Measurement of Urinary 1-Nitropyrene Metabolites as Biomarkers of Exposure to Diesel Exhaust in Underground Miners

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Abstract

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Exposure to diesel exhaust (DE) is prevalent in both occupational and environmental settings and has been associated with several adverse health outcomes including cancer and respiratory and cardiovascular disease. The ability to accurately quantify DE levels is therefore crucial for understanding and controlling exposures. DE is a complex mixture of particulate matter and gaseous components which complicates exposure measurement. Current methods rely on the use of elemental carbon (EC) to monitor DE exposure, however, EC is formed by multiple other sources in addition to DE which introduces the potential for exposure misclassification. 1-Nitropyrene (1-NP), a chemical component specific to DE, has been proposed as a potential marker for exposure to DE in air and biological samples. In this study the suitability of 1-NP urinary metabolites as biomarkers for monitoring occupational exposure to DE in underground miners was evaluated.

The study took place in a large underground metal mine that makes extensive use of diesel engines. Air and urine samples were collected from a cohort of 20 miners who performed a variety of underground and surface jobs within the mine. Four sampling campaigns were conducted, each 2-3 months apart. During each campaign personal air samples, pre- and post-shift urine, and job task/activity surveys were collected for each subject. Air samples (n=103) were analyzed for EC and 1-NP. Urine samples (n=170) were analyzed for 1-NP metabolites using an HPLC-MS/MS assay. The association between 1-NP metabolites in urine and exposure to

1-NP in air was assessed using a regression model to determine if 1-NP urinary metabolites are a suitable biomarker for DE. Additionally, the suitability of survey data as a surrogate estimate for DE exposure was evaluated using a predictive model for 1-NP metabolites based on job and time-activity covariates.

A range of EC and 1-NP exposures were observed within this cohort (EC: GM = 8.5 $\mu g/m^3$, GSD = 2.5 $\mu g/m^3$; 1-NP: GM = 47 pg/m^3, GSD = 2.9 pg/m^3). Levels of EC, 1-NP, and urinary metabolites in this cohort were high relative to environmental exposures, but were within the range of reported occupational levels. Underground workers tended to have higher 1-NP and EC exposures than surface workers, however none of the miners were overexposed to DE using the MSHA standard for EC. A predictive mixed effects model was generated to estimate exposure to 1-NP in air on unmonitored work shifts. This model included terms for time spent underground and time spent working around diesel exhaust as well as subject-specific random effects. The out-of-sample R^2 (RMSE) was 0.41 (0.80) for this model.

Of the measured 1-NP metabolites 6-OHNP and 8-OHNP were detected at the highest levels (6-OHNP: GM = 0.13 pg/mg creatinine, GSD = 2.9 pg/mg creatinine; 8-OHNP: GM = 0.006 pg/mg creatinine, GSD = 2.8 pg/mg creatinine). Very few workers reported off-shift exposure to DE, suggesting that metabolite levels reflect occupational exposures. A significant trend for increasing metabolite levels with day of work week was observed indicating that uptake, elimination, or both of 1-NP is delayed relative to the within-day variability in occupational exposure. To account for this delay an association model was developed that paired urine samples with air exposures lagged by 1 day. This model estimated that for every doubling of exposure to 1-NP on the day before sample collection there would be a 12% increase in geometric mean 6-OHNP concentration and a 10% increase in geometric mean 8-OHNP concentration.

A predictive model for estimating post-shift levels for 6-OHNP and 8-OHNP was developed. The models including job location, time exposed to diesel exhaust, respirator use, time since previous void, and day of week provided the most practical approach to estimating metabolite levels. These models had relatively poor out of sample predictive ability but were capable of identifying general trends between metabolite levels and predictors.

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INTRODUCTION

Background

Exposure to diesel exhaust (DE) is ubiquitous in both occupational and environmental contexts and has been associated with multiple adverse health outcomes. DE has been linked with respiratory and cardiovascular disease and was classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC) based on observed associations between exposure to DE and incidence of lung cancer (EPA, 2002; IARC, 2012). Although several epidemiological studies have reported associations between these adverse health outcomes and DE, their ability to quantitatively assess exposure levels has been limited. Due to the pervasiveness of diesel as a fuel source a large number of individuals are potentially exposed to DE on a daily basis, making the ability to accurately quantify DE levels crucial. Without a quantitative exposure measure for DE understanding the relationship between risk and exposure and ensuring that people are not exposed to hazardous levels of DE becomes extremely difficult.

Measurement of Exposure to Diesel Exhaust

DE is a complex mixture of particulate matter and vapor phase components making identification of a single measure of exposure difficult. Multiple agents have traditionally been used to measure DE including; elemental carbon (EC), organic carbon (OC), particulate matter (PM), carbon monoxide (CO), and oxides of nitrogen (NO_x) (MSHA, 2001; Pronk, Coble, & Stewart, 2009). However, many of these agents are also produced by other combustion sources or fuels and none are specific to DE, leaving the potential for exposure misclassification. Additionally, the correlation between each of these traditional exposure measures can vary greatly depending on engine load, exhaust treatment, and fuel composition among others which can make comparisons across studies difficult (EPA, 2002).

There is also limited consensus on measurement of exposure to DE in regulatory settings. The Environmental Protection Agency (EPA) regulates fuel and emissions for on road and non-road diesel engines. However, emissions requirements are for PM, NO_x, and CO all of which are non-specific components of DE (EPA, 2010). Similarly, the Occupational Safety and Health Administration (OSHA) does not have a unique standard or specific sampling method for diesel exhaust, although there are separate standards for various components

of DE including total particulate matter, CO_2, CO, NO_x species, and SO_2 (OSHA, 2014). The National Institute for Occupational Safety and Health (NIOSH) also has no recommended exposure limit specific to DE, however, NIOSH has listed DE as a potential occupational carcinogen with the general statement that exposure levels be kept as low as can be feasibly achieved using engineering controls. NIOSH has also proposed a method for measuring exposure to DE using diesel particulate matter (DPM) as EC (NIOSH, 2010).

The Mine Safety and Health Administration (MSHA) is the only regulatory body to have a unique standard for exposure to DE. Similar to the method proposed by NIOSH, exposure is determined using DPM, however, DPM is measured as total carbon (TC) rather than EC. MSHA has set a permissible exposure limit for DPM as 160μg/m^3 of TC. Compliance determination for this standard is not straightforward and requires using both EC and TC measurements from personal air samples and the ratio of TC to EC from an air sample taken from an area without diesel exhaust in order to adjust for non-diesel OC interferences (MSHA, 2001). Additionally this standard only applies to underground metal/non-metal for mines. MSHA has published separate standards for measurement of diesel exhaust and determination of compliance for surface mines and coal mines. It is therefore clear that in order to adequately measure exposure and protect the health of exposed individuals a more specific marker for DE is needed.

1-Nitropyrene as a Specific Marker for Diesel Exhaust

Nitro-polycyclic aromatic hydrocarbons (NPAHs), including 1-nitropyrene (1-NP), are among the byproducts formed during combustion of diesel fuel. 1-NP has been proposed as a marker for DE because it is enriched in DE relative to other combustion sources, it is present in DE at higher levels than other particle-associated NPAHs, and it is not formed to a significant extent through atmospheric photochemical reactions (Bamford, Bezabeh, Schantz, Wise, & Baker, 2003; Toriba et al., 2007). Because 1-NP is enriched in DE relative to other combustion sources and is present at higher levels than other NPAHs it is much more specific for diesel than other measures and is readily measured in DE (IARC, 2013b; Miller-Schulze et al., 2010, 2013; Toriba et al., 2007). Additionally, many NPAHs are mutagenic/carcinogenic and 1-NP has been implicated as one of the primary compounds contributing to the direct-acting mutagenicity of DE (Toriba et al., 2007). Therefore, use

of 1-NP could not only provide a more specific measure of DE exposure but more accurately reflect the carcinogenic properties of DE as well.

1-Nitropyrene Metabolites as Biomarkers of Exposure to Diesel Exhaust

In addition to the high specificity of 1-NP for DE, metabolic products of 1-NP can be measured in biological samples which introduces the potential for biological monitoring of internal DE dose. Biomarkers have shown considerable promise for exposure monitoring, especially for products like DE where the exposure is prevalent with multiple potential sources (Miller-Schulze et al., 2013). Health effects are related to the internal dose which is a consequence of total exposure. Because biomarkers integrate across all exposure pathways they are able to more accurately reflect dose as opposed to environmental samples which may capture only part of the exposure. However, it is important to note that the integrative nature of biomarkers can be a limitation if identification of dominant exposure sources is a goal of the exposure assessment.

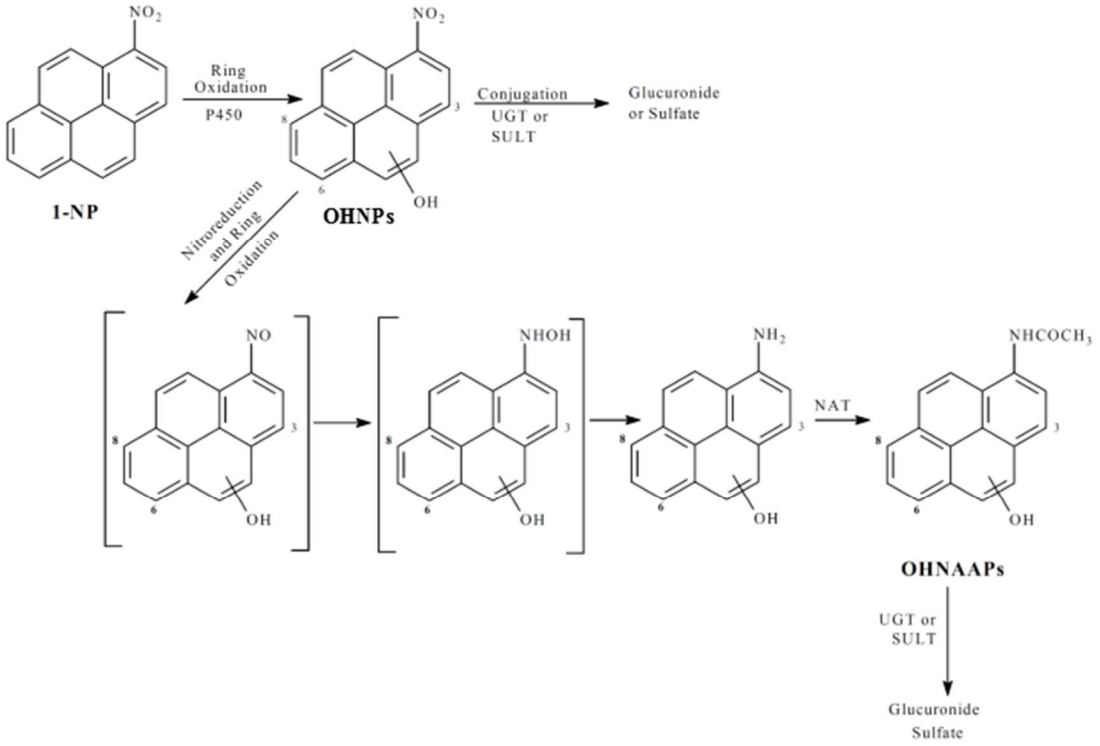

Figure 2: Major metabolites of 1-NP due to metabolism by cytochrome P450s, N-acetyltransferase (NAT), UDP-glucuronyltransfere (UGT), and sulfotransferase (SULT) (IARC, 2013).

Metabolism of 1-NP has been studied in various tissue and animal models and appears to proceed through two pathways: nitroreduction and cytochrome P450-mediated ring oxidation (Figure 1) (IARC, 2013). Excretion is

primarily through the feces, although metabolites in the form of glucuronide and sulfate conjugates are excreted in the urine. Nine metabolites have been detected in urine in *in vivo* studies; hydroxy-1-nitropyrenes (3-, 6-, and 8-OHNP), hydroxyl-*N*-acetyl-1-aminopyrenes (3-, 6-, and 8-OHNAAP), *N*-acetyl-1-aminopyrene (NAAP), 1-aminopyrene (1-AP), and *trans*-4,5-dihyro-4,5-dihydroxy-1-nitropyrene (Table 1).

For studies that measured multiple urinary metabolites only 6-OHNP, 8-OHNP, and 8-OHNAAP were consistently present at appreciable amounts in human urine samples (Table 1) (Miller-Schulze et al., 2013; Toriba et al., 2007). Three studies reported high levels of 1-AP (Laumbach et al., 2008; Neophytou et al., 2014; Seidel, Dahmann, Krekeler, & Jacob, 2002). 1-AP was not detected, or was only detected at very low levels in the studies that measured the levels of multiple metabolites (Miller-Schulze et al., 2013; Toriba et al., 2007). However, only two studies used the same sample preparation and analytical methods. Differences in measured levels could therefore reflect differences in sensitivity and specificity of the methods in addition to, or in place of, true differences in metabolite amounts (Table 1).

Underground Miners as a Study Population

Underground miners are a particularly vulnerable population for exposure to DE. The highest occupational DE exposure levels were observed in underground work sites using heavy equipment, such as mines and underground construction (Coble et al., 2010; Pronk et al., 2009; Stewart et al., 2010; Vermeulen, Coble, Lubin, et al., 2010; Vermeulen, Coble, Yereb, et al., 2010). However, exposures can be highly variable and are largely dependent on ventilation and exhaust treatment devices (EPA, 2002; Pronk et al., 2009; Stewart et al., 2010). Several previous studies have looked into the association between exposure to DE and adverse health outcomes within mine cohorts.

The association between exposure to DE and development of lung cancer shown in the Diesel Exhaust in Miners Study (DEMS) was highly influential in the 2012 decision of IARC to classify DE as a human carcinogen. The authors selected respirable elemental carbon (REC) as the primary DE exposure measure. Because historical REC measurements were unavailable the authors used previously measured CO levels along with REC and CO measurements made at the time of the study to predict retrospective exposures (Coble et al., 2010; Stewart et al., 2010; Vermeulen, Coble, Lubin, et al., 2010; Vermeulen, Coble, Yereb, et al., 2010).

However, a main criticism of DEMS was use of this extrapolation due to the issues with correlation between REC and CO within and between diesel engines. Although REC is currently used in conjunction with TC for determining exposure to DE in underground miners there is the potential for interference from non-diesel sources, which is reflected in the complexity of compliance determination under the MSHA DPM standard (MSHA, 2001).

A separate study evaluated urinary biomarkers in a small cohort of salt miners occupationally exposed to DE (Seidel et al., 2002). However, the authors' were unable to detect an association between air and biological levels of 1-NP metabolites. This could be due to the authors' selection of 1-AP as the target metabolite for 1-NP as some studies have shown that 1-AP is present at much lower levels than other metabolites (Table 1) (Seidel et al., 2002; Toriba et al., 2007; van Bekkum, van den Broek, Scheepers, & Bos, 1998). However, other studies have shown that 1-AP is present at much higher levels so the lack of association could reflect limited sensitivity of the analytical methods available at that time (Table 1) (Laumbach et al., 2008; Neophytou et al., 2014). Seidel and colleagues also observed a variation in the levels of metabolites for multiple DE constituents, including some 1-NP metabolites, by smoking status introducing the possibility that smoking could modify the metabolism of DE constituents (Seidel et al., 2002).

Motivation and Specific Aims

Diesel exhaust (DE) is classified as a Group 1 carcinogen by IARC as a result of studies indicating that exposure to DE is associated with the development of lung cancer (IARC, 2013). In addition to carcinogenicity, exposure to DE has also been associated with respiratory and cardiovascular disease (EPA, 2002). Although several epidemiological studies have reported associations between these adverse health outcomes with exposure to DE, the quantification of exposure is limited. Multiple agents are currently used to monitor DE exposure including EC, TC, PM, CO, and NO_x species (Pronk et al., 2009). However, there is limited consensus on the best agent to measure DE in scientific and regulatory settings. Additionally, none of these agents are specific for DE leaving the potential for exposure misclassification. In order to adequately measure exposure and protect the health of exposed individuals a more specific marker for DE is needed.
1- Nitropyrene is a DE specific chemical and has been proposed as a potential marker for exposure to DE in

Table 1: Previously Reported 1-NP Biomarker Levels

Author	Toriba	Miller-Schulze		Laumbach		Seidel	Neophytou [a]	Zwirner-Baier [b]		
Year	2007	2013		2008		2002	2014	1999		
Subjects	n = 22	n = 24		n = 55; 38 analyzed		n = 18	n = 95	n = 29	n = 20	n = 14
Location	Japan	China	Seattle	Canada			Northeastern US			
Exposure Type	Env	Occ		Controlled		Occ	Occ	Occ	Env	Env
Setting	Students	Taxi Drivers		Exposed	Unexposed	Underground Mine	Truck Drivers	Bus Garage	Urban	Rural
Analytical Method	HPLC-MS/MS [c]	HPLC-MS/MS [c]		HPLC-FD [d]		GC-MS [e]	HPLC-FD [f]	GC-MS [g]		
Air (ng/m3)										
1-NP		0.022 (0.011) – 0.097 (0.031)		2.68 (0.51)	ND < 0.006	0.50 – 3.00				
Urinary Metabolite (ng/g creatinine)										
3-OHNAAP	< DL									
6-OHNAAP	0.28 (0.26)									
8-OHNAAP	0.27 (0.22)	0.28 (0.20)	0.11 (0.14)							
3-OHNP	0.01 (0.02)									
6-OHNP	0.47 (0.27)	1.68 (1.52)	0.56 (0.70)							
8-OHNP	0.32 (0.16)	1.74 (1.53)	0.57 (0.69)							
OHNAAPs (total)	0.55 (0.45)									
OHNPs (total)	0.79 (0.42)									
1-AP	< DL			324.0 (442.4)	234.4 (852.9)	2-200 ng/24hr urine	28.7			
Hemoglobin Adduct (ng/g Hb)										
1-AP								0.028	0.035	0.022

values are mean (SD) unless otherwise noted

[a] average of medians, approximated from box plot

[b] median

[c – d] Additional information about analytical methods in Appendix Table 1

both air and biological samples (Miller-Schulze et al., 2010, 2013; Toriba et al., 2007). In this study we measured 1-NP metabolic products as DE-specific urinary biomarkers for monitoring exposure to DE in underground miners. The specific aims are:

(1) Compare levels of 1-NP metabolites in urine with 1-NP levels in personal air samples to determine the association between urinary metabolites of 1-NP and exposure to DE;

(2) Compare levels of 1-NP metabolites in urine with responses to job task surveys to determine if job classification and time-activity logs are reliable surrogate estimates of personal exposure to DE, as measured by 1-NP metabolites.

