Monitoring Contaminants of Emerging Concern in Global Wastewater Using Sewage

Epidemiology

by

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ABSTRACT

This dissertation applies wastewater-based epidemiology (WBE) to aqueous

process flows to gauge the public health status concerning exposure and potential abuse

of pharmaceuticals, antimicrobials, and narcotics. The masses of emerging contaminants

emitted into Indian aquatic and terrestrial environments were the highest for open

defecation (17 ± 12 mt/d), with non-steroidal anti-inflammatory drugs dominating

environmental loading (14 ± 10 mt/d), followed by antibiotics, antimicrobials, phthalates

and miscellaneous pharmaceuticals (Chapter 2). Fourteen wastewater treatment plants

sampled across the U.S. had a combined average mass loading of 71 ± 12 µg/d/capita for

the antimicrobials triclosan and triclocarban, with paraben compounds contributing $19 \pm$

5 µg/d/US capita. Risk models showed unfavorable hazard quotients (HQ>1) for

sensitive aquatic organisms (algae, zebra fish and rainbow trout) from predicted

exposures to antimicrobials of alternative use, i.e., chlorhexidine and benzalkonium

chloride (Chapter 3). Substances subject to licit and illicit use, monitored by WBE in a

medium-sized southwestern U.S. city before and during COVID-19-related lockdowns,

showed the highest mass loads for cocaine and its major metabolite benzoylecgonine

(2,207 total), methadone and its major metabolite 2-Ethylidene-1,5-dimethyl-3,3-

diphenylpyrrolidine (197), parent mitragynine (60), oxycodone and its major metabolite

noroxycodone (48), heroin and its major metabolite 6-acetylmorphine (45), and parent

codeine (37) in mg/1,000 capita/day. Heroin use during the lockdown increased ~10-fold

relative to the pre-lockdown baseline, whereas oxycodone and codeine mass loading

decreased 5-fold and 2.5-fold, respectively (Chapter 4). Experiments elucidating the

stability of stress hormones and their metabolites as a function of temperature and in-

sewer residence time revealed a rapid degradation to completion over 24 hours at 35°C, whereas lower temperatures of 25°C and 15°C were found to allow for successful tracking of indicators of stress at the population level; statistically significant differences in stress hormone decay rates were observed due to geographic locations at 25°C (p=0.009) but not due to redox conditions in the sewer pipe (Chapter 5). This thesis demonstrated the successful application of WBE for studying population health frequently and inexpensively, with the limitation that a lack of centralized wastewater infrastructure in developing countries may create barriers for at-risk populations to access and utilize this novel technology (Chapter 6).

DEDICATION

All of this work and anything meaningful I ever contribute is dedicated to my family. My mom and dad, Smita and Pushkaraj Kelkar, have had my back at each stage of my life, through the ups and the downs. Their support has been unwavering. I look up to my parents each day of my life, feeling privileged and grateful for having them as friends, role models and members of our larger family. Without their kind support and guidance, I would not be where I am today.

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CHAPTER 1

INTRODUCTION

An assessment of contaminants of emerging concern in wastewater and in the natural environment is crucial for protecting planetary health and human populations. Wastewater-based epidemiology (WBE), also referred to as urban metabolism metrology (UMM), is a rapidly evolving field of scientific study that can aid in monitoring, managing, and protecting ecosystem integrity and human wellbeing. The goal of this thesis was to harness this emerging public health assessment tool and help define its benefits and limitations.

1.1 Wastewater-based epidemiology and population health

Wastewater-based epidemiology (WBE), i.e., the analysis for biosignature compounds (having features that can be utilized to infer information about contributing individuals) in untreated composited municipal sewage representing the excreta from multiple to hundreds of thousands or even millions of people, promises to provide near real-time information related to health status, lifestyle, and the behaviors of populations served by a sewage collection and treatment system. Through this approach it may be possible to measure excretion products of human metabolism in wastewater in order to estimate consumption patterns of licit and illicit substances (e.g., cocaine, kratom, etc.)(Gushgari et al., 2018), determine inadvertent chemical exposures (e.g., to antimicrobials)(J. Chen et al., 2018), or to monitor other indicators of potential threats or human health status

(e.g., stress hormones)(Choi et al., 2018). Since WBE is a fairly young scientific discipline that was jumpstarted in 2020 by the onset of the COVID-19 pandemic, there are several aspects of this technology which require additional research to better understand the potential benefits and limitations of its use. Some of those aspects include (i) opportunities and potential barriers to an expansion of WBE use to serve vulnerable populations in the developing world (e.g., India, Africa, etc.), (ii) the suitability of WBE for monitoring the consumption of potentially harmful consumer products such as pharmaceuticals and antimicrobial personal care products, (iii) the detection of infectious disease agents such as SARS-CoV-2 and the impact of these health threats, and the corresponding effect on the behavior and wellbeing of affected populations (e.g., elevated stress levels, substance abuse, etc.), and (iv) a more nuanced understanding of the diagnostic value of wastewater biomarkers, which may be subject to decay as a function of both travel time from the location of excretion to the location of sampling as well as the environmental conditions that may govern their stability in wastewater (e.g., temperature, redox conditions, etc.).

1.2 Knowledge gaps concerning the applicability of WBE

WBE has the potential to be of great utility to study the health of large populations, to detect signs of early disease outbreaks, and to understand how the socio-cultural setting impacts the behavior and health status of (sub)population contributing to composited municipal wastewater. However, due to lack of resources, access to locations or inadequate wastewater treatment infrastructure, vulnerable and susceptible populations may be at risk of missing out on the application of WBE and the potential benefits to be

realized. One country for which limitations in the applicability of WBE are likely is India, a nation featuring the world's second largest population. In India, disease outbreaks are rampant and the government is struggling to provide adequate healthcare to potentially vulnerable populations. Moreover, the municipal wastewater infrastructure in this country may be inadequate for serving all people, thereby resulting in areas lacking sewage collection where untreated human waste may enter terrestrial and aquatic environments at a massive scale. Hence, understanding the mass loads of pharmaceuticals, personal care products, and other potentially toxic chemicals polluting the Indian water resources via wastewater is critical and has not yet been studied thoroughly.

Certain antibacterial compounds (triclosan and triclocarban) have been banned in the U.S. by the U.S. Food and Drug Administration (USFDA) since 2017 (Brose et al., 2019) due to the risk these chemical are known to pose to ecosystems and humans alike. Several WBE studies have documented the occurrence of parabens, triclosan, and triclocarban in wastewater with the goal of assessing the resultant risk posed to aquatic and terrestrial organisms (Tamura et al., 2013). However, after enaction of the FDA ban, newer antimicrobials were used as a substitute for which information on occurrence levels and toxicological impacts is sparse. These replacements to newly banned antimicrobial chemicals have been extensively used during the COVID-19 pandemic in the wake of a heightened need for better sanitation and hygiene (Rundle et al., 2019). A small number of toxicology studies have determined that some of the newer replacement chemicals (e.g. chlorhexidine and benzalkonium chloride) may be as toxic to flora and fauna as the ones banned under the new law (FDA, 2016; Rundle et al., 2019; Senate,

2019). But absent of more information on usage levels of antimicrobials and mass

loadings to wastewater, a determination of the risk they pose cannot be performed.

The application of WBE necessitates the collection of representative wastewater

samples, transportation of collected water to an analytical laboratory, and subsequent

analysis of biomarkers or chemicals of interest. However, several environmental factors

such as elevated ambient temperatures, redox conditions, as well as time spent in sewers

prior to sample acquisition may influence the detectability and concentration of

compounds and biomarkers of interest (Thai et al., 2019a). Some endogenous compounds

(e.g., 8-iso-prostaglandin F2a, metabolite dinor-11b-Prostaglandin F2a, Prostaglandin E2)

demonstrate unstable behavior when subjected to microbial activity in sewers for

extended periods of time at elevated ambient air and water temperatures (O'Brien et al.,

2019; Pandopulos et al., 2020; Thai et al., 2019b). Despite this knowledge of potential

biomarker decay, studies investigating the stability of commonly used wastewater-borne

biomarkers are lacking. One example of biomarkers of endogenous origin that are

potentially susceptible to in-sewer decay are the stress hormones cortisol and cortisone,

and their corresponding metabolites. As with other wastewater-borne biomarkers of

health and human wellbeing, a better understanding of their stability and degradation

kinetics in municipal wastewater is critical in order to enable a meaningful interpretation

of WBE data collected for these substances.

1.3 Primary goals and research strategies

The first Chapter of this thesis is concerned with a theoretical assessment of the type and

magnitude of contaminants of emerging concern that are presumed to enter Indian aquatic

and terrestrial environments contained in human excreta. This work involved a thorough literature review of published WBE studies investigating Indian wastewater treatment plants (WWTPs) and an analysis of locations at which surface water and groundwater sampling was performed in the past. The resultant concentration data for pharmaceuticals and personal care products abstracted from the literature were analyzed in conjunction with data from the Indian census and information provided in documents from the Indian Pollution Control Board data to estimate the magnitude of the total mass load of CECs across all Indian states and union territories. Study findings then were visualized spatially using ArcGIS.

In addition to this literature review and modeling exercise, the thesis features two chapters in which I performed original hands-on laboratory work. Both WBE studies concentrated on an analysis of flow-weighted 24-hour composite samples of wastewater from U.S. WWTPs that then were analyzed using solid phase extraction (SPE) in conjunction with liquid chromatography tandem mass spectrometry (LC/MS-MS). Captured analytes of interest included various antimicrobials, including five common parabens triclosan, and triclocarban, as well as controlled substances linked to misuse and addiction, e.g., cocaine, heroin, and prescription opioids) as well as substances used to manage substance addiction (e.g., kratom and methadone). Sampling strategies varied by project. For studies exploring use of antimicrobials and exposure of aquatic organisms, 14 states were sampled for one day, the samples were shipped on ice to the laboratory and stored at -20°C until analysis. In the drug monitoring study, however, I used samples collected locally in Tempe over several consecutive days each month from January to July 2020, a time period that covered pre-pandemic and COVID-19 situations.

Studies on the stability of stress hormones and their characteristic metabolites (i.e., cortisol, cortisone and their derivatives tetrahydrocortisol and tetrahydrocortisone) involved laboratory experiments in which I incubated known quantities of these stress biomarkers at three different temperatures (15, 25 and, 35°C) under both oxic and anoxic conditions for 24 hours. Samples of untreated wastewater that was obtained directly from local sewers and that was never frozen or thawed, were taken every 3 hours over the course of a 24-hour time period and then analyzed by LC/MS-MS for a determination of the impact of temperature, time and oxygen availability on the stability of these markers of population stress. To understand the biomarker behavior better, resulting data was fitted to first-order decay rates followed by an analysis of the half-lives and rates obtained.

1.4 Research Hypotheses

Research hypotheses informing the design and work documented in this thesis were as follows:

1. Chemical mass load polluting Indian terrestrial and aquatic environments is the highest from open defecation compared to three other modes of defecation.

2. Concentrations of antimicrobials such as triclosan, triclocarban, parabens in the treated effluent from U.S. wastewater treatment plants pose unacceptable risk to the freshwater biota.

3. Mass consumption of licit and illicit opioids is affected due to COVID-19 lockdowns and is statistically different ($\alpha=0.05$) between pre-lockdown and during lockdown periods.

4. Cortisol, cortisone, tetrahydrocortisol, and tetrahydrocortisone display significantly greater stability ($\alpha=0.05$) at 25°C compared to 35°C in raw wastewater

1.5 Specific Aims

The specific aims for this dissertation are to:

I.   Estimate the mass loadings of contaminants of emerging concern into the Indian aquatic and terrestrial environment due to improper handling of wastewater or inadequate treatment of wastewater generated.

II. Spatially plot the mass loadings for each Indian state and union territory in a manner that it highlights the most populated and vulnerable regions of India.

III. Determine the mass loadings via different modes of defecation such as open defecation, water closet, pit latrines, and other latrines.

IV. Measure the concentrations, removal efficiencies, mass loadings of parabens, triclosan, and triclocarban from influent and effluent wastewaters from 14 WWTPs and 12 U.S. states.

V.   Determine the risk posed by parabens, triclosan, and triclocarban to toxicological model organisms *Daphnia magna*, green algae, zebrafish embryo, *Caenorhabditis elegans*, and rainbow trout via treated effluent wastewater.

VI. Measure opioids and opioid medication consumption by the population of a southwestern U.S. city during the COVID-19 pre-pandemic period (January-March 2020) and during the pandemic (April-July 2020).

VII. Assess the stability of stress hormones (cortisol and cortisone) and the metabolites (tetrahydrocortisol and tetrahydrocortisone) in wastewater under oxic and anoxic

conditions over a period of 24 hours and at three different temperatures (15, 25, and 35°C).

TRANSITION 1

Much of the development and application of WBE has been conducted in and is focused on developed, industrialized nations. However, it is reasonable to presume that the biggest health benefits of WBE may be realized in settings in the developing world, where both infectious diseases and toxic exposures to harmful chemicals are known to impart a disproportionate burden on quality of life, human wellbeing, excess morbidity and premature death. Therefore, in the first chapter of my thesis, I elected to perform a literature-based evaluation of the prospects of using WBE in India, with a particular focus on the fate of CECs that are excreted in human waste, and that may enter the Indian environment by different modes of waste disposal, including bacteria, viruses and other potentially fatal pathogens.

This second thesis chapter is comprised of published literature that critically evaluates methods for assessing human and ecological health using wastewater-based epidemiology (WBE). I used published data on the occurrence of CECs in Indian wastewater, surface water, groundwater, and wastewater collected at sewage treatment plants as well as additional data on contaminants present in sediments and biosolids to investigate the loading of emerging contaminants including various pharmaceuticals, opioids, stimulants, personal care products, antibiotics, and painkillers. I then used the data from the literature analysis to estimate the mass loads of CECs entering the Indian natural environment via untreated wastewater and via different modes of defecation. Finally, the mass loading data was represented spatially on maps created using Geographic Information System (GIS) to interpret the findings and pinpoint the location of susceptible regions and the magnitude of vulnerable populations residing therein. This

study is intended to serve as a guiding map for epidemiologists, wastewater scientists, and disease modelers interested in studying major Indian cities, states, or union territories.

CHAPTER 2

NATIONWIDE LOADINGS OF CONTAMINANTS OF EMERGING CONCERN

INTO INDIAN AQUATIC AND TERRESTRIAL ENVIRONMENTS AS A

FUNCTION OF HUMAN

Abstract

Lacking or inadequate sewerage infrastructure and rapid population growth can exacerbate the release of contaminants of emerging concern (CECs) contained in human waste to the environment. We performed a literature review, meta-analysis, and modeling study to estimate the daily loadings into Indian aquatic and terrestrial environments for some 56 organic compounds, including antibiotics, painkillers, stimulants, artificial sweeteners, antimicrobials, and phthalates. Geospatial contaminant loading rates were determined as a function of the mode for human waste disposal, ranging from open defecation to pit latrines andwater closets with conventional wastewater treatment downstream. Rates of CEC loadings into the Indian environment in units of metric tons per day yielded the following rank order of mean values and associated standard errors: sum of ($\sum$) antibiotics (13.7 ± 9.81), $\sum$ painkillers (16 ± 5), $\sum$ antimicrobials (9.7 ± 9.2), $\sum$ phthalates (2.9 ± 0.7), and $\sum$ other pharmaceuticals (0.50 ± 0.27). As of 2015, seven (20%) of the 35 Indian states and union territories (UTs) had no wastewater treatment, and 17 states/UTs (48.6%) had no plans to increase treatment capacity. CEC loading rates were dominated by open defecation followed by water closet, pit latrines, and other latrines. Obtained data represent an initial order-of-magnitude estimate of chemical contamination nationwide, obtained from the scarce monitoring data available. Results highlight the urgent need for infrastructure

improvements. Study data further may aide in interpreting geospatial differences in disease prevalence (e.g., COVID-19) in India, with the mode of human waste disposal known to represent an important key driver of public health outcomes.

2.1 Abbreviations

AN: Andaman and Nicobar Islands

AP: Andhra Pradesh

AR: Arunachal Pradesh

AS: Assam

BR: Bihar

CH: Chandigarh

CH: Chhattisgarh

DL: New Delhi

DD: Diu Dadra and Nagar Haveli

GA: Goa

GJ: Gujarat

HP: Himachal Pradesh

HR: Haryana

JH: Jharkhand

JK: Jammu and Kashmir

KA: Karnataka

KL: Kerala

LD: Lakshadweep

LPD: Liters per day

MG: Meghalaya

MH: Maharashtra

MN: Manipur

MP: Madhya Pradesh

mt/d: metric ton per day

MZ: Mizoram

NL: Nagaland

NSAIDS: Non-steroidal anti-inflammatory drugs

OD: Open defecation

OH: Odisha

OL: Other latrines

PB: Punjab

PL: Pit latrines

PY: Puducherry

RJ: Rajasthan

SK: Sikkim

TG: Telangana

TN: Tamil Nadu

TR: Tripura

UK: Uttarakhand

UP: Uttar Pradesh

UT: Union Territory

WB: West Bengal

WC: Water closet

2.2 Introduction

India is an emerging economy that faces considerable environmental pollution issues. Among the still poorly defined and quantified sources of environmental pollution are contaminants of emerging concern (CECs) released into the environment as a result of human waste disposal (Subedi et al., 2014). Previous meta-analyses and literature reviews on CECs in India have focused primarily on environmental matrices, such as groundwater, surface water, and wastewater (Balakrishna et al., 2017; Philip et al., 2018). Whereas some studies emphasized the lack of wastewater treatment in India (Balakrishna et al., 2017; Subedi et al., 2015), quantitative and geospatial estimates on the release of CECs contained in human waste and wastewater are still scarce.

Current practices of human waste disposal and wastewater treatment in India are deemed to be inadequate, due to a reported lack of treatment infrastructure, improper disposal of septic tank sludge, lack of toilets, and lack of connectivity of water closets to wastewater treatment plant (WWTP) infrastructure (Central Pollution Control Board, 2015; CPCB, 2005; Gautam et al., 2009; Kamyotra & Bhardwaj, 2011; Kaur et al., 2012). Untreated wastewater is usually disposed of directly on the soil or into rivers, lakes or seawaters, thereby causing pollution of groundwater, freshwater and coastal environments. Among the CECs known to threaten human health and ecosystem integrity

are painkillers, sedatives, antibiotics, stimulant drugs, artificial sweeteners, antimicrobials, allergy medication, antidepressants, pesticides and other pharmaceuticals, personal care products and bioactive organic compounds.

In 2015, the Central Pollution Control Board of India (CPCB) published an inventory of existing WWTPs nationwide and their respective operational capacity (Central Pollution Control Board, 2015) using information obtained in a survey conducted in 2013-2014. According to this most recent inventory, India had 816 WWTPs nationwide by the end of 2014 that treated only about 33% of the total wastewater produced nationwide, with the balance of 67% being discharged untreated. Previously published reports focused only on cities classified as either Class I or Class II (CPCB, 2005; Gautam et al., 2009; Kaur et al., 2012), implying highly populated and more fully developed regions with a majority of people residing in densely populated urban areas. Limiting the analysis to metropolitan areas of Class I (220 million people) and Class II (40 million) left out a significant fraction of the Indian population, over 900 million people, who are living in less densely populated urban areas and rural settings. The relative lack of development in towns outside of Class I and II cities implies that there is little to no treatment of human waste and presumably a high degree of environmental contamination. Reports by the Government of India (CPCB, 2005; Kaur et al., 2012) typically exclude less developed cities, thereby contributing to a significant knowledge gap concerning the fraction of the population having access to robust sanitary infrastructure for disposal and treatment of human waste.

It is estimated that India's population will reach ~1.7 billion people by 2050 with 850 million people thereof living in urban areas (Bhardwaj, 2005). The population of

Indian major cities is increasing, which is evident from the Census data acquired in 1901, 2001, and 2011. In 1901, 11% (27 million people) (Territory, 1901) of the population was living in urban areas. That number increased to 29% (293 million) in 2001 (Affairs & India, 2001), 31% (377 million) in 2011 (Census 2011) and 34% in 2018 (Bank, 2018). A 2016 report of the United Nations (UN) concerning the state of the world predicts that the Indian urban population will be 41% by 2030 (Pawan, 2016). With over 100 million more people in urban areas within just a decade, (2001-2011) city's waste management resources will undergo periods of extreme stress. Additionally, the CPCB report from 2009-2010, states that existing WWTPs are experiencing problems with operation and maintenance, frequently not complying with the general standards of the environmental protection rules while also underutilizing the maximum designed capacity (Gautam et al., 2009).

As a consequence, open defecation is a major issue especially in rural India, affecting ~66% of the rural population as of 2018. The World Health Organization (WHO) reported that as of 2010 an estimated 626 million people have to opt for open defecation due to lacking sanitary infrastructure (WHO- UNICEF, 2012a). After initiating a campaign to rapidly add toilets nationwide, the Indian government claimed in 2018 that the country is 'open defecation free' (ODF), a claim that appears to be in stark contrast to the visible reality of many millions of Indian households (Mehrotra, 2019a). A survey by Research Institute for Compassionate Economics in 2018 contradicted government claims of an ODF status for India (Mehrotra, 2019b; Research Institute for Compassionate Economics (RICE), 2018). They reported that 42-57% of the people still defecated in the open in those states surveyed, and that India has not yet achieved the

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ODF status, although there has been a significant drop in open defecation across the country (Mehrotra, 2019b). Information on the type of toilets built and their acceptance and usage rates also are still lacking. With limited water resources and drinking water infrastructure in place, sanitary infrastructure choices may be restricted to pit latrines, waste lagoons and septic tanks as opposed to water closets that are connected to conventional biological WWTPs.

Pit latrines and improper disposal of the contents of septic tanks can often lead to waterborne infections. In 2010, the United Nations reported that 1.2 million children in India are dying each year due to water related diseases and one third of India's total districts does not have safe drinking water yet (Gupta, 2010). This situation is to blame for the steady increase (%) in cases of acute diarrhea (24%), typhoid fever (34%) and viral hepatitis (33%) from 2013 through 2016 in India (Bhasker Tripathi, 2018).

The COVID-19 pandemic has added to the many concerns over waterborne diseases. Bhowmick and team explored the possibilities of a SARS-CoV-2 outbreak in the Indian subcontinent due to lack of wastewater treatment (Dhar Bhowmick et al., 2020). Moreover, RNA of SARS-CoV-2 has been globally observed in untreated wastewater although the infection risks posed remain uncertain (Ahmed et al., 2020; Kitajima et al., 2020; Tran et al., 2021). Mixing of untreated wastewater with freshwater resources would put the Indian population at an elevated risk, since it more heavily relies on direct freshwater sources and engages in direct consumption of untreated surface water sources (Dhar Bhowmick et al., 2020). These hypotheses could be partly corroborated by the fact that despite having in place a longer than 3-month lockdown in

2020, SARS-CoV-2 cases continued to increase in India exponentially and resulted in a disease surge in 2021.

The present study leveraged data on CECs in human waste to assess vulnerabilities quantitatively and geospatially to human health on the Indian subcontinent. Whereas infectious disease risks are key concerns, chemicals may serve as an indicator of water quality issues that are more readily traceable and whose modeling is more robust than that of infectious disease agents which can self-propagate, may go into dormant stages, or can undergo genotypic or phenotypic changes, thereby complicating quantitative analyses of water quality, exposure, risk and human health impacts.

Study objectives were to provide an initial mass estimate of the nationwide releases into the environment of human waste-borne CECs for all of the nation's states and UTs and to estimate the mass load of the emerging contaminants entering the environment via different modes of defecation, specifically open defecation (OD), pit latrines (PL), water closets (WC) and other latrines (OL) based on the latest available robust data set from the 2011 Indian census (Padhi et al., 2015). The overarching goal of the work was to reveal the magnitude of wastewater-based pollution the Indian nation is facing and to establish an initial spatial distribution of the putatively most vulnerable regions.

2.3 Materials and Methods
2.3.1 Literature Survey

A literature survey was conducted via searching of the Scopus database until March 5, 2021. Detailed search terms were as follows, with individual searches being performed

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for title, abstract, and keywords of articles: India* AND wastewater* AND (treatment AND plant)* AND pharmaceuticals* AND (emerging AND contaminants) OR (ground AND water) OR wastewater OR (surface AND water).

2.3.2 Data analysis

For the papers reporting on sampling of WWTPs (*n*=14), mean (if reported), or a calculated average of minimum and maximum values of concentration (ng/L) were considered for further calculations. The mean concentrations (ng/L) were obtained for a variety of pharmaceuticals and personal care products, including artificial sweeteners, antibiotics, non-steroidal anti-inflammatory drugs (NSAIDS), stimulants, antihistamines, lipid regulators, antihypertensives, metabolites, serotonin uptake inhibitors, antimicrobials, sedatives, anti-convulsants, blood thinners, diarrhea medication, and contrast agents. Only chemicals with literature-documented (endocrine disruption) negative environmental impacts were further considered in the data analysis, i.e., antibiotics, NSAIDs, antimicrobials, phthalates and, other pharmaceuticals (antihistamines, lipid regulators, antihypertensives, anti-convulsant, blood thinners, diarrhea medication, and contrast agents). Considering the sample size and ease of calculations, we divided the mentioned classes of compounds into six major classes, i.e., antimicrobials *(n=8)*, antibiotics *(n=17)*, NSAIDs *(n=8)*, phthalates *(n=4)* and, other pharmaceuticals *(n=19)*, where *n* is the number of individual chemicals included in each class. Details on calculations and the literature review can be found in the Section 2.4. and Table 1 of appendix A respectively.

2.3.3 Mapping and GIS

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The software package QGIS 3.8.1 "Zanzibar"1 and ArcGIS pro 2.5.2. were used for creating geospatial graphics and plots. Outside data sources for this map included the NASA Socioeconomic Data and Applications Center2, OpenStreetMap3, and Central Pollution Control Board, 2015 (CPCB) Indian sewage treatment plant inventory. Information on WWTPs that were operational or under construction and their respective design capacities were taken from the CPCB report, entered into an Excel spreadsheet, and geospatially analyzed using GIS software. Outside data sources included Indian states shapefiles and 2011 Indian Census data.

2.3.4 Calculations

Estimates of the average mass contributed per person and average environmental loading via WWTP effluent per capita for each class of compound were obtained using Equations 1 and 2 respectively.

$$m_{Exc} = \frac{Q_L\, C_I}{P_{WWTP}} \tag{1}$$

$$m_{Env} = \frac{Q_L\, C_E}{P_{WWTP}} \tag{2}$$

Where C_I and C_E are the means of individual influent and effluent data obtained from the literature; Q_L is the amount of wastewater treated per day in liters (MLD); and P$_{WWTP}$ is the population served by each WWTP (Table 2, Appendix A). m_{Exc} and m_{Env} are the average mass excretion rate and average environmental loading via WWTP effluent rates, respectively. They were calculated for each class of chemical and have the units mg/d/capita. The average volume of wastewater generated per person was calculated using equation 3.

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$$Q' = \frac{Q_L}{P_{WWTP}} \tag{3}$$

Where Q' is the volume of wastewater treated per person per day (l/d/person). A detailed

Table consisting of literature sourced values used to calculate Q' can be found in

supplementary Table 3 The size of populations served by or without access to wastewater

treatment in each state were calculated using equations 4 and 5, respectively.

$$P_{served} = \frac{Q_{OC}}{Q'} \tag{4}$$

$$P_{unserved} = P_T - P_{served} \tag{5}$$

Where P_{Served} and $P_{Unserved}$ are the populations served or not served by a WWTP,

respectively. Q_{OC} represents the operational capacity (l/p/d) and P_T represents the total

state population (Table 3, Appendix A).

Mass loadings of chemicals (kg/d) for each state were calculated using equation 6

considering the contributions from human waste treated and unaccounted human waste.

Details on total served and unserved values with the background data for every state can

be found in Table 4.

$$m_T = \frac{m_{Exc} * P_{served} + m_{Env} * P_{unserved}}{10^6} \tag{6}$$

Where m_T represents the total mass load per state (kg/d) and a factor of 10^6 was used to

convert mg/d to kg/d. Mass loads of chemicals corresponding to each mode of human

waste disposal/treatment were calculated using equations 7, 8, 9, and 10.

$$m_{OD} = OD * m_T$$

(7)

$$m_{PL} = PL * m_T \tag{8}$$

$$m_{WC} = WC * m_T \tag{9}$$

$$m_{OL} = OL * m_T \tag{10}$$

Where *OD, PL, WC* and *OL* refer to open defecation, pit latrine usage, water closet usage, and other latrine usage percentages as obtained from the 2011 Indian census and m_{OD}, m_{PL}, m_{WC} and m_{OL} represent their respective mass loads (g/d).
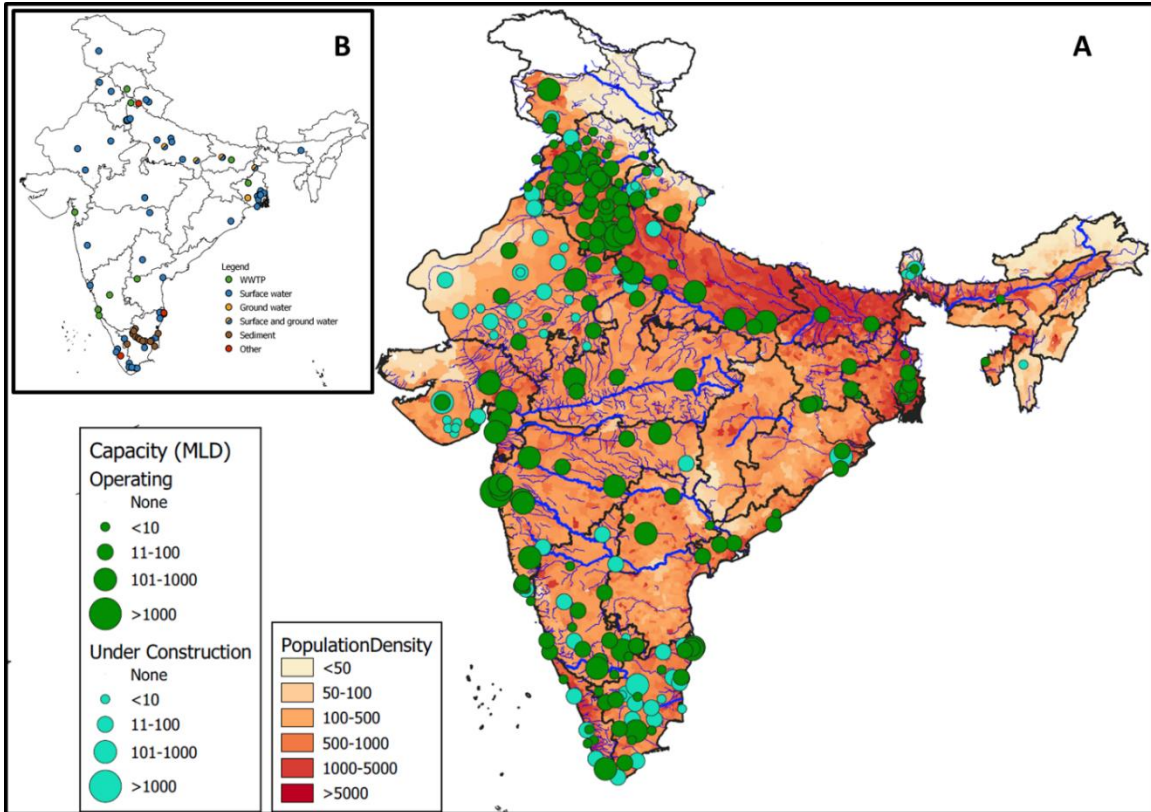
Fig. 1 - Panel A shows a heat map of population density overlain with data on wastewater treatment plant (WWTP) locations, capacities of plants in operation (dark green circles) and those under construction or proposed (blue circles) as well as major rivers; data was compiled from the Central Pollution Control Board (Central Pollution Control Board, 2015). Panel B shows a map of India indicating locations and sample matrices previously investigated in prior studies documented in the scientific literature.

2.4 Results and Discussion

2.4.1 Literature review results

A total of 886 results were obtained. We examined these publications individually and eliminated irrelevant articles focusing on antibiotic resistant genes, detection in animal organs/tissues, air sampling, human serum analysis, sludge analysis, and heavy metal monitoring. Only the research papers which sampled WWTPs, hospitals, surface waters and groundwaters in India were retained for analysis. Out of those, only 14 publications were used for quantitative analysis and 32 papers were used for qualitative analysis only. The remaining 840 publications were not included in this analysis.

2.4.2 Extent of wastewater treatment in India

According to a CPCB report, in 2013-2014 India had around 269 treatment plants which amounted to a total of ~18,885 million liters per day (MLD) for all the 35 states and UTs (Central Pollution Control Board, 2015). Although the Indian wastewater treatment capacity has increased 2.5 times from 1978 to 2007 (Kaur et al., 2012), data on the extent of WWTP infrastructure had been lacking prior to the CPCB report in 2015 (Central Pollution Control Board, 2015) and only data on the WWTP capacity of municipalities falling into the categories of Class 1 and Class 2 had been published by the Indian government up to that point.

The established operational capacities, in units of MLD, of WWTPs and those of facilities proposed or already under construction are plotted in Figure 1, which was created using data of the 2015 CPCB report. These data were overlaid with population density and river locations to understand the distribution of wastewater treatment infrastructure in the context of population density and rivers. This visual analysis of the CPCB data revealed that the UTs Diu, Daman (DD), Andaman and Nicobar Islands,

Lakshadweep islands and the states of Arunachal Pradesh, Manipur, Nagaland, and Chhattisgarh had no current operational treatment capacity and had no plans for adding such infrastructure in the immediate future. The population of these states as per the 2011 Indian census ranges from anywhere between 50,000 to 6 million people. This implies that all the wastewater and human excreta that are generated in these states had no appropriate disposal routes, thus rendering them destined to be deposited on soil or into nearby water bodies. The states of Assam, Meghalaya, Mizoram, Sikkim, Tripura, and the union territory of Puducherry had less than 20 MLD of operational treatment capacity with none of them having additional capacity proposed or an under construction.

2.4.3 Regional treatment scenario and previous studies

India can be divided into six regions based on culture and climate: the north, west, south, east, north-east, and central regions. These zones were adopted from (Philip et al., 2018). A discussion of the operating capacity, future capacity for wastewater treatment, the previous sampling for each region, and the environmental mass loadings in kg/d (Figure 2) is provided in the following section.

The landlocked north eastern states of Assam (AS), Tripura (TR), Sikkim (SK), Meghalaya (ML), Mizoram (MZ), Nagaland (NL), and Arunachal Pradesh (AR) have a collective population of ~46 million people (2011 data) with only 20 MLD operational capacity and 30 MLD in future capacity. One of India's major rivers, the Brahmaputra, flows through China, Arunachal Pradesh, Assam and Bangladesh supporting some 625 million people. With the rudimentary or non-existing treatment infrastructure documented, surface waters like the Brahmaputra River are at risk of serious

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contamination with biological and chemical hazards contained in human excreta and municipal effluent. In areas like northeastern India, epidemiology studies on chemical pollution are urgently needed but only two studies exist that could be classified as environmental monitoring studies here thus far, one detecting per- and polyfluoroalkyl substances (PFAS) compounds in ambient air samples (Jun Li et al., 2011) and the other reporting phthalates in surface waters (Roy & Kalita, 2011), with both studies being based in Assam.

The northern region is comprised of Chandigarh (CH), New Delhi (DL), Haryana (HR), Himachal Pradesh (HP), Jammu and Kashmir (JK), Punjab (PB), Uttar Pradesh (UP) and Uttarakhand (UK) and features a total population of about 300 million people according to the 2011 census, with 212 million people residing in rural areas. This region is rich in natural resources due to the presence of major rivers such as the Jhelum, Chenab, Ravi, Baes, Ganga, Yamuna etc. and an abundance of fertile agricultural land. Collectively, the northern region has only 7,400 MLD of operational capacity and 710 MLD of future capacity, sufficient to serve only 42 million people in total. This puts the rich water resources in the region at extreme risk of contamination. Since rural populations have less access to wastewater treatment when compared to urban ones, environmental contamination in rural parts of northern India may be severe even if the overall loading of pollutants is less on a per-capita basis. Out of the 8 states and UTs, only Chandigarh has enough operational capacity for the wastewater generated by its population to treat ~300 LPD/person, which is a little over the USEPA's required estimate of 227-264 LPD/person (USEPA, 2002).

