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Nitro-PAH exposures of occupationally-exposed traffic workers and associated urinary 1-nitropyrene metabolite concentrations

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ABSTRACT

The assessment of occupational exposure to diesel exhaust (DE) is important from an epidemiological perspective. Urinary biomarkers of exposure have been proposed as a novel approach for measuring exposure to DE. In this study, we measured the concentrations of two urinary metabolites of 1-nitropyrene (1NP), a nitrated polycyclic aromatic hydrocarbon that has been suggested as a molecular marker of diesel particulate matter. These two metabolites, 6-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene, were determined in urine samples (10 mL) from a small group of workers who were occupationally-exposed to vehicle exhaust in Trujillo, Peru, before and after their workshifts. Workshift exposures to 1NP, as well as $PM_{2.5}$, 2-nitropyrene and 2-nitrofluoranthene, were also measured. Exposures to 1NP were similar in all studied workers, averaging 105 ± 57.9 pg/m^3 (± standard deviation). Median urinary concentrations of the average of the pre- and post-exposure samples for 6-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene, were found to be 3.9 and 2.3 pg metabolite/mg creatinine, respectively in the group of occupationally-exposed subjects (n = 17) studied. A direct relationship between workshift exposure to 1NP and urinary 1NP metabolites concentrations was not observed. However, the 1NP exposures and the creatinine-corrected urinary concentrations of the hydroxynitropyrene metabolites in these Peruvian traffic workers were similar to occupationally-exposed taxi drivers in Shenyang, China, and were higher than biomarker levels in office workers from Trujillo without occupational exposure to vehicle exhaust.

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This study provides further evidence that urinary metabolites of 1NP are associated with exposure to DE and may serve as a useful exposure biomarker.

Introduction

Exposures of humans to particulate matter (PM), have been shown to cause adverse health effects (Dockery et al., 1993). These effects have been observed as a result of both acute and chronic exposures. Health risks from exposures to PM are elevated for particles that are less than 2.5 μm in diameter, also known as $PM_{2.5}$ (Russell and Brunekreef, 2009). $PM_{2.5}$ can be produced by a variety of processes, including internal combustion engines that use gasoline or diesel as fuel, coal combustion, biomass combustion, and secondary atmospheric reactions of gas-phase precursors. The relative impact of individual sources of $PM_{2.5}$ can be investigated through the use of molecular markers that are produced preferentially by specific processes (Schauer et al., 1996). The use of these molecular markers can be applied to assessments of PM source strength on a regional scale (Bergauff et al., 2009), or to assessments of the exposures of individuals to a specific type of PM (Sheesley et al., 2008). Exposure to specific types of $PM_{2.5}$ (or other types of pollution) can also be investigated through the use of exposure biomarkers — chemical species measured in biological samples, derived either from chemicals inherent in the exposure or produced by the body in response to a specific exposure (Jakubowski, 2012; Xue et al., 2014).

Diesel particulate matter (DPM) is a significant contributor to ambient PM, and epidemiological and toxicological studies have indicated that exposure to diesel exhaust (DE) is associated with a variety of adverse health effects including respiratory disease, cardiovascular disease and lung cancer (US EPA, 2002; Cassee et al., 2013; IARC, 2013). As such, there is particular interest in reliably measuring human exposures to DPM. 1-Nitropyrene (1NP) is a nitrated polycyclic aromatic hydrocarbon (nitro-PAH) that is predominately produced in the exhaust of a diesel engine and is highly enriched in DPM relative to PM from other sources (Scheepers et al., 1995). 1NP was classified as a probable human carcinogen (group 2A) by the International Agency for Research on Cancer (IARC) in 2012 (Benbrahim-Tallaa et al., 2012). 1NP has been suggested as a molecular marker of DE exposure (Scheepers et al., 1995). Recent work by Zimmermann et al. (2013) has shown that 1NP is also produced *via* heterogeneous reactions on particles at night. However, this atmospheric formation is expected to be relatively small in comparison to the levels of 1NP formed by DE. 1NP is metabolized in humans primarily through hydroxylation, nitro-reduction and N-acetylation pathways (Ball et al., 1984; Elbayoumy and Hecht, 1984; Elbayoumy et al., 1984; Ball and King, 1985; Howard et al., 1985, 1995; Toriba et al., 2007). The major urinary metabolites observed from human and animal exposure to DPM include the hydroxy-1-nitropyrenes, 3-, 6-, and 8-hydroxy-1-nitropyrene (OHNP), their N-acetylamino derivatives, *N*-acetyl-1-aminopyrene (NAAP), and 1-aminopyrene (1AP) (van Bekkum et al., 1998; Toriba et al., 2007). These metabolites have been suggested as biomarkers of DE exposure in humans (van Bekkum et al., 1998; Toriba et al., 2007).

2-Nitropyrene (2NP) and 2-nitrofluoranthene (2NFl) are nitro-PAHs that are produced *via* secondary reactions between pyrene and fluoranthene with ambient hydroxyl radical and/or NO_3 in the presence of NO_2 (Atkinson and Arey, 1994). Although these compounds are not directly produced by a specific combustion process, they are related both to atmospheric reaction conditions and emissions from combustion sources (Arey et al., 1989) and are toxicologically relevant (Durant et al., 1996).

Adetona et al. (2012) explored exposure to $PM_{2.5}$ and associated urinary hydroxy-PAH concentrations in a group of subjects with occupational exposures to traffic-associated $PM_{2.5}$, who lived and worked in the Peruvian city of Trujillo, a coastal city of over 700,000 people in the foothills of the Andes Mountains. As of the year 2000, the majority of the registered vehicle fleet in Trujillo was not equipped with emission controls. In the current study, we investigate the relationship between exposure to nitro-PAHs in $PM_{2.5}$ and the urinary metabolites of 1NP, in a subset of 13 workers from the Adetona cohort.

