1995

In vitro short-term human bioassays in environmental cytotoxicity and genotoxicity assessments.

Masumeh Abdolalipour
University of Windsor

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IN VITRO SHORT-TERM HUMAN BIOASSAYS IN ENVIRONMENTAL CYTOTOXICITY AND GENOTOXICITY ASSESSMENTS

by

MASUMEH ABDOLALIPOUR

A Thesis

Submitted to the Faculty of Graduate Studies and Research through the Department of Chemistry and Biochemistry in Partial Fulfilment of the Requirements for the Degree of Master of Science at the University of Windsor

August 1995
Windsor, Ontario, Canada
1995
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ABSTRACT

IN VITRO SHORT-TERM HUMAN BIOASSAYS IN ENVIRONMENTAL CYTOTOXICITY AND GENOTOXICITY ASSESSMENTS

by

MASUMEH ABDOLALIPOUR

In vitro short-term assays are becoming important tools in the preliminary evaluation of potential mutagenic and carcinogenic hazards associated with the release of chemical contaminants into the environment. In this study, an in vitro human cell culture, the HepG2 cell line, has been adapted and calibrated to be used as a surrogate human model system in environmental toxicology studies. To evaluate both cellular and genetic effects of test chemicals, a cytotoxicity test and a genotoxicity test were developed in the HepG2 cell line. A Neutral Red (NR) dye-uptake assay was used to assess cytotoxicity, and an alkaline unwinding, DNA break assay (AU) was developed to detect genotoxicity of both known toxicants as well as unknown environmental samples. The response of the NR and AU bioassays was calibrated by using known mutagens. Dose-response relationships were demonstrated in both assays for methyl methansulphonate, 4-nitroquinoline, 9,10-phenanthrenequinone, and benzo(a)pyrene. The effect of serum supplementation in the culture system on the toxic effects of tested chemicals were also studied. The toxicity of highly hydrophobic chemicals such as benzo(a)pyrene was enhanced when applied in serum-containing media.

Following the calibration of the HepG2 bioassays, the potential toxicity of a
number of environmental samples including lake sediments, fish tissues, and eagle eggs were evaluated. Sediments were collected from a number of sites in the Huron-Erie corridor and extracts were prepared in two fractions composed of OCCs (i.e., organochlorinated compounds) and PAHs (i.e., polycyclic aromatic hydrocarbons). Both contaminant fractions obtained from different sites in the Great Lakes Basin were cyto- and genotoxic in the HepG2 system to various degrees. Contaminants extracted from Lake Erie fish tissues and eagle eggs were composed of mostly pp'-DDE and Aroclor 1254:1260 and demonstrated significant cyto- and genotoxicity in HepG2 cells.

In conclusion, the HepG2 cell system combined with the NR and the AU bioassays appears to provide a suitable surrogate model system to evaluate the potential toxicity of environmental pollutants and the associated hazards posed to the human population. This model system could be applied to the assessment of environmental stress associated with any natural habitat.
ACKNOWLEDGMENTS

I would like to thank Dr Adeli for his supervision, support, and kindness. Also I like to thank Bruce Hasspieler who helped me through this research. Furthermore, I would like to thank Abbas Mohamadi and many others who made my stay at the University of Windsor much more easier.
DEDICATION

To my family and my friends
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</tr>
<tr>
<td>AHH</td>
<td>aryl hydrocarbon hydroxylase</td>
<td></td>
</tr>
<tr>
<td>AU</td>
<td>alkaline unwinding</td>
<td></td>
</tr>
<tr>
<td>B(a)P</td>
<td>benzo(a)pyrene</td>
<td></td>
</tr>
<tr>
<td>B(a)PDE</td>
<td>benzo(a)pyrene diolepoxide</td>
<td></td>
</tr>
<tr>
<td>BPQ</td>
<td>benzo(a)pyrene -7,8- dione</td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
<td></td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
<td></td>
</tr>
<tr>
<td>DDT</td>
<td>1,1,1-Trichloro-2,2-bis(p-chlorophenyl)-ethane</td>
<td></td>
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<tr>
<td>dG</td>
<td>2-deoxyguanosine</td>
<td></td>
</tr>
<tr>
<td>DMBAQ</td>
<td>7,12-dimethylbenz(a)anthracene-3,4-dione</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle's balanced salt solution</td>
<td></td>
</tr>
<tr>
<td>ECD</td>
<td>electron capture detector</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
<td></td>
</tr>
<tr>
<td>EROD</td>
<td>ethoxyresourufin O-deethylase</td>
<td></td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
<td></td>
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<tr>
<td>GC</td>
<td>gas chromatography</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
<td></td>
</tr>
<tr>
<td>GSSH</td>
<td>oxidized glutathione</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>HAQO</td>
<td>4-hydroxyaminoquinoline</td>
<td></td>
</tr>
<tr>
<td>HCB</td>
<td>hexachlorobenzene</td>
<td></td>
</tr>
<tr>
<td>HepG2</td>
<td>hepatoma G2 cells</td>
<td></td>
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<tr>
<td>HP</td>
<td>hydroxylapatite</td>
<td></td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
<td></td>
</tr>
<tr>
<td>MMS</td>
<td>methyl methanesulphonate</td>
<td></td>
</tr>
<tr>
<td>MSD</td>
<td>mass selective detector</td>
<td></td>
</tr>
<tr>
<td>MTase</td>
<td>methyltransferase</td>
<td></td>
</tr>
<tr>
<td>NADP+</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
<td></td>
</tr>
<tr>
<td>NPQ</td>
<td>naphthalene-1,2- dione</td>
<td></td>
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<tr>
<td>NQO</td>
<td>4-nitroquinoline 1-oxide</td>
<td></td>
</tr>
<tr>
<td>NR</td>
<td>neutral red</td>
<td></td>
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<tr>
<td>OCCs</td>
<td>organochlorinated compounds</td>
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</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>PCB</td>
<td>polychlorinated biphenyls</td>
<td></td>
</tr>
<tr>
<td>pp'-DDE</td>
<td>2,2- bis (p-chlorophenyl) 1,1-dichloroethylene</td>
<td></td>
</tr>
<tr>
<td>PPB</td>
<td>potassium phosphate buffer</td>
<td></td>
</tr>
<tr>
<td>PQ</td>
<td>9,10-phenanthrenequinone</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>SLS</td>
<td>sodium lauryl sarcosinate</td>
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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Toxic Chemicals and Great Lakes Ecosystem

The Great Lakes are the largest body of fresh water on earth with respect to surface area, and constitute 20% of the total earth's water. Large lakes are used as drinking water, waste assimilation, irrigation and as sources of minerals and petroleum. In North America, the Great Lakes are particularly important as a source of drinking water, fishing and as a source of water for manufacturing. In addition, large lakes contribute greatly to species diversity providing a variety of habitats. For instance, Lake Baikal in the USSR, the world's deepest lake (maximum depth; 1,741m), supports approximately 1,700 biological species, of which 1,200 are found nowhere else (Schmidtke, 1988).

Concern has been raised by chemical production and the fate of these chemicals in the aquatic and human environment in recent years. Production of about 1,000 new chemicals per year combined with the presence of over seven million known chemical compounds from which, over 1,000 chemicals are suspected carcinogens (Thomas, 1986) supports such concerns. According to Postel (1990), about 79% of the chemicals in commerce have no information on their toxic effects.

Toxic substances enter the Great Lakes via three major routes. These routes include point sources, non-point sources including agricultural practices (nutrients and pesticides), urban run off from streets, industrial waste, and sewage releases into
surface waters from combined-sewer over-flows. The third category comprises waste dump sites or land fills that can leach contaminants into the receiving water or contaminate groundwater. However, the fate of toxic chemicals in the Great Lakes depends on many factors such as solubility, hydrophobicity, lipophilicity, the extent of partitioning onto suspended matters, and susceptibility to photo- and biodegradation. The loss of chemicals from the aquatic ecosystem occurs through degradation processes such as hydrolysis, photolysis, and bacterial or fungal dechlorination, burial and volatilization to the atmosphere.

1.1.1 Great Lakes in North America and Environmental Contaminants

In North America (ranked by surface area), Lake Superior, Lake Huron, Lake Michigan, Lake Erie, and Lake Ontario are the 2nd, 5th, 6th, 13th, and 17th largest lakes in the world (Strachan et al., 1986), respectively. The Great Lakes flow from Lake Superior and Lake Michigan through the other Great Lakes and the St. Lawrence River to the Atlantic Ocean. The lakes are interconnected by short narrows at Mackinac and by short connecting channels of the St. Clair, Detroit and Niagara rivers. These channels have extremely high discharges of approximately 6,000m³/sec, putting them on scale with the world's largest rivers. The ecosystem quality of these lakes have been affected by chemical contaminants and according to the International Joint Commission, 42 areas in the Great Lakes have been identified as hot spots (Figure 1.1) (Great Lakes Water Quality Board, 1987). The lower Great Lakes, such as Lake Erie and Lake Ontario, are major concerns due to contaminant input from industries and agricultural use.
Figure 1.1  42 Areas of Concern in the Great Lakes (Great Lakes Water Quality Board, 1987)
Considering the fact that the annual flow through the St. Lawrence River to the Atlantic Ocean is less than 1% of the total volume of the lakes, this short retention time greatly endangers the quality of these lakes (Great Lakes Water Quality Board, 1985). The U.S. Fish and Wildlife Service, 1977 (International Joint Commission, 1993), has reported the presence of nearly 500 organic compounds in adult lake trout and walleye. Reports from the International Joint Commission’s Great Lakes Water Quality Board in 1987, have also listed 362 chemicals, to be present in the water, sediment, and biota of the Great Lakes Basin Ecosystem (Council of Great Lakes Research Managers, 1989). Eleven critical pollutants (Table 1.1), were identified by the Great Lakes Water Quality Board in 1985, based on their persistence, bioaccumulation potential and ability to induce adverse human and environmental health effects (Great Lakes Water Quality Board, 1985).

Table 1.1 Critical Pollutants Identified by the Water Quality Board

- Total polychlorinated biphenyls (PCB)
- DDT and metabolites
- Toxaphene
- 2,3,7,8-tetrachlorodibeno-p-dioxin (2,3,7,8-TCDD)
- 2,3,7,8-tetrachlorodibenzoferan(2,3,7,8-TCDF)
- Mirex
- Mercury
- Alkylated lead
- Benzo(a)pyrene
- Hexachlorobenzene

Source: (Great Lakes Water Quality Board, 1985)
1.1.2 Cause and Effect Linkages of Contaminants on Human and Wildlife

Both human and wildlife (birds, fish, reptiles, mammals) populations are exposed to similar types of contaminants. In general, Great Lakes wildlife have been shown to have very high tissue concentrations of chlorinated organic chemicals such as PCBs, dioxins, furans, mirex, HCB, DDT, and organometals such as methyl mercury and alkyl lead. Table 1. 2 illustrates seriously affected populations that eat primarily fish in the Great Lakes Basin. Contaminants are known to cause various types of problems through bioaccumulation of chemicals along the food chain. This has led to the suggestion that humans, who consume from the top of the food chain, are also at risk of developing similar types of effects (Council of Great Lakes Research Managers, 1989).

Fish are an excellent indicator of ecosystem health in that they can bioaccumulate many aquatic contaminants to many orders of magnitude greater than ambient concentration levels in the surrounding waters. Fish-eating animals, in turn, have shown a variety of adverse health effects linked to toxic chemicals. Several studies indicate a strong correlation between reduced population numbers of the bald eagle and elevated levels of a metabolite of DDT (i.e., DDE), and PCB (Blankenship, 1992; Colborn, 1991). The levels of DDE, PCBs and dieldrin in the eggs of the bald eagles, feeding on Great Lakes animals, have been linked to reduction in hatching success (Colborn, 1991; Gibertson et al., 1991). There are a number of studies, regarding the association between persistent toxic chemical (i.e., toxic substances with a half-life in water of greater than eight weeks) exposures and tumor frequency, deformities, and other lesions in fish, in particular bottom feeders. For example, according to Black et al., (1980), about 10% of brown
bullheads from the Buffalo River were affected by liver tumors (this river is contaminated by effluents from steel and textile industries).

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Species</th>
<th>Effect</th>
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<tr>
<td>dieldrin, PCB</td>
<td>Bald Eagle</td>
<td>Eggshell thinning; embryo mortality; adult mortality</td>
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<tr>
<td>PCB</td>
<td>Forester's tern</td>
<td>Embryonic mortality; deformities</td>
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<tr>
<td>Dioxin, PCB, DDT</td>
<td>Double-crested cormorant</td>
<td>Embryo deformities; eggshell thinning</td>
</tr>
<tr>
<td>PCB</td>
<td>Snapping turtle</td>
<td>Embryo abnormalities; embryo mortality</td>
</tr>
<tr>
<td>PCB, dioxin</td>
<td>Mink and otter</td>
<td>Reproductive dysfunction</td>
</tr>
<tr>
<td>PAH</td>
<td>Brown bullhead</td>
<td>Liver and skin tumors</td>
</tr>
<tr>
<td>PCB</td>
<td>Lake trout</td>
<td>Unable to reproduce normally; hatchability and fry mortality</td>
</tr>
<tr>
<td>Dioxin, PCB, DDT</td>
<td>Herring gull</td>
<td>Embryonic mortality, porphyria; thyroid hyperplasia; Vitamin A depletion; deformities; feminization; poor parenting</td>
</tr>
<tr>
<td>PCB</td>
<td>Human offspring</td>
<td>Short-term memory deficits (visual, verbal, quantitative, pictorial); growth retardation; activity retardation</td>
</tr>
<tr>
<td>Lead</td>
<td>Human offspring</td>
<td>Hyperactivity; permanently reduced intelligence; neurobehavioural abnormalities</td>
</tr>
<tr>
<td>Mercury</td>
<td>Human offspring</td>
<td>Learning and motor skill deficits</td>
</tr>
</tbody>
</table>

Source: (Great Lakes Water Quality Board, 1985)

Furthermore, an extensive survey of the Black River (Lake Erie) by Baumann et al., (1982), revealed that 37% of the Brown bullheads, three years and older, had liver tumors.
Carcinogenic PAHs, in the discharge to the Black River from steel and coke industries, were implicated as the cause of this event. Hepatocellular carcinoma (liver cell tumor) was detected in white suckers from Western Lake Ontario (Hamilton harbour, Sixteen Mile Creek, Humber River, the Rouge River and Toronto Harbour), Hby Cairns and Fitzsimons (1987). Although no specific carcinogen has been associated with liver tumours in white suckers, it is suggested that exposure to PAH's may play a role in the latter stages of cancer development. Many persistent contaminants such as DDT and its metabolites, dieldrin, PCBs, dioxin, PAHs, lead and mercury among numerous others, have been found to exert endocrine disruption both in wildlife and laboratory animals (Colborn et al., 1992). The same hormonally active chemicals found in fish have been reported to be present in human tissues as well (Thomas et al., 1992). Major human exposure to organochlorinated compounds, such as PCBs and DDT, is through food (80-90%). Although the atmosphere is the dominant medium of transport and deposition to land and water for many persistent toxic substances, human exposures from contaminated fish far out weigh exposure to contaminants through other routes (air, water, or soil). There is a known correlation between fish consumption by pregnant women and the quantity of PCBs in their milk, tissues, and children (Jacobson et al., 1990; Jacobson et al., 1984). Women who consumed 2 or 3 fish meals (Lake Michigan) per month, for at least six years prior to their pregnancies, had infants with lower birth weights, shorter gestational periods, and smaller head circumference. At seven months of age, these infants showed discernible cognitive, motor, and behavioral deficits. More recently, increased fetal exposure to estrogens or estrogenic chemicals (endocrine disruptors such as DDT, PCBs, dioxins, furans,
hexachlorobenzene, other organochlorine chemicals, and metals) (Carlsen et al., 1992),
might be correlated with sperm counts reduction and the rising incidence of abnormalities
in the male reproductive tract (Share, 1989).

