BENZENE ORAL BIOAVAILABILITY ASSESSMENT USING IN VITRO DIGESTION MODEL IN COMBINATION WITH CELL CULTURE METHODOLOGY

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ABSTRACT

The bioavailability of volatile organic compounds (VOCs) in a given situation often remains challenging to assess, and the lack of standard methods for introducing VOCs into *in vitro* bioassays can lead to poorly defined bioavailable concentrations. As a result, *in vitro* assays normally conducted in wells of cell culture plates for risk assessment of volatile and hydrophobic organic chemicals (VHOCs) have always faced significant experimental difficulties due to high volatility and high hydrophobicity. This compromises the true exposure concentration by: (i) causing the amount of test substances in the test medium to decline, (ii) limits the quality of toxicological responses and their extrapolation, and thus, can lead to interpretational errors. In the research herein, a dosing method was developed to assess the bioavailability of benzene (that served as a model for VHOCs) in aqueous tests and to better characterize exposure estimates for an improved risk assessment during *in vitro* biotests. This study hypothesizes that (1) benzene bioavailability to intestinal porcine enterocyte cell line (IPEC-1 cells) can be partially explained by phase partitioning, as measured by freely dissolved concentration that drives the diffusive uptake into the cell membranes, (2) benzene equilibrium partitioning between the donor and the cell membranes is dependent upon energetic state of the chemical concentration in the partitioning donor, which describes its chemical activity. Silicone polydimethylsiloxane (PDMS) was used as the partitioning donor for passive dosing in transwell plates. The buffering capacity of the donor compensates for routine loss against depletion processes during the toxicity tests, resulting in stable exposure concentrations of benzene freely available to cells at relatively constant chemical activity. For IPEC-1 cells in the passive dosing tests, the median effective concentration (EC50) was 4.82 mg/L. The obtained median effective activity (Ea_{50}) value is within the chemical activity range (0.01– 0.1) for baseline toxicity of several hydrophobic chemicals reported in the literature.

Cell inhibition ranged from 9.6 ± 2% to 97.7 ± 0.8% for freely dissolved concentrations of benzene, which ranged from 0.6 to 5.4 mg/L after 24 h exposure. The spiking tests result in an EC_{Spike}-50 projected to be greater than 5.4 mg/L, (highest spiked concentration) and reduced test sensitivity of benzene to IPEC-1 cells. This study introduces a new effective approach to passive dosing and demonstrates the utility of passive dosing over solvent spiking for *in vitro* toxicity testing of hydrophobic chemical (log *Kow* < 4.6) with high volatility. This has fundamental implications for a better understanding of the interactions between VHOCs exposure to humans and the toxic effects on the human intestine to help set remediation objectives and further the improve future risk assessment and standard setting for VHOCs.

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LIST OF ABBREVIATIONS

ALC	Absolute lymphocyte count
BMC	Benchmark concentration
BMD	Benchmark dose
BTEX	Benzene, Toluene, Ethylbenzene, and Xylene
CCME	Canadian Council of Ministers of the Environment
GCDWQ	Guidelines for Canadian Drinking Water Quality
COPC	Contaminants of potential concern
CYP	Cytochrome P-450
DMEM	Dulbecco modified eagle medium
EC50	Median effective concentration
Ea50	Median effective activity
US EPA	United State Environmental Protection Agency
C_{free}	Freely dissolved concentration
GC-MS	Gas chromatography-mass spectrometry
GI	Gastro-intestinal
GSH	Glutathione
HOCs	Hydrophobic organic chemicals
RfC	Inhalation reference concentration
IPEC-1 cells	Intestinal porcine enterocyte cell line
BMCL	Lower bound on the BMC
LOAELs	Lowest-observed-adverse-effect levels
MAC	Maximum acceptable concentrations

MCV	Mean corpuscular volume
MUC	Muconaldehyde
MA	Muconic acid
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
ROS	Reactive oxygen species
RBCC	Red blood cell count
RfD	Reference dose
PDMS	Silicone polydimethylsiloxane
SLsub	Subcooled liquid solubility
TDI	Tolerable daily intake
TRVs	Toxicity reference values
TEER	Transepithelial electrical resistance
VHOCs	Volatile and hydrophobic organic chemicals
VOCs	Volatile organic compounds
WBC	White blood cell count

1 INTRODUCTION

Benzene is a carcinogen and a widespread contaminant of groundwater. The human health risk associated with benzene exposure has been increasingly recognized (Boelsterli, 2003; Martínez-Velázquez et al., 2006). For pollutants, such as benzene, to be accessible to humans, it must be bioavailable and cross barrier membranes to enter the systemic circulation. Environmental risk assessors often suggest that all of the ingested pollutants reach the site of toxic action following the assumption the pollutant has the same bioavailability as what occurred in the reference study. This notion is widely held as a conservative estimate. However, the bioavailability of orally ingested hydrocarbons is commonly known to vary depending on the type of hydrocarbons, dose, carrier (vehicle), and host-species. Hence, the question of how bioavailable of a hydrophobic organic chemical is in a given situation often remains difficult to answer. For volatile hydrophobic chemicals such as benzene, it is technically challenging to determine true exposure concentration and maintain constant recharge of exposure concentrations during *in vitro* toxicity tests. The research of this Master thesis introduces a new approach to passive dosing for an improved *in vitro* exposure and quantitative assessment of potential health risk from chemicals with high volatility and high hydrophobicity. The passive dosing model uses a silicone PDMS loaded with the test substance to establish a partition-controlled delivery of test concentrations, and thus provides direct contact with the test medium and constant exposure level at maximum chemical activity to epithelial cells.

1.1 Objectives and Hypotheses

The overall objective of this master's research is to develop a dosing system to assess benzene bioavailability to gastrointestinal (GI) epithelial cells and to estimate benzene transfer across the

cell lines. Two hypotheses are evaluated for the overall objective stated above: (1) benzene bioavailability to intestinal porcine enterocyte cell line (IPEC-1 cells) can be partially explained by phase partitioning, as measured by freely dissolved concentration that drives the diffusive uptake into the cell membranes, (2) benzene equilibrium partitioning between the donor and the cell membranes is dependent upon energetic state of the chemical concentration in the partitioning donor, which describes its chemical activity.

For the two hypotheses, the freely dissolved bioavailable fractions of benzene in aqueous tests are determined, and their toxicity is linked to calculated chemical activity for the concentration exerting the toxic effect. This is presented in Chapter 3 (Development of a dosing system to assess benzene bioavailability in gastrointestinal cells).

2 LITERATURE REVIEW

2.1 Benzene

Benzene is the simplest aromatic hydrocarbon, an important element of environmental pollution and occurs in crude oil and gasoline (IRIS report, 2002; Boelsterli, 2003; Burbacher, 1993). Benzene is produced largely in the chemical industry and also a widely used industrial product as an intermediate in chemical syntheses and the manufacture of products like resins, dyes, plastics, etc. (IRIS report, 2002; McHale et al., 2012). Benzene contamination is commonly associated with various industrial operations such as petroleum production, distribution processing and storage. It has a long history for its carcinogenic potential, most evidently from human epidemiologic studies and animal data (Boelsterli, 2003). Toxicokinetic studies both in humans and in animal models indicate that benzene, due to its lipophilicity, is readily absorbed, distributed to several highly vascular tissues such as bone marrow and adipose tissue, and are eliminated most rapidly from the body (IRIS report, 2002; U.S report, 2004).

For benzene to become toxic, benzene must be biotransformed to reactive metabolites or intermediates (Boelsterli, 2003). Several benzene metabolites have been linked to benzene toxicity (IRIS report, 2002). The interactions of benzene metabolism and toxicity allow the prediction of toxic effects and can be assessed at a quantitative level only when the mode of action is understood (IRIS report, 2002). Benzene exposure is associated with multiple adverse health effects by all routes of administration (IRIS report, 2002; Burbacher 1993). The toxic effect most frequently observed after chronic exposure in human and animal models is the bone marrow toxicity (Boelsterli, 2003). Benzene has been consistently reported to produce neurotoxic effects in test animals and humans after exposure in a dose-dependent manner (IRIS report, 2002). It was shown to induce nephrotoxicity in the kidney (Boelsterli, 2003). Acute exposure to benzene has been

reported to cause long-lasting changes in motor behavior and cognitive process during gestational organogenesis (Burbacher, 1993). Benzene has also been shown to induce hematotoxicity in human over decades and reports from recent studies have confirmed hematological effect at varying levels of benzene exposure, including those that are below the USA Occupational Safety and Health Administration's permissible exposure limit of 1 part per million (ppm) in air (McHale et al., 2012).

Benzene is a contaminant of potential concern, and thus, is currently regulated federally, by provinces and territories. In Canada, contaminants of potential concern (COPCs) are regulated to provide a substantial margin of safety between the Canadian Council of Ministers of the Environment (CCME) permissible exposure levels and the levels potentially expected to result in adverse toxic effects in humans, by comparing the maximum measured on-site concentration to CCME Environmental Quality Guidelines for protection of human health (Health Canada, 2012). Depending on the route of exposure, chemicals in groundwater are regulated through the application of Health Canada Guidelines for Canadian Drinking Water Quality. Also, many provinces and territories have adopted the CCME Canada-Wide Standard (CWS) for Petroleum Hydrocarbons (PHCs) in Soil for risk assessment at various contaminated sites (Health Canada, 2012). In the United States, regulation for COPCs applies through the US Environmental Protection Agency, (US EPA) preliminary remediation goals (US EPA, 2004a) or risk-based concentrations (US FDA, 2006).

2.1.1 Benzene Interaction with Toluene, Ethylbenzene, and Xylenes – (BTEX), and Arsenic

Benzene contaminations frequently occurs as a joint mixture with toluene, ethylbenzene, and xylenes termed (BTEX). BTEX are volatile organic compounds and exhibit good solvent properties (U.S report, 2004). The joint toxic action of the mixture is dose-dependent based

metabolism that involves the cytochrome P-450 super family (CYP2E1). Exposure to BTEX is reported to produce neurological impairment, and further exposure to benzene reportedly causing hematological effects via the action of reactive metabolites. Though the carcinogenic potential of benzene is well established in the literatures, recent assessment by IARC (2000) have also suggested that ethylbenzene is possibly carcinogenic to humans, excluding toluene and xylenes that have been classified, by both EPA (IRIS, 2001) and IARC (1999a), as non-carcinogenic to humans (U.S report, 2004). Benzene's potential risk from interaction with potent toxicants such as arsenic (As) is also significant due to the carcinogenic potential of As. Arsenic is widely found in contaminated soil and groundwater, and a critical determinant of its toxic effects is the metabolism of trivalent inorganic arsenic, catalyzed by arsenic (+3 oxidation state) methyltransferase (As3mt), (Hughes et al., 2011). Comparisons between Health Canada and U.S. EPA classification on human toxicity reference values (TRVs) for BTEX, and Arsenic are presented in Table 2.1. The allowable concentrations of BTEX and Arsenic in surface water for generic uses: drinking water, aquatic life, and livestock based on Alberta Tier 1 Surface water quality guidelines are presented in Table 2.2. For residential/parkland water uses, the allowable chemical concentrations are reported in Table 2.3. Table 2.4 shows all exposure pathways for industrial land use and the permissible exposure concentrations based on Alberta Tier 1 Subsoil remediation guidelines.

