Tracking Chemical Indicators of Public Health in the Urban Water Environment

by

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ABSTRACT

This dissertation focuses on the application of urban metabolism metrology (UMM) to process streams of the natural and built water environment to gauge public health concerning exposure to carcinogenic *N*-nitrosamines and abuse of narcotics. A survey of sources of exposure to *N*-nitrosamines in the U.S. population identified contaminated food products (1,900 ± 380 ng/day) as important drivers of attributable cancer risk (Chapter 2). Freshwater sediments in the proximity of U.S. municipal wastewater treatment plants were shown for the first time to harbor carcinogenic *N*-nitrosamine congeners, including *N*-nitrosodibutylamine (0.2-3.3 ng/g dw), *N*-nitrosodiphenylamine (0.2-4.7 ng/g dw), and *N*-nitrosopyrrolidine (3.4-19.6 ng/g dw) were, with treated wastewater discharge representing one potential factor contributing to the observed contamination (p=0.42) (Chapter 3). Opioid abuse rates in two small midwestern communities were estimated through the application of wastewater-based epidemiology (WBE). Average concentrations of opioids (City 1; City 2) were highest for morphine (713 ± 38, 306 ± 29 ng/L) and varied by for the remainder of the screened analytes. Furthermore, concentrations of the powerful opioid fentanyl (1.7 ± 0.2, 1.0 ± 0.5 ng/L) in wastewater were reported for the first time in the literature for the U.S. (Chapter 4). To gauge narcotic consumption within college-aged adults the WBE process used in Chapter 4 was applied to wastewater collected from a large university in the Southwestern U.S. Estimated narcotics consumption, in units of mg/day/1,000 persons showed the following rank order: cocaine (470 ± 42), heroin (474 ± 32), amphetamine (302 ± 14) and methylphenidate (236 ± 28). Most parental drugs and their respective metabolites showed detection frequencies in campus wastewater of 80% or more, with the notable exception

of fentanyl, norfentanyl, buprenorphine, and norbuprenorphine. Estimated consumption of all narcotics, aside from attention-deficit/hyperactivity disorder medication, were higher than values reported in previous U.S. WBE studies for U.S. campuses (Chapter 5). The analyses presented here have identified variation in narcotic consumption habits across different U.S. communities, which can be gauged through UMM. Application of these techniques should be implemented throughout U.S. communities to provide insight into ongoing substance abuse and health issues within a community.

DEDICATION

I would like to dedicate this work to my fiancée and future wife, *Yer Jalan Atthirari Anni*, Dr. Lydia Meador, as well as my family, Shakir, Kathy, and Sara Gushgari. I would not be where I am today if it was not for your constant support, advice, and love that you all have shown me. I would also like to thank my friends Robbie, Jeff, Joe, and Richie for the camping, hunting, and beer consumption (not while hunting) that kept me grounded throughout my PhD studies. I am truly blessed to have you all in my life.

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CHAPTER 1

INTRODUCTION

Access to reliable data on human behavior and population health is vital for public health officials, scientific researchers, government officials, and those involved in the science and practice of healthcare delivery. Methods of data collection have traditionally included socio-epidemiological surveys (Kim et al. 2015), crime statistics and seizure data, and analysis of medical records (Van Nuijs et al. 2011). These methods have proven valuable but also are known to be susceptible to potentially substantial bias and thus do not always represent the true nature of human behavior and population health within a community of interest. Urban metabolism metrology (UMM) is the science of measuring and interpreting the occurrence and concentrations of signature compounds and biomarkers informative of human activities and human health in communities large and small (Halden 2016). The UMM approach encompasses analytical work on an array of environmental matrixes including raw and treated wastewater (Archer et al. 2018, Baz-Lomba et al. 2016, Zuccato et al. 2008, Zuccato et al. 2005), sewage sludges, freshwater and coastal sediments impacted by urban discharges (Gushgari et al. 2016), as well as dust, condensate (Roll et al. 2015) and other process streams of the natural and built environment (Fig. 1). The goal of UMM is to detect and quantify trends in population health using robust metrics that can be tracked in real-time or near-real time.

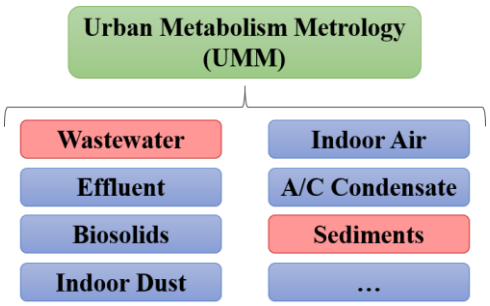


Fig. 1 - Possible matrices used in urban metabolism metrology approaches. Matrices studied in this thesis include wastewater and freshwater sediments.

Perhaps the most developed subset of UMM is wastewater-based epidemiology (WBE) - the process of analyzing samples of composited raw wastewater to identify compounds providing insight into human health and behavior (Fig. 2). This process typically begins with the collection of raw wastewater over a period of time (Irvine et al. 2011) sufficient enough to capture inputs from most or all people present in a sewershed. Collected samples can be flow rate-adjusted or time-adjusted to best reflect the characteristic temporal chemistry of a study population. These samples have historically been collected at the inlet of wastewater treatment plants (Kim et al. 2015) but samples have also been obtained from building discharge points or along pipes traversing the sewershed (Postigo et al. 2011) to obtain insights on specific geographic areas or neighborhoods. Wastewater samples then typically are processed and screened for the occurrence of target analytes with output data being reported either as a concentration, as a mass load per time, or as a mass load per time further adjusted for the number of people known or presumed to be present in the sampling area (Zuccato et al. 2008). The data derived from this analysis can then been viewed on its own or alongside other methods of

collecting population trend data to obtain a more complete understanding of sensitive topics such as use of prescription opioids and illicit narcotics.

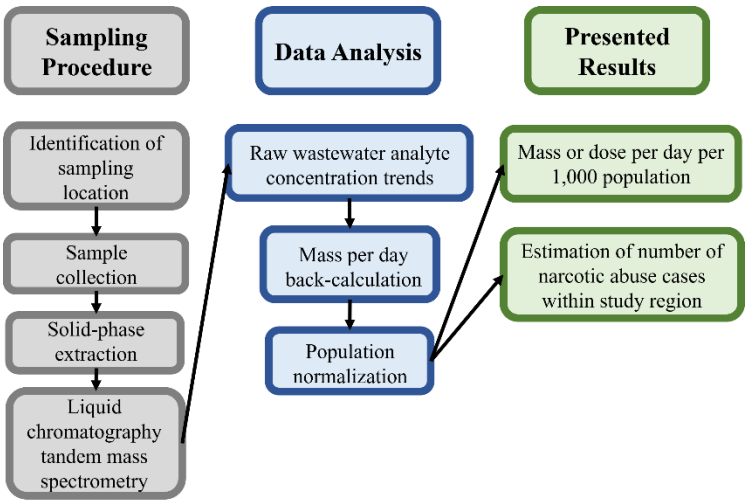


Fig. 2 - Sampling procedure, data analysis, and results presented in wastewater-based epidemiological monitoring.

1.1 *N*-Nitrosamines in wastewater

N-Nitrosamines are a class of carcinogenic water treatment disinfection byproducts that have seen increased research attention due to their formation during drinking water treatment and post-treatment distribution (Najm and Trussell 2001). *N*-Nitrosamines have historically been considered disinfection byproducts but also are contaminants of foods (Song and Hu 1988), tobacco (Hecht 2014b), certain alcoholic beverages (Goff and Fine 1979), and an array of personal care products (Shen and Andrews 2011). While over 300 congeners of the *N*-nitroso class of chemicals exist (Hecht 1997) research to date has focused only on a select number of *N*-nitrosamines known or suspected to play a role in causing cancer in humans. Congeners of *N*-nitrosamine have been shown in animal models to induce cancers of the liver, lung,

esophagus, nasal mucosa, bladder, tongue, forestomach, and pancreas (Hecht 1997), with site-specific tumor development being dependent on both the *N*-nitrosamine congener administered and the test species exposed (Hecht 1997).

Regulation of these contaminants has been slow to catch up with scientific findings but progress in this realm has been accomplished. The International Agency for Research on Cancer (IARC) has classified 24 different *N*-nitrosamines with respect to their carcinogenetic potential to humans, with two of these being classified as known human carcinogens and the remainder being split between the categories of probable carcinogen and possible carcinogen (Internation Agency for Research on Cancer 2015). The U.S. Environmental Protection Agency (USEPA) has also listed five *N*-nitrosamines on their Contaminant Candidate List 3 (CCL3) and its update, which is currently in draft format (CCL4) (U.S. Environmental Protection Agency 2014). The overall *N*-nitrosamine research focus has spurred the development of several water quality regulations for select *N*-nitrosamine congeners. The state of California has adopted stringent regulations regarding the presence of *N*-nitrosamines in drinking water, with response levels for *N*-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA) and *N*-nitrosodi-*n*-propylamine (NDPA) as 100 $ng*L^{-1}$, 300 $ng*L^{-}1$, and 500 $ng*L^{-1}$, respectively. Furthermore, Massachusetts drinking water guidelines outline a regulatory limit of 0.01 µg/L for NDMA (EPA 2015), and Arizona has set regulatory limits for NDMA (0.001 µg/L), *N*-nitrosodiphenylamine (NDPhA) (7.1 µg/L), and NDPA (0.005 µg/L) in their National Pollutant Discharge Elimination System Permit Program (Arizona Department of Environmental Quality 2015).

1.2 U.S. prescription and illegal narcotic use

Abuse of prescription medication and illegal narcotics has become an increasingly pervasive problem within the United States with 10.1% of U.S. residents ages 12 and older admitting to illicit narcotic consumption within the past month and 2.4% of U.S. residents ages 12 and older admitting to nonmedical use of a psychotherapeutic drug in the past month (CDC 2017). Prescription and illegally sourced opioids raise additional concern as opioid-related overdoses have been found responsible for 66.5% and 63.1%, respectively, of all drug overdose deaths reported in 2014 and 2015 (Rudd 2016, Warner et al. 2016). Opioids accounted for six of the ten narcotics most commonly involved in drug-overdose deaths, namely heroin (23.1%), oxycodone (11.5%), fentanyl (8.9%), morphine (8.5%), methadone (7.4%) and hydrocodone (7.4%) (Warner et al. 2016). From 2010 to 2015, U.S. death rates from drug overdoses increased from 12.3 to 16.3 per 100,000 population, driven primarily by consumption of heroin and fentanyl (Rudd 2016).

Geographical variance of narcotics abuse has been noted, likely is multi-factorial and deemed to be influenced by: resident narcotic tolerance, frequency of use, degree of dependence, social factors, and economic factors (Harocopos et al. 2016, Warner et al. 2016). Available U.S. narcotic abuse statistics may not accurately forecast the scope of drug addiction within a specific U.S. community due to these aforementioned factors. Due to the significant time delay associated with current analyses these sources of data can also be considered retrospective and may not capture the true scope of narcotic abuse within the target region at the present time. Researchers have speculated that the frequent implementation of WBE may add significant value as the process permits the collection

of data in near-real time for many communities. This sort of data acquisition via analysis of municipal sewage potentially can provide municipalities with the information needed to properly gauge community narcotic abuse and to track the efficacy of implemented programs designed to combat substance abuse.

1.3 Data gaps

Compared to European and Asian countries, wastewater epidemiology as an approach to study and diagnose narcotics consumption and abuse in the United States has seen limited use (Burgard et al. 2013, Heuett et al. 2015, Panawennage et al. 2011, Subedi and Kannan 2014). Studies which have examined U.S. wastewaters for drug use prevalence have primarily focused on US DEA schedule I and II narcotics (Banta-Green et al. 2009, Gerrity et al. 2011, Subedi and Kannan 2014). Few U.S. based studies screen wastewater for opioids aside from heroin, and to the author's knowledge no wastewater epidemiological study in the U.S. has screened for fentanyl use despite the recent drastic increase in fentanyl-related overdose deaths. WBE testing at U.S. universities has seen some application but analyte screening has primarily been limited to ADHD medication (Burgard et al. 2013, Moore et al. 2014). Two university-based studies have expanded on this to include a wider suite of narcotics (Heuett et al. 2015, Panawennage et al. 2011), but reported infrequent detections for many of their targeted analytes. In principle, the WBE approach can be applied to U.S. communities to study indicators of population health such as narcotic consumption and carcinogen exposure. Increased spatial screenings will provide national averages which individual municipalities can benchmark their data against and routine analysis will additionally provide a metric to gauge the efficacy of implemented substance abuse practices in near-real time.

The peer-reviewed WBE literature indicates that both parental drugs (Kim et al. 2015, Zuccato et al. 2005) and their specific metabolites (Gatidou et al. 2016, Subedi and Kannan 2014) can serve to estimate drug consumption. Specific analytes are chosen based on characteristics which favor WBE such as in-sewer stability (Castiglioni et al. 2014) and often both parent and metabolite compounds are used to estimate drug consumption (Baker et al. 2014). Some researchers have continued to use parent narcotics in their analyses due to unfavorable pharmacokinetic and degradation parameters associated with specific metabolites (Baker et al. 2014, Burgard et al. 2013). Direct comparison between two sampling locations may suffer from limitations due to differences in analytical approaches. The "elimination half-life" from the human body of various narcotics and metabolites may add further complexity.

WBE has seen limited application outside of narcotic use (Fattore et al. 2016, González-Mariño et al. 2017, Rousis et al. 2017) but could provide valuable insight into multiple parameters of human health and wellness including carcinogen exposure (Lai et al. 2017). Due to seemingly ubiquitous presence of carcinogenic *N*-nitrosamines in water (Ma et al. 2012, Schreiber and Mitch 2006) and wastewater (Krauss and Hollender 2008, Krauss et al. 2009) it is important to understand the average daily human exposure to the disinfection byproducts. Understanding average daily *N*-nitrosamine exposure could provide insight into the carcinogenic risk associated with the compounds and could be further studied through WBE approaches.

1.4 Primary goals and strategy

The goal of this PhD thesis was to investigate the occurrence and quantity of select harmful chemicals and chemical indicators of narcotic use in various environmental matrices and to explore the analytical value of UMM in evidence-formed public health decision making. *N*-Nitrosamine contamination in solid matrices was historically ignored due to the classes high affinity for aqueous matrices (Gushgari et al. 2016, Venkatesan et al. 2014) – but their recent quantification in biosolids (Venkatesan et al. 2014) identifies that contamination in other solid matrices related to water and wastewater treatment may exist. Nationwide existence and prevalence of *N*-nitrosamine contaminated freshwater sediments was examined by identifying contamination in freshwater sediment samples taken from multiple locations near wastewater treatment plants across the United States.

Wastewater-based epidemiology could be a valuable analytical tool in evidence-informed public health decision making (Yang et al. 2015) but its current capabilities are restricted due to its limited application within the United States (Subedi and Kannan 2014). Through solid phase extraction cleanup and pre-concentration followed by tandem mass spectrometry quantification concentrations of narcotic use indicator compounds were analyzed in 24-hour composite raw wastewater samples for three geographically distinct regions of the United States. Analyte concentrations in raw wastewater were then compared to average metabolization and excretion rates to estimate narcotic consumption in these regions and were compared to national statistics and relevant WBE literature.

1.5 Hypotheses

I hypothesize that (i) *N*-nitrosamine exposure from the ingestion pathway due to food and alcohol consumption constitutes a carcinogenic risk; (ii) freshwater sediments downstream of U.S. wastewater treatment plants contain higher levels of *N*-nitrosamines than sediments located upstream; (iii) high rates of opioid consumption in U.S. communities are reflected by drug indicator compounds identified in municipal wastewater; and (iv) analyte masses in campus-generated wastewater for known recreational use narcotics will show statistical differences (α=0.05) between weekday and weekend mass loads.

1.6 Specific aims

Specific aims of this dissertation were to:

(i) quantify the approximate *N*-nitrosamine daily exposure to human beings through the inhalation, ingestion, and dermal sorption pathways;

(ii) determine the attributable carcinogenic risk posed by *N*-nitrosamine congeners to the general population of the U.S. from main exposure sources;

(iii) determine the prevalence and profile of *N*-nitrosamine contamination within U.S. freshwater sediments local to wastewater treatment plants;

(iv) identify water quality parameters which show correlation with *N*-nitrosamine sediment contamination;

(v) implement WBE to identify concentrations of opioid consumption indicators in wastewater to estimate the prevalence of opioid use within two small (25,000-200,000 residents) midwestern U.S. communities; and

(vi) implement WBE to identify concentrations of narcotic consumption indicators in wastewater to estimate the incidence of prescription and illegal narcotic consumption at a southwestern U.S. university campus.

TRANSITION 1

This dissertation is comprised of individual studies focused on the fate and occurrence in the urban wastewater infrastructure of two classes of analytes related to human health: *N*-nitrosamines and narcotics of the class of opioids. At the beginning of this project sufficient literature pertaining to *N*-nitrosamine contamination in a variety of commonly encountered environmental and synthetic matrices existed – but few studies had attempted to model the carcinogenic impact of daily *N*-nitrosamine exposure from a combination of potable water, food products, beverages, tobacco use, and personal care products. Furthermore, no studies had attempted to address the potential reduction in daily *N*-nitrosamine loading that can be achieved through personal intervention.

In Chapter 2, published data on *N*-nitrosamine occurrence was compiled for eleven *N*-nitrosamines congeners, specifically *N*-nitrosonornicotine, 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosomorpholine, *N*-nitrosopiperidine, *N*-nitrosodi-n-butylamine, *N*-nitrosopyrrolidine, *N*-nitrosodiethanolamine, *N*-nitrosomethylethylamine, and *N*-nitrosodiphenylamine; data originated from five commonly monitored matrices, namely food, water, tobacco products, alcoholic beverages, and personal care products. Estimated daily exposure values were modeled using occurrence data obtained from a comprehensive literature review for six different scenarios of personal diets and lifestyle choices. A risk analysis also was conducted to determine the number of U.S. cancer cases attributable to exposure to the *N*-nitroso class of contaminants. Finally, reducible *N*-nitrosamine exposure achievable through personal intervention was estimated and areas

of suggested further research were identified to advance our current understanding of *N*-nitrosamine exposure and the likely effectiveness of exposure prevention strategies.

CHAPTER 2

CRITIAL REVIEW OF MAJOR SOURCES OF HUMAN EXPOSURE TO *N*-NITROSAMINES

ABSTRACT

More than 24 *N*-nitrosamine compounds contribute to the total *N*-nitrosamine (TNA) burden monitored routinely to assess human exposure to this important group of known and suspected human carcinogens. A literature review (n = 122) identified multiple sources of human exposure to TNAs, including waters (40 ± 10.5 ng/L; average and standard deviation), food and beverages (6.7 ± 0.8 ng/g), tobacco (16,100 ± 3,650 ng/g) and personal care products (1,500 ± 750 ng/g). Due to source control interventions, levels of TNAs in beer have dropped by about 96% between 1980 and 1990, whereas *N*-nitrosamine levels in other known sources have shown little to no change. Average daily TNA exposure in the U.S. in units of ng/d is estimated at 25,000 ± 4,950, driven by consumption of tobacco products (22,000 ± 4,350), food (1,900 ± 380), alcohol (1,000 ± 200), and drinking water (120 ± 24). Behavioral choices of individuals in non-occupational settings were calculated to result in a spectrum of exposure values ranging from a lower bound of 1,900 ± 380 ng/d to a higher bound of 25,000 ± 4,950 ng/d, indicating opportunities for a possible reduction in TNA exposure by up to 92% through deliberate choices in diet and lifestyle. Human exposure to TNAs from ingestion and (tobacco-smoke related) inhalation, respectively, are estimated to account for about 2,600 ± 1,050 and 3,400 ± 1,900 expected lifetime cancer cases per one million U.S. residents –

which translates to an expected 1,940,000 ± 950,000 attributable lifetime cancer cases across the United States.

2.1 Introduction

N-Nitrosamines have been identified as important environmental pollutants due to their near-ubiquitous presence in many environmental matrices, albeit at typically low concentrations in the nanogram per kilogram and nanogram per liter range. Characterized by a nitroso group bonded to an amine, this hydrophilic family of compounds consists of at least 300 previously documented congeners (Hecht 1997). While structural diversity is extensive, research has primarily focused on a small subset of *N*-nitrosamine congeners. *N*-nitrosamines are monitored and investigated for their site-specific carcinogenic impact noted in over 30 test animal species (Hecht 1997) and their well-documented occurrence in chlorinated and chloraminated waters (EPA 2011), food products (Park et al. 2015), tobacco products (Brunnemann and Hoffmann 2008), and personal care products (Shen and Andrews 2011). *N*-nitrosamine-induced tumors of the liver, lung, esophagus, nasal mucosa, bladder, tongue, forestomach, and pancreas have been documented (Hecht 1997), with site-specific tumor development being dependent on both the *N*-nitrosamine congener administered and the test species exposed (Hecht 1997). Site-specific *N*-nitrosamine induced tumors have been observed in specific target organs irrespective of the route of administration (Bartsch and Montesano 1984, Hill et al. 1973, Larsson et al. 2006, Lijinsky 1992, Wilkens et al. 1996), and a linear dose-response relationship of *N*-nitrosodimethylamine (NDMA) in the sub-parts-per-million exposure range has been noted along with absence of a discernible “safe threshold” concentration (Peto et al. 1991).

The chemical interaction of nitrous acid with primary aromatic amines was first observed and published by Peter Griess in 1864 (Griess 1864), and further researched through the work of Baeyer and Caro, and Otto Witt in the 1870s (Witt 1878). Now in extensive use, the term "nitrosamine" was first introduced by Otto Witt in his 1878 publication to describe "any substituted ammonia which contains, instead of at least one atom of hydrogen, the univalent nitrosyl group, -NO, in immediate connection with the ammoniacal nitrogen" (Witt 1878). Growth of malignant primary hepatic tumors in animal test species exposed to NDMA was observed in 1956 (Magee and Barnes 1956), which sparked the development of a large body of literature on the carcinogenicity and toxicity of the *N*-nitrosamine class of contaminants. Their role as environmental carcinogens was first proposed by William Lijinsky in 1970 (Lijinsky 1970), which fostered research on *N*-nitrosamine occurrence in environmental media, such as ambient water, aquatic sediments and municipal sewage sludge (Gushgari et al. 2016, Schreiber and Mitch 2006, Venkatesan et al. 2014, Zeng and Mitch 2015). Studies on *N*-nitrosamines mainly have been concerned with the quantification of *N*-nitrosamines from different sources, assessments of cancer impact using animal models, and the modeling of cancer risks related to specific *N*-nitrosamine/cell interactions. Cancer risk of select *N*-nitrosamines, most notably NDMA, has been shown to exceed that of many known potent carcinogens, including: asbestos, benzo[*a*]pyrene, and polychlorinated biphenyls (OEHHA 2009). Slope factors for cancers attributed to *N*-nitrosamine ingestion or inhalation are available for only for a select few *N*-nitrosamines, thus the proposed carcinogenic impact of the class of *N*-nitrosamines is still poorly defined and potentially underestimated.

The International Agency for Research on Cancer (IARC) has classified 24 different *N*-nitrosamines with respect to their carcinogenetic potential to humans, with two congeners being classified as known human carcinogens and the remainder being split between the categories of probably carcinogenic and possibly carcinogenic (Internation Agency for Research on Cancer 2015). The U.S. Environmental Protection Agency (USEPA) has also listed five *N*-nitrosamines in their two most recent Contaminant Candidate Lists (CCL3 and CCL4) (U.S. Environmental Protection Agency 2014). Overall, research on *N*-nitrosamines has spurred the development of a number of water quality regulations for select congeners. The State of California has adopted stringent regulations regarding the maximum levels of *N*-nitrosamines in drinking water, with response levels for *N*-Nitrosodiethylamine (NDEA), NDMA and *N*-Nitrosodi-*n*-propylamine (NDPA) as low as 100, 300, and 500 ng/L, respectively. Furthermore, drinking water guidelines for the State of Massachusetts outline a regulatory limit of 10 ng/L for NDMA (EPA 2015), and Arizona has set regulatory limits for NDMA (1 ng/L), *N*-nitrosodiphenylamine (NDPhA) (7,100 ng/L), and NDPA (5 ng/L) in their National Pollutant Discharge Elimination System Permit Program (Arizona Department of Environmental Quality 2015).

Whereas a fair amount of studies have documented the occurrence of *N*-nitrosamines in environmental matrices (De Mey et al. 2017, Kim et al. 2013, Qiu et al. 2017, Rattray and Cochran 2014) and attempts to correlate these exposures to site specific tumor occurrence (Fritschi et al. 2015, Gankhuyag et al. 2017, Kao et al. 2017, Stepanov et al. 2014), thus far still lacking are quantitative analyses of the relative importance of major TNA sources on cancer risk and an identification of opportunities

for source and exposure reduction. Therefore, the present analysis of the scientific literature was designed to identify sources of *N*-nitrosamine exposure which could be curtailed through individual lifestyle choices. Specifically, average *N*-nitrosamine levels within commonly contaminated matrices were evaluated in conjunction with dietary and lifestyle data to estimate average daily exposures for select *N*-nitrosamine congeners. These exposure values were then compared to oral or inhalation cancer slope factors, when available, to estimate specific carcinogenic risks as well as the number of expected cancer cases in the U.S. population attributable to the *N*-nitrosamine class of emerging contaminants.

2.2 Materials and methods

2.2.1 Literature search

Peer-reviewed literature published prior to 2017 was searched using Google Scholar and Arizona State University's Library One search engines. Search terms used individually and in combinations included chemical names (Nitrosamine, *N*-Nitrosamine, Nitrosamines, and *N*-Nitrosamines"), media of interest (water, food, personal care product, alcohol, or tobacco), and routes of exposure (ingestion, inhalation, dermal adsorption). We included journal articles focusing on *N*-nitrosamine concentrations in potable water, food, alcohol, tobacco, and personal care products. Peer-reviewed articles which did not present concentration data within the manuscript or supplemental information were omitted from the analysis, as were articles which were not translated to English from their original publishing language. Journal articles which presented concentrations of *N*-nitrosamines for products other than the aforementioned five major categories were omitted from analysis.

Literature for average U.S. smoking statistics, average daily water intake, and average food consumption statistics were searched for using the Google Scholar search engine. Oral cancer slope factors were obtained from the U.S. EPA Integrated Risk Integration System (IRIS) (USEPA 2017) for: NDMA, *N*-nitrosomorpholine (NMOR), *N*-nitrosomethylethylamine (NMEA), *N*-nitrosopyrrolidine (NPYR), NDEA, NDPA, and NDPhA. Inhalation cancer slope factors were also obtained from the U.S. EPA IRIS (USEPA 2017) database for: *N*-nitrosonornicotine (NNN), 4-(*N*-nitrosomethylamine)-1-(3-pyridyl)-1-butanone (NNK), and NDMA. *N*-Nitrosamine congeners which did not have cancer slope factors were omitted from the attributable risk analysis.

2.2.2 Data extraction and analysis

Publication literature reporting *N*-nitrosamineconcentrations by media meeting the eligibility criteria were extracted from Google Scholar and compiled into EndNote citation manager (vX7.7, Thomas Reuters, New York, USA). The final literature set (n = 122) was reviewed for establishing average *N*-nitrosamine concentrations in products within the five matrix categories, as well as average U.S. health and product usage statistics. Individual product concentrations were compiled in Microsoft Excel and analyzed using the JMP Pro 12.1.0 data analysis software and Microsoft Excel. Figures were created using a combination of Microsoft's Office Suite programs and Origin Pro.

2.2.3 Exposure and attributable risk analyses

For average exposure assessment purposes, average *N*-nitrosamine concentrations were considered alongside average U.S. citizen use data to estimate average exposure levels. Average daily smoking values were estimated as 14.2 cigarettes per day (FSPTCA

2010), average water intake was estimated to be 3 liters per day (Gleick 1998), and average food intake was estimated from the American Heart Association's 2,000-Calorie level dietary guidelines. Average exposure values were then compared to oral and inhalation cancer slope factors (when available), obtained from the U.S. Environmental Protection Agency (USEPA) for attributable risk analyses purposes.

To gauge the carcinogenic impact of the *N*-nitrosamine class of emerging contaminants, an attributable carcinogenic risk evaluation was completed for select *N*-nitrosamines when necessary data was available. Results obtained from this analysis detail the expected number of lifetime cancer cases that can be attributed to exposure to the *N*-nitrosamine congeners included in the analysis. Exposure concentration levels (in mg per kilogram body weight per day, mg/kg-d) from different routes of exposure were calculated from data ascertained from the comprehensive literature review using the two equations below:

$$C_{Ing}\left(\frac{mg}{kg-day}\right) = \frac{\left(C_{NW}*2\frac{L}{d}\right)+\left(C_{NM}*80\frac{g}{d}\right)+\left(C_{NF}*50\frac{g}{d}\right)+\left(C_{NC}*200\frac{g}{d}\right)+\left(C_{NV}*375\frac{g}{d}\right)}{60.55\ kg} \qquad \text{Eq. 1}$$

$$C_{Inhalation}\left(\frac{mg}{kg-day}\right) = \frac{\left(C_{NTC}*20\frac{cigarette}{d}\right)}{60.55\ kg} \qquad \text{Eq. 2}$$

Where:

C_{NW}: Average N − Nitrosamine Water Concentration (mg/day)

C_{NM}: Average N − Nitrosamine Meat Concentration (mg/day)

C_{NF}: Average N − Nitrosamine Fat, Oil, and Sweets Concentration (mg/day)

C_{NC}: Average N − Nitrosamine Carbohydrate Concentration (mg/day)

C_{NV}: Average N − Nitrosamine Vegetable Concentration (mg/day)

C_{NTC}: Average N − Nitrosamine Cigarette Smoke Concentration (mg/day)

Exposure concentrations were then multiplied by the oral slope factor or inhalation slope factor obtained from the U.S. Environmental Protection Agency (USEPA) in order to gauge individual cancer risk, and then multiplied by 1,000,000 for population normalization purposes. This process was repeated for each *N*-nitrosamine where USEPA oral and/or inhalation slope factors were available, and finally summed up to obtain an initial estimate of the "total *N*-nitrosamine risk" for both inhalation and ingestion exposure pathways.

2.3 Results

2.3.1 N-Nitrosamine contamination data

The exclusion criteria utilized in the literature review resulted in a pool of 122 relevant studies on *N*-nitrosamine occurrence, encompassing contamination of food products, water, tobacco, alcohol, and personal care products. Publications on *N*-nitrosamines have increased in number since the 1950s with a further uptick by 120% from 2000 to 2015, a time period during which regulatory activity also increased for these emerging contaminants and human carcinogens (Fig. 3).

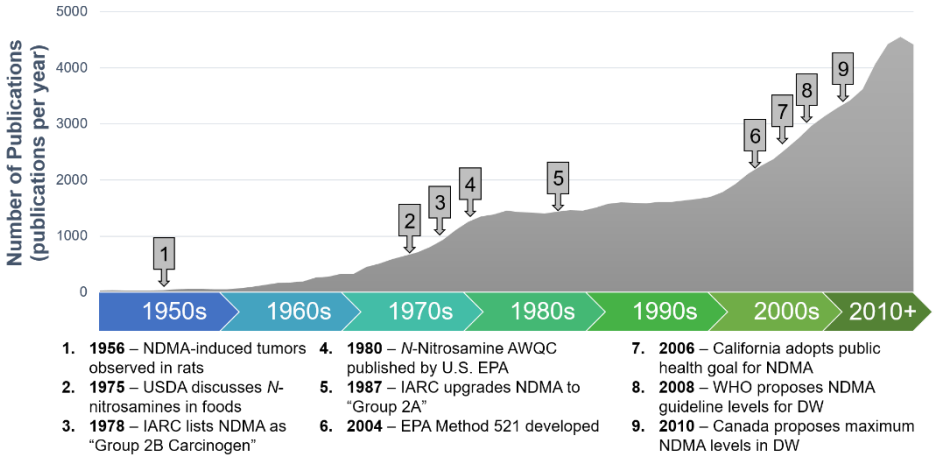
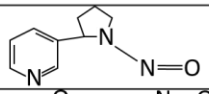
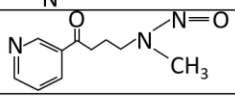
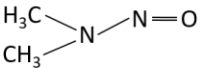
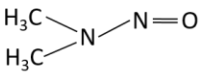
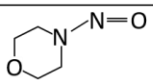
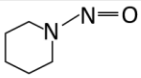
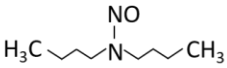
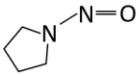
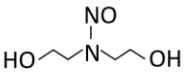
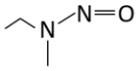
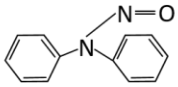


Fig. 3 - Publication activity (three-year moving average) and timeline of notable events of N-nitrosamine-directed research. Abbreviations: AWQC, Ambient water quality criteria; WHO, World Health Organization; DW, drinking water.

Out of the 122 studies considered, 56 studies provided quantitative information on some 262 *N*-nitrosamine contaminated food products (Campillo et al. 2011, Coffacci et al. 2013, DOMAŃSKA-BLICHARZ et al. 2005, Fajen et al. 1979, Gavinelli et al. 1988, Glória et al. 1997, Goff and Fine 1979, Hedler et al. 1979, Herrmann et al. 2015, Izquierdo-Pulido et al. 1996, Jawad 2012, Jo et al. 2010, Jurado-Sánchez et al. 2007, Kim and Shin 2013, Kocak et al. 2012, Kubacki et al. 1989, Mavelle et al. 1991, McWeeny 1983, Mitacek et al. 1999, Okafor and Nwogbo 2005, Oliveira et al. 1995, Ozel et al. 2010, Park et al. 2015, Scanlan 1983, Scanlan et al. 1990, Seo et al. 2015, Song and Hu 1988, Spiegelhalder and Preussmann 1984, Tricker et al. 1991b, Weston 1983, Yurchenko and Mölder 2006, 2007), 140 contaminated nicotine-containing products (Adams et al. 1987, Brunnemann and Hoffmann 2008, Ding et al. 2008, Fischer et al. 1990, Hoffmann et al. 1979, Kim and Shin 2013, Laugesen 2008, Mostafa et al. 1994, Österdahl et al. 2004, Rickert et al. 2008, Rühl et al. 1979, Stepanov et al. 2012, Tricker et al. 1991a, Tricker et al. 1991b, Wu et al. 2005, Xiong et al. 2010), 74 contaminated

personal care products (Fan et al. 1977, Schothorst and Somers 2005, Schothorst and Stephany 2001, Spiegelhalder and Preussmann 1984), 64 contaminated alcoholic beverages, and 36 potable water *N*-nitrosamine concentrations (Charrois et al. 2004, Planas et al. 2008, Wang et al. 2011, Zhao et al. 2006). Tobacco product concentrations, governed primarily by the tobacco-specific *N*-nitrosamines NNN and NNK (Brunnemann and Hoffmann 2008, Tricker et al. 1991a), were consistently reported to have the highest levels of *N*-nitrosamines (TNA: 16,100 ± 3,651 ng/g) of all media categories, followed by personal care products (TNA: 1,507 ± 752 ng/g), food products (TNA: 6.7 ± 0.8 ng/g), potable waters (TNA: 39.4 ± 10.5 ng/L), and alcoholic beverages (TNA: 2.9 ± 0.4 ng/L). Nicotine-containing products also constituted the largest range of concentrations of any media (range: 0-326,000 ng/g), followed by personal care products (range: 0-49,000 ng/g), food products (range: 0-120.8 ng/g), potable waters (range: 2.8-309 ng/L), and alcoholic beverages (range: 0-17.4 ng/L). It is important to note that significant variation exists in the concentration of *N*-nitrosamines populating the sub-classes within the matrix categories.