The International Joint Commission has recognized that the presence of persistent
toxic substances in the Great Lakes basin poses an ongoing threat to the health of the
ecosystem for the benefit of all users of this natural resource. The weight of the
evidence reinforces and strengthens the agreement to virtually eliminate the input of
persistent toxic substances into the Great Lakes Basin Ecosystem, and by that enhance and
restore the health and vitality of this precious resource.

Despite these concerns, there is yet no direct measure of the threat of toxic
chemicals to human populations. This research develops, calibrates and verifies the use
of a human cell line as a bioindicator to quantify the threat of toxic chemicals to human
populations.

1.2 Environmental Contaminants and Genotoxicity

1.2.1 Introduction

Many chemicals, as well as ionizing (x rays) and UV radiation, produce cyto-and
genetic damage. It would be impossible to discuss all these agents, but it is possible
to describe some chemical structures which are related to the present study and cause
cyto-and genotoxicity through direct or indirect metabolic pathways.
1.2.2 Direct-Acting Agents

Direct acting agents are intrinsically reactive and interact with biological components such as DNA, RNA, and proteins without biological activation. The Direct-acting chemicals such as alkylating agents are known to induce mutations and chromosome aberrations, and are also toxic and teratogenic (i.e., induce formation of developmental abnormalities in the fetus) (Ernst, 1971). Alkylation of cellular nucleophiles (i.e., phosphate groups, [forming semi stable triesters], ring nitrogens of the bases as well as sulfhydryl, thioester, ionized acid, and nonionized amino groups) occur through various reaction mechanisms. For instance, Monoalkylating agents, such as methyl methanesulphonate (MMS), involves $S_N2$ mechanism (i.e., the rate of the reaction depends on the concentration of both the MMS and the nucleophile, $Y^-$), whereas methyl nitrosourea (MNU), exerts its biological activities by $S_N1$ type of reaction (i.e., the rate of reaction is independent of the concentration of nucleophile) (O'Neill, 1979). Thus, the nucleophilic reaction follows as:

$$\text{RX} + Y^- \rightarrow \text{RY} + X^-$$

Covalently linked polyfunctional alkylating agents, such as sulphur or nitrogen mustards, can cross link DNA strands, causing either intrastrand cross link (i.e., two strands of one DNA molecule) or interstrand cross link (i.e., two strands of different DNA molecules). DNA cross linking (to a lesser extent) can occur by monofunctional agents that produce reactive DNA ends by the alkylation-induced backbone breakage.
1.2.3 Indirect-Acting Agents

Indirect-acting agents refer to classes of chemicals which require metabolic activation in order to exert biological effects. The main classes of procarcinogens/mutagens (i.e., inactive molecules) include: hydrocarbons (i.e., unsaturated alkenes, polynuclear aromatic, and heterocyclic), amines and amides (i.e., aromatic and heterocyclic primary, secondary, and tertiary amines, aliphatic amides and thioamides), azo and aminoazo compounds, nitroso- and nitro-compounds (i.e., aromatic C-nitroso- and C-nitro- compounds) (Bartsch et al., 1971).

Biotransformation of xenobiotics to become toxicants was first observed by Miller and Miller in 1966 (Miller et al., 1981). Metabolism of parent compounds takes place through multistep processes classified as Phase I and Phase II metabolism. Phase I reactions involve the introduction of polar groups to the molecule through oxidation, reduction, and hydrolysis reactions. For instance, the hydroxylation of various xenobiotics is catalyzed by mixed-function oxidases (MFOs). The MFOs system is a coupled electron-transport system which is composed of a flavoprotein (NADPH cytochrome P$_{450}$ reductase) and cytochrome P$_{450}$ which is known to act as a terminal oxidase. The basic reaction involves introduction of an oxygen atom into the substrate in the presence of NADPH and molecular oxygen. The overall reaction of these enzymes is such that:

\[
R\cdot H + O_2 + NADPH + H^+ \leftrightarrow R\cdot OH + NADP^+ + H_2O
\]

NADPH-cytochrome P$_{450}$ reductase transfers two single electrons that are supplied by NADPH
through a hydride ion (i.e., hydrogen atom carrying two electrons) to reduce cytochrome P₄₅₀ which binds to the substrate and molecular oxygen. The cytochrome P₄₅₀ then catalyses the incorporation of one of the oxygen atoms into the substrate and the second oxygen atom with water (Zakrzewski et al., 1991; Bartsch et al., 1971).

Phase I reactions are catalyzed by heme containing cytochromes, and are located on the surface of the endoplasmic reticulum. Cytochromes are composed of a complex system of 78 members, divided into 14 families (Nebert et al., 1978). In general P₄₄₈ (P₄₅₀₁) involves the metabolism of PAH's, while P₄₅₀ (P₄₅₀₂) metabolizes the environmental pollutants such as, PCB, and DDT. The term P₄₅₀ refers to the whole system when the identity of the cytochrome is not specified. Cytochrome P₄₅₀₁ dependent reactions include, N-oxidation and S-oxidation, and cytochrome P₄₅₀₂ dependent oxidations include, aromatic hydroxylation, acyclic hydroxylation, dealkylation and deamination. Among the widely studied enzymes are benzo(a)pyrene hydroxylase (BaPH), ethoxyresourufin O-deethylase (EROD), and aryl hydrocarbon hyroxylase (AHH) (Peakall, 1992). Other cytochrome dependent enzymes include: flavoprotein N-oxygenase (catalyzes the oxidation of nitrogen atoms in organic compounds) (Gorrod, 1978), epoxide hydrolase (hydrolyses a carbon-oxygen bond atom in epoxide ring (oxirane) to form a trans dihydrod’oi), hydrolases (i.e., esterase or amidase-catalyzed hydrolysis of N-C bond) and reductases (azoreductase-catalyzed cleavage of (di) azo bonds). Both MFOs and dependent enzymes have been identified in tissues of various species, including, mammals, birds, amphibians and fish. MFO activity has been demonstrated to be primarily concentrated in liver as well as in lung, skin, intestinal, epithelium, kidney, testis, placenta, mammary gland, and
other organs (Hutson, 1977).

In phase II reactions, the substrates or their metabolites are conjugated with endogenous agents (e.g., glucuronate, sulphate, acetate, glutathione), via the transferases (located mainly in the cytosol) to form more polar compounds. Conjugation reactions of chemicals often lead to the biological inactivation of substrates. However, the generation of active metabolites (e.g., electrophiles, free radicals, etc.) can result in cytotoxicity, mutagenicity or carcinogenicity. An example of indirect acting chemicals include many polycyclic aromatic hydrocarbons (PAHs), a major class of environmental pollutants. PAHs in general, require biotransformation prior to expression of any cyto- and or genotoxicity effects. Benzo(a)Pyrene (B(a)P), a common PAH, is a well known procarcinogen and mutagen (Osborne et al., 1986). The first biotransformation step in oxidation of B(a)P involves, monooxygenation by AHH (predominantly found in liver), to arene oxides (epoxides). In rats, mice and humans epoxidation of terminal benzo-ring takes place via cytochrome P₄₅₀, which requires molecular oxygen and NADPH as a cofactor. Hydration of this reactant by a second microsomal enzyme (epoxide hydrase), yields the trans-dihydrodiol a proximate carcinogen (i.e., intermediate metabolites of procarcinogen). Secondary oxidation (i.e., epoxidation) of the trans-dihydrodiol leads to the formation of the highly reactive anti-diolepoxide, an ultimate carcinogen. Although rodents use the same cytochrome (i.e., P₄₅₀₁) system in both primary and secondary epoxidation of B(a)P, the human epoxidation of trans-dihydrodiol is catalyzed through cytochrome P₄₅₀₁F, to yield the anti-diolepoxide (Penning, 1993). Consequently, anti-diolepoxide alkylates the N2 amino group on 2-deoxyguanosine (dG) to form anti-diol
epoxide (i.e., anti-BPDE-dG) adducts (Figure 1.2) (Miller et al., 1981).

Other PAH-metabolites such as PAH o-quinones have been studied, and these have been shown also to modify macromolecules (i.e., DNA, RNA, proteins). For example, Benzo(a)pyrene-7,8-dione (BPQ), is known to form 2'-deoxyguanosine adducts with calf thymus and plasmid DNA (Shoo et al., 1992). Oxidation of trans-dihydrodiols by dihydrodiol dehydrogenase (DD) (present both in rat and human hepatoma cells) has been shown to form intermediate hydroquinones (Lynn et al., 1993). Hydroquinones are known to be genotoxins (Solveig, 1992), that may undergo conjugation (glucuronide, sulpa te, methylated catechol), or autoxidation (i.e., air oxidation). Consumption of oxygen in the latter, leads to the formation of a semiquinone radical. Semiquinone then reacts with another oxygen molecule to form fully a oxidized o-quinone conjugate. A consequence of this autoxidation is the formation of hydrogen peroxide (H₂O₂), superoxide (O²⁻) and o-semiquinone radicals.

Quinone can interact with DNA, RNA, glutathione (GSH) and amino acids to form adducts. One-electron reduction of quinones, by a series of one-electron reductases such as NADPH-cytochrome c reductase and NADH cytochrome b₅ reductase, can lead to the formation of semiquinone, which in turn leads to the generation of various reactive oxygen species (e.g. O²⁻, H₂O₂, OH⁻). Active oxygen species, such as, hydroxy free radical, can induce various DNA lesions (i.e., strand breaks) by the destruction of deoxyribose moieties, the generation of apurinic and apyrimidinic sites, conversion of thymine to hydroxymethyluracil, as well as the formation of DNA adducts (e.g., thymine glycol, thymidine glycol and 8-hydroxyguanine) (Heflich, 1991; Tada et al., 1971).
Figure 1.2 Common metabolic activation pathways of PAHs
Source: (36)
Production of $\text{H}_2\text{O}_2$, via the dismutation of $\text{O}_2^-$, results in the depletion of GSH, a general loss in cellular reducing equivalents, perturbation of $\text{Ca}^{2+}$ homeostasis, and cell death (Kappus et al., 1981; O' Brien, 1991; Read, 1990).

Cytotoxicity of other toxic PAH metabolites have also been studied (Haliwell et al., 1989). Catalysis of naphthalene-1,2-dione, 7,12-dimethylbenz(a)anthracene-3,4-dione, and benzo(a)pyrene-7,8-dione (BPQ), by dihydrodiol dehydrogenase via oxidation of trans-dihydrodiol in human (HepG2) and rat hepatoma cells (H-4I1E) have been demonstrated. The observed cytotoxicity in cells (1-100 $\mu$M for 0-4 h), was related to formation of $\text{O}_2^-$, a decrease in glutathione (GSH) levels, and an increase in GSSG levels. The increased levels of oxidized glutathione (GSSG) have been related to the formation of reactive oxygen species, and the resultant elimination of $\text{H}_2\text{O}_2$ by the GSH peroxidase/GSSG reductase system (Kappus et al., 1981; O' Brien, 1991; Cathcart et al., 1984), as well as an increase in the cellular redox state $[\text{NAD(P)}^+ / \text{NAD(P)}\text{H}]$ (Lynn et al., 1993).

In addition to cytochrome $P_{450}$, mammalian systems have also shown to contain soluble reducing enzymes for the reduction of carbonyl, nitro, and azo groups, and esterases that hydrolyse esters and amides to their corresponding carboxylic acids and alcohols or amines, respectively (Zakrzewski, 1991). The potent carcinogen, 4NQO, is known to exert its mutagenic and carcinogenic effects by reduction to 4-hydroxylaminoquinoline 1-oxide (4-HAQO) via cytoplasmic diaphorase (flavoprotein) (Jeffrey et al., 1979). This primary metabolite of 4-NQO reacts primarily with nucleophilic sites in cellular macromolecules such as DNA, mainly with the guanine and

1.2.4 Mechanisms of DNA Damage

DNA is the repository of genetic information and is subjected to damage by various endogenous processes (e.g., errors during replication), and exogenous genotoxins (e.g., ultraviolet light, cosmic and ionizing radiation and chemicals such as PAHs and their metabolites, free radicals, and alkylating agents). The most common types of DNA damage are discussed below.

i. Hydrolytic damage. Occurs through the reaction with water leading to depurination, depyrimidination (i.e., loss of a DNA base which may be followed by phosphodiester bond breaks), and deamination of exocyclic amino groups.

ii. Electromagnetic radiation damage. Ionizing radiation (x-ray) is known to promote strand breakage as well as DNA base modification via free radicals. Ultraviolet radiation (e.g., 254 nm), however, forms cyclobutyl dimers between adjacent primidines (mostly thymine dimers), preventing normal DNA base pairing.

iii. DNA adducts. DNA damage caused by the covalent binding of various groups of chemicals (or their metabolites, e.g., alkylating agents, PAHs, aromatic amines, etc.), with nucleophiles such as phosphate, amino, sulfhydryl, hydroxyl, carbonyl, and imidazole groups, leading to the formation of DNA-adducts. DNA-adducts can enhance single strand breaks or point mutation frequencies (i.e., changes in the nucleotide sequence in one or few codons), as a result of base substitution, deletion or addition.

iv. DNA strand breaks. Depurination, depyrimidination and hydrolytic cleavage, as a
result of DNA-adducts, DNA alkylation, as well as ionizing radiation can result in DNA strand breaks. DNA breaks may lead to chromosomal damage and the prevention of DNA duplication (Venitt et al., 1984).

Biological consequences of DNA lesions varies with regard to the nature of reactive agents and their position on the DNA bases. Most DNA modifications have been demonstrated to occur with guanine atoms (e.g., N7, C8, O6, exocyclic N2) (Venitt et al., 1984). Alkylation by MMS, N-ethyl-N-nitrosourea, dimethylsulfate, in human lymphoblastic cell line (CCRF-CEM), at N7 and N3-purine results in AP sites (i.e., apurinic and / or apyrimidine sites) in the DNA (Venitt et al., 1984)). A methyl group at N7-G position, however, is much less mutagenic than methylation at O6-G which participates in hydrogen binding during DNA base pairing. Bulky adducts such as, aflatoxin B1 at N7-G position or B(a)P at N2-G have been shown to be highly mutagenic(Penning, 1993; Venitt et al., 1984). DNA lesions such as strand breaks can be removed by excision repair, replaced by recombination or retained, the latter leading to mutation or cell death (Ernst, 1971).

