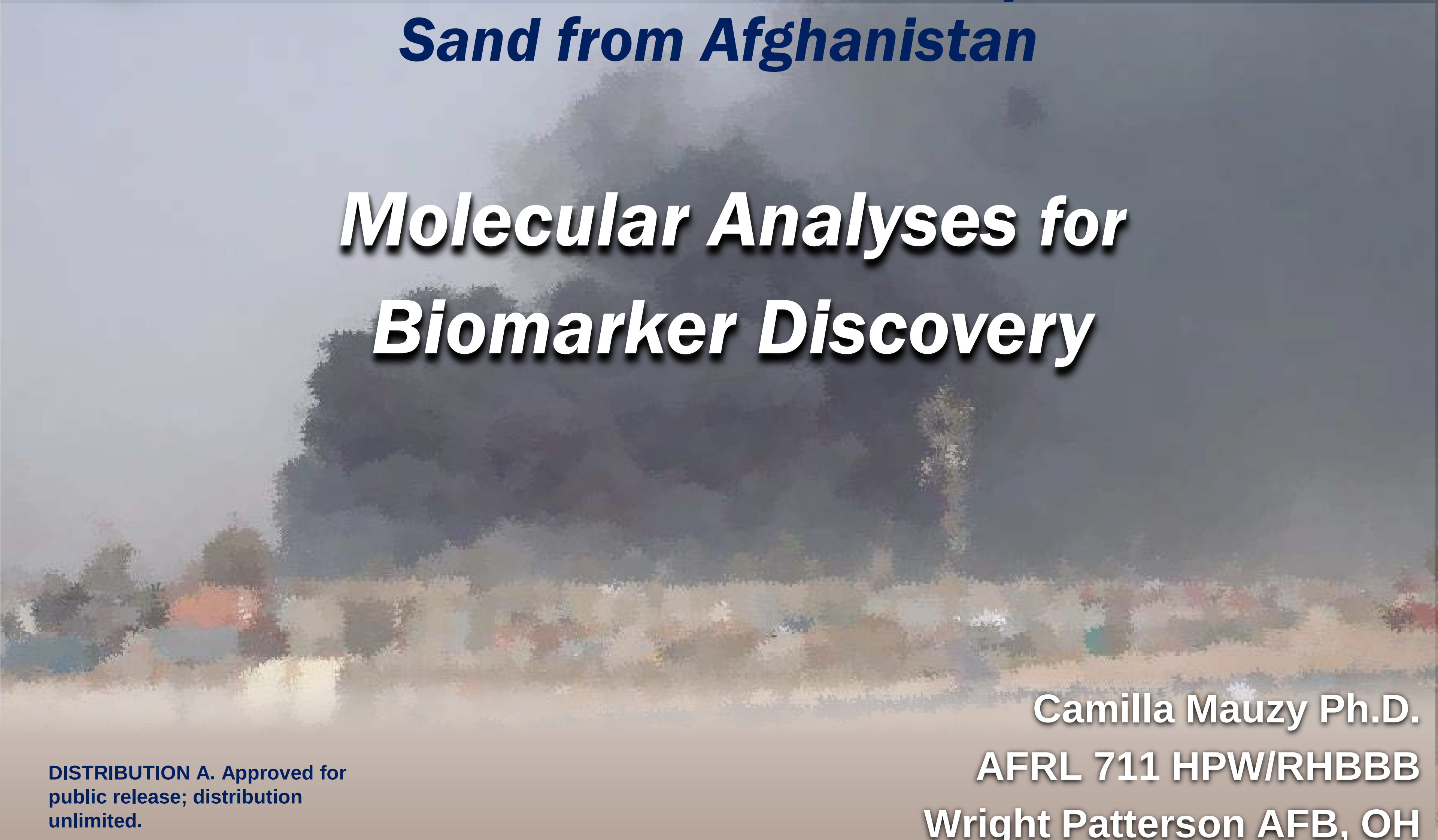
Toxicity Evaluation and Biomarker Identification in Rats Exposed to Burn Pit Emissions and Respirable Sand from Afghanistan



Molecular Analyses for Biomarker Discovery

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Information

Disclaimer

The views expressed in this presentation represent the personal views of the author and are not necessarily the views of the Department of Defense or of the Department of the Air Force.

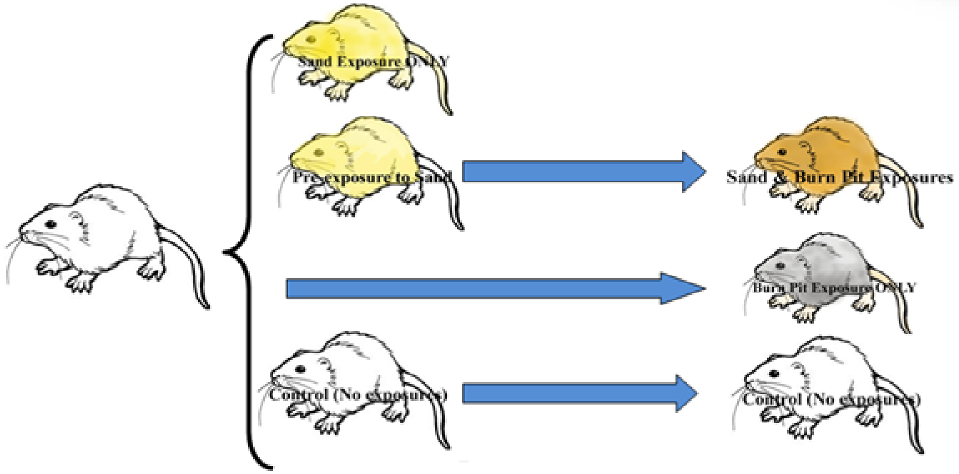
As such, this presentation focuses on molecular discovery and future utility only, and any discussion does not reflect official AF, Navy, or DOD policies.