Table 1 - Summary facts on N-nitrosamines covered in this critical review.*

IARC Classification	Congener Name	Congener Structure	U.S. EPA Oral Slope Factor
Group 1 (Carcinogenic to Humans)	*N*-Nitrosonornicotine ***(NNN)***		*Not Available*
	4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone ***(NNK)***		*Not Available*
Group 2A (Probably Carcinogenic to Humans)	*N*-Nitrosodimethylamine ***(NDMA)***		$51\ \frac{mg}{kg * d}$
	N-Nitrosodiethylamine ***(NDEA)***		$150\ \frac{mg}{kg * d}$
Group 2B (Possibly Carcinogenic to Humans)	*N*-Nitrosomorpholine ***(NMOR)***		*Not Available*
	N-Nitrosopiperidine ***(NPIP)***		*Not Available*
	N-Nitrosodi-*n*-butylamine ***(NDBA)***		$5.4\ \frac{mg}{kg * d}$
	N-Nitrosopyrrolidine ***(NPYR)***		$2.1\ \frac{mg}{kg * d}$
	N-Nitrosodiethanolamine ***(NDELA)***		$2.8\ \frac{mg}{kg * d}$
	N-Nitrosomethylethylamine ***(NMEA)***		$22\ \frac{mg}{kg * d}$
Group 3 (Not Classifiable as to its Carcinogenicity to Humans)	*N*-Nitrosodiphenylamine ***(NDPhA)***		$0.0049\ \frac{mg}{kg * d}$

* IARC classifications were obtained from the "IARC Monographs on the Evaluation of Carcinogenic Risks to Humans". USEPA Oral Slope Factors were obtained from OEHHA's "Technical Support Document for Cancer Potency Factors 2009 – Appendix A". *N*-Nitrosamine congener structures were recreated by the primary author from WHO's "Concise International Chemical Assessment Documents" when available, and from "PubChem."

While over 300 congeners of the *N*-nitrosamine class of contaminants are known to exist, the peer-reviewed literature focuses primarily on a select group of *N*-nitrosamines (Table 1). Studies concerned with the occurrence of *N*-nitrosamines within tobacco products mainly focused on four tobacco-specific *N*-nitrosamines: NNK, NNN, *N*-Nitrosoanatabine (NAT), and *N*-nitrosoanabasine (NAB) (Kim and Shin 2013, Stepanov et al. 2006, Stepanov et al. 2012, Xiong et al. 2010). Two of these *N*-

nitrosamines, NNN and NNK, are the only congeners of the *N*-nitroso class that have been identified as 'known human carcinogens' (Internation Agency for Research on Cancer 2015). Of these tobacco-specific *N*-nitrosamines, tobacco product nitrosamine concentrations were predominantly governed by NNN (*n*=140, mean: 7,400 ± 1,500 ng/g), followed by NAT (*n*=102, mean: 4,600 ± 1,550 ng/g), NNK (*n*=140, mean: 3,200 ± 1,150 ng/g), and NAB (*n*=102, mean: 950 ± 310 ng/g). Cigarettes were found to have the highest concentrations of the tobacco-specific *N*-nitrosamines (TNA: 52,600 ± 19,650 ng/g, range: 590-326,060 ng/g), followed by cigars (TNA: 45,900 ± 34,100 ng/g, range: 11,800-80,000 ng/g), chewing tobacco (TNA: 5,850 ± 2,450 ng/g, range: 270-41,400 ng/g), and snuff products (TNA: 5,400 ± 1,250 ng/g, range: 19-77,100 ng/g). Interestingly, tobacco-specific *N*-nitrosamines were also found in electronic cigarette fluid (TNA: 1,430 ± 700, range: 0-3,870 ng/g) and nicotine cessation products (TNA: 450 ± 150 ng/g, range: 0-983 ng/g) (Hoffmann et al. 1979, Kim and Shin 2013). Concentrations in these products were, respectively, 97.3% and 99.2% lower than the average concentration in traditional cigarettes (Brunnemann and Hoffmann 2008, Tricker et al. 1991a, Wu et al. 2005). Furthermore, *N*-nitrosamine concentrations measured in mainstream (TNA: 1,530 ± 670 ng/cigarette, range: 112.7-9,700 ng/cigarette) and sidestream (TNA: 6,550 ± 3,400 ng/cigarette, range: 340-32,300 ng/cigarette) significantly violate OEHHA's "no significant risk level" (NSRL) for NNN (500 ng/day) and of NNK (14 ng/day) (OEHHA 2009).

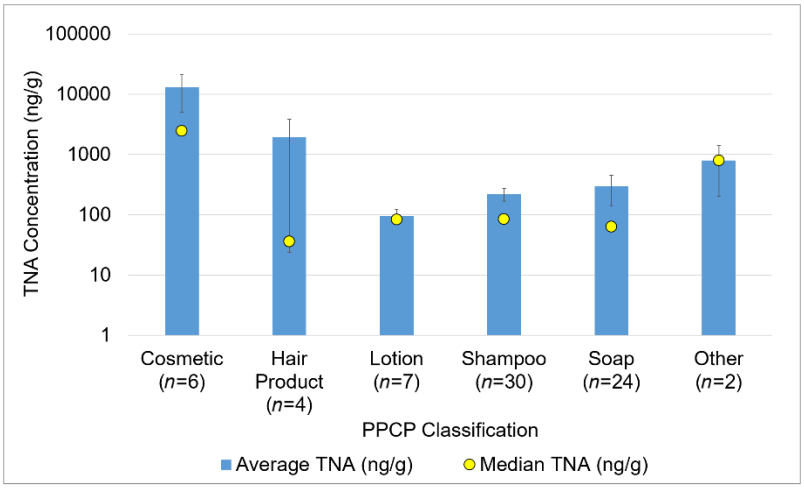


Fig. 4 - Average and median TNA concentrations in various categories of personal care products, plotted on a logarithmic scale. Term "n" denotes the number of individual product concentrations obtained through literature review. "Other" category denotes products which did not fit into additional categories. Only NDELA was examined in this analysis, due to the lack of testing additional N-nitrosamine congeners within PPCP's.

The high levels of *N*-nitrosamines observed in personal care products is primarily due to the presence of *N*-nitrosodiethanolamine (NDELA), accounting for 99% of all observed *N*-nitrosamines within care products. The remaining 1% of observed contamination stems from NMOR (~0.99%) and NDMA (~0.01%). Cosmetic products (Fig. 4) were found to have the highest average total *N*-nitrosamine concentration (TNA: 13,000 ± 8,100 ng/g, range: 400-49,000 ng/g), but were heavily weighted by two samples with concentrations above 20,000 ng/g. Hair care products (TNA: 1,900 ± 1,900 ng/g, range: 0-7,644 ng/g), soaps (TNA: 300 ± 150 ng/g, range: 0-3,746 ng/g), shampoos (TNA: 220 ± 50 ng/g, range: 23-1,287 ng/g), and lotions (TNA: 100 ± 25 ng/g, range: 22-230 ng/g) were all shown to have quantifiable *N*-nitrosamine concentrations with NDELA constituting the major congener in all cases. Two personal care products, an unidentified children's care product (TNA: 1,500 ng/g) and a facial cleaner (TNA: 200 ng/g), were averaged together to obtain the "other" category.

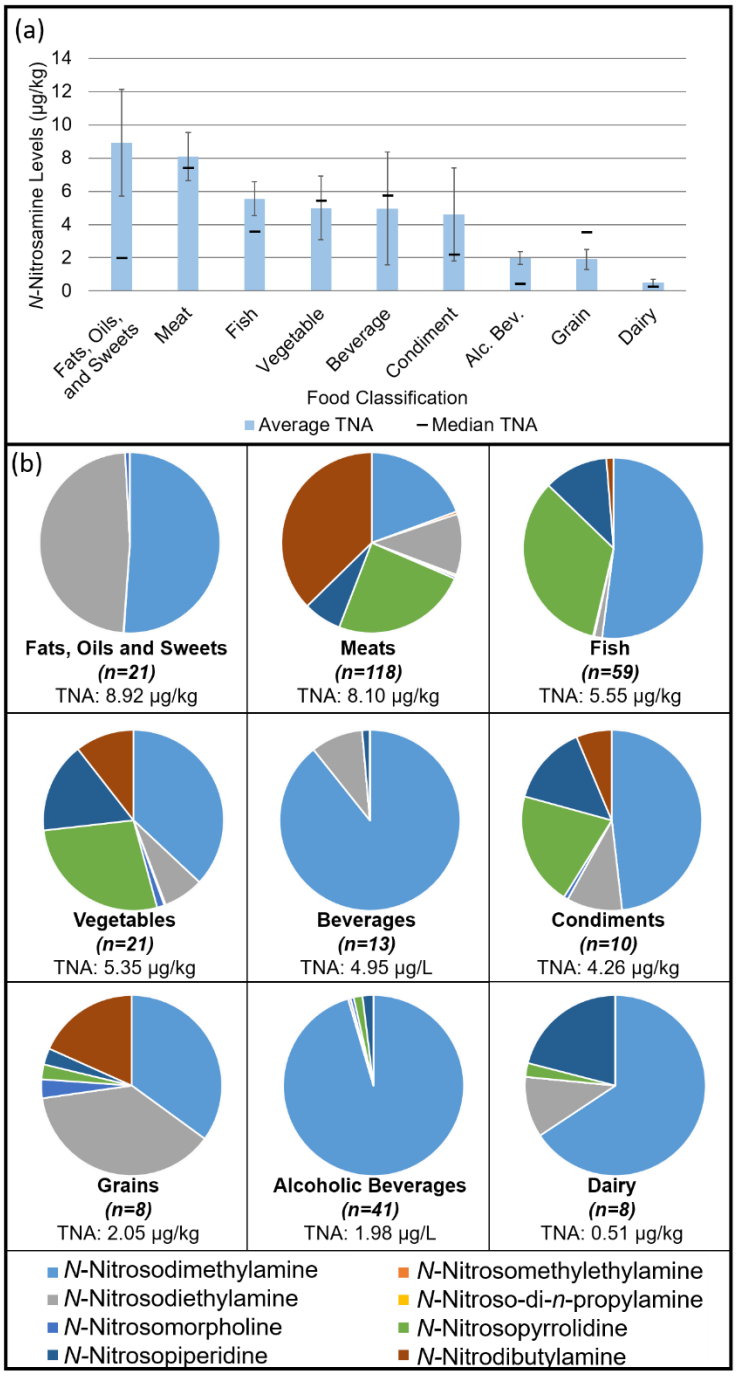


Fig. 5 - (a) Average and median concentrations (± standard error) of total N-nitrosamines (TNA) in various food categories. (b) Contribution of individual N-nitrosamine congeners to TNA levels detected in various food category, listed in descending order of concentrations reported. Term "n" denotes the number of studies one or more N-nitrosamines were detected.

N-Nitrosamine concentrations in food and alcohol products (Fig. 5) represented the largest category of data in this analysis, with data collected from 31 peer-reviewed articles. Currently available literature cites over 300 different reports of *N*-nitrosamine contaminated foods and beverages containing detectable levels of various *N*-nitrosamines, including: fats, oils, and sweets (TNA: 0-44 ng/g, *n=21*), meat products (TNA: 0.1-121 ng/g, *n=118*), fish products (TNA: 0-43.9 ng/g, *n=59*), canned vegetables (TNA: 0.02-40.5 ng/g, *n=21*), beverages (TNA: 0.2-45.7 ng/mL, *n=13*), condiments (TNA: 0.3-29.59 ng/g, *n=10*), grains (TNA: 0.2-4.6 ng/g, *n=8*), dairy products (TNA: 0-1.6 ng/g, *n=8*), fruit (TNA: 8.1 ng/g, *n=1*), rice (TNA: 1.5 ng/g, *n=1*), drink mixes (TNA: 0.9 ng/g, *n=1*), and tofu (TNA: 0.2 ng/ng, *n=1*) (Campillo et al. 2011, Coffacci et al. 2013, DOMAŃSKA-BLICHARZ et al. 2005, Gavinelli et al. 1988, Glória et al. 1997, Goff and Fine 1979, Hedler et al. 1979, Herrmann et al. 2015, Izquierdo-Pulido et al. 1996, Jawad 2012, Jo et al. 2010, Jurado-Sánchez et al. 2007, Kim and Shin 2013, Kocak et al. 2012, Mavelle et al. 1991, McWeeny 1983, Mitacek et al. 1999, Okafor and Nwogbo 2005, Oliveira et al. 1995, Ozel et al. 2010, Park et al. 2015, Scanlan et al. 1990, Seo et al. 2015, Song and Hu 1988, Tricker et al. 1991b, Yurchenko and Mölder 2006, 2007). The four food classes with the highest average *N*-nitrosamine concentration levels were identified as fats, oils, and sweets (average TNA: 8.9 ± 3.2 ng/g), meats (average TNA: 8.1 ± 1.4 ng/g), fish (average TNA: 5.6 ± 1.0 ng/g), and vegetables (average TNA: 5.4 ± 1.9 ng/g). NDMA (average: 2.2 ± 0.3 ng/g) was found to have the highest average concentration of all congeners across all food categories, followed by NDBA (average: 1.5 ± 0.5 ng/g), NPYR (average: 1.5 ± 0.2 ng/g), NDEA (average: 0.9 ± 0.3 ng/g), NPIP

(average: 0.5 ± 0.1 ng/g), NMOR (average: 0.05 ± 0.01 ng/g), NMEA (average: 0.04 ± 0.01 ng/g), and finally NDPA (average: 0.02 ± 0.01 ng/g).

N-Nitrosamine formation in potable water is a well-documented phenomenon (Charrois et al. 2004, Planas et al. 2008), and thus values for potable water were obtained specifically for a comparison to other matrices and for estimating the attributable risk. The average total *N*-nitrosamine concentration in U.S. potable waters was 39.4 ± 10.5 ng/L, with a range of values between 2.8-309.0 ng/L. The average NDMA concentration (17.7 ± 4.7 ng/L) in potable waters exceeded those of all other congeners, but notable levels also were observed for other congeners listed in the following as average concentrations ± standard deviation: NPIP (7.9 ± 4.0 ng/L), NPYR (5.5 ± 2.6 ng/L), NDEA (4.2 ± 0.8 ng/L), NDBA (1.7 ± 0.6 ng/L), NMOR (0.9 ± 0.2 ng/L), NMEA (0.6 ± 0.1 ng/L), NDPhA (0.6 ± 0.2 ng/L), and NDPA (0.4 ± 0.03 ng/L). While levels of *N*-nitrosamines have been identified in surface water (Schreiber and Mitch 2006), wastewater(Krauss and Hollender 2008, Krauss et al. 2009), biosolids, and freshwater sediments (Gushgari et al. 2016, Venkatesan et al. 2014), these sources are not expected to represent a direct route of human exposure, and thus were omitted from analyses.

In addition to the aforementioned contaminated matrices there are a number of additional potential sources of human exposure that warrant discussion. Firstly, a number of *N*-nitrosamines have been detected in commonly used rubber and latex products (Altkofer et al. 2005, Fajen et al. 1979, Havery and Fazio 1982). Car tires, child care products, rubber balloons, and condoms have all shown to not only contain, but readily release *N*-nitrosamines into surrounding matrixes. One source cites that human exposure

to *N*-nitrosamines from the use of condoms could exceed exposure from foods 1.5- to 3-fold (Altkofer et al. 2005). Occupational exposure is another important, but selective route of human *N*-nitrosamine exposure. While not applicable to the population as a whole, certain occupations (especially those involved in manufacturing processes) can be associated with a higher risk of *N*-nitrosamine induced tumor development (Cocco et al. 1996, De Vocht et al. 2007, Spiegelhalder and Preussmann 1983). Furthermore, a considerable number of additional exposure mechanisms have been postulated that theoretically could further increase the total human *N*-nitrosamine exposure, but many of these have not yet been verified and quantified in laboratory or field studies (Altkofer et al. 2005, Havery and Fazio 1982, Hecht 1997, Schothorst and Somers 2005).

2.3.2 Cancer incidence rate changes in nations consuming large quantities of beer

This literature review also reveals a notable decrease in *N*-nitrosamine concentrations in beers and other malt beverages from the 1980s to the 1990s (Fig. 6). This decrease in concentration has been attributed primarily to manipulation of manufacturing methods targeted at reducing *N*-nitrosamine occurrence (McWeeny 1983).

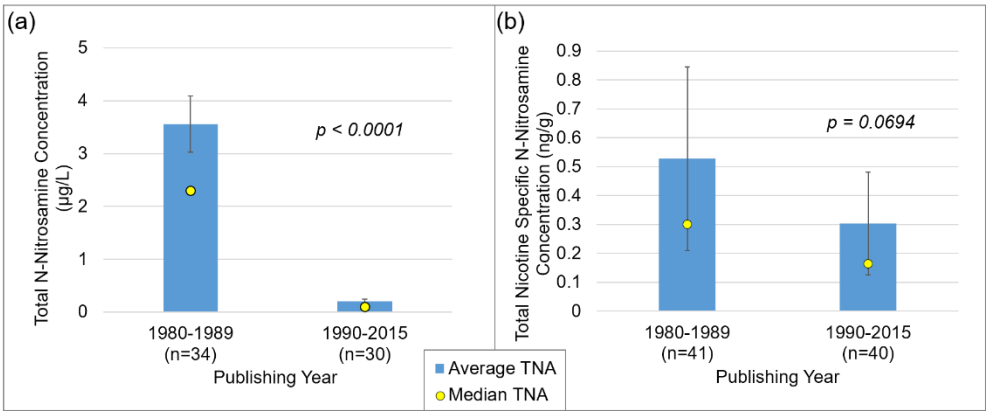


Fig. 6 - Representation of N-nitrosamine levels in domestic and international beer and tobacco products. (a) Comparison of TNA levels in beer products, from 1980-1989 and 1990-2015. Value "n" denotes number of reported values obtained from literature. (b) Comparison of TNA levels in mainstream cigarette smoke, from 1980-1989 and 1990-2015. Error bars represent ± standard error. Value "n" denotes number of reported values obtained from literature.

To gauge the impact of *N*-nitrosamine reduction in beer on cancer incidence, tumor occurrence rates were compared in two countries with high levels of per-capita beer consumption. In the Czech Republic, overall cancer incidence from 1977 to 2011 increased 32% for males and 22.8% for females (Dušek et al. 2010). In this same timeframe, the incidence of tumors of the pancreas, kidney and bladder increased by 56%, 171%, and 82%, respectively (Dušek et al. 2010). In contrast, a cancer registry of the Federal State of Saarland, Germany has noted a decrease in mortality from cancers from 1950-2002 for both male and female populations (Becker et al. 2007). However, the overall incidence of cancer (from 1970-2002) in this same region did not decrease, and the occurrence of certain site-specific cancers decreased only slightly (laryngeal, -3.3%; lung, -1.8%; stomach, -2.7%). In this same timeframe, lung cancer cases in females increased by 4.9% and prostate cancer cases in males by 5.7%, whereas normalized occurrence rates of all other site-specific cancers showed neither a significant increase nor a decrease (Becker et al. 2007). These findings suggest that while a significant

reduction in beer-borne nitrosamines has been achieved, total tumor occurrence and occurrence of site-specific tumors associated with NDMA exposure have nevertheless increased. Observations summarized here in regard to the occurrence of and mortality caused by cancer may be influenced by a variety of factors, including a demographic shift toward an increase in the average age of the general population over the study period and the advent of life-prolonging cancer treatments.

2.4 Discussion

2.4.1 N-Nitrosamine Exposure Estimations

Approximate daily *N*-nitrosamine exposure levels were estimated from the data ascertained from the comprehensive literature review and average American consumption habits. Average daily smoking values were estimated as 14.2 cigarettes per day (FSPTCA 2010), average water intake was estimated to be 3 liters per day (Gleick 1998), and average food intake was estimated from the American Heart Association's 2,000-Calorie level dietary guidelines. Daily intake values for food sub-classifications were estimated as 500 grams/day of vegetables, 170 grams/day of meats, and 168 grams/day of fats, sweets and oils (AHA 2016). This estimation of exposure deliberately omitted uptake from personal care products due to the large uncertainties associated with the use and type of personal care products and the highly variable level of *N*-nitrosamines found therein. Results from the *N*-nitrosamine intake estimates are presented in six categories of varying diets and lifestyle choices (Fig. 7).

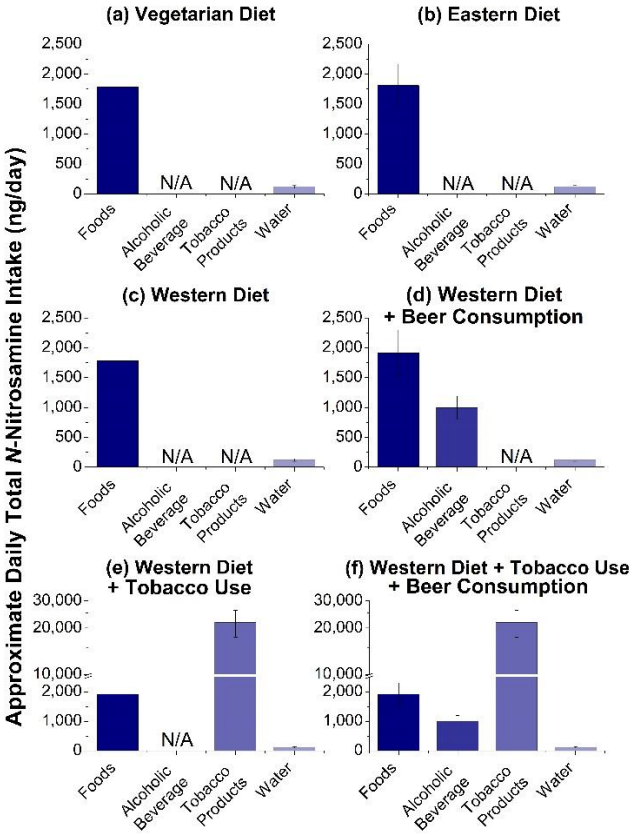


Fig. 7 - Estimations of total N-Nitrosamine exposure (TNE) by diet and lifestyle. Error bars represent +/- 20% of N-nitrosamine daily load from the corresponding source.

Not surprisingly, tobacco use was identified to constitute the largest source of daily *N*-nitrosamine intake across all considered categories, at a rate of 21,800 ± 4,350 ng/day. Uptake of *N*-nitrosamines from food intake, irrespective of dietary choices, was identified as the second largest source of *N*-nitrosamine exposure, with daily intake values ranging from 1,800 ± 350 ng/day (vegetarian diet) to 1,900 ± 380 ng/day (western diet). Consumption of beer or other malt beverages was found to contribute an estimated intake of 1,000 ± 200 ng/day of *N*-nitrosamine, whereas exposure from ingestion of potable water was consistently found to contribute the smallest daily dose of *N*-nitrosamine exposure at a rate of 120 ± 24 ng/day. Thus, individuals subscribing to a western diet, regularly consuming beer, and smoking tobacco are expected to incur the

majority of their daily exposure from tobacco use (88%), with food ingestion (8%), beer consumption (4%), and potable water ingestion (<1%) accounting for the remainder. In contrast, individuals electing a western diet but refraining from alcohol and tobacco consumption would have a 92% lower daily nitrosamine exposure, with the governing factors constituting ingestion of food (94%) and potable water (6%).

2.4.2 Attributable Risk Evaluation

Based on currently available data, we calculated that *N*-nitrosamines contribute 2,600 ± 1,050 and 3,400 ± 1,900 expected lifetime cancer cases per 1 million people in the U.S. from exposure through ingestion and inhalation pathways, respectively. This lifetime cancer incidence rate translates into 840,000 ± 340,000 and 1,100,000 ± 610,000 lifetime cases for the U.S population as a whole, or a total attributable number of lifetime cancer cases from *N*-nitrosamines of 1,940,000 ± 950,000. When compared to U.S. average cancer incidence rates, these values account for between 1-2% of the expected total lifetime U.S. cancer cases (NCI 2017). Inhalation was found to pose the most significant *N*-nitrosamine associated carcinogenic risk (58%), but the combined risk (42%) from ingestion of food and potable water nearly rivaled this value. Attributable cancer cases due to inhalation were limited to the tobacco-specific *N*-nitrosamines NNN and NNK, as additional congener data in mainstream cigarette smoke was unavailable. A significant number of attributable expected cancer cases from exposure to both NNN and NNK (approximately 450 ± 190 and 3,000 ± 1,700 per 1,000,000 population, respectively) was found, with NNK contributing 87% of the total *N*-nitrosamine risk through the inhalation pathway. This is due to the higher inhalation slope factor of NNK (19.2 $(mg/kg\text{-}d)^{-1}$) compared to that of NNN (1.4 $(mg/kg\text{-}d)^{-1}$). Attributable carcinogenic

risk due to ingestion of *N*-nitrosamines was dominated by the two congeners NDEA (55%) and NDMA (43%), and to a much lower extent NDBA (1.5%) (Fig. 8). The combined additional congeners, NMOR, NMEA, NPYR, and NDPA, accounted for less than 1% of the total attributable *N*-nitrosamine cancer risk. A similar analysis of attributable cancer risk from dermal exposure was attempted, but uncertainties regarding the parameters used in the equations made the values obtained from this analysis impractical.

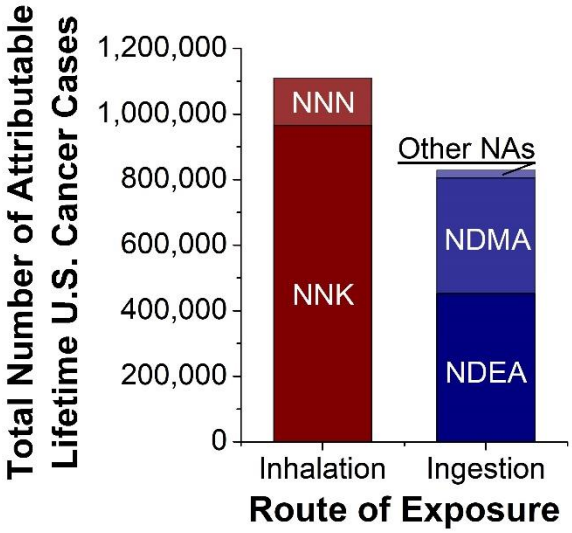


Fig. 8 - Number of expected cancer cases in the U.S. attributable to ingestion and inhalation of the N-nitrosamine class of emerging contaminants. "Other NAs" refers to the combined attributable cancer burden posed by: NDBA, NPYR, NMOR, NMEA, NDPA, and NDPhA.

2.4.3 Reduction in the Daily N-nitrosamine Load

Whereas exposure to *N*-nitrosamines appears to be both pervasive and largely unavoidable, certain lifestyle changes and municipal actions may help to potentially attenuate daily intake. Judging from currently available information, the most important lifestyle choice an individual can make clearly is to abstain from smoking and use of other tobacco products. Notwithstanding the abundance of adverse health effects and

consequences associated with the use of tobacco products, our analysis showed that daily tobacco use contributes a substantial daily concentration (average: 21,800 ± 4,350 ng/day) of tobacco-specific *N*-nitrosamines to users. Daily doses of tobacco-related exposure were calculated to exceed by a factor of 10 the baseline exposure from combined intake of water and food, and are also associated with exposure to the only two nitrosamines that are classified by the IARC as 'known human carcinogens' (Internation Agency for Research on Cancer 2015).

Altering the dietary lifestyle was found to constitute another, less important avenue for reducing the total daily *N*-nitrosamine exposure of individuals, but unlike cessation of tobacco products, this is a more difficult task that involves altering diets and cooking methods. The literature shows meats and fish products to contain notable concentrations of a wide array of *N*-nitrosamines, whose occurrences have been correlated with the use of preservatives (Herrmann et al. 2015) and cooking methods (Drabik-Markiewicz et al. 2009) and, lesser so, with additional factors such as pesticide use (Park et al. 2015). Somewhat unexpectedly, the vegetable food category also was found to be associated with a substantial intake of *N*-nitrosamines (Coffacci et al. 2013, Seo et al. 2015, Tricker et al. 1991b), but these concentrations appear to be dominated by preservatives added to vegetables rather than to chemistry innate to the plant itself (Coffacci et al. 2013). This modeling of exposure was conducted with the assumption that all vegetable food sources contain the average *N*-nitrosamine levels calculated from published data. In reality, some vegetables may contain negligible to no levels of *N*-nitrosamines, whereas others may greatly exceed the average value found through this analysis, leading to a potentially significant variation in exposure levels of individual

consumers. While a substantial portion of the population relies on meat and fish products for protein, intake of alternative, plant-based protein sources may aid in *N*-nitrosamine avoidance. Furthermore, following a teetotaler lifestyle has the potential to reduce daily *N*-nitrosamine exposure even further.

Proteins are rich in nitrogen and thus deserve consideration as potential vehicles of *N*-nitrosamine exposure. To further explore this notion, *N*-nitrosamine exposure from protein sources was examined quantitatively. An average daily protein intake of 51 ± 5 grams was assumed, and the resulting exposure from beef, lamb, pork, poultry, and tofu were estimated. Tofu as a protein source was determined to pose the lowest risk, constituting a *N*-nitrosamine load of 145 ± 10 ng per day per person. This was followed by lamb (1,100 ± 50 ng/day), pork (1,200 ± 75 ng/day), poultry (1,950 ± 230 ng/day), and beef (2,350 ± 350 ng/day). This analysis indicates that adhering to alternative sources of protein other than meat (and the cooking habits associated therewith) can reduce the total daily dose of *N*-nitrosamines an individual incurs. It should also be noted that studies which have examined *N*-nitrosamine contamination in tofu are rare when compared to studies focusing on other protein sources, and data regarding *N*-nitrosamine contamination in other vegetarian protein sources was not available, an important limitation of this analysis.

Careful use and avoidance of certain personal care products also has the potential to significantly reduce daily *N*-nitrosamine exposure, but further research is necessary to gauge how impactful this source of exposure actually may be. This literature review did

not uncover any notable trends regarding specific personal care products which should be avoided.

There also exist multiple sources of *N*-nitrosamine intake that are difficult or impossible to avoid, such as exposure from ingestion, absorption and inhalation of *N*-nitrosamines contained in municipal drinking water (Soltermann et al. 2012). Here, the responsibility for source control and monitoring lies with municipalities, water purveyors and regulatory agencies to protect the public. While advanced water treatment options have shown to remove *N*-nitrosamines and their respective precursors (Farré et al. 2011, Planas et al. 2008, Plumlee et al. 2008), the use of residual chlorine or chloramine within distribution lines may negate whatever TNA reduction may have been achieved upstream in the urban water cycle (Zhao et al. 2006).

Furthermore, manipulation of manufacturing methods, addition of stringent "notification" and "action" levels for contamination, and additional regulations all constitute theoretically viable methods of contaminant control, some of which have previously been shown to lead to risk reduction (EPA 2011). Successful implementation of these methods can be seen through the switch from chlorination to chloramination for the reduction of associated disinfection byproducts (Brodtmann Jr and Russo 1979), and through the reduction of *N*-nitrosamines in alcoholic beverages and beer products from the 1980s to the 1990s (McWeeny 1983).

Considering the high risk posed by these carcinogenic emerging contaminants, municipal regulation of the *N*-nitroso class of compounds at the Federal level within the United States is still slow to evolve. Many *N*-nitrosamine congeners have been included

in the EPA's contaminant candidate list (CCL), but no maximum contaminant levels or goals have been set for the contaminants within the national primary drinking water regulations. In contrast, a number of U.S. states and other countries have adopted action levels, public health goals, and regulatory limits for some *N*-nitrosamine congeners (Appendix A: Table 3). While limits for NDMA appear in all *N*-nitrosamine related regulation, the respective maximum limits of NDMA, as well as regulation of other *N*-nitrosamine congeners vary. Further regulation of a wider suite of *N*-nitrosamines at the federal level has the potential to positively impact the quality of life for millions of Americans, in addition to any economic benefits the implemented actions would entail.

Regulatory oversight in the cosmetics industry could result in a significant daily *N*-nitrosamine reduction – but regulation of *N*-nitrosamines in personal care products would be difficult due to the numerous existing laws and regulations which currently govern the manufacturing and sale of cosmetics and personal care products. *N*-nitrosamines in these media have the potential for human exposure through two pathways: (1) dermal sorption from applied personal care products and cosmetics through the skin (Bronaugh et al. 1981), and (2) black-water and gray-water contamination which introduces large quantities of *N*-nitrosamines to natural and man-made water systems (Shen and Andrews 2011, Zeng and Mitch 2015). Exposure levels due to dermal adsorption (DA) are dependent on many factors, including: type of cosmetic or care product used, volume of product applied, contact time of the product, and the solubility of constituents within product (Bronaugh et al. 1981). Under current U.S. law, no specific tests to demonstrate product safety are required prior to product sales (U.S. Food and Drug Administration 2016). Furthermore, companies are not required to share their

product safety information with the U.S. Food and Drug Administration (FDA) (U.S. Food and Drug Administration 2016). Addressing these product safety loopholes could lead to the reduction of some of the very high *N*-nitrosamine concentrations found in some cosmetic products (up to 49,000 ng/g) (Fan et al. 1977).

2.4.4 Potential Biases

While the results presented in this study are relatively well supported by current literature, it is important to consider the potential biases which may have propagated through this study.

Differing treatment of non-detect values within the studies is a potential concern. While many peer-reviewed articles have suggested multiple ways to treat non-detect values (Kayhanian et al. 2002, Krishnamoorthy et al. 2009), most studies which considered *N*-nitrosamine food concentrations treated non-detects as zero. This may not be a true representation of *N*-nitrosamine concentrations in these foods, and therefore may have indirectly caused an underestimate of the true average *N*-nitrosamine concentrations within food products. Extremely high concentrations of *N*-nitrosamines in food have been reported in the literature. If these values represent “outliers” rather than being representative, actual daily doses may be lower than the numbers presented here. A bias in food products routinely analyzed for *N*-nitrosamine also may propagate bias into this analysis, as monitoring efforts are very limited when considering the large number of food products available to consumers. It is possible that many more food items contain one or multiple *N*-nitrosamines, which would further increase the calculated daily doses and may affect the ranking of the various exposure sources considered here.

While the average *N*-nitrosamine potable water concentration determined through this meta-analysis is in-line with other literature (EPA 2011), this value is subject to some uncertainty due to *N*-nitrosamine formation in water distribution systems. It has been shown that users of municipal water located far downstream from the distribution point have significantly higher *N*-nitrosamine concentrations in their drinking water than users closer to the treatment plant (Zhao et al. 2006). Furthermore, few studies have examined *N*-nitrosamine concentrations in additional sources of potable water, such as bottled water or water subjected to point-of-use treatment. These factors suggest that the average exposure due to ingestion of potable water could be orders of magnitude higher for certain individuals based upon unreported factors such as affluence, or distance from the drinking water treatment plant. Similar to the literature centering on *N*-nitrosamines in food, most literature dealing with water-related contamination focused on a small percentage of the total *N*-nitrosamine congeners in their analyses, which impedes the ability to fully understand the overall impacts (both environmentally and healthwise) of the *N*-nitroso class. The omission from monitoring efforts of congeners potentially present and important frequently included but was not limited to NNN, NNK, and NDELA.

2.4.5 Future Scope

One aspect of *N*-nitrosamine exposure which was not examined in this analysis, but could have a large impact on nitrosamine exposure, is the *in vivo* microbial formation of *N*-nitrosamines within the gut and microbiome. Studies have shown that *in vivo* nitrate reduction to nitrite can increase the formation of mutagenic *N*-nitrosamines within the human body (Lundberg et al. 2004). Intake of high-protein and low-carbohydrate diets

have been hypothesized to alter the microbiome community and change intestinal fermentation, subsequently leading to increased levels of hazardous metabolites such as *N*-nitrosamines (Schwabe and Jobin 2013). This area of research represents a potentially important but poorly understood avenue of human exposure of *N*-nitrosamines.

N-Nitrosamine contamination in personal care products was also identified as an area where a significant push in research is needed. While some product concentrations and experimental dermal sorption values for select *N*-nitrosamines are available, the data is not sufficient enough to perform a meaningful analysis on the carcinogenic impact of using cosmetics and personal care products. It is possible that exposure due to these sources could exceed that of exposure from ingestion of food or water. Research literature has also shown that the *N*-nitroso class is likely much more pervasive than was previously thought, as contamination in unlikely media such as sediments (Gushgari et al. 2016), biosolids (Venkatesan et al. 2014), and fog particles (Wang et al. 2015) has been observed. For this reason, it is important for *N*-nitrosamine monitoring studies to further explore environmental matrices where contamination currently is not suspected or thought to be improbable.

2.5 Conclusion

N-nitrosamines are a diverse class of chemicals that feature over 300 congeners of known or suspected carcinogenicity. Environmental contamination with *N*-nitrosamines is widespread, including tobacco smoke, food, drinking water and personal care products as important exposure sources. Uptake of *N*-nitrosamines in humans occurs primarily through inhalation and ingestion routes, resulting in average total daily doses of 21,800 ±

4,350 ng/day in the U.S. Individual exposure burdens are known to vary significantly as a function of lifestyle choices such as smoking, elected diet and use of personal care products. In the U.S., approximately 6,050 ± 2,950 cases of cancer per one million people are expected to result from everyday exposure. This lifetime cancer incidence rate translates into 1,940,000 ± 950,000 cases per year for the U.S population of 323.1 million people, making *N*-nitrosamines to account for about 1-3% of all cancer cases observed in the nation. Avoidance of exposure to *N*-nitrosamine is possible through interventions at the federal, state, municipal, commercial and individual level, with simple interventions such as foregoing smoking and drinking leading to intake reductions of 88% and 4%, respectively. While personal care products have been hypothesized to represent a significant contributor to daily *N*-nitrosamine exposure, currently available data do not allow a calculation of the attributable risk from this source of exposure. Future research directions to explore include the monitoring of *N*-nitrosamines specific to personal care products (e.g., to NDELA), and an integration of these and other congeners into risk assessments.