1.2.5 DNA Repair Pathways

The ability of living cells to repair damaged DNA was first reported in 1964 (Kanter et al., 1980; Setlow et al., 1964). The enzymes involved in various repair processes and their mechanisms have been studied in detail. Although DNA repair in humans and yeast have been studied, most studies concerning DNA repair mechanisms and related enzymes have concentrated on Escherichia coli (Sancar et al., 1988). Repair mechanisms
consist of various pathways and include: error-free repair, excision repair, and error-prone repair as described below.

1.2.5.1 Error-Free Repair

Error-free DNA repair involves neither removal nor replacement of DNA bases. Chemical modification is simply reversed by photolyases or by DNA methyltransferases. In the former, the UV-induced cyclobutyl dimers are removed by photolyases. Photoreactivation of these enzymes by visible light (300-600 nm) breaks the cyclobutane dimer ring and restores the bases to their monomeric form. These enzymes have been identified in cells, ranging from bacteria (mycoplasma) to human leukocytes (Kornberg et al., 1991). Another enzyme of this class is O6-methylguanine-DNA methyltransferase (MTase) which is known to occur in Escherichia coli, yeast, and human cells. MTase transfers both methyl or ethyl groups from O6-methylguanine or phosphotriesters to a lysine residue of its own, thus becoming irreversibly inactivated upon alkylation (Kornberg et al., 1991). Therefore, both photoreactivation and dealkylation mechanisms are able to reverse the covalent modification in DNA by a direct repair mechanism.

1.2.5.2 Excision Repair

Major DNA lesions are repaired by excision processes. The AP site excision repair processes initially involves, the identification and removal of damaged base creating an AP site. This is followed by the removal of the fragment containing the AP site (i.e., AP sites are generated by spontaneous acid-catalyzed hydrolysis; this reaction is
enhanced upon base alkylation, or radiation). The correct base is then replaced by joint action of DNA polymerase and ligase. In this process, altered or improper bases are identified and removed by a specific N-glycosylase, by hydrolysing the N-glycosyl bond that links the base to the deoxyribose of the DNA backbone. Excision of fragments containing an AP site may proceed by either of two types of endonucleases, type I, and type II. Excision repair through type II endonuclease enzyme proceeds by introducing the initial nick next to the AP site followed by removal of the AP fragment by an exonuclease. The second pathway involves endonuclease type I with both N-glycosylase and endonuclease activities. Type I endonuclease catalyzes the incision of the phosphodiester bond to the 3' side of the AP site using its endonuclease activity. The AP fragment is then removed by type II endonuclease which hydrolysates the phosphodiester bond in the DNA backbone at the 5' side of the AP site. At this stage, the DNA double helix consists of a strand with a gap opposite to the intact strand. The gap is then filled with complementary base pairing by polymerases, using the undamaged strand as the template, and covalently joined by DNA ligase (Venitt et al., 1984; Kim et al., 1991).

Excision of the damaged fragment (oligonucleotide) (i.e., UV dimer, bulky adduct, or interstrand cross-link), is known to be accomplished by an excinuclease such as Uvr ABC complex (i.e., uvrA, uvrB, uvrC) in Escherichia coli (Sancar et al., 1988)). In this reaction, the enzyme removes DNA adducts by hydrolysing phosphodiester bonds on both sides of the altered nucleotides. However, the incision pattern varies with respect to the type of adduct. For example, furan and pyrrole, N-acetoxyacetyl aminofluorene, and 4-NQO adducts are removed by hydrolysis of the 8th phosphodiester bond 5' and the 5th
phosphodiester bond 3' to the damaged base leaving a gap with 3'-OH and 5'-P. In contrast, removal of pyrimidine dimers occurs by incising the 8th phosphodiester bond 5' and the 4th or 5th phosphodiester bond 3' to the damaged nucleotide. The resulting gap is then filled with action of DNA polymerase I and joined by DNA ligase (Sancar et al., 1988; Kim et al., 1991).

1.2.5.3 Error-Prone Repair

The error-prone repair mechanism is known to be the major source of mutation and involves DNA polymerase III (E. coli). Polymerase III replicates pyrimidine dimer or other DNA lesions by two replication pathways: 1) Translesion replication: the polymerase III fills the gap by non-template-directed replication across the lesion as it encounters a pyrimidine dimer or certain lesions which block the DNA replication in the template (Kornberg et al., 1991), thus, restoring the replication and converting the DNA lesion to an error-prone site. 2) Recombinational bypass: the DNA polymerase III stops at the point of lesions and then resumes 1000 nucleotides or so downstream, the discontinuity or postreplication gap may be filled with a complementary strand from the sister duplex (Kornberg et al., 1991). Both translesion replication and the recombination bypasses are induced by activation of SOS (a regulon containing about 20 genes). The SOS activation in turn can be induced by the ssDNA at the replication fork, that may be caused by UV damage or formed by repair processes of various DNA damage. Although both pathways increase cell survival, they are highly mutagenic and known as error-prone repair pathways. As the function of polymerase is modified in undefined ways (i.e., the
modification of polymerase affects its ability in the process of DNA synthesis or proofreading) this increases the level of mutagenicity at the site of damage (Kim et al., 1991).

1.3 Effects of Serum in Culture Media in Relation to Toxic Chemicals

Serum is an essential component of a complete culture media, and supplies many factors required for cell culture. These factors include: adhesion promoting factors (e.g., fibronectin) for the adhesion and the spreading of cells, nutrients and trace minerals, transport proteins (e.g., transferrin and albumin), growth factors and hormones, and protein (e.g., albumin) (Burdon et al., 1990). With respect to alkylating agents, the serum content of the medium is known to reduce the level of absorbed dose to the cell, due to the interaction of test agents and serum macromolecules (Regan et al., 1973). In a previous study, the level of cellular inactivation (i.e., colony-forming ability) was correlated to the amount of alkylation, rather than the initial dose (administered dose) of the test agent (Regan et al., 1973). O’Neill (1979), observed identical results for cytotoxicity and mutagenicity in Chinese hamster ovary cells (CHO/HGPRT) treated with MMS and ethyl methanesulphonate (EMS), N-methyl- and N-ethyl-N’-nitro-N-nitrosoguanidine (MNNG, ENNG), and methyl- and ethyl-nitrosourea (MNU, ENU) in the presence or absence of serum in culture media.

The effect of serum in relation to ion channels in the cell membrane was also investigated. Lubin (1993), has shown the loss of K⁺ and gain of Na⁺ in human fibroblast, bladder carcinoma, and breast carcinoma cells treated with a commercial serum free media.
The rapid $K^+$ loss was correlated to one or more impurities in phenol red in culture media, and he suggested, adding serum or albumin to media prevents $K^+$ loss.

The scope of this study is limited to the effects of serum in culture media in relation to the interaction of test agents with cell components. However, in order to estimate any biologically adverse effects, as well as the level of absorbed dose it is important to consider the effect of all the possible interactions of the test agent with various constituents of the test system (i.e., solvents, medium, serum, cell components).
CHAPTER TWO

SHORT-TERM BIOASSAYS IN CYTO- AND GENOTOXICITY ASSESSMENT

2.1 Introduction

In vitro systems have been used for the screening of potential anti-cancer agents since the beginning of clinical cancer chemotherapy in 1964, following the discovery of the anti-neoplastic activity of nitrogen mustard (Foley et al., 1964). Considering the increasing public concern with the presence of millions of chemicals and the decline in environmental quality, there is a great need for a more comprehensive evaluation of the toxic effects of chemicals by in vivo and in vitro bioassays. Although, whole animal studies play an important role in toxicological assessment, they are limited. These limitations include: financial considerations (1.5 million dollars per chemical tested), interspecies differences, as well as ethical consideration (17 to 22 million animals sacrificed per year in U.S. laboratories) (DelRaso, 1992). In vitro systems can be used in preliminary evaluation of chemical toxicity effects at a lower cost and a shorter time period. Furthermore, isolated systems in short-term assays are used to study the susceptibility, reversibility and dose-response relationship in different cell types, in different organs exposed to toxic chemicals. Thus, such studies are useful in obtaining comparisons between exposures of organs or tissues of experimental animals and humans to toxic chemicals and providing baseline data for the further extrapolation of data to intact organisms. With respect to the present study two short-term bioassays will be discussed in detail.
2.1.1 Cytotoxicity Assays

Initial cytotoxicity assays were largely qualitative and based on morphological damage, under undefined medium. Eagle and Foley (1956), were able to demonstrate a clear-cut correlation between in vitro and in vivo activity of neoplastic agents by measuring protein content of treated and untreated cells. Although there are numerous in vitro cytotoxicity assays, the final choice depends on various factors such as biological endpoints.

In genotoxicity studies selection of a proper concentration range for the chemical to be tested is an essential prerequisite. This can be accomplished by an initial cytotoxicity test. Current tests include: 1) dye exclusion methods (trypan blue, erythrosine B, eosin Y, naphthalene black and nigrosin green), 2) lactate dehydrogenase (LDH) test, 3) total protein content, and 4) Dye-uptake assays.

1) The dye exclusion methods, such as trypan blue, require identical conditions of staining and scoring of each individual cell culture of the experimental groups and involves subjective decisions. 2) The lactate dehydrogenase (LDH) test, involves measurement of lactate dehydrogenase (LDH) in culture supernatant as a result of leakage (Jauregui et al., 1981; McQueen et al., 1982), indicative of disorders of membrane functions. However, presence of LDH in serum media, may lead to falsely increased positive results with regard to extracellular LDH as a result of leakage. 3) The total protein content test, is an assay to measure total protein content as a index of cell number, which also could be affected by overestimation of cell number which may arise with some drugs which inhibit replication without inhibiting protein synthesis (e.g., 5-
bromodeoxyuridine, methotrexate). 4) Dye-uptake assays, which are based on lysosomal uptake such as the neutral red (NR) uptake assay offer a number of advantages over the above tests such as higher sensitivity, reproducibility, and greater turnaround time. This NR uptake bioassay has been described in detail below.

2.1.1.1 Neutral Red (NR) Uptake Assay

NR uptake assay provides a better alternative for cytotoxicity assessment. The NR assay, has been applied for a broad range of test agents including: chemotherapeutics, pharmaceuticals, biomaterials, natural toxins, food additives, preservatives, antimicrobial agents, industrial chemicals, and environmental pollutants, using a variety of cell types, from fish, rodents, and humans, (Babich et al., 1992). NR (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) a weakly-cationic dye, penetrates membranes by nonionic diffusion and binds intracellularly to carboxylic and/or phosphate groups of the lysosomal matrix. Changes in the accumulation and retention of NR occur when cell membranes (plasma, as well as the sensitive lysosomal membranes) are injured or damaged (Babich et al., 1988). It is possible that nonspecific damage such as ionic imbalance, energy depletion or protein denaturation can lead to lysosomal leakage causing unscheduled releases of lysosomal enzymes (Scott et al., 1991). Thus, dead cells cannot retain NR dye. The utility of the method was recently confirmed by Zhang et al., (1990) who demonstrated a good correlation between the NR technique and conventional methods such as trypan blue, LDH assay and protein determination of cell populations (Borenfreund et al., 1985). Cell viability, at each concentration of test agent, is
determined by absorbance of the extracted dye (NR) from both treated and untreated cells at 540nm as follows:

\[
\text{mean absorbance of toxicant-treated wells} \quad \frac{\% \text{ Cell Viability} = \text{X100}}{\text{mean absorbance of control wells}}
\]

2.1.2 Genotoxicity Assays

Short-term genotoxic methods also play an important role in molecular toxicology in assessing DNA damage (i.e., genetic mutation, chromosomal aberration, DNA strand breaks,... etc.). Most of the knowledge in this field has been obtained from microbial systems. The Salmonella/histidine reversion assay developed by Ames (Li et al., 1991) was among the first tests used to correlate mutagenicity and carcinogenicity. The assay measures mutations and require an exogenous metabolic system (Aroclor 1254-induced rat liver post-mitochondrial supernatent) for metabolic activation of xenobiotics. In spite of its limitations (i.e., test organism, exogenous metabolic system), the assay has been used extensively in genetic toxicology. During the development of the genotoxicology field, many other methods have been established for the measurement of genotoxic effect of toxic chemicals. Biochemical techniques that are currently available for genotoxicity testing are tabulated in Table 2.1, from which, selected methods related to the present study will be discussed in some detail.
Table 2.1 Techniques for Detecting Mutagenic and Carcinogenic Chemicals

- Bacterial mutation assays
- Genotoxicity studies using Yeast cultures
- In Vitro cytogenetics and sister-chromatid exchange
- In Vitro cell-mutation assays
- The use of higher plants to detect mutagenic chemicals
- Drosophila sex-linked recessive lethal assay (SLRL)
- In Vivo cytogenetics: Bone marrow metaphase analysis and micronucleus test
- Dominant lethal assay
- Unscheduled DNA synthesis in cultured mammalian cells
- DNA Break Bioassays/Alkaline-Unwinding Tests

2.1.2.1 DNA Break Bioassays/Alkaline-Unwinding Tests

Biomarkers, such as DNA single-strand breaks, that indicate DNA damage by a variety of chemicals are used both as molecular dosimeters (biomarkers of exposure) as well as to assess the genotoxic potential of chemicals (biomarkers of effect). The presence of breaks can be investigated by various analytical techniques. Most common assays include; alkaline sucrose gradient (McGrath et al., 1966), alkaline elution (Studier, 1965), and hydroxyapatite batch assay (Kanter et al., 1979).

The alkaline sucrose gradient method developed by McGrath and Williams (1966), involves cell lysis and complete denaturation of the DNA in alkali solution prior to centrifugation. However, if denaturation does not proceed to completion, the
sedimentation profile will indicate a random size distribution of single stranded DNA molecules. This is particularly important with the assessment of DNA strand breaks. Therefore, in order to achieve complete denaturation, varying conditions for different cell types are required. In general the technique is accurate only when small numbers of cells are used and is not well adopted to many runs on a routine basis. In addition, DNA damage produced by low levels of ionizing irradiation less than 1000 rad is not quantified accurately (Kanter et al., 1979).

The alkaline elution assay, where DNA is lysed onto cellulose triacetate filters, appears to be adaptable as a routine assay, and sufficient number of cells may be processed. The technique requires large sample numbers to accurately define elution patterns, and has variable recovery losses due to DNA retention on the filters. However, the method is sensitive and as few as 50 rad (i.e., 500 breaks / cell) x-irradiation-induced DNA damage can be quantified (Daniel et al., 1985).