This lack of wastewater treatment in the northern area of Uttar Pradesh is highlighted in Figure 1. The dark red areas have no green or blue circles, indicating zero current or planned future treatment. The lack of sanitary infrastructure combined with the land use of large-scale agriculture suggests an elevate risk of contaminants of emerging concern (CECs) entering into and contaminating the food chain. Moreover, antibiotic and non-steroidal anti-inflammatory drugs (NSAIDs) use is rampant in India, with 103% increase in antibiotic consumption since the year 2000 (DTE, n.d.) mainly due to ease of access (Farooqui et al., 2018; Paul & Chauhan, 2005). This was well demonstrated for common antibiotics like sulfamethoxazole whose concentration was reported as high as 1,340 µg/L in influent wastewaters of Delhi, followed by ciprofloxacin at 417 µg/L and 432 µg/L in Delhi and Nagpur ,respectively (Saxena et al., 2021). Common NSAIDs like ketoprofen, aspirin, naproxen and ibuprofen were also detected in the ranges 1,000 - 1,600 µg/L in Delhi wastewater (Thalla & Vannarath, 2020). Low to moderate levels (1-14 µg/L) of antibiotics were detected in the river Yamuna, which is a major tributary of the Ganges (Mutiyar & Mittal, 2014a). These high to moderate levels of pollutants in raw wastewater become more diluted when they reach the rivers without or upon sewage treatment. Hence, much lower values ranging from 0.041 – 6.9 µg/L levels of antibiotics and NSAIDs were found in the Ganges (Sharma et al., 2019). Pollutant concentrations in ground water were significantly lower compared to those in surface waters. NSAIDs were detected at levels between 0.002 and 0.049 µg/L in groundwater near or within the Ganges river basin (Sharma et al., 2019). Similarly, antibiotics were found at concentrations between 0.005 and 0.12 µg/L in northern India (Lapworth et al., 2018). The rivers Bhagirathi, Alakananda and Ganga were studied for the occurrence and

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concentrations of antibiotics (Sharma et al., 2019) and PFAS (Sharma et al., 2016). Commonly used compounds like caffeine were found consistently within a range of 9 to 750 ng/L range in the Ganges river and in ground water, whereas other commonly used pharmaceuticals occurred at much lower concentrations or were not detected, the latter finding attributable in part to high rates of dilution (Sharma et al., 2019). Similarly, PFAS concentrations were in the low ng/L ranges (ND to10 ng/L), with only a handful of polyfluorinated substances being detectable at all sampling sites (Sharma et al., 2016). In addition, elevated concentrations of phthalates of up to 218 µg/L were consistently detected in northern influent wastewaters (Gani et al., 2016). Mean concentrations of all congeners of phthalates ranged from 2 to 210 µg/L. The states and UTs of Chandigarh, Haryana, Jammu and Kashmir have not been studied for CECs but account for the majority of Indian water bodies susceptible to contamination with human waste.

Western India with Rajasthan (RJ), Gujarat (GJ), Maharashtra (MH), Goa (GA), Daman, Diu, Dadra and Nagar Haveli (DD) is of economic importance due to its booming trade and development activity. Despite its economic activities, this region does not meet basic sanitation requirements. With a total population of 243 million (148 million thereof in rural areas), western India only has an operational capacity of about 7,214 MLD and a future capacity of 1,106 MLD (Central Pollution Control Board, 2015). This still leaves about 200 million people without a connection to wastewater treatment. All calculations are performed using equations 3, 4, and 5. A small number of studies on water resource quality in the region are available. There have been three studies reported so far looking at antibiotics in Maharashtra (Archana et al., 2016), phthalates in Gujarat (Gani et al., 2016) and PFAS in rivers and beaches of Goa (Yeung et al., 2009). The large

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and populous state of Rajasthan and the coastal UTs of DD have not been studied yet for the occurrence of any CECs in environmental media. With water resources being limited in Rajasthan and zero capacity in operation or planned for the immediate future in DD, a significant discharge and potential accumulation of environmental pollutants is a concern in these regions.

The southern states of Andhra Pradesh (AP), Telangana (TS), Karnataka (KA), Kerala (KL), Tamil Nadu (TN) and UTs of Puducherry (PY), and Lakshadweep (LD) have 253 million people with approximately 150 million people residing in rural areas. Although the population count is high, the operational capacity for wastewater treatment is much lower, when compared to the Western and Northern regions: at only 3,174 MLD current capacity and 1,075 in planned future capacity, the infrastructure is sufficient to serve only about 18 million people currently and an additional 6 million people in the future. Southern India has a long coastline and is rich with rivers, such as the Kaveri, Godavari, Krishna, Tungabhadra, Penna, Kabini, Bhima, etc. Southern India by far has the largest amount of studies examining different environmental matrices for contamination. The only rivers which have been investigated for pharmaceuticals are the Musi where antibiotics were measured (Gothwal & Shashidhar, 2016), and the Kaveri, Vellar, Tamiraparani rivers, where surfactants (Selvaraj et al., 2014) and NSAIDs were investigated (Shanmugam et al., 2014). A study examining the water quality in a lake, river and wells in Telangana found high levels of ciprofloxacin (ND - 14,000 µg/L) and Cetirizine (ND-2100 µg/L) (Fick et al., 2009). Moderate levels (ND - 1.1 µg/L) of antibiotics and non-steroidal anti-inflammatory drugs were detected in WWTP effluents in Karnataka (Prabhasankar et al., 2016; Subedi et al., 2017). High levels of insect

repellents and triclosan were found in effluent of a WWTP in Tamil Nadu (Anumol et al., 2016a; Mohan & Balakrishnan, 2019). Surface waters and sediments from Tamil Nadu were analyzed for bisphenol A (BPA) (M et al., 2013), antimicrobials and antibiotics (Ramaswamy et al., 2011). Backwaters and the Periyar River of Kerala were investigated for chloroprene (Rayaroth et al., 2015) and for a variety of other common CECs (Khalid et al., 2018) with 2-dodecyl benzene sulfonic acid being the most abundant at 1012 ng/L followed by mefenamic acid (680 ng/L) and 16-Hydroxyhexadecanoic acid (325 ng/L).

The eastern part of India is comprised of Bihar (BR), Jharkhand (JH), Odisha (OH) and West Bengal (WB). West Bengal ranks 22nd in GDP per capita , followed by Odisha (23rd), Jharkhand (30th) and Bihar (32th) (Statista, 2019a). Except for West Bengal, the other states are economically less fortunate, a fact that is reflected in their degree of sanitary infrastructure. Collective operating capacity is at 610 MLD but the region has a population of 270 million people. This treatment capacity is very low and can serve only about 3.2 million people. Also, out of the 4 states, only Odisha has plans to extend its treatment capacity by 227.5 MLD in the nearer future. The overall treatment capacity in the eastern region is rudimentary only, given that over 260 million people have to dispose of their human waste without engineered treatment. Compared to the presumably high level of contamination with chemical pollutants, there has been very little research done concerning the determination of CECs in water resources. Antibiotics and PFAS were measured in the river Ganges flowing from Bihar (Subedi et al., 2015) and West Bengal (Corsolini et al., 2012; Sharma et al., 2019). Jharkhand and Orissa, which have little treatment infrastructure but a collective population of about 75 million people, have not been studied yet.

Central India has two states, Madhya Pradesh (MP) and Chhattisgarh (CG). The combined population count is about 100 million people, with an operational capacity estimated ay 475 MLD. Chhattisgarh has zero operational and zero planned future capacity, yet it features a population of some 25.5 million, all lacking access to WWTPs. In Madhya Pradesh, only 2.7 million people are served, with ~70 million people still not having access to sewage treatment. Antibiotics were measured in two cities in Madhya Pradesh in the effluents from two hospitals (Diwan et al., 2009). Apart from that no other sampling was done throughout the central Indian region.

In conclusion, chemical contamination of surface waters in India has not been studied sufficiently. The lack of sewage treatment infrastructure and monitoring data available to date suggest that many regions are at an elevated risk of contamination with chemicals. More comprehensive research is needed that ideally should cover all classes of CECs and
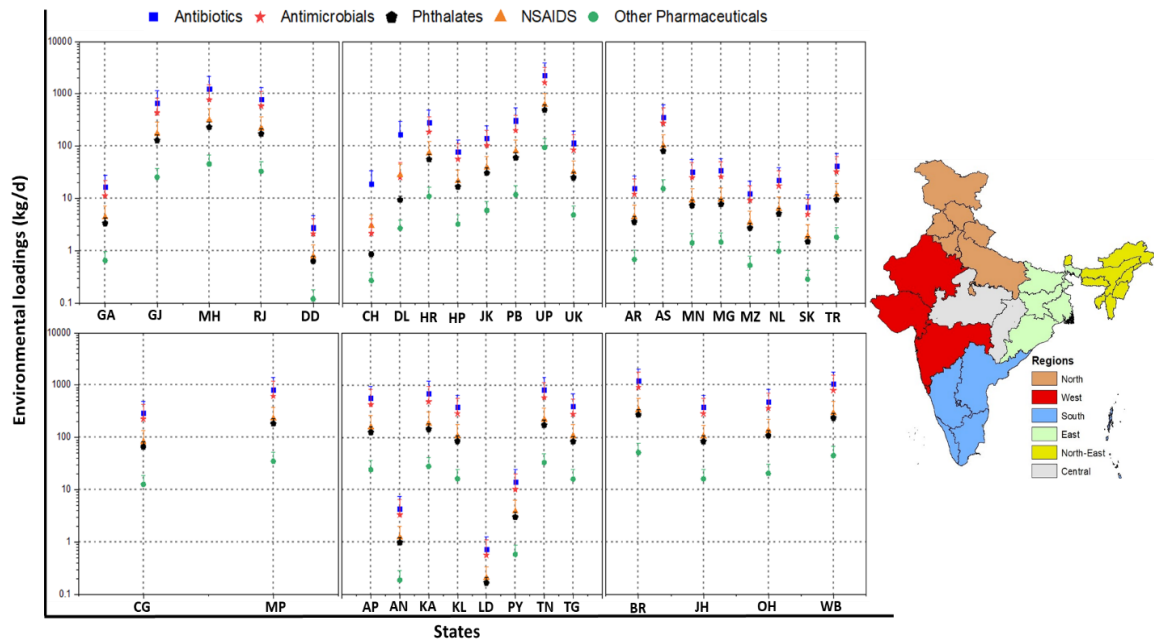
Fig. 4 - Environmental loadings (kg/d) via effluent and untreated wastewater for antimicrobials, artificial sweeteners, stimulant drugs, painkillers, antibiotics and other pharmaceuticals for six regions of India covering all states and union territories. Whiskers represent positive standard error.

all regions to inform the need for infrastructure upgrades. Special attention should be directed toward regions currently having no known treatment infrastructure while featuring significant population counts and rich natural resources.

2.4.4 Environmental mass loadings of emerging contaminants

Owing to the rampant use of potent pharmaceuticals often without the need to obtain a doctor's prescription and the lack of treatment of blackwater from human populations, an unknown number and presumably large quantities of emerging contaminants have been and currently are discharged into Indian aquatic and terrestrial environments. The total environmental loadings of six specific classes of compounds were evaluated in this work. Antibiotics by far have the highest loading in all six regions of India (Figure 2). The next high-ranking pollutant categories investigated here were antimicrobials, NSAIDs, phthalates, and miscellaneous pharmaceuticals captured in the "Other Pharmaceuticals" category. Environmental loadings, shown in Figure 2 in units of kg/d, are a function of population size, operational wastewater treatment capacity, discharge location of WWTP effluent, open defecation rates, connectivity to treatment plants and per capita use of the various compounds. Total loadings (all groups) by region (metric tons per day (mt/d) were determined to rank in the following order of decreasing magnitude: North (10.2 ± 6.1), East (10.1 ± 6.0), South (9.1 ± 5.3), West (8 ± 5), Central (3.6 ± 2.1), and North-east (1.7 ± 1.0). The hierarchies of sewage treatment capacity and population size did not align with this ranking. The north is the most populous region with approximately 300 million people, followed by the eastern region (270 million), the south (253 million), west (243 million), central India (98 million) and the north-eastern

region (46 million). The respective state governments are struggling to maintain environmental safety standards, which is reflected in the startling magnitude of the environmental loadings determined here.

The northern region had the highest loading of all regions with the eastern region being a close second, with only 4% lesser loading than the north. Uttar Pradesh in the north, was the state with the highest amount of total loading, at 7.2 ± 4.2 mt/d. This accounted for 69% of total loadings in the northern areas and was more than the central and northeastern regions combined. This could be attributed to its 200 million people, and not enough treatment infrastructure. Chandigarh on the other hand had the lowest total loading at 0.027 ± 0.019 mt/d in India. The top rank was occupied by UP, followed by Punjab (0.90 ± 0.54), Haryana (0.8 ± 0.5), Jammu and Kashmir (0.45 ± 0.26), Uttarakhand (0.36 ± 0.21), Himachal Pradesh (0.24 ± 0.12), New Delhi (0.23 ± 0.18), and Chandigarh (0.027 ± 0.019) all in mt/d. The eastern region with 270 million residents has a negligible treatment capacity of only 610 MLD. In the east, Bihar and West Bengal had the most loading among all the states at 3.9 ± 2.3 and 3.4 ± 2.0 mt/d, respectively. These were followed by the relatively smaller states of Jharkhand (1.2 ± 0.7) and Odisha (1.5 ± 0.1) mt/d. Out of the above 4 states, only Odisha has plans to build more WWTPs. The southern region, consisting of five major states and three UTs, had the third highest loading with Tamil Nadu featuring the highest value at 1.8 ± 1.3 mt/d, followed by Karnataka (2.1 ± 1.3), Andhra Pradesh (1.8 ± 1.2), Kerala (1.2 ± 0.8), Telangana (1.2 ± 0.8), Puducherry (0.03 ± 0.02), Lakshadweep (0.024 ± 0.012) and the lowest in the country was for Andaman-Nicobar Islands (0.014 ± 0.006) mt/d.

Western India had a similar scenario as the northern region. A total loading of 8.1 mt/d was determined for 4 states and 1 UT combined, with the highest loading being observed for Maharashtra (3.5 ± 2.2) which made up ~40% of the region's loading and lowest for the UT and Diu-Daman-Dadra Nagar Haveli (0.009 ± 0.004) mt/d. Despite being represented by just two states, the central region showed the highest loading mainly due to still rampant open defecation as the principal mode of human waste management. Chhattisgarh, having zero operational treatment capacity and had no plans to build treatment infrastructure, showed an environmental loading of 0.96 ± 0.6 kg/d. Madhya Pradesh on the other hand, had a treatment capacity of about 475.5 MLD and a loading value of 2.7 ± 1.5 kg/d.

The northeastern region had the smallest environmental loadings mainly due to lower population counts. The highest loading in the region was from Assam (1.2 ± 0.7 mt/d) which made up about 67% of the total regional loading. It was followed by Tripura (0.14 ± 0.8), Meghalaya (0.11 ± 0.07), Manipur (0.10 ± 0.05), Nagaland (0.07 ± 0.04), Arunachal Pradesh (0.05 ± 0.02), Mizoram (0.03 ± 0.02), and Sikkim (0.015 ± 0.008) all in mt/d. Lower loadings are accompanied with negligible treatment capacities. Three of eight states have zero operational capacity and two out of eight have ~30 MLD of proposed future capacity. These states are important due to the abundant natural resources in the area and its potential contamination.

2.4.5 Disposal of pharmaceuticals via different blackwater routes

The Government of India is actively undertaking efforts to counter the problem of OD but has been falling short of meeting its objectives, due in part to what has been

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considered a phenomenon of overpopulation of the nation (WHO- UNICEF, 2012b).

Figure 3 presents the total loadings in kg/d of pharmaceuticals via open defecation, water

closet, pit latrines and other latrines. The most recent census report indicates that 38% of
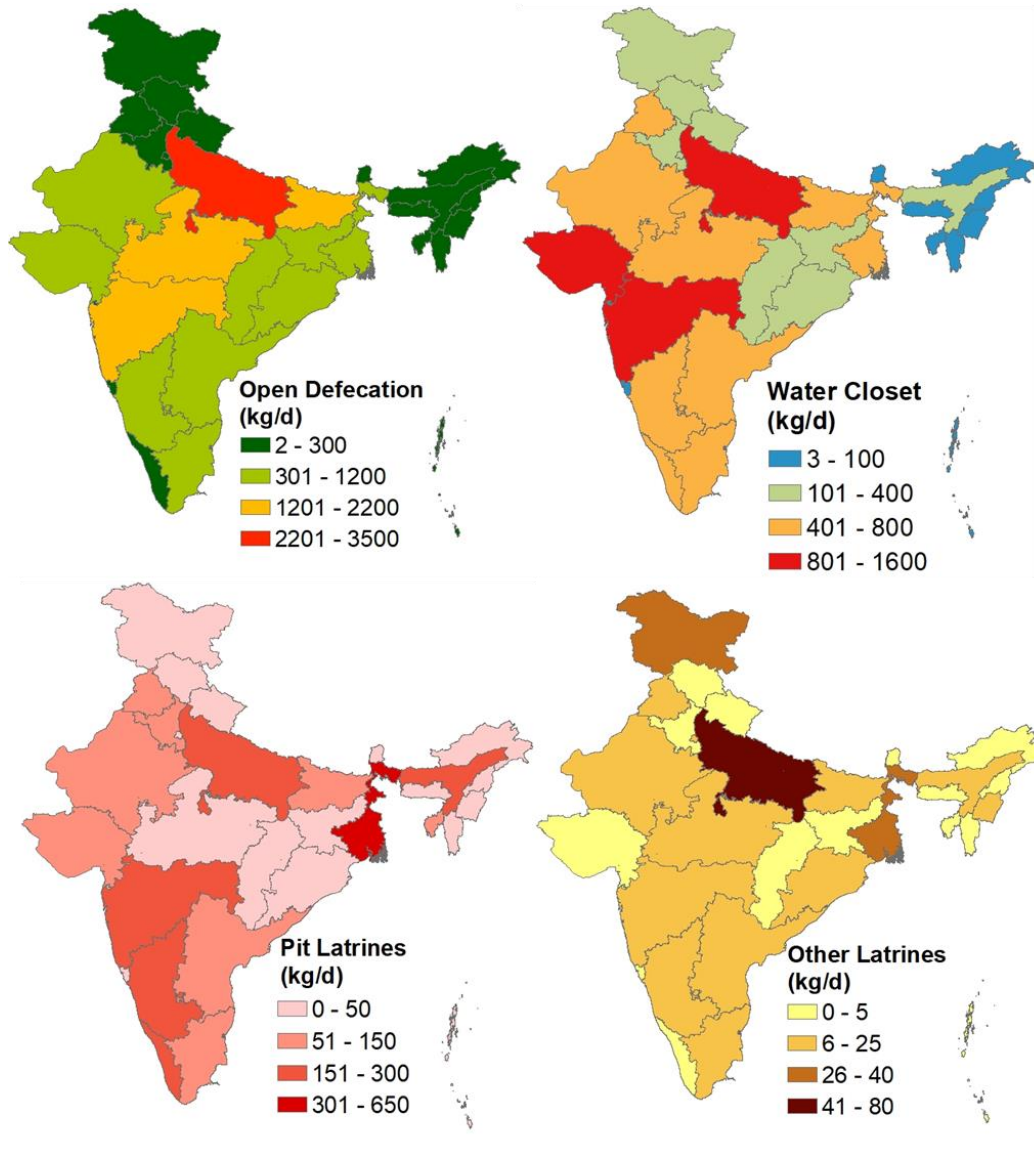
Fig. 6 - Spatial distribution of combined mass loadings (kg/d) of antimicrobials, antibiotics, pain killers and other pharmaceuticals via total and different modes of defecation: open defecation (OD), water closet (WC), pit latrines (PL), and other latrines (OL).

Indians have been defecating in the open as recently as 2011. The total loading of the specific chemicals investigated here released via open defecation was estimated at a maximum at 17.3 ± 12.7 mt/d, followed by 11.5 ± 8.5 mt/d via water closet, 3.0 ± 2.1 mt/d via pit latrines and 0.3 ± 0.2 mt/d via other types of latrines. Open defecation, as of 2011, was a major contributor toward environmental pollution in states like Jharkhand and Uttar Pradesh (see Fig. 3, OD panel) contributing some 24% of the total loading, with the next highest-ranking states including Maharashtra, Madhya Pradesh and Rajasthan. States with lowest release rates from OD were Uttarakhand, Mizoram, Manipur, Meghalaya, Tripura, Sikkim, Nagaland and Kerala. OD emissions in Jharkhand alone were 200% higher than the emissions of the lowest eight states combined, whereas its population was 20 million people lower than those eight states combined. Open defecation heightens the risk of transport of human excreta into drinking water sources, especially in rural India where drinking water treatment is scarce (Dhar Bhowmick et al., 2020). Hence, regions with high OD rates are more likely to be impacted by outbreaks and epidemics, including influenza and more recently, COVID-19, although a link between the SARS-CoV-2 detectability in environmental waters and disease transmission has not yet been established conclusively.

The highest percentage of WC use was in the UT of Lakshadweep at 97.4% followed by Chandigarh and DL at 87% and 85%, respectively. Lakshadweep represents an island, whose higher adoption rate of WCs is not matched by a similarly high rate of wastewater treatment capacity, thus implying that human excreta and the biological hazards as well as CECs contained therein enter the environment unattenuated to pose significant risks to human populations and local ecosystems. A higher prevalence of WC use can be an

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indicator of development and of a relatively higher standard of living. Yet, it does not guarantee a safe handling of the human waste generated. Risks of disease outbreaks remain if blackwater goes untreated.

Emissions from pit latrines typically represent a relatively lower risk to local drinking water but do have the potential to seep into shallow groundwater to potentially reach drinking water supplies and adversely impact human health. PL were less popular than OD and WC in India, as indicated by an implementation rate of only 9.3% of total emissions. West Bengal had the highest emissions via PL equaling to that of KA, MH and UP's combined. The rest of the states had and less significant amounts of PL emissions. Emissions via OL were negligible, since most states relied either on OD, WC and PL.

2.5 Assumptions and Limitations

A lack of reported data and relatively few studies conducted on the research topic made necessary several assumptions which may limit the robustness of this study. Industrial contributions to the reported influent concentrations were not included in this work, due to a lack of information in the publications used for this work, thereby implying that the reported data are conservative, with actual concentrations and loadings potentially being much higher in industrial regions. Excretion rates were assumed to be homogenous across the study population, irrespective of socioeconomic differences, a necessary assumption that may not hold for socio-economically depressed populations. Wastewater from the underserved population was assumed to be discharged completely

untreated, while rudimentary treatment with unreported means may exist in some places in the study area.

Sampling efforts in Indian environments would prove challenging particularly due to lack of infrastructure. Moreover, sampling open defecation and pit latrines locations followed by subsequent human/ecological health impact assessment would be difficult due lack of available data on population contributing to it, accumulation of human excreta, and the time normalized mass data.

2.6 Conclusions

This study presents an initial order-of-magnitude assessment of the release rate of six major groups of CECs into the Indian environment from disposal of human waste nationwide. While the numbers may be used to inform an initial risk assessment of chemical hazards in soils and surface waters, the data collected also have additional broader implications. Use of the absolute discharge rates and modes of human waste reported here may inform general public health assessments and the design management strategies to address principal threats to public health in India, most notably the discharge of untreated biological agents in the form of viruses, bacteria, parasites, drug-resistance gene and prions.

Human health risks posed by CECs should be viewed as a threat to public health second to biological threats from infectious pathogens. The present work represents a qualitative and quantitative assessment of the impact of lacking wastewater management infrastructure and the unwanted but significant emissions of chemicals contained in Indian sewage generated nationwide. The obtained data highlights India's need for a

more aggressive investment into wastewater treatment infrastructure. Investigating the chemical loadings can serve as an indicator and proxy to the quantity and magnitude of viruses and bacteria that may be entering into the Indian environment. Currently unaccounted for, the quantity and composition of human waste nationwide represents an important metric and determinant of disease outbreaks and spread, including the ongoing COVID-19 pandemic (Dhar Bhowmick et al., 2020).

With the current socio-economic situation in India, building centralized WWTP infrastructure may prove to be challenging if not impractical and cost prohibitive, given the common lack of access to water, financial resources and the logistical challenges of implementing large comprehensive infrastructure projects. Solutions are needed and may begin by implementing measures to sequester and age human waste to reduce the infectivity of the material and lessen the threat of transmission of infectious and parasitic diseases. Once these most pressing aspects have been successfully addressed, one may turn to managing the inherent risks posed by the release of pharmaceutically active substances, endocrine disruptors, bioaccumulative or persistent compounds and other chemical hazards that are captured under the umbrella term of contaminants of emerging concern.

TRANSITION 2

Among the CECs discussed in Chapter 2, antimicrobials constituted a major class of contaminants of concern given their high usage volume and widespread applications. Parabens and antimicrobials such as triclosan and triclocarban have long been used as effective antibacterial chemicals in consumer products such as soaps, food products and packaging, pharmaceuticals and miscellaneous personal care products. The ecological harm caused by triclosan and triclocarban rendered them banned in 2017 by the U.S. Food and Drug Administration (FDA). However, parabens were unaffected by the FDA ban and they see continued use in consumer products potentially today at quantities greater than those used in the past (Wolf, 2016). Moreover, the now banned chemicals (triclosan and triclocarban) may have been replaced with yet other antimicrobials of potential human health and ecological concern; the latter include quaternary chemicals such as benzalkonium chloride, benzethonium chloride, chlorhexidine, and chloroxylenol whose toxicological profiles are relatively unknown. Since the use of antimicrobials during the COVID-19 pandemic likely increased significantly, an evaluation of the traditional antimicrobials and their likely chemical substitutes is critical in understanding short-term and longer-term threats to ecosystem health.

In Chapter 3, I investigated 14 wastewater treatment plants from 12 states across the U.S. for a number of antimicrobials, including five parabens, triclosan and triclocarban. I determined their concentrations in raw influent and treated effluent streams to obtain data suitable for estimating chemical consumption and discharge into U.S. surface waters. For this purpose, I utilized LC/MS-MS to determine the concentration of analytes and subsequently estimated mass loads per 1,000 people per unit time based on

the removal efficiencies and effluent concentrations available. Moreover, I modeled the ecological risk posed to *Daphnia magna*, the green algae *Pseudokirchneriella subcapitata*, Zebrafish embryo (*Danio rerio*), the nematode *Caenorhabditis elegans* (*C. elegans*) and rainbow trout (*Oncorhynchus mykiss*) as a result of direct contact of these indicator species to both traditional and replacement antimicrobials.

CHAPTER 3

NATIONWIDE OCCURRENCE, TREATMENT EFFICIENCY, MASS LOADS OF
WASTEWATER-BORNE ANTIMICROBIALS AND ITS MODELED ECOLOGICAL
RISKS IN THE POST COVID-19 PANDEMIC ERA

Abstract

Parabens and polychlorinated antimicrobials incorporated in consumer products of daily use pose potential threats to human and ecological health due to their endocrine disrupting properties. In 2017, the U.S. Food and Drug Administration (FDA) banned the use of triclosan (TCS) and triclocarban (TCC) in hand soaps, shifting towards alternate antimicrobials like benzalkonium chloride (BAC), benzethonium chloride (BET), chlorhexidine (CHX), chloroxylenol (CXM) and parabens. This study assessed the concentrations, removal efficiency (RE), and mass loadings of TCS, TCC, and five parabens. Additionally, it also investigated the ecological risks due to alternate antimicrobials and their implications in the COVID-19 pandemic era. Methods included collection of raw influent and treated effluent from 14 WWTPs located across 12 U.S. states (2015-2016) and analyzed by liquid chromatography tandem mass spectrometry (LC/MS-MS) for target analytes. Higher REs were observed for antimicrobials (94-99%) relative to parabens (71-99%) throughout all WWTPs. Total TCS and TCC mass loadings (mean ± std. error) were 71 ± 11 µg/d/capita, total paraben loadings were 19 ± 5 µg/d/capita, and total annual U.S. loading for all 7 compounds are estimated at 11 ± 4 metric ton/year. Out of the newer antimicrobials assessed, CHX posed the highest risk to algae, zebra fish, and rainbow trout (>1), while BAC posed the lowest risk to the mentioned species.

3.1 Introduction

Parabens and antimicrobials (triclosan (TCS, 5-chloro-2-[2,4-dichloro-phenoxy]-phenol) and triclocarban (TCC, 3,4,4-trichlorocarbanilide)) are a class of compounds that have been widely used as preservatives in pharmaceutical and personal care products (PPCPs) in addition to food, beverages, and industrial products due to their broad spectrum of antimicrobial activity, stability over a wide pH range, and moderate solubility (Błędzka et al., 2014; J. Chen et al., 2017; Venkatesan et al., 2012). The potential risks of parabens (J. Chen et al., 2017; Soni et al., 2005; W. Wang & Kannan, 2016a), TCS and TCC (Venkatesan et al., 2012; Yao et al., 2018) on human and ecological health are well documented, as these compounds have been implicated in disturbances on metabolic systems, immune function, and hormone production through their endocrine disruption properties (Hanioka et al., 1996; Venkatesan et al., 2012). Studies have also shown that these chemicals can bioaccumulate in aquatic species, such as algae, fish and invertebrates (Venkatesan et al., 2012; Yao et al., 2018) as well as humans, possibly linking these effects to the onset and progression of breast cancer (Brausch & Rand, 2011; Charles & Darbre, 2013; P. D. Darbre et al., 2004; Philippa D. Darbre & Harvey, 2014; Yamamoto et al., 2011a).

These toxic chemicals primarily end up in wastewater treatment plants (WWTPs) and eventually in the environment in the form of wastewater effluent and sewage sludge. Although over 90% of parabens (Haman et al., 2015) and antimicrobials (Venkatesan et al., 2012) can be effectively removed during conventional wastewater treatment due to microbial activity, and concentrations as high as 5700 ng/L for TCS and 980 ng/L have

been reported (Blair et al., 2013) in treated effluent. Similarly, six parabens including

MePB, EtPB, PrPB, BuPB, BePB, and HePB (heptylparaben) were measured in the final

effluent from two WWTPs in Albany, NY, representing 1.57–8.03% of influent mass

loading (W. Wang & Kannan, 2016b). Studies with similar effluent paraben

concentrations found acceptable (hazard quotient < 1) risk values for certain ecological

species (Ahrens et al., 2016; DeLorenzo et al., 2008; Dobbins et al., 2009; Orvos et al.,

2002; Pedersen et al., 2010; Ramaswamy et al., 2011; Tamura et al., 2013; Yamamoto et

al., 2011a), however, a large scale U.S. nationwide study highlighting its removal

efficiency is still lacking.

Consequently, TCS, TCC, and 17 other antimicrobials were banned by the United

States Food and Drug Administration (USFDA) (effective from 2017) from inclusion in

over-the-counter antimicrobial products (Wolf, 2016). However, these compounds are

still allowed in personal care products for acne treatments, toothpaste, mouthwash, and

deodorant (Wolf, 2016). Parabens were not affected by this legislative mandate and

continue to be incorporated into commercial products. A recent study that monitored 7

WWTPs around the Chicago metropolitan area capturing over 5 million people from

2012-2017, concluded that there was a decreasing trend amongst the influent and effluent

concentrations of TCC and TCS compared to other pharmaceuticals. The concentrations

of TCC and TCS in all 7 treatment plants were reduced by approximately only 50% from

2015 to 2017 with considerable amounts of TCC and TCS still present in the effluent

streams of treatment plants (Brose et al., 2019).

Antimicrobials replacing TCC and TCS have moved into the market including

natural ingredients and other synthetic chemicals such as benzalkonium chloride (BAC),

benzethonium chloride (BET), chlorhexidine (CHX) and chloroxylenol (CXM) (Rundle et al., 2019; Sreevidya et al., 2018); the latter whose environmental risk has not been evaluated. Literature on the toxicology of these chemicals is lacking. However, Sreevidya and team reported that these new substitute chemicals are more toxic than TCC and TCS based on their effective concentration to kill 50% (EC50) and lethal dose to kill 50% (LD50) values for zebrafish embryos (Sreevidya et al., 2018). Moreover, the COVID 19 pandemic has led to increased dependence on antimicrobial products (Rusic et al., 2021) causing more antimicrobials (BAC, BET, CHX, CXM and parabens) to end up in the sewage and likely the environment. Ecological implications, removal efficiencies, and toxicological comparison values of the above chemicals are still lacking to continue their safe usage.

Due to continuous TCC/TCS presence, unaltered paraben regulations, and addition of new chemicals with a relatively unknown toxicology background, monitoring and risk assessment of antimicrobials is necessary. Moreover, the COVID 19 pandemic has led to increased public awareness for hygiene and a subsequent surge in antimicrobial use. Hence in this study, a removal assessment and mass loading of two antimicrobials (TCC and TCS) and five parabens (MePB, EtPB, PrPB, BuPB and BePB) has been performed for 14 WWTPs present in 12 U.S. states. Additionally, acute ecological risk posed by TCC, TCS, BAC, CXM and CHX on Water flea (*Daphnia Magna)*, green algae (*Pseudokirchneriella subcapitata*), zebrafish embryo (*danio rerio*), *Caenorhabditis elegans* (C. elegans) and rainbow trout (*Oncorhynchus mykiss*) is assessed considering a a direct exposure of effluent wastewater with the mentioned species.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

Methylparaben (MePB) was purchased from Aldrich (Sigma-Aldrich, St. Louis, MO), and $^{13}C_6$-MePB (99%) were obtained from Cambridge Isotope Laboratories (Andover, MA). Ethylparaben (EtPB), propylparaben (PrPB), butylparaben (BuPB), and benzylparaben (BePB) were purchased from RT Corp (Laramie, WY). LCMS-grade (99%) methanol, water, and acetic acid were obtained from Fluka and liquid chromatography (LC) -grade acetone was obtained from Sigma-Aldrich (St. Louis, MO). Individual stock solutions of the native and isotopically labeled compounds were prepared in methanol. High purity standards of native TCS and TCC, and LC-grade solvents were purchased from Sigma Aldrich (St. Louis, MO). Isotope-labeled standards $^{13}C_{13}$–TCC and $^{13}C_{12}$–TCS were purchased from Wellington Laboratories Inc. (Guelph, Canada). The working standards were prepared by serial dilution of stock solutions with methanol prior to use. All stock solutions were stored in glass vials with polytetrafluoroethylene septa at −20 °C. All glassware was washed with detergent, rinsed with ultrapure water, and heated at 550 °C for 4 h prior to use.

3.2.2 Study location and sampling

Single 24-hour composite samples of influent and effluent were collected from each of the 14 WWTPs located in 12 states (see Fig 4A) across the U.S. Samples were collected in clean disposable 2L high density polyethylene (HDPE) plastic bottles from August 2015 to June 2016. Samples were shipped in a Styrofoam cooler on ice to Arizona State University and stored at -20°C until processing. More about the study

locations, population captured, WWTP treatment details are listed in Table 6, Appendix B.

3.2.3 Sample processing

Aliquots of 500 milliliters of each sample (in duplicate) were extracted separately using a Dionex™ AutoTrace™ 280 Solid-Phase Extraction Instrument (Thermo Scientific, Waltham, MA). Wastewater was not filtered or centrifuged prior to processing but was spiked with 200 ng of the deuterated surrogate standards. Before loading, cartridges were conditioned with 3 mL of methanol, followed by 3 mL of water. The 500 mL of wastewater samples were loaded onto the cartridges at a flow rate of 2 mL/min, washed with water, and dried with nitrogen gas for 5 min. Elutions were performed, with 4 mL of a mixture (95:5, v/v) of methanol and formic acid (Sadaria et al., 2017). Equal volumes of serial eluates were combined, evaporated, and reconstituted to a water and methanol solution (50:50, v/v) for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

3.2.4 Sample analysis

Organic extracts of wastewater were analyzed using a Shimadzu 2100 high performance liquid chromatographer (HPLC), coupled to an AB Sciex APE 4000 triple quadrupole mass spectrometer (Applied Biosystems, Framingham, MA). Analytes were separated on a Symmetry C8 column (4.6 × 150 mm, 3.5 μm particle size) preceded by a Symmetry VanGuard Cartridge (3.9 × 5 mm, 3.5 μm particle size) (Waters, Milford, MA). Methanol was used as mobile phase A and water as the mobile phase B and the injection volume was 10 μL. Further information regarding gradients used and source

parameters can be found in the Table 7 and were adopted from Chen et al., 2017 (J. Chen et al., 2017). Analytes and labeled standards were identified using their specific retention time and multiple reaction monitoring transitions as reported earlier (J. Chen et al., 2019). Reported concentrations were determined based on a minimum 8-pt standard curve with minimum coefficients of determination $R^2 \geq 0.99$. Recoveries for parabens were determined based on spike-recovery experiments at three environmentally relevant concentrations, performed in triplicate as described in previously published manuscripts (J. Chen et al., 2017, 2019). As detection of background levels of parabens is a known issue (J. Chen et al., 2017) resulting from the ubiquity of the compounds, special care was taken to avoid possible contaminations by avoiding using products containing parabens and always wore gloves when handling samples. All extractions were performed along with method blanks (i.e., procedural controls), and a pure methanol/water mixture (50/50, v/v) was injected once per 10 samples as a check for carryover of parabens from sample to sample. No parabens were found in solvent blanks.

