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Wen-Li Wang Date

Development of a pooled-sample framework for a large-scale human biomonitoring program using urinary phthalate data from NHANES

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Taipei Medical University, Taiwan
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An abstract of
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Abstract

Development of a pooled-sample framework for a large-scale human biomonitoring program using urinary phthalate data from NHANES

By Wen-Li Wang

Background: Biomonitoring is an approach to assess human exposure to a chemical via its metabolite or reaction product in human tissues. To assess chemical exposure among a specific population, biological samples must be strategically taken from a sufficient number of subjects. However, not all studies can afford to measure levels of compounds in hundreds or thousands of subjects. Additionally, some samples may be below the limit of detection (LOD) due to extremely low exposure levels or insufficient quantity of biological samples. The pooled sample approach may be useful to address both problems above. However, one of the key considerations is the ambiguous pooled samples formulation strategy. The goal of this study is to investigate an optimal pooled sample framework that could be used for a large-scale human biomonitoring program aiming to assess exposure to phthalates.
Methods: We developed algorithms to address our pooled sample designs to achieve two aims: 1) recommend the number of samples per pool needed to obtain a grand mean phthalate metabolite concentration that is comparable to the average concentration of individual samples in the pools. 2) recommend a minimum sample size that can produce consistent grand mean concentrations of each phthalate metabolite.
Results: There was no significant differences in arithmetic mean (AM) concentrations between individual and pooled samples for all phthalate metabolites in each pool type. Majority of the pooled phthalate metabolites have no significant differences in the AMs across different pool types. The AMs, SDs, and corresponding CVs for all pooled phthalate metabolite concentrations are similar across sample sizes regardless of the pool type.
Conclusions: Our study suggests that using pooled urinary phthalate metabolite samples is a feasible approach for large-scale human biomonitoring to obtain the AM concentrations of individual urinary phthalates, regardless of the number of samples per pool. Taking into account the cost and time constraints, we recommend a minimum sample size of approximately 1500 to produce consistent AM and GM concentrations for MCPP, MEP, MiBP, and MEHP. For MBzP and MBP, a minimum sample size of approximately 1000 is recommended to produce consistent AM and GM concentrations.

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Development of a pooled-sample framework for a large-scale human biomonitoring program using urinary phthalate data from NHANES

1. Background and significance

1.1. Phthalates

Phthalates are a ubiquitous group of man-made chemicals mainly used as plasticizers and additives in various consumer products (Shin et al., 2020). Low-molecular-weight phthalates, such as di-ethyl phthalate (DEP), di-n-butyl phthalate (DnBP), and di-isobutyl phthalate (DiBP) are found in personal care products including cosmetics, fragrances, nail polish personal care products, while high-molecular-weight phthalates, such as Di(2-ethylhexyl) phthalate (DEHP), di-n-octyl phthalate (DnOP), and benzylbutyl phthalate (BzBP) are applied in food packaging, shower curtains, and polyvinyl chloride flooring (Bastiaensen et al., 2020). The widespread use of phthalate-containing products, combined with the substantial evidence of their potential toxicity to humans and endocrine-disrupting potential, emphasizes the importance of understanding the exposure pattern of these chemicals (Wang et al., 2019).

1.2. Biomonitoring

Biomonitoring is an approach used to assess human exposure to a chemical through its metabolite or reaction product (biomarker) in human tissues and fluids, such as blood, urine, breast milk, or hair (biomatrix) (Needham et al., 2007). With the growing concerns regarding the adverse health effects of exposure to emerging contaminants, there is an increasing need for the establishment of national or regional biomonitoring databases. Biomonitoring data provides a comprehensive perspective of all potential sources of exposure to a chemical, and as such, it is one of the key elements in epidemiological studies aimed at investigating whether exposure to a

chemical is associated with a given health outcome in humans. Although biomonitoring is useful in assessing both population-based and individual chemical exposures, several key challenges remain for this tool.

To assess chemical exposure among a specific population, biological samples must be strategically taken from a sufficient number of subjects. However, conducting studies to measure levels of compounds in hundreds or thousands of subjects is not always feasible due to cost constraints. Certain compounds can be expensive to measure, and the expense tends to rise as the accuracy of the assessment increases (Li et al., 2014). The cost of biomonitoring can become prohibitively high when analyzing large communities or populations. Biomonitoring programs for assessing environmental chemicals generally have lower concentrations compared to clinical and toxicology studies, which creates an additional challenge in characterizing exposure due to the potential for biological samples to be below the limit of detection (LOD) (Albertini et al., 2006). Despite continued improvements in analytical techniques, there is an increased percentage of results not detected by the instrumentation as the exposure level decreases or when the biological sample volume is limited, resulting in reporting biases (Caudill et al., 2007). The pooled sample approach may be useful to address the problems regarding results below the LOD, the cost of laboratory analysis, and limited biological sample volume.

1.3. Pooled sample approach

The pooled sample method is an approach that combines multiple individual biological samples into a single sample based on a set of grouping criteria such as age, sex, and race/ethnicity (Caudill, 2010). Pooling samples reduces the number of measurements required and, ultimately, decreases the overall expense of biomonitoring. Additionally, pooled samples often have a larger volume, lowering the likelihood of encountering LODs and may detect unexpected exposure

patterns. The use of pooled samples provides a grand mean concentration estimate of population exposure over time and eliminates the ethical and societal considerations surrounding the report of individual results. The benefits of the pooled sample approach have led to its application in biomonitoring programs.

In Australia, the National Research Centre for Environmental Toxicology (Entox) at the University of Queensland has measured certain persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), and parabens in the general Australian population by pooling biological samples (Aylward et al., 2014; Heffernan et al., 2015; Thai et al., 2016). In the US, the National Health and Nutrition Examination Survey (NHANES) has begun pooling serum samples in 2005 to increase the detection frequencies for dioxins, furans, polychlorinated biphenyls (PCBs), organochlorine pesticides and metabolites, and brominated flame retardants (MacDonald et al., 2022).

While the pooled sample method has its advantages, there are some potential concerns and limitations exist. Although the concentration of pooled samples is similar to the arithmetic mean concentration obtained from the individual samples making up the pools, the population geometric mean, median, and variance cannot be directly calculated using data from pooled samples (Heffernan et al., 2014). However, pooled sample concentration can be used to estimate the variability of exposures in a population when taking into account three statistical factors including measurement error and variation in pooled sample measurements, pooling error, and the shape of the underlying population's distribution (Heffernan et al., 2014). To estimate population variance and percentiles, Caudill used NHANES data and statistical approaches under the assumption of log-normality. The study analyzed multiple pools to estimate variance and repeated more analytic batches of each pool to measure error (Caudill, 2010). Pooling error

occurs when mistakes are made in extracting and transferring precise volumes of individual samples into pools. The greater the number of individual samples that are pooled, the higher the likelihood of error. Although pooling a larger number of samples can result in a more accurate estimation of the actual mean and reduce variance, it is important to balance the number of samples in each pool to accurately describe variance while avoiding errors that may arise from pooling a large number of individual specimens (Heffernan et al., 2014).

1.4. Pooled sample framework

The goal of this study is to investigate an optimal pooled sample framework that could be used for a large-scale human biomonitoring program aiming to assess exposure to phthalates. We selected urinary phthalate metabolites data from the NHANES 2017-2018 cycle to investigate the sufficient number of samples per pool (pool types) required to produce a grand mean concentration similar to the average concentration of the individual samples that make up the pools. Additionally, we randomly selected a variety of fixed sample sizes to explore the minimum sample size needed to have a consistent grand mean concentration for each phthalate metabolite. These research gaps are currently unknown and may depend on the exposure magnitudes (Heffernan et al., 2014).

2. Methods

2.1. Target analytes

The study focused on the six phthalates that are commonly found in the general population, including DnOP, DEP, DiBP, DBP, BzBP, and DEHP. We examined 2762 participants from NHANES 2017-2018 cycle who had available data for the six urinary phthalate metabolites. These metabolites listed in **Table 1** is served as biomarkers for assessing exposure to the six selected phthalates. NHANES is an ongoing program designed to assess the health and

nutritional status of the non-institutionalized US population, which is conducted by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC). The 2017-2018 cycle represents the US general population aged 2 years and older, and the participation rates of this cycle was 90.9% (Centers for Disease Control and Prevention, 2023).

Table1 Phthalates and their main metabolites used as biomarkers of exposure

Parent chemical	**Metabolite**
Di-n-octyl phthalate (DnOP)	Mono-(3-carboxypropyl) phthalate (MCPP)
Di-ethyl phthalate (DEP)	Mono-ethyl phthalate (MEP)
Di-isobutyl phthalate (DiBP)	Mono-isobutyl phthalate (MiBP)
Di-n-butyl phthalate (DnBP)	Mono-n-butyl phthalate (MBP)
Benzylbutyl phthalate (BzBP)	Monobenzyl phthalate (MBzP)
Di(2-ethylhexyl) phthalate (DEHP)	Mono(2-ethylhexyl) phthalate (MEHP)