In this project, a simple and sensitive technique to study induction of strand breaks in DNA of human liver cells is used which is similar to the hydroxylapatite batch assay. The technique is adapted from the Daniel et al., (1985) version of the alkaline unwinding (AU) assay, which has been used in various genotoxicity studies both in vivo and in vitro. The sensitivity of the assay, estimated by DNA strand breaks produced by doses of gama-radiation, was as low as one rad (Ryderberg et al., 1980). The principles of both hydroxylapatite batch assay and alkaline unwinding assay are the same, yet the latter requires extensive centrifugation processes, and is more time consuming.
2.1.3 Theory of Alkaline Unwinding Assay

The alkaline unwinding (AU) assay which was initially developed by Ahnstron and Erixon (1980) has been employed in assessing induction of DNA damage by various exogenous agents such as electromagnetic irradiation (i.e., ultraviolet light), and ionizing radiation (Dikomey et al., 1992; Ljungman et al., 1991; Heilmann et al., 1993) 51), chemical carcinogens and mutagens (Chang et al., 1992; Shugart et al., 1988; Daniel et al., 1985), in addition to complex environmental samples (Nacci et al., 1992; Meyers-Schone et al., 1993), in both in vitro and in vivo studies of human, rodents and aquatic species (Shugart, 1990).

The assay is based upon treatment of the cell with alkali which releases the DNA from membranes and other macromolecules (pH>11.4). Each single-strand break (SSB) serves as an unwinding point during strand separation in alkaline solution. The initial rate of separation is proportional to the number of breaks. As strand separation proceeds, the smaller molecular weight fragments are rapidly converted to single-stranded DNA (ssDNA) while the larger fragments continue to unwind. Thus, the rate of unwinding is inversely proportional to the number and size of fragment DNA and to the viscosity of the solvent. This suggests that the rate limiting factor in strand separation is the viscous drag of solvent molecules on the rotating DNA strands, so as the rotating portions become longer, the rate of unwinding decreases. The relationship between DNA duplex remaining after alkaline exposure and period of exposure (t) has been described by Rydberg (1980) as follows:
\[ \ln F = -K \frac{t^\beta}{M_n} \]  
\hspace{1cm} (\text{II})

Where \( F \) = amount of double stranded (ds-DNA), after unwinding at time \( t \); \( \text{ds-DNA} + \text{single stranded DNA (ss-DNA)} \); \( M_n \) = number of average molecular weight between strand breaks; \( K \) = proportionality constant; \( \beta \) = DNA unwinding constant as a function of particular unwinding conditions (solvent viscosity, temperature, pH, etc.). Kanter and Schwartz (Kanter et al., 1979) showed that the \( F \) value is related inversely to the number of DNA strand breaks (\( N \)) (i.e., Breaks / AU unit, i.e., one AU unit is the stretch of ss-DNA between breaks). An \( N \) is an increase in DNA strand breaks relative to the control. For example an \( N \) value of 5, indicates treated DNA had five times more strand breakage relative to the control as follows:

\[ N = \frac{\ln F_T}{\ln F_c} - 1 \]  
\hspace{1cm} (\text{III})

Where \( F_T \) = mean value from toxicant-treated cells, and \( F_c \) = mean values from control cells, and is calculated as (Daniel et al., 1985):

\[ F = \frac{\text{ds-DNA}}{\text{ds-DNA} + \text{ss-DNA}} \]
\hspace{1cm} (\text{IV})

The rate of unwinding is influenced, by concentration of alkali, exposure duration (\( t \)), temperature, ionic strength, solvent viscosity, the nucleotide base sequence and the number of single-strand breaks per molecule (Daniel et al., 1985). The effect of varying NaOH concentration in Chinese hamster cells preirradiated with gamma (\( \gamma \))
at 0°C temperature was studied by Ahnstrom and Erixon (1981). It was demonstrated that percent ds-DNA is inversely proportional to the concentration of NaOH. Similar observations have been made by Daniel (1985), using 0.1N NaOH. The rate of DNA unwinding in alkaline solution has been determined at different temperatures (at 4°C, 38°C, 80°C) by Shugart (1988). After a 20 min incubation at 80°C (0.05N NaOH), the measured fluorescence of the ssDNA was 50% of that observed for the ds-DNA (i.e., all the ds-DNA present at time zero was completely unwound as single stranded DNA). The decrease in fluorescence was correlated to the amount of ss-DNA during the time of incubation at any temperature. Furthermore, measurement of alkaline-induced DNA separation have also been shown to be affected by alkylated bases or phosphotriesters. For instance, for the quantitative hydrolysis of DNA-adducts of simple alkylating agents at N²-alkyl guanine such as methyl methanesulphonate, dimethyl sulphate, and N-ethyl-N-nitrosourea analysis, mild conditions (30 min, 0.1N NaOH, 22°C) are considered. On the other hand, analysis of breaks due to hydrolysis of DNA methyl- and or ethyl phosphotriesters, require rigorous conditions (60min, 0.5N NaOH, 37°C) (Daniel et al., 1985).

Considering that the F value is an intrinsic property of the DNA (GC content i.e., the larger the mole fraction of GC pairs, the higher the temperature or pH of melting) (Kornberg et al., 1991), thus, the F value may vary with each organism studied.

### 2.1.3.1 Separation of Single-and Double-Stranded DNA in the Alkaline-Unwinding Assay

Separation of both single- and double-stranded DNA can be accomplished by
hydroxylapatite (HP) chromatography using calcium phosphate gel \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\). A commercial HP preparation, Bio-Gel HTP (Bio-Rad, Richmond, Calif) has a high selectivity for double stranded DNA molecules. The interaction of HP gel, apparently takes place via calcium in gel, and phosphate groups in DNA, RNA, phosphoproteins. Small polynucleotides and oligonucleotides bind weakly and can easily be separated from samples of high molecular-weight nucleic acids. Both ss-DNA and ds-DNA adsorb to HP at low phosphate concentration and elution is performed by increasing the phosphate concentration in the eluting buffer (Freifelder, 1976). The HP column can be operated either by a peristaltic pump or by gravity alone (Ahnstrom et al., 1981) and up to one milligram of DNA per mL of gel can be processed (i.e., 200 mg / 2 X 10^4-10^5 cell, 0.1-0.5 μg DNA) (Kuchler, 1977). The HP chromatography has been used as the basis for rapid isolation and purification of DNA from cell extracts. Quantification of both ss-DNA and dsDNA, can be accomplished either by liquid scintillation (LS) counting (pre-radiolabeled DNA), or by fluorescence spectroscopy (non-radiolabeled DNA) procedures.

2.1.4 HepG2 cells as the Model system

Estimation of the risk associated with carcinogenic and mutagenic agents from long-term exposure to low doses of test chemical in humans, usually involves, extrapolation from laboratory animals. It is assumed that animals are good surrogates of human systems. The main disadvantage to this approach is that the exposure levels of interest are so low that to obtain a reliable data, large numbers of animals must be used. To avoid this problem, higher doses than those encountered in the environment are being
used to produce tumours at a higher frequency. Detection of the major types of genetic damage, such as mutation, chromosomal aberration, DNA strand breaks and DNA-adducts, have been investigated in various test organisms. Although more than a hundred test systems for investigating genotoxicity have been described in the literature, bacterial mutations tests have been deployed the most extensively. In addition, cultured mammalian cells such as mouse, rat, Syrian hamster (*Mesriets auratus*), Chinese hamster (*Gricetulus griseus*) and human have also been used. A majority of studies employ cultured Chinese hamster cell lines (CHO) (O’Neill et al., 1979), Human fibroblasts (Larsarow et al., 1992) and a mouse lymphoma cell line (L5178Y) (Cole et al., 1984). As liver possesses the highest activity and is the major site of xenobiotic biotransformations, this is the tissue of choice for metabolism or toxicity studies. Rat primary hepatocytes which are known to retain their *in vivo* characteristics for extended periods of times in culture have been used extensively for these type of studies (DelRaso, 1992). However, because of difficulties in cell isolation and culture, as well as exhibition of morphological changes and lack of active cell division, most researchers avoid using them. Furthermore, reports from Michalopoulos (Michalopoulos et al., 1976), indicates a significant decline in the levels of cytochrome P-450 within 24h in rat hepatocyte cultures. Also, considering that the degree of genotoxicity expressed depends on the activation and/or detoxification by phase I and phase II metabolisms which is affected by interspecies differences, extrapolation of data from such a test organism to a phylogenetically distant species such as man may result in substantial error. An experimental system using human cells reduces the number of assumptions and extrapolations between species. A
hepatocarcinoma cell line, HepG2, was established in 1979 from a human liver biopsy (taken from a 15 year old Caucasian male from Argentina) and has been used in the present study as a model system in the study of chemical carcinogens and mutagens. These cells are easy to culture and maintain expression of tissue specific genes such as AHH (microsomal mixed function oxidase), thiol proteinase (associated with biological processes such as cleavages of parathyroid hormone (PTH) (Granner, 1988; Dufresne et al., 1993), dihydriodiol dehydrogenase (catalyzes oxidation of trans-dihydriodiol of PAH o-quinones) (Lynn et al., 1993), oxidoreductase (DT-diaphorase) (involved in detoxifying processes) (Lind et al., 1982), NADPH-cytochrome c reductase and NADH cytochrome b5 reductase (involved in redox cycle of quinones) (Duthie et al., 1989) and apolipoproteins (Dufresne et al., 1993). Therefore, HepG2 cells can be used as a hepatocyte model system in the study of xenobiotics and their potential in cyto- and genotoxicity assessments.

2.1.5 Objectives of the Present Study

This project develops two in vitro short-term bioassays in HepG2 cells to investigate the cyto-and genotoxicity potential of xenobiotics. The Neutral Red uptake and Alkaline unwinding assays are used to measure cell viability and DNA strand breaks as cyto- and genotoxic endpoints, respectively. The HepG2 cell line was calibrated, using various well characterised carcinogens and mutagens such as methyl methanesulphonate, 4-nitroquinoline 1-oxide, 9,10-phenanthrenequinone, and benzo(a)pyrene. Following calibration of these bioassays, the cyto- and genotoxic induction potential of complex mixtures of contaminants recovered from the Great Lakes Basin was studied. Sediment
extracts from Trenton channel, Lake Erie, Lake St. Clair, and Peche Island and as well as contaminant extracts from various fish species and eagle eggs from Middle Sister Island in Lake Erie and Essex county respectively, were tested using the HepG2 cell line, and their cyto- and genotoxicity potential were compared.
2.2 Materials and Methods

2.2.1 Chemicals

Methyl methanesulphonate (MMS), 4-nitroquinoline 1-Oxide, 9,10-phenanthrenequinone, Benzo(a)Pyrene and other common laboratory reagents were purchased from Sigma Chemical Company. (St. Louise, MO). Dimethyl sulfate (DMSO) was from the British Drug Houses (BDH) Inc. (Toronto, ON). Sodium lauryl sarcosinate as bought from Sigma (St. Louis, MO).

Neutral Red Stain was bought from Difco (Detroit, Michigan, USA). Hydroxylapatite gel (DNA grade) was obtained from Bio Rad Laboratories (Richmond, CA). The radioisotope, thymidine, [methyl-\(^{3}H\)] (specific activity of approx. 89.4 Ci/mmole and concentration of 1.0 mCi/ml in 70% ethanol), was purchased from ICN Biochemicals. (Mississauga, Canada). Liquid scintillation cocktail for aqueous samples (Ready safe\textsuperscript{TM}) was purchased from Beckman (Wheaton, Millville, NJ).

HepG2 cells were obtained from (ATCC HB 8065) American Type Culture Collection. Eagle's minimum essential medium (alpha) powder, Waymouth's MB 752/2 medium powder, Earle's balanced salt solution powder, fetal bovine serum (certified grade), trypsin-EDTA (x1 liquid), antibiotic-antimycotic mix (x100) were obtained from Life Technologies (Paisley, UK).

2.2.2 Supplies

Disposable sterile tissue culture flasks (24-well, 35mm\(^{2}\) petridish, 25cm\(^{2}\) and 75cm\(^{2}\)), disposable sterile polystyrene centrifuge tubes (15 and 50 ml), disposable sterile serological pipettes (5 and 10 mL), were obtained from Corning Laboratory
Sciences Company (Richmond Hill, ON). Nalgene™ disposable syringes were purchased from Nalge Company (Rochester, NY). Graduated polypropylene microcentrifuge tubes (1.0 and 1.5 mL) were from Diamed Laboratory Supplies Inc. (Mississauga, ON). Disposable poly-Q scintillation vials were bought from Backman. (Wheaton, Millville, NJ). Disposable polyethylene columns (11×1 cm), were from Evergreen Scientific (Los Angeles, CA).

2.2.3 Apparatus

A homemade thermostated water bath (Model HAAKEDI), equipped with 24 disposable columns (11×1 cm) was constructed by the University of Windsor workshop. Scintillation counting was performed on a LS 6000 Liquid Scintillation System (Beckman Instruments Inc., Mississauga, Ontario).

All spectrophotometry measurements were carried out by a UV-Visible recording Spectrophotometer (UV-160 Shimadzu).

Mass determinations were made with Metler P1000, Fisher Scientific Ltd., (Toronto, ON) and an A&D electronic ER-60A balance, Johns Scientific Inc., (Toronto, ON).

pH measurement was by Corning pH meter (model 240) (Corning Science products, Coming, NY). The electrode was calibrated prior to each measurement with the pH standard solutions obtained from BDH chemicals (Toronto, ON).

A Nuaire class II, type A/B3 flowhood biological cabinet (Plymouth, MN) was used for tissue culture. A Nuaire Auto Flow CO2 incubator was used for maintaining cells in a 95% humidified air and 5% CO2 atmosphere at 37°C. Microscopic examination of cell culture was made by a TMS inverted phase-contrast microscope (Nikon Inc., Melville, NY).

Centrifugation was carried out by Sorvall® Ultra Pro™ 80 ultracentrifuge (Dupont
Co., Mississauga, ON). All microcentrifugations (less than 10,000xg) were performed by either Model 235-C microcentrifuge (Fisher Scientific Canada Ltd., Toronto, ON) or Model 24S Sorvali microspin (Dupont Co., Mississauga, ON)

Micropipetting were performed by Eppendorf pipettes (Germany) and Nichiryo pipettes (Japan). The micropipette tips were purchased from Canlab Scientific Products (Mississauga, ON), Diamed Laboratories Supplies Inc. (Mississauga, ON) and National Scientific Supply Inc. (San Rafael, CA). An Eppendorf pipettor with Eppendorf combitips (Brinkman Instruments Inc., Westbury, NY) was used for repetitive micropipetting.

2.2.4 Sediment, Fish and Eagle Egg Sampling and Extraction

Sediments were obtained from Peche Island, Lake Erie, Lake St. Clair, and Trenton Channel. Contaminant extraction was performed by the Environmental Analytical laboratories, GLIER (Great Lakes Institute for Environmental Research) according to procedures outlined by Ali et al (1993). Extracts were subfractioned into two fractions as eluted from a Florisil column. The first fraction was composed of a mixture of polychlorinated biphenyls (PCBs) and organochlorinated compounds, and was recovered from the column by hexane. The second fraction which consisted of polycyclic aromatic hydrocarbons was eluted from Florisil column with dichloromethane (DCM): hexane (1:1). The analyses of fraction I and fraction II was performed by GC-ECD and GC-MSD respectively. Table A.1 (Appendix. A), illustrates the concentration of individual chemicals in μg/g dry sediment.