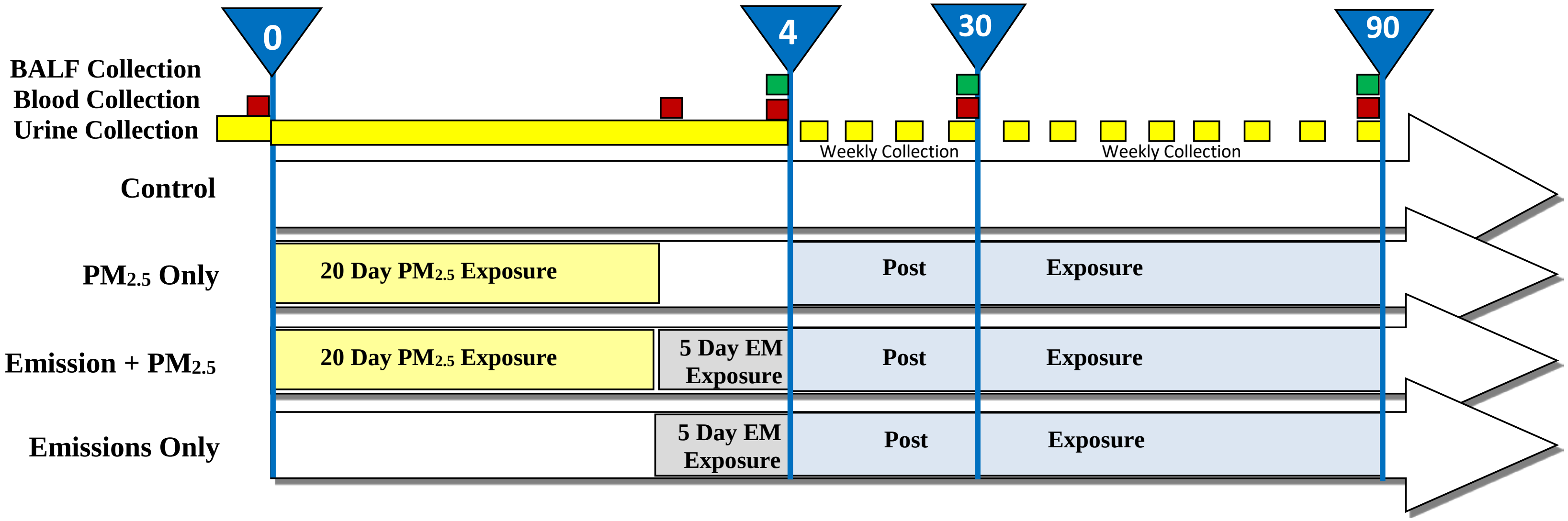
Animal Study

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.

Animal Study Design

- **Joint Air Force AFRL/Navy NAMRUD project Project: W81XWH-13-MOMJPC5-IPPEHA**
- **Funded through Military Operational Medicine Joint Program committee (JPC-5) Injury Prevention, Physiological and Environmental Health Award (IPPEHA), Department of the Army, Ft. Detrick, MD**

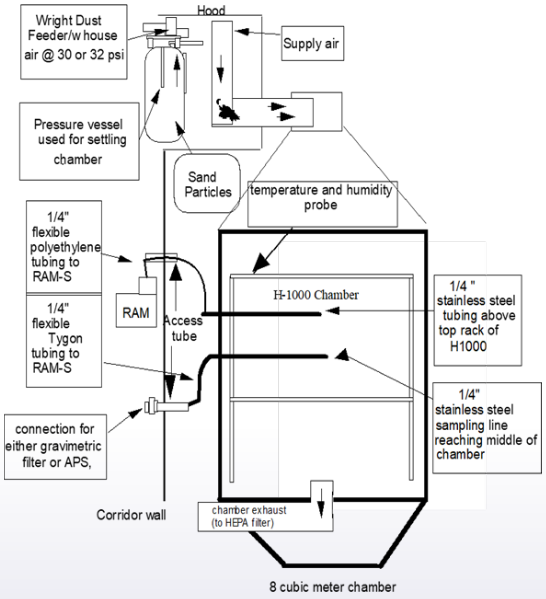




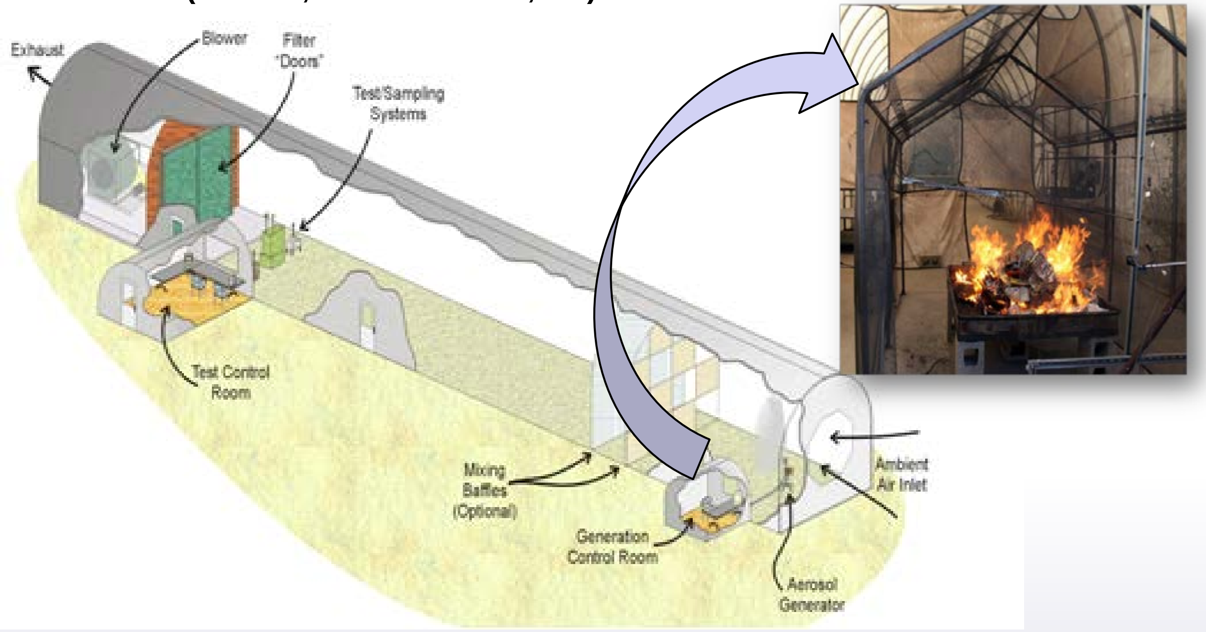
▼ = Days Post Exposure. Tests to include ventilation function tests, histopathology, clinical tests on animal subgroups.

Inhalation Exposures

Whole Body Exposure Chamber with Wright Dust Feeder apparatus.



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



Sand Particulate Matter Exposure

- 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

Burn Pit Emissions Exposure

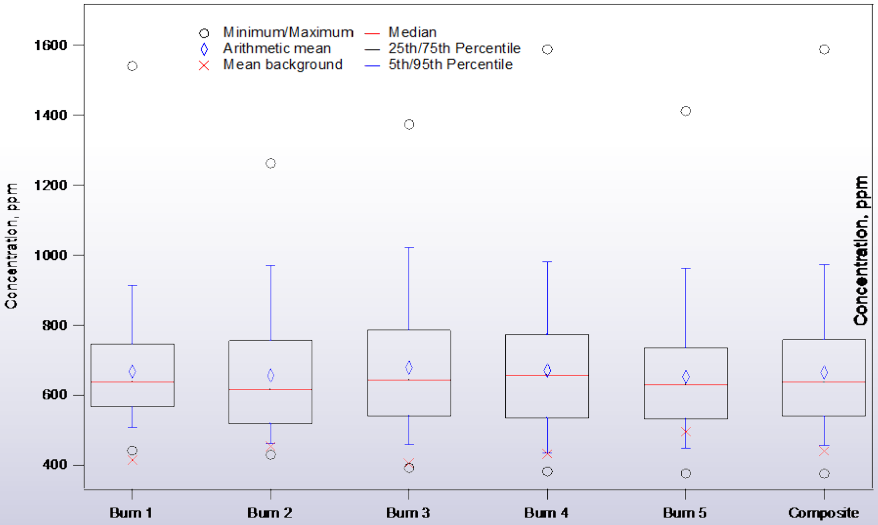
- Waste materials burned reflect solid waste combusted in-theater as based on \U.S. Army Central (USARCENT) Area of Responsibility (AOR) contingency Base Waste Stream Analysis (CBWSA). U.S. Army Logistics Innovation Agency (USALIA) 2013 report.
- Burned waste included cardboard, food waste, mixed paper,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