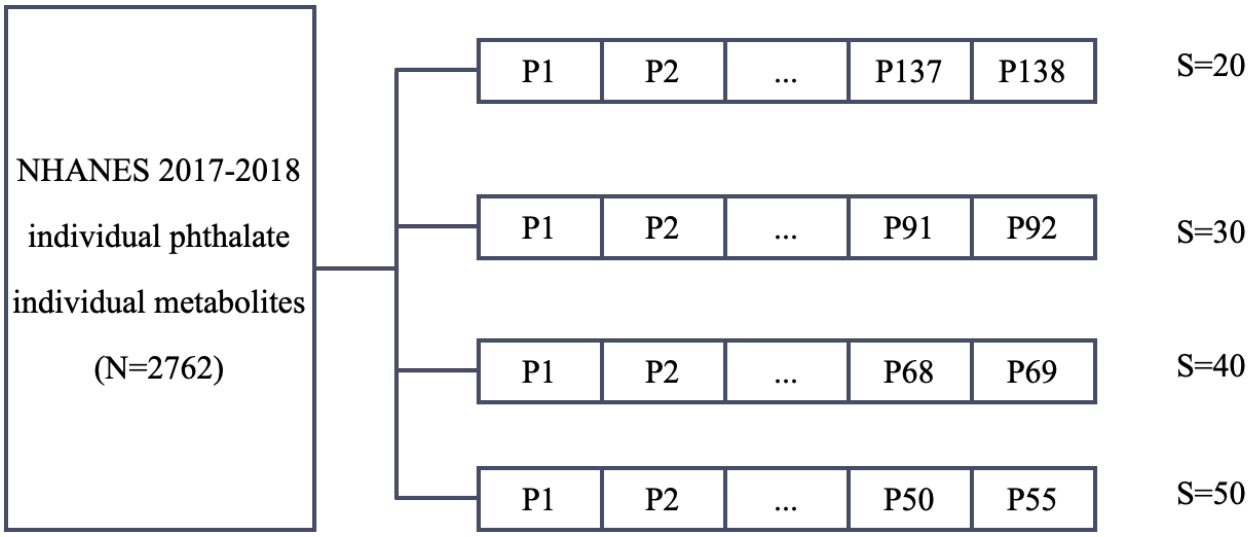
2.2. Pooled sample designs

2.2.1. Design 1

The aim of this design is to suggest a number of samples per pool required to produce a grand mean phthalate metabolite concentration that is similar to the average concentration of individual samples in the pools (**Figure1**). An algorithm was developed for each of the six phthalate metabolites to randomly pool individual samples into 20, 30, 40, or 50 samples per pool (pool types). The random pooling algorithm was replicated ten times for each pool type to reduce the potential impact of random variation in the pooling procedure and ensure the reliability and consistency of the results. These pool types are denoted by pooled $^{s=x}$, where s represents "samples per pool", and x represents the sample count per pool. We decided to use a fixed

sample count instead of relying on the algorithm to randomly determine the number of samples to be pooled, due to practical considerations. In theory, pooling more than 50 samples per pool can improve accuracy in estimates. However, we must also take into account pooling error. This design provided clear suggestions on the number of samples to be pooled, avoiding ambiguity and uncertainty in the pooling procedure.

Figure1 Pooled sample design 1

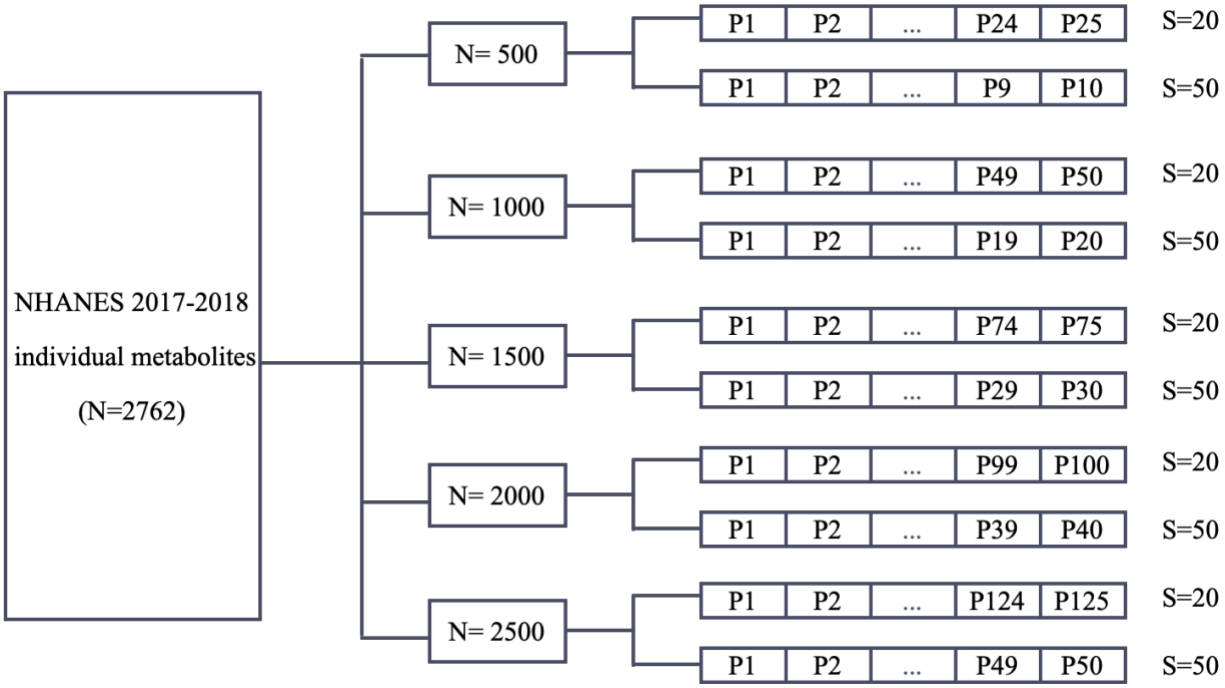


Note: N= sample size; S= samples per pool; P= pool count

2.2.2. Design 2

The aim of this design is to suggest a minimum sample size that would produce consistent grand mean concentrations of each phthalate metabolite (**Figure2**). An algorithm was developed to randomly select sample sizes of 500, 1000, 1500, 2000, and 2500 from the total sample size and replicate the process 10 times for each sample size. For each subset, the individual samples of the six phthalate metabolites were randomly pooled into 20 and 50 samples per pool. The naming convention for this pooled design is $pooled_N{}^{s=x}$, where N represents the sample size, s represents as "samples per pool", and x represents the sample count per pool.

Figure2 Pooled sample design 2



Note: N= sample size; S= samples per pool; P= pool count

2.3. Statistical analysis

Descriptive analyses were conducted on the six individual phthalate metabolites, including their detection frequencies, geometric means (GMs), geometric standard deviations (GSDs), arithmetic means (AMs), standard deviations (SDs), and distribution percentiles. Phthalate metabolite values that fell below the LODs were imputed by dividing the LOD value by the square root of two(Hornung & Reed, 1990). T-tests were used to compare the difference in AM concentrations of the six individual phthalate metabolites between males and females.

Under pooled sample design 1, GMs, AMs, and distribution percentiles were calculated for the concentration of the six pooled phthalate metabolites. T-tests were conducted to compare the differences in AM concentrations between the six individual and pooled phthalate metabolites. ANOVA was conducted to compare the differences in the AMs and GMs of the six pooled

phthalate metabolite concentrations among the different pool types. Further analysis was conducted using Tukey's Studentized Range Test to identify the pairs that contributed to the significant differences.

Under pooled sample design 2, GMs, GSDs, AMs, SDs , and coefficient of variations (CVs) were calculated for the six pooled phthalate metabolite concentrations. The consistency of the geometric and arithmetic means of the six pooled phthalate metabolite concentrations across the 10 replicates of each sample sizes was examined using GSDs, SDs, and CVs. All data analyses were performed using R (version 3.6.1).

3. Results

3.1. Individual phthalate metabolites

Descriptive statistics and t-test results for individual MCPP, MEP, MiBP, MBP, MBzP, and MEHP concentrations stratified by gender are presented in **Table 2**. MCPP, MEP, MiBP, MBP, and MBzP were detected at high frequencies (83–99%), except for MEHP, which has a lower detection frequency (56%). Outliers are observed in all phthalate metabolites, such as MEP, where the maximum concentration (102452 ng/mL) in females is approximately 270 times greater than the 95th percentile value (378 ng/mL). Although the MEP maximum concentration for females is over 10 times that of males (9029 ng/mL), the GM, GSD, and distribution percentiles are similar between genders. This pattern is consistent across other phthalate metabolites as well. The t-test results showed no statistically significant difference in all six of the AM phthalate metabolite concentrations by gender (p-values > 0.05).

Table2 Distribution of the individual phthalate metabolite concentrations (ng/mL)

		n	% > LOD	AM[b]	SD	GM	GSD	Min Conc.	P25	P50	P75	P95	Max Conc.
MCPP[a]	N	2762	83.16	3.09	42.00	1.15	2.75	0.28	0.60	1.10	2.20	6.10	2170.00
	F	1399	79.49	3.95	58.66	1.09	2.88	0.28	0.50	1.10	2.00	6.51	2170.00
	M	1363	86.94	2.21	6.50	1.22	2.61	0.28	0.60	1.20	2.30	5.80	185.00
MEP[a]	N	2762	99.53	149.00	2015.8	28.72	4.24	0.85	11.00	26.4	64.7	378.10	102452.00
	F	1399	99.30	192.00	2803.67	29.07	4.41	0.85	10.70	27.1	70.65	378.36	102452.00
	M	1363	99.80	104.86	406.36	28.37	4.06	0.85	11.45	25.5	59.35	372.07	9029.40
MiBP[a]	N	2762	97.83	14.67	27.23	8.10	2.90	0.57	4.20	8.30	16.40	42.78	513.60
	F	1399	97.50	13.83	22.67	7.64	3.00	0.57	3.75	8.10	16.40	40.13	431.70
	M	1363	98.17	15.54	31.21	8.61	2.79	0.57	4.60	8.80	16.30	44.10	513.60
MBP[a]	N	2762	99.31	16.86	26.04	9.91	2.89	0.28	5.20	10.80	20.00	49.30	649.10
	F	1399	99.14	16.99	24.86	9.60	3.07	0.28	4.70	10.6	20.65	50.33	487.00
	M	1363	99.49	16.72	27.21	10.25	2.71	0.28	5.70	10.9	19.40	47.96	649.10
MBzP[a]	N	2762	96.20	9.12	18.68	3.74	3.81	0.21	1.50	3.70	9.20	34.10	307.20
	F	1399	94.90	9.28	19.89	3.50	4.07	0.21	1.30	3.50	9.30	36.24	307.20
	M	1363	97.60	8.96	17.36	4.00	3.54	0.21	1.80	3.90	9.10	33.00	303.00
MEHP[a]	N	2762	56.23	1.82	3.37	1.17	2.26	0.57	0.57	1.00	2.00	5.69	115.90
	F	1399	52.60	1.72	2.65	1.12	2.25	0.57	0.57	0.90	1.90	5.80	57.30
	M	1363	59.94	1.93	3.98	1.22	2.28	0.57	0.57	1.10	2.10	5.60	115.90

Note: N= sample size; F= female; M= male; n = sample count; LOD = limits of detection; AM= arithmetic mean; SD = standard deviation; GM= geometric mean; GSD= geometric standard deviation; Min Conc.= minimum concentration; P25 = the 25th percentile; Max Conc.= maximum concentration.

