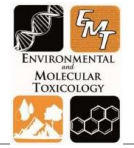
Toxicity and Mutagenicity of Polycyclic Aromatic Hydrocarbons in Contaminated Soils using a Mammalian In Vitro Assay



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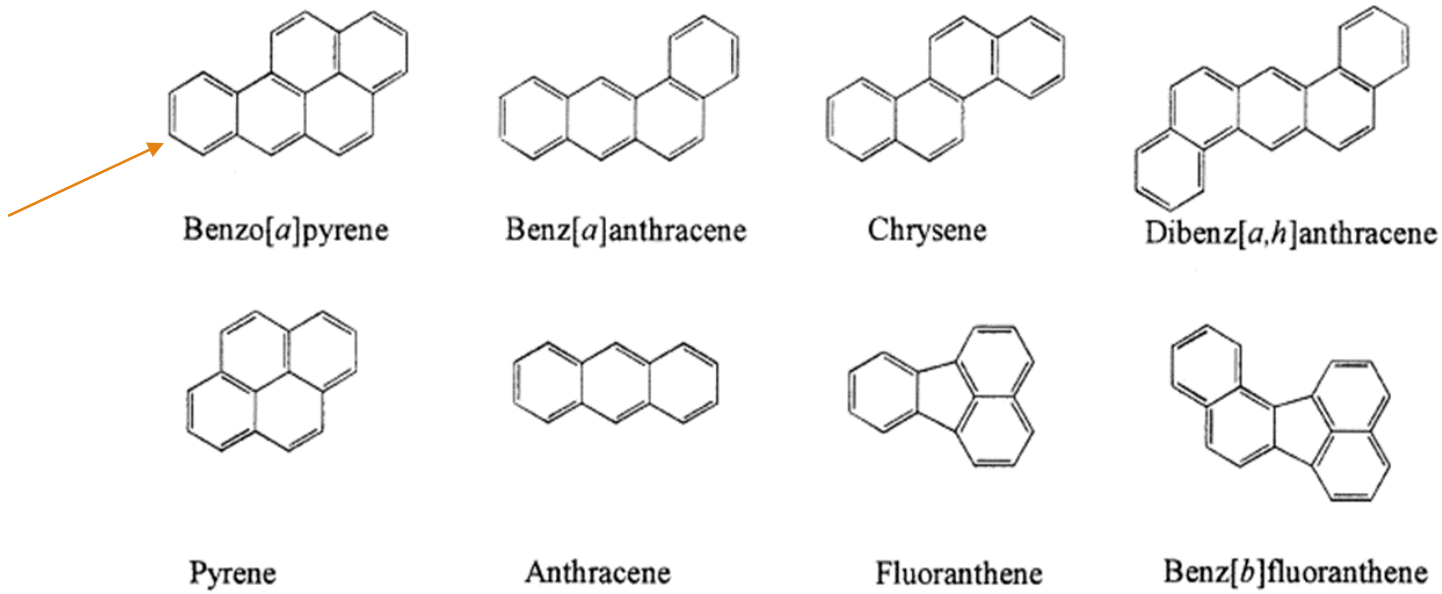
Outline for Presentation

- **Introduction**
  - Polycyclic Aromatic Hydrocarbons as Environmental Hazards
  - Risk Assessment of PAHs
  - Muta Mouse Model
  - Remediation Efforts
- **Hypothesis**
- **Study Goals**
  - Evaluate for:
    - Mutagenicity
    - Cytotoxicity
    - Biomarkers of Expression
- **Results**
- **Conclusions**

Polycyclic Aromatic Hydrocarbons (PAHs)

- Ubiquitous contaminants formed through incomplete combustion of organic material typically associated with air pollution
- Identified by large planar aromatic structure and 2 or more fused rings
- Formations of mixtures that contain hundreds of PAHs



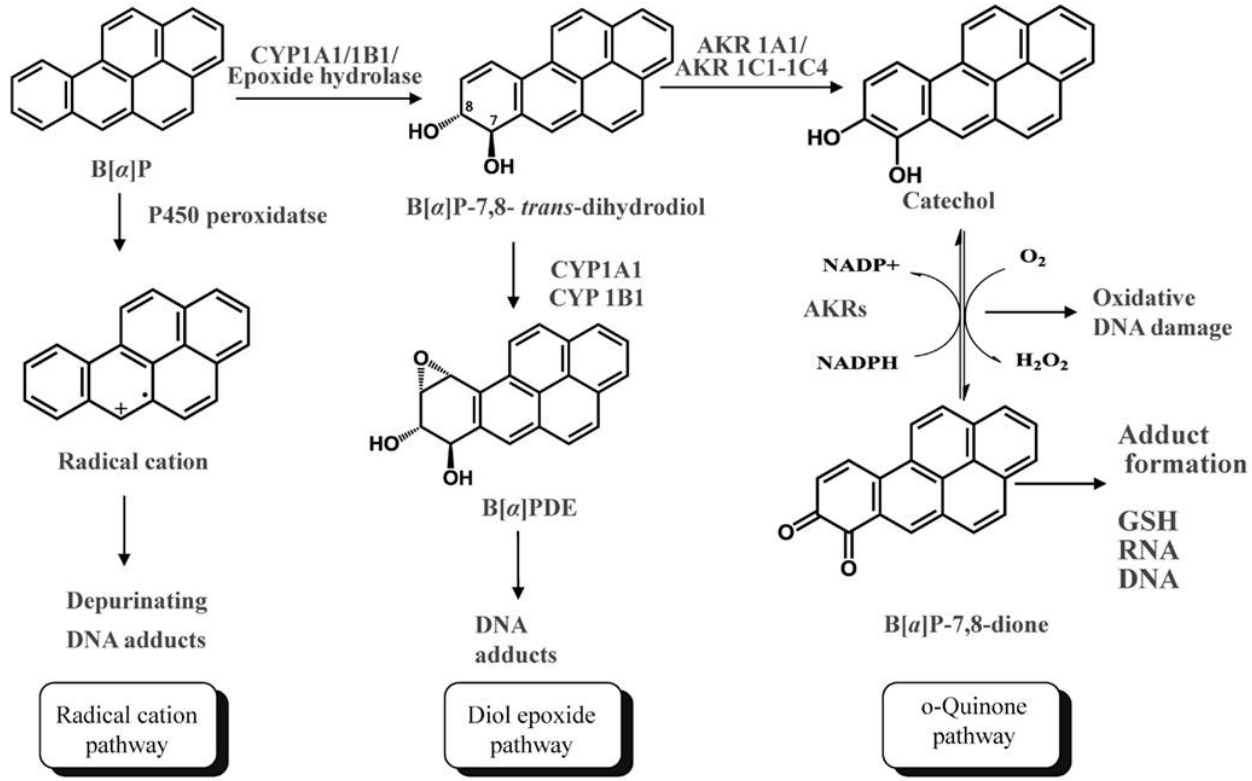
(Phillips, 1999)

PAH Sources

- PAHs can be derived from many sources
  - Natural and Man-made
- Naturally Occurring Sources
  - Forest Fires
  - Volcanoes
- Anthropogenic (human derived) Sources
  - Car exhaust
  - Tobacco smoke
  - Coal Tar
  - Asphalt production

Mechanism of PAH Toxicity

- Why are PAHs important?
- PAHs are made carcinogenic by actions of metabolism (Phase I)
  - P450 Induction
  - CYP enzymes
- Parental PAHs become toxic
- Generates metabolites for DNA-Binding and formation of DNA-Adducts implicated in lung cancer
- How do we identify the carcinogenic potential?