To address these aims a cohort of underground miners has been enrolled in the study and personal and biological monitoring of DE exposure was carried out. The outcomes for these specific aims will provide an important step forward in accurately monitoring and measuring human exposure to DE and the resulting internal dose. Improved measurement techniques for exposure and dose should pave the way for both an increased understanding of the adverse health effects associated with DE, and an improved ability to control exposures and reduce the associated adverse health effects.

METHODS

Study Setting and Subjects

This research focuses on a subset of the aims for a larger study under the direction of Dr. Christopher Simpson at the University of Washington and Dr. Dale Stephenson with Boise State University. The study is set in a large underground metal mine that employs approximately 1,300 workers and is capable of processing over 2,000 tons of ore per day. The mine makes extensive use of diesel engines for both surface and underground work. This study was an observational cohort design, 20 miners belonging to the same work group were selected from a pool of volunteers. The study subjects were selected from several job types and locations, including the mine face underground, maintenance and repair shops, and surface, to increase the likelihood of observing diversity in exposure and to improve the ability to estimate biomarker response over a range of exposure levels. Subjects were excluded if they had diagnosed kidney, liver, or bladder disease as these factors

could potentially influence 1-NP metabolism and the urinary levels of metabolites. Prior to sample collection industrial hygienists employed by the mine were consulted so that miners could be assigned to qualitative high, medium, or low exposure groups based on expert judgment and prior sampling by Stephenson et al (Stephensen, Spear, & Lutte, 2006). Demographic information for study subjects along with their job title, work location, and assigned exposure group is presented in Tables 2 and 3.

Table 2: Study Subject Job Title, Job Location, and Assigned Exposure Group

Subject ID	Job Title	Job Location	Exposure Group
1	Muck Hauler	Face	Medium
2	Sandplant Operator	Surface	Medium
3	Operator	Face	High
4	Miner I	Face	High
5	Miner III	Face	Low
6	Miner I	Face	High
7	Geologist	Surface & Face	Medium
8	Miner I	Face	High
9	Mechanic	Shop	Low
10	Diamond Driller	Face	Low
11	Electrician	Shop	Medium
12	Miner I	Face	High
13	Miner I	Face	High
14	Stationary Mechanic	Shop	Low
15	Operator	Face	Medium
16	Raise Bore I - Driller	Face	High
17	Geologist	Surface & Face	Low
18	Beat Mechanic	Shop	High
19	Surface Mill Operator	Surface	Low
20	Miner	Face	High

Table 3: Study Cohort Demographics by Exposure Group

	High n=9 (45%)	Medium n=5 (25%)	Low n=6 (30%)	All n=20
Age (years) *	40 (10, 30 – 59)	43 (9, 28 – 50)	47 (11, 36 – 60)	43 (10, 28 – 60)
Gender (% Male)	100%	60%	100%	90%
Years at Mine *	11 (8, 2 – 26)	3 (3, 1 – 9)	9 (6, 2 – 14)	9 (7, 1 – 26)
Years in Mining *	12 (7, 6 – 26)	5 (5, 1 – 13)	16 (14, 2 – 34)	11 (10, 1 – 34)
Smoke Cigarettes	11%	40%	50%	30%
Use Chewing Tobacco	11%	0%	17%	10%

* mean (standard deviation, min – max)

Most of the miners at this facility worked rotating shifts, with all the miners within a work group on the same schedule. The miners would alternate between day and night shifts, working 4 days followed by 4 days off. Each shift lasted 12 hours. Four sampling campaigns were carried out 2-3 months apart between March, 2014

and October, 2014. All sampling campaigns were scheduled for when the cohort miners were working day shifts. During each campaign personal air samples, pre-shift and post-shift urine samples, and job task/activity surveys were collected for each subject (Appendix II). Urine samples and surveys were collected daily. Due to a limited amount of personal air sampling equipment the personal air samples were only collected two of the four days for each subject (Table 4). Personal air samples were collected near the worker's breathing zone using co-located GS-1 and GS-3 respirable cyclones. A quartz fiber filter was used with the MSHA DPM impactor and GS-1 respirable cyclone to measure DPM components, including EC. Pump flow rate was calibrated to 1.7L/min. The 1-NP samples were collected using a 37mm Teflon filter with GS-3 respirable cyclones at a pump flow rate of 2.75L/min. All air samples were collected using SKC AirChek and PXCR personal sampling pumps. However, due to leakage problems with the Teflon filters discovered after sample collection the quartz filters were used to measure 1-NP in place of the Teflon.

The total urine void volume and specific gravity were measured in the field. When possible, samples of 110mL were taken from total void and the remainder of the urine was discarded. If the void volume was less than 110mL the entire volume was kept. Duplicate urine samples were collected for some urine voids with high volumes. Approximately 2 duplicates were collected for each set of pre-shift samples and each set of post-shift samples. The urine samples were stored and transported on dry ice and were frozen for long-term storage.

Table 4: Typical Sample Collection Schedule*

	Personal Air Sample	Pre-shift Urine Sample	Post-Shift Urine Sample	Job Task/Activity Survey
Day 1	Subjects 01 – 10	All Subjects	All Subjects	All Subjects
Day 2	Subjects 11 – 20	All Subjects	All Subjects	All Subjects
Day 3	Subjects 01 – 10	All Subjects	All Subjects	All Subjects
Day 4	Subjects 11 – 20	All Subjects	All Subjects	All Subjects

*Air sample collection occasionally varied to accommodate subject absences or other changes in work schedule

Selection of Urine Samples for Analysis

A total of 535 urine samples and 51 duplicate samples were collected over the four campaigns, each subject gave an average of 32 samples (range: 8 – 39). Of the 535 unique samples 170 were selected for measurement of 1-NP metabolite levels. Subjects 5 and 16 were excluded as they had dropped out of the study after the first session. Urine samples were eliminated if the urine void volume was less than 50mL. The remaining samples were divided into three pools for selection:

1. Pre-shift, day 1 of work week
2. Post-shift, day with valid personal air sample
3. Post-shift, day without a valid personal air sample

One pre-shift, day 1 sample was chosen for each subject. A second pre-shift, day 1 sample was chosen for 10 subjects. Urine samples were not selected for this pool if more than 7 hours had passed between the time of urine collection and the subject's previous void (Appendix I Figure 1). Personal air samples were considered invalid if the sample volume was unreliable, there was damage to the air filter, or there was filter leakage. Air volume was classified as unreliable is there were differences greater than 10% between pre-collection and post-collection pump flow or if any pump faults were recorded. All post-shift urine samples on days with a valid personal air sample were selected for analysis. The remaining samples were selected from post-shift urine samples on days without a valid personal air measurement. At least one sample was selected from this pool for each subject, more samples were selected from individuals with higher exposures to maximize the likelihood of detecting an association between air 1-NP exposures and 1-NP metabolite levels. A summary of the analyzed samples and all collected samples are shown in Appendix I Tables 2 and 3.

Air and Urine Sample Analysis

Air samples were analyzed using a method previously described by Miller-Schulze et al (Miller-Schulze et al., 2010). Urine samples were randomized prior to beginning analysis and extraction was carried out on batches of 20 or 32 samples. Four quality control samples were included with each batch, an internal standard spike (D), an internal standard and metabolite spike (DH), a water blank (B), and a benchmark sample (BM). The benchmark sample was a 50 mL aliquot taken from pre-existing pooled urine samples that were spiked with known levels of 1-NP metabolites and were used to evaluate the consistency of results across extraction batches. Deuterated analogues of the 1-NP metabolites were used as internal standards. Analysis was based on the method described by Toriba et al (Appendix III) (Toriba et al., 2007). Briefly, samples were filtered and the pH stabilized using a 4M acetate buffer (pH 5) then the metabolites were deconjugated using β-Glucuronidase/aryl sulfatase. The deconjugated metabolites were extracted using a blue rayon sorbent, eluted from the sorbent, then evaporated to near dryness and reconstituted. The reconstituted samples were cleaned

using Alumina Sep-Paks then evaporated to near dryness, the sample volume was then brought to roughly 300µL in methanol and filtered into HPLC vial inserts. Samples were then stored in the freezer until analysis. Prior to analysis the samples were evaporated to approximately 50µL then mixed with 150µL water. Samples were analyzed in the order they were prepared using HPLC/MS/MS (Agilent 1100 HPLC with a 6410 tandem mass spectrometer). The analysis method was optimized for samples volumes of 100mL, so any samples less than 100mL were brought to volume using DI water after filtering. Aliquots of 1mL were taken prior to each sample being filtered for measurement of creatinine.

The LOD for 1-NP in air samples was calculated for each batch by taking the average concentration detected in blank samples and adding 3 times the standard deviation across these samples. Similar to the approach taken for 1-NP air samples the analytical Limit of Detection (LOD) was calculated by taking the average metabolite level detected in the extract from deuterated blank samples and adding 3 times the standard deviation of the metabolite concentration.

Data Analysis

All data analysis was performed using Stata Version 13 statistical software, the basic code for the models used is shown in Appendix I Table 4. Descriptive statistics were generated for the air and urine data separately. Air samples were excluded from analysis using criteria similar to those used to select post-shift urine samples. Samples were excluded if the pump flow error exceeded the 95th percentile for all collected samples (26%) or the sample collection time was less than the 10th percentile (359min). All samples with concentrations less than the analytical LOD, were substituted with the LOD divided by the square root of two. Sample concentrations were also replaced with the LOD divided by the square root of two if no peak above noise was observed or an interfering peak was observed. The urinary metabolites 6-OHNP and 8-OHNP were selected for analysis as they were detected at the highest levels in the urine samples. Metabolite levels were normalized using creatinine to adjust for urinary dilution.

The distributions of the native and log-transformed 1-NP and EC air data were visually assessed for normality using histograms and Q-Q plots. Summary statistics were also generated for the 1-NP and EC data, including the number of observations, arithmetic mean, arithmetic standard deviation, geometric mean, geometric

standard deviation, range, and number of missing observations. Between and within subject variance was calculated and visually displayed using a box plot with air concentrations stratified by subject and job location.

This process was repeated for the creatinine adjusted metabolite concentrations. Their distributions were assessed for normality or log-normality using histograms and Q-Q plots and summary statistics were generated for both metabolites. The between- and within-subject variance was calculated and displayed using a box plot with metabolite concentrations stratified by subject and job location. Metabolite concentrations were also stratified by day of work week to assess any trends in metabolite accumulation. Both the air and metabolite concentrations were log-transformed for all further analyses.

Aim 1: Determine the association between 1-NP metabolites in urine with 1-NP in personal air samples

The association between 6-OHNP and 8-OHNP with 1-NP in air for each subject (n) and shift (t) was evaluated using a linear mixed model with the form:

$$\ln(6{,}8-OHNP)_{n,t} = \beta_0 + \beta_1 \times \ln(air\ 1NP)_{n,t} + b_n + \varepsilon_{n,t}$$

Equation 1: General form for linear mixed model

Where b_n is the subject-specific random effect and $\varepsilon_{n,t}$ is the residual error term for subject *n* and shift *t*. Because air samples were not collected for each shift a predictive model was generated to estimate exposure to 1-NP in air for unmonitored shifts. The predicted air concentrations were then used in place of the measured concentrations in the above model. Non-occupational exposures were assumed to be much lower than occupational exposures and were assigned a value of the LOD/5. A linear mixed model was also used to estimate concentrations of 1-NP in air. The model was in the form:

$$\ln(air\ 1NP)_{n,t} = \beta_0 + \beta_1 \times tUG + \beta_2 \times tDE + b_n + \varepsilon_{n,t}$$

Equation 2: General form for model used to predict 1-NP air concentrations

With fixed effects adjusting for reported time spent underground (tUG) and reported time spent working around diesel exhaust emissions (tDE). Again, b_n is a subject-specific random effect and $\varepsilon_{n,t}$ is a residual error term for subject *n* and shift *t*. The out-of-sample model performance was assessed using cross validation (CV). Air 1-NP observations were randomly assigned to mutually exclusive groups each containing approximately 10% of observations for the CV assessment. Out-of-sample RMSE and R^2 were estimated using CV with the

random grouping described above. All out-of-sample R^2 values were determined by assessing deviation around the 1:1 line.

In order to address increase in metabolite levels models were generated to test the association between metabolite levels with exposures from past shifts and exposures accumulated over multiple shifts. Datasets were generated that linked post-shift metabolite levels with air exposures that were lagged by 1, 2, and 3 shifts as well as exposures that were accumulated over the past 2, 3, and 4 shifts. These associations between 6-OHNP and 8-OHNP with 1-NP in air were evaluated using the linear mixed models described below.

Table 5: General equations for linear mixed models used to address accumulation of urinary metabolites

Shift Model	General Equation
Lagged by 1 day	$\ln(6,8OHNP)_{n,t} = \beta_0 + \beta_1 \times \ln(air\ 1NP)_{n,t-1} + b_n + \varepsilon_{n,t}$
Lagged by 2 days	$\ln(6,8OHNP)_{n,t} = \beta_0 + \beta_1 \times \ln(air\ 1NP)_{n,t-2} + b_n + \varepsilon_{n,t}$
Lagged by 3 days	$\ln(6,8OHNP)_{n,t} = \beta_0 + \beta_1 \times \ln(air\ 1NP)_{n,t-3} + b_n + \varepsilon_{n,t}$
Accumulated over 2 days	$\ln(6,8OHNP)_{n,t} = \beta_0 + \beta_1 \times \ln\left(\sum_{i=t-1}^{t} air\ 1NP_{n,t_i}\right) + b_n + \varepsilon_{n,t}$
Accumulated over 3 days	$\ln(6,8OHNP)_{n,t} = \beta_0 + \beta_1 \times \ln\left(\sum_{i=t-2}^{t} air\ 1NP_{n,t_i}\right) + b_n + \varepsilon_{n,t}$
Accumulated over 4 days	$\ln(6,8OHNP)_{n,t} = \beta_0 + \beta_1 \times \ln\left(\sum_{i=t-3}^{t} air\ 1NP_{n,t_i}\right) + b_n + \varepsilon_{n,t}$

t indicates the day the urine sample was collected on

Aim 2: Generate predictive model for 1-NP metabolites in urine using responses to job task surveys

The association between 6-OHNP and 8-OHNP with responses to survey questions for was evaluated using a linear regression model with the form:

$$\ln(6,8-OHNP) = \beta_0 + \beta_1 \times X_1 + \beta_2 \times X_2 + \cdots + \beta_n \times X_n$$

Equation 3: General form for linear regression model

Where X_{1-n} are predictors identified from job task/activity questionnaires. Predictors that were considered for inclusion in the model were time spent underground, time exposed to diesel exhaust, percent of time that a respirator was used, and integration time for urine samples. Job tasks were not considered for inclusion as there was an insufficient number of independent observations within the data to support parameter prediction for the number of reported tasks. The out-of-sample model performance was assessed using cross validation (CV). 6-OHNP and 8-OHNP observations were randomly assigned to mutually exclusive groups each containing approximately 10% of observations for the CV assessment. Out-of-sample RMSE and R^2 were estimated using

CV with the random grouping described above. All in-sample and out-of-sample R^2 values were determined by assessing deviation around the 1:1 line. A set of models was also created to assess the association between survey responses from the previous day with metabolite levels in urine.

RESULTS

Air and Urine Sample Analysis

The LOD for 1-NP on quartz filters differed for each sample analysis batch. The batch-specific LODs for 1-NP in the sample extract were 3.87fg/µL, 5.76fg/µL, and 17.75fg/µL. Sixteen of the 1-NP samples met the replacement criteria and their concentrations were substituted with the LOD over the square root of two. The LOD for the EC samples was reported as 1.7µg. Nine EC samples met replacement criteria and were substituted.

A single LOD was calculated for each metabolite as all urine samples were analyzed in a single batch. An LOD of 0.004pg/µL was calculated for 6-OHNP in the sample extracts and an LOD of 0.006pg/µL was calculated for 8-OHNP in sample extracts. Four 6-OHNP values and six 8-OHNP values met the replacement criteria and were substituted with the LOD over square root of two. A summary of the quality control data is presented for each metabolite in Tables 5 and 6 of Appendix I.

Air Data

Both 1-NP and EC appeared to be log-normally distributed (Appendix I Figure 3, 4). The 1-NP air concentrations showed variability between and within subjects (Figure 2, Table 6). Between subject standard deviation was estimated as 42.14 pg/m^3, 22% of the total variance in 1-NP, and the within subject standard deviation was estimated as 78.70 pg/m^3, 78% of the total variance. However, much of the between subject variability was explained by shift location. When shift location was adjusted for the between subject standard deviation decreased to 30.93 pg/m^3, 13% of the total variance in 1-NP air exposures. The within worker standard deviation was minimally affected by adjusting for shift location and showed a marginal increase to 79.45 pg/m^3 which now accounted for 87% of the total variance in 1-NP.

Although there was a large amount of overlap in the range of exposures for work shift locations the underground jobs, Face and Shop, tended to have higher exposures than surface jobs with the highest 1-NP and EC exposure levels for Face jobs. The measured 1-NP air concentrations for underground jobs were high relative to previously measured levels in ambient air, but were within the range of levels that have been observed in occupational settings (IARC, 2013). Using the MSHA standard for EC of 160µg/m^3 none of the study subjects were overexposed to diesel exhaust during monitored air shifts (Table 6). Concentration of 1-NP in air did not show any consistent trends with day of week (Appendix 1 Figure 5).

