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Toxicity Evaluation and Biomarker Identification in Rats Exposed to Burn Pit Emissions and/or Respirable Sand from Iraq

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The views expressed are those of the authors and do not reflect the official guidance or position of the United States Government, the Department of Defense or of the United States Air Force. As such, this presentation focuses on molecular discovery and future utility only, and any discussion does not reflect official Air Force, Navy, or DOD policies.

The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the *“Guide for the Care and Use of Laboratory Animals,”* Institute of Laboratory Animal Research, National Research Council, National Academies Press, 2011.



- **2.5 million military personnel have served in Southwest Asia (SWA) since 2002 as part of the Iraq and Afghanistan conflicts.**

Many returned with COPD, emphysema, and asthma, and later developed other diseases including cancers, interstitial lung disease, bronchitis, and pulmonary fibrosis.

- **In 2010**

Institute of Medicine committee examined the human health risks from burn pits.

- Recommended epidemiological studies of active duty and veterans to assess potential health effects related to burn pit emissions.
- Also recommended an examination of the potential adverse health effects resulting from mixed exposures, including exposure to respirable dust and combustion products.

- **In 2013**

VA established the Airborne Hazards and Open Burn Pit (AH&OBP) Registry.

- **Efforts to link burn pit exposure to adverse outcomes**

Epidemiology studies conducted, but limited ability to associate environmental exposures with specific adverse effects due to limited exposure or locale data.



Could Biomarkers of Exposure or Biomarkers of Effect be found?



Human Studies



Pro:

- Molecular responses are species appropriate
- Real Exposures

Con:

- Unknown and uncharacterized exposures, unknown doses
- No control, many variables
- Difficulty of sample collection in theater
- Later samplings miss early responses
- Linking adverse outcomes to exposure difficult

Modeling Burn Pit Exposures

How?

In vitro Modeling

Pro:

- Can run multiple exposures using human cell lines
- Relatively inexpensive
- Tightly controlled – limited variables

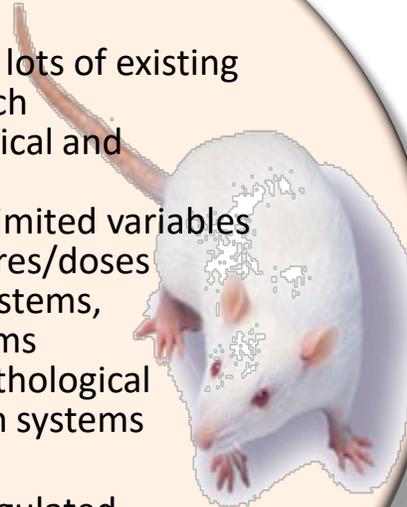
Con:

- Transformed cell lines may not reflect mechanisms
- Primary cells can be difficult to grow, expensive
- Cells may not grow in presence of toxin
- Misses organ-organ or organ-microbiome interactions.
- How do you model Burn pit emissions?

In vivo Modeling

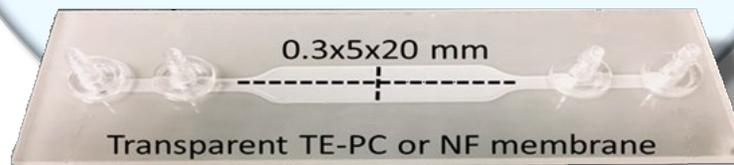
Pro:

- Murine models have lots of existing data for toxin research
- Well developed physical and cognitive tests
- Tightly controlled – limited variables
- Well defined exposures/doses
- Very similar organ systems, molecular mechanisms
- Can conduct histopathological examination of organ systems



Con:

- Expensive, strictly regulated
- Murine models tend to be more resistant to toxins
- How do you model Burn pit emissions?





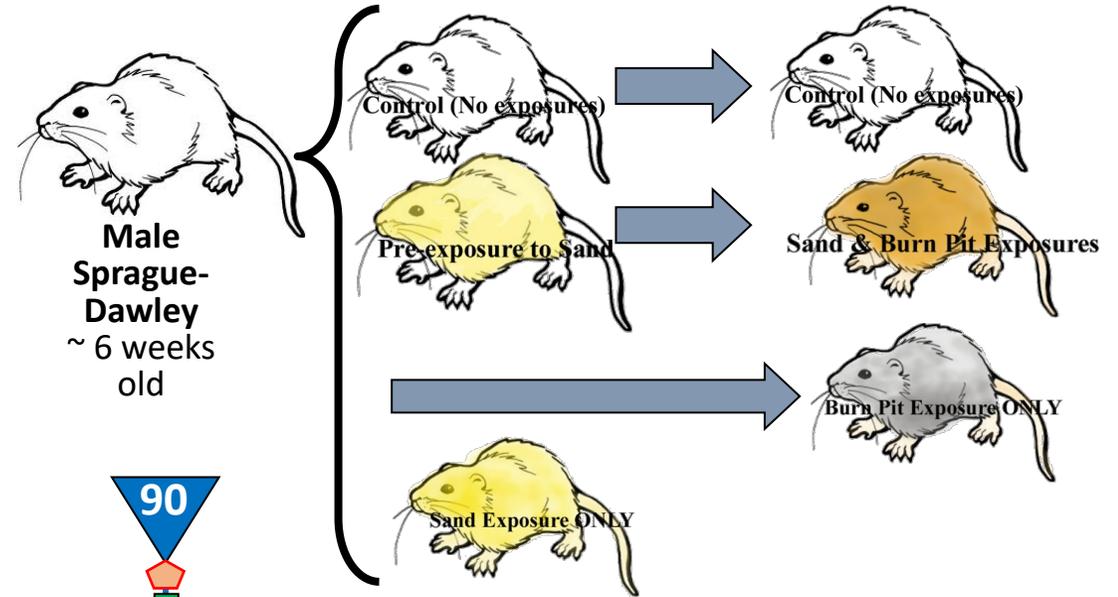
**Toxicity Evaluation and Biomarker
Identification in Rats (*Rattus norvegicus*)
Exposed to Burn Pit Emissions and
Respirable South West Asian (SWA)
Particulate Matter**



Animal Study Design

- Joint Air Force Research Laboratory/Naval Medical Research Unit Dayton Project
- Funded through Military Operational Medicine Joint Program committee (JPC-5)

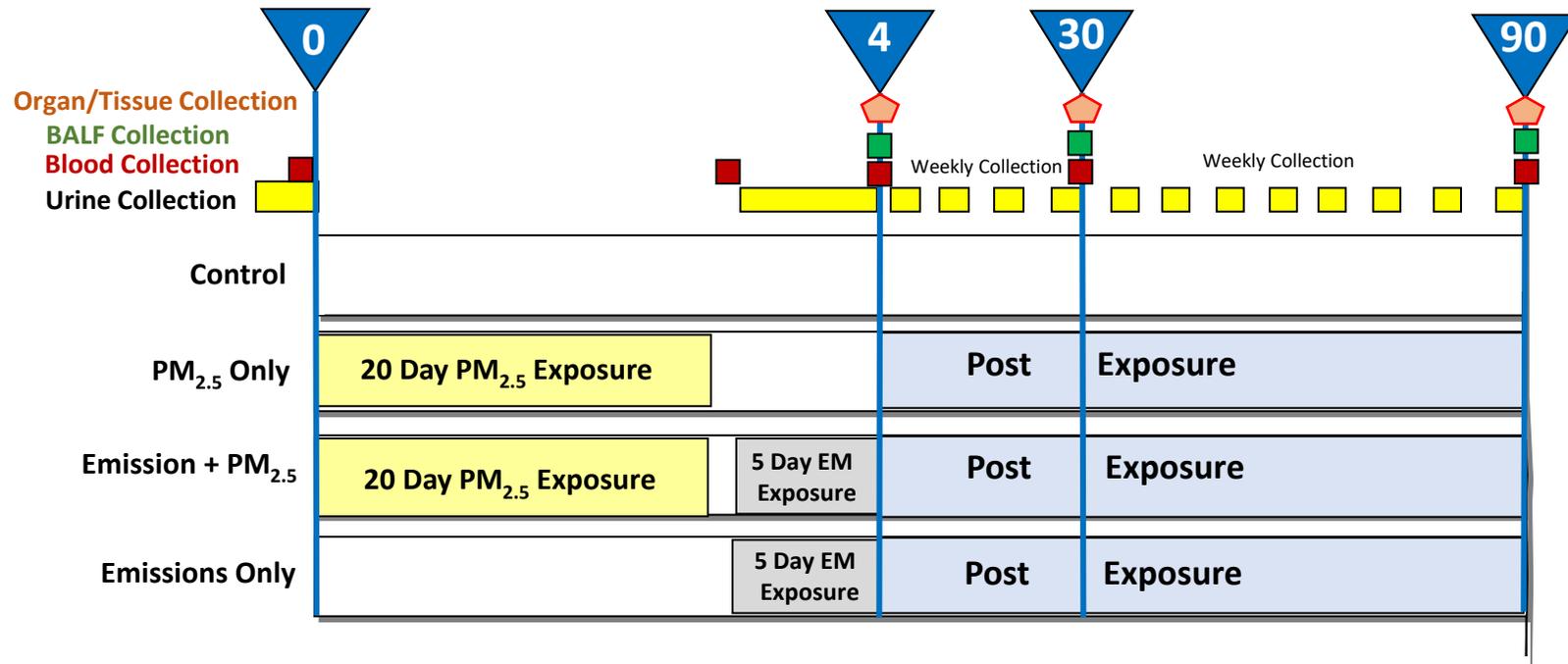
▼ = Days Post Exposure. Tests to include ventilation function tests, histopathology, clinical tests on animal subgroups.