Zhang et al (2012)

Risk Assessment for PAHs

- Relative Potency Factor Approach (RPF)
- A risk assessment standard for PAHs in mixture form

- An approach that analyzes each component in the mixture for additivity and scales the concentration to BaP

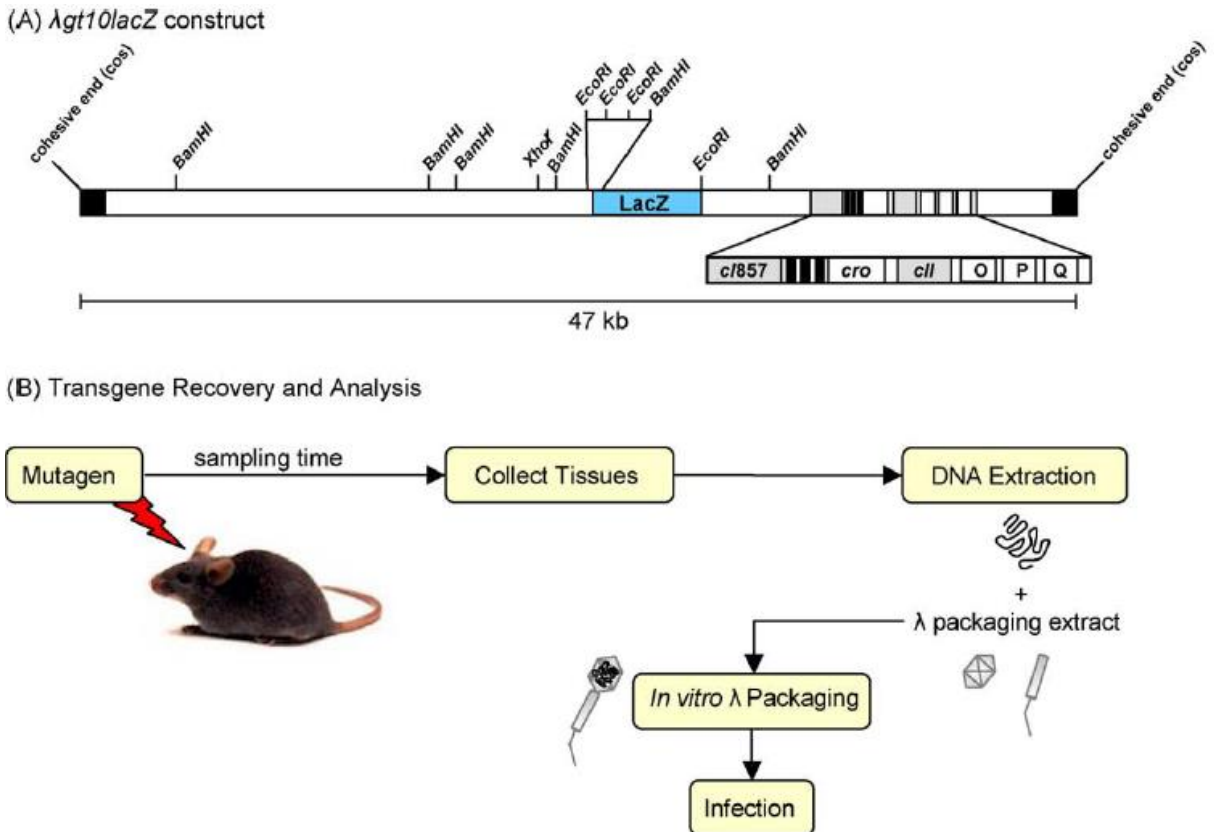
$$B[a]P_{eq} = \sum_{i=1}^{n} \left(C_{PAH_i} \times RPF_i \right)$$

EPA Development of a RPF Approach for PAH Mixtures (2010)

- BaP Equivalency; a measure of cancer potential for the mixture

Method for Evaluation of Risk

- A model that can be tested and that can prove mutagenic potential.
- **Muta™ Mouse Model**
  - Transgenic rodent mutation assay
  - Integration of bacterial *lacZ* gene into the chromosomes of mice
- Retrieval of potentially mutated *lacZ* transgene to assess mutagenicity
- Promotes only the formation of mutant plaques, eliminating the need for coloring screening



(Lambert, Singer, Boucher, & Douglas, 2005)

Muta™ Mouse Lung Epithelial Cell (FE1 Cells)

- Lung epithelial cells derived from MutaMouse model
- Resemble actual lung epithelial cells both in appearance and characteristics
- FE1 Cells are metabolically competent
  - Metabolize PAHs to their mutagenic intermediates
- Can analyze additional gene expression endpoints such as P450 enzyme activity due to metabolism
- Better stability and metabolic competency compared to other models (e.g. BigBlue Mouse embryonic fibroblast cell line and Chinese Hamster Ovary Cell Line)
- **What will this model test?**

PAHs in Soils and Remediation

- Remediation Methods
  - Speed the degradation of PAHs in the hopes of having less toxic constituents

Thermal Remediation

- High heat and pressure application to force the degradation of PAHs

- Steam Injection Platform
  - Performed by Simonich laboratory (OSU) on samples obtained from Eagle Harbor Superfund Puget Sound, Washington

- PAH Sample Soil Extracts
  - Pre-Remediated (**PreR**)
  - Post-Remediated (**PostR**)

("Monitoring helps to reveal hidden dangers in the food web | Encyclopedia of Puget Sound," n.d.)