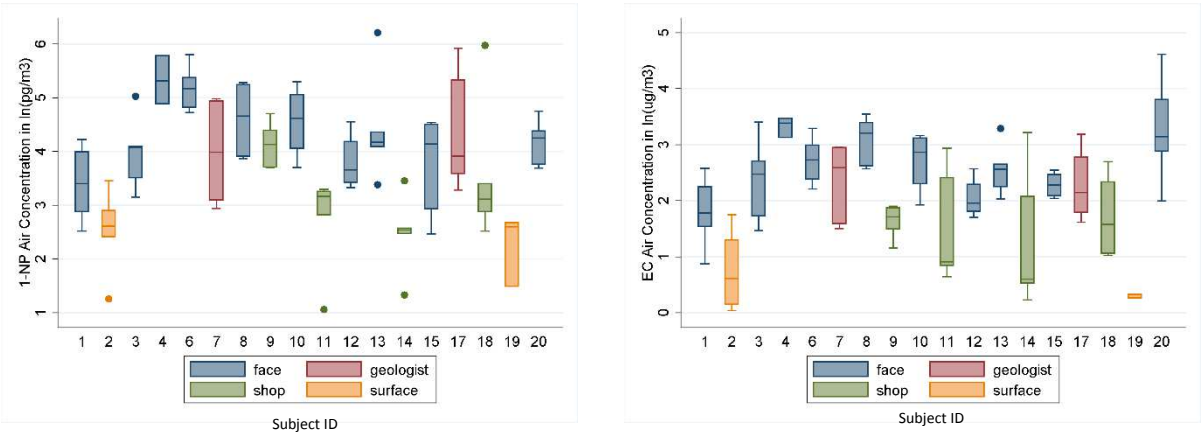

Figure 2: Distribution of log-transformed 1-nitropyrene and elemental carbon by subject ID and job location

Table 6: Summary statistics for 1-Nitropyrene and Elemental Carbon in air by shift location

	All Locations	Face	Shop	Surface
	(n = 103)	(n = 68)	(n = 21)	(n = 14)
1-Nitropyrene (pg/m^3)				
Range	2.89 – 497	11.74 – 497	3.78 – 392	2.89 – 115
AM(ASD)	78.0 (88.1)	96.9 (91.9)	52.0 (82.1)	25.5 (32.3)
GM(GSD)	47.0 (2.86)	67.9 (2.34)	30.7 (2.59)	14.8 (2.85)
Elemental Carbon (µg/m^3)				
Range	1.04 – 101	2.38 – 101	1.26 – 25.1	1.04 – 44.9
AM(ASD)	12.4 (12.5)	15.1 (13.3)	7.09 (6.06)	7.14 (12.1)
GM(GSD)	8.46 (2.51)	12.0 (1.92)	5.30 (2.17)	3.09 (3.30)

Predictive Air Model

Three predictive air models were generated using exposure predictors from Job/Task Activity Surveys and measured 1-NP air concentrations. The predictors selected for inclusion were shift location (Face, Shop, or Surface), time spent working in areas with diesel exhaust (Time DE), and time spent underground (Time UG).

Predictions were based on both the fixed effects and the random effect estimates. Model performance was evaluated using CV as described above and the out-of-sample model R^2 and RMSE were used to select the model. Out-of-sample RMSE represents prediction error in the generalized setting and the model with the lowest error (RMSE) and highest R^2 was selected. The model adjusting for time underground and time working around DE (Model C) showed the best out-of-sample predictive ability with an R^2 of 0.41 and RMSE of 0.80 and was selected for use in the metabolite level and air regression analysis (Table 7, Figure 3). This model predicts that the geometric mean air 1-NP exposure increases by 8% for each additional hour spent underground and increases by 3% for each additional hour spent working in areas with diesel exhaust (Table 7).

Table 7: Parameter estimates for predictive air models

	β (SE)	p	95% Conf. Interval	Subject-Specific Random Effect Est.(SE)	Residual Random Effect Est (SE)	Model R^2	Model RMSE
Model A: Shift Location (Appendix I, Figure 6)							
				0.48 (0.143)	0.78 (0.061)	0.37	0.83
Shop*	-0.72 (0.331)	0.03	(-1.37, -0.07)				
Surface*	-1.25 (0.320)	< 0.001	(-1.88, -0.62)				
Intercept	4.18 (0.168)	< 0.001	(3.85, 4.50)				
Model B: Shift Location and Time Spent Working in Areas with Diesel Exhaust (Appendix I, Figure 7)							
				0.49 (0.14)	0.78 (0.061)	0.36	0.83
Shop*	-0.72 (0.332)	0.029	(-1.37, -0.07)				
Surface*	-1.16 (0.328)	< 0.001	(-1.8, -0.52)				
Time DE (hr)	0.04 (0.031)	0.245	(-0.02, 0.10)				
Intercept	3.94 (0.264)	< 0.001	(3.42, 4.46)				
Model C: Time Spent Underground and Time Spent Working in Areas with Diesel Exhaust (Figure 3)							
				0.69 (0.152)	0.76 (0.059)	0.41	0.80
Time UG (hr)	0.08 (0.034)	0.023	(0.01, 0.14)				
Time DE (hr)	0.03 (0.033)	0.402	(-0.04, 0.09)				
Intercept	3.01 (0.346)	< 0.001	(2.34, 3.69)				

*reference location is Face
number of observations = 103 for all models

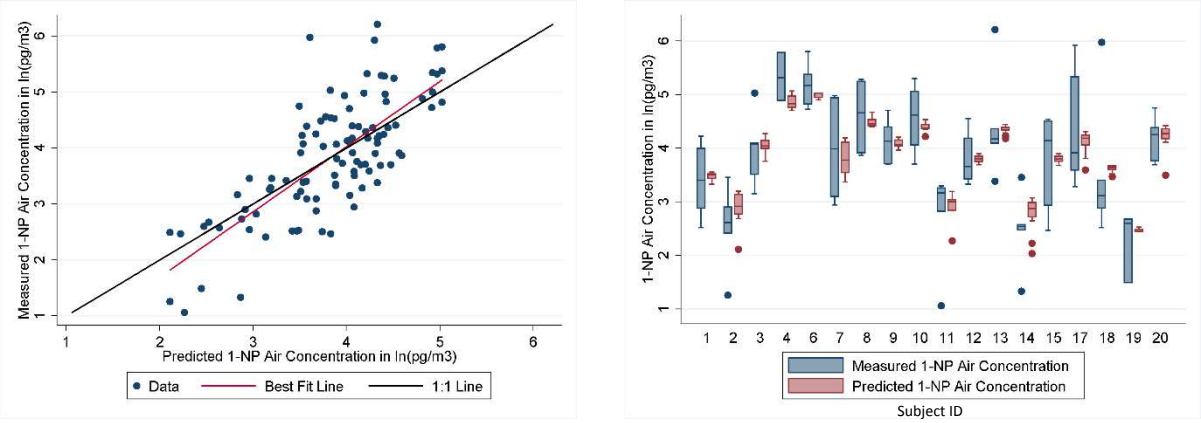

Figure 3: Comparison of predicted and measured 1-NP air concentrations in ln(pg/m^3) for Model C over all observations and by Subject ID

Urine Data

6-OHNP and 8-OHNP concentrations were adjusted for urinary dilution using creatinine and the adjusted concentrations were used in all analyses. Both 6-OHNP and 8-OHNP appeared to be log-normally distributed (Appendix I Figure 8, 9). Levels of 6-OHNP in urine tended to be higher than levels of 8-OHNP. The 6-OHNP and 8-OHNP metabolite concentrations showed variability between and within subjects (Figure 4, Table 8). For 6-OHNP the between subject standard deviation was estimated as 0.014 pg/mg creatinine, 23% of the total variance, and the within subject standard deviation was estimated as 0.026 pg/mg creatinine, 77% of total variance. For 8-OHNP the between subject standard deviation was estimated as 0.007 pg/mg creatinine, 23% of the total variance, and the within subject standard deviation was estimated as 0.013 pg/mg creatinine, 77% of the total variance. A consistent trend for higher metabolite levels by shift location was not observed, however, the metabolite levels were highly variable in all locations (Table 8).

Table 8: Summary statistics for post-shift metabolite levels by shift location

	All Locations (n = 142)	Face (n = 96)	Shop (n = 27)	Surface (n = 19)
Post-shift 6-OHNP Concentration (pg/mg creatinine)				
Range	0.0004 - 0.23	0.0007 - 0.16	0.0058 - 0.23	0.0004 - 0.095
AM(ASD)	0.024 (0.031)	0.022 (0.024)	0.031 (0.048)	0.023 (0.030)
GM(GSD)	0.014 (2.9)	0.014 (2.6)	0.017 (2.7)	0.009 (4.7)
Post-shift 8-OHNP Concentration (pg/mg creatinine)				
Range	0.0006 - 0.11	0.0006 - 0.092	0.0014 - 0.11	0.0006 - 0.072
AM(ASD)	0.012 (0.016)	0.011 (0.012)	0.014 (0.023)	0.015 (0.020)
GM(GSD)	0.007 (2.8)	0.007 (2.4)	0.006 (3.4)	0.006 (4.4)

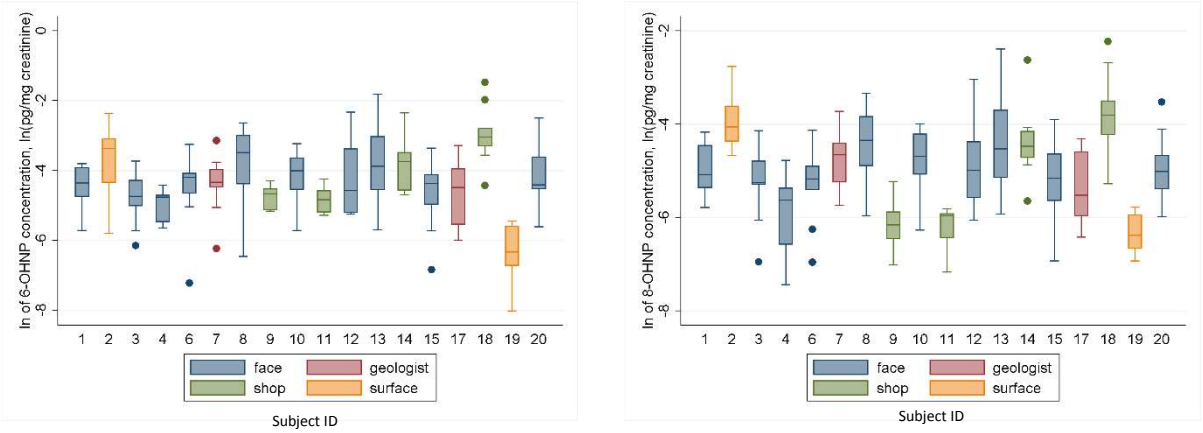

Figure 4: Distribution of log-transformed 6-OHNP and 8-OHNP by subject ID and job location

Metabolite levels tended to increase over the work week with an average increase in 6-OHNP of 0.007 pg/mg creatinine per day and an increase in 8-OHNP of 0.003 pg/mg creatinine per day (Table 9). Additionally, the summary statistics for the pre-shift day 1 metabolite concentrations were nearly identical to those of the post-shift day 1 metabolite concentrations for both 6-OHNP and 8-OHNP. This indicates that the elimination rate for these metabolites is slow relative to the within-day variability in occupational exposure.

Table 9: Metabolite levels by day of week in pg/mg creatinine and trend for increase in pg/mg creatinine per day

	All Samples (n = 170)	Pre-Shift Day 1 (n = 28)	Post-Shift Day 1 (n = 41)	Post-Shift Day 2 (n = 40)	Post-Shift Day 3 (n = 31)	Post-Shift Day 4 (n = 30)	Trend for Increase
6-OHNP	0.022 (0.029)	0.014 (0.012)	0.014 (0.012)	0.023 (0.029)	0.025 (0.024)	0.036 (0.049)	0.007 (0.002)*
8-OHNP	0.011 (0.015)	0.006 (0.005)	0.008 (0.006)	0.013 (0.018)	0.013 (0.013)	0.017 (0.023)	0.003 (0.001)*

Values are mean (SD)
* significant at 0.05 level

Association between Urinary Metabolites and Predicted 1-NP in Air

All non-occupational exposures were assumed to be low relative to occupational exposures and were assigned the LOD of 1-NP in air divided by 5. No association was detected between either 1-NP metabolite in post-shift urine with 1-NP in air for air measurements taken on the same day as urine sample collection (Model 1) (Tables 10, 11; Appendix I Figure 8). Inclusion of pre-shift samples (Model 2) marginally improved the significance of the association between urinary metabolites with air samples from that day, however the magnitude of the association was lower and still was not significant (Tables 10, 11; Appendix I Figure 9). This is likely due to the observed tendency for metabolites levels to increase over the work week. In order to

address the delay in uptake and elimination indicated by the trend for increasing metabolite levels across the week Models 3–5 were generated which tested the association between metabolite levels with air exposures lagged by 1–3 days and Models 6–8 which tested the association between metabolite levels with air exposures accumulated over the last 2–4 shifts, including the current day's shift.

Significant associations were detected between both 6-OHNP and 8-OHNP with 1-NP in air for in all three lagged air models (Tables 10, 11). The parameter estimates for the fixed effects were similar in each of these models as were the random effect estimates although the Model 3 (air lagged by 1 day) tended to have a lower subject-specific random effect for both metabolites indicating that this model accounted for more of the variability in metabolite levels between subjects. Using Model 3 we would estimate that for every doubling of exposure to 1-NP on the day before sample collection there would be a 12% increase in geometric mean 6-OHNP concentration and a 10% increase in geometric mean 8-OHNP concentration (Figure 5; Tables 10, 11; Appendix I Figures 10, 11).

None of the models that tested the association between accumulated 1-NP air exposures and urinary metabolite levels were significant at the 0.05 level, with the exception of Model 8 (air accumulated over 4 days) for both 6-OHNP and 8-OHNP (Tables 10, 11). Although these models were statistically significant the slope estimates are much shallower than those obtained for the lagged air models indicating that the association is weaker and of limited practical significance (Appendix I Figures 12–14).

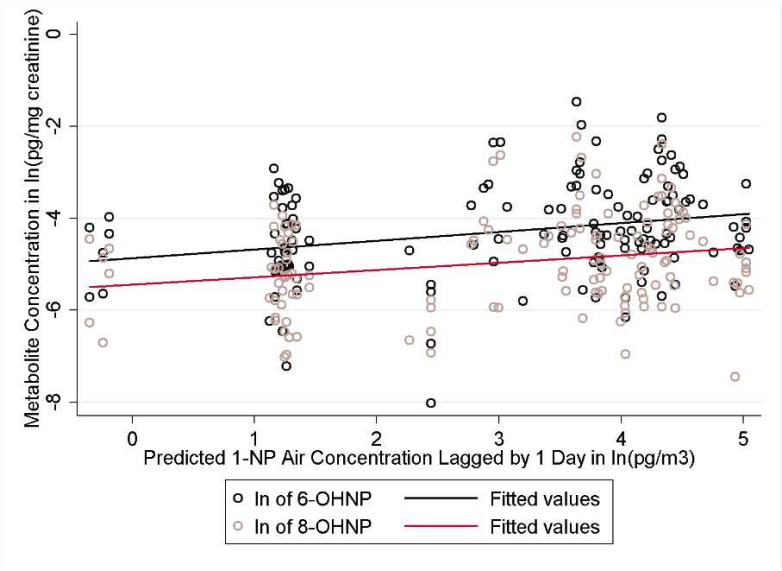

Figure 5: Estimated association between exposure to 1-NP in air and levels of 1-NP metabolites in urine (Model 3)

Table 10: Results for mixed effects models for association between log-transformed 6-OHNP in ln(pg/mg creatinine) and log-transformed 1-NP in air in ln(pg/m^3)

	β (SE)	p	95% Conf. Interval	Number of Observations	Subject-Specific Random Effect Est.(SE)	Residual Random Effect Est (SE)
Model 1: Post-Shift Urine with Predicted 1-NP in Air						
				142	0.66 (0.15)	0.88 (0.06)
ln of 1-NP in Air	0.13 (0.21)	0.519	(-0.27, 0.54)			
Intercept	-4.84 (0.82)	<0.001	(-6.43, -3.25)			
Model 2: Pre-shift and Post-shift Urine with Predicted 1-NP in Air						
				158	0.60 (0.14)	0.89 (0.05)
ln of 1-NP in Air	0.08 (0.07)	0.254	(-0.06, 0.22)			
Intercept	-4.63 (0.29)	<0.001	(-5.20, -4.05)			
Model 3: Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Lagged by 1 Day						
				138	0.58 (0.14)	0.89 (0.06)
ln of 1-NP in Air	0.16 (0.06)	0.003	(0.06, 0.27)			
Intercept	-4.79 (0.23)	<0.001	(-5.25, -4.34)			
Model 4: Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Lagged by 2 Days						
				116	0.72 (0.18)	0.86 (0.06)
ln of 1-NP in Air	0.17 (0.06)	0.002	(0.06, 0.28)			
Intercept	-4.73 (0.25)	<0.001	(-5.22, -4.25)			
Model 5: Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Lagged by 3 Days						
				106	0.72 (0.19)	0.87 (0.07)
ln of 1-NP in Air	0.18 (0.08)	0.024	(0.02, 0.34)			
Intercept	-4.67 (0.27)	<0.001	(-5.19, -4.15)			
Model 6: Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Accumulated over 2 Days						
				138	0.60 (0.15)	0.91 (0.06)
ln of 1-NP in Air	0.10 (0.06)	0.115	(-0.02, 0.22)			
Intercept	-4.69 (0.30)	<0.001	(-5.28, -4.10)			
Model 7: Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Accumulated over 3 Days						
				113	0.74 (0.19)	0.89 (0.07)
ln of 1-NP in Air	0.11 (0.06)	0.068	(-0.008, 0.22)			
Intercept	-4.72 (0.32)	<0.001	(-5.35, -4.09)			
Model 8: Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Accumulated over 4 Days						
				129	0.73 (0.19)	0.89 (0.06)
ln of 1-NP in Air	0.09 (0.05)	0.045	(0.002, 0.18)			
Intercept	-4.65 (0.27)	<0.001	(-5.17, -4.11)			

Table 11: Results for mixed effects models for association between log-transformed 8-OHNP in ln(pg/mg creatinine) and log-transformed 1-NP in air in ln(pg/m^3)

	β (SE)	P	95% Conf. Interval	Number of Observations	Subject-Specific Random Effect Est.(SE)	Residual Random Effect Est (SE)
Model 1: Post-Shift Urine with Predicted 1-NP in Air						
				142	0.75 (0.15)	0.77 (0.05)
ln of 1-NP in Air	0.07 (0.21)	0.725	(-0.34, 0.49)			
Intercept	-5.28 (0.84)	<0.001	(-6.91, -3.64)			
Model 2:Pre-shift and Post-shift Urine with Predicted 1-NP in Air						
				158	0.72 (0.14)	0.77 (0.05)
ln of 1-NP in Air	0.07 (0.06)	0.248	(-0.05, 0.19)			
Intercept	-5.27 (0.28)	<0.001	(-5.82, -4.71)			
Model 3:Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Lagged by 1 Day						
				138	0.68 (0.14)	0.77 (0.05)
ln of 1-NP in Air	0.14 (0.05)	0.004	(0.05, 0.24)			
Intercept	-5.41 (0.23)	<0.001	(-5.86, -4.96)			
Model 4:Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Lagged by 2 Days						
				116	0.85 (0.18)	0.72 (0.05)
ln of 1-NP in Air	0.13 (0.05)	0.007	(0.03, 0.22)			
Intercept	-5.33 (0.25)	<0.001	(-5.83, -4.83)			
Model 5:Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Lagged by 3 Days						
				106	0.83 (0.18)	0.72 (0.05)
ln of 1-NP in Air	0.14 (0.07)	0.042	(0.05, 0.28)			
Intercept	-5.27 (0.27)	<0.001	(-5.79, -4.75)			
Model 6:Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Accumulated over 2 Days						
				138	0.69 (0.15)	0.79 (0.05)
ln of 1-NP in Air	0.09 (0.05)	0.101	(-0.02, 0.20)			
Intercept	-5.34 (0.29)	<0.001	(-5.90, -4.77)			
Model 7:Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Accumulated over 3 Days						
				113	0.82 (0.18)	0.74 (0.05)
ln of 1-NP in Air	0.10 (0.05)	0.051	(-0.0005, 0.19)			
Intercept	-5.37 (0.30)	<0.001	(-5.95, -4.78)			
Model 8:Pre-Shift and Post-Shift Urine with Predicted 1-NP in Air Accumulated over 4 Days						
				129	0.80 (0.18)	0.75 (0.05)
ln of 1-NP in Air	0.08 (0.04)	0.034	(0.006, 0.16)			
Intercept	-5.31 (0.26)	<0.001	(-5.82, -4.80)			

Predictive Metabolite Models

Four models were generated using exposure predictors from Job/Task Activity Surveys and measured 1-NP metabolite concentrations to predict post-shift levels of 6-OHNP and 8-OHNP. The predictors selected for inclusion were shift location (Face, Shop, or Surface), time spent working in areas with diesel exhaust (Time DE), time spent underground (Time UG), respirator use, day of work week, and time between urine voids. A model (Model C) was also generated that paired metabolite levels with survey answers from the previous day to determine if this was a better approach to accounting for the observed increase in metabolite level over the work week. However, including day or week in the model appeared to result in better performance than lagging the survey responses (Tables 12, 13). Model performance was once again evaluated using CV with random groups as described above and the out-of-sample model R^2 and RMSE were used to select a model. The model with the lowest error (RMSE) and highest R^2 was selected.