Table 2.1 Human toxicity reference values (TRVs) for Benzene, Toluene, Ethylbenzene, and Xylene (BTEX), and Arsenic – Health Canada and U.S. EPA

Non-Carcinogenic TRVs *					Carcinogenic TRVs *			
	Oral TDI		Inhalation TC	Inhalation TDI	Oral slope factor		Inhalation slope factor	
	Health Canada	US EPA	Health Canada	US EPA	Health Canada	US EPA	Health Canada	US EPA
Unit	mg/kg/d		mg/m3		1/mg/kg/d			
Benzene	ND	4.0×10^{-3}	ND	3.0×10^{-2}	8.3×10^{-2}	5.5×10^{-2}	1.4×10^{-2}	2.7×10^{-2}
Toluene	2.2×10^{-1}	8.0×10^{-2}	3.8	5.0	ND	ND	ND	ND
Ethylbenzene	1.0×10^{-1}	1.0×10^{-1}	1.0	1.0	ND	ND	ND	ND
Xylenes	1.5	2.0×10^{-1}	1.8×10^{-1}	1.0×10^{-1}	ND	ND	ND	ND
Arsenic (inorganic)	ND	3.0×10^{-4}	ND	ND	1.8	1.5	27	15

ND – no data
TDI – tolerable daily intake
TC – tolerable concentration
US EPA – United States Environmental Protection Agency
* U.S. EPA Interaction profile for BTEX report, 2004 and guidance document on Federal Contaminated Site Risk Assessment in Canada, Part II, (2010).

Table 2.2 Alberta Tier 1 Surface water quality guidelines for generic uses of surface water and the allowable concentrations of (BTEX) and Arsenic in groundwater or surface water use

	Drinking water mg/L *	Aquatic life mg/L *	Livestock water mg/L *	DTED mg/kg-bw/d *
Benzene	0.005	0.04	0.088	0.08
Toluene	0.024	0.005	4.91	4.46
Ethylbenzene	0.0016	0.09	3.20	2.91
Xylenes	0.02	0.03	13.1	11.9
Arsenic (inorganic)	0.01	0.005	0.025	ND

DTED – daily threshold effect dose (mg/kg-bw/d)
ND – no data
* Alberta Tier 1 Soil and Groundwater Remediation Guidelines, 2016, No. 3.

Table 2.3 Alberta Tier 1 Groundwater remediation guidelines for residential/parkland – All water uses

Water use	Lowest guideline	Potable	Inhalation	Aquatic life
Unit	mg/L *	mg/L *	mg/L *	mg/L *
Benzene	0.005	0.005	2.8	3.6
Toluene	0.024	0.024	NGR	12,000
Ethylbenzene	0.0016	0.0016	NGR	NGR
Xylenes	0.02	0.02	80	NGR
Arsenic (inorganic)	0.005	0.01	NGR	0.005

NGR – no guideline required
* Alberta Tier 1 Soil and Groundwater Remediation Guidelines, 2016, No. 3.

Table 2.4 Alberta Tier 1 Subsoil remediation guidelines for industrial land use – All exposure Pathways

Receptor	Overall guideline	Human				Ecological
Pathways		Direct soil contact	Vapor inhalation	Protection of domestic use aquifer	Off-site migration	Protection of freshwater aquatic life
Unit	mg/kg *	mg/kg *	mg/kg *	mg/kg *	mg/kg *	mg/kg *
Benzene	0.046	120	11	0.046	1,100	7.9
Toluene	0.52	11,000	14,000	0.52	9,200	63,00
Ethylbenzene	0.073	24,000	6,700	0.073	24,000	NGR
Xylenes	0.99	8,100	1,800	0.99	6,900	NGR
Arsenic (inorganic)	ND	ND	ND	ND	ND	ND

NGR – no guideline required
ND – no data
* Alberta Tier 1 Soil and Groundwater Remediation Guidelines, 2016, No. 3.

2.1.2 Review of Benzene Epidemiological Studies

Toxicological parameters such as the tolerable daily intake (TDI), maximum acceptable concentrations (MAC), reference dose (RfD), and a number of other human health toxicity benchmarks are derivatives for the quantitative estimation of the threshold dose or exposure level that is free of detrimental effects to humans. Several case reports of adverse effect of death from acute oral exposure to benzene are documented in the literatures. Thienes and Haley (1972) and ATSDR (2007) estimate lethal oral doses of benzene in humans to be at approximately 125 mg/kg (125 ppm). Toxic responses such as the central nervous system depression, respiratory arrest, or cardiac collapse result at lethal doses to humans (Green- burg, 1926).

Benzene exposure occurs predominantly via inhalation, and this route of exposure is what has been used to set toxicological reference values (IRIS report, 2002). The principal study selected by the U.S. EPA for the derivation of the inhalation reference concentration (RfC) and oral reference dose (RfD) is the human occupational epidemiological study by Rothman et al. (1996a) (IRIS report, 2002, IRIS report, 2003). A cross-sectional study of 44 workers exposed to benzene at varying concentrations and 44 other people: age-and gender-matched unexposed controls, all from China, were conducted. Of the 44 subjects in the exposed and control groups, twenty-one were female. In the study, benzene exposure was monitored for a full workshift on 5 days in a 1-2 week period before blood sampling from the exposed subjects. Benzene exposure of control groups was monitored for 1 day. Six haematological parameters: absolute lymphocyte (ALC) count, total white blood cell (WBC) count, red blood cell count (RBCC), platelet count, hematocrit, and mean corpuscular volume (MCV) were evaluated in the study, and of all, reduced ALC was selected as the critical endpoint for the derivation of the RfC/RfD.

Rothman et al. (1996a) epidemiological study provides the lowest-observed-adverse-effect levels (LOAELs) suitable for the benchmark dose (BMD) modeling of ALC to derive a point of departure (POD) for the calculation of the RfC. The BMD modelling of the ALC from Rothman et al. (1996a) exposure-response data yielded a benchmark concentration (BMC) and a 95% lower bound on the BMC (BMCL) of 8.2 mg/m^3, and was performed using the U.S. EPA's BMD modelling software (version 1.2). The RfC is then obtained by dividing the adjusted BMCL by the overall uncertainty factor (UF) of 300: $BMCL_{ADJ}/UF = 8.2\ mg/m^3 \div 300 = 3 \times 10^{-2}\ mg/m^3$. As most commonly used in risk assessment, the UF or safety factor is considered a safety margin that accounts for individual differences in sensitivity to chemical exposure. Depending on the degree of uncertainty, a 10-fold UF is usually used but can also assume a range of other values.

The RfD derivation is based on route-to-route extrapolation of benzene inhalation results to oral exposures. The RfD is obtained by dividing the equivalent oral dose rate value calculated using the $BMCL_{ADJ}$ by the overall uncertainty factor (UF) of 300: RfD = equivalent oral dose ÷ UF = $1.2\ mg/kg/day \div 300 = 4 \times 10^{-3}\ mg/kg/day$. The equivalent oral dose rate value is obtained when the $BMCL_{ADJ}$ is multiplied by the default inhalation rate, multiplied by 0.5 to correct for the higher oral absorption, and divided by the standard default human body weight of 70 kg (U.S. EPA, 1988): $8.2\ mg/m^3 \times 20\ m^3/day \times 0.5 \div 70\ kg = 1.2\ mg/kg/day$.

The Health Canada guidance on human toxicity reference value for benzene had been developed based on a 2-year cancer study (experimental animal gavage study) in rats and mice by the National Toxicological Program (NTP, 1986). Health Canada guideline for benzene is developed based on cancer endpoints, and this guideline provides a substantial margin of safety between cancer and non-cancer endpoints. The approach for quantitative estimation of cancer risks from exposure to benzene in the gavage study, and because carcinogenicity is considered to be the

critical health effects among varying adverse outcomes from benzene exposure, were deemed sufficient by Health Canada to serve as the basis upon which the Guidelines for Canadian Drinking Water Quality (GCDWQ) is established. The MAC derivation for benzene in drinking water is 0.005 mg/L (5 μg/L) based on the NTP (1986) study. For cancer endpoint, the overall unit risk, given a lifetime exposure to benzene by ingestion of drinking water containing benzene at the MAC of 0.001 mg/L is reported as a range from 2.03×10^{-6} (based on malignant lymphoma in female mice) to 4.17×10^{-6} (based on bone marrow hematopoietic hyperplasia in male mice) (Health Canada, 2009). These unit risks assume a standard drinking water consumption rate of 3.5 L-eq/day for humans.

2.1.3 Benzene Kinetics and Metabolism

Benzene is readily absorbed from oral and inhalation exposures, but from dermal exposure, absorption is very low due to rapid evaporation from skin (IRIS report, 2002). Distribution is rapid throughout the body following exposure by all routes of administration. Animals experimentally exposed to benzene orally at low concentrations have shown complete absorption. In Sabourin et al. (1987) studies following oral gavage (in corn oil) or intraperitoneal injection, the absorption of benzene from the GI tract in all animal subjects was found essentially to be 100 %. Supporting studies by Mathews et al. (1998) on laboratory animals treated by oral gavage showed that, at a high dose of benzene estimated at 100 mg/kg bw, a fraction of benzene was significantly eliminated by exhalation. It is suggested that the excretion routes might be influenced by benzene dose and a weight of evidence that suggest a linear increase in total metabolite production with exposure level. A greater proportion of benzene metabolites, at the low doses, are excreted in urine, and a shift to a greater proportion of unmetabolized benzene, at the high doses, excreted in exhaled air. Like absorption following oral ingestion, benzene (through inhalation) is better absorbed at

lower exposures versus higher exposures. Since extrapolation of animal studies data from exposure to oral doses to which humans are likely exposed suggest a dose-dependent linear increase in metabolite production, the dose-related production of metabolites in humans from benzene exposure is remained inadequately understood, more particularly at exposure to low levels of benzene.