Study Objectives

- **Evaluate the hazard associated with PAHs in mixture form using:**
  - The RPF Approach
  - Muta Mouse Model for Mutagenicity and Toxicity
- PAH Mixture
  - PreR Soil Extract
  - PostR Soil Extract
- Compare to a Class 1 Human Carcinogen Benzo(a)pyrene (BaP)

- **Evaluate additional endpoints for toxicity of PAHs in soil pre and post-remediation:**
  - Cytotoxicity
    - Lactate Dehydrogenase Assay
    - Cell Plasma Membrane Damage
  - Biomarkers of toxicity (quantitative PCR)
    - Metabolic Enzymes
    - Oxidative stress
    - DNA damage

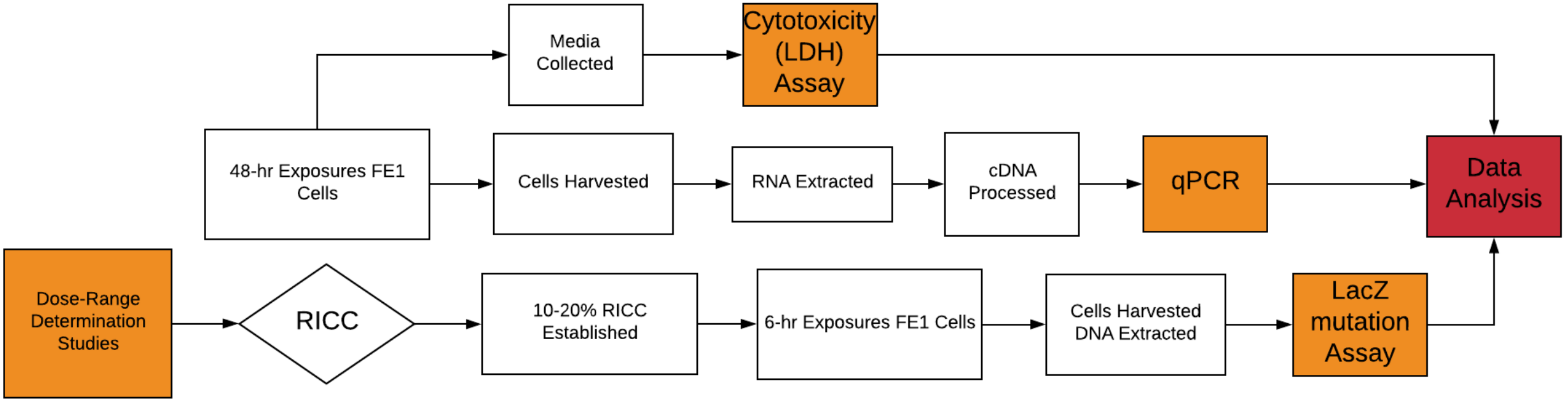
Hypothesis

PostR soil sample extract will be less mutagenic and cause less toxicity in transgenic FE1 cells than PreR sample extract

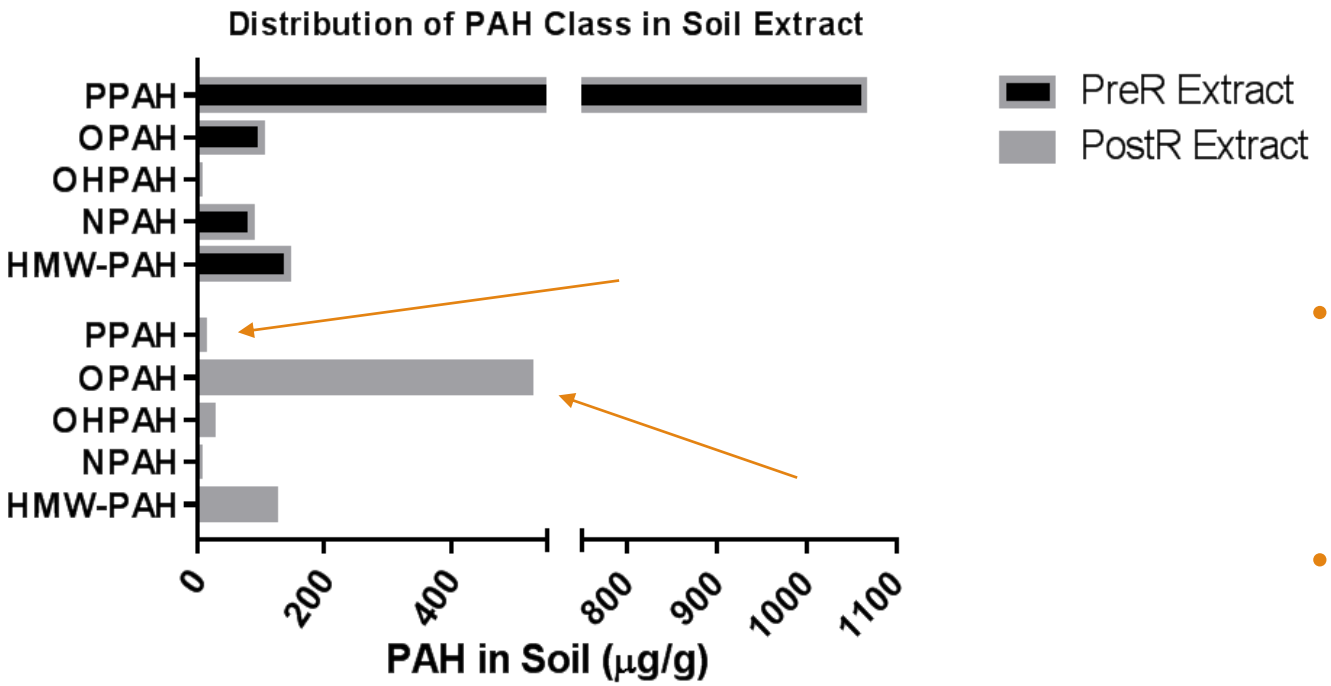
Material and Methods

- Media and Cells
  - FE1 Cells obtained from Environmental Health Sciences and Research Bureau, Health Canada (Ottawa, ON, Canada).
- Soil Extracts and Chemicals
  - PreR and PostR samples obtained from Simonich Laboratory
- Cell Culture and Maintenance
  - 37 ºC
  - 95% Humidity
  - 5% CO2

