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

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The experiments reported were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

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14. ABSTRACT
The present study, utilizing a rat model, investigated the unusual pulmonary morbidity that has been reported in military personnel deployed to the Middle East. It has been hypothesized that these adverse health effects are caused by inhalation exposure to combustion smoke emissions emitted from open burning of mixed solid waste ("burn pit" emissions) mixed with particulate matter (blowing desert sand) in the theatre of operation. This study involved four experimental animal groups over a 37-day protocol involving inhalation exposure to clean air (controls), aerosol sand for 20 days (sand alone), burn pit emissions for 5 days (emissions alone), or sand followed by burn pit emissions (combined exposure). A cohort of rats provided urine samples at specific times during the 127-day protocol. Overall, NMR analysis of rat urine indicated that the largest effect on urinary metabolite profiles was associated with time. This finding was observed for both the control group and experimental exposure groups. It was also observed that the greatest difference in urine metabolite profiles occurred during the exposure timeframe (day 1-38) and the least during the recovery period (day 39-97). However, the effects due to exposure (emission and sand plus emission) on urinary metabolite profiles became more evident when NMR data was evaluated using a paired analysis at day 33, which is the acclimation time point following sand exposure and preceding the emissions exposure protocol. Results from this study indicated that stress appeared to be a major contributing factor to the changes observed in urinary metabolite profiles during this experimental exposure protocol to sand and burn pit emissions, but minor effects due to exposures could be discerned through careful analyses of the data.

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PREFACE

Funding for this project was provided through the Military Operational Medicine Joint Program committee (JPC-5) FY13 Injury Prevention, Physiological and Environmental Health Award (IPPEHA), Department of the Army, Ft. Detrick, MD 21702 This research was conducted under cooperative agreements FA8650-10-2-6062 and FA8650-15-2-6608, both with the Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF). The program manager for the HJF cooperative agreements was David R. Mattie, PhD (711 HPW/RHXJ), who was also the technical manager for this project.

The protocol "Toxicity Evaluation and Biomarker Identification in Rats (*Rattus norvegicus*) Exposed to Burn Pit Emissions and Respirable South West Asian Particulate Matter" was approved by the Wright-Patterson AFB Installation Animal Care and Use Committee (IACUC) as protocol number F-WA-2014-0153-A. The study was conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), International, in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011). The study was performed in compliance with DODI 3216.1.

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1.0 SUMMARY

Since 2003, a significant number of the military personnel who have deployed to the Middle East have reported respiratory symptoms. An Institute of Medicine report in 2011 concluded that there was evidence suggesting an association between exposure to combustion products and reduced lung function. The present study, utilizing a rat model, investigated the unusual pulmonary morbidity that has been reported in military personnel deployed to the Middle East. It has been hypothesized that these adverse health effects are caused by inhalation exposure to combustion smoke emissions emitted from open burning of mixed solid waste ("burn pit" emissions) mixed with particulate matter (blowing desert sand) in the theatre of operation. With regard to this proposal, we hypothesized that changes in urinary metabolite profiles would correlate with exposures and adverse health effects. This study involved four experimental animal groups (rats) over a 37-day protocol involving inhalation exposure to clean air (controls), aerosol sand for 20 days (sand alone), burn pit emissions for 5 days (emissions alone), or sand followed by burn pit emissions (combined exposure). Animals were continuously monitored over an additional 90-day recovery period. A cohort of rats provided urine samples at specific times during the 127-day protocol. The present metabolomics investigation focused on samples selected at 12 specific time points. All animal studies were conducted at the Air Force Research Laboratory (AFRL), 711 Human Performance Wing, WPAFB, OH or at Battelle Laboratory (West Jefferson, OH). Rat urine samples were transported to the Wright State University Magnetic Resonance Laboratory, Dayton, OH, where proton Nuclear Magnetic Resonance (NMR) spectra was acquired on a Varian INOVA at 600 MHz. Overall, NMR analysis of rat urine indicated that the largest effect on urinary metabolite profiles was associated with time. This finding was observed for both the control group and experimental exposure groups. It was also observed that the greatest difference in urine metabolite profiles occurred during the exposure timeframe (day 1 through day 38) and the least during the recovery period (day 39 through day 97). However, the effects due to exposure (emission and sand plus emission) on urinary metabolite profiles became more evident when NMR data was evaluated using a paired analysis at day 33 (i.e. highlights changes in each animal relative to a specific time points), which is the acclimation time point following sand exposure and preceding the emissions exposure protocol. Results from this study indicated that stress appeared to be a major contributing factor to the changes observed in urinary metabolite profiles during this experimental exposure protocol to sand and burn pit emissions, but minor effects due to exposures could be discerned through careful analyses of the data. Further research involving longer emission exposure time will be necessary to attempt to identify salient metabolite biomarkers indicative of adverse pulmonary effects.

2.0 INTRODUCTION

Burn pit operations in the Mideast by the US military began in 2003 (NRC, 2011). Exposure of military personnel and contractors to these mixed waste emissions, comprised of a mixture of toxicants as well metals such as manganese released upon combustion, has occurred at Air Force (AF) locations such as those at Joint Base Balad and Tallil AFB (Military Times, 2008). A 2010 U.S. Government Accountability Office (GAO) report indicated that the military did not follow its own guidelines with regards to burn pits and possible exposures (GAO, 2010). These have been identified as a significant health hazard, although the levels or duration or exposure to the human system has been difficult to determine in a quantitative manner (Air Force Surgeon General, 2010). A recent Institute of Medicine (IOM) report indicated that there is yet insufficient evidence to develop any conclusions as to the long term health issues for military exposed to burn pits in theater, mostly due to inefficient methods for determination of exposure as well as data gathering methods (NRC, 2011; Tollerund et al., 2011). In addition, exposure data assessments conducted on specific chemical content did not address risk for multiple and cumulative exposures to the burn pit emissions (NRC, 2011). However, the report did indicate that the data was suggestive of associations between burn pit emission exposures and a reduction in pulmonary function (NRC, 2011). Other studies also have not found a link to burn pit exposures and health effects, although the severe limitations of the discussed epidemiology studies were clearly indicated, such as the need for individual-level exposure data [AFHSC/ NHRC/USAPHC, 2010). As such, burn pit exposures and its possible long term health repercussions in deployed personnel has garnered attention from Congress (Tollerund et al., 2011) to address better monitoring of troops to exposures. Indeed, a final conclusion of the IOM report suggested the possibility of biomonitoring as a possible means of quantitation of individual exposures (NRC, 2011; Tollerund et al., 2011). In addition, a point made in the IOM's report was that the potential for adverse health effects resulting from mixed exposures should also be investigated, including exposure to respirable dust and combustion products. Thus, the potential health effects of the combined inhalation of ambient particulate matter (PM) with the inhalation of emissions from burn pits is of interest to the U.S. Department of Defense (DoD) and Veterans Administration and is the subject of this study.

Respirable dust exposures may be exacerbated by sand storms, with such events occurring approximately 20% of the time in Kuwait (Draxler et al., 2001). Major dust events in which sustained particulate matter concentration exceeds 1 mg/m^3 can occur several times per year and persist for days; however, moderate dust events (with concentrations in the range 0.2-1 mg/m^3) can occur more frequently (Draxler et al., 2001). Previous Navy Aerospace Medical Research Unit-Dayton (NAMRU-D)-sponsored inhalation studies of sand dust from Iraq combined with cigarette smoke, and sand from Iraq and Afghanistan found no adverse health effects in rats exposed to sand dust concentrations at 1 mg/m^3 for 20 hours/day, 5 days/week, for 2 weeks (Dorman et al., 2012; Wong et al., 2011).

In order to conduct studies of burn pit emissions in a local area under controlled conditions, NAMRU-D funded a collaborative project with Battelle Memorial Institute (Columbus, OH) to reproduce burn pit combustion by using an ambient breeze tunnel (ABT) test facility operated by Battelle (West Jefferson, OH). The ABT consisted of a large tunnel with semi-cylindrical cross-section, 6 m high x 6 m wide x 45 m long, made from commercially available materials. A fan at the outlet end of the tunnel was used to generate an air flow through the tunnel to simulate a breeze of up to 5 mph. In the ABT burn pit model, materials that simulated in-theater waste were combusted in an open stainless steel pan located at the inlet end of the tunnel. Sampling or monitoring devices were placed at various points in the tunnel downwind of the source. In a pilot study, a set of three burn events showed that the ABT system could generate combustion emissions under near-real-world open burn conditions similar to those found in theater burn pits.

Using inhalation exposures of rats to simulated burn pit residue, this proposal utilizes combined Omics technologies and bioinformatics to gain an understanding of the molecular mechanism systems that are perturbed by particulate exposure toxicities, the temporal effects of such toxicities, how long these reactions are retained, and possible biological markers indicative of level and duration of exposure for risk analysis and prediction of potential biological consequences. In addition, the molecular signatures of burn pit emissions in the presence of sand, which may exhibit additive effects, will also be examined.

This technical report is focused on the identification of metabolite biomarkers in urine resulting from burn pit emission and sand exposures using nuclear magnetic resonance (NMR)-based metabolomics, which can be used as an indicator of potential hazardous exposures. Metabolomics is an approach used to characterize the metabolic profile of a specific tissue or biofluid. This approach can provide information about time-related metabolic responses to pathophysiological processes. Such processes may be related to physical, biological, or psychological stressors that induce perturbations in the concentrations and fluxes of endogenous metabolites involved in critical cellular pathways. Stressors, whether psychological, physical or chemical are expected to induce alterations in the biofluid (i.e. urine, blood, saliva, etc.) metabolite profiles that can be monitored by NMR-based metabolomics analyses. Metabolomics has the potential to give a more accurate assessment of the actual physiological state of an organism because metabolites are downstream of both gene transcription and enzyme activities. NMR-based metabolomics has shown great promise as a valuable tool for discovery of the metabolic response to toxin exposures and tissue injury. NMR metabolic analysis using biofluids is well documented, and the principles of this approach have been described in detail [Lenz et al., 2003; Lindon et al., 1999; Reo, 2002; Holmes et al., 1992a&b; Holmes et al., 2000; Lindon et al., 2004; Robertson, 2005; Anthony et al., 1994; Beckwirth-Hall et al., 1998; Holmes et al., 1998; Robertson et al., 2000 and Holmes and Shocker, 2000). In this particular study, NMR-based metabolomics will be used to monitor serial changes in urinary metabolite profiles during exposure protocols in a rat model of sand and burn pit emissions. This component of the project, under the direction of Nicholas V. Reo, Ph.D., will be conducted at the Wright State University Magnetic Resonance Laboratory.

This project is part of a larger collaboration of Air Force, Army, and Navy scientists to examining multi-level systemic changes that occur in a rat model upon inhalation exposure to emissions from a standardized burn pit waste mixture developed by the Navy (NAMRU-D). This waste mixture was developed using Army (USACHPPM/AFIOH) data to mimic the chemical composition of known burn residue (USACJPPM, 2008). In addition to this burn pit residue mimic, NAMRU-D also obtained actual burn pit soil residues from in-theater burn pits found in Bagram, Afghanistan, for comparison studies in the creation of a burn pit 'mimic.' Once the chronic exposures have been completed in a rat model, urine and blood will be collected for biomarker discovery at WPAFB. Navy research efforts at WPAFB will involve collection of several tissues, including the lungs, which will be examined for histopathology to enable linkage of tissue damage with marker response. We also anticipate examining peripheral blood mononuclear cells (PBMCs) as well as lung transcript modulations, although this may be accomplished in follow up studies. A combined bioinformatics effort of all data sets may reveal temporary and/or permanent perturbation of biological pathways. This large data set may also be used to propose biological markers indicative of both burn pit exposure level and length.