Benzene biotransformation results in the production of several metabolites in the liver, and interactions between these metabolites, are required for the expression of benzene toxicity (IRIS report 2002; Health Canada, 2009). Benzene is metabolized, primarily in the liver, by Cytochrome P450 2E1 (CYP2E1) (Health Canada, 2009; IRIS report, 2002; U.S. EPA, 1998; Snyder and Hedli, 1996). Two metabolic pathways for biotransformation are commonly hypothesized as being responsible for benzene toxicity. The first pathway involves a variety of hydroxylases products, the major hepatic metabolites of benzene (phenol, catechol, and hydroquinone), and the second pathway involves ring-opened products of benzene (muconate derivatives) (Health Canada, 2009; IRIS report, 2002; U.S. EPA, 1998; Snyder and Hedli, 1996), an iron-catalyzed metabolism that forms trans, trans-muconaldehyde (MUC), a precursor of trans, trans-muconic acid (MA). The primary event in benzene metabolism is the hepatic conversion of benzene to epoxide, benzene oxide. Follow the formation of the epoxide; benzene oxide spontaneously rearranges (nonenzymatically) to form phenol, the major hydroxylated product of the first step in benzene metabolism. Phenol formation from benzene can occur via an alternative route through the hydroxylation of benzene by hydroxyl radicals generated from hydrogen peroxide (H_2O_2) (Snyder and Hedli, 1996). The CYP2E1, and perhaps other cytochrome P450, when function as oxidases of nicotinamide adenine dinucleotide phosphate (NADPH) can generate H_2O_2, through which the hydroxyl radical ($^{\bullet}OH$) is formed. Benzene oxide, alternatively, may react with glutathione (GSH)

catalyzed by glutathione S-transferase to form phenylmercapturic acid, a urinary metabolite that can be excreted via urine. Catechol formation occurs when benzene oxide undergoes enzymatic conversion to benzene dihydrodiol by epoxide hydrolase in the liver with subsequent conversion to catechol by dehydrogenase enzymes (Health Canada, 2009; IRIS report, 2002; U.S. EPA, 1998; Snyder and Hedli, 1996). Also, catechol can be formed by the oxidation of phenol. Further oxidation of phenol by mixed-function oxidases leads to the formation of hydroquinone. The p-benzoquinone may be formed by the hydroxylation of hydroquinone catalyzed by peroxidases, essentially the myeloperoxidase (MPO) (Smith et al., 1989) that is reportedly present in bone marrow in high concentrations (Bainton et al., 1971). In bone marrow *in vitro*, bioactivation of hydroquinone by peroxidases to reactive quinone derivatives can result in covalent binding to protein (Subrahmanyam et al., 1989) and the formation of DNA adducts (Lévay et al., 1993). It has been postulated that interaction of phenolic metabolites may induce benzene toxicity or at least are a contributing factor to toxic effect. Phenol and hydroquinone interactions reproduce the myelotoxicity observed with benzene exposure (Eastmond et al., 1987). Catechol and hydroquinone interaction also have been observed to markedly promote a synergistic genotoxic effect in human lymphocytes (Robertson et al., 1991).

2.1.4 Mechanism from Redox Cycling of Benzene Metabolites-induced Oxidative Stress in the Bone Marrow

After chronic exposure, benzene is metabolized by a hepatic enzyme (CYP2E1) to the intermediate metabolite, phenol (monohydroxylated benzene). Hydroquinone, once formed from oxidation of phenol, is further oxidized by myeloperoxidases to p-benzoquinone and then accumulates in the bone marrow. The p-benzoquinone has been directly linked to bone marrow toxicity, because it is a protein-reactive species that is highly reactive and can covalently bind to

cellular macromolecules (Snyder and Hedli, 1996; Boelsterli, 2003) or deplete cellular levels of GSH (Snyder and Hedli, 1996; Brunmark and Cadenas, 1988). The bone marrow is richly oxygenated and contains myeloperoxidase in high concentrations (Bainton et al., 1971), which is the biological basis of the bone marrow-selectivity of oxidant-induced stress and toxicity (Boelsterli, 2003). The myeloperoxidases catalyze the transfer of electron from easily oxidizable substrates such as phenol to generate reactive oxygen species (ROS) through the four-electron stepwise reduction of molecular oxygen (O_2) that leads to the production of superoxide anion radical, hydrogen peroxide, and hydroxyl radical (Snyder and Hedli, 1996; Boelsterli, 2003). The oxidized benzene metabolite p-benzoquinone enzymatically undergoes reduction by CYP reductases of the mitochondria or endoplasmic reticulum via a one-electron transfer to yield the starting material (semiquinone anion radical) that may re-enter the redox cycle. Redox cycling between the benzene metabolites: semiquinone and quinone may generate superoxide anion radicals and oxidative stress in the bone marrow (Snyder and Hedli, 1996; Boelsterli, 2003). The ROS subsequently target macromolecules such as DNA and RNA, and exerts oxidative damage which may result in DNA strand breaks and chromosome damage (Boelsterli, 2003). Another metabolic pathway is the oxidative metabolism to the reactive trans-muconaldehyde, an open-ring microsomal metabolite of benzene. The toxicity of trans-muconaldehyde may stem from its interaction with key cells (macrophages and neutrophils) in the bone marrow, which may result in cell membrane damage, including loss of membrane integrity and alterations in membrane lipid fluidity (Witz et al., 1989).

2.1.5 Mechanisms of Benzohydroquinone –glutathione-S-conjugate Nephrotoxicity

Benzohydroquinone is a metabolite of benzene. Benzohydroquinone is a reactive electrophile that can covalently bind to nucleophilic sites of target organs. Benzohydroquinone is nephrotoxic

and may induce tumors by a mechanism involving cytotoxicity (Boelsterli, 2003). Following oxidation of benzohydroquinone to benzoquinone, the benzoquinone is conjugated to glutathione to form secondary metabolites (GS-conjugate isomers). The reactive GS-conjugate isomers are actively exported from the liver into bile. Intestinal reabsorption does occur after passing through the enterohepatic cyclic process, the GS-conjugates are released from the liver and transported to the systemic circulation. The GS-conjugate when delivered to the kidney, reaches the tubular lumen of the epithelial cells. Subsequently, the GS-conjugates are metabolically degraded by two peptidase enzymes that catalyse the removal of two amino acids off the GS complex leaving behind the cysteine conjugate and are taken up into the proximal tubular epithelia. The cysteine conjugate is bioactivated to the substituted benzoquinone by oxidation inside the tubular epithelial cells. The substituted benzoquinone (cysteine -S-conjugate of benzoquinone) retains the molecular characteristic of the parent compound (benzohydroquinone) and consequently exerts its nephrotoxic effects on the proximal tubular epithelium. The toxic effects may induce tumors or possible risk of cancer progression (nephrocarcinogenesis) in the tubular epithelia.

2.1.6 CYP2E1 Gene Expression and Benzene Metabolism

Cytochrome P450 2E1 (CYP2E1) gene expression and its ability to metabolically activate benzene may vary with inter-and intra-individual differences playing a significant role in human variability and the resultant differential risk from benzene exposure, since the enzyme is particularly involved in the metabolism of benzene (Hu et al., 1997; U.S. EPA, 1998). CYP2E1 gene expression may be regulated at the transcription, translation, mRNA stabilization, and protein depletion levels (Danko and Chaschin, 2005; Ingelman-Sundberg et al., 1993). It has been demonstrated that there exists a correlation between CYP2E1 catalytic activity, mRNA level, and transcriptional activity of polymorphic gene variants (Danko and Chaschin, 2005). It is likely that

changes in CYP2E1 catalytic activity may be a consequence of gene induction or inhibition by exposure to potent environmental toxicants. Toluene is well known competitive inhibitor of benzene and has been consistently held to significantly alter the metabolism of benzene and certain other chemicals, contributing to a decrease in benzene metabolite production and resulting bioactivity of other chemicals, potentially influencing their toxicities (Andrews et al., 1977; Hsieh et al., 1990; and Fishbein 1985). Ethanol-inducible CYP2E1 has been shown to potentially play an important toxicological role in metabolism. Induction of CYP2E1 enzyme after ethanol treatment results in greater formation of reactive metabolites and may contribute to oxidative stress and increase toxicity and carcinogenic responses in the target organ (Hu et al., 1997).

The physicochemical properties of toxicant, particularly lipophilicity (dissolved phase), and the dissolved species component of the toxicant also may largely influence the metabolism and bioavailability of the toxicant for a given animal or human, consequently determining the assimilation potential across barrier membranes before the reach of the target organ or systemic circulation. The present research aims to assess the physiologic parameters of the gastrointestinal tract such as dosage (bioavailable fraction), duration of exposure, and the transport-mediated mechanism (the integrity of cell monolayer by a measure of the transmembrane resistance) that drive the degree of uptake and transport of benzene through the gastrointestinal epithelium.

2.2 *In vitro* Bioavailability/Bioaccessibility in Chemical Risk Assessment

Two concepts of bioavailability (bioaccessibility and chemical activity) are fundamental in chemical risk assessment. A contaminant becomes accessible for biouptake when it is bioavailable. Hence, a look at bioavailability suggests two different divides: one that is readily available (termed bioavailable), and a portion of the total concentration that includes those molecules that can be mobilized and made available on desorption (termed bioaccessible) for diffusive uptake into an

organism (Reichenberg and Mayer, 2006). Chemical activity describes the energetic state of the test substance that determines the potential for spontaneous processes, such as diffusion, partitioning, and baseline toxicity (Reichenberg and Mayer, 2006; Niehus et al., 2018). Chemical activity is comparable to fugacity, which describes a "fleeing" or "escaping" tendency from a phase donor (McKay, 2001; Reichenberg and Mayer, 2006; Niehus et al., 2018) and it is also linearly related to freely dissolved concentrations (Table 2.5). The striking similarity of chemical activity to fugacity and freely dissolved concentration is mainly because all different parameters describe the energetic state of a chemical. Therefore, for an improved risk assessment of chemicals, bioavailability/bioaccessibility measurements can better characterize exposure estimates during *in vitro* bioassays.

In bioassays, the risk assessors think of total aqueous concentrations of hydrophobic organic chemicals (HOCs) and, in theory, assume an absorption factor of 100% for exposure assessment (Richardson et al. 2006; Juhasz et al. (2014a). This assumption overestimates bioavailability, as it may exceed the freely dissolved concentration of test substance and, therefore, can potentially lead to overestimating the risk to target cells. Only the freely dissolved fraction solubilized into GI fluids or test medium can potentially be absorbed by the GI epithelium.

For volatile hydrophobic organic chemicals (VHOCs), the freely available fraction (bioaccessible portion) is often difficult to measure, can exhibit significant losses through sorption and volatilization, and can vary over time. This can result in poorly defined exposure of test substances and undermine the risk assessment and data interpretation. A passive dosing method can be applied to provide stable exposure concentrations of the biologically available fraction in aqueous tests to overcome these challenges. Passive dosing of contaminants is based on a uniform resupply of test substance into the test medium via passive diffusion from a biocompatible polymer

preloaded as partitioning donor (Smith et al., 2010). In the present research, a novel *in vitro* exposure technique driven by partitioning from a biologically inert polymer, silicone PDMS, was utilized to produce defined and stable exposure concentrations of a VHOC such as benzene in aqueous biotests. For the experiment, the passive dosing model is set up in transwell plates (Figure 2.1). Then the silicone PDMS is preloaded with benzene and suspended in apical media via a plastic insert. Consequently, benzene is released into apical media by partitioning from a dominating polymeric phase, providing direct contact and constant exposure levels to the target cells at the maximum chemical activity.