Table 12: Parameter estimates for predictive models for log-transformed post-shift 6-OHNP in ln(pg/mg creatinine)

	β (SE)	p	95% Conf. Interval	In-sample R^2 (RMSE)	Out-of-sample R^2 (RMSE)
Model A				0.36 (1.02)	0.28 (1.05)
Shop*	-0.15 (0.23)	0.529	(-0.61, 0.31)		
Surface*	-0.89 (0.30)	0.004	(-1.48, -0.29)		
Time DE (hr)	0.10 (0.03)	<0.001	(0.05, 0.16)		
Respirator Use (%)	0.85 (0.34)	0.013	(0.18, 1.52)		
Time between Voids (hr)	0.20 (0.06)	0.001	(0.08, 0.32)		
Day of Week	0.30 (0.08)	<0.001	(0.14, 0.46)		
Intercept	-3.75 (0.39)	<0.001	(-4.53, -2.97)		
Model B				0.38 (1.01)	0.32 (1.03)
Time UG (hr)	0.12 (0.03)	0.001	(0.05, 0.18)		
Time DE (hr)	0.09 (0.03)	0.002	(0.03, 0.15)		
Respirator Use (%)	0.85 (0.33)	0.011	(0.20, 1.50)		
Time between Voids (hr)	0.23 (0.06)	<0.001	(0.11, 0.34)		
Day of Week	0.34 (0.08)	<0.001	(0.18, 0.49)		
Intercept	-4.98 (0.40)	<0.001	(-5.76, -4.20)		
Model C: Predictors from Survey Data Lagged by 1 Day				0.36 (1.06)	0.24 (1.08)
Time UG (hr)	0.20 (0.05)	<0.001	(0.10, 0.30)		
Time DE (hr)	0.02 (0.04)	0.554	(-0.05, 0.10)		
Respirator Use (%)	0.53 (0.43)	0.219	(-0.32, 1.37)		
Time between Voids (hr)	0.28 (0.07)	<0.001	(0.14, 0.43)		
Day of Week	0.20 (0.13)	0.141	(-0.07, 0.45)		
Intercept	-5.10 (0.65)	<0.001	(-6.39, -3.80)		

*reference location is Face
Number of observations = 141 for Models A, B and 96 for Model C

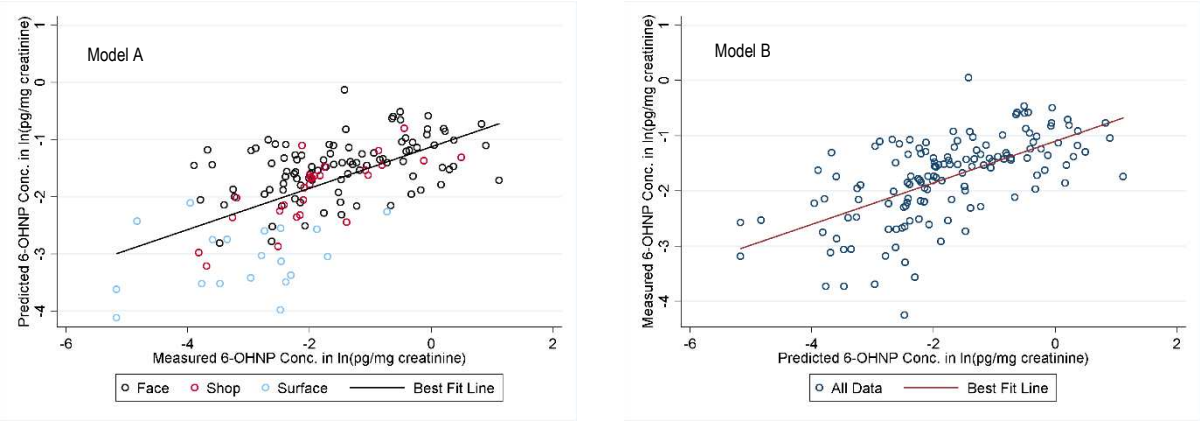

Figure 6: Comparison of predicted and measured log-transformed 6-OHNP concentrations for Models A and B

Table 13: Parameter estimates for predictive models for log-transformed 8-OHNP in ln(pg/mg creatinine)

	β (SE)	p	95% Conf. Interval	In-sample R2 (RMSE)	Out-of-sample R2 (RMSE)
Model A				0.32 (0.95)	0.24 (0.98)
Shop*	-0.55 (0.22)	0.012	(-0.98, -0.12)		
Surface*	-0.68 (0.28)	0.016	(-1.24, -0.15)		
Time DE (hr)	0.10 (0.03)	<0.001	(0.04, 0.15)		
Respirator Use (%)	0.76 (0.32)	0.017	(0.14, 1.39)		
Time between Voids (hr)	0.13 (0.06)	0.024	(0.02, 0.24)		
Day of Week	0.21 (0.07)	0.005	(0.06, 0.36)		
Intercept	-3.86 (0.37)	<0.001	(-4.58, -3.13)		
Model B				0.33 (0.94)	0.26 (0.96)
Time UG (hr)	0.11 (0.03)	0.001	(0.05, 0.17)		
Time DE (hr)	0.07 (0.03)	0.009	(0.02, 0.13)		
Respirator Use (%)	0.82 (0.31)	0.009	(0.21, 1.43)		
Time between Voids (hr)	0.17 (0.05)	0.002	(0.06, 0.27)		
Day of Week	0.26 (0.07)	0.001	(0.11, 0.40)		
Intercept	-5.08 (0.37)	<0.001	(-5.81, -4.33)		
Model C: Predictors from Survey Data Lagged by 1 Day				0.35 (0.97)	0.20 (1.01)
Time UG (hr)	0.18 (0.05)	<0.001	(0.09, 0.27)		
Time DE (hr)	0.04 (0.04)	0.324	(-0.04, 0.11)		
Respirator Use (%)	0.55 (0.39)	0.162	(-0.23, 1.33)		
Time between Voids (hr)	0.21 (0.07)	0.003	(0.08, 0.34)		
Day of Week	0.15 (0.12)	0.210	(-0.09, 0.40)		
Intercept	-5.29 (0.60)	<0.001	(-6.48, -4.09)		

*reference location is Face
Number of observations = 141 for Models A, B and 96 for Model C

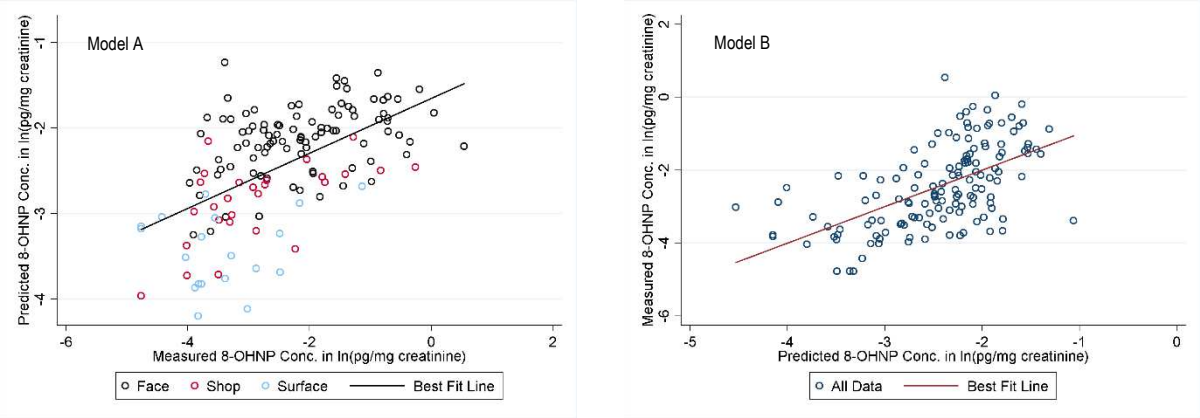

Figure 7: Comparison of predicted and measured log-transformed 8-OHNP concentrations for Models A and B

The model adjusting for time underground, time working around DE, respirator use, day of work week, and time between urine voids (Model B) showed the best out-of-sample predictive ability for both metabolites with out-of-sample R^2 of 0.32 and RMSE of 1.03 for 6-OHNP and an out-of-sample R^2 of 0.26 and RMSE of 0.96 for 8-OHNP (Tables 12, 13; Figures 6, 7). This model predicts an increase in metabolite concentration with increasing time spent underground, time working around diesel exhaust, time between voids, percent of shift a respirator is used, and day of work week (Tables 12, 13).

However, the model adjusting for work location (Model A) may provide the more practical approach to predicting metabolite levels (Tables 12, 13, Figures 6, 7). Although the out-of-sample predictive ability was slightly lower than what was seen for Model B, information on shift location may be easier to obtain and more reliable than information on amount of time spent underground. In this study time spent working underground was estimated from the Time-Activity Matrix in the Job Task/Activity questionnaire and was not directly reported by subjects. This model predicts a decrease in metabolite levels for shop and surface jobs relative to face jobs and an increase in metabolite levels with increasing time spent underground, increasing percent of shift that a respirator is used, increased time between voids, and day of work week (Tables 12, 13).

DISCUSSION

Although no trends were observed for increasing exposure to 1-NP by day of work week a significant increase in metabolite level across the week was observed for both 6-OHNP and 8-OHNP indicating that the time course of 1-NP in the body is slow relative to the frequency of exposure at occupational levels (Table 9). The

similarity in day 1 pre-shift and post-shift metabolite levels supports this conclusion. Very few workers reported off-shift exposure to diesel exhaust and the occupational DE levels measured in this cohort were high relative to previously reported ambient/environmental levels (Table 6). If the elimination was rapid relative to the occupational exposure frequency we would expect to see an increase in metabolite levels from the pre-shift day 1 sample, which represents 4 days of environmental level exposure, to the post-shift day 1 sample, which represents an occupational level exposure. As this increase is not seen the conclusion that time scale for elimination of 1-NP via urinary metabolites is longer than a single shift is supported. Therefore, an association between 1-NP exposure in air and metabolite levels in urine across a single shift would not be expected, as was shown in the regression analyses for Models 1 and 2 presented in Tables 10 and 11.

The strongest association was observed for models that lagged air exposures for 1, 2, or 3 days (Models 3–5) with the most between-subject variability accounted for in the model with air levels lagged by 1 day (Table 10, 11). The similarity in parameter estimates for these three models is likely due to the use of predicted air levels in this analysis. As not all shifts were monitored it was necessary to estimate air exposures for each subject and shift. However, the estimated air levels showed less variation than the measured levels (Figure 3). The model was based on amount of time that was reported for working underground and time that was reported for working around diesel exhaust exposures. Most subjects reported similar lengths of time each day which would reduce the variability in estimations and therefore variability in lagged exposures resulting in similar association estimates.

The lack of increase in metabolite levels from pre-shift day 1 to post-shift day 1 could also explain the poor association observed for models with accumulated air exposures. Almost none of the exposure from the day 1 shift was observed in the post-shift sample. Therefore, the inclusion of the shift on the day the sample was collected on probably resulted in overestimation of metabolite levels and the observed weak associations (Table 10, 11).

Although the magnitude of the association between 1-NP and air appears to be low, a 10%–12% increase in urinary metabolite level for doubling exposure, it could be because renal excretion is likely not the dominant elimination pathway. Dose recovery studies in rodents have found that 50%– 60% of the dose was excreted in

the feces and only 15%–20% was found in the urine (IARC, 2013). Additionally, this analysis looked at metabolite levels individually rather than in aggregate so each metabolite would represent an even smaller fraction of the dose. The modest increase in metabolite levels for each doubling of occupational exposure could also suggest that over the timescale that was examined in this study the increase is urinary biomarker levels was modest relative to baseline levels of the metabolites.

Subject responses to Job Task/Activity questionnaires do not appear to offer a viable approach to predicting urinary metabolite levels in this study. The models all had relatively poor predictive ability. However, they were capable of predicting differences in exposures between shift locations. Time spent underground was associated with higher levels as were underground face jobs. Levels predicted for shop jobs were lower than face jobs and higher than metabolite concentrations for surface jobs (Tables 12, 13: Model A). In addition, this study cohort was only 20 subjects with a relatively small number of independent observations. A larger study may allow for further refinement of predictions with specific task as well as location information that could not be reliably estimated with these data. Further refinement could also help identify if some covariates, such as respirator use, are reflecting mine-specific exposure patterns rather than generalizable trends. Contrary to what was expected, respirator use was associated with an increase in metabolite levels. However, in this mine subjects were required to wear respirators only for specific tasks that were associated with high DE exposures. Therefore, the respirator use covariate could be acting as a surrogate for highly exposed jobs in this model rather than representing use of a control device to reduce exposures.

Recommendations for Future Work

One of the primary limitations in this study is the uncertain elimination half-life for 1-NP which was compounded by the inability to collect air samples for each day of exposure. Future study could focus on better understanding the disposition of 1-NP by collecting daily air samples and collecting all voids during the exposure period and during washout. Collection of daily air samples would also eliminate dependence on predicted air levels for determining the association between 1-NP exposure in air and 1-NP metabolite levels in urine which may clarify the magnitude and timescale of the association.

There is also the potential for tobacco use to modify the association between 1-NP in air and metabolite levels in urine as well as the toxicity of 1-NP. Multiple studies have shown that the 1-NP is activated to a toxic intermediate by nitroreductive pathways and that oxidative pathways which are mediated by CYP450s are less toxic. Smoking tends to upregulate several CYP450s which could potentially alter the metabolite profile as well as the toxicity for a given exposure (IARC, 2013b). Future work could therefore look at the metabolite profiles between subjects who use tobacco and subjects who do not to determine if this modifies the association between 1-NP in air and biomarker levels which could have implications for both biomonitoring and predicting health outcomes for a given exposure.

CONCLUSIONS

Underground exposures to 1-NP in this cohort were higher than previously reported environmental levels but were within the range of measured occupational levels. Surface exposures tended to be lower and were within the range of high urban exposures and low occupational levels (IARC, 2013b). However, none of the collected samples exceed the MSHA standard for EC in underground metal/non-metal mines of 160µg/m^3. Of the 1-NP metabolites detected, 6-OHNP and 8-OHNP were measured at the highest levels in urine samples which is consistent with prior studies (Table 1) (Miller-Schulze et al., 2013; Toriba et al., 2007). Metabolite levels showed a significant increase across the work week indicating that uptake, elimination, or both of 1-NP is slow relative the frequency and level of exposure for this cohort.

A significant association between exposure to 1-NP in air and metabolite levels in urine was observed. However, due to the tendency for metabolites to accumulate the optimal exposure window appears to be longer than the shift preceding the spot urine sample. The association was strongest between exposures lagged by 1 day and metabolite levels, with a 12% increase in geometric mean creatinine adjusted 6-OHNP for every doubling in exposure to 1-NP on the day before sample collection and a 10% increase in the geometric mean of creatinine adjusted 8-OHNP. Although, significant associations were also detected when exposures were lagged by 2 or 3 days the selected model was also able to explain the greatest amount of between-subject variability.

Subject responses to Job Task/Activity Surveys did not appear to offer a viable approach to predicting urinary metabolite levels within this cohort as all models had relatively poor out of sample predictive ability. This could be due to how the surveys were structured or idiosyncrasies in what predictors acted as surrogates for in this mine. However, the models were capable of predicting general trends in metabolite levels with each of the predictors. Although Model B had the best out-of-sample predictive ability Model A may provide a more practical approach to predicting metabolite levels as information about shift location is likely to be more accurate and easier to obtain than total time spent underground.

REFERENCES

Bamford, H. A., Bezabeh, D. Z., Schantz, M. M., Wise, S. A., & Baker, J. E. (2003). Determination and comparison of nitrated-polycyclic aromatic hydrocarbons measured in air and diesel particulate reference materials. *Chemosphere*, *50*(5), 575–587. http://doi.org/10.1016/S0045-6535(02)00667-7

Coble, J. B., Stewart, P. A., Vermeulen, R., Yereb, D., Stanevich, R., Blair, A., … Attfield, M. (2010). The Diesel Exhaust in Miners Study: II. Exposure Monitoring Surveys and Development of Exposure Groups. *Annals of Occupational Hygiene*, *54*(7), 747–761. http://doi.org/10.1093/annhyg/meq024

EPA. (2002). Health Assessment Document For Diesel Engine Exhaust.

EPA. (2010). National Clean Diesel Campaign. Retrieved from http://www.epa.gov/cleandiesel/

IARC. (2012). Diesel Exhaust Monograph Press Release.

IARC. (2013a). 1NP Monograph (2013).

IARC. (2013b). Diesel Gas Exhausts and Nitroarenes (2013).

Laumbach, R., Tong, J., Zhang, L., Ohman-Strickland, P., Stern, A., Fiedler, N., … Zhang, J. (2008). Quantification of 1-aminopyrene in human urine after a controlled exposure to diesel exhaust. *Journal of Environmental Monitoring*, *11*(1), 153–159. http://doi.org/10.1039/B810039J

Miller-Schulze, J. P., Paulsen, M., Kameda, T., Toriba, A., Tang, N., Tamura, K., … Simpson, C. D. (2013). Evaluation of urinary metabolites of 1-nitropyrene as biomarkers for exposure to diesel exhaust in taxi drivers of Shenyang, China. *Journal of Exposure Science and Environmental Epidemiology*, *23*(2), 170–175. http://doi.org/10.1038/jes.2012.40

Miller-Schulze, J. P., Paulsen, M., Toriba, A., Tang, N., Hayakawa, K., Tamura, K., … Simpson, C. D. (2010). Exposures to Particulate Air Pollution and Nitro-Polycyclic Aromatic Hydrocarbons among Taxi Drivers in Shenyang, China. *Environmental Science & Technology*, *44*(1), 216–221. http://doi.org/10.1021/es802392u

MSHA. (2001, 2006). Diesel Exhaust Exposure Measurement.

