Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Justin Byun April 9, 2024

Utilizing wastewater-based epidemiology to assess the chemical exposome of residents in Louisville, Kentucky

By

Justin Byun

Douglas Walker

Adviser

Center for the Study of Human Health

Douglas Walker

Adviser

William Michael Caudle

Committee Member

Myra Woodworth-Hobbs

Committee Member

2024

Utilizing wastewater-based epidemiology to assess the chemical exposome of residents in Louisville, Kentucky

By

Justin Byun

Douglas Walker

Adviser

An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of the requirements of the degree of

Bachelor of Arts with Honors

Center for the Study of Human Health

2024

Abstract

Utilizing wastewater-based epidemiology to assess the chemical exposome of residents in Louisville, Kentucky

By Justin Byun

Exposure to chemicals has become an inescapable reality, with increasing evidence showing that environmental exposures are linked to non-communicable diseases among residents of high-income countries. There has been an increase in cases of chronic diseases that may be attributable to environmental exposures among the last decade. With the need for a more complete environmental exposure assessment to understand the association between environmental influences and their biological response, wastewater-based epidemiology holds potential to identify the chemical exposome without requiring individual samples from a study population, avoiding the costly and logistical constraints of human biomonitoring of biofluids.

The purpose of this study was to optimize and validate a solid phase extraction (SPE) method to prepare wastewater samples for untargeted screening using liquid chromatography-high resolution mass spectrometry (LC-HRMS) and compare wastewater exposome profiles from low to high socioeconomic neighborhoods, by population, sewershed area, and between residential and commercial areas to evaluate potential exposure disparities. We evaluated how SPE can enhance determination of the composition of analytes in wastewater and potentially identify previously unrecognized exposures for a more comprehensive chemical exposome profile.

Conducted in Louisville, Kentucky, which exhibits both industrial and residential areas, and a significant socioeconomic disparity between the west and east sides of the city, samples from 27 wastewater sites from both water quality treatment centers and sewershed areas leading to such centers were collected and analyzed by LC-HRMS.

SPE significantly increased the intensity of detected features in wastewater and the total number of detected features in wastewater compared to traditional sample preparation methods. Each site exhibited a larger number of detected features in wastewater prepared through SPE compared to wastewater prepared with traditional methods. However, no association was found between feature count and household income, population, and sewershed area of the wastewater sites. Features were annotated through xMSannotator, and the top five most concentrated chemicals detected in SPE wastewater, which was run on C18pos mode, were reported. This study demonstrated that untargeted analysis of exposure with annotation of detected features can detect previously known and potentially harmful exposures and delve further into problems of environmental injustice.

Utilizing wastewater-based epidemiology to assess the chemical exposome of residents in Louisville, Kentucky

By

Justin Byun

Douglas I. Walker

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of the requirements of the degree of

Bachelor of Arts with Honors

Center for the Study of Human Health

2024

Acknowledgements

I would like to thank Dr. Walker for his guidance and support throughout this project. I would also like to thank Catherine Mullins and the CLUES team in supporting me with instrumental analysis and data processing.

Table of Contents

Background

The presence of chemicals and pollutants in the environment contributes significantly to the development of complex chronic diseases (Cui, Balshaw et al. 2016). The last decade has seen a considerable increase in cases of allergies, infertility, impaired brain development of children, various cancers, metabolic changes, and neurological disorders that may be attributable to environmental exposures (Chin-Chan, Navarro-Yepes and Quintanilla-Vega 2015, Nurmatov, Tagiyeva et al. 2015, Russ and Howard 2016, Carre, Gatimel et al. 2017, Klotz and Goen 2017, Han, Kim and Song 2019). Many chemicals lurking in the environment have been found to be associated with adverse health outcomes, including Parkinson's disease, other neurological disorders, respiratory disease, and genetic damage in sperm (Baldi, Gruber et al. 2011, Mamane, Baldi et al. 2015, Moisan, Spinosi et al. 2015, Knapke, Magalhaes et al. 2022). Industrial products such as flame retardants persist in the environment, and have also been associated with various cancers, kidney diseases, diabetes, and metabolic and endocrine changes (Allen, Gale et al. 2016, Ji, Yao et al. 2021, Tsai, Cheng et al. 2022). Microplastics have also been detected in the environment, and there is emerging evidence that plastic exposures could impact health (Li, Ding et al. 2020, Zhang, He et al. 2022). Therefore, it is important to understand which exposures humans are subjected to help identify how these may contribute to chronic diseases. Patterns of environmental pollution and the diseases caused by such pollution vary from country to country. In higher income countries like the United States, ambient air pollution, toxic synthetic chemicals, pesticides, heavy metals, and other hazards of the urban environment are likely key risk factors for disease (Landrigan, Sly et al. 2016).

The concept of the exposome emerged due to the need for a more complete environmental exposure assessment to understand how complex exposures may be associated with health outcomes. The chemical exposome is defined as the cumulate measure of environmental influences and their corresponding biological responses, which requires consideration of both the nature of such exposures, the impacts they have in our bodies, and how they change during the course of a human lifetime (Miller and Jones 2014, Vermeulen, Schymanski et al. 2020). The exposome complements the genome by considering internal, specific external, and general external exposures (Wild 2012). Internal exposures include internal processes in the body like metabolism, endogenous hormones, and inflammation. Specific external exposures include radiation, infectious agents, chemical contaminants, environmental pollutants, diet, lifestyle factors, and occupation and medical interventions. General external exposures are the wider social, economic, and psychological influences on the individual, like social capital, education, financial status, and climate. The exposome allows research to connect epidemiology to psychological, sociological, and economic issues that impact humans every day.

Human biomonitoring is one of the primary methods for identifying exposures that may contribute to disease risk. Biomonitoring provides an accurate depiction of the exposome, as it directly measures the chemicals in the body using biofluids such as urine or blood (Yusa, Millet et al. 2015). However, most biomonitoring studies utilize targeted screening analytic strategies, which are likely biased since they only measure known chemicals. Human biomonitoring can also be arduous due to logistical constraints, requirements for collecting invasive samples, privacy concerns and high cost. Biomonitoring depends on selecting a biomarker that is relevant to the exposure of study. Urine and blood are the most common biofluids for measuring exposure biomarkers, but collection of both is laborious and inefficient; the excretion profiles of urine vary

throughout the day due to the short half-lives of exposures in the human body, and blood collection is time-consuming and invasive. Due to the large number of constraints of human biomonitoring, a call for a proxy that measures chemical exposure in humans is an urgent need for public health.

Wastewater-based epidemiology (WBE) is a new and promising approach that can identify the chemical exposure of populations without requiring individualized samples from study participants. Wastewater can be thought of as a collective, integrated biospecimen, as the wastewater pools anonymous samples from a population within a certain geographical region. The biggest advantage of WBE is it provides real-time, objective information on chemicals that were directly and indirectly ingested regularly by a population, and does not require collection of personalized identifying information, such as names or addresses. Wastewater has already been used to monitor specific substances, such as illicit drugs and pharmaceuticals, and has been used as a key monitoring tool for COVID-19 prevalence within populations (Castiglioni, Salgueiro-Gonzalez et al. 2021, Escola Casas, Schroter et al. 2021, Weidhaas, Aanderud et al. 2021, Cohen, Maile-Moskowitz et al. 2022). Recent studies have showcased the power of WBE in vast residential areas; sewage sludge from wastewater treatment plants was screened in Barcelona to show correlations with type and concentration of chemicals found in humans (Gil-Solsona, Nika et al. 2021).

Due the availability of samples and presence of both industrial and residential areas, Louisville, Kentucky was selected as the area of study for this project. In 2023, Louisville was one of the most polluted cities, ranked 22nd among the nation in year-round particulate pollution, or the pollution of tiny pieces of solid or liquid. Louisville also has a significant socioeconomic

disparity between different neighborhoods and communities. The east side of the city tends to be more affluent, with higher median incomes, lower poverty rates, and better access to resources like education, health care, and cultural amenities (United States Census Bureau 2020-2022). In contrast, the west side of the city contain many neighborhoods with high poverty rates, lower median incomes, and higher rates of unemployment (United States Census Bureau 2020-2022). West Louisville is predominantly an African American community made up of nine different neighborhoods and a large industrial plant called Rubbertown. Initial studies have already been conducted looking into the environmental impact of Rubbertown in the nearby neighborhoods, demonstrating the elevated pollutant levels in that area (Mukerjee, Smith et al. 2020). Samples were collected at 27 locations chosen to represent the geographic and demographic diversity of Louisville.

Advances in high-resolution mass spectrometry (HRMS) and the emergence of large chemical repositories have shown considerable promise to identify previously unknown chemicals in the environment (Chen, Hsu et al. 2023). Liquid chromatography coupled to mass spectrometry (LC-MS) is a combination of analytical techniques to enable determination of such chemicals through its selectivity and sensitivity in detecting trace chemicals in complex matrices. LC-MS also enables analysis through non-targeted screening, a method that detects and identifies unknown exposures in various matrices (Wright, Beach and McCarron 2022). One challenge in non-targeted screening is the occurrence of false-positive findings when two chromatographically coeluting compounds have very similar mass-to-charge (*m/z*) values, forming a single peak that would be mistaken as an overestimation of a single analyte concentration (Acena, Stampachiacchiere et al. 2015). HRMS is utilized so that potentially

interfering peaks in LC are resolved, and also provide accuracy in analyses of high *m/z* regions (Lai and Wang 2023).

The first goal of this project is to optimize a solid phase extraction (SPE) method to prepare wastewater samples for untargeted screening using liquid chromatography-high resolution mass spectrometry (LC-HRMS). We accomplish this goal by evaluating how SPE can enhance methods for determining the composition of analytes in wastewater and to the identification of previously unrecognized exposures for a more thorough view of the human exposome and compare concentrations of analytes in SPE with the concentrations of analytes in regular automated sample processing through Opentrons, which does not enrich the analytes. SPE is a commonly used sample preparation technique for liquid samples, as it enriches the analytes with high recovery and specificity through different sorbent cartridges (Plotka-Wasylka, Szczepanska et al. 2016). SPE can be combined with chromatographic techniques coupled with mass spectrometry in order to determine unknown compounds at trace levels within complex matrices, with research showcasing the power of the combination of SPE with LC/GC-MS methods (Richardson and Kimura 2016, Semreen, Shanableh et al. 2019). Through SPE, exposures in wastewater are assessed to benchmark how SPE improves detection of exposures compared to traditional sample preparation methods for measuring the exposome in human samples and improves detection of chemicals present in wastewater that may have a negative effect on residents of the different sewer sheds studied.

The second goal of this project is to compare wastewater exposome profiles from low to high socioeconomic neighborhoods, by population, sewershed area, and between residential and commercial areas to evaluate potential exposure disparities. The application of WBE opens

opportunities for meaningful public health initiatives in environmental justice. It has been long established that environmental pollutants have disproportionate effects of people of lower socioeconomic status than those of higher socioeconomic status because poorer communities were more likely to live near environmentally hazardous areas (Elliott, Wang et al. 2004, Strube, Thiede and Auch 2021). WBE can provide valuable insights by looking at the composition of exposures found in certain wastewater sheds near residential areas with varying socioeconomic status. During the COVID-19 pandemic, a study utilizing WBE was conducted to assess disease disparities between communities in California, opening the gate to utilizing WBE in tackling environmental disparities between communities (Medina, Kadonsky et al. 2022). By utilizing wastewater sites as a proxy for socioeconomic background of nearby neighborhoods, WBE unlocks the ability to delve into significant environmental exposure disparities for future public health initiatives.

The results of this work are relevant for understanding the human exposome and may have implications for public health initiatives in environmental justice and spatial inequality. There has been a larger call for extensive research about the exposome and WBE to assess human health. Multiple reports state how wastewater can serve as an effective proxy to analyze the chemical exposome for large area without conducting extensive human biomonitoring campaigns (Markosian and Mirzoyan 2019, Gracia-Lor, Zuccato et al. 2020, Saththasivam, El-Malah et al. 2021). Conducting WBE in Louisville will contribute to understanding the exposures that residents are exposed to on an everyday basis and compare the exposures between various locations in Louisville.

Methods

Study Site

The study took place in Louisville, Jefferson County, Kentucky, USA (Fig. 1). Twenty-seven sample locations were chosen throughout the county to represent the diversity of geographic and demographic characteristics at the sub-county level, with a focus on neighborhoods with known environmental exposures and risks. Five of these sites were flowing into the wastewater treatment plant, while the other 22 sites were upstream corresponding sewer shed sub-area catchments at community sewer line locations or intermediate pump stations, which eventually flowed to a water quality treatment center (WQTC). Some sample locations were both a sanitary sewer and storm water system, which may induce dilution or contribute toxicants from the environment during high rainfall events. One site was a water blank to test contamination of the sampling equipment (E6).