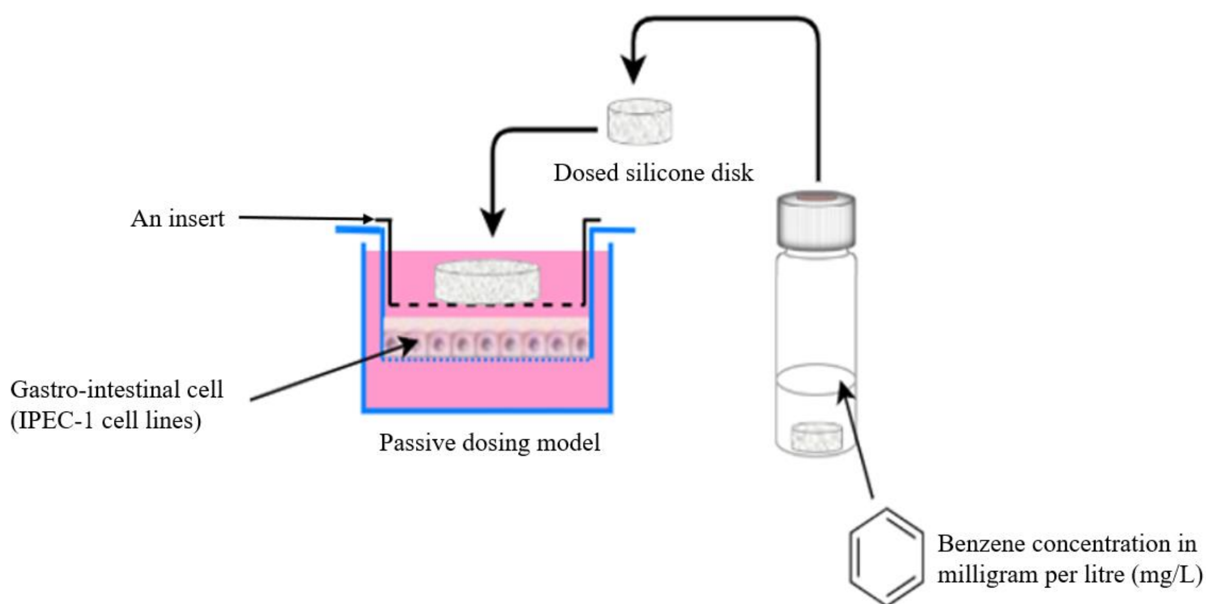

Figure 2.1 Experimental setup for the passive dosing model.

Table 2.5 Comparing the parameters of bioavailability: Chemical activity (*a*), fugacity (*f*), and freely dissolved concentration (C_{free})

Measure	Conversion [a]	Reference state
Fugacity	$f \approx P/P_{Lsub}$	Ideal gas
Chemical activity	$a \approx C_{free}/S_{Lsub}$	Liquid compound

[a] Vapor pressure (*P*), subcooled liquid vapor pressure (P_{Lsub}), and Subcooled liquid solubility (S_{Lsub}). Adapted from Reichenberg and Mayer, 2006.

PREFACE

The following data chapter will be submitted to the journal Environmental Science and Technology for publication. In the chapter, the primary focus is to develop and apply a new passive dosing approach for determining time-resolved freely dissolved fractions of the total benzene in aqueous tests for an improved assessment of benzene bioavailability to gastrointestinal cells during *in vitro* toxicity testing. A list of all the authors, their contributions, and affiliation can be found in the footnote[1].

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Olukayode O. Jegede: Provides context input to the introduction section of the original draft
Hamzat O Fajana: Contributes to the statistical analysis
Steven D Siciliano: Conceptualization, funding acquisition, supervision
Tom Van de Wiele: Co-supervised the research work

3 DEVELOPMENT OF A DOSING SYSTEM TO ASSESS BENZENE BIOAVAILABILITY IN GASTROINTESTINAL CELLS

3.1 Introduction

Bioavailable fractions of VHOCs in aqueous tests are often difficult to assess with regards to risk assessment and toxicity testing with currently applied methods for introducing VHOCs into *in vitro* bioassays. In recent times, transition from *in vivo* to *in vitro* test methods has gained increasing acceptance in risk assessment of industrial chemicals and toxicological investigations (Barile, 1994; Gad et al., 2000; Bakand et al., 2005; Bakand et al., 2006; Anadón et al., 2014). With an extremely large number (> 86,000) of industrial chemicals in commerce (U.S. EPA, 2019), the potential application of *in vivo* test methods for toxicity studies of this large number can be technically more challenging and practically unachievable from the economic viewpoint (Bakand et al., 2005; Anadón et al., 2014). *In vitro* exposure techniques have demonstrated significant potential for rapidly providing toxicity assessment and risk evaluation of industrial chemicals and volatile test chemicals (Bakand et al., 2005; Bakand et al., 2006).

Chemicals with characteristic high volatility (of air to water partition coefficients greater than one, i.e., $K_{\text{air, water}} > 1$) and highly hydrophobic (octanol to water partition coefficients above five, i.e., log Kow > 5) are generally difficult to test with currently available methods, such as direct solvent spiking and water accommodated fraction (Bakand et al., 2006; Trac et al., 2018; Trac et al., 2019; Hammershøj et al., 2020). As a result, toxicity testing and risk assessment of VHOCs face significant difficulties. In exposure assessment of chemicals *in vitro*, the ultimate goal is to estimate the biologically effective dose or assess optimal exposure concentrations and apply measures to minimize routine loss processes that otherwise would lead to a significant drop in exposure concentration during testing. Examples of such losses include compound losses to

headspace, sorption to the test vials or test vessels, walls of cell culture plates, and through volatilization, or degradation (Mayer et al., 1999; Riedl and Altenburger, 2007; Schreiber et al., 2008; Smith et al., 2010), making it difficult to control exposure in *in vitro* tests. In typical dosing scenarios, losses up to 35 % were reported for VHOCs with a log Kow < 4.6, such as naphthalene and acenaphthene (Niehus et al., 2018). Another challenge associated with volatiles or poorly soluble chemicals in *in vitro* experiments is encountered during efforts to ensure complete dissolution of the exposure concentrations in aqueous medium. Hydrophobic organic chemicals have very low aqueous solubilities (Mayer and Holmstrup, 2008). This physical-chemical characteristic limits their partitioning in the aqueous phase, thereby reducing the bioavailable concentration in aqueous test (Mayer et al., 1999; Mayer and Holmstrup, 2008). The bioavailability of a toxicant for a given animal or human is strongly dependent on the physicochemical substance properties, the exposure pattern, the duration, and frequency of the exposure. This phenomenon of reduced bioavailability compromises the quality of the risk assessment and estimation of the true exposure concentration of such chemicals (Mayer et al., 1999).

Different experimental strategies have been pursued to improve the risk assessment of volatile hydrophobic chemicals. To prevent or minimize compound losses through volatilization, cultured cells are overlayed with a mineral oil layer to forcibly inhibit the vaporization of test substances from the culture medium (Yang et al., 2002; Bakand et al., 2006). Static methods have been used to generate the standard test atmospheres of volatile organic compounds through a glass chamber for direct air sampling and static exposure to cultured cells at the air/liquid interface (Bakand et al., 2006). Furthermore, octadecyl Empore disk and Silicone O-rings, and polydimethylsiloxane (PDMS) are used as polymer phase reservoir for hydrophobic organic chemicals (HOCs) for passive dosing of test substances (Mayer et al., 1999; Mayer and Holmstrup, 2008; Smith et al.,

2010). The constant release of test substance into the test medium is then driven by continual partitioning from a dominating reservoir, following the principles of partitioning-driven administrations (Mayer et al., 1999; Brown et al., 2001; Mayer and Holmstrup, 2008; Kwon et al., 2009; Smith et al., 2010). Many of these test strategies have mainly been applied to semi-volatile and hydrophobic organic chemicals (SVHOCs) (Bakand et al., 2006; Smith et al., 2010; Trac et al., 2019) or HOCs with moderate volatility (Stibany et al., 2017) in *in vitro* assays. However, there is a need to develop new in vitro exposure techniques and time efficient dosing methods suited for HOCs with high volatility, such as benzene.

Benzene (log K_{ow}, 2.13), a highly volatile hydrophobic, lipophilic, carcinogenic compound (Boelsterli, 2003; Van Noort, 2009), was selected for this study as an important chemical with extensive industrial applications and widespread contamination in groundwater and soils. Oral exposure to benzene occurs through incidental ingestion of contaminated groundwater or soil is an important route of human exposure.

The basic *in vitro* cell culture techniques allow cells to be cultured primarily in flat-bottomed plates and then seeded in transwell or multi-well plates with permeable microporous membrane inserts. In the present study, we introduce a new passive dosing approach of highly volatile HOCs in aqueous tests for an improved risk assessment and toxicity testing during *in vitro* bioassays. A defining capability of this dosing model is the design for its compatibility. The passive dosing unit was designed to suit the physical dimension of transwell insert for cell culture plates, and the design is compatible with the standard multi-well plates, also practical and consistent with the operation of *in vitro* tests.

To perform *in vitro* testing of volatile HOCs in toxicity assays, the key points of consideration are summarized as follows: the bioavailability of chemical agents to be assessed, exposure

modeling of chemical agents, and the cell lines used should closely mimic the phenotypic attributes of cells within the human or animal target tissues (Anadón et al., 2014). A chemical becomes accessible to humans or animal target if it is bioavailable and cross barrier membranes to enter the systemic circulation. However, currently available *in vitro* testing strategies are limited in their potential for assessing volatile contaminant bioavailability and toxicity to target cells from chemical exposure due to lack of standard methods for introducing VHOCs into in vitro bioassays (Hattemer-Frey et al., 1990; Trac et al., 2019). Therefore, the present study aims (i) to develop a dosing system to assess benzene bioavailability in gastrointestinal cells and (ii) to establish test systems that linked a well-characterized toxicological response to a well-defined exposure concentration of benzene in aqueous tests.

3.2 Materials and Methods

3.2.1 Chemicals and Materials

Benzene (C_6H_6), CAS # 71-43-2, HPLC grade, ≥99.9%, was purchased from Sigma Aldrich Canada and served as a model chemical for this study. Silicone elastomer kit (MDX4-4210 kit) consisted of two components, a biomedical grade silicone polydimethylsiloxane (PDMS) and a cross-linking agent, was purchased from Krayden Inc., USA, and used for passive dosing in transwell plates. Silicone PDMS was chosen because it has been tested and proven a suitable biocompatible polymer material with excellent partitioning properties for hydrophobic organic chemicals (Mayer et al., 2000; Mayer and Holmstrup 2008; Smith et al., 2010). A standard 6-well cell culture-treated polystyrene microplates from Corning Life Sciences were used as supplied by the Fisher Scientific Canada. Also, all cell culture reagents consisted of Dulbecco modified eagle medium or DMEM/F-12, fetal bovine serum (FBS), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), epidermal growth factor (EGF), insulin-transferrin/selenium (ITS),

penicillin, and streptomycin were obtained from Fisher Scientific Canada. Milli-Q ultrapure water (Milli-Q® Direct Water Purification System) was used.