Method detection limit (MDL) was determined following USGS procedures (Childress et al., 1999) and USEPA guidelines. Method detection limits (MDLs) for parabens in wastewater ranged from 0.5 to 7.1 ng/L. The MDLs for TCC and TCS were determined to be 20 ng/L and 5 ng/L, respectively, which were adopted from Venkatesan et al. (2012).

3.2.5 Data analysis

LC-MS/MS data were acquired with Analyst 1.5 software (Applied Biosystem, Foster City, CA). Concentrations were calculated using the isotope-dilution method and

were reported as a ng/L. Concentrations were reported when the analyte peak height to background signal (signal-to-noise ratio) was greater than 3, and the concentrations were above the MDL. Non-detects failing to meet the above requirements were assigned a conservative value of MDL/$\sqrt{2}$ for statistical analysis, considering a worst-case scenario ("Estimation of Average Concentration in the Presence of Nondetectable Values," 1990). Equations for calculation of aqueous phase removal efficiency and effluent mass loadings are as follows.

$$RE = \frac{C_{inf} - C_{eff}}{C_{inf}} * 100 \text{ \%} \tag{11}$$

$$M_{eff} = C_{eff} * Q \tag{12}$$

where RE represents the aqueous phase removal efficiency, C_{inf} is the influent concentration (ng/L), C_{eff} is the effluent concentration (ng/L), M_{eff} is the effluent mass loading (g/day) and Q is the effluent flow rate (10^6 L/day).

3.2.6 Hazard Quotients and ecotoxicological risk assessment

Ecotoxicological risk assessment was evaluated considering a worst-case scenario, *i.e.* at zero dilution in the receiving water body as a screening-level analysis. The predicted environmental concentration (PEC) values were substituted with the respective WWTP effluent concentrations while the predicted no effect concentration was calculated using equation 13.

$$PNEC = \frac{EC50 \text{ or } LC50 \text{ value}}{Assessment \text{ factor (Acute)}} \tag{13}$$

EC50 and LC50 values were obtained from the literature with additional details in the Table 5, Appendix B. The assessment factor value was assumed to be 100 considering

acute toxicity (Tamura et al., 2013). Using the PEC and PNEC, the Hazard quotient (HQ) was calculated as the ratio of the two per equation 14.

$$HQ = \frac{PEC}{PNEC} \tag{14}$$

Species considered for risk assessment were *Daphnia Magna*, Green algae (*Pseudokirchneriella subcapitata*), Zebrafish embryo (*danio rerio*), *Caenorhabditis elegans* (*C. elegans*) and rainbow trout (*Oncorhynchus mykiss*). Species and toxicology parameter selection was based on their common occurrence in North American environments, and their literature availability. Risk was categorized as low risk (HQ<0.1), medium risk (0.1>HQ>1) and high risk (HQ>1) (Epa, 2015).

3.2.7 Modeling of Replacement Chemicals in Wastewater

Dependence on BAC, BET, CXM, and CHX has greatly increased as a antimicrobial substitute post-FDA ban on TCC and TCS (Sreevidya et al., 2018). Antimicrobial effective concentrations found in retail hand soaps vary greatly depending upon the antimicrobials used. Those in top selling antimicrobial soap brands such as Softsoap, Dawn, Dial, etc. were researched in combination with literature and online product specifications for TCC, TCS and new chemicals. Hand soap companies were selected based on their popularity and high sales figures (Statista, 2019b).

TCS and TCC have been used in the concentration ranges of 0.1-15% (Halden, 2014), and are either being replaced by BAC (0.13 -2%), CXM (0.3-3.75%), or CHX (0.5-4%) (Organisation, 2017). Looking at various product labels on Amazon.com and google searches, concentrations of BAC were close to the TCC/TCS concentrations and hence assumed a BAC:(TCC/TCS) ratio as 1.0 (Halden, 2014; Organisation, 2017).

CXM, a less effective antiseptic, was observed at higher concentrations between 0.3-
0.6% w/w and hence assumed a CXM:TCC/TCS ratio of 4.0. Additionally, CHX,
although used scarcely, was observed to be in the ranges 2-4% which gives it an average
CHX:TCC/TCS ratio of 30.0. The risk analysis followed the same pattern as shown in
equations 14 and 15 and is discussed in detail in further sections.

Since monitoring of every novel chemical in wastewater is cost prohibitive and
impractical, ecotoxicological risks to the above-mentioned species are modeled assuming
a 100% replacement of TCC and TCS by BAC, CXM and CHX individually at zero
dilution, hence the risk scenario assumes a worst-case scenario.

REs of BAC, CXM and CHX vary slightly compared to their previous
counterparts based on the published literature. BAC removal from 9 WWTPs averaged
$98.5 \pm 1.7\%$ (Clara et al., 2007) followed by CXM from 1 WWTP at $90.13 \pm 0.99\%$
(Kasprzyk-Hordern et al., 2009a) and CHX from 3 WWTPs at $98.23 \pm 0.25\%$ (Östman et
al., 2018). Combined influent concentrations of TCC and TCS were multiplied by the
removal rates listed above to obtain effluent concentrations.

$$Ratio_{X:TCC/TCS} = \frac{\% X}{\% TCC \ or \ TCS} \tag{15}$$

$Ratio_{X:TCC/TCS}$ in equation 15 is the ratio of effective concentration of replacement
antimicrobial X to individual TCC and TCS concentrations.

$$C'_X = C_{TCC+TCS} * (1 - RE_X) \tag{16}$$

C'_X in equation 16 is the predicted concentration of a replacement antimicrobial in
wastewater effluent, which is derived from sum of influent concentrations of TCC and
TCS ($C_{TCC+TCS}$) and removal efficiencies of new chemicals (RE_X) as in equation 16.

$$C_X = C'_X * Ratio_{X:TCC/TCS} \tag{17}$$

Corrected concentrations (C_X) for mass used is critical in ecological risk assessment due to their varying concentrations in hand soaps as shown in equation 17. Such modeling would give a better perspective of the hazards posed by each chemical in the case of a complete substitution in place of TCC and TCS and would help rank the substitutions from a toxicology standpoint. The risk analysis followed the same pattern as shown in equations 13 and 14 and is discussed in detail in further sections.

3.3 Results and discussion
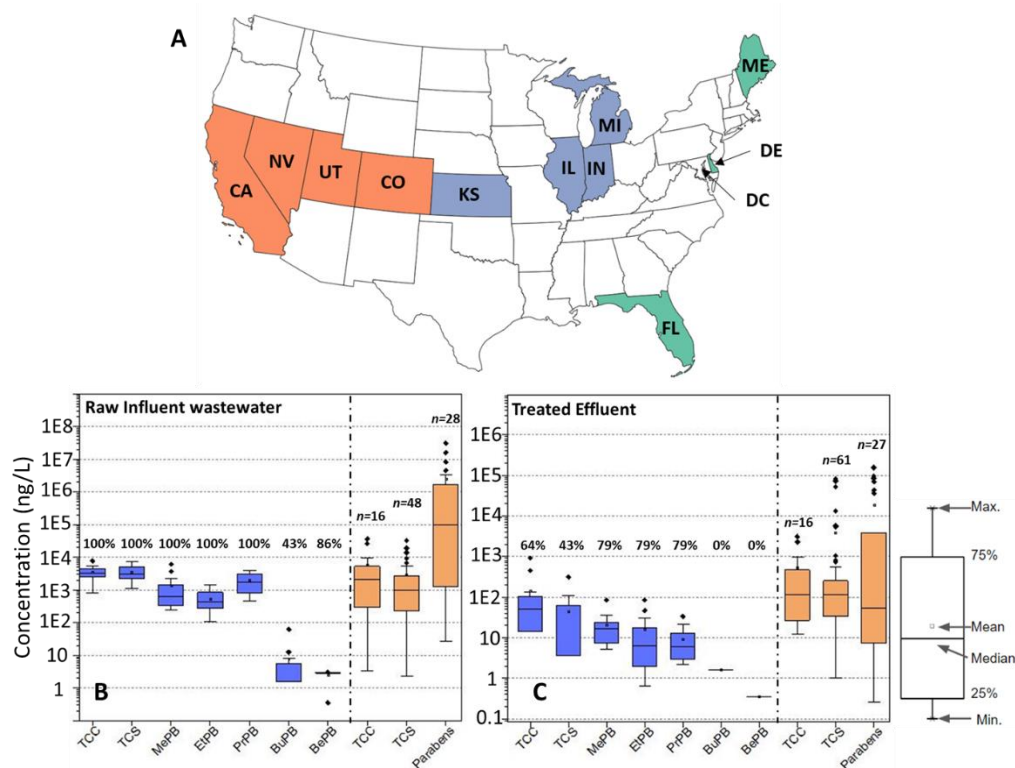
Fig. 8 - Map of the continental U.S. showing highlighted states where samples were collected from August 2015 and June 2016. Orange represents the west, blue for the mid-west and green for the east. (A) Measured influent (B) and effluent concentrations (C) (ng/L) of the seven compounds. Blue boxes represent data from this study and orange boxes represent the literature data. Error bars represent standard deviations and % represents the detection frequency. Non-detects were assigned an estimated value (method detection limit/$\sqrt{2}$).

3.3.1 Removal of Antimicrobials and Parabens in wastewater

TCC, TCS, MePB, EtPB and PrPB were detected in all the influent (dissolved + aqueous phase, ng/L) samples (100%) but BuPB and BePB were detected in only 43% and 86% of samples, respectively. The highest influent concentration (mean ± std. dev.) was observed for TCS at 3600 ± 1970 ng/L, followed by 3500 ± 1800 (TCC), 1970 ± 1180 (PrPB), 1300 ± 1000 (MePB), 530 ± 380 (EtPB), 15 ± 23 (BuPB), and 2.55 ± 0.93 (BePB), with all units in ng/L (see Fig 4). TCS was especially high at WWTP$_{CA2}$ influent streams with

a population of only 220,000, TCS concentration was highest at 6.58 ± 1.3 mg/L, which

is similar to that in Australia (Roberts et al., 2016) but 2-5 orders of magnitude higher

than some studies from Spain (Carmona et al., 2014), France (Gasperi et al., 2014),

Greece (Stasinakis et al., 2008) and China (Wu et al., 2007). Mean TCC concentrations in

the present study were 3 and 50 times greater than observed concentrations in India

(Anumol et al., 2016b) and Spain (Carmona et al., 2014), respectively, but many-fold

lower than certain studies in the US (Hedgespeth et al., 2012; Kumar et al., 2010).

Sampling year gaps could result in such differences due to the decreasing usage of
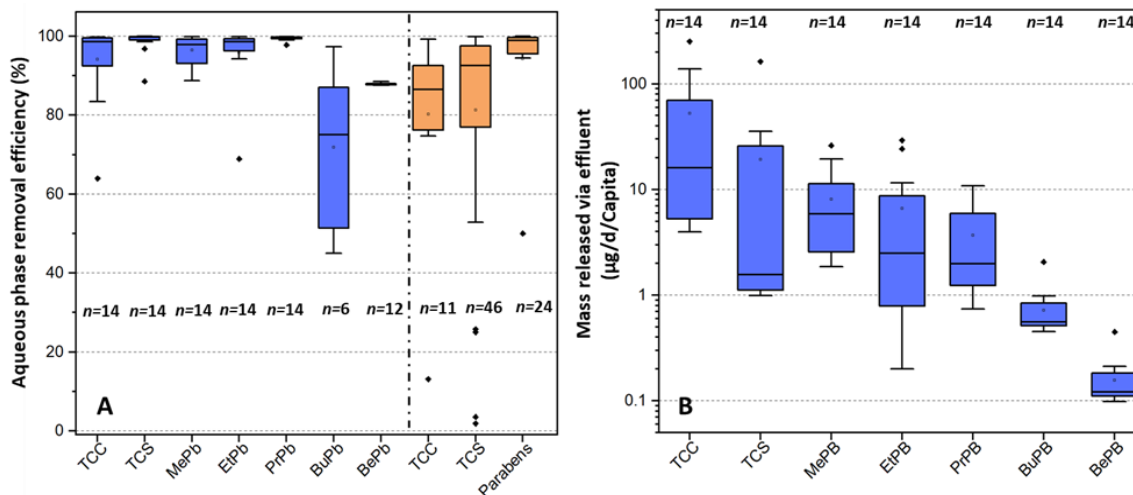
Fig. 10 - Aqueous phase removal efficiency (%) of various antimicrobials during full-scale municipal wastewater treatment. Error bars represent standard deviations and n the number of observations. (B) Mass loading determined per capita per day for all 7 compounds.

TCC/TCS over the years corroborating the study by Brose and team (Brose et al., 2019).

Several non-detects were found in the effluent streams and detectable concentrations

were much lower compared to its influent counterparts. Low effluent values can be

attributed to multiple pathways of degradation such as abiotic hydrolysis,

photodegradation, sorption to particulates and microbial activity (Heidler et al., 2006).

Due to the hydrophobic nature of TCC/TCS (log Kow>4) (Venkatesan et al., 2012) and a

majority of WWTPs having either conventional activated sludge process or an advanced

treatment, microbial activity and sorption are the most likely pathways for degradation.

The highest TCS effluent was in WWTP$_{CO}$ at 310 ng/L which accounted for 50% of the

total TCS concentrations among all WWTPs, despite sampling only 0.27% of the total

population. This value was still 50% lower than the highest effluent concentration found

in Spain (Gómez et al., 2009), 65% lower than in Antartica (Emnet et al., 2015) and 93%

lower than in Georgia, US (Kumar et al., 2010). TCC was the most prevalent with mean

± std. dev. at 141 ± 247 ng/L in the effluent streams from all the WWTPs followed by

TCS, PrPB, MePB, EtPB, BuPb, and BePB. Despite low paraben concentrations, MePB,

EtPB and PrPB were the most detected at 79% in effluent streams, suggesting a

ubiquitous presence throughout WWTP effluents. Paraben effluent concentrations were

similar to studies in India, China, Switzerland, United Kingdom and other US studies (see

Table 1). It should be noted that during sampling, the hydraulic retention times were not

accounted for, and preference was given towards capturing a 24-hour average

concentration. Differences in the concentrations between this study and the published

literature are evident in fig 4A and 4B.

Average removal efficiencies (see Fig. 5A) and sample sizes (%, *n*) in descending

order were PrPb (99 ± 0.6%, 11) followed by EtPB (95 ± 9%, 11), MePB (96 ± 4%, 11),

TCS (99 ± 3%, 14), TCC (94 ± 10%, 14), BePB (87 ± 26%, 6) and the lowest for butyl

paraben (BuPB) (71 ± 20, 13). Lower removal of parabens could be due a combination of

its persistent nature and hydrophilicity (Log Kow: 2-3.5). It should be noted that all non-

detects were assumed to take a conservative value of MDL/$\sqrt{2}$ and non-detects in the

influent stream were excluded from calculations. TCS removal has been consistently high

(>95%) worldwide (Halden & Paull, 2005) with 99.8% in Australia (Roberts et al.,

2016), 78-98% in Canada (Lishman et al., 2006),and >97% in China (Zheng et al., 2020)

while paraben removal efficiencies were a little lower and ranged between 84-98% (see

Table 7, Appendix B).

3.3.2 Mass released of the Antimicrobials and Parabens via effluent

Environmental mass emission loadings were calculated based on effluent

concentrations, averaged flow rates, and normalized using the population served by each

WWTP at the time of sampling (see Fig. 5B). Average mass loads (mean ± stdev) in

descending order were calculated for TCC (52 ± 70), TCS (19 ± 43), MePB (8 ± 7), EtPB

(7 ± 9), PrPB (4 ± 3), BuPB (0.7 ± 0.4), and BePb (0.2 ± 0.1), all in the units of

µg/d/capita. WWTP$_{CA2}$, with a population of just 95,000 contributed ~250 µg/d/capita of

emissions accounting for 25% of total TCC loadings. TCS loadings were lower compared

to TCC but WWTP$_{CO}$ with 108,000 people accounted for 65% of the total TCS emissions

at ~163 µg/d/capita. TCC and TCS mass loads can be influenced by the local

demographics and economy of the country. India followed a similar trend as this study

had higher dissolved effluent TCC loads than TCS. TCC and TCS loads from 5 sewage

treatment plants (STP) in India ranged from 0.34-50 and 3-60 µg/d/capita respectively

(Subedi et al., 2015). A study of two WWTPs in United Kingdom reported ~8-38

µg/d/capita of effluent TCS loadings (Kasprzyk-Hordern et al., 2009a). Conversely, a

Chinese study reported antimicrobial load values 3-4 orders of magnitude lower than

those reported in an Indian study (Y. Wang et al., 2018). A high environmental emission

for India can also be linked to a lack of efficient treatment in place and treatment

overload due to high population density. A study in Georgia from 2010 reported TCC

environmental emissions were between 1.6-76 g/d while TCS were higher between 1.6-

168 g/d (Kumar et al., 2010) which showed an opposite trend compared with the present

study having 13.8-408 g/day for TCC and 0.0015-23.21 g/d for TCS.

Observed paraben mass loadings were several orders of magnitude lower than

TCC and TCS. WWTPs in Illinois, Colorado and Delaware had the highest effluent

loading for total parabens at ~40 µg/d/capita with over 95% of it coming from MePB,

EtPB and PrPB. The range of effluent paraben loadings from this study ranged from 4-40

µg/d/capita. Paraben emissions were comparable to other studies performed in the U.S.

(W. Wang & Kannan, 2016a). Table 10 represents average mass loading (µg/d/capita)

and average effluent concentrations for MePB, EtPB, PrPB, BuPB and BePB calculated

from the literature. The lowest mass loads of 3 ± 0.7 µg/d/capita were obtained from

studies done in China (W. Li et al., 2015; Sun et al., 2016) while the effluent loadings

(MePB, EtPB, PrPB and BuPB) from United Kingdom were comparable to this study at

~0.3-16 µg/d/capita (Kasprzyk-Hordern et al., 2009b). The effluent stream values in the

UK were 1000 times higher than other studies performed thus far (Kasprzyk-Hordern et

al., 2009a). The average removal efficiencies reported from previous studies were $89 \pm$

8%(Sun et al., 2016), 89 ± 8%(W. Li et al., 2015), 98 ± 2% (Jonkers et al., 2009), $84 \pm$

3% (R. Karthikraj et al., 2017), 95 ± 3% (W. Wang & Kannan, 2016b), and 98 ± 2%

(Kasprzyk-Hordern et al., 2009a), which have remained consistent throughout literature

3.3.3 Risk posed to biota

Ecotoxicological risk posed by TCC, TCS, BAC, CXM, and CHX were evaluated for *Daphnia Magna*, Green algae, Zebrafish embryos, *C. elegans,* and rainbow trout, and expressed in terms of hazard quotients (see Fig.6). In the present study, the PECs assumed were corroborated with surface water concentrations of TCS and TCC across several countries. Concentration ranges of TCC (14.14-907 ng/L) and TCS (3.53-310 ng/L) from this study were comparable to TCS observed in rivers from China (35-1023
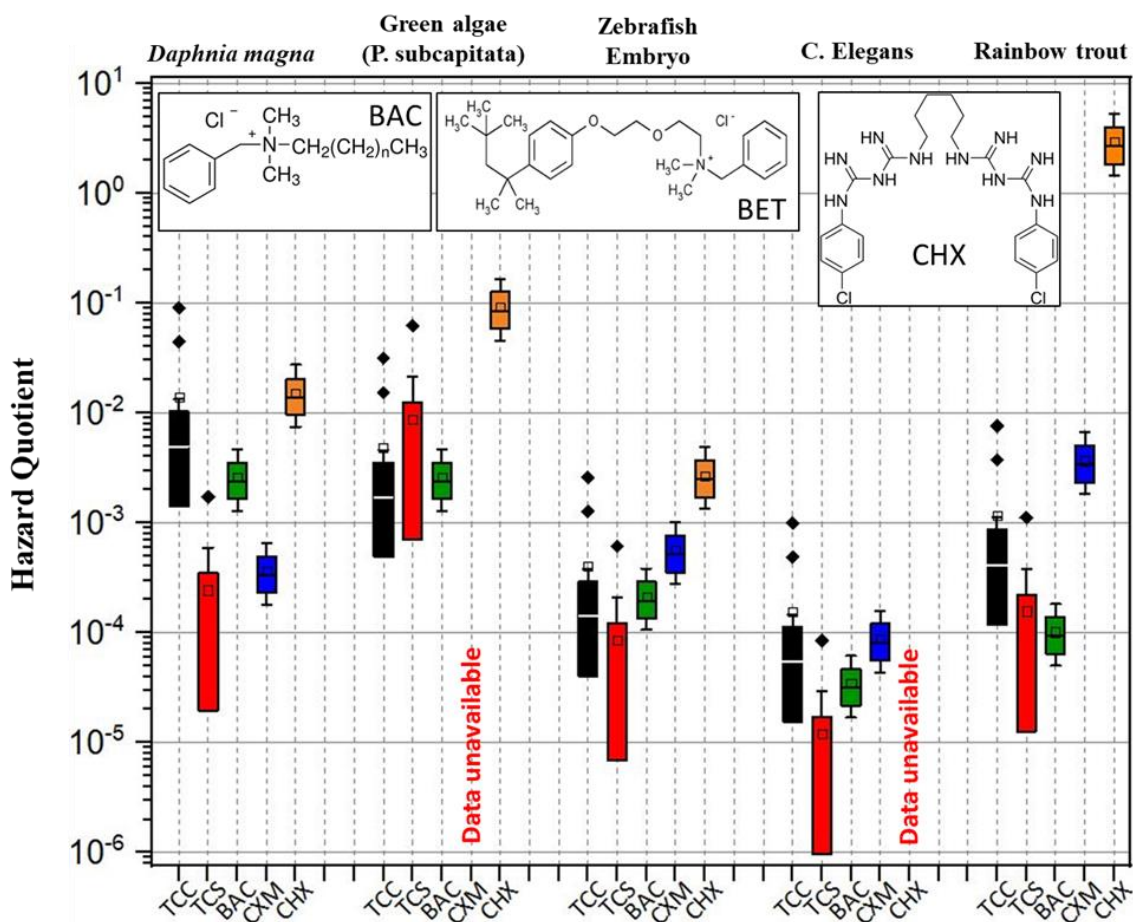
Fig.12 - Hazard quotients at a zero-dilution factor (worst case scenario) TCS and replacement chemicals BAC, CXM and CHX for different ecological species is presented here. HQ < 1 is considered an unacceptable risk. n=14 for all the box plots. Missing data is represented by 'Data unavailable'.

ng/L)(Peng et al., 2014), Spain (24-157 ng/L)(Gómez et al., 2009), Japan (2-177 ng/L) (K. Kimura, K. Kameda, H. Yamamoto, N Nakada, 2011) and to TCC in downstream from a WWTP in the United States (2-250 ng/L) (Sapkota et al., 2007).

Only CHX was observed to pose high risk to rainbow trout (0.4<HQ<23.4), assuming no dilution of treated wastewater effluent. Although not high, CHX posed a medium risk to green algae and *Daphnia magna* with its maximum HQ at 0.3 and 0.2 respectively. CHX proved to be ~28 times toxic to green algae and ~200 times more toxic to *Daphnia magna* compared to TCS. Wide ranges of HQs were seen in this study with CHX posing a hazard to the studies species. HQs of all chemicals studied here ranged from 10^{-6} to 23. Additionally, low risk was observed for zebrafish embryos, *C. elegans*, and rainbow trout, their HQs ranged from 10^{-6}-0.02-, 10^{-7}-10^{-4} respectively. A global ecotoxicological analysis on *Daphnia magna* and green algae due to TCS and TCS using surface water concentrations from several countries reported values in the range of 0.001-30 for both chemicals (Tamura et al., 2013). This could be possibly due to different environmental scenarios and usage patterns. Exclusion of paraben risk analysis from Fig. 3 was due to its EC50/LC50 values being 3-5 orders of magnitude higher than other chemicals analyzed and consequently diluted HQs. Studies from Japan (Yamamoto et al., 2011a) and India (Ramaswamy et al., 2011) had similar findings with paraben HQs of 2-4 orders of magnitude under the threshold value of 1, while HQs for TCS/TCC were several magnitudes above 1. Unfortunately, there are no studies which measure surface water concentrations of BAC, CXM, and CHX currently available, which could help compare it to TCC and TCS, helping us verify some of our assumptions. Considering all assumptions stated in section 2.6.1, CHX posed the highest risk (5×10^{-4}-23.4) while

CXM posed the least risk (10^{-6}-10^{-3}) to the biota studied here and can be a safer substitute for TCS and TCC in hand soaps. Although relatively safer, CXM mass loads would have to be 30x higher in order to match the antimicrobial effectiveness of TCC and TCS in personal care products. This action can cause unforeseen hazards and potentially a bigger threat to the environment which is largely unknown and demands attention. Some EC50/LD50 values were unavailable due to lack of research in the field and are listed as 'unavailable' in Fig. 6.

3.4 Limitations

New data and insights into the potential behavior of replacement chemicals currently used were presented here. However, there are some limitations and assumptions that should be considered when interpreting these results. A scarcity of studies focusing on occurrence and concentrations of BAC, BET, CXM, and CHX in surface and wastewaters made it difficult to gauge the trend and magnitude of its use in the US. Hence, the percentage concentration from the labels of hand soaps were used as a metric to calculate the substitution ratios between old and new chemicals. Ecotoxicological risk had to be limited to the mentioned species due to lack of toxicity data available for the new chemicals.

3.5 Conclusions

A ubiquitous presence of parabens and antimicrobial compounds was found across the US. High removal efficiencies and a federal ban on TCC/TCS might subdue the threat these chemicals pose to the environment but fail to mitigate potential concerns

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associated with new chemicals. This study showed that replacing the antimicrobial agents by CHX can be 30-2000 times more toxic to certain biota than TCC/TCS while CXM would be a lesser toxic substitute for the studied biota. The lack of information calls for research on removal efficiencies, wastewater chemistry, toxicity effects on biota and its propensity to enter the food chain to deem the current usage of these chemicals as safe.

TRANSITION 3

In Chapter 3, I discussed the loads of antibacterial compounds which were subject to change as a result of both the US FDA ban on antimicrobials in personal care products and the onset of the COVID-19 pandemic. While the use of antibacterial chemicals increased, other supply chains saw declines, as they were negatively impacted by the global spread of the virus and the subsequent lockdowns and public health response mounted (Ali et al., 2021). Opioids and prescription drugs of licit and illicit use as well as medications to combat addiction were some of the commodities presumed to be severely impacted by the lockdowns. However, the behavior of drug users during this assumed shortage, their reliance on other drugs, and potential risks that these may pose to substance users' health are still largely unknown. In Chapter 4, I applied WBE to measure the consumption of narcotics in near real-time in a non-invasive and anonymous way for the City of Tempe, Arizona. The study spanned from January 2020 to July 2020 and captured approximately 280,000 people covering the before and after lockdown periods.

I set out to measure two illicit drugs (cocaine and heroin), two legally prescribed drugs (codeine and oxycodone), and two opioid medications (mitragynine and methadone) and the metabolites for all but mitragynine and codeine in raw wastewater. Samples (24-hour flow-weighted composites) were obtained for 7-22 days for each month. The estimated consumption values were compared to related U.S. and international literature, as well as to four U.S. studies that similarly looked at drug

consumption in an urban setting with a large population of college students. I used

nonparametric statistical tests to evaluate consumption rates and to identify differences

between narcotic use before and during COVID-19 lockdown.

CHAPTER 4

TRACKING OPIOID USE AND CONSUMPTION OF RELATED PAIN

MEDICATIONS DURING THE 2020 COVID-19 EPIDEMIC IN A SOUTHWESTERN

U.S. CITY BY WASTEWATER-BASED EPIDEMIOLOGY

Abstract

The COVID-19 pandemic caused distress in communities worldwide and affected

the supply chains of both licit and illicit drugs. This study examines the occurrence in

wastewater of substances known to be subject of abuse and their respective metabolites

on a neighborhood scale. Results showed detectable quantities of psychoactive illegal

opioids (cocaine, benzoylecgonine, heroin, and 6-acetylmorphine), prescribed opioids

(oxycodone, noroxycodone, and codeine), and opioid medications (Kratom, Methadone,

and EDDP) prior and during the COVID-19 pandemic era using LC-MS/MS. 24-hour

composite wastewater capturing university campuses, student housing, and residential

areas for 7-10 days from the months of January to July 2020 each was collected from the

southwestern city in the U.S. Average mass loads (mg/day/1000 people) ranked as

follows: benzoylecgonine (1453), cocaine (754), EDDP (134), methadone (63), kratom

(60), oxycodone (41), codeine (37), 6-acetylmorphine (32), heroin (13) and

noroxycodone (7). A non-parametric Wilcoxon test for doses consumed (doses/day/1000

people) between the pre-lockdown period and during lockdown period yielded p values

and respective median changes suggesting significant differences in doses consumed for

heroin (1.3E-08, increased 10-fold), oxycodone (0.0003, decreased 5-fold), codeine

(0.0448, decreased 2.5-fold) and insignificant differences for cocaine (0.2944),

methadone (0.1987), kratom (0.2371) when α=0.05. This study yields a baseline for consumption patterns of two illegal opioids, two prescribed opioids and two opioid medications including Kratom for the first time in a major southwestern U.S. city.

4.1 Introduction

Opioids have been notorious for their psychoactive properties and their widespread use in medicinal and recreational settings. However, their mechanisms of binding to the opioid receptors in the central nervous system and the gastrointestinal tract can often lead to an increased dependence and a subsequent addiction (Campos-Mañas et al., 2018). Opioids were responsible for 67% and 63% of all drug overdose deaths in 2014 and 2015, respectively (Rudd, 2019). Moreover, use of prescription drugs such as codeine, morphine, and oxycodone for non-medicinal purposes has been increasing exponentially since the 1980s. Over 400,000 people reportedly abused prescribed opioids for non-medicinal uses in 1980 (Zacny et al., 2003), that number was 7 million for people aged 12 or older according to a 2006 national survey on drug abuse (Manchikanti & Singh, 2008), and over 10.3 million in 2014 (Gushgari et al., 2019).

As the opioid crisis continues to unfold, the novel coronavirus (SARS-COV-2) caused worldwide lockdowns as a strategy to reduce human to human infection spread and a required public health measure beginning end of March 2020. The lockdowns also affected supply chains of several crucial industries such as manufacturing, retail, public services, entertainment, media, and the transport industry (Xiang et al., 2021). These measures affected activities required to distribute drugs to consumers individually and affected the illegal drug markets from production to consumption (Mann, 2020; United

Nations Office on Drugs and Crime, 2020) These measures have shown to negatively affect the people who use drugs (PWUD) in Canada (Ali et al., 2021) and Germany (Scherbaum et al., 2021) in terms of supply frequencies and the quality. In Europe, heroin is mostly trafficked by land and thus was less impacted by COVID-19 restrictions compared to cocaine which mostly is transported by air (United Nations Office on Drugs and Crime, 2020). The U.S. drug enforcement administration (DEA) reported slight to no change in cocaine production coming in via south and central American countries. A subsequent decrease in cocaine availability was reported in the spring of 2020 but data on heroin availability is still lacking (Drug Enforcement Administration, 2020). In a major Austrian city, consumption of illicit drugs such as methamphetamine, cocaine, MDMA, and amphetamine did not follow a consistent increasing trend observed from 2016-2019 and instead showed a minor decrease in consumption during the COVID-19 lockdowns. Effects of supply chain disruptions were evident in the U.S. state of Kentucky as well where cocaine and methamphetamine consumption decreased by 40 and 16% respectively from March to July 2020 (Kentucky Substance Use Research and Enforcement, 2020).

In cases of illicit drug shortages, PWUD can often rely on other drug substitutes that imitate opioid like effects. This premise was corroborated by a study surveying 200 Canadian citizens, out of which 47% substituted their usual drug with an alternative substance during the COVID-19 lockdowns (Ali et al., 2021). As observed in the past, opioid users have relied on legally prescribed opioids such as morphine, oxycodone, and codeine and even commonly available opioid medications such as methadone (Brands et al., 2004) and kratom (Matson & Schenk, 2019) for similar psychoactive effects. A

clinical urine testing study with 150,000 subjects with opioid disorder revealed a consistent increase (%) in heroin (1%), cocaine (1.2%), fentanyl (4%), and methamphetamine (3%) consumptions during the pandemic (March 2020-July 2020) as compared to before pandemic (November 2019-February 2020) (Wainwright et al., 2020). An urban emergency department reported a 100% increase in opioid related visits compared to the pre-pandemic period (Ochalek et al., 2020; Slavova et al., 2020). Some surveys during the pandemic indicated increased anxiety, boredom, and fear, as well as increased cases of relapsed drug consumption in Canada and in Europe (Centre on Substance Use, n.d.; *EMCDDA Trendspotter briefing I Impact of COVID-19 on drug services and help-seeking in Europe*, 2020). However, most people surveyed/clinically tested were regular opioid users having history of opioid abuse and did not represent the nation as a whole. Studies investigating the change in opioid consumption as a consequence of lockdown restrictions for a large population over a long duration are still lacking.

Therefore, the goal of the present study was to examine the shift in opioid abuse trends caused due to COVID-19 lockdown restrictions in a moderately sized southwestern U.S. city (<300,000 inhabitants) over the period of 7 months (January 2020-July 2020). Two common illicit opioids, two legally prescribed opioids, and two opioid medications were selected due to their potential to be misused or relied on in cases of shortages. The list of compounds measured include cocaine, benzoylecgonine, heroin, 6-acetylmorphine, oxycodone, noroxycodone, and codeine, kratom, methadone, and EDDP. Specific objectives of the study were to (i) to measure the concentrations of listed compounds in wastewater from 10 different sewersheds before and during the COVID-19

lockdowns (ii) estimate the dose consumption per 1000 people of parent compounds

before and during lockdowns, and (iii) statistically analyze the difference in consumption

patterns caused due to the lockdowns.

4.2 Materials and Methods
4.2.1 Study locations and wastewater sampling methods

Influent from 10 sewer sheds in a southwestern U.S. city were collected in 24-h

time-weighted composites using automated samplers by WWTP personnel from January

2020 to July 2020. Cumulative population captured in all sewer sheds amounted to

approximately ~287,000 people. Sampling occurred on 7-10 days per month during the 7-

month study period; the weeks/days of collection varied and was entirely at the discretion

of sampling personnel. Sampled were processed immediately upon arrival at Arizona

State University.

4.2.1 Target analytes

Six parent opioids and 4 of their respective metabolites were monitored in raw

wastewater. The investigated opioids were codeine (COD), oxycodone (OXY), its major

metabolite noroxycodone (NOXY), heroin (HER), and its minor but exclusive metabolite

6-acetylmorphine (6-AM), methadone (MHT) and its exclusive metabolite 2-ethylidene-

1,5-dimethyl-3,3- diphenylpyrrolidine (EDDP), cocaine (COC) and its major metabolite

benzylecgonine (BCG), and mitragynine/kratom (KRT). High purity (>97%) standard

solutions of the target compounds were obtained from Sigma Aldrich (Milwaukee, WI)

and Cerilliant (Round Rock, TX, USA) as solutions in methanol or acetonitrile. Eight

deuterated compounds, one for each of the parent opioid and its metabolite compounds

were also purchased from Cerilliant for use as internal standards for quantification: codeine-d6 (COD-d6), oxycodone-d3 (OXY-d3), noroxycodone-d3 (NOXY-d3), heroin-d9 (HER-d9), 6-acetylmorphine-d6 (6AM-d6), methadone-d9 (MHT-d9), EDDP-d3, cocaine-d3 (COC-d3), benzylecgonine-d3 (BCG-d3), and mitragynine-d5 (KRT-d5)

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

Briefly, 100 mL of WWTP composite influent was loaded onto Oasis HLB 150 mg solid phase extraction (SPE) cartridges (Waters, Barcelona, Spain) at a rate of 1.5 mL/min using automated extraction with a Dionex Autotrace 280 (Sunnyvale, CA, USA). Prior to extraction, all composite influent samples were spiked with a mixture of the deuterated compounds at a concentration of 5 ng/mL for HER-d9, 6AM-d6, COD-d6, OXY-d3, NOXY-d6, MHT-d9, EDDP-d3, COC-d3, BCG-d3, and KRT-d5. Following sample loading, cartridges were washed with water at a rate of 5 mL/min for 5 min and dried under a stream of nitrogen gas for10 min. Drip-wise elution of analytes from the SPE 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), in series with 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 attained with a Symmetry C18 3.5mm by 6.4 mm by 75 mm analytical column 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 0.35 mL/min flow rate. Samples were

introduced into the mass spectrometer using an electrospray ionization probe operating in positive mode. Multiple reaction monitoring (MRM) was used for qualitative analyses.