Figure 1. **A map of Louisville that details the locations of the 27 sites where the wastewater samples were collected.**

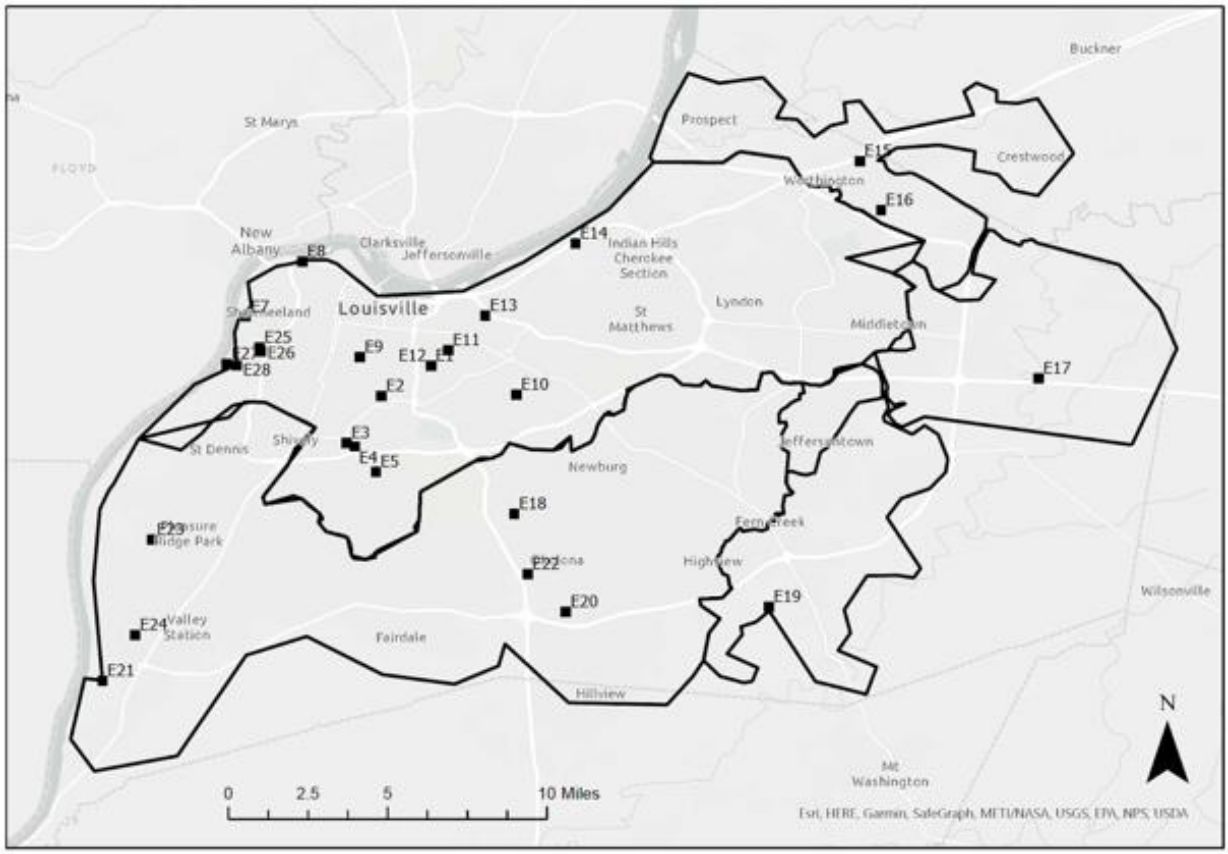

Table 1. **Sample site characteristics of Jefferson County, KY (USA).**

Sampling Site	Household income (USD) 2020 Median HH Income (ACS 5-Yr)	Population	Race: White (%)	Race: Black (%)	Hispanic Population (%)	Area (km^2)	Sample site type	Does sewershed include combined sewer overflow?	Material and year
E1	0	0	0	0	0	0.01	Nested sewershed leading to E27	Yes	Clay 1916
E2	34,490	8,258	51	32	10	4	Nested sewershed leading to E27	Yes	Clay 1916
E3	36,812	3,459	57	24	13	1	Nested sewershed leading to E27	Yes	Concrete 1928
E4	37,059	1,610	61	22	10	1	Nested sewershed leading to E27	Yes	Concrete 1938
E5	40,168	3,587	56	21	12	3	Nested sewershed leading to E27	Yes	Concrete 1930

E7	31,050	10,949	13	82	2	5	Nested sewershed leading to E27	Yes	Concrete 1924
E8	27,752	9,073	57	36	2	5	Nested sewershed leading to E27	Yes	Concrete 1950
E9/E12	27,517	23,751	55	32	6	12	Nested sewershed leading to E27	Yes	Brick 1910
E10	65,791	145,346	70	14	7	112	Nested sewershed leading to E27	Yes	Concrete 1932
E11	56,672	8,838	80	9	3	3	Nested sewershed leading to E27	Yes	Brick 1912
E13	74,500	95,603	80	7	5	80	Nested sewershed leading to E27	Yes	Concrete 1977
E14	101,140	11,444	91	2	3	12	Nested sewershed leading to E27	Yes	PVC 1993
E15	86,478	40,824	73	11	6	67	WQTC	-	

E16	81,994	5,781	70	12	5	11	Nested sewershed leading to E15	No	Concrete 1969
E17	108,021	37,193	78	8	4	88	WQTC	-	
E18	55,433	78,206	52	27	13	55	Nested sewershed leading to E21	No	Concrete 1954
E19	76,796	60,885	75	12	7	80	WQTC	-	
E20	72,401	24,969	70	15	9	23	Nested sewershed leading to E21	No	Concrete 1995
E21	55,436	309,998	61	21	11	332	WQTC	-	
E22	61,923	45,148	62	19	12	37	Nested sewershed leading to E21	No	Concrete 1994
E23	45,794	37,972	47	41	7	28	Nested sewershed leading to E21	No	Concrete 1978

E24	53,857	23,135	71	17	6	21	Nested sewershed leading to E21	No	Concrete 1979
E25	61,081	309,184	71	16	5	242	Nested sewershed leading to E27	Yes	Concrete 1958
E26	28,054	41,777	38	52	5	20	Nested sewershed leading to E27	Yes	Concrete 1912
E27	52,751	350,766	62	25	5	280	WQTC	-	
E28	20,000	74	11	80	4	3	Nested sewershed leading to E27	Yes	Concrete 1960

Note: E6 was a blank sample; E9/E12 was a duplicate

Sample Collection

Wastewater samples were collected from 27 locations in Louisville, Kentucky in late 2022. A grab sample was collected then divided into three 50 mL polypropylene tubes per site. Samples were transported on ice to the University of Louisville, where it was stored at -80ºC, and then transported to Emory University for analysis.

Sample Preparation

An Opentrons OT-2 liquid handling robot was purchased from Opentrons Inc. The automated liquid handler was used to efficiently prepare a large number of samples while minimizing potential errors. To a 96 well plate, 30uL of each sample was aliquoted, followed by 90uL of extraction solvent composed of LC-MS grade acetonitrile with 2.5uL of internal standard containing 13-C isotopically labelled compounds. The well plate was placed on top of a temperature module, which kept the samples at 4° C. After the extraction solvent was added, the well plate was sealed, vortexed at 4° C for 2 minutes at 700 rpm, equilibrated at 4° C for 30 minutes, then centrifuged at 4° C for 45 minutes at 4000 rpm. For HILIC/ normal phase analysis, 30ul of the supernatant diluted in 60uL 1:1 LCMS-grade acetonitrile/water. For C18/ reverse phase analysis, 30uL of the supernatant was diluted 60uL LCMS-grade water. Samples were placed in a freezer at -80° C until LC-HRMS analysis.

See Appendix for step-by-step detail on sample preparation.

Solid Phase Extraction

We next tested a modified sample preparation method using solid phase extraction to detect low level environmental chemicals in wastewater effluent. Methanol (MeOH, LC-MS grade), formic acid (FA), ammonium hydroxide (NH4OH), and deionized water (LC-MS grade)

were purchased from Fisher Chemical. Acetonitrile (ACN, LC-MS grade) was purchased from Thermo-Scientific.

OASIS HLB (6cc, 200mg sorbent per cartridge, 30um) and WAX (6cc, 150 mg sorbent per cartridge, 30um) cartridges were purchased from Waters. The OASIS HLB cartridge is considered the standard cartridge to use when running wastewater samples for SPE (Hajeb, Zhu et al. 2022). The OASIS WAX cartridge was also utilized in the extraction process to account for accurate PFAS concentrations. Each wastewater sample was thawed at 4° C, then was run on one OASIS WAX cartridge and one OASIS HLB cartridge. Once the SPE vacuum manifold was properly set up with the cartridges, all cartridges were conditioned with 10mL of MeOH followed by 10mL of deionized water. After conditioning, 10 mL of wastewater each was eluted through the cartridge to retain analytes and remove interferences, followed by 10 mL of wash solution (2% formic acid in water for the WAX cartridge, 5% methanol in water for the HLB cartridge). After the wash solution was discarded, 10 mL of the elution solvent (5% ammonium hydroxide in methanol for the WAX cartridge, 1:1 methanol acetonitrile for the HLB cartridge) was added to the cartridge, and the collected sample from the HLB cartridge and WAX cartridge were combined. Samples were evaporated in a BioTage TurboVap LP evaporator using high purity N_2 at a gas flow of 2.5 L per minute for one hour. Once fully evaporated, the sample was resuspended in 250uL of 1:1 water acetonitrile. Resuspended samples were placed in a freezer at -80° C for LC-HRMS analysis.

For a step-by-step details on the SPE method, see Appendix.

Liquid Chromatography with HRMS

LC-HRMS analysis is performed through two dual-channel liquid chromatography systems interfaced to Thermo-Scientific Exploris120 hybrid quadrupole-Orbitrap mass spectrometers. Untargeted analysis has been demonstrated on environmental chemicals in numerous publications (Walker, Uppal et al. 2016, Montone, Moneta et al. 2022). Both wastewater sample batches prepared using the Opentrons and SPE sample preparation methods were analyzed by LC-HRMS using dual-column chromatography incorporating a C18 column with positive and negative electrospray ionization (ESI) and hydrophilic (HILIC) interaction LC column with positive and negative ESI (Walker, Lane et al. 2019). This results in thousands of environmental chemicals detected within both Opentrons-ran and SPE-ran wastewater matrices. For the purposes of this study only C18 mode was used for comparison analysis between Opentrons sample processing and SPE.

Data Processing

Following analysis of all study and QA/QC samples, raw instrument files were converted to .mzXML and extracted using the two-stage hybrid feature detection and alignment procedure available in apLCMS using 5 different parameter settings optimized for a range of peak intensities. apLCMS is a specialized software tool designed for the processing and analysis of LC-HRMS data, and separates features whose *m/z* values overlap or are very close to each other through complex adaptive procedures (Yu, Park et al. 2009). The resulting feature tables were merged using xMsanalyzer. xMSanalyzer is a software framework that enhances the processing of exposomic data by improving feature detection, detect feature overlap between datasets, and characterize high-resolution *m/z* matches to exposures using multiple chemical databases (Uppal,

Soltow et al. 2013). Computational annotation was completed using xMSannotator using four databases. xMSannotator is a software tool designed for the annotation of exposomic data, identifying potential exposures by matching experimental data with known features (Uppal, Walker and Jones 2017). The Complete Norman Network chemical database, which includes environmental chemicals, commercial products, and predicted metabolites, was utilized for compound matching. xMSannotator only annotates the detected features, and there is still uncertainty to the chemical identification.

Data Analysis

Fold change factors (FCF) were calculated by measuring the ratio between the intensity, or concentration, of the detected analyte in a wastewater sample and the average intensity of the same analyte in the water blanks that was run alongside the wastewater sample. FCFs represent the change in intensity of a detected analyte, therefore a FCF less than five was considered not significantly increased in intensity, while a FCF greater than five is significantly increased and a reliably detected analyte in the sample. A FCF set at 1000 is an analyte that was detected in the wastewater sample, but not detected in the water blanks. Because C18 provides the best detection of environmental exposures, only data run on C18 mode was considered for comparison between the Opentrons and SPE sample preparation methods.

Results

SPE sample preparation method significantly increases the intensity of features compared to Opentrons sample preparation method at the 750-1000 bin.

There was a significant increase in detected features in wastewater samples run on SPE compared to wastewater samples run on Opentrons in the FCF of 750-1000 bin for both modes (Figure 4). In C18pos mode, the number of detected features is 10038 in the SPE-prepared wastewater samples (Figure 3A), compared to 2024 features detected in Opentrons-prepared wastewater samples (Figure 2A). In C18neg mode, the number of detected features is 7894 in the SPE wastewater samples (Figure 3B), compared to 1321 features detected in Opentrons wastewater samples (Figure 2B). There is also an increase in detected features in C18neg mode in the 5-10 FCF bin in SPE wastewater samples compared to Opentrons samples (Figure 4B), with 4357 features detected in SPE compared to 1495 features detected in Opentrons (Figures 3B and 2B).

There is a significant decrease in detected features in wastewater samples from SPE compared to wastewater samples from Opentrons in the FCF of 0-5 bin for C18pos mode (Figure 4A). 9417 features were detected in the Opentrons-run wastewater samples, compared to 5061 features detected in the SPE-run wastewater samples (Figures 2A and 3A). However, there is an increase in feature detection in wastewater samples run on SPE compared to wastewater samples run on Opentrons in the FCF of 0-5 bin for C18neg mode (Figure 4B).

SPE sample preparation method significantly increases the number of features detected at every site compared to the Opentrons sample preparation method.

There is a significant increase in the number of features detected with a FCF greater than 5 at every site for wastewater samples run on SPE compared to wastewater samples run on Opentrons (Figure 7). For Opentrons wastewater samples run on C18pos mode, the count of features with a FCF greater than 5 at each site do not surpass 3500 (Figure 5A). For Opentrons wastewater samples run on C18neg mode, the count of features with a FCF greater than 5 at each site do not surpass 3000 (Figure 5B). However, for SPE-run wastewater samples run on C18pos mode, the count of features with a FCF greater than 5 at each site surpasses at least 5000, with most sites surpassing 15000 detected features (Figure 6A). On C18neg mode, the count of features at each site surpasses at least 4800, with most sites surpassing 7500 detected features (Figure 6B). E06 did not see such significant increase like the rest of the sites because it is a blank sample.

No significant difference in feature count between sewershed areas leading to a WQTC and the WQTC itself.

Influent pipes for three water quality treatment centers (WQTC) were chosen and sampled for the study, which are E27, E21, and E15. E27 had 15 sewershed area sites leading up to it, E21 had 5 sewershed area sites leading up to it, and E15 had one sewershed area site leading up to it (Table 1). A similar number of features with a FCF greater than 5 were detected in both C18pos and C18neg from SPE-run wastewater samples between the sewershed nest sites leading to the WQTC, and the WQTC itself (Figures 8-10).

No association between feature count and the household income, population, and area of the wastewater sites.

There was no statistical significance when measuring the association between the number of features with a FCF greater than 5 detected in both C18pos and C18neg from SPE-run wastewater samples and the household income (Figure 11). The p-value for the association in C18pos mode was 0.8032 and the p-value for the association in C18neg mode was 0.52276, indicating no statistical significance between the count of feature detection and median household income of the wastewater sites. Median household income was measured in USD through the 2020 Median HH Income (ACS 5- Year).

There was no statistical significance when measuring the association between the number of features with a FCF greater than 5 detected in both C18pos and C18neg from SPE-run wastewater samples and the nearby population of the wastewater sites (Figure 12). The p-value for the association in C18pos mode was 0.18559 and the p-value for the association in C18neg mode was 0.89406, indicating no statistical significance between the count of feature detection and the nearby population count of wastewater sites.

There was also no statistical significance when measuring the association between the number of features with a FCF greater than 5 detected in both C18pos and C18neg from SPE-run wastewater samples and the area of the wastewater sites, measured in km^2 (Figure 13). The p-value for the association in C18pos mode was 0.15815 and the p-value for the association in C18neg mode was 0.45158, indicating no statistical significance between the count of feature detection and the area of the wastewater sites.