▪ **Four Groups:**

- Control
- Sand only
- Emissions Only
- 'Sand + Emissions'

- **Animals acclimatized to exposure cage units to minimize stress.**
- **Controls handled exactly same as exposed groups.**

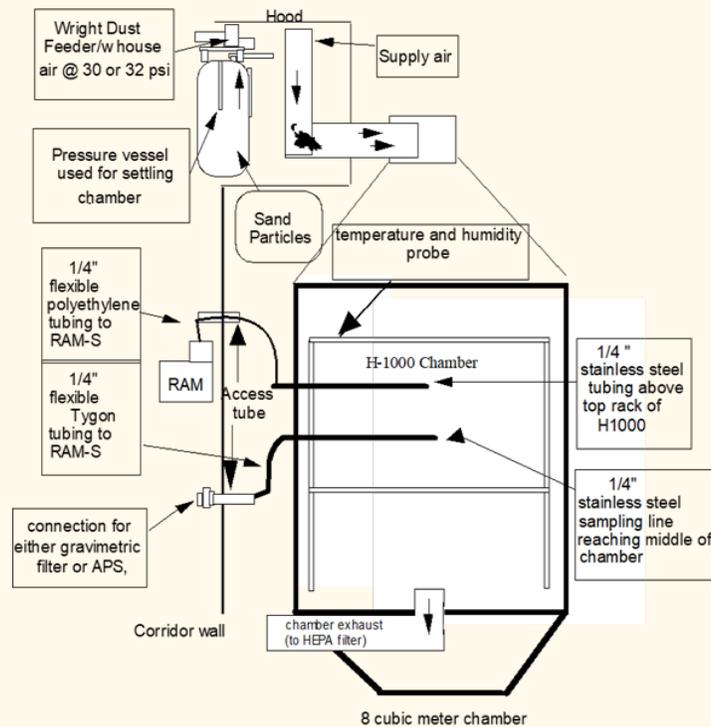




Inhalation Exposure Methodology

Sand Particulate Matter Exposure

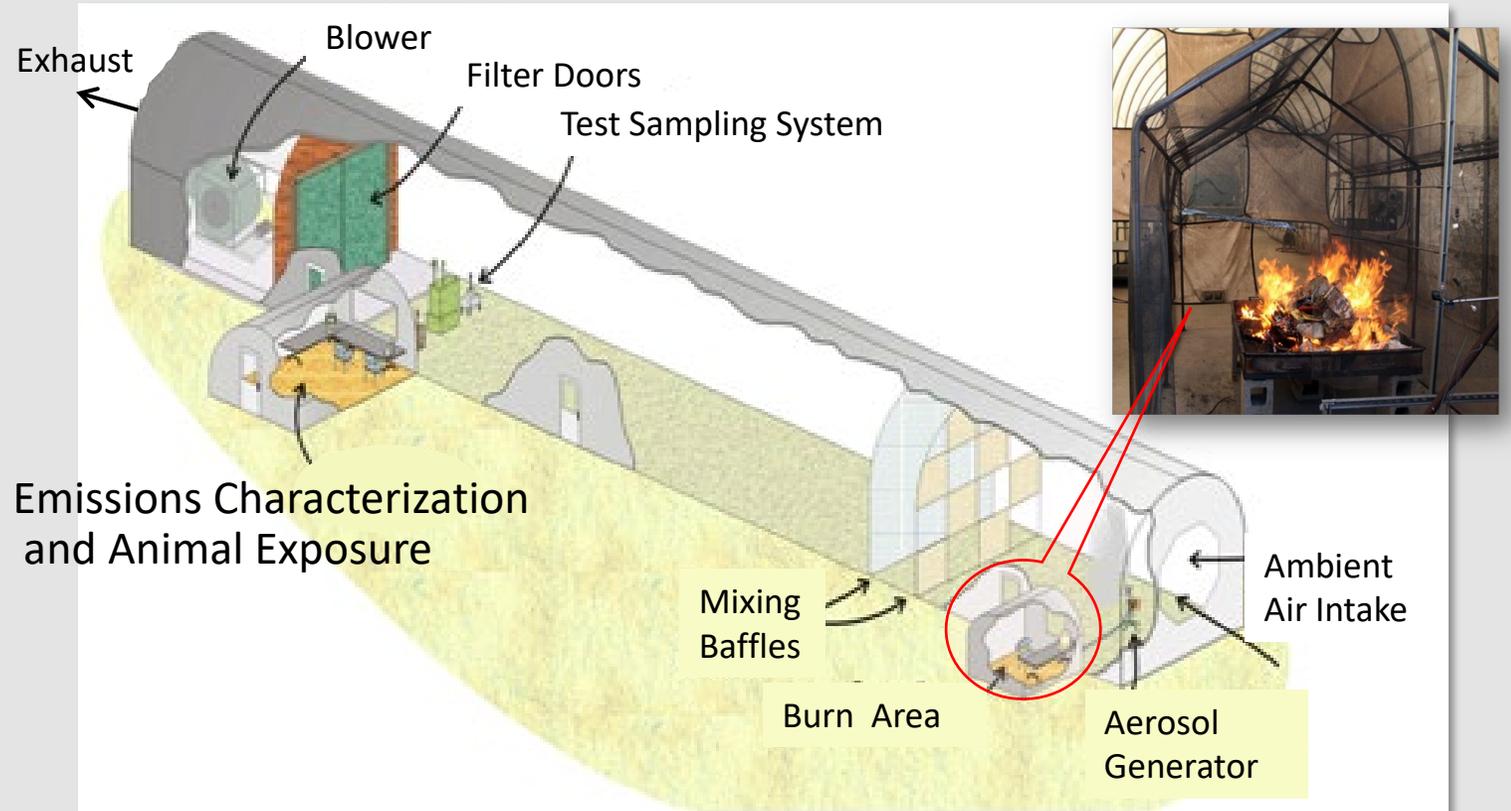
Whole Body Exposure Chamber with Wright Dust Feeder apparatus



- SWA PM Samples taken by US Army Corps of Engineers
 - Camp Slayer (in Camp Victory), Iraq
 - Soil sample from undisturbed area
- Samples prepared for NAMRU-D
 - 2 kg sieved material, autoclaved

Simulated Burn Pit Emissions Exposure

Simulated open air combustion using Ambient Breeze Tunnel (Battelle, W. Jefferson, OH).



Emissions Characterization and Animal Exposure

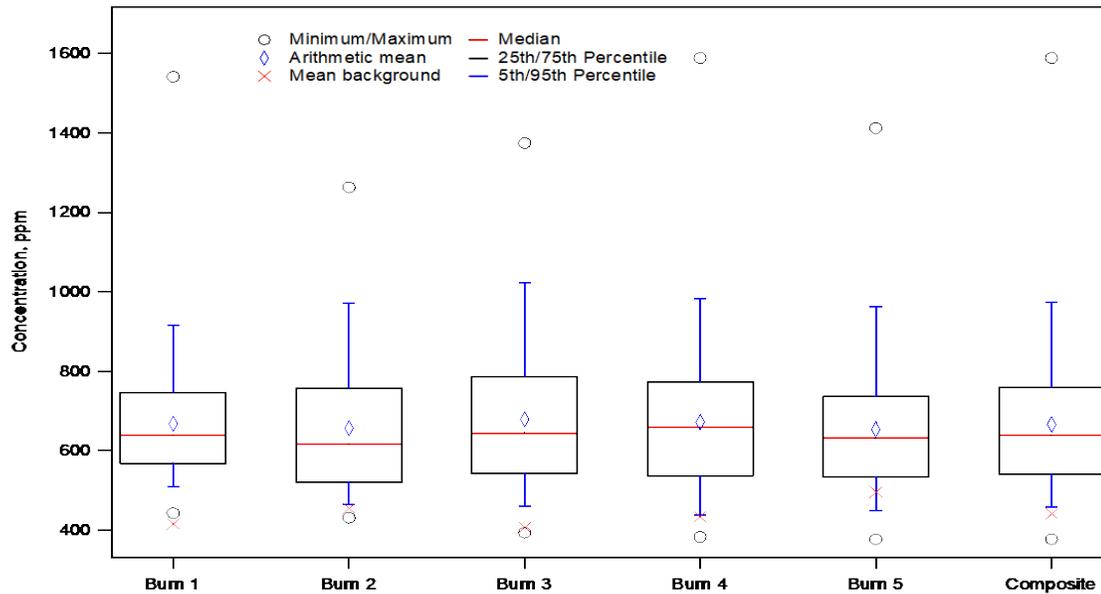
- Waste materials burned reflect solid waste combusted in-theater as based on U.S. Army Central (USARCENT) Area of Responsibility (AOR) contingency Base Waste Stream Analysis (CBWSA). U.S. Army Logistics Innovation Agency (USALIA) 2013 report.
- Burned waste included cardboard, food waste, mixed paper, non-combustibles, plastics, textiles, wood, and miscellaneous wastes.