[a] The values below LODs were replaced by LOD/$\sqrt{2}$.

[b] T-test examines the differences in mean concentrations of each phthalate metabolite between genders. *p-value < 0.05.

3.2. Pooled phthalate metabolites

3.2.1. Sample design 1

Descriptive statistics, t-test, and ANOVA results for pooled MCPP, MEP, MiBP, MBP, MBzP, and MEHP concentrations are presented in **Table 3** through **Table 8**, respectively.

Arithmetic means (AMs)

The t-test results indicated no significant differences in AM concentrations between individual and pooled samples for all phthalate metabolites in each pool type (p-values > 0.05). The ANOVA results indicated that, except for MCPP, there were no significant differences in the AMs of the other five pooled phthalate metabolite concentrations across different pool types. Post hoc analysis was conducted to explore which pool types contributed to the AM difference in MCPP. The results revealed that only pooled $^{s=50}$ had a significant difference compared to the other pool types (p-value < 0.05), while the other pairs of pool types did not show significant differences (p-values > 0.05).

Geometric means (GMs)

The ANOVA results indicate a significant difference between the GMs of all pooled phthalate metabolite concentrations across different pool types, suggesting that at least one pool type has a different GM compared to the others. Post hoc analysis was conducted for all pooled phthalate metabolites, revealing that for MCPP, MEP, MiBP, and MEHP, all pool types show significant differences from each other (p-values < 0.05). As for MBP and MBzP, the pair of pooled $^{s=40}$ and pooled $^{s=50}$ did not show significant differences, while the other pool type pairs showed significant differences.

Table3 Distribution of pooled MCPP concentrations (ng/mL) by pool types

MCPP		AM†	GM	Min Conc.	P25	P50	P75	P95	Max Conc.
	Individual	3.09	1.15	0.28	0.60	1.10	2.20	6.10	2170.00
20 subjects per pool (S=20) and 138 pools (P=138)	**Replication**								
	1	3.09	2.06	0.90	1.44	1.85	2.42	5.65	110.34
	2	3.09	2.03	0.73	1.49	1.81	2.37	5.58	110.80
	3	3.09	2.06	0.96	1.51	1.83	2.38	5.83	110.39
	4	3.09	2.07	0.79	1.53	1.82	2.31	5.92	109.80
	5	3.09	2.05	0.92	1.48	1.85	2.38	5.69	113.96
	6	3.09	2.05	0.80	1.48	1.83	2.30	6.29	110.19
	7	3.09	2.04	0.97	1.45	1.88	2.38	6.03	109.98
	8	3.09	2.03	0.99	1.49	1.88	2.32	5.25	114.44
	9	3.09	2.03	0.82	1.44	1.79	2.29	6.81	109.76
	10	3.09	2.05	0.97	1.49	1.83	2.30	6.16	110.58
	Average‡	3.09[ab]	2.05[ab]	0.89	1.48	1.84	2.34	5.92	111.02
30 subjects per pool (S=30) and 92 pools (P=92)	1	3.09	2.16	0.95	1.54	1.96	2.34	5.42	74.03
	2	3.09	2.15	1.17	1.57	1.87	2.31	5.19	74.30
	3	3.09	2.16	0.98	1.63	1.88	2.29	5.03	74.03
	4	3.09	2.14	1.01	1.53	1.89	2.52	5.15	73.83
	5	3.09	2.14	1.18	1.53	1.83	2.39	5.64	74.36
	6	3.09	2.13	1.10	1.62	1.95	2.37	5.77	77.33
	7	3.09	2.13	1.03	1.53	1.92	2.33	5.95	73.76
	8	3.09	2.11	1.04	1.60	1.85	2.26	4.89	78.76
	9	3.09	2.17	1.07	1.62	1.99	2.40	5.49	73.56
	10	3.09	2.10	1.00	1.56	1.93	2.37	4.30	84.59
	Average‡	3.09[ab]	2.14[c]	1.05	1.57	1.91	2.36	5.28	75.85

40 subjects per pool (S=40) and 69 pools (P=69)	1	3.09	2.19	1.21	1.67	1.91	2.24	5.25	56.08
	2	3.09	2.22	1.34	1.59	1.89	2.45	5.26	56.06
	3	3.09	2.23	1.20	1.65	1.97	2.52	5.01	56.23
	4	3.09	2.21	1.10	1.64	1.94	2.56	5.72	55.70
	5	3.09	2.18	1.03	1.60	1.90	2.33	5.84	56.29
	6	3.09	2.18	1.12	1.57	1.94	2.41	5.36	58.47
	7	3.09	2.18	1.06	1.53	1.96	2.49	6.33	56.09
	8	3.09	2.20	1.15	1.65	1.86	2.39	5.28	55.95
	9	3.09	2.25	1.08	1.77	1.93	2.42	5.18	55.77
	10	3.09	2.15	1.21	1.63	1.87	2.44	4.41	63.96
	Average‡	3.09[ab]	2.20[d]	1.15	1.63	1.92	2.43	5.36	57.06
50 subjects per pool (S=50) and 55 pools (P=55)	1	3.10	2.26	1.22	1.67	1.94	2.33	5.10	44.86
	2	3.10	2.27	1.23	1.67	1.91	2.67	5.08	45.17
	3	3.10	2.28	1.22	1.76	1.98	2.52	5.27	45.29
	4	3.10	2.24	1.14	1.59	1.96	2.57	5.88	44.98
	5	3.09	2.23	1.17	1.63	1.98	2.49	5.47	45.97
	6	3.10	2.26	1.27	1.72	1.93	2.34	4.99	45.19
	7	3.10	2.23	1.04	1.60	1.84	2.57	5.54	45.26
	8	3.10	2.21	1.17	1.59	1.91	2.30	5.25	47.97
	9	3.10	2.28	1.13	1.73	1.99	2.31	4.88	45.96
	10	3.10	2.20	1.31	1.65	1.98	2.49	4.10	51.40
	Average‡	3.10[c]	2.25[e]	1.19	1.66	1.94	2.46	5.15	46.21

Note: S= samples per pool; P= pool count; AM= arithmetic mean; GM= geometric mean; Min Conc.= minimum concentration; P25 = the 25th percentile; Max Conc.= maximum concentration.

† T-test examines the mean differences in MCPP concentrations between individual and pooled samples. *p-value < 0.05.

‡ ANOVA compares the mean differences in MCPP concentrations among each pool type. Tukey's Studentized Range Test for multiple comparisons among each pool type.

Table4 Distribution of pooled MEP concentrations (ng/mL) by pool types

MEP		AM†	GM	Min Conc.	P25	P50	P75	P95	Max Conc.
	Individual	149.00	28.72	0.85	11.00	26.4	64.7	378.10	102452.00
20 subjects per pool (S=20) and 138 pools (P=138)	**Replication**								
	1	149.06	87.70	25.53	52.72	73.95	121.79	311.76	5160.71
	2	149.10	83.95	22.86	52.67	72.30	105.39	344.16	5182.19
	3	149.07	85.75	29.18	52.13	71.04	116.36	376.95	5374.25
	4	149.08	87.51	21.38	55.28	75.37	122.05	301.63	5176.22
	5	149.00	86.94	17.45	54.07	80.22	124.47	303.66	5203.39
	6	149.09	87.27	27.00	48.27	79.09	123.48	294.44	5160.00
	7	148.65	83.81	21.04	47.96	74.84	112.21	432.38	5398.52
	8	149.10	87.95	20.77	53.51	79.10	132.86	331.75	5195.79
	9	149.03	87.60	17.81	50.38	80.51	130.00	300.79	5152.82
	10	149.09	85.57	14.53	51.54	77.50	118.83	361.07	5195.27
	Average‡	149.03[ab]	86.4[ab]	21.75	51.85	76.39	120.74	335.86	5219.91
30 subjects per pool (S=30) and 92 pools (P=92)	1	149.03	93.09	25.66	60.31	83.93	126.85	338.60	3468.31
	2	149.06	96.32	31.24	61.81	83.28	130.36	345.71	3458.86
	3	147.28	92.76	32.32	59.47	82.95	129.35	316.04	3456.50
	4	149.10	95.91	21.62	65.00	83.68	133.52	319.82	3463.35
	5	149.09	91.94	25.61	55.28	81.14	136.01	374.63	3475.35
	6	149.07	91.54	31.26	58.44	81.52	113.24	380.36	3580.29
	7	149.08	96.38	33.32	63.61	84.20	124.50	315.86	3555.52
	8	149.07	93.57	28.15	59.97	79.97	126.91	354.98	3551.33
	9	149.10	94.21	25.76	58.90	81.44	128.52	306.20	3513.31
	10	149.09	94.41	30.12	58.52	87.11	126.87	344.01	3544.22
	Average‡	148.90[ab]	94.01[c]	28.50	60.13	82.92	127.61	339.62	3506.70