Characterization of Emissions Plume from Simulated Burn Pit burns

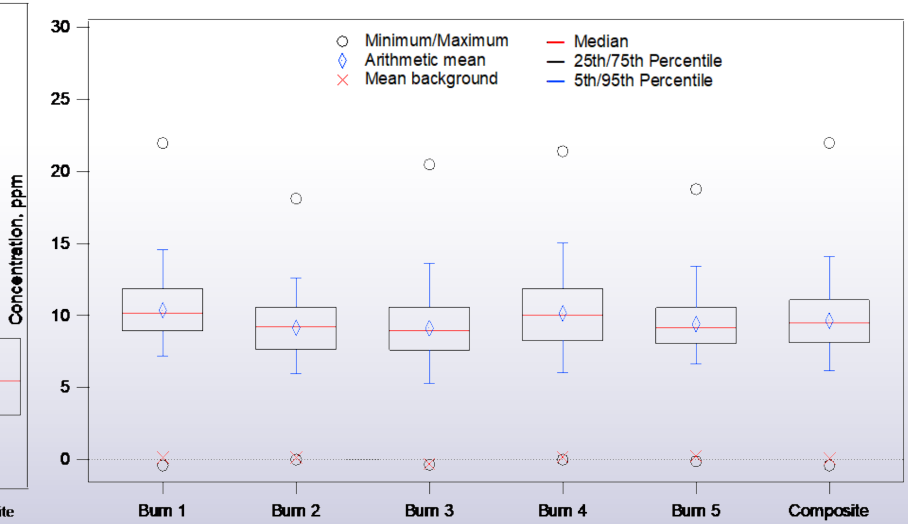
VOC Concentrations: (µg/m^3) of four carbonyl compounds

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 Exposure Day



Carbon Monoxide Levels per Exposure Day



Post Exposure Analyses

Lung Histopathology

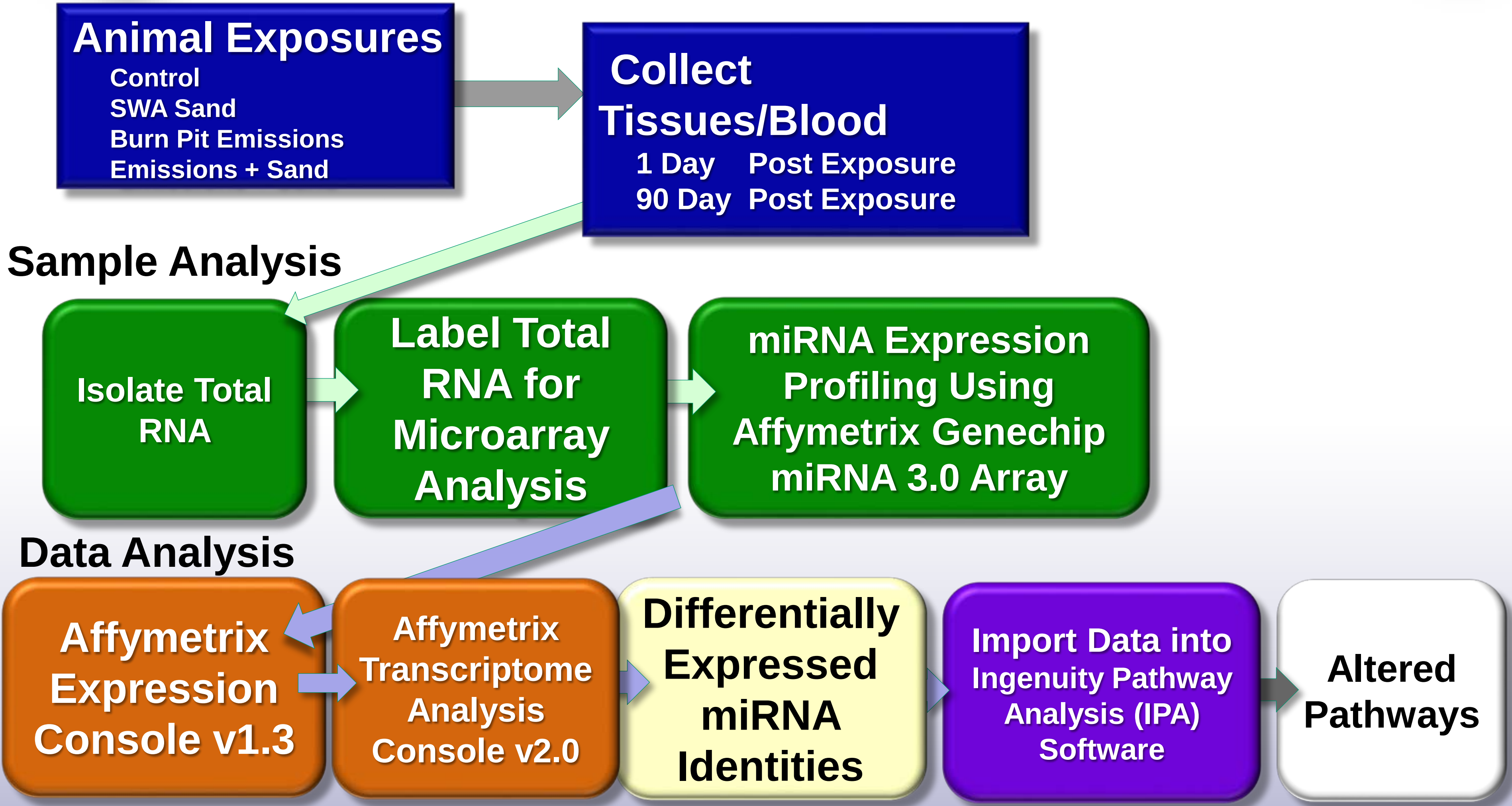
- **All Groups:**
  - Minimal to mild changes
  - Neutrophilic infiltration in all, but more frequent in Sand and Burn Pit Emissions Exposure Groups. However, very low and may not be clinically insignificant.
- **Sand Exposure Groups**: Foreign matter found in lung
- **Emissions and 'Emissions + Sand'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