1. Materials and methods

1.1. Description of subject cohorts

Two groups of individuals were monitored: those anticipated to have occupational exposure to DE, and a smaller group of individuals not anticipated to have occupational exposure to DE. The occupationally-exposed individuals had a variety of occupations (minivan drivers, minibus drivers, motorcycle police officers), in which the subjects' job duties were such that they were anticipated to inhale traffic exhaust throughout their workshift. Due to the small number of individuals in each category, data collected from these occupationally-exposed individuals were summarized as a single cohort. For occupationally-exposed subjects, filter samples for the $PM_{2.5}$ exposures corresponding to their workshifts were collected. Each subject was sampled once on a single day. Combi (minivan) vehicles are small van-size vehicles that operate like a commercial bus and were powered by diesel engines using high-sulfur diesel fuel (Han et al., 2005). Minibus drivers operated normal sized city buses. Neither vehicle type was air-conditioned. Non-occupationally exposed individuals were office workers, whose primary job duties did not place them in direct, prolonged contact with traffic exhaust.

Pre-exposure urine samples were collected immediately prior to the workshift from four minivan drivers, eight minibus drivers, two motorcycle police officers, and three office workers. Post-exposure urine samples were collected immediately after the workshift from four minivan drivers, nine minibus drivers, three motorcycle police officers, and

four office workers. In some cases, a post-exposure urine sample was not available although a pre-exposure urine sample had been collected, and *vice-versa*. For this reason, the number of pre- and post-exposure urine samples is not equal. In addition, filter samples were not available for every subject, and no filter samples were collected for the office worker subjects (*i.e.*, the non-exposed subjects). As such, thirteen paired urine samples from occupationally-exposed subjects, with associated filter samples were available for analysis: three minivan drivers, eight minibus drivers, and two motorcycle police officers. In addition, one unpaired pre-exposure urine sample (minivan driver) and three unpaired post-exposure urine samples (one motorcycle police office, one minivan driver, and one minibus driver) were available, resulting in 30 urine samples from a total of 17 occupationally-exposed subjects. For the non-occupationally exposed subjects, two paired urine samples were available, as well as one additional pre-exposure urine sample and two additional post-exposure urine samples, resulting in seven urine samples from five non-occupationally exposed subjects.

Institutional Review Board (IRB) approval was obtained from the University of Georgia (Approval #98-7729G/A-09), and the University of Washington (Approval #23097-E/G).

1.2. Collection and measurement of fine PM and urine samples

Details regarding subject recruitment and sample collection have been reported previously (Adetona et al., 2012). Briefly, subjects were recruited through the city of Trujillo municipal government. Subjects worked approximately 13 hr/day between 5:00 AM and 8:30 PM, 5 or 6 days/week. Subjects wore sampling equipment for the duration of their workshifts for $PM_{2.5}$ samples, consisting of AirChek 2000 pumps (SKC Inc., Eighty Four, PA, USA) with Triplex $PM_{2.5}$ cyclones (Model SCC1.062, BGI Inc., Waltham, MA, USA), which sampled at 1.5 L/min through 37 mm Teflon filters (Teflo 2.0 μm; Pall Corp., Ann Arbor, MI, USA). All filter samples were collected between November 8–11, 2003. Filters were transported back to the University of Georgia on dry ice for gravimetric analysis, and forwarded to the University of Washington for analysis of nitro-PAHs. Urine samples (50 mL) were collected from the subjects before and after their workshifts, frozen, and 10 mL of these urine samples was transported to the University of Washington by way of Atlanta, GA, USA.

1.3. Measurement of nitro-PAHs in PM samples

Nitro-PAHs were measured in the $PM_{2.5}$ samples using the methods published previously (Miller-Schulze et al., 2007, 2010). Briefly, filters were spiked with deuterated (d_9) 1NP, sonicated for 60 min in methylene chloride, after which the methylene chloride was evaporated under nitrogen. The extracts were reconstituted in 150 μL of ethanol/sodium acetate buffer, vortexed and then filtered through syringe filters into silanized vial inserts. Extracts were analyzed using two-dimensional high performance liquid chromatography tandem mass spectrometry (2D-HPLC-MS/MS). This method involves the separation and online derivatization of the nitro-PAH analytes using a Pt/Rh catalyzed online reduction, online trapping and concentration, and secondary HPLC separation of the amino-PAH derivatives. The amino-PAH analytes and deuterated 1-aminopyrene internal standard were then detected using tandem mass spectrometry (triple quadrupole) using selected reaction monitoring which monitored the transition corresponding to the loss of the protonated amine group (loss of 17 Da).

Recoveries of nitro-PAH analytes from positive control samples (blank filters spiked with nitro-PAH analytes and perdeuterated 1-nitropyrene (1dNP) internal standard and extracted as "normal" samples) were as follows: 2NP: 141%, 2NFl: 138%, and 1NP: 138% (average of two control samples).

1.4. Measurement of 1NP metabolites in urine samples

Metabolites of 1NP were measured as described in the work of Toriba et al. (2007) and applied in our previous study of Shenyang taxi drivers (Miller-Schulze et al., 2013), with minor modifications. Briefly, 10 mL of the urine sample was vacuum filtered through nylon membrane filters, transferred to silanized glass bottles, and then spiked with a suite of deuterated internal standards. The pH of the urine samples was adjusted to 5 with sodium acetate and HCl, followed by an enzymatic deconjugation of the 1NP metabolites using β-glucuronidase/aryl sulfatase. After 4 hr of incubation at 37°C, Blue Rayon was added to extract the deconjugated 1NP metabolites from the urine. The Blue Rayon was separated from the urine by filtration, after which the 1NP metabolites were extracted from the Blue Rayon using a solution of 50 parts methanol to 1 part ammonia followed by sonication. This methanol/ammonia mixture was evaporated to near dryness (with only 50 μL of DMSO keeper solvent remaining) under nitrogen and then reconstituted in 1:1 ethyl acetate:methanol, which was then passed through a Sep-Pak alumina A cartridge to remove impurities. The ethyl acetate/methanol mixture was then evaporated to near dryness, reconstituted in 300 μL methanol, filtered through a 0.45 μm PTFE syringe filter and reduced to a volume of 50 μL. Twenty microliters of water was added to bring the final extract volume to 70 μL. Ten microliters of this extract was injected into the HPLC-MS/MS system to be analyzed as described below.