TRANSITION 2

Average human exposure to the *N*-nitrosamine class of contaminants has been shown to account for a significant carcinogenic risk which potentially could be measured through UMM. Before UMM is applied to estimate human *N*-nitrosamine exposure it is important to understand the extent of environmental *N*-nitrosamine contamination. Due to the hydrophilic nature of the compounds they were not thought to partition onto solid matrices adjacent to natural and manmade watercourses. Municipal sewage sludge (biosolid) *N*-nitrosamine contamination was only recently shown through a comprehensive examination (*n*=74) of U.S. biosolid samples. The low *N*-nitrosamine method detection limits (0.06-5.7 ng/g dw) by LC-MS/MS allowed for the frequent detection at sub-parts per billion levels and called for further work in identification of *N*-nitrosamine contamination within additional solid matrices which exist within both natural and manmade watercourses.

In Chapter 3, the pervasion of eight IARC classified *N*-nitrosamines (*N*-nitrosodibutylamine, *N*-nitrosodiphenylamine, *N*-nitrosopyrrolidine, *N*-nitrosodimethylamine, *N*-nitrosomethylethylamine, *N*-nitrosodiethylamine, *N*-nitroso-di-n-propylamine, and *N*-nitrosopiperidine) was examined in 40 freshwater sediment samples upstream and downstream of U.S. wastewater treatment plants. Analyzed samples were obtained from the top 10 cm of surficial freshwater sediments. Samples were collected from the Southern, Midwestern and Western U.S. during spring, fall and winter seasons, respectively. *N*-Nitrosamines were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) featuring low method detection limits (0.06-5.7

ng/g dw). A principal component analysis (PCA) was also conducted using a comprehensive water quality dataset to identify water quality parameters which correlate with the occurrence of *N*-nitrosamines in freshwater sediments.

CHAPTER 3

OCCURRENCE OF *N*-NITROSAMINES IN U.S. FRESHWATER SEDIMENTS NEAR WASTEWATER TREATMENT PLANTS

ABSTRACT

In the present study, 40 freshwater sediments collected near 14 wastewater treatment plants (WWTPs) across the United States were analyzed for eight *N*-nitrosamines by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Three *N*-nitrosamines were detected for the first time in freshwater sediments in units of ng/g dry weight at the specified detection frequency: *N*-nitrosodibutylamine (NDBA; 0.2-3.3; 58%), *N*-nitrosodiphenylamine (NDPhA; 0.2-4.7; 50%), and *N*-nitrosopyrrolidine (NPYR; 3.4-19.6; 18%). At least one *N*-nitrosamine was detected in 70% (28/40) of sediments analyzed. Non-detect values in units of ng/g dw were obtained for *N*-nitrosodimethylamine (NDMA; <10.2), *N*-nitrosomethylethylamine (NMEA; <1.7), *N*-nitrosodiethylamine (NDEA; <3.9), *N*-nitroso-di-*n*-propylamine (NDPA; <1.7), and *N*-nitrosopiperidine (NPIP; <3.6). Principal component analysis specifically points to two of multiple potential pathways explaining *N*-nitrosamine occurrences in sediment: NDBA and NDPhA were positively correlated with bulk water ammonia and pH levels, and NPYR with sediment content of organic carbon and iron. Interestingly, *N*-nitrosamine occurrences up- and downstream of WWTPs were statistically indistinguishable (p >0.05). This is the first report on the occurrence of the carcinogenic *N*-nitrosamines NDBA, NDPhA, and NPYR in U.S. freshwater sediments. Discovery of this phenomenon

warrants further research on the compounds' origin, environmental persistence, aquatic toxicity, and risks posed.

3.1 Introduction

N-Nitrosamines are a large group of emerging contaminants of ecological and human health concern due to their carcinogenic potential. Over 300 congeners have been reported and may affect humans through a number of different exposure routes, including ingestion of food and water, use of tobacco products, occupational exposure, and the use of certain cosmetic or pharmaceutical products (Hecht 2014a). *N*-Nitrosamine sources in industrial, commercial and residential settings are known to increase the quantities detected in raw wastewater (Krauss et al. 2009). The International Agency for Research on Cancer (IARC) has classified 24 different *N*-nitrosamines with respect to their carcinogenic potential to humans. Two of these, *N*-nitrosonornicotine and 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, have been classified as Group 1 known human carcinogens (IARC). The remainder are classified as either probably carcinogenic to humans (Group 2A), possibly carcinogenic to humans (Group 2B), or not yet classifiable (Group 3) (IARC). Although the United States Environmental Protection Agency (U.S. EPA) recognizes the existence of sub-populations at risks of multiple exposures, quantifying the latter remains challenging and incomplete due to the ubiquitous nature of *N*-nitrosamines (Regulations 1980). As of today, a total of five *N*-nitrosamines are included in the U.S. EPA Contaminant Candidate List 3 (CCL-3), and the same five have been listed again in the U.S. EPA CCL-4 draft: these are *N*-nitrosodiethylamine (NDEA), *N*-nitrosodimethylamine (NDMA), *N*-nitroso-di-n-

propylamine (NDPA), *N*-nitrosodiphenylamine (NDPhA) and *N*-nitrosopyrrolidine (NPYR) (2014, Venkatesan et al. 2014).

One area of increasing concern is the unintentional formation of *N*-nitrosamines as disinfection byproducts (DBP) in drinking waters. For instance, chlorination and chloramination of waters containing secondary and tertiary amines can result in the formation of NDMA, a widely studied probable human carcinogen (2014, Mitch and Sedlak 2002, Wang et al. 2011). Other documented or hypothesized pathways of *N*-nitrosamine formation include generation in waters containing ammonia, organic nitrogen, or other inorganic nitrogenous substances; UV-induced formation in the presence of chlorinated dimethylamine and monochloramine; and formation via oxidation of dimethylacetamide to hydroxylamine in ozonation processes (Lee and Westerhoff 2009, Oya et al. 2008, Soltermann et al. 2012, Yang et al. 2009).

Because of the lower partitioning coefficients of most *N*-nitrosamines, it is generally believed that *N*-nitrosamine occurrence is limited to only aqueous matrices; therefore, little research has been conducted on the occurrence and sorption behavior of these compounds in solid environmental matrices. A recent study reported the occurrence of eight *N*-nitrosamines in nationally representative U.S. biosolids samples with a detection frequency of 88%, suggesting either *in situ* formation or sorption of *N*-nitrosamines to biosolids during secondary or sludge treatment in wastewater treatment plants (WWTPs) (Venkatesan et al. 2014). Detection frequency of *N*-nitrosamines in sludge samples linearly correlated with their *n*-octanol water partitioning coefficient (K_{OW}), suggesting hydrophobic sorption as a mechanism governing *N*-nitrosamines

accumulation in solid environmental matrices (Venkatesan et al. 2014). Numerous *N*-nitrosamines and their secondary amine precursors have been detected in WWTP effluent, as well as in wastewater-impacted aquatic environments (Akyuz and Ata 2006, Boyd et al. 2011, Krauss and Hollender 2008, Schreiber and Mitch 2006). It has also been shown that common water constituents, such as the presence of ammonia and chloramine, can increase the rate of *N*-nitrosamine formation (Kristiana et al. 2013). Motivated by a lack of data on *N*-nitrosamine occurrences in freshwater bed sediments proximal to WWTP discharges, we screened for the following eight *N*-nitrosamines: NDMA, *N*-nitrosomethylethylamine (NMEA), NDEA, NDPA, *N*-nitrosodibutylamine (NDBA), NPYR, *N*-nitrosopiperidine (NPIP), NDPhA. To the best of our knowledge, only one peer-reviewed study exist in the literature that reports the occurrence of NDMA in bulk sediments acquired from the Calumet River (Indiana Harbor, Indiana) (Hoke et al. 1993). Reported NDMA concentrations from this study ranged from 0.16 to 1.69 mg/kg dry weight with a detection frequency of 60% (n = 10) (Hoke et al. 1993). Another study that examined the extraction methods of *N*-nitrosamines in solid matrices screened for *N*-nitrosamines in freshwater sediments (n = 4), but were not able to detect levels above the method detection limit of the study (Jurado-Sánchez et al. 2013). In order to address this important knowledge gap, the objectives of the present study were to: (i) quantify and provide the first occurrence data of eight *N*-nitrosamines in freshwater bed sediments collected near WWTPs from three geographically distinct regions of the United States; and, (ii) apply principal component analyses of water and sediment quality parameters to inform on potential mechanisms explaining any given detections of harmful *N*-nitrosamines in freshwater sediments.

3.2 Materials and methods

Analytical standards of *N*-nitrosamines and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), including NDMA, NMEA, NDEA, NDPA, NDBA, NPYR, NPIP, NDPhA, dichloromethane (DCM) (HPLC grade), methanol (LC-MS grade), water (HPLC grade), ammonium acetate, and acetic acid. The deuterated isotopes NDMA-d_6, NDPA-d_{14} and NDPhA-d_6 were purchased from Cambridge Isotope Laboratories (Andover, MA). The deuterated isotope NPIP-d_{10} was purchased from C/D/N Isotopes Inc. (Quebec, Canada).

3.2.1 Sediment samples

Grab samples were collected from the top 10 cm of surficial freshwater sediments in 40 U.S. locations near 14 WWTPs between 2009 and 2015. Samples were collected from the Southern, Midwestern and Western U.S. during spring, fall and winter seasons, respectively. Due to confidentiality agreement with municipalities, the location of the facilities is not revealed in the present study and spatial analyses were not part of the scope of work. After collection, samples were stored in amber glass jars at -20 ^{o}C until analysis. The flow volume processed by the sampled WWTPs varied: three processed <3.8 million liters per day (ML/d), seven between 3.8 and 38 ML/d, two between 38 and 380 ML/d, and two treated >380 ML/d. The majority of sediment samples (39 of 40) were collected within 3,000 m of the corresponding WWTP, either upstream (30% of the samples) or downstream of the plant (60% of the samples).

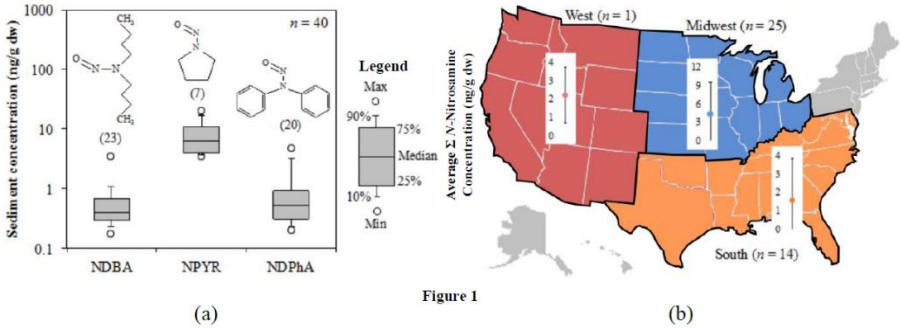


Fig. 9 - (a) Box and whisker plot of concentrations of N-nitrosamines in n samples of freshwater sediments collected near 14 U.S. wastewater treatment plants. Numbers above the boxes represent number of detects. (b) U.S. regional map showing average N-nitrosamine concentration for corresponding U.S. regions and the number of samples analyzed per region (n). Bars represent the standard deviation of the mean of all samples analyzed in a specific region of the U.S.

3.2.2 N-Nitrosamine analysis

Sediments were spiked with deuterated surrogates and extracted using dichloromethane (2 mL per g of sediments) utilizing an isotope dilution method similar to the method described previously for sludge, with slight modifications [5]. The extract was then subjected to analysis by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) in positive ionization mode. A detailed explanation of the extraction method is available in the supporting information.

3.2.3 Statistical analysis

Analytical data of detected *N*-nitrosamine concentrations were analyzed for interrelations with various physicochemical properties of the study locations by performing a principal component analysis (PCA) using version 21 of the IBM SPSS software package (IBM, Armonk, New York, U.S.). Water and sediment quality parameters used in PCA were available by public record for the sampling location and time for 25 of the 40 samples analyzed in the present study.

3.3 Results and discussion

3.3.1 Method performance

Method detection limits (MDLs) for the various *N*-nitrosamines ranged from 0.1 to 10.2 ng/g dw (Appendix B: Table 4). Method detection limits were determined based upon USEPA guidelines described in 40 CFR 136, Appendix B (USEPA 1984). Process control samples and blanks showed no laboratory contamination. Method performance and analyte detection were further confirmed by performing matrix spike experiments in selected samples showing positive detections of *N*-nitrosamines (Appendix B: Fig. 18). Final, normalized recoveries of analytes determined with deuterated surrogates ranged from 78 to 88%. Absolute recoveries for all analytes ranged from 54 to 108% and were consistent with values previously observed for complex environmental matrices (Venkatesan et al. 2014). A complete list of absolute recoveries, relative recoveries and method detection limits for each screened *N*-nitrosamine is available in Appendix B: Table 4 of this document. Analysis of archived samples over a period of eight months did not show any significant degradation or formation of *N*-nitrosamines under the storage conditions chosen (see SI for more info). Concentrations and detection frequencies reported for NPYR and NDBA in sediments should be considered conservative, *i.e.*, lower-bound estimates of the true value, because the concentrations were determined without labeled isotopes to correct for analyte losses and ion suppression during sample processing and analysis, respectively. Analysis precision expressed as relative percentage difference (RPD) was good at less than 20% (average) for NPYR and NDPhA, and slightly less favorable for NDBA at 38%. Similar RPD values (18 to 66%) have been reported in literature for organic and inorganic contaminants in sediments, and this high

value may be explained by the non-homogenous nature of wet sediment samples (Barber and Writer 1998, Gilmour et al. , Venkatesan et al. 2014).

3.3.2 Occurrence of N-nitrosamines in U.S. freshwater sediments

Out of the 40 freshwater sediment samples analyzed, 70% tested positive for at least one *N*-nitrosamine. Three of eight targeted *N*-nitrosamines were detected in freshwater sediments in three geographical regions of the United States (Fig. 9): NDBA, NPYR, and NDPhA. This is the first study to report the occurrence of the three aforementioned *N*-nitrosamines in freshwater sediment samples. The remaining smaller aliphatic *N*-nitrosamines (NDMA, NMEA, NDEA, NDPA and NPIP) were absent from all samples analyzed or present only at levels below the corresponding MDLs (Appendix B: Table 4). The most frequently detected *N*-nitrosamine was NDBA [58% detection frequency (DF)], with a concentration range of 0.2-3.3 ng/g dw, followed by NDPhA (50% DF) and NPYR (18% DF) with a concentration range of 0.2-4.7 ng/g dw and 3.4-19.6 ng/g dw, respectively (Fig. 9).

3.3.3 Potential sources of detected N-nitrosamines

The higher detection frequency observed for NDBA and NDPhA may be explained in part by the relatively higher potential for hydrophobic sorption of these two *N*-nitrosamines (log K_{OW} of 2.63 and 3.13, respectively). Similar partitioning properties of the corresponding secondary amines serving as precursors of these two *N*-nitrosamines (*i.e.*, dibutylamine and diphenylamine featuring log K_{OW} values of 2.83 and 3.50, respectively) also may have played a role; precursors accumulated in sediments may increase opportunities for *in situ* formation of the two *N*-nitrosamines detected. In

contrast to NDBA and NDPhA, NPYR has no hydrophobic sorption potential (log K_{OW} = -0.19), yet it was still detected in 18% of sediment samples analyzed. *In situ* formation from unknown precursors is one potential explanation for NPYR occurrences in sediments. A number of research studies have reported detectable concentrations of aliphatic and aromatic amines (including dibutylamine, pyrrolidine, and diphenylamine) in effluent discharges, raw waters (rainwater and ground water), surface waters and both saltwater and freshwater sediment samples (Akyuz and Ata 2006, Sacher et al. 1997, Wang et al. 2011, Wang and Lee 1990). Amine-containing pharmaceuticals and personal care products (PPCPs) also have been found to increase the occurrence of aliphatic and aromatic amines near WWTP effluent discharge locations, and thus have been linked to *N*-nitrosamine DBP formation (Shen and Andrews 2011). It should also be noted that microbes, such as fecal streptococci, has been shown to produce *N*-nitrosamines in the presence of nitrites and secondary amines (Ayanaba et al. 1973, Collins-Thompson et al. 1972, Okolie 2005). These conditions are typical in natural open water systems and could account for another potential pathway of *N*-nitrosamine sediment contamination. Since secondary amines have been detected in surface waters and have been shown to serve as precursors in the formation of stable *N*-nitrosamines (Mitch and Sedlak 2002, Padhye et al. 2011), *in situ* formation of *N*-nitrosamines in sediments of surface waters containing secondary amines is a plausible mechanism deserving future studies.

Because of the variation of *N*-nitrosamine levels detected at different sample locations, it is reasonable to hypothesize that *N*-nitrosamine formation is not due to one factor alone, but a result of many different chemical reactions. For this reason, it is also important to consider the atmospheric photo-oxidation of amines as a potential loading

source. One study showed that NDMA and *N*-nitrodimethylamine can be produced through atmospheric photo-oxidation of *N*-methylformamide and *N*,*N*-dimethylformamide (Bunkan et al. 2015). It is possible for these degradation products to sorb onto particulate matter and contribute to sediment contamination.

Although at least one *N*-nitrosamine was detected in 70% of the sediment samples, the individual *N*-nitrosamines and concentrations varied across the different sediments. Diverse and distinct sources could be at play, including unique treatment processes used in WWTP near the studied locations, river sediment-water composition, and contribution from other non-point sources. With respect to the treatment processes of the studied locations, 88% of the sampled WWTPs which employed a chlorination and de-chlorination disinfection process (n = 8) tested positive for at least one *N*-nitrosamine in the neighboring sediment samples. However, detectable levels of *N*-nitrosamines also were found in 50% of sediment samples from areas near WWTPs utilizing UV disinfection (n = 4). Although UV radiation is known to effectively degrade *N*-nitrosamines, irradiation with UV light also has been shown to result in the formation of NDMA in the presence of chlorinated dimethylamine and monochloramine (Soltermann et al. 2012). Therefore, both disinfection processes could potentially serve as a source to *N*-nitrosamine contamination in sediments.

A comparison of total detected *N*-nitrosamine concentrations between paired upstream and downstream sediment samples within 3,000 m from a WWTP showed neither appreciable differences (p = 0.42; 95% CI) nor any visual trends (Fig. 11). Additional reference sites (samples taken from distances greater than 3,000 m) also

showed varying levels of *N*-nitrosamine concentration. Determination of *N*-nitrosamine levels in the sediment sample local to the treated effluent discharge location of one WWTP showed no detectable concentrations of any of the three *N*-nitrosamines detected in other sediment. This observation and the detection of *N*-nitrosamines upstream of WWTPs suggest the existence of other, additional non-point sources of *N*-nitrosamines to sediments. For instance, urine has been shown to contain high levels of *N*-nitrosamines (NDMA, NPIP and NPYR) as well as ammonia (Mostafa et al. 1994, van Maanen et al. 1996) and thus represents one potential source of both surface and groundwater *N*-nitrosamine contamination (Ma et al. 2012). Urine may originate from human sources or wildlife. Discharge of untreated domestic wastewater at the study locations may have served as a source of *N*-nitrosamines in upstream sediments. The role of WWTPs in *N*-nitrosamine formation is important to consider and could contribute to *N*-nitrosamine loading in sediments, but results obtained here suggest that these may not necessarily be the most important sources contributing to the *N*-nitrosamine levels discovered. Regardless, it is still important to consider WWTP's as a source of *N*-nitrosamines in sediments due to the numerous reports of multiple *N*-nitrosamine detection in WWTP effluents (Krasner et al. 2009, Planas et al. 2008, Schreiber and Mitch 2006). Formation of *N*-nitrosamines in aquatic environments is a complex process that is still not yet fully understood and may vary in different environments due to differing environmental and anthropogenic loading factors. Sediment based microbial communities have been shown to induce degradation of NDMA precursors (which helps explain the lack of NDMA in our samples), but microbial presence and activity is highly dependent on water conditions (Woods and Dickenson 2016). Under appropriate conditions, it has been hypothesized

that sediments could be responsible for releasing NDMA precursors into water systems (Woods and Dickenson 2016). Despite a growing body of research findings on the formation and degradation of some *N*-nitrosamines such as NDMA, information on the occurrence and origin of other *N*-nitrosamine compounds is still limited. Therefore, further research regarding the complex formation of these contaminants is necessary, including some of the *N*-nitrosamines reported on in this work.

3.3.4 Principal component analysis of data relating to nitrosamine occurrences in sediments

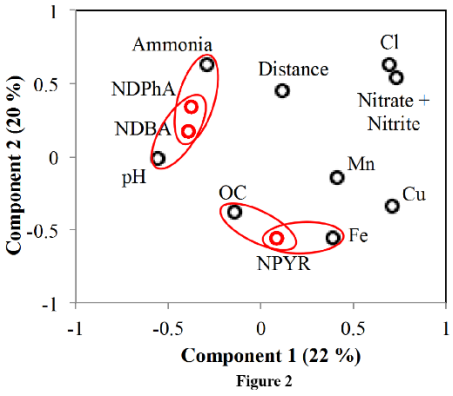


Fig. 10 - Principal component analysis of the N-nitrosamine levels and parameters of sediment and water quality from the corresponding sampling locations. The first two principal components accounted for 42% of total variance in the dataset. Highlighted circles represent clusters of parameters correlating with each other. OC – organic carbon fraction of sediments; Distance from the corresponding WWTP; concentrations of ammonia, nitrate + nitrite and chloride in surface water; pH of surface water; transition metals (Fe, Cu. Mn, Mo, Ni, V, Zn) concentration in surface water.

A PCA was performed using obtained concentration data in conjunction with water and sediment quality parameters of the study locations for which secondary data sources were available (n = 25), *i.e.*, water pH, distance of sampling site from WWTP (with upstream distance used as negative distance), organic carbon fraction (OC) of sediments, water concentration of ammonia, nitrate + nitrite, chloride and transition

metals – Fe, Cu, Mn, Zn, Ni, V, Mo (Fig. 10). The first two principal components explained the highest amount of variance in the dataset and combined accounted for 42% of the total observed variability. PCA determined that NDBA and NDPhA correlated positively with both bulk water pH and ammonia concentration (Fig. 10). Two different pathways of deposition or formation of *N*-nitrosamines in sediments may be envisioned based on the findings: a positive correlation of NPYR with OC suggests that its occurrence in sediments may be due to hydrophobic partitioning or electrostatic sorption following release of unidentified inputs that may include untreated wastewater; whereas hydrophobic interactions must be viewed as being a less likely occurrence pathway given the n-octanol-water partitioning coefficient of NPYR (Appendix B: Table 6), in conjunction with for example electrostatic interactions it may still be worth considering. Alternatively, organic carbon, which is well known to play a role in the formation of NDMA (Krasner et al. 2009, Kristiana et al. 2013), may have promoted the formation of NPYR in sediments through similar *in situ* reactions.

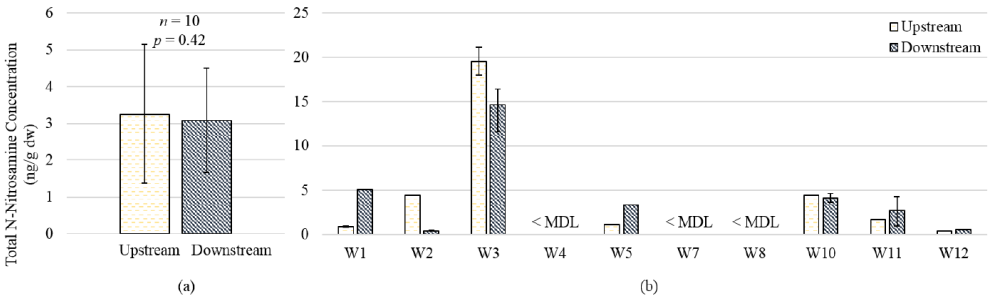


Figure 3

Fig. 11 - Comparison of concentrations of the sum of N-nitrosamines detected in paired sediment samples obtained within a distance of 3,000 m upstream and downstream from WWTP discharge locations; shown are average total N-nitrosamine concentrations (Panel A) and data pairs for individual sampling locations (n = 10; Panel B).

The effluent of WWTPs is known to contain detectable concentrations of *N*-nitrosamines as well as precursors and promoters of *N*-nitrosamine formation, including chlorine, ammonia, and secondary amines (Chen et al. 2009, Gersberg et al. 1986, Krasner et al. 2009). Chlorine and ammonia can react to form chloramines, which have been found to produce significantly greater yields of *N*-nitrosamines than chlorine alone (Kristiana et al. 2013). In surface waters with high concentrations of ammonia, residual chlorine would be more readily transformed to chloramine, which in turn would account for a higher transformation of secondary amine precursors to *N*-nitrosamines. Also, degradation or formation of NDMA is known to be influenced by pH conditions (Xu et al. 2009), an important aspect to consider when interpreting the observed positive correlation of NDPhA and NDBA with both ammonia concentration and pH of the bulk water.

Interestingly, the PCA determined that NPYR concentrations also correlate with bulk water iron content. Few studies have examined the role of transition metals in the formation of *N*-nitrosamines (Challis et al. 1978, Lunn and Sansone 1994, McCleverty 1977, Shehad 1993). Based on the observed chemical relationship between $[\mathrm{Fe(CN)_5(NO)}]^{2-}$, ammonia, aliphatic amines and a high pH, it has been hypothesized that under similar conditions secondary amines could undergo transformation to metal-bound *N*-nitrosamines (McCleverty 1977). Another study found a correlation between the rate of *N*-nitrosamine formation and the presence of metal salts (Challis et al. 1978). Reaction rates of NPIP increased substantially when metal salts (ferric nitrate and cupric chloride) were introduced to the system (Challis et al. 1978). Similarly, a patent focusing on the inhibition of *N*-nitrosamine formation suggests that some transition metals may

play a role in the formation of *N*-nitrosamines (Shehad 1993), although the pathway and mechanisms are not yet fully understood. Furthermore, absence of iron was found to inhibit cancerous growths known to be triggered by certain *N*-nitrosamines (Toyokuni 1995), suggesting a potential role of iron in carcinogen formation and adverse effects. Some metal nitrosyl compounds have been found to also react with both primary and secondary amines, promoting *N*-nitrosamine formation (Lee et al. 2001); these reactions are typically coupled with high temperature and pressure conditions, and thus may not be applicable to aquatic environments. Nevertheless, the observed PCA correlation makes transition metals an important consideration in determining the possible pathways of *N*-nitrosamine formation, with primary amines acting as chemical precursors in addition to secondary amines. Thus, the correlation between NPYR occurrence and sediment iron content is plausible, but more research is needed to substantiate or refute causal associations.

3.3.5 Carcinogenic potential and aquatic toxicity of N-nitrosamines in sediment porewater

To understand the potential carcinogenic potential of the reported *N*-nitrosamines residing in sediments, cancer potency values for NDBA, NDPhA and NPYR were obtained from the California Office of Environmental Health Hazard Assessment (OEHHA). Inhalation/oral slope factors for the three reported *N*-nitrosamines are as follows: 9.0 μg/kg-day (NDPhA), 2.1 mg/kg-day (NPYR), and 11 mg/kg-day (NDBA) (OEHHA 2009). At face value, *N*-nitrosamines in freshwater sediments may not pose an immediate cancer risk to humans because there is no direct route of oral or respiratory exposure; however, *N*-nitrosamine levels in waters in contact with the contaminated

sediments have the potential to create an indirect route of oral exposure. Furthermore, these areas are commonly used for recreational water uses (e.g., camping, swimming), which may contribute to inadvertent human exposure to *N*-nitrosamines from either accidental or deliberate water ingestion. In order to further quantify the carcinogenic risk, the pore water *N*-nitrosamine concentrations were estimated from the sediment concentrations by using equation 3:

$$C_{pore} = \frac{C_{sed}}{K_{OC} f_{OC}} \qquad \text{Eq. 3}$$

K_{OC} values were calculated using the equation: $\log K_{OC} = 0.72 * \log K_{OW} + 0.49$ (Essington 2015). Mathematically, average sediment pore water concentrations were determined to be: 0.063 µg/L (NDPhA, 0.004-0.31 µg/L), 0.092 µg/L (NDBA, 0.022-0.51 µg/L), and 201 µg/L (NPYR, 45.7-813.1 µg/L). When compared to the OEHHA data, it can be determined that these pore water levels alone do not exceed the given inhalation/oral exposure slope factors – but could still contribute to overall *N*-nitrosamine exposure. When compared to the California State Water Resource Control Board's *N*-nitrosamine notification limit of 10 ng/L (applicable to NDEA, NDMA and NDPA), it becomes clear that the carcinogenic potential of these sediment-based *N*-nitrosamines needs to be evaluated further.

Also, the toxicological implications of *N*-nitrosamine occurrences in freshwater sediment deserve a brief discussion in this study. Presently available LC_{50} values for a number of aquatic species seem to span a wide range (330 mg/l for *Gammarus limnaeus* versus 1,365 mg/l for *Dugesia dorotocephala*), and are several orders of magnitude higher than the pore water concentrations reported here (Draper and Brewer 1979). These

LC_{50} values are significantly higher than reported concentrations in sediments or waters, and thus suggest no imminent threat to aquatic organisms (Brooks and Wright 2008, Draper and Brewer 1979, Jurado-Sánchez et al. 2010, Poste et al. 2014, Regulations 1980). Certain chronic effects have been associated with NDMA and NDEA exposure, including hepatocellular carcinomas in rainbow trout *(Oncorhynchus mykiss)*, antennal gland degradation and hyperplasia of tubular cells in crayfish *(Procambarus clarkia)*, and adverse growth effects and DNA damage in multiple species of green algae (Brooks and Wright 2008). Severely toxic cellular and tissue responses have been shown in the Sheepshead Minnow *(Cyprinodont variegatus)* and the Japanese Rice Fish (*Oryzias latipes)* when subjected to contact with NDEA spiked waters (Hinton et al. 1988). Not all *N*-nitrosamines have been tested for their toxicity and for adverse mixture effects in aquatic ecosystems; and while they do not appear to pose a threat in terms of acute toxicity, chronic toxicity problems may be significant (Draper and Brewer 1979, Poste et al. 2014).

3.3.6 Study limitations, data gaps, and research needs

While providing important data on freshwater sediment quality, this study also featured a number of limitations. Data analysis was hampered by a lack of information on WWTP locations, lack of water quality quantification at certain test sites, unit operations employed, and treatment levels achieved for certain water quality parameters. Also, the number of nationwide sediment samples analyzed here was limited, rendering the representativeness of the study for the United States overall vulnerable to potential bias. The age of sediment samples analyzed in the present study varied between less than a few days to 5 years. To test for the stability of *N*-nitrosamines during storage at – 20°C,

freshly obtained sediment was analyzed repeatedly over eight months. Obtained data showed no appreciable differences in detectable concentrations of NDBA and NDPhA at the beginning and end of the experiment (8 months after sample collection), and the remaining six *N*-nitrosamines consistently showed non-detect values (<MDL) throughout the storage experiment (Appendix B: Fig. 19). Similar stability tests performed by our group in the past for sewage sludge samples using a methodology identical to that of the present study also had shown no significant differences in *N*-nitrosamine levels during prolonged storage (Venkatesan et al. 2014). Importantly, detection of NDBA, NPYR and NDPhA in freshly obtained sediments precludes in-storage generation of *N*-nitrosamines as a viable explanation of the contaminant occurrences reported here.

Though PCA analysis of the dataset provided some important correlations observed between sediment/water quality parameters and *N*-nitrosamine levels, further research is needed to confirm the role of such parameters. The determination of the formation pathways and mechanism was beyond the scope of the present study, and thus formation and partition pathways discussed in this study should be viewed as only potential pathways but constitutes an important research need. Carcinogenic potential of *N*-nitrosamines discussed in this paper are estimates based on values from present literature available and represents another important gap in the understanding of *N*-nitrosamines that needs to be filled. Since only one sample per sampling location was collected, a detailed analysis with respect to temporal and seasonal variability was not conducted. Future research on *N*-nitrosamine occurrence in aquatic environments would benefit from accounting for these variables. Furthermore, future research should focus on the environmental impacts of these *N*-nitrosamines in freshwater sediments and exposed

biota. Such work will be essential to further the currently limited understanding of the overall implications of the occurrence of these contaminants in U.S. aquatic environments.

3.4 Conclusion

This study constitutes the first report on the occurrence of three *N*-nitrosamines (NDBA, NPYR, and NDPhA) of a total of eight monitored for in freshwater sediments in three geographically distinct regions of the United States. Whereas the origin of this newly discovered contamination is yet uncertain, sharing this new knowledge with the research community is essential due to carcinogenic nature of these pollutants.

The number of *N*-nitrosamines reported in this study accounts for less than 5% of all known *N*-nitrosamine compounds and hence future research should consider additional *N*-nitrosamine analytes of ecological and human health concern. Furthermore, methods to screen for both *N*-nitrosamine and nitramine contamination should be implemented when possible to better understand the origination and routes of formation of these contaminants. Future research also will help determine in greater detail how widespread the occurrence of *N*-nitrosamines in freshwater sediments is in U.S. regions not covered here and internationally, which routes of sediment contamination are important, and what preventive measures can and should be taken to limit source terms and to protect ecosystems and human populations alike.

TRANSITION 3

Substance abuse is a longstanding public health challenge worldwide and the ongoing opioid abuse crisis has had a particularly severe adverse impact on the U.S. population. Many strategies have been proposed to assist in managing the U.S. opioid crisis but could benefit from a technology which produces opioid-related health data in near-real time. Wastewater-based epidemiology (WBE) could be applied to U.S. populations to obtain near real-time opioid use data in a non-invasive and anonymous way but has seen little application within U.S. communities to track narcotic consumption. Smaller communities could see additional benefits from this technology as they are more limited in their resources and would benefit from stronger evidence-formed public health decision making.

In Chapter 4, a two-year longitudinal study identifying concentrations of five opioids (morphine, codeine, oxycodone, fentanyl, and heroin) in raw wastewater was conducted in two Midwestern cities of medium population size (25,000 to 250,000) that previously had reported high rates of opioid abuse. In year two sample screening was expanded to include the opioid metabolites: norcodeine, noroxycodone, norfentanyl, morphine-3-glucuronide, and 6-acetylmorphine. Samples (24-hour time weighted composites) were obtained once per month over a two-year sampling period and analyzed by LC-MS/MS. Opioid concentrations in raw wastewater were compared to wastewater flow and pharmacokinetic values to estimate approximate consumption values for the two cities. Estimated consumption was then compared across the two cities, and to U.S. and international consumption estimates obtained from related literature.

CHAPTER 4

TRACKING OPIOID CONSUMPTION IN TWO UNITED STATES CITIES BY WASTEWATER-BASED URBAN METABOLISM METROLOGY

ABSTRACT

Access to robust near-real time opioid use data is essential to the effective management of the U.S. opioid crisis. Current narcotic data collection methods are limited by time delay and would be complimented by a rapid data acquisition technique. Urban metabolism metrology using wastewater diagnostics potentially offers access to near real-time data on opioid consumption but thus far has seen little application in the United States. From 2015-2017, we analyzed monthly 24-hour time-weighted composites of municipal raw wastewater from two Midwestern U.S. cities using isotope dilution liquid chromatography tandem mass spectrometry. Opioid consumption rates estimated from wastewater analytics were similar compared to reported WBE-based estimates from New York and a nationwide U.S. survey but exceeded reported estimated consumption in Italy, London, Finland, Norway, Spain, Belgium, UK, Netherlands, Switzerland, and Denmark. Opioids were routinely detected in units of ng/L concentrations in effluent from City 1 and 2, respectively, including morphine (713 ± 38; 306 ± 29; detection frequency (DF): 100%), codeine (332 ± 37; 100 ± 27; DF: 93%), oxycodone (17.8 ± 1.1; 78 ± 6; DF: 100%), fentanyl (1.7 ± 0.2; 1.0 ± 0.5; DF: 62%), and heroin (41 ± 16; 9 ± 11; DF: 81%). Opioid consumption rates estimated from wastewater analytics ranged between 9 (fentanyl) and 2,590 (morphine) mg/day/1,000 persons. This long-term U.S.

screening study of opioids in wastewater was the first to identify detectable levels of the powerful synthetic opioid fentanyl in wastewater, and (2) the first U.S. study to identify opioid consumption trends of small cities within the midwestern United States.