Fish species and eagle egg samples were collected from Lake Erie (Middle Sister
Island) and Essex county respectively. The extracts of fish tissues and eggs were obtained through liquid-solid extraction and gel permeation chromatography (GPC). The eluate from GPC separation was then passed through a Florisil column and mixtures of pp-DDE and Arochlor 1254:1260 were eluted with hexane. The analysis of this fraction was performed by gas chromatography with an electron capture detector. Table A.2 (Appendix A), demonstrates the concentration of individual chemicals in each fish species and eagle eggs extracts.

2.2.5 Cell Culture

Reagents

_Eagle’s Minimum Essential Medium-alpha modification (α-MEM):_ The powdered form of Eagle’s media (x1) was reconstituted at room temperature using distilled-deionized water and sodium bicarbonate (NaHCO₃) was added at a concentration of 2.2 g/L. The pH of the solution was adjusted to 0.2-0.3 below the desired final working pH. The α-MEM was sterilized by membrane filtration.

_Earle’s balanced salt solution (EBSS):_ EBSS was purchased in powder form (x1) and prepared as above. The solution contains inorganic salts, CaCl₂ (anhydrous), KCl, MgSO₄ (anhydrous), MgSO₄.7H₂O, NaCl, NaHCO₃, NaH₂PO₄.H₂O₂); D-glucose and phenol red.

_Fetal Bovine Serum (FBS):_ FBS was defrosted at 37°C and heat inactivated at 56°C for 30 minutes. _Complete Media:_ Consisted of α-MEM, 5% (v/v) FBS and 1% antibiotic-antimycotic mix (x100 stock contains 10,000 units penicillin, 10,000 µg streptomycin,
25 μg amphotericin B/mL and fungizone in 0.85% saline).

*Serum-Free Media:* Serum-Free media was prepared as reported by Adeli and Sinkevitch (1990). The basic media was composed of 3 parts α-MEM and one part Waymouth's MB 752/2, including 2 mM L-glutamine, x1 antibiotic-antimycotic solution (x100 solution with 10,000 units penicillin, 10,000 μg streptomycin, 25 μg amphotericin B/mL and fungizone in 0.85 saline), 30 nM sodium selenite, 1.0 mg/L l-inositol, 8.0 mg/L thymidine and trace elements (0.5 mg/L CuSO₄·5H₂O, 0.016 mg/L MnSO₄·H₂O, 0.03 mg/L ZnSO₄·7H₂O, 0.024 mg/L MoO₃·4H₂O, 0.0022 mg/L CoCl₂·6H₂O).

**Procedures**

Frozen Hepatomablastoma (HepG2), cells were defrosted quickly using a 37°C water bath, followed by a gradual dilution with 4 mL complete medium using a syringe (18g, 1.5 gauge). Cells were then inoculated into the culture flask with complete medium, and maintained at 37°C under a mixture of 95% humidified air and 5% carbon dioxide. The medium was replaced daily for the first week to remove any residues of dimethyl sulfoxide which was used as preservative. After this period, the medium was changed every two or three days. At confluence, a 80-85% monolayer was examined macroscopically and microscopically for any signs of bacterial contamination. Cells were subcultured by cold trypsinization (4°C) with 1-2 mL trypsin/EDTA (0.25% trypsin in 1.0 mM EDTA, [1 mL / 25cm² flask, 2 mL / 75cm² flask]). After 90 seconds (room temperature), trypsin solution was removed and the flask containing residual trypsin was kept at room temperature for 2-3 minutes. The cells were resuspended in a complete medium (4°C) (5 mL / 25cm² flask, 10 mL / 75cm²), and
dispersed gently by pipetting. The next step was to inoculate approximately 1.0 \times 10^5 to 1.6 \times 10^6 cells per flask (e.g., 75cm²) with 10 mL complete media. Cells were discarded after 20 consecutive passages (i.e., 20 subcultures), and a new vial containing HepG2 cells was thawed from the -80°C freezer.

**2.2.6 HepG2 DNA Radiolabeling**

**Reagents**

Complete medium, Thymidine, [methyl-³H] with specific activity of 89.4 Ci/mmol and concentration of 1.0 mCi/mL.

**Procedure**

Once cells reached about 60% confluency the medium was replaced with media containing thymidine, [methyl-³H] at a concentration of 1.0 μCi/mL. The cells were then incubated for 24-48h at 37°C in an atmosphere of 95% humidified air and 5% carbon dioxide.

**2.2.7 Chemical Treatment**

**Reagents**

Complete medium, Serum-free medium, EBSS solution, Test chemical

**Procedure**

Cells prelabeled with thymidine, [methyl-³H], were rinsed with EBSS solution and incubated in complete medium two hours prior to chemical treatment. Chemical treatment was performed with 1% solvent using complete and or serum free medium. The solvent was used as a negative control in all experiments. Treated cells were maintained in a
atmosphere of 95% humidified air and 5% carbon dioxide.

2.2.8 Neutral Red Uptake Assay

Reagents

Fixative solution: 0.5% (v/v) formaldehyde, 1% (w/v) CaCl₂

Solution of 1% (v/v) acetic acid:50% (v/v) ethanol.

Neutral Red Stain 25 µg/mL.

Procedure

A revised version of the Babish and Borenfreund (1992) technique was used in this study. HepG2 cells at 60% confluency in a 24-well tissue culture plate were treated with the test agent. Cells were then washed with EBSS solution x3 to remove any chemical residue, a 1.0 mL solution of neutral red stain (25 µg/mL) was added and incubated for an hour. After this period, cells were washed with 1.0 mL fixative solution and then dye extracted by adding 0.5 mL solution of acetic acid 1%:ethanol 5% to each well. Cell cultures were left for 2-3 min and then agitated for a minute. The absorbance of extracted dye, as a measure of cell viability, was then determined using a spectrophotometer equipped with a 540-nm filter. Percent cell viability of various test agent concentrations was calculated by equation 1 (see text, Pp 26).

2.2.9 Alkaline Unwinding Assay

Reagents

Phosphate-Buffered Saline (PBS): NaCl (8.0 gr/L), KCl (0.3 gr/L), Na₂HPO₄ (0.73...
gr/L), KH$_2$PO$_4$ (0.20 gr/L), glucose (2.0 gr/L), pH 7.35-7.4

PBS solution containing $0.02 \, M$ disodium EDTA, pH 7.35-7.4

Sodium hydroxide (NaOH, 0.1 N), hydrochloric acid (HCl, 0.1 N), sodium lauryl sarcosinate (2%) containing 0.02M disodium EDTA.

**Procedure**

Single stranded breaks in HepG2 cell DNA were determined using a modified version of Daniel *et al.*, (1985). Treated HepG2 cells were rinsed x3 with ice cold PBS and then treated with 0.5 mL solution of PBS containing 0.02 M disodium EDTA. 0.5 mL NaOH (0.1 N) was then forcefully expelled from an eppendorf pipettor into the solution. The cells were then kept in a dark chamber for 25-30 min to protect them from light and also to minimize vibration of the culture plate. After the unwinding period, lysates were neutralized by 0.5 mL HCl (0.1 N, [neutralization was determined with phenolphthalein indicator]). The DNA aggregates formed by neutralization were dispersed by addition of 250 μL 2% (w/v) sodium lauryl sarcosinate containing 0.02M disodium EDTA and the solution was then sonicated for 10 seconds. The lysates were then stored at 4°C for further analysis.

**2.2.10 Separation of Single and Double-Stranded DNA**

**Reagents**

Hydroxyapatite gel, potassium phosphate buffers (PPB) at 0.012M, 0.1M, and 0.5M, with pH adjusted to pH 7.0.

**Procedure**

2.4g Hydroxyapatite gel was suspended in 20 mL 0.012M PPB for 10 minutes, the
supernatant was then discarded and the gel resuspended in 28 mL 0.012M PPB solution and boiled vigorously for 2-3 minutes, after which it was thoroughly degassed. Aliquots of the resuspended gel (1mL) were added to the lysate in 24-well tissue culture plates. Following gentle agitation, the lysates were transferred to separate test tubes and incubated in a thermostated water bath (50-60 °C, 10 min). The suspension was then poured into a 11x1 cm polyethylene column in a water bath at 50-60°C. After the lysates were passed through the column, the column bed was washed with 10 mL 0.012 M PPB, the elution of single stranded DNA (ss-DNA) was performed with 2 mL 0.1 M PPB and double stranded DNA (ds-DNA) at 0.5 M PPB. Both fractions were stored at -4°C for further analysis.

2.2.11 Liquid Scintillation Method

Reagents

Concentrated HCl, liquid scintillation cocktail

Procedure

The quantification of both ssDNA and dsDNA fractions was accomplished by liquid scintillation assay. Prior to reading the activity or disintegration per minute (dpm), 0.5 mL aliquots of the hydroxylapatite column washes were added to miniature vials with 50 μL concentrated HCl, after which the vials were capped and incubated at 80°C (1hr) for nucleic acid digestion. Following this period, the vials were cooled and 2 mL of aqueous liquid scintillation cocktail was added and mixed vigorously. The activity of the samples were determined using a Packard LS 6000 Scintillation counter. While the unknown sample was being counted, the quench parameter was measured and the counting efficiency of the
sample was automatically determined from the stored quench curve. Activity (dpm i.e., disintegration per minute) is calculated as follows:

\[ \text{dpm} = \frac{\text{Measured cpm (count per minute)}}{\text{Determined counting efficiency}} \]

The F value is then determined by dividing dpm of ds-DNA by the sum of the dpm of ds-DNA and ss-DNA (equation iv, Pp 30).
2.3 Results and Discussion:

2.3.1 Calibration of the HepG2 Cell Culture System with Known Carcinogens and Mutagens

Prior to assessment of the induction potential of HepG2 cell lines by environmental contaminants, the cell line was calibrated by measuring its biological response to known chemical mutagens/carcinogens. These included simple direct acting agent such as MMS, and indirect acting agents such as, 4NQO, PQ, and B(a)P as a PAH representative.

The cytotoxicity potential of these chemicals was established by quantitative assessment of cell viability of HepG2 at various concentrations using the NR uptake assay. At minimal toxic concentration range of test agents, the genotoxicity potential of target chemicals was then investigated by measuring DNA strand breaks via the AU assay.

Cytotoxicity and genotoxicity data for all chemicals were obtained by growing a monolayer of HepG2 cells in complete medium to 60-70% confluency in 24-well tissue culture plates. The cell cultures were then treated with freshly prepared chemicals either in the presence of serum free medium or 5% heat inactivated fetal bovine (FBS) serum in complete medium. However, in the DNA strand break assay, cells were first labelled with radiolabelled precursor (1μCi/mL ³[H]thymidine) which is accumulated in the cell and converted to deoxythymidine three phosphate (dTTP). Prior to chemical treatment, the cells were rinsed with EBSS solution and incubated for 2h in complete medium and then treated with the test chemical(s) (final solvent concentration ≤ 1.0%) for a defined exposure period (i.e., 24-48h). The N, and F values were then determined
using equation II, and iv respectively (see text, Pp 30). The variability of both assays, the mean and the standard deviation of the OD$_{560}$ and F values were determined by the number of replicate cultures (n): for each concentration level. Also, the percentage of viable cells, were calculated relative to the negative control (solvent only) using equation I (see text, Pp 26).

2.3.1.1 Methyl methanesulphonate

Methyl methanesulphonate (MMS) is a simple monoalkylating agent which is characterized as a mutagen in cultures of Chinese hamster ovary (CHO) (K1-BH4) (O’Neill et al., 1979) and Salmonella Typhimurium (Eder et al., 1990) and a clastogen (i.e., causes chromosomal changes) in epithelial cells of the murine small intestine (Tao et al., 1993).

In this study, HepG2 cells responded to MMS in a concentration-dependent fashion with induction of cytotoxicity and genotoxicity after 24h exposure in serum-free medium (Figure 2.3A). MMS caused a significant (ANOVA, P<0.05) decrease in viable cells at concentration levels of 1.00 and 1.5mM corresponding to 11.41 and 10.7% cell viability, respectively. However, at lower concentrations i.e., 0.25 and 0.5mM, the cytotoxicity was not as significant. In studying the genotoxic effect of this chemical, the relative strand break potential within the concentration range of 0.125-0.5mM was tested. The N values were significantly (ANOVA, P<0.05) higher at 0.25 and 0.5 mM, corresponding to 0.87 (F=73%) and 1.68 (F=54.24%), respectively. At lower concentrations such as 0.125 mM no statistically significant strand breaks over the negative control was observed. A
detailed data both from cyto- and genotoxicity are listed in Table 2.3.1. In comparing both cyto- and genotoxic induction potential of MMS in HepG2 cells, the genotoxic effect was demonstrated at lower concentrations (i.e., 0.25 and 0.5 mM), whereas the cytotoxic effect was not as significant at these concentrations. Fig 2.3A compares both cyto- and genotoxic effects of MMS in HepG2 cells after 24h exposure in terms of percent cell viability and percent F value (relative to negative control i.e., serum-free media), respectively vs dose administered. A similar pattern of dose-response relationship, was observed by Ali et al., (1994), in HepG2 cell line exposed to MMS, using the NR uptake and unscheduled DNA synthesis assays.

MMS is known to form various DNA adducts with heterocyclic DNA bases (i.e., O and N atoms). DNA adducts at purine-N7 and purine-N3 result in a greatly iabilized N-glycosidic linkages (Daniel et al., 1985). The sequential action of glycosylase and then AP-site endonuclease can produce strand breaks in chemically treated cells. Thus, if the breaks are not repaired, this may lead to irreversible DNA damage, resulting in prevention of DNA duplication and eventually cell death (Ernst, 1971). Therefore, the cyto- and genotoxic effect of the MMS observed in HepG2 cells can be related to direct DNA damage as outlined above.
<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Neutral Red (OD.540nm)</th>
<th>DNA Strand Breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M±SD(n)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>%CV&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.00</td>
<td>0.7±0.05(2)</td>
<td>100</td>
</tr>
<tr>
<td>0.125</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.25</td>
<td>0.68±0.07(3)</td>
<td>97.31</td>
</tr>
<tr>
<td>0.50</td>
<td>0.59±0.08(3)</td>
<td>85.52</td>
</tr>
<tr>
<td>1.00</td>
<td>0.08±0.01(3)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>11.41</td>
</tr>
<tr>
<td>1.50</td>
<td>0.07±0.01(3)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>10.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> F = Mean fraction of DNA remaining double-stranded after unwinding.

<sup>b</sup> Values represent Mean±SD of independent cultures of n replicates.

<sup>c</sup> Percent cell viability values calculated relative to negative control (serum free media).

<sup>d</sup> N = Increase in DNA strand breaks (N value) relative to control.

<sup>*</sup> At α = 0.05 (P<0.05), determined by one way ANOVA test statistic.