Neophytou, A., Hart, J., Chang, Y., Zhang, J., Smith, T., Garshick, E., & Laden, F. (2014). Short-Term Traffic-Related Exposures and Biomarkers of Nitro-PAH Exposure and Oxidative DNA Damage. *Toxics*, *2*(3), 377–390. http://doi.org/10.3390/toxics2030377

NIOSH. (2010). NIOSH Pocket Guide to Chemical Hazards. Retrieved from http://www.cdc.gov/niosh/npg/default.html

OSHA. (2014). Safety and Health Topics: Diesel Exhaust. Retrieved from https://www.osha.gov/SLTC/dieselexhaust/standards.html

Pronk, A., Coble, J., & Stewart, P. A. (2009). Occupational exposure to diesel engine exhaust: A literature review. *Journal of Exposure Science and Environmental Epidemiology*, *19*(5), 443–457. http://doi.org/10.1038/jes.2009.21

Seidel, A., Dahmann, D., Krekeler, H., & Jacob, J. (2002). Biomonitoring of polycyclic aromatic compounds in the urine of mining workers occupationally exposed to diesel exhaust. *International Journal of Hygiene and Environmental Health*, *204*(5–6), 333–338. http://doi.org/10.1078/1438-4639-00116

Stephensen, D., Spear, T., & Lutte, M. (2006). Comparison of Sampling Methods to Measure Exposure to Diesel Particulate Matter in an Underground Metal Mine. *Mining Engineering*, *58*(8), 39 – 45.

Stewart, P. A., Coble, J. B., Vermeulen, R., Schleiff, P., Blair, A., Lubin, J., … Silverman, D. T. (2010). The Diesel Exhaust in Miners Study: I. Overview of the Exposure Assessment Process. *Annals of Occupational Hygiene*, *54*(7), 728–746. http://doi.org/10.1093/annhyg/meq022

Toriba, A., Kitaoka, H., Dills, R. L., Mizukami, S., Tanabe, K., Takeuchi, N., … Simpson, C. D. (2007). Identification and Quantification of 1-Nitropyrene Metabolites in Human Urine as a Proposed Biomarker for Exposure to Diesel Exhaust. *Chemical Research in Toxicology*, *20*(7), 999–1007. http://doi.org/10.1021/tx700015q

Van Bekkum, Y. M., van den Broek, P. H. H., Scheepers, P. T. J., & Bos, R. P. (1998). Sensitive and Selective Detection of Urinary 1-Nitropyrene Metabolites following Administration of a Single Intragastric Dose of Diesel Exhaust Particles (SRM 2975) to Rats. *Chemical Research in Toxicology*, *11*(11), 1382–1390. http://doi.org/10.1021/tx980162x

Vermeulen, R., Coble, J. B., Lubin, J. H., Portengen, L., Blair, A., Attfield, M. D., … Stewart, P. A. (2010). The Diesel Exhaust in Miners Study: IV. Estimating Historical Exposures to Diesel Exhaust in Underground Non-metal Mining Facilities. *Annals of Occupational Hygiene*, *54*(7), 774–788. http://doi.org/10.1093/annhyg/meq025

Vermeulen, R., Coble, J. B., Yereb, D., Lubin, J. H., Blair, A., Portengen, L., … Silverman, D. T. (2010). The Diesel Exhaust in Miners Study: III. Interrelations between Respirable Elemental Carbon and Gaseous and Particulate Components of Diesel Exhaust derived from Area Sampling in Underground Non-metal Mining Facilities. *Annals of Occupational Hygiene*, *54*(7), 762–773. http://doi.org/10.1093/annhyg/meq023

APPENDIX I – Supplemental Tables and Figures

Table 1: Detailed descriptions of analytical methods used for previously reported 1-NP biomarker levels

Author (year)	Method	Analytical Method Description
Toriba (2007) & Miller-Schulze (2013)	c	Creatinine used to adjust for diuresis; pH was adjusted to 5.0 with HCl and metabolites were deconjugated using β-glucuronidase/arylsulfatase and extracted using blue rayon, metabolites were extracted from blue rayon using a 50:1 methanol:ammonia solution. The extract was dried then reconstituted using 1:1 ethyl acetate:methanol and again using methanol. Sample extracts were analyzed using HPLC-MS/MS with a triple quadrupole mass spectrometer equipped with an electron spray ionization source operated in negative ion mode.
Laumbach (2008)	d	1-AP was hydrolyzed using HCl then the sample pH was adjusted to 7-8 using NaOH and acetic acid. The deconjugated metabolite was extracted by centrifugation using dichloromethane. The extract was dried then reconstituted using acetonitrile. Sample extracts were analyzed using HPLC equipped with a fluorescence detector.
Seidel (2002)	e	pH was adjusted to 5.67 with HCl and NaOH then metabolites were deconjugated using β-glucuronidase/arylsulfatase and benzene. The benzene phase was separated & dried then reconstituted using methanol. Samples extracts were analyzed using GC-MS with the MS operated in positive ion mode.
Neophytou (2014)	f	1-AP was hydrolyzed using β-glucuronidase then the sample pH was adjusted to >10 using NaOH. The deconjugated metabolite was centrifuged then the supernatant was dried and reconstituted using methanol. The sample extracts were then analyzed using HPLC equipped with a fluorescence detector.
Zwirner-Baier (1999)	g	HB adducts were hydrolyzed using SDS and NaOH then extracted using a solid phase column conditioned with hexane, 2-propanol, methanol, and NaOH and eluted with ethyl acetate. The amines were derivitised using PFPA then concentrated to half volume and dried then reconstituted using ethyl acetate. The sample extracts were analyzed using GC-MS with negative chemical ionization in single ion mode.

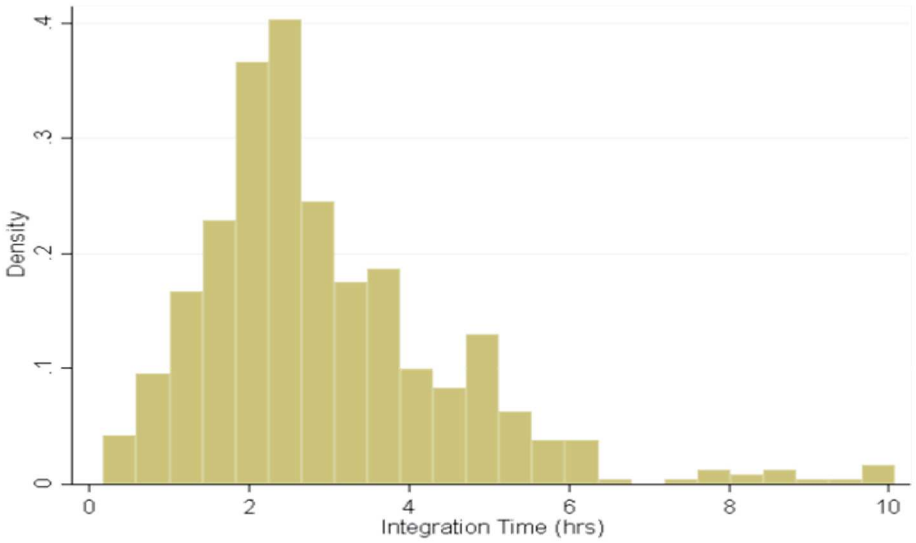

Figure 1: Distribution of Integration Times – time since previous void – for all Study Subjects

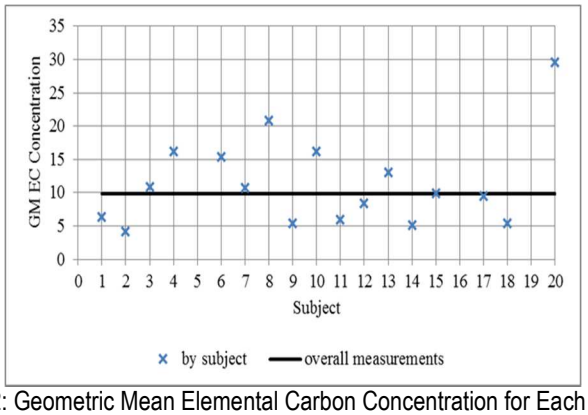

Figure 2: Geometric Mean Elemental Carbon Concentration for Each Subject

Table 2: Summary of Selected Samples by Subject, Session, and Day of Work Week

	Pool 1 n=28 (16%)	Pool 2 n=104 (61%)	Pool 3 n=38 (22%)	Total n=170
Subject				
01	1	6	1	8
02	1	6	1	8
03	2	5	2	9
04	2	4	3	9
05	-	-	-	-
06	2	6	3	11
07	2	5	2	9
08	2	6	4	12
09	2	8	1	11
10	2	5	3	10
11	1	6	1	8
12	1	4	2	7
13	2	7	3	12
14	1	6	1	8
15	2	8	3	13
16	-	-	-	-
17	2	6	3	11
18	1	7	1	9
19	1	4	1	6
20	1	5	3	9
Session				
1	0 (0%)	23 (14%)	0 (0%)	23 (14%)
2	11 (6%)	32 (19%)	17 (10%)	60 (35%)
3	8 (5%)	24 (14%)	10 (6%)	42 (25%)
4	9 (5%)	25 (15%)	11 (6%)	45 (26%)
Day of Work Week				
1	28 (16%)	27 (16%)	14 (8%)	69 (41%)
2	0 (0%)	28 (16%)	14 (8%)	42 (25%)
3	0 (0%)	25 (15%)	3 (3%)	28 (16%)
4	0 (0%)	24 (14%)	7 (4%)	31 (18%)

Table 3: Summary of Collected Samples by Subject and Session

		Session 1				Session 2				Session 3				Session 4			
Subject	Day	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
01	Pre Urine	pre	pre	pre	pre	pre	pre	pre	<50mL	pre	pre	pre	pre	pre	pre	pre	pre
	Post Urine	post	post	post	post	post	post	post	post	post	post	post	post	post	post	post	post
	Quartz 1-NP	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
02	Pre Urine	<50mL	pre	<50mL	pre	pre	pre	pre	pre	pre	<50mL	pre	pre	pre	pre	pre	pre
	Post Urine	post	post	post	post	post	.	post	post	post	post	post	post	post	post	post	post
	Quartz 1-NP	.	.	quartz	.	quartz	.	quartz	.	quartz	.	.	.	quartz	.	quartz	.
	Questionnaire	survey	survey	survey	survey	survey	.	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
03	Pre Urine	pre	pre	pre	pre	pre	pre	pre	pre	.	.	pre	pre	pre	pre	pre	pre
	Post Urine	post	post	post	post	post	post	post	post	.	.	post	post	post	post	post	>7 hr
	Quartz 1-NP	quartz	.	.	.	quartz	.	quartz	.	.	.	quartz*	.	quartz	.	quartz	.
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	survey	.	.	survey	survey	survey	survey	survey	survey
04	Pre Urine	pre	pre	pre	pre	pre	pre	pre	.	pre	pre	pre	pre	pre	pre	pre	pre
	Post Urine	post	post	post	post	post	post	>7 hr	.	post	>7 hr	post	>7 hr	post	post	post	post
	Quartz 1-NP	.	.	.	.	.	.	.	.	quartz	.	.	.	quartz	.	quartz	.
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	.	survey	survey	survey	survey	survey	survey	survey	survey
05		Cohort Dropout															
06	Pre Urine	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre
	Post Urine	post	post	post	post	post	post	post	post	post	post	post	post	post	post	post	post
	Quartz 1-NP	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	.	.	.	.
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
07	Pre Urine	pre	pre	pre	pre	pre	pre	pre	pre	.	.	.	.	pre	pre	pre	pre
	Post Urine	post	>7 hr	post	post	post	post	post	post	.	.	.	.	post	post	post	post
	Quartz 1-NP	quartz	quartz	.	.	quartz	.	quartz	.	.	.	.	.	quartz	.	quartz	.
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	survey	.	.	.	.	survey	survey	survey	survey
08	Pre Urine	pre	pre	<50mL	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre
	Post Urine	post	post	post	>7 hr	post	post	post	post	post	post	post	post	post	post	post	post
	Quartz 1-NP	quartz	.	.	.	quartz	.	quartz	.	.	.	quartz	.	quartz	.	quartz	.
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
09	Pre Urine	<50mL	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	<50mL	pre	pre	pre
	Post Urine	post	post	post	post	post	post	post	post	post	post	post	post	post	post	post	post
	Quartz 1-NP	quartz	.	.	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
10	Pre Urine	.	.	.	.	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre
	Post Urine	.	.	.	.	>7 hr	post	post	post	post	post	post	post	post	post	post	post
	Quartz 1-NP	.	.	.	.	quartz	.	quartz	.	quartz	.	quartz	.	.	.	quartz*	.
	Questionnaire	.	.	.	.	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
11	Pre Urine	.	pre	pre	pre	<50mL	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre
	Post Urine	.	post	post	post	post	post	<50mL	post	post	post	.	post	post	post	post	post
	Quartz 1-NP	.	quartz	.	.	.	quartz	.	quartz	.	.	.	quartz	.	quartz	.	.
	Questionnaire	.	survey	survey	survey	survey	survey	.	survey	survey	survey	.	survey	survey	survey	survey	survey
12	Pre Urine	pre	pre	pre	pre	pre	pre	pre	pre	<50mL	pre	pre	pre	pre	pre	pre	pre
	Post Urine	post	post	post	post	post	post	<50mL	post	post	post	<50mL	post	post	post	post	post
	Quartz 1-NP	.	.	.	.	.	quartz	.	quartz	.	.	.	quartz	.	quartz	.	.
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
13	Pre Urine	.	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	<50mL
	Post Urine	.	post	post	post	post	post	post	post	post	post	post	post	post	post	post	post
	Quartz 1-NP	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz*
	Questionnaire	.	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
14	Pre Urine	pre	pre	pre	pre	pre	pre	pre	.	.	pre	pre	pre	pre	pre	.	.
	Post Urine	post	post	post	post	post	post	post	.	.	post	<50mL	post	post	post	.	.
	Quartz 1-NP	.	quartz	.	quartz	.	quartz	.	.	.	quartz	.	quartz	.	quartz	.	.
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	.	.	survey	survey	survey	survey	survey	.	.
15	Pre Urine	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre
	Post Urine	post	post	post	post	post	post	post	post	post	post	post	post	<50mL	post	post	post
	Quartz 1-NP	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
16		Cohort Dropout															
17	Pre Urine	.	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre
	Post Urine	.	post	post	>7 hr	post	post	post	post	post	post	post	post	post	post	post	post
	Quartz 1-NP	.	.	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	.	quartz	quartz	.
	Questionnaire	.	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
18	Pre Urine	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	.	pre	pre	pre	pre	pre
	Post Urine	post	post	post	post	post	post	post	post	post	post	post	post	post	post	post	post
	Quartz 1-NP	.	quartz	.	quartz	.	.	.	quartz	.	quartz	.	.	.	quartz	.	quartz
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey
19	Pre Urine	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	pre	<50mL	.	.	.	.
	Post Urine	post	post	post	post	post	post	post	post	post	post	post	post	.	.	.	.
	Quartz 1-NP	.	.	.	quartz	.	.	.	quartz	.	.	.	quartz	.	.	.	.
	Questionnaire	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	survey	.	.	.	.
20	Pre Urine	pre	pre	pre	pre	pre	pre	.	.	pre	pre	pre	pre	pre	pre	.	.
	Post Urine	post	post	post	post	post	post	.	.	post	post	post	post	post	post	.	.
	Quartz 1-NP	.	quartz	.	quartz	.	quartz	.	.	.	quartz*	.	quartz	quartz	quartz	.	.
	Questionnaire	survey	survey	survey	survey	survey	survey	.	.	survey	survey	survey	survey	survey	survey	.	.