The specific aims of the metabolomics aspects of this project were to collect rat urine over a 127-day study period involving inhalation exposures to sand, various components of burn pit emissions and corresponding controls. Urine samples will be examined by NMR-based (600 MHz) metabolomics analyses and will address the following specific aims: 1) Acquire proton (^{1}H) NMR spectra of urine collected from rats belonging to four experimental groups (control, sand only, sand and emission, and emission only inhalation exposure groups), 2) Process and analyze NMR spectral data using multivariate data analysis tools, such as unsupervised *Principal Component Analysis* (PCA) to visualize metabolite patterns and search for unique metabolite profiles that are associated with experimental groups, and supervised discriminate analysis (*Orthogonal Projections onto Latent Structures Discriminate Analysis*; OLPS-DA) of urinary metabolite data for group classification and identification of specific spectral features that classify experimental groups with statistical confidence, 3) Examine the serial changes in metabolite profiles that may correlate with chronic exposure to PM, combustion emissions, or a combination of exposure, and be indicative of cumulative dose exposure. Furthermore, examine whether metabolite profiles correlate with other measures of toxicity as provided by the AFRL, and 4) Assign spectral features (NMR resonances) to identify specific metabolites that may serve as a biomarker of exposure and be correlated with other health-related indices of assessment.

3.0 METHODS

3.1 Animals

A total of 146 male Sprague-Dawley rats (approximately 6 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). Ten rats were used for training purposes prior to the start of the study. The rats were provided husbandry conditions consistent with the practices recommended by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and in compliance with guidelines for laboratory animal care (NRC, 2010). The rats were domiciled inside environmentally controlled facilities, provided food and water ad libitum, and kept on a 12 hour light/dark cycle throughout the study with exceptions during exposures as noted below.

3.1.1. Quarantine and Acclimation

After arrival at Wright-Patterson Air Force Base (WPAFB), the rats underwent a 7 to 10 day quarantine period in the vivarium, with an additional four days to acclimate to the exposure cage units. Exposure cage acclimation consisted of rats being placed in a stainless steel exposure cage units for increasing periods of time (2, 4, 6 and 7 hours) and then being returned to polycarbonate cages after the training period on four consecutive days during the week prior to the study start. Rats were placed into four experimental exposure groups and designated: G1) control, G2) sand, G3) sand and emission, and G4) emission

3.1.2. Exposures

Following acclimation, the rats were placed in stainless steel exposure cages for the duration of the 4-week sand inhalation portion of the study at NAMRU-D conducted in accordance with OECD Test Guidance 412 (2009), except when cages were changed (weekly) and for scheduled urine collections. A 90-day inhalation study was then conducted in accordance with the guidelines established in U.S. EPA 90-Day Inhalation Toxicity (EPA OPPTS 870, 1998), and OECD Subchronic 90-Day Inhalation Toxicity test guidelines (OECD 413, 2009). Following the 4-week sand exposure period, rats were transported approximately 55 miles to the surrogate burn pit facility (ABT) located on Battelle's campus in West Jefferson, OH. Exposure chambers and metabolism cages were housed in the Battelle Mobile Laboratory (ML) located adjacent to the ABT. During the 5-day burn pit emissions exposure period the rats were housed in stainless steel cage units when being exposed (6 hours/day) to either burn pit emissions or to clean air. Food was removed during the exposure period to prevent ingestion of food soiled with emissions deposits. Water was provided *ad libitum* during exposure periods. A designated cohort of rats were placed in polycarbonate metabolism cages between exposure periods for 18 hour urine collection. Rats were divided into four exposure groups (n=60 per group). This sample size was necessary since subgroups of rats were

sacrificed at specific time points for various analyses. For the cohort of rats used for metabolomics analyses, the experimental timeline involved 2 pre-exposure days (baseline), 37 days involving 25 exposure days, and 90 post-exposure days (recovery). During the 25-day exposure period, rats were exposed to clean air (control), PM for 20 days (sand only @ 5 days/wk for 4 wks), burn pit emissions for 5 contiguous days (emissions only), or sand for 20 days followed by emissions for 5 days (sand and emission; Table 1). Upon completion of the burn pit exposures and urine collection, the rats were returned to the WPAFB animal vivarium for toxicological assessment.

3.1.3. Sand Pre-exposure

Southwest Asian Particulate Matter (SWAPM), Camp Slayer (CSL-1-U): Surface sand (topsoil) was gathered approximately 5 years ago from Camp Slayer within Camp Victory, Iraq for the U.S. Army Corps of Engineers (ACE) (Vicksburg, MS) for their studies. Characterizations of the topsoil conducted by the ACE, including morphology, mineralogy and composition was used for reference in this study. A portion of the soil sample was sieved (#35 sieve) and autoclaved prior to shipment to NAMRU-D. These samples were characterized by inductively coupled plasma mass spectrometry (ICP-MS) analysis for metals. Sand particles were milled to a mass median diameter of 2.5 µm and aerosolized using a Wright Dust Feeder. Aerosol concentrations in the chamber were continuously monitored. Sprague-Dawley rats were subjected to whole body exposures of 1 mg/m3 prepared sand for a total of 10 days (20 hrs/day) in H-100 chambers. Ventilation function tests were conducted using whole body plethysmographs. At specific time points post exposure, a subset of this group was examined using clinical tests, histopathology, and bronchoalveolar lavage fluid (BALF) analysis.

3.1.4. Burn Pit Emission Exposure

Municipal solid waste (MSW) was combusted in the ABT to simulate the burn pits. A list of materials that were representative of the waste stream that was historically generated in theatre between 2006 and 2009 was compiled by the U.S. Army (USALIA, 2013). These materials, or suitable substitutes, were obtained by Battelle and NAMRU-D. A total of six test burns of 200 lbs of MSW each were performed, with Sprague-Dawley rats exposed (using nose-only towers) to the resultant MSW emissions for 6 hrs/day, for a total of 5 days. Appropriate controls were handled in the same manner, but with clean air exposure. NAMRU-D conducted clinical tests, histopathology, BALF analysis, and ventilation function tests on a subgroup of animals at 3 time points post exposure (7, 30, 90 days).

3.1.5. Transportation

In this study, animals were exposed at two different locations necessitating transportation

between the animal facility at WPAFB and the ABT facility at Battelle, West Jefferson, OH. These locations were approximately 55 miles apart, with an estimated driving time of one hour. Rats were transported using an approved animal transport vehicle operated by the designated NAMRU-D staff or WPAFB vivarium staff. The WPAFB Vivarium box truck was used to transport the animals and other assorted equipment and supplies required for the study. The box truck had an environmentally controlled interior where the animals were held during transport. The interior lighting was coordinated with the Vivarium housing light schedule. At the destination, cage units were unloaded from the truck and placed in the designated animal housing area. Animals being transported to the ABT facility were transported at least two days prior to the scheduled exposures to allow time to recover from the transport stress before undergoing exposures.

Table 1. Schedule of urine collections (U) and treatments (T) during the exposure protocol.
Treatments indicate the baseline period (BL), and time of sand exposure (S), emission exposure (E), and recovery (R). Urine collection times, and those samples selected for NMR analysis are marked ("x"). Transport days between WPAFB and Battelle Labs are indicated, as well as times to acclimate to the new environment. The recovery period extends for 90 days (full timeline not shown).

		U	T	Day	NMR	
Week 1	Su					
	M	x	BL	-3		
	T	x	BL	-2	X	
	W	x	BL	-1	X	
	Th		S	1		
	F		S	2		
	S		–	3		
Week 2	Su	x	–	4		
	M		S	5		
	T		S	6		
	W		S	7		
	Th		S	8		
	F		S	9		
	S		–	10		
Week 3	Su	x	–	11	X	
	M		S	12		
	T		S	13		
	W		S	14		
	Th		S	15		
	F		S	16		
	S		–	17		
Week 4	Su	x	–	18		
	M		S	19		
	T		S	20		
	W		S	21		
	Th		S	22		
	F		S	23		
	S		–	24		
Week 5	Su	x	–	25		
	M		S	26		
	T		S	27		
	W		S	28		**Transport to Battelle**
	Th	x		29	X	Acclimate
	F			30		Acclimate
	S	x		31		Acclimate
Week 6	Su	x		32	X	Acclimate
	M	x	E	33	X	
	T	x	E	34	X	
	W	x	E	35	X	
	Th	x	E	36	X	
	F	x	E	37	X	
	S		R	1		
Week 7	Su	x	R	2	X	**Transport to WPAFB**
	□		□	□		
Week 19	Su		R	86		
	M		R	87		
	T		R	88		
	W		R	89		
	Th	x	R	90	X	

3.2 Urine Collection and Sample Selection for NMR Analysis

Metabolism cages (Lab Products, Inc., Seaford, DE) were used to collect urine when rats were not being actively exposed. Urine samples were collected on weekends during the sand exposure periods, daily during the emissions exposures, and then weekly (24 h collections) over the 90-day recovery period. Urine samples were collected into plastic 50 mL conical tubes containing 1.0 mL of 1% sodium azide maintained at 6-10 °C using I-Cups (Bioanalytical Systems, Inc., West Lafayette, IN). All urine samples were stored at -80 °C until subsequent NMR analysis and validation of the results. A limited number of urine samples were selected (12 of 29 time points per rat) for metabolomics analysis in these initial studies as outlined below. Additional samples may be analyzed in the future depending upon the outcome of these initial studies. Samples from each of the four experimental groups (n=8 rats/group) were provided at the following time points (see Table 1):

- 2 days pre-exposure (baseline);
- 1 day during the sand exposure period, but pre-emission (burn pit) exposure;
- 2 days post sand exposure, but pre-emission exposure (during acclimation, post transport);
- 5 days during emission (burn pit) exposure;
- 2 day post emission exposure.

This provided a total of 12 urine samples per rat. Thus the total number of samples for NMR analysis was: 12 samples/rat x 8 rats/group x 4 groups = 384 urine samples. Based on this initial study design, we expected that analysis of these selected samples would provide sufficient data to identify changes in urinary metabolite profiles in response to exposures over the planned time course. Two baseline (pre-exposure) samples, 3 samples during or immediately post exposure to sand, and all 5 samples during exposure to burn pit emissions. During the sand exposure timeframe (day 1 – 28) urine samples were collected on weekends (4 samples), while during the recovery period (days 1 – 90) urine samples were collected weekly (14 samples). Initially, only two time points during recovery (day 2 and day 90 post emission exposure) were examined. Urine samples collected at other time points during sand exposure (days 1 – 28 during exposure) and recovery (days 1 – 90 during recovery) were stored at -80 °C for subsequent analyses if warranted.

3.3 Sample Preparation and NMR Analysis.

Urine samples were stored frozen at -80 °C and transported to Dr. Nicholas Reo's laboratory at the Neuroscience Engineering Collaboration building, WSU Magnetic Resonance Laboratory in Dayton, OH. On the day of NMR analysis, samples were thawed and centrifuged (5000 rpm for 10 min) to remove any particulate matter. A 0.550 ml aliquot of urine was transferred into a 5 mm NMR tube along with 0.150 ml of 9 mM trimethylsilylpropionic (2,2,3,3 d_4) acid (TSP) in D_2O. TSP was added as a chemical shift and concentration reference. Samples were analyzed using ^1H NMR spectroscopy conducted on a Varian Inova 600 NMR spectrometer. NMR spectra was acquired at 600 MHz and 25 °C using a pulse sequence designed to suppress the large resonance from water. Water suppression was achieved using the first increment of a NOESY pulse sequence, which incorporates saturating irradiation (on-resonance for water) during the relaxation delay (2 sec) and the mixing time (38 msec). These spectra provided a metabolite profile of the urine, which can be used as input to multivariate data analyses tools.

3.4 NMR Data Analyses and Processing

Multivariate data analyses were conducted on binned, scaled spectral data using MATLAB software. Binned NMR data were scaled to a chosen reference dataset by subtracting each bin value from the mean value for the corresponding bin in the reference data, then dividing this value by the standard deviation of the reference data (auto-scaling).