4.1 Introduction

The United States is in the midst of an unprecedented opioid epidemic that claims approximately 42,000 U.S. lives annually (Kounang 2017, Rudd 2016, Schuchat et al. 2017) and challenges the development of economic and rapid methods for tracking drug consumption in real-time or near real-time. Opioids were responsible for 67% and 63% of all drug overdose fatalities in 2014 and 2015, with death rate increases from 12.3 to 16.3 per 100,000 population being attributable to increased consumption of heroin (+21%) and the 50-times more powerful synthetic opioid, fentanyl (+72%) (Rudd 2016, Warner et al. 2016). In the U.S., 10.3 million residents reported using prescription opioids for nonmedical purposes in 2014, and a nine-fold increase of young adults using heroin has been observed from 2002 to 2014 (Martins et al. 2017). Correlations between non-medical opioid use and heroin use have also been observed (Compton et al. 2016). While exact percentages vary by study and city, studies cite that between 39% to 86% of heroin users admitted to nonmedical use of pharmaceutical opioids before beginning heroin use (Lankenau et al. 2012, Mateu-Gelabert et al. 2015, Peavy et al. 2012, Pollini et al. 2011, Siegal et al. 2003). Despite recent successful efforts by public health and medical professionals to curb opioid prescription rates (Dowell et al. 2016, Frieden and Houry 2016, Schuchat et al. 2017), drug related overdose deaths have continued to increase in the United States (Katz 2017).

With such widespread opioid use, obtaining relevant information related to opioid consumption is vital to developing effective substance abuse prevention strategies. Current data analysis involves a combination of population surveys, crime statistics, medical records and narcotic seizure data (Zuccato et al. 2008), but these methods are often costly, cumbersome, and may be subject to bias. Wastewater-based epidemiology (WBE) was first proposed in 2001 as a method for obtaining population health metrics (Daughton 2001). In 2005 it was tested as an alternative to current narcotic data collection methods of cocaine use (Zuccato et al. 2005), and since has experienced widespread use in Europe (Baker et al. 2014, Baz-Lomba et al. 2016, Gatidou et al. 2016, Kankaanpää et al. 2014, Lindberg et al. 2005, Postigo et al. 2011, Terzic et al. 2010, Van Nuijs et al. 2011, Vuori et al. 2014, Zuccato et al. 2008, Zuccato et al. 2005), Asia (Kim et al. 2015, Lai et al. 2013), Africa (Archer et al. 2018) and Australia (Lai et al. 2016, Tscharke et al. 2016) in order to obtain anonymous prescription and illicit narcotic consumption data in near-real time. The WBE approach has been further expanded under the umbrella of urban metabolism metrology (UMM), which studies multiple process flows within the natural and built water environment to obtain diagnostic information on activities, sustainability and the health statistics for a human population. Analysis of sample of flow-weighted composited sewage obtained from wastewater treatment plant (WWTP) may provide important epidemiological insights as usage prevalence statistics could theoretically be obtained for any commonly consumed product within a population (Dove 2006). The validity of this technique has been demonstrated through the comparison of wastewater epidemiological analysis of therapeutic drugs and known

amounts consumed by the population (Heberer and Feldmann 2005, Lindberg et al. 2005).

Compared to European and Asian countries, wastewater epidemiological analysis in the United States has seen limited use (Subedi and Kannan 2014). Studies which have examined U.S. wastewaters for drug use prevalence have primarily focused on US DEA schedule I and II narcotics (Banta-Green et al. 2009, Gerrity et al. 2011, Subedi and Kannan 2014). Some U.S. based studies have screened wastewater for prescription and illicit parent opioids and/or metabolites, with positive detections of morphine (Heuett et al. 2015, Subedi and Kannan 2014), codeine (Heuett et al. 2015), oxycodone (Chiaia et al. 2008, Heuett et al. 2015), and heroin (Heuett et al. 2015) being recorded. Despite the recent drastic increase in fentanyl-related deaths (CDC and University 2017), U.S. studies on the occurrence in wastewater of fentanyl are thus far lacking. Furthermore, small U.S. communities have been significantly impacted by the opioid crisis due to additional circumstances which do not impact larger communities, such as: outdated substance abuse infrastructure, shortages in emergency medical technician (EMT) personnel, long travel times of the same, lack of regional coordination, lack of physicians administering programs on substance abuse and medication-assisted treatment, and various administrative barriers (Hancock et al. 2017). Some of these locations have also been identified as areas with strikingly high opioid prescription rates compared to the number of residents within the service area (Whitaker 2017). Therefore, the objective of the present study was to examine opioid abuse trends in two moderately sized (<200,000 population) cities in the American Midwest, a U.S. region that has experienced the highest percentage increase of reported fentanyl abuse from 2014-2015 (CDC 2016).

Analytes investigated included morphine, morphine-3-glucuronide, codeine, norcodeine, oxycodone, noroxycodone, fentanyl, norfentanyl, heroin, and 6-acetylmorphine. The main objectives of the study were to: (i) obtain the first wastewater monitoring data for U.S. fentanyl use prevalence, (ii) to generate for participating municipalities wastewater-based data on opioid use prevalence for informed decision making, and (iii) to benchmark estimated opioid consumption to other previously published wastewater epidemiological literature.

4.2 Materials and Methods

4.2.1 Study locations and wastewater sampling methods

Raw wastewater arriving at central treatment plants in two Midwestern U.S. cities was collected in 24-hour time-weighted composites using automated samplers by WWTP personnel from March 2015 to March 2017. The WWTP of City 1 serves an approximate 130,000 residents, while that of City 2 serves an approximate 45,000 residents. The median age of residents was similar across both cities (City 1: 34.1 years; City 2: 37.1 years), and age distribution also was similar across both cities (Appendix C: Table 7). The percentage of white residents (City 1: 57%; City 2: 86.9%) and African Americans (City 1: 34.5%; City 2: 2.7%) varied across cities, but all other racial demographics were similar. Unemployment rate (City 1: 8.3%; City 2: 3.7%) and per capita income per year (City 1: $14,500; City 2: $29,400) also varied between cities. Average household size, homeowner vacancy rate, and rental vacancy rate were also similar across both cities (USCB 2010). Both cities feature a sewer system designed to separate municipal wastewater from stormwater inputs. Both climate range and reported water use per resident were similar across both participating cities. Sampling occurred on one day per

month during the 24-month study period; the day of collection varied and was entirely at the discretion of sampling personnel. Samples were stored in polyethylene terephthalate (PET) bottles and shipped to Arizona State University in Styrofoam shipping containers containing either ice or dry ice. Upon receipt, samples were stored at -20°C until analysis.

4.2.2 Target analytes

Five parent opioids and their respective metabolites were monitored in raw wastewater. The investigated opioids were morphine (MOR), its major metabolite morphine-3-glucuronide (M3G), codeine (COD), its major metabolite norcodeine (NCOD), oxycodone (OXY), its major metabolite noroxycodone (NOXY), fentanyl (FENT), its major metabolite norfentanyl (NFENT), heroin (HER), and its minor but exclusive metabolite 6-acetylmorphine (6-AM). High purity (>97%) standard solutions of the target compounds originated from Sigma Aldrich (Milwaukee, WI) and were prepared by Cerilliant (Round Rock, TX, USA) as solutions in methanol or acetonitrile. Five deuterated compounds, one for each of the parent opioid target compounds were also purchased from Cerilliant for use as internal standards for quantification, namely: heroin-d_9 (HER-d_9), morphine-d_6 (MOR-d_6), codeine-d_6 (COD-d_6), oxycodone-d_3 (OXY-d_3), and fentanyl-d_5 (FENT-d_5).

4.2.3 Isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS)

Briefly, 200 mL of raw wastewater was loaded onto Oasis HLB 150 mg cartridges (Waters, Barcelona, Spain) at a rate of 1.5 mL/min to determine the analytes measured in positive ionization (PI) mode. Prior to extraction, all wastewater samples were spiked

with a standard mixture of the deuterated compounds at a concentration of 5 ng/mL for HER-d_9, MOR-d_6, COD-d_6, OXY-d_3, FENT-d_5. After samples were loaded, cartridges were washed with water at a rate of 5 mL/min for five minutes and dried under a stream of nitrogen gas for 10 minutes. Slow, drip-wise elution of analytes from the solid phase extraction cartridges was accomplished using 4 mL of a 50:50 mixture of acetone and methanol containing 0.5% formic acid.

Mass spectrometric analyses were carried out on an API 4000 instrument (Applied Biosystems, Framingham, MA, USA), coupled to a Shimadzu Prominence HPLC (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) that was controlled by Analyst 1.5 software (Applied Biosystems, Framingham, MA, USA). Chromatographic separation was achieved with a Symmetry C_{18} 3.5 µm by 6.4 mm by 75 mm analytical column that was preceded by a guard column of the same material, both supplied by Waters (Massachusetts, USA), and a mobile phase consisting of gradient methanol/water with 0.2% formic acid at a 0.4 mL/min flow rate. Analytes were introduced into the mass spectrometer using an electrospray ionization probe operating in positive mode. Multiple reaction monitoring (MRM) was used for qualitative analysis (Appendix C: Table 8).

4.2.4 Analyte concentrations in raw wastewater and mass loads

Parent opioid compounds were selected as indicators of drug consumption in samples collected over the course of the sampling campaign, lasting from March 2015 to March 2017. Starting in June 2016, metabolite compound concentrations also were tracked as indicators of drug consumption until the end of the monitoring program in

March 2017. Potential loss of opioids and metabolites from wastewater during sample extraction was corrected for by using labeled internal standards and the isotope dilution method. Opioid mass loadings to the WWTP were calculated from concentration data for daily wastewater flows using equation 4:

$$Mass\ Load\ \left(\frac{mg}{day}\right) = Raw\ Concentration\ \left(\frac{ng}{L}\right) * Flow\ \left(\frac{L}{d}\right) * \left(\frac{1\ mg}{1{,}000{,}000\ ng}\right) \qquad \text{Eq. 4}$$

4.2.5 Estimation of mass and dose per-capita opioid consumption

Estimates of drug consumption were obtained by normalizing the mass load of opioids to the population serviced by the WWTP and were subsequently subjected to a correction factor which accounts for metabolic excretion of the compounds and the molar mass ratio of the indicator compound to the parent opioid (Appendix C: Table 10). For mass and dose population normalized values, the following equations were used:

$$M.C.\left(\frac{mg}{day*1{,}000\ persons}\right) = M.L.\left(\frac{mg}{day}\right) * \left(\frac{1{,}000}{Population}\right) * C.F. \qquad \text{Eq. 5}$$

$$D.C.\left(\frac{dose}{day*1{,}000\ persons}\right) = M.C.\left(\frac{mg}{day*1{,}000\ persons}\right) * Dose\ \left(\frac{dose}{mg}\right) \qquad \text{Eq. 6}$$

Where *M.C.* refers to mass consumption, *D.C.* refers to dose consumption, *M.L.* refers to mass load, and *C.F.* refers to the analyte correction factor. Wastewater epidemiological data was then compared to opioid use statistics to estimate the number of opioid users. The number of estimated opioid abusers were then compared to national opioid use statistics. Per the National Drug Intelligence Center's report on Heroin Consumption in the United States (NDIC 2000), average daily use of pure heroin mass was assumed to equal 50 mg/day per user. Prescription opioid mass use was obtained from Mayo Clinic prescription guidelines at an ingestion rate of two doses per day, equaling 60 mg/day for

morphine, 60 mg/day for codeine, and 20 mg/day for oxycodone (Mayo 2017). Since unknown exposure to fentanyl is thought to drive the increase in fentanyl use (CDC and University 2017) it is difficult to estimate the average dose a recreational user may receive. Therefore, fentanyl was omitted from this portion of the analysis.

4.2.6 Overdose-death and black-market value estimates

A ratio between opioid related overdose deaths and overdoses (Appendix C: Table 11) was computed from various state reported data (AZDHS 2017, CCPDAP 2017, MNDH 2017, OHA 2017, RIPO 2017, VDH 2017). The average of this ratio (5.35 overdoses/death) was then compared to current data on national opioid overdose death rates (total 2015 opioid deaths: 33,091; total 2015 heroin deaths: 13,000; total 2015 synthetic opioid deaths: 20,091) (CDC 2017) and U.S. opioid abuse prevalence (heroin: 3.8 million (Martins et al. 2017); prescription opioids: 11.5 million (Thompson 2017)) to estimate the number of deaths and overdoses that may be attributable to the addicted population of the two test cities. From these numbers it was estimated that one thousand estimated daily heroin users account for 3.4 heroin overdose deaths and 18.3 heroin related overdoses. One thousand estimated daily synthetic opioid users account for 1.8 synthetic opioid overdose deaths and 9.4 synthetic opioid related overdoses. The black-market value of heroin was calculated by comparing the observed mass load of heroin to its street value (NBC 2017). Furthermore, the following assumptions were factored into every portion of the study analysis: (i) no sewage loss due to leaks or pipe degradation; (ii) no transformation or degradation within sewer lines; and (iii) no direct drug addition to the sewer system (Zuccato et al. 2008). In most cases, the major drug metabolite was selected as the consumption indicator – with the exception of the heroin metabolite 6-

acetylmorphine, which is a minor but exclusive human metabolite of heroin (Postigo et al. 2011).

4.2.7 Statistical analysis

Statistical analysis of the data was performed with a combination of Microsoft Office suite products, Analyst 1.5 software (Applied Biosystems, Framingham, MA, USA), JMP Pro 12.1.0 (SAS, Phoenix, Arizona), and IBM SPSS 25 (IBM, Armonk, NY). Normality of the datasets was determined through two analyses run in IBM SPSS 25; (1) an analysis of skewness and kurtosis *z*-values, and (2) the Shapiro-Wilk test for normality. Following previously outlined wastewater epidemiological statistical testing (Brewer et al. 2016, Tscharke et al. 2016), two-tailed *t*-tests were used for comparison of parent-metabolite excretion rates, as well as opioid concentrations in raw wastewater between study locations.

4.3 Results and discussion

4.3.1 Method performance

Method detection limits (MDLs) for the various opioid parent compounds and metabolites ranged between 0.3 and 1.1 ng/L (Appendix C: Table 9, Appendix E), data that were in line with previous U.S. studies (Heuett et al. 2015, Subedi and Kannan 2014). All MDLs were determined based on EPA guidelines described in 40 CFR 136, Appendix B (EPA 1986). Potential loss of opioids and metabolites from wastewater during sample extraction was corrected for by using labeled internal standards and the isotope dilution method. Metabolite loss was assumed to be similar to parent compound loss, and therefore loss was calculated from respective parent opioid internal standards.

Recoveries from matrix spike experiments for the various analytes averaged 114%. Analysis precision, expressed as relative percent difference (RPD) for non-blinded duplicates of composite wastewater samples averaged ±30%.

4.3.2 Concentrations of opioids and metabolites in raw wastewater

Opioid parent compounds were identified in pooled wastewater samples for each city once per month from March 2015 to April 2017 (Fig. 12, Appendix C: Table 12). Ratios of concentrations in raw wastewater of the parent drug and its metabolite compounds were observed to be similar across both cities (Appendix C: Fig. 20). Average concentrations in wastewater of heroin, fentanyl and their respective metabolites were not statistically different in either city (p=0.05), and the metabolites norfentanyl and 6-acetylmorphine were both detected at a higher frequency than their respective parent compounds (Table 2). In both cities, concentrations of the fentanyl metabolite norfentanyl were significantly larger (2-times and 48-times) than the corresponding concentrations of parental fentanyl, a finding that potentially could be due to the previously observed rapid *in vivo* degradation and transformation following administration (Labroo et al. 1997). Average concentrations in raw wastewater of codeine (p<<0.001), oxycodone (p<<0.001), and their respective metabolites norcodeine (p=0.002) and noroxycodone (p=0.04) were all statistically different across cities with similar detection frequencies for both parent and metabolite compounds. Average concentrations of codeine were higher in City 1 compared to City 2, but interestingly average oxycodone concentrations in wastewater from City 2 were higher than those observed in City 1.

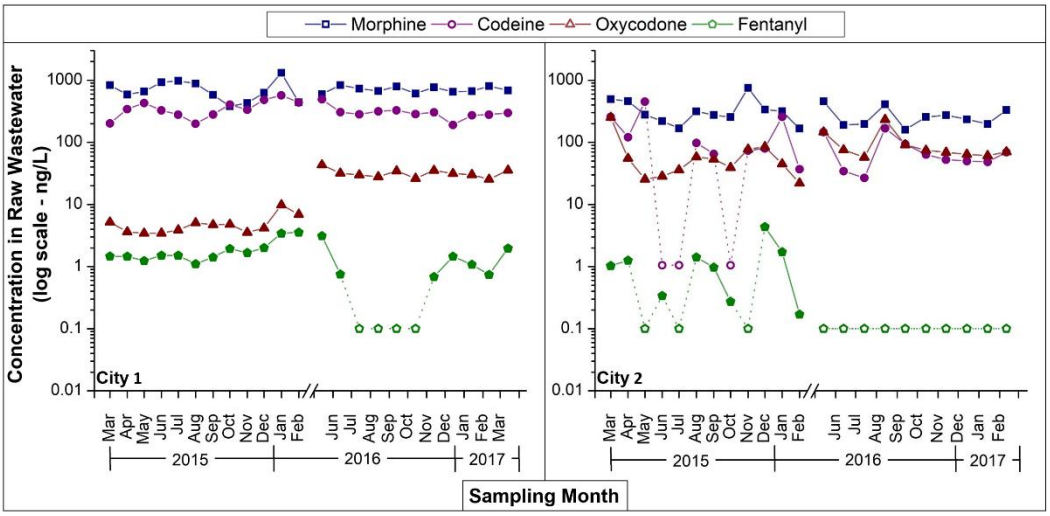


Fig. 12. Parent opioid concentrations determined in 24-hour time-weighted composite wastewater samples for the two cities over the sampling campaign from March 2015 to April 2017. Non-detects are represented by empty symbols within the graph.

Morphine concentrations in City 1 were significantly higher ($p<0.001$) than those observed in City 2, but interestingly the metabolite compounds ($p=0.644$) did not follow this same trend. Morphine presence in wastewater can be attributed to consumption of morphine (Hasselström and Säwe 1993), consumption of heroin (Cone et al. 1993), consumption of codeine (Vree and Wissen 1992), or as result of narcotic disposal (Daughton and Ruhoy 2009). Further analyte degradation (Skopp et al. 2001) and metabolization in the sewer system is likely and may influence parent-metabolite ratios (O'Brien et al. 2017). The discrepancy between the morphine parent and metabolite concentrations in raw wastewater suggest that the morphine concentrations are influenced by one of the alternative sources of morphine occurrence in wastewater and may point to illicit drug use.

Table 2 - Detection frequency, average analyte concentrations in raw wastewater ± standard deviations (SD), and maximum concentrations per opioid consumption indicator of all sample concentrations.

Consumption Indicator	Frequency of Detection (%)	Concentration (ng/L)	
		Average + SD	***Max.***
Morphine	100 (*n*=45)	514 ± 268	1,304
Morphine-3-glucuronide	90 (*n*=21)	7.3 ±6.6	26
Codeine	93 (*n*=45)	218 ± 154	571
Norcodeine	95 (*n*=21)	107 ± 90	397
Oxycodone	100 (*n*=45)	47 ± 52	251
Noroxycodone	100 (*n*=21)	88 ± 34	171
Fentanyl	62 (*n*=45)	1 ± 0.9	4.4
Norfentanyl	100 (*n*=21)	38 ± 49	198
Heroin	81 (*n*=21)	27 ± 30	120
6-Acetylmorphine	100 (*n*=21)	32 ± 28	115

Most opioids show a relatively consistent concentration pattern when compared over the two-year period. An exception of this is the dataset obtained for City 2 codeine concentrations from March 2015 – January 2016 where concentrations varied from 260 ng/L to below the method detection limit. This variation was not observed from June 2016 to March 2017 for City 2, but this observation lacks a definitive explanation. Results of *t*-tests comparing analyte concentrations in raw wastewater were confirmed by applying *t*-tests on converted mass loads (Appendix C: Table 13). Converting analyte concentrations in raw wastewater (ng/L) to mass loads (g/day) provides additional insight into consumption within a sampling population and allows the data to be used for population normalization and additional modeling.

4.3.3 Estimated opioid consumption

Opioid consumption was estimated from opioid mass loads and determined to be stable throughout the sampling campaign for all opioids (Appendix C: Table 15) aside

from City 1's oxycodone consumption which showed a statistically significant increase (565%, *p*-value: <0.01) from the March 2015 – Jan 2016 to the June 2016 – March 2017 sampling periods. Per-capita opioid consumption masses were then compared to pharmacokinetic excretion correction factors (Labroo et al. 1997, Lafolie et al. 1996, Lalovic et al. 2006, Postigo et al. 2011) and dosage guidelines (Mayo 2017) for both parent and metabolite compounds in order to obtain dose-estimated use rates (Appendix C: Table 16). Analysis of the parent opioids suggests that morphine consumption is likely higher than codeine or oxycodone consumption within these two cities (Appendix C: Table 14). Estimated morphine consumption values were in-line with other U.S. estimates (range: 1,610-2,240 mg/day/1,000 persons) (Subedi and Kannan 2014) but higher than consumption estimates outside the United States (range: 13.8-310 mg/day/1,000 persons) (Baker et al. 2014, Baz-Lomba et al. 2016, Tscharke et al. 2016, Vuori et al. 2014, Zuccato et al. 2008). Estimated morphine consumption from morphine-3-glucuronide was lower than other U.S. estimates, but in line with studies conducted outside the U.S. This discrepancy likely points to the influence of codeine and heroin use on wastewater morphine concentrations (Cone et al. 1993, Cone et al. 1991b), and further solidifies the idea that a stable morphine metabolite would be preferable for morphine consumption estimations.

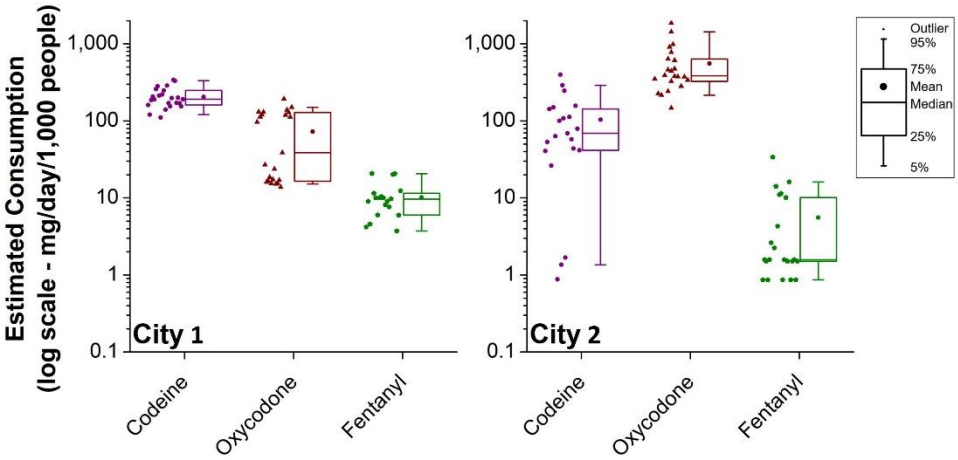


Fig. 13. Estimation consumption values for codeine, oxycodone and fentanyl derived from opioid parent compound analysis. Populations were estimated by population served by the wastewater treatment plants, and correction factors used are listed in Table 2.

Codeine consumption estimated from parent codeine was 2-times higher in City 1 compared to City 2, and oxycodone consumption estimated from parent oxycodone was nearly 8-times higher in City 2 compared to City 1. When compared to U.S. studies, oxycodone consumption estimates for City 1 (U.S. range: 8-170 mg/day/1,000 persons) (Chiaia et al. 2008) were in-line with other U.S. consumption estimates, but estimates for City 2 were significantly higher than reported values in other U.S. studies. When compared to international studies, oxycodone consumption estimates were higher across both cities (international range: 20-50.5 mg/day/1,000 persons), but codeine consumption estimates were in-line with international studies (international range: 164-927 mg/day/1,000 persons) (Tscharke et al. 2016, Vuori et al. 2014). Using norcodeine for consumption estimation purposes resulted in higher codeine consumption estimations across both cities (approximately 8-times higher) compared to parent codeine, which resulted in U.S. consumption estimation exceeding international values. This observation

was not mirrored with the noroxycodone:oxycodone relationship, as both values provided similar results (relative percent difference: 53-60%).

Heroin consumption estimates obtained from the metabolite 6-acetylmorphine were between 10 to 281 times higher than other estimated consumption values obtained from literature (range: 4.6-115 mg/day/1,000 population) (Heuett et al. 2015, Tscharke et al. 2016). This suggests that the scope of heroin abuse within these two midwestern cities may exceed both international and U.S. abuse rates. While comparison literature for U.S. fentanyl consumption is lacking, the estimated consumption unearthed by this analysis are still considerably higher than the average fentanyl consumption of 0.5 mg/day/1,000 persons identified in Adelaide, South Australia (Tscharke et al. 2016). While fentanyl concentrations were consistently the lowest of any analyte detected in this study, any detectable presence of synthetic fentanyl or its analogs should be considered significant due to the strength of the opioid (Donner et al. 1996), its prevalence in opioid-related fatalities (CDC and University 2017), and its ties to the illicit drug trade (CDC 2016). Furthermore, this study has provided the first U.S. wastewater concentrations for fentanyl and its metabolite norfentanyl – which is necessary for comparison purposes of future U.S. opioid-related wastewater epidemiological work.

4.3.4 User count, estimated overdose-deaths, and monetary black-market contribution

The number of heroin addicts within the two study locations were estimated at 3,400 (city 1) and 1,000 (city 2) persons. Considering the national average of 0.21% current habitual heroin users (SAMHSA 2013) these values are 1,135% and 982% higher than the calculated expectancy. These values were 61% and 41% higher than the national

average of lifetime heroin use of 1.6% (Martins et al. 2017). This suggests that current estimates of heroin use prevalence may be an underestimate of the true value. The number of codeine users were determined to be 3,600 (city 1) and 600 (city 2) persons. Oxycodone users were determined to be 800 (city 1) and 660 (city 2) persons. Number of morphine users estimated from parent morphine were determined to be 5,600 (city 1) and 1,500 (city 2) persons but does not account for morphine occurrence due to heroin or codeine consumption.

The number of estimated heroin users were compared to state opioid overdose death data to estimate the number of expected heroin and prescription opioid overdoses. From this analysis 12 heroin attributable deaths, 62 attributable heroin overdoses, 18 synthetic opioid attributable deaths, and 94 synthetic opioid attributable overdoses were estimated for City 1. City 2 was estimated to incur 4 heroin attributable deaths, 18 attributable heroin overdoses, 5 synthetic opioid attributable deaths, and 26 synthetic opioid attributable overdoses. When compared to reported coroner data, the estimated attributable death counts of both cities were within 30% of the true number identifying a correlation between the statistics unearthed through this analysis and municipal data. A cost estimate for black-market heroin consumption was also attempted for the two cities, with the average street value of heroin estimated to be $240/gram (NBC 2017). This analysis resulted in annual black-market contributions of $1.14 million (city 1) and $990 thousand (city 2) from heroin users. These estimates may be overly conservative, as a city with 100,000 individuals and a 0.21 addict rate could theoretically exceed an annual black-market contribution of 11.5 million USD from heroin alone. Cost estimations for

the remainder of the opioids were not attempted due to uncertainties with rates of medical vs. nonmedical use, and uncertainties in pharmaceutical vs. black market costs.

4.3.5 Study Limitations

While narcotic use and trend data collection via wastewater monitoring has been shown a viable tool both domestically and internationally (Baker et al. 2012, Kankaanpää et al. 2014, Subedi and Kannan 2014), there are shortcomings which factor in a level of uncertainty within the analysis. The most robust data that can be obtained from wastewater monitoring are analyte concentrations in raw wastewater (mass per volume) and daily mass loads (mass per day). These sources of data are not subject to significant error but also limit the knowledge that can be obtained from the dataset without further analysis. Previous literature has reported WBE data through usage statistics (in mass or doses per day per population) (Lai et al. 2013, Zuccato et al. 2008), monetary units (black market or overall economic impact) (Zuccato et al. 2011), and health statistics (attributable users, overdoses, or overdose deaths) (Terzic et al. 2010), but these analyses likely increase the associated error. Variations in individual narcotic mass usage (Harocopos et al. 2016, Warner et al. 2016), pharmacokinetic metabolization and narcotic excretion rates (Andes and Craig 2002, Cone et al. 1993, Jenkins et al. 1994, Schwartz 2003), and the extent of in-sewer analyte degradation and/or metabolization (Postigo et al. 2011) can have a marked effect on estimating drug use statistics from WBE data. Analysis of specific narcotics with various limiting factors such as low urinary and fecal excretion profiles or rapid *in*/*ex vivo* degradation may provide additional challenges for the quantification of certain narcotics in wastewater.

The use of time-weight samplers and the sampling frequency used in this study also constitutes limitations. The use of time-weighted sampling will not account for the diurnal wastewater flow patterns which could result in an underrepresentation of narcotic concentrations in raw wastewater. Due to budgetary constraints participating WWTP operators opted to sample for one 24-hour period per month. While this frequency of sampling can provide insights into long-term trends and baseline usage patterns obtaining more intricate trend analyses of the data (i.e. variation in weekly use trends) is not possible. An ideal study would sample for a set number of consecutive days throughout a longer timeframe to obtain data for both short and long-term drug use trends, and administration of self-reporting surveys for comparison purposes (Heuett et al. 2015, Moore et al. 2014). Large relative percent differences observed for some samples as well as increase in parent oxycodone observed for City 1 between the two sampling campaigns could have been impacted by errors in sample collection and processing, and possibly the hydrophobicity of the target analytes. While these factors contribute a level of uncertainty in this analysis data derived from these methods should still be considered a powerful analytical tool and considered alongside additional viable methods of data collection that are currently implemented within municipal communities.

4.4 Conclusion

The results of this study indicate that the higher opioid consumption in the United States is reflected in the higher opioid analyte concentrations observed in U.S. wastewaters, which have produced some of the highest opioid consumption estimations presented in WBE literature. While many methods have been implemented to track U.S. opioid use (Cicero et al. 2015, Dart et al. 2015, Kolodny et al. 2015) the WBE process

can complement current procedure by providing an additional analysis tool capable of producing data in near-real time. It is likely that specific opioid use varies between these two cities despite similar population demographics. Because of this observation it is reasonable to assume that estimating drug use for a population by forecasting data obtained from national statistics or data obtained from another sampling location may not be sufficient. Implementing WBE monitoring within a community requires minimal adjustment to wastewater infrastructure but would result in pertinent information related to opioid use. This study also provides the first reported U.S. occurrence of fentanyl and its metabolite norfentanyl in wastewater in published literature. These analytes were found in higher concentration and more frequently than in international studies which is likely due to the known increase in U.S. fentanyl use (CDC 2016, CDC and University 2017). Screening for fentanyl and its metabolites should be viewed as a mandatory practice in future U.S. WBE studies due to the association between fentanyl and the rise in opioid-related fatalities in the United States (Warner et al. 2016). WBE results could be further used to forecast opioid-related overdose and deaths attributable to a measurable concentration of drug analyte within wastewater. While the WBE process may be subject to some uncertainty the technology remains a valuable analytical tool to be used alongside current data acquisition approaches by providing location specific wastewater-based epidemiological data in near real-time.

TRANSITION 4

The two communities studied in Chapter 4 were both identified with higher rates of estimated opioid consumption compared to previously published international literature, but specific opioid use varied between communities. While this observation could be attributed to the likely variation in drug consumption between communities it could have also been impacted by known limitations associated with WBE when working at the city scale. Some of these limitations, such as analyte degradation and transformation in-sewer, could theoretically be reduced through sampling methods which reduce the long sewage transit time from the originating source to the wastewater treatment plant. Sampling within the sewershed would likely produce the most robust data extractable from community wastewater analysis for a sub-population or location serviced by a municipal wastewater treatment system.

In Chapter 5, a targeted wastewater sampling campaign was conducted at southwestern U.S. university for the identification of twelve prescription and illicit drugs in wastewater: morphine, codeine, oxycodone, fentanyl, diacetylmorphine (heroin), methadone, buprenorphine, amphetamine, methylphenidate, alprazolam, benzoylmethylecgonine (cocaine), and methylenedioxymethamphetamine (MDMA/Ecstasy). Seven consecutive 24-hour flow-weighted composite wastewater samples were collected once per month. Data analysis was completed in the same method presented in Chapter 4, and estimated consumption values were compared to related U.S. and international literature, as well as four similar U.S. campus studies. Two-tailed t-tests

on mass load calculations were also completed to identify differences between weekday and weekend narcotic use.

CHAPTER 5

APPLICATION OF WASTEWATER-BASED EPIDEMIOLOGY TRACKING NARCOTIC USE AT A SOUTHWESTERN U.S. UNIVERSITY

ABSTRACT

College-aged adults in the United States have been identified with the highest rates of drug abuse across all age categories but data collection for this age demographic relies heavily on self-reported surveys. Urban metabolism metrology using wastewater diagnostics potentially offers access to near real-time data on narcotic consumption but thus far has seen little application at U.S. universities. Furthermore, narcotic elimination half-lives which could have a marked effect on WBE results are often overlooked in the method development stages of WBE processes. From August 2017 to December 2017, seven consecutive 24-hour flow- or time-weighted composites of municipal raw wastewater were analyzed once per month from a Southwestern U.S. university using isotope dilution liquid chromatography tandem mass spectrometry. Estimated narcotic consumption (mg/day/1,000 persons) exceeded most U.S. and international WBE consumption estimates for the general population and were highest for cocaine (470 ± 42), heroin (474 ± 32), amphetamine (302 ± 14) and methylphenidate (236 ± 28). Aside from attention deficit/hyperactivity disorder medication estimated narcotic consumption and analyte detection frequency exceeded previously reported values from related U.S. campus literature. This campus-based U.S. screening study of narcotic analytes in wastewater yielded (1) sporadic but detectable campus-wastewater concentrations for the

powerful synthetic opioid fentanyl, and (2) the first study to consider drug analyte elimination half-life within method development.

5.1 Introduction

College-aged adults (ages 18-22) have historically been identified with the highest percentage of drug abuse compared to other age categories with 24-28% of respondents to a 2016 survey admitting to illicit drug use within the past 30 days (Schulenberg et al. 2017). While 48% of high school respondents to the same survey reported trying at least one illicit drug in their lifetime prevalence of drug use has been shown to be higher for those in their 20s indicating that drug use initiation continues for many individuals throughout the ages of 18-29 (Schulenberg et al. 2017). Americans between the ages of 15-24 have seen some of the lowest rates of overdose death (4-10 deaths per 100,000 population) across all age categories from 2000-2016, but the subsequent age group (25-34 years) has been identified with the highest number of drug overdose deaths in 2016 (35+ deaths per 100,000 population) (Hedegaard et al. 2017). This observation may be partially explained through drug-related associative learning where drug seeking habits are sustained later in life while the subjective effects that initially encouraged the drug use diminish (Robbins and Everitt 1999). Continued neurological development in the early 20s (Giedd et al. 1999) coupled with changes in brain chemistry due to drug use (Squeglia et al. 2009) may have a marked effect on this demographic group.