* collected quartz sample met exclusion criteria for selection of urine samples

Table 4: General Stata code used for predictive and association models

Model Type	General Stata Code	Included Covariates
Prediction of 1-NP in Air		
Mixed Effects Model		Shift location Time underground Time working around DE
xtmixed ln_air1np *[covariate$_1$, covariate$_2$,…,covariate$_n$]* || subjectid:, nolog reml var		
Prediction of Values		
predict ln_air1np_pred, fitted		
Association of Predicted 1-NP in air and 6-OHNP, 8-OHNP		
Mixed Effects Model		
xtmixed ln_6ohnpcreatadj ln_air1np_pred || subjectid:, nolog reml var		
xtmixed ln_8ohnpcreatadj ln_air1np_pred || subjectid:, nolog reml var		
Prediction of post-shift 1-NP Metabolite Levels		
Simple Linear Regression		Shift location Time underground Time working around DE Respirator use Time since previous void Day of work week
reg ln_pm6ohnpcreatadj *[covariate$_1$, covariate$_2$,…,covariate$_n$]*		
reg ln_pm8ohnpcreatadj *[covariate$_1$, covariate$_2$,…,covariate$_n$]*		
Prediction of Values		
predict ln_pm6ohnp_pred		
predict ln_pm8ohnp_pred		

Table 5: Summary of quality control samples for 6-OHNP

Sample Type	Peak Area	Concentration (pg/µL)	Recovery	% of Control	LOD (pg/µL)
					0.004
Lab Blank	65.1	-	-	-	
Lab Blank	56.7	-	-	-	
Lab Blank	71.1	-	-	-	
Lab Blank	238.7	-	-	-	
Lab Blank	55.2	-	-	-	
Lab Blank	56.4	-	-	-	
Deuterated	391.0	0.002	-	-	
Deuterated	243.0	0.001	-	-	
Deuterated	80.6	0.001	-	-	
Deuterated	144.0	0.001	-	-	
Deuterated	117.0	0.001	-	-	
Deuterated	220.0	0.002	-	-	
Deuterated	342.0	0.003	-	-	
Fortified	677060.0	5.876	235%	271%	
Fortified	255435.0	3.250	130%	150%	
Fortified	466905.0	3.623	145%	167%	
Fortified	593737.0	4.386	175%	202%	
Fortified	606311.0	5.199	208%	239%	
Fortified	503654.0	4.105	164%	189%	
100% Control	222019.4	2.039	82%	-	
100% Control	211424.9	1.938	78%	-	
100% Control	280051.0	2.539	102%	-	

Table 6: Summary of quality control samples for 8-OHNP

Sample Type	Peak Area	Concentration (pg/µL)	Recovery	% of Control	LOD (pg/µL)
					0.006
Lab Blank	223.0	-	-	-	
Lab Blank	96.9	-	-	-	
Lab Blank	724.0	-	-	-	
Lab Blank	769.3	-	-	-	
Lab Blank	261.7	-	-	-	
Lab Blank	140.0	-	-	-	
Deuterated	205.2	0.003	-	-	
Deuterated	330.1	0.003	-	-	
Deuterated	223.8	0.003	-	-	
Deuterated	261.6	0.003	-	-	
Deuterated	450.8	0.004	-	-	
Deuterated	602.2	0.005	-	-	
Deuterated	411.2	0.004	-	-	
Fortified	774869.2	2.737	109%	146%	
Fortified	227848.4	1.958	78%	104%	
Fortified	475127.2	2.324	93%	124%	
Fortified	546622.0	2.315	93%	123%	
Fortified	661846.1	3.478	139%	185%	
Fortified	604206.0	2.456	98%	131%	
100% Control	367066.4	1.875	75%	-	
100% Control	370068.5	1.928	77%	-	
100% Control	363566.9	1.831	73%	-	

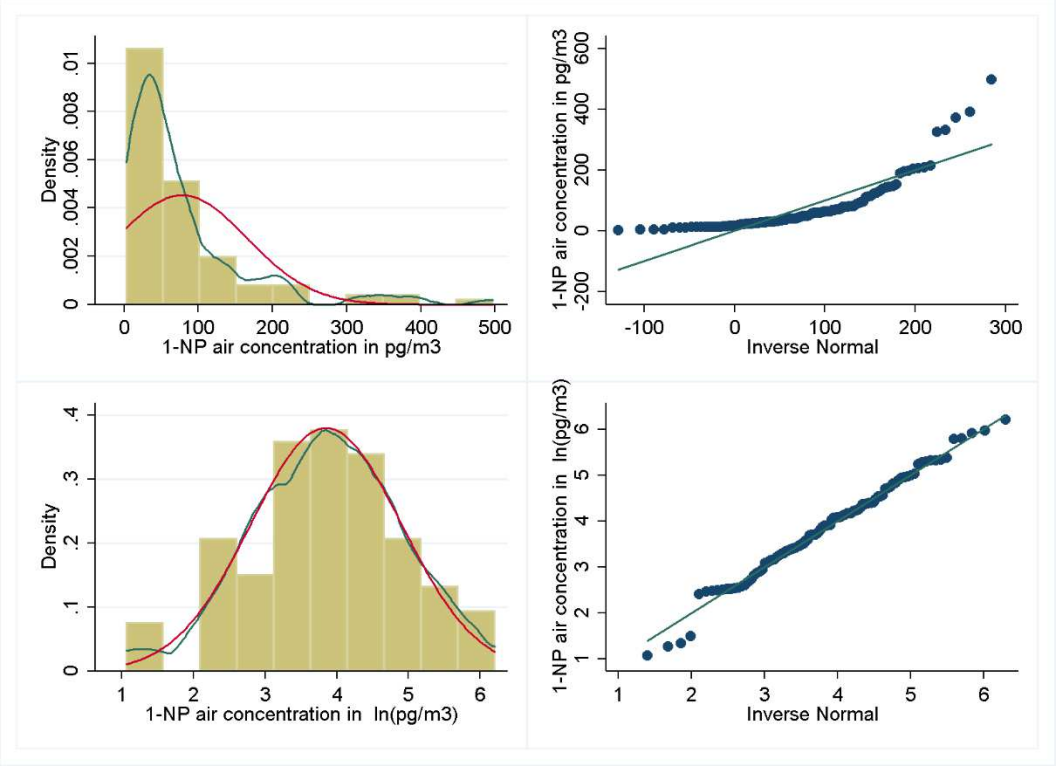

Figure 3: Distributions of native and log-transformed 1-nitropyrene

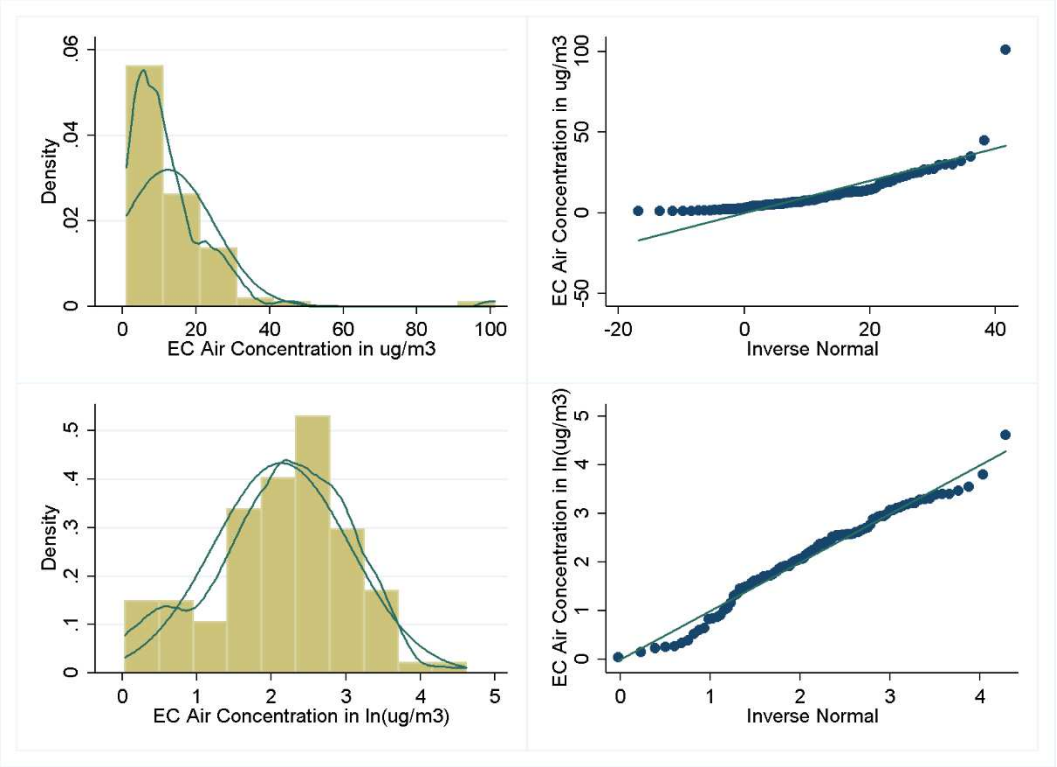

Figure 4: Distributions of native and log-transformed elemental carbon

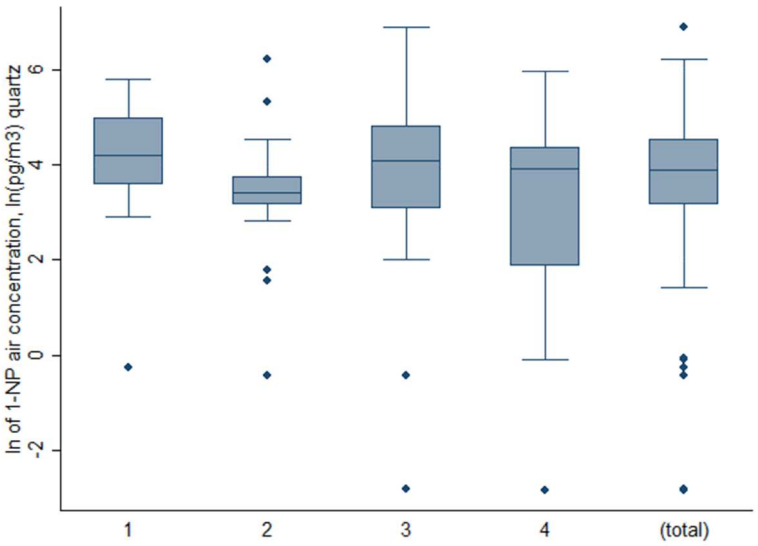

Figure 5: 1-NP in air by day of work week

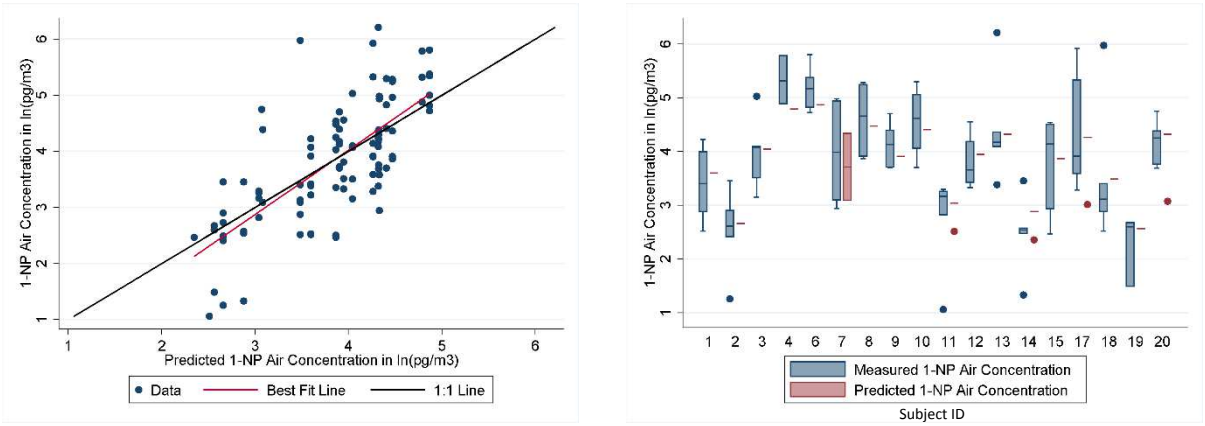

Figure 6: Comparison of predicted and measured 1-NP air concentrations in ln(pg/m^3) for Model A: Shift Location over all observations and by Subject ID

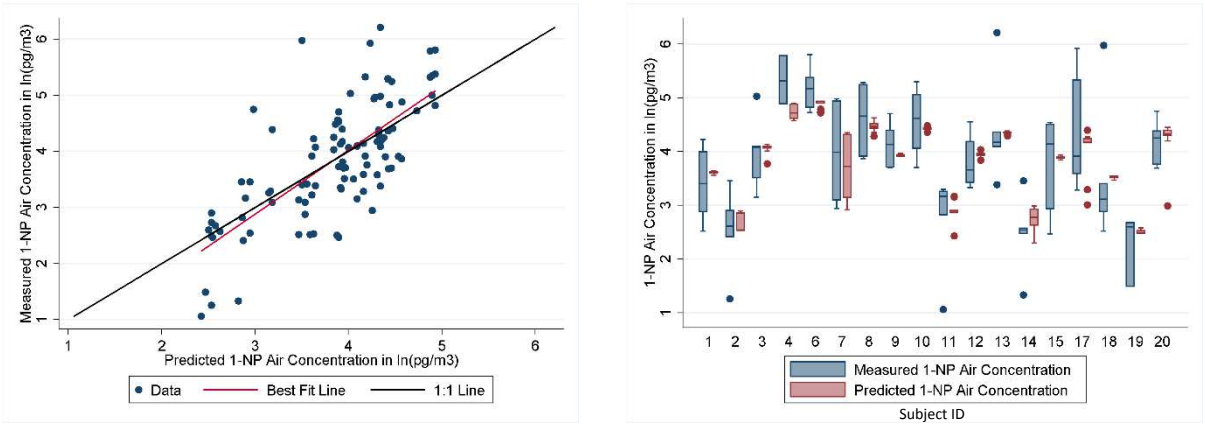

Figure 7: Comparison of predicted and measured 1-NP air concentrations in ln(pg/m^3) for Model B: Shift Location and Time Working near DE over all observations and by Subject ID

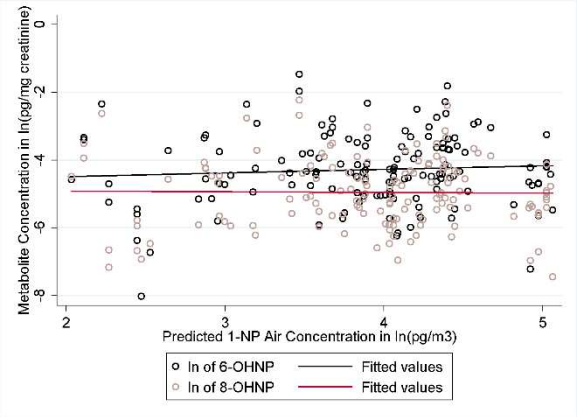

Figure 8: Estimated association between exposure to 1-NP in air and levels of 1-NP metabolites in urine (Model 1)

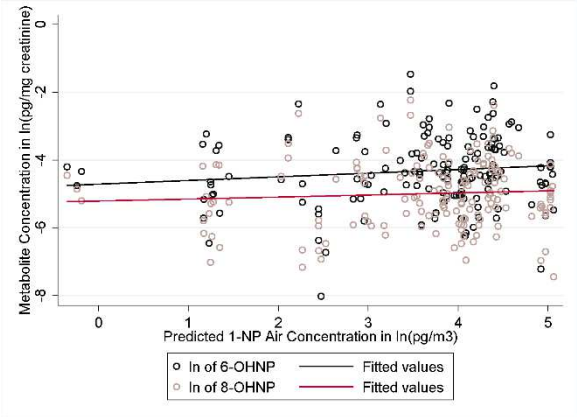

Figure 9: Estimated association between exposure to 1-NP in air and levels of 1-NP metabolites in urine (Model 2)

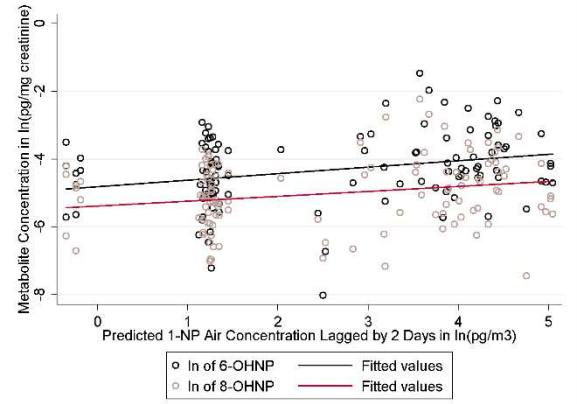

Figure 10: Estimated association between exposure to 1-NP in air and levels of 1-NP metabolites in urine (Model 4)

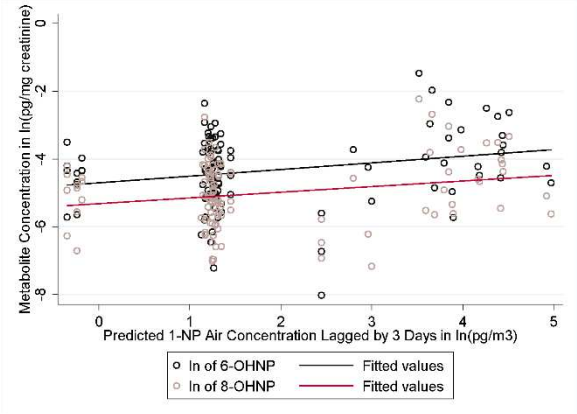

Figure 11: Estimated association between exposure to 1-NP in air and levels of 1-NP metabolites in urine (Model 5)

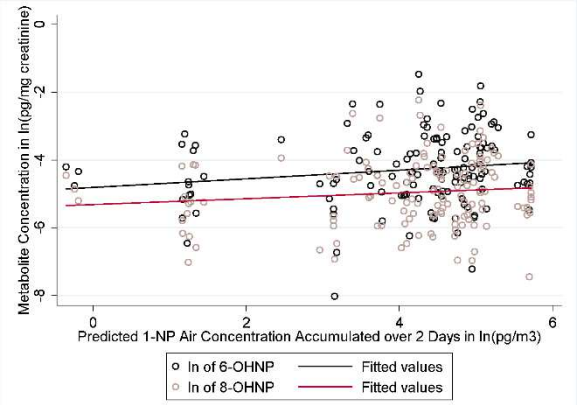

Figure 12: Estimated association between exposure to 1-NP in air and levels of 1-NP metabolites in urine (Model 6)

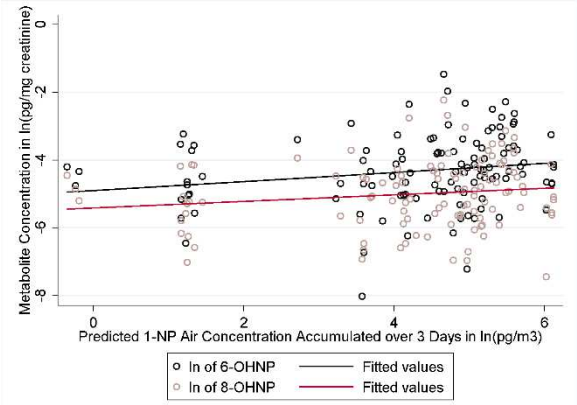

Figure 13: Estimated association between exposure to 1-NP in air and levels of 1-NP metabolites in urine (Model 7)

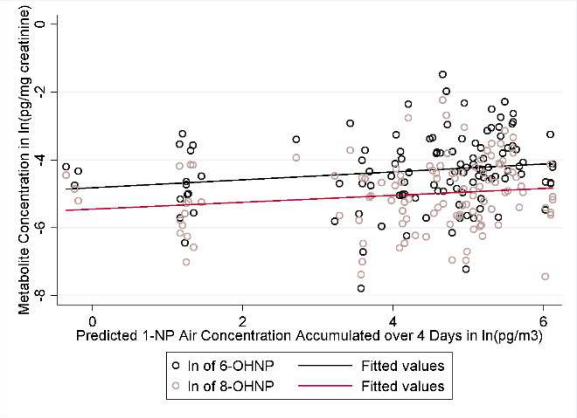

Figure 14: Estimated association between exposure to 1-NP in air and levels of 1-NP metabolites in urine (Model 8)

DE Exposure – Subject Daily Activity Log

Part 1: Technician - Complete time activity log each day based on observation and questioning of subjects. Estimate in 30-min time increments. List other activities in the Notes section at the end of log.

Activity	AM						PM						
	6	7	8	9	10	11	12	1	2	3	4	5	6
Work shift prep (inside miner change room)													
Travel from surface to underground mine site													
Working in areas with active DE emissions													
Working in areas having no active DE emissions													
Lunch/break in areas with active DE emissions													
Lunch/break in areas with no active DE emissions													
Note time, duration & cause of any work stoppage due to air quality problems													

Notes:

Part 2: Technician – Complete each day based on observation and questioning of subjects.