3.2.2 Preparation of the PDMS Mixture

The silicone mixture was prepared first by mixing the PDMS prepolymer and the cross-linking agent in a 10:1 (w/w) ratio using ultra power hand mixer for 15 min. A 1-mL aliquot of 200 mg/L toluene solution was then added to the resulting mixture to facilitate the reduction of viscosity and was further mixed thoroughly for 15 min in a fume hood. This step also ensured the removal of air entrapped during mixing and enhanced more adsorption capacity (sorptive sites) of silicone PDMS for benzene. Subsequently, the mixture was poured into 15 mL conical sterile polypropylene centrifuge tubes, leaving the tubes to stand for at least 48 h at room temperature to allow the mixture to cure. After thermal curing at 100 °C in an oven for 8 h, the tubes were cooled at room temperature before the cured silicone (silicone bar) was removed from the tube. Silicone disks of 1 g by weight were produced from the cured silicone using a sterile blade. Before use, silicone disks were cleaned to remove impurities. This was achieved by immersion in ethyl acetate for 24 h.

3.2.3 Adsorption Experiments

3.2.3.1 Loading of the PDMS with benzene

Silicone disks with a diameter of 17 mm were loaded by partitioning from methanol solution of benzene or a solution of benzene in water at 22 ^{o}C for 24 h. Loading was performed by adding 1 g of the silicone disk to a 20 mL vial (Amber glass bottles) filled with 10 mL of adsorbate solutions at varied nominal concentrations. One mL of each nominal loading solution was transferred into a 10 mL headspace vial for headspace analysis by the gas chromatography-mass

spectrometry (GC-MS) to measure the initial concentration of adsorbate solutions before loading. The partitioning coefficient ($K_{\text{MeoH:PDMS}}$) for the equilibrium benzene distribution between methanol and PDMS was determined using equation 1 below as described in Smith et al. (2010). The equilibrium partitioning ratio for benzene distribution between water and PDMS is expressed as $K_{\text{water:PDMS}}$ (eq 2). Concentrations of benzene in water and PDMS-sorbed benzene were from the loading experiments.

$$K_{\text{MeOH:PDMS}} = \frac{C_{\text{MeOH}}}{C_{\text{PDMS(eq)}}} \tag{1}$$

$$K_{\text{water:PDMS}} = \frac{C_{\text{water}}}{C_{\text{PDMS(eq)}}} \tag{2}$$

3.2.3.2 Adsorption Equilibrium

Adsorption of benzene onto PDMS from aqueous solution was studied in a series of 10 mL adsorbate solutions of different initial concentrations ranging from 30 to 350 mg/L and a fixed adsorbent dosage of 1 g. The concentration range of benzene solutions used in this study was prepared by mixing a known amount of benzene standard with Milli-Q water to yield various concentrations of choice. The experiments were performed in 20 replicate batches. Each batch of the 20 replicate disks was loaded with benzene according to the procedure outlined in the previous paragraph. Control disks were loaded with ultrapure water only to the same dosing volume. The experiments were conducted under laboratory conditions with temperature control to 22 ± 0.52 °C. Each batch of experiment was repeated at least twice, yielding reproducible results under similar conditions at different times. The amount of benzene adsorbed onto PDMS was determined from

the initial concentration of adsorbate solutions and equilibrium concentration using the equation below.

Amount adsorbed (q_e) = ($C_0 - C_e$) * V/m (3)

where q_e denotes the amount adsorbed onto adsorbent in mg/g, C_0 and C_e are the initial and equilibrium concentration (mg/L) of adsorbate solutions captured by the GC-MS respectively, V is the volume (L) of adsorbate solution and m is the mass (g) of the silicone PDMS.

The Freundlich equation is used in this study to describe the correlation of the liquid-phase adsorption data (Hindarso et al., 2001; Ravi et al., 2018). This equation is presented in the following form.

$$q_e = K_F C_e^{1/n} \quad (4)$$

where q_e is the equilibrium amount of benzene adsorbed to silicone in mg/g, C_e is the equilibrium concentration of benzene in water (mg/L), K_F is the Freundlich adsorption constant (L/g), and $1/n$ is the measure of intensity of adsorption.

3.2.4 Desorption Experiments

3.2.4.1 Initiating Exposure Concentrations in DMEM exposure medium

Freely dissolved fractions of benzene in the exposure medium were determined using passive dosing. The PDMS was loaded with benzene by adding 1 g of the silicone disk to a series of 20 mL vials filled with 10 mL solution of benzene in water at nominal loadings of 30, 40, 80, 120, 160, 240 mg/L for 24 h respectively. After loading, the silicone disks were rinsed with a small volume of DMEM medium. The loaded disks were then suspended in the apical medium via a plastic insert (see Figure 3.1) in six-well culture plates containing 6 mL apical medium (DMEM

only) and 4 mL of the same medium at the basolateral compartment. Benzene is released into the apical medium by partitioning from the stationary phase (PDMS), through direct contact with the aqueous medium, but with no direct contact between the PDMS donor and the target cell lines. The resulting release established a uniformly freely dissolved concentrations in the exposure medium. The equilibrium partitioning ratio for benzene distribution between PDMS and DMEM is expressed as $K_{\mathrm{PDMS:DMEM}}$ and was calculated using the equation (eq 5). The concentration of benzene freely dissolved (C_{free}) in the exposure medium was calculated using the equation as described (eq 6). The DMEM benzene concentration (mg L^{-1}) was from the desorption equilibrium experiments at 37°C.

$$K_{\mathrm{PDMS:DMEM}} = \frac{C_{\mathrm{PDMS(eq)}}}{C_{\mathrm{DMEM(eq)}}} \qquad (5)$$

$$C_{\mathrm{free}} = \frac{C_{\mathrm{PDMS}}}{K_{\mathrm{PDMS:DMEM}}} = C_{\mathrm{DMEM}} \qquad (6)$$

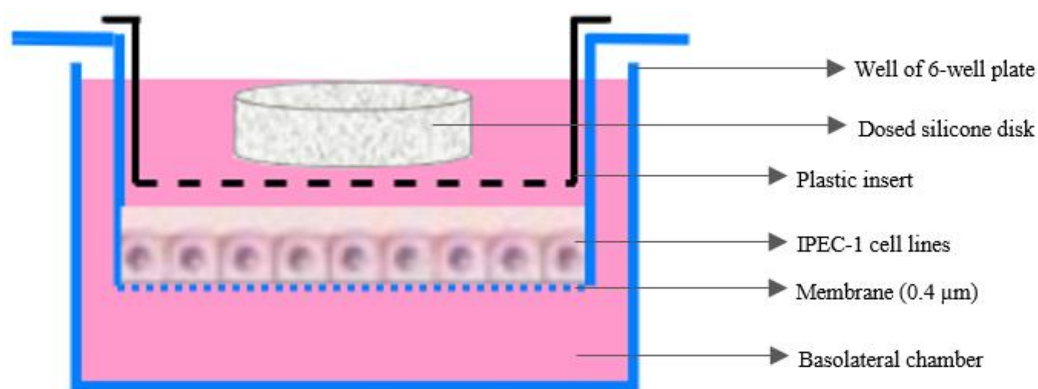

Figure 3.1 Passive dosing system for assessing benzene bioavailability in gastrointestinal cells.

3.2.4.2 Analytical Confirmation of Exposure Concentration

The released amount of benzene was determined from a 1 mL subsample of test medium taken at different times. Aliquot of 1 mL from each triplicate well was transferred into a 10 mL headspace GC injection vial containing a solution of methanol and Milli-Q water in a mixed ratio of 1:8 for analysis on a GC-MS equipped with a flame ionization detector (FID). All aqueous subsamples for headspace analysis were collected using a glass pipette. For quality control evaluation, a recovery spike of known benzene concentration (certified reference material) purchased from Sigma Aldrich Canada was added to the blank samples. The blank samples using Milli-Q water only were prepared following the same silicone loading and desorption equilibrium procedures. The percentage of recovery of the reference material was 90%, and the precision was 8% relative standard deviation for measured concentrations of the certified reference material. The GC-MS/FID system was operated with a split 20:1 for the whole run. Samples for analysis were incubated for 5 min at 82°C and agitated before subsamples of 500 µL were drawn from the headspace vials. The injector temperature was 200°C with a constant column flow of 1 mL min^{-1}.

The column employed was Bruker-BR86092 (30m × 0.25mm) with helium carrier gas at a flow rate of 27.5 mL min^{-1}, air at a flow of 300 mL min^{-1}, and hydrogen gas at a flow of 30 mL min^{-1}. The oven temperature was program to initially 60°C, then increasing 15 °C min^{-1} to 150°C.

3.2.5 Cell Viability and Cytotoxicity Tests

3.2.5.1 Cell Culture

The intestinal porcine enterocyte cell line (IPEC-1 cells) was chosen for this test because it mimics the phenotypic attributes of cells within the humans more closely than other cells of non-human origin (Geens and Niewold, 2011). The IPEC-1 cell is of swine small intestinal (jejunum/ileum) origin. However, IPEC-1 cell as a model for the human intestine has been widely used as an *in vitro* model of the small intestine for assessing the intestinal permeability and cellular response of orally ingested chemicals of natural or synthetic origin (Gonzalez-Vallina et al., 1996; Diesing et al., 2011). The IPEC-1 cell, when grown in culture, differentiates to exhibit a densely microvilli-like appearance on their apical surface characteristic of the small intestine enterocytes. For IPEC-1 cell culture, the cells were cultured in transwell membrane insert for passive dosing tests to assess benzene bioavailability and toxicity to gastrointestinal cell lines. IPEC-1 cells kindly donated by Dr. Natacha Hogan, Department of Animal and Poultry Science, University of Saskatchewan, Canada, at passage 44 were cultured in DMEM/F-12 (1:1). The culture medium was supplemented with 25 mL FBS (10 %, v/v), 5 mL HEPES, 100 μL EGF, 5 mL ITS, and 5 mL penicillin/streptomycin. The cell cultures were maintained in 75 cm^2 cell culture flasks at 37 °C in an incubator containing humidified air (95 %) and CO_2 (5 %). The culture medium was changed every three days. At 80-90 % confluence (day 4–5 of culture), cells were harvested from the flask by a trypsin wash with 5 ml solution of trypsin (0.5 mg/L) containing 0.22 g/L ethylenediamine tetra-acetic acid or EDTA. For propagation, cells were seeded at a density of 1.4×10^5 cells/3 mL

culture media into the apical chamber of the transwell membrane insert, and 4 ml culture media only to the basolateral compartment. The culture medium was routinely changed every three days, beginning the second day post-seeding. The transepithelial electrical resistance (TEER), expressed in ohm cm^2 (Ω cm^2), was measured using a Millicell STX probe until stable TEER values were reached after 12 d. After a total culture period of approximately 14 days, cells were exposed to benzene by passive dosing for cytotoxicity tests.