Sample extracts were analyzed by HPLC tandem quadrupole MS/MS (Toriba et al., 2007; Miller-Schulze et al., 2013). 1NP metabolites and deuterated internal standards were separated chromatographically using an Agilent Zorbax Extend-C18 column (2.1 × 100 mm, d_p = 3.5 μm) with a gradient of water and methanol, each of which contained 0.01% ammonia. The mass spectrometer employed as a detector was an Agilent Model 6410 triple quadrupole mass spectrometer (Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source operated in negative ion mode.

The transitions monitored to quantify the levels of 1NP metabolites were as follows: for the hydroxy-*N*-acetyl aminopyrene metabolites (OHNAAPs), the transition for the analytes was *m/z* 274 → 231 (loss of $COCH_3$), for the d_8-OHNAAP internal standards, *m/z* 282 → 239. For the hydroxynitropyrene metabolites (OHNPs), the transition monitored was *m/z* 262 → 232 (loss of NO), for the d_8-OHNP internal standards, *m/z* 270 → 240. For NAAP, the transition monitored was *m/z* 258 → 216 (loss of $COCH_2$), for d_9-NAAP, *m/z* 267 → 225.

The method used to quantify these 1NP metabolites is designed to quantify 3-, 6-, and 8-hydroxy nitropyrene, NAAP, and the 3-, 6-, and 8-hydroxy-*N*-acetyl-1-aminopyrenes.

However in the current data set, due to matrix interferences (OHNAAPs, 3-OHNP) or blank contamination (NAAP), only 6OHNP and 8OHNP were detected frequently enough and free from chromatographic interferences to yield useful data. This is likely due to interfering compounds that persisted through the sample preparation steps and chromatography as well as the relatively low volume of the urine samples (10 mL), as compared with the 100 mL sample volumes used in previous works by Toriba et al. (2007) and Miller-Schulze et al. (2013). In addition, the metabolite of 1NP produced by reduction of the nitro group, *i.e.*, 1AP, is not measured in our analysis method, but has been proposed as a marker of DPM exposure in other studies (Laumbach et al., 2009; Huyck et al., 2010).

In order to account for variation in diuresis, urinary creatinine was measured by the University of Washington Medical Center clinical laboratory using a colorimetric assay.

1.5. *Quantification of determinants and data analysis*

Chromatographic peaks corresponding to both nitro-PAHs and 1NP metabolites were integrated using Agilent's MassHunter software and quantified with a combination of SigmaPlot and Microsoft Excel. Statistical analysis was performed with SigmaPlot 12.0.

The quantification of the nitro-PAHs in the filter samples and 1NP metabolites in the collected urine samples was performed in somewhat different fashion. For the nitro-PAH analytes in the filter samples, a fixed mass of the perdeuterated 1-nitropyrene (d_9) was added to all samples (prior to extraction) and the calibration standards. Calibration curves were then created by regressing (with inverse concentration, "1/*x*", weighting) the relative response of the nitro-PAH analytes (response of either 2NP, 2NFl, or 1NP divided by the response of the deuterated 1NP) against the concentration of the calibration solution. Calibration solutions contained nitro-PAH analytes in concentrations of 2.5–5000 fg/μL.

For the 1NP metabolites in the urine samples, calibration solutions for each of the 1NP metabolites as well as their deuterated analogs were analyzed to create a calibration curve for each (again, using with 1/*x* weighting). The fraction of expected deuterated 1NP metabolite calculated to be in each calibration solution was used to "correct" the calculated concentration of the 1NP metabolite analytes to account for losses during the extraction process as well as matrix interferences affecting ionization. This correction was achieved by dividing the calculated concentration (based on a regression of 1NP metabolite response *versus* 1NP metabolite concentration) of the 1NP metabolite by the fractional recovery of the corresponding deuterated 1NP metabolite internal standard. For both 1NP metabolites and deuterated analogs, calibration solutions ranged from approximately 0.050 to 50 fg/μL.

Recoveries of the d_8-6OHNP and d_8-8OHNP internal standards from the urine samples were as follows: for d_8-6OHNP, 71% ± 24% (average ± standard deviation (SD)), for d_8-8OHNP, 65% ± 13%. The accuracy of 6- and 8OHNP recoveries from water samples spiked with these analytes and internal standards, and analyzed with each batch of samples, was 97% ± 20% for 6OHNP and 121% ± 21% for 8OHNP.

For both nitro-PAHs and 1NP metabolites, method limits of detection (LODs) were defined based on the presence of these analytes in laboratory blanks. In the case of the nitro-PAHs, laboratory blanks were vials identical to those used to hold the filters during sonication with solvent, spiked with 1dNP internal standard and processed as the other filter samples. For the 1NP metabolites, laboratory blanks were 100 mL deionized water spiked with deuterated internal standards and processed as the other urine samples. Method LODs were calculated as per Miller and Miller (1993), *i.e.*,

$$\text{Method LOD} = \text{Average Concentration}_{\text{LaboratoryBlanks}} + 3 \times \text{Standard Deviation}_{\text{LaboratoryBlanks}}$$

LODs were calculated for the nitro-PAHs on a per-analysis-batch basis and for the 1NP metabolites over the entire sample set. For the nitro-PAHs, LODs were approximately 0.5 pg/m^3 (2NP = 0.49 pg/m^3, 2NFl = 0.46 pg/m^3, 1NP = 0.48 pg/m^3). All nitro-PAH analytes were present at levels greater than their respective LODs in all collected filter samples. For the 1NP metabolites, LODs were below 1 pg 1NP metabolite/mL urine (6OHNP, 0.77 pg/mL, for 8OHNP, 0.24 pg/mL). In the 37 urine samples analyzed for 1NP metabolites, the measured metabolite concentration in urine was above this LOD in 86% of the samples for both 6OHNP and 8OHNP.

2. Results

2.1. *Measured concentrations of nitro-PAHs and fine PM*

Fig. 1 illustrates the $PM_{2.5}$ and nitro-PAH exposures (error bars represent the 95% confidence interval (CI)) for the occupationally-exposed subjects as a box and whisker plot. One anomalously high 1NP value (calculated to be 3868 pg/m^3, approximately 15 times greater than the next closest 1NP exposure) was removed from the data set presented in Fig. 1.