Methods Flow



Distribution of PAHs in Soil Extracts by Class



Data provided by Simonich Laboratory

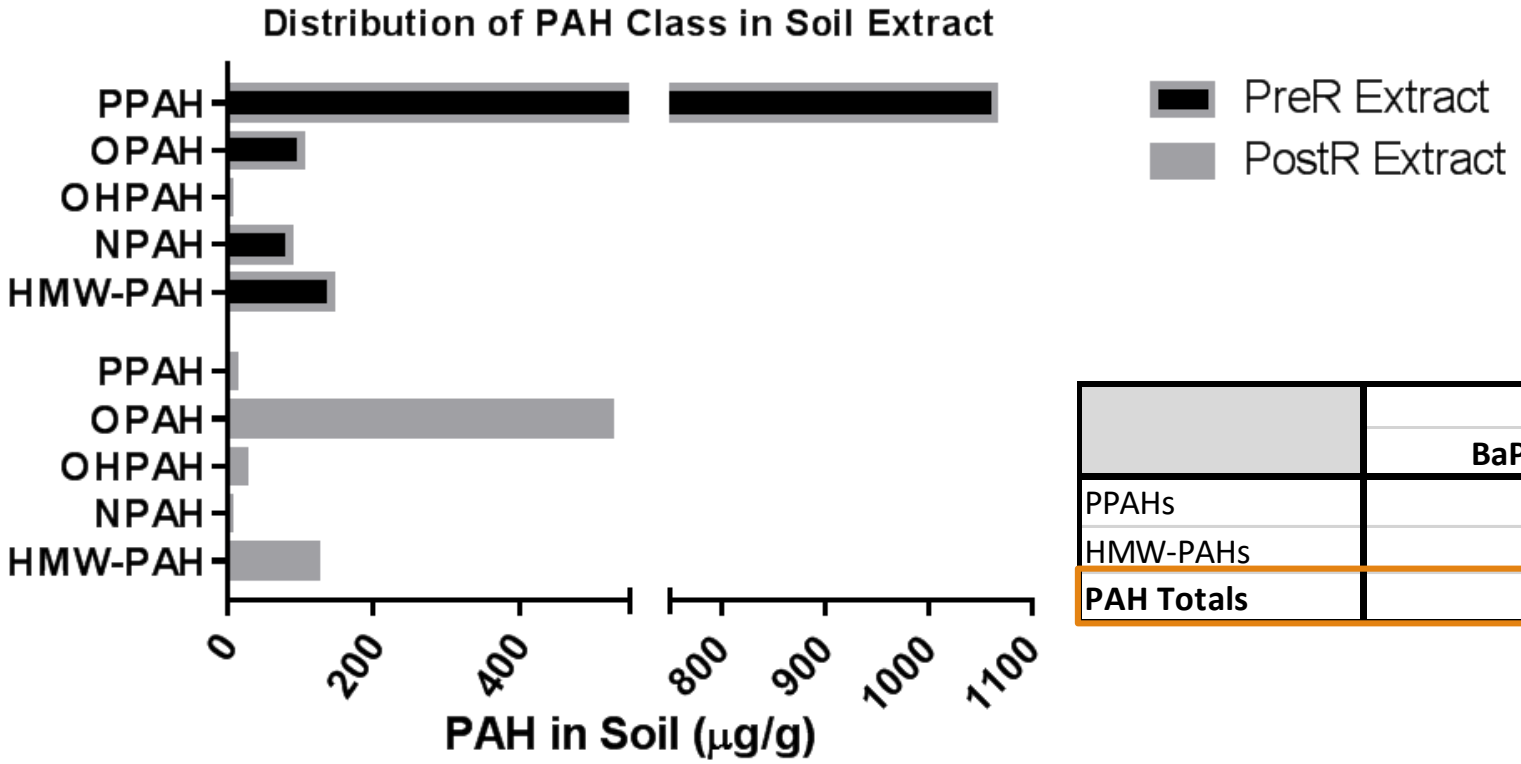
- Removal of parent PAHs (PPAH) from soil by remediation
- Formation of oxygenated PAHs (OPAHs) in soil by remediation

Calculations for BaP Equivalency

PAH Name:	Class	RPF	PreR Weight (µg g-1)	PostR Weight (µg g-1)	PreR BaP Equivalency (µg g-1)	PostR BaP Equivalency (µg g-1)
Dibenzo[a,e] + [b,k]fluoranthene	MW302-PAHs	0.90	14.7000	13.6000	13.23	12.24
Dibenzo[a,e]pyrene	MW302-PAHs	0.40	0.8690	0.8230	0.3476	0.3292
Dibenzo[a,h]pyrene	MW302-PAHs	0.90	0.6710	0.6060	0.7821	0.5454
Dibenzo[a,i]pyrene	MW302-PAHs	0.60	0.8760	0.8160	0.5214	0.4896
Dibenzo[a,l]pyrene	MW302-PAHs	30.00	0.8340	0.7490	25.02	22.47
Naphtho[2,3-e]pyrene	MW302-PAHs	0.30	0.6730	0.6660	0.2019	0.1998
Benzo(a)anthracene	PPAHs	0.20	17.9000	12.6000	3.58	2.52
Benzo(a)pyrene	PPAHs	1.00	5.7100	3.0700	5.71	3.07
Benzo(b)fluoranthene	PPAHs	0.80	8.6100	7.3300	6.888	5.864
Benzo(c)fluorene	PPAHs	20.00	7.7600	1.8500	155.2	37.00
Benzo(ghi)perylene	PPAHs	0.01	1.5900	0.9230	0.0159	0.00923
Benzo(k)fluoranthene	PPAHs	0.03	2.9800	1.3500	0.0894	0.0405
Chrysene + Triphenylene	PPAHs	0.10	25.3000	16.1000	2.53	1.61
Dibenz(a,h)anthracene + Dibenz(a,c)anthracene	PPAHs	10 and 4	7.8400	0.0263	54.88	0.1841
Fluorene	PPAHs	0.08	93.7000	0.5120	7.496	0.04096
Indeno(1,2,3-cd)pyrene	PPAHs	0.07	1.2500	0.8920	0.0875	0.06244

$$B[a]P_{eq} = \sum_{i=1}^{n} (C_{PAH_i} \times RPF_i)$$

Calculations for RPF and BaP Equivalency



	PreR Total BaP Equivalency (µg/g)	PostR Total BaP Equivalency (µg/g)
PPAHs	236.48	50.40
HMW-PAHs	40.10	36.27
PAH Totals	**276.58**	**86.67**

Data provided by Simonich Laboratory

Mutagenicity

- Are the levels of BaP equivalency for PreR and PostR extract Mutagenic?
- Determine the Dose-range necessary to treat *in vitro* cells
- Calculate Relative Increase in Cell Counts (RICC)
- Detect *lacZ* mutant and screen for plaque formations

Dose-Range Finding Studies

- Goal: To identify concentration that causes 20% cytotoxicity to choose concentrations for mutagenic assay

- Cells seeded into 6-well plates and incubated overnight
  - 20k cells/well

- Treatment Exposure times of 6-hrs
  - 5-7 concentrations of Soil Extract
    - PreR
    - PostR
    - BaP (Pos. Control)
    - DMSO (Neg. Control)

Dose-Range Finding Studies

- Treatment media removed after 6-hrs.
- 72-hr sampling period
- Cells extracted and counted
  - Estimation of Cytotoxicity using RICC

$$\mathrm{RICC} = \frac{N_{\mathrm{final}} - N_{\mathrm{initial}} \ \ (\mathrm{treated\ \ cells})}{N_{\mathrm{final}} - N_{\mathrm{initial}} \ \ (\mathrm{control\ \ cells})}$$

(Maertens et. al 2017)

