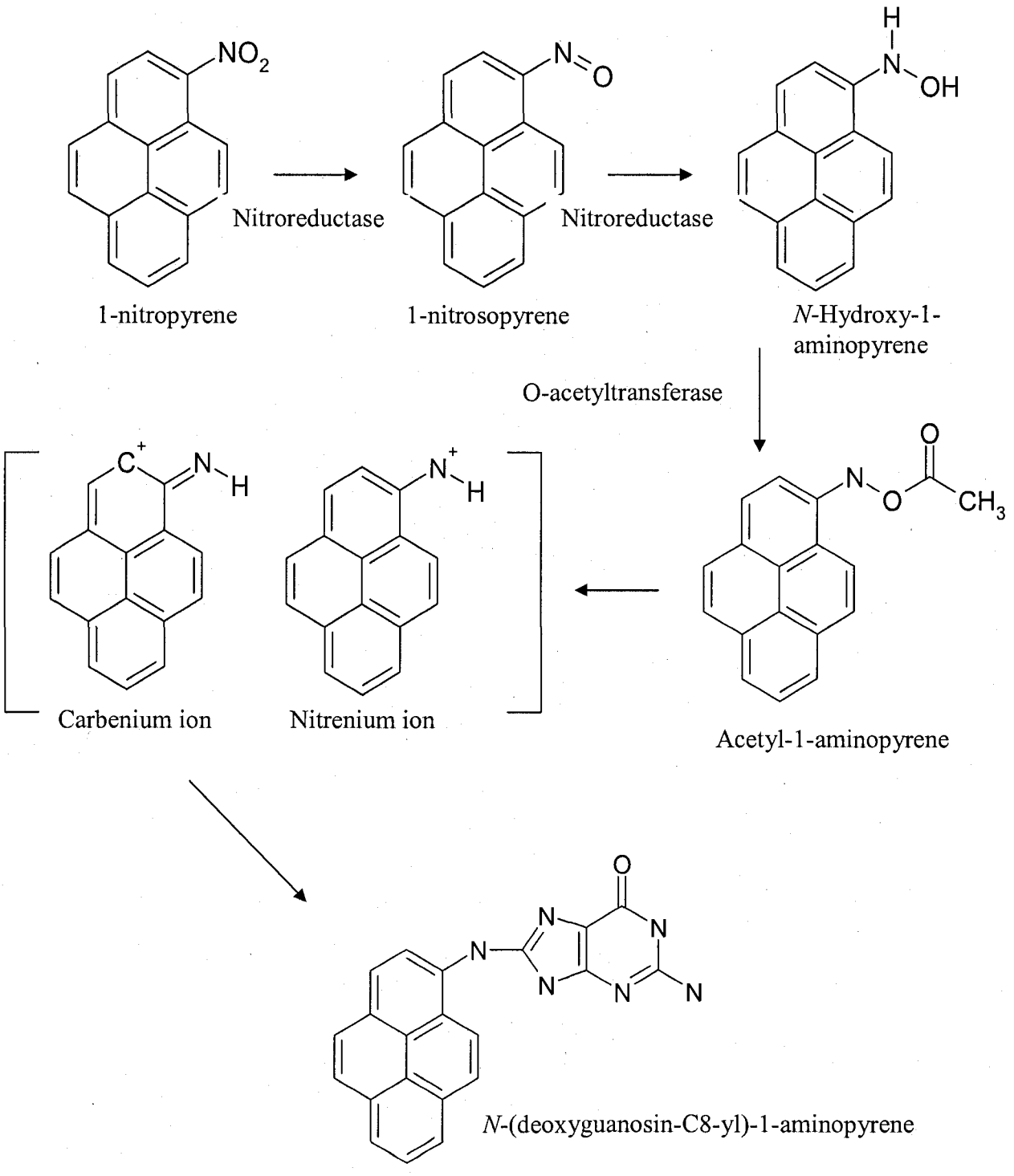


Figure 1.5 Metabolism of 1- nitropyrene into an active metabolite capable of forming the DNA adduct *N*-(deoxyguanosin-C8-yl)-1-aminopyrene. In Salmonella, nitroreduction is carried out by the classical nitroreductase (*Cnr*) or the major nitroreductase (*Snr*).

1.5 Toxicity Assessment of PACs

The first indication that exposure to combustion emissions is associated with increased risk of adverse health effects was put forward by Sir Percival Pott. In 1775, he found an association with exposure to soot and high incidences of scrotal cancer among chimney sweeps. Similar observations associated with occupational exposures to coal tar, vehicle exhaust, and cigarette smoke have confirmed that exposure to environmental agents increases the risk of developing neoplastic lesions [48-51]. It has since been well documented that PACs such as PAHs and nitro-PAHs are the cause of malignancies associated with exposure to cigarette smoke and vehicle exhaust [52,53]. Moreover, it has been shown that PACs, including PAHs and nitro-PAHs, exert their genotoxic effects through the induction of DNA mutations (i.e., DNA sequence changes) [54]. The knowledge that environmental contaminants have the ability to induce DNA mutations that can contribute to the initiation of neoplastic lesions has driven the scientific community to investigate the impacts of environmental and occupational exposures on human health.

The ability of chemicals and complex mixtures to induce mutations both *in vitro* and *in vivo* has been the focus of much research since the 1970s. Many assays have been developed to measure the ability of chemicals and complex mixtures to induce mutations. Key regulatory assays include the reverse gene mutation in *Salmonella* (*in vitro*), and the chromosomal aberration test (*in vitro).* Other useful bioassays focus on the induction of specific toxicologically-relevant pathways following exposure to selected compounds or complex mixtures. Several mammalian cell systems are useful *in vitro* indicators of pathway-specific responses, and are often used as cost-effective alternatives to *in vivo* models. Such systems include the DR-CALUX assay and the EROD assay, two assays that assess Ah-receptor agonism and the induction of enzymes involved in the Phase I response to

organic xenobiotics. For the purpose of this study, two *in vitro* toxicity assays were chosen to measure the mutagenic activity (i.e., the Salmonella reverse mutation assay) and Ah-receptor agonism (i.e., DR-CALUX assay) of diesel emissions.

1.5.1 Salmonella mutagenicity assay

The Salmonella mutagenicity test is an *in vitro* tool employed to assess the mutagenic activity of selected analytes. The test is frequently used as an initial screen to determine the mutagenic potential of new chemicals and drugs, and the results obtained have a high predictive value for rodent carcinogenicity [55]. The test uses histidine auxotrophs of *Salmonella typhimurium* to assess mutagenic activity. In the absence of histidine, these bacteria are unable to grow. However, when exposed to a mutagenic agent, mutations can occur that permit prototrophic growth in the absence of histidine. Revertant colonies (i.e., prototrophic colonies) are counted and expressed as revertants per unit concentration of chemical(s). The assay commonly employs an exogenous metabolic activation system (i.e., S9) that permits *in vitro* simulation of typical mammalian hepatic metabolism (i.e., Phase I), and the transformation/activation of the aforementioned PACs and PAHs. Figure 1.6 illustrates the basic steps of the Salmonella mutagenicity assay.

The *Salmonella* strains employed are engineered to detect frameshift mutations or base pair substitutions. The most common genotypes employed in the Salmonella mutagenicity test are the *hisG46* allele and the *hisD3052* allele. TA100, and the metabolically-enhanced derivatives of TA100 (e.g., YG1042), contain the *hisG46* allele that can only revert back to wild-type via base pair substitution. In contrast, TA98 and the metabolically-enhanced derivatives of TA98 (e.g., YG1041), contain the *hisD3052* allele that can only revert back to wild-type via frameshift mutations [56-58].

The strains employed in the Salmonella mutagenicity test also harbour plasmids and mutations to increase sensitivity to chemically-induced mutagenesis. Plasmid pKM101 harbours the β-lactamase gene for ampicillin resistance, as well as the *mucA/B* gene [55]. The *mucA/B* gene encodes PolR1, an error-prone DNA polymerase that permits DNA lesion bypass, and enhances susceptibility to chemical mutagenesis [59]. Furthermore, the strains contain a deep rough (*rfa*) mutation, which results in partial loss of the bacteria's outer lipopolysaccharide (LPS) layer and increases the cell's permeability to bulky chemicals. A deletion of the *uvrB* gene also increases sensitivity to chemically-induced mutagenesis by modifying the effectiveness of the nucleotide excision repair system. This deletion also results in the partial deletion of the *bio* gene (i.e., the biotin synthesis gene), a deletion that cannot be reverted [55].

Over the years, various *Salmonella* tester strains have been developed to detect specific classes of chemical mutagens and suspected carcinogens. Strain YG1041, a derivative of TA98, harbours the pYG233 plasmid that contains the *OAT* and *cnr* genes. This metabolic enhancement dramatically increases the sensitivity to nitroarenes, N-containing heterocyclics and aromatic amines [56]. YG5161 is a relatively new strain derived from TA1538, a frameshift strain related to TA98. This strain over-expresses the *E.coli dinB* gene that encodes Y class polymerase IV, thus making the strain more sensitive to DNA damage induced by homocyclic aromatic hydrocarbons [57].

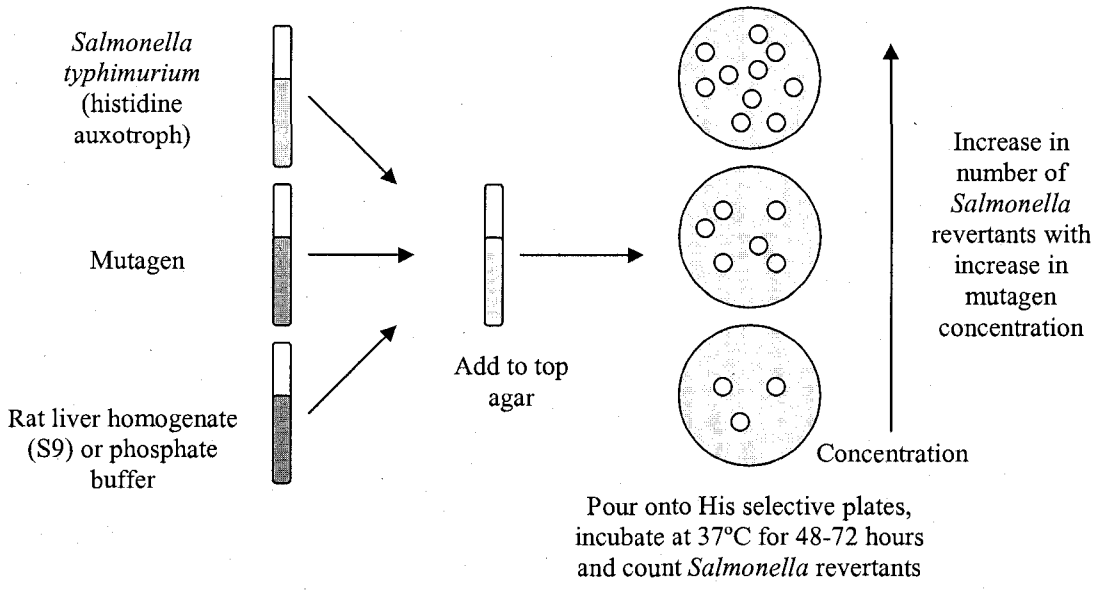


Figure 1.6 Schematic diagram of the Salmonella reverse mutation assay. The assay employs mutant *Salmonella* strains (i.e., histidine auxotrophs) and a metabolic activation system (i.e. S9) to assess the mutagenic activity of tested samples. This study employed three frameshift mutation strains (i.e., TA98, YG1041, YG5161), two of which are metabolically-enhanced to facilitate the detection of PAH-, nitroarene-, and aromatic amine-induced mutagenesis (i.e., YG5161 and YG1041 respectively).

1.5.2 DR-CALUX assay

A variety of bioassays have been developed to assess the dioxin-like effects of complex mixtures. One of most commonly used systems measures CYP450 enzyme activity via the EROD assay. This assay measures 7-ethoxyresorufin O-deethylasee activity, assessed via the conversion of ethoxyresorufin to resorufin in the presence of NADPH and oxygen [60]. Other more recently developed techniques involve the use of genetically engineered mammalian cell lines that employ a reporter system to record dioxin-like responses. Such assays include the P450 reporter gene system (RGS) and the CALUX (Chemically Activated LUciferase eXpression) assay. The DR-CALUX assay employs a mouse cell line (e.g., hepa1c1c7) modified to include a luciferase reporter plasmid (i.e.,

pGudLuc1.1) resulting in the H1L1.1c2 cell line that is responsive to Ah-receptor agonism and induction of the aryl hydrocarbon response pathway [61]. Figure 1.7 illustrates the pGudLuc1.1 plasmid. The plasmid and stably-transfected cell lines containing the plasmid, are useful for assessing the ability of complex mixtures, such as diesel emissions, to induce an Ah-receptor dependent response, and consequent upregulation of the cytochrome P450 system that is known to be responsible for the transformation of PAHs into active carcinogens [61,62].

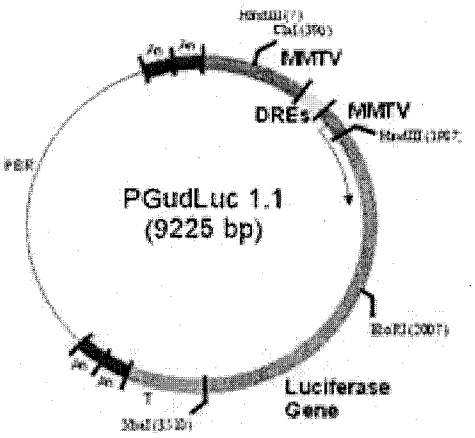


Figure 1.7 Illustration of the pGudLuc1.1 plasmid employed in the DR-CALUX assay. Four dioxin responsive elements, located upstream of a mouse mammary tumour virus promoter (MMTV), control the expression of the luciferase gene.

. The mechanism of action of dioxins has been well documented [62-65]. Briefly, the ligand-free Ah-receptor is a cytosolic heterodimer bound to a dimer of heat-shock proteins of 90 kD (HSP90) and a single molecule of the immunophilin-like protein hepatitis B virus X-associated protein 2 (XAP2) [62]. Once dioxins, co-planar PCBs and other dioxin-like chemicals enter the cell, they can bind the Ah-receptor releasing XAP2 [65]. The receptor-ligand complex is then free to enter the nucleus, release the HSP90 dimer and bind

the ARNT (aryl-hydrocarbon receptor nuclear translocator)[64]. This complex reacts with specific DNA sequences, namely, the dioxin responsive elements (DRE), resulting in an alteration in the expression of genes controlled by DREs [62]. Figure 1.8 illustrates the mechanism of action of dioxins, and provides a schematic of the CALUX assay. Briefly, binding of dioxins and dioxin-like compounds to the Ah-receptor results in increased transcription of firefly luciferase. Firefly luciferase reacts with Beetle luciferin *in vitro* to produce a luminescent product that is quantifiable with a luminometer.

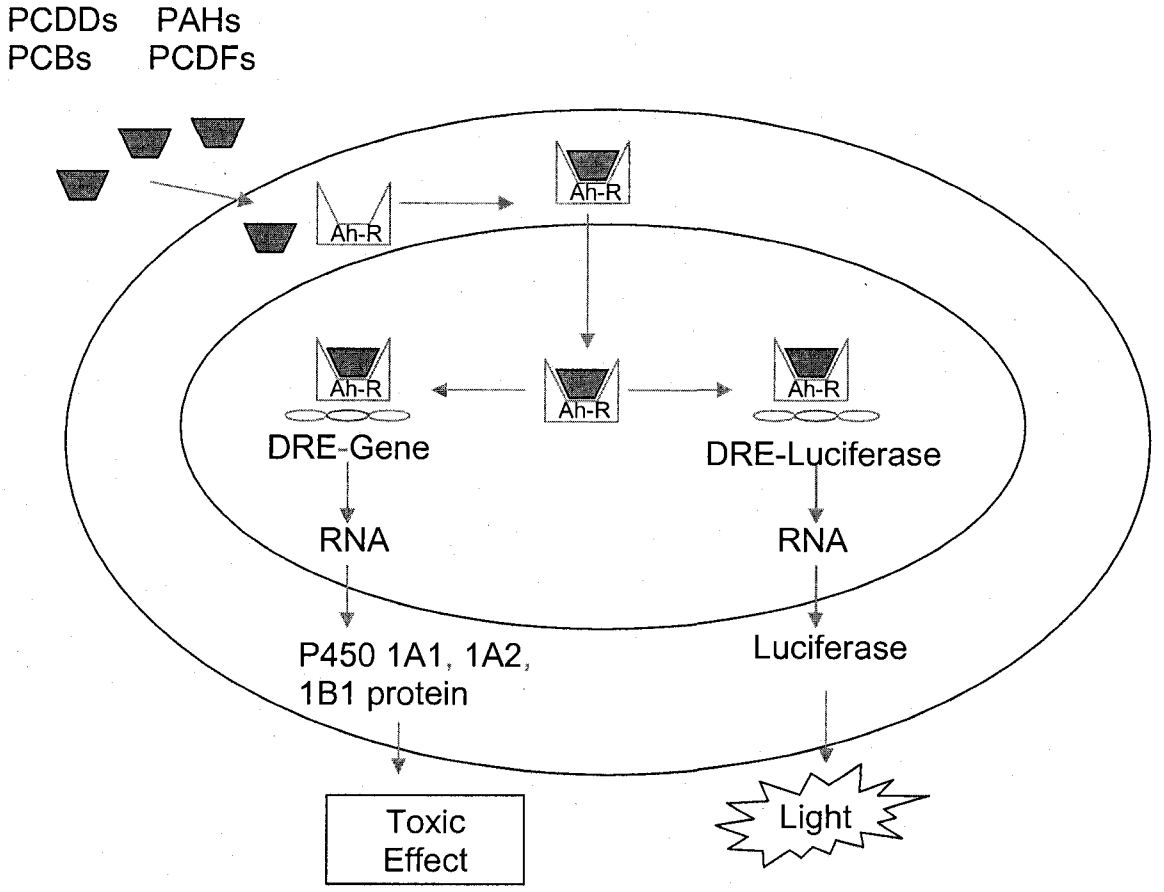


Figure 1.8 Schematic representation of the DR-CALUX assay and Ah-receptor pathway. This assay employs a mouse hepatoma cell line (i.e., H1L1.1c2) that contains the pGudLuc1.1 plasmid resulting in increased sensitivity to the Ah-receptor. Chemicals enter the cell and bind to the Ah-receptor. After the release of two 90 kD heat-shock proteins, the Ah-receptor/ligand complex enters the cell and heterodimerizes with the aryl-hydrocarbon receptor nuclear translocator (ARNT). The ARNT-ligand complex binds to dioxin-response elements (DRE) upstream of the luciferase gene. Upon binding to the DRE, the luciferase gene is transcribed and produces light in the presence of Beetle luciferin. The reaction produces light that can be quantified with a luminometer.

1.6 Objectives and hypothesis

The objectives of this study were to:

I. Employ *in vitro* bioassays; the Salmonella mutagenicity test and the DR-CALUX assay, to assess the comparative toxicological properties of the SOF of diesel engine emissions.

II. Compare the mutagenic and Ah-receptor agonism profiles of emissions generated from a single biofuel at various blend concentrations (i.e., 1 to 20%), as well as emissions generated from different biofuels at a single concentration (i.e., 20%).

III. Employ chemical fractionation to identify chemical groups/fractions that consistently yield positive responses on the aforementioned assay systems.

The null hypothesis for this thesis was that the addition of biodiesel in various concentrations and from different sources would not affect the ability of the SOF to induce mutations and the Ah-receptor pathway.

Chapter 2: Materials and Methods

2.1 Diesel and biodiesel particulate samples

The diesel samples investigated in this thesis were kindly donated by Lisa Graham (Environment Canada, Environmental Technology Centre, Emissions Research & Measurement Division) and Stuart Neill (National Research Council of Canada, Institute for Chemical Process and Environmental Technology). The samples were generated using novel fuels including ULSD and biodiesel. Details about the sample characteristics (e.g., fuel, driving cycle, etc.) are presented in Table 2.1.