2.2. *Concentrations of 1NP metabolites in occupationally-exposed subjects*

Table 1 details the concentrations of 6OHNP and 8OHNP in the occupationally-exposed and non-occupationally exposed subject cohorts monitored in this study. The average (of pre- and post-exposure samples) concentrations of 6OHNP and 8OHNP were calculated on a per-subject basis. In cases where only a pre-exposure sample was available for a particular subject (or post-exposure sample), the single value was used directly to represent the urinary hydroxynitropyrene concentration for that subject. To calculate the summary statistics of the 1NP metabolites, in cases where the concentration was determined to be less than the method LOD, a value of method LOD/2 was used in its place. In all, data for 30 urine samples from 17 occupationally-exposed subjects and seven urine samples from five non-occupationally exposed subjects are presented. In addition, the sum of the 6- and 8OHNP concentrations for each subject are presented.

In the occupationally-exposed subjects, over 90% of the urine samples were observed to contain 6- or 8OHNP at a concentration greater than the method detection limit. Median creatinine-corrected concentrations of both 6OHNP and 8OHNP

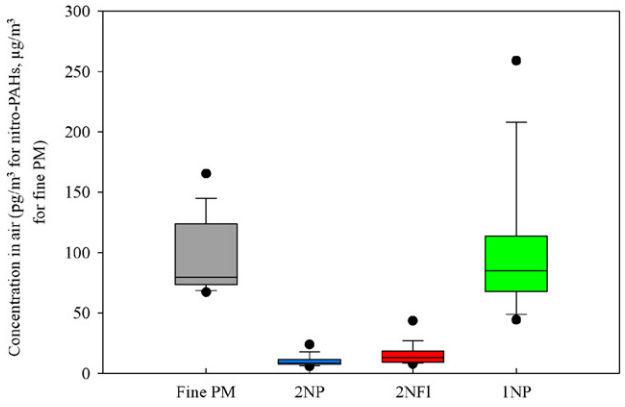

Fig. 1 – Concentration of fine particulate matter (PM), nitrated polycyclic aromatic hydrocarbon (nitro-PAH) for traffic exposed subjects. Middle line in box represents median, top and bottom of boxes represent 75th and 25th percentiles, respectively, and whiskers represent the 90th and 10th percentiles. Outliers are plotted as points outside the whiskers. One anomalously high 1-nitropyrene (1NP) concentration (3868 pg/m^3) was removed from the data set for plot.

were observed to be 3.9 and 2.3 pg metabolite/mg creatinine, respectively. In the smaller number of non-occupationally exposed subjects, 71% and 57% of the samples contained 6OHNP and 8OHNP, respectively, at concentrations greater than the method detection limit.

For the occupationally-exposed subjects, urinary concentrations of 6- and 8OHNP by pre- and post-shift sub-groups are detailed in Appendix A Table S1.

Appendix A Fig. S1 presents a scatter plot of the sum of 6- and 8OHNP for the pre-workshift samples *versus* the sum of 6- and 8OHNP for the post-workshift samples for the subjects where paired samples were available (13 occupationally-exposed subjects and two non-occupationally exposed subjects).

2.3. Comparison of 1NP metabolite levels with subjects from previous works

Fig. 2 shows the creatinine-corrected 8OHNP concentrations and corresponding 1NP exposures for occupationally-exposed (or individuals exposed to 1NP during commuting) subjects from previous studies. Only 8OHNP concentrations were available consistently through these studies. Galaviz et al. (2014) measured fine PM and 1NP exposures in a group of subjects who commuted between Tijuana and San Diego and also measured the 1NP metabolite concentrations of these subjects (Galaviz et al., 2015). Our previous study of taxi drivers from Shenyang, China, collected samples from 24 taxi drivers who were sampled before, immediately after, and the next day (first morning void) their workshifts (Miller-Schulze et al., 2013). In the Galaviz study, participants were assumed to have steady-state concentrations of 1NP metabolites. In the Shenyang taxi driver study, no significant associations with 1NP exposure or cross-shift change were found, so the three urine sample types were averaged together.

2.4. Correlation between 1NP metabolites and nitro-PAH exposure

Table 2 lists the correlations (Spearman's rho) between "cross-shift" changes in creatinine-corrected urinary concentrations of 6OHNP and 8OHNP in the occupationally-exposed subjects, as well as pre- and post-exposure and average (across pre- and post-exposure samples) for urinary concentrations of 6OHNP and 8OHNP and the corresponding $PM_{2.5}$, 2NP, 2NFl, and 1NP exposures. The population of paired subjects consisted of eight minibus drivers, three minivan drivers, and two motorcycle

Table 1 – Summary statistics for urinary 1NP metabolite concentrations in occupationally-exposed and non-occupationally exposed subjects.

	Average 6OHNP pg metabolite/mL urine	Average creatinine-adjusted 6OHNP pg metabolite/mg creatinine	Average 8OHNP pg metabolite/mL urine	Average creatinine-adjusted 8OHNP pg metabolite/mg creatinine	Average SUM 6, 8OHNP pg metabolite/mL urine	Average creatinine-adjusted SUM 6, 8 OHNP pg metabolite/mg creatinine
Occupationally-exposed subjects (30 urine samples from 17 individuals: 14 pre-exposure, 16 post-exposure, 13 sets of paired samples)						
% Detected	90	93				
Geomean	4.0	3.2	2.0	1.6	6.3	5.1
Geo SD	2.6	3.0	2.5	3.0	2.3	2.7
Median	5.4	3.9	2.4	2.3	8.9	5.2
Range*	<0.77–13	<0.77–28	<0.24–5.4	<0.24–5.4	<1.1–19	<1.1–37
Non-occupationally exposed subjects (7 urine samples from 5 individuals: 3 pre-exposure, 4 post-exposure, 2 sets of paired samples)						
% Detected	71	57				
Geomean	1.2	1.1	0.42	0.42	1.7	1.6
Geo SD	2.3	2.8	2.3	3.5	2.2	3.0
Median	1.1	1.8	0.64	0.58	2.1	2.4
Range*	<0.77–3.4	<0.77–3.7	<0.24–1.0	<0.24–1.7	<1.1–4.1	<1.1–4.7

1NP: 1-nitropyrene; LOD: limit of detection; SD: Standard deviation

* For samples where the measured urinary metabolite concentration was <LOD, a nominal creatinine concentration of 1.0 mg/mL was used to estimate the resulting creatinine-corrected concentration for that sample.