RICC

- Determinations of 10-20% RICC
- Tested multiple times with inconsistent dose-response curves
  - Potentially due to complexity of whole extracts
  - Recommendation to test less complex fractions in future tests
- Chose 0.8 µg/ml as highest concentration for mutagenicity assay

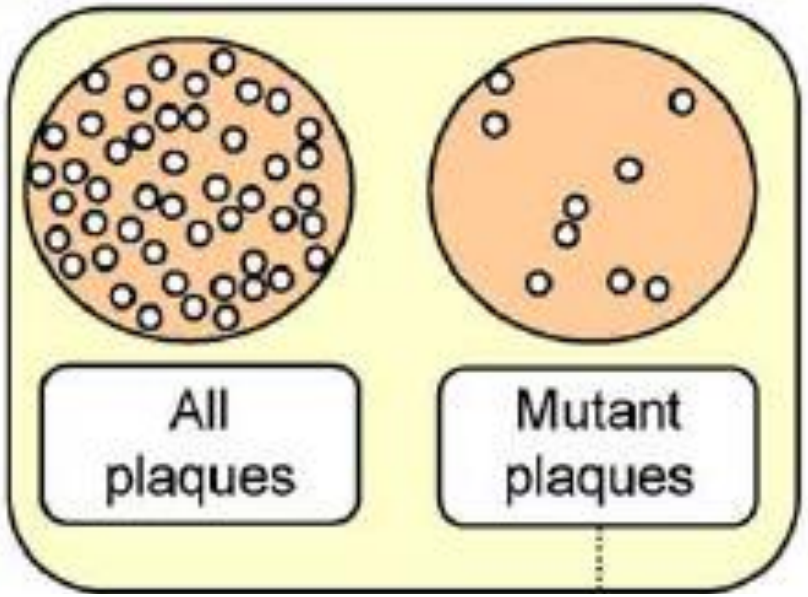
PreR Extract (µg/mL)	Mean RICC (%)	PostR Extract (µg/mL)	Mean RICC (%)
DMSO	1.00	DMSO	1.00
.049 µg/ml	0.52	0.1 µg/ml	0.84
.098 µg/ml	0.23	0.2 µg/ml	0.78
.195 µg/ml	0.63	0.4 µg/ml	0.38
0.39 µg/ml	0.56	0.6 µg/ml	0.63
0.78 µg/ml	0.31	0.8 µg/ml	0.60
		1.0 µg/ml	0.63
		2.0 µg/ml	0.57
		4.0 µg/ml	0.56

Treatment of Cells for Mutagenicity

- Dosing ranges for mutagenic screen PreR and PostR (n=2)
  - DMSO (Neg. Control)
  - BaP (Pos. Control)
  - 0.2 µg/mL
  - 0.4 µg/mL
  - 0.6 µg/mL
  - 0.8 µg/mL
- Cultured on 10cm Plates
  - 300k cells/well
- 6-hr Treatment Time
- 72-hr Sampling Period

Detection of LacZ Mutants

- Determined using P-Gal positive selection assay
- *lacZ* transgene excised from treated DNA Samples
- Packaged into phage heads
- Absorbed by host bacterium *E.coli*
- Calculate mutant frequency (pfu)
  - Proportion of mutant plaques containing *lacZ* mutations to non-selective plaques



(Lambert, Singer, Boucher, & Douglas, 2005)

Are PreR and PostR Samples Mutagenic?

Sample ID:	Mutant Freq. (x10^5)	Sample ID:	Mutant Freq. (x10^5)
preR DMSO-1	70	postR DMSO-1	55
preR DMSO-2	111	postR DMSO-2	49
preR 0.1 µg/ml-1	61	postR 0.1 µg/ml-1	73
preR 0.1 µg/ml-2	59	postR 0.1 µg/ml-2	51
preR 0.2 µg/ml-1	57	postR 0.2 µg/ml-1	53
preR 0.2 µg/ml-2	53	postR 0.2 µg/ml-2	51
preR 0.4 µg/ml-1	95	postR 0.4 µg/ml-1	71
preR 0.4 µg/ml-2	77	postR 0.4 µg/ml-2	66
preR 0.8 µg/ml-1	92	postR 0.8 µg/ml-1	68
preR 0.8 µg/ml-2	67	postR0.8 µg/ml-2	77
BaP-1 (0.10 µg/ml)	**637**	**BaP-2 (0.10 µg/ml)**	**713**

- PreR and PostR samples Non-mutagenic
- BaP (Pos. Control) Mutagenic
- DMSO (Neg. Control) also Non-mutagenic

Evaluation of Additional Endpoints for Toxicity

- Conducted 48-hr exposure treatments of soil extract on FE1 cells

- Cytotoxicity of soil sample extracts on FE1 Lung Epithelial Cells
  - Lactate Dehydrogenase Assay representing plasma membrane damage

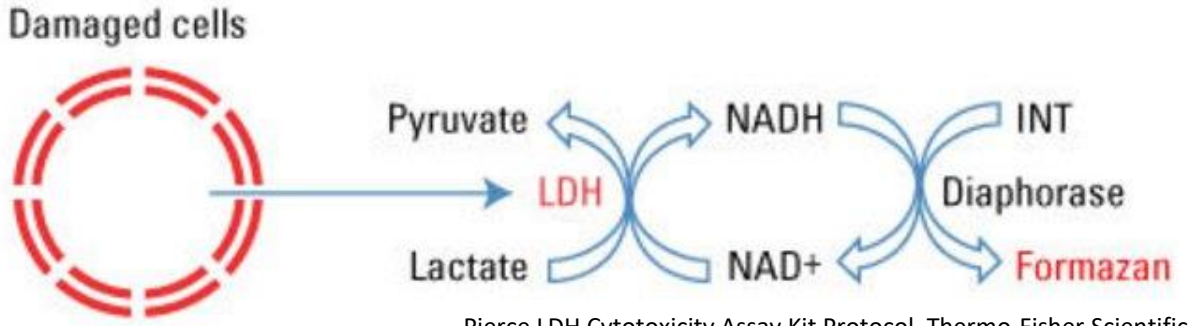
- Biomarkers of PAH Exposure and Toxicity through qPCR
  - Observing metabolism
  - Oxidative stress
  - DNA damage markers

Treatment of Cells for Biomarkers of Toxicity

- 6-Well plates
  - 170k cells/well
- Dosing-ranges (n=4)
  - NT (No Treatments) (n=2)
  - DMSO (Neg. Control)
  - 0.4 µg/mL
  - 0.8 µg/mL
  - 1.6 µg/mL
  - 3.2 µg/mL
- 48-hr Treatment times
- Media collected
- Cells harvested
  - 22 Samples Each (PreR and PostR)
  - Additional 16 samples for BaP