Principal Component Analysis (PCA) provided a first-approach, unsupervised technique, for data visualization. As previously described (Mahle et al., 2010), PCA model constructs were based on specific experimental groups to explore any systematic differences between groups that may exist. Once the model is constructed, other groups can then be superimposed into the visualization, by applying the model-specific bin coefficients (PCA loadings), to show how they compare. Thus PCA models were constructed to maximize visualization of specific responses based upon the nature of the effects being assessed, and PCA scores plots were used to help identify the time points of maximum effects for treatments.

Orthogonal Projection onto Latent Structures - Discriminant Analysis (OPLS-DA) was used as a supervised technique to classify data and identify salient features that allow class separation (Wold et al., 2001). Statistical differences in metabolite profiles can be assessed using OPLS-DA. In order to apply OPLS-DA, spectral data were collected into a matrix of variables or bins (X) and a vector of categorical labels (Y), representing the effects. These data were then analyzed and modeled as follows: (1) determine a specific time point of interest; (2) encode each treatment and corresponding control group as a two-group problem and analyze with OPLS; (3) using the model created for this specific two-group problem, project the remaining samples from other groups into the OPLS model. Therefore, OPLS enabled classification into specific groups. The OPLS model was evaluated on its predictive ability, using the coefficient of prediction (Q^2) metric. Q^2 was calculated as follows:

$$Q^2 = 1 - \frac{PRESS}{SSY} = 1 - \frac{\sum_{i=1}^{n} e_i^{\,2}}{\sum_{i=1}^{n} (y_i - \bar{y}_i)^2} = 1 - \frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{n} (y_i - \bar{y}_i)^2}$$

where PRESS is the Predicted Residual Sum of Squares calculated as the residual *ei* between the predicted and actual Y (class labels) during leave-one-out cross-validation, SSY is the Sum of Squares for *y*, $\bar{y}$ is the *y* mean across all samples, and $\hat{y}i$ is the *y* value for *ith* sample. As Q^2 approaches 1, the more predictive capability the model exhibits. A Q^2 value less than zero indicates that the model has no predictive power. A permutation test was performed to evaluate the significance of the Q^2 metric. The test involved repeatedly permuting the data labels and re-running the discrimination analysis, resulting in a distribution of the Q^2 scores (Westerhuis et al., 2008). The Q^2 from the correctly labeled data is then compared to the distribution to determine the significance of the model at a specified alpha (set herein at $\alpha = 0.01$).

Variable selection (salient bins) from OPLS-DA was also statistically evaluated. The bin loadings, commonly referred to as coefficients, were compared to calculated null distributions in order to select for significance. The null distribution for each bin was determined by refitting the OPLS model to datasets in which each bin was independently and randomly permuted to remove any correlation between it and the control/treatment groups. The true OPLS model loading was then compared to the resulting null distribution of loadings, and values in the tail (greater than 99.5% or less than 0.5% of the null distribution; corresponding to $\alpha = 0.01$) were assumed to contribute significantly to the model. The permutation was initially repeated 500 times for each bin and those near-significant loadings (greater than 92.5% or less than 7.5% of the null distribution; corresponding to $\alpha = 0.15$) were selected for 500 additional permutations (total 1000). Comparisons between exposure groups were used to help identify metabolite profiles or markers of exposure susceptibility. The salient spectral resonances were assigned to metabolites using Chenomx 5.1 software, on-line NMR databases (i.e., mmcd.nmrfam.wisc.edu; U Wisc, etc), and by "spiking" samples with known compounds, if necessary.

Shorthand notation was used for data analyses to generate a model as input to PCA or OPLS-DA. For example, the effects of sand exposure may be modeled by comparing groups 1 and 4 (G1 and G4) *versus* groups 2 and 3 (G2 and G3) during days 10 and 29 (d10, d29). The input data for this analysis may be auto-scaled (AS) to only the non-exposed groups (G1 and G4) at the times included in the analysis. Thus, a shorthand notation for this analysis is:

Model = allG at d(10,29); AS = G1G2 at d(10,29).

In the following results, this shorthand notation was used to describe the analysis that were conducted.

4.0 RESULTS AND DISCUSSION

All exposure groups (G1-G4) were processed identically, allowing for multivariate data analyses for comparisons among all experimental groups. The largest effects in the urinary profiles that were observed occurred across time. The largest trajectory along PC1 occurs from BL day -1 through d38; the trajectory from d38 through d97 is mostly along PC2 (Fig. 1). Thus, it appears that large changes occurred during the exposure timeframe, and much smaller changes were observed during the recovery period. However, this was not due to exposure since this model includes only the control group (G1). This result may be due to stress, as animals were handled and moved between cages and facilities (Dayton and Columbus) during the exposure timeframe. All exposure groups (G1-G4) showed this time-related effect, but the variability differed from one group to another.

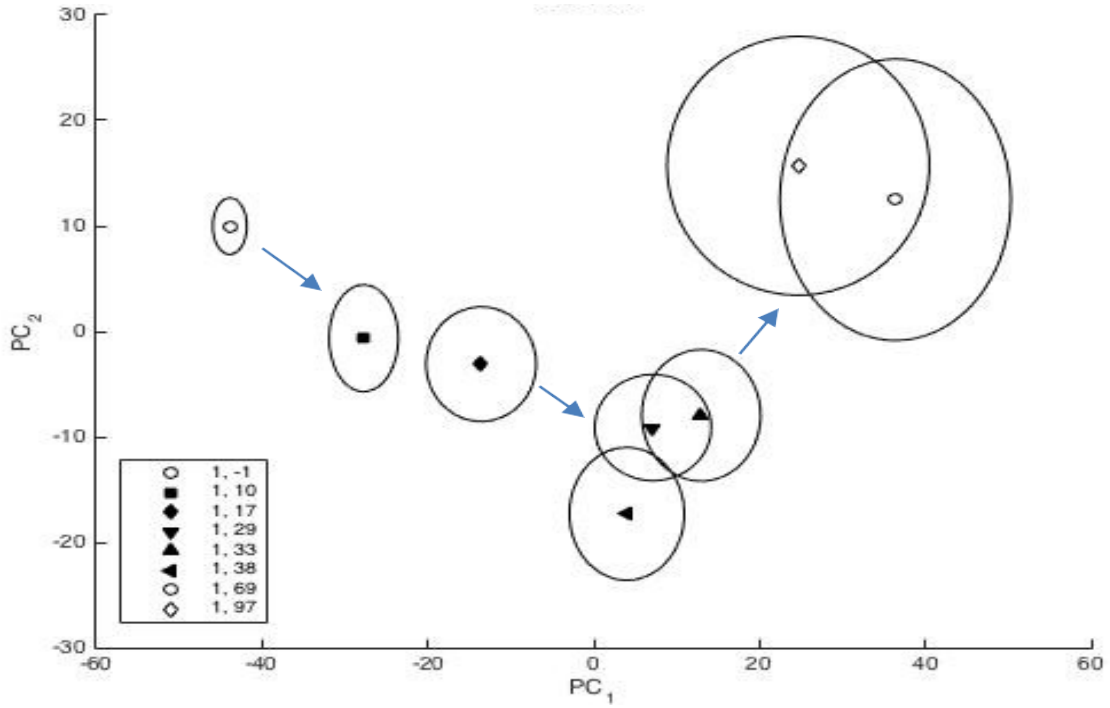

Figure 1. PCA modeling control (G1) across time from d(-1) through d(97). Data were auto-scaled (AS) to all groups during BL times. Data are represented as ellipses around a centroid mean ± 2SEM. The model excludes days 34, 35, 36, and 37 for clarity. Model = G1 at d(-1, 97); AS = allG at d(-1, -2).

Focusing on d29 and d38, it was hypothesized that we would observe differences in urinary metabolite profiles due to sand and emission exposures, respectively. Since G2 and G3 were exposed to sand, one might expect to observe a different profile at d29 (maximum day of sand exposure time course) for these groups versus the non-exposed rats (G1 and G4). However, this was not observed (Fig. 2). Again, the largest dispersion of data was due to time and not exposures. The same was also true for the groups exposed to emissions (G3 and G4), which

should have showed effects at d38 (maximum time following emissions exposure). Results appeared to show that the effects of exposure (if any) were more subtle than the effects of stress, manifested through the course of time. One observation that may be important was the variability seen at different times, especially at d97 for G2, which showed the greatest variability.

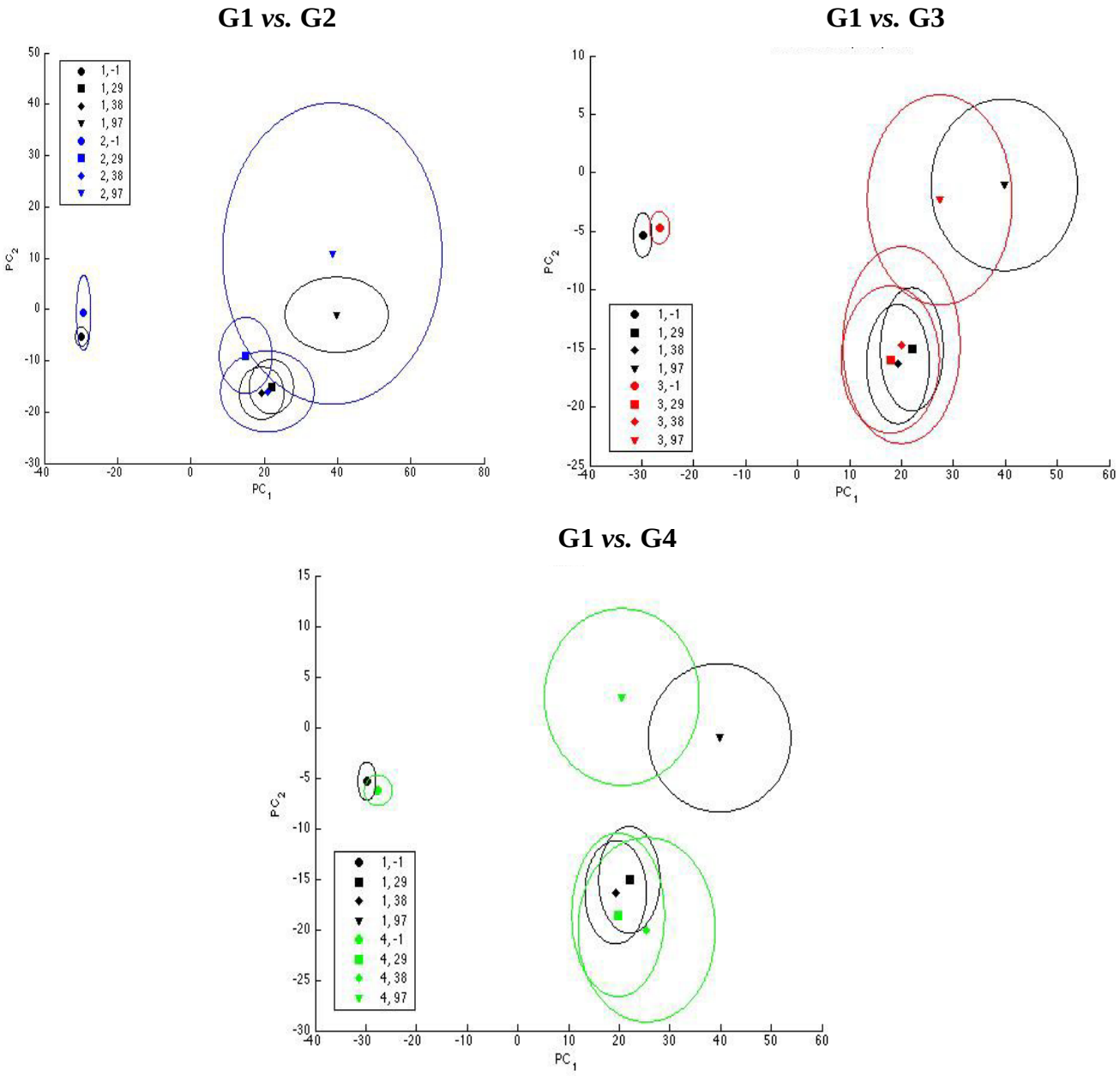

Figure 2. PCA modeling all groups at day -1 and 97 {d(-1,97)}. Data were auto-scaled (AS) using all groups at base line as reference. Data are represented as ellipses around a centroid mean ± 2SEM. Even though the model includes all groups, the plots show only the control group (G1) together with each treatment group separately for clarity and for comparison purposes. Colored ellipses identify G1 control (black), G2 sand only (blue), G3 sand and emission (red) and G4 emission only (green). Model = allG at d(-1, 97); AS = allG at d(-1, -2); d29 and d38 are superimposed into the model.