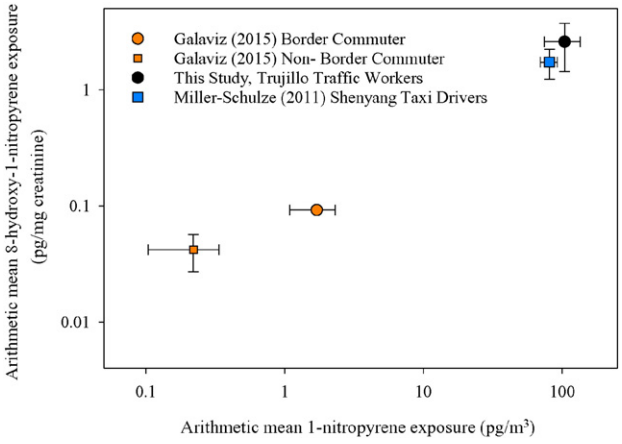

Fig. 2 – Relationship between urinary concentration of 8-hydroxy nitropyrene and 1-nitropyrene exposures. Error bars represent 95% CI; note log_{10} scale on *x*- and *y*-axes.

police officer (n = 13). In cases where the pre- or post-shift value was determined to be less than the method LOD, a value of the method LOD/2 was used. The data in Table 2 illustrate that no association was observed between urinary metabolite concentrations and workshift exposures measured on the same day as urine sample collection.

3. Discussion

3.1. *Context for nitro-PAH exposures and 1NP metabolite concentrations*

The 1NP exposures experienced by these Peruvian workers (mean ± 95% CI: 104 ± 31 pg/m^3) are similar to the workshift 1NP exposures from our previous study of taxi drivers in Shenyang, China (~80 pg/m^3) (Miller-Schulze et al., 2010), and are also similar to the ambient concentrations observed in traffic-impacted areas in Vietnam (~73 pg/m^3) (Hien et al., 2007) and Denmark (~127 pg/m^3) (Feilberg et al., 2001). Measured $PM_{2.5}$ exposures for the Peruvian workers (mean ± 95% CI: 97 ± 16 μg/m^3) are similar to our previous work in Shenyang, and much higher than the 24-hour ambient air quality standard of 35 μg/m^3 in the United States (US EPA, 2012), but are well below the United States Department of Labor's Occupational Safety and Health Administration (OSHA) occupational permissible exposure level of 5 mg/m^3 for respirable particles.

The concentrations of 2NP observed in this study (11 ± 5 pg/m^3, average ± SD) are similar to those measured in other areas of the world, including Riverside, CA and downtown Los Angeles, CA, USA (3.1–7.8 pg/m^3) (Reisen and Arey, 2005), or rural and urban Denmark (8 ± 4 and 20 ±5 pg/m^3, respectively) (Feilberg et al., 2001). However, the concentrations of 2NFl (17 ± 10 pg/m^3) observed are generally lower than those measured in these same studies: in the Riverside/Los Angeles work, the measured 2NFl was 63–153 pg/m^3, in the Denmark study, the rural and urban concentrations of 2NFl observed were 60 and 91 pg/m^3, respectively. There are some previous reports of relatively low 2NFl concentrations, consistent with the observations in the current study. For example, in Brazil, where Ciccioli et al. (1996) measured a mean 2NFl value of 15 pg/m^3. Much of the published work on the formation pathways of these nitro-PAHs suggests that ratios of 2NFl:2NP of 10:1 indicate the dominance of the hydroxyl radical initiated formation route, whereas 2NFl:2NP ratios of 100:1 indicate dominance of the nitrate-initiated pathway (Arey et al., 1989). In the current case, the ratio of 2NFl:2NP is well below 10:1 (1.5 ± 0.2, average ± SD), indicating almost equivalent prevalence of these two nitro-PAHs. The value of 2NFl:2NP measured in the current study (1.5 ± 0.2, average ± SD) is similar to the ratios of these two compounds measured in several other urban environments: average values of 2.1 were observed in Athens (Marino et al., 2000), 1.6 in Naples, 3.8 in Milan, 6.3 in Rome, and 1.9 in Brazil (Ciccioli et al., 1996).

Appendix A Fig. S1 and Fig. 2 present evidence that higher 1NP exposures result in higher concentrations of the hydroxylated 1NP metabolites, either in terms of the sum of 6- and 8OHNP urinary concentrations (Appendix A Fig. S1) or as urinary concentrations of 8OHNP (Fig. 2). Although no 1NP exposure data was available for the non-occupationally subjects in this study (corresponding to the purple square data points in Appendix A Fig. S1), it is reasonable to assume that their 1NP exposures were lower than the occupationally-exposed subjects, and Fig. 2 shows that the sum of the observed concentrations of 6- and 8OHNP in these subjects' urine samples were lower than most of the occupationally-exposed subjects. As Table 1 shows, median creatinine-corrected 6OHNP, 8OHNP, (and, consequently, 6 + 8OHNP) concentrations are lower in these subjects as compared with the occupationally-exposed Peruvian sub-cohort.