Addressing substance abuse within college-aged adults should be viewed as a principal task – but understanding the scope of abuse within this age category is met with significant difficulty. Current data analysis involves a combination of population

surveys, crime statistics, medical records and narcotic seizure data (Zuccato et al. 2008), but these analyses provide data on previous years and may not adequately capture the current state of drug use. The costly and cumbersome procedures may also induce unintentional bias into studies through misrepresentation in self-reporting surveys (Zuccato et al. 2008). First proposed in 2001 (Daughton 2001) wastewater-based epidemiology (WBE) has been shown as a viable alternative to current narcotic data collection methods (Zuccato et al. 2005), and has been applied worldwide to obtain narcotic abuse statistics in near-real time for varying population sizes (Kankaanpää et al. 2014, Kim et al. 2015, Lai et al. 2013, Postigo et al. 2011). This idea has been further expanded under the umbrella of urban metabolism metrology (UMM), which examines multiple environmental matrices to estimated health statistics for a population or area of interest. Analysis of time- or flow-weighted composite wastewater treatment plant (WWTP) samples may provide a unique epidemiological insight as consumption statistics could theoretically be obtained for any commonly consumed product within a population (Dove 2006) if target analyte properties are favorable for WBE testing. Sampling for wastewater epidemiological analyses generally focus at the inlet point of the WWTP to obtain statistics related to the population served by the WWTP (Kankaanpää et al. 2014, Kim et al. 2015, Terzic et al. 2010), but the technique has also been applied to obtain similar information for smaller population sizes such as college campuses (Burgard et al. 2013, Heuett et al. 2015, Moore et al. 2014) or prisons (Brewer et al. 2016, Postigo et al. 2011).

Application of urban metabolism metrology has seen limited application in the United States (Subedi and Kannan 2014). To the authors' knowledge four studies have

applied UMM technology at sampling points local to U.S. college campuses to obtain drug use statistics. Two of these studies were primarily interested in quantifying non-medical ADHD prescription drug use (Burgard et al. 2013, Moore et al. 2014), while the latter two studies screened for a wider suite of illicit and prescription drugs including: amphetamines, opioids, cocainics, cannabinoids, and lysergics (Heuett et al. 2015, Panawennage et al. 2011). None of these studies screened for the potent synthetic opioid fentanyl, despite its known association with increasing overdose rates and fatalities from drug abuse (Rudd 2016). These studies typically focus on a single university and thus are limited in their generalizability due to the social, economic, and circumstantial factors that cause variation in drug use across different locations (Harocopos et al. 2016, Warner et al. 2016). Therefore the main objectives of the present study were to: (i) develop a demographic-targeted liquid chromatography-tandem mass spectrometry UMM method for the detection of 12 drugs of abuse including some of their known metabolites in a university setting, (ii) obtain the first wastewater monitoring data for prevalence of fentanyl use in a university setting where the contributing population is predominantly college-aged adults, and (iii) assess and quantify potential consumption of targeted prescriptions and illicit narcotics within the campus population.

5.2 Materials and methods

5.2.1 Study location and methods of wastewater sampling

Seven (7) consecutive 24-hour flow-weighted wastewater samples were collected using automated samplers once per month from August 2017 to December 2017 from two sampling locations capturing 100% of campus-borne sewage. Sampling location 1 accounted for approximately 95% of the total campus-borne wastewater while sampling

location 2 accounted for approximately 5% of the total flow. The sewershed contributing population for both locations ranged between approximately 15,000 to 60,000 persons depending on the day of sampling. Mean age (26.5 years) of the catchment population was estimated by comparing average age of the population from years with available age data (2004-2009). Of the student population, approximately 53.6% of students are male and 46.4% female. Furthermore, 81.8% of the student population are pursuing an undergraduate degree, and 18.2% of students are pursuing a graduate degree. Undergraduate ethnicity demographics were as follows: white: 50.5%; Hispanic/Latino: 21.7%; international: 10.6%; Asian: 6.6%; African American: 4.3%; American Indian: 1.3%. Graduate ethnicity demographics are as follows: white: 45.5%; Hispanic/Latino: 10.9%; international: 30.8%; Asian: 4.9%; African American: 3.1%; American Indian: 1.2%. Population demographics were provided by the participating university. The campus features a sewer system designed to separate municipal wastewater from stormwater inputs. Ambient temperatures throughout the study period ranged from 3.4-42.8℃. The average sewage travel distance within the study catchment was estimated to be 2,700 m. Sewage retention time in the catchment system was estimated to average about 50 minutes but could range between 10-110 minutes depending on travel distance and sewer flow conditions (Appendix D: Table 17). Sampling occurred one week per month during the five-month study period through a joint effort between the study researchers and municipality personnel. Samples were stored in polyethylene terephthalate (PET) bottles for transport and storage and immediately processed through solid phase extraction upon receipt by laboratory personnel. Remaining samples and concentrated sample extracts were stored at -20°C until analysis.

5.2.2 Target analytes

Ten parent prescriptions and illegal narcotics and nine (9) metabolites were monitored in raw wastewater collected from two sampling locations on university campus accounting for a majority of campus-borne wastewater. The investigated drugs were morphine's major metabolite morphine-3-glucuronide (M3G), codeine (COD), its major metabolite norcodeine (NCOD), oxycodone (OXY), its major metabolite noroxycodone (NOXY), fentanyl (FENT), its major metabolite norfentanyl (NFENT), heroin (HER), its minor but exclusive metabolite 6-acetylmorphine (6-AM), methadone's major metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), buprenorphine (BUP), its metabolite norbuprenorphine (NBUP), amphetamine (AMP), methylphenidate (MPH), alprazolam (ALP), its metabolite α-OH-alprazolam (OH-ALP), cocaine (COC), its metabolite benzoylecgonine (BZE), and 3,4-methylenedioxy-methamphetamine (MDMA). High purity (>97%) standard solutions of the target compounds originated from Sigma Aldrich (Milwaukee, WI) and were prepared by Cerilliant (Round Rock, TX, USA) as solutions in methanol or acetonitrile. 18 deuterated compounds, one for each of the parent opioid target compounds were also purchased from Cerilliant for use as internal standards (IS) for quantification, namely: heroin-d_9 (HER-d_9), codeine-d_6 (COD-d_6), oxycodone-d_3 (OXY-d_3), fentanyl-d_5 (FENT-d_5), buprenorphine-d_4 (BUP-d_4), amphetamine-d_6 (AMP-d_6), methylphenidate-d_9 (MPH-d_9), alprazolam-d_5 (ALP-d_5), cocaine-d_3 (COC-d_3), 3,4-methylenedioxy-methamphetamine-d_5 (MDMA-d_5), morphine-3-glucuronide-d_3 (M3G-d_3), noroxycodone-d_3 (NOXY-d_3), norcodeine-d_3 (NCOD-d_3), 6-acetylmorphine-d_3 (6AM-d_3), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine-d_3 (EDDP-d_3), norbuprenorphine-d_3 (NBUP-d_3), α-OH-

alprazolam-d_5 (OH-ALP-d_5), and benzoylecgonine-d_8 (BZE- d_8). Instrument analyte loss for norfentanyl was estimated from fentanyl-d_5.

5.2.3 Isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS)

Briefly, 200 mL of raw wastewater was loaded onto Oasis HLB 150 mg cartridges (Waters, Barcelona, Spain) at a rate of 1.5 mL/min to determine the analytes measured in positive ionization (PI) mode. Prior to extraction, all wastewater samples were spiked with a standard mixture of the deuterated compounds at a concentration of 5 ng/mL for HER-d_9, COD-d_6, OXY-d_3, FENT-d_5, BUP-d_4, AMP-d_6, MPH-d_9, ALP-d_5, COC-d_3, MDMA-d_5, M3G-d_3, NOXY-d_3, NCOD-d_3, 6AM-d_3, EDDP-d_3, NBUP-d_3, OH-ALP-d_5, and BZE-d_8. After samples were loaded, cartridges were washed with D.I. water at a rate of 5 mL/min for five minutes and dried under a stream of nitrogen gas for 10 minutes. Slow, drip-wise elution of analytes from the solid phase extraction cartridges was accomplished using 4 mL of a 50:50 mixture of acetone and methanol containing 0.5% formic acid.

Mass spectrometric analyses were carried out on an API 4000 instrument (Applied Biosystems, Framingham, MA, USA), coupled to a Shimadzu Prominence HPLC (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) that was controlled by Analyst 1.5 software (Applied Biosystems, Framingham, MA, USA). Chromatographic separation was achieved with a Symmetry C18 3.5 µm by 6.4 mm by 75 mm analytical column that was preceded by a guard column of the same material, both supplied by Waters (Massachusetts, USA), and a mobile phase consisting of gradient

methanol/water with 0.2% formic acid at a 0.4 mL/min flow rate. Analytes were introduced into the mass spectrometer using an electrospray ionization probe operating in positive mode. Multiple reaction monitoring (MRM) was used for qualitative analysis (Appendix D: Table 18).

5.2.4 Analyte concentrations in raw wastewater and mass loads

Screened parent and metabolite narcotic compounds were all examined as potential indicators of drug consumption in samples collected over the course of the sampling campaign, lasting from August 2017 to December 2017. Potential loss of opioids and metabolites from wastewater during sample extraction was corrected for by using labeled internal standards and the isotope dilution method. Narcotic mass loadings within the study sewer catchment system were calculated from analyte concentrations in raw wastewater (in units of ng/mL) for daily wastewater flows provided by the city municipality using equation 7:

$$Mass\ Load\ \left(\frac{mg}{day}\right) = Raw\ Concentration\ \left(\frac{ng}{L}\right) * Flow\ \left(\frac{L}{d}\right) * \left(\frac{1\ mg}{1{,}000{,}000\ ng}\right) \qquad \text{Eq. 7}$$

5.2.5 Estimation of mass per-capita narcotic consumption

Estimates of drug consumption were obtained by normalizing the mass load of narcotics to the estimated contributing population and were subsequently subjected to a correction factor which accounts for metabolic excretion of the compounds and the molar mass ratio of the indicator compound to the parent opioid (Appendix D: Table 18). Number of contributing individuals was estimated through wastewater flow, and concentrations of caffeine, paraxanthine, and nicotine in raw wastewater. Population estimates from wastewater flow were obtained using design standards outlined in the

Arizona Department of Environmental Quality – Water Pollution Control design manual through equation 8:

$$C.I.(persons) = \frac{Average\ Flow}{(80\ GPM)(L.C.)+(20\ GPM)(O.C.)} \qquad \text{Eq. 8}$$

Where *C.I.* refers to the number of contributing individuals (in persons), *L.C.* refers the fraction of total population living on campus, and *O.C.* refers to the fraction of total population living off campus. Population estimates from analyte concentrations assumed 5.1 mg/day/person for caffeine (FDA 2012, Gracia-Lor et al. 2017), 13.8 mg/day/person for paraxanthine (Gracia-Lor et al. 2017), 0.125 mg/day/smoker for nicotine (Hukkanen et al. 2005), and 14% smoking prevalence in the population (AZ-DHHS 2016). All estimated population values were within the expected population range for the catchment (15,000 to 60,000 persons). Mass population normalized values were calculated through equations 9 and 10:

$$M.C.\left(\frac{mg}{day*1{,}000\ persons}\right) = M.L.\left(\frac{mg}{day}\right) * \left(\frac{1{,}000}{Population}\right) * C.F. \qquad \text{Eq. 9}$$

$$D.C.\left(\frac{dose}{day*1{,}000\ persons}\right) = M.C.\left(\frac{mg}{day*1{,}000\ persons}\right) * Dose\ \left(\frac{dose}{mg}\right) \qquad \text{Eq. 10}$$

Where *M.C.* refers to mass consumption, *D.C.* refers to dose consumption, *M.L.* refers to mass load, and *C.F*. refers to the analyte correction factor. Wastewater epidemiological data was compared to narcotic use statistics to estimate the number of users per narcotic of interest. Per the National Drug Intelligence Center's report on Heroin Consumption in the United States (NDIC 2000), average daily use of pure heroin mass was assumed to equal 50 mg/day per user. Average cocaine (50 mg/dose) and MDMA (100 mg/dose) dose estimates were obtained from relevant human pharmacokinetic studies (Breiter et al.

1997, De La Torre et al. 2000). The remainder of prescription mass use was obtained from Mayo Clinic prescription guidelines, equaling 30 mg/dose for morphine, 30 mg/dose for codeine, 10 mg/dose for oxycodone, 30 mg/dose for methadone, 30 mg/dose for amphetamine, 30 mg/dose for methylphenidate, and 2 mg/dose for alprazolam (Mayo 2017). Due to the lack of detection of buprenorphine or its metabolite, these compounds were omitted from this portion of the analysis. Since unknown exposure to fentanyl is thought to drive the increase in fentanyl use (CDC and University 2017) it is difficult to estimate the average dose a recreational user may receive. Therefore, fentanyl was omitted from this portion of the analysis.

5.2.6 Estimation of overdoses, overdose-deaths, and black-market value

Based upon the dose analysis, number of users per narcotic were estimated through the assumption that 1 user constitutes 2 doses per day. Overdose and overdose-death analysis for heroin was computed in the same method detailed in chapter 4. The black-market value of heroin and cocaine was calculated by comparing the average mass of narcotic compound consumption to the "street value" (Kucher 2018, NBC 2017). Furthermore, the following assumptions were factored into every portion of the study analysis: (i) no sewage loss due to leaks or pipe degradation, (ii) no transformation or degradation within sewer lines, and (iii) no direct drug addition to the sewer system (Zuccato et al. 2008).

5.2.7 Statistical analysis

Statistical analysis of the data was performed with a combination of Microsoft Office suite products, Analyst 1.5 software (Applied Biosystems, Framingham, MA,

USA), JMP Pro 12.1.0 (SAS, Phoenix, Arizona), and IBM SPSS 25 (IBM, Armonk, NY). Normality of the datasets was determined through two analyses run in IBM SPSS 25; (1) an analysis of skewness and kurtosis *z*-values, and (2) the Shapiro-Wilk test for normality. Following previously outlined wastewater epidemiological statistical testing (Brewer et al. 2016, Tscharke et al. 2016), two-tailed *t*-tests were used for comparison of weekend vs. weekday mass load observations.

5.3 Results and discussion

5.3.1 Method performance

Method detection limits (MDLs) for the various narcotic parent and metabolite compounds ranged between 0.2 to 1.7 ng/L (Appendix D: Table 19, Appendix E), data that were in line with previous U.S. studies (Burgard et al. 2013, Heuett et al. 2015, Moore et al. 2014, Subedi and Kannan 2014). All MDLs were determined based on EPA guidelines described in 40 CFR 136, Appendix B (EPA 1986). Potential loss of narcotics and metabolites from wastewater during sample extraction was corrected for by using labeled internal standards and the isotope dilution method. Recoveries from matrix spike experiments for the various analytes averaged 110%. Analysis precision, expressed as relative percent difference (RPD) for non-blinded duplicates of composite wastewater samples averaged 7.4%.

5.3.2 Concentrations of narcotics and metabolites in raw wastewater

Concentrations in raw wastewater (ng/mL) for all analytes of interest were identified for each sampling location seven consecutive days per month from August 2017 to December 2017 (Fig. 14, Appendix D: Table 21, Table 22). Analyte

concentrations in raw wastewater for heroin, fentanyl, and norfentanyl were detected sporadically throughout the sampling campaign while the analytes buprenorphine and norbuprenorphine were not detected. The remainder of the analyte concentrations were frequently detected in raw wastewater (detection frequency (D.F.)=80%+) at both sampling locations with the exception of 6-acetylmorphine (D.F.=30%) and alpha-hydroxyalprazolam (D.F.=33%) at one sampling location. The fentanyl and norfentanyl analytes were detected sporadically throughout the sampling campaign which may point to infrequent non-medical fentanyl consumption (CDC 2016, CDC and University 2017). No other narcotics considered in this analysis were identified with sporadic patterns within the catchment.

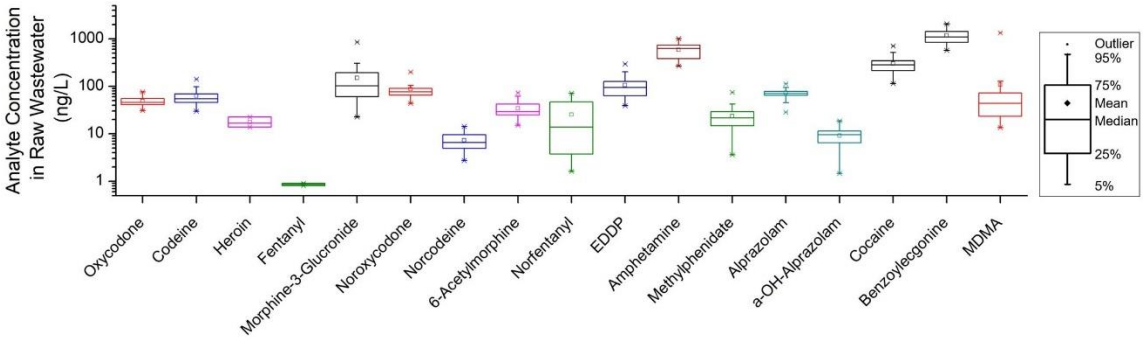


Fig. 14 – Box plots of analyte concentrations identified in raw wastewater (ng/L) of all analytes detected during the August 2017-December 2017 sampling campaign.

Following normalization of the data by wastewater flow data was analyzed to determine weekly consumption trends and patterns (Fig. 15, Appendix D: Table 23). Data was log-transformed for normality and tested with 2 tailed *t*-tests to check for statistical differences in weekday and weekend narcotic use. Mass loads did not vary significantly between weekend and weekday use (p>0.05) for any of the screened opioids confirmed through both the parent and metabolite analyses. These findings are similar to

previously reported opioid use trends derived from WBE analysis (Kankaanpää et al. 2014, Postigo et al. 2011, Zuccato et al. 2008). Weekday amphetamine mass loads were statistically higher ($p<0.001$) compared to weekend mass loads, but this trend was not observed for methylphenidate ($p=0.303$). These findings are supported by U.S. WBE literature which has identified a correlation between amphetamine (Adderall) use and times of high academic stress – for which the same relationship for methylphenidate (Ritalin) was not observed (Burgard et al. 2013, Moore et al. 2014). Weekday alprazolam (Xanax) mass loads were statistically higher ($p=0.005$) than weekend mass loads but this same trend was not observed for the metabolite alpha-hydroxyalprazolam ($p=0.747$). Due to the high urinary excretion percentage of alpha-hydroxyalprazolam (Fraser et al. 1991) this discrepancy may be explained by in-sewer degradation and transformation (O'Brien et al. 2017, Thai et al. 2014).

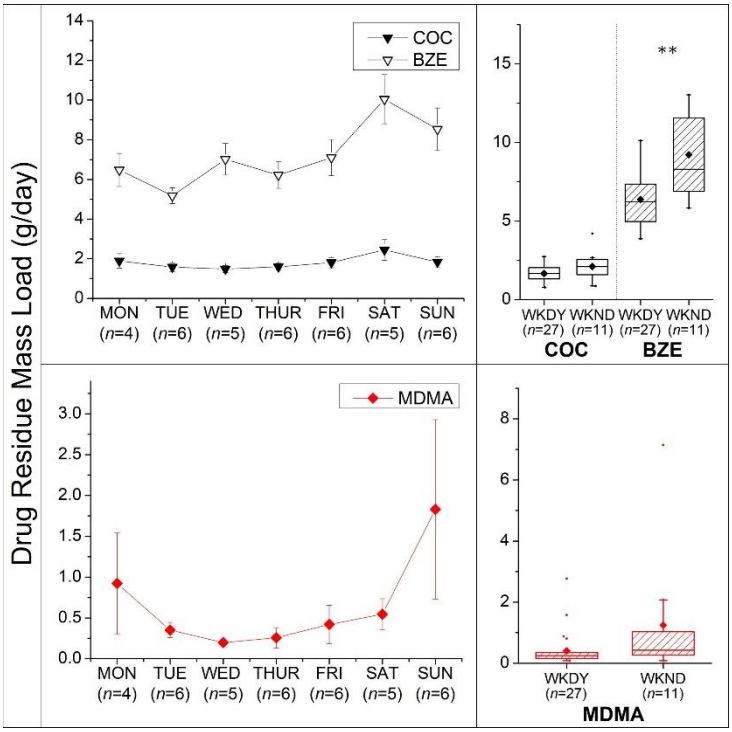


Fig. 15 - Average drug residue mass loads per day and a comparison between weekend and weekday mass load occurrence for cocaine (COC), its metabolite benzoylecgonine (BZE), and MDMA. Error bars represent the standard error of all measured values for a specific day. Weekend comparison was done by a two-tailed t-test (α=0.05). Asterisks (**) denote a statistically significant difference between weekday and weekend mass loads.

Weekend mass loads for the cocaine metabolite benzoylecgonine were statistically higher (p=0.006) compared to weekday mass loads which coincides with the higher observed rates of cocaine consumption on weekends (Kankaanpää et al. 2014, Tscharke et al. 2016, Zuccato et al. 2008). This same trend was not observed for parent cocaine within the sampling area (p=0.145). This discrepancy may be explained by the low excretion percentage of cocaine excreted as parent cocaine (Ambre et al. 1988) coupled with potential transformation and degradation in the sewer system. Previously reported concentrations of MDMA in raw wastewater have suggested higher weekend consumption (Kankaanpää et al. 2014, Tscharke et al. 2016) but our analysis did not

identify a statistically significant difference between weekday and weekend mass loads (p=0.204). This observation could be explained by the longer elimination half-life of MDMA (De La Torre et al. 2000, Torre et al. 2000) but is also likely impacted by metabolization rates and in-sewer degradation.

While analyte concentrations in raw wastewater are necessary for further modeling of the data they provide little insight above an analysis of long-term trends. This point is demonstrated by comparing the amphetamine concentrations in raw wastewater at the two sampling locations. By comparing amphetamine concentrations in raw wastewater at sampling location 1 (average AMP = 574 ± 30) to sampling location 2 (average AMP = 852 ± 66), one would assume the contributing population at the second sampling location has a higher usage of amphetamine than the first. When the data is normalized to the wastewater flow to obtain mass load values (mass/day), it becomes clear that first sampling location is responsible for most of amphetamine analytes entering the university sewer system – thus the aforementioned assumption would have been made in error. This demonstrates that comparisons of analyte concentrations in raw wastewater may not produce comparable results and exemplifies the necessity of normalization of analyte concentrations in raw wastewater to wastewater flow.

5.3.3 Substance consumption estimates

Narcotic analyte mass loads were population normalized and corrected using the pharmacokinetic correction factors (Appendix D: Table 20) to provide narcotic consumption estimates. Population normalized and corrected data was then compared to dosage guidelines to obtain dose estimated consumption (Appendix D: Table 23).

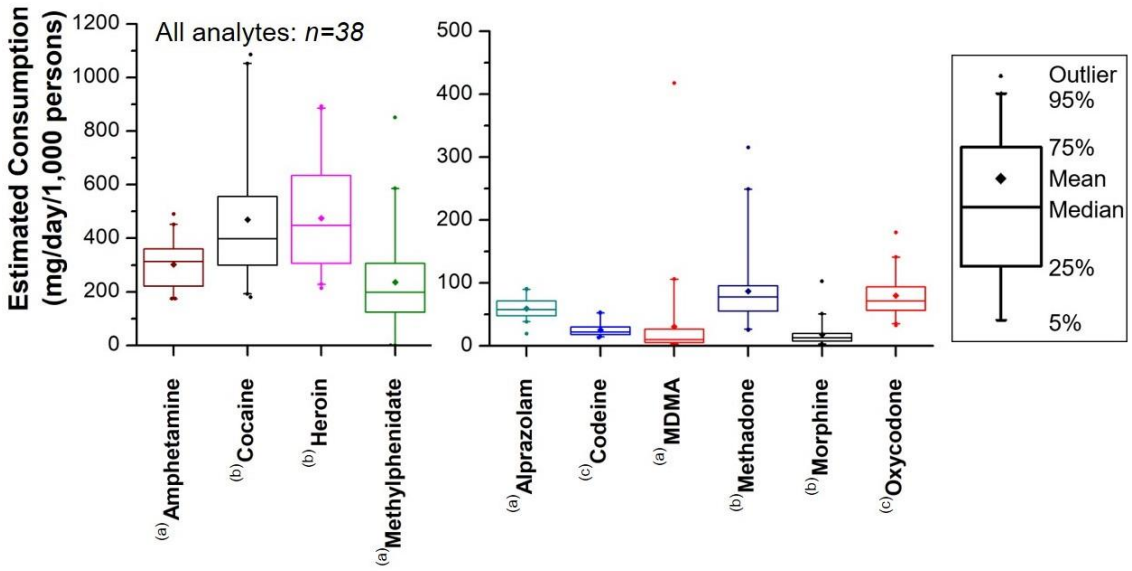


Fig. 16 - Estimated campus population consumption of the following substances of potential abuse. Superscripts [(a)] denotes estimation from parent compound, [(b)] denotes estimation from metabolite compound, and [(c)] denotes estimation from both parent and metabolite compounds.

Estimated cocaine consumption (Fig. 16) was the highest of any of the screened metabolites and was similar to previously reported cocaine consumption estimates in U.S. WBE studies (range: 100-1,500 mg/day/1,000 persons) (Chiaia et al. 2008, Subedi and Kannan 2014) and international WBE studies (range: 0.05-9,793 mg/day/1,000 persons). The cocaine consumption reported here also exceeds previously reported values in other U.S. universities both in terms of detected mass and detection frequency (Heuett et al. 2015). Heroin consumption, constituting the 2nd highest consumption estimation within this study, also exceeded previously reported U.S. campus consumption estimates by over 10-fold (Heuett et al. 2015). Both attention deficit/hyperactivity disorder analytes exceeded both U.S. (Chiaia et al. 2008, Subedi and Kannan 2014) and international (Baker et al. 2014, Baz-Lomba et al. 2016, Tscharke et al. 2016, Vuori et al. 2014, Zuccato et al. 2008) mass consumption estimates but were in-

line with similar studies identifying attention deficit/hyperactivity disorder stimulant use within the U.S. collegiate setting (Burgard et al. 2013, Heuett et al. 2015, Moore et al. 2014, Panawennage et al. 2011). Average consumption estimates for the remainder of the analytes were similar to reported consumption U.S. estimates and higher than estimates presented in international literature except for morphine estimated from morphine-3-glucuronide which was lower than U.S. estimates and in-line with values presented in international literature. It is possible that the instability of the metabolite morphine-3-glucuronide (Skopp et al. 2001) coupled with the impact of in-sewer degradation contributed to the discrepancy in morphine consumption noted here.

The frequent detection of attention deficit/hyperactivity disorder medication analytes (D.F.=88%+) in this study was echoed by all WBE-based U.S. campus studies showing near ubiquitous amphetamine and methylphenidate detection (Burgard et al. 2013, Heuett et al. 2015, Moore et al. 2014, Panawennage et al. 2011). This could suggest young adults within the collegiate setting may be more inclined to abuse attention deficit/hyperactivity disorder medication for recreational or educational purposes (McCabe et al. 2005, Teter et al. 2006) and non-medical ADHD stimulant use may not be limited to a single U.S. university or geographic location. Detection of the cocaine metabolite benzoylecgonine in this study (DF=100%) was also similar to previously reported U.S. campus literature (DF=97-100%) (Heuett et al. 2015, Panawennage et al. 2011). The remainder of the narcotic analytes were detected at a much higher frequency at this location compared to reported values from other U.S. campus studies. The heroin metabolite 6-acetylmorphine was detected infrequently within U.S. campus literature (DF=0-1%) which screened for the compound compared to the relatively high detection

frequency presented in this study (Appendix D: Table 21, Table 22). This higher detection frequency was similar for morphine, codeine, MDMA, oxycodone, and EDDP. This suggests that variation in narcotic use among different campus populations could be analyzed in near-real time through WBE analysis. Furthermore no U.S. campus studies have identified concentrations of fentanyl, alprazolam, or their metabolites within campus-borne wastewater which were identified in this study. The lack of detectable fentanyl and norfentanyl within the Southwestern university wastewater samples suggests that medical use of fentanyl at this location is low and any detection of either compound could point towards non-medical fentanyl use. The chemical data also does not provide any information regarding substance abuse control measures that may have been implemented on the campus.

5.3.4 Drug user count, estimated overdose-deaths, and estimated black-market value

An estimation of the number of narcotic users, presented in units of users/1,000 persons resulted in values between 0.15 ± 0.05 (MDMA) to 14.9 ± 0.6 (alprazolam) (Appendix D: Table 23), with notable values observed for oxycodone (4 ± 0.26), heroin (7.9 ± 0.6), amphetamine (5.1 ± 0.2), methylphenidate (3.9 ± 0.47), alprazolam (14.9 ± 0.6), and cocaine (4.9 ± 0.4). The number of calculated heroin users exceeded the national average of 0.21% by four-fold (SAMHSA 2013) but was under the national average lifetime heroin use of 1.6% (Martins et al. 2017). While heroin use is usually higher in young adults (Cerdá et al. 2015, SAMHSA 2013) compared to other age demographics the high estimated percentage of heroin users (1%) could have been impacted by the low excretion percentage (1.6%) used for 6-acetylmorphine calculations. If the higher-bound excretion percentage of 5% (Labroo et al. 1997) is used the resulting

number of estimated heroin users decreases by 68% and puts the estimation in-line with national averages. This identifies the importance of the pharmacokinetic and excretion rates within WBE approaches; metabolization of heroin to 6-acetylmorphine may be greater in younger adults compared to older populations which could account for the high user estimate. Samples collected for this study were subjected to much shorter sewage retention times compared to studies which sample at the WWTP inlet point theoretically reducing analyte degradation within the wastewater. This may have resulted in higher concentrations than what would have been observed if the wastewater had been subjected to a longer transit time. Estimated overdose and overdose-deaths due to heroin were estimated from 6-acetylmorphine concentrations. This resulted in 6 ± 0.5 expected heroin overdoses and 1.1 ± 0.1 expected heroin overdose-deaths for the campus population during the 2017 year. Overdose and overdose-deaths for other substances were not attempted due to unavailability of pertinent information.

An estimated black market monetary contribution for the 2017 year was also calculated for the narcotics cocaine and heroin. Assuming a price per gram of $240 for heroin (NBC 2017) and $33.8 for cocaine (Kucher 2018) the estimated black-market value of heroin was $1.6 million and the estimated black-market value of cocaine was $230 thousand. These estimates only account for street-value of the narcotics and do not constitute the economic impact of use of these drugs, which is likely much higher due to the additional strain drug use causes on communities due to crime rates, hospitalization, child abuse and neglect, and increased risk of HIV transmission (Hoffman and Goldfrank 1990).

5.3.5 Impact of elimination half-life

Following ingestion of a narcotic parent compounds and metabolites are retained within the body and excreted over time. This excretion, generally expressed as the narcotic elimination half-life, is governed by the average dose of drug, the route of administration, duration of use, rate of metabolism, and the chemical properties of the analyte (Cary 2006). Consideration of the elimination half-life in WBE method development offers an opportunity to refine analysis techniques and improve the value of data collected. The drug analytes used in this study were identified with elimination half-lives ranging from 0.6 to 39.5 hours (Appendix D: Table 24) (Ambre et al. 1988, Chan et al. 1983, Cone et al. 1991a, Cone et al. 1991b, DeVane et al. 1991, Greenhill et al. 2003, Hasselstrom et al. 1990, Kirvela et al. 1996, Kuhlman Jr et al. 1996, Olkkola et al. 1999, Schepers et al. 2003, Torre et al. 2000, Wolff et al. 1997), which could impact the presented results. The elimination half-life of 6-acetymorphine (0.6 hours) results in 99.9% body elimination of the compound within a 24-hour period, thus any detectable presence of 6-acetylmorphine can be reasonably attributed to heroin consumption within the 24-hour sampling period. The long elimination half-life of EDDP (39.5 hours) results in an excretion period exceeding 16 days from a single dose of methadone making estimations on daily consumption difficult.

Considering elimination half-lives within method development should be looked at as a necessary step in wastewater-based epidemiological approaches and analytes with specific elimination half-lives should be chosen to compliment the study design. If sampling occurs frequently analytes with lower elimination half-lives should be chosen so that day-to-day variance in consumption estimates can be easily identified. If sampling

occurs infrequently analytes with longer elimination half-lives should be used to increase the chance that a target analyte will be identified within the sample. Within this study 41% of the analytes were estimated to have 99.9% narcotic elimination within a 24-hour period and 47% of analytes were estimated to have 99.9% narcotic elimination between 2-4 days. Only two of the analytes were identified with 99.9% narcotic elimination exceeding 7 days. Due to the frequent sampling of this study it is more advantageous to select analytes with lower elimination half-lives if possible.

Factoring in elimination half-lives prior to population normalization could account for the variation in narcotic excretion but is met with significant limitation. Accounting for the long elimination half-life of EDDP (16.5 days) would require 17 days of continuous sampling to obtain corrected consumption for a single day. This is an obvious increase in time and cost to the researcher and may not constitute additional cost of such an analysis. Back-calculation becomes less cumbersome when considering analytes with shorter elimination half-lives but application to this study resulted in statistically insignificant changes to the individual mass loads and no change to the trends observed in the non-corrected dataset.

5.3.6 Study limitations

Analyte concentrations in raw wastewater and narcotic mass loads can be considered the most robust data that can be collected from WBE procedures as error mostly stems from deviance in sample collection, preparation, analyte loss, population variance, and instrument error. This error can be quantified through sample replication and use of proper controls. Narcotic consumption estimates may provide a more tangible

analysis of WBE data but also factor error into the analysis. Variation in narcotic use across the population (Harocopos et al. 2016, Lankenau et al. 2012), prevalence of specific narcotic abuse within a specific region (Harocopos et al. 2016, Warner et al. 2016), uncertainties in pharmacokinetic metabolization and excretion rates (Andes and Craig 2002, Jenkins et al. 1994, Schwartz 2003), uncertainties in the contributing population count (Been et al. 2014, Rico et al. 2017), and the extent of in-sewer narcotic degradation and/or biotic transformation (Postigo et al. 2011) can skew results by orders of magnitude. Analysis of specific narcotics with various limiting factors such as low urinary and fecal excretion profiles or rapid *in*/*ex vivo* degradation may provide additional challenges for the quantification of certain narcotics in wastewater. Furthermore, large relative percentage differences observed for some samples during the sampling campaign could be explained in-part by errors in sample collection, preparation, and analysis procedures, as well as the hydrophobicity of target analytes. The lack of detection of buprenorphine and its metabolite norbuprenorphine in any campus samples is likely due to method sensitivity for the compounds but could also reflect local prescribing practices for opioid withdrawal medication.

There are several factors which would have strengthened this analysis and should be considered in future WBE studies. The pharmacokinetic percentages used in this analysis were obtained for a normalized age range but the average age of the population within the study catchment is likely lower. This could lead to discrepancies in actual metabolization of the compounds which would be reflected in the estimated consumption data. The analytes used for population estimations suffer from the same limitations as the narcotic analytes and could also have impacted the population normalized results. The

addition population estimation from wastewater flow also suffers from the incorporated design safety factor in the flow values used and possible incorrect stormwater tie-in. While this study sampled more routinely (seven consecutive days per month for 5 months) than previous campus-related WBE projects daily sampling would have been ideal and may have identified trends of fentanyl and norfentanyl in wastewater which could have passed through the catchment unobserved. Expanding the analytical method to include more types of drug analytes or using methods which can identify classes of narcotics could provide additional insight into the scope of narcotic abuse on this campus. Inclusion of a self-reporting drug use survey would have also provided a comparison metric for the WBE data.

5.4 Conclusions

Except for ADHD medication, the concentrations and detection frequencies of the narcotics examined in this study exceeded values presented in previously published campus-related literature. Estimated consumption values varied by narcotic compared to U.S. estimations but were predominantly higher than international estimations from city-based WBE literature. These findings correlate with the observed higher drug consumption of college-aged young adults (Schulenberg et al. 2017) and may suggest that variations in drug use could be tracked and compared between geographically distinct U.S. regions through WBE analysis. The first sporadic detection of fentanyl and its metabolite in U.S. campus wastewater may also point to illicit non-medical consumption within the university population (CDC 2016, CDC and University 2017). Certain design factors such as reducing the sewage retention time and consideration of elimination half-life in analyte selection were chosen to improve the study design. This

could account for the high concentrations observed for some narcotic analytes but further examination into the degradation and metabolization of target analytes across different demographic representations is necessary to understand this observation. These results have demonstrated that implementation of WBE in the collegiate setting can provide useful temporal information pertaining to the use of a wide array of narcotics in near-real time and should be adopted by institutes which have a vested interest in the well-being of a collegiate population.

CHAPTER 6

RESEARCH IMPLICATIONS AND RECOMMENDATIONS

This work has shown that chemical data available from environmental matrices can potentially provide insight into health or related trends for a specific community but is still subject to many uncertainties and variants in data analysis which can cause often overlooked discrepancies between sampling locations and/or studies. Variations in sampling method, duration and frequency of sampling, metabolization and/or excretion rates used, and variation in population-normalization procedures can all induce unintended bias into the analyses. Despite these disadvantages application UMM can still provide relatively precise analyte concentrations and mass loads in municipal wastewater which can be used as a complimentary source of data to be considered alongside other viable methods of community health data collection. Furthermore, the technology has primarily been applied to drug analytes to provide hard-metrics on drug consumption – but this technology has seldom been applied to gauge other metrics related to human health and wellness.