4.2.3 Method Performance

Method detection limits (MDLs) for the various narcotic parent and metabolite compounds ranged between 0.2 to 10 ng/L (Table 9, Appendix C). Method detection limits (MDL) were determined following USGS procedures (Childress et al., 1999), Appendix B. 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 10 sample matrix spike experiments for the various analytes averaged 117% and relative standard deviation averaging 23% (Table 10, Appendix C). A detailed explanation of procedures to experimentally determine recovery rates is available in the Appendix C.

4.2.4 Calculation of opioid mass loadings

Opioid mass loadings were calculated from influent wastewater flow and corresponding concentration using equation 18.

$$Mass\ load\ \left(\frac{mg}{d}\right) = concentration\ \left(\frac{ng}{L}\right) * wastewater\ flow\ \left(\frac{L}{d}\right) * \frac{mg}{1,000,000\ ng} \quad (18)$$

$$Mass\ consumed\ \left(\frac{mg}{d*1000\ people}\right) = Mass\ load\ \left(\frac{mg}{d}\right) * \frac{1000}{Population} * C.F. \quad (19)$$

$$Doses\ consumed\ \left(\frac{doses}{d*1000\ people}\right) = Mass\ consumed\ \left(\frac{mg}{d*1000\ people}\right) * Dose\ \left(\frac{dose}{mg}\right)(20)$$

C.F. refers to the analyte correction factor. Wastewater epidemiological data was then compared to opioid consumption and excretion data obtained from peer-reviewed

literature to estimate the number of opioid users. The number of estimated opioid abusers were then compared to the national opioid use statistics.

Table 1- Parent drugs with their respective consumption indicators, excretion factors, correction factors, and average doses.

Drug	Consumption Indicator	Excretion Rate (%)	Correction factor	C.F. ref.	Average dose (mg)	Dose ref.
Codeine	Codeine	30	3.33	(PK et al., 2016)	30	(*Codeine (Oral Route) Proper Use - Mayo Clinic*, n.d.)
Oxycodone	Noroxycodone	22.1	4.58	(B et al., 2006)	10	(*Oxycodone (Oral Route) Proper Use - Mayo Clinic*, n.d.)
Cocaine	Benzylecgonine	39.1	2.55	(Castiglioni et al., 2013)	50	(RW et al., 2003)
Heroin	6-acetylmorphine	1.3	86.8	(C et al., 2011)	30	(*Methadone (Oral Route) Description and Brand Names - Mayo Clinic*, n.d.)
Methadone	EDDP	27.5	4.1	(PK et al., 2016)	30	(*Methadone (Oral Route) Description and Brand Names - Mayo Clinic*, n.d.)
Mitragynine	Mitragynine	70	1.42	(VK et al., 2014)	50	(EMCDDA, n.d.)

4.2.5 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), and R studio version 4.1.0. Normality of the datasets was determined through two analyses run in Microsoft excel; (1) an analysis of skewness and kurtosis z-values, and (2) the Shapiro-Wilk test for normality. A nonparametric Wilcoxon rank sum test was used for comparison of dose consumptions before and during the pandemic across all analytes.

4.3 Results and Discussion

4.3.1 Concentrations of narcotics and metabolites in raw wastewater

Concentrations in raw wastewater (ng/L) for all analytes of interest were screened for each sampling location seven to ten days per month from January 2020 to July 2020 (Fig. 7, 8, and 9) in a southwestern U.S. city. No analytes in raw wastewater were detected at 100% detection frequency across all locations and all dates. Detection frequencies (DFs) for all analytes fell in the range 77-88% sporadically throughout the sampling campaign. Cocaine and EDDP had the highest average DF (88%) across all months screened followed by benzoylecgonine (87%), oxycodone and codeine (86%), methadone (85%), 6-acetylmorphine and noroxycodone (80%), mitragynine (79%), and heroin (77%).

Sampling each month was sporadic and was primarily dictated by the city schedule and

uncertainties caused due to the pandemic. Seven days were sampled in January, February,
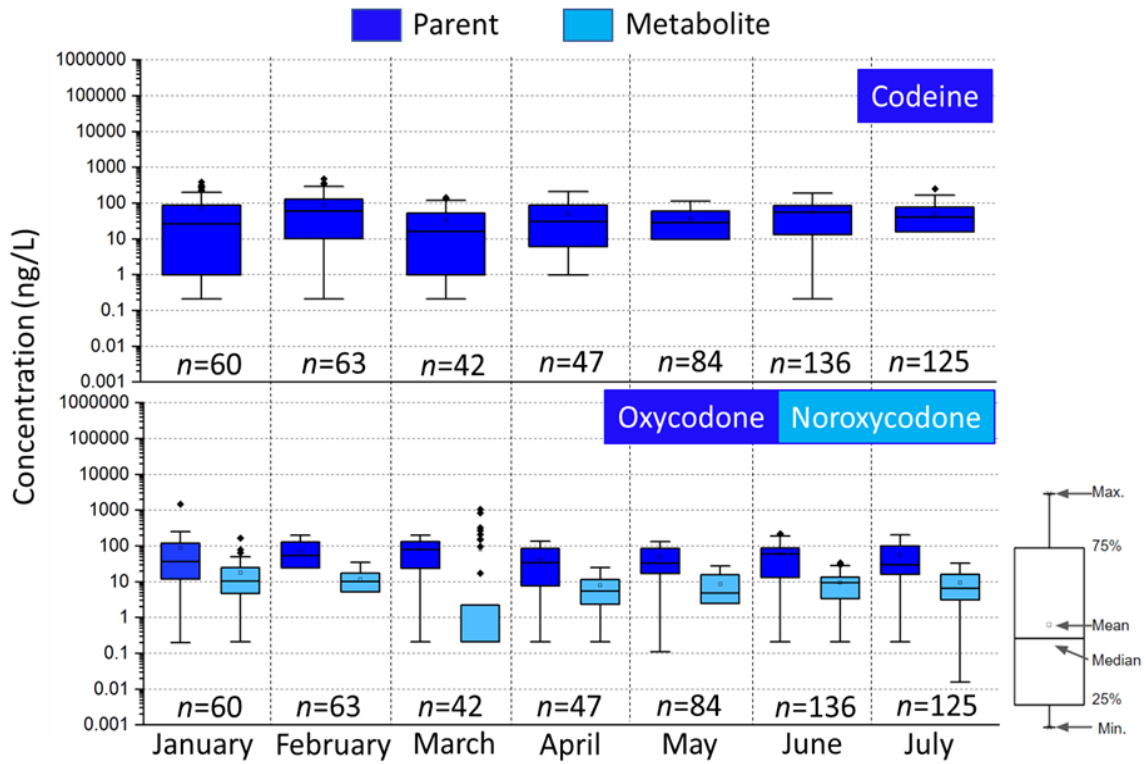
Fig.14 - Concentrations (ng/L) of common prescribed opioids, codeine, oxycodone
and its metabolite noroxycodone detected in wastewater collected from January 2020
to July 2020 sampling campaign.
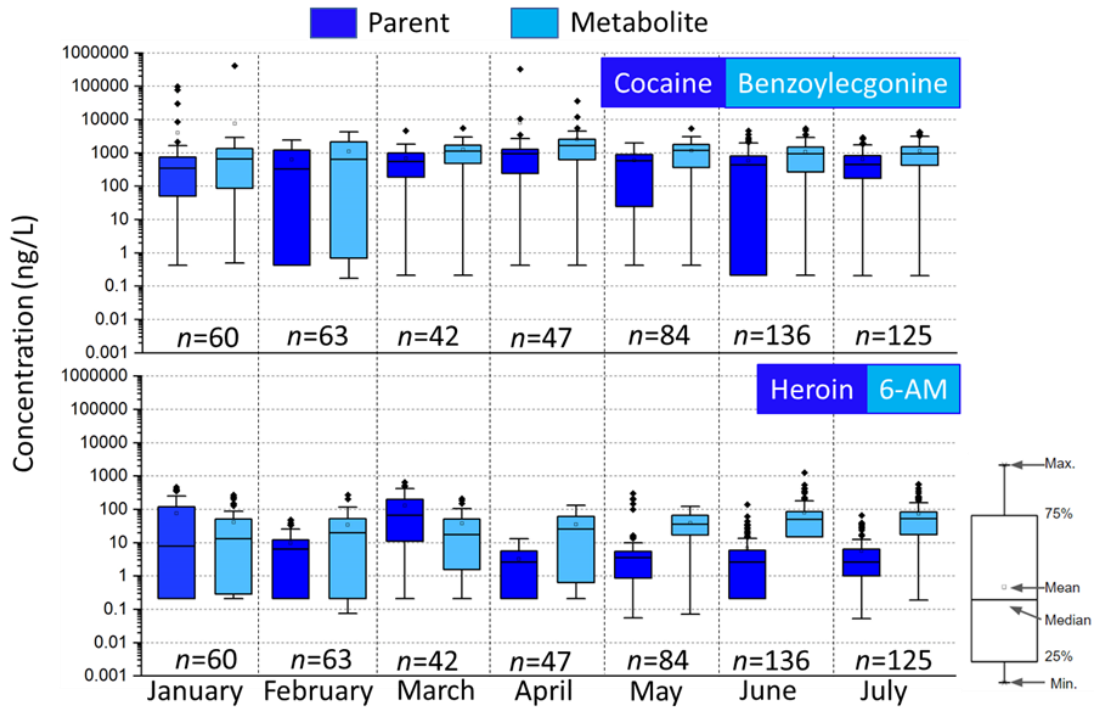
Fig. 17 - Concentrations (ng/L) of common illegal opioids, cocaine, heroin, their metabolites benzoylecgonine and 6-acetylmorphine respectively detected in wastewater collected from January 2020 to July 2020 sampling campaign.
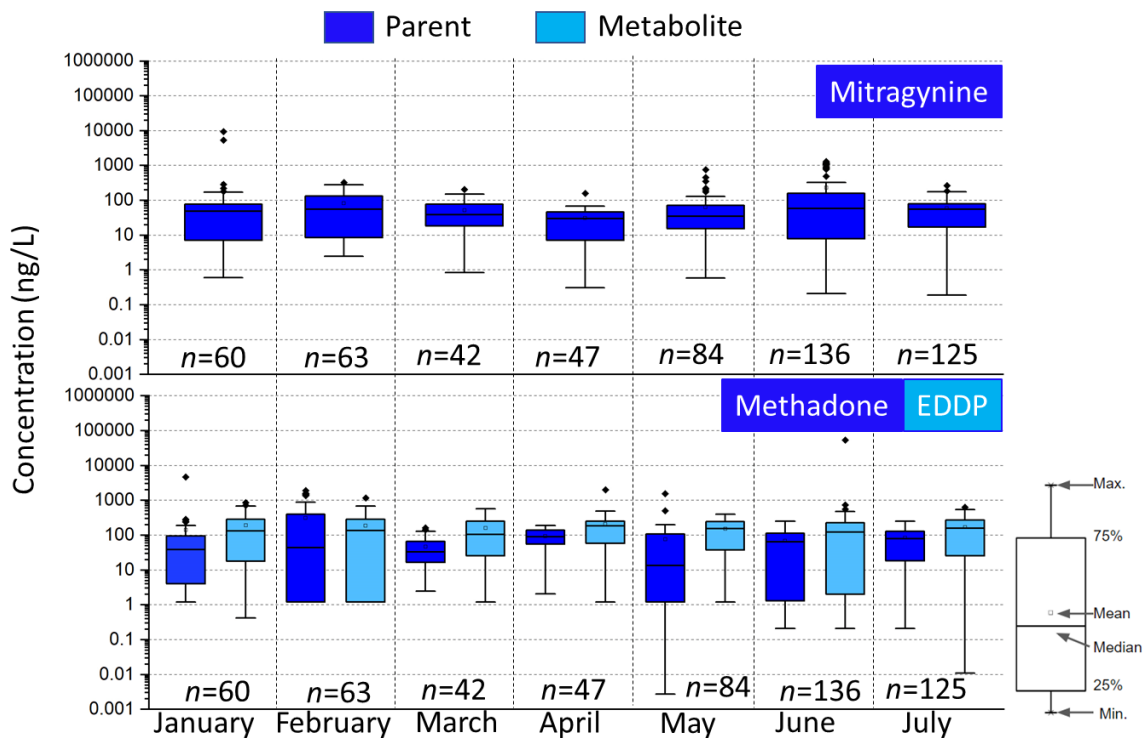
Fig. 20 - Concentrations (ng/L) of opioid medication, mitragynine, methadone, and its metabolite EDDP detected in wastewater collected from January 2020 to July 2020 sampling campaign.

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March, and April with sampling schedule increasing in the latter months with sampling

12 days in May, 20 days in June and 13 days in July. Benzoylecgonine concentrations in

raw wastewater were determined to be 4334 ± 3301 ng/L (before lockdown) and $1057 \pm$

57 ng/L (during lockdown), and cocaine concentrations in raw wastewater were

determined to be 2086 ± 1042 and 568 ± 38 ng/L before and during the lockdown

respectively. The parent to Metabolite ratio (PM ratio) of Cocaine to benzoylecgonine

peaked in the month of April at 538 and was the lowest in the month of June at 0.58 (Fig.

10). Since cocaine consumption yields parent/metabolite ratios in the range of 10-14

(Jones et al., 2008), current data can be attributed more towards higher consumption of

cocaine than dumping in all months but June. A large discrepancy was observed in heroin

concentrations before (75 ± 12) and during (6 ± 1) the lockdown, but 6-AM had opposite observations with 19 ± 3 and 52 ± 4 before and after the lockdown respectively. Since 6-AM could be contributed from consumption of morphine and codeine as well (Lötsch, 2005), their higher levels could not be solely attributed to the heroin consumption. Average concentrations in raw wastewater of codeine were determined to be 35 ± 4 ng/L (before) and 44 ± 2 ng/L (during); average concentrations in raw wastewater of oxycodone were determined to be 72 ± 12 ng/L (before) and 47 ± 3 ng/L (during); the codeine metabolite norcodeine concentrations were not analyzed during this sampling campaign; observed noroxycodone concentrations were determined to be 27 ± 9 ng/L (before) and 8.2 ± 0.4 ng/L (during). Oxycodone to noroxycodone ratios peaked in the month of March (288), February (34) and remained comparable in the rest months between 4-15. Opioid medications such as mitragynine concentrations before the lockdown were 171 ± 87 ng/L and decreased by ~25% during the lockdown period. Average concentrations in raw wastewater of methadone were determined to be 86 ± 38 ng/L (before) and 68 ± 6 ng/L (during); observed EDDP concentrations were determined to be 100 ± 10 ng/L (before) and 311 ± 183 ng/L (during). Most concentrations showed a significant decrease during the lockdown period and ranked as follows: heroin (1200%), benzoylecgonine (310%), cocaine (266%), noroxycodone (239%), oxycodone (52%), methadone and mitragynine (30%). Only codeine, 6AM, and EDDP increased in concentrations increased during the lockdown, by 20, 64, and 67% respectively.

4.3.2 Estimated opioid consumption

The first case of SARS-COV-2 in the study area was detected in the month of February followed by a lockdown in the latter half of March 2020. Normalized data by wastewater flow, population, and adjusting for the dosage, were analyzed to determine a monthly drug consumption baseline and changes during the pandemic in the units of doses/ day/1000 people (Fig. 10 and 11). Since the data was not normally distributed, it

was tested with nonparametric Wilcoxon rank sum test corrected with to check for

statistical differences in pre-lockdown and during lockdown narcotic

use. Illegal narcotics such as heroin and cocaine showed large variations before and

during the lockdown. Heroine consumption was 14 ± 3 doses/day/1000 people from

January-March 2020 and it increased ~10 times to 131 ± 41 doses/day/1000 people. As
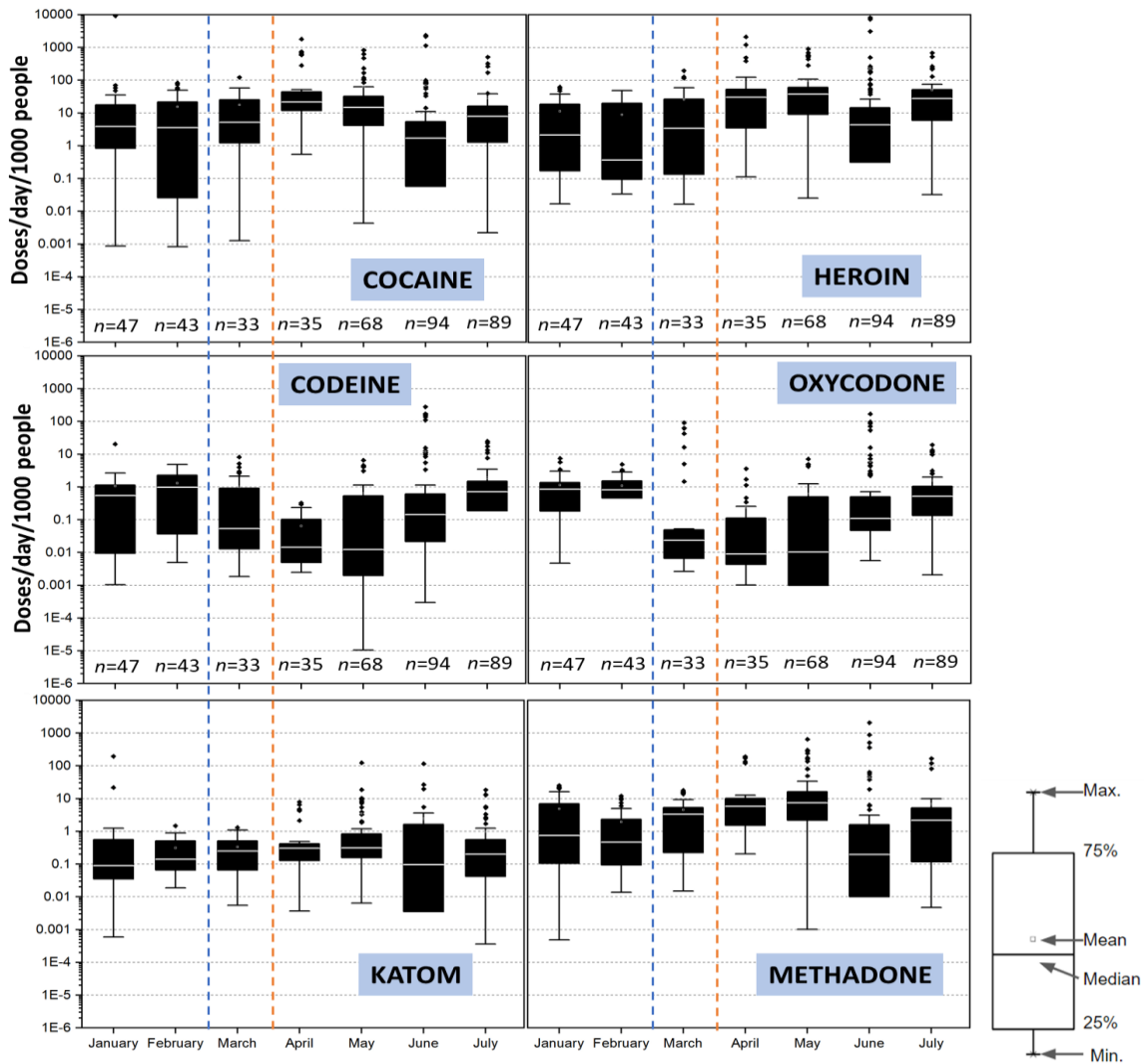
Fig. 23 - Dose consumption (doses/day/1000 people) of cocaine, heroin, codeine, oxycodone, mitragynine and methadone in wastewater collected from January 2020 to July 2020 sampling campaign. Blue vertical line denotes first detection of COVID-19 case and the orange line indicates beginning of the lockdown in the area sampled.

stated earlier, increased heroin dosage could be contributed due to morphine and codeine

consumption as well. While doses before the lockdown were similar to those observed on campus populations (15.8 ± 1.1) (Gushgari et al., 2018; Heuett et al., 2015), dose estimates during the pandemic were 3 times greater than those observed metropolitan cities across the US (38-43 doses/day/1000 people) (Baker & Kasprzyk-Hordern, 2013; Gushgari et al., 2019). Cocaine on the contrary decreased in dosage during the lockdowns from 89 ± 75 to 70 ± 15 doses/day/1000 people. Cocaine dose estimates in this study were several times higher than the campus populations (Gushgari et al., 2018; Heuett et al., 2015), but were comparable to other locations across the U.S. (30-180)(Baker & Kasprzyk-Hordern, 2013; Chiaia et al., 2008; Subedi & Kannan, 2014). Dose consumption varied significantly for heroin (p = 1.3E-08) but not for cocaine (p = 0.2944) between pre-lockdown and during lockdown usage. The average PM ratio for heroin-6AM decreased from 90 ± 16 to 11 ± 3 indicating an increased prevalence of 6AM compared to the heroin during the lockdowns.

Prescribed opioid oxycodone decreased significantly (p = 0.0003) from 3 ± 1 during the lockdown to 2.7 ± 0.8 doses/day/1000 people. Codeine on the other hand had significant increased consumption (p = 0.0448) and showed ~3 times greater doses/day/1000 people compared to the pre-lockdown period. It was hypothesized that an
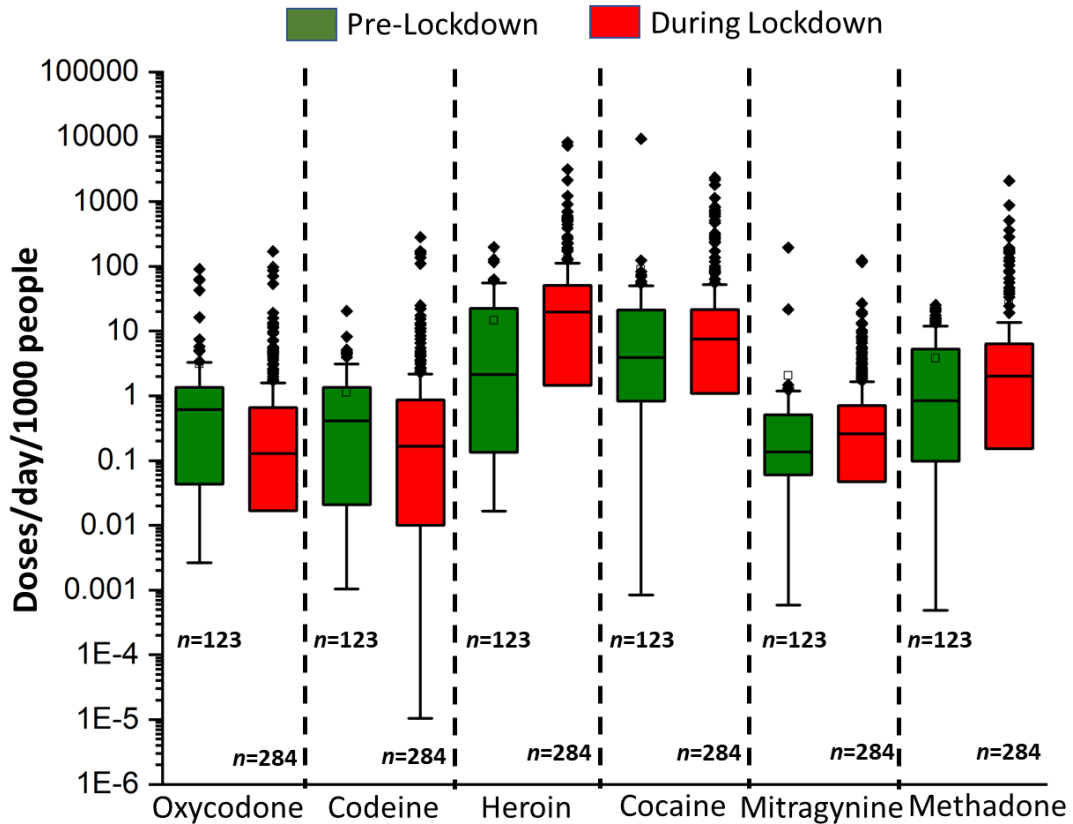
Fig. 25 - Box plots of doses per day per 1000 people consumed before pandemic (January-March 2020) in green and during lockdown (April-July 2020) in red for all parent drugs analyzed.

increased reliance on prescribed opioids would be observed due to the supply chain shortages that may be caused due to the pandemic and the lockdowns. Oxycodone concentrations in this study were an order of magnitude lower than the two midwestern cities (Gushgari et al., 2019) and comparable to the campus population (Gushgari et al., 2018). However, that was not observed with mitragynine and methadone. Mitragynine doses were determined to be 2.05 ± 1.58 before and 2.03 ± 0.61 doses/day/1000 people during the lockdowns. Average methadone doses increased from 3.7 ± 0.5 to 25 ± 8 doses/day/1000 people during the pandemic lockdowns. However, there was no statistical difference observed

between the two periods tested (p = 0.1987). That could be explained by a few outliers in the March-July 2020 dataset but comparable median values (Fig. 10). Methadone doses during the lockdown exceeded the previously set baselines in southwestern U.S. by an order of magnitude (Gushgari et al., 2018) but were comparable to the doses observed in other metropolitan cities (Baker & Kasprzyk-Hordern, 2013; Subedi & Kannan, 2014). Cocaine, heroin, and methadone were observed to increase in dose consumptions after the lockdown was in effect (see Fig. 10 panel COCAINE, HEROIN, and METHADONE). All their dose consumptions were observed to continue to increase in the month of May eventually decreasing by 2 orders of magnitude in the month of June and increased again in July. Levels observed in June for the above compounds were significantly lower than the pre-lockdown baseline levels measured. A possible explanation could be due to shortage of supply during the months of April and May trickling down into the month of June.

Prescription opioids like codeine and oxycodone showed order of magnitude fall in March 2020 compared to February 2020. The consumption levels showed a steady increase there after until July 2020 but failed to reach the pre-lockdown levels (see Fig. 10 panel CODEINE and OXYCODONE). Mitragynine consumption remained constant from January through April with no to slight variations. The consumption showed slight signs of increase in May and June eventually falling to original levels in July (see Fig. 10 panel KRATOM). A direct comparison for pre-lockdown to during lockdown consumption patterns can be seen in Figure 11.

Concentrations and subsequent dose consumption observed in this study are largely a function of community contributing, environmental conditions (wastewater

temperatures and matrix). Since this study sampled before lockdown and during lockdown periods, the possibility of change in contributing demographic could be significant. Lost employment, subsequent migration, decreased travel could all result in a shift in the concentrations studied. Moreover, pre-lockdown months (January, February, and March) are relatively cooler months compared to the during pandemic (April, May, June, July) especially in the southwestern U.S. Higher wastewater temperatures can negatively affect analyte concentrations as demonstrated in several stability studies on opioids(Baker & Kasprzyk-Hordern, 2011; C. Chen et al., 2013; Senta et al., 2014). Details on wastewater temperatures and demographic change was not available for the duration of this study and hence the data collected was not corrected for the mentioned factors.

4.4 Conclusions

No compounds measured in this study had 100% detection frequency across all months tested. Estimated consumption values before the lockdowns were comparable to other U.S. estimations but were predominantly higher in the lockdown period. These findings provide the first detection of mitragynine in wastewaters from a major U.S. city and an insight into drug consumption patterns under supply chain shortage scenarios. These results have demonstrated that implementation of WBE in a moderately sized city 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 population.

TRANSITION 4

Wastewater based epidemiology has been extensively used for exogenous compounds such as antibiotics, antibacterial, pharmaceuticals, and other emerging contaminants. However, measuring human endogenous chemicals could provide a deeper insight into human health, behavior, and be a robust indicator of human health. A subclass within the endogenous compounds are human stress hormones and metabolites, cortisone, cortisol, tetrahydrocortisol and tetrahydrocortisone. However, a major drawback of endogenous chemicals is their stability in the wastewater. Long in-sewer times, hot temperatures, and microbial activity can negatively affect the concentration of endogenous compounds and render them undetectable in wastewater. Lower ranges can lead to inaccurate interpretation of the data and can lead to inconclusive results unless the kinetics of decay are studied under different temperatures, time points and different sewer conditions and subsequent correction of the acquired data.

In chapter 4, the stability of cortisone, cortisol, tetrahydrocortisol and tetrahydrocortisone was examined at three temperatures (15, 25, and 35°C), two sewer types (oxic and anoxic), over 24 hours from two distinct locations. Wastewater samples were fortified with non-labeled standards, sampled every 2-3 hours, fortified with isotope labeled standards and analyzed using LC-MS/MS. Data collected was then normalized to the zero-time concentration and fitted to a first order decay model. Decay rate constants and half-lives were calculated to study the stability profiles of the stress hormones and metabolites.

CHAPTER 5

STABILITY OF THE HUMAN STRESS HORMONES AND HORMONE

METABOLITES IN WASTEWATER UNDER OXIC AND ANOXIC CONDITIONS

Abstract

Levels in wastewater of human stress biomarkers, such as cortisol (F), cortisone (E),
tetrahydrocortisol (THF), and tetrahydrocortisone (THE), may serve as indicators of
population wellbeing and overall health. This study aims to study these biosignature
compounds using wastewater-based epidemiology and to understand their stability in
wastewater. Wastewaters from two distinct locations were pre-spiked at 10 ppb, and the
decay of stress biomarkers in wastewater was studied at four temperatures (35, 25,15, and
6°C) over 24 hours in oxic anoxic conditions. The samples collected were analyzed on
liquid chromatography-tandem mass spectrometry (LC/MS-MS) using the isotope
dilution method. The results demonstrated that the decay of the biomarkers increased
with increasing temperatures. E, F, and THE degraded 100% at 35°C within 24 hours in
wastewaters from both locations, while detectable levels were observed at 25 and 15°C.
A non-parametric Wilcoxon test revealed no distinctive differences (p>0.05) between
oxic and anoxic decay rates in the period studied at all temperatures. In all the
temperatures studied, THF had the lowest decay rate observed. At 35 and 15°C, E had the
highest decay, while F decayed the most rapidly at 25°C.

5.1 Introduction

Wastewater-based epidemiology (WBE) has been gaining attention in recent years due to its wide range of applications such as monitoring public health, measuring population exposure to hazardous chemicals, estimating consumption of illicit drugs, and assessing human and ecological risk. Gradually, WBE applications are moving towards measuring endogenous chemicals produced as a result of human metabolism which could be used as early indicators of population behavioral changes. A common approach is to measure concentrations of biomarkers in different areas and to compare them by normalizing for flow rate and population served.

A major challenge in determining the concentrations and the endogenous chemicals is their stability in the sewer system. Several factors including but not limited to temperature, presence of oxygen, residence time, microbial activity (Thai et al., 2019b) etc. play a crucial role in influencing the in sewer stability. Previous studies evaluating stability of endogenous compounds in sewers concluded the instability of cortisol, androstenedione (Thai et al., 2019a), 5-hydroxy indole acetic acid (5-HIAA) (Thai et al., 2019b), and creatinine (Rico et al., 2017) in untreated wastewater.

One crucial class of endogenous compounds are the glucocorticoids, a class of natural steroid hormones commonly referred to as stress hormones with Cortisol (F) and Cortisone being one of the primary hormones. As shown in Figure 12, 11-α hydroxysteroid dehydrogenase type 2 (11α-HSD-2) is responsible for primarily converting cortisol to cortisone and 11α-HSD-1 for primarily converting cortisone to cortisol (Basu et al., 2004). Cortisone is also metabolized into tetrahydrocortisone using

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5β reductase during this process (Wassif & Ross, 2013). Cortisol on the other hand is metabolized to 5-alpha-Tetrahydrocortisol (5a-
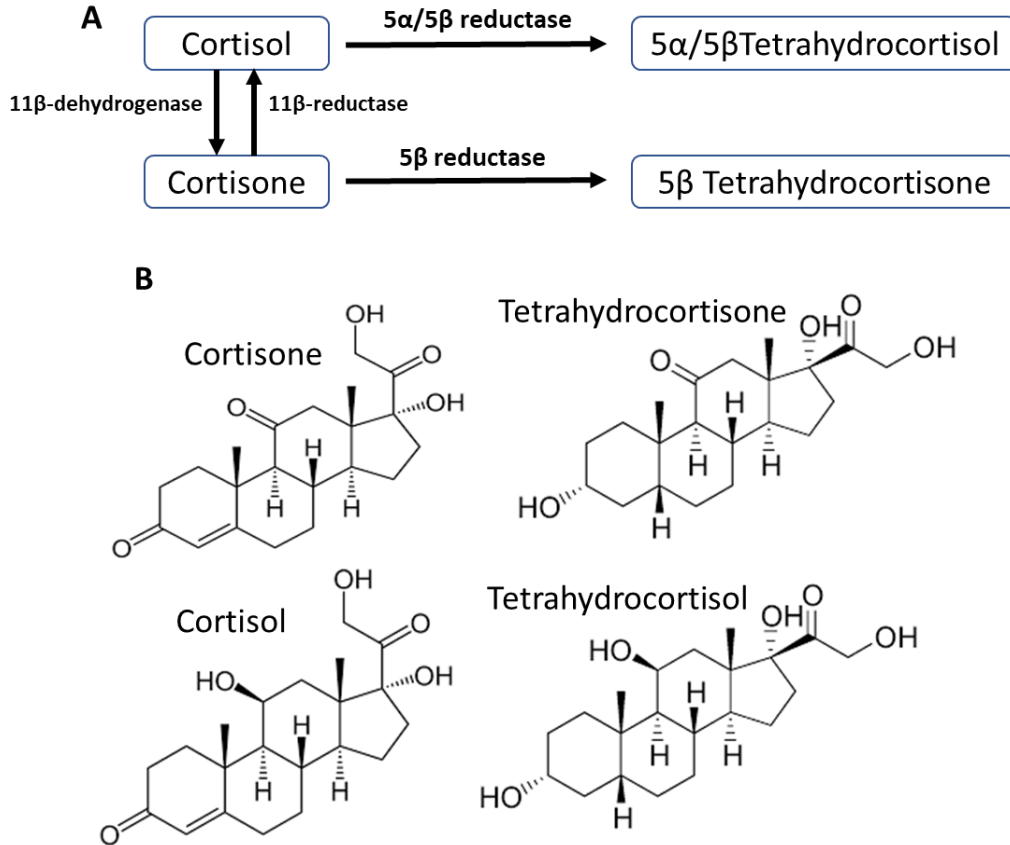
Fig. 28 - (A) Formation processes of THE, THF from E and F respectively. (B) Molecular structures of E, THE, F, and THF (clockwise) as adopted from PubMed.

THF) and 5-beta-Tetrahydrocortisol (5b-THF) using 5β reductase and 5α reductase respectively (Walker et al., 1997) (See Fig. 12).

It has been estimated that stress causes the U.S. 300 billion dollars every year (Brondolo et al., 2017). Moreover, stress is a major driving force in influencing the daily lives of individuals and has been directly connected to various health issues such as anxiety, depression (Chiba et al., 2012), heart ailments (Chandola et al., 2008), skin conditions (Alexopoulos & Chrousos, 2016), asthma (Marshall & Agarwal, 2000), and

arthritis (Huyser & Parker, 1998). Given the importance of stress and its implications, its accurate measurement and subsequent interpretation is essential. However, information on their stability in sewers, at high temperatures, in presence of biological agents is lacking. Moreover, sewer conditions can vary depending upon the region, demographics, design requirement. Sewer pump system and the sewer diameter can cause sewer design to vary greatly (US EPA, 2002). Smaller sewer diameter can often result in lack of oxygen availability due to complete filling of the pipes (Jelic et al., 2015) resulting in anoxic conditions (Thai et al., 2014).