Table 2.1 Fuel composition, driving cycle, after-treatment technology and particle mass information for samples provided by EC, NRC, and NIST. The Caterpillar (1Y700) engine was employed at NRC to generate diesel particulate samples. The Caterpillar (C11) engine was employed at EC to generate diesel particulate samples. Diesel and biodiesel particulates were given in the form of particles on filters for both EC and NRC samples. Furthermore, volatile compounds extracted from polyurethane foam plugs (PUFs) were given for EC samples only. SRMs were provided as particles in a vial

	Sample	Diesel Fuel Content (% v/v)	Biodiesel Fuel Content (% v/v)	Biodiesel Source[a]	Driving Cycle	After-Treatment Technology	Particle Mass (mg)	Final Extract Concentration (mg/ml DMSO)
NRC	B0[b]	100	0	NA	AVL 8-mode heavy-duty	EGR	38	76
	B1	99	1	Canola	AVL 8-mode heavy-duty	EGR	44	88
	B2	98	2	Canola	AVL 8-mode heavy-duty	EGR	36	72
	B5	95	5	Canola	AVL 8-mode heavy-duty	EGR	33	66
	B10	90	10	Canola	AVL 8-mode heavy-duty	EGR	42	84
	B20	80	20	Canola	AVL 8-mode heavy-duty	EGR	35	70
	Ref6[c]	100	0	Canola	AVL 8-mode heavy-duty	EGR	38	76
EC	1	80	20	Canola	HS Heavy-duty FTP transient	None	10	20
	2[d]	100	0	NA	HS Heavy-duty FTP transient	DOC	10	20
	3	80	20	Soy	HS Heavy-duty FTP transient	DOC	10	20
	4	80	20	Canola	HS Heavy-duty FTP transient	DOC	10	20
	5[d]	100	0	NA	Steady-state (100%, 75%, and 50% load)	DOC	30	60
	6	80	20	Canola	Steady-state (100%, 75%, and 50% load)	DOC	27	54
	7	80	20	Soy	Steady-state (100%, 75%, and 50% load)	DOC	30	60
	8	80	20	Animal	Steady-state (100%, 75%, and 50% load)	DOC	23	46
SRM	1650b	100	0	NA	Variety of cycles	None	40	80
	2975	100	0	NA	Not specified	None	40	80

NA=not applicable, HS=hot start, EGR=exhaust gas recirculation, DOC=diesel oxidation catalyst

[a] The biodiesel used to generate both NRC samples and EC samples was provided by Milligan Bio-Tech

[b] B0 is an ULSD provided by Shell and was used to create all NRC biodiesel blends.

[c] Ref6 is a winter grade Shell fuel and is used by NRC as a reference fuel.

[d] Samples 2 and 5 are ULSD and were used to prepare the EC B20 biodiesel blends.

2.2 Collection of diesel and biodiesel emission samples

2.2.1 EC samples

Diesel exhaust particles were generated using various fuels and after-treatment technologies. Samples 1 through 4 were generated using ULSD and biodiesel blended ULSD fuel at 20% blends (i.e., B20) derived from canola, no biodiesel (i.e., 100% ULSD), soy, and canola respectively. Furthermore, samples 2 through 4 were generated using a diesel oxidation catalyst (i.e., DOC); however sample 1 did not employ a DOC. These samples were generated using the hot-start portion of the Federal Testing Procedure (FTP) heavy-duty transient driving cycle. Figure 2.1 illustrates the FTP heavy-duty transient driving cycle. Samples 5 through 8 were also generated using ULSD (i.e., sample 5) and biodiesel blended ULSD fuel at 20% blends (i.e., B20, samples 6-8) derived from canola, soy, and animal fat respectively. These samples were generated using a DOC and operating on a steady-state driving cycle at 100%, 75% and 50% load.

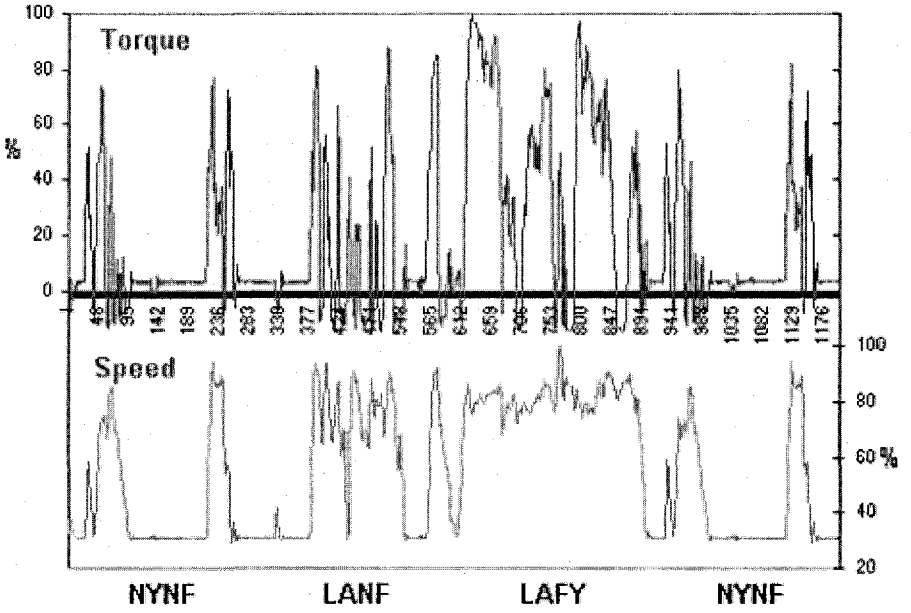


Figure 2.1 Variation of normalized speed and torque illustrating the heavy-duty FTP transient cycle used by EC to generate diesel emissions. This cycle is used in the US for emissions testing of heavy-duty vehicles. The cycle consists of four phases: New-York non-freeway (NYNF), Los Angeles non-freeway (LANF), Los Angeles freeway, and New-York non-freeway. The equivalent average speed is 30km/hr with a distance of 10.3 km, and a running time of 1200s (20 minutes). This figure was obtained from http://www.dieselnet.com/standards/cycles/ftp_trans.html.

Particulate material from each testing condition (i.e., samples 1 through 8) was generated using a Caterpillar six-cylinder test engine (model C11). Engine characteristics are shown in Table 2.2. The detailed collection method can be found in ERMD report # 2005-32 [66]. Briefly, engine exhaust was generated using an eddy-current dynamometer to control engine load and speed (i.e., based on the FTP heavy-duty transient cycle). Particulate matter was obtained by directing the engine exhaust through a double dilution diesel particulate sampling system (DPS), and allowing the particles to be deposited on pre-weighed 70 mm EMFAB Teflon coated glass fibre filters (Pallflex, East Hills, NY). Dilution tunnels (DPS) are used to simulate environmental exposures of diesel exhaust by mixing it with air prior to collection. Volatile components of diesel exhaust emissions were

collected on polyurethane foam plugs (PUFs) located downstream of filters. Figure 2.2 illustrates a schematic representation of the collection system.

Table 2.2 Characteristics of the Engines employed at EC and NRC to generate diesel PM.

	Engine Model	
	Caterpillar C11 (EC)	Caterpillar 1Y3700 (NRC)
Number of Cylinders	6	1
Bore x Stroke (mm)	130 x 140	137.2 x 165.1
Displacement (L)	11.1	2.44
Maximum Power Output	305 bhp at 2100 rpm	305 bhp at 2100 rpm

*bhp=brake horsepower, rpm=revolutions per minute

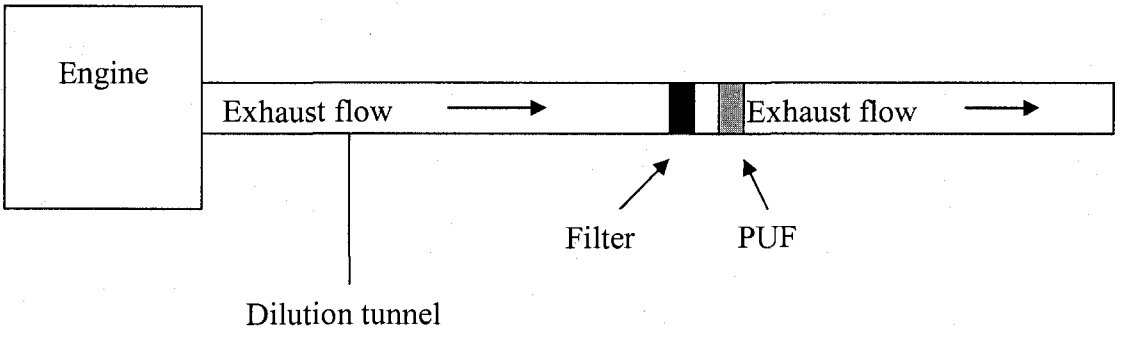


Figure 2.2 Schematic representation of the collection system. Diesel exhaust is passed through a dilution tunnel where it is mixed with air to represent environmentally-relevant conditions of exhaust emissions. Particulate material is collected on Teflon® coated filters and the volatile components are collected on the PUFs.

2.2.2 NRC samples

Diesel exhaust emissions were generated using ULSD (i.e. Shell B0) and ULSD-blended canola-derived biodiesel fuels at various concentrations (i.e., Milligan canola, B1, B2, B5, B10, and B20). A winter-grade Shell fuel (i.e., Ref6) was used as a reference fuel. All diesel particulate samples were generated using a modified Caterpillar single-cylinder test engine (model 1Y3700) fitted with an EGR system. Engine characteristics are shown in

Table 2.2. The collection method and engine description are fully described in Neill et al [67]. Briefly, the engine was connected to an eddy-current dynamometer to control engine load and speed. The AVL eight-mode steady-state simulation of the US EPA transient test procedure was employed for all tests. Engine emissions were measured at 8 speed/load conditions represented in Figure 2.3. Engine particulate emissions were collected using a fully automated particulate sampling system (model BG-2, Sierra Instruments Inc., Monterey, CA) that operates by diluting a portion of the exhaust gas with a measured amount of air in a dilution chamber and passing the diluted exhaust gas through a pair of 90 mm Teflon-coated filter membranes (fiberfilm T60A20, Pallflex, East Hills, NY). Emissions from each mode were collected separately and each mode was repeated in triplicate. This resulted in a total of 24 pairs of filters representing one sample series (i.e., one driving cycle).

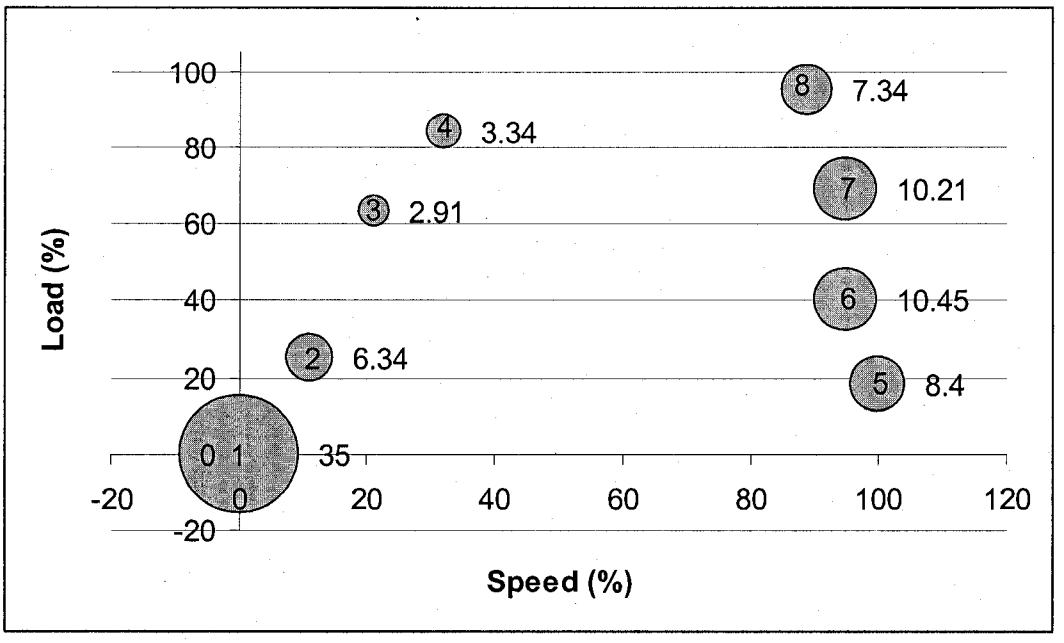


Figure 2.3 Speed, load and weight distributions of the AVL 8-mode heavy-duty driving cycle used by NRC to generate diesel emissions. The AVL test is a steady-state procedure that closely correlates with the exhaust emission results of the US FTP transient test cycle. The test involves collecting particulate material at 8 steady state modes, and the effect of each mode is calculated by applying weighing factors to the results. The relative weights of each mode are represented by the area of the bubble. Figure reproduced from: http://www.dieselnet.com/standards/cycles/ftp_trans.html.

2.3 Diesel filter and PUF extraction and fractionation

2.3.1 Chemicals

All chemicals used for extraction and fractionation of filter media were analytical grade chemicals (≥99%) obtained from EMD chemicals (Gibbstown, NJ).

2.3.2 Filter extractions

Organic compounds from both NRC and EC filter media were extracted using pressurized fluid extraction with the ASE 300 Accelerated Solvent Extractor (Dionex Corp., Sunnyvale, CA). For each extraction, filters containing diesel particulates were placed in 34 ml extraction cells filled to 1 cm from the top with inert sand (i.e., Ottawa Sand Standard 20-

30 Mesh, Fisher Scientific Canada, Ottawa, ON). For NRC filters, 48 filters representing one sample series were combined into one extraction cell whereas, for EC filters, 3 filters representing one sample were combined in one extraction cell. Filters were extracted with a solvent mixture consisting of hexane:acetone (50:50 v/v) at 1500 psi (10.3 MPa) and 150°C. The extraction process consisted of a dynamic extraction of 7 minutes and two static extractions of 5 minutes each. Extracts were evaporated with ultra-pure nitrogen gas to a final volume of approximately 1 ml prior to fractionation.

2.3.3 PUF extractions

PUF extractions were performed by Environment Canada at the Environmental Technology Centre (Ottawa, ON). Briefly, PUFs were placed in a clean Soxhlet body using clean tweezers and extracted overnight (16-20 hours) in cyclohexane (approximately 700 ml) at a rate of 2-5 cycles/hour. The extracts were then filtered through anhydrous Na_2SO_4 and concentrated to a volume of 5 ml by rotary evaporation at 45 °C. Extracts were kept at 4 °C until needed.

2.3.4 Filter and PUF fractionation

Filter and PUF extracts were fractionated on open silica gel columns into three fractions: aliphatics (not tested), non-polar neutral compounds (i.e., PAHs, alkyl-PAHs and O- and S-heterocyclics), and polar aromatic compounds (i.e., N-heterocyclics, nitroaromatics and oxy-PAHs). The method employed was developed and fully validated by Lundstedt et al [68]. Briefly, glass columns were packed with 10 g of silica gel (10% deactivated w/w) and 2 g of anhydrous sodium sulphate. The columns were rinsed with 30 ml of DCM and 10 ml of hexane before applying the sample. Each extract was evaporated onto 0.5 g of silica gel and added to the column. The columns were then eluted with 20 ml of hexane, 26 ml of hexane:DCM (3:1 v/v) and 60 ml of DCM. Each fraction was evaporated to 1 ml under a

stream of nitrogen gas and solvent exchanged to 500 µl of DMSO (Sigma-Aldrich Canada Ltd., Oakville, ON). Fractions were kept at 4 °C until needed.

2.4 Chemical analyses

2.4.1 EC samples

Limited chemistry was performed on these emission samples; however, the data were not available at the time this thesis was prepared.

2.4.2 NRC samples

NRC samples were not analyzed for their chemical composition. However, 5 mg of particulate (filter weight) extracts were kept for possible future analysis.

2.5 Salmonella mutagenicity assay

2.5.1 Media, solutions and reagents

All reagents and solutions used in the Salmonella reverse mutation assay, as well as the protocol followed for the microsuspension (Kado) version of the Salmonella reverse mutation assay, adhered to the Standard Operating Procedures of the Environmental Carcinogenesis Division of the US EPA (Research Triangle Park, NC), which were adapted from Mortelmans and Zeiger [55]. All chemicals and reagents were purchased from Sigma-Aldrich Canada Ltd unless otherwise specified. All water used to prepare solutions and reagents was prepared using a Milli-Q Ultrapure water purification system (Millipore Corp., Bedford, MA) and filtered at 0.45 µm. Autoclaving of all reagents and solutions was performed at 121 °C for 20 minutes using a Steris SV-120 Sterilizer (Amsco Scientific, Apex, NC).

Top agar, containing 0.6% w/v agar and 86 mM NaCl, was prepared the day of testing. The solution was autoclaved and placed in a water bath at 52 °C for the duration of the assay.

Stock solutions of 30% dextrose (1.7 M) were prepared, autoclaved, and stored at 4°C until needed. Stock solutions of biotin/histidine containing 0.4 M histidine and 0.02 mM biotin were prepared and sterilized by filtration with a 0.2 μm Nalgene bottle top filter (Fisher Scientific, Ottawa, Canada) and stored at 4°C until needed. Stock solutions of 50X Vogel-Boner Medium E (VBME) containing 0.04 M magnesium sulphate, 0.6 M citric acid monohydrate, 2.9 M potassium phosphate dibasic and 0.8 M sodium ammonium phosphate were filter sterilized with a 0.45 μm Nalgene bottle top filter (Fisher Scientific, Ottawa, Canada), autoclaved, and stored at room temperature until needed.

The bottom agar used for the Salmonella reverse mutation assay was glucose minimal medium prepared by autoclaving 5310 ml of water and 90 g of Difco™ granulated agar (Becton, Dickinson & Company, Le Pont de Claix, France) using a MediaClave sterilizer (Integra Biosciences, Switzerland) at 121 °C for 25 minutes. After the solution was cooled to 50 °C, 400 ml of dextrose solution, 120 ml of VBME and 60 ml of biotin/histidine solution were added to the media and allowed to mix for 10 minutes. 26 ml of the agar solution was dispensed into sterile 100mm Petri dishes using a Tecomat automatic plate pourer (Fernwald, Germany). Plates were allowed to solidify overnight on a level surface and stored at room temperature until needed.

Solutions of 0.15 M phosphate buffer were prepared the day of testing by adding 12.5 ml of water to 37.5 ml of 0.20 M phosphate buffer (pH 7.4). The mixture was kept on ice throughout the duration of the experiment.

2.5.2 Metabolic activation system

A metabolic activation system was used to simulate mammalian hepatic metabolism. The metabolic activation system, herein after referred to as the S9 mixture, contained 5% v/v post-mitochondrial supernatant from Aroclor-1254-induced male Sprague-Dawley rats (Moltox Inc., Boone, NC). Cofactor solutions for the S9 mixture were prepared in advance and stored at -30 °C until needed. The solutions contained 2% v/v microsomal salt solution (0.4 M $MgCl_2$ and 1.65 M KCl), 5 mM glucose-6-phosphate, 4 mM nicotinamide adenine dinucleotide (NADP) (Roche Diagnostics, Laval, QC), and 50 % v/v 0.2 M phophate buffer (pH 7.4). The mixture was sterilized by filtration using a 0.45 μm Nalgene bottle top filter, and 19 ml aliquots were placed in 50 ml conical tubes (Fisher Scientific Canada, Ottawa, ON). When metabolic activation was needed, one tube was removed from the freezer, allowed to thaw at room temperature, and mixed with the aforementioned post-mitochondrial supernatant to a final concentration of 5% v/v. The S9 mixture was kept on ice for the duration of the experiment.

2.5.3 Salmonella tester strains

The *Salmonella typhimurium* tester strains used in the reverse mutation assay were TA98, YG1041 and YG5161. TA98 was purchased from Moltox Inc (Boone, NC) and YG1041 and YG5161 were generously provided by Dr. Takehiko Nohmi (National Institute of Health Sciences, Tokyo, Japan). All three strains are derivatives of TA1538 and detect frameshift mutations. TA98 harbours plasmid pKM101 that expresses the *E. coli mucA/B* gene that encodes DNA polymerase R1. The expression of this error-prone polymerase increases the observed mutagenic potency of several mutagens. YG1041 harbours plasmid pKM101 in addition to plasmid pYG233. The latter harbours the *cnr* and *OAT* genes that encode the *Salmonella* classic nitroreductase and O-acetyltransferase genes, respectively. It

has been shown that overexpression of these genes dramatically increases the sensitivity to nitroarenes and aromatic amines [69]. YG5161 only contains the pYG768 plasmid. This plasmid harbours the *E. coli dinB* gene that encodes Y class DNA polymerase IV. This polymerase is part of the SOS response system, and has been shown to be involved in mutagenesis induced by 4-nitroquinoline and benzo[*a*]pyrene [57]. Table 2.3 describes the *Salmonella* tester strains in further detail.