As Fig. 2 shows, the creatinine-corrected arithmetic mean 8OHNP concentrations are markedly higher for the occupationally-exposed Peruvian subjects of this work compared to the U.S.–Mexico border commuters. Concentrations of 8OHNP are similar between the Shenyang and Peruvian subjects (Shenyang median 8OHNP: 1.5 pg/mg creatinine, Peru median 8OHNP: 2.3 pg/mg creatinine). The similarity between the urinary 8OHNP concentrations in these two subject cohorts is reasonable, given that the 1NP exposures for these two cohorts were also similar (Shenyang median 1NP: 77 pg/m^3, Peru median 1NP: 85 pg/m^3). The data in Fig. 2 strongly suggest a positive relationship between 1NP exposures and urinary concentrations of 8OHNP. However, with the small number of studies represented in Fig. 2, and the small number of subjects included in each of the studies, the apparent relationship between 1NP exposure and 1NP metabolite concentration should be interpreted with caution. Further studies of both occupationally and non-occupationally exposed subjects are necessary to firmly establish this relationship. For instance, the urinary concentrations of non-occupationally exposed Peruvian subjects are higher than the commuters studied by Galaviz et al. (2014, 2015), possibly due to a greater ambient exposure to 1NP (and, consequently, DE) of the Peruvian subjects. High background (non-occupational) exposures to DE, then, possibly obscure the relationship between occupational 1NP exposures and urinary concentrations of 1NP metabolites.

3.2. *Relationship between 1NP exposures and urinary 1NP metabolite concentrations in urine*

A positive correlation was observed between 6OHNP and 8OHNP, measured either as pre-shift, post-shift or cross-shift

Table 2 – Correlations (Spearman's rho) between 1NP urinary metabolite measures and workshift nitro-PAH and fine PM exposures.

n = 13	6OHNP X shift change	8OHNP X shift change	Pre-6OHNP	Pre-8OHNP	Post-6OHNP	Post-8OHNP	Average 6OHNP	Average 8OHNP	$PM_{2.5}$	2NP	2NFl	1NP
6OHNP X shift change	1	**0.670**	**−0.599**	**−0.599**	0.445	0.165	−0.137	−0.335	−0.269	−0.346	−0.214	−0.456
8OHNP X shift change		1	−0.214	**−0.714**	**0.566**	0.544	0.110	−0.231	−0.165	−0.050	0.005	−0.467
Pre-6OHNP			1	**0.648**	0.363	**0.549**	**0.786**	**0.753**	0.280	0.148	0.050	0.071
Pre-8OHNP				1	−0.099	0.088	0.434	**0.802**	0.121	−0.033	−0.154	0.181
Post-6OHNP					1	**0.725**	**0.769**	0.379	0.039	−0.039	0.110	−0.456
Post-8OHNP						1	**0.747**	**0.593**	0.132	0.154	0.088	−0.335
Average 6OHNP							1	**0.813**	0.258	0.209	0.242	−0.181
Average 8OHNP								1	0.121	0.082	0.005	−0.077
$PM_{2.5}$									1	**0.654**	0.505	**0.582**
2NP										1	**0.896**	0.440

Values in bold text are significant at the $p = 0.05$ level.
1NP: 1-nitropyrene; nitro-PAH: nitrated polycyclic aromatic hydrocarbon; PM: particulate matter; 2NP: 2-nitropyrene; 2NFl: 2-nitrofluoranthene.

change. This is not surprising given that these metabolites are produced *via* the same metabolic pathway (Toriba et al., 2007), and should rise and fall together. A strong positive correlation was also observed between the ambient concentrations of 2NP and 2NFl, which is also not surprising given the similar photochemical formation pathways for these compounds in the atmosphere (Arey et al., 1989; Atkinson et al., 1990; Miller-Schulze et al., 2010).

No consistent associations were observed between measured workshift exposure to 1NP and 6OHNP or 8OHNP levels in post-shift urine samples. Similarly, cross-shift changes in both 6OHNP and 8OHNP were not significantly correlated with measured workshift exposure to 1NP (Table 2). Scatter plots of the 1NP exposures and associated 6OHNP and 8OHNP cross-shift change are shown in Appendix A Figs. S2A and S2B. These plots demonstrate that the majority of subjects are clustered close together and that two subjects with relatively high 1NP exposures, one of which also had low measured pre-shift creatinine concentration (0.303 mg/mL) are separate from this cluster of points.

The lack of a clear relationship between measured exposure to 1NP and urinary metabolite levels is generally consistent with our prior study of Shenyang taxi drivers (Miller-Schulze et al., 2013), in which we did not see a relationship between workshift exposures to 1NP and urinary 6OHNP and 8OHNP concentrations over a similar time period (pre- and post-shift samples, with an additional "next-morning" urine sample). A likely explanation for this failure to observe a relationship between observed urinary 1NP metabolite levels and 1NP exposures within this cohort relates to a potential mis-match between the exposure window that was measured, and the time-window represented by the urinary biomarker. In a recent study by Ramsay (2015), serial urine samples were collected from a cohort of underground miners who are occupationally-exposed to DE. Ramsay reported a progressive increase in 6- and 8OHNP concentrations in miners' urine across the work week, indicating that the OHNP biomarker levels in a spot urine sample are derived from exposures integrated over several days prior — not just the most recent workshift. In the Ramsey study, urinary 6- and 8OHNP levels did show a significant positive association with 1NP exposures accumulated over three and four days prior to collection of the urine specimen, or when a lag time factor between exposure and urinary metabolite was incorporated. Further support for this hypothesis come from Adetona's study of hydroxy-PAH metabolites, in which it was speculated that the period of time between adjacent workshifts (11 hr) was insufficient to eliminate hydroxy-PAH metabolite associated with the previous day's workshift (Adetona et al., 2012).

4. Conclusions

These findings contribute to a larger body of data that support the potential of urinary 1NP metabolites as indicators of chronic exposure to DE by using 1NP exposure as a surrogate for DE exposure. Because DE has been associated with a variety of adverse health outcomes, including cancer, establishing a quantitative relationship between exposure to DE and a urinary biomarker of this exposure would be of great utility to the field of exposure science. To this end, we found that in this cohort of

workers from Trujillo, Peru who were occupationally-exposed to vehicle exhaust, subjects' exposures to 1NP were consistent with exposures reported previously amongst workers occupationally-exposed to vehicle exhaust and diesel particulate, and were higher than subjects exposed to lower ambient concentrations of 1NP. In combination with two other studies of 1NP exposure and 8OHNP biomarker levels, the results from this study indicate a positive relationship between 1NP exposure and 8OHNP in urine. However, further investigation is necessary to definitively describe the relationship between 1NP exposure and urinary concentrations of its hydroxylated metabolites. Similar to our earlier study of taxi drivers in Shenyang, we did not observe robust associations between individual-level measures of urinary 1NP metabolites and workshift exposure to 1NP in the current study, and this is consistent with recent work indicating that 6- and 8OHNP concentrations in urine are representative of exposure accumulated over multiple days prior to urine collection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.06.007.