Identification of the detected features in C18pos mode with the greatest FCF in WQTC sites.

Using xMSannotator, the detected features have been annotated with a chemical name, and the identities of the chemicals with the five highest FCFs were written in Table 2 for every WQTC site. The identities are only annotations, and there is still some uncertainty on whether identification is correct. The highest chemical in Site E15 was 1,19-bis(oxiranyl)-8,16-bis(oxiranylmethoxy)-2,6,10,14,18-pentaoxanonadecane-4,12-diol, which is found on the 2017 List of REACH Chemicals, which lists substances predicted to meet criteria for carcinogenicity, mutagenicity, or reproductive toxicity, or with dispersive or diffuse use to be environmentally hazardous (National Center for Biotechnology Information 2024). The second highest chemical was Disodium 3-[(5-chloro-2-phenoxyphenyl)azo]-4-hydroxy-5-[[(p-tolyl)sulphonyl]amino]naphthalene-2,7-disulphonate, which is found to be toxic to aquatic life with long-lasting effects (National Center for Biotechnology Information 2024). The third highest chemical was 4-[3-(4-Chlorophenyl)-2,1-benzisoxazol-5-yl]pyrimidin-2-amine, or PIM-1 INHIBITOR 2, which is involved in cancer treatment (National Center for Biotechnology Information 2024). The fourth highest chemical was CI Direct Violet 51 disodium salt, a basic dye that is soluble in organic solvents, and is a known irritant (National Center for Biotechnology Information 2024). The fifth highest chemical was not found on the PubChem database.

In site E17, the top four highest chemicals were found in site E15. The last chemical, 8,13-diethyl-3,7,12,17-tetramethyl-21H23H-porphine-2-propionic acid, is a known irritant (National Center for Biotechnology Information 2024).

In site E19, the first three and fifth highest chemicals were found in site E15. The fourth highest chemical, 4-fluoro-N-(2-fluoro-3-4-(1112333-heptafluoropropan-2-yl)-26-dimethylphenylcarbamoylphenyl)-N-methyl-2-nitrobenzamide, is a known irritant (National Center for Biotechnology Information 2024).

In site E21, all chemicals were found in previous sites. In site E27, all chemicals were found in previous sites.

Figures

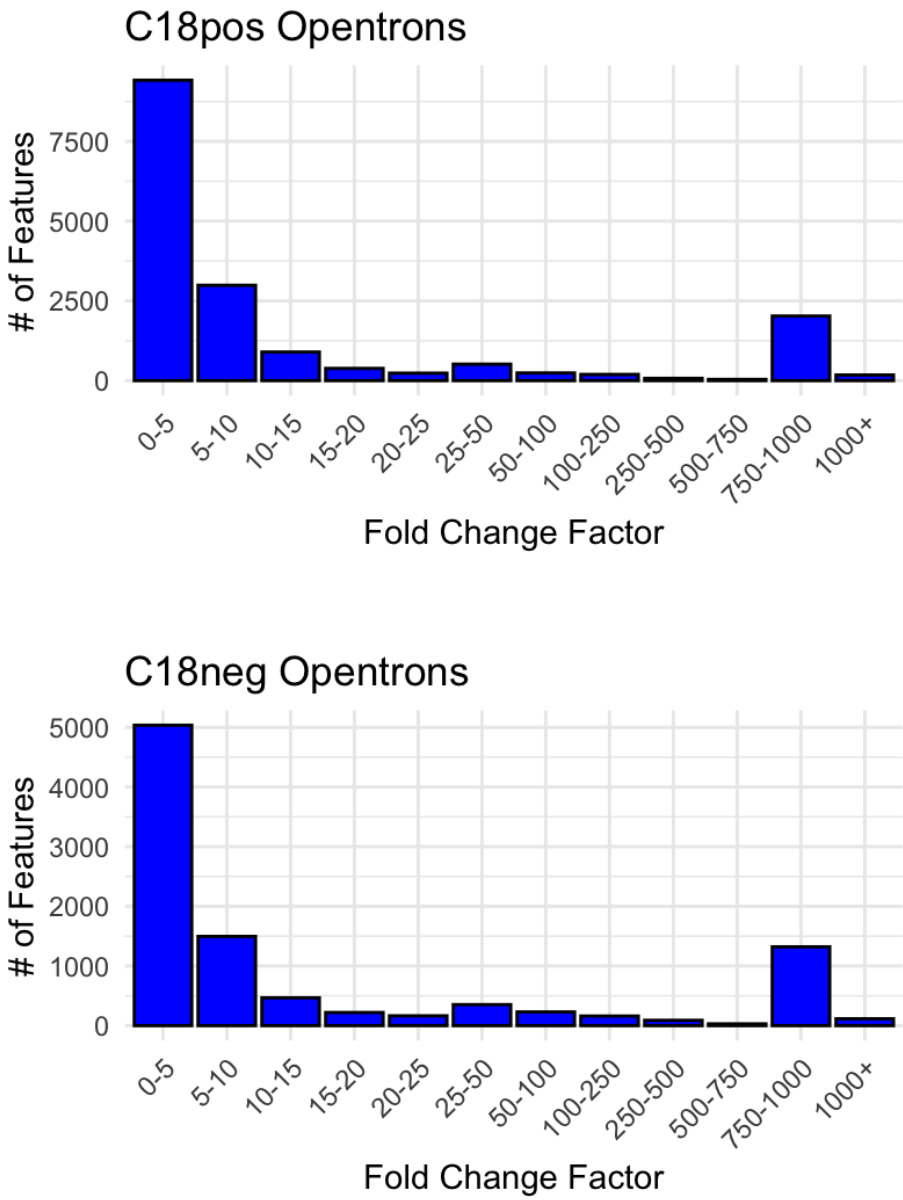

Figure 2. **Histograms displaying the count of features detected in Opentrons wastewater samples run on C18pos mode (A) and C18neg mode (B) at a certain fold change factor bin.**

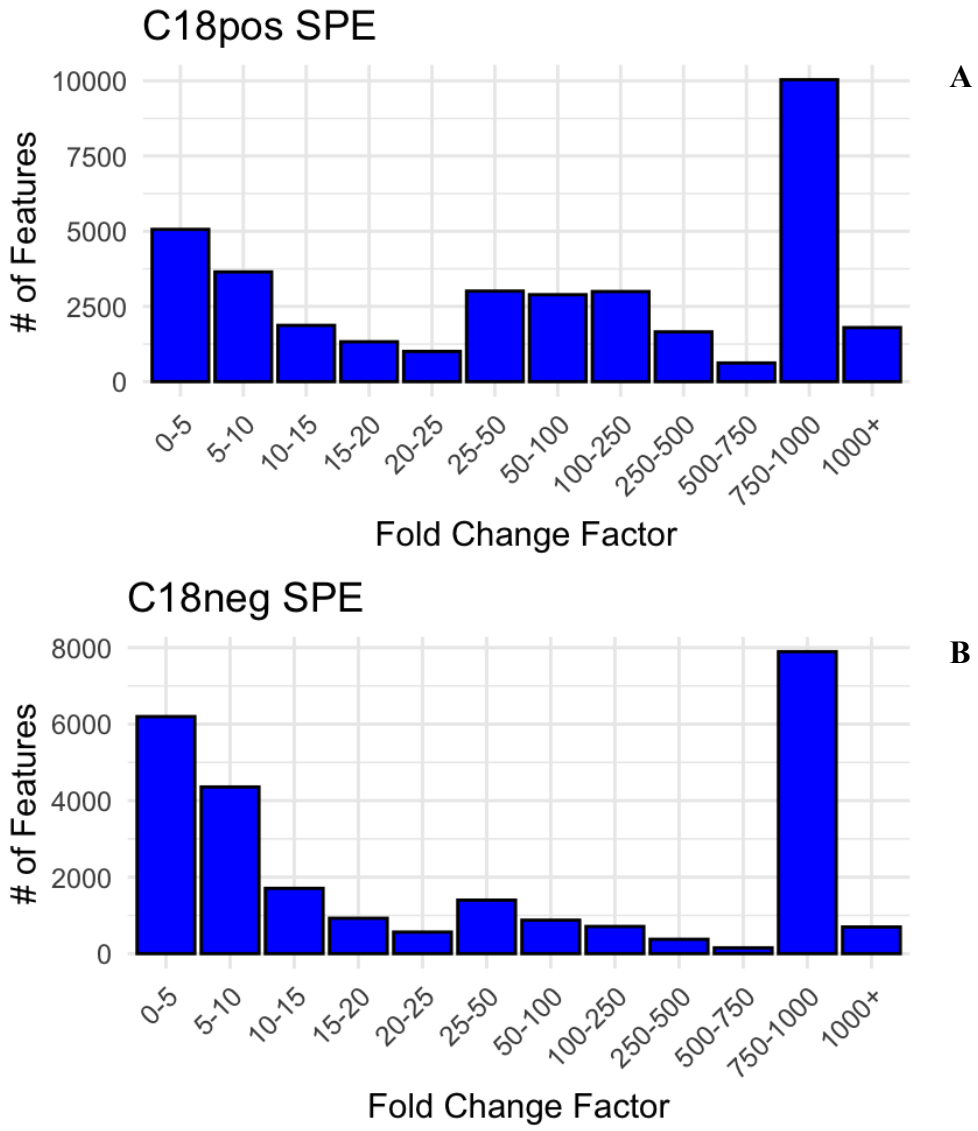

Figure 3. **Histograms displaying the count of features detected in SPE wastewater samples run on C18pos mode (A) and C18neg mode (B) at a certain fold change factor bin.**

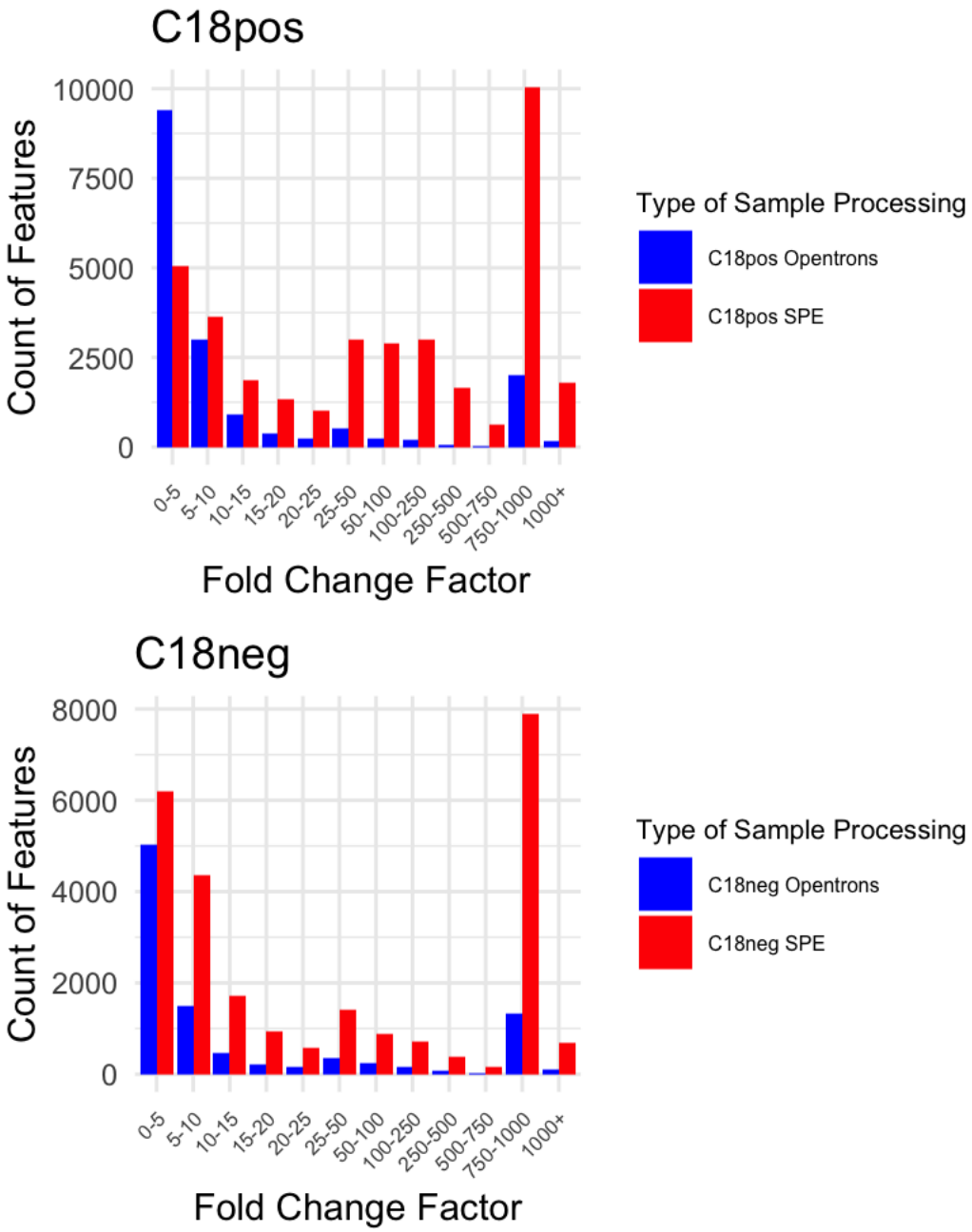

Figure 4. **Histograms showing the comparison of the count of features detected at a certain fold change factor bin between Opentrons and SPE sample preparation methods for C18pos mode (A) and C18neg mode (B)**