3.2.5.2 In Vitro Bioassays

Assays for determining cell viability and cytotoxicity were performed by employing cell proliferation reagent WST-1 (tetrazolium salts). The WST-1 cytotoxicity assay is based on the measurement of colorimetric differences that appear when cells start to die after exposure to *in vitro* treatments. For this assay, a new passive dosing approach for controlling benzene *in vitro* exposure regime was applied to determine the effective concentration and toxicity of benzene to IPEC-1 cells. Nominal concentrations of benzene were prepared by mixing benzene standards with ultrapure water to produce varied desired concentrations. Silicone disks were dosed in three replicate batches at nominal loadings of 40, 80, 120, 130, 140, 150, and 160 mg/L of benzene. Blanks were also prepared in three replicates, which were loaded with ultrapure water only in the same loading step. Before the cell viability test, cells were cultured in a transwell insert. Each treatment group consists of three replicate wells. At test start, media was aspirated off and to each well, 6 mL media (DMEM only) was added to the apical side of the cell insert and 4 ml of the same media to the basolateral compartment in a 6-well plate. We used DMEM medium only in the passive dosing tests (24 h exposure) to ensure the determination of the true exposure concentration freely available to cells. Sorption of the test compound to the medium constituents has been reported in previous studies. When sorption occurs, it can lead to altered release kinetics and an

enhanced diffusive transport into the culture medium (Smith et al., 2010). The dosed silicone disks were removed from the loading solutions and rinsed with a small volume of Milli-Q water to remove any solvent residues. The silicone disk was then transferred into the apical insert and remained suspended in the apical medium via a plastic insert. The toxicity of benzene on the viability of IPEC-1 cells was determined after 24 h of passive dosing at 37 °C in an incubator containing humidified air (95 %) and CO_2 (5 %).

After 24 h incubation, media (experimental treatments) were aspirated, and to each replicate well, 1 mL media (DMEM, supplemented with FBS, HEPES, EGF, ITS, and penicillin/streptomycin) containing 10% WST-1 reagent was added to the apical side of the cell insert. The culture plate was incubated for an additional 3 h and measurement of absorbance of the treatment samples against a background control as blank were taken at 0.5, 1, 2, and 3 h using a multi-well spectrophotometer plate reader (ELISA reader) at 440 nm and a background reference wavelength at 620 nm. Viable, metabolically active cells produce cellular reductase enzymes, NADPH. The presence of NADPH facilitates the intracellular conversion of the WST-1 tetrazolium salts cleavage to formazan dye (Berridge et al., 1996). The number of viable cells directly correlates with the absorbance of formazan dye, which was measured to quantify the degree of cytotoxicity that occur in cells resulting from *in vitro* treatments. The calculated mean % cell viability of the media control samples was used as the reference for estimating the cell inhibition resulting from benzene toxicity in the treatment groups. A dose-effect relationship between the freely dissolved concentration of benzene in the exposure medium and partitioning of benzene into the cell membranes was generated by plotting the measured equilibrium concentration of benzene freely available to cells (C_{DMEM}) as a function of cell viability. The

toxicity of benzene was also linked to calculated chemical activity (*a*) of benzene concentration exerting the observed effects:

$$\text{Chemical activity } (a) = \frac{C_{\text{DMEM}}}{S_{\text{Lsub}}} \tag{7}$$

where S_{Lsub} is the subcooled liquid solubility. The value for S_{Lsub} of benzene, 1.36 mmol/L (106.22 mg/L) was obtained from Van Noort, (2009).

In separate experiments, we estimated the viability of IPEC-1 cells from a direct spiking of benzene in aqueous tests using a glass syringe, for comparison between the two dosing methods (spiking and passive dosing). On both tests, the median effective concentration of passive dosing (EC_{PDMS}-50) and spiking (EC_{Spike}-50) applications in the cell viability tests were evaluated from the dose-response relationship. The median effective activity (Ea_{50}) value was calculated as a measure of the baseline toxic potential of benzene for IPEC-1 cells using the equation (eq 8).

$$Ea_{50} = \frac{EC_{\text{PDMS}}\text{-}50}{S_{\text{Lsub}}} \tag{8}$$

3.2.6 Statistical Analysis

Benzene adsorption to silicone between partitioning from a methanol suspension of benzene and a mixed benzene solution in water was compared by one-way analysis of variance (ANOVA) with the software OriginPro (2020b) to determine the preferential adsorption of benzene that differs significantly from the two loading solutions. A post hoc test was performed using the Bonferroni test to compare the mean difference of equilibrium benzene distribution between each aqueous partitioning phase and silicone. The Levene test was used to verify the homogeneity of variance assumption. The median effective concentration from passive dosing (EC_{PDMS}-50) and direct spiking (EC_{Spike}-50) applications in the cell viability tests was estimated by R software

package. All adsorption equilibrium modelling was carried out in SigmaPlot, Ver 14.0. Data from a dose-response relationship for the effect of benzene on the viability of IPEC-1 cell line after exposure to benzene were fitted by SigmaPlot using global curve fitting.

3.3 Results

3.3.1 PDMS Loading

Partitioning of benzene from water to PDMS was higher than from a methanol loading solution (one-way ANOVA, $F = 31.713$, $p = 0.001$), (Figure 3.2). The amount of actual loadings of benzene ranged from 80 to 88% of the nominal loading from a solution of benzene in water and were between 47 and 66% of the nominal loading from a methanol solution following loading of silicone disks for 24 h with benzene. The standard deviations ranged from 2.5 to 5.8% for calculated recoveries of the loading. For the passive dosing experiments, the silicone disks were loaded by partitioning from a water suspension of the respective benzene concentrations for 24 h. The amount of benzene adsorbed to PDMS increased with increasing solution concentration (up to 160 mg/L nominal concentration) of benzene (Table 3.1) and time. The results obtained from the adsorption experiments indicate that the adsorption equilibrium of benzene was reached about 98 min, and the sorption rate constant (k) (min^{-1}) was less than 1 min (Figure 3.3A and B). Chemical partitioning varied with concentration. At low concentration, benzene equilibrium distribution between liquid and solid phase silicone PDMS demonstrates excellent partitioning of benzene for silicone, partitioning pattern showed small deviations both at medium and high concentration, as shown in Figure 3.4. The best explanation for this deviation is that benzene partitioning to silicone were possible until a certain concentration, above which the concentrations in the silicone reach saturation.

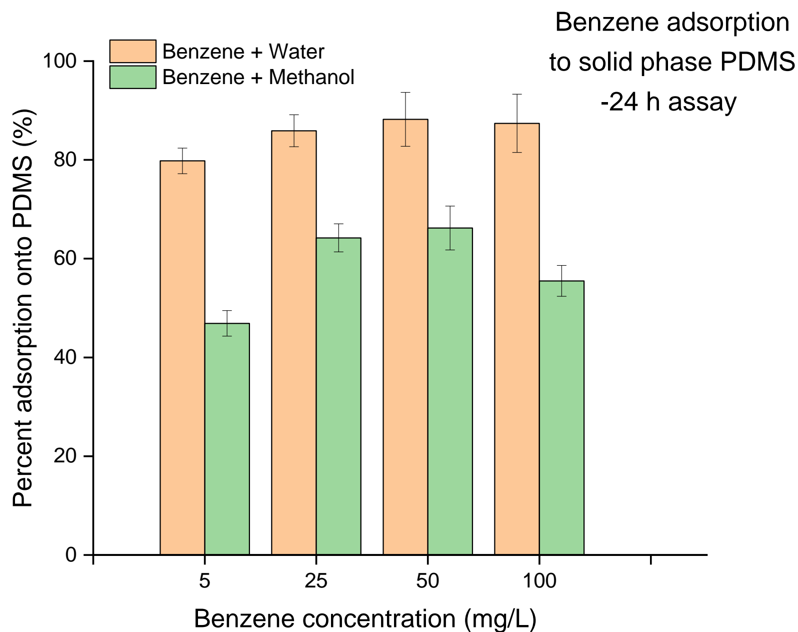

Figure 3.2 Loading of the silicone disks with benzene from a solution of benzene in water and methanol at a concentration range of 5 to 100 mg/L (error bars represent standard deviations of four replicates). At the 0.05 level, benzene adsorption to silicone is significantly different between the aqueous partitioning phases (one-way ANOVA, $p = 0.001$, followed by a post hoc Bonferroni test).

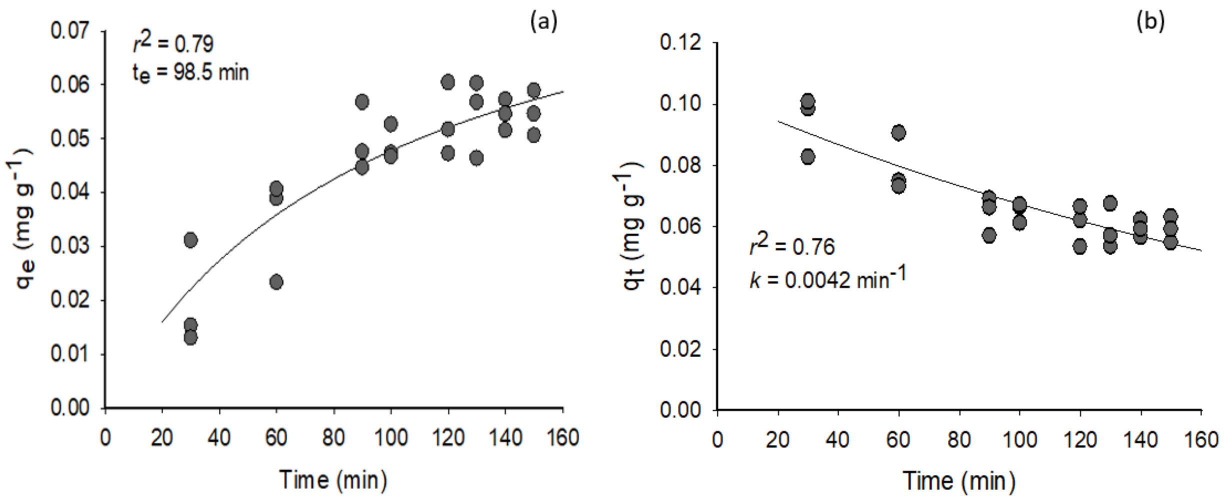

Figure 3.3 (a) Contact time on adsorption of benzene to silicone PDMS. Conditions: 1 g of silicone PDMS, 10 mL of mixed benzene solution (in water) at an initial nominal concentration of 30 mg L^{-1} and a temperature of 22 °C. (b) The sorption rate constant (k) of benzene to silicone PDMS. q_e = Equilibrium amount of benzene adsorbed (mg g^{-1}); q_t = Amount of benzene (mg g^{-1}) adsorbed to the silicone PDMS at time t; t_e= Time for adsorption to reach equilibrium (min); Number of replicates for each time point = 3. The regressions are significant ($p < 0.001$).