40 subjects per pool (S=40) and 69 pools (P=69)	1	149.03	95.87	25.32	64.99	86.80	130.44	394.50	2633.92
	2	149.06	100.15	38.68	66.68	86.42	130.31	338.41	2634.85
	3	147.28	98.53	32.23	65.96	90.23	123.80	311.05	2610.27
	4	149.10	101.32	29.63	70.81	89.95	137.18	305.68	2606.15
	5	149.09	98.31	26.65	60.87	90.09	130.59	363.21	2616.74
	6	149.07	95.59	33.21	60.54	85.23	118.35	328.22	2705.98
	7	149.08	99.75	28.97	65.78	83.55	139.94	317.53	2684.37
	8	149.07	98.74	33.80	60.21	85.20	133.61	375.52	2671.98
	9	149.10	100.87	33.95	64.09	94.95	149.70	288.43	2652.53
	10	149.09	97.84	31.46	68.52	91.90	119.65	281.78	2932.88
	Average‡	148.90[ab]	98.70[d]	31.39	64.84	88.43	131.36	330.43	2674.97
50 subjects per pool (S=50) and 55 pools (P=55)	1	149.29	99.68	24.88	65.87	92.75	134.83	323.74	2118.35
	2	148.12	102.45	41.03	69.93	87.94	122.90	299.72	2105.79
	3	147.62	102.22	36.02	69.01	93.65	127.05	302.65	2095.83
	4	149.45	104.26	35.64	70.16	95.57	130.96	278.65	2109.77
	5	149.52	103.78	36.53	66.26	104.04	130.19	318.00	2097.77
	6	149.57	100.35	45.37	62.42	82.71	127.89	304.78	2191.65
	7	149.33	100.96	40.06	68.10	87.21	137.04	291.14	2155.28
	8	149.05	101.07	33.81	70.48	90.99	137.69	314.40	2155.59
	9	149.35	105.45	43.71	67.27	96.80	136.40	264.90	2138.66
	10	149.53	102.25	36.44	68.46	85.94	141.91	298.27	2174.95
	Average‡	149.08[ab]	102.25[e]	37.35	67.79	91.76	132.69	299.63	2134.37

Note: S= samples per pool; P= pool count; AM= arithmetic mean; GM= geometric mean; Min Conc.= minimum concentration; P25 = the 25th percentile; Max Conc.= maximum concentration.

† T-test examines the mean differences in MEP concentrations between individual and pooled samples. *p-value < 0.05.

‡ ANOVA compares the mean differences in MEP concentrations among each pool type. Tukey's Studentized Range Test for multiple comparisons among each pool type.

Table5 Distribution of pooled MiBP concentrations (ng/mL) by pool types

MiBP		AM†	GM	Min Conc.	P25	P50	P75	P95	Max Conc.
	Individual	14.67	8.10	0.57	4.20	8.30	16.40	42.78	513.60
20 subjects per pool (S=20) and 138 pools (P=138)	**Replication**								
	1	14.68	13.72	7.68	10.71	12.85	16.08	29.04	41.24
	2	14.65	13.71	6.43	10.81	13.26	16.34	25.84	48.12
	3	14.68	13.64	6.86	10.63	12.99	16.73	26.60	52.09
	4	14.68	13.65	6.38	10.88	13.13	16.38	28.19	38.43
	5	14.68	13.79	7.63	10.86	13.01	17.16	25.38	39.45
	6	14.67	13.76	6.63	10.82	13.25	16.11	27.93	40.32
	7	14.67	13.70	6.72	10.68	12.94	16.63	28.61	37.36
	8	14.68	13.74	6.51	10.97	13.29	16.10	26.79	39.56
	9	14.68	13.73	5.73	10.86	13.41	16.66	25.66	37.51
	10	14.68	13.66	7.32	10.84	13.32	15.81	26.82	61.76
	Average‡	14.67[ab]	13.71[ab]	6.79	10.81	13.14	16.40	27.09	43.58
30 subjects per pool (S=30) and 92 pools (P=92)	1	14.67	14.04	7.54	11.67	13.66	16.19	26.13	32.32
	2	14.67	14.03	8.03	11.59	13.41	16.42	24.29	35.33
	3	14.67	13.93	7.95	11.24	13.30	16.23	26.08	34.40
	4	14.68	13.97	6.24	11.51	13.24	16.36	25.66	32.36
	5	14.67	13.89	7.90	11.15	13.33	15.58	25.65	43.08
	6	14.68	13.97	8.18	11.64	13.32	17.16	24.19	47.39
	7	14.68	13.98	7.99	11.15	13.50	16.97	24.21	32.48
	8	14.68	13.92	6.46	11.19	13.72	16.00	25.14	42.40
	9	14.68	14.02	6.26	11.70	13.80	16.38	26.10	30.28
	10	14.68	14.09	8.43	11.18	13.83	16.52	23.63	29.93
	Average‡	14.68[ab]	13.98[c]	7.50	11.40	13.51	16.38	25.11	36.00

40 subjects per pool (S=40) and 69 pools (P=69)	1	14.67	14.18	7.67	12.07	13.72	16.12	23.22	25.20
	2	14.67	14.23	9.09	12.19	13.82	16.66	21.92	28.73
	3	14.67	14.17	8.69	11.83	13.82	16.60	22.93	26.07
	4	14.68	14.20	9.18	11.92	13.43	16.02	23.79	26.64
	5	14.67	14.07	8.56	12.14	13.11	15.32	24.01	34.92
	6	14.68	14.17	8.07	12.31	13.71	15.93	22.42	37.74
	7	14.68	14.06	8.91	11.61	13.07	16.56	23.48	29.58
	8	14.68	14.09	8.68	11.87	13.17	16.22	24.58	33.67
	9	14.68	14.16	7.69	11.66	13.96	16.18	23.98	27.40
	10	14.68	14.17	8.11	11.80	13.39	17.01	22.64	29.80
	Average‡	14.68[ab]	14.15[d]	8.46	11.94	13.52	16.26	23.30	29.97
50 subjects per pool (S=50) and 55 pools (P=55)	1	14.68	14.23	9.28	11.90	13.50	16.09	22.64	25.44
	2	14.68	14.34	10.88	11.89	13.79	16.49	20.69	25.25
	3	14.68	14.28	9.72	12.18	13.89	16.79	21.48	24.40
	4	14.68	14.24	9.13	11.56	13.59	16.56	21.90	24.23
	5	14.69	14.11	9.41	11.81	13.49	15.46	23.25	31.30
	6	14.69	14.36	8.69	12.65	14.35	16.04	20.62	23.95
	7	14.68	14.17	8.64	11.34	13.89	17.02	22.76	26.08
	8	14.70	14.17	8.80	12.07	13.36	15.89	21.91	31.68
	9	14.66	14.28	8.50	12.56	13.94	16.73	21.85	23.84
	10	14.68	14.27	8.51	12.25	13.64	16.81	21.25	25.94
	Average‡	14.68[ab]	14.24[e]	9.16	12.02	13.74	16.39	21.84	26.21

Note: S= samples per pool; P= pool count; AM= arithmetic mean; GM= geometric mean; Min Conc.= minimum concentration; P25 = the 25th percentile; Max Conc.= maximum concentration.

† T-test examines the mean differences in MiBP concentrations between individual and pooled samples. *p-value < 0.05.

‡ ANOVA compares the mean differences in MiBP concentrations among each pool type. Tukey's Studentized Range Test for multiple comparisons among each pool type.

Table6 Distribution of pooled MBP concentrations (ng/mL) by pool types

MBP		AM†	GM	Min Conc.	P25	P50	P75	P95	Max Conc.
	Individual	16.86	9.91	0.28	5.20	10.80	20.00	49.30	649.10
	Replication								
20 subjects per pool (S=20) and 138 pools (P=138)	1	16.63	16.05	8.73	13.75	15.93	18.53	25.28	38.10
	2	16.86	16.14	7.50	13.40	15.54	19.06	26.87	43.10
	3	16.86	16.03	7.83	12.98	15.85	18.94	26.43	44.23
	4	16.86	16.08	8.76	12.89	16.10	19.74	26.46	45.13
	5	16.85	16.03	7.47	13.36	15.33	17.92	30.15	50.58
	6	16.85	16.06	9.30	13.17	15.84	18.37	26.77	45.99
	7	16.86	16.03	7.39	13.13	15.89	18.84	26.14	49.05
	8	16.86	16.03	8.94	13.00	15.73	19.32	26.95	46.75
	9	16.86	15.98	8.18	13.27	16.01	18.71	25.74	57.75
	10	16.86	16.14	9.49	13.57	15.39	18.24	27.39	57.95
	Average‡	16.84[ab]	16.06[c]	8.36	13.25	15.76	18.77	26.82	47.86
30 subjects per pool (S=30) and 92 pools (P=92)	1	16.86	16.26	8.40	13.85	15.45	18.68	28.00	34.67
	2	16.86	16.37	10.57	13.93	15.83	18.33	24.19	33.32
	3	16.85	16.28	9.67	13.69	16.05	19.78	24.54	36.01
	4	16.86	16.25	9.31	13.51	15.58	19.12	25.70	36.54
	5	16.84	16.20	10.14	13.19	16.11	18.71	24.84	45.95
	6	16.87	16.33	10.38	13.78	15.68	18.81	25.48	35.44
	7	16.86	16.32	9.87	13.88	15.84	18.49	25.28	36.95
	8	16.86	16.26	7.81	13.64	15.56	18.36	26.28	41.45
	9	16.86	16.25	9.17	13.96	16.04	18.42	23.82	43.62
	10	16.85	16.39	9.19	14.14	16.14	18.45	24.10	34.87
	Average‡	16.86[ab]	16.29[d]	9.45	13.76	15.83	18.71	25.22	37.88