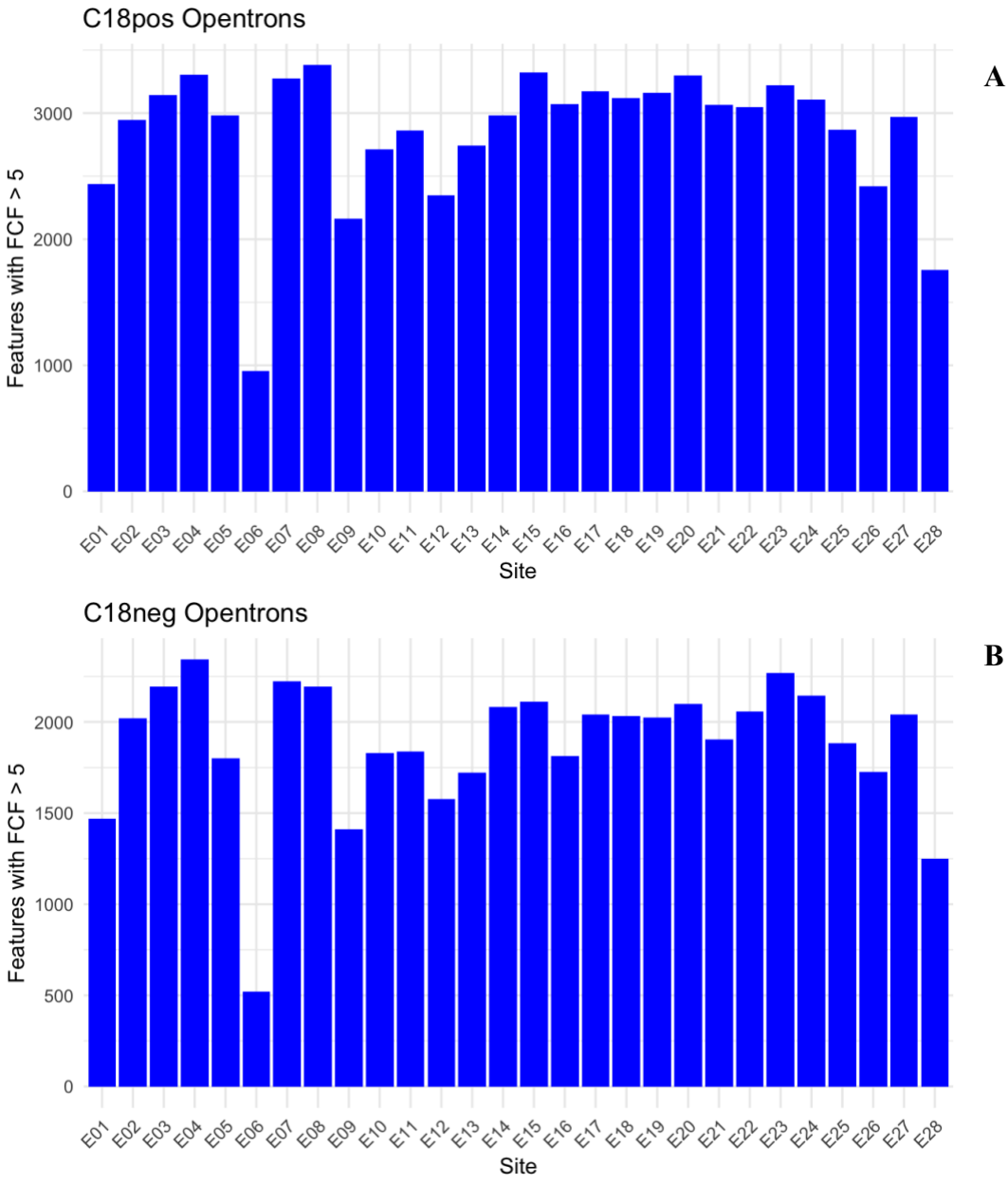

Figure 5. **Number of detected features in Opentrons wastewater samples at C18pos mode (A) and C18neg mode (B) with a FCF greater than 5 at each site.**

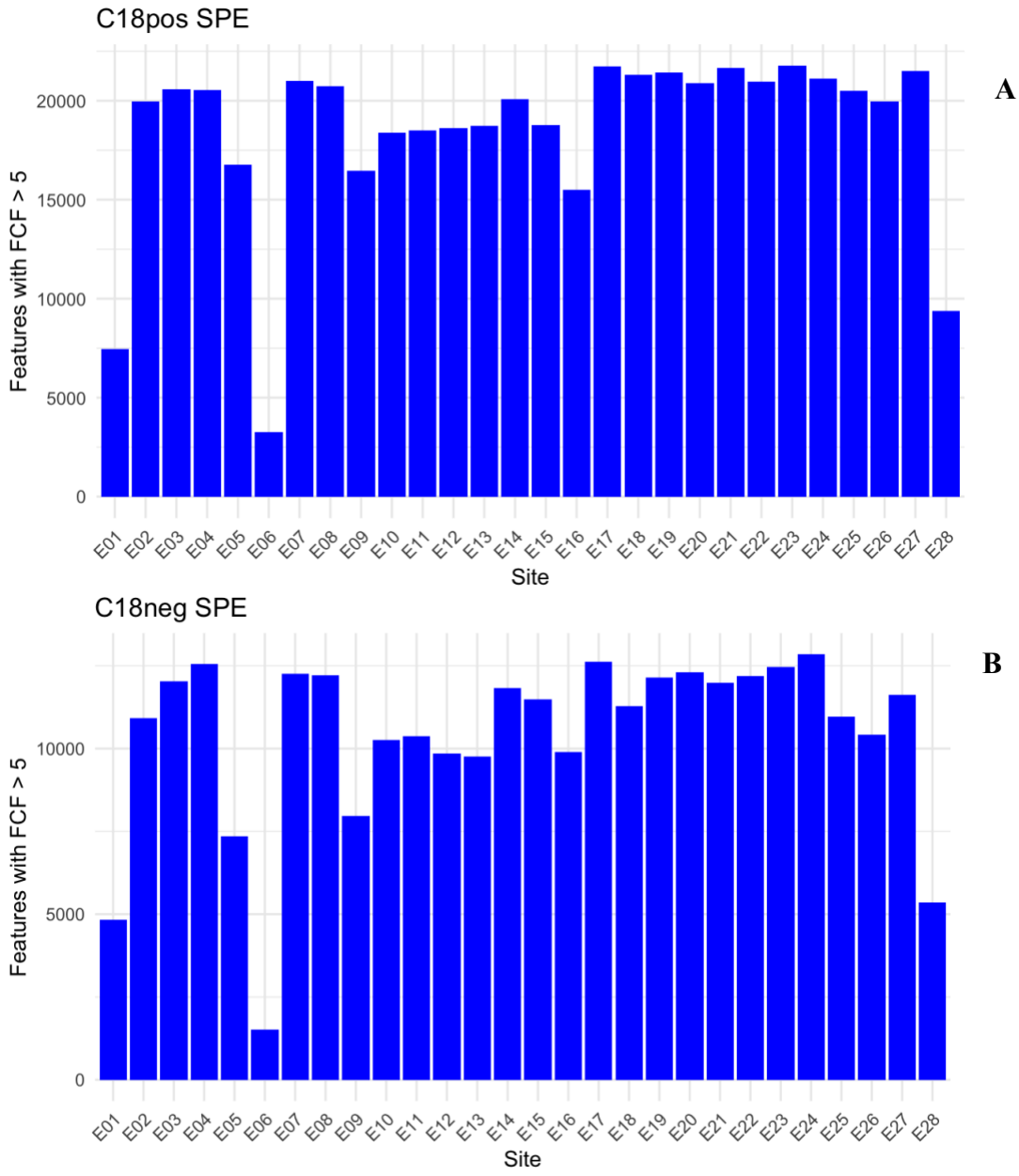

Figure 6. **Number of detected features in SPE wastewater samples at C18pos mode (A) and C18neg mode (B) with a FCF greater than 5 at each site.**

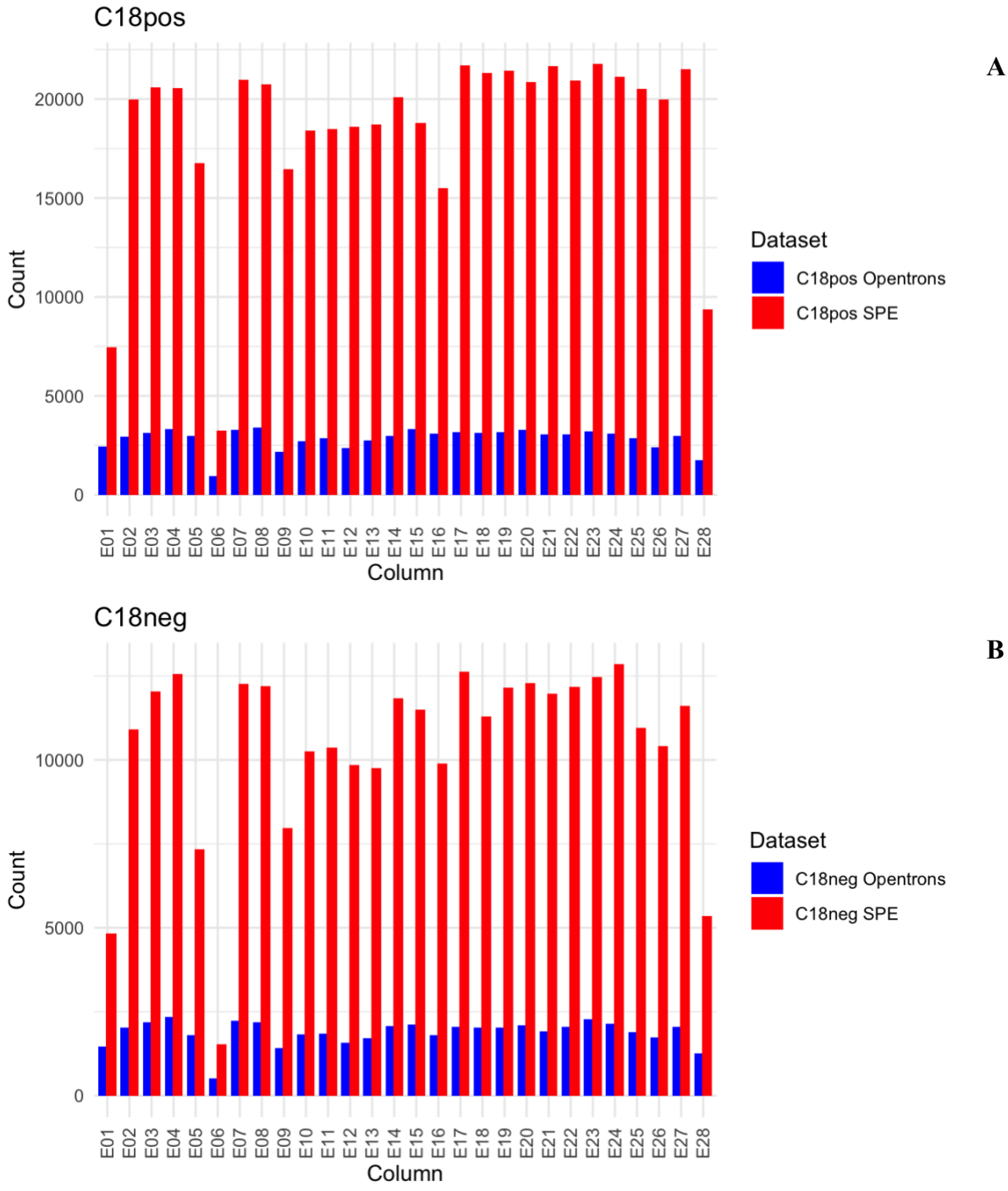

Figure 7. **Comparison of the number of detected features between Opentrons and SPE wastewater samples at C18pos mode (A) and C18neg mode (B) with a FCF greater than 5 at each site.**

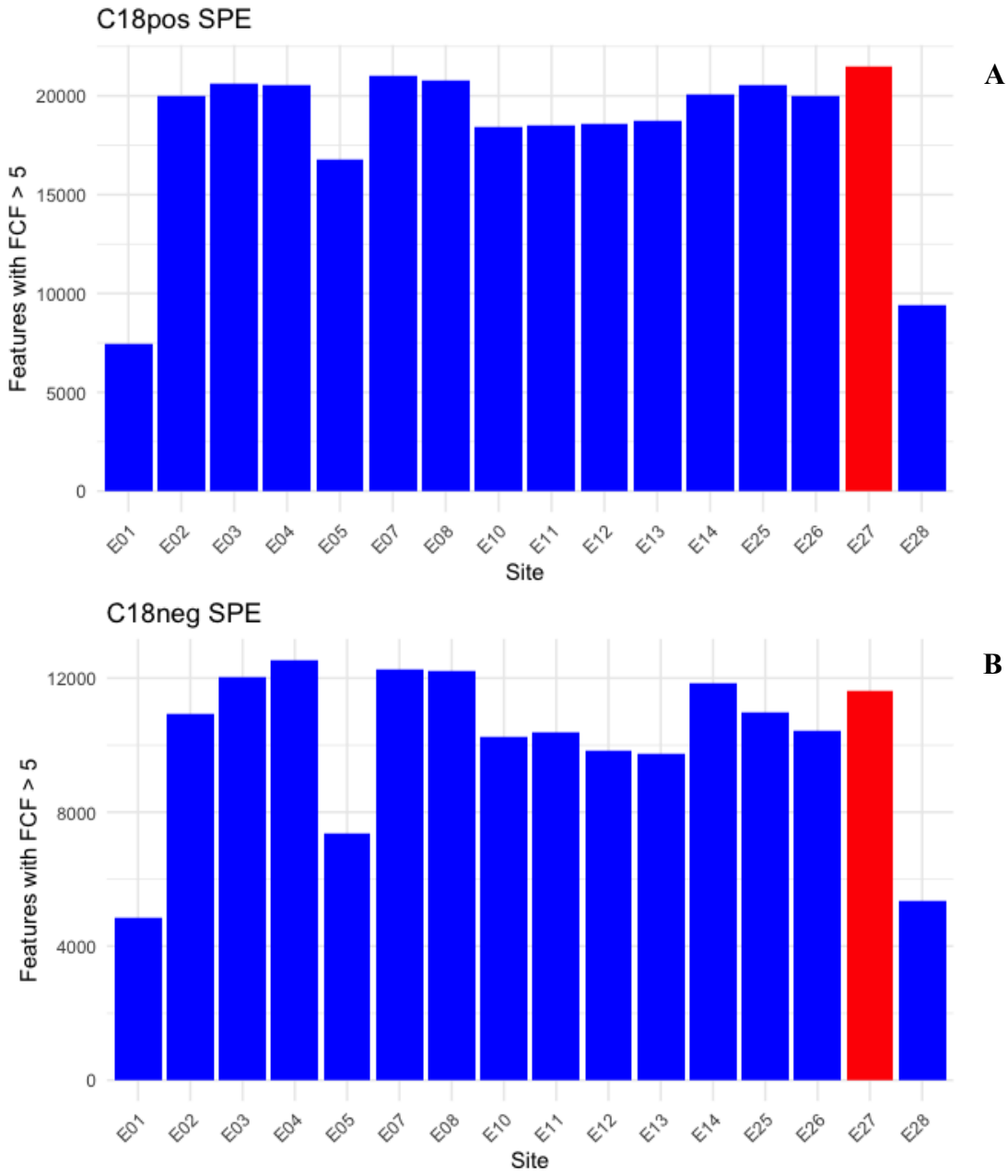

Figure 8. **Comparison of detected features with a FCF greater than 5 in SPE wastewater samples run on C18pos (A) and C18neg (B) mode between nested sewersheds leading to E27, a water quality treatment center (WQTC), and E27.**