1. What type of job activity did you perform today? What was the % time you spent on each activity? (Check all that apply and estimate to the nearest 5% - overall % must equal 100)

1a. ☐ Ore channeling_____% 1b. ☐ Jack leg drill operation _____% 1c. ☐ Load-Haul-Dump operation _____%

1d. ☐ Cage Tending _____% 1e. ☐ Diesel Engine Repair _____% 1f. ☐ Above ground office work _____%

1g. ☐ Other: _____ _____%

2. What percentage of the time that you were exposed to DE emissions did you wear a respirator?

_____%

Technician: Questions related to biosampling

Part 3: Technicians: Ask these questions at the end of the day about the previous day's activities, using the phrase, "In the last 24 hours have you…"

1. Smoked cigarettes?

☐ Yes ☐ No ☐ Don't know

→ _____ (number of cigarettes smoked)

2. Used chewing tobacco?

☐ Yes ☐ No ☐ Don't know

→ _____ (number of cans of chewing tobacco used)

3. Performed any off the job activities where you may have been exposed to DE emissions?

☐ Yes ☐ No ☐ Don't know

→ 3a. If yes, how long were you exposed? _____ (hrs, min)

3b. What types of activities were you doing?

Activities: _____

_____

3c. What percent of your time spent doing these activities did you use a respirator?

_____%

Technician: Only ask part 4 on the first day of the work week

Part 4: Technicians: Ask these questions at the end of the day about the previous day's activities, using the phrase, "On the day before yesterday, have you…"

1. Smoked cigarettes?

☐ Yes ☐ No ☐ Don't know

→ _____ (number of cigarettes smoked)

2. Used chewing tobacco?

☐ Yes ☐ No ☐ Don't know

→ _____ (number of cans of chewing tobacco used)

3. Performed any off the job activities where you may have been exposed to DE emissions?

☐ Yes ☐ No ☐ Don't know

→ 3a. If yes, how long were you exposed? _____ (hrs, min)

3b. What types of activities were you doing?
Activities: _____

_____

3c. What percent of your time spent doing these activities did you use a respirator?
_____**%**

Notes:

STANDARD OPERATING PROCEDURE (SOP) FOR EXTRACTION AND ANALYSIS OF NITROPYRENE METABOLITES IN URINE USING LC/MS/MS

Prepared by:	Mike Paulsen	Date:	11/23/07
Revised by:	Mike Paulsen	Date:	4/9/08
Revised by:	Mike Paulsen	Date:	5/5/08
Revised by:	Mike Paulsen	Date:	5/12/08
Revised by:	Mike Paulsen	Date:	6/28/12
Revised by:	Mike Paulsen	Date:	4/4/13
Revised by:	Mike Paulsen	Date:	12/3/13
Revised by:	Mike Paulsen	Date:	4/2/15
Revised by:		Date:	
Reviewed by:		Date:	
Approved by:	Chris Simpson Assistant Professor	Date:	

Table of Contents

List of Tables

Changes for Version 3.6:

1. Use solid form of glucuronidase that has higher sulfatase activity; also change from Roche to Sigma
2. Change volume from 100 ml urine to 50 ml urine, diluted with 50 ml water
3. Spike urine with D9-Hydroxypyrene-glucuronide as a deconjugation control
4. Add D9-Hydroxypyrene and Hydroxypyrene to calibrants
5. Analyze using MedChem LC/MS system for higher sensitivity
6. Change ammonium hydroxide supplier from Sigma to Fisher
7. Add ascorbic acid to urine during hydrolysis (during hydrolysis experiment in December 2013 – may become permanent part of SOP if demonstrated to be beneficial)

Supplies:

1. Silanized glassware: amber HPLC vials, HPLC vial micro inserts, Pasteur pipettes, TurboVap tubes
2. 125 mL glass bottle with Teflon-lined cap
3. HCl, Fisher, A144s-500
4. Sodium Acetate anhydrous, Sigma S8750-1KG
5. Acetic acid
6. β-Glucuronidase/Arylsulfatase
   a. Roche, 127 698, 100,000 Fishman units/mL / 800,000 Roy units/mL,
   b. Sigma, G0751 (H-1), Lot SLBG8609V (Rec. 11/20/13), ≥ 300,000 U/gram glucuronidase and ≥10,000 U/gram arylsulfatase
7. L(+)-Ascorbic acid, ACROS 401475000, CAS 50-81-7, Lot A0320409, Received 12/9/13
8. Blue Rayon
   a. MP Biomedicals, 808687 or
   b. Funakoshi Co (Japan), BR-001
9. SPE tube (6 cc size)
10. ~~Polyethylene frit for blue rayon filtration, Supelco 57181~~
11. Ammonium hydroxide,
    a. Sigma, ACS reagent, 28-30% NH_3, 221228-500ML-A
    b. Fisher, certified ACS PLUS, A669-500, 29.55%, Lot 131698, Received 12/2/13
12. TurboVap tubes, 50ml size (silanized and methanol-rinsed)
13. Sep-Pak Alumina A cartridge, Waters, WAT020500
14. Methanol, Fisher, optima, A454-4
15. Ethyl acetate (JT Baker, Baker Analyzed ACS Reagent, 9280-05)
16. Syringe for extract filtration, disposable, 1 cc, BD, with slip tip, 309602
17. Syringe filters, Pall Acrodisc 13 mm PTFE 0.2 µm, #4542
18. HPLC vials with caps
19. Nylon membrane filters 0.45µm 100 discs, cat #7404-004, Whatman
20. Disposable flow control valve liners for the visiprep-DL, cat#57059 pack 150, Supelco
21. D9-Hydroxypyrene-glucuronide
22. DMSO

Equipment:

1. Shaking Water-Bath
2. TurboVap (50 ml size)
3. TurboVap (5 ml size); modified to fit HPLC vials
4. Wrist-Action Shaker
5. Sonic Bath
6. SPE Vacuum Manifold
7. LC/MS/MS, Agilent 1100/6410 or MedChem system

Preparation of Solutions:

Note: All solutions should be stored in glassware dedicated for the NP metabolite assay and for that specific solution.

1. 1 M HCl (75µl per sample) (0.825 ml concentrated HCl + 9.175 ml H_2O) (Stable for couple of months)
2. 4 M acetate buffer (pH 5) (5 ml per sample) (Stable for couple of months). Discard: neutralize with baking soda and pour down drain
   a. 4 M sodium acetate (49.2 g/ 150 ml final volume). Sonicate to help dissolve
   b. 4 M acetic acid (34.5 ml glacial/ 150 ml final volume)
   c. Start out with 150 ml (a) in a 250 ml Wheaton bottle and then add (b) until pH 5. Requires approximately 1.5:1 (a:b)
3. 1 M acetate buffer, pH 5.5, for preparing glucuronidase solution from powder
   a. 1 M sodium acetate, 8.20 g/100 ml final volume
   b. 1 M acetic acid, 5.75 ml/ 100 ml final volume
4. Deuterated spike solution (see table)
5. D9-Hydroxypyrene-glucuronide (deconjugation control standard)
6. Methanol: Ammonium (50:1) (20 ml per sample)
   a. prepared by diluting ammonia water to 7% of concentrate →17.5 ml NH_4OH plus 232.5 ml MeOH
   b. Make day of (discard polar waste)
7. Methanol: ethyl acetate (1:1) (40 ml per sample)
   a. Measure equal volumes of each solvent using a MeOH-rinsed graduated cylinder and combine in a Wheaton bottle
   b. Stable for couple months (discard polar waste)
8. Mobile phase B: Methanol with 0.03% ammonium hydroxide (1050 µl of 28-30% stock per liter). Prepare fresh and keep refrigerated or on ice. (Make day of)
9. Mobile phase A: Water with 0.03% ammonium hydroxide (1050 µl of 28-30% stock per liter). Prepare fresh and keep refrigerated or on ice. (Make day of)
10. Glucuronidase: prepare fresh and only the amount needed. Prior to 2014, glucuronidase was purchased in solution form and 75 ul of 85 U/ul was added for a concentration of 6375 U/100 ml urine. Following the hydrolysis test in December, 2013, the procedure was altered to use a dry form of the enzyme, purchased from Sigma Aldrich. Note that each lot will have a different activity and the actual activity may be much higher than the minimum level stated on the product information sheet. The actual concentration must be obtained from the company web site so the correct amount of enzyme is used.
    a. Historic liquid enzyme amount used: 6375 U/100 ml urine
    b. Standard amount of dry enzyme used beginning 2014 (based on Hydrolysis test #2 Dec. 2013):
       i. Discover Exp. #2: 3000 U/50 ml urine _____
       ii. Stillwater Mine: 6000 U/100 ml urine _____
    c. Weigh enzyme into 1.5 ml glass autosampler vial. Use static discharger
    d. Pour powder into a 20 ml headspace vial _____
    e. Add 1 ml of 1 M sodium acetate buffer, pH _____
    f. Cap and mix by hand
~~11. Ascorbic acid, 1.25 mg/ml urine, 62.5 mg/ 50 ml urine, 625 mg/ 10 ml water, add 1 ml to 50 ml urine.~~
    ~~a. Weigh solid into clean, silanized 15 ml glass test tube and add appropriate volume of water.~~

Urine Sample Collection

1. Collect urine sample in 500 ml Nalgene polypropylene bottles (methanol-rinsed)
2. Store frozen at -20ºC until analysis

Urine Extraction

1. Thaw urine samples
2. Filter through 0.45 µm nylon membrane filters and transfer into 125 ml silanized glass Wheaton bottles (methanol-rinsed)
3. Total volume should be 100 ml. If the sample volume is less than 100 ml, add DI water to make a total volume of 100 ml.
4. Label bottles near the top using a sharpie and label caps using tape
5. Add 75 µl of 1 M HCl (can use plastic pipette tip)
6. Add 5 ml of 4 M acetate buffer (pH 5) (use 5 ml glass pipette)
7. Add 25 µl D spike (recover standards OHNPs-d_8, OHNAAPs-d8 and 1-NAAP-d9) (use positive displacement pipette)
8. Add 20 µl D-OHP-glucuronide spike
9. Add β-Glucuronidase/aryl sulfatase (75 µl for Roche solution **or** 1 ml for solution prepared from solid Sigma enzyme)
10. Prepare water spike samples and blanks (unspiked water)
    a. D Spike (N=2): 100 ml water amended with HCl, buffer, deuterated standards, and glucuronidase
    b. D+H Spike (N=2): 100 ml water amended with HCl, buffer, protonated and deuterated standards, and glucuronidase
    c. Blank (N=2): 100 ml water amended with HCl, buffer, and glucuronidase

8. Incubate at 37°C for 4 hours in a shaking water-bath (note: should start incubation by around 10:30 am)

Blue Rayon Extraction

1. Weigh 100 mg portions of blue rayon into clean weigh boats. Store individual portions in ziplock bags until samples finish deconjugation. Longer-term storage of 100 mg portions may be in clean glass headspace vials sealed with Teflon-lined caps
2. Add 100 mg blue rayon to each sample
3. Incubate samples at room temperature while shaking on wrist-action shaker for 1 hour
    a. Hand-tighten the clamps, then use pliers to tighten ¼ turn extra
    b. Cover with black plastic bag to protect from light
4. Extraction of blue rayon
    a. Prepare 6 cc empty SPE tubes by ~~inserting polyethylene frits and~~ rinsing twice with 6 ml methanol (tubes can be cleaned in advance)
    b. Pour the urine through a plastic funnel into the SPE tube
    c. Transfer blue rayon to the SPE tube using tweezers
    d. Rinse and shake the bottle with 2 X 25 ml water and pour through the blue rayon in the SPE tube
    e. Vacuum excess water out of blue rayon (20 seconds)
    f. Rinse the bottle with 2 X 5 ml methanol to remove residual water (Important to remove as much residual water as water will extend the evaporation step)
    g. Return the blue rayon to the bottle
    h. Add 20 ml extraction solvent to bottles containing blue rayon (50:1 methanol: ammonium hydroxide solution)
    i. Sonicate blue rayon for 30 minutes (covered to protect from light)
        a. Note: sonicator holds 10 X 125 ml Wheaton bottles, so two are needed. Use care to prevent bottles from tipping over in the sonicator bath.

j. Rinse the SPE tubes with water from squirt bottle to remove any remaining blue rayon fibers, followed by 6 ml MeOH
k. Place a TurboVap tube inside the vacuum manifold, using a small beaker to hold the tube upright. Use a spacer under the beaker to elevate the TurboVap tube to just below the SPE outlet.
l. Pour extract through a funnel and the SPE tubes into TurboVap tubes
    a. Keep Blue Rayon in the bottle
    b. Rinse the bottle and Blue Rayon with methanol (2 x 5 ml). Cap and shake. Pour the methanol through SPE tube to combine with extract in TurboVap tube.
    c. Transfer the Blue Rayon to the SPE tube and use vacuum to remove remaining MeOH from blue rayon
m. Add 50 µl DMSO to each sample as a keeper solvent
n. Evaporate extract to near-dryness in TurboVap at 45ºC
    a. After 40 minutes, add 10 ml acetonitrile (volume should be approximately 1-5 ml) to assist evaporation of water (Note→ TK did not do this step). Total time to evaporate to 50 µl should be approximately 70-80 minutes.
o. CAN STOP AT THIS STAGE TO CONTINUE THE FOLLOWING DAY. PLACE CAPS ON TURBOVAP TUBES AND STORE OVERNIGHT AT -20ºC
p. Re-dissolve residue in 5 ml methanol: ethyl acetate (1:1)
q. Vortex ~10 seconds individually then sonicate 15 minutes with plastic cap and Parafilm sealing the top of TurboVap tube

Alumina Sep-Pak Cleanup

1. Place new Alumina A Sep-Paks on a clean vacuum manifold
2. Precondition Sep-Paks with 5 x 5 ml methanol:ethyl acetate (1:1)
3. Place clean, silanized 15 ml test tubes inside manifold (flow control valve liners are not needed if a spacer is placed under the manifold rack to elevate tubes to positions just under the tips)
4. Clean silanized Pasteur pipets by drawing up and expelling methanol three times
5. Rinse sides of TurboVap tube ten times and transfer extract to Sep-Paks.
6. Add a second 5 ml portion of methanol: ethyl acetate (1:1) to TurboVap tubes and transfer to Sep-Paks
7. Add a third 5 ml portion of methanol: ethyl acetate (1:1) to TurboVap tubes and transfer to Sep-Paks
8. Pour extracts from test tubes back to original TurboVap tubes. Rinse test tubes with 5 ml methanol and pour into TurboVap tubes
9. Evaporate methanol:ethyl acetate to near-dryness (approximately 50 µl) in TurboVap at 45ºC. Set pressure to 12 psi. Should be around 0.5 to 1 ml after 15 minutes and 50 µl after around 25 minutes
10. Re-dissolve residue by adding 300 µl methanol to TurboVap tubes
11. Cover TurboVap tubes with red plastic caps (methanol-rinsed)
12. Vortex briefly (10 seconds)
13. Rinse apron of TurboVap tube 10 times using a silanized, methanol-rinsed Pasteur pipet
14. Transfer to 1 cc syringe with 13 mm, 0.2 µm PTFE filter and a valve liner tip (cut to 1 cm)
15. Filter into silanized HPLC vial inserts
16. Cap vials and store samples in freezer until day of analysis
17. Make 100% Control solutions
    a. Add 25 µl D spike and 25 µl H spike (AND OHP, BUT NOT D-OHP-GLUC?)

18. On day of analysis, remove samples from freezer and evaporate methanol to approximately 50 µl under nitrogen using the small volume TurboVap with the modified rack that holds autosampler vials (100% Controls don't need evaporation). 50 µl is at the top of the apron of the insert (use marked inserts as a guide to estimate the volume).
19. Add 150 µl water. Cap vial, flick vials several times, vortex, and gently invert vials to mix. It is important to completely mix the methanol extract with the water, but be careful not to allow any extract to get out of the insert.

Quality Control Samples

1. Water Blanks
   a. N=2 per batch
   b. 100 ml water process like urine, but no spike solutions are added
2. Deuterated spiked water
   a. N=2 per batch
   b. 100 ml water process like urine
   c. Spike with 25 µl deuterated internal standards (AND D-OHP-GLUC)
3. D and H spiked water
   a. N=2 per batch
   b. 100 ml water process like urine
   c. Spike with 25 µl deuterated internal standards and with 25 µl protonated spike mix (AND OHP AND D-OHP-GLUC)
   d.
4. 100% Controls: deuterated and protonated spike solution controls. Spike solutions added directly to solvent for injection
   a. N=2 per batch
   b. 25 µl deuterated spike and 25 µl protonated spike plus 150 µl water
5. Benchmark urine
   a. N=2 per batch

Instrumental Analysis

1. Analyze by LC/MS/MS (Agilent 1100 HPLC with a 6410 tandem mass spectrometer)
2. Method: NP Metab MRM Hi pH 042308.m (See attached method printout)
3. Prepare mobile phases by adding ~~350~~ 1050 µl ammonium hydroxide per liter of either water or methanol (0.03% NH3). Mobile phases should be prepared fresh and replaced daily.
4. Install column (Agilent Zorbax Extend-C18, 2.1 X 100 mm, 3.5 µm particles, 761753-902, with guard column)
5. Purge pumps with fresh mobile phase for 10 minutes at 5 ml/ min, 50% each channel A and channel B
6. Sample naming (examples, where MMDDYY is the preparation date of the sample or standard and AA is the run number):
   a. Std 10 MMDDYY AA.d
   b. D H2O Spk #1 MMDDYY AA.d
   c. D+H H2O Spk #1 MMDDYY AA.d
   d. D+H 100% Ctl #1 MMDDYY AA.d
   e. H2O Blk #1 MMDDYY AA.d
   f. BM #1 MMDDYY AA.d

g. Sample ID MMDDYY AA.d

7. Sample log: Analyze the samples and standards in the following order
   a. Instrument checkout standard (STD 2.5)
   b. Blank
   c. Std 0.01
   d. Std 0.025
   e. Std 0.1
   f. Std 0.25
   g. Std 1
   h. Std 2.5
   i. Std 10
   j. Std 25
   k. Std 100
   l. Blank
   m. Blank
   n. QC and Benchmark sample extracts plus samples to make 15 runs
   o. Std 2.5
   p. Blank
   q. 15 Samples
   r. Std 2.5
   s. Blank
   t. 15 Samples
   u. Std 2.5
   v. Blank
   w. Repeat pattern
   x. Reanalysis of 1 QC (D+H H2O Spk)
   y. Reanalysis of urine extracts (1 per 15 samples analyzed; for long sequences, can re-analyze earlier in the sequence)
   z. Std 2.5
   aa. Blank (shutdown method NP Metab Shutdown 050908.m)

Reporting, Data Analysis and QC Review

1. Set up calibration curves using 1/x weighting
2. Print the worklist
3. Generate quantitative analysis report
4. In Excel, print each report
5. Review reports
   a. Were peaks correctly chosen?
   b. Were peaks free of apparent interfering peaks?
   c. Were peaks integrated properly?
   d. Do values in summary report match the values in the printed reports being edited?
   e. Generate a summary table for export to Excel
6. Open file in Excel for assigning QC flags