The results above prompted an examination of each group across the 97-day time course employing a separate model for each group (Fig. 3).

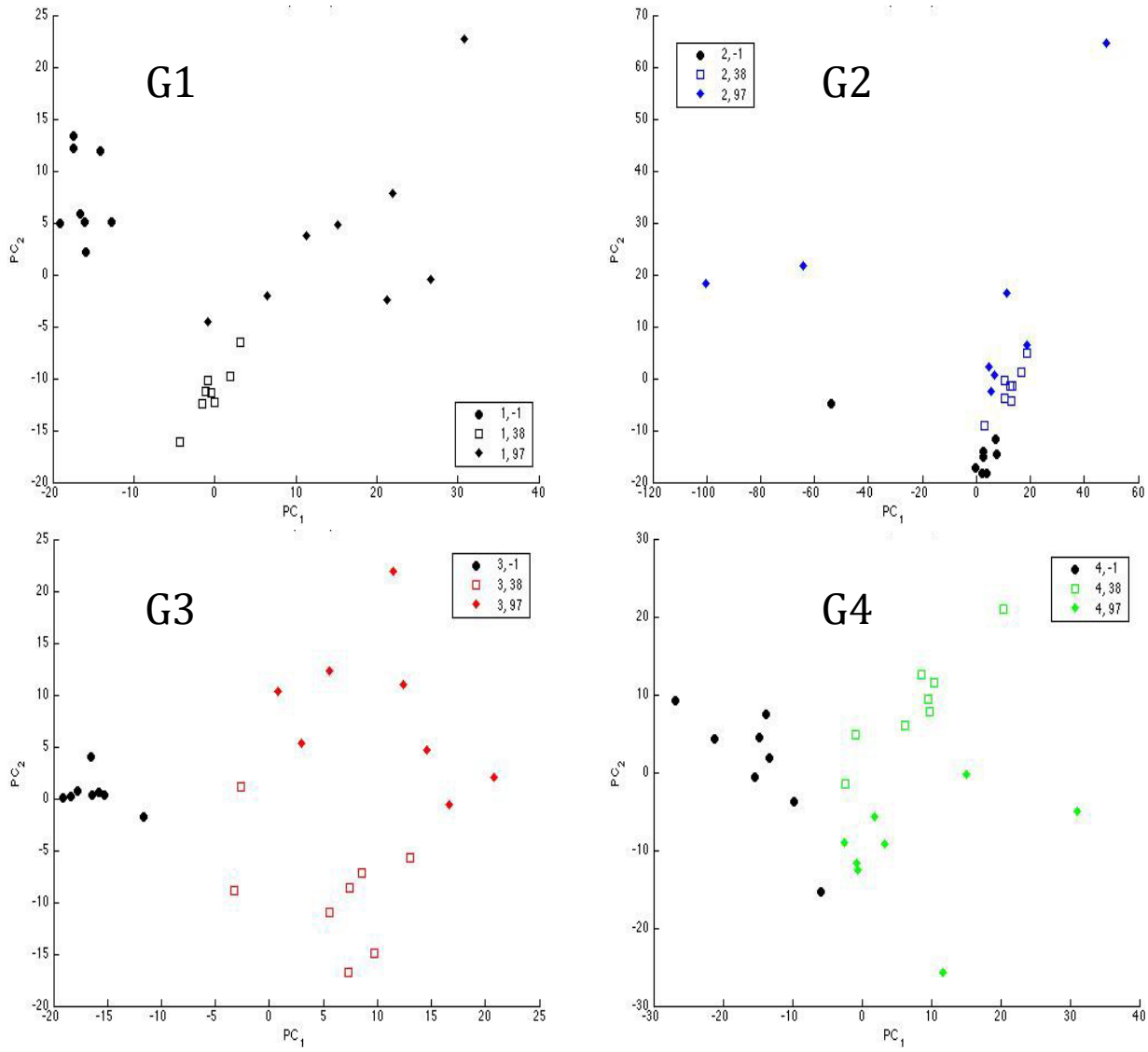

Figure 3. PCA scores plot of all exposure groups over entire time course. Each group was analyzed separately across the entire time course, but only included three time points in the model: days -1, 38, and 97. This encompassed the base line period, a time corresponding to the end of the exposure period (d38), and the last time point at the end of the recovery period (d97). The data were auto-scaled to the entire time course for the control group. Colored data points identify G1 control (black), G2 sand only (blue), G3 sand and emission (red) and G4 emission only (green). Model = G*n* at d(-1, 38, 97); AS = G1 (all times); Here G*n* refers to group *n* as identified in the plots.

All groups showed a similar pattern where the trajectory from BL through d38 was along PC1, while the trajectory from d38 through d97 was mainly along PC2. With the exception of G2, the trajectory for the control group (G1) is not distinguishable from the groups exposed to sand +/- emissions. In fact, the magnitude of change along PC1 from BL through d38 was very similar for G1, G3, and G4, indicating that this was a 'time-effect' and not an 'exposure-effect' (Figure 3). The data for G2 (sand only) is puzzling. Some of the G2 animals appeared to be outliers. Note that the scale in this plot (G2) is much greater than that seen for the other three group plots. In addition, one BL data point and 3 data points at d97 are well separated from others in the group. These data points have been identified as subjects #62 (BL), #65, #67, and #68. The three data points at the far left in the G2 plot are dominating this analysis, which looks very different from the similar analyses for G1, G3, and G4. A further investigation of the effects due to time is presented below in Figures 4 and 5.

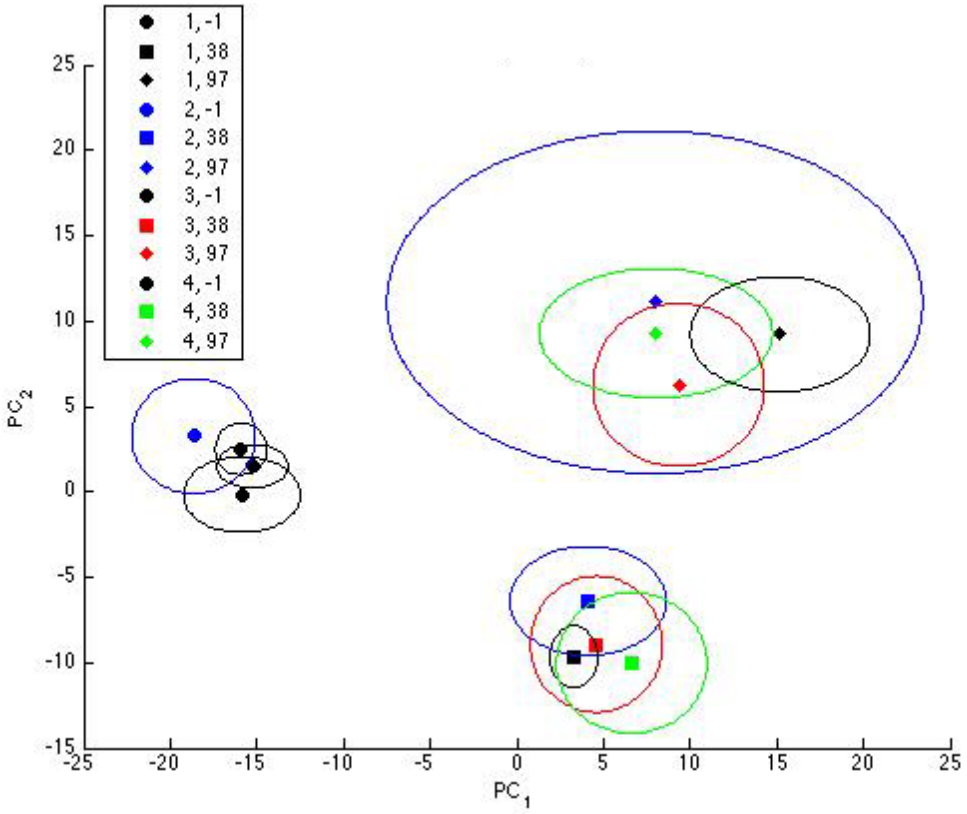

Figure 4. PCA scores plot with G1, G3, and G4 modeled across time. Plot generated using days -1, 38, and 97 in the model as above, and auto-scaling (AS) the data relative to the control (G1). Data are represented as ellipses around a centroid mean ± 2SEM. Colored ellipses identify G1 control (black), G2 sand only (blue), G3 sand and emission (red) and G4 emission only (green). G2 intentionally omitted in this analysis, but data was superimposed into this model, which is based on the other three groups. Model = G1G3G4 at d(-1,38,97); AS = G1 (all times).

As expected, we observed the largest trajectory along PC1 that occurred from BL through d38, with a secondary trajectory along PC2 from d38 through d97. However, it was also noticed that the data for G2 (blue symbols) was slightly displaced from the other groups at BL and d38, and showed a very large variability at d97 (Fig. 4).

Next, subjects #62, #65, #67, #68 (all belonging to G2) were omitted, as these appear to be outliers in Figure 3. The results of this analysis is shown below in Figure 5.

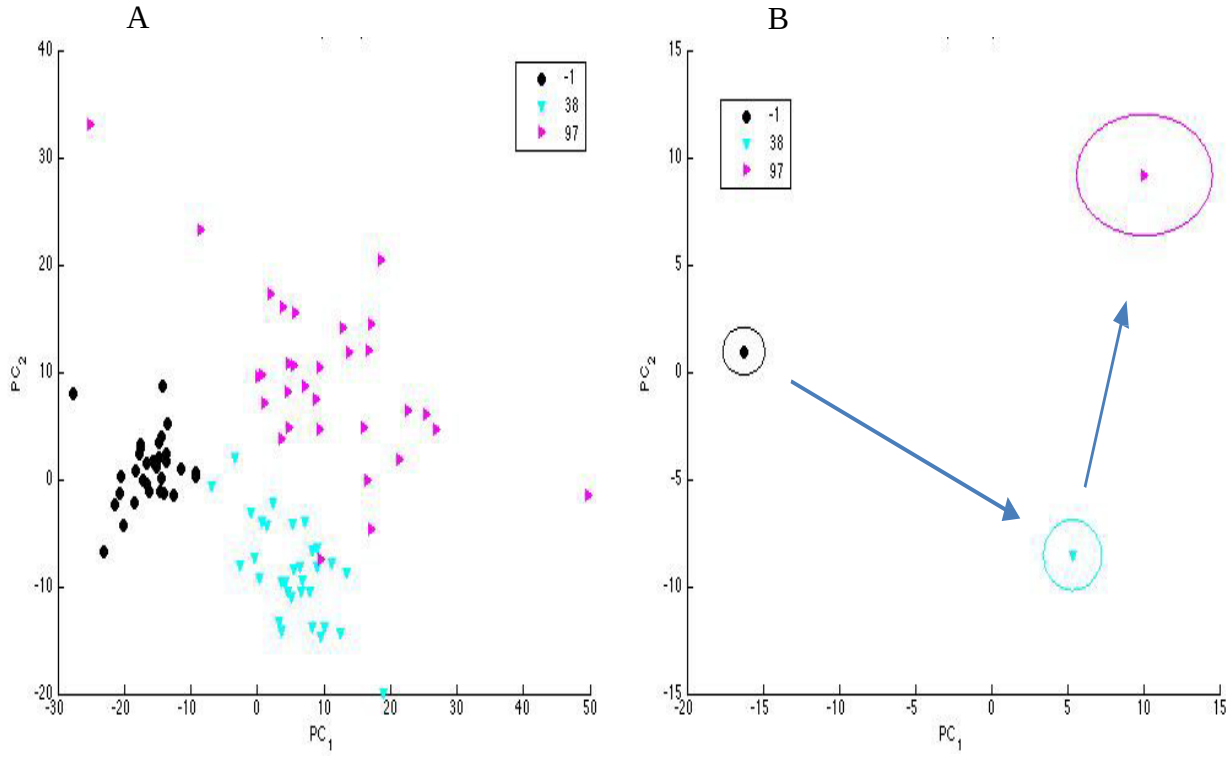

Figure 5. The PCA scores plots show all data points (A) and the centroid mean ± 2SEM (B).
Model = all groups at d(-1,38,97), subjects #62, #65, #67 and #68 omitted; AS = G1 (all times).