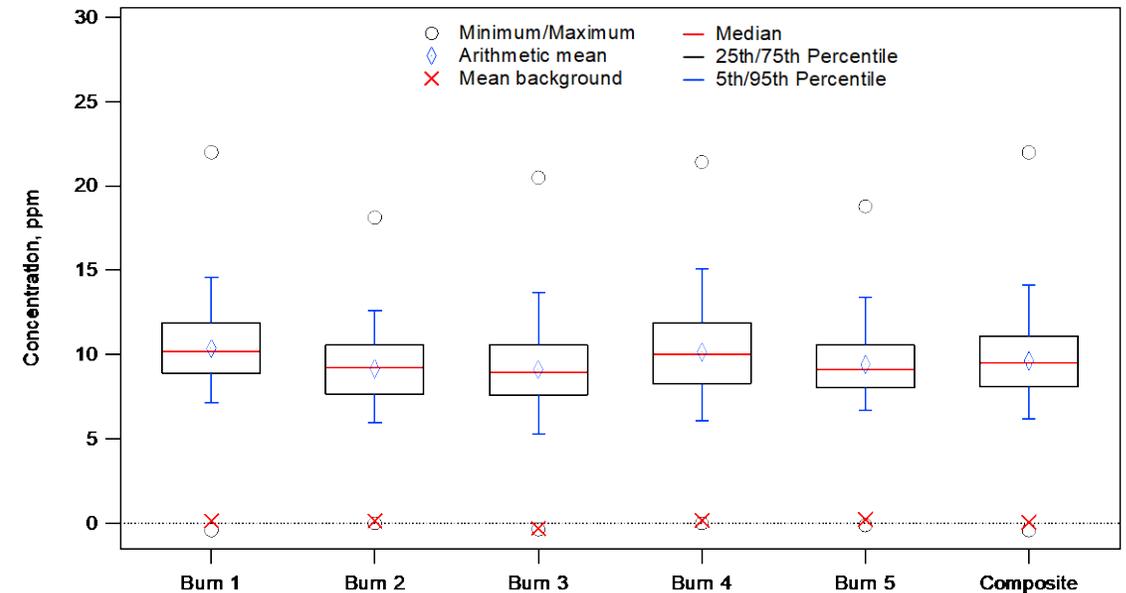
VOC Concentrations: ($\mu\text{g}/\text{m}^3$) of four carbonyl compounds

Compound	Burn 1	Burn 2	Burn 3	Burn 4	Burn 5	Observed Background Concentration	Mean	Standard Deviation
Formaldehyde	1.6	29	35	49	29	7.1	32	12
Acetaldehyde	6.5	4.1	5.3	5.8	5.8	2.8	5.5	0.9
Propionaldehyde	3.4	2.1	2.3	3.0	<0.4	0.9	2.2	1.1
Benzaldehyde	10	8.5	4.6	12	17	0.7	10	4.5

Carbon Dioxide Levels per Burn Day



Carbon Monoxide Levels per Burn Day





Lung Histopathology

- **All Groups:**
 - Minimal to mild changes seen in lung
 - Neutrophilic infiltration in all exposure groups, but more frequent in Sand and Burn Pit Emissions Exposure Groups. However, very low and may not be clinically insignificant.
- **Sand Exposure Group: Foreign matter found in lung**
- **Emissions and 'Emissions + Sand' Exposure Groups:**
 - Low amounts of hemorrhage associated with burn pit exposure found early post exposure
 - Hemorrhage is minor and significance was undetermined

Other Data

- **Body Weight differences:**
 - No significant differences seen
- **BALF total cell count and differential:**
 - No significant differences seen
- **BALF LDH and total protein:**
 - No significant differences seen
- **BALF TNF- α :**
 - Day 4 elevated in *Sand* group as well as '*Sand+Emissions*' group
- **Clinical Chemistry, Hematology:**
 - No biological significance to scattered parameters showing statistical significance
- **Resting Respiratory Physiology:**
 - Tidal Volume and Breathing Frequency have exposure related trends
- **Elemental Measurements in Lung and Brain**
 - No suggestions of transport from lung to brain





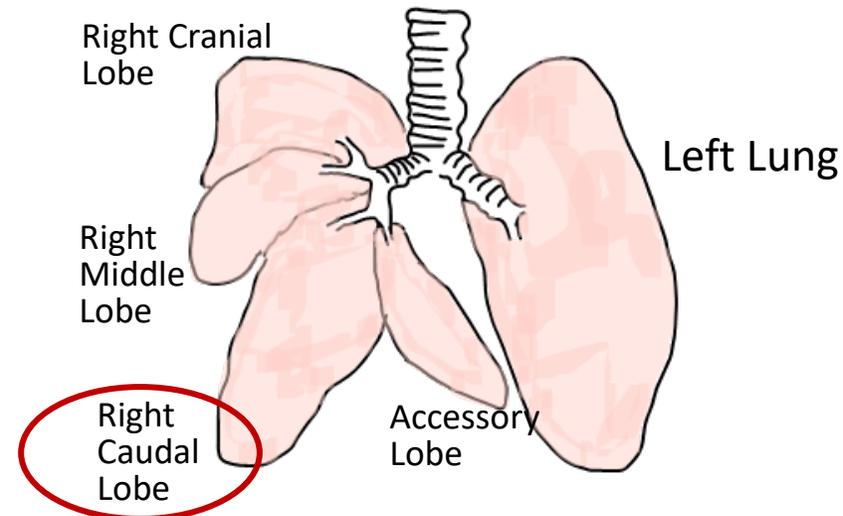
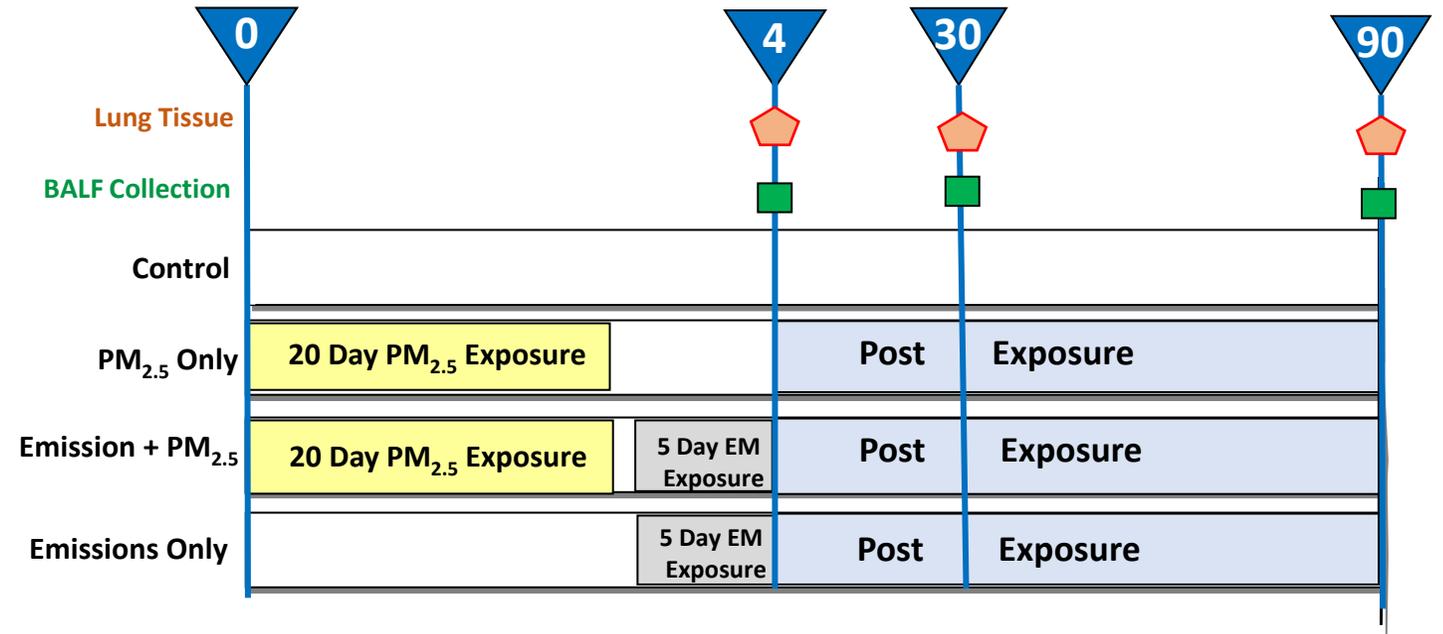
Epigenetics:

miRNA Analysis in Blood and Lung Tissue



miRNA Analysis

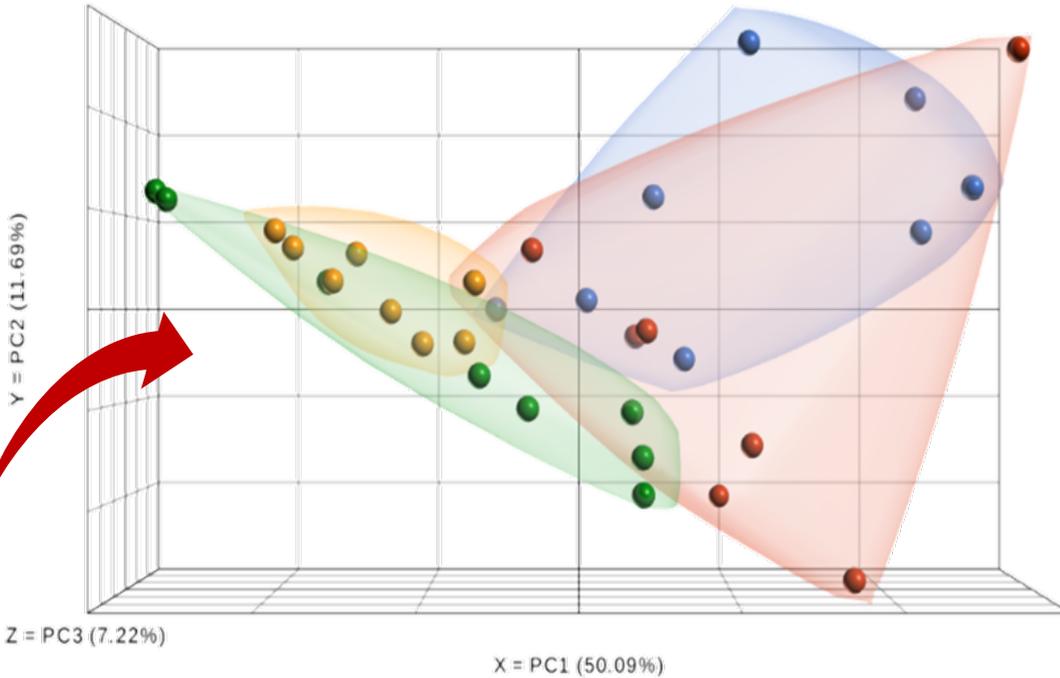
- Blood collected at terminal euthanasia by cardiac puncture into RNAprotect[®] Animal Blood Tubes (Qiagen)
- Tissues collected at terminal euthanasia at 4, 30, 90 day post exposure from Right caudal lung lobe
- RNA was extracted
 - From blood (RNAeasy MinElute)
 - From lung (Rneasy Mini Kit, Qiagen)
- Both Affymetrix Genechip miRNA 3.0 Arrays and Sequencing based methods utilized
- Identified differentially expressed miRNAs
 - 2 fold or greater change in expression
 - $p=0.05$
- Examine pathway alterations using Ingenuity Pathway Analysis



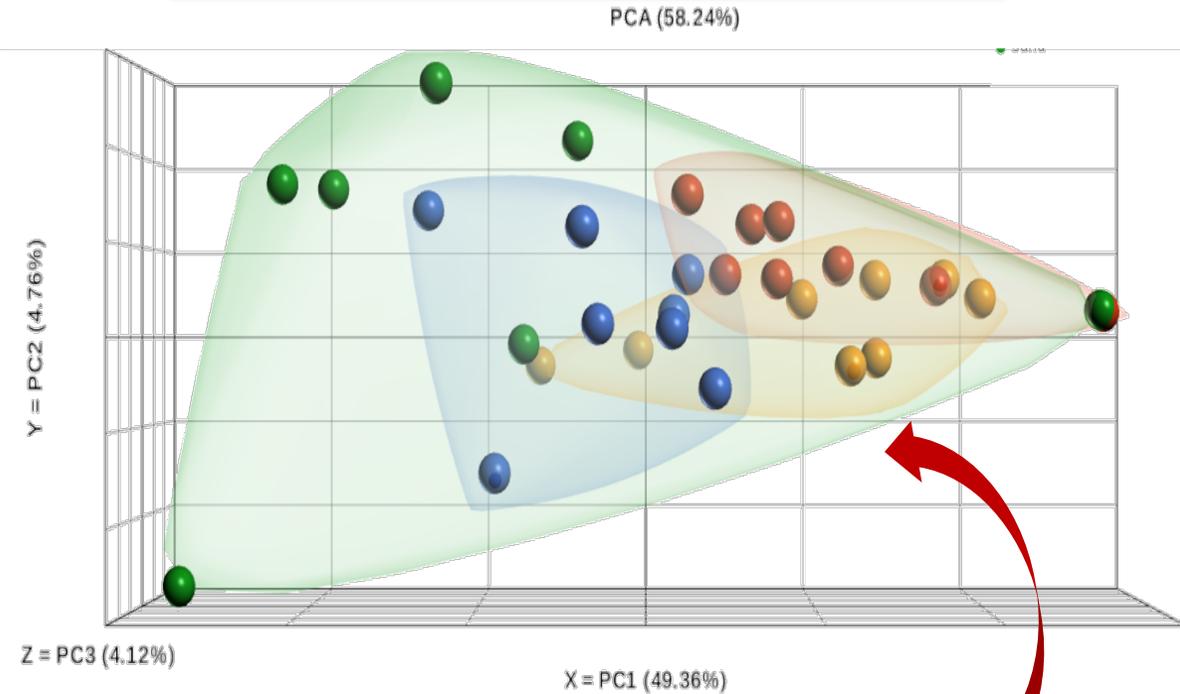
miRNA Sequence-based Discovery

PCA Plots

4 Day Post-Exposure, Lung Tissue



90 Day Post-Exposure, Lung Tissue



- Exposure-specific Distinct Grouping
- Burn Pit Emission impact > Sand
- Sand ≈ Controls