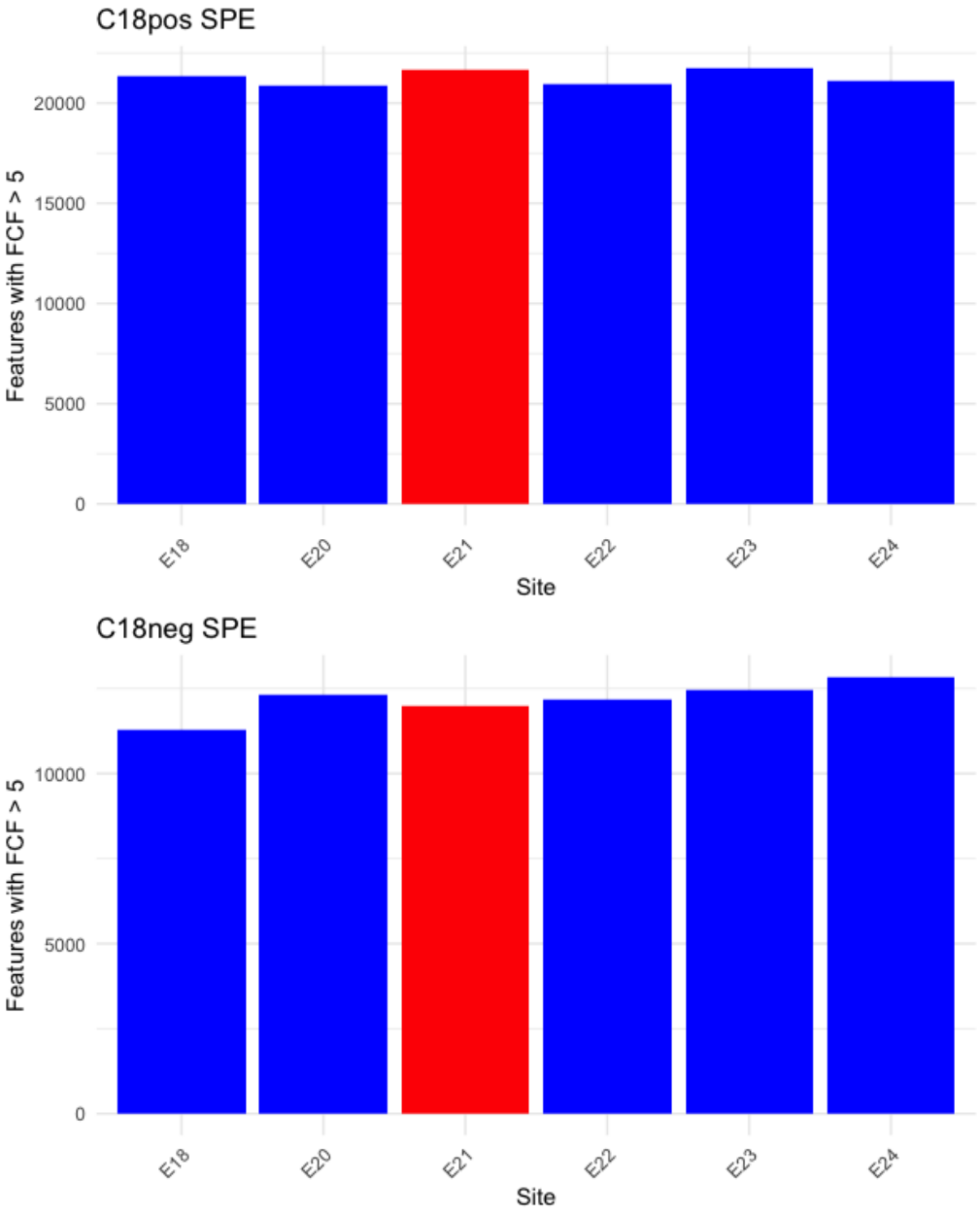

Figure 9. **Comparison of detected features with a FCF greater than 5 in SPE wastewater samples run on C18pos (A) and C18neg (B) mode between nested sewersheds leading to E21, a WQTC, and E21.**

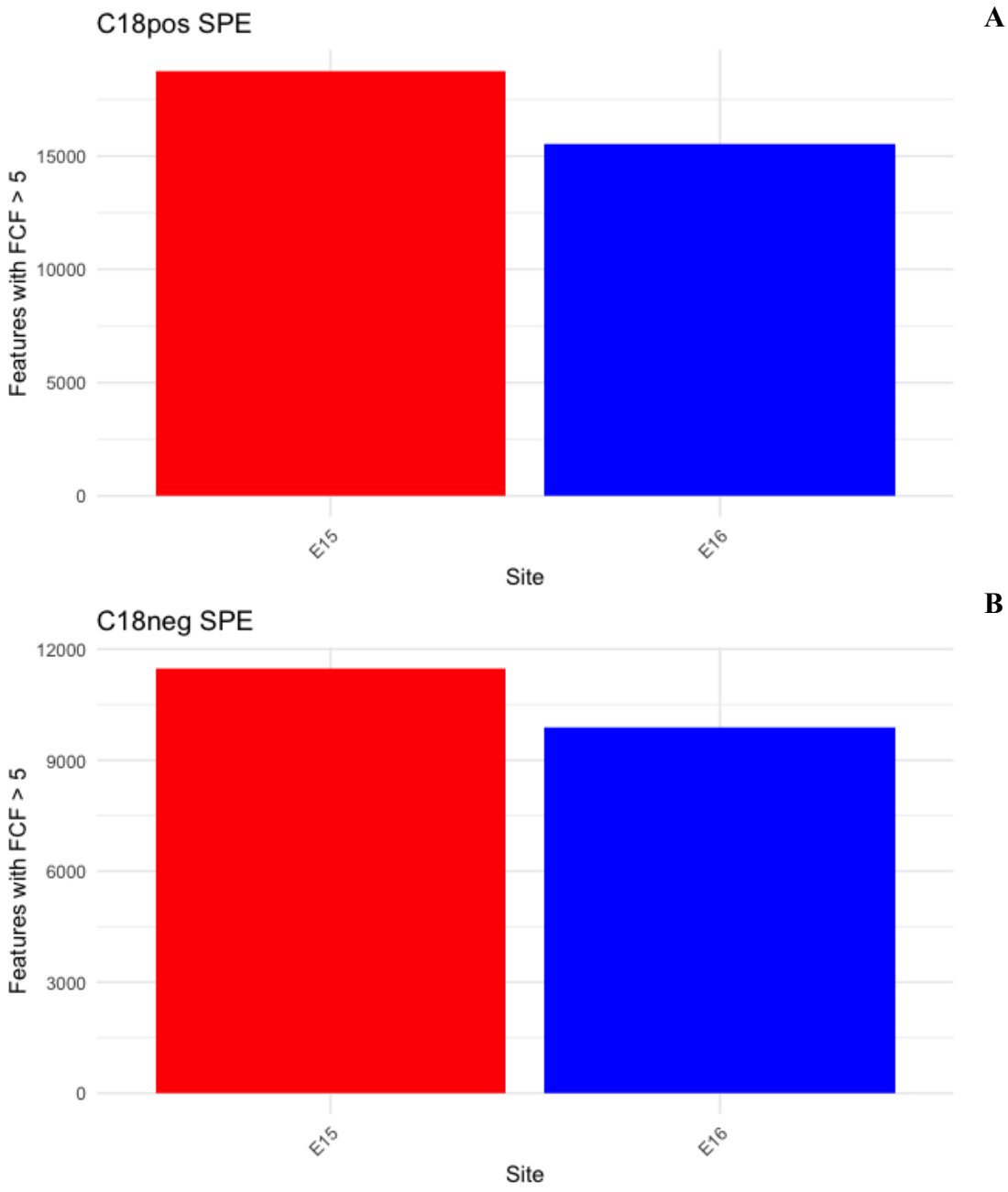

Figure 10. **Comparison of detected features with a FCF greater than 5 in SPE wastewater samples run on C18pos (A) and C18neg (B) mode between nested sewersheds leading to E15, a WQTC, and E15.**

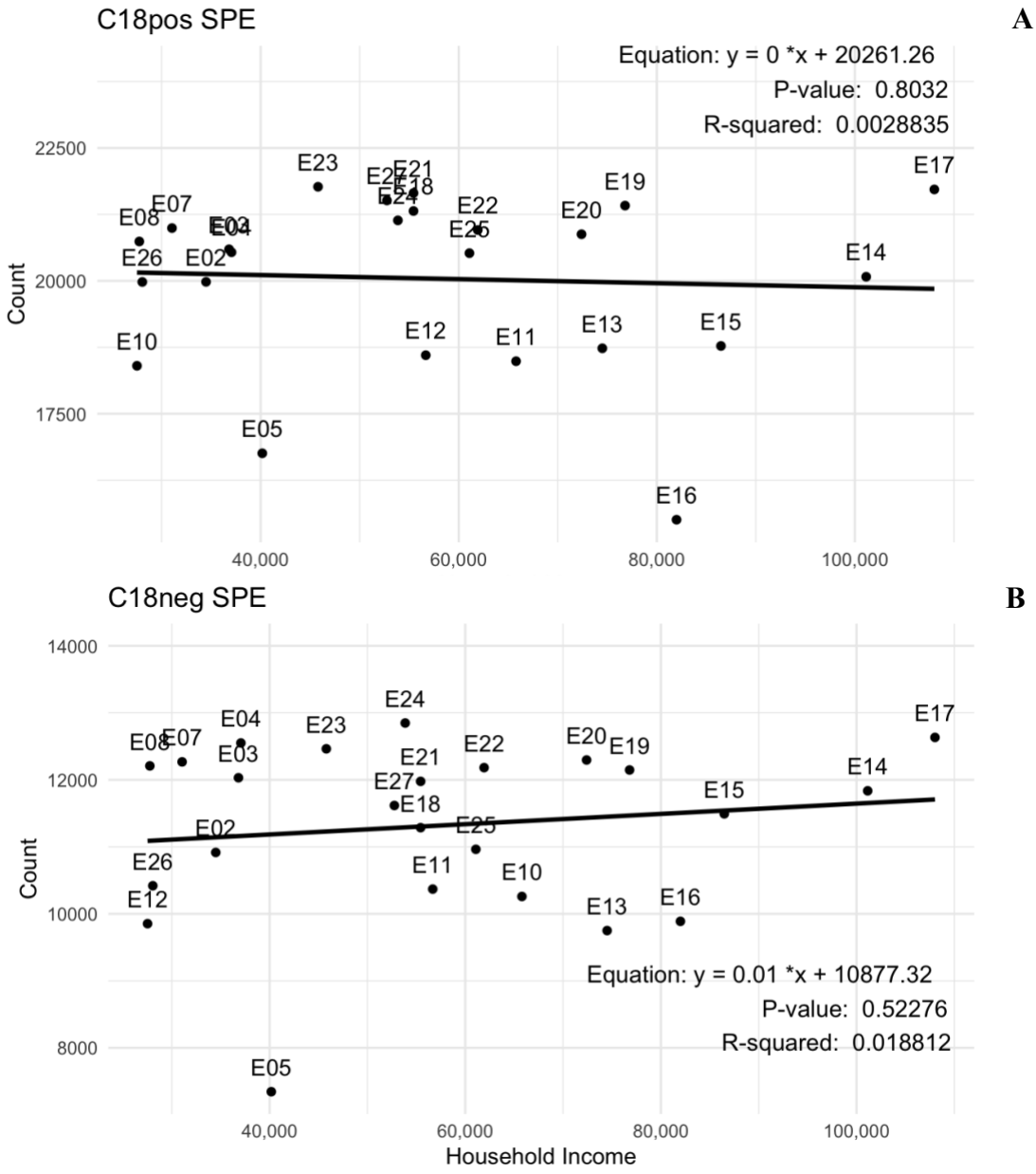

Figure 11. **Line graph showing the association between the count of features for SPE wastewater samples run at C18pos (A) or C18neg (B) with a FCF greater than 5 at each site's respective median household income in USD based on the 2020 Median HH Income (ACS 5-Yr).**

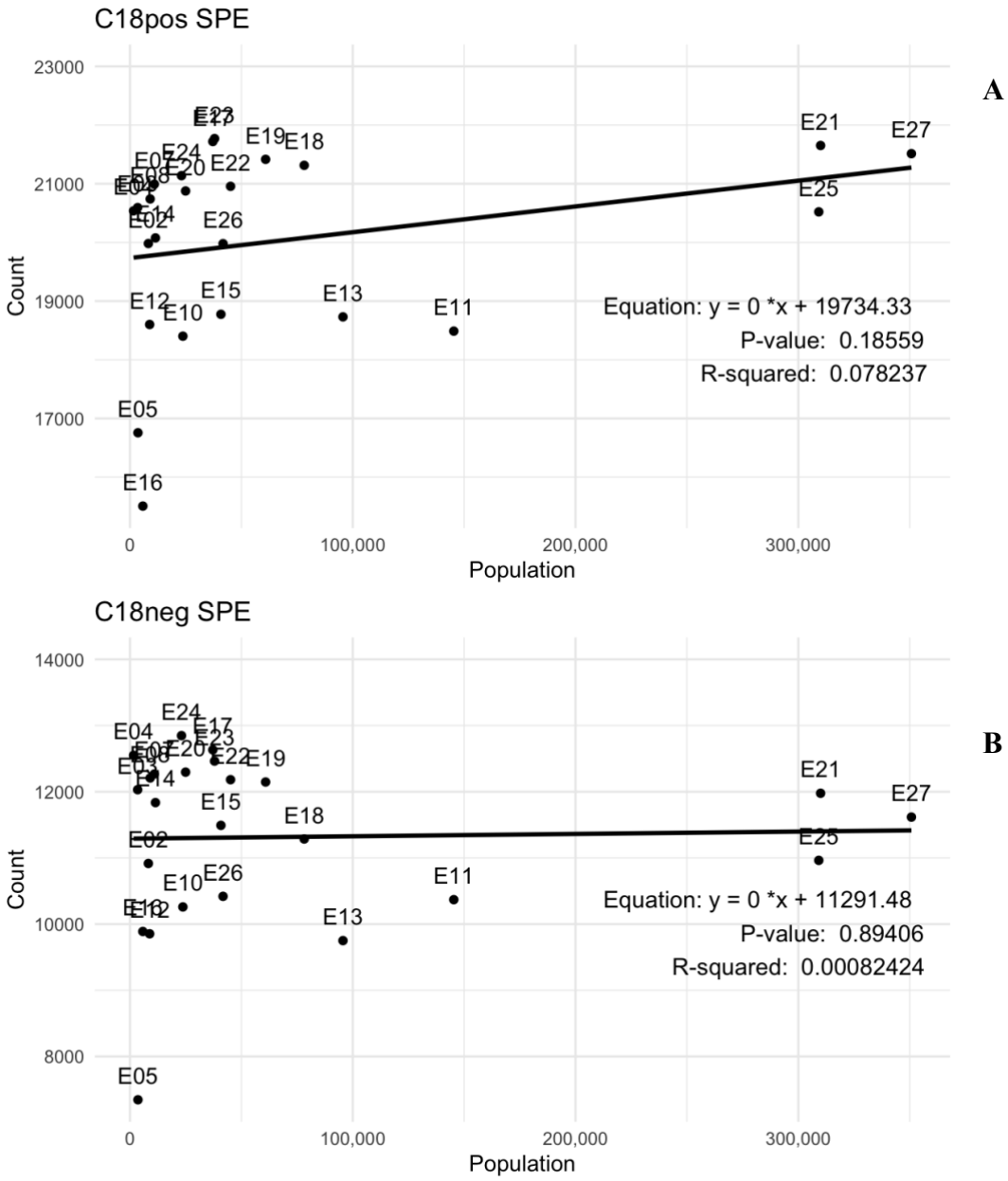

Figure 12. **Line graph showing the association between the count of features for SPE wastewater samples run at C18pos (A) or C18neg (B) with a FCF greater than 5 at each site's respective population.**