In the above analysis, the largest trajectory along PC1 occurred from d-1 through d38 and the trajectory from d38 through d97 occurred along PC2 (Fig. 5B). It is worth noting that this result was independent of 'group' in this model. Taken together, figures 3, 4 and 5 suggested that the changes in urinary metabolite profiles were greatest from BL through d38 (exposure period), with much smaller changes occurring from d38 through d97 (recovery period). However, this was true for all exposure groups and cannot be associated with the exposures. We hypothesize that this was due to stress imposed by handling of the animals (and transport of the animals) during this phase of the experimental time course. Any effects due to exposures to sand +/- emissions was much smaller than the stress induced effects observed, and are difficult to 'tease out' in the present experimental design. However, as shown below, we have uncovered some effects that we believe were due to exposures.

As mentioned above, the G2 data showed some anomalies in the PCA scores plots or possible outliers, which may obscure the effects of sand exposure. Three of the four outliers were observed during the recovery phase (d97 in Fig. 3). It is conceivable that any deleterious effects from sand exposure would be observed earlier during the experimental protocol, rather than after some 60 days following the exposure. Indeed, finding some distinctions between treated and control groups during the time of exposure proved to be rather challenging. However, some differences were found using a 'paired analysis.' This particular type of analysis emphasizes the change in metabolite profile within each animal referenced to a specific time point. This type of analysis may help to suppress the changes due to 'time' (or stress) since it considers the change for each animal from one time point to another. Thus, all of the effects reported below were found using a paired analysis, where data were paired-by d33 as a reference point. It should be noted that d33 corresponds to an acclamation period after completion of the sand exposure, but before the start of emissions exposure. The following figures summarize our findings.

The control group (G1) and emission only group (G4) served as the control from BL through d33 (no sand; S-; n=16), while the sand only group (G2) and sand & emission group (G3) were intermittently exposed to sand (S+; n=16) from d1 through d28. The last urine collection immediately following sand exposure was on d29. Therefore, d29 for S- and S+ groups was modeled, but data were paired-by d33. This paired-by analysis yielded a difference spectrum (d29 – d33) emphasizing the change between these two time-points (Fig. 6).

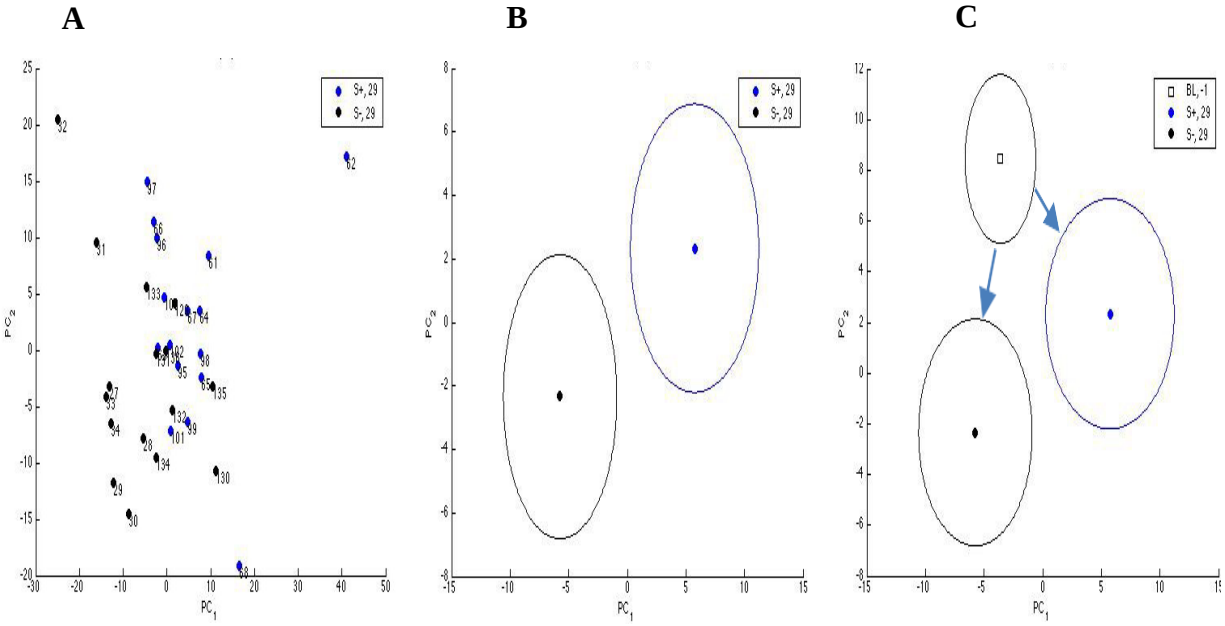

Figure 6. The PCA scores plots show all data points (A), the centroid mean ± 2SEM (B), and the same plot with the BL time point superimposed into the model (C). Model = S+S- at d29, paired-by d33; AS = S+S- at d29. The arrows depict the trajectory from BL through d29 for S-(G1 and G4) and S+ (G2 and G3)

A clear separation between the sand-treated and control groups, mostly along PC1, was observed (Fig. 6B). Approximately 33% of the total variance was captured in PC1 + PC2. The BL time included both groups (G1 and G4) at d-2 and d-1, and also separated from the groups at d29 (Fig. 6C). A similar PCA analysis as that described above in Figure 6 was performed using d10 and d17 superimposed into the model (Fig. 7). These earlier time points were within the sand exposure phase for which urine samples were obtained.

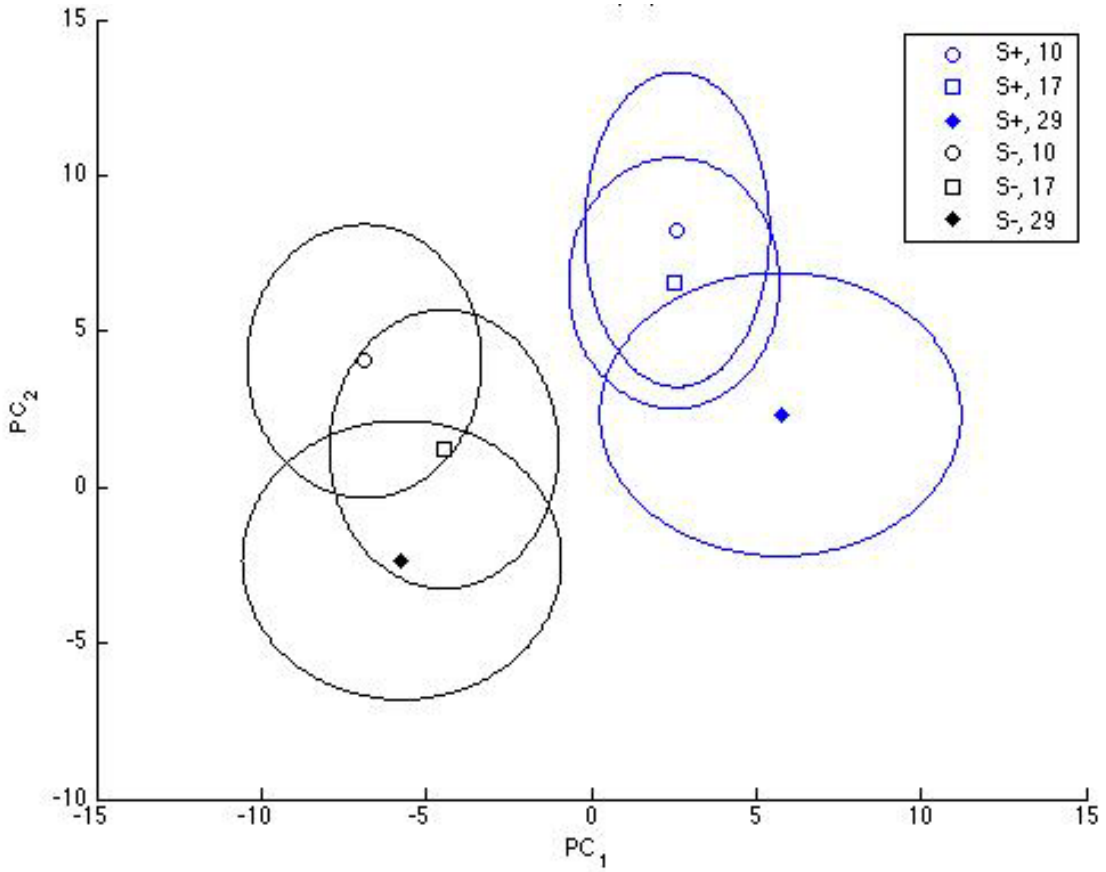

Figure 7. PCA scores plot using the same paired-by analysis described in Figure 6, but with d10 and d17 superimposed into the model. Data are represented as ellipses around a centroid mean ± 2SEM. Model = S+S- at d29, paired-by d33; AS = S+S- at d29; d10 and d17 are superimposed.

Although the three time points (d10, d17, d29) do not separate from one another within each exposure group, the two exposure groups (S- and S+) do remain separated across all time points (Fig. 7). This finding further supports the interpretation that potentially significant effects due to sand exposure were observed. This is further illustrated in Figure 8 below.