In this study, we aim to observe the stability of two stress hormones, cortisol and cortisone and two of their metabolites, tetrahydrocortisone and tetrahydrocortisol in wastewater. We observed the stability under two scenarios, oxic and anoxic to account for the sewer design variability. Fresh wastewater from two distinct locations to account for matrix variability were selected as well.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

Analytical grade methanol and water were purchased from Birch Biotech (Pennsylvania, PA) and formic acid (≥98%) was purchased from Honeywell (Muskegon, MI). Cortisol (≥99%), cortisone (≥99%), cortisol-D4 (≥99%), and cortisone-D4 (≥99%) were purchased from Sigma Aldrich (Round Rock, TX). Tetrahydrocortisone (≥99%), tetrahydrocortisol (≥99%), tetrahydrocortisone-D5, and tetrahydrocortisol-D5 were purchased from TRC (Ontario, Canada).

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5.2.2 Instrumentation

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was achieved with a Shimadzu LC/MS-MS 8060 (Shimadzu Corporation, Japan). 10 µL of each sample was injected using an autosampler. An ARC-18 guard column 5 µm, 5 x 4.6 mm column maintained at 35 °C provided chromatographic separation. Mobile phase A was LC grade water and 0.1% acetic acid. Mobile phase B was 100% methanol. The mobile phase gradient was as follows; t = 0: 35% B, t = 0.25: 35% B, t = 3: 75% B, t = 3.01: 100% B, t = 4: 100% B, t = 4.01: 35% B, t = 6.5: 35% B, STOP. Flow rate was kept constant at 0.400 mL/min. Nebulizing gas flow was held at 3L/min, heating gas flow at 10 L/min, and drying gas flow was set at 10 L/min. Interface, desolvation, DL, and heat block temperature were set at 300°C, 526°C, 250°C, and 400°C respectively. Data acquisition was performed using the LabSolutions postrun and browser functions. Quantification was achieved using deuterated analogues (Table S1).

5.2.3 Experimental design of batch tests replicating sewer conditions

24-hour batch tests were conducted in duplicates to study the stability of stress hormones and metabolites at four different temperatures (4, 15, 25, and 35°C) under anoxic and oxic conditions in wastewaters from two distinct locations. All experiments were conducted in an incubator (Thermo Scientific MaxQ 6000, USA). To replicate the movement of the wastewater within the sewers, all vials were secured inside the incubator and shaken at 150 rpm for the entire duration of the experiment. Specific time points slightly varied for each condition, but all started at 0 h and then progressed in steps of 2-3 hours intervals finally ending at 24 h.

Experiments for each temperature point were performed on a separate day. Fresh wastewater was collected the day of the experiment (never frozen) and four aliquots (2 for anoxic and 2 for oxic) of 7 mL were prepared for each location. All aliquots were spiked with 10 ppb of cortisone, cortisol, tetrahydrocortisone, and tetrahydrocortisol mixture prior to the beginning of the experiment.

Oxic experiments were conducted in 50 mL beakers to provide adequate space at the top for oxygen exchange. The tops of oxic containers were covered with parafilm to allow transport of oxygen and maintain an aerobic atmosphere. Anoxic experiments were conducted in 10 mL batch bottles fitted with blue butyl rubber stopper and clamped down with aluminum seal after wastewater and 10 ppb spike mixture addition. Each batch bottle was sparged with nitrogen gas using a 25-gauge sterile needle.

5.2.4 Sampling

Wastewater samples were collected fresh using a 24-hour flow weighted composite sampler. Samples at appropriate time points were removed from the incubator, transported on a cart and placed in a water bath set at the temperature of interest inside a chemical hood. All samples were shaken adequately before sampling. For oxic samples, 200 µL of solution was pipetted using a plastic pipette whereas anoxic samples were inverted and sampled 200 µL using a calibrated glass syringe fitted with 25-gauge sterile needle. The solutions are transferred into a 1.5 ml plastic centrifuge tube and spiked with 1 ppb labeled standard mixture and vortexed until completely homogenous. The centrifuge tubes are centrifuged at 10,000 RPM for 10 minutes at 4 °C, removed carefully, placed on a rack inside the chemical hood. 100 µL of the supernatant is

carefully pipetted into 350 µL amber vials with inserts and mixed with 100 µL mixture of

methanol and 0.2% formic acid. The samples are vortexed and stored in -20°C until

further analysis.

5.2.5 Method Performance

Method detection limit (MDL) was determined following USGS procedures

(Childress et al., 1999) and USEPA guidelines. Method detection limits (MDLs) for

stress compounds were determined to be 115 ng/L for E, 1435 ng/L for F, 70 ng/L for

THE, and 8 ng/L for THF. Recoveries from 10 sample matrix spike experiments for the

various analytes ranged from 80-126% and relative standard deviation ranging 10-28%

(Table 15, Appendix D)

5.2.6 Statistical Analysis

Data obtained was not normally distributed hence a nonparametric Wilcoxon test

was conducted on the dataset using R studio version 4.1 at a 95% confidence interval.

Tests were performed to find significant differences between decay rates between two

locations and between two different conditions (oxic and anoxic) for all compounds of

interests (Table 2)

Table 2 - Wilcoxon p values tested for differences in locations and conditions for each temperature.

Temperature (°C)	Number of Samples	Wilcoxon p-value (Location)	Wilcoxon p-value (Oxic/Anoxic)
35	8	0.72	0.23
25	8	.0009	0.71

5.2.7 Data Analysis

All areas under the curve were exported from LabSolutions browser system to Microsoft Excel for further analysis. Peak areas from all time points were normalized using the peak area from zero hour time point. The resulting normalized values were natural log transformed and plotted against the respective time points (hours). Detailed first order decay plots for 25 and 35°C can be found in Appendix D (Figures 50-57).

For zero order plots, a plot of concentration (µg/L) versus the time (hour) were plotted and rates resulting from a linear trendline are summarized in Table 20 (Appendix D). Also, zero order plots with respective R^2 values and decay rates can be found in Appendix D (Figures 42-49).

5.3 Results and Discussion

5.3.1 Half-life analysis

Four stress biomarkers' stability was studied at four temperatures. Since no change was observed at 15°C, its decay rates are denoted as 0 in the Figures 13-17. All decay curves presented in this section are assumed pseudo-first order kinetics and fitted to the following equation. Previous publications have utilized a similar approach in modeling the stability of WBE biomarkers (Jiaying Li et al., 2021; Senta et al., 2014)

$$C = C_0 * e^{-kt} \tag{1}$$

Where C_0 is the initial concentration, k is the decay rate constant, t is the exposure time, and C is the concentration at time t.

Subsequently, half-life ($t_{0.5}$) was calculated using the equation 2 and individual values are listed in Table 3.

$$t_{0.5} = \frac{\ln 2}{k} \tag{2}$$

Table 3 - Half-lives (hours) of all stress biomarkers for both locations, both conditions, and every temperature point.

	Location 1		Location 2		
	Anoxic	Oxic	Anoxic	Oxic	
E	3.00	2.77	2.31	3.30	35°C
F	3.01	4.08	4.08	3.85	
THE	3.47	4.08	3.01	2.77	
THF	4.33	5.33	3.65	3.85	
E	11.75	7.97	23.10	17.33	25°C
F	8.15	8.66	22.36	23.10	
THE	7.45	6.93	173.29	34.66	
THF	17.33	13.86	34.66	34.66	

5.3.2 Effect of temperature on decay rates

Declining concentrations of stress parent hormones and metabolites are shown in figures 13-16. Parent stress hormones, E and F decayed at (mean ± standard deviation) 0.24 ± 0.03 hr^{-1} and 0.18 ± 0.03 hr^{-1} respectively at 35°C in untreated wastewater. Their R^2 values ranged from 0.56-0.91. At 25°C, the decay rates dropped by 300-400% to 0.05

± 0.02 hr^{-1} for both parent compounds. R^2 values ranged from 0.70-0.91 were determined

to be 0.5 to 0.93 across all sewer conditions and locations. At 35°C, the average half-lives

for E and F for oxic and anoxic at both were determined to be between 3-4 hours (see

Table 3). At 25°C, half-life increased 5 fold to 15 ±6 for E and F. Decay rates and half-

lives varied slightly between wastewater collected from two different locations at 35 and

25°C however temperature proved to be the most significant environmental variable

contributing to the degradation.
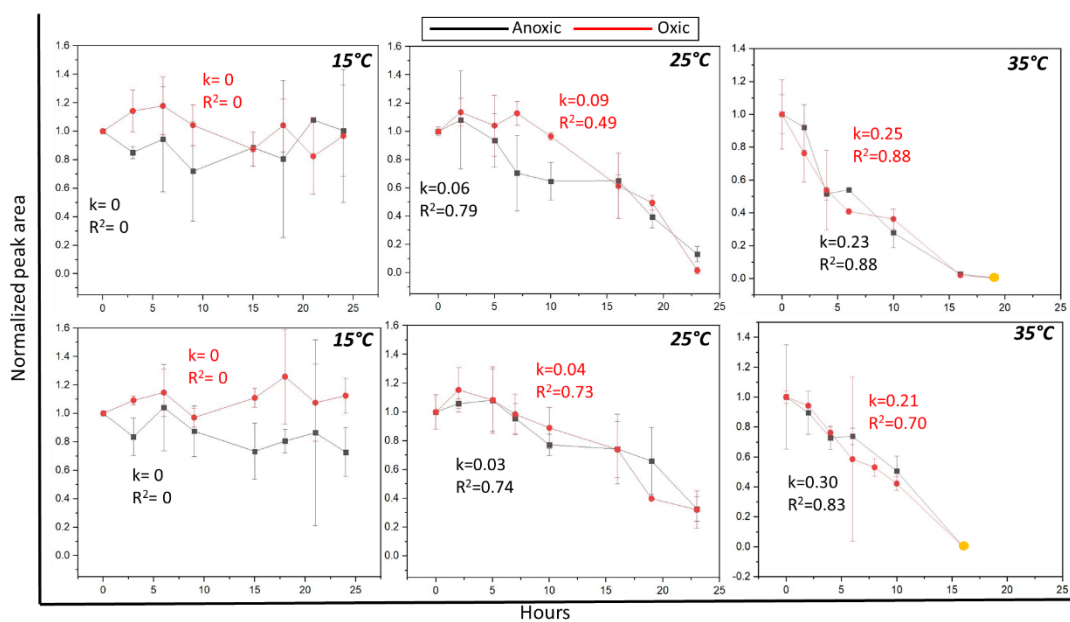
Fig. 30 - Stability profiles of cortisone (E) studied over 24-hour time period with the first order decay rates and the corresponding R^2 values. Error bars represent standard deviation of two measurements. Top and bottom rows represent location 1 and 2 respectively. Yellow dots represent non-detects.

Stress hormone metabolites THE and THF decay patterns are shown in Figures 15

and 16 respectively. THE and THF decayed at (mean ± standard deviation) 0.21 ± 0.03

hr^{-1} and 0.17 ± 0.03 hr^{-1} respectively at 35°C in untreated wastewater (R^2 values ranged

from 0.65-0.94). At 25°C, the decay rates dropped by 390% for THE to 0.05 hr^{-1} and

500% for THF to 0.02 for location 1 wastewater (R^2 values ranged from 0.78-0.97). THE

was observed to degrade less than 20% for the first 10 hours in all samples at 35°C and followed a sharp decline thereafter. Slow degradation was coupled with an increased concentration in the first 5 hours followed by a decay pattern similar to other observations. This increase in concentration could be due to formation of THE from E but the finding could not be validated due to absence of consistent evidence at other temperatures. At 25°C, less than 20% degradation was observed in the first 10 hours at 25°C and then a linear decline can be observed until the 24-hour mark for all compounds in location 1 wastewater. However, all compounds had 40-80% residual concentration remaining in location 2 wastewater samples at the 24-hour mark. Wilcoxon test to determine decay rate differences at 25°C between location 1 and 2 resulted in p value 0.04 ($\alpha=0.05$) indicating a significant difference. Overall, all compounds indicated a 70-80% fall in the degradation rate from 35 to 25 °C. E and F underwent an average 5-6-fold increase in the half-life whereas THE and THF half-lives increased by 17 and 6 times respectively.

As the temperature decreased to 15°C, an even more stable behavior was exhibited by all compounds. No visible degradation was observed at 15°C and hence the decay rates could not be determined.

5.3.3 Effects of oxygen availability on the decay rates
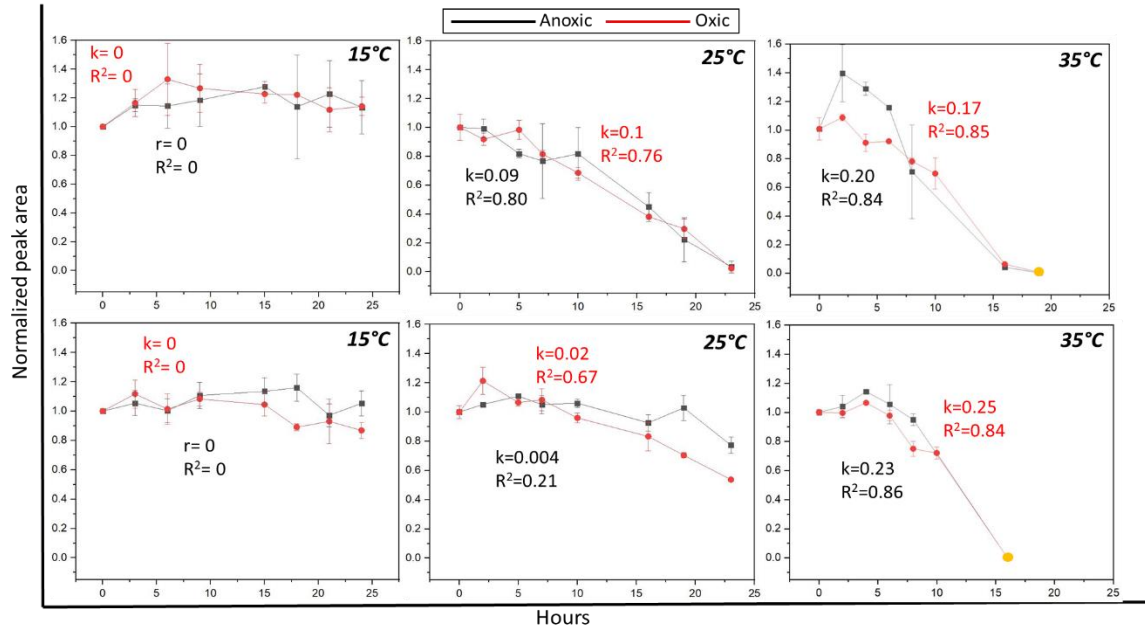
Fig. 34 - Stability profiles of tetrahydrocortisone (THE) studied over 24-hour time period with the first order decay rates and the corresponding R^2 values. Error bars represent standard deviation of two measurements. Top and bottom rows represent location 1 and 2 respectively. Yellow dots represent non-detects.
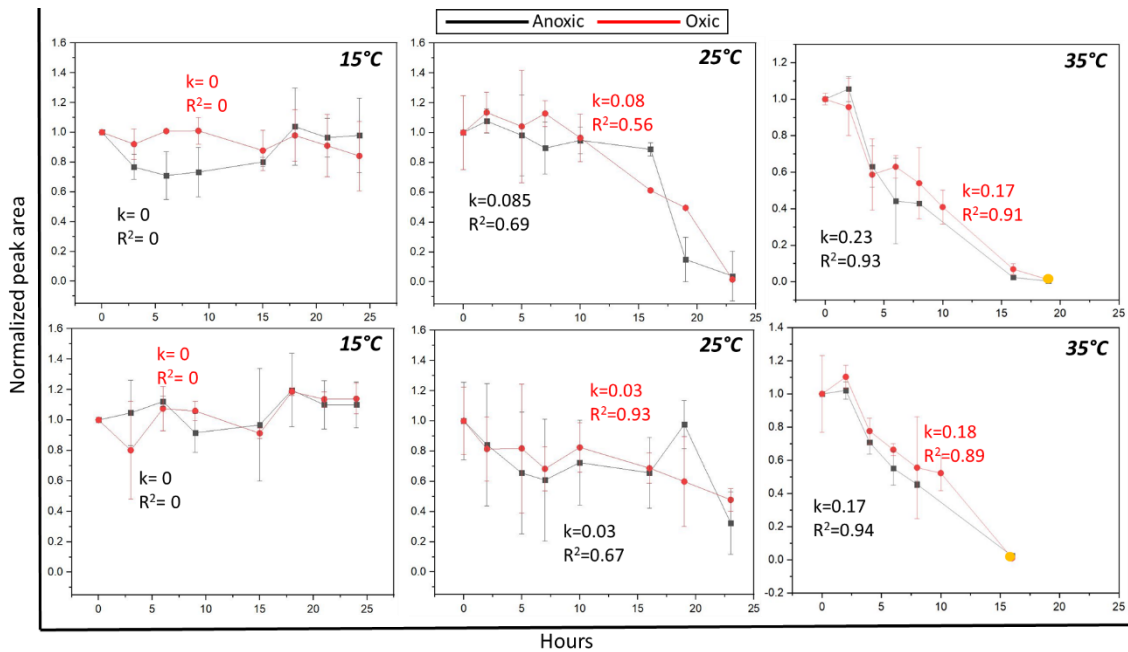
Fig. 32 - Stability profiles of cortisol (F) studied over 24-hour time period with the first order decay rates and the corresponding R^2 values. Error bars represent standard deviation of two measurements. Top and bottom rows represent location 1 and 2 respectively. Yellow dots represent non-detects.

94
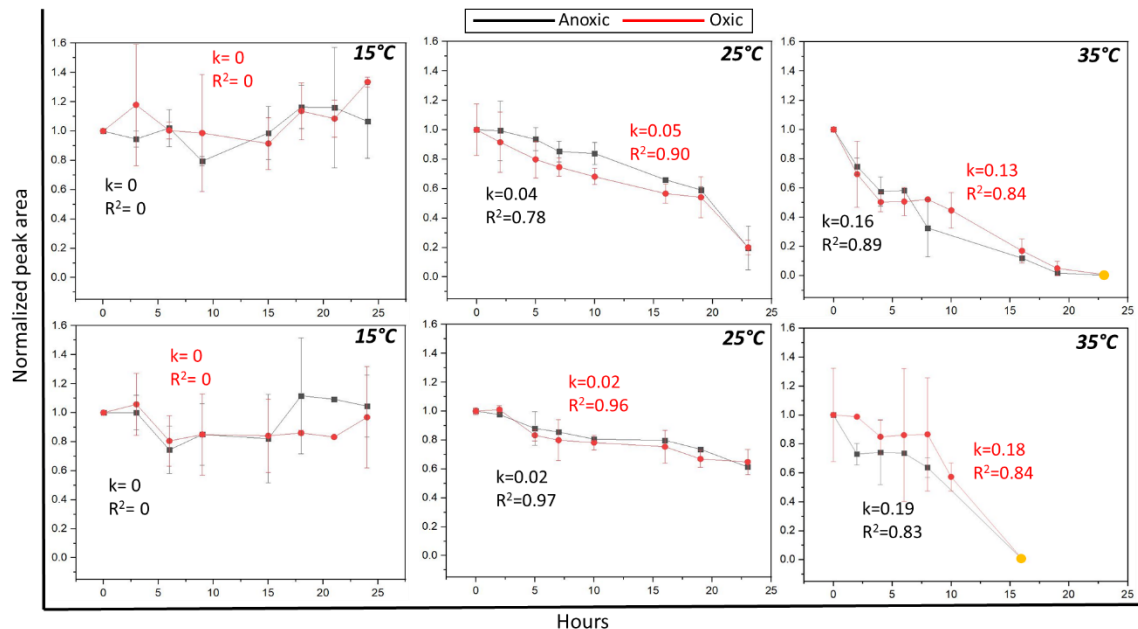
Fig. 37 - Stability profiles of tetrahydrocortisol (THF) studied over 24-hour time period with the first order decay rates and the corresponding R^2 values. Error bars represent standard deviation of two measurements. Top and bottom rows represent location 1 and 2 respectively. Yellow dots represent non-detects.

For the two oxygen conditions studied, no observable differences in patterns of degradation were observed. Relative percent differences (RPD) between anoxic and oxic decay rates for both locations at 35°C ranged between 0 and 28% with average RPD at 15%. RPD increased considerably at 25°C with mean RPD at 28%. Wilcoxon nonparametric analysis with the α value set at 0.05 yielded no significant differences at all temperatures and both locations (p-value: 0.71). Moreover, the trends displayed for oxic, and anoxic conditions are identical as shown in Figures 13-16. Data from this study was in accordance with the biotransformation results of cocaine, methyl ester, and benzoylecgonine where no significant difference was observed between anaerobic and aerobic decay rates (Plósz et al., 2013). However, a contrasting result demonstrating

higher decay rate for cocaine under aerobic scenario as compared to anaerobic was observed (Thai et al., 2014).

5.3.4 Zero order decay fit

Results obtained in this study were fitted to two kinetic models, zero-order and first-order decay. In sections 5.3.2 and 5.3.3 the decay rates for first-order are discussed in greater detail. A close match for zero-order fits was also observed for all the compounds studied. Since meaningful prediction of zero-order kinetics requires the modeling practitioner to have knowledge about the absolute concentration of the compound present (in order to know when they become nondetectable and to avoid prediction of negative concentrations), the use of zero-order kinetic models in environmental modeling is hampered and discouraged. For that reason, a first-order decay model was adopted throughout to the dataset in the current study, even if experimental data on occasion could be fitted successfully to zero-order kinetics, sometimes with results being superior to the first-order fit. Table 20 (Appendix D) summarizes zero-order decay rates for all compounds studied at 25 and 35°C. Also, zero-order plots with their respective R^2 values and decay rates can be found in Appendix D (Figures 42-49).

5.4 Discussion

This study provides a strong empirical dataset for researchers measuring stress biomarkers across the world. Parent hormones and their respective metabolites demonstrated identical half-lives at 35°C but displayed significantly different half-lives at 25 and 15°C, respectively. Degradation issues stability issues occurred immediately for

in-sewer residence times up to or even longer than 5 h at 35°C for E and F as contrary to THE and THF where a greater resilience was displayed for as long as 10 hours followed by a quick degradation onwards. At 15 and 25°C, all compounds showed a very stable behavior (<20% decay) at least for the first 10 hours.

For the sake of determining decay rates over a 24-hour period (and to avoid premature loss of detectable signal), samples for this study were fortified to a final concentration of approximately 10 µg/L of stress biomarkers in wastewater. Naturally occurring concentrations of stress hormones and metabolites in raw wastewater in units of µg/L were the highest for THF (0.81 ± 0.88) followed by THE (0.63 ± 0.58), F (0.26 ± 0.10), and E (non-detectable). Hence, concentrations in raw wastewater of the stress hormone metabolites THF and THE were 3-4 times higher then those of the parent compounds, which renders them more likely to be detectable even if they were to display decay rates and half-lives similar to the parental compounds. Method detection limits for all four compounds (0.008-1.4 µg/L) were determined using the EPA guidelines described in 40 CFR 136 (USEPA, 2017). The decay kinetics determined here at 25°C suggest that all biomarkers studied here require at least about ~15 hours to degrade by 30% from their initial concentration. At 35°C, it was calculated that 2.5-5 hours of in-sewer residence time have to pass to observe a similar loss in biomarker concentration. Hence, at temperatures of 25 and 35°C it is advisable to take into consideration and normalize for biomarker loss after 15 hours and 5 hours, respectively, of average in-sewer residence time or wastewater age in the pipe.

As reported by Hart and Halden, 2020, 75% of the world's wastewater temperatures lie between 7 and 35°C (Hart & Halden, 2020). In equatorial areas, the

Middle East, and southern Asia where temperatures range from 25-35°C throughout the year, shorter durations of in-sewer transport should be be maintained by an appropriately designed sampling network and sampling location spacing in order to avoid for concentrations to drop below detection levels. In cases where international sample transport might be involved, extra care must be taken to keep the temperatures of samples lower than 15°C during shipping to preserve biomarker levels at levels sufficient for detection in wastewater.

CHAPTER 6

RESEARCH IMPLICATIONS AND RECOMMENDATIONS

This work has shown that chemical data available from environmental matrices can prove to be a unique identifier for public and environmental health providing insight into health or related trends for a specific community but is still subject to many uncertainties and variants in data analysis. Important findings documented in this thesis are as follows:

1. Implementing WBE in the developing world presents unique challenges linked to the absence of centralized wastewater collection and treatment infrastructure.

2. Banning controversial chemicals from commercial products may be problematic if data are lacking on the safety of replacement chemistry that likely will be dropped in in order to meet consumer demand.

3. Monitoring effects of the COVID-19 pandemic on distribution, availability and consumption of illicit opioids, prescribed opioids, and opioid medication in a southwestern U.S. city applying wastewater-based epidemiology (WBE).

4. Evaluating stability of stress hormones and its metabolites in raw wastewater at common global wastewater temperatures and sewer conditions over 24-hour duration to aid interpretation of WBE results.

Concentrations of pharmaceuticals and personal care products such as antibiotics, antimicrobials, non-steroidal anti-inflammatory drugs etc. also commonly categorized as contaminants of emerging concern (CEC), occurring in the Indian environmental matrices were inventoried in Chapter 2. Over 75% of India's wastewater goes untreated and results in aquatic and terrestrial contamination. Amongst the bacterial load are also present CECs that are heavily consumed by the Indian population mostly due to lack of

restrictions and affordable costs. Moreover, lack of sanitation causes the public to resort to open defecation contributing a total of 17 ± 12.7 mt of CEC mass load daily. Spatial analysis revealed regions with heavy aquatic resources and natural reserves having little to no treatment infrastructure. This study is the first to expose the vulnerable regions of the densely populated country, India, and an estimate of the untreated CEC load via different blackwater routes for every geographical region.

Concentrations of five parabens, triclosan, and triclocarban were screened in 24-hour composite influent and effluent samples collected from 14 wastewater treatment plants across 12 states in the U.S. Despite of high removal efficiencies (>95%) observed for all compounds measured, triclosan and triclocarban had high mass loads (52 ± 70 µg/d/capita) posing a considerable risk to the biota studied here. Benzalkonium chloride, benzethonium chloride, chlorhexidine, chloroxylenol replaced triclosan and triclocarban for their antimicrobial properties post 2017. However, their toxicology research data largely lacking. This issue is particularly concerning due to the exacerbated use of antimicrobial products during the COVID-19 pandemic. A modeling analysis in this study revealed chlorhexidine posing 30-2000 times more toxicity to the biota studied here while benzalkonium chloride being a relatively safer choice.

Raw wastewater composited over 24-h from moderately sized southwestern city in Chapter 4. Sampling campaign covered 10 separate locations for the pre COVID-19 lockdown months (January-March 2020) and during lockdown period (April-July 2020). Consumption estimates within the city was for the most part similar compared to other parts of the U.S. for the pre-lockdown months observed but changed significantly for heroin (1.3E-08, increased 10-fold), oxycodone (0.0003, decreased 5-fold), codeine

(0.0448, decreased 2.5-fold) during the lockdown. Consumption of other analytes remained statistically unaltered dur the effects of the lockdown.

In Chapter 5, the WBE approach was applied to assess stability of stress hormones and their metabolites replicating three commonly observed wastewater temperatures (35, 25, and 15°C) and two common sewer conditions (oxic and anoxic) to wastewaters collected from two distinct locations. Wastewater samples were collected every 2-3 hours until 24 hours and data obtained was fitted to a first order decay model. Decay rates were similar for parent and respective metabolites studied. Cortisone and tetrahydrocortisone decayed at a greater rate (0.29 ± 0.03 hr^{-1}) compared to cortisol and tetrahydrocortisol (0.2 ± 0.03 hr^{-1}) at 35°C. However, the rate was similar across all compounds at 25 and 15°C. To summarize, researching stress biomarkers in hot environments would require greater precautions to keep the sample temperatures under 15°C over the entire duration of transport to have detectable levels during analysis.

6.1 Recommendations for future research

Several communities across the world are at risk of infectious diseases and other calamities due to lack of access to sanitation, clean drinking water, and exposure to chemicals of concern via different exposure routes (dermal, inhalation, ingestion). Many such countries often have high population density and sparse wastewater treatment infrastructure. Such places could be breeding grounds for novel disease outbreaks and often remain neglected by the epidemiology community. As a collective effort towards human advancement, an increased monitoring of mentioned regions needs to be

undertaken. This would give researchers an early diagnosis of population health and help prevent COVID-19 pandemic-like scenarios in the future.

As a modern society, we rely on and are exposed to a variety of different chemicals via personal care products, medicines, drinking water, packaging and recreational purposes. Several of these chemicals have questionable toxicology backgrounds and unknown long term health effects. Although with evolving research, hazardous substances are prohibited from further use in consumer goods, newer chemicals with similar or worse toxicological data are used to replace them. A recommendation from a sustainable point of view would be to develop greener, readily biodegradable chemicals to reduce the chemical burden on the ecosystem and potential human health hazards.

Application of WBE has been limited to monitoring community levels of drug consumptions and chemical exposure. However, that needs to be expanded to understand population health trends, habits, indicators of mental health to interpret overall population well-being. Moreover, in regions such as Arizona where temperatures can often reach and stay upwards of 110°F, a stability study of all the community well-being biomarkers is necessary. Hot temperatures and long duration in sewers could result in undetectable levels of crucial biomarkers as demonstrated in chapter 5. Since usage of degraded concentrations could lead to underestimation and misinterpretation of public health data, a reliable decay rate is necessary to correct for the lost concentrations and interpret the data accurately. This dissertation has shown that trends of analyte concentrations in raw wastewater can be identified through wastewater analysis and emphasized on the

importance of stability tests in conjunction with currently viable methods of public health

data collection across the United States.

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APPENDIX A

[TITLE OF APPENDIX IN ALL CAPS]

Table 4 - Tabulated wastewater treatment plant meta-data abstracted from the published literature.

Sr. No.	Capacity (MLD)	Population served	Liters treated/person/day	Reference
1	3.5	13000	269.23	(Prabhasankar et al., 2016)
2	2	9000	222.22	
3	0.05	200	250.00	
4	14.2	150000	94.67	(Subedi et al., 2017)
5	81.7	450000	181.56	
6	45	350000	128.57	(Subedi et al., 2015)
7	35	275000	127.27	
8	50	350000	142.86	
9	2	10000	200.00	
10	2	12000	166.67	
11	571	4500000	126.89	(Anumol et al., 2016b)
12	636	4003136	158.88	(Saxena et al., 2021)*
13	45	283241	158.88	
14	0.3	1888	158.88	
15	4.44	27946	158.88	(Gani et al., 2016)*
16	130	818251	158.88	
17	17	107002	158.88	
18	181.6	1143034	158.88	
19	27	169944	158.88	
20	4.54	28576	158.88	
21	136.4	858534	158.88	
22	20	125885	158.88	
23	30	188827	158.88	
24	29	182533	158.88	
25	50	314712	158.88	

26	38	239181	158.88	
27	100	629424	158.88	
28	6	37765	158.88	
29	10	62942	158.88	
30	45	350000	128.57	(Rajendiran Karthikraj & Kannan, 2017) (R. Karthikraj et al., 2017)
31	35	275000	127.27	
32	50	350000	142.86	
33	2	10000	200.00	
34	2	12000	166.67	
35	18	114696	158.88	(Saini et al., 2016)*
36	42	267624	158.88	(Mohapatra et al., 2016)*
37	60	382320	158.88	
38	14.2	500000	26.63	(Archana et al., 2016)
39	135	860220	158.88	(Mutiyar & Mittal, 2014a)*
40	636	4052592	158.88	(Mutiyar & Mittal, 2014b)*
41	2	12000	166.67	(Praveenkumarreddy et al., 2021)
42	12	150000	80.00	(Thalla & Vannarath, 2020)
43	43.5	450000	96.67	

*Not available in the literature hence assumed an average value from available literature values.

Table 5 - Estimation of the population size served or not by centralized wastewater treatment in every Indian state and union territory derived from data published by the Indian Central Pollution Control Board, 2015.

Regions	State/ UT	Urban population	Rural population	Population (2011)	Operational Capacity (MLD)	Under Construction Capacity (MLD)	Proposed Capacity (MLD)	Estimate of People served	Estimate of People Unserved
North	Chandigarh	1.03E+06	2.90E+04	1.06E+06	314.5	0	0	1.98E+06	0.00E+00
	Delhi	1.64E+07	4.20E+05	1.68E+07	2671.2		0	1.68E+07	0.00E+00
	Haryana	8.84E+06	1.65E+07	2.54E+07	805	45	0	5.07E+06	2.03E+07
	Himachal Pradesh	6.89E+05	6.18E+06	6.86E+06	79.51	0	0	5.00E+05	6.36E+06
	Jammu & Kashmir	3.43E+06	9.11E+06	1.25E+07	145.74	117	0	9.17E+05	1.16E+07
	Punjab	1.04E+07	1.73E+07	2.77E+07	921.45	276.7	32.1	5.80E+06	2.19E+07
	Uttar Pradesh	4.45E+07	1.55E+08	2.00E+08	2372.25	170	15	1.49E+07	1.85E+08
	Uttarakhand	3.14E+06	6.95E+06	1.01E+07	90.75	39.15	23	5.71E+05	9.53E+06

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West	Goa	9.07E+05	5.52E+05	1.46E+06	34.5	40.08	0	2.17E+05	1.24E+06
	Gujarat	2.57E+07	3.47E+07	6.04E+07	2111.64	359.5	93.78	1.33E+07	4.71E+07
	Maharashtra	5.08E+07	6.16E+07	1.12E+08	4683.9	131.96	0	2.95E+07	8.25E+07
	Rajasthan	1.71E+07	5.14E+07	6.85E+07	384.5	149.3	332.12	2.42E+06	6.61E+07
	Daman Diu & Dadra Nagar Haveli	1.82E+05	6.08E+04	2.43E+05	0	0	0	0.00E+00	2.43E+05
South	Andhra Pradesh	1.46E+07	3.48E+07	4.94E+07	156.27	91	0	9.84E+05	4.84E+07
	Andaman & Nicobar Islands	1.43E+05	2.37E+05	3.81E+05	0	0	0	0.00E+00	3.81E+05
	Karnataka	2.36E+07	3.75E+07	6.11E+07	1112.05	192.11	0	7.00E+06	5.41E+07
	Kerala	1.59E+07	1.75E+07	3.34E+07	112.87	37.1	0	7.10E+05	3.27E+07
	Lakshadweep	5.03E+04	1.41E+04	6.45E+04	0	0	0	0.00E+00	6.45E+04
	Puducherry	8.53E+05	3.95E+05	1.25E+06	17.5	51	0	1.10E+05	1.14E+06

	Tamil Nadu	3.49E+07	3.72E+07	7.21E+07	1140.83	521.08	132.64	7.18E+06	6.49E+07
	Telangana	1.37E+07	2.16E+07	3.53E+07	634.8	51	0	4.00E+06	3.13E+07
East	Bihar	1.18E+07	9.23E+07	1.04E+08	99.55	0	0	6.27E+05	1.03E+08
	Jharkhand	7.92E+06	2.51E+07	3.30E+07	117.24	0	0	7.38E+05	3.23E+07
	Odisha	7.01E+06	3.50E+07	4.20E+07	158.04	227.5	0	9.95E+05	4.10E+07
	West Bengal	2.91E+07	6.22E+07	9.13E+07	235.36	0	0	1.48E+06	8.98E+07
NE	Arunachal Pradesh	3.18E+05	1.07E+06	1.38E+06	0	0	0	0.00E+00	1.38E+06
	Assam	4.37E+06	2.68E+07	3.12E+07	0.21	0	0	1.32E+03	3.12E+07
	Manipur	9.27E+05	1.93E+06	2.86E+06	0	0	0	0.00E+00	2.86E+06
	Meghalaya	5.95E+05	2.37E+06	2.97E+06	1	0	0	6.29E+03	2.96E+06
	Mizoram	5.72E+05	5.25E+05	1.10E+06	10	10	0	6.29E+04	1.04E+06
	Nagaland	5.71E+05	1.41E+06	1.98E+06	0	0	0	0.00E+00	1.98E+06

	Sikkim	1.54E+05	4.57E+05	6.11E+05	8	18.88	0	5.04E+04	5.61E+05
	Tripura	9.61E+05	2.71E+06	3.67E+06	0.045	0	0	2.83E+02	3.67E+06
Central	Chhattisgarh	5.94E+06	1.96E+07	2.55E+07	0	0	0	0.00E+00	2.55E+07
	Madhya Pradesh	2.01E+07	5.26E+07	7.26E+07	475.48	0	0	2.99E+06	6.96E+07

APPENDIX B

SUPPLEMENTAL MATERIAL FOR CHAPTER 3

Table 6 - Optimized conditions for the ionization and fragmentation of the opioid parent and metabolite analytes screened for in this method adopted from (Chen et al., 2019)

Analyte	RT (min)	MS/MS Transition [a]	DP (V)	EP (V)	CE (V)	CXP (V)	DW (ms)
MePB	5.3	151 > **92**, 136	-60	-10	-30	-5	50
$^{13}C_6$-MePB	5.3	157 > **98**	-60	-10	-30	-5	50
EtPB	5.9	165 > **92**, 136	-55	-10	-30	-15	50
d_5-EtPB	5.8	170 > **92**	-55	-10	-30	-15	50
PrPB	6.4	179 > **92**, 136	-55	-10	-30	-13	50
d_4-PrPB	6.4	183 > **96**	-55	-10	-30	-13	50
BuPB	6.9	193 > **92**, 136	61	10	37	14	50
d_4-BuPB	6.9	197 > **96**	-55	-10	-38	-1	50
BePB	6.8	227 > **92**, 136	-65	-10	-36	-1	50
TCS	8.2	287 > **35**	-50	-10	-30	-3	50
$^{13}C_{12}$-TCS	8.2	300 > **35**	-55	-10	-28	-3	50
TCC	8.0	313 > **160**, 126	-80	-10	-18	-9	50
$^{13}C_6$-TCC	8.0	319 > **160**	-50	-10	-20	-25	50

[a] Parent ion > **Quantification ion**, Confirmation ion. RT: Retention Time; DP: Declustering Potential; EP: Entrace Potential, CE: Collision Energy; CXP: Collision Cell Exit Potential; DW: Dwell Time

Table 7 - Effective concentration affecting 50% of the organisms (EC50) and lethal concentration for 50% of the organisms (LC50) in mg/l values for compounds and the respective species.