- Sand Alterations 90 day > 4 day

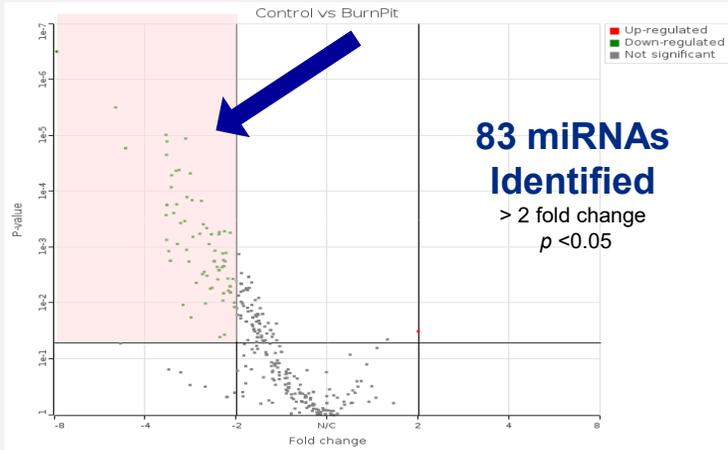




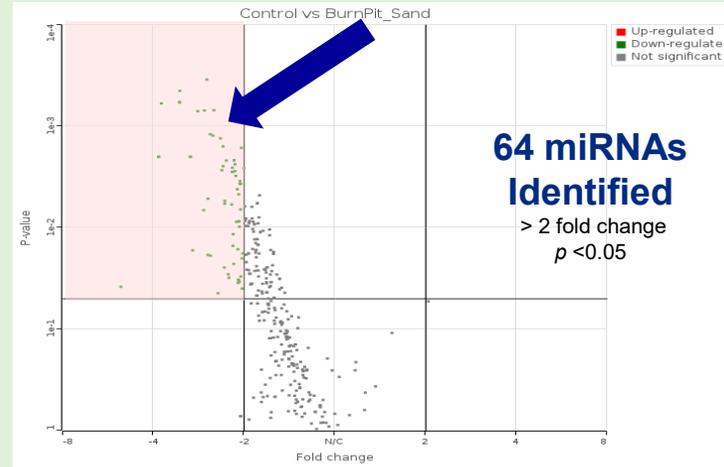
miRNA Expression

4 Day Post Exposure, Lung Tissue

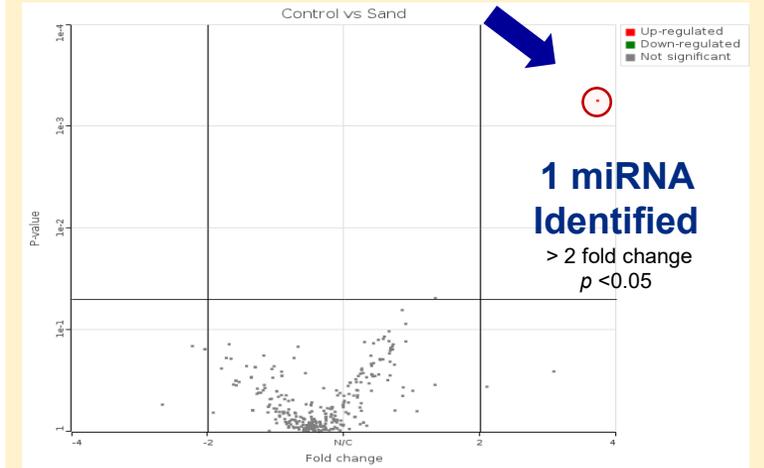
Burn Pit Emissions miRNA vs. Control



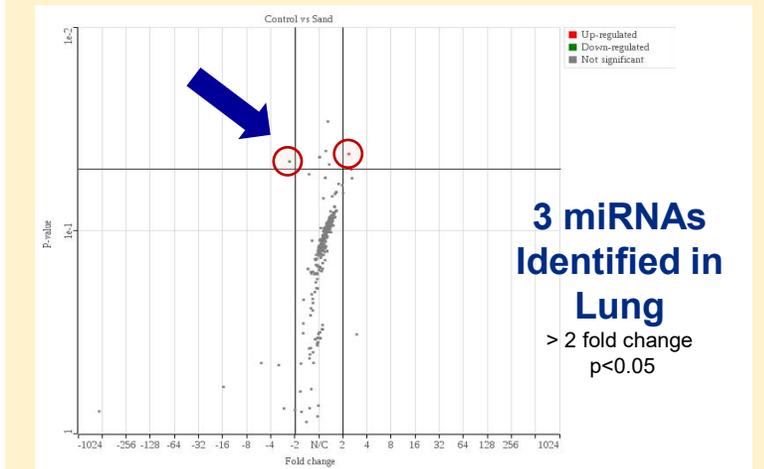
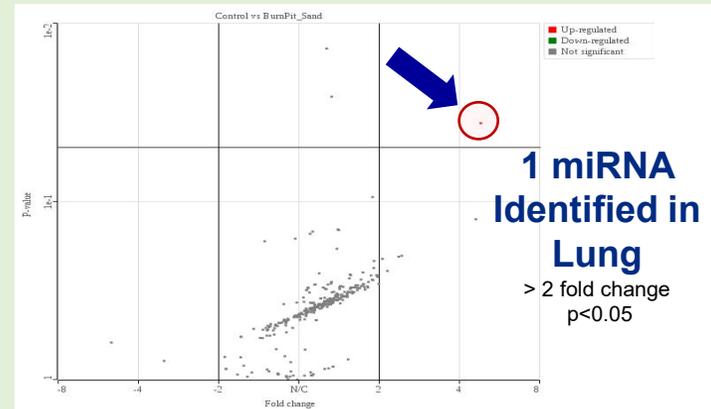
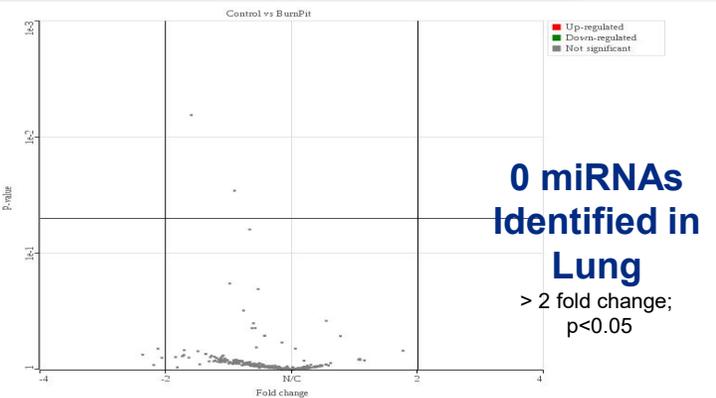
Burn Pit Emissions + Sand miRNA vs. Control



Sand miRNA vs. Control



90 Day Post Exposure, Lung Tissue





Conclusions

- **Burn pit emission exposures *strongly initiated* molecular host responses when compared to Sand inhalation exposures**
 - 5 days of emissions exposure impact >> than 4 weeks of sand exposure
 - Acute, and likely chronic, exposures initiate strong host response
- **Sand exposure response stronger at >90 days**
 - Host response to Emissions exposure relatively fast, whereas host response to sand inhalation is slower
 - Chronic exposure more likely responsive than acute exposures
- **Epigenetic data did support any additive effect of Sand plus burn emission exposures**
- **Sets of differentially-expressed miRNAs identified from Sequence Discovery in lung tissue**
- **Sequencing and Affymetrix Discovery completed**
 - Four species were found to decrease in expression by our criteria in both data sets: miR-92a-1-5p, miR-221-star, rno-miR-181c-3p, and rno-miR-93-3p/
 - miR-92a-1-5p, miR-221-star are seen in pathways leading to lung cancer or disease
 - Etiology not clear





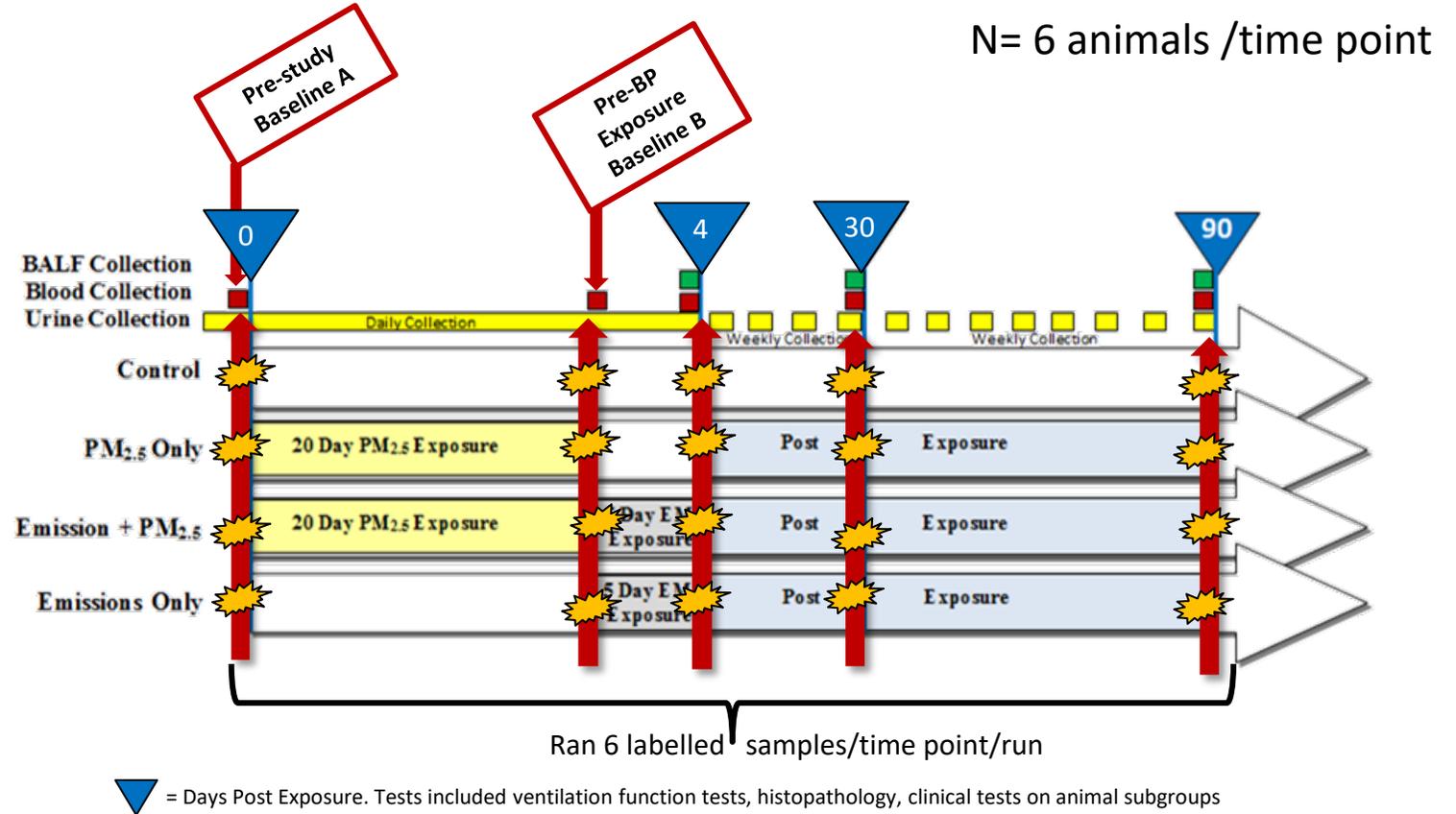
Proteomics:

iTRAQ Analysis of Blood



iTRAQ LC/MS Analysis

- **Plasma sample preparation:**
 - IgY immune-depleted
 - TMT6 labelled, then Trypsin digested
 - SCX spin column separation into fractions
- **Mass Spectrometry**
 - Reverse phase nanoAcquity UPLC-LTQ Orbitrap Velos mass spectrometer (MS)
- **MS/MS data Analysis**
 - SEQUEST algorithm in the Proteome Discoverer 2.2
 - Combine CID and HCD spectra prior to searching human, mouse, and rat proteins from the non-redundant NCBI protein database.
 - In-house developed Matlab-based program
 - To be included in the Final summary, the protein had to be identified in at least 3 of the 4 replicates and had to occur across all reporter ions within each exposure group