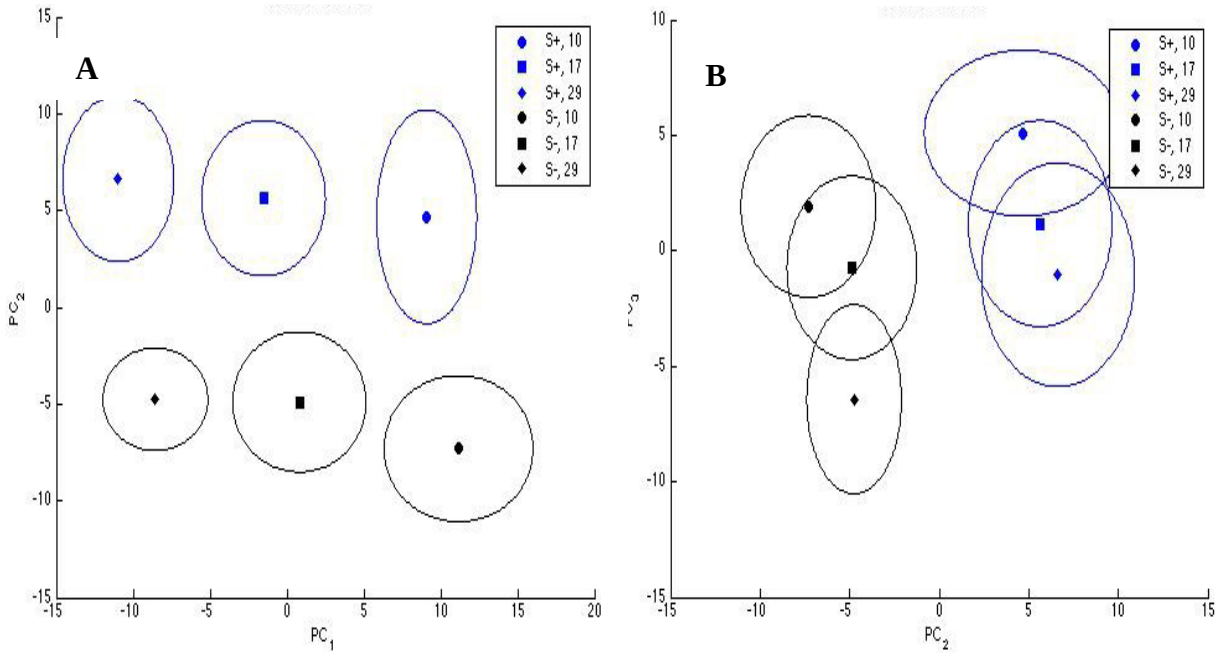

Figure 8. PCA Scores plots generated using three time points during sand exposure (d10, d17, d29). Exposure groups included the S- groups (G1 and G4; n=16) and S+ groups (G2 and G3; n=16). Data are represented as ellipses around a centroid mean ± 2SEM. A; PC1 *vs.* PC2. B; PC2 *vs.* PC3. These data were paired-by d33. Model = S+S- at d(10, 17, 29) paired-by d33; AS = S+S- at d29.

The major effect, or trajectory along PC1, was due to time as the data points moved from d10 through d17, and d17 through d29 (Fig. 8). This was true for both exposure groups (S- and S+). However, the exposed and unexposed groups separated along PC2. This was captured in the plot of PC2 *vs.* PC3 (Fig. 8B), where the 'time effect' was removed and the more subtle effects due to treatment become more evident.

Using the data derived from the PCA scores plot of Figure 6, which showed clear separation, an OPLS-DA was then performed on the S- and S+ groups at d29. The resulting T-score plot and plot of significant NMR metabolite bins is shown in Figure 9 below.

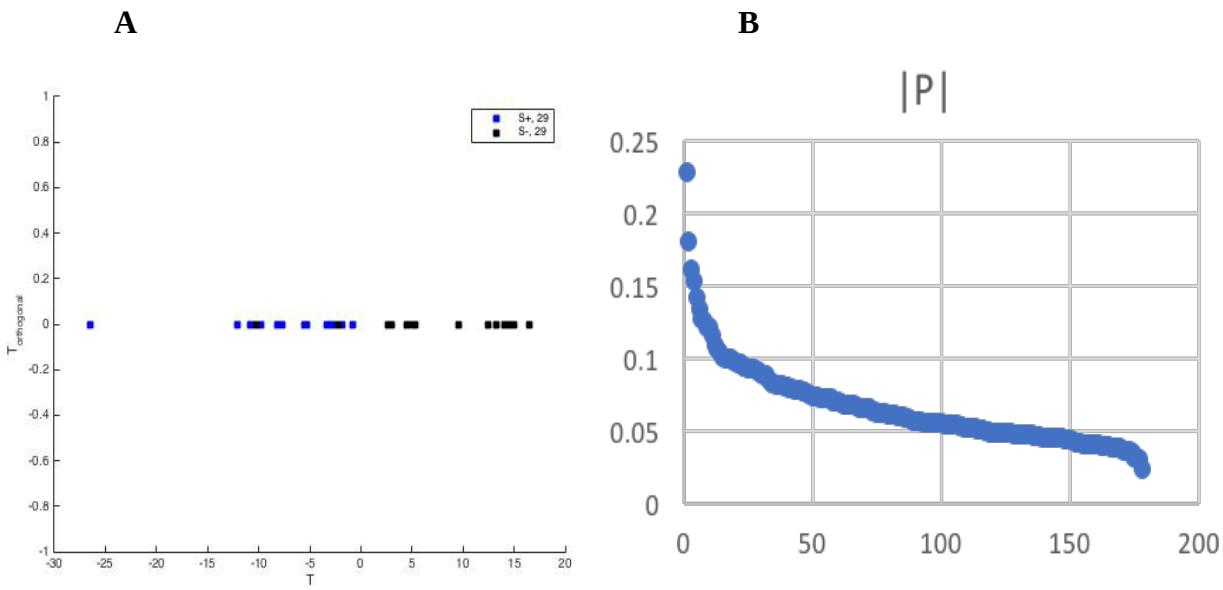

Figure 9. OPLS-DA on the S- and S+ exposure groups at d29. A; T-scores plot (Q^2 = 0.399), significant separation at p < 0.01. B; Plot of 178 significant metabolite bins identified from NMR analysis. OPLS-DA Model = S+ S- at d29, paired-by d33; AS = S+S- (d29).

Although the Q^2 value was found to be fairly low (0.399), the classification of S- and S+ exposure groups was statistically significant at p<0.01 (threshold Q^2 = 0.217). This was determined by computing a Q^2 distribution using 10,000 permutations. In addition, the area under the Rock curve (AUC) was found to be 0.910, and the accuracy was determined to be 100% by the leave-one-out cross-validation method. This analysis resulted in 178 significant variables (bins), which are plotted in rank order from most important to least important (Fig. 9B).

Although the classification of S- and S+ exposure groups at d29 was significant (p<0.01), there were overlapping data points in the T-score plot (Figure 9A) and the number of significant variables was considerably high (178). Therefore, many small differences in the NMR spectrum (metabolite profiles) contributed to this classification. However, as observed in the plot of significant features (Fig. 9B), the first ~15 points show a much steeper slope than the remainder of the data points; this indicates their relative greater importance. So, it may be possible to determine a few of the metabolites that were important factors for this exposure group classification. Although a more detailed analysis of the NMR spectra has not yet been performed, the top 12 features (NMR chemical shift values, δ, in ppm) are listed in Table 2 below.

Table 2. NMR chemical shift values, δ, in ppm with values derived from OPLS-DA on S- and S+ at d29. Most significant features listed in order from left to right.

δ, (ppm**)**	6.06	3.98	5.99	5.97	5.79	5.55	5.54	5.60	3.35	6.85	2.17	5.59

To examine the effects of emissions exposure, we focused on d38, which corresponds to the last day during the exposure period. Animals received five consecutive days of emissions just prior to this urine collection at d38. The data were again paired-by d33 (the acclimation period just prior to entering the emissions-phase of the protocol); data was also auto-scaled to the d33 time point. PCA models were developed for both the emission exposed and the sand and emission exposed animal groups, with the sand exposed group superimposed into the PCA models (Fig. 10). In both models, rats exposed to burn pit emissions separate along PC1 from the non-exposed groups. Interestingly, when G2 (sand) and G3 (sand and emissions) groups are superimposed into the PCA model based on G1+G4 (Fig. 10A), G2 (sand) overlaps with G4 (emissions), while G3 (sand and emissions) plots between the exposed and non-exposed groups. This suggests that the effects of sand alone and emissions alone yield similar metabolite profiles (unexpected); this may have resulted due to the effects of stress. However, the exposure combination of sand and emissions yielded a different urinary metabolite profile (expected). Furthermore, if G1 and G3 were model, and the other groups are superimposed into the plot, then the result was slightly different (Fig. 10B). Here, all the exposed groups clearly separate from the non-exposed control group (G1), but no differences were observed for the exposed groups (all overlap). Interestingly, G3 (sand and emissions) yielded a rather large variability in this plot. A PCA plot was also generated modeling all groups at d38, and similar results were obtained where the treated groups (G2, G3, and G4) completely separated from the control group (data not shown).

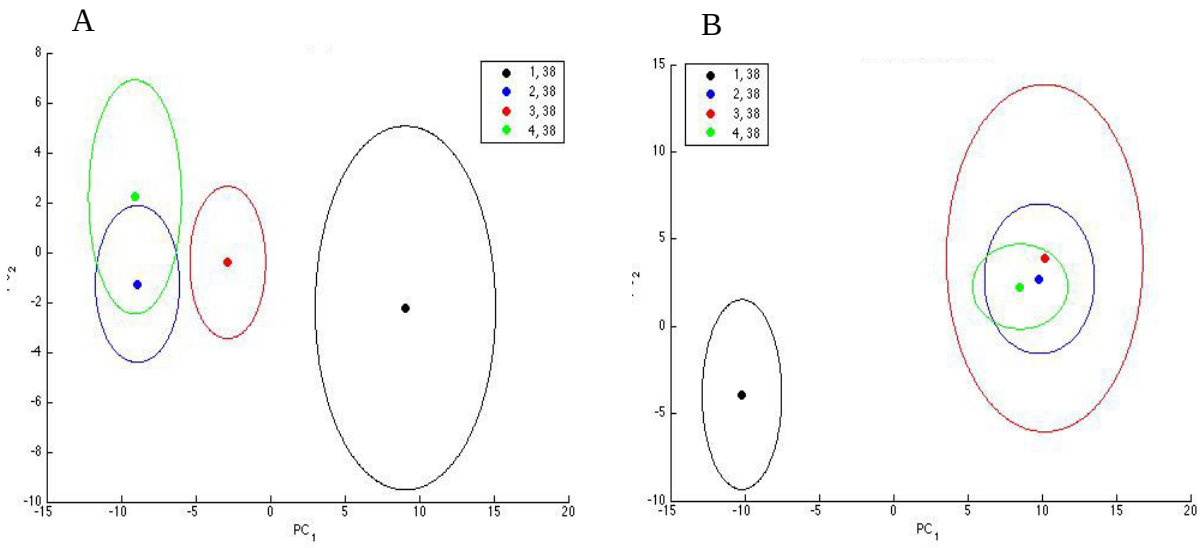

Figure 10. PCA scores plot using a similar paired-by analysis as described in Figure 6 using the same pair-by day (d33). Data are represented as ellipses around a centroid mean ± 2SEM. Colored ellipses identify G1 control (black), G2 sand only (blue), G3 sand and emission (red) and G4 emission only (green). A; PCA Model = G1 & G4 at d38 (paired-by d33); AS = G1 + G4 at d33 (both groups were identically treated up to this time point – no exposures). G2 and G3 were superimposed into the model. B; PCA Model = G1 & G3 at d38 (paired-by d33); Auto Scale = G1 at d33 (this group was not exposed). G2 and G4 were superimposed into the model.

Following the PCA, an OPLS-DA was conducted to classify the groups with regard to burn pit emissions exposure. This was initiated by performing a discriminate analysis for G1 *vs* G4 (Fig. 11A) and G1 *vs* G3 (Fig. 11B). These models tested whether the control was different from the group exposed to emissions alone (G4) or the combination of sand and emissions (G3). In both cases, the analysis was statistically significant and the T-score plots showed clear separation of groups without any overlapping data points. The analysis used 10,000 permutations and leave-one-out cross validation to determine the Q^2 distribution, p-value and accuracy of the model, respectively. Additionally, the significant features (variables) were determined using 1,000 permutation (alpha = 0.01).