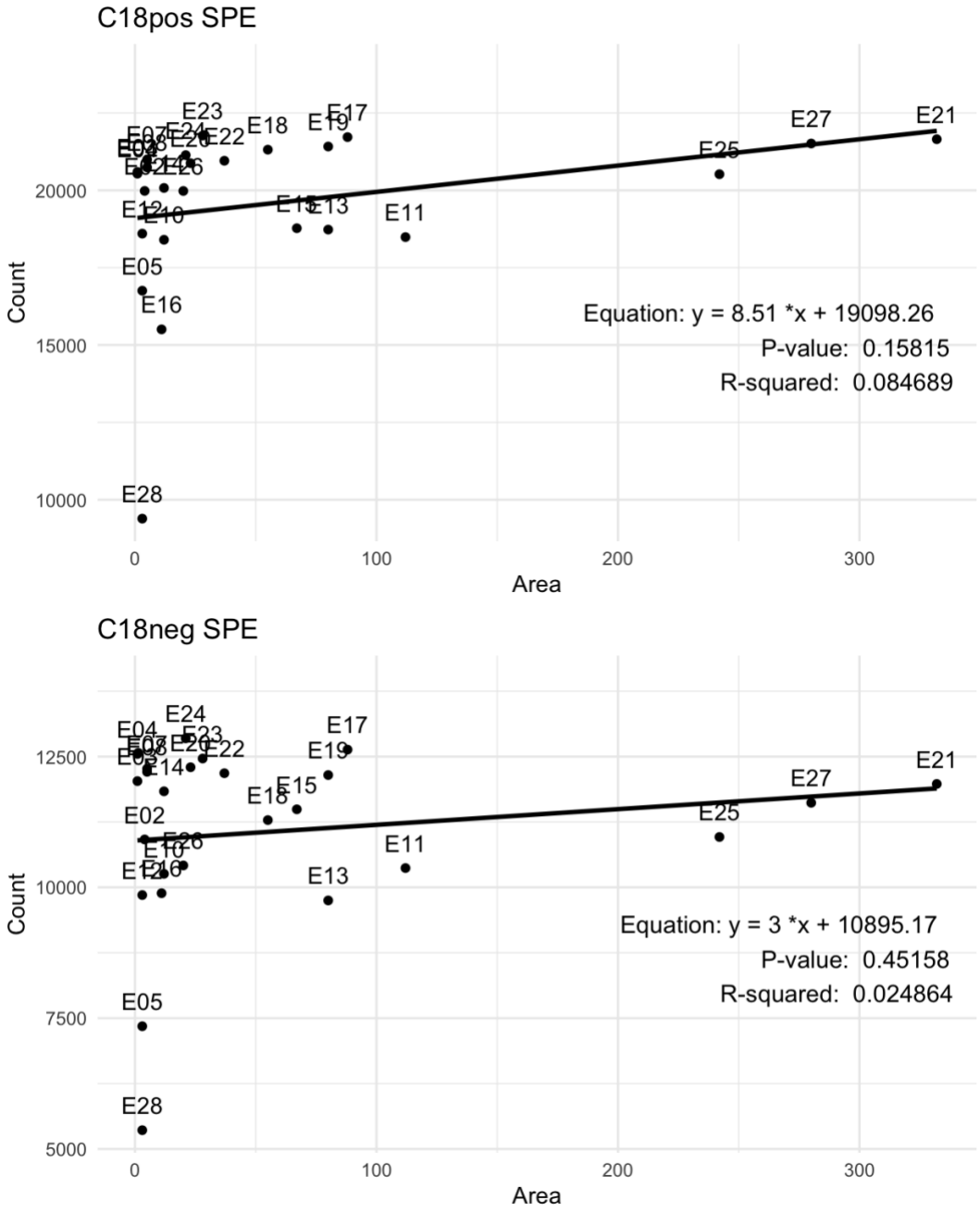

Figure 13. **Line graph showing the association between the count of features for SPE wastewater samples run at C18pos (A) or C18neg (B) with a FCF greater than 5 at each site's respective area in km^2.**

Table 2. **Identified exposures with the five highest FCF in SPE wastewater samples at WQTC sites in C18pos mode**

E15

Chemical Identity	Fold Change
119-bis(oxiranyl)-816-bis(oxiranylmethoxy)-26101418-pentaoxanonadecane-412-diol	103614.6177
Disodium 3-5-chloro-2-(phenylmethoxy)phenylazo-4-hydroxy-5-(p-tolyl)sulphonylaminonaphthalene-27-disulphonate	60831.35773
PIM-1 INHIBITOR 2	59048.52147
CI Direct Violet 51 disodium salt	51016.29912
369121518212427-nonaoxaoctatriacontan-1-ol	48378.59531

E17

Chemical Identity	Fold Change
CI Direct Violet 51 disodium salt	146566.7058
119-bis(oxiranyl)-816-bis(oxiranylmethoxy)-26101418-pentaoxanonadecane-412-diol	145395.0738
PIM-1 INHIBITOR 2	120046.9926
Disodium 3-5-chloro-2-(phenylmethoxy)phenylazo-4-hydroxy-5-(p-tolyl)sulphonylaminonaphthalene-27-disulphonate	76805.69616
813-diethyl-371217-tetramethyl-21H23H-porphine-2-propionic acid	71331.8097

E19

Chemical Identity	Fold Change
119-bis(oxiranyl)-816-bis(oxiranylmethoxy)-26101418-pentaoxanonadecane-412-diol	114016.4422
PIM-1 INHIBITOR 2	57968.06486
813-diethyl-371217-tetramethyl-21H23H-porphine-2-propionic acid	46793.27488
4-Fluoro-N-(2-fluoro-3-4-(1112333-heptafluoropropan-2-yl)-26-dimethylphenylcarbamoylphenyl)-N-methyl-2-nitrobenzamide	36015.21378
Disodium 3-5-chloro-2-(phenylmethoxy)phenylazo-4-hydroxy-5-(p-tolyl)sulphonylaminonaphthalene-27-disulphonate	33850.43094

E21

Chemical Identity	Fold Change
119-bis(oxiranyl)-816-bis(oxiranylmethoxy)-26101418-pentaoxanonadecane-412-diol	88450.97741
CI Direct Violet 51 disodium salt; 7-anilino-3-4-(24-dimethyl-6-sulphophenyl)azo-6-methoxy-m-tolylazo-4-hydroxynaphthalene-2-sulphonic acid	73518.07527
PIM-1 INHIBITOR 2	50000.40538
4-Fluoro-N-(2-fluoro-3-4-(1112333-heptafluoropropan-2-yl)-26-dimethylphenylcarbamoylphenyl)-N-methyl-2-nitrobenzamide	37310.83432
Disodium 3-5-chloro-2-(phenylmethoxy)phenylazo-4-hydroxy-5-(p-tolyl)sulphonylaminonaphthalene-27-disulphonate	33373.36722

E27

Chemical Identity	Fold Change
119-bis(oxiranyl)-816-bis(oxiranylmethoxy)-26101418-pentaoxanonadecane-412-diol	29519.66985
CI Direct Violet 51 disodium salt	24948.54839
813-diethyl-371217-tetramethyl-21H23H-porphine-2-propionic acid	20781.55473
4-Fluoro-N-(2-fluoro-3-4-(1112333-heptafluoropropan-2-yl)-26-dimethylphenylcarbamoylphenyl)-N-methyl-2-nitrobenzamide	20339.51603
PIM-1 INHIBITOR 2	17101.22817

Discussion

The solid phase extraction method was successful in increasing the intensity of detected features on both C18pos and C18neg modes compared to traditional sample preparation methods. Figures 2-4 display the increase in intensity by displaying the larger amount of FCF that are greater than 5, and the significant increase in the number of features with FCFs ranging between 750 to 1000. This increase could also indicate many new features identified that couldn't be identified before with traditional sample preparation methods for untargeted exposome analysis. Because the FCF of 1000 indicates a new feature detected that was not detected in the water blanks, a significant increase in FCF of 1000 shows early promise that many chemicals that weren't identified before could now be identified with the performed SPE sample preparation method. Figure 7 also shows a massive increase in the number of detected features with a FCF greater than five in wastewater samples prepared with SPE compared to wastewater samples prepared with traditional sample methods. All sites have shown increases in detected features, giving solid phase extraction methods a promising outlook on being utilized in future wastewater-based epidemiology studies.

The number of detected features with FCF greater than 5 in E27, a WQTC, compared to the sewershed nested areas that lead to E27 do not have a significant difference in both C18pos and C18neg modes. The same trend is seen with E21 and E15, the other two WQTCs with sewershed area sites leading to them. This indicates that exposures measured in sewershed nested area sites can also be detected in combined flows into a corresponding WQTC.

Despite no association detected between the number of detected features with FCF greater than 5 and household income, population, and area of the sites, WBE was explored to

tackle environmental health policy issues about the dangers of residing near industrial plants. This study serves as a stepping stone into the potential of WBE in determining how identification of chemicals in wastewater could serve to push future agendas in banning such chemicals from industrial usage. Wastewater is a key component of health equity discussions, and has potential to be utilized in monitoring vulnerable and underserved communities by facilitating inclusion and prompt action to public health threats (Holm, Osborne Jelks et al. 2023). Wastewater may offer a new environmental matrix to view how vulnerable residents are to environmental exposures and prioritize governmental regulations in monitoring such exposures. Finding no association in this study suggest the extent of exposures may be the same; however, since all individual exposures were not evaluated, it is not possible to determine if the exposures themselves are the same.

The annotations of detected chemicals in Table 2 showed that LC-HRMS analysis of wastewater can be useful in obtaining information on the risk of unknown exposures in human populations. This study has shown the potential and utility of untargeted analysis to identify chemicals that were unknown to human knowledge, potentially gearing for future studies to examine such chemicals and their potential hazard to humans and the environment.

Limitations

Chromatograms of the wastewater samples prepared through SPE has shown protein contamination. The water blanks that were run with the wastewater samples did not have protein contamination, so SPE did not introduce contamination to the samples. Analysis was continued with the assumption that protein contamination will not skew results, but revising the SPE method to include protein removal should be considered. Annotations for detected features at

C18neg mode is still in progress for xMSannotator analysis. The collected wastewater samples were collected as grab samples, which is a good representation of a county at a specific point in time, but a study analyzing wastewater composite samples across a county would be a more representative study and account for potential variability in wastewater characteristics and increase sample validity.

Next Steps

A study assessing the chemical exposome of residents utilizing wastewater composite samples should be conducted to account for the potential variability in wastewater composition and increase the validity of the sample. Although comparison of 24-hour composite and grab samples for exposure investigations of COVID-19 genetic material in wastewater has been conducted (Kmush, Monk et al. 2022), a comparison study of composite and grab samples should be conducted to investigate how wastewater characteristics may vary. Further research should be done investigating why and how the variability in feature detection with FCF greater than 5 is minimal between the WQTC sites and sewershed nested sites leading to the WQTC. Greater research should be conducted in analyzing highly concentrated chemicals in wastewater to better understand its environmental impact and its impact on humans. Lastly, a comparison study of the annotated chemical exposome profiles between wastewater samples and biofluid samples of a representative population should be conducted to analyze validity of wastewater as a potential proxy in determining the human chemical exposome.

Conclusion

Solid phase extraction has shown to enrich analytes of concern in wastewater samples and could be utilized in future WBE studies to detect more exposures that were previously undetected through traditional sample preparation methods. Despite no association was seen between the number of detected features and household income, population, and area, WBE has demonstrated its potential in investigating problems of environmental injustice. Utilization of untargeted analysis of exposures and annotation of detected features has shown to detect potential harmful exposures, which could push future agendas in investigating such exposures and banning such chemicals from industrial usage.

Appendix

Opentrons Method

*Adapted from the Standard Operating Procedure Automated Sample Prep Document

Sample Aliquoting

Overview: This is the first step of sample preparation. This can be performed for just LC, just GC, or for both LC and GC at once. The LC plate receives 30uL of sample, while the GC plate receives 100uL of sample. The LC only and GC only aliquoting protocols both include the solvent addition step. The combined LC+GC protocol includes solvent addition only for the LC plate.