Other Data

- **Body Weight differences:**
  - No significant differences
- **BALF total cell count and differential:**
  - No significant difference
- **BALF LDH and total protein:**
  - No significant differences
- **BALF TNF-α:**
  - Day 4 elevated in Sand group as well as 'Burn Pit Emissions + Sand' exposure 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

Epigenomics

Design and Methodology



Animal Exposures
Control
SWA Sand
Burn Pit Emissions
Emissions + Sand

Collect Tissues/Blood
1 Day Post Exposure
90 Day Post Exposure

Sample Analysis

Isolate Total RNA

Label Total RNA for Microarray Analysis

miRNA Expression Profiling Using Affymetrix Genechip miRNA 3.0 Array

Data Analysis

Affymetrix Expression Console v1.3

Affymetrix Transcriptome Analysis Console v2.0

Differentially Expressed miRNA Identities

Import Data into Ingenuity Pathway Analysis (IPA) Software

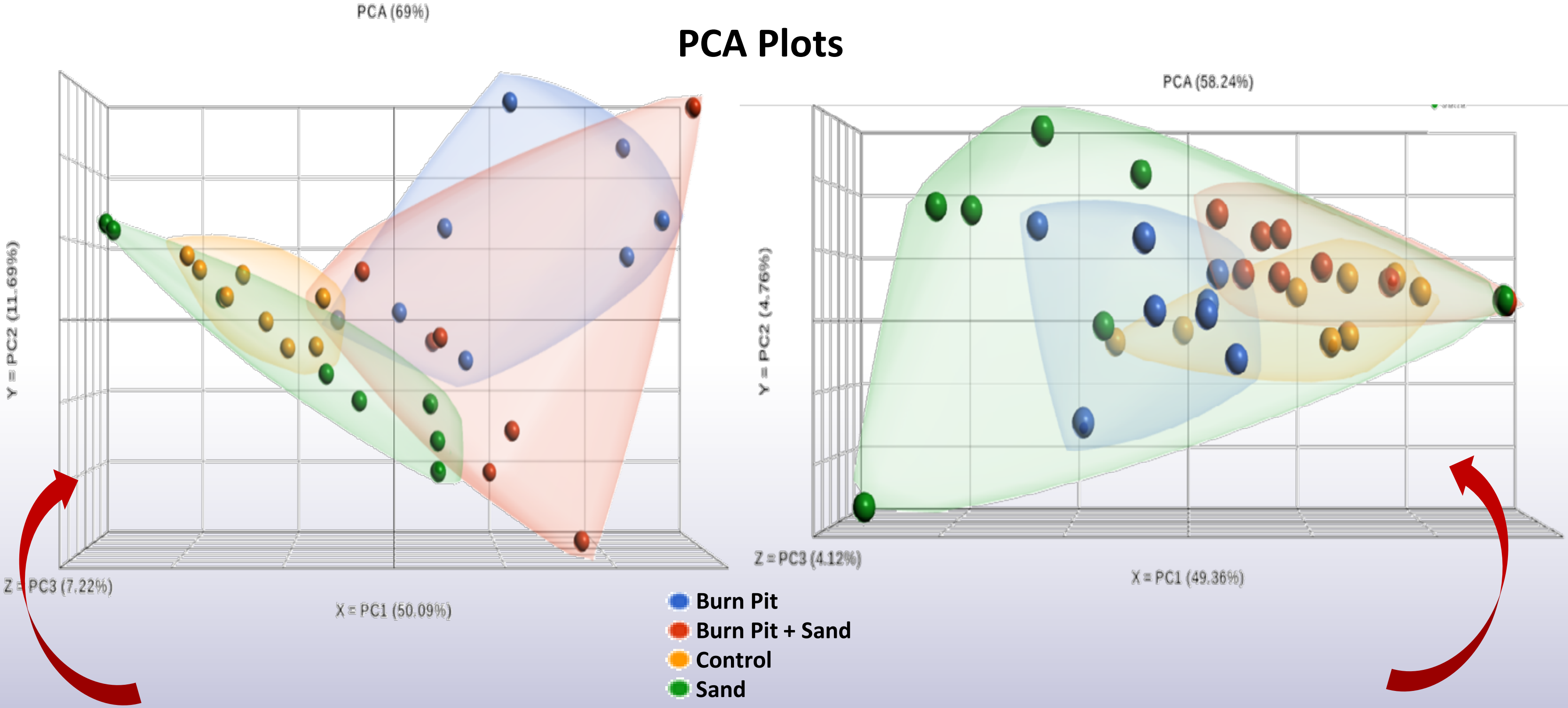
Altered Pathways

Results

miRNA Sequence-based Discovery

4 Day Post-Exposure, Lung Tissue | **90 Day Post-Exposure, Lung Tissue**

PCA Plots



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

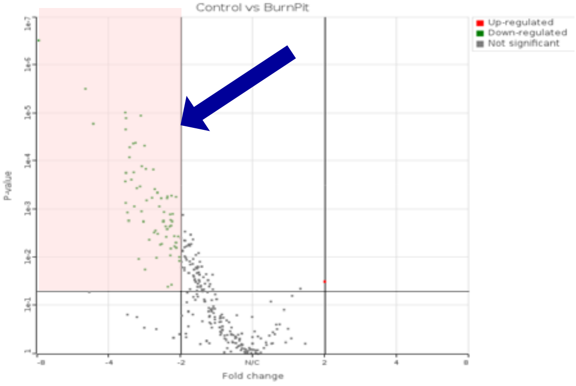
- Sand Alterations 90 day>4 day

Results

miRNA Sequence-based Discovery

4 Day Post Exposure, Lung Tissue

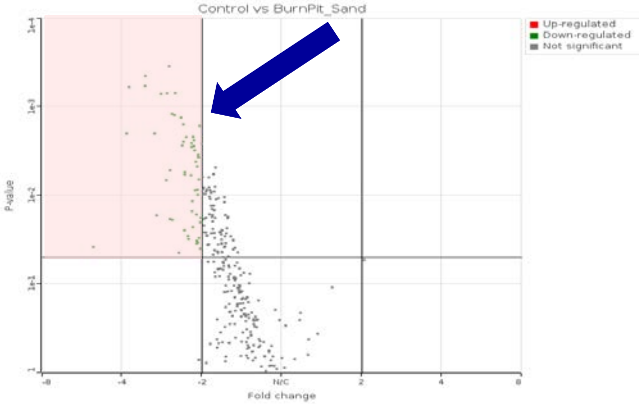
Burn Pit Emissions miRNA vs. Control



83 miRNAs Identified

> 2 fold change $p < 0.05$

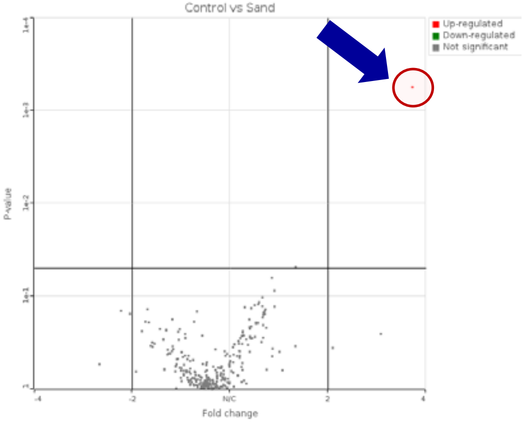
Burn Pit Emissions + Sand miRNA vs. Control