Strain checks were performed prior to preparing frozen permanent stocks of the tester strains. Histidine requirements for growth were confirmed by streaking a loopful of each strain across a minimal glucose agar plate supplemented with 0.1 ml of 0.5 mM biotin. No growth was observed for any strain after an incubation of 48 hours at 37 ℃. Biotin requirements were not confirmed because this mutation cannot be reversed [58]. The deep rough mutation (*rfa*), which leads to a defective LPS layer that confers the increased permeability to bulky chemicals, was confirmed by streaking a loopful of each strain on a nutrient agar plate containing sterile discs soaked with crystal violet (1 mg/ml). An inhibition zone was observed for each strain after an incubation of 48 hours at 37 ℃. The *uvrB* mutation was confirmed by demonstrating UV sensitivity in all strains. A loopful of each strain was streaked across a nutrient agar plate, half the plate was covered with aluminium foil, and the plate was subsequently irradiated with a 15-W germicidal UV lamp at a distance of 33 cm. No growth was observed on the irradiated half for all strains following a 48 hour incubation period at 37 ℃. Ampicillin resistance (i.e., presence of the desired plasmids) was confirmed by streaking a loopful of each strain across a nutrient agar plate supplemented with 24 μg/ml ampicillin. Growth was observed for all strains following an incubation of 48 hours at 37 ℃.

Frozen permanent cultures of the *Salmonella* tester strains were prepared by adding 150 µl of autoclaved glycerol (99.5 % ACS, Sigma Aldrich Canada Ltd.) to 850 µl of overnight cultures containing 25 µg/ml ampicillin in 1.5 ml Eppendorf tubes (Fisher Scientific, Ottawa, Canada). Frozen permanent cultures were stored at -80 °C until needed. Overnight cultures were prepared by adding 750 µl of the thawed *Salmonella* frozen permanent to 150 ml of Oxoid Nutrient Broth #2 in 500 ml Erlenmeyer flasks. The culture was incubated at 37 °C and 200 rpm in a shaking waterbath (Gyratory Water Bath Shaker, Model # GY6, New Brunswick Scientific, Edison, NJ) for 16 hours. After the incubation period, *Salmonella* cultures were transferred to sterile conical tubes and centrifuged at 4 °C and 343g for 15 minutes. The supernatant was decanted and the culture was resuspended in 15 ml of 0.15 M phosphate buffer and placed on ice until needed.

Table 2.3 Properties of *Salmonella typhimurium* strains used in the Salmonella reverse mutation assay.

Strain	Mutation	DNA Target	Plasmid-Encoded Genes	Use
TA98	*his*D3052	CGCGCCG	pKM101-*MucA/B, Amp*	Derived from TA1538. Detects frameshift mutagens.
YG1041	*his*D3052	GCGC	pKM101-*MucA/B, Amp* pYG233-*OAT, Cnr, Tet*	Derived from TA98. Sensitive to nitroarenes and aromatic amines.
YG5161	*his*D3052	GC	pYG768-*DinB, Amp*	Derived from TA1538. Sensitive to homocyclic aromatics.

2.5.4 Assay protocol

The microsuspension (i.e., Kado) version of the reverse mutation assay was used to test the mutagenic activity of each diesel exhaust fraction on all three *Salmonella* strains with and without metabolic activation. All experiments were completed under aseptic conditions in a laminar flow hood (Model # BM6-2B-49, Microzone Corporation, Nepean, ON).

A 100 µl aliquot of the appropriate *Salmonella* overnight culture, 10 µl of the appropriate diesel fraction dilution, and 100 µl of 0.15 M phosphate buffer or 100 µl of S9 were added to a sterile 13 x 75 mm glass tube (Fisher Scientific Canada, Ottawa, ON). The contents were vortexed, placed in a rack, and incubated at 37 ℃ for 90 minutes at 55 rpm in a shaking waterbath (Versa-Bath® S, Model # 224, Fisher Scientific Canada, Ottawa, ON). Following the incubation period, 2 ml of molten top agar was added to each glass tube, vortexed, and poured evenly onto a minimal media plate and set to solidify on a level surface. Once solidified, the plates were incubated at 37 ℃ for 72 hours.

Negative and positive controls were tested during each experiment. The negative solvent control used during each experiment was 10 µl of DMSO. The positive controls, purchased from Moltox Inc. (Boone, NC), were 2-aminoanthracene (0.5 µg per plate) when S9 was added and 2-nitrofluorene (1 µg per plate) when S9 was not added. Controls were tested in triplicate with each strain.

Each diesel extract fraction and both Standard Reference Materials were tested in triplicate. At least 5 concentrations were tested for each sample. Plates were counted twice using the ProtoCOL SR automated colony counter (Synbiosis, Frederick, MD), and the mean number of revertants per plate for each triplicate was used for data analysis.

2.5.5 Salmonella testing of diesel fractions

Both fractions of each diesel extract were tested on *Salmonella* strains TA98, YG1041 and YG5161 with and without metabolic activation (i.e., S9). At least five test concentrations were used and each concentration was tested in triplicate. Concentrations ranged from 20 µg of filter particle weight per plate to 880 µg per plate.

2.5.6 Statistical analyses

SAS v. 9.0 for Windows was used for statistical analysis of all concentration-response curves for all samples [70]. The mutagenic potency, defined as the slope of the linear portion of the concentration-response curve was calculated using ordinary least squares linear regression analysis. A response was considered positive only if a concentration-dependant increase in the number of revertants was observed, and at least a two-fold increase in the number of revertants compared to the DMSO solvent control was observed for two consecutive concentrations.

2.6 DR-CALUX assay

All experiments were carried out under aseptic conditions in a laminar flow hood (Canadian Cabinets Co. Ltd., Model # BM6-2B, Ottawa, ON). Incubations were carried out at 37 °C, 95% humidity, and 5% CO_2 in a Sanyo Scientific CO_2 incubator (Model # MC0-20A1C, Bensenville, IL).

2.6.1 Media, solutions and reagents

All media, solutions and supplements were purchased from Gibco-Invitrogen (Burlington, ON) unless otherwise indicated.

2.6.2 Mouse hepatoma (H1L1.1c2) cell line

A mouse liver hepatoma cell line, H1L1.1c2, derived from a BW7756 hepatoma that developed in a C57L mouse, was used as an *in vitro* model to asses the ability of diesel extracts to induce the aryl hydrocarbon receptor response [61]. H1L1.1c2 cells were maintained in α-MEM supplemented with foetal bovine serum (10 % v/v). Confluent cultures were split 1:10 (approximately every 3-4 days) using 0.25% trypsin.

2.6.3 DR-CALUX assay protocol

Cells were plated in 12-well culture dishes at a density of 6 x 10^4 cells/ml with a final volume of 1 ml/well and grown to approximately 90% confluency (i.e., 24-48 hours). Once confluent, the media was removed, and the cells were rinsed with phosphate buffered saline (PBS). Cells were exposed to 1 ml of media containing 1 µl of the appropriate sample dilution and were incubated for 4 hours at 37 °C. Following the exposure, the media was removed and cells rinsed twice with PBS. Cells were then lysed by applying 250 µl of passive lysis buffer (Fisher Scientific Canada, Ottawa, ON) and shaking (50 rpm) for 20 minutes. Cell lysates were collected and placed in 1.5 ml Eppendorf tubes and frozen at –80°C until needed.

Negative and positive controls were tested during each experiment. The negative solvent control used during each experiment consisted of 1 µl/ml of DMSO. The positive control consisted of 8 serial dilutions of benzo[*a*]pyrene (Sigma Aldrich Canada Ltd., Ottawa, ON). Controls were used in each experiment and were tested in triplicate.

2.6.4 Diesel fraction exposures

Both fractions of each diesel extract were tested for their ability to produce an Ah-receptor mediated effect using the DR-CALUX assay. At least five test concentrations were used and each concentration was tested in triplicate. Concentrations ranged from 2 µg of filter particle weight per well to 88 µg per well.

2.6.5 Luciferase assay

A 20 µl aliquot of thawed cell lysate from each triplicate was placed in an individual well of a white, flat bottom 96-well microplate (Fisher Scientific Canada, Ottawa, ON). Luminescence was measured using a Veritas Microplate Luminometer (Model # 9100-102, Turner Biosystems, Sunnyvale, CA) for 10 seconds after a 2 second delay following the

injection of 100 µl of luciferase assay substrate (Fisher Scientific Canada, Ottawa, ON). The results were compiled in an Excel worksheet using the Veritas software version 1.5.0 and the output was expressed as relative light units (RLU).

2.6.6 Bradford assay

Protein concentrations were measured using a Bradford assay kit (Fisher Scientific Canada, Ottawa, ON). A 2 µl aliquot of each cell lysate from each triplicate was placed in an individual well of a clear, flat bottom 96-well microplate (Fisher Scientific Canada, Ottawa, ON). Aliquots of known concentrations of bovine serum albumin were tested alongside samples to establish a protein concentration standard curve. Aliquots of 200 µl Bradford assay substrate (1x) were added to each well, and the optical density was measured at 595 nm using a Spectra Max 190 plate reader (Sunnyvale, CA) and SOFTmax Pro version 3.1.2 software.

2.6.7 Statistical analyses

SAS v. 9.0 for Windows was used for statistical analysis of all concentration-response curves for all samples [70]. Ah-receptor agonism, defined as the slope of the linear portion of the concentration-response curve was calculated using ordinary least squares linear regression analysis.

Chapter 3: Results

3.1 Salmonella mutagenicity assay

The Salmonella reverse mutation assay was employed to quantify the mutagenicity of non-polar neutral and polar aromatic fractions of the SOF of diesel emissions. Both the non-polar neutral fraction and the polar aromatic fraction were assayed on three Salmonella strains (i.e., TA98, YG1041, and YG5161) with and without metabolic activation (i.e., S9).

3.1.1 Positive and negative controls

Positive and negative controls were used during each Salmonella reverse mutation experiment in order to ensure consistent, reproducible responses. The solvent (DMSO) was used as a negative control and the frequency of spontaneous revertants for each *Salmonella* strain is presented in Table 3.1. Two different positive controls (i.e., 2-aminoanthracene and 2-nitrofluorene) were used, and the number of revertants induced by each positive control for all three *Salmonella* strains is presented in Table 3.2.

Table 3.1 Mean number of spontaneous *Salmonella* revertants induced by the negative solvent control (i.e. DMSO) in the Salmonella reverse mutation assay.

Strain	S9 activation	Mean number of revertants per plate	SEM[a]	N[b]
TA98	+	53.7	± 2.9	54
	-	46.0	± 1.7	54
YG1041	+	103	± 3.5	54
	-	96.7	± 4.4	54
YG5161	+	45.6	± 1.7	54
	-	45.2	± 1.8	54

[a] Standard error of the mean
[b] Number of observations

Table 3.2 Mean number of *Salmonella* revertants induced by the positive controls in the Salmonella reverse mutation assay.

Strain	S9 Activation	Chemical	Concentration (μg/plate)	Mean number of revertants per plate	SEM[a]	N[b]
TA98	+	2-aminoantracene	0.5	448	± 23.9	54
	-	2-nitrofluorene	0.5	651	± 15.7	54
YG1041	+	2-aminoantracene	0.1	1014	± 30.8	54
	-	2-nitrofluorene	0.1	387	± 6.7	54
YG5161	+	2-aminoantracene	1	1535	± 37.9	54
	-	2-nitrofluorene	1	724	± 25.6	54

[a] Standard error of the mean
[b] Number of observations

3.1.2 Mutagenicity of the SRM

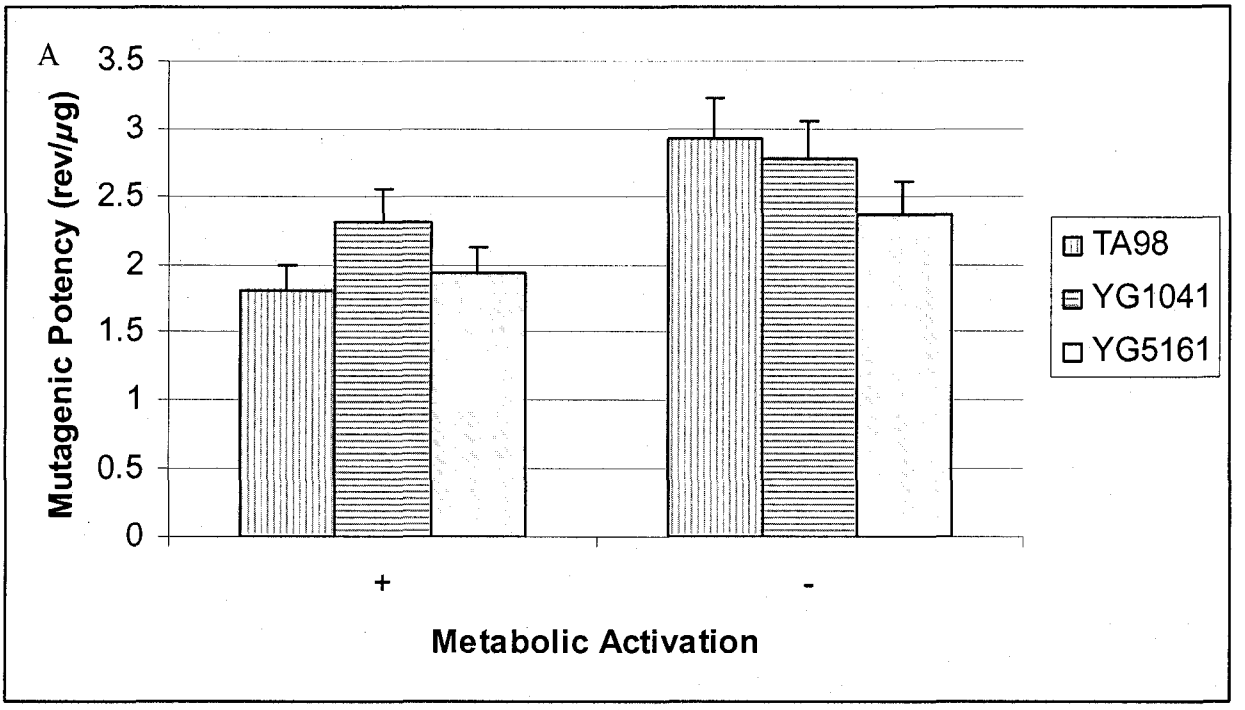
The mutagenic potency of the non-polar (i.e., PAH containing) and polar aromatic (i.e. nitro-PAH containing) fractions of two standard reference material (i.e., SRM 1650b and SRM 2975) extracts was determined using the Salmonella mutagenicity assay on strains TA98, YG1041, and YG5161. Although SRM 1650b does not represent a particular engine operating on a specific driving cycle, this material represents diesel emissions in general. SRM 2975 is intended to represent emissions of a diesel operated forklift. The mutagenic activities are presented in Table 3.3.

The non-polar fraction of both SRMs failed to yield a significant positive response on all three *Salmonella* strains assayed with and without metabolic activation. However, the polar aromatic fraction of both SRMs yielded positive responses on all three *Salmonella* strains assayed with and without metabolic activation. Figure 3.1 summarizes the mutagenic activities of polar aromatic fractions of extracts of SRM 1650b and SRM 2975 assayed with and without metabolic activation. The mutagenic activity of SRM 1650b was higher than that of SRM 2975 with the exception of the YG1041 response without metabolic activation.

Furthermore, the mutagenic activity without metabolic activation was higher than that with metabolic activation for both SRMs confirming the presence of direct-acting polar aromatic mutagens. The mutagenic potencies of both SRMs was the same (i.e., no statistical difference) for strains TA98 and YG5161 with metabolic activation ($p<0.01$). However, the mutagenic potency of both SRMs was lower with YG5161 compared to TA98 ($p<0.01$) without metabolic activation indicating that YG5161 is not as sensitive to polar aromatic compounds. For SRM 1650b, the mutagenic potency with metabolic activation was higher with YG1041 than TA98 ($p<0.01$); however, there was no statistical difference between both strains without metabolic activation ($p<0.01$). For SRM 2975, the mutagenic potency without metabolic activation was higher with YG1041 than TA98 ($p<0.01$); however, there was no statistical difference between the strains with metabolic activation ($p<0.01$).

Table 3.3 Mutagenic potencies of polar aromatic fractions of extracts of SRM 2975 and SRM 1650b determined using the Salmonella reverse mutation assay on strains TA98, YG1041, and YG5161 with and without metabolic activation.

Strain	Sample	Metabolic Activation	Mutagenic Activity (rev/μg)	Standard Error	r^2	p
TA98	1650b	+	1.8	0.18	0.99	<0.0001
		-	2.9	0.35	0.97	<0.0001
	2975	+	0.79	0.20	0.95	<0.0001
		-	2.4	0.45	0.88	<0.0001
YG1041	1650b	+	2.3	0.22	0.99	<0.0001
		-	2.8	0.41	0.95	<0.0001
	2975	+	0.94	0.22	0.96	<0.0001
		-	7.6	0.46	0.99	<0.0001
YG5161	1650b	+	1.9	0.29	0.97	<0.0001
		-	2.4	0.33	0.97	<0.0001
	2975	+	0.68	0.13	0.99	<0.0001
		-	1.3	0.29	0.92	<0.0001



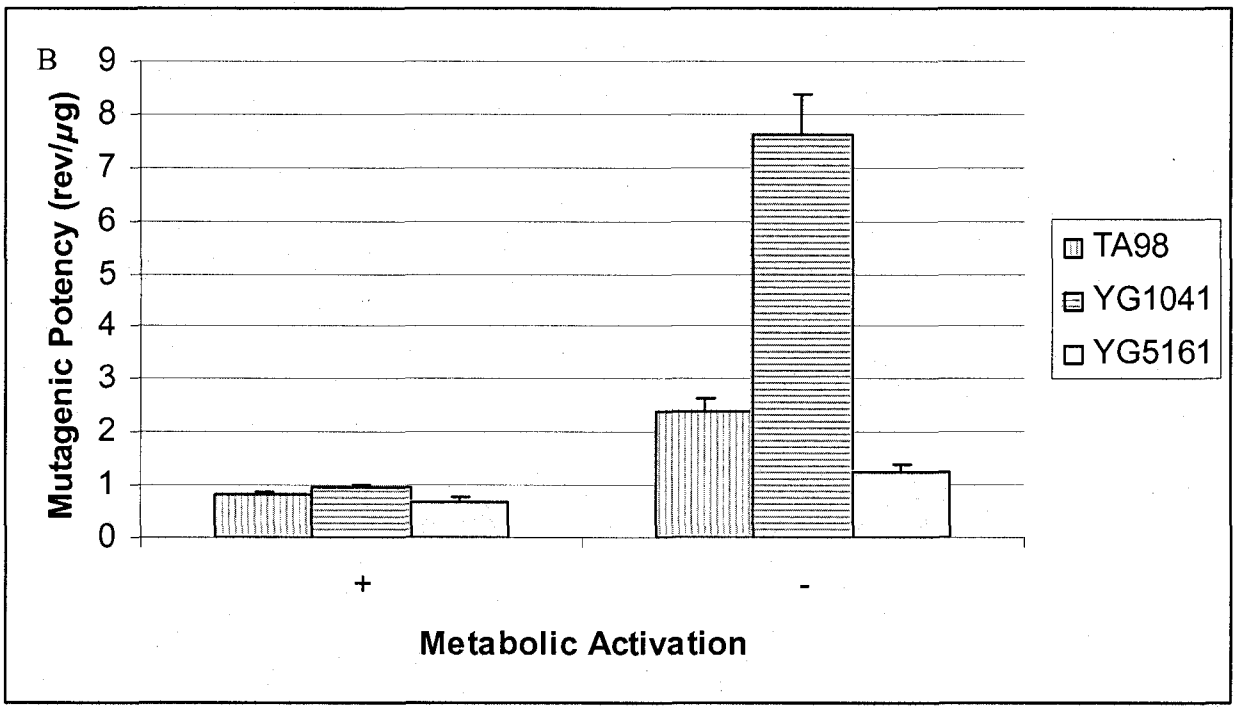


Figure 3.1 Comparison of mutagenic activities of polar aromatic fractions of (A) SRM 1650b and (B) SRM 2975 determined using the Salmonella reverse mutation assay on strains TA98 YG1041, and YG5161 with and without metabolic activation.