Chemicals	*Daphnia Magna*	Zebrafish Embroyo (*danio rerio*)	Green algae (P. subcapitata)	*C. elegans*	Rainbow trout (*Oncorhynchus mykiss*)
TCS	0.18(Tamura et al., 2013) (EC50)	0.51(Zhang et al., 2018)(LC50) 0.36 (EC50)	0.005(Tamura et al., 2013) (EC50)	3.65(Lenz et al., 2017)(LC50)	0.28(PubChem, 2006) (LC50)
TCC	0.013(PubChem, n.d.-a) (LC50)	0.30(Qimeng Shi, Yuhang Zhuang, Tingting Hu, 2019) (LC50)	0.029(Tamura et al., 2013) (EC50)	0.9(Lenz et al., 2017) (LC50)	0.12(PubChem, n.d.-a)(LC50)
BAC	0.041(Sreevidya et al., 2018)(EC50) 0.4 (LC50)	0.5(Sreevidya et al., 2018)(EC50)	0.041(Sreevidya et al., 2018)(EC50)	3.1(Sreevidya et al., 2018)(EC50)	1.05(Antunes et al., 2016)(LC50)
CXM	7.7(PubChem, n.d.-b)(EC50)	5(Sreevidya et al., 2018)(EC50)	NA	31.8(Sreevidya et al., 2018)(LC50)	0.76(PubChem, n.d.-b)(LC50)
CHX	0.25(PubChem, n.d.-c)(LC50)	1.4(Jesus et al., 2013)(EC50)	0.04(Jesus et al., 2013)(EC50)	NA	0.0013(PubChem, n.d.-c)(LC50)
MePB	25(Dobbins et al., 2009) (EC50)	0.065(Dambal et al., 2017) (EC50)	80000(Yamamoto et al., 2011b) (LC50)	0.278(Nagar et al., 2020) (EC50)	NA
EtPB	18.7(Dobbins et al., 2009) (EC50)	NA	52000(Yamamoto et al., 2011b) (LC50)	0.217(Nagar et al., 2020) (EC50)	NA
PrPB	12.3(Dobbins et al., 2009) (EC50)	NA	36000(Yamamoto et al., 2011b) (LC50)	0.169(Nagar et al., 2020) (EC50)	NA

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| BuPB | 5.3(Dobbins et al., 2009) (EC50) | NA | 9500(Yamamoto et al., 2011b) (LC50) | 0.131(Nagar et al., 2020) (EC50) | NA |
| BePB | 4(Dobbins et al., 2009) (EC50) | NA | 1200(Yamamoto et al., 2011b) (LC50) | NA | NA |

NA: data not available

Table 8 - Details of sampling locations, population served and treatment systems in each sampled wastewater treatment plant (WWTP).

WWTP	Avg. flow rate (MGD)	Population served	Percent solids (%)	Primary Treatment	Secondary Treatment	Advanced_Treatment	Sludge treatment 1	Dewatering	Sludge_disposal
CA1	260	35M	28.6	Primary Sedimentation	NA	NA	Anaerobic compost	Centrifuge	Land application; landfill; composting
IL	800	2.38M	25	Primary Sedimentation	Conventional Activated Sludge	Nitrification; Denitrification; Enhanced biological; phosphorous removal	Anaerobic Lagoons	Centrifuge	Land application
CA2	7	95K	55	Primary Sedimentation	Conventional Activated Sludge	Nitrification; Denitrification; Filtration	Anaerobic Lagoons	ess; filter press; ai	Landfill
CO	15	108K		Primary Sedimentation	Conventional Activated Sludge	Nitrification; Denitrification	Anaerobic	Centrifuge	Land application
ME	0.25	3K	4	Primary Sedimentation	Conventional Activated Sludge	Nitrification	Lime_stablization	None	Land application
FL	100	1.2M	60	NA	Pure Oxygen Activated Sludge	Filtration	Anaerobic Lagoons	Centrifuge	Land application
CA3	19	220K	27	NA		NA	Compost Incineration	Belt press	Incineration
DL	12	130K	40	None	Parkson Biolac Extended Aeration Process	Nitrification; Denitrification	Lime_stablization	Belt press	Land application
NV	100.36	921K	23.47	Chemically-Enhanced Primary Sedimentation	Conventional Activated Sludge	Nitrification; Denitrification; Enhanced biological phosphorous removal; Filtration	Other	Centrifuge	Landfill
DC	301	2.2M	32	Primary Sedimentation	Conventional Activated Sludge	NA	Anaerobic	Thermal hydrolysis	Land application
KS	11.3	127K	13.4	Primary Sedimentation	Conventional Activated Sludge	Nitrification; Denitrification	Anaerobic	Belt press	Land application
UT	4	48K	16	Primary Sedimentation	Attached Growth	NA	Anaerobic	Belt press	Land application
IN	7	44K	NA	NA	NA	NA	Anoxic aerobic	NA	Land application; landfill
MI	12.87671233	131K	NA	NA	NA	NA	Aerated settling lagoons	NA	Landfill

NA: information not available

Table 9 - Sum of paraben loadings calculated from the literature data.

Geographic location (Sampling period)	WWTPs studied	Aqueous removal efficiencies (%)	∑ Paraben emission mass loadings	Average effluent concentrations of MePb, EtPB,

		(mean ± std dev)	(g/d/100,000 people) (mean ± std dev)	PrPB, BuPB, BePB (ng/L)
United States (This study, 2015-16)	14	90 ± 11	148 ± 4	20 ± 20, 15 ± 23 9 ± 9 1.6 ± 0 0.3 ± 0
China (2014)	4	89 ± 8	0.3 ± 0.07	4.72 ± 3 0.14 ± 0.2 1.5 ± 1.5 0.04 ± 0.05 <MDL (0.1)
India (2012)	5	84 ± 3	3 ± 0.5	28 ± 14 6 ± 3 12 ± 7 1.5 ± 1 1 ± 1.3
New York, U.S. (2013)	2	95 ± 3	0.3 ± 0.01	0.14 ± 0 0.22 ± 0.1 0.8 ± 0.5 0.4 ± 0.33 0.07 ± 0
Switzerland (2006)	7	98 ± 2	4 ± 0.3	11 ± 4 0.4 ± 0.2 2 ± 2 0.6 ± 0.6 0.16 ± 0.06
United Kingdom (2007)	2	98 ± 2	6600 ± 1000	29500 ± 29000, 24000 ± 750, 1000 ± 500, 18 ± 0, NA

NA: not available; <MDL: below method detection limit
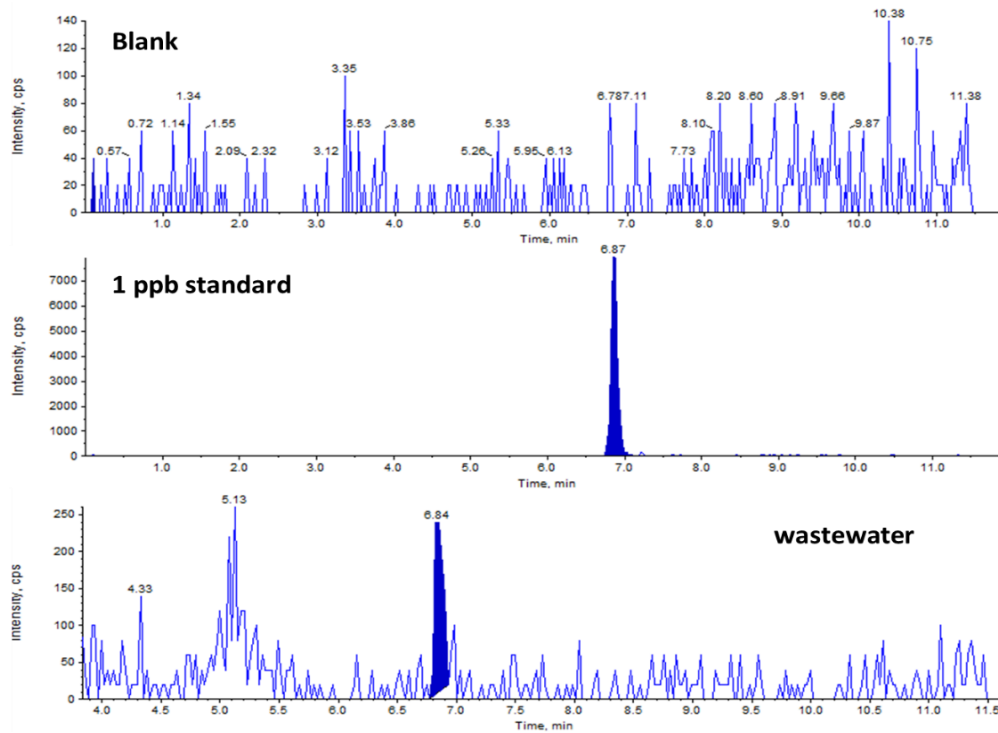
Fig. 40 - Transition (*m/z*) 227→92 Chromatograms for benzyl paraben: Deionized Water blank, 1 ppb standard, and wastewater.
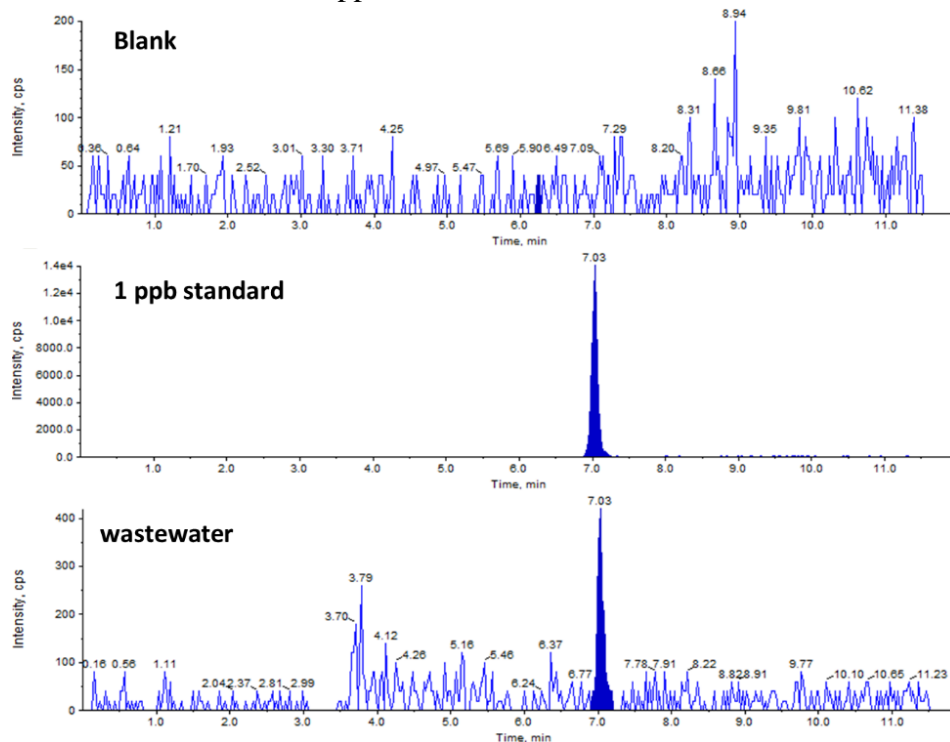
Fig. 43 - Transition (*m/z*) 192→92 Chromatograms for butyl paraben: Deionized Water blank, 1 ppb standard, and wastewater.
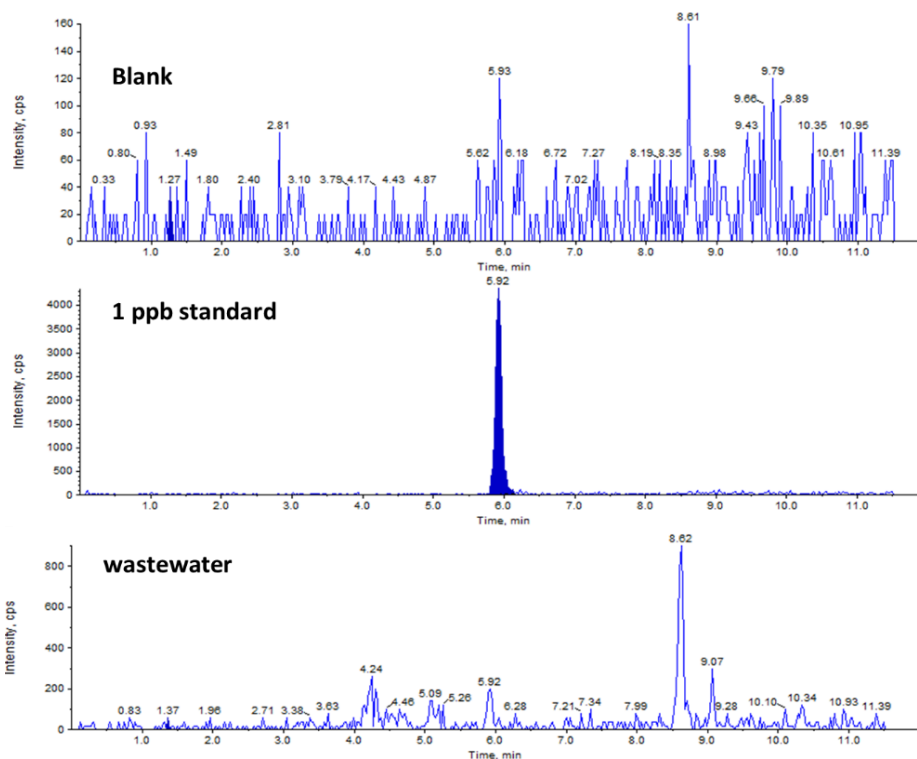
Fig. 45 - Transition (m/z) 165→92 Chromatograms for ethyl paraben: Deionized Water blank, 1 ppb standard, and wastewater.
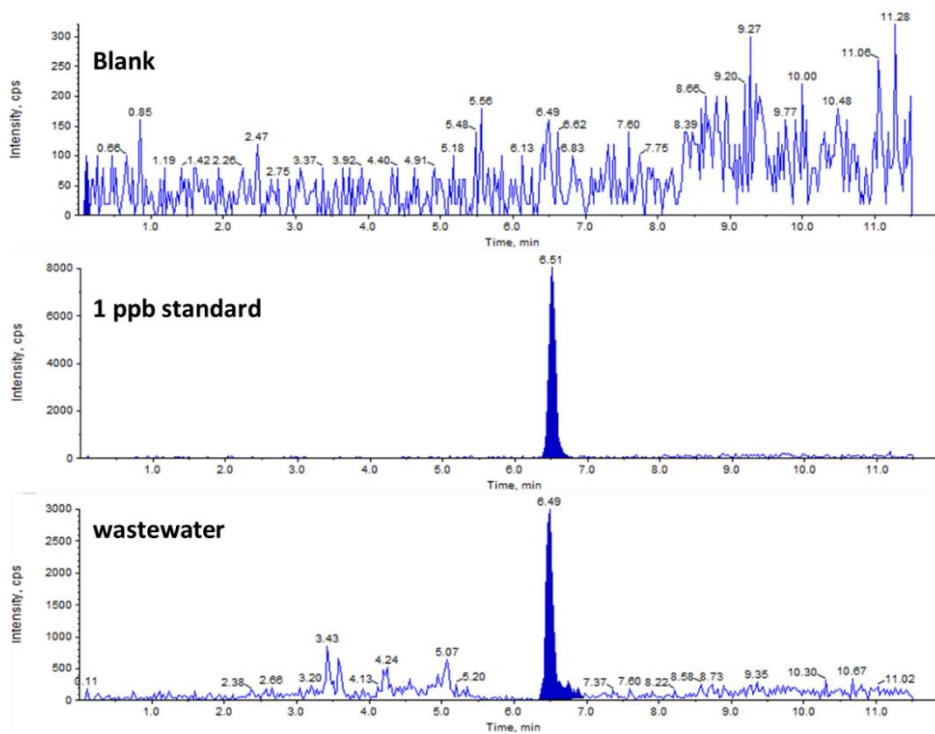
Fig. 48 - Transition (m/z) 151→92 Chromatograms for methyl paraben: Deionized Water blank, 1 ppb standard, and wastewater.
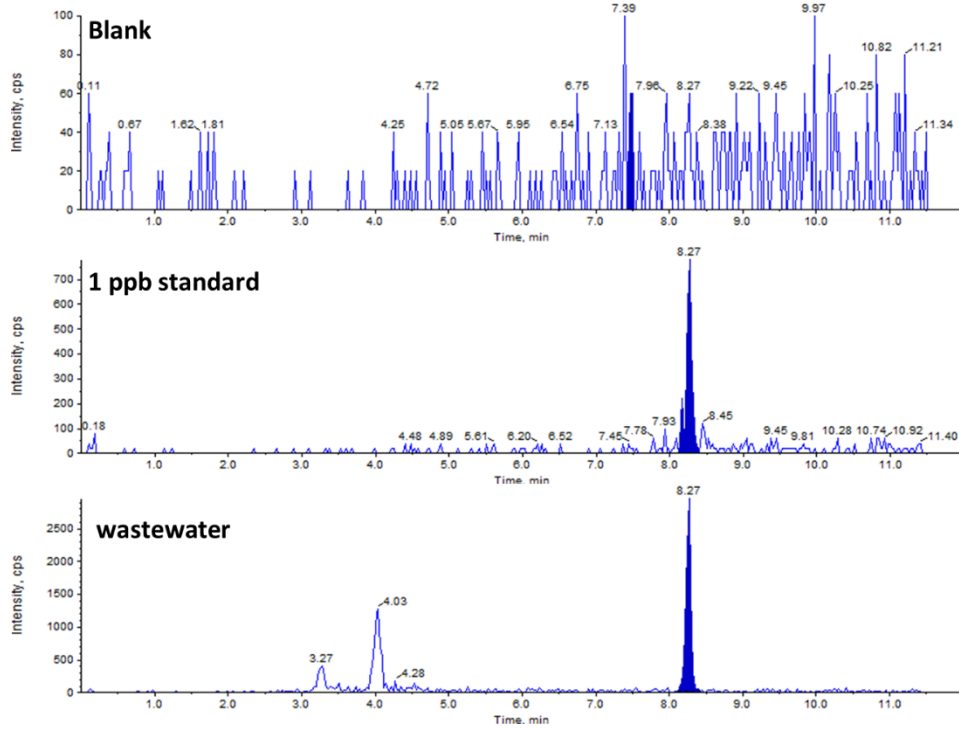
Fig. 51 - Transition (*m/z*) 313→160 Chromatograms for triclocarban: Deionized Water blank, 1 ppb standard, and wastewater.
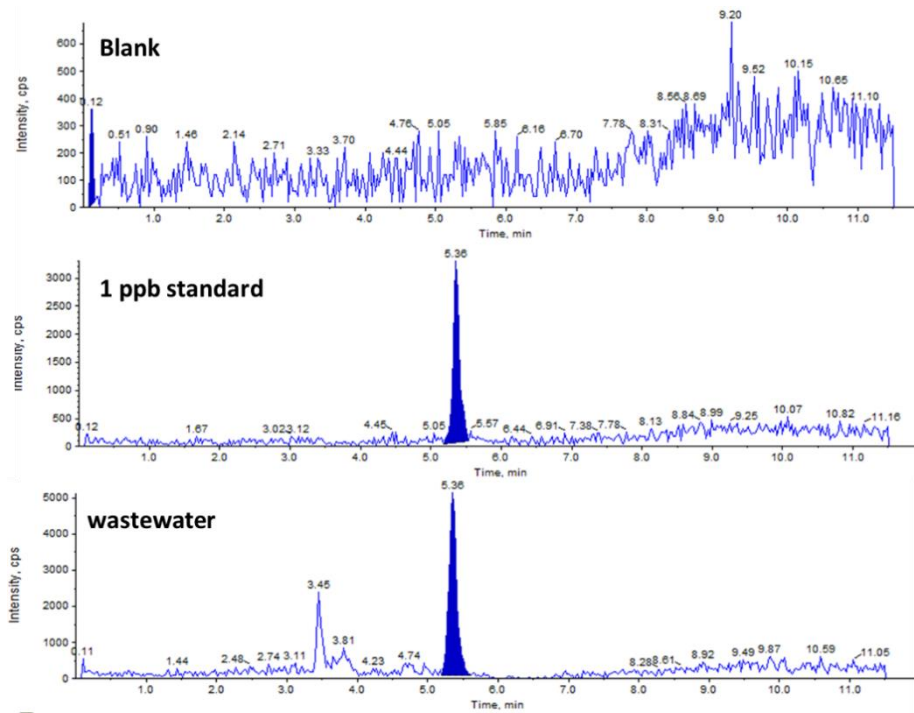
Fig. 54 - Transition (m/z) 179→92 Chromatograms for propyl paraben: Deionized Water blank, 1 ppb standard, and wastewater.
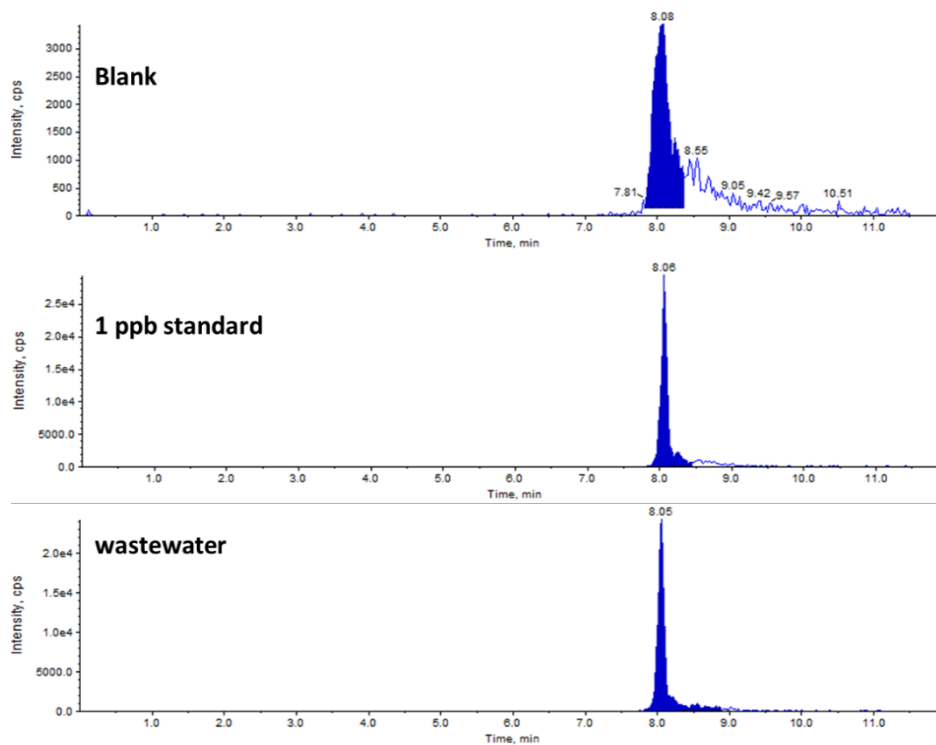
Fig. 57 - Transition (*m/z*) 287→35 Chromatograms for triclosan: Deionized Water blank, 1 ppb standard, and wastewater.

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APPENDIX C

SUPPLEMENTAL MATERIAL FOR CHAPTER 4

Recovery Experimental Procedure and Calculations

Analyte recoveries were determined by spiking native narcotic standards within D.I. water fortified with peat moss to simulate organics within samples. D.I. water samples were spiked with native standard concentrations which represent 10-times the method detection limit of the analyte. Fortified water samples were subjected to the same extraction and analysis procedure as wastewater samples and compared to the prepared native standard curve to determine the concentration of analyte within the fortified sample. Method recoveries were then calculated by comparing the known native standard spiking concentration to the concentration measured by LC-MS/MS. Relative standard deviation (%) was calculated using equation 1

$$RSD\ (\%) = \frac{Standard\ Deviation}{Mean} * 100 \qquad\qquad \text{Equation 1}$$

Sample relative percent difference (RPD) (Tables S10, S11) were adopted from the previous publication (Gushgari et al., 2018) except for Mitragynine. RPD from the individual concentrations obtained from the duplicate analysis through the equation 2.

$$RPD(\%) = \left[|C_1 - C_2| \Big/ \left(\frac{C_1 + C_2}{2}\right) \right] * 100 \qquad\qquad \text{Equation 2}$$

Where C_1 and C_2 represent the two measured sample concentrations.

Table 10 - 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) (volts)	Product ion 2 (*m/z*)	CE(2) (volts)
Alprazolam	Alprazolam	309	281	39	205	59
	alpha-hydroxyalprazolam	325	216	55	205	61
Amphetamine	Amphetamine	136	91	23	119	35
Buprenorphine	Buprenorphine	468	396	55	414	47
	Norbuprenorphine	414	101	57	115	125
Cocaine	Cocaine	304	182	29	105	45
	Benzoylecgonine	290	168	29	105	45
Codeine	Codeine	300	152	89	165	57
	Norcodeine	268	152	79	165	57
Fentanyl	Fentanyl	337	188	33	105	51
	Norfentanyl	223	84	25	55	59
Heroin	Heroin	370	165	67	58	59
	6-Acetylmorphine	328	165	51	211	37
MDMA	MDMA	194	163	19	105	35
Methadone	EDDP	278	234	43	186	49
Methylphenidate	Methylphenidate	234	84	35	56	40
Morphine	Morphine	268	152	81	165	57
	Morphine-3-Glucuronide	462	268	45	165	83
Oxycodone	Oxycodone	316	241	41	298	27
	Noroxycodone	302	284	25	187	35
Mitragynine	Mitragynine	399	174	41	159	65

Table 11 - Method detection limits for narcotic analytes.

Analyte	Method Detection Limit (ng/L)
Codeine	1.4
Oxycodone	0.2
Noroxycodone	0.3
Heroin	0.3
6-Acetylmorphine	0.3

2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	1.7
Cocaine	0.6
Benzoylecgonine	0.7
Mitragynine	10

Table 12 - Analyte recovery values from peat-moss water fortified with native standard. Concentrations were spiked at 10-times the method detection limit. Ten samples (n=10) were used to determine analyte recovery.

Indicator Compound	Percent Recovered	% RSD (n=10)
OXY	104	16
COD	99	11
HER	139	14
6-AM	116	12
NOXY	110	24
EDDP	109	19
COC	141	43
BZE	161	59
KRT	86	23
Average	**117**	**23**

Table 13 - Narcotic analyte sample extract concentration average ± standard error, minimum and maximum concentrations observed, and detection frequency for all locations and months.

Analyte	Average ± Standard Error	Minimum Concentration	Maximum Concentration	Detection Frequency
		(ng/L)		*(%)*
Oxycodone	55 ± 4	<0.2	1492	87
Codeine	42 ± 2	<1.4	277	86
Heroin	26 ± 4	<0.3	643	78
Noroxycodone	14 ± 3	<0.3	1051	80
6-Acetylmorphine	43 ± 3	<0.3	573	81
EDDP	250 ± 128	<1.7	53602	88
Cocaine	1021 ± 312	<0.6	99540	88
Benzoylecgonine	2034 ± 984	<0.7	5500	88

Methadone	71 ± 12	<1.7	4690	85
Mitragynine	144 ± 28	<10	1290	79

Table 14 - Minimum, average, and maximum relative percent difference (%) for all indicator compounds across the entirety of the sampling campaign.

Indicator Compound	Minimum RPD (%)	Maximum RPD (%)	Average RPD (%)
Oxycodone	0.01	179	25
Noroxycodone	0.78	198	33
Codeine	2.61	165	34
Heroin	0.00	199	50
6-Acetylmorphine	0.71	161	39
Methadone	0.18	188	30
EDDP	0.00	191	30
Cocaine	0.15	106	29
Benzoylecgonine	0.07	195	28
Mitragynine	1.36	194	40
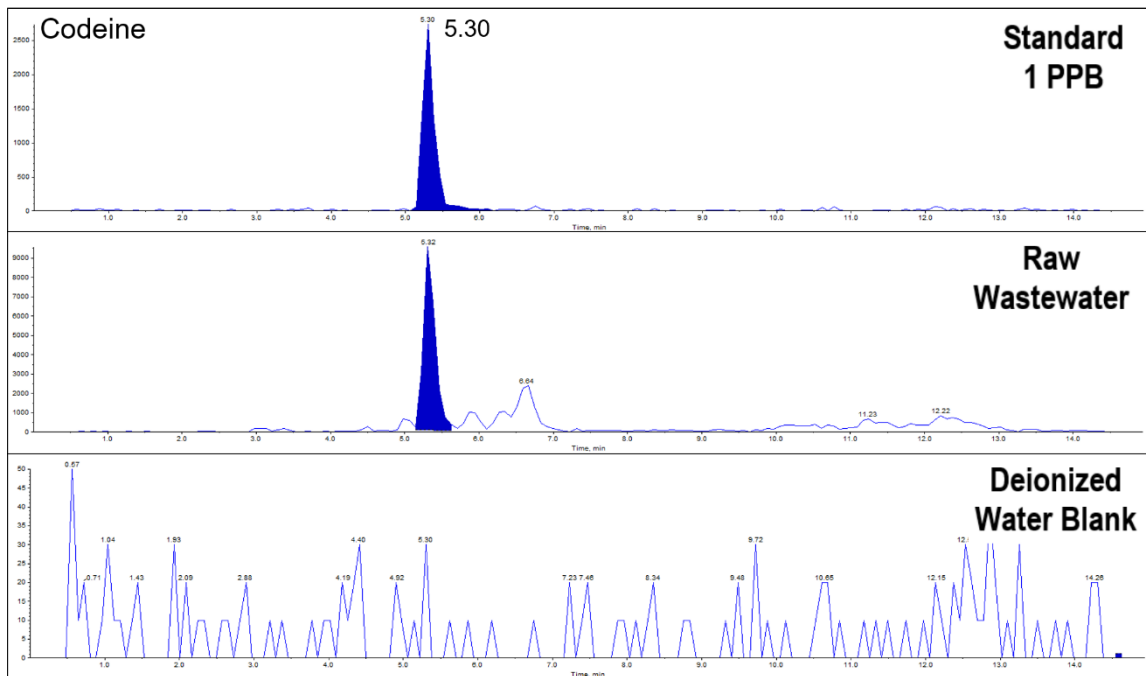
Fig. 59 - Transition (m/z) 300→152 Chromatograms for Codeine: Standard, Raw Wastewater, and the Deionized Water Blank.