Known concentrations of *N*-nitrosamines in matrices commonly related to human exposure were inventoried in Chapter 2, including: food, water, tobacco products, alcoholic beverages, and personal care products. Average daily total *N*-nitrosamine exposure in the U.S. in units of ng/d is estimated at 25,000 ± 4,950 and identified with 6,000 ± 2,950 attributable lifetime cancer cases per one million U.S. residents. Approximately 92% of total daily *N*-nitrosamine exposure can be reduced through

deliberate choices in lifestyle and diet, but some sources of unavoidable exposure exist. In Chapter 3, 40 freshwater sediments collected near 14 U.S. wastewater treatment plants were analyzed by LC-MS/MS to identify *N*-nitrosamine contamination. Three *N*-nitrosamines (NDBA, NDPhA, and NPYR) were detected for the first time in freshwater sediments with a 70% detection frequency across the entirety of the study. *N*-Nitrosamine occurrences up- and downstream of WWTPs were statistically indistinguishable ($p > 0.05$) – which led us to reject one of our initial hypotheses. The results from these two studies suggest that *N*-nitrosamine contamination may be prevalent within environmental matrices within to the urban water system which could provide challenges in application of UMM to track the prevalence of human *N*-nitrosamine exposure within the built environment.

Concentrations of parent and metabolite opioid compounds were screened for in 24-hour composite raw wastewater samples collected from two small midwestern cities in Chapter 4. Consumption estimates within the two small communities was for the most part similar compared to other parts of the U.S. but exceeds use in cities outside of the U.S. for most opioids. Despite similar demographic characteristics of the two regions, prevalence of specific opioid use varied between the two regions – calling into question the validity of forecasting national drug statistics onto a smaller population demographic. In Chapter 5, the WBE approach was applied to a campus population where samples were obtained along the sewer line to capture 100% of campus-borne wastewater and minimize analyte degradation within the sewer system. All analytes aside from buprenorphine and its metabolite were detected at least once during the sampling campaign but estimated narcotic consumption and analyte detection frequency exceeded

previous reported values from related U.S. campus literature. The results presented in Chapters 4 and 5 suggest that the variation in narcotic consumption between similar communities could be tracked through WBE approaches which could provide valuable insight for evidence-based public health decision making. The sensitive method detection limits developed for the LC-MS/MS analysis also allowed for the detection of fentanyl and its metabolite in all the sampling locations, which could yield clues into the prevalence of non-medical fentanyl consumption within the United States. Focusing on potent opioids responsible for the drastic increase of drug-induced overdose deaths, like fentanyl and its analogs, could have a large positive impact on the current U.S. opioid crisis – and thus would benefit from frequent and widespread wastewater monitoring.

To summarize, two parameters of UMM (freshwater sediments and wastewater) have been examined and results suggest that (i) *N*-nitrosamine exposure from the ingestion pathway due to food consumption (6.7 ± 0.8 ng/g) constitutes an important role in exposure which can be mitigated to some extent through deliberate diet and lifestyle choices; (ii) *N*-nitrosamine contamination (NDBA: 0.2-3.3 ng/g dw; NDPhA: 0.2-4.7 ng/g dw; NPYR; 3.4-19.6 ng/g dw) is prevalent (DF=70%) in freshwater sediments; (iii) upstream and downstream *N*-nitrosamine sediment contamination were not statistically different (p=0.42); (iv) estimated opioid consumption varied between similar communities, was in-line with previous U.S. consumption estimates, but exceeded estimates provided in international literature, and (v) narcotic consumption in the southwestern U.S. university was higher, and more frequently detected compared to similar WBE studies focusing on sampling at college campuses.

6.1 Considerations for further development of WBE analysis methods

Wastewater-based epidemiology has experienced significant worldwide implementation, especially within the European Union (Kankaanpää et al. 2014, Vuori et al. 2014, Zuccato et al. 2005) even though it is still a developing technology. While city-specific WBE data provides valuable information for internal validation and trend analyses, discrepancies in method development and analytical approaches can propagate error into the results (Fig. 17) (Thai et al. 2014). These discrepancies could be partially addressed if WBE researchers approach data analysis using standardized metabolite excretion rates, degradation rates, analyte elimination half-lives, population estimators, correction factors, and target analytes used for the estimation of narcotic consumption. While error will still propagate within analyses it would likely be relatively similar across studies and could strengthen trend observations between unrelated study locations. Furthermore, if the study catchment primarily consists of individuals from a specific sub-demographic (i.e. college-aged young adults) average population metabolization and excretion parameters used in the data analysis may need to be reevaluated.

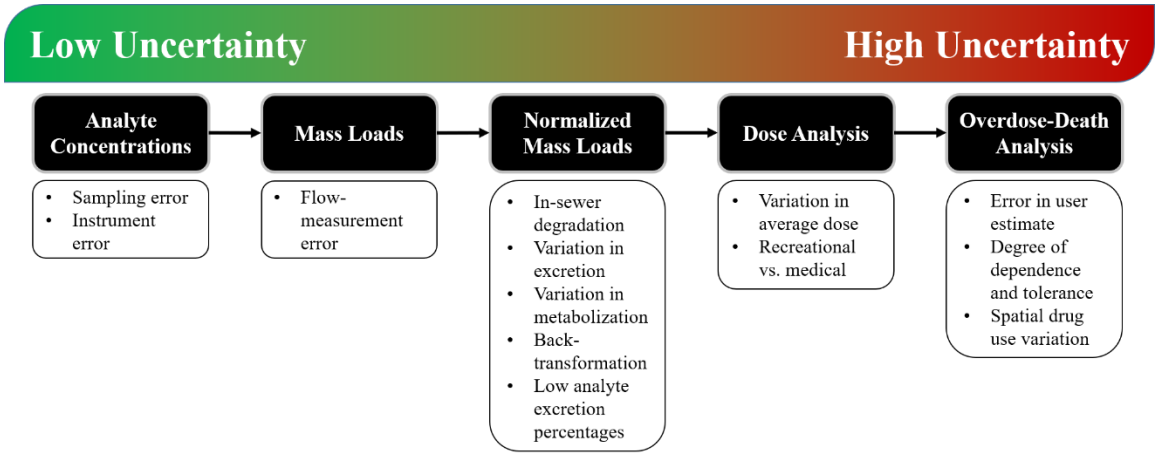


Fig. 17 - Uncertainty in various steps of WBE data modeling and estimations.

One assumption that is echoed through many WBE papers is the assumption of no analyte degradation in-sewer (Postigo et al. 2011, Zuccato et al. 2008), but papers which have examined analyte degradation rates note up to 90% degradation (O'Brien et al. 2017, Thai et al. 2014) under sewershed conditions. The frequent use of the negligible degradation assumption has likely contributed to the underestimation of narcotic abuse in some study regions, thus sewer degradation rates should be considered in the WBE approach. The frequency of sampling is also an important consideration in the analyte selection phase of method development. Analytes with short elimination half-lives compliment frequent sampling, as these analytes point to the recent ingestion of a narcotic. If possible, short elimination half-life analytes should not be used in sporadic sampling campaigns as there is a high chance that an analyte mass will pass through the catchment system unnoticed. Consequently, analytes with longer elimination half-lives will excrete slowly over time – and should be coupled with infrequent sampling as there is a high chance for analyte capture but less dissectible information regarding day-to-day consumption trends of the narcotic.

6.2 Application of wastewater-based epidemiology to *N*-nitrosamine exposure

Before WBE can be applied to the *N*-nitrosamine class of carcinogens it is important to understand at what rate humans are exposed to these chemicals, how they are degraded, formed, and metabolized *in vivo* (Lundberg et al. 2004), and how they impact matrices that are integral to urban water systems. These carcinogens are not knowingly ingested by individuals but instead are a result of unintentional exposure through ingestion, inhalation and dermal sorption. This provides a unique limitation because it becomes difficult to identify sources of *N*-nitrosamines wastewater occurrence

through *N*-nitrosamine congener analysis. It may be advantageous to identify *N*-nitrosamine metabolites in wastewater as these may provide a more accurate representation of human exposure. Literature related to the human metabolism of *N*-nitrosamines is limited (Carmella et al. 1993, Kozlovich et al. 2015), and studies that have examined human metabolism of *N*-nitroso compounds suggest a high variability between individuals (Camus et al. 1993). It would also be necessary to identify any potential additional routes of formation for these metabolites within the microbial communities encountered in sewer pipe biofilms.

Application of the WBE process to monitor *N*-nitrosamines and respective metabolites would result in a novel method of tracking carcinogen exposure within our communities. Estimated exposure concentrations founded in chemical data (in units of mass/day/person) could be calculated for a specific community and benchmarked against the theoretical exposure concentrations identified in Chapter 2. *N*-Nitrosamines WBE analysis could also provide an additional metric for smoking prevalence by monitoring tobacco-specific *N*-nitrosamines and their respective metabolites (Ma et al. 2017). As regulations and maximum contamination limits regarding the *N*-nitroso class are developed it will become important to understand if the imposed regulatory limits are sufficient. While testing for *N*-nitrosamine contamination at the drinking water treatment plant (DWTP) is necessary, concentrations reported here may not accurately reflect exposure levels due to the observed *N*-nitrosamine formation in water distribution systems after treatment discharge (Zhao et al. 2006). By sampling both at the DWTP discharge point and the WWTP inlet, we can understand if (i) drinking water is in

accordance with regulations, and (ii) if those regulations have any impact on exposure levels within the service community.

6.3 Widespread spatial and temporal WBE testing

Evidence-formed public health decision making is vital to addressing public health crises effectively, but this process is only effective if key players have a comprehensive understanding of the health crisis. The nature of the opioid epidemic makes this a difficult task as prevalence of opioid abuse can vary substantially between two similar regions, made evident in chapters 4 and 5. Targeted substance abuse programs have been implemented as tools to reduce addiction prevalence through education and intervention (Botvin et al. 1984, Jalilian et al. 2015, Walsh 2015) but the efficacy of these programs is subject to debate (West and O'Neal 2004). Some programs, such as needle exchanges (Lurie et al. 1993), have also been proposed as methods to reduce substance abuse or lessen some of the externalities associated with drug addiction, such as HIV transmission (Hurley et al. 1997) or overdose-death rates (Maxwell et al. 2006). Implementation of WBE approaches provides researchers and health officials with an analytical tool to monitor drug analyte concentrations in wastewater within a specific region and could be employed to provide near real-time information regarding the efficacy of implemented substance abuse programs. WBE also has the potential to be applied to screen for viral DNA (Bofill-Mas et al. 2006, Tamaki et al. 2012) and could be implemented to determine estimated rates of HIV prevalence before and after implementation of a needle-exchange program, although this would likely require long-term monitoring as reduction in HIV prevalence would not be an immediate response.

While WBE has traditionally been applied to gauge drug addiction, the technology has widespread potential application. As previously mentioned, health parameters such as carcinogenic exposure, prevalence of viral or bacterial infection within a community, prevalence of smoking and alcohol use, stress profiles of a community, and prevalence of antibiotic resistance all constitute potential applications of the WBE technology. In fact, if an event results in the deposition of a target analyte within the urban water environment it is likely that we can track that parameter through WBE analysis – which is perhaps the largest benefit of this technology. Between 70-90% of U.S. residents are serviced by a municipal wastewater treatment system (Westerhoff et al. 2015) so WBE analyses can be applied for most U.S. populations without any major infrastructure changes. These factors favor the widespread implementation of WBE testing across the United States, and data obtained from this practice will continue to improve as the technology continues to develop.

6.4 Recommendations for future research

While wastewater analysis can provide researchers with an understanding of health trends within a specific area, further development in the field is necessary to improve the validity and accuracy of the results obtained. Standardization of WBE method development, sampling, and analysis procedures is necessary to provide more uniform results across unrelated study locations. This includes (i) the development of standard target analytes, (ii) further understanding of the percentage and variation of analyte metabolization and excretion rates across the general population and sub-demographics, (iii) the development of robust population estimator compounds, (iv) use of frequent sampling as opposed to infrequent sampling, and (v) development of robust

analyte in-sewer degradation and/or transformation rates. Widespread application of WBE in the United States will also provide additional necessary benchmarks for narcotic abuse and identify areas where intervention is mandatory – thus constituting a necessary area of continued research. Perhaps more important is the expansion of the wastewater analysis approach to health parameters outside drug abuse, as it represents a small percentage of the total volume of information that can be derived from wastewater analyses. Expansion of the WBE process will likely require additional information regarding the *in vivo* occurrence, metabolization, and excretion of target analytes – but it is possible pertinent information already exists within literature.

The UMM analyses conducted in this dissertation represent a small subset of the potential information that can be derived from routine wastewater analysis. While WBE has traditionally been applied to track drug consumption within a community, the process could be applied to better understand population consumption habits (Baker et al. 2014, Kim et al. 2015), general wellness of a community (Fattore et al. 2016, Rousis et al. 2017), prevalence of personal care product use (Gao et al. 2016), and approximate carcinogen exposure (Lai et al. 2017). Expanding matrix analysis outside of wastewater could provide additional insight into some of these population health and wellness parameters. Benefits of UMM will only increase as the technology continues to develop and it is likely that the technology will experience wider implementation in the future, so continued development of UMM analytical methods should be viewed as incumbent for researchers involved in UMM and WBE analyses. Regardless, the UMM analyses conducted in this dissertation have shown that variation of analyte concentrations in raw wastewater can be identified through wastewater analysis and should be implemented in

conjunction with currently viable methods of public health data collection across the United States.

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APPENDIX A

SUPPLEMENTAL MATERIAL FOR CHAPTER 2

Table 3 - Existing regulations involving N-nitrosamine congener contamination within water sources.

Regulatory Country or State	Enacted Law	Water Type	Contaminant (Maximum Limit)	Source
United States Federal Government	Contaminant Candidate List	Drinking	NDMA (no limit) NDEA (no limit) NDPA (no limit) NDPhA (no limit) NPYR (no limit)	CCL 4, 2016
California (U.S. State)	Action Level Public Health Goal	Drinking	NDMA (2 ng/L) NDMA (3 ng/L)	EPA, 2011
Massachusetts (U.S. State)	Regulatory Limit	Drinking	NDMA (10 ng/L)	EPA, 2015
Arizona (U.S. State)	Regulatory Limit	Discharge	NDMA (1 ng/L) NDPA (5 ng/L) NDPhA (7,100 ng/L)	AZDEQ, 2015
Canada	Maximum Limit	Drinking	NDMA (40 ng/L)	Selin, 2011
Germany	Maximum Limit	Any Waters	NDMA (10 ng/L)	Selin, 2011
United Kingdom	Maximum Limit Emergency Action	Drinking	NDMA (10 ng/L) NDMA (200 ng/L)	UKDWI, 2001

APPENDIX B

SUPPLEMENTAL MATERIAL FOR CHAPTER 3

Quality Assurance and Quality Control

Calibration accuracy was verified for each batch using a calibration standard solution with labeled and native analytes. Retention times of native and labeled compounds in the sample had to be within ±12 s (0.2 min) of the respective retention time established during the previous calibration. Multiple lab blanks were analyzed for each batch to check for laboratory contamination. A duplicate sample was analyzed for every five samples in a batch to evaluate analysis precision. Precision between samples and duplicates was expressed as relative percentage difference (RPD), which was calculated using the following expression:

$$\mathrm{RPD}[\%] = \frac{\left|\mathrm{C_{sample}} - \mathrm{C_{duplicate}}\right| * 100}{\frac{\mathrm{C_{sample}} + \mathrm{C_{duplicate}}}{2}}$$

where S_{ample} and $C_{duplicate}$ are the concentrations detected in the original sample and in its duplicate, respectively. Matrix spikes were performed for selected samples to confirm analyte presence in the sample and to evaluate recovery rates for analytes without deuterated labeled analogues. The stability of *N*-nitrosamines under storage conditions was tested for freshly collected samples by frequent analysis over a period of eight months. Testing of these samples revealed no discernable degradation or formation of *N*-nitrosamines under the aforementioned storage conditions.

***N*-Nitrosamine Analysis.** All glassware used in the experiments were baked at 550 ºC, caps were acid washed using 10% HCl and thoroughly rinsed with ultrapure water prior to use to prevent contamination. About 6 g wet weight (ww) of sediment was weighed in amber glass (40 mL) vials, spiked with 250 ng each of deuterated surrogates and extracted using DCM (2 mL per g of sediments) as described for sludge elsewhere (Venkatesan et al. 2014). The extract was concentrated to near dryness under a gentle stream of nitrogen gas, reconstituted with 2 mL methanol and then sonicated for 15 minutes. The extract was then centrifuged at 440 G for 5 minutes, and 0.75 mL of the resulting supernatant was diluted 1+1 (v/v) with water prior to analysis by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) in positive ionization mode.

The tandem mass spectrometer (API 4000 instrument; Applied Biosystems, Framingham, MA, USA) used was coupled to a Shimadzu Prominence HPLC (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) for sample introduction and compound separation. Separation of analytes was carried out on an XBridge BEH C_8 column, (130 Å, 3.5 µm, 4.6 x 150 mm; Waters, Milford, USA). The mobile phase consisted of solvent A (10 mM ammonium acetate with 0.01% acetic acid) and solvent B (100% methanol) flowing at a rate of 400 µL/min with a total runtime of 16 minutes. The solvent gradient program consisted of 50% of solvent B for 2 min, followed by an increase from 50% to 90% over 11 min, and holding at 90% for 3 min, before returning back to 50% of solvent B over 0.1 min, followed by a 2-min equilibration period prior to injection of the next sample aliquot (100 µL volume). Analytes were introduced into the mass spectrometer using an electrospray ionization probe in positive mode. Multiple reaction monitoring (MRM) was used for qualitative analysis. Optimized conditions for the ionization and fragmentation of the analytes and QA/QC protocol are included as supporting information (see supporting information Table S-1). Wet weight concentrations obtained from the analysis were converted to dry weight (dw) concentrations using the solid content of the analyzed sediments. All concentrations are reported as ng/g dw.

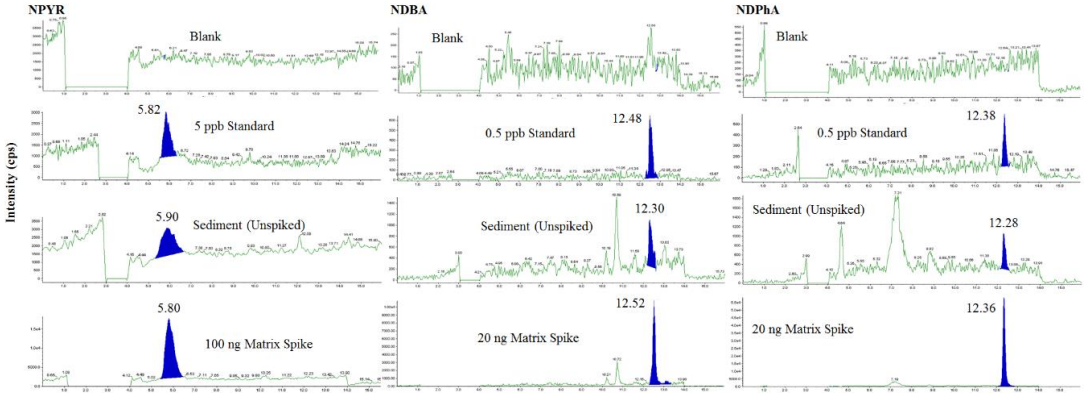

Fig. 18 - LC-MS/MS chromatograms of standards, sample extracts and matrix spike samples of three detected N-nitrosamines. The number next to the peak represents the retention time of the analyte in minutes.

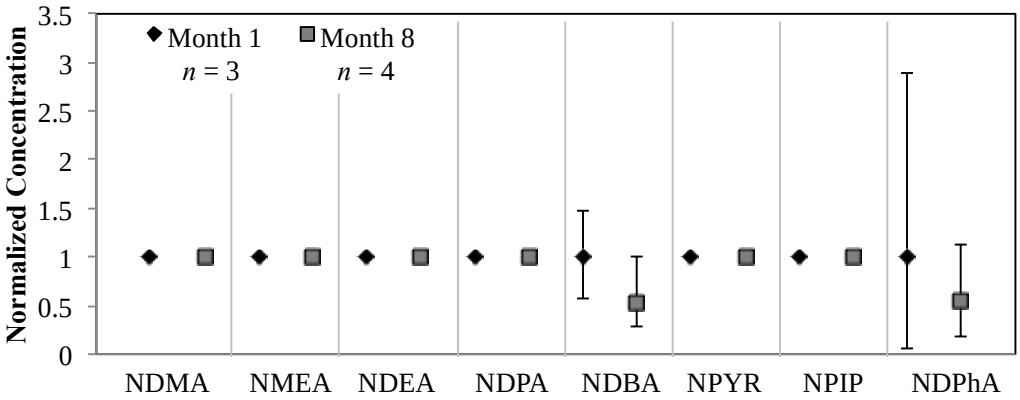

Fig. 19 - Stability test for the analyzed N-nitrosamines in fresh sediments during storage at –20C analyzed over a period of eight months. Concentrations were normalized to the average initial concentration detected in freshly collected sediment (Month 1). Half the corresponding MDL value was used for non-detects (NDMA, NMEA, NDEA, NDPA and NPIP). NDBA and NDPhA were present in native sediment and did not show appreciable changes in concentration during prolonged storage.

Table 4 - Method performance and concentrations of N-nitrosamines in freshwater sediments

Compound	Recovery (%)		MDL (ng/g dw)	Sediment concentration avg. (min, max) (ng/g dw)[c]	RPD (%)	Detection frequency (%)
	Absolute	Relative				
N-nitrosodimethylamine (NDMA)	54 ± 21	87 ± 2	10.2	<MDL (10.2)	-	-
N-nitrosomethylethylamine (NMEA)[a]	63 ± 34[b]	-	1.7	<MDL (1.7)	-	-
N-nitrosodiethylamine (NDEA)[a]	60 ± 28[b]	-	3.9	<MDL (3.9)	-	-
N-nitrosodi-*n*-propylamine (NDPA)	64 ± 23	78 ± 8	1.7	<MDL (1.7)	-	-
N-nitrosodibutylamine (NDBA)[a]	64 ± 17[b]	-	0.1	0.7 (0.2, 3.3)	38 ± 25	58
N-nitrosopyrrolidine (NPYR)[a]	108 ± 39[b]	-	3.5	8.7 (3.4, 20)	18 ± 16	18
N-nitrosopiperidine (NPIP)	78 ± 18	80 ± 17	3.6	<MDL (3.6)	-	-
N-nitrosodiphenylamine (NDPhA)	59 ± 14	82 ± 31	0.1	1.4 (0.2, 4.7)	11 ± 9	50

[a]Concentrations of analytes lacking stable-isotope labeled analogues are not recovery corrected. [b]Absolute recoveries of these analytes were determined from matrix spike studies. [c]Dry weight concentrations were calculated from wet weight concentrations using the solids content of the biosolids samples. "<MDL" represent not-applicable/non-detects. MDL: method detection limit. RPD: relative percentage difference.

Table 5 - LC-ESI-MS/MS parameters for analysis of N-nitrosamines

MS/MS parameter	
Ion source	Positive electrospray ionization
Collision Gas	6
Curtain Gas	50
Ion source Gas 1	80
Ion Source Gas 2	70
Ion Spray Voltage	4500 V
Source Gas Temperature	700 ^{o}C

Analyte	Parent ion (m/z)	Product ion (m/z)	Declustering potential (V)	Exit Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)	Retention Time (min)
NDMA	75	43	51	10	25	2	5.34
NMEA	89	61	51	10	17	10	5.86
NDEA	103	75	51	10	17	12	6.65
NDPA[a]	131	89	51	10	17	8	8.82
	131	43					
NDBA[a]	159	103	56	10	17	8	11.18
	159	57					
NPYR	101	55	61	10	23	8	5.64
NPIP[a]	115	69	61	10	23	12	6.73
	115	41					
NDPhA[a]	199	169	56	10	17	8	11.16
	199	168					
			Deuterated isotopes				
NDMA-d_6	81	46	51	10	25	2	5.35
NDPA-d_{14}	145	50	51	10	17	8	8.76
NPIP-d_{10}	125	78	61	10	23	12	6.70
NDPhA-d_6	205	175	56	10	17	8	11.11

[a]Two different transitions were used for these analytes for quantification and identification

Table 6 - n-Octanol-water partitioning coefficient of N-nitrosamines (source: SciFinder).

Compound	**Log K_{ow}**
N-nitrosodimethylamine (NDMA)	-0.5
N-nitrosomethylethylamine (NMEA)	0.01
N-nitrosodiethylamine (NDEA)	0.51
N-nitroso-di-*n*-propylamine (NDPA)	1.54
N-nitrosodibutylamine (NDBA)	2.56
N-nitrosopyrrolidine (NPYR)	-0.1
N-nitrosopiperidine (NPIP)	0.44
N-nitrosodiphenylamine (NDPhA)	3.13

APPENDIX C

SUPPLEMENTAL MATERIAL FOR CHAPTER 4

Table 7 - Demographic information for City 1 and City 2, obtained from the U.S. Census Bureau 2010 American fact finder statistics.

	City 1	City 2
Median Age	34.1	37.1
Under 18 (%)	27.7	27.6
18-29 (%)	12.4	12.8
30-64 (%)	46.3	45.2
65+ (%)	13.6	14.4
% White	57	86.9
% African American	34.5	2.7
% Asian	0.4	5.6
% Other/Mixed	8.1	4.8
% Hispanic or Latino (any race)	8.2	5.8
Average Household Size	2.53	2.43
Homeowner Vacancy Rate	2.8	2.9
Rental Vacancy Rate	10.6	14.2
Unemployment Rate (%)	8.3	3.7
Per Capita Income	14,500	29,400

Table 8 - Optimized conditions for the ionization and fragmentation of the opioid parent and metabolite analytes screened for in this method.

Opioid Type	Consumption Indicator	Precursor ion (m/z)	Product ion 1 (m/z)	CE(1)	Product ion 2 (m/z)	CE(2)
Morphine	Morphine	268.054	151.9	81	164.9	57
	Morphine-3-Glucuronide	462.184	268	45	164.9	83
Codeine	Codeine	300.153	151.9	89	164.8	57
	Norcodeine	268.084	152.1	79	164.9	57
Oxycodone	Oxycodone	316.029	240.8	41	297.9	27
	Noroxycodone	302.117	284	25	187	35
Fentanyl	Fentanyl	337.1	188.1	33	105.1	51
	Norfentanyl	223.144	84	25	55	59
Heroin	Heroin	370.018	164.8	67	58	59
	6-Acetylmorphine	328.162	165	51	210.9	37

Table 9 - Method detection limits for opioid analytes.

Analyte	Method Detection Limit (ng/L)
Morphine	0.9
Morphine-3-Glucuronide	0.2
Oxycodone	0.2
Noroxycodone	0.3
Codeine	1.4
Norcodeine	0.8
Heroin	0.3
6-Acetylmorphine	0.3
Fentanyl	0.3
Norfentanyl	0.2

Table 10 - Opioid narcotics, respective consumption indicator compounds, excretion rate of respective consumption indicators, correction factors used for each consumption indicator, and average prescribed oral dose per opioid per Mayo Clinic doctor guidelines.

Drug	Consumption Indicator	Excretion Rate *(%)*	Correction Factor	Average Dose *(mg)*
Morphine	Morphine	10	10.0	30
	Morphine-3-Glucuronide	75	0.8	
Codeine	Codeine	57.5	1.7	30
	Norcodeine	3.77	27.8	
Oxycodone	Oxycodone	8.9	11.2	10
	Noroxycodone	22.1	4.7	
Fentanyl	Fentanyl	6	16.7	0.1
	Norfentanyl	91.08	1.6	
Heroin	Heroin	*n/a*	*n/a*	30
	6-Acetylmorphine	1.3	86.8	

Table 11 - State overdose, overdose-deaths, and ratio information.

State	Overdose	Death	Ratio	Notes
Arizona	3920	538	7.29	
Virginia	8710	803	10.85	
Rhode Island	1499	335	4.47	
Minnesota	2074	395	5.25	
Oregon	9.6	6.829	1.41	*Pop Normalized Values
Colorado	22.3	7.8	2.86	*Pop Normalized Values

Table 12 - Average ± standard error, minimum, and maximum raw wastewater analyte concentrations across the two cities.

City 1			
Analyte	***Average Concentration***	***Minimum Concentration***	***Maximum Concentration***
	all concentration in ng/L		
Morphine	*713 ± 38*	*379*	*1,310*
Oxycodone	*17.8 ± 1.1*	*3*	*43*
Codeine	*322 ± 37*	*191*	*571*
Fentanyl	*1.7 ± 0.2*	*<MDL*	*3.6*
Heroin	*41 ± 16*	*<MDL*	*120*
Morphine-3-Glucuronide	*7.0 ± 2.5*	<MDL	26.1
Noroxycodone	*73 ± 5*	61	96
Norcodeine	*162 ± 27*	15	397
Norfentanyl	*30 ± 2*	12	136
6-Acetylmorphine	*43 ± 15*	13	136

City 2			
Analyte	***Average Concentration***	***Minimum Concentration***	***Maximum Concentration***
	all concentration in ng/L		
Morphine	*306 ± 29*	*159*	*750*
Oxycodone	*78 ± 6*	*22*	*251*
Codeine	*100 ± 27*	*<MDL*	*453*
Fentanyl	*1.0 ± 0.5*	*<MDL*	*4.4*
Heroin	*19 ± 11*	*<MDL*	*28*
Morphine-3-Glucuronide	*7.6 ± 1.8*	<MDL	23.8
Noroxycodone	*105 ± 7*	47	171
Norcodeine	*47 ± 8*	<MDL	103
Norfentanyl	*48 ± 2*	11	198
6-Acetylmorphine	*21 ± 3*	7	35

Table 13 - Average ± standard error, minimum, and maximum analyte daily mass loading across the two cities.

City 1			
Analyte	***Average Concentration***	***Minimum Concentration***	***Maximum Concentration***
	all concentration in mg/day		
Morphine	*30,000 ± 1,600*	*15,332*	*60,296*
Oxycodone	*850 ± 51*	*161*	*2,249*
Codeine	*15,800 ± 1,800*	*8,520*	*26,273*
Fentanyl	*80 ± 9*	*<MDL*	*162*
Heroin	*910 ± 335*	*<MDL*	*5,152*
Morphine-3-Glucuronide	*350 ± 109*	<MDL	1,360
Noroxycodone	*3,470 ± 230*	2,923	4,754
Norcodeine	*7,680 ± 1,320*	794	19,647
Norfentanyl	*1,450 ± 80*	569	7,097
6-Acetylmorphine	*1,950 ± 640*	665	4,915

City 2			
Analyte	***Average Concentration***	***Minimum Concentration***	***Maximum Concentration***
	all concentration in mg/day		
Morphine	*8,690 ± 790*	*4,287*	*29,718*
Oxycodone	*2,185 ± 170*	*578*	*7,305*
Codeine	*2,648 ± 600*	*<MDL*	*10,303*
Fentanyl	*24 ± 9*	*<MDL*	*89*
Heroin	*156 ± 92*	*<MDL*	*730*
Morphine-3-Glucuronide	*200 ± 44*	<MDL	630
Noroxycodone	*2,770 ± 176*	1,244	5,240
Norcodeine	*1,230 ± 190*	<MDL	2,713
Norfentanyl	*1,270 ± 60*	290	5,240
6-Acetylmorphine	*560 ± 75*	190	929

Table 14 - Average ± standard error, minimum, and maximum analyte population normalized mass load consumption across the two cities.

City 1			
Analyte	***Average Concentration***	***Minimum Concentration***	***Maximum Concentration***
	all concentration in mg/day/1,000 population		
Morphine	*2,590 ± 157*	*1,170*	*4,603*
Oxycodone	*72 ± 12*	*14*	*192*
Codeine	*204 ± 13*	*111*	*341*
Fentanyl	*10 ± 1.2*	*4*	*21*
Morphine-3-Glucuronide	26 ± 8	<MDL	103.8
Noroxycodone	124 ± 6	105	171
Norcodeine	1,630 ± 284	169	4,169
Norfentanyl	18 ± 7	7	87
6-Acetylmorphine	1,294 ± 296	441	3,257

City 2			
Analyte	***Average Concentration***	***Minimum Concentration***	***Maximum Concentration***
	all concentration in mg/day/1,000 population		
Morphine	*1,970 ± 255*	*974*	*6,754*
Oxycodone	*556 ± 89*	*147*	*1,859*
Codeine	*102 ± 21*	*0.9*	*398*
Fentanyl	*9 ± 2.7*	*0.9*	*34*
Morphine-3-Glucuronide	3.8 ± 1	<MDL	11.5
Noroxycodone	300 ± 35	128	487
Norcodeine	790 ± 180	<MDL	1,726
Norfentanyl	47 ± 18	10	191
6-Acetylmorphine	1,127 ± 163	404	1,844

Table 15 - Analyte concentration changes across the study period for City 1 and City 2.

City 1			
Analyte	***Increase/Decrease***	***Percent***	***P-Value***
Morphine	Decrease	1.9%	0.875
Oxycodone	Increase	565%	<0.01
Codeine	Decrease	13.50%	0.258
Fentanyl	Decrease	22.10%	0.307

City 2			
Analyte	***Increase/Decrease***	***Percent***	***P-Value***
Morphine	Decrease	25.80%	0.244
Oxycodone	Increase	39.10%	0.303
Codeine	Decrease	33.30%	0.327
Fentanyl	*n/a*	*n/a*	*n/a*

Table 16 - Dose-estimated usage for City 1 and City 2.

City 1			
Analyte	***Parent Compound Estimation***	***Metabolite Compound Estimation***	***Percent Difference***
	all concentration in dose/day/1,000 population		
Morphine	86 (range: 39-153)	1 (range: 0-3)	195.4%
Oxycodone	7 (range: 1-19)	12 (range: 10-17)	52.6%
Codeine	5 (range: 4-11)	54 (range: 6-139)	166.1%
Fentanyl	102 (range: 37-207)	177 (range: 70-867)	53.8%
Heroin	n/a	43 (range: 15-109)	n/a

City 2			
Analyte	***Parent Compound Estimation***	***Metabolite Compound Estimation***	***Percent Difference***
	all concentration in dose/day/1,000 population		
Morphine	66 (range: 32-225)	n/a	n/a
Oxycodone	56 (range: 15-186)	30 (range: 13-49)	60.5%
Codeine	3 (range: 0-13)	26 (range: 0-58)	158.6%
Fentanyl	91 (range: 9-339)	475 (range: 104-1,905)	135.7%
Heroin	n/a	38 (range: 13-61)	n/a

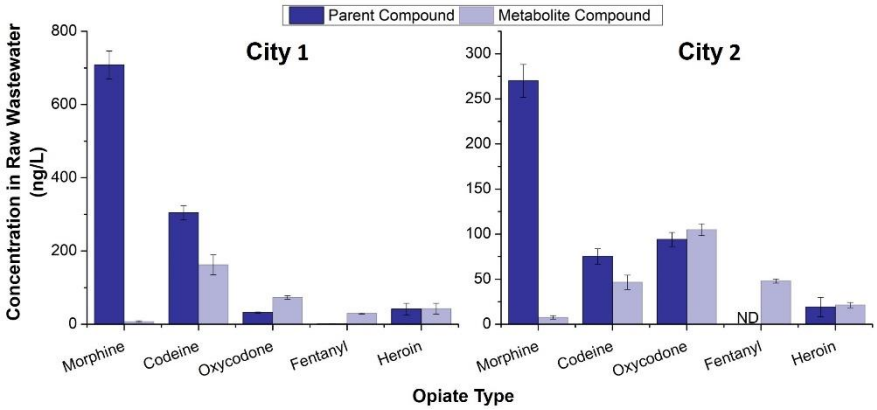

Fig. 20 - Comparison of raw wastewater parent opioid and opioid metabolite concentrations during the 2016-2017 sampling period. Error bars represent calculated standard error.