3.1.3 Mutagenicity of the non-polar neutral fraction of organic extracts of diesel particulates

The non-polar fraction of extracts of diesel emissions generated from both NRC and EC failed to yield any significant positive responses on all three *Salmonella* strains with and without metabolic activation (results not shown).

3.1.4 Mutagenicity of the polar aromatic fraction of organic extracts of diesel particulates provided by NRC

Mutagenic activities of the polar aromatic fractions of extracts of diesel PM generated using various canola-derived biodiesel blends were determined using the Salmonella mutagenicity assay on stains TA98, YG1041, and YG5161 with and without metabolic activation (i.e., S9). All samples generated a positive response with and without metabolic activation on strain TA98. However, extracts of PM generated from B10 blended biodiesel failed to yield a significant response on YG1041, and extracts of PM generated from B5 and B20 blended biodiesel failed to yield a significant positive response on YG5161 without the addition of S9.

The mutagenic potencies of the polar aromatic fractions of extracts of diesel PM generated using various canola-derived biodiesel blends determined using the Salmonella mutagenicity assay on strain TA98 are shown in Table 3.4. The potency of extracts of PM generated from various concentrations of biodiesel blends was similar with and without the addition of S9; however, the response was slightly higher without S9 addition. These results confirm the presence of both direct- and indirect-acting polar aromatic mutagens.

Table 3.4 Mutagenic potencies of polar aromatic fractions of extracts of diesel emission particles provided by NRC determined using the Salmonella reverse mutation assay on strain TA98 with and without metabolic activation.

Sample	Metabolic Activation	Mutagenic Activity (rev/μg)	Standard Error	r^2	p	p value Comparison with B0[a]
B0	+	0.06	0.09	0.72	<0.0001	NA
	-	0.33	0.13	0.95	<0.0001	NA
B1	+	0.27	0.22	0.96	<0.0001	<0.0001
	-	0.35	0.13	0.98	<0.0001	0.75
B2	+	0.17	0.15	0.85	<0.0001	0.04
	-	0.30	0.15	0.95	<0.0001	0.48
B5	+	0.17	0.09	0.96	<0.0001	0.02
	-	0.29	0.15	0.90	<0.0001	0.32
B10	+	0.26	0.13	0.96	<0.0001	0.0001
	-	0.15	0.12	0.92	<0.0001	0.0001
B20	+	0.17	0.10	0.96	<0.0001	0.03
	-	0.17	0.11	0.93	<0.0001	0.0009
Ref6	+	0.47	0.18	0.91	<0.0001	<0.0001
	-	0.18	0.20	0.68	<0.0001	0.53

[a]Based on ANCOVA with aliased B0 slope term.

Figure 3.2 summarizes the mutagenic potencies of all extracts of diesel PM generated from canola-derived biodiesel blends on TA98 with and without metabolic activation. An analysis of covariance revealed that the mutagenic potencies of the biodiesel generated PM extracts with S9 activation were more potent than that of the base fuel (i.e., B0 or 100 % ULSD). However, the mutagenic potencies of the PM extracts generated from biodiesel blends did not differ from each other, thus failing to confirm a relationship between biodiesel concentration and mutagenic activity. Nevertheless, the results do confirm increases in the mutagenic activity of indirect acting components of the SOF with the addition of biofuel. Conversely, the same comparison without S9 activation failed to identify significant difference between the components of the SOF generated using biodiesel blends B1, B2, or

B5, and the B0 base fuel. However, the mutagenic potencies of the components of the SOF generated using biodiesel blends B10 and B20 were lower than that of the base fuel, suggesting a decrease in the mutagenic activity of direct acting components of the SOF with increasing biofuel concentration.

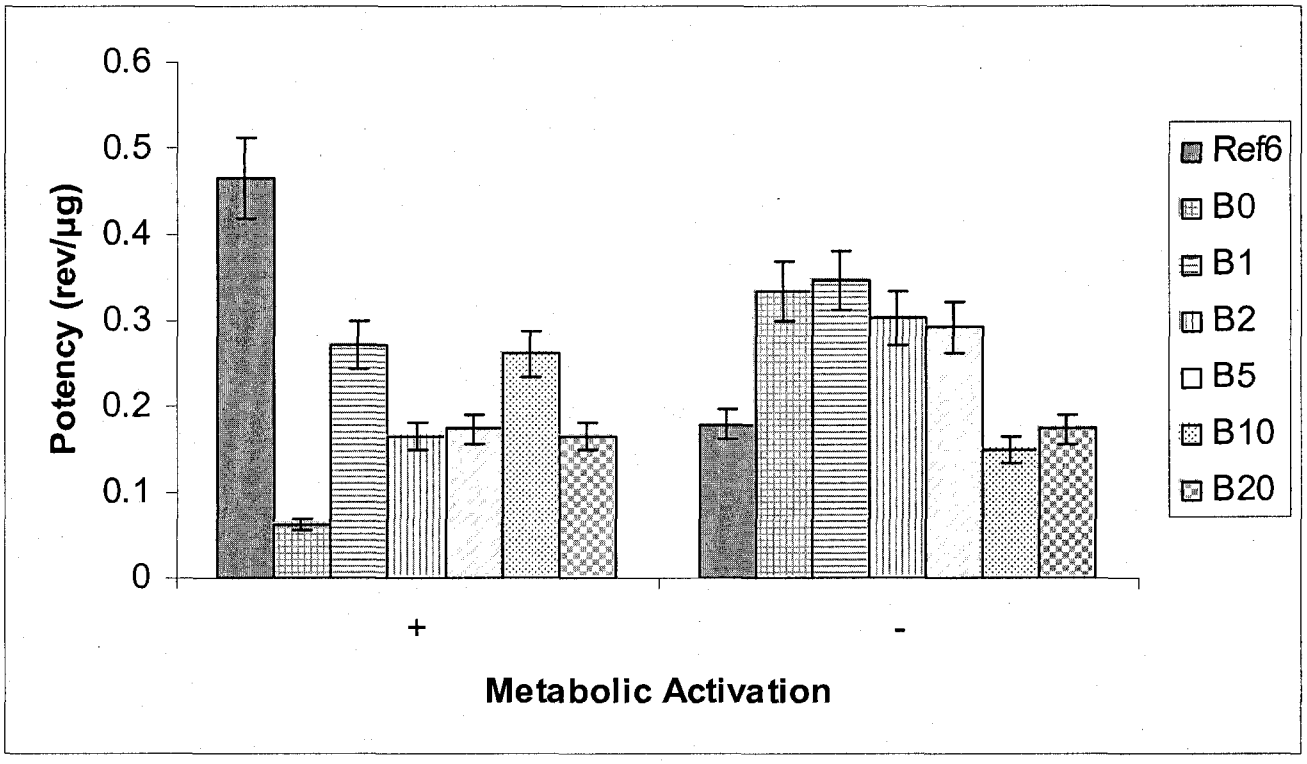


Figure 3.2 Comparison of mutagenic activities of polar aromatic fractions of diesel extracts (NRC) for all biodiesel blends determined using the Salmonella reverse mutation assay on strain TA98 with and without metabolic activation.

The mutagenic potencies of the polar aromatic fractions of extracts of diesel PM generated using various canola-derived biodiesel blends determined using the Salmonella mutagenicity assay on strain YG1041 are shown in Table 3.5. The potencies of extracts of PM generated from various concentrations of biodiesel blends were similar with and without the addition of S9; however, the response was slightly higher without S9 addition. These results confirm the presence of both direct- and indirect-acting polar aromatic mutagens.

Table 3.5 Mutagenic potencies of polar aromatic fractions of extracts of diesel emission particles provided by NRC determined using the Salmonella reverse mutation assay on strain YG1041 with and without metabolic activation.

Sample	Metabolic Activation	Mutagenic Activity (rev/μg)	Standard Error	r^2	p	p value Comparison with B0[a]
B0	+	0.22	0.2	0.76	<0.0001	NA
	-	0.31	0.11	0.97	<0.0001	NA
B1	+	0.20	0.14	0.91	0.0008	0.99
	-	0.42	0.10	0.99	<0.0001	0.1
B2	+	0.08	0.14	0.69	<0.0001	0.04
	-	0.25	0.16	0.91	<0.0001	0.5
B5	+	0.27	0.20	0.82	<0.0001	0.29
	-	0.37	0.14	0.97	<0.0001	0.41
B10	+	0.25	0.14	0.94	<0.0001	0.32
	-	NM	-	-	-	<0.0001
B20	+	0.21	0.13	0.93	<0.0001	0.91
	-	0.60	0.20	0.94	<0.0001	0.0006
Ref6	+	0.44	0.15	0.96	<0.0001	0.0002
	-	0.49	0.18	0.93	<0.0001	0.19

NM=not mutagenic at these concentrations
[a]Based on ANCOVA with aliased B0 slope.

Figure 3.3 summarizes the mutagenic potencies of all extracts of diesel PM generated from canola-derived biodiesel blends on YG1041 with and without metabolic activation. An analysis of covariance on the mutagenic potencies of the biodiesel generated PM extracts with S9 activation indicated that all the blends did not differ from each other or the base fuel (i.e., B0 or 100 % ULSD), with the exception of the SOF of diesel PM generated from biodiesel blend B2. Once more, these results failed to confirm a relationship between biodiesel concentration and mutagenic activity. Conversely, the same comparison without S9 activation failed to identify any trend in mutagenic potencies of the SOF of diesel PM generated from various concentrations of biodiesel blends. Potencies of the samples increased and decreased as biodiesel in the fuel increased. The potency of the SOF of diesel

PM generated from B20 biodiesel yielded the highest response; however, the SOF of diesel PM generated from B10 failed to induce a statistically significant positive response.

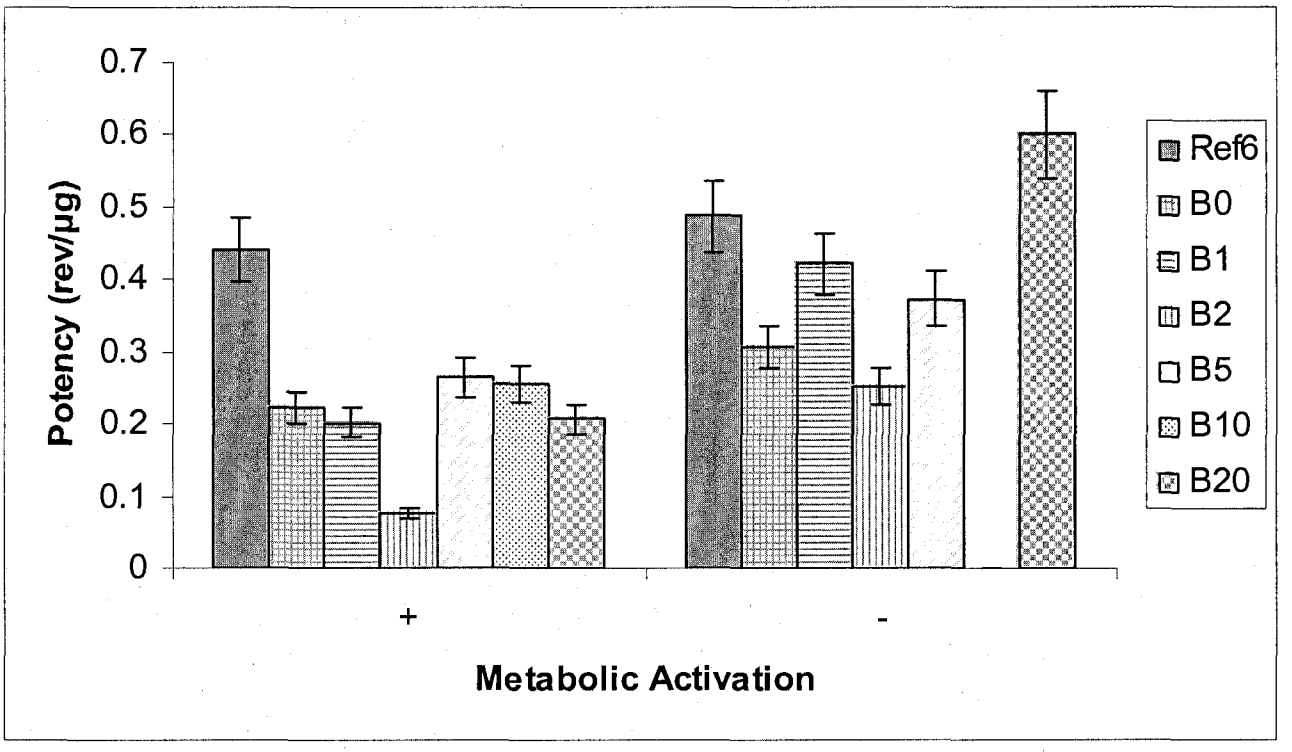


Figure 3.3 Comparison of mutagenic activities of polar aromatic fractions of diesel extracts (NRC) for all biodiesel blends determined using the Salmonella reverse mutation assay on strain YG1041 with and without metabolic activation.

The mutagenic potencies of the polar aromatic fractions of extracts of diesel PM generated using various concentrations of canola-derived biodiesel blends determined on strain YG5161 are shown in Table 3.6. The potencies of extracts of diesel PM generated with biodiesel fuel are comparable with and without the addition of S9. These results suggest the presence of slightly less potent direct and indirect acting polar aromatic mutagens.

Table 3.6 Mutagenic potencies of polar aromatic fractions of extracts of diesel emission particles provided by NRC determined using the Salmonella reverse mutation assay on strain YG5161 with and without metabolic activation.

Sample	Metabolic Activation	Mutagenic Activity (rev/μg)	Standard Error	r^2	p	P value Comparison with B0[a]
B0	+	0.09	0.11	0.87	0.0002	NA
	-	0.18	0.12	0.88	<0.0001	NA
B1	+	0.24	0.13	0.96	<0.0001	<0.0001
	-	0.14	0.13	0.88	<0.0001	0.09
B2	+	0.15	0.11	0.95	<0.0001	0.012
	-	0.16	0.11	0.94	<0.0001	0.36
B5	+	0.09	0.09	0.85	<0.0001	0.98
	-	NM	-	-	-	<0.0001
B10	+	0.17	0.10	0.94	<0.0001	0.002
	-	0.09	0.08	0.95	<0.0001	0.005
B20	+	0.22	0.11	0.96	<0.0001	<0.0001
	-	NM	-	-	-	<0.0001
Ref6	+	0.49	0.18	0.93	<0.0001	<0.0001
	-	0.67	0.18	0.96	<0.0001	<0.0001

NM=not mutagenic at these concentrations
[a]Based on ANCOVA with aliased B0 slope

Figure 3.4 compares the mutagenic potencies of all the extracts of the SOF of diesel PM generated using canola-derived biodiesel blends on strain YG5161 with and without metabolic activation. An analysis of covariance on the mutagenic potencies of these samples with S9 activation indicated that all the blends, with the exception of B5, were more potent than B0 (i.e., 100 % ULSD base fuel) However, the mutagenic potencies of the biodiesel blends did not differ from each other (again with the exception of B5). These results suggest a slight increase in the mutagenic activity of the indirect acting components of the SOF generated with the addition of biofuel, but no consistent trend in mutagenic activity with increasing biofuel concentration. In contrast, the same comparison without S9 activation indicated that extracts of the SOF of diesel PM generated with biodiesel blends B1 and B2 were not statistically different from the B0 base fuel. Furthermore, the mutagenic potencies

of the SOF of diesel PM generated with biodiesel blends B5, B10, and B20 were lower than that of the base fuel sample, suggesting a decrease in the mutagenic activity of direct acting components of the SOF as biofuel concentration in the fuel increases. These results are consistent with the results obtained on strain TA98; however, the mutagenic potencies were lower on strain YG5161.

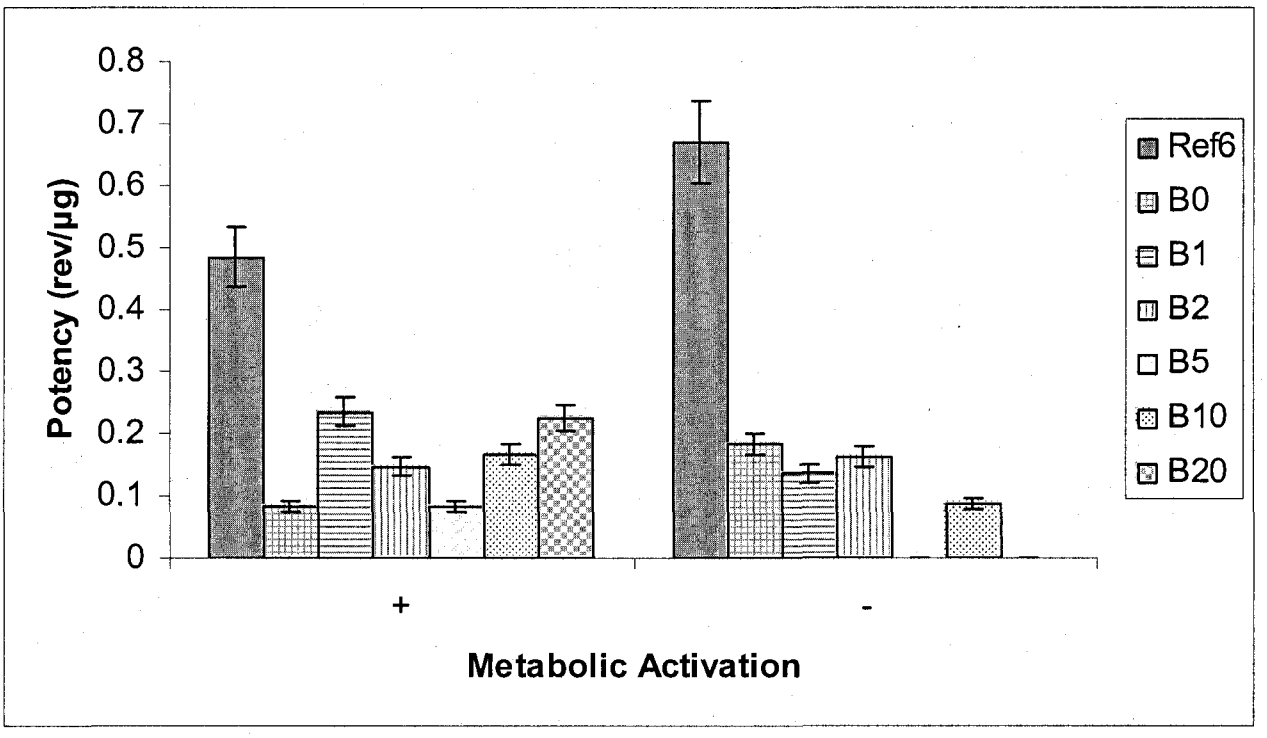
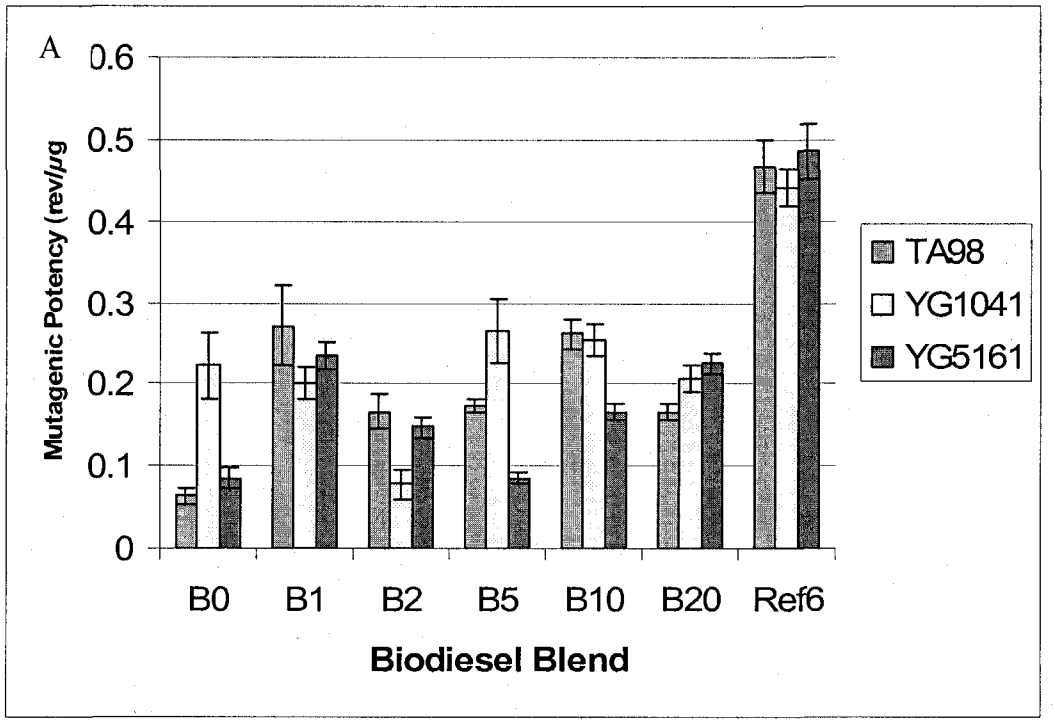


Figure 3.4 Comparison of mutagenic activities of polar aromatic fractions of diesel extracts (NRC) for all biodiesel blends determined using the Salmonella reverse mutation assay on strain YG5161 with and without metabolic activation.