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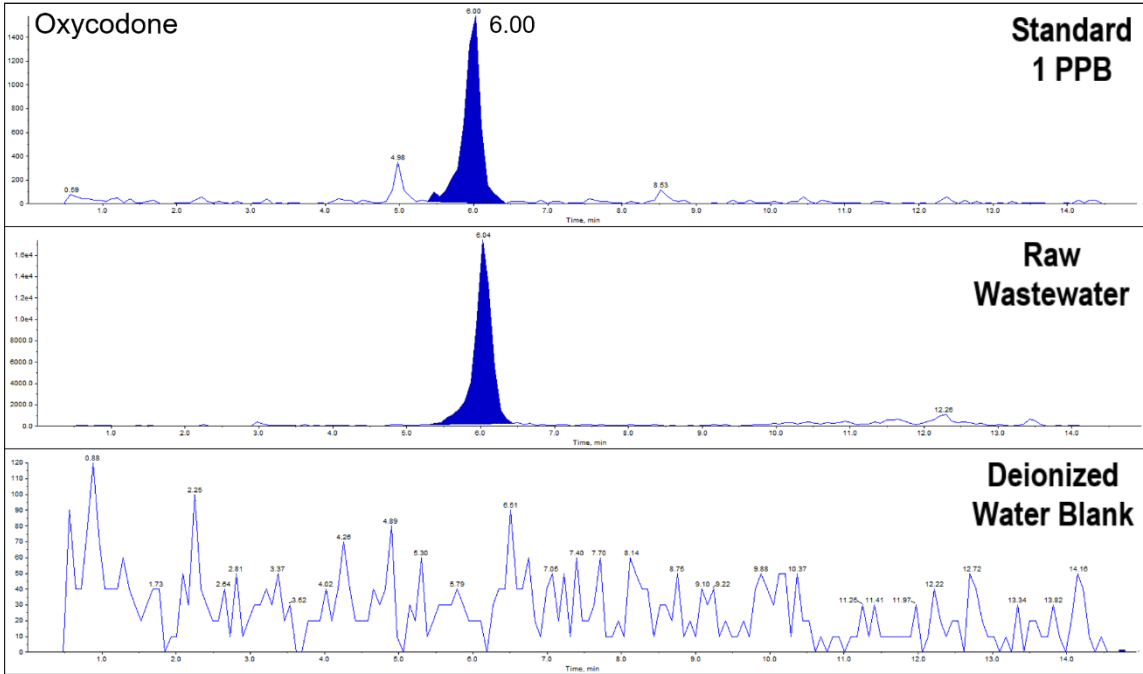
Fig. 61 - Transition (*m/z*) 316→241 Chromatograms for Oxycodone: Standard, Raw Wastewater, and the Deionized Water Blank.
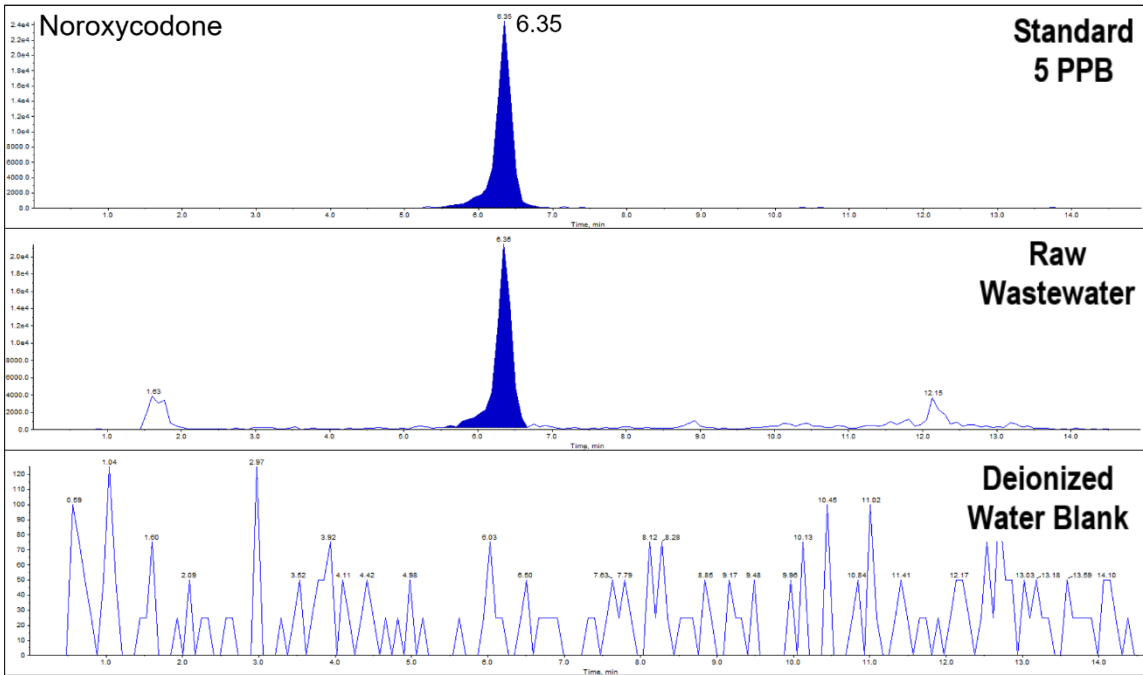
Fig. 64 - Transition (*m/z*) 302→284 Chromatograms for Noroxycodone: Standard, Raw Wastewater, and the Deionized Water Blank.
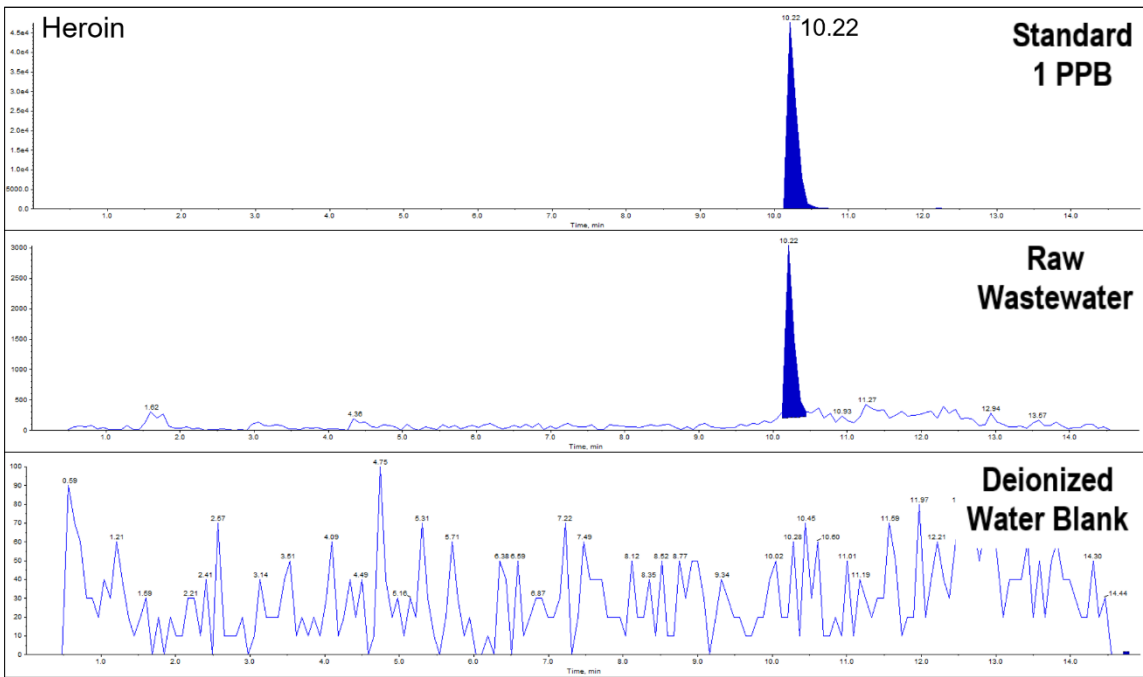
Fig. 70 - Transition (*m/z*) 370→165 Chromatograms for Heroin: Standard, Raw Wastewater, and the Deionized Water Blank.
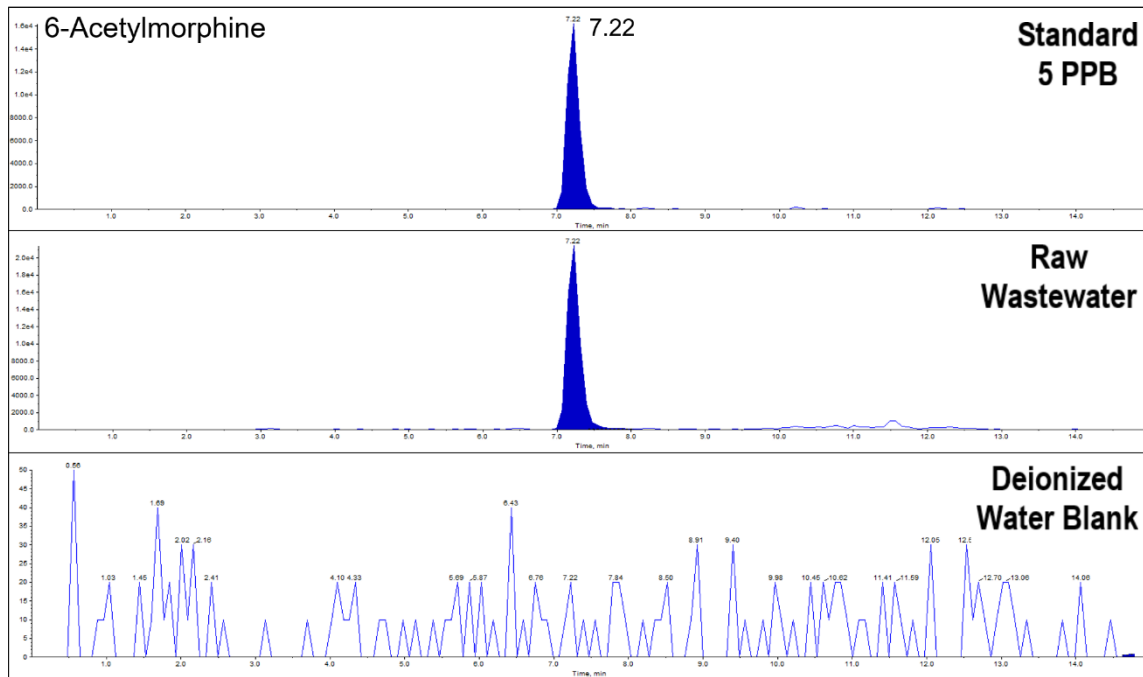
Fig. 67 - Transition (*m/z*) 328→165 Chromatograms for 6-Acetylmorphine: Standard, Raw Wastewater, and the Deionized Water Blank.
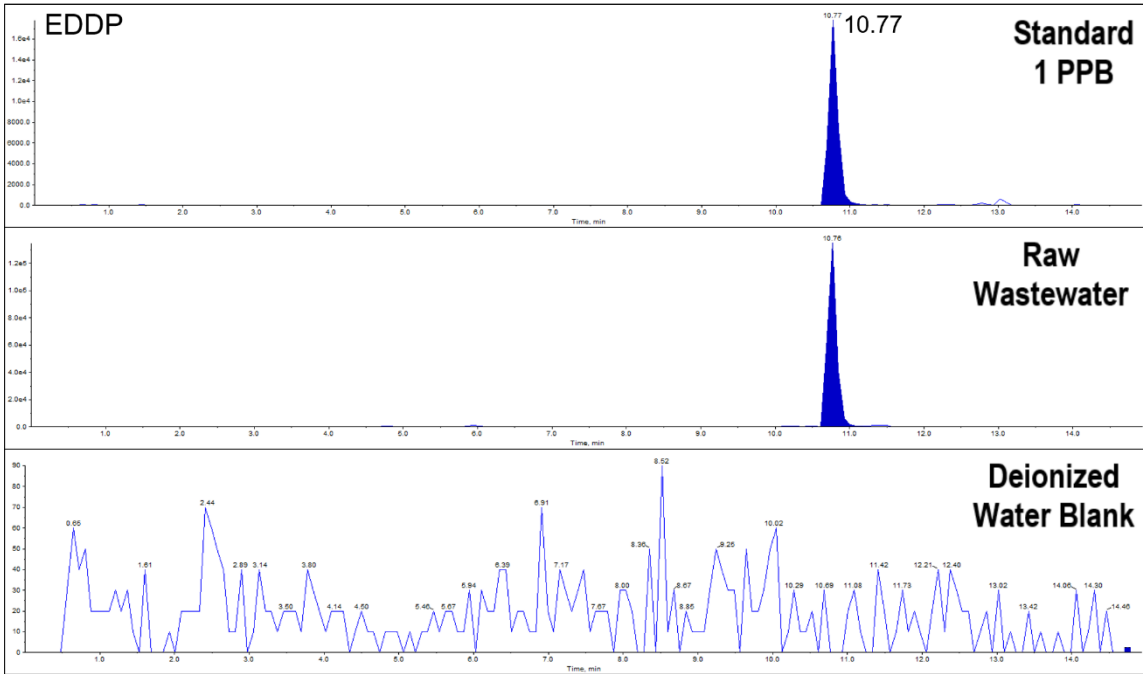
Fig. 73 - Transition (*m/z*) 278→234 Chromatograms for EDDP: Standard, Raw Wastewater, and the Deionized Water Blank.
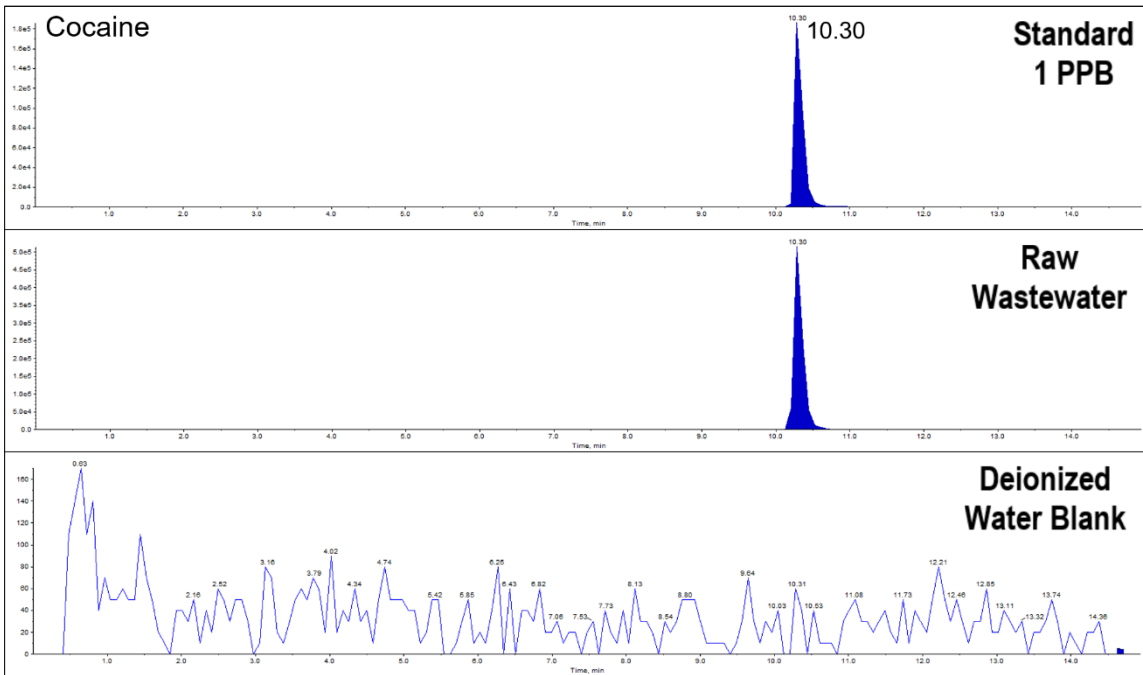
Fig. 76 - Transition (*m/z*) 304→182 Chromatograms for Cocaine: Standard, Raw Wastewater, and the Deionized Water Blank.
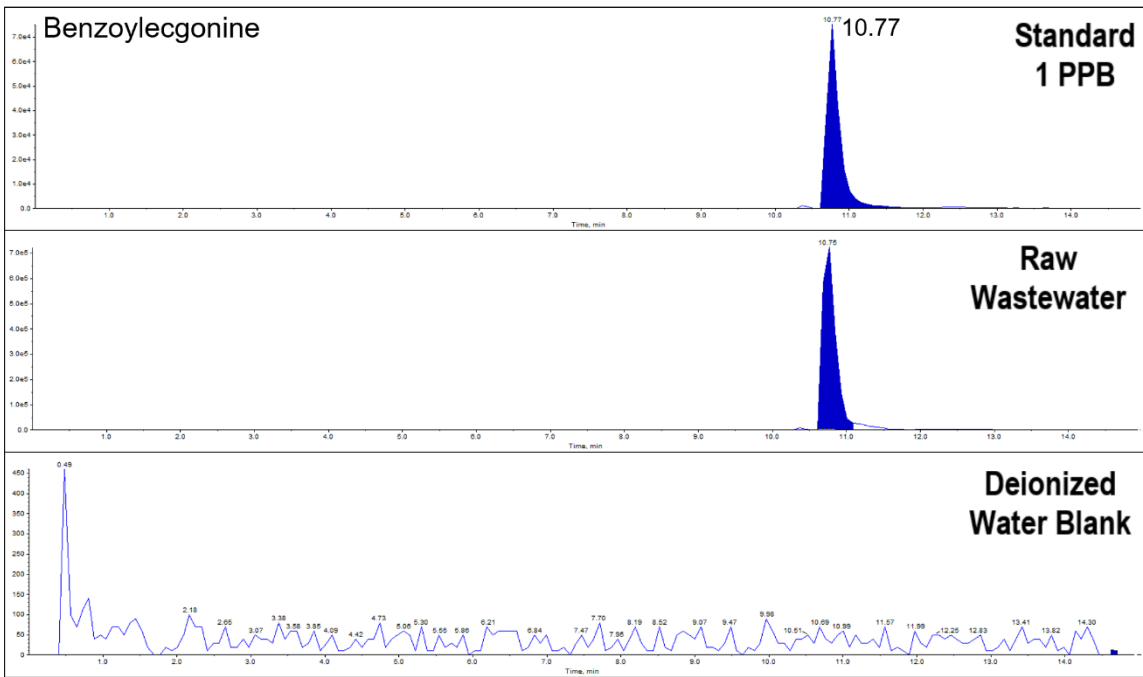
Fig. 79 - Transition (*m/z*) 290→168 Chromatograms for Benzoylecgonine: Standard, Raw Wastewater, and the Deionized Water Blank.
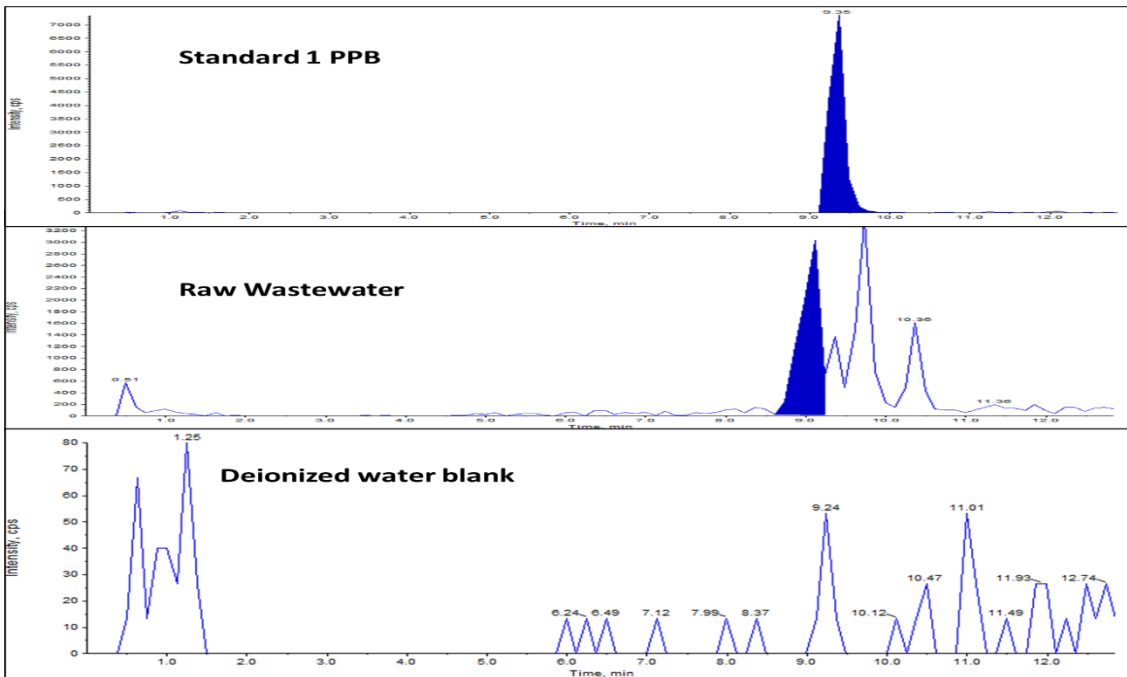
Fig. 81 - Transition (*m/z*) 399→174 Chromatograms for Mitragynine: Standard, Raw Wastewater, and the Deionized Water Blank.

APPENDIX D

SUPPLEMENTAL MATERIAL FOR CHAPTER 5

Recovery Experimental Procedure and Calculations

Analyte recoveries were determined by spiking native narcotic standards within D.I. water fortified with peat moss to simulate organics within samples. D.I. water samples were spiked with native standard concentrations which represent 10-times the method detection limit of the analyte. Fortified water samples were subjected to the same extraction and analysis procedure as wastewater samples and compared to the prepared native standard curve to determine the concentration of analyte within the fortified sample. Method recoveries were then calculated by comparing the known native standard spiking concentration to the concentration measured by LC-MS/MS. Relative standard deviation (%) was calculated using equation 3

$$RSD\ (\%) = \frac{Standard\ Deviation}{Mean} * 100 \hspace{3cm} \text{Equation 3}$$

Sample relative percent difference (RPD) (Table 16154) were adopted from the previous publication (Gushgari et al., 2018) except for Mitragynine. RPD from the individual concentrations obtained from the duplicate analysis through the equation 4.

$$RPD(\%) = \left[|C_1 - C_2| \Big/ \left(\frac{C_1 + C_2}{2}\right) \right] * 100 \hspace{2cm} \text{Equation 4}$$

Where C_1 and C_2 represent the two measured sample concentrations.

Table 15 - Optimized conditions for the ionization and fragmentation of the stress hormones meabolites and their internal standard analytes screened for in this method.

Compound	Precursor ion (*m/z*)	Product ion 1 (*m/z*)	CE(1) (volts)	Product ion 2 (*m/z*)	CE(2) (volts)
Cortisone	361	163.4	-25	93.3	-39
Cortisone-D8	369	169.4	-26	369.45	-12
Cortisol	363.1	91.3	-57	121.3	-27

	367	121.3	-26	163.5	-29
Cortisol-D4	367	121.3	-26	163.5	-29
Tetrahydrocortisol	411	335	18	301	37
Tetrahydrocortisol-D5	414.2	338.3	20	310	26
Tetrahydrocortisone	409	333.3	19	305.4	24
Tetrahydrocortisone-D5	416	306.4	40	340.4	19

Table 16 - Method detection limits for stress analytes.

Analyte	MDL (µg/L)
Cortisone	0.115
Cortisol	1.435
Tetrahydrocortisol	0.008
Tetrahydrocortisone	0.07

Table 17 - Analyte recovery values from de-ionized water fortified with native standard. Concentrations were spiked at 10-times the method detection limit. Ten samples (n=10) were used to determine analyte recovery.

Indicator Compound	Percent Recovered	% RSD (*n*=10)
Cortisone	114	9.6
Cortisol	126	9.5
Tetrahydrocortisol	82	28
Tetrahydrocortisone	80	24

Table 18 - Minimum, average, and maximum relative percent difference (%) for all indicator compounds across the entirety of the sampling campaign.

Indicator Compound	Minimum RPD (%)	Maximum RPD (%)	Average RPD (%)
Cortisone	1.1	64	32
Cortisol	1.2	100	30
Tetrahydrocortisol	2.63	63	27
Tetrahydrocortisone	1.18	82	18
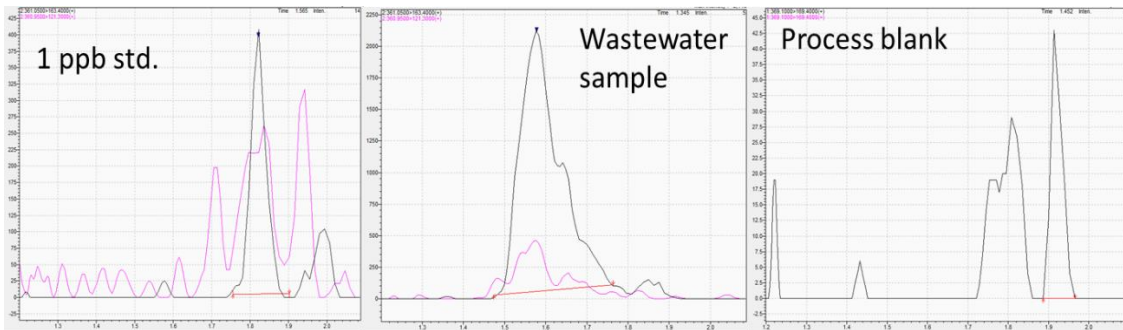
Fig. 84 - Transition (*m/z*) 361→163 Chromatograms for Cortisone: Standard, Raw Wastewater, and the Process blank.
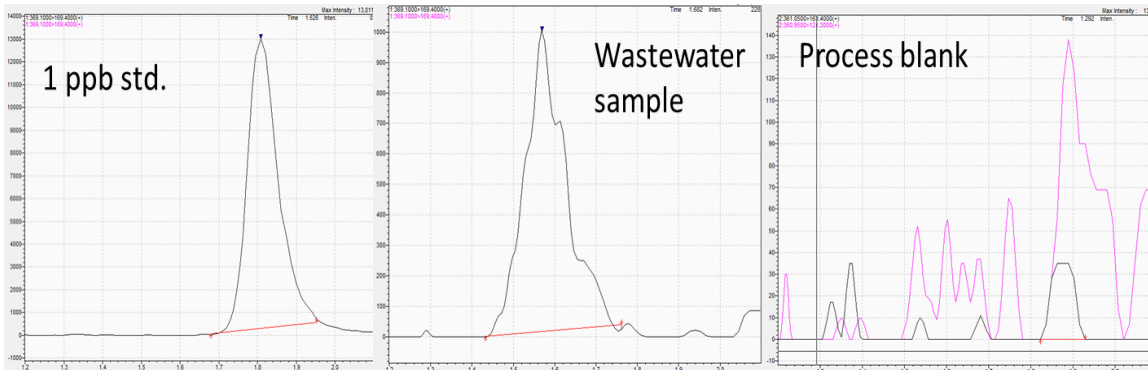
Fig. 87 - Transition (*m/z*) 369→169.4 Chromatograms for Cortisone-D8: Standard, Raw Wastewater, and the Process blank.
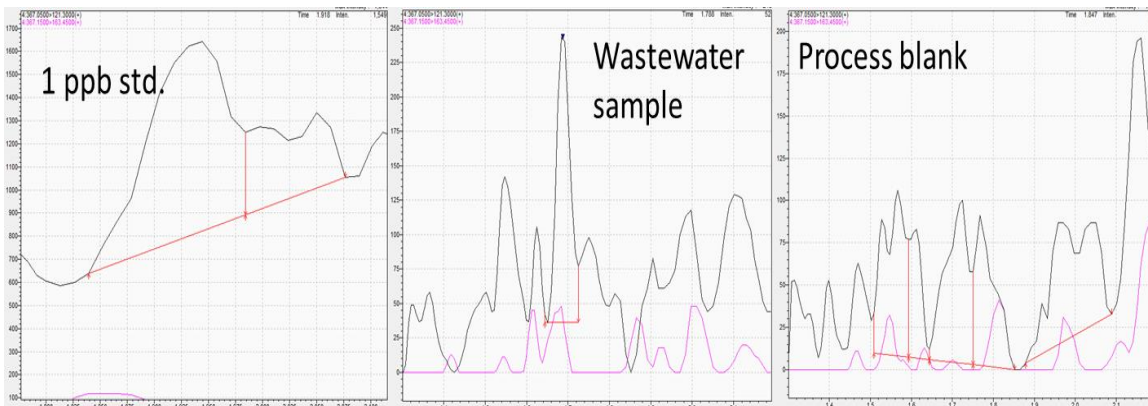
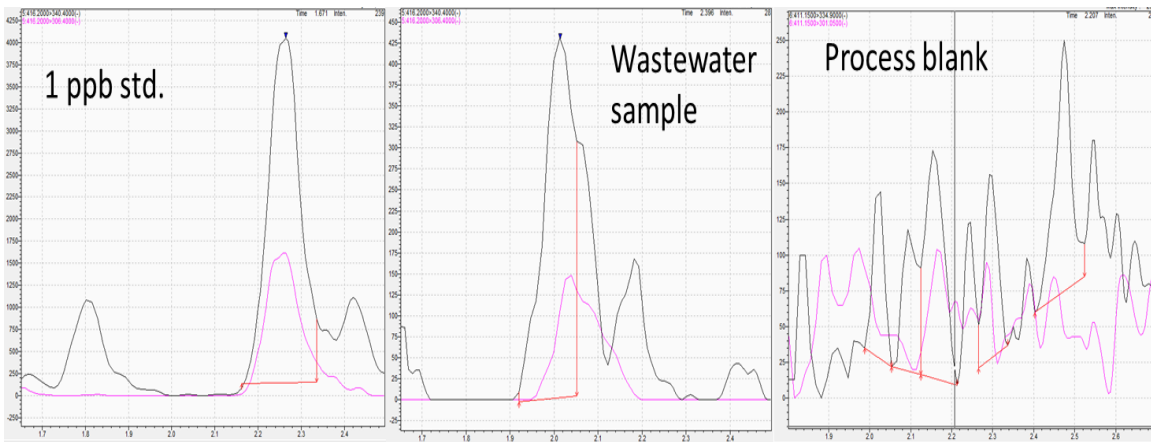**Fig. 89 -** Transition (*m/z*) 363→91 Chromatograms for Cortisol: Standard, Raw Wastewater, and the Process blank

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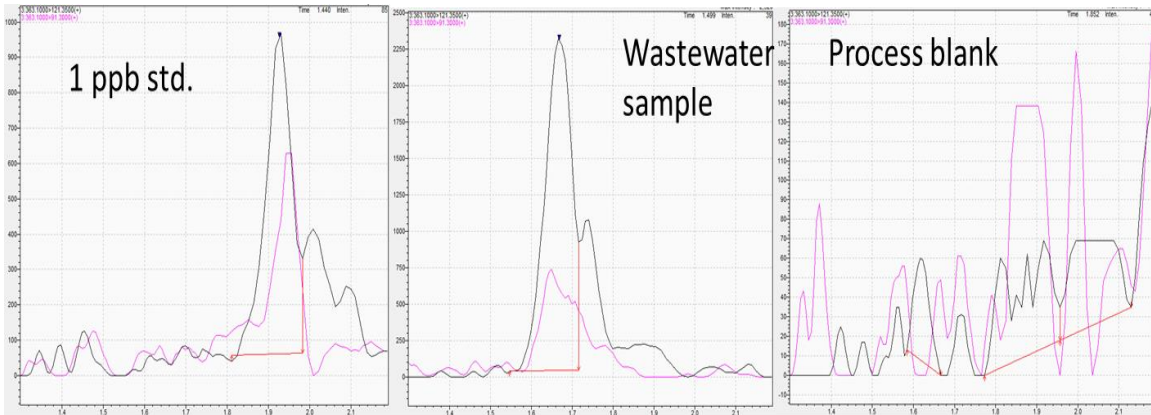
Fig. 91 - Transition (*m/z*) 367→121 Chromatograms for Cortisol-D4: Standard, Raw Wastewater, and the Process blank.
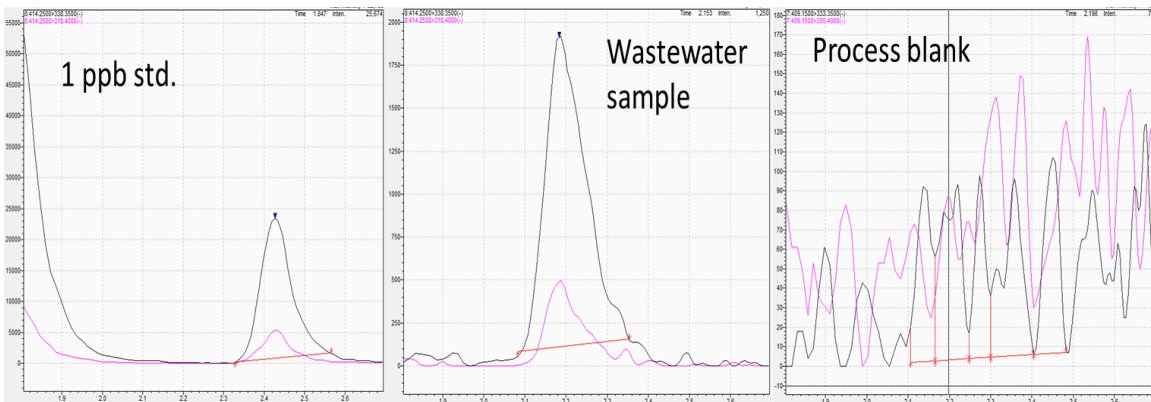
Fig. 94 - Transition (*m/z*) 409→333 Chromatograms for tetrahydrocortisone: Standard, Raw Wastewater, and the Process blank.
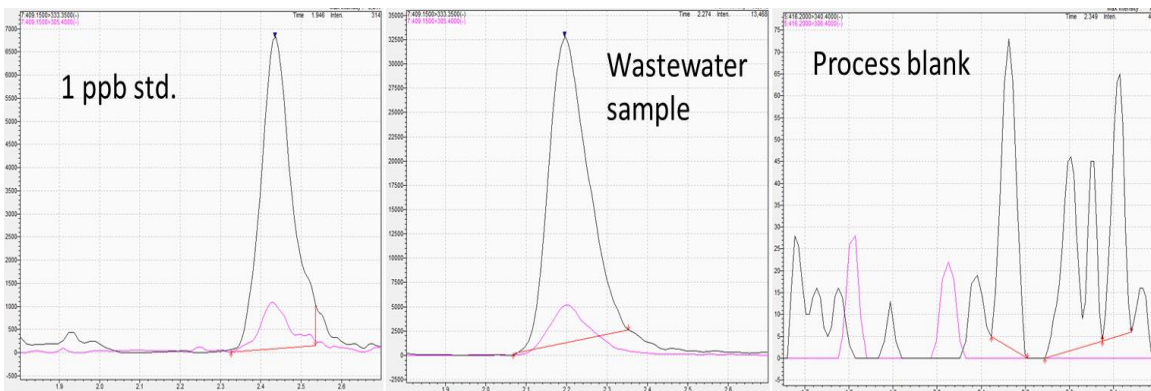
Fig. 96 - Transition (*m/z*) 416→306 Chromatograms for tetrahydrocortisone-D5: Standard, Raw Wastewater, and the Process blank.
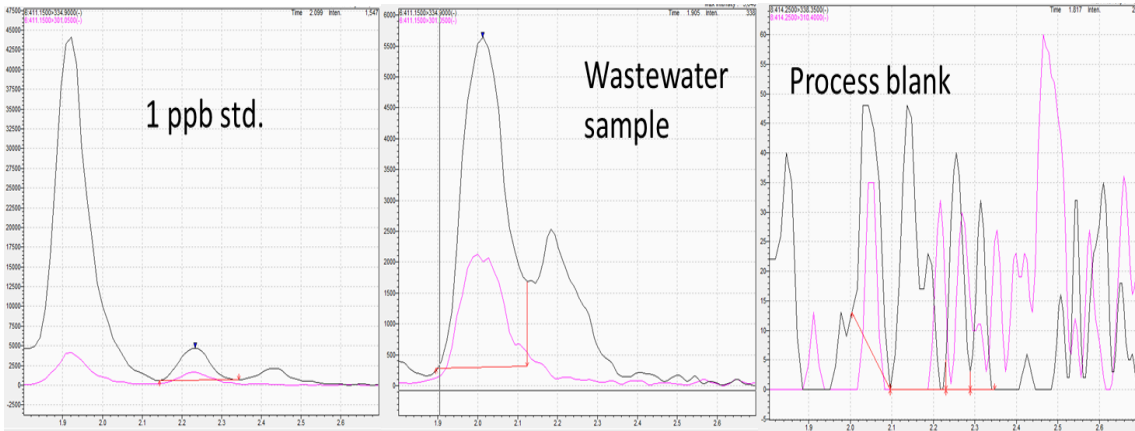
Fig. 99 - Transition (*m/z*) 416→306 Chromatograms for tetrahydrocortisol: Standard, Raw Wastewater, and the Process blank.