40 subjects per pool (S=40) and 69 pools (P=69)	1	16.86	16.42	9.69	14.07	15.95	18.42	25.84	31.78
	2	16.86	16.43	11.19	14.41	15.78	18.61	24.21	34.07
	3	16.85	16.41	9.77	14.53	16.15	19.01	24.26	29.96
	4	16.86	16.41	11.45	13.74	15.49	18.78	25.61	31.80
	5	16.84	16.33	9.11	14.34	16.12	18.04	26.24	36.07
	6	16.87	16.45	9.63	14.74	16.06	18.39	24.79	29.57
	7	16.86	16.47	10.73	14.37	16.07	18.50	22.58	39.41
	8	16.86	16.51	10.61	14.56	16.06	18.29	23.79	33.87
	9	16.86	16.36	9.97	13.83	16.04	18.74	24.10	38.21
	10	16.85	16.47	9.47	14.37	16.51	18.59	24.24	29.83
	Average‡	16.86[ab]	16.43[ab]	10.16	14.30	16.02	18.54	24.57	33.46
50 subjects per pool (S=50) and 55 pools (P=55)	1	16.87	16.48	11.09	14.63	15.94	18.47	25.28	28.21
	2	16.84	16.47	11.78	14.16	15.68	19.01	22.58	29.13
	3	16.85	16.47	10.26	14.17	16.03	19.11	23.10	26.82
	4	16.85	16.45	12.26	13.79	15.78	18.32	26.34	27.21
	5	16.85	16.44	10.46	14.39	15.78	17.97	24.53	30.00
	6	16.89	16.55	10.50	14.47	16.19	19.00	24.24	26.10
	7	16.87	16.47	11.42	14.25	15.91	18.54	22.51	34.00
	8	16.87	16.49	10.61	14.06	16.73	18.54	25.08	28.49
	9	16.79	16.39	10.67	14.29	16.26	18.52	24.29	30.55
	10	16.86	16.53	9.70	14.77	16.40	18.11	22.40	28.95
	Average‡	16.85[ab]	16.48[b]	10.88	14.30	16.07	18.56	24.03	28.94

Note: S= samples per pool; P= pool count; AM= arithmetic mean; GM= geometric mean; Min Conc.= minimum concentration; P25 = the 25th percentile; Max Conc.= maximum concentration.

† T-test examines the mean differences in MBP concentrations between individual and pooled samples. *p-value < 0.05.

‡ ANOVA compares the mean differences in MBP concentrations among each pool type. Tukey's Studentized Range Test for multiple comparisons among each pool type.

Table7 Distribution of pooled MBzP concentrations (ng/mL) by pool types

MBzP		AM†	GM	Min Conc.	P25	P50	P75	P95	Max Conc.
	Individual	9.12	3.74	0.21	1.50	3.70	9.20	34.10	307.20
20 subjects per pool (S=20) and 138 pools (P=138)	**Replication**								
	1	9.13	8.38	3.59	6.18	8.03	10.73	16.15	29.24
	2	9.12	8.26	2.98	6.14	8.05	10.92	17.32	30.71
	3	9.13	8.22	2.97	6.06	7.81	10.83	18.87	25.95
	4	9.12	8.28	2.81	6.13	7.98	10.86	18.01	24.78
	5	9.13	8.40	3.32	6.64	8.14	10.53	15.41	35.97
	6	9.12	8.35	2.84	6.49	8.07	11.11	17.00	23.31
	7	9.13	8.34	3.50	6.16	8.14	11.36	16.11	27.41
	8	9.10	8.34	2.54	6.56	8.49	10.42	17.16	26.36
	9	9.13	8.33	2.63	6.64	7.98	11.08	16.18	31.31
	10	9.13	8.29	3.22	6.38	8.27	10.87	18.04	25.62
	Average‡	9.12[ab]	8.32[c]	3.04	6.34	8.10	10.87	17.03	28.06
30 subjects per pool (S=30) and 92 pools (P=92)	1	9.13	8.55	3.73	6.59	8.75	10.78	14.28	28.96
	2	9.13	8.56	4.04	6.74	8.50	11.34	15.50	23.74
	3	9.12	8.58	4.13	6.75	8.52	10.72	14.72	22.48
	4	9.13	8.54	2.70	6.72	8.75	10.89	16.34	19.19
	5	9.13	8.44	4.00	6.08	8.54	11.38	17.18	20.99
	6	9.13	8.59	3.79	7.15	8.59	10.00	15.72	22.90
	7	9.13	8.50	4.18	6.67	8.25	10.89	15.15	27.82
	8	9.13	8.61	3.20	7.15	8.40	9.75	16.87	25.40
	9	9.13	8.61	4.02	6.82	8.60	10.62	15.72	23.87
	10	9.11	8.64	4.64	6.84	8.58	10.24	15.87	23.25
	Average‡	9.12[ab]	8.56[d]	3.84	6.75	8.55	10.66	15.74	23.86

40 subjects per pool (S=40) and 69 pools (P=69)	1	9.13	8.62	3.96	6.89	8.81	10.41	14.04	28.03
	2	9.13	8.71	4.55	7.15	8.37	10.59	14.85	17.25
	3	9.12	8.60	4.80	6.64	8.25	10.96	16.25	18.57
	4	9.13	8.65	4.07	7.02	8.67	10.86	14.98	17.87
	5	9.13	8.60	3.98	6.88	8.70	10.38	15.88	18.56
	6	9.13	8.72	4.85	7.09	8.45	10.34	15.49	18.97
	7	9.13	8.75	4.49	7.63	8.48	10.24	12.94	23.04
	8	9.13	8.80	5.09	7.41	8.91	10.26	14.75	15.53
	9	9.13	8.80	4.72	7.25	8.92	10.70	13.56	19.17
	10	9.11	8.72	4.37	7.35	8.46	9.84	15.32	18.41
	Average‡	9.12[ab]	8.70[ab]	4.49	7.13	8.60	10.46	14.81	19.54
50 subjects per pool (S=50) and 55 pools (P=55)	1	9.14	8.74	4.63	7.53	8.86	10.10	13.83	22.19
	2	9.13	8.75	5.46	6.98	8.45	10.56	14.71	17.04
	3	9.09	8.71	5.03	7.05	8.08	11.43	14.23	14.88
	4	9.12	8.73	4.52	7.09	8.14	10.81	14.17	19.70
	5	9.11	8.58	4.03	6.94	8.50	10.72	16.03	20.66
	6	9.14	8.84	4.88	7.46	8.63	10.19	13.96	16.57
	7	9.12	8.75	5.30	7.18	8.36	10.17	14.84	19.92
	8	9.09	8.72	4.69	7.26	8.36	9.81	14.99	17.95
	9	9.12	8.79	4.88	7.26	8.73	10.52	13.31	16.82
	10	9.13	8.82	4.71	7.44	8.41	10.01	13.69	16.10
	Average‡	9.12[ab]	8.74[b]	4.81	7.22	8.45	10.43	14.38	18.18

Note: S= samples per pool; P= pool count; AM= arithmetic mean; GM= geometric mean; Min Conc.= minimum concentration; P25 = the 25th percentile; Max Conc.= maximum concentration.

† T-test examines the mean differences in MBzP concentrations between individual and pooled samples. *p-value < 0.05.

‡ ANOVA compares the mean differences in MBzP concentrations among each pool type. Tukey's Studentized Range Test for multiple comparisons among each pool type.

Table8 Distribution of pooled MEHP concentrations (ng/mL) by pool types

MEHP		AM†	GM	Min Conc.	P25	P50	P75	P95	Max Conc.
	Individual	1.82	1.17	0.57	0.57	1.00	2.00	5.69	115.90
	Replication								
20 subjects per pool (S=20) and 138 pools (P=138)	1	1.82	1.72	0.91	1.38	1.69	2.04	2.96	7.24
	2	1.82	1.71	0.82	1.30	1.66	2.16	2.80	7.50
	3	1.82	1.71	0.75	1.36	1.65	2.15	2.96	7.03
	4	1.82	1.70	0.82	1.34	1.62	2.04	3.22	8.37
	5	1.82	1.72	0.81	1.39	1.65	2.06	3.05	6.99
	6	1.82	1.71	0.82	1.39	1.64	2.01	2.91	8.26
	7	1.82	1.73	0.65	1.43	1.68	2.01	2.94	7.15
	8	1.82	1.72	0.86	1.38	1.68	2.08	2.81	7.71
	9	1.82	1.74	0.88	1.41	1.70	2.09	2.63	6.87
	10	1.82	1.72	0.67	1.40	1.68	1.96	3.03	7.14
	Average‡	1.82[ab]	1.72[ab]	0.80	1.38	1.66	2.06	2.93	7.43
30 subjects per pool (S=30) and 92 pools (P=92)	1	1.82	1.74	1.01	1.42	1.67	2.06	2.80	6.08
	2	1.82	1.77	1.05	1.49	1.73	2.02	2.61	4.78
	3	1.82	1.75	1.11	1.44	1.65	2.09	2.75	5.27
	4	1.82	1.75	1.03	1.45	1.74	2.07	2.52	5.69
	5	1.82	1.76	0.97	1.46	1.70	2.08	2.57	5.32
	6	1.82	1.75	0.88	1.44	1.75	2.03	2.88	5.25
	7	1.82	1.75	1.00	1.46	1.68	1.95	2.71	5.81
	8	1.82	1.75	0.95	1.46	1.69	2.00	2.62	5.82
	9	1.82	1.75	0.98	1.46	1.68	2.00	2.84	5.89
	10	1.82	1.75	1.02	1.46	1.72	1.96	2.64	6.34
	Average‡	1.82[ab]	1.75[c]	1.00	1.46	1.70	2.03	2.69	5.63