Figure 3.5 compares the mutagenic activities of polar aromatic fractions of diesel extracts (NRC) for all biodiesel blends determined using the Salmonella reverse mutation assay on strains TA98, YG1041, and YG5161 with and without the addition of S9. ANCOVA (analysis of covariance) was employed to simultaneously analyze all the Salmonella mutagenicity data (by fraction, strain and S9) and examine interactions between the sample and concentration effect variates. Table 3.7 summarizes the results from the

analysis of covariance. The results confirmed heterogeneity of slopes and a significant effect of sample source on the concentration effect (i.e., a sample*concentration interaction) for selected assays (i.e., strain, S9 combinations).



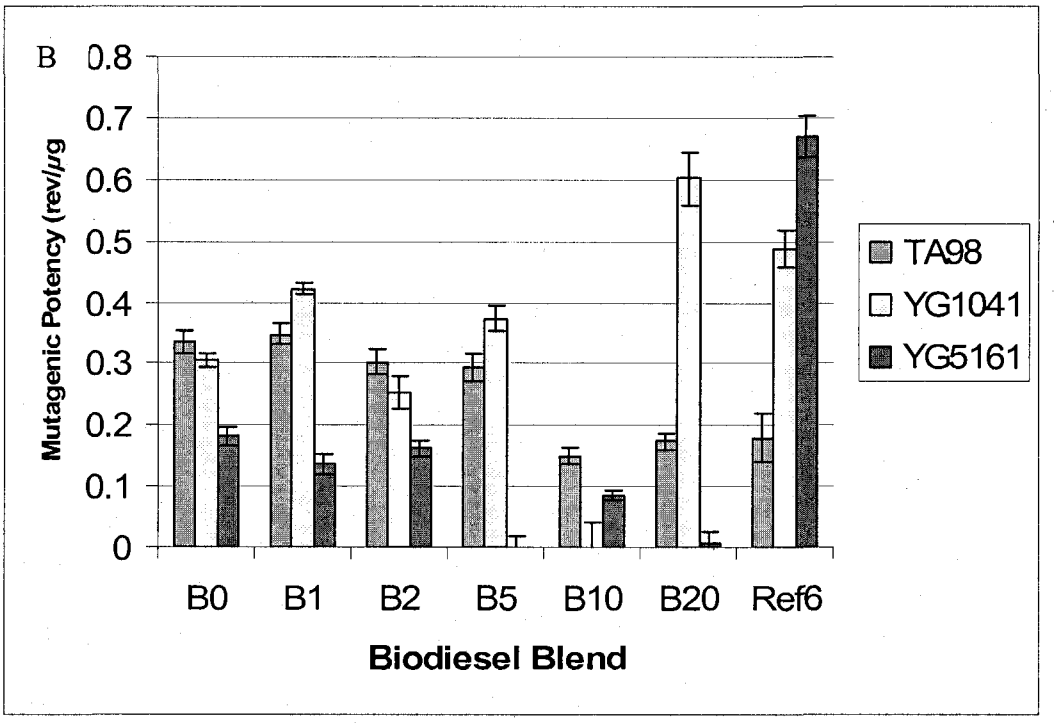


Figure 3.5 Comparison of mutagenic activities of polar aromatic fractions of diesel extracts (NRC) for all biodiesel blends determined using the Salmonella reverse mutation assay on strains TA98, YG1041, and YG5161 with (A) and without (B) metabolic activation.

Table 3.7 ANCOVA results showing the effects of concentration and the sample*concentration interaction on the response variable (i.e., revertants/plate).

Strain	Fraction	S9	Effect Variable	F ratio	p-value
TA98	2	Y	Concentration	0.1	0.76
			Concentration*Sample	0.89	0.52
		N	Concentration	1.06	0.31
			Concentration*Sample	0.94	0.49
	3	Y	Concentration	437.4	<0.0001
			Concentration*Sample	19.2	<0.0001
		N	Concentration	634	<0.0001
			Concentration*Sample	6.9	0.0004
YG1041	2	Y	Concentration	4.56	0.34
			Concentration*Sample	1.05	0.42
		N	Concentration	0.47	0.5
			Concentration*Sample	0.95	0.48
	3	Y	Concentration	314.2	<0.0001
			Concentration*Sample	10.6	<0.0001
		N	Concentration	294.1	<0.0001
			Concentration*Sample	12.2	<0.0001
YG5161	2	Y	Concentration	110.5	<0.0001
			Concentration*Sample	3.1	0.0236
		N	Concentration	0.51	0.48
			Concentration*Sample	1.01	0.44
	3	Y	Concentration	807.8	<0.0001
			Concentration*Sample	36.1	<0.0001
		N	Concentration	148	<0.0001
			Concentration*Sample	31.8	<0.0001

3.1.5 Mutagenicity of the polar aromatic fraction of organic extracts of diesel particulates and PUFs provided by EC

Extracts of PUFs, which constitute the volatile components of diesel emissions, failed to yield any positive responses on the three *Salmonella* strains with and without metabolic activation.

Five of the eight filter extracts of the SOF of diesel PM generated from ULSD and B20 blends of soy-, animal-, and canola-derived biodiesel provided by EC failed to yield any statistically significant responses. Two of the samples were generated from ULSD and soy-derived B20 biodiesel fuel combusted in an engine operating on the FTP transient driving cycle. The other three were samples generated from ULSD, soy- and canola-derived B20 biodiesel fuels combusted in an engine operating on the steady-state driving cycle. Two samples generated with the FTP driving cycle yielded a positive response: extracts of diesel PM generated from B20 canola biodiesel both with and without a DOC. The third sample to yield a positive response was an extract of PM generated from animal-derived B20 fuel combusted in an engine operating on a steady-state driving cycle. Table 3.8 summarizes the mutagenic potencies of the samples that generated a positive response.

Table 3.8 Mutagenic potencies of polar aromatic fractions of extracts of diesel emission particles provided by EC determined using the Salmonella reverse mutation assay with strains TA98, YG1041, and YG5161 with and without metabolic activation.

Sample[a]	Strain	Metabolic Activation	Mutagenic Activity (rev/μg)	Standard Error	r^2	p
B20 Canola No DOC (FTP)	TA98	+	0.57	0.04	0.94	<0.0001
B20 Canola No DOC (FTP)	TA98	-	0.71	0.02	0.95	<0.0001
B20 Canola No DOC (FTP)	YG1041	+	0.44	0.06	0.92	<0.0001
B20 Canola No DOC (FTP)	YG1041	-	0.77	0.05	0.93	<0.0001
B20 Canola (FTP)	TA98	+	0.39	0.03	0.92	<0.0001
B20 Canola (FTP)	TA98	-	0.39	0.02	0.96	<0.0001
B20 Canola (FTP)	YG1041	-	0.37	0.04	0.97	<0.0001
B20 Canola (FTP)	YG1041	+	NM	-	-	-
B20 Animal (SS)	TA98	-	0.14	0.009	0.90	<0.0001

NM=Not mutagenic. FTP=Federal Testing Procedure, SS=steady-state

[a] Letters in parenthesis refer to the driving cycle used to generate diesel PM. See section 2.2.1 and Table 2.1 for further details.

Both samples from the FTP driving cycle (i.e., B20 Canola without a DOC and B20 Canola with a DOC) yielded a positive mutagenic response on *Salmonella* TA98 and YG1041 both with and without metabolic activation. However, the sample from the SS driving cycle (i.e., B20 animal with DOC) only generated a positive mutagenic response on *Salmonella* TA98 without metabolic activation. These results suggest the presence of direct- and indirect-acting polar aromatic mutagens in the SOF of diesel PM generated from biodiesel blends. However, the nature and magnitude of the response depend on the source of the biodiesel used to generate the PM. Figure 3.6 illustrates the mutagenic potencies for the three samples that generated a positive response on *Salmonella* strains TA98 and YG1041 with and without metabolic activation. The results of an ANCOVA analysis comparing the slopes of the samples that elicited a positive response are shown in Table 3.9. The analysis reveals that all of the slopes are statistically different from one another (i.e., for each strain, S9 comparison). These results suggest that the use of a diesel oxidation catalyst and the choice of driving cycle play a role in reducing the mutagenicity of direct- and indirect-acting polar aromatic compounds found in the SOF of diesel PM.

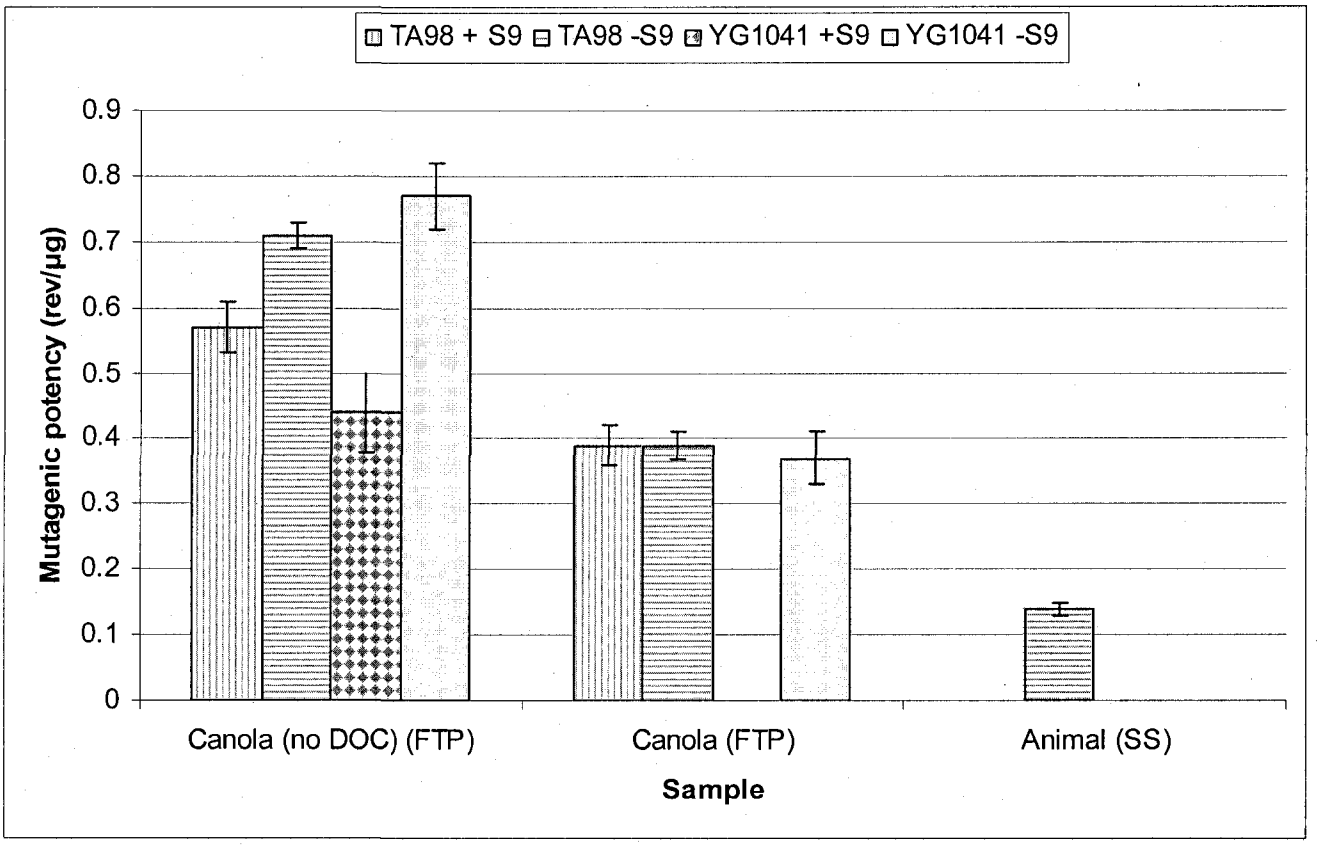


Figure 3.6 Comparison of mutagenic activities of polar aromatic fractions of diesel filter extracts (EC) for the three samples that generated a positive response on the Salmonella reverse mutation assay with TA98 with and without metabolic activation.

Table 3.9 Results of ANCOVA custom contrasts comparing the slopes of samples that elicited a positive mutagenic response in the Salmonella mutagenicity assay.

Strain	S9	Contrast	F ratio	p-value
TA98	Y	B20 canola (no DOC) versus B20 canola	3.22	0.09
		B20 canola (no DOC) versus B20 animal	72.31	<0.0001
		B20 canola versus B20 animal	37.61	<0.0001
	N	B20 canola (no DOC) versus B20 canola	22.22	<0.0001
		B20 canola (no DOC) versus B20 animal	113.91	<0.0001
		B20 canola versus B20 animal	18.68	0.0003
YG1041	Y	B20 canola (no DOC) versus B20 canola	4.32	0.05
		B20 canola (no DOC) versus B20 animal	43.22	<0.0001
		B20 canola versus B20 animal	15.04	0.0007
	N	B20 canola (no DOC) versus B20 canola	15.39	0.0006
		B20 canola (no DOC) versus B20 animal	89.31	<0.0001
		B20 canola versus B20 animal	19.04	0.0002

3.2 DR-CALUX assay

3.2.1 Positive and negative controls

Positive and negative controls were included during every experiment in order to ensure data quality. NRC samples were tested alongside a single concentration of benzo[*a*]pyrene (BaP) (0.5 µg/ml medium), whereas EC samples were tested alongside 8 concentrations in order to obtain a full concentration-response curve. The negative solvent control used during every experiment was 0.1% (v/v) DMSO. Results of luciferase induction by both positive and negative controls are shown in Table 3.10 and Figure 3.7. The average

EC_{50} for BaP was 3.1E-03 ± 2.1E-04 µg/ml, and the average maximum response was 24.34E-03 RLU/µg protein ± 0.24.

Table 3.10 Mean Ah-receptor agonism response of the negative solvent control (i.e., DMSO) and positive control determined by the DR-CALUX assay.

Control	Concentration	Response (RLU[a] E-03/µg protein)	SEM[b]	N[c]
Benzo[*a*]pyrene	0.5 µg/ml	23.7	±1.5	42
DMSO	1 µl/ml	0.39	±0.04	42

a Relative luciferase units
b Standard error of the mean
c Number of observations

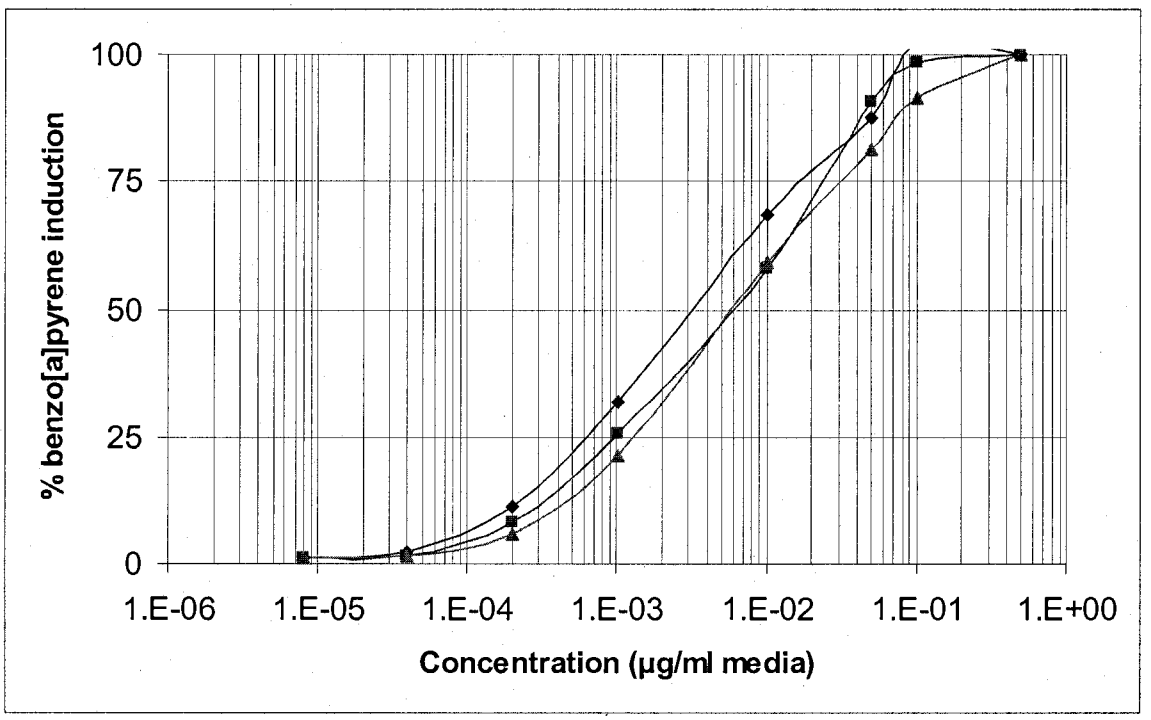


Figure 3.7 Ah-receptor response of benzo[*a*]pyrene determined using the DR-CALUX assay. The results of three separate experiments are shown.

3.2.2 Dioxin-like effects of the SRM

Two standard reference materials of diesel emissions were tested for their ability to induce the Ah-receptor response in the DR-CALUX assay. Extracts of SRM 2975 and SRM 1650b were separated into non-polar neutral and polar aromatic fractions, and tested at 6 concentrations. Figure 3.8 illustrates the concentration-response curves for both fractions of SRM 2975 and SRM 1650b. Both fractions of the organic extracts from SRM 2975 and SRM 1650b induced a positive response in this assay; however, all responses yielded by both fractions of the SOF of SRM 1650b were higher. Furthermore, the slopes for both fractions of the SOF of SRM 1650b were higher than those of SRM 2975, thus indicating that extracts of SRM1650b are more potent Ah-receptor agonists.