REFERENCES

Adetona, O., Sjodin, A., Zheng, L., Romanoff, L.C., Aguilar-Villalobos, M., Needham, L.L., Hall, D.B., Luis, A., Cassidy, B.E., Simpson, C.D., Naeher, L.P., 2012. Personal exposure to PM2.5 and urinary hydroxy-PAH levels in bus drivers exposed to traffic exhaust, in Trujillo, Peru. J. Occup. Environ. Hyg. 9 (4), 217–229.

Arey, J., Zielinska, B., Atkinson, R., Aschmann, S.M., 1989. Nitroarene products from the gas-phase reactions of volatile polycyclic aromatic hydrocarbons with the OH radical and N_2O_5. In. J. Chem. Kinet. 21 (9), 775–799.

Atkinson, R., Arey, J., 1994. Atmospheric chemistry of gas-phase polycyclic aromatic hydrocarbons: formation of atmospheric mutagens. Environ. Health Perspect. 102 (Suppl. 4), 117–126.

Atkinson, R., Arey, J., Zielinska, B., Aschmann, S.M., 1990. Kinetics and nitro-products of the gas-phase OH and NO_3 radical-initiated reactions of napthalene-d8, fluoranthene-d10, and pyrene. In. J. Chem. Kinet. 22 (9), 999–1014.

Ball, L.M., King, L.C., 1985. Metabolism, mutagenicity, and activation of 1-nitropyrene in vivo and in vitro. Environ. Int. 11 (2–4), 355–362.

Ball, L.M., Kohan, M.J., Inmon, J.P., Claxton, L.D., Lewtas, J., 1984. Metabolism of C14 1-nitropyrene in vivo in the rat and mutagenicity of urinary metabolites. Carcinogenesis 5 (12), 1557–1564.

Benbrahim-Tallaa, L., Baan, R.A., Grosse, Y., Lauby-Secretan, B., El Ghissassi, F., Bouvard, V., Guha, N., Loomis, D., Straif, K., 2012. Carcinogenicity of diesel-engine and gasoline-engine exhausts and some nitroarenes. Lancet Oncol. 13 (7), 663–664.

Bergauff, M.A., Ward, T.J., Noonan, C.W., Palmer, C.P., 2009. The effect of a woodstove changeout on ambient levels of PM2.5 and chemical tracers for woodsmoke in Libby, Montana. Atmos. Environ. 43 (18), 2938–2943.

Cassee, F.R., Heroux, M.E., Gerlofs-Nijland, M.E., Kelly, F.J., 2013. Particulate matter beyond mass: recent health evidence on the role of fractions, chemical constituents and sources of emission. Inhal. Toxicol. 25 (14), 802–812.

Ciccioli, P., Cecinato, A., Brancaleoni, E., Frattoni, M., Zacchei, P., Miguel, A.H., Vasconcellos, P.D., 1996. Formation and transport of 2-nitrofluoranthene and 2-nitropyrene of photochemical origin in the troposphere. Geophys. Res. Lett. 101 (D14), 19567–19581.

Dockery, D.W., Pope, C.A., Xu, X.P., Spengler, J.D., Ware, J.H., Fay, M.E., Ferris, B.G., Speizer, F.E., 1993. An association between air-pollution and mortality in 6 United States cities. New Engl. J. Med. 329 (24), 1753–1759.

Durant, J.L., Busby, W.F., Lafleur, A.L., Penman, B.W., Crespi, C.L., 1996. Human cell mutagenicity of oxygenated, nitrated and unsubstituted polycyclic aromatic hydrocarbons associated with urban aerosols. Mutat. Res. Genet. Toxicol. 371 (3–4), 123–157.

Elbayoumy, K., Hecht, S.S., 1984. A study of chemical carcinogenisis.69. Metabolism of 1-nitropyrene U-4,5,9,10-C14 pyrene in the F344 rat. Cancer Res. 44 (10), 4317–4322.

Elbayoumy, K., Reddy, B., Hecht, S.S., 1984. Identification of ring oxidized identification of ring oxidized metabolites of 1-nitropyrene in the feces and urine of germfree F344 rats. Carcinogenesis 5 (10), 1371–1373.

Feilberg, A., Poulsen, M.W.B., Nielsen, T., Skov, H., 2001. Occurrence and sources of particulate nitro-polycyclic aromatic hydrocarbons in ambient air in Denmark. Atmos. Environ. 35 (2), 353–366.

Galaviz, V.E., Quintana, P.J.E., Yost, M.G., Sheppard, L., Paulsen, M.H., Camp, J.E., Simpson, C.D., 2015. Urinary metabolites of 1-nitropyrene in US–Mexico border residents who frequently cross the San Ysidro Port of Entry. J. Expo. Sci. Environ. Epidemiol. http://dx.doi.org/10.1038/jes.2015.78 (December 16, 2015).

Galaviz, V.E., Yost, M.G., Simpson, C.D., Camp, J.E., Paulsen, M.H., Elder, J.P., Hoffman, L., Flores, D., Quintana, P.J.E., 2014. Traffic pollutant exposures experienced by pedestrians waiting to enter the US at a major US–Mexico border crossing. Atmos. Environ. 88, 362–369.

Han, X.L., Aguilar-Villalobos, M., Allen, J., Carlton, C.S., Robinson, R., Bayer, C., Naeher, L.P., 2005. Traffic-related occupational exposures to PM2.5, CO, and VOCs in Trujillo, Peru. Int. J. Occup. Environ. Health 11 (3), 276–288.