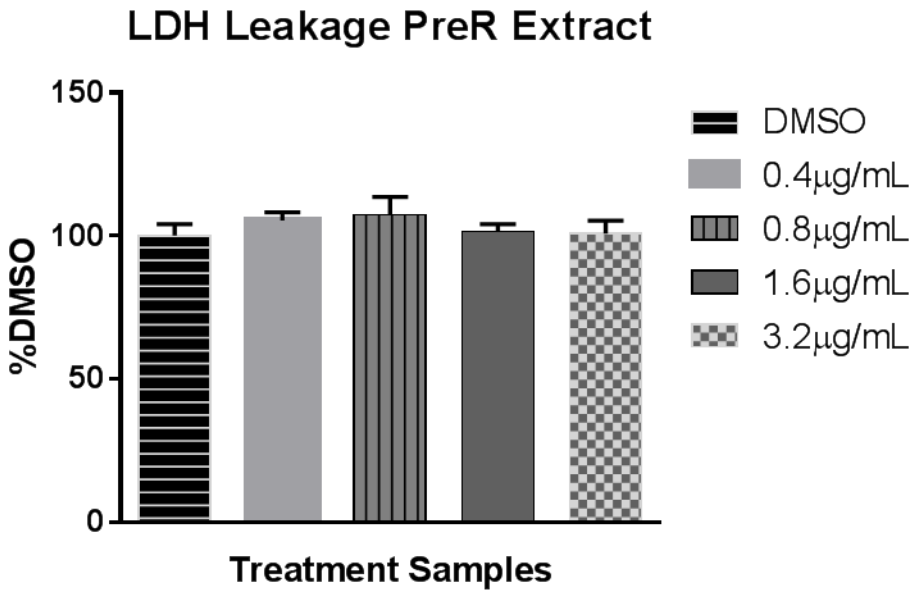
Cytotoxicity Assay

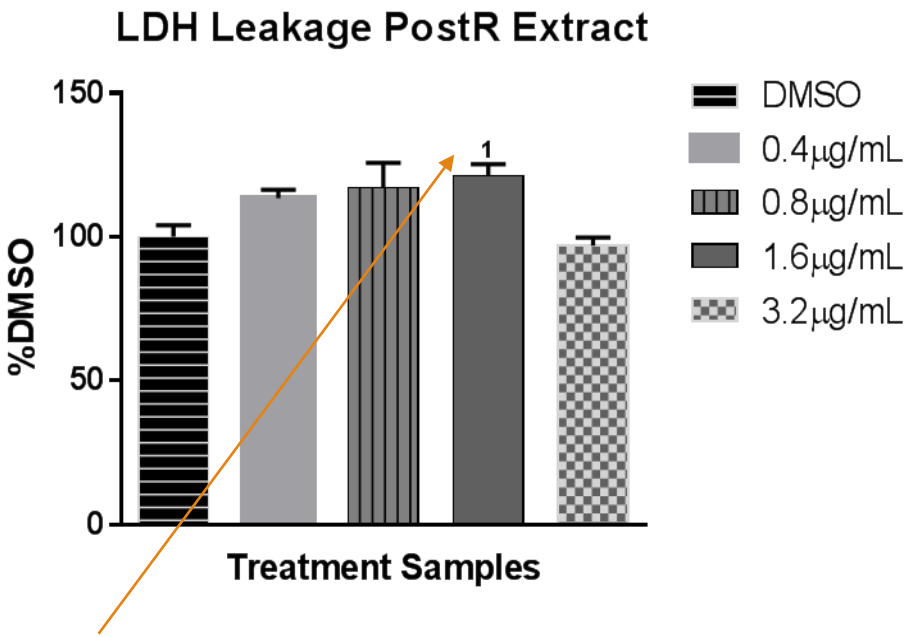
- Collected media from 48-hr exposure
- Max Level LDH (Pos. Control)
- 96-Well plate setup
- Absorbance measure
  - 490nm (Formazan)
  - 680nm (Background)
- LDH leakage into cell media when quantified gives an idea the cell damage