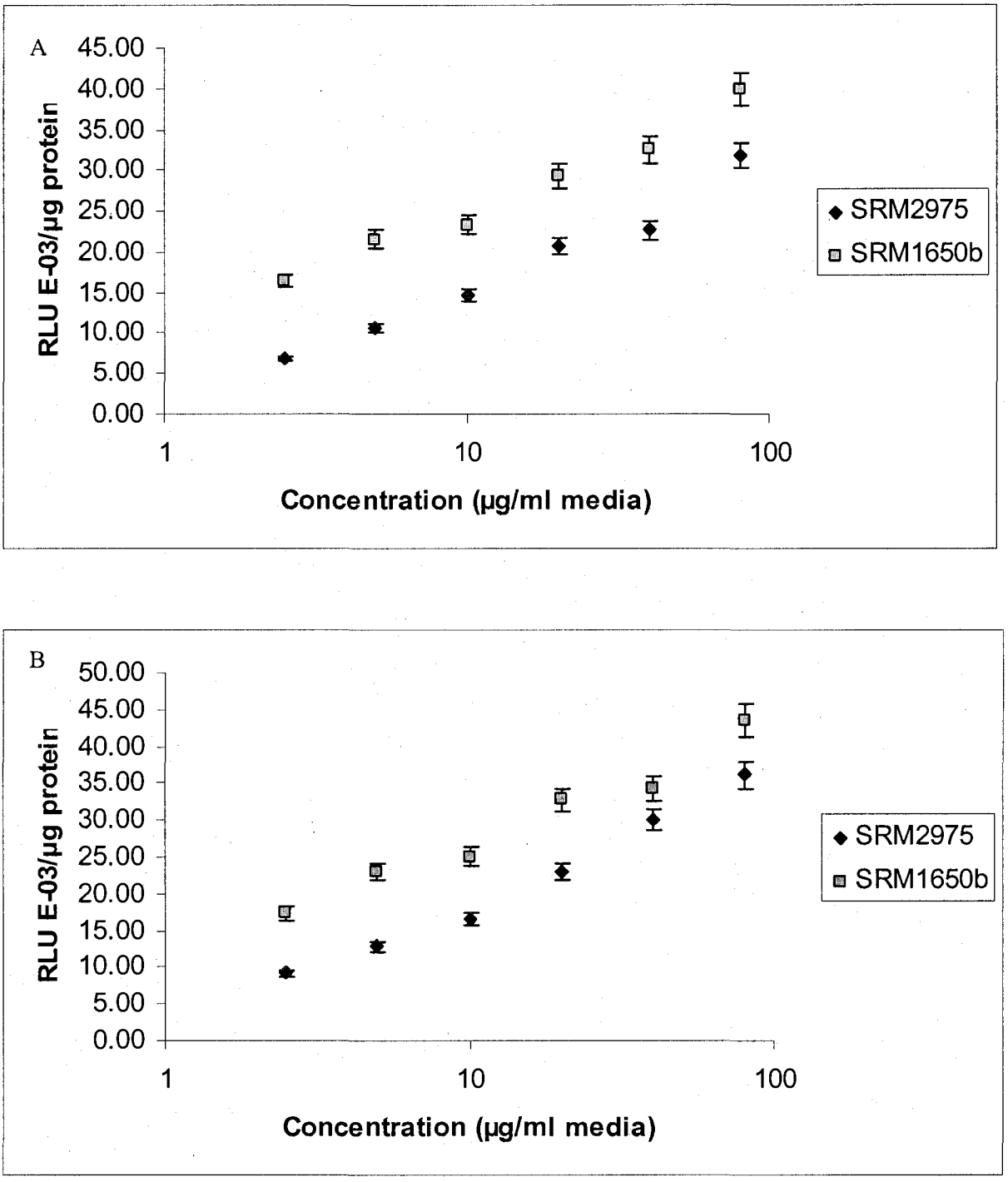


Figure 3.8 Comparisons of the Ah-receptor response of non polar fractions (A) and polar aromatic fractions (B) of the SOF of SRM1650b and SRM 2975 determined using the DR-CALUX assay.

3.2.3 Dioxin-like effects of the polar aromatic and non-polar neutral fractions of organic extracts of diesel particulates generated from various canola biodiesel blends

Diesel emissions generated from ULSD and five canola-derived biodiesel blends (i.e., B1, B2, B5, B10, B20), as well as a reference fuel (i.e., Ref6, a winter grade Shell fuel used as an internal standard), were tested for their ability to induce the Ah-receptor pathway using the DR-CALUX assay. Non-polar neutral and polar aromatic fractions of each sample were tested at six concentrations in triplicate, and Figures 3.9 and 3.10 illustrate the concentration-response curves for the non-polar fraction and polar aromatic fraction respectively.

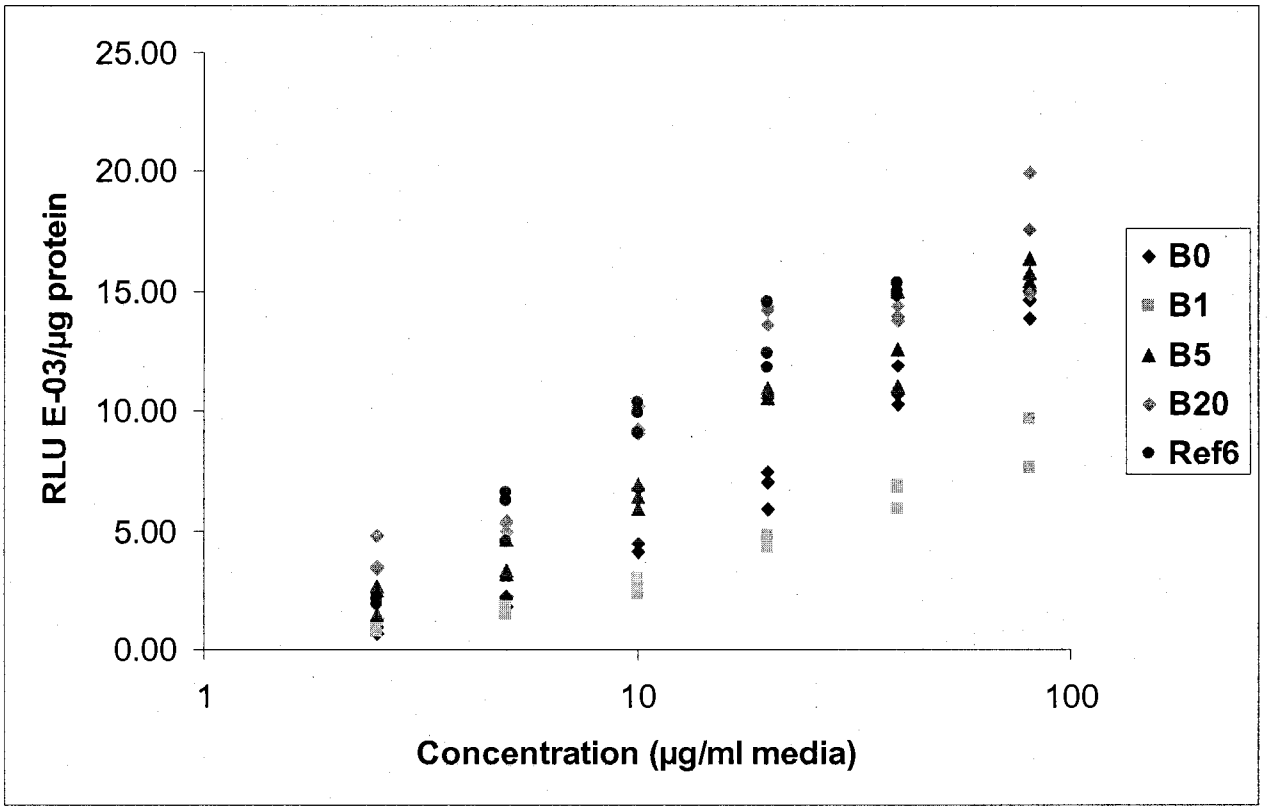


Figure 3.9 Comparisons of Ah-receptor response for the non-polar aromatic fractions of diesel extracts (NRC) for all biodiesel blends determined using the DR-CALUX assay. Each concentration was tested in triplicate.

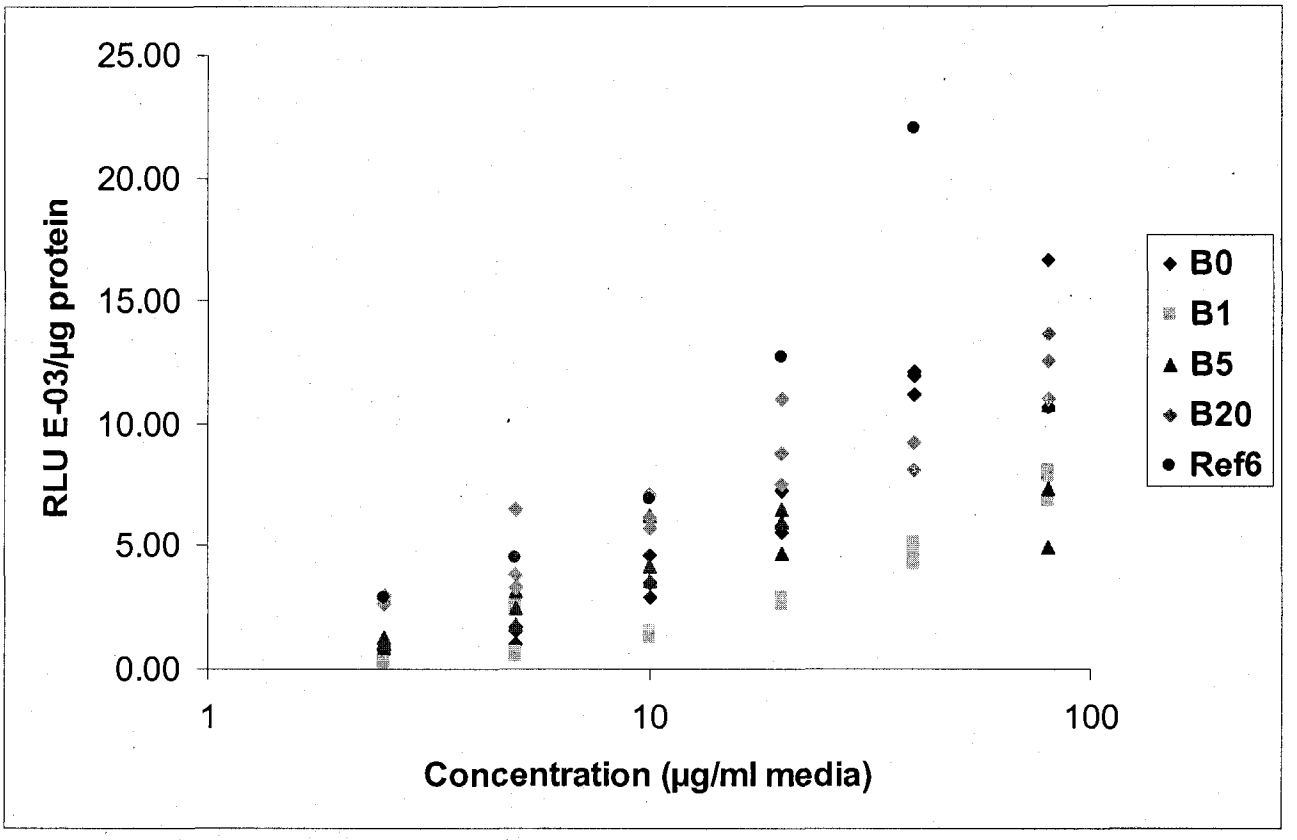


Figure 3.10 Comparisons of the Ah-receptor response for the polar aromatic fractions of diesel extracts (NRC) determined using the DR-CALUX assay. Each concentration was tested in triplicate.

Filter blanks (i.e., blank filters extracted and fractionated using the identical methodology) were also assayed for their ability to induce the Ah-receptor response. The responses obtained were equivalent to that of the negative solvent control, and therefore, the filter medium and extraction protocol did not affect sample responses.

Both the non-polar and polar aromatic fractions were able to induce the Ah-receptor pathway and generate a luciferase product, thus indicating the presence of non-polar and polar aromatic Ah-receptor agonists in the samples. However, PM generated from B2 and B10 biodiesel blends were not able to induce an Ah-receptor response. Table 3.11 presents the slope values for both fractions of the SOF generated from biodiesel blends and both

SRMs determined using the DR-CALUX assay. None of the samples reached a response plateau when plotted on a log scale suggesting that the response pathway was not saturated at the highest exposure level.

Table 3.11 Potency of the Ah-receptor response for the non-polar and polar aromatic fractions of the SOF of filters provided by NRC. Results are expressed as the slope of the linear portion of the concentration-response curve. Ah-receptor agonism was determined using the DR-CALUX reporter-gene assay.

Sample	Fraction	r^2	p	Slope (RLU[b] E-03/µg protein/ µg/ml media)	Standard Error	p value Comparison with B0[c]
B0	non-polar	0.95	0.0009	0.27	0.031	NA
	polar	0.92	0.0007	0.19	0.025	NA
B1	non-polar	0.96	0.0006	0.15	0.016	0.02
	polar	0.98	<0.0001	0.09	0.006	0.0003
B5	non-polar	0.99	0.0002	0.50	0.024	0.0046
	polar	0.91	0.124	0.28	0.051	0.24
B20	non-polar	0.96	0.003	0.66	0.074	<0.0001
	polar	0.93	0.0073	0.40	0.061	0.0073
Ref6	non-polar	0.92	0.009	0.62	0.105	0.0001
	polar	0.99	0.0003	0.59	0.031	<0.0001
SRM1650b	non-polar	0.67	0.0867	1.1	0.46	NA
	polar	0.72	0.0699	1.3	0.47	NA
SRM2975	non-polar	0.91	0.0124	0.93	0.17	NA
	polar	0.87	0.0215	1.0	0.23	NA

NA=not applicable
[a] Number of observations
[b] Relative luciferase units
[c] Based on ANCOVA with aliased B0 slope

The most potent sample for both fractions, as determined by the slope of the linear portion of the concentration response curve, was the SOF of PM from the B20 blend; the least potent was the sample generated from the B1 blend. A slope comparison for both fractions is illustrated in Figure 3.11. The non-polar fractions yielded better induction of the Ah-receptor response in comparison to the polar aromatic samples (i.e., a greater slope value). An analysis of covariance revealed that although there was no significant difference between slopes of the B0 and B1 blends, there was a significant difference between the slope of the B0 blend and the slopes of the B5 and B20 blends for both fractions. These results

suggest that increasing concentrations of biodiesel in the fuel enhance the ability of components of the SOF of diesel emissions to stimulate the Ah-receptor response pathway.

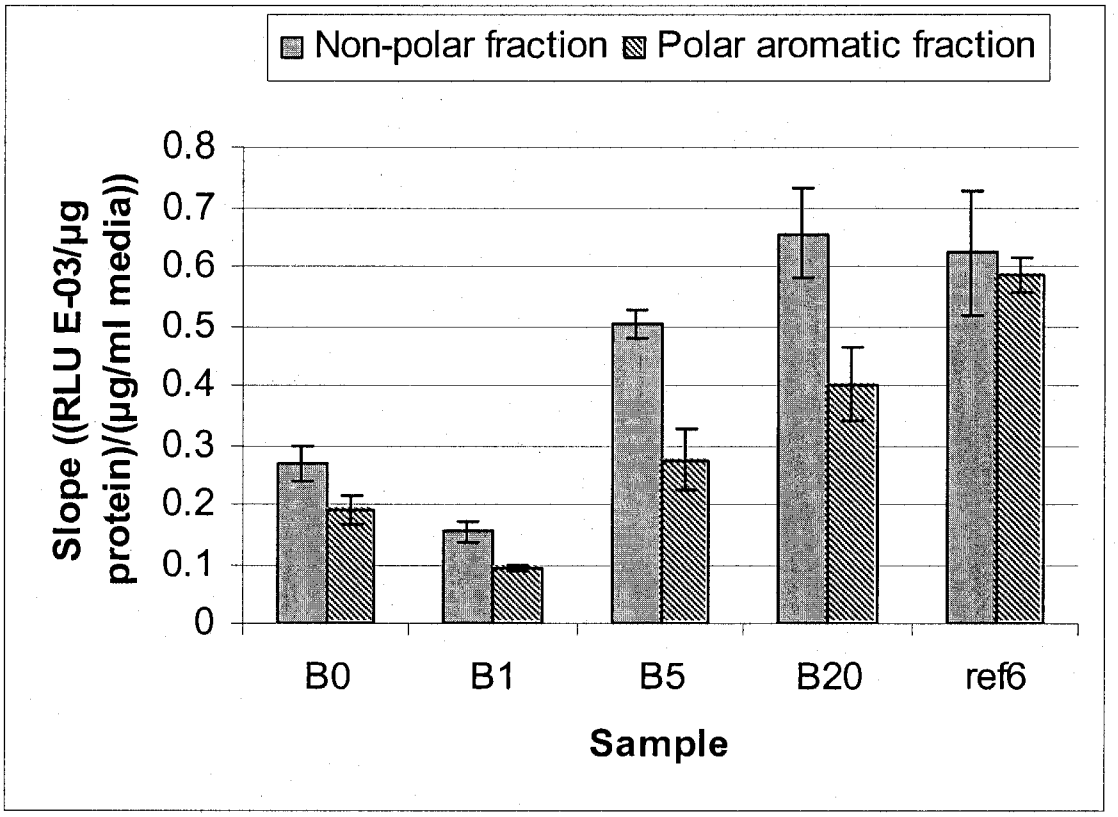


Figure 3.11 Slope comparisons for the Ah-receptor response (i.e., DR-CALUX response) of polar and non-polar aromatic fractions of diesel extracts (NRC) for all biodiesel blends examined.

3.2.4 Dioxin-like effects of the polar aromatic and non-polar fractions of organic extracts of diesel particulates and PUFs generated from various biodiesel fuels

Non-polar and polar aromatic fractions of the SOF of diesel emissions generated using three biodiesel blended fuels (i.e., B20 canola, soy, and animal) and one ULSD with the FTP and SS driving cycles (see section 2.2.1 and Table 2.1 for further sample information) were tested for their ability to induce the Ah-receptor response using the DR-CALUX assay. Neither fraction of the PUF extracts was able to induce an Ah-receptor response. Filter blanks (i.e., blank filters extracted and fractionated using the identical

methodology) were also assayed for their ability to induce the Ah-receptor response. The responses obtained for both the polar aromatic and non-polar fractions indicated that the filter medium and/or extraction protocol affected sample responses, and therefore filter blank responses (different for both fractions) were subtracted from the sample response in order to account for background induction of the response pathway.

Non-polar and polar aromatic fractions of each sample were tested in triplicate at six concentrations, and Figures 3.12 and Figure 3.13 illustrate the concentration-response curves for the non-polar fraction and polar aromatic fraction, respectively. Three samples did not induce the Ah-receptor response: the SOF of PM generated from soy-derived B20 (FTP) and animal fat-derived B20 (SS) and ULSD (SS). Five of the eight filter samples were able to induce a statistically significant Ah-receptor response: the SOF of PM generated from ULSD (FTP), B20 biodiesel derived from Canola (no DOC, FTP), Canola (FTP), Canola (SS) and Soy (SS).

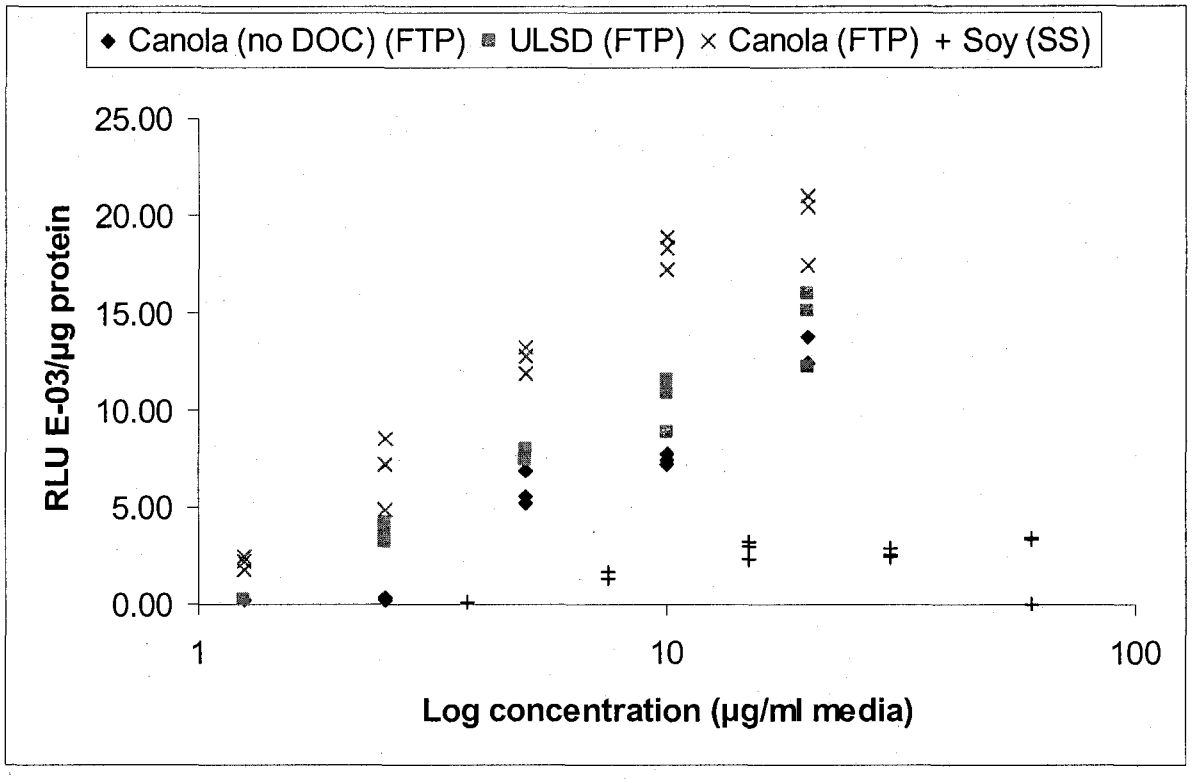


Figure 3.12 Comparisons of the Ah-receptor response for the non-polar aromatic fractions of diesel extracts (EC) determined using the DR-CALUX assay. Each concentration was tested in triplicate.