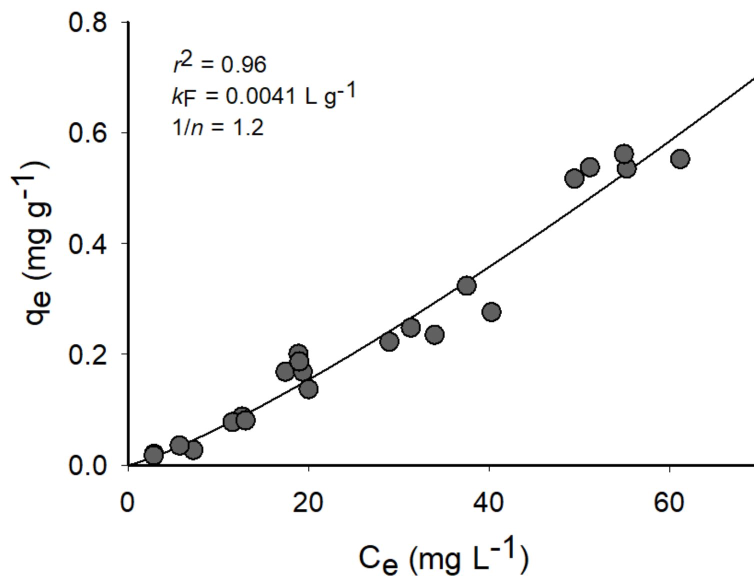

Figure 3.4 Freundlich adsorption isotherm for the adsorption behavior of benzene to silicone PDMS. q_e = Equilibrium amount of benzene adsorbed (mg g^{-1}), C_e= Equilibrium concentration of benzene in water (mg L^{-1}), K_F = Freundlich adsorption constant (L g^{-1}), $1/n$ = Measure of intensity of adsorption, Number of replicates = 20. The estimation of K_F was significant (p = 0.0038).

3.3.2 Initiating Freely Dissolved Concentrations

Freely dissolved concentration of benzene in the exposure medium was established within 30 minutes of passive dosing and maintained throughout a normal exposure period of IPEC-1 cells (Figure 3.5). A linear relationship between the desorbed concentration of benzene freely available to cells in the culture medium and nominal concentration adsorbed by the silicone after 90 min is provided in the appendix (Figure A1). The DMEM concentrations of benzene remained stable for 24 h. The buffering capacity of the dosed silicone disk freely compensates for routine losses that otherwise would lead to a decreasing benzene concentration. The measured concentrations of the free fraction of benzene in the exposure medium (C_{free}), with their calculated chemical activity (a), are summarized in Table 3.1.

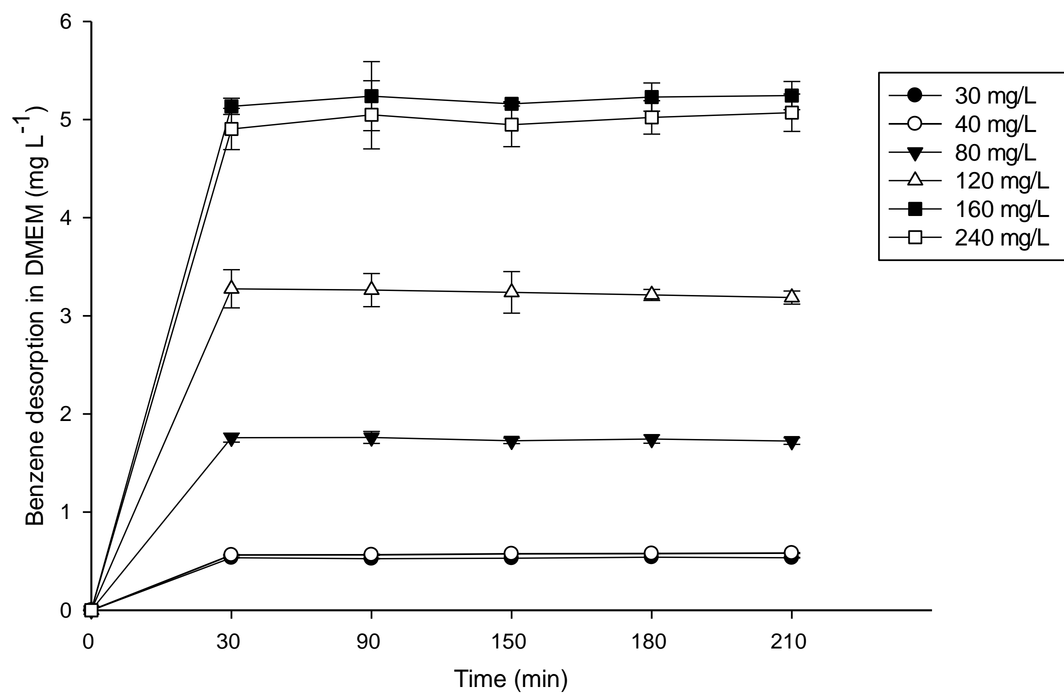

Figure 3.5 Release of benzene into cell culture medium by passive dosing plotted against time. Error bars represent standard deviations of four replicates, and non-visible error bars are smaller than symbols.

Table 3.1 Benzene loading concentrations, octanol-water coefficient (log K_{ow}) and (subcooled) liquid solubility (S_{Lsub}). Also shown are equilibrium concentrations of benzene in the silicone PDMS (C_{PDMS}), equilibrium partitioning ratios for Water:PDMS ($K_{water:PDMS}$, L L $^{-1}$), PDMS:DMEM ($K_{PDMS:DMEM}$, L L $^{-1}$) at 37 °C, freely dissolved concentrations (C_{free}) measured in DMEM medium at test termination as well as the calculated chemical activity (a).

Log K_{ow}	S_{Lsub} (mmol/L)
2.13	1.36 [a]

Nominal loading mg/L	$C_{initial}$ (measured) mg/L*	C_{PDMS} mg/L* [b]	C_{free} (measured) mg/L*	$K_{water:PDMS}$	$K_{PDMS:DMEM}$	(a)
40	20.65 ± 0.42	18.24 ± 0.27	0.6 ± 0.01	1.13	30.41	0.01
80	35.16 ± 0.44	30.32 ± 0.83	1.7 ± 0.07	1.16	17.84	0.02
120	60.17 ± 1.84	54.46 ± 1.79	3.2 ± 0.95	1.10	17.02	0.03
130	60.77 ± 1.79	54.93 ± 1.73	3.4 ± 0.51	1.11	16.15	0.03
140	61.52 ± 2.78	54.85 ± 2.61	4.2 ± 0.68	1.12	13.06	0.04
150	70.47 ± 1.60	62.07 ± 1.24	4.8 ± 0.74	1.14	12.93	0.05
160	75.94 ± 1.77	67.22 ± 1.57	5.4 ± 1.04	1.13	12.45	0.05

[a] Taken from Van Noort, (2009) at 25 °C but converted to 106.22 mg L $^{-1}$ for calculation purposes.
[b] Based on equation (eq. 2).
* Given are mean values (± absolute deviation) of replicate measurements from four independent replicates.

3.3.3 Cytotoxicity Test

With the silicone model, there is more than 90% cell inhibition, resulting in an EC_{PDMS}-50 of 4.82 mg/L (Figure 3.6A). Without silicone, we did not see any response at all, resulting in an EC_{Spike}-50 projected to be greater than 5.4 mg/L, (highest spiked concentration), and reduced test sensitivity of benzene to IPEC-1 cells. This highlights the significance of passive dosing in risk assessment and toxicity testing of VHOCs. The effective chemical activity causing 50% toxicity (Ea_{50}) was calculated to be 0.0454 (0.048 mg/L, predicted value), which reflects the sensitivity of IPEC-1 cells to benzene (Figure 3.6B). The median effective activity (Ea_{50}) was determined as the ratio between the calculated EC_{PDMS}-50 value and the S_{Lsub} of benzene. The Ea_{50} value for IPEC-1 cells from the present study is within the chemical activity range (0.01– 0.1) for baseline toxicity of several hydrophobic chemicals reported in the literature (Schmidt and Mayer, 2015).

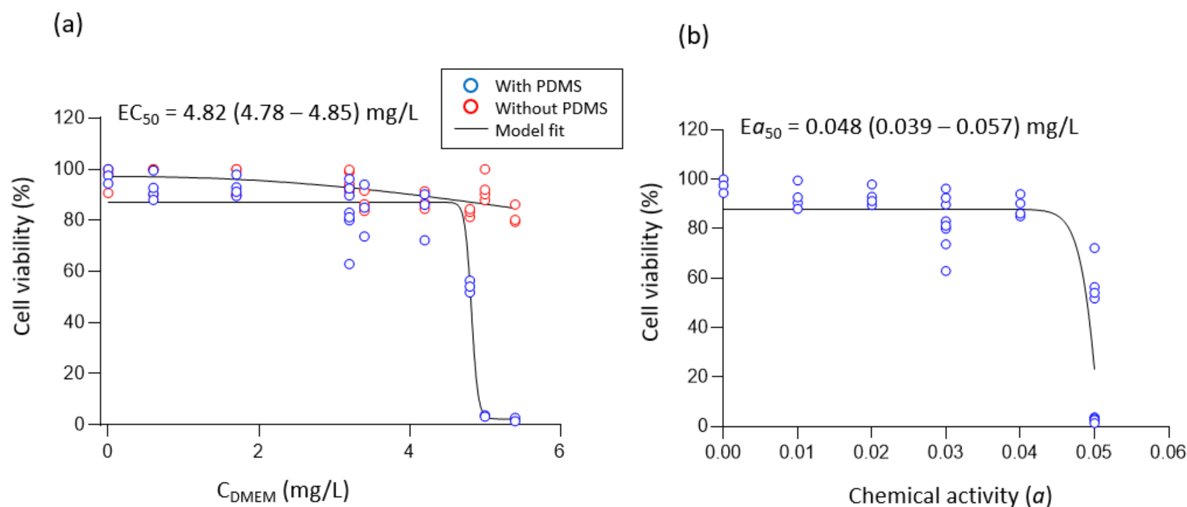

Figure 3.6 A dose-response relationship for the effect of benzene on the viability (%) of IPEC-1 cell lines after exposure to benzene (0.6, 1.7, 3.2, 3.4, 4.2, 4.8, 5.0, and 5.4 mg/L) for 24 h via the passive dosing silicone model approach. Cell inhibition ranged from 9.6 ± 2% to 97.7 ± 0.8%. The total number of replicates = 6, from two experiments conducted in three replicate batches under similar conditions at different times, and the repeats were taken as replicates. The dose-response was significant ($p < 0.001$).

3.4 Discussion

This study introduces a unique passive dosing architecture compatible with the standard 6-well transwell plates for excellent passive dosing performance *in vitro* tests. Passive dosing techniques establish and control the partitioning of single compounds and simple mixtures between different phases (Mayer et al., 1999; Mayer and Holmstrup, 2008; Brown et al., 2001; Smith et al., 2010a; Stibany et al., 2017; Trac et al., 2018; Trac et al., 2019; Smith et al., 2010). More recently, the use of passive dosing has been explored in exposure control of complex mixtures of hydrocarbons in aquatic tests (Redman et al., 2017; Bera et al., 2018; Hammershøj et al., 2020). However, no system has yet been developed for VHOCs. For *in vitro* testing of VHOCs, passive dosing application has several advantages: (1) true exposure concentration in the test medium can be established, (2) a

resupply of test substance through phase partitioning from a dominating polymer phase donor can be achieved to compensate for compound losses during the test, (3) controls *in vitro* exposure concentrations at a relatively constant chemical activity, (4) offers the possibility of estimating the actual dose or biologically effective dose at the target site, and (5) the chemical potential that drives the equilibrium partitioning of the test chemicals into the cell membranes is determined by the chemical concentration in the partitioning donor and can be quantified by its chemical activity.