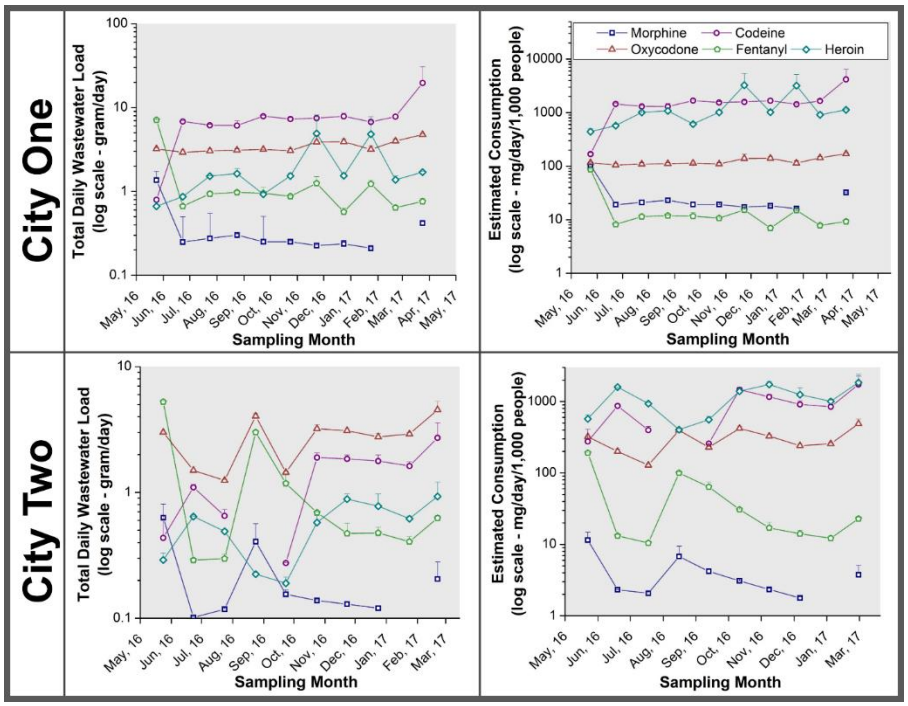

Fig. 21 - Total daily wastewater loading and estimation consumption values for the suite of opioids derived from opioid metabolite analysis. Populations were estimated by population served by the wastewater treatment plants, and correction factors used are listed in Table S2.

APPENDIX D

SUPPLEMENTAL MATERIAL FOR CHAPTER 5

Table 17 - Sewer length and approximate sewage retention time (SRT) information.

	Location 1	**Location 2**	**Location 3**	**Longest Distance**
Max (ft)	4528	5808	1624	9951
Min (ft)	1341	2882	1519	6764
Average (ft)	3463.375	4572.4	1571.5	8886.375
Minimum Velocity (ft/s)	1.5	1.5	1.5	1.5
Average Velocity (ft/s)	3	3	3	3
Maximum Velocity (ft/s)	10.7	10.7	10.7	10.7
Max Time (min)	50.3	64.5	18.0	110.6
Min Time (min)	2.1	4.5	2.4	10.5
Average Time (min)	**19.2**	**25.4**	**8.7**	**49.4**

Table 18 - Optimized conditions for the ionization and fragmentation of the opioid parent and metabolite analytes screened for in this method.

Opioid Type	Consumption Indicator	Precursor ion (m/z)	Product ion 1 (m/z)	CE(1)	Product ion 2 (m/z)	CE(2)
Morphine	Morphine	268.054	151.9	81	164.9	57
	Morphine-3-Glucuronide	462.184	268	45	164.9	83
Codeine	Codeine	300.153	151.9	89	164.8	57
	Norcodeine	268.084	152.1	79	164.9	57
Oxycodone	Oxycodone	316.029	240.8	41	297.9	27
	Noroxycodone	302.117	284	25	187	35
Fentanyl	Fentanyl	337.1	188.1	33	105.1	51
	Norfentanyl	223.144	84	25	55	59
Heroin	Heroin	370.018	164.8	67	58	59
	6-Acetylmorphine	328.162	165	51	210.9	37
Methadone	EDDP	278.192	234.1	43	186	49
Buprenorphine	Buprenorphine	468.281	396.1	55	414.3	47
	Norbuprenorphine	414.328	101.1	57	115.1	125
Amphetamine	Amphetamine	136.039	91	23	119	35
Methylphenidate	Methylphenidate	234.2	84	35	56.1	40
Alprazolam	Alprazolam	309.105	281	39	205	59
	alpha-hydroxyalprazolam	325.112	215.9	55	205	61
Cocaine	Cocaine	304.117	182	29	104.9	45
	Benzoylecgonine	290.103	168	29	105	45
MDMA	MDMA	194.098	162.8	19	105	35

Table 19 – Method detection limits for narcotic analytes.

Analyte	Method Detection Limit (ng/L)
Morphine	0.9
Morphine-3-Glucuronide	0.2
Oxycodone	0.2
Noroxycodone	0.3
Codeine	1.4
Norcodeine	0.8
Heroin	0.3
6-Acetylmorphine	0.3
Fentanyl	0.3
Norfentanyl	0.2
EDDP	1.7
Buprenorphine	140
Norbuprenorphine	120
Amphetamine	0.9
Methylphenidate	0.3
Alprazolam	0.5
Alpha-hydroxyalprazolam	0.2
Cocaine	0.6
Benzoylecgonine	0.7
MDMA	0.5

Table 20 - Screened narcotics, respective consumption indicator compounds, excretion rate of respective consumption indicators, correction factors used for each consumption indicator, and average prescribed oral dose per opioid per Mayo Clinic doctor guidelines.

Drug	Consumption Indicator	Excretion Rate *(%)*	Molar Mass Ratio	Correction Factor	Average Dose *(mg)*
Morphine	Morphine	10	1	10.0	30
	Morphine-3-Glucuronide	75	0.62	0.8	
Codeine	Codeine	57.5	1	1.7	30
	Norcodeine	3.77	1.05	27.8	
Oxycodone	Oxycodone	8.9	1	11.2	10
	Noroxycodone	22.1	1.05	4.7	
Fentanyl	Fentanyl	6	1	16.7	0.1
	Norfentanyl	91.08	1.45	1.6	
Heroin	Heroin	*n/a*	n/a	*n/a*	30
	6-Acetylmorphine	1.3	1.13	86.8	
Methadone	EDDP	23	1.12	4.9	30
Amphetamine	Amphetamine	30	1	3.3	30
Methylphenidate	Methylphenidate	1.5	1	66.7	30
Alprazolam	Alprazolam	20	1	5.0	2
	Alpha-OH-Alprazolam	n/a	n/a	n/a	
Cocaine	Cocaine	n/a	n/a	n/a	50
	Benzoylecgonine	45	1.05	2.3	
MDMA	MDMA	65	1	1.5	100

Table 21 – Sampling location 1 narcotic analyte sample extract concentration average ± standard deviation, minimum and maximum concentrations observed, and detection frequency.

Analyte	Average ± Standard Deviation	Minimum Concentration	Maximum Concentration	Detection Frequency
		(ng/mL)		*(%)*
Oxycodone	2.4 ± 0.1	1.6	3.9	100
Codeine	3.1 ± 0.2	1.5	7.1	100
Heroin	0.07 ± 0.04	<MDL	1.2	7
Fentanyl	N.D.	<MDL	<MDL	0
Morphine-3-Glucuronide	7.7 ± 1.3	<MDL	44.6	98
Noroxycodone	2.4 ± 0.3	<MDL	9.5	95
Norcodeine	0.37 ± 0.03	0.14	0.73	100
6-Acetylmorphine	1.7 ± 0.1	0.8	3.6	100
Norfentanyl	0.13 ± 0.09	<MDL	3.74	7
EDDP	5.4 ± 0.4	2	15	100
Amphetamine	28.7 ± 1.5	13.3	50	100
Methylphenidate	1.0 ± 0.1	<MDL	3.8	90
Alprazolam	3.6 ± 0.1	1.4	5.6	100
alpha-OH-Alprazolam	0.48 ± 0.03	0.07	0.96	100
Cocaine	15.2 ± 1	5.8	35.9	100
Benzoylecgonine	59.8 ± 3.2	29	104.4	100
MDMA	5.7 ± 1.8	0.6	67.9	100

Table 22 – Sampling location 2 narcotic analyte sample extract concentration average ± standard deviation, minimum and maximum concentrations observed, and detection frequency.

Analyte	Average ± Standard Deviation	Minimum Concentration	Maximum Concentration	Detection Frequency
		(ng/mL)		*(%)*
Oxycodone	1.3 ± 0.1	0.4	3	100
Codeine	2.8 ± 0.5	0.4	15.3	100
Heroin	N.D.	<MDL	<MDL	0
Fentanyl	0.05 ± 0.03	<MDL	1.05	5
Morphine-3-Glucuronide	2.2 ± 0.5	<MDL	9.9	50
Noroxycodone	4.3 ± 0.3	2.3	10.2	100
Norcodeine	0.37 ± 0.03	0.14	0.73	100
6-Acetylmorphine	0.34 ± 0.15	<MDL	5.2	30
Norfentanyl	N.D.	<MDL	<MDL	0
EDDP	0.80 ± 0.15	<MDL	4.6	85
Amphetamine	42.6 ± 3.3	6.7	87.6	100
Methylphenidate	0.77 ± 0.11	<MDL	2.68	88
Alprazolam	4.8 ± 0.2	2.5	7	100
alpha-OH-Alprazolam	0.21 ± 0.03	<MDL	0.52	33
Cocaine	2.0 ± 0.2	0.2	6	100
Benzoylecgonine	17.1 ± 2.2	0.5	61.7	100
MDMA	3.0 ± 0.9	0.2	26.7	100

Table 23 - Average estimated consumption, dose consumption, and estimated number of users within the university study area.

Analyte	Estimated Consumption *(mg/day/1,000 persons)*	Dose Consumption *(dose/day/1,000 persons)*	Estimated Number of Users *(Users/1,000 persons)*
Morphine	18 ± 2.8	0.6 ± 0.1	0.3 ± 0.5
Oxycodone	80 ± 6	8 ± 0.5	4 ± 0.26
Codeine	26 ± 2	0.85 ± 0.06	0.43 ± 0.03
Heroin	474 ± 32	15.8 ± 1.1	7.9 ± 0.55
Fentanyl	N.D.	N.D.	N.D.
Methadone	86 ± 10	2.9 ± 0.3	1.5 ± 0.15
Amphetamine	302 ± 14	10.1 ± 0.4	5.1 ± 0.2
Methylphenidate	236 ± 28	7.8 ± 0.95	3.9 ± 0.47
Alprazolam	60 ± 2	29.7 ± 1.2	14.9 ± 0.6
Cocaine	470 ± 42	9.4 ± 0.8	4.9 ± 0.4
MDMA	30 ± 12	0.3 ± 0.1	0.15 ± 0.05

Table 24 - Analyte elimination half-lives.

Analyte	Classification	Elimination Half-Life (hours)
Morphine	2-day	4.2
Morphine-3-Glucuronide	2-day	4.2
Codeine	24-hour	2.3
Norcodeine	24-hour	2.3
Oxycodone	24-hour	2.3
Noroxycodone	24-hour	2.3
6-Acetylmorphine	24-hour	0.6
Norfentanyl	3-day	9.4
EDDP	7+ day	39.5
Buprenorphine	24-hour	3.21
Norbuprenorphine	7+ day	35.56
Amphetamine	3-day	8
Methylphenidate	24-hour	2
Alprazolam	4-day	12
Cocaine	2-day	5.1
Benzoylecgonine	2-day	5.1
MDMA	3-day	8.5

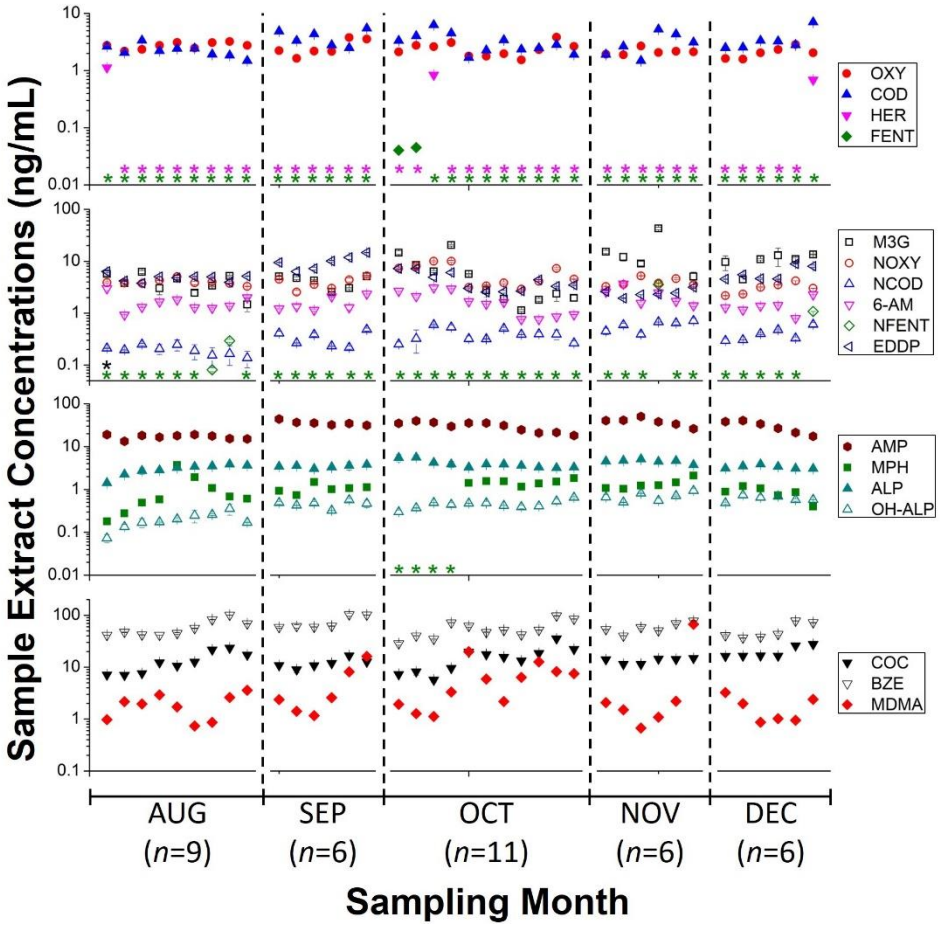

Fig. 22 – Uncorrected sample extract concentrations (ng/mL) of all analytes detected during the August 2017-December 2017 sampling campaign. MOR: morphine; OXY: oxycodone; COD: codeine; HER: heroin; FENT: fentanyl; M3G: morphine-3-glucuronide; NOXY: noroxycodone; NCOD: norcodeine; 6-AM: 6-acetylmorphine; NFENT: norfentanyl; EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; AMP: amphetamine; MPH: methylphenidate; ALP: alprazolam; OH-ALP: alpha-hydroxy-alprazolam; COC: cocaine; BZE: benzoylecgonine; MDMA: 3,4-Methylenedioxymethamphetamine; METH: methamphetamine. Non-detects are represented by an asterisk (*).

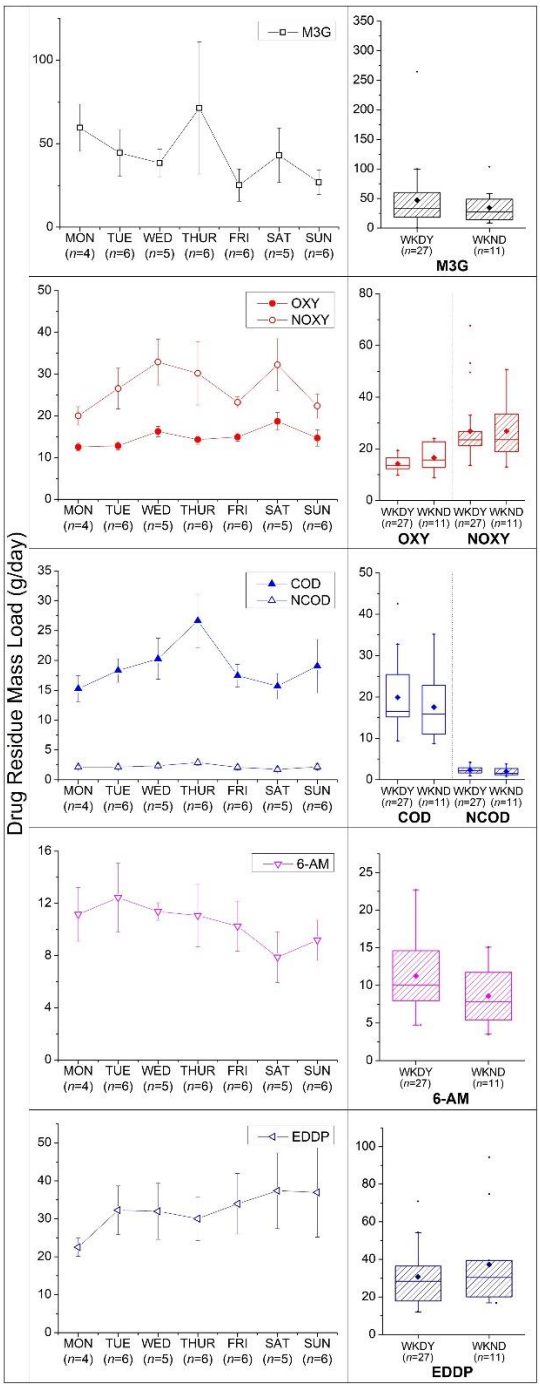

Fig. 23 - Average drug residue mass loads per day and a comparison between weekend and weekday mass load opioid occurrence for morphine-3-glucuronide (M3G), oxycodone (OXY), noroxycodone (NOXY), codeine (COD), norcodeine (NCOD), 6-acetylmorphine (6-AM), and EDDP. Error bars represent the standard error of all measured values for a specific day. Weekend comparison was done by a two-tailed t-test (α=0.05). Asterisks (**) denote a statistically significant difference between weekday and weekend mass loads.

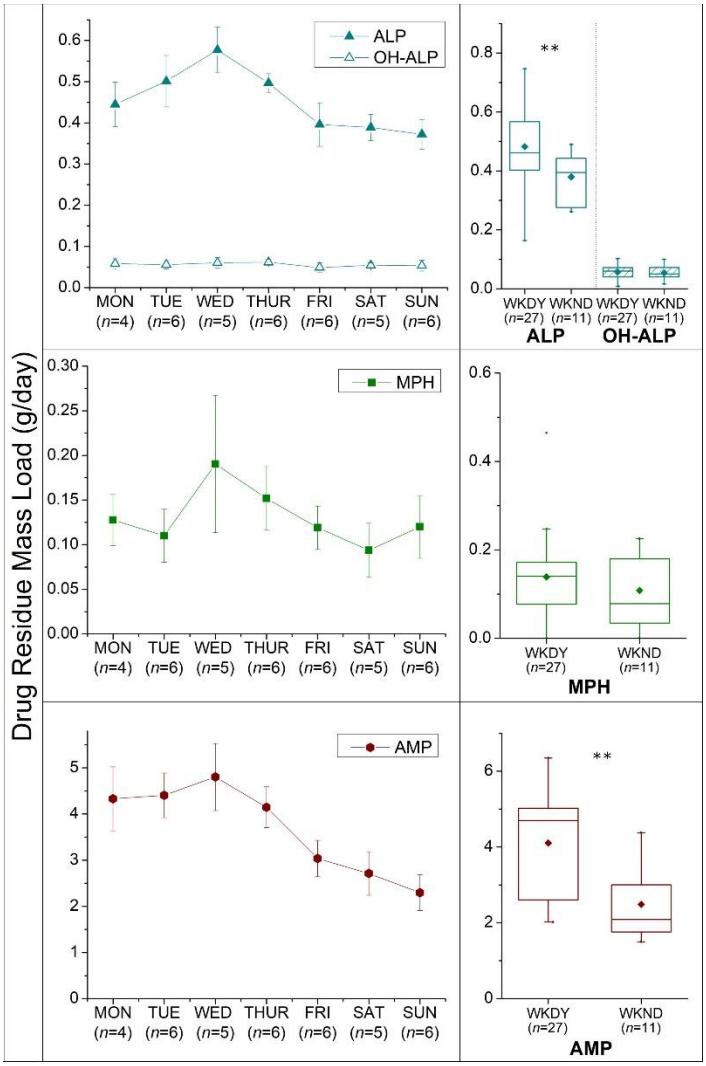

Fig. 24 - Average drug residue mass loads per day and a comparison between weekend and weekday mass load prescription occurrence for alprazolam (ALP), alpha-hydroxyalprazolam (OH-ALP), methylphenidate (MPH), and amphetamine (AMP). Error bars represent the standard error of all measured values for a specific day. Weekend comparison was done by a two-tailed t-test (α=0.05). Asterisks (**) denote a statistically significant difference between weekday and weekend mass loads.

APPENDIX E

NARCOTIC ANALYTE CHROMATOGRAMS FOR CHAPTERS 4 AND 5

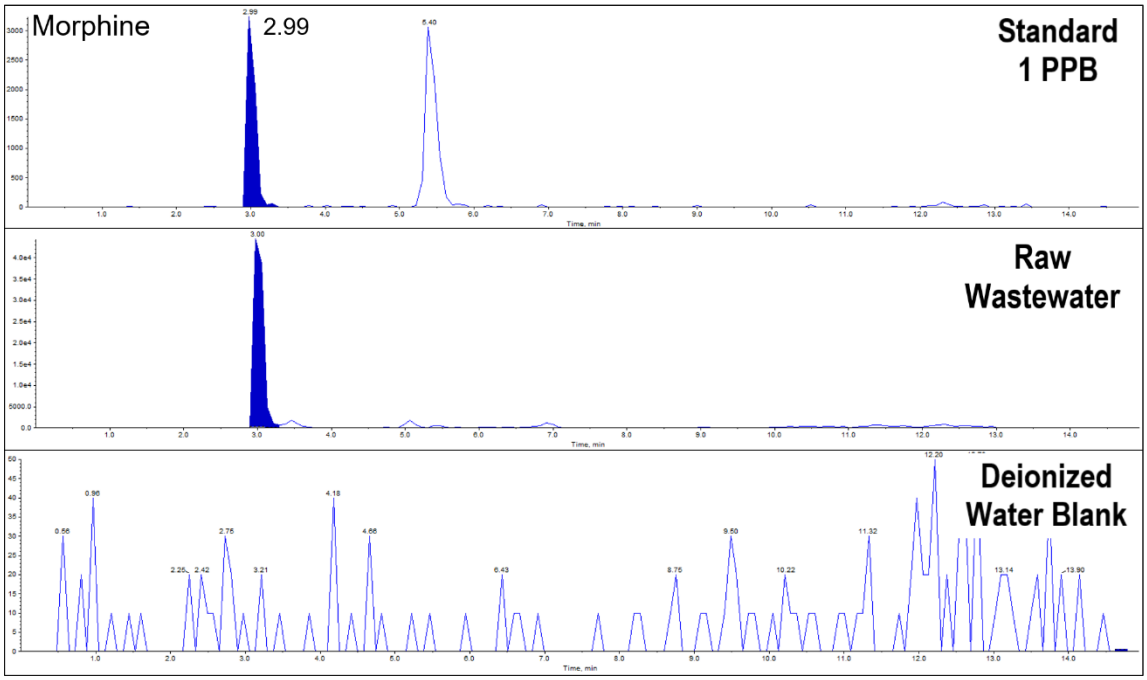

Fig. 25 – Total Ion Count Chromatograms for Morphine: Standard, Raw Wastewater, and the Deionized Water Blank.

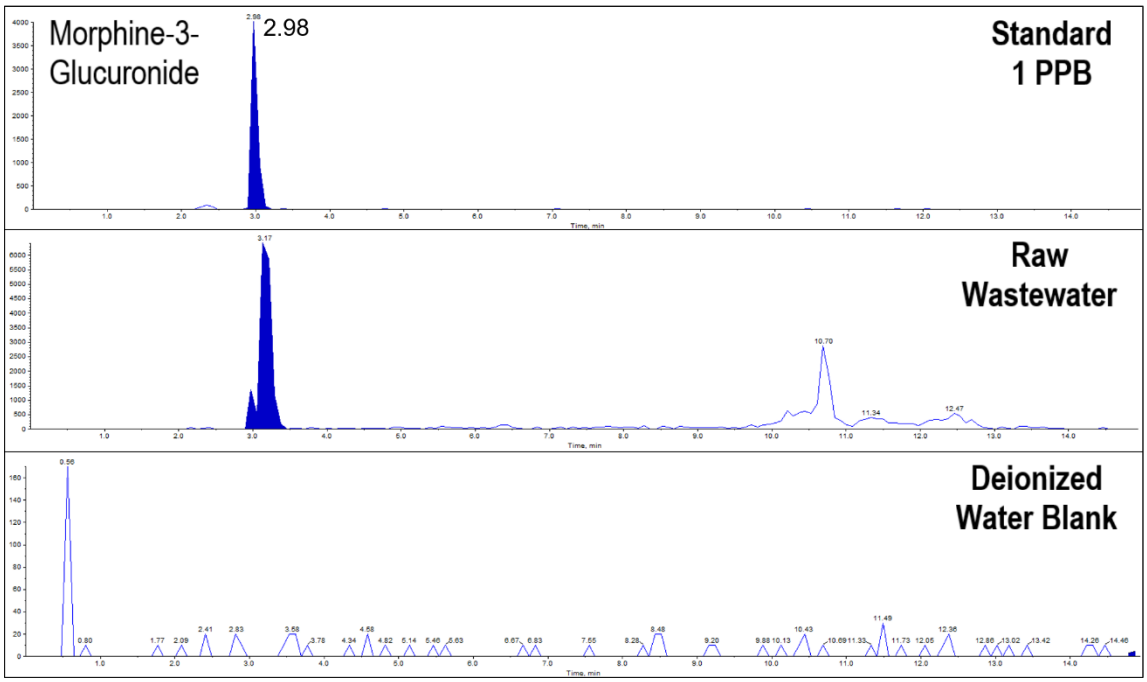

Fig. 26 - Total Ion Count Chromatograms for Morphine-3-Glucuronide: Standard, Raw Wastewater, and the Deionized Water Blank.

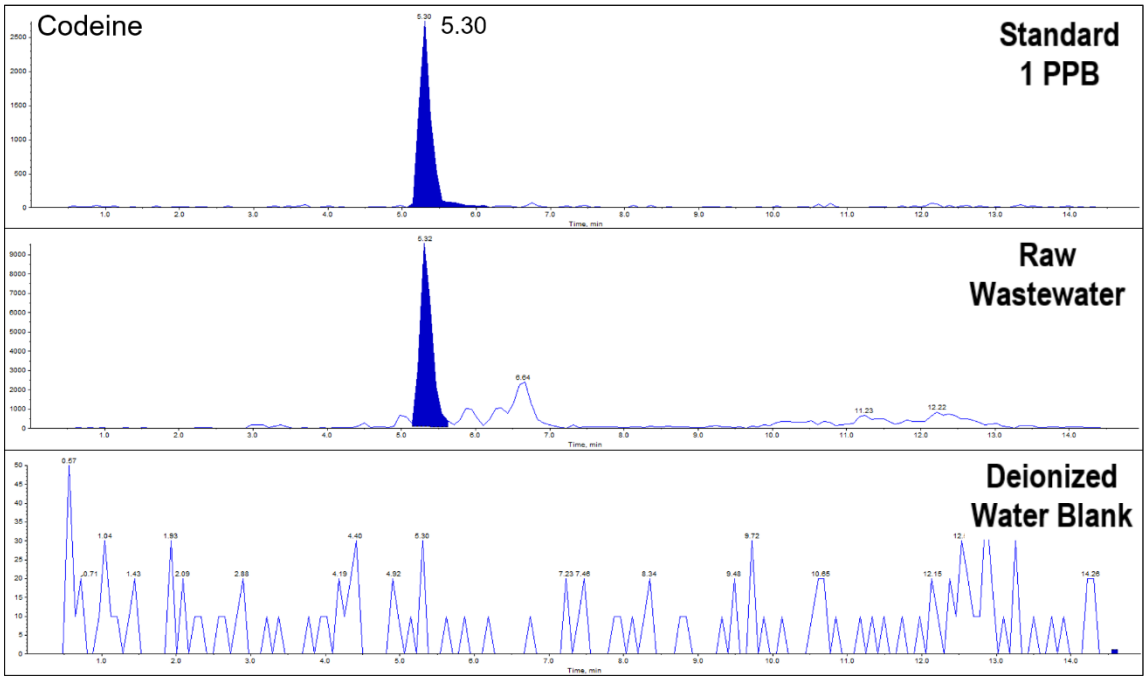

Fig. 27 - Total Ion Count Chromatograms for Codeine: Standard, Raw Wastewater, and the Deionized Water Blank.

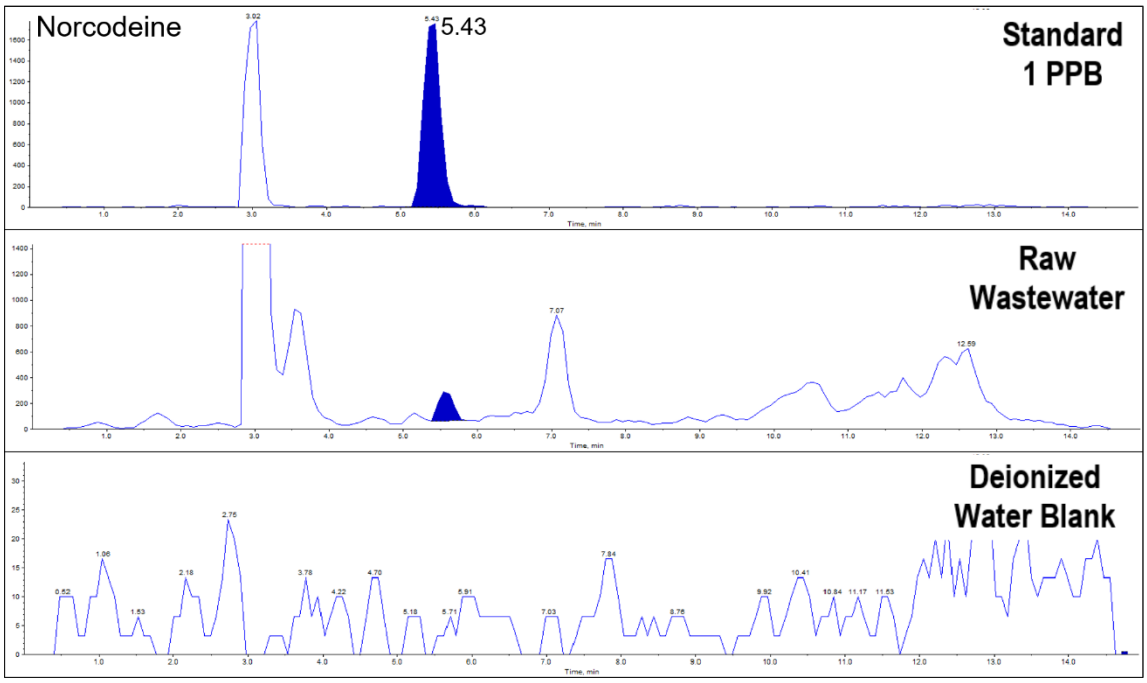

Fig. 28 - Total Ion Count Chromatograms for Norcodeine: Standard, Raw Wastewater, and the Deionized Water Blank.

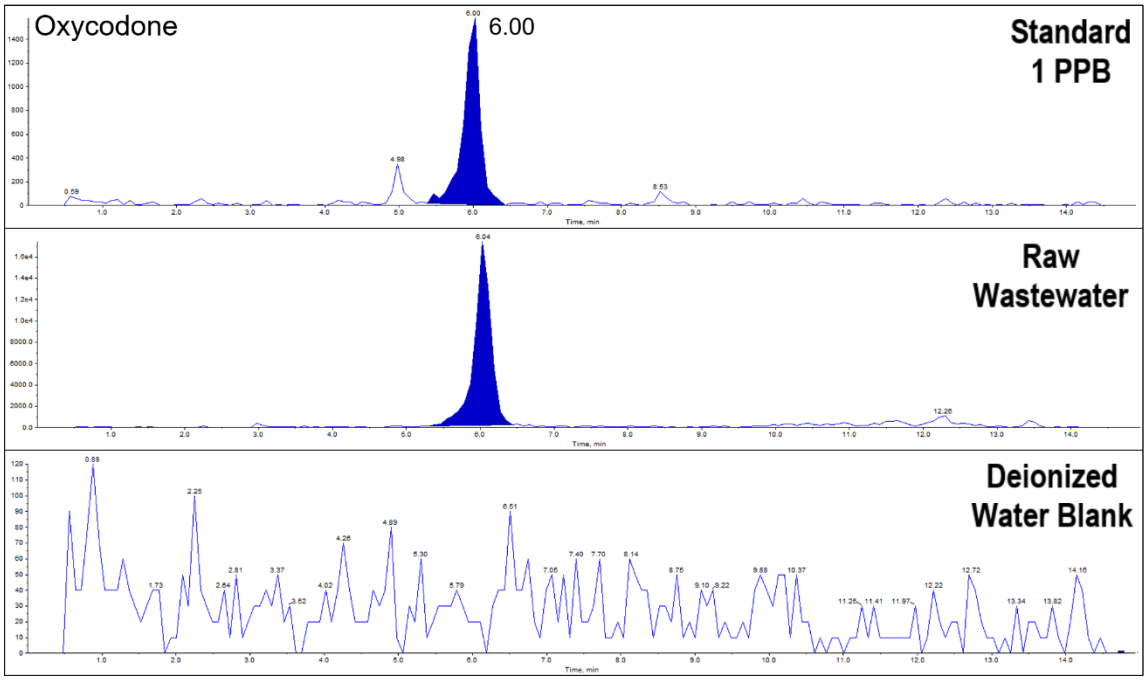

Fig. 29 - Total Ion Count Chromatograms for Oxycodone: Standard, Raw Wastewater, and the Deionized Water Blank.

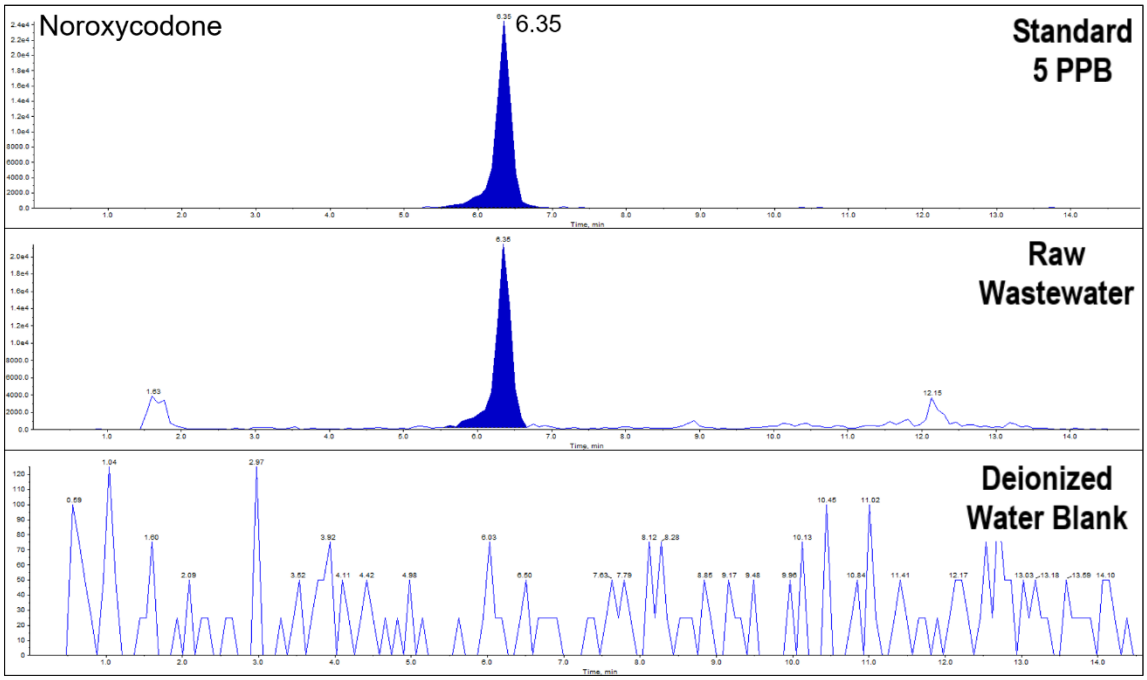

Fig. 30 - Total Ion Count Chromatograms for Noroxycodone: Standard, Raw Wastewater, and the Deionized Water Blank.