64 miRNAs Identified

> 2 fold change $p < 0.05$

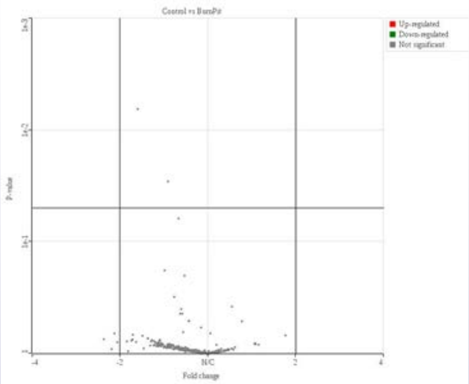
Sand miRNA vs. Control



1 miRNAs Identified

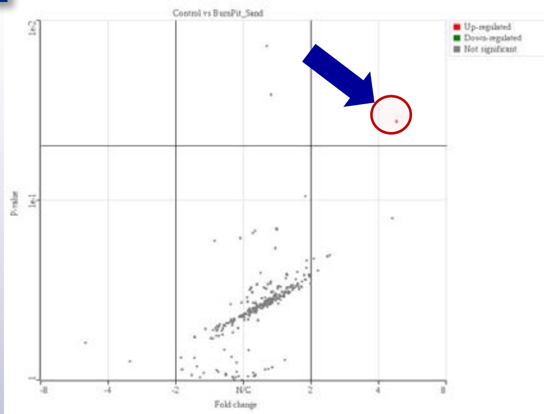
> 2 fold change $p < 0.05$

90 Day Post Exposure, Lung Tissue



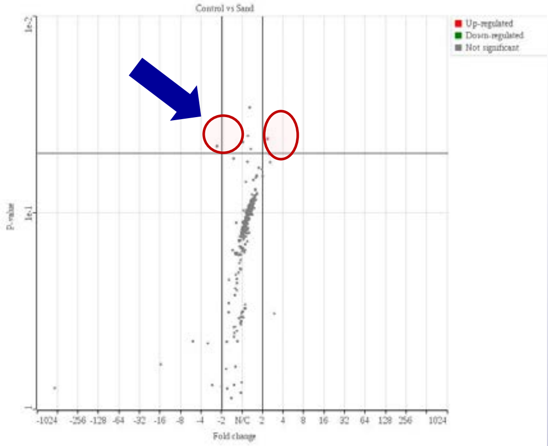
0 miRNAs Identified in Lung

> 2 fold change; p<0.05



1 miRNAs Identified in Lung

> 2 fold change; p<0.05

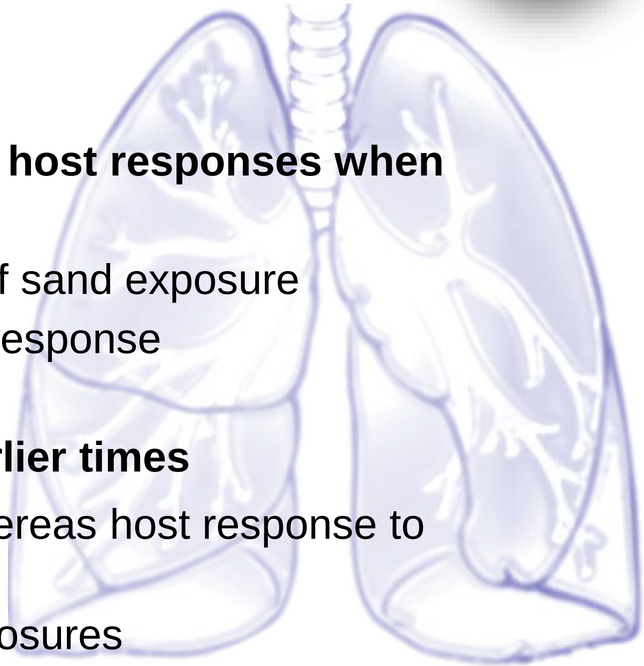


3 miRNAs Identified in Lung

> 2 fold change; p<0.05

Understanding Airborne Hazard Inhalation Effects

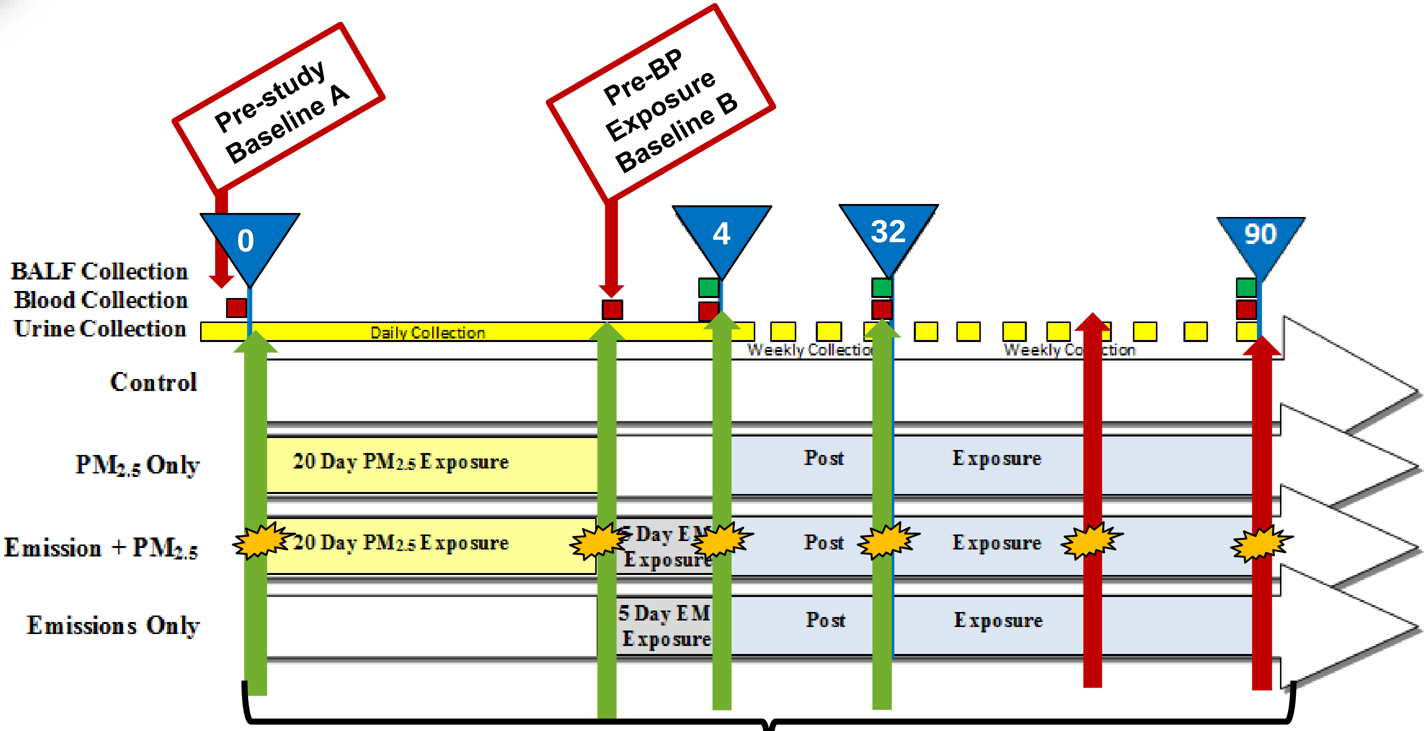
What did we learn from this Study?