40 subjects per pool (S=40) and 69 pools (P=69)	1	1.82	1.76	1.09	1.47	1.72	1.95	2.72	5.10
	2	1.82	1.77	1.14	1.54	1.72	1.91	2.70	4.04
	3	1.82	1.77	1.13	1.52	1.71	2.12	2.44	4.58
	4	1.82	1.76	1.07	1.49	1.72	1.99	2.48	5.15
	5	1.82	1.77	1.08	1.49	1.67	2.09	2.47	4.11
	6	1.82	1.76	1.08	1.51	1.69	1.97	2.78	4.52
	7	1.82	1.76	1.05	1.50	1.65	1.98	2.84	4.65
	8	1.82	1.76	1.09	1.49	1.70	2.02	2.60	4.96
	9	1.82	1.77	1.03	1.53	1.72	1.92	2.62	4.95
	10	1.82	1.76	1.08	1.49	1.72	1.96	2.36	5.37
	Average‡	1.82[ab]	1.76[d]	1.09	1.50	1.70	1.99	2.60	4.74
50 subjects per pool (S=50) and 55 pools (P=55)	1	1.83	1.77	1.19	1.50	1.72	2.06	2.63	4.31
	2	1.82	1.78	1.22	1.54	1.69	1.96	2.62	3.45
	3	1.82	1.78	1.27	1.52	1.74	2.00	2.49	3.91
	4	1.83	1.77	1.15	1.49	1.81	2.06	2.48	4.24
	5	1.82	1.78	1.09	1.55	1.74	2.03	2.34	3.51
	6	1.83	1.77	1.19	1.50	1.75	2.05	2.61	3.65
	7	1.82	1.77	1.27	1.50	1.69	1.90	2.74	4.44
	8	1.83	1.78	0.97	1.54	1.78	2.02	2.44	4.22
	9	1.83	1.78	1.07	1.59	1.74	1.91	2.69	3.67
	10	1.83	1.77	1.22	1.48	1.70	2.05	2.33	4.28
	Average‡	1.82[ab]	1.78[e]	1.16	1.52	1.74	2.00	2.54	3.97

Note: S= samples per pool; P= pool count; AM= arithmetic mean; GM= geometric mean; Min Conc.= minimum concentration; P25 = the 25th percentile; Max Conc.= maximum concentration.

† T-test examines the mean differences in MEHP concentrations between individual and pooled samples. *p-value < 0.05.

‡ ANOVA compares the mean differences in MEHP concentrations among each pool type. Tukey's Studentized Range Test for multiple comparisons among each pool type.

3.2.2. Sample design 2

Descriptive statistics for pooled MCPP, MEP, MiBP, MBP, MBzP, and MEHP concentrations are presented in **Table 9** through **Table 14**, respectively. Ideally, as the sample size gets larger, we would expect to observe a trend of increasing GMs and AMs, as well as decreasing SDs, GSDs, and CVs.

Arithmetic means (AMs) related statistics

The AMs, SDs, and corresponding CVs for all pooled phthalate metabolite concentrations are similar across sample sizes regardless of the pool type ($pooled_N{}^{s=20}$ & $pooled_N{}^{s=50}$). The trend of increasing AMs and decreasing SDs as sample size gets larger is consistent for pooled MCPP and MEP, but not for pooled MiBP, MBP, MBzP, and MEHP. However, the trend of decreasing CVs as the sample size gets larger is consistent for pooled MCPP, MEP, MiBP, MBP, and MBzP, except for pooled MEHP.

Geometric means (GMs) related statistics

The GMs, GSDs, and corresponding CVs values for all pooled phthalate metabolites vary across sample sizes and pool types. There is no consistent trend observed for GMs as the sample size increases for all pooled phthalate metabolites. However, there is a consistent trend of decreasing GSDs and CVs as the sample size gets larger for pooled MCPP, MEP, MBzP, and MEHP, but not for pooled MiBP and MBP.

Table9 Distribution of pooled MCPP concentrations (ng/mL) by sample size types

MCPP		N=500		N= 1000		N=1500		N=2000		N=2500	
	Replication	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**
20 subjects per pool (S=20)	1	2.04	1.84	2.20	1.89	2.32	1.95	2.35	1.96	3.22	2.09
	2	1.74	1.67	1.96	1.79	3.73	2.06	2.30	1.99	3.12	2.07
	3	2.07	1.87	2.13	1.87	3.56	1.96	2.36	2.00	3.13	2.02
	4	2.11	1.85	4.33	2.08	2.53	2.13	2.32	2.01	3.15	2.05
	5	1.89	1.72	1.99	1.83	2.48	2.10	3.49	2.10	3.13	2.04
	6	2.20	1.83	2.56	2.08	2.24	1.98	3.49	2.13	3.10	2.02
	7	6.39	2.27	2.40	1.98	3.73	2.11	2.23	1.94	3.17	2.01
	8	2.26	2.03	4.30	2.00	3.78	2.11	3.19	1.92	3.22	2.07
	9	2.08	1.92	2.20	1.91	2.00	1.84	3.24	2.01	3.19	2.07
	10	2.67	2.14	4.28	2.04	2.37	2.00	3.53	2.14	3.20	2.05
	Average	2.54	1.92	2.84	1.95	2.87	2.03	2.85	2.02	3.16	2.05
	SD or GSD	1.37	1.10	1.03	1.06	0.73	1.05	0.58	1.04	0.04	1.01
	CV	54.02	9.48	36.23	5.43	25.33	4.75	20.26	3.76	1.38	1.23
50 subjects per pool (S=50)	1	2.04	1.95	2.20	2.01	2.32	2.10	2.35	2.12	3.22	2.30
	2	1.74	1.71	1.96	1.86	3.73	2.33	2.30	2.15	3.12	2.26
	3	2.07	1.98	2.13	1.99	3.56	2.19	2.36	2.11	3.13	2.24
	4	2.11	1.97	4.33	2.38	2.53	2.32	2.32	2.14	3.15	2.23
	5	1.89	1.77	1.99	1.91	2.48	2.25	3.49	2.35	3.13	2.22
	6	2.20	1.97	2.56	2.31	2.24	2.07	3.49	2.38	3.10	2.23
	7	6.39	2.83	2.40	2.19	3.73	2.35	2.23	2.08	3.17	2.24
	8	2.26	2.16	4.30	2.27	3.78	2.37	3.19	2.13	3.22	2.29
	9	2.08	2.01	2.20	2.04	2.00	1.93	3.24	2.19	3.19	2.22
	10	2.67	2.41	4.28	2.36	2.37	2.17	3.53	2.39	3.20	2.28
	Average	2.54	2.08	2.84	2.13	2.87	2.21	2.85	2.20	3.16	2.25
	SD or GSD	1.37	1.16	1.03	1.10	0.73	1.07	0.58	1.05	0.04	1.01
	CV	54.02	14.76	36.23	9.13	25.33	6.54	20.26	5.34	1.38	1.39

Note: N= sample size; S= samples per pool; AM= arithmetic mean; GM= geometric mean; SD = standard deviation; GSD= geometric standard deviation; CV= coefficient of variation.

Table10 Distribution of pooled MEP concentrations (ng/mL) by sample size types

MEP		N=500		N= 1000		N=1500		N=2000		N=2500	
	Replication	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**
20 subjects per pool (S=20)	1	106.74	82.28	119.92	82.47	113.31	88.10	164.66	90.69	150.94	87.44
	2	348.08	98.60	102.63	80.99	100.21	76.91	159.63	86.24	154.67	86.99
	3	116.86	80.89	101.80	81.82	200.57	98.91	111.16	80.35	152.75	86.24
	4	102.33	73.54	224.18	104.55	185.62	90.63	155.53	84.97	156.79	89.30
	5	96.80	77.66	104.00	75.20	168.98	82.10	171.91	92.92	148.05	87.38
	6	73.36	67.64	208.69	85.38	175.52	92.28	111.06	82.67	155.06	87.93
	7	322.51	113.98	193.64	79.11	126.02	89.76	101.21	77.20	115.07	85.30
	8	108.31	92.81	112.97	81.86	181.58	89.38	171.31	88.93	151.60	89.44
	9	94.78	75.41	218.54	94.72	185.83	96.31	172.63	92.85	153.09	88.81
	10	109.59	83.82	121.33	88.80	174.55	82.31	166.50	91.80	152.30	83.68
	Average	147.94	84.66	150.77	85.49	161.22	88.67	148.56	86.86	149.03	87.25
	SD or GSD	99.62	1.17	53.04	1.10	34.74	1.08	28.76	1.07	12.17	1.02
	CV	67.34	15.47	35.18	9.61	21.55	7.67	19.36	6.45	8.17	2.09
50 subjects per pool (S=50)	1	106.74	96.52	119.92	99.22	113.31	100.97	164.66	107.50	150.94	101.61
	2	348.08	153.15	102.63	94.62	100.21	86.00	159.63	102.87	154.67	103.74
	3	116.86	103.60	101.80	89.20	200.57	121.62	111.16	92.00	152.75	101.00
	4	102.33	81.48	224.18	122.69	185.62	110.79	155.53	101.64	156.79	101.14
	5	96.80	83.91	104.00	86.69	168.98	97.66	171.91	107.28	148.05	100.25
	6	73.36	70.12	208.69	102.54	175.52	107.18	111.06	95.72	155.06	105.14
	7	322.51	139.92	193.64	91.97	126.02	100.32	101.21	87.74	115.07	97.56
	8	108.31	102.40	112.97	91.67	181.58	107.06	171.31	107.89	151.60	106.38
	9	94.78	83.59	218.54	121.96	185.83	115.19	172.63	108.77	153.09	105.80
	10	109.59	94.73	121.33	102.30	174.55	98.96	166.50	112.40	152.30	100.30
	Average	147.94	100.94	150.77	100.29	161.22	104.57	148.56	102.38	149.03	102.29
	SD or GSD	99.62	1.27	53.04	1.13	34.74	1.10	28.76	1.08	12.17	1.03
	CV	67.34	24.58	35.18	12.22	21.55	9.76	19.36	8.14	8.17	2.79

Note: N= sample size; S= samples per pool; AM= arithmetic mean; GM= geometric mean; SD = standard deviation; GSD= geometric standard deviation; CV= coefficient of variation.