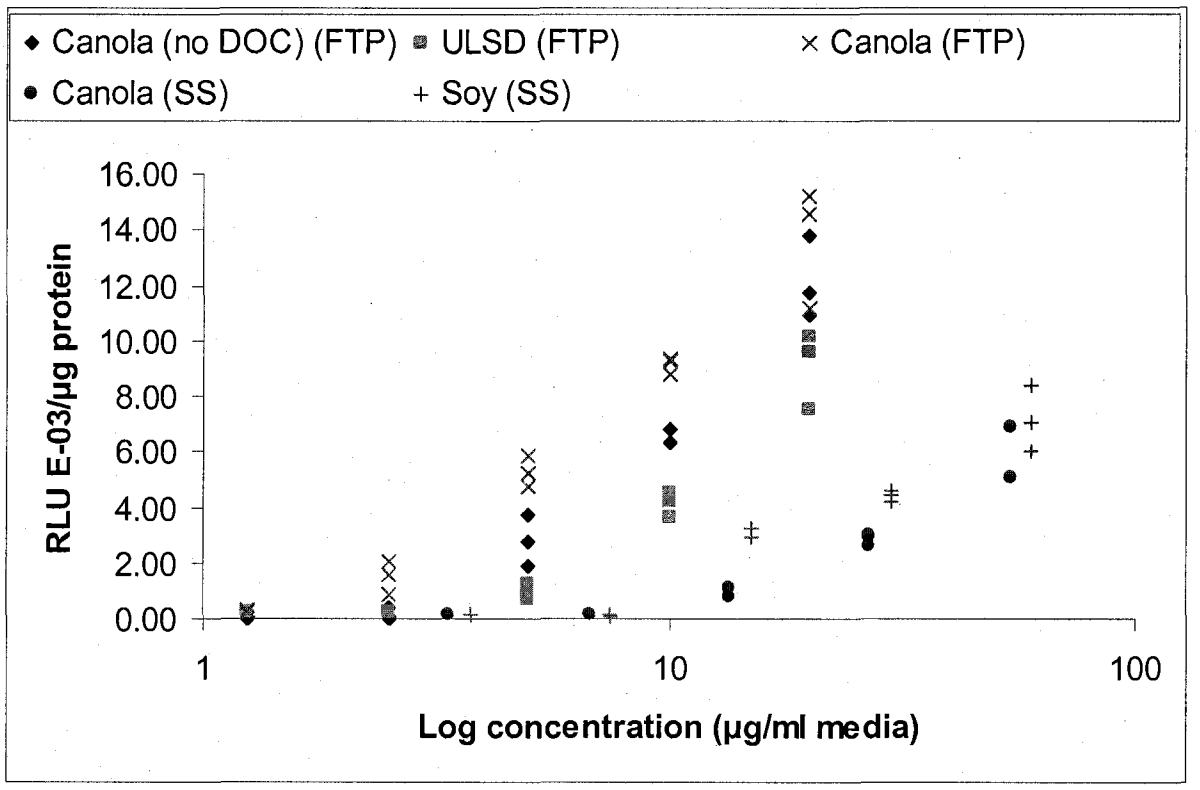


Figure 3.13 Comparisons of the Ah-receptor response for the polar aromatic fractions of diesel extracts (EC) determined using the DR-CALUX assay. Each concentration was tested in triplicate.

Table 3.12 presents the slopes of the concentration response curves that elicited a positive response. None of the samples reached a plateau of maximum induction, suggesting that the response pathway was never saturated.

Table 3.12 Potency of the Ah-receptor response for the non-polar and polar aromatic fractions of the SOF of filters provided by EC. Results are expressed as the slope of the concentration-response curve. Ah-receptor agonism was determined using the DR-CALUX reporter-gene assay. Only samples that yielded a positive response are shown. Refer to section 2.2.1 and Table 2.1 for sample information.

Sample	Fraction	N[a]	r^2	p	Slope (RLU[b] E-03/μg protein/ μg/ml media)	Standard Error
B20 Canola (no DOC) (FTP)	non-polar	18	0.96	0.039	0.33	0.04
	polar	18	0.99	0.0023	0.30	0.014
ULSD (FTP)	non-polar	18	0.88	0.018	0.32	0.067
	polar	18	0.98	0.012	0.23	0.026
B20 Canola (FTP)	non-polar	18	0.95	0.0049	0.92	0.12
	polar	18	0.96	0.004	0.34	0.043
B20 Canola (SS)	polar	18	0.99	0.0065	0.06	0.0047
B20 Soy (SS)	non-polar	18	0.99	0.05	0.07	0.006
	polar	18	0.93	0.0018	0.06	0.008

FTP=Federal Test Procedure, SS=steady-state
[a] Number of observations
[b] Relative luciferase units

The most potent sample, as determined by the slope of the linear portion of the concentration-response curve, was the non-polar fraction of the SOF of PM generated from B20 Canola (FTP) and the polar aromatic fraction of the SOF of PM generated from B20 Canola (no DOC). Figure 3.15 illustrates the slope comparisons for both fractions of samples that generated a positive response in the DR-CALUX assay. The non-polar fraction was more potent than the polar aromatic fraction for the SOF of PM generated from B20 Canola (FTP) and B20 Canola (SS); however, there was no statistical difference for the

samples generated from B20 Canola (no DOC) (FTP), ULSD (FTP) and B20 Soy (SS). These results suggest that both fractions are equally capable of inducing the Ah-receptor response pathway. Furthermore, the slope values were lower for PM generated using the steady-state driving cycle suggesting that driving conditions might play a role in the ability of the components of the SOF of biodiesel emissions to induce an Ah-receptor response.

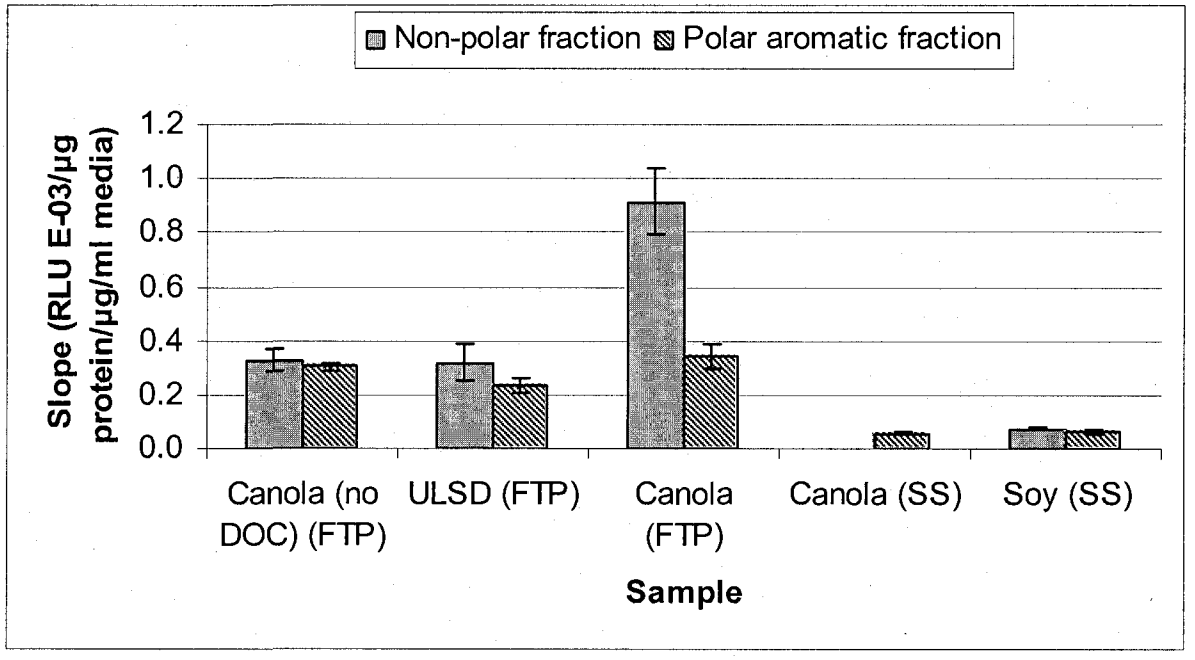


Figure 3.14 Slope comparisons of the Ah-receptor response (i.e., DR-CALUX response) for polar aromatic and non-polar fractions of diesel extracts (EC) for the biodiesel blends examined.

These results suggest that the potency of the SOF of PM generated from B20 biodiesel blends is dependant on the source of the biofuel, the driving cycle, and the use of a diesel oxidation catalyst. Furthermore, the SOF of PM generated from the canola-derived biodiesel fuels seem to be more potent inducers of the Ah-receptor response than that of other biodiesel fuels examined in this study,.

Table 3.13 Results of ANCOVA custom contrasts comparing the slopes of samples that elicited a positive response in the DR-CALUX assay.

Fraction	Contrast	F ratio	p-value
non-polar	B20 Canola (no DOC) (FTP) versus ULSD (FTP)	0.02	0.89
	B20 Canola (FTP) versus ULSD (FTP)	24.01	0.0006
	B20 Canola (no DOC) (FTP) versus B20 Canola (FTP)	23.16	0.0007
	B20 Canola (no DOC) (FTP) versus B20 Soy (SS)	6.76	0.03
	B20 Canola (FTP) versus B20 Soy (SS)	38.25	0.0001
	ULSD (FTP) versus B20 Soy (SS)	6.2	0.03
polar	B20 Canola (no DOC) (FTP) versus ULSD (FTP)	2.79	0.12
	B20 Canola (FTP) versus ULSD (FTP)	7.15	0.02
	B20 Canola (no DOC) (FTP) versus B20 Canola (FTP)	0.9	0.36
	B20 Canola (SS) versus B20 Soy (SS)	0.08	0.78
	ULSD (FTP) versus B20 Canola (SS)	29.9	0.0001
	B20 Canola (FTP) versus B20 Canola (SS)	89.01	<0.0001
	B20 Canola (no DOC) (FTP) versus B20 Canola (SS)	59.01	<0.0001
	ULSD (FTP) versus B20 Soy (SS)	29.87	0.0001
	B20 Canola (FTP) versus B20 Soy (SS)	91.09	<0.0001
	B20 Canola 9no DOC) versus B20 Soy (SS)	59.76	<0.0001

Chapter 4: Discussion

. Exposures to combustion-derived materials in environmental and occupational settings have been associated with a range of health effects. The most alarming and oft-cited effect is cancer (i.e., induction of malignant neoplasia). These combustion-derived materials, including coal tar, vehicle emissions, etc, contain a wide range of toxic agents including benzene, metals, and PACs. The PACs, and more specifically the PAHs, are the focus of a great deal of attention due to the fact that many are carcinogenic in experimental animals, and exert their effect via the induction of gene mutations. These combustion-derived materials are highly complex mixtures and comparative hazard assessment using chemical analyses has been criticized (i.e., hazard may be related to hitherto unknown components). For this reason bioassays are extremely useful for the comparative toxicological assessment of combustion-derived materials such as vehicular exhaust.

The ability of complex mixtures to induce mutations and dioxin-like effects can have serious repercussions. DNA mutations in specific genes (i.e., tumour suppressor genes, cell cycle regulators, etc) can alter the expression of proteins resulting in the development of neoplastic lesions (i.e., cancer). Furthermore, the ability of a complex mixture to induce an Ah-receptor response can also lead to negative outcomes. The Ah-receptor regulates the transcription of various genes encoding Phase 1 and Phase II enzymes of xenobiotic metabolism, as well as several proteins involved in the control of cell growth (e.g., plasminogen activator inhibitor) [62]. Modifications in the expression of these proteins/enzymes can affect cellular detoxification pathways and cell growth. Binding of PAHs to the Ah-receptor upregulates the transcription of P450 enzymes, which in turn metabolise PAHs into potent mutagens capable of forming DNA adducts.

In vitro bioassays are convenient tools used to evaluate the ability of complex mixtures to induce gene mutations and/or induce the Ah-receptor pathway and exert a

dioxin-like effect. They are employed to provide an indication of potential hazard, and are often used to justify the use of more complex *in vivo* systems for further investigation. Two *in vitro* assays that have been extensively used in recent years to investigate the toxicological hazards of complex matrices are the Salmonella mutagenicity assay and the DR-CALUX assay. The former measures the ability of the mixture and/or the mixture components to induce DNA mutations in genetically modified strains of *Salmonella*. The latter measures the ability of the mixture and/or the mixture components to bind to the Ah-receptor and induce a pathway response (i.e., dioxin-like effect). This study employed these *in vitro* assays to provide a rapid, comparative assessment of the mutagenic and dioxin-like activity of the SOF of diesel emissions generated using biodiesel blended fuels.

4.1 Mutagenicity of the standard reference materials

The non-polar fraction of both SRMs failed to yield a positive response on all three *Salmonella* strains. The fractionation technique employed to separate the components of the SOF of diesel PM was tailored to isolate the PAHs and alkyl-PAHs in the non-polar fraction [68]. It has been previously shown in the literature that the mutagenic potency of a complex PAH mixture can be attributed to the mutagenic effect of benzo[*a*]pyrene in that mixture [71,72]. However, the concentration of BaP adsorbed to PM of SRM 2975 and 1650b was very low (i.e., 0.05 mg/kg and 1.17 mg/kg for SRM 2975 and SRM 1650b respectively) [33,34]. At the concentrations examined in this study (i.e., maximum of 800 μg of particles per plate), the concentration of PAHs in the extracts was below the detection limit for S9-activated mutagenicity.

The polar aromatic fraction of both standard reference materials generated a positive response with and without metabolic activation on all three *Salmonella* strains. The

mutagenic potency of whole extracts of SRM 1650b and SRM 2975 has been previously determined in many studies [33,34]. The mutagenic activity of whole extracts of SRM 1650b and SRM 2975 was determined as part of an international collaborative study sponsored by the International Program on Chemical Safety (IPCS). Twenty laboratories from across the world participated and the average mutagenic potencies were determined. Particulate material was extracted using soxhlet or sonication procedures with DCM. The observed mutagenic potency for SRM 1650b with S9 metabolic activation ranged from 1.5 to 3.5 revertants per μg of extracted particles, and 2.1 to 3.8 revertants per μg of extracted particles without S9 on *Salmonella* TA98 [33,73,74]. Similarly, the observed mutagenic potency for SRM 1975 (i.e., a soxhlet DCM extract of SRM 2975) with S9 activation ranged from 125 to 147 revertants per μg of organic extract (3.4 to 4.0 revertants per μg particle), and 462 to 587 revertants per μg of organic extract (12.4 to 15.8 revertants per μg particle) without S9 activation on *Salmonella* TA98 [75-77]. The results obtained in this study for the polar aromatic fraction of SRM1650b fall within the range observed in the IPCS study both with and without metabolic activation for strain TA98 (i.e., 1.8 rev/μg particles with S9 and 2.9 rev/μg particles without S9). On the other hand, the mutagenic potency of the polar aromatic fraction of SRM 2975 obtained in this study is substantially different from the values observed in the IPCS study (i.e., 0.79 rev/μg particles and 2.4 rev/μg particles). Although the potencies determined in this study illustrate the same general trends (i.e., mutagenic potency without metabolic activation is higher than that with metabolic activation), the values are approximately 4-5 fold lower that those noted in the certificate of analysis [34]. A separate study by DeMarini et al. [78] determined the mutagenic potency of a DCM extract (i.e., soxhlet extraction) of SRM 2975 separated on open silica into hexane, 50:50 hexane:DCM, DCM, and methanol fractions. The average mutagenic potency

obtained on *Salmonella* strain TA98 for the hexane/DCM fraction (i.e., polar aromatic fraction) was 4.4 rev/μg particles without S9 and 1.9 rev/μg particles with S9. DeMarini et al. concluded that the mutagenic potency per μg particle is dependant on the efficiency of the extraction process (i.e., % extractible mass), and reduced extraction efficiency may be the cause of the lower potencies obtained for SRM 2975 in this study. Nevertheless, the body of published evidence, in addition to the results obtained in this study, indicates that the mutagenicity of diesel emissions is primarily determined by polar aromatic compounds, including nitro-PAHs, present in the SOF. In contrast, the less polar compounds including homocyclic, unsubstituted PAHs appear to play a relatively minor role in determining the Salmonella mutagenic activity of extractable organics associated with diesel particulate matter [78].

4.2 Dioxin-like effects of the standard reference material

Both the polar and non-polar fractions of SRM 1650b and SRM 2975 yielded a positive response in the DR-CALUX assay. These results suggest that both polar and non-polar compounds associated to the SOF of diesel PM emissions can bind to the Ah-receptor and induce transcription of genes regulated by dioxin response elements (i.e., CYP 1A). Thus, it is clear that organic extracts of diesel PM emissions can induce dioxin-like effects.

There are no published studies that have evaluated the dioxin-like effect of SRM 1650b and SRM 2975 using the DR-CALUX assay. However, one study by Mahadevan et al. [79] evaluated the ability of a recreated mixture of PAHs representing SRM1650b to induce the expression of CYP 1A1 in MCF-7 cells, measured using the EROD assay. Cells were exposed to 50 μl of an 8 mg/ml (i.e., 400 μg) mixture (i.e., sufficiently similar to 1650b) for 24 hours. The mixture induced an approximately 13-fold expression of CYP 1A1

over the DMSO control. Thus, these results confirm that PAHs in a complex mixture can induce dioxin-like effects.

The DR-CALUX slope values indicate that both fractions of SRM1650b and SRM2975 were equally effective at generating an Ah-receptor-mediated response (i.e., slope for the non-polar fraction did not differ from the slope of the polar aromatic fraction). However, SRM1650b was more effective than SRM2975 at inducing a positive response (i.e., higher slope value). These results suggest that the non-polar neutrals and polar aromatic components of the SOF of diesel particulates are equally capable of binding to the Ah-receptor to induce a dioxin-like effect; moreover, the higher PAH concentrations (per unit mass of particles) in SRM1650b is likely responsible for a greater response at a lower concentration (i.e., higher slope).

4.3 Mutagenicity of organic extracts from diesel and biodiesel emission particulates

Both the polar and non-polar fractions of PUF extracts (i.e., EC samples) that represent the volatile component of diesel emissions failed to generate a positive response in the Salmonella mutagenicity assay. The lack of response from the volatile (i.e., PUF) components of diesel emission extracts was not unexpected. Previous studies have shown that approximately 90% of the mutagenic compounds (i.e., PAHs of 4 or more rings) are found in the particulate phase of diesel PM. The PAHs present in the volatile component of the SOF of diesel PM are usually of low molecular weight (i.e., 3 rings or less), and have been shown to be less potent and non-mutagenic [80]. However, it is also possible that the concentration of PAHs and nitro-PAHs in the PUF extracts was below the detection limit for mutagenicity. Perhaps repeating the experiment using *Salmonella* strain TA100, and at

higher concentrations could provide more insight on the ability of these extracts to induce a mutagenic response.