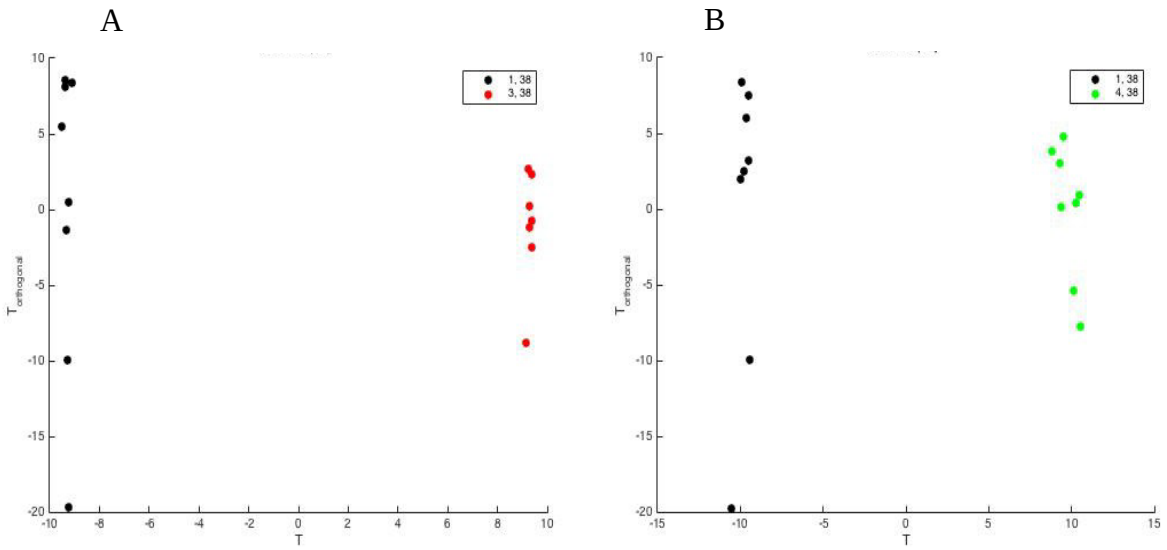

Figure 11. OPLS-DA scores plot using data from G1 *vs* G4 (A) and G1 *vs* G3 (B). A; G1 *vs* G3: Q2 = 0.872; AUC = 1.000; ($p<0.01$); accuracy = 100%. B; G1 *vs* G4: Q2 = 0.786; AUC = 1.000; ($p<0.01$); accuracy = 100%. Colored data points identify G1 control (black), G3 sand and emission (red) and G4 emission only (green).

Whether rats were exposed to emissions alone (G4), or exposed to both sand and emissions (G3), the data can be classified separately from the control group (non-exposed; G1). However, G3 and G4 groups could not be separated from one another. This was confirmed in a separate OPLS-DA in which all three groups (G1, G3, and G4) were included into the model (data not shown). The G1-G3 analysis yielded 37 significant variables (bins), while the G1-G4 analysis produced 66 significant variables. Among these variables, 21 were common to both analyses (Table 3). Possibly these common features were associated with effects due to emissions (the common treatment). Furthermore, 7 of the 21 bins (highlighted in yellow) were identical to significant features/bins identified from the sand analysis (Table 2). At present, we have not traced these variables (bins) to the actual NMR spectra to identify specific metabolites; the chemical shift values (δ in ppm) for these 21 bins are listed in Table 3.

Table 3. NMR chemical shift values, δ, in ppm with values derived from OPLS-DA of G1-G3 and G1-G4; paired-by d33. The 21 bins represented in the Table were common to both the G1-G3 and G1-G4 analyses. Most significant features listed in order from left to right. Seven of the identified significant peaks (highlighted) were the same as those identified for sand only exposures (see Table 2).

δ, (ppm)	2.96	5.54	5.79	5.58	5.59	5.60	5.55	3.80	6.06	5.99	5.57	5.56	5.97	8.00	7.96	3.98	1.09	6.50	2.68	5.39	3.21

The recovery time period (days 39-97) for which we had urine samples collected included days 39, 69, and 97. Day 39 was immediately following the last day of emissions exposure (d38).

Thus, d39, d69, and d97 can be designated as 0, 30, 58 days post-exposure, respectively. A PCA for all groups at each of these time points is shown below with the data auto-scaled to the unexposed animals (G1 and G4) on d33. At d39 (0 day post-exposure) the spread of the data points along PC1 was mostly driven by one G3 (sand and emission) animal (Fig. 12A; #96; red data point, far right). There were also several other rats belonging to G2 (sand), G3 (sand and emission), and G4 (emission) that were displaced from the main clustering of samples. Presumably, all these rats were showing effects due to exposures. At d69 and d97, the PC1 and PC2 plot scales were much smaller than that at d39 (Fig. 12B and C), indicating a tighter clustering and more uniform urinary profile. Surprisingly, at d97 there were several rats belonging to G2 (sand) that were displaced from most other animals (particularly #67 and #68; Fig. 12C). This was observed above in Fig 4 as well, where G2 showed a large variability at d97.

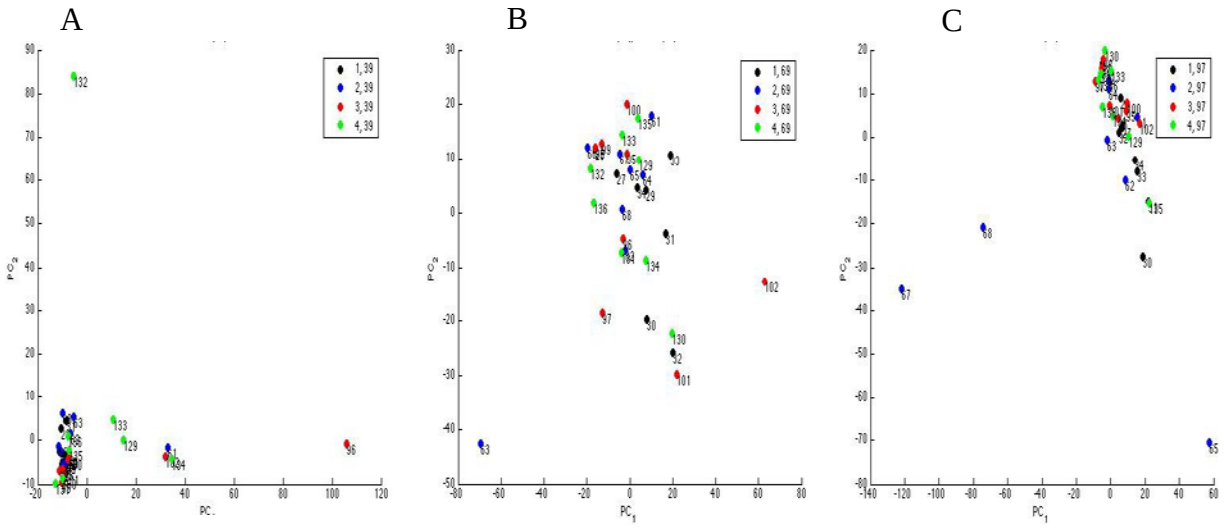

Figure 12. The PCA scores plots showing all subject data points for the recovery phase (d38-d97). A; Plot immediately following the last burn pit emission exposure (d38; 0d post-exposure). B; Plot 30 days following the last emission exposure (d69; 30 days post-exposure). C); Plot 58 days following the last emission exposure (d97; 58 days post-exposure). Colored data points identify G1 control (black), G3 sand and emission (red) and G4 emission only (green). Model = all Groups at d39 (A); at d69 (B); at d97 (C). AS = G1 + G4 at d33.

We further analyzed the recovery times by scaling the data to each specific time point for all exposure groups, or for G1 (control) only (Fig. 13). At d39, rat #96 (G3) is seemingly an outlier again when the data are scaled to the control group (G1; Fig. 13B). However, if the data are scaled to all groups, then this animal is clustered with all others (Fig. 13A). This animal was not an outlier at the later time points (d69 and d97). What was surprising, however, was the G2 (sand) animals at d97. The G2 exposure group showed a large variability, especially at d97 (Fig. 13E & F). Rats #67 and #68 were displaced from others in the group regardless of whether the data were auto-scaled to the control group (G1) or all exposure groups. These were the same rats that were outliers in Fig. 12 where the data were auto-scaled to the d33 time point. Therefore, these two sand-exposed rats were consistently proving to be uniquely different from others in

their group. Surprisingly, all of the G3 rats (exposed to both sand and emissions) appeared to recover by d97 (Fig. 13E & F).

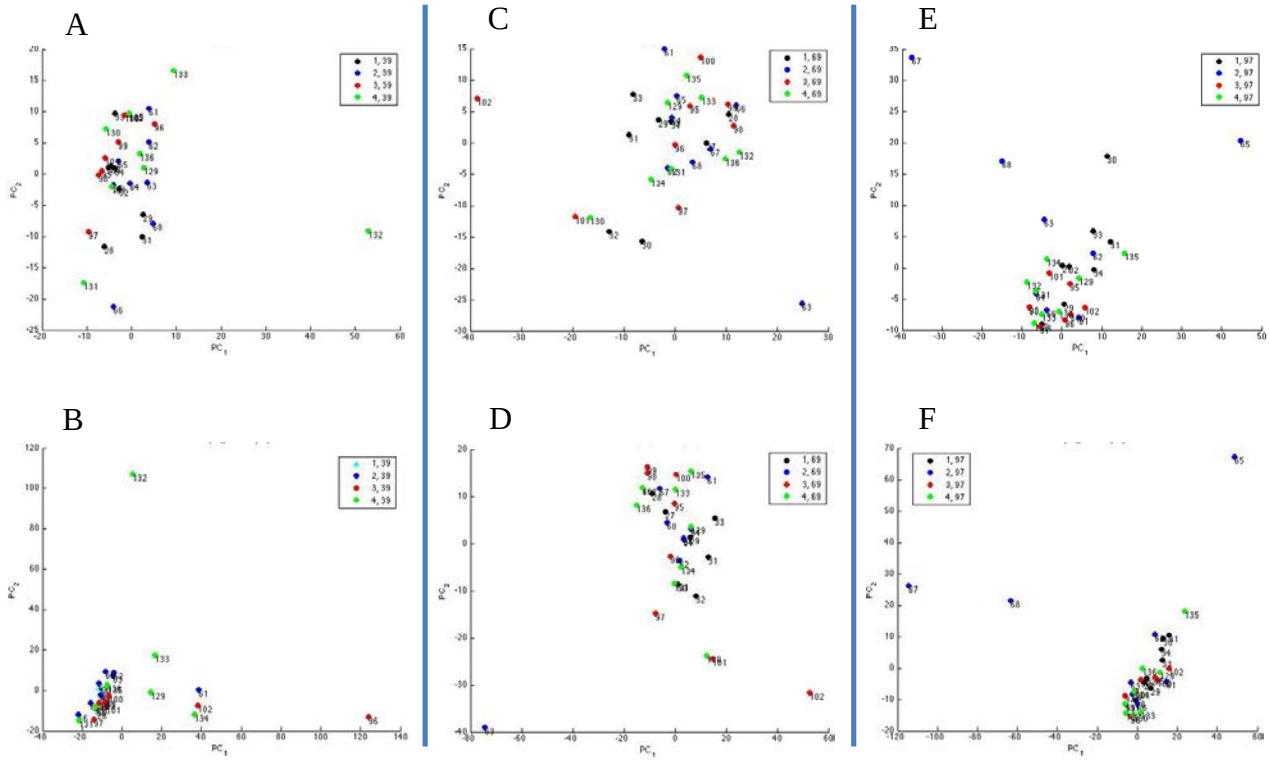

Figure 13. The PCA scores plots showing all subject data points for the recovery phase (d38-d97) auto scaled to all groups or control. A and B; Scores plots immediately following the last burn pit emission exposure (d38; 0d post-exposure). C and D; Scores plots 30 days following the last emission exposure (d69; 30 days post-exposure). E and F); Scores plots 58 days following the last emission exposure (d97; 58 days post-exposure). Scores plots A, C and E were auto-scaled (AS) to all exposure groups. Scores plots B, D, and F were AS to G1 controls only. Colored data points identify G1 control (black), G2 sand (blue), G3 sand and emission (red) and G4 emission only (green). Model = all Groups at d39 (A & B); at d69 (C & D) and at d97 (E & F). AS = all groups (A, C, & E) or G1control (B, D, & F).