Table11 Distribution of pooled MiBP concentrations (ng/mL) by sample size types

MiBP		N=500		N= 1000		N=1500		N=2000		N=2500	
	Replication	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**
20 subjects per pool (S=20)	1	17.15	15.91	14.75	13.62	14.85	13.82	14.59	13.52	14.52	13.49
	2	13.90	13.32	14.44	13.45	14.67	13.60	14.80	13.87	14.41	13.48
	3	14.13	13.33	15.04	13.86	14.18	13.40	14.81	13.79	14.73	13.85
	4	13.19	12.74	14.49	13.63	14.75	13.74	14.87	13.75	14.79	13.95
	5	15.77	14.84	13.56	13.11	14.12	13.37	14.76	13.81	14.77	13.75
	6	13.18	12.08	14.85	14.01	14.91	14.10	14.63	13.72	14.76	13.78
	7	14.12	13.62	13.46	12.88	14.76	13.74	15.31	14.01	14.69	13.72
	8	14.02	13.08	15.28	14.11	14.29	13.45	14.88	13.73	14.82	13.84
	9	13.48	13.04	14.22	13.30	14.70	13.75	14.48	13.54	14.78	13.80
	10	14.39	13.91	14.47	13.59	14.58	13.61	14.91	13.83	14.65	13.79
	Average	14.33	13.59	14.46	13.55	14.58	13.66	14.80	13.76	14.69	13.75
	SD or GSD	1.24	1.08	0.59	1.03	0.28	1.02	0.23	1.01	0.13	1.01
	CV	8.63	7.83	4.07	2.86	1.94	1.62	1.53	1.06	0.90	1.10
50 subjects per pool (S=50)	1	17.15	16.45	14.75	14.33	14.85	14.36	14.59	14.19	14.52	14.01
	2	13.90	13.67	14.44	13.95	14.67	14.05	14.80	14.40	14.41	14.02
	3	14.13	13.59	15.04	14.44	14.18	13.73	14.81	14.29	14.73	14.31
	4	13.19	13.01	14.49	14.19	14.75	14.15	14.87	14.34	14.79	14.42
	5	15.77	15.44	13.56	13.36	14.12	13.86	14.76	14.39	14.77	14.35
	6	13.18	12.82	14.85	14.50	14.91	14.40	14.63	14.16	14.76	14.26
	7	14.12	13.83	13.46	13.27	14.76	14.38	15.31	14.67	14.69	14.14
	8	14.02	13.89	15.28	14.83	14.29	13.97	14.88	14.15	14.82	14.33
	9	13.48	13.26	14.22	13.74	14.70	14.13	14.48	14.05	14.78	14.20
	10	14.39	14.20	14.47	14.16	14.58	14.16	14.91	14.26	14.65	14.33
	Average	14.33	14.02	14.46	14.08	14.58	14.12	14.80	14.29	14.69	14.24
	SD or GSD	1.24	1.08	0.59	1.04	0.28	1.02	0.23	1.01	0.13	1.01
	CV	8.63	7.73	4.07	3.59	1.94	1.59	1.53	1.22	0.90	0.99

Note: N= sample size; S= samples per pool; AM= arithmetic mean; GM= geometric mean; SD = standard deviation; GSD= geometric standard deviation; CV= coefficient of variation.

Table12 Distribution of pooled MBP concentrations (ng/mL) by sample size types

MBP		N=500		N= 1000		N=1500		N=2000		N=2500	
	Replication	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**
20 subjects per pool (S=20)	1	16.27	15.81	15.63	15.24	16.83	16.33	17.04	16.19	16.99	16.18
	2	15.72	15.24	16.74	15.99	17.78	16.71	16.99	16.23	16.70	16.03
	3	17.54	16.51	16.52	15.97	16.16	15.40	16.92	16.04	16.96	16.09
	4	17.71	15.88	16.40	15.59	17.46	16.64	16.92	16.09	16.87	16.13
	5	17.53	16.52	16.46	15.76	17.13	16.26	16.93	16.38	16.88	16.10
	6	14.90	14.61	17.89	17.08	17.02	16.10	16.92	16.07	16.77	16.02
	7	16.53	15.54	16.75	16.08	17.06	16.13	16.78	15.99	16.57	15.76
	8	17.24	16.42	16.51	15.66	16.50	15.78	16.69	15.78	17.05	16.30
	9	15.33	14.96	16.76	15.93	16.96	16.19	16.90	15.98	16.70	16.04
	10	17.25	16.81	16.81	15.89	16.45	15.69	16.97	16.09	16.89	16.16
	Average	16.60	15.83	16.65	15.92	16.94	16.12	16.91	16.08	16.84	16.08
	SD or GSD	1.01	1.05	0.55	1.03	0.48	1.03	0.10	1.01	0.15	1.01
	CV	6.09	4.72	3.33	2.94	2.85	2.55	0.60	1.00	0.90	0.87
50 subjects per pool (S=50)	1	16.27	16.05	15.63	15.48	16.83	16.68	17.04	16.73	16.99	16.60
	2	15.72	15.48	16.74	16.44	17.78	17.36	16.99	16.54	16.70	16.43
	3	17.54	16.95	16.52	16.36	16.16	15.81	16.92	16.46	16.96	16.61
	4	17.71	16.85	16.40	15.96	17.46	17.12	16.92	16.57	16.87	16.48
	5	17.53	17.23	16.46	16.19	17.13	16.82	16.93	16.70	16.88	16.48
	6	14.90	14.71	17.89	17.48	17.02	16.68	16.92	16.48	16.77	16.29
	7	16.53	16.08	16.75	16.51	17.06	16.66	16.78	16.43	16.57	16.17
	8	17.24	16.94	16.51	16.02	16.50	16.08	16.69	16.24	17.05	16.80
	9	15.33	15.19	16.76	16.37	16.96	16.57	16.90	16.32	16.70	16.41
	10	17.25	17.15	16.81	16.49	16.45	16.13	16.97	16.70	16.89	16.59
	Average	16.60	16.26	16.65	16.33	16.94	16.59	16.91	16.52	16.84	16.48
	SD or GSD	1.01	1.06	0.55	1.03	0.48	1.03	0.10	1.01	0.15	1.01
	CV	6.09	5.60	3.33	3.11	2.85	2.87	0.60	1.00	0.90	1.07

Note: N= sample size; S= samples per pool; AM= arithmetic mean; GM= geometric mean; SD = standard deviation; GSD= geometric standard deviation; CV= coefficient of variation.

Table13 Distribution of pooled MBzP concentrations (ng/mL) by sample size types

MBzP		N=500		N= 1000		N=1500		N=2000		N=2500	
	Replication	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**
20 subjects per pool (S=20)	1	9.52	8.74	9.13	8.38	9.81	9.04	9.63	8.89	9.23	8.55
	2	7.58	7.07	8.24	7.60	9.24	8.34	9.20	8.46	8.99	8.37
	3	8.19	7.63	9.30	8.47	8.67	8.05	9.01	8.22	8.88	8.15
	4	8.55	7.76	8.60	7.60	9.47	8.74	8.92	8.04	9.10	8.38
	5	9.39	8.74	8.46	8.01	9.12	8.40	9.25	8.49	9.27	8.52
	6	9.27	8.50	9.58	8.63	8.88	8.25	9.14	8.40	9.12	8.32
	7	8.27	7.65	8.73	8.08	9.40	8.53	9.12	8.44	8.94	8.23
	8	9.15	8.17	8.79	7.94	8.37	7.69	8.72	8.06	9.30	8.51
	9	8.52	8.11	9.20	8.48	9.19	8.37	8.89	8.16	9.12	8.27
	10	9.43	8.94	8.97	8.19	8.54	7.98	9.07	8.30	9.17	8.33
	Average	8.79	8.13	8.90	8.14	9.07	8.34	9.10	8.35	9.11	8.36
	SD or GSD	0.66	1.08	0.41	1.05	0.45	1.05	0.25	1.03	0.14	1.02
	CV	7.51	7.52	4.62	4.46	4.97	4.62	2.72	3.00	1.53	1.61
50 subjects per pool (S=50)	1	9.52	9.24	9.13	8.69	9.81	9.46	9.63	9.32	9.23	8.95
	2	7.58	7.53	8.24	7.97	9.24	8.75	9.20	8.93	8.99	8.69
	3	8.19	8.06	9.30	9.02	8.67	8.43	9.01	8.63	8.88	8.57
	4	8.55	8.27	8.60	8.13	9.47	9.26	8.92	8.54	9.10	8.74
	5	9.39	8.98	8.46	8.29	9.12	8.80	9.25	8.94	9.27	8.84
	6	9.27	9.00	9.58	9.13	8.88	8.62	9.14	8.78	9.12	8.75
	7	8.27	8.06	8.73	8.50	9.40	8.97	9.12	8.76	8.94	8.64
	8	9.15	8.65	8.79	8.33	8.37	7.90	8.72	8.51	9.30	9.02
	9	8.52	8.37	9.20	9.02	9.19	8.82	8.89	8.59	9.12	8.74
	10	9.43	9.31	8.97	8.63	8.54	8.39	9.07	8.73	9.17	8.87
	Average	8.79	8.55	8.90	8.57	9.07	8.74	9.10	8.77	9.11	8.78
	SD or GSD	0.66	1.07	0.41	1.05	0.45	1.05	0.25	1.03	0.14	1.02
	CV	7.51	6.91	4.62	4.65	4.97	5.16	2.72	2.74	1.53	1.58

Note: N= sample size; S= samples per pool; AM= arithmetic mean; GM= geometric mean; SD = standard deviation; GSD= geometric standard deviation; CV= coefficient of variation.