Materials:

Protocol Name	Manufacturer	Item	Item number
Plate	Thermo Scientific	1mL Plate+ well plates	60180-P343
Heat Seal	Thermo Scientific	Easy Pierce 20um Foil	AB-1720
Reservoir	Opentrons	NEST 12-Well Reservoir, 15mL	999-00076
Microfuge tube	USA Scientific	1.5mL Microcentrifuge Tubes	1415-2500
15mL centrifuge tube	Corning	Falcon 15 mL PP Conical Tube	352097
Acetonitrile*	Fisher	"PFAS-free" UHPLC-MS Acetonitrile	A956-1
Water	Fisher	Optima Grade LC-MS Water	W64
Formic Acid**	Fisher	Optima LC-MS Formic Acid	117-50
4:1 Hexane/ Ethyl Acetate **	Fisher	Optima Grade Hexane and Ethyl Acetate	H306-1 and E19462
30 mL tube**	Fisher	30mL Borosilicate Glass Tubes with Threaded ends	1495776E
Cap**	Fisher	Black Phenolic caps for 30mL glass tubes	1495782E

**For LC plate, only needed for LC+GC and LC only aliquoting protocols
**For GC plate, only needed for GC only aliquoting protocol.

Notes: The 30mL tube & cap may be reused for extraction solvent preparation. Rinse before and after used with hexanes and ethyl acetate. Each reservoir may also be reused 1x. After first used, cover loosely in foil to allow the used side to evaporate and the unused side to stay clean. Store on Opentron deck. Flip the reservoir around to reuse the opposite side.

General procedure outline:

1. **Thaw samples (20-30min):** use StationOne in cold room (4°C).
2. **While samples are thawing:**
   a. Print hard copy of plate loading order.
   b. Turn on Opentron and computer.
   c. Make sure temperature module(s), centrifuge, and thermomixers are set to 4C
   d. Turn on the heat sealer and start preheating to 165C
   e. Prepare Opentrons protocol:
      i. Update CSV: Add sample IDs, Delete extra rows, Edit the “Source pick up height from top”
      ii. Save edited protocol to study folder: R:\diwalke\Run Lists
   f. Prepare any extraction solvents needed:
      i. LC: make fresh each batch in 15ml Falcone tube; vortex + store at 4C while aliquoting.

1. 10.8mL LC-MS Acetonitrile
2. 300uL Emory LC Internal Standard
3. 180uL PFAS Internal Standard (for PFAS studies only!)

ii. GC (ONLY for GC only protocol!): prepare in 30mL tube with cap

1. 20mL Hexane
2. 5 mL Ethyl Acetate
3. 2.5mL GC internal standard (stored in cold room at 4°C)

g. Perform labware position check
h. Make sure Opentron trashbin is emptied and workspace is clean
i. Prepare tube of LCMS water as water blank.
j. Fill out Sample Prep Worksheet.

3. **Prepare samples for run:** vortex & position in racks/blocks. Double check ID positions.
4. **Run protocol in Opentrons (1hr):** after aliquoting, protocol will pause for solvent addition. Add solvent to Nest Reservoir and resume protocol.
   a. For LC (LC and LC+GC aliquoting protocols): add to first column of reservoir
   b. For GC (GC only aliquoting protocol):
      i. Add 2.5mL formic acid to the first column of reservoir.
      ii. *Protocol pause*
      iii. Add 13.5 mL extraction solvent to columns 2 and 3 of reservoir
5. **Aliquoted plates:**
   a. **GC:**
      i. If prepared using GC+LC aliquoting protocol: proceed to **GC Solvent Addition**.
      ii. If prepare using GC only aliquoting protocol:
         1. Heat seal
         2. Vortex: 1100rpm/ 4C /1 hour.
         3. Centrifuge: 4000rpm/ 4C/ 10 minutes.
         4. Use plate dispenser to add 30ul of sorbent into a clean plate.
         5. Proceed to GC Supernatant Transfer #1
   b. **LC**
      i. Heat seal
      ii. Vortex: 4°C/2 min/700rpm
      iii. Equilibrate: 4°C/ 30 min/ 0rpm
      iv. Centrifuge: 4°C/15 minutes/4000rpm
      v. Continue to **LC Supernatant Transfer.**

LC Supernatant Transfer

Materials

Protocol Name	Manufacturer	Description	Part number
Plate	Thermo Scientific	96 Well Plate, Coated PP, Round, U bottom, 7mm dia, 1.0mL	60180-P343
Clear silicone webseal mat*	Thermo Scientific	Micromat Clr, 96RD Flat, 7mm, Clear Silicone, non-slit	60180-M149
Blue PFTE webseal mat*	Thermo		60180-M115
Reservoir	Opentrons	NEST 12-Well Reservoir, 15mL	999-00076
Acetonitrile	Fisher	Optima grade LCMS Acetonitrile	A9554
Water	Fisher	Optima grade LCMS Water	W64

*****For prepared C18 plate
**For prepared HILIC plate
Notes: The reservoir used in the aliquoting step may be reused for this step—simply flip the reservoir around to reuse the opposite side. Discard after both sides have been used.

Procedure

1. Upload the most up to date LC Supernatant Transfer protocol in the Opentrons app.
2. Set up the deck as specified in the protocol (see "Labware Setup").
3. Label the C18 and HILIC wellplates
4. Run labware position check.
5. Add solvents to the reservoir:
    a. **Column 1:** 10mL LCMS water
    b. **Column 2:** 10mL 50:50 LCMS water/acetonitrile
6. Click "Start Run."
7. After transfers are complete, cap C18 plate with a silicone webseal mat and the HILIC plate with a blue PFTE webseal mat.
8. Vortex: 2 minutes/ 700 rpm/ 4°C.
9. Return remaining unused samples to storage in the appropriate freezer location.

Solid Phase Extraction Protocol for Wastewater

Solvent Preparation (for one sample)

- 2 tubes of methanol, 10 mL each (conditioning solvent)
- 2 tubes of DI water, 10 mL each (equilibration solvent)
- Wastewater samples, 5 mL each
- 10 mL of 2% formic acid in water (wash solvent WAX)
- 10 mL of 5% ammonium hydroxide in methanol (elution solvent WAX)
- 10 mL of 5% methanol in water (wash solvent HLB)
- 10 mL of 1:1 methanol acetonitrile (elution solvent HLB)

Vacuum Manifold Preparation

1. Insert a tube from the vacuum source into the vacuum port. Make sure the vacuum gauge is turned tightly to the right so that the vacuum port is completely closed and the barometer reads 0 inHg.
2. Attach a stainless steel liner guide needle onto the ceiling of the white top. Make sure the needle is attached to the same attachment point as the cartridge.
3. Attach a Disposable Liner (for Visiprep) onto an Oasis WAX and HLB cartridge tips and insert the cartridge onto the cartridge attachment point of the vacuum manifold.
    a. Place the Oasis WAX cartridges on one row, and the HLB cartridges on the other row. Each row should have 12 vacuum manifolds.
    b. Make sure the cartridges are positioned correctly so the sample will flow through in the desired direction.
    c. Make sure the manifold valves are all configured to block the flow of any fluids.
4. Place collection tubes to collect the methanol (conditioning solvent) when conditioning the cartridge and the deionized water for equilibrating.

Oasis WAX – for PFAS and acids

5. Condition the WAX cartridge of methanol. Use the correct amount of methanol depending on the size of the cartridge.
    a. Set the vacuum to 5 inHg. Do not go past 20 inHg.
    b. For a 6 cc (6 mL) WAX cartridge, use 10 mL of methanol.
    c. Insert 5 mL of methanol into the cartridge, then twist the manifold valves loose enough that the eluent is coming out of the cartridges in drops.
    d. Repeat 1 more time, for a total of 10 mL.
    e. Release vacuum, remove collection tubes, discard the solvent, and place the collection tubes back for equilibration.
    f. Double check that the manifold valves are closed tightly before moving onto next step.
6. Equilibrate the WAX cartridges with deionized water. Use the correct amount of water depending on the size of the cartridge.

   a. Set the vacuum to 5 inHg. Do not go past 20 inHg.
   b. For a 6 cc (6 mL) WAX cartridge, use about 10 mL of water.
   c. Insert 5 mL of water into the cartridge, then twist the manifold valves loose enough that the eluent is coming out of the cartridges in drops.
   d. Repeat 1 more time, for a total of 10 mL.
   e. Release vacuum, remove collection tubes, discard the solvent, and place new collection tubes for wastewater sample collection to use for HLB.
   f. Double check that the manifold valves are closed tightly before moving onto next step.
7. Load 5mL wastewater sample onto the WAX cartridge slowly and evenly to prevent channeling. The sample should flow through the cartridge under the vacuum so the sorbent retains the target compounds.
   a. Switch on or open valve at the lowest possible vacuum and gradually increase as needed to load the entire sample onto the sorbent bed.
   b. Load the full 5 mL of wastewater sample.
   c. Accomplish a slow sample flow rate to ensure that wanted compounds are embedded in the cartridge. Twist the manifold valves loose enough that the eluent is coming out of the cartridges in drops.
8. Once the entire sample has gone through the WAX cartridge, release the vacuum.
   a. Double check that the manifold valves are closed tightly before moving onto next step.
   b. Collect the waste collection tube and place in safe area. This tube will be used for the Oasis HLB cartridge. Start the conditioning and equilibration steps for the Oasis HLB cartridges here.
9. Apply 2% formic acid in water or other suitable acid (such as 0.1 N HCl) as the wash solvent.
   a. Set the vacuum to 5 inHg. Do not go past 20 inHg.
   b. For a 6 cc (6 mL) WAX cartridge, use 10 mL of wash solvent.
   c. Insert 5 mL of methanol into the cartridge, then twist the manifold valves loose enough that the eluent is coming out of the cartridges in drops.
   d. Repeat 1 more time, for a total of 10 mL.
10. Pull vacuum for another 30 seconds to a minute to eliminate any residual wash solvent.
11. Release vacuum and discard waste fluids and insert new collection tubes for sample collection.
12. Apply 5% ammonium hydroxide in methanol as elution solvent and let it flow through by gravity before switching on the vacuum pump.
   a. Turn on the vacuum valve at the lowest possible vacuum and gradually increase as needed.
   b. For a 6 cc (6 mL) WAX cartridge, use 10 mL of elution solvent.
   c. Insert 5 mL of 5% ammonium hydroxide into the cartridge, then twist the manifold valves loose enough that the eluent is coming out of the cartridges in drops.
   d. Repeat 1 more time, for a total of 10 mL.
   e. Pull vacuum for another 30 seconds to a minute to collect all of the elution solvent.

13. Remove the collection tubes and place in a safe area.

Oasis HLB – for general collection of compounds

14. Refer to steps 3 and 4 for conditioning and equilibration of Oasis HLB cartridge.
15. Load 5mL waste sample from step 6 onto the HLB cartridge slowly and evenly to prevent channeling. The sample should flow through the cartridge under the vacuum so the sorbent retains the target compounds.
    a. Switch on or open valve at the lowest possible vacuum and gradually increase as needed to load the entire sample onto the sorbent bed.
    b. Load the full 5 mL of wastewater sample.
    c. Accomplish a slow sample flow rate to ensure that wanted compounds are embedded in the cartridge. Twist the manifold valves loose enough that the eluent is coming out of the cartridges in drops.
16. Once the entire sample has gone through the HLB cartridge, release the vacuum.
    a. Double check that the manifold valves are closed tightly before moving onto next step.
    b. Ensure that the collection tube can collect 10 mL without spilling. Otherwise, remove the collection tubes, discard the waste fluids, and replace the collection tubes with an empty one.
17. Apply 5% methanol in water as the wash solvent.
    a. Set the vacuum to 5 inHg. Do not go past 20 inHg.
    b. For a 6 cc (6 mL) HLB cartridge, use 10 mL of wash solvent.
    c. Insert 5 mL of 5% methanol into the cartridge, then twist the manifold valves loose enough that the eluent is coming out of the cartridges in drops.
    d. Repeat 1 more time, for a total of 10 mL.
18. Pull vacuum for another 30 seconds to a minute to eliminate any residual wash solvent.
19. Release vacuum and discard waste fluids and insert new collection tubes for sample collection.
20. Apply 10mL of 1:1 methanol acetonitrile or a 100% organic solvent as the elution solvent and let it flow through by gravity before switching on the vacuum pump.
    a. Turn on the vacuum valve at the lowest possible vacuum and gradually increase as needed.
    b. Pull vacuum for another 30 seconds to a minute to collect all the elution solvent.
21. Remove the collection tubes and place in a safe area.

Sample Evaporation and Resuspension

22. Carefully transfer the extracted sample from the Oasis WAX cartridge and the Oasis HLB cartridge into a 50mL Falcon tube.

23. Prepare water bath for the Turbovap by placing the indicated amount of water level (marked by the yellow tape on the side of the machine).
24. Adjust the settings of the Turbovap.
    a. Set the mode to “Manual”.
    b. Set the water bath temperature to 40 degrees Celcius.
    c. Set the gas flow to 2.0 L/min.
    d. Adjust the nozzle setup so that the gas flows into the necessary rows.
25. Place the extracted sample into the tube rack.
    a. Double check the tube rack is the correct size to fit 50mL tubes into.
    b. Adjust the depth of the tube rack accordingly so that the gas nozzle snugs right into the tube.
26. Place the tube rack into the water bath. Double check the front side of the tube rack is facing the correct direction.
    a. Double check the water level is above the sample solvent level so that the water is completely surrounding the solvent for proper evaporation.
27. Adjust the gas nozzles so that the unused nozzles are covered and only the used nozzles aren’t covered.
    a. Adjust the manifold so that the nozzles is snuggled right into the tube close to the middle.
    b. Double check that the nozzle setup is properly set up. If improperly set up, the gas flow will blow into the water bath, which could cause contamination of sample.
28. Press start. Evaporation of sample will approximately take ~50 minutes.
29. For each sample, prepare 250 uL of 1:1 water acetonitrile to resuspend the remaining compounds in the evaporated sample.
30. Once evaporation is complete, pipette 250 uL of 1:1 water acetonitrile into the Falcon tube, then continuously pipette the solvent to properly resuspend the compounds with the solvent.
31. Store the resuspended sample in -80 degrees Celcius or begin LC-MS analysis of the samples.

References

Acena, J., S. Stampachiacchiere, S. Perez and D. Barcelo (2015). "Advances in liquid chromatography-high-resolution mass spectrometry for quantitative and qualitative environmental analysis." Anal Bioanal Chem **407**(21): 6289-6299.

Allen, J. G., S. Gale, R. T. Zoeller, J. D. Spengler, L. Birnbaum and E. McNeely (2016). "PBDE flame retardants, thyroid disease, and menopausal status in U.S. women." Environ Health **15**(1): 60.

Baldi, I., A. Gruber, V. Rondeau, P. Lebailly, P. Brochard and C. Fabrigoule (2011). "Neurobehavioral effects of long-term exposure to pesticides: results from the 4-year follow-up of the PHYTONER study." Occup Environ Med **68**(2): 108-115.