Benzene at high exposure levels can act as a narcotic (Bingham et al., 2001), and nonpolar narcotic chemicals such as highly hydrophobic organic substances are known to exert baseline toxicity in aquatic organisms (Mayer and Reichenberg, 2006b; Schmidt and Mayer, 2015; Trac et al., 2019). Baseline toxicity or nonpolar narcosis describes the minimal effect exerted at the site of toxic action in membranes by partitioning of nonpolar (lipophilic) chemicals into the lipid bilayer of cell membranes at a relatively constant exposure concentration (Van Wezel and Opperhuizen, 1995; Reichenberg and Mayer, 2006; Mayer and Reichenberg, 2006b). Based on our study, the obtained value for the EC_{PDMS}-50/ S_{Lsub} ratio, an indicator of the baseline toxic potential of benzene for IPEC-1 cells, is within the chemical activity range (0.01– 0.1) for baseline toxicity of several non-polar narcotic chemicals, such as Toluene, Xylenes, and Benzophenone reported in previous study (Schmidt and Mayer, 2015). Comparison to other previously published studies also suggests that the EC_{PDMS}-50 from the present study is lower than the EC50 values reported in the range of 4.97 – 5.55 mg/L for IEC-6 cells (rat intestinal epithelial cell line) (Upreti et al., 2007), following experimental exposure to arsenic. In real-world exposure scenarios, risk assessors commonly adopt a conservative estimate that assumes an absorption factor of 100% for exposure assessment. But, gastrointestinal absorption of orally ingested hydrocarbons is known to vary based on the type of hydrocarbons (the physiochemical substance properties). The test system developed in this study

produced a more realistic estimate of the effective concentration of benzene that is present in a biologically available form by measuring the amount of benzene molecules that became available on desorption and solubilized into simulated GI fluids (DMEM). In exposure assessment of chemicals normally conducted *in vitro*, high hydrophobicity and volatility of chemicals may result in a series of pulses of high concentrations or poorly defined exposure concentrations. This study produced a suitable passive dosing method for *in vitro* tests in multi-well plates and demonstrated excellent passive dosing efficiency over solvent spiking, thus improving the predictive power of toxicity data from the *in vitro* tests.

For risk assessment of human exposure to benzene at contaminated sites, *in vivo* models will best simulate the exposure conditions and bioavailability. However, due to ethical considerations in the use of animal models in toxicity tests, the development of new *in vitro* models simulates the exposure conditions as *in vivo* and ensure reliable results can be extrapolated to the population of interest, including humans. Essentially, for benzene to be accessible to humans, it must be bioavailable and cross barrier membranes to exert toxic effects at target sites. In the present study, we developed and applied a new passive dosing approach for assessing the bioavailable concentrations of benzene in aqueous tests. From a risk assessment level, acute toxicity of benzene was assessed by estimation of the EC_{50} using the toxicity test data from the cell viability assay. Correlating the effective concentration exerting the observed cell inhibition of 97% at maximum chemical activity thus suggests that benzene has acute toxicity towards IPEC-1 cells at the maximum aqueous exposure level (Bingham et al., 2001).

Further in risk assessment of benzene, the potential risk commonly associated with benzene exposure was demonstrated in a previous study by Martinez-Velazquez et al. (2006), using the cultured hepatic cell line HepG2. Martinez-Velazquez et al. (2006) have reported that benzene

exposure to cultured cells induced oxidative stress, cell cycle alterations, and a higher decrease in cell viability was found. The low viability was reportedly due to apoptosis - a mechanism commonly known as programmed cell death in cultured cells exposed to such toxicants as benzene.

To assess benzene risk to humans from oral exposure, using *in vitro* cell models to predict benzene metabolism in humans may have important implications for risk assessment. In risk assessment of benzene oral ingestion, the gut microbiota may play a critical role in metabolism. Mounting evidence has shown that bacteria-dependent metabolism of chemicals influences the *in vitro* bioaccessibility and absorptive properties of the gut wall (Laird et al., 2007; Laird et al., 2013) and modulates the toxicity for the host (Claus et al., 2016). It has been postulated that the human health risk assessment that eliminates the inclusion of microbial activity may underestimate *in vitro* bioaccessibility of ingested chemicals (Laird et al., 2013). It highlights the importance of gut microbes to biotransform environmental chemicals, influencing their partitioning across the intestinal wall and altering the toxicokinetics (Claus et al., 2016; Laird et al., 2013).

4 SYNTHESIS, CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS

4.1 Synthesis and conclusions

The bioavailability of contaminant for a given animal or human is largely dependent on the physicochemical substance properties, the exposure route along which the chemical is absorbed by the body, the exposure pattern, the duration, and frequency of the exposure. A primary concern is human health risk assessment for the oral absorption through incidental ingestion of VHOCs like benzene at downstream hydrocarbon sites, either from groundwater or from adhered soil. However, it is ethically difficult to justify animal use to estimate oral bioavailability at a contaminated site. Apart from ethical complications, animal models are costly and time consuming. Consequently, in-vitro test system is increasingly recognized as alternative to animal model. Currently, a major challenge in *in vitro* testing of VHOCs is the commonly observed decrease in bioavailable concentration in aqueous tests as a result of (bio) sorption, volatilization, or degradation, and more challenging to control chemical activity during the test. This compromises the quality of the risk assessment of such test chemicals. Hence, the goal of this research was to develop a partition-controlled delivery model via passive dosing to enable a well-defined exposure for an improved risk assessment of VHOCs during *in vitro* bioassays. Chapter 3 reported a novel finding on how passive dosing was utilized for determining time-resolved freely dissolved fractions of the total benzene in aqueous tests. This chapter concludes that in aqueous tests, the utility of passive dosing to control *in vitro* exposure of benzene produces a defined exposure level, resulting in a well-characterized toxicological response in gastrointestinal epithelial cells. The dosing approach used in chapter 3 was novel because it provides a direct and quantitative determination of the freely available concentration of benzene to cells at its maximum chemical activity, thus improving the predictive power of toxicity data from the *in vitro* tests. This

passive dosing approach is promising and could be applied to future toxicity testing of VHOC in complex mixtures.

4.2 Limitations

In the present study, the passive dosing method produced the best in vitro assessment of benzene bioavailability in aqueous tests and demonstrated significant potential for improving the risk assessment of benzene. Though, *in vivo* animal bioavailability models, such as rat or swine, are the best standard for assessing contaminant bioavailability across the gastrointestinal epithelium. The lack of gut bacterial in *in vitro* cell exposure posed a limitation to *in vitro* models predicting *in vivo* bioavailability. The gastrointestinal (GI) microbiota is increasingly recognized for its importance in the metabolism of orally ingested chemicals and a variety of gastrointestinal functions (such as intestinal absorption) of the host (Blaut and Clavel, 2007). As a consequence, the absence of gut microbiota in *in vitro* cell models to mimic *in vivo* models may be a critical limitation to estimating benzene bioavailability to IPEC-1 cells.

4.3 Future Directions

In aquatic environments, the quantitative assessment of potential risk from benzene exposure in complex mixtures is significant, given that benzene contaminations frequently occur as a joint mixture with chemicals of potential environmental concerns. Future research may be directed toward investigating the potential risk of benzene from interactions with environmental contaminants.

For future research direction, further study on interactions of VHOCs with potent toxicants such as heavy metals, for instance, arsenic that is commonly found in contaminated soil and groundwater, is required to investigate how metals interact with benzene to influence toxicity responses in IPEC cells. The most toxicological concern of arsenic is its carcinogenic potential, as

the case for benzene. Studies of the interaction between arsenical and other elements have suggested that one can ultimately alter the metabolism or toxicity of the other (Kraus & Ganther, 1989). However, the interactive effects of arsenic on benzene metabolism and transport in epithelial cells remain unknown. Crucial to the future study is how arsenic and other environmental carcinogens may influence benzene transport process in the intestinal epithelial cells. This has fundamental implications for understanding the interaction between VHOCs, metals, and the human intestinal cells to help set remediation objectives in future risk assessment.

Further, the silicone dosing format developed in this study was applied to improving *in vitro* toxicity testing of single VHOC exposure. The silicone passive dosing *in vitro* test system can also play a vital role in the risk assessment of VHOC mixtures, such as benzene in a joint mixture with Toluene, Ethylbenzene and Xylenes (BTEX). Hence, the baseline toxicity of the chemical mixtures will generally assume concentration additivity. Also, the estimation of true exposure concentration of each mixture constituent can be highly compound-specific, depending on the compound being a HOC with moderate or high volatility (Riedl et al., 2007, Schreiber et al., 2008; Smith et al., 2010). With benzene being a highly VHOC, the passive dosing system developed in this study demonstrated excellent passive dosing performance for benzene. It will also be best suited for improving the testing of VHOC in mixture risk assessment.

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APPENDIX

CHAPTER 3 SUPPLEMENTAL INFORMATION

The following figure presents a linear regression model for deriving equilibrium benzene concentrations (C_e) of nominal in cell culture medium (DMEM) after equilibration with the silicone PDMS. The simple linear regression statistically relates C_e to nominal through an equation of the form C_e= -8.332 + 5.699 ^{x} $\log C_{\mathrm{nominal}}$. This equation can be used as a predictive model for calculations of exposure concentrations to enhance estimations of benzene bioavailability based on partitioning theory.

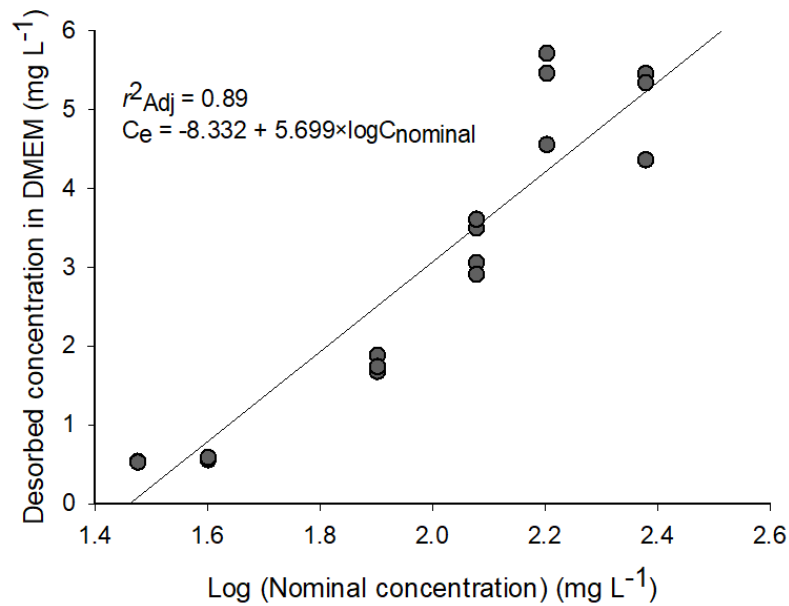

Figure A1 A linear regression model of the relationship between the desorbed concentration in DMEM and nominal concentration adsorbed by the silicone PDMS after 90 min. C_e= Equilibrium concentration of benzene in water (mg L^{-1}); r^2_{Adj} = Adjusted coefficient of determination.