ND = Not determined.
2.3.1.2 4-nitroquinoline 1-Oxide

4-NQO a potent carcinogen known to induce cancer in various tissues such as lung, pancreas, and stomach (Bailleul et al., 1989), induced significant cyto-and genotoxicity in HepG2 cells in a dose-dependent manner (Figure 2.3. B) after 24h exposure in serum-free medium. Cytotoxicity data, from the NR uptake assay, indicate a significant (ANOVA, P<0.01) decrease in absorbance of extracted dye at concentrations of 20, 100, and 1000 μM corresponding to 75, 66.2, and 46.1% cell viability, respectively (Table 2.3.2). The genotoxic induction potential as measured by single strand breaks via AU assay showed a concentration-related increase in DNA strand breaks within concentration range of 0.5-100 μM after 24h exposure. Significant (ANOVA, P<0.05) levels of strand breaks were found in a concentration range of 1.0 to 100μM corresponding to N values of 0.99 (F=73.3%) and 4.74 (F=31.1%) relative strand breaks in HepG2 cell DNA, respectively. Table 2.3.2 summarizes the data for cyto- and genotoxicity of 4NQO in a 24h study in the presence of serum free medium. Also, Figure 2.3. B compares both cyto- and genotoxic effect of 4NQO (24h exposure) in terms of % F value and % cell viability relative to the negative control (DMSO final concentration ≤ 1%). This figure demonstrates that the DNA damage as measured by single strand breaks (in terms of % F value) occurs at a lower concentration range compared to cytotoxicity response which takes place at higher concentrations of 4-NQO.

Cyto- and genotoxicity of 4-NQO in HepG2 cells have previously been demonstrated (Hasspieler et al., 1995) using the NR uptake, UDS, and the AU assays. 4NQO was also mutagenic in a bacterial assay (Fukuda et al., 1972), and induced cyto- and genotoxic effects in a wide range of biological systems such as Escherchia coli (Ikenaga et al.,
1975), cultured mouse cells (Ikenaga et al., 1977), and human cells (Ikenaga et al., 1977). The cytotoxicity and carcinogenicity of 4NQO is governed by its metabolic activation to 4-hydroxyaminoquinoline 1-oxide (4HAQO). The initial reaction, involves reduction of 4NQO to 4HAQO by cytosolic enzyme such as DT diaphorase which is a NAD(P)H quinolineoxido reductase. This primary metabolite can undergo further reduction by enzymes localized in the endoplasmic reticulum and mitochondria to form 4-aminoquinoline 1-oxide, a non-toxic metabolite (Jeffrey et al., 1976; Sugimura, 1981). 4HAQO is believed to be the carcinogenic form of 4NQO. Stable 4-NQO-purine adducts with guanine and adenine moieties of DNA have been related to toxicity and mutation in Escherichia Coli, in cultured mouse cells, and also in human cells (Ikenaga et al., 1977).
<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Neutral Red (OD.540nm)</th>
<th>%CV</th>
<th>DNA Strand Breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M±SD(n)(^b)</td>
<td></td>
<td>F±SD(n)(^b)</td>
</tr>
<tr>
<td>0.00</td>
<td>1.00±0.02(4)</td>
<td>100</td>
<td>0.78±0.05(20)</td>
</tr>
<tr>
<td>0.02</td>
<td>0.98±0.02(3)</td>
<td>97.5</td>
<td>ND</td>
</tr>
<tr>
<td>0.5</td>
<td>ND</td>
<td>ND</td>
<td>0.70±0.01(2)</td>
</tr>
<tr>
<td>1.0</td>
<td>ND</td>
<td>ND</td>
<td>0.61±0.01(2)*</td>
</tr>
<tr>
<td>20</td>
<td>0.75±0.07(4)**</td>
<td>75.0</td>
<td>ND</td>
</tr>
<tr>
<td>100</td>
<td>0.66±0.05(4)**</td>
<td>66.2</td>
<td>0.24±0.03(2)**</td>
</tr>
<tr>
<td>1000</td>
<td>0.46±0.04(3)**</td>
<td>46.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) F = Mean fraction of DNA remaining double-stranded after unwinding.
\(^b\) Values represent Mean±SD of independent cultures of n replicates.
\(^c\) Percent cell viability values calculated relative to negative control (dimethyl sulfoxide 1%).
\(^d\) N = Increase in DNA strand breaks (N value) relative to control.
* At \( \alpha = 0.05 \) (P<0.05), ** at \( \alpha = 0.01 \) (P<0.01), the level of significance determined by one way ANOVA test statistic.
ND = Not determined.
2.3.1.3 9,10-Phenanthrenequinone

Quinones, such as 9,10-phenanthrenequinone (PQ), are ubiquitous environmental pollutants formed by various combustion processes (e.g., cigarette smoke, automobile and diesel exhaust, and urban air particulates) (O’Brien, 1991). HepG2 cells displayed metabolic competency after exposure to PQ. Data from cytotoxicity, and DNA strand breaks studies, revealed a concentration-dependent response after a 24h exposure to PQ in serum-free medium. At the minimum cytotoxicity level (i.e., 0.3 μM with 88.5% cell viability), the N value was 0.53 corresponding to 91.2% F value. At higher concentrations, such as 0.36 and 1.44 μM, a significant decrease in % cell viability was observed. Also, the induction of strand breaks in HepG2 cells was highest at 0.5 and 3.0 μM corresponding to relative strand breaks (i.e., N value) of 3.3 (F value = 59%) and 2.2 (F value = 69%), respectively. However, a decrease in relative strand breaks and a corresponding higher F value at 3.0 μM was observed, possibly as a result of the saturation of the metabolic system and/or the repair enzymes. Table 2. 3. 3 presents the data on cyto- and genotoxicity of PQ in a 24h study in the HepG2 cell line in serum free medium. A plot of % F value and % cell viability vs concentration (Figure 2. 3. C) demonstrates a concentration-dependent relationship both in cyto- and genotoxicity induction potential in HepG2 cells. DNA strand breaks and cell viability plots indicate a similar sensitivity with the same concentration range in contrast to MMS and 4-NQO which induced genotoxic response at lower concentrations compared to cytotoxicity.

Mutagenicity of PQ has been demonstrated in bacteria (Salmonella strain), which are sensitive to a wide variety of oxidative mutagens (Chesis et al., 1984). PQ
as a quinone, may exert its cyto- and genotoxic effects by one-electron reduction of PQ. Semiquinone radical, as a product of this reaction, then reduces oxygen to superoxide radicals and reforms the quinone. One-electron reduction of quinones which is known as the cytotoxic pathway which results in oxidative stress by the production of oxygen species (O$_2^-$, H$_2$O$_2$, OH$^-$), leading to oxidative damage to DNA bases, as well as perturbation of Ca$^{2+}$ homeostasis, and eventually cell death (Kappus et al., 1981; O'Brien, 1991).
Table 2.3.3 Cyto- and genotoxicity of 9,10-phenanthrenequinone in HepG2 Cells after 24h Exposure

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Neutral Red (OD. 540nm)</th>
<th>DNA Strand Breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M±SD(n)</td>
<td>%CV</td>
</tr>
<tr>
<td>0.00</td>
<td>0.44±0.02(2)</td>
<td>100</td>
</tr>
<tr>
<td>0.30</td>
<td>0.38±0.03(2)</td>
<td>88.5</td>
</tr>
<tr>
<td>0.36</td>
<td>0.33±0.01(2)*</td>
<td>77.0</td>
</tr>
<tr>
<td>0.50</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1.44</td>
<td>0.29±0.01(2)*</td>
<td>66.5</td>
</tr>
<tr>
<td>3.00</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a F = Mean fraction of DNA remaining double-stranded after unwinding.
b Values represent Mean±SD of independent cultures of n replicates.
c Percent cell viability values calculated relative to negative control (acetone 1%).
d N = Increase in DNA strand breaks (N value) relative to control.
* At α = 0.05 (P<0.05), ** at α = 0.01 (P<0.01), the level of significance determined by one way ANOVA test statistic.
ND = Not determined.
2.3.1.4 Benzo(a)Pyrene

Benzo(a)pyrene (B(a)P), a polynuclear aromatic hydrocarbon (PAH), is formed by incomplete combustion of fossil fuels, organic matter and garbage. In addition, it is a component of many petroleum products, asphalt, cigarette smoke and vehicle exhaust (Chesis et al., 1984). B(a)P has been shown to be a carcinogen and a mutagen to bacterial strains in the presence of liver microsomal activation systems (Green et al., 1976). Both cyto- and genotoxicity effects of B(a)P-exposed HepG2 cells, were studied in 24h and 48h treatments in serum free media and 5% FBS in complete medium at concentrations of 7.8, 15.5, 31.2 and 62.4 µM. Treatment of cells with B(a)P in serum-free culture medium did not show any significant cyto- and genotoxic effects (data not shown). However, 48h treatments of HepG2 cell lines with B(a)P in complete media (5% FBS) resulted in DNA strand breaks in a dose-dependent manner, up to 31.2 µM (Table 2.3.4). Also, a significant (ANOVA, P<0.05) decrease in cell viability was demonstrated at 31.2 µM, corresponding to 97.2% cell viability, and a N value of 0.48, corresponding to a F value of 74.7%. Exposing the cells with higher concentrations of B(a)P (62.4 µM) yielded a lower N value of 0.28, corresponding to a F value of 82.5% relative to lower concentrations. This observation was similar to the effect seen with PQ, and may be related to the saturation of metabolic systems or the repair enzymes at high concentrations of B(a)P. A graphical presentation of %F value and % cell viability vs concentration (48h exposure, 5% FBS in complete media) demonstrates a dose-dependent relationship respond only in the DNA strand breaks assay (Figure 2.3. D), whereas the cytotoxicity response was not dose-dependent. The increased cell viability at 7.8, and
15.5 μM, may be due to experimental variations (i.e., uneven cell number).

The increased cyto- and genotoxic effect of a highly hydrophobic chemical such as B(a)P in the presence of 5% FBS compared with serum-free media, may be due to the presence of transport proteins such as transferrin and albumin in the serum, thereby, increasing accessibility to the cell.

In general, PAHs such as B(a)P require metabolic activation to highly reactive metabolites, such as anti-diol epoxide, reactive o-quinone and oxygen species (O2-, H2O2, OH·), in order to exert any biological effect. Thus, the observed genotoxic effect in HepG2 cells can be related to interaction of these reactive metabolites and macromolecules in the cell, resulting in DNA adduct formation, strand breakage, and cell toxicity (for detailed mechanism refer to text, Pp 10). Table 2. 3. 4 summarizes the cyto- and genotoxicity data after 48h exposure of HepG2 cells to B(a)P in 5% FBS containing complete medium.
<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Neutral Red (OD.540nm)</th>
<th>DNA Strand Breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M±SD(N)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>%CV&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.00</td>
<td>0.40±0.004(2)</td>
<td>100</td>
</tr>
<tr>
<td>7.80</td>
<td>0.42±0.01(2)</td>
<td>104.8</td>
</tr>
<tr>
<td>15.5</td>
<td>0.44±0.01(2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>109.8</td>
</tr>
<tr>
<td>31.2</td>
<td>0.39±0.004(2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>97.2</td>
</tr>
<tr>
<td>62.4</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> F = Mean fraction of DNA remaining double-stranded after unwinding.
<sup>b</sup> Values represent Mean±SD of independent cultures of n replicates.
<sup>c</sup> Percent cell viability values calculated relative to negative control (Dioxane 1%).
<sup>d</sup> N = Increase in DNA strand breaks (N value) relative to control.
<sup>*</sup> At α = 0.05 (P<0.05), the level of significance determined by one way ANOVA test statistic.
ND = Not determined.
FIGURE 2.3

Comparative Cyto- and Genotoxicity of Methyl methanesulphonate, 4-nitroquinoline 1-oxide, 9,10-phenanthrenequinone, and Benzo(a)Pyrene in HepG2 cells

Legend

Plot of percent double-stranded DNA (F value) and percent cell viability vs dose administered for methyl methanesulphonate, 4-nitroquinoline 1-oxide, and 9,10-phenanthrenequinone after 24h exposure in serum-free medium, and benzo(a)pyrene after 48 exposure in complete medium in the HepG2 cells.
FIGURE 2.3

A. Methyl methanesulphonate (mM)

B. 4-nitroquinoline 1-oxide (μM)

C. 9,10-phenanthrenequinone (μM)

D. Benzo(a)pyrene (μM)
2.4.1 Cyto- and Genotoxicity Assessment of Sediment Extracts in the Great Lakes

Sediments and suspended particulate matter play important roles in the dynamics of organic compounds in an aquatic environment. Hydrophobic contaminants enter an aqueous environment by adsorbing and accumulating on sediment particles and suspended particulates. In this manner, fine-grained organic sediments can be contaminated from historic, unregulated discharges. Thus sediment-associated contaminants as in-place pollutants will continuously expose aquatic biota to toxic substances (Smith et al., 1985). The International Joint Commission (International Joint commission, 1983) has identified over 1000 industrially-derived chemical contaminants in the biota, water column, and sediment of the Great Lakes. Another report from the same agency has identified 42 contaminated locations in the Great Lakes as areas of concern or hot-spots (International Joint Commission, 1988).

For the purpose of this study, contaminated sediments were sampled (Summer 1993) at four sites in the Great Lakes. These include Peche Island, Lake St. Clair, Lake Erie (Middle Sister Island) and Trenton Channel. The extracts from contaminated sediments were subfractioned into fractions I and II. Fraction I consisted of organochlorinated pesticides and polychlorinated biphenyls (Arochlor Mix 1254/1260). Fraction II comprised of a mixture of PAHs. Table A.1 (Appendix A), summarizes the concentration of extracted contaminants in both fractions.

The responses of HepG2 cells to in-place pollutants were evaluated using both NR uptake and AU assays. The HepG2 cells were grown to 60-70% confluency in 24-well
tissue culture plates. Both fractions at all sites were cyto- and genotoxic in HepG2 cells to various degrees in serum-free medium. 24h or 48h incubation of both fractions from Peche Island (Table 2. 4. 1), Lake St. Clair (Table 2. 4. 2), Lake Erie (Table 2. 4. 3) and Trenton Channel (Table 2. 4. 4) only showed decreased % cell viability at higher concentration (100 fold dilution). Also, data from DNA strand breaks in HepG2 cells revealed a decrease in F value and an increase in N value at the maximal concentration of the extracts (100 fold dilution), in both fractions and at all sites. After 48h exposure of fraction I to HepG2 cells at 100 fold dilution, the observed cytotoxicity was Trenton Channel (91.0%), Lake St. Clair (94.4%), Lake Erie (103%), Peche Island (107%). Similarly, fraction II extracts induced cytotoxicity as follows: Trenton Channel (86%), Lake St. Clair (92.0%), Lake Erie (94%), and Peche Island (105%). Higher toxicity was induced by fraction II which comprised numerous PAHs. Induced DNA strand breaks (N values) by fraction I as measured by the AU assay were, Lake St. Clair (0.97), Lake Erie (0.88), Peche Island (0.25), Trenton Channel (0.00). Similarly fraction II N values were, Lake St. Clair (1.26), Lake Erie (0.97), Peche Isl (0.51), and Trenton Channel (0.51). Cyto- and genotoxicity data indicate fraction II from all sites was more potent than fraction I. Interestingly, genotoxicity of sediment extracts in both fractions I and II was inversely correlated to percent organic matter in sediments, which were Lake St. Clair (3.97%), Lake Erie (8.9%), Peche Island (9.15%), and Trenton Channel (11.06%). With 24h exposure of HepG2 cells to Trenton Channel contaminant extracts (fractions I and II) using 200 fold dilution (10.97 g/mL, dry basis) of the extracts, marginal strand breaks were observed (Table 2. 4. 5). Furthermore, HepG2 cells treated in complete medium,
instead of serum-free medium, did not significantly respond to Trenton Channel extracts (fraction I, II) both in the NR and AU assays after 48h exposure (100 fold dilution from 19.75 g/ml stock solution) compared to controls (data not shown).