- **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
  - Indicate a need for concern with even shorter exposures
- **Sand exposure response stronger at >90 days than at earlier times**
  - Host response to Emissions exposure relatively fast, whereas host response to sand inhalation is slower
  - Chronic exposure more likely responsive than acute exposures
- **Data did not indicate any additive effect of Sand + Burn pit emission exposures**
- **Sets of differentially-expressed miRNAs identified from Sequence Discovery in lung tissue**
- **Two species were found in both Affymetrix and Sequence based discovery data sets**
  - Both are seen in pathways leading to lung cancer or disease
  - Etiology not clear

Blood Proteomics

iTRAQ LC/MS Analysis



Ran 6 labelled samples/run

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

<u>Baseline A</u>' data was normalized to blood collected from the same animal prior to study initiation

'<u>Baseline B</u>' data was normalized to blood collected from the same animal prior to burn pit emissions exposure

Orbitrap LC/MS Protein Discovery

Sample Prep

- IgY immune-depletion, TMT6 labelled, Trypsin digested
- SCX spin column separation into fractions
- Reverse phase nanoAcquity UPLC-LTQ Orbitrap Velos mass spectrometer (MS)

MS and MS/MS data Analysis

- Analyzed using the SEQUEST algorithm in the Proteome Discoverer 2.2 software suite (Thermo Fisher, San Jose, CA).
- The CID and HCD spectra were combined prior to searching human, mouse, and rat proteins from the non-redundant NCBI protein database.
- Used in-house developed Matlab-based program
  - ➢ batch calculates the weighted ratios by summing the total intensity across all peptides in a protein
  - ➢ The program batch processed the data and calculated the weighted ratio of the protein.
- To be included in the final summary, the protein had to be identified in at lest 3 of the 4 replicates, and had to occur across all reporter ions within each exposure group.

Plasma Protein Identifications

Group 1:

7 emissions-based 4 sand-based markers were seen across single or combination exposures.

Response in Group 1 indicates that molecular alterations are not observably changed by inclusion of a secondary exposure type and may target a single key pathway or regulatory point.

Group 2:

24 Sand + Emissions exposure-based markers were seen.

As these markers are only found in the combination exposure, we hypothesize that the complex exposure induces an additive effect, altered more than one key pathway and/or regulatory point.

Group 3:

20 other markers were found.

Seen within the single exposure groups but not reflected in the combination exposure set.

Urine Metabolomics

Urine Metabolite Technical Report

AFRL-RH-WP-TR-2018-0061 APRIL 2018

BURN PIT EMISSION AND RESPIRABLE SAND EXPOSURES IN RATS: NMR-BASED URINARY METABOLOMIC ASSESSMENT

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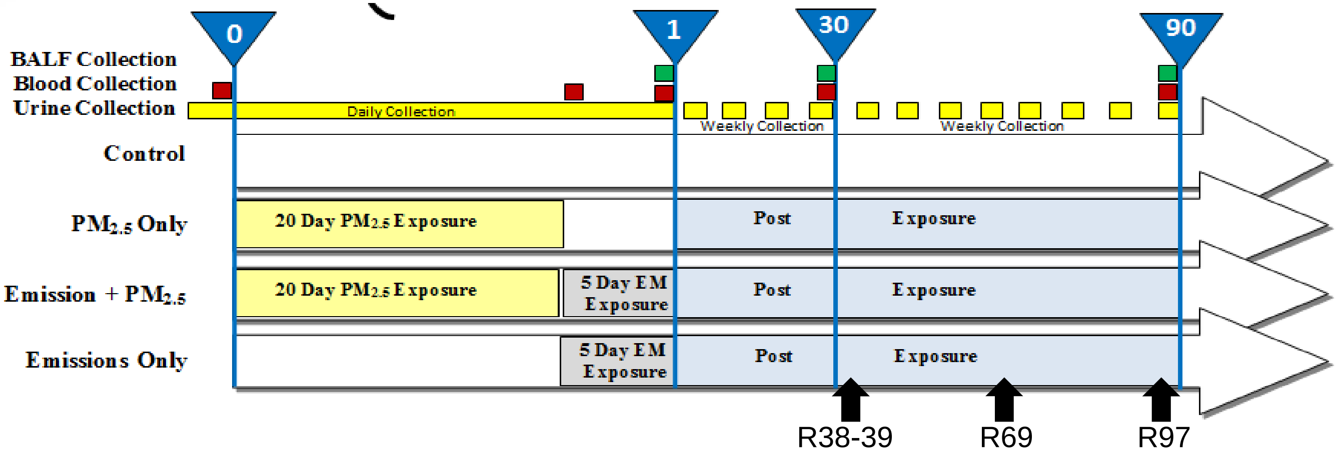
Report found at: https://apps.dtic.mil/dtic/tr/fulltext/u2/1064148.pdf

NMR Based Metabolomics Methods

- NMR-based (600 MHz) metabolomics conducted on urine samples
- Acquired NMR spectral data
  - Zeroed water & urea regions
  - Sum normalized
  - Binned (577 bins)
- Analyzed NMR spetral data using multivariate data analysis tools
  - Unsupervised *Principal Component Analysis* (PCA)
  - Supervised discriminate analysis
- Autoscale data using various time points as reference (i.e., BL, E34-35, etc.)
  - PC1 + PC2 accounted for 67% of total variance.