Baseline A data was normalized to blood collected from the same animal prior to study initiation

Baseline B data was normalized to blood collected from the same animal prior to burn pit emissions exposure



Lon protease homolog, mitochondrial (LonP1)

- Changes in 'Sand+Emissions' exposure group, increases at 4 day post exposure, then drops at 30 day post exposure, dropping further at 90 day
- Significant increases (1110%) but some due to aging
- Normalization to the pre-emissions exposure (*Baseline B*), still exhibits increases >455%
- LONP1 triggered by inflammation or oxidative stress
 - removes denatured proteins in mitochondria to attenuate cellular apoptosis
- Increases are also seen in asbestos exposures
- See *Annu Rev Pathol* 2013 8:161

Gelsolin

- Changes in Emissions and 'Sand+Emissions' exposure groups
- Ave 15% increase in 3 time points (*Emissions* Group), but 93% increase 4 day post exposure, down to 17% increase at 30 day post exposure (*Baseline B*)
- Sand exposure exacerbate response?
- Proposed biomarker to inflammation thought to scavenge actin from damaged tissue
- Hypogelsolinemia in response to many types of injury, precedes a second wave of organ injury
- See *Arthritis Res Ther* 10:124 (2008). <https://doi.org/10.1186/ar2547>



Data suggest that LonP1 and Gelsolin may not only be appropriate blood biomarkers for inhalation exposure but may also suggest a possible mechanism for disease progression.

Hypothesis that Gulf War Syndrome-based 'combination exposures' may initiate ROS buildup and disruptions in the mitochondrial respiratory chain, initiating cellular disruption.

Chen et al. (*PLoS One* 2017 12(9): e0184832) and Koslik et al. (*PLoS One*. 2014 9(3): e92887)

Need to use Secondary Analytic Method ←



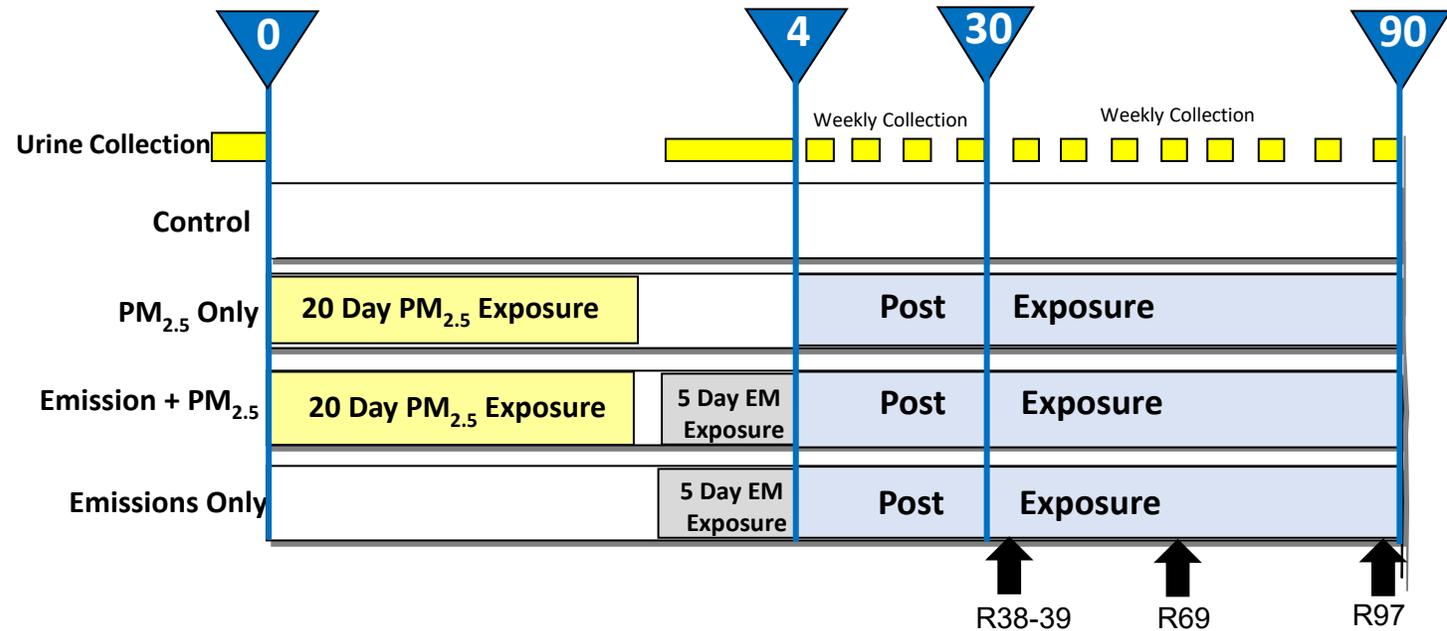
Metabolomics:

NMR Metabolomic Analysis of Urine



NMR Metabolomics

- Metabolism cages used to collect urine when not being exposed
- Conducted NMR Metabolomics using 600 MHz Varian Inova 600 NMR Spectrometer
- NMR spectra acquired at 600 MHz at 25 °C using a pulse sequence designed to suppress the large resonance from water.
 - Water suppression achieved using the first increment of a NOESY pulse sequence
- Multivariate data analyses were conducted on binned, scaled spectral data using MATLAB software
 - Principal Component Analysis (PCA) – unsupervised
 - Orthogonal Projection onto Latent Structures - Discriminant Analysis (OPLS-DA) - supervised

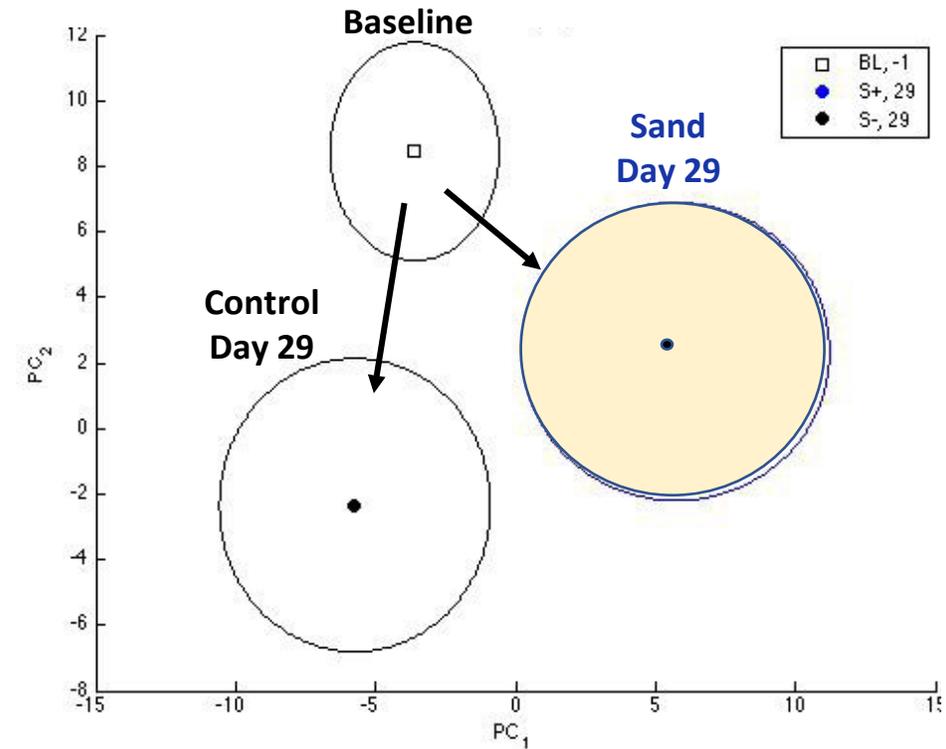


Urine Samples Analyzed

BL	Baseline	Combines days (-2) + (-1)
S10-17	Sand	Combines days 10 + 17
S29-33	Sand	Combines days 29 + 33
E34-35	Emissions	Combines days 34 + 35
E36-37	Emissions	Combines days 36 + 37
R38-39	Post exposure	Combines days 38 + 39
R69	Post exposure	Day 69
R97	Post exposure	Day 97