Fig. 102 - Transition (*m/z*) 414→338 Chromatograms for tetrahydrocortisol-D5: Standard, Raw Wastewater, and the Process blank.
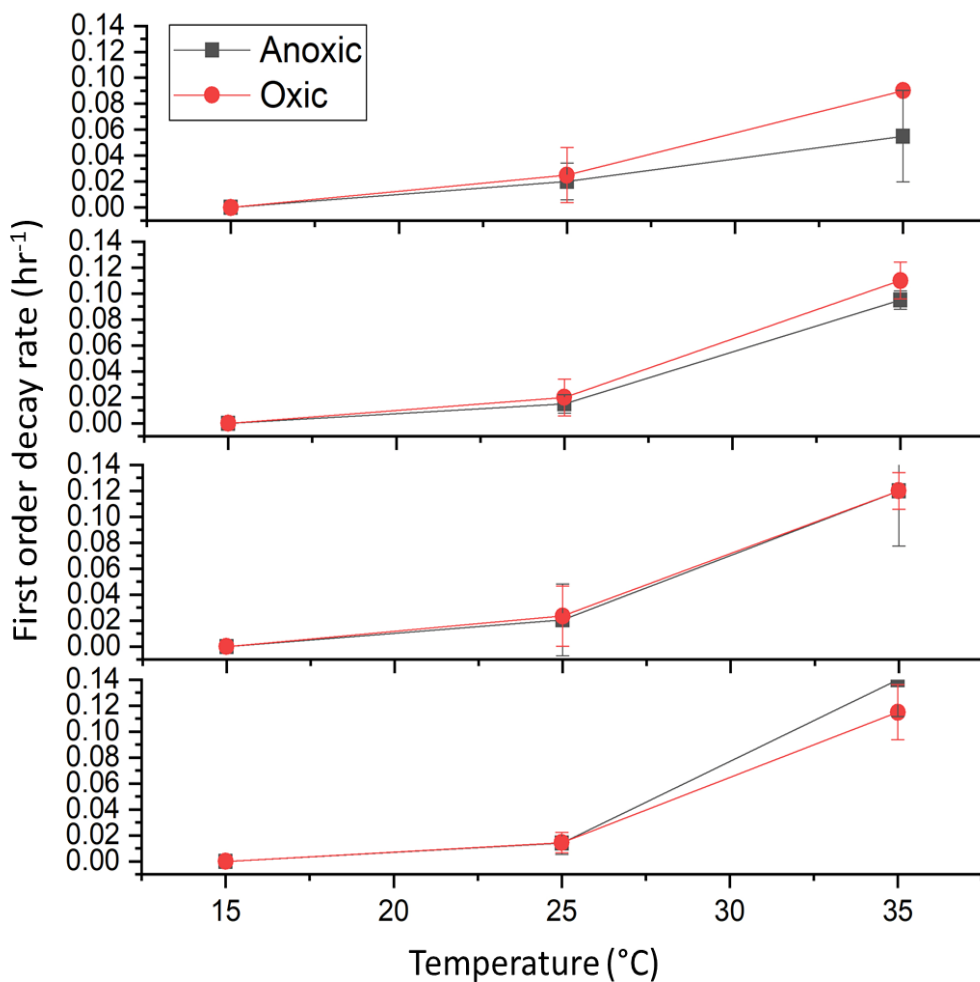
Fig. 104-Graph of first order decay rate versus the incubation temperatures for oxic and anoxic conditions. Each data point is an average of all four stress biomarkers studied and the whiskers represent the standard deviation.
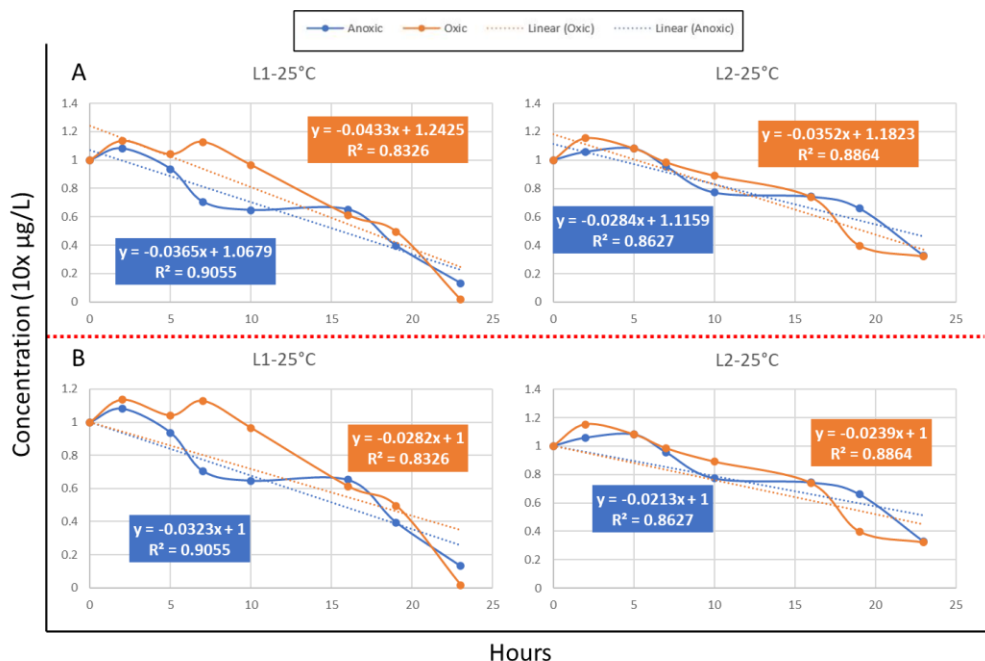
Fig. 106- Zero order decay plots for cortisone at 25°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
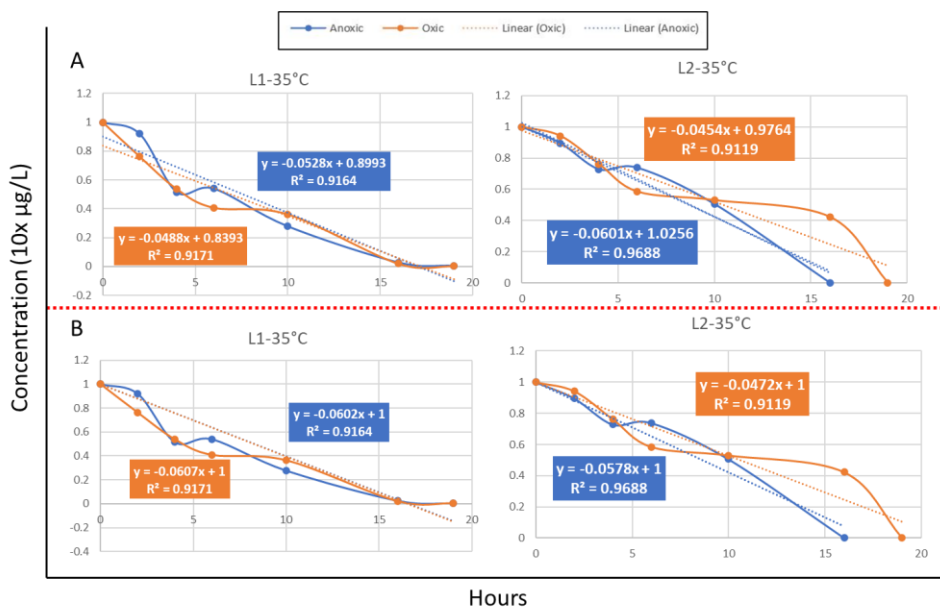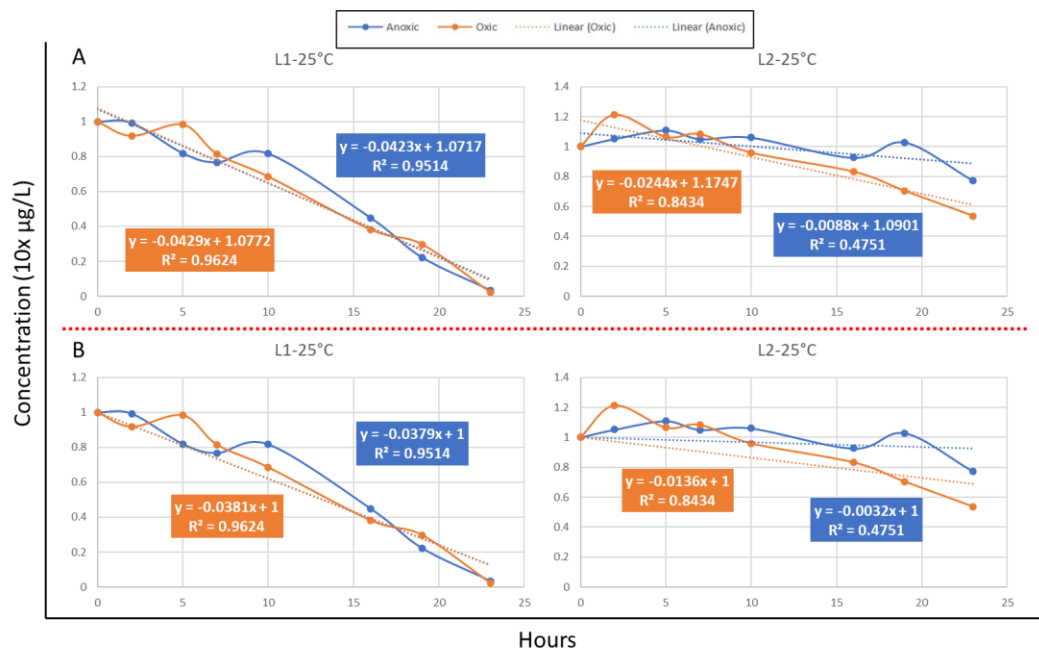

Fig. 43- Zero order decay plots for cortisone at 35°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
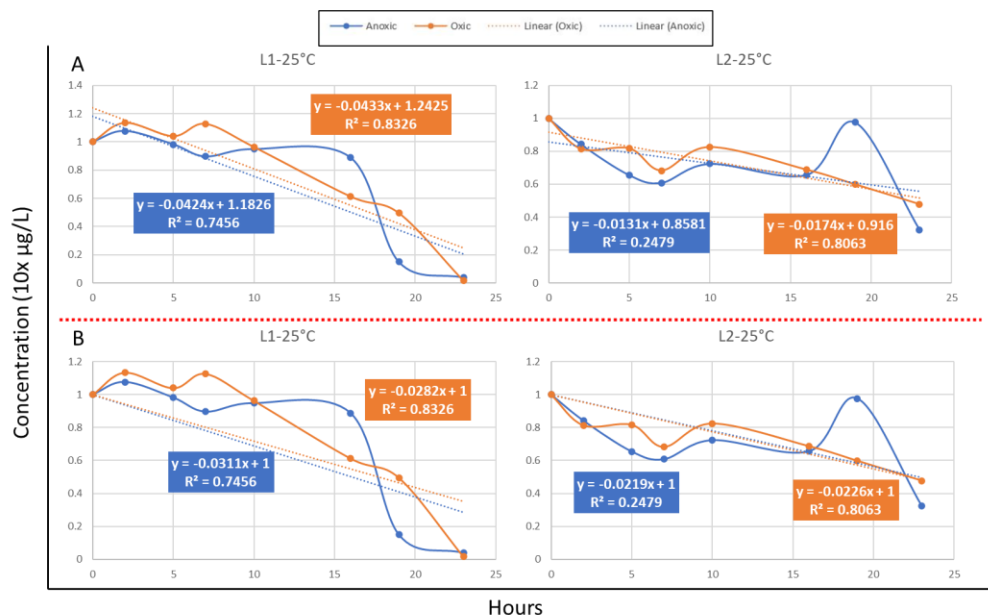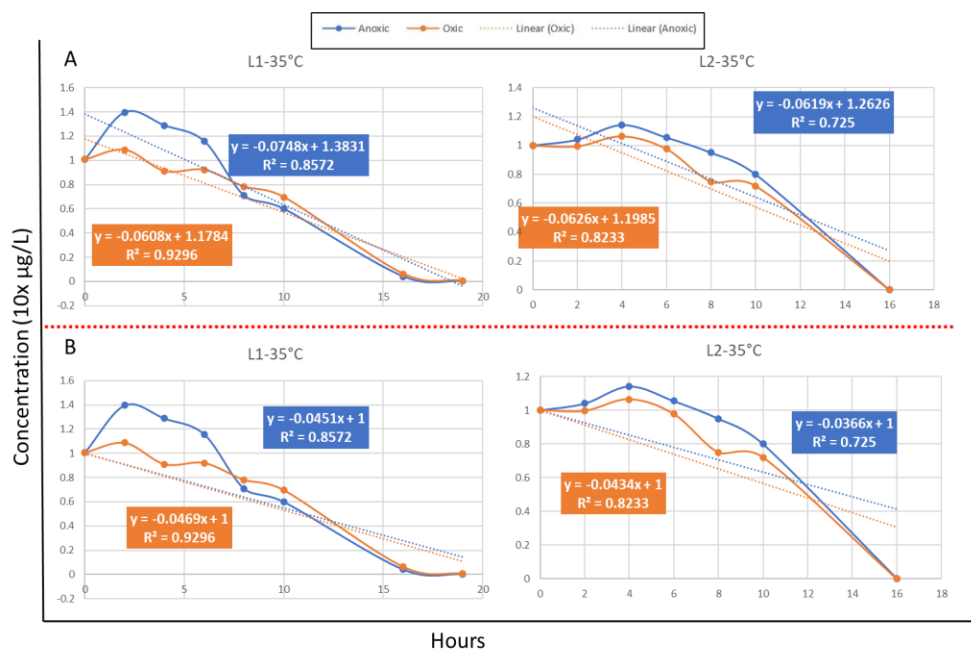
Fig. 44- Zero order decay plots for cortisol at 25°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
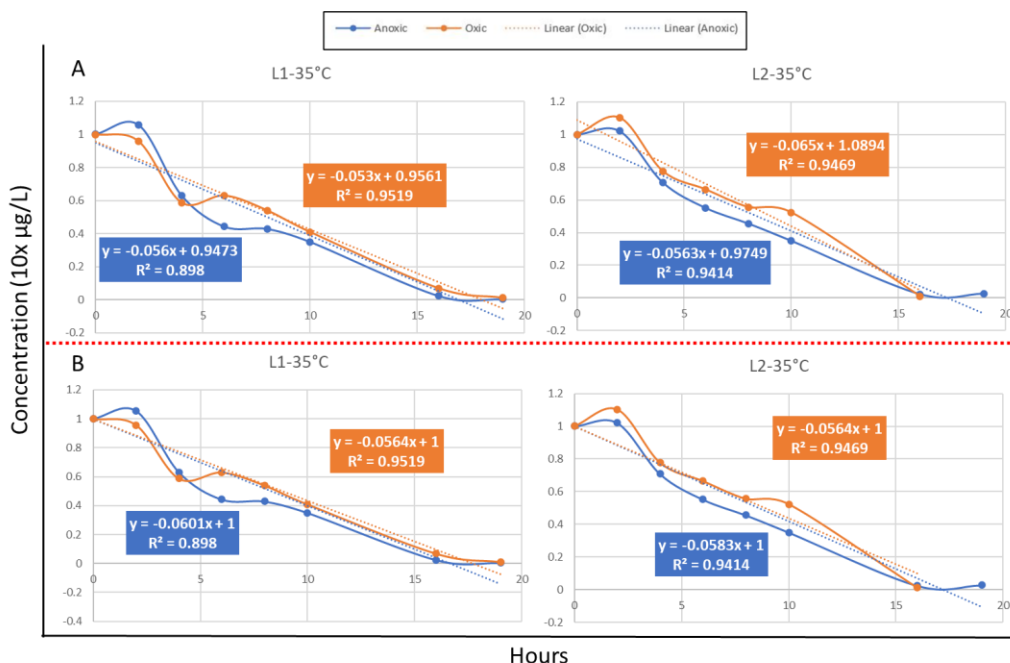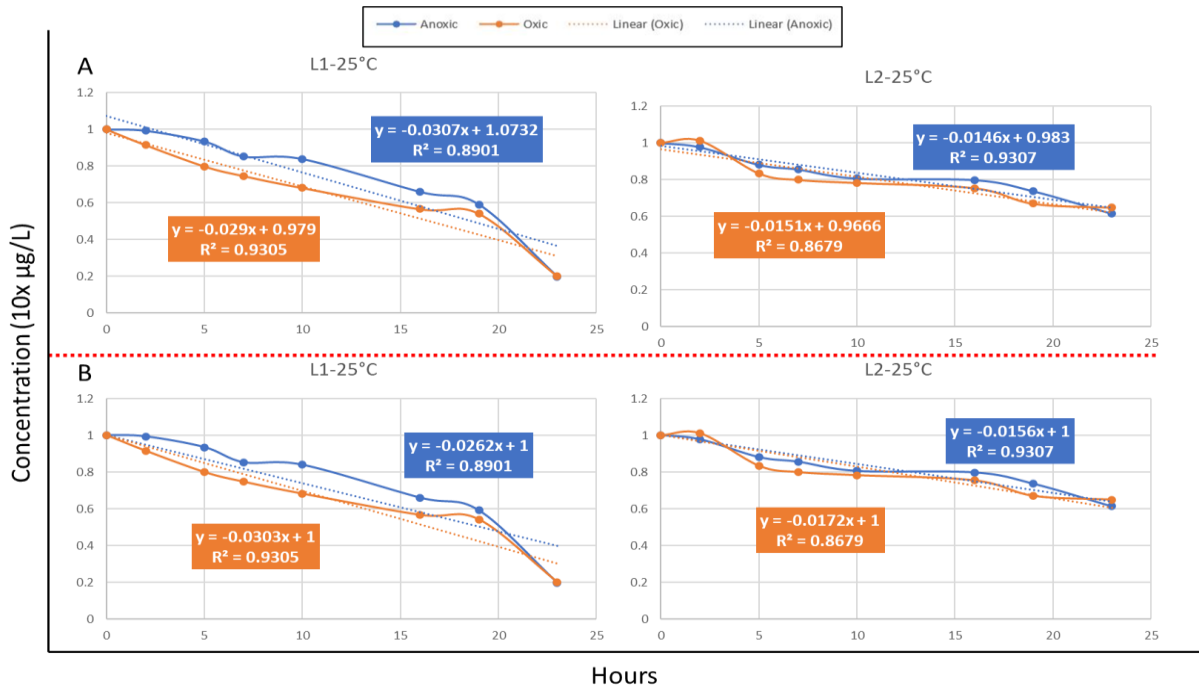

Fig. 45- Zero order decay plots for cortisol at 35°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.

Fig. 46- Zero order decay plots for tetrahydrocortisone at 25°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
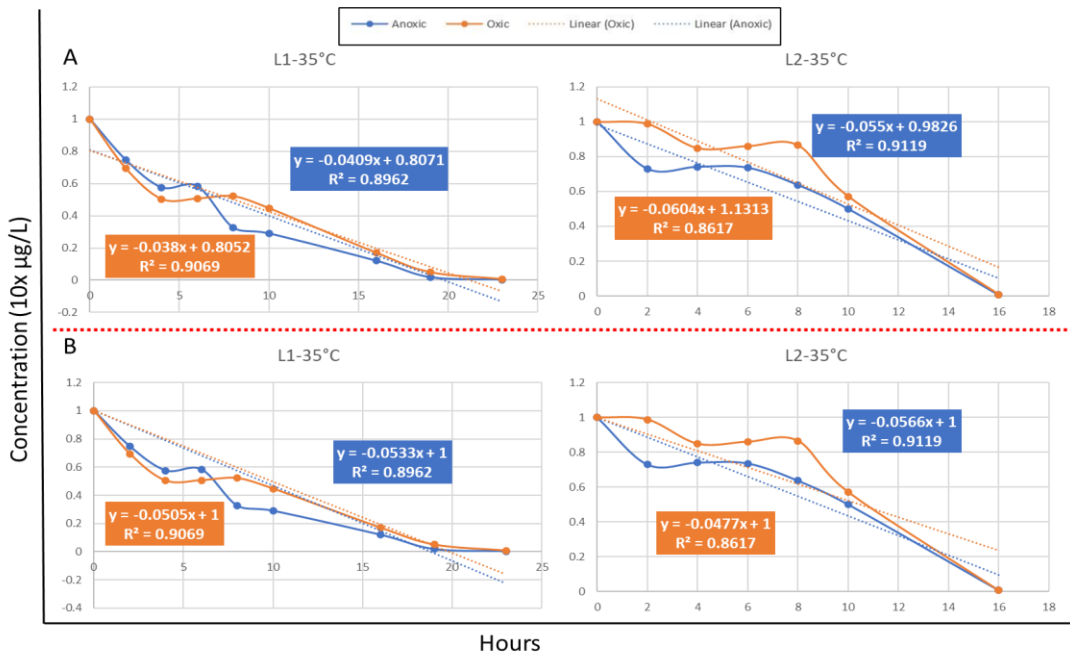

Fig. 47- Zero order decay plots for tetrahydrocortisone at 35°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.

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Fig. 48- Zero order decay plots for tetrahydrocortisol at 25°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
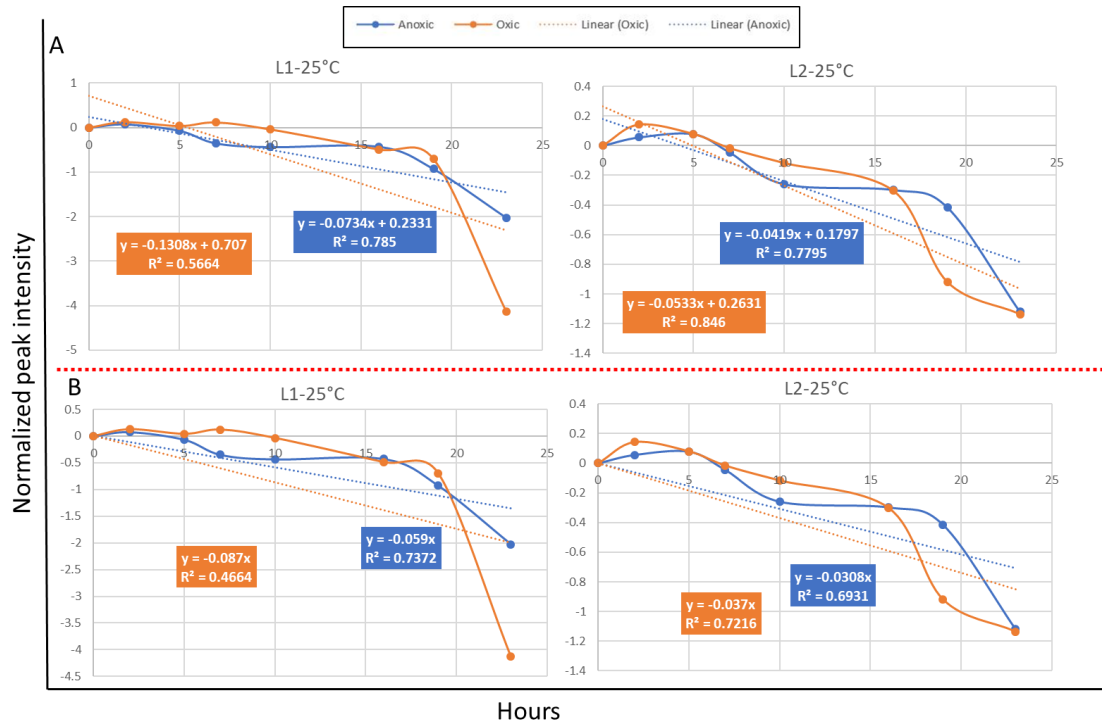

Fig. 49- Zero order decay plots for tetrahydrocortisol at 35°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.

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Table 20. Zero order decay rates (concentration/hour) summarized for all compounds studied under anoxic and oxic conditions.

	25°C				35°C			
	Anoxic		Oxic		Anoxic		Oxic	
Compounds	L1	L2	L1	L2	L1	L2	L1	L2
E	0.032	0.021	0.028	0.023	0.067	0.057	0.067	0.047
F	0.031	0.021	0.028	0.022	0.06	0.058	0.056	0.056
THE	0.038	0.013	0.037	0.003	0.047	0.043	0.045	0.036
THF	0.03	0.017	0.026	0.015	0.053	0.056	0.05	0.056

First order decay fits

Fig. 50- First order decay plots for cortisone (log scale) at 25°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
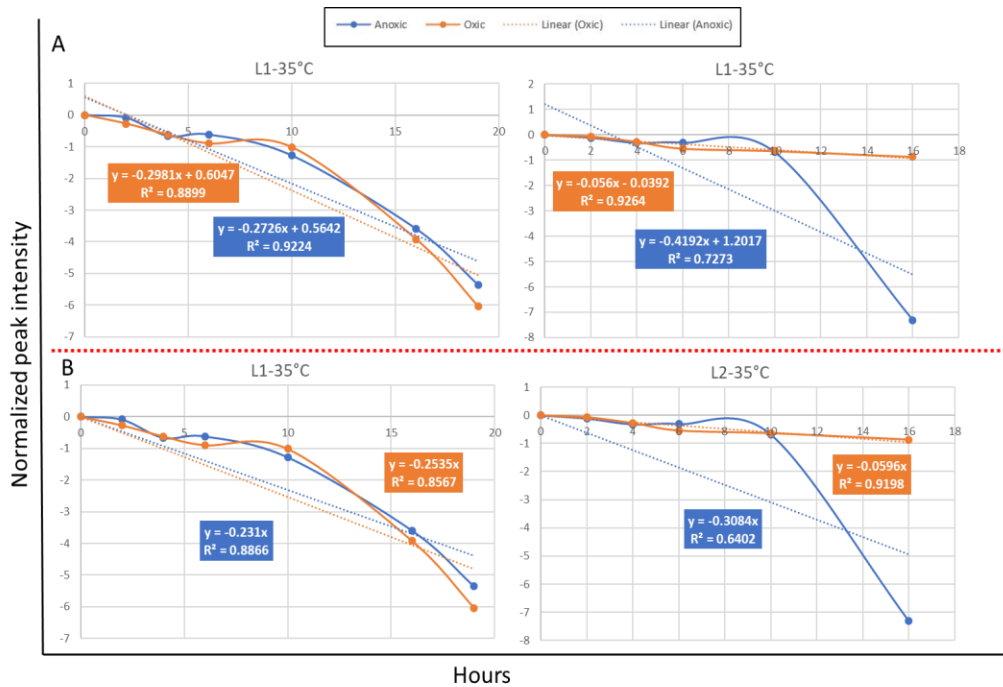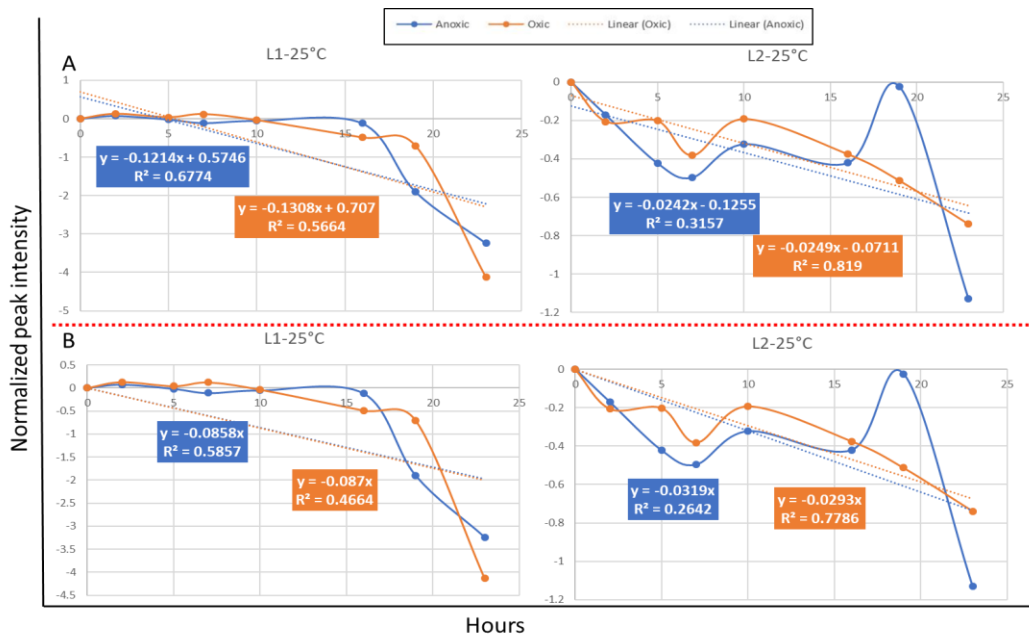
Fig. 51- First order decay plots for cortisone (log scale) at 35°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
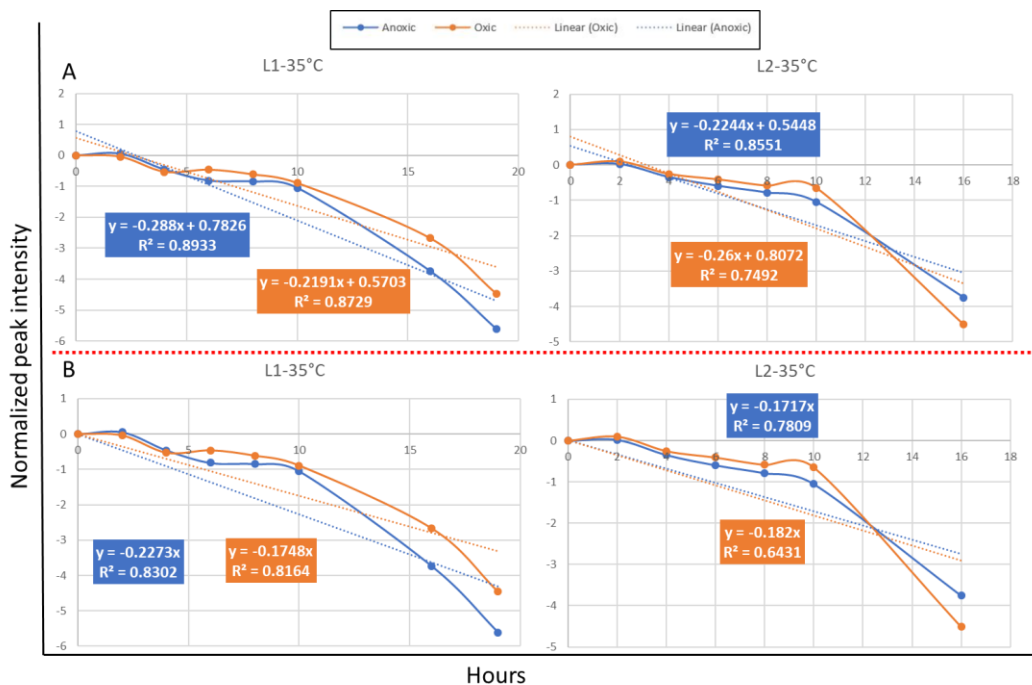

Fig. 52- First order decay plots for cortisol (log scale) at 25°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
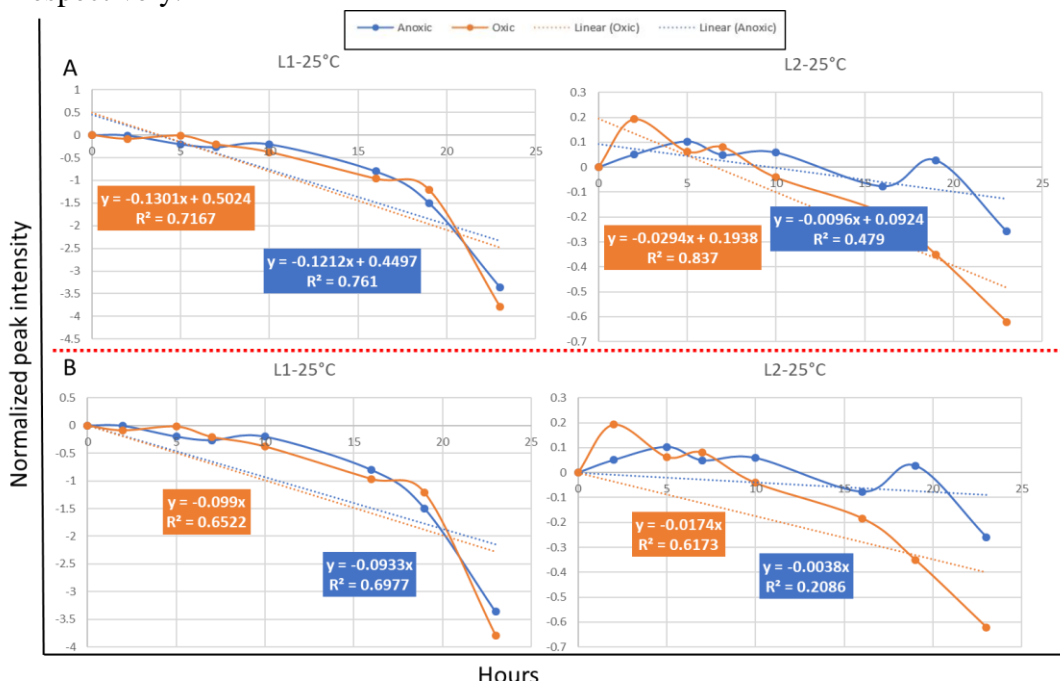
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Fig. 53- First order decay plots for cortisol (log scale) at 35°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
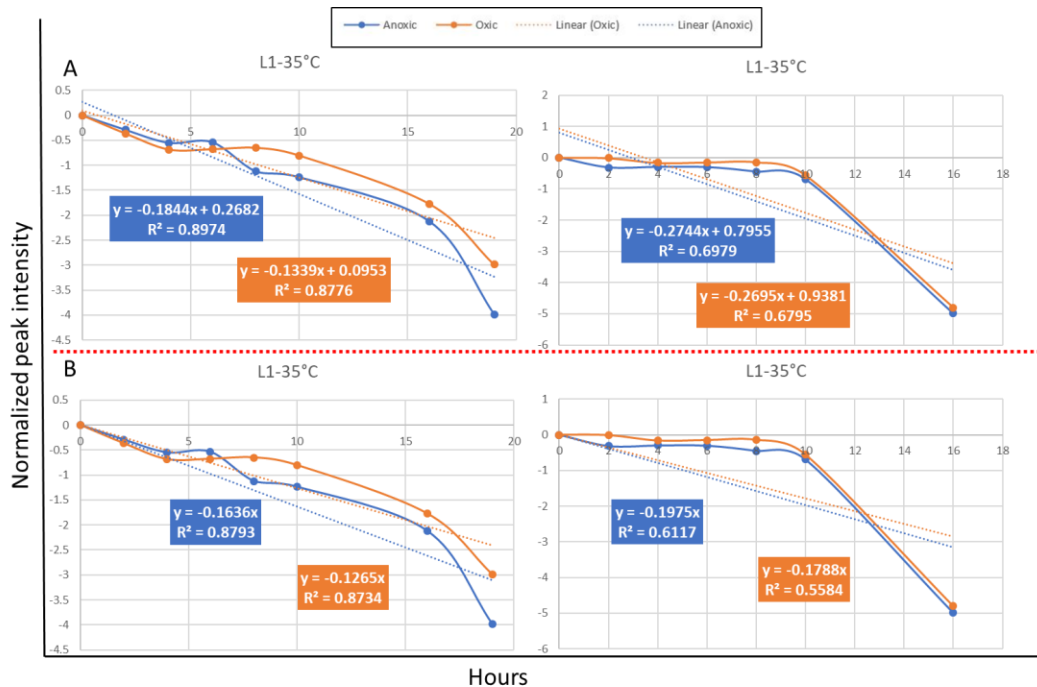

Fig. 54- First order decay plots for tetrahydrocortisone (log scale) at 25°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.

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Fig. 55- First order decay plots for tetrahydrocortisone (log scale) at 35°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
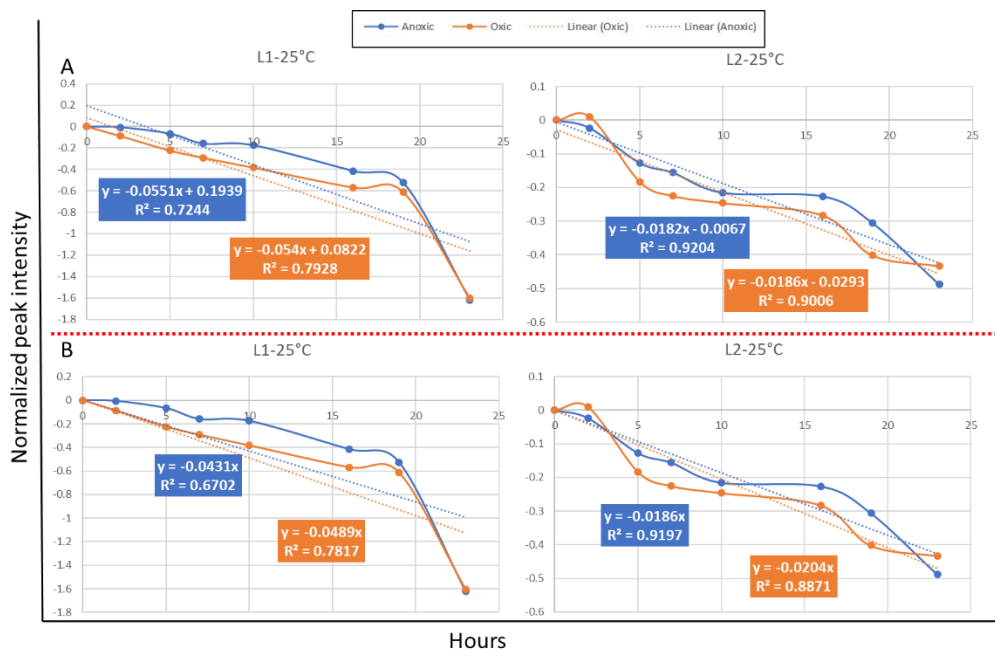

Fig. 56- First order decay plots for tetrahydrocortisol (log scale) at 25°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.
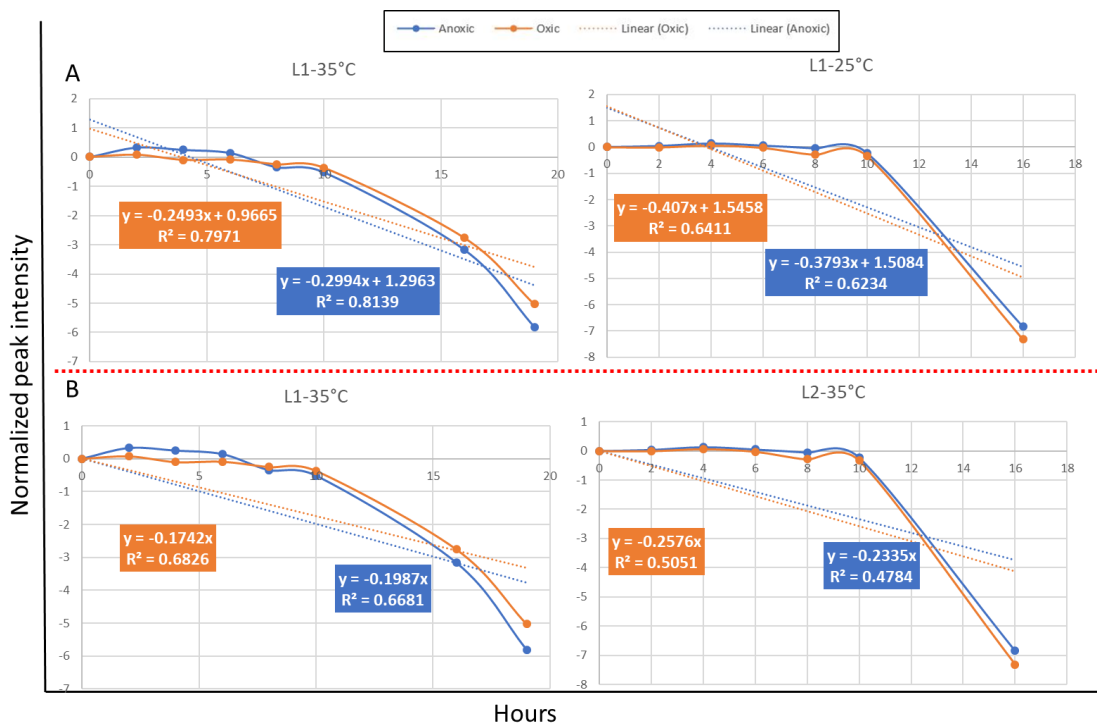
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Fig. 57- First order decay plots for tetrahydrocortisol (log scale) at 35°C for location 1 (L1) and location 2 (L2) with respective trendlines at both anoxic and oxic conditions. Panel A and B represent plots where the trendline is not forced through 1 and trendline forced through 1 respectively.