Sample Collections

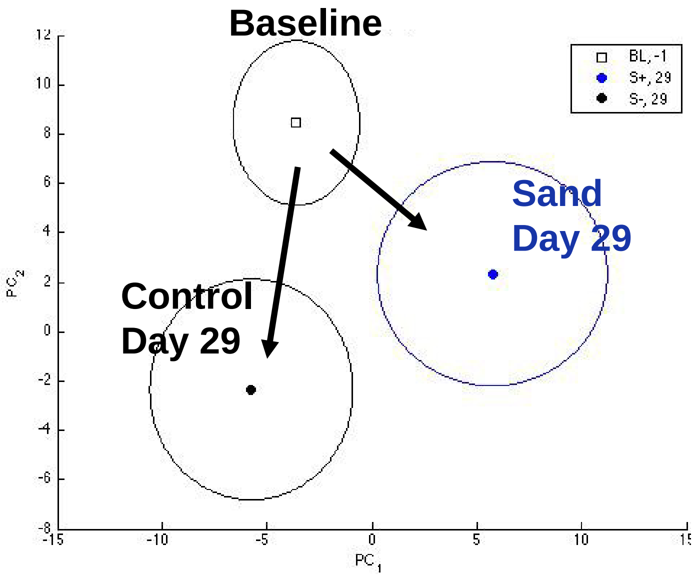


Urine Samples Analyzed

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**

Results

- **Large effect over time, possibly due to stress, found in data sets**
- **Some 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



The arrows depict the trajectory from BL through d29 for S-(G1 and G4) and S+ (G2 and G3)

- **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

Lung Microbiome

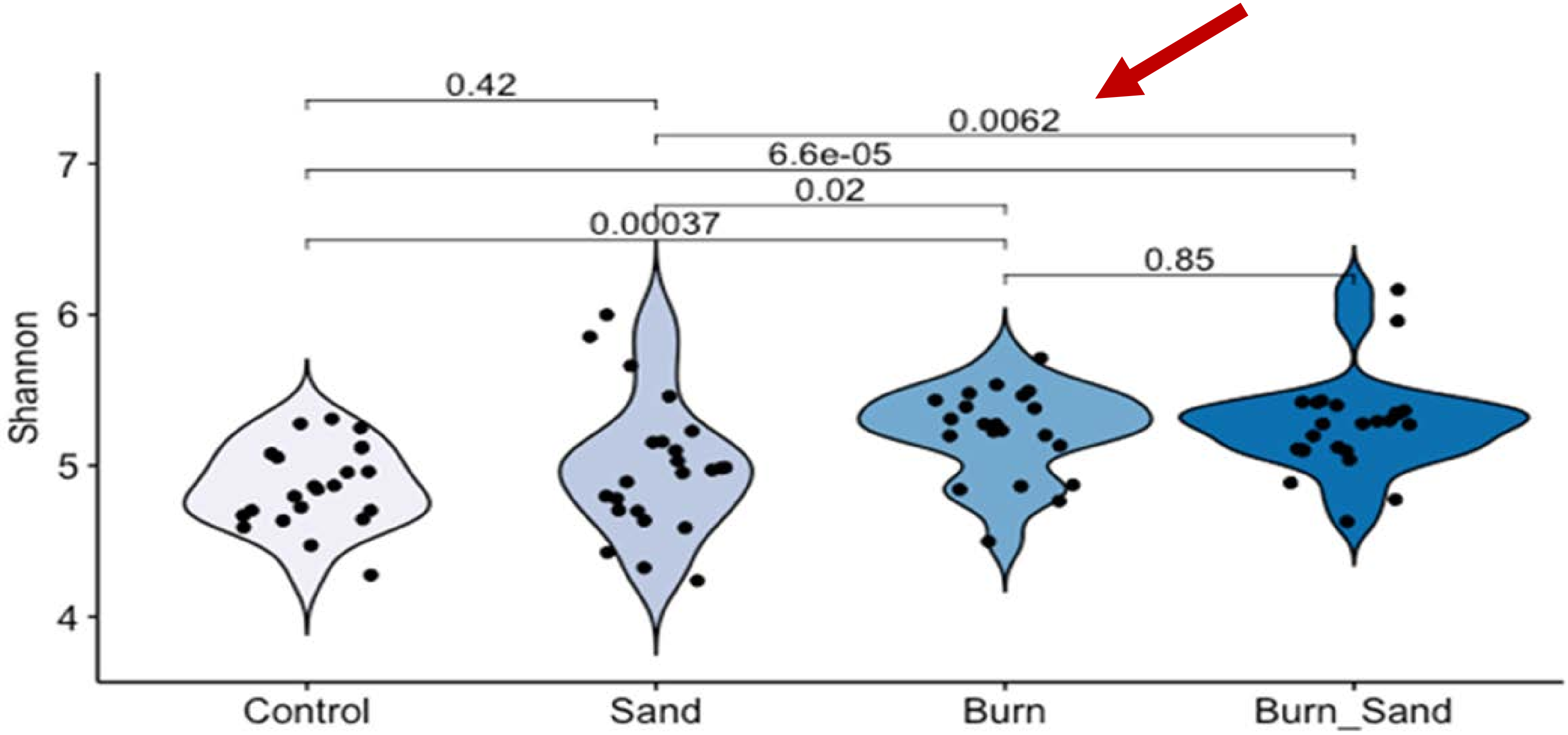
Methods

- Bronchoalveolar lavage was removed from the left lung
- DNA from the BALF pellets was extracted
- Bacterial 16S hypervariable regions were amplified
- Sequencing was performed on an Ion S5 System (ThermoFisher) using the Ion 520 & 530 Kit-Chef and Ion 530 Chip Kit
    - Base calling and demultiplexing were performed using Torrent Suite software, v 5.0 with default parameters. ISP loading was greater than 85% with a polyclonal amount lower than 35%. Total reads were greater than 19,500,000 with the final percentage of usable reads greater than 55%. 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
    - Open-reference OTU picking involves matching reads with 85% identity of any 16S bacterial gene and discard the remaining. The surviving reads are clustered based on 97% identity to the Greengenes reference and assigned the corresponding OTU. However, surviving non-matching reads are then clustered de novo to 97% identity and assigned a new OTU reference. Each sample was analyzed for alpha diversity and beta diversity using weighted UniFrac distances using their respective OTUs.