- **Large effect over time, likely due to stress, found in data sets**
- **Exposure differences found using a ‘paired analysis’**
 - emphasizes the change in metabolite profile within each animal referenced to a specific time point
 - helps suppress the changes due to ‘time’ (or stress) since it considers the change for each animal from one time point to another
- **Greatest difference in urine metabolite profiles occurred during the exposure timeframe day 1-38 and the least during the recovery period day 39-97.**
- **Recovery is not observed.**
 - Large spread in data points during the recovery period but remain clustered in data seen immediately following exposures (Day 36-37).
 - Data *do not* return to the baseline plotting region in exposure groups
- **Data reported in “Burn Pit Emission and Respirable Sand Exposures in Rats: NMR-Based Urinary Metabolomic Assessment” Nicholas DelRaso et al. Technical Report 2018 Accession No. AD1064148 found at <https://apps.dtic.mil/sti/citations/AD1064148>**





Microbiomics: ***Microbiome Analysis of Lung*** ***Lavage***



Bacterial Identification

Sample Preparation

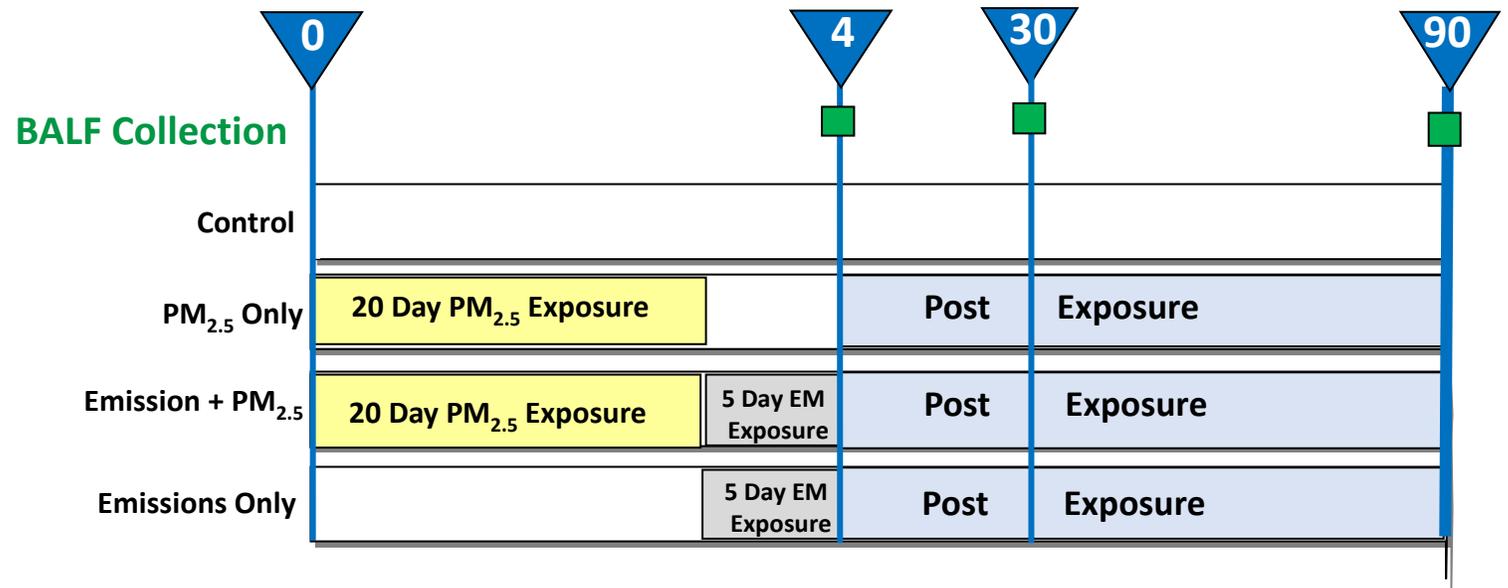
- Bronchoalveolar lavage was removed from the left lung
- DNA from the BALF pellets extracted

16S rRNA Sequencing

- Bacterial 16S hypervariable regions amplified
- Ion S5 System (ThermoFisher) with the Ion 520 & 530 Kit-Chef and Ion 530 Chip Kit

Data Analysis

- QIIME was used for identification of operational taxonomy units (OTUs) using the 16S rRNA region of DNA.
- These regions were matched to the rRNA database Greengenes
- OTUs were picked by open-reference method

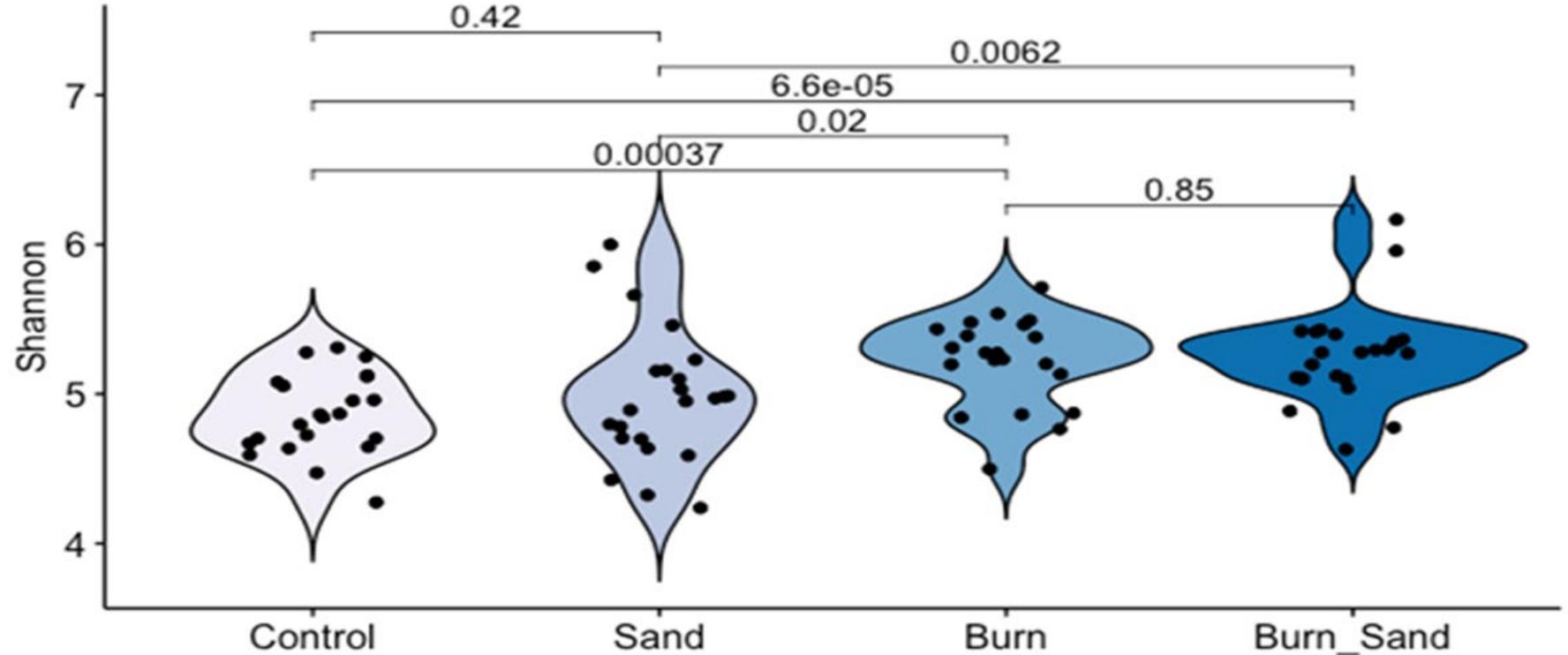




Alpha Diversity:

How *many* different species could be detected in each group?