Preparation of Calibrants and Spike Solutions

1. Calibrants:
   a. Prepare standard 200 according to Table 1
   b. Prepare calibrants by diluting Standard 200 with methanol and water according to **Table 2**
   c. Store at -20°C in freezer FZ-S2

Table 1 Preparation of High Calibration Standard (200 pg/µl)

~~Stock Solution ID~~	~~Chemical~~	~~Stock Conc. (µg/ml)~~	~~Conc. of 1:100 dil (pg/µl)~~	~~Calculated Vol of 1:100 dilutions into STD 200 (µl)~~	~~Actual Vol into STD 200 (µl)~~	~~Conc. In STD 200 (pg/µl)~~
~~9~~	~~3-OHNP~~	~~235~~	~~2350~~	~~170.2~~	~~170.2~~	~~200.0~~
~~6~~	~~6-OHNP~~	~~505~~	~~5050~~	~~79.2~~	~~79.2~~	~~200.0~~
~~8~~	~~8-OHNP~~	~~51~~	~~510~~	~~784.3~~	~~784.3~~	~~200.0~~
~~13~~	~~3-OHNAAP~~	~~77~~	~~771~~	~~519~~	~~200~~	~~77.1~~
	~~6-OHNAAP~~	~~650~~	~~6504~~	~~61.5~~	~~200~~	~~650.4~~
	~~8-OHNAAP~~	~~478~~	~~4782~~	~~83.6~~	~~200~~	~~478.2~~
	~~NAAP~~	~~444~~	~~4440~~	~~90.1~~	~~90.1~~	~~200.0~~
		~~Vol MeOH (µl)~~			~~676.2~~	
		~~Total vol (µl)~~			~~2000~~	

1/27/2014

Standard No.	Chemical	Stock Conc. (ug/ml)	Conc. of 1:100 dil (pg/ul)	Calculated Vol of 1:100 dilutions into STD 200 (ul)	Actual Vol into STD 200 (ul)	Conc. In STD 200 (pg/ul)
9	3-OHNP	235	2350	170.2	170.2	200
6	6-OHNP	505	5050	79.2	79.2	200
8	8-OHNP	51	510	784.3	784.3	200
13	3-OHNAAP	77	771	519	200	77.1
	6-OHNAAP	650	6504	61.5	200	650.4
	8-OHNAAP	478	4782	83.6	200	478.2
	NAAP	444	4440	90.1	90.1	200
	OHP	25	Not diluted	Not diluted	200	2500
		Vol MeOH (µl)			476.2	
		Total vol (µl)			2000	

Table 2 Dilution of Calibrants from 200 to 0.025 pg/µl

STD	Nominal STD Conc (pg/µl)	Vol Standard (µl)	STD Used *	Vol Methanol Diluent (µl)	Final Vol Before Adding ISTD and Water (µl)	Volume ISTD (µl)	Vol Water (µl)	Final Volume (µl)	Final STD Conc. (µl)
1	100	200	200	0	200	200	1200	1600	25.0
2	25	55	200	165	198	198	1188	1584	6.25

3	10	22	200	198	198	198	1188	1584	2.50
4	2.5	22	50	198	198	198	1188	1584	0.625
5	1	22	20	198	200	200	1200	1600	0.250
6	0.25	22	5	198	200	200	1200	1600	0.0625
7	0.10	20	2	180	200	200	1200	1600	0.0250
8	0.025	20	0.5	180	200	200	1200	1600	0.00625
9	0.000			200	200	200	1200	1600	0.0000

* Concentration is before adding ISTD

Procedure:
Starting with STD 200, dilute with methanol to make standards 1-9
There should be 198-200 µl each standard
Add 198-200 µl ISTD spike solution (same proportion as 25 µl per 50 µl final sample volume in methanol)
Add 1188-1200 µl water to each standard (75% water; same as adding 150 ul water to 50 ul sample)

Table 3 Concentration of Calibrants

Concentration of Standards after adding ISTD and Water (1/27/14)

Compound	Stock STD Concentration (pg/µl)	Nominal conc after adding ISTD, before diluting 1:4 with water 100*	25*	10*	2.5*	1*	0.25*	0.1*	0.025*	0*
3-OHNP	200	25.000	6.250	2.500	0.625	0.250	0.0625	0.0250	0.00625	0.000
6-OHNP	200	25.000	6.250	2.500	0.625	0.250	0.0625	0.0250	0.00625	0.000
8-OHNP	200	25.000	6.250	2.500	0.625	0.250	0.0625	0.0250	0.00625	0.000
3-OHNAAP	77	9.633	2.408	0.963	0.241	0.0963	0.0241	0.0096	0.00241	0.000
6-OHNAAP	650	81.306	20.326	8.131	2.033	0.813	0.2033	0.0813	0.02033	0.000
8-OHNAAP	478	59.781	14.945	5.978	1.495	0.598	0.1495	0.0598	0.01495	0.000
NAAP	200	25.000	6.250	2.500	0.625	0.250	0.0625	0.0250	0.00625	0.000
OHP	2500	312.500	78.125	31.250	7.813	3.125	0.7813	0.3125	0.07813	0.000

*Nominal concentrations before diluting with water

~~Compound~~	~~Stock STD Conc. (pg/µl)~~	~~100*~~	~~25*~~	~~10*~~	~~2.5*~~	~~1*~~	~~0.25*~~	~~0.1*~~	~~0.025*~~	~~0*~~
~~3-OHNP~~	~~200~~	~~25.0~~	~~6.25~~	~~2.5~~	~~0.625~~	~~0.25~~	~~0.0625~~	~~0.025~~	~~0.00625~~	~~0.0000~~
~~6-OHNP~~	~~200~~	~~25.0~~	~~6.25~~	~~2.5~~	~~0.625~~	~~0.25~~	~~0.0625~~	~~0.025~~	~~0.00625~~	~~0.0000~~
~~8-OHNP~~	~~200~~	~~25.0~~	~~6.25~~	~~2.5~~	~~0.625~~	~~0.25~~	~~0.0625~~	~~0.025~~	~~0.00625~~	~~0.0000~~
~~3-OHNAAP~~	~~77~~	~~9.633~~	~~2.408~~	~~0.963~~	~~0.241~~	~~0.0963~~	~~0.0241~~	~~0.00963~~	~~0.00241~~	~~0.0000~~
~~6-OHNAAP~~	~~650~~	~~81.306~~	~~20.326~~	~~8.131~~	~~2.033~~	~~0.8131~~	~~0.2033~~	~~0.08131~~	~~0.02033~~	~~0.0000~~
~~8-OHNAAP~~	~~478~~	~~59.781~~	~~14.945~~	~~5.978~~	~~1.495~~	~~0.5978~~	~~0.1495~~	~~0.05978~~	~~0.01495~~	~~0.0000~~
~~NAAP~~	~~200~~	~~25.0~~	~~6.25~~	~~2.5~~	~~0.625~~	~~0.25~~	~~0.0625~~	~~0.025~~	~~0.00625~~	~~0.0000~~

*Nominal concentrations before diluting with water

Spike Solutions

Table 4 Preparation of Deuterated Spike Solution

(Old solution with 1X OHNAAPs)

Standard No.	Chemical	Stock Conc. (ug/ml)	Conc. of 1:10 dil (pg/ul)	Calculated Vol into ISTD Spk Solution (ul)	Actual Vol into ISTD Spk Solution (ul)	Actual Conc ISTD Spk Solution (pg/ul)	Actual Conc in Sample (pg/ul)
17	d8-3-OHNP	190	18967	36.91	10	7.6	0.95
17	d8-6-OHNP	604	60352	11.60	10	24.1	3.02
17	d8-8-OHNP	509	50928	13.74	10	20.4	2.55
25	d8-3-OHNAAP	19	1910	367	440	33.6	4.20
23	d8-6-OHNAAP	282	28193	24.83	75	84.6	10.57
23	d8-8-OHNAAP	201	20067	34.88	75	60.2	7.53
-	d9-NAAP	117	11700	59.83	150.0	70.2	8.78
-							-
-			Vol MeOH (µl)			24230	-
-	-	-	Total vol (µl)	-	-	25000	-

Table 5 Preparation of Deuterated Spike Solution with 5X Higher OHNAAPs

Standard No.	Chemical	Stock Conc. (µg/ml)	Conc. of 1:10 dil (pg/µl)	Calculated Vol into ISTD Spk Solution (µl)	Actual Vol into ISTD Spk Solution (µl)	Actual Conc ISTD Spk Solution (pg/µl)	Actual Conc in 100 ml Urine (pg/ml)
17	d8-3-OHNP	190	18967	36.91	10	7.6	1.90
17	d8-6-OHNP	604	60352	11.60	10	24.1	6.04
17	d8-8-OHNP	509	50928	13.74	10	20.4	5.09
25	d8-3-OHNAAP	19	Use stock	367	220	168.1	42.02
23	d8-6-OHNAAP	282	Use stock	24.83	37.5	422.9	105.72
23	d8-8-OHNAAP	201	Use stock	34.88	37.5	301.0	75.25
	d9-NAAP	117	11700	59.83	150.0	70.2	17.55
			Vol MeOH (µl)			24230	
			Total vol (µl)			25000	

Table 6 Preparation of Protonated Spike Solution

Compound	Actual Used Vol of 1:100 dil stock for 100 samples (ul)	Actual Used Vol of 1:1000 dil stock for 100 samples (ul)	Corrected Stock Conc. (ng/ul)	Mass in 2.5 ml Spike solution (pg)	Actual High Spike Mass per sample (pg)	Actual High Spike Extract Conc (pg/ul)
3-OHNP	21.3		235	50000	500	2.50
8-OHNP	98.0		51	50000	500	2.50
6-OHNP		99.0	505	50000	500	2.50
3-OHNAAP	20.0		77		154	0.77
8-OHNAAP			478		956	4.78

6-OHNAAP			650		1301	6.50
NAAP		112.6	444	50000	500	2.50
Spike volume	25					
Volume MeOH	2149					
Final volume = 2.5 ml						

Table 7 Preparation of Hydroxypyrene and D9-Hydroxypyrene-glucuronide Spike Solutions

Hydroxypyrene stock:

- Prepared 5/18/13
- 25 µg/ml in _____

D9-Hydroxypyrene-glucuronide

- Prepared _____
- 5 µg/ml in _____

Table 8 Preparation of Benchmark Urine #1

Batch #1

The first NP metabolites benchmark urine sample was prepared on _____ from leftover urine from the Shenyang study plus urine from lab staff members at the UW. As of 5/10/13, there are 9-100 ml aliquots remaining and we would like to use some to conduct experiments related to evaluating the hydrolysis step of the sample preparation.

Compound	Stock Conc. (ng/ul)	1:100 dilution Stock Conc. (pg/ul)	Vol of 1:100 dil used for 5 L (ul)	Make 10 X Volume (ul)	Conc in Benchmark (pg/ml)
3-OHNP	235	2350	2.6	25.5	1.2
8-OHNP	51	510	11.8	117.6	1.2
6-OHNP	505	5050	1.2	11.9	1.2
3-OHNAAP	77	771	2.0	20.0	0.31
8-OHNAAP	478	4782			1.9
6-OHNAAP	650	6504			2.6
NAAP	444	4440	1.4	13.5	1.2
			Total vol (ul)	188.6	
			Vol added to 5 L (ul)	18.9	

Batch #2

Use CDC 2004 Peru Worker Study urine (Trujillo driver cohort). Approximately 170 tubes with around 10-15 ml each will be combined (total volume = 1500 ml). To increase the volume, additional urine from UW researchers will be added (volume = 2500). Total volume is 4000 ml; 80 X 50 ml tubes.

Procedure:

1. Thaw CDC Peru Worker Study urine
2. Rinse 4 liter bottle (only used for new Optima Methanol) with MeOH
3. Transfer urine to bottle
4. Add UW researcher urine
5. Spike with protonated analytes according to the table below
6. Aliquot into 50 ml plastic tubes (Falcon _____)

Table 9 Preparation of Benchmark Urine #2 May 2013

Compound	Stock Conc. (ng/ul)	1:100 dilution Stock Conc. (pg/ul)	Vol of 1:100 dil used for 4 L (ul)	Make 20 X Volume (ul)	Conc in Benchmark (pg/ml)	Date 1:100 dilution prepared	Notes
3-OHNP	235	2350	2.04	40.9	1.2	4/2/2007	a
8-OHNP	51	510	9.41	188.2	1.2	4/9/2007	a
6-OHNP	505	5050	0.95	19.0	1.2	4/2/2007	a
3-OHNAAP	77	771	1.61	32.2	0.31	4/2/2007	a, b
8-OHNAAP	478	4782	1.59		1.9		a, b
6-OHNAAP	650	6504	1.60		2.6		a, b
NAAP	444	4440	1.08	21.6	1.2	4/2/2007	a, c
			Total vol (ul)	302			
			Vol added to 4 L (ul)	15.1			

a) Concentrations chosen to match Banchmark #1

b) Wrong units are written on the 1:100 dilution vial. Correct units are ng/ul.

c) Wrong concentration is written on the 1:100 dilution vial. Correct conc is 4440 pg/ul.

D9-Hydroxypyrene-glucuronide

Table 10 LC/MS/MS Method from Mass Hunter Software

Acquisition Method Info

Method Name NP Metab MRM Hi pH 042308_40.m
Method Path D:\MassHunter\methods\2010 Methods\NP Metabolites methods\ NP Metab MRM Hi pH 042308_40.m
Method Description ESI Positive MS2 Background Scan Method

Device List

ALS
Quat
Pump

Column

MS QQQ

QQQ Mass Spectrometer

Ion Source ESI
Tune File atunes.tune.xml
Stop Mode No Limit/As Pump
Stop Time 20
Time Filter On
Time Filter Width 0.07

Time Segments

Time Seg #	Time	Scan Type	Ion Mode	Polarity	Div Valve	Delta EMV	Store
1	0	MRM	ESI	Negative	To Waste	0	☐
2	1	MRM	ESI	Negative	To MS	800	☑
3	11.5	MRM	ESI	Negative	To MS	800	☑
4	19.5	MRM	ESI	Negative	To MS	800	☑
5	23.5	MRM	ESI	Negative	To Waste	0	☐

Time Segment 1

Scan Segments

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)
Compound1	☐	350	Unit	200	Unit	500	140	0

Source Parameters

Parameter	Value
Gas Temp (°C)	350
Gas Flow (l/min)	11
Nebulizer (psi)	50
Capillary (V)	1500

Time Segment 2

Scan Segments

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)
d8-OHNAAP	☐	282	Unit	239	Unit	400	170	25
OHNAAP	☐	274	Unit	231	Unit	400	170	25

Source Parameters

Parameter	Value
Gas Temp (°C)	350
Gas Flow (l/min)	10
Parameter	**Value**
Nebulizer (psi)	40
Capillary (V)	2000

Time Segment 3

Scan Segments

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)
d8-OHNP	☐	270	Unit	240	Unit	400	160	25
OHNP	☐	262	Unit	232	Unit	400	160	25

Source Parameters

Parameter	Value
Gas Temp (°C)	350
Gas Flow (l/min)	11
Nebulizer (psi)	50
Capillary (V)	1500

Time Segment 4

Scan Segments

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)
d9-NAAP	☐	267	Unit	225	Unit	400	150	25
NAAP	☐	258	Unit	216	Unit	400	150	25

Source Parameters

Parameter	Value

Gas Temp (°C)	350
Gas Flow (l/min)	11
Nebulizer (psi)	50
Capillary (V)	1500

Time Segment **5**

Scan Segments

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)
Compound1	☐	350	Unit	200	Unit	200	135	0

Source Parameters

Parameter	Value
Gas Temp (°C)	350
Gas Flow (l/min)	11
Nebulizer (psi)	50
Capillary (V)	1500

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	0	10000000

Instrument Curves

Actual

#N/A

Autosampler

Name	ALS	**Model**	G1313A
Ordinal #	1	**Options**	

Stop Time (min)	As Pump	**Post Time (min)**	Off

Injection Type	Standard Injection	**Injection Volume**	50
Overlap Time	Overlap when flushed	**Draw Position**	3
Draw Speed	100	**Eject Speed**	100
Wash Vessel	N/A		
Ready Temp. Range		**Temp.**	

Contact 1	0
Contact 2	0
Contact 3	0
Contact 4	0

Quaternary Pump

Name	Quat Pump	**Model**	G1311A
Ordinal #	1	**Options**	

Stop Time (min)	24.5	**Post Time (min)**	Off

Flow (µl/min)	0.2	**Pressure Min (bar)**	0
Pressure Max (bar)	400	**Max Flow Gradient (ml/min)**	100

Solvent A	water w/0.03% NH3	**Solvent B**	MeOH w/0.03% NH3
Solvent C		**Solvent D**	
Solvent Ratio A	70	**Solvent Ratio B**	30
Solvent Ratio C	0	**Solvent Ratio D**	0
Compress. A (*10-6/bar)	50	**Stroke A**	Auto
Primary Channel	0		

Contact 1 0
Contact 2 0
Contact 3 0
Contact 4 0

Pump Time Table

Time	Flow	Pressure	Solv Ratio B	Solv Ratio C	Solv Ratio D
0	0.2	300	30	0	0
17	0.2	No Change	70	0	0
17.01	0.2	No Change	90	0	0
20	0.2	No Change	90	0	0
20.1	0.3	No Change	30	0	0
24	0.3	No Change	30	0	0
24.1	0.2	No Change	30	0	0

Thermostated Column Compartment

Name Column **Model** G1316A
Ordinal # 1 **Options** CSV

Stop Time (min) As Pump **Post Time (min)** Off

Left Temp. 30 **Right Temp.** 30
Left Ready Off **Right Ready** Off
Valve Position 1

Contact 1 0
Contact 2 0
Contact 3 0
Contact 4 0

Signals Selected

Description

Temperature of left heat exchanger

Table 11 Data QC Flags

Symbol	Meaning	Action
ND	Compound not detected	Replace value with <LOD
NQ	Compound not quantifiable but detected	delete value/censor value cell
NR	Concentration has error	delete value/censor value cell
NS	ISTD Missing	delete value/censor value cell
N<	Conc is < LQL	no action
AI	Area not valid ˆ Interference	delete value/censor value cell
N>	Conc is > UQL	no action
AX	Area not valid	delete value/censor value cell
N1	Compound not analyzed for	no action
RY	Recovery outside QA limits or was indeterminate	delete value/censor value cell
MR	Value less than minimum reported level	replace with <LOD
R2	Calibration outside QA limit	delete value/censor value cell
M03	outlier	no action