The non-polar fraction of both the EC and the NRC samples failed to generate a positive response in the Salmonella mutagenicity assay when tested both with and without metabolic activation. The lack of response from the non-polar (i.e., PAH-containing) fraction without S9 activation was not unexpected. Numerous studies have shown that PAHs require conversion to active intermediates by cytochrome P450 isozymes (e.g., 1A1 and 1A2) in order to elicit a mutagenic response [81,82]. However, the lack of response for the non-polar fraction in the presence of metabolic activation was unexpected. Although most studies show that the mutagenic activities of extracts of diesel emissions can be attributed to substances in more polar fractions, positive responses from the non-polar fraction have been observed [83]. There are many possible explanations for the failure to detect a positive mutagenic response from this particular fraction. First, it is possible that the compounds in the non-polar neural fractions (i.e., PAHs and alkyl-PAHs) of the tested emissions are not mutagenic, or unable to elicit a significant positive response at the concentrations examined in this study. Second, competition by both non-mutagenic and mutagenic compounds in the fraction could have limited the amount of P450 isozymes (i.e., S9 activation enzymes) available to transform the mutagenic PAHs into active metabolites [84]. In other words, the concentration of S9 employed in this study may have been sub-optimal for the detection of mutagenic activity in the non-polar neutral fraction. Numerous studies have noted that the standard concentration of S9 extract recommended by Maron and Ames [58] and Mortelmans and Zeiger [55] are not necessarily optimal for the examination of extracts from complex matrices such as diesel particulate matter. However, the limited supply of collected particulate material did not permit an examination of the optimal S9 concentration required

to examine the diesel samples studied. Moreover, since there is no information on the chemical composition of the samples studied (i.e., chemical composition and concentration), it is difficult to pinpoint the exact reason for the lack of mutagenic response. Nonetheless, the lack of responses for the non-polar fractions is consistent with that obtained for SRM 1650b and SRM 2975.

The hypothesized absence of potent mutagenic PAHs and alkyl-PAHs in the non-polar fraction of extracts of diesel emissions is supported by the lack of enhanced mutagenic activity on *Salmonella* strain YG5161. This strain harbours the pYG768 plasmid that allows over-expressions of the *dinB* gene [57]. The *dinB* gene encodes a Y class DNA polymerase [85] that participates in translesion synthesis at damaged G:C base pairs [86]. However, since it lacks exonuclease activity, it is error-prone [86] and the polymerase has 10- to1000-fold less fidelity than replicative DNA polymerases acting on undamaged DNA templates [86]. Therefore, translesion synthesis by Y-family polymerases (i.e., polymerase IV) carries an implicit mutagenic potential and the addition of the *dinB* gene to *Salmonella* strain YG5161 confers enhanced sensitivity to a variety of chemicals including PAHs such as benzo[*a*]pyrene and its oxygenated biotransformation products [57,87,88]. However, the strain does not possess added sensitivity to nitroarenes such as 1-nitropyrene and its derivatives [57]. As a result, the mutagenic potencies of the polar aromatic fractions determined using this strain were comparable to those obtained using strain TA98.

The Salmonella mutagenic activity of the non-polar aromatic fractions of diesel emissions generated from various blends of canola-derived biodiesel fuels ranged from 60 to 470 revertants per mg of particles with S9 activation, and 90 to 770 revertants per mg particles without S9 activation. These values are very similar to those observed for unfractionated extracts of diesel emissions generated under similar conditions [74,89,90].

For example, Bunger et al. [90] examined the mutagenic activity of a DCM extract of diesel PM emissions generated from diesel fuel (fuel not specified) and biodiesel (i.e., 100% rape-seed methyl esters) on strain TA98 with and without metabolic activation. Although the extracts were not fractionated, the mutagenic responses were similar to those obtained in this study. The mutagenic activity for the diesel fuel was 596 revertants per mg of particles without S9, and 569 revertants per mg of particles with S9. The activity of the biodiesel fuel was 333 revertants per μg of particles without S9 and 293 revertants per μg of particles with S9.

Earlier work by Lundsetdt et al. [68] showed that the polar aromatic fraction will contain nitro-PAHs, oxy-PAHs (e.g., ketones and quinones), aromatic amines, and N-heterocyclics. The use of metabolically enhanced strains like YG1041 can provide information on the degree to which the response is influenced by nitro-PAHs and aromatic amines (including N-heterocyclics). The addition of plasmid pYG233 to the YG1041 strain allows over-expression of the *cnr* and *OAT* genes that encode the *Salmonella* classical nitroreductase and O-acetyltransferase respectively. These enzymes play an important role in the transformation of nitro-PAHs and aromatic amines into active mutagens (refer to Figure 5). The results obtained in this study confirm the presence of direct- and indirect-acting mutagens in the polar aromatic fractions, and the direct acting mutagenicity is likely due to potent nitro-PAHs present in the sample. Nitro-PAHs do not require metabolic activation in order to produce DNA adducts in *Salmonella* [91,92] and these results are supported by the slightly higher response obtained on strain YG1041 in comparison to TA98. However, the indirect-acting mutagenicity is likely a result of aromatic amines and N-heterocyclics that must be converted to hydroxylamines by P450 enzymes in order to elicit a mutagenic response [93]. The presence of nitroarenes and aromatic amines in extracts of

diesel emissions is not surprising since earlier studies have shown that they are the primary inducers of mutagenicity in such samples [78,94,95].

4.4 Dioxin-like effects of organic extracts of diesel and biodiesel emission particulates

Neither fraction of the volatile components of diesel emissions (i.e., PUFs) induced a dioxin-like effect. A study by Bagley et al. [32], showed that the use of an oxidation catalyst not only reduces the amount of PM in diesel emission, it can also reduce the volatile components of the emission including PAHs and their derivatives by 90% [32]. It is possible that the lack of response from the volatile component of the exhaust samples examined in this study may be explained by the reduction of PAHs associated to the volatile component of diesel/biodiesel emissions. Furthermore, it is also possible that the concentrations of the components of the volatile compounds are below detection limit to induce a dioxin-like response.

Both the polar aromatic and non-polar neutral extracts of diesel particulates generated using biodiesel blended fuels were able to induce an Ah-receptor response (i.e., a dioxin-like effect). PAHs, alkyl-PAHs, and nitro-PAHs alone or in complex mixtures have been shown to bind to the Ah-receptor and induce a dioxin-like response in numerous bioassays [48,96-100]. Dioxin-like responses are usually expressed as TCDD equivalents which are a ratio of the EC_{50} of the compound or mixture relative to the EC_{50} of TCDD. However, for the purpose of this study, benzo[*a*]pyrene was chosen as a positive control. PAHs such as BaP are present in the samples investigated and therefore, such a comparison is more relevant. Furthermore, in biological systems, PACs are metabolized and cleared more rapidly, therefore, using TCDD as a positive control probably has little relevance to estimating the potential toxicity of mixtures primarily composed of PAHs [98]. In order to fully illustrate

the nature of the responses obtained in this study, it would have been appropriate to express the data using three assessment endpoints: EC_{50} value, the slope of the linear portion of the concentration response curve (i.e., induction potency), and the maximum response observed. However, since none of the responses induced an induction plateau, it was impossible to calculate an accurate and reliable maximum response or EC_{50} value. Therefore, the results obtained were solely expressed as the slope of the linear portion of the concentration response curve.

The slope values of the non-polar fractions were generally higher than those observed for the polar aromatic fraction. These results suggest that non-polar neutrals such as PAHs and alkyl-PAHs present in extracts of diesel particulates generated using various blended biodiesel fuels are more potent Ah-receptor agonists than the polar compounds such as oxy-PAHs, nitroarenes, N-heterocyclics and aromatic amines. Although the results obtained suggest that non-polar neutrals in biodiesel particulates are potent Ah-receptor agonists, it is interesting to note that a study by Bunger et al. [101] showed that despite lower concentrations of monitored PAHs and nitro-PAHs, diesel particulates generated using methyl-ester fuels had a higher percentage of extractable material. Moreover, they noted that the higher amount of extractable material was likely due to the higher amounts of unburned fuel adsorbed onto the solid black carbon. Therefore, it seems unlikely that the increased Ah-receptor response observed for the non-polar neutral fraction is due solely to PAHs and similar compounds. Rather, the Bunger et al. study suggests that the increased response of the non-polar neutral fraction may be caused by a higher concentration of olefins (unburned fuel) present in the exhaust. Additional analyses would be required to confirm this assertion. Nevertheless, it should be noted that S- and O-heterocyclics may also contribute to the potent response of the non-polar neutral fraction. These compounds elute in the non-polar fraction

[68,102], and although their concentrations in extracts of diesel emissions are unknown, a thorough chemical analysis is warranted in order to determine their possible contributions to the observed Ah-receptor response.

4.5 Relationship between biodiesel fuel source/concentration and the toxicity of organic extracts of particulate emissions

Extracts of particulate emissions generated from five canola-derived biodiesel blends (i.e., B1, B2, B5, B10, and B20) and one ULSD fuel (i.e., B0) were assayed for their ability to induce a mutagenic or dioxin-like response. All six polar aromatic fractions of these extracts yielded a positive mutagenic response in the Salmonella mutagenicity assay on all three strains with and without metabolic activation. Although chemical analyses of the emission samples was not available for this study, other research has shown that PAH and nitro-PAH concentrations in the SOF of diesel PM emissions decrease when combusting blended biodiesel fuels (i.e., B20 blends), regardless of the driving cycle [103-105]. This relationship is confirmed by the results obtained on *Salmonella* strains TA98 and YG5161 without metabolic activation. Although these strains do not possess additional copies of the *cnr* and *OAT* genes, *Salmonella* is inherently capable of producing the nitroreductase required to transform nitro-PAHs into active mutagens [106]. The mutagenic activities of the samples on these two strains decreased as biodiesel concentration in the fuel increased, and it seems likely that the trend is related to decreasing concentrations of nitroarenes in the samples. As mentioned previously, nitro-PAHs are direct-acting mutagens that do not require the addition of a metabolic activation system to elicit a mutagenic response in the Salmonella mutagenicity assay [92,95]. Therefore, these results are consistent with earlier results, and support the contention that nitro-PAH concentrations are reduced in the SOF of

diesel particulates generated using bio-blended fuels. Despite the agreement between the published, chemical-specific studies of nitro-PAHs in biodiesel particulates, and the Salmonella mutagenicity results observed on TA98 and YG5161, the YG1041 results did not show a consistent relationship with biodiesel concentration. The mutagenic activities of the samples on the metabolically-enhanced YG1041 both increased and decreased as biodiesel concentration in the fuel blend increased (i.e., there was no apparent trend). The lack of mutagenic activity for PM generated from the B10 blend and the increased mutagenic activity for PM generated from the B20 blend were unexpected and can be considered contradictory. Thus, although the results obtained in this study do provide some evidence to support the contention that biodiesel supplementation can decrease the mutagenic activity of particulate emissions, a more detailed examination on the effect of biodiesel supplementation on the mutagenic activity of emissions seems a promising area for further research. Unfortunately, sample limitations did not permit re-examination of the biodiesel particulates studied here.

All three polar aromatic and non-polar neutral fractions of extracts of diesel particulates generated from canola-derived biodiesel blends (i.e., B1, B5, and B20), and one ULSD fuel (i.e., B0) yielded a positive response in the DR-CALUX assay. For both the non-polar and polar fractions the slope values increased as biodiesel concentrations in the fuel increased. These results suggest that extracts of particulate emissions generated from blends with higher concentrations of biofuel have an increased ability to induce an Ah-receptor response. As already noted, the concentrations of PAHs and nitro-PAHs in the SOF of diesel emissions decrease with increasing concentrations of biodiesel in the fuel [90,103,104]. Therefore, it is unlikely that these compounds are responsible for the increasing dioxin-like response observed. However, ligands for the Ah-receptor include a variety of hydrophobic

aromatic compounds [62], and these could include compounds such as oxy-PAHs and olefins. These compounds are rarely included in routine chemical analyses and investigations into the production and emission of these compounds in biodiesel emissions seems a promising area for further research. Moreover, future research could employ an effect-directed fractionation approach to determine the physical-chemical properties of the putative Ah-receptor agonists in biodiesel emissions.

Extracts of PM emissions generated using three biodiesel B20 blends (i.e., B20 canola (no DOC), B20 soy, and B20 canola) and one ULSD operating on the FTP transient driving cycle and three biodiesel B20 blends (i.e., B20 canola, B20 soy, and B20 animal) and one ULSD operating on a steady-state driving cycle were assayed for their ability to induce a mutagenic and dioxin-like response. Not only did these samples provide information on the profiles of PM generated from various biodiesel sources, it also provided information of the influence of different driving cycle (i.e., FTP versus SS), and the influence of a diesel oxidation catalyst on the toxicity of diesel PM. Only three of the eight polar aromatic fractions of diesel particulate extracts yielded a positive response on *Salmonella* strains TA98 and YG1041 (i.e., B20 canola (no DOC, FTP), B20 canola (FTP) and B20 animal (SS)). It should be noted that both extracts of PM from ULSD fuels (i.e., both FTP and SS driving cycles) failed to yield a positive mutagenic response; however during this particular experiment (i.e., one day of testing) the positive controls (i.e., 2AA and 2NF) also failed to elicit a significant positive. Thus, it would be necessary to repeat this assay with PM generated from the ULSD fuels. However, lack of sufficient sample prevented retesting in this study

The two extracts of diesel particulates generated using canola-derived biodiesel from FTP cycles yielded a significant positive response in the Salmonella mutagenicity assay on

strains TA98 and YG1041 both with and without metabolic activation. The mutagenic activity was higher without S9 (i.e., 710 revertants per mg particles without S9 compared 570 revertants per mg particles with S9 for TA98 and 770 revertants per mg particles without S9 compared 440 revertants per mg particles with S9 for YG1041). These results further confirm the presence of direct-acting mutagens in the SOF of diesel PM. However, the mutagenic response was comparable on strains TA98 and YG1041 and the lack of an increased response on the metabolically enhanced YG1041 strain indicate that the response is not due to the presence of nitro-PAHs in the SOF of these particular samples.

Diesel oxidation catalysts have been shown to reduce particulate matter emissions by 20%, and emissions of unburned hydrocarbons by 50% [107]. The mutagenic potency of an extract of PM generated with B20 canola derived fuel and a DOC was lower than that obtained from PM generated from the same fuel without a DOC (i.e., 710 revertants per mg particles (no DOC) compared to 390 revertants per mg particles (with DOC) on TA98 without S9). Oxidation catalysts are small metal-coated ceramic structures (e.g., palladium, rhodium) that reduce emissions of carbon monoxide and unburned hydrocarbons by catalyzing a reaction with oxygen [19,35]. The results obtained in the study support the evidence that diesel oxidation catalysts reduce the toxicity of the SOF PM generated using biodiesel blended fuels.

Only one sample generated using the steady-state driving cycle with varying loads yielded a positive response in the Salmonella mutagenicity assay (i.e., B20 animal). A very low mutagenic response was elicited by this extract on TA98 without S9 activation (140 revertants per mg particles). Few studies have investigated emissions of PM generated from animal derived biofuel. One study by Graham et al. [108] compared regulated and unregulated emissions generated using ULSD diesel and 20% blends of animal-, vegetable-

and restaurant oil-derived biodiesel fuels. Their results showed comparable reductions in the SOF of PM and PAH content in the SOF of diesel emissions generated from all three sources of biodiesel. The results obtained in this study suggest that the use of canola and soy derived biodiesel might yield emissions that are less toxic than those from animal derived biodiesel. However, unknown influences of biodiesel fuel quality and stability on the results obtained make it difficult to compare across studies, or even across experiments. It is difficult to generalize in the absence of a clear understanding regarding the composition and temporal stability of biodiesel fuels.

Both the polar and non-polar fractions of PM extracts generated using ULSD, B20 canola, and B20 canola (no DOC) fuels (i.e., FTP cycle) induced an Ah-receptor response. Similarly, both polar and non-polar fractions of PM extracts generated using B20 canola and B20 Soy (i.e., SS cycle) induced an Ah-receptor response. The slope values for the non-polar fraction were comparable among all samples for each driving cycle. The same trend was observed for the polar aromatic fraction. The small differences observed are most likely due to the composition of PAHs and nitro-PAHs associated with the SOF from the combustion of similar fuels with slightly different chemistry. Without any chemical analyses, it is impossible to know whether the differences are simply attributable to the fuel storage time. Vegetable oils are more readily oxidized by atmospheric oxygen than conventional diesel, and oxidation of biodiesel can produce shorter-chain fatty acids and even insoluble polymers. The oxidation of oils and fats by atmospheric oxygen is known as rancidity, and rancidity would likely effect the chemistry and toxicology of the biodiesel emissions.

The results obtained indicate that both the polar aromatic and non-polar neutral fractions of extracts of biodiesel particulates contain Ah-receptor agonists. The ability to yield a positive response and the magnitude of the response appear to depend on fuel type,

driving cycle, and after-treatment technology, with the most potent responses corresponding to PM generated from canola-derived B20 without a diesel oxidation catalyst. Conversely, the best combination, which appears to reduce both mutagenicity and dioxin-like effects, correspond with soy-derived biodiesel combusted using a steady state driving cycle on an engine fitted with a diesel oxidation catalyst.

It would prove interesting to compare the results obtained in this study with *in vivo* assessments of biodiesel emission toxicity. However, only one study has examined the acute *in vivo* toxicity of biodiesel emissions in rats. Finch et al. [109] exposed F33 rats to biodiesel exhaust (i.e., 100 % soy-derived) by inhalation for 6 hours/day, 5 days/week for 15 weeks to 0.04, 0.2 and 0.5 mg particles/m^3. Finch et al noted a modest induction of macrophage activity at the highest exposure level; however, there was no change in survival, micronuclei in bone marrow, sister chromatid exchanges in peripheral lymphocytes, fertility, and reproductive toxicity. Nonetheless, the ability of diesel and biodiesel emissions to contribute to adverse effects in human populations is an important topic, and the subject of considerable controversy.

4.6 General conclusions

There exist many advantages to using diesel engines as opposed to gasoline engines. Diesel engines are more efficient resulting in lower fuel consumption and lower emissions of carbon monoxide. However, diesel particulate emissions continue to be problematic for human health and the environment. Diesel emissions are mutagenic and carcinogenic in various *in vitro* and *in vivo* bioassays, and have been classified by IARC and the US NTP [9,29] as probable human carcinogens. Current efforts to reduce the adverse health effects of diesel emissions include the use of novel fuels, modified engine designs, and after-treatment

technologies. Biodiesel-based fuels are being explored as a safer alternative to diesel fuel. Biodiesel is a cleaner burning fuel that is generally thought to produce less toxic emissions. From an economic point of view, biodiesel yields 93 % more energy than that invested in its production, and, in comparison with conventional diesel, reduces greenhouse gas emissions by 41% [110]. Although the use of biodiesel as an alternative fuel can reduce the output of regulated emissions such as CO, THC, PM, and NO_x, their impact on the chemical composition and toxicological profile of the soluble organic fraction of exhaust emissions is unknown. In this study, extraction and fractionation of exhaust emissions (i.e., particulates and PUFs) generated from diesel and biodiesel fuel blends yielded polar aromatic and non-polar neutral mixtures/fractions that elicited significant mutagenic and dioxin-like effects. The results obtained suggest that the observed mutagenicity was associated with polar aromatic compounds such as nitroarenes, N-heterocyclics and aromatic amines; however, mutagenicity of the SOF decreased with increasing concentrations of biodiesel in fuel blends. Furthermore, the results obtained confirm the presence of polar and non-polar Ah-receptor agonists in the SOF of diesel/biodiesel exhaust particulates. Moreover, the ability of particulate extracts to induce dioxin-like effects increased with increasing concentrations of biodiesel in fuel blends. Finally, the mutagenic activity and potency of the Ah-receptor response associated with biodiesel particulates appears to depend on the source of the biodiesel, with soy-derived biodiesel blends yielding a reduced response relative to a canola-derived biofuel blend. However, issues regarding the stability and quality control of biodiesel remain to be addressed, and thus, it is difficult to issue general statements regarding the toxicological activity of biodeiesel emissions.

This study demonstrated that there are clear advantages and disadvantages to using alternative fuels (e.g., biodiesel blends) and after-treatment devices that are expected to

reduce to the risk of adverse health effects associated with diesel exhaust emissions. However, chemical analysis, further *in vitro* testing and follow-up *in vivo* testing, including inhalation exposures, will be necessary to reliably assess the relative hazards of diesel particulates generated under different fuel formulation, engine design and after-treatment scenarios.

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