Table14 Distribution of pooled MEHP concentrations (ng/mL) by sample size types

MEHP		N=500		N= 1000		N=1500		N=2000		N=2500	
	Replication	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**	**AM**	**GM**
20 subjects per pool (S=20)	1	1.88	1.69	1.90	1.74	1.90	1.77	1.86	1.73	1.83	1.72
	2	1.81	1.72	1.93	1.74	1.90	1.78	1.75	1.67	1.79	1.71
	3	1.96	1.75	1.72	1.65	1.84	1.70	1.76	1.69	1.83	1.72
	4	1.95	1.82	1.89	1.76	1.87	1.75	1.80	1.67	1.83	1.74
	5	1.75	1.68	1.72	1.65	1.79	1.68	1.83	1.73	1.83	1.73
	6	1.67	1.62	1.98	1.85	1.82	1.71	1.88	1.78	1.82	1.71
	7	1.83	1.74	1.84	1.70	1.89	1.80	1.74	1.68	1.81	1.70
	8	1.89	1.79	1.69	1.65	1.86	1.73	1.85	1.74	1.83	1.72
	9	1.61	1.55	1.81	1.70	1.81	1.70	1.81	1.69	1.77	1.68
	10	1.76	1.72	1.92	1.77	1.82	1.72	1.83	1.73	1.85	1.74
	Average	1.81	1.71	1.84	1.72	1.85	1.73	1.81	1.71	1.82	1.72
	SD or GSD	0.11	1.05	0.10	1.04	0.04	1.02	0.05	1.02	0.02	1.01
	CV	6.33	4.65	5.43	3.70	2.09	2.28	2.66	2.06	1.24	1.00
50 subjects per pool (S=50)	1	1.88	1.77	1.90	1.84	1.90	1.85	1.86	1.80	1.83	1.77
	2	1.81	1.79	1.93	1.83	1.90	1.84	1.75	1.72	1.79	1.75
	3	1.96	1.84	1.72	1.69	1.84	1.77	1.76	1.73	1.83	1.78
	4	1.95	1.87	1.89	1.83	1.87	1.81	1.80	1.73	1.83	1.79
	5	1.75	1.72	1.72	1.69	1.79	1.74	1.83	1.79	1.83	1.78
	6	1.67	1.64	1.98	1.93	1.82	1.76	1.88	1.84	1.82	1.77
	7	1.83	1.79	1.84	1.77	1.89	1.84	1.74	1.71	1.81	1.76
	8	1.89	1.84	1.69	1.68	1.86	1.80	1.85	1.80	1.83	1.77
	9	1.61	1.59	1.81	1.75	1.81	1.76	1.81	1.75	1.77	1.73
	10	1.76	1.75	1.92	1.83	1.82	1.78	1.83	1.78	1.85	1.80
	Average	1.81	1.76	1.84	1.78	1.85	1.79	1.81	1.77	1.82	1.77
	SD or GSD	0.11	1.05	0.10	1.05	0.04	1.02	0.05	1.02	0.02	1.01
	CV	6.33	5.10	5.43	4.57	2.09	2.08	2.66	2.45	1.24	1.11

Note: N= sample size; S= samples per pool; AM= arithmetic mean; GM= geometric mean; SD = standard deviation; GSD= geometric standard deviation; CV= coefficient of variation.

4. Discussions

4.1. Individual phthalate metabolites

This is the first study to explore different pooled sample designs for measuring urinary phthalate metabolite concentrations using the NHANES data. Initially, we considered pooling by gender, but **Table 2** revealed no significant difference in the six individual phthalate metabolite concentrations between genders, rendering pooling by gender uninformative. Due to the scope of the study, we solely focused on overall phthalate metabolite levels without further stratification by other variables.

4.2. Pooled sample design 1

4.2.1. Individual vs. Pooled

The results from the pooled sample design 1 (**Table 3 –Table 8**) support that the measured value of a pooled sample is comparable to the AM concentration of the individual samples that make up the pool (Caudill, 2010). We observed no significant difference in AM concentrations between individual and pooled samples for any of the six phthalate metabolites, regardless of the type of pool used. The GM concentrations and most of the distribution percentiles of the six pooled phthalate metabolites were not comparable to those of the individual samples for all types of pools. This result was expected since pooled sample data allows for the measurement of only the AM, and additional statistical descriptors such as the GM or median of a population cannot be calculated (Heffernan et al., 2014). However, the 75th percentile (P75) values of the pooled and individual MCPP, MiBP, MBP, MBzP, and MEHP are similar, except for MEP, where the 95th percentile (P95) value is closer. As MEP has the greatest outliers among all phthalate metabolites, we attempted to remove them and observe whether the similar values between pooled and individual phthalate metabolites move toward P75. However, the trend remained the

same. Further exploration, such as observing the distribution pattern, is needed to investigate the reason for the similar P75 values between most of the pooled and individual phthalates.

4.2.2. Pool types

When comparing the AM concentrations of MEP, MiBP, MBP, MBzP, and MEHP across pool types, no differences were found, except for MCPP. However, there were significant differences in the geometric mean (GM) concentrations of all pooled phthalate metabolites. It is important to note that there were only 10 data points (10 replications) for each pool type among each pooled phthalate metabolite, indicating low variability in the data. Therefore, even small differences between groups may be statistically significant. For instance, although the pair differences were only about 0.1, the pooled $^{s=50}$ had a significant difference with other pooled types in the AM concentrations of MCPP. Overall, the findings suggest that the choice of pooled $^{s=n}$ does not have a significant impact on AM values. However, there appears to be a trend where larger pooled $^{s=n}$ values result in larger GM values.

4.3. Pooled sample design 2

We will suggest a minimum sample size needed to produce consistent AMs and GMs concentrations for each pooled phthalate metabolite by observing SDs, GSDs, and CVs. It is important to note that the acceptable level of variability in the data depends on the purpose of the analysis and the required level of precision. As a result, there is no standard criterion to determine whether SDs, GSDs and CVs are small enough. In general, pooled $_{N=2500}$ $^{s=n}$ provides the most consistent AMs and GMs for each pooled phthalate metabolite. However, due to cost and resource constraints, we will suggest a minimum sample size that is acceptable for each phthalate metabolite based on the results from **Table 9 –Table 14** and the trend observation described in the result section. The findings suggest that pooled $_{N=1500}$ $^{s=n}$ produce a consistent

AM and GM concentrations for MCPP, MEP, MiBP, MEHP. Moreover, Pooled $_{N=1000}{}^{s=n}$ produce a consistent AM and GM concentrations for MBzP, MBP.

4.4. Limitations and Strengths

Our study provides novel insights into the different pooled sample designs using NHANES, one of the largest biomonitoring programs globally. While the large sample size offers significant advantages, we acknowledge certain limitations of our study. Only six among the nineteen phthalate metabolites with relatively high frequency of detection from the dataset are selected for analysis. Additionally, not stratifying age, race/ethnicity, and other demographic variables by phthalate metabolites may result in biased estimates if there are systematic differences in phthalate exposure among subgroups. Another limitation of the study is that we only replicated each algorithm 10 times. However, the optimal number of replications required to obtain reliable and accurate standard deviation values for this study remains unknown. Last but not least, we did not test the sufficient number of pool (pool counts) required to produce a grand mean concentration comparable to the average concentration of the individual samples within each pool. This aspect of the pooled sample design may yield interesting patterns and warrants further investigation.

4.5 Future study recommendations

In addition to the limitations mentioned previously, we suggest addressing several other aspects in future studies. Firstly, the pooled sample approach yields only the AM as a reliable statistic for comparison with individual sample statistics. Therefore, we can only draw conclusions based on the AM for our finding in pooled sample design 1, which indicates that varying the number of samples per pool does not result in differences in the AM concentrations between individual and pooled samples for any of the six phthalate metabolites. In future studies aiming to suggest a

number of samples per pool required to produce a GM phthalate metabolite concentration similar to the GM concentration of individual samples in the pools, it is recommended to use the pooled sample estimated geometric mean method described by Caudill et al., 2007. This method can provide a reliable estimate of the GM from pooled samples and may help address this question more accurately. Additionally, there is no standard criterion to decide what level of consistency is acceptable for AMs and GMs concentrations of phthalate metabolites for different sample sizes. In future studies, we will need to test and validate the suggested minimum sample size for each phthalate metabolite on other datasets.

5. Conclusions

Our study suggests that using pooled urinary phthalate metabolite samples is a feasible approach for large-scale human biomonitoring to obtain the AM concentrations of individual urinary phthalates, regardless of the number of samples per pool. Taking into account the cost and time constraints, we recommend a minimum sample size of approximately 1500 to produce consistent AM and GM concentrations for MCPP, MEP, MiBP, and MEHP. For MBzP and MBP, a minimum sample size of approximately 1000 is recommended to produce consistent AM and GM concentrations.

5.1. Public Health significances

This study may improve the accuracy of the pooled-sample method by providing guidance on the pooled-sample formulation. This work is crucial as it will assist countries with limited financial and laboratory resources to implement large-scale human biomonitoring programs to assess exposure to environmental toxicants among their populations. Furthermore, since the populations of middle- and low-income countries are often facing with high magnitude of environmental chemical exposure due to either the lack of or ineffective control plans, biomonitoring studies

will provide valuable data leading to the development of effective control measures and prevention of health burdens associated with chemical exposure.

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