Pierce LDH Cytotoxicity Assay Kit Protocol, Thermo-Fisher Scientific

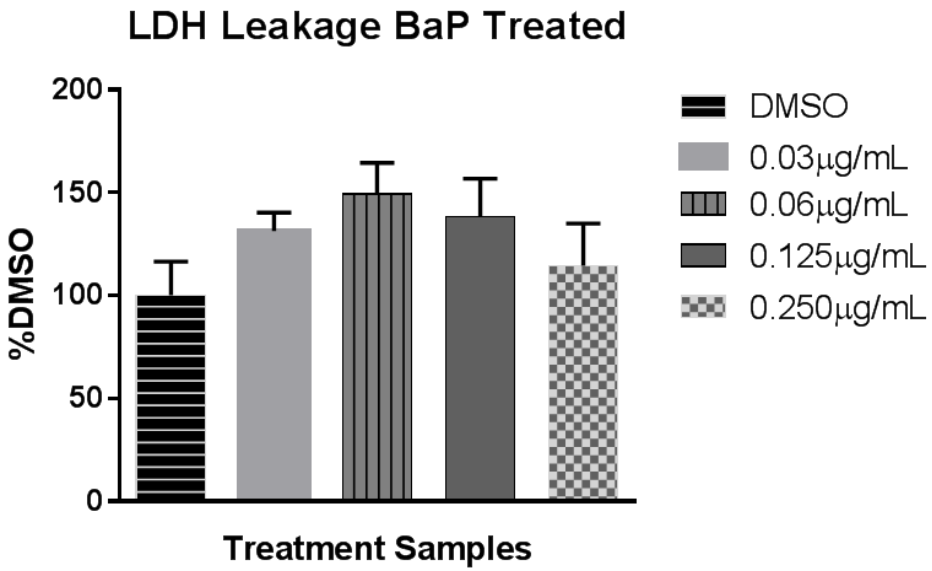
Cytotoxicity Assay Results

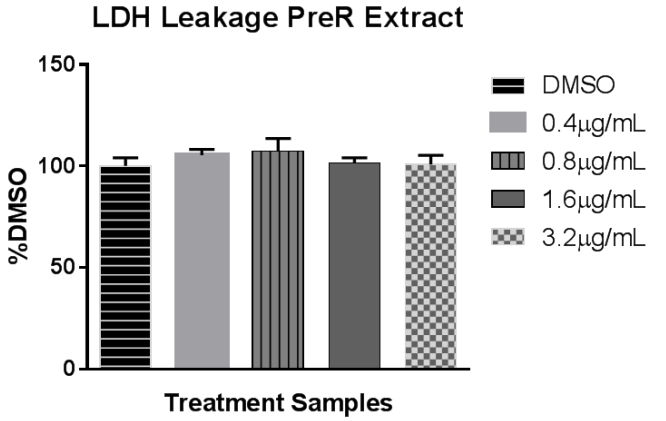


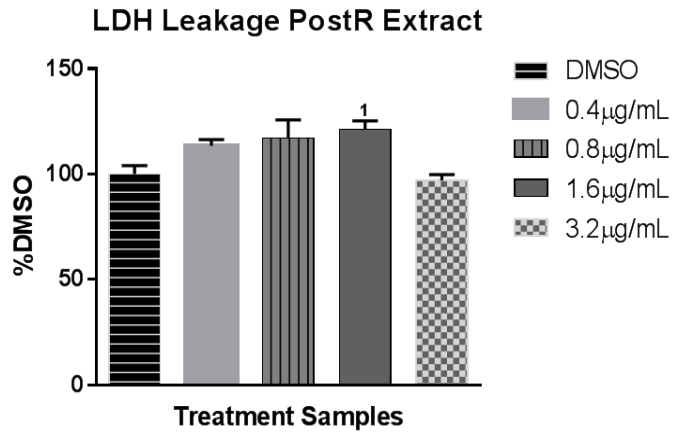


(1) Represents a P-value where, $p<0.05$

Cytotoxicity Results Compared to BaP







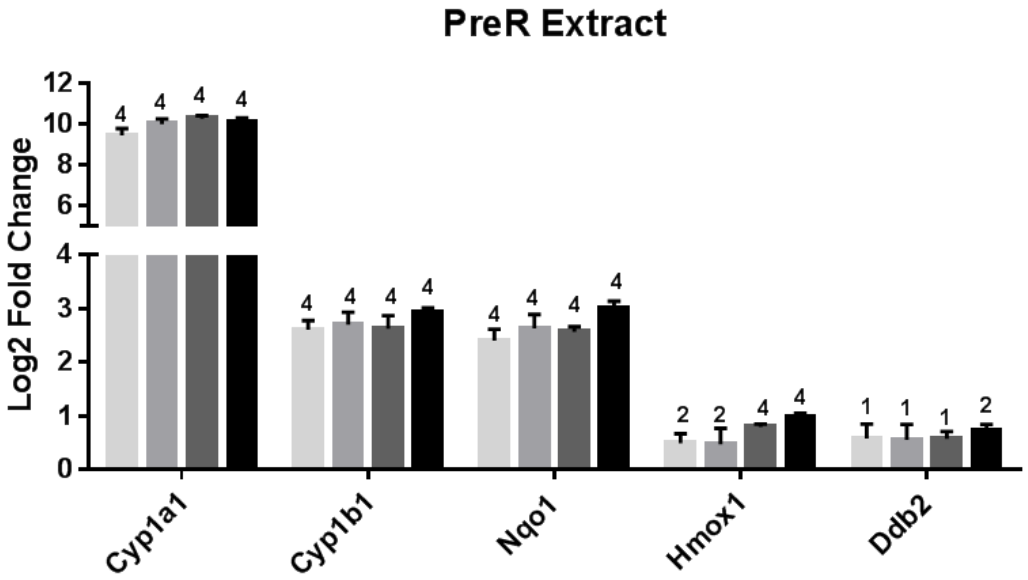
- Levels of LDH for **PreR** insignificant
- Levels of LDH for **PostR** significant for only one dosing range (1.6 µg/mL)
- Levels of LDH for **BaP** high, but not statistically significant

qPCR Prep

- 22 Samples Each (PreR) and (PostR)
- 16 Samples for BaP
- RNA Extraction
  - Using Qiagen RNeasy mini spin kit
- cDNA Processing
  - .25µg total RNA for 10µL Rxns
  - Dilution 1:10
- Gene expression was calculated using: ΔΔCt to determine Log2Fold Change

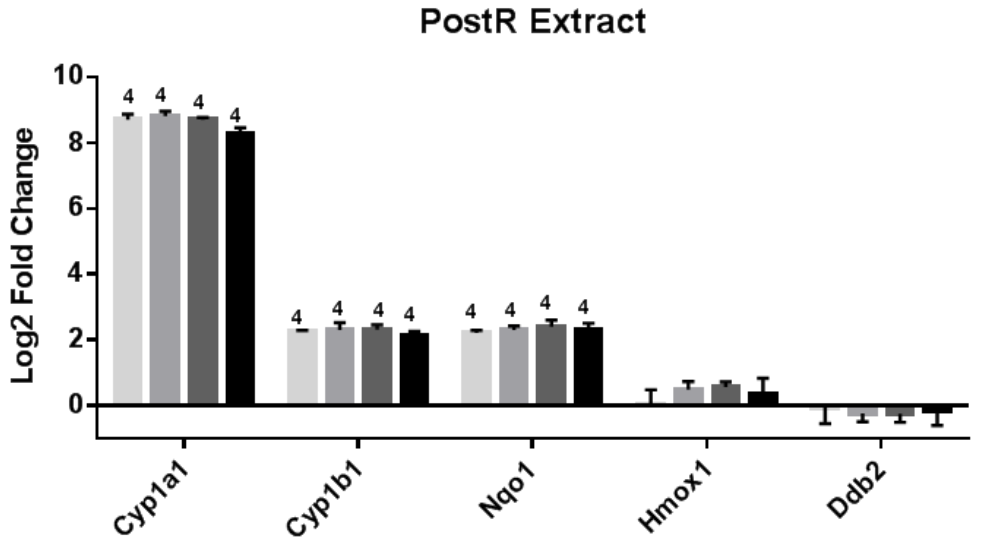
- Primers used for gene expression analysis
- Housing Keeping Gene
  - Gapdh
- Cytochrome P450 monoxygenases
  - Cyp1a1
  - Cyp1b1
- NAD(P)H quinone oxidoreductase
  - Nqo1
- Heme oxygenase
  - Hmox1
- DNA Damage-Binding Protein 2
  - Ddb2