Hien, T.T., Thanh, L.T., Kameda, T., Takenaka, N., Bandow, H., 2007. Nitro-polycyclic aromatic hydrocarbons and polycyclic aromatic hydrocarbons in particulate matter in an urban area of a tropical region: Ho Chi Minh City, Vietnam. Atmos. Environ. 41 (36), 7715–7725.

Howard, P.C., Consolo, M.C., Dooley, K.L., Beland, F.A., 1995. Metabolism of 1-nitropyrene in mice-transport across the placenta and mammary tissues. Chem. Biol. Interact. 95 (3), 309–325.

Howard, P.C., Flammang, T.J., Beland, F.A., 1985. Comparison of the invitro and invivo hepatic-metabolism of the carcinogen 1-nitropyrene. Carcinogenesis 6 (2), 243–249.

Huyck, S., Ohman-Strickland, P., Zhang, L., Tong, J.A., Xu, X., Zhang, J.F., 2010. Determining times to maximum urine excretion of 1-aminopyrene after diesel exhaust exposure. J. Expo. Sci. Environ. Epidemiol. 20 (7), 650–655.

IARC, 2013. Diesel and gasoline engine exhausts and some nitroarenes. World Health Organization. 105. http://monographs.iarc.fr/ENG/Monographs/vol105/mono105.pdf.

Jakubowski, M., 2012. Biological monitoring versus air monitoring strategies in assessing environmental–occupational exposure. J. Environ. Monit. 14 (2), 348–352.

Laumbach, R., Tong, J., Zhang, L., Ohman-Strickland, P., Stern, A., Fiedler, N., Kipen, H., Kelly-McNeil, K., Lioy, P., Zhang, J.F., 2009. Quantification of 1-aminopyrene in human urine after a controlled exposure to diesel exhaust. J. Environ. Monit. 11 (1), 153–159.

Marino, F., Cecinato, A., Siskos, P.A., 2000. Nitro-PAH in ambient particulate matter in the atmosphere of Athens. Chemosphere 40 (5), 533–537.

Miller, J.C., Miller, J.N., 1993. Statistics for Analytical Chemistry. third ed. Horwood PTR Prentice Hall, New York.

Miller-Schulze, J.P., Paulsen, M., Kameda, T., Toriba, A., Tang, N., Tamura, K., Dong, L.J., Zhang, X.M., Hayakawa, K., Yost, M.G., Simpson, C.D., 2013. Evaluation of urinary metabolites of 1-nitropyrene as biomarkers for exposure to diesel exhaust in taxi drivers of Shenyang, China. J. Expo. Sci. Environ. Epidemiol. 23 (2), 170–175.

Miller-Schulze, J.P., Paulsen, M., Toriba, A., Hayakawa, K., Simpson, C.D., 2007. Analysis of 1-nitropyrene in air particulate matter standard reference materials by using two-dimensional high performance liquid chromatography with online reduction and tandem mass spectrometry detection. J. Chromatogr. A 1167 (2), 154–160.

Miller-Schulze, J.P., Paulsen, M., Toriba, A., Tang, N., Hayakawa, K., Tamura, K., Dong, L.J., Zhang, X.M., Simpson, C.D., 2010. Exposures to particulate air pollution and nitro-polycyclic aromatic hydrocarbons among taxi drivers in Shenyang, China. Environ. Sci. Technol. 44 (1), 216–221.

Ramsay, J., 2015. Measurement of Urinary 1-Nitropyrene Metabolites as Biomarkers of Exposure to Diesel Exhaust in Underground Miners Masters of Science Thesis University of Washington, Seattle, Washington, USA.

Reisen, F., Arey, J., 2005. Atmospheric reactions influence seasonal PAH and nitro-PAH concentrations in the Los Angeles basin. Environ. Sci. Technol. 39 (1), 64–73.

Russell, A.G., Brunekreef, B., 2009. A focus on particulate matter and health. Environ. Sci. Technol. 43 (13), 4620–4625.

Schauer, J.J., Rogge, W.F., Hildemann, L.M., Mazurek, M.A., Cass, G.R., Simoneit, B.R.T., 1996. Source apportionment of airborne particulate matter using organic compounds as tracers. Atmos. Environ. 30 (22), 3837–3855.

Scheepers, P.T.J., Martens, M.H.J., Velders, D.D., Fijneman, P., Vankerkhoven, M., Noordhoek, J., Bos, R.P., 1995. 1-Nitropyrene as as marker for the mutagenicity of diesel exhaust-derived particulate matter in workplace atmospheres. Environ. Mol. Mutagen. 25 (2), 134–147.

Sheesley, R.J., Schauer, J.J., Smith, T.J., Garshick, E., Laden, F., Marr, L.C., Molina, L.T., 2008. Assessment of diesel particulate matter exposure in the workplace: freight terminals. J. Environ. Monit. 10 (3), 305–314.

Toriba, A., Kitaoka, H., Dills, R.L., Mizukami, S., Tanabe, K., Takeuchi, N., Ueno, M., Kameda, T., Tang, N., Hayakawa, K., Simpson, C.D., 2007. Identification and quantification of 1-nitropyrene metabolites in human urine as a proposed biomarker for exposure to diesel exhaust. Chem. Res. Toxicol. 20 (7), 999–1007.

US EPA, 2002. Health Assessment Document for Diesel Engine Exhaust. National Center for Environmental Research. Environmental Protection Agency, Washington, DC, United States.

US EPA, 2012. National Ambient Air Quality Standards (NAAQS). Retrieved March 28, 2014, from http://www.epa.gov/air/criteria.html.

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. Chem. Res. Toxicol. 11 (11), 1382–1390.

Xue, X., You, Y., Wu, J., Han, B., Bai, Z., Tang, N., Zhang, L., 2014. Exposure measurement, risk assessment and source identification for exposure of traffic assistants to particle-bound PAHs in Tianjin, China. J. Environ. Sci. 26 (2), 448–457.

Zimmermann, K., Jariyasopit, N., Simonich, S.L.M., Tao, S., Atkinson, R., Arey, J., 2013. Formation of nitro-PAHs from the heterogeneous reaction of ambient particle-bound PAHs with $N2O_5/NO_3/NO_2$. Environ. Sci. Technol. 47 (15), 8434–8442.