Alpha Diversity

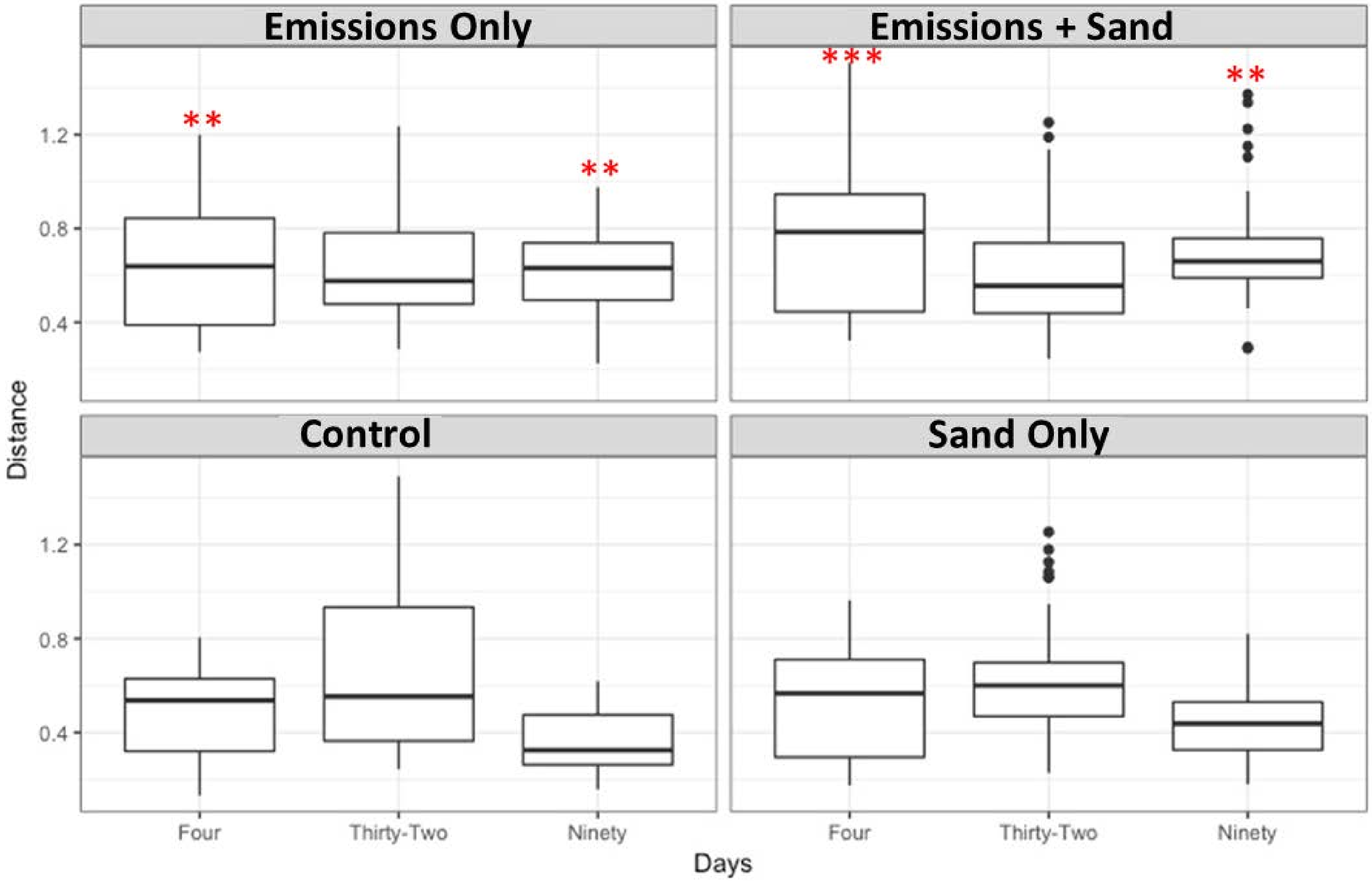
How many different species could be detected in each group?

Comparisons Based on Exposure Type



Beta Diversity

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

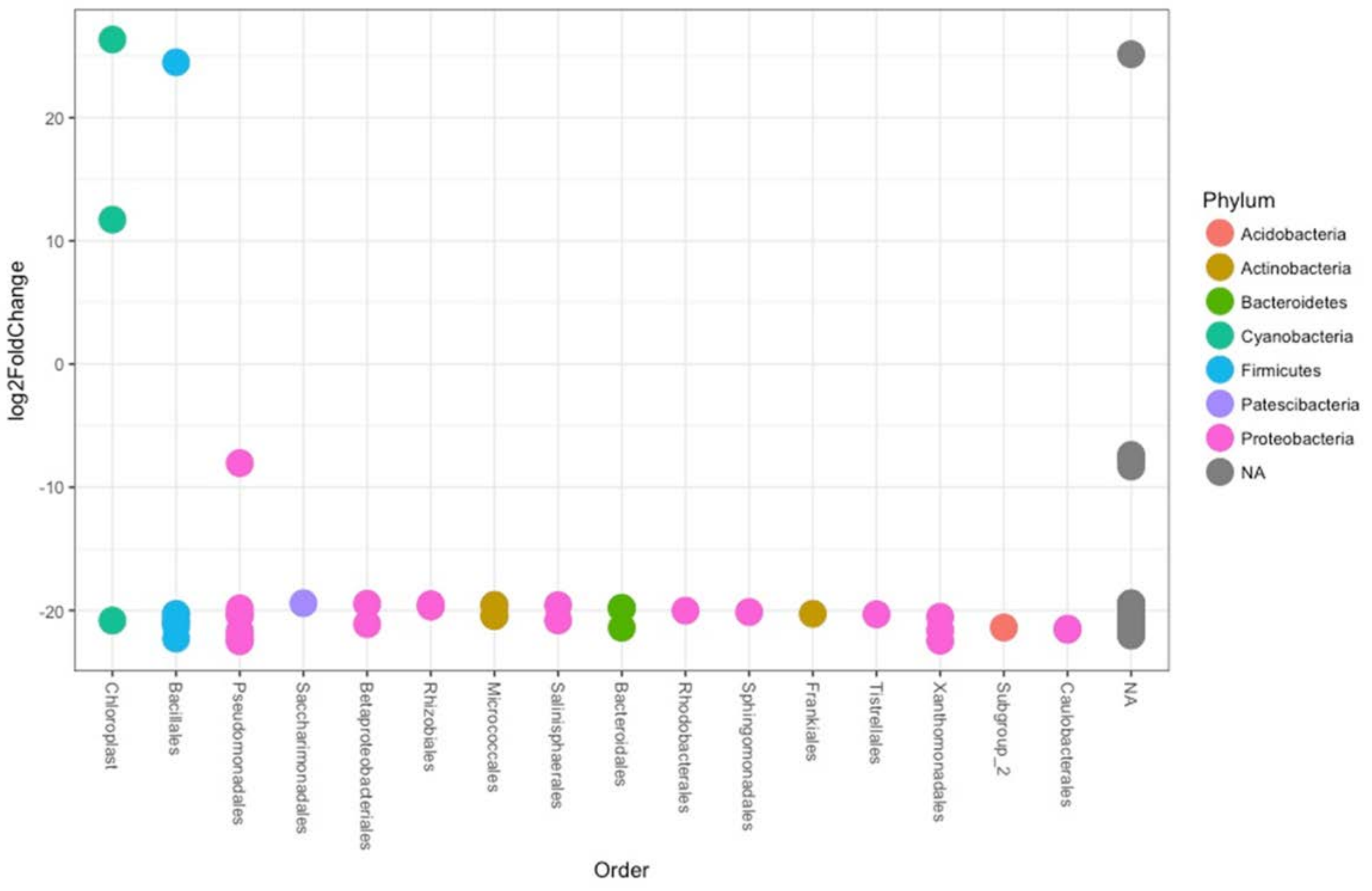


Statistically significant alterations compared to Control (PERMANOVA) = *** p ≤ 0.001, ** p ≤ 0.01, * p ≤ 0.05

Major Taxonomic Shifts

Emissions Group

4 Day Post Exposure



Questions?

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