The largest effects in urinary metabolites observed in this study resulting from sand and burn pit emission exposures were across time. Indeed, the largest trajectory in Principle Component space occurred from BL (d-1) through day 38, while a much smaller trajectory was observed from d38 to d97. This was clearly depicted in Figs. 3, 4 and 5. Therefore, it appears that the largest changes in urinary metabolite profiles occurred during the exposure timeframe, and much smaller urinary metabolite changes were observed during the recovery period. This same result was seen in the control group (G1), as well as the sand and emission exposure groups. Therefore, these changes in urinary metabolite profile effect were likely unrelated to exposures, and were rather an effect of stress due to animal handling and transport to the emission exposure facility during the exposure time course of the experimental protocol.

The sand exposure (G2) showed a high degree of variability in comparison to the other groups. At d97, all groups overlapped with the control (G1), but the variability for G2 was much larger than other groups (Figs. 2 and 13). Various other PCA plots also depicted this high degree of variability for G2 (Fig. 4). G2 samples #67 and #68 seemed to be consistent outliers in various PCA plots (Figs. 3 and 13). Effects due to sand and emission (G3) exposures become evident using a 'paired analysis,' which highlights changes in each animal relative to a specific time points.

Changes in urinary metabolites due to the effects of sand exposure were revealed when data were 'paired-by' d33, which was the acclimation time point following sand exposure and preceding the emissions exposure protocol. A PCA for d29 'paired-by d33' (Fig. 6) showed separate profiles for rats exposed to sand (S+) and those not exposed (S-). The d29 time point corresponded to the last day of sand exposure. OPLS-DA of these two groups (S+ and S-) yielded a significant result, but the Q^2 value was fairly low ($Q^2 = 0.399$; $p<0.01$), and a large number of features were needed to reach significance (178 bins; Fig. 9). In addition, the T-score plot showed overlapping data points indicating that this group classification was weak.

The effects of pseudo-burn pit emissions exposure, or sand and emission exposure, on urinary metabolite profiles were much more convincing than that observed for sand exposure. Again, the data were "paired-by" d33 for the analysis, which focused on changes immediately before exposure to emissions (d33) compared with post-exposure on d38. Day 38 was the final time point following five consecutive days of emissions exposure. PCA modeling of the control group (G1) and each of the treated groups (G3 or G4) at d38 showed that rats exposed to emissions clearly separated along PC1 from non-exposed animals (Fig. 10). Indeed, an OPLS-DA yielded highly significant results for group classification (Fig. 11; G1 *vs.* G4 (emissions only): $Q^2 = 0.786$; AUC = 1.000; ($p<0.01$); accuracy = 100%; G1 *vs.* G3 (sand and emissions): $Q^2 = 0.872$; AUC = 1.000; ($p<0.01$); accuracy = 100%). These OPLS-DAs yielded much fewer numbers of significant features (≤ 66) than what was observed for the sand analysis. There were 21 significant features/bins that were shared by the two models. This was a much more tractable problem, and will likely result in the identification of some of the metabolites that contributed to this classification in the future.

An analysis of the recovery time period (d39 – d97) allowed for an evaluation of possible persistent effects on urinary metabolite profiles due to exposure. As expected, the earliest recovery time point (d39) showed that several rats belonging to the exposure groups (G2, G3, and G4) were displaced from the main cluster of data points (Fig 13A and B). Day 39 was only one day post-emissions for G3 and G4, and 10 days post-sand exposure for G2. Four rats in G4 (#129, #132, #133 and #134) and two rats in G3 (#96 and #102) were outliers, presumably resulting from lingering effects due to exposures (Fig. 13). This was observed only when the data were scaled relative to the control group (G1; Fig 13B); these effects were resolved by the next time point at day 69 (Fig 13D). As noted above, G2 (sand) showed a high degree of variability, particularly during the late recovery time (d97; Fig. 13E and F). This was rather perplexing.

Therefore, another analysis was conducted to focus on the variability of G2 (sand) at d29, which was the time point corresponding to the end of the sand-exposure period (Fig 14).

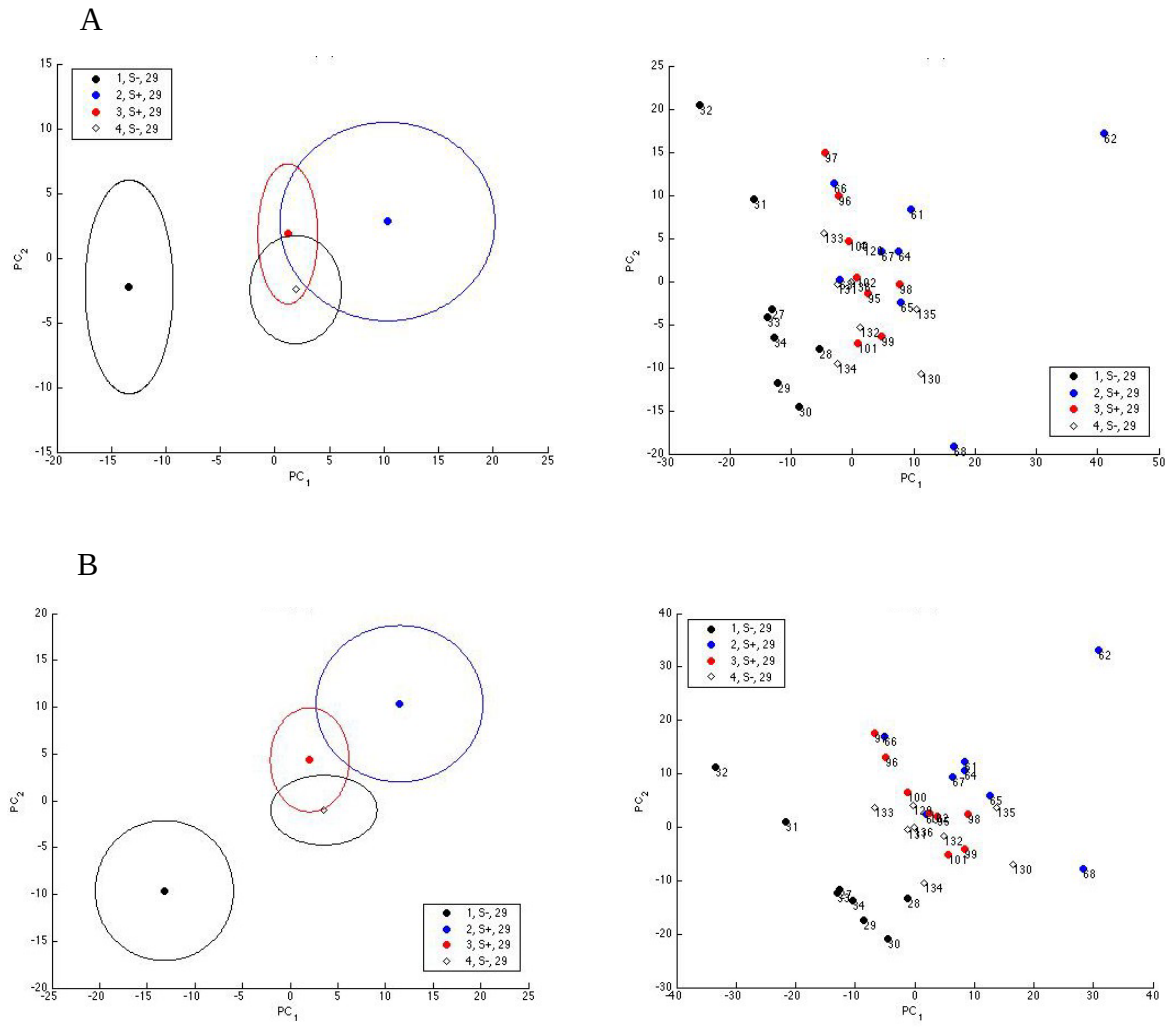

Figure 14. A PCA was constructed modeling all unexposed rats on day 29 (G1 + G4 at d29) and all sand exposed rats on day 29 (G3 + G4 at d29). The data were again paired-by d33, but were auto-scaled (AS) two different ways: A); scaled to all groups at d29 (Model = S+S-(d29), paired-by d33; AS = S+S-(d29)), and B); scaled to only the unexposed groups at d29 (Model = S+S-(d29), paired-by d33; AS = S-(d29); subject #62 was omitted from the PCA model, but superimposed into the plot). The PCA scores plots show the centroid mean ± 2SEM (left) and all data points (right). Colored data points identify G1 control (black), G2 sand (blue), G3 sand and emission (red) and G4 emission only (green).

Two features emerged in this final analysis (Figure 14): (1) G2 variability was greater than G3 variability (blue and red symbols, respectively), and (2) G1 and G4 showed separate clusters (filled and open black symbols, respectively). Groups G1 and G4 were unexposed 'controls' at this time point. With regard to the observation above in Figure 14B (right), animal #62 was an extreme outlier when the data were scaled to unexposed rats on d29 (S-(d29)), and resulted in it

having to be omitted from the model in order to observe any more subtle effects. Even with animal #62 superimposed into the plot, it was still the furthest displaced along PC1. Animal #62 was not one of the rats that was displaced from the main grouping on d97 of recovery (Fig. 13). Instead, animals #65, #67, and #68 were outliers at d97, and of these, animal #68 was the only one showing separation from the main group clustering at d29. However, animal #68 was not displaced on d39 or d69. Therefore, any differences in comparison to other rats in the sand-exposed groups (G2) did not appear to be due to an exposure effect. Instead, this group (G2) only showed a much greater variability in comparison to all other exposure groups. This may have resulted due to animal handling during the experimental protocol. The second observed feature from the above analysis showing separate clusters for G1 and G4 was also somewhat disconcerting, and again brings into question the experimental protocol. The control group (G1) and emission exposure group (G4) were unexposed during the timeframe from BL through d29. Therefore, these animals can serve as controls, and should display very similar urinary metabolite profiles. However, this was not observed. It is possible that if these rats were not handled identically during this timeframe of the experimental protocol and they would be expected to yield different urinary metabolite profiles.

5.0 CONCLUSIONS

To our knowledge, this is the first time that NMR-based urinary metabolomics has been used to assess the effects of sand and pseudo-burn pit emission exposure in rats. In summary, rats exposed to sand only, or sand and burn pit emissions, displayed major changes in urinary metabolite profiles that were attributed to stress (i.e. handling and transport). However, minor urinary metabolite profile changes due to exposures could be discerned through careful paired-analyses of the data. Further data analyses will be required prior to proceeding with a more detailed spectral analyses to attempt to identify salient metabolites or biomarkers of sand exposure and sand with emission exposures.

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LIST OF ACRONYMS

^{1}H	proton
AAALAC	Assessment and Accreditation of Laboratory Animal Care
ABT	ambient breeze tunnel
ACE	Army Corps of Engineers
AF	Air Force
AFIOH	Air Force Institute for Operational Health
AFRL	Air Force Research Laboratory
AS	auto scaled
AUC	area under the curve
BALF	bronchoalveolar lavage fluid
BL	baseline
CSL-1-U	Camp Slayer
DoD	Department of Defense
G1	control group
G2	sand only group
G3	sand and emission group
G4	emission group
GAO	Government Accountability Office
ICP-MS	inductively coupled plasma mass spectrometry
IACUC	Installation Animal Care and Use Committee
MHz	megahertz
MSW	municipal solid waste
NAMRU-D	Navy Aerospace Medical Research Unit-Dayton
NMR	Nuclear Magnetic Resonance
OECD	Organization for Economic Cooperation and Development
OPLS-DA	Orthogonal Projections onto Latent Structures Discriminate Analysis
PBMC	peripheral blood mononuclear cells
PCA	Principal Component Analysis
PM	particulate matter
Q^2	coefficient of prediction
RSC	Research Support Center
SEM	standard error of mean
SWPM	Southwest Asian Particulate Matter
TSP	trimethylsilylpropionic acid
USACHPPM	US Army Center for Health Promotion and Preventive Medicine
WPAFB	Wright-Patterson Air Force Base