Although there is variable information concerning the acute or chronic effects of a number of individual chemicals, the information is not useful for evaluating the toxicological significance of a complex chemical mixture. The components of a complex mixture may interact to produce synergistic, antagonistic, or additive effects. For example, mutagenicity of binary mixtures of isomers of nitrobenzo(a)pyrene was shown to be greater than the sum of the mutagenicity produced by the individual compounds (Hass et al., 1987). Moreover, the biological effects from such mixtures can also be affected by inhibition (reversible or irreversible), or induction of metabolic activating enzymes such as cytochrome P<sub>450</sub> by components of the mixture. Reversible inhibitors such as α-naphthoflavon can occupy the active site of the enzyme and thus retard the processing of other xenobiotics. Alternatively, an irreversible inhibitor such as carbon tetrachloride could cause peroxidation of lipids, which in turn destroys cell membrane integrity, with subsequent loss of P<sub>450</sub> activity (Zakrzewski, 1991). Mutagenicity and carcinogenicity of many PAHs are well established and the majority of mutagenic effects of organic extracts of sediments from industrial areas in Salmonella Typhimurium mutagenicity tests was accounted for by fractions containing PAH and nitro-PAH. In a study by Sato et al., (1983) on testing constituents of the iso-octane-benzene fraction of Arata River (Gifu, Japan) sediments, indicated benzo(b)fluoranthene and B(a)P as the only mutagens, which were correlated to observed
mutagenicity. The amount of benzo(b)fluoranthene and B(a)P in the sediment were 3.4 and 2.2 μg/g (dry weight). Mutagenicity of PAHs has also been correlated to the number of benzene rings. West et al., (1986) showed that fractions containing PAHs with four to six aromatic rings, in particular the fraction composed of five-ring compounds, largely accounted for mutagenic activity in the presence of exogenous metabolizing enzymes. Additionally, among the organic extracts of sediments from an Eastern Massachusetts pond, historically shown to be mutagenic to human cells such as, B-lymphoblastoid cells (MCL-3), only one compound, B(a)P, was found mutagenic (Durant et al., 1994).

The response of HepG2 cells to fraction II which was composed of carcinogenic and mutagenic PAHs, further confirmed the cyto- and genotoxic potential of these mixtures compared to fraction I which consisted of mostly OCCs.
<table>
<thead>
<tr>
<th>FR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutral Red (OD.540 nm)</th>
<th>DNA Strand Breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24h</td>
<td>48h</td>
</tr>
<tr>
<td>(I)</td>
<td>M±SD(n)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>%CV&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.00</td>
<td>0.59±0.02(4)</td>
<td>100</td>
</tr>
<tr>
<td>500</td>
<td>0.59±0.03(4)</td>
<td>100</td>
</tr>
<tr>
<td>400</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>200</td>
<td>0.61±0.04(4)</td>
<td>103</td>
</tr>
<tr>
<td>100</td>
<td>0.51±0.08(4)</td>
<td>86</td>
</tr>
<tr>
<td>(II)</td>
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<td></td>
</tr>
<tr>
<td>500</td>
<td>0.53±0.03(4)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>90</td>
</tr>
<tr>
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<td>0.53±0.06(4)</td>
<td>90</td>
</tr>
<tr>
<td>100</td>
<td>0.51±0.03(4)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>86.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> F = Fraction of DNA remaining double stranded after unwinding.
<sup>b</sup> Values represent Mean±SD of independent cultures of n replicates.
<sup>c</sup> Percent cell viability calculated relative to control (dimethyl sulfoxide 1%).
<sup>d</sup> N = Increase in DNA strand breaks (N value) relative to control (dimethylsulfoxide).
<sup>e</sup> FR = Sediment extract fraction.

*At α = 0.05 (P<0.05), the level of significance determined by one way ANOVA test statistic.
ND = Not determined.
NB: Aliquots are made from 7.056 gr eq/mL dry weight.
<table>
<thead>
<tr>
<th>FR&lt;sup&gt;e&lt;/sup&gt;</th>
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<td></td>
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<td>%CV&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.00</td>
<td>0.59±0.01(4)</td>
<td>100</td>
</tr>
<tr>
<td>500</td>
<td>0.50±0.1(4)</td>
<td>85.0</td>
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<td>0.55±0.02(3)*</td>
<td>93.0</td>
</tr>
<tr>
<td>100</td>
<td>0.60±0.03(3)</td>
<td>102</td>
</tr>
<tr>
<td>500</td>
<td>0.57±0.01(3)</td>
<td>97.0</td>
</tr>
<tr>
<td>200</td>
<td>0.64±0.01(3)*</td>
<td>109</td>
</tr>
<tr>
<td>100</td>
<td>0.51±0.03(4)**</td>
<td>86.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> F = Mean fraction of DNA remaining double-stranded after unwinding.
<sup>b</sup> Values represent Mean±SD of independent cultures of n replicates.
<sup>c</sup> Percent cell viability calculated relative to control (dimethyl sulfoxide).
<sup>d</sup> N = Increase in DNA strand breaks (N value) relative to control (dimethyl sulfoxide).
<sup>e</sup> FR = Sediment extract fraction

* At α = 0.05 (P<0.05), ** at α = 0.01(P<0.01), the level of significance determined by one way ANOVA test statistic.

ND = Not determined.

NB; aliquots are made from 11.92 g eq/mL dry weight.
<table>
<thead>
<tr>
<th>FR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutral Red (OD 540 nm)</th>
<th>DNA Strand Breaks</th>
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<tbody>
<tr>
<td>(I)</td>
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<tr>
<td>0.00</td>
<td>0.36±0.01(3)</td>
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</tr>
<tr>
<td></td>
<td>0.36±0.01(3)</td>
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</tr>
<tr>
<td>500</td>
<td>0.43±0.01(3)**</td>
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<tr>
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<td>0.43±0.01(3)</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0.39±0.01(3)*</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>0.39±0.01(3)</td>
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<tr>
<td>100</td>
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<td>100</td>
</tr>
<tr>
<td></td>
<td>0.36±0.01(3)</td>
<td></td>
</tr>
<tr>
<td>200</td>
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</tr>
<tr>
<td></td>
<td>0.35±0.01(3)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.34±0.01(3)</td>
<td>94</td>
</tr>
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</table>

<sup>a</sup> F = Mean fraction of DNA remaining double-stranded after unwinding.

<sup>b</sup> Values represent Mean±SD of independent cultures of n replicates.

<sup>c</sup> Percent cell viability calculated relative to control (dimethyl sulfoxide).

<sup>d</sup> N = Increase in DNA strand breaks (N value) relative to control (dimethyl sulfoxide).

<sup>e</sup> FR = Sediment extract fraction

ND = Not determined.

NB: aliquots are from 6.37 g eq/mL dry weight.

*At α = 0.05 (P<0.05), ** at α = 0.01 (P<0.01), the level of significance determined by one way ANOVA test statistic.
Table 2.4.4 Cytotoxicity of Sediment Extracts from Trenton Channel in HepG2 cells.

<table>
<thead>
<tr>
<th>FR&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Neutral Red (OD 540 nm)</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M±SD(n)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>%CV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>M±SD(n)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(I)</td>
<td>0.00 0.54±0.09(3)</td>
<td>100</td>
<td>1.10±0.04(3)</td>
</tr>
<tr>
<td></td>
<td>500 0.59±0.04(3)</td>
<td>109</td>
<td>1.15±0.03(3)</td>
</tr>
<tr>
<td></td>
<td>200 0.56±0.02(3)</td>
<td>104</td>
<td>1.15±0.02(3)</td>
</tr>
<tr>
<td></td>
<td>100 0.55±0.05(3)</td>
<td>102</td>
<td>1.00±0.04(2)</td>
</tr>
<tr>
<td>(II)</td>
<td>0.00 0.5±0.02(3)</td>
<td>100</td>
<td>1.09±0.05(3)</td>
</tr>
<tr>
<td></td>
<td>500 0.53±0.01(3)</td>
<td>106</td>
<td>1.08±0.02(3)</td>
</tr>
<tr>
<td></td>
<td>200 0.54±0.04(3)</td>
<td>108</td>
<td>1.00±0.02(3)</td>
</tr>
<tr>
<td></td>
<td>100 0.50±0.01(3)</td>
<td>100</td>
<td>0.94±0.05(3)**</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent Mean±SD of independent cultures of n replicates.

<sup>b</sup> Percent cell viability calculated relative to parallel control (hexane).

<sup>c</sup> FR= Sediment extract fraction.

NB: aliquots are made from 19.75 g eq/mL dry weight sediment.

**At α = 0.01(P<0.01), the level of significance determined by one way ANOVA test statistic.
<table>
<thead>
<tr>
<th>FR / I</th>
<th>DNA Strand Breaks</th>
<th>DNA Strand Breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24h</td>
<td>48h</td>
</tr>
<tr>
<td></td>
<td>F ± SD(n)</td>
<td>N</td>
</tr>
<tr>
<td>0</td>
<td>0.83±0.01(2)</td>
<td>0</td>
</tr>
<tr>
<td>800</td>
<td>0.84±0.3(2)</td>
<td>-0.30</td>
</tr>
<tr>
<td>400</td>
<td>0.79±0.01(2)</td>
<td>0.95</td>
</tr>
<tr>
<td>200</td>
<td>0.74±0.10(2)</td>
<td>0.21</td>
</tr>
<tr>
<td>100</td>
<td>ND</td>
<td>0.46±0.02(4)</td>
</tr>
<tr>
<td></td>
<td>FR = Sediment extract fraction.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N = Increase in DNA strand breaks (N value) relative to control (acetone in 24h, hexane in 48h treatment).</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ND = Not determined.</td>
<td></td>
</tr>
</tbody>
</table>

NB: Aliquots are made in 24h treatment from 10.97 g eq/mL [fractions I, II], in 48h treatment from 19.95 g eq/mL [fraction I], 24.4 g eq/mL [fraction II] dry sediment.). *At α = 0.05 (P<0.05), the level of significance determined by one way ANOVA test statistic.
2.5.1 Cyto- and Genotoxicity Evaluation of Contaminant Extracts from Fish Tissues and Eagle Eggs

Fish are excellent indicators of ecosystem health, they are exposed to aquatic contaminants and bioaccumulate (process by which chemical substances are accumulated by aquatic organisms from water directly or through consumption of food containing the chemical) many toxic chemicals that cannot be detected in water since they are present at concentration levels below the minimum detection levels of standard analytical procedures. Chemicals concentrations in fish, can be used to assess bioaccumulation, and biomagnification (the efficient transfer of chemicals from food to consumer, so that residue concentration increases systematically from one trophic level to the next) factors. Both marine and fresh water species have been affected by the presence of contaminants in the Great Lakes. These include: ulcers, lesions, fin rot, gill deformities, skeletal abnormalities and tumours in the wild fish population (Sindermann et al., 1980; Warwick, 1988). The presence of tumours in Ontario fish was reported by Wiggins and Hadwen as early as 1909 (Sonstegard, 1977). Numerous reports have been made on fish tumours. In an extensive survey of Black River in Lake Erie, Baumann (1987) found that 37% of the brown bullheads that were 3 years or older had liver tumours. The author correlated the occurrence of tumours in fish to the levels of carcinogenic PAHs in the sediments.

Other wildlife populations, in this ecosystem, have also been affected by exposure to environmental contaminants. The most seriously affected animal population are those in closest proximity to the most contaminated lakes. The bald eagle population
which feeds on large fish, and herring gulls are seriously affected in terms of population decline and eggshell thinning. Nisbet (1978) and Wiemeyer (1991), collected over 100 eagle eggs from more than 12 states (not from nests in the Great Lakes shorelines) in 1970, and correlated eggshell thinning and reproductive impairment to the presence of DDE. These authors suggested PCBs may have also contributed to reproductive problems. Although the concentration of certain restricted or banned toxicants (e.g., DDT, dieldrin, heptachlor, and PCBs) in water have declined considerably over the past decade, biomagnification in fish feeding near sediments containing PCBs, dioxins, and other OCs pose a continuing hazard to the Great Lakes wildlife.