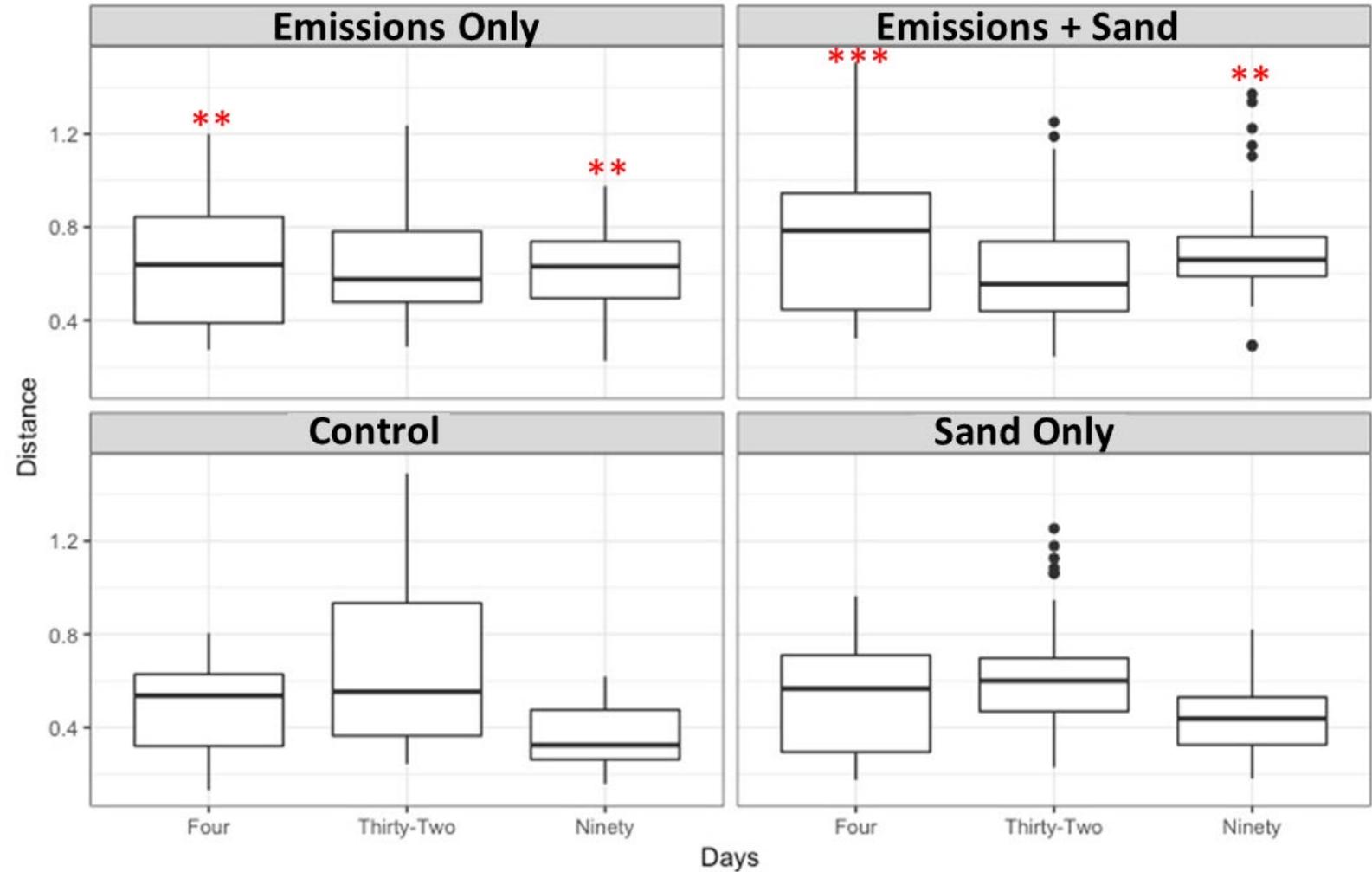
Comparisons Based on Exposure Type





Beta Diversity:

How *different* is the microbial composition in one group compared to another?

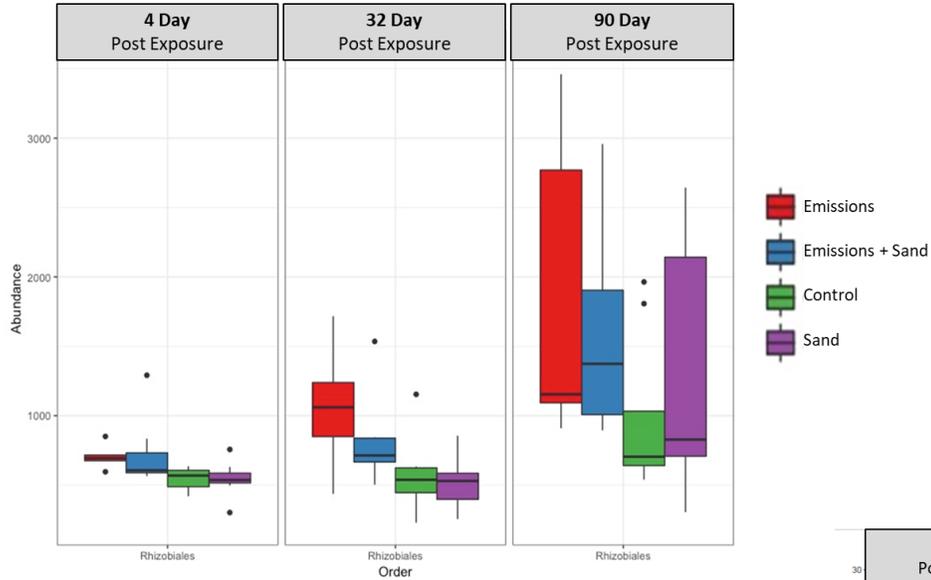


Statistically significant alterations compared to Control (PERMANOVA) = *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$



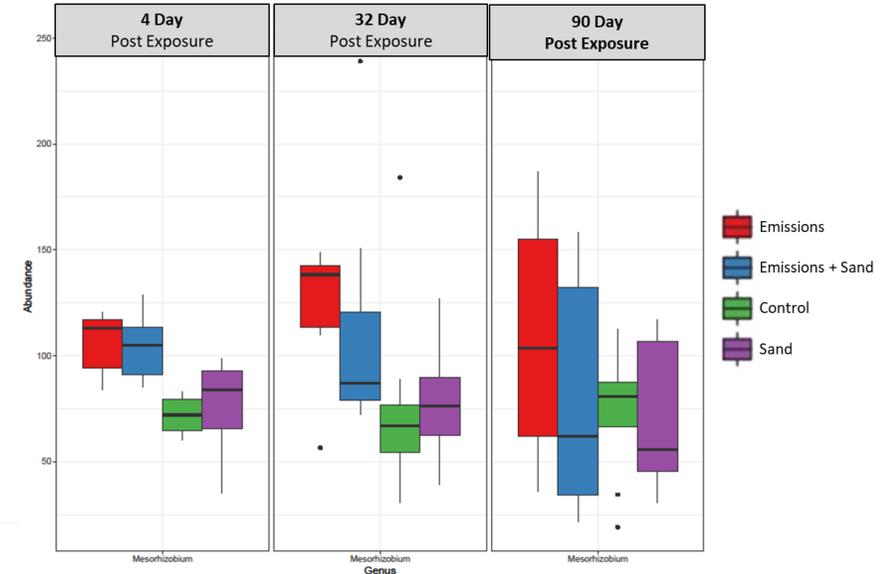
Specific Bacterial Populations

Increases Seen in Emissions Exposures

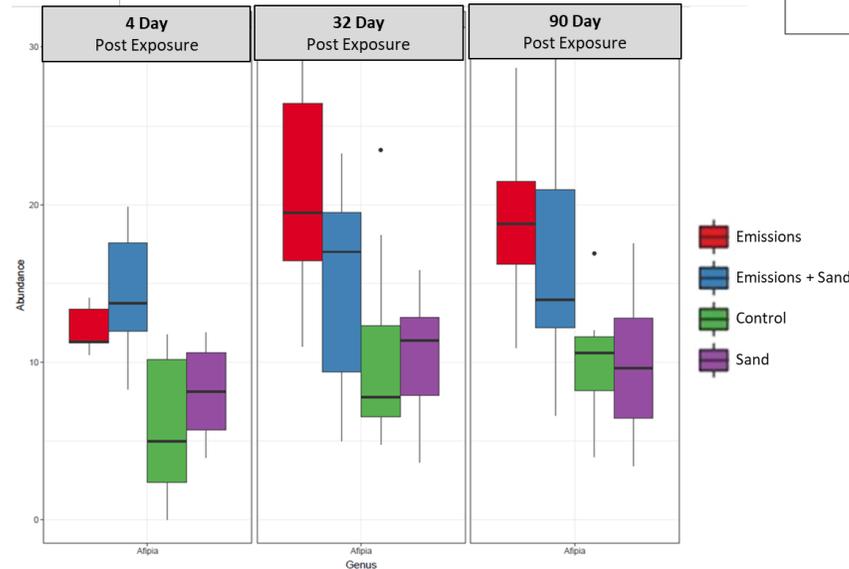


Order: Rhizobiales
Bacterial Taxonomy:
Proteobacteria;
Alphaproteobacteria

Genus: Afipia
Bacterial Taxonomy:
Proteobacteria;
Alphabacteria;
Rizobiales;
Bradyrhizobiaceae



Proteobacteria;
Alphabacteria; Rizobiales;
Phyllobacteriaceae





- **Modeling Burn Pit exposures is not easy, and there is no one model that works best.**
 - Capturing complex mixtures exposures – ‘*did you get the batteries*’ moments
 - For molecular discovery, deployment near Burn Pit \neq high level exposure.
 - Presented *in vivo* exposure study was sub-chronic \rightarrow full chronic study?
- **Molecular analyses indicate that sub-chronic exposures alter molecular expression at least 90 days post exposure.**
 - No return to baseline.
- **Proteomics indicate that sand effects may be additive, but not supported by other Omic analyses.**
- **Omic analyses indicate that inflammatory and ROS protection mechanisms were triggered by Emissions exposure, and these may initiate secondary organ injury.**
- **Lung Microbiome analyses indicate that Emissions inhalation altered commensal communities and specific bacterial orders (*Rhizobiales*) increased. Sand inhalation did not alter alpha or beta diversity.**
 - Individualized link to increased risk?





Questions?