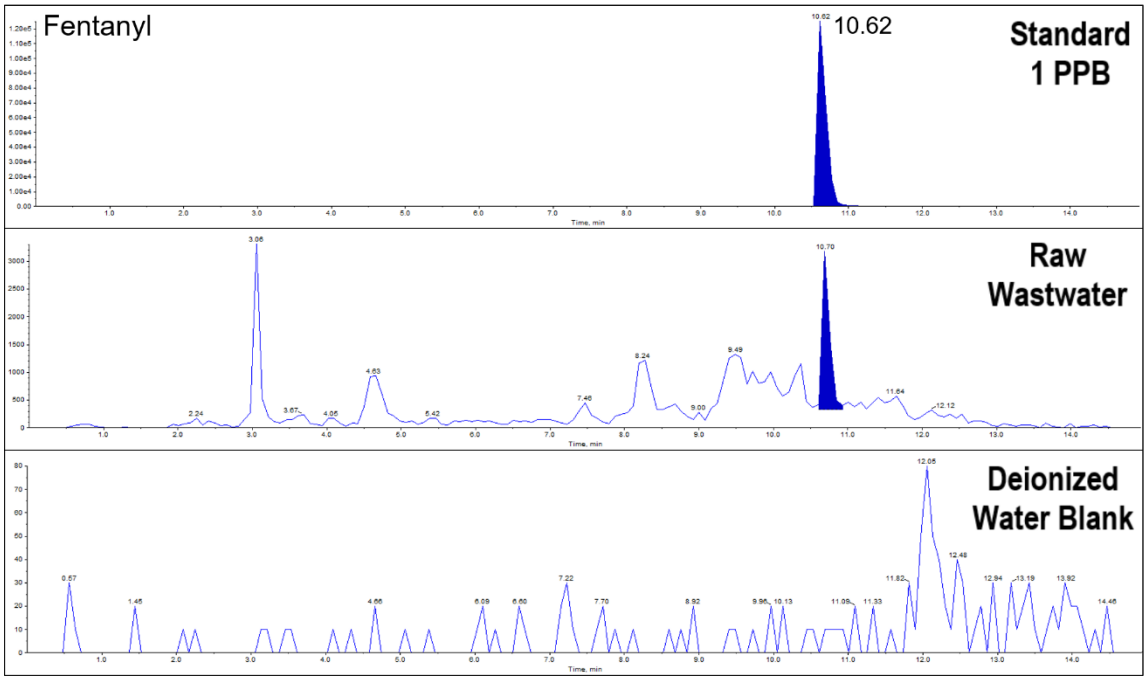

Fig. 31 - Total Ion Count Chromatograms for Fentanyl: Standard, Raw Wastewater, and the Deionized Water Blank.

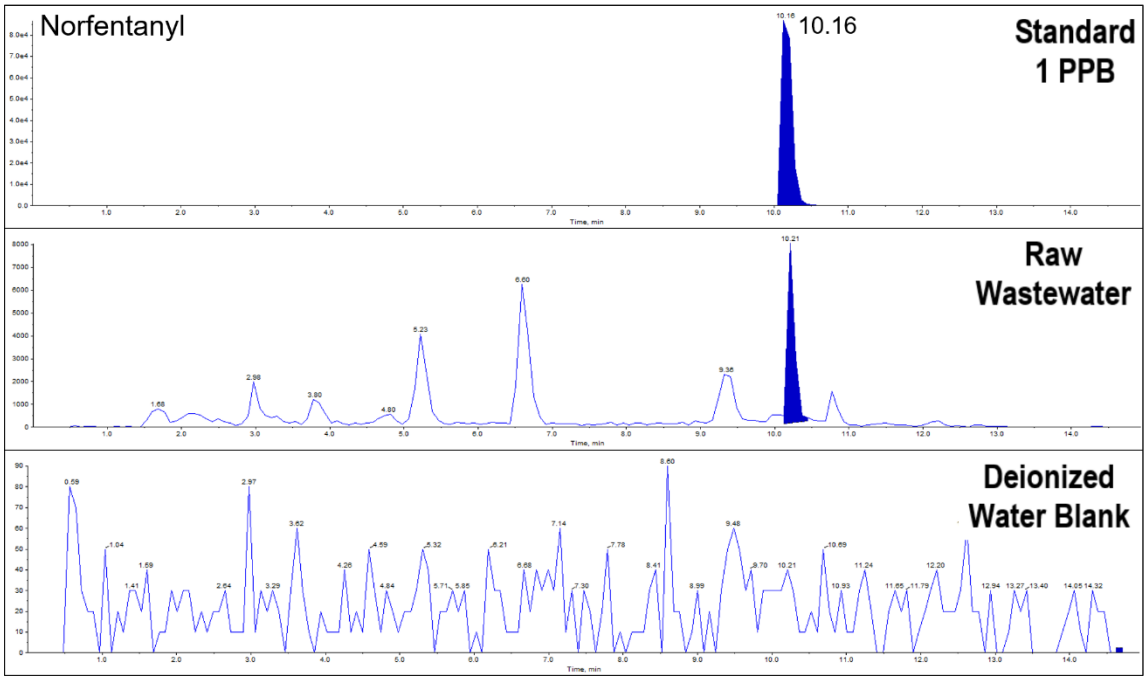

Fig. 32 - Total Ion Count Chromatograms for Norfentanyl: Standard, Raw Wastewater, and the Deionized Water Blank.

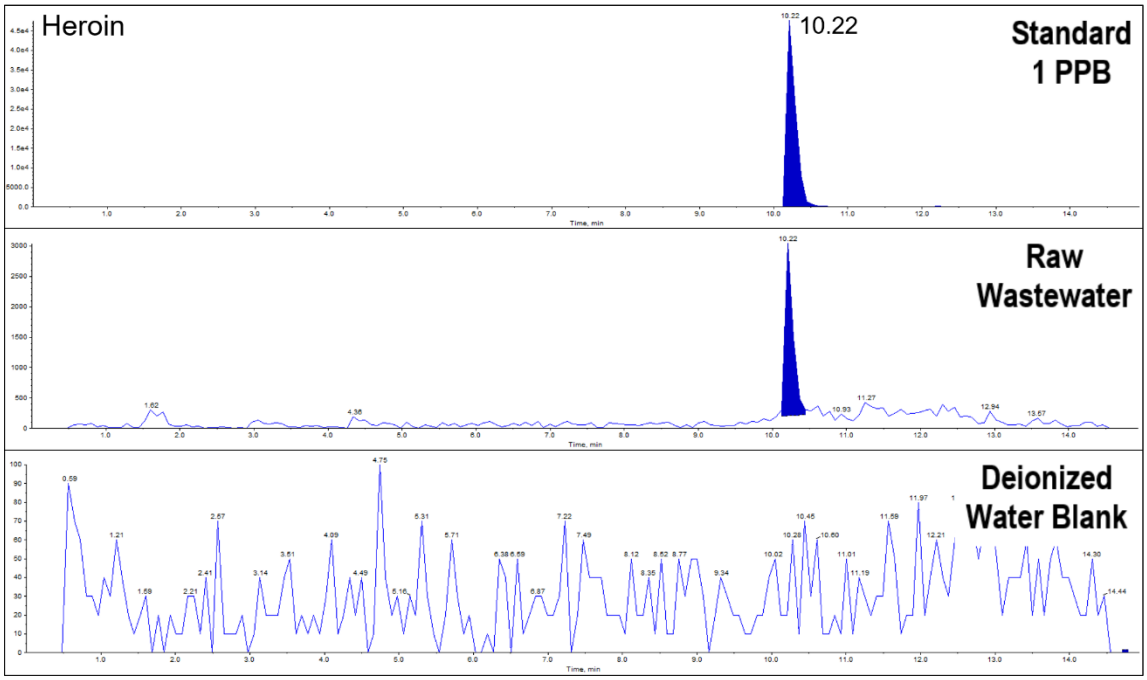

Fig. 33 - Total Ion Count Chromatograms for Heroin: Standard, Raw Wastewater, and the Deionized Water Blank.

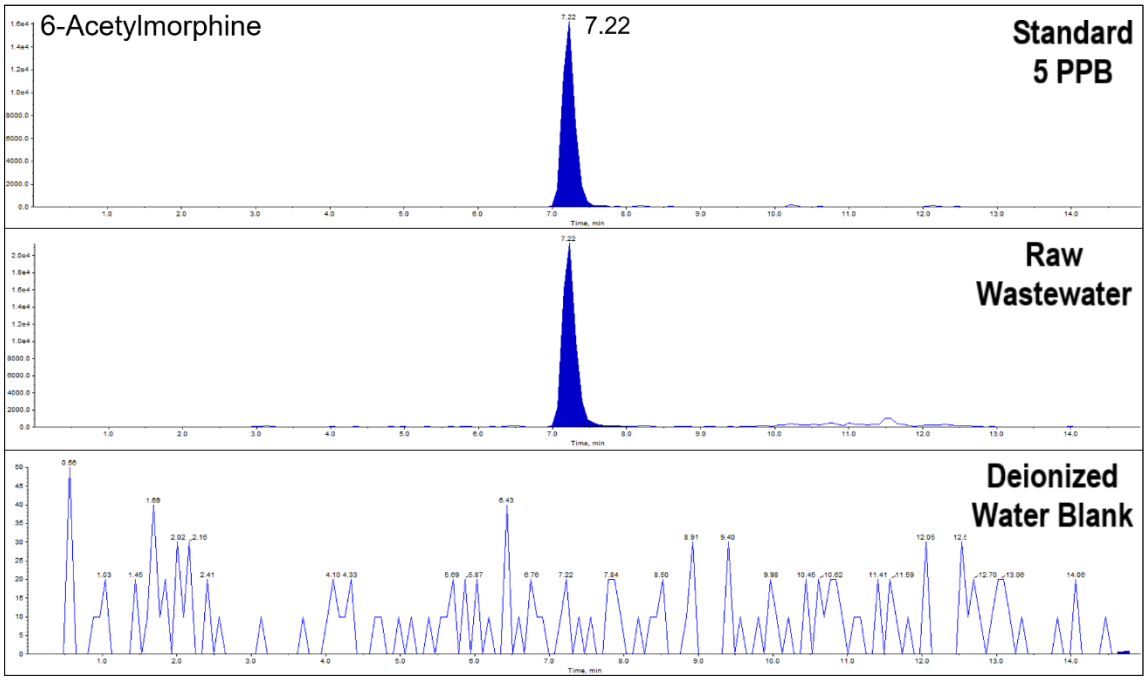

Fig. 34 - Total Ion Count Chromatograms for 6-Acetylmorphine: Standard, Raw Wastewater, and the Deionized Water Blank.

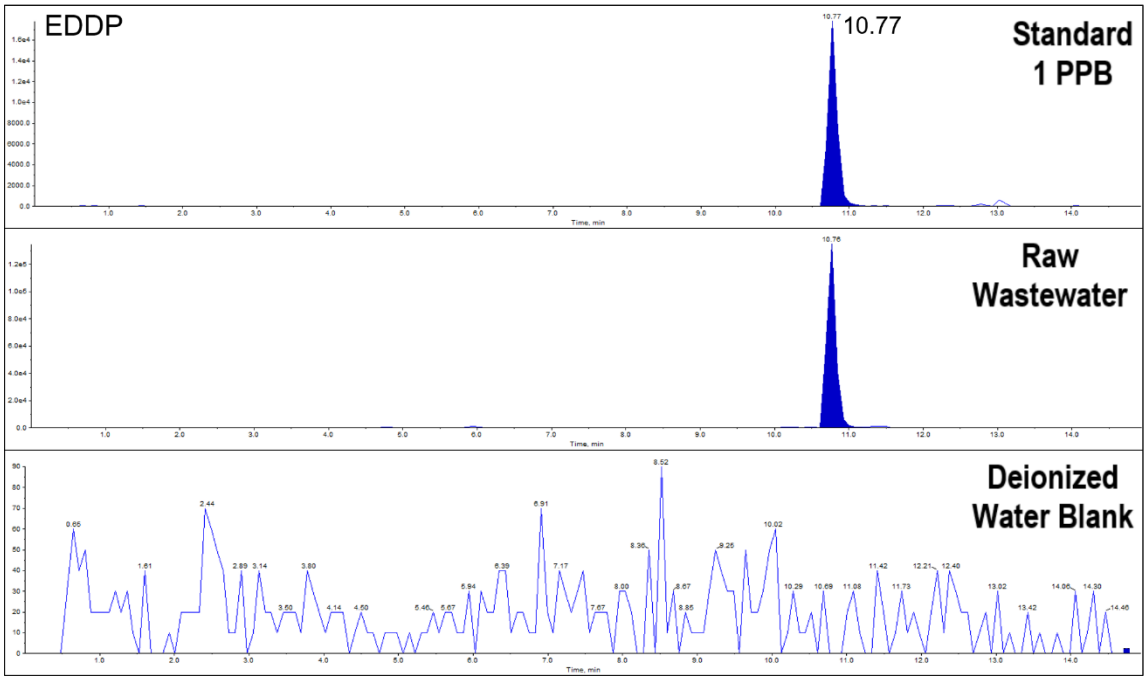

Fig. 35 - Total Ion Count Chromatograms for EDDP: Standard, Raw Wastewater, and the Deionized Water Blank.

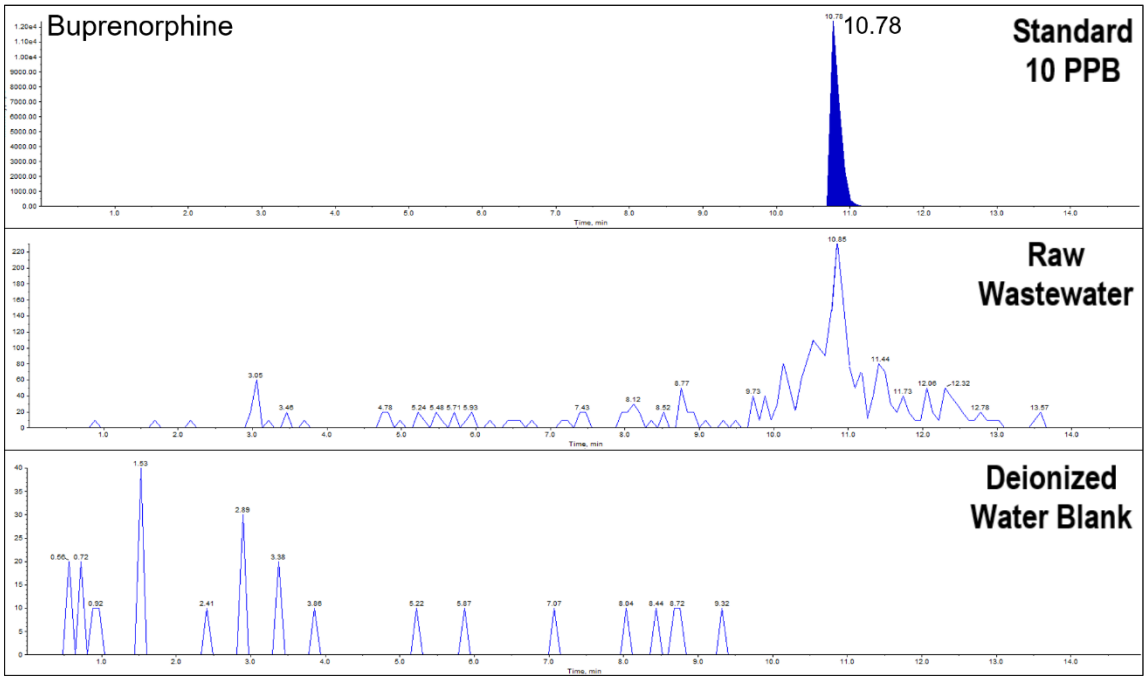

Fig. 36 - Total Ion Count Chromatograms for Buprenorphine: Standard, Raw Wastewater, and the Deionized Water Blank.

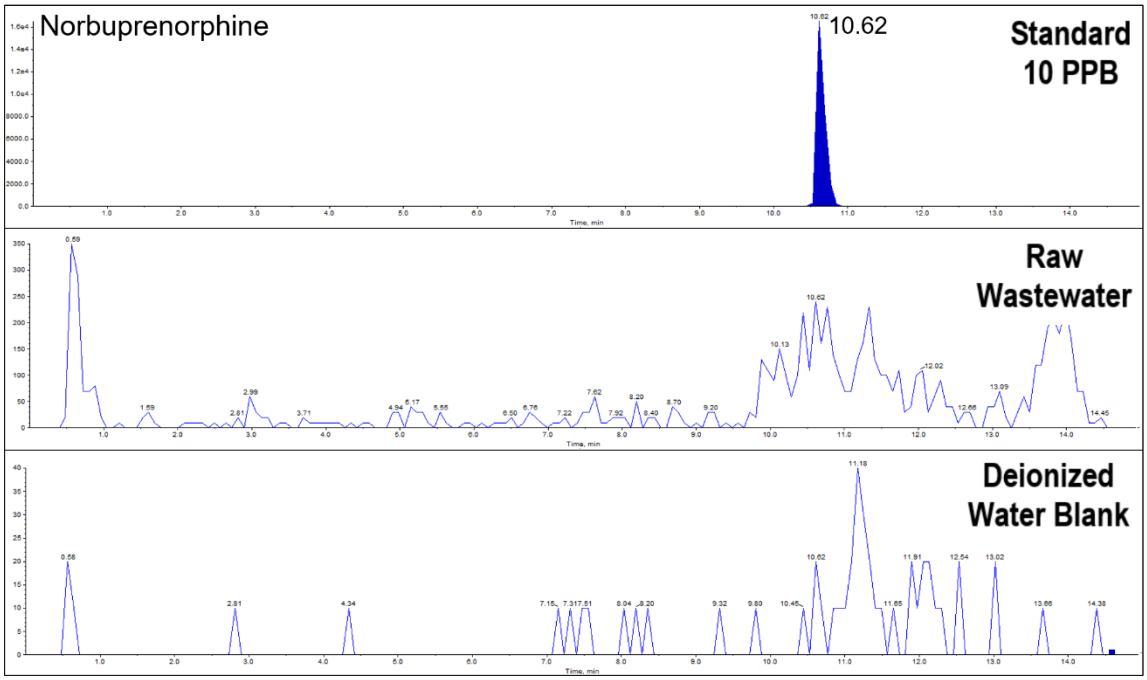

Fig. 37 - Total Ion Count Chromatograms for Norbuprenorphine: Standard, Raw Wastewater, and the Deionized Water Blank.

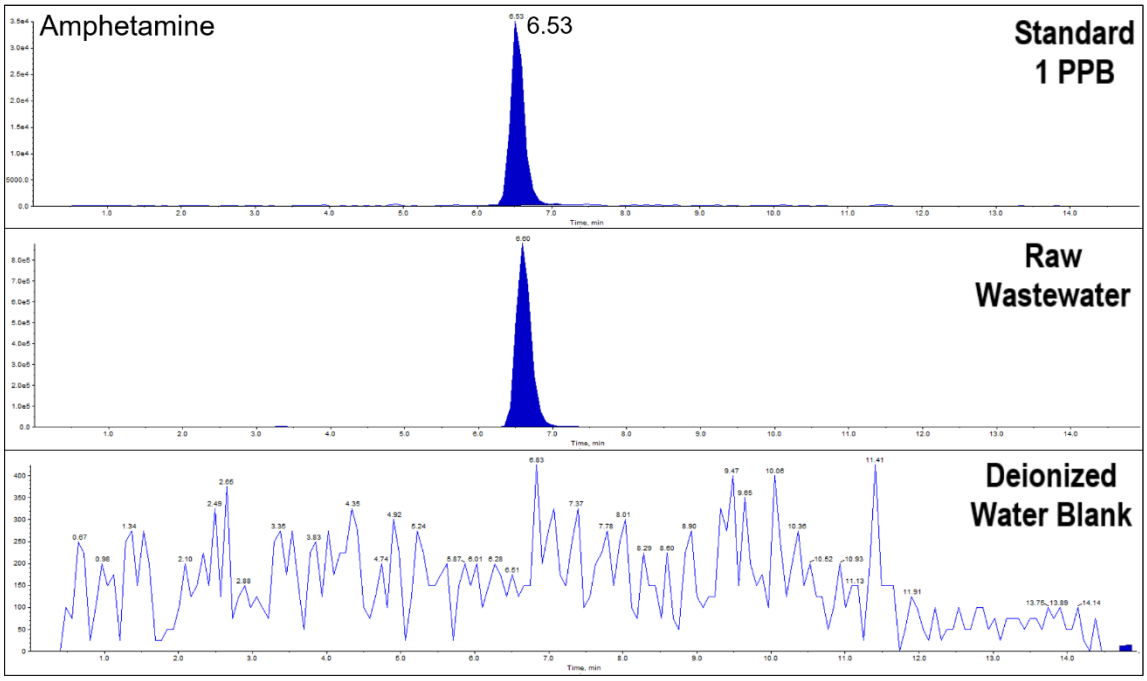

Fig. 38 - Total Ion Count Chromatograms for Amphetamine: Standard, Raw Wastewater, and the Deionized Water Blank.

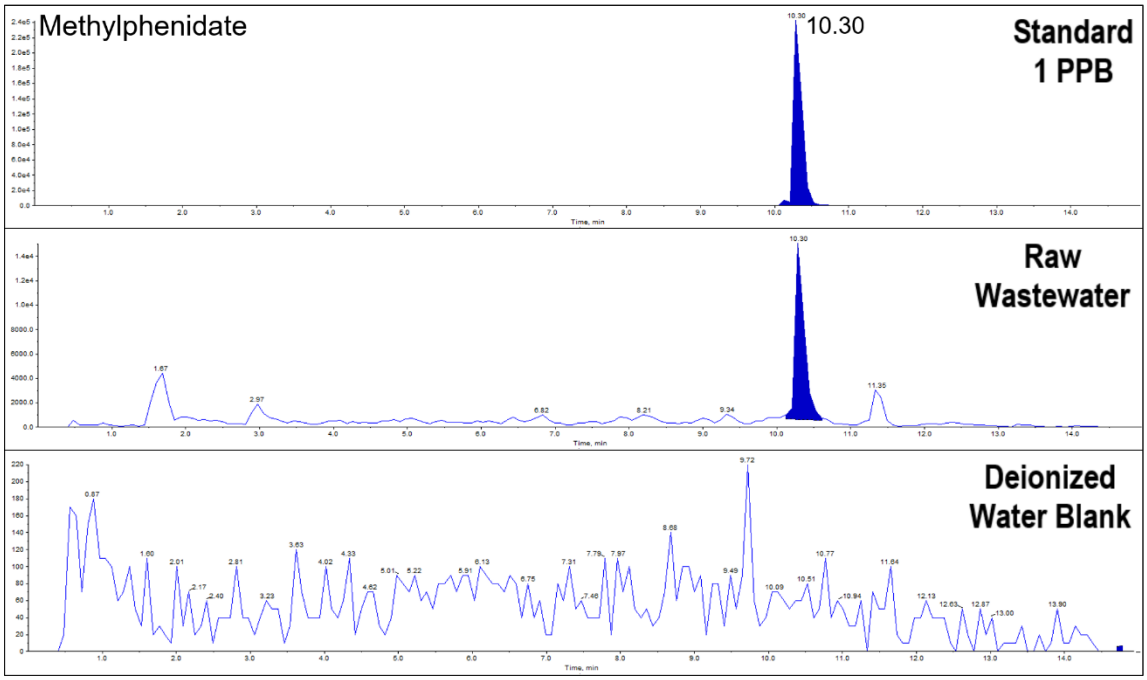

Fig. 39 - Total Ion Count Chromatograms for Methylphenidate: Standard, Raw Wastewater, and the Deionized Water Blank.

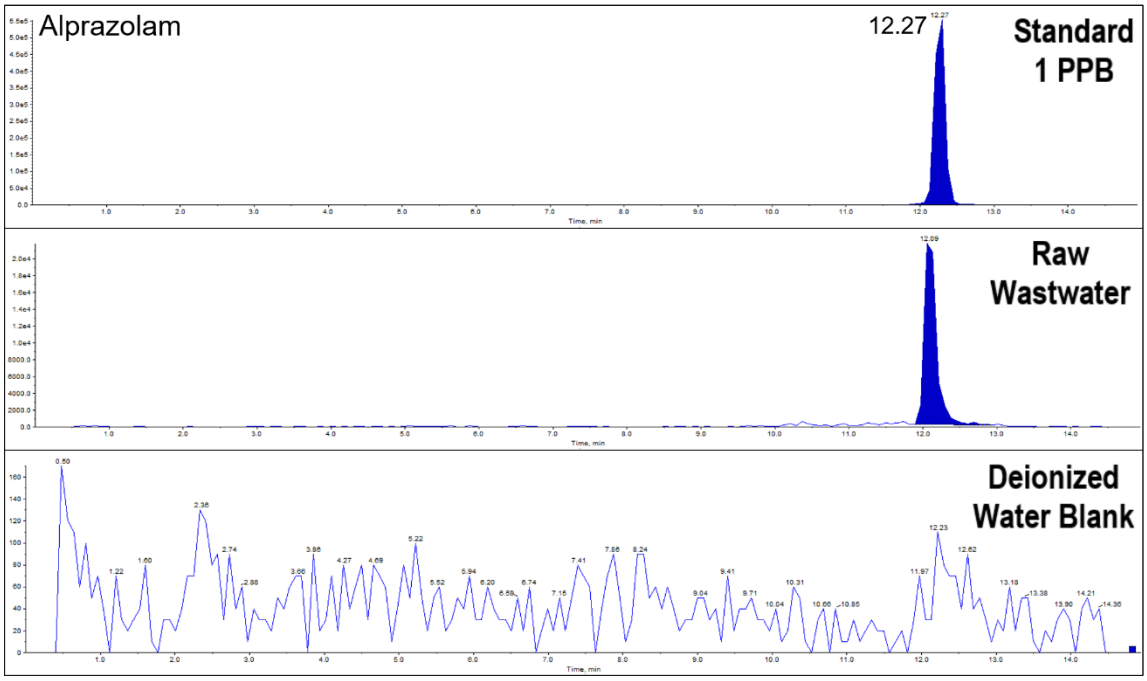

Fig. 40 - Total Ion Count Chromatograms for Alprazolam: Standard, Raw Wastewater, and the Deionized Water Blank.

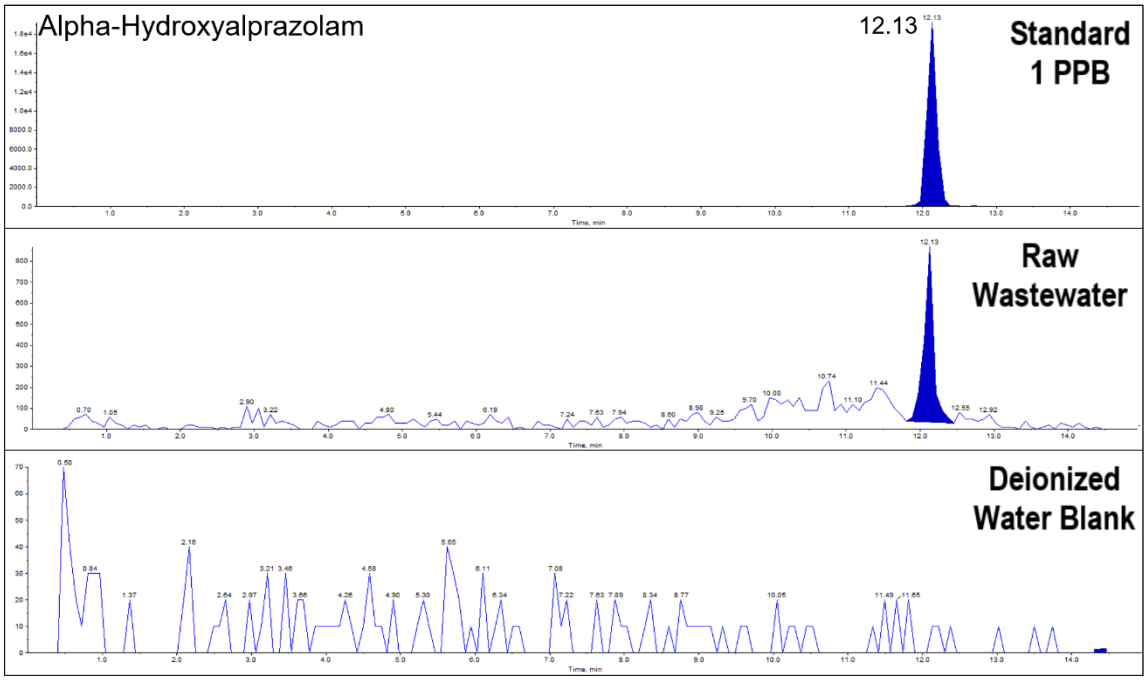

Fig. 41 - Total Ion Count Chromatograms for Alpha-Hydroxyalprazolam: Standard, Raw Wastewater, and the Deionized Water Blank.

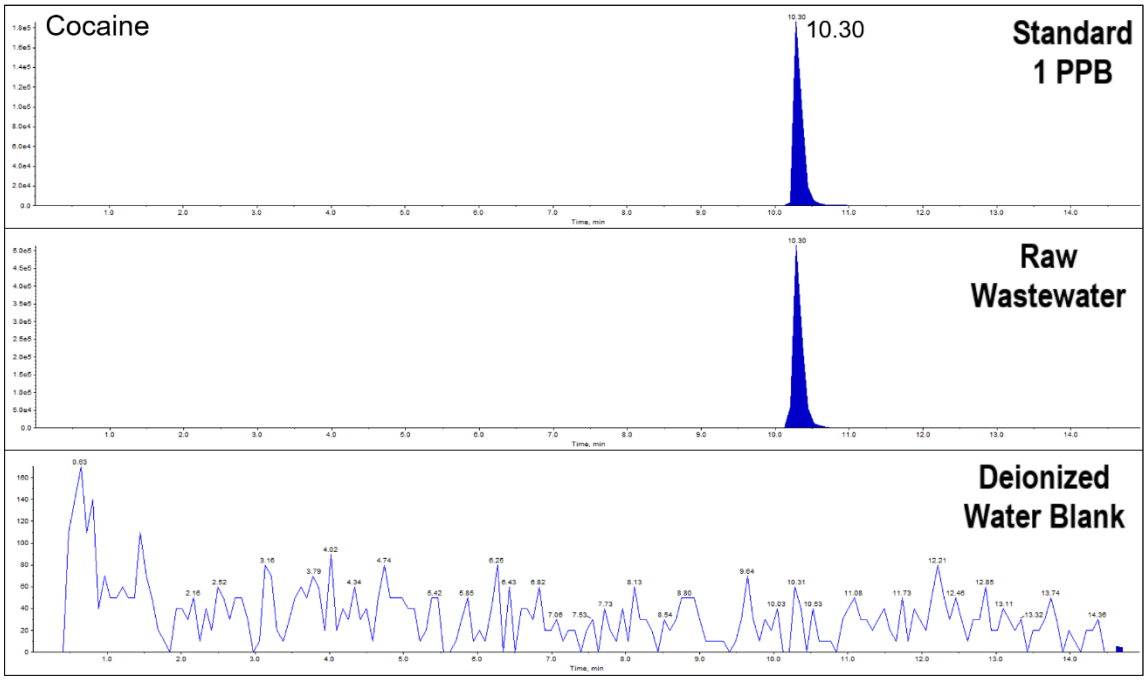

Fig. 42 - Total Ion Count Chromatograms for Cocaine: Standard, Raw Wastewater, and the Deionized Water Blank.

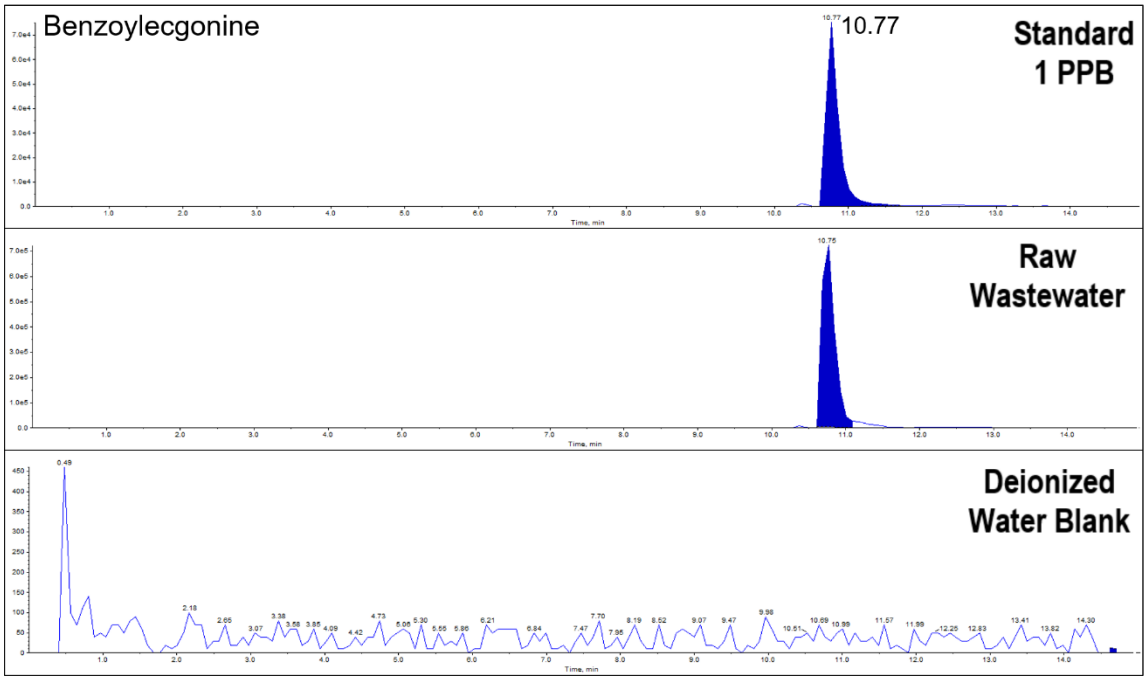

Fig. 43 - Total Ion Count Chromatograms for Benzoylecgonine: Standard, Raw Wastewater, and the Deionized Water Blank.

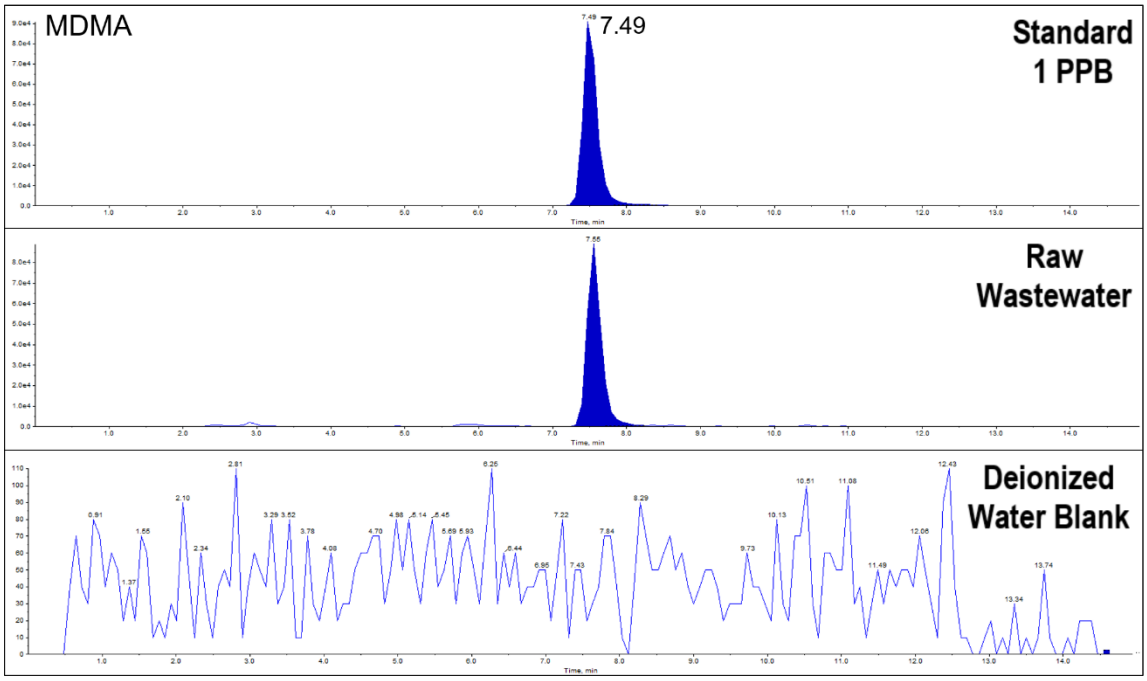

Fig. 44 - Total Ion Count Chromatograms for MDMA: Standard, Raw Wastewater, and the Deionized Water Blank.