In this study, contaminant extracts were obtained from tissues of various fish species such as, fresh water drum (Aplodinotus grunniens), small mouth bass (Micropterus dolomieu), white sucker (Catostomus commersoni), as well as eagle (Haliaeetus leucocephalus) eggs. Fish samples were collected from the Lake Erie (Middle Sister Island) and eagle eggs from the Essex county (September, 1994). Cyto- and genotoxic effects of the contaminants in fish tissue and eagle egg extracts, consisting of pp'-DDE and Arochlor 1254:1260 were determined in HepG2 cells using the NR uptake and AU assays, respectively. HepG2 cell cultures were grown to 60-70% confluency in 24-well tissue culture plates. The induction of cytotoxicity in HepG2 cells by fish tissues and eagle egg extracts in serum-free and 5% FBS medium was studied after a 48h exposure at 100 fold dilution (Table A. 2, Appendix. A). Figure 2. 5. 1 demonstrates the cytotoxic effects of these extracts, as measured by cell viability in NR uptake assay. With all extracts from fish tissues and eagle eggs, no significant cytotoxic effect regardless of the cell
culture medium was observed after 48h exposure. The extent of DNA damage, was measured as strand breaks by the AU assay under similar conditions (both culture media, 48h exposure, 100 fold dilution). Induction of strand breaks by contaminants in all extracts were significantly (ANOVA, P<0.05) higher in extract treated-cells maintained in 5% FBS medium than those in serum-free medium (which did not show any DNA damage). Figure 2.5. 2 compares the effects of serum in relation to the extent of DNA strand breaks in HepG2 cells induced by contaminant extracts from fish tissues and eagle egg samples. Data from cyto- and genotoxicity studies indicate that contaminant extracts from fish tissues and eagle eggs resulted in significant DNA strand breaks in HepG2 cells, while the % cell viability was not significantly affected under similar conditions (48h exposure, 5% FBS). This observation can be related to the fact that DNA damage such as DNA strand breaks can occur at lower chemical concentration than cell cytotoxicity, as demonstrated by standard chemicals (MMS, 4-NQO, PQ, B(a)P) in this study. The decreasing order of cytotoxicity (% cell viability), although not statistically significant, in both fish tissues and eggs extracts (ranked by species) was, fresh water drum (94.1%), eagle eggs (95%), white sucker (96.4%), small mouth bass (104.7%). Genotoxicity potential in decreasing sequence (as N and %F values) was, eagle egg (N=2.97, F=27%), fresh water drum (N=2.59, F=30%), small mouth bass (N=2.28, F=35%), white sucker (N=1.57, F=50%). The genotoxic effect of both pp'-DDE and the PCBs mixture in fish tissues and eagle eggs extracts was higher from that in sediment extracts fraction I which also contained these chemicals (pp'-DDE 4.64 µg/mL, and Arochlor 1254:1260 47.8 µg/mL) using similar conditions (both culture medium, 48h exposure, 100 fold dilution) (data not shown). This
may indicate, that the presence of other chemicals in Trenton Channel sediment extracts may interfere with cellular interactions and metabolisms of these chemicals. Therefore, no direct relationship can be derived from the concentration of individual components of mixtures and their biological effects. The toxicity of pp'-DDE as prime metabolite of DDT and PCBs mixtures are known to be related with their ability to induce P_{450} (Environment Canada, 1991; Hass et al 1987). However, as mixtures they may act synergistically or additively in order to exert any biological adverse effects.
FIGURE 2.5.1

Comparative Cytotoxicity of Contaminant Extracts in Lake Erie Fish Tissues and Eagle Eggs

Legend

HepG2 cells were grown in complete medium to 60-70% confluency. Cultures were then treated by fish tissue and eagle egg contaminant extracts (100 fold dilution) for 48h in a serum-free and a serum containing media. The 100 fold dilution was prepared from stock solutions composed of contaminants including pp'-DDE and Aroclor 1254:1260. The cytotoxicity was then measured by the NR uptake assay. The absorbance of extracted dye was determined at 540nm. The % cell viability was calculated relative to the negative control. Standard deviations for the data (treatment with complete medium), presented in this figure are given in Table 2.9.6
FIGURE 2.5.1

% Cell Viability

0% FBS
5% FBS

Concentration (x100 Fold)

White Sucker  S.Mouth Bass  F.Water Drum  Eagle Egg
FIGURE 2.5.2

Comparative Genotoxicity of Contaminant Extracts in Lake Erie Fish Tissues and Eagle Eggs

Legend

HepG2 cells were grown in complete medium to 60-70% confluency, and then radiolabeled with 1.0µCi/mL [³H]TdR for 24h. At the end of the labeling period, the cells were rinsed with EBSS solution for two hours prior to treatment. Cultures were then treated with contaminant extracts from fish tissues (hexane as solvent) and eagle eggs (acetone as solvent) in a serum-free medium and a 5% fetal bovine serum in complete medium for 48h exposure. The 100 fold dilution concentrations were prepared from stock solutions. The extent of DNA damage was measured by strand breakage in HepG2 cells was determined by the AU assay conditions. The %F value was measured relative to the negative controls. Standard deviations for the data (treatment with complete medium), presented in this figure are given in Table 2.9.6
FIGURE 2.5.2

Concentration (x100 Fold)
Table 2.4.6 Cyto- and Genotoxicity of Fish Tissues (Middle Sister Island), and Eagle Eggs (Essex) Extracts in HepG2 Cells after 48h exposure.

<table>
<thead>
<tr>
<th>Extract 100 x F.D.</th>
<th>Neutral Red (OD.540nm) 48h M±SD(n)ᵇ</th>
<th>%CVᶜ</th>
<th>DNA Strand Breaks 48h Fⁿ±SD(n)ᵇ</th>
<th>Nᵈ</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Sucker</td>
<td>0.70±0.05(3)</td>
<td>96.4</td>
<td>0.31±0.12(3)*</td>
<td>1.57</td>
</tr>
<tr>
<td>S.Mouth Bass</td>
<td>0.76±0.04(3)</td>
<td>104.7</td>
<td>0.22±0.01(3)**</td>
<td>2.28</td>
</tr>
<tr>
<td>F.Water Drum</td>
<td>0.68±0.05(3)</td>
<td>94.1</td>
<td>0.19±0.03(3)**</td>
<td>2.59</td>
</tr>
<tr>
<td>Eagle Egg</td>
<td>0.69±0.08(3)</td>
<td>95.5</td>
<td>0.16±0.004(3)**</td>
<td>2.97</td>
</tr>
</tbody>
</table>

ᵃ F = Fraction of DNA remaining double-stranded after unwinding.
ᵇ Values represent Mean±SD of independent cultures of n replicates.
ᶜ Percent cell viability values calculated relative to solvent (fish tissues, eagle eggs extracts in hexane [eagle eggs extracts in acetone only in genotoxicity assessment]).
ᵈ N = Increase in DNA strand breaks (N value) relative to control.
* At α = 0.05 (P<0.05), ** at α = 0.01 (P<0.01), the level of significance is determined by one way ANOVA test statistic.

NB. The 100 fold diluted solutions are made from stocks containing OCCs (refer to Appendix A. 2).
2.6 Conclusion

In this project two *in vitro* short-term assays were employed to evaluate potential environmental contaminants using the human HepG2 cell line as the model system. A number of known carcinogens and mutagens were used to calibrate the response of the HepG2 cell system to a toxic dose, and its potency in activating indirectly acting chemical agents. HepG2 cells have been shown to metabolize chemical carcinogens and mutagens in a dose-response manner, and have been employed as a model system in studies involving the metabolism of a wide range of xenobiotics. These include bromobenzene, a hepatotoxic chemical (Duthie *et al.*, 1994), adriamycin, a quinoid anthracycline drug which is used widely in the treatment of a wide range of human malignancies, mitoxantrone an anthraquinone drug and menadione (quinone drug) (Smith *et al.*, 1985), PAH o-quinone (Lynn *et al.*, 1993), methyl methanesulphonate, 4-nitroquinoline, 9-10-phenanthrenequinone and also environmental contaminants (Ali *et al.*, 1994; Hasspieler *et al.*, 1995).

This study further confirmed the potential applicability of the HepG2 cell line in assessing the potential toxicity of chemical toxicants and provides evidence for the competency of this system to activate indirectly acting chemicals. Overall, the data lends support to the notion that the HepG2 cell system can be used as an *in vitro* human model system and can be used in combination with NR and AU bioassays to assess the potential cyto- and genotoxicity of environmental chemicals. The validity of both tests was established by using well-characterized carcinogens and mutagens such as methyl methanesulphonate as direct agent and 4-nitroquinoline 1-oxide and 9-10,
phenanthrenequinone, and benzo(a)pyrene as indirect agents. Thus, the utility of these chemicals as standard test agents in toxicology studies can be valuable in estimating metabolic activation potential of a test organism.

The HepG2 system was also used to evaluate the cyto- and genotoxic potential of contaminant extracts from lake sediments, fish species, and eagle eggs, obtained from the Great Lakes Basin. The system was capable of detecting extracts that were toxic and its response appeared to correlate the levels of contaminants in the extracts tested. These results suggest that the HepG2 NR and AU bioassays can be used as a model to estimate the risks posed to the human population living in the Great Lakes Basin.

Therefore on the basis of this study and previous works, the HepG2 cell line could be used as a bioindicator in screening environmental contaminant mixtures using the NR uptake assay in conjunction with the AU assay. The experimental protocols outlined here were designed to provide optimum experimental conditions for testing cyto- and genotoxicity of known carcinogens and mutagens as well as environmental contaminants with respect to human liver cells. Both the NR uptake and AU assays were shown to be highly sensitive, reproducible and rapid.

The AU bioassay appeared to be an excellent test for genotoxicity and showed an excellent dose-dependent response to a variety of known mutagens. It is important to note however that in estimating chemical genotoxicity, one test is not enough to examine every chemical because some assays of genetic damage may respond better than others to various classes of chemical carcinogens and mutagens, or they may give positive results to some non-carcinogens (e.g., Bacterial mutation tests). It is
recommended that "batteries" of tests be used when assessing the toxicity of a mixture of unknown suspected toxicants. Such a test battery may consist of bacterial mutation assays, chromosome changes, DNA damage, and eukaryotic gene mutation assays (Environmental Health Criteria 51, 1985). Although HepG2 in the AU bioassay appears to be a sensitive bioindicator of DNA breakage, further supplementary tests are required to investigate other biological effects of tested chemicals such as mutation and chromosomal abrasions. Then the results can be used to supplement in vivo assays and establish correlations providing a basis for making assessments of possible human hazards associated with exposure to chemical contaminants.

In addition, this study demonstrates increased genotoxic activity of highly hydrophobic chemicals such as Benzo(a)pyrene and environmental contaminants when tested in the HepG2 system in the presence of 5% fetal bovine serum. The observed increase in genotoxic activity could be related to the presence of transport proteins (i.e., transferrin and albumin) in the serum which may nonspecifically bind toxicants and thus promote faster accessibility to the target sites in the cell through the aqueous culture medium. In many studies, the presence of serum in the culture medium has been shown to have no marked effects on the toxicity of chemicals tested (such as, alkylating agents). However, cultured cells may not respond appropriately to highly hydrophobic agents when applied in serum free media, leading to false negative results.

In conclusion, the HepG2 cell system combined with the NR and the AU bioassays provides a suitable surrogate model system to evaluate the potential toxicity of environmental pollutants and the associated hazards posed to the human population. This
model system could be applied to the assessment of the environmental stress associated with any natural habitat.
APPENDIX A

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peche Isl.</th>
<th>Lake St. Clair</th>
<th>Lake Erie</th>
<th>Trenton Channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCCs (fraction I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penta chlorobenzene</td>
<td>0.00036</td>
<td>0.0076</td>
<td>0.0028</td>
<td>0.0215</td>
</tr>
<tr>
<td>Hexa chlorobenzene</td>
<td>0.0123</td>
<td>0.0231</td>
<td>0.0065</td>
<td>0.1344</td>
</tr>
<tr>
<td>Octa chlorostyrene</td>
<td>0.0022</td>
<td>0.0055</td>
<td>0.0036</td>
<td>0.0801</td>
</tr>
<tr>
<td>pp'-DDE</td>
<td>0.0029</td>
<td>0.0022</td>
<td>0.0083</td>
<td>0.2348</td>
</tr>
<tr>
<td>Aroclor Mix 1254/1260</td>
<td>0.0204</td>
<td>0.0243</td>
<td>0.3406</td>
<td>2.4191</td>
</tr>
<tr>
<td>PAHs (fraction II)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthalene</td>
<td>0.01</td>
<td>0.13</td>
<td>0.16</td>
<td>0.58</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>0.03</td>
<td>0.09</td>
<td>0.06</td>
<td>1.22</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>0.03</td>
<td>0.08</td>
<td>0.05</td>
<td>2.57</td>
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<tr>
<td>Fluorene</td>
<td>0.11</td>
<td>0.24</td>
<td>0.17</td>
<td>10.01</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>0.59</td>
<td>0.82</td>
<td>0.58</td>
<td>24.26</td>
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<tr>
<td>Anthracene</td>
<td>0.10</td>
<td>0.13</td>
<td>0.16</td>
<td>6.64</td>
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<tr>
<td>Fluoranthene</td>
<td>0.97</td>
<td>1.06</td>
<td>1.13</td>
<td>36.05</td>
</tr>
<tr>
<td>Pyrene</td>
<td>0.85</td>
<td>1.2</td>
<td>1.22</td>
<td>29.89</td>
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<tr>
<td>Compound</td>
<td>0.4</td>
<td>0.55</td>
<td>0.73</td>
<td>24.39</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----</td>
<td>------</td>
<td>------</td>
<td>-------</td>
</tr>
<tr>
<td>Benzo(a)Anthracene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chrysene/Triphenylene</td>
<td>0.66</td>
<td>0.87</td>
<td>1.05</td>
<td>24.49</td>
</tr>
<tr>
<td>Benzo(b)fluoranthrene</td>
<td>0.52</td>
<td>0.72</td>
<td>1.34</td>
<td>28.19</td>
</tr>
<tr>
<td>Benzo(k)fluoranthrene</td>
<td>0.41</td>
<td>0.42</td>
<td>0.92</td>
<td>14.98</td>
</tr>
<tr>
<td>Benzo(a)Pyrene</td>
<td>0.31</td>
<td>0.44</td>
<td>0.86</td>
<td>21.19</td>
</tr>
<tr>
<td>Indeno(1,2,3,c,d)Pyrene</td>
<td>0.39</td>
<td>0.52</td>
<td>1.54</td>
<td>30.75</td>
</tr>
<tr>
<td>Dibenzo(a,b)Anthracene</td>
<td>ND</td>
<td>ND</td>
<td>0.44</td>
<td>4.45</td>
</tr>
<tr>
<td>Dibenzo(g,h,i)Perylene</td>
<td>0.42</td>
<td>0.61</td>
<td>1.16</td>
<td>20.35</td>
</tr>
</tbody>
</table>

PCBs presented as 1:1 mixture of 1254/1260
<table>
<thead>
<tr>
<th>Extracts from:</th>
<th>pp'′-DDE  (µg/mL)</th>
<th>Aroclor 1254:1260 (µg/mL)</th>
<th>g. wet sample / mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Water Drum</td>
<td>0.24</td>
<td>5.92</td>
<td>51.71</td>
</tr>
<tr>
<td>Small Mouth Bass</td>
<td>0.8</td>
<td>15.9</td>
<td>51.71</td>
</tr>
<tr>
<td>White Sucker</td>
<td>0.8</td>
<td>16.7</td>
<td>59.18</td>
</tr>
<tr>
<td>Eagle Egg</td>
<td>41.1</td>
<td>142.9</td>
<td>1.85</td>
</tr>
</tbody>
</table>

NB: Both fish tissue and egg extracts contained mixtures of pp′-DDE and Aroclor 1254:1260
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Montreal, Quebec

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Canada Inc. Amherstburg, Ontario
Fall 1989 - Winter 1990

research assistant (soil and water analysis)

project title: The development and implementation of an integrated soil, crop and water management system to abate pesticide and of nitrate contamination of the Great Lakes.

Agriculture Canada Research Station Branch
Harrow, Ontario

Summer 1989

research assistant (research & development)

project title: In Vivo Study of Fatty Acid Composition in Plant Root System. McGill University, Montreal, Quebec

Skills & Techniques

- Experienced in advanced chemistry, biochemistry and microbiology laboratories.

Responsibilities:

- chemical analysis using automated instrumentations
- growing subcultures from stock cultures and maintenance under sterile technique
- medium preparation and general autoclaving

- Cell Culture:
  - two years experience with human liver cell culture (HepG2)
  - work experience with bacterial cell culture (salmonella), and plant cell (tomato, cucumber, lettuce, spinach)
- DNA Purification: preparation of crude DNA (Sephadex G-25 column)
- Chromatography: absorption, paper, thin layer, gel, and gas chromatography
- Industrial Experience:
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  - data management

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**Abstracts:**

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