Gene Expression Data



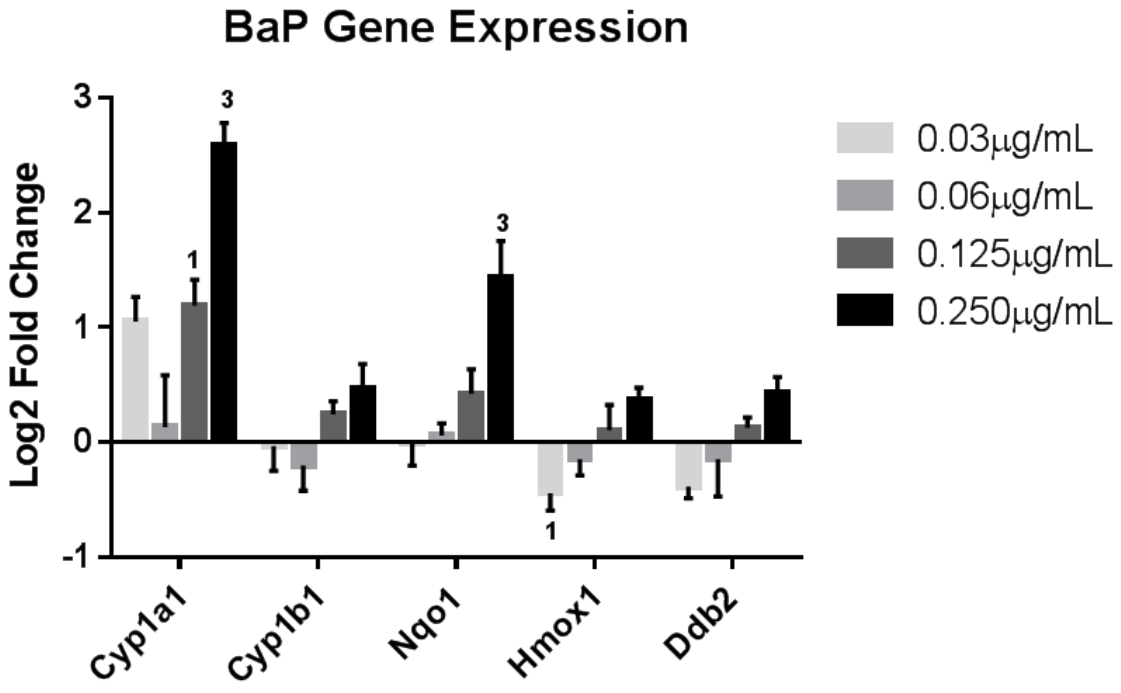
Pre 0.4µg/mL
Pre 0.8µg/mL
Pre 1.6µg/mL
Pre 3.2µg/mL

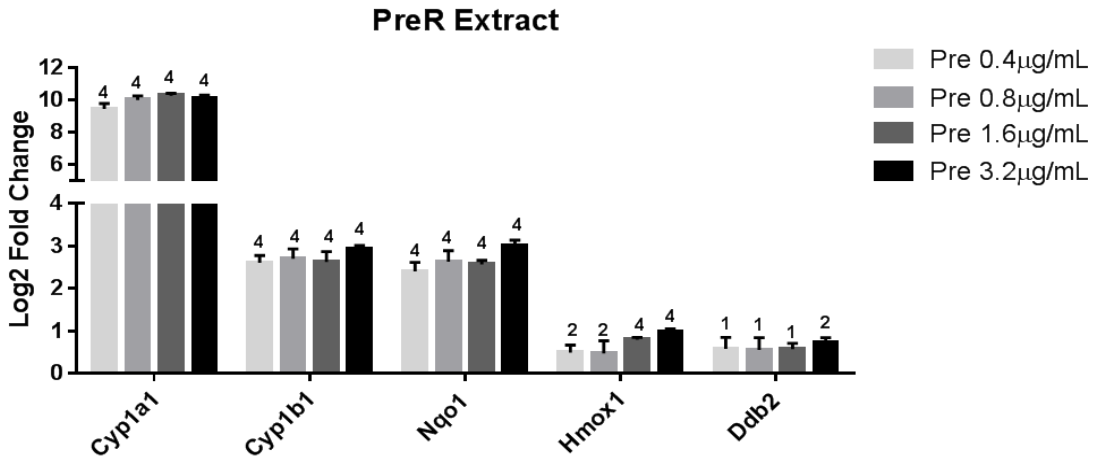
[1]p<0.05
[2]p<0.01
[3]p<0.001
[4]p<0.0001

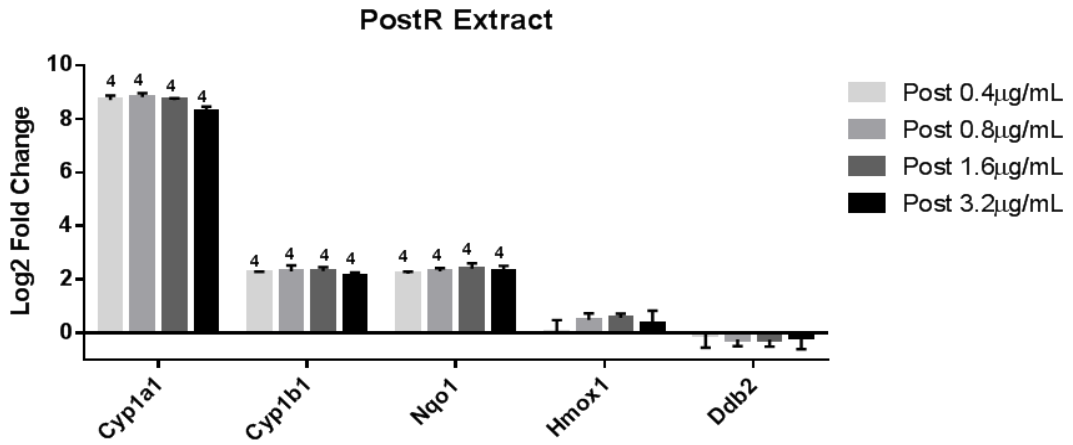


Post 0.4µg/mL
Post 0.8µg/mL
Post 1.6µg/mL
Post 3.2µg/mL

Gene Expression Compared to BaP







Conclusion

- Thermal remediation reduced quantities of known carcinogenic parent PAHs from soil, but not quantities of known carcinogenic high-molecular weight PAHs. It also increased formation of oxygenated PAHs (OPAHs) post-remediation (of unknown carcinogenic risk).
- Thermal remediation resulted in reduction of estimated carcinogenic risk of soils samples using the relative potency factor approach for postR compared to preR
- Observed mutation frequencies in FE1 cells were similar to control levels for whole extracts from soil preR and postR at tested concentrations
- Common biomarkers of PAH exposure (metabolic enzymes Cyp1A1, 1B1 and Nqo1) were significantly induced in FE1 cells by both preR and postR extracts at similar levels indicating continued exposure to PAHs after thermal remediation
- Biomarkers for oxidative stress (Hmox1) and DNA damage (Ddb2) were significantly induced by extracts preR and not postR indicating potential reduction in toxicity by these mechanisms after thermal remediation

Future Studies

- **What can we do in the future to better conclude PAH mixture toxicity?**
- Repeat mutagenicity assay in transgenic FE1 cells
  - Advised by Health Canada to reduce mixture complexity
  - Difficulty in dose determination of full mixtures
  - Instead attempt fractionation of soil samples could give more consistent RICC trends (or create synthetic mixtures of PAHs found in soil)
- Potentially compare outcomes to other assays for genotoxicity (e.g. Ames assay)
- Expand BaP dose-response for gene expression studies to more directly compare to biomarkers by soil samples
  - Test higher BaP concentrations that overlap with dosing of soil samples based on calculation of BaP equivalents to better compare potential mechanisms of toxicity

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