Carre, J., N. Gatimel, J. Moreau, J. Parinaud and R. Leandri (2017). "Does air pollution play a role in infertility?: a systematic review." Environ Health **16**(1): 82.

Castiglioni, S., N. Salgueiro-Gonzalez, L. Bijlsma, A. Celma, E. Gracia-Lor, M. S. Beldean-Galea, T. Mackulak, E. Emke, E. Heath, B. Kasprzyk-Hordern, A. Petkovic, F. Poretti, J. Rangelov, M. M. Santos, M. Sremacki, K. Styszko, F. Hernandez and E. Zuccato (2021). "New psychoactive substances in several European populations assessed by wastewater-based epidemiology." Water Res **195**: 116983.

Chen, Y. C., J. F. Hsu, C. W. Chang, S. W. Li, Y. C. Yang, M. R. Chao, H. C. Chen and P. C. Liao (2023). "Connecting chemical exposome to human health using high-resolution mass spectrometry-based biomonitoring: Recent advances and future perspectives." Mass Spectrom Rev **42**(6): 2466-2486.

Chin-Chan, M., J. Navarro-Yepes and B. Quintanilla-Vega (2015). "Environmental pollutants as risk factors for neurodegenerative disorders: Alzheimer and Parkinson diseases." Front Cell Neurosci **9**: 124.

Cohen, A., A. Maile-Moskowitz, C. Grubb, R. A. Gonzalez, A. Ceci, A. Darling, L. Hungerford, R. D. Fricker, Jr., C. V. Finkielstein, A. Pruden and P. J. Vikesland (2022). "Subsewershed SARS-CoV-2 Wastewater Surveillance and COVID-19 Epidemiology Using Building-Specific Occupancy and Case Data." ACS ES T Water **2**(11): 2047-2059.

Cui, Y., D. M. Balshaw, R. K. Kwok, C. L. Thompson, G. W. Collman and L. S. Birnbaum (2016). "The Exposome: Embracing the Complexity for Discovery in Environmental Health." Environ Health Perspect **124**(8): A137-140.

Elliott, M. R., Y. Wang, R. A. Lowe and P. R. Kleindorfer (2004). "Environmental justice: frequency and severity of US chemical industry accidents and the socioeconomic status of surrounding communities." J Epidemiol Community Health **58**(1): 24-30.

Escola Casas, M., N. S. Schroter, I. Zammit, M. Castano-Trias, S. Rodriguez-Mozaz, P. Gago-Ferrero and L. Corominas (2021). "Showcasing the potential of wastewater-based epidemiology to track pharmaceuticals consumption in cities: Comparison against prescription data collected at fine spatial resolution." Environ Int **150**: 106404.

Gil-Solsona, R., M. C. Nika, M. Bustamante, C. M. Villanueva, M. Foraster, M. Cosin-Tomas, N. Alygizakis, M. D. Gomez-Roig, E. Llurba-Olive, J. Sunyer, N. S. Thomaidis, P. Dadvand and P. Gago-Ferrero (2021). "The Potential of Sewage Sludge to Predict and Evaluate the Human Chemical Exposome." Environ Sci Technol Lett **8**(12): 1077-1084.

Gracia-Lor, E., E. Zuccato, F. Hernandez and S. Castiglioni (2020). "Wastewater-based epidemiology for tracking human exposure to mycotoxins." J Hazard Mater **382**: 121108.

Hajeb, P., L. Zhu, R. Bossi and K. Vorkamp (2022). "Sample preparation techniques for suspect and non-target screening of emerging contaminants." Chemosphere **287**(Pt 3): 132306.
Han, M. A., J. H. Kim and H. S. Song (2019). "Persistent organic pollutants, pesticides, and the risk of thyroid cancer: systematic review and meta-analysis." Eur J Cancer Prev **28**(4): 344-349.
Holm, R. H., N. Osborne Jelks, R. Schneider and T. Smith (2023). "Beyond COVID-19: Designing Inclusive Public Health Surveillance by Including Wastewater Monitoring." Health Equity **7**(1): 377-379.
Ji, Y., Y. Yao, Y. Duan, H. Zhao, Y. Hong, Z. Cai and H. Sun (2021). "Association between urinary organophosphate flame retardant diesters and steroid hormones: A metabolomic study on type 2 diabetes mellitus cases and controls." Sci Total Environ **756**: 143836.
Klotz, K. and T. Goen (2017). "Human Biomonitoring of Lead Exposure." Met Ions Life Sci **17**.
Kmush, B. L., D. Monk, H. Green, D. A. Sachs, T. Zeng and D. A. Larsen (2022). "Comparability of 24-hour composite and grab samples for detection of SARS-2-CoV RNA in wastewater." FEMS Microbes **3**: xtac017.
Knapke, E. T., D. P. Magalhaes, M. A. Dalvie, D. Mandrioli and M. J. Perry (2022). "Environmental and occupational pesticide exposure and human sperm parameters: A Navigation Guide review." Toxicology **465**: 153017.
Lai, Y. H. and Y. S. Wang (2023). "Advances in high-resolution mass spectrometry techniques for analysis of high mass-to-charge ions." Mass Spectrom Rev **42**(6): 2426-2445.
Landrigan, P. J., J. L. Sly, M. Ruchirawat, E. R. Silva, X. Huo, F. Diaz-Barriga, H. J. Zar, M. King, E. H. Ha, K. A. Asante, H. Ahanchian and P. D. Sly (2016). "Health Consequences of Environmental Exposures: Changing Global Patterns of Exposure and Disease." Ann Glob Health **82**(1): 10-19.
Li, B., Y. Ding, X. Cheng, D. Sheng, Z. Xu, Q. Rong, Y. Wu, H. Zhao, X. Ji and Y. Zhang (2020). "Polyethylene microplastics affect the distribution of gut microbiota and inflammation development in mice." Chemosphere **244**: 125492.
Mamane, A., I. Baldi, J. F. Tessier, C. Raherison and G. Bouvier (2015). "Occupational exposure to pesticides and respiratory health." Eur Respir Rev **24**(136): 306-319.
Markosian, C. and N. Mirzoyan (2019). "Wastewater-based epidemiology as a novel assessment approach for population-level metal exposure." Sci Total Environ **689**: 1125-1132.
Medina, C. Y., K. F. Kadonsky, F. A. Roman, Jr., A. Q. Tariqi, R. G. Sinclair, P. M. D'Aoust, R. Delatolla, H. N. Bischel and C. C. Naughton (2022). "The need of an environmental justice approach for wastewater based epidemiology for rural and disadvantaged communities: A review in California." Curr Opin Environ Sci Health **27**: 100348.
Miller, G. W. and D. P. Jones (2014). "The nature of nurture: refining the definition of the exposome." Toxicol Sci **137**(1): 1-2.
Moisan, F., J. Spinosi, L. Delabre, V. Gourlet, J. L. Mazurie, I. Benatru, M. Goldberg, M. G. Weisskopf, E. Imbernon, C. Tzourio and A. Elbaz (2015). "Association of Parkinson's Disease and Its Subtypes with Agricultural Pesticide Exposures in Men: A Case-Control Study in France." Environ Health Perspect **123**(11): 1123-1129.
Montone, C. M., B. G. Moneta, S. E. Aita, F. Aulenta, C. Cavaliere, A. Cerrato, S. Fazi, A. Lagana, V. Paolini, F. Petracchini, S. Piovesana and A. L. Capriotti (2022). "Untargeted analysis of contaminants in river water samples: Comparison between two different sorbents for solid-phase extraction followed by liquid chromatography-high-resolution mass spectrometry determination." Microchemical Journal **172**.

Mukerjee, S., L. A. Smith, E. D. Thoma, D. A. Whitaker, K. D. Oliver, R. Duvall and T. A. Cousett (2020). "Spatial analysis of volatile organic compounds using passive samplers in the Rubbertown industrial area of Louisville, Kentucky, USA." Atmos Pollut Res **11**(6): 81-86.
National Center for Biotechnology Information (2024). "PubChem Compound Summary for CID 79631, Direct Violet 51."
National Center for Biotechnology Information (2024). PubChem Compound Summary for CID 113595, 1,19-Bis(oxiranyl)-8,16-bis(oxiranylmethoxy)-2,6,10,14,18-pentaoxanonadecane-4,12-diol.
National Center for Biotechnology Information (2024). "PubChem Compound Summary for CID 160832."
National Center for Biotechnology Information (2024). "PubChem Compound Summary for CID 173165, 21H,23H-Porphine-2,7,12,18-tetrapropanoic acid, 3,8,13,17-tetramethyl-, dihydrochloride."
National Center for Biotechnology Information (2024). "PubChem Compound Summary for CID 2765355, 4-[3-(4-Chlorophenyl)-2,1-benzisoxazol-5-yl]pyrimidin-2-amine."
National Center for Biotechnology Information (2024). "PubChem Compound Summary for CID 59296547, 4-((2-cyanopropan-2-yl)amino)-2-fluoro-N-methylbenzamide."
Nurmatov, U. B., N. Tagiyeva, S. Semple, G. Devereux and A. Sheikh (2015). "Volatile organic compounds and risk of asthma and allergy: a systematic review." Eur Respir Rev **24**(135): 92-101.
Plotka-Wasylka, J., N. Szczepanska, M. de la Guardia and J. Namiesnik (2016). "Modern trends in solid phase extraction: New sorbent media." Trac-Trends in Analytical Chemistry **77**: 23-43.
Richardson, S. D. and S. Y. Kimura (2016). "Water Analysis: Emerging Contaminants and Current Issues." Anal Chem **88**(1): 546-582.
Russ, K. and S. Howard (2016). "Developmental Exposure to Environmental Chemicals and Metabolic Changes in Children." Curr Probl Pediatr Adolesc Health Care **46**(8): 255-285.
Saththasivam, J., S. S. El-Malah, T. A. Gomez, K. A. Jabbar, R. Remanan, A. K. Krishnankutty, O. Ogunbiyi, K. Rasool, S. Ashhab, S. Rashkeev, M. Bensaad, A. A. Ahmed, Y. A. Mohamoud, J. A. Malek, L. J. Abu Raddad, A. Jeremijenko, H. A. Abu Halaweh, J. Lawler and K. A. Mahmoud (2021). "COVID-19 (SARS-CoV-2) outbreak monitoring using wastewater-based epidemiology in Qatar." Sci Total Environ **774**: 145608.
Semreen, M. H., A. Shanableh, L. Semerjian, H. Alniss, M. Mousa, X. Bai and K. Acharya (2019). "Simultaneous Determination of Pharmaceuticals by Solid-phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry: A Case Study from Sharjah Sewage Treatment Plant." Molecules **24**(3).
Strube, J., B. Thiede and W. Auch (2021). "Proposed pipelines and environmental justice: Exploring the association between race, socioeconomic status, and pipeline proposals in the United States." Rural Sociol **86**(4): 647-672.
Tsai, K. F., F. J. Cheng, W. T. Huang, C. T. Kung, C. T. Lee, B. C. Cheng, J. B. Chen, S. H. Li, C. C. Wang, L. J. Wang, Y. C. Ou and W. C. Lee (2022). "The associations between renal disease severity and exposure to organophosphate flame retardants in patients with chronic kidney disease." Environ Int **170**: 107573.
United States Census Bureau. (2020-2022). "Census Data for Louisville East CCD, Jefferson County, Kentucky." from

https://data.census.gov/profile/Louisville_East_CCD,_Jefferson_County,_Kentucky?g=060XX00US2111192108.
United States Census Bureau. (2020-2022). "Census Data for Louisville West CCD, Jefferson County, Kentucky." from https://data.census.gov/profile/Louisville_West_CCD,_Jefferson_County,_Kentucky?g=060XX00US2111192110.
Uppal, K., Q. A. Soltow, F. H. Strobel, W. S. Pittard, K. M. Gernert, T. Yu and D. P. Jones (2013). "xMSanalyzer: automated pipeline for improved feature detection and downstream analysis of large-scale, non-targeted metabolomics data." BMC Bioinformatics **14**: 15.
Uppal, K., D. I. Walker and D. P. Jones (2017). "xMSannotator: An R Package for Network-Based Annotation of High-Resolution Metabolomics Data." Anal Chem **89**(2): 1063-1067.
Vermeulen, R., E. L. Schymanski, A. L. Barabasi and G. W. Miller (2020). "The exposome and health: Where chemistry meets biology." Science **367**(6476): 392-396.
Walker, D. I., K. J. Lane, K. Liu, K. Uppal, A. P. Patton, J. L. Durant, D. P. Jones, D. Brugge and K. D. Pennell (2019). "Metabolomic assessment of exposure to near-highway ultrafine particles." J Expo Sci Environ Epidemiol **29**(4): 469-483.
Walker, D. I., K. Uppal, L. Zhang, R. Vermeulen, M. Smith, W. Hu, M. P. Purdue, X. Tang, B. Reiss, S. Kim, L. Li, H. Huang, K. D. Pennell, D. P. Jones, N. Rothman and Q. Lan (2016). "High-resolution metabolomics of occupational exposure to trichloroethylene." Int J Epidemiol **45**(5): 1517-1527.
Weidhaas, J., Z. T. Aanderud, D. K. Roper, J. VanDerslice, E. B. Gaddis, J. Ostermiller, K. Hoffman, R. Jamal, P. Heck, Y. Zhang, K. Torgersen, J. V. Laan and N. LaCross (2021). "Correlation of SARS-CoV-2 RNA in wastewater with COVID-19 disease burden in sewersheds." Sci Total Environ **775**: 145790.
Wild, C. P. (2012). "The exposome: from concept to utility." Int J Epidemiol **41**(1): 24-32.
Wright, E. J., D. G. Beach and P. McCarron (2022). "Non-target analysis and stability assessment of reference materials using liquid chromatography–high-resolution mass spectrometry." Anal Chim Acta **1201**: 339622.
Yu, T., Y. Park, J. M. Johnson and D. P. Jones (2009). "apLCMS--adaptive processing of high-resolution LC/MS data." Bioinformatics **25**(15): 1930-1936.
Yusa, V., M. Millet, C. Coscolla, O. Pardo and M. Roca (2015). "Occurrence of biomarkers of pesticide exposure in non-invasive human specimens." Chemosphere **139**: 91-108.
Zhang, Q., Y. He, R. Cheng, Q. Li, Z. Qian and X. Lin (2022). "Recent advances in toxicological research and potential health impact of microplastics and nanoplastics in vivo." Environ Sci Pollut Res Int **29**(27): 40415-40448.